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Isolation and characterization of novel glycoproteins from fish epidermal mucus: correlation between their pore-forming properties and their antibacterial activities

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Abstract

In fish, a layer of mucus covers the external body surface contributing therefore, among other important biological functions, to the defense system of fish. The prevention of colonization by aquatic parasites, bacteria and fungi is mediated both by immune system compounds (IgM, lysozyme, etc.) and by antibacterial peptides and polypeptides. We have recently shown that only the hydrophobic components of crude epidermal mucus of fresh water and sea water fish exhibit strong pore-forming properties, which were well correlated with antibacterial activity [N. Ebran, S. Julien, N. Orange, P. Saglio, C. Lemaitre, G. Molle, Comp. Biochem. Physiol. 122 (1999)]. Here, we have isolated novel glycosylated proteins from the hydrophobic supernatant of tench (*Tinca tinca*), eel (*Anguilla anguilla*) and rainbow trout (*Oncorhynchus mykiss)* mucus. The study of their secondary structure was performed by circular dichroism and revealed structures in random coil and α-helix in the same proportions. When reconstituted in planar lipid bilayer, they induced the formation of ion channels. This pore-forming activity was well correlated with a strong antibacterial activity (minimal inhibitory concentration < 1 μM for the three proteins) against both Gram-negative and Gram-positive bacteria. Our results suggest that fish secrete antibacterial glycoproteins able to kill bacteria by forming large pores (several hundreds to thousands of pS) in the target membrane.

Keywords: Antibacterial activity; Ion channel; Glycoprotein; Circular dichroism; Mucus; Fish

1. Introduction

The resistance to infection is mediated by two systems, acquired and innate. Antimicrobial substances belong to the innate system and insure a first line of defense. They were isolated in invertebrates, especially in insects [2], and since the first cecropin from *Hyalophoar* [3] many other antimicrobial peptides were isolated from different insects. In addition to the cecropin family, three other families were found, such as insect defensins with cysteine bridges [4], or proline-rich peptides like drosocin [5], or glycine-rich polypeptides like attacin (20 kDa) [6].

Abbreviations: DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; HS, hydrophobic supernatant; LEP, LiDS-electroeluted protein; LiDS, lithium dodecyl sulfate; OPOE, octylpolyoxyethylene; PLB, planar lipid bilayer; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SEP, SDS-electroeluted protein

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These antimicrobial peptides were also found in vertebrates and were shown to be active against both Gram-negative and Gram-positive bacteria. The first isolated peptide was the bombinin from the skin secretion of the frog *Bombina variegata* [7] followed by several other peptides from the frog skin, such as magainins [8], brevinins [9] or dermaseptins [10]. Antimicrobial peptides, like defensins [11], cathelicidins [12] or protegrins [13], are also present in higher vertebrates including humans. The number of such antibiotic peptides is rapidly increasing (for review see [14–17]). Many of these peptides act by forming ion channels across the cell membrane, causing a disruption of the membrane and leading to the death of the infectious agents [18,19].

With the expansion of fish farms, the immune system of fish has been extensively investigated. To survive in its aquatic environment full of parasites, fish possess, like other vertebrates, an immune system with antibodies (IgM), lysozyme and precipitins [20] but also an innate system with fast induction of antimicrobial peptides and proteins localized in the mucus layer. While the antibacterial role of mucus has been known for many years [20–23] the isolation of antimicrobial molecules from mucus is much more recent. These antibacterial compounds could be proteinases isolated in numerous fish, like trout [24], salmon [25] and eel [26], or peptides like pardaxin, a 33-amino acid peptide found in the sole *Pardachirus marmoratus* [27], or pleurocidin, a 25-amino acid peptide isolated from the winter flounder *Pleuronectes americanus* [28]. Beside these small compounds (MW $<$ 10 kDa), antibacterial polypeptides with higher molecular weight could also be found such as 27- and 31-kDa proteins isolated from the epidermal mucus of carp [29] and, more recently, three antimicrobial proteins (15, 15.5 and 30 kDa) from the skin of the channel catfish *Ictalarus punctatus* [30].

We previously showed that the hydrophobic component of epidermal mucus of fresh and seawater fish exhibited antibacterial activity [1]. Our studies using artificial lipid bilayers strongly suggested that the antibacterial activity could be ascribed to the ability to form ion channels in bacterial membranes. The aim of this work was to isolate and characterize the antibacterial components of epidermal mucus of three fish (tench, eel and trout) in order to generalize the mode of action of defence proteins.

