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Enzymes responsible for the bactericidal effect in extracts of vitelline and fertilisation envelopes of rainbow trout eggs

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Summary

Extracts from both the vitelline envelope (VE) and fertilisation envelopes (FE) of rainbow trout eggs have the ability to exert a bactericidal effect on Gram-positive and -negative bacteria. The effect may be due to the presence of phospholipase D (PLD), lysozyme, proteinase and DNases, as the extracts contain these enzyme activities. The intensity of chorionic PLD and lysozyme activities in the VE extract was maintained in the FE without any alteration in activity even after transformation in the course of the cortical reaction, as components of a fundamental architecture of the envelope. Both extracts also contain different types of proteinase activities. Treatment with VE or FE extract seriously damaged the outer membrane of Gram-negative bacteria and the plasma membrane of Gram-positive and -negative bacteria at the ultrastructural level. Chorionic DNases probably degrade DNA of bacterial cells killed by virtue of the action of PLD and/or lysozyme and contribute to the transmigration of nucleosides and/or nucleotides produced by degrading bacterial DNA after degradation of bacterial components by the actions of the chorionic PLD, lysozyme and proteinase. These results suggest that the bactericidal process manifested by the VE or FE extract may start with the action of PLD and/or lysozyme against bacteria and be completed by subsequent degradation of constitutive proteins and DNA by the action of proteinases and DNases, respectively. Thus the VE and FE are able to protect the egg itself and the embryo, respectively, from bacterial infection in the internal or external environments.

Keywords: Bactericide, Chorionic enzyme, Chorionic role, Fish egg

Introduction

The vitelline and fertilisation envelopes of animal eggs are considered to physically protect the egg or the embryo itself as an extracellular matrix. After transformation of the vitelline envelope (VE) into the fertilisation envelope (FE), the latter becomes resistant to various drugs and enzymes except hatching enzymes. In addition, there is no doubt that the completed FE contributes to protection of the early embryo by maintaining the specific microenvironment of the perivitelline space. The VE or FE has also to protect the egg or the embryo from noxious drugs and bacterial infection. Indeed, since both envelopes have the ability to bind or trap bacterial toxins and antibiotics (Kudo & Yazawa, 1995, 1997), the egg or embryo would be protected by this ability from noxious substances originating in microbes. That the VE or FE manifests bactericidal and fungicidal activities is considered to be the most effective means for the protection of the egg or the embryo. Extracts from purified FEs, but not VEs, of fish eggs have been demonstrated to exert a bactericidal effect on either *Aeromonas hydrophila* or *Vibrio anguillarum*, never both, and a fungicidal or antifungal effect on the fungus *Saprolegnia parasitica* (Kudo & Inoue, 1986, 1989, 1991; Kudo *et al*., 1988; Kudo & Teshima, 1991; Kudo, 1992). An extraction method hitherto used on purified VEs or FEs enabled the verification of the presence of enzymes responsible for degrading carbohydrates and casein (Kudo & Teshima, 1991).

However, the enzymes responsible for the direct bactericidal or fungicidal action remain unknown, except for lysozyme in FE extracts. Therefore, experiments were undertaken to attempt to identify these

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enzymes using a new method of extraction from purified VEs or FEs. A first extraction was performed as described previously, and a second extraction was carried out using VE or FE residues after the first extraction. The second extraction resulted in the discovery of several enzymes responsible for the direct bactericidal action of both VE and FE extracts. These were PLD, lysozyme, proteinase and DNases. Thus, this paper addresses the bactericidal effect of VE or FE extracts on Gram-positive and -negative bacteria on the basis of the presence of the above-mentioned enzymes.

Materials and methods

Preparation of VE and FE extracts

Mature eggs of rainbow trout (*Oncorhynchus mykiss*) were obtained by courtesy of Gunma Prefectural Fisheries Experimental Station in Maebashi, Japan. VEs and FEs were purified from the mature eggs and artificially activated eggs, respectively, by repeatedly crushing gently and washing the VEs or FEs with 5 mM Tris-HCl buffer (pH 7.0–7.1) containing 0.15 M NaCl, 5 mM EDTA (Kanto Chemicals, Tokyo, Japan) and 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma Chemical Co., St Louis, MO) under ice-cold conditions. A first extraction from purified VEs or FEs was performed as described previously (Kudo & Inoue, 1986, 1989), and the residues were intermittently sonicated at 20 kHz and 200 W for 20 min with a 1-s interval timer under ice-cold conditions after suspension in 5 mM Tris-HCl buffer (pH 7.0–7.1). Electron microscopy revealed that the sonicated VEs or FEs lacked the outermost layer (Kudo, unpublished data). A second extraction was carried out overnight at 4 °C using the sonicated residues in the following extraction buffer: 50 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl, 1% Nonidet P-40 (Iwai Kagaku Co., Tokyo, Japan), 5 mM EDTA, 2 mM *N*-ethylmaleimide (*N*-EM) and 2 mM PMSF. The VE and FE extracts obtained by the second extraction were dialysed against 5 mM Tris-HCl buffer (pH 7.0–7.1) and lyophilised.

