

Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*

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Skin exudates of rainbow trout contain a potent 13.6 kDa anti-microbial protein which, from partial internal amino acid sequencing, peptide mass fingerprinting, matrix-associated laser desorption/ionization MS and amino acid analysis, seems to be histone H2A, acetylated at the N-terminus. The protein, purified to homogeneity by ion-exchange and reversed-phase chromatography, exhibits powerful anti-bacterial activity against Gram-positive bacteria, with minimal inhibitory concentrations in the submicromolar range. Kinetic analysis revealed that at a concentration of 0.3 μ M all test bacteria lose viability after 30 min incubation. Weaker activity is also displayed against the yeast *Saccharomyces cerevisiae*. The protein is salt-sensitive and has no

haemolytic activity towards trout erythrocytes at concentrations below 0.3 μ M. Reconstitution of the protein in a planar lipid bilayer strongly disturbs the membrane but does not form stable ion channels, indicating that its anti-bacterial activity is probably not due to pore-forming properties. This is the first report to show that, in addition to its classical function in the cell, histone H2A has extremely strong anti-microbial properties and could therefore help contribute to protection against bacterial invasion.

Key words: anti-bacterial protein, epithelium, mucosal immun-ity, nucleosome.

INTRODUCTION

The last few years have seen a great burgeoning of reports of the occurrence and characterization of low-molecular-mass anti-microbial peptides from a wide variety of organisms [1]. These molecules have attracted much research interest because of their biochemical diversity, broad specificity against bacteria and/or fungi [1] and also because some have anti-viral [2], anti-tumoural [3] or wound-healing effects [4]. Undoubtedly, they are important components of the innate host defences and represent a source of potentially useful natural antibiotics for pharmaceutical application. To date, over 750 such eukaryotic peptides, mostly isolated from mammals, amphibians, insects or other invertebrates, have been described and listed on the anti-microbial sequences database (<http://www.bbcm.univ.trieste.it/~tossi/>). Certainly, the teleostei is an important and worthy taxon for further study in this respect. Fish are of great economic importance in aquaculture throughout the world. They have a long evolutionary history, display huge species diversity and have wide distribution in marine and other aquatic habitats. Moreover, they live in a microbe-rich environment and are vulnerable to invasion by pathogenic or opportunistic micro-organisms. Non-specific microbicidal compounds are also likely to be especially important for fish, as their adaptive immune system is structurally simpler than that of mammals or amphibians, and is not fully effective in young fry or at low environmental temperature [5]. The skin epithelium and other mucosal surfaces of fish are the sites in which anti-microbial peptides are particularly likely to be abundant, as the mucosae are constantly exposed to water-borne micro-organisms, rendering the fish at risk of infection through grazes or cuts. In many other animal groups the surface mucosa

produces a battery of non-specific microbicidal proteins to prevent harmful systemic infections that would result if microbes breached this barrier [6,7].

It is well established that skin secretions of fish contain a number of innate humoral defence factors, including complement factors [8], lectins [9], proteases [10] and lysozymes [11,12]. More recent investigations have started to reveal that the surface mucosa of some fish also contains a range of anti-microbial peptides [12–17]. So far the number of 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. To address this issue we initiated an investigation into the presence, character and diversity of low-molecular-mass anti-microbial proteins in skin secretions of fish, focusing on the rainbow trout *Oncorhynchus mykiss* as the model species. In a previous paper [12] we reported the isolation of two constitutively expressed anti-bacterial proteinaceous factors from detergent extracts of skin mucus from this species: a novel muramidase with an unusually low isoelectric point and a cationic peptide of approx. 3 kDa. As our studies also indicated that other anti-microbial factors might be present, the present investigation was aimed at examining the presence and character of acid-soluble anti-microbial proteins in *O. mykiss* skin secretions.

EXPERIMENTAL

Animals

Female rainbow trout, weighing 400–500 g, were purchased from College Mill Trout Farm, Perthshire, U.K. They were maintained in flow-through freshwater tanks (14 ± 1 °C) and fed daily with

Abbreviations used: MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight MS; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; MHB, Mueller–Hinton broth; TFA, trifluoroacetic acid; c.f.u., colony-forming units.

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The partial internal amino acid sequence of histone H2A from trout skin has been deposited in the Swiss-Prot database under accession number P83327.

Table 1 List of the micro-organisms used for anti-bacterial assays

Original sources, identification codes and culture conditions are also indicated. Yeastrel medium: 0.5% (w/v) Lab-Lemco (Oxoid), 0.7% (w/v) yeastrel (Natex, Gloucs., U.K.), 0.95% (w/v) peptone (Difco, West Molesey, Surrey, U.K.) and 0.5% (w/v) NaCl. KDM-2 medium: 1% (w/v) peptone, 0.05% (w/v) yeast extract (Sigma), 0.1% (w/v) cysteine hydrochloride (Sigma) and 20% (v/v) foetal calf serum (Invitrogen, Paisley, U.K.), pH 6.5. Yeast extract medium (ATCC 1067): 0.05% (w/v) yeast extract and 1% (w/v) D-(+)-glucose (Sigma). Blood base was from Difco. BKD, bacterial kidney disease; ERM, enteric red mouth disease.

Species	Identification code	Original source	Culture conditions (medium, temperature)
<i>Aerococcus viridans</i>	NCIMB 1120	Moribund lobsters	Nutrient (Oxoid), 30 °C
<i>Aeromonas hydrophila</i>	NCIMB 1134	Mouth lesion of rainbow trout	Nutrient, 30 °C
<i>Aeromonas salmonicida</i> 004	MT 004*	—	Yeastrel, 20 °C
<i>A. salmonicida</i> 849	MT 849*	Diseased salmon	Yeastrel, 20 °C
<i>Bacillus subtilis</i>	ATCC 6051	—	Nutrient, 30 °C
<i>Escherichia coli</i>	NCIMB 12210	—	Nutrient, 37 °C
<i>Listonella anguillarum</i>	NCIMB 2129	Farmed rainbow trout	Blood base, 20 °C
<i>L. anguillarum</i> 01	MT 1637*	—	Blood base, 20 °C
<i>Micrococcus luteus</i>	NCIMB 376	—	Blood base, 20 °C
<i>Planococcus citreus</i>	NCIMB 1493	—	Nutrient/1.5% NaCl, 20 °C
<i>Renibacterium salmoninarum</i>	NCIMB 1114	Salmonid with BKD	KDM-2, 15 °C
<i>Staphylococcus aureus</i>	NCIMB 6571	—	Nutrient, 37 °C
<i>Saccharomyces cerevisiae</i>	—	—	Yeast extract, 37 °C
<i>Yersinia ruckeri</i>	MT 252*	Salmonid with ERM	Nutrient, 25 °C

* Strains kindly provided by Dr Tony Ellis (Marine Laboratory, Aberdeen, U.K.).

the commercial diet Dynamic Red M (Ewos, Bathgate, West Lothian, U.K.).