2. Materials and methods

2.1. Isolation of skin mucus

Rainbow trout (*Oncorhynchus mykiss*) were bred in an aquatic laboratory (SCRIBE, INRA de Rennes). European eel (*Anguilla anguilla*) and tench (*Tinca tinca*) were provided from natural pounds in Briere (France). Before collecting the epidermal mucus, fish were kept for 1 week in the laboratory in running water tanks in order to decrease the bacterial contamination and to increase the mucus production rate (which reaches a maximum after a week in tank). Mucus was carefully scraped from the dorsal body surface using a plastic spatula. Ventral skin mucus was not collected to avoid intestinal and spermal contaminations. Mucus samples were immediately frozen, lyophilized and stored at −73°C.

2.2. Purification of epidermal mucus

One gram of crude lyophilized mucus was solubilized in 50 ml of Ringer buffer (10 mM HEPES, 112 mM NaCl, 3.4 mM KCl, 2.4 mM $NaHCO_3$, pH 7.4) with an antiprotease cocktail (5 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 1 μM pepstatin A, 1 μg/ml leupeptin and 1 μg/ml aprotinin) then homogenized on ice with a potter. After centrifugation at 30 000 rpm for 45 min at 4°C (Sigma 2K-15, Rotor 12139), the supernatant was discarded and the pellet was resuspended in the above solution. This step was repeated as long as water-soluble proteins were extracted (extraction monitored by UV spectrometry). The final pellet was solubilized at 4°C for 2 h by magnetic stirring in 5 ml of lithium dodecyl sulfate (LiDS) 1% (Sigma) in Ringer's buffer and in the presence of an antiprotease cocktail (5 mM EDTA, 1 mM EGTA, 0.1 mM PMSF and 1 μM pepstatin A), then centrifuged at 30 000 rpm for 45 min at 4°C (Sigma 2K-15, Rotor 12141). This step was repeated five times. The supernatants obtained were collected and stored at −18°C.

2.3. Ultrafiltration

Ultrafiltration was performed using a microcon 10 filter (Amicon) with a 10-kDa cut-off filter. Each microcon was centrifuged at $10\,000 \times g$ for 15 min at 4°C (Sigma 2K-15, Rotor 12145).

2.4. Protein purification

The active proteins were purified from hydrophobic supernatant (HS) as follows: LiDS supernatants were mixed (vol. 1:1) with sample buffer (0.01 M Tris–glycine buffer (pH 6.8), 0.1% sodium dodecyl sulfate (SDS), 1% 2-βmercaptoethanol and 20% glycerol), and heated at 100°C for 4 min and applied to a 12% gel SDS–polyacrylamide gel electrophoresis (PAGE), topped with 7% stacking gel [31]. The separation was performed at 30 mA (7%) and 50 mA (12%). Gels were stained with Coomassie brilliant blue G250 for at least 2 h and then destained by several washes with a mixture of methyl alcohol/acetic acid (50:10 for the initial washes and 8:7 for the final washes). After washing with deionized water, the protein bands were excized and electroeluted. The electroelution was carried out in 25 mM Trizma-base, 192 mM glycine and 0.025% SDS (pH 6.8) at 120 mV for 2 h. Proteins purified by this procedure were called SDS-electroeluted protein (SEP). As SDS, acrylamide and Coomassie blue inhibit bacterial growth, the above protocol was modified. In a first step, SDS was replaced by LiDS in all buffers used for the purification. To decrease the concentration of acrylamide monomers in the final samples, a pre-migration was performed overnight under a constant current of 10 mA. Furthermore, to limit protein unfolding, the sample buffer was devoid of 2-βmercaptoethanol and samples were not heated. Finally, Ponceau S red was used instead of Coomassie blue for gel staining. Gels were incubated in a 0.025% (w/v) Ponceau S solution for 1 min and destained with deionized water until gels were colorless. A protein purified by this procedure was called LiDS-electroeluted protein (LEP). Lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (BSA; 67 kDa) and phosphorylase b (94 kDa) were used as molecular weight markers (Pharmacia).

2.5. Planar lipid bilayer (PLB) experiments

Planar phospholipid bilayer membranes were formed by apposition of two monolayers using the technique of Montal and Mueller [32]. The membrane was formed across a thin film of Teflon (10 μm) with a 0.15–0.20 mm hole pre-treated with 1:40 (by vol.) hexadecane/hexane separating two half glass cells. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Avanti Polar) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti Polar) were used in a 7:3 molar ratio diluted to 0.5% (w/v) in hexane. The compartments were connected to an adjustable potential source (BLM 120 Amplifier, Biologic). The current flowing through the membrane as a result of the applied potential was simultaneously monitored on an oscilloscope and stored on a DATA recorder DTR 1202 (Biologic) for subsequent analysis by Intracel software (Royston (UK) for single channel recording (curve $I = f(t)$) and on X–Y plot for macroscopic experiments (curve $I = f(U)$). SEP were diluted 10-fold in 2% octylpolyoxyethylene (OPOE) (Bachem), incubated for a day at 4°C in order to allow protein refolding. After membrane formation, 3 μl of samples were added to one compartment. Experiments were run at room temperature. OPOE and LiDS were also tested in the reincorporation cell and no activity was detected. Each SEP was tested and only those able to induce ion channel activity into PLB were kept for the subsequent studies. KCl (1 M) was used as electrolyte for both single channel and macroscopic experiments.

