Antimicrobial Peptides and Antibacterial Proteins from Rainbow Trout, *Oncorhynchus mykiss*

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"There is something fascinating about science. One gets such wholesale returns of conjecture out of such a trifling investment of fact."

Mark Twain (1835 - 1910)

To my Mother

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Declarations

(i) I, Jorge Manuel de Oliveira Fernandes, hereby certify that this thesis, which is approximately 70 000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 30th September 2002 Signature of candidate

(ii) I was admitted as a research student in October 1998 and as a candidate for the degree of Doctor of Philosophy in October 1999; the higher study for which this is a record was carried out in the University of St Andrews between 1998 and 2002.

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

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Abstract

Antimicrobial peptides are potent natural antibiotics present in myeloid cells or mucosal tissues of most multicellular organisms. It is now well established that these peptides are crucial components of the innate immune system, operating as a first-line of host defence against potential pathogens, either individually or in synergism with other defence factors, such as lysozyme.

This thesis reports the purification and characterisation of 5 low molecular weight, cationic antimicrobial proteins present in skin secretions, liver or erythrocytes of rainbow trout, *Oncorhynchus mykiss*. One is a novel 3 kDa peptide with a predicted amphipathic α-helical structure and active against the Gram-(+) bacterium *Planococcus citreus*. Another is a 13.6 kDa protein, active against Gram-(+) bacteria, which was identified as being histone H2A. A third is a 6.7 kDa peptide that displays activity against *P. citreus* and is likely to be the 40S ribosomal protein S30. The fourth is a 7.2 kDa C-terminal fragment of histone H1, with broad-spectrum activity and the fifth is a 6.7 kDa N-terminal fragment of histone H6 that is also active against both Gram-(+) and Gram-(-) bacteria. Histone H2A and the histone H6-derived peptide are inhibited by high NaCl concentrations (maximum 3.2 % (w/v)), do not display significant haemolytic activity to trout erythrocytes and are unable to form stable pores on artificial membranes.

Additionally, skin mucus was found to contain two muramidases: a novel muramidase with an acidic isoelectric point and a c-type lysozyme that was purified and characterised. Antibacterial activity was likewise detected in liver ribosomes and in erythrocyte extracts; the antibacterial, cationic factors were partially purified.

Taken together, these data demonstrate that rainbow trout expresses a multitude of novel antimicrobial peptides and proteins that are likely to confer protection against microbial exploitation, particularly at the mucosal surfaces.

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Chapter 1

Introduction

1.1. The Immune System of Vertebrates

Under the tremendous selective pressure imposed by a multiplicity of pathogens with outstanding plasticity, all multicellular organisms have developed several host-defence mechanisms that allow them to recognise and eliminate invading infectious agents (reviewed by Medzhitov & Janeway, 1997a; Du Pasquier, 2001). The host-defence mechanisms range from simple phagocytosis, probably originating from a nutritive function in Protista (reviewed by Ratcliffe, 1989), to the complex and sophisticated adaptive immune system of vertebrates. Since many of the responses initiated by the immune system are destructive, it is crucial that they are triggered solely in response to molecules that are foreign to the host. This ability of the body to distinguish "self" from "non-self" is a fundamental feature of the immune system, and has been proposed by Sir Frank Macfarlane Burnet in 1949 as a definition of immunity (Burnet & Fenner, 1949). This definition of "self" has proved unsatisfactory, for it does not account for phenomena such as tolerance and auto-immunity responses. In spite of the various models of "self/ non-self" discrimination developed in recent years, the concept of "self" still remains a controversial one (reviewed by Anderson & Matzinger, 2000; Langman & Cohn, 2000).

The vertebrate immune system comprises two distinct recognition and effector mechanisms: the innate system and the adaptive system. The innate immune system does not directly involve antibodies and has a non-clonal system of antigen recognition based on pattern recognition receptors (reviewed by Medzhitov & Janeway, 2000; Medzhitov & Janeway, 2002); this later property is

advantageous, for it permits the mobilisation of large numbers of cells and molecules in a short period of time (reviewed by Medzhitov & Janeway, 1997b). For this reason it is crucial as the first line of host defence. The main disadvantage of the innate system is its lack of memory (reviewed by Hultmark, 1993), i.e. a further exposure to the same antigen does not lead to faster or stronger responses. By contrast, the adaptive system, which is exclusive of vertebrates, is characterised by a precise discriminative ability and immunological memory (reviewed by Borghans *et al.*, 1999).

Complement is a response mechanism involved in both the innate and adaptive recognition and effector systems. It is composed of interacting soluble proenzymes that are sequentially activated upon antigenic challenge by three distinct pathways, stimulated by different factors. The classical pathway is directly related to adaptive immunity since it is activated by antigen-antibody complexes, whilst the alternative pathway is triggered by bacterial lipopolysaccharides; the lectin pathway is activated by ficolins and mannose-binding lectins (reviewed by Muller-Eberhard, 1988; Fujita, 2002). Activation of this system initiates a cascade of limited proteolytic reactions that result in release of anaphylatoxins, activation of the C3 complement component, assembly of the membrane attack complex and covalent linkage of late complement components to the antigen, thus inducing its opsonisation and digestion by phagocytes or lysis (reviewed by Muller-Eberhard, 1988). Besides being a major effector of innate immunity in vertebrates, the complement system is important in the adaptive immune response (reviewed by Carroll *et al.*, 1998).

For many years the adaptive immune response was considered to be independent of innate immunity, which was considered by many to be of secondary importance. Research over the past few years has proven that there is an intricate relation between the innate and adaptive immune systems (reviewed by Medzhitov & Janeway, 1997a; Medzhitov & Janeway, 1998). It is now well established that the non-adaptive system triggers the initiation of the adaptive immune response and influences the type of effector response by releasing effector cytokines (reviewed by Medzhitov & Janeway, 1997a; Medzhitov & Janeway, 1998). Moreover, recent studies have indicated a new role for complement in efficient clonal selection and maintenance of $CD5^+$ B1 lymphocytes (reviewed by Carroll *et al.*, 1998; Barrington *et al.*, 2001) as well as in regulation of T lymphocyte activation (Wagner *et al.*, 2001). Taken together, these findings show that in vertebrate immunity the innate and adaptive immune responses coalesce into a single, effective system to control "self/ non-self discrimination".

The epithelia, which include the epidermis and the surfaces of the digestive, respiratory, urinary and reproductive tracts, are constantly exposed to microbes. Therefore, the microenvironment in these mucosal barriers provides the first line of host defence against potential pathogens (reviewed by Nagler-Anderson, 2001). In mammals, mucosal defence is attained by special organs, such as the gut-associated lymphoid tissue (GALT) and secretory glands, which constitute a regional (mucosal) immune system (reviewed by Ouellette, 1999; Nagler-Anderson, 2001).

1.1.1. Adaptive Immunity

The mammalian adaptive immune response involves two classes of lymphocytes: T cells, which are produced in the thymus and are responsible for cell-mediated immune responses, and B cells, that are generated in the adult bone marrow and make antibodies (collectively called immunoglobulins, Igs). The antibody effector mechanisms comprise neutralisation of the antigen function, precipitation or agglutination of antigens, opsonisation of antigens and the above referred complement-mediated functions. In higher vertebrates, B lymphocytes display Igs on their surface (reviewed by Meffre *et al.*, 2000), whereas T cells are characterised by another type of antigen-specific receptor, termed T-cell receptor (TCR) (reviewed by Fowlkes & Schweighoffer, 1995). Class I and class II major histocompatibility complex (MHC) molecules are highly polymorphic glycoproteins that are essential in presentation of foreign antigens to T cells (reviewed by Fowlkes & Schweighoffer, 1995). During T-lymphocyte development, lymphocytes that react strongly with self antigens are eliminated, ensuring that the immune system will react only against foreign antigens. When a lymphocyte encounters an antigen to which it is committed, it will proliferate and mature. In this process, termed clonal expansion, it gives rise to two types of cells: activated cells, that will trigger a response, and memory cells with the ability to react more readily than virgin cells and to further proliferate into either activated cells or memory cells (reviewed by Tonegawa, 1983; Sprent, 2002).

1.1.2. Innate Immunity

The innate (non-adaptive) immune system, although lacking the sharp discriminative and memory properties of the adaptive system, is perfectly capable of distinguishing "self" from "non-self" (reviewed by Medzhitov & Janeway, 1997a). It comprises not only the two broad classes of immune responses (cell-mediated immune and humoral) but also the physical and biological barriers provided by protective organs like the skin and other epithelial surfaces.

The cells involved in the non-adaptive immune response are different types of leukocytes, namely macrophages, granulocytes (neutrophils, eosinophils and basophils) and natural killer (NK) cells. Besides being involved in direct elimination of pathogens these leukocytes produce cytokines, namely interleukin-1 (IL-1), IL-6, tumour necrosis factor α (TNF-α), transforming growth factor β (TGF-β), type I interferons (IFNs) and other chemokines. As well as mediating the inflammatory response, these co-stimulatory molecules function as co-stimulators of lymphocytes involved in the adaptive immune response and control the induction of effector functions (reviewed by Medzhitov & Janeway, 1997a). Interferons are proteins or glycoproteins that induce the expression of IFN-stimulated genes on virus-infected cells (reviewed by Katze *et al.*, 2002) and coordinate all the machinery required to present viral antigens to cytotoxic T cells of the adaptive system (reviewed by Medzhitov & Janeway, 1997a).

The inflammatory response is a complex process that contributes to fighting infection and/ or wound healing, being induced by a diverse array of signalling molecules produced by leukocytes, mast cells and platelets, as well as by the activation of complement. Inflammation is accompanied by an increase in blood supply to the affected area, followed by an increase in capillary permeability and migration of white blood cells out of the blood stream (reviewed by Ley, 2002). Macrophages and granulocytes are mobile phagocytic cells that play an important role in inflammation. After engulfing an intruder by phagocytosis or pinocytosis they neutralise it both by oxidative and non-oxidative mechanisms (reviewed by Levy, 2000). Once phagocytosis is initiated there is a dramatic increase in oxygen uptake, unrelated to mitochondrial respiration (reviewed by Verhoef, 1991). During this oxidative burst, oxygen is converted via NAPDH oxidase and myeloperoxidase into reactive oxygen and halogen species, such as superoxide anions, hydrogen peroxide, singlet oxygen and hydroxyl radicals (reviewed by Neumann *et al.*, 2001; Chapman *et al.*, 2002). Concomitantly, there is also the formation of reactive nitrogen species, for example nitrogen dioxide, nitrogen trioxide and nitronium ions, all of them potent microbicidal agents (reviewed by Neumann *et al.*, 2001). The oxygen-independent mechanisms involve a variety of endogenous bactericidal and bacteriostatic factors that are directed to phagosomes containing the ingested particles. These antimicrobial molecules include, amongst others, lysozyme, bactericidal permeability increasing factor, lactoferrin, lectins and antimicrobial peptides (reviewed by Lehrer *et al.*, 1991; Levy, 2000).

The humoral defences comprise a series of soluble factors (predominantly proteins and glycoproteins) that intervene at various levels of the immune response, playing a crucial role in host protection (reviewed by Yano, 1996; Carroll *et al.*, 1998). The arsenal of soluble antimicrobial agents present in the serum and other extracellular fluids includes, amongst others, C-reactive protein (CRP), cytokines, transferrin, lectins, proteases, lysozyme and antimicrobial peptides. The latter will be discussed in greater detail in sections 1.2 and 1.3.

C-reactive protein (CRP) is an acute phase protein that reacts with molecules at the cell surface of microorganisms and acts as an opsonin, facilitating phagocytosis and activating the complement system (reviewed by Carroll *et al.*, 1998). Additionally, it enhances NK cell-mediated cytotoxicity and helps to solubilise chromatin and other products resulting from cell lysis (reviewed by Kilpatrick & Volanakis, 1991). The synthesis of CRP by hepatocytes is up-regulated by tissue damage, inflammation or infection, with the consequent immediate increase of its concentration in the plasma (reviewed by Yano, 1996).

In vertebrates, the transport of iron between locations of absorption, storage and utilisation is made chiefly by transferrin, an iron-binding glycoprotein (reviewed by Irie & Tavassoli, 1987). The protective role of transferrin is connected to its ability to chelate iron, thus limiting the amount of free iron available to bacteria during infection (Weinberg, 1974).

Lectins, also known as natural haemagglutinins, are a group of proteins that display high affinity for simple sugars, e.g. mannose, and have the ability to agglutinate cells and precipitate glycoconjugates. They serve several biological functions, including the neutralisation of bacterial components released by pathogens during infection and the immobilisation of microorganisms (Yano, 1996; Buchmann, 2001).

1.1.3. Comparison Between the Immune Systems of Teleosts and Higher Vertebrates

Teleostei (teleost fishes, bony fishes) is a subgroup of the Actinopterygii (ray-finned bony fishes) class, an extremely diverse and relatively recent group of primitive vertebrates, with the first fossil records dating back to the late Silurian, *circa* 400 million years ago (Basden *et al.*, 2000). Two decades ago an expanding interest in the immune system of teleosts has emerged, connected to the exponential expansion of fish farming (reviewed by Ellis, 1982) (Fig. 1.1). With the continuing decline in fishery resources and the concomitant increase in fish demand for human consumption, aquaculture became an attractive alternative for the supply of finfish (Fig. 1.1), of which teleosts are the group of major commercial importance (reviewed by Tacon, 1997).

The main finfish species groups farmed in Europe are salmonids (salmon and trout), demersal marine fish (sea bass and sea bream) and cyprinids (reviewed by Varadi *et al.*, 2001). Within these, the most important is by far the salmonid group, with the total European production in 2000 ascending to $9.14 \cdot 10^5$ metric tons, equivalent to *circa* 72 % of total finfish production (Fig. 1.2). Salmonids are mainly produced at high stocking densities in intensive cage-, tank- or pond-based farming systems, with the two major farmed species being the Atlantic salmon, *Salmo salar*, and the rainbow trout, *Oncorhynchus mykiss* (Tacon, 1997).

High population densities, poor water quality, temperature extremes and other adverse conditions to which fish are subjected in intensive aquaculture, facilitate the growth of fish pathogens. Bacterial, viral or parasitic infections can have devastating effects on both wild and cultured stocks of teleosts, resulting in serious economic losses. It is therefore crucial to understand the immune mechanisms in teleosts in order to better control inflammation and disease.

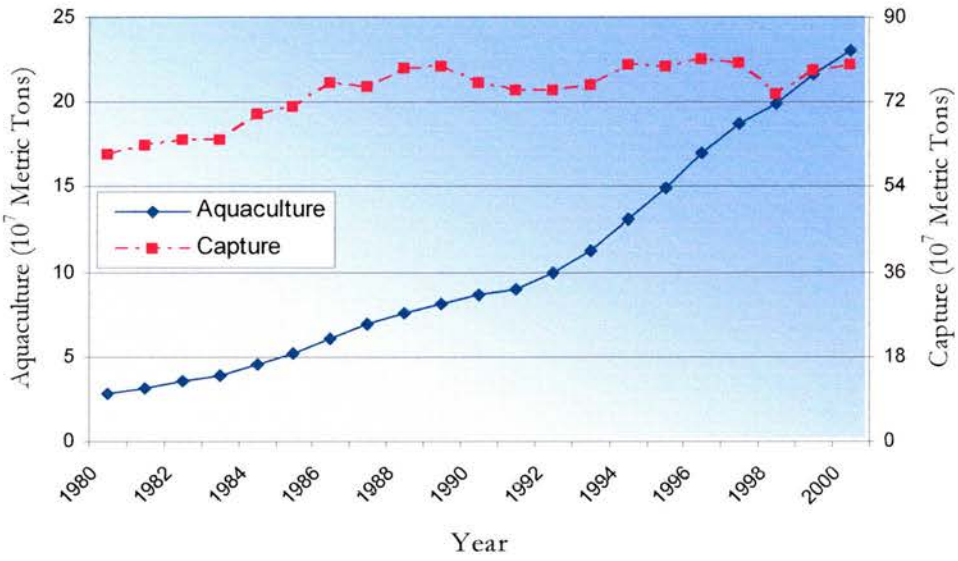


Figure 1.1. Evolution of the worldwide wild fish capture (Capture) and farmed fish production (Aquaculture) during the last two decades. Data are expressed as live weight, i.e. nominal weight of the fish at time of capture (Source: FISHSTAT databases, FAO).

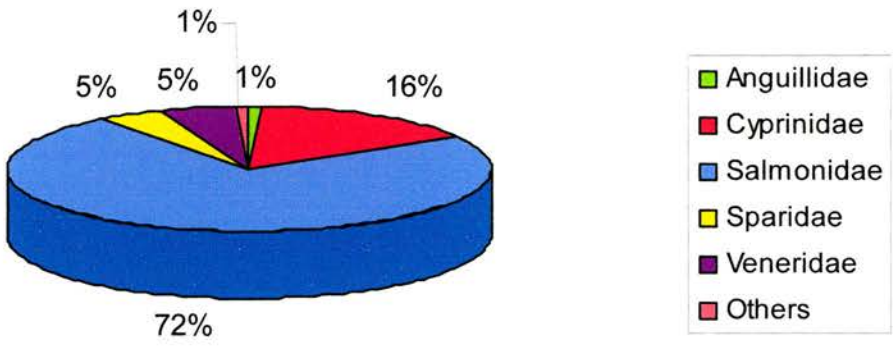


Figure 1.2. Distribution (in percentage) of the most significant finfish species groups in European aquaculture production in 2000 (Data source: FISHSTAT databases, FAO).

Besides their practical importance for aquaculture, teleosts have proven to be valuable animal models for study by comparative immunologists, especially in the fields of immune system ontogeny and T cells antigen-receptors (reviewed by Ellis, 1982).

As fish are ectothermic animals, their immune response is strongly regulated by ambient temperature (reviewed by Manning & Nakanishi, 1996). In particular, the adaptive immune response is severely impaired at low environmental temperatures by mechanisms which are yet to be clarified (Miller & Clem, 1984; reviewed by Bly *et al.*, 1997). The "winter kill" outbreaks, observed in commercial fish ponds when the water temperature decreases abruptly, seems to

be associated with a compromised immunocompetence at low temperatures (reviewed by Bly & Clem, 1992). For salmonids, the immunologically permissive temperature, i.e. the temperature below which the adaptive immune response is suppressed, is as low as 4 °C (reviewed by Bly & Clem, 1992). In general, a better adaptive immune response is attained at elevated water temperatures, within the physiological range for each species (reviewed by Bly & Clem, 1992; Manning & Nakanishi, 1996).

Teleosts have a complement system that is structurally and functionally comparable to that of mammals, comprising the classical (CCP), alternative (ACP) and lectin complement pathways (reviewed by Nonaka & Smith, 2000; Fujita, 2002). The ACP activity in teleosts, which is an important effector response mechanism during early stages of infection, is extremely high when compared to mammalian ACP activity measured in equivalent optimum conditions (reviewed by Yano, 1996). Moreover, bony fish have multiple forms of C3 that have arisen from gene duplication events (reviewed by Nonaka, 2001) and that display different affinities to several complement activators (Sunyer *et al.*, 1996). This unique amplification strategy suggests that the complement system has a primordial importance in teleost host defence (reviewed by Nonaka, 2001).

The adaptive immune system of teleosts is, in many aspects, similar to that of higher vertebrates, but there are also some significantly different features that need to be emphasised (Fig. 1.3). Unlike anurans and all other higher vertebrates, teleosts lack bone marrow (reviewed by Zapata *et al.*, 1996; Du Pasquier, 2001).

Nevertheless, they possess lymphohaemopoietic bone marrow-like microenvironments in distinct but equivalent organs, where blood cell formation occurs (reviewed by Zapata *et al.*, 1996). The kidney of teleosts is an important primary lymphoid organ, performing functions homologous to those associated with the bone marrow and lymph nodes in mammalians. It is formed by two distinct regions: the opisthonephros or trunk kidney, and the pronephros or head kidney, which is the first to develop during ontogeny (reviewed by Zapata *et al.*, 1996). Both zones display lymphohaemopoietic capacity although the head kidney, which loses its excretory functions as the trunk kidney evolves, is richer in haemopoietic tissue (reviewed by Zapata *et al.*, 1996). In contrast to bone marrow, lymphoid cells, granulocytes, T-like cells, B-like cells and phagocytes can be found within the kidney haemopoietic tissue (reviewed by Ellis, 1982). Cell separation techniques based on the use of monoclonal antibodies have shown that teleosts have lymphocyte subpopulations that are similar in many respects to mammalian T cells and B cells, with the distinction that different subsets of T cells are absent (reviewed by Manning & Nakanishi, 1996).

In the vast majority of teleost species, the thymus is remarkable for its peculiar location below the epithelium near the dorso-posterior part of the branchial cavity (reviewed by Ellis, 1982). It is widely accepted that the thymus is the first organ to become lymphoid and in the rainbow trout, *O. mykiss*, for instance, this occurs 5 days pre-hatch (reviewed by Zapata *et al.*, 1996). Experiments on the migration of labelled thymocytes to peripheral organs and studies about the effects of early thymectomy on the maturation of the immune system indicate that the teleost thymus, despite its strikingly different morphology, has the same

function as in higher vertebrates, i.e. the provision of immunocompetent T cells (reviewed by Zapata *et al.*, 1996).

The spleen matures after the kidney and thymus develop, and only acquires a lymphoid population about the time of free feeding (reviewed by Ellis, 1982). Populations of antibody-producing cells are present in the teleost spleen (Rijkers *et al.*, 1980), suggesting that this secondary lymphoid organ plays an important role in antigen trapping and processing (reviewed by Zapata *et al.*, 1996).

In common with all other vertebrates, teleosts contain lymphoid aggregates in the intestinal epithelium, known as gut-associated lymphoid tissue, GALT (Fig. 1.3). The GALT is composed chiefly of lymphocytes, plasma cells, macrophages and granulocytes (reviewed by Brandtzaeg *et al.*, 1999). There is histological evidence that teleosts have lymphoid tissue associated not only with the gut but also with other mucosal surfaces, including the reproductive tract, skin and gills (reviewed by Zapata *et al.*, 1996). Furthermore, there are several reports of the presence of mucosal antibody without concurrent serum antibody (reviewed by Kaattari & Piganelli, 1996), suggesting the existence of a mucosal immune system similar to that found in mammals.

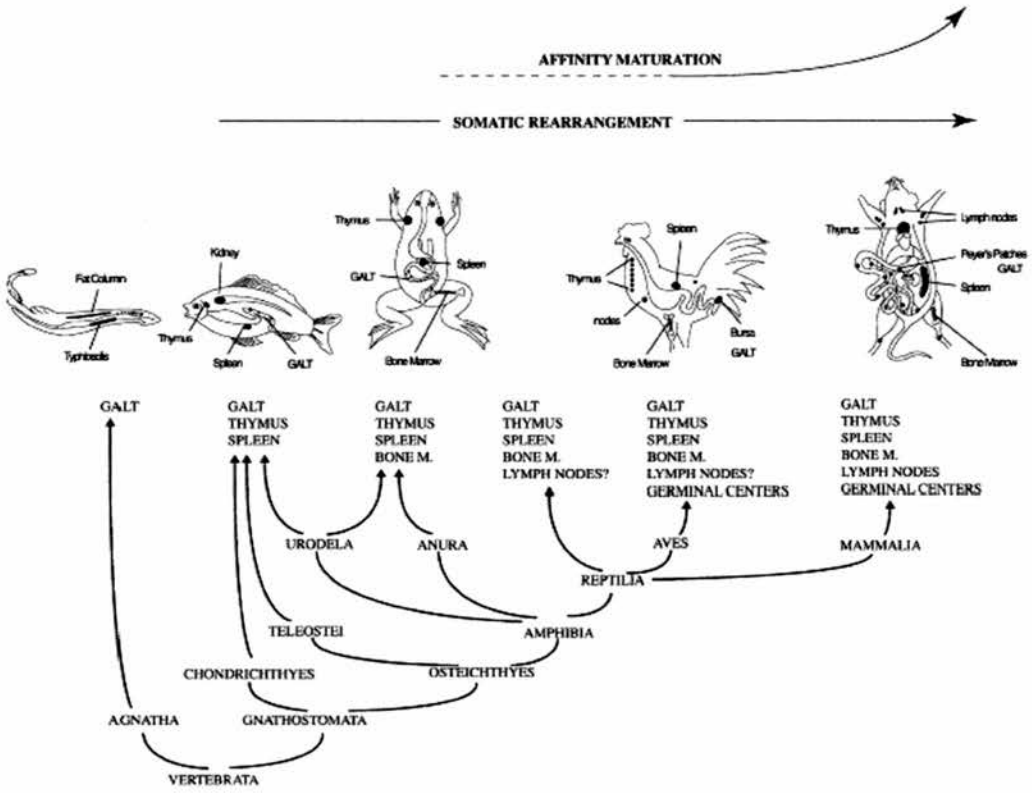


Figure 1.3. Evolution of lymphoid organs in vertebrates. Following antigen stimulation, B lymphocytes undergo somatic gene rearrangement of the antigen receptor variable genes and hypermutation, thus originating a diverse repertoire of immunoglobulins. Affinity maturation is the increase in average antibody affinity, as frequently seen during a secondary immune response. This process is not well developed in teleosts, partially due to the lack of specialised lymphoid organs, such as lymph nodes and germinal centres (Reprinted from *Comparative Biochemistry and Physiology B*, Vol. 129, Louis Du Pasquier, The immune system of invertebrates and vertebrates, Pages 1-15, Copyright (2001), with permission from Elsevier Science).

In contrast to mammals, teleosts do not express the functionally specialised five immunoglobulin classes; instead they only seem to express one type of Ig that is present in the plasma, lymph, skin and gut mucus and bile (reviewed by Ellis, 1982). The Ig from teleosts resembles mammalian IgM and it is a tetramer of four units, each composed of two heavy (~ 72 kDa) and two light (~ 27 kDa) protein chains (reviewed by Kaattari & Piganelli, 1996). Electrophoretic mobility studies have revealed that each piscine B lymphocyte routinely assembles structurally varied forms of one IgM chain, thus implying that teleost IgM may have functional diversity (Kaattari *et al.*, 1998).

The adaptive immune system of fish has memory but the secondary immune response in teleosts, when compared to that of mammals, is feebler and short-lived (reviewed by Du Pasquier, 2001), and only takes place if the animals are of sufficient age, i.e. 4-5 weeks for rainbow trout (reviewed by Tatner, 1996), and are above the permissive temperature (reviewed by Bly & Clem, 1992; Bly *et al.*, 1997). It is therefore likely that fish have greater reliance on the innate defences, particularly during ontogeny and at immunosuppressive water temperatures or physiological conditions.

The innate immune system of teleosts displays most features of that of higher vertebrates. Like mammals, they have a number of different leukocyte types distributed throughout the serum and other extracellular fluids that are involved in innate cell-mediated defence (reviewed by Secombes, 1996). These include macrophages, several types of granulocytes and "non-specific" cytotoxic cells that are equivalent to NK cells of mammals (reviewed by Secombes, 1996).

In teleosts, the living epidermal surface of the skin is protected by secretions from mucous gland cells, also termed goblet cells (reviewed by Hawkes, 1974; Shephard, 1994). This mucosal epithelial layer is the interface with an austere external environment and, besides being involved in osmoregulation and locomotion, it provides a physical and biochemical barrier that is crucial as a first line of host defence (reviewed by Shephard, 1994; Ellis, 2001). The biological relevance of the mucus has been demonstrated by experiments conducted on carp, *Cyprinus carpio*. It was found that loss of the skin mucus rendered the carps highly susceptible to infections, ultimately leading to death (Lemaître *et al.*, 1996). The skin mucus contains a collection of antibacterial substances, also found in other epithelial surfaces and in the serum and eggs of fish (reviewed by Yano, 1996), that impede pathogens from entering to the body through the skin, gills, gastrointestinal and reproductive tracts. The existence of these soluble antimicrobial factors in mucosal surfaces, together with the presence of the above-referred antibody-secreting cells, strengthens the hypothesis that a separate mucosal or local immune system does exist in teleosts (reviewed by Kaattari & Piganelli, 1996).

The humoral defence factors of teleosts comprise, amongst others, two types of lysozyme (Grinde *et al.*, 1988; Grinde, 1989), cytokines (reviewed by Secombes *et al.*, 1998; Secombes *et al.*, 1999), C-reactive protein (Winkelhake & Chang, 1982), transferrin (Lee *et al.*, 1998), lectins (Buchmann, 2001; Tasumi *et al.*, 2002), proteinases (Hjelmeland *et al.*, 1983), α_2-macroglobulin (Starkey *et al.*, 1982), chitinase (Leiro *et al.*, 1997), α-precipitin (Alexander, 1980) and antimicrobial peptides (reviewed by Ellis, 2001).

1.2. Antimicrobial Peptides

Antimicrobial peptides are a diverse group of constitutively expressed or induced cationic, low molecular weight peptides (less than *circa* 100 residues) that display a broad-spectrum antimicrobial activity (reviewed by Boman, 1995; Lehrer & Ganz, 1999; Zasloff, 2002), with a specificity that is usually related to the natural flora of the animal (reviewed by Boman, 1995; Boman, 2000). Their potent microbicidal properties allied to their strategic location in phagocytes and epithelial surfaces indicate that these peptides play an active role in resistance against infection (reviewed by Lehrer & Ganz, 1999; Hancock, 2001). The value of antimicrobial peptides as first line effectors of innate immunity lies on their small size and on their ability to function without either specificity or memory. This enables them to be effortlessly synthesised without complex tissues or cells, and readily diffusible to sites of injury or infection well before the adaptive immune system is mobilised (Boman, 1991). The *in vivo* survival value of antimicrobial peptides in host defence and regulation of the natural flora has first been demonstrated in *Drosophila* (Lemaître *et al.*, 1997; Engstrom, 1999) and in the frog, *Rana esculenta* (Simmaco *et al.*, 1997; Simmaco *et al.*, 1998).

The quest of antimicrobial peptides dates back to the 1960s with the discovery of the bee venom toxin melittin and the peptide isolated from frog skin, bombinin. However, antibacterial activity was only really documented for bombinin, which was in fact a mixture of several peptides (reviewed by Boman, 1994). The first animal antimicrobial peptides to be purified and characterised were cecropins and defensins, isolated in the early 1980s from the cecropia moth, *Hyalophora*

cecropia, by Boman and collaborators (Hultmark *et al.*, 1982) and from rabbit macrophages by Lehrer and co-workers (Selsted *et al.*, 1985), respectively. Zasloff discovered magainins in 1987, following the observation that after surgical removal of the ovaries from the African clawed toad, *Xenopus laevis*, the wounds healed without significant inflammation or infection (Zasloff, 1987).

1.2.1. Structural Classification

The finding of magainin in 1987 (Zasloff, 1987) has greatly stimulated research on this field and in the following years a large number of antimicrobial peptides have been isolated from a variety of species, establishing that antimicrobial peptides are widespread throughout the animal and plant Kingdoms (Zasloff, 2002). The global distribution of antimicrobial peptides suggests that they have played a crucial role in survival and successful evolution of multicellular eukaryotes (reviewed by Zasloff, 2002). To date, over 750 such eukaryotic peptides have been described and listed on the antimicrobial sequences database (http://www.bbcm.univ.trieste.it/~tossi/), mostly isolated from mammals, amphibians, insects and plants (Fig. 1.4).

Despite the vast variety of sequences and structures known so far, most antimicrobial peptides are cationic and have a three dimensional amphipathic structure, with the hydrophilic residues spatially distant from the hydrophobic ones (Hancock, 1997; Hancock, 2001; Zasloff, 2002). Antimicrobial peptides have been grouped in four structural classes (Hancock, 2001), based on the classification system proposed by Boman (1995). These four classes include β-sheets containing two or more disulfide bonds, amphipathic α-helices, extended coils and β-sheets with a single disulfide bond (Fig. 1.5). Some examples of representative peptides belonging to each of these structural classes are listed in Table 1.1. Despite its usefulness as a categorisation system, the grouping of antimicrobial peptides in four structural classes is not able to accommodate all peptides discovered so far, in particular heterodimeric peptides (Baptista *et al.*,

2001). On the other hand, some peptides, such as cathelicidins, fit more than one category (reviewed by Lehrer *et al.*, 2002; Ramanathan *et al.*, 2002). Therefore, it is necessary to develop a more general classification system for antimicrobial peptides.

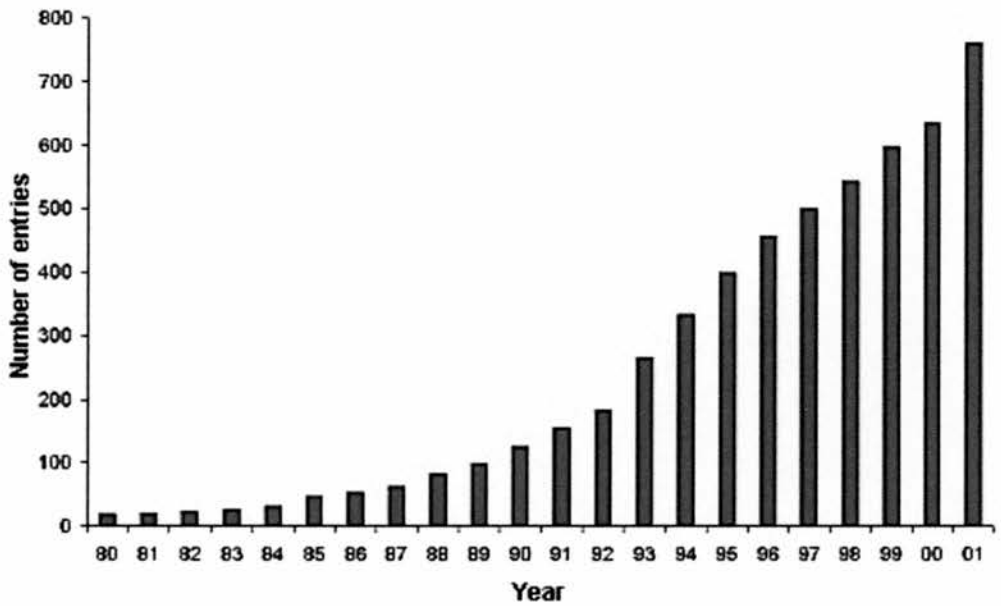


Figure 1.4. Evolution of the total number of antimicrobial peptide sequences lodged in the Antimicrobial Sequences Database (reproduced with permission from the Antimicrobial Sequences Database, http://www.bbcm.univ.trieste.it/~tossi/).

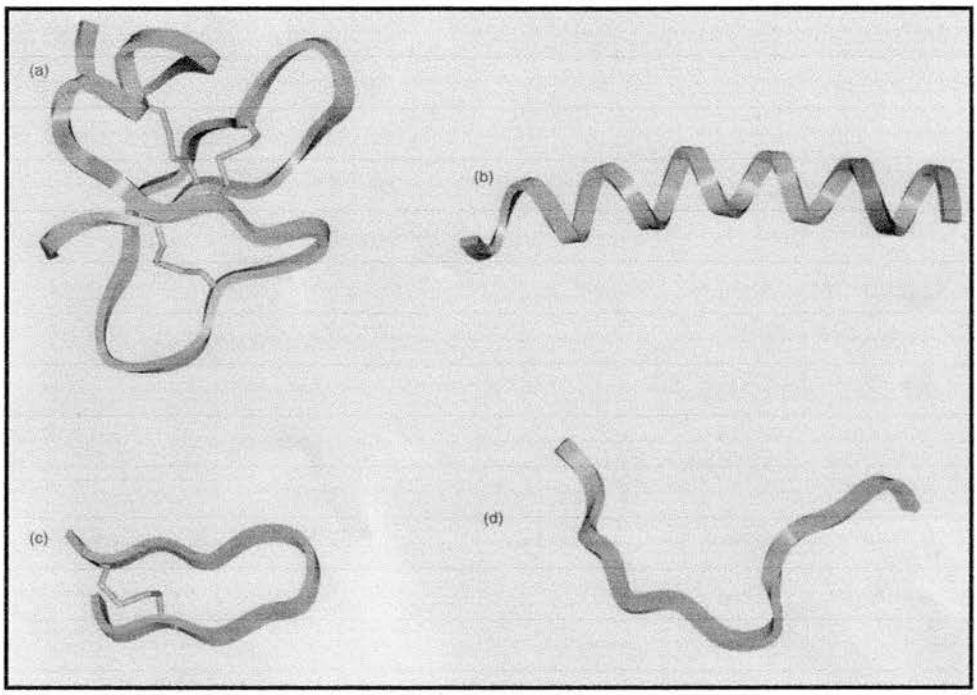


Figure 1.5. Molecular models of four structural classes of antimicrobial peptides: (a) β-stranded human β-defensin-2, (b) amphipathic α-helix of magainin, (c) β-turn loop and tail of bovine bactenecin and (d) extended coil of bovine indolicidin (Reprinted with permission from Elsevier Science (*The Lancet*, 2001, Vol. 1, No. 3, Pages 156-164)).

Table 1.1. Classification of antimicrobial peptides according to their secondary structure and representative examples of each category.

Class	Representative Peptides	References
Peptides containing two or more disulfide bonds and mainly or only β-sheets	Defensins	Lehrer *et al.*, 1991; Lehrer *et al.*, 1993; Selsted & Ouellette, 1995; Yang *et al.*, 2002
	Protegrins	Kokryakov *et al.*, 1993; Zhao *et al.*, 1994
	Tachyplesins	Nakamura *et al.*, 1988; Miyata *et al.*, 1989
	Insect Defensins	Chalk *et al.*, 1994; Hoffmann, 1995; Bulet *et al.*, 1999
Linear α-helical peptides	Cecropins	Hultmark *et al.*, 1982; Qu *et al.*, 1982
	Magainins	Zasloff, 1987
	Clavanins	Lee *et al.*, 1997
	Citropins	Wegener *et al.*, 1999
	Buforins	Kim *et al.*, 1996; Park *et al.*, 1998a
Peptides with one disulfide bond, forming a loop with tail	Bactenecin	Raj & Edgerton, 1995
	Brevinins	Kwon *et al.*, 1998; Wang *et al.*, 1998
	Ranalexin	Clark *et al.*, 1994
	Esculentin	Simmaco *et al.*, 1993
Linear peptides without Cys and with dispro-portionate presence of one or two residues, usually Pro, Arg, Trp or Gl	Apidaecins	Casteels *et al.*, 1989
	Drosocin	Bulet *et al.*, 1993
	PR-39	Gudmundsson *et al.*, 1995
	Indolicidin	Selsted *et al.*, 1992

1.2.2. Biosynthesis

Antimicrobial peptides are usually synthesised in a precursor form as a direct product of ribosomal translation from an RNA template (reviewed by Nissen-Meyer & Nes, 1997). In some cases, like magainins, this precursor is composed of several inter-spaced copies of the mature peptide, suggesting the presence of multi-copy genes (Zasloff, 1987; Bevins & Zasloff, 1990). Some antimicrobial peptide genes, e.g. the human β-defensin-1 gene, are known to exhibit intragenic polymorphism (Dörk & Stuhrmann, 1998).

Despite some similarities in the modes of action of the various families of antimicrobial peptides (Section 1.2.3), their precursors differ greatly, but are usually composed of an active, variable fragment and a conserved prepro piece, which is cleaved during peptide processing to yield the mature peptide (reviewed by Ganz & Lehrer, 1994; Boman, 1995). The cDNA sequences for the dermaseptin family, which includes dermaseptins (Ammar *et al.*, 1998), brevinins (Wang *et al.*, 1998), temporins (Simmaco *et al.*, 1996), ranalexin (Clark *et al.*, 1994), gaegurins (Park *et al.*, 1994) and esculentin (Simmaco *et al.*, 1993), revealed that the precursor proteins contain an N-terminal prepro region of approximately 50 amino acids which is highly conserved, even amongst different species (Amiche *et al.*, 1999). All preprodermaseptins contain a 16-25 residue acidic propiece (Amiche *et al.*, 1999) that may inactivate the peptide until proteolytic maturation and release to sites of infection. Cathelicidin genes have a similar structure, being organised in 4 exons that code for a 123-144 residue precursor with an anionic pro segment (reviewed by Ramanathan *et al.*, 2002).

Proteolytic processing of cathelicidin precursors is mediated by elastase (Panyutich *et al.*, 1997) or proteinase 3 (Sorensen *et al.*, 2001), upon degranulation of activated neutrophils.

The expression of antimicrobial peptides can be either constitutive (reviewed by Yang *et al.*, 2002) or induced by microbial products, e.g. bacterial lipopolysaccharide (Russell *et al.*, 1996) and L-isoleucine (Fehlbaum *et al.*, 2000), proinflammatory cytokines (reviewed by Lehrer & Ganz, 1999) or injury (Park *et al.*, 1998b; Cho *et al.*, 2002).

The regulation of antimicrobial peptide gene expression has been extensively studied in *Drosophila*, where an immediate and transient synthesis of antimicrobial peptides is observed as a response to infection (Lemaître *et al.*, 1997; Engstrom, 1999). The regulatory regions of these genes contain multiple binding sites for the *Rel*-like transactivators *Dorsal*, *Dif* and *Relish* (Levashina *et al.*, 1998; Khush *et al.*, 2001). These three transcription factors are homologous to the NF-κB family of transcriptional activators in mammals, showing that the regulation of immune response genes has been conserved over millions of years of evolution (reviewed by Medzhitov & Janeway, 1998). NF-κB consensus sequences have also been identified in other families of antimicrobial peptides, namely defensins (Diamond *et al.*, 1993) and cathelicidins (reviewed by Ramanathan *et al.*, 2002).

1.2.3. Mode of Action

The primary target of action of antimicrobial peptides is the bacterial cytoplasmic membrane, which, unlike eukaryotic cell membranes, contains mainly negatively charged phospholipid heads on the outermost leaflet of the bilayer (reviewed by Hancock & Lehrer, 1998; Zasloff, 2002). The problem of self-destruction of host cells by antimicrobial peptides is solved either by compartmentalisation, as occurs with defensins (reviewed by Lehrer *et al.*, 1991), or by some degree of selectivity, as observed in magainins (Jacob & Zasloff, 1994). This selectivity is achieved by attacking targets that are absent in the host or by the ability to kill pathogens at concentrations that are not toxic for the host (reviewed by Boman, 1995; Hancock & Lehrer, 1998).

All peptides studied so far act stoichiometrically and there is no reported data of an antimicrobial peptide with catalytic activity (reviewed by Boman, 1995). The mechanism of binding, insertion and destabilisation of membranes by most antimicrobial peptides can be explained by the Shai-Matsuzaki-Huang two-state model (Matsuzaki *et al.*, 1997; reviewed by Shai, 1999; Huang, 2000). Membrane permeabilisation can occur by two distinct processes, termed "barrel stave" or "carpet" (see Huang, 2000 for review). In the "barrel-stave" model, the amphipathic α-helices penetrate into the hydrophobic core of the membrane and form stable transmembrane pores (reviewed by Shai, 1999). By contrast, in the "carpet" model the peptides interact with the membrane surface via their hydrophobic regions until a local threshold concentration is reached; at this stage

the membrane's integrity is disrupted by the formation of transient "wormholes" (reviewed by Shai, 1999).

In spite of the irrefutable permeabilisation capability of antimicrobial peptides, the mechanism by which they ultimately exert their microbicidal action is still subject of debate. Some peptides are known to form pores that cause the cell contents to leak whilst others simply disrupt the ionic gradients of the membrane, with the consequent bioenergetic collapse of the cell (reviewed by Zasloff, 2002). An increasing number of peptides have been found to kill bacteria without any detectable initial lysis, thus indicating that they must be impairing critical cellular targets, such as protein or DNA synthesis. Examples of peptides that kill bacteria by arresting bacterial DNA synthesis are PR-39 (Boman *et al.*, 1993) and indolicidin (Subbalakshmi & Sitaram, 1998). Pyrrhocoricin, an inducible antimicrobial peptide from the sap-sucking bug, *Pyrrhocoris apterus*, inhibits the ATPase activity of DnaK and prevents chaperone-assisted protein folding (Otvos *et al.*, 2000; Kragol *et al.*, 2001).

To date, there are no published reports of complete studies directed at ascertaining the mechanism of action of antimicrobial peptides isolated from teleosts.

1.2.4. Epithelial Antimicrobial Peptides

Most of our knowledge about antimicrobial peptides derives from studies carried on peptides isolated from circulating phagocytes, haemolymph or other intracellular fluids. However, epithelial surfaces are the first and critical biological boundary, where most pathogens are effectively eliminated. Recent years have seen an increasing number of reports of antimicrobial peptides from mucosal tissues, and there is evidence that they play a key role in host defence at epithelial surfaces (reviewed by Bevins, 1994; Ouellette, 1999; Ganz, 2002).

Ceratotoxin A is a sex-specific antimicrobial peptide isolated from secretions of the female reproductive accessory glands of the medfly, *Ceratitis capitata* (Marri *et al.*, 1996). Andropin, is a male-specific antimicrobial peptide expressed solely in the cells of the ejaculatory duct and induced by mating but not by infection (Samakovlis *et al.*, 1991). Samakovlis and collaborators (1991) have speculated that this peptide confers protection for semen and the male reproductive tract.

The bovine tracheal mucosa contains an abundant defensin, TAP, which has *in vitro* microbicidal activity against fungus or bacteria, including *Klebsiella pneumoniae* (Diamond *et al.*, 1991). There is evidence that inhibition of antimicrobial peptides of the human respiratory epithelium is connected with the impairment of pulmonary defences against *Pseudomonas aeruginosa*, leading to respiratory infections characteristic of patients with cystic fibrosis (reviewed by Hancock & Lehrer, 1998).

Cryptdins are enteric defensins expressed by the mammalian Paneth cells (reviewed by Ganz & Lehrer, 1998; Ouellette, 1999). The presence of defensins in the gastrointestinal tract may be important in regulating the natural luminal flora, as well as in mucosal defence against microbial exploitation (reviewed by Bevins, 1994). An epithelial β-defensin, LAP, has been found in the lingual epithelium of bovine. Schonwetter and co-workers have demonstrated that LAP transcripts are much more abundant near naturally occurring tongue lesions where acute and chronical inflammation signals may also be detected, sustaining the role of epithelial antimicrobial peptides as components of inflammatory response (Schonwetter *et al.*, 1995).

Simmaco and collaborators (1997; 1998) have inhibited the *de novo* synthesis of skin antimicrobial peptides in frogs, *Rana esculenta*, by skin treatment with a glucocorticoid cream, and then induced mouth infections with *Aeromonas hydrophila*. They observed that, unlike the pre-treated frogs, an untreated frog could effectively eliminate all the bacteria within 15 minutes (Simmaco *et al.*, 1997; Simmaco *et al.*, 1998). These *in vivo* experiments provide further evidence for the involvement of antimicrobial peptides in control of the natural flora. Several peptides of the magainin family are also expressed in cells of the gastric mucosa and intestinal tract of *Xenopus*, perhaps forming the basis of a more general amphibian mucosal system (reviewed by Bevins, 1994).

Non-specific antimicrobial compounds, namely antimicrobial peptides, are likely to be especially important for fish, as the adaptive immune system in these animals is structurally less sophisticated than that of higher vertebrates and can

be seasonally or physiologically suppressed (Section 1.1.3). There are a few reports regarding the bacteriostatic or bactericidal properties of fish mucus (Magarinos *et al.*, 1995; Lemaître *et al.*, 1996). Studies to ascertain the precise nature of the factors responsible for antimicrobial activity on mucosal surfaces of fish have rarely been performed. In particular, few studies have been directed at ascertaining the presence and character of antimicrobial peptides in skin mucus of teleosts. At the onset of this project, only two antimicrobial peptides, pardaxin and pleurocidin (Section 1.2.7), had been isolated from skin secretions of teleosts. Moreover, there were no published reports of antimicrobial peptides from salmonids, albeit these be a group of major commercial importance (Section 1.1.3).

1.2.5. Additional Biological Activities

In addition to their antibacterial activity, many of these peptides also possess antifungal (Skerlavaj *et al.*, 1996; Lugardon *et al.*, 2000; Rollins-Smith *et al.*, 2002), antitumoural (Yamazaki, 1993; Winder *et al.*, 1998; Yang *et al.*, 2002) or antiviral properties (Zerial *et al.*, 1987; Murakami *et al.*, 1991; Lehrer *et al.*, 1993; Robinson *et al.*, 1998; Wachinger *et al.*, 1998; Cole *et al.*, 2002). Several antimicrobial peptides from North American ranid frogs, namely ranatuerins, brevinins, esculentins and ranalexin, have been found to inhibit the growth of *Batrachochytrium dendrobatidis*, the skin fungal pathogen associated with global amphibian declines (Rollins-Smith *et al.*, 2002). Winder and collaborators have shown that expression of cecropin in a human bladder carcinoma derived cell line resulted in reduction or even complete loss of tumourigenicity (Winder *et al.*, 1998). It has also been recently reported that cecropins inhibit replication of human immunodeficiency virus serotype 1 (HIV-1) by direct suppression of HIV-1 gene expression (Wachinger *et al.*, 1998).

It is now well established that the involvement of antimicrobial peptides in immunity is not only limited to their role as microbicidal agents but also to their functions as signalling molecules (reviewed by Salzet, 2002). Some of these peptides are known to induce apoptosis (Risso *et al.*, 1998), stimulate polymorphonuclear leukocytes (Ammar *et al.*, 1998), inhibit protein phosphorylation by protein kinase C (see Salzet, 2002 for review) or repress activation of the classical complement pathway (Berg *et al.*, 1998). These modulatory roles for antimicrobial peptides indicate that the role of antimicrobial

peptides in immunity is much wider than initially expected and is not only confined to innate immune responses.

1.2.6. *In vivo* Role in Immunity

Numerous *in vitro* and *in vivo* studies have shown that the expression of antimicrobial peptides is induced by antigenic or environmental challenge (Schonwetter *et al.*, 1995; Sugiyama *et al.*, 1995; Lemaître *et al.*, 1997; Hiratsuka *et al.*, 1998; Liu *et al.*, 1998; Welling *et al.*, 1998; Bals *et al.*, 1999; Destoumieux *et al.*, 2000; Matutte *et al.*, 2000; Cho *et al.*, 2002; Hoffmann & Reichhart, 2002), thus providing circumstantial evidence that antimicrobial peptides are important molecular effectors of innate immunity, contributing to the homeostatic control of the normal flora (reviewed by Boman, 2000) and conferring protection against potential pathogens (reviewed by Zasloff, 2002). This hypothesis has been recently confirmed by a series of experiments performed in insects, amphibian and mammals.

Two recent reports have clearly demonstrated the irrefutable importance of antimicrobial peptides in host defence of *Drosophila* (Tzou *et al.*, 2002) and mice (Nizet *et al.*, 2001). It has been previously reported that *Drosophila* mutants with deficient *Toll* and *imd* pathways do not express any known antimicrobial peptides and are extremely susceptible to bacterial or fungal infections (Lemaître *et al.*, 1996). Tzou and collaborators (2002) have clearly demonstrated that this immunodeficiency in *Toll/ imd* mutant flies can be overcome by the expression of a single antimicrobial peptide, which is sufficient to restore a wild-type level of protection against infection.

The study by Nizet *et al.* (2001) has shown, using transgenic mice with a null mutation for the *Cnlp* gene that codes for the mouse cathelicidin CRAMP, that cathelicidins are important innate defence factors and confer protection against necrotic skin infections caused by Group A *Streptococcus*.

The *in vivo* role of teleost antimicrobial peptides in immunity is yet to be investigated, even though they are known to be crucial components of the innate immune system of other animals.

1.2.7. Antimicrobial Peptides from Teleosts

Despite the vast number of living teleost species, few antimicrobial peptides have been isolated from these animals. Antimicrobial peptides are likely to be especially important epithelial defence factors for aquatic animals because these surfaces are in constant contact with an environment crowded with a wide variety of potential pathogens. Pardaxins were the first antimicrobial peptides to be purified from a teleost. Originally isolated from skin secretions of the Red Sea Moses sole, *Pardachirus marmoratus*, (Lazarovici *et al.*, 1986; Shai *et al.*, 1988) and Western Pacific peacock sole, *Pardachirus pavoninus*, (Thompson *et al.*, 1986) on the basis of their pore-forming and cytotoxic properties, these 33-residue peptides were later acknowledged to have antibacterial properties (Oren & Shai, 1996). Pardaxins have a helix-hinge-helix structure (Zagorski *et al.*, 1991) and are thought to permeabilise cell membranes by a "barrel-stave" mechanism (Shai *et al.*, 1990).

Following the finding of pardaxins, Cole and collaborators reported the finding of pleurocidin, a 25-amino acid peptide with broad-spectrum antibacterial activity present in the skin mucus of the Winter flounder, *Pleuronectes americanus* (Cole *et al.*, 1997). This linear, α-helical peptide with structural similarity to ceratotoxin is constitutively expressed by epidermal mucous gland cells and intestinal goblet cells (Cole *et al.*, 1997; Cole *et al.*, 2000).

The skin of the catfish, *Parasilurus asotus*, has been found to contain parasin I, a potent 19-residue antimicrobial peptide (Park *et al.*, 1998b). Similarly to

pleurocidin, this peptide is predicted to form an amphipathic α-helix and is active against Gram-(+) or Gram-(-) bacteria at micromolar concentrations (Park *et al.*, 1998b). Furthermore, it has recently been shown to derive from cytoplasmic, unacetylated histone H2A present in epithelial mucus cells of the catfish via proteolytic cleavage by cathepsin D (Cho *et al.*, 2002).

Piscidins are 22-amino acid antimicrobial peptides purified from the hybrid striped bass, *Morone chrysops* x *M. saxatilis* (Silphaduang & Noga, 2001). These linear α-helical peptides display broad-spectrum antibacterial activity and significant haemolytic activity against human erythrocytes (Silphaduang & Noga, 2001). Piscidins are present in gill mast cells, suggesting that these cells may participate directly in microbial killing (Silphaduang & Noga, 2001).

Two isoforms of novel, 22-residue, C-amidated antimicrobial peptides have been isolated this year from the skin and gill of immunologically challenged hybrid striped bass (Lauth *et al.*, 2002). These broad-spectrum antimicrobial peptides also have an amphipathic α-helical structure in solution, as confirmed by circular dichroism analysis (Lauth *et al.*, 2002).

Apart from a 21 kDa antibacterial protein isolated from the liver of Atlantic salmon, *Salmo salar* (Richards *et al.*, 2001) and HSDF-1, a 26-residue antimicrobial peptide purified from the serum and mucus of immunostimulated coho salmon, *Oncorhynchus kisutch* (Patrzykat *et al.*, 2001), there are no other published reports of antimicrobial peptides from salmonids, in spite of the commercial importance of this group in aquaculture (Section 1.1.3).

As far as I know, there are no published reports of antimicrobial peptides isolated from peripheral blood cells of salmonids. Moreover, the potential changes in expression of antimicrobial peptides under conditions where the adaptive immune response is suppressed, namely at low environmental temperatures and during the reproductive cycle, have never been reported.

1.2.8. Application as Alternative Antibiotics in Human Clinical Medicine and Aquaculture

The widespread use of antibiotics has resulted in the emergence of an increasing number of multi-drug resistant bacteria, particularly methicillin-resistant *Staphylococcus aureus*, MRSA (Hiramatsu *et al.*, 2001). This serious issue has greatly stimulated research in the development of antimicrobial peptides as pharmaceuticals. Besides being potent antibiotics, antimicrobial peptides also display other biological activities (Section 1.2.5), such as wound healing, stimulation of monocyte chemotaxis and antiendotoxin activity (Jacob & Zasloff, 1994). These properties make antimicrobial peptides attractive candidates as alternative antibiotics. *De novo* analogues of natural antimicrobial peptides have been developed in order to obtain potent, stable, non-cytotoxic, salt-resistant peptides that might be appropriate for use in human clinical medicine (Javadpour *et al.*, 1996; Friedrich *et al.*, 1999). Importantly, and unlike conventional antibiotics, acquisition of resistance by sensitive bacterial strains is unlikely (Zasloff, 2002). Moreover, attempts to induce resistance in several bacterial species through repeated exposure to subinhibitory concentrations of pexiganan, a magainin analogue, (Ge *et al.*, 1999a; Ge *et al.*, 1999b) or chemical mutagenesis were unsuccessful (Zasloff, 2002).

The antimicrobial peptide derivatives used in clinical medicine have been developed as topical agents. Pexiganan, for instance, entered clinical trials for the treatment of polymicrobial foot-ulcer in diabetes patients (Ge *et al.*, 1999a), although it did not obtain approval by the United States Food and Drug

Administration (FDA) in 1999. The antitumoural activity of some antimicrobial peptides raises the possibility of their use for antitumoural therapy (Winder *et al.*, 1998; Williams *et al.*, 2001). In fact, squalamine, an aminoesterol from the dogfish shark, *Squalus acanthias*, (Moore *et al.*, 1993) has already entered phase I clinical trials as an antiangiogenic agent (Bhargava *et al.*, 2001).

Antibiotic resistance is also a serious problem in both wild and farmed fish (Miranda & Zemelman, 2001). For farmed fish, vaccination has become standard to control or prevent disease, albeit with limited success. Only a limited range of vaccines is commercially available, they are often ineffective and must be administered at the right time of year if protection is to be successful. A classic example of the ineffectiveness of prophylactic immunisation is provided by the numerous unsuccessful vaccines developed to prevent bacterial kidney disease (Kaattari & Piganelli, 1997). Fish mortalities are particularly common during the winter, probably due to outbreaks of infection propitiated by immune incompetence of the adaptive system (Manning & Nakanishi, 1996). There is also a considerable amount of anecdotal information, mainly from fish farms, that fish after spawning tend to suffer greater incidence of disease than non-reproducing fish. Thus, there is an urgent need to find new ways of potentiating other aspects of host defence in fish, especially juveniles, in order to limit losses due to disease. Several antimicrobial peptides have been found to display potent *in vitro* antibacterial, antifungal or antiparasitic activity against fish pathogens, such as *Aeromonas salmonicida*, *Cytophaga aquatilis*, *Leucothrix mucor*, *Listonella anguillarum*, *Vibrio salmonicida*, *Yersinia ruckeri*, *Amyloodinium ocellatum* and *Saproglenia* sp. (Cole *et al.*, 1997; Robinette *et al.*, 1998; Kjuul *et*

al., 1999; Noga *et al.*, 2001). Moreover, Jia *et al.* (2000) have shown that continuous delivery of an antimicrobial peptide (cecropin-melittin hybrid or pleurocidin amide) by miniosmotic pumps placed in the intraperitoneal cavity of *O. kisutch* protects the fish against *L. anguillarum* infection. Clearly, antimicrobial peptides have great potential as alternative, natural antibiotics for use in aquaculture and yet their application in fish farming has never been evaluated.

1.3. Lysozyme

Lysozyme is a humoral enzyme that is capable of hydrolysing β(1→4) linkages between N-acetylmuramic acid and N-acetylglucosamine, the two components of the glycan backbone in murein. The ability of lysozyme to destroy bacterial cell walls, and its connection to the complement system, makes it an important defensive factor against invasion by prokaryotes. Since its discovery by Alexander Fleming in 1921, lysozyme has been extensively studied with respect to its action, structure and occurrence in different organisms (reviewed by Jollès, 1996). Comparative studies have shown that animals express multiple forms of lysozyme that exhibit marked differences in primary structure and substrate specificity (Jollès, 1996; Matsumura & Kirsch, 1996; Prager & Jollès, 1996; Ito *et al.*, 1999). These various lysozymes have been grouped in three families: chicken-type (c-type), goose-type (g-type) and invertebrate-type (i-type) lysozymes (Jollès & Jollès, 1975; Jollès, 1996; Prager & Jollès, 1996; Ito *et al.*, 1999; Nilsen *et al.*, 1999; Nilsen & Myrnes, 2001; Bachali *et al.*, 2002).

Recent studies have further started to show that lysozyme may augment the activity of antimicrobial peptides through a synergistic mechanism (Chalk *et al.*, 1994; Patrzykat *et al.*, 2001), and that, in addition to enzymatic bacteriolytic activity, some lysozymes have independent non-enzymatic bactericidal domains (Düring *et al.*, 1999).

Muramidase activity has been detected in several fish species, distributed especially in tissues rich in leukocytes or at sites where infection risk is high,

such as the mucosal surfaces and the eggs (reviewed by Yano, 1996). Two c-type lysozymes, differing by just one amino acid (aspartate/ alanine) at position 86, have been purified from the kidney of *O. mykiss* and characterised (Grinde *et al.*, 1988; Dautigny *et al.*, 1991). These lysozymes were active not only against Gram-(+) bacteria, but also Gram-(-) bacteria in the absence of complement (Grinde, 1989). Skin mucus of *O. mykiss* has also been found to display muramidase activity (Hjelmeland *et al.*, 1983), but the exact nature of the proteins responsible for this lysozyme-like activity has never been ascertained. Indeed, there are no published reports of lysozymes purified from skin secretions of a salmonid, despite the known importance of lysozyme in mucosal immunity.