Preparation of skin mucus extracts

A total of 10 fish were humanely killed by immersion in an anaesthetic bath containing 0.6 g of 3-aminobenzoic acid ethyl ester/l of solution, 4 h after gentle surface stimulation with ultrafine sandpaper to enhance mucus secretion. Samples of mucus and associated epidermal cells were collected by scraping the skin dorso-lateral surfaces (total volume of approx. 150 ml) and homogenized 1:4 (v/v) in a solution of 50% (v/v) ethanol (Merck, Poole, Dorset, U.K.), 3.3% (v/v) trifluoroacetic acid (TFA; Sigma, Poole, Dorset, U.K.) and 2% (v/v) general-use protease inhibitor cocktail (Sigma). Following extraction by stirring for 60 min at 4 °C, the preparation was centrifuged at 29000 g for 60 min at 4 °C and the supernatant lyophilized. The resulting extract was resuspended in 100 ml of 20 mM Hepes (Acros, Loughborough, Leics., U.K.) and the pH adjusted to 7.0 with 5 M NaOH (BDH, Poole, Dorset, U.K.) before centrifuging at 29000 g for 30 min at 4 °C.

Test micro-organisms

The various strains of micro-organisms, their original source and culture conditions used in the present study are listed in Table 1. Each micro-organism was grown to exponential phase before washing in sterile saline [approx. 3.2% (w/v) NaCl (Sigma) for marine strains; 0.8% (w/v) NaCl for non-marine strains] and resuspension in Mueller–Hinton broth (MHB) (Oxoid, Basingstoke, Hants., U.K.) to a concentration of 10⁵ colony-forming units (c.f.u.)/ml.

Anti-bacterial assays

Anti-bacterial activity was assessed using a modification of the two-layer radial diffusion assay of Lehrer et al. [18], as described previously by Smith et al. [12]. The Gram-positive bacterium *Planococcus 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 1 were performed by microtitre broth dilution, based on that devised by Friedrich et al. [19]. Briefly this entailed diluting the test protein in 0.2% (w/v) BSA (Sigma)/0.01% (v/v) acetic acid (BDH) to give a series of eight 2-fold dilutions. Volumes of 11 µl of each dilution were added to individual wells of a polypropylene 96-well microtitre plate (Corning Costar, Cambridge, U.K.). Each suspension of washed bacteria (100 µl), grown under the conditions in Table 1 and diluted in MHB to a concentration of 10⁵ c.f.u. · ml⁻¹ (as above), was then added to each well in duplicate, and the trays were incubated at the appropriate temperature for each bacterium (Table 1) until the attenuation at 570 nm (read with an MRX II plate reader; Dynex, Billingham, West Sussex, U.K.) reached 0.2 in the positive control well, containing bacteria and diluent only. The MIC was considered to be the lowest concentration of protein that reduced growth by 50% compared with 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.

Protein purification

The reconstituted protein extract was fractionated by cation-exchange chromatography using a CM Macro-Prep 1 cm × 10 cm Econo-column (Bio-Rad, Hemel Hempstead, Herts., U.K.), previously equilibrated with 20 mM Hepes/0.1 M NaCl, pH 7.0 (buffer A). Elution was performed with a linear gradient of buffer A to buffer B (where buffer B is 20 mM Hepes/1 M NaCl, pH 7.0) over 90 min, followed by 35 min of buffer B, at a flow rate of 1 ml · min⁻¹. Fractions eluting between 80% and 100% buffer B were collected and applied to Sep-Pak Vac 5 g C₁₈ cartridges (Waters, Watford, Herts., U.K.) 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) in acidified water. The latter fraction was lyophilized, resuspended in acidified deionized water (Elga, High Wycombe, Bucks.,

U.K.) and loaded on to an ODS2-Inertpak C_{18} reversed-phase HPLC column (particle size $5\ \mu\text{m}$, $4.6\ \text{mm} \times 250\ \text{mm}$; Capital HPLC, Broxburn, U.K.). The HPLC system comprised a series 410 LC pump (Perkin Elmer, Beaconsfield, Bucks., U.K.) coupled with a 996 photodiode array detector (Waters) and a 2110 fraction collector (Bio-Rad). Elution was executed at $25\ ^\circ\text{C}$ with a biphasic gradient of $0.1\ \%$ (v/v) TFA in water and $0.09\ \%$ (v/v) TFA in acetonitrile (see Figure 1, top left-hand panel, below) at a flow rate of $1\ \text{ml} \cdot \text{min}^{-1}$. Active fractions of interest were further chromatographed by reversed-phase HPLC on the same column but under a shallower gradient ($30\text{--}55\ \%$ acetonitrile over 50 min at a flow rate of $1\ \text{ml} \cdot \text{min}^{-1}$).

At each step, protein profiles were determined by SDS/PAGE using the Tris/Tricine system with a $16\ \%$ separating gel, $14\ \%$ spacer gel and $5\ \%$ stacking gel, as described by Schägger and von Jagow [20].

Protein quantification

Total protein was estimated by the method of Bradford [21] using BSA (Pierce, Rockford, IL, U.S.A.) as standard. Amino acid analysis of the purified protein was performed at the Protein and Nucleic Acid Chemistry Facility, University of Cambridge, Cambridge, U.K., using the post-column ninhydrin method or the pre-column AccuTag system when picomolar sensitivity was required.

Matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS)

Information concerning the molecular mass and purity of the anti-bacterial factors was determined by MALDI-TOF MS. The sample of interest ($0.5\ \mu\text{l}$, estimated concentration $10\ \text{pmol} \cdot \mu\text{l}^{-1}$ in deionized water) was applied to the target plate along with $0.5\ \mu\text{l}$ of a saturated solution of α -cyano-4-hydroxycinnamic acid (Fluka, Poole, Dorset, U.K.) in acetonitrile (Ultrafine, Manchester, U.K.)/ $0.1\ \%$ TFA ($35:65$, v/v). Cytochrome *c* and haemoglobin (both from Sigma) were similarly applied to an adjacent spot for calibration. Mass spectra were obtained on a ToFSpec 2E instrument (Micromass, Manchester, U.K.) using a $337\ \text{nm}$ laser and operated in linear mode. Peptide mapping of tryptic digests was performed by MALDI-TOF in reflectron mode.

Partial primary structure determination

In order to determine partial N-terminal amino acid sequence, the purified protein was subjected to standard automated Edman degradation on a Procise Sequencer (Applied BioSystems, Warrington, Cheshire, U.K.). Internal sequence information was obtained following digestion of the purified protein with chymotrypsin for 18 h at $37\ ^\circ\text{C}$ using an enzyme/substrate ratio of approx. 1:20. The digests were fractionated on a $100\ \text{mm} \times 1\ \text{mm}$ C_{18} microbore column (Brownlee, Cheshire, U.K.) using a linear biphasic gradient of $0.1\ \%$ (v/v) TFA in water (buffer C) and $0.1\ \%$ (v/v) TFA in acetonitrile (buffer D), in the range $5\ \%$ buffer D to $35\ \%$ buffer D over 30 min. The purified peptide was also sequenced by standard automated Edman degradation on a Procise Sequencer.

Sequence analysis

Homology searches were performed against the SwissProt, NR and Month databases with the basic local alignment search tool (BLAST) [22], provided by the NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>). The Mascot algorithm [23] was

employed for protein identification by peptide mass fingerprinting. 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/>). Protein identification by amino acid composition and molecular mass was performed with the second constellation of the AACompIdent tool (ExPASy). Sequence alignments were executed with the Omega 2.0 sequence analysis software (Oxford Molecular/Accelrys, Cambridge, U.K.), using the Clustal W 1.6 algorithm [24]. The Omega 2.0 software was also used to search for proteolytic cleavage sites within the protein sequence.

Proteolytic digestion

Anti-bacterial activity of the purified protein was assessed by radial diffusion assay as above, following digestion with a final concentration of $60\ \mu\text{g} \cdot \text{ml}^{-1}$ proteinase K (Sigma) for 60 min at $37\ ^\circ\text{C}$.

Kinetic assay

Washed *P. citreus* cell suspensions ($90\ \mu\text{l}$) containing $10^5\ \text{c.f.u.} \cdot \text{ml}^{-1}$ (as above) were incubated at $20\ ^\circ\text{C}$ for different time periods with $10\ \mu\text{l}$ of anti-bacterial protein at two different concentrations (mean value of MIC interval or MBC). As a control, the anti-bacterial protein solution was replaced with $10\ \mu\text{l}$ of 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 $20\ ^\circ\text{C}$.

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 (Sigma; 500 units/ml of blood) and extensively washed with $10\ \text{mM}$ PBS containing $0.9\ \%$ NaCl, pH 7.4, to remove the leucocytes and plasma. Erythrocytes were then packed by centrifugation at $800\ \text{g}$ for 10 min at $4\ ^\circ\text{C}$. For the assay, the test sample was serially diluted to give a range of protein concentrations from 0.2 to $12\ \mu\text{M}$. Aliquots of $11\ \mu\text{l}$ of each dilution were added to $100\ \mu\text{l}$ of a $2\ \%$ (v/v) packed cell volume of trout erythrocyte suspension in PBS and incubated for 30 min at $37\ ^\circ\text{C}$. For negative controls, $11\ \mu\text{l}$ of PBS replaced the protein sample under test, while for positive controls it was substituted with $0.2\ \%$ (v/v) Triton X-100 (Sigma). Following incubation, all samples were centrifuged at $1000\ \text{g}$ for 5 min at room temperature and $100\ \mu\text{l}$ of the supernatant from each well was diluted with $800\ \mu\text{l}$ of PBS before absorbance measurement at $545\ \text{nm}$ using a Ultrospec 3300pro spectrophotometer (Amersham Biosciences, Little Chalfont, Bucks., U.K.). The supernatant from the negative control served as a reference. Percentage haemolysis was defined as the ratio of absorbances between each sample and the positive control.

Muramidase activity

Muramidase activity was tested by radial diffusion assay as described in Smith et al. [12] using hen egg-white lysozyme (Sigma; $50\ \mu\text{g} \cdot \text{ml}^{-1}$) as standard.

Planar lipid bilayer assay

To test the ion-channel behaviour of the anti-microbial protein purified from trout mucus, macroscopic and single-channel

