Journal of Cellular Biochemistry 99:816–823 (2006)

# Proteolytic Processing of a Sea Urchin, ECM-Localized Protein into Lower Mol Mass Species Possessing Collagen-Cleavage Activity

**John J. Robinson***

Department of Biochemistry, Memorial University of Newfoundland, St John's, Newfoundland, Canada A1B 3X9

**Abstract** The hyaline layer is an apically located extraembryonic matrix, which blankets the sea urchin embryo. Using gelatin substrate gel zymography, we have identified a number of gelatin-cleaving activities within the hyaline layer and defined a precursor–product processing pathway which leads to the appearance of 40- and 38-kDa activities coincident with the loss of a 50-kDa species. Proteolytic processing of the precursor required the presence of both $CaCl_2$ and NaCl at concentrations similar to those found in sea water. The cleavage activities utilized both sea urchin and rat tail tendon gelatins as substrates but demonstrated a species-specific cleavage activity towards sea urchin collagen. The gelatin-cleaving activities were refractory to inhibition by 1, 10-phenanthroline but were inhibited by benzamidine. This latter result defines the serine protease nature of the cleavage activities. Both the 40- and 38-kDa activities were found to comigrate with gelatin-cleaving activities present in the sea urchin embryo. J. Cell. Biochem. 99: 816–823, 2006.


**Key words:** extraembryonic matrix; proteolytic processing; collagenase

The extracellular matrix (ECM) in both vertebrates and invertebrates is a ubiquitously distributed proteinaceous coat, which surrounds tissues and embryos. In addition to contributing to structural integrity, the ECM is a dynamic entity, which indirectly modulates cell behavior by mediating interactions between the cell surface and extracellular signaling molecules. Also, a number of the constituent components of the ECM can directly bind receptor molecules on the surface of cells. Thus, by acting as a reservoir of cell interactive molecules, the ECM modulates many cellular properties including shape, migration, proliferation, and differentiation. Not surprisingly, cells have evolved elaborate mechanisms for controlling the biogenesis of the ECM. Composition is controlled at several levels including de novo synthesis of constituents, regulation of secretory pathways, and protease-mediated turn-over of components.

The regulation of ECM composition occurs at many levels including de novo synthesis, post-translational modification and proteolytic processing. Identifying and characterizing the proteolytic activities responsible for remodeling the ECM has emerged as a major area of investigation. This level of interest is derived, in part, from the recognition that cleavage-activities directed against components of the ECM contribute to the homeostatic balance required for normal development and physiology [Vu and Werb, 2000]. Consequently, changes to the level of cell surface proteolytic activity can lead to a number of pathological conditions [Coussens et al., 2002; Garcia-Touchard et al., 2005]. The activities responsible for

Abbreviations used: ECM, extracellular matrix; MMP, matrix metalloproteinase; MFSW, Millipore-filtered and UV-irradiated sea water; EDTA, ethylenediaminetetraacetic acid.

Grant sponsor: Natural Sciences and Engineering Research Council of Canada.

*Correspondence to: John J. Robinson, Department of Biochemistry, Memorial University of Newfoundland, St John's, Newfoundland, Canada A1B 3X9.
E-mail: johnro@mun.ca

Received 21 February 2006; Accepted 31 March 2006
DOI 10.1002/jcb.20975

regulating protein composition in the pericellular space belong to the metallo- and serine families of proteases. Recent studies have broadened the spectrum of molecules recognized as substrates for these proteases. Non ECM constituents such as growth factors, cell surface receptors, and cell–cell adhesion molecules are utilized as substrates for extracellular metallo- and serine proteases [Del Rosso et al., 2002; Somerville et al., 2003]. The former family is the best studied and consists of four subfamilies, the matrix metalloproteases (MMP), the adamalysins, seralysins, and astacins [Bode et al., 1996].

