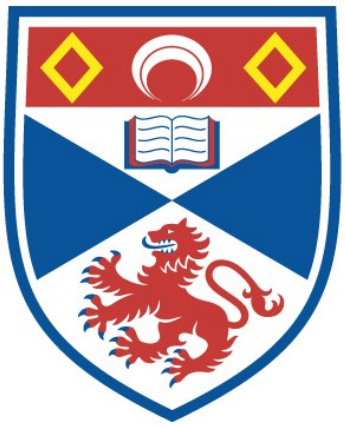
SOME STUDIES ON BETA-AMYLASE FROM BARLEY MALT

Diane Cook

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1975

SOME STUDIES ON β-AMYLASE FROM BARLEY MALT

by

Diane Cook

A thesis

submitted to the University of St. Andrews in application for the degree of Doctor of Philosophy.

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ABSTRACT

1. β-Amylase was prepared from barley malt by salt extraction and purified by ammonium sulphate fractionation and gel filtration on Sephadex G-25 followed by chromatography on DEAE-cellulose. Traces of α-amylase activity were removed by low temperature storage under acid conditions. β-Amylase so prepared released no soluble blue dye from chromogenic α-1,4-glucans.

2. The kinetic parameters (K_m and V_m) of β-amylase hydrolysing α-1,4-glucan polymers were found to increase in the presence of α-amylase. This was explained in relation to the chain length of the substrate.

3. Amyloses of different degree of polymerization ($\bar{D}P$) were prepared by ethanol fractionation of thymol-precipitated amylose from soluble starch. The values of K_m and V_m for β-amylase were shown to decrease with an increase in the $\bar{D}P$ of the substrate. The values of K_m for the non-reducing terminal and of K_i for the internal portions of the substrate were found to be 0.001mM and 0.00015mM, respectively.

4. Series of dialdehyde amyloses and borohydride-reduced dialdehyde amyloses were prepared. An explanation was given for the dependence of K_m and V_m upon the degree of oxidation of dialdehyde and reduced dialdehyde substrates.

5. Inhibition studies involving maltose, dialdehyde and reduced dialdehyde amyloses were carried out. A theory to explain the inhibitory effect of oxidized amyloses upon β-amylase activity was put forward.

6. Immobilised β-amylase derivatives prepared using AE-cellulose and polyaminostyrene supports were found to retain 18% and 9.1%, respectively, of the original activity of the soluble enzyme. The effects of pH and temperature

upon the immobilised derivatives was compared with the effects upon soluble β-amylase and the apparent values of K_m and V_m (K_m' and V_m') for soluble starch and of K_i (K_i') for maltose were determined for each preparation.

7. The action patterns of the soluble enzyme and the immobilised derivatives on amylose were investigated by plotting the decrease in blue value of the amylose substrate against the increase in the reducing power of the solution as hydrolysis proceeded and were confirmed by chromatographic analysis of the reaction products and intermediates of maltoheptaose hydrolysis and explained in terms of the alteration of enzyme affinity towards the substrate upon immobilisation.

DECLARATION.

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry of the University of St. Andrews, under the direction of Dr. W.M. Ledingham.

CERTIFICATE.

I hereby declare that Diane Cook has spent nine terms in research work under my direction, and that she has fulfilled the conditions of Ordinance No. 16 (St. Andrews), and that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD.

I matriculated at the University of St. Andrews in October 1968, and graduated with the degree of Bachelor of Science, Upper Second Class Honours, in Biochemistry in June 1972.

In October 1972, I matriculated as a research student at the University of St. Andrews.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. W.M. Ledingham for his encouragement and guidance throughout this work and Dr. L.B. de Carvalho for the many helpful discussions.

My thanks also to Dr. W.E. Hornby and Dr. D.L. Morris for the provision of ethyl adipimidate.

Finally, I wish also to thank the University of St. Andrews for a Research Studentship.

ABBREVIATIONS AND SYMBOLS.

Abbreviations and symbols used in this thesis are as follows:

AE-cellulose:-	aminoethyl cellulose
DMSO:-	dimethyl sulphoxide
DNSA:-	3,5-dinitrosalicylic acid
$\bar{DP}$:-	degree of polymerization
G_n:-	malto-oligosaccharides with degree of polymerization n
PAS:-	polyaminostyrene
PS:-	polystyrene

All other abbreviations are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature as published in the Biochemical Journal Instructions to Authors (revised) 1975 (1).

C O N T E N T S.

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

First reported by Ohlsson (2), β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) is an exo-enzyme which attacks alternate linkages of α-1,4-glucan substrates from the non-reducing end, releasing β-maltose by an inversion mechanism (Fig. 1.1.1).

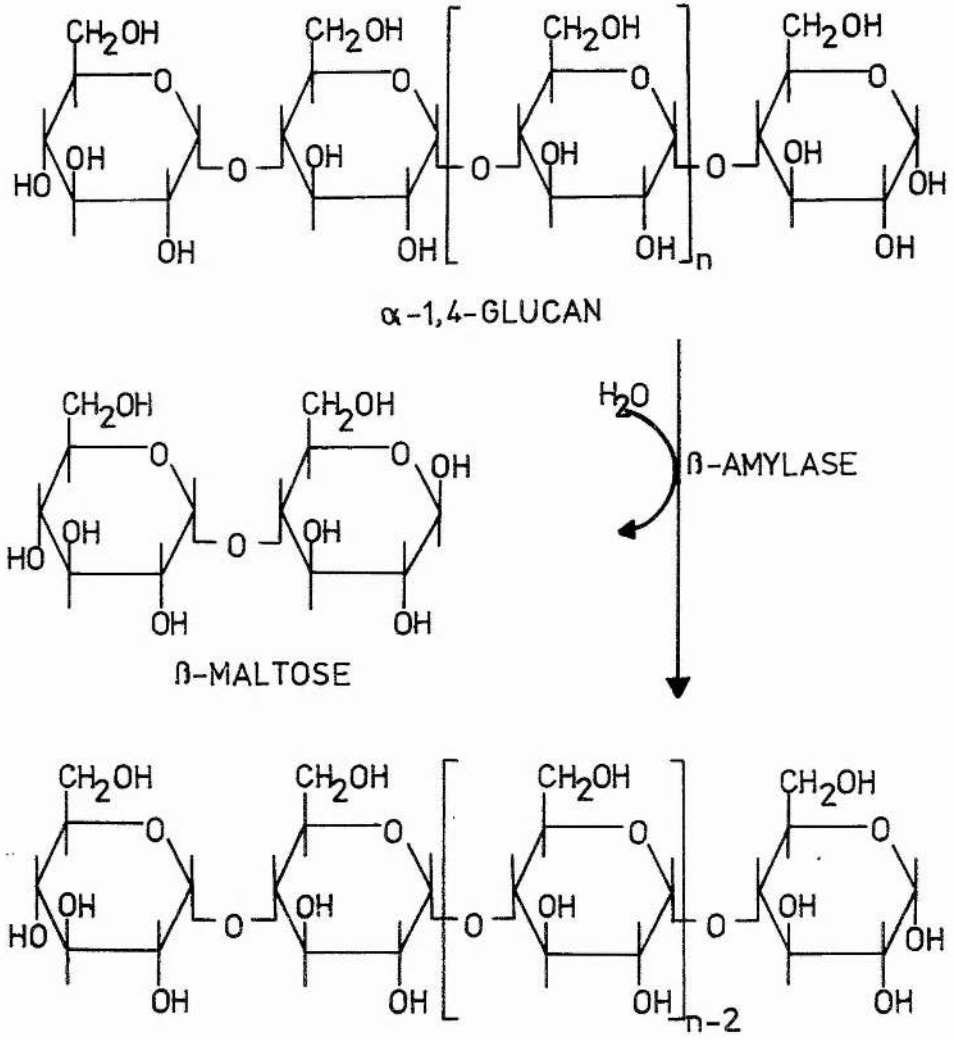
The enzyme has been found in higher plants, especially in cereal grains and sweet potato, and, more recently, β-amylase has been located in certain bacteria. (3, 4, 5).

Amylases from a number of sources (6) appear to be simple proteins with no requirement for essential, low molecular weight cofactors. Thoma et al. (7) found β-amylase to be a tetramer, with a monomer molecular weight of 50,000 and one inhibitor binding site per monomer molecule. The binding site was thought to correspond to the catalytic site.

There exist three classes of amylases, which have been extensively reviewed, (8, 9); α-amylases, β-amylases and amyloglucosidases, which differ only in their position of attack upon their substrates. These enzymes can be described as endo- or exo-amylases. Exo-amylases act upon the terminal α-1,4 bonds in the substrate, systematically degrading the glucan chain, whereas an endo-amylase is one which cleaves internal bonds in the substrate, rapidly decreasing the chain length and producing small oligo-saccharides which then function as secondary substrates.

Amyloglucosidase, like β-amylase, is an exo-amylase, releasing glucose from the non-reducing terminal

Fig. 1.1.1. Illustration of the action of β-amylase on α-1,4-glucan substrates to release β-maltose by an inversion mechanism.


α-1,4-GLUCAN
H2O
β-AMYLASE
β-MALTOSE
α-1,4-GLUCAN

of the substrate, whereas α-amylase acts by endo-attack. On account of its exo-amylolytic nature, β-amylase is unable to by-pass an α-1,6 branch point or any other than minor modifications in its substrate. Although amyloglucosidase has exo-amylolytic activity, the Rhizopus delemar enzyme is able to cleave α-1,6 bonds, so that this enzyme, together with α-amylase, has the ability to by-pass such anomalies.

Until recently there has been some dispute over the mode of action of β-amylase. Cleveland and Kerr (10) and Kerr and Gehman (11) produced evidence for a single chain action pattern for barley β-amylase acting on corn amylose with a degree of polymerization ($\bar{D}P$) of 235. (Fig. 1.1.2) Cowrie et al (12) obtained similar results with a larger ($\bar{D}P$ 3200) amylose sample. The requirement for a single chain mechanism is that the enzyme-substrate complex is retained until the substrate is hydrolysed as completely as possible before forming a new enzyme-substrate complex with another substrate chain.

However, Bourne and Whelan (13) advocated a multi-chain action pattern (Fig. 1.1.2) in which one catalytic event occurred per enzyme-substrate encounter.

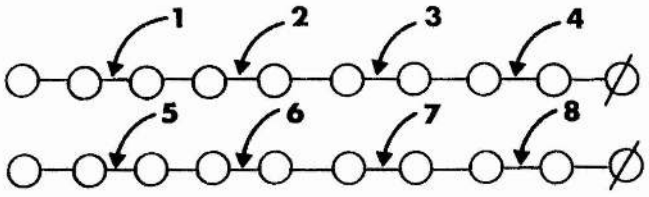
Poorly characterized substrates and impure enzyme preparations had been frequently employed to investigate the mode of action of the enzyme. Apart from obvious anomalies introduced by traces of an endo-acting enzyme or a non-specific α-glucosidase, the products of hydrolysis of a substrate with a Poisson distribution and one with a most probable distribution could differ greatly, although both

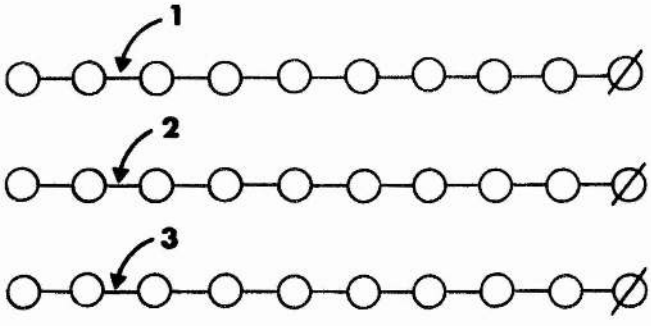
Fig. 1.1.2. Illustration of the attack mechanisms of α-1,4-glucanases:

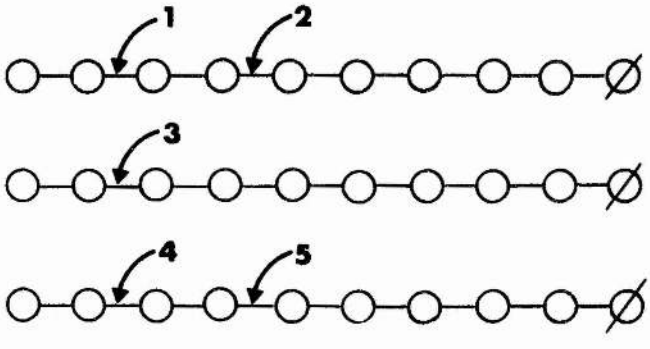
Single chain attack

Multi-chain attack

Multiple attack







position of enzymic attack

α-1,4-linked glucose residues

reducing terminal

distributions may have the same average DP (14). Hence, it was realized that only polymer mixtures with narrow chain length distributions were suitable for the study of action patterns. Also, the chemical and physical properties of amylose samples can differ markedly, depending not only on their origin, but also on the maturity of the plant and the method of separation. Both for ease of comparison of enzyme preparations and for interpretation of kinetic data, much work has been carried out to investigate the activity of β-amylase upon characterized malto-oligosaccharides or amyloses of known $\bar{DP}$.

The action pattern of β-amylase has been well reviewed recently (15, 16). The use of chromatographically pure malto-oligosaccharides (17) led to the conclusion that β-amylase quantitatively acts in a similar manner on small and medium-sized substrates and that chain-end effects were important only for smaller substrates. The accumulation of intermediates (maltotriose and maltopentaose) during the β-amylolysis of maltoheptaose provided evidence for a multi-chain attack mechanism. However, single and multi-chain attack mechanisms are extreme examples of multiple attack (Fig. 1.1.2) and it has been found (18) that the action pattern of β-amylase can be shifted towards either extreme by variations in pH and temperature.

Inhibition studies, using substrate analogues, have been invaluable in providing information concerning the mechanism of β-amylase action. Substrate analogues have been employed to attempt to discern the requirements for

binding and for β-amylase activity. The catalytic specificity of β-amylase has been of interest because of its ability to distinguish between internal segments and the non-reducing terminal of the substrate chain.

Koshland et al (19) proposed a mechanism of induced fit for the catalysis of substrates by β-amylase, which was thought to have a specific requirement for an unsubstituted C_4 hydroxyl group at the non-reducing terminal of the substrate which is considered to allow correct alignment by its small steric bulk (20).

Weill and Caldwell (21) investigated the nature of the active site of β-amylase and concluded that the sulphydryl groups and the tyrosine residues were essential for activity, but that the free amino groups were probably not essential.

The intramolecular nature of oxidative inactivation of β-amylase by thiol-selective reagents led Englard et al (22) to propose that ageing of the enzyme resulted in auto-oxidation of one thiol group not essential for activity but sufficiently close to an essential group to react with it in mercaptide formation.

Thoma and Koshland (23) investigated the inactivation of β-amylase by iodoacetamide and found that alkylation of sulphydryl groups resulted in a modified enzyme with 2% of the original activity. Inactivation was prevented in the presence of cyclohexaamylose. The pH-activity profiles of the native and modified enzymes revealed pK values indicative of a carboxyl group (pK 3.7) and a protonated

imidazole group (pK 7.0), evidence for the latter being substantiated by photo-oxidation studies.

Thoma et al (24) concluded that the surface thiol groups of sweet potato β-amylase are not essential for activity. Gertler and Birk (6) further suggested that the thiol groups of soya bean and sweet potato β-amylase do not participate in binding the substrate but do have a role in the catalytic step. Spradlin and Thoma (25) speculated that β-amylase thiol groups are regulatory entities in vivo, a proposal supported by the fact that the enzyme can be reversibly inactivated by disulphide interchanges.

An involved investigation of the catalytic site of malt β-amylase was carried out by Zherebtsov (26) who found results indicating that the effect of pH was not only upon the active groups but also upon the structure of the catalytic site. His experiments demonstrated that imidazole and carboxyl groups were present at the active site, possibly in close proximity to each other, and led him to propose a mechanism for the effect of β-amylase on α -1,4 glucosidic bonds.

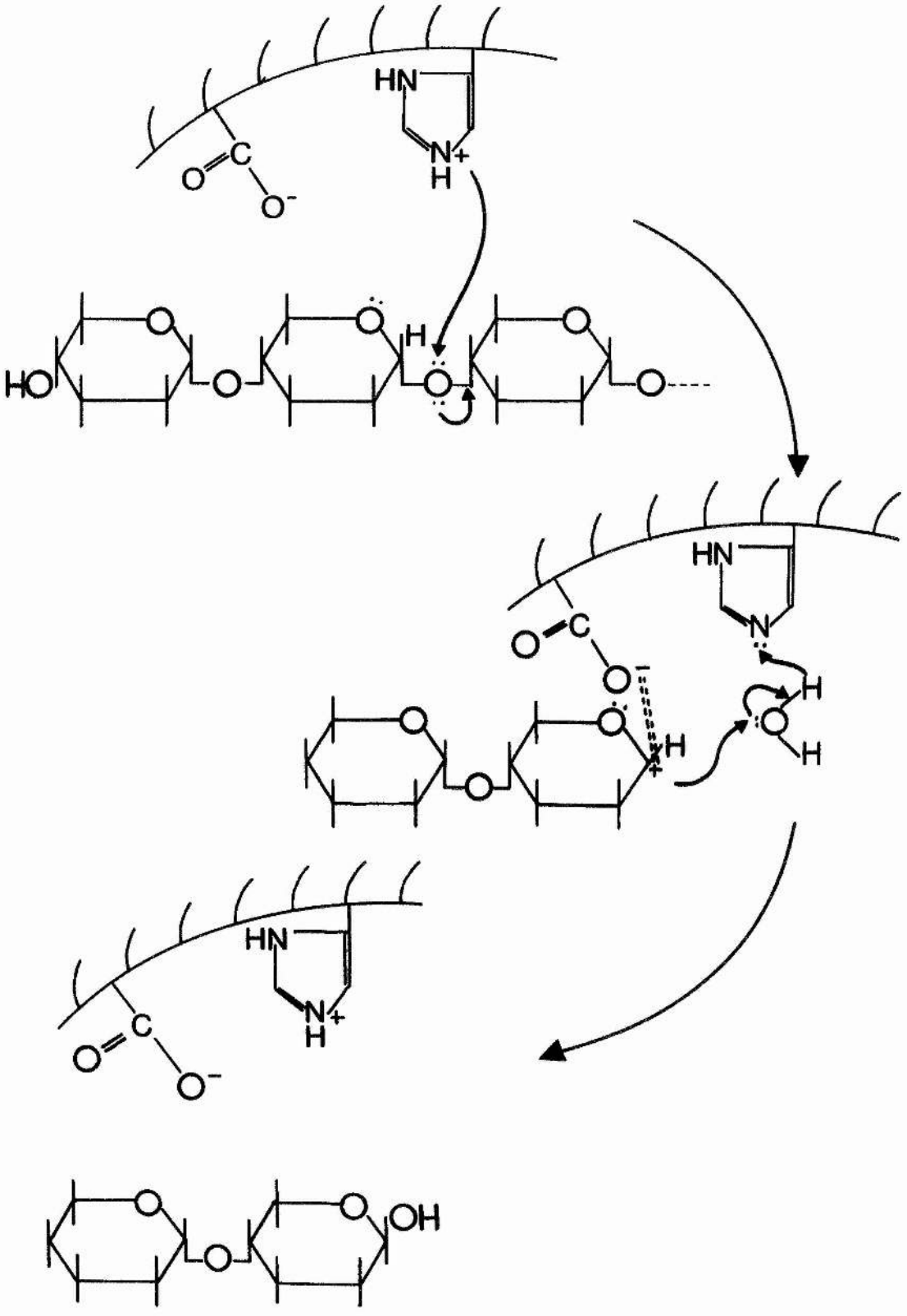
The active site of β-amylase is thought to have a distinct hydrophobic region (19) which enhances the polarizing effect of the imidazoliun ion, leading to a decrease in the activation energy of the reaction. Since β-amylase catalysis cannot be explained by a simple acid catalysis mechanism, an ion-pair mechanism operating in a hydrophobic environment may account for the large added rate needed to explain the enzyme action.

Marshall (27) reported that the expected conformational change in β-amylase on interaction with cycloamyloses did not occur, and offered evidence in support of the involvement of sugar ring distortion during β-amylase catalysis, similar to that occurring with lysozyme. Marshall suggested that the shape of the binding site of β-amylase is such that it optimally accommodates a complete turn of the amylose helix at the non-reducing terminal, which may account for the inhibitory effect of cyclohexaamylose, a cyclic compound with six glucose residues bound by α-1,4 linkages. He proposed that evidence was more consistent with a template-type specificity pattern.

Thoma (20) revised some of his earlier theories to explain the dependence of V_m upon chain length in terms of both the degree of multiple attack and the self-inhibition caused by internal residues of the substrate chain.

A current hypothesis for amylase action was proposed earlier by Thoma (28), in which the chair conformation of a glucopyranoside residue is distorted towards the half-chair conformation when it binds at the active site, making the ring more susceptible to nucleophilic attack. Imidazole is thought to act as a general acid, donating a proton to the acyclic oxygen while the carboxylate anion at the active site possibly stabilizes the developing oxycarbonium ion. Removal of a portion of the solvation sheath may occur during binding of the substrate, enhancing the field of the anion and the resultant electrostatic

Fig. 1.1.3. Illustration of the mechanism of α-1,4-glucan hydrolysis by β-amylase involving imidazole and carboxyl functions at the active site.


HN
N+
H
C
O
O-
HO
H
HN
N
H
H
H
HN
N+
H
C
O
O-
OH

shielding (Fig. 1.1.3).

The theory of induced fit was supported by Holló et al (29). On the basis of their experimental results, these workers defined the mechanism of amylases in three stages.

1. The weak negative charge on the hydrated substrate and the hydration and weak positive charge at the active site attract each other, eventually bringing about a partial dehydration.
2. Hydrogen bonding develops between the partially dehydrated groups of enzyme and substrate. Structural alterations in the enzyme molecule are induced by the partially spiral structure of the substrate and, owing to the slow formation of hydrogen bonds, a gradual fitting of the enzyme occurs.
3. The induced fit causes alterations to occur in the spatial structure of the enzyme, making possible further hydrogen bonds. Consequently, the conformation of the glucosidic bond to be split is deformed and can be cleaved readily.

Chemically modified malto-oligosaccharides have been studied as substrates for β-amylase action. Malto-tetraose, the smallest rapidly cleaved substrate, modified at either terminal has been found to inhibit enzyme action (30).

Modification of the internal residues of a polymer substrate also adversely affects the activity of the enzyme, as seen by the action of β-amylase on periodate-oxidized amyloses with a low degree of oxidation (31).

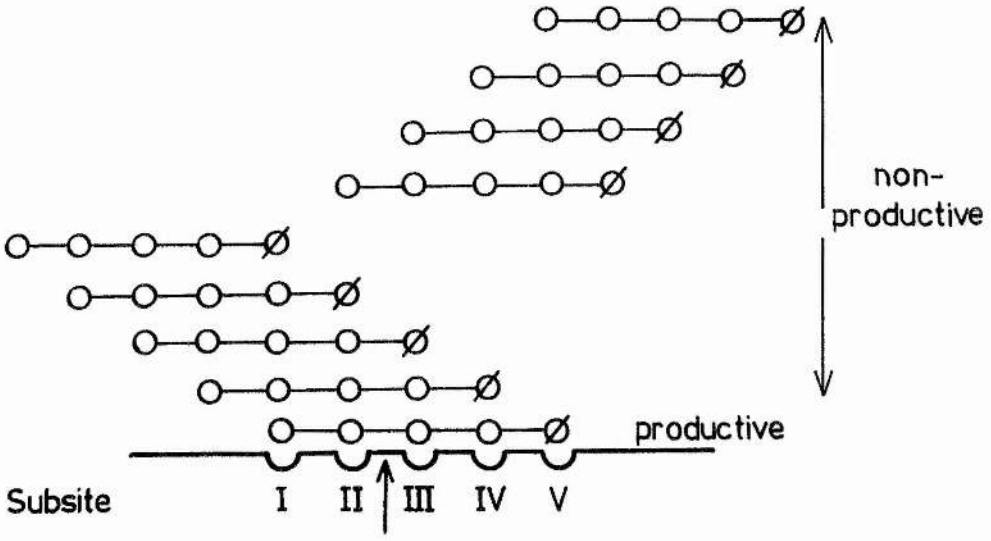
Cyclohexaamylose was found by Thoma and Koshland (32) to be a good competitive inhibitor and was thought to act by effectively mimicking the internal residues of an amylose chain. These authors proposed that on forming a reactive enzyme-substrate complex, the substrate induced the active site of β-amylase into the correct configuration with the help of the C_4 hydroxyl group at the non-reducing terminal. The internal residues of the substrate chain and cyclohexaamylose are considered to be bound by the enzyme in an unreactive conformation by inhibiting the alignment of the catalytic groups into the active conformation. As the chain length is increased, the number of internal segments able to form unreactive enzyme-substrate complexes also increases and the extent of the so-called "self-inhibition" would be expected to rise. This aspect will be dealt with more fully in section 4.5.

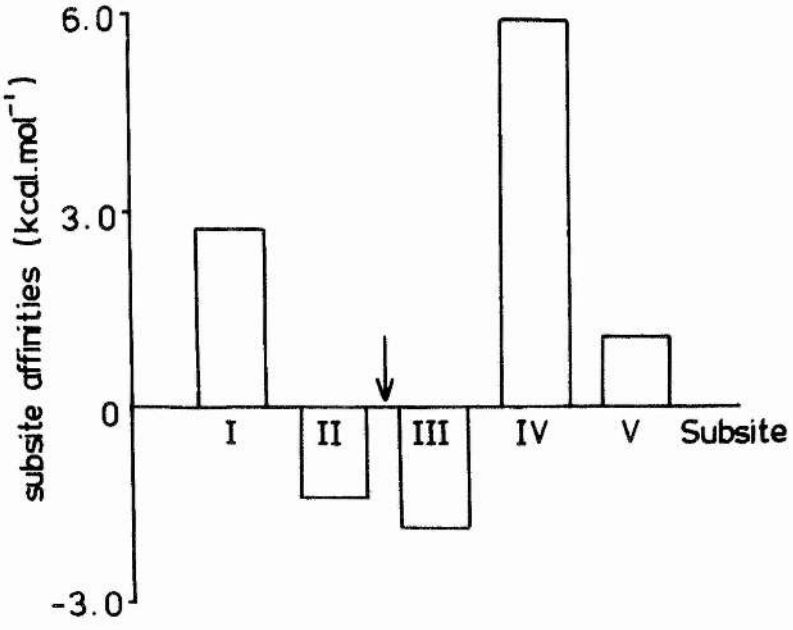
The predicted dependence of k_m upon $\bar{D}P$ was experimentally found by Thoma and Koshland (32) and by Hüsemann and Pfannemüller (33), who also found the expected variation in Vm for barley β-amylase, using large polymer substrates.

Such dependence of the Michaelis parameters of depolymerases upon substrate chain length originally led to the speculation that such enzymes have a binding region composed of a set of related subsites geometrically complementary to the monomer units of the polymer substrate (34). Lysozyme, which cleaves N-acetyl glucosamine polymer substrates, has been extensively investigated with respect to subsites and it was found that a substrate polymer composed of six N-acetyl glucosamine units fully occupied the active site

Fig. 1.1.4. Schematic representation of the productive and non-productive binding modes at the subsites of the active site of β-amylase. ↓ represents the catalytic site.

Fig. 1.1.5. Histogram of the affinities of the subsites of β-amylase for α-1,4-glucan substrates. ↑ represents the catalytic site.


non-
productive
productive
Subsite
I
II
III
IV
V


6.0
3.0
0
-3.0
subsite affinities (kcal.mol⁻¹)
I
II
III
IV
V
Subsite

cleft and that some distortion of the substrate occurred. It is now thought that substrate distortion plays an important role in the catalytic mechanism of amylases. (28, 35).

It is generally thought that between four and six subsites exist for β-amylase (32). Recently, Kato et al (36) estimated the number of subsites to be five for wheat bran β-amylase. A schematic representation of the binding modes at the subsites, showing the formation of productive and non-productive complexes together with self inhibition is shown in Fig. 1.1.4.

By using a series of linear substrates with a DP range of 2 to 12.5, Kato et al (36) established the presence of the catalytic site between subsites 2 and 3. These two subsites were found to have negative subsite affinities, therefore the contributions made to the positive affinity for the substrate by subsites 1 and 4 is important and indicates that the smallest most readily-cleaved substrate is maltotetraose.

Fig. 1.1.5 displays a histogram of the affinity of each subsite towards a maltopentaose substrate.

The subsite model could be used to explain the variation in km and Vm with substrate chain length (37) if it is assumed that enzyme action is directly proportional to the fraction of productive complexes and that all bonds lying across the catalytic site are cleaved at equal rates. As the substrate chain length is increased, the ratio of internal residues to terminal residues also increases and

the possibility of the correct bond being positioned across the catalytic site becomes less probable. Experimentally, (32, 36) it was found that the enzyme has a greater affinity for larger substrates, since the ratio of internal to external residues increases, but Vm decreases with increasing chain length due to self-inhibition as already described. Table 1.1.1 shows the values of Km and Vm collected from various authors and determined from the action of β-amylase upon substrates of increasing DP.

Amylases are considered to have a catalytic function in reversion or condensation reactions of oligosaccharides. Abdullah and French (39) reported that the action of β-amylase on maltose resulted in a weak maltotetraose spot after chromatography. Subsequent work on the reverse action of β-amylase was carried out by Hehre et al (40) who found that sweet potato β-amylase rapidly synthesized maltotetraose from β-maltose with a much slower rate of condensation using α-maltose. This corresponds to the known hydrolytic specifity of β-amylase in releasing β-maltose as exclusive product from α-1,4 glucans. The synthesis of maltosaccharides from β-maltose involves a product inversion comparable to that for the hydrolytic action, showing the condensation mechanism to be one of maltosyl transfer.

Table 1.1.1. KINETIC PARAMETERS FOR β-AMYLASE HYDROLYSIS OF SUBSTRATES OF INCREASING CHAIN LENGTH.

SUBSTRATE	$\bar{D}P$	K_m (M)	V_m (M/min)	Reference
Maltotriose	3	9.5×10^{-3}	1.2×10^{-5}	36
Maltopentaose	5	12.5×10^{-4}	8.7×10^{-4}	38
Maltohexaose	6	10.5×10^{-4}	8.6×10^{-4}	38
Maltoheptaose	7	2.9×10^{-4}	2.1×10^{-5}	36
Amylose	24	2.2×10^{-4}	7.2×10^{-4}	38
"	48	1.3×10^{-4}	6.0×10^{-4}	38
"	250	2.8×10^{-5}	3.1×10^{-4}	38
"	250*	1.0×10^{-5}	5.2×10^{-3}	38
"	760**	1.8×10^{-5}	2.5×10^{-4}	36
Amylopectin	24***	0.36****	1.33×10^{-2}	27

* bound to insoluble albumen for "infinite" molecular weight.

** number average $\bar{D}P$.

*** average outer chain length.

**** expressed as $mg.ml^{-1}$

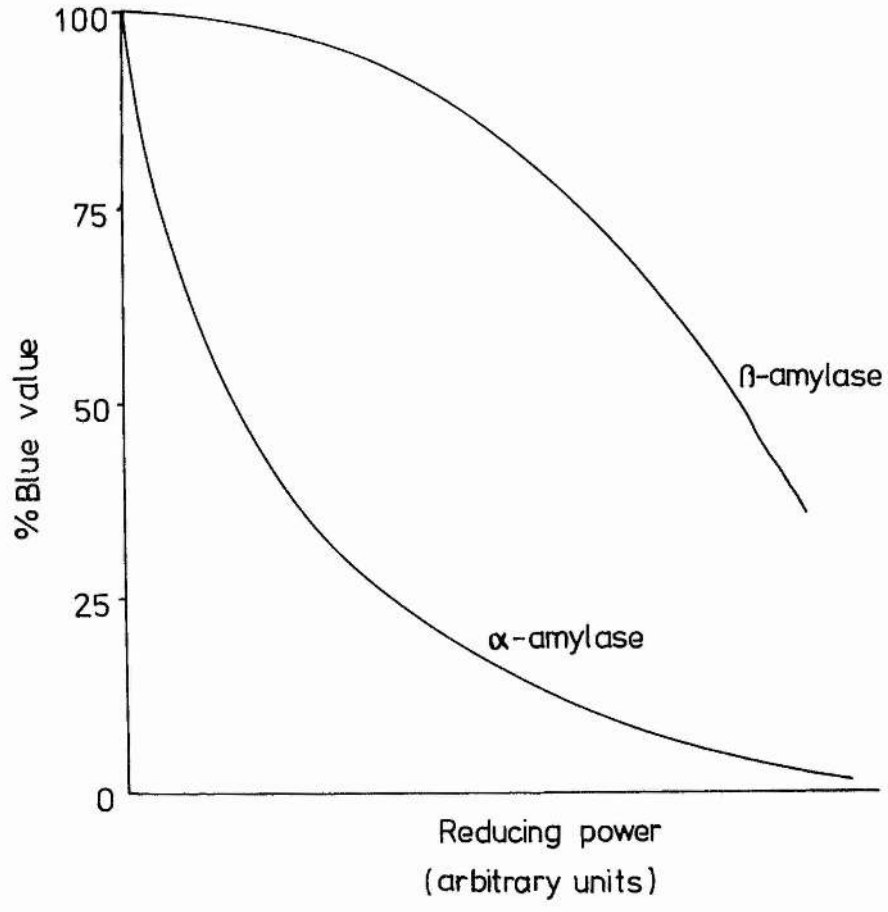
1.2. ASSAY OF β-AMYLASE ACTIVITY.

On account of the heterogeneous nature of polysaccharides, it is more usual to assay β-amylase activity by measuring the rate of release of β-maltose from an α-1,4 glucan substrate. Since there is no rapid method to specifically measure β-maltose, it is monitored by the more general methods for reducing sugars. Many of these are alkaline-oxidation methods, the simplest being the use of 3,5-dinitrosalicylic acid (D.N.S.A.) (41), which suffers from the disadvantage of being inaccurate at low sugar concentrations and requiring a large sample volume.

Other methods involve alkaline-copper reagents (42, 43), the most sensitive being the copper-neocuproine method (44) where neocuproine (2,9-dimethyl-1,10-phenanthroine) acts as a chelating agent for cuprous ions.

Useful information concerning the action patterns of amylases has been furnished by plots of the substrate blue value against the product reducing power during the course of hydrolysis. The blue value, or iodine absorption, of amylase is dependent upon the length of the helical regions in the polysaccharide, (45) a minimum of 25-40 glucose residues in helical arrangement being required for the blue colour, which has an absorption maximum at 610nm. The profiles obtained for different amylases are characteristic for their action patterns. α-Amylase displays an initially rapid decrease in blue value with a small increase in reducing power due to its initial random cleavage of internal bonds of the substrate, resulting in a decrease in the length of

Fig. 1.2.1. Illustration of the action patterns for α-amylase and β-amylase.


100
75
50
25
0
% Blue value
β-amylase
α-amylase
Reducing power
(arbitrary units)

helical regions. The second stage of α-amylase action, where shorter chain length substrates are hydrolysed more slowly by the enzyme, results in a rapid increase in reducing power compared to the relatively slow decrease in blue value (Fig. 1.2.1).

β-Amylase, which is an exo-acting enzyme, is thought to act by a multiple attack mechanism (Fig. 1.1.2). This results in virtually no change in the blue value of the amylose substrate during the initial stages of the reaction, but a large increase in reducing power is apparent, since approximately three maltose residues are removed per enzyme-substrate encounter (45). However, as the hydrolysis proceeds, the blue value steadily decreases with an increase in reducing power, resulting in an action pattern profile as shown in Fig. 1.2.1.

1.3. DETECTION OF ENDO-AMYLASE CONTAMINATION IN β-AMYLASE PREPARATIONS.

In the past, the detection of α-amylase contamination in an exo-amylase sample has depended upon product distribution patterns, thermal and pH-stabilities, differences in action patterns, or action upon limit dextrins.

As improved methods for detecting trace amounts of contaminants were developed, the purification techniques for enzymes also improved. The more successful of these analytical methods, involving the use of modified substrates, depend upon the ability of endo-amylases to by-pass structural modifications which otherwise block exo-amylase action.

1.3.1. CHROMOGENIC SUBSTRATES.

Those substrates which, after enzymic hydrolyses, release a coloured product either directly or upon the addition of a single reagent can be defined as chromogenic substrates (47).

The introduction of dye molecules at random points into glucan chains produces a substrate which is insoluble, chromogenic and upon which exo-amylases have virtually no activity.

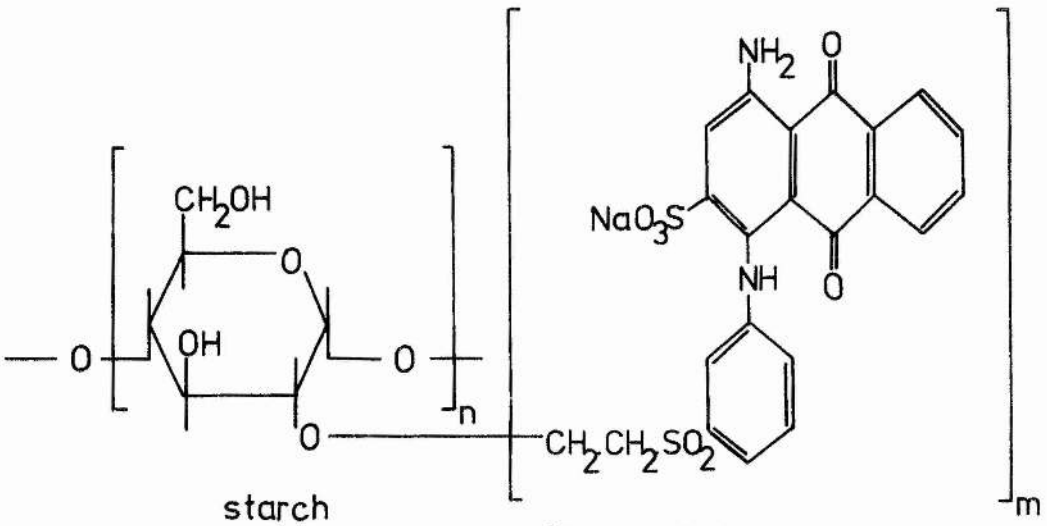
Rinderknecht et al (48) prepared a Remazolbrilliant Blue derivative of starch (Fig. 1.3.1.1.).

A second, commonly used, chromogenic substrate, Cibachron Blue amylose (Fig. 1.3.1.1) was shown by Marshall (49) to be almost inaccessible to amyloglucosidase but able to be hydrolysed by endo-amylases.

Fig. 1.3.1.1. Illustration of the structures of chromogenic substrates.

Remazolbrilliant Blue Starch

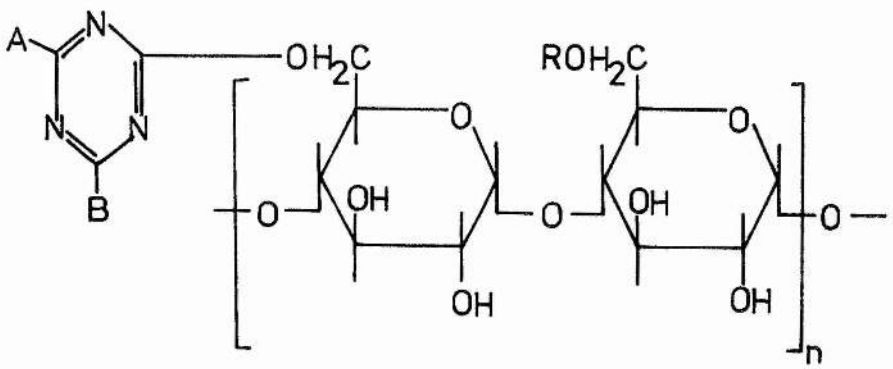
Cibachron Blue Amylose



starch

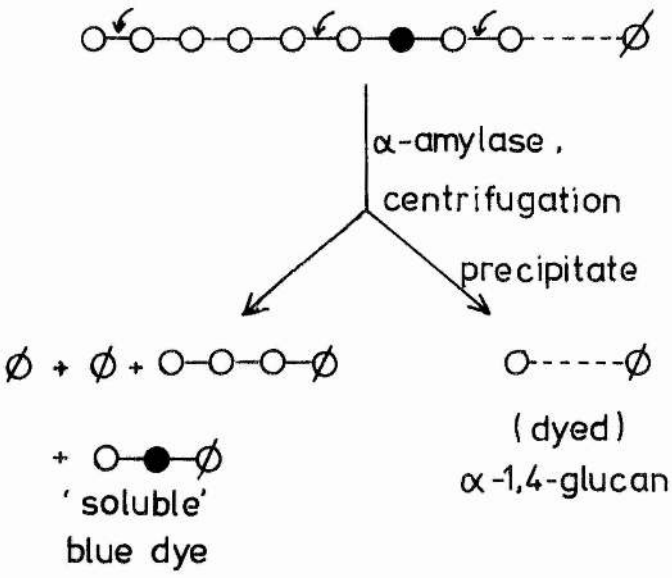
Remazolbrilliant Blue

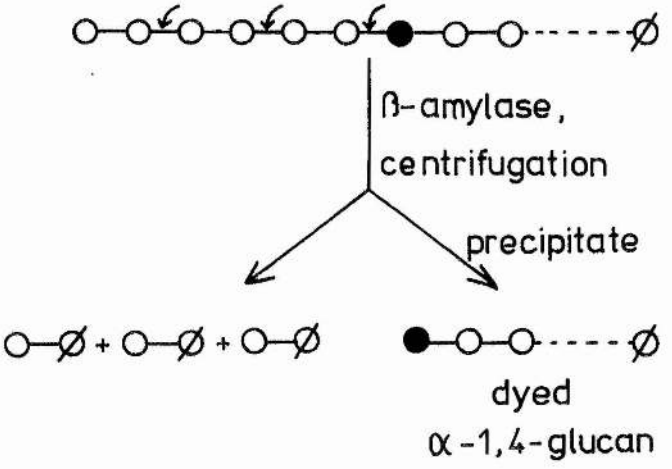
[m≪n]



R = H, triazinyl

Fig. 1.3.1.2. Schematic representation of the action of α- and β-amylases upon chromogenic substrates.





O—O α-1,4-linked glucose residues

Ø reducing terminal

● glucose residue with dye substitution

↙ position of enzymic attack

The action of α- and β-amylases upon such substrates is shown in Fig. 1.3.1.2. β-Amylase is able to release maltose units from the non-reducing terminal until it approaches a substituted glucose residue, where the bulk of the dye molecule sterically blocks further hydrolysis. On the other hand, α-amylase, through its ability to cleave internal bonds, can effectively by-pass the modifications, releasing short "dyed" oligosaccharide chains which are soluble and which can be monitored spectrophotometrically.

A combination of the older β-limit dextrin assay for the detection of α-amylase, and of the use of chromogenic substrates was proposed by Bilderback, (50) who prepared an amylopectin azure β-limit dextrin. However, this does not appear to have any advantage over the usual chromogenic substrates which β-amylase is unable to hydrolyse to any great extent, as already described. The disadvantage in preparing the "dyed" limit dextrin would be the prerequisite for an endo-amylase-free β-amylase preparation, with apparently no criteria for establishing the purity of the exo-amylase sample.

1.3.2. PARTIALLY OXIDIZED SUBSTRATES.

The controlled action of sodium metaperiodate upon α -1,4 glucans results in the random oxidation of a number of the glucosyl residues, (Fig. 1.3.2.1) the extent of oxidation depending upon the amount of periodate used.