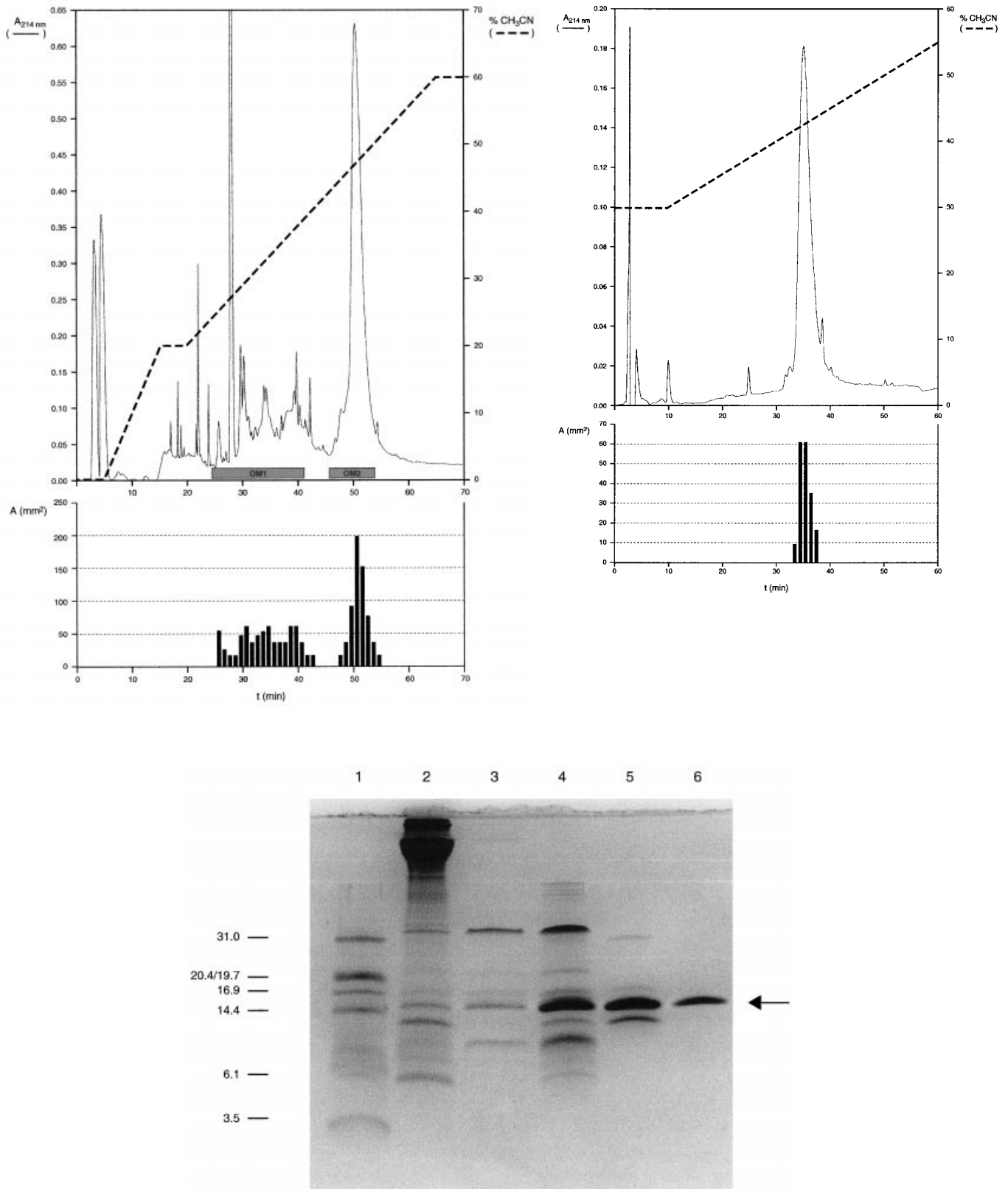


Figure 1 Purification of an anti-microbial protein from skin secretions of rainbow trout

Top left-hand panel: acidic protein extracts of skin mucus were fractionated by cation-exchange chromatography on a CM Macro-Prep column and the active fractions were concentrated by solid-phase extraction using C_{18} Sep-Pak cartridges; the 70% acetonitrile (CH_3CN) eluate was subjected to C_{18} reversed-phase HPLC. The chromatogram was obtained at 214 nm (solid line). Acetonitrile concentration gradient is represented by the dashed line. Anti-bacterial activity profile against *P. citreus* is denoted by the histogram, which shows the area of the clear zones (A) on a radial diffusion assay. Top right-hand panel: active fractions labelled OM2 were pooled and fractionated by C_{18} reversed-phase HPLC using a shallower water/acetonitrile gradient. Absorbance was monitored at

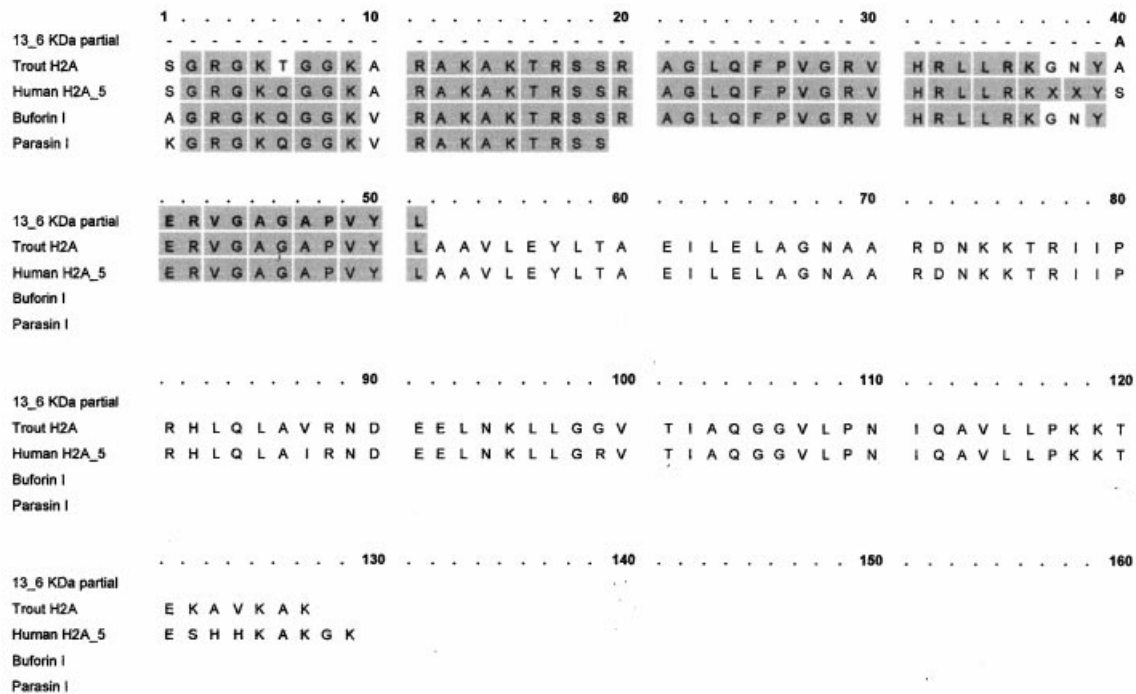


Figure 2 Partial internal sequence of the anti-microbial protein purified from skin mucus of rainbow trout and its homology with other histones or histone-derived peptides

Identical residues between the purified anti-microbial protein, histone H2A from rainbow trout [26], human histone H2A.5 [44], buforin I [33] and parasin I [15] are represented by shaded boxes.

experiments were carried out. For the conductance experiments, virtually solvent-free planar lipid bilayers were formed using the Montal and Mueller technique [25]. 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 the 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 soya bean (Sigma) was used as the lipid. Voltage was applied through an Ag/AgCl electrode in the *cis* side.

With regard to the macroscopic conductance experiments, the 13.6 kDa protein was added in a concentration range between 5×10^{-8} and 5×10^{-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/s) and transmembrane currents were fed to a Keithley amplifier (model 427; Cleveland, OH, U.S.A.). Current–voltage curves were recorded on an *x–y* plotter.