2.6. Protein concentration

As samples contain detergent, determination of the protein concentration is not as precise as with hydrosoluble proteins. In order to reduce this inaccuracy, an average was made of values obtained from two methods. Firstly, the bicinchoninic acid method (Pierce) was performed and the absorbance was measured at 562 nm (colorless samples only). Secondly, the concentration was determined after analytical SDS–PAGE (10 μl of sample were used) by staining with Coomassie brilliant blue G250 (all samples). In both methods BSA was used as a standard.

2.7. Antimicrobial assays

Different bacterial groups were selected for this study: *Aeromonas hydrophila* CIP 7430, *Escherichia coli* CIP 54127 and *Pseudomonas fluorescens* MF0 from raw milk for Gram-negative bacteria and *Staphylococcus aureus* CIP 53156 for Gram-positive bacteria. Strains were grown on broth nutrient medium. LEP was used without dilution and was tested in a concentration range of 1 to 5 μg/ml. LEP was made up to 100 μl with nutrient medium on microtiter plates and 100 μl of bacterial culture (10^5 bacteria/ml) was added. The microplates were incubated for 24 h at 30°C. The minimal inhibitory concentration (MIC) was the lowest concentration necessary to inhibit bacterial growth. The control for LEP did not inhibit bacterial growth at the concentrations used for these assays.

2.8. Glycosylation and sialic acid determination

The determination of glycosylated motifs was performed using an Immuno-Blot System for glycoprotein (Bio-Rad) which can detect both glycoproteins and sialic acids. The carbohydrate moiety of a glycoprotein was oxidized with a solution of sodium periodate (10 mM at room temperature for glycoprotein detection and 0.1 mg/ml at 4°C for sialic acid detection) to form aldehyde groups which can react with hydrazide. Biotin X hydrazide was used to incorporate the biotin into the oxidized carbohydrate. The biotin was then coupled to streptavidin and this complex was stained with NTB/BCIP solution. The 49-kDa protein (tench), 45-kDa protein (eel) and 65-kDa protein (trout) from SEP samples, were transferred onto a nitrocellulose membrane (CERALABO) and the membrane was treated as described above. Biotinylated proteins from Bio-Rad were used as positive control and native proteins (except for ovalbumin) were used as negative control.

2.9. Circular dichroism (CD) spectrometry

SEP stained with Ponceau S red were used for this experiment. In a first step, 500 μl of SEP were dialyzed against 2% OPOE in a microdialysis cell (PIERCE) at 4°C for 2 h. Then 200 μl (at 200 μg/ml) were introduced into a quartz optical cell with 0.1 mm path length (121.000 QS, JOBIN YVON). The CD spectra of these proteins were performed at room temperature with a CD6 (Jobin Yvon), at wavelengths ranging between 190 and 260 nm. Ten scans were made per spectra with a speed of 10 s per point between 190 to 205 nm and at a speed of 5 s per point between 205 to 260 nm. The protein secondary structure was determined by the following two methods: K2D [33] and one custom made in our laboratory by Brachais and Brullemans and based on Chang's method [34].

3. Results

Only the hydrophobic component of the mucus that exhibits pore-forming properties, which was well correlated with antibacterial activity [1], was used for the purification of the antibacterial compounds.

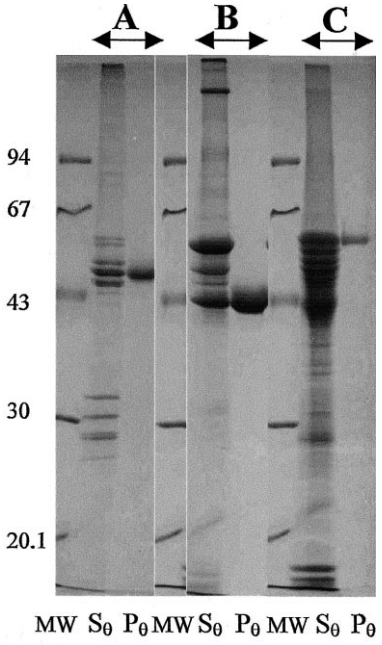

Fig. 1. Analytical electrophoresis of LiDS supernatant and electroeluted protein. Samples were applied to a 12% gel SDS–PAGE after mixing in sample buffer and heating for 5 min at 100°C. (A) Tench samples. (B) Eel samples. (C) Trout samples. Lanes S contain LiDS supernatants used for purification. Lanes P contain electroeluted proteins. Gels were stained with Coomassie Brilliant blue. Molecular weight markers are 94, 67, 43, 30 and 20.1 kDa.