The idea of the random oxidation of polysaccharide residues by periodate was applied to α-1,4 glucans by

Drummond et al (31) who found that α-amylase hydrolysed an amylose sample, which had 5% of its glucose residues oxidized, at only a slightly lower rate than the unoxidized substrate, whereas β-amylase cleaved only 6% of the maximum available maltosidic bonds compared with 75% for the original substrate (Fig. 1.3.2.2).

Marshall and Whelan (51) developed the principle into a general method for the detection of endo-acting amylases in the presence of exo-enzymes acting on the same substrate.

Fig. 1.3.2.1. Illustration of the effect of periodate oxidation upon the glucosyl residues of α -1,4-glucans.

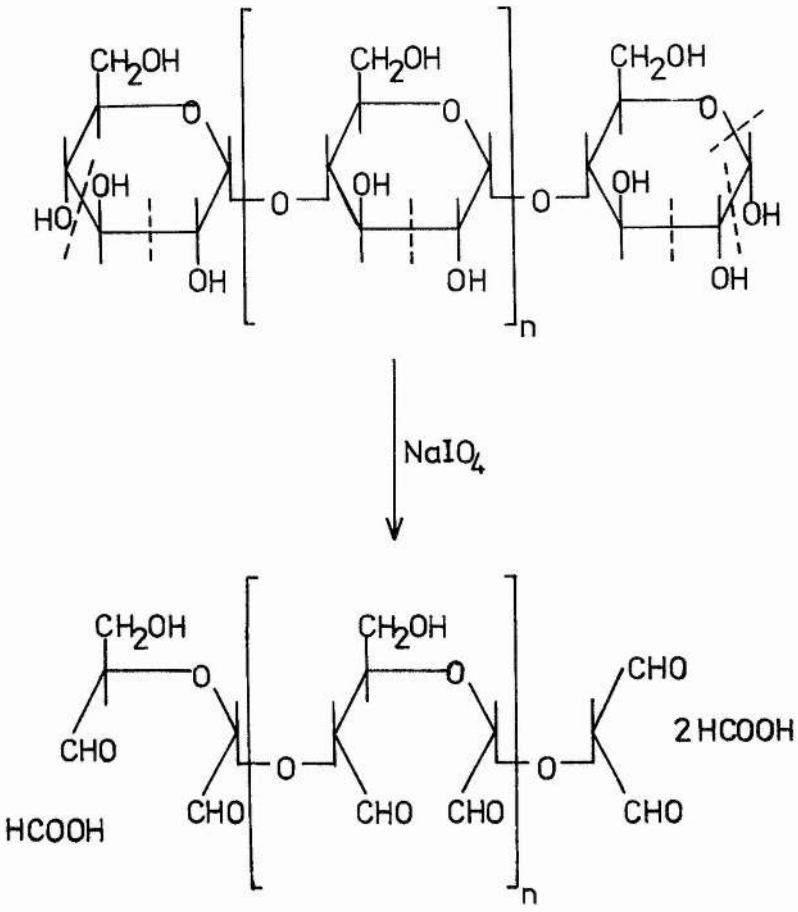
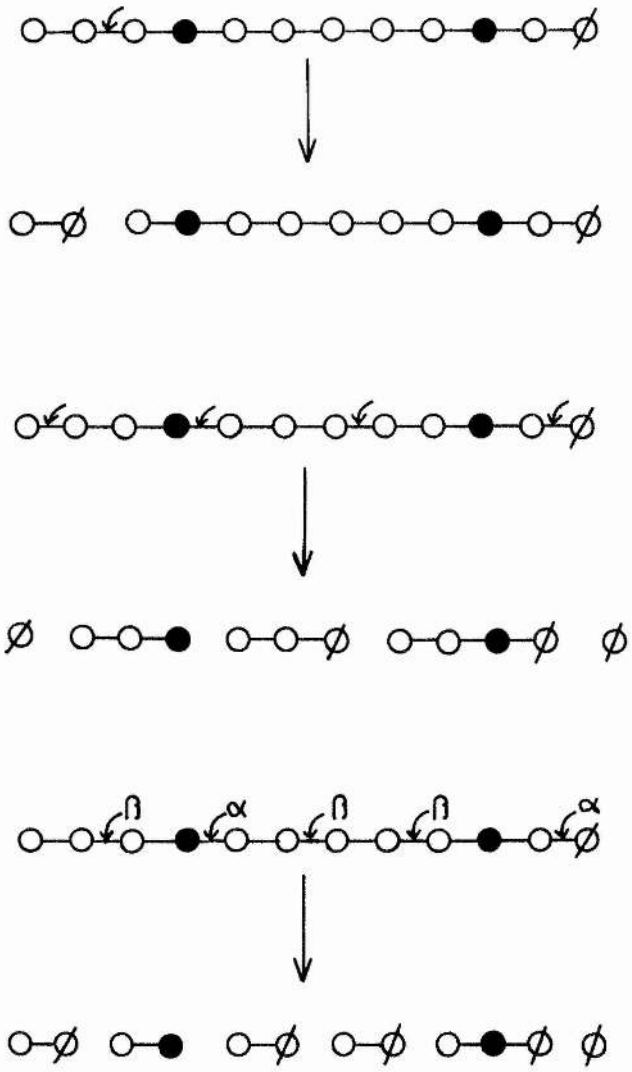

CH2OH
O
OH
HO
OH
n
NaIO4
CHO
2 HCOOH
HCOOH

Fig. 1.3.2.2. Schematic representation of the combined and separate actions of α- and β-amylases on oxidized α-1,4-glucans:

β -amylase

α -amylase

α -amylase + β-amylase



○—○ α-1,4-linked glucose residue

Ø reducing terminal

● oxidized glucose residue

↙ position of enzymic attack

1.4. RECENT PURIFICATION TECHNIQUES.

In the past, the separation of α-and β-amylases has posed problems, although techniques frequently used took advantage of their different physical and chemical properties. α-Amylase could be selectively denatured at acidic pH and low temperatures, or by proteolytic digestion coupled with the removal of stabilizing Ca^{++} ions by EDTA.

In the mature seed, β-amylase is stored adjacent to the aleurone layer, covalently attached to glutenin. The increase of β-amylase activity in water or salt extracts during the malting process is generally regarded as a transformation of "bound" barley enzyme into "free" β-amylase of malt. Both forms are considered to be present in the ungerminated grain and can be preferentially extracted (52), the bound enzyme being released from a larger protein aggregate or from cross-linked sulphydryl bonds by the use of papain or thiols, resulting in an increase in enzyme activity of the extract.

Previous work has indicated that barley β-amylase is a multicomponent system (53) when isolated in the absence of thiols. Addition of thioglycollic acid to the heterogeneous system resulted in only the smallest component being present, suggesting that the larger components were aggregates of the smallest.

The origin of cereal β-amylase has been investigated. It has been shown (54) that no new β-amylase synthesis occurs during the germination of wheat and it is widely accepted that the bound enzyme in barley is released by proteolytic enzymes acting specifically at disulphide bonds and that the enzyme is not synthesized de novo.

β-Amylase has been extracted from barley with reducing agents and has been purified and crystallized by Visuri and Nummi (55), but no comparable crystalline preparation has been carried out for the malt preparation, although β-amylase has been isolated from malt by LaBerge and Meredith (55). Both methods involved the sequential use of gel filtration and ion-exchange chromatography.

Little kinetic data has been accumulated for comparably purified preparations, making it difficult to compare the enzyme purified in different laboratories, although the specific activity (222U.mg dry weight^{-1}) obtained by Visuri and Nummi (54) compares favourably with that obtained by Meyer et al (57).

TABLE 1.5.1. SUMMARY OF IMMOBILISATION TECHNIQUES USED IN THE PREPARATION OF INSOLUBLE β-AMYLASE DERIVATIVES.

METHOD OF COUPLING	ENZYME UNITS. mg FREE PROTEIN^{-1}	BOUND PROTEIN (mg.100mg derivative^{-1})	ENZYME UNITS. mg BOUND PROTEIN^{-1}	ACTIVITY (%) RETAINED AFTER COUPLING	REF
Chemical coupling to microcrystalline cellulose	488.7	1.75	1232	25.1	63
Chemical coupling to polyacrylamide beads	431.2	3.2	6.4	1.5	64
Covalent coupling to acrylamide-acrylic acid copolymer	212.4	0.68	48.6	23*	62
Covalent coupling to cross-linked Sepharose beads	60	8.0	12.0	20	61
Covalent coupling to Sephadex G-200	n.s.	2.3	inactive	inactive	60

*calculated from data
n.s. not stated.

1.5. IMMOBILISATION OF β-AMYLASE

The preparation and uses of immobilised enzymes have been extensively reviewed in recent years (58, 59). Four principal methods have been used to prepare biologically active, immobilised enzymes:

1. entrappment within gel matrices, the pores of which are too small to allow escape of the protein,
2. physical adsorption on to solid surfaces of inert carriers,
3. covalent attachment to a water-insoluble carrier or to itself via a suitable bifunctional reagent, and
4. microencapsulation within semi-permeable membranes.

Immobilisation of an enzyme and the study of its resulting activity could facilitate the detection and isolation of intermediates formed at the early stages of an enzymic reaction. It may also be expected that the properties of the enzyme would provide information about the mode of action of enzymes present in cell membranes since a large number of enzymes are thought to exist bound to the membrane or other subcellular particles.

Numerous insoluble derivatives of amylolytic enzymes have been prepared, but only those employing β-amylase will be dealt with here. Information on insoluble β-amylase derivatives is given in Table 1.5.1.

One of the first insoluble β-amylase derivatives was prepared by Axén and Porath (60) using isothiocyano-Sephadex G-200 as a support coupled to the enzyme through α-amino groups or the ε-amino group of lysine residues. The preparation was biologically inactive with large

polymer substrates, since the substrate was excluded from the active site due to steric hinderance. However, it was found that low molecular weight substrates were hydrolysed more efficiently.

Such steric restrictions have partially been overcome by the use of "spacer" molecules to remove the enzyme from close proximity to the carrier surface. By coupling cyanogen bromide-activated Sepharose with aromatic spacers, Vretblad and Axén (61) obtained an active β-amylase derivative which was used for the continuous hydrolysis of dilute starch solutions over a period of 45-50 days at 23°C, and which retained 90% of its original activity over this time.

Mårtensson (62) prepared a β-amylase derivative leading to an immobilised preparation of β-amylase and pullulanase, which is considered to be of industrial interest for the complete hydrolysis of starch.

Barker et al (63, 64, 65) have undertaken a large number of investigations into the immobilisation of various amylolytic enzymes, including β-amylase, which they immobilised using cellulose or polyacrylamide supports.

A wide range of hydrophobic and hydrophilic supports can be used for immobilisation. The nature of the support is often important in determining the activity of the bound enzyme (66, 67, 68). The effect of steric hinderance and charge effects upon enzyme activity are important for polymer-degrading enzymes and will be discussed later (section 4.9) with respect to β-amylase. However, proteolytic enzymes have been found to display a lower specific activity towards proteins after immobilisation, but virtually no change in

their activity towards lower molecular weight substrates (58).

The maximum percentage of activity retained after coupling (35%) is reported by Vretblad and Axén (61) using Sepharose 6B and 4,4' methylene dianiline with crude β-amylase. Contamination of the preparation by α-amylase or a low-specificity α-glucosidase may account for such a high retention of activity after the conditions of coupling (6h, 23°C, pH 6.0).

The stability of immobilised β-amylase preparations would appear to depend upon the method of coupling and the nature of the support. Preparations of greater thermal stability compared with the free enzyme were reported using Sepharose beads (61), acrylamide-acrylic acid copolymers (62) and microcrystalline cellulose (63). The use of polyacrylamide beads (64) produced an enzyme derivative which was less stable at 45°C than the soluble enzyme.

Few reports exist on the effect of immobilisation upon the pH optima and Michaelis constants, which are important in assessing the potential use of an immobilised preparation. Vretblad and Axén (61) reported a broader pH profile for the immobilised barley β-amylase compared with the free enzyme. In the β-amylase-pullulanase system (62) conditions were such that the β-amylase component was rate-limiting. Mårtensson found an apparent Michaelis constant (Km') six times larger than the Km value (1.2mg.ml^{-1}) for the corresponding free enzyme system, and a k_i' value for maltose (14.6 mM) approximately twice the k_i value (7.6mM) for the free enzyme.

However, it is difficult to compare and contrast different immobilised β-amylase preparations, since the substrates and assay conditions were not standardized. Until standardization of basic experimental conditions is achieved, little significance can be placed on results originating from different sources.

2. MATERIALS.

The chemicals used in this thesis are listed below in the relevant sections, together with abbreviated names of the suppliers. The full names and addresses of the suppliers are given at the end of this section.

Unless otherwise specified, the chemicals listed are of laboratory grade.

Substrates

Amylopectin (potato starch)	B.D.H.
Amylopectin azure (A Grade)	Calbiochem
Amylose type I (potato)	Sigma
Amylose azure (B Grade)	Calbiochem
Maltose	B.D.H.
Soluble starch (Analar)	B.D.H.

Enzymes and proteins.

α-Amylase, type IIA	Sigma
β-Amylase, crude, type IIA (barley)	Sigma
β-Amylase, α-amylase-free (B Grade)	Calbiochem
Bovine serum albumen, fraction V	Sigma
Glucose oxidase, type II	Sigma
Lysozyme (grade I)	Sigma
Peroxidase, horseradish, crude	Sigma

Chromatography components.

Amberlite resins	B.D.H.
Celite 535	Hopkin and Williams
Charcoal	May and Baker
DEAE-cellulose (DE 32)	Whatman
Dithiothreitol	Sigma
EDTA	B.D.H.

2-Mercaptoethanol, type I	Sigma
Sephadex G-25 (fine)	Pharmacia

Supports and coupling agents.

AE-cellulose (Cellex-AE)	Bio Rad Laboratories
N-ethyl morpholine	B.D.H.
Polystyrene beads	Shell
Sodium dithionite	Fisons

Miscellaneous.

Dimethyl sulphoxide, grade I	Sigma
DNSA	B.D.H.
Folin-Ciocalteau reagent	B.D.H.
Glycine	B.D.H.
Neocuproine-hydrochloride	Sigma
o-Dianisidine-dihydrochloride	Sigma
Sodium borohydride	Fisons
Sodium metaperiodate	B.D.H.
Thymol	B.D.H.

All other reagents used were of Analar grade. All solvents were redistilled before use and dried over anhydrous sodium sulphate, except in the case of pyridine, which was stored over sodium hydroxide pellets.

Bio-Rad Laboratories Ltd., Bromley, Kent, England

B.D.H. Chemicals Ltd., Poole, England

Calbiochem, Los Angeles, U.S.A.

Fisons Scientific Apparatus Ltd., Loughborough, England

Hopkin and Williams, Chadwell Heath, Essex, England

May and Baker Ltd., Dagenham, Essex, England

Pharmacia (G.B.) Ltd., London, England

Shell Chemical Co. Ltd., London, S.E.1, England

Sigma (London) Chemical Co. Ltd., Surrey, England

Whatman Biochemicals Ltd., Kent, England.

3. METHODS.

3.1. ASSAY OF β-AMYLASE ACTIVITY.

β-Amylase activity was measured routinely by assaying the rate of release of reducing equivalents (maltose) during incubation at 37° with an α-1,4-glucan substrate. Typically, the composition of the incubation medium was 1% (w/v) soluble starch in 0.1M-acetate buffer, pH 4.8.

At appropriate time intervals, an aliquot of the incubation mixture was withdrawn and the maltose content assayed by either the dinitrosalicylic acid method (3.3.1) of Bernfeld (41) or the copper-neocuproine method (3.3.2) of Dygert et al (44).

The unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of maltose per minute under the specified conditions.

3.2. METHODS FOR PROTEIN DETERMINATION.

Protein determinations were carried out by one of several methods which generally involved alkaline-copper solutions.

During the course of enzyme purification (3.8), β-amylase was recovered by dialysis against ammonium sulphate solutions. To determine the protein content of solutions or suspensions of the enzyme, measurements were made together with a control to take into account the ammonium salt present in solution, since such determinations are considered to be affected to some degree by the presence of ammonium sulphate. The same is true for thiol-containing

compounds, therefore a similar control was prepared using suitable dilutions of mercaptoethanol or dithiothreitol.

All protein determinations for β-amylase were carried out together with one of a standard protein solution.

3.2.1. FOLIN-LOWRY ESTIMATION.

This method was developed by Lowry et al (69) involving the reaction of copper in alkaline solution and the reduction of Folin-Ciocalteau reagent.

Reagents:

Solution A: 2% (w/v) sodium carbonate in 0.1M NaOH.

Solution B: 1g sodium tartrate was dissolved in water with 0.5g cupric sulphate pentahydrate, 10 ml 1M NaOH were added and the solution made up to 100 ml.

Solution C: 50 ml A + 1 ml B.

Solution D: Folin-Ciocalteau reagent, diluted to 1M with distilled water.

Assay:

A calibration plot (Fig. 3.2.1.1) was prepared using a standard solution (500 $\mu g.ml^{-1}$) of lysozyme in distilled water. A 0.5 ml aliquot of protein solution (10-100μg) was added to 5 ml solution C, mixed well and left for 10 min, after which time 0.5 ml solution D was added. The solutions were mixed again, left for 30 min and the absorbance measured at 500 and 750 nm against a control in which distilled water replaced the protein solution.

3.2.2. BIURET ESTIMATION.

Llayne (70) first described the Biuret method which depends upon a reaction between an alkaline-copper solution and peptides or proteins, with a minimum requirement for two peptide bonds, to produce a purple colour with an absorption maximum at 540 nm.

Reagents:

Biuret reagent was prepared as follows:

Cupric sulphate pentahydrate (0.15g) was dissolved in 50 ml distilled water with sodium potassium tartrate (0.6g). To this solution was slowly added 10% (w/v) NaOH solution (30 ml) and the solution made up to 100 ml with distilled water.

Assay:

A 1 ml aliquot of protein solution, not exceeding 10 $mg.ml^{-1}$, was mixed with 4 ml Biuret reagent and left at room temperature (20-25°) for 30 min. The absorbance of the resulting solution was read at 540 nm against a control in which distilled water replaced the protein solution.

A calibration plot (Fig. 3.2.2.1) was prepared for a range (1-9 $mg.ml^{-1}$) of lysozyme concentrations.

3.2.3. MODIFIED BIURET ESTIMATION.

A disadvantage of the Biuret and especially of the Folin-Lowry methods for protein determination is the variability of colour intensity between different proteins, therefore making the choice of protein for the calibration plot important.

Itzhaki and Gill (71) suggested an alternative method for protein estimation, essentially a modification of the Biuret method, which gave less variability between different proteins but at the same time gave a reduction in accuracy.

Reagents:

Solution 1: 0.21% (w/v) cupric sulphate pentahydrate in 30% (w/v) NaOH.

Solution 2: 30% (w/v) NaOH.

All protein standard solutions were made up in 12% (w/v) NaOH.

Assay:

Sets of solutions were prepared as follows:

A_1 : - 2 ml H_2O + 1 ml Solution 1.

A_2 : - 2 ml protein solution + 1 ml Solution 1.

B_1 : - 2 ml H_2O + 1 ml Solution 2.

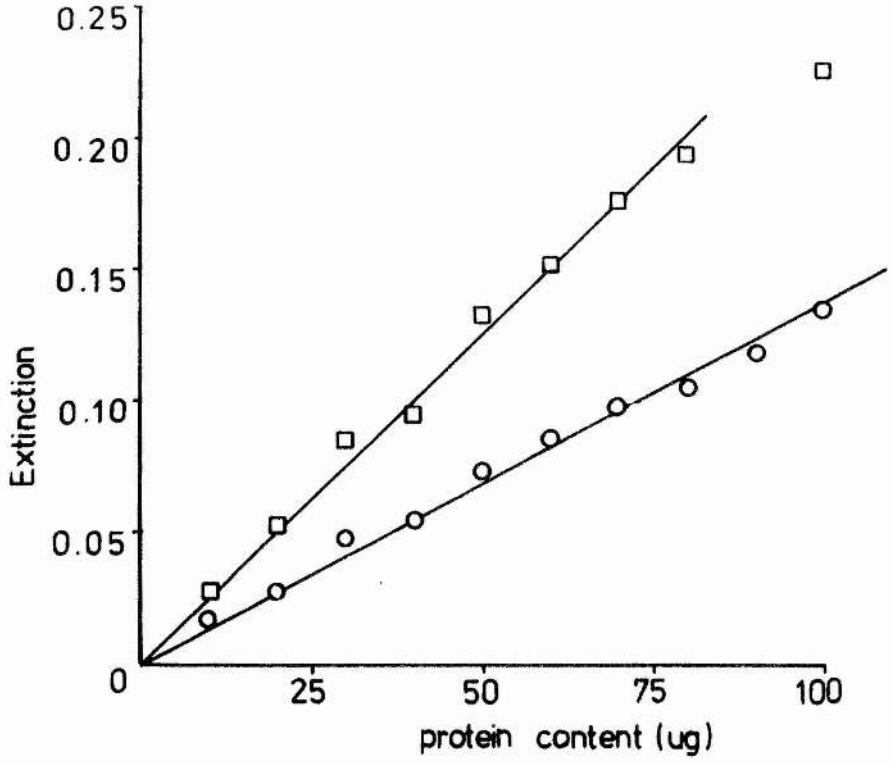
B_2 : - 2 ml protein solution + 1 ml Solution 2.

The solutions were mixed well and left for 5 min to allow for colour development. The absorbances were read at 310 nm, reading A_2 against A_1 and B_2 against B_1, giving values D_A and D_B respectively.

A calibration plot (Fig. 3.2.3.1) was prepared for lysozyme, bovine serum albumen and α-amylase in a concentration range of 0.2 - 1.5 mg. The difference, $D_A - D_B$, was plotted as Δ absorbance against protein concentration for each protein.

Fig. 3.2.1.1. Calibration plot for the estimation of protein by the method of Lowry *et al.*, at 500nm (○) and at 750nm (□).

Fig. 3.2.2.1. Calibration plot for the estimation of protein by the Biuret method.


0.25
0.20
0.15
0.10
0.05
0
Extinction
25
50
75
100
protein content (ug)

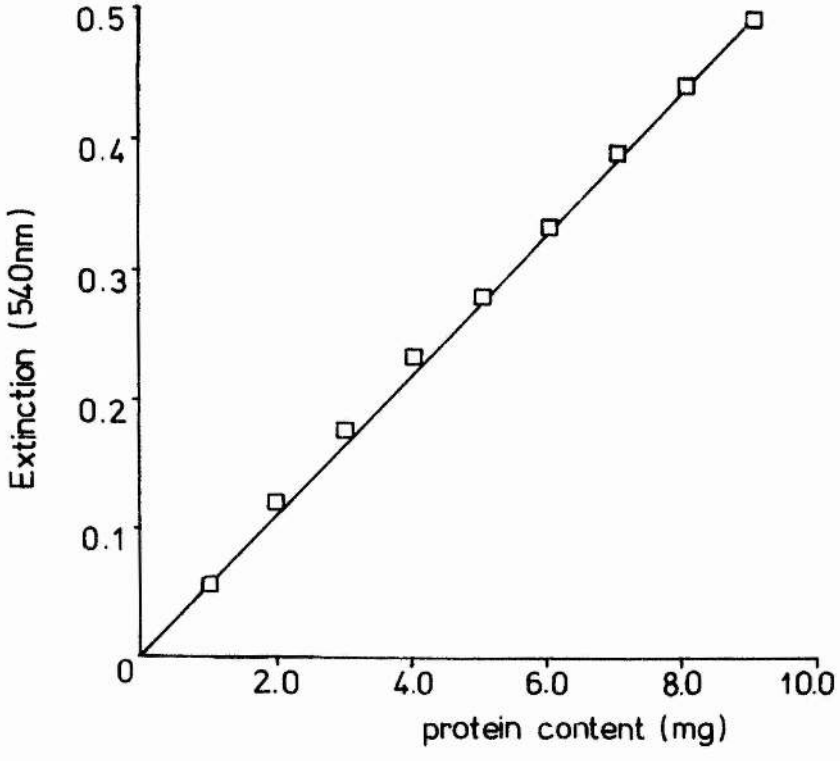
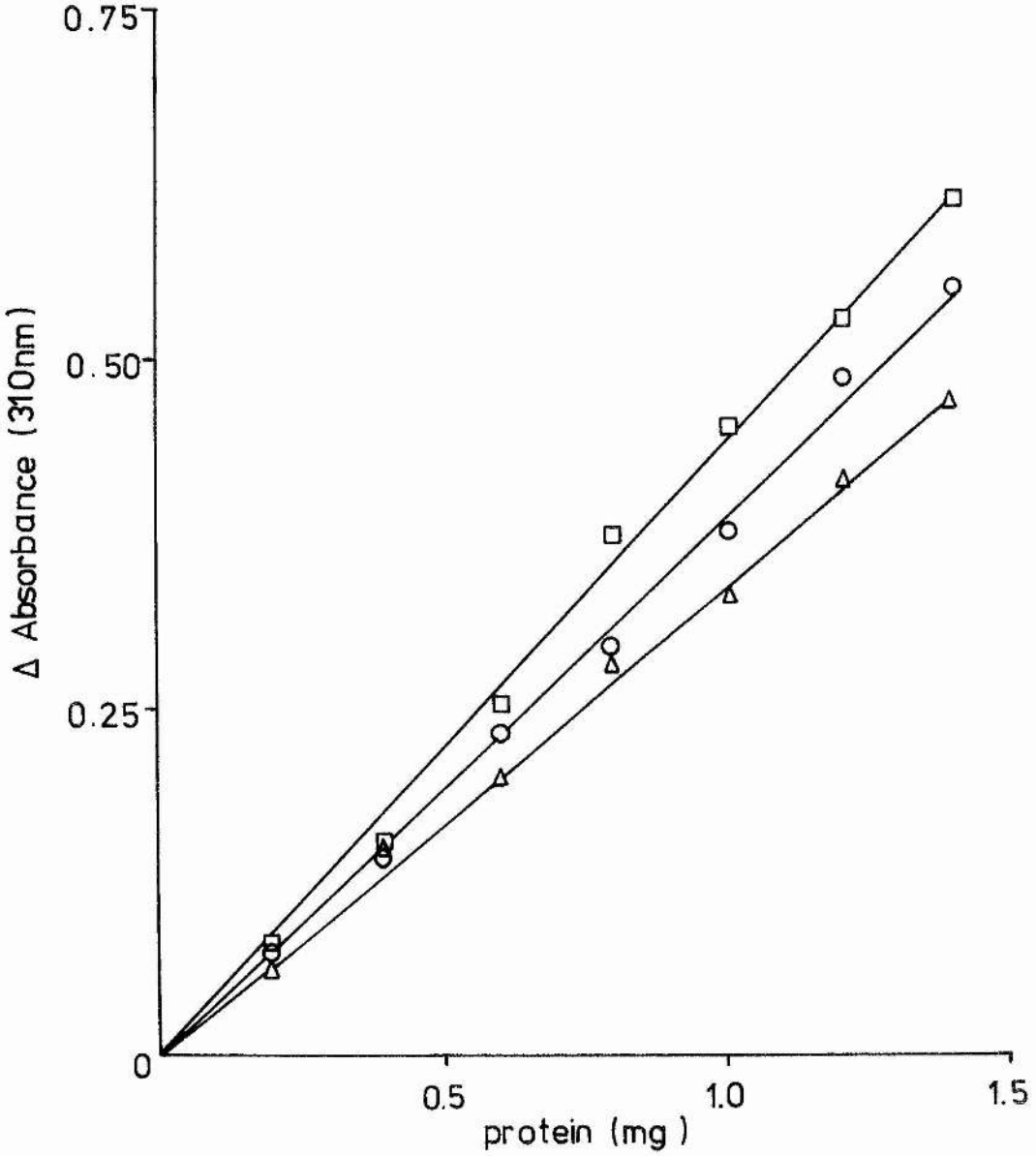

0.5
0.4
0.3
0.2
0.1
0
Extinction (540nm)
2.0
4.0
6.0
8.0
10.0
protein content (mg)

Fig. 3.2.3.1. Calibration plot for the estimation of protein by the modified Biuret method using lysozyme (□), bovine serum albumen (Δ) and α-amylase (O).


0.75
0.50
0.25
0
Δ Absorbance (310nm)
0.5
1.0
1.5
protein (mg)

3.2.4. ULTRAVIOLET ABSORBANCE.

During later stages of enzyme purification, to quantitatively assay the column effluent, the absorbance of the β-amylase solution at 280 nm was measured. According to Visuri and Nummi (55) pure β-amylase has a specific extinction coefficient

$$E_{1cm}^{1\%} = 15.0 \text{ at } 280 \text{ nm.}$$

This information was used to determine the concentration of β-amylase in solution.

3.3. DETERMINATION OF REDUCING SUGARS.

3.3.1. DINITROSALICYLIC ACID (DNSA) METHOD.

The DNSA method of Bernfeld (41) is based upon the reduction of 3,5-dinitrosalicylic acid by reducing sugars to 3-amino, 5-nitrosalicylic acid. Although the reduced compound has a maximum absorbance at 440 nm, its appearance was measured at 540 nm, where 3,5-dinitrosalicylic acid gave a minimum reading and at which wavelength there was most difference between the spectra of the two compounds.

Reagents:

DNSA reagent was prepared as follows:

100 ml NaOH (2M) was heated almost to boiling point and 5g DNSA added with stirring. When this was completely dissolved, 50 ml distilled water and 150g sodium potassium tartrate were added. The cool solution was made up to 500 ml with distilled water.

Assay:

A 2 ml aliquot of reducing sugar solution was mixed with an equal volume of DNSA reagent, heated for 5 min in a boiling water bath and cooled in ice and water. The solutions were diluted to 25 ml with distilled water and the absorbances measured at 540 nm against a control in which distilled water replaced the sugar solution.

A calibration plot (Fig. 3.3.1.1) was prepared for a range (0.5-12.5μmol) of maltose solutions in distilled water.

To assay for the production of maltose in an incubation mixture, account was taken of the control values produced both by α-1,4-glucan substrate and by mercapro-

ethanol or dithiothreitol, which, on account of possessing reducing groups, interfered with the colour reaction.

3.3.2. NEOCUPROINE METHOD.

The method was devised by Dygert et al (44) with the advantage of requiring a small sample volume and resulting in increased sensitivity compared to the DNSA method.

Due to the greater sensitivity, thiols and α-1,4-glucans produced substantial interference in the reduction of the cuprous ions involved in the colour reaction. Therefore, controls were used as in the previous method.

Reagents:

Solution A: 40g anhydrous sodium carbonate were dissolved in 600ml distilled water, followed by 16g glycine then 0.45g cupric sulphate pentahydrate. The solution was made up to 1 litre with distilled water.

Solution B: 0.12g neocuproine-HCl was dissolved in 100ml distilled water.

Assay:

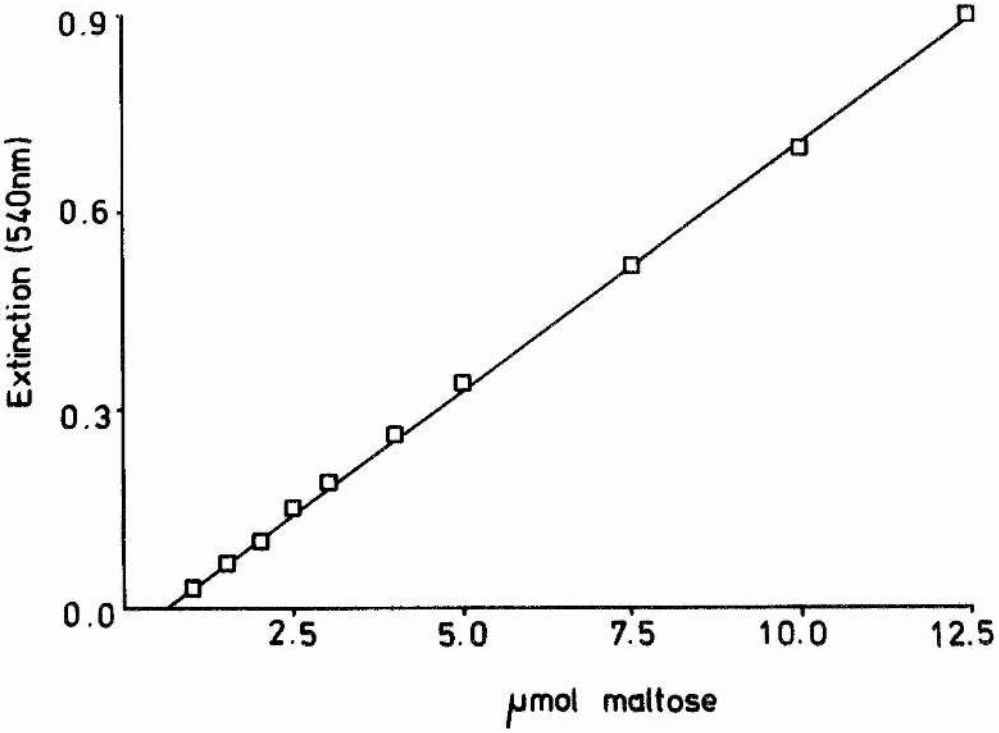
Aliquots (0.1ml) of reducing sugar solutions were mixed with equal volumes of solutions A and B as indicated below, heated for 10 min in a boiling water bath and cooled to room temperature under running water. The solutions were diluted to 25 ml and the absorbance measured at 450 nm against a control in which distilled water replaced the sugar solution.

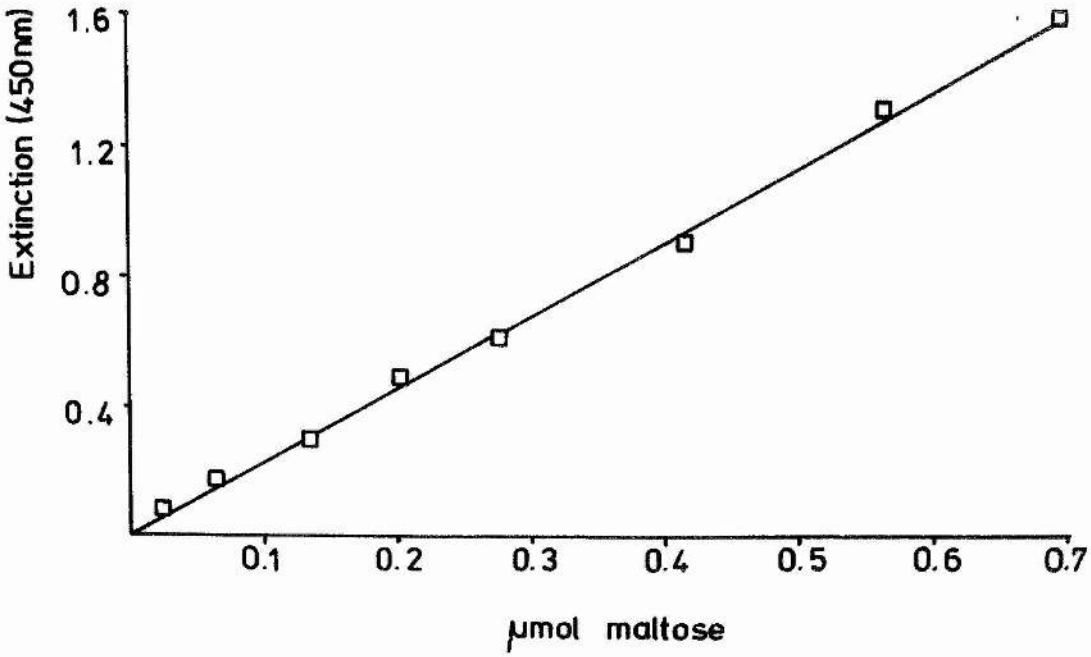
A calibration plot (Fig. 3.3.2.1) was prepared using maltose solutions (0.02-0.75 µmol), with volumes of solutions A and B as follows:

µmol maltose	ml A	ml B
0.02 - 0.15	1	1
0.16 - 0.3	2	2
0.31 - 0.45	3	3
0.46 - 0.6	4	4
0.61 - 0.75	5	5

Fig. 3.3.1.1. Calibration plot for the estimation of maltose by the DNSA method.

Fig. 3.3.2.1. Calibration plot for the estimation of maltose by the neocuproine method.


Extinction (540nm)
0.9
0.6
0.3
0.0
2.5
5.0
7.5
10.0
12.5
µmol maltose


Extinction (450nm)
1.6
1.2
0.8
0.4
0.1
0.2
0.3
0.4
0.5
0.6
0.7
µmol maltose

The glucose oxidase method for the estimation of glucose levels in solution (72) is based on the following sequence of reactions:

$$\text{glucose} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{oxygen acceptor} \xrightarrow{\text{peroxidase}} H_2O + \text{oxidized acceptor}$$

In this method, the oxygen acceptor used is o-dianisidine.

Commercially - available samples of glucose oxidase often contain traces of α-1,4-glucanase activity which may cause anomalous results if glucose is assayed in the presence of α-1,4-glucans. Lloyd and Whelan (72) overcame this problem by the use of Tris as a component of Tris-phosphate buffer.

Reagents:

Tris-phosphate-glycerol buffer:

36.3g Tris and 50g sodium dihydrogen phosphate monohydrate were dissolved in distilled water and 400ml glycerol added. The solution was made up to 1 litre with distilled water and titrated to pH 7.0 by the addition of solid sodium dihydrogen phosphate.

Glucose oxidase reagent:

30mg glucose oxidase, 3mg horseradish peroxidase and 100mg o-dianisidine dihydrochloride were dissolved in 100ml Tris-phosphate-glycerol buffer.

Assay:

A 1ml sample of a solution containing not more than 70µg of glucose was mixed with 2ml glucose oxidase reagent

and incubated at 40° for 30 min. After this time, 4ml 5M-HCl were added to stop the reaction and the absorbance was measured at 525nm against a reagent blank.

The calibration plot is shown in Fig. 3.4.1.

3.5. DETERMINATION OF TOTAL CARBOHYDRATE BY THE PHENOL-SULPHURIC ACID METHOD.

The phenol-sulphuric acid method of Dubois et al (73) is based on the dehydration of pyranose and furanose ring structures in sugars by concentrated sulphuric acid. On account of the sensitivity of this method, great care is necessary in avoiding contact with cellulosic materials which can affect the intensity of the colour produced.

Reagents:

Solution A: 5% (w/v) phenol solution in distilled water.

Solution B: 96% sulphuric acid (sp.gr. 1.84)

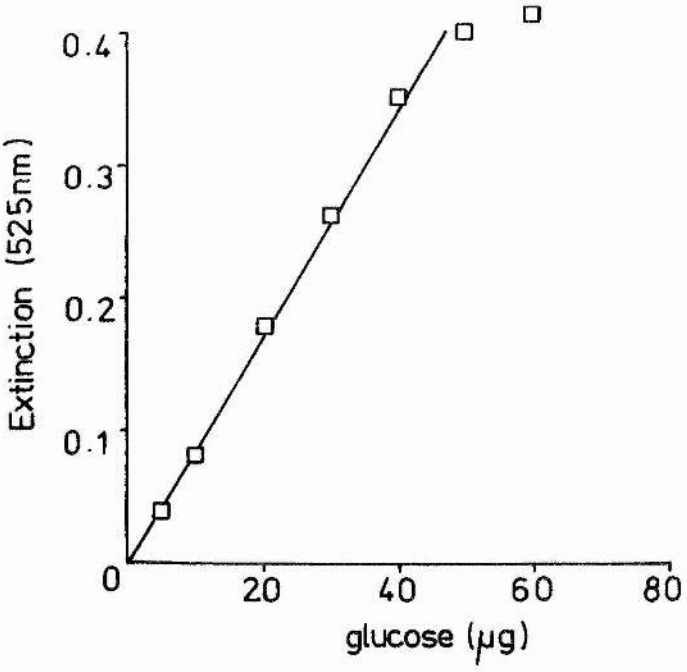
Assay:

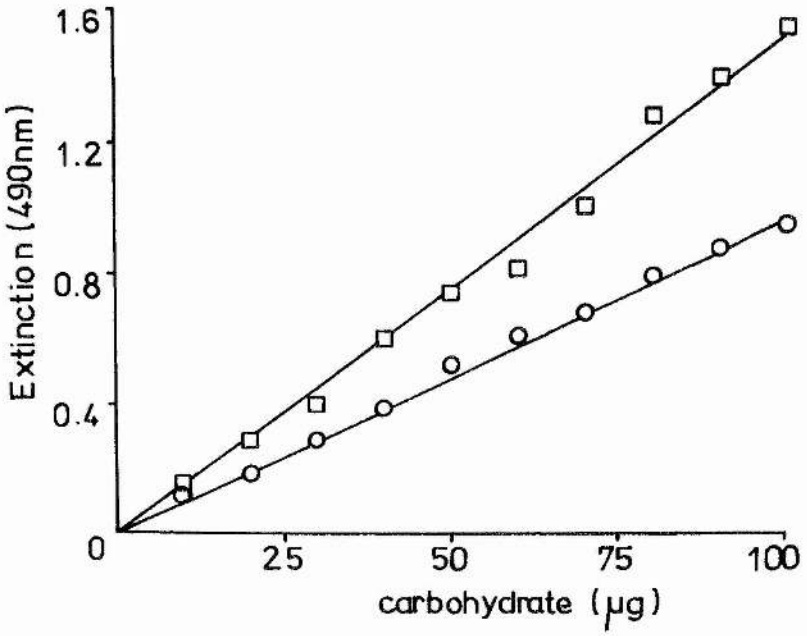
A 1ml sample of carbohydrate solution (10-100µg) in distilled water was mixed with 1ml solution A and placed in ice. From a fast-flowing pipette, 5ml solution B was added to ensure good mixing and even heat distribution. A control was prepared, replacing the carbohydrate solution with distilled water. After 10 min the tubes were shaken and incubated at 25° for 20 min. The absorbance of the resulting solutions was measured at 490 nm. against the control.

A calibration plot, prepared for glucose and soluble starch to compare the effects of chain length, is shown in Fig. 3.5.1.

Fig. 3.4.1. Calibration plot for the estimation of glucose by the glucose oxidase method.

Fig. 3.5.1. Calibration plot for the estimation of total carbohydrate by the phenol-sulphuric acid method using glucose (○) and soluble starch (□).


Extinction (525nm)
0.4
0.3
0.2
0.1
0
20
40
60
80
glucose (µg)


Extinction (490nm)
1.6
1.2
0.8
0.4
0
25
50
75
100
carbohydrate (µg)

3.6. PREPARATION OF MODIFIED SUBSTRATES.

3.6.1. DIALDEHYDE AMYLOSES.

The action of sodium metaperiodate on α-1,4-glucans results in the random oxidation of the glucose residues as already described in section 1.3 (Fig. 1.3.2.1), producing dialdehyde (oxidized) α-1,4-glucans.

Dialdehyde amyloses have been used to detect traces of α-amylase activity in an exo-amylase preparation (51) by Marshall and Whelan who produced an amylose substrate with 5% of its glucose residues oxidized (5% oxidation). From their data, amyloses with various degrees of oxidation could be prepared.

Amylose type I (500mg) of $\bar{D}P$ 790, was wetted with ethanol and dissolved by warming with 1M-NaOH solution, then titrated to pH 6.0 with acetic acid and made up to 40ml with distilled water. A calculated number of mols of sodium metaperiodate, determined from the data given below, was dissolved in 10ml distilled water and slowly added to the amylose with continuous stirring. The solution was covered and stored in the dark at 20° for 4h, after which time it was exhaustively dialysed against distilled water and freeze-dried.

From the following data, amyloses of varying degrees of oxidation were prepared:

% oxidation required	mmol $NaIO_4$.µmol amylose^{-1}
2	0.16
5	0.39
10	0.79
30	2.39
45	3.56
60	4.79

3.6.2. DETERMINATION OF THE DEGREE OF OXIDATION OF DIALDEHYDE AMYLOSES.

The determination of the degree of oxidation of α-1,4-glucans was carried out by complete acid hydrolysis of the amylose preparations followed by the determination of the glucose content of the hydrolysate. (74).