The sea urchin has long been used as a paradigm for embryonic development. In this context, a number of laboratories have identified and partially characterized gelatin and collagen cleaving activities in the egg and embryo [Karakiulakis et al., 1993; Quigley et al., 1993; Vafa and Nishioka, 1995]. While these studies were devoid of structural details on the proteases, they did describe cleavage activities that were both expressed in a developmentally regulated pattern and inhibited by the zinc chelator, 1, 10-phenanthroline. This latter result identifies these activities as metalloproteases. In an additional study BB94, a vertebrate MMP inhibitor, was shown to inhibit spiculogenesis in the sea urchin embryo [Ingersoll and Wilt, 1998]. Analysis of a zinc and calcium-dependent protease responsible for dissolution of the sea urchin fertilization envelope revealed a structure similar to that reported for vertebrate MMPs [Roe and Lennarz, 1990; Nomura et al., 1997]. These results identify MMP-like activities in the sea urchin egg and embryo. In parallel studies, we have characterized a serine protease, which is prominent in the sea urchin egg and exported to the ECM following fertilization [Mayne and Robinson, 1998, 2002; Robinson, 2000]. Clearly, a number of proteases, belonging to at least two different families, are required to maintain the proper composition of the sea urchin ECM.

In this study, we report on two gelatin-cleaving activities, which appear in the sea urchin extraembryonic matrix, the hyaline layer, following proteolytic processing of a 50-kDa precursor. These activities possess a species-specific, collagen-cleaving activity and, interestingly, display a serine, rather than the more common metalloprotease character.

## MATERIALS AND METHODS

### Materials

All materials were of the highest grade available.

### Growth of Embryos

*Strongylocentrotus purpuratus* were purchased from Seacology, Vancouver, Canada, and gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were washed three times in ice-cold Millipore-filtered and UV-irradiated seawater (MFSW; 0.45 μm) and fertilized with an approximate 100-fold numerical excessive of sperm. Embryos were cultured with constant aeration, at 12°C, in cylindrical chambers containing paddles rotating at 40 rpm. Samples were harvested at the times indicated after fertilization (HPF).

### Isolation of Hyaline Layers

To facilitate the isolation of hyaline layers, unfertilized eggs were dejellied by titration with HCl to pH 5.5 and left on ice for 2 min. The pH was then returned to 8.0 with the addition of 1 M Tris-HCl (pH 8.0). After extensive washing in ice-cold MFSW, the eggs were resuspended in MFSW to give a final concentration of 10% (v/v) and their vitelline layer was disrupted with 10 mM dithiothereritol [Epel et al., 1970]. After extensive washing in ice-cold MFSW, the eggs were fertilized with a 100-fold numerical excess of sperm. Hyaline layers were isolated as follows. One-hour-old embryos were washed several times in ice-cold MFSW and resuspended to give a final concentration of 10% (v/v) in MFSW containing 10 mM benzamidine hydrochloride as a proteinase inhibitor. The resuspended embryos were homogenized with 100 strokes in a hand-held Dounce homogenizer (type A) at 0°C. The homogenate was passed by gravity filtration through a 28-μm-pore-size Nitex mesh (B, D, and H Thompson and Co., Mount Royal, Quebec), the retarded layers were resuspended in ice-cold MFSW and the filtration step repeated twice more. Each preparation was examined by phase-contrast microscopy and showed no evidence of contaminating cytoplasmic debris. The isolated hyaline layers were harvested by centrifugation and stored as pellets at −70°C.

### Substrate Gel Zymography

Substrate gel zymography was performed essentially as described previously [Huessen

and Dowdle, 1980]. Gels containing sodium dodecyl sulfate were prepared by copolymerizing acrylamide and gelatin or a test substrate at a final concentration of 0.1% (w/v). Samples of embryos or the gelatinase(s) were dispersed for 30 min at room temperature in Laemmli solubilizing solution from which both DTT and bromophenol blue had been omitted [Laemmli, 1970]. Electrophoresis was performed at a constant current of 10 mA at 4°C for 4 h. After electrophoresis, the gels were incubated for 60 min at room temperature in 50 mM Tris-HCl, pH 8.0, containing 2.5% (v/v) Triton X-100 followed by 16 h incubation at room temperature in 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$. The gels were stained with Coomassie Brilliant Blue R-250 using a standard protocol.

