SUCROSE METABOLISM IN STORAGE ORGANS OF 'SOLANUM TUBEROSUM L.', 'VICIA FABA L.', AND 'BETA VULGARIS L.'

Heather A. Ross

A Thesis Submitted for the Degree of PhD
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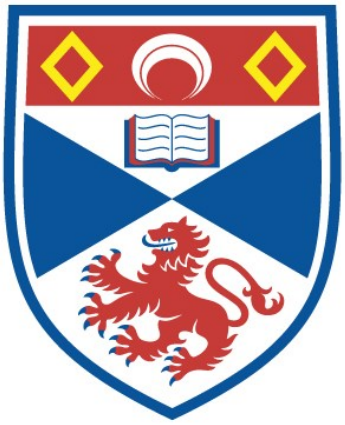

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by

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A thesis submitted to the University of St. Andrews
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LIST OF ABBREVIATIONS

AMP, ADP, ATP	adenosine 5' -mono, -di, -triphosphate
ADPGlc	adenosine 5' -diphosphate-glucose
ATPase	adenosine triphosphatase
Arg	arginine
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
Bq	becquerel
CDP	cytidine 5'-diphosphate
cDNA	complementary deoxyribonucleic acid
Ci	curie $1Ci = 3.77 \times 10^{10}$ Bq
DAA	days after anthesis
DAF	days after flowering
DEAE	diethylaminoethyl
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
DW	dry weight
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycol-bis (ß-aminoethylether)-*N,N'*-tetra-acetic acid
Fru	fructose
Fru 1,6 bis P	fructose 1,6-bisphosphate
Fru 6 P	fructose 6-phosphate

FW	fresh weight
G	giga- (10^9)
GDP	guanosine 5'-diphosphate
Glc	glucose
Glc 1 P	glucose 1-phosphate
Glc 6 P	glucose 6-phosphate
Glu	glutamic acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
IEF	isoelectric focussing
k	kilo- (10^3)
kD	kilodalton
K_{eq}	equilibrium constant
K_i	inhibition constant
K_m	Michaelis constant
M	mega- (10^6)
μ	micro- (10^{-6})
M_r	relative molecular weight
MW	molecular weight
mRNA	messenger RNA
n	nano- (10^{-9})
NAD/NADH	nicotinamide adenine dinucleotide and its reduced form

NADP/NADPH	nicotinamide adenine dinucleotide phosphate and its reduced form
NBT	nitro blue tetrazolium
NDP,NTP	nucleoside 5'-di, -triphosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCMBS	*p*-chloromercuribenzenesulfonic acid
PDA	piperazine di-acrylamide
PFK	phosphofructokinase
PGA	3-phosphoglyceric acid
PGI	phosphoglucose isomerase
PGM	phosphoglucomutase
pI	isoelectric point
P_i	inorganic phosphate
PMSF	phenylmethylsulphonyl fluoride
PP_i	inorganic pyrophosphate
PVP	polyvinylpyrrolidone
Q_{10}	temperature quotient (10^oC)
R_f	retardation factor
RGR	relative growth rate
RNA	ribonucleic acid
Rubisco	ribulose-1, 5-bisphosphate carboxylase/oxygenase
RuBP	ribulose-1, 5-bisphosphate
SDS	sodium dodecyl sulphate
SE-CC	sieve element - companion cell complex

SEM	standard error of mean
Sh	*shrunken*
SPS	sucrose phosphate synthase
Sus	*sucrose synthase*
TBS	tris-buffered saline
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
TTC	triphenyl-tetrazolium chloride
UDP	uridine 5'-diphosphate
UDPGlc	uridine 5'-diphosphate-glucose
V_e	eluted volume
V_{max}	maximal velocity
V_o	void volume

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I am indebted to both Dr Peter Waister and Dr Terry Hegarty for instilling an early interest and enthusiasm in scientific research.

Finally, I like to think of this thesis as dedicated to both my late father, who taught and cared for me so much and to my mother who has shown such love and patience with me throughout my life (even during the course of this study!).

DECLARATION

I, Heather A. Ross, hereby certify that this thesis, which is approximately 60,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

16th March, 1994

I was admitted as a research student under Ordinance No. 12 in October, 1989 and as a part-time candidate for the degree of Doctor of Philosophy in October, 1989; the higher study for which this is a record was carried out in the Cellular and Environmental Physiology Department of the Scottish Crop Research Institute, Invergowrie, Dundee and in the School of Biological and Medical Sciences, University of St. Andrews between 1989 and 1994.

16th March, 1994

We hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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SUMMARY

The involvement of the sucrose-cleaving enzymes, acid and alkaline invertases and sucrose synthase in carbohydrate metabolism, was investigated in three different developing sink organs: 1) the starch-storing tubers from *Solanum tuberosum* L., 2) the starch- and protein-storing cotyledons from *Vicia faba* L., and 3) the sucrose-storing taproots from *Beta vulgaris* L. subsp. *altissima*.

In potato, tuberisation is characterised by a change from an invertase-dominated sucrolytic pathway in stolons to one dominated by sucrose synthase in developing tubers. This pathway continues to be the major route for sucrose breakdown during tuber growth but only in tubers receiving a ready supply of photoassimilate. Sucrose flux to the tuber was shown to regulate sucrose synthase activity, excision of developing tubers from the mother plant resulting in a rapid decrease in sucrose synthase activity and an increase in acid invertase. Acid invertase was by far the major sucrolytic enzyme in stored tubers.

In contrast, acid invertase does not play a major role in sucrose cleavage in developing bean cotyledons. Sucrose synthase is the dominant sucrolytic enzyme during the early stages of seed growth but in the later stages of development alkaline invertase predominates.

During sugar beet development, high acid invertase activity in very young roots declines rapidly when taproot swelling commences, to be replaced by both sucrose synthase and alkaline invertase. Neither enzyme predominates during taproot growth. No significant increase in the activity of any of the sucrolytic enzymes occurred in taproots stored for 80 d at 8°C.

Sucrose synthase was purified to homogeneity from bean cotyledons and characterised. Polyclonal antibodies were raised to both native and denatured sucrose synthase protein. Similarly alkaline invertase was purified from bean cotyledons and sugar beet taproots and polyclonal antibodies raised to both denatured proteins. Isoforms of bean and sugar beet alkaline invertases were separated by anion-exchange chromatography but were not immunologically distinct. The antibodies produced were used throughout this study to confirm enzyme levels.

CHAPTER 1

INTRODUCTION

1.1 Synthesis and properties of sucrose

The analogy of the role of sucrose in plants to that of glucose in animals has often been used to stress the central and unique importance of this disaccharide in plant metabolism. Indeed, studied analysis of plant biochemistry supports the statement that "plants run on sucrose" (ap Rees, 1993). Not only is sucrose one of the major products of photosynthesis and as such is translocated from source to sink organs, but also, it is the main form of carbohydrate storage, it provides energy during growth and is a source of carbon for biosynthetic processes.

Carbohydrate synthesis commences with the reduction of carbon dioxide by the reductive pentose phosphate pathway or Calvin cycle within the stroma of the chloroplasts. The energy required for the pathway is generated during the light reactions of photosynthesis and is in the form of the reduced pyridine nucleotide (NADPH) and ATP. Within the cycle, atmospheric carbon dioxide and water are combined with ribulose 1,5-bisphosphate to yield two molecules of 3-phosphoglycerate. After phosphorylation by ATP, the products are reduced using NADPH to yield triose phosphate (glyceraldehyde 3-phosphate). The triose phosphate is then either transported out of the chloroplast for sucrose synthesis or used within the chloroplast for starch synthesis or for the regeneration of ribulose 1,5-bisphosphate (Stitt *et al.*, 1987a). This latter set of reactions, commencing with the conversion of glyceraldehyde 3-phosphate to dihydroxyacetone 3-phosphate which then undergoes aldol condensation to yield fructose 1,6-bisphosphate, and finally

through a series of reactions involving C3, C4, C5, C6 and C7 compounds to the regeneration of ribulose 1,5-bisphosphate, is essential for the continued fixation of carbon dioxide. The activity of enzymes in the pathway is regulated by light, and by changes in the stromal pH, Mg^{2+} concentration, and ATP:ADP ratio (Buchanan, 1980). However, advances in plant molecular studies has led to the use of genetically manipulated plants in which the expression of a particular enzyme has been decreased in order to investigate whether it is rate-limiting. The enzyme catalysing the initial carboxylation step, ribulose bisphosphate carboxylase/oxygenase (Rubisco) has been investigated recently using transgenic tobacco plants transformed with 'antisense' rbcS - the nuclear-encoded gene for the small subunit of Rubisco (Lauerer *et al.*, 1993). The results show that the enzyme does not exert major control over the rate of photosynthesis in growth conditions over a wide range of light intensities. The maximum control coeffficient calculated was less than 0.4, thus confirming that Rubisco only exerts partial control and that the overall control of the pathway is shared.

The excess (one sixth) of triose phosphate generated during the cycle may be exported for sucrose synthesis in the cytosol (Stitt *et al.*, 1987b). This step is highly regulated as it determines the rate of starch and sucrose synthesis during photosynthesis and ensures that sufficient triose phosphate remains for regeneration of ribulose 1,5-bisphosphate (Stitt and Heldt, 1985). The main factors involved in this regulation are the relative concentrations of inorganic phosphate (Pi) and triose phosphate in the chloroplast and cytosol and the concentration of fructose 2,6 bisphosphate in the cytosol (Stitt, 1990). Communication between the two compartments is via a transport protein, the phosphate triosephosphate 3-phosphoglycerate translocator, which is located in the chloroplast envelope (Heldt and Flügge, 1987). Transport by the phosphate translocator is facilitated by counterexchange so that for each

molecule transported out of the chloroplast another is transported inwards. Thus the export of triose phosphate to the cytosol is in exchange for Pi released during sucrose synthesis. However, it also allows an exchange of triose phosphate and 3-phosphoglycerate so that ATP and NADPH are transferred to the cytosol (Stitt *et al.*, 1987b). Therefore the ability of the phosphate translocator to adjust the concentrations of Pi, 3-phosphoglycerate and triose phosphate in the chloroplast in response to changes in the cytosol, provides a mechanism allowing for precise coordination in the metabolic processes of the two compartments (Stitt *et al.*, 1987b). Thus, when the expression of the triose phosphate translocator was decreased by approximately 30%, the level of starch increased 2 to 3-fold while sucrose synthesis decreased (Riesmeier *et al.*, 1993).

The first major regulatory step in the synthesis of sucrose in the cytosol is that catalysed by cytosolic fructose 1,6-bisphosphatase. This enzyme catalyses the irreversible conversion of fructose 1,6-bisphosphate to fructose 6-phosphate, but is itself highly regulated (Stitt *et al.*, 1987a). A list of the complex regulatory properties of the enzyme is given in the previously mentioned paper but of particular interest is the strong inhibition by fructose 2,6-bisphosphate which not only inhibits the enzyme but also causes a synergistic response to other effectors. Fructose 2,6-bisphosphate is synthesized from fructose 6-phosphate by a specific fructose 6-phosphate 2-kinase and is degraded by fructose 2,6-bisphosphate phosphatase. Each of these enzymes are regulated by metabolite levels e.g. spinach leaf fructose 6-P 2-kinase is activated by Pi and fructose 6-P but is inhibited by 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, dihydroxyacetone phosphate and the intermediate glycollate-2-phosphate (Stitt *et al.*, 1984). The fructose 2,6-bisphosphate phosphatase exists in two forms in spinach leaves, as a monofunctional and bifunctional enzyme (Stitt, 1990). Inhibition of the monofunctional enzyme

occurs with fructose 6-phosphate, Pi, fructose 1,6-bisphosphate and divalent cations. The bifunctional form is also inhibited by fructose 6-phosphate and Pi but to a different extent to the monofunctional enzyme. Due to their different properties, both forms of the enzyme are thought to be required (Stitt, 1990). The overall effect of the above interactions means that the activity of cytosolic fructose 1,6-bisphosphatase will be regulated primarily by the relative amounts of triose phosphates, Pi and hexose 6-phosphates.

The activity of sucrose phosphate synthase, catalysing the formation of sucrose phosphate from fructose 6-phosphate and UDP-glucose, is also of primary importance in controlling the rate of sucrose synthesis. Its activity is regulated *in vivo* by fine and coarse control mechanisms. An important contribution to the fine control of the enzyme is by the ratio of glucose 6-phosphate : Pi as glucose 6-phosphate activates the enzyme while Pi is a competitive inhibitor with respect to both fructose 6-phosphate and UDP-glucose. Both effectors act at allosteric sites in spinach leaf sucrose phosphate synthase as they require sulfhydryl groups that are absent in the catalytic site (Doehlert and Huber, 1985).

The level of sucrose phosphate synthase also changes in response to light/dark transitions and to alterations in the requirement by sink organs for assimilate. Both types of response are thought to reflect a "coarse" level of control (Stitt *et al.*, 1987b). The biochemical process involved is thought to be protein phosphorylation but the extremely complex cascade of reactions has still to be clarified (Huber *et al.*, 1992). Consequently, the results of overexpressing or reducing the expression (approx. 2-fold) of the gene encoding sucrose phosphate synthase in potato plants, did not yield large increases or decreases in sucrose levels as might be expected (Stitt, 1993 - reported at Autumn School, Wageningen). The plants apparently compensated for reduced levels of the enzyme by having more of the enzyme present in its activated state. It is

considered necessary to target steps in the cascade of reactions affecting the enzyme in order to produce a significant effect on the rate of sucrose synthesis.

Investigation of the expression of genes encoding Rubisco, fructose 1,6 bisphosphatase and sucrose phosphate synthase showed that the level of transcripts of each of the enzymes changed during sugar beet leaf transition from sink to source and was also correlated with leaf photosynthetic rates and sucrose content (Harn *et al.*, 1993). The results were taken as evidence of the molecular coordination of sucrose synthesis and photosynthesis.

The final step in the pathway of sucrose synthesis is catalysed by sucrose phosphate phosphatase with the release of Pi into the cytosol. While the cytosolic fructose 1,6-bisphosphatase and the sucrose phosphate synthase are important points in the pathway for regulation, the interactions between all components and the flux through the entire pathway need to be taken into account. Recent investigations have considered the effect on sucrose synthesis of reduced expression of phosphoglucose isomerase (PGI) (Stitt, 1993 - reported at Autumn School, Wageningen). This enzyme catalyses the isomerisation reaction of Fru 6P to Glc 6P and was not thought to contribute significantly to the overall control of the pathway. However, when activity of PGI was reduced a substantial decrease in sucrose synthesis resulted. This was due to an increase in Fru 6-P leading to the production of more Fru 2,6-bisP and as a consequence reduced activity of Fru 1,6 Pase. This finding highlights how individual steps within a pathway are closely integrated and the importance of investigating the overall control of the pathway. The concept that metabolic control is shared by enzymes rather than being restricted to a single rate-limiting enzyme in a pathway was emphasised by Kacser and Burns, (1973). They suggested that the determination of the amount of control exerted by an enzyme on the flux through a particular pathway, the 'flux control coefficient', would provide a more accurate measurement of the

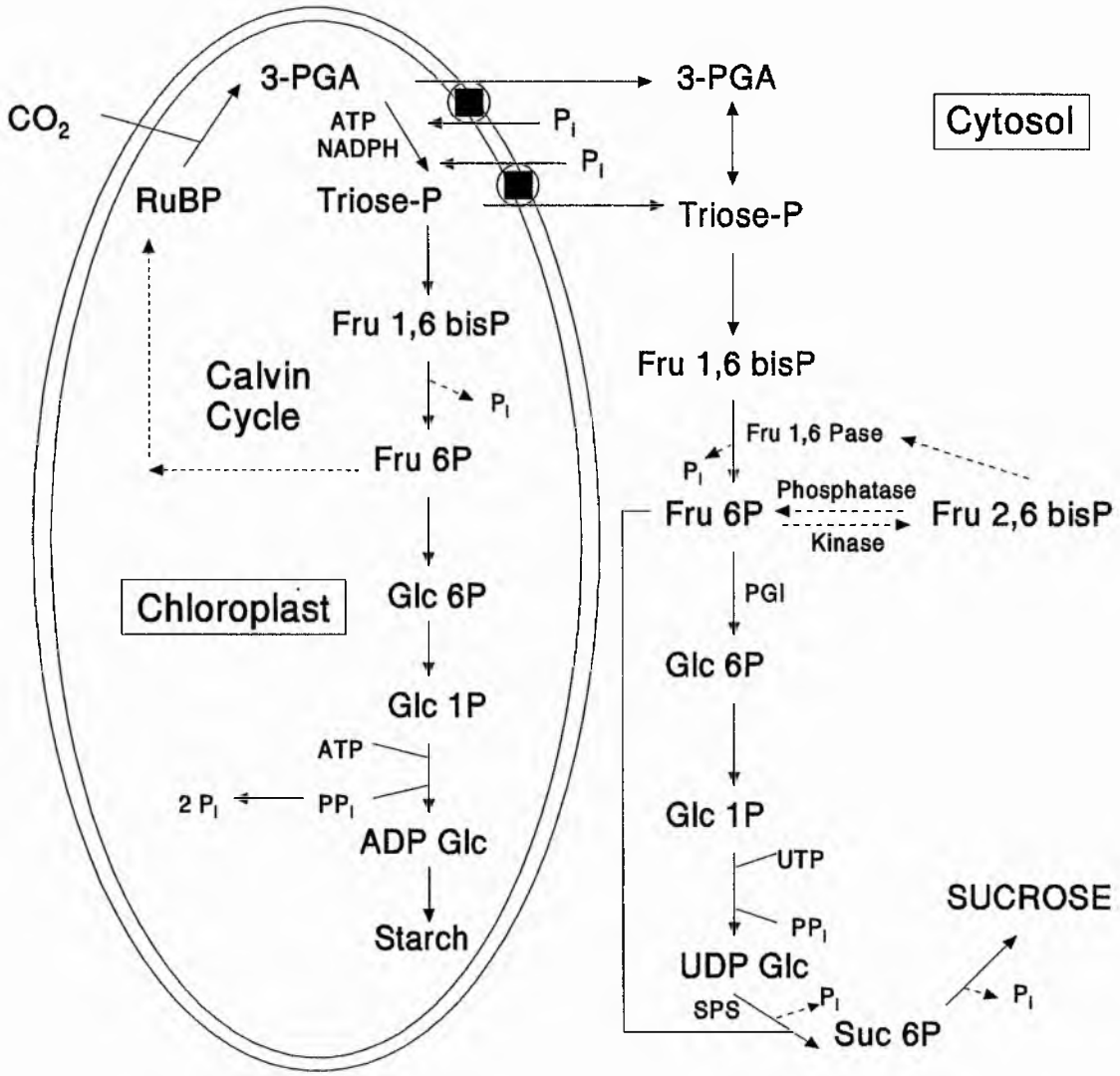


Fig. 1.1 Schematic representation of sucrose synthesis showing the regulatory interdependence with photosynthesis.

contribution of that enzyme to the pathway *in vivo*. The equation defining the flux control coefficient (*C*) where the flux is *J* and the enzyme concentration is *E* is shown below:-

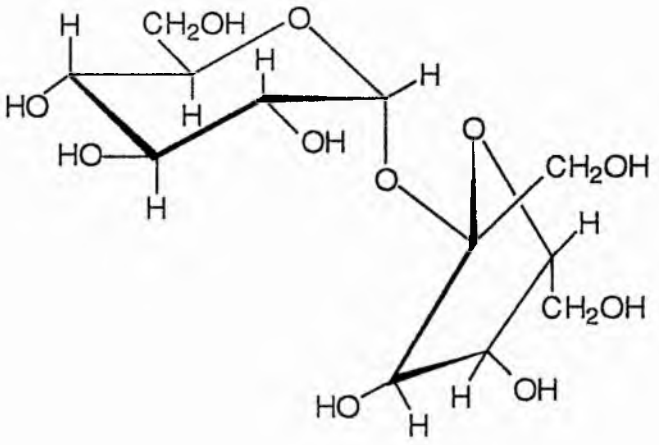
$$C = \frac{\delta J / J}{\delta E / E}$$

(Kacser, 1987). A flux control coefficient is therefore, the fractional change in the flux through a pathway induced by a fractional change in the activity of a particular enzyme. A low flux control coefficient means that any change in the activity of that particular enzyme will have minimal effect on the flux and as a result the enzyme would have little control on the pathway, whereas a large coefficient is indicative of a high level of control. The sum of all the flux control coefficients for a particular pathway is always unity. Thus, the aim in investigating metabolic control is to alter the concentration or activity of a particular enzyme by a small amount and then to measure the effect that change has on the flux through the pathway, as determined for Rubisco (Lauerer *et al.*, 1993).

A schematic representation of sucrose synthesis with the reactions discussed above is shown in Figure 1.1.

Sucrose thus formed during photosynthesis and from the breakdown of chloroplast starch during the night is ready for transport from the cytosol of the mesophyll cells to sink organs in the rest of the plant. However, the question that has often been raised is why sucrose is transported in higher plants rather than glucose. Pontis (1977) considered the ubiquity of sucrose in the plant kingdom as a "riddle" as the reasons were not immediately obvious, although on further discussion of its properties the mystery was partly revealed. One of the main reasons is thought to involve its relatively unreactive chemical structure (see below).

SUCROSE - α-D-glucopyranosyl-ß-D-fructofuranoside (C1>C2 linkage)



While reducing sugars such as glucose and fructose contain an exposed reactive aldehyde or ketone group, within the sucrose molecule the anomeric carbons from both monosaccharide residues are effectively sequestered. This confers on the sucrose molecule a relative degree of chemical inertness. It has been suggested that in animal systems the more reactive monosaccharide, glucose, can be used for transport as the transport system is extracellular (Lucas and Madore, 1988). This ensures that blood glucose is separated from cellular enzymes until it is taken up across a plasma membrane, whereas in plant cells, as discussed above, sucrose is synthesised within the cytosol in the presence of glycolytic enzymes.

The presence of a fructose residue in the furanose configuration in a ß-linkage with glucose confers extreme lability to the fructose moiety, consequently giving the sucrose molecule a high free energy of hydrolysis (approx. -30 kJ mol^{-1}). This property, in combination with its chemical inertness, would allow sucrose to remain intact until it came in contact with specific enzymes, when it would be readily degraded (Lucas and Madore, 1988). The high free energy of hydrolysis is also of significance when considering later the contribution of the

enzymes involved in sucrose cleavage (see section **1.4**). Another important feature of the sucrose molecule in relation to its transport function is its three dimensional structure which is recognised by specific membrane proteins. These sucrose-specific carriers are responsible for the uptake and transport of sucrose across cell membranes. The recognition by the carrier proteins of a sucrose derivative 1'-fluorosucrose (α-D-glucopyranosyl-ß-D-1-deoxy-1-fluorofructofuranoside) has proved advantageous in studying sucrose transport as fluorosucrose is not readily hydrolysed by invertase (Hitz *et al.*, 1985).

The physicochemical properties of the sucrose molecule (discussed above) can partly explain its central role in plant metabolism while further biochemical characteristics important to developing sink organs will be considered later.

1.2 Sucrose transport from source to sink tissues

The main form of carbon transported from source leaves to developing sink organs has been identified as sucrose in many plant species, for example, tubers of *Solanum tuberosum* (Oparka and Prior, 1987), developing seeds of *Glycine max, Phaseolus vulgaris, Vicia faba* - (Thorne, 1980; Patrick, 1983; Wolswinkel and Amerlaan, 1983) and taproots of *Beta vulgaris* (Giaquinta, 1979). The transport of photoassimilate (mainly sucrose) from source to sink tissues may be subdivided into several steps:- movement out of the mesophyll cell towards the phloem and loading into the sieve element-companion cell complex, transport through the phloem towards sink organs, unloading from the sieve elements into the sink organ, and finally, either storage or metabolism within the sink tissues.

1.2.1 Phloem loading

Attempts at identification of the pathway from the mesophyll cells to the phloem led to ultrastructural studies of the phloem loading zone. In this detailed work, the type of plasmodesmatal connections of the sieve element - companion cell complex in the minor veins of 700 species distributed over 140 families was classified (Gamalei, 1985, 1989, 1991). Additionally, the construction of 'plasmodesmograms' based on plasmodesmatal frequencies between mesophyll and sieve element-companion cell complexes (Van Bel *et al.*, 1988) has led to the concept of multiprogrammed phloem loading (van Bel and Gamalei, 1991). Three main modes of phloem loading have been identified as 1) strictly symplastic, 2) strictly apoplastic or 3) a combination of both (van Bel, 1992). The symplastic pathway requires the presence of plasmodesmata between mesophyll cells and the sieve element - companion cell complex but the actual mechanism by which it occurs is uncertain. The problem is in explaining the transport of sucrose against a concentration gradient. The apoplastic pathway involves the active transport of solutes out of the symplast of the mesophyll cells into the apoplast. Within the apoplast the possibility exists of hydrolysis by an extracellular acid invertase prior to uptake by the sieve element - companion cell complex. However, insertion of a yeast-derived invertase into the apoplast of potato plants confirmed that phloem loading involved the apoplast but that reduced sucrose export from the mesophyll cells was due to the presence of apoplastic invertase (Heineke *et al.*, 1992). This supports the view that sucrose does not normally undergo hydrolysis within the apoplast but is loaded intact into the phloem. Apoplastic phloem loading is an active energy-requiring process, in which sucrose in the apoplast binds to a membrane carrier, and together with a proton, is released inside the symplast of the sieve element - companion cell complex against a sucrose gradient. This sucrose-proton cotransport model utilises the energy derived from a proton

gradient produced by a plasma membrane ATPase to drive sucrose into the symplast (Giaquinta *et al.*, 1983). A cDNA from spinach leaves encoding a sucrose carrier has been identified by functional expression in yeast (Riesmeier *et al.*, 1992). The carrier is pH dependent, is inhibited by thiol-modifying agents such as PCMBS, the activity is dependent on the pH gradient and the membrane potential and the K_m value of the carrier for sucrose is estimated to be approximately 1.5 mM. It was also shown to have the ability to transport maltose. More recently, Riesmeier and coworkers (1993) isolated a sucrose transporter cDNA from potato and showed it had 68% identity to the spinach sucrose transporter on the basis of deduced amino acid sequence. In addition, many of the properties of the potato transporter were similar to those found for spinach. The tissue-specificity expression of the sucrose transporter in potato was studied by RNA gel blot analysis and *in situ* hybridisation. These results show very high expression of the gene in mature source leaves but much lower expression was found in stems and sink tissues, such as roots and tubers. The *in situ* hybridisation studies showed that the transporter is expressed to high levels specifically in the phloem although further experimentation will be needed to identify in which cell types. The results are consistent with an apoplastic phloem loading pathway in source leaves as discussed above.

1.2.2 Phloem translocation

The pressure-flow hypothesis is now widely accepted as the most probable mechanism of phloem translocation (Münch, 1930). This model proposes that the flow of solution in the phloem sieve elements is driven by an osmotically generated pressure gradient between source and sink cells. Active phloem loading into sieve elements at the source end of phloem vessels increases the osmotic pressure, water from the xylem enters the sieve elements and results in high turgor pressure. At the sink end, active phloem unloading causes a

decrease in the osmotic pressure, water flows out and the turgor pressure decreases. Thus a pressure gradient is created throughout the phloem.

1.2.3 Phloem unloading

The complexity of the phloem loading pathways, as discussed above, is repeated in sink organs, where a number of phloem unloading pathways may be operational (Oparka *et al.*, 1992). The first step in the phloem unloading pathway is the exit of assimilates from the sieve element-companion cell complex. Solutes can leave through the symplast via plasmodesmata and this would represent the pathway of least resistance. Evidence for symplastic sieve element unloading is widespread, for example, in importing leaves of sugar beet (Gougler Schmalstig and Geiger, 1985) and tobacco (Turgeon, 1987), developing potato tubers (Oparka and Prior, 1986), maternal tissues of developing seeds (both legumes and cereals) (Thorne, 1985) and pea roots (Dick and ap Rees, 1975). The alternative exit out of the sieve element-companion cell complex into the apoplast has not been so evident and has proved difficult to demonstrate conclusively. The model of phloem unloading proposed for sugar beet taproots (Fieuw and Willenbrink, 1990) assumes that sucrose is released first from the SE-CC complex into the apoplast where a cell wall bound invertase causes its hydrolysis. Their assumption is based on uptake studies which show that glucose is transported preferentially, the presence of an active acid invertase and absence of symplastic connections between sieve elements and sink cells. However, the cellular site of unloading has yet to be identified.

The examples given above are of unloading either symplastically or apoplastically from the SE-CC complex. However, as explained by Oparka and van Bel (1992) four possible pathways of phloem unloading (i.e. the entire pathway from the SE-CC complex to the cells of the sink organ) may exist: 1)

symplastic phloem unloading in conjuntion with symplastic SE unloading; 2) apoplastic phloem unloading in conjunction with apoplastic SE unloading; 3) apoplastic phloem unloading but symplastic SE unloading; and 4) apoplastic phloem unloading in which symplastic continuity is interrupted at some distance away from the SE-CC complex. Unequivocal evidence supporting a particular pathway is only available for a few plant species.

The phloem unloading pathway is perhaps best understood in developing seeds where the embryo is symplastically isolated from the maternal tissue and assimilates have to pass into the apoplast prior to uptake by the seed (Thorne, 1985). The pathway of photoassimilate movement in developing seeds of *Vicia faba* has been studied in detail (Offler *et al.*, 1989; Offler and Patrick, 1993). The proposed pathway is one in which photoassimilate moves away from the SE-CC complex radially and laterally in the symplast to thin-walled parenchyma/transfer cells which form the main site for membrane exchange to the seed apoplast. These transfer cells, bordering the entire inner surface of the seed coat, have 43% of their cell wall length exhibiting ingrowths which results in a 58% increase in plasma membrane surface area. This characteristic of the transfer cells, along with an increase in the number of mitochondria and ribosomes found within the cytoplasm, are thought to have developed to facilitate exchange to the seed apoplast. It is thought that a similar pathway of transfer occurs in the seed coats of most grain legumes.

Information on the phloem unloading pathways in vegetative sinks is not so detailed. In potato tubers, evidence of symplastic continuity between the SE-CC complex and surrounding parenchyma cells and the inhibition of tracer efflux by induced plasmolysis suggest that the phloem unloading pathway is symplastic (Oparka, 1986: Oparka and Prior, 1987). However, when apoplastic yeast invertase was expressed in potato tubers, the resulting accumulation of glucose and fructose and decrease in the level of sucrose indicates that

movement into the apoplast occurs in growing potato tubers (U. Sonnewald pers. comm. to H. V. Davies). At what stage in the phloem unloading pathway this takes place is not clear, therefore the possibility of either a symplastic or apoplastic route of phloem unloading in potato tubers still exists. As yet, no clear pattern associating specific vegetative sink organs with a particular route of phloem unloading has been identified.

According to the pressure-flow hypothesis (Münch, 1930), as described above (**1.2.2**), the movement of solutes will be driven by a pressure gradient between source and sink cells. Thus sink cells would need to maintain a low sucrose concentration in comparison to the SE-CC complex to ensure the continued transport of sucrose into the sink organ. Several mechanisms have been proposed to allow the continued import by the sink organ. When apoplastic SE unloading occurs, hydrolysis of sucrose by apoplastic acid invertase would cause a decrease in the apoplastic sucrose level, prevent reloading and enhance SE unloading (Eschrich, 1980, 1986). However, the hydrolysis of sucrose prior to uptake by parenchyma cells is not a prerequisite for the continuance of SE unloading (Gougler Schmalstig and Hitz, 1987; Lingle, 1989). A gradient may also be maintained by compartmentation of sucrose into the vacuoles of storage cells or by the conversion of sucrose to starch (Ho, 1988).

This traditional view of phloem unloading being enhanced by low apoplastic sucrose concentrations needs reviewing in the light of results with developing legume seeds (Wolswinkel, 1992). No inhibition of sucrose unloading from the testa or sucrose transport into the empty seed occurred when solutions of high sucrose concentration were present in empty ovules (Patrick, 1984; Wolswinkel and Amerlaan, 1984). Phloem unloading in legume seeds, rather than being inhibited by high apoplastic solute concentrations, appears to be stimulated. The suggestion is that a high concentration of osmotically active solutes in the sink apoplast will result in an osmotic efflux of water from the sieve elements

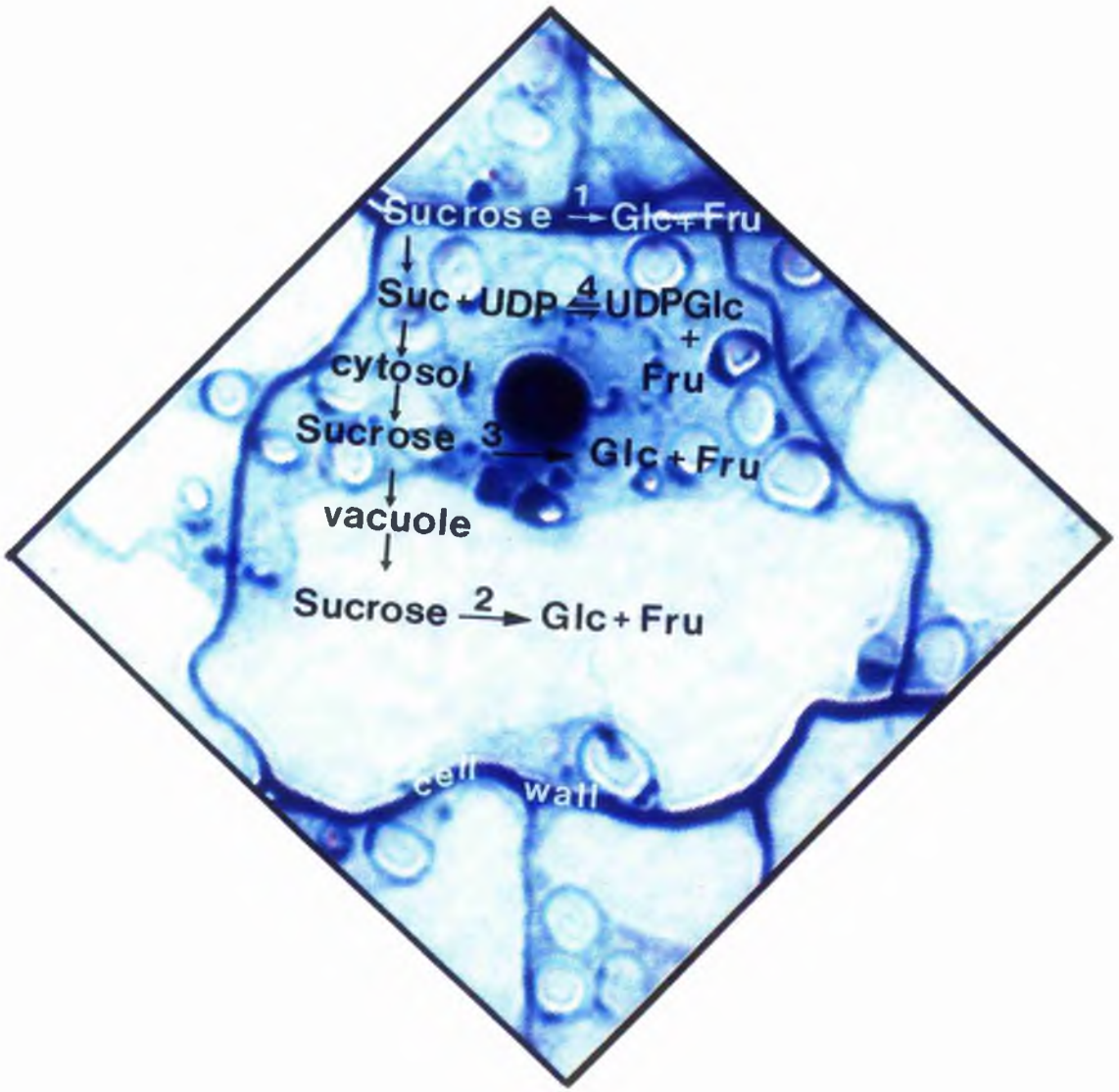


Fig. 1.2 Sites of sucrose cleavage shown in a storage parenchyma cell from a young potato tuber with possible enzymes involved:- 1) cell wall acid invertase, 2) vacuolar acid invertase, 3) alkaline invertase, and 4) sucrose synthase.

and other cells in the seed-coat, further reducing the turgor pressure at the sink end of the phloem pathway and thus promoting phloem transport into developing sinks (Wolswinkel, 1992). Restricted xylem connections between developing seeds and transpiring leaves is thought to prevent the movement of apoplastic solutes back along the water potential gradient (Wolswinkel, 1992).

While no generalizations on the most common phloem unloading pathway can be made, the form of carbon most likely to be delivered and taken up by sink organs is sucrose. It appears likely from the studies of uptake with fluorosucrose (Gougler Schmalstig and Hitz, 1987; Lemoine *et al.*, 1988) and the use of [^{14}C]fructosyl sucrose (Lingle, 1989; Thom and Maretzki, 1992) that while hydrolysis in the apoplast may occur it is not a prerequisite of uptake and that sucrose is taken up intact by maize endosperm cells and by parenchyma cells in sugarcane internode tissue. Similarly, translocated sucrose in sugar beet enters the storage parenchyma cells in mature taproots without apoplastic hydrolysis (Giaquinta, 1977, 1979).

Sucrose thus delivered to the storage cells of sink organs may be cleaved, prior to storage or utilisation in cell metabolism, by four possible routes involving the following enzymes: 1) cell wall acid invertase, 2) vacuolar acid invertase, 3) alkaline/neutral invertase or 4) sucrose synthase (see Figure 1.2).

1.3 Properties of the enzymes involved in sucrose cleavage

1.3.1 Invertases

Invertases (EC 3.2.1.26, ß-D-Fructofuranoside fructohydrolases) are widespread throughout the plant kingdom and catalyse sucrose hydrolysis to yield the reducing sugars, glucose and fructose.

$$\text{SUCROSE} + H_2O \rightarrow \text{GLUCOSE} + \text{FRUCTOSE}$$

$\Delta G'$ for the reaction = -27.6 kJ

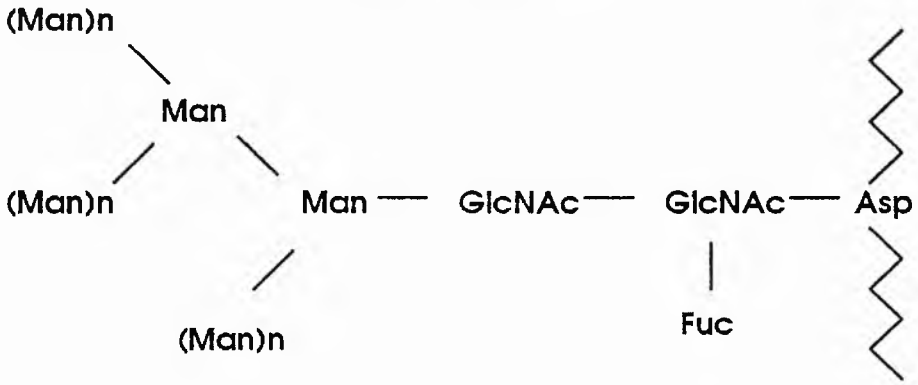
While sucrose, the usual substrate for invertase, is a ß-fructofuranoside it is also an α–glucoside. Plant invertases are predominantly ß-fructofuranosidases which attack the sucrose molecule from the fructose end and may also cleave the trisaccharide, raffinose. α–Glucosidases are not so common in plant tissues but may be characterised by their hydrolytic action on melezitose and other α–glucosides and their inability to hydrolyse raffinose (Myrbäck, 1960).

The main classification of invertase enzymes is based on their optimum activity at either acid pH of 4.0 to 5.3 or at pH 7.0 to 7.8 (ap Rees, 1974). The acid and alkaline invertases are distinct proteins and their properties will be discussed separately below.

1.3.1.1 Acid invertases

Acid invertases are active in most plants at some stage during their development and growth and they may be distinguished by their location within the cell (see Figure 1.2). Cell fractionation studies found some forms of the enzyme to be readily soluble while others appeared to be tightly bound to the cell wall (Ruffner *et al.*, 1990; Getz, 1991; Ranwala *et al.*, 1992). Doubts were cast on some of the earlier cell fractionation studies when it was shown that acid invertase could be released from cell wall preparations of Jerusalem artichoke tuber tissue if buffers with high ionic strength were used (Little and Edelman, 1973). It was suggested that soluble acid invertases could bind to cell wall preparations by salt linkages during the extraction procedure. However, immunocytochemical localisation of acid invertase bound to cell walls in radish seedlings (Faye and Ghorbel, 1983) and purification of cell wall acid invertases from tobacco crown gall cells (Weil and Rausch, 1990), from carrot cells (Laurière *et al.*, 1988), from leaves of *Urtica dioica* (Fahrendorf and Beck, 1990) and from a number of other plants (see Table 1.1), provide ample

evidence to confirm their presence *in vivo*. Soluble acid invertases are generally considered to be localised within the vacuole where they are able to operate at a pH close to their optimum. Direct evidence of their vacuolar location has been demonstrated for the storage roots of red beet and for potato tubers (Leigh *et al.*, 1979; Milling *et al.*, 1993; Isla *et al.*, 1992). All purified acid invertases tested are glycoproteins. Usually the purification protocols employ a column step using the lectin, Concanavalin A (e.g. Krishnan *et al.*, 1985; Bracho and Whitaker, 1990a; Moriguchi *et al.*, 1991). This lectin, isolated from *Canavalia ensiformis* - jack bean, specifically binds α-D-glucopyranosyl, α-D-mannopyranosyl and ß-D-fructofuranosyl groups and forms precipitates with branched glycoproteins and polysaccharides containing these residues at the non-reducing end (Faye and Berjonneau, 1979). A 7.7% carbohydrate content of radish acid invertase has been reported (Faye *et al.*, 1981) but in grapes the value was as high as 33% (Nakanishi and Yokotsuka, 1990). While the detailed structures of the carbohydrate chains in plant invertases have not been identified, studies with radish seedlings show that cell wall and soluble invertases have different carbohydrate moieties (Faye *et al.*, 1986). The glycans, however, were shown to be asparagine-linked oligosaccharides with the main difference between the forms due to the size of the carbohydrate moiety (as shown below with n=1 and n>1 for soluble and cell wall forms respectively).



It was suggested that a fucose residue may be present on the core sequence of the soluble invertase due to its retardation on lentil lectin-Sepharose columns as fucose had been shown to be essential for high affinity binding to both lentil and pea lectin-Sepharose (Kornfeld *et al.*, 1981). This detailed structural study of radish invertases by Faye and coworkers led them to suggest that the soluble and cell wall forms of invertase have common precursor polypeptides and that differences between the forms are a result of post-translational events of processing (Faye *et al.*, 1986). It may be that plant invertases are analagous to yeast invertases and undergo processing while associated with the endoplasmic reticulum and Golgi membranes before being targeted either to the vacuole where the acidic conditions allow the enzyme to be active or to the cell wall to hydrolyse sucrose in the apoplast.

Nearly all the purified acid invertases (reported in table 1.1) cleave raffinose but at much slower rates (10% to 50%) than sucrose confirming that plant acid invertases are predominantly ß-fructofuranosidases with a high degree of specificity for sucrose. They have also a relatively high affinity for sucrose (K_m values range from 0.4 to 55 mM with the average K_m value approximately 6 mM).

A wide range of molecular weights for the native enzyme has been reported (28,000 to 360,000) with subunit weights from 11,000 to 70,000 (Table 1.1). The average subunit molecular weight is approximately 58,000 and the enzyme, in general, is considered to be either a monomer or dimer.

Recent advances in molecular biology have led to the cloning and characterisation of a number of genes encoding plant invertases. cDNAs corresponding to the mRNAs encoding both extracellular and vacuolar invertases have been cloned from carrot (Sturm and Chrispeels, 1990, EMBL accession number X67163) and for a vacuolar invertase from tomato fruits

TABLE 1.1 Properties of purified acid invertases

Source	Fraction	Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	pH optimum	Km (sucrose) mM	Nature of protein	Specificity	Reference
Bamboo shoots (*Leleba oldhami*)								
Type I	Soluble	240	70	4.5	7.9	All glycoproteins and possible tetramers	–	Cheng *et al.* (1990)
II	Soluble	195	60	4.8	22.5			
III	Cell wall	360	–	4.2–4.8	–			
IV	Cell wall	195	–	3.9–4.2	1.3			
Barley leaves (*Hordeum vulgare*)								
Type I	Soluble	64	64	~5.0	8.1	All glycoproteins	All ß-fructofuranosidases	Obenland *et al.* (1993)
IIA	Soluble	116	23–56	~5.0	1.0			
IIB	Soluble	155	25,47 & 64	~5.0	1.7			
Barley internodes	Soluble	120	60	4.5	5	Both glycoproteins	Both ß-fructofuranosidases	Karuppiah *et al.* (1989)
	Cell wall	120	60	5.5	12			
Beet (red) storage roots (*Beta vulgaris*)								
Peak I	Soluble	65	65	5.0	2.1	Both glycoproteins and monomers	Both ß-fructofuranosidases	Milling *et al.* (1993)
II	Soluble	–	46	5.0	–			
Beet (sugar) seedlings and mature roots (*Beta vulgaris*)	Cell wall	28	–	3.5–5.0	1.33	–	ß-fructofuranosidase	Masuda & Sugawara (1980)

TABLE 1.1 Properties of purified acid invertases contd.

Source	Fraction	Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	pH optimum	Km (sucrose) mM	Nature of protein	Specificity	Reference
Carnation petals (*Dianthus caryophyllus*)								
Type I	Soluble	215	–	5.5	3.3	Both glycoproteins	Both	Woodson & Wang
II	Soluble	–	–	5.5	–		ß–fructofuranosidases	(1987)
Carrot (*Daucus carota*) seedlings and suspension cells	Soluble	–	58,52	5.6	–	Both glycoproteins	Both	Laurière *et al.*
	Cell wall	63	63	4.6	–		ß–fructofuranosidases	(1988)
Carrot seeds & seedlings								
Type I	Soluble	68	68,43 & 25	5.2	5	Both glycoproteins	Both	Unger *et al.*
II	Soluble	68	68,43 & 25	–	–	pI values different for I & II	ß–fructofuranosidases	(1992)
Carrot cell cultures								
Type I	Soluble	–	61	4.5	–	Both glycoproteins	–	Stommel & Simon
II	Soluble	–	49	4.5	–			(1990)
Castor bean leaves (*Ricinus communis*)	Soluble	78	11	3.5 & 5.5	7.22	Heptamer	ß–fructofuranosidase	Prado *et al.* (1985)
Cherry fruit (*Prunus avium*)	Soluble	400	63	5.0	4	Glycoprotein	ß–fructofuranosidase	Krishnan & Pueppke (1990)

TABLE 1.1 Properties of purified acid invertases contd.

Source	Fraction	Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	pH optimum	Km (sucrose) mM	Nature of protein	Specificity	Reference
Chicory leaf lamina (*Cichorium intybus*)								
Type I	Soluble	–	–	5.0	0.4	–	Both	Kaur *et al.*
II	Soluble	–	–	5.5	1.1		ß-fructofuranosidases	(1992)
Chicory roots	Soluble	93	50	5.0–5.5	55	Glycoprotein dimer	Sucrose:sucrose fructosyltransferase activity in addition to invertase activity	van den Ende & van Laere (1993)
Grass leaves (*Lolium temulentum*)	Soluble (vacuole)	–	68	–	–	Glycoprotein	ß-fructofuranosidase	Walker *et al.* (1989)
Maize	Soluble	–	–	5.0	2.83	Both glycoproteins	Both	Doehlert & Felker
(*Zea mays*)	Cell wall	40	–	5.0	1.84		ß-fructofuranosidases	(1987)
Melon	Soluble	–	70	5.5	4.2	Both glycoproteins	Both	Iwatsubo *et al.*
(*Cucumis melo*)	Cell wall	–	70	4.5	1.7		ß-fructofuranosidases	(1992)
Mung bean	Soluble	70	30 & 38	–	–	Both glycoproteins	–	Arai *et al.*
(*Phaseolus aureus*)	Soluble	70	70	–	–			(1991)
Nettle leaves	Soluble	56	58	5.5	5	Both glycoproteins	Both	Fahrendorf & Beck
(*Urtica dioica*)	Cell wall	56	58	4.5	1.2		ß-fructofuranosidases	(1990)

TABLE 1.1 Properties of purified acid invertases contd.

Source	Fraction	Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	pH optimum	Km (sucrose) mM	Nature of protein	Specificity	Reference
Oat seedlings (*Avena sativa*)								
Type I	Soluble	59	–	4.3	2.4	–	Both	Pressey & Avants
II	Soluble	108	–	5.0	6.7	–	ß-fructofuranosidases	(1980)
Oat leaf								
Type I	Soluble	143	–	–	–	–	–	Greenland & Lewis
II	Soluble	85	–	–	–	–	–	(1981)
Orange leaves (*Citrus sinensis*)	Soluble	200	–	4–4.5	3.3	–	ß-fructofuranosidase	Schaffer (1986)
Peach fruits (*Prunus persica*)	Soluble	120	64	5.0	4.2	Glycoprotein	ß-fructofuranosidase	Moriguchi *et al.* (1991)
Pepper (sweet) (*Capsicum annum*)	Soluble	63	42	4.5	–	Glycoprotein	ß-fructofuranosidase	Michaud *et al.* (1993)
Potato tubers (*Solanum tuberosum*)	Soluble	–	–	4.5–5.0	6.1	–	–	Pressey (1966)
Potato tubers	Soluble	–	–	~5.0	–	Glycoprotein	–	Anderson & Ewing (1978)

TABLE 1.1 Properties of purified acid invertases contd.

Source	Fraction	Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	pH optimum	Km (sucrose) mM	Nature of protein	Specificity	Reference
Potato tubers	Soluble	60	30	5.0	16	Glycoprotein dimer	–	Bracho & Whitaker (1990)
Potato tubers	Soluble	60	58	5.0	7.9	Both glycoproteins	α-glucosidase	Burch *et al.* (1992)
leaves	Soluble	60	58	5.0	2.4	monomers	α-glucosidase (maltotetrahydrolase based on sequence homology)	
Radish seedlings (*Raphanus sativus*)	Soluble	48.5	48.5	5.0	–	Glycoprotein	ß-fructofuranosidase	Faye *et al.* (1981)
Satsuma Mandarin fruit (*Citrus* 'Unshu Marcovitch')	Soluble	69	–	4.8–5.3	7.3	–	ß-fructofuranosidase	Kato & Kubota (1978)
Spruce root cells	Cell wall	60.3	61	4.5	15.8	Both glycoproteins	Both	Salzer & Hager
(*Picea abies*)	Cell wall	–	–	4.5	8.3		ß-fructofuranosidases	(1993)
Strawberry fruit	Soluble	–	–	4.6	3.5	–	All	Ranwala *et al.*
(*Fragaria x ananassa*)	Cell wall 1	–	–	5.0	3.7	–	ß-fructofuranosidases	(1992)
	Cell wall 2	–	–	4.2	4.4	–		
Sugar cane (*Saccharum officinarum*)	Soluble	380	–	5.5	45	–	–	Batta *et al.* (1991)

TABLE 1.1 Properties of purified acid invertases contd.

Source	Fraction	Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	pH optimum	Km (sucrose) mM	Nature of protein	Specificity	Reference
Tobacco cells (*Nicotiana tabacum*)	Soluble	76	73	4.0	–	Glycoprotein	ß-fructofuranosidase	Nakamura *et al.* (1988)
Tobacco crown gall cells	Cell wall	63	63	4.7	0.6	Glycoprotein	–	Weil & Rausch (1990)
Tomato fruit	Cell wall	~75	–	4.5	3.45	Both glycoproteins	Both	Nakagawa *et al.*
(*Lycopersicon esculentum*)	Cell wall	~75	–	4.5	4.76		ß-fructofuranosidases	(1974)
Tomato fruit	Soluble	50	52	4.8	4.35	Glycoprotein monomer	ß-fructofuranosidase	Konno *et al.* (1993)
Wheat coleoptiles	Soluble	158	53	5.5	3.5	Glycoprotein	ß-fructofuranosidase	Krishnan *et al.*
(*Triticum aestivum*)	Cell wall	–	–	4.5	1.7	–	–	(1985)
Wheat coleoptiles	Soluble	50	50	5.0	1.0	Glycoprotein	ß-fructofuranosidase	Walker & Pollock (1993)

(Klann *et al.*, 1992; Elliott *et al.*, 1993). A cDNA for the mRNA encoding the heterodimeric form of soluble intracellular acid invertase identified in etiolated seedlings of mung bean, has also been characterised (Arai *et al.*, 1992). Several cDNA clones encoding potato invertases have been identified: 1) a clone isolated from a potato leaf cDNA library and thought to be an apoplastic invertase based on its similarity to carrot cell wall invertase (Hedley *et al.*, 1993); 2) a related gene, with 53% nucleotide identity to the apoplastic gene and considered to be a vacuolar invertase (U. Sonnewald, communicated by H. V. Davies); and 3) another apoplastic invertase which differs from the other clone but is isolated from the same cDNA library (Hedley *et al.*, 1994). Comparing the entire polypeptide sequence of each of the cloned enzymes it was shown that plant invertases may be divided into distinct apoplastic and vacuolar sub-families on a sequence basis alone (Hedley *et al.*, 1994). This is the first direct molecular evidence confirming the separate identity of these two different classes of invertase enzymes. The isolation of these invertase clones will now allow the production of transgenic plants to explore the physiological functions of these various enzymes.

Acid invertases do not appear to be highly regulated although end product inhibition by glucose and fructose has been reported for several of the purified enzymes, e.g. fructose inhibition (Salzer and Hager, 1993; Walker and Pollock, 1993); glucose and fructose inhibition (Isla *et al.*, 1991; Burch *et al.*, 1992). However, a naturally occurring acid invertase inhibitor has been detected in a number of plant tissues including sugar beet roots (Burakhanova *et al.*, 1987), potato tubers (Schwimmer *et al.*, 1961; Pressey, 1966; Anderson and Ewing, 1978; Bracho and Whitaker, 1990a, b; Isla *et al.*, 1992), red beet, sugar beet and sweet potato roots (Pressey, 1968), maize endosperm (Jaynes and Nelson, 1971) and flower petals (Winkenbach and Matile, 1970; Halaba and Rudnicki, 1988, 1989). The first suggestion of the presence of an invertase

inhibitor in plant tissues resulted from the kinetic studies of Schwimmer *et al.*, (1961) in which the rate of the reaction did not increase linearly with the volume of crude invertase extract from potato tubers. Ironically, further tests carried out on the potato tuber invertase inhibitor (Pressey, 1966; Ewing, 1971; Ewing and McAdoo, 1971) showed that it formed an essentially undissociable complex with the enzyme and therefore no detectable effect on the linearity of the reaction rate should be observed. The potato tuber invertase inhibitor is the only inhibitor reported to have been partially purified and characterised and its discovery has provided the impetus not only to investigate whether it is involved in invertase regulation *in vivo*, but also to identify other possible plant invertase inhibitors and their function. Pressey (1966, 1967) showed that the inhibitor is a protein with a molecular weight of approximately 17,000 and it acts as a noncompetitive inhibitor having greatest inhibitory effect at pH 4.5. Additionally, the slow inhibition with mercaptoethanol was thought to indicate that a disulphide bridge was necessary for activity. The amino acid composition of the inhibitor protein was determined and one cystine residue is present per 17,000 molecular weight. The discovery by Pressey (1966) that highest activity of crude invertase extracts is obtained after vigorous "foaming" led him to suggest that the inhibitor can be surface denatured without inactivation of invertase. It is possible, in the light of the finding that purified inhibitor is inactivated by foaming (Ewing *et al.*, 1977), that the denaturation of the inhibitor protein results from the destruction of the disulphide bridge which may be essential for invertase binding. The effect of the potato invertase inhibitor was tested on invertases isolated from various plants and shown to inhibit all but three, birch, tulip and sunflower, and it also had no effect on yeast or *Neurospora* invertases (Pressey, 1967). In addition, invertase inhibitors were purified from red beet, sugar beet and sweet potato roots and found to be small molecular weight proteins (18,000 to 23,000) that inhibited potato tuber invertase and other plant invertases (Pressey, 1968). More recent purification

and characterisation of the potato tuber invertase inhibitor (Bracho and Whitaker, 1990a, b) confirmed both the size of the inhibitor protein and many of the binding characteristics of the inhibitor/enzyme complex. However, despite all the available evidence, the possibility that it may be an artifact of enzyme extraction has often been raised to counter its effect *in situ*. The first evidence in support of its physiological role in potato tubers came from studies of sugar accumulation in cold-stored potatoes (Pressey and Shaw, 1966; Pressey, 1969). These showed that 1) freshly harvested tubers contain low levels of total invertase and high levels of invertase inhibitor; 2) invertase activity increases when inhibitor levels decrease; and 3) high reducing sugar content is always associated with low inhibitor levels. Further evidence came from studies of potato tuber slices (Ewing *et al.*, 1977) used as experimental material as it had been shown previously that invertase activity increased in washed disks of red beet, carrot and potato (Vaughan and Macdonald, 1967a, b). Ewing and coworkers (1977) found that the invertase activity estimated by immersing thin tuber slices directly into the sucrose assay medium corresponds to the activity of extracts from the same tissues which contain inhibited enzyme, not to that of extracts from which the inhibitor has been selectively removed. It was also shown that the inhibitor disappears from slices after 2 days and that its disappearance is blocked by the use of inhibitors of RNA or protein synthesis, actinomycin D and cycloheximide (Ewing *et al.*, 1977). However, in direct opposition in support of an *in vivo* role for the inhibitor in potato tubers were the results of Isla *et al.*, (1992) who found that, while acid invertase is in the vacuole, the proteinaceous inhibitor could only be extracted from the cell wall fraction. This uncertainty of the physiological role of the inhibitor in potato tubers can only be solved by direct immunolocalisation experiments using labelled antibodies raised against the purified protein.

In the other plants investigated, the inhibitor is envisaged as having an important role in the regulation of acid invertase activity. The activity of the sugar beet inhibitor increases proportionally with root growth so that in very young roots acid invertase activity is high but rapidly decreases as the roots start to store sucrose (Burakhanova *et al.*, 1987). Increased invertase activity was obtained in washed sugar beet slices and it was shown that this activation was associated with a decrease in inhibitor activity when the degree of inhibition of the protein fractions containing the inhibitor from sugar beet slices before and after activation were compared (Burakhanova *et al.*, 1987). Thus the endogenous sugar beet inhibitor is thought to regulate invertase activity, allowing sucrose to accumulate in the developing tap roots.

The senescing flower petals of *Ipomoea purpurea* - "morning glory", carnation, alstroemeria, dahlia, gladiolus, petunia and rose were all shown to contain an invertase inhibitor (Winkenbach and Matile, 1970; Halaba and Rudnicki, 1988, 1989). The carnation petal inhibitor was shown to be a low molecular weight protein (18,200) with similar properties to the potato tuber, red beet, sweet potato and sugar beet invertase inhibitors (Halaba and Rudnicki, 1989). The synthesis of the invertase inhibitor was blocked in both *Ipomoea* and carnation when petals were treated with cycloheximide (Winkenbach and Matile, 1970; Halaba and Rudnicki, 1988). The physiological significance was investigated by following the movement of ^{14}C-sucrose supplied to the petals (Halaba and Rudnicki, 1988). In untreated flowers, ^{14}C-sucrose is transported from the senescing petals to both the ovary and receptacle but after cycloheximide treatment only about a tenth of the labelled sucrose was detected in these flower parts. The later discovery of the presence of the inhibitor in wilting petals of various flowering species led Halaba and Rudnicki (1989) to suggest that the synthesis of an invertase inhibitor after flower pollination is a general phenomenon to allow for sucrose transfer from the wilting petals to

neighbouring organs. The involvement of the inhibitor in regulating acid invertase levels in plants in general, however, is still controversial and requires further investigation.

1.3.1.2 Alkaline invertases

Although in comparison with acid invertase not nearly so much is known about alkaline invertase, reports indicate that it is present in many plant tissues but is inclined to be more active in mature organs (for example mature orange leaves - Schaffer, 1986). It is thought to be a cytosolic enzyme (Ricardo and ap Rees, 1970) although no direct evidence is available as yet. To date, only one report on alkaline invertase purified to homogeneity is available although other workers describe partial purification of the enzyme (see Table 1.2). Conclusive evidence shows that alkaline invertases, unlike acid invertases, are not glycoproteins (Chen and Black, 1992; Stommel and Simon, 1990). All reported enzymes are ß-fructofuranosidases with an even higher degree of specificity for sucrose than the acid invertases. While some may cleave raffinose, the rate was generally less than 6% of that with sucrose as substrate (Matsushita and Uritani, 1974; Morrell and Copeland, 1984). The affinity of alkaline invertase for sucrose is generally lower than that of acid invertase and K_m values range from approximately 10 to 40 mM (Table 1.2).

The molecular weights of native alkaline invertase proteins range from 60,000 to 280,000 but only in the case of soybean hypocotyls has the subunit molecular weight been determined (Chen and Black, 1992). This estimate of 58,000 from denaturing SDS-PAGE indicates that the soybean alkaline invertase is a homotetramer.

When antibodies were raised against both acid and alkaline soybean invertases it was shown that they were immunochemically distinct from each other (Chen and Black, 1992). These results indicate that the two classes of enzymes are

TABLE 1.2 Properties of purified alkaline invertases

Source	Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	pH optimum	Km (sucrose) mM	Nature of protein	Specificity	Reference
Chicory (*Cichorium intybus*)	–	–	8.0	20.0	–	ß-fructofuranosidase	Kaur *et al.* (1992)
Orange leaves (*Citrus sinensis*)	60	–	6.8–7.2	20.0	–	ß-fructofuranosidase	Schaffer (1986)
Pea leaves (*Pisum sativum*)	–	–	7.3	42.0	–	ß-fructofuranosidase specific for sucrose	Storr & Hall (1992)
Satsuma Mandarin Fruit (*Citrus* 'Unshu Marcovitch')	200	–	7.2–7.7	35.7	–	ß-fructofuranosidase	Kato & Kubota (1978)
Soybean hypocotyl (*Glycine max*)	240	58	7.0–7.6	10.0	Not a glycoprotein homotetramer	ß-fructofuranosidase specific for sucrose	Chen & Black (1992)
Soybean nodules	–	–	7.6	10.0	–	ß-fructofuranosidase specific for sucrose	Morrell & Copeland (1984)
Sugar beet roots (*Beta vulgaris*) I	280	–	~8.0	33.3	–	ß-fructofuranosidases	Masuda *et al.*
II	280	–	~8.0	biphasic	–	specific for sucrose	(1987)
Sweet Potato (*Ipomoea batatas*)	–	–	7.6	8.3 at low [sucrose] 32 at high [sucrose]	–	ß-fructofuranosidase specific for sucrose	Matsushita & Uritani (1974)

encoded by separate genes. Information on the cloning of any genes encoding alkaline invertase is not, as yet, available although this would prove valuable in studies to assess the main physiological role of the enzyme in plant metabolism.

Alkaline invertase, like acid invertase, is inhibited by the end products of the reaction - glucose and fructose. Sweet potato alkaline invertase was inhibited by 2 mM glucose and glucose-6-P (12 and 20% inhibition, respectively) while the enzyme from soybean nodules was inhibited only 7% by 5 mM glucose. Fructose (15 mM) competitively inhibited the soybean nodule alkaline invertase by 50% and a K_i value for fructose of 11 mM was determined. No inhibition, however, was found with the metabolites - ATP, ADP, UDP, ADPglucose, UDPglucose, glucose-1-P, glucose-6-P and fructose-6-P, all at a concentration of 5 mM (Morell and Copeland, 1984). The enzyme does not appear to be highly regulated, although this may be due, in part, to a lack of knowledge of the operation of the enzyme *in planta*. All reported alkaline invertase enzymes are strongly inhibited by Tris with, for example, the nodule enzyme inhibited 50% with a concentration as low as 0.7 mM.

1.3.2 Sucrose synthase

Sucrose synthase (UDP-D-glucose:D-fructose 2-α-glucosyltransferase, EC 2.4.1.13) catalyses the reversible reaction shown below:

$$\mathbf{UDP^{3-} + SUCROSE + H^{+} \leftrightarrow UDP\text{-}GLUCOSE^{2-} + FRUCTOSE}$$

$\Delta G'$ for the reaction = -4.2 kJ

The reaction is shown with uridine diphosphate as the nucleoside although the reaction may occur with adenosine-, guanosine-, thymidine-, cytidine-, or inosine-diphosphates but generally at a much slower rate, if at all.

The enzyme is ubiquitous in higher plants and is generally considered to be most active in non-photosynthetic tissues (Delmer, 1972a). The expression of the sucrose synthase gene in potato tubers was shown to be at least ten times higher than in photosynthetically active tissues analysed (Salanoubat and Belliard, 1987). Sucrose synthase was initially considered to be the dominant pathway for sucrose synthesis in plants and therefore the enzyme was named UDPG-fructose transglycosylase (Cardini *et al.*, 1955). Later studies showed that the enzyme catalyses the cleavage of sucrose more effectively than its synthesis (Pressey, 1969a; Nomura and Akazawa, 1973). Values reported for the theoretical equilibrium constant (K_{eq}) in the direction of sucrose degradation [UDP-glucose] [fructose] / [UDP] [sucrose] are 0.15 for mung bean sucrose synthase (Delmer, 1972a) and 0.39 for soybean nodule sucrose synthase (Morell and Copeland, 1985). In the synthesis direction values for K_{eq} are between 1.3 and 2.0 (Avigad, 1982). Recent estimates of the mass-action ratio from measurements of the sucrose, fructose, UDP-glucose and UDP content in potato tubers and *Ricinus* cotyledons of 0.42 and 0.23 respectively (similar to the K_{eq} values of 0.15 and 0.39 cited above) confirm that sucrose synthase catalyses a near-equilibrium and readily reversible reaction *in vivo* (Geigenberger and Stitt, 1993).

The enzyme was found to be extravacuolar and hence considered cytosolic in Jerusalem artichoke (Keller *et al.*, 1988) and a cytosolic location was further confirmed by immunogold labelling studies in soybean root nodules (Gordon *et al.*, 1992). In general, the molecular weight of the native protein ranges from 360,000 to 400,000 with identical subunits ranging from 87,000 to 100,000 (Table 1.3). While the native protein is usually present as a homotetramer, several oligomeric forms have been detected (Su and Preiss, 1978; Sakola and Lobov, 1988). Optimum pH values for sucrose cleavage reported in Table 1.3 are on average ≅ 6.6 which is considerably lower than pH values of

Table 1.3 Properties of purified sucrose synthases

Plant source		Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	Substrate	Km (mM)	pH optimum	Reference
Jerusalem Artichoke	A	–	–	Sucrose	56	–	Wolosuik & Pontis
(*Helianthus tuberosus*)	B	–	–	Sucrose	200	–	(1971)
Bamboo		280	–	Sucrose	100	5.8–6.8	Su *et al.*
(*Leleba oldhami*)				UDP	2		(1977)
Mung bean		375	94	Sucrose	17	–	Delmer
(*Phaseolus aureus*)			(homotetramer)	UDP	0.19		(1972)
Sugar beet	I	400	95	–	–	–	Sakalo & Lobov
(*Beta vulgaris*)	II	550	(tetra–, hexa– & octamers	–	–	–	(1988)
	III	750	with tetramer the main form)				
Sugar beet		–	–	UDP	0.7	7.0–8.0	Silvius & Snyder (1979)
Maize		365	88	Sucrose	30–40	6.0	Su & Preiss
(*Zea mays*)		550	(tetramer & hexamer)	UDP	0.04–0.06		(1978)
Peach		360	87	Sucrose	62.5	7.0	Moriguchi & Yamaki
(*Prunus persica*)			(homotetramer)	UDP	0.08		(1988)

Table 1.3 Properties of purified sucrose synthases contd.

Plant source	Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	Substrate	Km (mM)	pH optimum	Reference
Pinyon (*Pinus edulis*)	350	90 (homotetramer)	Sucrose UDP	149 0.17	–	Hammer & Murphy (1993)
Potato (*Solanum tuberosum*)	290	–	Sucrose UDP	130 1.7	6.6	Pressey (1969)
Sweet Potato (*Ipomea batatas*)	540	–	Sucrose UDP	31 –	5.5	Murata (1971a & 1971b)
Rice (*Oryza sativa*)	400	100 (homotetramer)	Sucrose UDP	290 0.8	6.0	Nomura & Akazawa (1973)
Rice	400	90 (homotetramer)	–	–	–	Juang 1986
Soybean nodules (*Glycine max*)	400	90 (homotetramer)	Sucrose UDP	31.3 0.005	6.0	Morell & Copeland (1985)
Soybean nodules	–	90	–	–	–	Thummler & Verma (1987)
Soybean nodules	–	-90	–	–	–	Gordon *et al.* (1992)

Table 1.3 Properties of purified sucrose synthases contd.

Plant source	Approx. MW x 10^{-3}	Subunit MW x 10^{-3}	Substrate	Km (mM)	pH optimum	Reference
Tapioca (*Manihot utilissima*)	–	–	Sucrose UDP	10 6.6	7.5	Shukla & Sanwal 1971
Tomato (*Lycopersicon chmielewskii*)	380	89	Sucrose UDP	53 0.0189	6.2–7.3	Sun *et al.* (1992)
Wheat leaves (*Triticum aestivum*)	380	–	Sucrose UDP	40–60 1.1–2.0	7.3	Larsen *et al.* (1985)
Wheat germ	370	–	Sucrose UDP	40–60 1.1–2.0	7.3	Larsen *et al.* (1985)

approximately 8.5-8.8 given for the enzyme when catalysing sucrose synthesis (Moriguchi and Yamaki, 1988; Su and Preiss, 1978).

In general only UDP of the nucleoside diphosphates is active as the substrate for the reaction, with (in some cases) ADP giving activity but less than 20% of that with UDP (Moriguchi and Yamaki, 1988, Su and Preiss, 1978). The enzyme was characterised by a high affinity for its substrate UDP with, in general, a K_m value of less than 1 mM but a very low affinity for sucrose - K_m values range from 10 to 290 mM (Table 1.3).

While the earliest biochemical studies on sucrose synthase are on *Vicia faba* (de Fekete, 1969) and mung bean (Delmer, 1972a, 1972b), the most detailed molecular and genetic investigations have been carried out using maize. Maize sucrose synthase is a tetrameric protein composed of subunits encoded by the *Sh* and *Sus* genes (Chourey and Nelson, 1976; Su and Preiss, 1978; Chourey 1981). Initially two isozymes of sucrose synthase , SS1 and SS2 encoded by the *Sh* and *Sus* genes respectively, were identified in maize (Chourey and Nelson, 1976; Chourey, 1981). In the *shrunken* (*sh*) mutant of maize, the *Sh* locus on chromosome nine fails to encode sucrose synthase activity as it does in the normal wild-type maize and only the SS2 protein is expressed (Chourey and Nelson, 1978). Biochemical investigations of these mutant maize endosperms, confirmed that sucrose synthase activity was less than 10% of normal and it was shown that in one of the mutants sucrose cleavage by sucrose synthase was more affected than sucrose synthesis (Chourey and Nelson, 1978). At the molecular level, the *Sh* gene has been cloned and analysed (Geiser *et al.*, 1982; Werr *et al.*, 1985) and using *Sh1* as a probe, McCarty *et al.*, (1986) cloned the *Sus1* gene which was mapped to chromosome 9 approximately 32 map units from *Sh1*. A total of five sucrose synthase isozymes have now been identified in maize plants (Rowland and Chourey, 1990). The *Sh* and *Sus*-encoded protein subunits, SS1 and SS2, polymerize randomly in seedling roots and shoots to

produce the five different sucrose synthase isozymes, two homotetramers (S1S1S1S1 and S2S2S2S2) and three heterotetramers (S1S1S1S2, S1S1S2S2 and S1S2S2S2) (Chourey, 1988; Rowland and Chourey, 1990). Developing endosperm cells contain the two homotetramers, whereas in young roots and shoots, the three heterotetramers are also present (Chourey *et al.*, 1986). Similarly, five isozymes have been detected in sorghum but, in contrast to maize, both sucrose synthase genes are expressed simultaneously in the endosperm, leading to the additional presence of the heterotetramers in this tissue (Chourey *et al.*, 1991).

A cDNA encoding sucrose synthase in potato has been cloned and sequenced and a comparison made with the maize sucrose synthase cDNA (Salanoubat and Belliard, 1987; Werr *et al.*, 1985). The nucleotide sequence and deduced amino acid sequence for the two clones show 70% and 75% identity, respectively. Three amino acid regions are about 90% homologous and two are considered important regions for enzyme function due to strong constraints at the amino acid sequence level.

A sucrose synthase cDNA was also cloned and sequenced from mung bean seedlings (Arai *et al.*, 1992a). The extent of the similarity between mung bean sucrose synthase and the enzymes from maize and potato was 71% and 72%, respectively, at the nucleotide level, and 76% and 81% at the deduced amino acid level. The deduced amino acid sequence from a cDNA sucrose synthase clone from *Vicia faba* cotyledons (Heim *et al.*, 1993) was closely related to both the mung-bean and potato sucrose synthase (95% homology) and to a lesser extent the maize sucrose synthase (approx. 75% homology). These molecular studies indicate a high degree of homology between the sucrose synthase genes from different plant species.

Sucrose synthase appears to be highly regulated both at the level of fine and coarse control. Discussion of possible effectors of sucrose synthase activity will be limited to those involved in sucrose cleavage only. The products of the reaction, UDP-glucose and fructose, both inhibit the enzyme. Pinyon (*Pinus edulis*) sucrose synthase was non-competitively inhibited by UDP-glucose (K_i = 0.011 mM) when tested over a range of UDP concentrations (Hammer and Murphy, 1993); whereas sucrose synthase from Jerusalem artichoke (*Helianthus tuberosus*) was competitively inhibited by UDP-glucose (Wolosuik and Pontis, 1974). Fructose acts as a competitive inhibitor with respect to sucrose but as an uncompetitive inhibitor with respect to UDP (Wolosuik and Pontis, 1974). Fructose and glucose both uncompetitively inhibited maize endosperm sucrose synthase by binding to the enzyme-UDP complex (Doehlert, 1987). Soybean nodule sucrose synthase was inhibited 25% and 50% by glucose concentrations of 2 and 5 mM, respectively (Morell and Copeland, 1985). It is of physiological significance that the enzyme shows inhibition to the products of sucrose hydrolysis by invertase. By contrast, none of the following metabolites (at a final concentration of 5 mM) had any effect on soybean nodule sucrose synthase: galactose, mannose, maltose, raffinose, glucose-1-P, glucose-6-P, fructose-6-P, fructose-1,6-P_2, 3-P-glycerate, P-enolpyruvate, ethanol, succinate, 2-oxoglutarate, glutamate, glutamine, NAD, AMP, and PPi (Morell and Copeland, 1985).

A cDNA sequence encoding a nodule-specific protein, nodulin-100, from soybean nodules was identified as a subunit of sucrose synthase (Thummler and Verma, 1987). Nodule sucrose synthase protein was shown to dissociate into its monomers in the presence of heme and it has been suggested that the availability of free heme may regulate the activity of the nodule sucrose synthase.

At the gene level, sucrose synthase expression is not only spatially controlled within the plant (Heinlein and Starlinger, 1989; Chourey *et al.*, 1991) but is also regulated by tissue carbohydrate levels (Koch *et al.*, 1992; Salanoubat and Belliard, 1989; Sowokinos and Varns, 1992) and by anaerobic conditions (Salanoubat and Belliard, 1989; Taliercio and Chourey, 1989; Chourey *et al.*, 1991). The results of Koch and coworkers (1992) demonstrate for the first time that there is differential regulation of the sucrose synthase isozyme genes within a plant organ. Metabolisable sugars had both positive and negative effects on the sucrose synthase gene system with *Sh1* transcript levels decreasing in the presence of 4% glucose while *Sus1* levels increased. Sucrose was shown to down regulate the *Sh1* message to an equal or greater extent than did glucose but had little effect on *Sus1*. Fructose affected transcript levels of both *Sh1* and *Sus1* in a similar manner to glucose although the negative and positive changes in the expression of the two genes with fructose was not so great as with glucose.

In potato leaf petioles supplied with a range of sucrose solutions (50, 100, 150, 200 and 250 mM), an almost linear increase in sucrose synthase transcript levels was observed by Northern blotting indicating that sucrose is involved in the coarse control of the enzyme (Salanoubat and Belliard, 1989). Additional evidence on sucrose induction of sucrose synthase was demonstrated with cultured potato cells (Sowokinos and Varns, 1992). Anaerobic incubation conditions had a stimulating effect on the level of sucrose synthase mRNA in potato tubers (Salanoubat and Belliard, 1989) and on the level of both sucrose synthase genes in sorghum (Chourey *et al.*, 1991).

The importance of a high degree of metabolic regulation will be evident when considering the involvement of sucrose synthase activity in carbohydrate metabolism.

1.4 The roles of the sucrose-cleaving enzymes in carbohydrate metabolism

1.4.1 Cell wall acid invertase

Evidence presented in section 1.3.1.1 clearly identifies a distinction between cell wall and vacuolar acid invertases based on their properties and structure. Studies on a number of the cell wall invertases, whose properties are listed in Table 1.1, have helped to elucidate the function of these enzymes *in planta*. In maize kernels, 60 to 80% of acid invertase activity was found to result from insoluble acid invertase which was apparently bound to cell walls by ionic interactions (Doehlert and Felker, 1987). In addition, most of the activity was restricted to the pedicel tissues and the basal region of the endosperm (Felker, 1986), suggesting a role for the cell wall invertase in the unloading of sucrose from the phloem and subsequent uptake into the basal endosperm. This possibility has been further investigated using the maize invertase-deficient *miniature-1* seed mutation which has abnormal pedicel and endosperm development (Miller and Chourey, 1992). Biochemical and histochemical analysis of the mutant maize kernels showed that extremely low (<0.5% of the wild type) to undetectable levels of both soluble and wall-bound invertase activities were present. The data is thought to provide strong evidence that invertase in basal endosperm cells is required for the cleavage of sucrose to allow its subsequent mobilisation to the upper parts of the maize endosperm. In its absence, the normal movement of photoassimilate between the pedicel and endosperm is disturbed resulting in cell degeneration and the characteristic small seed size of the *miniature-1* mutants.

Evidence for the involvement of cell wall acid invertase in the cleavage of apoplastic sucrose prior to uptake into the parenchyma cells in sugar beet taproots, based on protoplast studies, is provided by the preferential uptake of glucose (Fieuw and Willenbrink, 1990). The glucose influx, mediated by a

H^+/glucose symport mechanism, has an apparent K_m of 1.4 mM in contrast to 7.8 and 18.6 mM for fructose and sucrose, respectively. They also suggested that the *in vitro* activity of cell wall acid invertase (5120 nmol gFW^{-1} h^{-1}), would far exceed the rate of sucrose hydrolysis that may limit the hexose uptake system in the sugar beet taproots. In further support of these results is the work reported on cell wall bound acid invertase of mature red beet roots (Getz, 1991). Cell wall acid invertase activity is highest in the youngest peripheral tissue regions of the mature red beet and lowest within the storage parenchyma. It is thought, therefore, that cell wall acid invertase is important for apoplastic sucrose cleavage for the maintenance of a high sucrose concentration gradient between phloem symplast and apoplast.