50mg of each dialdehyde amylose preparation was dissolved in 5ml 1M-hydrochloric acid and heated in sealed hydrolysis tubes in a boiling water bath for 3h. The tubes were cooled and approximately 5ml 1M-NaOH was added to each to bring the pH to 7.0. The solutions were diluted to 20ml with distilled water.

A control containing 1.1mg.ml^{-1} glucose was treated in the same way to determine the loss of glucose residues during the hydrolysis.

The solutions were diluted ten-fold with distilled water and 1ml aliquots of each were assayed in duplicate by the glucose oxidase method (3.4) to determine the concentration of glucose residues.

3.6.3. PREPARATION OF REDUCED DIALDEHYDE AMYLOSES.

The disadvantage of the neocuproine method (3.3.2) for the determination of reducing equivalents is that high background readings are obtained when dealing with α-1,4-glucans, and this is greatly increased when dealing with dialdehyde amyloses since the large number of aldehyde groups which are produced on oxidation react with the chelating agent.

Strumeyer (75) has described a method for reducing the terminal reducing groups of soluble starch with sodium

borohydride to decrease the interference in the assay by the substrate.

An adaptation of this method was used to prepare reduced dialdehyde amyloses. Sodium borohydride in 5 ml cold, distilled water was added to a cooled, stirring solution of dialdehyde amylose (200 mg) in 20 ml distilled water. The quantity of borohydride used depended upon the degree of oxidation of the dialdehyde amylose being reduced, as indicated below.

The solutions were left at room temperature for 10h, after which time all effervescence had ceased, and were stored at 4^{o}.

% oxidation of amylose	mmol $NaBH_4$. 100mg amylase^{-1}
5	10
10	20
30	100
45	300
60	400

Prior to use, a 10 ml aliquot of the solution was withdrawn and 0.2 ml acetone was added dropwise to destroy excess borohydride. The solution was titrated to pH 7 with 1M-acetic acid and suitably diluted to a standard volume with 0.1M-acetate buffer, pH 4.8.

3.7. USE OF MODIFIED SUBSTRATES TO DETECT ENDO-AMYLASE ACTIVITY IN EXO-AMYLASE PREPARATIONS.

3.7.1. DIALDEHYDE AMYLOSES.

A 1% (w/v) solution of dialdehyde amylose of each degree of oxidation was prepared by warming the amylose with a suitable volume of 0.1M-acetate buffer, pH 4.8. The solutions were incubated with α-1,4-glucanases at 37° and at various time intervals 0.1ml aliquots were withdrawn and assayed for the presence of reducing sugars by the neocuproine method (3.3.2).

3.7.2. CHROMOGENIC SUBSTRATES.

A suspension of amylose or amylopectin azure ($2mg.ml^{-1}$) in 0.1M-acetate buffer, pH 4.8 was prepared and heated for 3 min in a boiling water bath. The colloidal solution was allowed to cool to room temperature and an aliquot of 5% (w/v) bovine serum albumen in distilled water was added to produce a 0.1% (v/v) protein solution. The addition of bovine serum albumen was to stabilize the dispersed substrate.

Assay:

A suspension of dyed substrate prepared as described was incubated, with stirring, at 37° with α-1,4-glucanases. At various time intervals, aliquots of 1ml were withdrawn, the reaction stopped with three drops of 5M NaOH and the suspension centrifuged at 7,000g for 1 min. The increase in absorbance of the supernatant, due to the release of soluble blue dye, was measured at 595nm against a substrate control. The supernatants were then assayed for the presence of reducing sugars by the neocuproine method (3.3.2).

3.8. ENZYME PURIFICATION.

3.8.1. EXTRACTION FROM BARLEY MALT.

The preparation of barley malt has been described by Whitmore and Sparrow (76).

A suitable sample of barley (Hordeum vulgare, var. Golden Promise) was steeped in tap water at 37° for 48h with three changes of water. After this time the grain was removed from the water, spread out on a tray and maintained at 37° for several days. The grain was turned and watered twice a day to prevent unequal heat distribution and possible drying effects.

β-Amylase was extracted from the malt in an initial extraction by a combination of the methods of LaBerge and Meredith (56) and Visuri and Nummi (55).

The grain was first ground by hand in a cooled mortar and pestel, then slurried for 5 min in a homogenizer with cold 0.1M-citrate buffer, pH 6.0 containing 0.35% (w/v) EDTA, 0.5% (w/v) sodium chloride and 1mM mercaptoethanol. The slurry was filtered through muslin and re-extracted with more buffer. The combined filtrates were centrifuged at 9000g for 20 min and the supernatant retained.

The volume of the supernatant was measured and the solution was assayed for protein content by the Folin-Lowry method (3.2.1) and for total α-1,4-glucanase activity by the neocuproine method for estimating reducing sugars (3.3.2).

3.8.2. AMMONIUM SULPHATE PRECIPITATION.

The supernatant from 3.8.1 was treated with ammonium sulphate to a concentration of 20% (w/v) with respect to the salt (1.05M). The ammonium sulphate was added slowly with stirring, to prevent high local concentrations due to large amounts of undissolved ammonium salt.

The precipitate was removed by centrifugation at 4^{o} and 18000g for 20 min and discarded, and the supernatant was treated with more ammonium sulphate to 40% saturation (2.1M), centrifuged as before and the precipitate resuspended in distilled water containing 10mM-mercaptoethanol.

3.8.3. GEL FILTRATION ON SEPHADEX.

Gel filtration was carried out using Sephadex G-25 to remove ammonium sulphate and any traces of globulins (77) from the redissolved crude protein solution obtained in 3.8.2. Globulins are relatively insoluble in distilled water and are therefore retarded on the column.

Preparation of the Sephadex G-25 column:

50g of Sephadex G-25 (fine) were suspended in 500 ml distilled water and allowed to stand at room temperature for 3h to swell. The finings were removed by successive decantations and the swollen gel was made into a slurry with distilled water and packed into a 3 x 100cm Pharmacia column. The Sephadex column was washed with 2 l of distilled water containing 10mM-mercaptoethanol and had a final bed volume of 3 x 80cm.

Application of the sample:

The protein solution from 3.8.2 was applied in 20ml samples to the Sephadex column and eluted with cold distilled water containing 10mM mercaptoethanol. The column effluent was monitored by an L.K.B. U.V. recorder and 5 ml fractions were collected by an L.K.B. fraction collector.

The fractions containing protein were assayed for total α-1,4-glucanase activity (3.1), for α-amylase activity using amylose azure (3.7.2) and for protein by the Folin-Lowry method (3.2.1).

The contents of the appropriate tubes were pooled and dialysed at 4^{o} against 2.1M-ammonium sulphate solution containing 10mM-mercaptoethanol. The protein precipitate was removed by centrifugation at 4^{o} and 10000g for 20 min and dialysed into 5mM-phosphate buffer, pH 7.0, containing 10mM-mercaptoethanol.

3.8.4. ION-EXCHANGE CHROMATOGRAPHY ON DEAE-CELLULOSE.

Preparation of the DEAE-cellulose (DE 32):

30g of DEAE cellulose (DE 32) were swollen overnight in 2 l distilled water and washed several times by sedimentation in water to remove the finings. The cellulose was then precycled, as recommended by the manufacturers, by washing alternately with 1M-NaOH (300ml) and 0.5M-sodium dihydrogen phosphate solution (300ml) a number of times. The cellulose was made into a slurry with 0.5M-sodium dihydrogen phosphate solution and packed into a 5 x 60cm Pharmacia column and washed with 700ml 0.05M-phosphate buffer, pH 7.0, to a final bed volume of 5 x 15 cm. The column was then equilibrated

with 5mM-phosphate buffer, pH 7.0, containing 10mM-mercapto-ethanol.

3.8.4.1. CHROMATOGRAPHY BY GRADIENT ELUTION.

An aliquot of the dialysed enzyme solution, containing about 100mg of protein, was applied to the column and washed in with 20ml of the equilibration buffer. The column was washed with 500ml of the same buffer to remove any unbound material, and two linear gradients were applied, successively, of 5mM-50mM and 50mM to 500mM-phosphate buffer, pH 7.0, with mercapto-ethanol as before, such that 800ml of buffers were used in the gradient.

The column effluent was monitored, collected and assayed as previously described.

3.8.4.2. CHROMATOGRAPHY BY STEP-WISE ELUTION.

A suitable sample of dialysed protein solution, containing 100-150mg protein, was applied to the DEAE-cellulose column which was washed, as in 3.8.4.1, with 500ml of the equilibration buffer. β-Amylase was eluted either by 0.05M-or by 0.1M-phosphate buffer, pH 7.0.

Fractions were monitored, collected and assayed as previously described and those containing β-amylase activity were pooled and dialysed against a 2.1M- ammonium sulphate solution containing 10mM-mercaptoethanol.

The protein was removed by centrifugation, as before, resuspended in 2.1M-ammonium sulphate solution, and stored as a suspension at $4^{o}C$ in the presence of 10mM-mercaptoethanol or 5mM-dithiothreitol.

3.9. PREPARATION OF PURE MALTOSE.

Maltose was purified by the method of Wolfrom and Thompson (78).

Preparation of β-maltose octa-acetate.

A suspension of 20g anhydrous sodium acetate in 200g acetic anhydride was heated to boiling in a 500ml flask and 40g commercial maltose monohydrate was slowly added. The exothermic reaction was induced by gently heating the flask in a flame and the mixture was maintained at boiling point by the gradual addition of maltose. After the addition of all of the disaccharide and when the reaction had subsided, the mixture was vigorously boiled then allowed to cool to 50-75°, when it was poured, with stirring, into 1 litre of ice and water. Stirring was continued for 5h.

The water was decanted from the solid acetate and extracted with 150ml redistilled chloroform. The solid material was dissolved in the chloroform extract and washed with distilled water again. The chloroform solution was dried with anhydrous sodium sulphate and evaporated under reduced pressure to a syrup. Residual chloroform was removed by repeatedly dissolving the acetate in redistilled ethanol and concentrating the solution under reduced pressure. The octa-acetate derivative was crystallized from 95% (v/v) ethanol and three times recrystallized from 95% (v/v) ethanol until pure.

The melting point of the derivative was 157° (uncorr.) compared with 159-160° (corr.) reported by Wolfrom and Thompson (78).

Preparation of β-maltose monohydrate.

10g of β-maltose octa-acetate were suspended in 100 ml absolute methanol to which was added 30 ml sodium methoxide, prepared from 0.15g sodium in 30 ml absolute methanol. The suspension was stirred at room temperature for 20 min and sufficient cation exchange resin (Amberlite IRC-50), pretreated with 0.1M-hydrochloric acid and washed free of acid by distilled water, was added to bring the pH to 7.0. The solution was filtered and the resin washed with absolute methanol.

The methanol fraction was concentrated to 25 ml, decolourized with carbon, filtered and concentrated to 5 ml. It was then left to crystallize at room temperature.

The crystals were filtered off from the mother liquor and dried at 60° in a vacuum oven.

3.10. PREPARATION OF CHARACTERIZED SUBSTRATES WITH A KNOWN DEGREE OF POLYMERIZATION.

Characterized α-1,4-glucans are those with a narrow distribution range and of a known chain length. They are not commercially available and therefore had to be prepared by standard methods in the laboratory, as described below.

3.10.1. PREPARATION OF AMYLOSE BY THYMOL PRECIPITATION.

Commercial amylose is thought to be oxidized to a small extent (79), the oxidation occurring during its fractionation from starch. The effect of the oxidation of α -1,4-glucans has been mentioned in 1.3.2 and since such modifications in the glucose residues of its substrate can seriously affect β-amylase activity, it was important to prepare a sample which was protected against oxidation during its fractionation for use in further studies with β-amylase.

The linear amylose component of starch can be separated from amylopectin in a number of ways, the one most used being the dispersion of the starch granule in an aqueous solution with subsequent precipitation of the amylose by the addition of polar organic solvents. The procedure used was as follows, with all solutions being gassed with N_2/CO_2 before use to prevent oxidation of the amylose component.

Soluble starch (40g) was refluxed three times with 200ml redistilled methanol for 1h to remove any lipid material. The methanol was decanted from the starch, which was air dried.

Defatted starch (30g) was mixed to a cream with 150ml distilled water and poured into 1.5 l boiling 2% (w/v) sodium

chloride solution. The solution was stirred until it was homogeneous and filtered through hot muslin.

To the filtrate was added 4.5g powdered thymol and the mixture was mechanically stirred for 36h. Amylose was precipitated as a thymol-amylose complex and the supernatant discarded.

The precipitate was washed six times with thymol-saturated water and four times with absolute ethanol and finally was air dried.

A sample of the amylose was found to be insoluble in boiling water, but to dissolve slowly in warm, dilute alkali solutions and to undergo 59% hydrolysis with β-amylase.

3.10.2. FRACTIONATION OF THYMOL-PRECIPITATED AMYLOSE.

The method of Everett and Foster (80) was employed with the amylose prepared in the previous section.

Amylose (7.5g) was dissolved in 500ml dimethyl sulphoxide (DMSO) and 200ml absolute ethanol was added at 4°. The precipitate was removed by centrifugation and discarded.

The supernatant was treated with gradually increasing volumes of ethanol to precipitate amylose fractions of decreasing $\bar{D}P$. The precipitates were removed by centrifugation at 2500g, washed several times with absolute ethanol and freeze dried.

3.10.3. PREPARATION OF MALTO-OLIGOSACCHARIDES.

A mixture of malto-oligosaccharides was prepared by a partial acid hydrolysis of amylose, rather than of soluble

starch, to prevent the production of branched products. Separation of the hydrolysis products was attempted either by gel filtration, using Sephadex G-25 (81), or by chromatography on a charcoal-celite column (82).

3.10.3.1. PREPARATION OF PARTIAL ACID HYDROLYSATE OF AMYLOSE.

Amylose (500mg), prepared as described in section 3.10.1, was heated with 250ml 0.1M-hydrochloric acid in a boiling water bath for approximately 1h, until the degree of apparent hydrolysis to glucose, measured by the glucose oxidase method (3.4), was 33%. The solution was then cooled, neutralized with 0.1M-NaOH and reduced to a small volume by rotary evaporation under reduced pressure. The concentrate was de-ionized by treatment with anion exchange resin (Amberlite IRA-400), pretreated with 0.1M-NaOH and washed free of alkali with distilled water, followed by cation exchange resin (Amberlite IRC-50), pretreated with 0.1M-hydrochloric acid and washed free of acid with distilled water. Each of the resins was washed with distilled water and the washings combined with the de-ionized solution which was then reduced to a syrup by rotary evaporation under reduced pressure.

3.10.3.2. FRACTIONATION OF ACID HYDROLYSATE BY GEL FILTRATION ON SEPHADEX G-25.

The method used was an adaptation of that used by Flodin and Aspberg (81).

A Sephadex G-25 (fine) column was prepared as previously described to produce a packed bed of dimensions 3 x 75cm, pre-equilibrated with distilled water.

The hydrolysate syrup prepared in the previous section was made up to 50ml with distilled water and a 25ml sample was applied to the Sephadex G-25 column. Elution was effected with distilled water and the effluent collected in 5ml fractions using an L.K.B. fraction collector.

The total carbohydrate content of the fractions was estimated by the phenol-sulphuric acid method (3.5).

3.10.3.3. FRACTIONATION OF ACID HYDROLYSATE USING A CHARCOAL-CELITE COLUMN.

Charcoal columns have often been used because of the adsorptive properties of charcoal. However, charcoal-Celite columns were adopted because they were found to be more effective in separating the components of malto-oligosaccharide mixtures. The method of Whelan et al (82) was used in this work.

Preparation of the column.

Equal weights (25g) of acid-washed charcoal and Celite 535 were mixed and washed with distilled water by filtration using a Buchner funnel. The powders were made into a thick slurry with water and poured into a column (2 x 40cm) containing a sintered glass filter, previously overlaid with a filter paper disc and 2cm compressed glass wool. The slurry was allowed to settle and the surface covered with another filter paper disc and 1cm compressed

glass wool to prevent disturbance of the charcoal particles. The column was washed with 0.2M-citrate buffer, pH 7.0 (500mls), to remove an iron-citrate complex reported to be an efficient amylase inhibitor (82), and with 1 litre of distilled water to a final bed volume of 2 x 30cm.

Separation of malto-oligosaccharides.

A sample (10ml) of the hydrolysate solution was applied to the column, washed in with two 10ml samples of distilled water and the column washed with 7.5% (v/v) ethanol (200ml) to remove glucose and maltose. The effluent was discarded.

Selective elution of larger malto-oligosaccharides was carried out using a range (15-28% v/v) of ethanol concentrations (82). Fractions of 10ml were collected and assayed for carbohydrate content by the phenol-sulphuric acid method (3.5) appropriate fractions pooled and evaporated to a syrup by rotary evaporation under reduced pressure. Each malto-oligosaccharide syrup was made up to a standard concentration with distilled water.

3.11. DETERMINATION OF SUBSTRATE CHAIN LENGTH.

One of two methods was used to determine the $\bar{D}P$ of prepared substrates. The first was based on the measurement of formic acid released after periodate oxidation of the α-1,4-glucan (74), and suffered from the disadvantage of requiring a relatively large sample of substrate for the estimation.

The second method was proposed by Hiromi et al (83) using a modified Nelson-Somogyi method to relate the reducing power of the α-1,4-glucan chain to its molecular weight.

3.11.1. ESTIMATION OF CHAIN LENGTH BY PERIODATE OXIDATION.

A sample (400mg) of substrate was dissolved in a small volume of 0.1M-sodium hydroxide solution, neutralized with 0.1M-hydrochloric acid and diluted to 25ml with distilled water. To this solution, 25ml sodium metaperiodate solution (0.05M) was added, with stirring.

Immediately, 25ml was withdrawn, mixed with 1ml ethylene glycol to degrade the periodate and left for 30 min at room temperature, after which time the pH of the solution was measured.

The other 25ml was stored in the dark at 4^{o} for 24h, after which time 1ml ethylene glycol was added and the solution left as before. The solution was then titrated to the same pH value as the control with 1mM-sodium hydroxide solution.

From the volume of alkali used in the titration the molarity of formic acid released could be determined, from which could be calculated the $\bar{D}P$ of the sample as follows:

25ml XM-formic acid $\equiv$ yml 1mM-NaOH

mmol formic acid $\equiv y \times 10^{-3} \times \frac{40}{25}$ mmol.

Since 3 mol formic acid are produced per polymer chain,

$$\text{no. of chains} = \frac{\text{mol formic acid}}{3}$$

$$\text{and } \bar{DP} = \frac{\text{mol glucose in sample}}{\text{no. of chains}}$$

3.11.2. ESTIMATION OF CHAIN LENGTH BY END-GROUP ANALYSIS.

This method was used to determine the number-average molecular weight ($\bar{M}_n$) of polymer samples and was found by Hiromi et al (83) to compare favourably with viscosity determinations.

The reducing power of α-1,4-glucan solutions of equal concentration, measured by the Nelson-Somogyi method, is dependent upon chain length. Hiromi et al (83) adapted this method to determine the reducing power of solutions of α-1,4-glucans of high molecular weight, using glucose or maltose as standards for comparison.

3.11.2.1. MODIFIED NELSON-SOMOGYI ASSAY.

This was an adaptation of the methods of Nelson (42) and Somogyi (43).

Solution A: The following were dissolved in 500ml of distilled water:

2g cupric sulphate pentahydrate

12g anhydrous sodium carbonate

8g sodium bicarbonate

6g sodium potassium tartrate

90g anhydrous sodium sulphate.

Solution B: Arsenomolybdate colour reagent was prepared by dissolving 25g ammonium molybdate in 450ml distilled water, adding 21ml conc. H_2SO_4 followed by 3g sodium hydrogen arsenate heptahydrate dissolved in 25ml water. The solution was mixed, incubated at 37^{o} for 24h and stored in a dark bottle.

Assay:

A 2ml aliquot of sample was mixed with 3ml Solution A and the mixture deoxygenated by bubbling with nitrogen for 10 min. The solution was then placed in a boiling water bath for 20 min, to form a cuprous oxide precipitate, and cooled in ice and water. To this suspension was added 3ml solution B to redissolve the precipitate and the solution was diluted to 20ml with distilled water. The absorbance was measured at 500nm against a reagent control.

A calibration plot (Fig. 3.11.2.1.1.) was prepared using glucose and maltose standard solutions in a range of 10 - 100$\mu g.ml^{-1}$.

3.11.2.2. DETERMINATION OF NUMBER-AVERAGE MOLECULAR WEIGHT ($\bar{M}_n$).

A sample of each amylose fraction (100mg) was dissolved in 1ml 0.1M-NaOH overnight at 4^{o}, then diluted to 10ml with water. This solution was used as described in 3.11.2.1. The absorbance at 500nm was measured using glucose or maltose as a reference

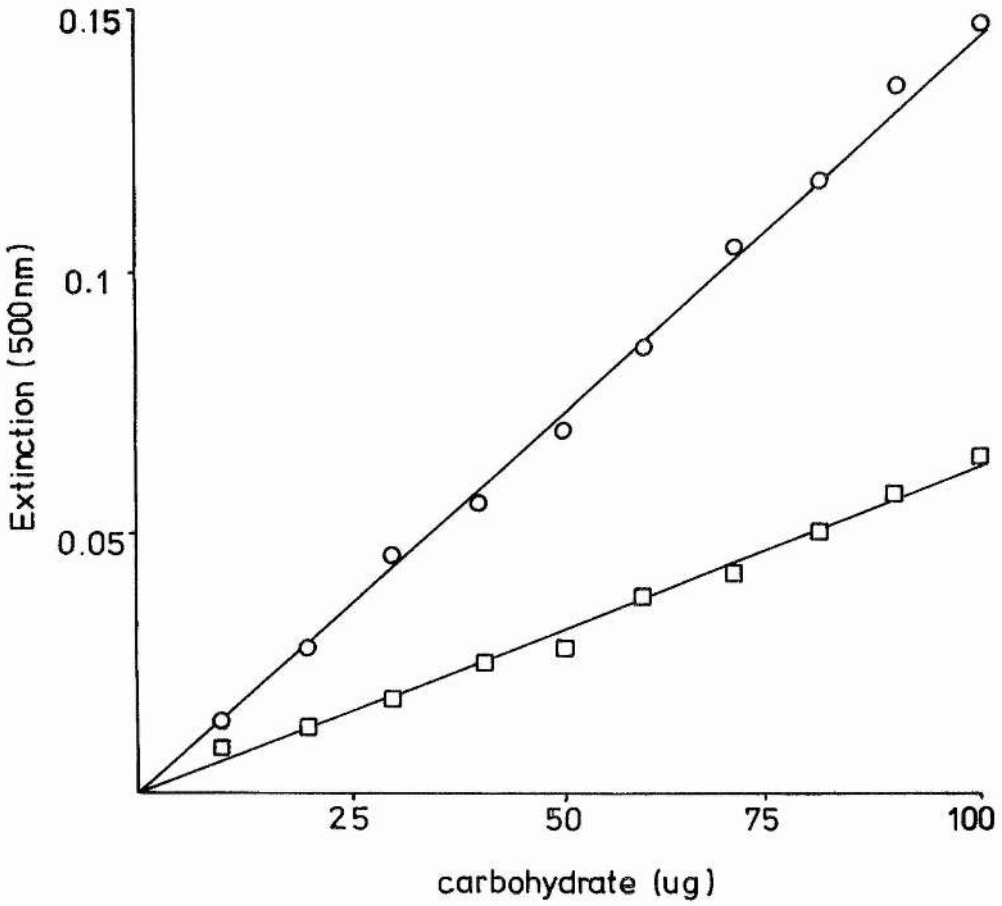
$\bar{M}_n$ could be calculated from:

$$\bar{M}_n = \frac{10\ c}{R}$$

where c = concentration of amylose in $g.100ml^{-1}$

and R = molar concentration of glucose or maltose giving the same absorbance as the amylose sample.

Fig. 3.11.2.1.1. Calibration plot for glucose (O) and maltose (□) estimated by the modified Nelson-Somogyi method.


0.15
0.1
0.05
Extinction (500nm)
25
50
75
100
carbohydrate (ug)

3.12. PAPER CHROMATOGRAPHIC TECHNIQUES.

3.12.1. SAMPLE PREPARATION.

A partial acid hydrolysate of amylose, prepared as described in section 3.10.3.1, was used as a standard for paper chromatograms. To avoid "tailing" of spots and uneven staining, the hydrolysate was de-ionized as before (3.10.3.1).

Where possible, all enzyme incubations with α-1,4-glucan substrates were carried out in distilled water containing 1mM-mercaptoethanol. All solutions of β-amylase were dialysed against distilled water and mercaptoethanol before use and all insoluble β-amylase derivatives (3.13) were washed with the same solution.

Samples removed from an incubation mixture were either placed in a boiling water bath for 3 min in the case of the soluble enzyme or centrifuged at 7000g for 1 min in the case of immobilized derivatives.

All samples were then reduced in volume and applied as small spots to Whatman 3mm chromatography paper (30 x 35cm), 2cm above the bottom edge. The paper was then rolled into a cylinder, stapling the two side edges together so that the paper did not overlap.

3.12.2. SOLVENT SYSTEM.

For optimum separation of malto-oligosaccharide mixtures from enzymic and partial acid hydrolyses, a one-phase n-butanol:pyridine:water (6:4:3, v/v) system was chosen. Chromatography was carried out in a sealed tank using a multi-ascent technique. Each chromatogram was left in the solvent system for 5h, dried and rechromatographed.

The process was repeated until at least four ascents had been carried out. Finally, the chromatograms were dried.

This method ensured the good separation of malto-oligosaccharides, enabling them to be identified more easily.

3.12.3. VISUALIZATION PROCEDURE.

A silver nitrate-sodium hydroxide system (84) was chosen for staining the chromatograms.

The visualization solvent was prepared by diluting a saturated silver nitrate solution (0.1ml) with 20ml acetone and adding sufficient distilled water dropwise to redissolve the white precipitate.

After the final drying of the chromatogram, it was passed through the silver nitrate-acetone solution and allowed to dry before being sprayed with 0.5M-NaOH solution in ethanol. Black or dark brown spots on a brown background were further clarified by removal of excess silver oxide from the paper with 6M-ammonium hydroxide.

The chromatogram was washed in running water for 1h and dried.

3.13. PREPARATION OF IMMOBILISED DERIVATIVES OF β-AMYLASE.

Two immobilised derivatives of β-amylase were prepared by similar coupling techniques but using different supports. In both cases the enzyme was coupled to the support by means of the bifunctional reagent, ethyl adipimidate (85).

3.13.1. PREPARATION OF SUPPORTS.

Aminoethyl cellulose (Cellex AE) with a degree of substitution of 0.315 mequiv.g^{-1} was washed successively with 0.5M-NaOH and 0.5M-HCl before use. The cellulose was finally washed free of acid and alkali and stored as a suspension (10mg.ml^{-1}) in distilled water at 4°.

Polystyrene beads were suspended in 47% (v/v) HNO_3 (sp. gr. 1.42) in H_2SO_4 (sp. gr. 1.84) at 0° and vigorously stirred for 20 min. The reaction was stopped by pouring the suspension into a large volume of water. The beads were washed well with distilled water and suspended in 6% (w/v) $Na_2S_2O_4$ in 2M-KOH and refluxed overnight. The polyaminostyrene so prepared (Fig. 3.13.1.1) was washed well with dilute (0.1M) HCl and water and air-dried.

3.13.2. COUPLING PROCEDURE.

The coupling agent, ethyl adipimidate was susceptible to hydrolysis, therefore coupling to the supports was carried out in non-aqueous solvents. The procedure is outlined in Fig. 3.13.2.1.

100mg of prepared support material was suspended in a solution of 20% N-ethyl morpholine in dry methanol and to

Fig. 3.13.1.1. Illustration of the preparation of polyaminostyrene (PAS).

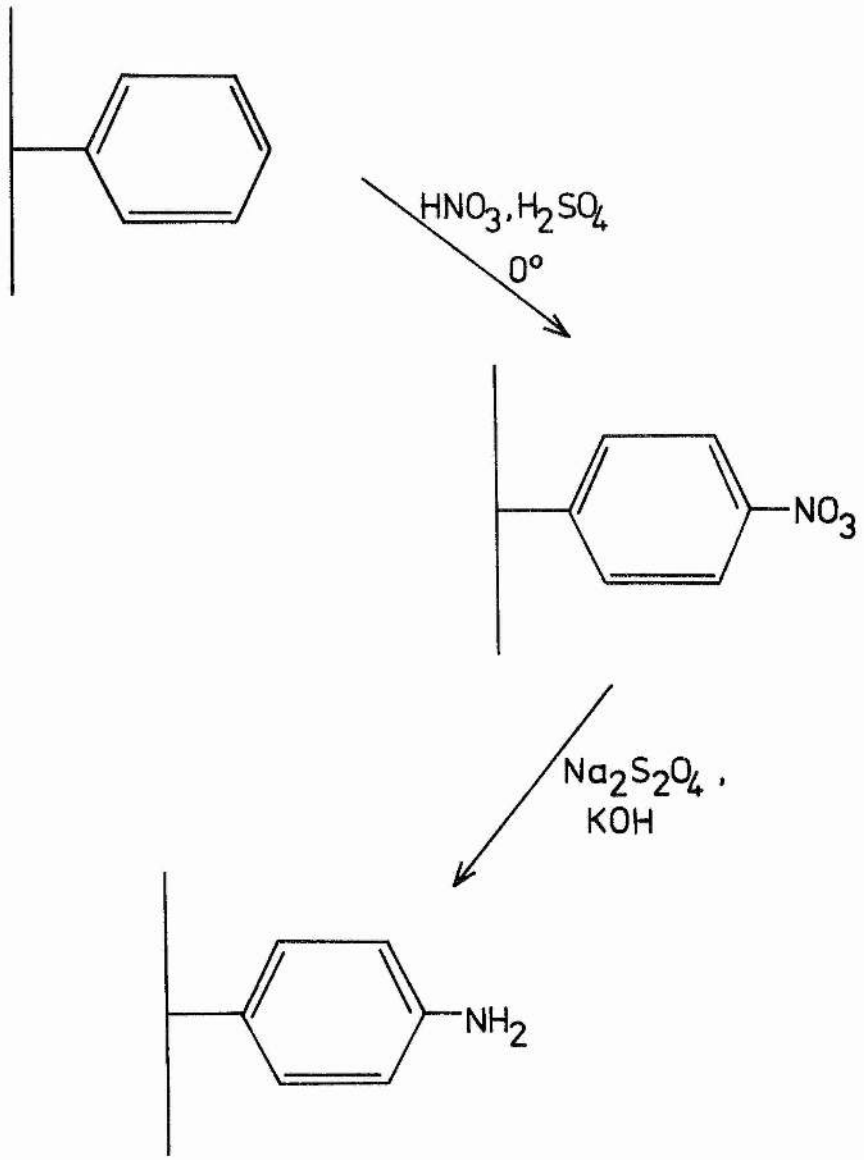
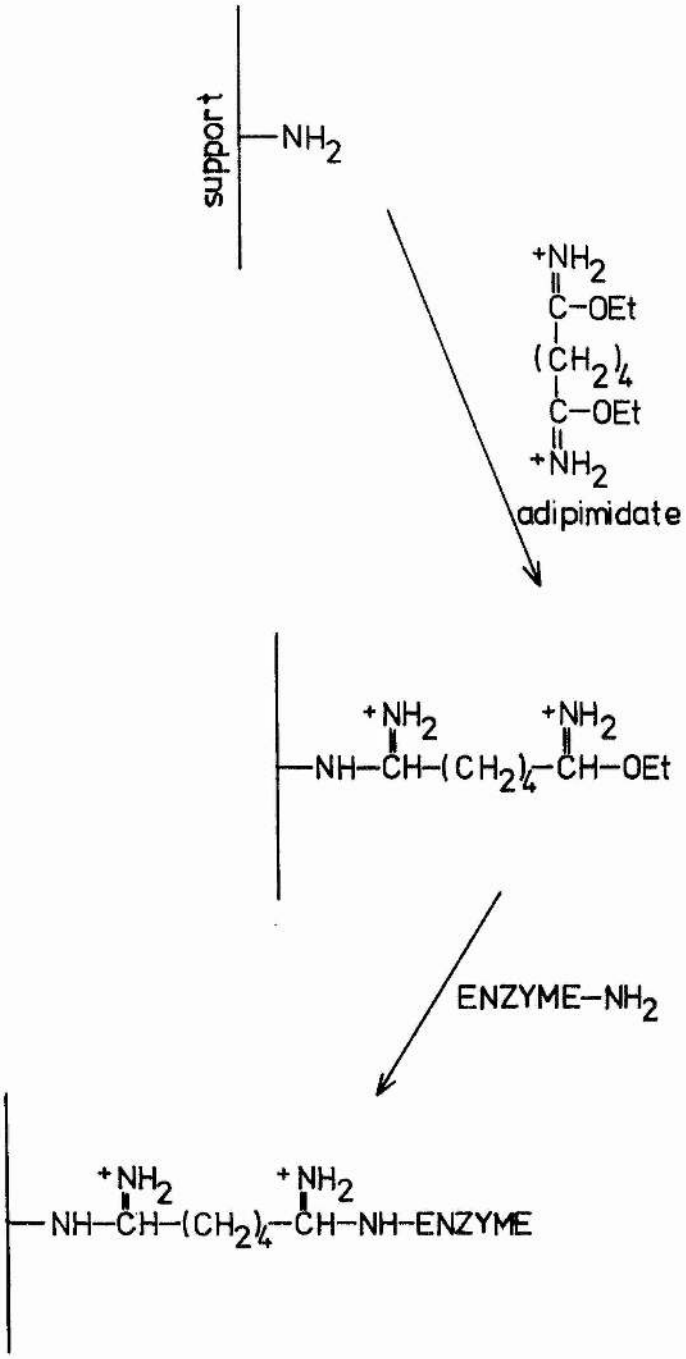

HNO3, H2SO4
0°
NO3
Na2S2O4 ,
KOH
NH2

Fig. 3.13.2.1. Illustration of the coupling of β-amylase to an aminated support (AE-cellulose or PAS) via ethyl adipimidate.


support
NH2
+NH2
C–OEt
(CH2)4
C–OEt
+NH2
adipimidate
+NH2
+NH2
NH–CH–(CH2)4–CH–OEt
ENZYME–NH2
+NH2
+NH2
NH–CH–(CH2)4–CH–NH–ENZYME

this suspension was added a solution of 100mg ethyl adipimidate in the same solvent.

The suspension was stirred in the dark at room temperature for 15 min then washed with methanol (x5) and once with 0.1M-N-ethyl morpholine-HCl buffer, pH 8.5.

A 1ml sample of β-amylase ($0.866 mg.ml^{-1}$), suspended in 2.1M-ammonium sulphate solution, was centrifuged and the pellet redissolved in a small volume of N-ethyl morpholine-HCl buffer, and the solution added to the support suspension, which was stirred at 4^{o} for 2h.

After this time, the immobilised enzyme was washed with 100ml 0.5M-NaCl in 0.1M-acetate buffer, pH 4.8, in 5ml aliquots. All aliquots were assayed for protein content (3.2.1), the amount of bound protein being determined from the difference between the amount originally used and the amount present in the washings.

The immobilised enzyme derivatives were finally washed with 0.1M-acetate buffer, pH 4.8 and stored as suspensions in the same buffer at 4^{o}.

3.13.3. ASSAY OF IMMOBILISED β-AMYLASE ACTIVITY.

β-Amylase activity was measured by the same method as the soluble enzyme (3.1). Typically, an aliquot of enzyme suspension was incubated at 37^{o} with 1% (w/v) soluble starch in 0.1M-acetate buffer, pH 4.8, in a continuously stirred reactor, to reduce diffusional effects, which will be considered in later sections.

The appearance of reducing sugars in the suspension was measured as before (3.1) after removing the enzyme derivative by centrifugation.

Activity was expressed in terms of U.mg bound enzyme^{-1}.

3.14. DETERMINATION OF THE ACTION PATTERNS OF β-AMYLASE AND IMMOBILISED DERIVATIVES OF β-AMYLASE.

The characteristic action pattern profiles for α-1,4-glucanases related to their mechanism of action has already been mentioned (1.2). A measure of the appearance of product plotted against the decrease in chain length of the substrate results in a graph considered to be characteristic of the action pattern of the particular amylase. (86).

In the present work, the appearance of reducing sugars was measured by the neocuproine method (3.3.2) whilst the decrease in chain length of α-1,4-glucan substrate (amylose) was measured for equivalent samples by the iodine absorption power or "blue value" of the amylose at 610nm.

Iodine Reagent:

This was prepared as a 0.01M-iodine solution in 0.02M-potassium iodide solution.

Assay of blue value.

An aliquot of enzyme solution or suspension was incubated at 37°, with stirring, with 10ml 0.1% (w/v) amylose solution ($\bar{D}P$ 1340) in 0.1M-acetate buffer, pH 4.8. Aliquots (0.1ml) were removed at time intervals and mixed with 1ml iodine reagent. The solutions were made up to 3ml with distilled water and the blue value estimated by measuring the absorbance at 610nm against a reagent blank. The blue values were expressed as % blue value of the original amylose solution and plotted against the % maximum reducing power of aliquots withdrawn at the same time.

4. RESULTS AND DISCUSSION.

4.1. ACTION OF α-1,4-GLUCANASES ON PARTIALLY OXIDIZED α-1,4-GLUCANS.

As already mentioned (sections 1.3 and 3.6.1), Drummond et al (31) reported that amylose preparations with a low degree of oxidation were incompletely hydrolysed by exo-amylases, whereas an endo-amylase, such as α-amylase, was able to hydrolyse the substrate to almost the same extent as the unoxidized α-1,4-glucan.

The effect of the oxidative modification of amylose upon the action of α-1,4-glucanases was investigated using α-amylase, pure β-amylase and crude β-amylase (containing traces of α-amylase activity).

Suitable quantities of α-amylase, type IIA (1U), α-amylase-free β-amylase (1.5U) and crude β-amylase, type IIA (1.3U) were each incubated at 37° with a 1ml aliquot of a 1% (w/v) solution of 5% oxidized amylose, prepared as previously described in section 3.6.1. At time intervals, 0.1ml aliquots were withdrawn and assayed for the release of reducing sugars by the neocuproine method (section 3.3.2). After 30 min, a trace of α-amylase (1U) was added to the pure β-amylase system and assaying was continued for a further 20 min.

The course of hydrolysis of the different enzyme systems is represented in Fig. 4.1.1. It can be seen that the pure β-amylase preparation was able to hydrolyse only 10% of the bonds in dialdehyde amylose, in agreement with the results of Drummond et al (31). The value of 10%, a percentage of the total number of glucose residues in the

amylose, is proportional to the average length of amylose chain between the non-reducing terminal residue and the first oxidized residue.

The actions of α-amylase and of crude β-amylase on 5% oxidized amylose were similar, the presence of endo-amylase in each preparation cleaving internal α-1,4 bonds and therefore by-passing the oxidation points. In the case of crude β-amylase, this effectively made available new non-reducing chain ends susceptible to renewed β-amylase attack.

The addition of a trace of α-amylase to pure β-amylase rendered the substrate accessible to further β-amylase attack by internally cleaving bonds as before.

4.2. ACTION OF α-1,4-GLUCANASES ON CHROMOGENIC SUBSTRATES.

Similar enzyme preparations to those in the previous section were used to investigate the action of endo- and exo-amylases on amylose azure and amylopectin azure.

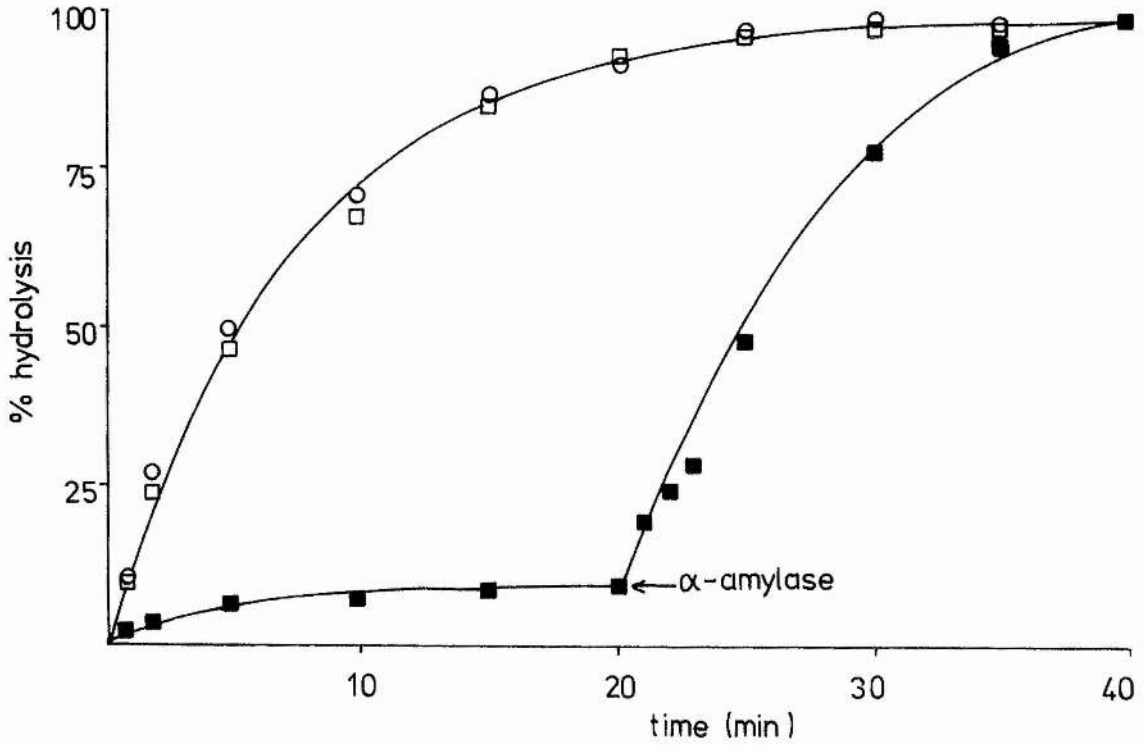
The incubations were set up as before using 5ml aliquots of 0.2% (w/v) solutions of the dyed substrates prepared as previously described in section 3.7.2. Aliquots were removed and assayed for the release of soluble blue dye (section 3.7.2) and for reducing sugars (section 3.3.2). As in the previous section, after 20 min a trace of α-amylase (1U) was added to the pure β-amylase preparation and assaying was continued for a further 20 min. The course of hydrolysis of the different enzyme preparations was similar for amylose azure and amylopectin azure. Fig. 4.2.1. represents the release of soluble blue dye from amylose azure

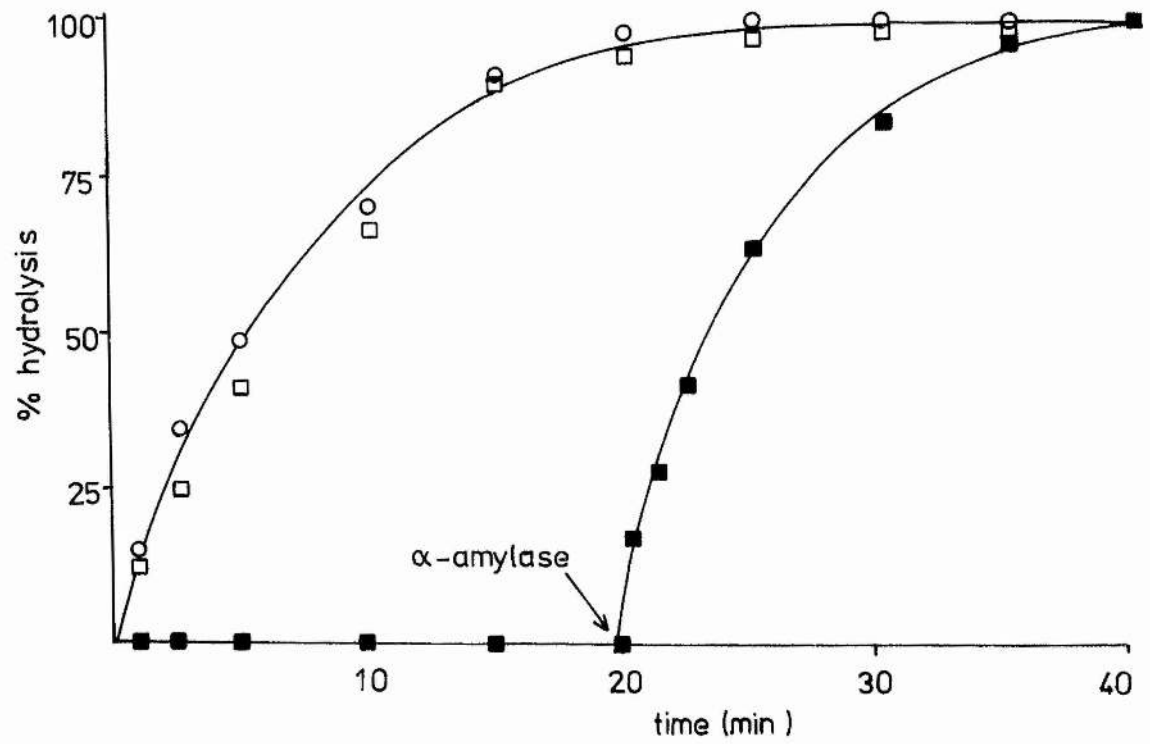
over a 40 min period. The plots for the release of reducing sugars from dyed substrates for the three enzyme preparations were similar to those obtained using 5% oxidized amylose. The explanation for the release of soluble blue dye during the incubation of each enzyme preparation with dyed substrates is as explained previously (section 4.1).

