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Assembly *in vitro* of vitelline envelope components induced by a cortical alveolus sialoglycoprotein of eggs of the fish *Tribolodon hakonensis*

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Summary

Assembly *in vitro* of vitelline envelope (VE) components, which were precipitated by 50–70% saturated ammonium sulphate from VE extracts, was induced by the action of a sialoglycoprotein that is immunohistochemically localised in cortical alveoli of fish eggs and has serine proteinase activity. The VE components consisted of major bands of molecular mass about 150–120, 110–100, 70 and 27 kDa in addition to about 20 minor bands and contained a chorionic transglutaminase, visualised as two fluorescent bands by monodansylcadaverine staining. The VE component assembly *in vitro* was Ca^{2+}-dependent, not induced if the sialoglycoprotein was pretreated with a serine proteinase inhibitor, and inhibited by the presence of *p*-chloromercuribenzoate, iodoacetamide or l-cysteine in the reaction medium system. Electron microscopy revealed that assembly *in vitro* of the VE components consisted of aggregates of network sheets, consisting of branching and anastomosing thin (approximately 27–52 nm) and thick (approximately 137–376 nm) filamentous substances. Separation by SDS-PAGE showed that a considerable number of VE components participated in the assembly *in vitro* in various amounts. These results suggest at least partial reproduction of the phenomena that occur in the process of fertilisation envelope (FE) formation, and provide a new approach to investigation of the process of FE assembly *in vitro*.

Keywords: Assembly *in vitro*, Cortical alveolus sialoglycoprotein, Fish egg, Serine proteinase, Vitelline envelope components

Introduction

The vitelline envelope (VE) in fish eggs is relatively thick, consisting of three to five layers that are ultrastructurally distinguishable from each other, and differ in different fish species (Kudo, 1976, 1982; Kudo & Inoue, 1986; Schmehl & Graham, 1987; Kudo *et al.*, 1988). The VE is transformed into the fertilisation envelope (FE) by the action of cortical alveolus exudates released into the perivitelline space from the egg following normal fertilisation or artificial activation. The process of VE–FE transformation is a sequence of events that biochemically involve catalytic conversion of the VE to a highly cross-linked and insoluble FE, and replacement of the VE outermost layer with cortical alveolus exudates. The latter event has been demonstrated by electron microscopy and cytochemical and immunohistochemical techniques (Kudo & Inoue, 1986, 1988; Kudo *et al.*, 1988; Kudo & Yazawa, 1995). The functional significance of the replacement has also been clarified by the demonstration of bactericidal and fungicidal activities of FE extracts which mainly consisted of the FE outermost layer components; VE extracts had no such activity (Kudo & Inoue, 1986, 1988, 1989, 1991; Kudo, 1991, 1992; Kudo & Teshima, 1991). Furthermore, the full-grown, complete FEs have increased chemical and mechanical resistance, including resistance to enzymatic degradation. These events in FE formation contribute to the protection of the embryo mechanically and functionally.

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In sea urchin eggs it has been shown that FE hardening involves transglutaminase (TGase) activity in the early stages of FE assembly before the cross-linking of dityrosine residues occurs by catalysation of ovoperoxidase (Battaglia & Shapiro, 1986). Such a TGase-mediated FE hardening has also been reported in fish eggs (Oppen-Berntsen *et al.*, 1990; Iuchi *et al.*, 1991). Indeed, a TGase has recently been shown to localise in the VEs of rainbow trout eggs (Ha *et al.*, 1995). The analogy between FE hardening and blood coagulation has made it possible to presume that thrombin or a thrombin-like enzyme may take part in the process of cortical reaction by activating a native chorionic TGase. If this presumption is indeed the case, it would be possible partially to reproduce *in vitro* the phenomena that may occur during the process of FE formation, using extracts from purified VEs and thrombin or a thrombin-like enzyme. Therefore, extracts containing a chorionic TGase are essential for successful *in vitro* experiments, and it is also very important to identify an enzyme that substitutes for thrombin.

Thus the main objectives of the present study were to reproduce FE assembly *in vitro*, which normally occurs in the matrix of elevating FEs, to clarify the histochemical localisation of a chorionic TGase and the presence of the enzyme in VE extracts on slab gels and to identify an enzyme that may substitute for thrombin. Here it is reported that a sialoglycoprotein, which is immunolocalised in cortical alveoli of fish eggs, contains serine proteinase activity and may substitute for thrombin. It has the ability to assemble VE components containing chorionic TGase activity, implying a partial reproduction of FE assembly *in vitro*.