### Gel Exclusion Chromatography

The supernatant containing the displaced gelatin-cleavage activities was applied to a $1 \times 28$ cm column of the gel exclusion resin, Agarose 1.5 M (BioRad). Chromatography was performed at a flow rate of 1.8 ml/h at 4°C and 0.3 ml fractions were collected.

### Quantitative Gelatinase Assay

Gelatinase activity was assayed by quantitating the cleavage of gelatin into trichloroacetic acid (TCA)-soluble peptides. Aliquots of gelatinase were incubated in a total volume of 100 μl containing 50 mM Tris-HCl, pH 8.0, 0.1% (w/v) gelatin and 10 mM $CaCl_2$ for 60 min at 37°C. A control was set up as above, except that the gelatinase was omitted, to ensure that cleavage was dependent upon the gelatinase. At the end of the incubations, an equal volume of 20% TCA was added and the samples left at 0°C for 30 min. A control for measuring the total amount of protein prior to cleavage contained all the reagents listed above and the 20% TCA was added at zero time. Samples were centrifuged at 16,000*g* for 10 min and the protein content of the pellet was determined [Lowry et al., 1951]. A unit of gelatinase activity is defined as 0.1 μg gelatin cleaved/min.

### Quantitation of Collagen-Cleavage Activity

Following incubation with the collagen-cleaving activities, the digestion products were fractionated by SDS–PAGE, stained with Coomassie Brilliant Blue, destained and gel slices containing the collagen polypeptide chains were excised. The dye was eluted in 25% (v/v) pyridine and quantitated by optical density measurements at 605 nm. Gel slices without protein were also excised, the dye eluted and the optical density at 605 nm was used to correct for background binding of stain. Values obtained for the collagen samples incubated in the absence of cleavage activities were normalized to 100%.

## RESULTS AND DISCUSSION

The ECM is a proteinaceous structure, which interfaces between the cell surface and the extracellular environment. Direct interactions between ECM components and cell surface receptors as well as indirect interactions through the sequestering of signaling molecules serve to direct cell behavior. The composition of the ECM is intimately related to its functional capabilities and the turn-over of components is controlled both at the level of new synthesis and regulated protease activities. The hyaline layer is an apically located, sea urchin extraembryonic matrix, which surrounds the embryo and larva from the time of fertilization until metamorphosis occurs. Within 60 min following fertilization, the hyaline layer is assembled through the timed sequential release of components stored within the egg [Alliegro and McClay, 1988; Alliegro et al., 1988]. The layer consists of two distinct structural domains which are maintained by protein–protein interactions [Robinson, 1991] and this structure is sufficiently robust to allow for its isolation and visualization (Fig. 1). Functionally, the layer is required to maintain the structural integrity of the early embryo and antibodies directed against a major protein component of the layer, hyalin, resulted in the arrest of development prior to gastrulation [Adelson and Humphreys, 1988]. Failure to deposit cross-linked collagen into the layer also resulted in the arrest of development [Wessel and McClay, 1987]. Arrested embryos could be rescued by the extracellular signaling molecules, platelet-derived growth factor, or transforming growth factor-α [Ramachandran et al., 1993; Govindarajan et al., 1995]. Also, antibodies prepared against vertebrate ECM components cross-react with species within the hyaline layer [Wessel et al., 1984; Spiegel et al., 1989]. Collectively, these results attest to the need