In single-channel recordings, the protein concentrations ranged from 2×10^{-9} to 10^{-8} M. Currents were amplified and potentials were applied simultaneously by a patch-clamp amplifier (RK 300; Biologic Science Instruments SA, Claix, France). Single-channel currents were monitored on an oscilloscope (R5103N;

Tektronix, Beaverton, OR, U.S.A.) and stored on a DAT recorder (DTR 1202; Biologic Science Instruments SA) for off-line analysis. Satori (V3.1; Intracel Software, Royston, Herts., U.K.) was used for downstream analysis. All experiments were performed at room temperature.

RESULTS

Protein purification

Cation-exchange chromatography of the skin secretion extracts yielded active fractions eluting between 0.8 and 1.0 M NaCl (results not shown). These active fractions were pooled and concentrated by solid-phase extraction on C_{18} Sep-Pak cartridges. Subsequent chromatography by C_{18} reversed-phase HPLC resulted in two groups of active fractions, designated OM1 (25–41 min) and OM2 (47–54 min; Figure 1, top left-hand panel). Refractionation of OM2 by HPLC with the same column, but using a shallower gradient, yielded a single peak with a retention time of 34.6 min (corresponding to 42.3% acetonitrile) that exhibited anti-bacterial activity (Figure 1, top right-hand panel). SDS/PAGE revealed a single band with an apparent molecular mass of approx. 15 kDa (Figure 1, bottom panel). This activity was thermostable, remaining present even after

214 nm (solid line). The peak fraction eluting at 34.6 min was found to be anti-bacterial to *P. citreus* (see histogram). Bottom panel: Tris/Tricine SDS/PAGE analysis of the active fractions. Lane 1, markers; lane 2, crude extract; lane 3, pooled active ion-exchange fractions; lane 4, 70% acetonitrile eluate from solid-phase extraction; lane 5, OM2 C_{18} reversed-phase HPLC fractions; lane 6, purified anti-microbial protein after second HPLC. Each lane contains 7.5 μl of sample. The numbers on the left correspond to the molecular-mass markers (kDa). Protein of interest is indicated by an arrow.

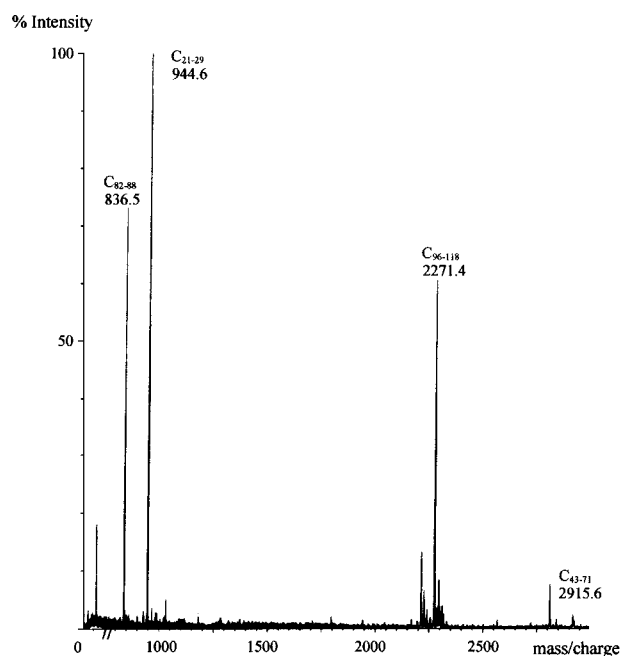
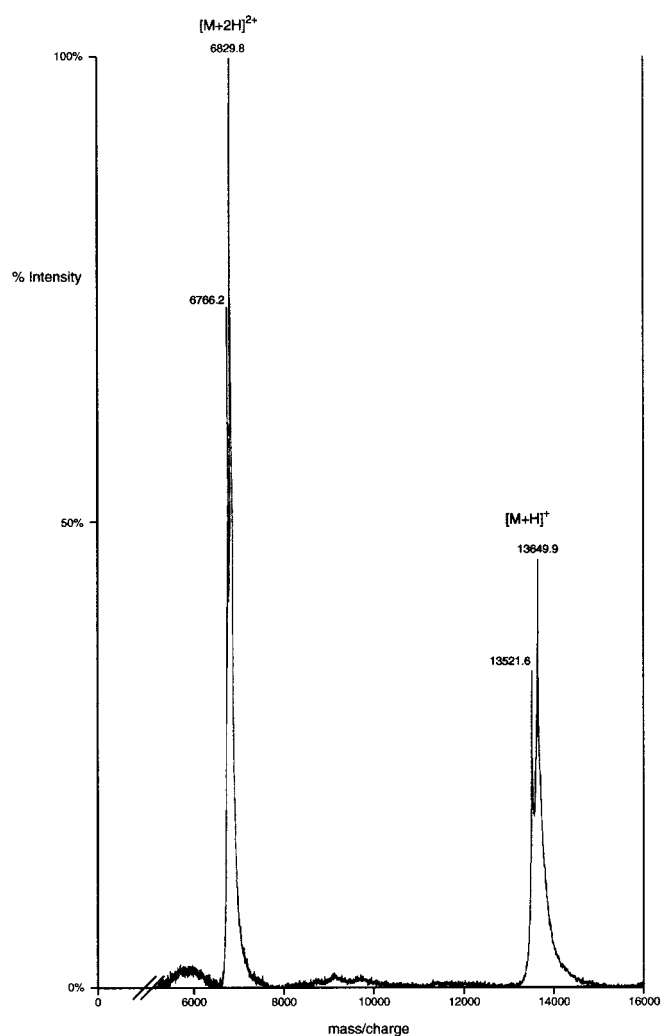


Table 2 Amino acid analysis of the anti-microbial protein purified from rainbow trout skin

The experimentally determined number of residues for the 13.6 kDa trout protein is shown and compared with predicted values for gonadal trout histone H2A (Swiss-Prot P02264). Norleucine was used as an internal standard. Data analysis using the AAComplndent tool (EXPASY) revealed that the experimental results match the amino acid composition of gonadal trout histone H2A.