α-Amylase, by cleaving internal bonds, rapidly decreases the substrate chain length, resulting in the release of small, soluble oligosaccharides carrying dye molecules, the presence of which could be assayed spectrophotometrically at 595nm. Crude β-amylase, containing endo-amylase activity is also able to release soluble blue dye. However, pure β-amylase, with no ability to by-pass the large dye molecules in the substrate chain, releases no blue dye into solution, although small amounts of reducing sugars are released by pure β-amylase action (cf. 10% hydrolysis of oxidized amylose by pure β-amylase in section 4.1) from chain sections between the non-reducing terminal residue and the first dye-substituted residue.

Fig. 4.1.1. Time course of the hydrolysis of 5% oxidized amylose with respect to the release of reducing groups by α-1,4-glucanases, using crude β-amylase type IIA, 1.3U (□), α-amylase, type IIA, 1U (○) and pure β-amylase, 1.5U (■), α-Amylase (1U) was added to the pure β-amylase system after 20 min as indicated.

Fig. 4.2.1. Time course of the hydrolysis of amylose azure with respect to the release of soluble blue dye by α-1,4-glucanases, using crude β-amylase, type IIA, 1.3U (□), α-amylase, type IIA, 1U (○) and pure β-amylase, 1.5U (■). α-Amylase (1U) was added to the pure β-amylase system after 20 min as indicated.


100
75
50
25
% hydrolysis
α-amylase
10
20
30
40
time (min)


100
75
50
25
% hydrolysis
α-amylase
10
20
30
40
time (min)

4.3. ASSESSMENT OF PURIFICATION TECHNIQUES.

4.3.1. DETERMINATION OF OPTIMUM MALTING TIME.

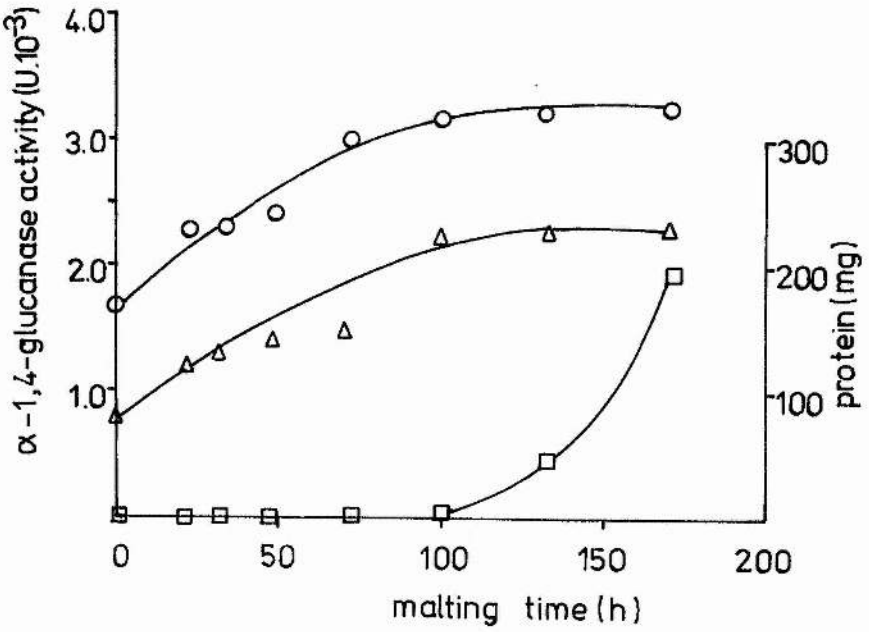
Since germination characteristics will differ with various strains of barley, the protein content, total amylase levels and amylase levels after heat treatment were assayed during germination as described below. In order to correlate the protein and enzyme levels throughout germination, seeds were sampled by number rather than by weight, since moisture content and grain weight would alter during the course of germination.

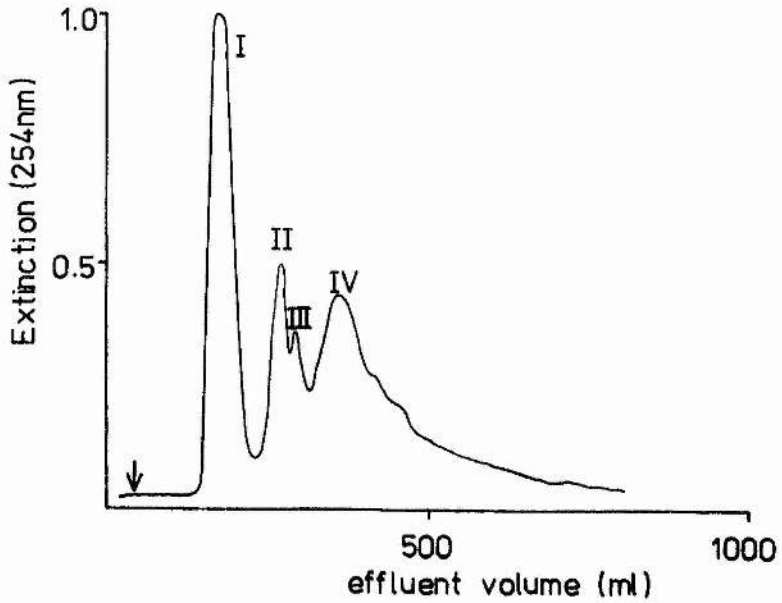
Samples of 200 seeds were taken before steeping and at approximately 12h intervals thereafter. The grain was treated as already described in section 3.8.1, resulting in the supernatant obtained after centrifugation of the extract being assayed for protein content by the Folin-Lowry method (section 3.2.1) and for total amylase activity by the neocuproine method (section 3.3.2). An aliquot of the supernatant was heated in a water bath at 70° for 15 min to inactivate β-amylase (87), cooled in an ice bath and assayed as before to determine the residual amylase activity, taken to be a measure of α-amylase activity.

The results are plotted in Fig. 4.3.1.1, from which it can be seen that β-amylase activity reaches a plateau level after approximately 72h, after which time, α-amylase activity becomes detectable. Therefore, to obtain maximum levels of β-amylase activity and reduced levels of α-amylase activity, the malt was treated to extract β-amylase after 96h of malting.

Fig. 4.3.1.1. Plot showing the release of soluble protein (Δ), β-amylase activity (O) and α-amylase activity (□) from barley during the course of germination.

Fig. 4.3.2.1. Elution profile of the chromatography of the redissolved precipitate obtained after ammonium sulphate fractionation of a crude extract of malted barley on a Sephadex G-25 (fine) column (3 x 80cm). An aliquot of protein solution (20ml, $5mg.ml^{-1}$) was applied to the column and elution was performed at a flow rate of $4ml.min^{-1}$ with 10mM-marcaptoethanol in distilled water and the effluent monitored for protein at 254nm and for α-1,4-glucanase activity by the neocuproine method. Elution of α-1,4-glucanase activity occurred in the first peak (peak I). ↓ indicates point of sample application.


α-1,4-glucanase activity (U.10⁻³)
4.0
3.0
2.0
1.0
300
200
100
protein (mg)
0
50
100
150
200
malting time (h)


Extinction (254nm)
1.0
0.5
I
II
III
IV
500
1000
effluent volume (ml)

4.3.2. FRACTIONATION OF MALT PROTEINS BY GEL FILTRATION ON SEPHADEX G-25.

The application of a crude β-amylase extract from malted barley to a Sephadex G-25 column, and the elution of the enzyme from the column, was carried out as described in section 3.8.3. The elution profile obtained is shown in Fig. 4.3.2.1, with the majority of α-1,4-glucanase activity occurring in the first peak.

A similar result was obtained by Nummi et al (77) with β-amylase activity being eluted in the first peak, although fewer peaks were obtained than with the malt proteins, partly due to a thiol-free water extract being used in the initial extraction, and also due to barley being used as opposed to malt, since the level of soluble proteins in the latter increased during germination, resulting in the profile obtained upon gel filtration showing the presence of a number of protein peaks.

4.3.3. CHROMATOGRAPHY ON DEAE-CELLULOSE BY GRADIENT ELUTION.

The chromatography of the protein sample obtained in the first peak eluted from Sephadex G-25 was carried out by gradient elution as previously described in section 3.8.4.1, and the resulting elution profile is presented in Fig. 4.3.3.1.

A peak containing no amylase activity was eluted as unbound protein by washing the column with the equilibration buffer. Upon application of the gradients, a large number of poorly-separated protein peaks were eluted. The large,

broad peak (VII), eluted at the boundary of the two gradients, contained the majority of β-amylase activity.

Using 0.2% (w/v) amylose azure (section 3.7.2) in 0.1M-acetate buffer, pH 4.8, as an indicator of the presence of α-amylase, peak IV was found to contain α-amylase activity, which "tailed" into peak VII.

However, the specific activity ($5U.mg^{-1}$) of the pooled peak VII fractions emphasized that an unsatisfactory separation was obtained by this method of gradient elution, with the β-amylase fraction containing substantial amounts of other proteins.

4.3.4. CHROMATOGRAPHY ON DEAE-CELLULOSE BY STEP-WISE ELUTION.

To attempt a more efficient separation between peaks, stepwise elution was used.

Nummi *et al* (77) suggested washing unbound protein from the column with 5mM-phosphate buffer, pH 7.0, followed by elution of β-amylase with 100mM-phosphate buffer. However, Visuri and Nummi (55) recommended elution with 50mM-phosphate buffer.

Both of these methods were used to attempt to improve the separation of β-amylase from other proteins, and the elution profiles for the two methods are shown in Figs. 4.3.4.1 and 4.3.4.2 for 100mM- and 50mM-phosphate buffers, respectively.

Each of the elution buffers gave a separation which was more satisfactory than the gradient elution, with 80% of the total β-amylase activity being eluted in

peak I.

However, elution with 100mM-phosphate buffer was found to result in a β-amylase peak with better resolution than the 50mM-phosphate buffer, possibly due to minor differences in the charge distribution at pH 7.0 between malt β-amylase used in this work and barley β-amylase used by Visuri and Nummi (55).

Also of possible significance is the increase in soluble (extractable) proteins which occurs during germination, and the presence of which would demand different conditions of purification compared to the barley enzyme.

Traces of α-amylase activity were found to be present in the β-amylase fraction, therefore causing an increase in the specific activity of β-amylase ($166U.mg^{-1}$) eluted with 100mM-phosphate buffer. If the possible presence of other proteins is taken into account, this value is comparable to that of $222U.mg^{-1}$ quoted by Visuri and Nummi (55) for the barley enzyme.

Further purification by chromatographic techniques was considered unnecessary, although a trace of α-amylase activity was present. The object of the purification procedure was to obtain a β-amylase preparation free of other α-1,4-glucanase activities, but not necessarily free of other contaminating proteins.

4.3.5. REMOVAL OF FINAL TRACES OF α-AMYLASE ACTIVITY

The removal of traces of α-amylase activity from enzyme preparations has been achieved in the past by the use of EDTA (88), acid treatment (89) or by a combination

Fig. 4.3.3.1. Elution profile obtained for the chromatography of protein from peak I in Fig. 4.3.2.1 upon a DEAE-cellulose (DE 32) column (5 x 15cm) by gradient elution using a sample volume of 10ml containing 8mg.ml^{-1} protein. Elution was performed at a flow rate of 2.5ml.min^{-1} and as indicated in the chromatogram, washing unbound protein from the column with 5mM-phosphate buffer, pH 7.0, containing 10mM-mercaptoethanol, prior to application of the gradients: 1, 5mM-50mM-phosphate buffer, pH 7.0; 2, 50mM-500mM-phosphate buffer, pH 7.0. The effluent was monitored as previously described.

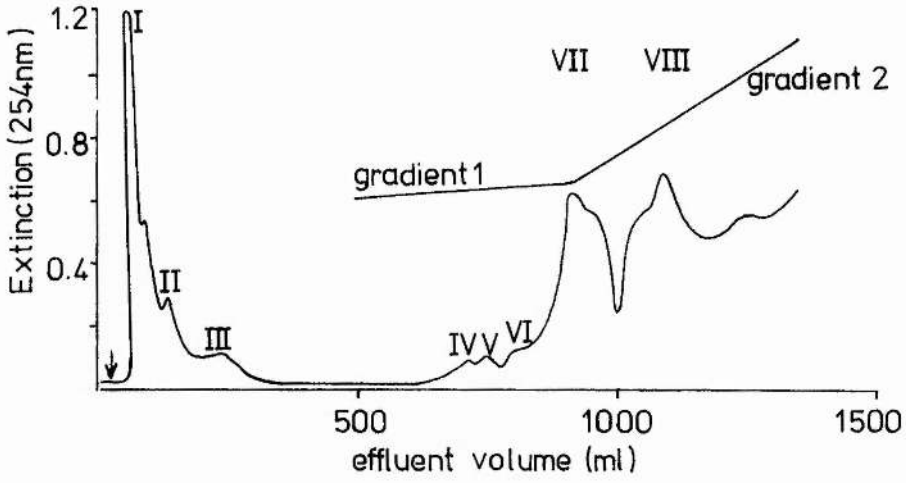
↓ indicates the point of sample application.

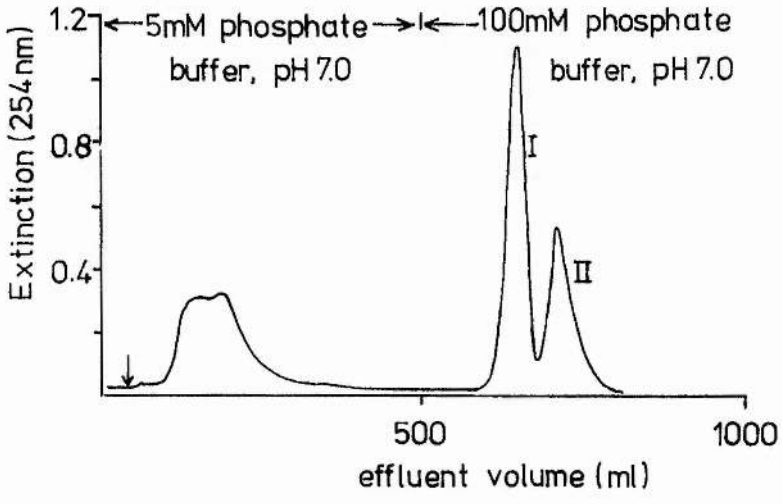
Fig. 4.3.4.1. Elution profile obtained for the chromatography of protein (as above) on DEAE-cellulose with 100mM-phosphate buffer, pH 7.0, after washing the column as before with 5mM-phosphate buffer. All other conditions were as above and the effluent was monitored as described in Fig. 4.3.2.1. β-Amylase activity was found in peak I.

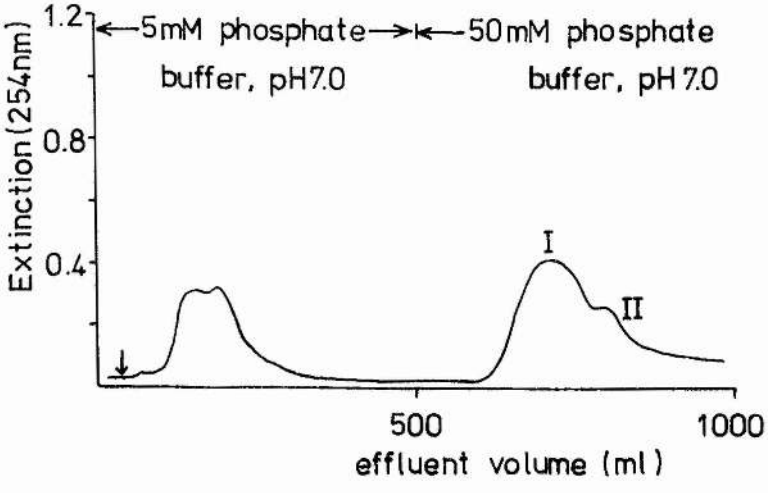
↓ indicates point of sample application.

Fig. 4.3.4.2. Elution profile obtained for the chromatography of protein (as above) on DEAE-cellulose with 50mM-phosphate buffer, pH 7.0 after washing the column with 5mM-phosphate buffer. All other conditions were as above. β-Amylase activity was found in peak I.

↓ indicates point of sample application.


1.2
0.8
0.4
Extinction (254nm)
I
II
III
IV
V
VI
VII
VIII
gradient 1
gradient 2
500
1000
1500
effluent volume (ml)


1.2
0.8
0.4
Extinction (254nm)
←5mM phosphate buffer, pH 7.0→
←100mM phosphate buffer, pH 7.0→
I
II
500
1000
effluent volume (ml)


1.2
0.8
0.4
Extinction (254nm)
←5mM phosphate buffer, pH7.0→
←50mM phosphate buffer, pH 7.0→
I
II
500
1000
effluent volume (ml)

of EDTA and proteases (90).

Cereal α-amylases in dilute solutions are easily inactivated by titrating the solutions to below pH 4 and storing at 4° for 12-18h.

The β-amylase fraction from DEAE-cellulose chromatography was dialysed against 2.1M-ammonium sulphate solution containing 10mM-mercaptoethanol and the protein precipitate was redissolved in a minimum volume of 0.1M-acetate buffer, pH 4.8, containing 10mM-mercaptoethanol. The solution was titrated to pH 3.6 with 0.1M-acetic acid and stored at 4° for 12h. During this time, 0.2ml aliquots were withdrawn and assayed for the presence of α-amylase activity by incubating with 1.8ml 0.2% (w/v) amylose azure in 0.1M-acetate buffer, pH 4.8, at 37° for 30 min. The inactivation profile is shown in Fig. 4.3.5.1.

To indicate the effect of the introduction of a trace of α-amylase activity into a preparation of purified β-amylase, β-amylase (1.5U) was incubated with 1ml 1% (w/v) solution of 5% oxidized amylose in 0.1M-acetate buffer, pH 4.8, and with 5ml 0.2% (w/v) amylose azure in a similar buffer, as previously described in sections 4.1 and 4.2, with the addition of α-amylase (1U) after 20 min. The release of reducing sugars or of soluble blue dye was assayed as previously described (sections 3.3.2 and 3.7.2).

Until the addition of α-amylase, β-amylase was able to hydrolyse up to 9% of the bonds in 5% oxidized amylose and to release no soluble blue dye from amylose azure, as shown in Figs. 4.3.5.2 and 4.3.5.3, respectively, confirming the presence of β-amylase alone.

Fig. 4.3.5.1. Time course of the inactivation of α-amylase by acid treatment at 4^{o} and pH 3.6, monitored by the release of soluble blue dye from chromogenic α-1,4-glucans.

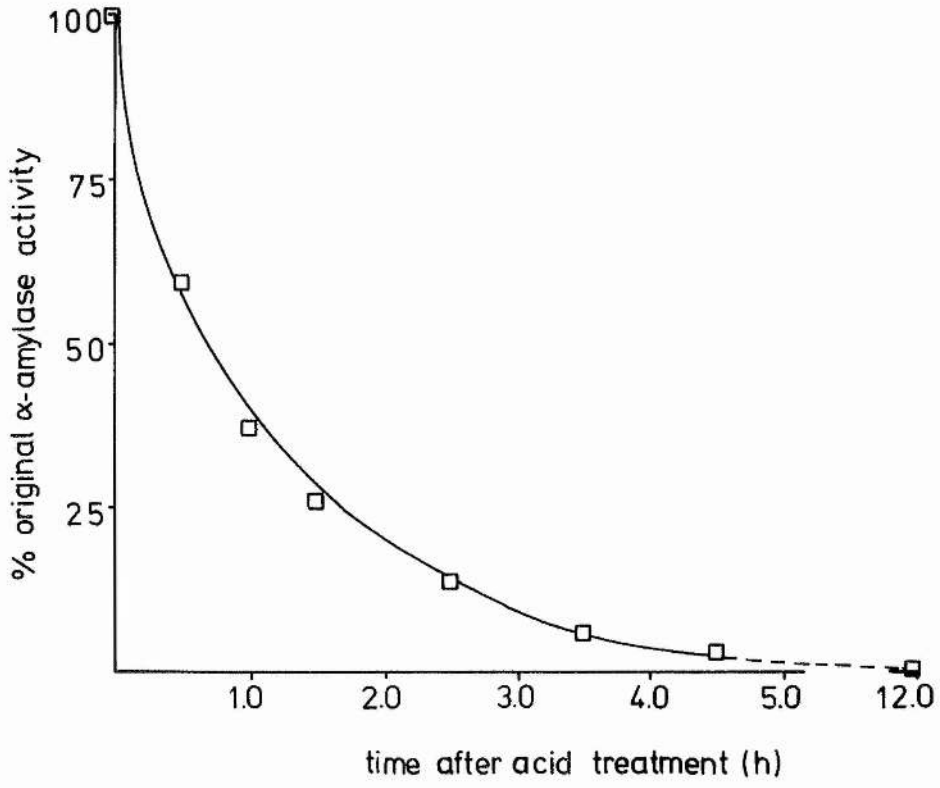
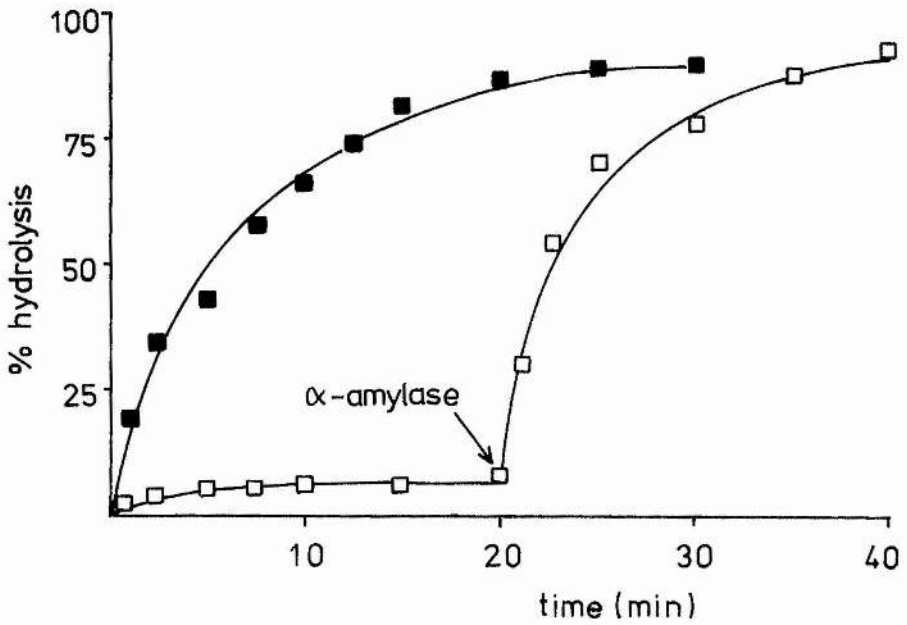
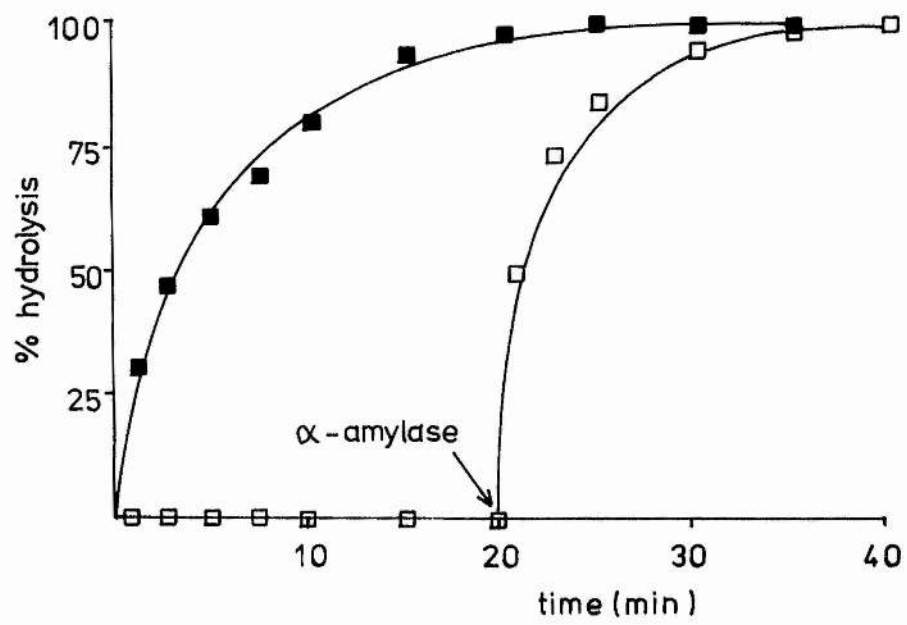

100
75
50
25
% original α-amylase activity
1.0
2.0
3.0
4.0
5.0
12.0
time after acid treatment (h)

Fig. 4.3.5.2. Time course of the hydrolysis of 5% oxidized amylose with respect to the release of reducing groups by α-amylase (■) and by peak I (□) from DEAE-cellulose chromatography (Fig. 4.3.4.1) after acid treatment. α-Amylase (1U) was added to the β-amylase system after 20 min as indicated.

Fig. 4.3.5.3. Time course of the hydrolysis of amylose azure with respect to the release of soluble blue dye by α-amylase (■) and peak I (□) from DEAE-cellulose chromatography (Fig. 4.3.4.1) after acid treatment. α-Amylase (1U) was added to the β-amylase system after 20 min as indicated.


100
75
50
25
% hydrolysis
α-amylase
10
20
30
40
time (min)


100
75
50
25
% hydrolysis
α-amylase
10
20
30
40
time (min)

To finally determine the enzymic purity of the β-amylase preparation, an aliquot of the enzyme (1.5U) was incubated with 1ml 1% (w/v) maltose solution in 0.1M-acetate buffer, pH 4.8, at 37° for 5h to assay for the presence of α-glucosidase (maltase) contamination. The incubation mixture was assayed for glucose release after this time by the glucose oxidase method of Lloyd and Whelan (section 3.1.4). No glucose was found to be released, indicating that no detectable α-glucosidase activity was present.

An example of the progress of purification is given in Table 4.3.5.1.

TABLE 4.3.5.1. PURIFICATION OF β-AMYLASE FROM BARLEY MALT*

PURIFICATION STEP	TOTAL ACTIVITY (U)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY ($U.mg^{-1}$)	YIELD %
Citrate extract	19232	22319	0.86	100
Ammonium sulphate fractionation (20-40%)	16198	985.4	16.43	84.2
Sephadex G-25	15211	291.7	52.15	79.1
DEAE-cellulose	9545	57.5	166.1	49.6
Acid treatment	8625	57.5	149.9	44.8

*Results obtained using 500g barley.

4.4. KINETIC STUDIES ON β-AMYLASE USING UNMODIFIED α-1,4-GLUCANS.

4.4.1. pH-ACTIVITY PROFILE.

The pH-activity profile of pure β-amylase was determined by incubating an aliquot of the enzyme (0.75U) in suitable buffer with 1ml 1% (w/v) soluble starch solution in 0.1M-citrate-phosphate buffer at various pH values (2.5-8.5) at 37°. The activity of the enzyme was assayed as previously described in section 3.1.

The results were plotted as % maximum activity obtained against pH values (Fig. 4.4.1.1). The optimum pH was found to lie between 4.5 and 5.0, in agreement with Zherebtsov (26) who also discussed the shape of the curve, attributing pK_a and pK_b values of 8.1 and 3.4 to imidazole and carboxyl groups, respectively, for malt β-amylase. Similar values (pK_a = 8.2, pK_b = 3.2) were found for pure malt β-amylase used in the present work.

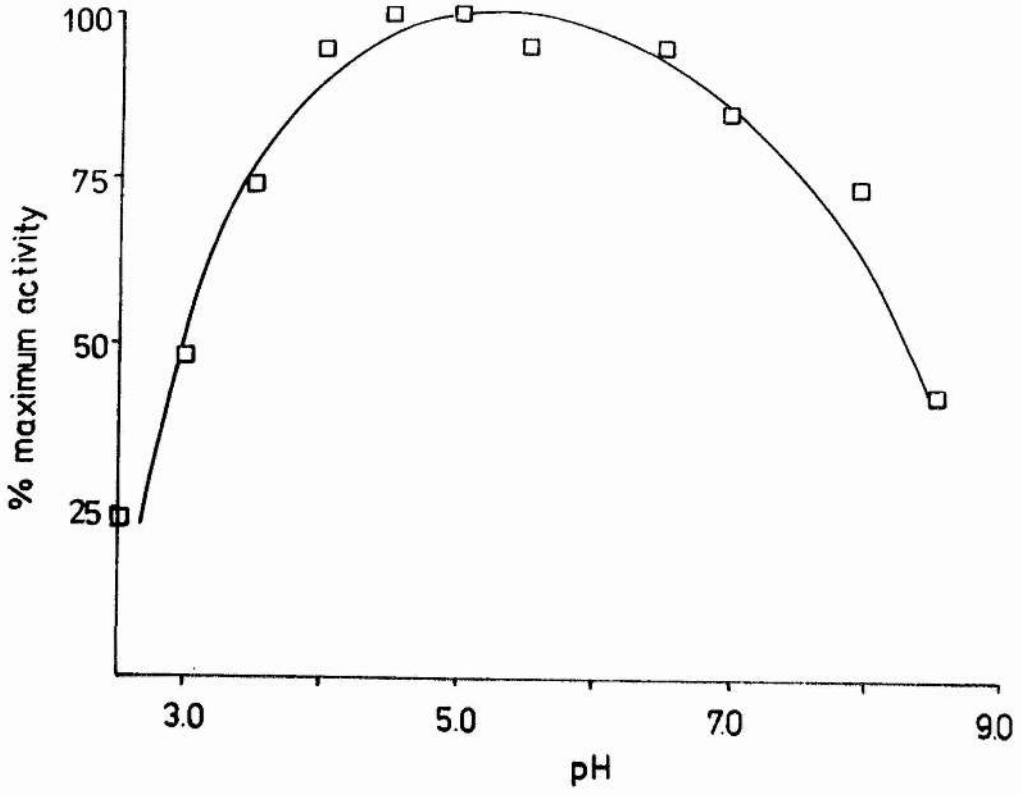
Zherebtsov suggested that the difference between the theoretical and experimental pH-activity profiles indicated that H^+ ions influenced not only the catalytically active groups but also the structure of the catalytic site itself.

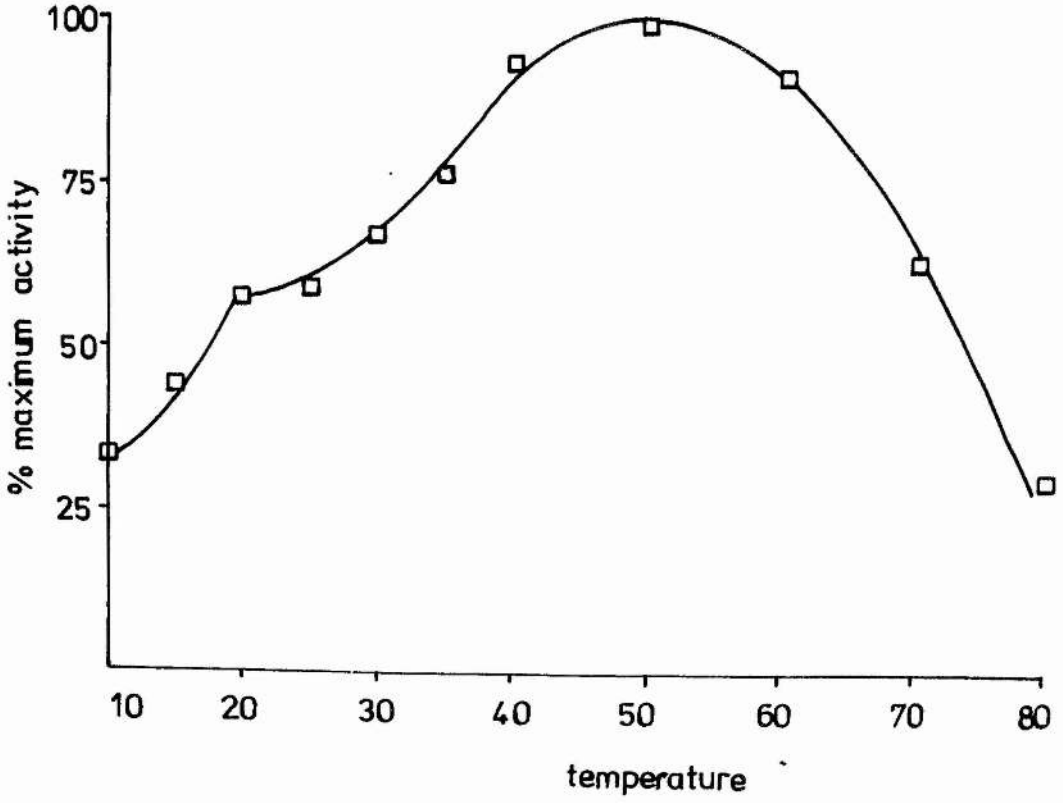
4.4.2. TEMPERATURE-ACTIVITY PROFILE.

Suitable aliquots (0.75U) of β-amylase were incubated at various temperatures with 1ml 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8, and the initial velocity measured as in section 3.1 at each temperature.

Fig. 4.4.1.1. pH-Activity profile obtained for pure β-amylase acting on 1% (w/v) soluble starch solution in 0.1M-citrate-phosphate buffer at 37°.

Fig. 4.4.2.1. Temperature-activity profile for pure β-amylase acting on 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8.


100
75
50
25
% maximum activity
3.0
5.0
7.0
9.0
pH


100
75
50
25
% maximum activity
10
20
30
40
50
60
70
80
temperature

Results were plotted as % maximum activity obtained against temperature (Fig. 4.4.2.1).

The optimum temperature for enzyme activity was found to lie between 40° and 50°, which appeared comparatively high for an enzyme isolated from a plant source.

Temperature-activity profiles of this kind are not very meaningful since the optima are due to two competing effects, namely the normal increase in rate with increasing temperature and the inactivation of the enzyme at elevated temperatures. However, such determinations indicate the region of optimum activity, and when used together with thermal stability data, to be discussed in later sections, they can be used to determine a compromise temperature for optimum enzyme activity and stability.

The profile obtained in this case has one interesting feature in that a break occurs in the profile at 20°, in agreement with Piguet and Fischer (89) and corresponding to a change in activation energy.

4.4.3. DETERMINATION OF KINETIC PARAMETERS FOR PURE β-AMYLASE AND β-AMYLASE IN THE PRESENCE OF α-AMYLASE.

The K_m and V_m values for the two enzyme preparations were determined graphically by the method of Lineweaver and Burk (91) and also by a FORTRAN IV program (92) fitting data to the Michaelis-Menten equation, calculating standard errors and presented as an Appendix to this work.

Solutions (2ml) of soluble starch and amylose type I in 0.1M-acetate buffer, pH 4.8, were incubated at 37° with

pure β-amylase (2.35U) and with "impure" β-amylase (2.7U) from DEAE-cellulose chromatography, prior to acid treatment.

Double reciprocal plots for each enzyme preparation with soluble starch and amylose are shown in Figs. 4.4.3.1 and 4.4.3.2, respectively.

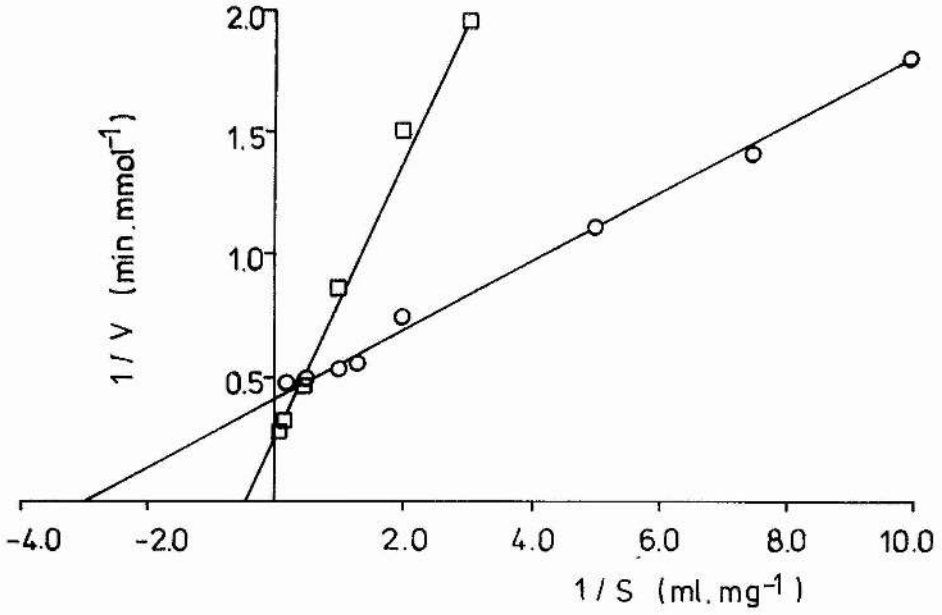
For both substrates, the K_m for pure β-amylase ($0.33 \pm 0.04 mg.ml^{-1}$ for soluble starch; $0.088 \pm 0.04 mg.ml^{-1}$ for amylose) is smaller than for the preparation containing α-amylase ($2.21 \pm 0.109 mg.ml^{-1}$ for soluble starch, $31.7 \pm 1.14 mg.ml^{-1}$ for amylose), although V_m values were higher for the impure enzyme ($4.607 \pm 0.106 mmol.min^{-1}$ for soluble starch, $4.8 \pm 1.3 mmol.min^{-1}$ for amylose) than for the pure enzyme ($2.27 \pm 0.08 mmol.min^{-1}$ for soluble starch, $1.35 \pm 0.11 mmol.min^{-1}$ for amylose).

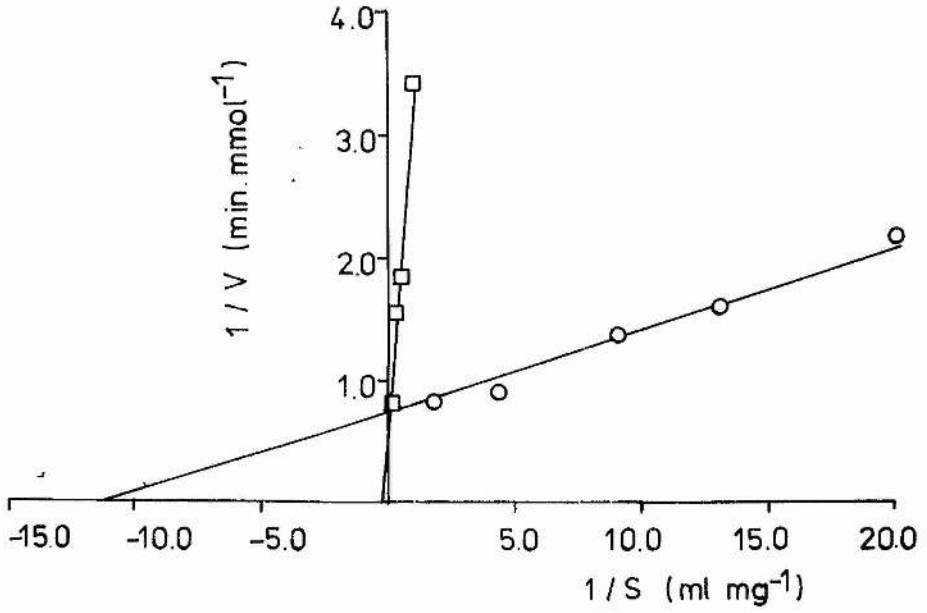
The apparent decrease in affinity of β-amylase for polymer substrates in the presence of α-amylase can be explained in terms of the relative chain lengths of the substrates. β-Amylase is known to have a greater affinity for larger substrate molecules (32) since there is a greater number of binding modes (both productive and non-productive) than with shorter chain length substrates. The introduction of α-amylase into a system containing β-amylase and a large polymer α-1,4-glucan results in the rapid internal cleavage of the molecules, essentially producing short-chain substrates and resulting in a lower affinity for the chain and an increase in K_m values.

The effect of the presence of α-amylase in preparations of β-amylase is to aid in the complete hydrolysis of the substrate molecule to maltose and maltotriose, with

Fig. 4.4.3.1. Double reciprocal plots obtained for pure β-amylase (o) and β-amylase containing traces of α-amylase (□) acting on soluble starch solutions in 0.1M-acetate buffer, pH 4.8, at 37°.

Fig. 4.4.3.2. Double reciprocal plots obtained for pure β-amylase (o) and β-amylase containing traces of α-amylase (□) acting on amylose (type I) solutions in 0.1M-acetate buffer, pH 4.8, at 37°.


2.0
1.5
1.0
0.5
1 / V (min.mmol^{-1})
-4.0
-2.0
2.0
4.0
6.0
8.0
10.0
1 / S (ml. mg^{-1})


4.0
3.0
2.0
1.0
1 / V (min.mmol^{-1})
-15.0
-10.0
-5.0
5.0
10.0
15.0
20.0
1 / S (ml mg^{-1})

some short chain, branched oligosaccharides in the case of branched α-1,4-glucans, and would therefore be expected to be of industrial interest.

A similar effect upon K_m and V_m values was found by Carvalho (92) for amyloglucosidase, another exo-amylase. In this case the $\bar{D}P$ of the substrate samples withdrawn during α-amylase hydrolysis was estimated and these α-1,4-glucans of different chain lengths were used as substrates for amyloglucosidase action, where K_m values were found to decrease with increasing substrate chain length.

Conversely, the effect of smaller chain length substrates is to increase the V_m values for β-amylase since a larger proportion of productive to non-productive complexes are formed.

These effects will be considered more fully in section 4.5.3.