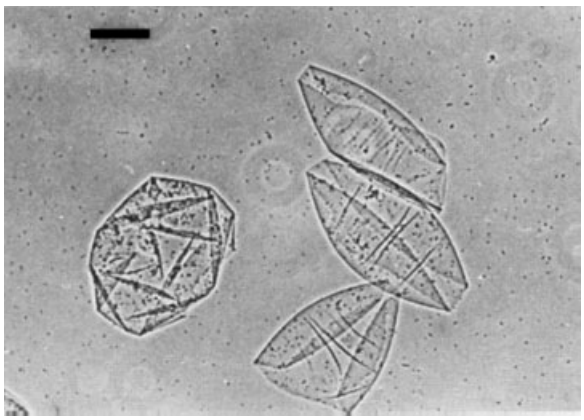

**Fig. 1.** Phase contrast microscopy of isolated hyaline layers. Hyaline layers were prepared as described in Materials and Methods, resuspended in MFSW and examined in a Zeiss Photomicroscope. Scale bar = 40 μm.

for a properly constituted hyaline layer to drive normal development and also suggest the utility of the sea urchin ECM as a model to examine cell surface signaling during embryonic development. Although the biogenesis, composition, and function of the hyaline layer has been extensively studied, little attention has been paid to the processes which regulate the composition of the layer.

We have previously analyzed the sea urchin extraembryonic matrix, the hyaline layer, for gelatin-cleavage activities. These analyses revealed that both serine- and metalloprotease activities utilize substrates within the apical ECM. In addition, our work with isolated hyaline layers suggested that most ECM-localized, gelatin-cleavage activities were deposited within the layer as larger precursor molecules, which were subsequently processed to their native forms through proteolytic cleavage [Ranganathan et al., 2004]. In a recent study, we documented the appearance of two serine-type proteases of apparent mol mass 40- and 50 kDa [Robinson et al., 2003]. These activities possessed many shared characteristics. While neither activity was detected in layers prepared from 1-h-old embryos, both appeared when these layers were incubated for up to 18 h at 37°C in MFSW. Both species were first detected following 7–9 h of incubation and required $Ca^{2+}$ but not $Mg^{2+}$ for activity. In addition, both displayed substrate specificity for gelatin and failed to cleave bovine serum albumin, hemoglobin, or casein.

In the study reported here, we have extended this work and increased the incubation time beyond 18 h. In initial experiments, isolated hyaline layers were incubated for up to 51 h at 37°C in the presence of 500 mM NaCl and 10 mM $CaCl_2$ and analyzed by gelatin substrate gel zymography (Fig. 2). After 8 h incubation, a number of gelatin-cleavage activities were detected at 90-, 70-, 50-, and 40 kDa (lane 1). Following 24 h of incubation, new species appeared at 55- and 38 kDa while the activity of the 40 kDa species appeared to increase. A particularly faint species was also seen at 80 kDa. In addition, the activity of the 90- and 70-kDa species appeared to decrease while the 50-kDa activity was no longer detected. This pattern of activities remained stable following up to 51 h of incubation (lane 5). The coincidental loss of the 50-kDa species and the appearance of the 38-kDa activity suggests a precursor–product relationship.

To explore the biochemical nature of the 50- and 40-kDa species detected in this study, we used gel exclusion chromatography to fractionate a mixture of the activities displaced from hyaline layers in the absence of NaCl and $CaCl_2$. We have previously shown that the structural integrity of the layer is compromised by the removal of $CaCl_2$ [Robinson, 1991]. Hyaline layers were incubated at 37°C for 8 h in the presence of 10 mM $CaCl_2$ and 500 mM NaCl,

