The epidermis of fish is covered with a layer of mucus, which contributes to the defence of the species against parasites, bacteria and fungi. We have previously extracted glycoproteins from various mucus samples from fish and have shown that they present pore-forming activities well correlated with strong antibacterial properties [Ebran, Julien, Orange, Saglio, Lemaitre and Molle (2000) Biochim. Biophys. Acta 1467, 271–280]. The present study focuses on the 65 kDa glycoprotein, Tr65, from the rainbow trout (Oncorhynchus mykiss, formerly Salmo gairdneri). Enzymatic digestion of Tr65 yielded a fragment pattern with strong homology with that of trout type II cytokeratin. Sequence analysis of the cDNA clone obtained by PCR confirmed this homology. We thus constructed a plasmid to overproduce the recombinant Tr65. We extracted and purified this recombinant Tr65, using it for multichannel and single-channel experiments in azolectin bilayers. Our results with recombinant Tr65 confirmed the pore-forming properties already shown with native antibacterial Tr65. These findings offer new insights into the function of keratin proteins present in various mucosal surfaces of animals and human beings.

Key words: bilayer, channel activity, fish defence, rainbow trout, recombinant keratin, skin mucous layer.

INTRODUCTION

The skin mucous layer and epidermis are important in fish defence as they are the first sites of interaction between the host and potential pathogens. Within these layers are many antimicrobial compounds involved in innate immunity of the fish [1–3]. These non-specific microbialic compounds are of critical relevance for fish as their adaptive immune system is less sophisticated than those of mammals and amphibians [4,5], and is not fully effective in young fry [6]. Moreover, it has been proposed that innate immunity tends to replace specific immunity at low temperatures [7,8]. Therefore innate immunity is assumed to be more important for fish than for other endothermic vertebrates [2]. A variety of antibacterial proteins are present in the mucus of various fish species (for reviews, see [1–3,9,10]), including complement factors, lectins and enzymes, e.g. lysozyme (also called muramidase), alkaline phosphatase and proteases. The surface mucosa of fish also contains a broad range of antibacterial proteins from catfish (Punctatus pleurocidin from winter flounder (Pardachirus marmoratus) [11,12], pleurocidin from winter flounder (Pleuronectes americanus) [13] and grannistsins from Grammistes selineatus and Pogonoperca punctata [13–16], and histone-derived peptides, e.g. parasin-I from catfish (Parasilius asotus) [17], oncorhycin II from rainbow trout (Oncorhynchus mykiss, formerly Salmo gairdneri) [18,19] and hippoixin from Atlantic halibut (Hippoglossus hippoglossus L.) [20]. Novel antibacterial factors will probably be identified in the future, as isolation and characterization of defence substances from the epidermal layer of fish are relatively recent events. We have previously reported the isolation of several antibacterial glycoproteins from the epidermal mucus of carp, tench, eel and rainbow trout skin secretions [21,22]. The present study aimed to identify one of these proteins (with a molecular mass of 65 kDa) from the skin mucus of rainbow trout. This protein, subsequently named Tr65, was purified from mucus samples and its gene was cloned. Analysis of internal sequences of Tr65 indicated that it significantly matched the trout O. mykiss type II epidermal keratin E1 (GenBank® accession number AJ272369). We produced recombinant keratin in Escherichia coli and purified it from inclusion bodies. Conductance experiments carried out with the recombinant protein yielded the same results as those observed with the protein extracted from mucus, confirming the identification of the protein. This is the first report showing that a protein from the keratin family has pore-forming activity.

EXPERIMENTAL

Protein purification

The Tr65 protein was purified from epidermal mucus of rainbow trout (O. mykiss) as previously described [21]. Briefly, hydrophobic proteins were extracted from the mucus with a Ringer buffer containing 1% (w/v) LiDS (lithium dodecyl sulphate) and were separated by SDS/PAGE. The protein band of interest (65 kDa) was excised from the gel and then electroeluted with the Biotrap system (Ceralabo). Tr65 was also analysed by two-dimensional electrophoresis. Protein (20 μg) was precipitated by trichloroacetic acid and resolubilized in an IEF (isoelectric focusing) buffer of 5 M urea, 2 M thiourea, 0.5% (w/v) ASB14 (amidosulfofetaine-14), 2 mM tributyl phosphine, 10 mM dithiothretiol, 2% (v/v) carrier ampholytes (pH 3.5–10; Sigma) and 0.025% (w/v) Coomassie Brilliant Blue G-250 (Sigma) [23] (final volume, 400 μL). The first-dimension gel separation was carried out with Immobiline Dry Strips NL.
(non-linear) (18 cm, pH 3–10; Amersham Biosciences, Uppsala, Sweden). IEF was carried out using an IEF cell apparatus (Bio-Rad, Hercules, CA, U.S.A.) as follows: 50 V for 12 h, 250 V for 15 min, 3 h of a linear increase to 10 and 10 kV (1 mA constant) for 10 h for a total of 105.6 kV. The second dimension was obtained by SDS/PAGE using a 12% (w/v) polyacrylamide gel (width, 16 cm; length, 20 cm; thickness, 0.75 mm). After migration, proteins were visualized by silver staining.