4.5. KINETIC STUDIES ON β-AMYLASE USING SUBSTRATES OF DIFFERENT CHAIN LENGTH.

4.5.1. PREPARATION AND CHAIN LENGTH ESTIMATION OF AMYLOSE SUBFRACTIONS.

Amylose subfractions of various degrees of polymerization ($\bar{D}P$) were prepared as described in section 3.10.2, and their $\bar{D}P$ values estimated by the method of Hiromi et al (83). The yield of each component and average DP are shown in Table 4.5.1.1 and a distribution profile for the total amylose fraction is shown in Fig. 4.5.1.1. The first fraction produced an anomaly in the profile and could be considered to have a broader distribution range than expected. No further fractionation was attempted with this sample.

TABLE 4.5.1.1. PREPARATION OF AMYLOSE SUBFRACTIONS OF DIFFERENT $\bar{D}P$ VALUES.

AMYLOSE SUBFRACTION	SAMPLE WEIGHT (mg)	$\bar{M}_n$ $\times 10^{-3}$	$\bar{D}P$
Thymol amylose	7.5×10^3	246.51*	1369*
		240.84**	1338**
A	2.53×10^3	263.7	1465
B	2.78×10^3	161.3	906
C	215.2	38.0	211
D	99.7	20.7	115
E	39.2	17.2	95
F	35.9	12.8	71
G	20.8	10.4	58

* estimation by periodate oxidation

** estimation by end-group analysis.

4.5.2. HYDROLYSIS OF AMYLOSE SUBFRACTIONS BY β-AMYLASE.

β-Amylase (2.4U) was incubated at 37° with 1ml aliquots of 1% (w/v) solutions of each amylose preparation in 0.1M-acetate buffer, pH 4.8. Aliquots of 0.1ml were removed at intervals and assayed for the release of reducing sugars (section 3.3.2).

Results were expressed as % maximum hydrolysis and plotted against time for the initial stages of the reaction (Fig. 4.5.2.1).

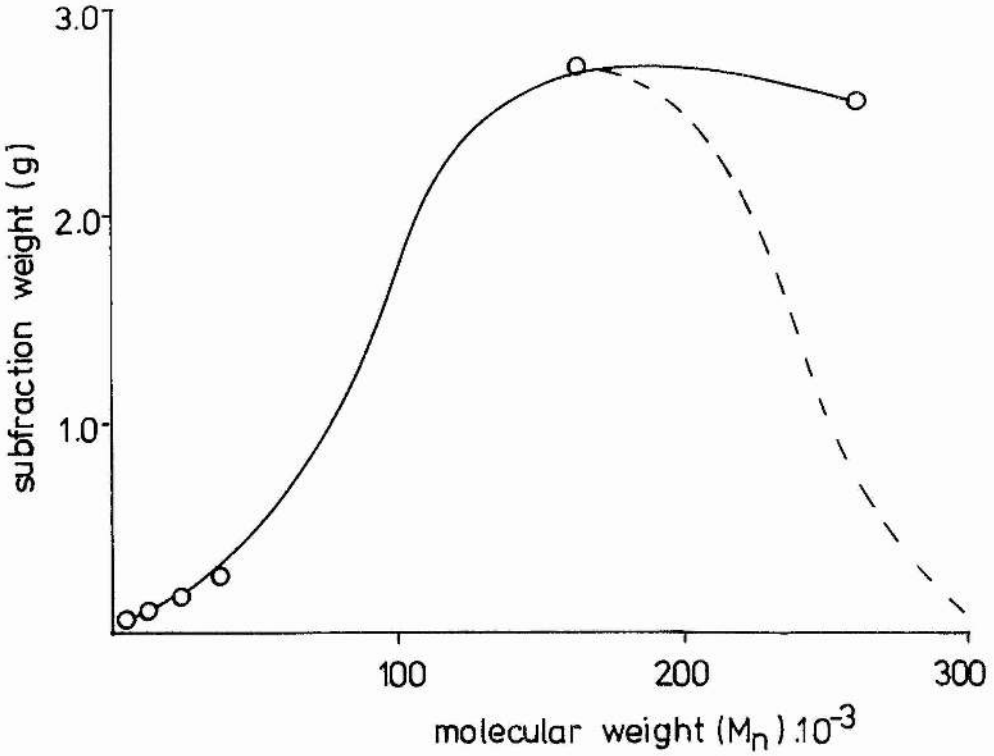
During the period of time in which the assays were performed, the enzyme displayed a more rapid attack upon smaller substrates than upon ones with a longer chain length. If it is remembered that Thoma and Koshland (32) predicted a dependence of K_m and V_m upon substrate size, the observation made for the amylose subfractions can be explained.

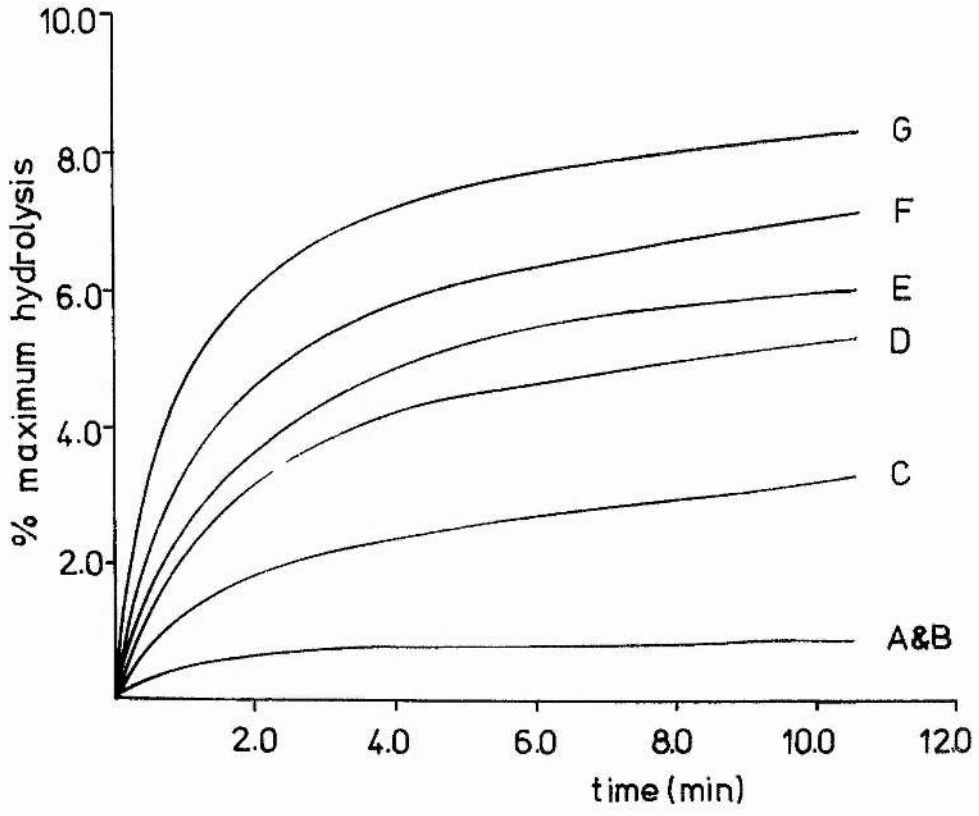
In section 4.4.3, β-amylase was found to have an increased affinity and a decreased V_m for large molecular weight α-1,4-glucans and this was explained in terms of the ratio of productive to non-productive enzyme-substrate complexes. This effect is reflected in the initial velocity of β-amylase hydrolysing the different amylose subfractions.

The hydrolyses were left for 18h at 37° and after this time, fractions A, B, C, D, E, F and G had undergone 61, 65, 73, 76, 82 and 84% hydrolysis, respectively.

Fig. 4.5.1.1. Distribution profile obtained for the amylose subfractions during the fractionation of amylose with ethanol (section 3.10.2). --- represents the shape of the expected profile.

Fig. 4.5.2.1. Time course of the initial release of maltose from amylose subfractions by the action of β-amylase.


3.0
2.0
1.0
subfraction weight (g)
100
200
300
molecular weight (M_n) $.10^{-3}$


10.0
8.0
6.0
4.0
2.0
% maximum hydrolysis
G
F
E
D
C
A&B
2.0
4.0
6.0
8.0
10.0
12.0
time (min)

4.5.3. DETERMINATION OF KINETIC PARAMETERS FOR β-AMYLASE HYDROLYSING AMYLOSES OF DIFFERENT CHAIN LENGTH.

The determination of the values of K_m and V_m for each amylose subfraction was carried out at 37° and pH 4.8 with β-amylase (0.95U) as previously described in section 4.4.3, and results were expressed as double reciprocal plots (Fig. 4.5.3.1).

As expected from section 4.4.3 and from the reports of other workers (32, 36, 93), the K_m values decreased as substrate chain length was increased due to the binding of β-amylase with internal residues of the substrate chain (Fig. 4.5.3.2). The corresponding values of V_m decreased as the chain length increased, due to the higher proportion of non-productive to productive enzyme-substrate complexes formed with larger substrate chains (Table 4.5.3.1).

Ono *et al* (93) considered this effect with respect to amyloglucosidase and Thoma and Koshland (32) and Kato *et al* (36) with respect to β-amylase, expressing the variations in K_m and V_m as a function of "self-inhibition" by the internal residues of the α-1,4-glucan chain.

The effective inhibition of the enzyme by the internal regions of the substrate cannot be represented by the conventional equations (equations 1-5 below) since the concentration of the inhibitor is directly proportional to the concentration of the substrate.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad \ldots\ldots\ldots (1)$$

$$E + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI \qquad \ldots\ldots\ldots (2)$$

$$K_m = \frac{k_{-1} + K_2}{k_1} \qquad \ldots\ldots\ldots (3)$$

$$k_i = \frac{k}{k_3} \qquad \ldots\ldots\ldots (4)$$

$$\frac{1}{v} = \frac{1}{V_m}\left[\, 1 + \frac{K_m}{[S]} \quad \left(1 + \frac{I}{K_i}\right) \right] \qquad \ldots\ldots\ldots (5)$$

Thoma and Koshland (32) stated that for an exo-amylase combining with a substrate of n units there will be one active and (n-m) inactive complexes where m represents the number of substrate units spanned by the active site, ie. the number of subsites, which has been estimated as five by Kato et al (36). Therefore;

$$I = (n-m)\ [S] \qquad \ldots\ldots\ldots (6)$$

which can be substituted into equation (5):-

$$\frac{1}{v} = \frac{1}{V_m}\left[\, 1 + \frac{K_m}{[S]} + (n-m) \quad \frac{K_m}{K_i} \right] \qquad \ldots\ldots\ldots (7)$$

It can be seen that equation (7) has the same form as the classical Michaelis-Menten equation,

$$\frac{1}{v} = \frac{1}{V_m} \quad \left(1 + \frac{K_m}{[S]}\right) \qquad \ldots\ldots\ldots (8)$$

where

$$V_m' = V_m \left[\, \frac{1}{1 + (n-m)\ \frac{K_m}{K}} \right] \qquad \ldots\ldots\ldots (9)$$

and

$$K_m' = K_m \left[\frac{1}{1 + (n-m)\frac{K_m}{K_i}} \right] \quad \ldots\ldots\ldots \quad (10)$$

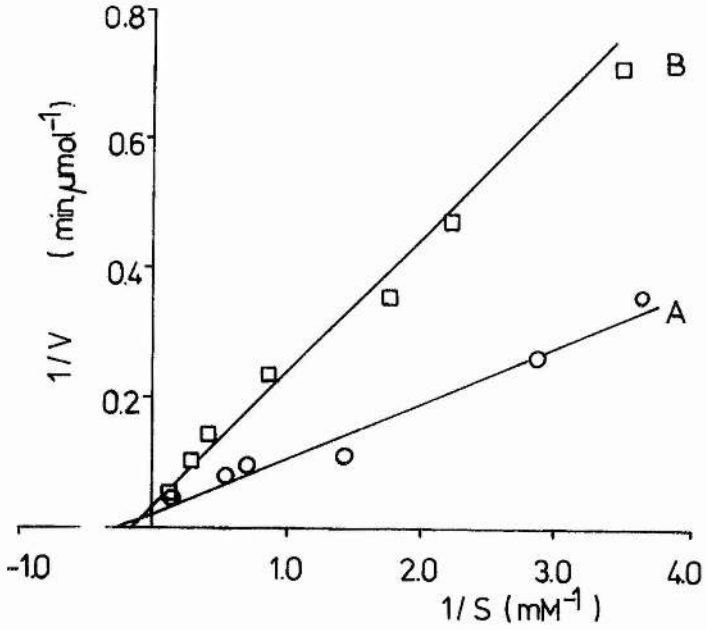
From these derivations, the inhibition constant, K_i, for the internal regions of the substrate chain can be estimated from the slope of a plot of $1/K_m$ against $(n-m)$ values for each chain length (Fig. 4.5.3.3). From the graph the K_m value for the non-reducing terminal was found to be 0.001mM and the K_i value for the internal portions of the chain, calculated from the reciprocal of the slope, was 0.00015mM. Holló *et al* (29) performed similar experiments using shorter amylose subfractions and assuming *m* to be equal to four, and found that the value of K_i was 6.75 times greater than the K_m value, drawing attention to the fact that each spiral of the amylose molecule contains approximately 6.8 glucose units. From the above values of K_m and K_i found for malt β-amylase, a 6.7-fold increase was found for K_i over K_m, indicating that, on average, 6.8 internal glucose units possess an affinity identical to that of the non-reducing end.

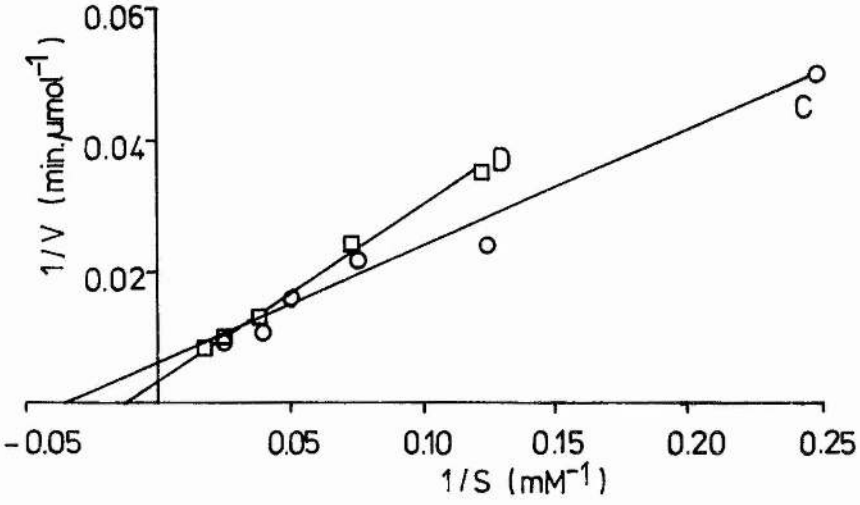
Holló *et al* (29) concluded that the inner spirals of the amylose molecule can bind to β-amylase with the same probability as a spiral occurring at the non-reducing terminal, a conclusion which appears to be supported by results from the present work.

TABLE 4.5.3.1. VARIATION IN KINETIC PARAMETERS FOR β-AMYLASE HYDROLYSING AMYLOSE SAMPLES OF DIFFERENT $\bar{D}P$.

$\bar{D}P$	K_m (mM)	V_m (mmol.min^{-1})
1465	3.5 ± 0.6	4.27 ± 0.33
906	5.2 ± 0.52	27.8 ± 1.92
211	42.0 ± 13.8	163.4 ± 44.4
115	79.0 ± 7.2	277.7 ± 41.4
71	140.0 ± 20.9	396.9 ± 37.5
58	200.0 ± 13.3	694.4 ± 59.2

Fig. 4.5.3.1. Double reciprocal plots obtained for pure β-amylase acting on solutions of amylose subfractions in 0.1M-acetate buffer, pH 4.8, at 37°.


0.8
0.6
0.4
0.2
1/V (min.μmol⁻¹)
B
A
-1.0
1.0
2.0
3.0
4.0
1/S (mM⁻¹)


0.06
0.04
0.02
1/V (min.μmol⁻¹)
C
D
-0.05
0.05
0.10
0.15
0.20
0.25
1/S (mM⁻¹)

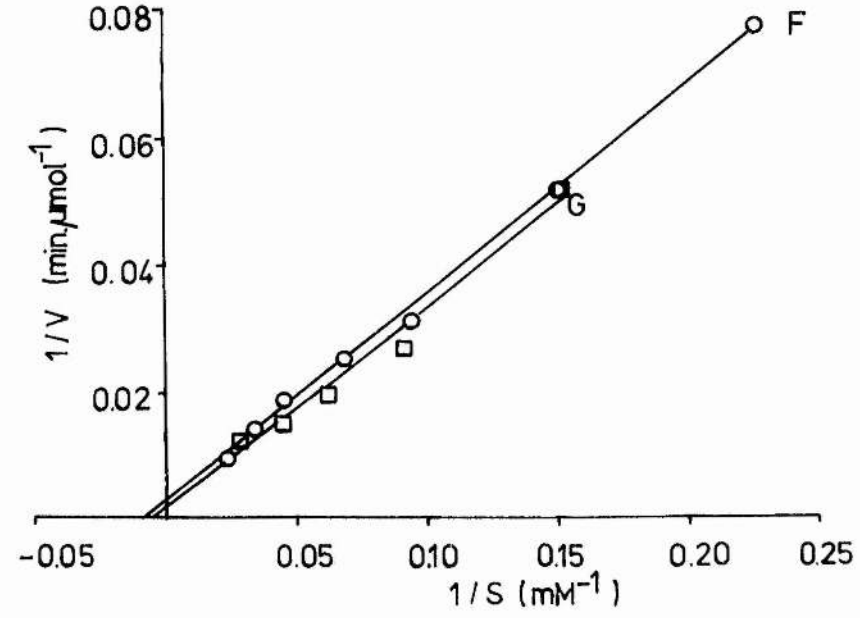
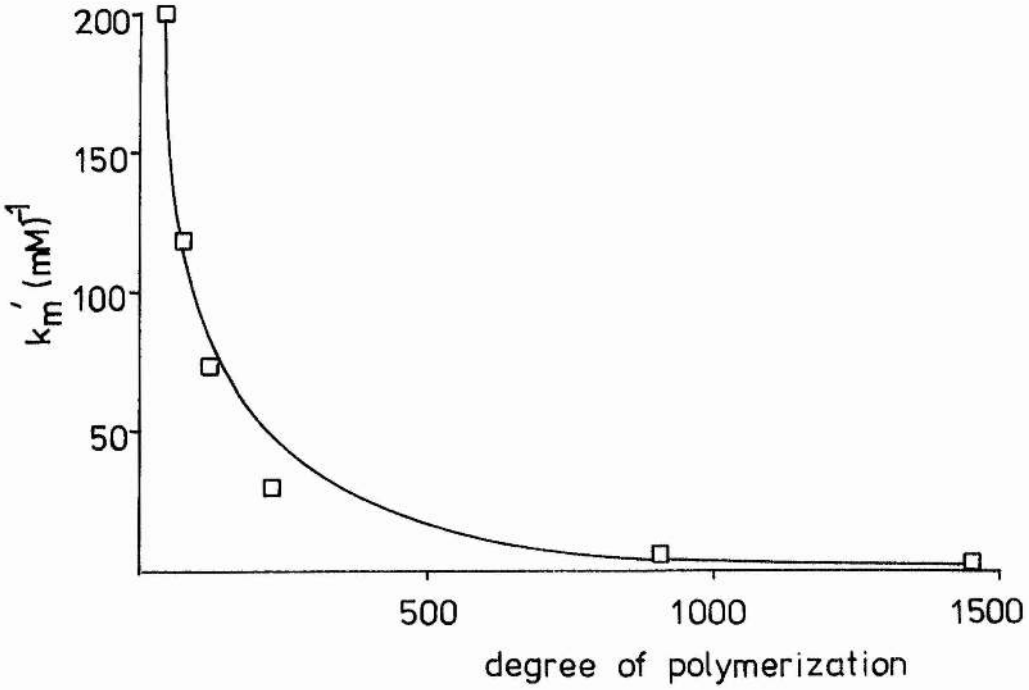
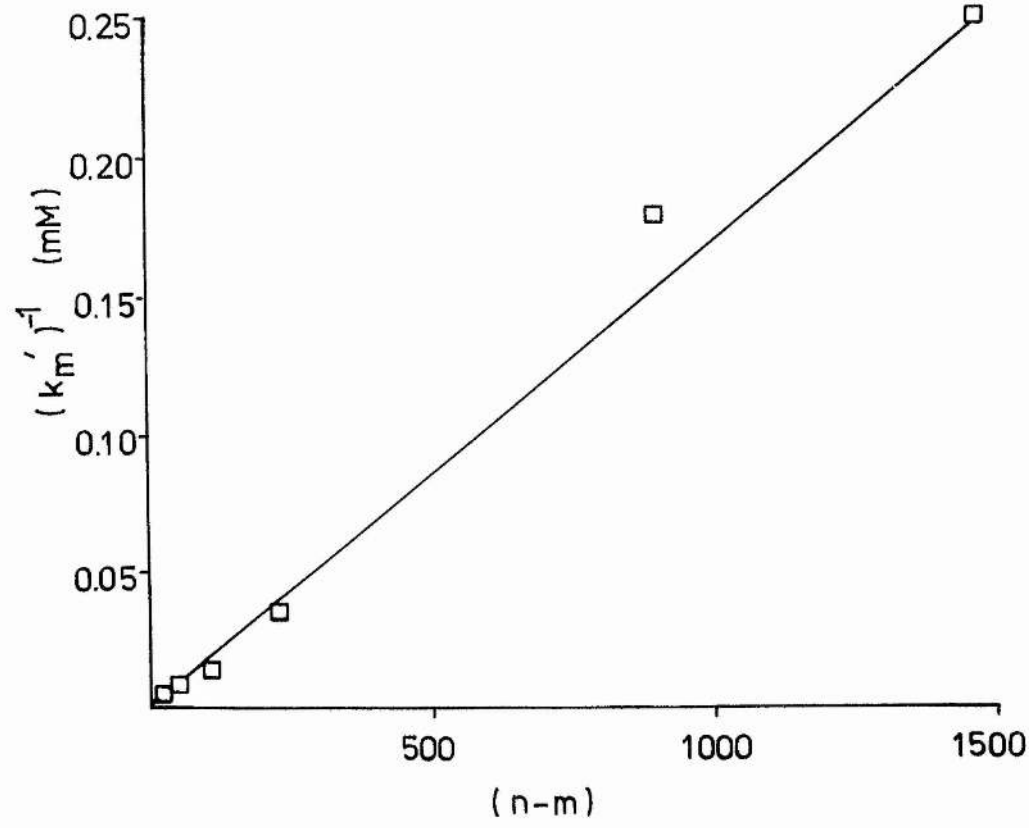

0.08
0.06
0.04
0.02
1/V (min.μmol⁻¹)
F
G
-0.05
0.05
0.10
0.15
0.20
0.25
1/S (mM⁻¹)

Fig. 4.5.3.2. Plot to show the behaviour of K_m' with variation in the degree of polymerization ($\bar{D}P$) of amylose substrates for β-amylase.

Fig. 4.5.3.3. Plot of $1/K_m'$ against (n-m) for β-amylase hydrolysing different $\bar{D}P$ amyloses, where n is the number of glucose residues in the substrate chain ($\bar{D}P$) and m is the number of subsites, and taken to be equal to 5 (36).


200
150
100
50
k_m' (mM)$^{-1}$
500
1000
1500
degree of polymerization


0.25
0.20
0.15
0.10
0.05
$(k_m')^{-1}$ (mM)
500
1000
1500
(n-m)

4.6. KINETIC STUDIES ON β-AMYLASE USING MODIFIED α-1,4-GLUCANS.

4.6.1. DETERMINATION OF THE DEGREE OF OXIDATION OF DIALDEHYDE AMYLOSES.

Complete acid hydrolysis, followed by the estimation of the glucose content of the dialdehyde amylose hydrolysates was carried out as described in section 3.6.2, and the results are presented in Table 4.6.1.1.

From the results obtained for the glucose control, it can be seen that approximately 9% of glucose residues were hydrolysed by the acid during the course of hydrolysis, and this value was taken into account in estimating the % original glucose for the dialdehyde amylose hydrolysates.

4.6.2. DETERMINATION OF KINETIC PARAMETERS FOR β-AMYLASE HYDROLYSING DIALDEHYDE AMYLOSES.

The oxidation of α-1,4-glucans results in a decrease in chain length available to β-amylase, as illustrated with 5% oxidized amylose in section 4.1. An investigation into the effect of a decrease in chain length on β-amylase activity (section 4.5.3) demonstrated a dependence of kinetic constants (K_m and V_m) upon chain length. These two points led to an investigation of the effects of increasing degrees of oxidation upon β-amylase activity, with respect to the values of K_m and V_m for each dialdehyde amylose preparation.

The determination of the values of K_m and V_m for each substrate was carried out at pH 4.8 and 37° with β-amylase (0.558U).

Due to the large proportion of aldehyde groups present in these modified substrates (Fig. 1.3.2.1), the

TABLE 4.6.1.1. DETERMINATION OF THE DEGREE OF OXIDATION OF DIALDEHYDE AMYLOSES.

THEORETICAL DEGREE OF OXIDATION	GLUCOSE PRESENT IN SAMPLE ($mg.ml^{-1}$)	GLUCOSE PRESENT AFTER HYDROLYSIS ($mg.ml^{-1}$)	% ORIGINAL GLUCOSE	% OXIDATION
2	1.11	1.001	98.2	1.8
5	1.11	0.97	95.3	4.7
10	1.11	0.90	88.6	11.4
30	1.11	0.71	68.9	32.1
45	1.11	0.55	54.1	45.9
60	1.11	0.38	37.4	63.6
(glucose)	1.11	0.98	*	-

* 8.9% glucose lost during hydrolysis.

neocuproine assay was not used initially, since very high substrate background measurements were obtained. Although the measurement of the decreasing blue value of amylose (section 3.14) with time during β-amylolysis is not generally used for the determination of β-amylase activity, this method was employed for an initial investigation into oxidation effects upon K_m and V_m values.

However, the initial (100%) blue values of the dialdehyde amylose samples were found to decrease markedly as the degree of oxidation was increased (Fig. 4.6.2.1). According to Erlander et al (94), the oxidation of amylose results in the disturbance of the stabilizing hydrogen bonds which normally hold the polymer in a helical conformation. Between 5 and 30% oxidation, rapid "unfolding" of the molecule occurs. If it is remembered that the blue value of the amylose sample is dependent upon the length of the helical regions in the molecule, the decrease in blue value mentioned above can be interpreted as a typical helix-coil transition.

To avoid inaccuracies resulting from a decrease in the blue value for dialdehyde amyloses, the determination of the values of K_m and V_m was carried out for each amylose preparation up to 30% oxidation using the neocuproine method (section 3.3.2), withdrawing samples after a short period of incubation to estimate the initial velocity of the reaction. Amylose samples of 45 and 60% oxidation could not be used since the background reading was too high.

Double reciprocal plots for each dialdehyde amylose were plotted in Fig. 4.6.2.3. It can be seen that K_m values

increase with the degree of oxidation, whereas V_m values remain constant.

The values of $1/K_m'$ were plotted against the degree of oxidation (Fig. 4.6.2.2.) and were found to result in a profile similar to that obtained with amylose preparations of different chain length (Fig. 4.5.3.2), if a high degree of oxidation is regarded as being equivalent to a low degree of polymerization.

Although these results were subject to large errors because of the nature of the substrates (Table 4.6.2.1) they showed a definite trend in that K_m values increased for substrates with higher degrees of oxidation. This would apparently agree with the K_m dependence upon chain length (section 4.5.3), since as the degree of modification (oxidation) of the residues is increased, the chain length available for β-amylase action is decreased, although theoretically, the molecule has the same chain length as the original unoxidized sample, but with an altered chemical structure.

Holló *et al* (29) postulated that the C_3 hydroxyl group of glucose residues was concerned with the fitting of α-1,4-glucanases. In unmodified α-1,4-glucans, this might partially explain the self-inhibitory effects induced by the binding of β-amylase with internal substrate residues, where the absence of a C_4 hydroxyl group (32) may prevent complete fitting of the active site of β-amylase for hydrolysis to occur.

Periodate oxidation of internal glucose residues in an α-1,4-glucan results in the cleavage of the bond

between C_2 and C_3, producing an aldehyde function at each of these positions (Fig. 1.3.2.1).

The absence of a C_3 hydroxyl group may effectively make the substrate "unattractive" to the enzyme and it may not be regarded as a substrate molecule in the region of disturbed helical structures. Therefore, the degree of self-inhibition would decrease, resulting in an increase in the K_m value.

The values of V_m remained essentially constant for all dialdehyde amylose samples. If the decreasing available chain length is considered to be the only factor affecting β-amylase action, V_m would be expected to increase as the degree of oxidation increased, ie. as the available chain length decreased, as found for amylose samples of decreasing $\bar{D}P$ (section 4.5.3). However, a strong interaction possibly occurs between amino groups in the enzyme molecule and the aldehyde functions in the α-1,4-glucan, which may compensate for the increasing V_m values as oxidation increases, to result in no apparent affect on V_m. Since barley β-amylase has been shown by an analysis of its amino acid composition to contain basic amino acids as 15% of the total amino acid residues (55), and since the amino side chains of these residues will be positively charged at the pH of β-amylase incubations (pH 4.8), it seems possible that the existence of a number of protonated amino groupings on the surface of the enzyme would bind with the aldehyde groups of the substrate molecule.

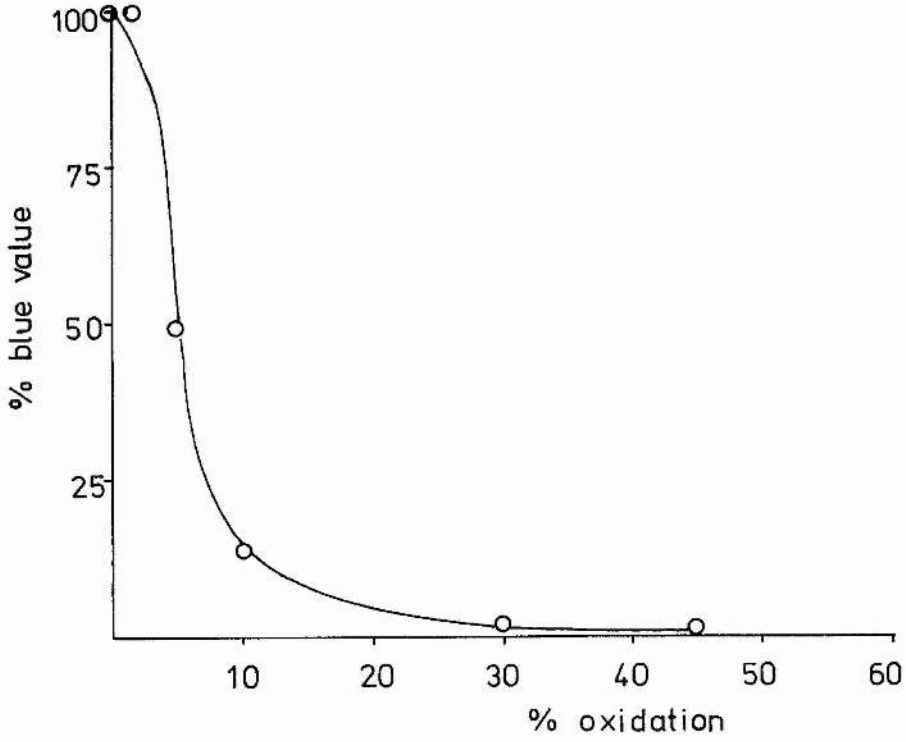
TABLE 4.6.2.1. KINETIC PARAMETERS ± STANDARD ERRORS FOR β-AMYLASE HYDROLYSING DIALDEHYDE AMYLOSES.

% OXIDATION	K_m ($mg.ml^{-1}$)	V_m ($\mu mol.min^{-1}$)
0	0.0818 ± 0.015	246.7 ± 9.6
1.8	0.208 ± 0.022	272.0 ± 9.2
4.7	0.286 ± 0.012	311.9 ± 4.9
11.4	1.666 ± 0.018	396.9 ± 17.3
32.1	3.000 ± 0.018	308.6 ± 26.1
45.9	n.d.	n.d.
63.6	n.d.	n.d.

n.d. not determined.

Fig. 4.6.2.1. Plot of the decrease in the blue value of amylose ($\bar{D}P$ 1340) as the degree of oxidation of the amylose preparation is increased.

Fig. 4.6.2.2. Plot to show the behaviour of $1/K_m$ for β-amylase with variation in the degree of oxidation of dialdehyde amylose substrates.


100
75
50
25
% blue value
10
20
30
40
50
60
% oxidation

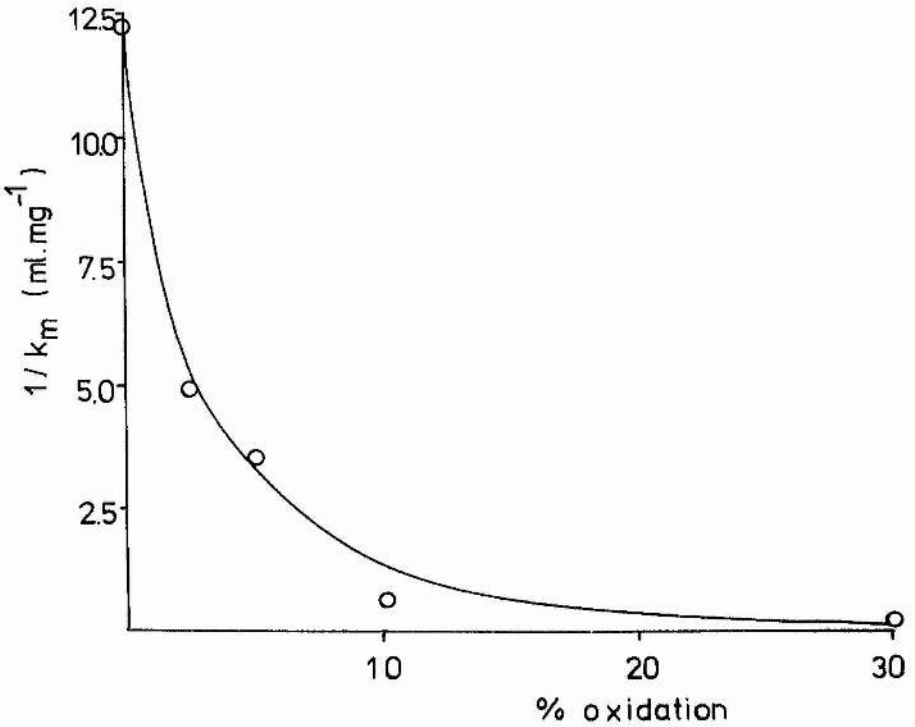
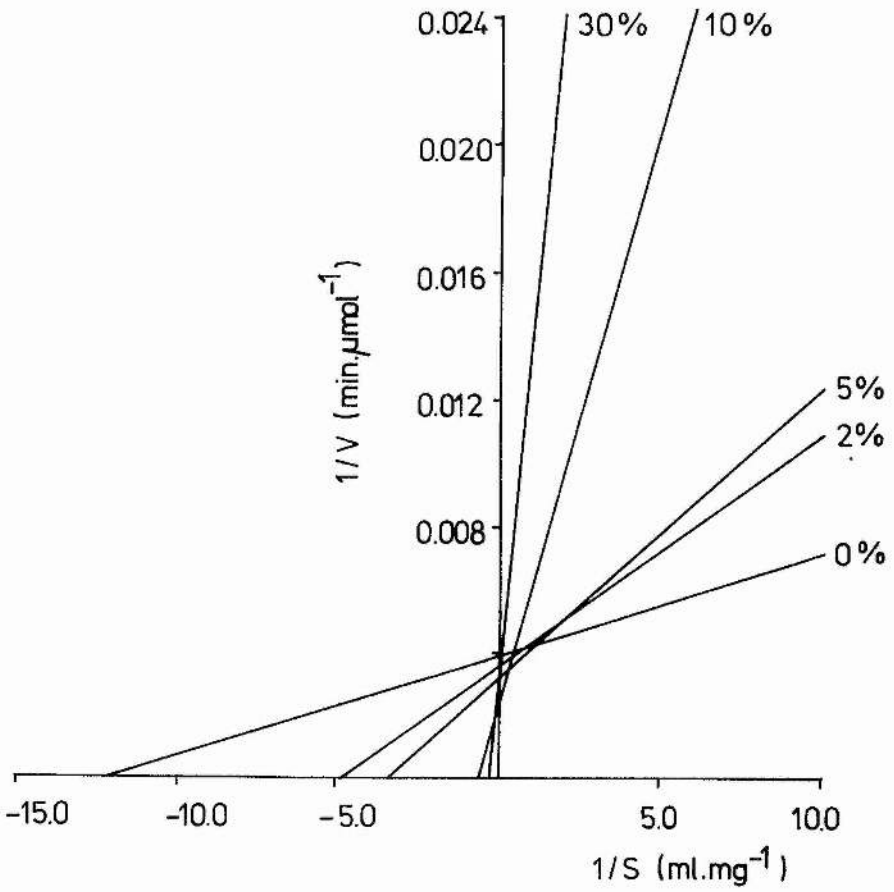

12.5
10.0
7.5
5.0
2.5
1/ k_m (ml.mg^{-1})
10
20
30
% oxidation

Fig. 4.6.2.3. Double reciprocal plots obtained for pure β-amylase acting on dialdehyde amylose substrates in 0.1M-acetate buffer, pH 4.8, at 37°.


0.024
0.020
0.016
0.012
0.008
1/V (min.µmol^{-1})
30%
10%
5%
2%
0%
-15.0
-10.0
-5.0
5.0
10.0
1/S (ml.mg^{-1})

4.6.3. DETERMINATION OF KINETIC PARAMETERS FOR β-AMYLASE HYDROLYSING REDUCED DIALDEHYDE AMYLOSES.

Dialdehyde starches have been used as supports for enzyme immobilisation, utilizing the property of the aldehyde groups to strongly bind to the amino groups of the enzyme protein or of "spacer" molecules (95, 96).

This same property may have induced anomalous binding of β-amylase to dialdehyde amyloses, resulting in a decrease in the activity of the enzyme.

The dialdehyde amylose substrates were treated by borohydride reduction (section 3.6.3) to produce derivatives with a corresponding number of alcohol groups. The reduction also resulted in the removal of the high background reading obtained using dialdehyde amylose substrates with the neocuproine method (section 3.3.2) as mentioned earlier.

The values of K_m and V_m were determined as before for each substrate (section 4.6.2) using pure β-amylase (1.2U) and the results displayed as double reciprocal plots in Fig. 4.6.3.1. A plot of $1/K_m$ against the degree of oxidation (Fig. 4.6.3.2) displayed a similar trend as that for dialdehyde amyloses, the values of $1/K_m$ decreasing as the degree of oxidation increased. However, from Table 4.6.3.1, the K_m values for each amylose sample are several times greater than for the unreduced dialdehyde substrates.

It would appear that the aldehyde groups play some part in binding the enzyme more strongly to internal residues of the substrate chain, but the reduction of these groups

resulted in the decreased affinity of the enzyme for the reduced dialdehyde substrates, as can be seen from the larger values of K_m for each reduced dialdehyde amylose compared with those for the corresponding unreduced substrate.

After the reduction of the aldehyde functions, the behaviour of the V_m values was as expected if the amylose was considered to decrease its available chain length as the degree of oxidation increased, until 30% oxidation when no further increase in V_m was found, probably due to the large proportion of distorted areas of the amylose molecule as a result of the breakdown of hydrogen bonding as already mentioned. The degree of distortion above 30% oxidation may be such that the enzyme has difficulty in approaching the amylose molecule.

TABLE 4.6.3.1. COMPARISON OF THE KINETIC PARAMETERS FOR β-AMYLASE HYDROLYSING DIALDEHYDE AND REDUCED DIALDEHYDE AMYLOSES.

% OXIDATION	K_m (mg.ml^{-1})		V_m (μmol.min^{-1})	
	dialdehyde	reduced	dialdehyde	reduced
0	0.0818	1.75	246.7	4.44
1.8	0.208	n.d.	272.0	n.d.
4.7	0.286	3.93	311.9	4.763
11.4	1.666	5.714	396.9	7.216
32.1	3.000	16.66	308.6	11.55
45.9	n.d.	20.00	n.d.	9.72
63.6	n.d.	22.70	n.d.	9.72

Fig. 4.6.3.1. Double reciprocal plots obtained for pure β-amylase acting on reduced dialdehyde amylose substrates in 0.1M-acetate buffer, pH 4.8, at 37°.

Fig. 4.6.3.2. Plot to show the behaviour of $1/K_m$ for β-amylase with variation in the original degree of oxidation of reduced dialdehyde amylose substrates.

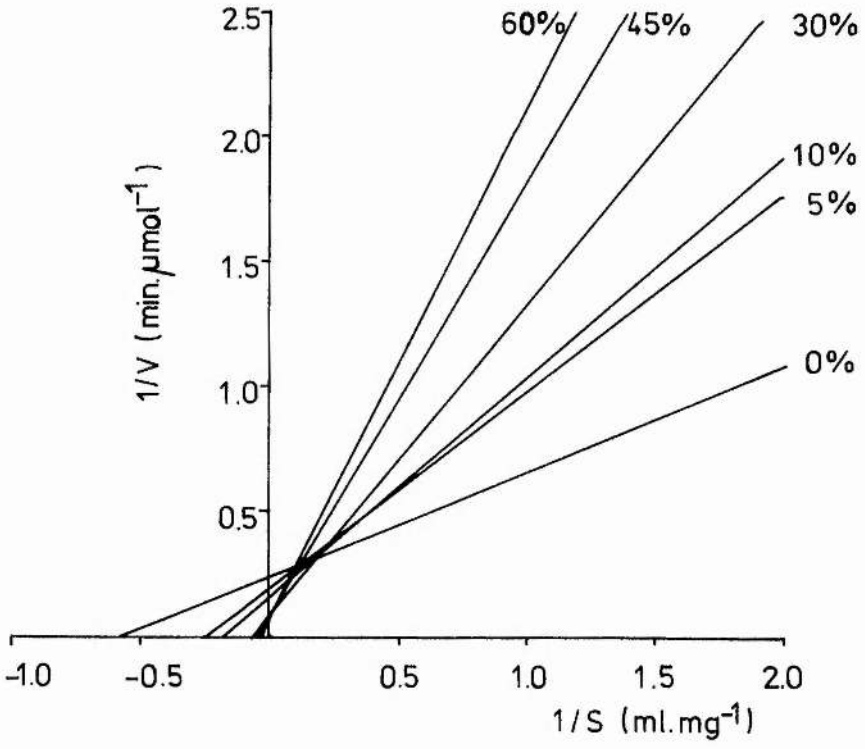
C ← FOR COMMENT

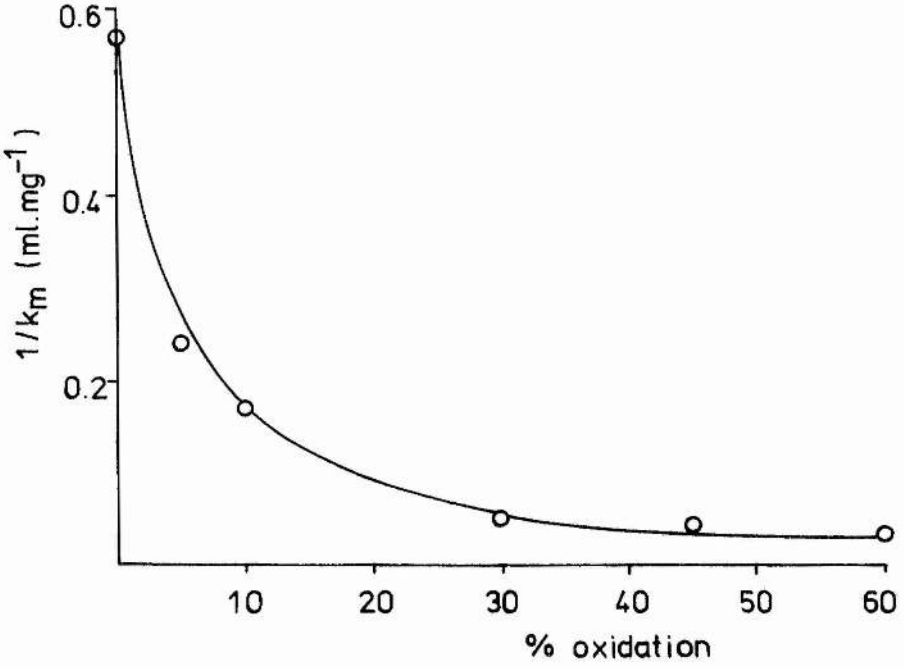
STATEMENT NUMBER

CONTINUATION

FORTRAN STATEMENT

LABEL


60%
45%
30%
10%
5%
0%
2.5
2.0
1.5
1.0
0.5
1/V (min.µmol^{-1})
-1.0
-0.5
0.5
1.0
1.5
2.0
1/S (ml.mg^{-1})


0.6
0.4
0.2
1/k_m (ml.mg^{-1})
10
20
30
40
50
60
% oxidation

4.6.4. DISCUSSION

β-Amylase activity is dependent upon several interacting factors when hydrolysing dialdehyde amylose substrates:-

1. aldehyde-amino group interaction, which may be expected to enhance the affinity of the enzyme for its substrate,
2. increasing disruption of the helical regions of the amylose molecule which would decrease the affinity due to "non-recognition" of the internal segments of the substrate by β-amylase, resulting in a decrease in self-inhibition, and
3. the oxidative removal of the C_3 hydroxyl group from internal glucose residues, which is thought to be required for the binding of the enzyme, but not to induce correct fitting of the active site for hydrolysis (29). Therefore, if this group could be considered to be partly responsible for self-inhibition by internal residues, its removal would be expected to decrease the affinity of β-amylase for dialdehyde substrates.