Materials and methods

Extraction of VE components from purified VEs

Mature eggs of the fish *Tribolodon hakonensis* were obtained by courtesy of the Gunma Prefectural Fisheries Experimental Station. The VEs were purified by repeatedly crushing gently in, and washing with, 5 mM Tris-HCl buffer (pH 7.0–7.1) containing 0.15 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA; Wako Pure Chemicals, Japan) and 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma) under ice-cold conditions. The purified VEs were extracted overnight at 4 °C with buffer containing 8 mM 2-mercaptoethanol (Kanto Chemicals, Japan), 2 mM EDTA, 0.3 M lactose, 0.3 M glucose and 0.15 M NaCl. After gauze filtration, the solution was dialysed against 5 mM Tris-HCl buffer (pH 7.0–7.1) and then lyophilised. The lyophilised sample (490 mg) was dissolved in 200 ml of 5 mM Tris-HCl buffer (pH 7.0–7.1), and subsequently fractionationed at 0–30%, 30–50% and 50–70% saturation by adding solid ammonium sulphate on ice. The precipitate formed in 50–70% saturated ammonium sulphate occurred mainly as a floating cake after centrifugation at 12 000 rpm for 1 h. A mixture of a small amount of sediment and the floating cake was dissolved in 5 mM Tris-HCl buffer (pH 7.1) and dialysed against the same buffer, and the sample was lyophilised after dialysis and used as VE components in the present experiments.

Immunohistochemical localisation of the sialoglycoprotein

An antibody was produced against a sialoglycoprotein purified from eggs of the fish *Tribolodon hakonensis* by immunising rabbits. The sialoglycoprotein was a generous gift of Dr S. Inoue (School of Pharmaceutical Sciences, Showa University, Hatanodai, Tokyo 142, Japan). For partial purification of the anti-sialoglycoprotein antibody, the immunoglobulin was precipitated from the antiserum with 50% ammonium sulphate, and the precipitate suspended and dialysed against phosphate-buffered saline (PBS). After removal of any precipitate by centrifugation at 10 000 *g* for 20 min at 4 °C, the supernatant was used for immunostaining. Immunostaining with the antibody was performed using mature eggs that had been fixed for 1.5 h with 0.1 M cacodylate-buffered 0.1% glutaraldehyde–4% paraformaldehyde containing 3% sucrose. The mature eggs had been embedded at –20 °C in JP-4 resin (Polysciences, USA) after washing with buffer containing 5% sucrose and subsequent dehydration. Sections were cut at a thickness of 1–2 µm using glass knives and mounted on glass slides, and then immersed for 30 min in methanol containing 0.3% H_2O_2 after rinsing in absolute ethanol to inhibit endogenous peroxidase activity, followed by rehydration in decreasing concentrations of ethanol and final immersion in PBS. The sections thus prepared were immunostained using an indirect enzyme-labelled antibody method as described previously (Kudo, 1989). The antibody was used at a dilution of 1 : 50 or 1 : 100 in PBS containing 1% bovine serum albumin (BSA). Control reactions included (1) initial incubation with preimmune rabbit serum instead of antiserum, and (2) incubation with antiserum that had been absorbed by the antigen.

Proteinase activity of the sialoglycoprotein

To determine whether the sialoglycoprotein contained a proteinase activity, two assay methods were used: agarose plate assay and zymography. The plate assay was carried out in 1.5% agarose using casein (1 mg / ml) as substrate in 20 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. When the agarose (18 ml / Petri dish) had solidified, five 4 mm diameter wells were punched in the agarose

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plate and filled with 25 µl of solution containing 100 µg of sialoglycoprotein and one of the following proteinase inhibitors, except for the central well of each group of five. The inhibitors used were 100 µM diisopropyl fluorophosphate (DFP), 1 mM PMSF, 2 mM EDTA, 1 mM 1, 10-phenanthroline (PHEN; Sigma), 2 mM *N*-ethylmaleimide (N-EM; Nakari Chemicals, Japan), 0.2 mM *p*-chloromercuribenzoate (p-CMB; Sigma), 2 mM iodoacetamide (IAA; Wako Pure Chemicals, Japan), and 1 mM l-cysteine. A mixture of the inhibitor and the sialoglycoprotein was preincubated for 1 h at 37 °C before pouring it into each well. The Petri dishes thus prepared were incubated for 18 h or 20 h at 37 °C. After incubation, lysis haloes were photographed after fixation with 10% acetic acid. A few Petri dishes were stained for 30 min with Coomassie Brilliant Blue R-250, followed by several destaining washes with a solution containing methanol and acetic acid to visualise the lysis haloes more clearly.

For zymography, polyacrylamide resolving slab gels were prepared using 1% casein as substrate according to the method of Huessen & Dowdle (1980). The sample (approximately 50 µg/lane), not boiled, was electrophoresed at 4 °C, and the slab gels were shaken gently for 1 h in two changes of 2.5% Triton X-100 in distilled water at room temperature to remove SDS. The slab gels were then transferred to a bath containing 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$, and incubated for 15 h at 37 °C. After staining with 0.25% Coomassie Brilliant Blue R-250 dissolved in a solution containing methanol and acetic acid, the gels were destained in the same solution without the dye.