**Determination of carbohydrates and sialic acid groups**

The Immuno-blot kit for the detection of glycoproteins obtained from Bio-Rad was used to detect the presence of carbohydrates and sialic acids. Following SDS/PAGE of the samples, the gels were transferred on to nitrocellulose membranes. After washing (10 min) with 30 ml of PBS (9 mM sodium phosphate and 27 mM NaCl, pH 7.2), the membrane was treated with 30 ml of 10 mM sodium periodate [the concentration of the solution was 0.1 g/l for the sialic acid oxidation (0.16 mM)], 100 mM sodium acetate and 5 mM EDTA for 20 min in the dark. The membrane was then washed three times for 10 min each with PBS buffer before adding 30 ml of a mixture containing 6 μl of hydrazide, 30 ml of sodium acetate (100 mM) and 5 mM EDTA. The membrane was then washed three times for 10 min with 30 ml of TBS (50 mM Tris and 27 mM NaCl, pH 7.2) under stirring. In order to block the non-specific sites, 0.25 g of gelatin in 50 ml of TBS was added during one night at 4 °C without stirring. After new washings (three times for 10 min) with TBS, the complex was revealed by 30 ml of TBS containing 150 μl of NBT (Nitro Blue Tetrazolium) and 112.5 μl of BCIP (5-bromo-4-chloroindol-3-yl phosphate). The staining was stopped with distilled water.

**Protein digestion and MS**

Following electrophoresis and Coomassie Brilliant Blue staining, the 65 kDa band was excised from the gel and was washed twice with water and twice with 100 mM sodium hydrogen carbonate (NaHCO₃) and 50% acetonitrile before complete dehydration by vacuum centrifugation. Gel pieces were rehydrated with trypsin solution [10 litres of 15 ng/l sequencing-grade trypsin (Boehringer–Mannheim, Manheim, Germany) in 100 mM NaHCO₃ and 5 mM CaCl₂]. Tryptic digestion was carried out overnight at 25°C. The trypic digest was extracted successively with a 100% acetonitrile, 100 mM NaHCO₃ solution and 5% formic acid. The digest solution and the extracts were pooled and dried in a vacuum centrifuge before nanospray MS/MS (tandem MS) analysis. A QSTAR-Pulsar-i instrument (Applied Biosystems, Foster City, CA, U.S.A.) was used with a Z-Spray ion source working in the nanospray mode. Amino acid sequences were analysed with Protein Prospector (http://prospector.ucsf.edu). These sequences were used for BLAST and FASTA homology searches. This experiment was repeated with endoprotease Asp N (Boehringer–Mannheim), staphylococcal (Staphylococcus aureus) V8 protease (Roche Applied Science, Basel, Switzerland) and with CNBr instead of trypsin. The conditions were identical except that the incubation temperature was 37°C and the solution of NaHCO₃ did not contain CaCl₂. Moreover, the incubation period was reduced to 1 h for digestion by V8 protease. Some of the peptides were separated by HPLC and the N-terminal amino acid sequence was determined by automated Edman degradation with the Procise 494A protein sequencing system (Applied Biosystems).