1.4. Aims

The rainbow trout, *Oncorhynchus mykiss*, is a member of the *Salmonidae* family, which includes salmon and char (Fig. 1.6). Originally native from North America, rainbow trout has been successfully introduced to many regions worldwide and it is now the most important trout species in aquaculture. Rainbow trout is carnivorous and requires cold fresh water to spawn, even though it can grow in brackish or even sea water.

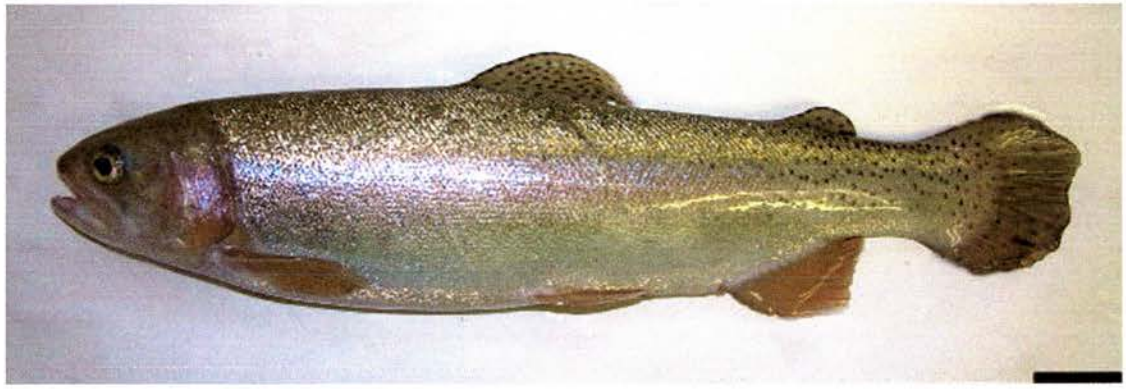


Figure 1.6. Rainbow trout, *Oncorhynchus mykiss*. Rainbow trout have the typical streamlined salmonid shape but their coloration can vary dramatically depending on the habitat, age, sex and reproductive status. Scale bar equals 5 cm.

As fish have greater reliance on their innate defences and their living skin is one of the first sites of contact with potential pathogens, it is reasonable to expect that the skin epithelium expresses one or more antimicrobial peptides and/ or proteins. Hence, the present investigation was conducted in order to examine the

presence, character, expression and biological significance of antimicrobial peptides and antibacterial proteins in skin secretions of this commercially important salmonid, the rainbow trout. As lysozyme is an important bacteriolytic enzyme with the ability to potentiate the activity of antimicrobial peptides, studies were directed at characterising this enzyme from skin exudates of rainbow trout. Specific aims include:

i) Investigation of the antibacterial properties of skin mucus;

ii) Isolation the antimicrobial peptide(s) responsible for antibacterial activity;

iii) Biochemical characterisation of the purified antimicrobial peptide(s);

iv) Ascertaining the spectrum of activity and haemolytic properties of the skin antimicrobial peptide(s);

v) Analysis of the mode of action of the purified antimicrobial peptide(s);

vi) Isolation of the protein(s) with muramidase activity from skin exudates;

vi) Enzymatic and biochemical characterisation of the lysozyme-like protein(s) purified from skin mucus and

vii) Characterisation of the antibacterial or haemolytic activities of the purified muramidases.

Chapter 2

Isolation of Oncorhyncin I, a 3 kDa Antimicrobial Peptide Present in Skin Secretions of Rainbow Trout, *Oncorhynchus mykiss*

2.1. Synopsis

Antimicrobial peptides are evolutionarily ancient defence factors widespread throughout the animal Kingdom. Their strategic location in phagocytes or mucosal tissues, allied to their powerful broad-spectrum antimicrobial activity, makes them crucial components of the innate immune system. They have been extensively studied in mammals, amphibians and invertebrates, but have received only scant attention in teleosts. The aim of the work described in the present chapter was to screen for antimicrobial peptides potentially present in skin secretions of rainbow trout, *Oncorhynchus mykiss*. *In vitro* analyses demonstrated that mucus extracts from adult naïve fish exhibited antibacterial activity against the Gram-(+) bacterium *Planococcus citreus*. Fractionation of proteins by ion exchange chromatography and RP-HPLC revealed that at least one antibacterial peptide is constitutively expressed in trout skin. This cationic peptide, provisionally named oncorhyncin, has an apparent molecular weight of approximately 3 kDa, as estimated by SDS-PAGE. Its partial N-terminal sequence was found to be Ser-Lys-Gly-(Gly/Lys)-Lys-Ala-Asn-Lys-(Asp/Thr)-Val-Glu-Leu-Ala-Arg-Gly. Similarity searches revealed that this sequence bears no significant similarity to any known protein. Moreover, oncorhyncin is predicted to form an amphipathic α-helical conformation, in common with many other antimicrobial peptides. Oncorhyncin is a novel antimicrobial peptide present in the skin mucosa, where it may play a first-line role in innate host defence.

2.2. Introduction

It is currently well established that teleosts rely heavily on their innate immune response for protection against microbial exploitation (reviewed by Yano, 1996), especially under certain environmental conditions that suppress their adaptive immunity (Bly & Clem, 1992; Tatner, 1996). The innate humoral defence factors of teleosts comprise, amongst others, two types of lysozyme, C-reactive protein, transferrin, lectins, proteinases, α_2-macroglobulin, chitinase and antimicrobial peptides (reviewed by Yano, 1996). Their value in innate host defence lies in their broad spectrum of activity against microorganisms, their low toxicity for eucaryotic cells, their ease of synthesis and rapid diffusion rates to sites of wounding or infection (Boman, 1995).

To date the presence of antimicrobial peptides on mucosal surfaces of rainbow trout has not been demonstrated. The present chapter describes the preliminary study undertaken to screen for antimicrobial peptides constitutively expressed in skin secretions from rainbow trout, *Oncorhynchus mykiss*.

2.3. Experimental Procedures

Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are aqueous and prepared with deionized water (Elga), except when stated otherwise. The list of suppliers and respective addresses is presented in appendix B.

2.3.1. Animals

Female rainbow trout, weighing 400 to 500 g, were purchased from College Mill Trout Farm. They were maintained in flow-through freshwater tanks (10 ± 2 °C) and fed daily *ad libitum* with commercial fish pellets Dynamic Red M (Ewos).

2.3.2. Sample Collection and Preparation of Epidermal Extracts

Fish were sacrificed by immersion in a solution containing 0.6 $g \cdot l^{-1}$ MS222, neutralised with sodium bicarbonate (0.6 $g \cdot l^{-1}$ final concentration), until the opercula movements ceased and no reflexes could be observed.

Mucous skin secretions and associated epidermal cells were collected by scraping the dorso-lateral surfaces of 10 freshly sacrificed fish and sluicing with sterile deionized water (approximately 140 ml per fish) containing 0.7 % (v/v) reconstituted general-use protease inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride, sodium EDTA, leupeptin, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane, bestatin and aprotinin). The pooled, diluted secretions were stirred on ice for 1 h before centrifuging at 11,000 x g for 30 min at 4 °C to remove scales and other insoluble material. The pellet was resuspended in 250 ml of sterile deionized water and spun again at 11 000 x g (30 min, 4 °C). The resulting supernatants were lyophilised, yielding *circa* 7 g of dried material from 25 fish. The lyophilised material was resuspended in 20 mM MES, pH 6.4,

constituting the water-soluble extract. The pellet remaining after the second centrifugation was extracted in 150 ml of 0.3 % (v/v) Triton X-100 at 5 °C for 2 h with stirring and finally re-centrifuged at 4,000 x g for 30 min at 4 °C. The supernatant remaining after this treatment was designated the detergent-soluble extract.

2.3.3. Test Bacteria

The Gram-(-) bacterium, *Psychrobacter immobilis* (NCIMB 308), and the Gram-(+) bacterium, *Planococcus citreus* (NCIMB 1493), were used as test bacteria. Members of the genus *Planococcus* are believed to be pathogenic for freshwater fish (Austin & Stobie, 1992). They were grown at 20 °C to logarithmic phase, harvested, washed as described in Schnapp *et al.* (1996) and finally resuspended in 3.2 % (w/v) NaCl at a final concentration of approximately 10^8 cfu·ml^{-1}.

2.3.4. Antibacterial Assay

Antibacterial activity was assessed by a modification of the two-layer radial diffusion method of Lehrer *et al.* (1991). Briefly, a 14 ml bacterial underlay of 1 % (w/v) agarose in 1/10 strength 2216E broth (Difco) supplemented with 0.02 % (v/v) Tween 20 and seeded with $2 \cdot 10^6$ washed bacteria were prepared in 144 cm^2 square Petri dishes. Three microlitres of the test sample were pipetted into 2 mm diameter wells punched in the agarose with a plastic Pasteur pipette. Positive controls comprised 3 µl of 50 µg·ml^{-1} penicillin G. Negative controls comprised 3 µl of buffer only. Each plate was incubated at 4 °C for 3 h and then overlaid with 14 ml of sterile 1 % (w/v) agarose containing double strength

2216E broth before further incubation for 12-24 h at 20 °C. Clear zones in the agar underlays indicate antibacterial activity. Their diameters were measured and activities were expressed as clear zone area (in mm^2) minus the area of the well.

2.3.5. Muramidase Assay

Muramidase activity was determined by radial diffusion assay (Lehrer *et al.*, 1991b) using a 1% (w/v) agarose plate buffered with 40 mM sodium phosphate, pH 6.2, containing 0.33 $mg \cdot ml^{-1}$ (final concentration) lyophilised *Micrococcus luteus* cell walls. Wells of 2 mm diameter were punched in the agarose with a plastic Pasteur pipette and 3 µl sample were applied in the wells. Hen egg white lysozyme (50 $\mu g \cdot ml^{-1}$) was used as standard. Clear zones were measured following incubation for 3 h at 37 °C and muramidase activity was represented as clear zone area (in mm^2) minus the area of the well.

2.3.6. Electrophoresis

Protein profiles were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) employing the Tris-Tricine system described by Schägger and von Jagow (1987), with a 16 % separating gel, 14 % spacer gel and 5 % stacking gel (please see Appendix C for details). Low molecular weight markers (Promega) were used. The gels were stained with the Bio-Rad silver staining kit.

2.3.7. Purification of a 3 kDa Antibacterial Peptide

Fifty millilitres of the detergent-soluble mucus extract were dialysed against 20 mM MES, pH 6.4, at 16 °C using cellulose benzoylated tubing (nominal

molecular weight cut off, NMWCO, 2 kDa) and then subjected to cation exchange on a CM-Sepharose 1 cm x 10 cm Econo-column (Bio-Rad) equilibrated in the same buffer. After a 20 min wash with 20 mM MES, pH 6.4, bound proteins were eluted at a flow rate of 1.0 $ml \cdot min^{-1}$ with a 20 mM MES, pH 6.4 (buffer A)/ 20 mM MES, 1M NaCl, pH 6.4 (buffer B) gradient as follows: 0 to 50 % B over 50 min, followed by 50 % B for 15 min and finally from 50 % to 100 % B in 15 min. Fractions of 1 ml were collected, lyophilised, reconstituted in 100 µl water and assayed for antimicrobial activity against *P. citreus* as above. Cation exchange fractions displaying antimicrobial activity were pooled and further purified by RP-HPLC on a 4.6 x 250 mm RSiL C_{18} HL column (Bio-Rad) using a biphasic gradient of 0.1 % trifluoroacetic acid (TFA) in water and 0.1 % TFA in acetonitrile at a flow rate of 1 $ml \cdot min^{-1}$, as depicted in Fig. 2.2. Fractions of 1 ml were collected, lyophilised, reconstituted in 50 µl sterile deionized water and assayed against P. *citreus* or *M. luteus* cell walls as described beforehand.

At each step, purity of samples and estimation of the molecular weight of the peptides was determined by high resolution SDS-PAGE as detailed above.

2.3.8. Protein Quantification

Total protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin (Pierce) as standard.

2.3.9. Partial Primary Structure Determination

N-terminal amino acid sequencing was performed by standard automated Edman degradation on a Procise Sequencer (Applied BioSystems) at the Centre for Biomolecular Sciences (University of St Andrews, UK).

2.3.10.Sequence Analyses

Homology searches were performed against the Swiss-Prot, NR and Month databases with the Basic Local Alignment Search Tool (Altschul *et al.*, 1990), BLAST, provided by the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). Amino acid composition, protein mass and isoelectric point were predicted by the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/). Schiffer-Edmundson helical wheel modelling was executed with the Antheprot sequence analysis software (Institute of Biology and Chemistry of Proteins, Lyon, France).

2.3.11.Thermal Stability Assay

Heat stability of the antibacterial activity against *P. citreus* was tested by comparing clear zone areas produced by samples incubated for 10 min at 50 °C, 65 °C or 100 °C. Samples were also tested for activity against *P. citreus* after 7 days storage at -20 °C. Each experiment was performed in triplicate.

2.3.12.Proteolytic Digestion

Antibacterial activity of the purified peptide was assessed by radial diffusion assay as above, following ten-fold concentration on a 3 kDa cut-off ultrafiltration

device (Microcon, Millipore) and digestion with 60 $\mu g \cdot ml^{-1}$ (final concentration) proteinase K for 60 min at 37 °C. The assay was done in triplicate.

2.4. Results

2.4.1. Antibacterial Properties of Trout Skin Secretions

Aqueous mucus extracts from unstimulated rainbow trout were tested in triplicate against the Gram-(+) bacterium *P. citreus*, Gram-(-) bacterium *P. immobilis* and also against *M. luteus* cell walls. At a total protein concentration of 6.5 $mg \cdot ml^{-1}$, the mucus extracts were found to display *in vitro* antibacterial activity against *P. citreus* with an average clear zone area of 32 ± 2 mm^2. No activity was noted against *P. immobilis* at the concentrations tested. Muramidase activity was observed against lyophilised *M. luteus* cell walls, with an average clear zone area of 117 ± 7 mm^2, indicating the presence of lysozyme or proteins with lysozyme-like activity in trout skin secretions.

Antibacterial activity of mucus aqueous extracts against *P. citreus* was thermosensitive, showing a reduction of *circa* 55 % after heating to 50 °C (Table 2.1). Heating to 65 °C diminished antibacterial activity to a level close to the limit of detection and boiling for 10 min completely abolished antibacterial properties of skin mucus (Table 2.1). Activity was cryostable to freezing at -20 °C for 7 days (Table 2.1).

Table 2.1. Stability of antibacterial activity in rainbow trout skin mucus. Antibacterial activity against *P. citreus* of skin mucus samples (total protein concentration of 2.0 $mg \cdot ml^{-1}$) incubated at different temperatures is represented as the clear zone area (A) in mm^2. Data are denoted as mean ± SE, n=3.

	Untreated	50 °C 10 min	65 °C 10 min	100 °C 10 min	-20 °C 7 days
A (mm^2)	22 ± 8	10 ± 2	2 ± 2	0	22 ± 10

2.4.2. Purification of a 3 kDa Antimicrobial Peptide

Fractionation of the detergent-soluble epidermal extract by cation exchange chromatography yielded several groups of fractions displaying antimicrobial activity against *P. citreus* (Fig. 2.1). Antibacterial and muramidase assays showed that fractions eluting between 120 mM and 280 mM NaCl possessed antibacterial activity against *P. citreus* but not *M. luteus* cell walls, indicating that these samples did not have muramidase activity (Fig. 2.1).

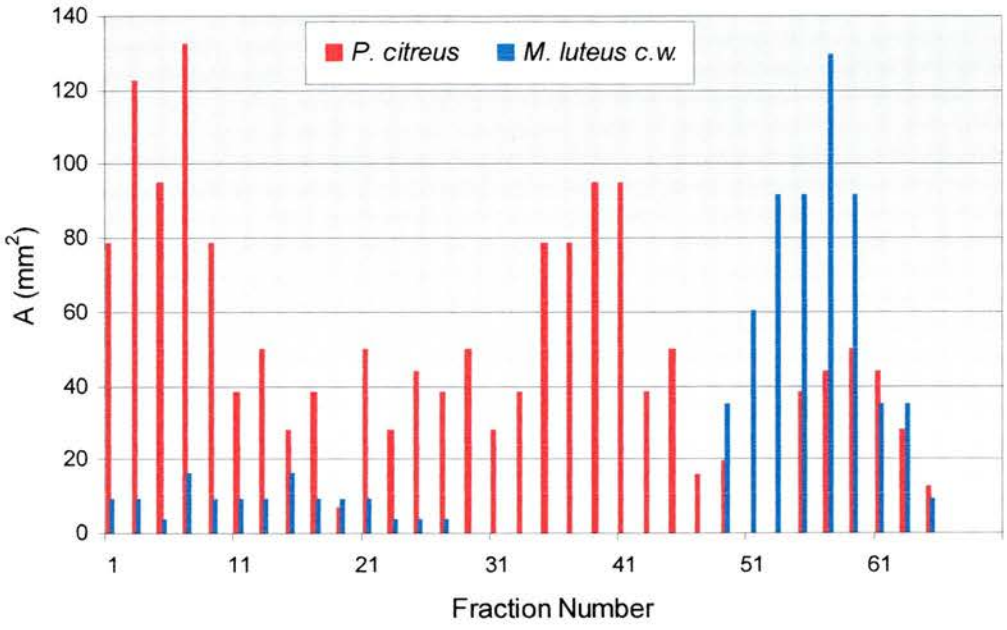


Figure 2.1. Activity profile of the cation exchange chromatography fractions. The detergent-soluble epidermal extract was subjected to cation exchange chromatography on CM-Sepharose, pH 6.4, and the resulting fractions assayed for antibacterial activity against *P. citreus* and muramidase activity against *M. luteus* cell walls. Fractions 29 to 47 displayed antibacterial but not muramidase activity. The histogram does not include fractions 71 to 120, as these were devoid of activity.

Fractions without muramidase activity were pooled and further resolved by C_{18} RP-HPLC, yielding a single fraction (number 47) that exhibited antibacterial activity against *P. citreus* (Fig 2.2). Treatment of fraction 47 with proteinase K completely abolished its antibacterial activity (Fig. 2.3). SDS-PAGE analyses showed that this fraction contained a single protein with an apparent molecular weight of approximately 3 kDa (Fig 2.4).

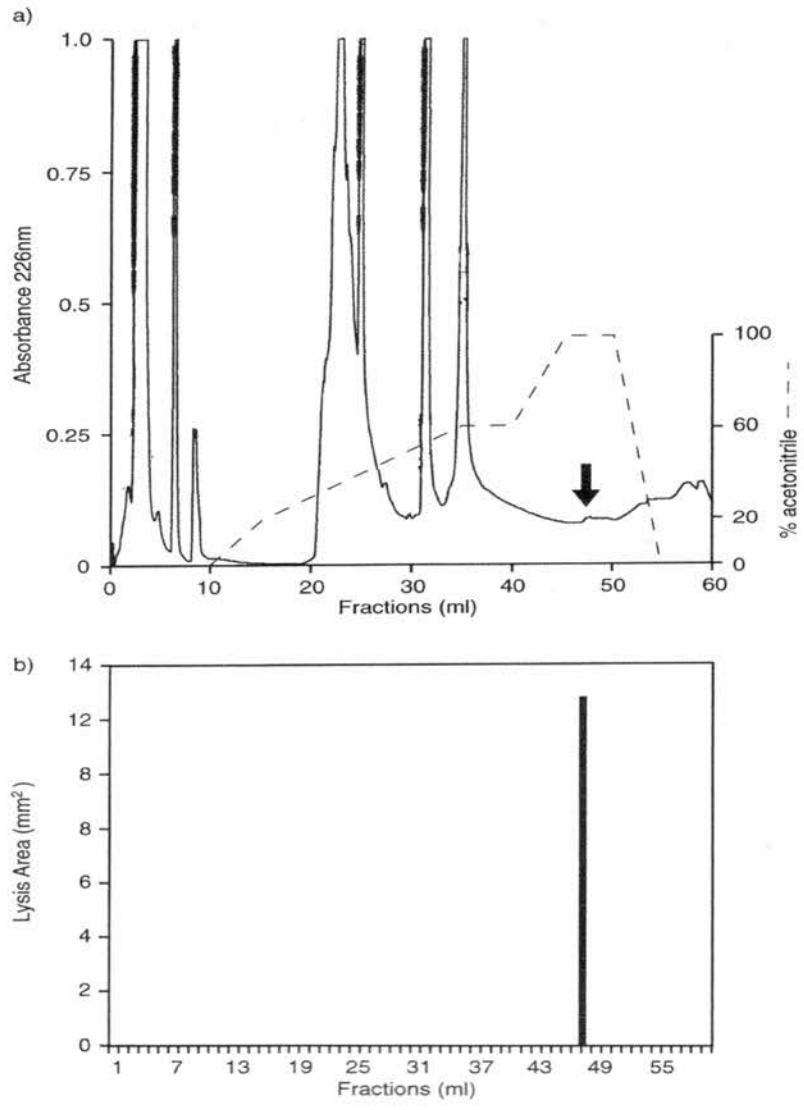


Figure 2.2. Purification of an antimicrobial peptide from skin secretions of rainbow trout by C_{18} RP-HPLC. (a) C_{18} RP-HPLC of the active fractions after partial purification by cation exchange chromatography on CM-Sepharose column. Active fractions eluted with 120 to 280 mM NaCl were separated on a RSiL C_{18} HL column using a biphasic water/ acetonitrile gradient in the presence of 0.1 % TFA (dashed line). Absorbance was monitored at 226 nm (solid line). The point of elution of the antimicrobial peptide is indicated by a bold arrow (b) Activity profile against P. *citreus* of RP-HPLC fractions, after lyophilisation and reconstitution in 50 µl of sterile deionized water. Antimicrobial activity is present in fraction 47.

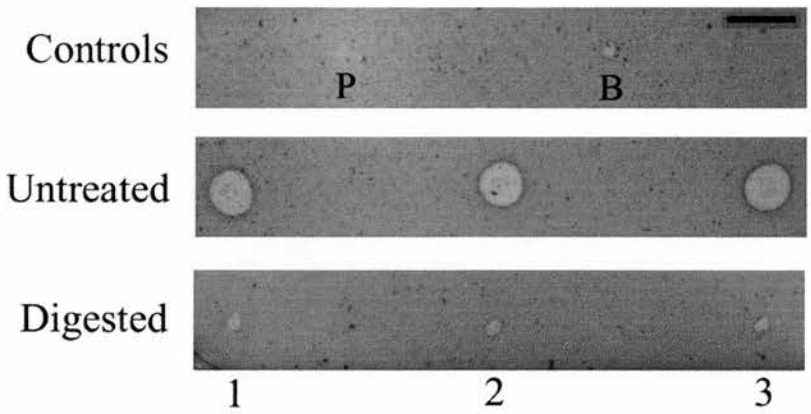


Figure 2.3. Proteinase K digestion assay. Radial diffusion assay of the purified antimicrobial peptide against *P. citreus*, before and after proteolytic digestion with 60 $\mu g \cdot ml^{-1}$ proteinase K for 60 min at 37 °C. The experiment was performed in triplicate (columns 1, 2 and 3). Negative controls comprised (P) 60 $\mu g \cdot ml^{-1}$ proteinase K or (B) buffer. Scale bar = 1 cm.

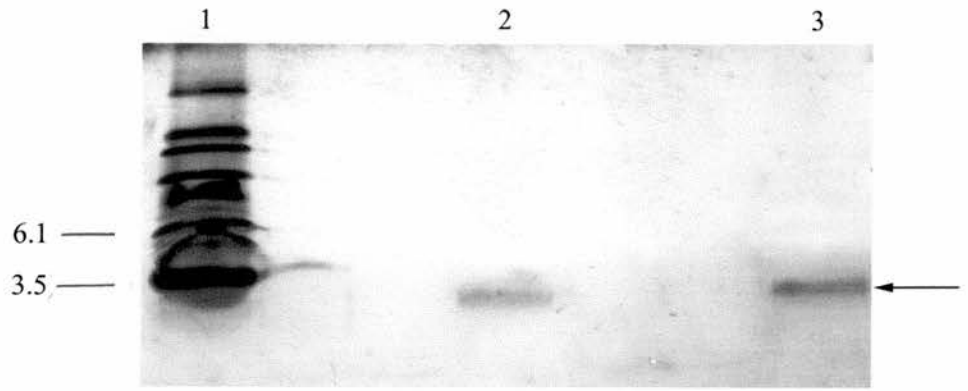


Figure 2.4. SDS-PAGE profile of the active C_{18} RP-HPLC fraction from the detergent-soluble fraction of skin mucus. After silver-staining a band with an apparent molecular weight of *circa* 3 kDa can be observed in the lanes containing fraction 47 (i.e., lanes 2 and 3). Lane 1 contains 2 µl of low-range markers. The peptide of interest is indicated by an arrow. The numbers on the left-hand side represent the molecular mass of the markers in kDa.

2.4.3. Structural characterisation of the Purified 3 kDa Antimicrobial Peptide

The partial amino acid sequence[#] of the isolated 3 kDa antimicrobial peptide, determined by automated Edman degradation, is Ser-Lys-Gly-(Gly/Lys)-Lys-Ala-Asn-Lys-(Asp/Thr)-Val-Glu-Leu-Ala-Arg-Gly. BLAST searches revealed no significant similarity to any known protein listed on the Swiss-Prot, NR or Month databases. Schiffer-Edmundson helical wheel modelling shows that oncorhyncin may adopt an amphipathic α-helical conformation (Fig 2.5).

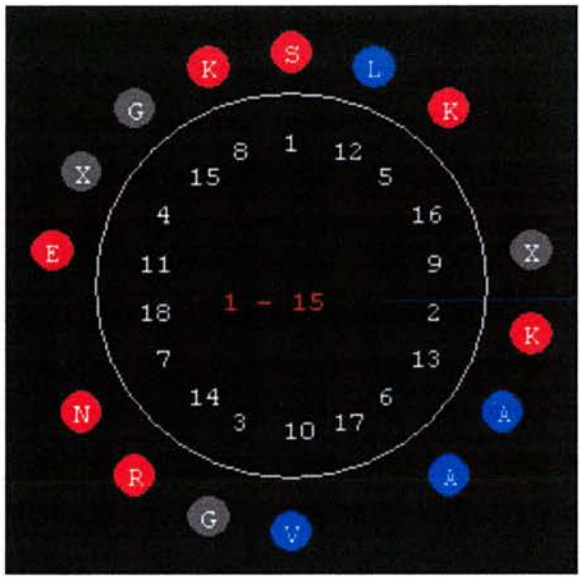


Figure 2.5. Schiffer-Edmundson helical wheel projection showing the probable amphipathic structure of the 3 kDa antimicrobial peptide. Red and blue circles indicate hydrophilic and hydrophobic residues, respectively. Glycine and unknown amino acids are represented by a grey circle. Residue numbers starting from the amino terminus are shown.

[#] The amino acid sequence data herein reported have been submitted to the Swiss-Prot database under the accession number P83287.

2.5. Discussion

This chapter demonstrates that skin secretions of rainbow trout, *O. mykiss*, display antibacterial activity against the Gram-(+) *P. citreus* but do not affect the growth of the Gram-(-) bacterium *P. immobilis*. Attempts to isolate the proteinaceous factors responsible for the antibacterial properties from the water-soluble fraction were unsuccessful. Nevertheless, a cationic peptide with an apparent molecular weight of *circa* 3.0 kDa was purified from the detergent-soluble mucus extract by a combination of cation exchange chromatography and C_{18} RP-HPLC. Proteolytic digestion of the purified peptide with proteinase K completely abolished its antibacterial activity, thus showing that the activity is indeed due to a proteinaceous factor. This antimicrobial peptide was tentatively designated oncorhyncin, after the genus of rainbow trout, *Oncorhynchus*.

Oncorhyncin exhibited antibacterial activity against *P. citreus* but was not active against *P. immobilis* and did not display detectable muramidase activity. Its primary structure has no significant similarity to any known protein. Schiffer-Edmundson helical wheel modelling predicted that oncorhyncin may adopt an amphipathic α-helical conformation, in common with other antimicrobial peptides (Park *et al.*, 1996; Cole *et al.*, 1997; Park *et al.*, 1998). The hydrophilic surface has a cationic nature, a feature that might allow this peptide to interact with the negatively charged phospholipids of the bacterial membranes (Zasloff, 2002). The limited amount of material obtained during the present study did not permit further structural and functional characterisation of oncorhyncin. The yield of purified peptide varied considerably between independent experiments,

suggesting that expression of this peptide may be seasonal or may require antigenic stimulation.

Despite the large number of antimicrobial peptides purified from varied animal sources (Zasloff, 2002), relatively few have been isolated from epithelial surfaces of aquatic animals. Pleurocidin was one of the first antimicrobial peptides isolated from a teleost. It is a 25-amino acid peptide with a broad-spectrum of activity and is expressed by the mucous cells of flounder skin (Cole *et al.*, 1997; Cole *et al.*, 2000). In common with oncorhyncin, it represents a novel antimicrobial peptide that is predicted to form an amphipathic α-helical structure (Cole *et al.*, 1997).

To the best of my knowledge, this is the first report of an antimicrobial peptide isolated from skin secretions rainbow trout. Inasmuch as oncorhyncin inhibits the growth of a bacterial strain associated with a pathogenic condition (Austin & Stobie, 1992) it may play a potential role in mucosal immunity of *O. mykiss*.

Chapter 3

Antimicrobial Properties of Histone H2A from Skin Secretions of *Oncorhynchus mykiss*

3.1. Synopsis

A 13.6 kDa antimicrobial protein was purified to homogeneity by solid phase extraction, cation exchange and C_{18} reversed phase chromatography of acid-extracted skin secretions from rainbow trout. The purified protein exhibited potent antibacterial activity against Gram-(+) bacteria, with minimal inhibitory concentrations in the submicromolar range. Kinetic analysis revealed that at a concentration of 0.3 μM all test bacteria lost viability after only 30 min incubation. Some activity was also observed against the yeast, *Saccharomyces cerevisiae*, although the antifungal effects are less potent. The protein is inhibited by NaCl and has no haemolytic activity towards trout erythrocytes at concentrations below 0.3 μM. Partial internal sequencing, combined with matrix-assisted laser desorption ionization mass spectrometry, peptide mass fingerprinting and amino acid analysis revealed that the protein appears to be histone H2A, acetylated at the N-terminus. Thus, in addition to its classical function in the cell, this histone may play a role in innate host defence, either by active secretion or by passive release following infection-induced cell lysis or apoptosis.

3.2. Introduction

The last few years have seen a great burgeoning of reports of the occurrence and characterization of low molecular weight antimicrobial peptides from a wide variety of organisms (Zasloff, 2002). These molecules have attracted much research interest for their biochemical diversity, broad specificity against bacteria and/or fungi (Zasloff, 2002) and also because some have antiviral (Wachinger *et al.*, 1998), antitumoural (Winder *et al.*, 1998) or wound healing properties (Murphy *et al.*, 1993).

Certainly, the Teleostei is an important and worthy taxon for further study in this respect, as bony fish have a long evolutionary history and are of great economic importance in aquaculture throughout the world. Moreover, they live in a microbe-rich environment and are vulnerable to invasion by pathogenic or opportunistic microorganisms. In many other animal groups, the surface mucosa is known to contain a battery of non-specific microbicidal proteins to prevent harmful systemic infections that would result if microbes breached this barrier (Bevins, 1994; Simmaco *et al.*, 1998).

So far the number of teleost species studied and the range of proteins recorded remains small and relatively few have been directed at salmonids, one of the chief groups of fish intensively farmed for commercial purposes. In the previous chapter, the purification and partial characterisation of a cationic antimicrobial peptide of approximately 3 kDa was described. The present study was aimed at investigating the presence and character of acid-soluble antimicrobial proteins in skin secretions of rainbow trout, *Oncorhynchus mykiss*.

3.3. Experimental Procedures

Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are aqueous and prepared with deionized water (Elga), except when stated otherwise. The various suppliers and respective addresses are listed in appendix B.

3.3.1. Animals

Adult female rainbow trout (College Mill Trout Farm), weighing 400 to 500 g, were maintained in flow-through freshwater tanks (14 ± 1 °C) and fed daily with an amount of Dynamic Red M trout pellets (Ewos) equivalent to 1 % (w/w) of their body weight.

3.3.2. Effect of Surface Stimulation on the Antibacterial Properties of Skin Mucus Extracts

Three groups of 3 fish were anaesthetised by immersion in a solution containing 0.06 $g \cdot l^{-1}$ MS222 neutralised with sodium bicarbonate (0.06 $g \cdot l^{-1}$ final concentration). The skin surface was then physically stimulated by gentle stroking with fine sand paper. The fish from one of the groups were immediately sacrificed as described in Chapter 2 (Section 2.3.2). The remaining two groups were allowed to recover and euthanized 2 or 4 hours post-stimulation.

One millilitre of skin mucus and associated epidermal cells was collected from each fish and homogenised in 3 ml of a solution containing 50% (v/v) ethanol (Merck), 3.3% (v/v) trifluoroacetic acid (TFA) and 2% (v/v) general-use protease inhibitor cocktail (please consult Section 2.3.2 for its detailed composition). Proteins were extracted by stirring for 60 min at 4 °C and the preparation was

centrifuged at 29 000 x g for 60 min at 4 °C. The resulting supernatant was lyophilised and reconstituted in 1 ml of 20 mM HEPES (Acros), pH 7.0. All samples were normalised to a total protein concentration of 150 $\mu g \cdot ml^{-1}$ by dilution with 20 mM HEPES, pH 7.0, and assayed for antibacterial activity against *Planococcus citreus* as described below.

3.3.3. Sample Collection and Preparation of Epidermal Extracts

Samples of skin mucus and associated epidermal cells were collected by scraping the dorso-lateral surfaces of 10 fish, sacrificed 4 hours after gentle surface stimulation as above. The mucous and cell scrapings were pooled, yielding a total volume of approximately 150 ml and homogenised 1:4 (v/v) in a solution of 50 % (v/v) ethanol, 3.3 % (v/v) TFA and 2 % (v/v) general-use protease inhibitor cocktail (constituents listed in Section 2.3.2). Following extraction by stirring for 60 min at 4 °C, the preparation was centrifuged at 29 000 x g for 60 min at 4 °C and the supernatant lyophilised. The resulting extract was resuspended in 100 ml of 20 mM HEPES and the pH adjusted to 7.0 with 5 M NaOH (BDH) before centrifuging at 29 000 x g for 30 min at 4 °C.

3.3.4. Test Microorganisms

The various strains of microorganisms used in the present study, as well as their original sources, are listed in Table 3.1. The culture conditions used for each microorganism are shown in Table 3.2. Each microorganism was grown to logarithmic phase before washing in sterile saline (*circa* 3.2 % (w/v) NaCl for marine strains, 1.5 % (w/v) for *P. citreus* and 0.8 % (w/v) NaCl for non-marine

strains) and resuspension in sterile saline (please consult Section 2.3.4) or Mueller-Hinton broth (Oxoid) (MHB) as described below in Section 3.3.5.

3.3.5. Antibacterial Assays

Antibacterial activity was assessed using a modification of the two-layer radial diffusion assay of Lehrer *et al.* (1991b) as previously described in Section 2.3.4. Nevertheless the following modifications were introduced: 1) Tween 20 was omitted, as it was found to negatively affect bacterial growth; 2) 0.6 % (w/v) agarose was used, in order to decrease the melting point below 37 °C and thus improve the survival rate of thermosensitive bacteria; 3) LB broth supplemented with NaCl replaced the 2216E marine broth because the latter tends to develop a precipitate during autoclaving, which can impair the visualisation of the clear zones. The Gram-(+) bacterium, *P. citreus*, was used as the test organism throughout the protein purification procedure.

Minimal inhibitory concentration (MIC) assays of the purified protein against each of the bacteria listed in Table 3.1 were performed by microtitre broth dilution (Friedrich *et al.*, 1999). In brief, this entailed diluting the test protein in 0.2 % (w/v) bovine serum albumin, 0.01 % (v/v) acetic acid (BDH) to give a series of eight two-fold dilutions. Volumes of 11 µl of each dilution were added to individual wells of a polypropylene 96-well microtitre plate (Corning Costar). Washed bacteria, grown under the conditions described in Table 3.2, were diluted in MHB to a concentration of 10^5 colony forming units (cfu) per ml. One hundred microlitres of each bacterial suspension were then added to each well in duplicate and the trays were incubated at the appropriate temperature for each bacterium (Table 3.2) until the optical density at 570 nm (read with a Dynex

MRX II plate reader) reached 0.2 in the positive control well, containing bacteria and diluent only. The MIC is defined in this thesis as being the lowest concentration of protein that reduced growth by 50 % compared to the control well. The minimal bactericidal concentration (MBC) was obtained by plating out the contents of each well showing no visible growth. The MBC was taken as the lowest concentration of protein that prevents any residual colony formation after incubation for 24 h at the appropriate temperature. Cecropin P1 (Sigma) was used as reference.

To investigate the influence of NaCl in antibacterial activity of the purified protein, MIC assays were performed using MHB supplemented with NaCl to a final concentration of 1.5 % (w/v) or 3.2 % (w/v).

Table 3.1. List of the microorganisms used for the antibacterial assays. Their original source and identification code are also indicated.

Species	Identification Code	Isolated From
Aerococcus viridans	NCIMB 1120	Moribund lobsters
Aeromonas hydrophila	NCIMB 1134	Mouth lesion of rainbow trout
Aeromonas salmonicida 004	MT 004 [1]	Unknown
Aeromonas salmonicida 849	MT 849 [1]	Diseased salmon
Bacillus subtilis	ATCC 6051	Unknown
Escherichia coli	NCIMB 12210	Unknown
Listonella anguillarum	NCIMB 2129	Farmed rainbow trout
Listonella anguillarum 01	MT 1637 [1]	Unknown
Micrococcus luteus	NCIMB 376	Unknown
Planococcus citreus	NCIMB 1493	Unknown
Renibacterium salmoninarum	NCIMB 1114	Salmonid with BKD
Staphylococcus aureus	NCIMB 6571	Unknown
Saccharomyces cerevisiae	Unknown	Unknown
Yersinia ruckeri	MT 252 [1]	Salmonid with ERM

1 Strains kindly provided by Dr. Tony Ellis (Marine Laboratory, Aberdeen, UK).

2 List of abbreviations used: ATCC, American type culture collection; NCIMB, National collections of industrial, food and marine bacteria; MT, Strain isolated by Dr Mary Tatner (University of Glasgow, U.K.)

Table 3.2. List of culture conditions for the microorganisms used in the antibacterial assays.

Species	Culture Conditions (Medium/Temperature)
Aerococcus viridans	Nutrient (Oxoid)/ 25 °C
Aeromonas hydrophila	Nutrient/ 30 °C
Aeromonas salmonicida	Yeastrel [1]/ 20 °C
Bacillus subtilis	Nutrient/ 30 °C
Escherichia coli	Nutrient/ 37 °C
Listonella anguillarum	Blood base (Difco)/ 20 °C
Listonella anguillarum 01	Blood base/ 20 °C
Micrococcus luteus	Blood base/ 20 °C
Planococcus citreus	Nutrient, 1.5 % NaCl/ 20 °C
Renibacterium salmoninarum	KDM-2 [2]/15 °C
Staphylococcus aureus	Nutrient/ 37 °C
Saccharomyces cerevisiae	Yeast extract [3]/ 37 °C
Yersinia ruckeri	Nutrient/ 25 °C

1 Yeastrel medium: 0.5 % (w/v) Lab-Lemco (Oxoid), 0.7 % (w/v) yeastrel (Natex), 0.95 % (w/v) peptone (Difco) and 0.5 % (w/v) NaCl.

2 KDM-2 medium: 1 % (w/v) peptone, 0.05 % (w/v) yeast extract, 0.1 % (w/v) cysteine hydrochloric acid and 20 % (v/v) foetal calf serum (Invitrogen), pH 6.5.

3 Yeast Extract medium (ATCC 1067): 0.05 % (w/v) yeast extract, 1 % (w/v) D-(+) glucose.

3.3.6. Muramidase Assay

Muramidase activity was tested by radial diffusion assay as described in Section 2.3.5.

3.3.7. Protein Purification

The reconstituted protein extract was fractioned by cation exchange chromatography using a CM Macro-Prep 1 cm x 10 cm Econo-column (Bio-Rad), previously equilibrated with 20 mM HEPES, 0.1 M NaCl, pH 7.0 (buffer A). Following a 35 min wash with 10 % 20 mM HEPES, 1 M NaCl, pH 7.0 (buffer B) to remove unbound material, elution was performed with a linear AB gradient from 10 % B to 100 % B over 90 min, followed by 35 min of B, at a flow rate of 1 $ml \cdot min^{-1}$. Active fractions eluting between 80 % and 100 % B were collected, acidified to a final concentration of 0.15 % (v/v) TFA and applied to Sep-Pak Vac 5g $^{t}C_{18}$ cartridges (Waters), previously equilibrated in 0.15 % (v/v) TFA. Two successive stepwise elutions were performed with 20 ml of 20 % (v/v) and 70 % (v/v) acetonitrile (BDH) containing 0.15% (v/v) TFA. The latter fraction was lyophilised, resuspended in acidified deionized water (0.1 % (v/v) TFA) and loaded onto an ODS2-Inertpak C_{18} reversed phase HPLC column (particle size 5 μm, 4.6 mm x 250 mm, Capital HPLC). The HPLC system comprised a series 410 LC pump (Perkin Elmer) coupled with a 996 photodiode array detector (Waters) and a 2110 fraction collector (Bio-Rad). Elution was executed at 25 °C with a biphasic gradient of 0.1 % (v/v) TFA in water and 0.09 % (v/v) TFA in acetonitrile (please see Fig. 3.5) at a flow rate of 1 $ml \cdot min^{-1}$. Active fractions of interest were further chromatographed by reversed phase

HPLC on the same column but under a shallower gradient (30 % to 55 % acetonitrile over 50 min at a flow rate of 1 $ml \cdot min^{-1}$).

At each step, protein profiles were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE), as described in Section 2.3.6. The gels were stained with Coomassie brilliant blue (Appendix C).

3.3.8. Protein Quantification

Total protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin (Pierce) as standard. Amino acid analysis of the purified protein was performed at the Protein and Nucleic Acid Chemistry facility (University of Cambridge, UK) using the post-column ninhydrin method or, when pmol sensitivity was required, the pre-column AccuTag system.

3.3.9. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

Information concerning the molecular weight and purity of the antibacterial factors was determined by MALDI-TOF MS at the Centre for Biomolecular Sciences (University of St Andrews, UK). The sample of interest (0.5 µl, estimated concentration 10 $pmol \cdot \mu l^{-1}$ in deionized water) was applied to the target plate along with 0.5 µl saturated solution α-cyano-4-hydroxycinnamic acid (Fluka) in acetonitrile (Ultrafine)/ 0.1% TFA (35:65, v/v). Cytochrome c and haemoglobin were similarly applied to an adjacent spot for calibration. Mass

spectra were obtained on a TofSpec 2E instrument (Micromass) using a 337 nm laser and operated in linear mode.

For peptide mapping the purified cationic protein was digested with trypsin and the digestion products analysed by MALDI-TOF MS in reflectron mode using ACTH clip (residues 18-39), angiotensin I and renin substrate as external standards.

3.3.10.Partial Primary Structure Determination

Following an unsuccessful attempt to directly sequence the N-terminus of the purified protein, it was digested with chymotrypsin for 18 h at 37 °C using an enzyme:substrate ratio of approximately 1:20. The digests were fractionated on a 100 mm x 1 mm C_{18} microbore column (Brownlee) using a linear biphasic gradient of 0.1 % (v/v) TFA in water (A) and 0.1 % (v/v) TFA in acetonitrile (B), in the range 5 % B to 35 % B over 30 min. One of the purified digestion fragments was sequenced by standard automated Edman degradation on a Procise Sequencer (Applied BioSystems).

3.3.11.Sequence Analyses

Homology searches were performed against the SwissProt, NR and Month databases with the Basic Local Alignment Search Tool (Altschul *et al.*, 1990), BLAST, provided by the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The Mascot algorithm (Perkins *et al.*, 1999) was employed for protein identification by peptide mass fingerprinting. Protein and peptide masses, amino acid composition and isoelectric point were predicted by the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of

Bioinformatics (http://www.expasy.ch/). Sequence alignments were executed with the Omiga 2.0 sequence analysis software (Oxford Molecular/ Accelrys, Cambridge, UK), using the Clustal W 1.6 algorithm (Thompson *et al.*, 1994). The Omiga 2.0 software was also used to search for proteolytic cleavage sites within the protein sequence.

3.3.12.Statistical Analysis

The significance of the difference between mean values of the clear zone areas from two independent test samples was determined by the Mann-Whitney U-test using the Analyse-It software for Excel, with an interval of confidence of 95 %.

3.3.13.Proteolytic Digestion

Antibacterial activity of the purified protein was assessed by radial diffusion assay as above, with or without digestion with 60 $\mu g \cdot ml^{-1}$ (final concentration) proteinase K for 60 min at 37 °C.

3.3.14.Kinetic Assay

Ninety microlitres of *P. citreus* cell suspension containing 10^5 $cfu \cdot ml^{-1}$ were incubated at 20 °C for different time periods with 10 µl of antibacterial protein at two different concentrations (mean value of MIC interval or MBC). As a control, the antibacterial protein solution was replaced with 10 µl MHB. The reactions were terminated by diluting the samples 1:100 in MHB and plating on MHB agar plates in triplicate. Plates were incubated for 18 h at 20 °C.

3.3.15.Haemolysis Assay

Haemolytic activity of the purified protein was tested against trout erythrocytes. The erythrocytes were obtained from whole, freshly collected *O. mykiss* blood withdrawn into syringes coated with heparin (500 IU per ml of blood) and extensively washed with 10 mM phosphate buffered saline containing 0.9 % (w/v) NaCl, pH 7.4 (PBS) to remove the leucocytes and plasma. Erythrocytes were then packed by centrifugation at 800 g for 10 min at 4 °C. In a preliminary experiment to ascertain the appropriate wavelength for absorbance measurements, the UV-visible absorption spectrum of trout erythrocyte lysate was obtained using an Ultrospec 3300pro spectrophotometer (Amersham-Pharmacia). For the assay, the test sample was serially diluted to give a range of protein concentrations from 0.2 to 12 μM. Eleven microlitre aliquots of each dilution were added to 100 μl of a 2 % (v/v) packed cell volume of trout erythrocyte suspension in PBS and incubated for 30 min at 37 °C. For negative controls, 11 μl of PBS replaced the protein sample under test, while for positive controls it was substituted with 0.2 % (v/v) Triton X-100. Following incubation, all samples were centrifuged at 1000 x g for 5 min at room temperature and 100 μl of the supernatant from each well was diluted with 800 μl PBS before absorbance measurement at 545 nm. The supernatant from the negative control served as reference. Percent haemolysis is defined as the ratio of absorbances between each sample and the positive control.

3.3.16.Planar Lipid Bilayer Assay

In order to test the ion channel behaviour of the antimicrobial protein purified from trout mucus, macroscopic and single-channel experiments were carried out

by Dr Gérard Molle (Centre de Biochimie Structurale, University of Montpellier, France). For the conductance experiments, virtually solvent-free planar lipid bilayers were formed using the Montal and Mueller technique (Montal & Mueller, 1972). The membrane was formed over a 100-150 μm hole in a Teflon film (10 μm thick), pre-treated with a mixture of 1:40 (v/v) hexadecane/hexane, separating two half glass cells. Lipid monolayers were spread on top of electrolyte solution (1 M KCl, 10 mM Tris, pH 7.4) in both compartments. Bilayer formation was achieved by lowering and raising the electrolyte level in one or both sides and monitoring capacity responses. Asolectine IV-S from soybean was used as lipid. Voltage was applied through an Ag/AgCl electrode in the *cis*-side.).

With regard to the macroscopic conductance experiments, the purified protein was added in a range of concentration between $5 \cdot 10^{-8}$ M and $5 \cdot 10^{-7}$ M from a 10^{-5} M stock solution in 0.3 % (v/v) Triton X-100. The doped membranes were subjected to slow voltage ramps (6.6 $mV \cdot s^{-1}$) and transmembrane currents were fed to a 427 Keitley amplifier. Current-voltage curves were recorded on an X-Y plotter.

In single channel recordings, the protein concentrations ranged from $2 \cdot 10^{-9}$ M to 10^{-8} M. Currents were amplified and potentials were applied simultaneously by a RK 300 patch clamp amplifier (Biologic Science Instruments SA). Single channel currents were monitored on a R5103N oscilloscope (Tektronix) and stored on a DTR 1202 DAT recorder (Biologic Science Instruments SA) for off-line analysis. Satori (V3.1, Intracel Software) was used for downstream analysis. All experiments were performed at room temperature.

3.4. Results

3.4.1. Effect of Skin Stimulation on its Antibacterial Properties

Trout skin secretions, collected either immediately after physical surface stimulation or 2 h and 4 h post-stimulation, yielded the protein levels shown in Table 3.3. Calibration was based on the standard curve depicted in Fig. 3.1. All samples were diluted to a uniform total protein concentration of 150 $\mu g \cdot ml^{-1}$ before assaying for antibacterial activity. A comparison between extracts from stimulated *versus* unstimulated fish revealed that there is a significant (2-tailed p value = 0.2), albeit small, difference in the mean values of antibacterial activity against *P. citreus* (Fig. 3.2). The difference between the mean antibacterial activities of the samples collected 2 h or 4 h post-stimulation are less marked and their significance is questionable (1-tailed p = 0.5). Nevertheless, as the extracts collected 4 h post-stimulation had a mean antibacterial activity greater than the ones obtained 2 h after stimulation, it was decided to collect the samples 4 hours post-stimulation of the skin surface.

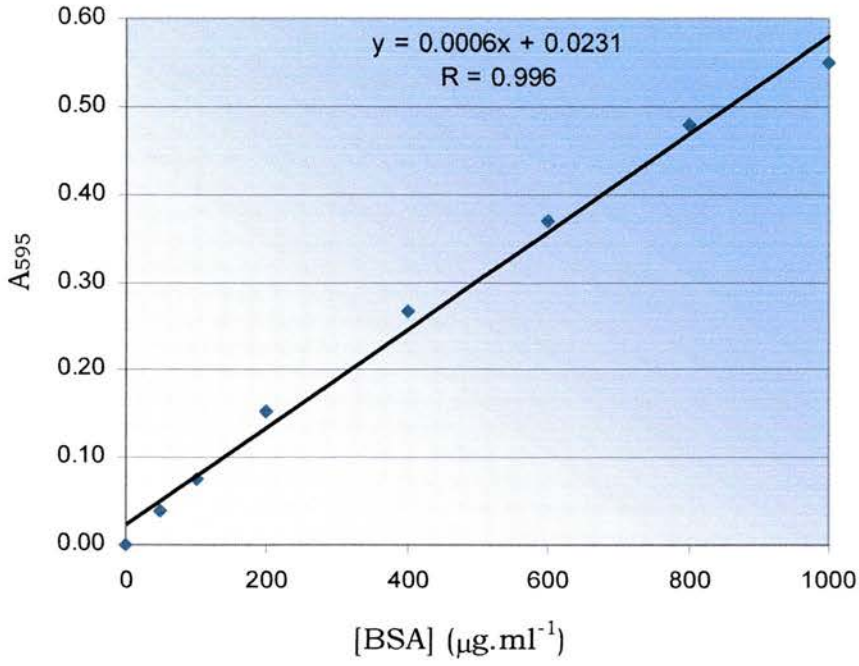


Figure 3.1. Calibration curve for determination of total protein concentration ($\mu g \cdot ml^{-1}$) by the method of Bradford. The absorbance at 595 nm (A_{595}) of the bovine serum albumin (BSA) standards is represented. The linear regression equation and the corresponding Pearson's coefficient (R) are also shown.

Table 3.3. Total protein concentration ([Protein]) of skin mucus extracts from samples collected either immediately after stimulation or 2 h or 4 h post-stimulation with fine sand paper. Each experimental group comprised 3 individuals (n=3).

Time post-stimulation (h)	0			2			4		
[Protein] ($\mu g \cdot ml^{-1}$)	280	300	190	160	280	240	350	290	210

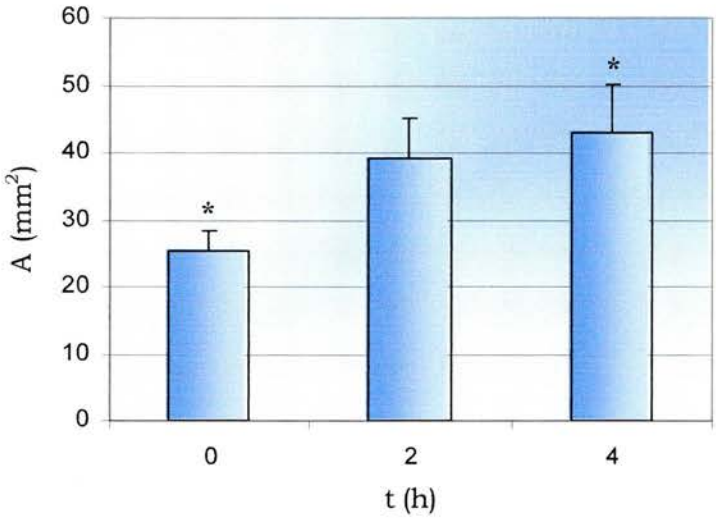


Figure 3.2. Antibacterial activity against *P. citreus* of trout skin mucus extracts from samples collected either immediately after stimulation (0 h) or 2 h or 4 h post-stimulation with fine sand paper. All samples were standardised to a total protein concentration of 150 $\mu g \cdot ml^{-1}$ before assaying for antibacterial activity. The y-axis represents the area of the clear zones (mm^2) obtained on a radial diffusion assay against *P. citreus*. Data are represented as the mean clear zone areas (A) ± SE, n=3. There is a significant difference between the mean area values labelled with an asterisk (2-tailed P = 0.20).

3.4.2. Protein Purification

Cation exchange chromatography of the skin secretion extracts yielded several active fractions, including a group with activity against both *P. citreus* and *E. coli*, eluting between 0.8 M and 1.0 M NaCl (Fig. 3.3). These active fractions were pooled and concentrated by solid phase extraction on $^{t}C_{18}$ Sep-Pak cartridges. The antimicrobial profiles of the reconstituted eluates are shown in Fig. 3.4.

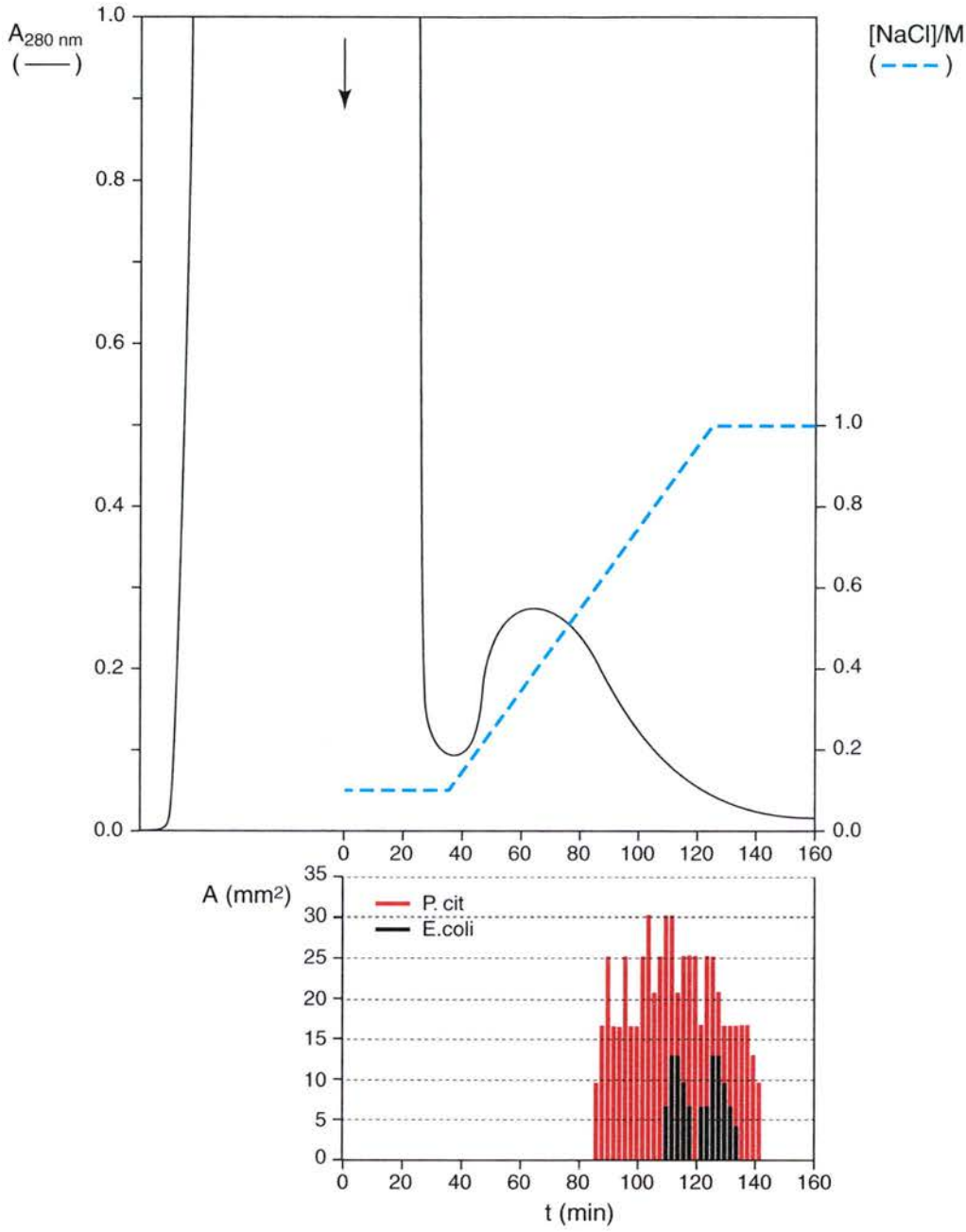


Figure 3.3. Fractionation of acidic skin mucus extract by cationic exchange chromatography. The chromatogram was obtained at 280 nm (solid line). The blue dashed line indicates the NaCl concentration gradient. The arrow indicates the end of sample application. Antibacterial activity profiles against *P. citreus* or *E. coli* are denoted by the histograms, which show the clear zone areas (mm^2) on a radial diffusion assay.

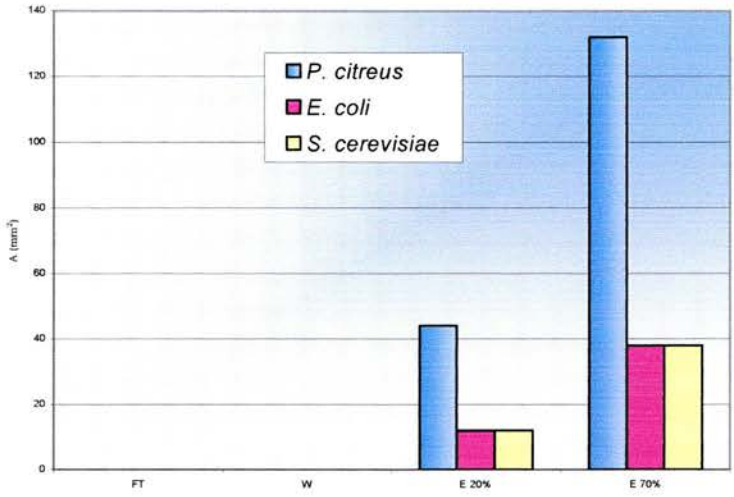


Figure 3.4. Antimicrobial profiles of the solid phase extraction eluates. Cation exchange active fractions eluted with NaCl concentrations between 0.8 M and 1.0 M were pooled and concentrated by $^{t}C_{18}$ solid phase extraction. Two stepwise elutions were performed with 0.15 % TFA in 20 % acetonitrile (E 20%) and 0.15 % TFA in 70 % acetonitrile (E 70%). Following lyophilisation and reconstitution in water, the eluates were assayed for antimicrobial activity by radial diffusion assay. The flow-through (FT) and the wash (W) fractions were also tested. The y-axis shows the area of the clear zones (mm^2) obtained on a radial diffusion assay against *E. coli*, *P. citreus* or *S. cerevisiae*.

Subsequent chromatography of the 70 % acetonitrile eluate by C_{18} reversed-phase HPLC, resulted in two groups of active fractions, designated OM1 and OM2 (Fig. 3.5). Re-fractionation of OM2 by HPLC with the same column but with a shallower gradient yielded a single peak with a retention time of 34.6 min (corresponding to 42.3 % acetonitrile) that exhibited antibacterial activity (Fig. 3.6).