PAS reaction of the sialoglycoprotein

Polyacrylamide resolving slab gels were prepared as described above without casein. The sample (approximately 50 µg), not boiled, was electrophoresed at room temperature. After electrophoresis, the slab gels were either stained weakly with 0.25% Coomassie Brilliant Blue, or left unstained. The gels were subsequently subjected to the periodic acid Schiff reaction according to the method of Zacharius *et al.* (1969).

Chorionic TGase histochemistry

VEs and FEs were purified by the same method as that used for extraction. The purified VEs and FEs were cryosectioned and fixed to silane-coated glass slides without any aldehyde fixation. The FE sections were coated with 1% gelatin solution to prevent their separation from the slides before washing with 50 mM Tris acetate buffer (pH 7.5); without the use of gelatin, the majority of them were washed off. After washing with the buffer, the sections were incubated at room temperature (25 °C) in darkness for 3–6 h in the following medium (Curtis & Lorand, 1976): 50 mM Tris acetate buffer (pH 7.5) containing 2 mM monodansylcadaverine (Sigma), 5 mM $CaCl_2$, 5 mM dithiothreitol (DTT; Wako Pure Chemicals, Japan), 1% *N*,*N*-dimethylcasein (Sigma) and bovine thrombin (2.5 units; Sigma). This medium containing no bovine thrombin was also used to detect the presence of chorionic TGase (see below). The incubated preparations were twice washed with 50 mM Tris acetate buffer, and then fixed for 20 min in 10% trichloroacetic acid solution at room temperature, followed by three washes with the same buffer. The sections thus prepared were embedded in 5% 1,4-diazobicyclo(2.2.2)octane (Kanto Chemicals, Japan)–50% glycerol in PBS (pH 8.6), and observed using a fluorescence microscope. Control preparations were pretreated for 1 h with 50 mM Tris acetate buffer (pH 7.5) containing 0.5 mM *p*-aminophenylmercuric acetate (p-APMA; Sigma)–10 mM $CaCl_2$, or 2 mM l-cysteine–1mM $CaCl_2$, or 2 mM IAA, or 0.1 mM p-CMB, or 2 mM N-EM, or 100 µM DFP, and then incubated in the same medium as described above.

Chorionic TGase on PAGE gels

Aliquots (*c.* 50 µg) of VE components, precipitated in 50–70% saturated ammonium sulphate, were subjected to PAGE using 4/20% gradient gel plates and a 0.025 M Tris–0.192 M glycine buffer (pH 8.5). To visualise enzyme activity, the gels were immersed immediately after electrophoresis for 4–6 h at 25 °C in the medium for revealing chorionic TGase described above according to the method of Curtis & Lorand (1976). The gels were then fixed in 10% trichloroacetic acid to remove free monodansylcadaverine. The gels were immersed in 50 mM Tris acetate buffer (pH 7.5) to visualise the casein-bound amine, and then photographed under UV light.

VE component assembly *in vitro*

The sample (1 mg/ml) was dissolved in 50 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl and 20 mM $CaCl_2$. A small amount of insoluble material was removed by centrifugation for 15 min at 3000 rpm. An aliquot (20–40 µg/ml) of the sialoglycoprotein described above was added to the supernatant in a test tube, and then mixed by vortexing. Immediately after vortexing, a fibrous substance appeared in the test tube. This substance was removed with the aid of a pipette and placed on formvar- and carbon-coated grids. Within 15–30 s an equal amount of uranium acetate solution was dropped on the sample. The uranium solution was a twofold dilution of a saturated solution. Excess solution was removed using filter paper, and the sample dried in an oven at 37 °C and observed in an electron microscope. Controls were prepared using Ca^{2+}-free medium, or medium containing p-CMB, IAA, l-cysteine or DFP.

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Results

Histochemical localisation and slab gel electrophoresis of chorionic TGase

Chorionic TGase activity was histochemically demonstrated in the VEs with an intense fluorescence, but little or no fluorescence was seen in the FEs (Fig. 1). The inhibitory effect of the proteinase inhibitors on the chorionic TGase may be divided into three classes on the basis of the intensity of fluorescence. l-Cysteine, p-CMB and DFP showed the strongest inhibitory effect, IAA and p-APMA exerted considerably less strong inhibition, and DTT, N-EM, PHEN, EDTA and