**Isolation and sequencing of the tr65 gene**

Total mRNA was extracted from rainbow trout skin (Mirwart strain from Pisciculture Expérimentale du Drennec, Sizun, France) with TRIzol® (Gibco BRL) according to the manufacturer’s instructions, and cDNA was synthesized using the Universal RiboClone cDNA synthesis system (Promega, Madison, WI, U.S.A.). The degenerated primers 5’-GCTGACCGCAGCTGACGATTCTATG-3’ and 5’-TGTCACAGCAGCTGACCAATAC-3’ designed from the corresponding peptide sequences were used for PCR amplification using cDNA template. The PCR products were then ligated directly into the pCR2.1 plasmid (Invitrogen, Carlsbad, CA, U.S.A.). *E. coli* One Shot Cells (Invitrogen) were transformed and grown overnight on an LB (Luria–Bertani) plate supplemented with ampicillin. Colonies containing the recombinant plasmid were grown overnight in LB–ampicillin broth and the plasmid DNA was isolated with a Qiagen plasmid purification kit and stored at −20°C. The cloned PCR products were verified by DNA sequencing (Cybergene, Evry, France).

**Bacterial strains and growth conditions**

Strains used for cloning and production of recombinant proteins were *E. coli* DH5α (Clontech Laboratories, Palo Alto, CA, U.S.A.) and *E. coli* BL21(DE3)omp8, a universal expression host lacking genes for the major *E. coli* Omp8 (outer membrane proteins) LamB (maltoporin precursor/lambda receptor protein), OmpA, OmpC and OmpF [24]. *E. coli* strains were maintained and grown in LB medium at 37°C. When required, the media were supplemented with 100 μg/ml ampicillin and/or 50 μg/ml kanamycin.

**Amplification and cloning of tr65**

The 1740-bp full-length tr65 gene, with appropriate sites at both ends, was synthesized by PCR amplification of genomic DNA with primer no. 245, 5’-ATTGATCTCATGAGCTGATTAC- AAGAGCAG-3’ and primer no. 246, 5’-TTAAGCTTTAGAAGCCTGCAATGAGGAGG-3’ (containing a BamHI site indicated in boldface and an initiation codon indicated by underlining). The PCR products were digested with BamHI and HindIII and ligated into the pETSIG vector prepared by previous digestion with the same enzymes, yielding pETSIG-tr65. The pETSIG vector is a pET28a (Novagen) derivative that enables production of the His-tagged protein of interest as an N-terminal fusion product with the signal peptide of LamB (maltoporin precursor/lambda receptor protein), a second BamHI cloning site, an additional 4 h at 37°C and the solution of NaHCO₃ did not contain CaCl₂. Moreover, the incubation period was reduced to 1 h for digestion by V8 protease. Some of the peptides were separated by HPLC and the N-terminal amino acid sequence was determined by automated Edman degradation with the Procise 494A protein sequencing system (Applied Biosystems).

**Production and purification of recombinant Tr65**

*E. coli* BL21(DE3)omp8 cells were transformed with pETSIG-tr65. The recombinant strains were selected on LB agar supplemented with ampicillin and kanamycin. Liquid cultures were incubated at 37°C with shaking until a DO₉₀ (attenuance at 600 nm) of 0.5 was reached. IPTG was added at a final concentration of 0.5 mM and the incubation was continued for an additional 4 h at 37°C with shaking. Cells were harvested by centrifugation for 10 min at 6000 g and resuspended in lysis buffer [50 mM Tris/HCl, pH 8, 300 mM NaCl, 10% (v/v) glycerol, 1 mM mercaptoethanol, antiprotease cocktail; Roche Applied Science]. Cells were then disrupted by sonication and the resulting suspension was centrifuged for 1 h at 55000 g at 4°C.

The membrane pellet was resuspended and incubated at room temperature for 2 h in a buffer [50 mM Tris/HCl, pH 8, 300 mM NaCl and 3% OPOE (n-octyl-polyoxyethylene)]. The final suspension was centrifuged at 55000 g for 1 h at 4°C. A second step of purification included resuspension of the pellet, which was then incubated for 2 h at room temperature in a buffer
(50 mM Tris/HCl, pH 8, 300 mM NaCl and 6 M urea). The final suspension was centrifuged at 55 000 g for 1 h at 4 °C. The supernatants were incubated with Ni-NTA (Ni2+-nitriotriacetate)–agarose suspension (Qiagen, Hilden, Germany). The protein–resin complex was packed into a column and washed extensively with a buffer consisting of 50 mM Tris/HCl (pH 8), 300 mM NaCl, 2 M urea and 15 mM imidazole. The proteins were eluted with a buffer consisting of 50 mM Tris/HCl (pH 8), 300 mM NaCl, 1 M urea and 300 mM imidazole. The eluted fractions were analysed by SDS/PAGE. Recombinant Tr65 with the His6 (hexahistidine) tag removed was produced by the same protocol but with the final step modified as follows. The protein–resin complex was treated overnight with protease (Amersham) to cleave the thrombin-recognition sequence of the linker fusing the tag to the recombinant Tr65. The protein was eluted with 50 mM Tris/HCl (pH 8), 300 mM NaCl and 1 M urea. Protein concentrations were determined by measuring the absorbance at 280 nm before the addition of the antiprotease cocktail (Roche Applied Science).