3.1. Purification of the active compounds

The extraction of the hydrophobic component of the mucus was performed using LiDS (CMC 7 mM). In contrast to SDS, LiDS allowed the extraction of hydrophobic proteins that were suitable for reconstitution experiments in PLB as well as for antibacterial assays [1]. In a first step, the ultrafiltration technique was carried out with a 10-kDa cut-off filter. The ultrafiltrate of the HS did not show any ionophore activity in PLB, in comparison to the upper fraction (>10 kDa) which induced ion channels under an applied voltage (data not shown). This result shows that the active compounds have molecular masses higher than 10 kDa.

The electrophoretic profiles obtained from the three fish supernatants displayed the largest protein concentration in the region around 50 kDa (Fig. 1, lanes S_θ). The most concentrated proteins were purified by LiDS–PAGE followed by an electroelution. Each electroeluted protein was tested in preliminary membrane reconstitution experiments and only those able to restore the ionophore activity observed previously with the supernatant were kept for further large scale purification. Three major pore-forming proteins were purified from the HSs: a 49-kDa protein for tench (49Te, Fig. 1, lane A, P_θ), a 45-kDa protein for eel (45Ee, Fig. 1, lane B, P_θ) and a 65-kDa protein for trout (65Tr, Fig. 1, lane C, P_θ). Protein analysis was also performed under denaturing conditions, 4 M urea were added to the SDS–PAGE gel. Samples were boiled (for a complete denaturation) or not, to determine the effect of urea. As shown in Fig. 2, the presence of urea and a heat treatment did not induce any modification in the profiles of the silver-stained SDS–PAGE gel. This result shows that the high MW could not be due to the oligomerization of hydrophobic peptides.

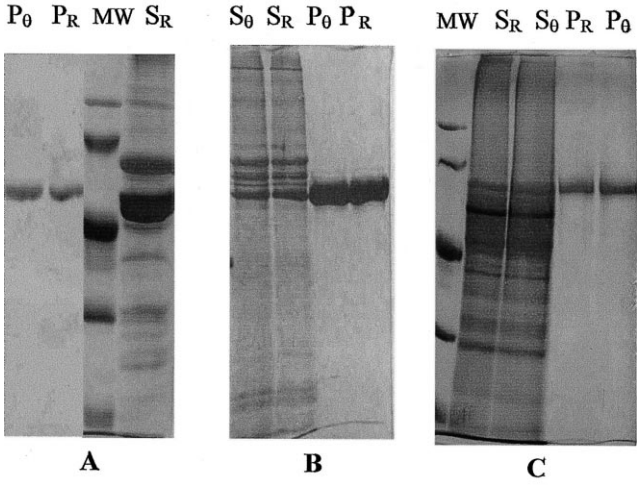

Fig. 2. Analytical electrophoresis of supernatants and proteins in 4 M urea. Urea was added to the SDS–PAGE gel to reach a final concentration of 4 M. Lanes S contain LiDS supernatants used for the purification. Lanes P contain electroeluted protein. In lanes θ, samples were heated at 100°C for 5 min before loading and in lanes R samples were prepared at room temperature. (A) Tench samples. (B) Eel samples. (C) Trout samples. Gels were silver-stained. Molecular weight markers are 94, 67, 43, 30 and 20.1 kDa.

3.2. Pore-forming activity

In order to improve the insertion of proteins in PLB and to decrease the lytic action of LiDS on the membrane, the proteins were diluted in a 2% solution of the non-ionic detergent OPOE (CMC 6.6 mM). After addition in a reconstitution cell, all proteins induced current fluctuations under an applied voltage. The pore-forming behavior consisted of the opening of a large conductance of several thousands of pS, followed by smaller fluctuations of this open state. The 49Te induced fast fluctuations of about 110 pS in 1 M KCl solution (Fig. 3,A1). For 45Ee, multiple large levels of conductance were observed (1400 and 300 pS) but smaller levels of 100 pS could also be occasionally recorded (Fig. 3,A2). Like 45Ee, the 65Tr induced several levels of conductance in addition to the large open state (Fig. 3,A3). Thus, in this range of concentration, (~ 5 ng/ml) it is difficult to determine the unitary conductance for these proteins. The multi-levels of conductance resulting from the opening of several ion channels at the same time were probably due to a massive insertion of the protein into the PLB. In Fig. 3,B1, a weak voltage dependence was detectable for the 49Te. In contrast, the same experiment performed with 45Ee and 65Tr showed an ohmic behavior for both proteins (respectively Fig. 3,B2,B3).