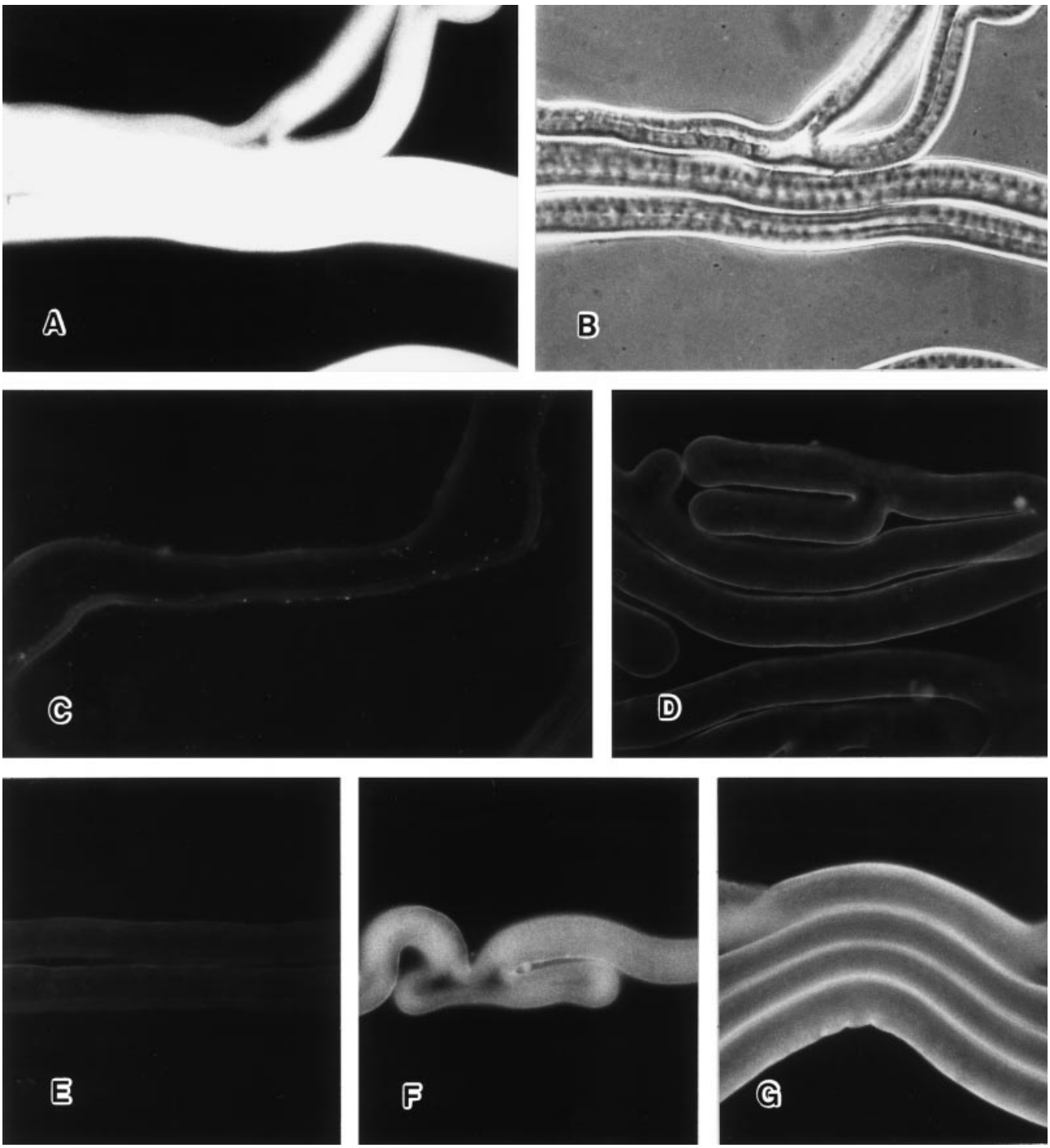

Figure 1 Fluorescence histochemistry of chorionic transglutaminase (TGase) in vitelline envelopes (VEs) and fertilisation envelopes (FEs). (*A*) The VEs are fluorescing strongly. (*B*) A phase-contrast photomicrograph of (*A*). (*C*) The FEs show little fluorescence. (*D*), (*E*), (*F*) and (*G*) show the fluorescence of VEs pretreated with l-cysteine, DFP, IAA and p-APMA, respectively. These control preparations show considerably reduced fluorescence as a result of the pretreatment. (*A*)–(*G*) ×440.

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Table 1 The effect of the inhibitors on proteinase activity on the cortical alveolus sialoglycoprotein

Inhibitors:	5 μM DFP	2 mM PMSF	1 mM EDTA	1 mM PHEN	2 mM N-EM	1 mM p-CMB	1mM IAA	1mM l-cysteine
Inhibitory effect:	+	+	+	–	–	–	–	–

+, inhibitory; -, non-inhibitory. The inhibitory effect was evaluated by agarose plate assay. The sialoglycoprotein may be Ca^{2+}-dependent, and a member of the serine proteinases.