An essential function for cell wall acid invertase has been identified in roots of conifer trees which form a symbiotic relationship with ectomycorrhiza-forming fungi (Salzer and Hager, 1993). The ectomycorrhizal fungi can only grow when supplied with glucose and fructose and not with sucrose as the sole carbohydrate source. Salzer and Hager (1993) purified cell wall acid invertase from cultured spruce cells and showed that two wall bound invertase isoforms were present and both were inhibited by fructose. Thus, *in vivo*, the wall bound invertases in spruce will hydrolyse sucrose to produce glucose and fructose required by the fungi in exchange for mineral nutrients. The competitive inhibition by fructose of both cell wall invertase isoforms may be involved in regulating the flow of carbohydrates from the host to the fungus.

These few examples are sufficient to illustrate the essential and important functions of cell wall acid invertases in carbon transport and uptake.

1.4.2 Soluble acid invertase

As already discussed (section **1.3.1.1**) soluble acid invertases are generally considered to be located within or associated with the plant vacuole and no

evidence has been provided to the contrary. Examination of available information on acid invertase activity in a number of different plant species leads to the identification of several possible roles for the enzyme in cellular carbohydrate metabolism. It is generally known that high acid invertase activity is associated with tissues at a rapid stage of growth and cell division (Morris and Arthur, 1984). In oat seedlings the distribution of invertase activity is highest in the area near the apical region, indicating that the enzyme is associated with rapidly elongating cells (Pressey and Avants, 1980). Acid invertase activity was also found to be highest in the most rapidly elongating stem internodes and expanding leaf tissue of *Phaseolus vulgaris* (Morris and Arthur, 1985, 1984a). In sugar-cane a highly significant correlation ($r = 0.88$) was obtained between internode elongation and vacuolar acid invertase activity (Gayler and Glasziou, 1972). The cloning and sequencing of soluble acid invertase from mung bean seedlings led to the investigation of the expression of the gene in conjunction with activity measurements (Arai *et al.*, 1992). The mRNA for acid invertase was most abundant in hypocotyls that were elongating rapidly in the dark in comparison with those from the slower-growing seedlings kept in the light.

Thus high acid invertase activity is associated with plant tissues where there is a rapid requirement for hexoses to: a) supply substrate for increased respiration to release the energy required for growth; b) supply substrates for synthesis of new cellular material; and c) to supply osmotically-active substances to help maintain the osmotic pressure of the cell sap during rapid water uptake (Hellebust and Forward, 1962).

As evidence suggests that both acid invertase and sucrose are compartmentalised within the vacuole (Isla *et al.*, 1992), the loss of soluble acid invertase activity is a general prerequisite for sucrose-storing organs. Thus in developing muskmelon fruit a highly significant negative correlation exists

between sucrose content and acid invertase activity (Lingle and Dunlap, 1987). Activity of a soluble acid invertase is very high in young muskmelon fruits, at which stage of development it is considered important in hydrolysing sucrose to glucose and fructose to maintain the osmotic pressure of the cell (McCollum *et al.*, 1988). High osmotic pressure is required for the accumulation of water during fruit growth. Again it was shown that only when acid invertase activity had declined to a low level during the later stages of fruit development was it possible for sucrose to start accumulating in the fruit (McCollum *et al.*, 1988).

In contrast to the example given above, sink organs that do not store sucrose may retain their soluble invertase activity. An interesting example of such an organ is the tomato (*Lycopersicon esculentum*) fruit which accumulates glucose and fructose. However, a wild relative of tomato (*Lycopersicon chmielewskii*) accumulates sucrose and evidence from genetic analysis indicates that a single recessive gene controls sucrose accumulation in these fruits (Yelle *et al.*, 1991). In order to characterise the biochemical and molecular aspects of the trait of sucrose accumulation, progeny resulting from crosses of both tomato species were examined (Klann *et al.*, 1993). The results demonstrated that acid invertase was the only enzyme that differed significantly between the sucrose and hexose accumulating fruits. The sucrose accumulating families showed the lowest invertase activity throughout fruit development, while the hexose accumulating families showed moderate activity up to 6 weeks after anthesis and then increased over 10-fold during fruit ripening. The levels of mRNA encoding acid invertase were undetectable in the sucrose accumulating fruit, leading to the suggestion that the *L. chmielewskii* invertase gene is transcriptionally silent in fruit.

The physiological importance of acid invertase activity during cherry fruit development was shown when an active form of soluble acid invertase was purified from cherry mesocarp and its properties and activity examined in the

ripening fruits (Krishnan and Pueppke, 1990). While sucrose is the main sugar translocated to the fruits, it is absent during fruit growth and development, during which time there is a steady increase in glucose and fructose. Acid invertase activity and acid invertase protein (detected on SDS-PAGE) both increased during the rapid phase of cherry fruit growth, accounting for the increase in the hexoses.

These results clearly demonstrate the importance of acid invertase in the regulation of sugar accumulation in developing sink organs. The commercial significance of this function of acid invertase is not only reflected in fruit quality during development, but may also contribute to the post-harvest quality of a number of different crop species. One such example that has been studied in detail is the potato tuber due to its importance to the processing industry. During the processing of crisps or French fries, browning of the fried product may occur due to a complex reaction (Maillard reaction) between the reducing sugars, glucose and fructose and the α-amino groups of nitrogenous compounds. When potato tubers are stored at low temperatures to prevent sprouting, reducing sugars accumulate resulting in an increase in the extent of browning. This accumulation of reducing sugars in tubers exposed to low temperatures occurs with a concomitant increase in soluble acid invertase activity (Pressey and Shaw, 1966; Richardson *et al.*, 1990). Recent results (Zrenner and Sonnewald, 1993) investigating transgenic potato plants in which the level of vacuolar acid invertase was reduced by 90% have confirmed the importance of this enzyme in controlling tuber hexose levels. During development, the tubers appeared normal with similar starch levels to those from control plants but when cold-stored, the hexose level was 60% lower than in control tubers although total sugars remained the same.

While measurements of maximum catalytic activity of acid invertases are often difficult due to the presence of inhibitors, the consistent results from a number

of species with the additional corroboration of molecular studies is now yielding an insight into the physiological importance of vacuolar acid invertase.

1.4.3 Alkaline invertase

In contrast to acid invertase, alkaline invertase is thought to be cytosolic. Sucrose transported to the cell may either pass directly into the vacuole for storage or cleavage by acid invertase, or may be cleaved directly in the cytosol by either alkaline invertase or sucrose synthase. Alkaline invertase has never been shown to reach the very high levels often found for acid invertase activity (ap Rees, 1974). A definitive role in cellular carbohydrate metabolism has not yet been assigned to alkaline invertase. In mature tuberous roots of sugar-beet, wild carrots, carrot cvs Nantes and Blanche, radish and turnip a positive correlation was observed between alkaline invertase activity of each root and its reducing sugar content (Ricardo, 1974). In addition, measurements of both acid and alkaline invertases in the same plant tissues, for example, pea roots (Lynne and ap Rees, 1971) and pea pods (Estruch and Beltrán, 1991) indicate an inverse relationship between the two enzymes. It may be that the presence of alkaline invertase allows cells that store sucrose within their vacuoles to retain a capacity for cleavage of sufficient sucrose to meet respiratory and metabolic demands for hexoses (ap Rees, 1974). The capacity of a plant to produce two different invertases that are spatially separated within the cell may allow the plant cell to regulate sucrose storage independently from sucrose breakdown.

In plant cells many enzyme activities are duplicated and it has been suggested that cleavage of sucrose by alkaline invertase offers an alternative route to breakdown via sucrose synthase (Black *et al.*, 1987). This possibility was investigated by comparing the activities of the enzymes involved in the two pathways from the endosperms of both wild type and *Sh1* shrunken mutant of

maize (Dancer and ap Rees, 1989). The sucrose synthase pathway dominated in the wild type maize but in the mutant, alkaline invertase activity increased to compensate for the deficiency in sucrose synthase, showing that either pathway may operate if conditions required.

In many plant tissues, alkaline invertase activity has been characterised by small and slow changes in specific activity associated with development or in response to environmental changes (Sung *et al.*, 1989). The possibility of differentiating between enzymes on the basis of their response in tissues subject to changing conditions led to the classification by Black and coworkers (1987) of adaptive and maintenance enzymes. While alkaline invertase would be identified as a maintenance enzyme, sucrose synthase shows the properties of an adaptive enzyme. Until futher information is obtained from characterisation studies of purified alkaline invertases and of the gene/s encoding the enzyme, only the general role of a maintenance-type enzyme can be assigned to alkaline invertase.

1.4.4 Sucrose synthase

The presence within plant cells of two cytosolic sucrose-cleaving enzymes - alkaline invertase and sucrose synthase - has caused considerable research into the differences between the pathways and when and why one pathway is preferred to the other. To help in discussing the results of this work, the two pathways are represented in Figure 1.3. Comparison shows that while the invertase pathway requires the input of four ATP to metabolise one sucrose molecule to the level of triose-P, the sucrose synthase pathway requires only three ATP. The sucrose synthase pathway is more energetically efficient as the energy contained in the glycosidic bond in the sucrose molecule is preserved (Huber and Akazawa, 1986). However, the sucrose synthase route requires an efficient cycling of uridylates from the NTP-dependent fructokinase and/or

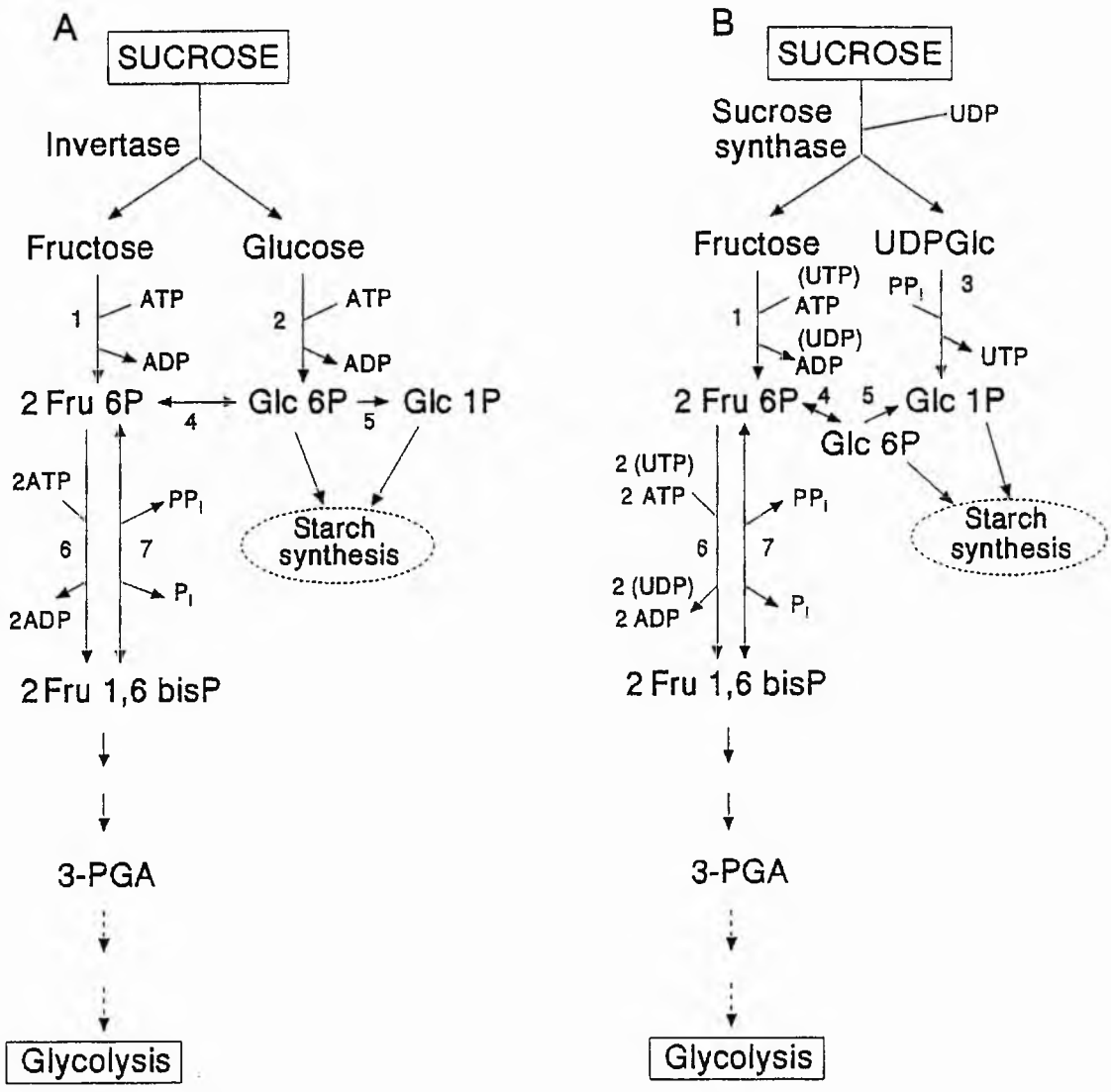


Fig. 1.3 Sucrose cleavage via A) Invertase pathway and B) Sucrose synthase pathway. Enzymes numbered are:- 1) Fructokinase, 2) Glucokinase, 3) UDP glucose pyrophosphorylase, 4) Phosphohexoisomerase, 5) Phosphoglucomutase, 6) (ATP) PFK, and 7) (PP_i) PFK

NTP-dependent PFK and of PP_i from the action of PP_i-dependent PFK in the reverse direction, to drive sucrose cleavage through UDP-glucose pyrophosphorylase (Xu *et al.*, 1989). As ATP is generally present within plant cells in greater amounts than UTP, the enzyme - nucleoside-5'-diphosphate kinase may be required to catalyse the transfer of the phosphoryl group of ATP to regenerate UTP (Raymond *et al.*, 1987). While direct recycling of UDP from the phosphorylation of fructose by fructokinase has been suggested (Huber and Akazawa, 1986; Xu *et al.*, 1989; ap Rees and Morrell, 1990), results on the nucleotide specificity of purified fructokinases from potato tubers (Gardner *et al.*, 1992; Renz and Stitt, 1993) do not support such a scheme.

Sucrose cleavage via sucrose synthase appears to be the preferred route in many rapidly developing starch-storing sink organs such as young potato tubers and lima bean seeds (Sung *et al.*, 1989), maize endosperm (Chourey and Nelson, 1976), and in cotyledons and hypocotyls of pinyon, *Pinus edulis* (Hammer and Murphy, 1993). In these organs, sucrose synthase activity was highest at the time of maximum growth and starch accumulation and responded to the level of imported sucrose (Sung *et al.*, 1989, 1990). Unlike alkaline invertase, this ability to adapt to changing levels of sucrose and to increase in activity in developing sink organs has led to the suggestion that the enzyme could be used as a biochemical determinant of the sink strength of an organ (Sung *et al.*, 1989). Of the sucrose-cleaving enzymes, only sucrose synthase activity followed similar patterns to growth in the several plants tested (Black, 1993). Sucrose synthase was also shown to be the dominant enzyme in metabolising imported sucrose in growing tomato fruit and in support of the above concept, the enzyme correlated positively with tomato fruit relative growth rate (RGR) and starch content (Wang *et al.*, 1993). While sucrose-cleaving enzymes have previously been considered important in determining sink strength by rapidly mobilising sucrose and thus increasing

the sucrose gradient and driving further import, the general consensus of opinion is that plant growth is of such high complexity that it is unlikely to be regulated by a single enzyme (see Farrar and following papers, 1993).

Direct evidence, however, of the importance of the sucrose synthase pathway in starch synthesis was first obtained from studies of the *shrunken* maize mutants (discussed in section 1.3.2). In these mutants, enzyme activity is <10% of the normal endosperm resulting in a reduction of the starch content to approximately 40% of the wild type, thus leading to the shrunken phenotype of the kernels (Chourey and Nelson, 1978). A recent study of transgenic potato plants in which the level of sucrose synthase activity in the tubers was reduced to approximately 5% of that found in wild type tubers further confirms the importance of the enzyme in regulation of sucrose import and conversion of sucrose to starch in developing sink organs (Zrenner and Sonnewald, 1993, pers. comm. to H. V. Davies). The level of starch in these transgenics was reduced by approximately 70% but the sucrose content apparently remained unchanged.

In the examples of developing sink organs mentioned above, sucrose synthase has been the dominant enzyme present, but in some tissues the activity of alkaline invertase may equal or exceed that of sucrose synthase. The presence of both enzymes showing similar activities has been demonstrated in sycamore cells (Huber and Akazawa, 1986), soybean nodules (Morell and Copeland, 1984, 1985), and sweet potato (Matsushita and Uritani, 1974; Murata 1971a, b). For these examples it is difficult to assess the relative activities of the two pathways. While it was suggested for sycamore cells that sucrose synthase (K_m=15 mM) may be more important than alkaline invertase (K_m=65 mM) when sucrose availability is limiting (Huber and Akazawa, 1986), the affinities of the enzymes from soybean and sweet potato for sucrose were the opposite to the sycamore enzymes with alkaline invertase having K_m values 8-10 mM and

sucrose synthase approximately 31 mM. Thus a general hypothesis that one pathway may operate at low sucrose concentrations and the other at high concentrations is not supported by the contrasting enzyme affinities.

Although the major role of sucrose synthase lies in the conversion of sucrose to starch (as shown above), UDP-glucose produced in the cleavage reaction is important for the biosynthesis of cell wall polysaccharides, glycoproteins and many other glycosides (Feingold and Avigad, 1980 and references therein).

In addition to the functions of sucrose synthase in providing precursors for starch and cell wall biosynthesis and for respiration, evidence suggesting its involvement in phloem metabolism is now being reported. While alkaline invertase and sucrose phosphate synthase showed highest activity in nonvascular sink structures of grapefruit, sucrose synthase was most active in extracts of transport tissues, where extensive phloem unloading and subsequent transfer are known to occur (Lowell *et al.*, 1989; Tomlinson *et al.*, 1991). Localization studies of sucrose synthase showing high levels of the enzyme in the vascular endodermis and cortical cells near to the vascular bundles in soybean root nodules (Gordon *et al.*, 1992) and in phloem companion cells in maize and citrus leaves (Nolte and Koch, 1993) indicate its involvement in sucrose transport. A sucrose synthase gene isolated from roots of *Arabidopsis thaliana* is expressed in the phloem tissue of leaves, and in roots (Martin *et al.*, 1993). The gene is inducible in the vascular leaf tissue by cold and anaerobic treatments and the results provide further evidence in support of a transport function for sucrose synthase. The phloem sieve tubes of *Ricinus communis* seedlings were shown to contain both sucrose synthase (operating close to equilibrium *in vivo*) and an operational glycolytic pathway, thus implicating its involvement in phloem metabolism (Geigenberger *et al.*, 1993). This equilibrium reaction catalysed by sucrose synthase is thought to provide a mechanism within the phloem of the castor-bean seedlings to: a) ensure that

sucrose cleavage is controlled in the presence of high sucrose concentrations, while b) maintaining the capacity to allow for rapid sucrose breakdown, whenever necessary, to provide energy for transport processes and UDP-glucose as a precursor for callose synthesis.

1.5 Aims of the work

While the importance of sucrose in plant metabolism has been discussed in this introduction, obvious gaps in our knowledge on the functions of the enzymes involved in its cleavage and entry into cell metabolism are apparent. The main purpose of this work, therefore, was to investigate the activity of each of the sucrose-cleaving enzymes in sink organs during development and maturation. In order to obtain a more general insight into the control of sucrose breakdown, three different types of developing storage organs were examined: 1) potato (*Solanum tuberosum*) tubers which are underground stems that store approximately 70% of their dry weight as starch; 2) field bean (*Vicia faba*) cotyledons which store almost equal amounts of both starch and protein (approximately 35% DW); and 3) sugar beet (*Beta vulgaris*) taproots which are predominantly sucrose-storing sinks (sucrose - 70% DW). To further this study it was necessary to purify the enzymes and obtain polyclonal antibodies for confirmation of enzyme activity measurements and characterisation of the purified enzymes. This was most important for alkaline invertase as, prior to the start of this work, it had not been purified to homogeneity or its properties fully characterised. As acid invertase was being purified concurrently at SCRI in a research project supported by ECSA Research Ltd and the EC under the Eclair programme, antibodies raised to the purified enzyme were available for use in this study.

CHAPTER 2

GENERAL MATERIAL AND METHODS

2.1 Reagents

The following enzymes listed below were obtained from either 1) - Boehringer Mannheim UK Ltd (Lewes, East Sussex, UK) or 2) - Sigma Chemical Co. Ltd (Poole, Dorset, UK). These enzymes were used in coupled enzyme assays and carbohydrate determinations.

	Enzyme	Source
1)	Phosphoglucomutase	rabbit muscle
	Glucose-6-phosphate dehydrogenase	NAD-utilising *Leuconostoc mesenteroides*
	Phosphoglucose isomerase	yeast
	Hexokinase/Glucose-6-P dehydrogenase	suspension from yeast
	ß-Fructosidase	yeast
2)	Amyloglucosidase	*Aspergillus niger*
	Uridine-5'-diphosphoglucose pyrophosphorylase	bakers yeast
	ß-Fructosidase	*Candida utilis*

All nucleoside di- and triphosphates and metabolites were obtained from Sigma with the exception of ADP which was from Boehringer Mannheim. The reagent for glucose determination was obtained from Technicon Bayer Diagnostics Manufacturing S.A. (Tournai, Belgium). This product was supplied in desiccated form and when reconstituted contained in one litre:-

Hexokinase (yeast)	≥ 1000 U (1U ≡ 1 μmole product min^{-1} at 37°C)
G-6-P dehydrogenase (*Leuconostoc mesenteroides*)	≥ 800 U
ATP	1.4 mM
NAD	0.8 mM
Mg^{2+}	10 mM
buffer	
stabilizer	

A 30% solution of Brij-35 (polyoxyethylene lauryl ether) used for the autoanalyser (Technicon model AA I) was also obtained from Technicon.

Sucrose used throughout was Aristar grade from BDH Merck Ltd (Poole, Dorset, UK) as it contained less than 50 ppm reducing sugars.

Most of the chromatographic media and columns used for enzyme purification were obtained from Pharmacia Biosystems Ltd (Biotechnology Division, Central Milton Keynes, UK).

All ^{14}C isotopes used were from ICN Biomedicals Ltd (High Wycombe, Bucks., UK) and the scintillation fluid used was Ecoscint-A (biodegradable) from National Diagnostics (Aylesbury, Bucks., UK). All other chemicals required were supplied by BDH, Sigma, or Bio-Rad Laboratories Ltd (Hemel Hempstead, Herts., UK). Liquid nitrogen was supplied by Cryoservice Ltd (Worcester, UK).

2.2 Enzyme extractions

During enzyme extractions, the composition of the extraction buffer varied with the type of plant tissue being used and details will be given in the

relevant chapters. Fresh or frozen plant tissue was ground to a fine powder in liquid nitrogen before adding 3-5 volumes ice-cold extraction buffer. After centrifugation (20,000g for 20 min at 4°C) samples were re-extracted to obtain high enzyme recovery values. Samples were desalted either by dialysis or gel filtration using Pharmacia PD-10 columns (Sephadex G-25 Medium).

2.3 Enzyme assays

2.3.1 Sucrose synthase

The method used for the assay of sucrose synthase in the cleavage direction was based on that initially used by Xu *et al.* (1986) but optimised for each of the tissues measured with respect to pH and concentration of substrates. The assay contained 0.1 M Tris-HCl (pH 7-7.5 depending on the resulting optimum pH required for the reaction mixture for the plant tissue), 5 mM DTT, 3 mM magnesium acetate, 2 mM sodium fluoride, 2 mM PPi (tetrasodium salt), 0.5 mM NAD, 10 µM glucose 1,6 diphosphate, 200 mM sucrose, 1 unit ml^{-1} UDP glucose pyrophosphorylase, 1 unit ml^{-1} glucose-6-P dehydrogenase and 2 units ml^{-1} phosphoglucomutase. The assay was started by the addition of UDP to a final concentration of 1 mM. The increase in absorbance at 340 nm produced by enzyme-coupled NADH production was measured using a Kontron (Uvikon-930) double beam spectrophotometer equipped with a multicell holder and thermostating fluid connection block (Kontron Instruments Ltd, Watford, Herts., UK). The assays were carried out at 30°C in a final total volume of 1 ml.

2.3.2 Acid invertase

Acid invertase activity was assayed by incubating an equal volume of extract with assay buffer (200 mM sodium acetate buffer pH 5.0 containing 250 mM sucrose and 20 mM EDTA) at 37°C for 1 hour. Boiled enzyme extracts were

treated in the same way and used as controls. After incubation, the reaction was terminated by boiling all samples for 5 minutes. The amount of glucose produced during the reaction was determined on an autoanalyser (details given in section **2.4.1.**) and used to calculate enzyme activity.

2.3.3 Alkaline invertase

Alkaline invertase was assayed as for acid invertase activity but sodium acetate was replaced by 200 mM sodium phosphate (pH 7.5) unless otherwise stated.

2.4 Carbohydrate Determination

2.4.1 Sucrose, Glucose and Fructose

Plant samples requiring sugar measurements were weighed, diced and quickly frozen in liquid nitrogen and kept at -80°C prior to freeze-drying. The samples were transferred quickly to a freeze-drier (Birchover Instruments Ltd, Letchworth, Herts., UK) to prevent any thawing prior to the vacuum forming in the drying chamber. When samples were completely dry (usually after 2-3 days), they were re-weighed and ground to a fine powder. Depending on the size of the sample, grinding was either by hand with a mortar and pestle or using a micro-hammer mill (Glen Creston, Stanmore, Berks., UK, sieve size 1 mm). Sugars were extracted from weighed samples of the finely ground powder in 80% ethanol at 55°C for 3 hours. Enzymic determination of glucose, fructose and sucrose based on the method described by Bergmeyer *et al.* (1974) and Bernt and Bergmeyer, (1974) was carried out using an autoanalyser. Glucose was assayed with the glucose reagent (described in **2.1**) which involved incubation of sample at 37°C with excess amounts of ATP, NAD, hexokinase and glucose-6-P dehydrogenase to allow the conversion of glucose

+ ATP ↔ glucose-6-P + ADP and glucose-6-P + NAD ↔ 6-phosphogluconate + NADH. This latter reaction catalysed by glucose-6-P dehydrogenase causes the concomitant reduction of stoichiometric amounts of NAD to NADH. This is then determined spectrometrically by following the rate of increase in absorbance at 340 nm and is measured on a chart recorder by peak heights. By comparison with the peak height of glucose standards (linear up to a concentration of 25 μg ml^{-1}) the amount of glucose in the samples can be calculated. Total reducing sugars (mainly glucose and fructose in the plant tissues examined) were determined by the addition of 100 μl PGI (350 U), 250 μl hexokinase/glucose-6-P dehydrogenase suspension (70 U hexokinase, 35 U glucose-6-P dehydrogenase) and 50 mg ATP to each 100 ml glucose reagent. The addition of phosphoglucose isomerase was required to convert the fructose phosphorylated by hexokinase to glucose-6-P which is then converted to 6-phosphogluconate as above. Additional hexokinase, glucose-6-phosphate dehydrogenase and ATP were added to provide non-limiting conditions when assaying for total reducing sugars (D. Richardson, pers. comm.). Fructose standards were used in addition to the glucose standards to confirm the assay. The amount of fructose was calculated by subtraction of the glucose values from the total reducing sugar values. Total sugar determination, to include sucrose, required the addition of ß-fructosidase (750 Units ml^{-1}) which was supplied through a separate line on the autoanalyser. Sucrose standards were included to confirm the completion of the reaction and the amount of sucrose calculated by subtraction of total reducing sugar values from total sugar values.

2.4.2 Starch

After extraction of soluble sugars from the freeze-dried samples, the pellets obtained after centrifugation (3,500 g for 30 min) were resuspended in distilled water (10 ml) and boiled for 1-2 hours with regular shaking to gelatinise the starch. Following gelatinisation, 1 ml was removed after thorough mixing and added to an equal volume of amyloglucosidase (from *Aspergillus niger*) at a concentration of 10 units ml^{-1} made up in 400 mM sodium acetate buffer (pH 4.7). The samples were incubated at 40°C and after 1 hour a small aliquot was removed and tested with Lugol's iodine to confirm complete starch breakdown. When this was confirmed, the samples were centrifuged (3500 g for 30 min) and the glucose in the supernatant, produced by the action of amyloglucosidase, determined as previously.

2.5 Electrophoresis

2.5.1 One-dimensional SDS-PAGE

SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) but using the Bio-Rad Mini-Protean II system. Piperazine di-acrylamide (PDA) obtained from Bio-Rad Laboratories Ltd was used as the crosslinker in preference to N,N'-methylene-bis-acrylamide (bis) resulting in improved resolution and reduced background when silver staining. The percentage of acrylamide was altered to give the best separation of the protein of interest. The majority of the gels were stained with 0.2% Coomassie Brilliant Blue R-250 made up in 40% methanol and 10% acetic acid. After approximately 1 hour at room temperature, the gels were destained with a solution containing 40% methanol and 10% acetic acid. Nanogram quantities of protein were stained using the improved protocol of Blum *et al.* (1987) for silver staining.

2.5.2 Two-dimensional SDS-PAGE

Two-dimensional polyacrylamide gel electrophoresis was carried out according to the method of O'Farrell (1975). The isoelectric focussing gels and sample buffer contained 2% (v/v) ampholines (3:2 ratio of pH ranges 3.5-10 and 5-7, Pharmacia Biosystems Ltd). The Mini-Protean II 2-D cell (Bio-Rad Laboratories Ltd) was used and the instructions in their manual followed. The second dimension was carried out as outlined for one-dimensional SDS-PAGE.

2.6 Electrophoretic transfer and Immunoblotting

Using the mini trans-blot electrophoretic cell (Bio-Rad Laboratories Ltd), polypeptides were transferred from SDS-polyacrylamide gels on to nitrocellulose - 0.45 μm pore size (Schleicher & Schuell, Dassel, Germany) according to the method of Towbin *et al.* (1979). A standard protocol was used with blocking solution (to prevent nonspecific binding) containing both dried milk (2%) and BSA (3%). Blots were washed then incubated with the antibodies diluted with either TBS (25 mM Tris, 137 mM NaCl, 2.7 mM KCl) or PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) and antigen-antibody complexes were detected using goat anti-rabbit immunoglobulin conjugate labelled with alkaline phosphatase. The chromogenic substrate for alkaline phosphatase used for detection was bromochloroindolyl phosphate / nitro blue tetrazolium (BCIP/NBT: Bio-Rad).

2.7 Protein assay

Protein concentrations were determined using the dye-binding Bio-Rad method with bovine serum albumin (BSA) as the standard. Both the microassay (0 - 10 μg) and the standard method (0 - 100 μg) were used depending on the amount of protein available for assay. Both assay methods are described in the instructions supplied with the Bio-Rad dye concentrate.

CHAPTER 3

SUCROSE METABOLISM IN POTATO STOLONS AND TUBERS

Tuber initiation and early development

3.1 Introduction

The potato tuber is a modified stem that normally develops at the tip of an underground lateral shoot termed a stolon (Cutter, 1992). Tuber formation commences in the sub-apical region of the stolon and involves both extensive cell division (Artschwager, 1918, 1924; Plaisted, 1957; Reeve *et al.*, 1969) and cell expansion (Booth, 1963). Additionally, a number of significant biochemical changes occur as the rapidly elongating stolon tip is changing into a starch-storing sink organ (Ewing and Struik, 1992). Investigation of the process of tuberisation using single-node potato cuttings, showed that the earliest detectable changes were starch deposition and mitosis (Duncan and Ewing, 1984). Significant changes in the content and composition of the soluble sugar pool in tuberising stolon tips also occur (Davies, 1984).

Sucrose is the main form in which carbon required for biosynthesis and energy is translocated to potato tubers. This was demonstrated after supplying leaves of potato plants with $^{14}CO_2$ and analysing the distribution of radiolabel arriving into the tuber (Oparka and Prior, 1987). Over 85% of the label in the water-soluble fractions was sucrose.

Within the developing tuber, the translocated sucrose is metabolised and partitioned primarily between respiration, starch and structural polysaccharide

biosynthesis. A proportion is stored as sucrose or hexoses. It has been estimated that 50-70% of the sucrose carbon is utilised in starch synthesis, 5-10% or less for synthesis of structural polysaccharides with the remainder divided between respiration and storage (ap Rees and Morrell, 1990). However, before conversion into starch, the sucrose must be cleaved by either sucrose synthase or invertase. The sucrose synthase pathway predominates in actively filling sinks and its activity is considered to give a measure of the sink strength of the organ (Sung *et al.*, 1989; Wang et al., 1993).

High acid invertase activity is usually associated with tissues undergoing rapid growth and elongation. Hence, acid invertase in the stem of *Phaseolus vulgaris* is highest in the most rapidly elongating internode (Morris and Arthur, 1985). In corn radicle tips, activity per cell increases 40-fold as the meristematic cell advances to the stage of most rapid elongation and decreases as the cell stops elongating (Hellebust and Forward, 1962).

Less information is available on the physiological role of alkaline invertase. It has been classified as a maintenance-type enzyme which does not appear to change in response to increased sucrose levels (Sung *et al.*, 1990). In maize mutants deficient in sucrose synthase, alkaline invertase activity increases to compensate for the very low activity of sucrose synthase thus providing an alternative pathway for sucrose cleavage within the cytosol (Dancer and ap Rees, 1989).

Tuber formation is usually characterised by a cessation in stolon elongation and accompanied by the radial swelling of the sub-apical region (Vreugdenhil and Struik, 1989). The following investigation, therefore, was designed to identify potential changes in the pathways of sucrose degradation associated with this developmental switch.

3.2 Materials and Methods

3.2.1 Plant material

3.2.1.1 Onset of tuberisation and early tuber development

Field-grown potato plants cv. Record were harvested on the 1st and 2nd July, approximately 10 weeks after planting, and the stolons and tubers carefully washed while still attached to the plant. The plant canopy was not yet fully developed but the plants were at, or close to, the stage of maximum light interception (Mackerron, pers. comm.). To provide sufficient replication and uniformity of samples, 100 plants were harvested. From these plants, non-tuberised stolons and those subtending small tubers were removed, measured and classified as below and immediately frozen in liquid nitrogen. Only primary stolons, i.e. the first, unbranched stolons formed, were used, with the aim of reducing variability. Samples were stored frozen, prior to measurements. The material was assigned to six different developmental stages described below and shown in plate 3.I.

<u>Classification of developmental stage</u>

Stage 0 - no visible signs of swelling at stolon tip

Stage 1 - swelling 1-4 mm greater than stolon diameter

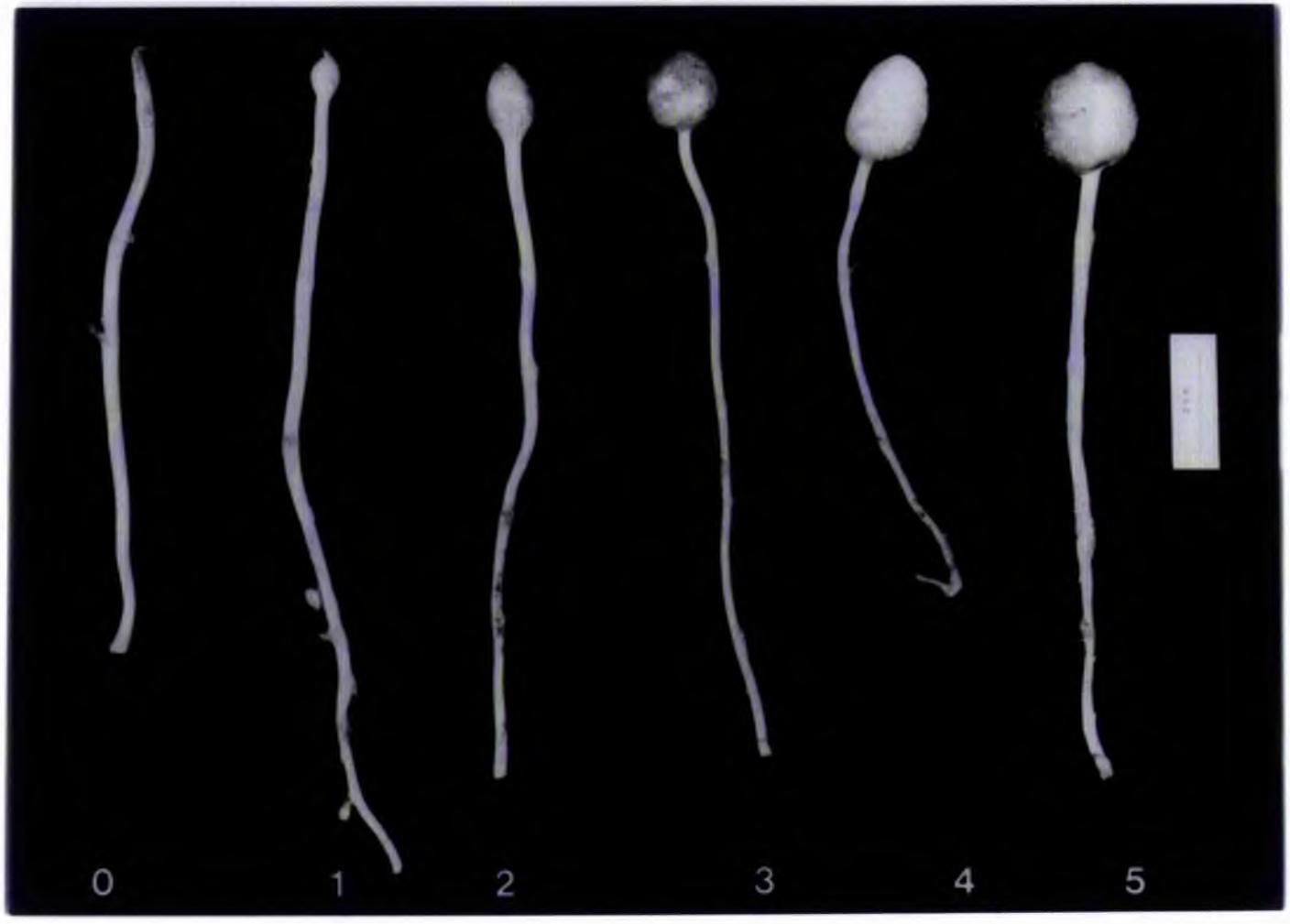
Stage 2 - " 4-8 mm " " " "

Stage 3 - " 8-12 mm " " " "

Stage 4 - " 12-16 mm " " " "

Stage 5 - " 16-20 mm " " " "

Plate 3.I Stages of tuber development showing from left to right, stage 0 to stage 5 (as described in section 3.2.1.1).


0
1
2
3
4
5

3.2.1.2 Mature stored tubers

Field-grown mature potato tubers (cv. Record) were harvested on 8th October and stored at 4°C for 3 months in the dark.

3.2.1.3 Tuber sprouts

Potato tubers cv. Record, stored as above at 4°C, were transferred to 10°C in the dark to encourage sprouting. To further stimulate vigorous elongation of the sprouts, the tubers were planted in a peat/sand compost (University of California formulation [Baker, 1957]) at a depth of 25 cm and grown at 18°C for 2 weeks, again without lighting.

3.2.2 Enzyme determinations

At each stage, the apical/subapical region of the stolon (whether swollen or not) was extracted separately from a 2 cm length of stolon excised 2 cm distal from the tuberising tip. Five replicates of two or more stolons were extracted to provide sufficient material for assay. In general, two samples were required for tuber extracts but six for stolon extracts. All samples were ground to a powder in liquid N_2 with a mortar and pestle and extracted with insoluble polyvinylpolypyrrolidone (1% w/v) and acid washed sand in four volumes of extraction solution (extracted twice to ensure maximum recovery).

Prior to starting this experiment, enzyme extracts were prepared from each of the stages of development from both the developing tubers and from the stolons subtending them. Samples of bean cotyledons (*Vicia faba*) were extracted at the same time and used in mixing experiments with tuber and stolon tissues. Measurements of activity of individual extracts could be used to

predict the values that should be obtained after extracting along with the other tissue. These recombination experiments, using the method described by Morrell and ap Rees (1986), were used to test possible losses during extraction for both acid and alkaline invertase and sucrose synthase activity.

The activity in the mixture is expressed as a percentage of the value calculated from the assays of the individual components of the mixture. The mean values for each of the enzymes shown below indicate no major losses during extraction.

Tuber sucrose synthase - 102%; stolon sucrose synthase - 89%
Tuber acid invertase - 110%; stolon acid invertase - 120%
Tuber alkaline invertase - 92%, stolon alkaline invertase - 86%

When cold-stored mature tubers were used, enzymes were extracted from longitudinal slices (approx. 2 mm thick).

When sprouts were extracted, 3 regions were used (as shown in plate 3.II).

1) apical 2cm (approx.) including the apical and subapical node

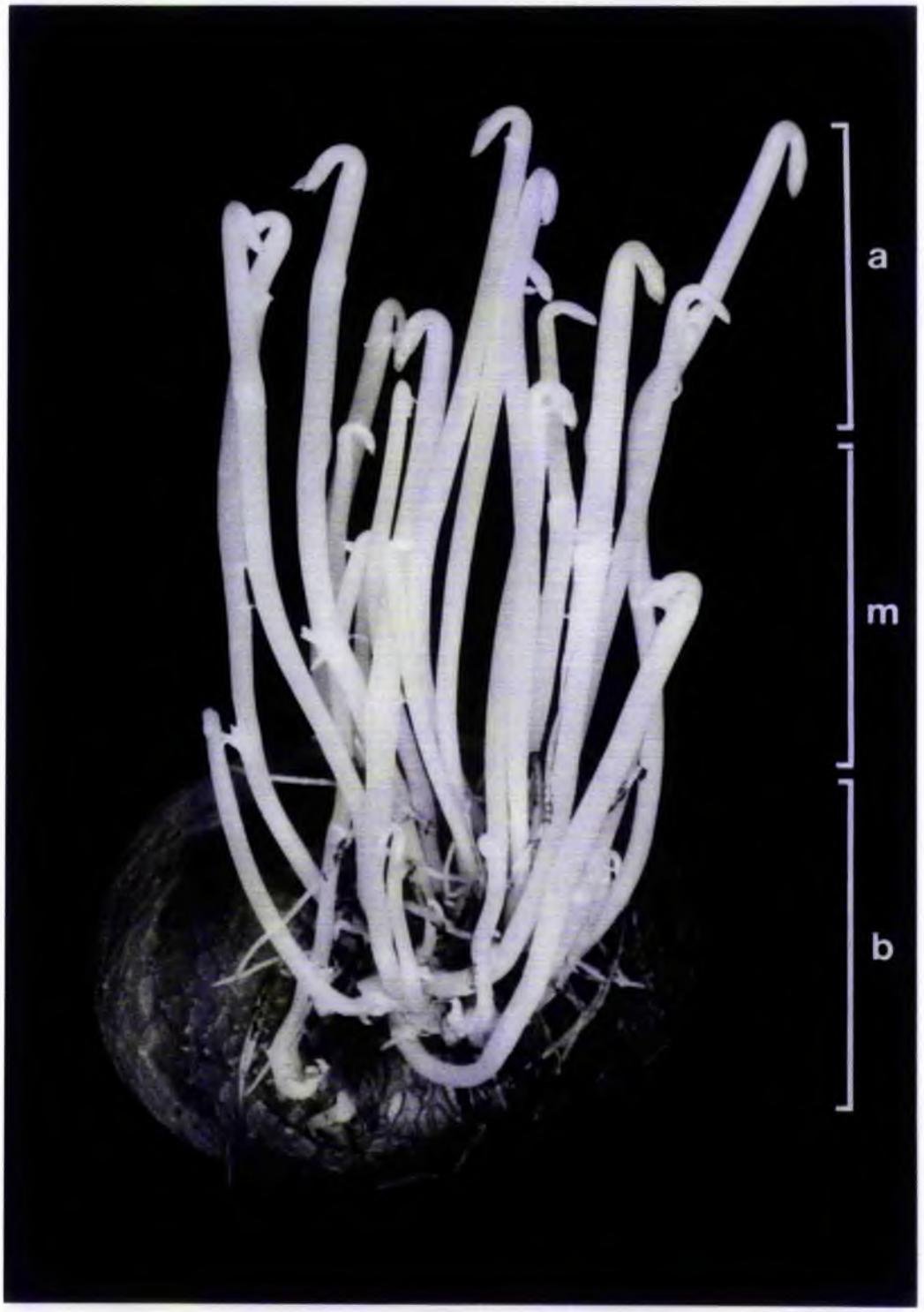
2) middle 2cm (approx.) including the mid node

3) basal 2cm (approx.) including the basal node

3.2.2.1 Sucrose synthase

For sucrose synthase, the extraction buffer contained 100 mM Tris-HCl (pH 7.5), 5 mM DTT, 3 mM magnesium acetate, 2% (w/v) glycerol and 2 mM PMSF. After centrifugation at 20,000g for 20min at 4°C, extracts were dialysed for 18h against two changes of 10 mM Tris-HCl (pH 7.5).

Plate 3.II Sprouting potato tuber indicating the three regions used for sampling (as described in section 3.2.2). a) - apical region including the apical and subapical node; b) - middle region including the mid node and c) basal region including the basal node.


a
m
b

Sucrose synthase activity was assayed in the cleavage direction under optimised conditions as described previously (**2.3.1**).

In addition to the recombination experiments already described (**3.2.2**) activities within the resuspended pellets from each of the extracts were tested and found to contain less than 5% of total sucrose synthase activity.

3.2.2.2 Acid invertase

For the determination of acid invertase, samples (as described previously in section **3.2.2**) were extracted in 100 mM sodium acetate (pH 5.0) containing 10 mM sodium sulphite, 1 M NaCl and 2 mM PMSF. After centrifugation at 20,000g for 20min at 4°C, extracts were dialysed against two changes of 10 mM sodium acetate buffer (pH 5.0) for 18h at 4°C.

Invertase activity was assayed as described previously (**2.3.2**) and activity determined before (basal activity) and after (total activity) destroying the endogenous invertase inhibitor by extensive foaming. This involved vortexing the extracts for 90 min in test-tubes fixed to a flask-shaker operating at maximum speed. The duration of the foaming treatment required to yield maximum catalytic enzyme activity was determined prior to the start of the experiment. No invertase activity was detected in the insoluble pellets when resuspended and assayed after dialysis.

3.2.2.3 Alkaline invertase

Alkaline invertase was extracted in the same buffer as used for the acid invertase. The enzyme was assayed at 37°C under optimum pH and substrate conditions in 0.2 M phosphate buffer (pH 7.5) containing 250 mM sucrose. As

for acid invertase, no activity was found in the resuspended pellet. Invertase activity at each of the developmental stages was determined over a pH range from 4-8 using 0.2 M sodium acetate and 0.2 M sodium phosphate buffers.

3.2.2.4 Fructokinase and Glucokinase

Both fructokinase and glucokinase were extracted using the same extraction buffer as used for sucrose synthase. Their activity was determined using a continuous spectrophotomeric assay at 25°C as described by Gardner *et al.*, (1992). The reaction mixture (1 ml final volume) for the fructokinase assay contained 100 mM Tris-HCl (pH 8.5), 5 mM $MgSO_4$, 2 mM ATP, 0.2 mM NAD, 2 units of phosphoglucose isomerase and 1.5 units of glucose 6-P dehydrogenase (from. *Leuconostoc mesenteroides*). The reaction was started by the addition of fructose (2 mM final concentration). For glucokinase activity, phosphoglucoisomerase was omitted and fructose replaced with glucose. Previous extractions from developing potato tubers using this method showed no major losses were incurred (Gardner, pers comm).

3.2.3 Protein determination

Protein in each of the extracts was quantified using the standard Bio-Rad protein assay with BSA as a standard (section 2.7).

3.2.4 Electrophoresis and Immunoblotting

Protein was extracted in 100 mM Hepes-KOH (pH 7.5) containing 10 mM sodium sulphite, 1 M NaCl, 2 mM PMSF, 2% glycerol (w/v), 20 mM EDTA, 5 mM DTT and PVP (1% w/v) and desalted on Sephadex G-25M PD10 columns

eluting with 20 mM Hepes-KOH (pH 7.5) containing 5 mM DTT. The extracts were kept at 4°C throughout and extracted as rapidly as possible to prevent proteolytic breakdown. Approximately 15µg of soluble protein (after boiling for 10 min in SDS-denaturing buffer) was subjected to SDS-PAGE (10 or 12.5% acrylamide) as described by Laemmli (1970) and the gels stained with Coomassie blue. In parallel gels, polypeptides were transferred to nitrocellulose membrane and after immunoblotting with polyclonal antibodies following the method of Towbin *et al.*, (1979), crossreacting bands were identified using anti-rabbit immunoglobulin conjugate labelled with alkaline phosphatase. The polyclonal antibodies used for immunodetection were antibodies raised against :

1) Sucrose synthase purified from developing cotyledons of *Vicia faba* (Ross and Davies, 1992);

2) A synthetic peptide derived from the N-terminal sequence of a purified acid invertase from potato tubers (Burch *et al.* 1992);

3) A polypeptide expressed in *E. coli* from an open reading frame representing part of a gene encoding a potato apoplastic invertase (Hedley *et al.* 1993);

4) Alkaline invertase purified from developing cotyledons of *Vicia faba* (Chapter 7);

5) Fructokinase (FKP-I) purified from pea seeds (provided by D.D. Randall, University of Missouri, Columbia, USA).

3.2.5 Determination of starch, sucrose, glucose and fructose

Samples used for carbohydrate measurements were frozen in liquid nitrogen and freeze-dried. The determinations were carried out as described in sections **2.4.1** and **2.4.2.**

3.3 Results

3.3.1 Carbohydrate levels and enzyme activities

3.3.1.1 Tuberising stolons

The changes in glucose, fructose, sucrose and starch along with the activities of enzymes involved in sucrose metabolism were measured over the six stages of tuberisation shown in Plate 3.I. Over this period the mean fresh weights of the stolon apex and developing tubers were:- 0.012g, 0.21g, 0.51g, 1.50g, 3.17g and 5.09g (SEM < 10% of the mean [n=10]).

The changes in glucose, fructose, sucrose and starch over the six stages of tuberisation are shown in Fig. 3.1. While the glucose and fructose content was higher in the stolon itself than in the subapical region prior to tuberisation (stage 0), in both tissues the levels decreased with the onset of tuberisation. Decreases in the level of both hexoses were far more pronounced in the stolon than in the developing tuber. The fructose content of the developing tubers decreased nearly fivefold over the developmental stages examined, resulting in an increase in the glucose:fructose ratio from 2.5:1 at stage 0 to over 9:1 at stage 5 in the developing tubers. In the non-tuberising stolon region the ratio changed from 1.9:1 to 6.5:1.

The sucrose and starch content increased in both the stolon and tubers during development reaching a plateau in both tissues by stage 3 (tubers 1.5 g fresh weight). The tubers attained the higher sucrose and starch content (approx. 32% and 42% higher, respectively, than stolons). However, sucrose and starch increased (14-fold and 60-fold) for stolons and (3-fold and 22-fold) in tubers over the stages examined.

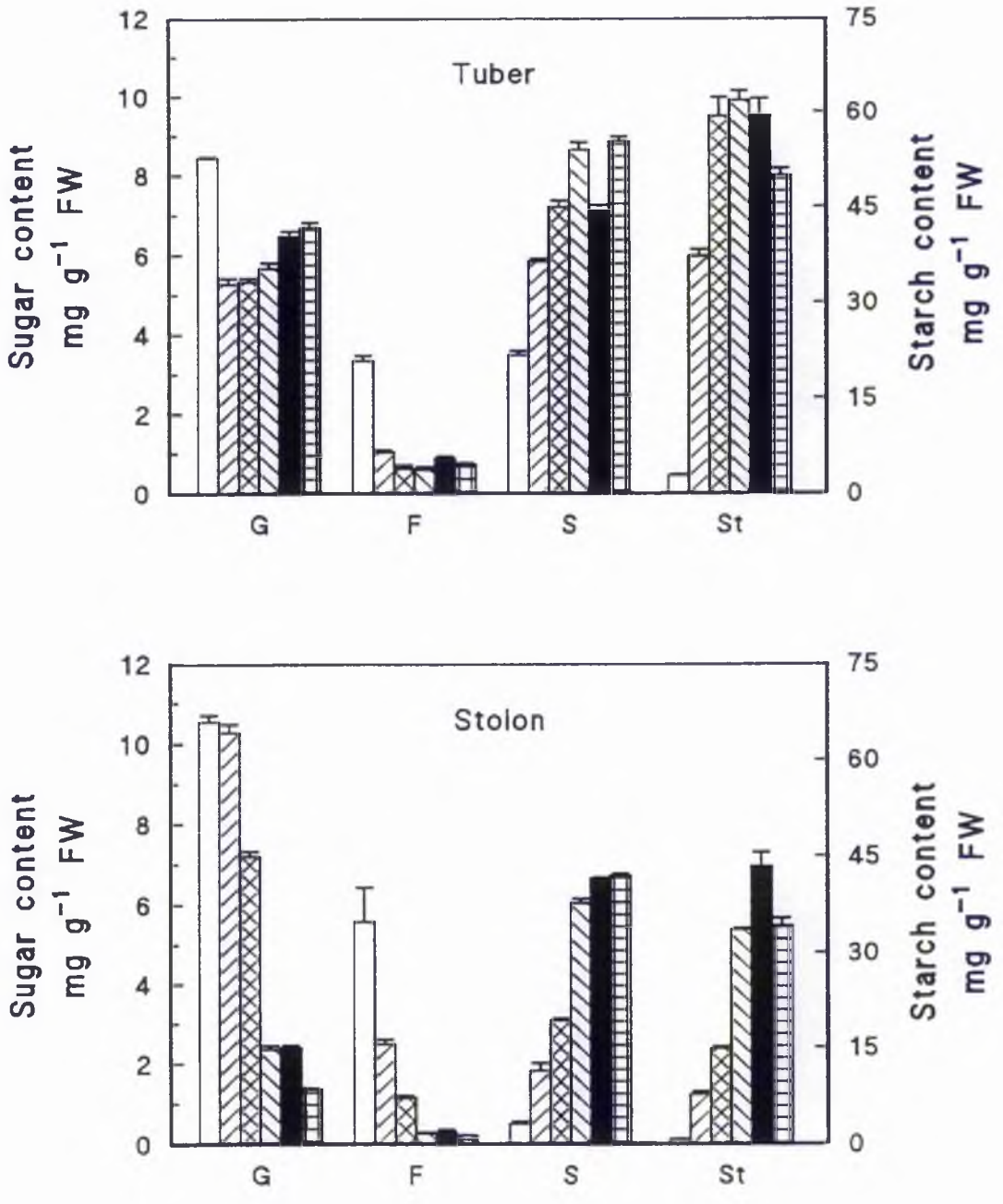


Fig. 3.1 Glucose (G), fructose (F), sucrose (S) and starch (St) content of both developing tubers and the stolons subtending them. □ - stage 0, ▨ - stage 1, ▩ - stage 2, ▧ - stage 3, ■ - stage 4, ⊞ - stage 5. Mean values (n = 5) + sem's are shown.

At stage 0, prior to tuberisation, sucrose synthase activity was very low in the stolon and sub-apical region (approx. 55 and 200 nmol min^{-1} g^{-1} FW, respectively) (Fig. 3.2). This activity increased rapidly in the swelling stolon tip during tuberisation, reaching a maximum catalytic activity of 3,730 nmol min^{-1} g^{-1} fresh weight, nearly 20-fold higher than in the non-tuberised stolon region where the activity increased just over 10-fold.

By contrast, acid invertase activity was high in the stolon, just prior to any visible swelling and particularly in the meristematic sub-apical region (Fig. 3.3A & B). On tuberisation, acid invertase activity decreased 10-fold and activities were reduced to 225 nmol min^{-1} g^{-1} fresh weight. Basal (before foaming the enzyme extract) and total (after foaming extract) invertase activities were not significantly different between tuberisation stages 0-2. However, between stage 3 and 5, basal invertase activity in developing tubers had decreased to half the values obtained for total activity. For the non-swelling stolon region there were no significant differences between basal and total activities at any of the stages examined.

Alkaline invertase activity, while over 6-fold lower than acid invertase in the stolon apices prior to tuberisation, also decreased during tuber development (Fig. 3.4). To confirm the presence of a distinct alkaline invertase, pH curves were constructed for invertase activity at each stage of tuberisation. These results shown for four stages of development (Fig. 3.5) clearly identify a specific alkaline invertase with optimum activity at pH 7.5-7.6, activity at this pH decreasing during tuber development.

In addition to the measurements of the sucrose-cleaving enzymes, both fructokinase and glucokinase were assayed to provide information on the potential for phosphorylation of the hexoses produced during sucrose

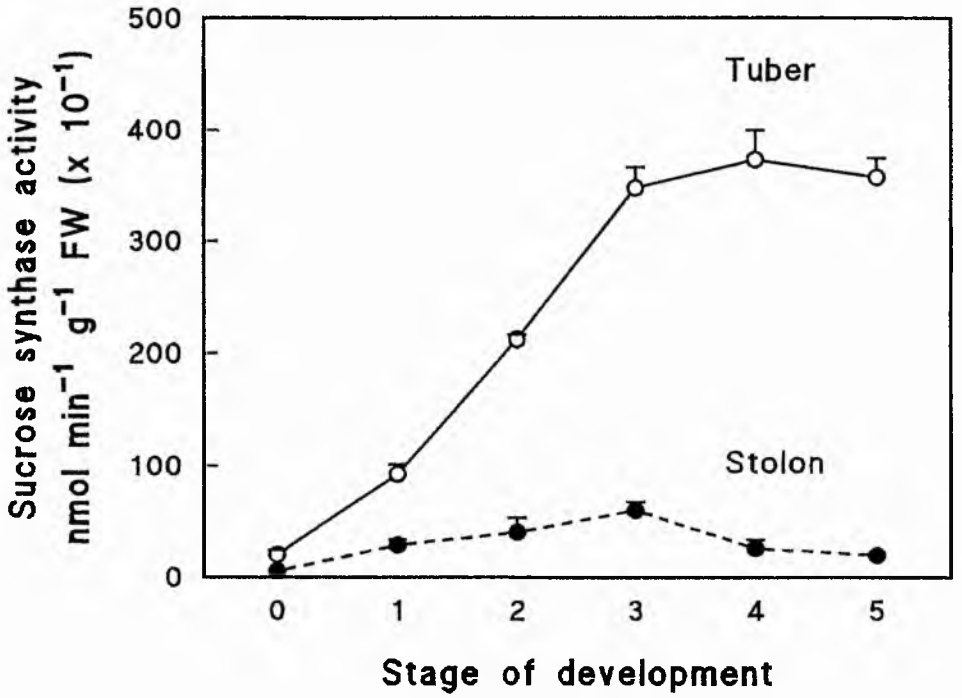


Fig. 3.2 Sucrose synthase activity in stolons and developing tubers over the six stages. Each value is shown as the mean (n = 5) + sem. The range of the y axis is from 0 to 5000 nmol min^{-1} g^{-1} FW.

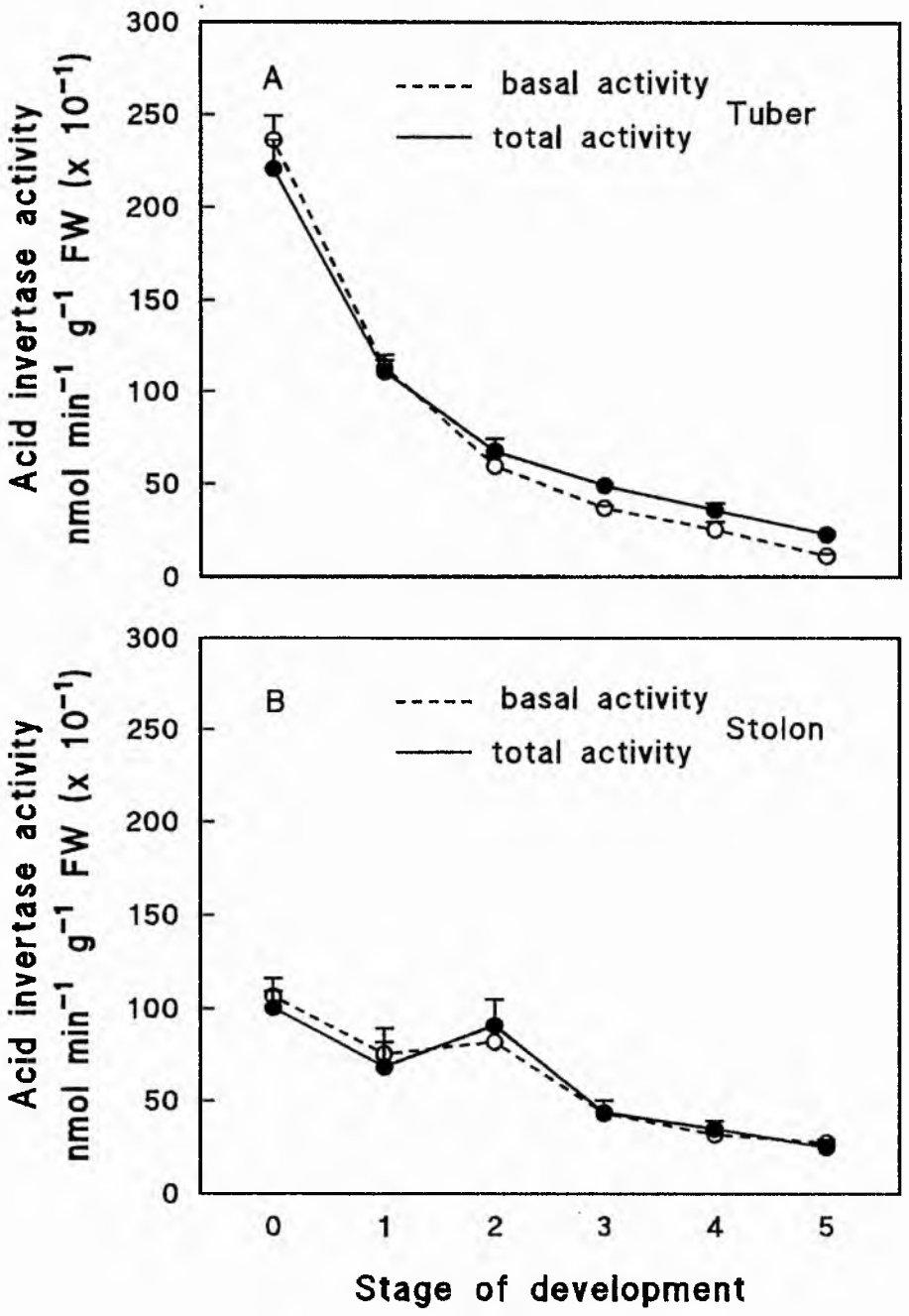


Fig. 3.3 Basal and total acid invertase activity in A) developing tubers and B) stolons over the six stages. Each value is shown as the mean (n = 5) + sem. The range of the y axes is from 0 to 3000 nmol min^{-1} g^{-1} FW.

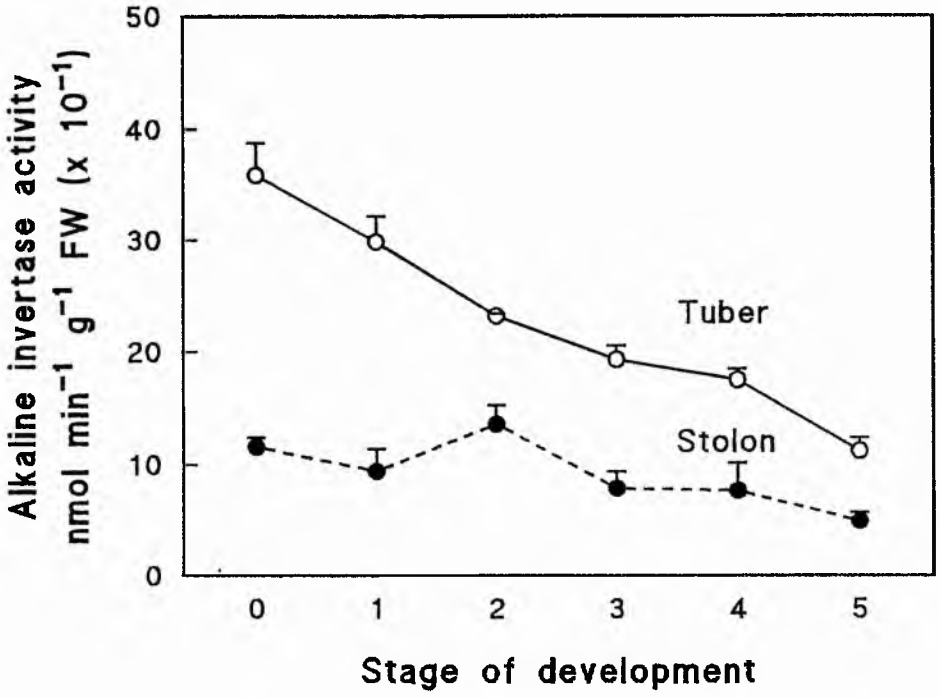


Fig. 3.4 Alkaline invertase activity in stolons and developing tubers over the six stages. Each value is shown as the mean (n = 5) + sem. The range of the y axis is from 0 to 500 nmol min^{-1} g^{-1} FW.

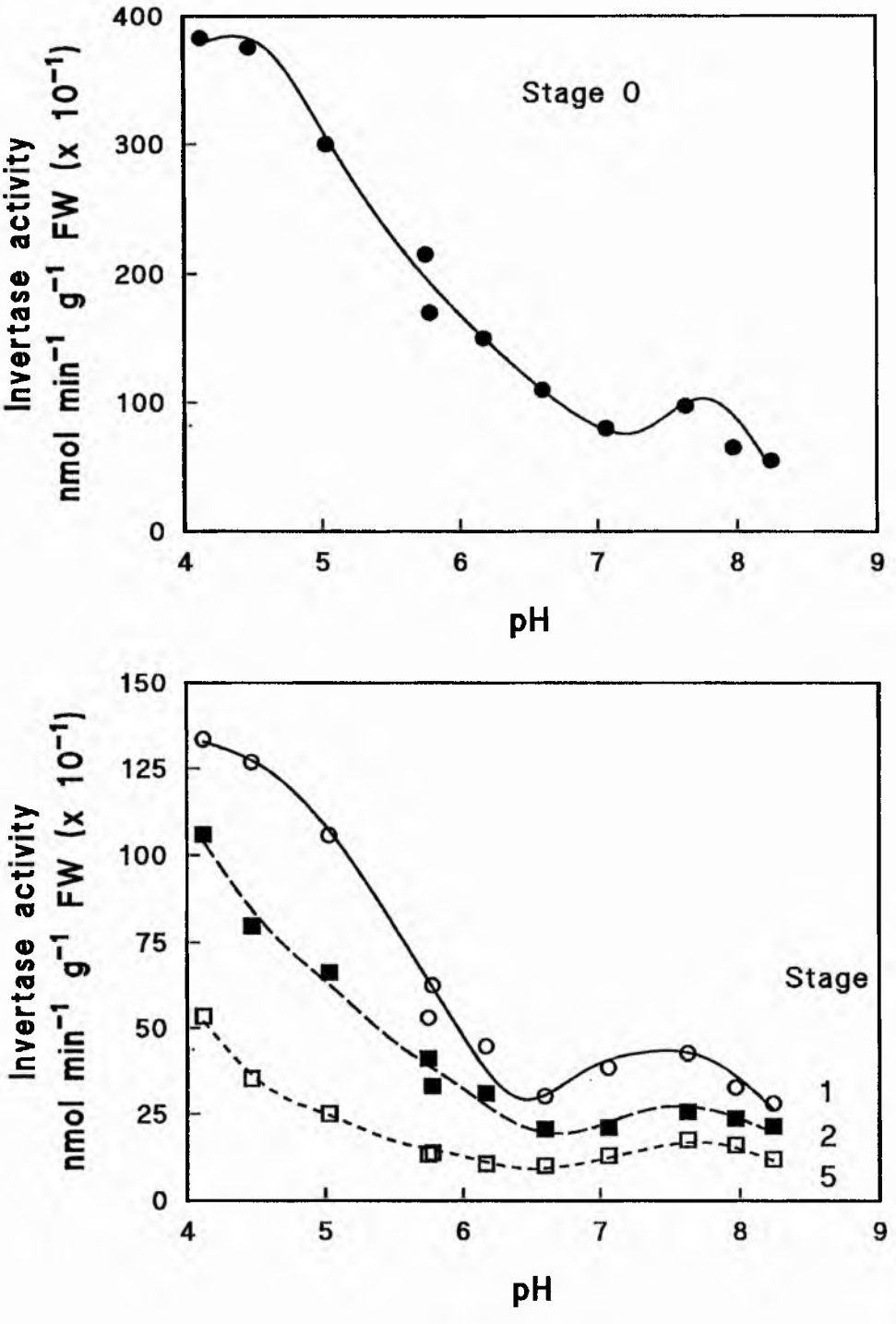


Fig. 3.5 Invertase activity for stages 0, 1,2 and 5 showing acid and alkaline pH optima. (n=3) sem's < 10% mean. The range of the top y axis is from 0 to 4000 nmol min^{-1} g^{-1} FW and of the lower y axis from 0 to 1500 nmol min^{-1} g^{-1} FW.

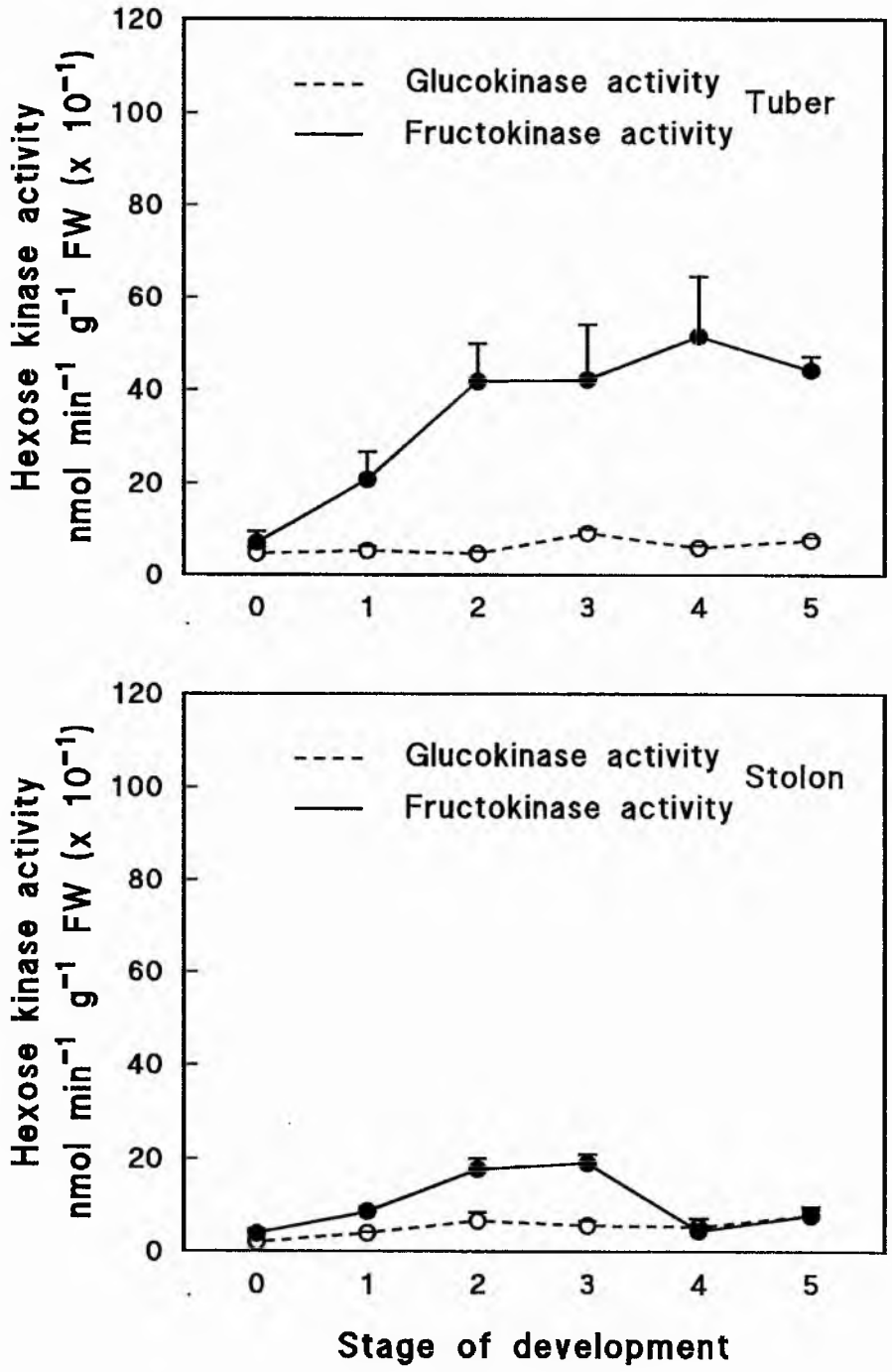


Fig. 3.6 Glucokinase and fructokinase activity in stolon and developing tuber over the six stages. Each value is shown as the mean (n=5) +sem. The range of the y axes is from 0 to 1200 nmol min^{-1} g^{-1} FW.

cleavage. Glucokinase activity remained low (approximately 60 nmol min^{-1} g^{-1} FW) throughout the developmental stages examined, while fructokinase activity increased over sevenfold in the developing tubers (Fig. 3.6). Fructokinase activity also increased in the non-swelling stolon region but maximum activity attained was approximately 40% of that in tubers. Fructokinase activity declined in the non-swelling region by stage 4 but remained high in the developing tuber. Glucokinase activity in the non-swelling region increased over 3-fold between stages 0 and 2.

Regression analysis showed that both sucrose synthase and fructokinase activities are positively correlated with sucrose content (r=0.965 and 0.957 respectively), whereas acid and alkaline invertases give significant negative correlations (r=-0.974 and r=-0.992 respectively). Similarly, both sucrose synthase and fructokinase are positively correlated with starch content (r=0.891 and 0.978 respectively) and the invertases negatively correlated (r=-0.996 for acid and r=-0.966 for alkaline invertase).

3.3.1.2 Mature tubers and potato sprouts

Both mature cold-stored tubers and potato sprouts were used to provide a comparison with young developing tubers. By testing the extracts from these different potato samples by immunoblotting with the range of antibodies available, the developmental stage at which the enzyme proteins were most highly expressed could be identified. The sugar contents and enzyme activities of the mature cold-stored tubers and potato sprout segments are shown in Table 3.1. The sucrose content of the mature tubers is just slightly lower than the maximum obtained by the developing tubers. By contrast, the glucose content is approximately half and the fructose content 4-fold higher than that of the young tubers (stage 5). This change in the levels of glucose and fructose

TABLE 3.1 Sugar contents and enzyme activities of mature cold-stored tubers and apical, mid and basal segments of potato sprouts

	Mature tubers	Apical segment	Mid segment	Basal segment
Sugar content				
(mg g^{-1} FW)				
Sucrose	7.83±0.03	1.15±0.17	1.83±0.23	2.70±0.15
Glucose	3.35±0.08	14.53±0.17	7.55±0.11	20.98±0.24
Fructose	3.08±0.12	5.61±0.46	0.46±0.06	0
Starch	64.40±0.46	1.93	-	5.33
Enzyme activities				
(nmol min^{-1} g^{-1} FW)				
Acid invertase	525.2 ±18.3	1729.6±43.2	1980.9±77.1	2848.1±40.5
Alkaline invertase	n.d.	n.d.	n.d.	n.d.
Sucrose synthase	24.6 ± 5.7	126.2± 5.3	342.8±11.7	689.7±17.0

Values are means ± s.e. from 3 replicates
n.d. - not detected

in the mature stored tubers resulted in a glucose : fructose ratio of just over one. The starch concentration of the mature tubers was only slightly higher than in the young tubers.

Invertase activity was was determined over the pH range (4 - 8) as before, in order to identify specific alkaline invertase activity. However, in this experiment, as in previous investigations with mature tubers (Richardson *et al.*, 1990), no alkaline invertase activity was detected. Sucrose synthase activity was detectable but 150-fold lower than in the developing tubers.

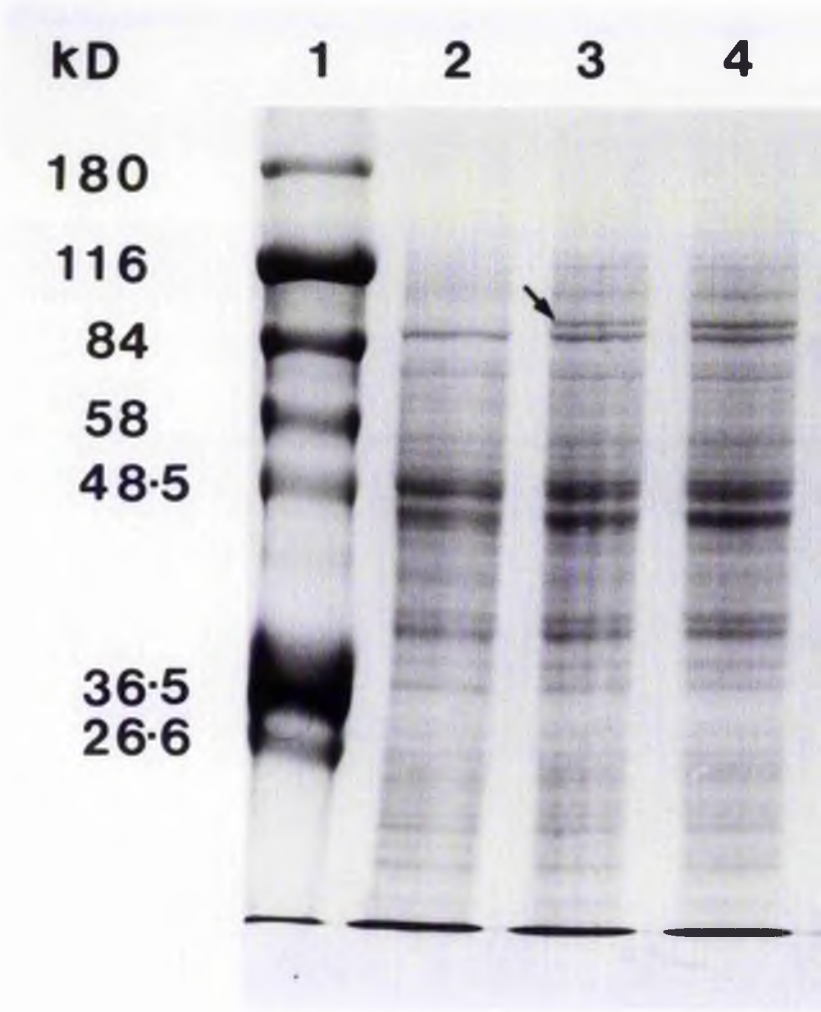
In sprout segments, the highest sucrose and glucose content was found in the basal region nearest the tuber but no fructose was found in this segment. Fructose content was highest in the apical region of the sprout. Starch content of the sprouts is low and only single values were obtained for the apical and basal regions. However, the measurements indicate a higher level of starch (>2.5-fold) in the basal compared to the apical region.