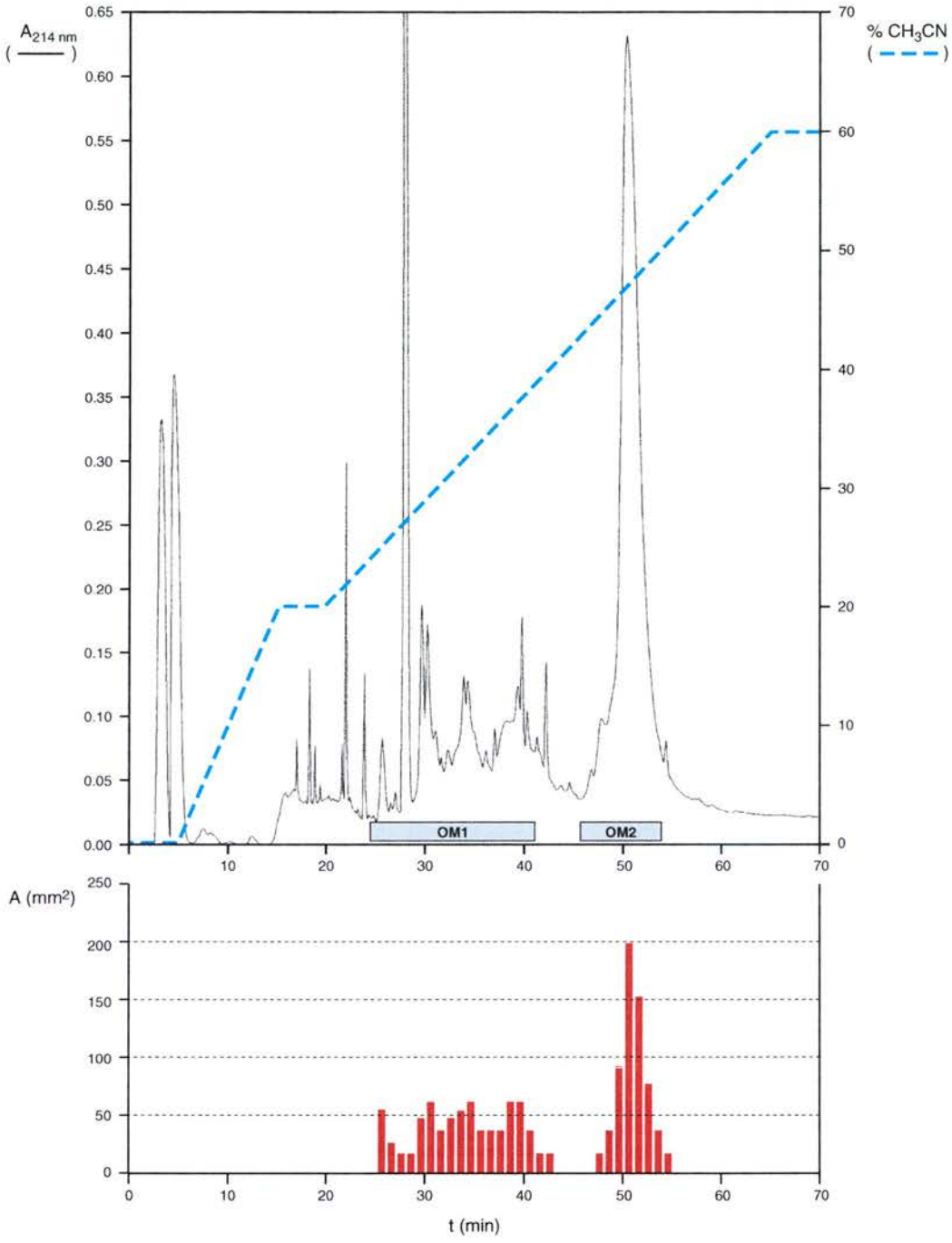


Figure 3.5. C_{18} reversed phase HPLC of the 70 % acetonitrile eluate from $^{t}C_{18}$ solid phase extraction. The chromatogram was obtained at 214 nm (solid line). The acetonitrile concentration gradient is represented by the blue dashed line. Antibacterial activity profile against *P. citreus* is denoted by the histogram. Activity was observed in two groups of fractions, labelled OM1 (fractions 25 to 43) and OM2 (fractions 47 to 55).

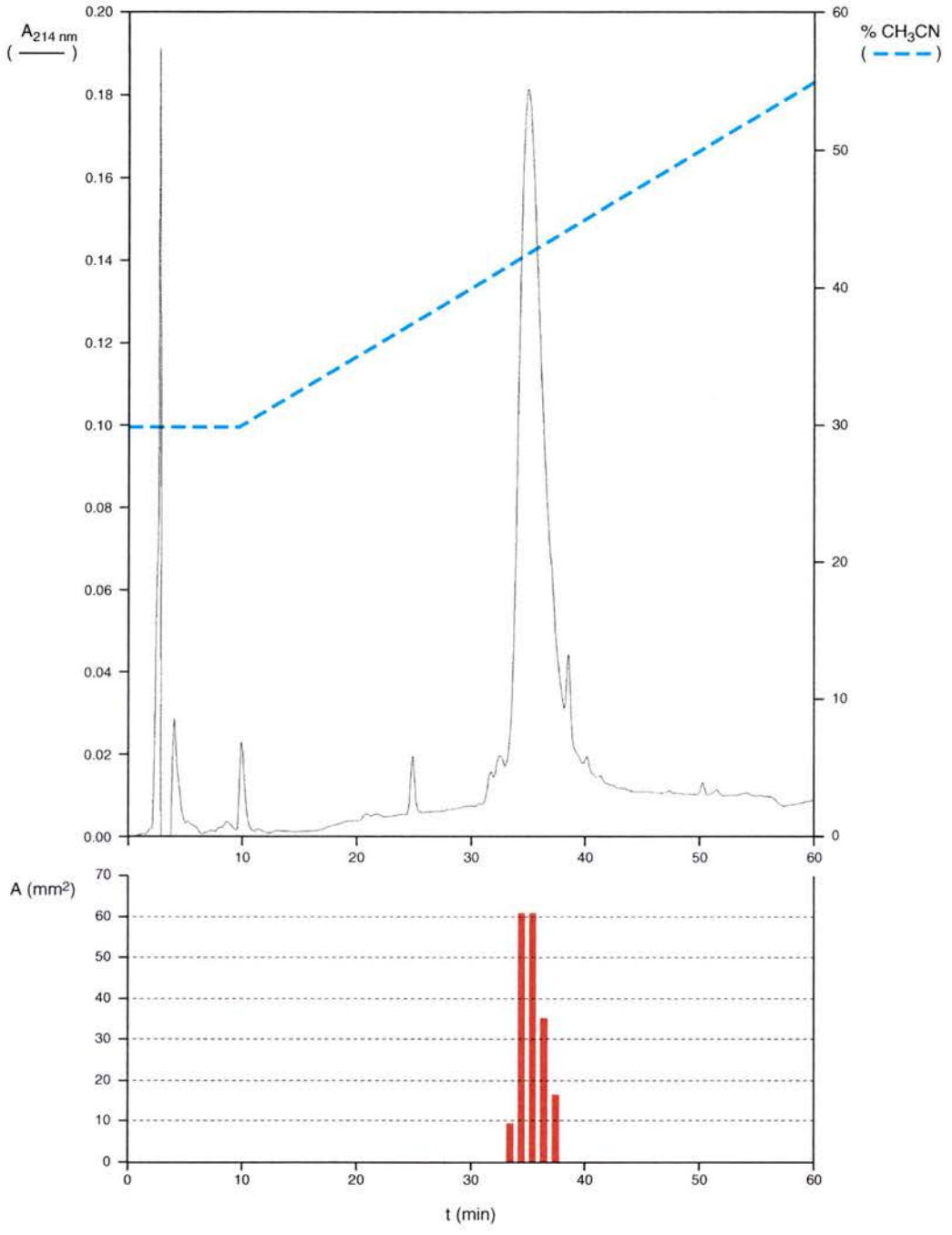


Figure 3.6. Final step in the isolation of an antimicrobial protein from skin secretions of rainbow trout. Active fractions labelled OM2 in fig. 3.5 were pooled and fractionated by C_{18} reversed phase HPLC using a shallower water/acetonitrile gradient. Absorbance was monitored at 214 nm (solid line). The blue dashed line indicates the proportion of acetonitrile. Fractions 33 to 37, corresponding to the peak eluting at 34.6 min, were found to be antibacterial to *P. citreus* (histogram).

SDS-PAGE revealed a single protein band with an apparent molecular weight of approximately 15 kDa (Fig. 3.7). The antibacterial activity of the purified protein was thermostable, remaining present even after incubation at 99 °C for 5 min. Proteolytic treatment with proteinase K completely abolished antibacterial activity, confirming that the active factor had a proteinaceous nature. The total amount of purified protein was approximately 1 $\mu g \cdot g^{-1}$ mucus.

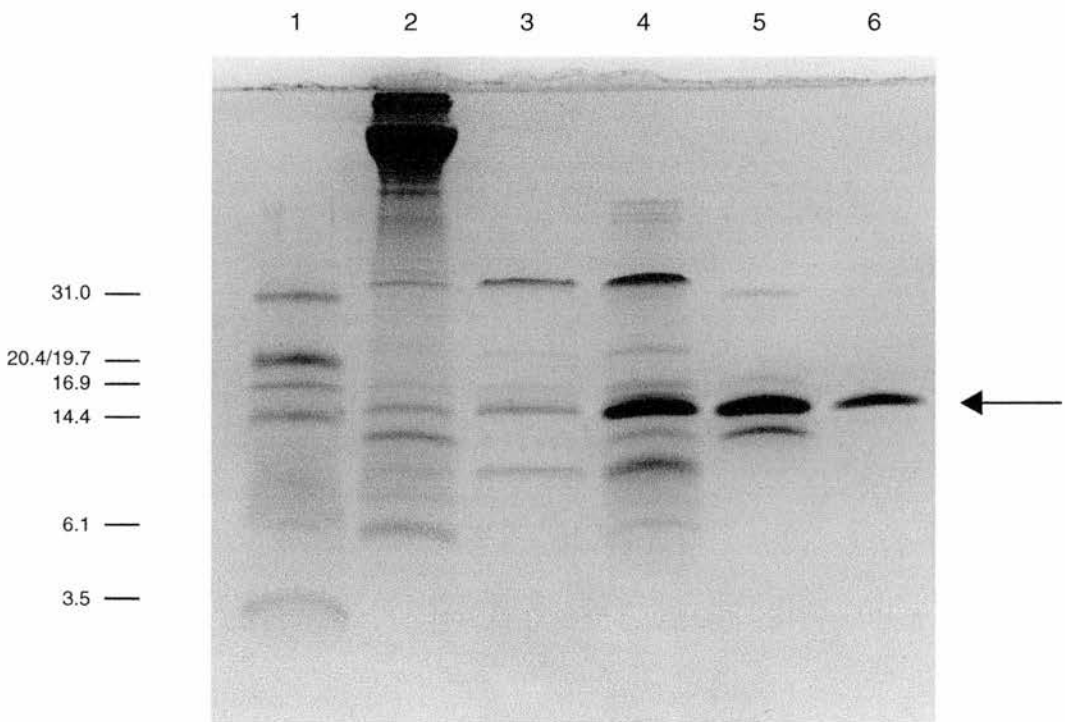


Figure 3.7. Tris-Tricine SDS-PAGE analysis of the active fractions obtained during the purification of an antimicrobial protein from skin secretions of rainbow trout. Lane 1: markers; lane 2: crude extract; lane 3: pooled ion exchange fractions; lane 4: 70 % acetonitrile eluate from solid phase extraction; lane 5: OM2 C_{18} reversed-phase HPLC fractions; lane 6: purified antibacterial protein after 2nd HPLC. Each lane contains 7.5 µl of sample. The numbers on the left hand side correspond to the molecular mass of the markers in kDa. The protein of interest is indicated by an arrow.

3.4.3. Protein Characterisation

Attempts to determine the primary structure of the protein by automated Edman degradation were unsuccessful because the N-terminus is blocked. Digestion with chymotrypsin generated a number of fragments, including a peptide with a mass of 1201.64 Da, as determined by MALDI-TOF MS. The N-terminal sequence[#] of this peptide, obtained by automated Edman degradation was: AERVGAGAPVYL. The true monoisotopic mass of this peptide is 1201.645 Da. Thus, there is an excellent agreement between the MALDI-TOF MS and the sequencing data. BLAST homology searches established that it is a perfect match with part of histone H2A from rainbow trout (Fig 3.8).

Mass spectrometry analysis of the purified protein revealed two signals at 13,512 Da and 13,639 Da (Fig 3.9) corresponding to the single charged molecular ions. These two peaks are consistent with the acetylation of the N-terminal residue of trout histone H2A, which accounts for the frustrated attempts to directly sequence the protein. Truncation of the protein at the last lysine would explain the lower intensity peak (13,512 Da). The MALDI-TOF spectrum of the protein following digestion with trypsin is shown in Fig. 3.10. Peptide mass fingerprinting using this set of peptide masses showed that histone H2A was the only significant protein candidate, with 4 matching peptides out of a total of 6 potential peptide masses (Table 3.4). The amino acid analysis results

[#] This partial internal amino acid sequence has been deposited in the Swiss-Prot database under the accession number P83327.

were in good agreement with the expected composition of histone H2A from trout gonads (Table 3.5).

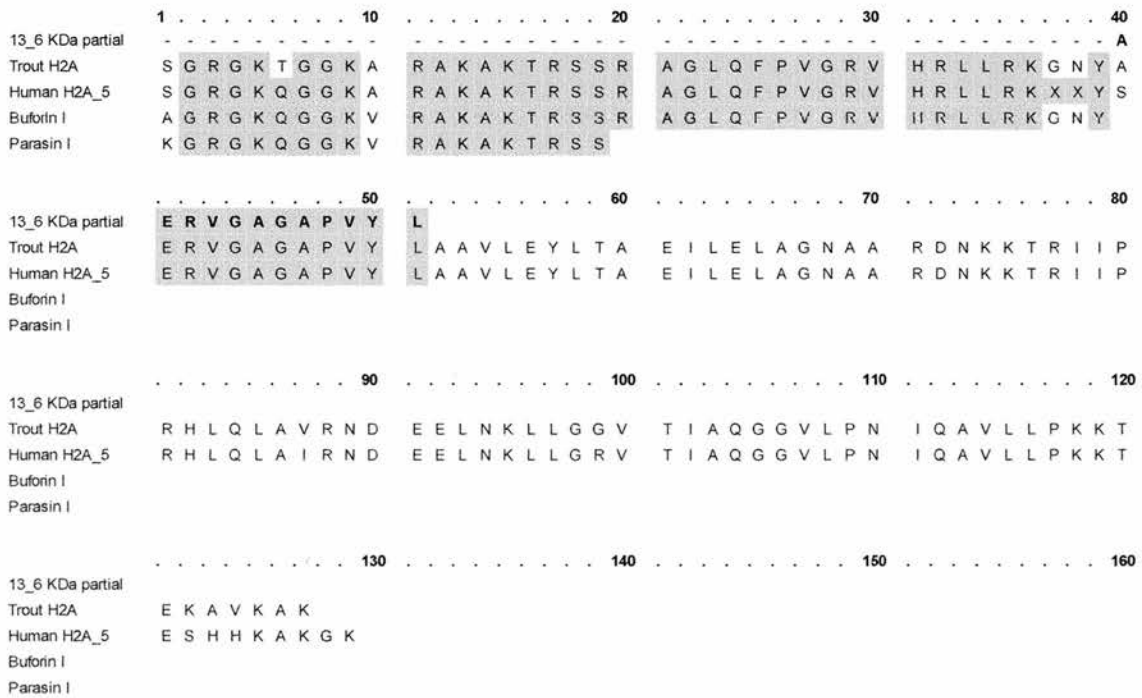


Figure 3.8. Partial internal sequence of the antimicrobial protein purified from skin mucus of rainbow trout and its homology with other histones or histone-derived peptides. Identical residues between the purified antimicrobial protein, histone H2A from rainbow trout (Connor *et al.*, 1984), human histone H2A.5 (Zhong *et al.*, 1983), buforin I (Park *et al.*, 1996) and parasin I (Park *et al.*, 1998b) are represented by a shaded box.

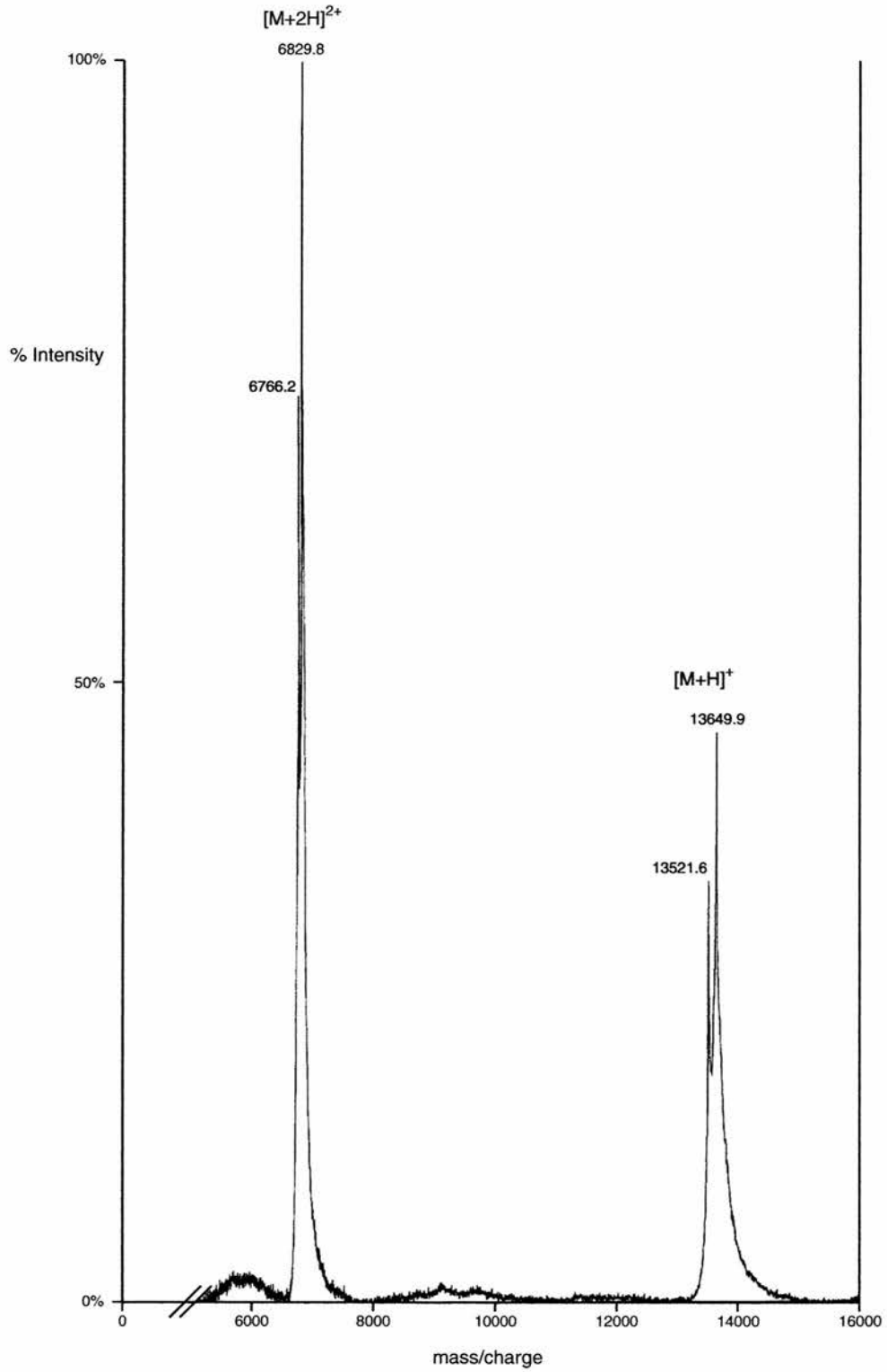


Figure 3.9. Representative mass spectrum of the pooled peak fractions 35 and 36 (Fig. 3.6) determined by MALDI-TOF. The single and double charged molecular ions are labelled. The experiment was performed in triplicate.

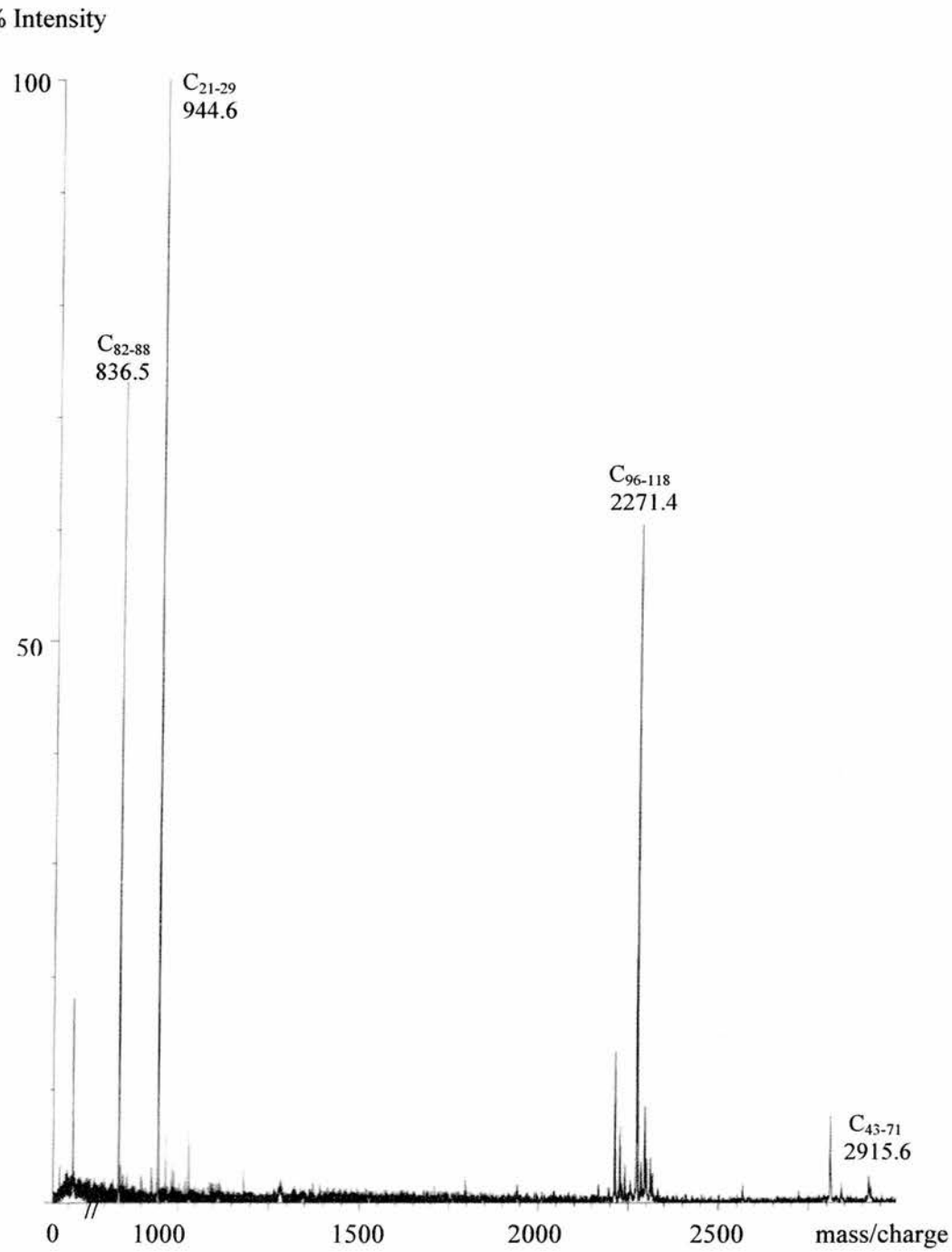


Figure 3.10. Peptide mapping by MALDI-TOF MS of the pooled peak fractions 35 and 36 (Fig. 3.6), following digestion with trypsin. The masses of the identified monovalent peptide ions in Dalton are indicated; their position within the protein are represented as C_{x-y}, where x and y indicate the first and last residue (respectively) for each peptide.

Table 3.4. Theoretical monoisotopic masses of the peptides generated by digestion of gonadal trout histone H2A (Swiss-Prot P02264) with trypsin and assuming no missed cleavages. The peptides which have been experimentally identified by peptide mass fingerprinting of histone H2A from trout skin are highlighted in red.

Mass (Da)	Position	Peptide Sequence
2915.59	43-71	VGAGAPVYLAAVLEYLTAEILELAGNAAR
2271.38	96-118	LLGGVTIAQGGVLPNIQAVLLPK
944.53	21-29	AGLQFPVGR
861.39	89-95	NDEELNK
836.51	82-88	HLQLAVR
709.33	37-42	GNYAER

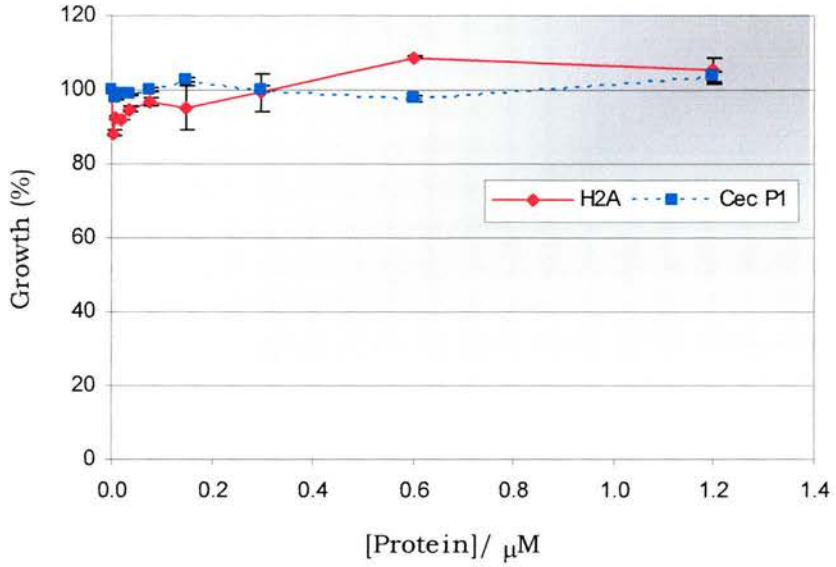
Table 3.5. Amino acid analysis of the antimicrobial protein purified from rainbow trout skin. Norleucine was used as internal standard. The results are in good agreement with the predicted amino acid composition of gonadal trout histone H2A (Swiss-Prot P02264).

Amino Acid	No Residues	Expected Composition
Ala	14	18
Arg	10	12
Asx	8	8
Cys	0	0
Glx	11	11
Gly	16	14
His	2	2
Leu	14	16
Lys	10	13
Ile	5	5
Met	excluded	0
Phe	1	1
Pro	7	5
Ser	8	3
Thr	5	5
Trp	not determined	0
Tyr	3	3
Val	9	10

3.4.4. Antimicrobial and Haemolytic Activity

Quantification of antibacterial activity using the microtitre broth dilution method revealed that the purified trout histone H2A is active at submicromolar concentrations against the Gram-(+) bacteria *A. viridans*, *B. subtilis*, *M. luteus* and *P. citreus* (Fig. 3.11, Table 3.6). In contrast, Cecropin P1 displays MICs approximately ten fold higher against the same bacterial strains (Fig. 3.11, Table 3.6). No activity of the trout histone H2A was observed against any of the Gram-(-) bacteria at the concentrations tested. The yeast, *S. cerevisiae*, showed susceptibility with a MIC similar to that observed against *B. subtilis* (Fig. 3.11, Table 3.6). The purified histone had no detectable muramidase activity.

(A)



(B)

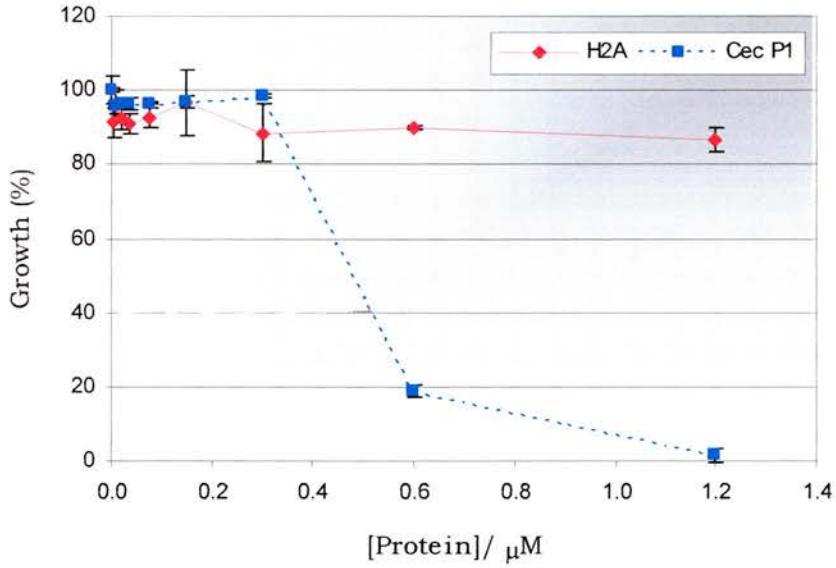
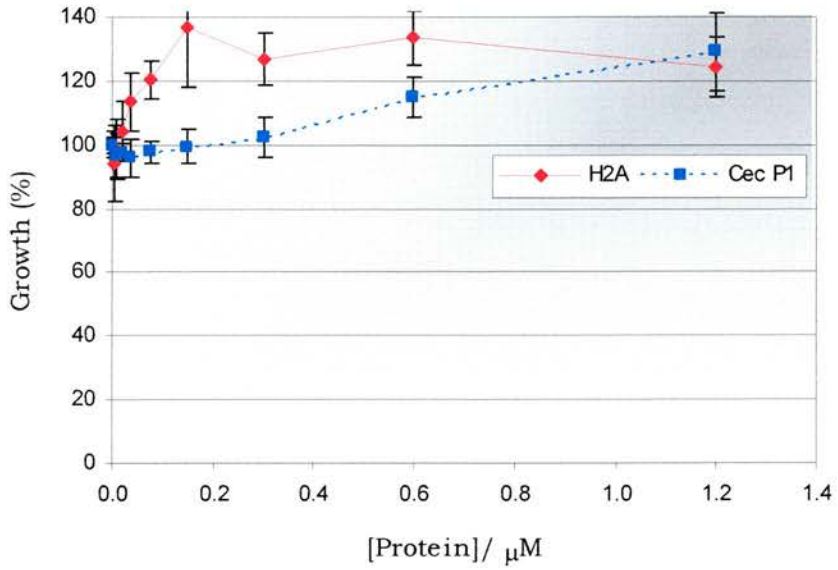


Figure 3.11. Quantification of antibacterial activity against (A) *A. hydrophila* or (B) *A. salmonicida* 004. For the complete legend please consult page 99.

(C)



(D)

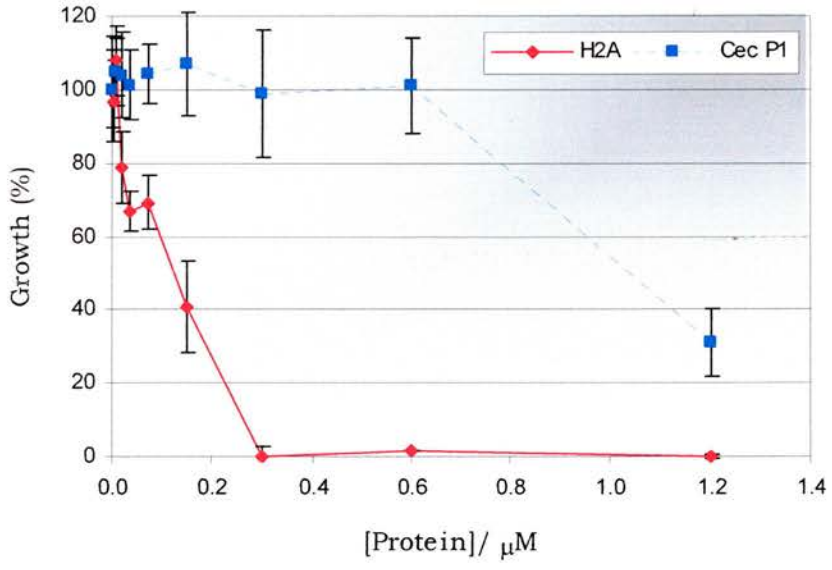
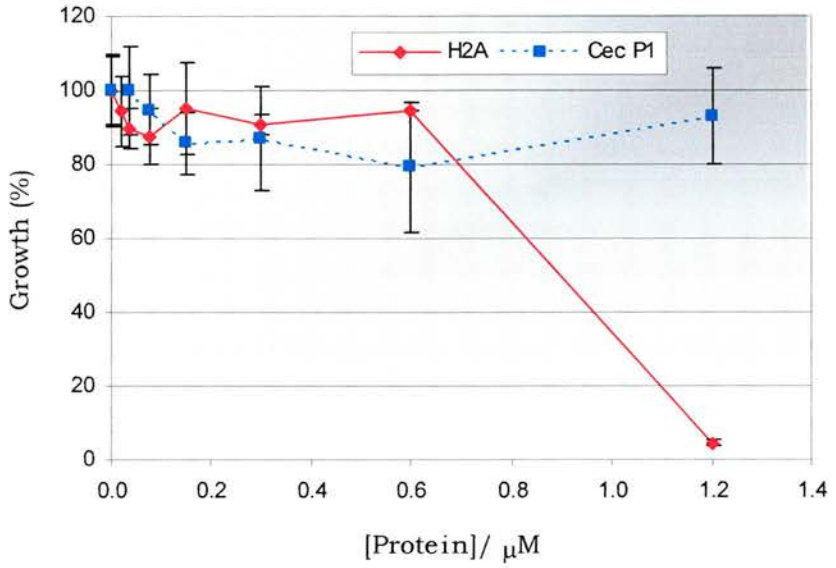


Figure 3.11. (continued) Quantification of antibacterial activity against (C) *A. salmonicida* 849 or (D) *A viridans*. For the complete legend please consult page 99.

(E)



(F)

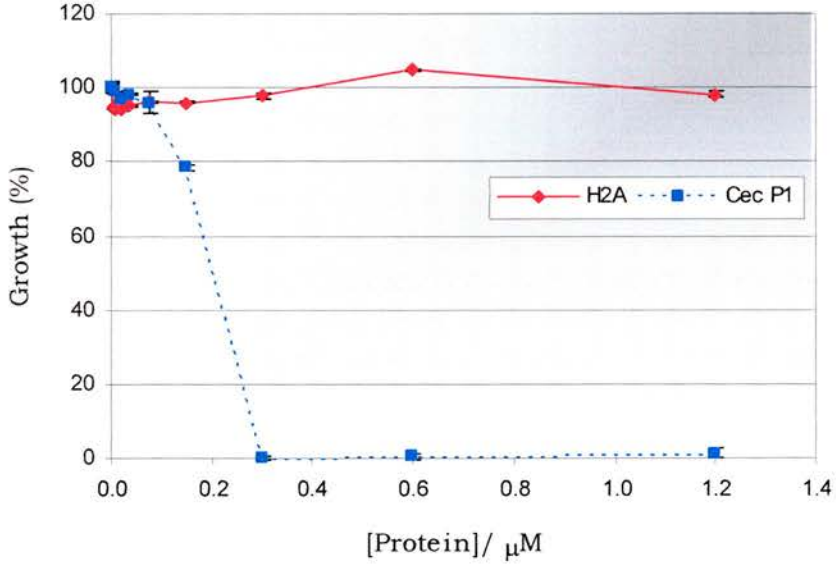
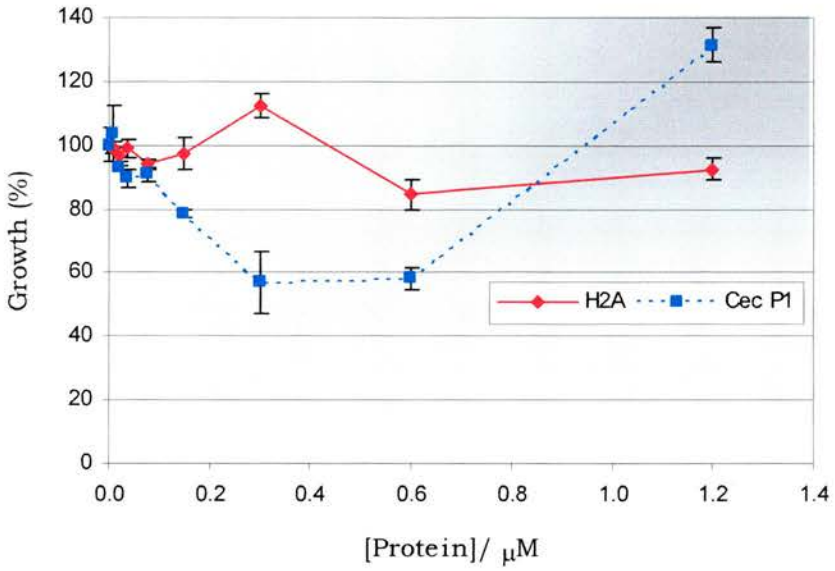


Figure 3.11. (continued) Quantification of antibacterial activity against (E) *B. subtilis* or (F) *E. coli*. For the complete legend please consult page 99.

(G)



(H)

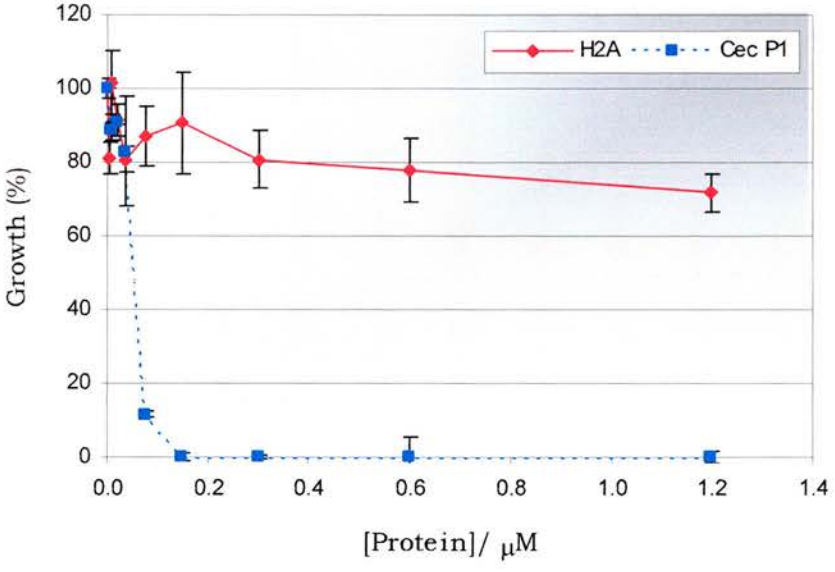
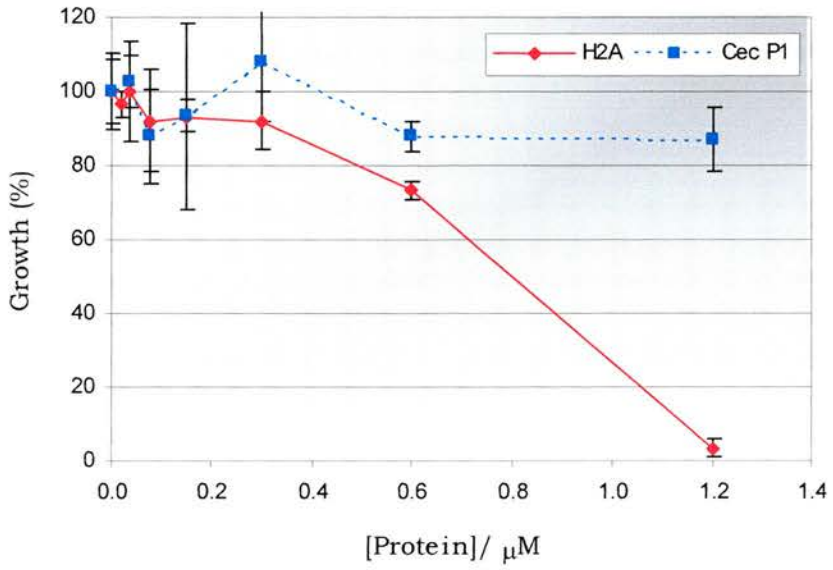


Figure 3.11. (continued) Quantification of antibacterial activity against (G) *L. anguillarum* 1637 or (H) *L. anguillarum* 2129. For the complete legend please consult page 99.

(I)



(J)

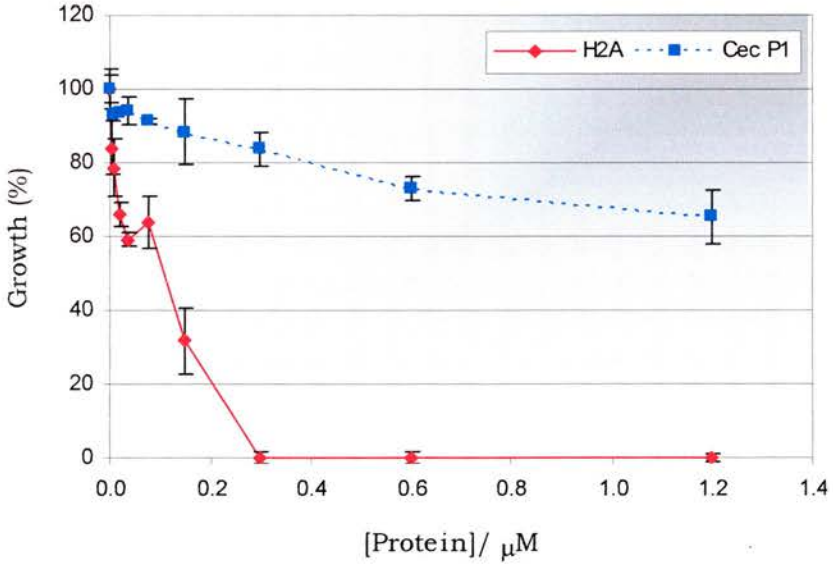
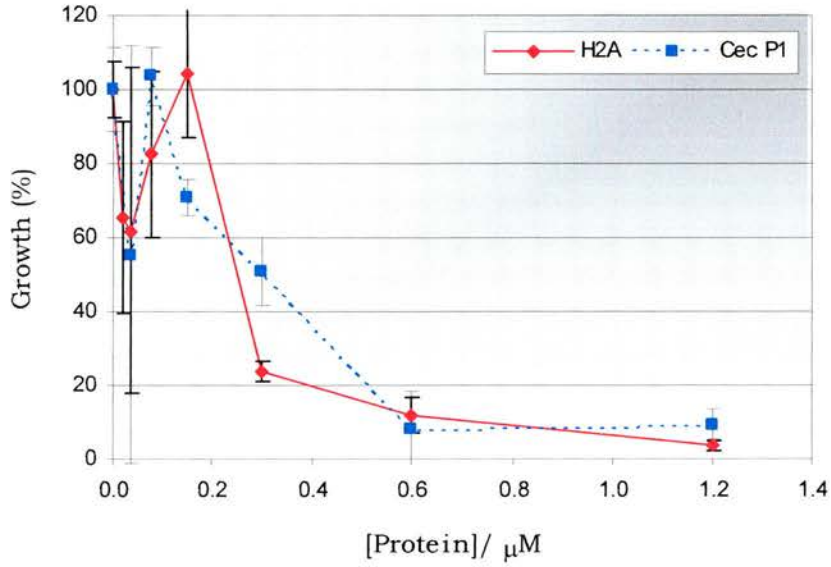


Figure 3.11. (continued) Quantification of antibacterial activity against (I) *M. luteus* or (J) *P. citreus*. For the complete legend please consult page 99.

(K)



(L)

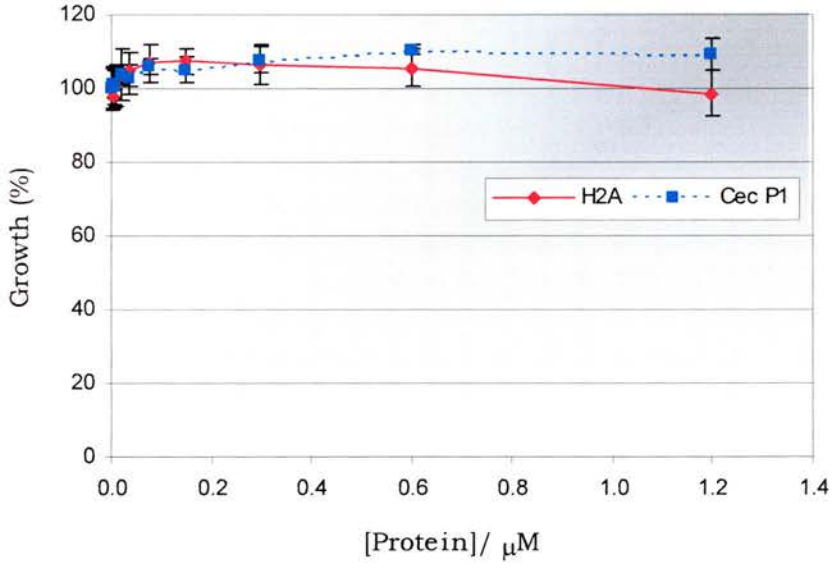
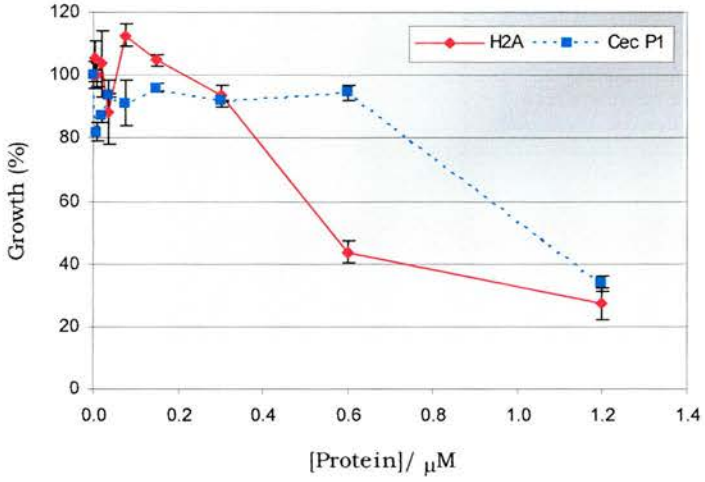


Figure 3.11. (continued) Quantification of antibacterial activity against (K) *R. salmoninarum* or (L) *S. aureus*. For the complete legend please consult page 99.

(M)



(N)

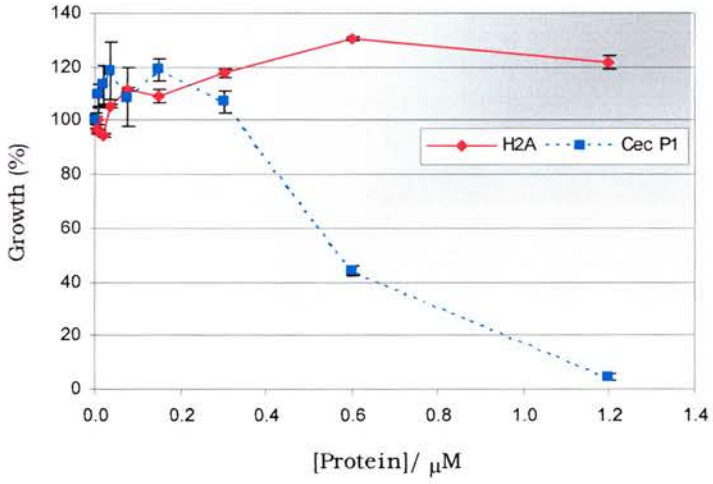


Figure 3.11. (continued) Quantification of antibacterial activity against (M) *S.cerevisiae* or (N) *Y. ruckeri*. Bacterial suspensions containing 10^5 cfu·ml^{-1} were incubated at the appropriate temperature (please see Table 3.2) with serially diluted cecropin P1 (Cec P1) or histone H2A from trout skin (H2A). Microbial growth is expressed as the ratio of optical densities read at 570 nm (OD_{570}) between each test sample and the control (no peptide added). The average final OD_{570} of the control was approximately 0.2. Data are represented as means ± SE, n=3.

Table 3.6. Summary of the bacteriostatic (MIC, μM) and bactericidal (MBC, μM) activities of trout skin histone H2A and cecropin P1. The MIC data are derived from the growth inhibition curves shown in Fig. 3.11.

		Histone H2A		Cecropin P1	
Species	**Gram Staining**	**MIC**	**MBC**	**MIC**	**MBC**
A. viridans	+	0.08-0.15	0.3	0.6-1.2	> 2.4
B. subtilis	+	0.6-1.2	1.2	> 2.4	> 2.4
M. luteus	+	0.6-1.2	1.2	> 2.4	> 2.4
P. citreus	+	0.08-0.15	0.3	1.2-2.4	> 2.4
R. salmoninarum	+	0.15-0.3	> 1.2	0.15-0.3	> 2.4
S. aureus	+	> 1.2	> 1.2	> 2.4	> 2.4
A. hydrophila	-	> 1.2	> 1.2	> 2.4	> 2.4
A. salmonicida 004	-	> 1.2	> 1.2	0.3-0.6	2.4
A. salmonicida 849	-	> 1.2	> 1.2	> 2.4	> 2.4
E. coli	-	> 1.2	> 1.2	0.15-0.3	0.6
L. anguillarum	-	> 1.2	> 1.2	0.04-0.08	0.3
L. anguillarum 01	-	> 1.2	> 1.2	> 2.4	> 2.4
Y. ruckeri	-	> 1.2	> 1.2	0.3-0.6	1.2
S. cerevisiae	n/a	0.3-0.6	> 1.2	0.6-1.2	> 2.4

The UV-visible absorption spectrum of trout haemoglobin shows several absorption maxima, including a peak at approximately 545 nm (Fig. 3.12), similar to human haemoglobin. This was the wavelength selected to perform the haemolytic assay. Haemolytic activity was not observed against trout erythrocytes at concentrations of the purified protein below 0.3 μM, although at higher concentrations it displayed a considerable dose-dependent haemolytic activity, comparable to that of melittin (Fig. 3.12). This haemolytic activity against *O. mykiss* erythrocytes is less potent than its bactericidal effect against the susceptible Gram-(+) bacteria (Fig. 3.13, Table 3.6).

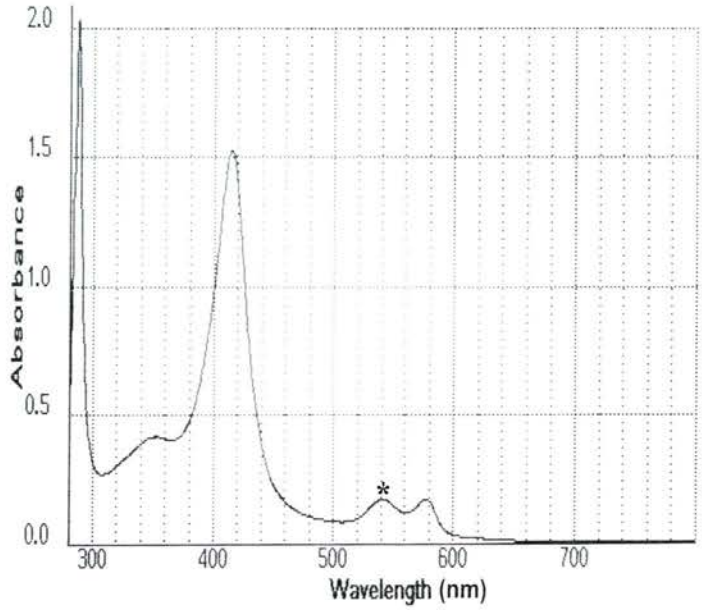


Fig. 3.12. UV-visible absorption spectrum of trout erythrocyte lysate. Absorption was measured against a reference comprising 0.2 % (v/v) Triton X-100. The peak labelled with an asterisk has an absorption maximum at approximately 545 nm.

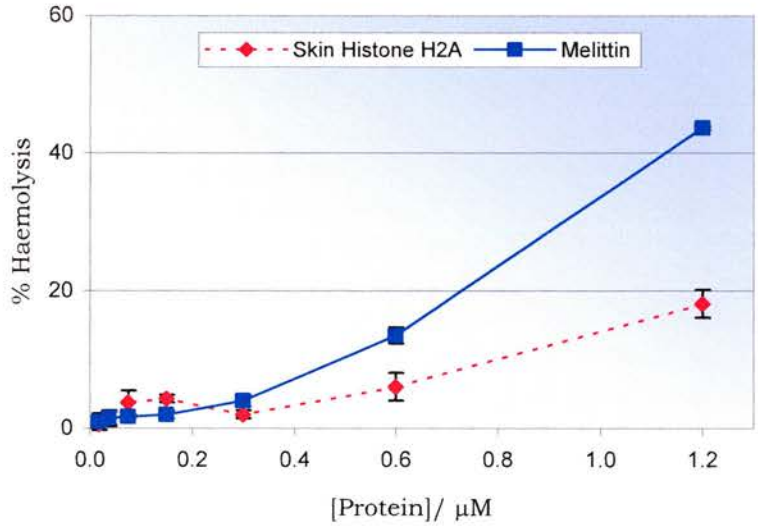


Figure 3.13. Haemolysis assay against trout erythrocytes. Serially diluted test samples were incubated with a 2 % (v/v) suspension of erythrocytes for 30 min at 37 °C. Percent haemolysis is defined as the ratio of absorbances (read at 545 nm) between each sample and the positive control (Triton X-100). Values are represented as means ± SE, n=3.

3.4.5. Kinetics

The kinetic study against *P. citreus* demonstrated that bacteria were killed in a dose and time-dependent manner, with increasing concentrations of antibacterial protein or increasing incubation times leading to higher loss of viability (Fig. 3.14). At 0.12 μM (MIC) antibacterial activity was noticed after 10 min incubation, where approximately 30 % bacteria were non-viable. Within 60 min incubation no colony forming units could be observed. After only 1 min, *circa* 40 % bacteria were killed by incubation with 0.3 μM (MBC) antibacterial protein. All bacteria had lost viability following 30 min incubation at this concentration.

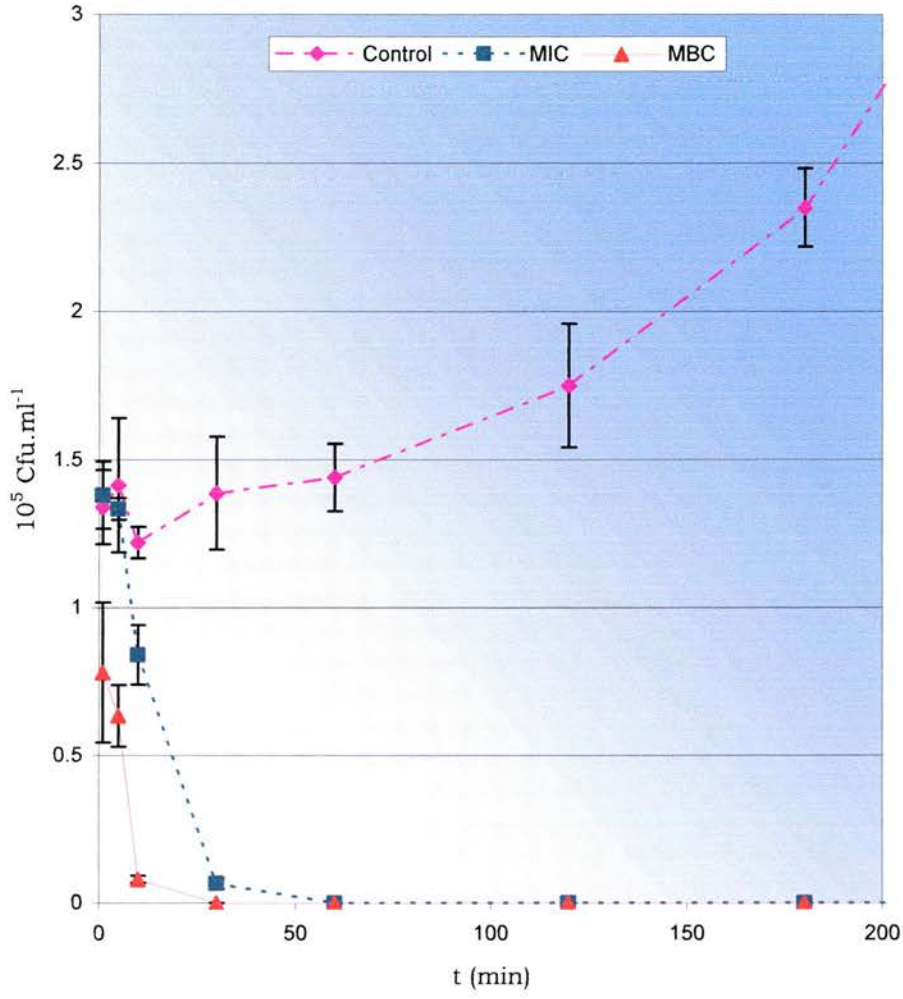


Figure 3.14. Kinetic analysis of bacterial killing. *P. citreus* cell suspensions (10^5 cfu·ml^{-1}) were incubated with purified histone H2A at 0.12 μM (MIC) or 0.3 μM (MBC) and plated in triplicate after the indicated times. The control comprised bacteria incubated in the absence of antimicrobial protein. Data are denoted as means ± SE.

3.4.6. Salt Sensitivity

The assays to investigate the influence of NaCl on antibacterial activity showed that, despite being active against *P. citreus* at concentrations up to 3.2 % (w/v) NaCl, the MIC value augmented 4-fold when the NaCl concentration increased from 0.8 % (w/v) to 1.5 % (w/v). Furthermore, a 16-fold increase in the MIC value (relative to 0.8 % (w/v) NaCl) was observed in the presence of 3.2 % (w/v) NaCl (Fig. 3.15).

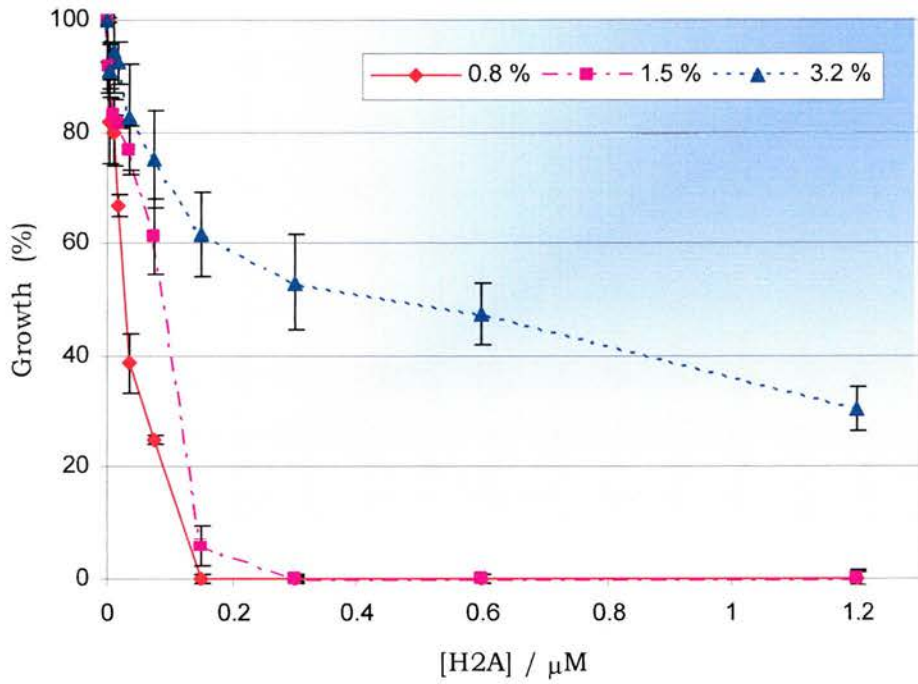


Figure 3.15. Influence of NaCl concentration on antibacterial activity of histone H2A against *P. citreus*. Bacterial suspensions containing different concentrations of sodium chloride were incubated with serially diluted purified histone H2A. The results are expressed as the ratio of absorbances (read at 570 nm) between each sample and the control (no peptide added). Values are shown as means ± SE, n=3.

3.4.7. Pore-forming Activity

It was found by Dr. Gérard Molle that in the concentration range between $5 \cdot 10^{-8}$ M and $5 \cdot 10^{-7}$ M, strong destabilisation of membranes occurred when the purified histone H2A was added to the measurement cell and no typical I-V curves could be recorded. This behaviour was confirmed by independent single channel experiments. Indeed, with a protein concentration ranging from $2 \cdot 10^{-9}$ to 10^{-8} M, and under a large range of applied voltages, a strong destabilisation of planar lipid bilayers was observed. It was also possible to observe large bursts showing the permeabilisation of the membrane.

3.5. Discussion

The present investigation shows that a 13.6 kDa antimicrobial protein is expressed by rainbow trout skin epithelia. The protein has a broad spectrum of activity, is highly potent and salt sensitive. Mass spectrometry combined with peptide mass fingerprinting, amino acid analysis and sequence alignments further reveal that it is, or is very likely to be histone H2A, acetylated at the amino terminus.