Figure 2 The chorionic TGase after PAGE slab gel. Two fluorescent bands are seen, but their molecular masses were not determined.

PMSF had little or no inhibitory effect (Fig. 1). No fluorescence was seen after treatment with the first group, except for slight fluorescence at the outer margin of the VE which was much weaker than that of control preparations. The inhibitory effect of IAA resulted in a striking decrease in fluorescence throughout the whole of the VE section, and after p-APMA treatment only weak fluorescence was seen at the outer margin of the VEs. With the other inhibitors used, the intensity of fluorescence was almost the same as that of control preparations. Electrophoresis of the chorionic TGase on PAGE slab gels revealed two fluorescent bands (Fig. 2).

Immunohistochemical localisation and proteinase activity of the sialoglycoprotein

The sialoglycoprotein used was demonstrated immunohistochemically only in cortical alveoli, not in the VE, yolk granules and in the control preparations (Fig. 3). The sialoglycoprotein was almost unstained by Coomassie Brilliant Blue R-250, and was subsequently revealed by the PAS reaction as a brand band after SDS-PAGE (Fig. 4). The major portion of the band had a molecular mass of 230–170 kDa, extending to approximately 66 kDa like the tail of a comet, probably due to carbohydrate compositions. Zymography revealed that the sialoglycoprotein had proteinase activity (Fig. 4). The agarose plate assay for the proteinase indicated that the activity was inhibited by DFP, PMSF and EDTA, but not by PHEN, N-EM, p-CMB, IAA or l-cysteine, implying that the enzyme was a member of serine proteinases (Table 1).

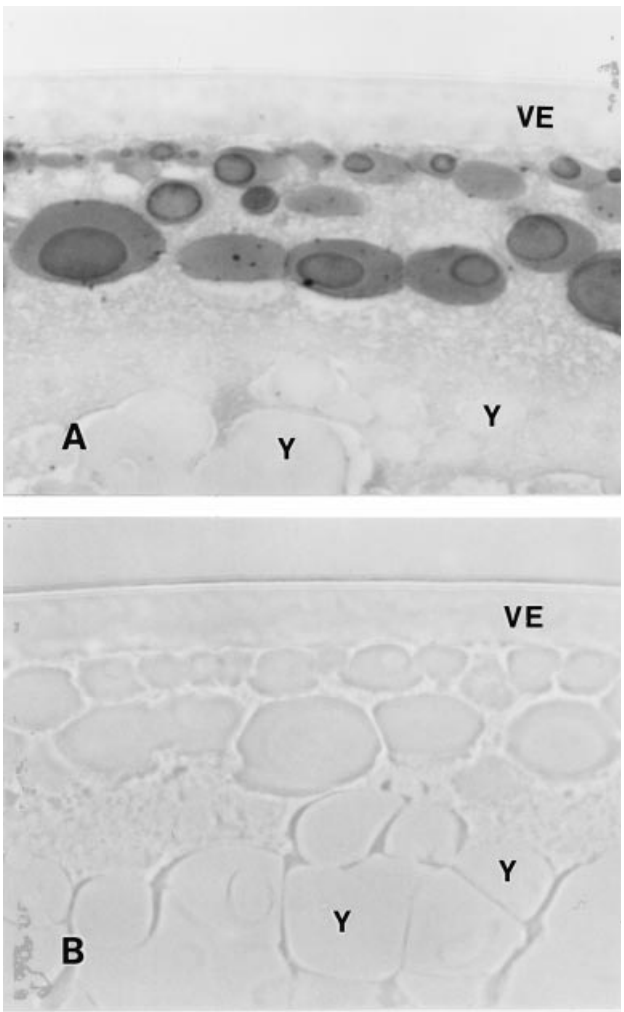

Figure 3 Immunohistochemical localisation of the sialoglycoprotein. (*A*) Cortical alveoli are immunoreactive, but the VE and yolk granules (Y) are not. (*B*) Control preparation. ×880.

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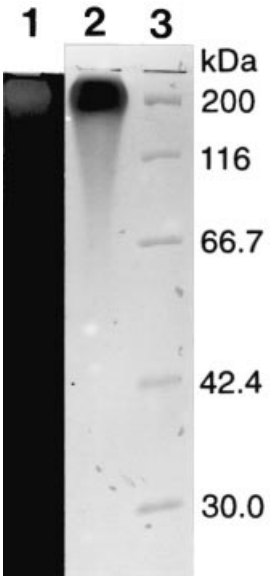

Figure 4 Purity and proteinase activity of the sialoglycoprotein. Lanes 1, 2 and 3 show zymography of the sialoglycoprotein, Coomassie Brilliant Blue stained-PAS reaction and molecular mass standards, respectively. The sialoglycoprotein used was of a high purity and exhibited proteinase activity.