Assay of bactericidal action

Two methods were employed to determine bactericidal action: agar plate assay and electron microscopy. For the agar plate assay, an agar solution (1.5%) containing culture medium for each bacterial species was spread in Petri dishes, and 2 ml of a bacterial cell suspension (1×10^9/ml) was overlaid when the agar had solidified. The superfluous bacterial suspension was immediately removed after being spread on the agar, and 4 mm diameter wells were punched into the bacteria-overlaid agar layer and filled with 25 µl of lyophilised VE or FE extract solution (*c*. 300 µg per well). The preparations were then incubated for 20 h at 30 °C for *Aeromonas hydrophila* and *Vibrio anguillarum* and at 35 °C for *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* PAO and *Staphylococcus aureus*.

For electron microscopy, bacteria (1×10^9/ml) were incubated for 18 h at 30 °C or 35 °C in 20 mM Tris-HCl buffer, pH 7.4, containing VE or FE extract (*c*. 300 µg), 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. Controls were incubated for the same period without the extract. After incubation, Gram-negative bacteria were washed twice with 0.85% NaCl solution and incubated for 1 h in the saline solution containing horseradish peroxidase (HRP, 2 mg/ml; Sigma Chemical). The Gram-positive and -negative bacteria were twice washed with 0.1 M cacodylate buffer (pH 7.4) containing 3% sucrose and fixed for 2 h in 0.1 M cacodylate-buffered 2% paraformaldehyde–0.1% glutaraldehyde containing 3% sucrose. HRP associated with Gram-negative bacterial cells was visualised using the technique of Graham & Karnovsky (1966), after washing with 50 mM Tris-HCl buffer (pH 7.6). The Gram-positive and -negative bacteria were subjected to electron microscopical observation after osmication and dehydration.

Assay of phospholipase activity

Phospholipase activity was usually assayed according to the method of Imamura & Horiuti (1978). The reaction mixture consisted of 0.1 ml of 10 mM Tris-HCl (pH 7.0–7.2) containing 0.1% bovine serum albumin, 0.05 ml of 0.1 M $CaCl_2$, 0.1 ml of 10 mM L-α-phosphatidylcholine (Sigma Chemical) emulsion, 0.2 ml of distilled water and 50 µl (10–250 µg) of the VE or FE extract solution. The reaction was started by adding the extract, continued for 30 min at 25 °C and then stopped by adding 0.2 ml of 50 mM EDTA in 1 M Tris-HCl buffer (pH 7.0–7.2). The resulting mixture was used for choline assay (Hayashi & Kornberg, 1954), and the optical density was determined at 365 nm in a spectrophotometer.

Assay of lysozyme activity

The lytic activity of lysozyme was determined according to the technique of Hayashi *et al*. (1963). Ethylene glycol chitin (0.4 mg/ml; Sigma Chemical) was used as a substrate in 0.1 M phosphate buffer (pH 5.6) and 0.1 ml (*c*. 200 µg) of the VE or FE extract was added to 1 ml of the substrate. The reaction was recorded continuously for 10 min at 293.5 nm at 18 °C. For controls, the extract was added to 0.1 M phosphate buffer containing 0.5 or 1.0 mM of *N*-bromosuccinimide (Sigma Chemical), and the reaction mixture allowed to stand for 30 min at room temperature. To this reaction mix-

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ture, 1 ml of the substrate was added and the reaction continuously recorded under the conditions described above.

Assay of proteinase activity

A first extract from purified VEs or FEs was used to examine proteinase activity by zymography. To concentrate the activity, the first VE (or FE) extract (500 mg) was dissolved in 200 ml of 5 mM Tris-HCl buffer (pH 7.1), and subsequently fractionated at 0–30%, 30–50% and 50–70% saturation by adding solid ammonium sulphate on ice. The precipitates formed in 30–50% saturated ammonium sulphate were lyophilised after dialysis against the same buffer and used as a VE (or FE) extract. Zymography for proteinase assay was performed as described previously (Kudo & Teshima, 1998) but with a modification: about 5–10 min before addition of SDS sample buffer lacking 2-mercaptoethanol, one of the inhibitors was added to the samples at the concentration shown in Tables 2 and 3, except for the control samples.