Possibly a combination of these factors resulted in an increase in K_m values as the degree of oxidation was increased. The effect of the aldehyde interaction upon V_m values has already been discussed in section 4.6.2, and resulted in virtually no change in V_m.

The values of K_m were substantially larger for reduced dialdehyde substrates, where the interaction between amino and aldehyde functions had been removed, and where the disruption of the helical regions and the removal of internal C_3 hydroxyl groups exerted a combined influence upon the affinity of β-amylase for the reduced substrate, resulting

in large increases in K_m values.

It is interesting to note that the decrease in affinity for the substrate as the degree of oxidation is increased, corresponds with the decrease in blue value mentioned earlier (Fig. 4.6.2.1).

Therefore, it might be supposed that the disruption of the helical regions is more important in the effect on K_m values than the removal of C_3 hydroxyl groups from internal glucose residues.

The removal of the anomalous binding situation by the reduction of dialdehyde amyloses also affected the V_m values, showing an increase in V_m until the degree of oxidation (30%) was such that the disruption of the helix probably imposed severe restrictions upon the approach of β-amylase to the substrate molecule, and a slight decrease in V_m was found.

4.7. INHIBITION STUDIES ON β-AMYLASE.

Many enzymes are regulated in the cell by inhibition by their immediate products or by compounds several steps removed from their products in a multi-enzyme system.

β-Amylase has been reported to be inhibited by β-maltose (97), and more recently by the internal regions of its substrate, resulting in competitive self-inhibition (32), often compared with the strongly competitive inhibition displayed by cyclohexa- and cyclohepta-amyloses (7).

The K_i for internal glucose units was estimated by Holló *et al* (29) for sweet potato β-amylase and has been calculated for malt β-amylase in section 4.5.3.

To further characterise the malt enzyme, inhibition studies were carried out using maltose, to compare the enzyme with sweet potato β-amylase used by Misra and French (97) and also using dialdehyde and reduced dialdehyde amyloses of high degrees of oxidation.

The determination of K_i values was carried out by the method of Dixon (98) from the equations

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \quad \ldots\ldots\ldots \quad (1)$$

$$E + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI \quad \ldots\ldots\ldots \quad (2)$$

$$K_i = \frac{k_{-3}}{k_3} \quad \ldots\ldots\ldots \quad (3)$$

and if $$v = \frac{V_m [S]}{K_m + [S]} \quad \ldots\ldots\ldots \quad (4)$$

then $$v = \frac{V_m [S]}{K_m \left(1 + \frac{I}{K_i}\right) + [S]}$$

where I is the concentration of inhibitor. Therefore

$$\frac{1}{v} = \frac{K_m}{V_m[S]} + \frac{1}{V_m} + \frac{K_m}{V_m[S]} \cdot \frac{I}{K_i} \quad \ldots\ldots\ldots (6)$$

If 1/v is plotted against I at a constant value of S (equation 6), a straight line is obtained. If the velocity of the reaction is measured in the presence of inhibitor at two substrate concentrations, S_1 and S_2, the lines obtained by plotting 1/v against I will intercept at a point above the abscissa for competitive inhibition, so that

$$\frac{K_m}{[S_1]} + 1 + \frac{K_m}{[S_1]} \cdot \frac{I}{K_i} = \frac{K_m}{[S_2]} + 1 + \frac{K_m}{[S_2]} \cdot \frac{I}{K_i} .$$

or,

$$\frac{1}{[S_1]} \left(1 + \frac{I}{K_i}\right) = \frac{1}{[S_2]} \left(1 + \frac{I}{K_i}\right)$$

which is true only if $S_1 = S_2$ or if $I = -K_i$.

Therefore the intersection of the two lines occurs at a point equal to $-K_i$.

4.7.1. INHIBITION BY MALTOSE.

Pure β-maltose monohydrate was prepared as already described in section 3.9 and the K_i value for maltose was determined by incubating β-amylase (1.3U) at 37° with 2ml aliquots of 0.4 and 0.04% (w/v) amylose ($\bar{D}P$ 1340) solutions in 0.1M-acetate buffer, pH 4.8, containing a range of concentrations (5-55mM) of maltose. Aliquots of 0.1ml were withdrawn and the blue value measured as previously described in section 3.14. The decrease in the blue value of the

amylose preparation was taken as a measure of β-amylase activity, because in the presence of maltose, extremely high background readings were obtained using the neocuproine assay method (section 3.3.2). The decrease in the blue value of $\bar{D}P$ 1340 amylose was found to be linear under the conditions used in the investigations into the inhibition of β-amylase by maltose and modified amyloses.

The results were plotted in Fig. 4.7.1.1 as the maltose concentration against the reciprocal of the velocity. The intercepts of each line with the ordinate and abscissa were computed using a FORTRAN IV bivariance program for correlation analysis and discussed in an appendix to this work.

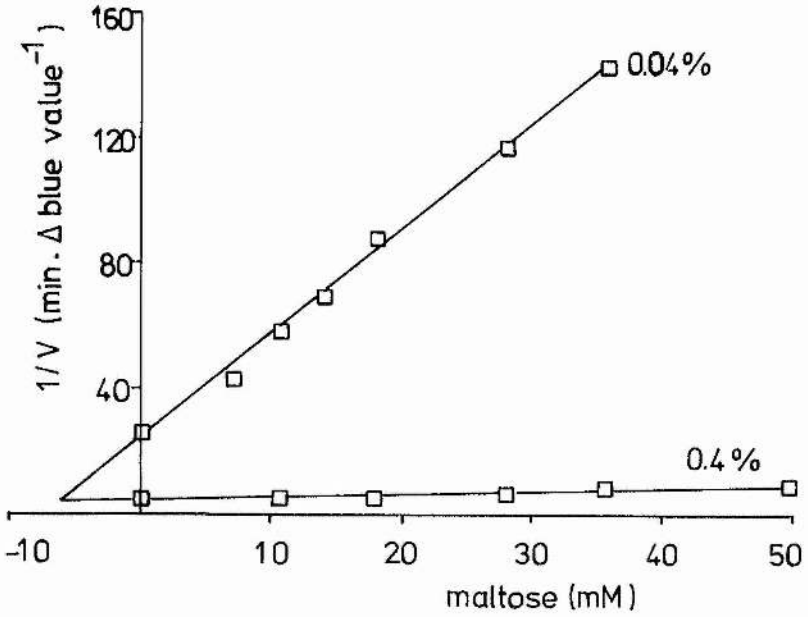
The lines were found to intersect above the abscissa (98), indicating competitive inhibition by maltose, with a K_i of 6.1mM, in agreement with the value (6mM) obtained by Misra and French (97).

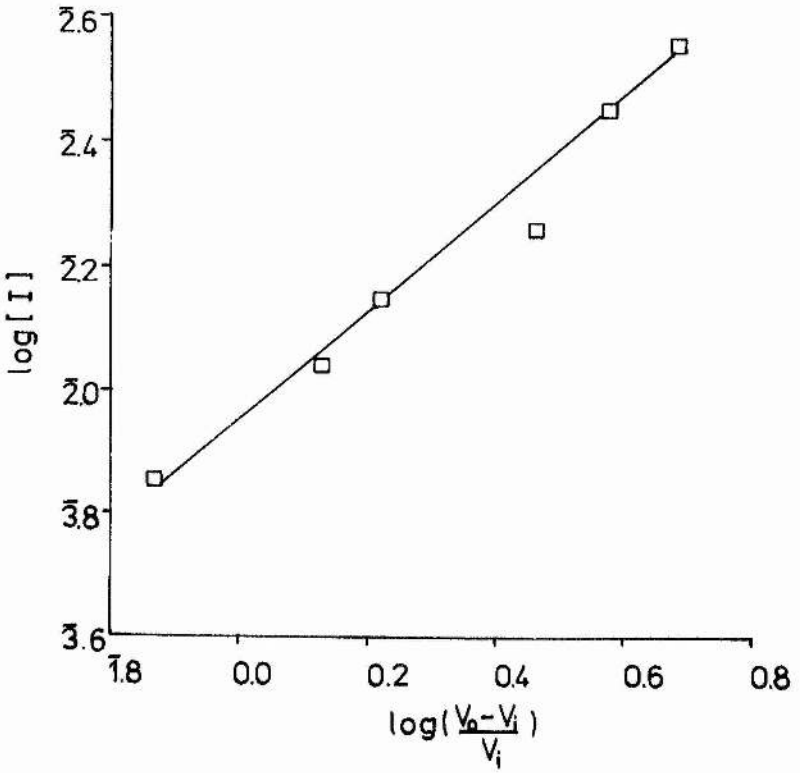
It is thought (9) that maltose acts as an inhibitor by combining with an enzyme form other than that which complexes with the substrate. Possibly the maltose product combines with β-amylase to produce a non-productive complex which is usually formed after bond rupture has occurred but before maltose leaves the active site. Evidence for the suggestion that subsites must exist on both sides of the catalytic site has been provided by Kato et al (36) who showed that the catalytic site is situated between subsites 2 and 3.

In their studies on the binding site of sweet potato β-amylase, Thoma et al (7) reported that one molecule of cycloamylose inhibitor bound with each binding, or catalytic

Fig. 4.7.1.1. Dixon plot of maltose concentration against the reciprocal of the velocity for β-amylase hydrolysing 0.04% and 0.4% (w/v) amylose ($\bar{D}P$ 1340) solutions in 0.1M-acetate buffer, pH 4.8, containing 10-50mM-maltose.

Fig. 4.7.1.2. Plot of log [I] against log $((V_o-V_i)/V_i)$ for β-amylase hydrolysing 0.4% (w/v) amylose solution as above.


160
120
80
40
1/V (min. Δ blue value^{-1})
0.04%
0.4%
-10
10
20
30
40
50
maltose (mM)


2.6
2.4
2.2
2.0
3.8
3.6
log [I]
1.8
0.0
0.2
0.4
0.6
0.8
log($\frac{V_o - V_i}{V_i}$)

site as estimated from the slope, n, of a plot of log [I] against $\log((V_o-V_i)/V_i)$, where V_i and V_o are the velocities in the presence and absence of inhibitor, I, respectively.

A similar plot (Fig. 4.7.1.2) was made for malt β-amylase inhibited by maltose and the slope of the line was calculated to be 0.95, ie. one molecule of maltose was effective in blocking each binding site of β-amylase.

4.7.2. INHIBITION BY DIALDEHYDE AMYLOSES.

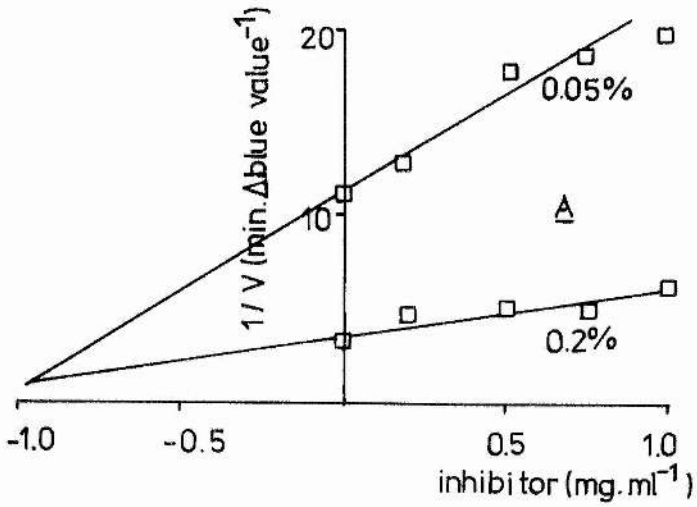
The internal helices of α-1,4-glucans are generally regarded as displaying self-inhibition towards β-amylase action (32), resulting in a decrease in both K_m and V_m with an increase in chain length. However, after oxidation with periodate, α-1,4-glucans tend to lose their helical structure (94) and a large number of aldehyde groups are introduced into the molecule, with effects on the K_m and V_m values as already discussed in sections 4.6.2 and 4.6.4. Dialdehyde amyloses can be described as modified substrates and they may display a type of inhibition other than the self-inhibitory action of internal residues in native amylose preparations.

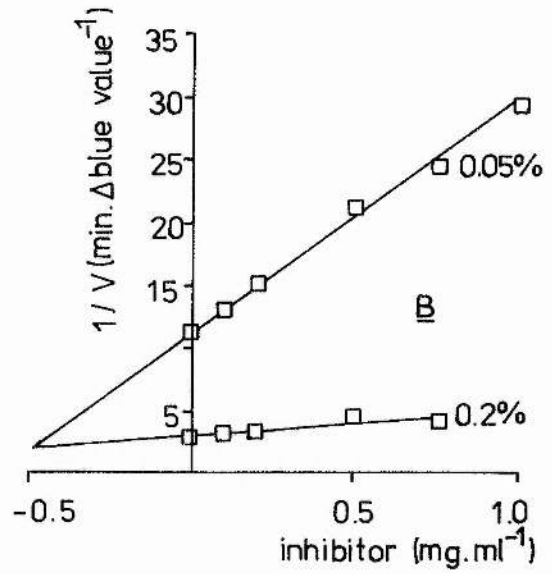
The value of K_i for internal chain portions (section 4.5.3) leads to the supposition that a complete turn of the amylose helix is involved in internal binding, therefore the effects of the oxidation of amylose upon the kinetic parameters of β-amylase may be influenced by the lack of helical regions, as previously mentioned, or by the presence of aldehyde functions (section 4.6.4).

Therefore inhibition studies were carried out by incubating β-amylase (1.3U) at 37° with 2ml aliquots of

Fig. 4.7.2.1. Dixon plots of dialdehyde amylose concentration against the reciprocal of the velocity for β-amylase hydrolysing 0.05% and 0.2% (w/v) amylose ($\bar{D}P$ 1340) solutions in 0.1M-acetate buffer, pH 4.8, containing 0.1-1mg.ml^{-1} dialdehyde amylose.

A, 10% oxidation; B, 30% oxidation;
C, 45% oxidation; D, 60% oxidation.


1/V (min. Δblue value^{-1})
20
10
0.05%
A
0.2%
-1.0
-0.5
0.5
1.0
inhibitor (mg.ml^{-1})


1/V (min. Δblue value^{-1})
35
30
25
20
15
5
0.05%
B
0.2%
-0.5
0.5
1.0
inhibitor (mg.ml^{-1})

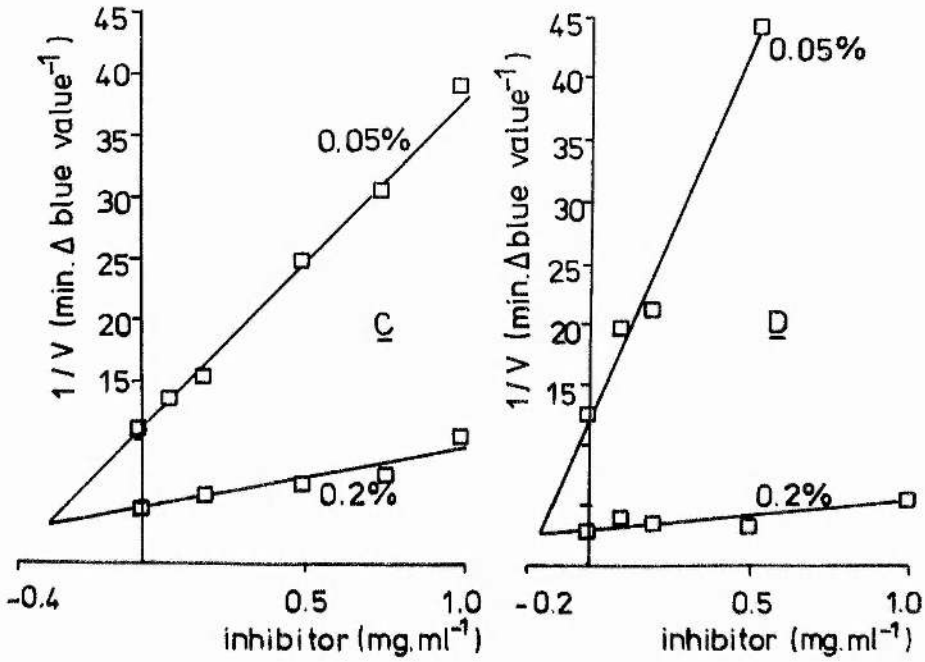
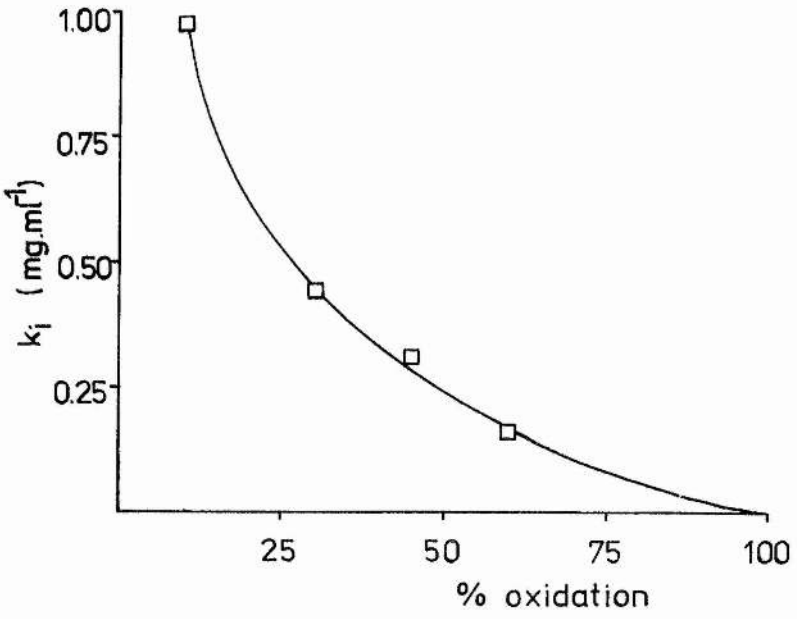
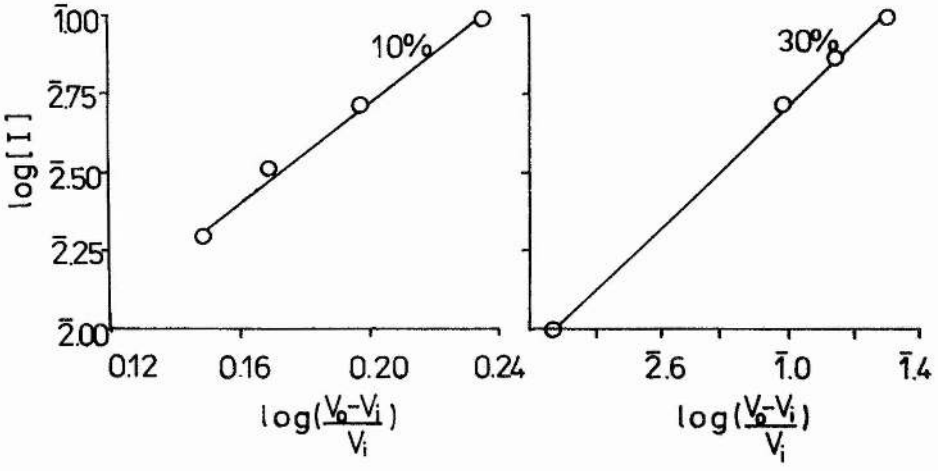
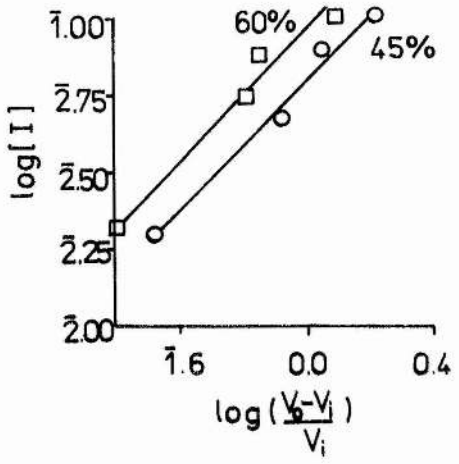

1/V (min. Δ blue value^{-1})
45
40
35
30
25
20
15
0.05%
C
0.2%
-0.4
0.5
1.0
inhibitor (mg.ml^{-1})
1/V (min. Δblue value^{-1})
45
40
35
30
25
20
15
0.05%
D
0.2%
-0.2
0.5
1.0
inhibitor (mg.ml^{-1})

Fig. 4.7.2.2. Plot to show the behaviour of K_i with variation in the degree of oxidation of dialdehyde amyloses acting as competitive inhibitors of β-amylase action.

Fig. 4.7.2.3. Plots of log [I] against log $((V_o-V_i)/V_i)$ for β-amylase hydrolysing 0.2% (w/v) amylose solution in 0.1M-acetate buffer, pH 4.8 containing 0.1-1mg.ml^{-1} dialdehyde amylose for each dialdehyde amylose preparation.


1.00
0.75
0.50
0.25
k_i (mg.ml^{-1})
25
50
75
100
% oxidation


10%
30%
log[I]
$\bar{1}$.00
$\bar{2}$.75
$\bar{2}$.50
$\bar{2}$.25
$\bar{2}$.00
0.12
0.16
0.20
0.24
$\bar{2}$.6
$\bar{1}$.0
$\bar{1}$.4
$\log(\frac{V_0-V_i}{V_i})$


60%
45%
log[I]
$\bar{1}$.00
$\bar{2}$.75
$\bar{2}$.50
$\bar{2}$.25
$\bar{2}$.00
$\bar{1}$.6
0.0
0.4
$\log(\frac{V_0-V_i}{V_i})$

0.05 and 0.2% (w/v) amylose ($\bar{D}P$ 1340) solutions in 0.1M-acetate buffer, pH 4.8, containing a range of approximately 10, 30, 45 and 60% oxidized amylose samples (0.1 - 1mg.ml^{-1}) as described for maltose in the previous section. Lower degrees of oxidation were not used since β-amylase was able to hydrolyse 2 and 5% oxidized amyloses to approximately 10-15% hydrolysis. The velocity of β-amylase action was measured by the decrease in the blue value of the amylose sample as before in section 4.7.1.

Dixon plots of dialdehyde amylose concentration against the reciprocal of the velocity (Fig. 4.7.2.1) showed each oxidized preparation to be a competitive inhibitor of β-amylase action on amylose.

It was interesting to note that the K_i values for each sample (Table 4.7.3.1) decreased as the degree of oxidation increased (Fig. 4.7.2.2), ie. the amyloses became more efficient inhibitors, with K_i tending to zero at 100% oxidation.

This would seem to emphasize that the dependence of β-amylase action on dialdehyde amyloses was related either to the intramolecular structure, such as the helical conformation, or to the presence of aldehyde groups, since the efficiency of inhibition was directly related to the degree of oxidation.

As in the case of maltose, plots of log [I] against log $((V_o-V_i)/V_i)$ were made (Fig. 4.7.2.3) for each dialdehyde amylose preparation. From the slopes, the values of 0.81, 1.0, 0.99 and 1.03 were obtained for 10, 30, 45 and 60%

oxidation, respectively, showing that one molecule of modified amylose was bound per binding site. The low value for 10% oxidation may be due to its being a less efficient inhibitor.

4.7.3. INHIBITION BY REDUCED DIALDEHYDE AMYLOSES.

As already mentioned, dialdehyde starches have been used to immobilise enzymes (95, 96) by virtue of the strong interaction between aldehyde and amino functions. To determine whether the inhibitory effect, like the effect on kinetic parameters, of dialdehyde amyloses was attributable to the loss of internal structure rather than to the strong binding forces already mentioned between the substrate and enzyme, reduced dialdehyde amyloses were used in inhibition studies.

The inhibitory effect of reduced dialdehyde amyloses upon β-amylase action was investigated as described in sections 4.7.1 and 4.7.2 by incubating β-amylase (1.3U) at 37° with 2ml aliquots of 0.1% and 1% (w/v) amylose ($\bar{D}P$ 1340) solutions in 0.1M-acetate buffer, pH 4.8, containing a range of reduced dialdehyde amylose concentrations (0.2-2mg.ml^{-1}) for approximately 10, 30, 45 and 60% oxidation.

The velocity of β-amylase action was measured by assaying the rate of release of maltose at 37° from amylose by the neocuproine method (section 3.3.2), and also by measuring the reduction in blue value of the amylose substrate (section 3.14) to compare the accuracy of the two methods.

The results obtained by reducing sugar estimation and by blue value measurements were found to be comparable,

with a variation of ±5%.

The K_i values for reduced dialdehyde amyloses were found to be virtually the same as for the unreduced preparations (Table 4.7.3.1), varying as before, with amyloses of higher degrees of oxidation being more efficient inhibitors of β-amylase action.

The values of the slope, n, obtained from plots of log [I] against log $((V_o-V_i)/V_i)$ for each reduced dialdehyde amylose preparation (cf. Fig. 4.7.2.3) were 0.78, 0.92, 0.94 and 0.95 for 10, 30, 45 and 60% oxidation, respectively.

Therefore, it would again appear that one molecule of inhibitor is effective in blocking one catalytic site. As before (section 4.7.2), a low value was found for the 10% oxidized preparation, and may be due to the presence in the preparation of helical regions, making the molecule a less efficient inhibitor and resulting in errors for the velocity of β-amylase action.

TABLE 4.7.3.1. INHIBITION CONSTANTS FOR DIALDEHYDE AND REDUCED DIALDEHYDE AMYLOSES ACTING BY COMPETITIVE INHIBITION ON β-AMYLASE.

% OXIDATION	K_i (mg.ml^{-1})	
	DIALDEHYDE	REDUCED DIALDEHYDE
11.4	0.975	0.9
32.1	0.45	0.4
45.9	0.3	0.34
63.6	0.15	0.15

4.7.4. DISCUSSION.

β-Amylase has been reported to be inhibited by a number of compounds, including maltose (97), α-methyl glucoside (99) and cycloamyloses (32).

The effect of maltose as a competitive inhibitor has been discussed by Misra and French (97) and will not be considered further here. However, the inhibitory effect of dialdehyde amyloses has not been mentioned previously. The investigations into the inhibitory effect of dialdehyde amyloses on β-amylase activity were carried out by assaying the incubation mixtures at time intervals when virtually no hydrolysis of the inhibitors would have occurred, since the rates of hydrolysis of dialdehyde amyloses were much lower than those of unmodified amylose preparations.

Since the K_i values for the dialdehyde and the reduced dialdehyde amyloses (Table 4.7.3.1) are similar, it would appear that the basic effect of inhibition by these modified amyloses is not dependent upon the presence of aldehyde functions, in common with the behaviour of K_m values, which have been suggested to be independent of such functions (section 4.6.4) but to be affected by the loss of internal helical structure.

Thoma and Koshland (32) proposed that β-amylase was self-inhibited by the internal glucose residues of its substrate chain. The competitive inhibition displayed by dialdehyde amyloses cannot be interpreted as an enhanced self-inhibitory state, since oxidation leads to a disruption of the intramolecular helical structure which has been important in self-inhibition.

Hiromi (100) has suggested that the active site of amylases may resemble that of lysozyme in being a cleft on the surface of the protein and in which the α-1,4-glucan substrate would lie. β-Amylase binds in a productive complex with the non-reducing terminal of an α-1,4-glucan chain, breaking and forming hydrogen bonds (29) as the substrate slides over the active site cleft during its sequential hydrolysis.

The effect of the oxidation of its substrate upon β-amylase activity can be considered with respect to the oxidation either of terminal glucose residues or of internal glucose residues. As the degree of oxidation is increased, the probability of glucose residues at or near the non-reducing terminal being oxidized is increased. Therefore, the possibility of β-amylase recognizing the non-reducing terminal as a potential substrate with α-1,4-linked maltose units, is decreased. However, since the active site is considered to be composed of five subunits (36), the modified non-reducing terminal may bind with these subsites in a non-productive complex, blocking the access of true (unmodified) amylose substrate molecules to the active site.

Between 2 and 40% oxidation, the internal structure of amylose changes from an ordered helical arrangement to a random coil (94), due to the oxidation of internal glucose residues. Since dialdehyde and reduced dialdehyde amyloses are competitive inhibitors of β-amylase action, their action must involve their binding to the enzyme at a binding site or the catalytic site (7) to block the access of the true substrate to the catalytic site. It is possible that the

random coil nature of the modified amyloses may result in their blocking the active site cleft by steric hinderance due to the size of the molecule rather than by specific binding with the subsites. Secondary binding may then occur between the aldehyde or alcohol functions on the inhibitor molecule and groups on the surface of the enzyme protein.

From the plots in Fig. 4.7.2.3 and from section 4.7.3, it was found that one molecule of each modified amylose bound with one binding site, which is possibly identical with the catalytic site (7). A similar result was found for maltose (Fig. 4.7.1.2).

However, until the exact nature of the subsites of β-amylase is known, the action of dialdehyde and reduced dialdehyde amyloses in inhibiting β-amylase can only be suggested as being concerned with the breakdown in the internal structure of the amylose molecules.

4.8. PREPARATION OF MALTO-OLIGOSACCHARIDES.

4.8.1. FRACTIONATION BY GEL FILTRATION ON SEPHADEX G-25

The preparation of a partial acid hydrolysate of amylose and its application to a Sephadex G-25 column was described in section 3.10. The elution profile obtained is shown in Fig. 4.8.1.1.

The number of fractions containing unhydrolysed amylose extended over such a wide range that the amylose peak probably masked the elution of malto-oligosaccharides with $\bar{D}P$ greater than 3.

Therefore, this method, carried out as described, was considered to be unsatisfactory.

4.8.2. FRACTIONATION ON CHARCOAL-CELITE.

The method used for the fractionation of malto-oligosaccharides on a charcoal-Celite column was described in section 3.10.3.3. The elution profile obtained with increasing ethanol concentration is shown in Fig. 4.8.2.1. The fractions obtained for each ethanol concentration were pooled and centrifuged at room temperature and 15000g for 20 min to remove charcoal which was present in all preparations. As reported by Whelan *et al* (82) the fractions were all a pale yellow colour.

Since a more satisfactory separation of malto-oligosaccharides was obtained by this method, the pooled fractions were stored at 4^{o} for further studies after their $\bar{D}P$ and the yield of each fraction had been calculated. The number-average molecular weight was determined by the method of Hiromi *et al* (83) as described in section 3.11.2, and the

Fig. 4.8.1.1. Elution profile obtained for the separation of malto-oligosaccharides on a Sephadex G-25 (fine) column (3 x 80cm).

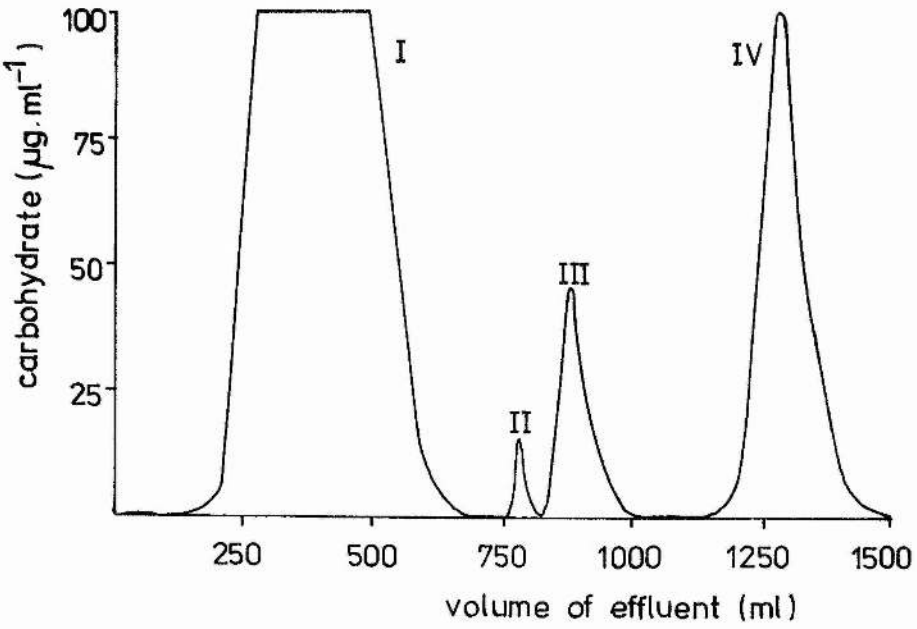
I amylose

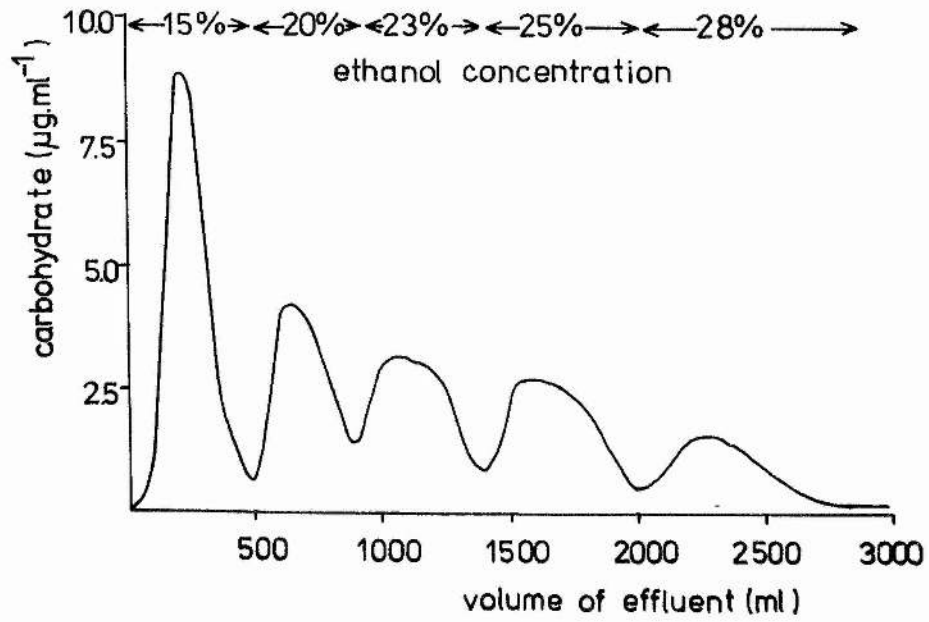
II maltotriose ?

III maltose

IV glucose

Fig. 4.8.2.1. Elution profile obtained for the separation of malto-oligosaccharides on a charcoal-Celite column with ethanol.


100
75
50
25
carbohydrate (µg.ml⁻¹)
I
II
III
IV
250
500
750
1000
1250
1500
volume of effluent (ml)


10.0
7.5
5.0
2.5
carbohydrate (µg.ml⁻¹)
←15%→ ←20%→ ←23%→ ←25%→ ←28%→
ethanol concentration
500
1000
1500
2000
2500
3000
volume of effluent (ml)

$\bar{D}P$ of each sample calculated from this data and presented in Table 4.8.2.1. Within experimental error, the values for $\bar{D}P$ obtained for the malto-oligosaccharides are comparable to the expected values.

TABLE 4.8.2.1. AVERAGE DEGREE OF POLYMERIZATION OF MALTO-OLIGOSACCHARIDE FRACTIONS.

% (v/v) ETHANOL FOR ELUTION	WT. OF SAMPLE (mg)	$\bar{M}n$	$\bar{D}P$	EXPECTED $\bar{D}P$
15	4.074	633.6	3.52	3
20	1.666	730.8	4.06	4
23	1.676	955.8	5.31	5
25	1.204	1024.0	5.8	6
28	2.166	1278.1	7.1	7

4.9. STUDIES ON IMMOBILISED β-AMYLASE DERIVATIVES.

Two different supports were used to immobilise β-amylase to investigate any differences between the two enzyme preparations and between each preparation and preparations of other immobilised enzymes on similar supports.

All experiments were carried out using β-amylase preparations in continuously stirred vessels to reduce diffusional effects, which will be considered in the discussion.

The advantage of preparing immobilised derivatives of β-amylase for kinetic studies lies in the fact that the enzyme can be recovered after each assay, washed with buffer and re-used, therefore eliminating the need to prepare large amounts of the derivatives.

4.9.1. AMINOETHYL CELLULOSE (AE-CELLULOSE) - β-AMYLASE.

An AE-cellulose derivative of β-amylase was prepared as described in section 3.13.2.

The specific activity of the enzyme before and after coupling was estimated as previously described (sections 3.1 and 3.13.3), the amount of protein bound (section 3.13.2) was determined by the Folin-Lowry method (section 3.2.1) and the values presented below:-

PROTEIN BOUND (mg. 100mg support^{-1})	SPECIFIC ACTIVITY (U.mg bound enzyme^{-1})	SPECIFIC ACTIVITY RETAINED (%)
0.4766	4.129	18.7

4.9.1.1. pH-ACTIVITY PROFILE OF AE-CELLULOSE - β - AMYLASE.

The pH-activity profile was determined by incubating an aliquot of the immobilised enzyme suspension (0.39U) in 0.1M-citrate-phosphate buffer in a continuously stirred vessel at 37° with 2.5ml 1% (w/v) soluble starch solution in 0.1M-citrate-phosphate buffer at a range of pH values (3.0-8.5).

Aliquots of 0.5ml were withdrawn at time intervals, centrifuged at 7000g for 1 min and the supernatant assayed for the release of maltose by the neocuproine method (section 3.3.2). The results were plotted as % maximum activity obtained against pH (Fig. 4.9.1.1.1), and compared with the pH-activity profile obtained for the soluble enzyme.

The optimum pH was found to lie between 4.5 and 5.5, in good agreement with the soluble enzyme, but the alkali limb of the profile was shifted towards lower pH values.

This effect was reported by Goldstein et al (101) and explained by the polycationic nature of the support resulting in an unequal distribution of protons and hydroxyl ions between the "polyelectrolyte phase", within which the enzyme was situated, and the outer solution. The local hydrogen ion concentration in the domain of the polycationic β-amylase derivative is lower than that measured in the external solution and consequently the pH-profile of β-amylase within a positively-charged carrier is displaced towards more acid pH values.

4.9.1.2. TEMPERATURE-ACTIVITY PROFILE OF AE-CELLULOSE-β-AMYLASE.

The temperature-activity profile was determined by incubating an immobilised enzyme suspension (0.39U) in a continuously stirred vessel with 2.5ml 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8 at various temperatures. Assays were carried out as described in the previous section.

The results were plotted at % maximum activity against temperature and compared with the soluble enzyme (Fig. 4.9.1.2.1). AE-cellulose-β-amylase appears to be less stable at higher temperatures than the soluble enzyme, with the temperature for optimum activity between 30° and 50°.

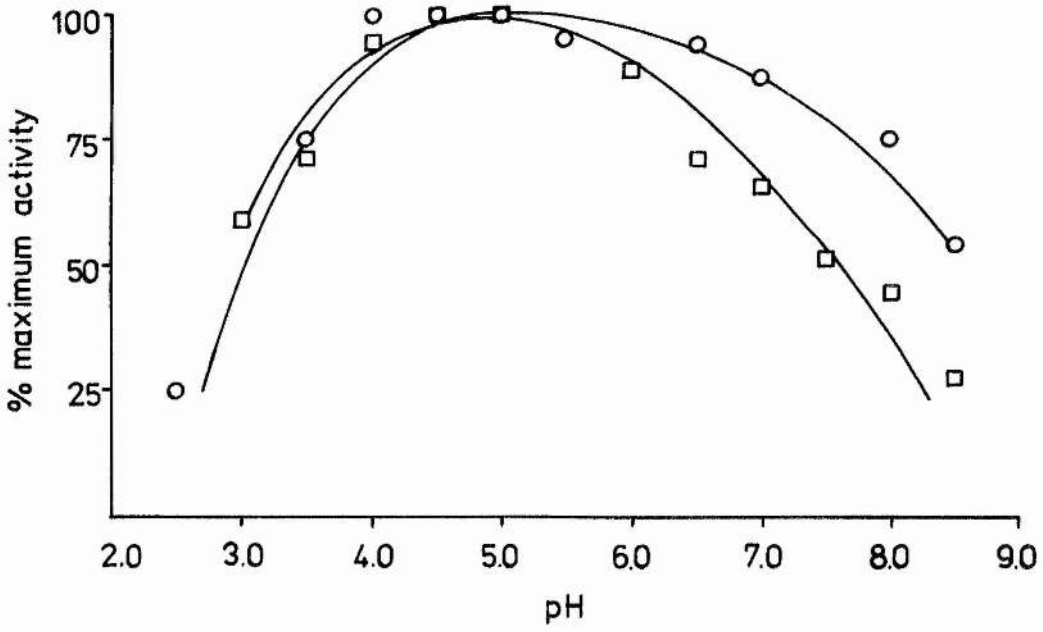
4.9.1.3. THERMAL STABILITY OF AE-CELLULOSE-β-AMYLASE.

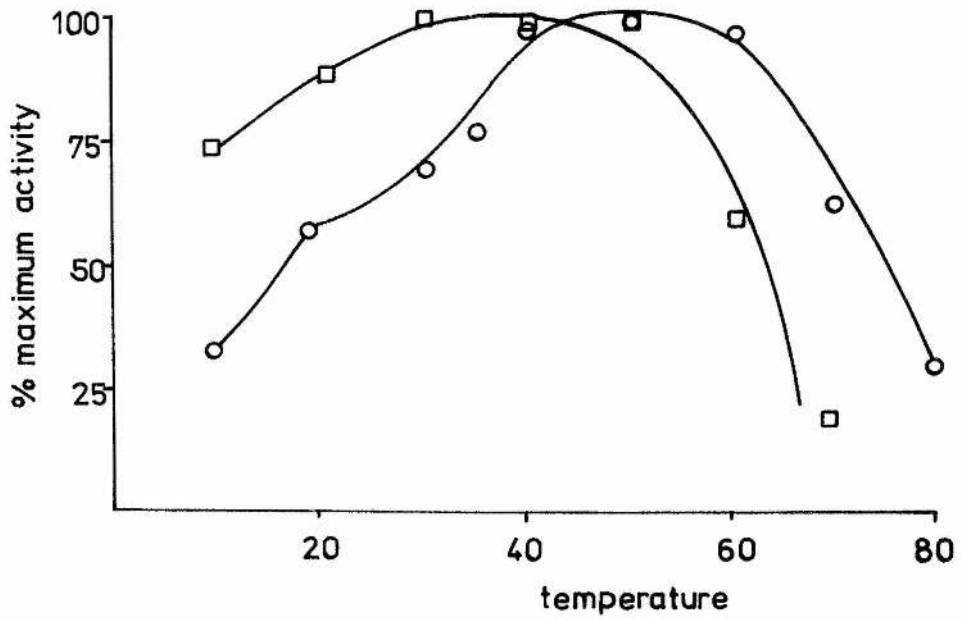
An aliquot (0.195U) of the immobilised enzyme suspension was pre-incubated at various temperatures (20-60°) for 1h, cooled in ice and assayed at 37° by incubation in a continuously stirred vessel with 2.5ml 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8, monitoring the release of maltose as previously described in section 4.9.1.1.