Amino acid	No. of residues determined for 13.6 kDa trout protein	Predicted no. of residues for histone H2A
Cys	0	0
Asx	8	8
Thr	5	6
Ser	8	3
Glx	11	11
Gly	16	14
Ala	14	18
Val	9	10
Met	Excluded	0
Ile	5	5
Leu	14	16
Tyr	3	3
Phe	1	1
His	2	2
Lys	10	13
Arg	10	12
Pro	7	5
Trp	Not determined	0

incubation at 99 °C for 5 min. Proteolytic treatment with proteinase K completely abolished anti-bacterial activity, confirming that the active factor had a proteinaceous nature. The total amount of purified protein was approx. 1 µg · g of mucus⁻¹.

Protein characterization

Attempts to determine the primary structure of the protein by automated Edman degradation were unsuccessful because the N-terminus was blocked. Digestion with chymotrypsin generated a peptide with a mass of 1201.64 Da, as determined by MALDI-TOF MS. The sequence obtained by automated Edman degradation of this peptide was AERVGAGAPVYL. BLAST homology searches established that it is a perfect match with part of histone H2A from rainbow trout (Figure 2).

MS analysis of the purified protein revealed two signals at 13 512 and 13 639 Da (a representative experiment is shown in Figure 3, top panel) corresponding to the single charged molecular ions. These two peaks are consistent with the acetylation of the N-terminal residue of trout histone H2A [26], 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 Figure 3 (bottom panel). Peptide mass fingerprinting using this set of peptide masses showed that histone H2A was the only significant protein candidate, with four matching peptides out of a total of six potential peptide masses. Database searches using

Figure 3 MALDI-TOF MS analysis of the pooled peak fractions 35 and 36 (Figure 1, top right-hand panel)

Top panel: representative mass spectrum of the intact protein. The singly and doubly charged molecular ions are labelled. The experiment was performed in triplicate. Bottom panel: peptide mapping of the tryptic digest. The masses of the identified monovalent peptide ions as well as their positions within the protein are indicated.

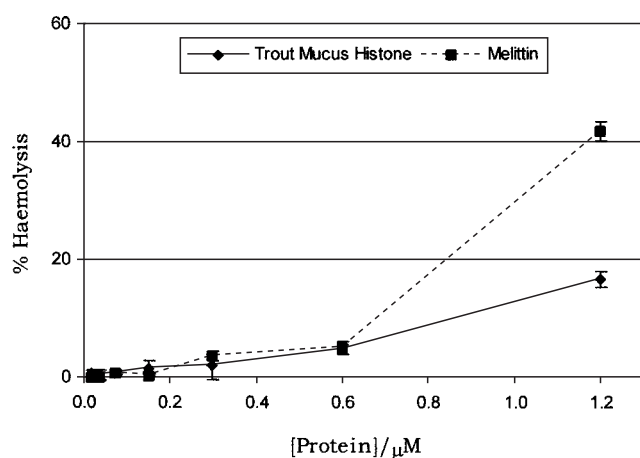


Figure 4 Haemolysis assay against trout erythrocytes

Serially diluted test samples were incubated with a 2% (v/v) suspension of erythrocytes for 30 min at 37 °C. Percentage 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 \pm S.E.M., $n = 3$.

the AACCompIdent tool revealed that the experimental amino acid analysis results were also in good agreement with the theoretical composition of gonadal histone H2A from trout (Table 2). The discrepancies observed for Ala, Ser and Lys can be attributed to a low concentration of protein and consequent decrease in the method's sensitivity.

Anti-microbial and haemolytic activity

The purified histone was found to have anti-microbial activity against the Gram-positive bacteria *Aerococcus viridans*, *Bacillus subtilis*, *Micrococcus luteus* or *P. citreus*, with MICs in the submicromolar range (Table 3). Only feeble activity was observed against Gram-negative bacteria at the concentrations tested ($16 \mu\text{g} \cdot \text{ml}^{-1}$ maximum concentration). The yeast *Saccharomyces cerevisiae* showed susceptibility with a MIC similar to that observed against *B. subtilis* (Table 3). The purified histone had no detectable muramidase activity.

Haemolytic activity was not observed against trout erythrocytes at concentrations of the purified protein below $0.3 \mu\text{M}$ ($4 \mu\text{g} \cdot \text{ml}^{-1}$), although at higher concentrations it displayed a considerable dose-dependent haemolytic activity comparable with that of melittin (Figure 4). This activity against *O. mykiss* erythrocytes is less potent than its bactericidal effect against susceptible Gram-positive bacteria (Figure 4, Table 3).

Kinetics

The kinetic study against *P. citreus* demonstrated that bacterial growth was affected in a dose- and time-dependent manner, with increasing concentrations of anti-bacterial protein or increasing incubation times leading to higher loss of viability (Figure 5). At $0.12 \mu\text{M}$ (MIC) anti-bacterial activity was noticed after 10 min incubation, where approx. 30% of the bacteria were non-viable. Within 60 min incubation no colony-forming units could be observed. After only 1 min, approx. 60% of the bacteria were actively growing following incubation with $0.3 \mu\text{M}$ (MBC) anti-bacterial protein. Bacterial growth was totally impaired after 30 min incubation at this concentration.

Table 3 Bacteriostatic (MIC) and bactericidal (MBC) activities of histone H2A and cecropin P1

Species	Gram staining	Histone H2A		Cecropin P1	
		MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)
<i>A. viridans</i>	+	0.08–0.15	0.3	0.6–1.2	> 2.4
<i>B. subtilis</i>	+	0.6–1.2	1.2	> 2.4	> 2.4
<i>M. luteus</i>	+	0.6–1.2	1.2	> 2.4	> 2.4
<i>P. citreus</i>	+	0.08–0.15	0.3	1.2–2.4	> 2.4
<i>R. salmoninarum</i>	+	0.15–0.3	> 1.2	0.15–0.3	> 2.4
<i>S. aureus</i>	+	> 1.2	> 1.2	> 2.4	> 2.4
<i>A. hydrophila</i>	–	> 1.2	> 1.2	> 2.4	> 2.4
<i>A. salmonicida</i> 004	–	> 1.2	> 1.2	0.3–0.6	2.4
<i>A. salmonicida</i> 849	–	> 1.2	> 1.2	> 2.4	> 2.4
<i>E. coli</i>	–	> 1.2	> 1.2	0.15–0.3	0.6
<i>L. anguillarum</i>	–	> 1.2	> 1.2	0.04–0.08	0.3
<i>L. anguillarum</i> 01	–	> 1.2	> 1.2	> 2.4	> 2.4
<i>Y. ruckeri</i>	–	> 1.2	> 1.2	0.3–0.6	1.2
<i>S. cerevisiae</i>	+	0.3–0.6	> 1.2	0.6–1.2	> 2.4

Salt sensitivity

The assays to investigate the influence of NaCl on anti-bacterial activity showed that, despite being active against *P. citreus* at NaCl concentrations up to 3.2%, the MIC value increased 4-fold when the NaCl concentration was raised from 0.8 to 1.5%. Furthermore, a 16-fold increase in the MIC value (relative to 0.8% NaCl) was observed in the presence of 3.2% NaCl (Figure 6).