Assay of DNase

DNase was assayed by immunoblot analysis and zymography, since the presence of DNase activity in VE and FE extracts had been confirmed using an agarose plate assay in a preliminary experiment.

For immunoblot analysis, the samples (*c.* 30–40 μg per lane) of VE or FE extract were electrophoresed using 7.5% or 11% slab gels and subsequently transferred onto PVDF membranes. Each PVDF membrane was processed for immunoblot analysis using a polyclonal antibody produced against a synthesised peptide with 15 amino acids of the N-terminus (nos. 6–20) of rat DNase I (Shimada *et al.*, 1998). The antibody was produced by immunising rabbits.

For DNase zymography, polyacrylamide resolving slab gels were prepared using 1% DNA from salmon testes (Sigma Chemical) as substrate according to the method of Huessen & Dowdle (1980). The samples (*c.* 30–50 μg per lane), not boiled, were electrophoresed at 4 °C, and the slab gels were shaken gently for 2 h in three changes of 25% 2-propanol in 0.01 M Tris-HCl buffer, pH 7.5, at room temperature to remove SDS. The slab gels were then transferred either to a bath containing 0.1 M acetate buffer, pH 4.7, containing 5 mM EDTA overnight at 37 °C after three washings (30 min each) with 0.01 M Tris-HCl, pH 7.5, and subsequently with 0.1 M acetate buffer, pH 4.7, containing 5 mM EDTA, or to a bath containing 0.1 M Tris-HCl, pH 7.5, 20 mM $MgCl_2$ and 2 mM $CaCl_2$ after three washings with 0.01 M Tris-HCl, pH 7.5, and incubated for 16–18 h at 37 °C. After washing with distilled water, the slab gels were stained with 1% ethidium bromide and DNA-degraded bands were visualised with the aid of ultraviolet illumination.

Results

Bactericidal action

The VE and FE extracts exerted a bactericidal action on the Gram-positive and -negative bacteria used (Table 1). In the agar plate assay, a halo was seen around the wells which was generally bactericidal close to the wells and bacteriostatic at the periphery (Fig. 1). The cell wall of Gram-negative bacteria was completely removed by treatment with the VE or FE extract, as revealed by electron microscopy: the lack of the HRP-labelled cell wall and the plasma membrane contrasted with the appearance in the control specimen (Fig. 2). Both extracts also exerted similar effects on Gram-positive bacteria. These were probably caused by the presence of PLD and lysozyme against Gram-negative

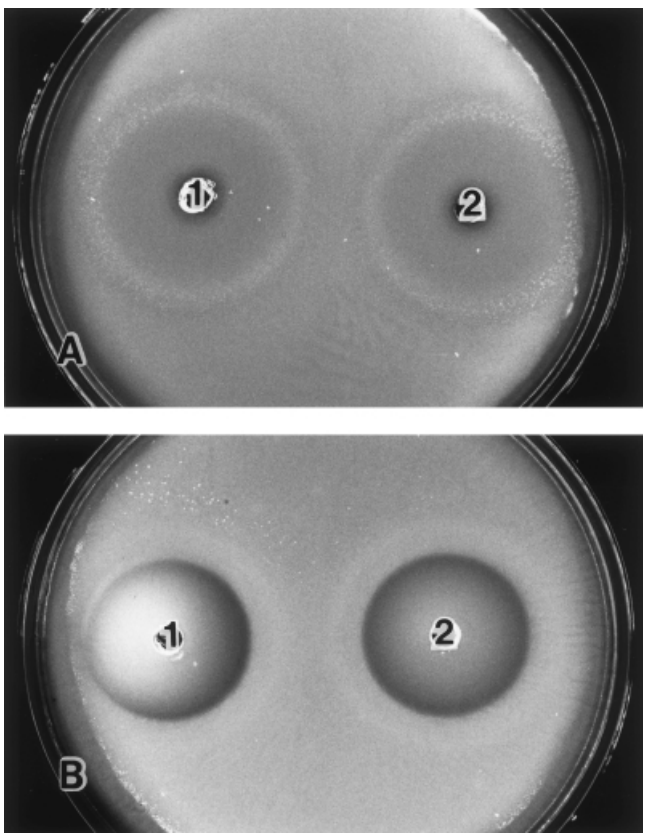

Figure 1 Agarose plate assay for bactericidal activity in vitelline envelope (VE) or fertilisation envelope (FE) extract. (*A*) Bactericidal effect of the VE (1) and FE (2) on *E. coli*. (*B*) Bactericidal effect of VE (1) and FE (2) on *Staphylococcus aureus*. Halo areas show absence of growth of bacterial colonies by virtue of the bactericidal action of the extracts, which were obtained by secondary extraction from the residues of the first extraction.