Results were plotted as % original activity against temperature of pre-incubation (Fig. 4.9.1.3.1) and were compared with results obtained using the soluble enzyme (1.24U). It can be seen that AE-cellulose-β-amylase has a lower thermal stability than the soluble enzyme over this range of temperatures.

Fig. 4.9.1.1.1. pH-Activity profile for AE-cellulose-β-amylase (□) and soluble β-amylase (O) hydrolysing 1% (w/v) soluble solution in 0.1M-citrate-phosphate buffer at 37°.

Fig. 4.9.1.2.1. Temperature-activity profile for AE-cellulose-β-amylase (□) and soluble β-amylase (O) hydrolysing 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8.


100
75
50
25
% maximum activity
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pH


100
75
50
25
% maximum activity
20
40
60
80
temperature

Although a number of immobilised enzymes show enhanced thermal stability over the soluble enzyme, there have been reports of the immobilisation of enzymes to carriers (59) which resulted in a decrease in thermal stability. This may be related to there being less probability of an enzyme regaining its active conformation after incubation at certain temperatures, due to novel hydrogen-bonding possibilities with the support.

4.9.1.4. DETERMINATION OF KINETIC PARAMETERS FOR AE-CELLULOSE-β-AMYLASE.

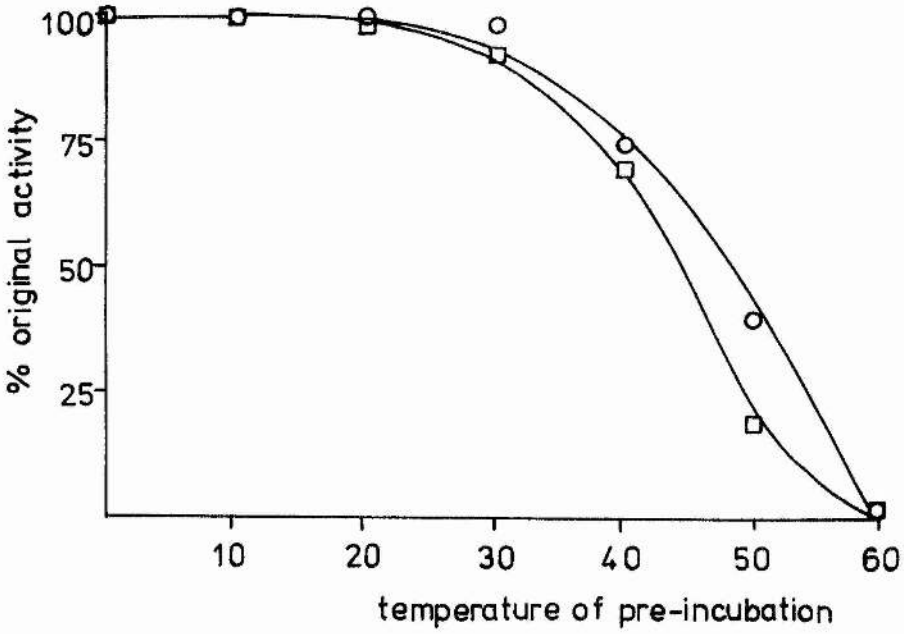
The values of the apparent Michaelis constant (K_m') and the apparent maximum velocity (V_m') for AE-cellulose-β-amylase were determined by incubating the immobilised enzyme suspension (0.398U) with 2.5ml aliquots of soluble starch solutions (0.1-10mg.ml^{-1}) in 0.1M-acetate buffer, pH 4.8, at 37^{o} in a continuously stirred vessel. The release of maltose was measured as previously described (section 4.9.1.1) and the results expressed as a double reciprocal Lineweaver-Burk plot (91) in Fig. 4.9.1.4.1 and compared with that for the soluble enzyme.

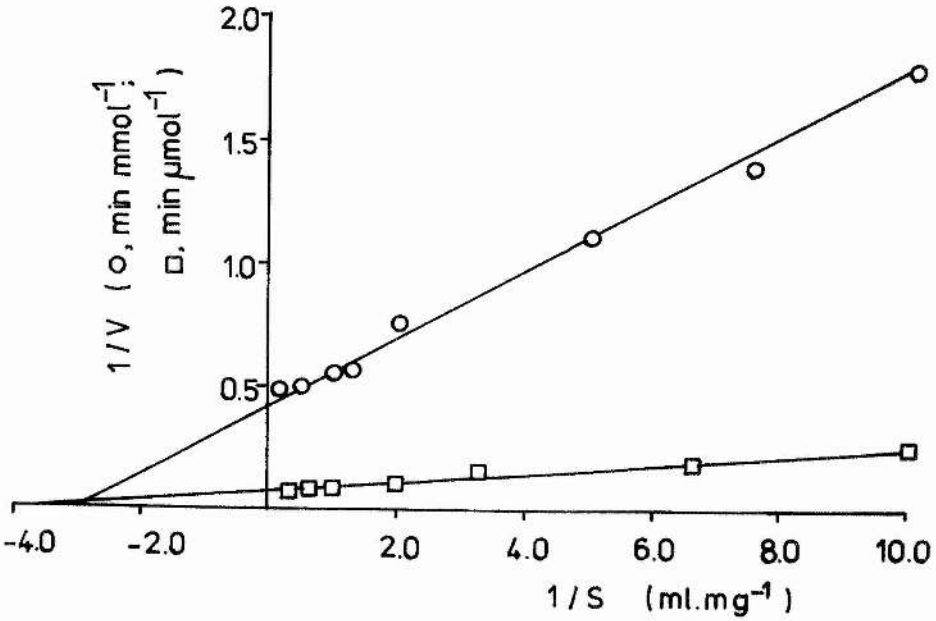
The value of K_m' was found to be 0.217 $\pm$ 0.017 mg.ml^{-1} compared with 0.33 mg.ml^{-1} for the soluble enzyme, and the value of V_m' was 15.98 $\pm$ 0.36 μmol.min^{-1} compared with a V_m value of 2.4195 mmol.min^{-1}.

From these results, it would appear that β-amylase immobilised to an AE-cellulose support has a greater affinity for soluble starch than the soluble enzyme and a V_m' value 150 times smaller than the soluble enzyme.

Fig. 4.9.1.3.1. Thermal stability of AE-cellulose-β-amylase (□) and soluble β-amylase (○) pre-incubated for 1h at various temperatures cooled and assayed by incubating at 37° with 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8.

Fig. 4.9.1.4.1. Double reciprocal plots for AE-cellulose-β-amylase (□) and soluble β-amylase (○) acting on soluble starch solutions in 0.1M-acetate buffer, pH 4.8, at 37°.


100
75
50
25
% original activity
10
20
30
40
50
60
temperature of pre-incubation


2.0
1.5
1.0
0.5
1/V (o, min mmol^{-1}; □, min μmol^{-1})
-4.0
-2.0
2.0
4.0
6.0
8.0
10.0
1/S (ml.mg^{-1})

These effects can be explained in terms of micro-environmental and steric hinderance effects and will be considered in section 4.9.3.

4.9.1.5. DETERMINATION OF INHIBITION CONSTANT OF AE-CELLULOSE-β-AMYLASE USING MALTOSE.

The method used for the determination of the inhibition constant (K_i') for AE-cellulose-β-amylase using maltose as inhibitor was essentially the same as that used for the soluble enzyme in section 4.7.1.

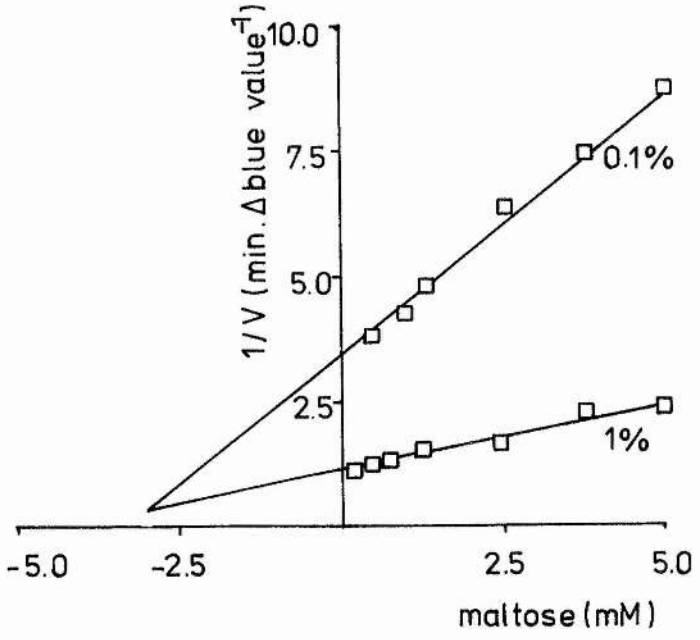
An aliquot of immobilised enzyme suspension (0.195U) was incubated, with stirring, at 37° with 2.5ml aliquots of 0.1 and 1% (w/v) amylose solutions in 0.1M-acetate buffer, pH 4.8 containing a range of maltose concentrations (0.5-6mM). The measurement of the release of reducing sugars as maltose was limited for the same reasons as before, therefore the velocity of the reaction was followed by measuring the decrease in blue value at 610nm of the amylose substrate (section 3.14).

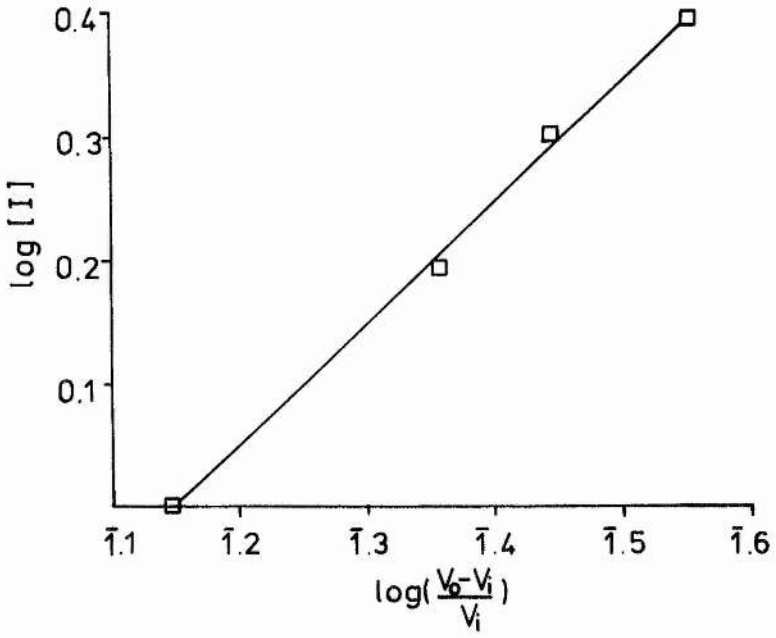
The results were expressed as Dixon plots (98) of maltose concentration against the reciprocal of the velocity for each substrate concentration (Fig. 4.9.1.5.1). The two lines were found to intersect above the abscissa, indicating competitive inhibition as found for the soluble enzyme (section 4.7.1), but the K_i' for AE-cellulose-β-amylase was 3mM maltose, half the value of K_i for the soluble enzyme.

As before (section 4.7.1), a plot of log [I] against log $((V_o-V_i)/V_i)$ was made (Fig. 4.9.1.5.2) and from the value

Fig. 4.9.1.5.1. Dixon plot of maltose concentration against the reciprocal of the velocity for AE-cellulose-β-amylase hydrolysing 0.1% and 1% (w/v) amylose ($\bar{D}P$ 1340) solutions in 0.1M-acetate buffer, pH 4.8 containing 0.1-5mM-maltose.

Fig. 4.9.1.5.2. Plot of log [I] against log $((V_o-V_i)/V_i)$ for AE-cellulose-β-amylase hydrolysing 1% (w/v) amylose ($\bar{D}P$ 1340) solutions containing maltose as above.


1/V (min. Δ blue value^{-1})
10.0
7.5
5.0
2.5
0.1%
1%
-5.0
-2.5
2.5
5.0
maltose (mM)


log [I]
0.4
0.3
0.2
0.1
$\bar{1}.1$
$\bar{1}.2$
$\bar{1}.3$
$\bar{1}.4$
$\bar{1}.5$
$\bar{1}.6$
$\log(\frac{V_o - V_i}{V_i})$

of 1.06 obtained for the slope of the plot (7), it was found that one molecule of inhibitor binds with each catalytic site, therefore the basic mode of inhibition appears unaffected by immobilisation to a cationic support, although the value of K_i' is decreased.

4.9.1.6. ACTION PATTERN OF AE-CELLULOSE-β-AMYLASE.

To compare the effects of immobilisation to a cationic support upon the mode of β-amylase action with the mode of action of the soluble enzyme, the action patterns were determined for both enzyme preparations (sections 1.2 and 3.14).

Soluble β-amylase (0.107U) and AE-cellulose-β-amylase (0.198U) were each incubated as previously described (sections 3.1 and 3.13.3) with 10ml 1% (w/v) amylose ($\bar{D}P$ 1340) solution in 0.1M-acetate buffer, pH 4.8. Aliquots of 0.25ml were withdrawn at intervals and, after removing the immobilised derivative by centrifugation, were assayed for reducing sugars (section 3.3.2) and for blue value (section 3.14).

The results were plotted as % maximum blue value against % maximum reducing power (Fig. 4.9.1.6.1) for both enzyme preparations.

The soluble enzyme displayed an action pattern characteristic of a multiple attack mechanism (section 1.2, Fig. 1.2.1). However, AE-cellulose-β-amylase appeared to display an action pattern tending towards a single chain attack mechanism (section 1.2, Fig. 1.2.1), similar to that shown by α-amylase.

4.9.1.7. ACTION OF AE-CELLULOSE-β-AMYLASE ON MALTO-HEPTAOSE.

To attempt to confirm a movement towards single chain attack as a result of the immobilisation of β-amylase on a polycationic support, the soluble enzyme and the immobilised derivative were each incubated with a solution of maltoheptaose (17) and the malto-oligosaccharide components of the incubation mixture were qualitatively determined by paper chromatographic techniques (section 3.12).

Assuming that the action pattern of AE-cellulose-β-amylase was shifted towards a single chain attack mechanism, the hydrolysis of maltoheptaose by the enzyme derivative would be expected to result in the presence only of the substrate and maltose in solution, with no detectable amounts of malto-pentaose and maltotriose intermediates since the enzyme would tend to more completely hydrolyse each maltoheptaose molecule before complexing with a new substrate molecule.

An aliquot of AE-cellulose-β-amylase (0.198U) was washed with 5ml volumes of distilled water (x 10) to remove all traces of salts and was resuspended in 0.5ml distilled water.

Soluble β-amylase (0.4U), stored as a suspension in 2.1M-ammonium sulphate solution was removed by centrifugation at 10000g and 4^{o} for 20 min, redissolved in a small volume of 5mM-dithiothreitol in distilled water and dialysed at 4^{o} against the same solution to remove traces of ammonium sulphate.

Both enzyme preparations (0.5ml) were incubated with continuous stirring, at 37^{o} with 2.5ml maltoheptaose solution

($208.3 ug.ml^{-1}$) in distilled water. At 30, 60, 120 and 240 min, 0.75ml aliquots were withdrawn, the immobilised enzyme derivative removed by centrifugation at 7000g for 1 min and the soluble enzyme inactivated by placing the sample in a boiling water bath for 5 min. The aliquots were concentrated by rotary evaporation under reduced pressure and applied to Whatman 3mm paper as previously described (section 3.12.1) together with a similarly concentrated aliquot of malto-heptaose solution and a sample of de-ionized amylose acid hydrolysate (section 3.10.3.1). The chromatograms were treated as previously described using a multi-ascent technique and staining the malto-oligosaccharide components with an NaOH-silver nitrate system (sections 3.10.3.2 and 3.10.3.3).

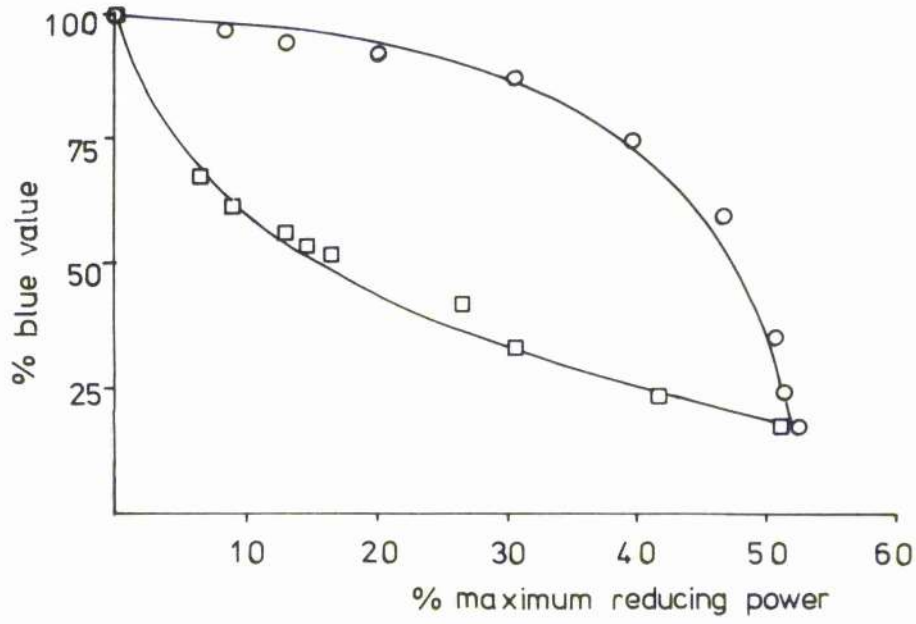
The chromatograms obtained were copied to display the spots more clearly and are presented in Fig. 4.9.1.7.1.

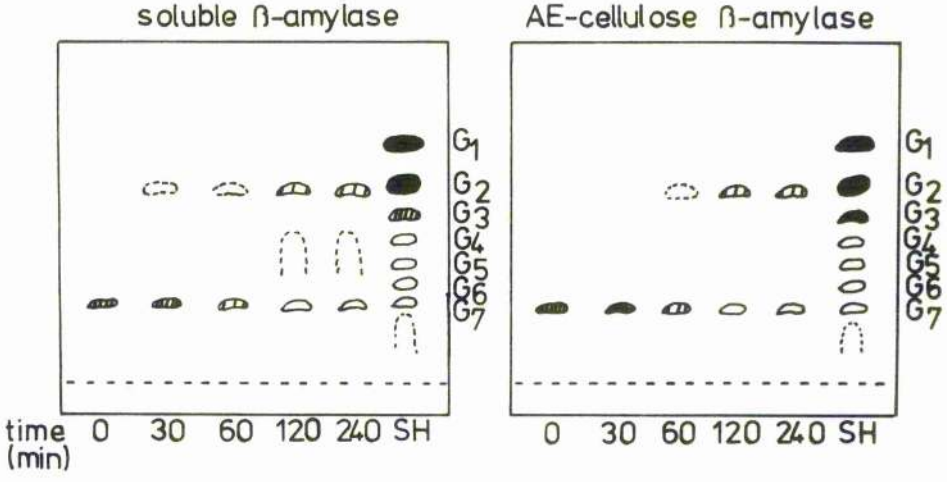
The soluble enzyme, during the course of hydrolysis, produced small quantities of maltotriose and maltopentaose in the incubation mixture as the maltoheptaose spot decreased in intensity and the maltose spot increased in intensity. However, no detectable amount of maltotriose and maltopentaose was found for AE-cellulose-β-amylase, although the substrate and product spots behaved as those for the soluble enzyme.

It would therefore appear that the action pattern of β-amylase is shifted towards a single chain attack mechanism on immobilisation to AE-cellulose.

Fig. 4.9.1.6.1. Action patterns of AE-cellulose-β amylase (□) and soluble β-amylase (○) hydrolysing 1% (w/v) amylose ($\bar{D}P$ 1340) solution in 0.1M-acetate buffer, pH 4.8 at 37°.

Fig. 4.9.1.7.1. Diagrams showing the separation of reaction intermediates and products by paper chromatography during the hydrolysis of maltopentaose by soluble β-amylase and AE-cellulose-β-amylase.

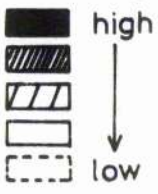




G_1, G_2, etc. glucose, maltose, etc.

SH starch hydrolysate

intensity of staining:



4.9.2. POLYAMINOSTYRENE (PAS)-β-AMYLASE.

A PAS-β-amylase derivative was prepared as described in section 3.13.2.

The specific activity of the enzyme preparation before and after coupling was estimated as previously described (sections 3.1 and 3.13.3), the amount of protein bound (section 3.13.2) was determined by the Folin-Lowry method (section 3.2.1) and the values presented below:-

PROTEIN BOUND ($mg.100mg\ support^{-1}$)	SPECIFIC ACTIVITY ($U.mg\ bound\ enzyme^{-1}$)	SPECIFIC ACTIVITY RETAINDD (%)
0.1423	2.002	9.1

4.9.2.1. pH-ACTIVITY PROFILE OF PAS-β-AMYLASE.

The pH-activity profile was determined by incubating an aliquot of the immobilised enzyme suspension (0.072U) in 0.1M-citrate-phosphate buffer in a continuously stirred vessel at 37^{o} with 2.5ml aliquots of 1% (w/v) soluble starch solution in 0.1M-citrate-phosphate buffer at a range of pH values (3.0-8.5). Aliquots of 0.5ml were removed, centrifuged at 7000g for 1 min and the supernatant assayed for the release of maltose by the neocuproine method (section 3.3.2). The results were plotted as % maximum activity obtained against pH (Fig. 4.9.2.1.1) and compared with the profile obtained for the soluble enzyme.

The profile for PAS-β-amylase is constricted compared to that for the soluble enzyme. A similar effect was obtained by Filippusson and Hornby (68) for polystyrene-β-fructofuranosidase and for the soluble enzyme in the

presence of dioxan and is considered to be attributable to the hydrophobic environment, which will be discussed in section 4.9.3.

4.9.2.2. TEMPERATURE-ACTIVITY PROFILE OF PAS-β-AMYLASE.

The temperature-activity profile was determined by incubating an aliquot (0.072U) of the immobilised enzyme suspension in a continuously stirred vessel with 2.5ml aliquots of 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8, at various temperatures. Assays were carried out as described in the previous section.

The results were plotted for PAS-β-amylase and for the soluble enzyme as % maximum activity against temperature (Fig. 4.9.2.2.1).

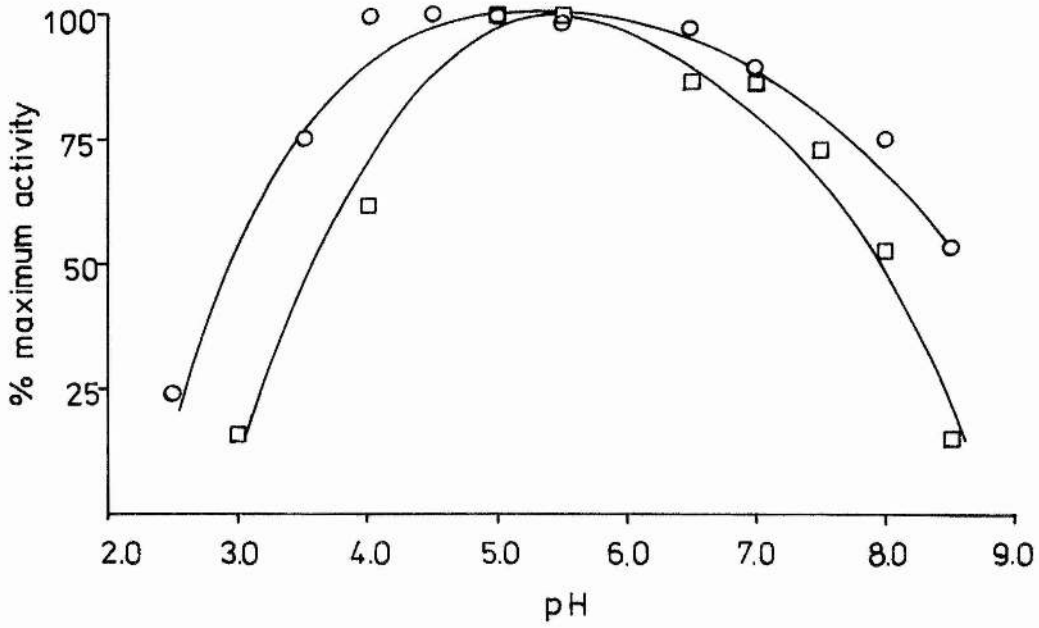
Although the temperature at which the immobilised enzyme displays optimum activity is unaltered, PAS-β-amylase is less stable than the soluble enzyme at temperatures above 50°, and displayed a constricted profile. However, the derivative showed an inflexion in the profile at 20°, corresponding to that of the soluble enzyme and indicating a change in the energy of activation.

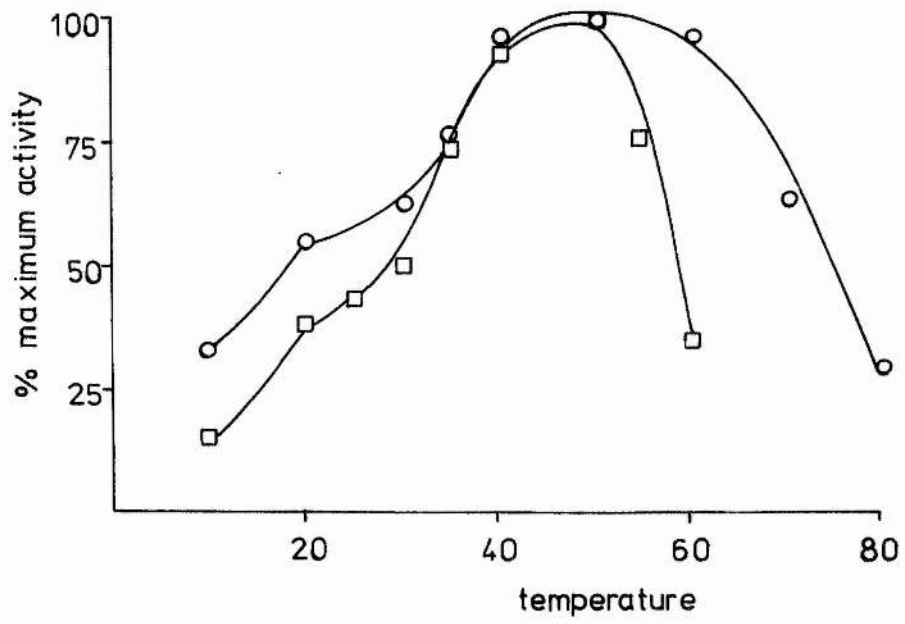
4.9.2.3. THERMAL STABILITY OF PAS-β-AMYLASE.

An aliquot (0.072U) of the immobilised enzyme suspension was pre-incubated at a range of temperatures (20-60°) for 1h, cooled in ice and assayed by incubation at 37° with 2.5ml 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8, in a continuously stirred vessel, monitoring the release of maltose as previously described in section 4.9.1.1.

Fig. 4.9.2.1.1. pH-Activity profile for PAS-β-amylase (□) and soluble β-amylase (o) hydrolysing 1% (w/v) soluble starch solution in 0.1M-citrate-phosphate buffer, at 37°.

Fig. 4.9.2.2.1. Temperature-activity profile for PAS-β-amylase (□) and soluble β-amylase (o) hydrolysing 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8.


100
75
50
25
% maximum activity
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pH


100
75
50
25
% maximum activity
20
40
60
80
temperature

The results were plotted for PAS-β-amylase and for the soluble enzyme as % original activity against temperature of pre-incubation, (Fig. 4.9.2.3.1). PAS-β-amylase was found to be more stable than the soluble enzyme at temperatures above 30°, but to show a similar retention of activity to the soluble enzyme at lower temperatures.

Since identical procedures were used for coupling β-amylase to AE-cellulose and PAS supports, it is possible that the hydrophobic environment imposed by the polystyrene matrix is responsible for the increase in thermal stability mentioned above.

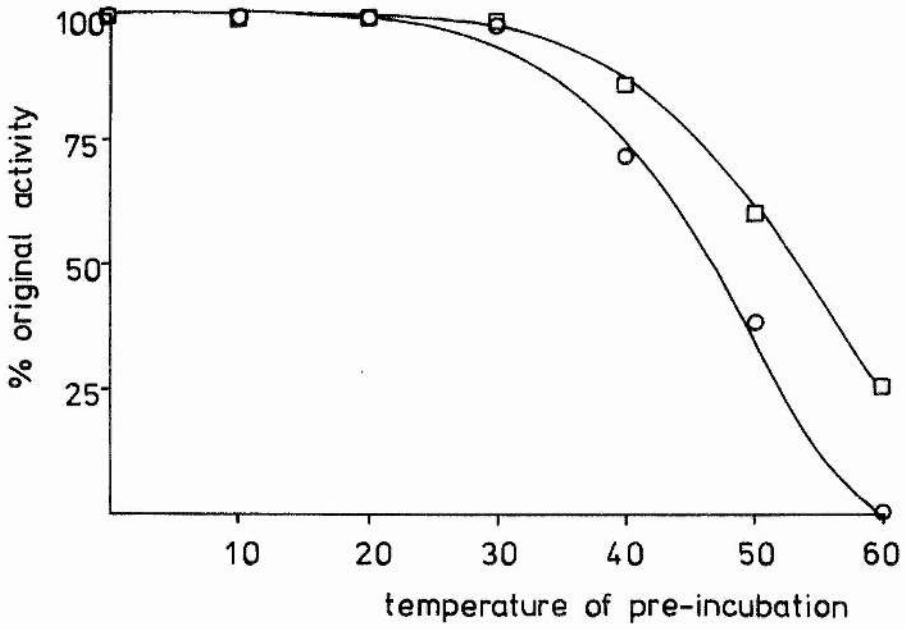
4.9.2.4. DETERMINATION OF KINETIC PARAMETERS FOR PAS-β-AMYLASE.

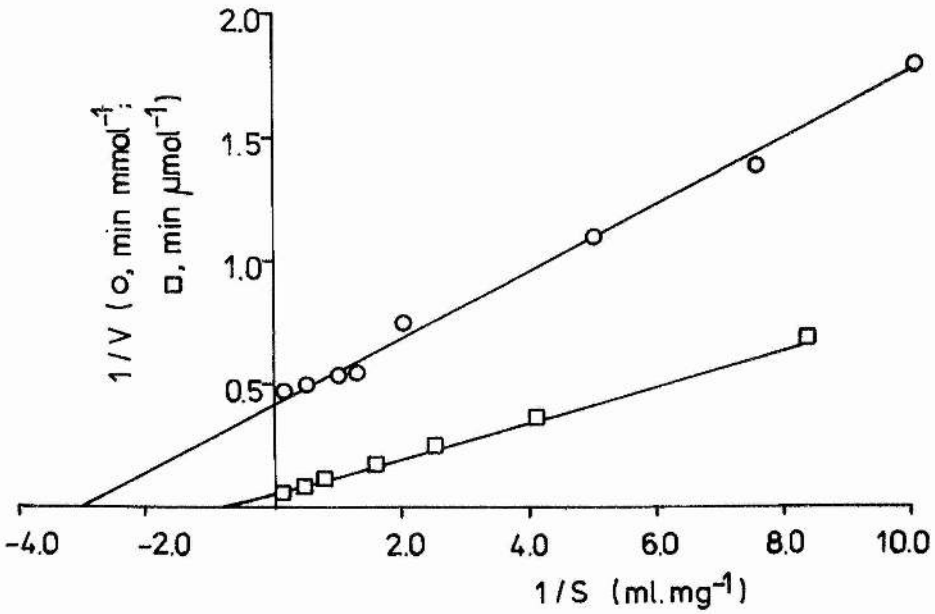
The values of the apparent Michaelis constant (K_m') and the apparent maximum velocity (V_m') for PAS-β-amylase were determined by incubating the immobilised enzyme suspension (0.072U) with 2.5ml aliquots of soluble starch solutions (0.1-8mg.ml^{-1}) in 0.1M-acetate buffer, pH 4.8 at 37° in a continuously stirred vessel. The release of maltose was measured as previously described (section 4.9.2.1) and the results, together with those for the soluble enzyme (section 4.4.3) were expressed as double reciprocal plots (91) as shown in Fig. 4.9.2.4.1.

The value of K_m' for PAS-β-amylase (1.25 ± 0.41mg.ml^{-1} was four times larger than that for the soluble enzyme (0.33mg.ml^{-1}) and almost five times larger than the K_m' value (0.275 mg.ml^{-1}) for AE-cellulose-β-amylase. The PAS derivative had a V_m' value of 8.79 ± 0.07μmol.min^{-1} compared with

Fig. 4.9.2.3.1. Thermal stability of PAS-β-amylase (□) and soluble β-amylase (○) pre-incubated at various temperatures, cooled and assayed at 37° by incubating with 1% (w/v) soluble starch solution in 0.1M-acetate buffer, pH 4.8.

Fig. 4.9.2.4.1. Double reciprocal plots for PAS-β amylase (□) and soluble β-amylase (○) hydrolysing soluble starch solutions in 0.1M-acetate buffer, pH 4.8 at 37°.


100
75
50
25
% original activity
10
20
30
40
50
60
temperature of pre-incubation


2.0
1.5
1.0
0.5
1/V (o, min mmol^{-1}; □, min μmol^{-1})
-4.0
-2.0
2.0
4.0
6.0
8.0
10.0
1/S (ml.mg^{-1})

2.419mmol.min^{-1} for soluble β-amylase and 15.98μmol.min^{-1} for AE-cellulose-β-amylase.

From these results, PAS-β-amylase appears to have less affinity for its substrate than the soluble enzyme and to have a V_m' value 300 times smaller than soluble β-amylase. As with AE-cellulose-β-amylase, these variations will be considered in section 4.9.3.

4.9.2.5. DETERMINATION OF INHIBITION CONSTANT OF PAS-β-AMYLASE USING MALTOSE.

The method used to determine the inhibition constant (K_i') for maltose was the same as that described in section 4.9.1.5.

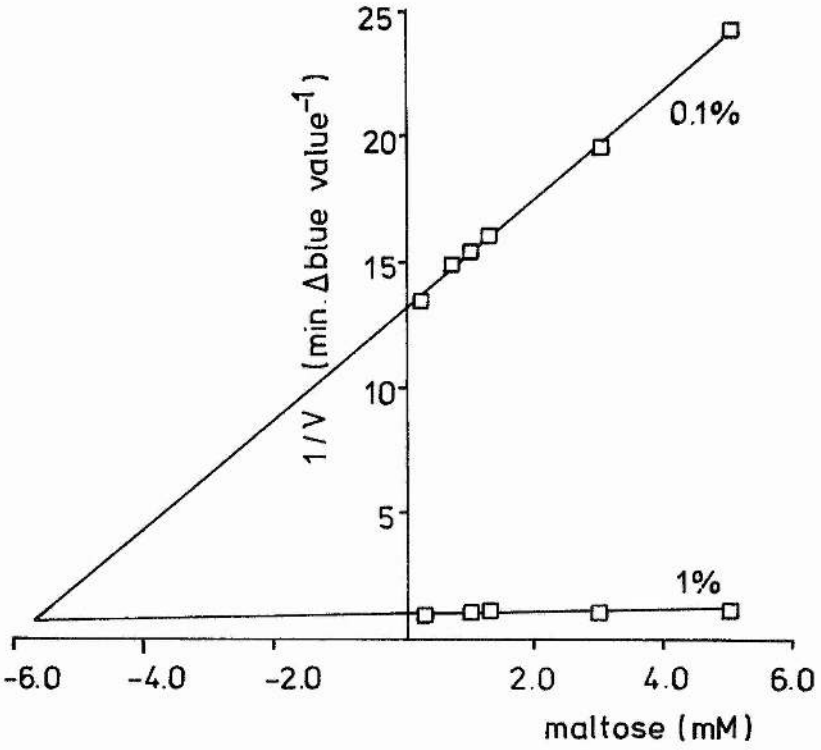
An aliquot of immobilised enzyme suspension (0.072U) was incubated, with stirring, at 37^o with 2.5ml aliquots of 0.1 and 1% (w/v) amylose ($\bar{D}P$ 1340) solutions in 0.1M-acetate buffer, pH 4.8, containing a range of maltose concentrations (0.25-6mM). On account of the interference by maltose in methods for reducing sugar assays, the velocity of the reaction was measured spectrophotometrically by monitoring the decrease in blue value of the amylose substrate (section 3.14) at 610nm.

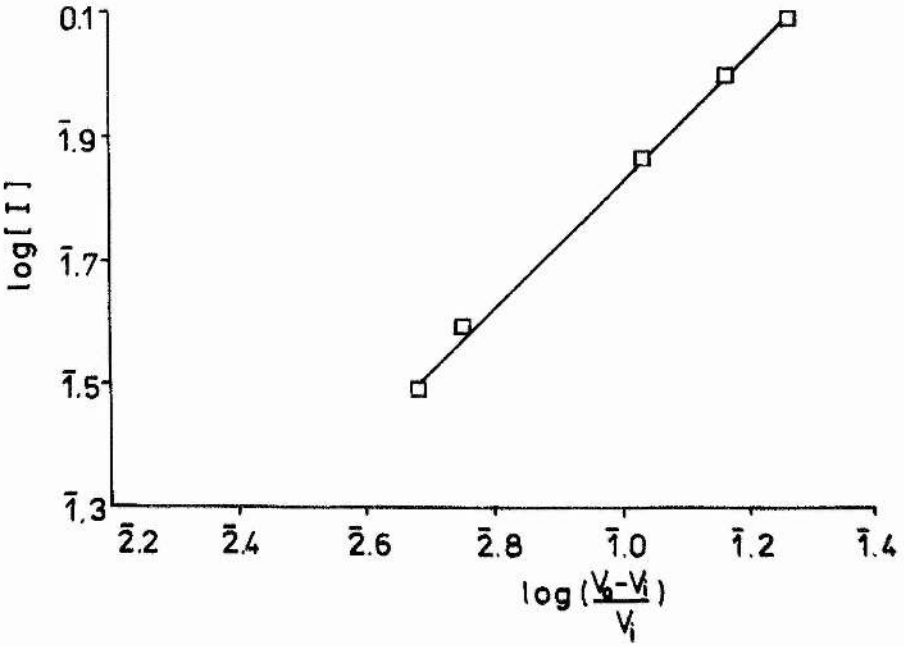
The results were plotted as maltose concentration against the reciprocal of the velocity (98) for each substrate concentration (Fig. 4.9.2.5.1).

The two lines were found to intersect above the abscissa, indicating that maltose acted as a competitive inhibitor upon PAS-β-amylase, with a K_i' value of 5.8mM, comparable to that obtained for the soluble enzyme (6.1mM).

Fig. 4.9.2.5.1. Dixon plot of maltose concentration against the reciprocal of the velocity for PAS-β-amylase hydrolysing 0.1% and 1% (w/v) amylose ($\bar{D}P$ 1340) in 0.1M-acetate buffer, pH 4.8, containing 0.1-5mM-maltose.

Fig. 4.9.2.5.2. Plot of log [I] against log $((V_o-V_i)/V_i)$ for PAS-β-amylase hydrolysing 1% (w/v) amylose ($\bar{D}P$ 1340) as above.


25
20
15
10
5
1/V (min. Δblue value^{-1})
0.1%
1%
-6.0
-4.0
-2.0
2.0
4.0
6.0
maltose (mM)


0.1
$\bar{1}$.9
$\bar{1}$.7
$\bar{1}$.5
$\bar{1}$.3
log [I]
$\bar{2}$.2
$\bar{2}$.4
$\bar{2}$.6
$\bar{2}$.8
$\bar{1}$.0
$\bar{1}$.2
$\bar{1}$.4
$\log\left(\frac{V_0-V_i}{V_i}\right)$

As in previous sections, a plot was made of log [I] against log $((V_o-V_i)/V_i)$ for PAS-β-amylase inhibited by maltose, and the slope, n, was found to have a value of 0.97, indicating that one molecule of maltose binds with one catalytic site in the enzyme (Fig. 4.9.2.5.2).

From these results, PAS-β-amylase displays identical properties to the soluble enzyme with respect to competitive inhibition by maltose.

4.9.2.6. ACTION PATTERN OF PAS-β-AMYLASE.

The kinetic parameters and pH-activity profile of β-amylase were found to be markedly affected by immobilisation upon a hydrophobic support (sections 4.9.2.1 and 4.9.2.5). β-Amylase has been reported to display a shift in its action pattern at extremes of pH and temperature (17), and upon immobilisation to AE-cellulose (section 4.9.1.6) and therefore the action pattern of PAS-β-amylase was determined to study the effect of a hydrophobic environment on the mode of action of the enzyme.

PAS-β-amylase (0.143U) was incubated with 10ml 1% (w/v) amylose ($\bar{D}P$ 1340) solution in 0.1M-acetate buffer, pH 4.8, at 37° in a continuously stirred vessel. Aliquots of 0.25ml were withdrawn at intervals and assayed for the release of reducing sugars and for a decrease in blue value of the amylose substrate as previously described in section 4.9.1.6.

The results were plotted as % original blue value against % maximum reducing power (Fig. 4.9.2.6.1) together with the results obtained in section 4.9.1.6 for the soluble enzyme preparation.

It was found that the action pattern of PAS-β-amylase was shifted towards a multi-chain attack mechanism (section 1.1, Fig. 1.1.2).

4.9.2.7. ACTION OF PAS-β-AMYLASE ON MALTOHEPTAOSE.

As in section 4.9.1.7, the incubation of PAS-β-amylase with a solution of maltoheptaose was carried out to attempt to confirm the shift in action pattern, the samples being taken at time intervals to qualitatively determine the malto-oligosaccharide nature of the incubation mixture.

The mode of action of β-amylase upon immobilisation to a PAS support appeared to be shifted towards a multi-chain attack mechanism, and therefore, with a decreased number of bonds being hydrolysed per enzyme-substrate encounter, it would be expected that maltotriose and maltopentaose intermediates would be detectable during the course of maltoheptaose hydrolysis.

An aliquot of PAS-β-amylase (0.072U) was washed with 5ml volumes of distilled water (x 10) to remove all traces of salts and resuspended in 0.5ml distilled water. The enzyme suspension was incubated, with continuous stirring, at 37^{o} with 2.5ml maltoheptaose solution ($208.3 mg.ml^{-1}$) in distilled water. At 30, 60, 120 and 240 min, 0.75ml aliquots were withdrawn, centrifuged, concentrated and applied to Whatman 3mm paper together with untreated maltoheptaose and de-ionized amylose acid hydrolysate as a standard as previously described in section 4.9.1.7.