Pore-forming activity

In the concentration range between 5×10^{-8} and 5×10^{-7} M, strong destabilization of membranes occurred when the purified protein was included in the cell measurement and no typical current–voltage curves could be recorded. This behaviour was confirmed by independent single-channel experiments. Indeed, with the protein concentration ranging from 2×10^{-9} to 10^{-8} M, and under a large range of applied voltages, a strong destabilization of planar lipid bilayers was observed, as revealed by noisy traces and a current leak (results not shown).

DISCUSSION

The present investigation shows that a 13.6 kDa anti-microbial protein is expressed by rainbow trout skin epithelium. The protein has a broad spectrum of activity, is highly potent and is inhibited by NaCl. MS combined with peptide mass fingerprinting, amino acid analysis and sequence alignments further reveal that it is likely to be histone H2A, acetylated at the N-terminus.

Amongst the numerous reports of anti-microbial peptides published in the literature, a few appear to be known proteins or protein fragments not previously thought to have anti-microbial properties. The bactericidal properties of histones have long been acknowledged [27]. Nevertheless, only recently have more complete reports describing the microbicidal effects of histones or histone-derived fragments (mainly H1 or H2B) started to appear in the literature [16,17,28,29]. To the best of our knowledge, the present study on *O. mykiss* is the first to directly demonstrate that histone H2A has anti-microbial properties.

Histone H2A is one of the core histones which, together with histones H2B, H3 and H4, forms the nucleosome [30], and it has therefore been classically associated with DNA packaging and regulation of transcription. Notwithstanding, recent reports have

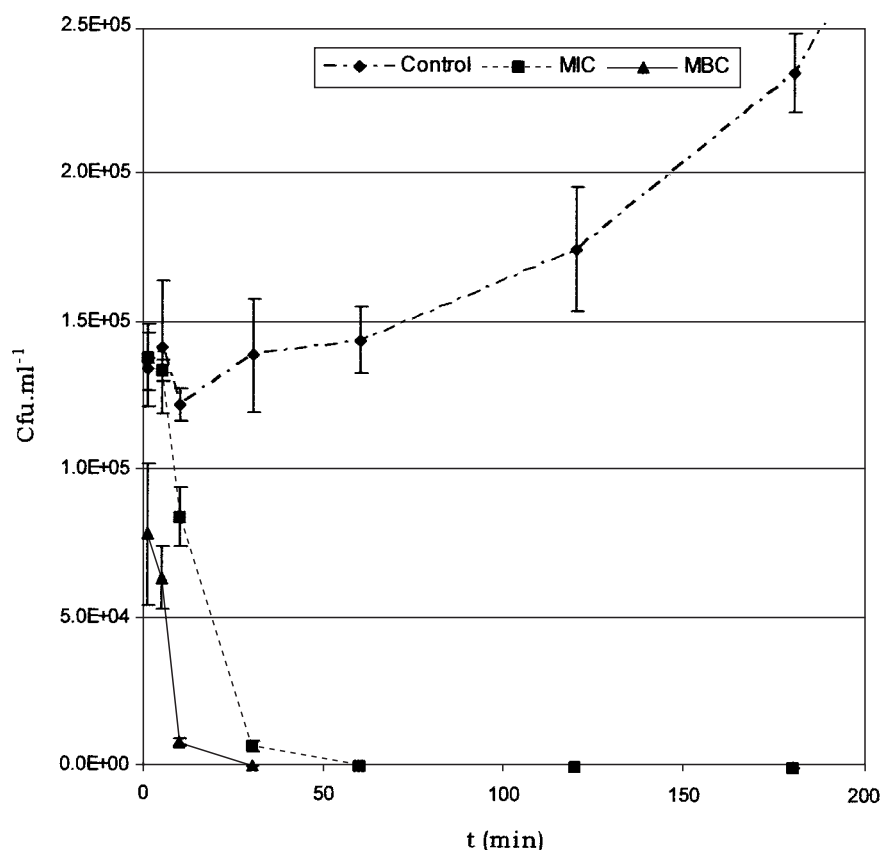


Figure 5 Effect of histone H2A on bacterial cell viability

P. citreus cell suspensions (10^5 c.f.u. · ml⁻¹) were incubated with purified histone H2A at 0.12 μ M (MIC) or 0.3 μ M (MBC) and plated in triplicate after the indicated times. The control comprised bacteria incubated in the absence of anti-microbial protein. Data are denoted as means \pm S.E.M., $n = 3$.

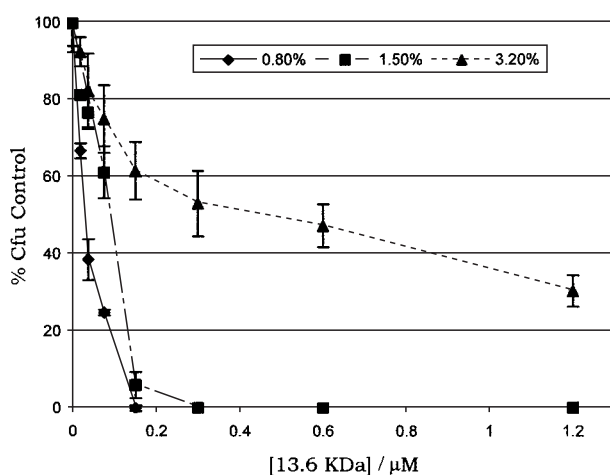


Figure 6 Influence of salt concentration on anti-bacterial activity of histone H2A against *P. citreus*

Bacterial suspensions containing different concentrations of NaCl were incubated with serially diluted histone H2A (13.6 kDa protein). 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 \pm S.E.M., $n = 3$.

demonstrated that histones or histone-derived fragments can have a cytosolic as well as a nuclear localization [28,31]. In

particular, Cho et al. [32] have clearly shown by immunohistochemistry that unacetylated histone H2A is present in the cytoplasm of mucous gland cells from catfish skin mucosa and that parasin I is released upon injury. These data support the hypothesis that, besides its nucleosomal involvement, histone H2A could also aid in protection of the cell against bacterial attack. It may function either in the cytoplasm against intracellular pathogens or extracellularly through release to mucosal surfaces or tissue fluids after infection-induced cell lysis or apoptosis. We are currently investigating the cellular localization of histone H2A in trout skin mucous gland cells and monitoring changes in its expression after immune challenge.