3.3. Antibacterial activity

Taking into account that many antibacterial com-

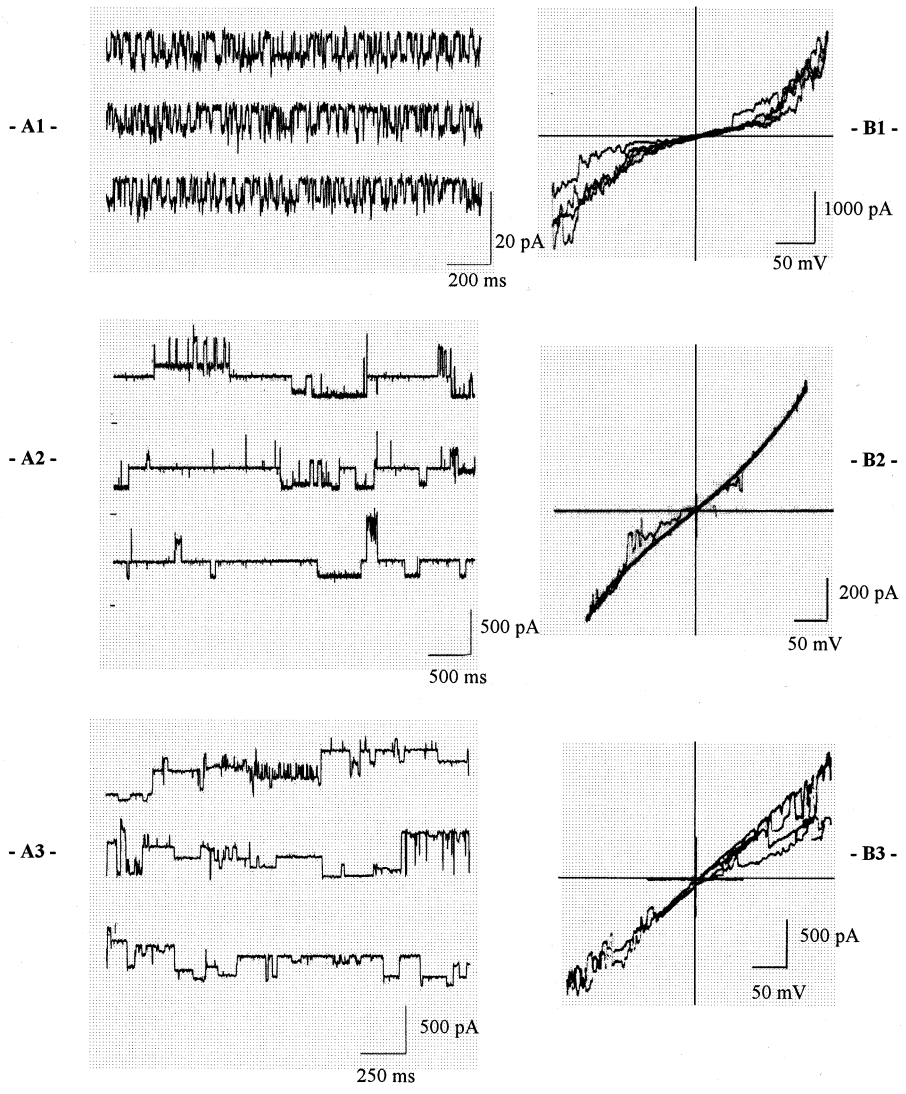

Fig. 3. Single channel traces (A) and *I/V* curves (B) of the 49Te, 45Ee and 65Tr proteins. (A1)–(A3) After dilution with 2% OPOE (1/10 for A1 and A3 and 1/20 for A2) and incubation for 24 h at 4°C, to refold the protein after the steps of purification, 3 μl of the solution was added to the *cis*-side of the cell. (A1) 49-kDa electroeluted protein from tench. Applied voltage +50 mV. (A2) 45-kDa electroeluted protein from eel. Applied voltage +150 mV. (A3) 65-kDa electroeluted protein from trout. Applied voltage −150 mV. (B1)–(B3) 10 μl of sample solution (see above) were added to the *cis*-side of the cell. (B1) Macroscopic behavior of the 49-kDa protein of tench with a potential ramp from −200 to +200 mV in 8 s. For the 45-kDa protein of eel (B2) and the 65-kDa protein of trout (B3) the potential ramp was from −150 to +150 V in 8 s. For each experiment (A and B) the lipid bilayer was formed with POPC/DOPE (7:3) in 1 M KCl.