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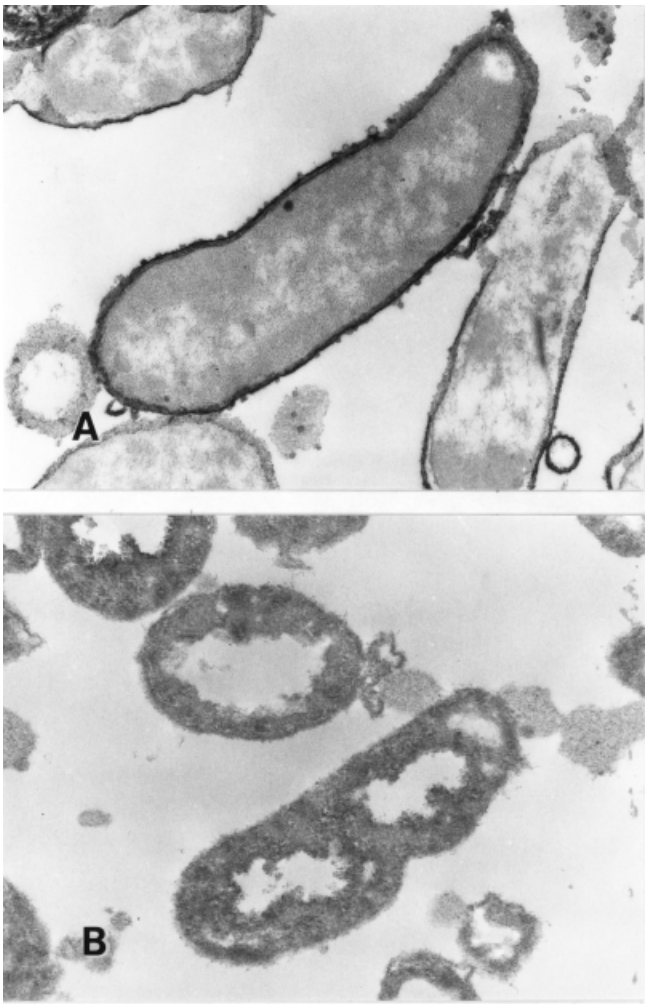

Figure 2 Bactericidal effect of VE extract on *Aeromonas hydrophila*. A control preparation shows the outer membrane decorated with horseradish peroxidase and the plasma membrane of the bacterial cells (*A*), as revealed by electron microscopy. Both membranes have disappeared after treatment with the extract, resulting in considerable swelling (*B*). ×22 800.

Table 1 Bactericidal action in vitelline envelope (VE) and fertilisation envelope (FE) extracts

	Extract	
Bacteria	VE	FE
Escherichia coli NIHJ	+++	+++
Pseudomonas aeruginosa PAO	+	+
Aeromonas hydrophila	+++	+++
Vibrio anguillarum	+++	+++
Staphylococcus aureus	+++	+++
Staphylococcus aureus (MRSA)	+++	+++

The bactericidal effect was examined by agar plate assay. Bactericidal action: +++, very strong; +, weak.

bacteria and lysozyme against Gram-positive bacteria, and other enzymes in the extracts, as described below.

Phospholipase D activity

An agarose plate assay using L-α-phosphatidylcholine as substrate suggested the presence of phospholipase activity in the envelope extracts. The phospholipase D activity in both envelope extracts was determined by the biochemical assay of released choline, suggesting the presence of the activity both before and after the cortical reaction, irrespective of VE–FE transformation (Fig. 3).

Lysozyme activity

Lysozyme activity, using ethylene glycol chitin as a substrate, was measured with positive results in both envelope extracts, suggesting little alteration in activity intensity in the process of VE–FE transformation (Fig. 4). The activity was completely inhibited in the presence of *N*-bromosuccinimide.