Amongst the numerous reports of antimicrobial peptides published in the literature, a few appear to be known proteins or protein fragments not previously thought to have antimicrobial properties. Histones are one such group of proteins, with reports of microbicidal activities for histones or histone-derived fragments (mainly H1 or H2B) starting to emerge (From *et al.*, 1996; Robinette *et al.*, 1998; Patrzykat *et al.*, 2001; Richards *et al.*, 2001). To the best of my knowledge, the present study on *O. mykiss* is the first to directly demonstrate that histone H2A has antimicrobial properties. Moreover, sequence alignments of the 13.6 kDa protein characterised in the present chapter with buforin I, parasin I, human histone H2A.5 and a 13.6 kDa histone H2A from rainbow trout testes reveal a high degree of homology amongst them (Fig. 3.8). This raises the possibility of a conserved role for histone H2A in immunity throughout the vertebrate subphylum. There are two previous reports of antimicrobial peptides derived from histone H2A: buforin I, a potent 39-residue antibacterial peptide from skin of the Asian toad, *Bufo bufo gargarizans*, (Park *et al.*, 1996) and parasin I, a 19-residue N-terminal fragment present in the skin of the catfish, *Parasilurus asotus* (Park *et al.*, 1998b). Monoclonal antibodies to this latter

peptide have some cross-reactivity with a peptide of similar size that is present in skin mucus of rainbow trout (Cho *et al.*, 2002). Nevertheless, the present investigation did not identify such a peptide in skin mucus of physically stimulated fish, suggesting that if it is present it may not be active against the test bacterium used. It would be interesting to investigate if the primary structure differences of this potential peptide (particularly the acetylation of the first residue) are enough to impair its antimicrobial activity. Moreover, the search for proteolytic sites for cathepsin D or pepsin using the PABASE database of proteolytic agents did not identify any potential recognition sequence.

Histone H2A is one of the core histones which, together with histones H2B, H3 and H4, forms the nucleosome (Dong & van Holde, 1991) and it has therefore been classically associated with DNA packaging and regulation of transcription. Nevertheless, recent reports have demonstrated that histones or histone-derived fragments can have a cytosolic as well as nuclear localisation (Kashima, 1991; From *et al.*, 1996). In particular, Cho *et al.* (2002) have clearly shown by immunohistochemistry that unacetylated histone H2A is present in the cytoplasm of mucous gland cells from catfish skin mucosa. These data support the hypothesis that besides its nucleosomal involvement, histone H2A may also aid in protection of the cell against bacterial attack. It probably functions either in the cytoplasm against intracellular pathogens or extracellularly through release to mucosal surfaces or tissue fluids after infection-induced cell lysis or apoptosis.

Different antimicrobial peptides kill bacteria by different mechanisms, either by forming pores that induce a disruption in the electroosmotic gradients and the

subsequent bioenergetic collapse of the cell (Ebran *et al.*, 2000), or by inhibiting cellular functions, such as DNA synthesis (Park *et al.*, 1998a) and chaperone-assisted protein folding (Kragol *et al.*, 2001). Macroscopic current-voltage (I-V) curves are useful to screen the functional properties of new potential channel-formers. In this configuration, hundreds to thousands of ion channels can be expressed in large bilayers submitted to slow voltage ramps and at relatively high protein concentration. The histone H2A isolated from trout mucus in the present study, induces a strong destabilisation of planar lipid bilayers but is unable to form stable channels, indicating that its main targets are not the bacterial membranes. Its ability to destabilise membranes may allow it to enter the cell where it exerts its antibacterial action, probably by inhibiting cellular functions.

The H2A histone isolated from *O. mykiss* skin displays a 16-fold reduction in potency against *P. citreus* when the salt concentration increases from 0.8 % (w/v) to 3.2 % (w/v) NaCl. Given that this histone is expressed in the skin mucosa, it is somewhat surprising that it is salt-sensitive. However, as histones are intracellular they may be shielded to some extent from the inactivating effect of direct exposure to high salt concentrations. Moreover, their role in saltwater could be protection against intracellular pathogens, whereas in freshwater their role could extend to surface disinfection. It is notable that the salmonid intracellular pathogen, *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease, is sensitive to killing by the histone H2A purified in the present study.

The likely importance in mucosal defence of the histone H2A purified from trout mucus in the present study is indicated by its very high potency. Its minimal inhibitory concentrations are in the submicromolar range and *circa* ten times lower than those of cecropin against sensitive bacteria. There is a direct proportionality between antibacterial activity, protein concentration and incubation time, in common with other antimicrobial peptides (Cole *et al.*, 1997). Furthermore, its antibacterial action is exerted rapidly, i.e. a 30 min incubation at 0.3 μM is sufficient to totally inactivate the Gram-(+) test bacteria. Importantly, at this concentration the protein is not lytic for trout erythrocytes, although it has haemolytic effects at concentrations over 0.3 μM. Thus, for the fish the protein could exert its biological effects *in vivo* against bacteria, and possibly also yeasts, without damage to its own tissues. Certainly fish have great reliance on the innate defences, as their adaptive immune system is less sophisticated than that of higher vertebrates, and is strongly regulated by environmental temperature. Amongst the innate humoral defences, complement is highly evolved in teleosts. Nevertheless, the alternative complement pathway in bony fish is rather insensitive to activation by Gram-(+) bacteria (Yano, 1996). Whilst Gram-(-) bacteria tend to dominate in marine or aquatic environments, Gram-(+) are still present and represent a potential threat to fish as pathogens or opportunistic invaders and it is important for fish to have good effector mechanisms to deal with them. Histone H2A may be one such factor, for it is active mainly against Gram-(+) bacteria. It is not improbable that histone H2A operates in synergism with other antibacterial factors such as lysozyme, a glycosyl hydrolase known to be abundant in fish blood and mucosa; indeed, a few studies have begun to reveal

synergistic effects of combining antibacterial peptides, including a histone H1-derived peptide, with lysozyme (Patrzykat *et al.*, 2001).

Chapter 4

A Novel Antimicrobial Function for the 40S Ribosomal Peptide S30 from Skin Exudates of *Oncorhynchus mykiss*

4.1. Synopsis

An antimicrobial peptide was purified from skin secretions of rainbow trout by $^{t}C_{18}$ solid phase extraction cation exchange and C_{18} reversed phase chromatography. Partial N-terminal amino acid sequence of the purified peptide revealed 100 % identity with the first eleven residues of a 40S ribosomal peptide from medaka fish. Its molecular mass, determined by matrix-assisted laser desorption ionization mass spectrometry, was found to be 6676.6 Da. These results indicate that this antimicrobial peptide is likely to be the 40S ribosomal protein S30. It is active at submicromolar concentrations, with a minimal inhibitory concentration of 0.02 to 0.04 μM against *Planococcus citreus*. Thus, in addition to its conventional function in the cell as part of the small ribosomal subunit, this peptide may play a role in innate host defence against both intracellular and extracellular pathogens. Moreover, as it is expressed in all eukaryotic, translationally active cells, it is likely to be a ubiquitous innate defence factor.

Preliminary experiments have also revealed that ribosomes from trout liver contain at least one additional peptide or protein that displays antibacterial activity against *P. citreus*.

4.2. Introduction

Gene-encoded antimicrobial peptides are crucial components of the innate immune system. Relatively few antimicrobial peptides have been purified from mucosa of aquatic organisms, particularly teleosts. Nevertheless, some of the teleost peptides found so far are unusual in being peptides or protein fragments with known function, not previously directly associated with immunity (Boman, 1995). Parasins, for instance, are potent antimicrobial peptides derived from catfish histone H2A (Park *et al.*, 1998b).

With regard to salmonids, two antimicrobial peptides have been reported: oncorhyncin, the 3 kDa peptide from rainbow trout (*Oncorhynchus mykiss*) described in Chapter 2, and HSDF-1 (Patrzykat *et al.*, 2001), a 26-residue fragment of histone H1 from coho salmon (*Oncorhynchus kisutch*). The preceding chapter reported the characterisation of a 13.6 kDa protein with antimicrobial activity from skin secretions of *O. mykiss*. During that study, it was noted that the 70 % acetonitrile eluate from solid phase extraction contained at least one additional antimicrobial factor. In the present chapter the isolation and characterisation of this peptide is described.

4.3. Experimental Procedures

Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are aqueous and prepared with deionized water (Elga), except when stated otherwise. The list of suppliers and respective addresses is presented in appendix B.

4.3.1. Animals

Adult female rainbow trout were maintained under the conditions described in Section 3.3.1.

4.3.2. Sample Collection and Preparation of Epidermal Extracts

Mucous skin secretions and associated epidermal cells were collected as reported in Section 3.3.3. Acid-soluble protein extracts were prepared as detailed in Section 3.3.3.

4.3.3. Test Bacteria

The following bacterial strains were used: *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (NCIMB 12210), *Listonella (Vibrio) anguillarum* (NCIMB 2129) and *Planococcus citreus* (NCIMB 1493). All were grown to logarithmic phase in Mueller-Hinton broth (MHB) (Oxoid) at the appropriate temperature (please see Tables 3.1 and 3.2).

4.3.4. Antibacterial Assays

Antibacterial activity was assessed using a modified version of the two-layer radial diffusion assay of Lehrer *et al.* (1991b) as stated in Section 3.3.5, using the Gram-(+) bacterium *P. citreus* as the test organism during the protein purification procedure.

Determination of the minimal inhibitory concentration (MIC) of the purified peptide against each of the bacteria listed above was performed using the microtitre broth dilution assay (Friedrich *et al.*, 1999) using the protocol described in Section 3.3.5.

4.3.5. Muramidase Assay

Muramidase activity was tested by radial diffusion assay as described in Section 2.3.5.

4.3.6. Peptide Purification

The reconstituted lyophilised extract was subjected to cation exchange chromatography and $^{t}C_{18}$ solid phase extraction, as detailed in Section 3.3.7. The fraction eluted with 0.15 % (v/v) trifluoroacetic acid (TFA) in 70 % (v/v) acetonitrile (BDH) was lyophilised, resuspended in acidified deionized water and fractionated by C_{18} reversed phase HPLC under the conditions stated in Section 3.3.7. The active fractions eluting between 25 and 40 min were lyophilised, reconstituted in 1 ml acidified water and further purified by C_{18} reversed phase HPLC on the same column but under a shallower gradient (0 to 30 % acetonitrile over 75 min at a flow rate of 1 $ml \cdot min^{-1}$). One millilitre fractions were collected, lyophilised and reconstituted in 100 µl deionized water.

At each step, the purity and molecular weight of the proteins were estimated by high resolution polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE), as described in Section 2.3.6. The gels were stained with silver under alkaline conditions (Appendix C).

4.3.7. Protein Quantification

Estimation of total protein concentration by the method of Bradford (1976) and amino acid analysis of the purified peptide were performed as stated in Section 3.3.8.

4.3.8. Matrix-Associated Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

The molecular mass of the purified antimicrobial peptide was determined by MALDI-TOF MS at the Centre for Biomolecular Sciences (University of St Andrews, UK) according to the protocol described in Section 3.3.9.

4.3.9. Partial Primary Structure Determination

N-terminal amino acid sequencing was performed by standard automated Edman degradation on a Procise Sequencer (Applied BioSystems) at the Centre for Biomolecular Sciences (University of St Andrews, UK).

4.3.10.Sequence Analyses

The proteomics tools mentioned in Section 2.3.9 were employed to perform homology searches and prediction of protein parameters. Sequence alignments

were executed with the Omiga 2.0 sequence analysis software, as stated in Section 3.3.11.

4.3.11.Proteolytic Digestion

Susceptibility of the purified antimicrobial peptide to proteolytic digestion was ascertained as described in Section 3.3.13.

4.3.12.Preparation of Ribonucleoprotein Particles (RNPs) from Trout Liver

Three rainbow trout were fasted for 24 h in order to minimise contamination by particulate glycogen and then sacrificed by a Schedule 1 procedure as described in Section 2.3.2. Livers were collected and the RNPs isolated according to the protocol reported by Siekevitz (1962). In summary, 10 g of liver were homogenised in 10 ml of 1.46 M sucrose solution using a glass homogeniser and then diluted to 10 % (w/v) with 0.88 M (30 % (w/v)) sucrose solution. The homogenate was clarified by centrifugation at 4 °C for 30 min at 20 000 x g and the supernatant further centrifuged at 4 °C for 60 min at 105 000 x g (equivalent to $31 \cdot 10^3$ rpm on a 50.1 titanium rotor, Beckman). The microsomes were resuspended by homogenising in 90 ml 30 % sucrose. To this suspension 9.0 ml of 3 % (w/v) sodium deoxycholate, pH 7.5 - 7.8, was added with shaking and the clarified suspension was promptly centrifuged at 105 000 x g for 2 h at 4 °C.

The resultant pellet consisted almost entirely of free RNPs, with very little apparent membranous material (Siekevitz, 1962). This pellet was resuspended in

10 ml 20 mM HEPES, 100 mM KCl, 2 % (v/v) protease inhibitors cocktail, pH 7.0.

4.3.13. Extraction and Preliminary Fractionation of Antibacterial Proteins from Liver Ribonucleoprotein Particles

One millilitre suspension of RNPs prepared as above was acidified with 20 µl of 10 % (v/v) TFA. Following addition of 20 µl 10 % (v/v) Triton X-100, proteins were extracted by stirring for 30 min at 4 °C. The homogenate was then clarified by centrifugation at 13 000 x g for 20 min at 4 °C and the supernatant subjected to reversed phase HPLC on an ODS2-Inertpak C_{18} column. Proteins were eluted with a biphasic linear gradient of 0.1 % (v/v) TFA in deionized water and 0.09 % (v/v) TFA in acetonitrile, as follows: 0 to 60 % acetonitrile over 60 min, at a flow rate of 1 $ml \cdot min^{-1}$. One millilitre fractions were collected, lyophilised, reconstituted in 100 µl 20 mM HEPES, pH 7.0, and assayed for antibacterial activity as described in Section 3.3.5. Active fractions were analysed by SDS-PAGE as above.

4.4. Results

4.4.1. Purification of a 6.5 kDa Antimicrobial Peptide

The active fractions eluted with 0.8 M to 1.0 M NaCl during cation exchange chromatography were concentrated by solid phase extraction and subsequently subjected to C_{18} reversed phase HPLC, resulting in two groups of active fractions designated OM1 and OM2 (Fig. 3.5). Subsequent chromatography of OM1 fractions by C_{18} reversed phase HPLC on the same column but with a shallower water/acetonitrile gradient yielded a single peak with a retention time of 60.1 min that exhibited antibacterial activity against *P. citreus* (Fig. 4.1).

Activity was abolished after proteolytic digestion with proteinase K, confirming that this antibacterial factor has a proteinaceous nature. Only one protein band with an electrophoretic mobility of approximately 6.5 kDa could be observed on an SDS-PA gel after silver staining (Fig. 4.2). The yield of purified peptide, determined by amino acid analysis, was approximately 2 $ng \cdot g^{-1}$ mucus.

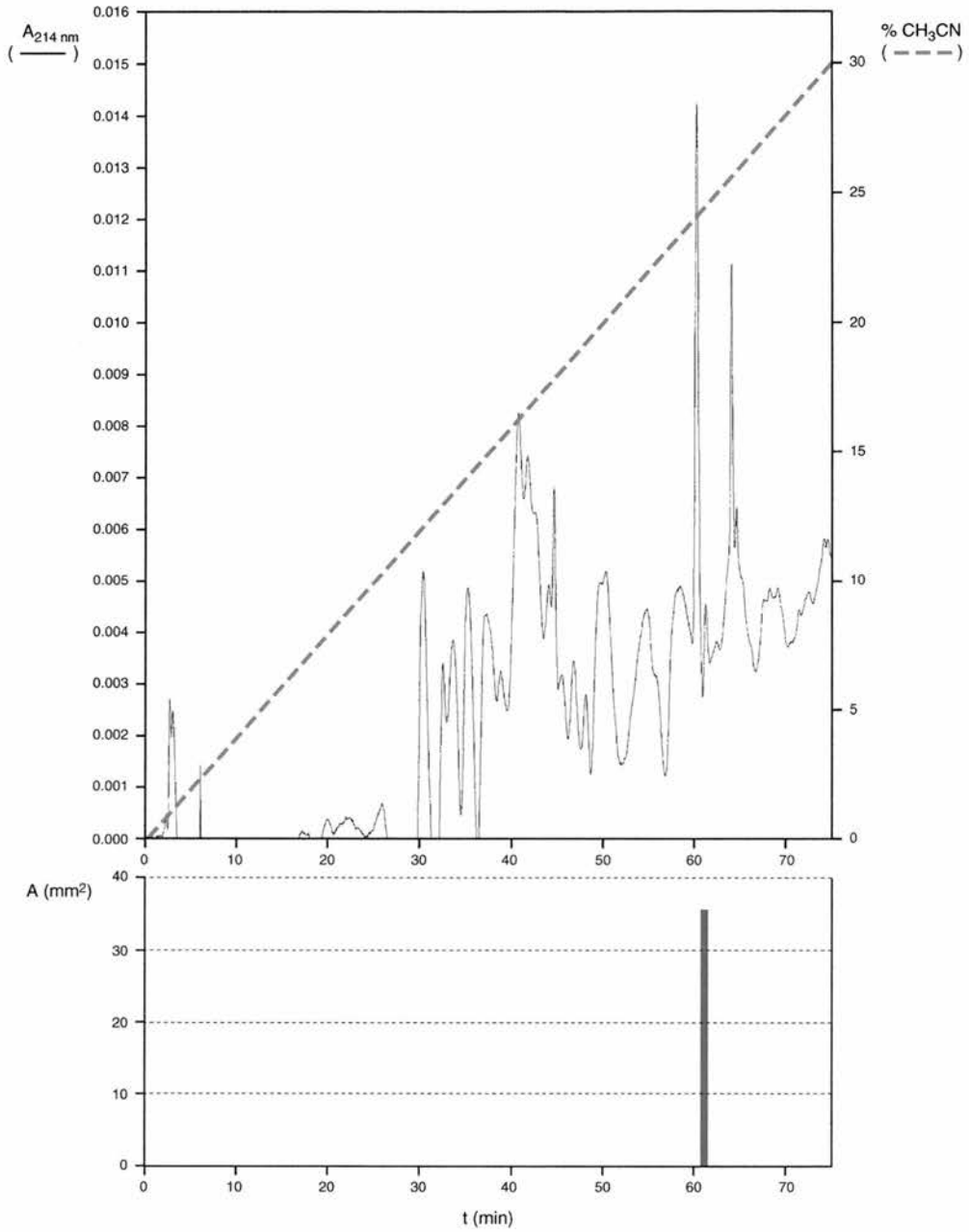


Figure 4.1. Purification of an antimicrobial peptide from trout skin extracts by C_{18} reversed phase chromatography. Acidic protein extracts of skin mucus and epidermal cells were fractionated by cation exchange chromatography on a CM Macro-Prep column and the active fractions subjected to solid phase extraction using $^{t}C_{18}$ Sep-Pak cartridges; the 70 % acetonitrile eluate was then applied to a C_{18} reversed phase column for HPLC. Fractions eluting between 24.4 % and 37.8 % acetonitrile were concentrated by lyophilisation, reconstituted in acidified water and subjected to another C_{18} reversed-phase HPLC using a shallower water/acetonitrile gradient, as indicated by the dashed line. The solid line represents the absorbance, monitored at 214 nm. The peak eluting at 60.7 min was found to be antibacterial to *P.citreus* (histogram).

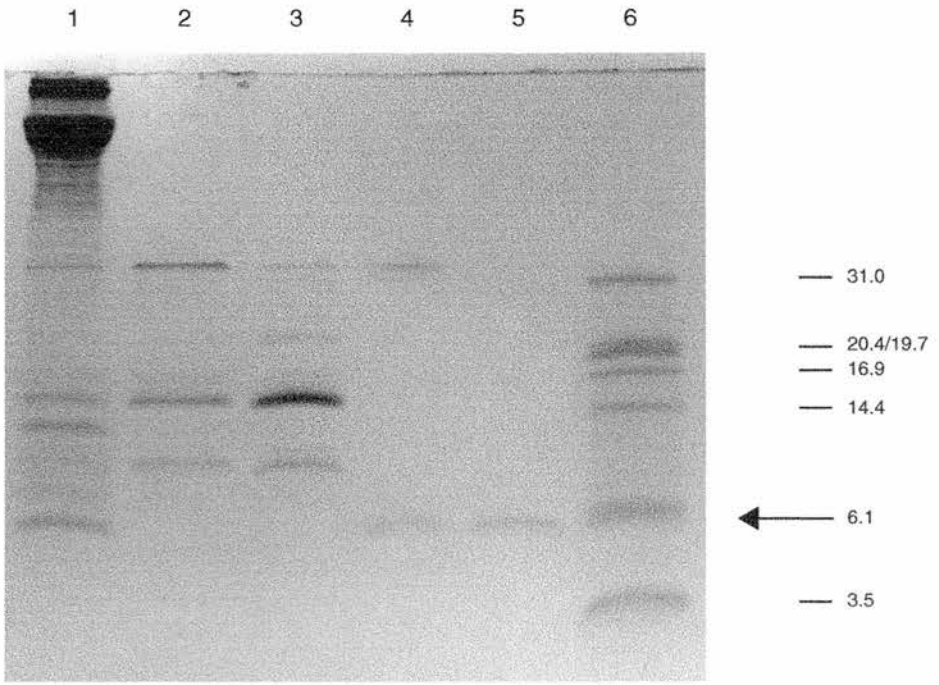


Figure 4.2. Protein profiles of the active fractions determined by Tris-Tricine SDS-PAGE. Lane 1: crude extract; lane 2: pooled cation exchange fractions; lane 3: 70 % acetonitrile eluate from solid phase extraction; lane 4: active fractions after the 1st C_{18} reversed-phase HPLC; lane 5: purified antimicrobial peptide after 2nd HPLC; lane 6: markers. Each lane contains 7.5 µl of sample. The numbers on the right hand side represent the molecular mass of the markers in kDa. Peptide of interest in lane 5 is indicated by an arrow.

4.4.2. Peptide Characterisation

Partial N-terminal amino acid sequencing of the purified peptide yielded the following sequence[#]: KVHGSLARAGK. BLAST homology searches showed that the first 11 amino acids of the purified peptide were an exact match to those of 40S ribosomal protein S30 (40S Rp S30) from medaka fish (Fig. 4.3). As

[#] This partial internal amino acid sequence has been deposited in the Swiss-Prot database under the accession number P83328.

shown in Table 4.1, the amino acid analysis results for the purified antimicrobial peptide are in good agreement with the expected amino acid composition of 40S Rp S30 from medaka fish, *Oryzias latipes*. Matrix-assisted laser desorption ionization/ time of flight (MALDI-TOF) mass spectrum showed a single signal at 6676.6 Da (Fig. 4.4).

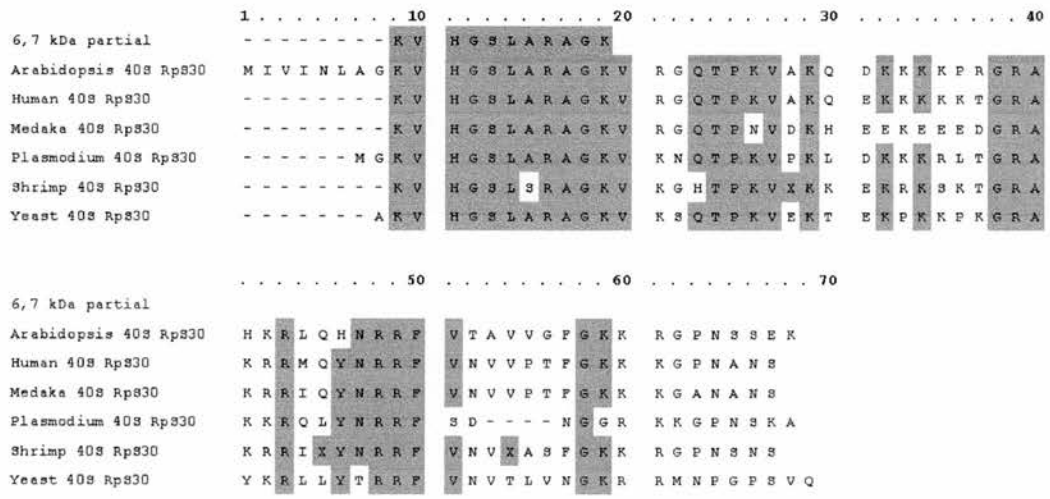


Figure 4.3. Partial N-terminal sequence of the antimicrobial peptide purified from skin of rainbow trout and its homology with 40S ribosomal protein S30. Identical residues between the purified antimicrobial peptide and the deduced sequences for the 40S ribosomal protein S30 from *Arabidopsis thaliana* (Swiss-Prot P49689), human (Swiss-Prot Q05472), medaka fish (Swiss-Prot Q9W6Y0), *Plasmodium falciparum* (Swiss-Prot O96269), Atlantic white shrimp (GenBank BF024580) and *Saccharomyces cerevisiae* (Swiss-Prot Q12087) are shaded.

Table 4.1. Amino acid analysis of the 6.5 kDa antimicrobial peptide purified from trout skin. Norleucine was used as internal standard. The results are compared with the predicted amino acid composition of 40S Rp S30 from medaka (Swiss-Prot Q9W6Y0).

Amino Acid	# Residues	Expected Composition
Ala	6	5
Arg	5	7
Asx	4	7
Cys	0	0
Glx	5	7
Gly	10	6
His	2	2
Ile	3	1
Leu	3	1
Lys	5	8
Met	0	0
Phe	2	2
Pro	2	2
Ser	5	2
Thr	5	2
Trp	not determined	0
Tyr	1	1
Val	3	6

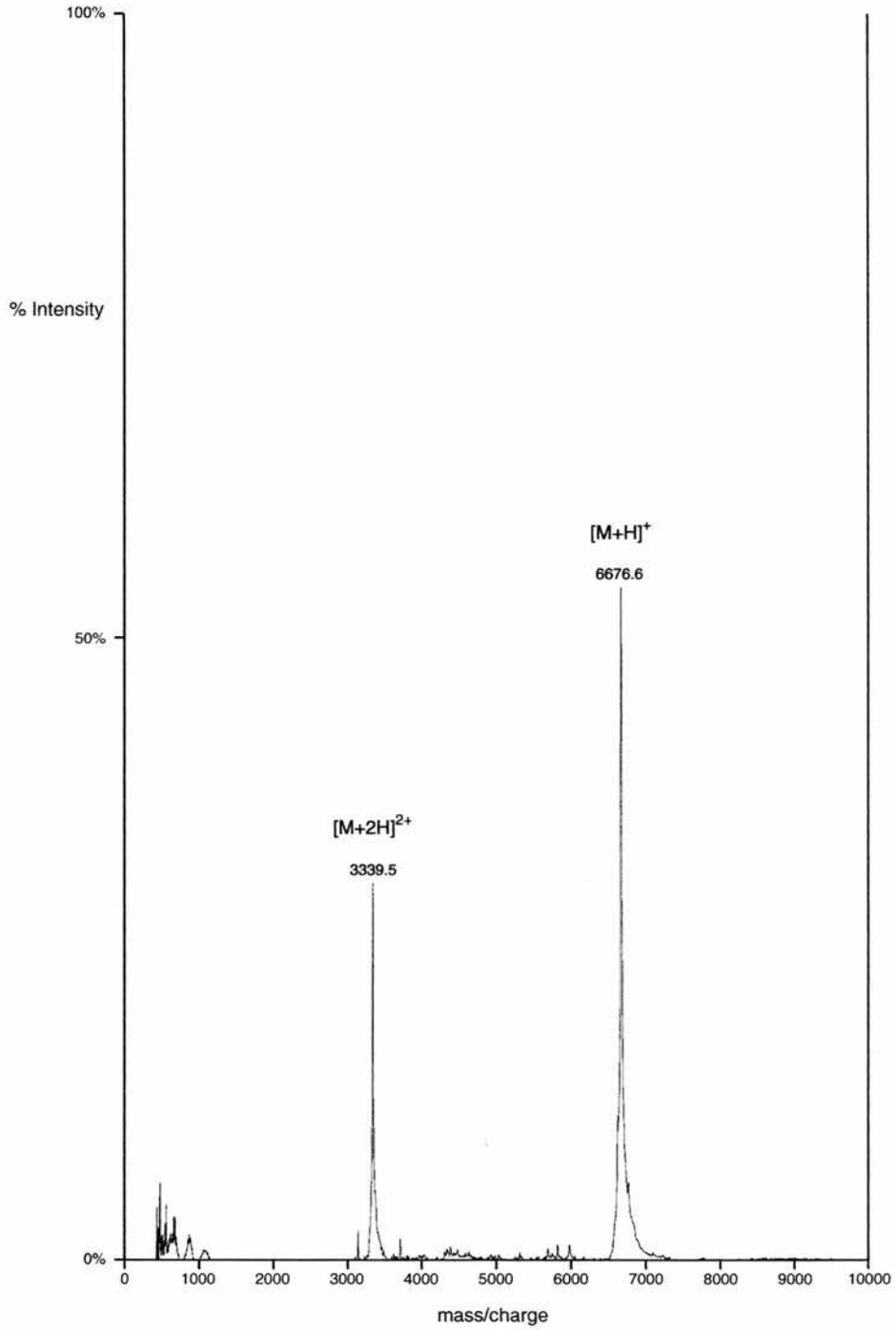
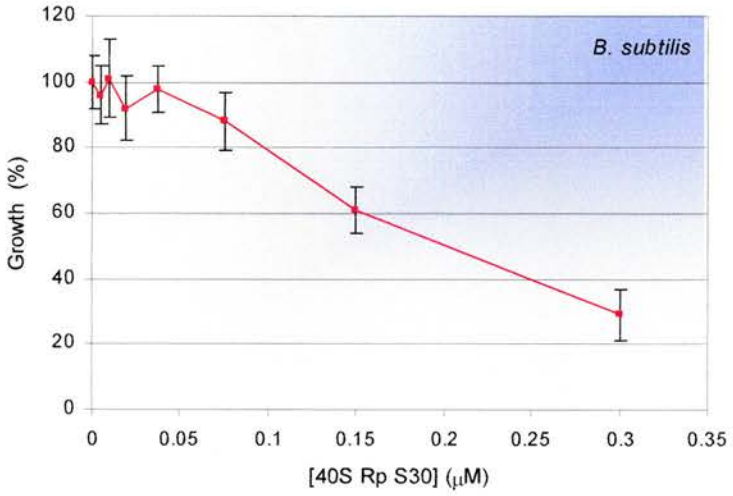


Figure 4.4. Mass spectrum of the purified peptide, determined by MALDI-TOF. The single ($[M+H]^+$) and double charged ($[M+2H]^{2+}$) molecular ions are labelled.

4.4.3. Antibacterial Activity

Quantification of activity using the microtitre broth dilution method revealed that the purified 6.5 kDa peptide is active at submicromolar concentrations against *B. subtilis* and *P. citreus*, with MICs in the range 0.15 - 0.3 μM and 0.02 - 0.04 μM, respectively (Fig. 4.5, Table 4.2).In addition, it is bactericidal to *P. citreus* with an MBC of 0.08 μM. The purified peptide was not active against Gram-(-) bacteria at the concentrations tested (maximum 0.3 μM, Table 4.2) and it did not have muramidase activity. By contrast, Cecropin P1 displays MIC values approximately 10 to 60 times higher against the same susceptible bacterial strains and is active mainly against Gram-(-) bacteria (Table 3.6).

(A)



(B)

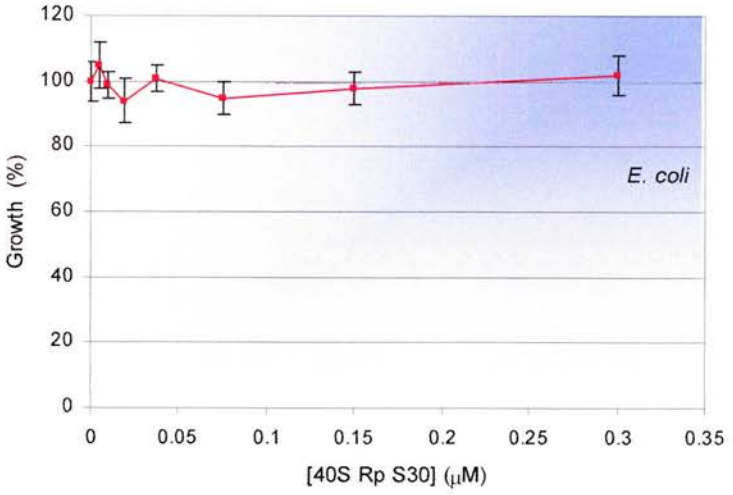
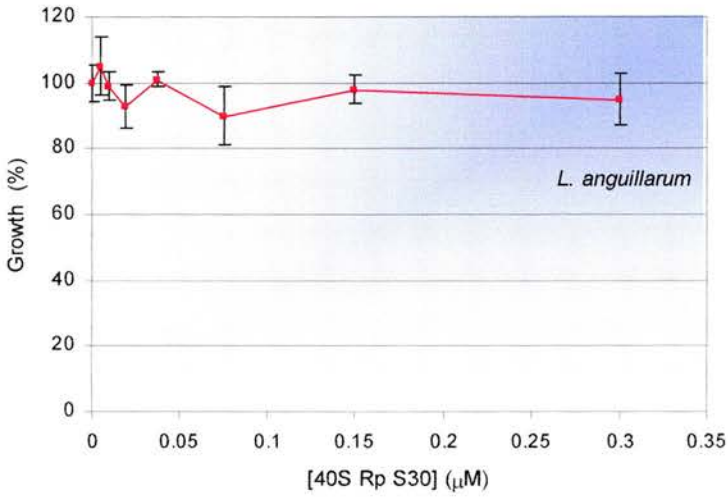


Figure 4.5. Antibacterial activity of the purified 6.5 kDa antimicrobial peptide against (A) *B. subtilis* or (B) *E. coli*. For the complete legend please consult the following page.

(C)



(D)

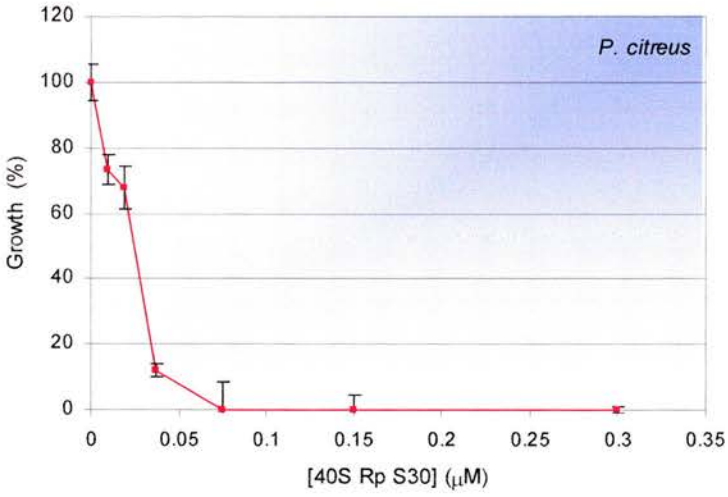


Figure 4.5. (continued) Antibacterial activity of the purified 6.5 kDa antimicrobial peptide against (C) *L. anguillarum* or (D) *P. citreus*. Bacterial suspensions containing 10^5 cfu·ml^{-1} were incubated at the appropriate temperature with serially diluted antimicrobial peptide. Bacterial growth is expressed as the ratio of optical densities read at 570 nm (OD_{570}) between each test sample and the control (no peptide added). The average final OD_{570} of the control was 0.2. Data are represented as mean ± SE, n=3.

Table 4.2. Bacteriostatic (MIC, μM) and bactericidal (MBC, μM) activities of the purified antimicrobial peptide 40S Rp S30. The MIC data for the 40S Rp S30 are obtained from the growth inhibition curves represented in Fig. 4.5. Cecropin P1 was used as standard.

		40S Rp S30		Cecropin P1	
Bacterium	**Gram Staining**	**MIC**	**MBC**	**MIC**	**MBC**
B. subtilis	+	0.15-0.3	> 0.3	> 2.4	> 2.4
P. citreus	+	0.02-0.04	0.08	1.2-2.4	> 2.4
E. coli	-	> 0.3	> 0.3	0.15-0.3	0.6
L. anguillarum	-	> 0.3	> 0.3	0.04-0.08	0.3

4.4.4. Antibacterial Factors from Liver Ribonucleoprotein Particles

A protein extract prepared from trout liver RNPs (total protein concentration of approximately 0.8 $mg \cdot ml^{-1}$) was fractionated by C_{18} reversed phase HPLC (Fig. 4.6). A group of fractions eluted with 37 % to 43 % acetonitrile was found to display antibacterial activity against *P. citreus* (Fig. 4.6) without detectable muramidase activity. The proteinaceous nature of the antibacterial factor(s) was confirmed by proteolytic digestion with proteinase K. All the active fractions contained several proteins with apparent molecular weights ranging from *circa* 8 kDa to 31 kDa (Fig. 4.7).

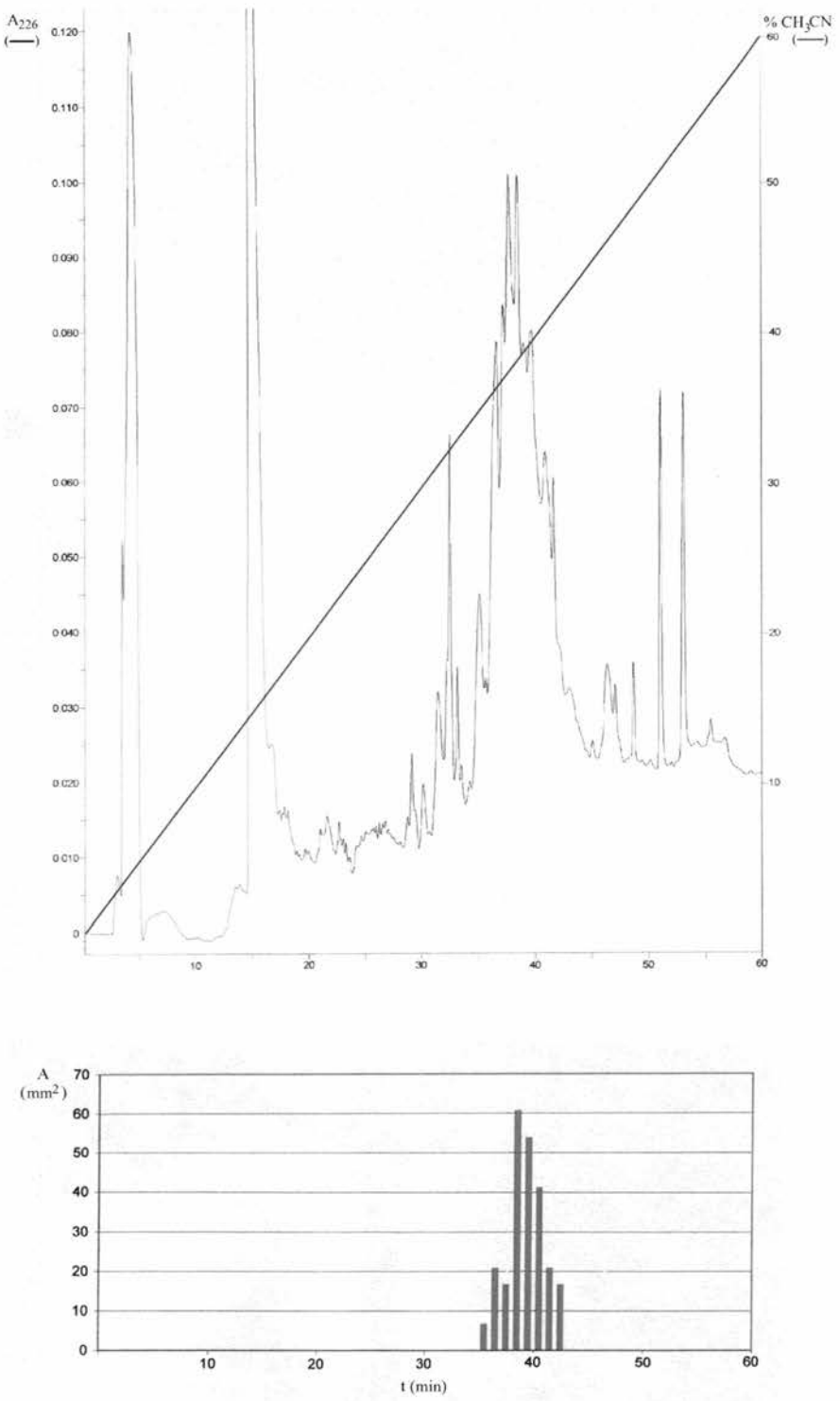


Figure 4.6. Fractionation of antibacterial proteins from liver RNPs by C_{18} reversed phase chromatography. Acidic protein extracts of trout liver RNPs were fractionated by C_{18} reversed phase HPLC on an ODS-2 column. The black line represents the absorbance, monitored at 226 nm. The proportion of acetonitrile is indicated by the blue line. Fractions eluted with 37 % to 43 % acetonitrile were found to be antibacterial to *P. citreus* (histogram).

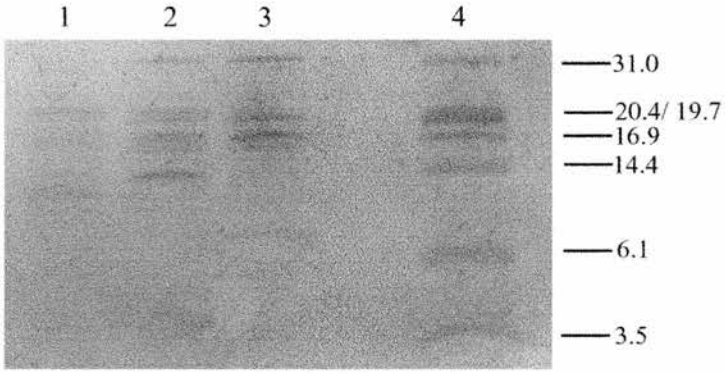


Figure 4.7. Protein profiles of the active C_{18} reversed phase HPLC fractions determined by Tris-Tricine SDS-PAGE. Acidic protein extracts of trout liver RNPs were fractionated by C_{18} reversed phase HPLC. The peak fractions displaying antibacterial activity against *P. citreus* were analysed by SDS-PAGE. Lane 1: fraction 39; lane 2: pooled fraction 40; lane 3: fraction 41; lane 4: markers. Each lane contains 7.5 µl of sample. Proteins were visualised by silver staining. The numbers on the right hand side represent the molecular mass of the markers in kDa.

4.5. Discussion

The present chapter describes the purification and partial characterisation of a cationic antimicrobial peptide from skin secretions of rainbow trout, collected following gentle surface abrasion. It also demonstrates the presence of a proteinaceous antibacterial factor in trout liver ribosomes, which has a molecular mass different from the peptide present in skin mucus. To the best of my knowledge, antimicrobial activity of ribosomal peptides or proteins from a non-mammalian animal has not been previously reported.

The purified antimicrobial peptide displays potent antibacterial activity against the Gram-(+) bacteria tested, with MIC values up to 60 times smaller than cecropin P1. The full identity between its partial primary structure and that of 40S Rp S30 from medaka fish indicates that the antimicrobial peptide purified from trout is identical or highly similar to 40S ribosomal protein S30 (40S Rp S30), a component of the small subunit of eukaryotic ribosomes. This finding is further supported by mass spectrometry analysis, which showed that this peptide's molecular mass (6676.6 Da) is very similar to 40S Rp S30 from medaka (6660 Da) (Henrich & Wittbrodt, 2000). Moreover, preliminary experiments have detected antibacterial activity in RNPs isolated from liver cells of rainbow trout. Further work is required to optimise the protocol for isolation of both the 40S Rp S30 and the additional antibacterial factor(s) from trout liver ribosomes. The lack of success of these early purification attempts can probably be attributed to an inefficient extraction procedure. It has proven extremely difficult to dissociate the ribosomes and precipitate the rRNA without the concomitant precipitation of the associated proteins.

Previous reports have shown that cecropin-like peptides found in *Helicobacter pylori* actually originate from the N-terminal region of the Rp L1 (Pütsep *et al.*, 1999a), indicating that cecropins may have evolved from Rp L1 of an ancestral intracellular pathogen (Pütsep *et al.*, 1999b). The involvement of ribosomal proteins in innate immunity is corroborated by the recent finding of ubiquicidin in the cytosolic fraction of IFN-γ activated murine macrophages (Hiemstra *et al.*, 1999). In that paper Hiemstra and collaborators have shown that the antimicrobial peptide is likely to be 40S Rp S30. The present finding of 40S Rp S30 in skin secretions of an ectothermic lower vertebrate demonstrates that it is confined neither to mammals nor to peripheral leukocytes. As shown in Fig. 4.3, 40S Rp S30 appears to be conserved throughout widely separated taxa, raising the possibility of a ubiquitous role for 40S Rp S30 in innate immunity. Moreover, this is one of the differentially expressed genes in immunostimulated Atlantic white shrimp, *Litopenaeus setiferus* (Gross *et al.*, 2001), a result that lends further credence to the potential role of 40S Rp S30 as a defence factor. In mammals, 40S Rp S30 from murine macrophages has been found to be active against the intracellular pathogen *Listeria monocytogenes* (Hiemstra *et al.*, 1999). As several bacterial pathogens induce apoptosis (Hilbi *et al.*, 1998) or cell lysis, 40S Rp S30 could be released from the cytosol to extracellular locations where it could exert its role as an antimicrobial peptide.

Chapter 5

Isolation and Characterisation of Oncorhyncin II, a Histone H1-derived Antimicrobial Peptide from Skin Secretions of *Oncorhynchus mykiss*

5.1. Synopsis

The present chapter reports the purification and characterisation of a potent 7.2 kDa antimicrobial peptide from an acid extract of the epithelial mucus layer of rainbow trout skin. This peptide, which is tentatively named oncorhyncin II, was purified to apparent homogeneity through cation exchange chromatography and C_{18} reversed phase HPLC. Amino acid sequence analysis showed that the first 17 residues of oncorhyncin II are identical to those of histone H1 from rainbow trout. Matrix-assisted laser desorption ionization mass spectrometry revealed that the purified peptide has a molecular mass of 7195.3 Da. Taken together, these data imply that oncorhyncin II is a 69-residue C-terminal fragment of histone H1, probably phosphorylated at two residues. Oncorhyncin II has minimal inhibitory concentrations in the submicromolar range against Gram-(+) as well as Gram-(-) bacteria and it does not display significant haemolytic activity towards trout erythrocytes. The purified peptide was found to induce a marked destabilisation of planar lipid bilayers without the formation of stable ion channels, suggesting that its antibacterial properties are not directly dependent on its pore-forming ability. Oncorhyncin II is a cleavage product of histone H1 that has the potential to play an important role in mucosal defence of rainbow trout.

5.2. Introduction

Relatively few antimicrobial peptides have been isolated from epithelial surfaces of teleosts. In the two preceding chapters the purification and characterisation of two antimicrobial factors from skin exudates of *Oncorhynchus mykiss* was described. These proteins were present in the 70 % acetonitrile eluate from solid phase extraction of the acid-soluble skin mucus extract. The current investigation was directed to ascertain the presence and character of additional antimicrobial agents in the fraction eluted with 20 % acetonitrile during $^{t}C_{18}$ solid phase extraction.

5.3. Experimental Procedures

Chemicals were acquired from Sigma-Aldrich, except when specified otherwise. Unless stated elsewise, all solutions are aqueous and prepared with deionized water (Elga). The various suppliers and their respective addresses are listed in appendix B.

5.3.1. Animals

Adult female rainbow trout were maintained under the conditions detailed in Section 3.3.1.

5.3.2. Sample Collection and Preparation of Epidermal Extracts

Trout skin exudates were collected as reported in Section 3.3.3. Acid-soluble protein extracts were prepared as described in Section 3.3.3.

5.3.3. Test Bacteria

Antibacterial activity was tested against the following strains: *Escherichia coli* (NCIMB 12210), *Listonella (Vibrio) anguillarum* (NCIMB 2129), *Micrococcus luteus* (NCIMB 376) and *Planococcus citreus* (NCIMB 1493). Each microorganism was grown to logarithmic phase in Mueller-Hinton broth (MHB) (Oxoid) at the appropriate temperature (please consult Table 3.2) before washing in sterile saline (*circa* 1.5 % (w/v) NaCl for *P. citreus*; 0.8 % (w/v) NaCl for non-marine strains) and resuspension in MHB as described below.

5.3.4. Antibacterial Assays

Antibacterial activity was assessed during the protein purification procedure using a modified version of the two-layer radial diffusion assay of Lehrer *et al.* (1991b) as reported in Section 3.3.5. The Gram-(+) bacterium *P. citreus* was employed as the test organism during the protein purification procedure.

Minimal inhibitory concentration (MIC) assays of the purified peptide against each of the bacteria listed above were performed by microtitre broth dilution assay (Friedrich *et al.*, 1999), according to the protocol described in Section 3.3.5.

5.3.5. Muramidase Assay

Muramidase activity was examined by lysoplate assay as described in Section 2.3.5.

5.3.6. Peptide Purification

The reconstituted lyophilised extract was applied to a CM Macro-Prep column for cation exchange chromatography at pH 7.0, as described in Section 3.3.7. The active fractions with retention times between 90 and 110 min were acidified to a final concentration of 0.15 % (v/v) trifluoroacetic acid (TFA) and subjected to solid phase extraction on Sep-Pak Vac 5g $^{t}C_{18}$ cartridges (Waters), previously equilibrated with 0.15 % (v/v) TFA. The proteins of interest were eluted with 0.15 % (v/v) TFA in 20 % (v/v) acetonitrile, lyophilised, reconstituted in deionized water containing 0.1 % (v/v) TFA and fractionated by C_{18} reversed phase HPLC using a biphasic gradient of 0.1 % (v/v) TFA in water and 0.09 % (v/v) TFA in acetonitrile, as depicted in Fig. 5.2. Fractions displaying

antimicrobial activity and eluting between 39 and 41 min were lyophilised, reconstituted in 250 µl acidified water and further purified by C_{18} reversed phase HPLC on the same column but using a linear gradient with a lower slope (0 to 20 % acetonitrile over 60 min at a flow rate of 1 $ml \cdot min^{-1}$). One minute fractions were collected, lyophilised and reconstituted in 200 µl deionized water. A single peak eluting at 50.9 min was found to contain the antimicrobial activity.

The purity and molecular weight of the protein fractions obtained throughout the purification procedure were estimated by Tris-tricine polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE), as described in Section 2.3.6. The gels were silver stained under alkaline conditions (please consult appendix C).

5.3.7. Protein Quantification

Total protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin (Pierce) as standard. The purified peptide was quantified by amino acid analysis at the Protein and Nucleic Acid Chemistry facility (University of Cambridge, UK) using the post-column ninhydrin method.

5.3.8. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

Information concerning the molecular weight of the purified antimicrobial peptide was obtained by MALDI-TOF MS at the Centre for Biomolecular Sciences (University of St Andrews, UK) according to the protocol detailed in Section 3.3.9.

5.3.9. Partial Primary Structure Determination

Amino acid sequencing of the purified peptide was performed by standard automated Edman degradation on a Procise Sequencer (Applied BioSystems) at the Centre for Biomolecular Sciences (University of St Andrews, UK).

5.3.10.Sequence Analyses

The proteomics tools listed in Section 4.3.10 were used to predict protein parameters and to perform homology searches sequence alignments. The NetPhos 2.0 prediction server (Blom *et al.*, 1999) was employed to prevision serine and threonine phosphorylation sites.

5.3.11.Proteolytic Digestion

Proteolytic digestion of the purified antimicrobial peptide with proteinase K (please consult Section 3.3.13) was used to confirm its proteinaceous nature.

5.3.12.Haemolysis Assay

The assay of haemolytic activity of the purified peptide against trout erythrocytes was performed according to the protocol detailed in Section 3.3.15.

5.3.13.Planar Lipid Bilayer Assay

Macroscopic and single-channel experiments were used to investigate the ion channel properties of the purified antimicrobial peptide. These were performed by Dr Gérard Molle at the Centre de Biochimie Structurale (University of Montpellier, France), as described in Section 3.3.16.

5.4. Results

5.4.1. Purification of a 7.2 kDa Antimicrobial Peptide

The active fractions with retention times between 90 and 110 min on a cation exchange column (Fig. 3.3) were pooled and concentrated by solid phase extraction. The 20 % acetonitrile eluate, found to be active against *P. citreus*, *E. coli* and *S. cerevisiae* (Fig. 5.1), was fractionated by C_{18} reversed phase HPLC (Fig. 5.2). Subsequent chromatography of the active fractions by C_{18} reversed phase HPLC on the same column, but with a shallower water/ acetonitrile gradient, yielded a single peak with a retention time of 50.9 min (equivalent to 17 % acetonitrile) that corresponded to the fractions exhibiting antibacterial activity against *P. citreus* (Fig. 5.3). Only one protein band with an electrophoretic mobility of approximately 8 kDa could be observed on an SDS-PA gel after silver staining (Fig. 5.4). The concentration of purified peptide, determined by amino acid analysis, was *circa* 60 $\mu g \cdot ml^{-1}$ and its yield was approximately 80 $ng \cdot g^{-1}$ skin mucus.

Proteolytic treatment with proteinase K depleted the active fractions of their antibacterial properties, thus confirming that the active agent has a proteinaceous nature. The purified antimicrobial peptide was found to be thermostable, retaining its activity even after incubation at 80 °C for 5 min.

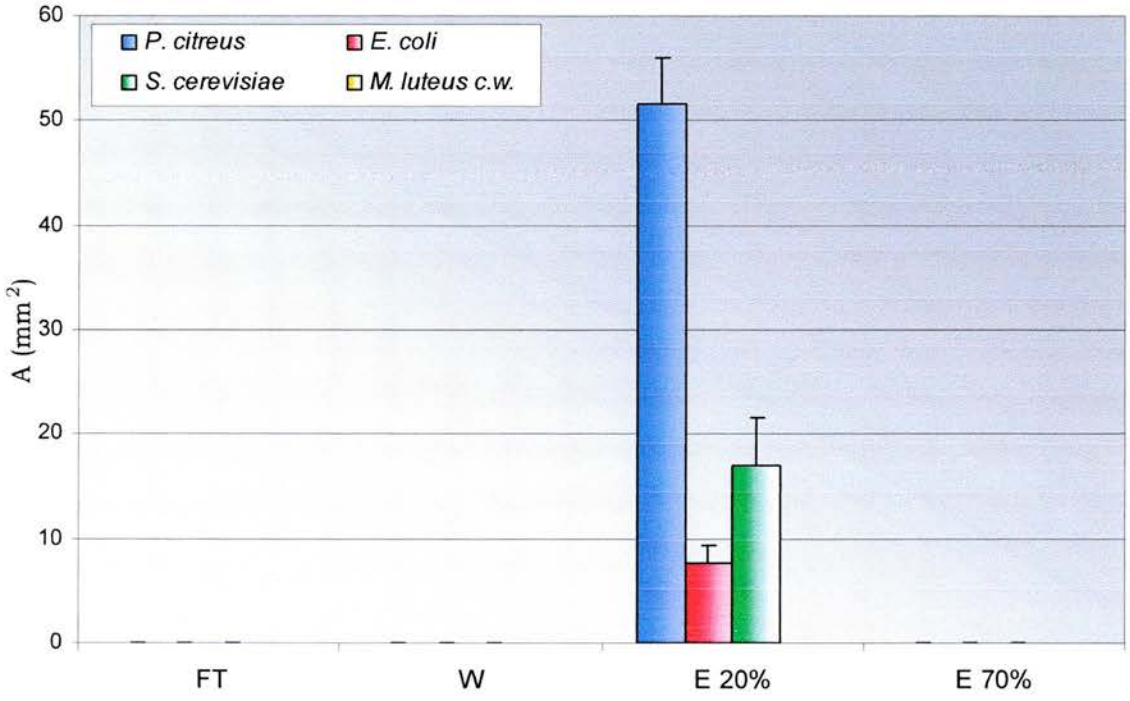


Figure 5.1. Antimicrobial activity profile of the $^{t}C_{18}$ solid phase extraction fractions. The acid-soluble skin mucus extract was subjected to cation exchange chromatography and the active fractions eluting between 90 and 110 min (Fig. 3.3) were pooled and concentrated by solid phase extraction on $^{t}C_{18}$ Sep-Pak cartridges. Following a wash with 0.15 % (v/v) TFA in water, proteins were sequentially eluted with 0.15 % (v/v) TFA in 20 % (v/v) acetonitrile (E 20 %) and 0.15 % (v/v) TFA in 70 % (v/v) acetonitrile (E 70 %). The histogram shows the antimicrobial activity profiles of the different solid phase extraction fractions, expressed as the area of the clear zones (in mm^2) obtained on a radial diffusion assay against *P. citreus*, *E. coli*, *S. cerevisiae* or *M. luteus* cell walls. Data are represented as means ± SE, n = 3.

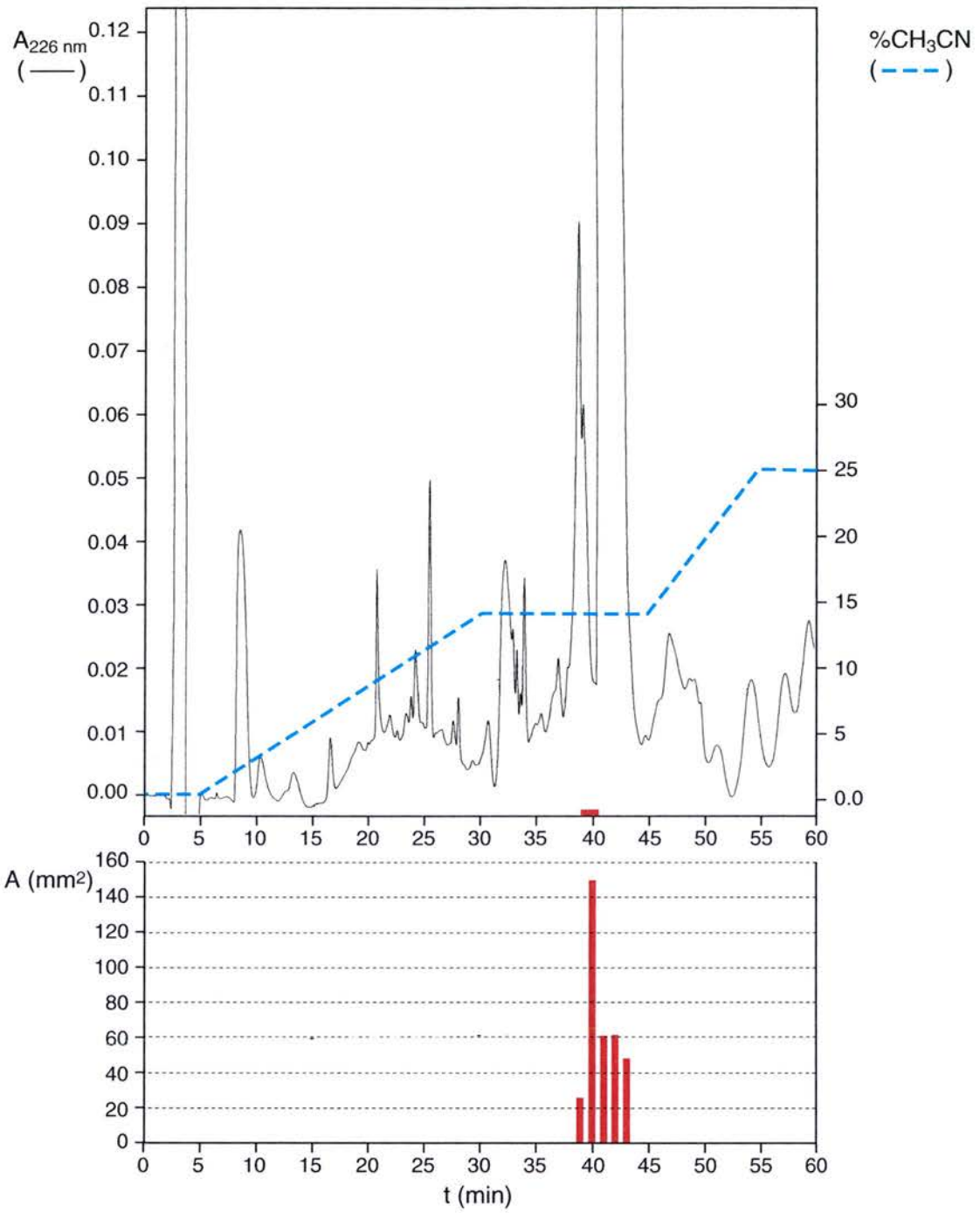


Figure 5.2. C_{18} reversed phase HPLC of the 20 % acetonitrile eluate from $^{t}C_{18}$ solid phase extraction. Absorbance was monitored at 226 nm (solid line). The red bar indicates the peak of interest. The histogram denotes the antibacterial activity profile against *P. citreus*. The biphasic water/ acetonitrile gradient is represented by the blue dashed line.