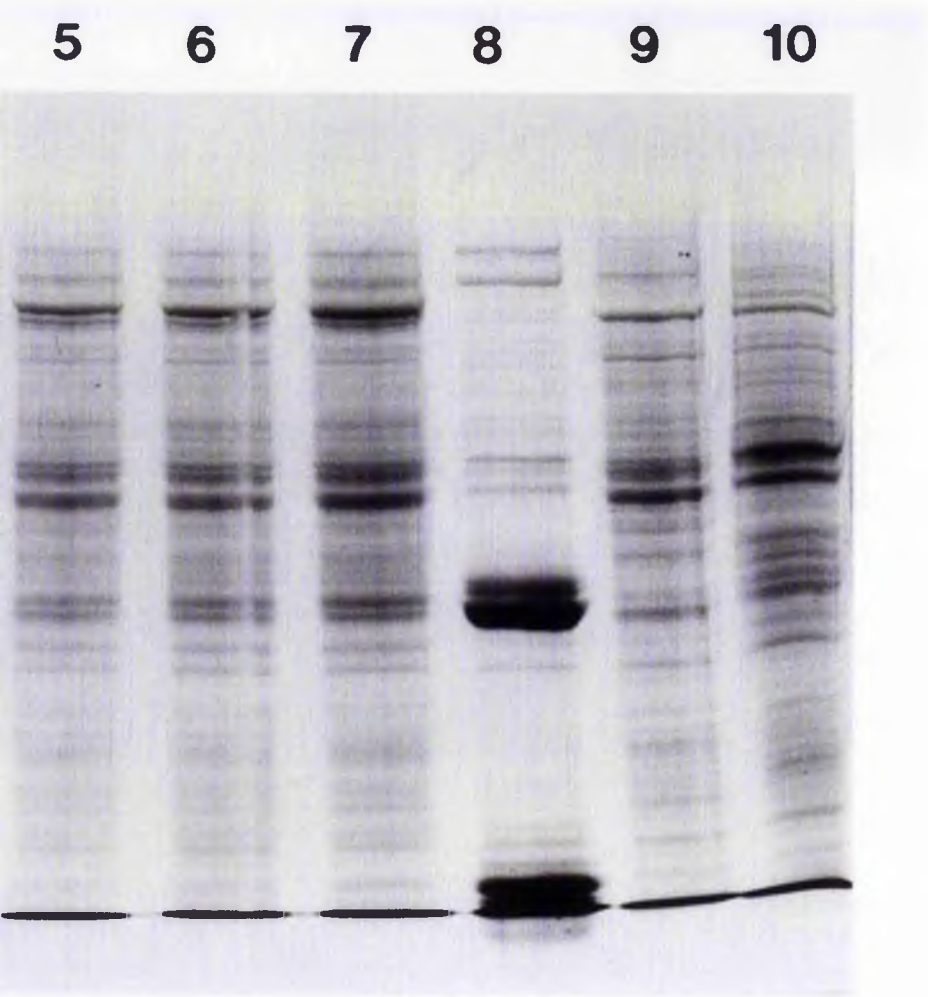
Acid invertase activity, as expected for rapidly elongating tissues, was high in the sprouts, with activity increasing from the apical to basal segments. Alkaline invertase was not detected in any of the sprout segments. The activity of sucrose synthase, while relatively low in the apical segment, increased to over 5-fold in the basal segment.

3.3.2 Immunoblotting

Samples of equal protein content were electrophoresed on SDS-PAGE (10% acrylamide) and the resulting gel (Plate 3.III) shows changes in the polypeptide profiles occurring during development. Polypeptides from mature cold-stored tubers and elongating sprouts were separated in parallel. The changes in the level of the storage protein patatin (approx. 42-43 kD) are not

Plate 3.III 10% SDS-PAGE. 15 μg protein loaded for each sample. Lane 1 - MW markers (SDS-7B Sigma), Lane 2-7 - developmental stage 0-5, Lane 8 - cold-stored mature tuber, Lane 9 - sprout apical region, Lane 10 - sprout basal region.


kD
1
2
3
4
180
116
84
58
48·5
36·5
26·6


5
6
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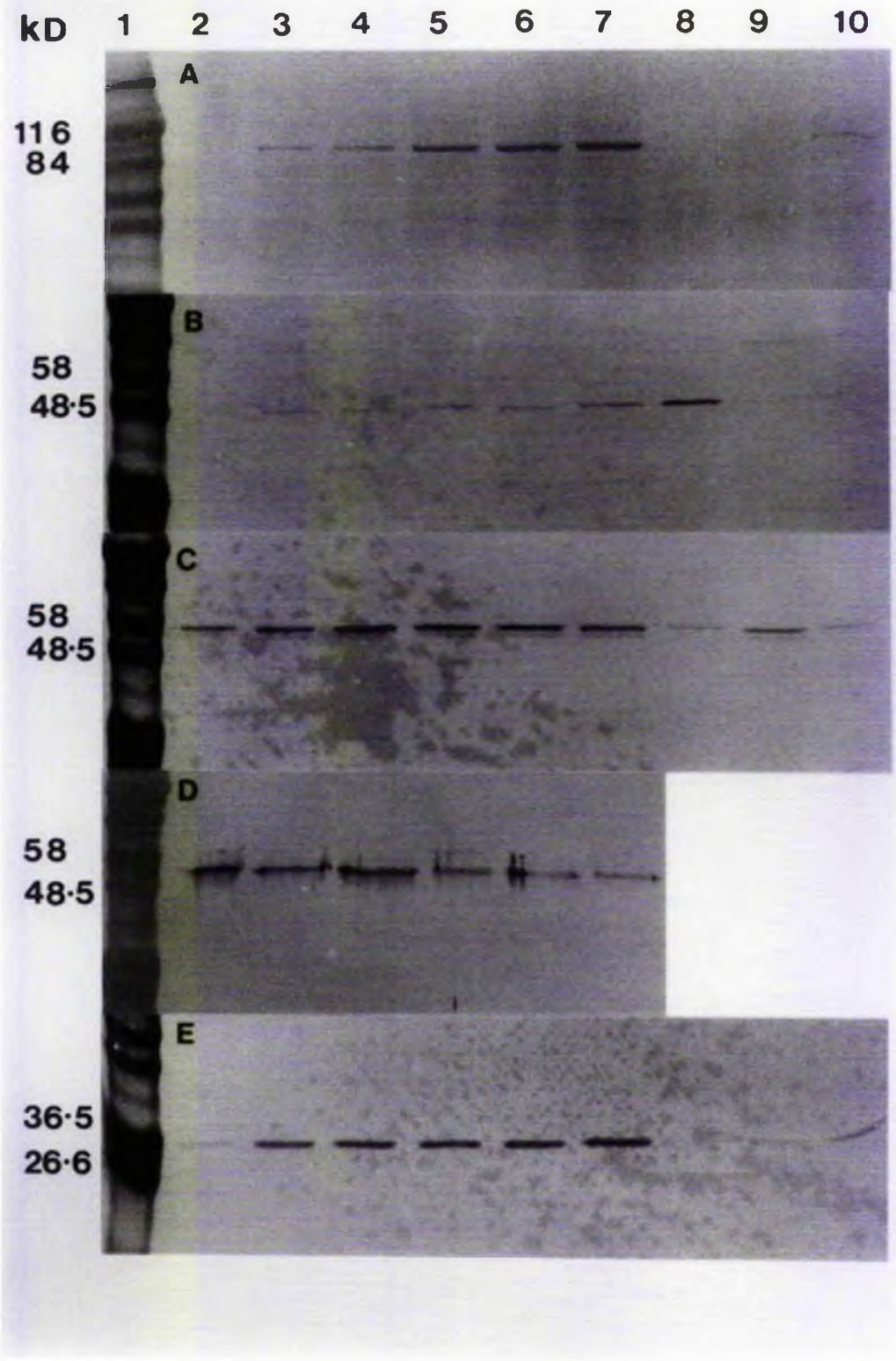
10

very evident during early tuber development although it is highly expressed in the mature tuber. The most obvious change occurring on tuberisation and during early tuber development is the appearance of a 90 kD polypeptide which is also present in basal sprout segments but virtually absent from apical sprout segments and mature tubers (shown by an arrow in Plate 3.III). This was identified as a subunit of potato sucrose synthase by immunoblotting with antibodies raised against sucrose synthase from bean cotyledons (Plate 3.IVA). The intensity of the sucrose synthase polypeptide band corresponded with the measured enzyme activity. The results of immunoblotting with the acid invertase antibodies are more difficult to interpret, as while both reacted with specific polypeptides, (antibody 2 with a 48 kD polypeptide and antibody 3 with a 58 kD polypeptide) neither corresponded with the decrease in acid invertase activity during tuber development (Plate 3.IV B,C). The 48 kD polypeptide was not expressed in the stolon but increased during tuber development, was at its highest in the mature tuber but virtually absent from the sprouts (Plate 3.IVB). By contrast, the 58 kD polypeptide was more heavily expressed in the stolons and young developing tubers than in the mature tuber. Furthermore, the level of the 58 kD polypeptide was higher in the apical sprout segment than in the basal, thus showing an increase in the opposite direction to acid invertase activity (Plate 3.IVC).

The alkaline invertase antibodies crossreacted specifically with a polypeptide (approximately 54 kD) and it appeared to be most heavily expressed in stolons, decreasing as tubers developed (Plate 3.IVD). This pattern followed the measured enzyme activity. No alkaline invertase activity was detected in mature stored tubers or in potato sprouts.

The FKP-I polyclonal antibodies specifically crossreacted with a single polypeptide (approximately 36 kD) (Plate 3.IVE), previously identified as a subunit of the 70 kD potato fructokinase protein (Gardner *et al.*, 1992). As with

Plate 3.IV Immunoblots of gel shown in Plate 3.III. A) sucrose synthase (antibody 1; dilution 1:10,000) Corresponding enzyme activities in lanes 2-10 respectively 10.4, 87.4, 121.9, 307.0, 341.7, 323.1, 10.4, 12.1, 137.9; B) potato acid invertase (antibody 2; dilution 1:200). Corresponding enzyme activities in lanes 2-10 respectively 405.8, 194.2, 110.8, 77.8, 60.7, 31.6, 43.9, 176.5, 550.7; C) potato acid invertase (antibody 3; dilution 1:1000) (activities in lanes 2-10 as for B); D) alkaline invertase (antibody 4; dilution 1:1000). Corresponding enzyme activities in lanes 2-7 respectively 61.8, 50.9, 43.6, 40.6, 42.3, 31.6; E) fructokinase (antibody 5; dilution 1:3000). Corresponding enzyme activities in lanes 2-10 respectively 6.9, 20.7, 49.0, 43.8, 73.6, 63.8, 2.7, -, -; activity not measured in sprout samples (lanes 9 and 10). All enzyme activities expressed as nmol min^{-1} mg^{-1}protein are mean values (n=5) SEM values <10% mean.


kD
1
2
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4
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A
116
84
B
58
48·5
C
58
48·5
D
58
48·5
E
36·5
26·6

sucrose synthase the level of expression of the polypeptide, low in the stolon, mature tuber, apical sprout region and increasing during development, also reflects the enzyme activity.

3.4 Discussion

The onset of tuberisation provides an important transition from elongating and meristematic tissues to rapidly developing starch-storing organs. It is a period over which the levels of the hexoses (glucose and fructose) and sucrose are subject to substantial changes with sucrose increasing several-fold to provide the energy requirements of the developing tubers. The expression of class-I patatin, proteinase inhibitor II and sucrose synthase genes in potato can all be enhanced by increased sucrose levels (Park and Du, 1991; Müller-Röber *et al.*, 1990; Kim *et al.*, 1991; Salanoubat and Belliard, 1989). Tuber formation is also promoted on axillary buds of potato cultured *in vitro* when the sucrose concentration increased from 88 mM to 234 mM (Levy *et al.*, 1993). Thus the process of tuberisation provides the opportunity to study enzyme activities in tissues where sucrose levels increase naturally.

While a number of studies have examined starch-synthesising enzymes during tuberisation (Obato-Sasamoto and Suzuki, 1979; Hawker *et al.*, 1979; Tsay and Kuo, 1980) no detailed investigation has been carried out on the sucrose-cleaving enzymes and how their activities interrelate with the changing levels of carbohydrates within the tissues.

Not surprisingly, tuberisation is accompanied by starch deposition in the swelling sub-apical region. During the rapid phase of starch accumulation (stages 0 to 2) the starch content of the tuberising stolon region increased by 56 mg while the total hexose content decreased by only 5.8 mg. Over the same

stages the starch in the non-tuberising region of the stolon region increased by 14 mg and the hexose content declined by 7.8 mg. The onset of starch synthesis in the non-tuberising stolon region may possibly correlate with the development of a strong metabolic sink for hexoses. Since the levels of the hexose-phosphorylating enzymes within this tissue did not change substantially throughout development (Fig. 3.6) this suggests the possibility of feed-back regulation of hexose-phosphorylation. Fine metabolic control of potato tuber hexose kinases may operate via nucleoside triphosphate content and composition and via ADP, fructose and fructose-6-phosphate contents (Gardner *et al.*, 1992; Renz and Stitt, 1993).

In the young, developing tuber, starch is apparently accumulated following the metabolism of imported sucrose rather than from stored hexoses. A change in the pathway of sucrose mobilisation during the early stages of tuberisation can be deduced from the rapid decline in both acid and alkaline invertases and the substantial increases in both sucrose synthase and fructokinase activities. This is confirmed, indirectly, by the increase in the G : F ratio. However, direct evidence for such a switch in metabolism during tuberisation is still lacking. Morrell and ap Rees (1986) calculated the rate of sucrose mobilisation in developing tubers of plants grown at 20°C to be approximately 3 μmol hexose h^{-1} g^{-1} fresh weight and estimated that acid and alkaline invertase could, at the most, account for < 50% of the total sucrose mobilised. In the present study the activities of both acid and alkaline invertases were sufficient to sustain such a rate, although the potential contribution of alkaline invertase becomes borderline as the tubers develop. It may well be that such discrepancies are due to the developmental stages analysed as previous results had shown a decline in acid invertase to very low levels and alkaline invertase to non-detectable levels in larger developing tubers (Ross and Davies, unpublished data). Additionally, differences in the levels of these enzymes present during

tuber development may result from the conditions under which the potato plants are grown. The samples used for this investigation were field-grown and were sampled at the stage of maximum light interception and most rapid growth, in contrast to the samples used by Morrell and ap Rees (1986), which were grown in compost under controlled conditions with artificial lighting. Measurements of total invertase activity (foaming treatment) rather than basal activity contributed to higher levels of acid invertase than found by Morrell (1984). In this present study, the significant differences between total and basal invertase activity that develop from stage three onwards, emphasise the importance of determining maximum catalytic activity. It may also suggest that either the invertase inhibitor is not present in stolons and very young tubers or, supported by the results from the immunoblots, a different form of the invertase enzyme, more susceptible to inhibition, is being synthesised in the growing tubers. However, the involvement of the proteinaceous inhibitor *in vivo* has been questioned by localisation studies (Isla *et al.*, 1992) which showed that its site within the cell, and that of a lectin inhibitor, were compartmentally separated from the enzyme. Alkaline invertase activity remained higher in swelling than non-swelling stolon regions and provides one explanation for the maintenance of a higher glucose content in the former. Starch turnover via amylase may also contribute to the glucose pool in developing tubers. Alternatively, respiration rates may be higher in stolons than in developing tubers.

The sucrose content in tuberising and non-tuberising regions by stage 4 had increased to similar levels. It may be argued that in both tissues the decline in acid invertase (presumed to be vacuolar) is responsible for the rise in sucrose content. However, the substantial increase in the maximum catalytic activity of sucrose synthase in the young developing tuber maintains a high sucrolytic potential. This potential, however, may not be realised since sucrose synthase

has a much lower affinity for sucrose (K_m - 130 mM, Pressey, 1969a) than invertases (tuber acid invertase - 8 mM, Burch *et al.*, 1992; plant alkaline invertases - between 9 and 25 mM, Avigad, 1982). The calculated sucrose concentrations in this present study range between 1.5 and 20 mM in the non-tuberising region and between 12 and 24 mM in the developing tuber. The concentration of sucrose, however, found in the cytosol is clearly important but is, as yet, unknown. The low affinity of sucrose synthase for sucrose together with an increase in sucrose influx into the swelling tuber, would help to explain the early increase in tuber sucrose content. An elevated sucrose concentration, or the concentrations of derived metabolites, would be expected to modify the expression of genes involved in the starch biosynthetic pathway, including sucrose synthase (Salanoubat and Belliard, 1989; Müller-Röber *et al.*, 1990; Kim *et al.*, 1991; Ross and Davies, 1992). This would increase sink strength further and, as a result, sucrose influx.

Increases in sucrose in the non-swelling stolon region were not accompanied by large increases in sucrose synthase activity, as was the case for developing tubers. By stage 3, the sucrose content of the stolons was nearly 70% of that in the tubers whereas the maximum catalytic activity of sucrose synthase in the stolons was only 17% of that in the tubers. This may be explained if most of the sucrose in the stolon is "in transit" via the phloem. It has been shown that a phloem-specific sucrose synthase exists in maize and citrus (Nolte and Koch, 1993) and also in *Ricinus* (Geigenberger and Stitt, 1992), thus allowing the removal of carbon for energy supply within the sieve element-companion cell complex.

Immunoblots showed that the developmental changes in sucrose synthase, alkaline invertase and fructokinase proteins were correlated with enzyme activity. There is good evidence from other sources that for sucrose synthase this reflects increased transcription (Salanoubat and Belliard, 1989). A potato

fructokinase gene has been cloned recently (Smith *et al.*, 1993) and preliminary Northern analyses have again indicated the importance of transcriptional control (unpublished data). Alkaline invertase has not yet been cloned, although it has been purified from soybean hypocotyls (Chen and Black, 1992), faba bean cotyledons (Ch. 7) and sugar beet taproots (Ch. 8).

In contrast, immunoblots with antibodies raised against acid invertases failed to demonstrate a correlation between protein content and enzyme activity. Antibodies raised against the N-terminal sequence of a purified soluble enzyme (Burch *et al.*, 1992) consistently detected a polypeptide in mature tubers only, where invertase activity is high (Richardson *et al.*, 1990). Antibodies raised against the *in vitro* expression product of a gene encoding a leaf apoplastic invertase (Hedley *et al.*, 1993) detected a polypeptide mainly in immature tubers and from the apical sprout region. RT-PCR (Reverse Transcription/Polymerase Chain Reaction) has confirmed that this gene is expressed at extremely low levels in tubers (Hedley *et al.*, 1993). There is little convincing physiological evidence that an apoplastic invertase exists in young developing tubers (Morrell, 1984; own unpublished data). Significant homologies exist between genes encoding apoplastic and vacuolar invertase genes in carrot (Unger *et al.*, 1992) and between vacuolar genes isolated from carrot, mungbean and tomato (Hedley *et al.*, 1994 and references therein). Polyclonal antibodies used in Plate 3.IVC may be detecting related epitopes on a vacuolar rather than an apoplastic protein. If this is the case then, given the different expression patterns detected by the two potato antibodies used, there is likely to be more than one gene encoding soluble invertase activity - one active in elongating stolons, young tubers and sprouts, the other only in mature tubers.

The comparison was made with cold-stored mature tubers and elongating sprouts as the former represented a sink to source transition and the latter an

elongating and developing sink. Although starch to sucrose interconversion occurs during tuber storage (Isherwood, 1973), the similar level of starch and sucrose (g^{-1} FW) found in the mature tubers in comparison with the young developing tubers (stage 5) would indicate that early in development a relatively fixed concentration of these carbohydrates is reached. Thus, in the young tubers, both starch and sucrose reached their plateau level by stage 3 (1.5 g FW). The almost equal amounts of glucose and fructose in the mature stored tubers support the results of enzyme activities, indicating a switch from a sucrose synthase dominated pathway in young tubers to one dominated by acid invertase in stored tubers. The lack of any detectable alkaline invertase activity and low level of sucrose synthase in mature tubers would suggest that sucrose cleaved at this stage is predominantly vacuolar.

As would be expected in the elongating sprouts, the basal region nearest to the tuber (source) has the highest level of both starch and sucrose. The sucrose synthase activity increasing from the apical to basal region, follows the gradient of sucrose content. While the activity of fructokinase was not determined for these samples, the expected values would be likely to follow a similar pattern to the activity of sucrose synthase and would explain the higher level of fructose found in the apical region where sucrose synthase activity is at its lowest. Thus, while both sucrose synthase and acid invertase are higher in the basal region, the fructose being produced is likely to be rapidly phosphorylated resulting in no detectable fructose in that region. The high levels of acid invertase in the sprouts were similar to those in the stolons prior to tuberisation, as expected for elongating tissues. Potato sprouts grown in culture also showed high acid invertase activity although variable results from two trials were presented (Blanc, 1983). In one trial a decreasing gradient in invertase activity was obtained from the apical to basal region, while in the other, acid invertase activity in the basal region was very similar and after

culture for 24 h, higher, than in the apical region. While the apical/subapical meristematic region might be considered to be the region most metabolically active, a possible explanation for higher acid invertase activity in the basal region may be due to the development of root meristems.

Unlike the stolons, no alkaline invertase activity is detected in the sprouts but with a moderate level of sucrose synthase available, no immediate requirement for alkaline invertase can be envisaged.

3.5 Conclusions

The comparisons between stolons during tuberisation (developing sinks), mature cold-stored tubers (sink to source transition) and elongating sprouts (sinks) provide evidence of the relative importance of the pathways of sucrose cleavage in potato plants. The process of tuberisation is shown to be characterised by a switch from a pathway of sucrose cleavage dominated by invertase (both acid and alkaline) within the stolons to one dominated by sucrose synthase within the developing tuber. The rapid accumulation of starch shown in young tubers is dependent on the efficiency of the mobilisation of imported sucrose. Thus sucrose synthase, influenced at the transcriptional level by high sucrose concentrations and catalysing the initial step in starch biosynthesis, is well suited to achieve this role and maintain the sink strength of the growing tuber.

In mature cold-stored tubers, the pathway of sucrose cleavage is via acid invertase thus providing the requirement of hexoses from stored sucrose within the vacuole. Significantly, results from immunoblots with acid invertase antibodies provide evidence for the presence of more than one gene coding for soluble acid invertase in the potato plant. Thus, in the elongating sprouts where the acid invertase pathway again predominates, a different form of the enzyme from that in mature tubers appears to be present.

CHAPTER 4

SUCROSE METABOLISM IN POTATO TUBERS

Effects of modifying assimilate supply to developing tubers

4.1 Introduction

In starch-storing sink organs such as potato tubers, incoming sucrose is degraded predominantly by sucrose synthase as described in the previous chapter and by others (Morrell and ap Rees, 1986; Sung *et al.*, 1989). The importance of this enzyme in sucrose cleavage *en route* to starch biosynthesis has been clearly shown in the maize *sh* mutants, which are characterised by shrunken kernels due to a reduced starch content in the endosperm (40% less than in the normal endosperm, [Chourey and Nelson, 1976]). Sucrose synthase activity in a number of these *sh* mutants was less than 5% of that in normal endosperms (Chourey and Nelson, 1978). The significant positive correlations found between sucrose synthase activity and starch levels in tuberising potato (see Chapter 3) provide further evidence that high enzyme activity is required to maximise starch synthesising potential. As potato tubers mature on the plant and the rate of starch synthesis decreases, a significant decline in sucrose synthase activity occurs (Pressey, 1969a). In mature, stored tubers, the level of sucrose synthase is barely detectable and there is an apparent switch in the pathway of sucrose cleavage to one dominated by acid invertase (Pressey, 1970; Richardson *et al.*,1990; see Chapter 3). Oparka *et al.*, (1990) showed that the gradual decline in sucrose synthase activity found during tuber maturation could be accelerated by excising the tuber from the mother plant. Following tuber excision there was a rapid loss in the starch-synthesising capacity of

storage parenchyma cells which was accompanied by a 3-fold reduction in sucrose synthase activity. Acid invertase activity decreased 4-fold. In this Chapter, this work has been extended to examine, in detail, the consequence of excising tubers at various stages of development on sugar balance and the activities of sucrose-cleaving enzymes. The effect of genotype on the responses to tuber excision has also been examined.

The work of Claussen *et al.* (1986) implied that the concentration of sucrose in the cytoplasm of sink leaves of *Solanum melongena* regulated the rate of sucrose cleavage by sucrose synthase, by changing both the activity and amount of the enzyme. The concomitant rise in sucrose synthase activity and sucrose concentrations in tuberising potato also implies that such a mechanism operates in starch-storing sinks. The possibility that sucrose flux regulates sucrose synthase in potato tubers is examined in this Chapter.

4.2 Materials and Methods

4.2.1 Plant material

4.2.1.1 Tuber excision and postharvest storage experiments

Seed tubers of potato (*Solanum tuberosum* L.) cvs Cara, Record and Brodick were planted in the field on 6th May 1988 at the Scottish Crop Research Institute at a density of 4 plants m^{-2}. Plots were fertilized prior to planting with N, P and K at rates of 187, 187 and 262 kg ha^{-1}. Four sequential harvests were taken between 1st August and 26th September to provide tubers of varying maturity. Mean individual tuber weights were about 70 g fresh weight at the first harvest and 125 g fresh weight at the end of September. After tuber excision, five tubers of each genotype were selected at random for immediate

analysis (within 2 h). The remainder were stored at 10 ± 1°C and analysed at - regular intervals over a period of up to 18 d.

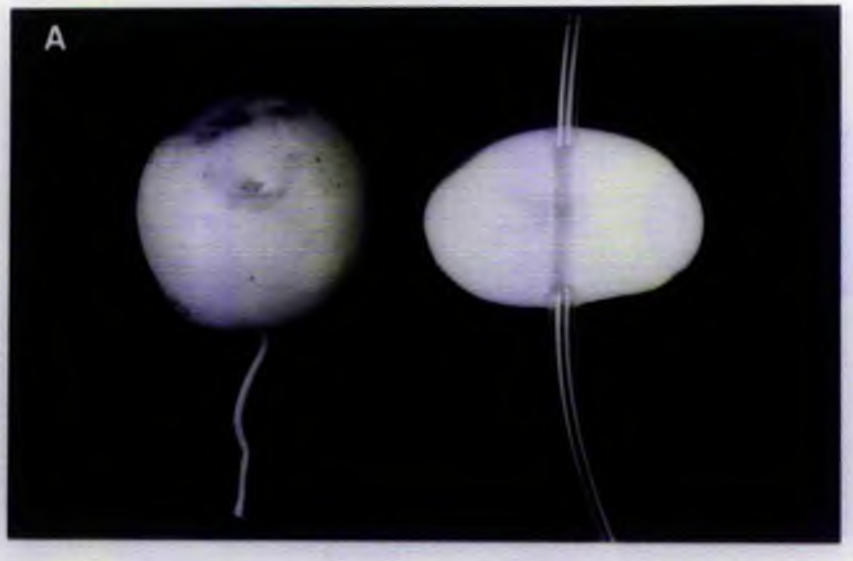
4.2.1.2 Effect of minimising tuber assimilate supply by light exclusion experiments

Unsprouted seed tubers of cv. Maris Piper were planted in the field on 14th June 1989 in a randomised block design. Tuberised plants (tubers approximately 90 g FW) were used to test the effect of manipulating sucrose flux to the tubers. On the 18th September, 40 potato plants, 10 from each of 4 plots, were selected at random and covered with two black polythene sacks to exclude, completely, any light from the plants. Each plot contained 60 plants at a density of 7.5 plants m^{-2}. Ten uncovered plants (controls) were harvested on the 19th September and two tubers from each plant used for measurements of sucrose, hexoses and the activities of sucrose synthase and acid invertase. Similar harvests from both covered and uncovered plants were taken after 7, 15, 22 and 28 days.

4.2.1.3 Exogenous application of sugars to intact, detached tubers

Seed tubers of cvs Cara and Record were planted in a peat/sand compost (University of California formulation [Baker, 1957]) in 30 cm square pots in an unheated glasshouse. Developing tubers with a mean fresh weight of 40 g (±10%) were excised at the point of attachment of the stolon with the stem and used in experiments to test the effect of supplying sugars exogenously over a period of up to 12 days. The effectiveness of two methods for supplying sugars was compared. 1) The excised stolons, each attached to a small tuber (Plate 4.IA), were immersed immediately in distilled water or in solutions of either

Plate 4.I A) The two methods used to supply sugars to developing tubers either via the attached stolon (left) or via a channel cut through the tuber (right); B) Experiment showing the method of supplying sugar solutions to tubers via their stolons; C) Experimental set-up used for supplying solutions via a channel cut through the tubers. Adjustable syringes were used to maintain a constant level of solution in each of the tubes supplying the tubers.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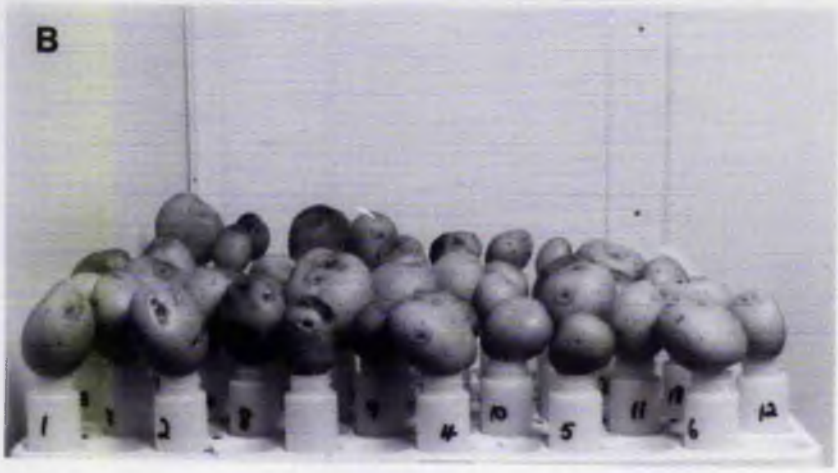
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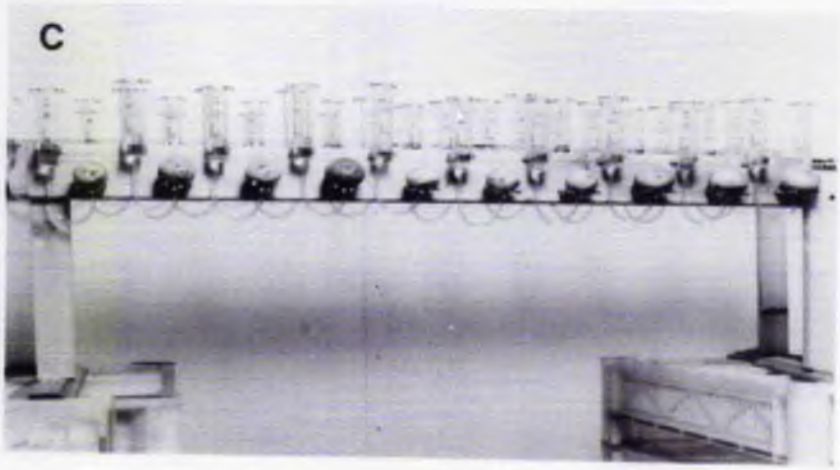

C

sucrose or maltose (method 1 - see Plate 4.IB). 2) Using a cork-borer, a channel 3.5 mm in diameter was made transversely through each of the tubers (Plate 4.IA) and connected by tubing to a movable syringe to maintain a constant level (method 2 - see Plate 4.IC). For both methods, 10 individual tubers were used as replicates for each treatment. To determine the rate of carbohydrate flux into the tubers, solutions were spiked with either [U-^{14}C]-D-maltose (specific activity of stock isotope, 15.5 GBq mmol^{-1}) or [U-^{14}C]sucrose (specific activity of stock isotope, 13.4 GBq mmol^{-1}). In the solutions supplied the final concentration of isotope was 37 KBq ml^{-1}. Using this ^{14}C method, sugar uptake was quantified for 5 of the 10 individual tubers.

To determine whether the effect of sucrose on sucrose synthase and acid invertase could be achieved by supplying glucose or fructose at the same osmolarity, tubers of cv. Record were supplied with these hexoses via cut stolons. Measurements of osmolarity were taken using an automatic micro-osmometer (Hermann Roebling).

4.2.2 Enzyme determinations

Enzyme extracts were prepared from fresh tissue. Three adjacent longitudinal slices (0.5 cm thick) were taken from each tuber sampled. This allowed a direct comparison of data on enzyme activities, carbohydrate concentrations and, where relevant, [^{14}C] uptake.

4.2.2.1 Sucrose synthase

Slices of fresh potato tissue were extracted with insoluble Polyvinylpolypyrrolidone (1% w/v) and acid-washed quartz sand in 3

volumes of extraction solution.

Extraction solution:- 100 mM Tris-HCl (pH 7.5) containing 5 mM Dithiothreitol (DTT), 3 mM Magnesium acetate and 2% (w/v) glycerol. The enzyme extracts were kept on ice until all samples were prepared. After centrifugation at 20,000 g for 20 min at 4°C, extracts were dialysed for 18 h at 4°C against 10 mM extraction buffer (2 x 8 litres used). Sucrose synthase activity was assayed in the cleavage direction using the method described in section **2.3.1** at the optimum pH for the samples (6.8-6.9). The reliability of the extraction method was tested by measurement of any remaining activity found in the pellet after centrifugation and in addition by a mixing experiment with bean tissue. In the latter test, developing cotyledons of bean (*Vicia faba* L.) were extracted together with tuber tissue and the activity of sucrose synthase compared with that from independent extractions of tuber and bean material. The recovery of sucrose synthase was 95% of that expected. It was concluded that no major losses of tuber sucrose synthase activity occurred during the extraction procedure. Less than 5% of the sucrose synthase activity remained in the pellet after extraction. The importance of testing the reliability of enzyme determinations to allow meaningful interpretation of results has been stressed by ap Rees (1974).

4.2.2.2 Acid invertase

For acid invertase determination, slices of fresh potato tissue were extracted as above but in 100 mM sodium acetate buffer (pH 5) containing 10 mM sodium sulphite. After centrifugation, extracts were dialysed against 10 mM sodium acetate buffer (pH 5) for 18 h at 4°C. Dialysis was chosen as the method of de-salting extracts due to the large number of samples processed. Initial comparisons with an alternative method of rapid de-salting using Sephadex G-

25M (Pharmacia PD10 columns) indicated almost identical recoveries of both sucrose synthase and invertase activities with both desalting methods.

Invertase activity was assayed as described previously (**2.3.2**) but activity was determined both before (basal) and after (total) destroying the endogenous invertase inhibitor by extensive foaming. Total invertase activity was measured after foaming de-salted extracts as described in the previous chapter (**3.2.2.2**). The recovery of yeast invertase added to tuber extracts and taken through the entire process was determined. Greater than 80% of the activity was recovered after foaming. For non-foamed extracts, > 90% activity was recovered. The activity of yeast invertase added was approximately equal to the activity of invertase present in tubers. Both developing tubers detached from the mother plant for 12 days (high invertase samples) and tubers detached for only 5 minutes (low invertase samples) were used for recovery experiments. Results indicated no effect of tuber treatment on the recoveries of invertase activity. No acid invertase activity was detected in the pellet after extraction. For the various treatments used throughout this chapter, pH curves of invertase activity gave no indication of a distinct alkaline invertase.

4.2.3 Protein determination

Protein in enzyme extracts was quantified using the standard Bio-Rad protein assay with BSA as a standard (section **2.7**).

4.2.4 Electrophoresis and Immunoblotting

Approximately 10 µg of soluble protein extracted with the sucrose synthase extraction medium was subjected to SDS-PAGE (7.5% acrylamide) as described

previously (section **2.5.1**) and stained with Coomassie blue. In parallel gels, polypeptides were transferred to nitrocellulose and, after immunoblotting with polyclonal antibodies raised against maize sucrose synthase, cross-reacting bands were identified using anti-rabbit immunoglobulin conjugate labelled with alkaline phosphatase. The sucrose synthase antibody was raised against protein extracted from whole kernels of wild type maize and kindly supplied by Dr. Karen Koch, University of Florida.

4.2.5 Determination of starch, sucrose, glucose and fructose

Slices of potato tissue were frozen in liquid nitrogen and freeze-dried. The determinations were carried as described in sections **2.4.1** and **2.4.2.**

4.2.6 Quantification of ^{14}C taken up by tubers

Longitudinal slices from tubers supplied with radiolabelled sugars were frozen immediately and freeze-dried. Ethanol extracts were prepared by refluxing 0.5 g of freeze-dried powder with 10 ml 80% (v/v) ethanol at 70°C for 30 min (with shaking). After centrifugation (5000 g for 30 min) the resulting pellet was washed twice with boiling 80% ethanol and extracts and washings were combined. Extracts were reduced in volume to 5 ml on a hot-plate at 80°C and total [^{14}C] in each of the samples determined by liquid scintillation spectrometry using a Tri-Carb 2000 scintillation counter (Hewlett Packard). Ecoscint-A fluid (1 ml) was added to 100 µl of extract and mixed thoroughly prior to counting. Five replicates of each extract were measured. To increase the concentration of [^{14}C] prior to analysis of the ethanol-soluble carbohydrates by HPLC the samples were freeze-dried and resuspended in distilled water (1 ml).

For the determination of [^{14}C] incorporation into starch, distilled water (10 ml) was added to the ethanol-extracted pellets and the samples maintained at 100°C for 1-2 h (with regular shaking) to gelatinise the starch. After cooling and thorough, rapid mixing, a 1 ml sample was removed from each tube and incubated with amyloglucosidase as described in section 2.4.2. After the starch was completely degraded to glucose, 5 replicate samples from each incubation were removed for scintillation spectrometry.

4.2.7 Distribution of [^{14}C] in ethanol-soluble carbohydrates

The sugars - glucose, fructose, sucrose and maltose were separated on a 15 cm Apex reverse-phase amino column (5 µ particle size) using a Gilson HPLC. The samples were eluted isocratically using acetonitrile : H_2O (85 : 15 v/v) at a flow rate of 2 ml min^{-1}. Sample (90 µl) was mixed with acetonitrile (100 µl) and standard mixture of 250 mg ml^{-1} glucose, fructose, sucrose and maltose (10 µl). The mixture (200 µl) was injected and sugar peaks monitored with a refractive index detector (Fig. 4.1). Individual sugar peaks were collected and radioactivity in each sugar determined by liquid scintillation counting. Preliminary studies with [U-^{14}C] sucrose and maltose showed that 2.4% of the sucrose and 2.3% of the maltose was degraded during the extraction procedure. The efficiency of the [^{14}C] separation was also tested by injecting known activities of ^{14}C glucose, fructose, sucrose and maltose. Results shown in Table 4.1 indicate good recoveries of both glucose and sucrose. However, with ^{14}C fructose nearly 15% of activity loaded was recovered in the glucose peak and ca 2% in maltose. This is likely to be due either to contamination of supplied isotope, a problem with complete separation of glucose and fructose peaks, or a combination of both. Similarly, over 9% was recovered in glucose and approximately 6% in sucrose when the ^{14}C maltose stock isotope was

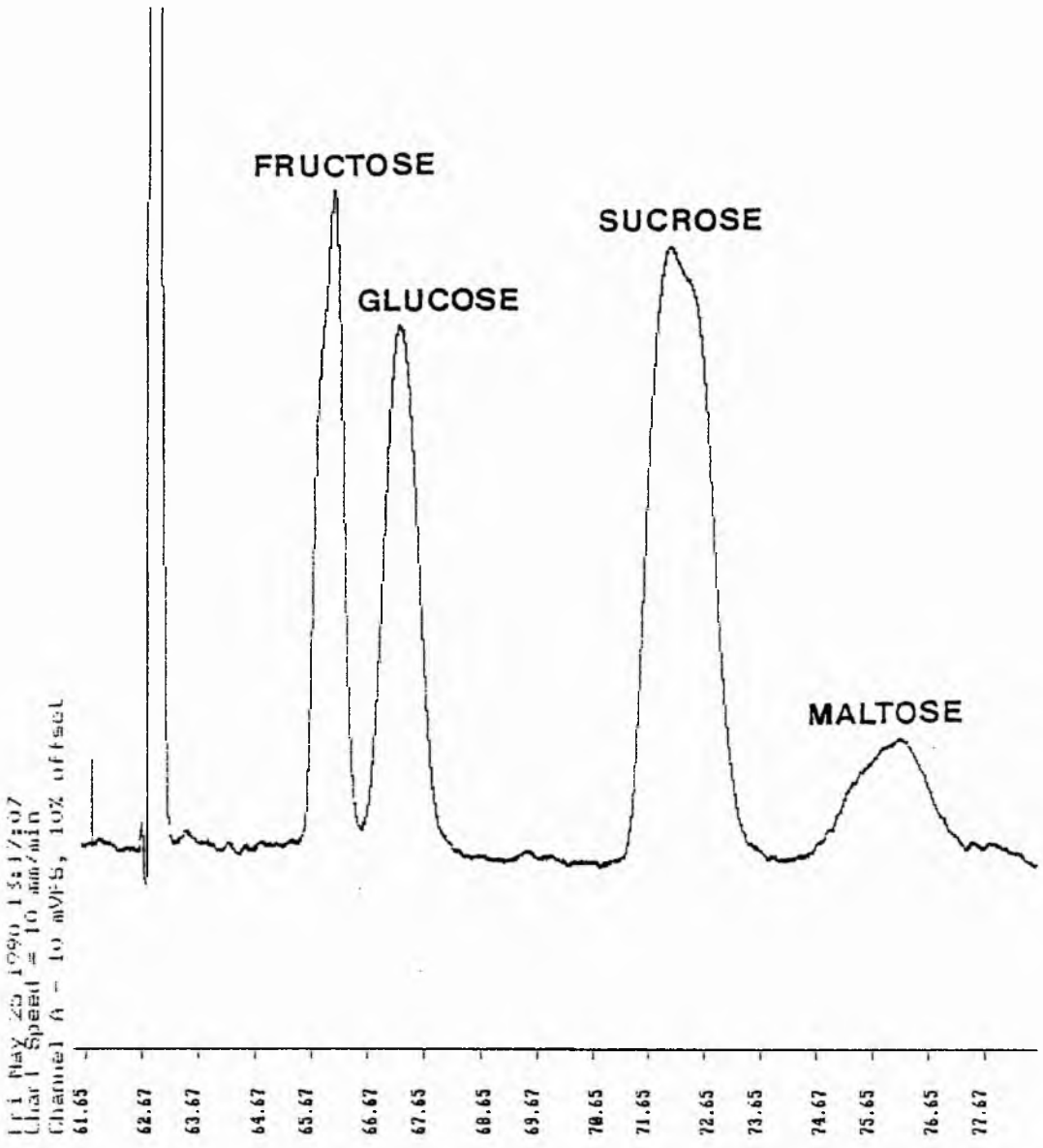


Fig. 4.1 Separation by HPLC of a mixture of sugars with a 15 cm reverse-phase NH_2 column. Mobile phase was acetonitrile : water 85 : 15 (v / v) ; flow rate was 2 ml min^{-1}.

chromatographed. The ^{14}C recovered in glucose may be due to partial maltose breakdown during storage. Incomplete separation between maltose and sucrose may explain the label recovered in the latter. The reduced recovery of maltose, however, does not affect the interpretation of the results.

Table 4.1

Test of separation	% distribution of ^{14}C between ethanol-soluble sugars			
	Fructose	**Glucose**	**Sucrose**	**Maltose**
Spiked with:-				
^{14}C fructose	82.4	14.9	0.7	2.0
^{14}C glucose	0.8	97.3	1.2	0.7
^{14}C sucrose	0.3	0.7	98.3	0.7
^{14}C maltose	0.8	9.4	5.9	83.9

4.3 Results

4.3.1 Sugar balance and enzyme activities in tubers after detachment

Tubers from field-grown plants of cvs Cara, Record and Brodick were excised on four occasions during the growing period to provide samples differing in physiological status and chronological age. The maximum catalytic activity of sucrose synthase decreased rapidly on each occasion after tuber detachment and this pattern was consistent for each cultivar (Fig. 4.2). After excision and

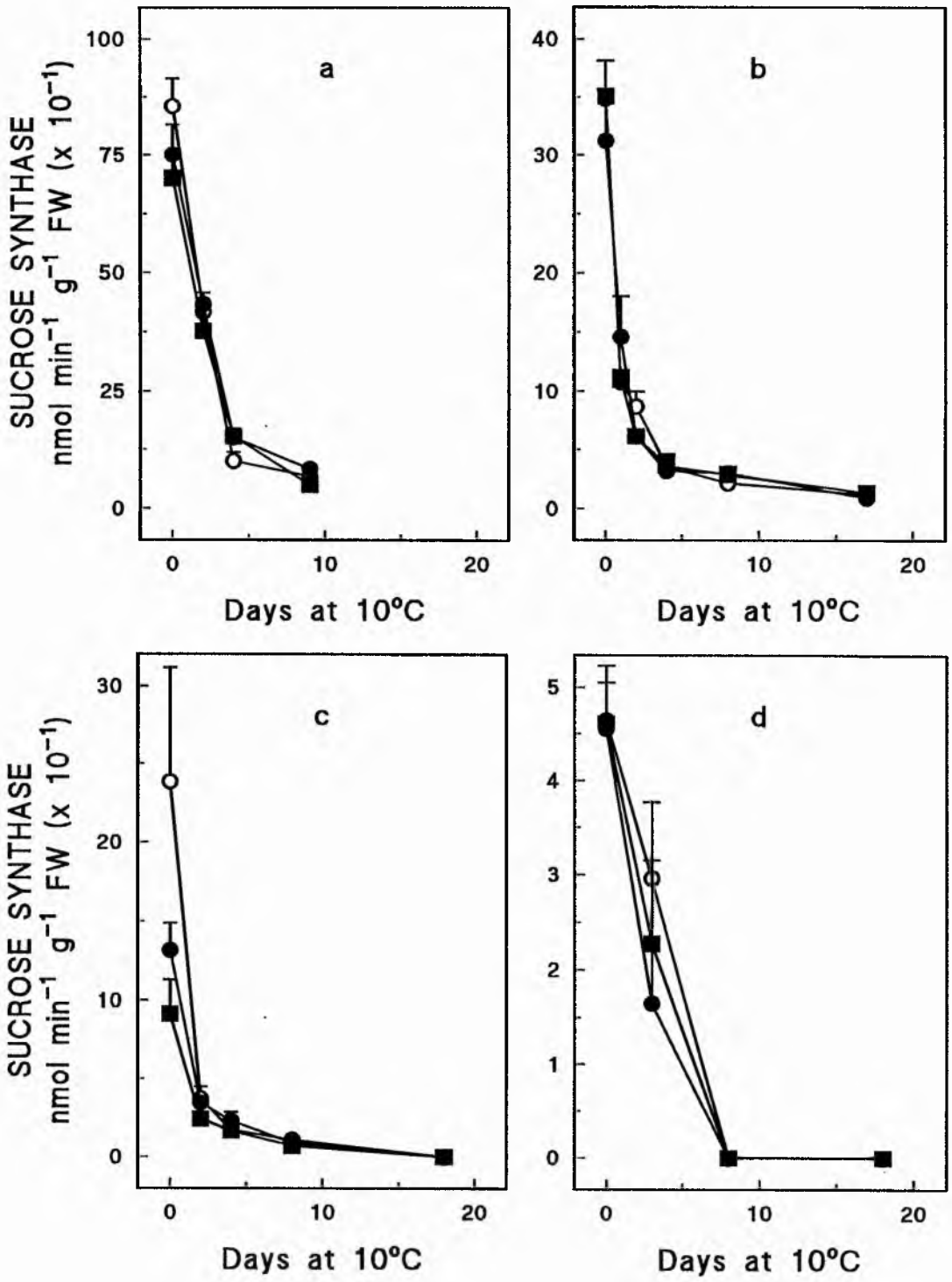


Fig. 4.5 Effect of excising developing tubers from the mother plant on tuber glucose content (——), and fructose content (----), in storage at 10°C. Tubers were excised from growing plants on August 1 (a), August 15 (b), August 29 (c) and September 26 (d). The cvs used were Cara (●), Record (○), and Brodick (■). Bars indicate SE of the mean. The ranges of the y axes are from 0 to 1000 nmol min^{-1} g^{-1} FW (a); 0 to 400 nmol min^{-1} g^{-1} FW (b); 0 to 300 nmol min^{-1} g^{-1} FW (c) and 0 to 50 nmol min^{-1} g^{-1} FW (d).

transfer to storage at 10°C, similar activities were found in all three genotypes, often declining to levels that were no longer measurable. Sucrose synthase activity measured in the tubers immediately after detachment also decreased between the first and final harvest dates.

Changes in sucrose content in tubers after excision were rather variable with no consistent differences between genotypes (Fig. 4.3). However, as with sucrose synthase activity, the sucrose content declined over the same harvest intervals (Fig. 4.4).

By contrast, at each harvest date and for each genotype, glucose and fructose concentrations were low at the time of excision but increased substantially within the first few days in storage (Fig. 4.5). As expected from previous results (Richardson *et al.*, 1990), the three genotypes showed consistent differences in the rate and extent of glucose and fructose accumulation, with cv. Cara accumulating the highest concentrations of both hexoses and cv. Brodick the lowest (Fig. 4.5). Additionally, tubers sampled on the first 2 occasions accumulated more glucose and fructose during subsequent storage than tubers from the later harvests. The glucose content of the tubers was always higher than that of fructose with the G : F ratio decreasing in tubers during storage as shown for harvest 3 and 4 (Table 4.2).

Tuber excision also resulted in a rapid and substantial increase in acid invertase activity at each stage of tuber development examined. This was evident when both basal and total activities were determined (Figs. 4.6 and 4.7). In some cases, a 10-fold increase in activity occurred within 3 days after excision. In general, invertase activity in the high hexose accumulating genotype Cara was significantly and consistently higher than in the lowest hexose accumulator, Brodick. Vortexing the extracts, in an attempt to achieve maximum catalytic activity, always resulted in higher levels of acid invertase

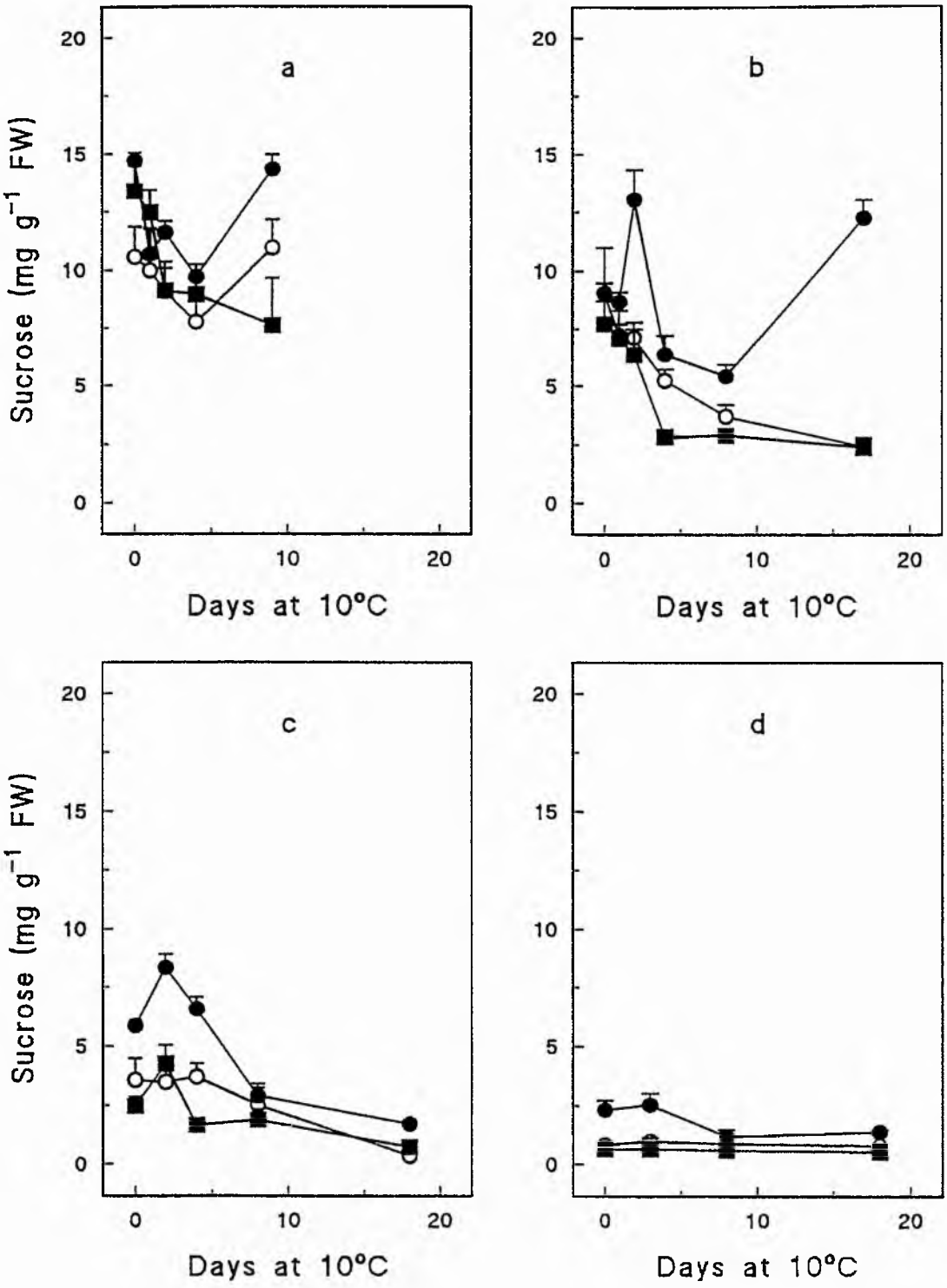


Fig. 4.3 Effect of excising developing tubers from the mother plant on tuber sucrose content in storage at 10°C. Tubers were excised from growing plants on August 1 (a), August 15 (b), August 29 (c) and September 26 (d). The cvs used were Cara (●), Record (○), and Brodick (■). Bars indicate SE of the mean.

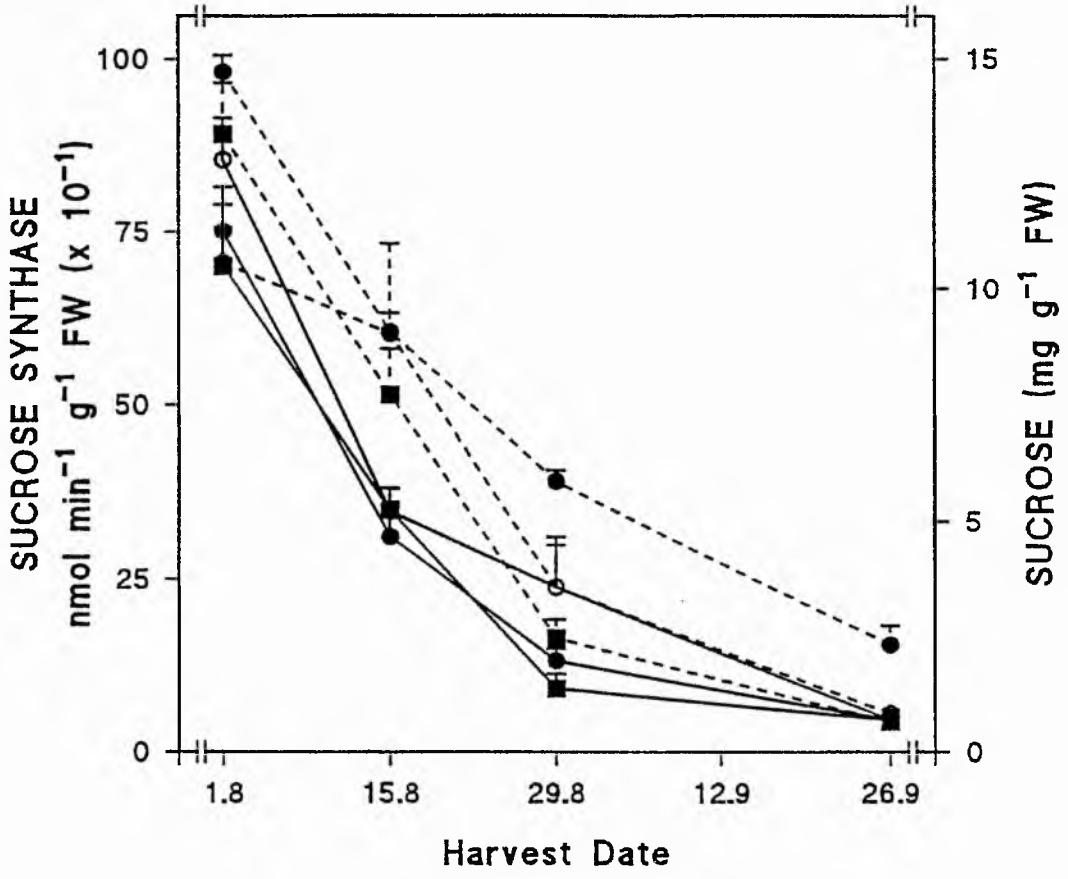


Fig. 4.4 Sucrose synthase activity in (——), and sucrose content of (-----), tubers sampled at four stages during the growing season. Key to genotypes: Cara (●), Record (○), and Brodick (■). Values are shown as means + SE.

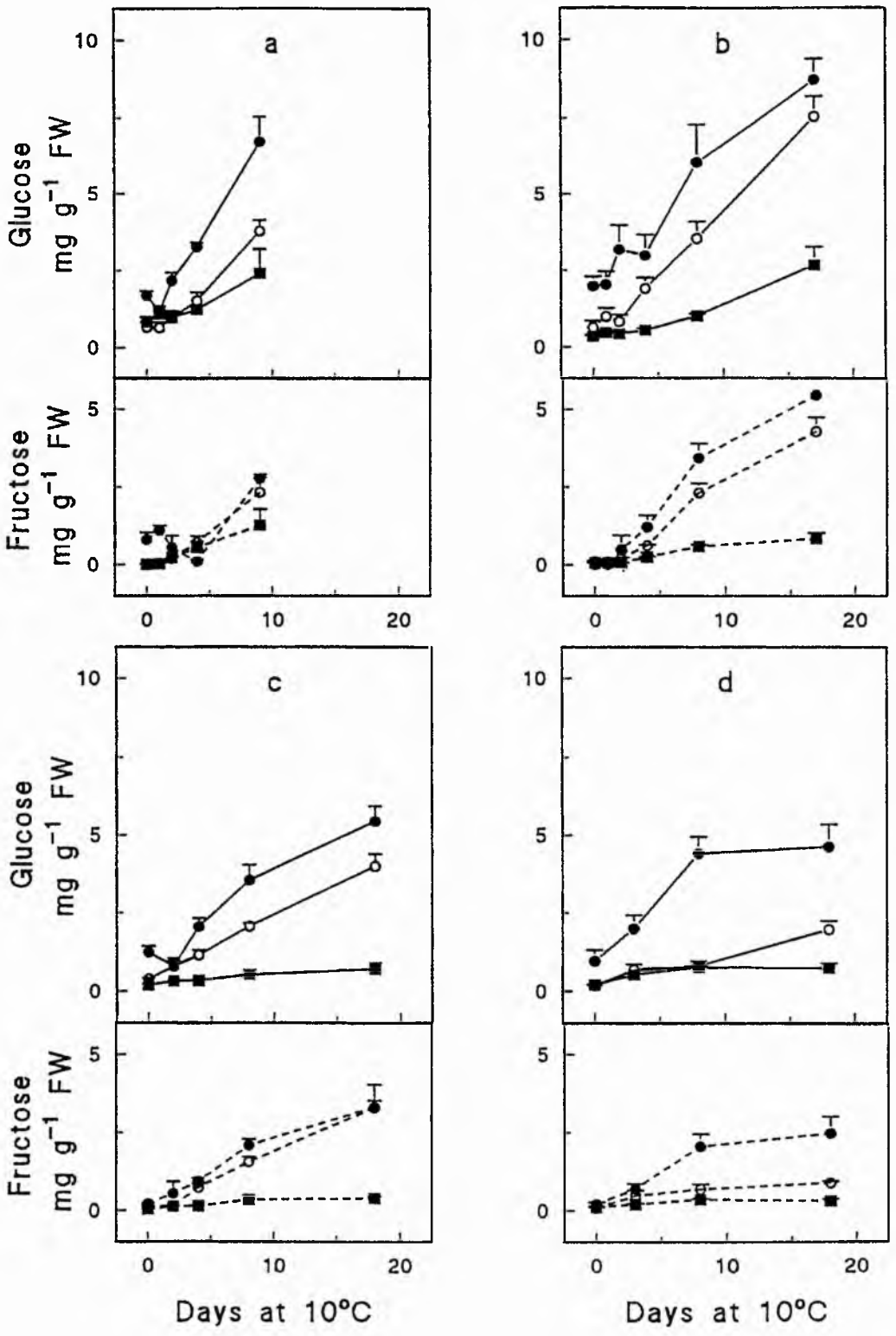


Fig. 4.5 Effect of excising developing tubers from the mother plant on tuber glucose content (——), and fructose content (----), in storage at 10°C. Tubers were excised from growing plants on August 1 (a), August 15 (b), August 29 (c) and September 26 (d). The cvs used were Cara (●), Record (○), and Brodick (■). Bars indicate SE of the mean.

Table 4.2 Glucose:Fructose ratios in tubers from harvest 3 and 4 at and after excision from the mother plant. Values are means of five replicates and are shown ± SE

	Harvest 3 (August 29)				
	Days at 10°C after excision				
Cultivar	0	2	4	8	18
Record	3.57±0.75	5.05±1.02	1.57±0.08	1.36±0.08	1.21±0.05
Cara	6.39±0.59	4.49±1.72	2.40±0.17	2.08±0.44	1.88±0.21
Brodick	4.45±1.03	2.48±0.16	2.64±0.50	1.50±0.12	1.65±0.08
	Harvest 4 (September 26)				
	0	3	8	18	
Record	2.03±0.25	1.91±0.54	1.21±0.06	2.12±0.29	
Cara	5.27±1.05	2.95±0.21	2.73±0.54	2.32±0.55	
Brodick	1.13±0.32	1.53±0.09	1.12±0.06	1.53±0.11	

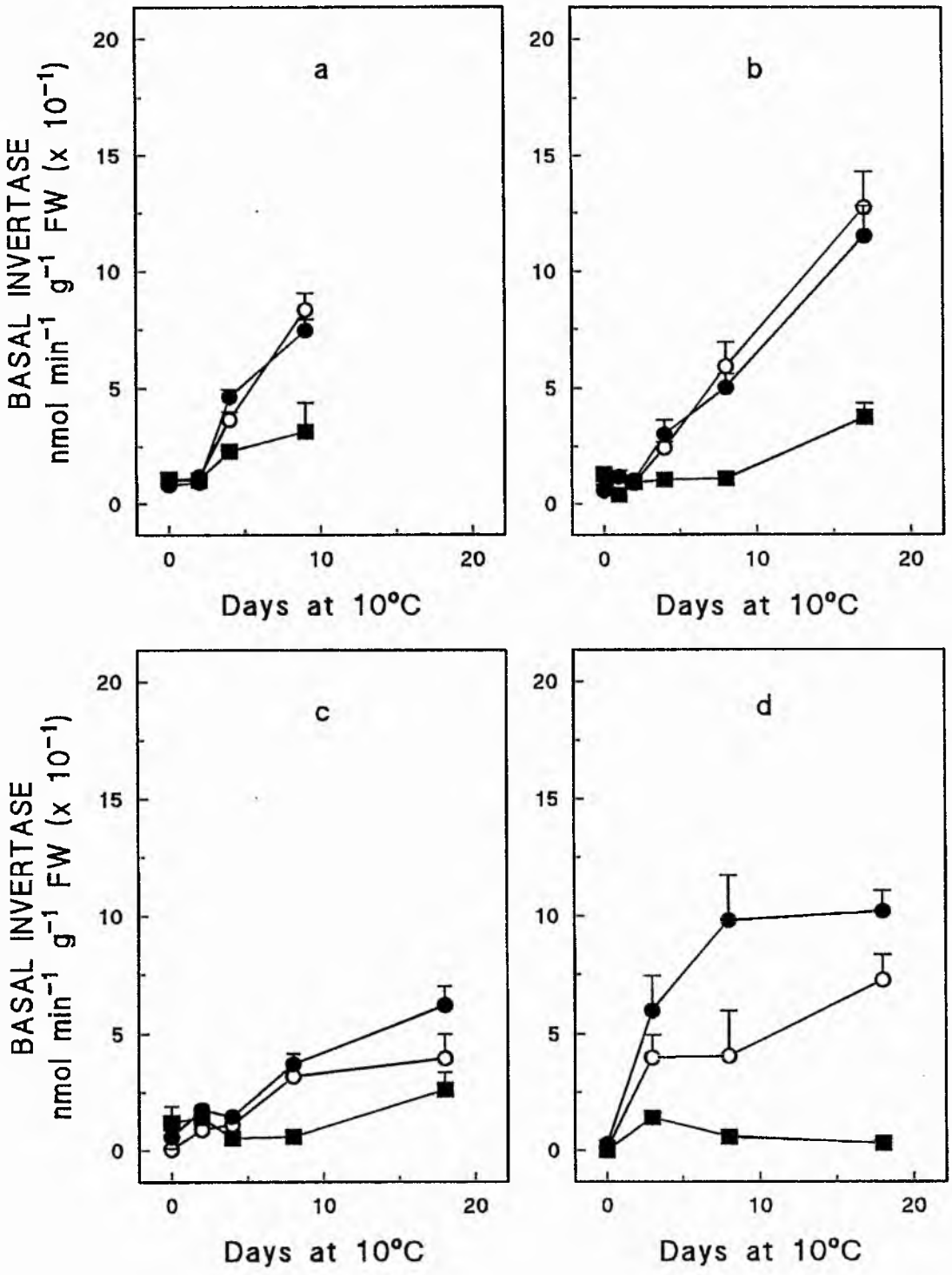


Fig. 4.6 Effect of tuber excision on the development of basal invertase activity in storage at 10°C. Tubers were excised from growing plants on August 1 (a), August 15 (b), August 29 (c) and September 26 (d). The cvs used were Cara (●), Record (○), and Brodick (■). Bars indicate SE of the mean. The range of all y axes is from 0 to 200 nmol min^{-1} g^{-1} FW.

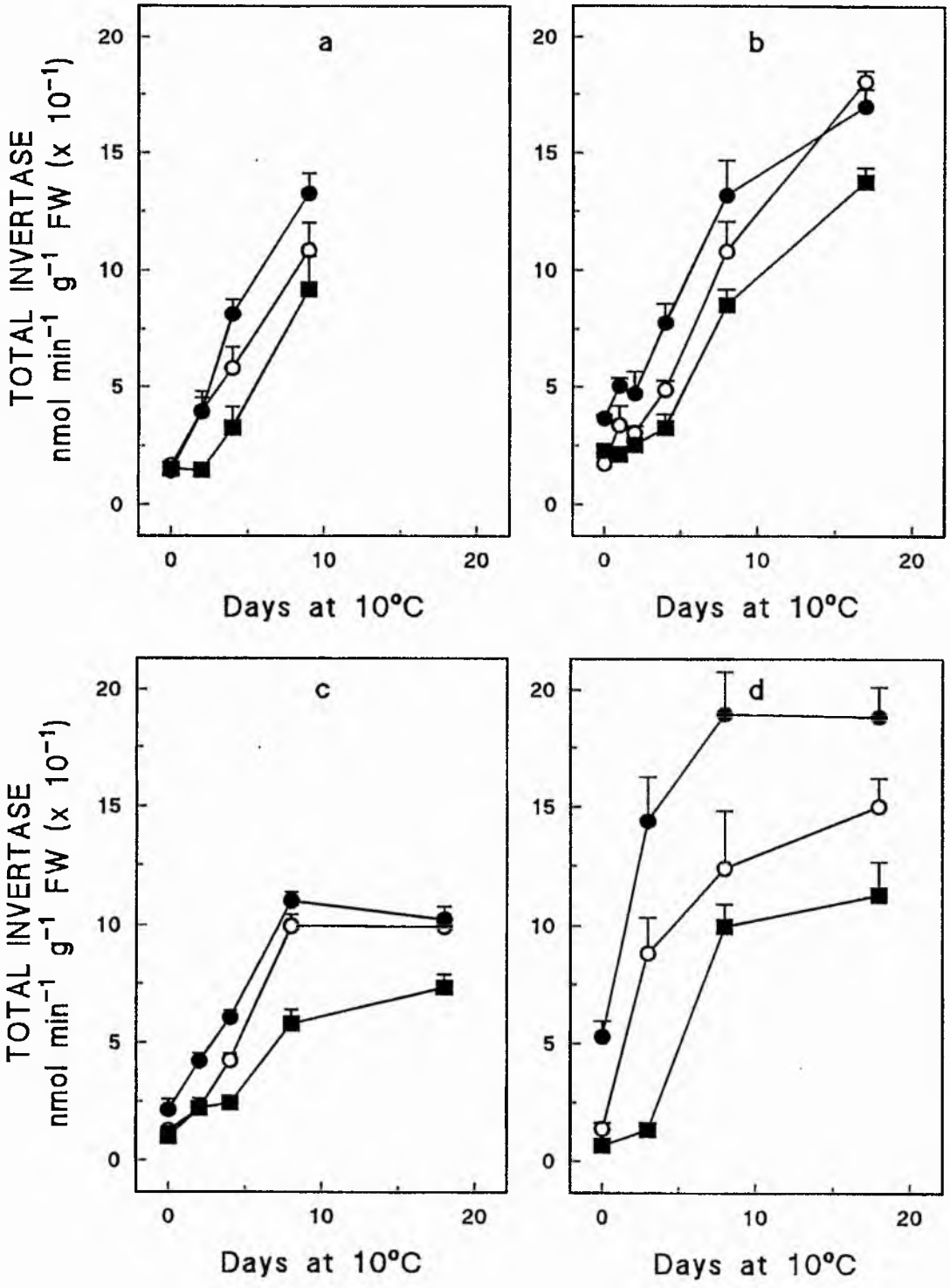


Fig. 4.7 Effect of tuber excision on the development of total invertase activity in storage at 10°C. Tubers were excised from growing plants on August 1 (a), August 15 (b), August 29 (c) and September 26 (d). The cvs used were Cara (●), Record (○), and Brodick (■). Bars indicate SE of the mean. The range of all y axes is from 0 to 200 nmol min^{-1} g^{-1} FW.

activity, although the degree of activation varied with tuber age, genotype and duration of tuber storage at 10°C. For example, increases in activity after vortexing extracts from tubers of cv. Record, Cara and Brodick, sampled immediately after the first harvest, were approximately 1.5-fold for all three cultivars. For tubers sampled immediately after the last harvest, increases were approximately 40-fold, 20-fold and 70-fold for each cultivar, respectively. Overall, the differences between basal and total activity were highest for the lowest hexose accumulating genotype, Brodick. During storage at 10°C, the extent of the difference between total and basal activity decreased. Thus by the end of the storage period for the last harvest, increases induced by vortexing were only 2-fold for Record and Cara and 30-fold for Brodick. This contrasts significantly with the increases obtained with freshly harvested tubers referred to above.

4.3.2 Effect of modifying sucrose supply to tubers

Eliminating light interception by plants produced the same effect as tuber detachment. Sucrose content and sucrose synthase activity both decreased and hexose content and total acid invertase activity both increased (Fig. 4.8). While the sucrose synthase activity and sucrose content of tubers from control plants decreased between the first and second sample dates, thereafter, the sucrose synthase activity and sucrose content of darkened plants were significantly lower. Acid invertase activity and hexose concentrations of tubers from both darkened and control plants increased between the second and third sample dates, but thereafter were significantly higher in darkened plants. The magnitude of the effect of covering plants on basal and total invertase activities was similar, although basal activity was almost threefold lower than total (data not shown).

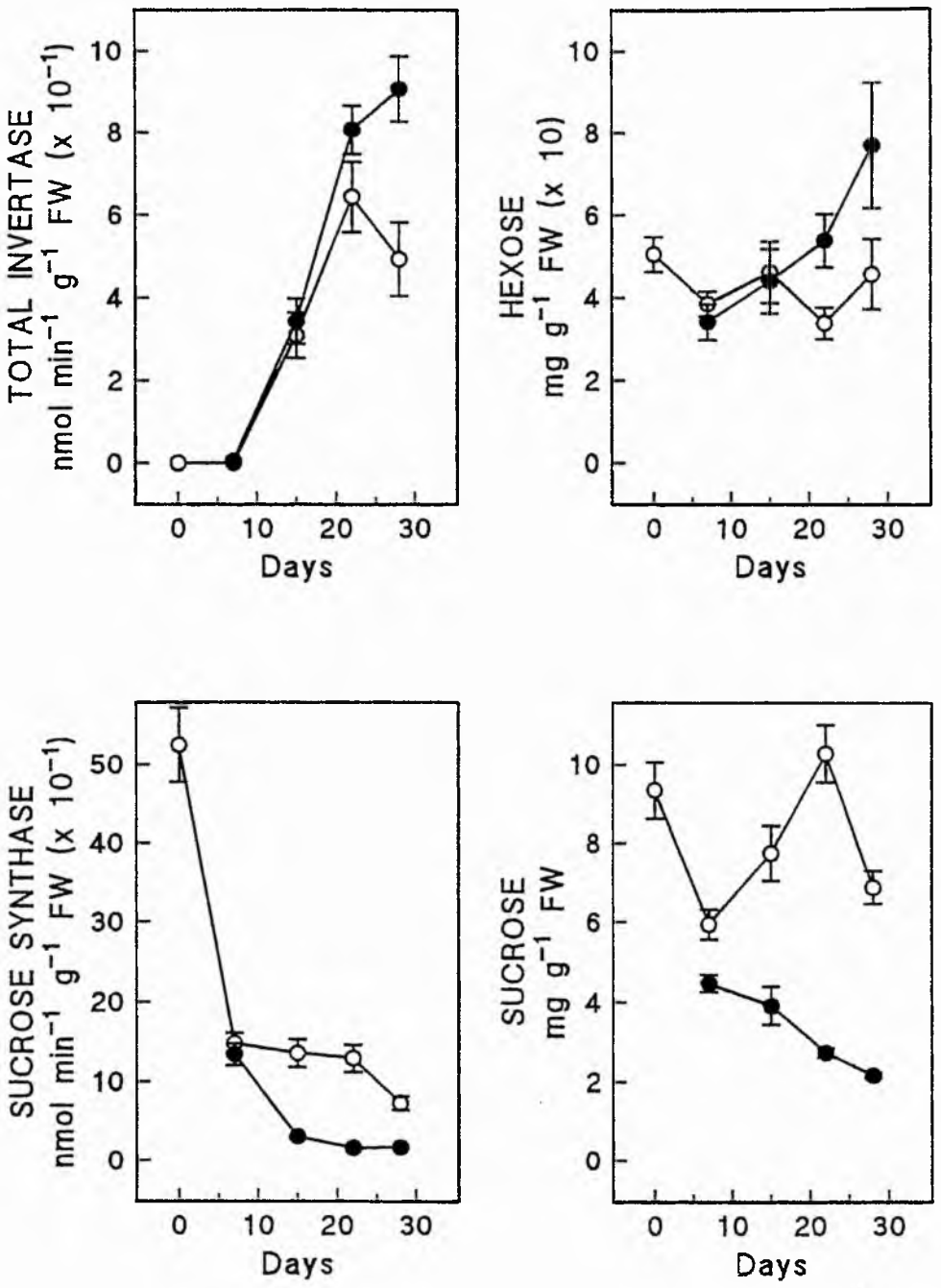


Fig. 4.8 Effects of eliminating light interception for up to 28 days on tuber invertase (total) and sucrose synthase activities and sugar content. Light interception prevented (●); control (○). Bars show SEM.

4.3.3 Effect of supplying sucrose exogenously to intact tubers

Initially, detached tubers (cv Record) were supplied through the cut stolon surface with either water alone or with sucrose solutions at a range of concentrations. The activities of both sucrose synthase and acid invertase were determined after 12 days (Table 4.3). The differences between basal and total invertase activities were not significant so only total activity is shown. Highest acid invertase activity occurred in the tubers supplied with water (control) for 12 days. This activity was nearly five-fold higher than at day 0 (immediately after tuber detachment). In contrast, sucrose synthase activity decreased almost three-fold. Increasing the concentration of exogenously supplied sucrose from 150 mM to 750 mM resulted in lower acid invertase activity and higher sucrose synthase activity (relative to H_2O control). A concentration of sucrose as high as 1500 mM disturbed this relationship, possibly due to serious osmotic effects.

To test the effect of other sugars (supplied as above to tubers) on tuber enzyme activities, the experiment was repeated but in addition to supplying sucrose at three concentrations (300 mM, 750 mM, 1500 mM), treatments with glucose (750 mM) and fructose (750 mM) were also used. The concentrations of sucrose, glucose and fructose (all at approximately 750 mM) were adjusted to allow a comparison of solutions at similar osmolarities (Table 4.4). Total acid invertase activity once again increased significantly (over ten-fold) after the tubers were detached and supplied with water (control) for 12 days while sucrose synthase activity decreased 15-fold in the same tubers (Table 4.4). Highest invertase activity occurred as before (Table 4.3) when tubers were supplied with water but decreased significantly when tubers were supplied with sucrose, glucose or fructose. Sucrose synthase activity on day 0 for these samples was considerably higher than for the first trial (Table 4.3). This may be because the tubers were at a physiologically more active stage in development or due to environmental factors, e.g. high light intensity on the day of harvest,

Table 4.3 Effect of sucrose concentration supplied to detached tubers (maintained at 10°C) on sucrose synthase and acid (total) invertase activities. Day 0 – enzyme activities immediately after tuber detachment. Day 12 – enzyme activities 12 days after supplying sucrose. Values are ± SE of the mean (n = 5)

Treatment	Sucrose synthase	Total invertase
	nmol min^{-1} g^{-1} fresh wt	
Day 0	154± 32	30±17
Day 12 control (H_2O)	57± 7	143±12
150 mM sucrose	85± 21	111±44
300 mM sucrose	106± 10	66± 8
750 mM sucrose	260±103	71±18
1500 mM sucrose	93± 22	122±29

Table 4.4 Effect of sucrose, glucose and fructose supplied to detached tubers on sucrose synthase and acid (total) activities (stored at 10°C). Day 0 – enzyme activities immediately after tuber detachment. Day 12 – enzyme activities 12 days after supplying sugars. Values are ± SE of the mean (n = 10 except for glucose and fructose for which n = 3)

Treatment	Sucrose synthase	Total invertase
	nmol min^{-1} g^{-1} fresh wt	
Day 0	1389±167	57±16
Day 12		
Air	44± 5	528±54
H_2O	88± 19	722±48
300 mM sucrose (342.5 mosmol Kg^{-1})	149± 33	506±54
750 mM sucrose (1050 mosmol Kg^{-1})	163± 23	430±64
1500 mM sucrose (3846 mosmol Kg^{-1})	222± 33	444±49
750 mM glucose (1157 mosmol Kg^{-1})	174± 37	378±10
750 mM fructose (1119 mos mol Kg^{-1})	122± 10	531±54

resulting in increased sucrose flux to the developing tubers. However, a positive effect of supplying sucrose on sucrose synthase activity was observed on both occasions. Increased sucrose synthase activity (relative to H_2O control) also occurred in tubers supplied with glucose or fructose. However, this data was obtained from only three tubers due to problems in maintaining the stolons in a viable condition when immersed in glucose or fructose solutions.

In the final trial, the two methods of uptake were used as shown in Plate 4.I and the effect of the disaccharide maltose (an α (1→4) glucan) compared with that of sucrose. Again, total invertase activity increased (almost 7-fold) when tubers were excised and supplied with water via their stolons (Table 4.5). The invertase activity also increased when the tubers were supplied with water through a channel (approximately 5-fold). The increase in invertase was less when tubers were supplied with maltose or sucrose. Conversely, sucrose synthase activity decreased ten-fold after tuber excision when the tubers were supplied with water and once again the decline was minimised by supplying sucrose. Maltose did not have a significant effect on sucrose synthase activity.