Conductance experiments

We used the Montal–Mueller technique [26] to form virtually solvent-free planar lipid bilayers for macroscopic and single-channel conductance experiments. The membranes were formed over a 100–150 μm hole in a Teflon film (10 μm thick) and pretreated with a 1:40 mixture (v/v) of hexadecane/hexane, separating two half cells. Lipid monolayers from a 5 mg/ml azolectin solution (soya-bean azolectin IV-S; Sigma) were spread on top of an electrolyte solution (1 M KCl and 10 mM Hepes, pH 7.4) in each of the two compartments. Bilayers were formed by lowering and raising the level of electrolyte in one side or both sides. A voltage was applied by an Ag/AgCl electrode on the cis-side. For the macroscopic conductance experiments, the doped membranes were subjected to slow voltage ramps (10 mV/s) and the transmembrane currents were amplified (BBA-01; Eastern Scientific, Rockville, MD, U.S.A.). The current–voltage curves were stored on a computer and analysed with Scope software (Bio-Logic, Claix, France). For single-channel recording, the potentials were applied and the currents amplified simultaneously with an amplifier (BLM 120; Bio-Logic). Single-channel currents were stored on a compact disc (DRA 200; Bio-Logic) for off-line analysis. Windac32 (http://www.shareit.com) and Biotools (Bio-Logic) software were used to analyse the compact disc data. All experiments were carried out at room temperature. Data were filtered at 1 kHz before digitizing at 11.2 kHz for analysis. In both cases, the proteins were incubated in 1% OPOE or in DDM (dodecyl maltoside) in different dilutions before incorporation into the membrane.

RESULTS

We have previously [21] isolated the antibacterial protein Tr65 from trout mucus and established a good correlation between its antimicrobial activity and pore-forming properties. Unfortunately, the amounts of recovered protein were too small for determining its structure. The present study aimed to determine the gene sequence of this protein, enabling the construction of a recombinant Tr65 that can be overproduced in E. coli.

Extraction, purification and characterization of the native Tr65 protein

We carried out a differential extraction from freeze-dried mucus by LiDS (see [21]). The protein Tr65 was isolated from the supernatant by preparative LiDS–PAGE followed by electrophoresis. We obtained only one band (Figure 1A), indicating high purity of Tr65. Nevertheless, we conducted two-dimensional electrophoresis (i) to monitor the integrity of the protein and (ii) to detect the presence of isomorphic proteins. We observed four spots with the same molecular mass on the two-dimensional gel (Figure 1B) at pH 3.3, 3.5, 5.1 and 5.2. These findings seem to indicate that Tr65 has various functional groups. As numerous proteins in the trout mucus are glycosylated, we performed experiments in order to determine the presence of carbohydrates in Tr65. The carbohydrate groups were specifically oxidized and then labelled with biotin before treatment with streptavidin–alkaline phosphatase. The coloration was made with NBT–BCIP. The purity control of the Tr65 protein is shown in Figure 1(C) (left panel), while Figure 1(C) (right panel) shows unambiguously the presence of carbohydrates (lane 2) and sialic acids (lane 3).

We conducted an antibacterial test and reconstitution experiments in planar lipid bilayers in order to determine the functionality of Tr65. We observed the same minimal inhibitory concentration as in our previous study (results not shown; see [21]). Although we could not determine unitary conductance for Tr65 in our preliminary study, we obtained here well-defined single channels in azolectin bilayers. The trace on Figure 2 shows a 515 pS level due to simultaneous insertions of Tr65 and fluctuations of 35 pS corresponding to unitary conductance values of Tr65 channels in 1 M NaCl. A similar behaviour was observed in 1 M KCl, but the conductance values were 40 pS.
Table 1 Sequencing of proteolytic fragments of native Tr65