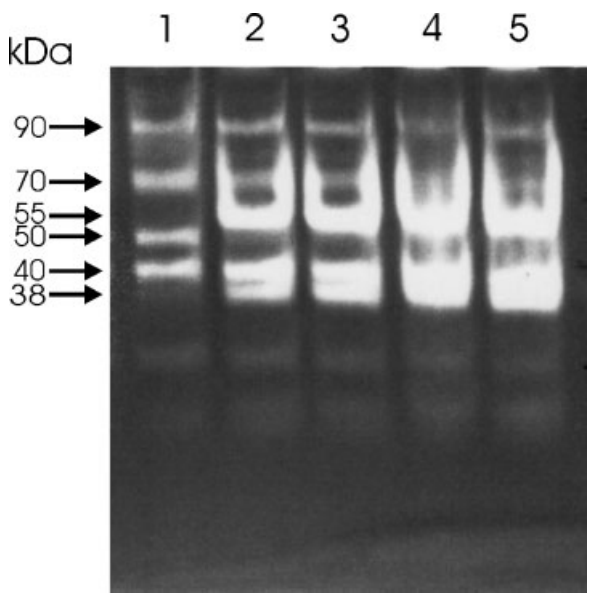


**Fig. 2.** Gelatin substrate gel analysis of isolated hyaline layers. Aliquots of isolated hyaline layers were incubated in 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, and 500 mM NaCl at 37°C for 8 h (**lane 1**), 24 h (**lane 2**), 36 h (**lane 3**), 45 h (**lane 4**), or 51 h (**lane 5**). Aliquots (5 μg) were then fractionated in a gelatin substrate gel.

harvested by centrifugation and the activities displaced by resuspending the layers in 50 mM Tris-HCl, pH 8.0 and 5 mM ethylenediaminetetraacetic acid (EDTA). The displaced activities were fractionated in a column packed with agarose 1.5 M gel exclusion resin and fractions enriched in the 40- and 50-kDa activities or the 40-kDa activity alone were obtained. Fractions enriched in the 40- and 50-kDa species were analyzed by gelatin substrate gel zymography and the gel strips processed in the presence of either the $Zn^{2+}$-dependent, metalloprotease inhibitor, 1, 10-phenanthroline, or the serine protease inhibitor, benzamidine (Fig. 3). Both species were active in the control sample and the 1, 10-phenanthroline-treated sample (lanes 2 and 3, respectively). However, when the gel slice was processed in the presence of benzamidine, a substantial decrease in activity occurred (lane 1). These results establish the serine protease nature of the gelatin-cleaving activities and confirm that they are the same species as those identified in our previous study [Robinson et al., 2003]. In addition, as previously reported, we have found that the appearance of the 40- and 50-kDa activities required the incubation of layers in the presence of both 10 mM $CaCl_2$ and 500 mM NaCl (data not shown).

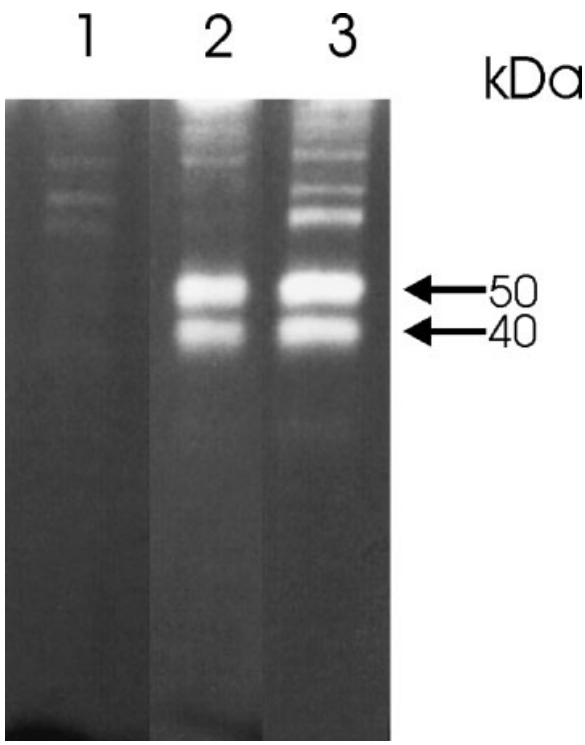


**Fig. 3.** Definition of the catalytic nature of the 40- and 50-kDa species. Aliquots, 0.5 μg, of fractions enriched in the 40- and 50-kDa cleavage activities were fractionated in a gelatin substrate gel followed by dissection of the gel into strips which were then processed in the presence or absence of selected protease inhibitors. One gel strip was processed in the absence of added inhibitior (**lane 3**), a second strip was processed in the presence of 2 mM 1, 10-phenanthroline (**lane 2**) while a third strip was processed in the presence of 10 mM benzamidine (**lane 1**).