From samples of tubers used in the above experiment and supplied with [^{14}C] sucrose or [^{14}C] maltose, the radioactivity recovered in the soluble and insoluble fractions was used to quantify sugar uptake (Table 4.6). These results show a much higher rate of uptake via the stolons than via a channel cut through the tubers. When uptake values for individual tubers supplied with sucrose via their stolons was calculated for the 12 day period and plotted against the sucrose synthase activity in the same tuber, there was a significant correlation ($r = 0.75$) (Fig. 4.9A). When tubers were supplied via the stolons with maltose, the correlation between maltose uptake and sucrose synthase activity was poor ($r = -0.38$) (Fig. 4.9B). Similarly, there was no significant correlation between invertase activity and sucrose uptake (total activity - $r = -0.28$, basal activity - $r = -0.061$) and the scatter of points showed only a slight

Table 4.5 Effect of sucrose and maltose supplied to detached tubers via stolons or via a channel cut in the tuber on sucrose synthase and acid invertase (total) activities. Day 0 – enzyme activities immediately after tuber detachment. Day 12 – enzyme activities 12 days after supplying sugars. Values are ± SE of the mean (n = 10)

Treatment	Sucrose synthase	Total invertase
	nmol min^{-1} g^{-1} fresh wt	
Day 0	545±95	25± 3
Day 12 via stolons		
H_2O	54± 9	171± 8
750 mM maltose	89± 8	84±14
750 mM sucrose	129±26	65±15
1500 mM sucrose	170±36	111±22
Day 12 via channel		
H_2O	98±11	117±12
300 mM maltose	95±15	73± 9
300 mM sucrose	173±23	61±12

Table 4.6 [^{14}C] Uptake by tubers supplied with [U–^{14}C] sucrose or [U–^{14}C] maltose via stolons or via a channel cut in the tuber over a period of 12 days. Values shown are ± SE of the mean (n = 5)

	^{14}C Uptake mg sucrose or maltose equivalent g^{-1} FW 12 $days^{-1}$
via stolons	
750 mM [U–^{14}C] maltose	5.28±0.57
750 mM [U–^{14}C] sucrose	8.04±1.20
1500 mM [U–^{14}C] sucrose	12.24±1.24
via channel	
300 mM [U–^{14}C] maltose	1.31±0.39
300 mM [U–^{14}C] sucrose	1.38±0.41

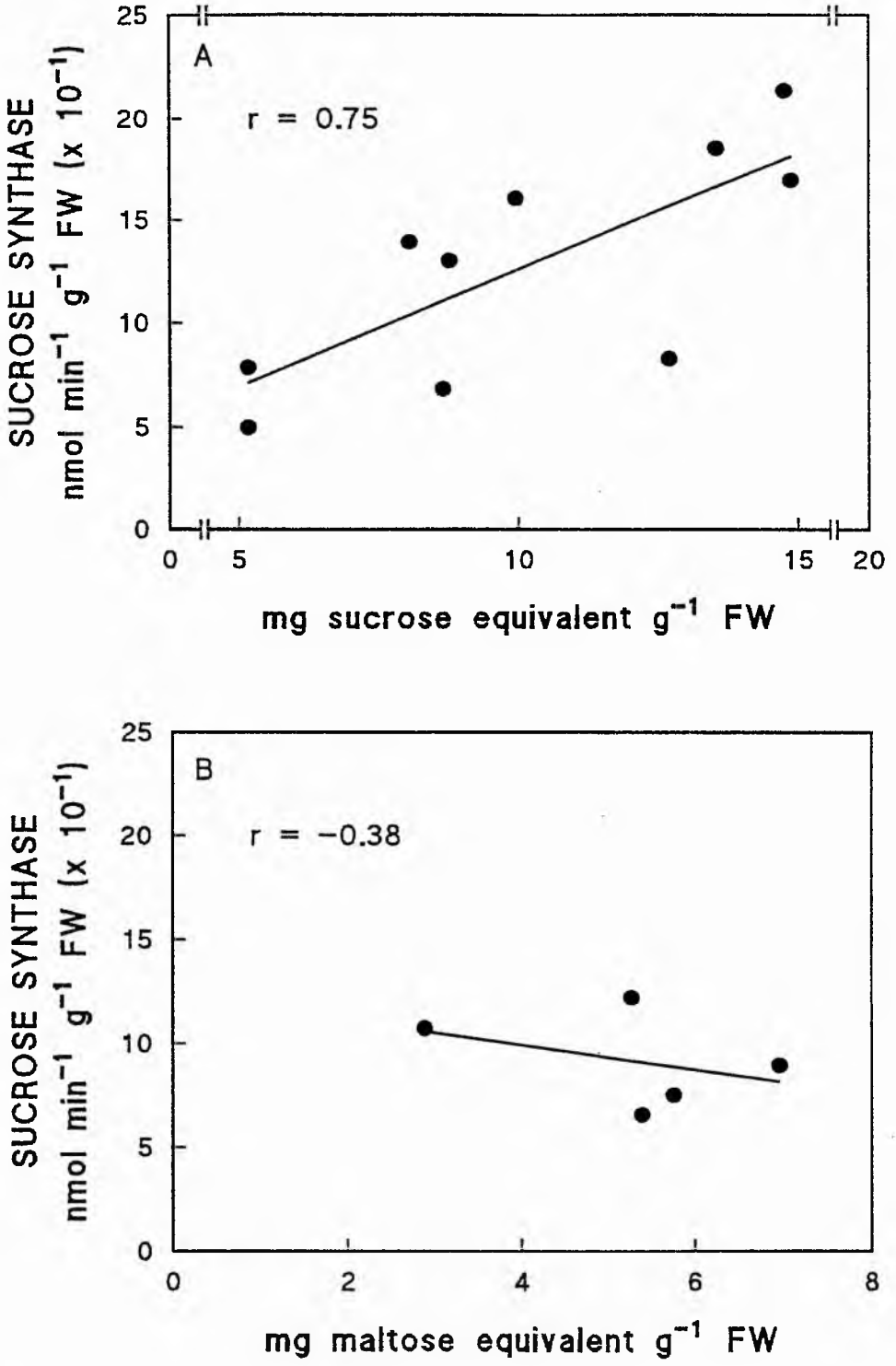


Fig. 4.9 Correlation between sucrose synthase activity and overall sucrose (A) or maltose (B) flux into individual tubers. Tubers were excised from the mother plant and the cut stolon surface immersed in 750 mM sucrose, 1500 mM sucrose or 750 mM maltose containing a known specific activity of [U-^{14}C] sucrose or [U^{14}C] maltose. ^{14}C incorporated after 12 days' incubation was used to determine the quantity of sucrose or maltose transported.

tendency of lower activity with higher sucrose uptake (data not shown). No significant relationship was found when values for either sucrose or maltose taken up via a channel (method 2) were plotted against sucrose synthase or invertase activity (data not shown).

The concentrations of glucose, fructose and sucrose in tubers used in the above experiment were also determined (Table 4.7). Tuber excision stimulated substantial increases in hexose levels while sucrose levels remained relatively constant. There was a significant decrease in the G : F ratio from 15:1 to an average of 1.3:1 in control tubers after 12 days (data derived from Table 4.7). Supplying maltose and sucrose resulted in significant increases in sucrose levels. Hexose levels were also increased in these treatments although the effect was not as marked as with tuber excision.

HPLC analysis showed that at the end of the incubation period with [^{14}C]sucrose more than 50% of radiolabel in the ethanol soluble fraction was present as sucrose, 1 to 2% as maltose and the remainder equally distributed between fructose and glucose. With [^{14}C]maltose, 50% of the label was recovered as sucrose, 7% as maltose and the remainder again equally distributed between glucose and fructose (Table 4.8). Despite the substantial conversion of maltose into sucrose, supplying maltose did not significantly reduce the decline in sucrose synthase activity (Table 4.5).

To determine whether the effect of sucrose on sucrose synthase activity reflected changes in the level of enzyme protein, samples from the above experiment were immunoblotted with maize sucrose synthase antibody as previously described (section 4.2.4). This revealed a cross-reacting polypeptide (M_r 90,000) which was visible in a sample from a tuber prior to detachment and from tubers after detachment when supplied with sucrose (Plate 4.IIA). The respective activities of sucrose synthase in extracts electrophoresed in

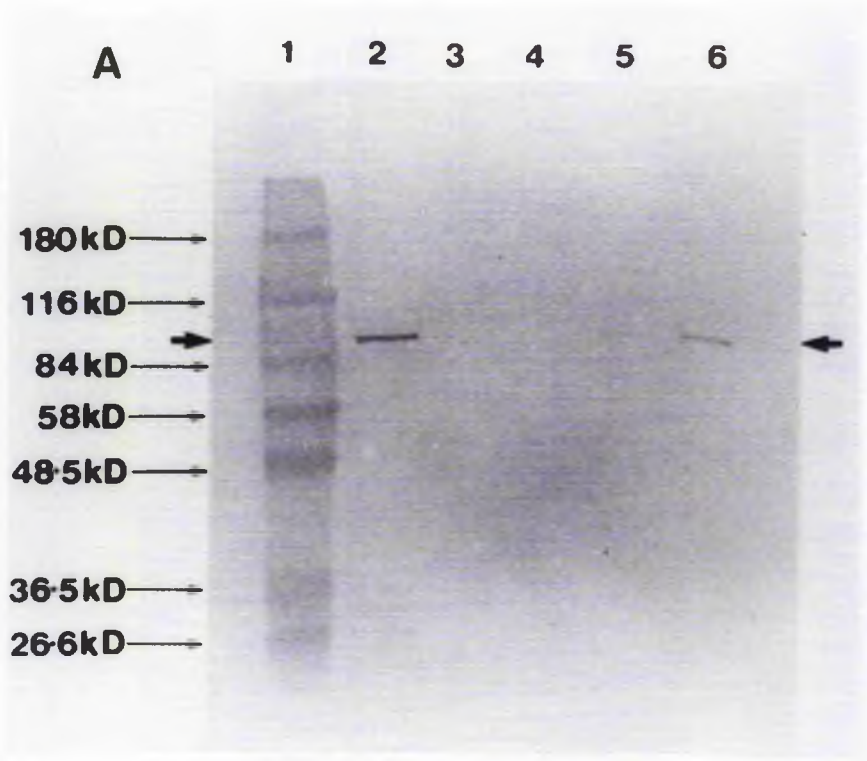
Table 4.7 Effect of sucrose and maltose supplied to detached tubers via stolons or via a channel cut in the tuber on glucose, fructose and sucrose contents. Day 0 – sugar content immediately after tuber detachment. Day 12 – sugar content 12 days after supplying sugars. Values are ± SE of the mean (n = 10)

Treatment	Glucose	Fructose	Total Hexoses	Sucrose
		mg g^{-1} FW		
Day 0	1.98±1.00	0.13±0.04	2.09±1.04	3.96±1.66
Day 12 via stolons				
H_2O	4.07±0.47	3.10±0.28	7.17±0.66	4.33±0.43
750 mM maltose	5.51±0.57	5.58±0.63	11.09±1.18	9.12±1.04
750 mM sucrose	6.12±0.75	5.63±0.69	11.76±1.43	10.74±1.47
1500 mM sucrose	8.45±1.36	5.78±0.76	14.11±2.05	10.40±1.95
Day 12 via channel				
H_2O	4.85±0.88	2.91±0.42	7.76±1.21	4.80±0.46
300 mM maltose	5.71±0.53	4.09±0.46	9.89±0.99	9.88±1.18
300 mM sucrose	5.90±0.52	4.19±0.32	10.08±0.83	12.37±1.00

Table 4.8 Distribution of radiolabel between various ethanol soluble sugars in tubers supplied with [U-^{14}C] sucrose or [U-^{14}C] maltose via stolons or via a channel cut in the tuber over a period of 12 days at 10°C. Values shown are means (n = 3). SEMs ≤ 10% of values

	% distribution of ^{14}C between ethanol soluble sugars			
Treatment	Fructose	Glucose	Sucrose	Maltose
via stolons				
750 mM [U-^{14}C] maltose	19.5	22.6	50.6	7.3
750 mM [U-^{14}C] sucrose	23.8	27.3	47.0	1.8
1500 mM [U-^{14}C] sucrose	17.2	20.3	60.9	1.5
via channel				
300 mM [U-^{14}C] maltose	16.5	23.6	53.4	6.5
300 mM [U-^{14}C] sucrose	17.9	19.0	59.5	3.5

Plate 4.II A) Immunoblot of crude potato extracts with antibodies raised against maize sucrose synthase. Lanes: 1, MW markers (SDS-7B Sigma); 2, newly harvested tuber; 3, 4, 5, and 6, 12 days after supplying excised tubers with water, 750 maltose, 750 mM sucrose, and 1500 mM sucrose, respectively. B) Coomassie-stained 7.5% SDS-polyacrylamide gel of crude potato extracts (protein loading 10 μg). Lane numbers correspond with treatments described in panel A). Arrow indicates sucrose synthase polypeptide.


A
1
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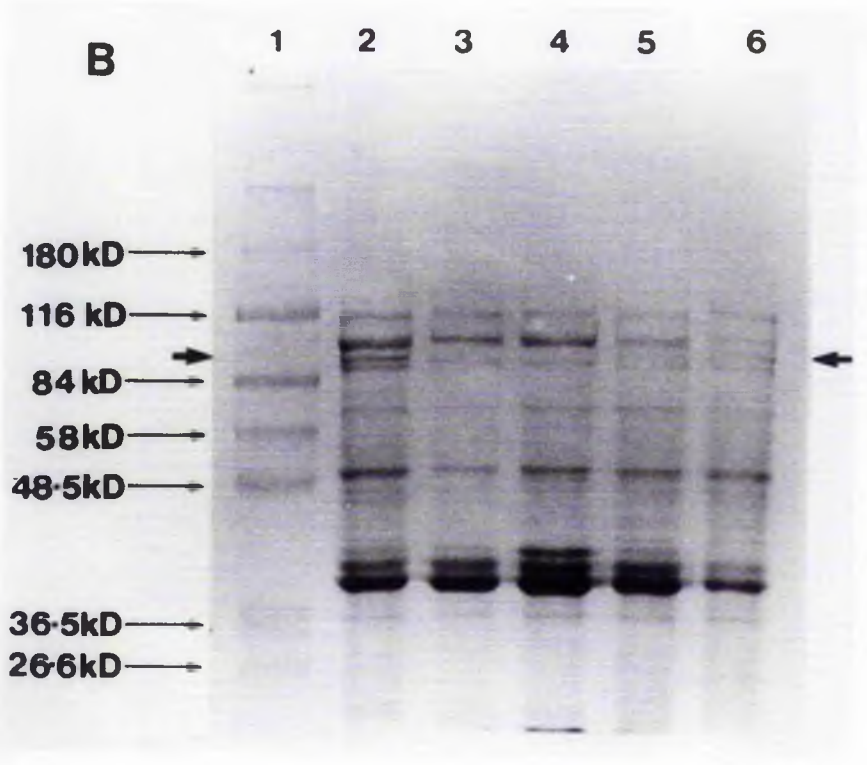
116kD
84kD
58kD
48·5kD
36·5kD
26·6kD


B
1
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180kD
116 kD
84 kD
58kD
48·5kD
36·5kD
26·6kD

lanes 2, 3, 4, 5 and 6 were 412, 45, 39, 281 and 445 nmol min^{-1} g^{-1} fresh weight. The protein content of each of the samples was determined and approximately 10 µg of each was loaded on to the gel (Plate 4.IIB). Although lane 6 appears to be underloaded by comparison with lanes 2-5, Coomassie staining also revealed a polypeptide (M_r - approximately 90,000), which was only visible in the extracts containing high sucrose synthase activity.

At this stage of the investigation, no acid invertase antibody was available to test the effect of the treatments on the relative amounts of invertase protein.

4.4 Discussion

4.4.1 The effect of tuber excision on carbohydrate metabolism

Excision of developing tubers from the plant caused a rapid decrease in sucrose synthase activity irrespective of plant age or genotype. As evidence from the studies of maize mutants low in sucrose synthase activity implies the involvement of the sucrose synthase pathway in starch synthesis (Chourey and Nelson, 1978), a decrease in the capacity for starch synthesis would be expected in excised tubers. The results of Oparka *et al.* (1990) demonstrated a 70% reduction in the incorporation of [U-^{14}C]sucrose and [U-^{14}C]glucose into starch following the excision of developing tubers from the mother plant. However, a 45% reduction in ADP-glucose pyrophosphorylase also occurred which may be the primary cause for the decline in the capacity for starch synthesis. Nevertheless, their results showing 4-fold higher incorporation into starch with glucose rather than sucrose suggests that sucrose synthase represents a rate-limiting step for starch synthesis *in vivo*. The expression of the genes encoding both sucrose synthase and ADP-glucose pyrophosphorylase are strongly sucrose-inducible (Salanoubat and Belliard, 1989; Müller-Röber *et*

al., 1990). The decrease in tuber sucrose content with chronological age (Fig. 4.4) occurs in parallel with a decline in sucrose synthase activity in all genotypes tested. This again implies a link between sucrose level or flux and the level of sucrose synthase protein and activity. A decline in sucrose synthase activity with tuber age and a more rapid decline in activity in tubers after detachment from the plant were also reported by Pressey, (1969a) for two potato cultivars, Norchip and Kennebec.

The variable sucrose content in tubers after harvest (Fig. 4.3) may be explained in several ways, as follows.

1) Variable rates of starch breakdown providing a fluctuating rate of supply of glucose-1-P (via phosphorylase) or glucose (via amylases and α-glucosidase) to support sucrose formation (Davies, 1990). In general, the overall decline in sucrose content does not always accompany, and is insufficient to account for, the extent of hexose accumulation in the stored tubers (Fig. 4.5). This again indicates that the sucrose pool is replenished via starch breakdown (Isherwood, 1973). The loss of starch needed to account for observed increases in hexoses is not measurable with any accuracy (70% of tuber dry weight is starch, but less than 1-2% is soluble sugar).

2) Variations in the rate of starch synthesis in stored tubers. Stored tubers, can, for some as yet undefined period of storage, incorporate ^{14}C glucose into starch (up to 50% of label taken up by the storage parenchyma) as shown by Viola, (1991). Since hexose phosphates are used to support starch synthesis (Viola *et al.*, 1991) this would limit the supply of intermediates for sucrose formation.

3) Changes in the phosphorylation status of sucrose phosphate synthase and hence its activation status (Huber *et al.*, 1992).

4) For the first three harvest dates (when sucrose synthase activity is in excess of 100 nmol min^{-1} g^{-1}FW) a futile cycling of sucrose via sucrose synthase (breakdown) and sucrose phosphate synthase (synthesis) may be occurring. Sucrose phosphate synthase activity is reported to be high in tubers after harvest (Pressey, 1970). A similar hypothesis has been postulated by Müller-Röber *et al.* (1992) to explain the high levels of sucrose accumulating in tubers from transgenic plants in which ADP-glucose pyrophosphorylase activity is downregulated. In these tubers the expression of sucrose synthase mRNA was unchanged whilst the expression of sucrose phosphate synthase was increased substantially.

5) Variable rates of sucrose cleavage occurring due to compartmentation. When, as following the last harvest date, sucrose synthase activity is extremely low in stored tubers, the major sucrose-cleaving enzyme, acid invertase, will only cleave sucrose if the sucrose is in the vacuole. Little is known about the compartmentation of newly synthesised sucrose generated from starch breakdown products in stored tubers. However, if the residence time of newly synthesised sucrose in the cytosol is significant then acid invertase will not function. The results showing that after tuber excision invertase activity (both basal and total) increases concomitantly with hexoses and that, across genotypes, the highest invertase activity coincides with highest hexose concentrations provide evidence that acid invertase mediates sucrose cleavage in storage. Additional evidence for this is a decrease in the G : F ratio from ca 4 : 1 at harvest (e.g. harvest 3, Table 4.2) to almost 1 : 1 after storage for 18 days at 10°C.

In developing tubers only a very small amount of incoming sucrose is thought to be stored as sucrose within the vacuole (Mares and Marschner, 1980; Morrell and ap Rees, 1986). If this is the case then, as shown in the previous chapter, the major fate of sucrose arriving in the storage cells of the developing tuber is

conversion to fructose and UDP-glucose by sucrose synthase. Following the excision of developing tubers from the mother plant any sucrose remaining in the cytosol should be rapidly metabolised via sucrose synthase.

6) Acid invertase is not functioning *in vivo*. The *in vitro* activity of acid invertase may be affected by a proteinaceous invertase inhibitor (Pressey, 1966; Pressey, 1967; Anderson and Ewing, 1978). The role of such an inhibitor in regulating invertase *in planta* is still open to question. Early evidence suggested it was involved in regulation of activity *in vivo* as fluctuations in invertase activity, in tubers stored at alternating temperatures (18°C and 4°C), could be attributed to changing inhibitor levels (Pressey and Shaw, 1966). In addition, the finding that the differences between basal and total invertase activities (Figs. 4.6 and 4.7) were greatest for cv. Brodick (the low hexose accumulator) would indicate inhibitor involvement. However, Isla *et al.* (1992) showed that the inhibitor was located in the cell wall fraction of tubers, spatially separated from vacuolar acid invertase. This casts doubt on the physiological significance of the inhibitor, therefore differences between basal and total invertase activity, shown in Figures 4.6 and 4.7, may be artifactual. Results from the previous chapter which showed significant differences between basal and total acid invertase activity when tubers were greater than 1.5 g FW suggest that inhibition increases with tuber development. This is further confirmed by the increasing differences between basal and total acid invertase activities measured in tubers from harvest 1 to harvest 4 (Figs. 4.6 and 4.7). While the proteinaceous inhibitor has a molecular weight of 17,000 (Bracho and Whitaker, 1990; Pressey, 1966), invertase inhibition was also shown to occur with a potato lectin with subunit M_r = 54,000 (Isla *et al.*, 1991; Matsumoto *et al.*, 1983). No attempt was made in this chapter to provide a qualitative analysis of the levels of such polypeptides by SDS-PAGE. This would require significant protein purification to provide meaningful data. Despite the problems in

measuring, unequivocally, maximum catalytic activity of acid invertase, the fact that both basal and total acid invertase activities increase together with hexoses following tuber excision indicates that acid invertase rather than sucrose synthase regulates sucrose hydrolysis in stored tubers.

While the above list is not exhaustive and permutations of some or all of the points raised are entirely possible, it does indicate the potential complexity of the situation.

4.4.2 The effect of reducing the supply of photosynthate to the tubers

The effect of reducing the sucrose flux to the tubers by preventing light interception by the canopy was studied in relation to sugar contents and sucrolytic enzyme activities. This was in an attempt to confirm that the results of tuber excision experiments were not due to wounding effects. While only the point of attachment to the stolon is severed, it could be argued a wound response is initiated and translocated. Wound respiration is induced when plant tissues are mechanically cut and the increased respiration is linked to wound-healing reactions involving the formation of lignin and suberin (Uritani and Asahi, 1980). Wounding of either potato leaves or tubers leads to specific, systemic induction of proteinase-inhibitor-II expression in both upper and lower leaves and upper parts of the stem (Peña-Cortes *et al.*, 1988). The inducing factor may be composed of pectic polysaccharides or oligosaccharides released from the plant cell wall (Bishop *et al.*, 1981).

The effect of reducing the assimilate supply to the tubers (4.3.2) was very similar to that obtained with tuber excision. This does not provide completely unequivocal evidence that "wound type" or "stress related" factors are not involved, but does imply that the supply of photosynthate, i.e. sucrose, is

important. The major constituents of assimilate transported from leaf cells to sink tissues via the phloem are sugars, mainly sucrose, and amino acids. While little is known of the effects of amino acids on gene regulation, induction of proteinase inhibitor-II genes occurs in detached potato leaves kept in the dark when supplied with both glutamine and sucrose (Peña-Cortes *et al.*, 1992) and specific responses have been observed with a range of sugars (Johnson and Ryan, 1990; Duke, 1991; Kim *et al.*, 1991). The control of expression of genes encoding the sucrose synthase isozymes in maize (*Sh1* and *Sus1*) differs. Glucose stimulated expression of the *Sus1* gene in excised maize roots whilst the expression of the *Sh1* gene is reduced (Koch *et al.*, 1989; Duke, 1991). The effects of fructose on gene expression were similar to those of glucose (but less pronounced), whereas mannitol and non-metabolisable sugars such as L-glucose were ineffective in eliciting any response (Koch *et al.*, 1992). These results indicate that sucrose or sucrose breakdown products may be involved in regulating sucrose synthase *in vivo*.

In tests with detached eggplant (*Solanum melongena*) leaves with petioles submerged in sterile sucrose solutions, sucrose synthase activity increased with increasing sucrose concentration (Claussen *et al.*, 1986). There was no effect of hormones such as ABA, kinetin or IAA. Acid invertase activity was not enhanced by sucrose; of the hormones used, only ABA caused a significant increase in activity.

4.4.3 The effect of supplying water and sugars to detached tubers

The results of supplying detached tubers of cvs Record and Cara with water or a range of sugars showed a number of consistent and significant features. The prevention of a rapid decline in sucrose synthase activity in detached tubers by supplying sucrose via the cut stolon (Table 4.3) confirms the potential role of

sucrose flux in regulating sucrose synthase activity. This is further substantiated by the correlation between sucrose synthase activity and sucrose uptake by individual tubers (Fig. 4.9). Glucose and fructose both elicited a similar response to sucrose, again implying that other metabolisable sugars can affect the level of sucrose synthase as found for maize (Koch *et al.*, 1992). However, despite substantial conversion of maltose into sucrose, the decline in sucrose synthase activity in tubers after detachment was not prevented by an exogenous supply of maltose. One reason may be that the rate of maltose conversion into sucrose may be low by comparison with the rate of sucrose influx from sucrose supplied exogenously. However, one might expect at least some positive correlation between sucrose synthase activity and maltose flux (Fig. 4.9B). As no significant positive correlation was observed using tubers supplied with sucrose via a cut channel, the effectiveness of sugars in eliciting a response may depend on the efficiency of transport into the cells and on the site of action.

The identification of the sucrose synthase polypeptide by gel electrophoresis and immunoblotting confirmed that not only the activity of the enzyme but also the level of sucrose synthase protein can be modified by sucrose supply. This response has significant physiological implications providing a mechanism by which cells of sink tissues can adjust their sucrose-metabolising capacity to changes in available photosynthate supply, as shown previously (section 4.3.2). It also has major implications for the role of sucrose synthase as an important determinant of sink strength (Sung *et al.*, 1989). Of the three sucrolytic enzymes studied, sucrose synthase, acid and alkaline invertases, only the activity of sucrose synthase was related to growth. Black (1993) therefore concluded that sucrose synthase activity could potentially be used as an indicator of sink strength. However, the concept of sink strength is now under review as no single measurement is likely to give a true indication of the

ability of a storage organ to regulate its import of assimilate (Farrar, 1993). If, as shown, sucrose synthase is sucrose inducible then one may ask how does it control sink strength? The answer is probably that the regulation of import to developing sinks is too complex a process to be dependent on the action of one enzyme only. While high sucrose synthase activity in a sink organ is indicative of a high rate of import, other processes, occurring concurrently with sucrose cleavage via sucrose synthase, are also likely to be involved in control of assimilate import. Stitt (1993) suggests that rapidly growing sink tissues ensure that the activity of sucrose synthase is sufficient to prevent a "bottleneck" at that stage. This explanation was preferred to the possibility that the enzyme drives import and growth via the more rapid mobilisation of sucrose.

Recent work, using antisense technology, has reduced the level of sucrose synthase activity to ca 5% of that found in wildtype tubers (Zrenner and Sonnewald, 1993 and pers. comm. to H. V. Davies). The level of starch in these transgenics was reduced by ca 70% but the sucrose content remained unchanged. This is unlike the situation in antisense ADP-glucose pyrophosphorylase transgenic tubers which accumulate high levels of sucrose (Müller-Röber *et al.*, 1992). The data imply an important role for sucrose synthase in regulation of sucrose import and the conversion of sucrose to starch in developing tubers.

Unlike sucrose synthase, soluble acid invertase activity increases rapidly following tuber detachment, but the highest activity is always found when the stolons are subsequently supplied with water rather than sugars (Tables 4.3, 4.4 and 4.5). Increased invertase activity appears to result in hexose accumulation and in a decrease in the G : F ratio (from 15:1 to almost 1:1). The reason why invertase activity is highest in tubers supplied with water is not altogether clear but it may be that storage cells need to regulate their turgor by increased sucrose hydrolysis. The vacuole, the major storage pool for

metabolites such as organic acids and sucrose, plays an important role in the osmoregulation of the cell (Leigh and Jones, 1986; Matile, 1987). Increased acid invertase activity in the vacuoles of the tuber cells would lead to an increase in hexoses which may be required to balance the solute concentration after the uptake of water (see also Moll, 1970). The control of turgor pressure during the mobilisation of vacuolar sucrose has been investigated using excised storage-roots of red beet (Perry *et al.*, 1987). Their results imply the involvement of acid invertase in the accumulation of hexoses following a range of osmotic treatments.

The consistent increase in acid invertase activity in tubers after detachment is evidence that this enzyme, rather than sucrose synthase, regulates hexose accumulation in stored tubers. Recent results (Zrenner and Sonnewald, 1993) investigating transgenic potato plants in which the level of vacuolar acid invertase was reduced by 90% have confirmed the importance of this enzyme in controlling tuber hexose levels. The tubers produced from these transgenic plants appeared normal with similar starch levels to those from control plants. However, when mature tubers from the transgenic plants were cold-stored, the hexose level was 60% lower than in control tubers but total sugars remained the same. It appears, therefore, that soluble vacuolar invertase is not involved to a significant extent in providing carbon for starch biosynthesis. Rather, it regulates the sugar balance of tubers in the post harvest phase. This is in full agreement with the findings of this chapter.

4.5 Conclusions

Treatments that interfere with the supply of photosynthate to developing potato tubers stimulate hexose accumulation, cause a rapid increase in acid

invertase activity, but result in a substantial depletion of sucrose synthase activity and sucrose synthase protein.

There were no significant effects of genotype on sucrose synthase activity in detached tubers during a subsequent period at 10°C storage. Differences between genotypes in the rate of hexose accumulation were related to invertase activity. After tuber detachment and during storage at 10°C, the G : F ratio decreased from over 5 : 1 to ca 1 : 1. Together the data are taken as evidence that acid invertase rather than sucrose synthase regulates hexose accumulation in mature, stored tubers.

Maintaining a flux of sucrose into excised tubers prevents the decline in sucrose synthase activity and sucrose synthase protein. This substantiates the view that sucrose or its metabolites have a positive effect on the expression of the gene in potato.

CHAPTER 5

CARBOHYDRATE METABOLISM IN DEVELOPING COTYLEDONS OF *Vicia faba* L.

5.1 Introduction

The field bean (*Vicia faba* ssp. *minor* L.) crop is an important source of seed protein and for this reason is currently grown in many countries. The seeds store approximately 36% (dry weight) of their food reserves as protein and 35% (dry weight) as starch. The major storage proteins are legumin, which consists of six acidic and six basic disulphide-bonded subunit pairs with MW of about 350-400,000, and vicilin, a glycosylated protein (MW about 170,000), made up of four subunits of approximately 50,000 MW with no disulphide bonding between subunits (Boulter, 1981). These proteins, accounting for 75% of the total proteins, are made on the rough endoplasmic reticulum (Bollini and Chrispeels, 1979) and stored in single membrane-bound protein bodies (Briarty *et al.*, 1969) within the cotyledons, to be remobilized during seed germination (Briarty *et al.*, 1970).

The developing seeds of *Vicia faba* also become an important sink for assimilated carbon and both the pathway of photosynthate transfer and the nature of the assimilate transferred have been studied in detail. Recent results (Offler and Patrick, 1993) suggest that photoassimilate within the seed coat of *Vicia faba* is transferred radially and laterally in the symplast from the sieve element-companion cell complexes to the thin-walled parenchyma/transfer cells. These cells are thought to act as the principal cellular site for membrane exchange to the seed apoplast.

Using the "empty seed" technique, in which the developing embryo is removed and the seed is filled with a substitute medium to trap the solutes released from the seed coat, it has been shown that sucrose is the principal form in which reduced carbon is delivered to the seed coat through the phloem (Wolswinkel, 1988). Although sucrose hydrolysis represents the first metabolic step *en route* to starch biosynthesis, no detailed investigation of the activities of sucrose-cleaving enzymes during seed development has been carried out. The work of de Fekete (1969) indicates that sucrose synthase rather than invertase catalyses sucrose breakdown in immature cotyledons of *Vicia faba*. The work reported in this chapter was aimed at identifying the potential roles played by the separate sucrose-degrading enzymes during seed development. Additionally, the uptake and incorporation of ^{14}C-glucose and ^{14}C-sucrose into starch was examined to provide an indication of potential rates and changes in sugar-starch interconversion.

Samples of beans at a range of developmental stages obtained during the course of this work were used for the later purification of sucrose synthase, alkaline invertase and fructokinase. The purification of the fructokinase was carried out by A. Gardner, (1992).

5.2 Materials and Methods

5.2.1 Plant material

Seeds of *Vicia faba* cv. Maris Bead were sown in the field at SCRI on the 28th March using an Øjord drill to give a final plant density of 45 plants m^{-2}. Flowers on the first podding node from the bottom of the plant had reached anthesis by the 25th June with the second and third podding nodes reaching the same stage three and six days later respectively. The first samples were

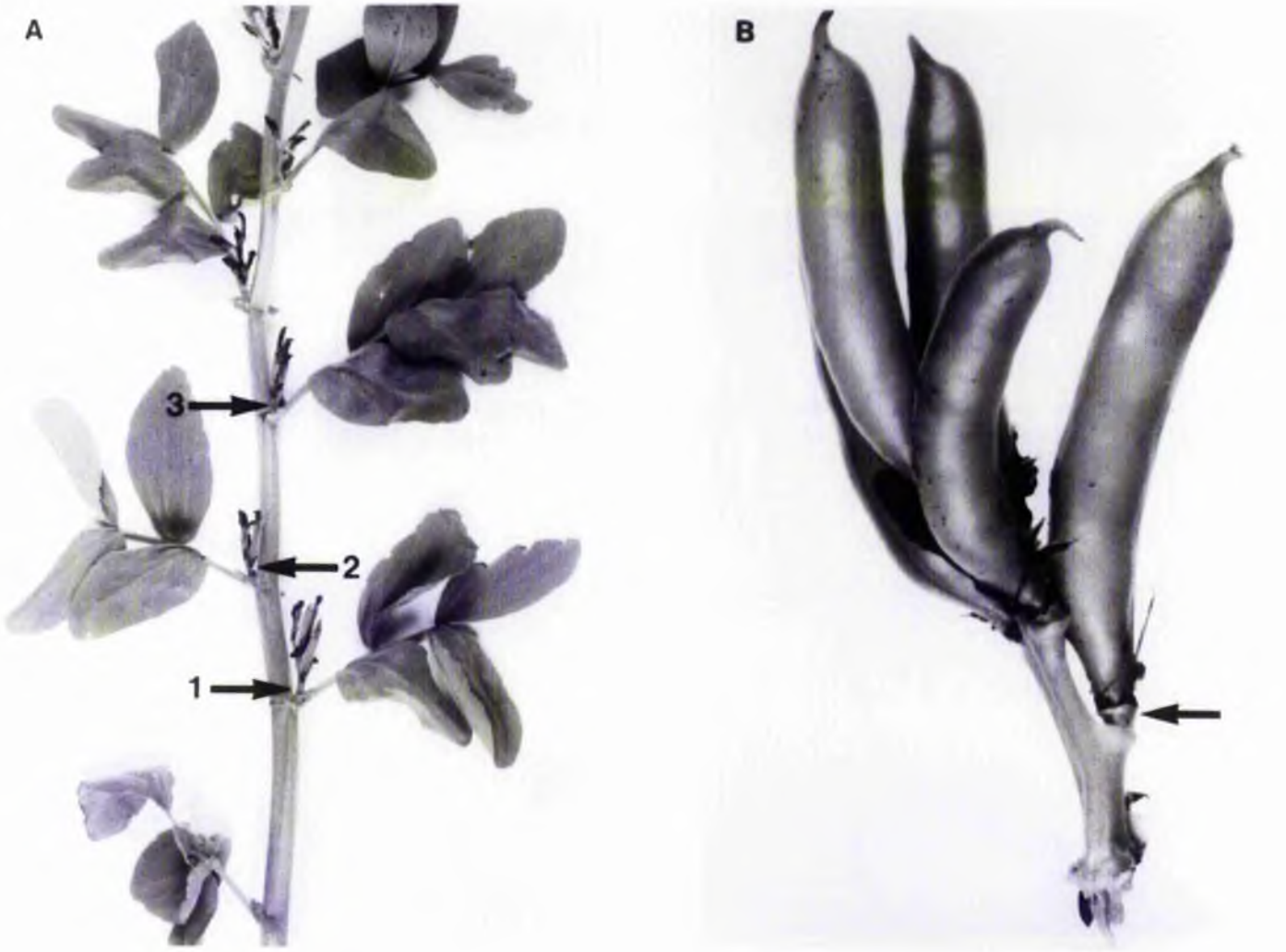
harvested on the 31st July when pods were removed from the first three podding nodes from fifty plants taken at random from the field plot. Beans from the single pod at each node (Plate 5.IA & IB) were used for enzyme determinations and other assays. These pods, being the first to develop at each node, were selected to provide as uniform a sample as possible. The remaining pods from each of the nodes were frozen and stored at -20°C for later enzyme purification work. Samples were taken on a further seven occasions and treated in the same manner.

SAMPLE DATES	DAYS AFTER ANTHESIS (DAA)		
	NODE 1	NODE 2	NODE 3
31st July	36	33	30
3rd August	39	36	33
7th August	43	40	37
10th August	46	43	40
15th August	51	48	45
23rd August	59	56	53
29th August	65	62	59
12th September	79	76	73

5.2.2 Enzyme determinations

Enzyme extracts were prepared from fresh bean samples from the first three nodes. Testas were removed and the embryonic axes excised prior to the

Plate 5.I A) *Vicia faba* plant showing the position of the first three podding nodes which were used for sampling. B) Arrow indicates the single pod from which beans were taken for assays.


A
3
2
1
B

weighing and extraction of five cotyledons for each individual enzyme sample. Four replicate extracts were assayed.

5.2.2.1 Sucrose synthase

The cotyledons were ground to a fine powder in liquid nitrogen and extracted with insoluble Polyvinylpolypyrrolidone (1% w/v) and acid-washed quartz sand in 3 volumes of extraction solution as described previously (**3.2.2.1**). To confirm the reliability of the extraction method, samples of potato tubers and of bean cotyledons were extracted separately and together and the % recovery calculated. No major losses were experienced with 95% activity recovered from the mixture in comparison with the independent extractions of both tissues. The same assay procedure was used as described previously (**2.3.1**) except for adjusting the buffer to give a final pH for the reaction of 6.8 (optimised pH). The linearity of the enzyme activity with time and proportionality with extract volume was tested for each set of measurements. Four replicates for each nodal sample were assayed with three determinations per assay.

5.2.2.2 Acid invertase

Acid invertase was extracted as above but in 2 volumes 100 mM acetate buffer (pH 5.0) containing 10 mM sodium sulphite and 5 mM DTT. Samples were re-extracted in 2 volumes of the same buffer but with the addition of 1 M NaCl to yield maximum extractable activity. After centrifugation and dialysis, both sets of extracts were assayed as described in section **2.3.2**. For each enzyme extraction, pellets were tested for residual activity and in all cases less than 10% was found in this fraction. The foaming treatment, used when

determining the total activity of potato acid invertase, had no effect on bean acid invertase.

5.2.2.3 Alkaline invertase

Alkaline invertase was extracted as described above for acid invertase. Preliminary investigations had shown maximum activity of both invertases when extractions were carried out at pH 5.0. The activity was also assayed in the same way as used for acid invertase but the assay buffer was replaced with 200 mM sodium phosphate to give a final pH in the reaction mixture of 7.5, the optimum for bean alkaline invertase.

5.2.2.4 Hexose kinases

Both fructokinase and glucokinase were extracted from the developing bean cotyledons in 100 mM Tris/HCl (pH 7.5) containing 20 mM cysteine HCl, 20 mM ethylenediaminetetra-acetic acid (EDTA), 15 mM dithiothreitol (DTT), 5 mM phenylmethanesulphonyl fluoride (PMSF) with the addition of 0.1% w/v insoluble PVP and acid-washed quartz sand during grinding. After centrifugation at 20,000g for 20 min at 4°C, extracts were desalted on Sephadex G-25M columns (Pharmacia PD-10) and eluted with 20 mM Tris-HCl (pH 8.0) containing 5 mM DTT. The activity of both enzymes were determined as described in section **3.2.2.4** and shown to be linear with time and proportional to volume of enzyme extract.

5.2.2.5 ADP-glucose pyrophosphorylase

ADP-glucose pyrophosphorylase activity was determined from the same samples extracted for fructokinase and glucokinase measurements. The enzyme was assayed spectrophotometrically at 30°C using the method outlined by Smith *et al.* (1989). The assay contained, in 1 ml, 100 mM Tris/HCl (pH 7.9), 5 mM $MgCl_2$, 5 mM DTT, 0.4 mM NAD, 1 unit of both phosphoglucomutase and glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides)* and 1 mM adenosine 5'-diphosphoglucose (ADPG). The reaction was initiated by the addition of pyrophosphate (PPi) at a final concentration of 2 mM. Activity was again shown to be linear with time and proportional to extract volume.

5.2.3 Protein determination

Protein was extracted from the cotyledons at each date after grinding the samples in 100 mM acetate buffer (pH 5.0) containing 10 mM sodium sulphite and 1 M NaCl. Insoluble PVP (1% w/v) and acid-washed quartz sand were included in the grinding medium. To ensure total protein recovery, the precipitates obtained after centrifugation at 20,000g for 20 min were re-extracted and the process repeated until no further protein was found in the samples. More than 90% was recovered after the first extraction and a further 8-9% after re-extraction of the pellet. The protein concentration in each of the samples was determined as described in section 2.7.

5.2.4 Electrophoresis and Immunoblotting

Protein samples were subjected to SDS-PAGE (10 or 12.5% acrylamide) as in

section 2.5.1. Rather than loading on an equal protein basis, the amount of sample used was calculated on an equal cotyledon basis. Hence, during development, the increasing protein content per cotyledon can be visualised by both the increasing polypeptide bands on the gel and their intensity. Parallel gels were run and the polypeptides transferred to nitrocellulose to allow immunoblotting with polyclonal antibodies raised against sucrose synthase (purified from bean cotyledons, Chapter 7), alkaline invertase (purified from sugar beet taproots, Chapter 8), fructokinase (purified from pea seeds (FKP-I) and kindly supplied by D. D. Randall, USA) and ADP-glucose pyrophosphorylase (purified from pea seeds - polypeptide unique to enzyme from RbRb embryos) - kindly provided by A. Smith, Norwich). The method described previously for detection of cross-reacting polypeptides on the membranes (section 2.6) was used throughout. However, for the sucrose synthase immunoblot, the enhanced chemiluminescence (ECL) detection method outlined by the suppliers (Amersham, UK) was used as a comparison. This method is considered to be 10x more sensitive than other methods commonly used and to give higher resolution. It is based on the oxidation of luminol in the presence of hydrogen peroxide. The secondary antibody is conjugated with horseradish peroxidase which catalyses this oxidation reaction to yield luminol in an excited state which decays via a light emitting pathway. This emitted light is recorded on autoradiography film (Hyperfilm ECL, from Amersham, UK).

5.2.5 Determination of starch, sucrose, glucose and fructose

Bean cotyledon samples from each developmental stage were frozen in liquid nitrogen and freeze-dried. The carbohydrate determinations were carried out as described in sections 2.4.1 and 2.4.2.

5.2.6 Determination of moisture content

At each sample date, cotyledons were weighed prior to freezing and subsequently freeze-dried and reweighed. The moisture content of each of the samples was calculated and also the mean cotyledon dry weight to show the pattern of dry matter accumulation.

5.2.7 ^{14}C-Glucose and ^{14}C-Sucrose uptake measurements

Prior to ^{14}C-glucose and ^{14}C-sucrose uptake measurements, the testas were removed from samples of thirty freshly harvested beans and the cotyledons were separated into two batches, one for each of the sugars. The two batches of thirty cotyledons from each of the three nodes at all stages were pre-incubated and aerated in 50 ml of 500 mM sorbitol buffered with 25 mM Mes/KOH (pH 6.0) for 5 min. Sorbitol was used in preference to mannitol as the latter was partially metabolised during previous experiments with bean cotyledons (K. Wright, pers comm.). After preincubation, the cotyledons were blotted, weighed and incubated for 1 h in 50 ml of 450 mM sorbitol buffered with 25 mM Mes/KOH (pH 6.0) containing either 50 mM sucrose radiolabelled with [U-^{14}C] sucrose (0.05 μCi ml^{-1} or 1.85 KBq ml^{-1}) or 50 mM glucose radiolabelled with [U-^{14}C] D-glucose (0.05 μCi ml^{-1} or 1.85 KBq ml^{-1}. The final specific activity of the sucrose and glucose used was 37 MBq mol^{-1}. Samples were incubated at 25°C in the dark for 1 h. Cotyledons were then washed (3 x 1 min washes in 25 ml of pre-incubation buffer). Each of the samples were subdivided to give 5 replicates of 6 cotyledons. These were blotted dry, weighed and frozen in liquid nitrogen prior to freeze-drying and the determination of distribution of label between the soluble, starch and pellet fractions.

5.2.8 Analysis of ^{14}C distribution

Ethanol extracts were prepared from the freeze-dried samples ground to a fine powder. The analysis of the ^{14}C distribution between the soluble and starch fractions was the same as described in section **4.2.6**. The radioactivity of the insoluble fraction not found as starch was determined after resuspending and counting the pellet from the starch samples.

5.3 Results

5.3.1 Dry matter accumulation

Over the period of bean development investigated, the moisture content of the cotyledons decreased from 80% at the first sample date to 17% at the end of sampling. At any one date the cotyledons from the different nodes did not vary significantly in their moisture content (Fig. 5.1A). The mean dry weights of 20 cotyledons at each sample date for each of the three nodes showed a similar pattern of dry matter accumulation (Fig 5.1B). Despite the differences in time of pod development the dry weights of the cotyledons from nodes 1 and 2 at, and after, the second sample date were identical. At node 3, however, the mean dry weights were lower at all but the last sample date but higher when compared on the basis of time after anthesis. The final dry matter of the cotyledons from each of the nodes reached a similar maximum with cotyledon dry weights of 173, 183 and 185 mg for nodes 1, 2 and 3 respectively.

5.3.2 Protein, starch and sugar accumulation

The protein content of the developing cotyledons increased rapidly with 20-fold increases from the first to last sample date (Fig. 5.2A). The cotyledons from node 3 have a significantly lower protein content at the start than nodes 1

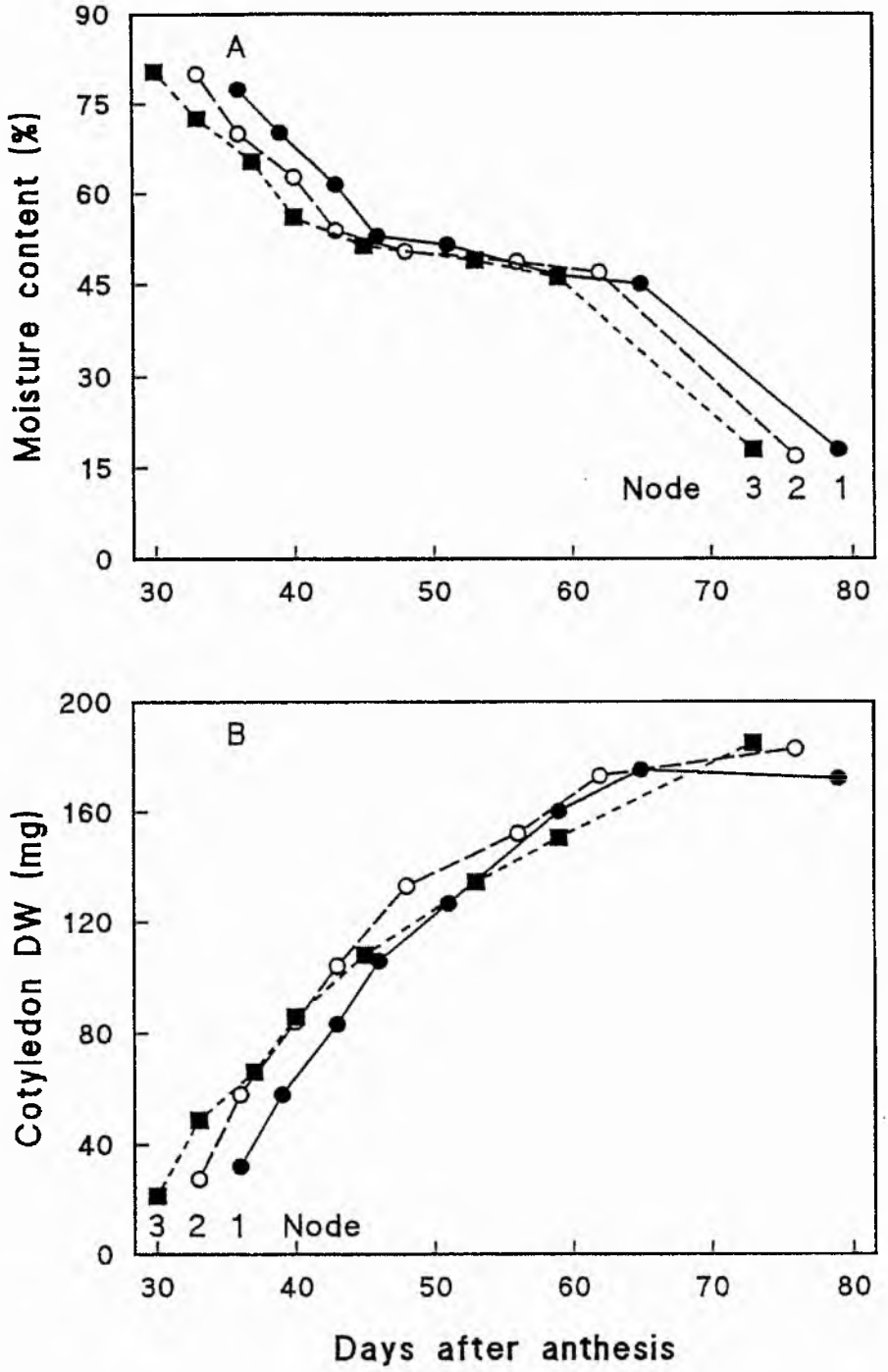


Fig. 5.1 A) Moisture content and B) dry weight of *V.faba* cotyledons sampled from the three nodal positions during bean development. Values shown are means (n = 20).

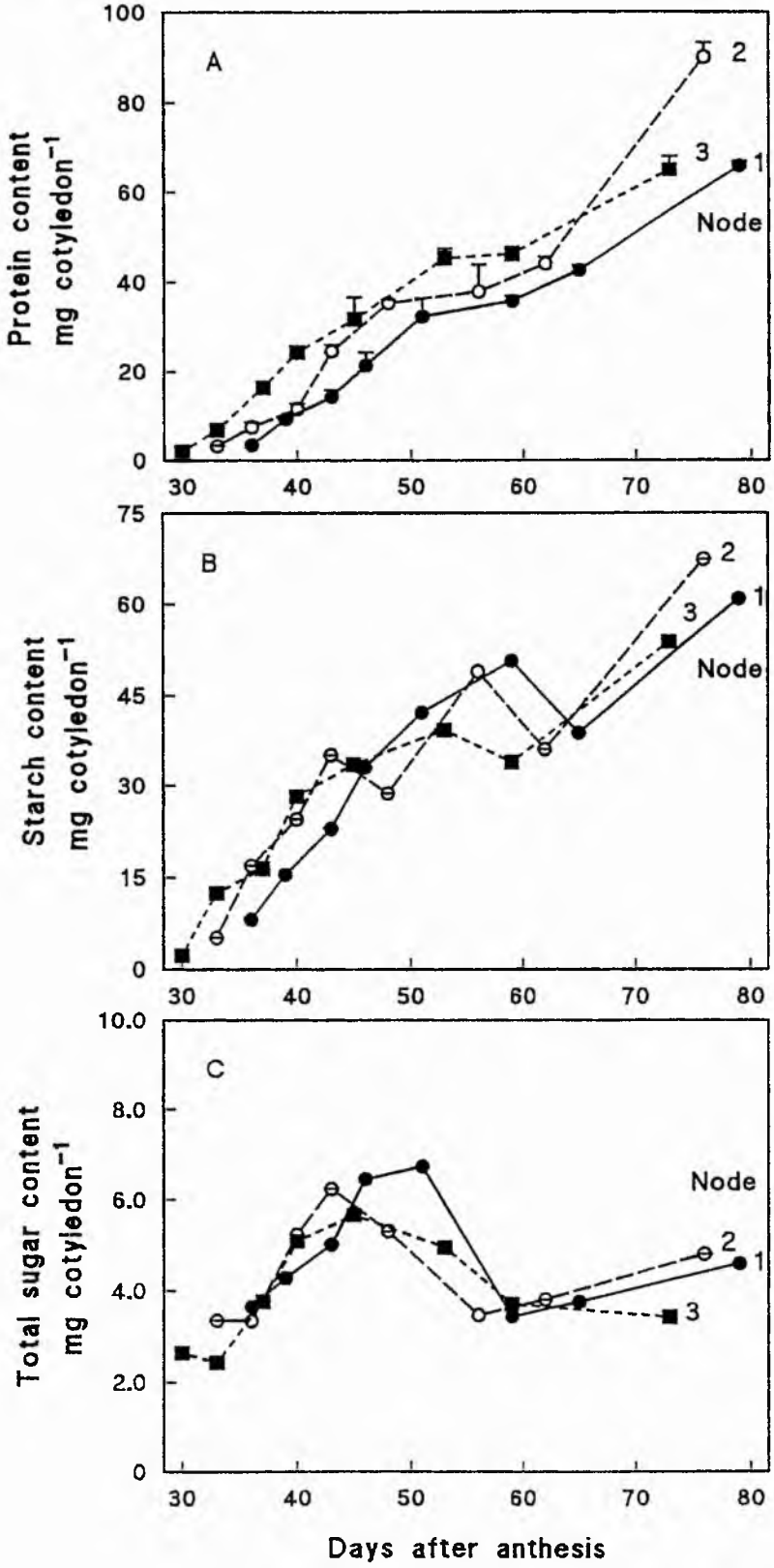


Fig. 5.2 A) Protein content, B) starch content and C) total sugar content of *V. faba* cotyledons sampled from the three nodal positions during bean development. Values shown are means (n = 3).

and 2. However, the final protein content of cotyledons from node 2 was significantly higher than from nodes 1 and 3.

Starch content increased approximately 15-fold during seed development (Fig. 5.2B). By the final sample date the starch contents of the cotyledons from the three nodal positions were only slightly lower than their respective protein contents. The highest final starch content was in the sample from node 2. When the rates of starch accumulation were calculated over the first four sample dates (from 31st July - 10th August) the values obtained were 575, 692 and 602 nmol (hexose equivalent) h^{-1} cotyledon^{-1}, but overall (from first to last sample date) were 284, 335 and 278 nmol (hexose equivalent) h^{-1} cotyledon^{-1} for samples from nodes 1, 2, and 3 respectively.

The total sugar content increased between ca. 36-50 days after anthesis then declined. The levels of the individual sugars, glucose, fructose and sucrose along with the G : F ratio are shown in table 5.1. The glucose and fructose levels remain low throughout development and, with the exception of the first sample date, glucose was generally 4-fold higher than fructose. Sucrose levels increased to reach a peak between 40-55 days after anthesis.

5.3.3 ^{14}C-glucose and ^{14}C-sucrose incorporation

The patterns of ^{14}C-sucrose and glucose uptake and incorporation are shown in Figs. 5.3 & 5.4 for samples from each of the three nodes. Total uptake of ^{14}C-sucrose and ^{14}C-glucose by the developing cotyledons decreased over 8-fold and approximately 2.5-fold respectively over the sampling period. However, for both sugars the patterns of uptake into the ethanol-soluble constituent and incorporation into the starch and pellet fractions were similar. Additionally, no major differences were found in the pattern of uptake by the samples from

Table 5.1 Glucose, fructose and sucrose content and glucose:fructose ratio in developing cotyledons from the first three nodes at eight sample dates. Values shown are means (n = 2)

Sample No.	Node No.	Glucose	Fructose	Sucrose	G:F ratio
			mg cotyledon^{-1}		
1	1	0.22	0.11	3.33	2:1
	2	0.14	0.14	3.07	1:1
	3	0.17	0.19	2.28	1:1
2	1	0.18	0.05	4.05	4:1
	2	0.23	0.06	3.11	4:1
	3	0.12	0.03	2.28	4:1
3	1	0.19	0.04	4.79	5:1
	2	0.18	0.03	5.02	6:1
	3	0.18	0.05	3.53	4:1
4	1	0.26	0.07	6.13	4:1
	2	0.22	0.04	5.98	5:1
	3	0.20	0.04	4.86	5:1
5	1	0.21	0.04	6.49	5:1
	2	0.17	0.03	5.11	6:1
	3	0.18	0.04	5.44	4:1
6	1	0.22	0.04	3.19	5:1
	2	0.23	0.05	3.19	5:1
	3	0.18	0.04	4.73	4:1
7	1	0.14	0.05	3.57	3:1
	2	0.10	0.08	3.64	1:1
	3	0.14	0.06	3.51	2:1
8	1	0.16	0.04	4.40	4:1
	2	0.19	0.04	4.58	5:1
	3	0.13	0.02	3.27	6:1

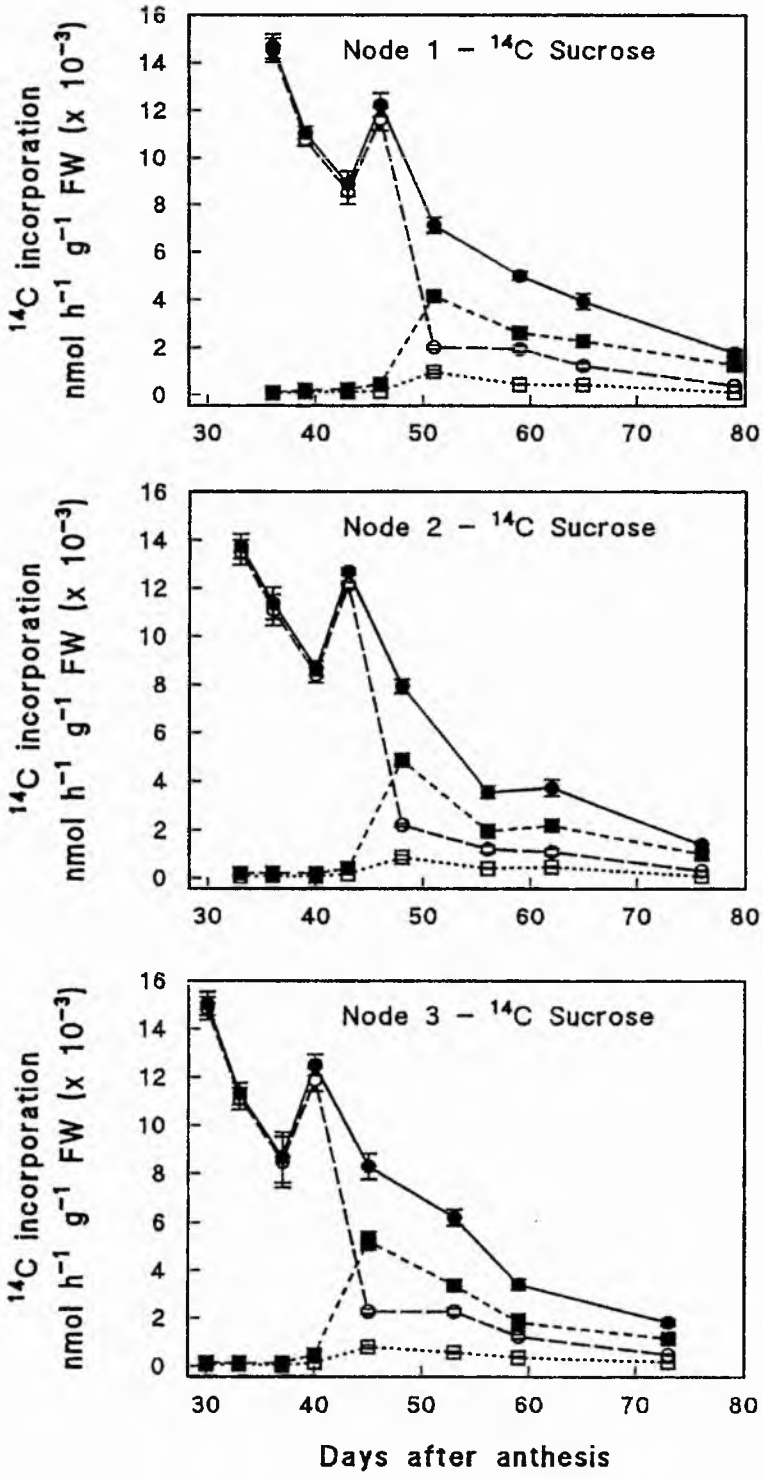


Fig. 5.3 Partitioning of ^{14}C sucrose in cotyledons sampled from the three nodal positions during bean development. ● - total uptake, ○ - incorporation into ethanol soluble constituent, ■ - incorporation into starch, and □ - incorporation into tissue residue. Values shown are means (n = 5) ± sem.

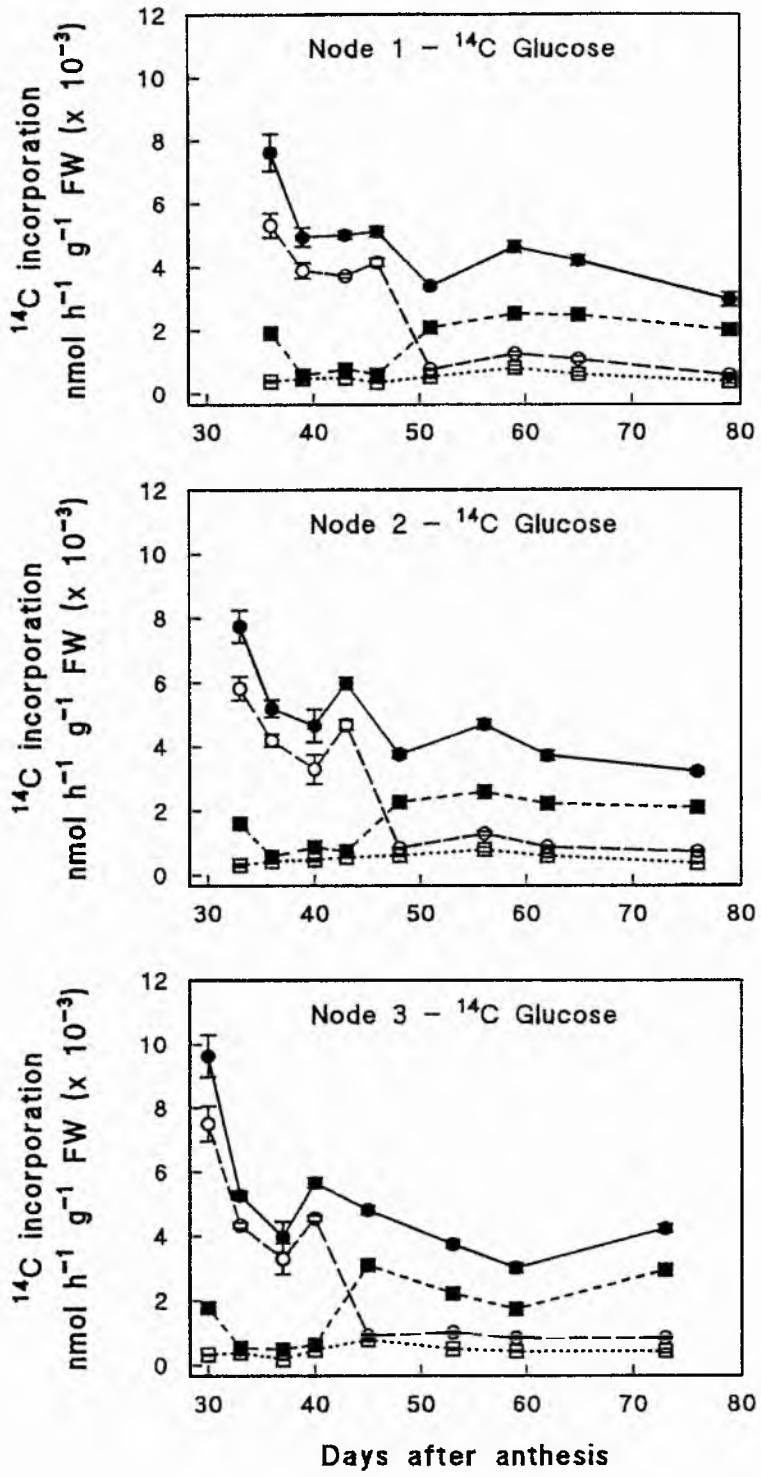


Fig. 5.4 Partitioning of ^{14}C glucose in cotyledons sampled from the three nodal positions during bean development. ● - total uptake, ○ - incorporation into ethanol soluble constituent, ■ - incorporation into starch, and □ - incorporation into tissue residue. Values shown are means (n = 5) ± sem.

different nodes. When supplied with ^{14}C-sucrose, the proportion of label incorporated into starch increases significantly between 40-50 DAA. Temporally, this increase occurs first in node 3. Increased incorporation of label into the pellet fraction also occurred at 40-50 DAA when supplied with either sugar. When supplied with ^{14}C-glucose, increases in partitioning into starch similarly occurred around 40-50 DAA (Fig. 5.4), although there was evidence for a relatively high but transient incorporation into starch around 30 DAA. Rates of glucose uptake were lower than for sucrose between 30-60 DAA.

Due to the large decrease in the moisture content of the developing cotyledons from 80 to 17% and the 20-fold increases in protein content, it was considered necessary to express the results per cotyledon. The results of ^{14}C-glucose and ^{14}C-sucrose uptake, expressed as nmol glucose or sucrose equivalents h^{-1} $cotyledon^{-1}$ for samples from node 2, are shown in Table 5.2. The percentages of the total uptake found in each of the soluble, starch and pellet fractions have been calculated. These values show a rapid decrease in the % of label incorporated into the ethanol soluble fraction over a period of only 5 days from 78 to 23% for ^{14}C-glucose and from 96 to 28% for ^{14}C-sucrose. Similarly, increases in the percentage of label recovered in the starch fraction from 12 to 61% and 3 to 61% when supplied with labelled glucose and sucrose respectively, are shown. The increase in the percentage of label recovered in the pellet fraction was from 9 to 16% and 1 to 11%, when supplied with labelled glucose and sucrose respectively.

5.3.4 Enzyme activities during seed development

Due to the problems of changing moisture and protein contents during seed development, all enzyme activity values were calculated per cotyledon after

Table 5.2 ^{14}C-glucose and ^{14}C-sucrose uptake and partitioning by developing cotyledons from node 2. Values shown are means (n = 5) ± s.e. and are expressed cotyledon^{-1}. The percentages of total radiolabel incorporated into ethanol soluble, starch and pellet fractions are shown in brackets

Treatment DAA	Total Uptake nmolh^{-1}cot^{-1}	Soluble Fraction nmolh^{-1}cot^{-1}	Starch Fraction nmolh^{-1}cot^{-1}	Pellet Fraction nmolh^{-1}cot^{-1}
^{14}C-glucose				
33	970.92 ± 62.99	730.35 (75.22) ±46.21	201.49 (20.75) ±12.56	39.07 (4.03) ± 5.57
36	1016.69 ± 51.99	819.45 (80.60) ±39.06	112.89 (11.10) ± 6.76	84.35 (8.30) ± 4.08
40	968.78 ±107.18	684.83 (70.76) ±95.55	183.02 (18.91) ±15.23	99.92 (10.32) ±10.08
43	1431.65 ± 44.72	1120.35 (78.26) ±38.14	178.58 (12.47) ±10.37	132.72 (9.27) ± 4.94
48	954.35 ± 29.91	216.80 (22.72) ± 7.67	580.60 (60.84) ±22.10	156.95 (16.45) ± 9.49
56	1384.88 ± 44.11	384.32 (27.75) ±18.12	763.70 (55.15) ±14.61	236.87 (17.10) ±21.09
62	1130.84 ± 45.87	269.89 (23.87) ±12.61	677.02 (59.87) ±24.75	183.93 (16.26) ±16.97
76	681.72 ± 18.92	156.82 (23.00) ± 8.68	445.12 (65.29) ±14.45	79.79 (11.70) ± 5.41

Table 5.2 contd.

Treatment DAA	Total Uptake $nmolh^{-1}cot^{-1}$	Soluble Fraction $nmolh^{-1}cot^{-1}$
^{14}C-sucrose		
33	1721.62 ± 65.53	1689.77 (98.15) ± 64.92
36	2223.19 ±132.01	2167.97 (97.52) ±126.11
40	1802.06 ± 63.94	1746.98 (96.94) ± 67.33
43	3031.76 ± 33.05	2898.03 (95.59) ± 30.74
48	2017.04 ± 74.53	561.59 (27.84) ±16.83
56	1047.39 ±101.13	354.59 (33.85) ±40.53
62	1135.18 ±104.20	332.05 (29.25) ±40.80
76	294.18 ± 5.77	64.25 (21.84) ± 1.75

Starch Fraction $nmolh^{-1}cot^{-1}$	Pellet Fraction $nmolh^{-1}cot^{-1}$
23.35 (1.36) ± 2.25	8.50 (0.49) ± 1.21
38.90 (1.75) ± 6.39	16.32 (0.73) ± 1.34
39.75 (2.21) ± 4.29	15.34 (0.85) ± 1.56
95.40 (3.15) ± 2.40	38.33 (1.26) ± 3.75
1235.64 (61.26) ±55.74	219.81 (10.90) ±24.53
575.96 (54.99) ±31.39	116.83 (11.15) ± 6.07
663.76 (58.47) ±52.93	139.37 (12.28) ±12.48
212.05 (72.08) ± 3.19	17.88 (6.08) ± 1.98

correction for changes in moisture content. This correction is required since at the start of sampling (31st July) each cotyledon provided approximately 100 µl water during extraction but this was be reduced to only 30 µl at the end (12th September).

5.3.4.1 Sucrose synthase

The activity of sucrose synthase increased rapidly from the first sample date to reach a peak (over 900 nmol min^{-1} $cotyledon^{-1}$) on the fifth sample date (45-51 DAA), thereafter declining rapidly to approximately 100 nmol min^{-1} $cotyledon^{-1}$ (Fig. 5.5A). The pattern of change in activity was similar for all three nodal positions but for the first four sample dates activity values were lowest for node 3.

5.3.4.2 Alkaline invertase

Preliminary experiments which tested a range of extraction buffers showed that maximum catalytic activity of alkaline invertase was obtained if samples were extracted in sodium acetate buffer (pH 5.0) containing 1 M NaCl and followed by dialysis against 10 mM acetate buffer at the same pH. The differences between the values obtained for alkaline invertase activity of the samples with or without NaCl in the extraction buffer are shown in Figure 5.5B. Clearly, NaCl is required to extract significant alkaline invertase activity. The maximum catalytic activity obtained for alkaline invertase was 3 to 4-fold lower than that determined for sucrose synthase. However, the pattern of activity of the two enzymes differed, with sucrose synthase most active between 37-51 DAA but alkaline invertase activity highest between 45-65 DAA. At the last two sample dates alkaline invertase activity was higher than that of sucrose synthase.

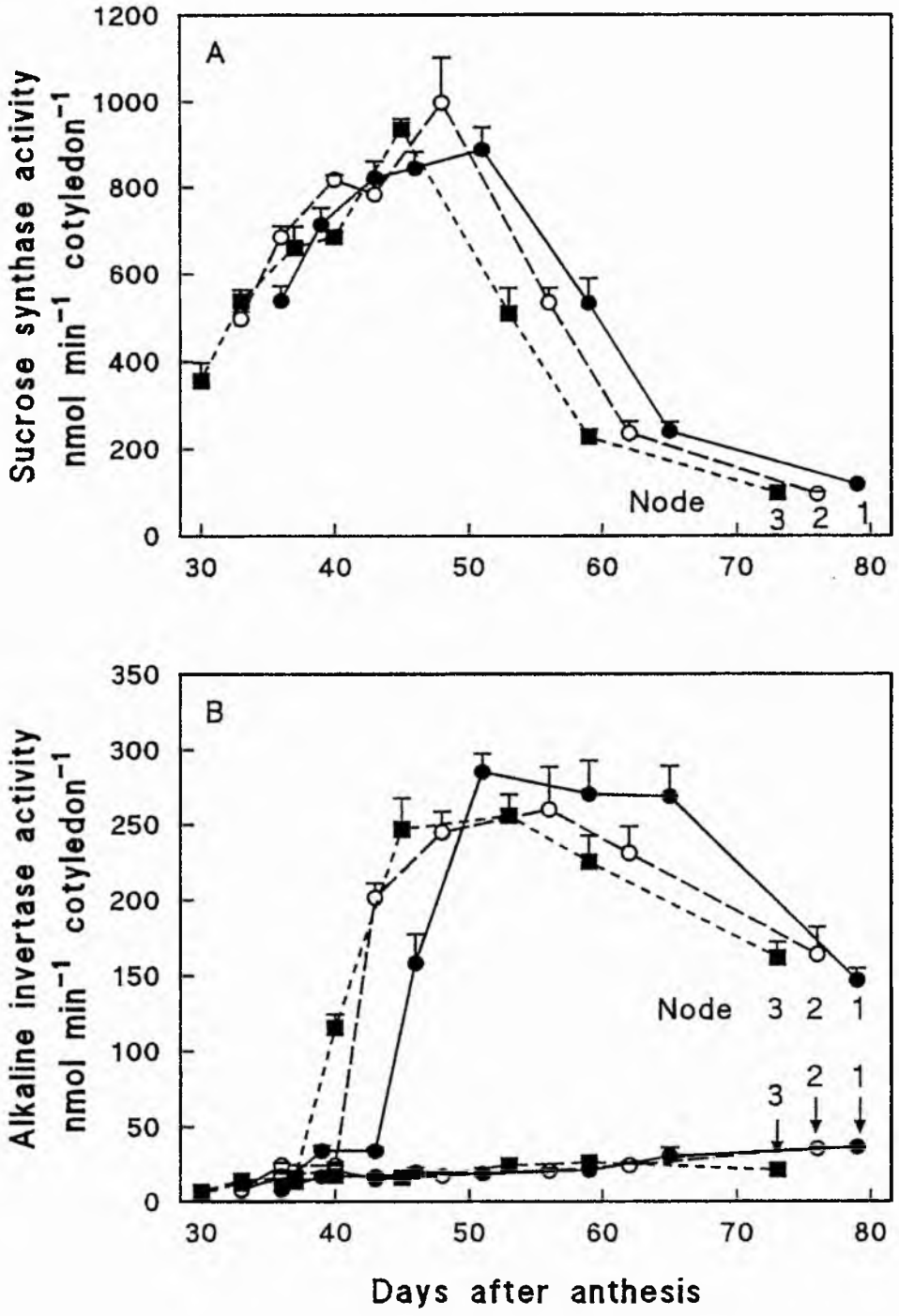


Fig. 5.5 Activity of A) sucrose synthase and B) alkaline invertase in *V. faba* cotyledons sampled from the three nodal positions during bean development. Lower alkaline invertase values, shown with arrows, result from the absence of NaCl in the extraction buffer. Values shown are means (n = 4) + sem.

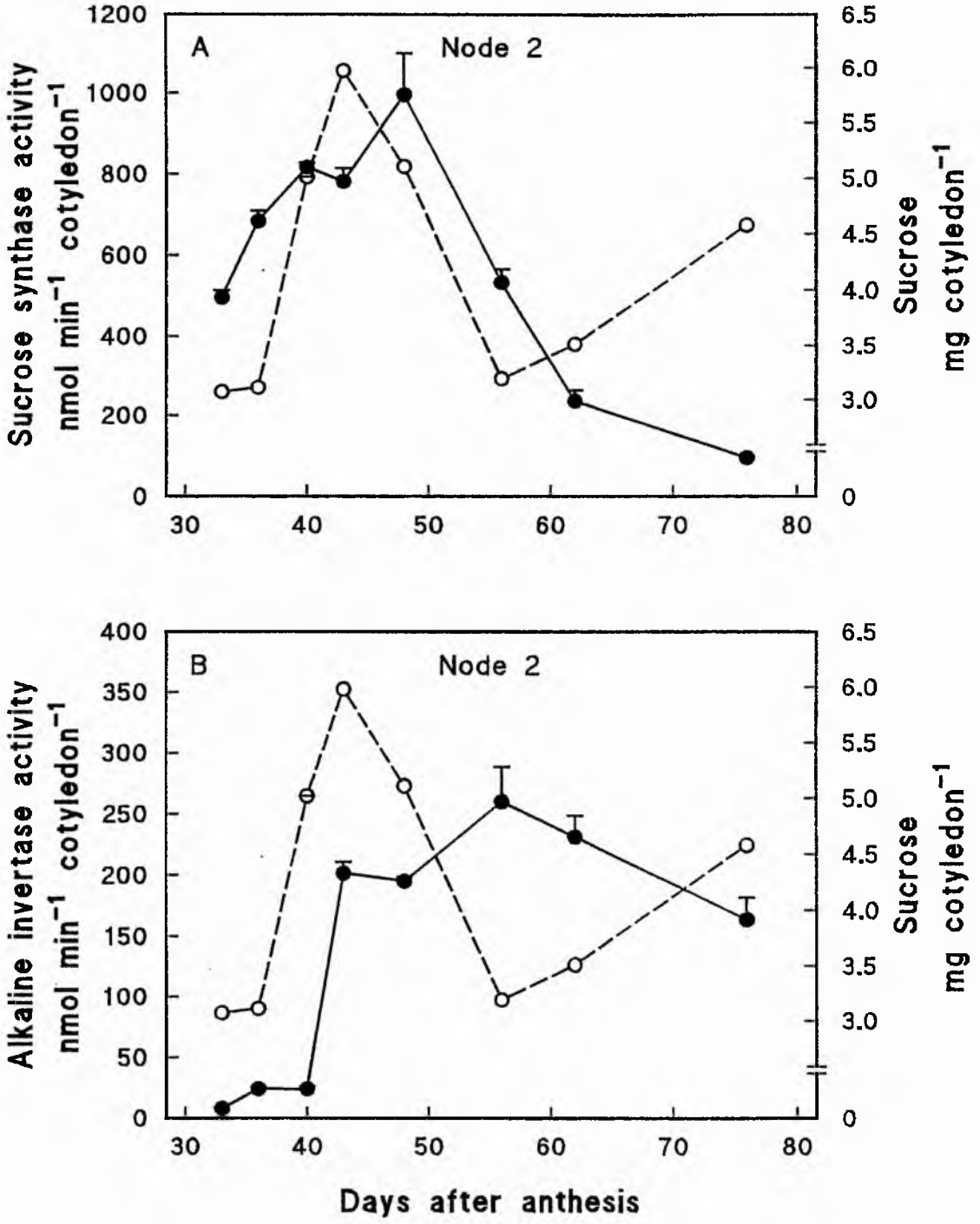


Fig. 5.6 Activity of A) sucrose synthase and B) alkaline invertase in *V. faba* cotyledons sampled from node 2 during bean development. Activities are shown in relation to sucrose content. Values shown are means (n=4) + sem. (●) - enzyme activity, (○) - sucrose content.