While the precise contribution of histones to innate immunity remains to be clarified, evidence is accumulating that some histones and histone-derived fragments isolated from a wide range of vertebrate species have anti-bacterial properties [15–17,29,33]. Indeed, sequence alignments of the 13.6 kDa protein described in the present paper with buforin I, parasin I, human histone H2A.5 and a deduced cDNA sequence for histone H2A from rainbow trout reveal a high degree of homology amongst them (Figure 2). There are two previous reports of antimicrobial peptides derived from histone H2A. One is buforin I, a potent 39-residue anti-bacterial peptide from skin of the Asian toad, *Bufo bufo gargarizans* [33]. The activity of buforin I against Gram-positive bacteria ranges from 4 to 8 μ g · ml⁻¹ [15], values that are within the interval of 1–16 μ g · ml⁻¹ obtained for rainbow trout H2A in the present study, despite the higher molecular

mass of trout H2A compared with buforin I. The other histone H2A-derived anti-microbial peptide is parasin I, a 19-residue N-terminal fragment present in the skin of the catfish *Parasilurus asotus*, which has activity against Gram-positive bacteria in the range $1\text{--}2\ \mu\text{g}\cdot\text{ml}^{-1}$ [15]. Moreover, monoclonal antibodies to this catfish peptide display some cross-reactivity with a peptide of similar size present in skin mucus of rainbow trout [32]. Our investigation, however, did not identify such a peptide in skin mucus of *O. mykiss*, indicating that, if it is present, it may not be active against the test bacterium used. The primary structure differences between parasin I and the equivalent trout peptide (particularly its N-terminal acetylation) might be enough to justify their dissimilarities in anti-microbial 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.

Different anti-microbial peptides kill bacteria by different mechanisms. They either form pores that induce disruption in the electro-osmotic gradients and the subsequent bioenergetic collapse of the cell [34], or they inhibit cellular functions, e.g. DNA synthesis [35] and chaperone-assisted protein folding [36]. Nevertheless, it is now well established that permeabilization of the cell membrane is the mechanism employed by a vast number of anti-microbial peptides. In spite of extensive research, the mode of action of these membrane-lytic peptides is still under discussion and their selectivity towards specific targets is not fully understood (reviewed in [37,38]). Macroscopic current-voltage 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 marked destabilization of planar lipid bilayers, but is unable to form stable channels. This result is in accordance with a previous study by Ebran et al. [39], which showed that, unlike the hydrophobic proteins, the water-soluble proteins from trout mucus (as histone H2A) did not induce ion channels in artificial membranes, but rather caused direct disintegration or micellization of the cell membrane. These results support the hypothesis that histone H2A disrupts the cell membrane through the 'carpet' mechanism. This model predicts that the protein adsorbs to the target membrane and covers it in a carpet-like fashion, maintaining the contact with the phospholipid head groups throughout the entire permeation process [38]. At a high-enough local concentration of protein the formation of transient holes in the membrane may occur, consistent with the observed ion-channel activities [40,41]. These toroidal holes [38,40] might allow histone H2A to reach the cytosol or the nucleus, where it could also exert its anti-bacterial action by inhibiting cellular functions.

In many animal species anti-microbial proteins are inhibited by high NaCl concentrations, because the salt interferes with the electrostatic interaction of the cationic proteins and the negatively charged microbial surface. However, some of the anti-bacterial peptides expressed by marine or aquatic animals appear to be salt-tolerant, for example pleurocidin from the Winter flounder *Pleuronectes americanus* [14]. A few are salt-dependent, as exemplified by an 11.5 kDa anti-bacterial protein purified from the shore crab, *Carcinus maenas* [42]. As shown in the present study, 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 to 3.2% NaCl. Given that this H2A is expressed in the skin mucosa, it is surprising that it is salt-sensitive. Some wild rainbow trout, also known as steelhead, are anadromous and migrate seasonally from freshwater to the sea,

where they spend several years before returning to their home streams for spawning [43]. It is therefore likely that during the seawater phase, the fish might be deprived of the protective effect of any of its anti-microbial proteins that are 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, in seawater their anti-bacterial activity could be directed against intracellular pathogens, whereas when the fish is in freshwater their presence in epithelial cells may also contribute to surface disinfection. It is notable that the salmonid intracellular pathogen, *R. salmoninarum*, the causative agent of bacterial kidney disease, is sensitive to killing by the histone H2A purified in the present study.

Histone H2A purified from trout mucus in the present study was found to be a remarkably potent anti-bacterial agent. Its minimal inhibitory concentrations are in the submicromolar range and approx. 10 times lower (on a molar basis) than those of cecropin against sensitive bacteria. There is a direct proportionality between anti-bacterial activity, protein concentration and incubation time, in common with other anti-microbial peptides [14]. Furthermore, its anti-bacterial action is exerted rapidly, i.e. a 30 min incubation at $0.3\ \mu\text{M}$ is sufficient to totally inactivate the Gram-positive test bacteria. Importantly, at this concentration the protein is not lytic for trout erythrocytes, although it has haemolytic effects at concentrations over $0.3\ \mu\text{M}$. 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 [5]. Amongst the innate humoral defences, complement is highly evolved in teleosts [8]. Nevertheless, the alternative complement pathway in bony fish is less sensitive to activation by Gram-positive bacteria than Gram-negative ones [8]. While Gram-negative bacteria tend to dominate in marine or aquatic environments, Gram-positives 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-positive bacteria. It is not improbable that histone H2A operates in synergy with other anti-bacterial 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 anti-bacterial peptides, including a histone H1-derived peptide, with lysozyme [16].

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