Proteinase activity

Zymography revealed that the VE extract showed seven proteinase-active bands with molecular masses of 180, 70, 45, 41, 36, 34–33 and 20 kDa, and the FE extract had seven active bands with molecular masses

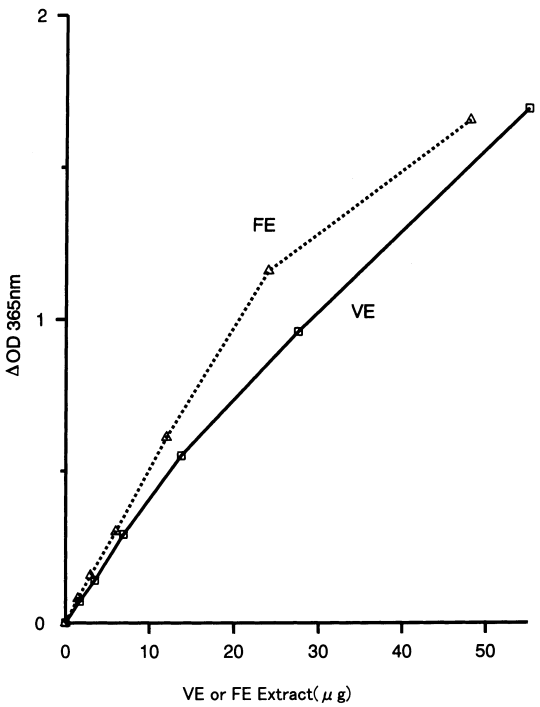

Figure 3 Phospholipase D activity in VE and FE extracts. The curves showing the activity are similar to each other, with little alteration in the process of VE–FE transformation.

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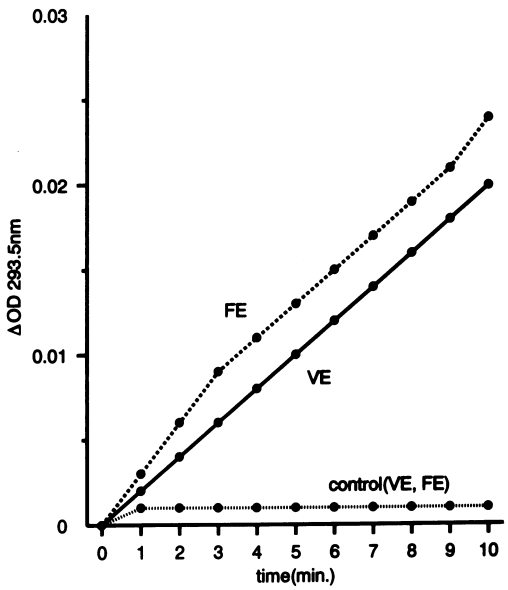

Figure 4 Lysozyme activity in VE and FE extracts. The activity is maintained in the process of VE–FE transformation with little alteration.

of 70, 50, 41, 36–32, 28, 25 and 20 kDa. In addition to the proteinase-active bands of similar molecular mass common to both extracts, other active bands existed in both extracts. More interestingly, subjecting both samples to EDTA, *p*-chloromercuribenzoic acid (*p*-CMB), *N*-EM, PMSF or diisopropyl fluorophosphate (DFP) revealed the occurrence of a new proteinase-active protein band with a molecular mass of 20 kDa (Tables 2, 3). In particular, DFP was the most effective in activating this 20 kDa protein in the VE and FE extracts (Fig. 5). Activation due to other inhibitors was considerably weaker.

In the VE extract, the proteinase activity of a protein with a molecular mass of 180 kDa was completely inhibited by PMSF and DFP, that of a 70 kDa protein was completely inhibited by DFP and L-cysteine and considerably by PMSF, that of a 45 kDa protein was inhibited considerably by DFP and completely by L-cysteine, and that of 41, 36 and 34–33 kDa proteins were inhibited considerably or completely by DFP. A 20 kDa protein, occurring as a band with proteinase activity, was activated by EDTA, *p*-CMB, *N*-EM, PMSF and DFP (Fig. 5, Table 2). In the FE extract, the proteinase activity of a 70 kDa protein was inhibited completely by EDTA and L-cysteine and considerably by DFP; that of a 50 kDa protein was inhibited completely by L-cysteine and its electrophoretic pattern was shifted to that of a 54 kDa protein by DFP; the activity of a 41 kDa protein was considerably inhibited by DFP and L-cysteine, as was that of a 36–32 kDa protein by DFP, *p*-CMB and L-cysteine; a 28 kDa protein was inhibited completely by L-cysteine and considerably by PMSF; and a 25 kDa protein was inhibited completely