In our previous study, we failed to detect the 38-kDa activity in layers incubated for up to 18 h. The data in Figure 2 suggests a coincidental relationship between the appearance of the 38-kDa activity and the loss of the 50-kDa species, an event triggered by increasing the incubation time from 18 to 24 h. We explored the relationship between the 50-, 40- and 38-kDa activities using fractions enriched in the 40- and 50-kDa species or the 40-kDa species alone. Fractions were made 10 mM in $CaCl_2$ and 500 mM in NaCl and either frozen immediately or incubated at 37°C for 48 h followed by analysis using gelatin substrate gel zymography (Fig. 4A). Following incubation of the fraction enriched in the 40- and 50-kDa species, the activity at 38 kDa appeared concomitant with the loss of the 50-kDa activity (lanes 1 and 2). Interestingly, the intensity of the 40-kDa activity appeared to increase following incubation (lane 2). These results suggest a precursor–product relationship between the 50-, 40-, and 38-kDa species. When the fraction enriched in the 40-kDa species alone (lane 3) was incubated in the presence of both $CaCl_2$ and NaCl, the 380-kDa activity was detected (lane 4). We also noted an apparent decrease in the activity of the 40-kDa species. Collectively, these results define a processing pathway involving the species at 50-kDa, which can be converted into an active species of 40 kDa. The 40-kDa species can in turn be converted to a third active species of 38 kDa. Processing required the presence of the major salts, NaCl and $CaCl_2$, present in sea water (data not shown). Interestingly, and in contrast with the 50-kDa species, the 40-kDa activity was not completely processed but remained detectable at the end of the incubations (lanes 2 and 4).

We next probed the biochemical nature of the activity responsible for processing the 50- and 40-kDa species. We have shown that these species themselves are only detected in zymogen gels processed in the absence of benzamidine (Fig. 3). When a fraction enriched in the 50- and 40-kDa activities was incubated in the