There is a reasonable relationship between tissue sucrose synthase activity and sucrose content (Fig. 5.6A). However, alkaline invertase activity is highest when the sucrose content is at its lowest value (Fig. 5.6B).

The importance of the sucrose content is more apparent when the K_m values for sucrose synthase and alkaline invertase (see Chapter 7) are considered. The rate of sucrose synthase and alkaline invertase activity at the measured sucrose content, converted to sucrose concentration, (assuming sucrose to be evenly distributed throughout the cell) was calculated as a percentage of V_{max} from the Hanes-Woolf plots (Figs. 7.3B and 7.8B) for node 2 during bean development. Using these values, the estimated enzyme activities occurring *in vivo* were determined and are shown in Figure 5.7. Values for the final sample date are omitted from this graph as by that stage of development the plants had senesced and the flux of sucrose into the cotyledons was very low. Consequently, reliable estimates of the sucrose concentration in the cytosol, where both enzymes are considered to be active, could not be calculated.

5.3.4.3 Acid invertase

Acid invertase activity is not shown as it was barely detectable during seed development. The maximum activity obtained was only 7 nmol min^{-1} $cotyledon^{-1}$. Inclusion of NaCl in the extraction buffer had no effect and no specific pattern of activity was observed.

5.3.4.4 Hexose kinases

The activities of the hexose kinases, fructokinase and glucokinase, are shown in Figs. 5.8A & 5.8B. As with sucrose synthase activity, fructokinase activity was

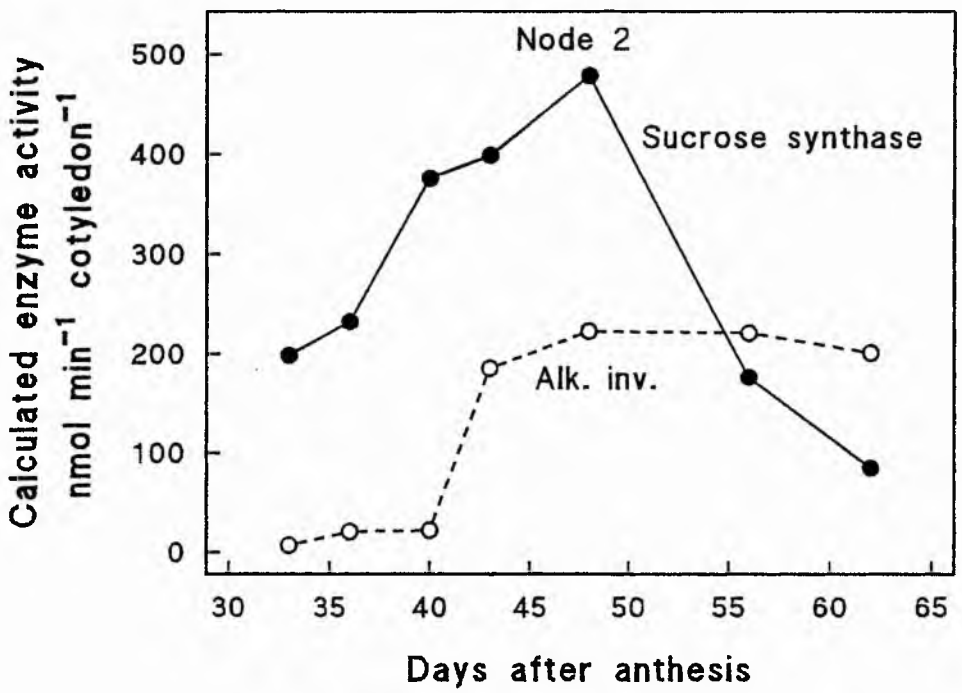


Fig. 5.7 Sucrose synthase and alkaline invertase activity calculated on the basis of measured sucrose concentration in cotyledons from node 2 during bean development.

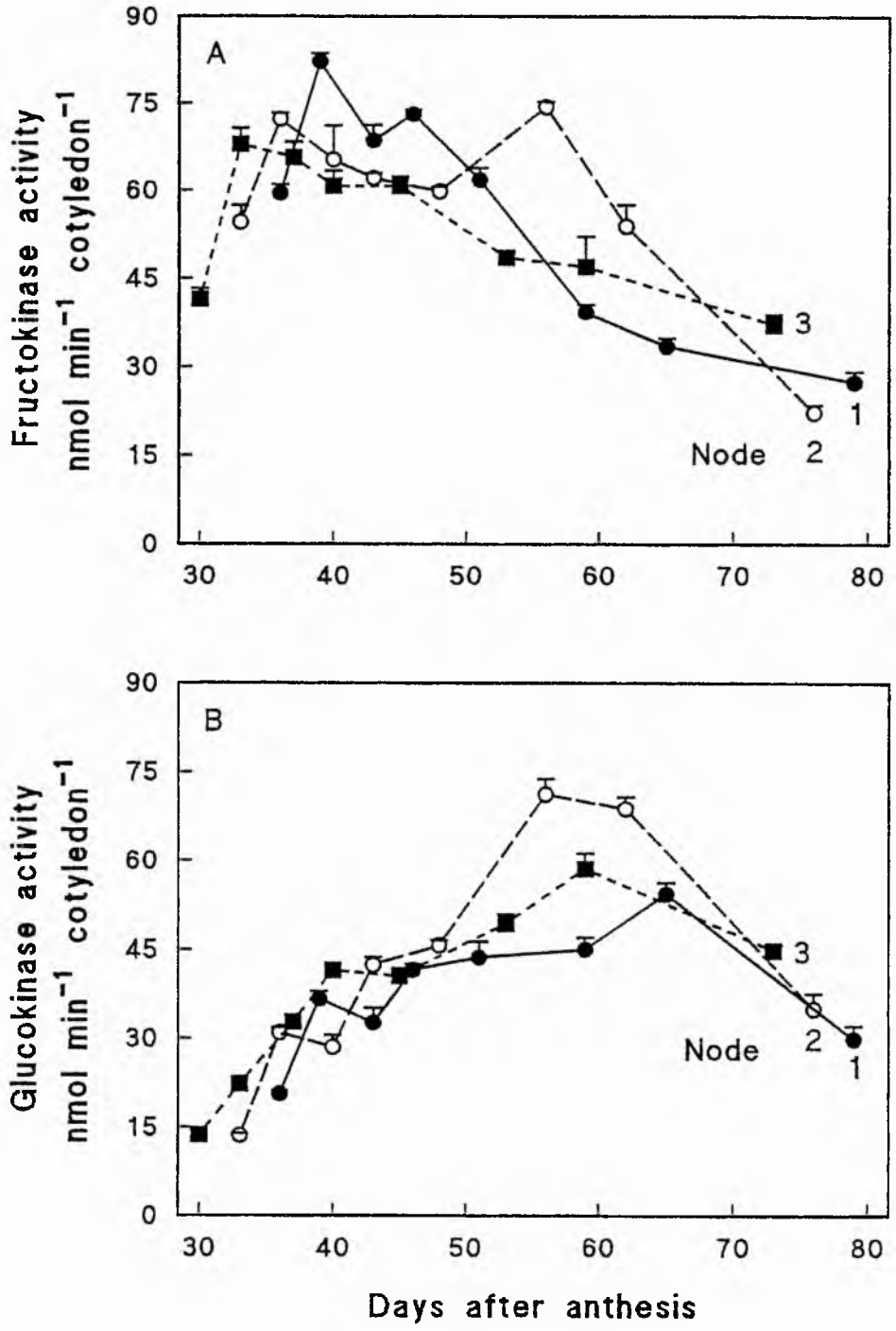


Fig. 5.8 Activity of A) fructokinase and B) glucokinase in *V. faba* cotyledons sampled from the three nodal positions during bean development. Values shown are means (n = 4) + sem.

highest early on in seed development, decreasing from about 50 to 60 DAA onwards. The maximum catalytic activity determined was 80 nmol min^{-1} $cotyledon^{-1}$ (node 1). By contrast, glucokinase activity increased from 14 nmol min^{-1} $cotyledon^{-1}$ to peak at 70 nmol min^{-1} $cotyledon^{-1}$ around 60 DAA. The pattern of activity is similar to that of alkaline invertase with the values only decreasing significantly at the last sample date.

5.3.4.5 ADP-glucose pyrophosphorylase

Extractable ADP-glucose pyrophosphorylase activity was erratic during seed development although the pattern obtained was almost identical for all nodal positions (Fig. 5.9). Changes for nodal positions 2 and 3 were consistently several days behind position 1. Highest enzyme activities were measured from samples taken 45-55 DAA.

5.3.5 Electrophoresis and Immunoblotting

Protein extracts from beans from node 2 for each of the sample dates were electrophoresed on 10 and 12.5% SDS-polyacrylamide gels (Plates 5.IIA & 5.IIB respectively). The increasing level of total protein $cotyledon^{-1}$ and the changing pattern of polypeptides are clearly shown. The presence of a polypeptide (ca. 90 kD) increasing in intensity on the gel during development and decreasing for the final sample (Plate 5.IIA marked with arrows), was subsequently identified as a subunit of sucrose synthase. It was not possible to identify any of the other enzymes as they are present in very small amounts relative to the storage proteins, legumin and vicilin. The major bands observed were subunits of these storage proteins.

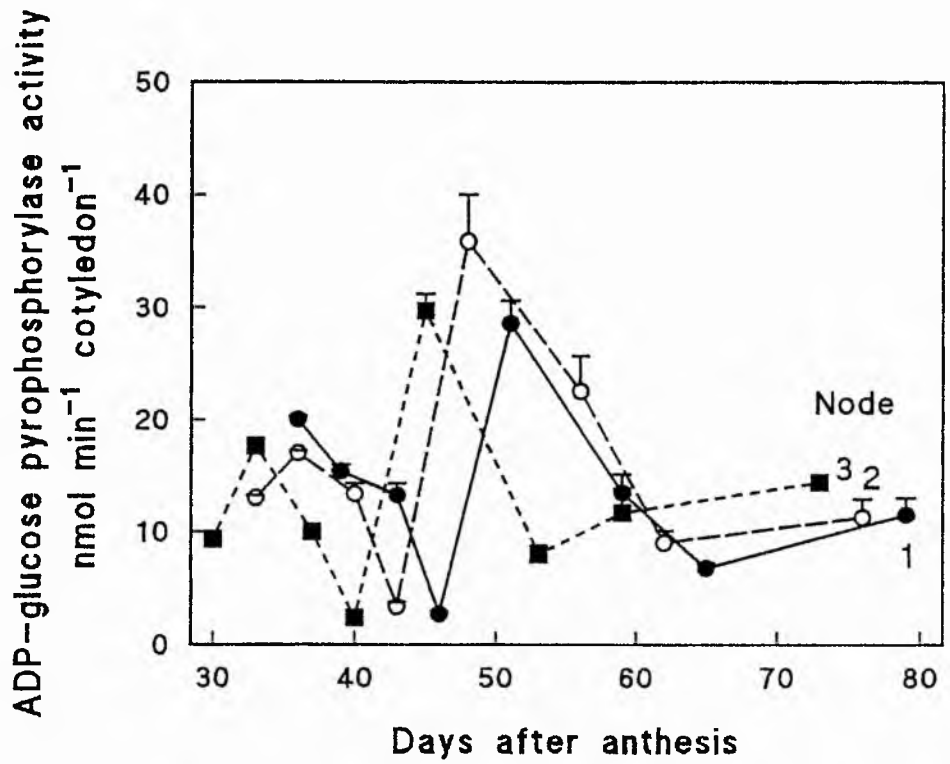
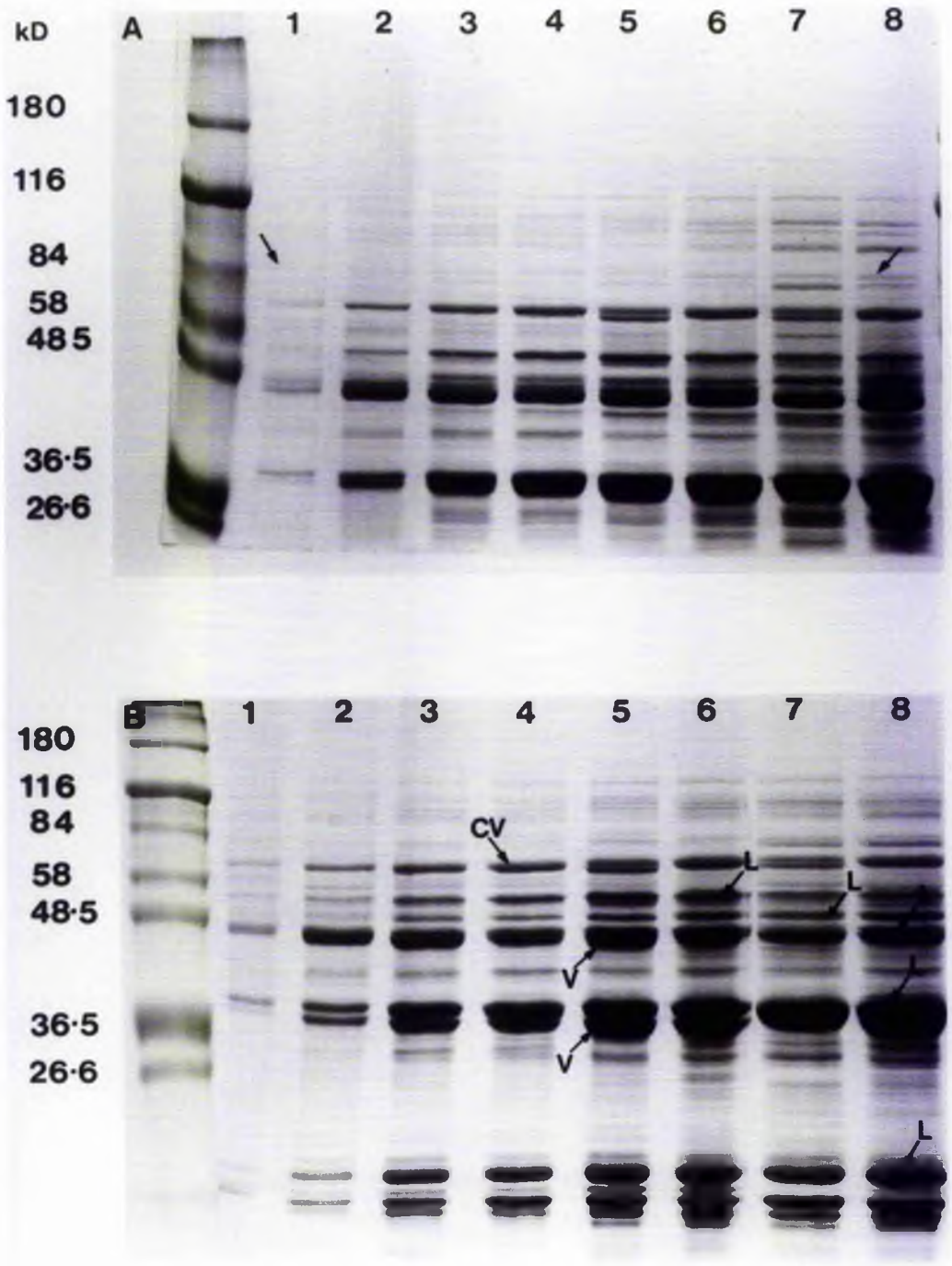


Fig. 5.9 ADP-glucose pyrophosphorylase activity of *V. faba* cotyledons sampled from the three nodal positions during bean development. Values shown are means (n = 4) + sem.

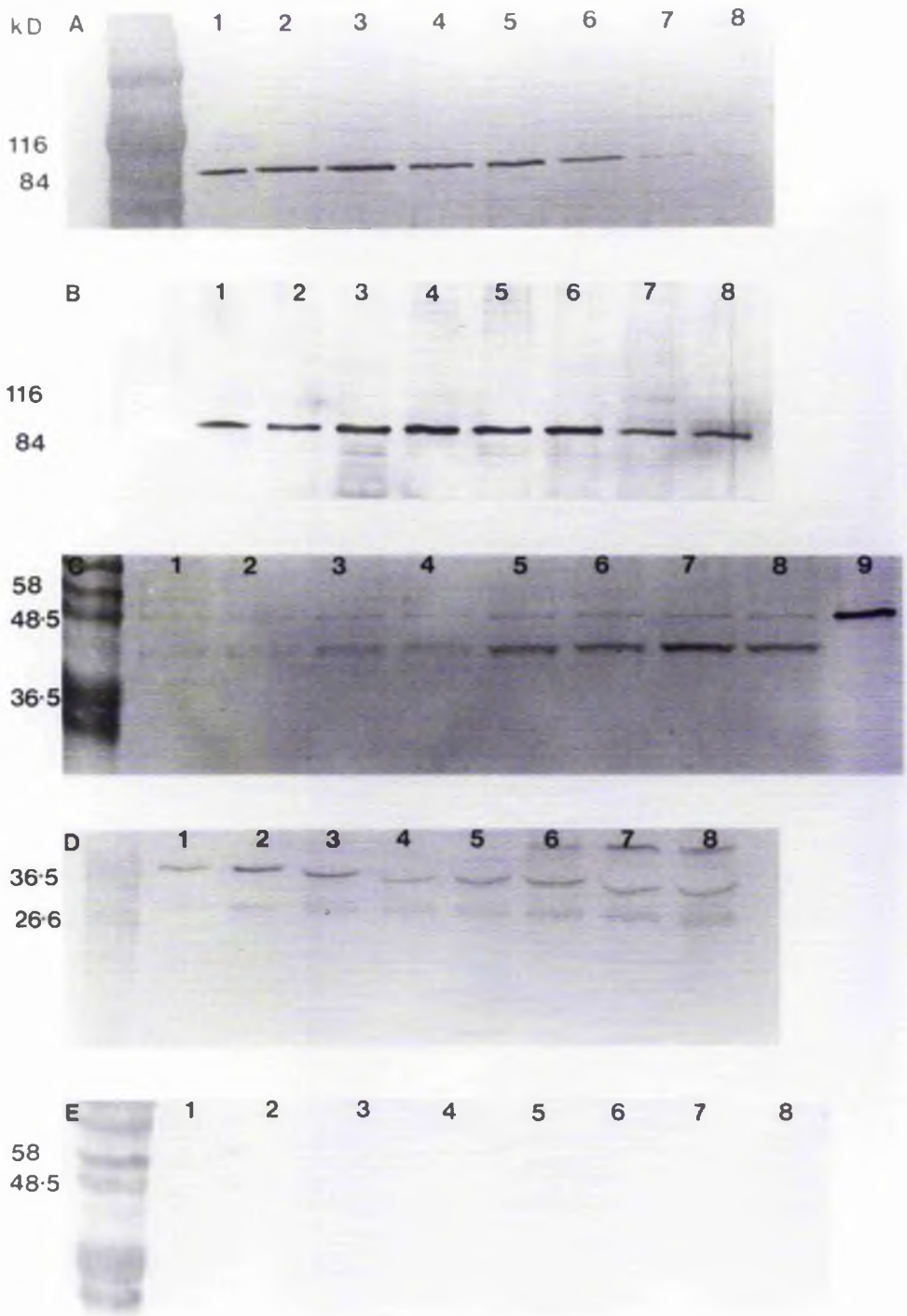
Plate 5.II Crude protein extracts from developing bean cotyledons with lanes 1-8 corresponding to sample dates 1-8 in both gels. A) 10% SDS-polyacrylamide gel with arrows indicating the position of the sucrose synthase polypeptides. B) 12.5% SDS-polyacrylamide gel with arrows indicating the position of polypeptides of convicilin (CV) ca 64 kD, legumin (L) ca 56, 52, 46.5, 37.5, and 21 kD and vicilin (V) ca 48 and 32 kD. The outer lane in both A) and B) contains MW markers (SDS-7B Sigma).


kD
A
1
2
3
4
5
6
7
8
180
116
84
58
48·5
36·5
26·6
B
CV
V
V
L
L
L

Legumin is cleaved into two components by reduction with 2-mercaptoethanol during electrophoresis - an acidic polypeptide (a), M_r 37,000, and a basic polypeptide (b), M_r 20,000 (Matta *et al.* 1981). These polypeptides are clearly visible, as are bands corresponding to vicilin (identified on Plate 5.IIB).

Immunoblots for sucrose synthase (two methods), alkaline invertase, fructokinase and ADP-glucose pyrophosphorylase are shown on Plates 5.IIIA, B, C, D & E respectively. The sucrose synthase antibodies, raised against purified faba bean cotyledon sucrose synthase protein (see chapter 7), recognised a single polypeptide at ca. 90 kD (Plate 5.IIIA). The intensity of this band increased to reach a peak in lane 5 (corresponding to sample date 5 - 48 DAA) before declining to a low intensity band in the mature cotyledons. The results using the alternative ECL method of detection were similar but considerably more sensitive (Plate 5.IIIB). While the usual method showed cross-reaction with a single band only, due to the increased sensitivity using the ECL method a number of minor bands were also visible. The alkaline invertase antibodies raised against the subunit of the sugar beet alkaline invertase protein (see Chapter 8) cross-reacted with two polypeptides, one of ca. 54kD and the other of ca. 40kD (Plate 5.IIIC). To aid in the identification of the polypeptide subunit of alkaline invertase, a partially purified sample of the enzyme from sugar beet was loaded in lane 9. The antibodies cross-reacted specifically with a polypeptide of ca. 54 kD from this sample. The same results for each of the samples (lanes 1-9) were obtained when the antibodies raised against alkaline invertase from *Vicia faba* were used (immunoblot not shown). Samples of potato tissue when extracted under certain conditions in which the level of protease inhibitors was insuffient, when immunoblotted cross-reacted with a polypeptide at ca. 40 kD. The same potato samples when extracted rapidly and with the inclusion of protease inhibitors resulted in a single band at 54kD when imunoblotted with alkaline invertase antibodies (Plate 3.IVD).

Plate 5.III Immunoblots of SDS-polyacrylamide gels shown in Plate 5.II with bean sucrose synthase antibodies (dilution 1:10,000), cross-reacting polypeptides detected by normal BCIP/NBT method A) or ECL method B); C) sugar beet alkaline invertase antibodies (dilution 1:500) - lane 9 contains a partially purified sugar beet sample; D) pea seed fructokinase antibodies (dilution 1:3000) and E) ADP-glucose pyrophosphorylase (dilution 1:750). Outer lane in each immunoblot contains MW markers (SDS-7B Sigma).


kD
A
1 2 3 4 5 6 7 8
116
84
B
1 2 3 4 5 6 7 8
116
84
C
1 2 3 4 5 6 7 8 9
58
48·5
36·5
D
1 2 3 4 5 6 7 8
36·5
26·6
E
1 2 3 4 5 6 7 8
58
48·5

These results (see section 3.3.2) would indicate that the lower polypeptide is a breakdown product of alkaline invertase. The fact that the intensity of this lower band follows activity further supports this theory.

The pea seed fructokinase reacted with a number of polypeptides, one of which at 36 kD (marked with arrow) was identified as the likely subunit of the fructokinase protein (Gardner, 1992). The intensity of this band followed the level of fructokinase activity (Plate 5.IIID).

The ADP-glucose pyrophosphorylase antibody was highly specific, reacting with a single polypeptide at ca. 48 kD (Plate 5.IIIE). The intensity of this band increased during the developmental time course reaching a peak at sample date 5 - 48 DAA (lane 5) and then declining. The maximum enzyme activity determined was also from this stage in development although the pattern of enzyme activity, as mentioned previously, was somewhat erratic.

5.4 Discussion

In contrast to potato tubers, the developing cotyledons of *V. faba* accumulate almost equal quantities of both starch and protein. Although a steady rise in the starch content of the developing cotyledons (at least over the first four sample dates) are shown (Fig. 5.2B) the potential for incorporation into starch (from the ^{14}C-glucose and ^{14}C-sucrose uptake results) remains low over the first four sample dates and only rises rapidly between 40-50 DAA. A similar pattern of uptake and incorporation of ^{14}C-sucrose into starch in cotyledons of *V. faba* has previously been observed (K. Wright, pers. comm.). Total uptake of ^{14}C-sucrose by soybean cotyledons declined during seed development in parallel with leaf photosynthetic rate and very slow incorporation of label into the ethanol-insoluble fraction was observed 35 and 46 days after flowering

(DAF) (VerNooy *et al.*, 1986). In their study, they followed the rate of incorporation of label into the insoluble fraction over a chase period of 40 h after a 50 min pulse with ^{14}C-sucrose. At the end of the incubation period, 50% of ^{14}C-sucrose taken up by the cotyledons at 35 DAF was incorporated into ethanol-insoluble components in contrast to nearly 100% by the cotyledons at 46 DAF. If similarly slow rates of incorporation of ^{14}C-sucrose into starch are present in *V. faba*, then the absence of a long chase period would explain the apparent contradictory results discussed above.

If the value of starch accumulation for samples from node 2 (692 nmol h^{-1} $cotyledon^{-1}$) is taken into account, the level of sucrose synthase (measured or calculated, see Fig. 5.7) would be considerably in excess of this rate. Apart from the level of alkaline invertase at the first sample date, activity of this enzyme would be sufficient or, at later stages in bean development, in excess of that needed to support the measured rate of starch accumulation. The measured activity of ADP-glucose pyrophosphorylase, a key enzyme in starch synthesis, was highest at sample date 5 and this peak in activity was further confirmed by immunoblotting. This activity (36 nmol min^{-1} $cotyledon^{-1}$) measured at 30°C, and assuming a Q_{10} of 2, would support a rate of starch accumulation of 720 nmol (hexose equivalent) h^{-1} $cotyledon^{-1}$. This value is very close to the actual rate observed. The results of maximum catalytic activity of ADP-glucose pyrophosphorylase at sample date 5, would support the sudden increase in ^{14}C- incorporation into starch at this stage. However, it does not explain the steady rise in starch content between sample date 1-4 when the measured enzyme activity is lower. In making these calculations a number of assumptions are made which may be incorrect. This enzyme, however, does not seem to have the same level of importance in controlling the flux of carbon into starch in pea embryos as in leaves (Smith & Denyer, 1992) and in this respect the faba bean cotyledons may be similar. The exact role of

starch phosphorylase in pea embryos has not been elucidated but it is thought that the plastidial form may be involved in the synthesis of low-molecular-weight glucans that could act as primers for starch synthesis during embryo development (Smith & Denyer, 1992). If this was possible in the developing bean cotyledons, it would explain the relatively high rate of starch accumulation when the activity of ADP-glucose pyrophosphorylase was still low.

The dependence of sucrose synthase activity on the sucrose flux to the sink tissues (shown in potato tubers, chapter 3 & 4) is also apparent in bean cotyledons with the activity following the rise and fall in sucrose content. During early development, the pathway of sucrose-cleavage via sucrose synthase appeared to dominate supported by high levels of fructokinase activity to prevent endproduct inhibition. During the development of lima bean (*Phaseolus lunatus*) seed, sucrose synthase was classified as a strongly adaptive enzyme responding rapidly to changes during development while both acid and alkaline invertase showed maintenance-type enzyme activity (Xu *et al.*, 1989). However, the pattern of activity of alkaline invertase and glucokinase in developing faba bean cotyledons indicates that these enzymes may have a significant role when sucrose levels are low. The affinity of sucrose synthase for sucrose is nearly seventeen-fold lower than alkaline invertase (K_m ≅ 169 mM for sucrose synthase, K_m ≅ 10.1 mM for alkaline invertase; Km determinations for both enzymes from bean cotyledons shown in chapter 7). Thus when calculating the possible *in vivo* enzyme activity (Fig. 5.7), the level of sucrose synthase is only 33-51% of the maximum catalytic activity while alkaline invertase is 85-92%. Acid invertase does not appear to be involved in sucrose cleavage during bean development but was very active in the elongating plumule during bean seed germination (previous experimental results). Although the interpretation of *in vitro* enzyme determinations needs to

be guarded, support for the pattern of enzyme activity from the level of enzyme protein subunits is obtained from the immunoblots.

5.5 Conclusions

The study of carbohydrate metabolism in developing bean cotyledons supports the hypothesis that the sucrose synthase pathway dominates in actively-filling sink tissues. In contrast, sucrose-cleavage via the invertase pathway (alkaline invertase) is more likely to dominate when sucrose levels are declining. The maximum catalytic activity and calculated activity of both enzymes is in excess of requirements for sucrose cleavage to support starch synthesis. The pattern of fructokinase activity is similar to that of sucrose synthase while glucokinase is more active at the time of high alkaline invertase activity. Both starch and protein accumulate in a relatively linear pattern and to a similar level.

CHAPTER 6

SUCROSE METABOLISM IN DEVELOPING TAPROOTS OF *Beta vulgaris* L.

6.1 Introduction

The taproot of sugar beet (*Beta vulgaris* L.) forms the sink tissue for the accumulation of sucrose within the vacuoles of the storage parenchyma cells. The sucrose concentration can increase to over 500 mM. Consequently, it is an important source of sugar, contributing approximately 40% of the world's supply (figure obtained from British Sugar plc, 1994).

Anatomical studies on the growth and development of the taproots have shown that concentric rings are laid down, the youngest at the periphery, and that these rings continue to develop simultaneously by both cell division and cell expansion (Artschwager, 1930; Milford, 1973). Each of the rings comprises a band of vascular tissue and a broader band of storage parenchyma cells.

Photoassimilate produced within the sugar beet leaves is transported to the phloem cells within the taproot in the form of sucrose, to be unloaded either symplastically or apoplastically. The precise route is still uncertain although a recent hypothesis suggests that sucrose is released from the sieve element-companion cell complex (SE-CC) into the apoplast of the vascular tissue, where a cell wall bound acid invertase may cleave sucrose into glucose and fructose thus preventing a backflow of sucrose into the sieve tubes (Fieuw & Willenbrink, 1990). Support for an apoplastic route rather than symplastic comes from microscopic investigation using the callose-staining dye, aniline blue (0.01%) (D. Prior, pers. comm.). Callose deposits on the cell walls of the

storage cells are usually indicative of plasmodesmatal connections (Oparka, 1986). Examination of transverse and longitudinal sections of sugar beet in comparison with potato tuber sections has revealed a very low frequency of callose-staining sites on storage cells (D. Prior, pers comm.).

To understand the fate of sucrose entering the cells of the sugar beet taproot it is essential to know more about the potential activity of each of the sucrose-cleaving enzymes - the acid and alkaline invertases and sucrose synthase. Additionally, sucrose inversion can occur during the storage of taproots and during the sucrose extraction process (Oldfield *et al.*, 1980, 1981). These result in a loss of sucrose yield and in reduced quality of the crystalline structure and colour of the sucrose (Oldfield *et al.*, 1980). Acid invertase has been implicated in this postharvest cleavage of sucrose (Wyse, 1974) although its activity is very low in mature taproots (Masuda *et al.*, 1987). An endogenous invertase inhibitor is thought to regulate acid invertase activity thus allowing the accumulation and maintenance of high sucrose levels during taproot development (Burakhanova *et al.*, 1987).

The experiment described in this chapter was designed to investigate the carbohydrate levels and the activity of the sucrose-cleaving enzymes in sugar beet taproots during development and postharvest storage.

6.2 Material and Methods

6.2.1 Plant material

Pelleted seeds of sugar beet cv. Celt (supplied by British Sugar plc, UK) were sown in the field at SCRI on the 27th May using an Øjord drill to give a final plant density of 7 plants m^{-2}. The first sampling of taproots was taken 7 weeks later. Due to the variation in time of seedling emergence, it was necessary to

select the taproots by fresh weight rather than on sample date alone. At the first date, samples taken ranged from 2-40 g FW. Further samples were taken at regular intervals until 16 weeks after sowing, when the average fresh weight of the taproots was over 500g. At this stage, sugar beet were harvested and stored on trays in a temperature-controlled room at 7-8°C (the average clamp temperature obtained in a study on beet storage [Oldfield *et al.*, 1981]) while at the same time samples of sugar beet in the field were defoliated by cutting off the tops. Samples from these treatments, along with control plants (left growing intact in the field), were used for measurements on a further 4 occasions (9, 24, 52 and 80 days after the start of the treatments).

6.2.2 Enzyme determinations

The sugar beet taproots were washed and cut longitudinally down the centre before fine longitudinal slices (to eliminate effects of possible gradients from top to bottom of root) were taken for enzyme extracts. The remainder of the root was chopped into small pieces, weighed, frozen and later freeze-dried for starch and sugar determinations.

6.2.2.1 Sucrose synthase

The samples were ground to a fine powder in liquid nitrogen and extracted in four volumes of extraction solution as described previously (**3.2.2.1**). The same assay method was used as outlined in section **2.3.1** except for a change in the pH of the assay buffer. Prior testing over a pH range had shown the optimum for sugar beet sucrose synthase in a crude extract to be pH 6.7-6.8. Three

separate replicates for each treatment were assayed with four determinations per assay. Additionally, samples from the vascular and storage regions were extracted separately to test any possible differences in their sucrose synthase activity.

6.2.2.2 Acid invertase

Acid invertase was extracted as above but in 4 volumes of extraction solution as described previously (**3.2.2.2**). For each enzyme extraction, pellets were tested for residual activity. While in most cases the activity was zero or less than 10%, in the earlier samples some pellets retained 20-25% of total measurable activity. The final values were therefore expressed as total activity recovered both in the soluble and pellet fractions. The foaming treatment, used previously for determining potato acid invertase activity, had no significant effect on sugar beet acid invertase.

6.2.2.3 Alkaline invertase

Alkaline invertase was extracted as described above for acid invertase. The activity was then assayed as in section **5.2.2.3**. The optimum pH for sugar beet alkaline invertase (pH 7.5-7.6) was determined from pH curves prior to starting the experiment.

6.2.3 Protein determination

The protein content of each of the samples was determined using the Bio-Rad method as described previously (section **2.7**).

6.2.4 Electrophoresis and Immunoblotting

Protein samples from each of the treatments were subjected to SDS-PAGE (12.5% acrylamide) as described previously (section **2.5.1**). Parallel gels were run and the polypeptides transferred to nitrocellulose to allow immunoblotting with polyclonal antibodies raised against sucrose synthase (purified from bean cotyledons, chapter **7**) and alkaline invertase (purified from bean cotyledons, chapter **7**).

6.2.5 Determination of starch, sucrose, glucose and fructose

All carbohydrate determinations of the sugar beet taproots were carried out after freeze-drying. The methods used are described in sections **2.4.1** and **2.4.2**.

6.3 Results

6.3.1 Enzyme activity

The activity of acid and alkaline invertase and sucrose synthase in taproots of increasing fresh weight are shown in Fig. **6.1**A, B & C. Maximum acid invertase activity (75 nmol min^{-1} g^{-1}FW) occurred in the smallest taproots

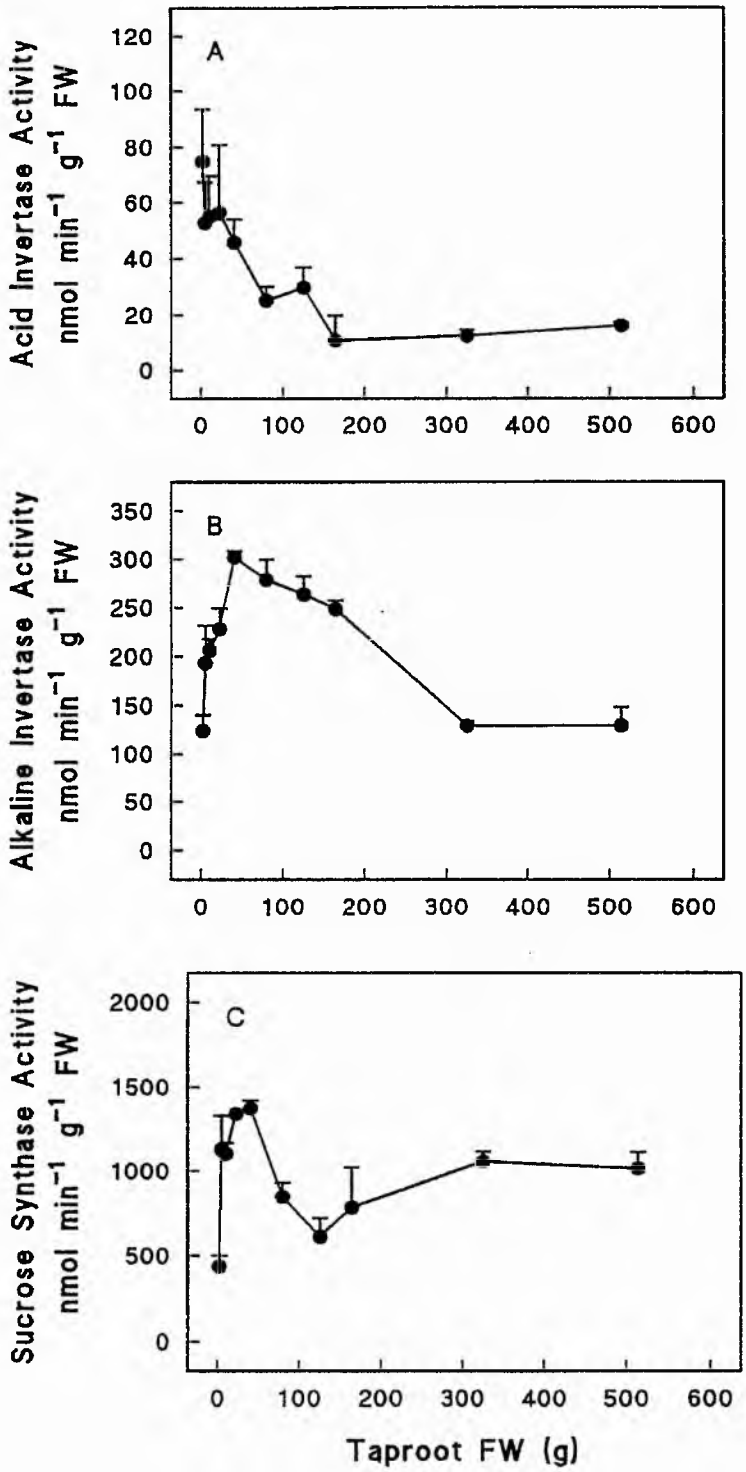


Fig. 6.1 Activity of A) acid invertase , B) alkaline invertase and C) sucrose synthase in sugarbeet taproots sampled from field-grown plants during the growth period. Root fresh weight range from 2- > 500 g. Values shown are means (n = 3) + sem.

(approx. 2g FW). Activity decreased rapidly during development to reach values of approximately 10-15 nmol min^{-1} g^{-1}FW. In contrast, alkaline invertase activity increased rapidly to reach a peak of ca 300 nmol min^{-1} g^{-1}FW in taproots weighing 40 g. Thereafter the activity decreased to 130 nmol min^{-1} g^{-1}FW in the mature taproots. Sucrose synthase showed maximum activity (1,371 nmol min^{-1} g^{-1}FW) also in 40 g FW taproots, but then declined to approximately 1,000 nmol min^{-1} g^{-1}FW in the mature sugar beet. Values obtained for sucrose synthase activity in the storage and vascular regions of the root, after separate extractions, are shown below:-

	nmol min^{-1} g^{-1}FW	nmol min^{-1} mg^{-1} protein
Vascular region	783.5 ± 128.0	223.2 ± 9.4
Storage region	558.9 ± 98.5	233.5 ± 12.1

While higher activity was present in the vascular region when values were expressed on a fresh weight basis there was no significant difference in the specific activity of the samples due to the higher protein level within the vascular region (3.46 mg g^{-1}FW cf. 2.35 mg g^{-1}FW in storage tissue).

A comparison of the activities of the three enzymes after storage and defoliation treatments are shown in Fig. 6.2A, B & C. The level of acid invertase increased in all treatments after 9 days, then decreased to barely detectable levels. There were no significant differences in the activities from the intact and defoliated plants in the field and stored taproots. Consequently, measurement of acid invertase activity was omitted for the sample (+52 days) but checked for the final sample (+80 days). Although a very slight rise occurred in the final sample, the activity was very low (approx. 7-12 nmol min^{-1} g^{-1} FW) in all treatments. The pattern of alkaline

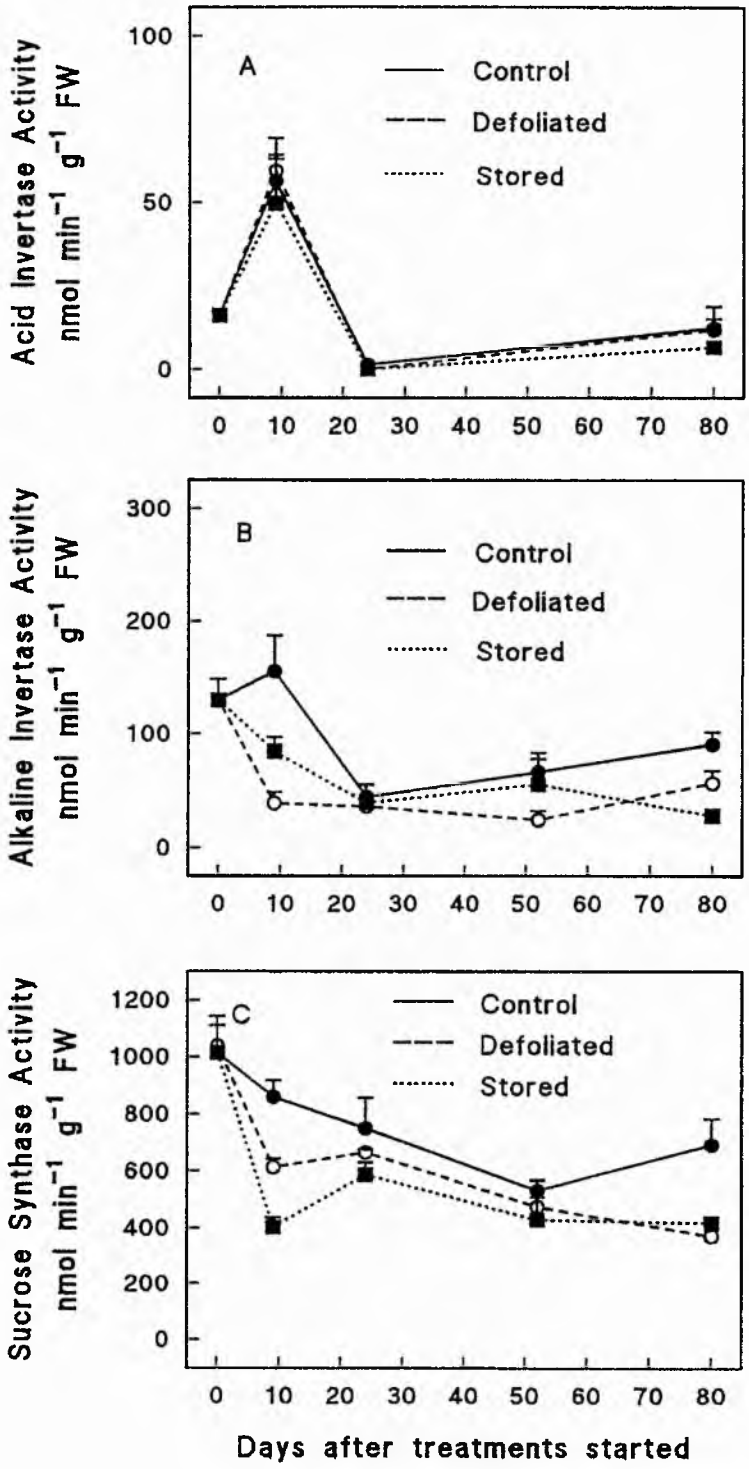


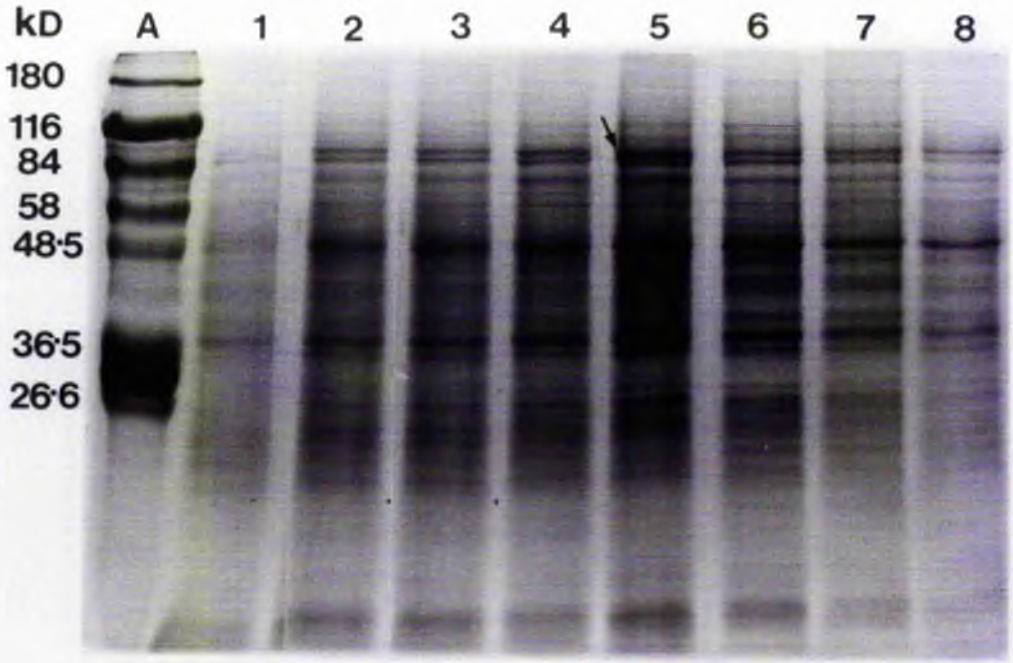
Fig. 6.2 Activity of A) acid invertase, B) alkaline invertase and C) sucrose synthase in sugarbeet samples from roots stored at 7-8°C, from defoliated plants left in the field and from control field-grown plants. Values shown are means (n = 3) + sem.

invertase activity was more variable between treatments with highest activity expressed throughout in the control samples (significantly higher than both treatments at +9 and +80 days). In contrast the lowest activity was found in the defoliated samples at 3 out of the 4 sample dates. By the final harvest date, however, significantly lower activity was found in the stored samples. Similarly, the highest sucrose synthase activity occurred in control samples (significantly higher at +9 and +80 days) and lowest in the stored samples. Sucrose synthase activity declined continuously and significantly following the start of all treatments.

6.3.2 Electrophoresis and Immunoblotting

When equal amounts of protein from extracts of taproots ranging from 2.5g to 160g FW were loaded on 12.5% SDS-polyacrylamide gel, no major qualitative differences were visible during development (Plate 6.IA). By the final sample date more bands were clearly visible but, unlike potato and bean tissues which both accumulate specific storage proteins, the sugar beet samples showed the absence of any major protein subunits (Plate 6.1B). On the same gel, no treatment differences were visible. A parallel gel to that shown in Plate 6.1A was immunoblotted with the sucrose synthase antibody and a specific cross-reacting polypeptide was visible at approximately 90 kD. The quantity of immunoreactive protein increased during development, reaching a peak in roots of ca. 40 g FW (Plate 6.IIA). A fainter crossreacting polypeptide (~94 kD) was visible in the samples from larger taproots (Plate 6.IIA, lanes 3 to 8). A parallel gel immunoblotted with alkaline invertase antibodies showed a specific crossreaction with a polypeptide of approximately 54 kD. Again the intensity of crossreactivity increased during

Plate 6.1 A) 12.5% SDS-PAGE of crude samples from developing sugar beet taproots. Lane 1 to 8, are loaded with 16 μg of protein extracted from taproots weighing (FW) 2.5g, 5g, 10g, 20g, 40g, 80g, 120g, and 160g respectively. An arrow indicates the sucrose synthase polypeptide which is at its highest intensity in the 40g FW taproots. B) 12.5% SDS-PAGE of crude samples of mature taproots from each of the treatments on the final sample date (10th December - +80 days after start of treatments). Lane 1 - sample from control sugar beet taproots left growing in the field (ca 1000g FW); Lane 2 - sample from defoliated sugar beet taproots left in the field (ca 700g FW); Lane 3 - sample from sugar beet taproots stored at 7-8^{o}C (ca 500g FW). Each of the samples contained approximately 16μg protein. The outer lane of both gels contain MW markers (SDS-7B Sigma).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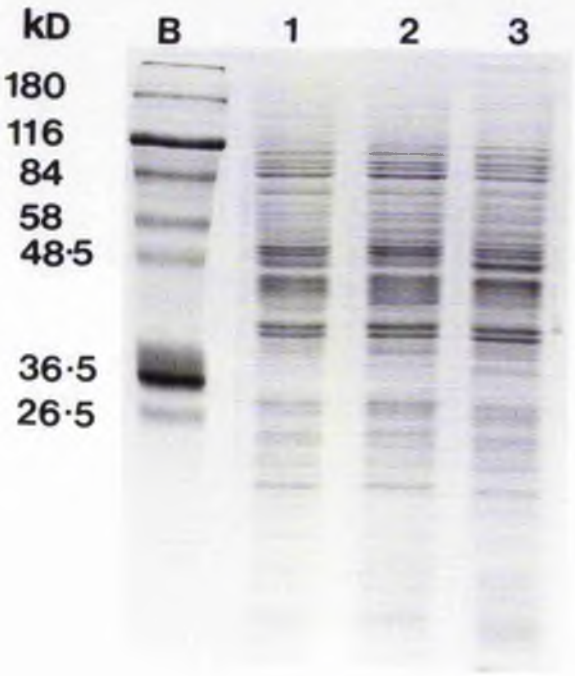
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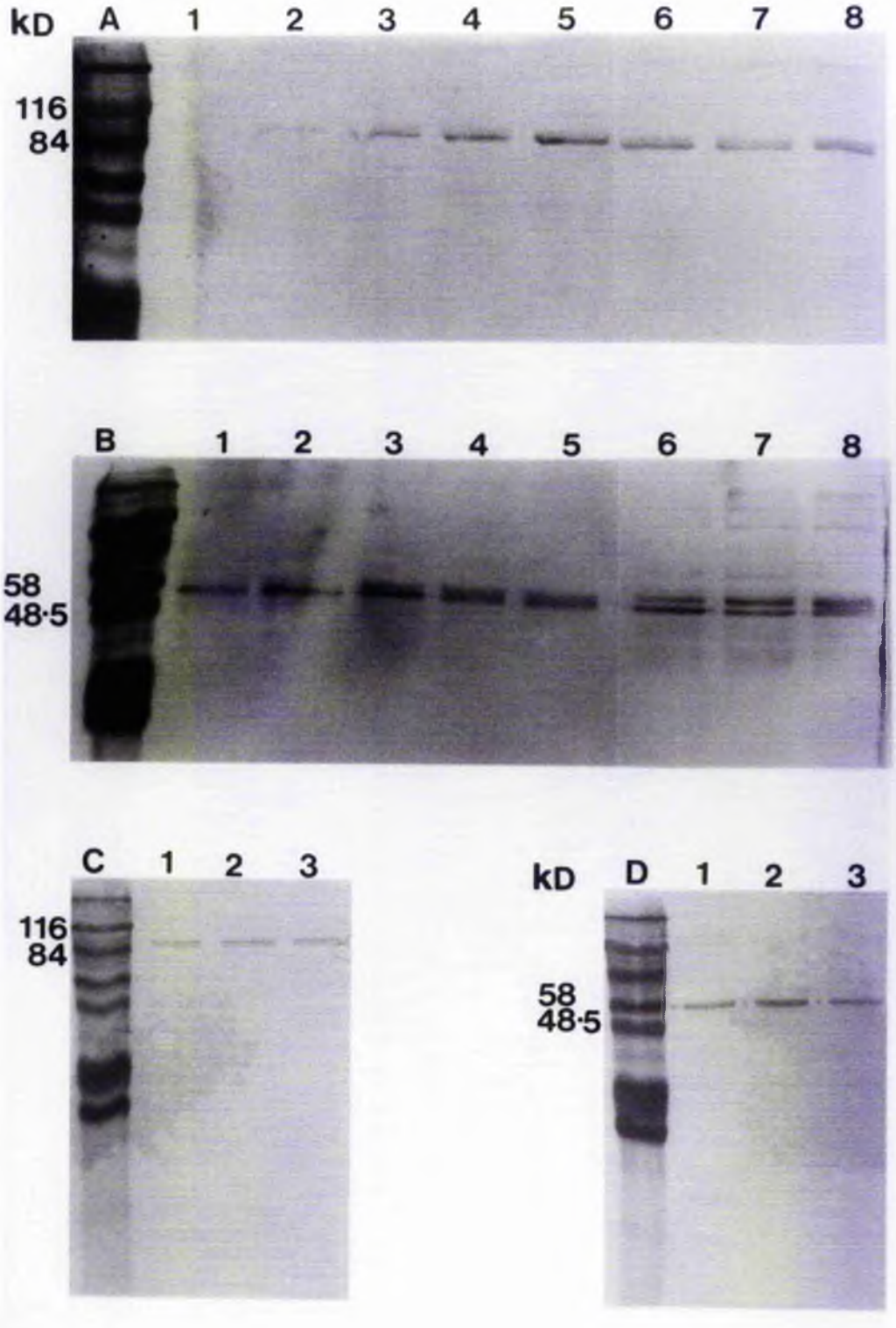
Plate **6.**II A) and B) Immunoblots of SDS-PAGE shown in Plate **6.**I A) with A) bean sucrose synthase antibodies (dilution 1:5000) and B) bean alkaline invertase antibodies (dilution 1:1000). C) and D) Immunoblots of SDS-PAGE shown in Plate **6.**1 B) with C) bean sucrose synthase antibodies (dilution 1:5000) and D) bean alkaline invertase antibodies (dilution 1:1000).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taproot growth (Plate 6.IIB). Larger taproots again contained a larger polypeptide (~58 kD) detected by the alkaline invertase antibody which increased in developing taproots (Plate 6.IIB, lanes 6, 7 and 8). Parallel gels of extracts from the final sample date (80 days after start of treatments) were immunoblotted with both sucrose synthase and alkaline invertase antibodies and showed no treatment differences (Plate 6.IIC & D). As noted for the previous blots, two crossreacting bands are detected with each antibody, although, in both cases, the upper band appears more intense than the lower. This may be due to the development of different isoforms of the enzymes or simply due to either re-oxidation of the protein samples during the period of electrophoresis or partial proteolytic cleavage.

6.3.3 Sugar and starch levels during development and after storage

During sugar beet taproot development the level of sucrose increased rapidly to reach concentrations at maturity of over 600 mM (Table 6.1 - value calculated after correction for moisture content). In contrast, the levels of both hexoses, glucose and fructose, were low and decreased substantially with glucose thirteen-fold lower and fructose over four-fold lower at maturity. The G : F ratio was > 12:1 in the smallest roots but decreased to just > 4:1 in the largest roots. The level of starch was low and decreased during development.

After the start of the treatments, the levels of the sugars and starch were again determined with the aim of identifying and quantifying possible sucrose losses (Table 6.2). In the control plants (i.e. left growing in the field), the sucrose content continued to rise to > 230 mg g^{-1}FW (a concentration of over 900 mM) in the plants finally sampled on the 10th December. In the plants defoliated but left growing in the field, the sucrose content remained at ca 170

Table 6.1 Fresh weight and sucrose, glucose, fructose and starch content of developing sugar beet taproots

FW (g)	Sucrose	Glucose	Fructose	Starch
		(mg g^{-1} FW)		
2.4± 0.6	73.75±8.24	2.180±0.342	0.177±0.089	1.897±0.265
5.3± 0.5	76.24±5.27	1.690±0.631	0.263±0.021	1.348±0.499
10.3± 0.5	88.81±2.78	0.907±0.116	0.134±0.037	0.859±0.047
22.7± 2.1	80.05±5.85	0.527±0.096	0.077±0.017	0.539±0.038
40.6± 0.9	58.45±5.68	0.303±0.007	0.051±0.005	0.341±0.084
80.0± 4.4	100.75±5.76	0.243±0.023	0.166±0.035	0.545±0.055
125.7± 0.7	98.84±6.52	0.267±0.018	0.061±0.005	0.400±0.018
164.6± 5.7	96.07±0.73	0.243±0.040	0.091±0.004	0.419±0.049
325.2± 2.2	165.37±9.46	0.237±0.017	0.043±0.005	0.769±0.007
513.9±29.4	140.80±4.20	0.168±0.046	0.039±0.009	0.860±0.101

Table 6.2 Sucrose, glucose, fructose and starch content of developing and stored sugar beet taproots

Treatment	Sucrose	Glucose	Fructose	Starch	Glc:Fru Ratio
		(mg g^{-1} FW)			
+ 9 days					
Control	144.22± 9.34	0.245±0.044	0.057±0.018	0.857±0.121	4.3
Defoliated	151.02± 3.18	0.204±0.057	0.032±0.009	0.928±0.137	6.4
Stored	136.91± 8.43	0.461±0.094	0.050±0.021	0.782±0.179	9.2
+24 days					
Control	186.03± 3.50	0.144±0.017	0.054±0.002	0.933±0.103	2.7
Defoliated	136.35± 0.91	0.194±0.040	0.058±0.002	0.589±0.077	3.3
Stored	155.05± 3.16	0.304±0.068	0.069±0.002	0.589±0.026	4.4
+52 days					
Control	195.35± 2.07	0.274±0.106	0.039±0.014	1.053±0.110	7.0
Defoliated	117.85± 2.63	0.115±0.021	0.080±0.004	0.579±0.030	1.4
Stored	203.38±18.47	0.153±0.034	0.084±0.009	0.761±0.050	1.8
+80 days					
Control	232.37± 4.04	0.398±0.024	0.231±0.008	0.422±0.032	1.7
Defoliated	171.65±21.15	0.335±0.069	0.189±0.018	0.187±0.012	1.8
Stored	248.16±11.03	0.935±0.199	0.170±0.029	0.401±0.064	5.5

mg g^{-1}FW (approximately 600 mM), significantly lower than in the control plants. In the stored sugar beet taproots the sucrose content increased to ca 250 mg g^{-1}FW (almost 1M). While calculations need to involve correcting values for moisture content, significant differences in moisture levels between treatments are present only for the last two sample dates. The (%) moisture content for each of the treatments for the last sample date are shown below:-

Control - 75.28 ± 0.18; Defoliated - 83.49 ± 0.39; Stored - 72.71 ± 1.02

The small differences in moisture content between control and stored taproots does not explain the occurrence of similar sucrose concentrations in taproots from these treatments.

The glucose and fructose levels remain low with no consistent major differences between treatments apart from a significantly higher glucose content in the stored taproots at the final sample date. By this time the content of both hexoses has increased significantly in all treatments. This may be due to exposure to low temperatures as, by the final date, the roots sampled from the field were partially frozen, although this does not account for the increased hexose levels in the stored taproots. Although the G : F ratio is quite variable for the treatments during the storage period, by the final date (+80 days), the ratio is significantly higher in the stored taproots (Table 6.2). For each of the treatments there were significant decreases in the starch content at the final sample date.

6.4 Discussion

While previous investigations on the activity of sucrose-cleaving enzymes during the growth and development of the sugar beet taproot have concentrated on one or two of the enzymes, no complete study of the activity

of each of the enzymes, measured from the same samples, has been reported. However, the pattern of activity observed in this present study confirms those reported from several separate investigations. High acid invertase (both soluble and cell-wall bound) in young roots decreases as the taproot develops as supported by the work of Burakhanova *et al.* (1987), Éngel & Kholodova (1970), Pavlinova & Prasalova (1973) and Silvius & Snyder (1979). Although direct comparisons are difficult to make due to the different methods of expressing the results, the data of Burakhanova *et al.* shows total acid invertase activity decreasing from over 300 nmol min^{-1} g^{-1} FW (8 day old seedlings) to a level just above zero by day 30. They provide evidence that an endogenous invertase inhibitor, previously found in sugar beet by Pressey (1968), suppresses the activity of acid invertase during the period of sucrose accumulation. When the protein fraction containing the inhibitor was isolated from either control taproots or from slices washed for 3 days to activate invertase, the former inhibited invertase activity 100% while the latter protein fraction caused only 34% inhibition. Silvius & Snyder (1979) showed that acid invertase is present in fibrous roots but in the taproot it is restricted to the peripheral meristematic tissue which produces cells for both taproot and fibrous root growth.

During the period of rapid sucrose accumulation, acid invertase activity decreases, whilst sucrose synthase activity increases. This crossover in activity in the early stages of root development has also been demonstrated by Pavlinova and Prasolova (1973), who showed maximum sucrose synthase activity around the middle of the growing season. Their maximum value for sucrose synthase activity was approximately 420 nmol min^{-1} mg^{-1}protein while the maximum activity reported in this chapter was ca 400 nmol min^{-1} mg^{-1}protein. They concluded that cleavage via sucrose synthase was the principal pathway during root growth and development, but did not consider

any involvement of alkaline invertase. By comparing enzyme activities in protoplasts and vacuoles from different parts of sugar beet roots, Fieuw & Willenbrink (1990) concluded that higher sucrose synthase activity was found in conducting tissue than in storage tissue. However, this conclusion was based on activities expressed on a fresh weight basis and as shown in this chapter, was no longer true when specific activities were calculated due to higher protein levels within the conducting tissues.

Masuda *et al.* (1986) showed that alkaline invertase activity was barely detectable in very young sugar beet roots but increased to approximately 150 nmol min^1 $g^{-1}FW$ during the period of rapid sucrose accumulation, before decreasing to reach a steady level of approximately 50 nmol min^{-1} $g^{-1}FW$ in the mature roots. These values are approximately half the activity values reported in the present study, but the pattern of activity is very similar during taproot growth and development. Masuda *et al.* (1986) partially purified the enzyme and characterised two forms, one which increased during sucrose accumulation, while the other remaining relatively constant. They concluded that alkaline invertase was involved in the regulation of accumulation and use of sucrose in developing sugar beet taproots. The exact role of alkaline invertase in sugar beet is uncertain as significant activity occurs alongside sucrose synthase. A similar situation is found in the sucrose-storing stem of sugar cane (Batta and Singh, 1986). While acid invertase is very active during stem elongation and decreases rapidly during ripening to reach a low level at maturity, activities of both alkaline invertase and sucrose synthase are high in mature canes. Incorporation studies using [U-^{14}C] labelled glucose, fructose and sucrose supplied exogenously to sugar cane storage tissue disks, resulted in equal labelling of both the glucose and fructose moieties of sucrose. Batta and Singh (1986) proposed a scheme in which sucrose is first inverted in the apoplast, and the hexoses so formed are transported across the plasmalemma

to be rapidly phosphorylated and synthesised into sucrose by the action of both sucrose synthase and sucrose phosphate synthase. They do not, however, identify a specific role for alkaline invertase. Future work with transgenic plants expressing very low levels of alkaline invertase, would hope to identify its specific function in cell metabolism in sugar beet taproots.

High sucrose synthase and sucrose phosphate synthase activities have also been measured in both the central and peripheral tissues of sugar beet taproots and results indicate higher sucrose phosphate synthase activity in the central region where the sucrose concentration is reported to be higher (Fieuw and Willenbrink, 1987). Clearly, the higher sucrose concentration found in this present study in the stored taproots cannot be attributed to continued import of assimilate. However, shrivelling of the outer, peripheral tissues occurred in stored beets so the re-translocation of previously stored sucrose may explain the phenomenon. The overall decrease in starch content is insufficient to account for the increase alone.

Relating the activity of the sucrose-cleaving enzymes in the control, defoliated and stored roots to sucrose and hexose levels is difficult as there was no effect of treatment on acid invertase activity, whilst activities of both alkaline invertase and sucrose synthase were generally higher in the control samples. In no case was there any indication of significant sucrose breakdown in stored roots. Previous data (Wyse, 1974) showed that acid invertase activity increased over ten-fold and thirty-fold in sugar beet roots stored for 30 days at 2°C and 21°C respectively and over sixty-fold in taproots stored at 5°C for 160 days. While the increase in acid invertase activity followed an increase in reducing sugars in stored roots at 5°C, the final level of acid invertase in samples stored for 160 days was still lower than alkaline invertase and sucrose synthase (Wyse, 1974). Additionally, Wyse's data indicates that 10-fold increases in acid invertase can occur in stored sugar beet with no effect on reducing sugar

content. This led him to suggest that acid invertase was required for the production of glucose and fructose to support postharvest respiration. In the present study, storage at 8°C for 80 days resulted in no significant increase in acid invertase activity (Fig. 6.2A) although the hexose levels doubled. Furthermore the high G : F ratio in the stored taproots after 80 days is not indicative of acid invertase activity (Table 6.2).

The results of clamping trials (Oldfield *et al.*, 1981), in which sugar beet taproots were stored by the growers in straw-covered mounds at temperatures averaging 7°C for 22-69 days, resulted in losses between 1.6-10.8% of total sucrose. Expressed on a daily basis, this amounted to losses of between 0.04-0.18%. The lowest losses occurred in clamps with temperatures below 10°C and highest losses where the high level of dirt on the roots had restricted ventilation and where temperatures had risen to as high as 16°C. If more time had been available, this present investigation would have provided better information on enzyme involvement with respect to sucrose losses, if a range of storage temperatures had been tested.

6.5 Conclusions

As sugar beet taproots develop and accumulate high sucrose concentrations, the level of acid invertase decreases rapidly while sucrose synthase and alkaline invertase activities increase. Thus both alkaline invertase and sucrose synthase are implicated in sucrose metabolism during taproot development. The possibility exists that sucrose synthase may be operating in the direction of synthesis in developing sugar beet taproots as postulated for the enzyme in sugar cane (Batta and Singh, 1986).

While no further sucrose accumulated in taproots after defoliation, no losses were detected in stored sugar beet after 80 days at 8°C. To explain this situation in stored taproots either a) the rate of respiration must be too low to affect the sucrose content or b) any sucrose breakdown must be accompanied by resynthesis via sucrose phosphate synthase and/or by sucrose synthase.

Future work with transgenic plants expressing very low levels of the sucrose-cleaving enzymes should be able to identify their precise role in sucrose metabolism during development and storage of sugar beet.

CHAPTER 7

PURIFICATION AND CHARACTERISATION OF SUCROSE SYNTHASE AND ALKALINE INVERTASE FROM THE COTYLEDONS OF *Vicia faba* L.

7.1 Introduction

The possible involvement of sucrose synthase and alkaline invertase in the cleavage of sucrose *en route* to starch biosynthesis, has been shown in Chapter 5. While the activity of both these enzymes was sufficient to support starch synthesis during the development of bean cotyledons, acid invertase activity was barely detectable.

The first genetic evidence indicating an important role for sucrose synthase in starch biosynthesis came from a study of the maize endosperm *sh* mutant (Chourey, 1976). Here enzyme activity is reduced to about 10% of the normal endosperm, and starch content to 40% of the wild type. In maize, a total of five sucrose synthase isozymes have been identified (Rowland & Chourey, 1990). Developing endosperm cells contain the two homotetramers (S1S1S1S1 and S2S2S2S2) whereas in young roots and shoots, the three heterotetramers are also present (Chourey *et al.*, 1986). Similarly, five isozymes have been detected in sorghum but, in contrast to maize, both sucrose synthase genes are expressed simultaneously in the endosperm, leading to the additional presence of the heterotetramers in this tissue (Chourey *et al.*, 1991).

Previous work (de Fekete, 1969) had implicated the involvement of the sucrose synthase pathway in starch biosynthesis in developing *Vicia faba* seeds.

Although Pridham *et al.* (1968) partially purified the protein, sucrose synthase has never been fully purified or characterised from the species.

Recent work has questioned the previous consensus that UDP is the principal nucleoside diphosphate in the sucrose cleavage reaction catalysed by sucrose synthase. In sycamore suspension cells and spinach leaves, ADP-specific sucrose synthase has been reported (Pozueta-Romero *et al.*, 1991). Previous work with relatively crude sucrose synthase preparations of *V. faba* cotyledons (de Fekete, 1969) showed no ADP specificity, although activity with ADP is clearly dependent on the assay conditions employed (Pozueto-Romero *et al.*, 1991).

Still less is known, however, about the properties of alkaline invertase from *V. faba*. While partial purification of alkaline invertase from soybean nodules (Morrell and Copeland, 1984), sugar beet root (Masuda *et al.*, 1987), and sweet potato (Matsushita and Uritani, 1974) has been reported, only from soybean hypocotyls has the enzyme been purified to apparent electrophoretic homogeneity (Chen and Black, 1992). To be able to understand how sucrose metabolism is regulated it is important to characterise both these enzymes after purification and to raise polyclonal antibodies for immunological studies. This forms the basis of the following chapter.

7.2 Material and Methods

7.2.1 Plant material

The pods from developing field beans (*Vicia faba* L. cv. Maris Bead) were harvested from field plots 40-50 and 50-65 days after anthesis (DAA) to provide cotyledons for the purification of sucrose synthase and alkaline

invertase respectively. Previous experiments (Chapter 5) revealed the highest activity of the enzymes at these stages of bean seed development.

7.2.2 Extraction and purification of Sucrose synthase

7.2.2.1 Extraction

Seeds (400 g total fresh weight), with their testas and embryonic axes removed, were extracted in ice-cold 200 mM Tris-HCl buffer (pH 8.5) containing 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, and 2 mM PMSF in a prechilled blender (Atomix). Insoluble polyvinylpolypyrrolidone was included during the extraction at 0.1% w/v. The homogenate was filtered through cheesecloth and re-extracted three times before centrifugation of the combined extracts at 10,000g and 4°C for 30 min.

7.2.2.2 Fractionation of extract with $(NH_4)_2SO_4$

After centrifugation of the combined extracts, the supernatant was fractionated by the addition of $(NH_4)_2SO_4$, and the fraction that precipitated between 30-80% saturation was collected by centrifugation at 10,000g for 10 min. The precipitate was resuspended in 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol and 2 mM PMSF (buffer A) and was dialysed against the same buffer overnight.

7.2.2.3 Hydrophobic interaction chromatography

Sufficient $(NH_4)_2SO_4$ was added to the dialysate to make the solution 0.5 M with respect to the salt, and the sample was applied to a 500 ml Phenyl

Sepharose XK-50 (50 x 300 mm) column (Pharmacia LKB, UK) previously equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$ (buffer B). Phenyl Sepharose was chosen, as the phenyl ligand is intermediate in hydrophobicity and even strongly hydrophobic proteins that would be difficult to elute from Octyl Sepharose can generally be eluted. Proteins bound to the column were eluted with a stepped gradient of buffer B and buffer A. The protein expressing sucrose synthase activity was tightly bound and eluted at 100% of buffer A. The active fractions were dialysed overnight against 50 mM Hepes-KOH (pH 8.5) containing 5 mM sucrose, 10 mM $MgCl_2$ and 5 mM 2-mercaptoethanol in preparation for the next column step.

7.2.2.4 Affinity chromatography

The dialysate was applied to a 5 mm x 100 mm phenyl boronate agarose-60 affinity column (Amicon, Stonehouse, UK), prewashed with 20 column volumes of 50 mM Hepes-KOH (pH 8.5), containing 200 mM sucrose, 10 mM $MgCl_2$ and 5 mM 2-mercaptoethanol, followed by further washes with 5 volumes of the same buffer containing only 5 mM sucrose. After unbound protein was eluted, sucrose synthase protein showing activity was eluted with 0.1 M Tris-HCl (pH 8.5), containing 5 mM 2-mercaptoethanol (Morrell and Copeland, 1985). Active fractions were pooled and dialysed against 20 mM Tris-HCl (pH 7.2) containing 5 mm 2-mercaptoethanol.

7.2.2.5 Anion exchange chromatography

The dialysate was applied at a flow rate of 0.5 ml min^{-1} to an anion exchange (5 x 50 mm) column (Mono Q; Pharmacia LKB, UK) previously equilibrated with dialysis buffer. The protein was eluted with a KCl gradient (0-1M) over

20 column volumes. Active fractions were concentrated using Centriprep concentrators with MW cutoff of 10,000 (Amicon, UK) to approximately 1 ml in preparation for the final column step.

7.2.2.6 Gel filtration chromatography

From the concentrated sample, 200 µl volumes were applied at 0.2 ml min^{-1} to a Superose 6 (10 x 30 mm) gel filtration column (Pharmacia LKB) pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 100 mM KCl and 5 mM 2-mercaptoethanol. This gel filtration column was used as it has an optimal separation range of 5,000 - 5,000,000 (MW). A mixture of blue dextran (V_0), thyroglobulin (M_r 669,000), apoferritin (M_r 443,000), ß-amylase (M_r 200,000), BSA (M_r 66,000) and carbonic anhydrase (M_r 29,000) were used to calibrate the column. Highly purified sucrose synthase preparations from this gel filtration column were used for kinetic studies. Active fractions were also dialysed against 10 mM Tris-HCl (pH 7.2) containing 5 mM 2-mercaptoethanol and subjected to both denaturing SDS-PAGE and native PAGE.

7.2.3 Extraction and purification of Alkaline invertase

7.2.3.1 Extraction

The same method of extraction was used for alkaline invertase but the buffer was replaced with 200 mM Acetate buffer (pH 5.0) containing 1 M NaCl, 10 mM sodium sulphite, 1 mM PMSF and 5 mM DTT.

7.2.3.2 Fractionation with $(NH_4)_2SO_4$

The extract was again fractionated with $(NH_4)_2SO_4$ but both the 0-30% and 30-80% fractions were collected and taken separately through the purification procedure.

7.2.3.3 Column chromatography

With the omission of the affinity column (PBA-60) the same columns and conditions as described for the purification of sucrose synthase were used and the highly purified preparation after gel filtration was used for characterisation studies.

7.2.4 Gel electrophoresis

7.2.4.1 SDS-PAGE

SDS-PAGE was performed as described in section 2.5.1 using 10 and 12.5% acrylamide. Gels used during the sucrose synthase purification were stained with 0.1% Coomassie brilliant blue R-250 while those used for detecting alkaline invertase required silver staining (both methods described in section 2.5.1).

7.2.4.2 Non-denaturing PAGE

Non-denaturing PAGE (SDS omitted) was performed essentially as described for SDS-PAGE but with the following exceptions: a) 100 mM sucrose was

included in the gels to maintain the enzyme in its active form; b) the pH of the resolving gel was reduced to 7.5 and c) 7.5% acrylamide was used. The gel filtration molecular weight markers (MW-GF-1000 from Sigma Chemical Co.) were used to help identify the sucrose synthase protein on the gel. Non-denaturing gels were not used during the purification of alkaline invertase as it was not possible to obtain an active protein by elution from the gel due to inhibition by Tris buffer. Some sucrose synthase samples were also run on a 4.5 to 7.0% linear gradient non-denaturing polyacrylamide gel for 20 h at 4°C (Chourey *et al.*, 1988) and used for immunoblotting.

7.2.4.3 Two-dimensional SDS-PAGE

To further confirm the presence or absence of isozymes of sucrose synthase and alkaline invertase, samples partially purified by $(NH_4)_2SO_4$ fractionation only were subjected to two-dimensional electrophoresis as described previously (section **2.5.2**) and immunoblotted.

7.2.5 Preparation of Antisera

7.2.5.1 Sucrose synthase

Initially, active fractions from gel filtration chromatography were run on 7.5% non-denaturing gels and a section of the gel stained with Coomassie brilliant blue R to identify the major protein band. The adjacent nonstained region of the gel was excised and eluted at 4°C for 48 h in 5 gel volumes of water and assayed for sucrose synthase activity to confirm the identity of the protein. A parallel aliquot was subjected to SDS-PAGE. An additional lyophilised preparation was redissolved in 1 ml of Tris-buffered saline (10 mM Tris/HCl,

10 mM borate [pH 7.3] and 0.9% NaCl) and divided into three aliquots, each containing approximately 50 µg of protein. An equal volume of complete Freund's adjuvant was mixed with one sample before injecting, intramuscularly, into a New Zealand White rabbit. Two booster injections with the addition of an equal volume of incomplete Freund's adjuvant were given 20 and 41 days later. Serum was collected 11 days after the final injection.

Antisera raised against denatured sucrose synthase protein were prepared in a similar way to those against the native protein. Serum (both immune and preimmune) was centrifuged at 16,000g for 30 min, diluted 10-fold with water, and partially purified by the addition of an equal volume of saturated $(NH_4)_2SO_4$ followed by gentle stirring overnight at 4°C. After centrifugation at 10,000g for 30 min, the resulting pellets were resuspended in phosphate-buffered saline (PBS). The antibody solutions were dialysed against PBS overnight and stored at -80°C.

7.2.5.2 Alkaline invertase

Initial attempts to gel purify sufficient amounts of alkaline invertase were not successful as this enzyme is present as a very minor protein in *Vicia faba*. Consequently, it was decided to try to raise antisera in mouse by the method of intrasplenic insertion of small portions of nitrocellulose into a New Zealand Black/BALB/ c cross mouse (Forrest and Ross, 1993). From SDS gels, the band that followed enzyme activity during purification was identified and its position relative to the molecular weight markers noted. Parallel gels (12.5% acrylamide) were run and the proteins blotted on to a nitrocellulose membrane. From this, a thin strip of nitrocellulose (50 x 1 mm) corresponding to the position of the denatured alkaline invertase on the blot was excised.

Prior to each immunisation, a 7-week-old female mouse was anaesthetized by subcutaneous injection (0.25 ml) of a solution of Hypnorm (Janssen Pharmaceuticals, UK), Hypnovel (Roche Products, UK) and sterile distilled water in the ratio of 1:1:2. The spleen was exposed as described by Nilsson *et al.* (1987). A third of the nitrocellulose strip was impaled on the point of a pin and 3-4 µl DMSO were applied by micropipette along the strip and the pin rotated gently to gather the dissolving nitrocellulose into a viscous droplet on the point. The nitrocellulose was placed on a small cut on the spleen which was then returned to the abdominal cavity. The abdominal wall and skin were sutured separately with silk sutures (Mersilk W500, Ethicon, UK). The second and third immunisations were carried out 14 and 42 days later and the final bleed was made 13 days after the third immunisation. Serum obtained (both pre-immune and immune) were treated in the same way as described previously (section **7.2.5.1**).

After concentration of several samples of purified alkaline invertase (post Superose 6), SDS 12.5% acrylamide gels were run and the identified band (≈54 kD) excised and the protein eluted as described for sucrose synthase. This required the elution from several gels to obtain sufficient protein (estimated from gel staining as a total of approximately 40 µg). After gel elution the protein was lyophilised and redissolved in 1.5 ml TBS to provide 3 aliquots (0.5 ml). An equal volume of complete Freund's adjuvant was mixed with one sample before injecting, intramuscularly, into a New Zealand White rabbit. Two booster injections with the addition of an equal volume of incomplete Freund's adjuvant were given 27 and 56 days later. Serum was collected 11 days after the final injection and treated as described above.

7.2.6 Protein blotting

Immunoblots using the polyclonal antibodies raised against denatured sucrose synthase from soybean nodules, and both native and denatured sucrose synthase from *Vicia faba* cotyledons, were carried out as described previously (section **2.6**). The antibodies raised against purified sucrose synthase from soybean nodules (Gordon *et al.*, 1992) were a kind gift from Dr A. J. Gordon and proved valuable in confirming the purified bean sucrose synthase protein. Blots were incubated with the antibodies (1:2,000 - 1:10,000 dilution with TBS). Similar immunoblots using the polyclonal antibodies raised against alkaline invertase (in mouse and rabbit) were used but at a lower dilution of 1:500 or 1:1,000.