<table>
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<tr>
<th>Method</th>
<th>Fragment sequence</th>
<th>Percentage similarity with Q90W76</th>
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<tr>
<td>Edman degradation</td>
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<td>91</td>
</tr>
<tr>
<td></td>
<td>MVKLADIEIAT</td>
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</tr>
<tr>
<td></td>
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<td>VILKVEEGAVMIN</td>
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<td></td>
<td>ALYDVKRIKDL</td>
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<td>MS</td>
<td>YEVEFVLLK</td>
<td>89</td>
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<td>VDSDELVNRIR</td>
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<td></td>
<td>DOTTSTRNL</td>
<td>100</td>
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</table>

Extraction, purification and characterization of recombinant Tr65

The amounts of Tr65 extracted from the mucus of trout were too low to conduct structural studies. Thus we attempted to produce a recombinant Tr65 in various strains of E. coli.

We used E. coli BL21(DE3)omp8 to produce His$_{6}$–Tr65. This strain is devoid of many porins [24] and is thus an ideal tool for the production and subsequent characterization of proteins with pore-forming activities, as contamination with endogenous porins is not possible. However, our attempt to produce His$_{6}$–Tr65 by transformation of E. coli BL21(DE3)omp8 with pET28a-tr65 was unsuccessful, probably due to toxicity of the eukaryotic Tr65 (results not shown). We also tried to produce GST (glutathione transferase)-tagged Tr65 with a pGEX(M) [27] vector. However, GST–Tr65 was only present in insoluble fractions (results not shown). Furthermore, high concentrations of urea are required to solubilize the target protein and thus glutathione beads cannot be used. We then tested another vector for significant expression. The pETSIG vector is derived from pET28a, as described previously [25]. This vector allows the His$_{6}$-tagged protein to be produced as an N-terminal fusion product with the signal peptide of E. coli OmpA porin, targeting the protein to the outer membrane. Introduction of this vector into E. coli BL21(DE3)omp8 led to production of a satisfactory amount of the fusion protein, although most recombinant Tr65 was found in inclusion bodies (results not shown). Some recombinant Tr65 was correctly targeted to the membrane fraction. We tried to extract this fraction using 3 % OPOE, a mild detergent compatible with later purification steps and conductance experiments. However, this extraction did not recover the recombinant protein; neither did Empigen, a detergent known to solubilize keratins [28]. We succeeded in recovering the fusion protein from membrane extract with 2 % (w/v) SDS. Unfortunately, SDS appears to be incompatible with further purification with Ni-NTA beads and with biophysical analysis of pore-forming properties. Thus we focused on the fusion protein accumulated in insoluble inclusion bodies and we finally chose the His$_{6}$–Tr65 construct including the E. coli OmpA signal peptide to produce recombinant Tr65 because Ni-NTA beads used for purification are compatible with high.

Determinations of the tr65 gene sequence

Sequencing of native Tr65 by Edman degradation was unsuccessful, probably due to N-terminal protection. We obtained proteolytic fragments of native Tr65 by enzymatic digestion with trypsin, V8 protease and endoproteinase Asp N and by chemical degradation with CNBr. These fragments were sequenced by Edman degradation and analysed by MS (Table 1). Similarity searches in data banks (Pattin Prot) identified a very high degree of similarity to a cytokeratin of rainbow trout (Swiss-Prot Protein Sequence database accession number Q90W76). Eleven of 18 proteolytic fragments were 100 % identical and six of 18 were approx. 90 % identical with the sequence of this keratin. We synthesized primers based on these sequences. We constructed a cDNA library from RNA extracted from trout skin. We used PCR at various hybridization temperatures with appropriate primers to generate several tr65 clones. Sequence analysis showed that only one matched all the sequences described in Table 1. The protein translated from this cloned gene is very similar to the keratin Q90W76 (Figure 3). However, residues from 105 to 140 of the keratin Q90W76 are absent from the Tr65 sequence. Keratin Q90W76 has a molecular mass of 62 344 Da for its 618 residues and Tr65 has a molecular mass of 59 574 Da for its 579 residues. The amino acid composition shows a strong proportion of serine and glycine residues (11.4 and 22.3 % respectively).
Concentrations of denaturant. We then extracted recombinant Tr65 from the inclusion bodies with 6 M urea. This fusion protein was precipitated after traditional dialysis to remove the denaturant. Therefore we used Ni-NTA resin to immobilize recombinant Tr65, while it was refolded by gradually decreasing the concentration of denaturant (final concentration 1 M). We then eluted recombinant Tr65 with a buffer containing 300 mM imidazole. We also produced recombinant Tr65 from which the His tag was removed by thrombin cleavage, while the refolded recombinant Tr65 was still bound to the Ni-NTA resin. The tag was cleaved successfully on overnight incubation at room temperature. SDS/PAGE analysis of both proteins (Figure 4) showed that we obtained His6–Tr65 and Tr65 with a high purity.