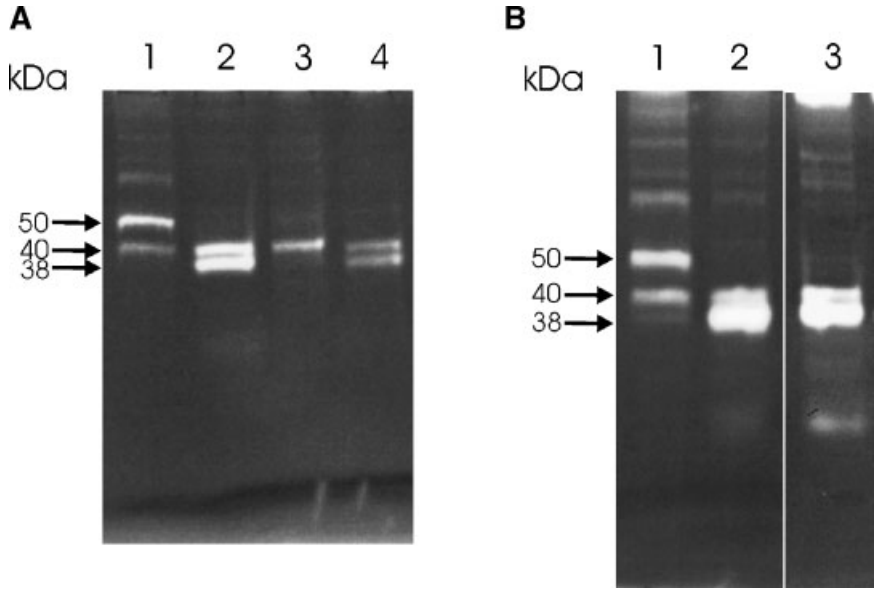


**Fig. 4.** Proteolytic processing of the 40- and 50-kDa species into the 38-kDa activity. **A**: Processing of the 40- and 50-kDa species. Aliquots of fractions recovered from the agarose 1.5 M column, enriched in the 40- and 50-kDa activities or the 40-kDa activity alone, were made 10 mM in $CaCl_2$ and 500 mM in NaCl and either frozen immediately or incubated for 48 h at 37°C. Following incubation, the samples were analyzed in a gelatin substrate gel. Aliquots enriched in the 40- and 50-kDa species (**lanes 1** and **2**) or the 40-kDa species alone (**lanes 3** and **4**) were analyzed without (lanes 1 and 3) or after (lanes 2 and 4) incubation. **B**: Processing of the 40- and 50-kDa species in the presence of protease inhibitors. Aliquots from the agarose 1.5 M column, enriched in the 40- and 50-kDa species, were incubated as described above in the absence (**lane 1**) or the presence of 2 mM 1,10-phenanthroline (**lane 2**) or 10 mM benzamidine (**lane 3**).

presence of $CaCl_2$, NaCl, and the $Zn^{2+}$-dependent metalloprotease inhibitor, 1, 10-phenanthroline followed by fractionation in a gelatin substrate gel, the 38-kDa species was detected (Fig. 4B, lanes 2). This result eliminates an active role for a metalloprotease-like activity in the processing pathway leading to the appearance of the 38-kDa species. Similarly, when the serine protease inhibitor benzamidine was present during the incubation, the 38-kDa species was again detected in a gelatin substrate gel (lane 3) eliminating the possibility of auto-catalytic processing. This latter result contrasts with an earlier study in which we demonstrated the auto-catalytic processing of a 90-kDa metalloprotease activity to species of 55- and 32-kDa [Ranganathan et al., 2004]. To date, our collective data suggest that two classes of proteases, serine- and metallo-, are responsible for modulating the composition of the apically located ECM in the sea urchin embryo. In addition, the data presented here identify a second, distinct processing pathway, which is responsible for the genesis of the serine protease activities.

In an effort to identify a physiological substrate for the 38-kDa activity, this species was assayed for collagen-cleavage activity. Following fractionation in a gelatin substrate gel, the 38-kDa activity was recovered by electroelution from a gel slice and assayed in a quantitative gelatin-cleavage assay. Aliquots, containing 0.12 units of gelatin-cleavage activity, were then incubated with collagen purified from either sea urchin peristome or rat tail tendon. Peristome collagen consists of two polypeptide chains of 116- and 140-kDa while rat tail tendon collagen is composed of polypeptide chains of 123- and 126 kDa. In a previous study, we reported that sea urchin peristome collagen possessed amino acid compositional features characteristic of vertebrate collagens [Robinson, 1997]. Peristome collagen was found to contain 28.1 mol % glycine as well as hydroxyproline and hydroxylysine. Following incubation for 2 h at 37°C in the absence (lane 1) or the presence (lane 2) of the gelatinase, both chains of the peristome collagen were cleaved (Fig. 5A). Quantification of chain cleavage revealed that $40 \pm 5\%$ of the 140 kDa and $37 \pm 7\%$ of the 116 kDa chains were cleaved (values represent means $\pm$ SEM, $n = 3$). In contrast, rat tail tendon collagen was refractory to cleavage (lanes 3 and 4). In additional experiments gelatins,