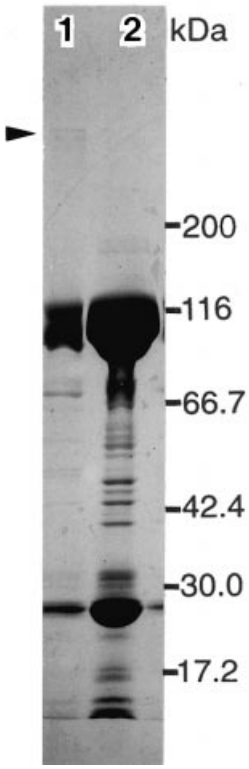

Figure 5 VE components and components assembled *in vitro* analysed by SDS-PAGE under reducing conditions. Lanes 1 and 2 show components assembled *in vitro*, and VE components respectively. The major bands of the assembled material are closely similar to those of the VE components. The arrowhead indicates a double band of molecular masses of approximately 400 and 370 kDa.

Electrophoretic pattern of VE components and assembly *in vitro*

The VE components consisted of four major bands and about 20 minor bands, as revealed by SDS-PAGE on 2–15% gradient polyacrylamide gels under reducing conditions. The major bands represent molecular masses of about 150–120, 110–100, 70 and 27 kDa (Fig. 5).

Electron microscopy revealed that the fibrous material, which was produced *in vitro* from VE components by the sialoglycoprotein with proteolytic activity, consisted of aggregates of network sheets, frequently expanded to various sizes. The main skeleton of the networks consisted of thick filamentous substances (*c.* 137–376 nm) and thin filamentous substances (*c.* 27–52 nm) (Fig. 6). The thick filamentous substances appeared to be bundles of thin filaments (Fig. 7), which branched out of the former to form smaller networks by anastomosing with each other. Furthermore, the smaller networks were composed of a number of even finer networks. Adding p-CMB, IAA or l-cysteine to the full reaction medium failed to produce fibrous material after mixing by vortexing. Bovine thrombin can substitute for the sialoglycoprotein but not trypsin (data not shown). Pretreatment of the sialoglycoprotein with DFP or PMSF resulted in failure to induce production of the fibrous material. Addition of thrombin to the VE components dissolved in 50 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl and 2 mM EDTA, and subsequent vortexing resulted in the appearance of flocculent material, but no fibrous material was detectable either macroscopically or electronmicroscopically. The electrophoretic pattern of the elements which participated in assembly *in vitro* indicated that the fibrous material (electron microscopical networks) might be composed of the major and a few minor bands of the VE components. In addition, bands with molecular masses of 400 and 370 kDa appeared faintly in the assembled material, suggesting that they were polymerised products (Fig. 5).

Discussion

The results indicate that VE components are assembled *in vitro* by the action of a sialoglycoprotein, which contains serine proteinase activity and immunolocalises in cortical alveoli of fish eggs, producing sheets of various sizes consisting networks of filamentous substance and their aggregates. VE component assembly *in vitro* may reproduce the initial events, cross-linking and reorganisation, of FE formation. This assembly is analogous to blood coagulation: it is Ca^{2+}-dependent, thrombin substitutes for the sialoglycoprotein, and ser-

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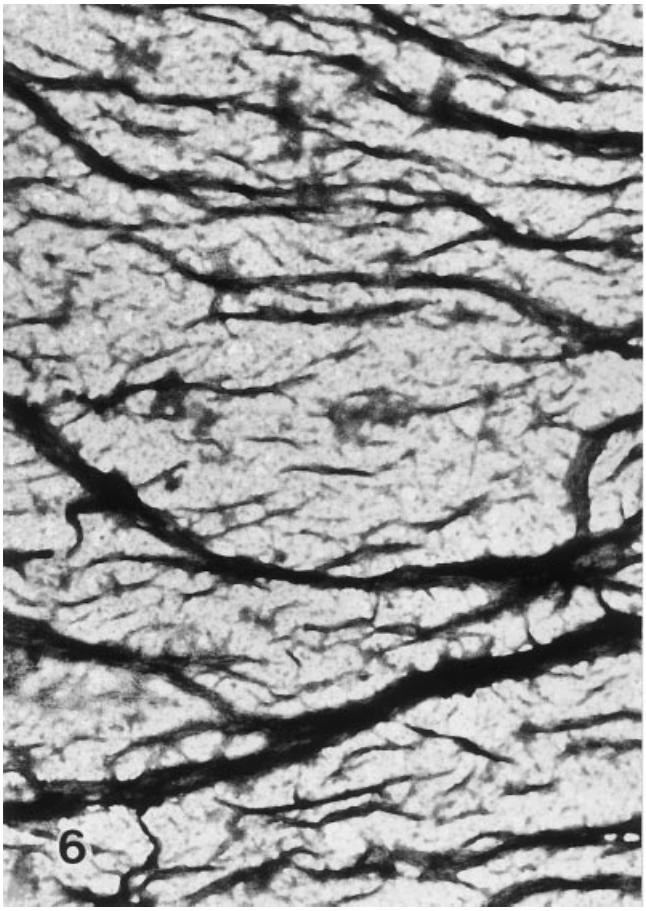