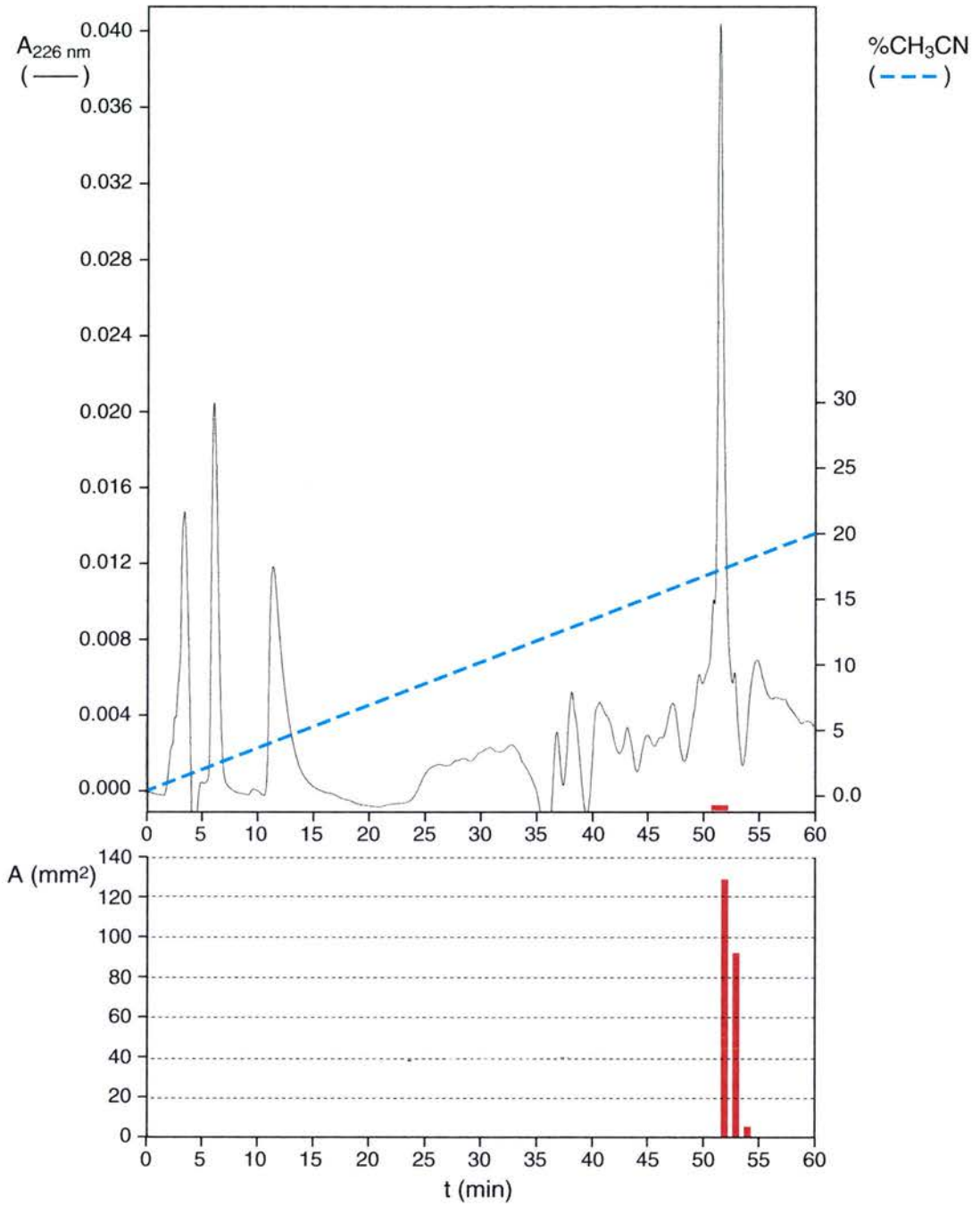


Figure 5.3. Final step in the isolation of a 7.2 kDa antimicrobial protein from skin secretions of rainbow trout. The active fractions eluted between 39 min and 42 min during C_{18} reversed phase HPLC (Fig. 5.2) were pooled and further fractionated by C_{18} reversed phase HPLC using a shallower water/acetonitrile gradient (blue dashed line). The chromatogram was obtained at 226 nm (solid line). The peak eluting at 34.6 min as indicated by the red bar corresponded to fractions that were antibacterial to *P. citreus* (histogram).

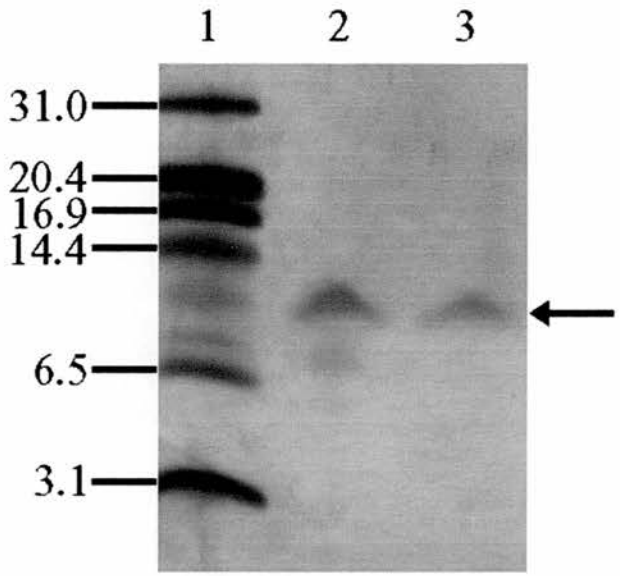


Figure 5.4. Tris-Tricine SDS-PAGE analysis of the active fractions obtained during the last two purification steps of an antimicrobial protein from skin secretions of rainbow trout. Lane 1: markers; lane 2: pooled active fractions from the 1st HPLC (fractions 40 - 43, Fig. 5.2); lane 3: purified antimicrobial peptide after 2nd HPLC (pooled fractions 52 and 53, Fig. 5.3). Each lane contains 7.5 µl of sample. The numbers on the left hand side correspond to the molecular mass of the markers in kDa. The peptide of interest is indicated by the arrow.

5.4.2. Peptide Characterisation

N-terminal amino acid sequencing of the antimicrobial peptide purified from trout skin secretions in the present study yielded the following sequence[#]: KAVAAKKSPKKAKKPAT. BLAST homology searches showed that this sequence shares 100 % identity with residues 138 to 154 of histone H1 from rainbow trout (Fig 5.5).

Mass spectrometry analysis by MALDI-TOF of the purified protein revealed a signal corresponding to the single charged molecular ion at 7195.3 Da (Fig 5.6). The difference between the predicted mass, based on the amino acid sequence of the trout histone H1 fragment comprising residues 138 to 206, and the experimental mass obtained for this peptide may be due to possible post-translational modifications, namely phosphorylations. Prediction of phosphorylation sites in this peptide using the NetPhos 2.0 server identified 3 plausible serine phosphorylations at positions 8, 24 and 41, as well as a possible phosphorylated threonine at position 17 (Fig. 5.7). Therefore, this 7.2 kDa antimicrobial peptide is likely to be a 69-residue C-terminal fragment of histone H1 (residues 138 to 206) containing two phosphorylated amino acids.

[#] This partial internal amino acid sequence has been lodged in the TrEMBL database under the accession number P83374.

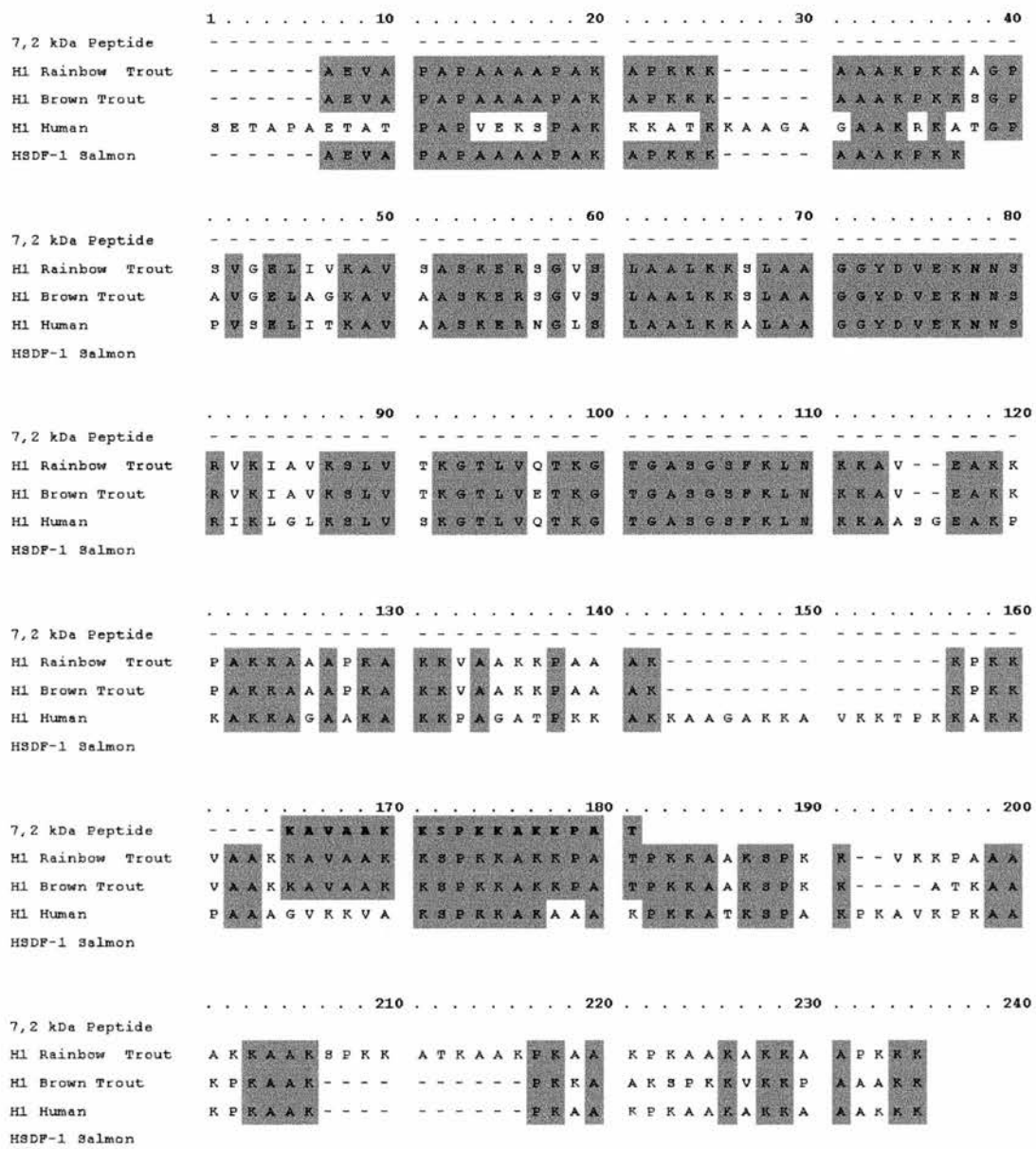


Figure 5.5. Partial N-terminal amino acid sequence of the 7.2 kDa antimicrobial peptide purified from skin mucus of rainbow trout and its similarity with histone H1. Identical residues between the purified antimicrobial peptide, histone H1 from rainbow trout (Mezquita *et al.*, 1984), brown trout histone H1 (Macleod *et al.*, 1977), human histone H1a (Ohe *et al.*, 1989) and a histone H1-derived antimicrobial peptide (HDSF-1) from coho salmon (Patrzykat *et al.*, 2001) are indicated by a shaded box.

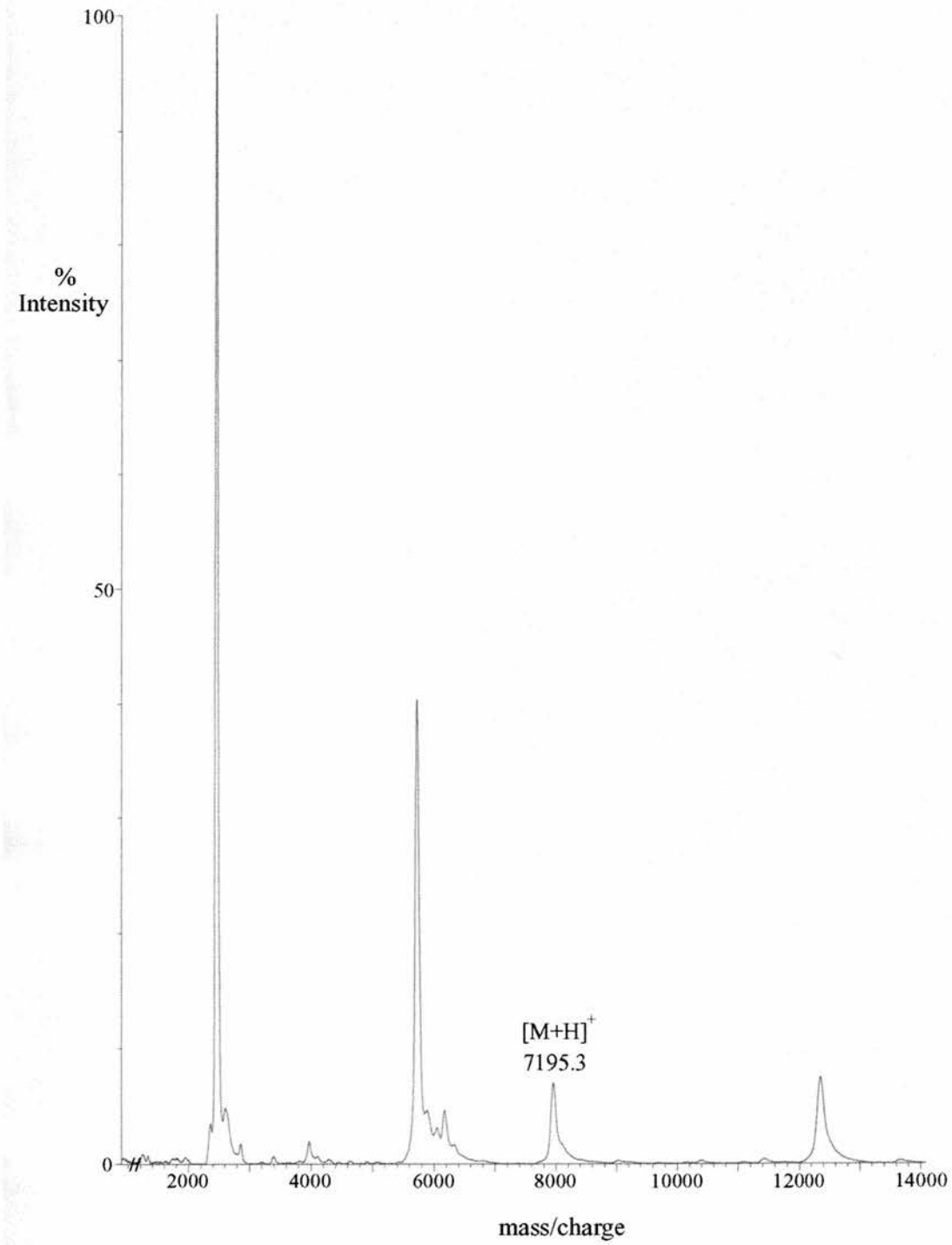


Figure 5.6. MALDI-TOF mass spectrum of the purified antimicrobial peptide. The peak corresponding to the single charged molecular ion is labelled. The remaining peaks correspond to the following internal standards: equine cytochrome c (12360.1 Da), bovine insulin (5733.5 Da) and ACTH "clip" peptide (residues 18 - 39, 2465.7 Da).

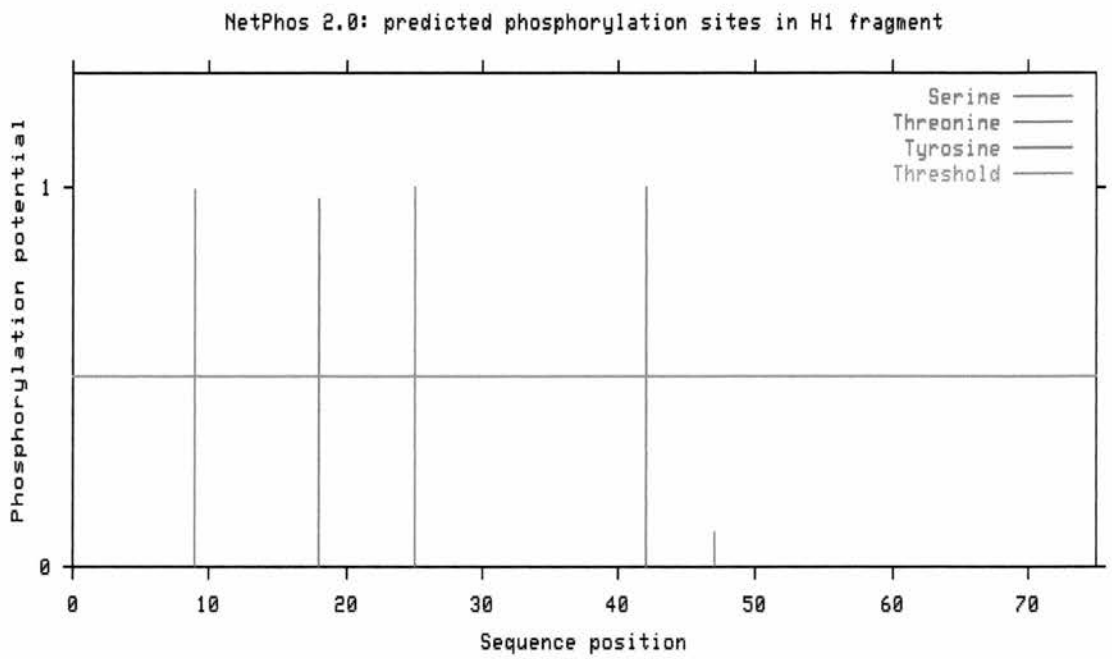


Figure 5.7. Prediction of phosphorylation sites in the C-terminal fragment of rainbow trout histone H1. The peptide sequence comprising residues 138 to 206 of trout histone H1 (Mezquita *et al.*, 1984) was submitted to the NetPhos 2.0 server for detection of possible phosphorylation sites. Three serine phosphorylation sites are predicted in positions 8, 24 and 41 with scores of 0.995, 0.997 and 0.998, respectively. In addition, one plausible threonine phosphorylation site is also present at position 17 with a score of 0.968.

5.4.3. Antibacterial and Haemolytic Activity

Quantification of antibacterial activity using the microtitre broth dilution method showed that the purified 7.2 kDa peptide is active at submicromolar concentrations, with MICs in the ranges 0.2 μM to 0.4 μM against *M. luteus* and *P. citreus* (Fig. 5.8A, B). These values are over 10-fold lower when compared with the MICs of cecropin P1 against the same bacteria. The 7.2 kDa peptide was

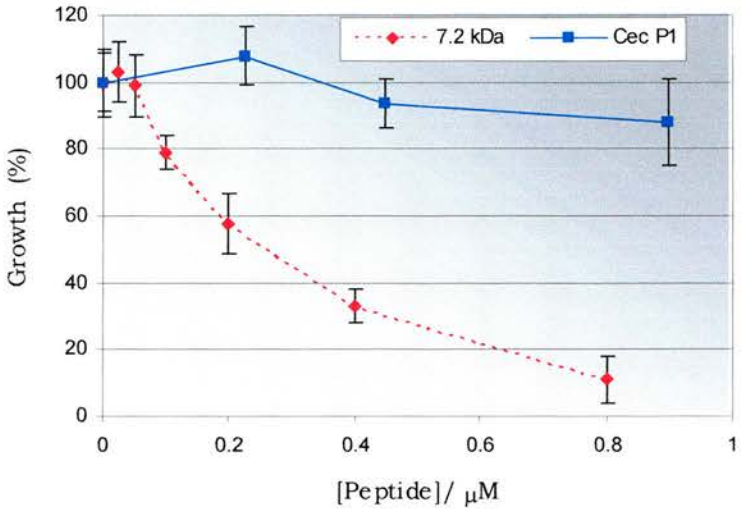
also found to be active against the Gram-(-) bacteria *E. coli* and *L. anguillarum*, with MICs of 0.4 μM to 0.8 μM (Fig. 5.8C, D). The purified histone fragment had no detectable muramidase activity. No significant haemolytic activity against trout erythrocytes was observed at concentrations of the purified peptide up to 0.8 μM. At this concentration the 7.2 kDa trout peptide caused only *circa* 7 % haemolysis, whilst the haemolytic peptide melittin (DeGrado *et al.*, 1982) lysed approximately 43 % of the erythrocytes (Fig. 5.9).

5.4.4. Pore-forming Activity[#]

Reconstitution of the purified 7.2 kDa antimicrobial peptide ($5 \cdot 10^{-8}$ M - $5 \cdot 10^{-7}$ M) on lipid bilayers induced a strong destabilisation of membranes without the formation of stable pores (no I-V curves could be recorded). Similarly, at a peptide concentration ranging from $2 \cdot 10^{-9}$ M to 10^{-8} M, single channel experiments showed large bursts that corresponded to the permeabilisation of the membrane.

[#] These results were obtained by Dr Gérard Molle at the Centre de Biochimie Structurale (University of Montpellier, France).

(A)



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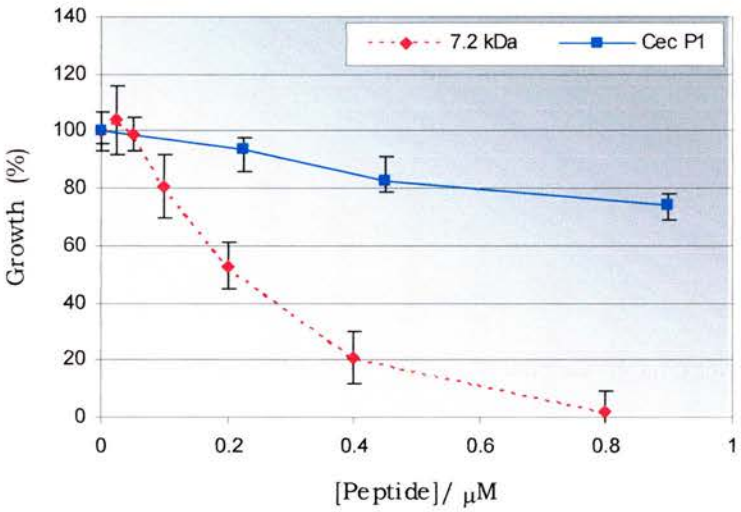
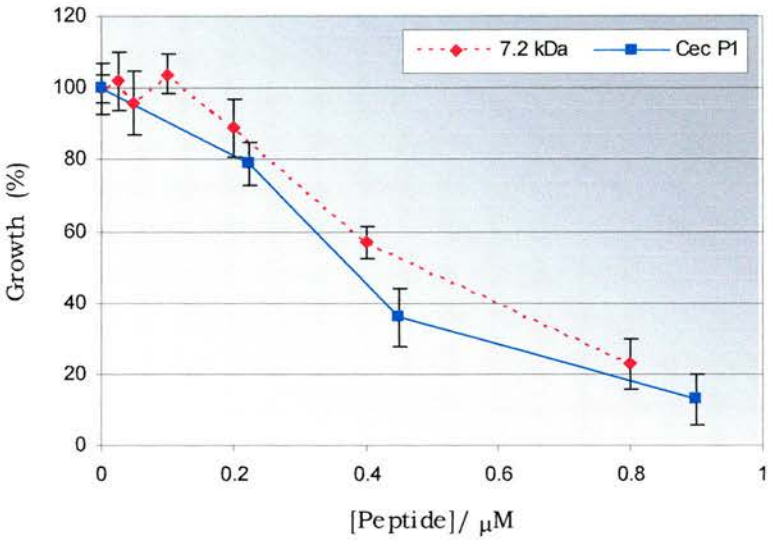


Figure 5.8. Quantification of antibacterial activity of the purified 7.2 kDa peptide against (A) *M. luteus* or (B) *P. citreus*. For the complete legend please consult the following page.

(C)



(D)

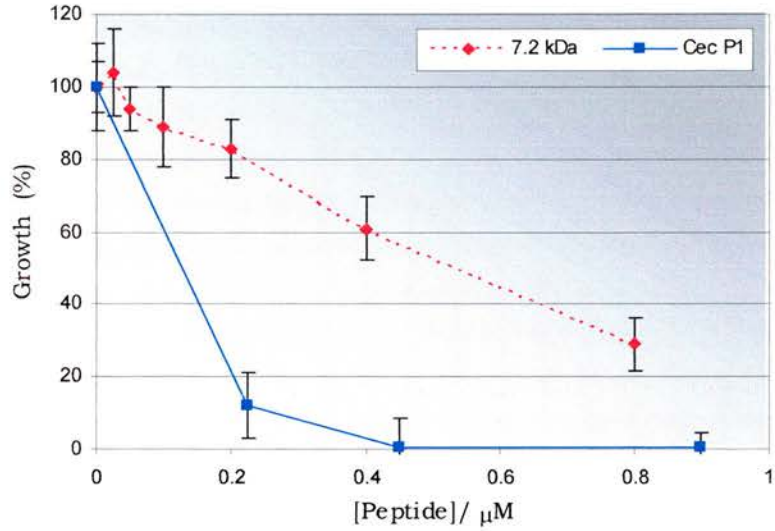


Figure 5.8. (continued) Quantification of antibacterial activity of the purified 7.2 kDa peptide against (C) *E. coli* or (D) *L. anguillarum*. Bacterial suspensions containing 10^5 cfu·ml^{-1} were incubated with serially diluted cecropin P1 (Cec P1) or with the purified 7.2 kDa antimicrobial peptide at the appropriate temperature (please see Table 3.2). Bacterial growth is expressed as the ratio of optical densities read at 570 nm (OD_{570}) between each test sample and the control (no peptide added). The average final OD_{570} of the control was approximately 0.2. Data are represented as means ± SE, n=3.

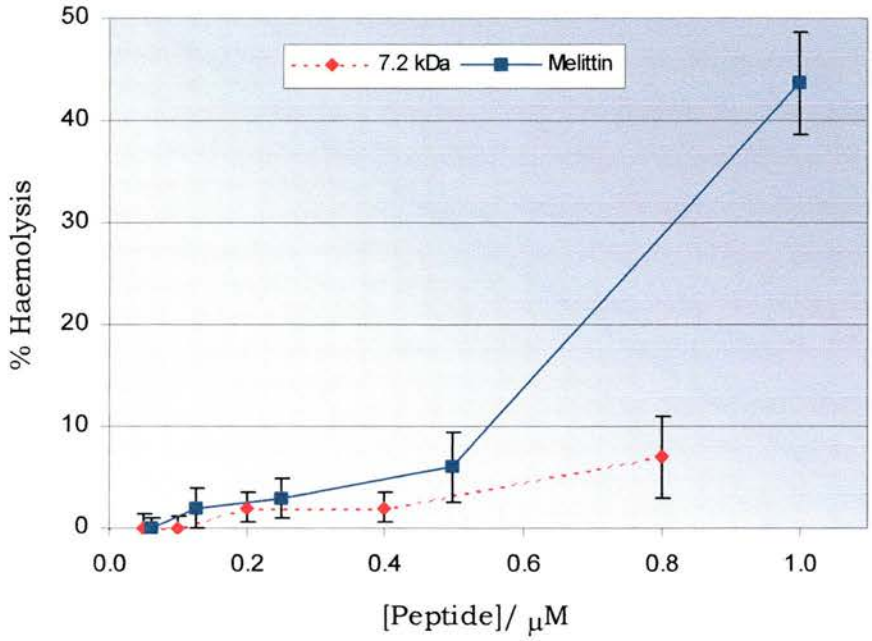


Figure 5.9. Haemolysis assay of the purified 7.2 kDa antimicrobial peptide against trout erythrocytes. A 2 % (v/v) suspension of erythrocytes was incubated with serially diluted test samples for 30 min at 37 °C. Percent haemolysis is defined as the ratio of absorbances (read at 545 nm) between each sample and the positive control (Triton X-100). Data are represented as means ± SE, n=3.

5.5. Discussion

The present study describes the purification and characterisation of a 7.2 kDa antimicrobial peptide that is expressed at the skin mucosal surface of *O. mykiss*. The peptide is active against both Gram-(+) and Gram-(-) bacteria at submicromolar concentrations. Amino acid sequencing and mass spectrometry data jointly suggest that it is likely to be a 69-residue C-terminal fragment of histone H1, phosphorylated at two residues. This peptide was named oncorhyncin II, after the genus of rainbow trout (*Oncorhynchus*). Phosphorylation is a very common post-translation modification of histone H1 (Linnala-Kankkunen *et al.*, 1988). In fact, histone H1 from brown trout (*Salmo trutta*) is known to contain three phosphorylated serines at positions 145, 161 and 182 (Macleod *et al.*, 1977). The level of phosphorylation of histone H1 modulates its ability to interact with DNA (Hill *et al.*, 1991) and also regulates its susceptibility to proteolytic degradation (Morin *et al.*, 1999). Morin *et al.* (1999) have demonstrated that the phosphorylated forms of sperm-specific histone H1 were resistant to proteolysis, whilst the non-phosphorylated forms were digested. It is plausible that the phosphorylated residues in oncorhyncin II protect it from proteolytic attack by bacterial proteases.

The H1 histones belong to the H1/ H5 family and are acknowledged as essential components of chromatin structure. Histone H1 links nucleosomes into higher order structures, inducing the formation of a compact chromatin structure that is inaccessible to the transcription machinery (Davie & Delcuve, 1991). Therefore, H1 histones serve as general repressors of gene expression (Davie & Delcuve, 1991). Besides its involvement in the regulation of transcription, histone H1 from

murine macrophages has been found to display broad-spectrum antibacterial properties (Hiemstra *et al.*, 1993). Moreover, it has been identified on the surfaces of murine macrophages (Brix *et al.*, 1998), showing that histone H1 can have an extracellular as well as a nuclear localisation.

More recently, histone H1 has been proposed to play a role in fish immunity. A 20.7 kDa antibacterial protein isolated from Atlantic salmon (*Salmo salar*) liver was identified by tandem nanoelectrospray mass spectrometry as being histone H1 (Richards *et al.*, 2001). This protein is active against *E. coli* D31 with a minimal inhibitory concentration of 31 $\mu g \cdot ml^{-1}$ (Richards *et al.*, 2001). Patrzykat and collaborators (2001) have reported the purification of an antimicrobial peptide (HSDF-1) from the mucus and serum of coho salmon, *Oncorhynchus kisutch*. HSDF-1 is a 26-residue N-terminal fragment of histone H1 (Fig. 5.5) that displays activity against *A. salmonicida*, *V. anguillarum* or *Salmonella enterica* serovar *typhimurium*. The authors of this study have shown that the expression of HSDF-1 is up-regulated following immunological stimulation and that it coincided with an increase in antibacterial activity of both mucus and serum (Patrzykat *et al.*, 2001). Importantly, it was also demonstrated that synthetic HSDF-1 potentiated the antimicrobial activities of the winter flounder antimicrobial peptide pleurocidin and hen egg white lysozyme (Patrzykat *et al.*, 2001). The present study on *O. mykiss* provides the first example of an antimicrobial peptide derived from the C-terminus of histone H1. Oncorhyncin II is a non-haemolytic peptide with potent broad-spectrum antibacterial properties and may therefore be an important component of the mucosal innate immune system.

Planar lipid bilayer experiments have shown that the peptide is able to induce a strong destabilisation of the membranes but is unable to form stable channels, indicating that the main target for its antibacterial action is not the bacterial membrane. Nevertheless, its potential to destabilise the membranes may allow it to enter the cell through a carpet mechanism (Shai, 1999) and then exert its antibacterial action against intracellular targets. The mode of action of oncorhyncin II contrasts with that of histone H1 purified from salmon liver, which is thought to interact with the cell surface and cause direct damage to the membranes (Richards *et al.*, 2001).

Oncorhyncin II is a very potent antimicrobial agent, with minimal inhibitory concentrations *circa* ten times lower than those of cecropin P1 against sensitive bacteria. At these concentrations oncorhyncin II is not lytic for trout erythrocytes. Therefore, this peptide may exert its biological effects *in vivo* without damage to the host. The potent antimicrobial properties of this 69-residue C-terminal fragment of histone H1 and its lack of haemolytic activity support the hypothesis that it may be involved in mucosal innate immunity.

Chapter 6

Oncorhyncin III: a Potent Antimicrobial Peptide Derived from the Non-histone Chromosomal Protein H6 of *Oncorhynchus mykiss*

6.1. Synopsis

A 6.7 kDa antimicrobial peptide was isolated from trout skin secretions using acid extraction followed by cation exchange chromatography, $^{t}C_{18}$ solid phase extraction, and C_{18} reversed phase HPLC. The molecular mass of this peptide, which is provisionally named oncorhyncin III, is 6671 Da, as determined by matrix-assisted laser desorption ionization mass spectrometry. N-terminal amino acid sequencing revealed that the first 13 residues of oncorhyncin III are identical to those of the non-histone chromosomal protein H6 from rainbow trout. These data combined with the mass spectrometry results indicate that oncorhyncin III is likely to be a fragment of the non-histone chromosomal protein H6 (residues 1 to 66) and that it probably contains two methylated residues or one double methylation. The purified peptide exhibits potent antibacterial activity against both Gram-(+) and Gram-(-) bacteria, with minimal inhibitory concentrations in the submicromolar range. The peptide is inhibited by NaCl and displays no haemolytic activity towards trout erythrocytes at the concentrations tested (maximum 1 μM). Scanning electron microscopy revealed that oncorhyncin III does not cause direct disruption of bacterial cells. Reconstitution of the peptide in a planar lipid bilayer strongly disturbs the membrane but does not induce the formation of stable ion channels, thus supporting the hypothesis that its antibacterial activity is not due to pore-forming properties. Taken together, these results suggest that oncorhyncin III is produced by proteolytic cleavage of the non-histone chromosomal protein H6 and that it may play a role in mucosal innate host defence.

6.2. Introduction

Antimicrobial peptides are increasingly recognised as playing a crucial role in innate immunity (Zasloff, 2002). They are thought to be particularly important at the mucosal surfaces, where the animals are constantly exposed to a wide variety of pathogens and opportunistic microorganisms (reviewed by Bevins, 1994).

The previous chapter reported the characterisation of oncorhyncin II, a histone H1-derived antimicrobial peptide from skin secretions of *O. mykiss*. During that study, it was noted that the 20 % acetonitrile eluate from $^{t}C_{18}$ solid phase extraction contained at least one additional antimicrobial agent. The present chapter describes the purification and characterisation of this antimicrobial peptide.

6.3. Experimental Procedures

Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are aqueous and prepared with deionized water (Elga), except when specifically mentioned. The list of suppliers and respective addresses is presented in appendix B.

6.3.1. Animals

Adult female rainbow trout were maintained under the conditions described in Section 3.3.1.

6.3.2. Sample Collection and Preparation of Epidermal Extracts

Mucous skin secretions and associated epidermal cells were collected as reported in Section 3.3.3. Acid-soluble protein extracts were prepared as detailed in Section 3.3.3.

6.3.3. Test Bacteria

Antibacterial activity was tested against the following strains: *Aerococcus viridans*, *Aeromonas hydrophila*, *Aeromonas salmonicida* (004), *Bacillus subtilis*, *Escherichia coli*, *Listonella (Vibrio) anguillarum*, *Micrococcus luteus* and *Planococcus citreus*. Their original sources and identification codes are listed in Table 3.1. Each microorganism was grown to logarithmic phase in Mueller-Hinton broth (MHB) (Oxoid) at the appropriate temperature (please see Table 3.2) before washing in sterile saline (*circa* 1.5 % (w/v) NaCl for *P. citreus*;

0.8 % (w/v) NaCl for the remaining non-marine strains) and resuspension in saline or MHB as described in Section 3.3.5.

6.3.4. Antibacterial Assays

Antibacterial activity was assessed using a modified version of the two-layer radial diffusion assay of Lehrer *et al.* (1991b) as reported in Section 3.3.5, using the Gram-(+) bacterium *P. citreus* as the test organism throughout the protein purification procedure. Qualitative evaluation of antibacterial activity of the purified peptide against each of the strains listed above was also achieved by radial diffusion assay.

Determination of the minimal inhibitory concentration (MIC) of the isolated peptide against *E. coli* or *P. citreus* was performed using the microtitre broth dilution assay (Friedrich *et al.*, 1999), using the protocol described in Section 3.3.5.

To ascertain the effect of NaCl concentration in the antibacterial activity of the purified peptide, MIC assays were performed using MHB supplemented with NaCl to a final concentration of 1.5 % (w/v) or 3.2 % (w/v).

6.3.5. Muramidase Assay

Muramidase activity was tested by radial diffusion assay as described in Section 2.3.5.

6.3.6. Peptide Purification

The lyophilised extract was reconstituted in 20 mM HEPES, pH 7.0, and subjected to cation exchange chromatography as detailed in Section 3.3.7. The fractions eluted between 85 and 90 min were subjected to $^{t}C_{18}$ solid phase extraction as stated in Section 5.3.6. The lyophilised eluate was resuspended in deionized water containing 0.1 % (v/v) TFA and fractionated by C_{18} reversed phase HPLC using the system described in Section 3.3.7 and the biphasic gradient of 0.1 % (v/v) TFA in water and 0.09 % (v/v) TFA in acetonitrile shown in Fig. 6.1. The active fractions eluting between 49 and 51 min were lyophilised, reconstituted in 200 µl acidified water (0.1 % (v/v) TFA) and further purified by C_{18} reversed phase HPLC on the same column but under the shallower gradient depicted in Fig. 6.2. One millilitre fractions were collected over 60 min, lyophilised and reconstituted in 200 µl deionized water.

At each step of the purification procedure, the purity and molecular weight of the proteins were estimated by high resolution polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE), as detailed in Section 2.3.6. The gels were stained with silver under alkaline conditions (Appendix C).

6.3.7. Protein Quantification

Total protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin (Pierce) as standard. Amino acid analysis of the purified peptide was performed at the Protein Lab and Proteomics facility (University of Aberdeen, UK) employing the PTC derivatization method on an Applied Biosystems 420A amino acid analyser.

6.3.8. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

The molecular weight of the purified antimicrobial peptide was determined by MALDI-TOF MS at the Centre for Biomolecular Sciences (University of St Andrews, UK) according to the protocol described in Section 3.3.9.

6.3.9. Partial Primary Structure Determination

N-terminal amino acid sequencing of the purified peptide was performed by standard automated Edman degradation on a Procise Sequencer (Applied BioSystems) at the Centre for Biomolecular Sciences (University of St Andrews, UK).

6.3.10.Sequence Analyses

The proteomics tools listed in Section 4.3.10 were used to perform homology searches, prediction of protein parameters and sequence alignments.

6.3.11.Proteolytic Digestion

The proteinaceous nature of the purified antimicrobial factor was tested by proteolytic digestion with proteinase K, as detailed in Section 3.3.13.

6.3.12.Haemolysis Assay

The purified peptide was assayed for haemolytic activity against trout erythrocytes according to the protocol described in Section 3.3.15. The initial maximum concentration of peptide was approximately 5 μM.

6.3.13.Scanning Electron Microscopy (SEM)

Ninety microlitres of an *E. coli* culture in exponential growth at $5 \cdot 10^6$ cfu·ml^{-1} were incubated at 37 °C for 1 h with 10 µl of purified trout antimicrobial peptide (10 µM) or cecropin P1 (20 µM). After washing with 0.8 % (v/v) NaCl the specimens were fixed in 2.5 % (v/v) glutaraldehyde and then dehydrated in ascending concentrations of ethanol, ranging from 70 % (v/v) to absolute ethanol (Merck). They were then transferred to a Samdri 780 (Emscope) for critical point drying with liquid CO_2 (BOC Gases). Dry specimens were mounted on 3 cm diameter aluminium SEM stubs (Agar Aids) using double-sided sticky tape, placed in a sputter coater with a gold target (Emscope SC500) and coated for 3 min at 15 µA under an argon (BOC Gases) atmosphere. The samples were then observed under a JEOL 35-CF scanning electron microscope, using an accelerating voltage of 10 KV. Photographs were taken using 125 asa FP4+ Ilford professional film (H.A. West), which was developed in Ifotec LC29 (H.A. West) developer.

6.3.14.Planar Lipid Bilayer Assay

The ion channel properties of the purified antimicrobial peptide were tested by macroscopic and single-channel experiments carried out by Dr Gérard Molle (Centre de Biochimie Structurale, University of Montpellier, France), as described in Section 3.3.16.

6.4. Results

6.4.1. Purification of a 6.7 kDa Antimicrobial Peptide

The active fractions eluted between 85 and 90 min during cation exchange chromatography (Fig. 3.3) were concentrated by solid phase extraction and the 20 % acetonitrile eluate subsequently subjected to C_{18} reversed phase HPLC (Fig. 6.1). Active fractions were further fractionated by C_{18} reversed phase HPLC on the same column but with a shallower water/acetonitrile gradient, yielding one main fraction that exhibited antibacterial activity against *P. citreus* and that corresponded to two peaks with retention times of 33.2 min and 33.5 min (Fig. 6.2). The antibacterial activity of the purified peptide was thermostable, remaining present even after incubation at 80 °C for 5 min. Activity was abolished after digestion with proteinase K, confirming that this antibacterial factor has a proteinaceous nature. One protein band with an electrophoretic mobility of approximately 6.5 kDa could be observed on an SDS-PA gel after silver staining (Fig. 6.3). The yield of purified peptide, determined by amino acid analysis, was approximately 30 $ng \cdot g^{-1}$ mucus.

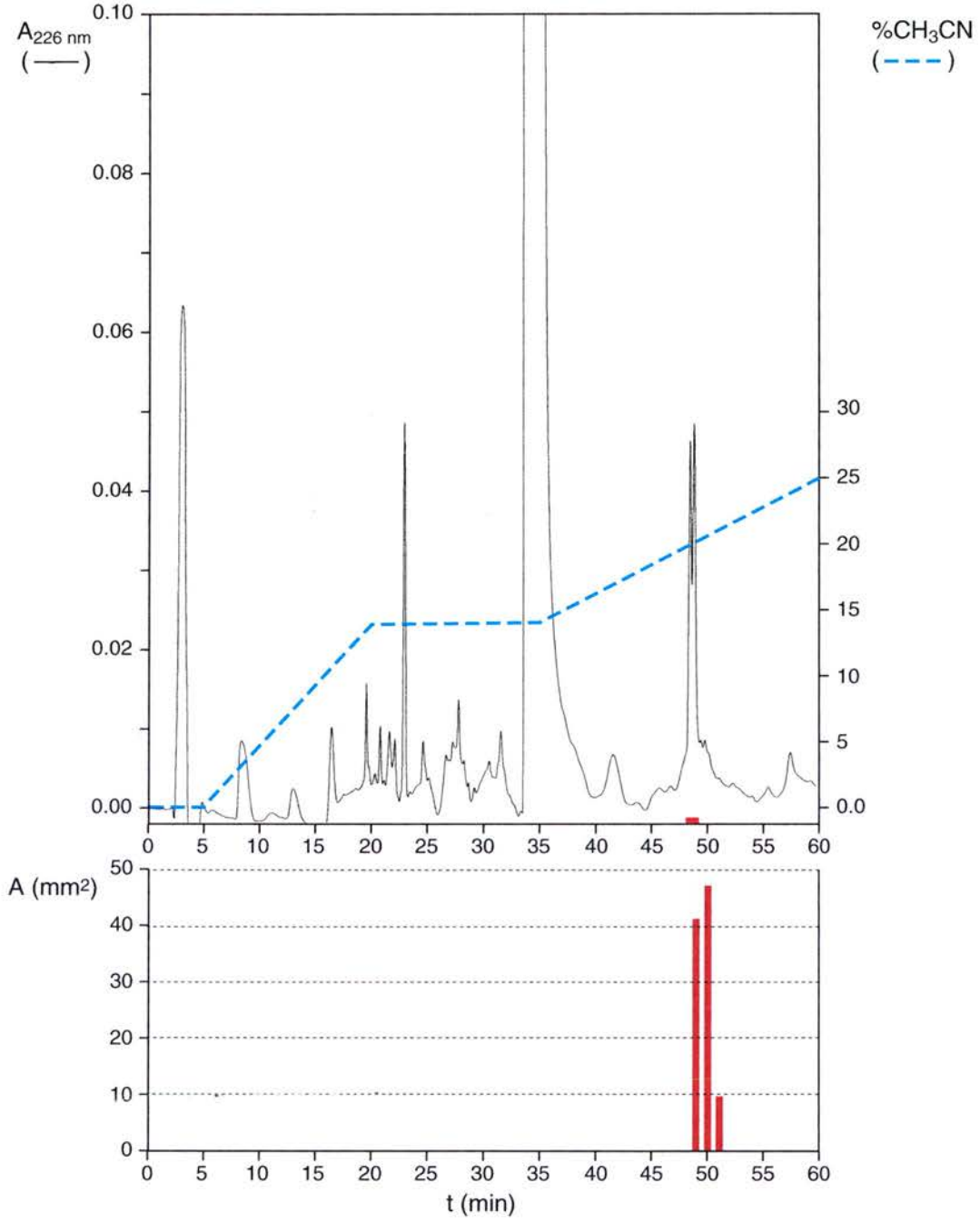


Figure 6.1. C_{18} reversed phase HPLC of the 20 % acetonitrile eluate from $^{t}C_{18}$ solid phase extraction. The active fractions eluted between 85 and 90 min during cation exchange chromatography (Fig. 3.3) were pooled and concentrated by $^{t}C_{18}$ solid phase extraction prior to fractionation by C_{18} reversed phase HPLC. The chromatogram was obtained at 226 nm (solid line). Antibacterial activity profile against *P. citreus* is represented by the histogram. The blue dashed line shows the acetonitrile concentration gradient. The peaks of interest are indicated by the red bar.

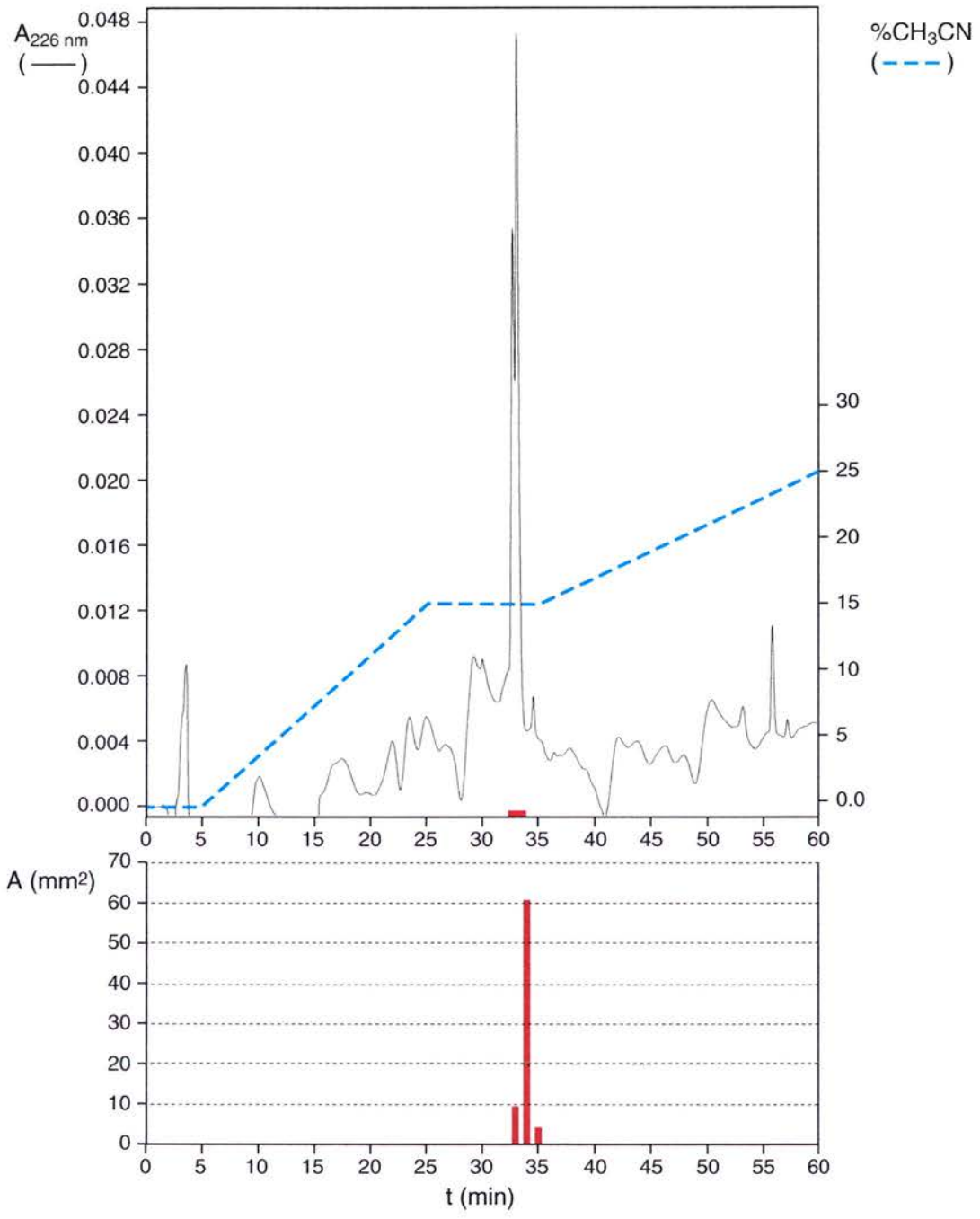


Figure 6.2. Final step in the isolation of a 6.7 kDa antimicrobial peptide from skin secretions of rainbow trout. The active C_{18} reversed phase HPLC fractions eluted between 20 % and 21 % acetonitrile (Fig. 3.5) were pooled and further subjected to C_{18} reversed phase HPLC under a shallower water/acetonitrile gradient (blue dashed line). Absorbance was monitored at 226 nm (solid line). The fractions corresponding to the peaks eluting at 33.2 min and 33.5 min were found to be antibacterial to *P. citreus* (histogram). The red bar emphasizes the peaks of interest.

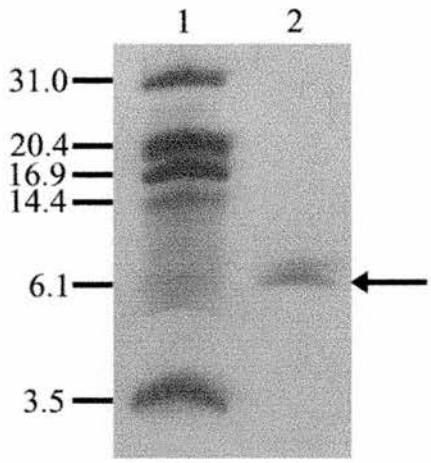


Figure 6.3. Tris-Tricine SDS-PAGE profile (silver stained) of an antimicrobial peptide purified from skin secretions of rainbow trout. Lane 1: markers; lane 2: purified antimicrobial peptide following C_{18} reversed phase HPLC (fraction 34, Fig. 6.2). Each lane contains 7.5 µl of sample. The numbers on the left hand side correspond to the molecular mass of the markers in kDa. The protein of interest is indicated by an arrow.

6.4.2. Peptide Characterisation

Automated Edman degradation of the purified peptide yielded the following partial N-terminal sequence[#]: PKRKSATKGDEPA. BLAST homology searches established that it is a perfect match with the N-terminus of the non-histone chromosomal protein H6 (histone T) from rainbow trout (Fig 6.4).

The molecular mass of this antimicrobial peptide is 6671 Da, as determined by MALDI-TOF mass spectrometry (Fig 6.5). This result revealed that it is a fragment of the non-histone chromosomal protein H6 (residues 1 to 66) and that

[#] This partial amino acid sequence has been deposited in the TrEMBL database under the accession number P83338.

it is likely to contain two methylated residues or one double methylation. The amino acid analysis data were in good agreement with the expected composition of the non-histone chromosomal protein H6 from trout gonads (Table 6.1).

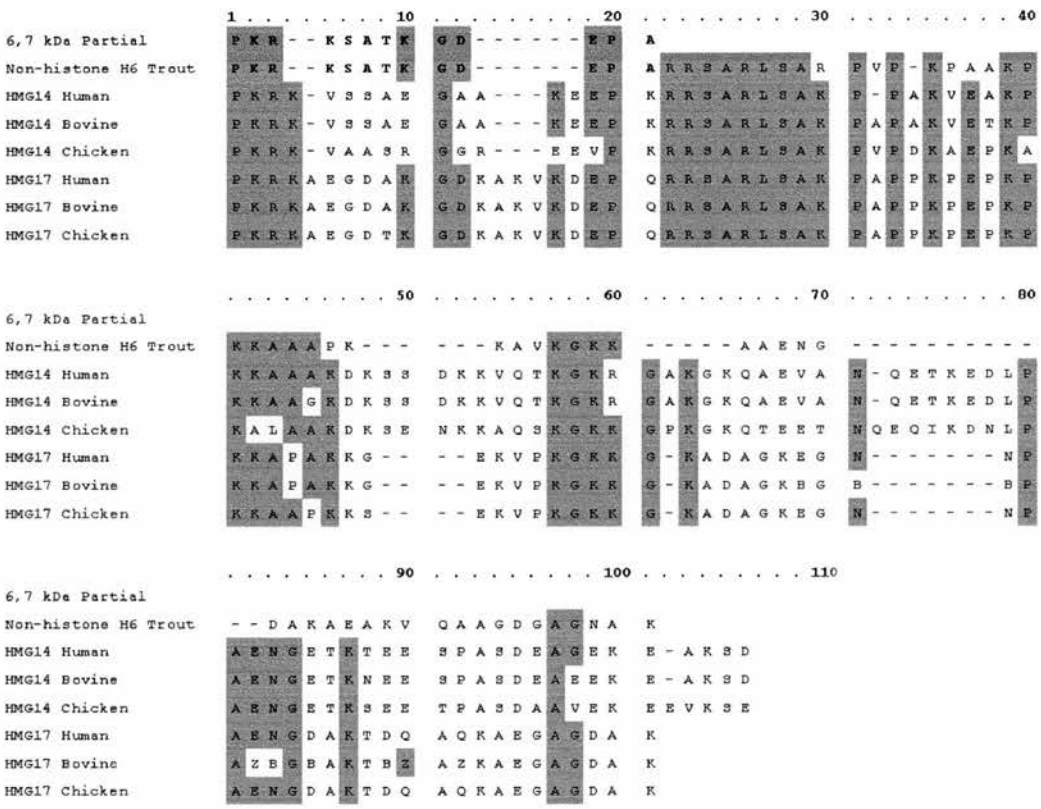


Figure 6.4. Partial N-terminal sequence of the 6.7 kDa antimicrobial peptide purified from skin mucus of rainbow trout and its homology with high mobility group (HMG) proteins. Identical residues between the purified antimicrobial peptide, non-histone chromosomal protein H6 from rainbow trout (Watson *et al.*, 1979), human HMG-14 (Landsman *et al.*, 1986b), bovine HMG-14 (Swiss-Prot P02316), chicken HMG-14 (Srikantha *et al.*, 1988), human HMG-17 (Landsman *et al.*, 1986a), bovine HMG-17 (Swiss-Prot P02313) and chicken HMG-17 (Landsman & Bustin, 1987) are represented by a shaded box.

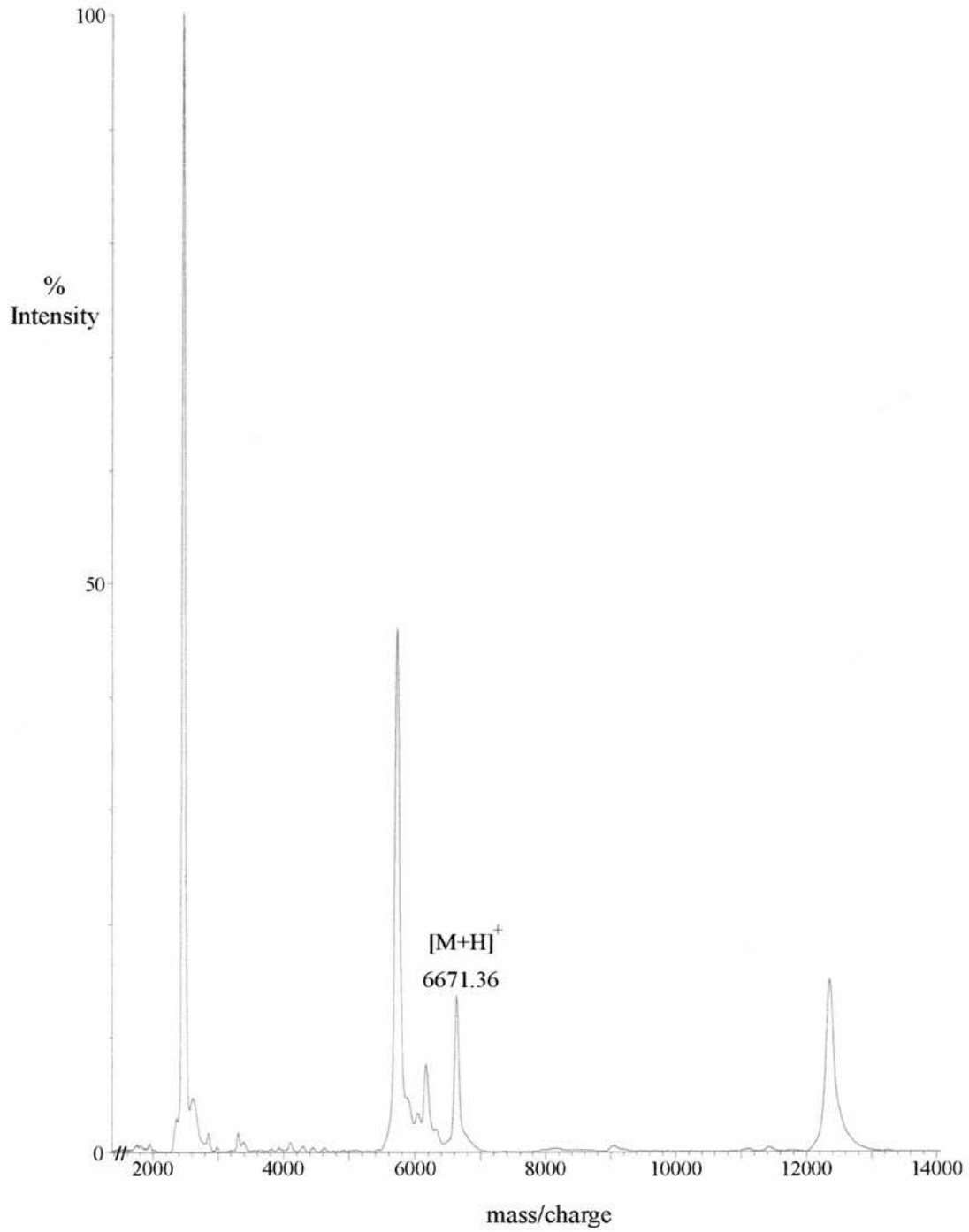


Figure 6.5. MALDI-TOF mass spectrum of the 6.7 kDa antimicrobial peptide purified from skin exudates of rainbow trout. The single charged molecular ion is labelled. The other peaks that can be observed correspond to the following internal standards: equine cytochrome c (12360.1 Da), bovine insulin (5733.5 Da) and ACTH "clip" peptide (residues 18 - 39, 2465.7 Da).

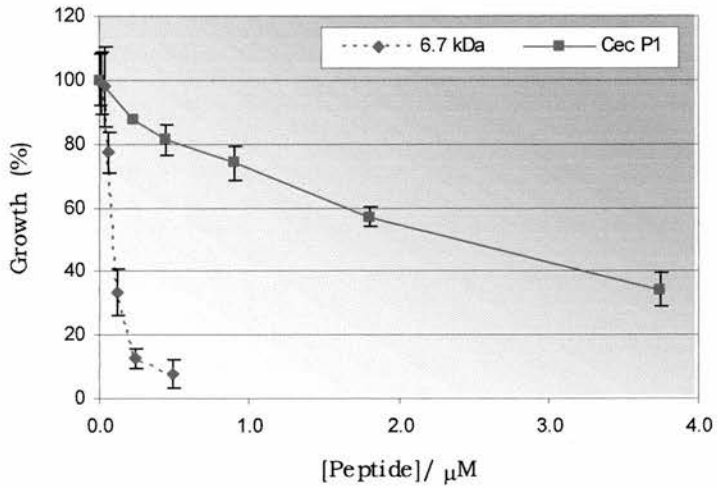
Table 6.1. Amino acid analysis of the 6.7 kDa antimicrobial peptide purified from rainbow trout skin. Norleucine was used as internal standard for derivatization and myoglobin was employed as the hydrolysis standard. The results are in good agreement with the expected amino acid composition for gonadal trout non-histone chromosomal protein H6 (Swiss-Prot P02315).