7.2.7 Immunoprecipitation

Antisera raised in mouse and rabbit against alkaline invertase were used to test for precipitation of enzyme activity. Both pre-immune and immune serum were diluted with PBS to give 0 to 80-fold dilutions. Equal volumes of the diluted sera were added to partially purified (post Superose 6) alkaline invertase (50 µl volumes) and incubated on a rotating wheel at 4°C overnight. Pre-swollen protein A agarose (Sigma, UK [50 µl]) was added and the mixture was further incubated as above for 4-6 h. The antigen/antibody/protein A complex was precipitated by centrifugation at 12,000 g for 10 min at 4°C. The supernatants were then assayed for alkaline invertase activity and results expressed as % of the control pre-immune serum values.

7.2.8 Protein sequencing

Purified sucrose synthase and alkaline invertase were subjected to SDS-PAGE using the improved method of Yuen *et al.* (1986) to give higher yields for sequencing. This method involves pre-running the gel with the inclusion of 10 mM glutathione in the electrophoresis buffer in the upper reservoir. After decanting the pre-run buffer, sodium thioglycollate (100 mM) was added to fresh electrophoresis buffer in the upper reservoir. Both glutathione and sodium thioglycollate act as sulphydryl protective agents and as free radicle scavengers thus helping to prevent oxidation during electrophoresis. The proteins were then electroblotted on to Problot membrane (Applied Biosystems, UK), stained with amido black (0.1%), and sequenced on an Applied Biosystems model 477A sequencer (Matsudaira, 1987). The NH_2 terminus of both proteins were blocked, necessitating the use of proteases to cleave them in order to obtain a partial sequence. Both Glu-C and Arg-C proteases were used according to the method of Cleveland *et al.* (1977). The endoproteinase Glu-C from *Staphylococcus aureus* V8 hydrolyses peptide and ester bonds specifically at the carboxyl end of glutamic and aspartic residues. Endoproteinase Arg-C from the submaxillaris glands of mice cleaves arginine specifically. Both proteases were obtained from Boehringer Mannheim, UK.

7.2.9 Enzyme assay

7.2.9.1 Sucrose synthase

Throughout the purification, sucrose synthase was assayed in the cleavage direction as described previously (2.3.1). Additionally, sucrose cleavage activity with nucleoside diphosphates other than UDP was determined using a stopped assay system. The 1 ml reaction mixture contained buffer (either 20

mM Tris/HCl or 20 mM Hepes/KOH, both at pH 7.0), 200 mM sucrose, 10 µl of purified faba bean sucrose synthase, and nucleosides in the range from 0.025 to 4.0 mM (all buffered at pH 7.0). The reaction was stopped after 3, 6, or 9 min by heating in boiling water. Fructose released was determined using an autoanalyser system as described in section **2.4.1.** Boiled enzyme extracts treated in the same way were used as controls. To determine the optimum pH for the purified enzyme, 100 mM Tris/HCl was used to give a final pH range of between approximately 6 to 8 during the reaction in the cuvette. The unit of enzyme activity is defined as 1 nmol min^{-1}.

7.2.9.2 Alkaline invertase

Alkaline invertase activity was determined as outlined in section **2.3.3.** However, during the purification a quicker but non-quantitative assay requiring only 10 - 50 µl of sample was developed. This involved incubation of equal volumes of samples with substrate for 1 h at 37°C as normal but using a microplate. After incubation, an equal volume of 0.1% 2,3,5-triphenyltetrazolium chloride (TTC) in 1 M NaOH was added to the wells and left at 55°C in the dark for 10 min to allow colour development. The reducing sugars produced due to the action of alkaline invertase caused the reduction of TTC to a red formazan. Due to the light sensitivity of this reaction, it was not possible to quantify activity but the assay proved useful during the purification process when a large number of samples required testing.

To determine the optimum pH for the purified enzyme, 200 mM phosphate buffer was used to give a final pH range of approximately 6 to 9 and assayed using the normal method. The specificity of alkaline invertase for substrate was tested with raffinose, lactose, stachyose, trehalose and maltose over a

range of concentrations (1.25 mM to 150 mM). Inhibition of alkaline invertase activity by fructose (0 to 20 mM) and by Tris/HCl buffer (0 to 10 mM) was assessed. Where used, the unit of enzyme activity is defined as 1 nmol min^{-1}.

7.2.10 Determination of pI

The pI of both sucrose synthase and alkaline invertase was determined on a Rotofor Isoelectric Focusing Cell (Bio-Rad) using Ampholines (Pharmacia LKB) in the pH range of 3.5 to 10.0. To confirm the pI, the active fractions were collected and refocused.

7.2.11 Protein assay

Protein concentrations were determined as described in section 2.7.

7.3 Results

7.3.1 Sucrose synthase

7.3.1.1 Purification of enzyme

The protocol developed resulted in a 270-fold purification of sucrose synthase (Table 7.1). The purified preparation consisted of one major protein when visualised on non-denaturing PAGE (Plate 7.IA) and one major polypeptide (approx. M_r 92,600) and additional minor polypeptides on SDS-PAGE (Plate 7.II, lane 1). The major polypeptide reacted strongly in immunoblots with antisera raised against sucrose synthase from soybean nodules (Plate 7.II, lane

Table 7.1 Purification of faba bean cotyledon sucrose synthase

Fraction	Total Activity U (nmol min^{-1})	Total Protein (mg)	Specific Activity (U mg^{-1} protein)	Yield (%)	Purification Fold
Crude	679,112	22,263	30.5	100	-
30–80% $(NH_4)_2SO_4$	586,367	18,042	32.5	86.3	1.07
Phenyl Sepharose	337,190	408.3	825.8	49.6	27.1
PBA–60	232,117	124.5	1864.4	34.2	61.1
Mono–Q	113,945	19.53	5834.4	16.8	191.3
Superose–6	74,124	8.89	8337.9	10.9	273.4

Plate 7.I A) Identification on a 7.5% non-denaturing polyacrylamide gel of the native sucrose sythase protein (3 central lanes) from the final purified protein sample. Outer lanes show position of MW markers (Sigma - MW-GF-1000 kit); B) Immunoblot of a gradient non-denaturing polyacrylamide gel (4.5 to 7.0%) showing the presence of single cross-reacting bands (marked with arrows). Antibodies used were raised against bean sucrose synthase (native protein), dilution 1:10,000; C) Two-dimensional immunoblot of bean sucrose synthase using first-dimension ampholines ranging from pH 3 to 10 and second dimension run on 10% SDS-PAGE. Antibodies used as in B).

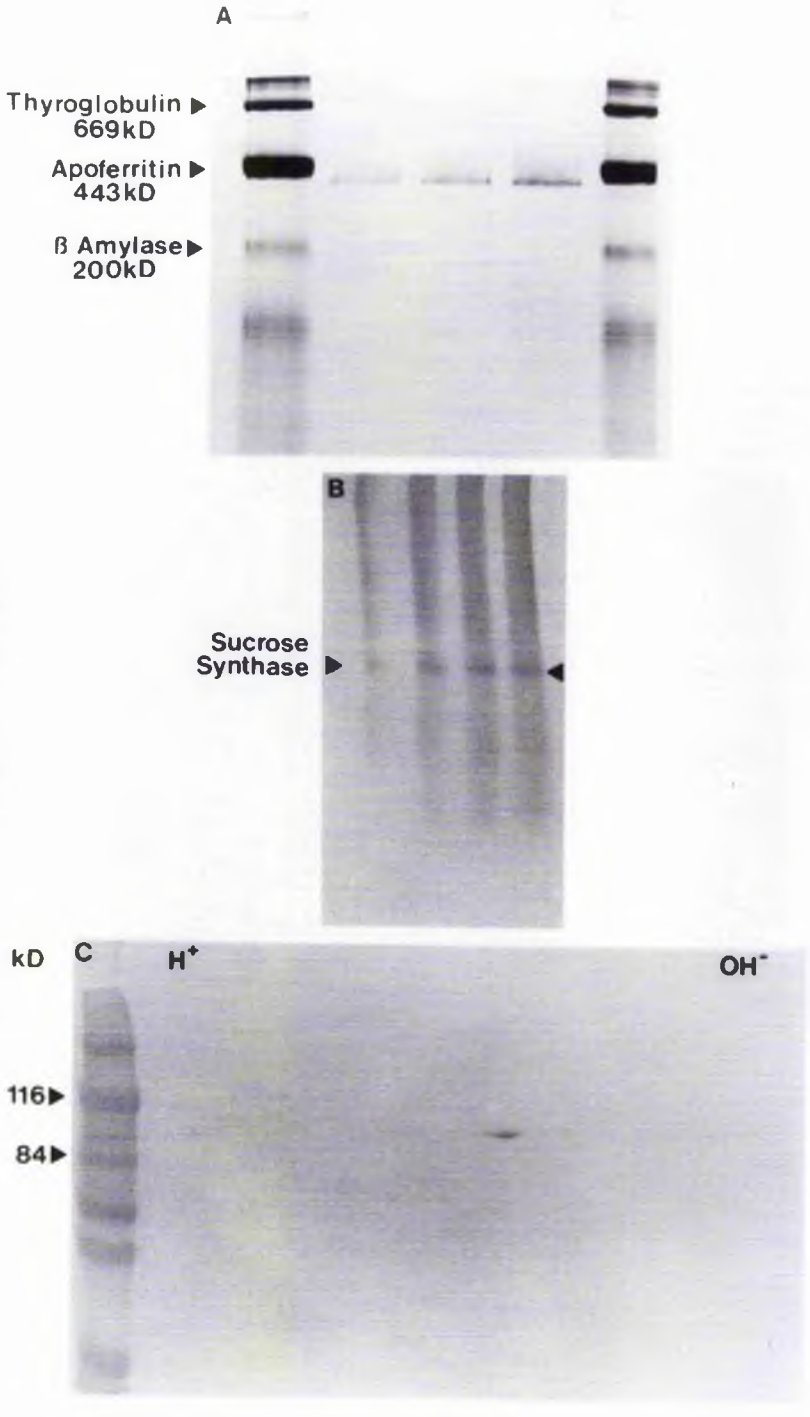
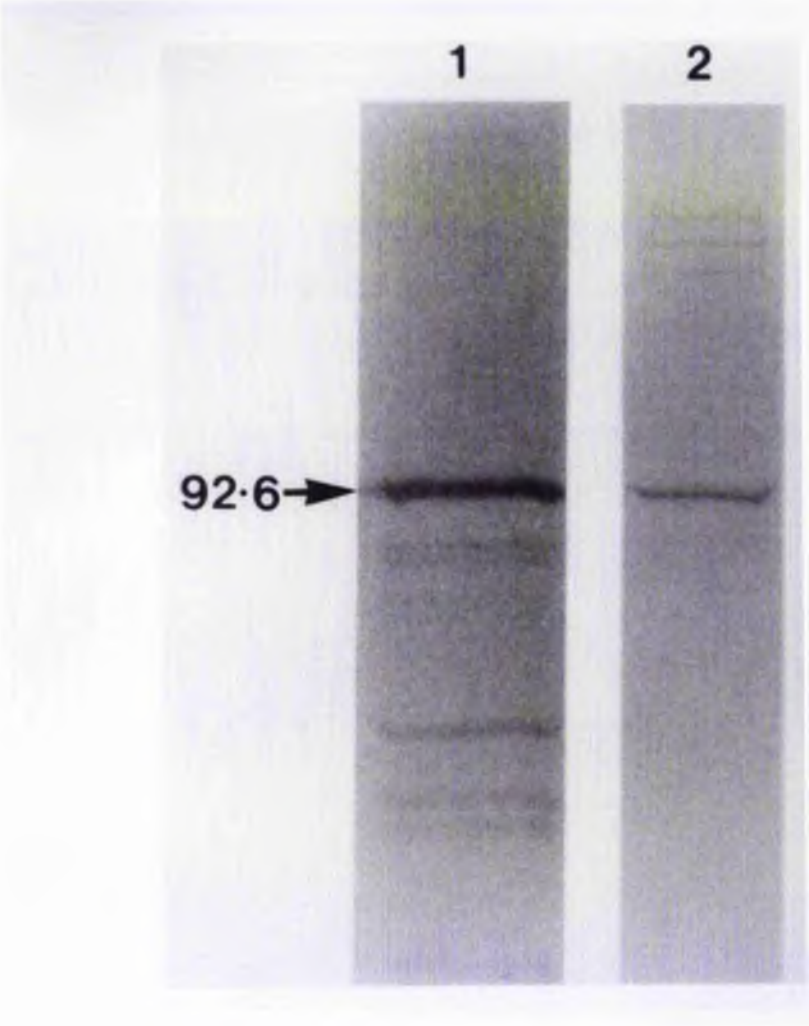
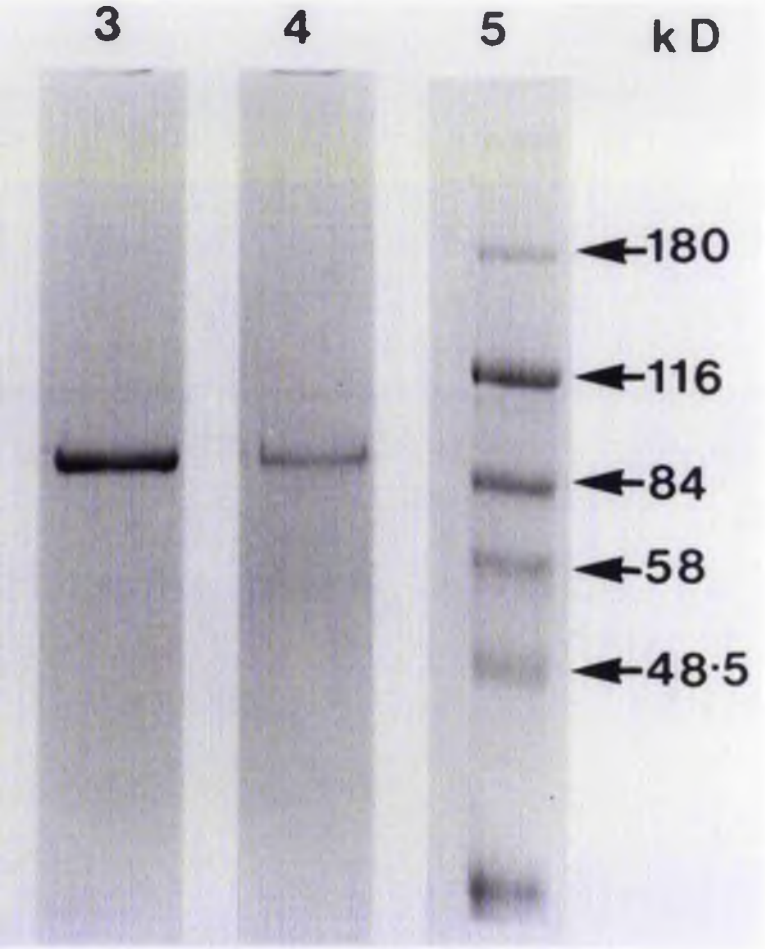

A
Thyroglobulin ▶
669kD
Apoferritin ▶
443kD
ß Amylase ▶
200kD
B
Sucrose
Synthase ▶
kD
C
H+
OH-
116▶
84▶

Plate 7.II Identification (10% SDS-PAGE gel) of the sucrose synthase subunit (92.6 kD). Lane 1, Enzymically active protein fraction recovered from Superose-6. Lane 2, Immunoblot of crude faba bean extract using polyclonal antibodies raised against soybean nodule sucrose synthase (1:2000 dilution). Lane 3, Denaturing gel of polypeptide purified following elution from SDS-PAGE. Lane 4, Denaturing gel of active sucrose synthase protein eluted from native-PAGE. Lane 5, Prestained markers (SDS-7B Sigma).


1
2
92·6


3
4
5
kD
180
116
84
58
48·5

2). The polypeptide eluted from the SDS-denaturing gel and the enzymically-active protein eluted from the non-denaturing gel were both homogeneous when visualised on SDS-PAGE (Plate 7.II, lane 3 and 4 respectively) revealing a single polypeptide (M_r 92,550 ± 370 [estimated from five samples]) as shown in Fig. 7.1. Antisera raised against both the gel-purified polypeptide and native protein cross-reacted specifically with the polypeptide on immunoblots at a dilution as high as 1:10,000 (Plate. 7.IIIA shows only results with native protein; results with gel-purified polypeptide were identical).

The mean molecular weight of sucrose synthase was calculated at 353,000 ± 19,000 following gel filtration on Superose 6 (Fig. 7.1). The protein therefore appears to be a homotetramer. Immunoblot of a gradient non-denaturing gel revealed a single band (Plate 7.IB) and further confirmation that only one form of the enzyme was present in *Vicia faba* cotyledons was provided by the two-dimensional immunoblot (Plate 7.IC). Several blots were prepared with a range of protein loadings, but, in every case, only one cross-reacting polypeptide was observed.

Both V8 and Arg-C endoproteases were tested as a means of producing peptide fragments from the 92.6 kD polypeptide for amino acid sequencing, but only V8 provided a product (≈78 kD) in sufficient quantity (Plate 7.IIIB). A sequence of 13 amino acid residues was obtained (see below) that showed substantial homology (about 50%) with potato sucrose synthase (Salanoubat & Belliard, 1987).

```
AMINO ACID
POSITION   130                  140                  150
POTATO     N F V L E L D F E P F T A S F P K P T L T
                   : : : : : :   :
BEAN               L D F E P F S A G G L - G V
```

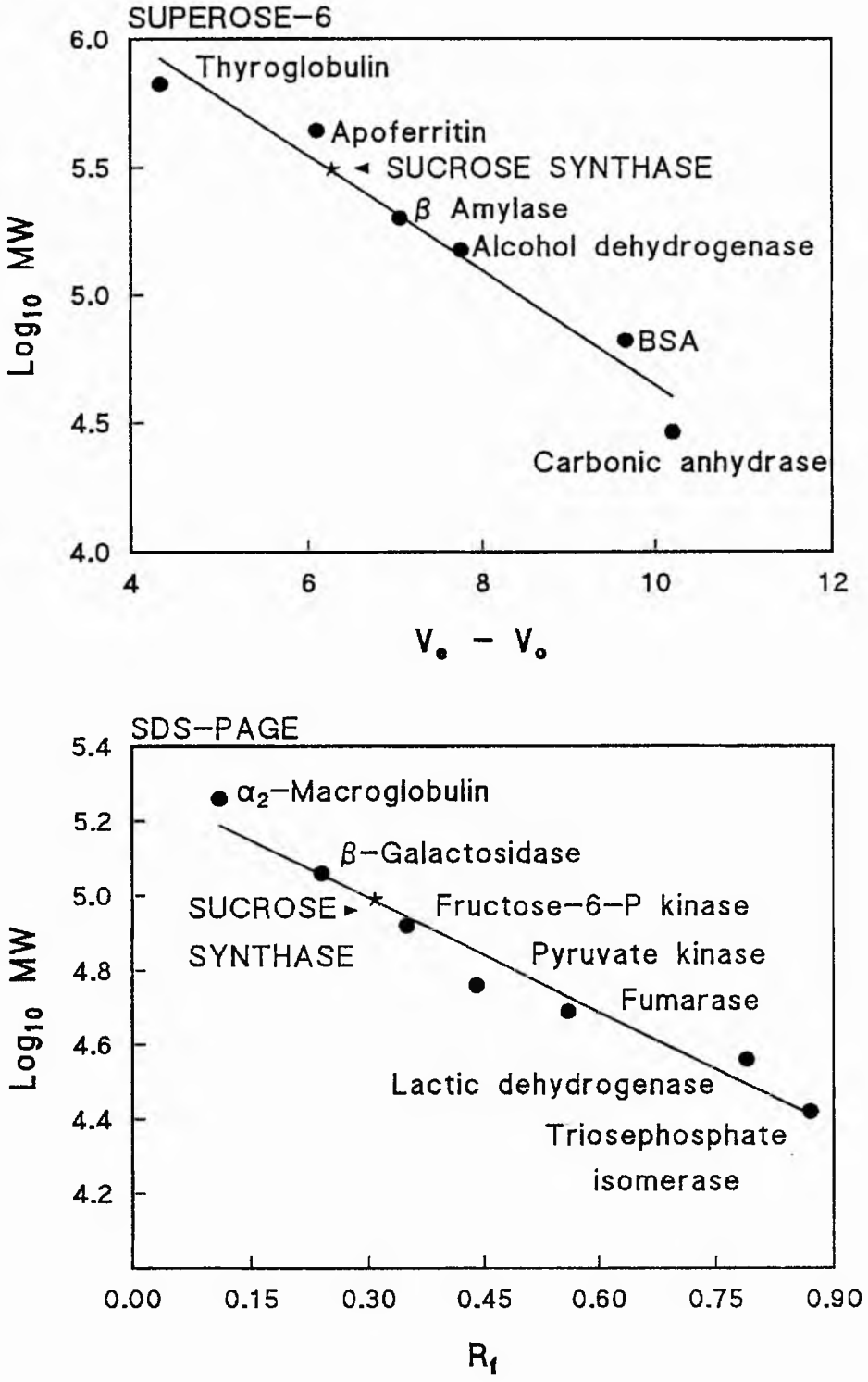
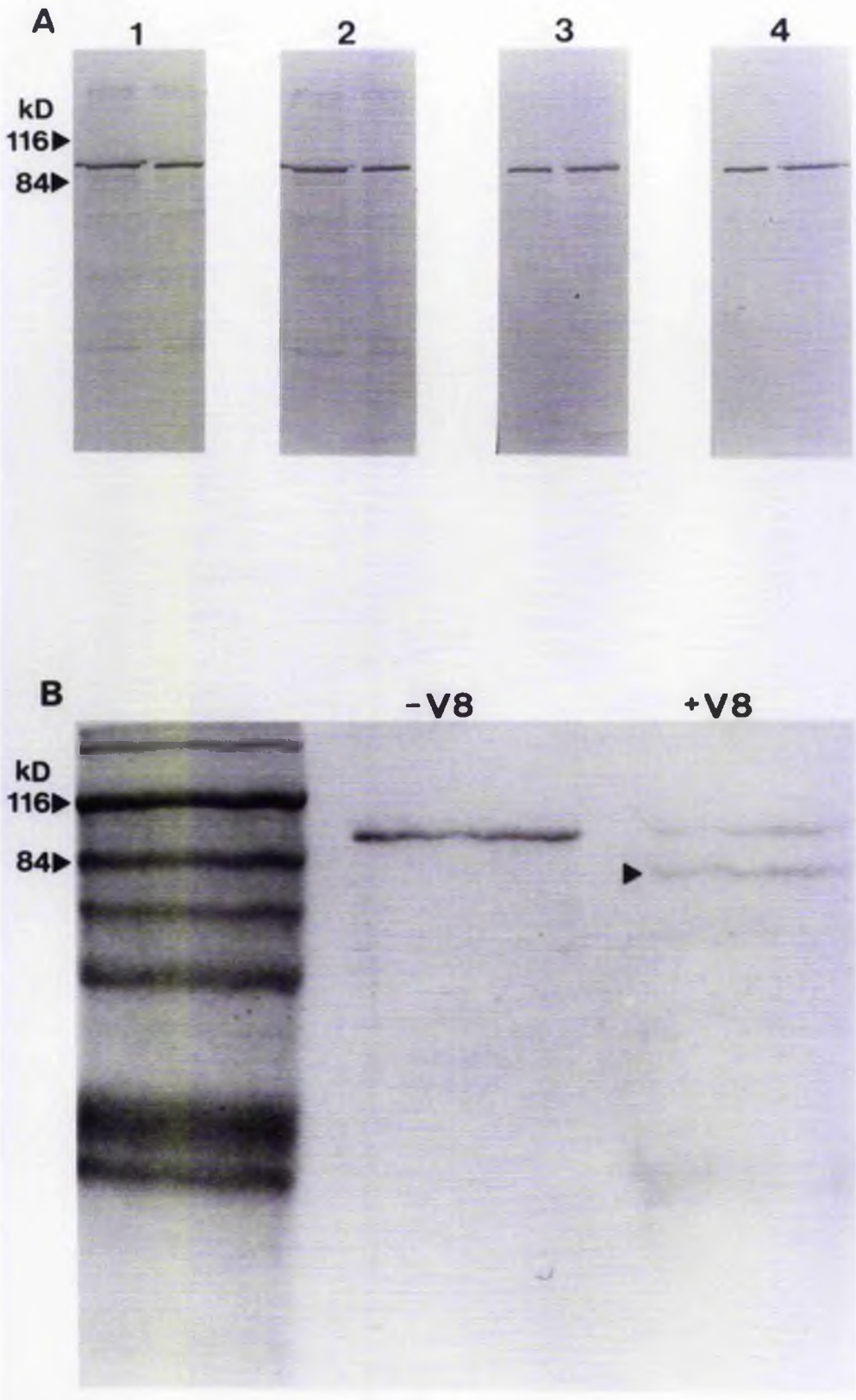


Fig. 7.1 Determination of the native and subunit weight of purified sucrose synthase from *Vicia faba* cotyledons on Superose-6 and SDS-PAGE respectively.

Plate 7.III A) Immunoblots of crude faba bean extracts loaded on 10% SDS-PAGE. Antibodies used were raised against bean sucrose synthase (native protein), dilution - 1) 1:1000; 2) 1:2000; 3) 1:5000; 4) 1:10,000. B) Purified sucrose synthase on 10% SDS-PAGE sequencing gel ± Glu-C endoprotease (0.5 μg *Staphylococcal aureus* V8). Lower polypeptide band marked with arrow was used to obtain amino acid sequence.


A
1
2
3
4
kD
116
84
B
-V8
+V8
kD
116
84

All the evidence therefore indicates that the bean protein purified is indeed sucrose synthase.

7.3.1.2 Enzyme properties and kinetics

The purified enzyme has a pH optimum of 6.4 (Tris/HCl buffer) in the cleavage direction, but there was less than a 5% decrease in activity between pH 6.2 and 6.6 (Fig. 7.2A). The pI was calculated at between 5.4 to 5.5 (Fig. 7.2B).

The K_m value for sucrose synthase was estimated from the Michaelis-Menten equation to be 169 mM ± 26 mM. However, neither the V_{max} nor the K_m for sucrose with UDP as a nucleoside diphosphate could be determined accurately because complete saturation did not occur, even with 500 mM sucrose (Fig. 7.3A). The sucrose saturation curve was sigmoidal rather than hyperbolic necessitating the construction of a Hanes plot to obtain an accurate estimate of V_{max} (Fig. 7.3B). This enabled a Hill plot to be drawn and a Hill coefficent to be determined ($h \approx 1$) (Fig. 7.3C). Similar kinetic properties for sucrose saturation have been reported for maize kernel sucrose synthase by Su and Preiss (1978). They offered the hypothesis that this is due to different quaternary structural forms of the enzyme in solution and, to my knowledge this is the only explanation offered so far.

Standard Michaelis-Menten type kinetics were observed for UDP when using 200 mM sucrose (V_{max} [UDP] 1,294 ± 28 nmol min^{-1} mg^{-1} protein).

Of the four nucleoside diphosphates tested (UDP, ADP, CDP, and GDP [0.025-4 mM]) using the stopped assay, sucrose synthase activity was only detected with UDP and ADP. In agreement with the findings of Pozueta-Romero *et al.*

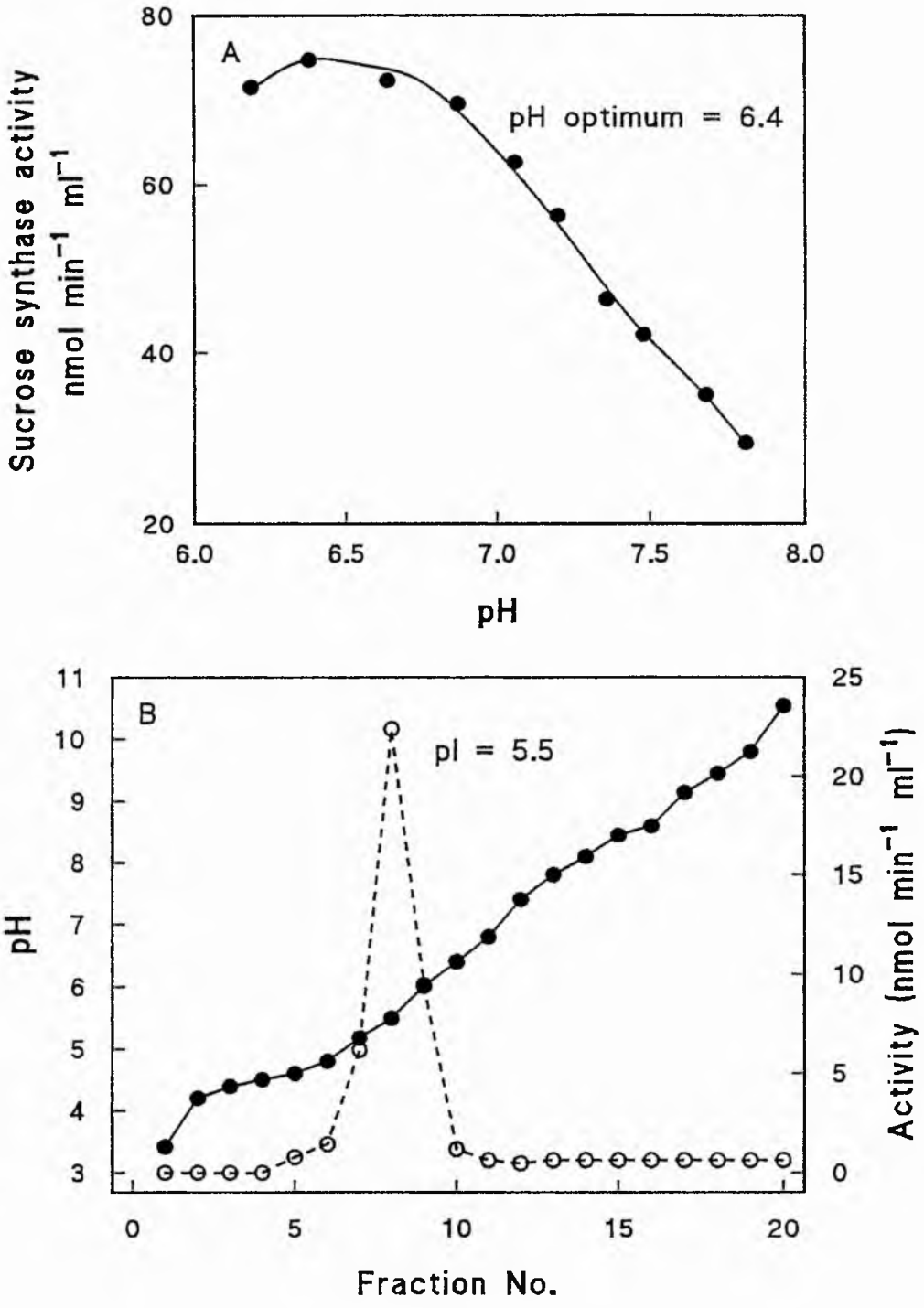


Fig. 7.2 A) pH curve of purified bean sucrose synthase activity. Buffer used throughout was 100 mM Tris/HCl. The reactions were carried out at 30°C and values shown are means (n=3). SEM's omitted but <10% of mean values. B) Determination of the isoelectric point of bean sucrose synthase on a Rotofor isoelectric focusing cell. ●—● - pH, o---o - sucrose synthase activity.

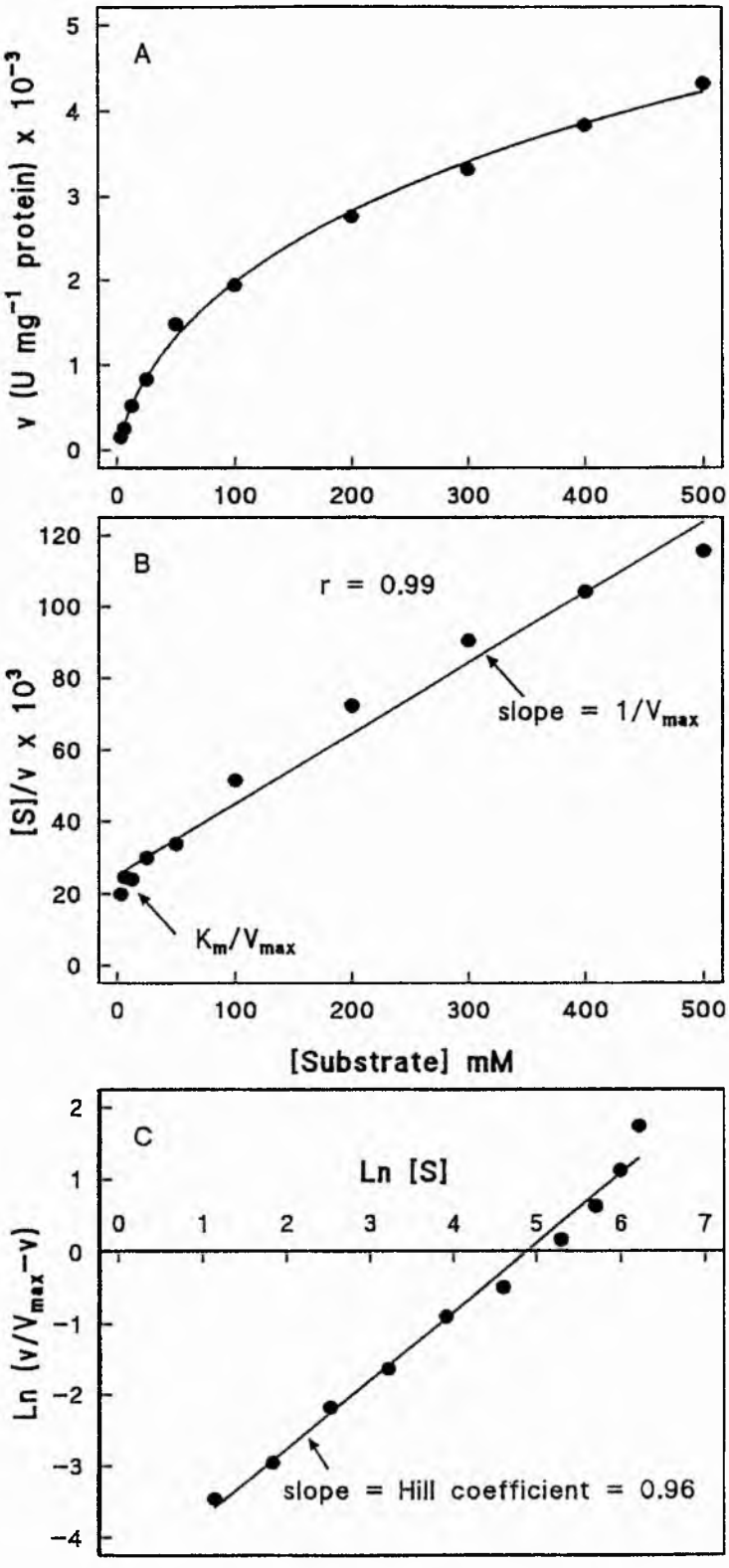


Fig. 7.3 A) Sucrose saturation curve of purified *V. faba* sucrose synthase. B) Hanes-Woolf plot derived from above. C) Hill plot using the estimated V_{max} derived from B). (Sucrose concentration 3 - 500 mM).

(1991), the reaction with ADP as substrate was strongly inhibited by Tris-HCl buffer (70-80% reduction in rate), whereas with UDP, the reaction was only slightly affected (< 1% reduction). With Hepes buffer, the K_m for UDP was 0.212 ± 0.004 mM and the V_{max} was 2031 ± 121 nmol min^{-1} mg^{-1}protein (Fig. 7.4). With Tris buffer, the K_m for UDP was 0.149 ± 0.003 mM and the V_{max} was 1,720 ± 107 nmol min^{-1} mg^{-1}protein. With Hepes buffer and ADP as the nucleoside diphosphate, the K_m for ADP was identical to UDP, but the V_{max} was only 496 ± 27 nmol min^{-1} mg^{-1}protein (Fig. 7.4). No values could be obtained for ADP in the presence of Tris buffer due to the high level of inhibition.

Fructose (10 mM) inhibited sucrose cleavage by 74% (inhibition was competitive). A Dixon plot provided an estimated K_i value of 2.48 mM at sucrose concentrations of 25, 50, 100, and 200 mM (Fig. 7.5).

7.3.2 Alkaline invertase

7.3.2.1 Purification of enzyme

Alkaline invertase was purified approximately 140-fold by column chromatography (Table 7.2). The 0-30% $(NH_4)_2SO_4$ fraction, while yielding lower total activity, had nearly 4-fold higher specific activity and was therefore used as well as the 30-80% fraction for further purification. The first column step (Phenyl Sepharose) increased the specific activity of the enzyme by 17 and 5-fold for the two $(NH_4)_2SO_4$ fractions respectively. Samples showing enzyme activity were eluted from a Mono Q column in the unbound fraction and during a salt (KCl) gradient at a concentration of approximately 0.4 M (bound activity). Activity found in the unbound fraction was considered to be due to column-overload as repeated re-applications of this fraction to the column

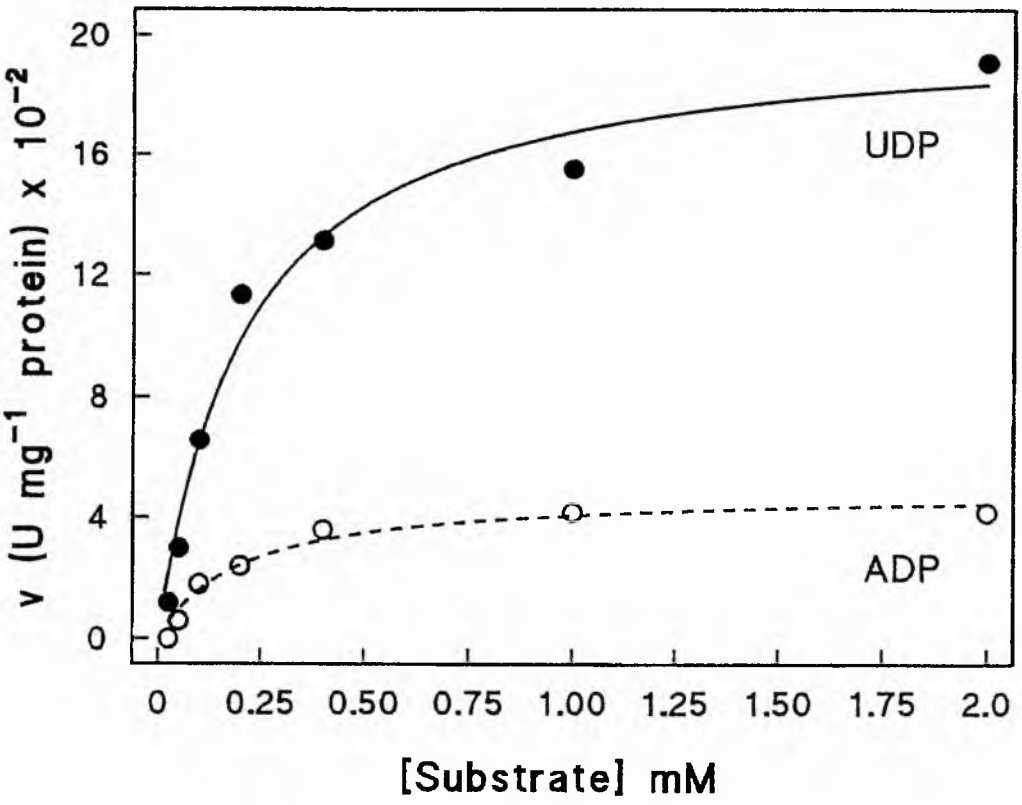


Fig. 7.4 UDP and ADP saturation curve of purified *V. faba* sucrose synthase using Hepes/KOH buffer. Data points fitted using the Michaelis-Menten equation.

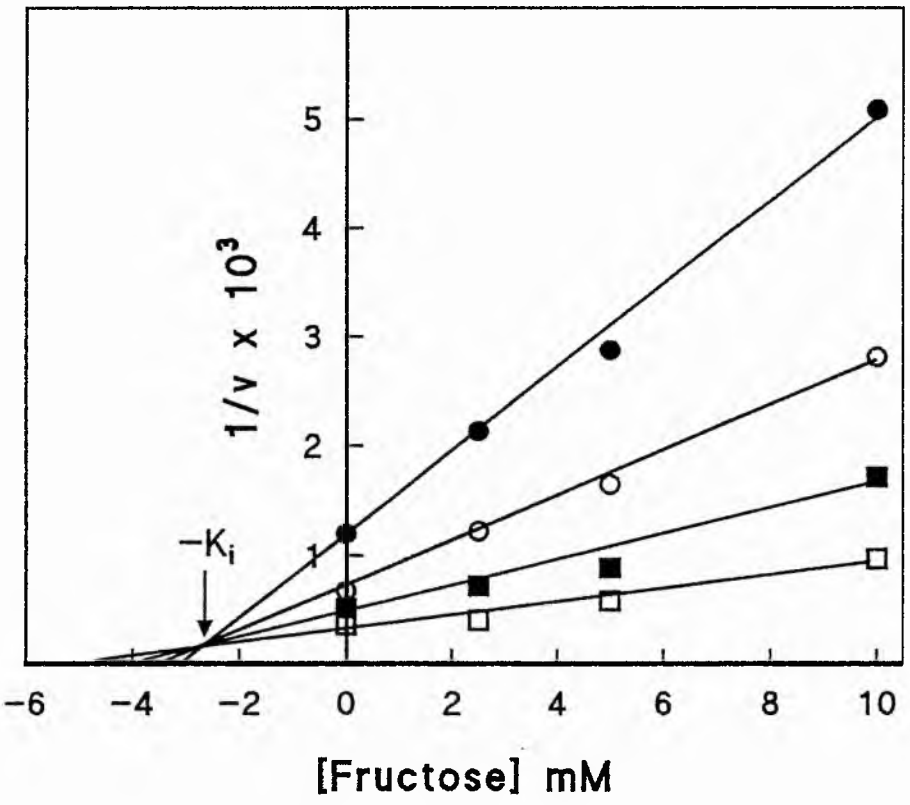


Fig. 7.5 Dixon plot showing competitive inhibition of purified *V. faba* sucrose synthase by fructose. ●, 25 mM; ○, 50 mM; ■, 100 mM; and □, 200 mM sucrose (v [units mg^{-1} protein]).

Table 7.2 Purification of faba bean cotyledon alkaline invertase

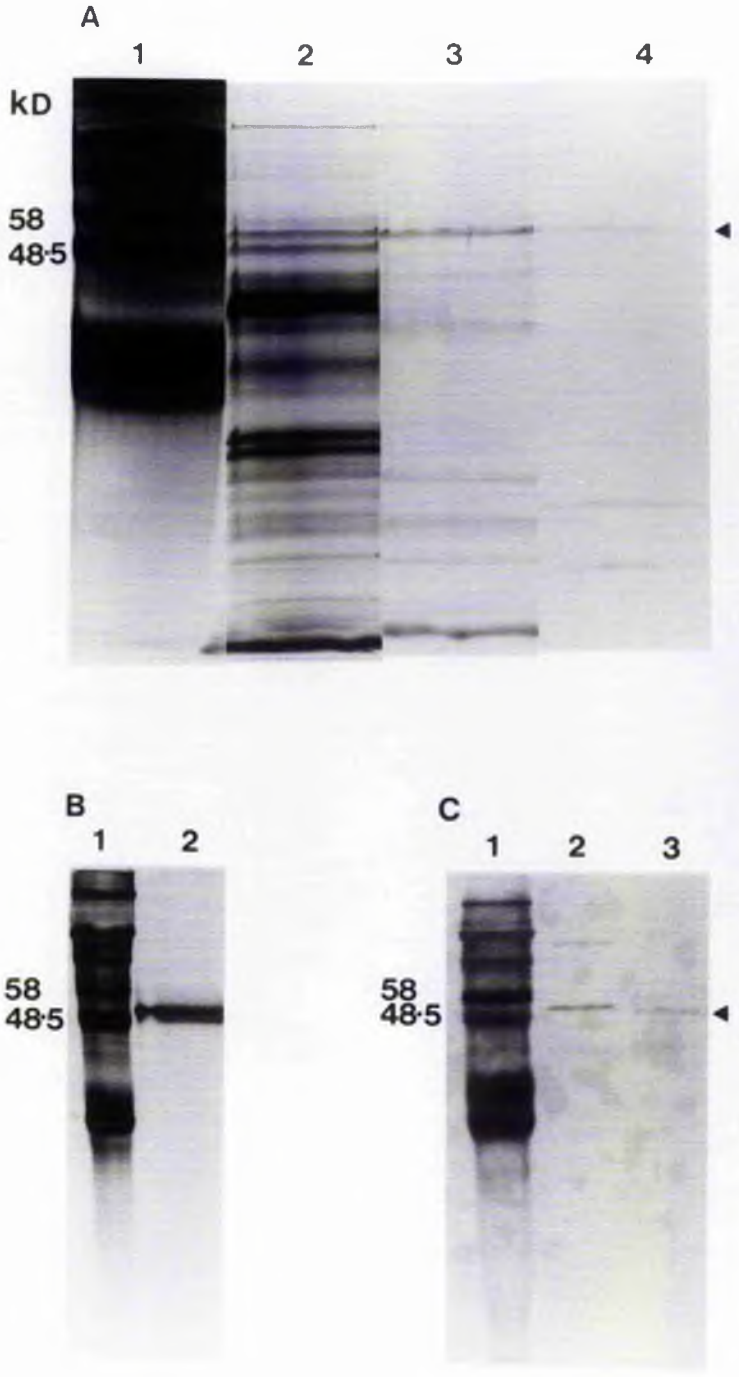
Fraction	Total Activity U (nmol min^{-1})	Total Protein (mg)
Crude	53,616	34,608
0–30% $(NH_4)_2SO_4$	7,362	1,815
30–80% $(NH_4)_2SO_4$	24,581	21,000
>80% $(NH_4)_2SO_4$	2,835	3,342
0–30% fraction		
Phenyl Sepharose – active fraction collected and freeze dried	1,953	74
Mono Q	547	3.4
Superose 6	294	1.4
30–80% fraction		
Phenyl Sepharose – active fraction collected and freeze-dried	4,337	511
Mono Q	4,218	312
Superose 6	1,792	7.8

Specific Activity (U mg^{-1} protein)	Yield (%)	Purification Fold
1.6	100	-
4.1	14	2.6
1.2	46	0.8
0.9	5	0.6
26.4	4	17.0
160.8	1	103.7
213.3	0.5	137.6
8.5	8.1	5.3
13.5	7.9	8.5
229.8	3.3	143.6

resulted in more of the enzyme binding to the column and less being eluted in the unbound fraction. The bound active fractions were applied to the gel filtration column and the molecular weight of the native active protein determined as 238,400 ± 4,500 (data not shown but determined as for sucrose synthase). Active fractions obtained from the Mono Q step (both unbound and bound) and following gel filtration columns were electrophoresed on SDS-PAGE. One major polypeptide, with an estimated molecular weight of 53,400 ± 900, was associated with alkaline invertase activity (Plate 7.IVA). When parallel gels were prepared and the proteins transferred to nitrocellulose, the antisera raised in mice against this major polypeptide precipitated alkaline invertase activity from partially purified preparations of the enzyme (Fig. 7.6A). On immunoblots a single faint band was just visible at approximately 54 kD (data not shown) with immune serum but no crossreaction occurred with pre-immune serum. Alkaline invertase was further purified by gel elution. The eluted polypeptide produced a single band when subjected to SDS-PAGE and visualised by silver-staining (Plate 7.IVB). Antisera raised in rabbit using this gel-purified polypeptide again precipitated alkaline invertase activity (Fig. 7.6B).

To test for the presence of alkaline invertase isoforms, samples from the 30-80% $(NH_4)SO_4$ fraction were applied to the Mono Q column but the salt gradient was decreased by eluting with 40 column volumes over the range 0 to 1 M KCl. This resulted in the separation of two forms of the enzyme, one eluting at approximately 0.37 M and the other at 0.42 M (Fig. 7.7A). When these isoforms were immunoblotted with the immune serum raised in rabbit, single bands were visible at approximately 54 kD (Plate 7.IVC). The presence of alkaline invertase isoforms was further confirmed by two-dimensional immunoblots which showed the presence of two distinct cross-reacting polypeptides separated by slight differences in their isoelectric points (data not shown).

Plate 7.1V A) 12.5% SDS-PAGE of samples from successive stages in the purification of bean alkaline invertase. Lane 1, Prestained markers (SDS-7B Sigma); Lane 2 - Mono Q, unbound sample; Lane 3 - Mono Q, bound sample (isoforms combined); Lane 4 - Superose 6. Alkaline invertase polypeptide indicated with arrow. B) 12.5% denaturing gel. Lane 1, Prestained markers as in A); Lane 2, bean alkaline invertase polypeptide purified following elution from SDS-PAGE. In both A) and B), polypeptides were visualized by silver staining. C) Immunoblot of 12.5% SDS-PAGE. Lane 1, Prestained markers as in A); Lane 2, Mono Q sample (isoform 1); Lane 3, Mono Q sample (isoform 2). Arrow shows the position of the cross-reacting alkaline invertase polypeptides.


A
1
2
3
4
kD
58
48·5
B
1
2
58
48·5
C
1
2
3
58
48·5

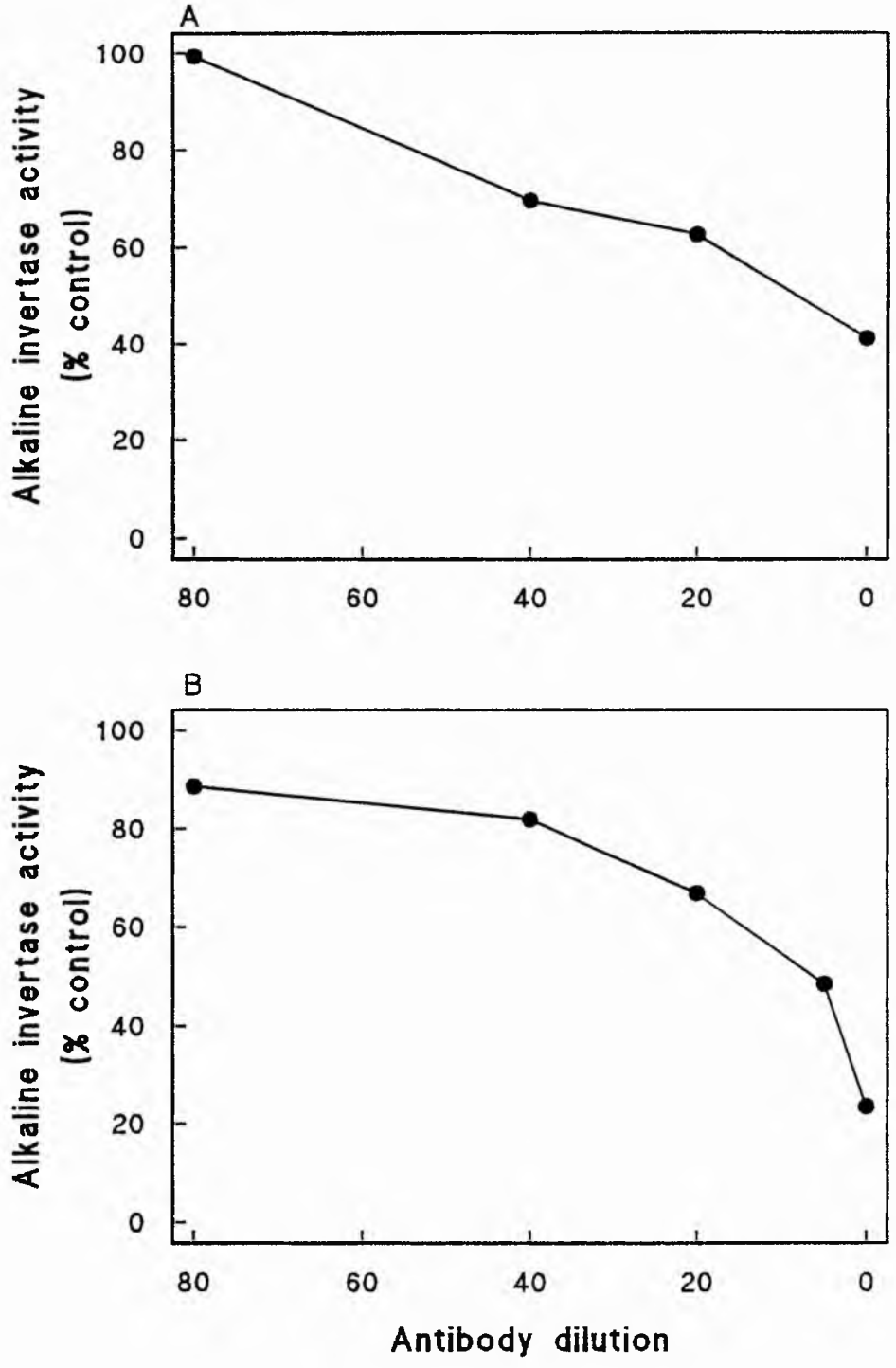


Fig. 7.6 Inhibition of alkaline invertase activity by immunoprecipitation with antisera to the polypeptide from *Vicia faba*. Antisera raised in A) mouse by intrasplenic immunisation and B) rabbit intramuscularly. Preimmune serum from both was used as controls.

When antibodies were used to immunoblot crude extracts from potato tubers and sugar beet taproots (Plates 3.IVD and 6.IIB,D respectively) specific cross-reaction occurred.

The first attempt at obtaining an amino acid sequence using the gel-purified bean polypeptide revealed that the NH_2 terminus was blocked. Despite numerous attempts at cleaving the protein with V8, insufficient amounts of polypeptides were produced to permit sequence determination.

7.3.2.2 Enzyme properties and kinetics

The purified enzyme has a broad peak of activity from pH 7.0 to 7.5 (sodium phosphate buffer) with a pH optimum at 7.4 (Fig. 7.7B). The isoelectric point (pI) was calculated to be 5.2. One peak of activity was detected following isoelectric focussing of a crude enzyme extract (data not shown). This indicates that the two isoforms have very similar pI values.

Standard Michaelis-Menten kinetics was observed for alkaline invertase with a K_m value for sucrose of 10.1 ± 1.5 and a V_{max} of 585.9 ± 20.3 (Fig. 7.8A). A secondary Hanes plot confirms linearity (r = 0.996 [Fig. 7.8B]).

The purified enzyme was strongly inhibited by Tris (50% inhibition with 5 mM Tris under standard assay conditions). The inhibition caused by Tris was of a linear mixed-type obeying Michaelis-Menten kinetics but with both the K_m and V_{max} altered (data not shown). The slope on a Dixon plot decreased with increasing substrate concentration (Fig. 7.9A).

Fructose (10 mM) inhibited the purified alkaline invertase by 32%. It acted as a competitive inhibitor with the K_m increasing but the V_{max} unchanged on a Michaelis-Menten plot (data not shown). The lines on a Dixon plot intersected at a point beyond the y-axis = $-K_i$ (Fig. 7.9B). The value for K_i determined for fructose was 8.6 mM.

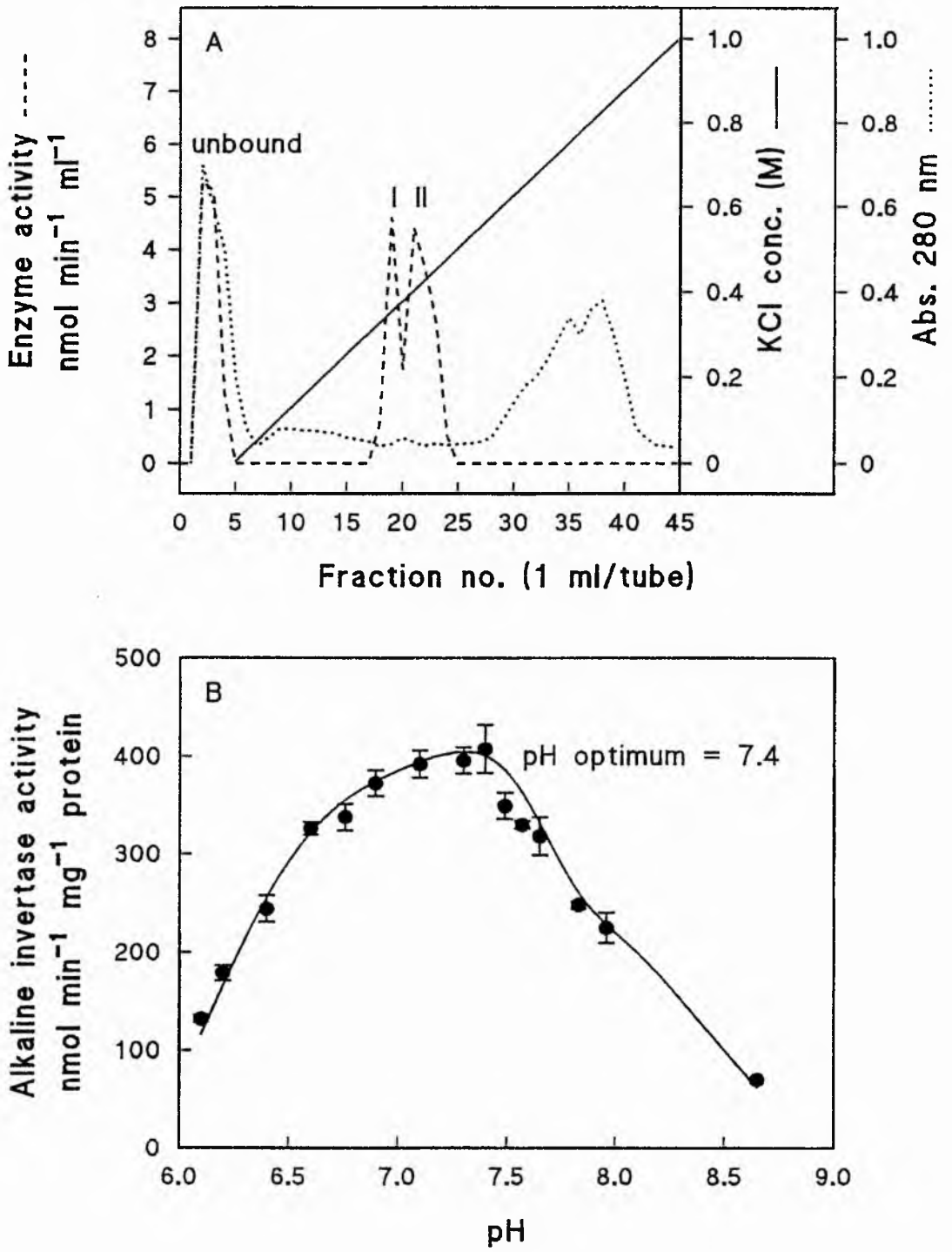


Fig. 7.7 A) Anion-exchange chromatography (mono Q) of alkaline invertase from bean cotyledons. B) pH curve of purified bean alkaline invertase activity. 200 mM sodium phosphate buffer was used throughout. The reactions were carried out at 37°C and values shown are means (n = 3) ± SEM.

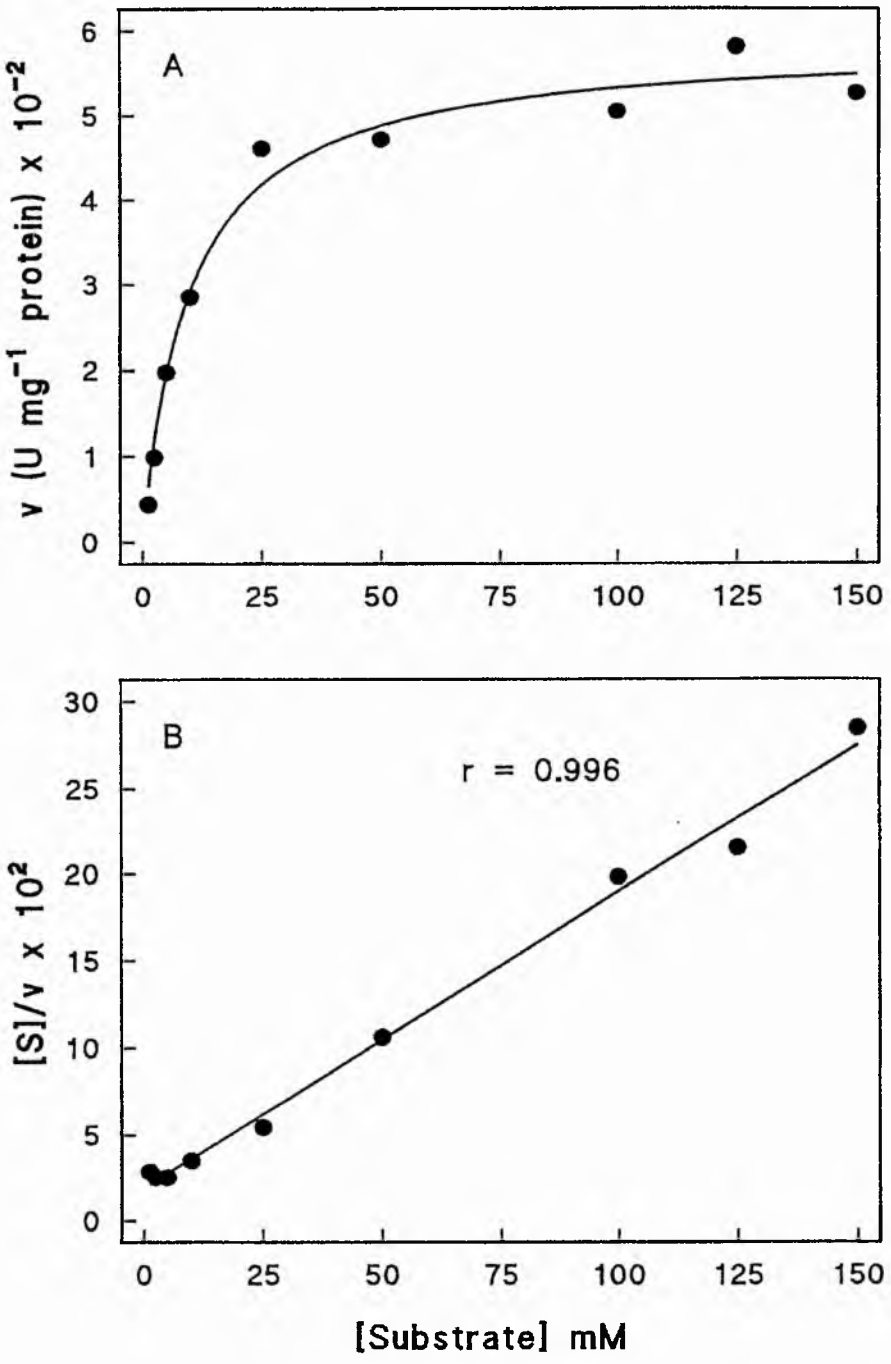


Fig. 7.8 A) Sucrose saturation curve of purified *V. faba* alkaline invertase fitted using the Michaelis-Menten equation. B) Hanes-Woolf plot derived from above. (Sucrose concentration 1.25 mM - 150 mM).

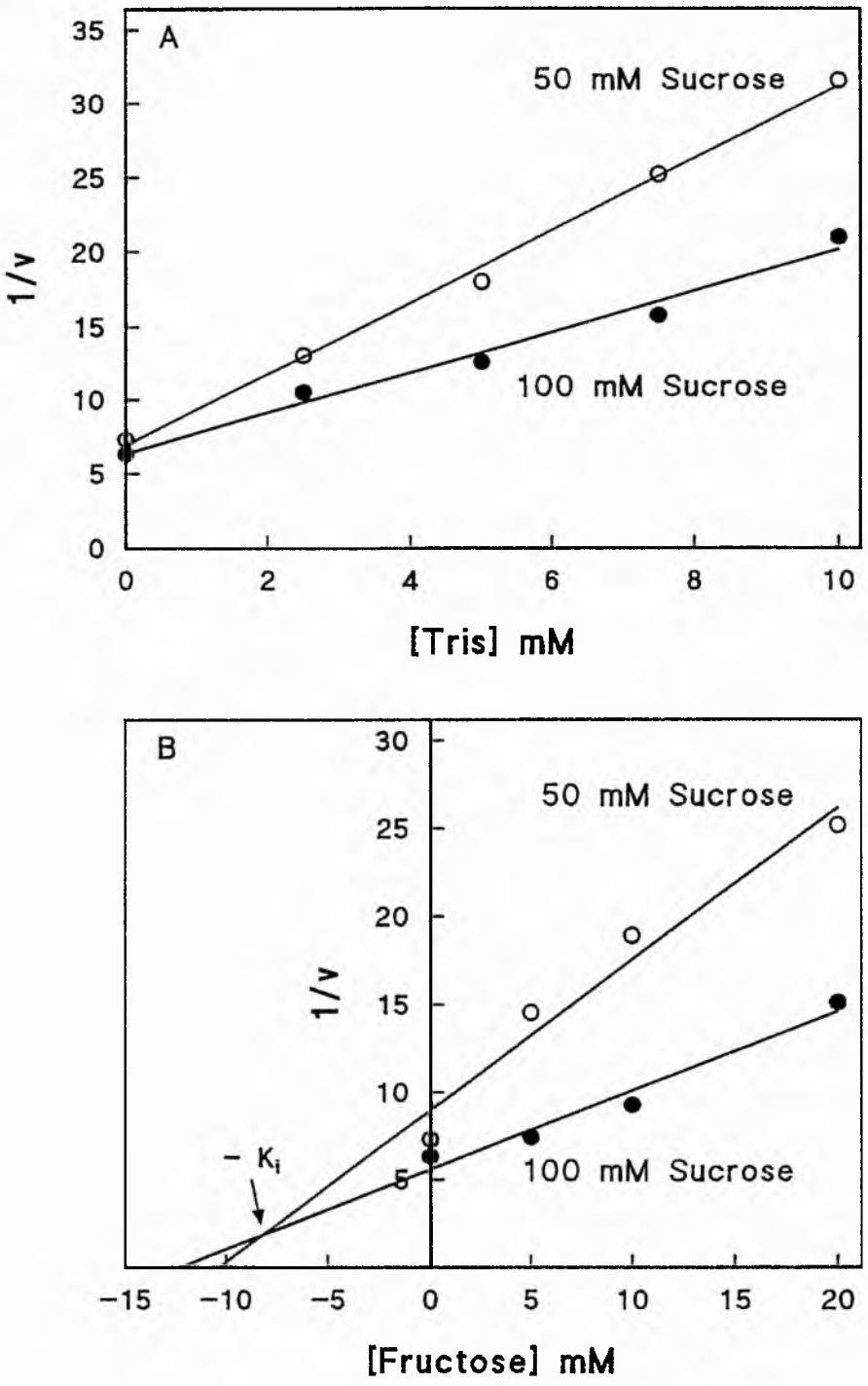


Fig. 7.9 Dixon plots showing A) linear mixed-type inhibition of purified *V. faba* alkaline invertase by Tris and B) competitive inhibition of purified *V. faba* alkaline invertase by fructose. (v [units mg^{-1} protein]).

The test of substrate specificity of the purified enzyme showed that alkaline invertase is a ß-fructofuranosidase specific for sucrose. No significant hydrolysis occurred with Raffinose (Gal α 1,6 Glc ß 1,2 Fru), Stachyose (Gal α 1,6 Gal α 1,6 Glc ß 1,2 Fru), Trehalose (Glc α 1,1 Glc), Maltose (Glc α 1,4 Glc) or Lactose (Gal ß 1,4 Glc).

7.4 Discussion

7.4.1 Sucrose synthase

The purification to homogeneity of sucrose synthase from *V. faba* cotyledons showed that the enzyme has a molecular mass of 360 kD and is composed of four subunits of 92 to 93 kD. The tetrameric structure of the native protein is similar to that observed with mung bean seedlings (Delmer, 1972), rice grains (Nomura & Akazawa, 1973), maize kernels (Su & Preiss, 1978), soybean nodules (Morrell & Copeland, 1985), and peach fruit (Moriguchi & Yamaki, 1988). In these instances, the molecular mass of the protein ranges between 360 and 400 kD, with the molecular masses of identical subunits ranging between 87 and 100 kD. As with soybean nodules (Morrell & Copeland, 1985), *V. faba* cotyledons contain only one (detectable) form of the enzyme. Polyclonal antibodies raised against sucrose synthase from whole kernels of wild-type maize (kindly supplied by Dr. Karen Koch, University of Florida) showed no specific cross-reaction on immunoblots with *V. faba* sucrose synthase protein, unlike those raised against soybean nodule sucrose synthase, which reacted specifically with the 92 kD polypeptide subunit. The maize antibody does, however, recognize potato sucrose synthase (Chapter 4 and Ross & Davies [1992]).

The amino acid sequence data, although only for a small portion of the faba bean sucrose synthase protein, show distinct homology with potato tuber sucrose synthase, which itself has a 75% overall identity with maize sucrose synthase (Salanoubat & Belliard, 1987). Additionally, the polyclonal antibodies raised against the faba bean sucrose synthase protein detect a single polypeptide (≈ 90 - 93 kD) from a crude tuber extract on an immunoblot (Plate 3.IVA), a crude sugar beet taproot extract (Plate 6.IIA and C) and crude extracts from *Pinus edulis* (Hammer & Murphy, 1993).

Recent work (Pozueta-Romero *et al.*, 1991) has suggested that ADP rather than UDP is the principal nucleoside diphosphate utilised in the sucrose synthase reaction. Previous work with relatively crude extracts of *V. faba* cotyledons demonstrated that the activity of sucrose synthase with ADP was only 16% of that with UDP (de Fekete, 1969). This has essentially been confirmed in the present study using purified enzyme. Although significant inhibition of the faba bean enzyme with Tris buffer and ADP confirms the results of Pozueta-Romero *et al.* (1991) with spinach leaves and sycamore cell suspensions, the faba bean enzyme is unable to utilise other nucleoside diphosphates as effectively as UDP, even when Tris is replaced by Hepes. The bean enzyme is certainly not ADP specific. The data do not therefore, agree with the hypothesis that ADP is the principal substrate for faba bean sucrose synthase, at least as far as maximum catalytic activity is concerned. Physiologically, the proportion of sucrose synthase activity driven by ADP and/or UDP *in vivo* will clearly depend on the concentration of the nucleoside diphosphates in the cytosol. It will also depend on whether or not ADP activity is suppressed by the presence of UDP. According to Pozueta-Romero *et al.* (1991) this is not the case. The hypothesis has not been tested for the purified faba bean enzyme. It should be noted that the kinetic data obtained for faba bean with UDP and ADP are similar to those reported for peach (Moriguchi & Yamaki, 1988).

Fructose acts as a competitive inhibitor of faba bean sucrose synthase with respect to sucrose (as shown previously with *Helianthus tuberosus* [Wolosuik & Pontis, 1974]). Calculations from values given in Chapter 5 have shown that during bean seed development, the concentration of fructose in cotyledons (on a whole tissue basis) decreases from about 7.5 mM 30 days after anthesis to about 2 mM 20 days later (at the time of maximum sucrose synthase activity). At the sucrose concentrations prevailing in the tissue at the same time, calculations show that sucrose synthase activity (cleavage direction) may be inhibited between 70 and 30% (assuming that sucrose, fructose, and sucrose synthase are within the same cellular compartment). Fructose-specific hexokinase, known to be present in a range of tissues, including developing *V. faba* cotyledons (de Fekete, 1969; Chapter 5; Gardner and Davies, pers.comm.), may therefore play an important role in regulating sucrose synthase activity *in vivo*.

7.4.2 Alkaline invertase

The purification of alkaline invertase from *V. faba* cotyledons showed that the enzyme, like sucrose synthase, is a homotetramer but with estimated subunit mass of 53.4 kD and native molecular mass of 238 kD. This structure is very similar to that of alkaline invertase purified to homogeneity from soybean hypocotyls (Chen and Black, 1992). They estimated the native molecular weight to be approximately 240,000 with subunits of about 58,000. While these authors only detected one form of the enzyme in soybean hypocotyls, others (Masuda *et al.*, 1987) have detected two forms in sugar beet taproots. At least two forms of faba bean alkaline invertase that bound to the anion exchange column were detected, although eluting very close together on the salt

gradient. Both forms were detected by the polyclonal antibodies produced. The ability of these antibodies to cross-react specifically with alkaline invertase from other sources (e.g. potato tubers and sugar beet taproots) is supported by the immunological studies with the soybean antibodies which reacted specifically with alkaline invertase from snow pea seeds, pole bean seeds, mung bean sprouts, cucumber fruit, corn roots and carrot roots (Chen & Black, 1992). Additionally, they found that alkaline invertase was immunochemically distinct from acid invertases.

The method of raising antiserum intrasplenically in mice proved advantageous when only nanogram amounts of purified antigen were available. The ability of the antiserum to specifically immunoprecipitate alkaline invertase confirmed the success of the technique.

The pH optimum of 7.4 falls within the range 7.0 to 8.0 usually reported for maximum activity, supporting the belief that it is a cytosolic enzyme (Avigad, 1982). The calculated K_m value (sucrose) was 10.1 mM which is in close agreement with that obtained for soybean hypocotyls (Chen and Black, 1992) and for soybean nodules (Morrell and Copeland, 1984) of 10 mM in both cases. This difference between sucrose synthase and alkaline invertase in their affinity for sucrose is important when considering the separate roles played by these enzymes during bean development. As shown previously (Chapter 5), maximum sucrose synthase activity occurred when the sucrose content of the developing bean was at its highest while alkaline invertase activity was high even when the sucrose content was at its lowest.

The strong inhibition of alkaline invertase by Tris even at low concentrations, reported by several authors (Huber and Akazawa, 1986; Chen and Black, 1992; Morrell and Copeland, 1984), was shown for the bean enzyme. Product inhibition with fructose acting as a competitive inhibitor of alkaline invertase

has been shown for a number of different species: carrot and turnip (Ricardo, 1974), sugar beet (Masuda *et al.*, 1987) and soybean nodules (Morrell and Copeland, 1984). The K_i value of 8.6 mM determined for fructose is similar to the value of 11 mM determined by Morrell and Copeland (1984) and additionally is similar to its K_m value for sucrose. This would indicate that the enzyme has similar affinities for fructose and sucrose as found for alkaline invertase from carrot and turnip (Ricardo, 1974). In a comparison of acid and alkaline invertases, Ricardo (1974) concluded that fructose inhibition was likely to be physiologically significant only for alkaline invertase and suggested that alkaline invertase may be involved in the regulation of the hexose level of the cell in growing storage roots.

The bean alkaline invertase in common with those already discussed, is a ß-fructofuranosidase specific for sucrose, lending support to the above suggestion that it has an essential role in maintaining the hexose levels within the cytosol.

7.5 Conclusions

Both sucrose synthase and alkaline invertase were purified to homogeneity and found to be homotetramers with native molecular weights of 360,000 and 240,000 respectively. While sucrose synthase shows a sigmoidal curve for sucrose saturation with an estimated K_m value of 169 mM, alkaline invertase follows standard Michaelis-Menten kinetics with a K_m value of 10 mM. Polyclonal antisera raised against sucrose synthase cross-reacted specifically in immunoblots with a single polypeptide (ca 93 kD). Antibodies raised in both mouse and rabbit against purified alkaline invertase cross-reacted specifically in immunoblots with single polypeptides (ca 53 kD) and immunoprecipitated

alkaline invertase activity. The antisera raised also specifically cross-reacted with the corresponding enzymes from other plant sources. The enzymes were inhibited by their product, fructose, which at a concentration of 10 mM caused 74% and 32% inhibition of the activity of sucrose synthase and alkaline invertase respectively.

CHAPTER 8

PURIFICATION AND CHARACTERISATION OF ALKALINE INVERTASE FROM THE TAPROOTS OF SUGAR BEET (*Beta vulgaris* L.)

8.1 Introduction

Despite the significant commercial importance of sugar beet and the need for both storing more sucrose during taproot development and preventing post-harvest sucrose cleavage, little is known about the properties of the enzymes involved. While previous work (Wyse, 1974) implicated acid invertase as one factor responsible for post-harvest sucrose inversion, acid invertase activity remains low even when mature taproots are stored at 7-8°C for up to 80 days (Chapter 6). In contrast, the levels of both alkaline invertase and sucrose synthase were high, especially during the period of rapid sucrose accumulation (Chapter 6).

The correlation obtained by Masuda *et al.* (1987) between sucrose accumulation and alkaline invertase activity led to the suggestion that alkaline invertase was involved in the regulation of sucrose accumulation. By partial purification of this enzyme, they found that two forms, separable by DEAE-cellulose column chromatography, were present and on the basis of their activity during growth, may be involved in different physiological functions in sucrose metabolism. Alkaline invertase I was very active at the stage of sucrose accumulation but decreased (ca 6-fold) when sucrose reached a constant level, suggesting that it regulates sucrose storage within the taproot. By contrast, the level of enzyme II

was lower and remained relatively constant throughout the development of the taproots.

Alkaline invertase is also present in mature storage tissue of sugar cane where it is considered to regulate sugar levels (Hatch and Glasziou, 1963). A linear relationship was obtained when changes in total sugar content were plotted against changes in alkaline invertase activity. The implication from these results, that alkaline invertase is controlling the amount of sucrose stored in both sugar beet and sugar cane, stresses the need to target this enzyme for more detailed studies.

The aim of the work described in this chapter was to purify alkaline invertase and obtain sufficient of the purified protein to raise antisera, characterise the properties of the enzyme and obtain a partial amino acid sequence. At present, to my knowledge, alkaline invertase antibodies have been raised only to the enzyme purified from developing cotyledons of *Vicia faba* (Chapter 7) and from sprouting soybean hypocotyls (Chen and Black, 1992). While antibodies raised against both *V. faba* and soybean proteins show immunological cross-reactivity with other plant alkaline invertases, it was considered important to raise antibodies against the sugar beet alkaline invertase to initiate work on the isolation of a sugar beet taproot gene.

8.2 Material and Methods

8.2.1 Plant material

2.5 Kg of taproots (mean FW per taproot = 60 g) from field-grown sugar beet (*Beta vulgaris* L. cv. Celt) were used for the purification of alkaline invertase.

Taproots at this stage of development were previously shown to contain high enzyme activity (Chapter 6).

8.2.2 Extraction and purification of Alkaline invertase

8.2.2.1 Extraction

After harvesting, the sugar beet taproots were washed and chopped prior to extraction in ice-cold buffer in a pre-chilled blender (Atomix). The extraction buffer used was 50 mM Hepes/KOH (pH 7.5) containing 5 mM DTT, 0.1% Triton X-100, 10% glycerol, 2 mM benzamidine, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and 2 mM ε-amino-caproic acid. Insoluble Polyvinylpolypyrrolidone was included during the extraction at 0.1% w/v. The homogenate was filtered through cheesecloth and re-extracted prior to centrifugation of the combined extracts at 16,000 g and 4°C for 30 min.

8.2.2.2 Fractionation of extract with $(NH_4)_2SO_4$

After centrifugation extracts were fractionated with $(NH_4)_2SO_4$ producing 0-20%, 20-50% and 50-80% saturation fractions. The precipitated proteins from each fraction were recovered by centrifugation at 16,000 g and 4°C for 30 mins and resuspended in 20 mM Hepes/KOH (pH 7.5) containing 5 mM DTT (buffer A). All fractions, including the final supernatant obtained after 80% saturation and a sample of the crude extract prior to fractionation, were dialysed against buffer A at 4°C overnight.