Figure 6 Filamentous substance networks of VE components assembled *in vitro* by the sialoglycoprotein. The networks are composed of the branching and anastomosing thin and thick filamentous substances. ×17 200.

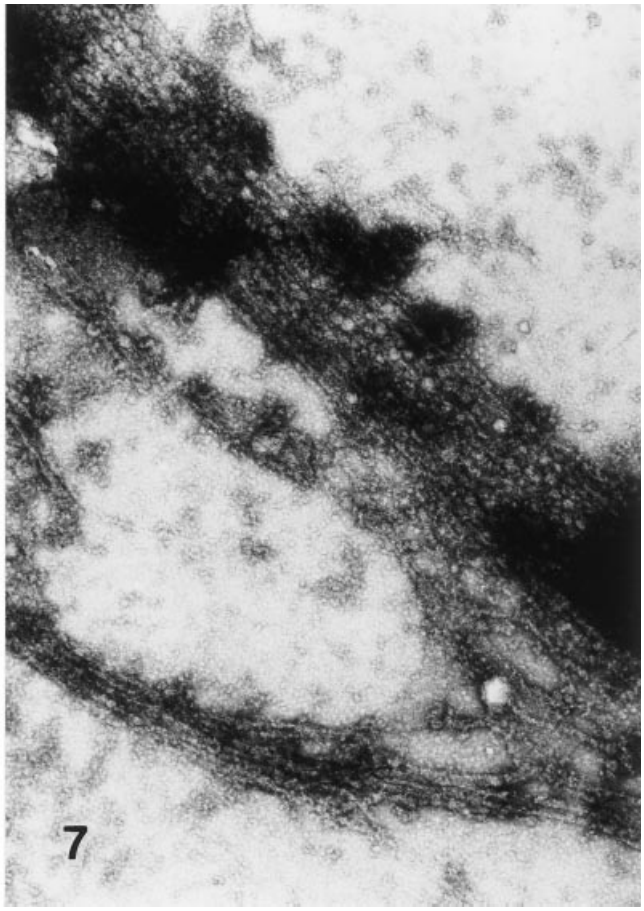

Figure 7 Negative staining of the filamentous substance, which consists of fine filaments. ×45 000.

ine proteinase inhibitors (DFP and PMSF) inhibit the assembly *in vitro* of VE components, although it is not clear whether VE components contain fibrinogen or its subunits (Hantgan *et al*., 1983; Lorand & Conrad, 1984).

In experiments to visualise the presence of the chorionic TGase, pretreatment with DFP of unfixed and frozen VE sections inhibited the fluorescence of monodansylcadaverine-stained sections almost completely, as was also the case with l-cysteine and p-CMB. Although the mechanism of the abolition of fluorescence is unclear, the inhibitory effect of DFP on both chorionic TGase and sialoglycoprotein proteinase activities may result in significant inhibition of FE elevation (Kudo, unpublished data). It is not clear whether the failure to demonstrate choionic TGase in the FEs is due to inactivation of the enzyme or its removal from the FE during the cortical reaction, but this question should be resolved by production of an antibody against the enzyme.

Defined on a physicochemical basis, the transformation of the VE into the FE involves a major change involving a remarkable increase in both the rigidity and stability of structural integrity. This can be accepted as a general feature of the process of FE formation after fertilisation of animal eggs. The rigidity and stability are Ca^{2+}-dependent, as a feature of complete FE hardening, but this Ca^{2+} requirement occurs in only an early, short period of the total time required for hardening (Zotin, 1958). It is apparent that the Ca^{2+} requirement may be essential for activation of a Ca^{2+}-dependent enzyme that corresponds to the sialoglycoprotein immunolocalised in cortical alveoli in the present experiments. The action of the enzyme may activate a chorionic TGase so as to form covalent ε-(γ-glutamyl)-lysine crosslinks between constituents of adjacent proteins or glycoproteins, resulting in structural stabilisation of the FEs. This is also supported by the fact that Glu-Lys isopeptides are present in the FEs, but not in the VEs, of trout eggs (Lonning *et al*., 1984).