Amino Acid	No. of Residues Determined for the 6.7 kDa Trout Peptide	Predicted No. of Residues for Trout Non-histone Chromosomal Protein H6
Ala	16	19
Arg	4	5
Asx	6	5
Cys	0	0
Glx	4	4
Gly	5	6
His	0	0
Ile	0	0
Leu	1	1
Lys	14	15
Met	0	0
Phe	1	0
Pro	8	7
Ser	4	3
Thr	3	1
Trp	not determined	0
Tyr	0	0
Val	2	3

6.4.3. Antibacterial and Haemolytic Activity

Quantification of antibacterial activity using the microtitre broth dilution method revealed that the purified 6.7 kDa antimicrobial peptide derived from trout non-histone chromosomal protein H6 is active at submicromolar concentrations, with MICs of 0.06 - 0.12 μM and 0.25 - 0.5 μM against *P. citreus* (Fig. 6.6A) and *E. coli* (Fig. 6.6B), respectively. By contrast, cecropin P1 has a MIC against *P.citreus* approximately 30-fold higher than that of the antimicrobial peptide from trout skin. Radial diffusion assays revealed that at a concentration of 5 μM the 6.7 kDa peptide also displays antimicrobial activity against *A. hydrophila*, *A. salmonicida*, *A. viridans*, *B. subtilis*, *L. anguillarum* and *M. luteus* (Table 6.2). As expected, the purified peptide had no detectable muramidase activity. As shown in Fig. 6.7, at a concentration of 1 μM oncorhyncin III caused only 5 % haemolysis, whereas melittin, a known haemolytic peptide (DeGrado *et al.*, 1982) induced 38 % haemolysis under the same conditions.

(A)



(B)

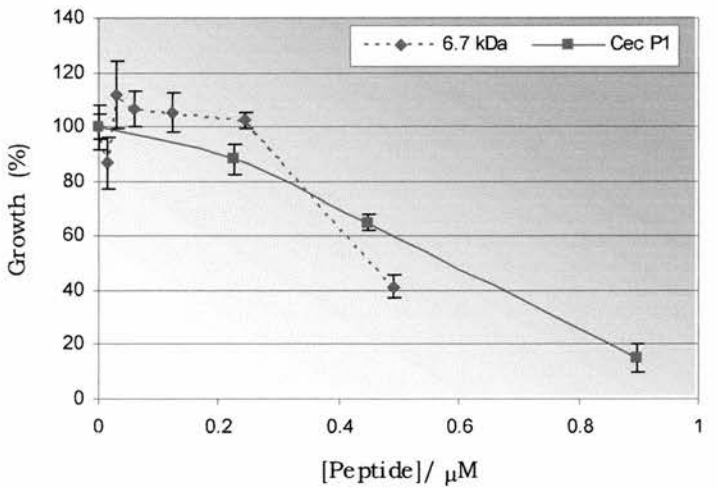


Figure 6.6. Quantification of antibacterial activity of the purified 6.7 kDa antimicrobial peptide against (A) *P. citreus* and (B) *E. coli*. Bacterial suspensions containing 10^5 cfu·ml^{-1} were incubated at 20 °C (*P. citreus*) or 37 °C (*E. coli*) with serially diluted cecropin P1 (Cec P1) or with the 6.7 kDa antimicrobial peptide from trout skin (6.7 kDa). Growth inhibition is expressed as the ratio of optical densities read at 570 nm (OD_{570}) between each test sample and the control (no peptide added). The average final OD_{570} of the control was approximately 0.2. Data are represented as means ± SE, n=3.

Table 6.2. Bacteriostatic activity of the 6.7 kDa antimicrobial peptide purified from trout skin secretions. Antibacterial activity of the purified peptide (5 μM) was assessed by radial diffusion assay as described in Section 3.3.5. The results are expressed as the corrected mean area of the clear zone (mm^2) ± SE, n=3.

Species	Gram Staining	A ± SE (mm^2)
A. viridans	+	57 ± 12
B. subtilis	+	12 ± 2
M. luteus	+	26 ± 6
P. citreus	+	70 ± 5
A. hydrophila	-	6 ± 2
A. salmonicida 004	-	8 ± 2
E. coli	-	17 ± 5
L. anguillarum	-	20 ± 5

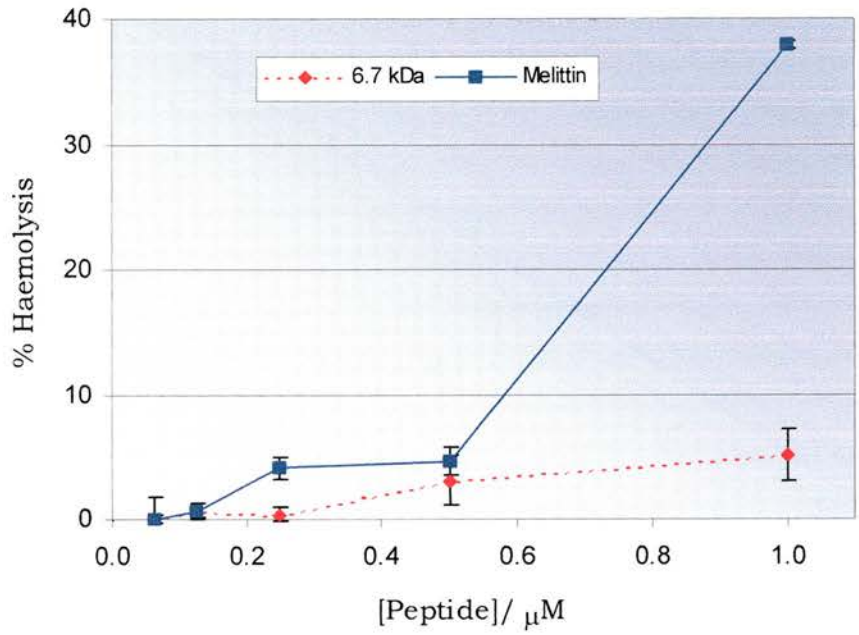


Figure 6.7. Haemolysis assay of the purified 6.7 kDa antimicrobial peptide against trout erythrocytes. Serially diluted test samples were incubated with a 2 % (v/v) suspension of erythrocytes for 30 min at 37 °C. The initial concentrations of 6.7 kDa peptide and melittin were approximately 10 μM. Percent haemolysis is defined as the ratio of absorbances (read at 545 nm) between each sample and the positive control (Triton X-100). Values are represented as means ± SE, n=3.

6.4.4. Salt Sensitivity

The assays to investigate the effect of salt on the antibacterial activity of the 6.7 kDa peptide revealed that, despite being active against *P. citreus* at concentrations up to 3.2 % (w/v) NaCl, the MIC value increased 2-fold when the NaCl concentration increased from 0.8 % (w/v) to 1.5 % (w/v); furthermore, an 8-fold increase in the MIC value (relative to 0.8 % (w/v) NaCl) was observed in the presence of 3.2 % (w/v) NaCl (Fig. 6.8).

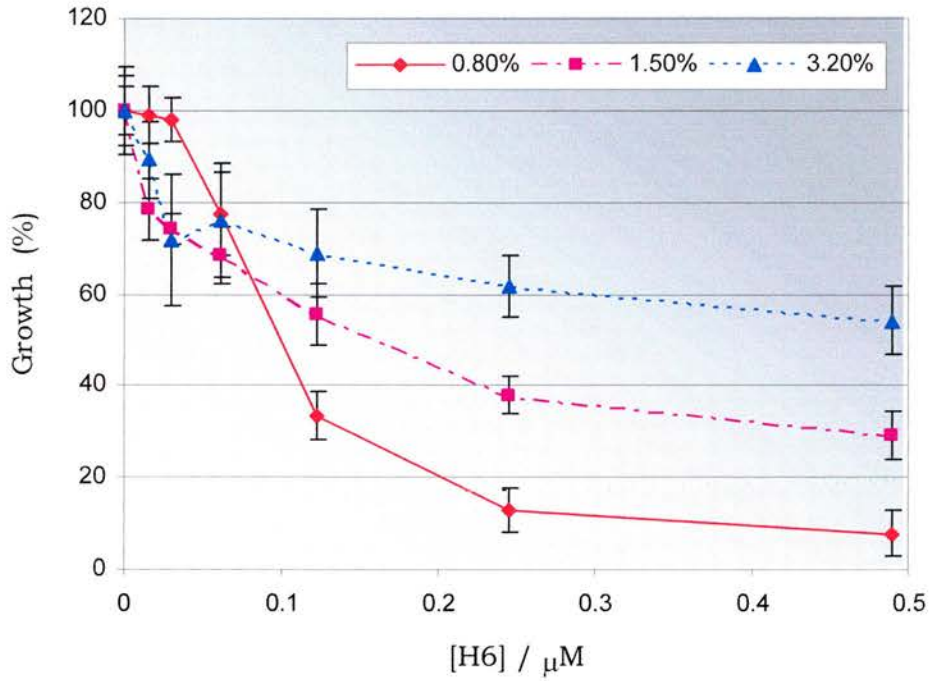


Figure 6.8. Effect of NaCl concentration on antibacterial activity of the 6.7 kDa trout antimicrobial peptide against *P. citreus*. Bacterial suspensions containing different concentrations of sodium chloride were incubated with serially diluted purified peptide. The results are expressed as the ratio of absorbances (read at 570 nm) between each sample and the control (no peptide added). Values are shown as means ± SE, n=3.

6.4.5. Scanning Electron Microscopy (SEM)

The effect of the purified 6.7 kDa peptide on *E. coli* cells was visualised by SEM, following incubation of bacteria with the peptide. Control bacteria incubated in the absence of peptide had an appearance characteristic of bacterial rods (Fig. 6.9A). *E. coli* cells retained their typical morphology following incubation with the 6.7 kDa trout peptide at a concentration two-fold greater than the MIC, albeit they had a smaller size (Fig. 6.9B). In contrast, incubation with cecropin P1 at a concentration two-fold greater than the MIC induced marked changes in cell shape and even disruption of bacterial integrity (Fig. 6.9C).

6.4.6. Pore-forming Activity

Experiments conducted by Dr. Gérard Molle showed that when the purified 6.7 kDa antimicrobial peptide was added to the measurement cell at a concentration between $5 \cdot 10^{-8}$ M and $5 \cdot 10^{-7}$ M no typical I-V curves could be recorded. Nevertheless, a strong destabilisation of the membranes occurred. This behaviour was also observed in independent single channel experiments. Indeed, at a protein concentration ranging from $2 \cdot 10^{-9}$ to 10^{-8} M, it was also possible to observe large bursts reflecting the permeabilisation of the membrane.

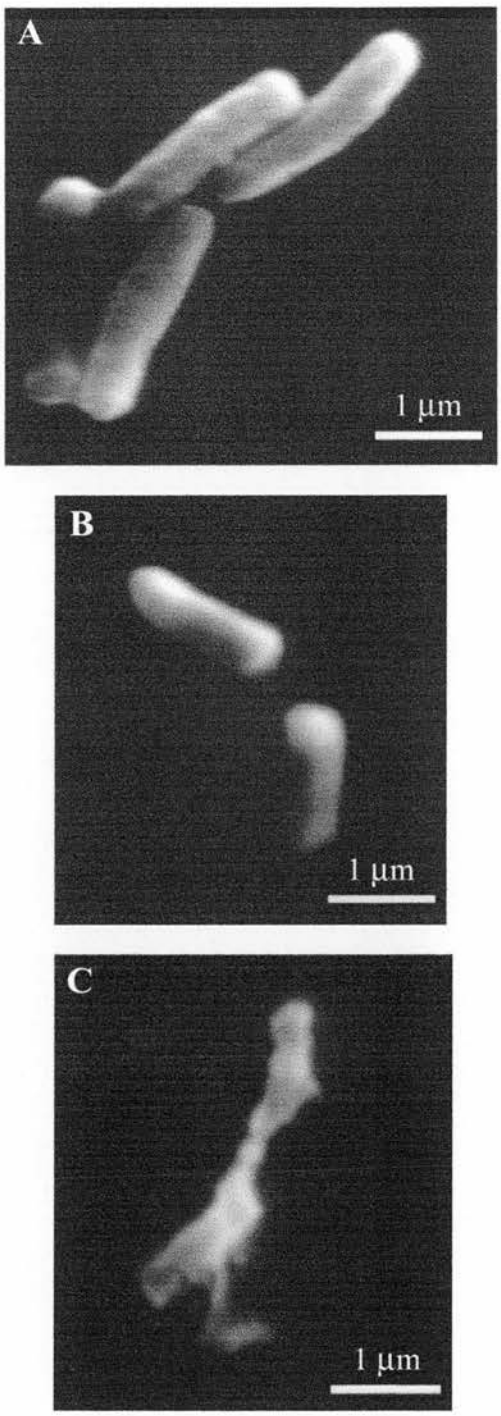


Figure 6.9. Scanning electron micrographs of *E. coli* following treatment with cecropin P1 or with the trout 6.7 kDa antimicrobial peptide. Bacteria in exponential growth at $5 \cdot 10^6$ cfu·ml^{-1} were incubated at 37 °C for 1 h with (A) medium only, (B) 1 µM (final concentration) 6.7 kDa trout antimicrobial peptide or (C) 2 µM (final concentration) cecropin P1. The scale bar represents 1 µm.

6.5. Discussion

The present study shows that the skin epithelium of rainbow trout expresses a 6.7 kDa antimicrobial peptide. This peptide displays a broad spectrum of activity at submicromolar concentrations and its activity is inhibited by increasing concentrations of sodium chloride. Mass spectrometry analysis combined with N-terminal sequence data showed that oncorhyncin III is a 66-residue N-terminal fragment of the non-histone chromosomal protein H6 from *O. mykiss*. Moreover, its molecular mass indicates that it is likely to contain two methylated residues or one double methylation, probably two N6-methyl-L-lysines or one N6,N6-dimethyl-L-lysine. The non-histone chromosomal protein H6 contains the consensus sequence Arg-Lys-Ser (residues 3 to 5) present in histone H3, which is methylated at lysines 9 and 27 (Honda *et al.*, 1975). In order to confirm the presence of methyllysines in the non-histone chromosomal protein H6 from skin epithelia, nanoelectrospray mass spectrometry experiments were to be performed. Unfortunately, these were made impossible due to time constraints.

To the best of my knowledge, this is the first report to directly demonstrate that a 66-residue N-terminal fragment of the non-histone chromosomal protein H6 displays potent antimicrobial properties and may therefore be an important component of the mucosal innate immune system. Moreover, the non-histone chromosomal protein H6 belongs to the high mobility group (HMG) of proteins, which are present throughout the vertebrate subphylum (Brown & Goodwin, 1983). BLAST homology searches and sequence alignments of oncorhyncin III with H6 from rainbow trout testes, human HMG14, bovine HMG14, avian HMG14, human HMG17, bovine HMG17 and avian HMG17 reveal a high

degree of similarity amongst them (Fig. 6.4). These proteins constitute a family of relatively low molecular weight non-histone components in chromatin. The proteins bind to the inner side of the nucleosomal DNA, altering the interaction between the DNA and the histone octamer (Bustin & Reeves, 1996). Levy-Wilson & Dixon (1979) have shown that H6 is selectively solubilised by limited digestion of trout testis nuclei with micrococcal nuclease, suggesting that it is associated with transcriptionally active chromatin. Indeed, it is currently accepted that the HGM proteins may be involved in the process that confers specific chromatin conformations to transcribable regions in the genome (Bustin & Reeves, 1996). It would be interesting to investigate if non-histone chromosomal proteins can have a cytosolic as well as nuclear localisation, similarly to other histones or histone-derived fragments (Kashima, 1991; From *et al.*, 1996; Kim *et al.*, 2000; Cho *et al.*, 2002). These data would support the hypothesis that besides their nucleosomal involvement, histones may also aid in protection of the cell against bacterial attack.

Most antimicrobial peptides found throughout the animal and plant Kingdoms are small, functionally specialised peptides (Boman, 1994). Nevertheless, an increasing number of antimicrobial peptides are discovered to be generated by proteolysis of larger proteins with previously known function (Zasloff, 2002). For instance, the murine 40S ribosomal protein S30 (Hiemstra *et al.*, 1999) and the trout equivalent (please see Chapter 4) have been found to have potent antimicrobial properties. Two other examples are provided by histone H2A from rainbow trout skin secretions (Chapter 3) and parasin I, a 19-residue N-terminal fragment of histone H2A present in the skin of the catfish, *Parasilurus asotus*

(Park *et al.*, 1998b). In fact, histone-derived antimicrobial peptides are relatively frequent amongst the group of proteins that have a secondary antimicrobial function. The 6.7 kDa antimicrobial peptide isolated from trout skin secretions was provisionally named oncorhyncin III, after the genus of rainbow trout, *Oncorhynchus*.

The mechanism by which antimicrobial peptides kill microbes is not yet clear and it is currently thought that different peptides employ different strategies. These include the fatal depolarisation of the cell membrane (Westerhoff *et al.*, 1989), the formation of pores and subsequent leakage of the cell contents (Yang *et al.*, 2000) and the damaging of critical intracellular targets after internalisation of the peptide (Subbalakshmi & Sitaram, 1998; Kragol *et al.*, 2001). Nonetheless, it is widely accepted that the primary target of antimicrobial peptides is the bacterial membrane. Regardless of the actual bactericidal mechanism the peptide has to interact with the membrane, either to physically disrupt it or to access the intracellular space. SEM imaging of *E. coli* cells incubated with oncorhyncin III revealed that the general cell morphology remains unchanged, thus showing that the peptide does not cause immediate disintegration of the cell membrane. This hypothesis is further corroborated by the ion channel experiments, which show that the H6-derived peptide isolated from trout mucus in the present study induced a marked destabilisation of planar lipid bilayers but was unable to form stable channels. These results are in accordance with the Shai-Matsuzaki-Huang model of the mechanism of action of antimicrobial peptides, whereby the peptide adsorbs to the target membrane and covers it in a carpet-like fashion (Shai, 1999). The formation of "wormholes" occurs at high enough local concentration

of peptide (Ludtke *et al.*, 1996; Matsuzaki *et al.*, 1997), consistent with the observed transient ion channel activities. Following this phase transition oncorhyncin III may diffuse onto intracellular targets (Shai, 1999; Zasloff, 2002), where it can exert its antibacterial function. Similarly, buforin II, a linear α-helical peptide derived from histone H2A from *Bufo bufo gargarizans*, does not cause *E. coli* cell lysis at concentrations up to five times its MIC (Park *et al.*, 1998a). Instead, buforin II penetrates the cell membrane and accumulates inside the cell, where it is likely to interact with bacterial nucleic acids (Park *et al.*, 1998a). The mode of action of oncorhyncin III contrasts with that suggested for another antimicrobial histone, histone H1, from Atlantic salmon (*Salmo salar*). Histone H1 interacts with the cell surface and directly compromises the integrity of the cell membrane, as visualised by SEM (Richards *et al.*, 2001).

Since the initial step of permeabilisation of the bacterial membrane involves the electrostatic interaction between the cationic peptides and the negatively charged phospholipid head groups of the outermost leaflet of the lipid bilayer (Shai, 1999), several antimicrobial peptides are inhibited by high sodium chloride concentrations (Bals *et al.*, 1998; Turner *et al.*, 1998; Bals *et al.*, 1999), albeit there are some examples of peptides which seem to be salt-resistant (Cole *et al.*, 1997; Lauth *et al.*, 2002). The H6-derived antimicrobial peptide purified from *O. mykiss* skin secretions in the present investigation is sensitive to the concentration of NaCl, displaying an 8-fold reduction in potency against *P. citreus* when the salt concentration increases from 0.8 % (w/v) to 3.2 % (w/v) NaCl. This implies that during their seasonal migrations to the sea, the protective antimicrobial effect of H6 may be greatly diminished in steelhead rainbow trout.

Nevertheless, the likely intracellular location of oncorhyncin III suggests that even under high NaCl concentrations it might still play an important role in host defence against intracellular pathogens, albeit this has yet to be confirmed.

The conceivable relevance of oncorhyncin III as a molecular effector of the innate immune system is indicated by its very high potency. It is active against all the Gram-(+) and Gram-(-) bacteria tested, with minimal inhibitory concentrations in the micromolar range. In particular, its MIC against *P. citreus* is in the range 0.06 to 0.12 μM and approximately 30-fold lower than those of cecropin P1 against the same microorganism. Importantly, the peptide is not significantly lytic for trout erythrocytes at the concentrations tested. Hence, this antimicrobial peptide could exert its biological effects *in vivo* against bacteria without damage to the host tissues.

Chapter 7

Purification and Preliminary Characterisation of a Novel Muramidase from Skin Mucus of *Oncorhynchus mykiss*

7.1. Synopsis

Epithelial surfaces of teleosts are known to contain a number of innate humoral defence factors, including muramidases. These enzymes are generally cationic and inhibit bacterial growth by degrading the peptidoglycan of bacterial cell walls. Acid-urea gel overlay assays revealed that skin mucus of rainbow trout contains two muramidases with clearly distinct isoelectric points. This chapter describes the purification and preliminary characterisation of one of these enzymes, a novel muramidase with an unusually acidic isoelectric point; it is the first anionic muramidase to be reported for fish. Isoelectric focusing of the partially purified muramidase has shown that its isoelectric point is between 4.7 and 6.0. The purified enzyme was found to have a molecular mass of 14268 Da, as determined by matrix-associated laser desorption/ ionization time of flight mass spectrometry. Albeit this novel muramidase does not exhibit antibacterial activity against *Planococcus citreus* at the concentrations tested, it may well act in synergism with other innate antimicrobial factors to prevent microbial exploitation of trout skin mucosa.

7.2. Introduction

Lysozyme exerts its bacteriolytic activity by hydrolysing the β-(1→ 4) glycosidic linkages between N-acetylmuramic and N-acetylglucosamine in peptidoglycan. In addition to its ability to digest the bacterial cell wall, lysozyme promotes phagocytosis as an opsonin or by direct activation of polymorphonuclear leukocytes (Jollès & Jollès, 1984). Comparative studies have shown that animals express multiple forms of lysozyme that exhibit marked differences in primary structure and substrate specificity (Jollès, 1996; Matsumura & Kirsch, 1996; Prager & Jollès, 1996; Ito *et al.*, 1999).

Most investigations regarding lysozyme from skin secretions of teleosts are based solely on muramidase activity of crude protein extracts (Hjelmeland *et al.*, 1983; Aranishi *et al.*, 1998; Fevolden *et al.*, 1999). The limitations of these studies are clearly illustrated by the known existence of multiple isoforms of lysozyme with rather different enzymatic and antibacterial properties (Grinde *et al.*, 1988). Muramidase activity in skin mucus of rainbow trout, *Oncorhynchus mykiss*, has been reported (Hjelmeland *et al.*, 1983; Fevolden et al., 1999) but no lysozymes have been isolated from epithelial surfaces of salmonids, one of the main groups of fish raised in aquaculture. Therefore, the present investigation was conducted to purify and characterise lysozymes or lysozyme-like proteins from *O. mykiss*. This chapter reports the isolation and preliminary characterisation of a novel muramidase with an anionic nature.

7.3. Experimental Procedures

Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are aqueous and prepared with deionized water (Elga), except when stated otherwise. The various suppliers and respective addresses are listed in appendix B.

7.3.1. Animals

Adult female rainbow trout were maintained as described in Section 2.3.1.

7.3.2. Preparation of Skin Mucus Extracts

Mucous secretions and associated epidermal cells were collected from 4 fish by scraping their dorso-lateral surfaces with a glass slide, yielding a total volume of approximately 40 ml. This sample was homogenised 1:5 (v/v) in 40 mM sodium phosphate buffer, pH 6.2, containing 2 % (v/v) general use protease inhibitors cocktail. Following extraction by stirring at 4 °C for 60 min, the homogenate was acidified to pH 4.0 with 1 M acetic acid (BDH) and then centrifuged at 29 000 x g for 20 min at 4 °C to remove insoluble material.

7.3.3. Gel Overlay Assay

An indication of the number of muramidases present in skin mucus extracts was obtained by a modified version of the acid-urea gel overlay assay described by Lehrer *et al.* (1991). Proteins were fractionated on 16.6 % T/ 0.43 % C polyacrylamide gels (Anachem) in the presence of 38.8 % (w/v) urea and 7 % (v/v) acetic acid (please consult appendix D for details). Following neutralisation by soaking for 10 min in 50 mM HEPES (Acros), pH 7.0, gels

were overlaid on a 1 % (w/v) agarose plate containing 0.33 $mg \cdot ml^{-1}$ lyophilised *Micrococcus luteus* cell walls and incubated for 3 h at 37 °C.

7.3.4. Enzymatic Assays

Muramidase activity was quantified either by radial diffusion assay, as detailed in Section 2.3.5, or by a modified version of the turbidometric assay described by Grinde *et al.* (1988). In the latter method, enzymatic activity was assessed by incubating 50 µl test sample with 950 µl substrate (0.25 mg/ml lyophilised *M. luteus* cell walls in 40 mM phosphate buffer, pH 6.2) at 20 °C. The reference cell contained 950 µl substrate and 50 µl deionized water. The optical density at 450 nm (OD_{450}) was measured every second for one minute on an Ultrospec 3300pro spectrophotometer (Amersham-Pharmacia), using a cuvette with a 1 cm light path (Fisher). Initial rates of muramidase activity were determined from the first 20 s of linear decrease in OD_{450}. One unit of activity (U) is defined as the amount of enzyme that generates a decrease in optical density of 0.001 min^{-1} at 450 nm under the above described conditions.

7.3.5. Antibacterial Assay

Antibacterial activity against *Planococcus citreus* was tested by radial diffusion assay using the protocol described in Section 3.3.5.

7.3.6. Partial Protein Purification

The lyophilised water-soluble mucus extract prepared as detailed in Section 2.3.2 was resuspended at a total protein concentration of 20 $mg \cdot ml^{-1}$ in 0.1 % (v/v) Triton X-100, 20 mM MES, pH 6.4, and applied to a CM-Sepharose column equilibrated in the same buffer. The flow-through was dialysed against deionized water at 16 °C using a cellulose benzoylated tubing (nominal molecular weight cut off, NMWCO, 2 kDa) and loaded onto a Sep-Pak 2 g C_{18} cartridge (Waters). The non-binding fraction was lyophilised, reconstituted in 5 ml of 20 mM HEPES, pH 8.0 (buffer A), and applied to a DEAE-Sepharose fast protein liquid chromatography (FPLC) column (Pharmacia), previously equilibrated with this buffer. Following a 10 min wash with the equilibration buffer, bound material was eluted with a linear gradient of 40 % to 65 % 1 M NaCl, 20 mM HEPES, pH 8.0 (buffer B) over 60 min followed by 100 % B for 10 min at a flow rate of 1.0 $ml \cdot min^{-1}$. The eluted fractions were screened for activity against *P. citreus* or *M. luteus* cell walls by radial diffusion assay as described above. Fractions displaying muramidase activity (eluted between 520 and 540 mM NaCl) were pooled and further fractionated by reversed phase high performance liquid chromatography (RP-HPLC) on a 4.6 x 250 mm RSiL C_{18} HL column (Bio-Rad) using a linear biphasic gradient of 0.1 % trifluoroacetic acid (TFA) in deionized water (buffer A) and 0.1 % (v/v) TFA in acetonitrile (BDH) (buffer B), as follows: 0 to 60 % B over 60 min followed by 100 % B for 10 min, at a flow rate

of 1 ml·min^{-1}. Sixty 1 ml fractions were collected, lyophilised and resuspended in 50 µl 20 mM MES, pH 6.4.

Each fraction was tested for activity against M. *luteus* cell walls and analysed by SDS-PAGE as described in Section 2.3.6. Fractions exhibiting muramidase activity, as assessed by radial diffusion assay, were filtered through a 20 kDa NMWCO membrane (Vectaspin 3, Whatman) and subjected to isoelectric focusing as detailed below.

7.3.7. Purification of the Anionic Muramidase to Apparent Homogeneity

The phosphate protein extract, prepared as above, was fractionated by successive precipitation with increasing amounts of ammonium sulphate (BDH) (Englard & Seifter, 1990). Following a cut off at 50 % saturation, the 85 % saturation precipitate was collected by centrifugation at 4 000 x g for 30 min (4 °C) and resuspended in 50 ml of 20 mM HEPES, pH 7.0. It was then centrifuged at 29 000 x g for 30 min at 4 °C and the supernatant subjected to anionic exchange chromatography using a DEAE-Sepharose 1 cm x 10 cm Econo-column (Bio-Rad), previously equilibrated with 20 mM HEPES, pH 7.0 (buffer A). After a 30 minute wash to remove non-specifically bound material, elution was performed with a linear AB gradient (where B is 20 mM HEPES, 1 M NaCl, pH 7.0) from 0 to 30 % B over 50 min, followed by 10 min of 30 % B, at a flow rate of 1 ml·min^{-1}. Fractions displaying muramidase activity and eluting between 7.5 % and 15 % B were pooled, acidified with TFA to a final concentration of 0.15 % (v/v) and concentrated by solid phase extraction on Sep-Pak Vac 5g $^{t}C_{18}$ cartridges equilibrated in 0.15 % (v/v) TFA in water. Bound material was eluted with 15 ml of 70 % acetonitrile in acidified water and freeze-dried. The

lyophilised eluate was reconstituted in 0.1% (v/v) TFA in deionized water before loading onto an ODS2-Inertpak C_{18} RP-HPLC column (particle size 5 μm, 4.6 mm x 250 mm, Capital HPLC). Elution was executed at 25 °C with a linear biphasic gradient of 0.1 % (v/v) TFA in deionized water (A) and 0.09 % (v/v) TFA in acetonitrile (B), ranging from 0 to 70 % B over 70 min at a flow rate of 1 $ml \cdot min^{-1}$.

At each step of the purification procedure protein profiles were determined by SDS-PAGE as detailed in Section 2.3.6. The gels were stained with Coomassie brilliant blue (Appendix C).

7.3.8. Protein Quantification

Total protein concentration was estimated by the method of Bradford as described in Section 2.3.8.

7.3.9. Isoelectric Focusing

An estimate for the isoelectric point (pI) of the partially purified protein was obtained by isoelectric focusing (IEF), using a pre-cast IEF acrylamide gel with a pH gradient ranging from 3 to 10 (Bio-Rad).

7.3.10.Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

The molecular mass of the purified protein was determined by MALDI-TOF MS at the Centre for Biomolecular Sciences (University of St Andrews, UK) as detailed in Section 3.3.9. Three independent experiments were performed for each sample.

7.3.11.Partial Primary Structure Determination

N-terminal amino acid sequencing was performed by standard automated Edman degradation as detailed in Section 4.3.9.

7.4. Results

7.4.1. Partial Protein Purification

The water-soluble mucus extract was fractionated by cation exchange chromatography on CM-Sepharose at pH 6.4, yielding two groups of fractions with lytic activity against *M. luteus* cell walls (Fig. 7.1). The fractions corresponding to the flow-through were subsequently applied to a DEAE-Sepharose column, pH 8.0. Fractions eluted with 520 to 540 mM NaCl displayed activity against M. *luteus* cell walls but not *P. citreus* (Fig. 7.2). Muramidase activity was found to be present in fractions eluting with 57 % to 60 % acetonitrile during further fractionation by C_{18} RP-HPLC (Fig 7.3).

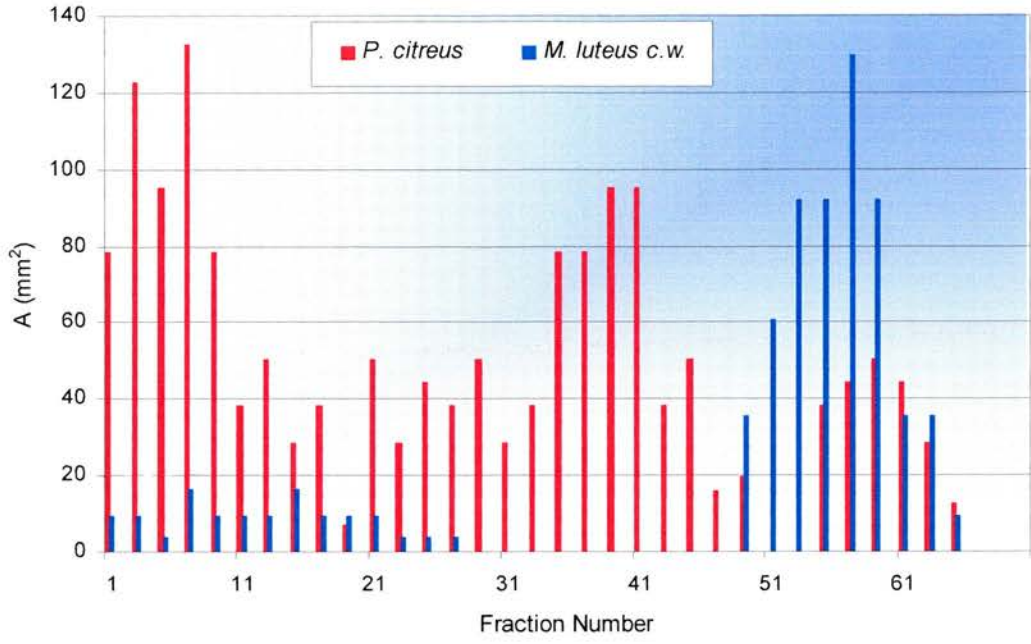


Figure 7.1. Activity profile of the cation exchange chromatography fractions. The water-soluble epidermal extract was subjected to cation exchange chromatography on CM-Sepharose, pH 6.4, and the resulting fractions assayed for antibacterial activity against *P. citreus* and muramidase activity against *M. luteus* cell walls. Fractions 1 to 27 displayed muramidase activity and did not bind to the matrix.

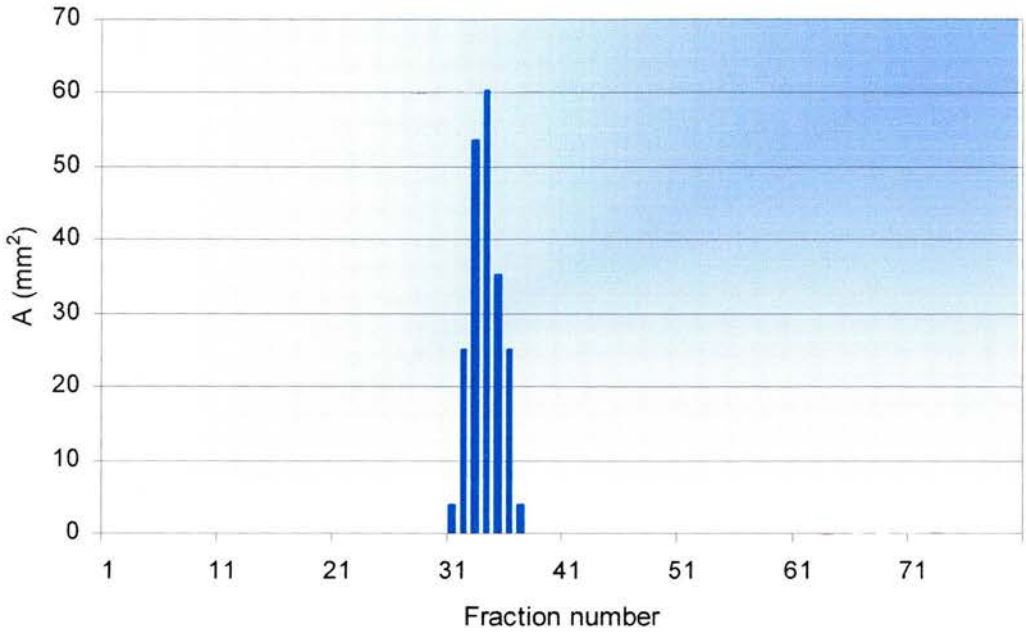


Figure 7.2. Activity profile of the anion exchange chromatography fractions. The flow-through from the cation exchange chromatography was dialysed and subjected to solid phase extraction. The unbound fraction was further fractionated by anion exchange chromatography on DEAE-Sepharose, pH 8.0, and the resulting fractions assayed for antibacterial activity against *P. citreus* and muramidase activity against *M. luteus* cell walls. Fractions 31 to 37 displayed muramidase but not antibacterial activity.

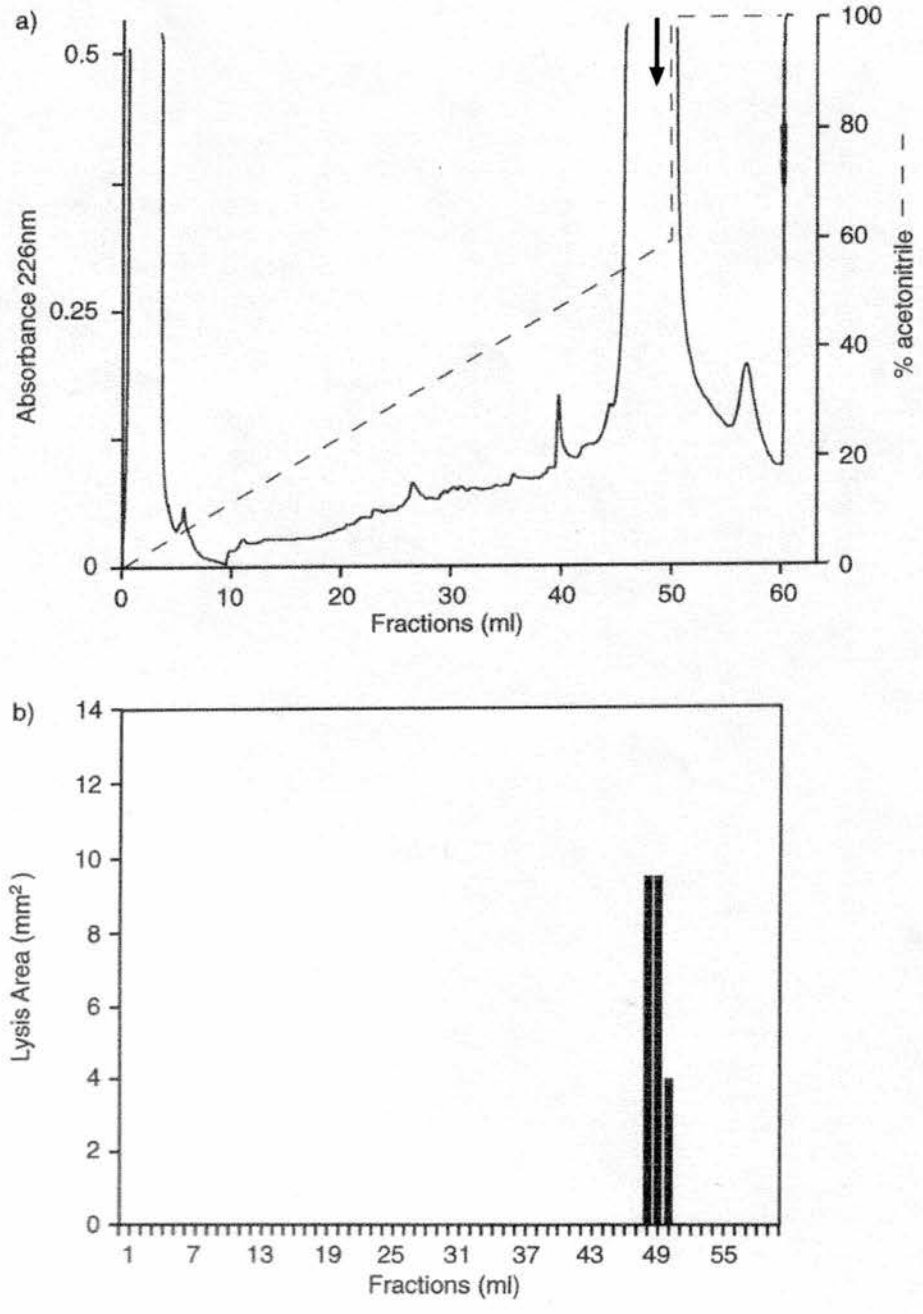


Figure 7.3. Chromatographic analyses of the antibacterial proteins in skin mucus. (a) C_{18} RP-HPLC elution profile of the DEAE-Sepharose FPLC active fractions, obtained at 226 nm (solid line). The proportion of acetonitrile is indicated by the dashed line. Muramidase-positive fractions are shown by the arrow. (b) Activity profile against M. *luteus* cell walls of C_{18} RP-HPLC fractions after lyophilisation and reconstitution in 50 µl of sterile deionized water. The active fractions were eluted with 57 % to 60 % acetonitrile.

7.4.2. Isoelectric Focusing

Isoelectric focusing of the partially purified muramidase after C_{18} RP-HPLC resulted in several bands focusing between *circa* 4.6 and 6.0 (Fig. 7.4).

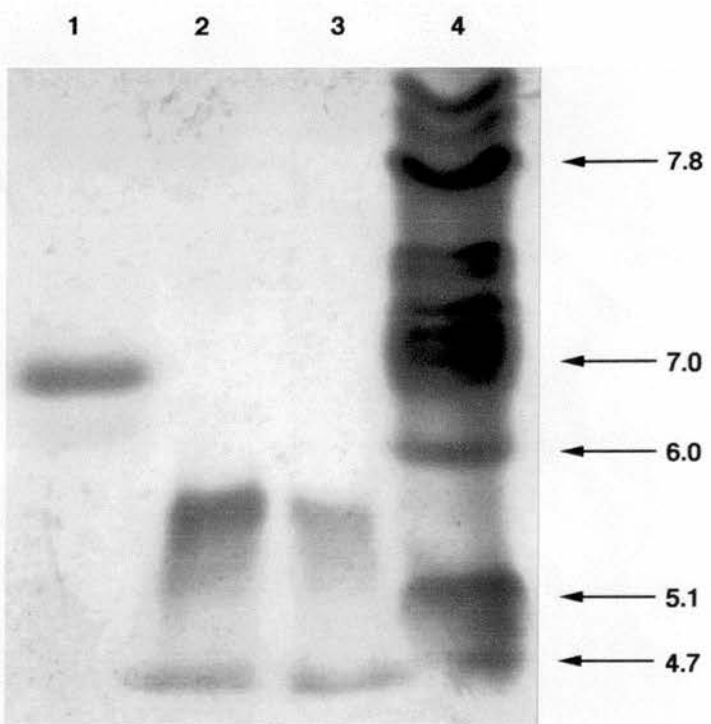


Figure 7.4. Isoelectric focusing of the muramidase-positive fractions obtained after C_{18} RP-HPLC of the water-soluble fraction of skin mucus. Samples were applied to a pre-cast IEF gel, pH 3-10,. Lane 1: myoglobin standard; lane 2: C_{18} RP-HPLC fraction 48, lane 2: C_{18} RP-HPLC fraction 49; lane 4: Bio-Rad IEF markers. The numbers on the right-hand side indicate the isoelectric point of the markers. Proteins were visualized by Coomassie staining. All the proteins present in the fractions displaying muramidase activity focus between 4.6 and 6.0.

7.4.3. Purification of an Anionic Muramidase

As shown in Fig. 7.4, the several chromatographic methods employed to isolate this anionic muramidase from trout skin mucus were not sufficient to purify it to apparent homogeneity. It was therefore necessary to refine this purification protocol. The optimised procedure was based on a distinct protein extraction method with phosphate buffer. When this protein extract was subjected to ammonium sulfate fractionation, muramidase activity was predominant in the 85 % ammonium sulfate precipitate (Table 7.1), with an activity recovery of 27.3 %.

Acid-urea gel overlay of the resuspended 85 % ammonium sulphate precipitate on a plate of *M. luteus* cell walls showed two clear bands corresponding to two proteins with muramidase activity and different electrophoretic mobility (Fig. 7.5).

Table 7.1. Recovery of muramidase activity from trout skin extract following ammonium sulfate fractionation. The phosphate-soluble extract was successively precipitated with increasing amounts of ammonium sulfate, corresponding to 20 %, 35 %, 50 %, 85 % and 100 % of the saturation value. Fractions were assayed for muramidase activity using a turbidometric assay with *M. luteus* cell walls as substrate. One unit of activity (U) is defined as the amount of enzyme that generates a decrease in optical density of 0.001 min^{-1} at 450 nm, 20 °C and pH 6.2. Protein concentration was estimated by the method of Bradford using bovine serum albumin as standard.

	Extract	20 %	35 %	50 %	85 %	100 %
V (ml)	180	4	4	8	50	4
[Protein] ($mg \cdot ml^{-1}$)	1.8	8.0	6.8	6.8	2.5	5.0
Total Protein (mg)	324	32	27	54	125	20
Protein Recovery (%)	100	9.9	8.3	16.7	38.6	6.2
[Muramidase] ($U \cdot ml^{-1}$)	240	125	50	75	240	125
Total Activity (10^3 U)	43.2	0.5	0.2	0.6	12.0	0.5
Activity Recovery (%)	100	1.2	0.5	1.4	27.3	1.2

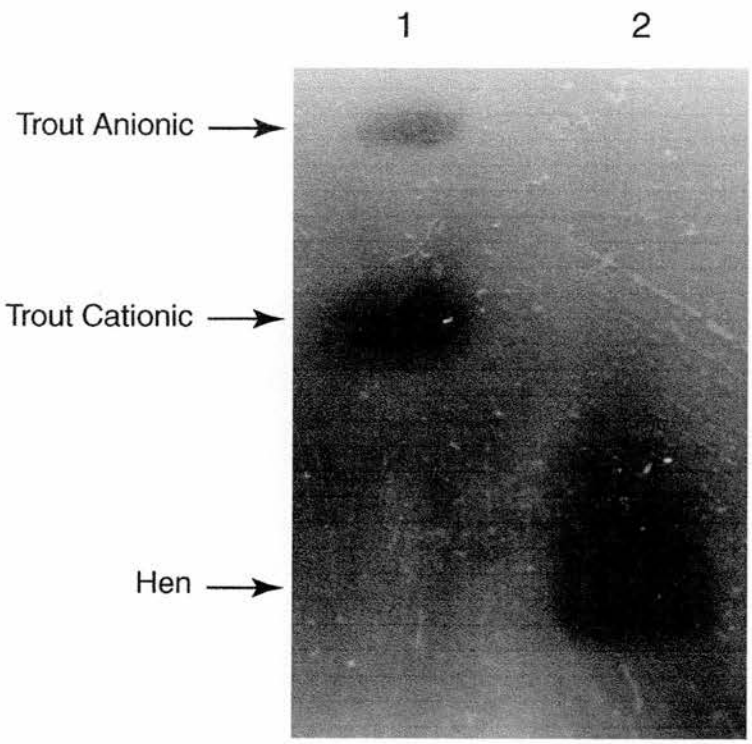


Figure 7.5. Acid-urea gel overlay of trout skin muramidases on *M. luteus* cell walls plate. The plate was incubated for 3 h at 37 °C. Lane 1, 85 % ammonium sulphate precipitate of trout skin extract; lane 2, 25 $\mu g \cdot ml^{-1}$ hen egg white lysozyme. Each lane contains 10 µl of sample. Arrows indicate the clear zones produced by trout skin muramidases or hen egg white lysozyme (standard).

These two muramidases were separated by anionic exchange chromatography (Fig. 7.6). The group of muramidase-positive fractions eluting between 75 mM and 150 mM NaCl (Fig. 7.6) was further fractionated by C_{18} reversed phase HPLC, yielding muramidase activity in two fractions corresponding to a single peak eluting at 45.7 min (Fig. 7.7). SDS-PAGE analysis showed a single band with an apparent molecular weight of approximately 14 kDa (Fig. 7.7 inset).

7.4.4. Protein Characterisation

Attempts to determine the N-terminal sequence of the purified protein were unsuccessful, probably due to a blocked N-terminus. Mass spectrometry of this protein revealed a single ion cluster at 14 268 Da (Fig. 7.8).

The purified muramidase did not exhibit activity against *P. citreus* at the concentrations tested (maximum 20 $\mu g \cdot m^{-1}$). Unfortunately, the low yield of purified protein made it impossible to obtain further internal sequence, antibacterial or enzymatic activity data.

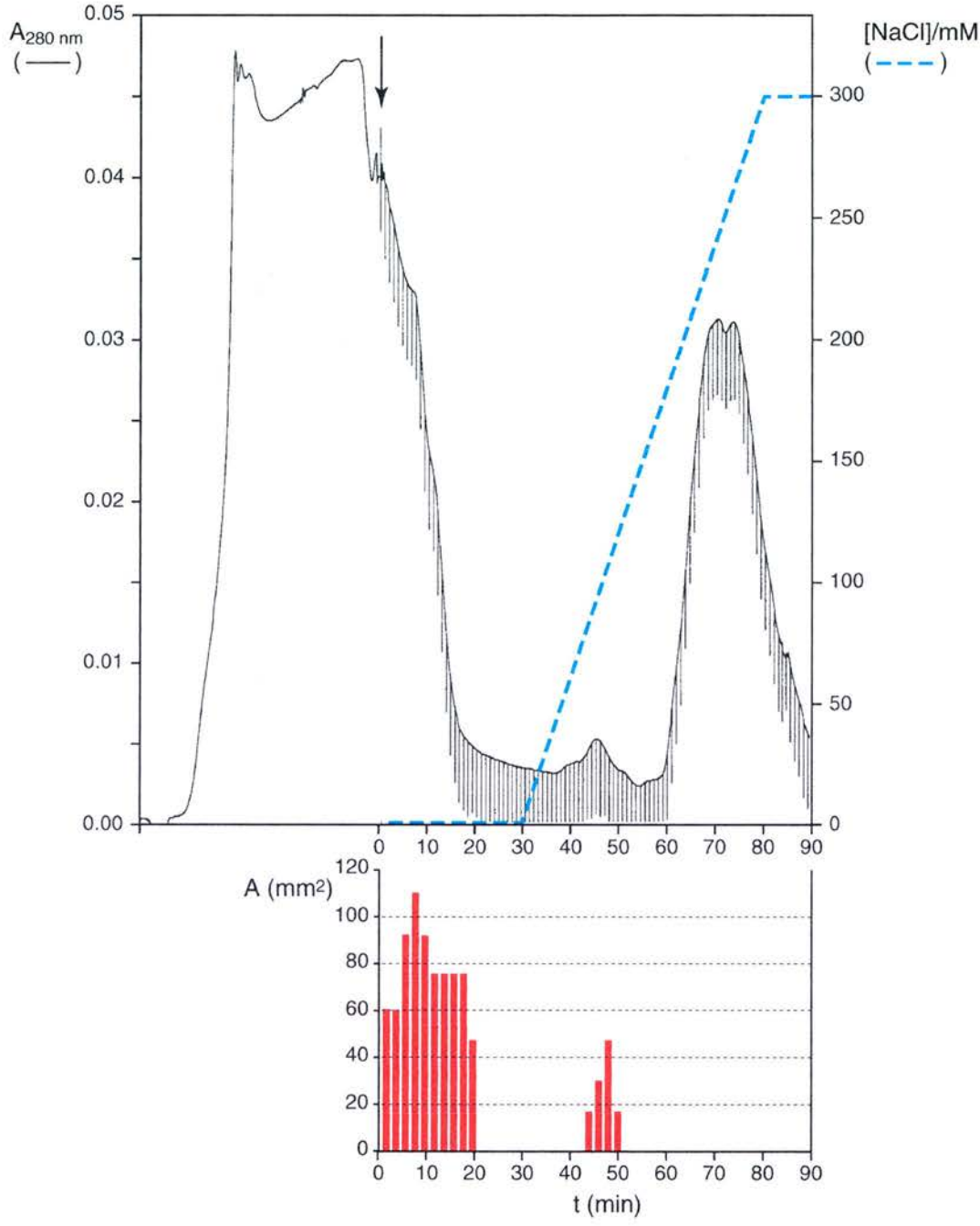


Figure 7.6. Purification of an anionic muramidase from skin secretions of rainbow trout. Protein extracts of skin mucus were fractionated by ammonium sulfate precipitation and the 85 % fraction was applied to a DEAE column for anionic exchange chromatography. The chromatogram was obtained at 280 nm (solid line). Dashed blue line indicates the NaCl concentration gradient. The arrow indicates the end of sample application. Muramidase activity profile is denoted by the histogram.

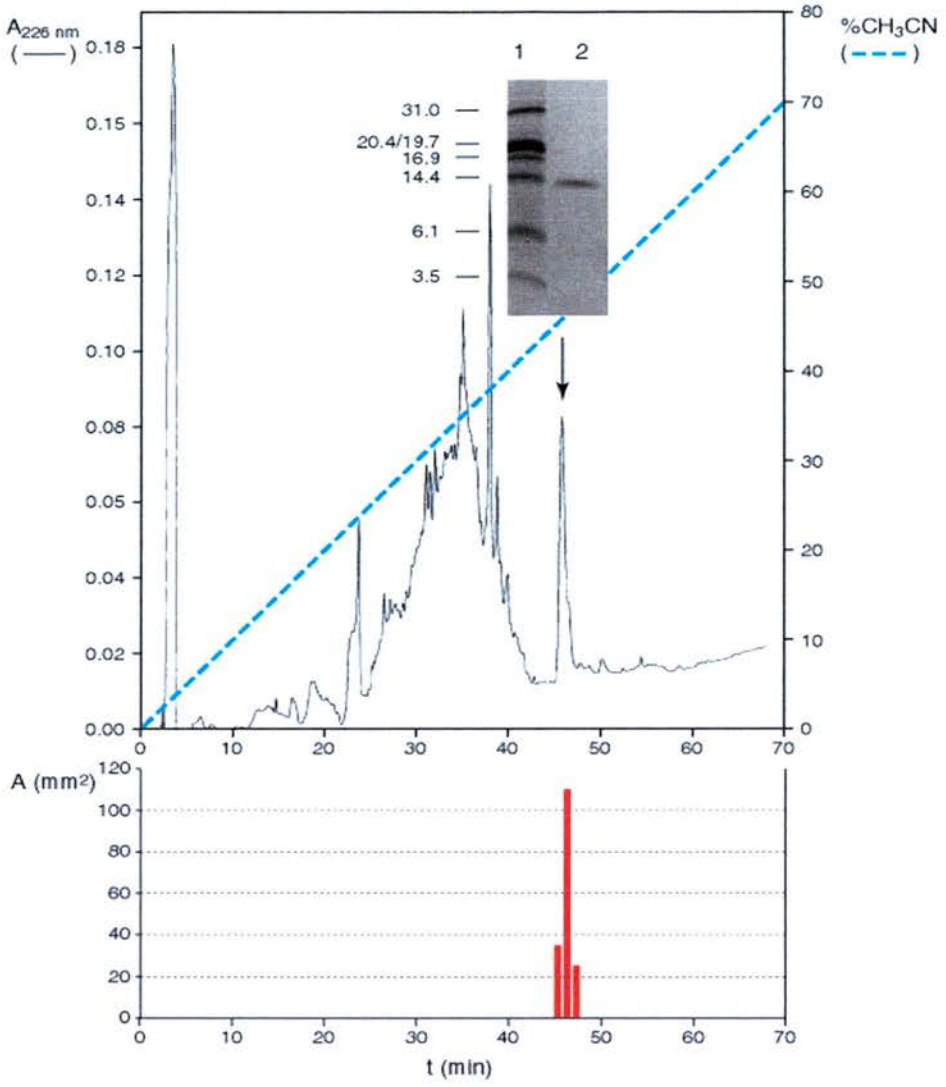


Figure 7.7. Final step in the isolation of an anionic muramidase from skin secretions of rainbow trout. The active fractions eluting between 75 mM and 150 mM NaCl on a DEAE column were concentrated by solid phase extraction using $^{t}C_{18}$ Sep-Pak cartridges; the 70% acetonitrile eluate was subjected to C_{18} reversed phase HPLC. Absorbance was measured at 214 nm (solid line) and the acetonitrile concentration gradient is represented by the dashed blue line. The histogram represents the muramidase-positive fractions, which are also indicated by an arrow in the chromatogram. The inset shows the SDS-PAGE analysis of the purified protein. Ten microlitres of sample (lane 2) were applied and the gel was stained with Coomassie Blue. The numbers on the left hand side indicate the molecular weight of the markers (lane 1) in kDa.

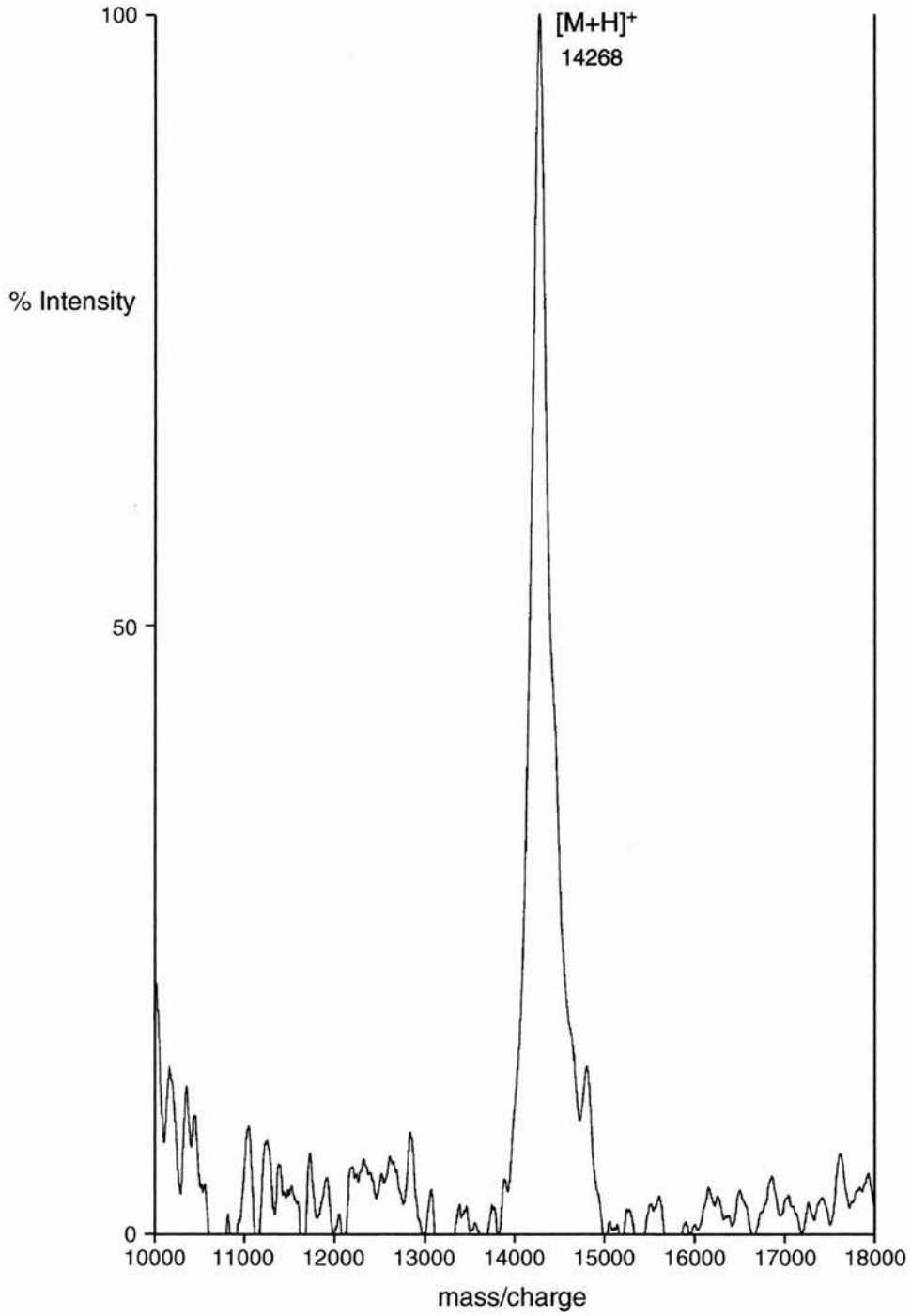


Figure 7.8. Mass spectrum of the purified anionic muramidase determined by MALDI-TOF. The single ion cluster is labelled.

7.4. Discussion

The present chapter shows that skin secretions of rainbow trout contain two proteins with muramidase activity. One has a low electrophoretic mobility on acid-urea gel and is able to bind DEAE-Sepharose at pH 7.0, indicating that, unlike other lysozymes reported for fish (Grinde *et al.*, 1988; Dautigny *et al.*, 1991; Hikima *et al.*, 1997; Fujiki *et al.*, 2000; Liu & Wen, 2002), it has an anionic nature. Isoelectric focusing of the partially purified anionic muramidase demonstrated that the pI of this muramidase must be below 7.0. In the present study it was purified to apparent homogeneity and found to have a molecular mass of 14 268 Da, as determined by MALDI-TOF with an experimental error of approximately 0.02 %. Attempts to directly obtain primary structure data were unsuccessful, probably due to a blocked N-terminus. Unusually, lysozyme from cassowary has an N-terminus blocked by a pyroglutamate residue (Thammasirirak *et al.*, 2002). Alas, the low yield of purified enzyme did not permit further biochemical or enzymatic characterisation. Until more data are obtained for this unusual, low pI lysozyme-like protein from *O. mykiss* mucus its status as a novel type of lysozyme remains unproven.

The presence of two muramidases in trout skin secretions suggests that they may be important components of trout mucosal immunity. Even though this anionic muramidase is not active against *P. citreus* at the concentrations tested, it may act in synergism with other innate humoral defence factors, such as lysozymes or antimicrobial peptides (Chalk *et al.*, 1994; Patrzykat *et al.*, 2001), to protect the skin epithelium from microbial exploitation.