pounds act by forming pores in the membrane of bacteria, proteins from a LiDS purification (LEP) were tested against various strains. In order to define their antibacterial activity, a strain representing Gram-positive bacteria (*S. aureus*) and three strains representing Gram-negative bacteria (*E. coli, P. fluorescens* and *A. hydrophila*) were tested. The last one belongs to bacterial flora which contaminate the epidermal mucus of the three fish. The MIC was determined by adding an increasing concentration of LEP (ranging from 1 to 5 μg/ml) into microtiter plates containing the strain (10^5 bacteria/ml) and the nutrient medium. Control experiments showed that these concentrations have no effect on bacterial growth. The MIC values are summarized in Fig. 4. The 49Te, 45Ee and 65Tr proteins inhibited the

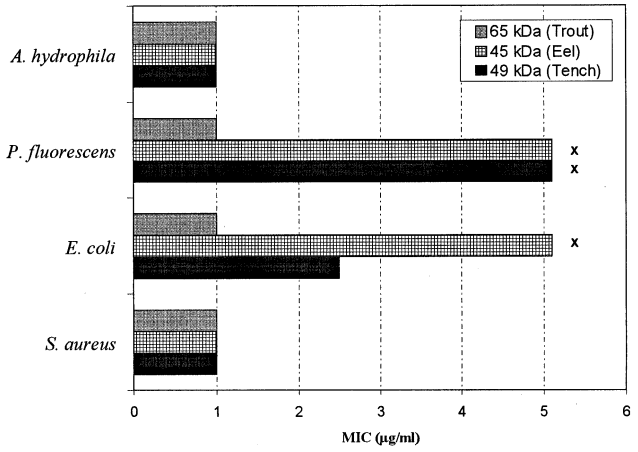

Fig. 4. Antibacterial activity of proteins. The MIC was determined against three Gram-negative bacteria (*E. coli*, *P. fluorescens* and *A. hydrophila*) and one Gram-positive bacterium (*S. aureus*). The concentrations tested for these three proteins were between 1 and 5 µg/ml in order to stay largely below the limit of the control. MIC = minimal inhibitory concentration. ⊠ means that the MIC was not reached for this sample at a concentration ≤5 µg/ml.

growth of *S. aureus* and *A. hydrophila* at approximately 1 µg/ml. Only the 65Tr inhibited the growth of *P. fluorescens* and *E. coli* at about 1 µg/ml while 49Te had a MIC of about 2.5 µg/ml against *E. coli* and higher than 5 µg/ml against *P. fluorescens*. For 45Ee, the growth of these two strains was not inhibited by concentrations less than 5 µg/ml. The greatest inhibition was exhibited by 65Tr (MIC = 1 µg/ml or ~8 nM). This protein was not selective and inhibited both Gram-positive and Gram-negative bacteria. 49Te and 45Ee were more selective against the Gram-positive bacteria and against *A. hydrophila*, a Gram-negative strain of the aquatic environment of fish (MIC = 1 µg/ml) while against the two other strains of Gram-negative bacteria, the MIC was increased.

3.4. Structural approach

3.4.1. Determination of the secondary structure

Proteins used in these experiments were separated on a SDS–PAGE and visualized with Ponceau S red coloration. The excized protein bands were washed before electroeluting in order to obtain colorless samples. Proteins (at 200 µg/ml) were dialyzed against 2% OPOE to remove optically active compounds, like glycine, present in our buffers. Spectra were performed at room temperature and an average of 10 acquisitions between 190 and 260 nm was performed per spectrum (Fig. 5). Structural analysis of these samples showed the presence of both α-helix and random coil structures. For 49Te (Fig. 5A), the following structural distribution was found: 36% α-helix, 17% β-sheet and 47% random coil. For 45Ee, we found 55% α-helix, 12% β-sheet and 33% random coil (Fig. 5B) and for 65Tr, 38% α-helix, 16% β-sheet and 46% random coil (Fig. 5C). The large value obtained in the prediction of random coil could result from a denaturation of an α-helical part of the protein during the various purification steps when proteins were in contact with a denaturing detergent like SDS or LiDS.