Pore-forming properties

We incorporated Tr65 (after dilution in 1% OPOE) into azolectin planar lipid bilayers formed by the Montal–Mueller technique [26]. Typically, after allowing the membrane to stabilize (30 min), we subjected the bilayers to repetitive triangular ramps (10 mV/s). Figure 5 shows current–voltage (I–V) curves recorded in 1 M KCl electrolyte solution according to progressive incorporation of Tr65 into the membrane. The various I–V curves show ohmic behaviour, indicating non-voltage dependence for Tr65, i.e. the formation of channels is independent of the applied voltage. This behaviour is similar to that of porins of the outer membrane of Gram-negative bacteria. These porins induce the opening of water-filled channels independent of voltage. We carried out single-channel experiments with purified His6–Tr65 and Tr65 inserted into planar lipid bilayers. Under an applied voltage, both these proteins induced well-defined fluctuations of current (Figure 6). We observed conductance values of 180±15 pS for Tr65 and 210±20 pS for His6–Tr65 in 1 M KCl. We occasionally observed a conductance value of 220 pS for Tr65. These findings suggest that the presence of the His tag does not significantly modify the behaviour of His6–Tr65. We tested also both proteins at increasing dilutions in OPOE or in DDM in order to improve

Figure 3 Alignment of Tr65 and Q90W76 sequences

Each boldface underlined letter corresponds to an amino acid residue substitution.

Figure 4 Coomassie Brilliant Blue-stained gel of purified recombinant His6–Tr65 and Tr65 produced in E. coli

Lane 1, Tr65; lane 2, His6–Tr65. Molecular-mass markers for 97, 66, 45, 30 and 20.1 kDa are shown.
we focused on the trout glycoprotein Tr65 to determine its primary role in fish defence. Previously, we showed that several glycoproteins isolated from mucus have pore-forming properties that correlated with hydrophobic regions.

It is well established that mucus, along with having other functions, contributes to the defence system of fish. Previously, we showed that several glycoproteins isolated from the mucus of trout have pore-forming properties that correlated with hydrophobic regions.

**DISCUSSION**

It is well established that mucus, along with having other important biological functions, contributes to the defence system of fish. Previously, we showed that several glycoproteins isolated from hydrophobic supernatants of epidermal mucus of freshwater fish have pore-forming properties that correlated with hydrophobic regions. In the present paper, we focused on the trout glycoprotein Tr65 to determine its primary role in fish defence. Previously, we showed that several glycoproteins isolated from mucus have pore-forming properties that correlated with hydrophobic regions.

We tested several plasmids to identify one that would adequately produce recombinant Tr65. Finally, we chose the pETSIG vector, derived from pET28a as described previously [25]. With this vector, the His6-tagged protein was produced mainly in inclusion bodies. We extracted the recombinant protein from the inclusion bodies with 6 M urea and immobilized the protein on a column of Ni-NTA resin. Gradually decreasing the concentration of denaturant allowed the recombinant protein to refold. We recovered His6-Tr65 by elution with imidazole and Tr65 after cleavage by thrombin. We carried out multichannel experiments with both these proteins and observed the same behaviour as that of native Tr65, i.e. ohmic $I$–$V$ curves indicating voltage-independent behaviour.

Single-channel experiments with both recombinant proteins gave similar values of conductance of approx. 200 pS in 1 M KCl. These results indicate that the presence of the His6 tag does not influence the pore-forming properties of His6-Tr65. However, these conductance values were greater than that for native Tr65 (40 pS). Additional refolding experiments did not display modifications of conductance value of Tr65 (180 pS). This difference suggests that the N-terminal sequencing of this protein was unsuccessful, as observed for other glycoproteins extracted from tench, carp, eel and turbot (Y. Bessin and G. Molle, unpublished work), suggesting that the N-terminal part of these proteins is protected. Proteolytic fragments from Tr65 were obtained by digesting the protein with various enzymes and chemical degradation reactions. We determined the sequences of these fragments by Edman degradation and by mass spectroscopy (Table 1). We found a very high degree of similarity between these internal sequences and the trout O. mykiss type II epidermal keratin E1 (Swiss-Prot accession number Q90W76). Major functions of this subfamily of keratins include epidermis architecture and regulation of epidermal cell development [29]. This type of structural protein has already been observed in the mucus of other teleost fish, including Atlantic salmon (Salmo salar) [30] and discus fish (Symphysodon spp.) [31]. Keratin protein content in parental mucus of discus fish (Symphysodon spp.) is higher than that in mucus from juveniles. This difference may be due to various factors, including the stressful condition of losing epidermal layers of mucus during larval feeding activities [27]. Type II homologues of epidermal keratin have been detected also in the external mucus of Pacific hagfish (Eptatretus stouti, class Myxini) [32], a primitive jawless vertebrate of ancient lineage that can produce voluminous quantities of mucus when stressed.