The VE of the present fish eggs consists of five layers (Kudo *et al*., 1988), and the fifth layer is composed of an aggregated complex of filaments (*c*. 86–137 nm in diameter; *c*. 110 nm on average), between which the

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matrix appears to consist of an amorphous substance. However, the main frameworks of the filamentous networks, as revealed by electron microscopy, which were reconstituted by the assembly *in vitro* of VE components, seem not to be identical to the VE fifth layer filaments, except for some similarity in thickness to the thick filamentous substances. Indeed, filamentous substance networks are also formed during the assembly *in vitro* of carp VE components induced by both thrombin and the presently described sialoglycoprotein (Kudo, unpublished data) and are indistinguishable in texture from those of the present fish, in spite of there being no filamentous constituent of carp VEs. Therefore, it seems a reasonable assumption to consider the assembly *in vitro* of VE components to be an approximate model for FE reconstruction, which is induced as the result of an interaction between VE components and a cortical alveolus component in the process of FE hardening.

The variability in thickness of the filamentous substance constituting the networks appears to be due to the aggregation to various degrees of the thin filamentous substances. Nevertheless, it remains to be determined whether or not the aggregated filamentous substances are identical in composition. The approximate similarity in basal ultrastructure of individual filamentous substance networks suggests that the quantitative interaction among VE components participating in the network construction may be controlled by kinetics rather than by an equilibrium binding process. Since the VE components that mainly participated in assembly *in vitro* have been clarified, the molecular construction of the networks consisting of filamentous substances should be immunocytochemically elucidated by the use of a combination of the antibodies against each element.

Serine proteinases are released from the eggs of sea urchins during the cortical reaction (Vacquier *et al.*, 1973) and from amphibian eggs (Lindsay & Hedrick, 1989). They are involved in removal of sperm receptors from the VE in sea urchin eggs, in the separation of the envelope from the plasma membrane, and in the modification of the envelope (Kay & Shapiro, 1985). In *Xenopus* eggs, a chymotrypsin-like proteinase, one of two proteinase activities, is involved in envelope conversion (Lindsay & Hedrick, 1989). These data indicate that a serine proteinase participates in VE modification, due to the limited proteolysis and FE hardening reaction. Presumably, involvement of a serine proteinase in the modification of the VE may be common to the process of FE formation in animal eggs, as has also been shown for the present sialoglycoprotein which contains serine proteinase activity. Involvement of the sialoglycoprotein in FE assembly was apparent in the present experiments, but it is not clear whether it was responsible for removal of the VE outermost layer during FE elevation.

It is well known that Ca^{2+} plays an important role in FE hardening. The mechanism of FE hardening and its Ca^{2+} requirement have been considerably clarified by studies on the interaction between VE components and cortical granule components in eggs of sea urchins (Weidman *et al.*, 1985, 1987; Weidman & Shapiro, 1987). In fish eggs, however, such studies remains insufficient. The present data seem to contribute to counteracting this insufficiency by providing a simple system for further analysis of FE hardening and its Ca^{2+} requirement, since Ca^{2+} dependence in the assembly *in vitro* of VE components containing chorionic TGase activity may have an essential role in exerting the proteolytic action of the sialoglycoprotein itself. There is no doubt that the sialoglycoprotein acts as a trigger for activation of the chorionic TGase in the presence of Ca^{2+}, because of the absence of any assembly by the VE components alone in a medium containing $CaCl_2$. This means that the sialoglycoprotein discharged from the cortical alveoli may activate the chorionic TGase by its proteolytic action. Furthermore, the fact that thrombin can substitute for the sialoglycoprotein indicates a similarity in chemical properties between chorionic TGase and plasma TGase (Mosker et al., 1979, 1980; Lorand & Conrad, 1984). It is therefore possible that the chorionic TGase might be present in the VE or VE components as an inactive proenzyme (or zymogen). If this assumption is true, the chorionic TGase might be activated as a result of limited proteolysis, as is known to occur with plasma TGase, and Ca^{2+} may be required for the sialoglycoprotein-catalytic activity of the active chorionic TGase but not for the sialoglycoprotein-catalysed activity of the inactive chorionic TGase, since a Ca^{2+} requirement for catalytic activity of active plasma TGase has been demonstrated (Tyler, 1970; Lorand *et al.*, 1979). Thus, activated chorionic TGase may contribute to FE assembly due to cross-linking of the VE component in the presence of Ca^{2+}, implying that this is at least part of the mechanism of FE hardening. The Ca^{2+} requirement is assumed from the fact that TGases catalyse a Ca^{2+}-dependent acyltransfer reaction (Folk & Chung, 1973). The assembly *in vitro* of VE components is only a first step in the analysis of the mechanism of FE hardening and integration, but purification and identification of each VE component and the cortical alveolus component would contribute to the full analysis of the entire mechanism.

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