Chapter 8

Purification and Characterisation of a C-type Lysozyme from Skin Mucus of *Oncorhynchus mykiss*

8.1. Synopsis

An antibacterial muramidase was purified to homogeneity from skin secretions of rainbow trout by cation exchange chromatography and reversed phase high performance liquid chromatography. This muramidase is cationic and has a molecular mass of 14252 Da, as determined by matrix-associated laser desorption/ ionization time of flight mass spectrometry. Partial N-terminal amino acid sequencing combined with peptide mapping indicated that it is likely to be a c-type lysozyme, the first to be purified and characterised from skin of a salmonid. Its optimum pH is in the range 4.5 to 5.5 and its optimum temperature, at pH 5.0, is between 33 °C and 49 °C, although it still exhibits considerable activity at 5 °C. At a concentration of 100 $U \cdot ml^{-1}$ it is bactericidal to the Gram-(+) bacterium *Planococcus citreus* but it is not haemolytic to trout erythrocytes and does not display significant chitinolytic activity. Comparison with hen egg white lysozyme revealed that the purified trout lysozyme has a higher rate of diffusion in a lysoplate assay. The presence of this antibacterial lysozyme in skin mucus suggests that it may be involved in mucosal innate immunity, acting either by itself or in synergism with other antibacterial factors such as antimicrobial peptides.

8.2. Introduction

The ability of lysozyme to kill bacteria by hydrolysing the peptidoglycan layer in the bacterial cell wall, coupled with its abundance and broad distribution across a wide range of animal phyla (Canfield, 1963; Canfield *et al.*, 1971; Jollès & Jollès, 1975; Snyder & Harrison, 1977; Grinde *et al.*, 1988; Nilsen *et al.*, 1999; Hikima *et al.*, 2001), has firmly established its role as a "natural" disinfectant (Jollès, 1996).

Multiple isoforms of lysozyme may occur within a single species. For example, two types of lysozyme, designated type I and type II, have been isolated from the head kidney of rainbow trout, *Oncorhynchus mykiss* (Grinde *et al.*, 1988). In type II alanine is substituted for aspartic acid at position 86 (Dautigny *et al.*, 1991). It might be expected that both forms are also expressed at the epithelial surface, which, in teleosts, is living and not shielded by a keratinised epidermis (Hawkes, 1974) and therefore at risk from opportunistic or pathogenic invasion.

There are several reports of muramidase activity in crude protein extracts of the skin mucus of teleosts (Hjelmeland *et al.*, 1983; Aranishi *et al.*, 1998; Fevolden *et al.*, 1999) but, surprisingly, no protein sequences for lysozymes from fish epithelia have been lodged on international databases, and no biochemical characterisations have been made of lysozyme from the skin mucus of salmonids, one of main groups of fish raised in aquaculture. In the present chapter the purification procedure of the cationic lysozyme identified in the previous study (Chapter 7) is described. Moreover, its structural and enzymatic characterisation is also reported.

8.3. Experimental Procedures

Unless declared otherwise, chemicals were acquired from Sigma-Aldrich. All solutions are aqueous and prepared with deionized water (Elga), except when specified elsewise. The list of suppliers and respective addresses is presented in appendix B.

8.3.1. Enzymatic Assays

Muramidase activity was quantified either by radial diffusion assay, as described in Section 2.3.5, or by a modified version of the turbidometric assay reported by Grinde *et al.* (1988), as detailed in Section 7.3.4. In order to ascertain the effect of pH on muramidase activity, turbidometric assays were performed at 20 °C in 50 mM sodium acetate, 15 mM sodium phosphate, 50 mM ethanolamine or 50 mM N-cyclohexyl-3-aminopropanesulfonic acid buffers, for pH ranges of 3.8 - 5.8, 6.2 - 8.0, 8.5 - 10.5 and 10.5 - 11.0, respectively. All buffers were adjusted to 50 mM ionic strength with NaCl according to the information obtained with the Twigger & Beynon's buffer maker software (http://www.biochem.mcw.edu/~simont/java/BufferMaker.html). To determine the optimal temperature for enzyme activity, solutions containing approximately 10^3 U·ml^{-1} at 25 °C were used. For each enzyme, substrate was prepared in the buffer that corresponded to its optimal pH and the suspension was allowed to equilibrate at the required temperature (ranging from 5 °C to 85 °C) prior to the turbidometric assay described above.

Chitinolytic activity of muramidase solutions was assayed using 0.2 $mg \cdot ml^{-1}$ chitin azure in acetate buffer (pH 4.0 - 7.0) as substrate, according to the protocol described by Düring *et al.* (1999).

8.3.2. Antibacterial Assay

Antibacterial activity was assessed by a modified microtitre broth dilution assay (Friedrich *et al.*, 1999), as described in Section 3.3.5. The following bacterial strains were used: *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (NCIMB 12210), *Planococcus citreus* (NCIMB 1493) and an isolate of *Yersinia ruckeri* kindly gifted by Dr Anthony Ellis (Marine Laboratory, Aberdeen, UK).

8.3.3. Purification of a Cationic Muramidase

The phosphate protein extract prepared as described in Section 7.3.2 was subjected to anion exchange chromatography on DEAE-Sepharose (please consult Section 7.3.7). The resulting flow-through and wash fractions (Fig. 7.6) were pooled and applied to a CM-Sepharose 1 cm x 10 cm Econo-column for cation exchange chromatography. Following a 30 minute wash with 20 mM HEPES, pH 7.0 (buffer A) to remove non-specifically bound material, elution was performed with a linear AB gradient (where B is 20 mM HEPES, 1 M NaCl, pH 7.0) from 0 to 40 % B over 60 min, followed by 10 min of 40 % B, at a flow rate of 1 $ml \cdot min^{-1}$. Muramidase-positive fractions eluting between 20 % and 32.5 % B were pooled, acidified to 0.15 % (v/v) trifluoroacetic acid and subjected to solid phase extraction on Sep-Pak Vac 5g $^{t}C_{18}$ cartridges equilibrated in acidified water (0.15 % (v/v) TFA). The 70 % acetonitrile eluate was lyophilised, reconstituted in acidified (0.1 % (v/v) TFA) deionized water and

loaded onto an ODS2-Inertpak C_{18} reversed phase HPLC column using the HPLC system above described. Elution was performed at 25 °C with the following biphasic gradient of 0.1 % (v/v) TFA in acidified deionized water (A) and 0.09 % (v/v) TFA in acetonitrile (B): 0 % to 30 % B over 15 min, 30 % B for 5 min and finally 30 % to 60 % B over 40 min, at a flow rate of 1 $ml \cdot min^{-1}$.

At each step of the purification procedure protein profiles were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) using the Tris-Tricine system described by Schägger and von Jagow (1987), as described in Section 2.3.6. The gels were stained with Coomassie brilliant blue (Appendix C).

8.3.4. Protein Quantification

The concentration of purified cationic muramidase was determined by the method of Bradford (1976) using hen egg white lysozyme as standard. Since lysozyme does not follow Beer-Lambert's law, standards were fitted to a third order polynomial equation and concentration of test samples ascertained by interpolation using the TableCurve 2D software (Jandel Scientific).

8.3.5. Matrix-Associated Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

The molecular masse of the purified cationic muramidase was determined by MALDI-TOF MS at the Centre for Biomolecular Sciences (University of St Andrews, UK) according to the protocol described in Section 3.3.9. Three independent experiments were performed for each sample. For peptide mapping

the purified protein was digested with trypsin and the digestion products analysed by MALDI-TOF MS as above.

8.3.6. Partial Primary Structure Determination

N-terminal amino acid sequencing of the purified cationic muramidase was performed as described in Section 4.3.9.

8.3.7. Sequence Analysis

The proteomics tools alluded to in Section 2.3.9 were employed to perform homology searches and prediction of protein parameters. Sequence alignments were executed with the Omiga 2.0 sequence analysis software, as described in Section 3.3.11. Identification of proteins by peptide mass fingerprinting was done with the Mascot algorithm (Perkins *et al.*, 1999), as detailed in Section 3.3.11.

8.3.8. Haemolytic Assay

The purified protein was tested for haemolytic activity against trout erythrocytes according to the protocol described in Section 3.3.15.

8.4. Results

8.4.1. Purification of a Cationic Muramidase

The flow-through from the anionic exchange chromatography, after pooling with the wash fractions (Fig. 7.6) and application to a CM matrix for cationic exchange chromatography, yielded active fractions eluting between 200 mM and 325 mM NaCl (Fig. 8.1). Following solid phase extraction on $^{t}C_{18}$ Sep-Pak cartridges and C_{18} reversed phase HPLC, a peak eluting at 35.9 min matched to the fractions displaying muramidase activity (Fig. 8.2). SDS-PAGE analysis of these fractions showed a single band with an apparent molecular weight of *circa* 14 kDa (Fig. 8.3).

The calibration curve shown in Fig. 8.4 was used to estimate the concentration of the purified muramidase, which was found to be approximately 40 $\mu g \cdot ml^{-1}$. The total yield of purified protein was approximately 150 μg.

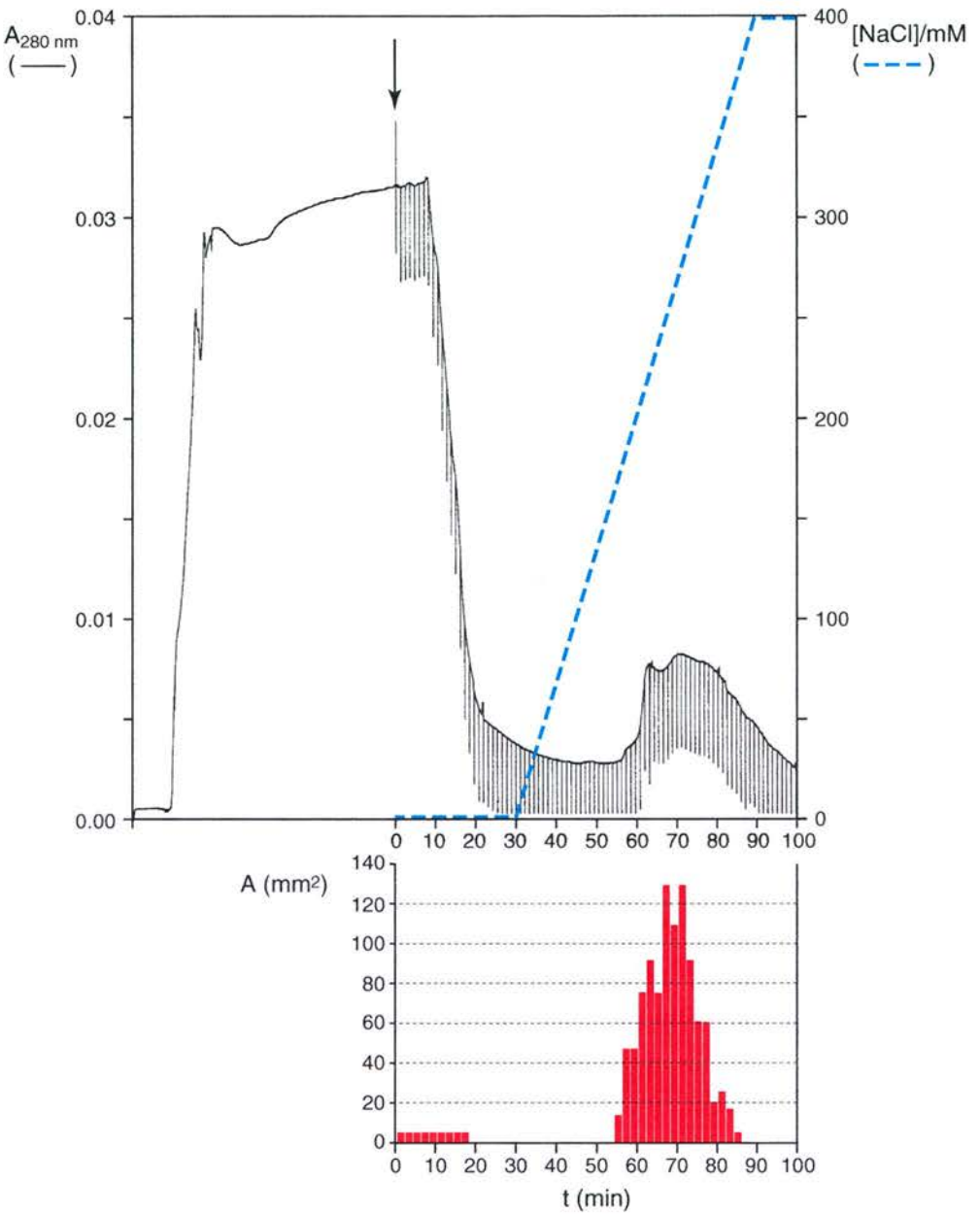


Figure 8.1. Purification of a cationic muramidase from skin secretions of rainbow trout. The flow-through from the anionic exchange chromatography (Fig. 7.6) was subjected to cationic exchange chromatography on a CM column using the depicted NaCl gradient (dashed line). Absorbance was monitored at 280 nm (solid line). Histogram represents the muramidase activity profile, determined by radial diffusion assay. The end of sample application is shown by an arrow.

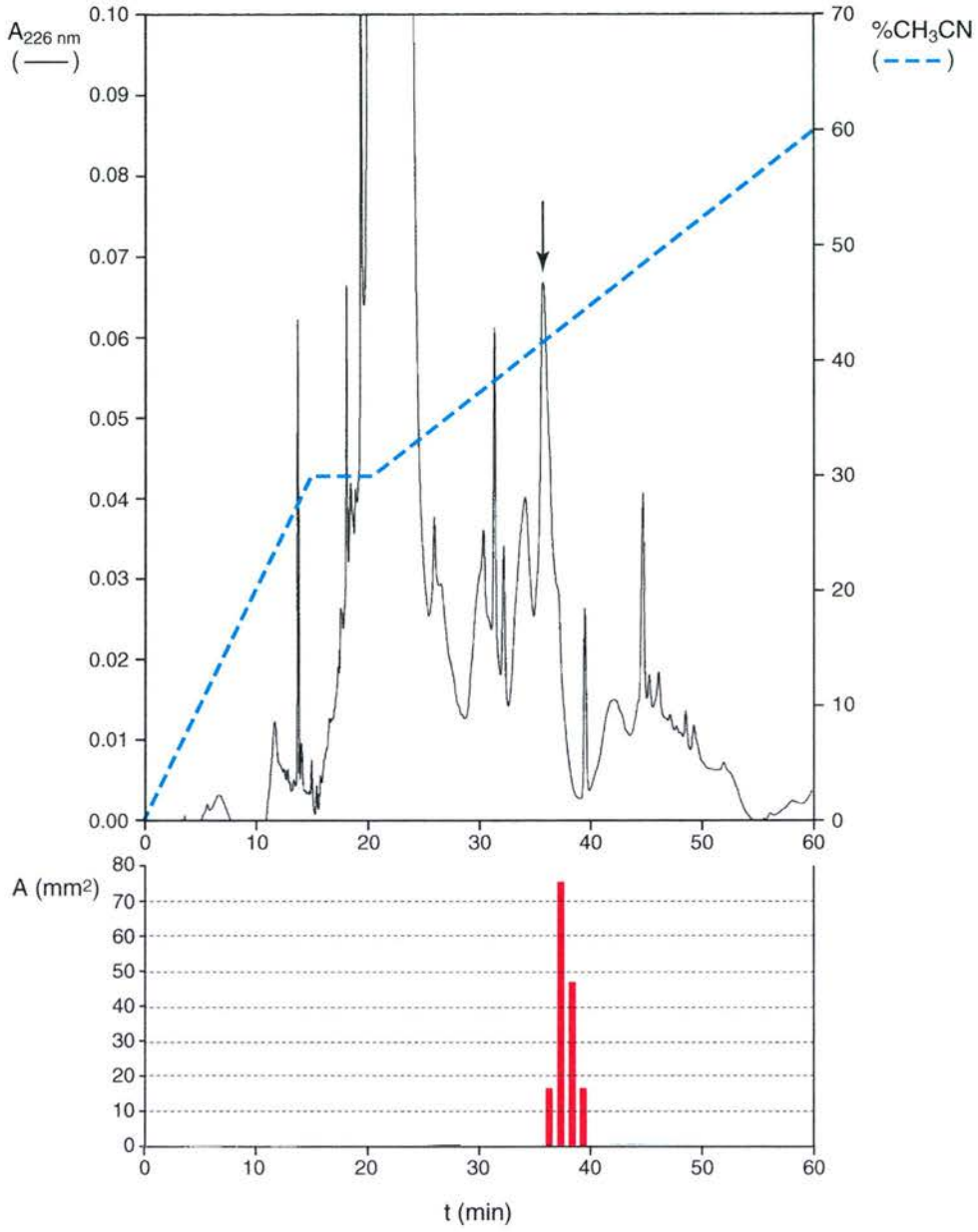


Figure 8.2. Final step in the isolation of a cationic muramidase from skin secretions of rainbow trout. The active fractions eluting between 200 mM and 325 mM NaCl were applied to C^t_{18} Sep-Pak cartridges for solid phase extraction. Following reconstitution, the lyophilised 70 % acetonitrile eluate was subjected to C_{18} reversed phase HPLC using the acetonitrile concentration gradient represented by the dashed line. The chromatogram was obtained at 214 nm (solid line). Muramidase-positive fractions are denoted by the histogram and indicated by an arrow in the chromatogram.

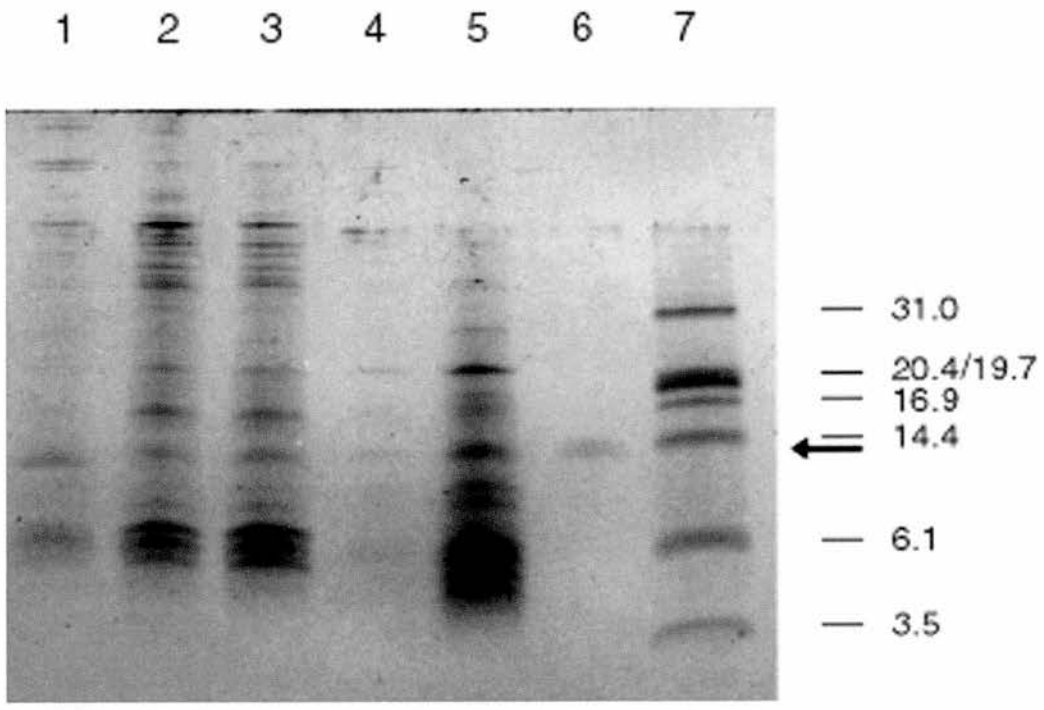


Figure 8.3. Tris-Tricine SDS-PAGE analysis of the active fractions obtained during the purification of a cationic muramidase from skin secretions of rainbow trout. Lane 1: crude extract; lane 2: 85 % ammonium sulfate precipitate; lane 3: pooled flow-through and wash fractions from the anion exchange chromatography; lane 4: cation exchange fractions; lane 5: 70 % acetonitrile eluate from solid phase extraction; lane 6: purified muramidase after C_{18} reversed-phase HPLC (fraction 37); lane 7: markers. Each lane contains 10 µl of sample. The numbers on the right hand side correspond to the molecular mass of the markers in kDa. The protein of interest is indicated by the arrow.

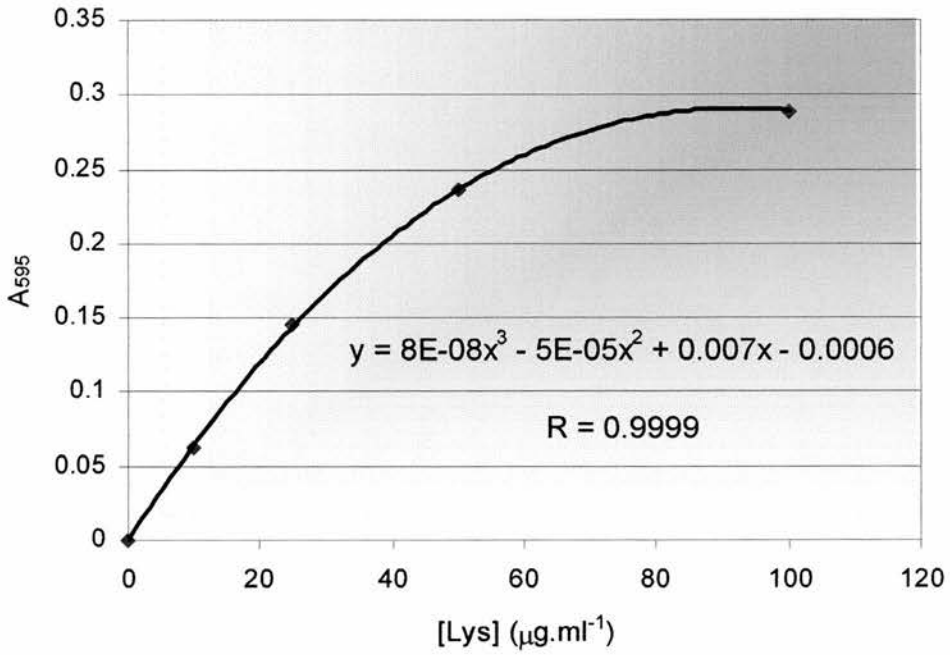


Figure 8.4. Calibration curve for determination of total protein concentration ($\mu g \cdot ml^{-1}$) by the method of Bradford. The absorbance at 595 nm (A_{595}) of the hen egg white lysozyme standards is represented. The equation of the third order polynomial regression and the corresponding Pearson's coefficient (R) are also shown. As the absorbance of the test sample was found to be 0.21, its concentration was estimated to be 40 $\mu g \cdot ml^{-1}$.

8.4.2. Structural Characterisation

MALDI-TOF analysis of the intact protein revealed a signal at 14252 Da corresponding to the single charged molecular ion (Fig. 8.5). Edman degradation of the cationic muramidase showed that its N-terminal sequence[#] is: KVXXRCELARALKAS, where X is an undetermined residue, likely to be cysteine. BLAST homology search showed that this sequence matches c-type lysozyme from the head kidney of rainbow trout (Fig 8.6). The MALDI-TOF mass spectrum of the tryptic digest of this protein shows a number of single charged ions with masses ranging from 750 Da to 2070 Da (Fig. 8.7). The peptide masses indicated in Fig. 8.7 were used to find protein candidates in the Swiss-Prot database using the Mascot fingerprinting algorithm. The only significant protein candidate was lysozyme c-type precursor from rainbow trout head kidney, with 6 significant matches (Table 8.1). Peptide mapping combined with sequencing data demonstrates that trout skin lysozyme and trout head kidney type II lysozyme share at least 69.8 % identity (Fig. 8.8).

[#] This partial amino acid sequence has been deposited in the TrEMBL database under the accession number P83333.

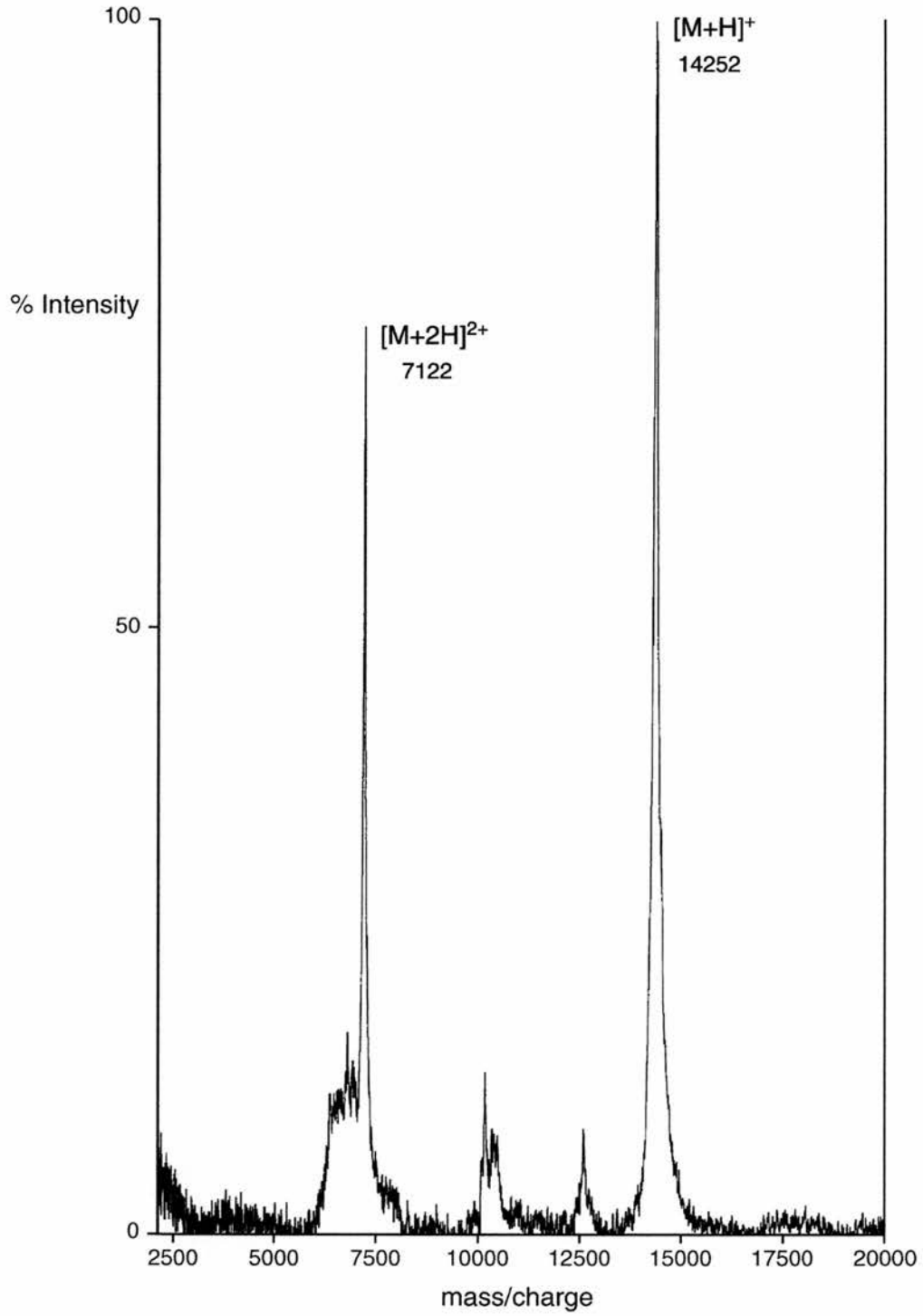


Figure 8.5. MALDI-TOF mass spectrum of the pooled muramidase-positive fractions (Fig. 8.2). The single and double charged ion clusters are labelled.

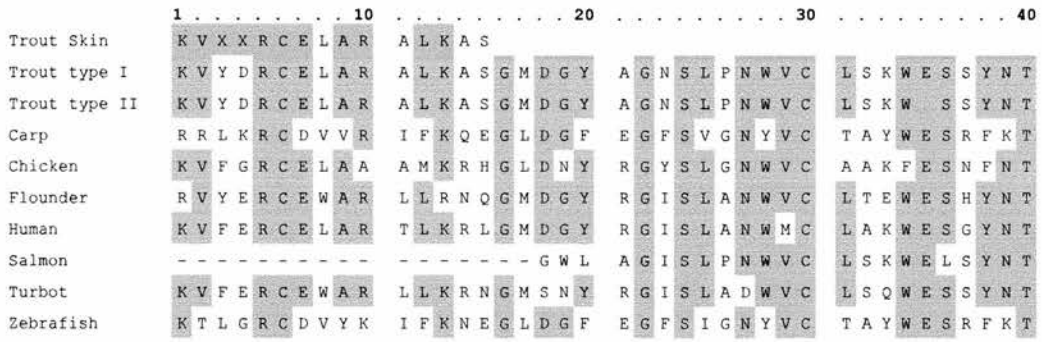


Figure 8.6. Comparison between the 15 N-terminal residues of the cationic trout skin muramidase and the N-terminal sequences of c-type lysozymes isolated from trout head kidney (type I and type II) (Dautigny *et al.*, 1991), chicken (Canfield, 1963) and human (Canfield *et al.*, 1971) or deduced from cDNA clones from carp (Fujiki *et al.*, 2000), flounder (Hikima *et al.*, 1997), salmon (TrEMBL Q9DD55), turbot (TrEMBL Q9PU28) and zebrafish (Liu & Wen, 2002). Identical residues are represented by a shaded box.

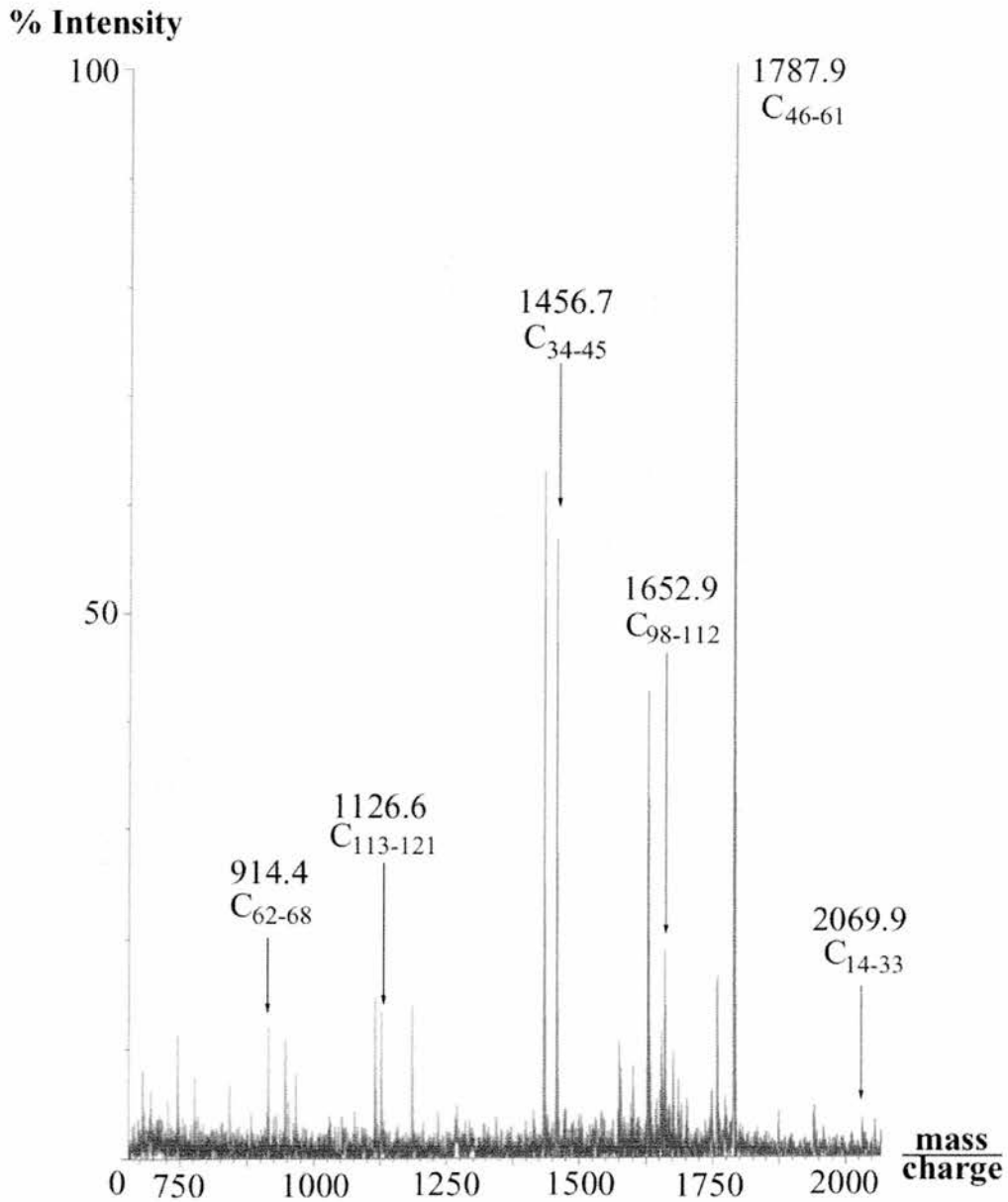


Figure 8.7. Peptide mapping of cationic trout skin muramidase. The purified protein was digested with trypsin and the digestion products analysed by MALDI-TOF MS. The masses (Da) of the identified monovalent peptide ions are indicated; the corresponding position of the fragments in trout head kidney lysozyme (Dautigny *et al.*, 1991) is represented as C_{x-y}, where x and y indicate the first and last residue (respectively) of each peptide.

Table 8.1. Theoretical monoisotopic masses of the peptides generated by digestion of trout kidney type II lysozyme (Swiss-Prot P11941) with trypsin assuming no missed cleavages. The peptides which have been experimentally identified by peptide mass fingerprinting of the cationic muramidase from trout skin are highlighted in red.

Mass (Da)	Position	Peptide Sequence
2069.94	29-48	ASGMDGYAGNSLPNWVCLSK
1787.82	61-76	NTDGSTDYGIFQINSR
1652.89	113-127	VVLDPNGIGAWVAWR
1503.82	95-108	CSQLLTADLTVAIR
1456.64	49-60	WESSYNTQATNR
1126.54	128-136	LHCQNQDLR
914.35	77-83	YWCDDGR
755.34	137-144	SYVAGCGV
661.34	89-94	NVCGIR
591.29	21-25	CELAR
552.28	17-20	VYDR

KVYDRCELARALKASGMDGYAGNSLPNWVCLSKWESSYNTQAT
NRNTDGSTDYGIFQINSRYWCDDGRTPGAKNVCGIRCSQLLTA
DLTVAIRCAKRVVLDPNGIGAWVAWRLHCQNQDLRSYVAGCGV

Figure 8.8. Comparison between the primary structures of trout skin lysozyme and trout head kidney type II lysozyme. The complete amino acid sequence (129 residues) of trout kidney type II lysozyme (Swiss-Prot P11941) is shown. The residues which have been experimentally identified by peptide mass fingerprinting or amino acid sequencing of the cationic muramidase from trout skin are highlighted in red, revealing an overall identity of at least 69.8 % between these two lysozymes.

8.4.3. Enzymatic Characterisation

The purified cationic muramidase had a specific activity against *M. luteus* cell walls of $7 \cdot 10^5$ U·mg^{-1} protein at pH 5.0 and 20 °C (Fig. 8.9C). Under the same conditions the specific activity of hen egg white lysozyme was $5 \cdot 10^5$ U·mg^{-1} (Fig. 8.9B). At a concentration of 20 µg·ml^{-1} and pH values ranging from 4.0 to 7.0, it had almost negligible chitinase activity compared with hen egg white lysozyme at the same concentration (Fig. 8.10).

The optimal pH for trout skin muramidase was found to be between 4.5 and 5.5 (Fig. 8.11). At pH values above 8.0 no enzymatic activity was observed (Fig. 8.11). Its temperature dependence at pH 5.0 and 50 mM ionic strength are

shown in Fig. 8.12. The enzyme has an optimal temperature in the range 33 °C to 49 °C.

Radial diffusion assays revealed that for concentrations in the interval 250 $U \cdot ml^{-1}$ to 10^3 $U \cdot ml^{-1}$, the area of the clear zones created by trout lysozyme is *circa* 2 times greater than those produced by hen egg white lysozyme (Fig. 8.13A, B). Moreover, for the same enzyme concentration the area of the clear zones was directly proportion to the natural logarithm of lysozyme concentration (Fig. 8.13 B).

Dilute solutions (10 $\mu g \cdot ml^{-1}$) of the cationic muramidase from trout skin had a half-life of one day at room temperature. The enzyme displayed muramidase activity following incubation at 80 °C for 10 min (Fig. 8.14) but did not retain its antibacterial properties against *P. citreus*.

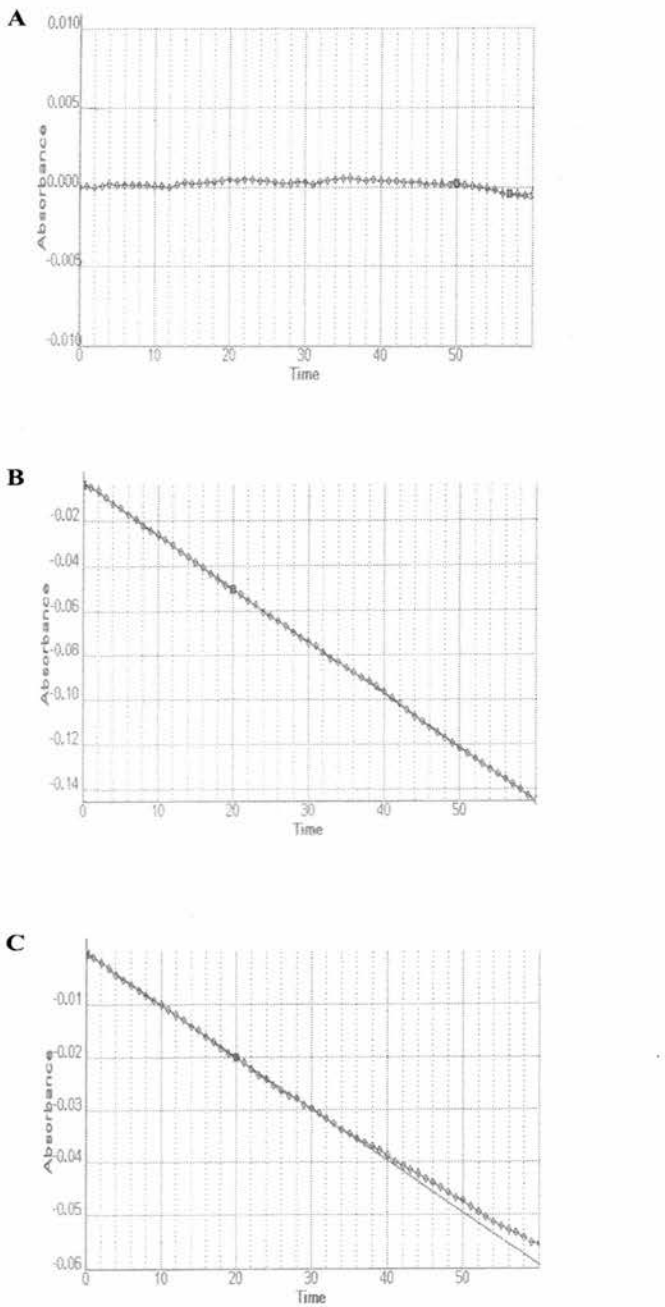


Figure 8.9. Time course of *M. luteus* cell walls hydrolysing activity. Muramidase test solutions were incubated with *M. luteus* cell walls at pH 5.0 and ionic strength of 50 mM and the OD_{450} was measured every second for 60 s. Initial rates of muramidase activity were determined from the first 20 s of linear decrease in OD_{450} (A) *M. luteus* cell walls suspension used as reference. (B) The muramidase activity of 25 $\mu g \cdot ml^{-1}$ of hen egg white lysozyme was $2.4 \cdot 10^3$ $U \cdot ml^{-1}$. (C) The purified trout skin lysozyme (20 $\mu g \cdot ml^{-1}$) had an activity equivalent to $2.8 \cdot 10^3$ $U \cdot ml^{-1}$.

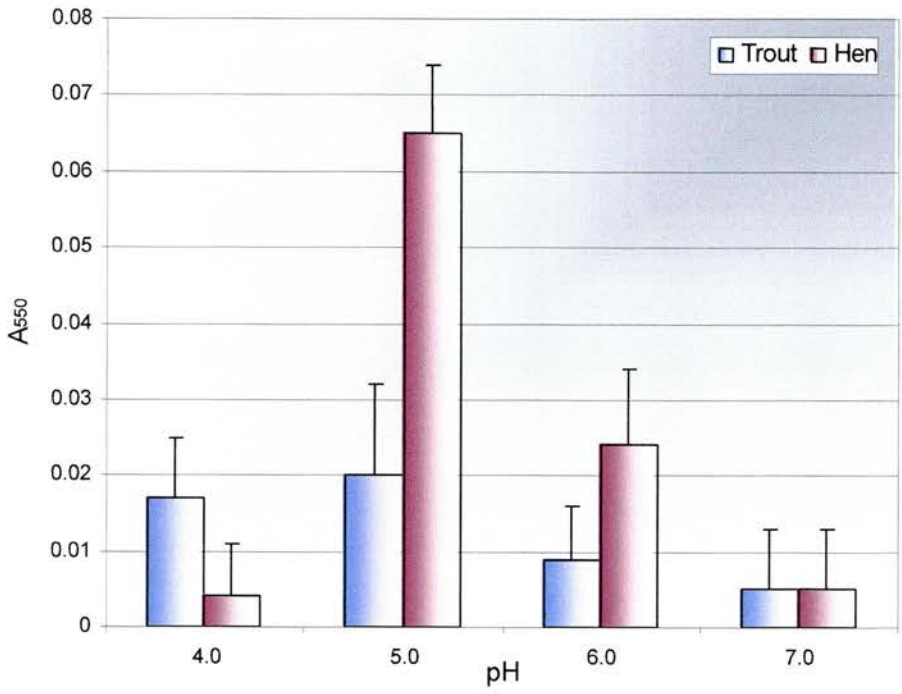


Figure 8.10. Chitinolytic activity of trout skin lysozyme. Two hundred units of either trout skin lysozyme (Trout) or hen egg white lysozyme (Hen) were incubated with 40 μg chitin azure in acetate buffer (pH ranging from 4.0 to 7.0) for 24 h at 37 °C. The soluble dye released by enzymatic hydrolysis was measured spectrophotometrically at 550 nm. Data are shown as means ± SE, n=3.

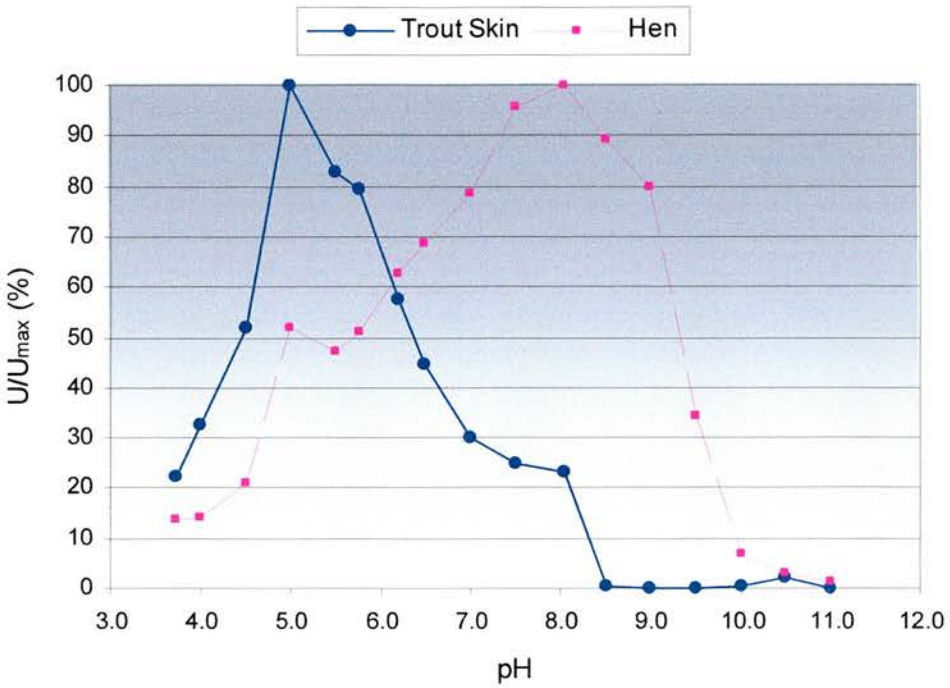


Figure 8.11. Influence of pH on muramidase activity. Purified cationic trout skin muramidase or hen egg white lysozyme were assayed for muramidase activity using a turbidometric assay at pH values ranging from 3.8 to 11.0 and constant ionic strength (50 mM). The results are expressed as a percentage of the activity at each enzyme's optimal pH. Each point represents the average of two independent experiments.

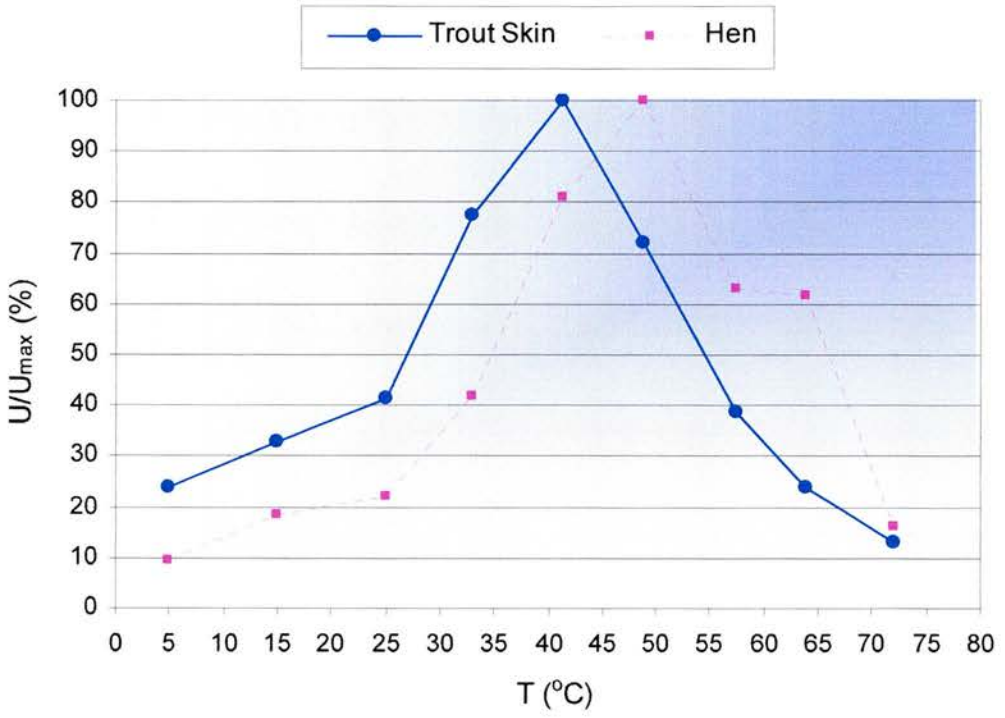
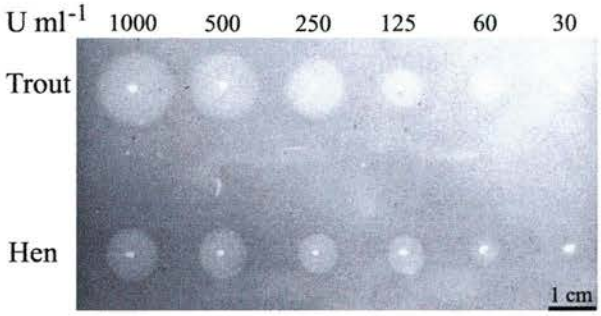


Figure 8.12. Profile of muramidase activity *versus* temperature. The cationic trout skin muramidase or hen egg white lysozyme were assayed for muramidase activity in a 5 °C to 85 °C interval, at pH 5.0 and 8.0, respectively. The temperatures depicted in the graph are averages that account for temperature fluctuations during the experiments. Data are represented as a percentage of maximum activity at its optimal temperature. Each point represents the average of two independent experiments.

A



B

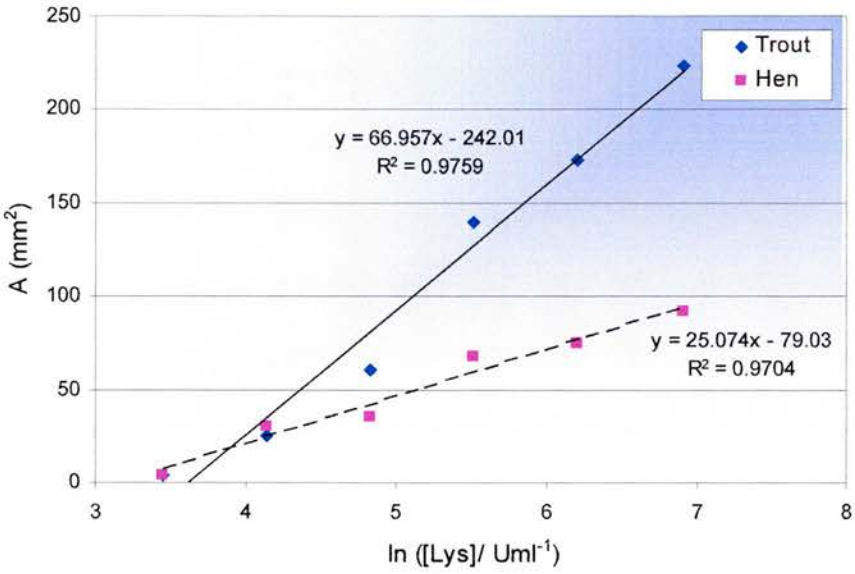


Figure 8.13. Diffusibility assay. (A) Two-fold dilutions of hen egg white lysozyme (Hen) or lysozyme purified from trout skin mucus (Trout) were tested for lysozyme activity by radial diffusion assay. Both original solutions had the same activity (10^3 U·ml^{-1}) in a turbidometric assay. The plate was incubated for 5 h at 37 °C. (B) Correlation between the zone area and lysozyme concentration. For each enzyme the linear regression equation and the corresponding squared Pearson's coefficient (R^2) are indicated.

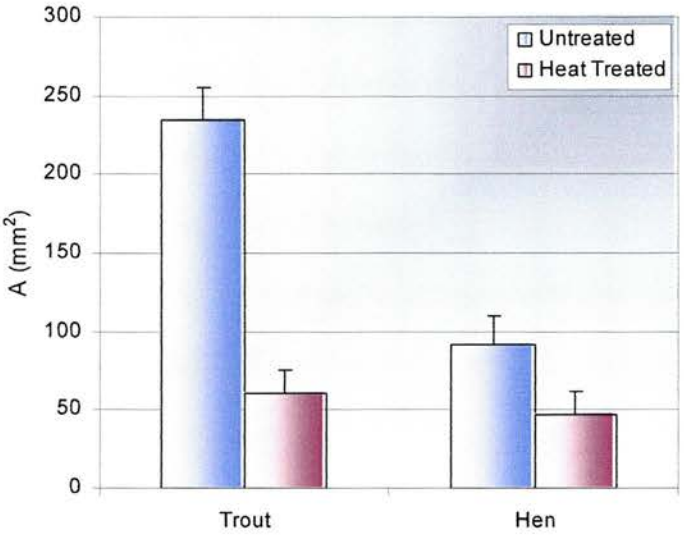


Figure 8.14. Thermal stability assay. Twenty microlitres of 10^3 U·ml^{-1} of either trout skin lysozyme (Trout) or hen egg white lysozyme (Hen) or were incubated at 80 °C for 10 min and assayed for muramidase activity by radial diffusion assay. Trout skin lysozyme retained approximately 25 % of its original activity, whilst hen egg white lysozyme lost only *circa* 50 % of its initial activity. The results shown are expressed as means ± SE, n=3.

8.4.4. Antibacterial and Haemolytic Activity

The purified cationic muramidase was found to be bactericidal to *P. citreus* at a concentration of 100 U·ml^{-1} (Table 8.2). Moreover, it reduced the growth of this bacterium by 50 % at concentrations as low as 6 U·ml^{-1} (Fig. 8.15). No significant activity was observed against the Gram-(-) bacteria at the concentrations tested (Table 8.2). It was not haemolytic against trout erythrocytes at concentrations up to 100 U·ml^{-1} (Fig. 8.16).

Table 8.2. Antibacterial activity of the cationic muramidase purified from trout skin. Results are shown as percentages of bacterial survival following incubation with 100 $U \cdot ml^{-1}$ muramidase under the conditions described in Table 3.2 until the OD_{570} reached 0.2 in the control wells. Hen egg white lysozyme was used as standard. Data are represented as means ± standard error, n=3.

Bacterium	Gram Staining	Trout Skin	Hen Egg White
B. subtilis	+	100 ± 8	90 ± 6
P. citreus	+	0 ± 8	0 ± 1
E. coli	-	95 ± 4	32 ± 6
Y. ruckeri	-	76 ± 5	96 ± 13

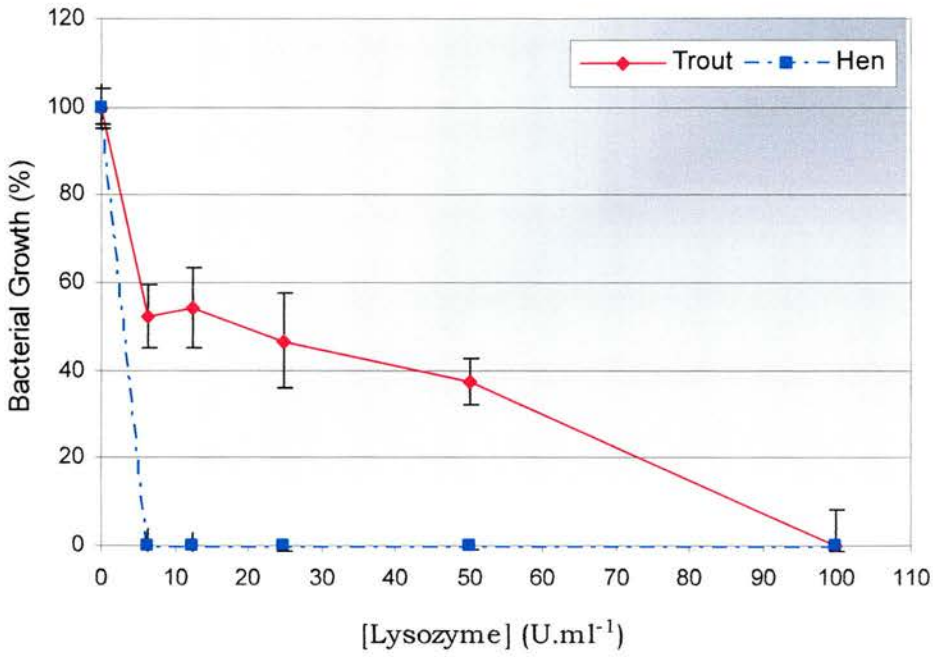


Figure 8.15. Quantification of antibacterial activity against *P. citreus*. Bacterial suspensions containing 10^5 *P. citreus* cfu·ml^{-1} were incubated at 20 °C for 18 h with serially diluted hen egg white lysozyme (standard) or trout skin lysozyme. Bacterial growth is expressed as the ratio of optical densities read at 570 nm (OD_{570}) between each test sample and the control (no lysozyme added). The average final OD_{570} of the control was approximately 0.2. Data are represented as means ± SE, n=3.

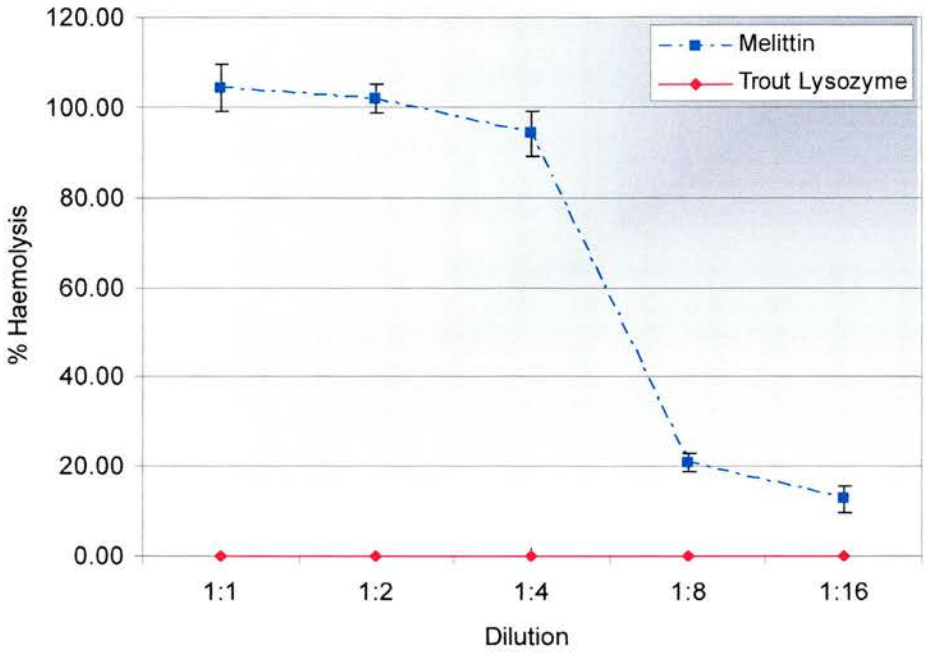


Figure 8.16. Haemolysis assay against trout erythrocytes. Serially diluted test samples were incubated with a 2 % (v/v) suspension of erythrocytes for 30 min at 37 °C. The initial concentrations for trout skin muramidase and melittin were 100 $U \cdot ml^{-1}$ and 25 $\mu g \cdot ml^{-1}$, respectively. Percent haemolysis is defined as the ratio of absorbances (read at 545 nm) between each sample and the positive control (Triton X-100). Values are represented as means ± SE, n=3.

8.5. Discussion

The present study shows that skin secretions of rainbow trout contain a cationic, c-type lysozyme. This muramidase has high similarity to the two lysozymes already isolated and characterised from the head kidney of *O. mykiss* (Grinde *et al.*, 1988).

Peptide mass fingerprinting using the Mascot algorithm identified 6 fragments identical to those expected from tryptic digestion of trout head kidney lysozymes. When combined with the sequencing data, these results show that the trout skin lysozyme shares approximately 70 % identity with trout head kidney lysozymes (Fig. 8.6). Furthermore, sequence alignments show that trout c-type lysozymes are homologous to cDNA-deduced lysozyme sequences from other teleosts (Fig. 8.6). To the best of my knowledge, this is the first report describing the purification and characterisation of a c-type lysozyme from skin mucus of a salmonid. On the other hand, mass spectrometry analysis demonstrates that the skin epithelial lysozyme differs from the kidney-derived forms in size. Its molecular mass was determined by three independent experiments (with an experimental error of *circa* 0.024%) to be 14252 Da, whereas Dautigny *et al.* (1991) have shown that the kidney-derived type I and type II lysozymes from *O. mykiss* have predicted molecular masses of 14288 Da and 14244 Da, respectively. The trout skin epithelial lysozyme reported here further differs from the trout kidney lysozymes in its pH *vs* activity profile. The skin-derived lysozyme, has a pH optimum of 4.5 - 5.5, similar to that displayed by the kidney-forms (Grinde *et al.*, 1988) but the skin enzyme, unlike those from the kidney can still hydrolyse *M. luteus* at pH 8. It is bactericidal to the Gram-(+) bacterium

P. citreus at a concentration at or above 100 $U \cdot ml^{-1}$ but displays neither chitinolytic activity nor haemolytic activity to *O. mykiss* erythrocytes. Importantly, the purified cationic lysozyme displays different activity *versus* pH or temperature profiles than the activity studied in crude skin extracts of *O. mykiss* by Hjelmeland *et al.* (1983).

The head kidney lysozymes type I and type II differ only by one residue at position 86 (Dautigny *et al.*, 1991) and yet have very different characteristics, with type II having a catalytic muramidase activity approximately 3 times higher than type I, and being active against fish pathogens, unlike type I (Grinde, 1989).The differences in mass between the lysozyme from trout skin mucus reported in the present paper and the head kidney lysozymes described by Dautigny *et al.* (1991) indicate that it is unlikely to be identical to type I or type II. We therefore suggest that it could it represent a third isozyme, tentatively designated type III, with different enzymatic and bactericidal properties.

The cationic skin lysozyme reported in the present paper has a higher turnover number than hen egg white lysozyme at low temperatures, with an optimum temperature in the range 33 °C - 49 °C. Its high catalytic activity at low temperatures points to its plausible importance as a defence factor in fish, particularly at low environmental temperatures that compromise their adaptive immune system (Bly & Clem, 1992).

Comparison of turbidometric *versus* radial diffusion assay to quantify lysozyme activity demonstrated that skin trout lysozyme generates much larger zones on a

lysoplate than hen egg white lysozyme. Importantly, therefore extraneous standards might not be appropriate to quantify lysozyme activity by radial diffusion assay.