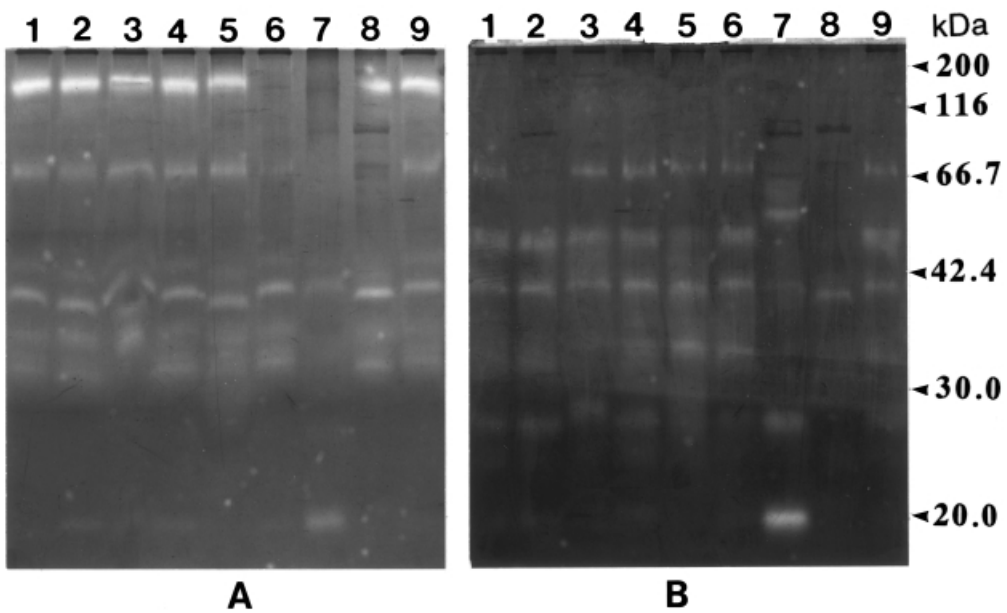

Figure 5 Proteinase activity in VE and FE extracts revealed by zymography. (*A*) Proteinase activity in VE extract is seen in six protein bands with molecular masses of 180, 70, 45, 41, 36 and 33 kDa before treatment with a proteinase inhibitor, but a further band (20 kDa) with proteinase activity is revealed after treatment with an inhibitor. Lanes 1 to 9 represent a control sample (1) of VE extract and samples treated with EDTA (2), *p*-CMB (3), *N*-EM (4), PHEN (5), PMSF (6), DFP (7), L-cysteine (8) and iodoacetamide (9). DFP was the most effective in activation of the proteinase (20 kDa protein band) among the inhibitors used. (*B*) Lanes 1 to 9 of the FE extract correspond to those of the VE extract. Treatment of the control sample with EDTA, *p*-CMB, *N*-EM, PMSF or DFP revealed the occurrence of a new protein band (20 kDa) with proteinase activity. DFP was again the most effective in activation.

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by PMSF, DFP and L-cysteine and considerably by *p*-CMB and 1,10-phenanthroline (PHEN). Treatment of the FE extract with EDTA, *p*-CMB, *N*-EM, PMSF or DFP revealed the occurrence of a 20 kDa protein with proteinase activity. Among them, DFP was the most effective. Furthermore, treatment of the FE extract with DFP seemed to cause a shift of the molecular masses of 70 and 50 kDa proteins to 64–44 and 54 kDa, respectively.

DNase activity

The VE and FE extracts degraded DNA from salmon testes, as revealed by zymography. The extent of DNA degradation was determined using buffers at different pH values for the incubation of slab gels after SDS-PAGE. DNA degradation was intense in the bands with molecular masses of 340–220 and 105–70 kDa in the VE extract and 340–220, 125 and 105–90 kDa in the FE extract at pH 4.7, and in those with molecular masses of 340–260, 94, 70 and the front of the lane (< 40 kDa) in the VE extract and 340–220, 125 and < 40 kDa in the FE extract at pH 7.5 (Fig. 6). The molecular masses of these DNase-active bands were not necessarily the same as those obtained by immunoblot analysis. This may have been due to non-reduction of the samples. Immunoblot analysis with the antibody produced against a synthesised rat DNase I peptide of the N-terminus region revealed reactive bands with molecular masses of 340–240, 200, 94, 46 and < 40 kDa and other minor bands in the VE extract, and 340–240, 125, 105, 50, 48 and < 40 kDa and other minor bands in the FE extract (Fig. 7). The 340–240 kDa protein band in the FE extract was very weak in reactivity. Reactive bands in the VE and FE extracts were not necessarily identical, but the reason for this is unclear. The electrophoretic patterns in the zymograms were not necessarily the same as those obtained by immunoblot analysis, since the envelope extracts were not reduced. Immunoblot analysis with the anti-DNase I peptide antibody revealed that some of the immunoreactive bands were identical to those immunoblotted with an anti-heparan sulphate monoclonal antibody which reacted with heparan sulphate alone, but not with heparin, hyaluronic acid or chondroitin sulphates, suggesting that the chorionic DNases are heparan sulphate proteoglycans. This problem will be described in detail in another paper.