3.4.2. Glycoprotein detection

The immuno-blot detection kit for glycoproteins (Bio-Rad) was used for this analysis. SEP were transferred onto a nitro-cellulose membrane and the presence of carbohydrate moieties was revealed for 49Te, 45Ee and 65Tr proteins. The presence of sialic acids

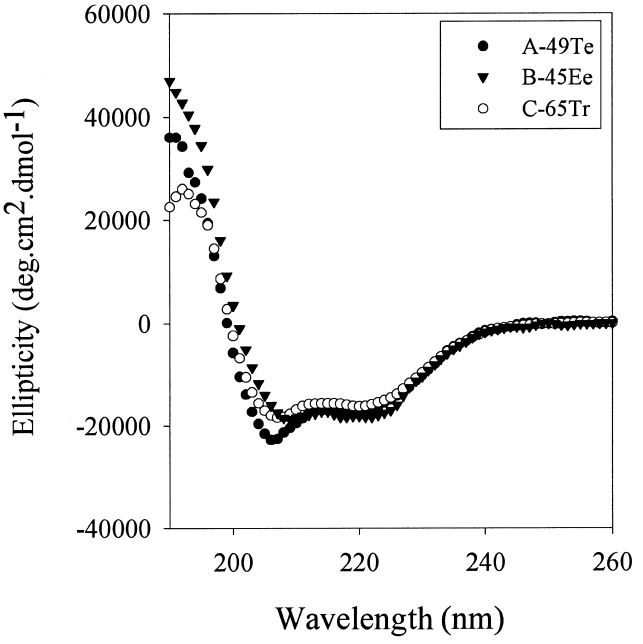

Fig. 5. CD spectra of the tench, eel and trout proteins. Electroeluted proteins were dialyzed against 2% OPOE. 200 µl of sample were introduced in a cell with an optical path of 0.1 mm. Measurements were performed between 190 and 260 nm at 25°C and under nitrogen atmosphere. Number of cycles per spectrum = 10.

was detected only for both the 49Te and 65Tr proteins (data not shown).

4. Discussion

Several antimicrobial compounds have been isolated from both plants and animals over the last decades. These compounds, belonging to the innate system, could act in different ways. Proteins such as PR-39 can cross cell membranes, bind to intracellular molecules and inhibit protein synthesis [35]. Other proteins, such as defensins, can induce ion channels into the cell membranes and disrupt the ion gradient, which may lead to cell death [36].

It is now well established that the epidermal mucus of fish plays a significant role in the mechanical and physiological protection of fish against unfavorable environmental conditions and pathogenic infections. This layer of mucus is essential in the prevention of colonization by bacteria, fungi and other aquatic parasites [20]. Moreover, an increase of secretion was generally observed when the fish were stressed (handling, contact with an infectious agent, etc.).

These antimicrobial properties of the mucus originated from the presence of compounds from the immune system like immunoglobulin [37], lysozyme [38] or precipitin [39], as well as peptides or polypeptides, which belong to the innate system. These antimicrobial compounds could be found in the epidermal secretions of fresh water fish [29] as well as in seawater fish [27,28].

Here we report the isolation and characterization of three novel antibacterial proteins from the hydrophobic component of the skin secretion of three fresh water fish: a 49-kDa protein from tench mucus (Cyprinidae), a 45-kDa protein from eel mucus (Anguillidae) and a 65-kDa protein from rainbow trout mucus (Salmonidae).

These three proteins are strongly hydrophobic and their extraction from epidermal mucus required an anionic detergent (like LiDS). All proteins exhibited strong pore-forming properties when tested in PLB under an applied voltage. When the concentration of these proteins is equal to or higher than 5 ng/ml, there is a massive insertion of the protein into the PLB and the formation of oligomers occurs with channel conductance values ranging from several hundreds to thousands of pS. Furthermore, a study performed with a higher concentration (>10 ng/ml) of proteins into the reconstitution cell showed that these proteins were slightly (49te) or not at all (45Ee and 65Tr) voltage-dependent (Fig. 3B).

In order to correlate these pore-forming properties with antibacterial activity, pharmacological tests were performed against one strain of Gram-positive bacteria (*S. aureus*) and three strains of Gram-negative bacteria (*E. coli*, *P. fluorescens* and *A. hydrophila*). For 65Tr, MIC values were about 1 μg/ml and thus an activity of a few nanomolar was effective

Table 1
Comparison of antibacterial activity of several peptides and proteins from epidermal secretion on the strains of Gram-positive and Gram-negative bacteria used in this study