Unlike the buffered, stable conditions of the blood (Szebedinszky & Gilmour, 2002), the skin epithelium of teleosts is directly exposed to an environment with variable conditions. Hence, it may be advantageous to have an isozyme expressed in epithelial surfaces with the plasticity to function in diverse pH and ionic strength conditions. The presence of two muramidases in trout skin, one of which with bactericidal properties, indicate that they may be important components of trout mucosal immunity, acting in synergism with other innate humoral defence factors, such as antimicrobial peptides (please consult Chapters 2 to 6), to protect the skin epithelium from microbial exploitation.

Chapter 9

Partial Purification of Antibacterial Proteinaceous Factors from Erythrocytes of *Oncorhynchus mykiss*

9.1. Synopsis

Antimicrobial peptides are present in both myeloid cells and epithelial surfaces of vertebrates. Nevertheless, there are no published reports of antimicrobial peptides isolated from blood cells of teleosts. The first evidence that rainbow trout erythrocytes contain cationic antibacterial factors is presented in the present chapter. Acid-soluble erythrocyte extracts were found to display antibacterial activity against *Planococcus citreus* on a radial diffusion assay. Following $^{t}C_{18}$ solid phase extraction, cation exchange chromatography and C_{18} reversed phase HPLC two groups of fractions with antibacterial properties were obtained. This activity was thermostable and susceptible to digestion by proteinase K, thus showing that the antibacterial factors have a proteinaceous nature. The group of partially purified proteins eluted from a C_{18} column with *circa* 33 % acetonitrile were active against *P. citreus* and *E. coli*, with minimal inhibitory concentrations in the range 7 - 14 $\mu g \cdot ml^{-1}$ and 14 - 28 $\mu g \cdot ml^{-1}$, respectively; the ones eluted with approximately 44 % acetonitrile on the same column only displayed activity against *P. citreus* with a minimal inhibitory concentration of 1 - 2 $\mu g \cdot ml^{-1}$. These results raise the possibility that trout erythrocytes may contain antimicrobial factors not previously considered to be part of the innate immune system.

9.2. Introduction

Antimicrobial peptides are potent natural antibiotics usually present in mucosal surfaces (reviewed by Bevins, 1994) and / or leucocytes (reviewed by Boman, 1991; Lehrer *et al.*, 1991) of vertebrates. With regard to teleosts several antimicrobial peptides have been purified from skin secretions (Oren & Shai, 1996; Cole *et al.*, 1997; Park *et al.*, 1998b), mast cells (Silphaduang & Noga, 2001), liver (Richards *et al.*, 2001) and gills (Lauth *et al.*, 2002; Shike *et al.*, 2002). Only recently an antimicrobial peptide has been purified from the serum of immunologically challenged salmon, *Oncorhynchus kisutch* (Patrzykat *et al.*, 2001). Therefore, the present study was conducted to investigate the presence of antimicrobial peptides and proteins in erythrocytes from rainbow trout, *Oncorhynchus mykiss*. The reasons for selecting the erythrocytes are two-fold: first, previous experiments performed in our laboratory failed to identify any antimicrobial peptides in leucocyte preparations from *O. mykiss*; secondly, the erythrocytes of teleosts are nucleated and therefore contain histones, which are known to display antibacterial activity. The present chapter describes the partial purification and preliminary characterisation of two groups of antibacterial proteins from trout erythrocytes.

9.3. Experimental Procedures

Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are aqueous and prepared with deionized water (Elga), except when stated otherwise. The list of suppliers and their respective addresses is presented in appendix B.

9.3.1. Animals

Adult female rainbow trout were maintained under the conditions described in Section 3.3.1.

9.3.2. Blood Collection and Preparation of Erythrocyte Extracts

Three fish were sacrificed as detailed in Section 2.3.2 and 30 ml of blood were collected by caudal vein puncture in the presence of heparin (500 IU per ml of blood) and 10 % (v/v) protease inhibitors cocktail (please consult Section 2.3.2 for its detailed composition). Following a 1:2 dilution with 10 mM phosphate buffered saline, pH 7.4 (PBS), the blood was centrifuged at 200 x g for 10 min at 4 °C. The plasma was discarded and the buffy coat removed with a glass Pasteur pipette before resuspension of the red blood cells in 100 ml PBS. This wash was repeated four times until a homogenous erythrocyte suspension was obtained. The erythrocytes were then packed by centrifugation at 500 x g and lysed in 30 ml deionized water. A protein extract was prepared by addition of 60 ml of 45 % (v/v) ethanol/ 1.5 % (v/v) trifluoroacetic acid (TFA), following by stirring for 60 min at 4 °C. The preparation was then centrifuged at 29 000 x g for 60 min at 4 °C to remove insoluble material and the supernatant lyophilised. For further protein purification, the resulting extract was resuspended in 20 ml of

0.20 M sodium acetate/ 0.1 % (v/v) Triton X-100, acidified to 0.1 % (v/v, final concentration) TFA and centrifuged at 29 000 x g for 30 min at 4 °C. Antibacterial activity was tested using a small proportion of the dry extract resuspended solely in 20 mM HEPES, pH 7.0.

9.3.3. Test Bacteria

Antibacterial activity was tested against *Escherichia coli* (NCIMB 12210) and *Planococcus citreus* (NCIMB 1493). Each microorganism was grown to logarithmic phase in the appropriate culture conditions (please consult Table 3.2) before washing in sterile saline (1.5 % (w/v) NaCl for *P. citreus*; 0.8 % (w/v) NaCl for *E. coli*) and resuspension in saline or MHB as described in Section 3.3.5.

9.3.4. Antibacterial Assays

Antibacterial activity was assessed using a modified version of the two-layer radial diffusion assay of Lehrer *et al.* (1991) as described in Section 3.3.5, using the Gram-(+) bacterium *P. citreus* as test organism throughout the protein purification procedure. Determination of the minimal inhibitory concentration (MIC) of the partially purified proteins against *E. coli* or *P. citreus* was performed using the microtitre broth dilution assay (Friedrich *et al.*, 1999), according to the protocol detailed in Section 3.3.5.

9.3.5. Muramidase Assay

Muramidase activity was tested by radial diffusion assay as described in Section 2.3.5.

9.3.6. Protein Purification

The supernatant of the reconstituted protein extract prepared as above was applied to two Sep-Pak Vac 5g $^{t}C_{18}$ cartridges (Waters), previously equilibrated in 0.1 % (v/v) TFA, for solid phase extraction. The proteins of interest were eluted with 0.1 % (v/v) TFA in 60 % (v/v) acetonitrile, lyophilised and reconstituted in 20 ml of 20 mM HEPES, pH 7.0. This eluate was then fractioned by cation exchange chromatography on a 1 cm x 10 cm CM Macro-Prep Econo-column (Bio-Rad), previously equilibrated with 20 mM HEPES, pH 7.0 (buffer A). Following a 20 min wash with buffer A to remove unbound material, elution was performed with a linear AB gradient (where B is 20 mM HEPES, 1M NaCl, pH 7.0) ranging from 0 to 100 % B over 70 min, followed by 50 min of 100 % B, at a flow rate of 1 ml·min^{-1}. The active fractions were pooled, acidified to 0.1 % (v/v) TFA and a 1 ml aliquot was subjected to C_{18} reversed phase HPLC on an ODS2-Inertpak column (particle size 5 μm, 4.6 mm x 250 mm, Capital HPLC). Elution was performed at 25 °C with a linear biphasic gradient of 0.1 % (v/v) TFA in water and 0.09 % (v/v) TFA in acetonitrile (0 to 50 % acetonitrile over 80 min) at a flow rate of 1 ml·min^{-1}.

At each step, protein profiles were determined by Tris-tricine polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) and β-mercaptoethanol, as described in Section 2.3.6. The gels were stained with Coomassie brilliant blue (Appendix C).

9.3.7. Protein Quantification

Protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin (Pierce) as standard.

9.3.8. Proteolytic Digestion

The proteinaceous nature of the partially purified antimicrobial factors was tested by proteolytic digestion with proteinase K, as detailed in Section 3.3.13.

9.4. Results

9.4.1. Protein Purification

Acid-soluble erythrocyte extracts resuspended in 20 mM HEPES, pH 7.0, to a total protein concentration of approximately 1 $mg \cdot ml^{-1}$ displayed antibacterial activity against *P. citreus* on a radial diffusion assay, with a typical clear zone area of 75 mm^2. The reconstituted 60 % acetonitrile eluate from solid phase extraction of the erythrocyte acid-soluble proteins displayed antibacterial activity against *P. citreus* and *E. coli* on a radial diffusion assay at a total protein concentration of *circa* 3 $mg \cdot ml^{-1}$ (Fig. 9.1). This activity was abolished by proteinase K digestion, showing that the antibacterial activity is entirely due to proteinaceous factors. Cation exchange chromatography of the 60 % acetonitrile eluate yielded several active fractions eluting between 70 and 110 min, corresponding to 0.7 M to 1.0 M NaCl (Fig. 9.2). Two major bands could be observed on a Coomassie-stained SDS-PA gel at approximately 14.4 kDa and just above the 31 kDa marker (Fig. 9.3). The active ion exchange fractions were pooled and further fractionated by C_{18} reversed phase HPLC, originating 2 groups of active fractions labelled E1 and E2 (Fig. 9.4). The peak fraction of group E1 (fraction 41) was eluted with 33 % acetonitrile whilst the one from group E2 (fraction 53) was eluted with 44 % acetonitrile (Fig. 9.4). SDS-PAGE analyses revealed a protein band in fractions E1 with an apparent molecular weight of approximately 28 kDa and a major band just above the 31 kDa marker (Fig. 9.5). The lanes corresponding to fractions E2 showed three protein bands with apparent molecular weights of 13 kDa, 16 kDa and 31 kDa (Fig. 9.5). The

antibacterial activity of the partially purified proteins was thermostable, remaining present even after incubation at 80 °C for 5 min.

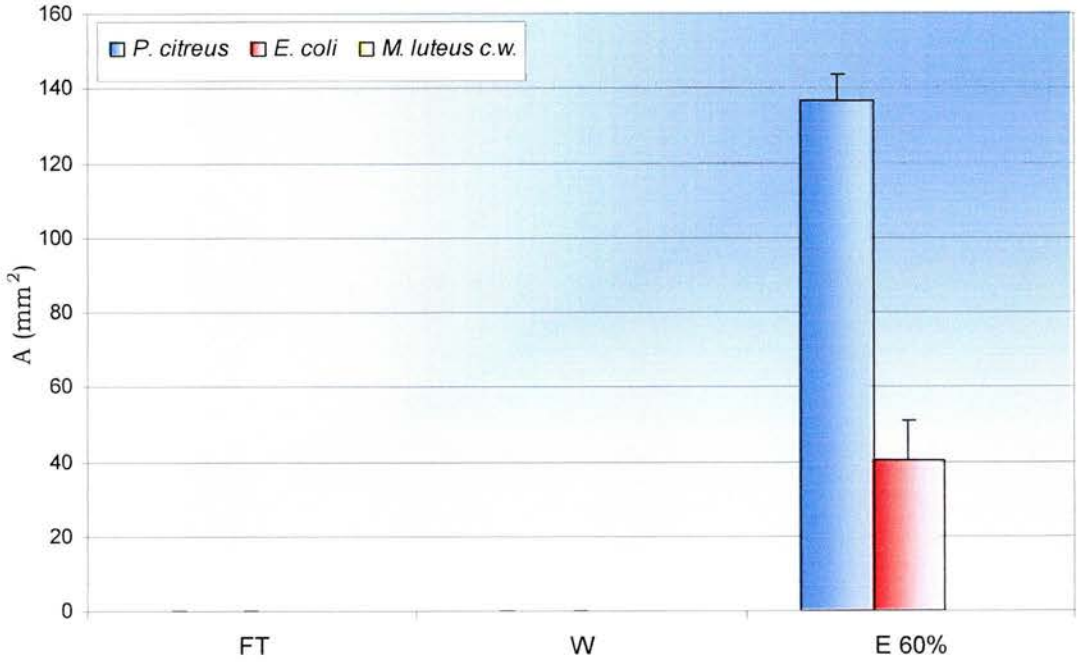


Figure 9.1. Antimicrobial profile of the solid phase extraction eluate. Acid-soluble proteins from trout erythrocytes were subjected to $^{t}C_{18}$ solid phase extraction and eluted with 0.1 % (v/v) TFA in 60 % (v/v) acetonitrile (E 60%). Following lyophilisation and reconstitution in 20 mM HEPES, pH 7.0, the eluate was assayed for antimicrobial activity by radial diffusion assay against *P. citreus*, *E. coli* and *Micrococcus luteus* cell walls. The flow-through (FT) and the wash (W) fractions were also tested. Total protein concentration in the reconstituted eluate was approximately 3 $mg \cdot ml^{-1}$. Data are represented as the mean clear zone areas (A) ± SE, n=3.

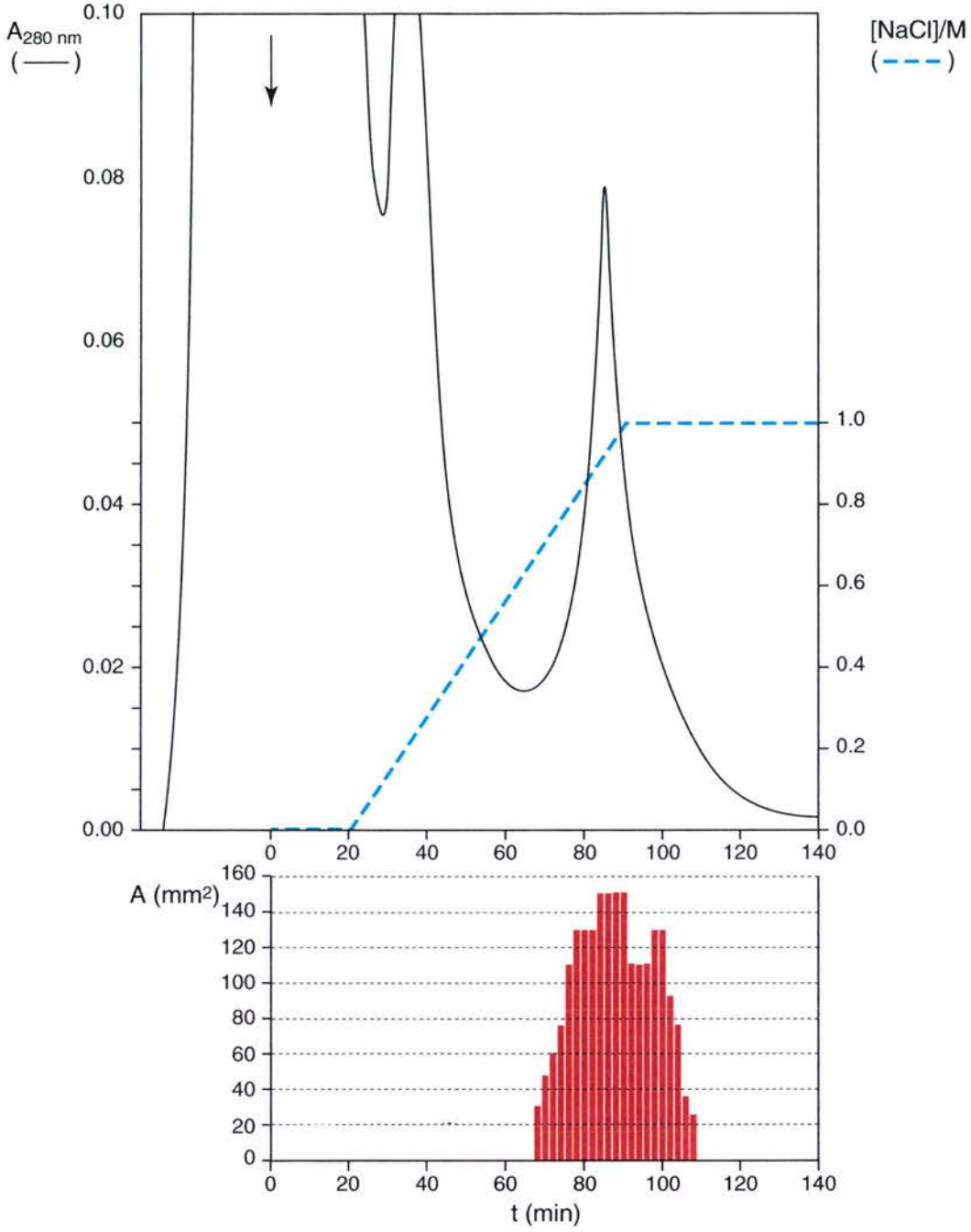


Figure 9.2. Fractionation of the solid phase extraction eluate by cationic exchange chromatography. Proteins eluted with 0.1 % (v/v) TFA in 60 % (v/v) acetonitrile (E 60%) were lyophilised, reconstituted in 20 mM HEPES and subjected to cationic exchange chromatography at pH 7.0. The chromatogram was obtained at 280 nm (solid line). The blue dashed line indicates the NaCl concentration gradient. The arrow indicates the end of sample application. Antibacterial activity profile against *P. citreus* is represented by the histogram.

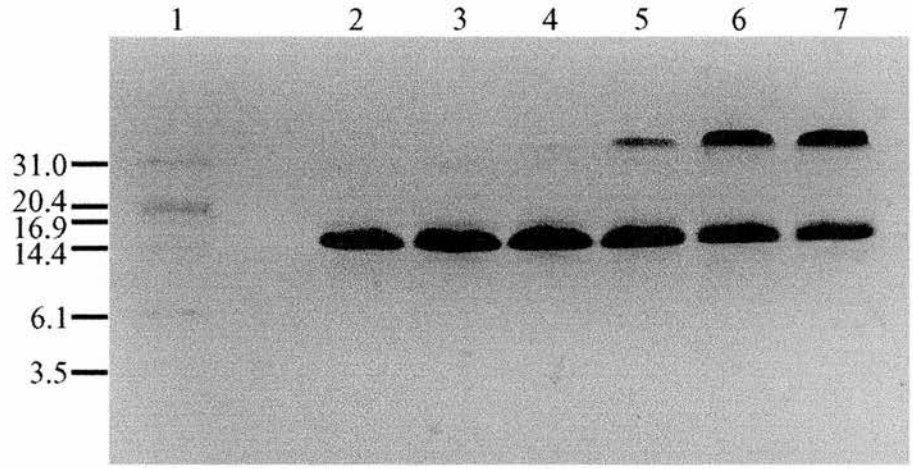


Figure 9.3. Coomassie stained SDS-PAGE of the active fractions obtained during cation exchange chromatography of a partially purified extract from erythrocytes of rainbow trout. Lane 1: markers; lane 2: fraction 75; lane 3: fraction 80; lane 4: fraction 85; lane 5: fraction 90; lane 6: fraction 95 and lane 7: fraction 100. Each lane contains 7.5 µl of sample. The numbers on the left hand side correspond to the molecular mass of the markers in kDa.

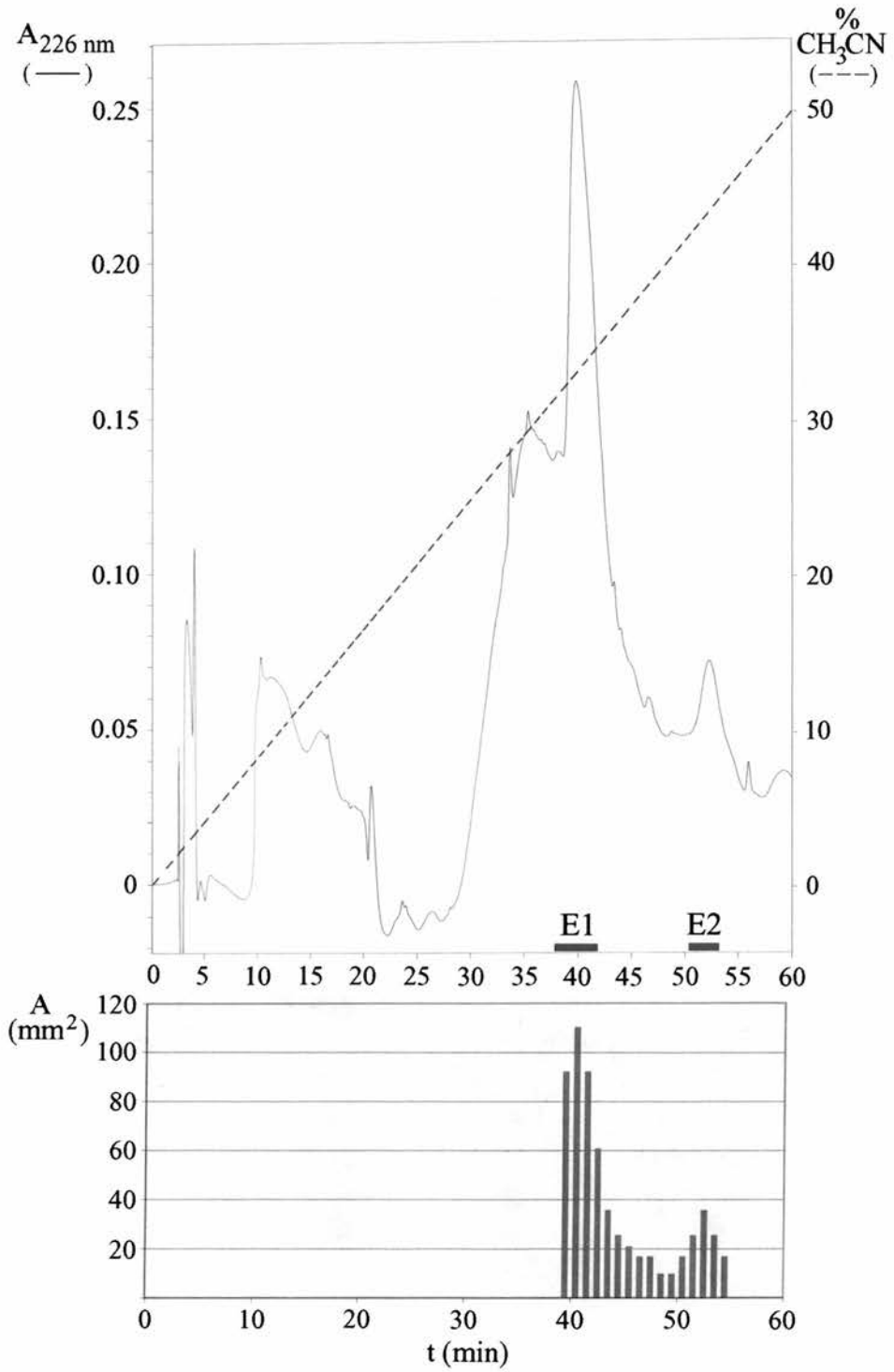


Figure 9.4. C_{18} reversed phase HPLC of the pooled cation exchange chromatography fractions. The active cation exchange fractions were pooled and further fractionated by C_{18} reversed phase HPLC using a linear, biphasic water/ acetonitrile gradient (dashed blue line). Absorbance was monitored at 226 nm (solid line). Two groups of fractions, labelled E1 and E2, were found to be antibacterial to *P. citreus* (histogram).

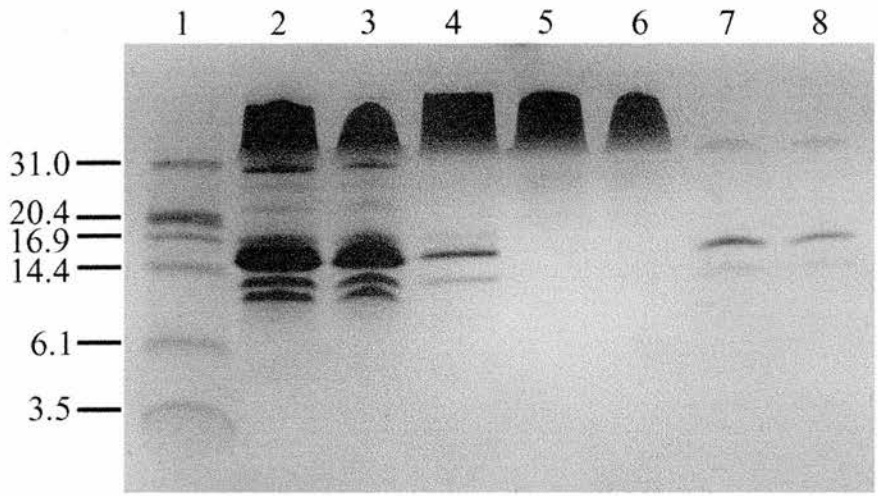
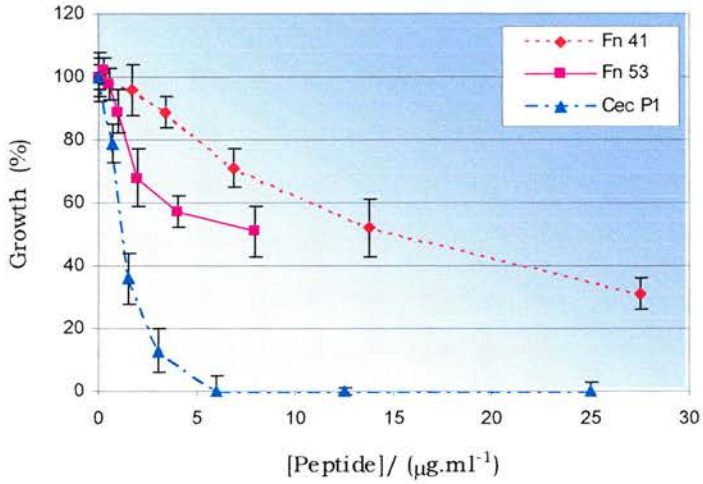


Figure 9.5. Tris-Tricine SDS-PAGE analysis of the active C_{18} reversed phase HPLC fractions obtained during purification of antibacterial proteins from rainbow trout erythrocytes. Lane 1: markers; lane 2: crude extract; lane 3: 70 % acetonitrile eluate from solid phase extraction; lane 4: pooled ion exchange fractions; lane 5: HPLC peak fraction 41; lane 6: HPLC fraction 42; lane 7: HPLC peak fraction 53 and lane 8: HPLC fraction 54. Each lane contains 7.5 µl of sample. The numbers on the left hand side indicate the molecular mass of the markers in kDa. Proteins were visualised by staining with Coomassie brilliant blue.

9.4.2. Antimicrobial Activity of the Partially Purified Proteins

Quantification of antibacterial activity using the microtitre broth dilution method revealed that the partially purified proteins from trout erythrocytes are active at submicromolar concentrations. The peak fraction from group E1 (fraction 41, Fig. 9.4) is active against both *P. citreus* and *E. coli*, with minimal inhibitory concentrations in the range 7 - 14 $\mu g \cdot ml^{-1}$ and 14 - 28 $\mu g \cdot ml^{-1}$, respectively (Fig. 9.6). Activity against *P. citreus* was also observed in the peak fraction from group E2 (fraction 53, Fig. 9.4) with a minimal inhibitory concentration of 1 - 2 $\mu g \cdot ml^{-1}$ (Fig. 9.6). These MIC values are within the same order of magnitude as those of cecropin P1 against the same sensitive bacteria (Fig. 9.6). The partially purified proteins did not display muramidase activity.

(A)



(B)

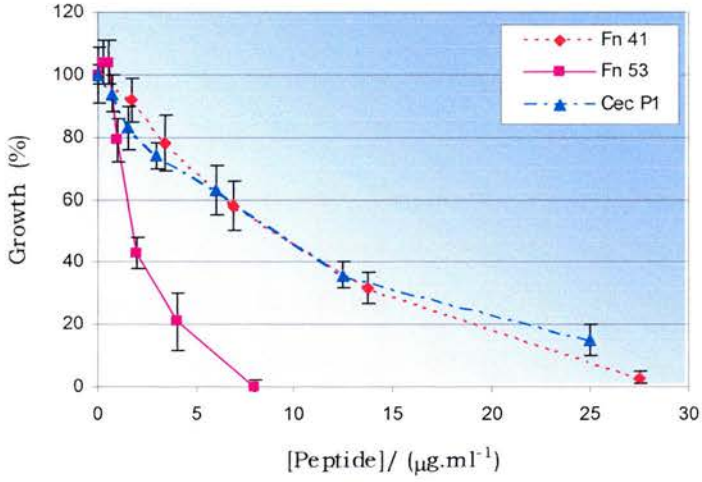


Figure 9.6. Quantification of antibacterial activity of the partially purified proteins from rainbow trout erythrocytes against *E. coli* and *P. citreus*. (A) *E. coli* or (B) *P. citreus* bacterial suspensions containing 10^5 cfu·ml^{-1} were incubated with serially diluted cecropin P1 (Cec P1) or with the partially purified proteins from trout erythrocytes (fraction 41, fraction 53) at the appropriate temperature until the average final OD_{570} of the control reached approximately 0.2. Bacterial growth is expressed as the ratio of optical densities read at 570 nm (OD_{570}) between each test sample and the control (no peptide added). Data are represented as means ± SE, n=3.

9.5. Discussion

The present study demonstrates that trout erythrocytes contain at least two potent, thermostable antibacterial proteins, of which one may be histone H2A. One group of fractions is active against both Gram-(+) and Gram-(-) bacteria at micromolar concentrations whilst the other seems to be more active against Gram-(+) bacteria.

It has long been established that the blood of vertebrates contains a number of innate defence factors that mediate rapid responses to infection (Levy, 2000). In particular, the cytoplasmic granules of mammalian neutrophils are known to contain various antimicrobial peptides, namely defensins and cathelicidins (Lehrer & Ganz, 2002). It is therefore surprising that only 3 antimicrobial peptides have been found in blood cells of teleosts. These are HSDF-1, a 26-residue antimicrobial peptide derived from the N-terminus of histone H1 of coho salmon, *O. kisutch* (Patrzykat *et al.*, 2001) and piscidins 1 and 3, 22-residue α-helical peptides isolated from mast cells of the hybrid striped bass, *Morone saxatilis* x *M. chrysops* (Silphaduang & Noga, 2001). To the best of my knowledge, the investigation reported in the current chapter provides the first evidence that the erythrocytes of rainbow trout contain relatively low molecular weight antibacterial proteins. It is highly unlikely that these antibacterial proteins were derived from contaminant leucocytes, as previous extensive research conducted in our laboratory on antimicrobial peptides from trout leucocytes showed that, apart from lysozyme, no other antimicrobial peptides could be found in leucocytes from immunologically naïve fish (Smith *et al.*, 2000).

The fractionation of erythrocyte extracts by C_{18} reversed phase HPLC resulted in two groups of fractions containing cationic antibacterial proteins. The predominant component in group E2 has an apparent molecular weight similar to that of histone H2A from trout skin, as well as an analogous chromatographic behaviour on a C_{18} column (please consult Chapter 3). It is necessary for the antibacterial protein to be purified to homogeneity, in order to proceed with its identification and biochemical characterisation. Group E1 contains two proteins which do not seem to match any of the other antimicrobial proteins and peptides described in the previous chapters. The apparent molecular weight of the major E1 protein is similar to that of the haemoglobin tetramer, which is known to display broad-spectrum antimicrobial activity (Parish *et al.*, 2001). However, the hypothesis that the protein from trout erythrocytes is not haemoglobin is supported by the following facts: i) the extraction method employed caused visible precipitation of haemoglobin, and ii) it is highly unlikely that the haemoglobin molecule would preserve its quaternary structure under the strong (5 % (v/v) β-mercaptoethanol) reducing conditions under which the electrophoresis was performed. In order to ascertain the exact nature of the erythrocyte E1 protein, it would obviously be necessary to purify this protein to homogeneity and characterise it.

The plausible relevance in host defence of the antibacterial proteins partially purified from trout erythrocytes in the present study is suggested by their very high potency. With minimal inhibitory concentrations in the submicromolar range these proteins might well act alone or in synergism with other antibacterial factors like lysozyme to protect the fish against systemic infections.

Chapter 10

General Discussion

The general aim of this research project was to investigate the presence and character of innate antibacterial factors, particularly antimicrobial peptides, in rainbow trout, *Oncorhynchus mykiss*. Trout skin secretions were found to contain five cationic, low molecular weight antimicrobial proteins and two muramidases, which, after isolation to apparent homogeneity, were structurally and functionally characterised. In addition, several antibacterial proteinaceous agents have been partially purified from a liver ribosomal preparation and from erythrocyte extracts. Therefore, the aims of this thesis have been successfully fulfilled.

The cationic, antibacterial factors were purified by multiple chromatographic methods, namely ion exchange chromatography, $^{t}C_{18}$ solid phase extraction and C_{18} reversed phase HPLC, and detected on the basis of their antibacterial activity against the Gram-(+) bacterium, *Planococcus citreus*. Unexpectedly, four of the five low molecular weight antimicrobial proteins purified from skin mucus were proteins with previously known functions or fragments thereof, more specifically a ribosomal peptide, a histone and histone-derived peptides. As there is no need for novel synthesis, these peptides are relatively inexpensive for the cell and they can be made readily available, providing a plausible first-line of host defence.

The three novel peptides purified from skin exudates were provisionally named after the genus of rainbow trout, *Oncorhynchus*. This terminology was selected because the vast majority of peptides discovered so far have been termed after the genus of the organism from which they were isolated. However, this nomenclature is limited, in the sense that it does not provide any information

about the structure, localisation or function of the antimicrobial peptide. Moreover, not all organisms from the same genus have similar antimicrobial peptides, e.g. *O. mykiss* (please see Chapters 2 to 6) and *O. kissutch* (Patrzykat *et al.*, 2001). On the other hand, analogous peptides can be present in evolutionary distant organisms. Cecropins, for instance, have been isolated from insects (Hultmark *et al.*, 1982) as well as from mammals (Lee *et al.*, 1989). Therefore, there is the obvious need of developing a standard nomenclature system. In the meantime, the 3 novel antimicrobial peptides from trout skin are tentatively referred to as oncorhyncins I, II and III.

Oncorhyncin I is a 3 kDa peptide active against the potential fish pathogen, *P. citreus* (Austin & Stobie, 1992) at submicromolar concentrations. Its primary structure has no similarity to any other known protein listed on the NCBI protein and nucleotide databases, revealing that oncorhyncin I represents a novel antimicrobial peptide, the first to be isolated from skin exudates of a salmonid. Schiffer-Edmundson helical wheel modelling predicts that oncorhyncin may form an amphipathic α-helix, in common with many other linear antimicrobial peptides (Zasloff, 2002). Such a conformation, with the hydrophilic domain well separated from the hydrophobic one, facilitates the interaction of the peptide with the negatively charged head groups and hydrophobic cores of bacterial membranes. The total yield of purified peptide varied considerably between different samples, suggesting that the expression of oncorhyncin I is either regulated by environmental variables or requires immunostimulation.

A 69-residue C-terminal fragment of histone H1, probably phosphorylated at two residues, was the second antimicrobial peptide to be purified from trout skin and it was therefore named oncorhyncin II. This peptide is very potent, displaying broad-spectrum antibacterial activity at submicromolar concentrations. In particular, oncorhyncin II is active against *L. anguillarum*, the causative agent of vibriosis in fish. Vibriosis, also known as "red plague" or "saltwater furunculosis", is a systemic bacterial infection that affects primarily marine and estuarine fishes (Bullock, 1987). The potential role of histone H1 in fish immunity is further supported by two recent reports. Richards *et al.* (2001) have isolated a 20.7 kDa antibacterial protein from the liver of Atlantic salmon, *Salmo salar*. This protein, which is active against *E. coli* D31 at submicromolar concentrations, was identified by tandem nanoelectrospray mass spectrometry as being histone H1 (Richards *et al.*, 2001). A 26-residue antimicrobial peptide has also been purified from the mucus and serum of coho salmon, *O. kisutch* (Patrzykat, 2001). This N-terminal fragment of histone H1 displays antibacterial activity against *A. salmonicida*, *V. anguillarum* and *Salmonella enterica* serovar *typhimurium* (Patrzykat, 2001). Moreover, this study also showed that the expression of the antimicrobial peptide was up-regulated following immunological stimulation and that it coincided with an increase in antibacterial activity of both mucus and serum.

Oncorhyncin III is a 66-residue N-terminal fragment of the non-histone chromosomal protein H6, containing two probable methylations. This report provides the first evidence that a non-histone chromosomal protein from trout skin may be involved in innate immunity. This peptide was found to display

potent activity against both Gram-(+) and Gram-(-) bacteria, including, *L. anguillarum* and *A. viridans*. The latter (formerly *Gaffkya homari*) is the aetiologic agent of gaffkemia, a septicaemic disease of the lobster. Gaffkemia is responsible for a considerable mortality rate during holding of lobsters in captivity, which results in economic losses estimated at 10 to 15 % per year (Lavallee *et al.*, 2001).

A low molecular weight antimicrobial protein present in skin secretions was also found in the present study to display antimicrobial properties. This 13.6 kDa protein was identified as being histone H2A, acetylated at the N-terminus. Histone H2A is active against Gram-(+) bacteria at submicromolar concentrations but at the concentrations tested (maximum 1.2 μM) it did not inhibit the growth of any of the Gram-(-) bacteria used. Amongst the sensitive bacteria is *R. salmoninarum*, the causative agent of the intractable bacterial kidney disease in salmonids (Kaattari & Piganelli, 1997). The yeast, *S. cerevisiae*, was also inhibited by histone H2A. Kinetic analysis revealed that at a concentration of 0.3 μM *P. citreus* cells lose viability after only 30 min incubation with the protein. To the best of my knowledge, the antimicrobial properties of histone H2A as a whole molecule have not been previously reported. However, three antimicrobial peptides derived from the N-terminus of histone H2A have been isolated. Buforins I and II are potent antimicrobial peptides purified from the stomach of the Asian toad, *Bufo bufo gargarizans* (Park *et al.*, 1996) and parasin I is a 19-residue antimicrobial peptide present on the mucosal surface of the catfish, *Parasilurus asotus* (Park *et al.*, 1998b; Cho *et*

al., 2002). The antimicrobial properties of histone H2B-like proteins have also been reported in the catfish, *Ictalurus punctatus* (Robinette *et al.*, 1998).

The 6.7 kDa antimicrobial peptide purified from trout skin turned out to be the 40S ribosomal protein S30. This finding is corroborated by the recent isolation of ubiquicidin, a ribosomal protein-like peptide, in the cytosolic fraction of IFN-γ activated murine macrophages (Hiemstra *et al.*, 1999). The 40S ribosomal protein S30 from trout is active at submicromolar concentrations against *P. citreus*. Unfortunately, the yield of purified peptide did not permit further structural and functional characterisation. Several attempts to isolate it from ribosomal preparations obtained from trout liver were fruitless. Nevertheless, these experiments revealed the presence of at least one additional antibacterial proteinaceous factor with an apparent molecular weight between 8 kDa and 31 kDa.

Piscine erythrocytes, unlike their mammalian counterparts, are permanently nucleated, oval cells (Glomski *et al.*, 1992) that contain eosinophilic granules (Lieschke *et al.*, 2001). During this investigation it was discovered that trout erythrocytes contain cationic, antimicrobial factors. The partially purified antibacterial proteins are active at submicromolar concentrations against Gram-(+) or Gram-(-) bacteria, indicating that they have the potential to play a role in innate host defence. To the best of my knowledge, the involvement of fish erythrocytes in innate immunity has not been previously proposed, albeit there be a report of myeloperoxidase activity in erythrocytes from zebrafish, *Brachydanio rerio* (Lieschke *et al.*, 2001).

Due to their notable sensitivity, erythrocytes are frequently used as test cells to investigate cytotoxic effects of various substances (Zeni *et al.*, 2002). In the present study, it was found that oncorhyncins I and II and histone H2A are not significantly haemolytic for trout red blood cells at concentrations that inhibit bacterial growth. Hence, these antimicrobial peptides could exert their biological effects *in vivo* against bacteria without damage to the host tissues, unlike several other antimicrobial peptides, e.g. defensins (Lehrer *et al.*, 1993) and cathelicidins (Oren *et al.*, 1999), which exhibit non-selective cytotoxicity.

Many antimicrobial proteins are inhibited by high sodium chloride concentrations, probably because the salt interferes with the electrostatic interaction between the cationic proteins and the negatively charged microbial surface. However, some of the antibacterial peptides expressed by marine or aquatic animal appear to be salt-tolerant, such as pleurocidin from the Winter flounder *Pleuronectes americanus* (Cole *et al.*, 1997), and a few are potentiated by salt, as exemplified by an 11.5 kDa antibacterial protein purified from the shore crab, *Carcinus maenas* (Relf *et al.*, 1999). Structure-activity studies have revealed that minor alterations in hydrophobicity, amphipathicity or charge of an antimicrobial peptide can have a profound effect on its sensitivity to salt and permeabilisation activity (Friedrich et al., 1999). Oncorhyncin III and histone H2A isolated from *O. mykiss* skin are inhibited by NaCl, displaying an 8-fold to 16-fold reduction in potency against *P. citreus* when the salt concentration increases from 0.8 % to 3.2 % NaCl. This salt-sensitivity may have significant consequences in innate host defence, as some wild rainbow trout, also known as

steelhead, are anadromous, migrating seasonally from freshwater to the sea, where they spend several years before returning to their home streams for spawning (Deutschlander *et al.*, 2001). It is therefore likely that during the seawater phase, the fish might be deprived to some extent of the protective effect of any of its antimicrobial proteins that are salt-sensitive.

For oncorhyncin III and histone H2A to function *in vivo*, they must be active in the presence of several ions, other than Na^+ and Cl^-. Of particular interest is the effect of divalent cations, such as Mg^{2+}, for several NaCl-resistant peptides are known to be strongly inhibited by relatively low Mg^{2+} concentrations (Friedrich et al., 1999). Unfortunately, time did not permit the realisation of these experiments.

Histone H2A from trout mucus, as well as oncorhyncins II and III, induces a marked destabilisation of planar lipid bilayers but are unable to form stable channels. These results are in accordance with the Shai-Matsuzaki-Huang model of the mechanism of action of antimicrobial peptides and support the hypothesis that these antibacterial factors disrupt the cell membrane through a "carpet" mechanism (Shai, 1999). This model predicts that the proteins adsorb to the target membrane and cover it in a carpet-like fashion, maintaining the contact with the phospholipid head groups throughout the entire permeation process (Shai, 1999). At a high enough local concentration of protein the formation of transient toroidal holes in the membrane may occur (Ludtke et al., 1996; Matsuzaki et al., 1997), as indicated by the observed ion channel activities. These "wormholes" (Shai, 1999; Zasloff, 2002) might allow the low molecular weight

antimicrobial proteins to reach the cytosol or the nucleus, where they could also exert their antibacterial action by impairing intracellular targets.

Imaging of *E. coli* cells by scanning electron microscopy following incubation with oncorhyncin III showed that the general cell morphology remains unchanged, thus demonstrating that the peptide does not cause immediate disintegration of the cell membrane and further supporting the hypothesis that oncorhyncin III exerts its antibacterial action by inhibiting cellular functions. Oncorhyncin I and the 40S ribosomal protein S30 were not tested for their pore-forming properties due to insufficient availability of purified peptide.

In addition to the isolation of antimicrobial peptides that act in a stoichiometric rather than enzymatic manner (Boman, 1995), two proteins with muramidase activity were purified from trout skin mucus. As far as I know, there are no precedent reports describing the purification and characterisation of muramidases from the skin of a salmonid. One of the isolated proteins is a muramidase with an unusually acidic isoelectric point, which suggests that this may represent a novel type of muramidase. Alas, the amounts of purified, active enzyme obtained were too low to perform structural or enzymatic studies and until further characterisation, its status as a novel muramidase remains unproven. The other muramidase is likely to be a c-type lysozyme that differs from the head kidney-derived forms in mass (Dautigny et al., 1991) and has therefore been tentatively designated as a third isozyme. The type III lysozyme from trout skin is bactericidal to *P. citreus* without being haemolytic to trout erythrocytes. As the epithelial surfaces are exposed to a variable environment, the presence of

multiple skin isozymes with the plasticity to function in diverse pH and ionic strength conditions may be an advantage. The importance of lysozyme as a bacteriolytic enzyme involved in innate immunity has long been acknowledged (Jolles, 1996). In addition, recent studies have further started to show that lysozyme may augment the activity of antibacterial peptides through a synergistic mechanism (Chalk *et al.*, 1994; Patrzykat *et al.*, 2001), and that some lysozymes also have independent non-enzymatic bactericidal domains (Düring *et al.*, 1999).

Innate host defence factors are unarguably crucial for teleosts, as their adaptive immune system is less sophisticated than that of higher vertebrates and is markedly constrained by developmental or environmental variables (Tatner, 1996). The adaptive immune response in teleosts only takes place if the animals are above a certain size or age and are above their immunological permissive temperature (Bly & Clem, 1992; Tatner, 1996). For salmonids the permissive temperature is close to or above average winter temperature in the United Kingdom. Therefore, the fish spend a considerable proportion of their life relying solely upon the innate immune system to prevent microbial exploitation. The potent activity against bacteria, namely potential pathogens, and lack of haemolytic activity of the antimicrobial factors from *O. mykiss* reported in the present thesis point to their potential significance in the mucosal innate immune system of rainbow trout.

Moreover, the high degree of similarity between histones from different organisms raises the possibility that the role of histones and histone-derived

peptides in immunity may be a more general phenomenon, not just confined to teleosts. In fact, this idea is supported by the findings of parasin I (Park *et al.*, 1996) and buforin II (Park *et al.*, 1998b), two histone H2A-derived peptides isolated from a teleost and an amphibian, respectively. In order to further test the hypothesis of a conserved role for histones in immunity, it would be necessary to investigate the antimicrobial properties of histones purified from other eukaryotes, namely plants and invertebrates, for so far there are no published reports in this area.

Similarly, the theory of a possible ubiquitous role for the 40S ribosomal protein S30 in innate immunity is corroborated by a previous report on the antimicrobial properties of a similar peptide isolated from a mammal (Hiemstra *et al.*, 1999). A recent investigation by Gross and collaborators (2001) showed that the 40S ribosomal protein S30 gene is differentially expressed in immunostimulated Atlantic white shrimp, *L. setiferus*, suggesting that its potential function in host defence is not confined to the vertebrate phylum.

The 40S ribosomal protein S30 might not only protect the host against intracellular pathogens, like *R. salmoninarum*, but also act extracellularly following infection-induced cell lysis.

As most antimicrobial agents function extracellularly, the potential in vivo role for histones and histone-derived peptides might seem incompatible with their assumed classical localisation in the nucleus. However, several histones have been found in the cytosol (From *et al.*, 1996; Rose *et al.*, 1998; Kim *et al.*, 2000

Cho *et al.*, 2002) or even extracellularly (Brix *et al.*, 1998). The known multiple extracellular/ cytosolic/ nuclear localisation of histones suggests that oncorhyncin II and III as well as trout histone H2A, may function extracellularly through active secretion to mucosal surfaces or by passive release after infection-induced cell lysis or apoptosis and may therefore play an important role in protection of skin epithelia against microbial colonisation. Their ability to destabilise membranes may allow them to enter the cell, where they could exert their antibacterial action. Even within the nucleus, these histones and histone-derived peptides with antimicrobial properties may contribute to protect the nuclear integrity against viruses, perhaps by binding to viral DNA and inhibiting its transcription.

The antibacterial proteinaceous factors partially purified from trout erythrocytes may have an important *in vivo* role in host defence against several intraerythrocytic parasites, such as bacteria, viruses and protists (Davies & Johnston, 2000).

Certainly, the antimicrobial peptides isolated in the present investigation have the potential, as individuals, to prevent bacterial growth *in vitro*. Nevertheless, they probably participate in a synergistic relationship amongst themselves and involving other antibacterial factors, such as lysozyme, in order to efficiently protect the trout skin epithelium against microbial exploitation. A similar synergistic relationship has been reported for histone H1-derived peptides, pleurocidin and lysozyme (Patrzykat *et al.*, 2001), and between the antimicrobial peptides magainin 2 and PGLa (Matsuzaki *et al.*, 1998)

Besides their obvious relevance for the innate immune system of trout, the antimicrobial peptides and muramidases purified from trout skin secretions may also be used as biomarkers to monitor fish health or to study aspects of immunity related to development and environmental changes. This idea is supported by a previous report, which showed that healthy unstressed *I. punctatus* had consistently high levels of histone H2B-like protein, whilst fish subjected to overcrowding and elevated ammonia concentrations for one week had significantly depressed levels of this antibacterial protein, as determined by enzyme-linked immunosorbent assay, ELISA (Robinette & Noga, 2001). The finding of several antimicrobial peptides derived from constitutively expressed proteins conserved in *Teleostei*, expands the range of potential molecular markers and raises the possibility that these might be used for other bony fish.

With the continuing decline in fishery resources, aquaculture is becoming an attractive alternative for the supply of fish (Tacon, 1997). High population densities, poor water quality and temperature extremes propitiate the development of fish pathogens in fish farms, where infections are usually epidemic and have devastating effects on cultured stocks of fish which result in serious economic losses (Roberts, 1986). With a limited range of vaccines available and the emergence of antibiotic-resistant bacteria in fish (Miranda & Zemelman, 2001), there is an urgent need to develop novel antibiotics. The antimicrobial peptides isolated from trout skin could be used as alternative, natural antibiotics for use in aquaculture. Moreover, they might also find potential application in clinical medicine as replacements of conventional antibiotics. Pexiganan, for instance, is an analogue of magainin (Ge *et al.*, 1999),

an antimicrobial peptide isolated from the African clawed frog, which is currently being used in the treatment of diabetic foot ulcers.

In summary, the present thesis has demonstrated that skin secretions of *O. mykiss* contain a multitude of constitutively expressed low molecular weight antimicrobial proteins, the first to be purified and characterised from this species. I believe that these results provide a valuable contribution towards a better understanding of the mucosal innate immune system of rainbow trout. In particular, they raise the possibility that antimicrobial peptides act not only extracellularly but also participate in intracellular immunity. Moreover, they raise the possibility of using these natural antimicrobial agents as alternative antibiotics in aquaculture or clinical medicine.

The results presented in this thesis provide the basis for a series of possible studies regarding the biological significance of antimicrobial peptides in mucosal immunity of teleosts. Antibodies could be raised against the various antimicrobial peptides and antibacterial proteins characterised in the present investigation, and used as biomarkers to monitor fish health. Furthermore, as the gene sequences for the majority of the histone precursors are known, oligonucleotide probes could be developed to examine expression changes during development and in relation to different environmental and physiological conditions. It would also be relevant to investigate the changes in expression of these antimicrobial factors in response to antigenic challenge, particularly viral infections. One might even discover that immunostimulation induces the expression of novel antimicrobial peptides…

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Appendices

Appendix A

Nomenclature of Common Amino Acids

The following table lists the trivial and systematic names of the α-amino acids that are commonly found in proteins and their one-letter and three-letter abbreviations, as recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature.

Table A1. Trivial and systematic names of commonly found α-amino acids and corresponding one-letter and three-letter abbreviations.

Trivial name	Symbols		Systematic name
Alanine	Ala	A	2-Aminopropanoic acid
Arginine	Arg	R	2-Amino-5-guanidinopentanoic acid
Asparagine	Asn [a]	N [a]	2-Amino-3-carbamoylpropanoic acid
Aspartic acid	Asp [a]	D [a]	2-Aminobutanedioic acid
Cysteine	Cys	C	2-Amino-3-mercaptopropanoic acid
Glutamine	Gln [a]	Q [a]	2-Amino-4-carbamoylbutanoic acid
Glutamic acid	Glu [a]	E [a]	2-Aminopentanedioic acid

[a] The symbol Asx denotes Asp or Asn; likewise B denotes N or D. Glx and Z likewise represent glutamic acid or glutamine or a substance, such as 4-carboxyglutamic acid, Gla, or 5-oxoproline, Glp, that yields glutamic acid on acid hydrolysis of peptides.

Table A1 (continued). Trivial and systematic names of commonly found α-amino acids and corresponding one-letter and three-letter abbreviations.

Trivial name	Symbols		Systematic name
Glycine	Gly	G	Aminoethanoic acid
Histidine	His	H	2-Amino-3-(1*H*-imidazol-4-yl)-propanoic acid
Isoleucine	Ile	I	2-Amino-3-methylpentanoic acid
Leucine	Leu	L	2-Amino-4-methylpentanoic acid
Lysine	Lys	K	2,6-Diaminohexanoic acid
Methionine	Met	M	2-Amino-4-(methylthio)butanoic acid
Phenylalanine	Phe	F	2-Amino-3-phenylpropanoic acid
Proline	Pro	P	Pyrrolidine-2-carboxylic acid
Serine	Ser	S	2-Amino-3-hydroxypropanoic acid
Threonine	Thr	T	2-Amino-3-hydroxybutanoic acid
Tryptophan	Trp	W	2-Amino-3-(l*H*-indol-3-yl)-propanoic acid
Tyrosine	Tyr	Y	2-Amino-3-(4-hydroxyphenyl)-propanoic acid
Valine	Val	V	2-Amino-3-methylbutanoic acid
Unspecified	Xaa	X	------

Appendix B

List of Suppliers

Accelrys	Cambridgeshire, U.K.
Accugel Acrylamide	Yorkshire, U.K.
Acros	Leicestershire, U.K.
Agar Aids	Essex, U.K.
Analyse-It Software	Yorkshire, U.K.
Amersham-Pharmacia	Buckinghamshire, U.K.
Anachem	Bedfordshire, U.K.
Applied BioSystems	Cheshire, U.K.
ATCC	Virginia, U.S.A.
BDH	Dorset, U.K.
Biologic Science Instruments	Claix, France
Bio-Rad	Hertfordshire, U.K.
BOC Gases	Surrey, U.K.
Brownlee	Cheshire, U.K.
Capital HPLC	West Lothian, U.K.
College Mill Trout Farm	Perthshire, U.K.
Corning Costar	Cambridgeshire, U.K.
Difco	Surrey, U.K.
Dynex	West Sussex, U.K.
Elga	Buckinghamshire, U.K.
Emscope	Maryland, U.S.A.

Ewos	West Lothian, U.K.
Fisher Scientific	Leicestershire, U.K.
Fluka	Dorset, U.K.
Hybaid	Middlesex, U.K.
H. A. West	Edinburgh, U.K.
Intracel Software	Hertfordshire, U.K.
Invitrogen	Renfrewshire, U.K.
JEOL	Tokyo, Japan
Keitley	Cleveland, U.S.A.
Merck	Dorset, U.K.
Micromass	Manchester, U.K.
Millipore	Hertfordshire, U.K.
Natex	Gloucestershire, U.K.
NCIMB	Aberdeen, U.K.
Oxoid	Hampshire, U.K.
Perkin Elmer	Buckinghamshire, U.K.
Pierce	Rockford, U.S.A.
Sigma-Aldrich	Dorset, U.K.
Tektronix	Beaverton, USA
Ultrafine	Manchester, U.K.
Waters	Hertfordshire, U.K.

Appendix C

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

(Modified from Schägger & von Jagow, 1987)

Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are aqueous and were prepared in deionised water (Elga), except when stated otherwise. The Mini-PROTEAN II protein electrophoresis cell (Bio-Rad) was used.

The discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) method described hereinafter is appropriate to separate peptides or proteins with molecular masses in the range 2.5 kDa to 35 kDa. The formulation of the gels is detailed in table C1. The separating gel should have a height of approximately 4.0 to 4.5 cm, and the spacer gel a height of *circa* 1 cm and be 1 cm below the bottom of the wells on the stacking gel.

An equal volume of protein sample was added to the sample buffer (0.61 % (w/v) Tris, 4 % (w/v) SDS, 12 % (w/v) glycerol, 0.01 % (w/v) bromophenol blue, 2% (v/v) β-mercaptoethanol, pH 6.8) prior to denaturing by heating at 100 °C for 5 min. Anode (2.42 % (w/v) Tris, pH 8.90) and cathode (1.21 % (w/v) Tris, 1.79 % (w/v) Tricine (BDH), 0.1 % (w/v) SDS, pH 8.25) buffers were poured in the respective chambers and the samples were pipetted into the wells of the stacking gel.

The gels were run at a constant current of 34 mA, supplied by a Power Pac 300 (Bio-Rad), until the dye front was 1 cm above the end of the separating gel.

Table C1. Composition of polyacrylamide gels for a 16 % separating gel, 14 % spacer gel and 5 % stacking gel. The quantities indicated are enough for 2 mini-gels with 0.75 mm thickness.

	Separating	**Spacer**	**Stacking**
40% Acrylamide:Bis (29:1), Accugel (ml)	3.1	0.77	0.62
Gel buffer[1] (ml)	2.5	1.0	1.55
Water (ml)	0.65	1.23	4.08
80 % (w/v) Glycerol (ml)	1.25	-----	-----
10 % (w/v) APS (µl)	75	30	100
TEMED (µl)	7.5	3.0	10.0
$\approx V_{total}$ (ml)	7.5	3.0	6.25

1. Gel Buffer: 36.33 % (w/v) Tris, 0.3 % (w/v) SDS, pH adjusted to 8.45 with HCl.

Following electrophoresis the gels were Coomassie or silver stained, according to the following protocols:

1. Coomassie Staining

Gels were immersed for 10 min in staining solution (0.25 % (w/v) Coomassie brilliant blue R250, 45 % (v/v) methanol and 10 % (v/v) glacial acetic acid) and then destained in a solution containing 25 % (w/v) methanol and 7.5 % (w/v) acetic acid.

2. Silver Staining

Gels were silver stained using either the Bio-Rad silver staining kit or the following method: gels were fixed for 20 min in 40 % (v/v) methanol/ 10 % (v/v) acetic acid and then washed for 10 min in 40 % (v/v) methanol; following two 5-minute washes with water, the gels were sensitised with 0.02 % (w/v) $Na_2S_2O_4$ for 1 min, rinsed for 30 s with water and then stained with 0.1 % (w/v) chilled $AgNO_3$ for 20 min; finally, they were developed with a solution of 0.05 % (w/v) formalin/ 3 % (w/v) Na_2CO_3 until appearance of protein bands and the reaction was stopped by addition of 5 % (v/v) acetic acid.

Appendix D

Acid/ Urea Polyacrylamide Gel Electrophoresis

Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are aqueous and were prepared in deionised water (Elga), except when stated otherwise. The Mini-PROTEAN II protein electrophoresis cell (Bio-Rad) was used.

The acid-urea polyacrylamide gel electrophoresis (AU-PAGE) method described hereinafter is appropriate to resolve peptide and/ or protein mixtures for subsequent "bug blots" (Lehrer *et al.*, 1991), as it retains the antibacterial properties of the native peptides or proteins. Separation is achieved on the basis of both charge and mass.

The composition of the gels is detailed in table D1. As dissolution of urea is endothermic, it is advisable to allow the mixture to reach room temperature before starting the polymerisation reaction. The anode and cathode chambers were filled with 5 % (v/v) acetic acid and the polymerised gels were pre-run for 30 min at a constant current of 15 mA per gel to remove acrylamide and bis-acrylamide monomers and excess APS.

An equal volume of protein sample was added to the sample buffer (36 % (w/v) urea, 10 % (v/v) glacial acetic acid, 20 % (w/v) glycerol and 1 % (w/v) methyl green). After rinsing the pre-run gels with 5 % acetic acid, the anode and cathode

chambers were re-filled with fresh 5 % acetic acid and the samples were applied in the wells of the gel. The gels were run at reversed polarity with a constant current of 15 mA per gel, supplied by a Power Pac 300 (Bio-Rad), until the green dye front reached the end of the gel.

Following electrophoresis the gels were rinsed with deionised water and Coomassie stained by immersing for 10 min in staining solution (0.25 % (w/v) Coomassie brilliant blue R250, 45 % (v/v) methanol and 10 % (v/v) glacial acetic acid). They were then destained in a solution containing 25 % (w/v) methanol and 7.5 % (w/v) acetic acid.

Table D1. Formulation for 16.6 % T/ 0.43 % C[#] acid-urea polyacrylamide gels. The quantities indicated are sufficient for 2 mini-gels of 0.75 mm thickness.

30 % Acrylamide : Bis (37.5 : 1), Anachem (ml)	8.9
Urea (g)	6.4
Water (ml)	4.55
45 % (v/v) Acetic Acid (ml)	2.56
10 % (w/v) APS (ml)	0.4
TEMED (μl)	100

[#] T is the total concentration of gel components, in percentage, and equals the % Acrylamide + % Bis. C denotes the concentration of crosslinkers (%) and is given by the % Bis × 100 ÷ T.

Appendix E

Accession Numbers of the Peptides and Proteins Purified from Trout Skin Secretions

The following table lists the novel antibacterial proteins or peptides purified from skin secretions of rainbow trout, *O. mykiss*, during the present investigation.

Table E1. Names and corresponding primary accession numbers on the Swiss-Prot or TrEMBL databases of the antibacterial proteins or peptides isolated from skin mucus of rainbow trout.

Protein Name	Accession Number
40S Ribosomal Protein S30	Swiss-Prot P83328
Histone H2A	Swiss-Prot P83327
Lysozyme C Type III	TrEMBL P83333
Oncorhyncin I	Swiss-Prot P83287
Oncorhyncin II (Histone H1 Fragment)	TrEMBL P83374
Oncorhyncin III (Histone H6-like Protein)	TrEMBL P83338