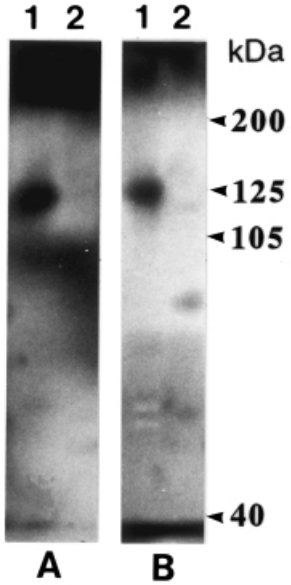

Figure 6 Zymography of DNA degradation by VE and FE extracts. (*A*) DNA degradation activity of the VE extract is seen in the bands with molecular masses of 340–220 and 105–70 kDa (lane 2), and 340–220, 125 and 105–90 kDa (lane 1) in the FE extract at pH 4.7. (*B*) At pH 7.5, DNA degradation due to the VE extract is seen in the bands with molecular masses of 340–260, 94, 70 and < 40 kDa (the front of the lane) (lane 2), and that due to the FE extract in the bands with molecular masses of 340–220, 125 and < 40 kDa (lane 1).

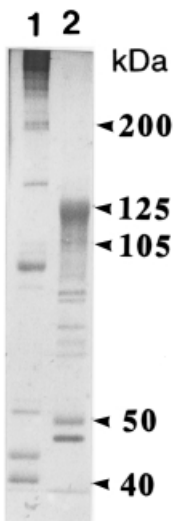

Figure 7 Immunoblot analysis of VE and FE extracts using anti-DNase I peptide antibody. Reactive bands in both VE (1) and FE (2) extracts are not necessarily identical to each other. The cause and significance of this remain unclear.

Discussion

The results indicate that the extracts from purified VEs and FEs of rainbow trout eggs possess phospholipase D, lysozyme, proteinase and DNase activity and exert a bactericidal action on all species of Gram-positive and -negative bacteria examined, involving complete degradation of the cell wall and plasma membrane of the bacteria. The degradation may have progressed from the cell wall to the plasma membrane by virtue of the action of phospholipase D followed by the destruc-

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Table 2 Effects of inhibitors on proteinase activity in a vitelline envelope extract of rainbow trout eggs

kDa	Control sample	1 mM EDTA	1 mM *p*-CMB	2 mM *N*-EM	1 mM PHEN	2 mM PMSF	10 μM DFP	1 mM L-Cys	1 mM IAA
180	+	+	+	+	+	−	−	+	+
70	+	+	+	+	+	±	−	−	+
45	+	+	+	+	+	+	±	−	+
41	+	+	+	+	+	+	±	+	+
36	+	+	+	+	+	+	−	+	+
34–33	+	+	+	+	+	+	−	+	+
20	*	+	+	+	*	+	+	*	*

+, positive proteinase activity; –, complete inhibition of the activity; ±, considerable inhibition; *, absence of any protein band with proteinase activity.
L-cys, L-cysteine; IAA, iodoacetamide.
The 20 kDa protein band with proteinase activity was seen only after treatment with EDTA, *p*-CMB, *N*-EM, PMSF or DFP. The activity was very weak, except after treatment with DFP. Treatment of the protein band with a molecular mass of 41 kDa with EDTA appears to have altered its molecular mass to 40 kDa, and treatment with PMSF, DFP or IAA appears to have changed it to 42 kDa. The 34–33 kDa protein band appears to be a doublet. See also Fig. 5.