8.2.2.3 Hydrophobic interaction chromatography

The dialysed protein solutions from the 20 to 50% $(NH_4)_2SO_4$ saturation fractions were pooled and used in the further purification steps. Sufficient $(NH_4)_2SO_4$ was added to the combined dialysate to make the solution 0.75 M with respect to the salt. The solution was applied to a Phenyl Sepharose column (Pharmacia LKB, UK) previously equilibrated with buffer A containing 0.75 M $(NH_4)_2SO_4$ (buffer B). Proteins bound to the column were eluted with a stepped gradient of buffer B and buffer A. The protein expressing alkaline invertase activity was tightly bound and eluted at 100% of buffer A. The fractions containing alkaline invertase activity were dialysed overnight at 4°C against buffer A.

8.2.2.4 Anion exchange chromatography

The dialysate was applied at a flow rate of 0.5 ml min^{-1} to an anion exchange column (Mono Q - as used in section **7.2.2.5**) previously equilibrated with the same buffer used for dialysis. The protein was eluted with a gradient of 0 to 1M KCl over 80 column volumes. Two alkaline invertases were detected and the respective fractions were concentrated using Macrosep and Microsep centrifugal concentrators with a 10,000 MW cutoff (supplied by Flowgen, UK) in preparation for the final column step. On the basis of their order of elution from the Mono Q column, the two forms were designated as alkaline invertases I and II.

8.2.2.5 Gel filtration chromatography

From the concentrated samples, 200 µl volumes were applied at a flow rate of 0.3 ml min^{-1} to a Superose 6 gel filtration column (Pharmacia LKB) pre-equilibrated with 20 mM Hepes (pH 7.5) containing 100 mM KCl and 5 mM 2-mercaptoethanol. The gel filtration column was calibrated using the same molecular weight markers as described previously in section **7.2.2.6**. Active fractions were pooled and dialysed against 20 mM Hepes (pH 7.5) containing 5 mM DTT. The purified samples were either further concentrated using the Microsep concentrators or used in their present form for characterisation studies or protein sequencing and antibody production.

8.2.3 Gel electrophoresis - SDS-PAGE

SDS-PAGE was performed as described in section **2.5.1** using 12.5% acrylamide. Both methods of staining as described in section **2.5.1** (Coomassie brilliant blue and silver staining) were used to detect the alkaline invertase polypeptide subunits.

8.2.4 Preparation of Antisera

SDS-PAGE of samples eluted from the gel filtration column allowed the identification of a polypeptide associated with alkaline invertase activity. Concentrated samples with high alkaline invertase activity were electrophoresed on 12.5% gels and the polypeptide identified as alkaline invertase excised and eluted at $4^{o}C$ for 48 h in 5 gel volumes of water. This process was repeated several times to provide sufficient protein to raise

antisera in rabbits. Due to the higher activity (on a volume basis) of alkaline invertase II, this was used in preference to alkaline invertase I. After gel elution, the protein was lyophilised and redissolved in TBS to provide 4 aliquots. Initially, an equal volume of complete Freund's adjuvant was mixed with one aliquot before injecting, intramuscularly, into a New Zealand White rabbit. Three booster injections with the addition of an equal volume of incomplete Freund's adjuvant, were given 14, 49 and 84 days later. Serum was collected 14 days after the final injection. Both pre-immune and immune sera were partially purified as described previously (**7.2.5.1**).

8.2.5 Immunoblotting

Immunoblots using the polyclonal antibodies raised against denatured alkaline invertase purified from developing cotyledons of *V. faba* were carried out as described previously in section **2.6**. Blots were incubated with the antibodies diluted 1:500 or 1:1000 with PBS.

8.2.6 Protein sequencing

Purified alkaline invertase II was subjected to SDS-PAGE using the recent, improved method of Dunbar and Wilson (1994), designed to enhance the degree of resolution of the sequencing gel. Thereafter, the proteins were electroblotted on to Problott membrane (Applied Biosystems, UK), stained with amido black (0.1%) and the alkaline invertase polypeptide loaded on to an Applied Biosystems model 477A sequencer (Matsudaira, 1987).

8.2.7 Enzyme assays

8.2.7.1 Sucrose synthase

Sucrose synthase activity was assayed in the cleavage direction as described previously (section **2.3.1**). Additionally, the optimum pH of partially purified sugar beet sucrose synthase was determined over the pH range 6.5 - 8.0 using 100 mM Tris/HCl buffer.

8.2.7.2 Alkaline invertase

Alkaline invertase activity was assayed as described in section **7.2.9.2.** The optimum pH for the partially purified enzyme was determined using 200 mM sodium phosphate buffer over the pH range of 7 to 8. The substrate specificity of alkaline invertase was tested with raffinose, lactose, stachyose, trehalose, and maltose (each at concentrations of 50, 100 and 150 mM).

8.2.8 Protein assay

Protein concentrations were determined as described in section **2.7.**

8.3 Results

8.3.1 Purification of alkaline invertase

As the principle objective was to obtain sufficient purified enzyme to raise antisera and to sequence the protein, no samples were taken for assessment of the degree of purification in order to conserve the amount of protein.

However, activity values and protein contents of crude and $(NH_4)_2SO_4$ precipitated fractions were determined so that the fractions with the highest alkaline invertase activity could be selected for further purification (Table 8.1). The proteins precipitated at 20 and 50% $(NH_4)_2SO_4$ saturation were combined and used in the further purification steps.

Sugar beet alkaline invertases showed a high degree of hydrophobicity and were only eluted from the Phenyl Sepharose column in the absence of $(NH_4)_2SO_4$. Active alkaline invertase protein applied to the anion-exchange column (Mono Q) was eluted at approximately 0.28 M KCl (alkaline invertase I) and at 0.56 M KCl (alkaline invertase II) (Fig. 8.1A). Whilst maximum enzyme activity for alkaline invertase I was ca 60-fold lower than for alkaline invertase II, the small amount of protein present in the first peak resulted in a higher specific activity for alkaline invertase I. Thus, when samples collected from the anion-exchange column after 5 separate runs were concentrated, the specific activity of alkaline invertase I was nearly 25-fold higher than that for the crude enzyme extract. The value for alkaline invertase II was only 4-fold higher. Further purification by gel filtration chromatography increased the purification of alkaline invertase I 26-fold by comparison with the crude extract, while alkaline invertase II was increased 12-fold (data not shown). Due to the low final yield of protein after this stage, further purification by column chromatography was not deemed possible. However, the mean molecular weight of the active protein corresponding to alkaline invertase I, determined by gel filtration, was 234,672 ± 7,638 and that of alkaline invertase II, 254,059 ± 12,324.

When fractions collected over a peak of enzyme activity (alkaline invertase I) eluted from Superose 6 were electrophoresed, the intensity of staining of a polypeptide with a mass of ca 52 kD corresponded with enzyme activities in each fraction (Plate 8.IA shown with arrow). The two most active fractions, eluted from several gel filtration runs, were pooled separately and

Table 8.1 Alkaline invertase activity and protein content of sugar beet samples before and after fractionation with $(NH_4)_2SO_4$ showing specific activity, yield and overall purification

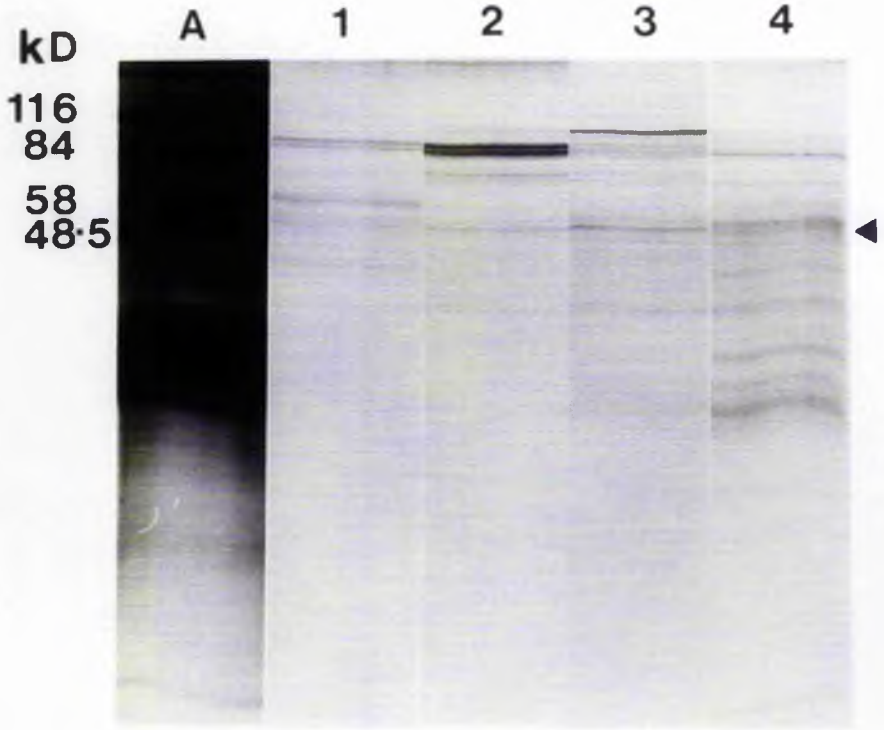
Fraction	Total activity (nmol min^{-1})	Total protein (mg)	Specific activity (nmol min^{-1} mg^{-1})	Yield %	Purification (Fold)
Crude	347,650	10,102	34.4	-	-
20% precip.	2,460	31	79.4	0.7	2.3
50% precip.	102,820	2,989	34.4	29.6	1.0
80% precip.	93,110	5,202	17.9	26.8	0.5
>80%	19,410	1,341	14.5	5.6	0.4

concentrated before electrophoresis and Coomassie-staining (Plate 8.IB). This gel revealed approximately 7 polypeptides with distinct bands in both lanes visible at positions corresponding to molecular masses of ≈96 kD and 52 kD. Previous experience obtained during the purification of sucrose synthase and alkaline invertase from bean cotyledons suggested that these bands may correspond to the subunits from these two enzymes. The bean alkaline invertase antiserum (undiluted) immunoprecipitated approximately 50% of alkaline invertase activity from a crude extract of sugar beet taproot (data not shown) and was useful, therefore, for testing cross-reactivity on a blot. A duplicate gel, immunoblotted with the bean alkaline invertase and sucrose synthase polyclonal antibodies (Plate 8.IIC, lanes 1 and 2 respectively) confirmed the identification of the two polypeptides arrowed in Plate 8.IB. Hence the purification protocol used for alkaline invertase resulted in partial co-purification of sucrose synthase. The molecular mass of the sucrose synthase subunit was estimated from several denaturing gels to be 96 ± 1.2 kD. The partially purified sucrose synthase fraction was used for later characterisation studies.

Having identified the alkaline invertase subunit polypeptide (M_r for both forms = 52,007 ± 1,960) it was important to confirm whether the bean antibodies recognised both forms of the enzyme. Hence, post Mono Q samples of alkaline invertases I and II (equal protein loadings) were immunoblotted with the faba bean antibodies. Both forms of the enzyme specifically cross-reacted with a single polypeptide of ≈52 kD (Plate 8.IIA and B - alkaline invertase I and II respectively).

Alkaline invertase II was further purified by gel elution and tested for homogeneity on SDS-PAGE, yielding a single band when visualised by silver-staining. Antisera raised in rabbits using this purified preparation cross-reacted specifically with a single polypeptide (approx. 52 kD) from a relatively crude sugar beet sample and from crude *V. faba* cotyledon samples (Plate

Plate 8.I A) Identification on 12.5% SDS-PAGE of alkaline invertase I from Superose-6 fractions collected over a peak of activity. Specific activities were:- Lane 1 - 74; Lane 2 - 784; Lane 3 - 502; Lane 4 - 67. All values are expressed as nmol min^{-1} mg^{-1}protein. An arrow marks the identified alkaline invertase polypeptide. Silver-staining was used to visualise the polypeptides. B) 12.5% SDS-PAGE of concentrated active fractions collected from Superose-6 with arrows indicating possible position of sucrose synthase polypeptides (ca 96 kD) and alkaline invertase polypeptides (ca 52 kD). Polypeptides visualised by coomassie-blue stain. In both A) and B) the outer lane contains MW markers (SDS-7B Sigma).


kD
A
1
2
3
4
116
84
58
48·5

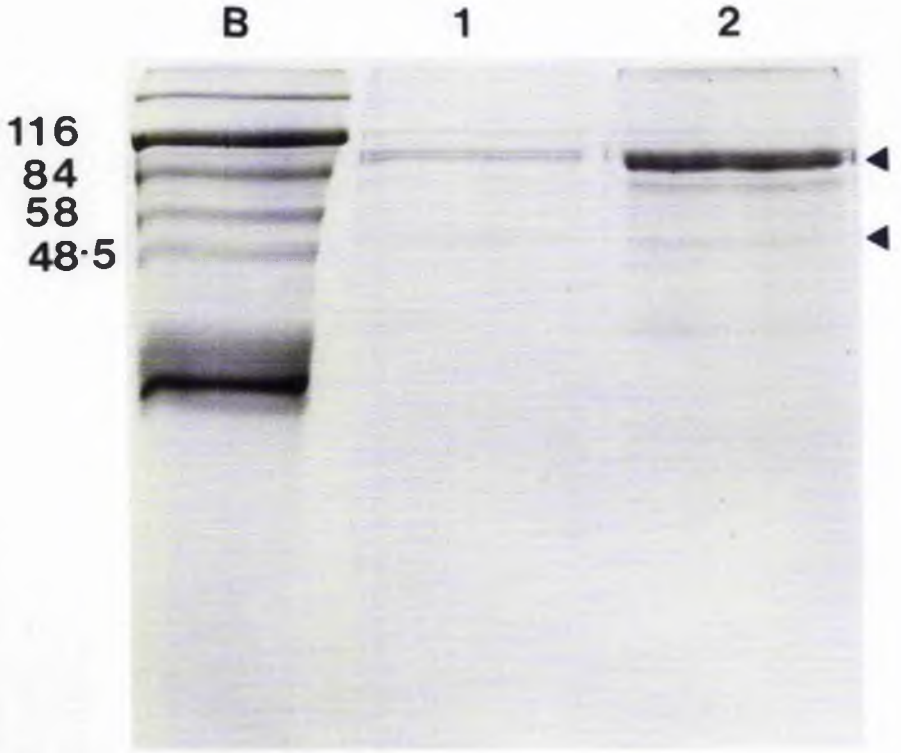
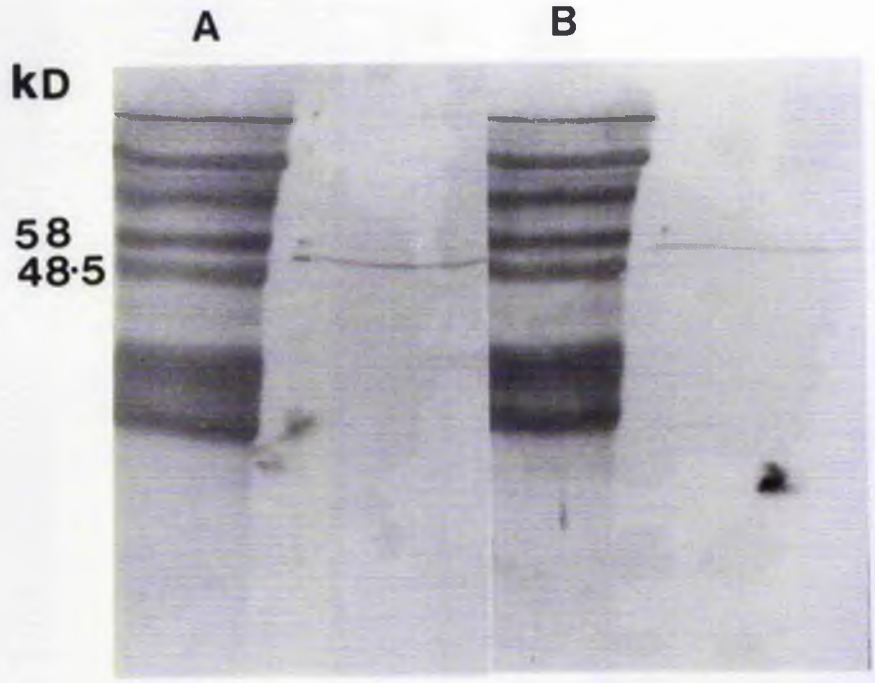

B
1
2
116
84
58
48·5

Plate 8.II A) and B) Immunoblots of post Mono Q samples of alkaline invertase I and II respectively with bean alkaline invertase antibodies. (dilution 1:1000). For comparison of the relative positions of the alkaline invertase polypeptides, MW markers (SDS-7B Sigma) are shown in both A) and B). C) Immunoblots of SDS polyacrylamide gel shown in Plate 8.1B. Lane 1 - immunoblotted with bean alkaline invertase antibody (dilution 1:1000); Lane 2 - immunoblotted with bean sucrose synthase antibody (dilution 1:10,000). MW markers in first lane as detailed above.


A
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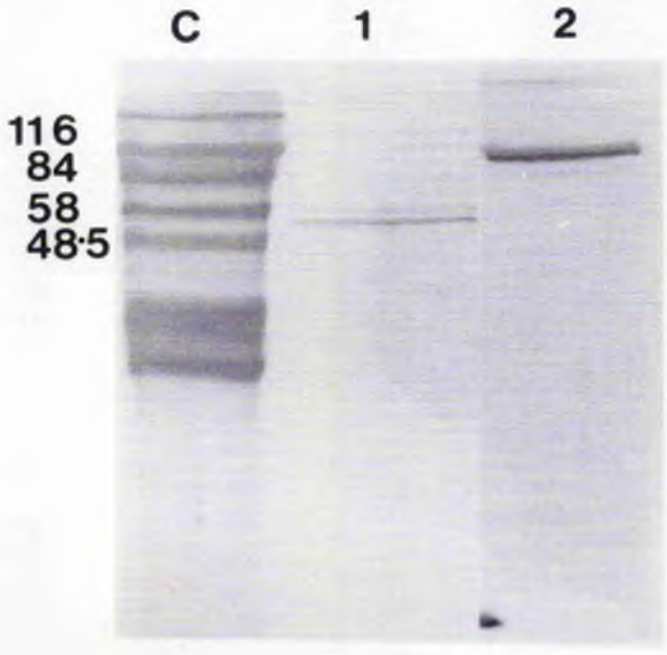
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5.IIIC). In addition, the sugar beet antibodies cross-reacted specifically with a single polypeptide (ca 52 kD) from spinach leaves and the intensity of the band on the immunoblot corresponded with enzyme activity (data not shown). These results confirm that the purified polypeptide is a subunit of alkaline invertase.

Attempts to obtain an amino acid sequence of this purified polypeptide were hindered by the blocking of the NH_2-terminus (as previously found for the purified alkaline invertase polypeptide from *V. faba* cotyledons). The small amount of the purified protein obtained was insufficient to yield significant polypeptides after proteolytic cleavage with the endoproteinase Glu-C.

8.3.2 Enzyme properties and kinetics

8.3.2.1 Alkaline invertase

Both alkaline invertases I and II showed pH optima at approximately 7.4 although the effect of pH on the two forms was not identical (Fig. 8.1B). The activity of alkaline invertase I continued to decrease after pH 7.6, whereas for alkaline invertase II, activity remained relatively constant between pH 7.6-7.9.

Standard Michaelis-Menten kinetics was observed for both forms of the enzyme with K_m values for sucrose of 56.2 ± 7.3 mM and 43.5 ± 7.7 mM for alkaline invertase I and II respectively. The corresponding values of V_{max} were 1,113.9 ± 46.6 and 455.7 ± 25.3 nmol min^{-1} mg^{-1} protein. A Michaelis-Menten plot and secondary Hanes plot, the latter drawn to confirm linearity, show the differences between the two forms (Fig. 8.2).

The test of substrate specificity showed that both forms of alkaline invertase were β-fructofuranosidases specific for sucrose. No significant hydrolysis was measured with lactose, maltose, trehalose, stachyose and raffinose.

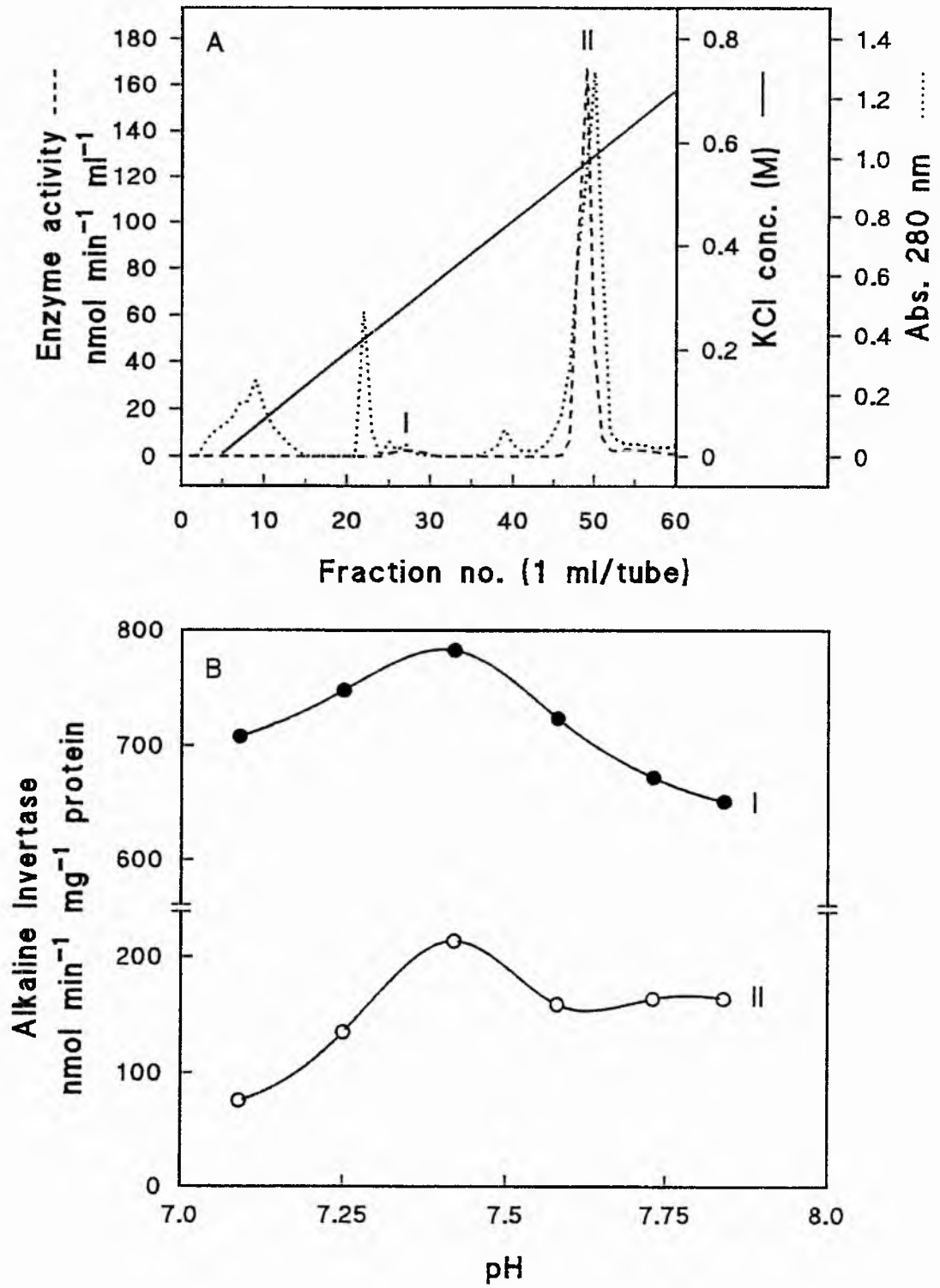

Fig. 8.1 (A) Anion-exchange chromatography (mono Q) of alkaline invertase from sugar beet taproots. (B) pH curves of alkaline invertase I and II, using 200 mM sodium phosphate buffer.

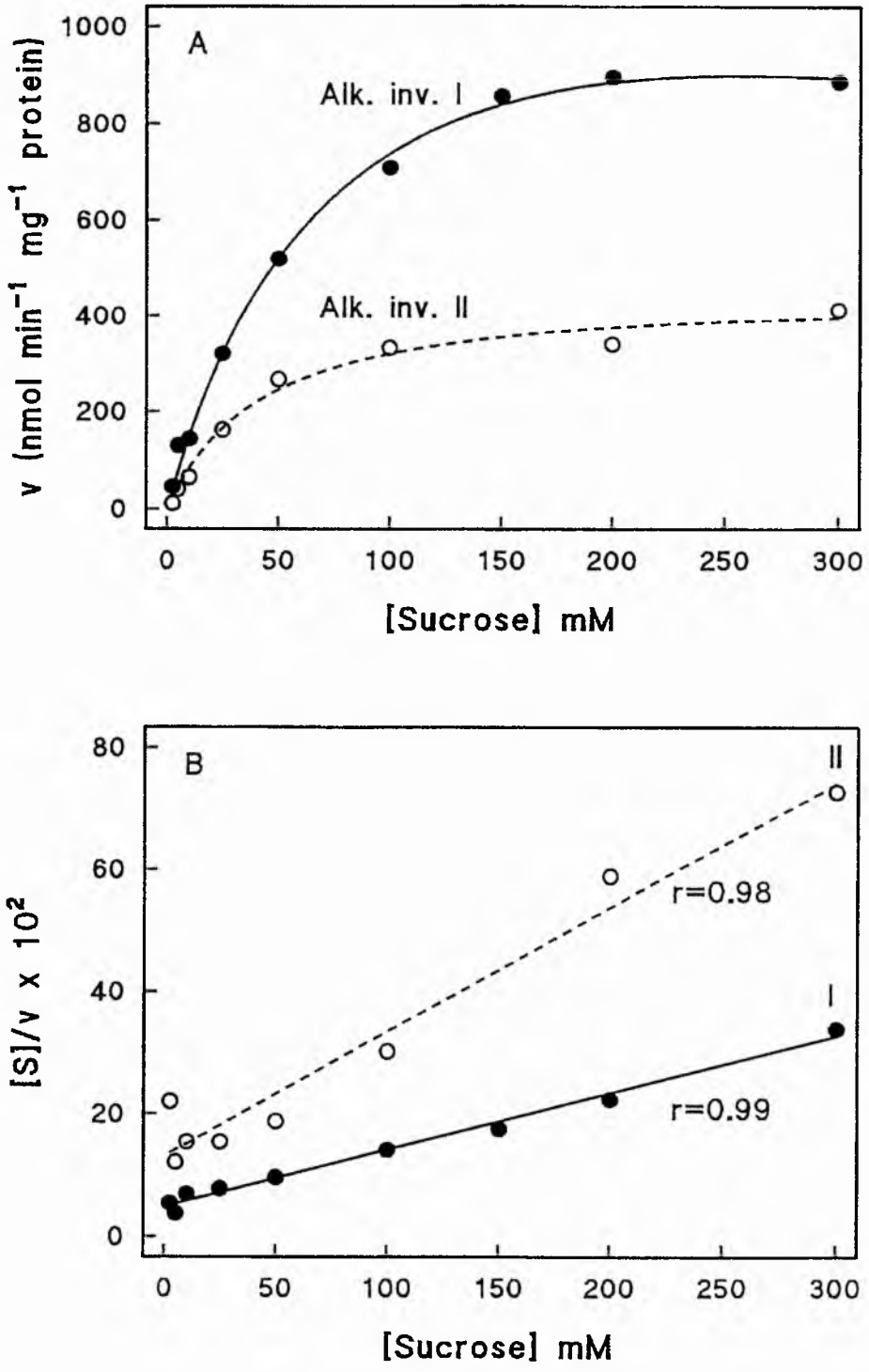

Fig. 8.2 Michaelis-Menten sucrose saturation curves (A) and derived Hanes-Woolf plots (B) of alkaline invertase I and II purified from sugar beet taproots.

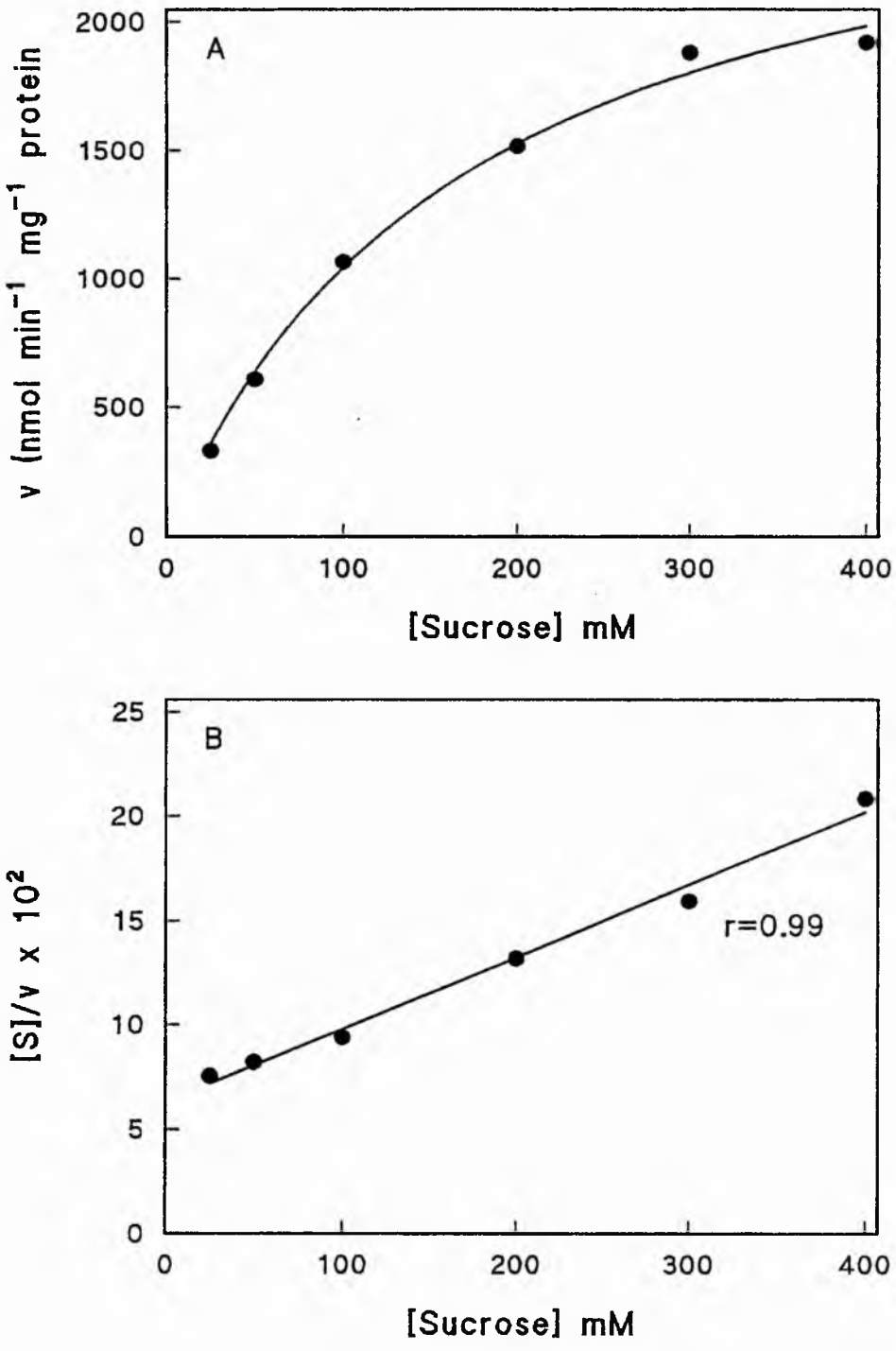

Fig. 8.3 Michaelis-Menten sucrose saturation curve with UDP (A) and derived Hanes-Woolf plot (B) of sucrose synthase purified from sugar beet taproots.

8.3.2.2 Sucrose synthase

The pH optimum of 6.7, of the partially purified sucrose synthase obtained during the purification of alkaline invertase, was determined in the cleavage direction with Tris/HCl buffer (data not shown).

The K_m value for sucrose, with UDP as the nucleoside diphosphate was estimated from the Michaelis-Menten equation to be 172.3 ± 21.1 with a V_{max} value of 2,837.8 ± 150.5 nmol min^{-1} mg^{-1} protein (Fig. 8.3). The secondary Hanes plot confirmed linearity with sucrose concentration (Fig. 8.3).

8.4 Discussion

8.4.1 Alkaline invertase

Sugar beet alkaline invertase, as found for the purified enzyme from *V. faba* cotyledons, is a homotetramer, with native molecular masses of 235 and 254 kD for alkaline invertases I and II respectively. The estimated subunit mass for both isoforms was 52 kD. The difference in mass of the native proteins for the two isoforms is not significant. Both forms of the enzyme, purified from mature sugar beet roots by Masuda *et al.* (1986), have a molecular mass of 280 kD but no subunit mass was determined in their report. However, Chen and Black (1992) have estimated that the molecular weight of purified alkaline invertase from soybean is 240,000 with a subunit weight of 58,000. Therefore, the values obtained for the sugar beet enzyme lie within the expected size range. A high degree of homology seems to exist between subunits of alkaline invertase from a number of plants. Bean alkaline invertase antibodies cross-react specifically with both isoforms of the sugar beet enzyme, while the sugar beet antibodies cross-react with bean cotyledon and spinach leaf alkaline invertases. This degree of subunit similarity, both in size and epitope

homology, was also shown for alkaline invertases from a number of plants when using the soybean antibodies (Chen and Black, 1992).

The presence of isoforms, separable by ion-exchange chromatography, confirms the occurrence of two forms of alkaline invertase in sugar beet roots as previously shown (Masuda *et al.*, 1987). Multiple forms of the enzyme from wounded root tissue of sweet potato (*Ipomoea batatas* Lam.) have also been separated by ion-exchange chromatography (Matsushita and Uritani, 1974).

The inability to obtain an amino acid sequence from both the bean (Chapter 7) and sugar beet alkaline invertases, implies that the block at the NH_2-terminus may be a feature common to plant alkaline invertase proteins. This would explain why no plant alkaline invertases, to my knowledge, have been sequenced.

The optimum pH for sugar beet alkaline invertase I and II (pH 7.4) is identical to that of the bean enzyme and falls within the expected range for plant alkaline invertases of 7.0 to 7.8 (Avigad, 1982). Both forms of the sugar beet enzyme characterised by Masuda *et al.* (1987) showed optimal pH values in the region of 8.0 while sweet potato and pea leaf alkaline invertases had pH optima of 7.6 and 7.3 respectively (Matsushita and Uritani, 1974; Storr and Hall, 1992).

Standard Michaelis-Menten hyperbolic saturation curves were obtained with both forms of the purified sugar beet enzyme. In contrast, Masuda *et al.* (1987) reported a straight line on a Lineweaver-Burk plot for alkaline invertase I but a biphasic curve for alkaline invertase II. However, examination of their data for alkaline invertase II suggest that the slight deviation from a straight line, shown for these values, is a consequence of using the double-reciprocal plot. As stressed by Cornish-Bowden (1981), this type of plot exaggerates experimental errors for small values of v and underestimates errors for large values of v. The preferred Hanes plot provides a more accurate representation of errors for all values of v. A biphasic curve was also obtained for alkaline

invertase from sweet potato but only when measured in potassium phosphate buffer and not in sodium phosphate buffer (Matsushita and Uritani, 1974). Chen and Black (1992) showed monophasic curves with purified alkaline invertase extracts, whereas with crude extracts biphasic curves were obtained. In addition, they found when combining purified alkaline invertase with partially purified acid invertase, a change from a monophasic to a biphasic curve could be obtained when the ratio of alkaline to acid invertase decreased from 2:1 to 1:2. They therefore suggest that the combination of more than one form of the enzyme or the co-existence of both acid and alkaline invertases in extracts could lead to biphasic kinetics. The calculated K_m values for alkaline invertase I and II of 56.2 and 43.5 mM respectively are similar to those found for sycamore cells - 65 mM (Huber and Akazawa, 1986), pea leaves - 42 mM (Storr and Hall, 1992), sugar beet - 33.3 mM (Masuda *et al.*, 1987) and sweet potato - 32 mM (Matsushita and Uritani, 1974). However, alkaline invertases purified from both the nodules and hypocotyls of soybean have a much lower K_m value of 10 mM (Morrell and Copeland, 1984; Chen and Black, 1992).

In general, reported alkaline invertases are highly specific for sucrose and this degree of specificity was also shown for both forms of the sugar beet alkaline invertase. This confirms that the purified enzyme is a true ß-fructofuranosidase.

8.4.2 Sucrose synthase

Partial co-purification of sugar beet sucrose synthase along with alkaline invertase provided a relatively pure extract for a very limited characterisation of the enzyme. While a small amount of sucrose synthase protein was eluted from the gel filtration column in extracts containing alkaline invertase I, the majority of sucrose synthase protein should have been eluted earlier if the

enzyme, like most other plant sucrose synthases, is a homotetramer. However, the native molecular weight was not confirmed as it was only after immunoblotting the gel that sucrose synthase was detected. Thus, only the subunit mass of 96 kD was determined. As discussed previously in section **7.4.1**, the molecular mass of native sucrose synthase proteins, characterised from a number of plants, ranges from 360 to 400 kD. The enzyme is generally considered to be a tetramer with identical subunits ranging in molecular mass from 87 to 100 kD. The subunit molecular mass of sucrose synthase purified previously from sugar beet tap roots was 95 kD (Sakalo and Lobov, 1988), the same, allowing for error, as reported in this chapter. However, the authors found that the native enzyme could exist in a number of oligomeric forms. While the tetrameric form occurred predominantly, dimeric, hexameric and octameric forms were also detected with molecular weights ranging from 160,000 to 750,000. The existence of lower molecular weight forms, would explain the co-purification of sucrose synthase and alkaline invertase by gel filtration chromatography (section **8.3.1**).

A high degree of homology exists between sucrose synthases from several plants and this was further demonstrated by specific cross-reaction of the polyclonal antibodies (raised against the sucrose synthase protein, either native or denatured, from *V. faba* cotyledons) with sugar beet sucrose synthase denatured protein on immunoblots. The ability of the bean antibodies to specifically recognise the sugar beet protein proved valuable in confirming enzyme activity during development (Chapter 6).

The affinity of the sugar beet sucrose synthase for sucrose was only tested in the presence of UDP as it was shown previously to give the highest V_{max} of any of the nucleoside diphosphates (Silvius and Snyder, 1979). The calculated K_m value of 172 mM was similar to that of the bean enzyme - 169 mM (Chapter 7). The major difference between the two enzymes is the stage at which they become saturated. For sugar beet sucrose synthase the rate of the reaction

saturates between 300 - 400 mM sucrose, whereas the bean enzyme is still not saturated at 500 mM. However, the affinity of sucrose synthase for sucrose was once again much lower than for alkaline invertase.

8.5 Conclusions

Alkaline invertase was purified to homogeneity by column chromatography and by elution from SDS-denaturing gels. Two isoforms were separated by anion-exchange chromatography. These forms, alkaline invertase I and II, were similar in size and optimum pH but had slightly different affinities for sucrose with K_m values of 56.2 and 43.5 mM respectively. The enzymes were homotetramers with subunit molecular weights of 52,000. Polyclonal antibodies raised against the purified denatured enzyme cross-reacted specifically with a single polypeptide from a crude extract from sugar beet taproots and with extracts from other plants. The enzyme was a ß-fructofuranosidase with a high specificity for sucrose.

Partial co-purification of sucrose synthase along with alkaline invertase showed the sugar beet sucrose synthase enzyme to have a subunit molecular weight of 96,000. The K_m value for sucrose was 172 mM when UDP was supplied as the nucleoside diphosphate during the determination.

While no amino acid sequence was obtained for alkaline invertase, the further production of polyclonal antibodies has proved, and will continue to prove, useful in understanding the involvement of the enzyme in sink metabolism.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

This current work, involving the comparison of sucrose breakdown in three different sink organs, has produced valuable tools with which to examine the roles played by specific sucrose-cleaving enzymes in carbohydrate metabolism. The successful purification to homogeneity of both sucrose synthase and alkaline invertases and the subsequent raising of polyclonal antibodies, particularly to alkaline invertase, will assist the cloning of corresponding genes. Their further exploitation in transgenic plants should identify the physiological importance of each of the enzymes in developing sink organs. As highlighted by ap Rees (1974), and ap Rees and Morrell (1990), reliable extraction and assay of plant enzymes is inherently difficult, re-emphasising the value of specific antibodies to enable the confirmation of the enzyme measurements made in this work.

9.1 The role of acid invertase

High acid invertase activity was associated with potato stolons, potato tuberisation, elongating potato sprouts and young developing sugar beet taproots. In contrast, low activity was found in developing bean cotyledons and in older, but growing and developing sugar beet taproots and potato tubers. The consensus of opinion (Morris and Arthur, 1984) is that acid invertase functions to supply the rapidly expanding and dividing cells of meristematic tissues with hexoses, as discussed in Chapter 1. However, the fact that extremely low acid invertase activity is associated with developing tubers

and for a significant period of sugar beet taproot development indicates that such statements must be viewed with care. Cell division and cell expansion in potato tubers continue throughout the period of tuber growth and both the number of tuber cells and cell volume exhibit an exponential function of the form:

$$y = a.x^b$$

where y represents either number of cells or cell volume and x the tuber weight (Conghua, 1989). Likewise, in sugar beet taproots both cell division and cell expansion continue until maturity (Milford, 1973). Conversely, acid invertase activity increases in stored, mature tubers to significant levels - in a tissue where cell growth has ceased. Similarly, whilst a good inverse correlation between sucrose and acid invertase levels in the vacuole has been shown (Leigh *et al.*, 1979), substantial increases in sucrose in cold-stored tubers can occur alongside an increase in acid invertase activity. This may or may not be related to sucrose compartmentation, as discussed in Chapter 4.

Acid invertase activity may be regulated *in planta* by its end products: glucose and fructose. Potato tuber acid invertase is non-competitively inhibited by glucose, but competitively inhibited by fructose (Isla *et al.*, 1991; Burch *et al.*, 1992). The inhibition by fructose is thought to occur through two interacting sites on the enzyme and proteins were unable to suppress the inhibitory effect produced by either fructose or glucose (Isla *et al.*, 1991). During the rapid decrease in acid invertase activity in tuberising potato stolons the approximate concentrations of glucose and fructose were 58 mM and 23 mM respectively (Chapter 3). These levels decreased during tuber development to reach concentrations of 46 mM glucose and 5 mM fructose in small tubers (5 g FW). At glucose and fructose concentrations of 25 mM, partially purified tuber invertase was inhibited ca 20% and 25% by the two hexoses, respectively

(L. Burch, pers. comm.). Hence, glucose and fructose levels *in vivo* may contribute to invertase regulation, although it is always difficult to extrapolate from *in vitro* measurements. In stored mature tubers (Chapter 4), however, when acid invertase activity is increasing the corresponding levels of glucose and fructose are as high or higher than in the young developing tubers in which acid invertase activity is decreasing. Hence the extent to which acid invertase activity may be regulated by end product inhibition *in vivo* is uncertain.

A second hypothesis to explain the control of acid invertase *in planta* is the presence of an invertase inhibitor protein. The initial indication of an endogenous invertase inhibitor in stored potato tubers (Schwimmer *et al.*, 1961) was followed by the identification and partial purification of a small molecular weight protein which inhibits potato tuber invertase non-competitively (Pressey, 1966). During measurements of acid invertase in stored potato tubers, Pressey (1966) obtained maximum activity when extracts were prepared by prolonged high speed blending at room temperature, suggesting that the inhibitor can be denatured without inactivation of the enzyme. When the effect of alternating potato storage temperatures on tuber invertase activity was studied, Pressey and Shaw (1966) observed a pattern of low inhibitor levels with high acid invertase activity and conversely high inhibitor levels with low enzyme activity. Further characterisation of the inhibitor protein confirmed its molecular weight at ca 17,000 and showed that it could effectively inhibit not only potato tuber invertase but also many other plant invertases (Pressey, 1967). The inhibitor forms an essentially undissociable complex with the enzyme which is noncompetitive with substrate and dependent on temperature with respect to rate of complex formation (Ewing and McAdoo, 1971). Investigation of invertase activity in potato tuber disks showed that 2 days after slicing, the inhibitor level decreased while enzyme activity increased

(Ewing *et al.*, 1977). This increase in enzyme activity was not caused by decreased susceptibility of the enzyme to the inhibitor but due to an increase in the first peak of invertase activity eluted from a DEAE-cellulose column. Both the loss of inhibitor protein and the increase in invertase activity could be completely blocked by cycloheximide. In addition, invertase activity of leachates from potato disks was much closer to values of the unfoamed extracts than to foamed extracts indicating that the inhibitor is operating in the whole slice and it is not an artifact of extraction (Ewing *et al.*, 1977).

While most of the early invertase inhibitor studies have concentrated on potato tuber invertase, similar small molecular weight protein inhibitors have been partially purified from red beets, sugar beets and sweet potatoes (Pressey, 1968). The sugar beet inhibitor (MW - 18,100) inhibited potato tuber invertase 90% and, like the potato inhibitor, was most effective at pH 4.5. Sugar beet acid invertase activity increased and inhibitor levels decreased in aged sugar beet slices in a similar manner to potato tuber disks (Burakhanova *et al.*, 1987). Increased invertase activity in both potato tuber disks and sugar beet slices are considered to be due to both loss of inhibitor protein and to *de novo* synthesis of invertase protein (Ewing *et al.*, 1977; Burakhanova, 1987). The increase in inhibitor levels in developing sugar beet taproots over the period of sucrose accumulation when acid invertase activity is low indicates that the inhibitor may exert a degree of control *in vivo* (Burakhanova *et al.*, 1987). While the above studies support involvement of the inhibitor in regulating acid invertase activity, the general consensus of opinion is that the inhibitor is not the sole controlling factor. This hypothesis is further confirmed by the data presented in Chapter 3, where a decline in acid invertase activity is observed prior to any indication of the presence of an inhibitor as defined by activity before and after foaming of extracts. Similarly no inhibitor was detected in stolons from potato

plants (*Solanum demissum*) grown under both long and short day conditions or in small tubers from *S. demissum* (Helder, 1994).

In older tubers foaming treatments designed to selectively denature the inhibitor whilst it is asssociated with the enzyme results in increased invertase activity although the extent of the increase is variable and can be dependent on genotype and tuber age (Chapter 4). Similarly, Helder (1994) showed that in both one and two year-old potato tubers, cv. Bintje, acid invertase activity increased two-fold in foamed extracts but no significant increase occurred in extracts from freshly-harvested tubers. Given the fact that Isla *et al.* (1992) found that the inhibitor was located in the cell-wall fraction, spatially separated from the enzyme in the vacuole, the role of the inhibitor in regulating acid invertase is very much open to question. Recently, a highly purified preparation of potato invertase inhibitor has been obtained which yields single bands on both SDS-PAGE and IEF-PAGE (Ovalle *et al.*, 1993). The purified active inhibitor protein, with pI 5.1, is currently being used to raise antibodies and should help to resolve the present uncertainty surrounding endogenous invertase inhibitors.

Potato tubers undergo a clear and significant switch in the pathway of sucrose cleavage during their various stages of development. Principally, a substantial decline in the role of acid invertase occurs during tuber formation phasing into a key role for the enzyme in mature, stored and sprouting tubers (Chapters 3 and 4; Morrell, 1984; Pressey, 1969). Commercially, the enzyme is believed to play a major role in modifying the processing potential of tubers used in the snack food industry (Davies and Viola, 1992). At the high processing temperatures prevailing during the production of crisps and French fries, the hexoses interact with ∝-amino groups of nitrogenous compounds in a Maillard reaction resulting in darker, off-flavoured products. If the hexoses are derived from sucrose directly then downregulating invertase activity should have

significant commercial benefits. Certainly, genotypes which accumulate low levels of sugars in storage have low invertase activity (Chapter 4; Richardson *et al.* 1990). Furthermore, recent data confirming that antisensing vacuolar acid invertase inhibits hexose accumulation in cold-stored potato tubers by ca 60-70% adds weight to this argument (Zrenner and Sonnewald, 1993). In terms of the complement of acid invertase genes, in potato five have been cloned so far. Two genomic clones (GE and GF) and two leaf cDNA clones (CD111 and CD141) have been isolated at the SCRI (Hedley *et al.*, 1994; P. Hedley, pers. comm.). The genomic clones show 80% nucleotide sequence identity and, from their sequences, all apparently encode apoplastic invertases. Recently, a vacuolar invertase gene has been cloned by Zrenner and Sonnewald (1993) and the same gene has now been isolated at the SCRI. Homologies between the potato leaf cDNA clones and apoplastic and vacuolar invertase genes from other species are shown in Fig. 9.1. Clearly there are significant homologies between all plant invertases isolated so far. However, there is good evidence that at least some of the potato genes are differentially expressed. CD111, for example, is expressed in sink leaves and CD141 in source leaves (Hedley *et al.*, 1994). Promoter analysis will be required to identify the factors causing a switch in their expression during leaf maturation. Physiologically, apoplastic invertases have been identified in potato stolons, leaves, stems and roots (L. Burch, pers comm.; Helder, 1994). Apoplastic invertase is apparently absent from developing and stored tubers (Chapters 3 and 4), adding weight to the argument that phloem unloading is symplastic (see Chapter 1) and that sucrose metabolism is driven predominantly by sucrose synthase.

Acid invertase has received considerable attention in another Solanaceous species - tomato. A soluble vacuolar invertase protein with a molecular mass of 52 kD has been purified to homogeneity (Konno *et al.*, 1993) and in addition a number of tomato vacuolar genes have been cloned and sequenced (Klann *et*

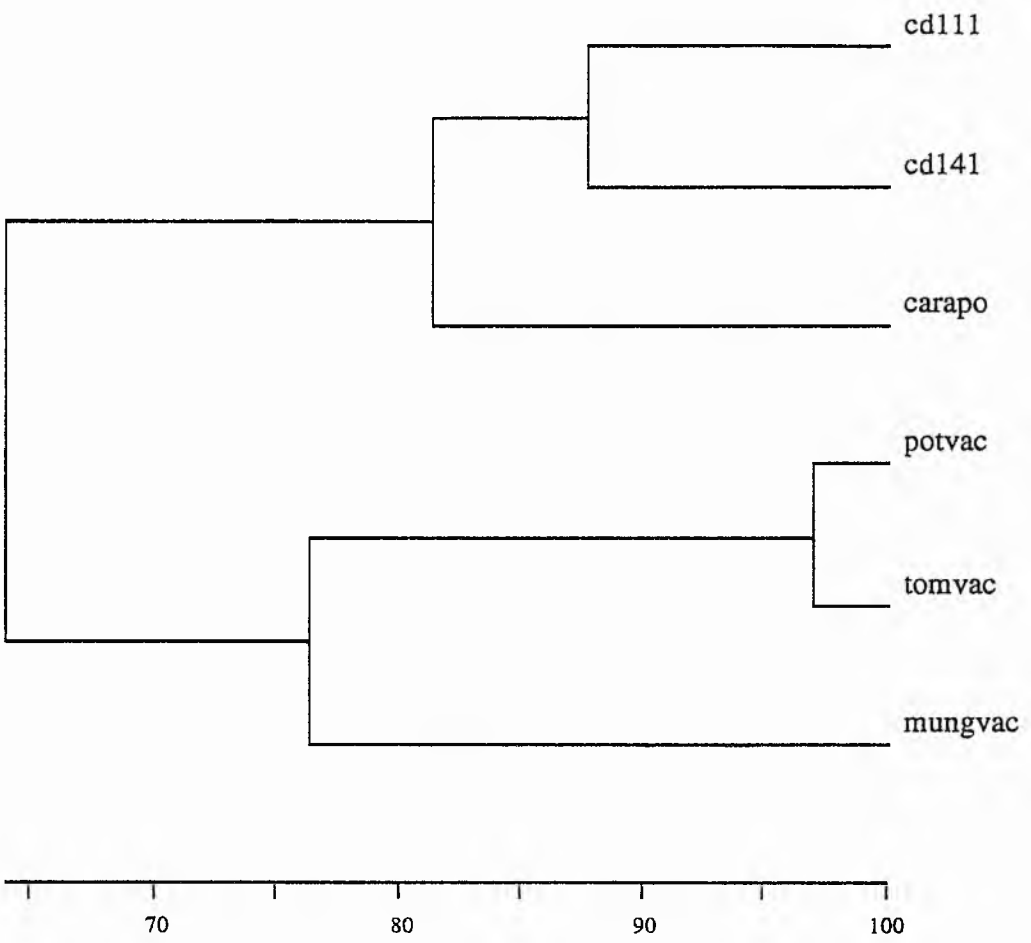

Fig. 9.1 Dendogram representing pairwise comparisons of the deduced amino acid sequence of pCD141 and those of other plant invertases. cd111 - potato apoplastic (Hedley *et al.*, 1993); carapo - carrot apoplastic (Sturm and Chrispeels, 1990); potvac - potato vacuolar (Sonnewald, pers. comm. to H. V. Davies); tomvac - tomato vacuolar (Klann *et al.*, 1992; Elliott *et al.*, 1993); mungvac - mung bean vacuolar (Arai *et al.*, 1992). Scale represents percentage amino acid similarity. (After Hedley *et al.*, 1994).

al., 1992; Elliot *et al.*, 1993). These vacuolar genes have high homology with the potato vacuolar invertase gene (Fig. 9.1). In studying the expression of these genes a comparison was made between the wild tomato species (*Lycopersicon chmielewskii*) which accumulates sucrose in its fruit and the domestic species (*Lycopersicon esculentum*) which accumulates the hexoses, glucose and fructose (Klann *et al.*, 1993). The trait of sucrose accumulation in *L. chmielewskii* is thought to be controlled by a single recessive gene (Yelle *et al.*, 1991). This trait has now been mapped to a location near the centromere of chromosome 3 and the evidence strongly suggests that it is an allele of the invertase gene (Chetalat *et al.*, 1993). Further biochemical studies showing both the absence of invertase activity and invertase mRNA transcripts in sucrose-accumulating fruits has confirmed that the acid invertase gene is not expressed in the wild species (Klann *et al.*, 1993).

In sugar beet taproots during sucrose accumulation, acid invertase activity decreases to very low levels and is always lower, both during development and post-harvest storage, than alkaline invertase or sucrose synthase (Chapter 6). This suggests that acid invertase is regulated by repression of synthesis of invertase protein and/or by the action on invertase of a specific inhibitor protein. While no definitive conclusions have been reached on its control, Burakhanova *et al.* (1987) has shown that the endogenous invertase inhibitor increases during sugar beet development and acts to suppress acid invertase activity during the period of high sucrose accumulation, post-harvest storage and into the second year of regrowth. This would agree with the data presented in this thesis (Chapter 6) but unlike the potato data, no increases in acid invertase activity resulted from foaming crude sugar beet extracts. Hence, in this study no direct indication of inhibitor levels was obtained. Acid invertase activity increases rapidly in washed and aged slices of sugar beet taproots (Éngel and Kholodova, 1970; Burakhanova *et al.*, 1987) and may

possibly contribute to sucrose breakdown in wounded taproots during storage. This latter hypothesis needs testing particularly as sucrose losses may be experienced by the processors.

In a parallel sucrose-storer, sugarcane, acid invertase is considered to be the principal enzyme responsible for sucrose inversion in harvested sugarcane and in juice (Batta *et al.*, 1991). A soluble acid invertase has been partially purified and characterised and the pH of sugarcane juice ca 5.0-5.5 is close to the optimum pH of the enzyme. During development, soluble acid invertase is only active during the stage of rapid stem elongation and activity is not detected after cessation of internode growth (Gayler and Glasziou, 1972). The factors regulating acid invertase levels within this sucrose-storing organ are unknown and, unlike sugar beet, no endogenous inhibitors have been reported.

9.2 The role of alkaline invertase

The purification of alkaline invertases from both bean cotyledons (Chapter 7) and sugar beet taproots (Chapter 8) has provided valuable information on the properties of a relatively uncharacterised enzyme. By the use of the polyclonal antibodies, the presence of alkaline invertase in each of the sink organs has been confirmed. The highest activity occurred in developing bean cotyledons in which the level was ca 3-fold higher than in either developing potato tubers or in sugar beet taproots. In all three sink organs, however, the pattern of enzyme expression is different. Firstly, in developing potato tubers alkaline invertase activity decreases as sucrose accumulates and sucrose synthase activity increases. A significant negative correlation between potato alkaline invertase activity and sucrose content was observed over the early stages of tuber development ($r = -0.99$). In contrast, alkaline invertase activity in bean

cotyledons appeared unrelated to sucrose content, increasing when the sucrose content was at its highest and reaching a peak in activity when sucrose had decreased to its lowest level. The peak in bean alkaline invertase activity also coincided with a significant decrease in sucrose synthase activity. Finally, in sugar beet taproots, the pattern of alkaline invertase and sucrose synthase were very similar. While the activity increased over the early stages of development, the later decrease occurred over a period of active sucrose accumulation by the sugar beet taproots.

In each of these sink organs, alkaline invertase is not acting as a typical maintenance-type enzyme as defined by Sung *et al.* (1989) and Xu *et al.* (1989). They envisage very small and slow changes in enzyme activity in response to changing conditions. In stolon tips during tuberisation, alkaline invertase activity decreases over 3-fold before reaching a low level in small tubers. At later stages of tuber development, no detectable alkaline invertase is present. This indicates that the enzyme may have a specific function in providing a ready supply of hexoses within the cytosol at certain stages of sink development - in this case prior to and during the onset of tuberisation. Thereafter, it would appear to act as a maintenance enzyme with very low activity during tuber growth. Alkaline invertase activity increases ca 15-fold (activity calculated on a FW basis) and >30-fold (activity calculated on a cotyledon basis) during faba bean development and it is active at a time of low sucrose synthase activity. These results differ substantially from those reported for developing lima bean (*Phaseolus lunatus*) seeds which showed very low activity throughout seed development (Xu *et al.*, 1989). This discrepancy may be due to the difficulty in determining maximum catalytic activity, which was only obtained for the faba bean enzyme after testing a range of buffers and including NaCl during extraction. The simultaneous increase in faba bean glucokinase with alkaline invertase, both reaching a maximum during the later

stages of development when sucrose levels are decreasing, indicates that the invertase enzyme is fulfilling a specific requirement of the cells during that period. Both the magnitude of the increase in alkaline invertase activity and the definite pattern in enzyme protein levels confirmed by immunoblotting are atypical of a maintenance enzyme. In sugar beet taproots the concomitant increase of alkaline invertase activity with sucrose synthase is difficult to reconcile with the role of the enzyme as one of solely catalysing an alternative pathway of sucrose breakdown. While Masuda *et al.* (1987) showed a parallel increase of sucrose with alkaline invertase activity in sugar beet taproots, in this present study enzyme activity decreased while sucrose was still accumulating. Thus their hypothesis of a role for alkaline invertase in regulating sucrose accumulation within taproots is not supported in this present study. The comparison of the three different sets of conditions under which alkaline invertase is active do not immediately highlight a common function for the enzyme nor how it may be regulated in sink organs. To identify what its role may be, one has to envisage a specific requirement for a ready supply of cytosolic glucose or rather glucose phosphates as any free glucose is likely to be rapidly phosphorylated.

The characterisation of the purified alkaline invertases as β-fructofuranosidases, highly specific for sucrose and subject to product inhibition, tends to add support to the notion of the enzyme having a definite function in the metabolism of sink cells. However, identification of this function(s) is only likely to be solved by the availability of mutants or by comparing the metabolism of transgenic plants in which the alkaline invertase gene(s) has been differentially expressed. One of the points raised by the use of mutants and transgenic plants is that for many key reactions, more than one enzyme exists which can catalyse that step: e.g. PFP and PFK (Black *et al.*, 1987); sucrose synthase and alkaline invertase (Dancer and ap Rees, 1989); and

pyruvate kinase and PEP phosphatase (Duff *et al.*, 1989). Furthermore, many enzymes are present in excess: e.g. downregulating the cytosolic form of pyruvate kinase by 100% has little or no effect on plant phenotype or metabolism (Gottlob-McHugh *et al.*, 1992). Similarly, when PFP expression is decreased up to 90% in transformed potato plants, no significant changes were observed (Hajirezaei *et al.*, 1994). In this respect the effect of downregulating alkaline invertase cannot be predicted. During endosperm development of the *shrunken* maize mutant, while sucrose synthase activity is less than half that found in the normal wild type endosperm, alkaline invertase increases to compensate (Dancer and ap Rees, 1989). These examples illustrate the flexibility of plant metabolism and the problems encountered in trying to gain an insight into the roles of specific enzymes.

9.3 The role of sucrose synthase

The highest sucrose synthase activity in the developing sink organs occurred in the bean cotyledons in which levels of >4,000 nmol min^{-1} g^{-1}FW were determined. The enzyme level was only slightly lower (ca 3,700 nmol min^{-1} g^{-1}FW) in young potato tubers but over 3-fold lower in sugar beet taproots. This pattern of activity, very high in starch-storing sink organs but lower in the predominantly sucrose-storing organ, emphasises the importance of the enzyme in catalysing the initial step of sucrose breakdown *en route* to starch biosynthesis. Thus over the early stages of tuber and bean development, with the onset of starch accumulation, there is a rapid rise in sucrose synthase activity. As shown for potato tubers (Chapter 4), the enzyme activity and protein levels are controlled by the flux of sucrose to the tuber cells. From the close similarity in the pattern of sucrose content and enzyme activity in the

developing bean cotyledons, this mechanism of regulation may be associated with most starch-storing sink organs. This is further supported by the general observations that sucrose synthase activity decreases in mature and senescing plants and high levels of activity only occur in young importing sink organs (Xu *et al.*, 1989). Sucrose breakdown within a sink organ is considered to be a biochemical determinant of the sink strength of that organ with sucrose synthase acting as a reliable indicator over the period of maximum growth (Sung *et al.*, 1989; Sun *et al.*, 1992). The evidence in Chapters 3 and 4 supports the theory that sucrose synthase regulates sucrose breakdown in young developing sinks but also confirms the regulation of the enzyme itself, by sucrose. Hence it is unlikely that the activity of this enzyme completely controls the amount of assimilate imported to individual sinks.

In both the starch-storing sink organs, bean cotyledons and potato tubers, sucrose synthase is considered to be operating predominantly in the direction of cleavage to provide intermediates for rapid starch synthesis. While the level of glucokinase remained relatively low in these tissues, fructokinase increased concomitantly with sucrose synthase. These results further confirm the action of sucrose synthase in the cleavage of sucrose in these organs, as fructokinase is required to phosphorylate the fructose produced and prevent end-product inhibition (see Chapter 7).

In the sugar beet taproots, however, the level of starch is very low (ca 30 to 150-fold lower than in potato tubers and bean cotyledons) and both sucrose synthase and alkaline invertase activities increase while starch levels decrease. This pattern of sucrose synthase activity would indicate its participation in sucrose synthesis as outlined by Batta and Singh, (1986) for sugar cane although estimation of the mass-action ratio would be required to confirm the reversibility of the sugar beet enzyme *in vivo*. Determining the relative contribution of sucrose synthase to sucrose synthesis and breakdown in sugar

beet taproots is of vital importance in targeting the enzymes involved in sucrose losses in stored taproots.

Over recent years, there has been a rapid increase in techniques available for allowing the production of transgenic plants exhibiting differential expression of selected genes. Recent results obtained from transgenic potato plants having reduced activities of sucrose synthase and acid invertase (Zrenner and Sonnewald, 1993) confirmed the results obtained in this present study (see Chapter 4). The main aims of future work as a direct result of this thesis are to clone the gene(s) encoding alkaline invertase, to produce transgenic plants in which the enzyme has been over- or under-expressed and identify how this may affect metabolic fluxes during the growth and development of the sink organ.

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Sucrose Metabolism in Tubers of Potato (*Solanum tuberosum* L.)

Effects of Sink Removal and Sucrose Flux on Sucrose-Degrading Enzymes

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ABSTRACT

Excision of developing potato (*Solanum tuberosum* L.) tubers from the mother plant, followed by storage at 10°C, resulted in a rapid, substantial decrease in sucrose synthase activity and considerable increases in hexose content and acid invertase activity. A comparison of the response of three genotypes, known to accumulate different quantities of hexoses in storage, showed that both sucrose synthase activity and the extent to which activity declined following excision were similar in all cases. However, there was significant genotypic variation in the extent to which acid invertase activity developed, with tubers accumulating the highest hexose content also developing the highest extractable activity of invertase. Similar effects were found in nondetached tubers when growing plants were maintained in total darkness for a prolonged period. Furthermore, supplying sucrose to detached tubers through the cut stolon surface prevented the decline in sucrose synthase activity. Maltose proved to be ineffective. Western blots using antibodies raised against maize sucrose synthase showed that the decline in sucrose synthase activity was associated with the loss of protein rather than the effect of endogenous inhibitors. Although there were indications that maintaining a flux of sucrose into isolated tubers could prevent the increase in acid invertase activity, the results were not conclusive.

Sucrose cleavage is catalyzed either by sucrose synthase (UDP-glucose: D-fructose-2-glucosyl transferase, EC 2.4.1.13) or by invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26). Sucrose synthase is a cytosolic enzyme (8), whereas invertase can be located in the cell wall, vacuole, or cytosol (9, 11). Neutral or alkaline invertases are generally believed to be cytosolic enzymes (12), although there are reports of association with the cell wall (3).

In starch-storing sinks such as potato tubers, in-coming sucrose is degraded predominantly via the sucrose synthase pathway (15, 24). An important feature of the sucrose synthase reaction is the conservation of the energy in the glycosidic bond of sucrose, thus making it more efficient, energetically, than the invertase pathway (5, 27). Sucrose synthase activity declines as tubers mature on the plant (19) and in mature, stored tubers, acid invertase activity predominates (20). In a previous article, it was reported that the decline in sucrose synthase can be accelerated by excising the tuber from the mother plant (18), although the effects on sugar balance and of tuber maturity in response to excision were not addressed.

In the present study, we examine in detail the consequence of excising tubers at various stages of development on the change in sugar balance and the activities of sucrose-cleaving enzymes during a subsequent storage period at 10°C. The effect of genotype on the response is also reported. This complements work published previously on the influence of genotype on sugar accumulation in tubers stored at low temperature (21) and on sugar metabolism in developing tubers (15, 23). Furthermore, whereas Claussen *et al.* (2) suggested that sucrose regulates sucrose synthase activity, this hypothesis has not been adequately tested for starch-storing sink tissues such as potato tubers. The present study addresses the question of regulation of enzyme levels by sucrose (assimilate) flux.

MATERIALS AND METHODS

Tuber Excision and Postharvest Storage Experiments

Potatoes (*Solanum tuberosum* L.) cvs Cara, Record, and Brodick, were grown in the field at the Scottish Crop Research Institute as described previously (21). Four sequential harvests were taken between August 1 and September 26 to provide tubers of varying maturity. Mean individual tuber weights were about 70 g fresh weight at the first harvest and 125 g fresh weight at the end of September. After tuber excision, five tubers of each genotype were selected at random for immediate analysis (within 2 h). The remainder were stored at 10 ± 1°C and analyzed at regular intervals over a period of up to 18 d.

Exogenous Application of Sugars to Intact, Detached Tubers

Developing tubers with a mean fresh weight of 40 g (±10%) were excised at the point of attachment of the stolon with the stem. The stolons were then immersed in distilled water or in solutions of either sucrose or maltose (10 individual tubers per treatment). To determine the rate of carbohydrate flux into the tubers, solutions were spiked with either [*U*-^{14}C]-D-

maltose (specific activity of stock isotope, 15.5 GBq $mmol^{-1}$) or [U-^{14}C]sucrose (specific activity of stock, 13.4 GBq $mmol^{-1}$). The concentration of isotope for all solutions was 37 KBq ml^{-1}.

Tuber Analysis

The majority of analyses were carried out on material frozen in liquid N_2 and stored at −80°C. Frozen samples were lyophilized and finely ground before carbohydrate extractions and ^{14}C analyses. Enzyme extracts were prepared from fresh tissue. Three adjacent longitudinal slices (0.5-cm-thick) were taken from each tuber to provide sufficient material for analyses. This allowed a direct comparison of data on carbohydrate balance, enzyme activities, etc.

Determination of Sucrose, Glucose, and Fructose

Lyophilized and finely ground samples were extracted in 80% ethanol at 70°C and the concentration of glucose, fructose, and sucrose determined using enzyme-coupled reaction systems (21). In ^{14}C experiments, ethanol extracts were prepared as above and total ^{14}C in the soluble fraction determined. The remaining tissue pellet was gelatinized, digested with amyloglucosidase (25), the entire sample shaken thoroughly, and several aliquots removed for scintillation spectrometry (insoluble fraction). The ethanol extracts were freeze-dried and the distribution of label between glucose, fructose, sucrose, and maltose determined by HPLC. Sugars were separated on a 15-cm reverse-phase amino column using acetonitrile:H_2O (85:15 v/v) as the mobile phase (flow rate, 2 mL min^{-1}). Radioactivity in sugar peaks (refractive index detection) was measured by liquid scintillation counting. Preliminary studies with ^{14}C sugars showed that less than 2% of maltose or sucrose added to tuber tissue before extraction was degraded as a result of the procedures used.

Enzyme Determinations

Samples of fresh potato tissue were extracted with insoluble PVP (1% w/v) and acid-washed sand in 3 volumes of extraction solution. For sucrose synthase, the extraction solution contained 100 mM Tris-HCl (pH 7.5), 5 mM DTT, 3 mM magnesium acetate, and 2% (w/v) glycerol. For invertase determination, tissue was extracted in 100 mM acetate buffer (pH 5.0) containing 10 mM sodium sulfite. After centrifugation at 20,000g for 20 min at 5°C, extracts were dialyzed for 18 h at 3°C against 10 mM extraction buffer. Recoveries of sucrose synthase and invertase activities were unaffected by choice of de-salting method (dialysis or rapid de-salting with Sephadex G-25M [PD10; Pharmacia, United Kingdom]). Due to the number of samples processed, dialysis was chosen for convenience.

Invertase activity before (basal) and after (total) destroying the endogenous invertase inhibitor by extensive foaming was determined as described previously (21). Essentially, total invertase was measured after foaming de-salted extracts by vortexing for 90 min in test tubes fixed to a flask shaker operating at maximum speed. The recovery of yeast invertase added to tuber extracts and taken through the entire process was 82% for foamed extracts and 90% for nonfoamed extracts. The activity of yeast invertase added was approximately equal to the activity of tuber invertase. Both developing tubers detached from the mother plant for 12 d (high invertase samples) and tubers detached for only 5 min (low invertase samples) were used for recovery experiments. Results indicated no effect of tuber treatment on the recoveries of invertase activity. Invertase activity was assayed under optimum pH and substrate conditions in 0.1 M acetate buffer, pH 5, containing 250 mM sucrose. These conditions were optimal for both developing and stored tubers. Numerous pH curves were produced from the various treatments imposed. These revealed no specific alkaline invertase. Throughout the text, any reference to tuber invertase therefore refers to acid invertase only. Sucrose synthase was assayed in the cleavage direction under optimized pH (7.2) and sucrose (200 mM) and UDP (1 mM) concentrations. The method used was essentially that of Xu *et al.* (27). When developing cotyledons of bean (*Vicia faba L.*) were extracted together with tuber tissue, the recovery of sucrose synthase was 95% of that expected from independent extractions of tuber and bean material. It is concluded that no major losses of tuber sucrose synthase activity occurred during the extraction procedure.

Protein Determination, Electrophoresis, and Immunoblotting

Protein in enzyme extracts was quantified using the Bio-Rad protein assay with BSA as a standard. Approximately 10 μg of soluble protein extracted with the sucrose synthase extraction medium was subjected to SDS-PAGE (7.5% acrylamide) as described by Laemmeli (10) and polypeptides stained with Coomassie blue. In parallel gels, polypeptides were transferred to nitrocellulose and, after immunoblotting with polyclonal antibodies raised to maize sucrose synthase, cross-reacting bands were identified using anti-rabbit immunoglobulin conjugate labeled with alkaline phosphatase. The sucrose synthase antibody was raised against protein extracted from whole kernels of wild type maize and kindly supplied by Dr. Karen Koch, University of Florida.

RESULTS AND DISCUSSION

Sugar Balance and Enzyme Activities in Tubers after Detachment

Tubers from field-grown plants of cvs Cara, Record, and Brodick were excised on four occasions during the growing period to provide samples differing in physiological status and chronological age. Genotypic variation in tuber sugar balance, particularly during the postharvest period, is well known. The cvs used were chosen because of their differential accumulation of hexoses in storage (21). The hexose (glucose + fructose) and sucrose content of tubers after detachment and during a subsequent storage period at 10°C is shown in Figure 1. Data for harvests 1 and 4 only are presented. Similar patterns were observed with harvests 2 and 3, but the data were omitted for clarity. At each harvest date and for each genotype, the tuber hexose content was low at excision but increased substantially within the first few days in storage. As predicted, the three genotypes showed consistent differences in the rate and extent

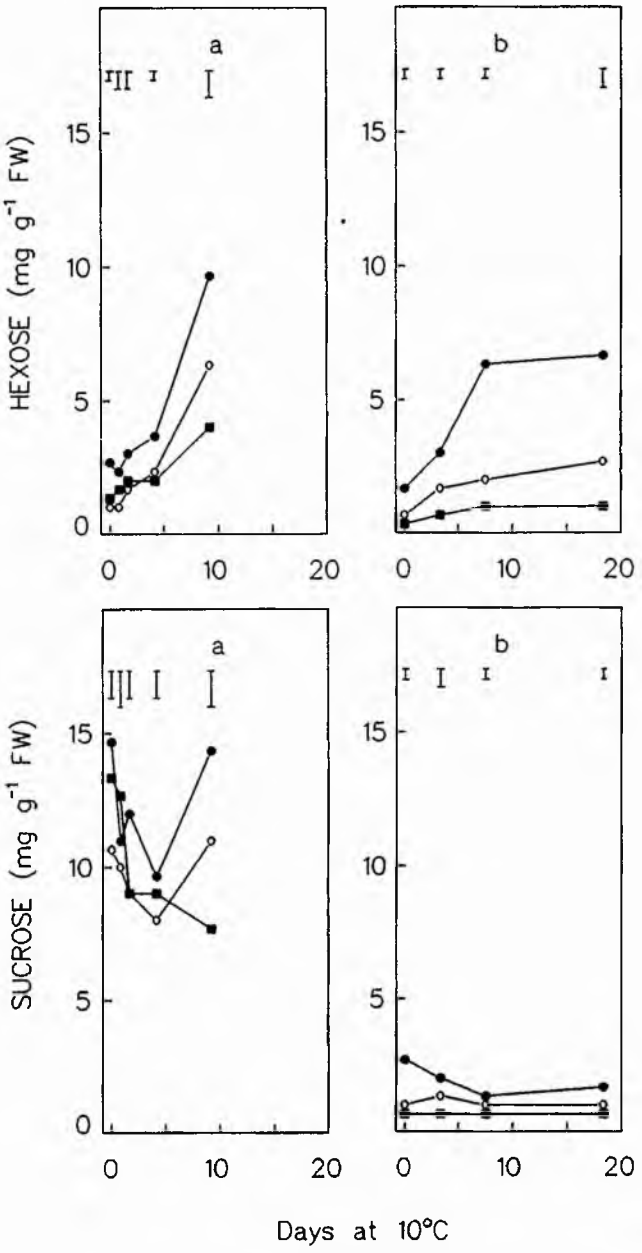

Figure 1. Effect of excising developing tubers from the mother plant on tuber hexose and sucrose content in storage at 10°C. Tubers were excised from growing plants either on August 1 (a) or September 26 (b). The cv used were Cara (●), Record (○), and Brodick (■). Bars indicate SE of the mean.

of hexose accumulation, with cv Cara accumulating the highest concentration and cv Brodick the lowest. Changes in sucrose content after excision were far more variable with no consistent differences between genotypes. The fact that a decline in sucrose content did not always accompany hexose accumulation implies that the sucrose pool may be replenished as a result of starch breakdown (6). The loss of starch required to deliver the observed increase in hexoses is not measurable with any accuracy (70% of tuber dry matter is starch, but less than 1–2% is soluble sugar).

Tuber excision also resulted in a rapid and substantial increase in acid invertase activity at each stage of tuber development examined. This was evident when both total and basal activities were plotted (Fig. 2). Again, data for harvests 2 and 3 are omitted for clarity. In some cases, a 10-fold increase in activity occurred within 3 d after excision. In general, invertase activity in the high sugar accumulating genotype Cara was significantly and consistently higher than in the lowest sugar accumulator, Brodick. Often, the most substantial difference in invertase activity between genotypes

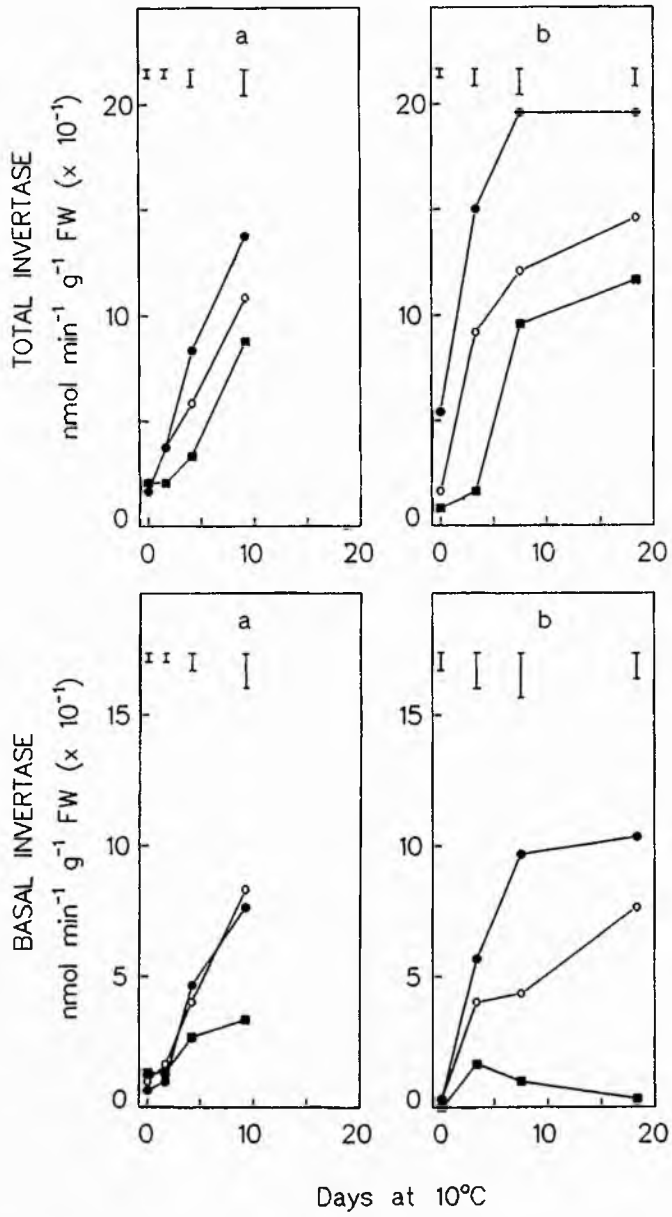

Figure 2. Effect of tuber excision on the development of total and basal acid invertase activity in storage at 10°C. Basal activity was determined in the presence of invertase inhibitor protein. Total activity, in the same extract, was determined after the destruction of inhibitor by rapid vortexing. Letters and symbols used to identify excision dates and potato genotypes are as in Figure 1.