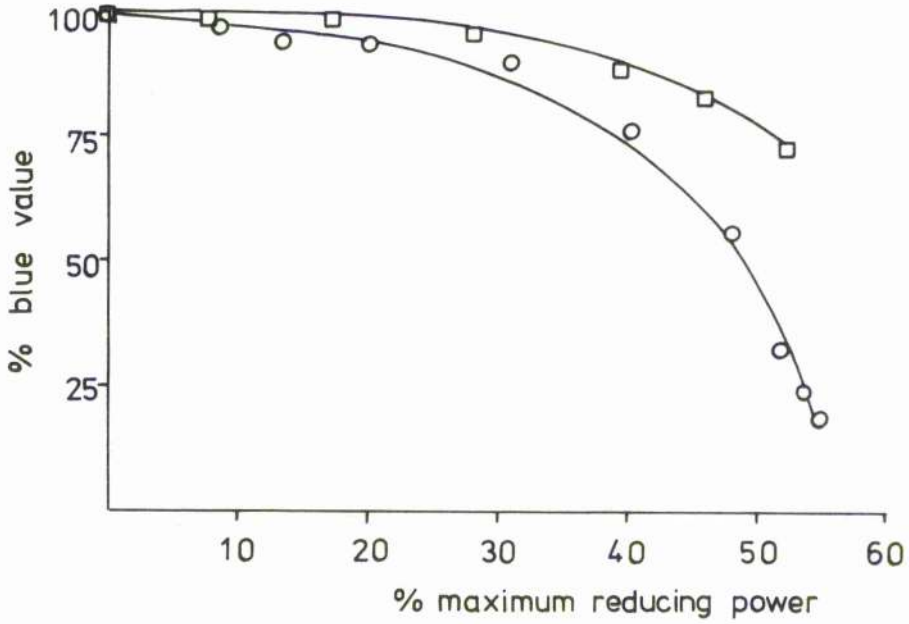
The chromatogram was treated as previously described, using a multi-ascent separation technique and staining as

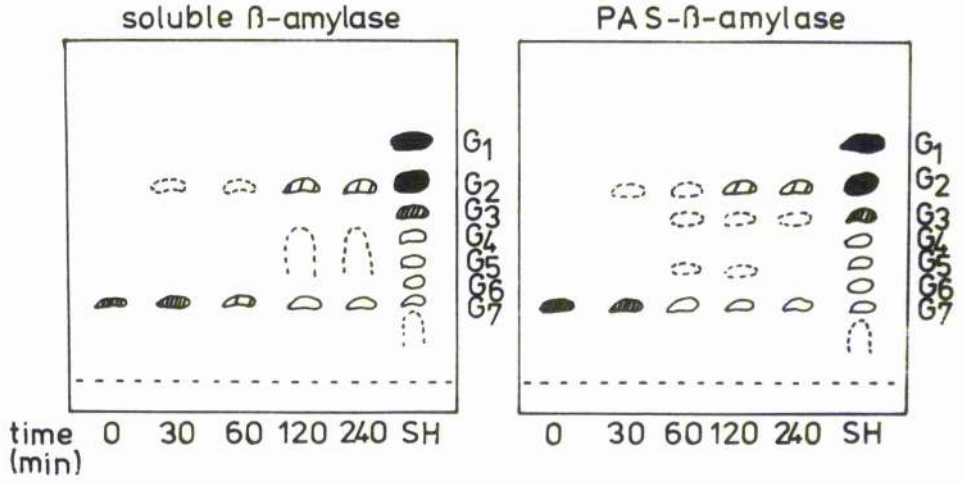
before (sections 3.10.3.2 and 3.10.3.1). A copy of the chromatogram is shown in Fig. 4.9.2.7.1, together with the chromatogram obtained for the soluble enzyme in section 4.9.1.7.

Maltotriose and maltopentaose intermediates were detectable during the course of hydrolysis by PAS-β-amylase, and produced darker spots on staining than the traces of intermediates detected during the course of maltopentaose hydrolysis by the soluble enzyme. Since the experimental conditions were identical, it appears that more intermediates were produced by PAS-β-amylase action as a result of the shift in its action pattern towards a multi-chain attack mechanism after immobilisation.

Fig. 4.9.2.6.1. Action patterns of PAS-β-amylase (□) and soluble β-amylase (○) hydrolysing 1% (w/v) amylose ($\bar{D}P$ 1340) solution in 0.1M-acetate buffer, pH 4.8, at 37°.

Fig. 4.9.2.7.1. Diagram showing the separation of reaction intermediates and products by paper chromatography during the hydrolysis of maltoheptaose by soluble β-amylase and PAS-β-amylase.

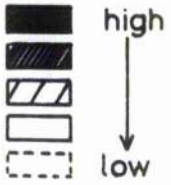




G_1, G_2, etc. glucose, maltose, etc.

SH starch hydrolysate

intensity of staining:



4.9.3. DISCUSSION OF THE EFFECTS OF IMMOBILISATION ON β-AMYLASE ACTIVITY.

The immobilisation of β-amylase both on a cationic (AE-cellulose) and a hydrophobic (PAS) support resulted in changes in a number of the enzyme's characteristics, which will be considered in this section.

1. pH-Activity profile.

The alkaline limb of the pH-activity profile for AE-cellulose-β-amylase was shifted towards lower pH values than the corresponding limb for the soluble enzyme, although the same optimum pH values were obtained for each preparation. This effect was explained in section 4.9.1 with respect to the local proton concentration around the immobilised enzyme being lower for a polycation-supported enzyme than for the bulk solution (101). Zherebtsov (26) proposed that an electrophilic-nucleophilic pair formed between an imidazole and carboxyl function at the active site was responsible for the shape of the pH-activity curve for malt β-amylase, with the extended alkaline limb being due to a possible buffering action at the active site between these functions, reducing the effect of removing the proton of the positively-charged imidazole group, important in the hydrolysis of the substrate (Fig. 1.1.3). As the pH value of the solution containing AE-cellulose-β-amylase was increased, the nature of the cationic support would cause an increase in the distribution of hydroxyl ions around the immobilised enzyme surface compared with the concentration in the bulk solution, resulting in an apparent decrease in β-amylase activity at a given pH value due to the hydroxyl ions removing

the electrophilic-nucleophilic buffering action mentioned above. The pH activity profile for AE-cellulose-β-amylase therefore appears to approach the theoretical profile for the soluble enzyme mentioned by Zherebtsov (26).

PAS-β-amylase displayed a constricted pH-activity profile, compared to the soluble enzyme, an effect previously reported for polystyrene-β-fructofuranosidase (68) and considered to be due to the hydrophobic environment imposed by the matrix.

2. Thermal Stability.

Immobilisation often results in a decrease in thermal stability of the enzyme, as found for AE-cellulose-β-amylase. It has been suggested (59) that such a decrease may be due to the difficulty of the enzyme in regaining its active conformation after heat treatment, possibly because of steric hinderance from the support and also because of hydrogen bonding introduced between the support and the enzyme in an inactive conformation.

β-Amylase coupled to cellulose carriers has been reported to show increased thermal stability by Barker et al (63) and by Vretblad and Axén (61) using Sepharose.

PAS-β-amylase was found to be more thermally stable than the soluble enzyme, possibly due to the conformation of the enzyme on the hydrophobic support. However, Campbell (102) reported that polystyrene immobilized glucose oxidase was less thermally stable than the soluble enzyme and presumed the decrease to be due to the proximity of the hydrophobic matrix to the enzyme. Epton et al (103) found that hydrophilic carriers conferred greater thermal stability

on thermolysin preparations than did hydrophobic carriers and suggested that the latter may compete for amino acid side chains involved in the hydrophobic bonds maintaining the tertiary structure of the enzyme.

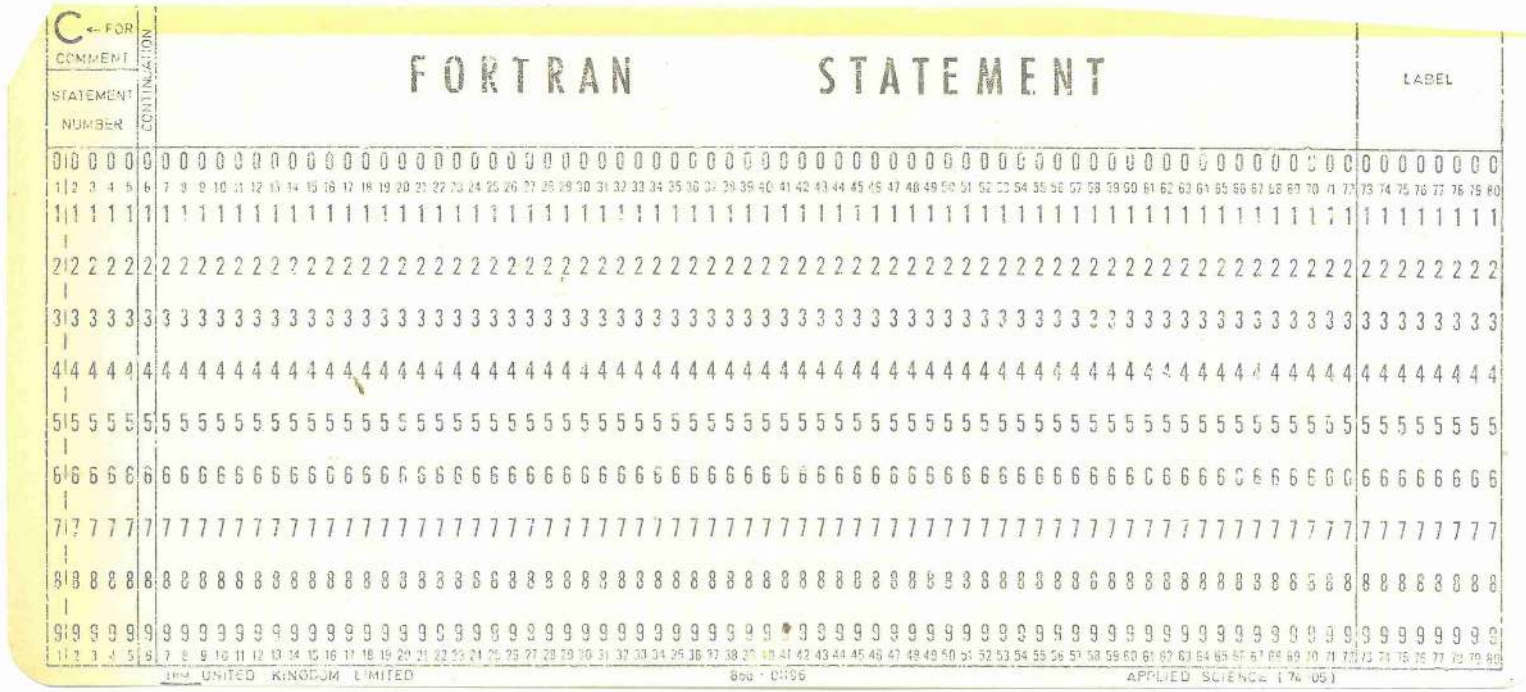
3. Kinetic parameters.

The nature of the support was significant with respect to the kinetic parameters (K_m' and V_m') for the immobilised β-amylase derivatives.

AE-cellulose-β-amylase displayed a lower K_m' value ($0.274 mg.ml^{-1}$) and a lower V_m' value ($15.98 \mu mol.min^{-1}$) than the soluble enzyme ($0.33 mg.ml^{-1}$; $2.4195 mmol.min^{-1}$). Bayne (104) found that the V_m' values for AE-cellulose-yeast alcohol dehydrogenase preparations decreased on immobilisation and the K_m' values increased for a number of substrates.

A polyacrylamide-immobilised derivative of another hydrolase, thermolysin, (103) displayed extremely large decreases in K_m' and V_m' values.

However, PAS-β-amylase showed an increase in K_m' value ($1.25 mg.ml^{-1}$) and a decrease in V_m' value ($8.79 \mu mol.min^{-1}$). Polystyrene-β-fructofuranosidase (68) was found to display a two-fold increase in K_m' and a nine-fold decrease in V_m', possibly due to the particulate nature of the support isolating the enzyme from the bulk solution (66) or due to the hydrophobic environment affecting the distribution of the substrate around the enzyme. Geynes and Sehon (105) found that the behaviour of polystyrene-antigen complexes depended on the degree of amination of the polystyrene, since this would determine the steric configuration of the complex. If the polystyrene was highly aminated, it may result in an


C ← FOR COMMENT
STATEMENT NUMBER
CONTINUATION
FORTRAN STATEMENT
LABEL
IBM UNITED KINGDOM LIMITED
APPLIED SCIENCE

unfolded structure, whereas a low degree of amination may produce a coiled structure stabilized by hydrophobic bonding. If this theory is extended to PAS-β-amylase, it can be seen that the degree of amination, by affecting the structure of the PAS-enzyme complex, will affect the access of substrate to the active site of the enzyme. A large α-1,4-glucan molecule will be less able to approach β-amylase if the immobilised enzyme is involved in a coiled structure, where a certain amount of enzyme may be trapped inside the coil. Therefore, if the value of V_m' is taken as a measure of substrate saturation of the enzyme, it can be seen that if less enzyme is available, ie. a proportion is enclosed within a strongly hydrophobic structure, substrate saturation would occur at lower substrate concentrations, and the apparent maximum velocity would decrease.

Few reports exist concerning the effect of immobilisation upon the kinetic parameters of β-amylase, but Mårtensson (62) reported a K_m' value of 7.0mg.ml^{-1} for soluble starch, compared with 1.2mg.ml^{-1} for the soluble enzyme in a β-amylase-pullulanase system immobilised to an acrylic copolymer.

The K_i value for maltose was found to decrease for AE-cellulose-β-amylase (3mM) and to remain constant for PAS-β-amylase (5.8mM) compared with the soluble enzyme (6.1mM). Mårtensson (62) reported a K_i' of 14.6mM compared with a K_i value of 7.6mM for the immobilised β-amylase-pullulanase system, but made no comments upon this increase. Filippusson and Hornby (68) reported an increase in the K_i'

value for Tris and a decrease in K_i' for aniline by β-fructofuranosidase. Both compounds were affected by the hydrophobic support, since aniline is an aromatic compound and would be in a favourable environment around the enzyme, whereas Tris, being a highly charged molecule, would be partitioned such that its concentration around the enzyme would be lower than that in the bulk solution.

If the nature of the support is considered, in a simple case, to be the only factor affecting the kinetic parameters of β-amylase, the variations in K_m' and V_m' can be explained as follows.

AE-cellulose-β-amylase, by virtue of its cationic nature, places the enzyme in a positively-charged environment, where it is attached to a large polymeric support. In the soluble state, the enzyme is small compared to its α-1,4-glucan substrates and is considered to carry a slight positive charge at its active site which attracts the overall slight negative charge of its substrate. The enhanced positively-charged environment created by immobilisation to AE-cellulose increases the attractive force between the enzyme and its substrate and results in an increased affinity, or lower K_m' value (101).

This decrease in K_m' would also be contributed to by the effect of substrate distribution between the environment around the supported enzyme and the main solution, since the charge effects mentioned above would result in a higher concentration of substrate around the enzyme than in the main solution, leading to β-amylase apparently reaching its maximum velocity at a lower substrate concentration and there-

fore displaying a lower K_m' value.

However, the size of the supported enzyme would be such that a large polymer substrate would be sterically hindered in its approach to the active site. Also, some weak binding may occur between the substrate and the support, resulting in a decrease in V_m' for the immobilised enzyme.

The decrease in K_i' obtained using maltose can also be explained in this way, since maltose carries a weak negative charge, and its distribution between the main solution and the environment around the enzyme would be such that its concentration would be greater at the surface of the enzyme particles and inhibition would occur at apparently lower maltose concentrations.

PAS-β-amylase showed less affinity for its substrate than the soluble enzyme since β-amylase was situated in a hydrophobic environment and hydrolysing a hydrophilic substrate. The concentration of α-1,4-glucan substrates at the enzyme surface would therefore be expected to be lower than in the bulk solution and, for reasons similar to those already mentioned, the K_m' value would increase. Similarly, an increase in K_m' was found for polystyrene-β-fructofuranosidase (68) hydrolysing sucrose.

Steric hinderance by the support and the distribution of the substrate between the environment around β-amylase and the bulk solution, as mentioned above, resulted in a lower V_m' value.

However, the K_i' value obtained for PAS-β-amylase using maltose was unaltered. Possibly maltose is a sufficiently small molecule, with little charge, so that it

may act as an uncharged molecule and be unaffected by the strongly hydrophobic environment imposed by the polystyrene matrix.

4. Action patterns.

The action patterns found for β-amylase preparations were each different from the multiple attack pattern found for the soluble enzyme.

AE-cellulose-β-amylase displayed an action pattern which was shifted towards a single chain attack mechanism. This effect can explain the variation in kinetic parameters already discussed. For a single chain attack mechanism to occur (an extreme case), the life time of the enzyme-substrate complex formed between β-amylase and an α-1,4-glucan chain must be sufficient to allow the enzyme to completely hydrolyse the α-1,4-glucan chain, and would be more favoured under conditions where the affinity of the enzyme for its substrate is increased. Therefore, in the case of AE-cellulose-β-amylase, the enzyme may be expected to bind more strongly with its substrate.

During the course of hydrolysis, dissociation of the active enzyme-substrate complex may occur, but unless the substrate molecule (the residual α-1,4-glucan chain) diffuses sufficiently far from the active site to be indistinguishable from other α-1,4-glucan chains, the dissociation is not regarded as such, since the "dissociated" substrate has a greater possibility of binding, or re-complexing with β-amylase than an average substrate chain in the remaining solution. For this situation, where the formation of an enzyme-substrate complex is favoured more

than its dissociation, the equilibrium constant, K_{eq}, is larger, and therefore the k_m' value is smaller, than in the situation where there is less affinity between enzyme and substrate. To extend the idea of "true" dissociation to the AE-cellulose-β-amylase system, the number of "true" dissociations occurring during the action of the immobilised enzyme upon its α-1,4-glucan substrate would therefore be less than for the soluble enzyme, since the positively-charged environment retains the substrate near the active site of the enzyme. Also, less competition would occur between substrate molecules for access to the active site due to steric hinderance imposed by the size of the matrix. Therefore, more catalytic events, ie. removal of maltose units, would occur for each productive enzyme-substrate complex than in the case of the soluble enzyme.

A similar explanation can be given for the shift towards a multichain attack mechanism for PAS-β-amylase. Similar dissociations would occur during the course of hydrolysis of the substrate as those described above, but the nature of the environment would be such that the hydrophilic substrate would be less likely to recombine with the enzyme in a hydrophobic environment and would tend to diffuse from the active site so that the next productive complex formed could be with any substrate molecule, since all would be indistinguishable from each other. Therefore, fewer catalytic events would be expected to occur for each productive enzyme-substrate complex and the action pattern would show a shift towards a multi-chain attack mechanism. Similar results have been reported for polystyrene-α-

amylase (106) and for polystyrene-amyloglucosidase (107), which both showed a shift in action pattern towards a multi-chain attack mechanism for similar reasons to those proposed for β-amylase.

In the discussion above, the nature of the support has been regarded as the sole factor in determining the activity of immobilised enzymes, whereas the effects of immobilisation upon the kinetic behaviour of enzymes can be classified as follows:-

1. Conformational changes, where the enzyme may be in a different conformation on the support (108) due to chemical modification induced by its linkage to the support and also to the type and number of bonds and the nature of the support. Adipimidate introduces a positive charge into the enzyme environment and therefore the retention of activity found for AE-cellulose-β-amylase (18%) and for PAS-β-amylase (9.1%) may be due to the stabilization of the positive charge of lysine residues, for which adipimidate is specific.

2. Steric hinderance. The activity of immobilised depolymerases has been found to be decreased for polymer substrates but almost unaffected for smaller substrates. This was demonstrated for proteases acting on protein and peptide substrates (109) and was mentioned by Axén and Porath (60) to explain the lack of activity of β-amylase immobilised to Sephadex. This may also partly explain the low V_m' values found in this work for immobilised β-amylase derivatives.

3. Environmental effects, where the enzyme is subjected to a change in reaction environment, induced by the nature of the support. This has already been mentioned with respect to the environments created around β-amylase by AE-cellulose and PAS supports, and is related also to the next factor.

4. Partitioning effects. Several factors can affect the distribution of the substrate around an immobilised enzyme.

i) Specific interactions may occur between the substrate and the matrix and between protons and the matrix (110). This effect can explain the shifts in pH-profiles found for a number of immobilised enzymes on charged supports (101) and for AE-cellulose-β-amylase (section 4.9.1.1). If the matrix and substrate have opposite charges the distribution of the substrate around the immobilised enzyme would be increased and this in turn would affect the K_m' value as found for AE-cellulose-β-amylase in section 4.9.1.5.

ii) The physical nature of the support may be different from the nature of the surrounding solution. A large number of enzyme reactions occur in aqueous solution with hydrophilic substrates, as in the case of β-amylase. The immobilisation of β-amylase upon a hydrophobic support, eg. polystyrene, would be expected to affect the distribution of the substrate around the immobilised enzyme so that the enzyme is placed in an environment where the substrate concentration is less than in the bulk solution and therefore an increase in K_m' is obtained (68).

iii) Diffusional limitations may affect the distribution of substrate around the enzyme. Swollen gel particles can bind protein within their structure and therefore the binding capacity of a swollen gel is a function of the total number of its residues. Non-enzymic heterogeneous catalysis is limited by the rate of diffusion of the reactants to the active centre and therefore diffusion is rate-limiting to the kinetics of the catalytic reaction. In solution, few enzymic reactions are sufficiently fast that they are affected by diffusion, but solid-supported enzymes, ie. those in a heterogeneous system, are generally considered to be subject to diffusional limitations unless their activities are extremely slow (111).

Two types of diffusion are important in immobilised enzyme systems; interparticulate, or film, diffusion and intraparticulate, or pore, diffusion.

a. Film diffusion

Substrate molecules diffuse from the bulk solution to the surface of the support, to reach the immobilised enzyme, through a diffusional layer which is a film around each particle.

Lilly et al (66) studied the kinetics of CM-cellulose-ficin both in a stirred reactor and in packed beds and found that the K_m' of the enzyme preparation in packed beds was dependent upon the flow rate, the K_m' decreasing as the flow rate was increased, and at high flow rates the K_m' value tended towards that for a stirred reactor. If each enzyme particle is regarded as being surrounded by a diffusion layer,

the thickness of which is inversely proportional to the flow rate, the rate of diffusion of substrate to the enzyme is therefore directly proportional to the flow rate. Similarly, Lilly *et al* (112) observed that the rate of hydrolysis of substrate by CM-cellulose-chymotrypsin particles increased with the rate of stirring since the diffusional film contracted with increased stirring.

However, it can be assumed that the β-amylase systems investigated in this work were not diffusion-limited since all experiments were carried out with the enzyme in a rapidly-stirred reactor.

b. Pore diffusion

As already mentioned, certain porous supports can bind enzyme protein internally within their structure, creating a lattice structure round the enzyme with pores through which the substrate can diffuse.

In the case of β-amylase derivatives, both AE-cellulose and PAS beads are porous and theoretically it could be expected that diffusional limitations would occur. However the size of the macrosubstrate is such that virtually no diffusion of such a large molecule could be expected through the pores of the matrix and therefore any internally bound enzyme would make a negligible contribution to the overall activity of the preparation.

From the above considerations it can be seen that the effect of immobilisation upon the kinetic parameters and the action patterns of β-amylase are due to a number of interacting effects and not to such a simple system as that considered earlier.

The use of immobilised amylases is now gaining industrial importance, with uses in the treatment of effluents from a number of industries and also in food processing where a number of α-1,4-glucanase preparations have been immobilised on a single support (62) for use in the breakdown of soluble starch to fermentable sugars.

5. SUMMARY.

1. β-Amylase was purified from barley malt by ammonium sulphate fractionation and gel filtration on Sephadex G-25 followed by chromatography on DEAE-cellulose. Traces of α-amylase activity were removed by low temperature storage under acid conditions.

2. Pure β-amylase was shown to release no soluble blue dye from chromogenic α-1,4-glucans.

3. The kinetic parameters (K_m and V_m) of β-amylase were found to increase in the presence of traces of α-amylase. This was explained in relation to the chain length of the substrate.

4. The values of K_m and V_m for β-amylase were shown to decrease with an increase in the degree of polymerization of the substrate. The values of K_m for the non-reducing terminal and K_i for the internal portions of the substrate were determined.

5. An explanation was given for the dependence of K_m and V_m upon the degree of oxidation of dialdehyde and reduced dialdehyde substrates.

6. Inhibition studies involving maltose, dialdehyde and reduced dialdehyde amyloses were carried out. A theory to explain the inhibitory effect of oxidized amyloses upon β-amylase activity was put forward.

7. Immobilised β-amylase derivatives were prepared using AE-cellulose and PAS supports. The apparent values of K_m and V_m for soluble starch and of K_i for maltose were

determined for each preparation.

8. The action patterns of the soluble enzyme and immobilised derivatives on amylose were investigated, confirmed by chromatographic analysis of the reaction products and intermediates of maltoheptaose hydrolysis and explained in terms of the alteration of enzyme affinity towards the substrate upon immobilisation.

6. APPENDIX.

DATA PROCESSING (1)

All K_m and V_m values throughout this work were estimated using a FORTRAN IV program modified by Carvalho (92) from an original program by Cleland (113).

Symbols used in the program were:

V(1), V(2), etc. - Experimental velocities.

A(1), A(2), etc. - Corresponding substrate concentrations.

W(1), W(2), etc. - Weighting factors for velocities.

S(1,1), S(1,2), etc. - The array in which ϵ, δ, β, γ and α are solved.

Q(1), Q(2) etc. - The array used to calculate the S array.

SV2 - Summation of V^2.

JJ - Number of data sets processed.

NP - Number of data cards following title card.

M - With M = 1, the matrix solution subroutine uses statements 15 and 16 to make provisional estimates of K_m and V_m. With M = 2, statements 17 and 18 are used to make fine estimates of K_m and V_m.

CK - K_m.

VM - V_m.

NT - Number of iterations.

DD - Denominators used to simplify arithmetical operations.

S2 - Experimental variance.

S1 - Square root of S2 (Sigma).

SECK, SEV - Standard errors of estimates of K_m and V_m, respectively.

I,J,K - Counting indices for DO-loops.

```
C       PROGRAM HYPER
        DIMENSION V(100),A(100),W(100),S(2,3),Q(3),SS(6)
        PRINT 100
  100 FORMAT(35H FIT TO HYPERBOLA V = VMAX*A/(K+A)///)
   11 FORMAT(I3,17X,46H ANYTHING HERE WILL BE PRINTED DURING OUTPUT)
    1 FORMAT(3F10.5)
        JJ = 0
   14 READ 11,NP
        IF(NP) 99,99,12
   12 M = 1
        SV2 = 0
        P = NP - 2
        JJ = JJ + 1
        PRINT 11,JJ
        PRINT 37
   37 FORMAT(5H DATA,5X,8HVELOCITY,5X,9HSUBSTRATE,5X,6HWEIGHT)
        GO TO 2
   15 READ 1, V(I),A(I),W(I)
        PRINT 38,V(I),A(I),W(I)
   38 FORMAT(10X,F10.5,3X,F10.5,2X,F10.5)
        IF(W(I)) 19,19,20
   19 W(I) = 1
   20 Q(1) = V(I)**2/A(I)
        Q(2) = V(I)**2
        Q(3) = V(I)
        SV2 = SV2 = Q(2)
        GO TO 13
   16 DD = (SS(6)*SS(1) - SS(5)*SS(2))
        CK = ((SS(4)*SS(5)) - (SS(6)*SS(2)))/DD
        NT = 0
        M = 2
```

```
      GO TO 2
   17 D = CK + A(I)
      Q(1) = A(I)/D
      Q(2) = -Q(1)/D
      Q(3) = V(I)
      GO TO 13
   18 DD = (SS(1)*SS(4) - SS(2)**2)
      B1 = ((SS(4)*SS(5)) - (SS(2)*SS(6)))/DD
      B2 = ((SS(1)*SS(6)) - (SS(2)*SS(5)))/DD
      CK = CK + B2/B1
      VM = B1
      NT = NT + 1
      IF(NT-3) 2,21,21
   21 S2 = SV2 - B1*SS(5) - B2*SS(6)
      S2 = S2/P
      S1 = SQRT(S2)
      SECK = (S1/B1)*SQRT(SS(1)/DD)
      SEV = S1*SQRT(SS(4)/DD)
      PRINT 30,CK,SECK
      PRINT 31,VM,SEV
      PRINT 35,S2,S1
   30 FORMAT(7HOK    = F12.6,13H S.E.(K)    = F11.6)
   31 FORMAT(7H K    = F12.6,13H S.E.(V)    = F11.6)
   35 FORMAT(12H VARIANCE = E14.5,10H  SIGMA = F12.7//)
      GO TO 14
C     MATRIX SOLUTION SUBROUTINE
    2 DO 3 J = 1,3
      DO 3 K = 1,2
    3 S(K,J) = 0
      DO 4 I = 1,NP
      GO TO (15,17),M
```

```
13 DO 4 J = 1,3
   DO 4 K = 1,2
 4 S(K,J) = S(K,J) + Q(K)*Q(J)*W(I)
   I = 0
   DO 7 J = 1,3
   DO 7 K = 1,2
   I = I + 1
 7 SS(I) = S(K,J)
   GO TO (16,18),M
36 FORMAT(23H PROGRAM COMPLETED FOR I4,6H LINES)
99 PRINT 36,JJ
   STOP
   END
```

Statements 14 and 15 formulate the input according to formats 11 and 1. An initial card used in front of each data set contains the number of data points (columns 1-3) and any desired title (columns 21-69). The data set follows, with a data card for each point, providing velocity, substrate concentration and weighting information in columns 1-10, 11-20 and 21-30, respectively. In this way, any number of data sets may be used in the data deck, with data input terminated by using a blank card after the last data set.

A full explanation of the program has been given by Carvalho (92), involving rough (provisional) estimations of K_m and V_m values, followed by three iterations resulting in finely adjusted values together with standard errors for K_m and V_m and the experimental variance for the data set.

DATA PROCESSING (2).

A number of investigations during the course of this work required plots drawing a best fit line through a set of data points to give accurate information of the intercepts of the line at the ordinate and abscissa (section 4.5.3) and also to allow an evaluation of the point of intersection of two lines (sections 4.7.1, 4.7.2, 4.7.3, 4.9.1.5 and 4.9.2.5).

A standard FORTRAN IV bivariance statistical program (ESBIVAR; author, W.E. Stephens, Department of Geology, University of St. Andrews), stored in the University of St. Andrews computer was used for correlation analysis.

7. BIBLIOGRAPHY.

1. Policy of the Journal and Instructions to Authors (1975) Biochem.J. 145, 1-20

2. Ohlsson, E. (1930) Z. Physiol. Chem. 189, 17-63.

3. Griffin, P.J. and Foggarty, W.M. (1973). J. Appl. Chem. Biotechnol. 23, 301-308.

4. Shinke, R., Nishira, H. and Mugibayashi, N. (1974). Agric. Biol. Chem. 38, 665-666.

5. Marshall, J.J. (1974). FEBS (Fed. Eur. Biochem. Soc.) Letters 46, 1-4.

6. Gertler, A. and Birk, Y. (1966). Biochim. Biophys. Acta 118, 98-105.

7. Thoma, J.A., Koshland, D.E., Ruscica, J. and Baldwin, R. (1963). Biochem. Biophys. Res. Commun. 12, 184-188.

8. French, D. (1960) in The Enzymes, 2nd edn., (Boyer, P.D., Lardy, H. and Myrback, K., eds), vol. 4, pp. 345-368, Academic Press, London and New York.

9. Thoma, J.A., Spradlin, J.E. and Dygert, S. (1971) in The Enzymes, 3rd edn., (Boyer, P., ed.), vol. 5, pp. 115-189, Academic Press, London and New York.

10. Cleveland, F.C. and Kerr, R.W. (1948) Cereal Chem. 25, 133-139.

11. Kerr, R.W. and Gehman, H. (1951) Staerke 3, 271-278.

12. Cowie, J.M.G, Fleming, I.D, Greenwood, C.T. and Manners, D.J. (1958) J. Chem. Soc. (Lond) 697.

13. Bourne, E.J. and Whelan, W.J. (1950). Nature (Lond.) 166, 258-259.

14. French, D. (1961). Nature (Lond.) 190, 445-446.

15. Thoma, J.A. and Spradlin, J.E. (1970). Brew Dig. 45(1), 58-67.

16. Thoma, J.A. and Spradlin, J.E. (1970). Brew Dig. 45(2), 66-75.

17. French, D. and Youngquist, R.W. (1963). Staerke 15, 425-431.

18. French, D., Knapp, D.W. and Pazur, J.H. (1950). J. Am. Chem. Soc. 72, 1866-1867.

19. Koshland, D.E., Yankeelov, J.A. and Thoma, J.A. (1962). Fed. Proc. 21, 1031-1038.

20. Thoma, J.A. (1974). Eur. J. Biochem. 44, 139-142.

21. Weill, C.E. and Caldwell, M.L. (1945). J. Am. Chem. Soc. 67, 212-214, 214-217.

22. Englard, S., Sorof, S. and Singer, T.P. (1951). J. Biol. Chem. 189, 217-226.

23. Thoma, J.A. and Koshland, D.E. (1960). J. Mol. Biol. 2, 169-170.

24. Thoma, J.A., Koshland, J.E., Shinke, R. and Ruscica, J. (1965). Biochemistry 4, 714-722.

25. Spradlin, J.E. and Thoma, J.A. (1970). J. Biol. Chem. 245, 117-127.

26. Zherebtsov, N.A. (1968). Biokhimiya 33, 435-444.

27. Marshall, J.J. (1973). Eur. J. Biochem. 33, 494-499.

28. Thoma, J.A. (1968). J. Theor. Biol. 19, 297-310.

29. Holló, J., László, E. and Hosche, A. (1973). Staerke 25, 1-12.

30. Wing, R.E. and BeMiller, J.N. (1969). Carbohydr. Res. 10, 371-377.

31. Drummond, G.S., Smith, E.E. and Whelan, W.J. (1971). FEBS (Fed. Eur. Biochem. Soc.) Letters 15, 302-304.

32. Thoma, J.A. and Koshland, D.E. (1960). J. Am. Chem. Soc. 82, 3329-3333.

33. Hüsemann, E. and Pfannemüller, B. (1965). Makromolekulare Chem. 87, 139-151.

34. Thoma, J.A., Brothers, C. and Spradlin, J.E. (1970). Biochemistry 9, 1768-1775.

35. Wakim, J., Robinson, M. and Thoma, J.A. (1969). Carbohydr. Res. 10, 487-503.

36. Kato, M., Hiromi, K. and Morita, Y. (1974). J. Biochem. (Tokyo) 55, 315-320.

37. Hanson, K.R. (1964). Biochemistry 1, 723-734.

38. Holló, J., László, E. and Tóth-Békés, Zs. (1972). Acta Alimentaria Acad. Sci. Hung. 1, 105-117.

39. Abdullah, M. and French, D. (1966). Nature (Lond.) 210, 200.

40. Hehre, E.J., Okado, G. and Genghof, D.S. (1969). Arch. Biochem. Biophys. 135, 75-89.

41. Bernfeld, P. (1955) in Methods in Enzymology (Collowick, S.P. and Kaplan, N.O., eds.), vol. 1, pp. 149-158, Academic Press, London and New York.

42. Nelson, N. (1944). J. Biol. Chem. 153, 375-380.

43. Somogyi, M. (1952). J. Biol. Chem. 195, 19-23.

44. Dygert, S., Li, L.H., Florida, D. and Thoma, J.A. (1965). Anal. Biochem. 13, 367-374.

45. Holló, J. and Szeitli, J. (1968) in Starch and its Derivatives, 4th edn., (Radley, J.A., ed.), ch. 7, Chapman and Hall, London.

46. Bailey, J.M. and French, D. (1957) J. Biol. Chem. 226, 1-14.

47. Klein, B., Foreman, J.A. and Searcy, R.L. (1969). Anal. Biochem. 43, 316-320.

48. Rinderknecht, H., Wilding, P. and Haverback, B.J. (1967). Experientia (Basel) 23, 805.

49. Marshall, J.J. (1970). Anal. Biochem. 37, 466-470

50. Bilderback, D.E. (1973). Plant Physiol. 51, 594-595.

51. Marshall, J.J. and Whelan, W.J. (1971). Anal. Biochem. 43, 316-320.

52. LaBerge, D.E., Clayton, J.W. and Meredith, W.O.S. (1967). Amer. Soc. Brew. Chem., Proc. 18-23.

53. Nummi, M., Vilhunen, R. and Enari, T-M. (1965). Eur. Brew. Conv., Proc. Congr. 10th 52-61.

54. Daussant, J. and Corvazier, P. (1970). FEBS (Fed. Eur. Biochem. Soc.) Letters. 7, 191-194.

55. Visuri, K. and Nummi, M. (1972). Eur. J. Biochem. 28, 555-565.

56. LaBerge, D.E. and Meredith, W.O.S. (1971). J. Inst. Brew. 77, 436-442.

57. Meyer, K.H., Fischer, E.H. and Piguet, A. (1951). Helv. Chim. Acta 34, 316-324.

58. Silman, I. and Katchalski, E. (1966). Annu. Rev. Biochem. 35, 873-908.

59. Katchalski, E., Silman, I. and Goldman, R. (1971). Adv. Enzymol. Relat. Areas Mol. Biol. 34, 445-536.

60. Axén, R. and Porath, J. (1966). Nature (Lond.) 210, 367-369.

61. Vretblad, P. and Axen, R. (1973). Biotechnol. Bioeng. 15, 783-794.

62. Mårtensson, K. (1974). Biotechnol. Bioeng. 16, 567-577, 579-591, 1567-1587.

63. Barker, S.A., Somers, P.J. and Epton, R. (1969). Carbohydr. Res. 9, 257-263.

64. Barker, S.A., Somers, P.J., Epton, R. and McLaren, J.V. (1970). Carbohydr. Res. 14, 287-296.

65. Barker, S.A., Somers, P.J. and Epton, R. (1970). Carbohydr. Res. 14, 323-326.

66. Lilly, M.D., Hornby, W.E. and Crook, E.M. (1966). Biochem. J. 100, 718-723.

67. Hornby, W.E., Lilly, M.D. and Crook, E.M. (1968). Biochem. J. 107, 669-674.

68. Filippusson, H. and Hornby, W.E. (1970). Biochem. J. 120, 215-219.

69. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275.

70. Llayne, E. (1957) in Methods in Enzymology (Collowick, S. P. and Kaplan, N.O., eds.), vol. 3, pp.450-451, Academic Press, London and New York.

71. Itzhaki, R.F. and Gill, D.M. (1964). Anal. Biochem. 9, 401-410.

72. Lloyd, J.B. and Whelan, W.J. (1969). Anal. Biochem. 30, 467-470.

73. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Anal. Chem. 28, 350-356.

74. Bobbitt, J.M. (1956) in Advances in Carbohydrate Chemistry (Wolfrom, M.L., ed.), vol. 11, pp. 1-41, Academic Press, New York.

75. Strumeyer, D.H. (1967). Anal. Biochem. 19, 61-71.

76. Whitmore, E.T. and Sparrow, D.H.B. (1957). J. Inst. Brew. 397-398.

77. Nummi, M., Vilhunen, R. and Enari, T-M. (1965). Fin. Kemistsamf. Medd. 74, 49-61.

78. Wolfrom, M.L. and Thompson, A. (1962), in Methods in Carbohydrate Chemistry (Whistler, R.L. and Wolfrom, M.L., eds.), vol. 1, pp. 334-335, Academic Press, London and New York.

79. Baum, H., Gilbert, G.A. and Scott, N.D. (1956). Nature (Lond.), 177, 889.

80. Everett, W.W. and Foster, J.F. (1959). J. Am. Chem. Soc. 81, 3459-3464.

81. Flodin, P. and Aspberg, K. (1961) in Biological Structure and Function (Goodwin, T.W. and Lindberg, O, eds.), vol. 1, pp. 345-349. Academic Press, London and New York.

82. Whelan, W.J., Bailey, J.M. and Roberts, P.J.P. (1953). J. Chem. Soc. (Lond.), pp. 1293-1298.

83. Hiromi, K, Ogawa, K., Nakanishi, N. and Ono, S. (1966). J. Biochem. (Tokyo) 60, 439-449.

84. Trevelyan, W.E., Proctor, D.B. and Harrison, J.S. (1950). Nature (Lond.), 166, 444.

85. Hornby, W.E. and Morris, D.L. (1973). U.K. Patent No. 16148/73.

86. Tung, K.K. and Nordin, J.H. (1969). Anal. Biochem. 29, 84-90.

87. Greenwood, C.T. and MacGregor, A.W. (1965). J. Inst. Brew. 71, 405-417.

88. Greenwood, C.T. and Milne, E.A. (1968). Staerke 20, 139-150.

89. Stein, E.A. and Fischer, E.H. (1958). J. Biol. Chem. 232, 867-879.

90. Piguet, A. and Fischer, E.H. (1952). Helv. Chim. Acta 35, 257-262.

91. Lineweaver, H. and Burk, D. (1934). J. Am. Chem. Soc. 56, 658-666.

92. Carvalho, L.B. de (1974), Ph.D. Thesis, University of St. Andrews.

93. Ono, S., Hiromi, K. and Zinbo, M. (1964). J. Biochem. (Tokyo) 55, 315-320.

94. Erlander, S.R., Griffin, H.L. and Senti, F.R. (1965). Biopolymers 3, 497-508.

95. Goldman, R., Goldstein, L. and Katchalski, E. (1971) in Biochemical Applications of Reactions on Solid Supports (Stark, G.R., ed.), pp. 1-78. Academic Press, London and New York.

96. Zaborski, O.R. and Ogletree, J. (1974). Biochem. Biophys. Res. Commun. 61, 210-216.

97. Misra, U.K. and French, D. (1960). Biochem. J. 77, 1p.

98. Dixon, M. (1953). Biochem. J. 55, 170-171.

99. Thoma, J.A. and Koshland, D.E. (1960). J. Biol. Chem. 235, 2511-2517.

100. Hiromi, K., Nitta, Y., Numata, C. and Ono, S. (1973). Biochim. Biophys. Acta, 302, 362-375.

101. Goldstein, L., Levin, Y. and Katchalski, E. (1964). Biochemistry 3, 1913-1919.

102. Campbell, J. (1974). Ph.D. Thesis, University of St. Andrews.

103. Epton, R., McLaren, J.V. and Thomas, T.H. (1973). Biochim. Biophys. Acta 328, 418-427.

104. Bayne, S.J. (1974). Ph.D. Thesis, University of St. Andrews.

105. Geynes, L. and Sehon, A.H. (1960). Can. J. Biochem. 38, 1235-1248.

106. Ledingham, W.M. and Hornby, W.E. (1969). FEBS (Fed. Eur. Biochem. Soc.) Letters 5, 118-120.

107. Ledingham, W.M. and Ferreira, M. do S.S. (1973) Carbohydr. Res. 30, 196-201.

108. Gabel, D., Steinberg, I.Z. and Katchalski, E. (1971) Biochemistry 10, 4661-4669.

109. Hornby, W.E., Lilly, M.D. and Crook, E.M. (1966). Biochem. J. 98, 420-425.

110. Gestrelius, S., Mattiasson, B. and Mosbach, K. (1973). Eur. J. Biochem. 36, 89-96.

111. Laidler, K.J. and Bunting, P.S. (1973). The Chemical Kinetics of Enzyme Action, 2nd edn., pp. 382-412, Clarendon Press, Oxford.

112. Lilly, M.D., Kay, G., Sharp, A.K. and Wilson, R.J.H. (1968) Biochem. J. 107, 5p.

113. Cleland, W.W. (1967). Adv. Enzymol. Relat. Areas Mol. Biol. 29, 1-32.