Table 3 Effects of inhibitors on proteinase activity in a fertilisation envelope extract of rainbow trout eggs

kDa	Control sample	1 mM EDTA	1 mM *p*-CMB	2 mM *N*-EM	1 mM PHEN	2 mM PMSF	10 μM DFP	1 mM L-Cys	1 mM IAA
70	+	−	+	+	+	+	±	−	+
50	+	+	+	+	+	+	−	−	+
41	+	+	+	+	+	+	±	±	+
36–32	+	+	±	+	+	+	±	±	+
28	+	+	+	+	+	±	+	−	+
25	+	+	±	+	±	−	−	−	+
20	*	+	+	+	*	*	+	*	*

Symbols are as in Table 2.
Treatment of the 70 and 50 kDa proteins with DFP appears to have caused a shift molecular mass to 64–44 and 54 kDa, respectively. See also Fig. 5. Treatment with *p*-CMB appears to have also caused a shift from 36–32 kDa to 37–34 kDa, and from 28 kDa to 29 kDa.

tion of the peptidoglycan layer by lysozyme action in Gram-negative bacteria, and from the peptidoglycan layer to the plasma membrane in Gram-positive bacteria as the result of the action of lysozyme and, subsequently, PLD. Presumably, proteinase and DNase activities participated in the final degradation of bacterial components such as proteins and DNA after heavy damage resulting from the action of PLD and/or lysozyme. These chorionic enzymes may contribute to the transmigration of substances in the natural world by degrading dead bacterial cells as the results of bactericidal action in the VE or FE, in addition to protecting the egg or the embryo itself from bacterial infection.

Little alteration was seen in PLD and lysozyme activities in the process of VE–FE transformation. This indicates that these enzymes are continuously maintained in the FE, as components of the fundamental architecture of the envelope. Therefore, it is possible that the enzymes in the VE extract act as a defence mechanism in the transformed FE against different bacteria in the external environment. It remains unknown whether these enzymes are able to function to the same extent in both envelopes without being affected by changes in the internal or external environments.

Proteinase activities in the VE extract were not necessarily identical to those in the FE extract in molecular mass or in the response to the inhibitors used. However, this would not be caused by contamination of cytoplasmic proteinases, since the purified VEs or FEs showed no cytoplasmic contamination, as rigorously checked by electron microscopy, and a final buffer obtained by washing the purified envelopes showed no proteinase activity after lyophilisation, as examined by agarose plate assay. The 180 kDa protein

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in the VE extract is a serine proteinase, but proteinase activity corresponding to this is absent from the FE extract. The activity of the 70 kDa protein in the FE is inhibited by EDTA, but not that in the VE extract, although it is not clear whether the proteinase activity changed in nature during VE–FE transformation. The activity of the 25 kDa protein band in the FE extract was inhibited by *p*-CMB, PHEN, PMSF, DFP and L-cysteine. However, it is unknown whether the protein possesses two active domains of thiol and serine proteinases, and whether proteinase-active bands in the FE extract, lacking in the VE extract, were due to the participation of materials originating in the cortical alveoli as constituents of the FE. Although it is at least certain that the envelope extracts possess different types of proteinase activities, it is not clear whether these proteinases are only necessary for degradation of the proteins of dead bacterial cells by virtue of the action of an enzyme system in the envelopes or whether they enhance the activity of a hatching enzyme, or both. These problems will be investigated further.

Using a previous method of extraction from purified VEs and FEs, FE extracts alone from several fish species exerted a bactericidal action on either *Aeromonas hydrophila* or *Vibrio anguillarum*, never both, and also *E. coli*, and a fungicidal or antifungal action on *Saprolegnia parasitica*. However, VE extracts did not exert any such effect (Kudo & Inoue, 1986, 1989, 1991). These extracts consisted mainly of the outermost layer components of the envelopes. The outermost layer of the FE is formed by the deposition of cortical alveolus exudates immediately after or simultaneously with removal of the VE's outermost layer. Therefore, the bactericidal action of the previous extracts was considered to be caused mainly by the components of the outermost layer originating in cortical alveoli, although lysozyme activity in previous FE extracts may have originated in the FE matrix, as inferred by the presence of the enzyme in the present VE and FE extracts. Since transmission electron microscopy revealed that the VE or FE outermost layer was almost completely removed by the method of extraction and by subsequent ultrasonication, both the present extracts contained almost none of the outermost layer components. Therefore, the extracts used may be major matrix components of the envelope architecture. Indeed, the present lyophilised extracts were rather sticky in nature and not fluffy, thus differing from those obtained previously.

When we used 2-*N*-(hexadecanoyl)-amino-4-nitrophenyl-phosphorylcholine (Sigma Chemical) as a substrate, we were unable to demonstrate sphingomyelinase activity in the VE or FE extracts. Furthermore, agarose plate assay for phospholipase activity showed that the formation of a halo due to degradation of the substrate was not inhibited by the inhibitors of phospholipase A_2, *p*-bromophenacyl bromide and quinacrine (data not shown). These observations provide further support for the suggestion that the phospholipase activity in both extracts may be due to PLD, in addition to evidence of choline release.

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