We synthesized various primers and constructed a cDNA library to determine the complete amino acid sequence of Tr65 by gene sequencing. We generated and sequenced clones of PCR products from reactions carried out at various hybridization temperatures. Only one matched with all sequences described in Table 1. The protein sequence translated from this gene confirms the very strong similarity previously observed with a trout keratin (subtype IIE) expressed in epidermal keratinocytes (Swiss-Prot accession number Q90W76), except for the absence of the area observed in the mucus of other teleost fish, including Atlantic salmon (Salmo salar) [30] and discus fish (Symphysodon spp.) [31]. Keratin protein content in parental mucus of discus fish (Symphysodon spp.) is higher than that in mucus from juveniles. This difference may be due to various factors, including the stressful condition of losing epidermal layers of mucus during larval feeding activities [27]. Type II homologues of epidermal keratin have been detected also in the external mucus of Pacific hagfish (Eptatretus stouti, class Myxini) [32], a primitive jawless vertebrate of ancient lineage that can produce voluminous quantities of mucus when stressed.

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may be explained by a slight structural difference due to refolding of the recombinant Tr65 and to the presence of post-translational modifications in the native Tr65. This later hypothesis was confirmed by the detection of carbohydrate groups in the native Tr65 (Figure 1C). Moreover, native Tr65 probably has various levels of phosphorylation, as we observed several spots at various pI values by two-dimensional electrophoresis. Finally, sequencing of native Tr65 by Edman degradation was unsuccessful due to N-terminal protection. All these post-translational modifications must also be important for folding of the native Tr65, as 6 M urea is not required to solubilize native Tr65, but is required to solubilize recombinant Tr65. These slight structural differences between the native and recombinant Tr65 would be responsible for the modification of pore-forming properties. Numerous studies showed that the dephosphorylation or deglycosylation of membrane proteins could lead to dramatic changes in their channel properties [33–36]. Nevertheless, the present study clearly shows for the first time that a protein from the keratin family has pore-forming properties in an artificial membrane.

Our findings suggest that type II epidermal keratins in the mucus of fish could contribute to host defence against water-borne micro-organisms. Indeed, Atlantic salmon have been shown to have a higher epidermal keratin content in the mucus after being exposed to sea lice (Lepeophtheirus salmonis) [30]. Additionally, cytokeratins (or fragments of cytokeratins) are consistently found at higher levels after infection of the mucosal surfaces of mammals [37,38]. For example, a 5.7 kDa peptide with similarity to the tail of human cytokeratin 7 has been found in the bladder mucosal surface of pig. This peptide, PiBP-5, is clearly involved in the defence mechanisms of the urinary bladder against bacterial infections [37]. PiBP-5 has a high content of glycine (20.0%) and serine (16.3%) residues, as does Tr65 (22.3% glycine and 11.4% serine). Substantial transcriptional changes for keratin genes have also been observed in the human airway epithelium in response to infection by Pseudomonas aeruginosa [38]. The human airway epithelium is continuously exposed to potentially harmful micro-organisms, as is the epidermal surface of fish, and plays a central role in innate immunity. The authors proposed that increased expression of keratin genes after exposure to Ps. aeruginosa plays a crucial role in maintaining the physical epithelial barrier. Although it now appears that teleost Iκ keratins diversified independently of mammalian Iκ keratins [39], it would be interesting to investigate whether mammalian cytokeratins have similar pore-forming and antibacterial properties. Perhaps the differential expression of keratin genes in human airway epithelium in response to Ps. aeruginosa is due to a direct contribution of cytokeratins to innate immunity.

### REFERENCES


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