Origin	Composition	MW (Da)	MIC (μg/ml)		
			S. aureus	*E. coli*	*A. hydrophila*
Skin secretion of xenope *Xenopus leavis*	magainin 2 [8]	2467	50	100	n.d.
Skin of bullfrog *Rana catesbeiana*	ranalexin [47]	2103	4	32	n.d.
Albumin gland of sea hare *Aplysia kurodai*	aplysianin [43]	62 376[a]	n.d.	2–7.4	2–7.4
Mucus of giant African snail *Achatina fulica* Férussac	achacin [45]	59 086[a]	7	42	n.d.
Skin mucus of winter flounder *Pleuronectes americanus*	pleurocidin [28]	2711	48–95[b]	6–9[b]	n.d.
Mucus of catfish *Paralilurus asotus*	parasin I [40]	2000	2	4	n.d.
Skin secretion of Moses sole *Pardachirus marmoratus*	pardaxin [27]	3324	n.d.	43[b]	n.d.
Skin of channel catfish *Ictalarus punctatus*	HLP-1 [30]	15 500	no	5.7[b]	50
Mucus of tench *Tinca tinca*	49 kDa	49 000	1	5	1
Mucus of eel *Anguilla anguilla*	45 kDa	45 000	1	n.d.	1
Mucus of trout *Oncorhynchus mykiss*	65 kDa	65 000	1	1	1

[a]This is the MW of the unprocessed precursor.
[b]MIC given in μM in the literature and converted to μg/ml in this table.

against both Gram-negative and Gram-positive bacteria. For 49Te and 45Ee, the weakest values of MIC (1 μg/ml) were obtained against *S. aureus* (Gram-positive) and *A. hydrophila* which belong to the contaminant flora of the epidermal mucus of these three fish [1]. The growth of both other strains (*E. coli* and *P. fluorescens*) was less inhibited by the 49Te and by 45Ee in the concentration range of 1–5 μg/ml. It was not possible to test these two proteins at higher concentrations because the control begins to inhibit the bacterial growth.

The MIC values found for these three novel antibacterial proteins are compared with those described in the literature for several compounds from the secretion of amphibian, molluscs and fish. Table 1 shows that the best results are obtained with 49Te, 45Ee and 65Tr and only parasin I, a peptide of 2 kDa from the catfish secretions, yields similar MIC [40], while both proteins aplysianin and achacin give higher MIC values.

These proteins, like many compounds in fish epidermal mucus, are glycosylated. In mucus and in various other functions, the glycoproteins are known to provide gel properties [41], but to this date, their chemical composition and properties are still fragmentary. Several studies have shown that the structural motifs of these glycoproteins are characteristic of each fish species and often possess sialic acid [42]. We have shown that in addition to being glycosylated, these proteins comprized sialic acid components (except 45Ee). Using concanavalin A, we have shown that 49Te also contained mannose motifs. An antibacterial and pore-forming glycoprotein of 27 kDa was previously isolated from the skin secretion of carp (*Cyprinus carpio*) [29]. Two other antibacterial glycoproteins were also found in the skin secretion of mollusks; aplysian A from the sea hare *Aplysian kurodai* [43] or achacin from the giant African snail *Achatina fulica* [44]. The physiological role of the carbohydrate moiety in these structures is not yet well understood. For achacin, the carbohydrate moiety is not essential to the antibacterial activity of this protein [45], however, a loss of the carbohydrate part in the drosocin, an antibacterial peptide from *Drosophila melanogaster*, decreases the antibacterial activity [5].

Generally, two main mechanisms [19] could be suggested to explain these antibacterial properties: (i) the formation of classic transmembrane channels into the membrane of the target cells or (ii) the solubilization of the cell membrane by a 'carpet-like' mechanism. These two modes of action could induce membrane permealization that may lead to cell death. In our case, we can suggest that the first mechanism would occur since our fish proteins present a strong correlation between pore-forming properties and antibacterial activity. Thus, the interactions between the hydrophobic surface of transmembrane segments of the protein and the lipid membrane allow the insertion of protein fragments into the hydrophobic core of the membrane while the hydrophilic surfaces face each other to form the hydrophilic channel. Such a mechanism has often been seen with antimicrobial compounds from the innate system [46]. For example, pardaxin 1 [27] induces the formation of voltage-dependent channels, which produce the lysis of the membrane. Two hydrophobic proteins (27 and 31 kDa) from carp mucus induce ion channels in PLB and this property was correlated with antibacterial activity [29]. The results obtained by CD support this mechanism in which the presence of α-helix segments was essential for the insertion of the compounds in the membrane [19].

In summary, we have isolated three novel antibacterial glycoproteins from the skin secretion of three fresh water fish and showed their ability to form ion channels and to lyze bacteria. The presence of these proteins in mucus of fish leads us to speculate that they may serve as a systemic antibacterial agent in order to limit the colonization by micro-organisms.

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