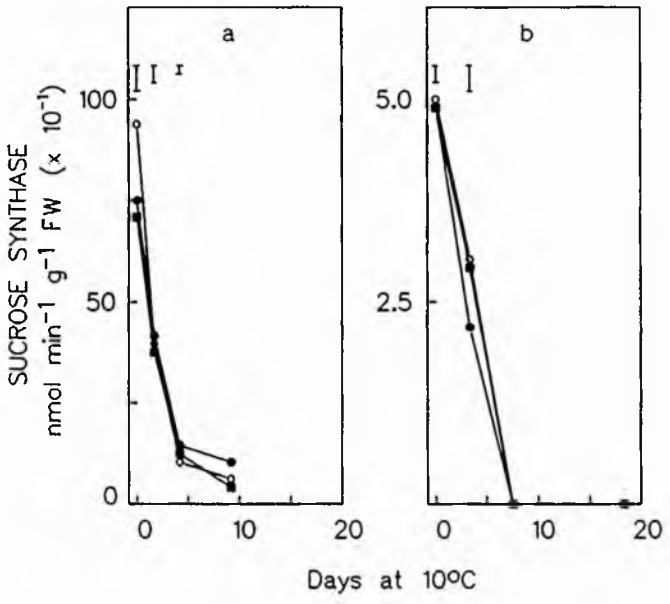

Figure 3. Sucrose synthase activity in tubers immediately detached from the mother plant and during a subsequent storage period at 10°C. Activity was determined in the cleavage direction. Letters and symbols are identified as in Figures 1 and 2.

was observed when basal rather than total activity was measured. This could imply that the proteinaceous invertase inhibitor present in tubers plays a role in regulating invertase activity *in vivo* (20). It must be pointed out, however, that the use of techniques such as rapid vortexing and foaming of extracts, developed to destroy the inhibitor, are severe, and the possibility that losses of invertase as well as inhibitor protein occur cannot be ruled out. It should also be noted that no alkaline invertase activity was detected in tubers.

In contrast to acid invertase, sucrose synthase activity declined rapidly after tuber detachment and by as much as 84% in 2 d (Fig. 3). Experiments in which extracts from attached and detached were mixed showed that this was not due to the presence of an inhibitor in excised tubers. Similar activities were found in all three genotypes. As with sucrose content, enzyme activity declined between the first and final harvests (Fig. 4) and in many cases there was no measurable activity in stored tubers. The results suggest that acid invertase rather than sucrose synthase regulates sucrose hydrolysis in stored tubers and that sucrose concentration or sucrose flux modifies sucrose synthase activity. Sucrose is known to regulate the expression of a number of genes in plants (4, 7). Of particular relevance is the positive effect of sucrose on the expression of potato genes including those encoding patatin (26), sucrose synthase (22), and ADP-glucose pyrophosphorylase (16). The latter experiments were carried out with leaf and stem tissue.

Effect of Modifying Assimilate (Sucrose) Supply to Tubers

As with tuber detachment, excluding light completely from growing plants (cv Record), by covering them with two layers of black polythene produced increases in tuber hexose content and invertase activity and decreases in sucrose content and sucrose synthase activity (Fig. 5). The magnitude of the effect on basal and total invertase activities was similar, although basal activity was about threefold lower than the total (data not shown). Compared with the excision experiment, the effects were not as rapid, with substantial differences between covered and uncovered plants occurring more than 10 d after eliminating light interception. The result again implies that the rate of assimilate supply to developing tubers modifies sucrose-hydrolyzing potential via both sucrose synthase and invertase pathways. The delayed response referred to above may be attributed to the mobilization of starch and sucrose supplies in leaf, stem, or root tissues.

Effect of Supplying Sucrose Exogenously to Intact Tubers

Detached tubers were supplied with sucrose at a range of concentrations through the cut stolon surface, and enzyme activities were determined after 12 d (Table I). The loss of sucrose synthase activity after tuber detachment was prevented by sucrose. A concentration of 750 mM was most effective. The effect of sucrose concentration on invertase activity was more variable. The large SEs obtained with certain treatments probably reflect variation in the rate of sucrose uptake between individual tubers. As Figure 6 clearly shows, there was a good correlation ($r = 0.75$) between sucrose uptake by, and sucrose synthase activity in, individual tubers. Clearly, a proportion of incoming [^{14}C]sucrose will either be compartmentalized in the vacuole or converted into starch. It was beyond the scope of this study to determine the concentration

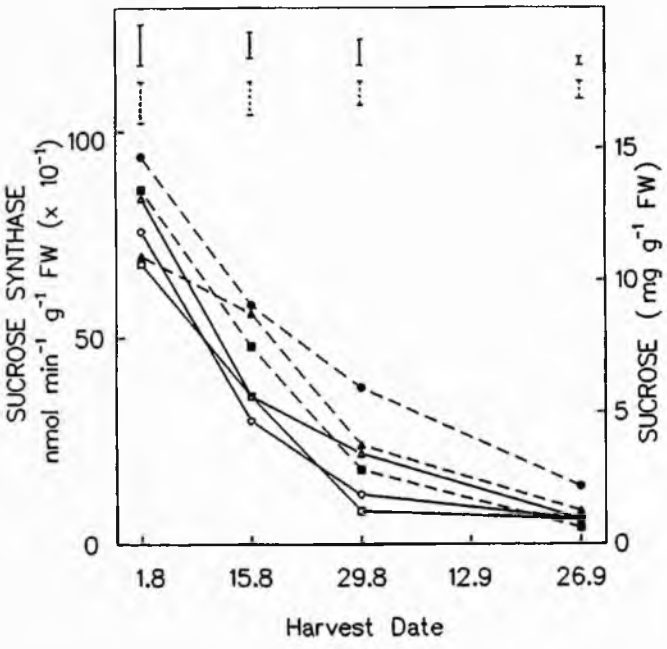

Figure 4. Sucrose synthase activity in (——), and sucrose content of (– – – –), tubers collected at various stages of the growing season. Analyses were carried out immediately after tubers were collected. Key to genotypes: Cara (○, ●), Record (△, ▲), and Brodick (□, ■). Bars represent SE of the mean.

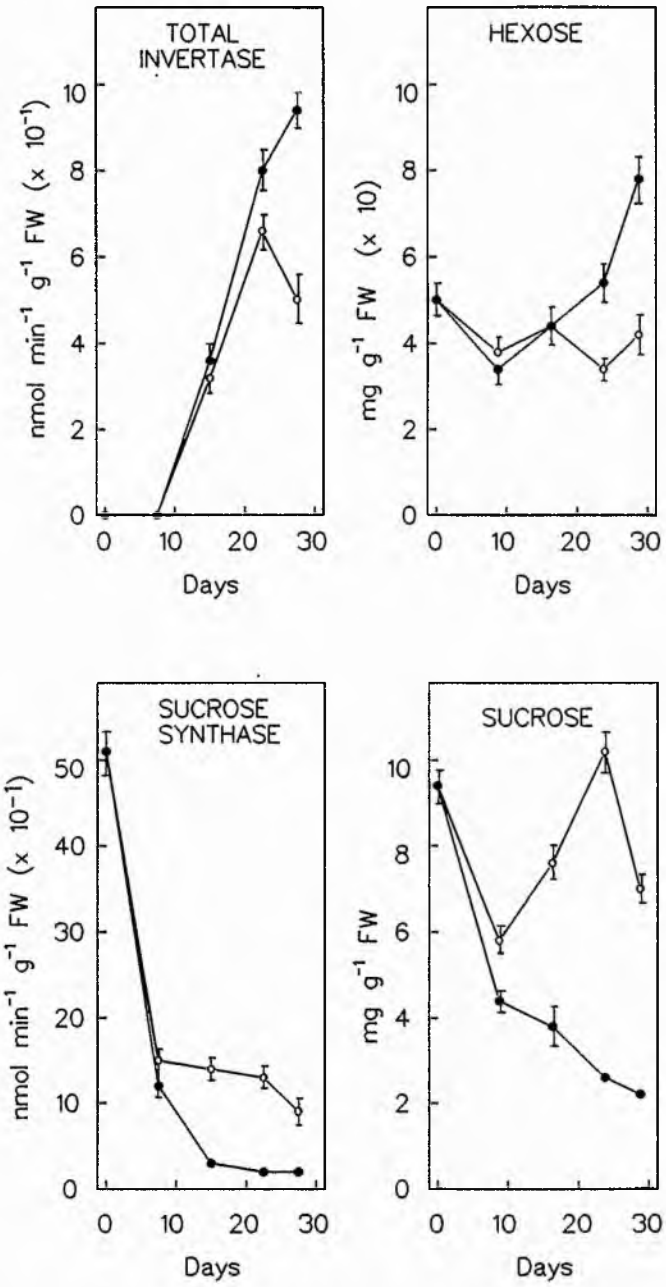

Figure 5. Effects of eliminating light interception for up to 30 d on tuber invertase (total) and sucrose synthase activities and sugar content. ●, Light interception prevented; ○, control.

of sucrose in the cytosol and vacoule and to relate these to sucrose synthase activity. When tubers were supplied with maltose, the correlation between maltose uptake and sucrose synthase activity was poor ($r = -0.35$). Similarly, there was no significant correlation between invertase activity and sucrose uptake (total $r = -0.28$, basal $r = -0.061$). HPLC analysis showed that at the end of the incubation with [^{14}C] sucrose, more than 50% of radiolabel in the soluble sugar fraction was present as sucrose, 1 to 2% as maltose, and the remainder equally distributed between fructose and glucose.

Table I. *Effect of Sucrose Concentration Supplied to Detached Tubers on Sucrose Synthase and Acid (Total) Invertase Activities after Storage at 10°C*

Day 0, enzyme activities immediately after tuber detachment. Day 12, enzyme activities 12 d after supplying sucrose. Values are ± SE of the mean ($n = 5$).

Treatment	Sucrose Synthase	Total Invertase
	nmol min^{-1} g^{-1} fresh wt	
Day 0	154 ± 32	30 ± 17
Day 12 control (H_2O)	57 ± 7	143 ± 12
150 mM sucrose	85 ± 21	111 ± 44
300 mM sucrose	106 ± 10	66 ± 8
750 mM sucrose	260 ± 103	71 ± 18
1500 mM sucrose	93 ± 22	122 ± 29

With [^{14}C]maltose, 50% of the label was recovered as sucrose, 7% as maltose, and the remainder again equally distributed between glucose and fructose. Despite the substantial conversion of maltose into sucrose, the decline in sucrose synthase was not prevented. This result is difficult to interpret in the light of the sucrose effect. On a daily basis, the rate of maltose conversion may be low compared with the rate of sucrose influx directly from the external medium. This would not prevent an accumulation of significant amounts of sucrose by the end of the incubation period, but this is most likely to occur in the vacuole.

Immunoblotting of protein extracts from the above experiment with maize sucrose synthase antibody revealed a cross-reacting polypeptide (M_r 90,000) (Fig. 7). The molecular mass of potato tuber sucrose synthase has been estimated at 290 kD (19), but generally the enzyme is a tetramer consisting of subunits ranging from 87 to 100 kD (14, 17). In Figure 7, the respective activities of sucrose synthase in extracts electropho-

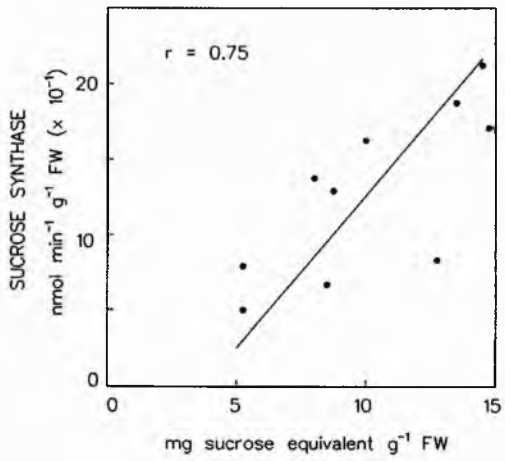

Figure 6. Correlation between sucrose synthase activity and sucrose flux into individual tubers. Tubers were excised from the mother plant and the cut stolon surface immersed in 750 mM sucrose containing a known specific activity of [*U*-^{14}C]sucrose. ^{14}C incorporated by tubers after 12-d incubation was used to determine the quantity of sucrose transported.

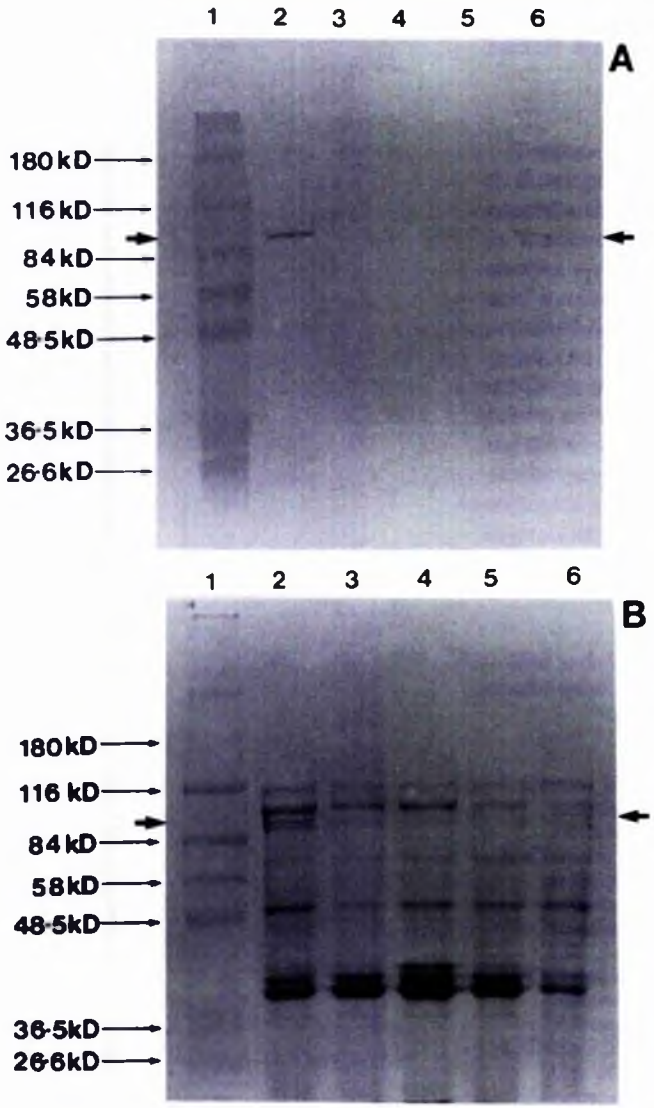

Figure 7. A, Western blot of crude potato extracts with antibodies raised against maize sucrose synthase. Lanes: 1, mol wt markers; 2, newly harvested tuber; 3, 4, 5, and 6, 12 d after supplying excised tubers with water, 750 mM maltose, 750 mM sucrose, and 1500 mM sucrose, respectively. B, Coomassie-stained gel of crude potato extracts (protein loading 10 μg). Lane numbers correspond with treatments described in panel A. Arrow indicates sucrose synthase polypeptide.

rested in lanes 2, 3, 4, 5, and 6 were 412, 45, 39, 281, and 445 nmol min^{-1} g^{-1} fresh weight. The relative abundance of sucrose synthase protein therefore follows sucrose synthase activity. It can be concluded that in potato tuber storage parenchyma, the level of sucrose synthase protein, and hence activity, is regulated by sucrose supply. The work of Salanoubat and Belliard (22) has shown that transcription of the gene in potato leaf and stem is modified by sucrose supply. One must conclude that an identical control mechanism is operating in the tuber. This is unlike the situation in maize protoplasts in which sucrose appears to affect, negatively, transcription of the NTP 11 reporter gene fused to the maize sucrose synthase promoter (13).

CONCLUSIONS

Treatments that interfered with the supply of photosynthate to developing potato tubers stimulated hexose accumulation, caused a rapid increase in acid invertase activity, but resulted in a substantial depletion of sucrose synthase activity and sucrose synthase protein. The data are taken as evidence that acid invertase rather than sucrose synthase regulates hexose accumulation in mature, stored tissues. Differences between genotypes in the rate of hexose accumulation were related to invertase activity. Maintaining a flux of sucrose into excised tubers prevented the decline in sucrose synthase, substantiating the view that sucrose has a positive effect on the expression of the gene in potato. The mechanism by which sucrose regulates gene expression is not known as yet. There is no convincing evidence that sucrose has a negative effect on the synthesis of invertase protein.

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Purification and Characterization of Sucrose Synthase from the Cotyledons of *Vicia faba* L.

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ABSTRACT

Partial purification (approximately 270-fold) of sucrose synthase (EC 2.4.1.13) from developing cotyledons of *Vicia faba* L. cv Maris Bead was achieved by ammonium sulfate fractionation and hydrophobic, affinity, anion-exchange, and gel filtration chromatography. Further purification to homogeneity resulted from gel elution of single bands from native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was identified as a homotetramer with a total molecular mass of 360 kD and subunits of 92 to 93 kD. Antibodies were raised to both native and denatured protein. The identity of the polypeptide was confirmed in western blots using antibodies raised against soybean nodule sucrose synthase. The enzyme has a pH optimum of 6.4 (cleavage direction) and an isoelectric point of 5.5. The affinity of the enzyme for sucrose (K_m) was estimated at 169 mM, and for UDP at 0.2 mM. With uridine diphosphate as the nucleoside diphosphate, the V_{max} is 4-fold higher than with adenosine diphosphate. Fructose acts as a competitive inhibitor with an inhibitor constant (K_i) of 2.48 mM.

Seeds of faba bean (*Vicia faba L.*) store approximately 35% (dry weight) of their food reserves as starch and 36% (dry weight) as protein. Since sucrose is the major carbohydrate transported into developing cotyledons (23), sucrose hydrolysis represents the first metabolic step en route to starch biosynthesis. Sucrose cleavage is catalyzed either by SS[1] (UDP-D-glucose:D-fructose 2-α-glucosyltransferase, EC 2.4.1.13) or invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26). The invertases can be categorized as acidic or neutral/alkaline on the basis of their pH optima (1).

The first genetic evidence indicating an important role for SS in starch biosynthesis came from a study of the maize endosperm *sh* mutant (6). Here enzyme activity is reduced to about 10% of the normal endosperm, and starch content to 40% of the wild type. In maize, a total of five SS isozymes have been identified (19). Developing endosperm cells contain the two homotetramers (S1S1S1S1 and S2S2S2S2), whereas in young roots and shoots, the three heterotetramers are also present (5). Similarly, five isozymes have been detected in sorghum but, in contrast to maize, both SS genes are expressed simultaneously in the endosperm, leading to the additional presence of the heterotetramers in this tissue (7).

The work of de Fekete (9) indicates that SS rather than invertase catalyzes sucrose breakdown in developing *Vicia faba* cotyledons. Although Pridham et al. (16) partially purified the protein, SS has never been fully purified or characterized from the species.

Recent work has questioned the previous consensus that UDP is the principal nucleoside diphosphate in the sucrose cleavage reaction catalyzed by SS. In sycamore suspension cells and spinach leaves, ADP-specific SS has been reported (17). Previous work with relatively crude SS preparations of *V. faba* cotyledons (9) showed no ADP specificity, although activity with ADP is clearly dependent on the assay conditions employed (17). The purification and characterization of faba bean SS is reported in this article.

MATERIALS AND METHODS

Plant Material

Developing field beans (*Vicia faba* cv Maris Bead) were grown in field plots at a density of 45 plants m^{-2}, and pods were harvested 40 to 50 d after anthesis. Previous experiments revealed the highest activity of SS at this stage of bean seed development.

Extraction and Purification of SS

Seeds (400 g total fresh weight), with their testas and embryonic axes removed, were extracted in ice-cold 200 mM Tris-HCl buffer (pH 8.5) containing 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, and 2 mM PMSF in a prechilled blender (Atomix). Insoluble polyvinylpolypyrrolidone was included during the extraction (at 0.1% w/v). The homogenate was filtered through cheesecloth and re-extracted three times before centrifugation of the combined extracts at 10,000*g* (4°C) for 30 min. The supernatant was fractionated by the addition of $(NH_4)_2SO_4$, and the fraction that precipitated between 30 and 80% saturation was collected by centrifugation at 10,000*g* for 10 min. The precipitate was resuspended in 20 mM Tris-HCl buffer, pH 8.0, containing 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, and 2 mM PMSF (buffer A), and was dialyzed against the same buffer overnight. Sufficient $(NH_4)_2SO_4$ was added to make the solution 0.5 M with respect to the salt, and the sample was then applied to a Phenyl Sepharose column (Pharmacia LKB, UK) previously equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$ (buffer B).

[1] Abbreviations: SS, sucrose synthase; Vo, void volume; pI, isoelectric point; V8, endoprotease Glu-C from *Staphylococcus aureus* V8; Arg-C, endoprotease Arg-C from mice glands.

Proteins bound to the column were eluted with a stepped gradient of buffer B and buffer A. SS activity was tightly bound and eluted at 100% of buffer A. The active fractions were dialyzed overnight against 50 mM Hepes-KOH (pH 8.5) containing 5 mM sucrose, 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol.

The dialysate was applied to a 5 mm × 100 mm phenyl boronate agarose-60 affinity column (Amicon, Stonehouse, UK), prewashed with 20 column volumes of 50 mM Hepes-KOH (pH 8.5) containing 200 mM sucrose, 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol, followed by further washes with 5 volumes of the same buffer containing only 5 mM sucrose. After unbound protein was eluted, SS activity was eluted with 0.1 M Tris-HCl, pH 8.5, containing 5 mM 2-mercaptoethanol (14). Active fractions were pooled, and following dialysis against 20 mM Tris-HCl (pH 7.2) containing 5 mM 2-mercaptoethanol, they were applied at 0.5 mL min^{-1} to an anion exchange column (Mono Q; Pharmacia LKB, UK) previously equilibrated with the same buffer. The protein was eluted with a KCl gradient (0–1 M) over 20 column volumes.

Active fractions were concentrated to about 1 mL, and 200-μL volumes were applied at 0.2 mL min^{-1} to a Superose 6 gel filtration column (Pharmacia LKB) pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 100 mM KCl and 5 mM 2-mercaptoethanol. The column was calibrated with a mixture of blue dextran (Vo), thyroglobulin (M_r 669,000), apoferritin (M_r 443,000), β-amylase (M_r 200,000), BSA (M_r 66,000), and carbonic anhydrase (M_r 29,000). Highly purified SS preparations from this gel filtration column were used for kinetic studies. Active fractions were also dialyzed against 10 mM Tris-HCl (pH 7.2) containing 5 mM 2-mercaptoethanol and subjected to both denaturing SDS-PAGE and native PAGE.

SDS-PAGE

SDS-PAGE was performed using a Bio-Rad mini-gel apparatus according to the method of Laemmli (11) and using 10% polyacrylamide. Gels were stained with 0.1% Coomassie brilliant blue R (Sigma) in methanol:acetic acid:water (45:10:45) and destained in methanol:acetic acid:water (30:5:65).

Nondenaturing PAGE

Nondenaturing PAGE (SDS omitted) was performed essentially as described above with the following exceptions: (a) 100 mM sucrose was included in the gels to maintain the enzyme in its active form; (b) the pH of the resolving gel was reduced to 7.5; and (c) 7.5% polyacrylamide was used. Some samples were also electrophoresed on a 4.5 to 7.0% linear gradient nondenaturing polyacrylamide gel for 20 h at 4°C (4). To confirm the presence or absence of isozymes of SS, samples partially purified by $(NH_4)_2SO_4$ fractionation only were subjected to two-dimensional electrophoresis for western blotting.

Preparation of Antisera

Initially, active fractions from gel filtration chromatography were run on 7.5% nondenaturing gels and a section of the gel was stained with Coomassie brilliant blue R to identify the major protein band. The adjacent nonstained region of the gel was excised and eluted at 4°C for 48 h in 5 gel volumes of water. The aqueous extract was lyophilized, redissolved in a small volume of water, and assayed for SS activity to confirm the identity of the protein. A parallel aliquot was subjected to SDS-PAGE. An additional lyophilized preparation was redissolved in 1 mL of Tris-buffered saline (10 mM Tris/HCl, 10 mM borate [pH 7.3], and 0.9% NaCl) and divided into three aliquots, each containing approximately 50 μg of protein. An equal volume of complete Freund's adjuvant was mixed with one sample before injecting, intramuscularly, into a New Zealand White rabbit. Two booster injections with the addition of an equal volume of incomplete Freund's adjuvant were given 20 and 41 d later. Serum was collected 11 d after the final injection.

Antisera raised against denatured SS protein were prepared in a similar way to those against the native protein. Serum was centrifuged at 16,000*g* for 30 min, diluted 10-fold with water, and partially purified by the addition of an equal volume of saturated $(NH4)_2SO_4$ followed by gentle stirring overnight at 4°C. After centrifugation at 10,000*g* for 30 min, the resulting pellets were resuspended in PBS. The antibody solutions were dialyzed against PBS overnight and stored at −80°C .

Protein Blotting

Western blots using polyclonal antibodies raised against denatured SS from soybean nodules, and both native and denatured SS from *Vicia faba* cotyledons were carried out according to instructions issued by Biorad (UK). Blots were incubated with the antibodies (1:2,000 to 1:10,000 dilution with Tris-buffered saline), and antigen-antibody complexes were detected using goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (1:8,000). The chromogenic substrate for alkaline phosphatase used for detection was bromochloroindoyl phosphate/nitro blue tetrazolium.

Protein Sequencing

Purified SS was subjected to SDS-PAGE using the improved method of Yuen et al. (24) to give higher yields for sequencing. The protein was electroblotted on to Problot membrane (Applied Biosystems, UK), stained with amido black, and sequenced on an Applied Biosystems model 477A sequencer (12). The NH_2 terminus of the protein was blocked, necessitating the use of proteases to cleave the protein to obtain a partial sequence. Both V8 and Arg-C proteases were used according to the method of Cleveland et al. (8).

Enzyme Assay

Throughout the purification, SS activity was assayed in the cleavage direction (18). Additionally, sucrose cleavage activity with nucleoside diphosphates other than UDP was determined using a stopped assay system. The 1-mL reaction mixture contained buffer (either 20 mM Tris/HCl or 20 mM Hepes/KOH, both at pH 7.0), 200 mM sucrose, 10 μL of purified faba bean SS, and nucleosides in the range from

Table I. *Purification of Faba Bean Cotyledon Sucrose Synthase*

Fraction	Total Activity	Total Protein	Specific Activity	Yield	Purification
	units μmol min⁻¹	*mg*	*units mg⁻¹ protein*	%	*fold*
Crude	679	22,263	0.031	100	
30–80% $(NH_4)_2SO_4$	586	18,042	0.032	86.3	1.1
Phenyl sepharose	337	408.3	0.83	49.6	27
Phenyl boronate agarose-60	232	124.5	1.86	34.2	61
Mono-Q	114	19.53	5.83	16.8	191
Superose-6	74	8.89	8.34	10.9	273

0.025 to 4.0 mM (all buffered at pH 7.0). The reaction was stopped after 3, 6, or 9 min by heating in boiling water. Fructose released was determined using an autoanalyzer system based on the method of Bergmeyer and Bernt (2, 3). Boiled enzyme extracts treated in the same way were used as controls. The unit of enzyme activity is defined as 1 μmol·min^{-1}.

Determination of pI

The pI of SS was determined on a Rotofor apparatus (Biorad) using Ampholines (Pharmacia LKB, UK) in the pH range of 3.5 to 10. To confirm the pI, the active fractions were collected and refocused.

Protein Assay

Protein concentrations were determined using the dye-binding Biorad method with BSA as the standard (0–100 μg).

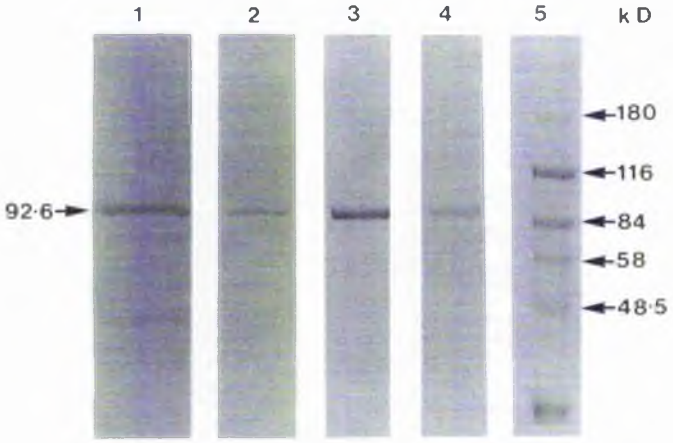

Figure 1. Identification (10% SDS-PAGE gel) of the SS subunit (92.6 kD). Lane 1, Enzymically active protein fraction recovered from Superose-6. Lane 2, Western blot of crude faba bean extract using polyclonal antibodies raised against soybean nodule SS (1/2000 dilution). Lane 3, Denaturing gel of polypeptide purified following elution from SDS-PAGE. Lane 4, Denaturing gel of active SS protein eluted from native-PAGE. Lane 5, Prestained markers (SDS-7B Sigma).

RESULTS

Purification of Enzyme

The protocol developed resulted in a 270-fold purification of SS (Table I). The purified preparation contained one major polypeptide (M_r 92, 600) and additional minor polypeptides on SDS-PAGE (Fig. 1, lane 1). The major polypeptide reacted strongly in western blots with antisera raised against SS from soybean nodules (Fig. 1, lane 2). This polypeptide was gel purified to homogeneity from a denaturing gel (Fig. 1, lane 3). A denaturing gel of enzymically active protein eluted from native PAGE also revealed a single polypeptide (M_r 92,600) (Fig. 1, lane 4). Antisera raised against gel-purified native faba bean SS also cross-reacted specifically with the polypeptide (data not shown).

Both V8 and Arg-C proteases were tested as a means of producing peptide fragments from the 92.6-kD polypeptide for amino acid sequencing, but only V8 provided a product (78 kD) in sufficient quantity (data not shown). A sequence of 13 amino acid residues was obtained that showed substantial homology (about 50%) with potato SS (20) (Fig. 2). All the evidence therefore indicates that the protein purified is SS.

Determination of Relative mol wt

The mean mol wt of SS was calculated at 353,000 ± 19,000 following gel filtration on Superose-6 (data not shown). The protein therefore appears to be a homotetramer. This is confirmed by the two-dimensional western blot shown in Figure 3. Several blots were prepared with a range of protein loadings, but in every case, only one cross-reacting polypeptide was observed.

```
AMINO
ACID
POSITION 130                 140                 150

POTATO   N F V L E L D F E P F T A S F P K P T L T
                 : : : : : :   :
BEAN             L D F E P F S A G G L - G V
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Figure 2. Internal amino acid sequence (13 residues in total) of bean SS (V8 digest) showing homology with potato SS.

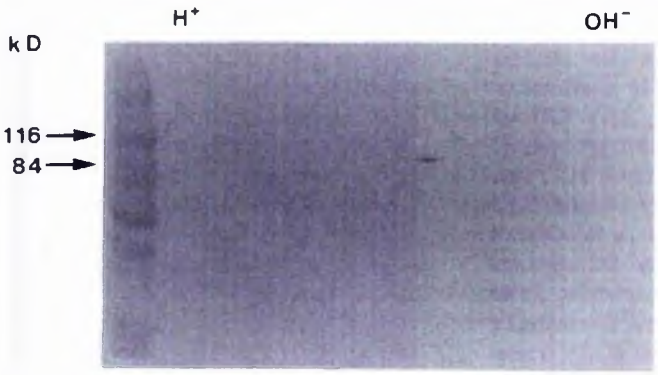

Figure 3. Two-dimensional western blot of bean SS using first-dimension ampholines ranging from pH 3 to 10 and second-dimension run on 10% SDS-PAGE. Antibodies used were raised against bean SS (native protein), dilution 1:10,000.

Enzyme Kinetics

The enzyme has a pH optimum of 6.4 (Tris-HCl buffer) in the cleavage direction, but there was less than a 5% decrease in activity between pH 6.2 and 6.6 (data not shown). The pI was calculated at 5.4 to 5.5 (data not shown).

The K_m value for sucrose was estimated from the Michaelis-Menten equation to be 169 mM ± 26 mM. However, neither the V_{max} nor the K_m for sucrose with UDP as a nucleoside diphosphate could be determined accurately because complete saturation did not occur, even with 500 mM sucrose (Fig. 4). Similar kinetic properties for sucrose saturation have been reported for maize kernel SS by Su and Preiss (21). They offered the hypothesis that this is due to different quaternary structural forms of the enzyme in solution and, to our knowledge, this is the only explanation offered so far. The sucrose saturation curve was sigmoidal rather than hyperbolic with a Hill coefficient (n = 1.1) (Fig. 4).

Standard Michaelis-Menten type kinetics were observed for UDP (V_{max} [UDP] 1.29 ± 0.03 μmol·min^{-1}·mg^{-1} protein). Of the four nucleoside diphosphates tested (UDP, ADP, CDP, and GDP [0.025–4 mM]), SS activity was only detected with UDP and ADP. In agreement with the findings of Pozueta-Romero et al. (17), the reaction with ADP as substrate was strongly inhibited by Tris-HCl buffer (70–80% reduction in rate), whereas with UDP, the reaction was only slightly affected (<1% reduction). With Hepes buffer, the K_m for UDP was 0.212 ± 0.004 mM and the V_{max} was 2.03 ± 0.12 μmol·min^{-1}·mg^{-1} protein. With Tris buffer, the K_m for UDP was 0.149 ± 0.003 mM and the V_{max} was 1.72 ± 0.11 μmol·min^{-1}·mg^{-1} protein. With Hepes buffer and ADP as the nucleoside diphosphate, the K_m for ADP was identical to UDP, but the V_{max} was only 0.496 ± 0.027 μmol·min^{-1}·mg^{-1} protein (25% of UDP). No values could be obtained for ADP in the presence of Tris buffer due to the high level of inhibition.

Fructose (10 mM) inhibited sucrose cleavage by 74% (inhibition was competitive). A Dixon plot provided an estimated K_i value of 2.48 mM at sucrose concentrations of 25, 50, 100, and 200 mM (Fig. 5).

DISCUSSION

The purification to homogeneity of SS from *V. faba* cotyledons showed that the enzyme has a molecular mass of 360 kD and is composed of four subunits of 92 to 93 kD. The tetrameric structure of the native protein is similar to that observed with mung bean seedlings (10), rice grains (15), maize kernels (21), soybean nodules (14), and peach fruit (13). In these instances, the molecular mass of the protein ranges between 360 and 400 kD, with the molecular masses

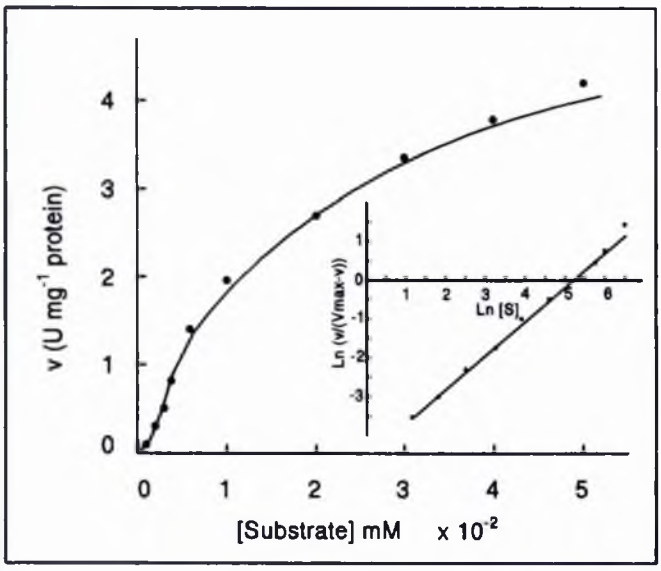

Figure 4. Sucrose saturation curve of purified faba bean SS (inset: Hill plot [n = 1.1]) (sucrose concentration 3–500 mM).

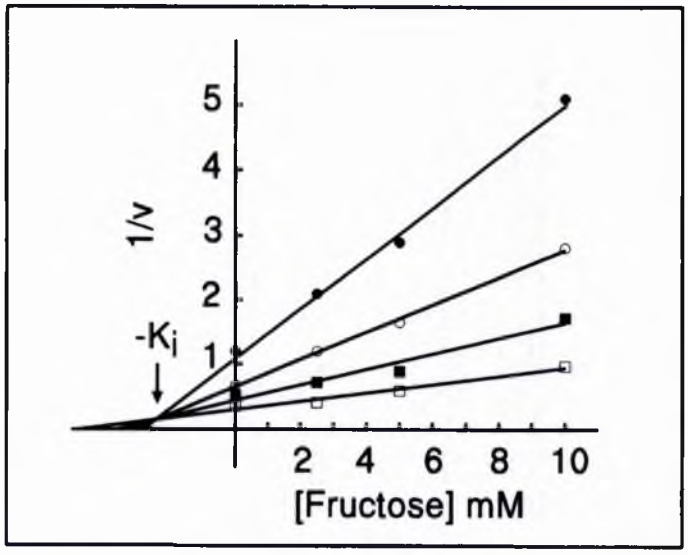

Figure 5. Dixon plot showing competive inhibition of SS by fructose: ●, 25 mM; ○, 50 mM; ■, 100 mM; and □, 200 mM Sucrose (v [units mg^{-1} protein]).

of identical subunits ranging between 87 and 100 kD. As with soybean nodules (14), *V. faba* cotyledons contain only one (detectable) form of the enzyme. Polyclonal antibodies raised against SS from whole kernels of wild-type maize (kindly supplied by Dr. Karen Koch, University of Florida) showed no specific cross-reaction on western blots with *V. faba* SS protein, unlike those raised against soybean SS, which reacted specifically with the 92-kD polypeptide subunit. The maize antibody does, however, recognize potato SS (18).

The amino acid sequence data, although only for a small portion of the faba bean SS protein, show distinct homology with potato tuber SS, which itself has a 75% overall identity with maize SS (20). Additionally, the polyclonal antibodies raised against the faba bean SS protein detect a single polypeptide (90 kD) from a crude tuber extract on a western blot (data not shown).

Recent work (17) has suggested that ADP rather than UDP is the principal nucleoside diphosphate utilized in the SS reaction. Previous work with relatively crude extracts of *V. faba* cotyledons demonstrated that the activity of SS with ADP was only 16% of that with UDP (9). This has essentially been confirmed in the present study using purified enzyme. Although significant inhibition of the faba bean enzyme with Tris buffer and ADP confirms the results of Pozueta-Romero et al. (17) with spinach leaves and sycamore cell suspensions, the faba bean enzyme is unable to utilize other nucleoside diphosphates as effectively as UDP, even when Tris is replaced by Hepes. The bean enzyme is certainly not ADP specific. The data do not, therefore, agree with the hypothesis that ADP is the principal substrate for faba bean SS, at least as far as maximum catalytic activity is concerned. Physiologically, the proportion of SS activity driven by ADP and/or UDP in vivo will clearly depend on the concentration of the nucleoside diphosphates in the cytosol. It will also depend on whether or not ADP activity is suppressed by the presence of UDP. According to Pozueta-Romero et al. (17), this is not the case. The hypothesis has not been tested for the purified faba bean enzyme. It should be noted that the kinetic data obtained for faba bean with UDP and ADP are similar to those reported for peach (13).

Fructose acts as a competitive inhibitor of faba bean SS with respect to sucrose (as shown previously with *Helianthus tuberosus* [22]). We have calculated (unpublished data) that during bean seed development, the concentration of fructose in cotyledons (on a whole tissue basis) decreases from about 7.5 mM 30 d after anthesis to about 2 mM 20 d later (at the time of maximum SS activity). At the sucrose concentrations prevailing in the tissue at the same time, we calculate that SS activity (cleavage direction) may be inhibited between 70 and 30% (assuming that sucrose, fructose, and SS are within the same cellular compartment). Fructose-specific hexokinases, known to be present in a range of tissues, including developing *V. faba* cotyledons (9, A. Gardner and H.V. Davies, unpublished data), may therefore play an important role in regulating SS activity in vivo.

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Developmental changes in carbohydrate content and sucrose degrading enzymes in tuberising stolons of potato (*Solanum tuberosum*)

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Tuberising stolon tips of potato (*Solanum tuberosum* L. cv. Record) accumulate starch and sucrose but the hexose content, particularly fructose, declines rapidly. Similar changes occur in the region 2 cm behind the swelling apex but the decline in glucose is far more pronounced than in the developing tuber. Tuberisation is characterised by an apparent switch from an invertase-dominated sucrolytic system (both acid and alkaline invertases [EC 3.2.1.26] are present) to one dominated by sucrose synthase (EC 2.4.1.13). Sucrose synthase and fructokinase (EC 2.7.1.4) activities were, at a maximum, ca 10- and 5-fold higher, respectively in the swelling stolon tip compared with the non-tuberising region. At the highest starch contents attained, the starch level in the young developing tuber was approximately double that in the adjacent non-tuberising stolon region. Immunoblots revealed that developmental changes in sucrose synthase, fructokinase and alkaline invertase polypeptides corresponded with enzyme activities. Antibodies raised against the N-terminal amino acid sequence of a soluble invertase purified from mature tubers did not detect significant quantities of a polypeptide in stolons and young, developing tubers. Antibodies raised against an in vitro expression product of an apoplastic invertase cloned from a leaf cDNA library detected a polypeptide in developing tubers but not in mature ones. However, expression of the protein did not correlate well with acid invertase activity during early tuber formation.

Key words – Carbohydrates, hexose kinase, invertase, potato, *Solanum tuberosum*, sucrose synthase, tuberisation.

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Introduction

Tuberisation in potato is a complex process that results in the differentiation of a specialised shoot, the stolon, into a storage organ, the tuber. The process is characterised by significant anatomical, hormonal and biochemical changes (Ewing and Struik 1992), some of the earliest including an increase in starch deposition and in the percentage frequency of cells in mitosis (Duncan and Ewing 1984). In addition, there are significant changes in the content and composition of the soluble sugar pool in tuberising stolon tips (Davies 1984). Increased starch deposition is accompanied by elevated activities of both plastidic and cytosolic enzymes, including ADP-glucose pyrophosphorylase, starch synthase, UDP-glucose pyrophosphorylase and sucrose synthase (Sowokinos 1976, Hawker et al. 1979, Obata-Sasamoto and Suzuki 1979, Tsay and Kuo 1980, Helder et al. 1991). Sucrose metabolism during tuber formation is of particular interest as it is known that carbohydrates (directly or indirectly) affect the transcription of genes encoding carbohydrate metabolising enzymes (Salanoubat and Belliard 1989, Krapp et al. 1993) including those involved in starch synthesis e.g. ADP-glucose pyrophosphorylase (Müller-Röber et al. 1990). It is well established that the sucrose synthase pathway predominates in developing tubers (Pressey 1969), and indeed, in other starch-storing sinks (Sung et al. 1989, Wang et al. 1993), whereas high acid invertase

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is common in rapidly elongating tissues such as corn radicle tips (Hellebust and Forward 1961), bean internodes (Morris and Arthur 1985) and potato sprouts (Blanc 1983). Tuber formation is characterised by a cessation in stolon elongation accompanied by the radial swelling of the sub-apical region (Vreugdenhil and Struik 1989). This implies that significant changes in the pathways of sucrose degradation are associated with the developmental switch. This is examined in the present manuscript.

Abbreviations – Q_{10}, temperature coefficient; K_m, Michaelis Menten coefficient; RT-PCR, Reverse Transcription/Polymerase Chain Reaction; V_{max}, maximum velocity.

Materials and methods

Plant material

Field-grown potato plants (*Solanum tuberosum* L. cv. Record) were harvested approximately 10 weeks after planting and the stolons and tubers washed while still attached to the plant. To provide sufficient material for replicated analyses, 100 plants were harvested and a range of tuberisation/tuber developmental stages selected. The material was assigned to 6 different developmental stages (Fig. 1): 0 – no visible swelling at stolon tip; 1 – swelling 1–4 mm; 2 – swelling 4–8 mm; 3 – swelling 8–12 mm; 4 – swelling 12–16 mm; 5 – swelling 16–20 mm greater than stolon diameter. For stages 1 to 5 the mean tuber fresh weights were, respectively, 0.21, 0.51, 1.50, 3.17, 5.09 g with SEM's < 10% of the mean (n = 10). After measuring and weighing, stolons and tubers were immediately frozen in liquid nitrogen and stored at −80°C prior to analysis.

Enzyme extractions and assays

The apical/subapical region of the stolon (whether swollen or not) was extracted separately from a 2 cm length of stolon excised 2 cm distal to the tuberising tip. Five replicates of two or more stolons were extracted, to provide sufficient material for assay. In general, two or three samples were sufficient for tuber extractions but six were required for stolon extractions. Tissue was ground to a fine powder in liquid N_2 in a mortar and pestle and extracted in 4 volumes of extraction medium together with 1% w/v insoluble polyvinylpolypyrrolidone (PVP) and acid-washed sand.

For sucrose synthase, glucokinase and fructokinase the extraction buffer contained: 100 m*M* Tris-HCl (pH 7.5), 5 m*M* dithiothreitol (DTT), 3 m*M* magnesium acetate, 2% (w/v) glycerol and 2 m*M* phenylmethylsulfonylfluoride (PMSF). For both acid and alkaline invertases, tissue was extracted in 100 m*M* sodium acetate (pH 5.0) containing 10 m*M* sodium sulphite, 1 *M* NaCl and 2 m*M* PMSF. After centrifugation at 20000 *g* for 20 min at 4°C, extracts were dialysed for 18 h at 3°C against two changes of 10 m*M* extraction buffer.

Sucrose synthase was assayed in the cleavage direction under optimised conditions as described previously (Ross and Davies 1992a). Fructokinase and glucokinase activities were determined as described by Gardner et al. (1992). Acid invertase activity before (basal) and after (total) destroying the endogenous invertase inhibitor by extensive foaming was measured as described by Ross and Davies (1992a). Alkaline invertase activity was assayed at 37°C under optimum pH and substrate conditions in 0.2 *M* phosphate buffer (pH 7.5) containing 250 m*M* sucrose. For each of the enzymes extracted, no residual activity was detected in the insoluble pellets when

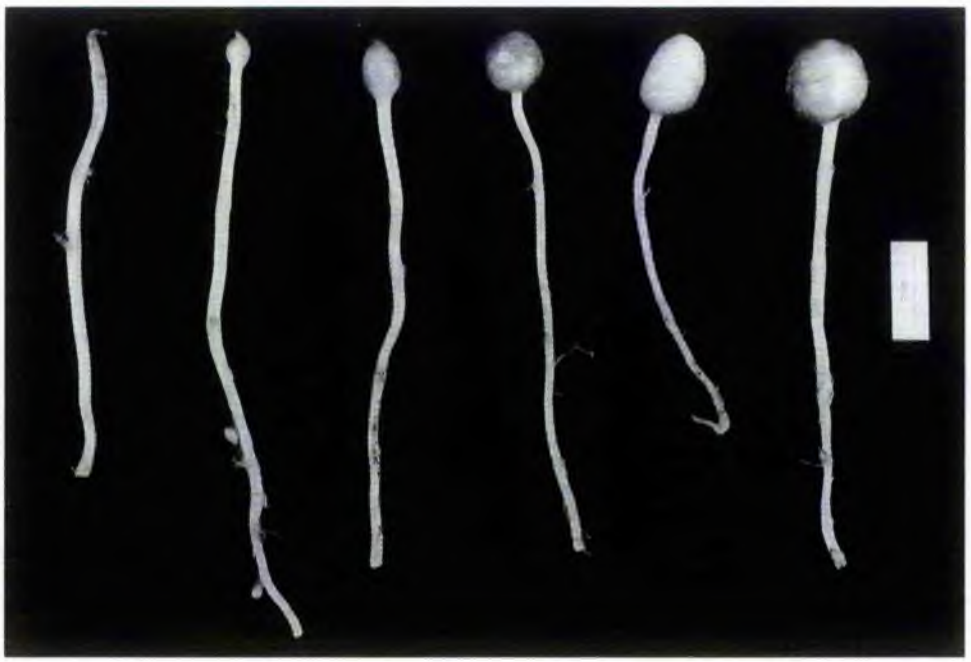

Fig. 1. Stages of tuber development showing from left to right, stage 0 to stage 5 (as described in text).

resuspended and assayed after dialysis. Possible losses of activity during preparation of enzyme extracts were assessed, for each of the tuberisation stages examined, by preliminary recombination experiments with extracts from developing cotyledons of *Vicia faba*. The % recoveries of tuber enzyme activities were: tuber sucrose synthase 102%, stolon sucrose synthase 89%; tuber acid invertase 110%, stolon acid invertase 102%; tuber alkaline invertase 92%, stolon alkaline invertase 86%.

Previous extractions of fructokinase and glucokinase from different stages of developing potato tubers had shown no major losses when extracted under the above conditions (A. Gardner, 1992. PhD Thesis, Univ. of Dundee, Scotland, UK). It was concluded that the procedures used did not affect, significantly, enzyme recovery.

Carbohydrate determinations

Freeze-dried and finally ground tissue samples were extracted in 80% ethanol at 55°C for 3 h (Ross and Davies 1992a). The concentrations of glucose, fructose and sucrose were determined using enzyme-coupled reaction systems (Richardson et al. 1990). Starch remaining in the tissue pellets after centrifugation was gelatinised, digested with amyloglucosidase and the glucose determined as above (Davies and Oparka 1985).

Protein determination

Protein in each of the enzyme extracts was quantified using the Bio-Rad protein assay (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts., UK) with bovine serum albumin (BSA) as a standard (0–100 μg).

Electrophoresis and immunoblotting

Protein was extracted in 100 m*M* HEPES-KOH (pH 7.5) containing 10 m*M* sodium sulphite, 1 *M* NaCl, 2 m*M* PMSF, 2% glycerol (w/v), 20 m*M* EDTA and 5 m*M* DTT and extracts were desalted on sephadex G-25M PD10 columns (Pharmacia). Extracts were maintained at 4°C throughout and all procedures were performed as rapidly as possible to prevent proteolysis. Solubilised protein (15 μg) was subjected to SDS-PAGE (10 or 12.5% acrylamide) as described by Laemmli (1970) and the gels were stained with Coomassie blue. In parallel gels, polypeptides were transferred to nitrocellulose membrane and, after immunoblotting with polyclonal antibodies following the method of Towbin et al. (1979), cross-reacting bands were identified using anti-rabbit immunoglobulin conjugate labelled with alkaline phosphatase. The polyclonal antibodies used for immunodetection were raised against:

(1) sucrose synthase purified from developing cotyledons of *Vicia faba* (Ross and Davies 1992b); (2) a synthetic peptide derived from the N-terminal sequence of a purified acid invertase from potato tubers (Burch et al. 1992); (3) a polypeptide expressed in *E. coli* from an open reading frame representing part of a gene encoding an apoplastic invertase (Hedley et al.1993); (4) alkaline invertase purified from *Vicia faba* cotyledons (H. A. Ross and H. V. Davies, unpublished data); (5) antibodies raised against fructokinase (FKP-I) purified from pea seeds (provided by D. D. Randall, Univ. of Missouri, Columbia, MO, USA).

Results

Carbohydrate content

The changes in glucose, fructose, sucrose and starch, as measured over the 6 stages of tuberisation, are shown in Fig. 2. The glucose and fructose content was higher in the stolon itself than in the subapical region prior to tuberisation (stage 0), but the converse was true for sucrose. In both tissues hexose levels decreased with the onset of tuberisation, concomitantly with an increase in sucrose and starch content. After stage 1, in the developing tuber, the glucose content increased while the fructose content remained relatively constant. In contrast, in the stolon, the levels of both glucose and fructose declined. The glucose:fructose ratio increased from 1.9 to 6.5 in stolons and from 2.5 to 9.3 in tubers from stages 0 to 5. Sucrose and starch content reached a plateau in both tissues by stage 3 (tubers 1.5 g fresh weight), with tubers attaining the highest sucrose and starch content (ca 32%

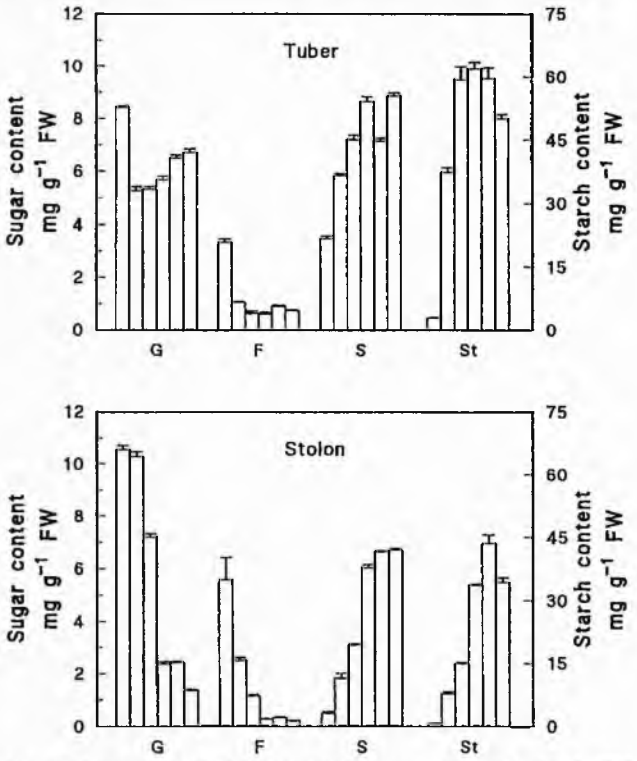

Fig. 2. Glucose (G), fructose (F), sucrose (S) and starch (St) content of both developing tubers and the stolons subtending them. Values for each (shown by open bars) are from left to right, stage 0 to stage 5. Mean values (n = 5) + SEM values are shown.

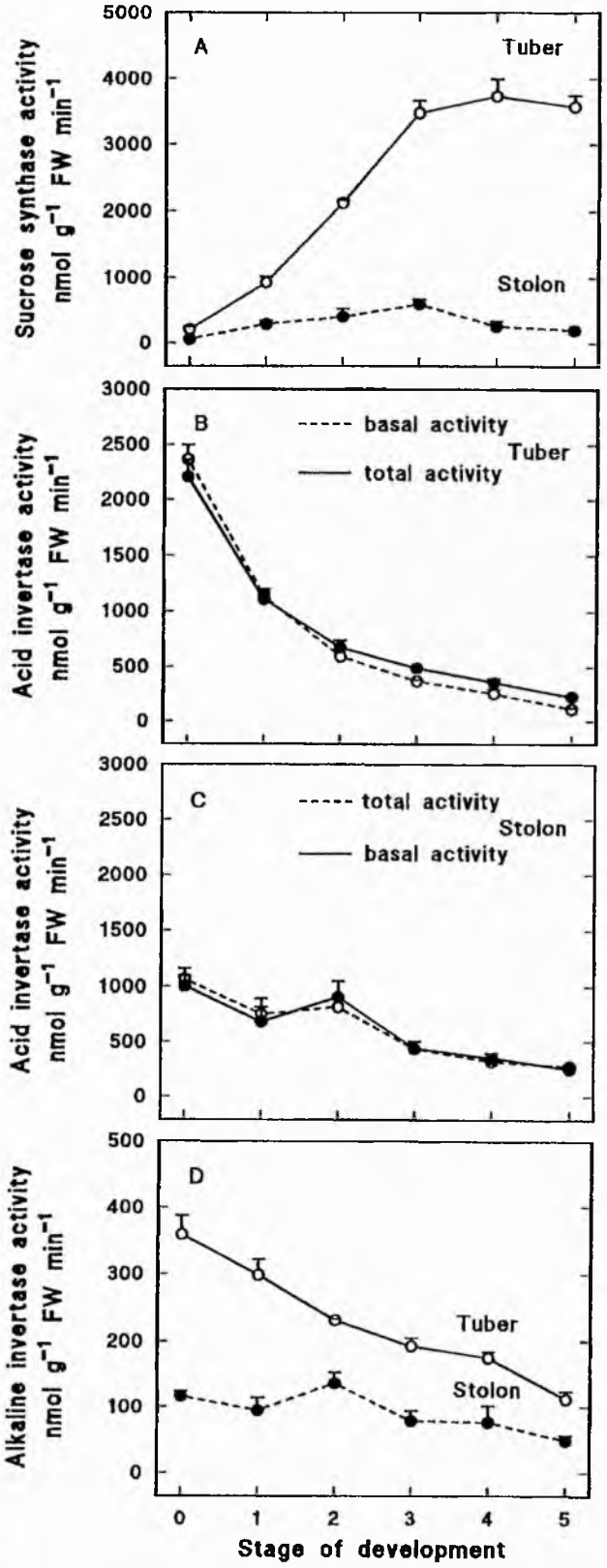

Fig. 3. Enzyme activity in stolons and developing tubers over the 6 stages. A – Sucrose synthase, B & C – total and basal acid invertase, D – alkaline invertase. Each value is shown as the mean (n = 5) + SEM.

and 42% higher, respectively, than stolons). However, overall sucrose and starch increased (14-fold) and (60-fold) for stolons and (3-fold) and (22-fold) for tubers.

Enzyme activities

Sucrose synthase activity increased rapidly in the swelling stolon tip during tuberisation, reaching a maximum catalytic activity of 3 730 nmol g^{-1} fresh weight min^{-1}, nearly 20-fold higher than in the non-tuberised stolon (Fig. 3A). In the non-swelling stolon, sucrose synthase activity remained low, increasing just over 10-fold in the region behind the swelling tip during tuberisation. By contrast, acid invertase activity was high in the stolon, just prior to any visible swelling and particularly in the meristematic sub-apical region (Fig. 3B, C). On tuberisation, acid invertase activity decreased 10-fold and activities were reduced to 225 nmol g^{-1} fresh weight min^{-1}. Basal (before foaming the enzyme extract) and total (after foaming extract) invertase activities were not significantly different between tuberisation stages 0–2. However, between stage 3 and 5, basal invertase activity in developing tubers had decreased to half the values obtained for total activity. For the non-swelling stolon region there were no significant differences between basal and total activities at any of the stages examined.

Alkaline invertase activity was more than 6-fold lower than acid invertase prior to tuberisation, but activity also decreased during tuber development (Fig. 3D). To confirm the presence of a distinct alkaline invertase pH curves were constructed for sucrolytic activity at each stage of tuberisation. Results for three of the stages (Fig. 4) clearly identifies a specific alkaline invertase with a pH optimum of 7.5–7.6, activity at this pH decreasing during tuber development.

Fructokinase and glucokinase were included in the enzymes quantified to provide information on the potential for hexose phosphorylation. Glucokinase activity remained low (at approximately 60 nmol g^{-1} fresh weight min^{-1}) throughout the developmental stages but fructo-

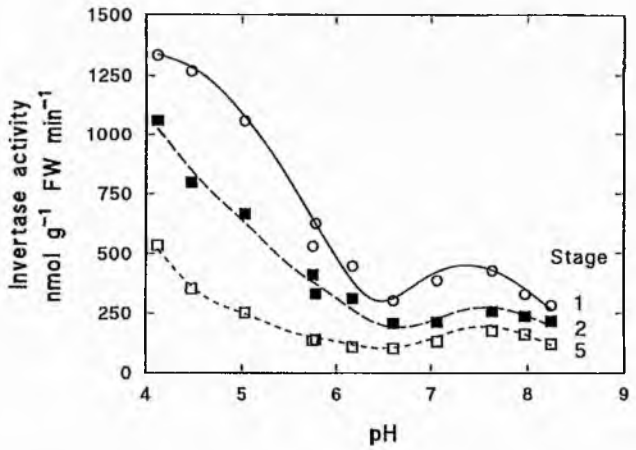

Fig. 4. Invertase activity for stages 1, 2 and 5 showing acid and alkaline pH optima. (n = 3) SEM values <10% mean.

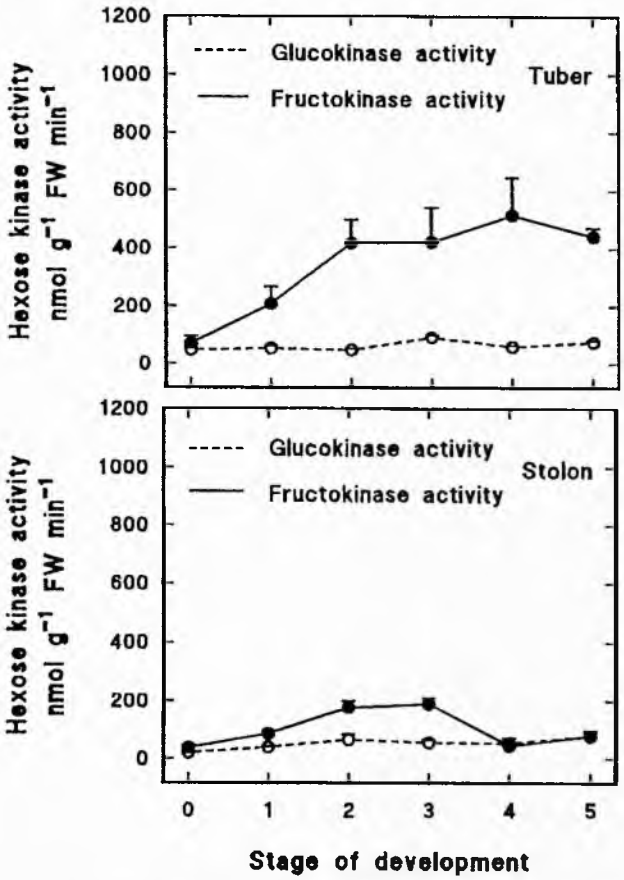

Fig. 5. Glucokinase and fructokinase activity in stolon and developing tuber over the 6 stages. Each value is shown as the mean (n = 5) + SEM.

kinase increased over 7-fold in the developing tubers (Fig. 5). Fructokinase activity also increased in the non-swelling stolon region but maximum activity attained was approximately 40% of that in tubers. Fructokinase activity declined in the non-swelling region by stage 4 but remained high in the developing tuber. Glucokinase activity in the non-swelling region increased over 3-fold between stages 0 and 2.

Regression analysis showed that both sucrose synthase and fructokinase activities were highly positively correlated with sucrose content ($r = 0.965$ and 0.957, respectively), whereas acid and alkaline invertases were negatively correlated with sucrose ($r = -0.974$ and -0.992, respectively). Similarly, both sucrose synthase and fructokinase activites were positively correlated with starch content ($r = 0.891$ and 0.978, respectively) and acid and alkaline invertases negatively correlated with starch ($r = -0.996$ and -0.966, respectively).

Immunoblotting

Polypeptides extracted from developing tubers were separated by SDS-PAGE (Fig. 6). Protein extracted from a mature, stored tuber, of cv. Record was also included (lane 8; Fig. 6). The most obvious change, occurring on tuberisation and during early development, was the appearance of a polypeptide (approximately 90 kDa) which decreases in the mature tuber, as shown by the arrows in Fig. 6. This was identified as a subunit of potato sucrose synthase by immunoblotting with antibodies raised against sucrose synthase from bean cotyledons (Fig. 7A). The level of sucrose synthase polypeptide corresponded with the measured enzyme activity. Changes in the level of the storage protein, patatin (around 42 kDa) are not particularly evident during early tuber development although patatin is clearly the major protein in the mature tuber.

Both acid invertase antibodies (antibodies 2 and 3) produced specific immunogenic responses with crude extracts. Antibody 2 recognised a polypeptide of approximately 48 kDa (Fig. 7B) and antibody 3 a 58 kDa polypeptide (Fig. 7C). Visually, in neither case did the quantity of protein detected correspond with extractable acid invertase activity. The 48 kD polypeptide was not expressed in the stolon but increased during tuber development and was most abundant in the mature tuber. By contrast, the 58 kDa polypeptide was detected in the stolon, increased during early tuber development but was virtually absent from the mature tuber. Polyclonal antibodies raised against *Vicia faba* cotyledon alkaline invertase cross-reacted specifically with a polypeptide of 54 kDa (Fig. 7D). The protein was heavily expressed in non-tuberising stolons but decreased during tuber development along with enzyme activity. No alkaline invertase activity was detected in mature stored tubers so lane 8 was omitted for this blot.

The FKP-I polyclonal antibodies specifically cross-reacted with a single polypeptide (36 kDa; Fig. 7E) which had been previously identified as a subunit of the 70 kDa potato fructokinase protein (Gardner et al. 1992). The level of expression of the polypeptide – low in the stolon and increasing during early development – reflects the enzyme activity.

Discussion

During the rapid phase of starch accumulation (stages 0 to 2) the increase in the starch content of the tuberising stolon region amounted to 56 mg. The decrease in the total hexose content amounted to only 5.8 mg. Over the same stages the starch in the non-tuberising stolon region increased by 14 mg whilst the hexose content declined by 7.8 mg. It seems plausible that the onset of starch synthesis in the non-tuberising region of the stolon correlates with the development of a strong metabolic sink for the hexoses. Since the hexose phosphorylating potential of this tissue did not change substantially throughout the developmental stages (see Fig. 5) this raises the possibility of feed-back regulation of hexose-phosphorylation. Fine metabolic control of potato tuber hexose kinases is likely to operate via nucleoside triphosphate content and composition, and via ADP, fructose and fructose-6-phosphate contents (Gardner et al. 1992, Renz and Stitt 1993).

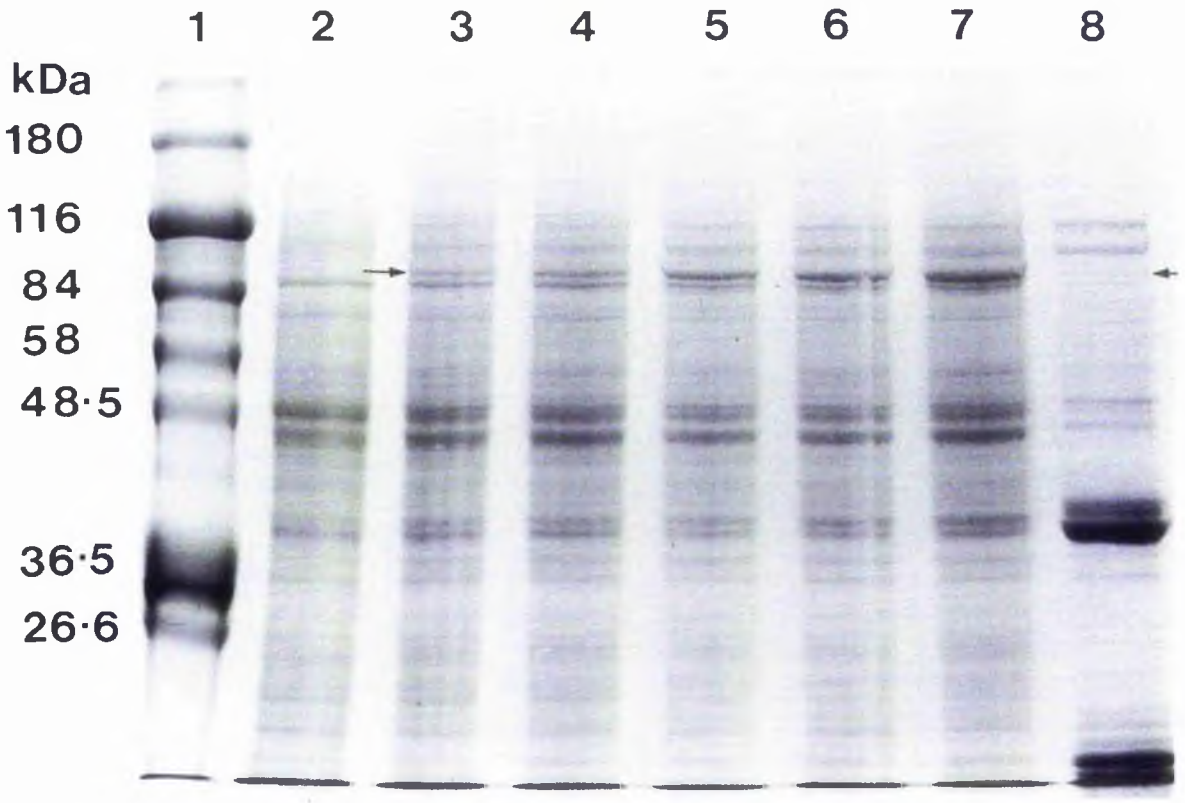

Fig. 6. 10% SDS-PAGE. 15 μg protein loaded for each sample. Lane 1 – MW markers (SDS-7B Sigma), Lane 2–7 – developmental stage 0–5, Lane 8 – cold-stored mature tuber.

In the young, developing tuber, starch is apparently accumulated following the metabolism of imported sucrose rather than from stored hexoses. A change in the pathway of sucrose mobilisation during the early stages of tuberisation can be deduced from the rapid decline in both acid and alkaline invertases and the substantial increases in both sucrose synthase and fructokinase activities. This is confirmed, indirectly, by the increase in the G:F ratio. However, direct evidence for such a switch in metabolism during tuberisation is still lacking. Morrell and ap Rees (1986) calculated the rate of sucrose mobilisation in developing tubers of plants grown at 20°C to be approximately 3 μmol hexose g^{-1} fresh weight h^{-1} and estimated that acid and alkaline invertases could, at best, account for <50% of the total sucrose mobilised. In the present study the activities of both acid and alkaline invertases were sufficient to sustain such a rate, although the potential contribution of alkaline invertase becomes borderline as the tubers develop. It may well be that such discrepancies are due to the developmental stages analysed since, in our experience, acid invertase declines to very low levels and alkaline invertase to non-detectable levels in larger developing tuber (H. A. Ross and H. V. Davies, unpublished data). Alkaline invertase activity remained higher in swelling than non-swelling stolon regions and provides one explanation for the maintenance of a higher glucose content in the former. Starch turnover via amylase may also contribute to the glucose pool in developing tubers.

By stage 4 the sucrose content in tuberising and non-tuberising regions had increased to similar levels. It may be argued that in both tissues the decline in acid invertase (presumed to be vacuolar) is responsible for the rise in sucrose content. However, the marked increase in the maximum catalytic activity of sucrose synthase in the young developing tuber maintains a high sucrolytic potential. The potential may not be realised since sucrose synthase has a much lower affinity for sucrose (K_m 130 m*M*, Pressey 1969) than invertases (tuber acid invertase 8 m*M*; plant alkaline invertases between 9 and 25 m*M* [Avigad 1982]). The concentration of sucrose in the cytosol of developing tubers is clearly important but is, as yet, unknown. However, the low affinity of sucrose synthase for sucrose together with an increase in sucrose influx into the swelling tuber, would help to explain the early increase in tuber sucrose content. Elevated sucrose concentration, or the concentrations of metabolites derived, would be expected to modify the expression of genes involved in the starch biosynthetic pathway, including sucrose synthase (Salanoubat and Belliard 1989, Müller-Röber et al. 1990, Kim et al. 1991, Ross and Davies 1992a). This would increase sink strength further and, as a result, sucrose influx.

Increases in sucrose in the non-swelling stolon region

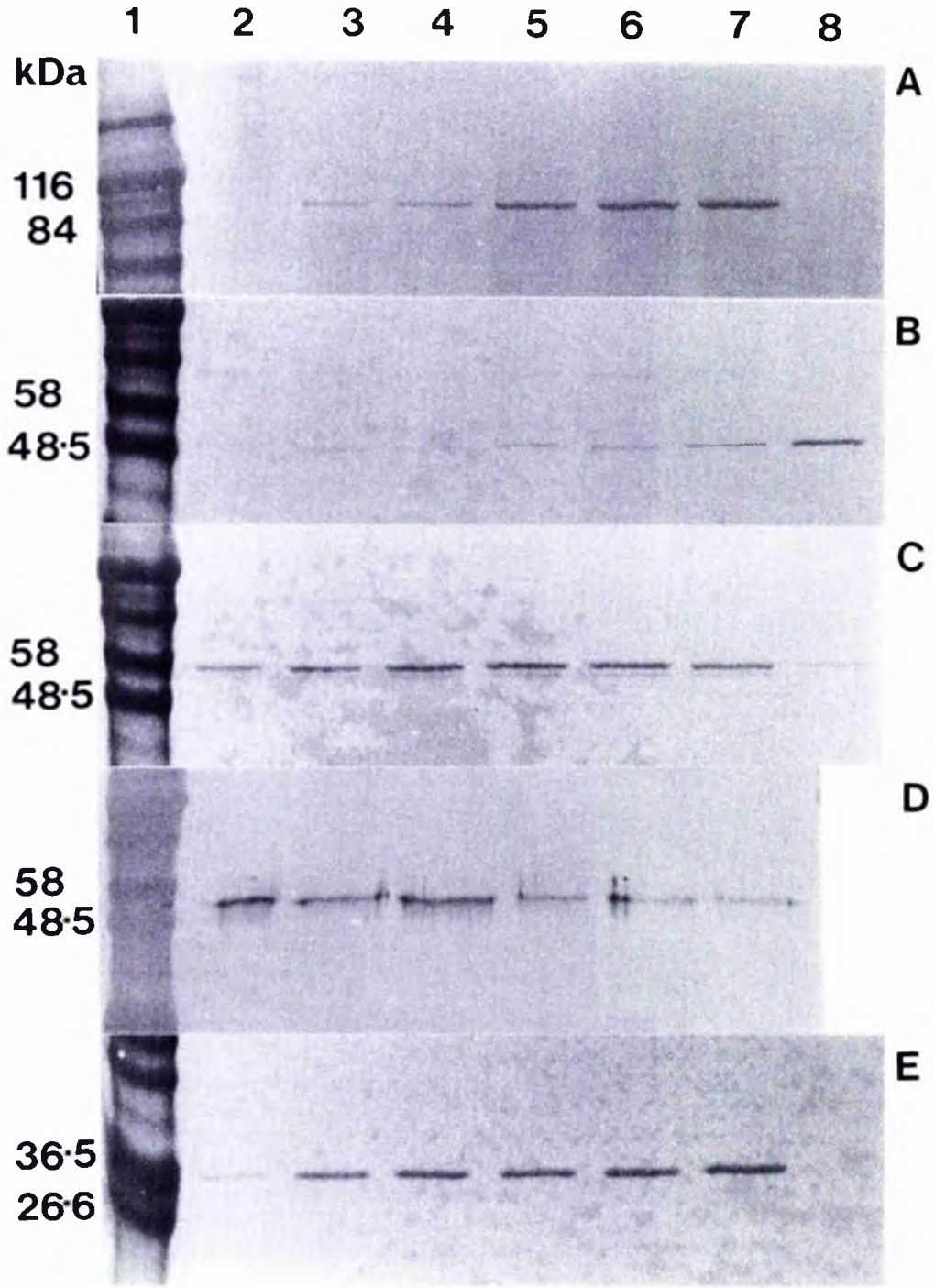

Fig. 7. Immunoblots of gel shown in Fig. 6. A) sucrose synthase (antibody 1; dilution 1:10 000). Corresponding enzyme activities in lanes 2–8 respectively 10.4, 87.4, 121.9, 307.0, 341.7, 323.1, 10.4; B) potato acid invertase (antibody 2; dilution 1:200). Corresponding enzyme activities in lanes 2–8 respectively 405.8, 194.2, 110.8, 77.8, 60.7, 31.6, 43.9; C) potato acid invertase (antibody 3; dilution 1:1000) (activities in lanes 2–8 as for B); D) alkaline invertase (antibody 4; dilution 1:1000). Corresponding enzyme activities in lanes 2–8 respectively 61.8, 50.9, 43.6, 40.6, 42.3, 31.6; E) fructokinase (antibody 5; dilution 1:3000). Corresponding enzyme activities in lanes 2–8 respectively 6.9, 20.7, 49.0, 43.8, 73.6, 63.8, 2.7. All enzyme activities expressed as nmol mg^{-1} protein min^{-1} are mean values (n = 5) SEM values <10% mean.

were not accompanied by large increases in sucrose synthase activity – as was the case for developing tubers. By developmental stage 3 the sucrose content of the stolon approached 70% of that in the tubers. The maximum catalytic activity of sucrose synthase in the stolons was only 17% of that in the tubers. This is explicable if the bulk of the sucrose in the stolon is "in transit", i.e. located in the phloem. It has been shown that a phloem-specific

sucrose synthase exists in maize and citrus (Nolte and Koch 1993) and also in *Ricinus* (Geigenberger and Stitt 1992) thus allowing the removal of carbon for energy supply within the sieve element – companion cell complex. The fact that the capacity for starch synthesis in the non-tuberising region is apparently lower than in the developing tuber could also be explained by a more limited supply of sucrose to parenchymatous tissue, although a reduced expression of sucrose-inducible genes in the pathway of starch synthesis would clearly contribute to the overall effect. This last point needs to be tested experimentally. However, the hypothesis does fit with the statement made earlier i.e. that a significant proportion of the starch in the non-tuberising region is accumulated at the expense of endogenous hexoses.

Immunoblots showed that the developmental changes in sucrose synthase, fructokinase and alkaline invertase proteins were correlated with enzyme activity. A potato fructokinase gene has recently been cloned (Smith et al. 1993) and preliminary Northern analyses has again indicated the importance of transcriptional control (S. B. Smith, unpublished data). Alkaline invertase has, to our knowledge, not been cloned, although it has been purified from soybean (Chen and Black 1992), sugarbeet taproots and faba bean cotyledons (H. A. Ross and H. V. Davies, unpublished data). Evidence for the physiological role of alkaline invertase is meagre. Some consider it a maintenance-type enzyme which does not change in response to increased sucrose levels (Sung et al. 1990). In maize mutants deficient in sucrose synthase, alkaline invertase activity apparently increases to compensate, thus providing an alternative pathway for sucrose cleavage within the cytosol (Dancer and ap Rees 1989).

Immunoblots with antibodies raised against acid invertases failed to demonstrate a correlation between protein content and enzyme activity in the early stages of tuberisation. Antibodies raised against the N-terminal sequence of a purified soluble enzyme (Burch et al. 1992) consistently detected a polypeptide in mature tubers only, where invertase activity is high (Richardson et al. 1990). Antibodies raised against the in vitro expression product of a gene encoding a leaf apoplastic invertase (Hedley et al. 1993) detected a polypeptide only in immature tubers. Reverse Transcription/Polymerase Chain Reaction (RT-PCR) has confirmed that this gene is expessed at extremely low levels in tubers (Hedley et al. 1993). However, there is little convincing physiological evidence that an apoplastic invertase exists in young developing tubers (S. Morrell, 1984. PhD Thesis, Univ. of Cambridge, UK). Significant homologies exist between genes encoding apoplastic and vacuolar invertase genes in carrot (Unger et al. 1992) and between vacuolar genes isolated from carrot, mungbean and tomato (Hedley et al. 1993 and references therein). Polyclonal antibodies used in Fig. 7C may therefore be detecting related epitopes on a vacuolar rather than an apoplastic protein. If this is the case then, given the different expression patterns detected by the two potato antibodies used, there is likely to be more than one gene encoding soluble invertase activity – one active in elongating stolons and young tubers, the other only in mature trubers. This question is currently being addressed.

A proteinaceous inhibitor has been reported as a potential regulator of acid invertase activity in planta (Pressey 1966) and a "foaming" procedure has been developed to quantify invertase activity in association with the inhibitor (basal activity) or dissociated from the inhibitor (total activity; see Richardson et al. 1990). Basal and total invertase activities did not differ in extracts from the non-tuberising stolon region but in developing tubers, from stage 3 onwards, total activity was double basal activity. However, there is some doubt that this has any relevance, physiologically. Isla et al. (1992) showed that the inhibitor was located in the cell wall of mature tubers, whilst invertase activity was located in the vacuole. In this case the two components are likely to be brought together on tissue extraction only. Whatever the reality, the pattern of change in extractable invertase activity remained the same whether the inhibitor-invertase complex was destroyed or not.

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