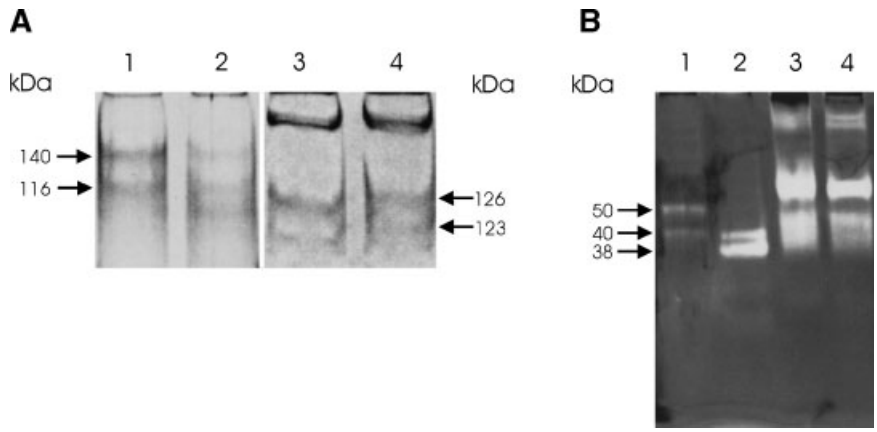


**Fig. 5.** **A**: Identification of a physiological substrate for the 38-kDa activity. Aliquots containing 5 μg of either sea urchin peristome (**lanes 1** and **2**) or rat tail tendon (**lanes 3** and **4**) collagens were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 0.12 units of the 38 kDa, gelatin-cleavage activity. Following incubation for 2 h at 37°C, the reactions were stopped by the addition of equal volumes of ice-cold, 20% (w/v) TCA. Protein pellets were harvested by centrifugation, solubilized and fractionated in an 8% (w/v) polyacrylamide gel [Laemmli, 1970]. The gel was stained with Coomassie brilliant blue R-250 and destained. **B**: Comigration of the 40- and 38-kDa activities with species present in gastrula stage sea urchin embryos. Samples containing the 50-, 40- and 38-kDa activities (**lanes 1** and **2**) were fractionated in a gelatin substrate gel alongside aliquots of 44- and 51 h-old sea urchin embryos (**lanes 3** and **4**, respectively).

generated by heat denaturation of either peristome or rat tail tendon collagen, were both cleaved by the 38-kDa activity (data not shown). These results clearly identify a species specific, collagen-cleavage activity as well as a less specific gelatin-cleavage activity associated with the 38-kDa species. In a related experiment, a mixture of the 40- and 50-kDa activities cleaved peristome collagen but not rat tail tendon collagen (data not shown).

Gelatin substrate gel zymography was used to examine sea urchin embryos for the presence of cleavage activities comigrating with the 50-, 40- and 38-kDa species (Fig. 5B). Gastrula stage embryos (lanes 3 and 4) were found to contain a broad band of gelatin-cleavage activity, which comigrated with the 40- and 38-kDa species. In additional experiments, blastula stage embryos were also found to possess the same broad band of cleavage activity (data not shown). This finding clearly establishes the 40- and 38-kDa species as biologically relevant activities in the sea urchin embryo. Interestingly, the 50-kDa species was not detected in the embryos examined (lane 1). This latter result would be expected if the 50 kDa, gelatin-cleaving activity is in fact a transient precursor to the 40- and 38-kDa species.

The data presented here document the proteolytic processing of a 50-kDa precursor into 40- and 38-kDa gelatin- and collagen-cleaving activities in the sea urchin extraembryonic matrix. Interestingly, the 50-kDa protein can utilize gelatin and, most probably, collagen as cleavage substrates. Processing, therefore, appears not to be required for the activation of cleavage activity. This result is similar to that seen during the proteolytic conversion of chymotrypsinogen to α-chymotrypsin. The intermediate species, π, δ, and κ are all catalytically active but less stable than α-chymotrypsin. Our failure to detect the 50-kDa protein in sea urchin embryos, both here and in a previous study Robinson et al. [2003], suggests that this species may be inherently unstable. The conversion of the 50-kDa protein into more stable 40- and 38-kDa activities may be a response to the need for sustained gelatin- and collagen-cleavage activities during periods of embryonic development requiring extensive remodeling of the extraembryonic matrix. However, the need for two stable, active species remains unclear.

## ACKNOWLEDGMENT

I thank Roy Ficken for performing the photography.

## REFERENCES

Adelson DC, Humphreys T. 1988. Sea urchin morphogenesis and cell-hyalin adhesion are perturbed by a monoclonal antibody specific to hyalin. Development 104:391–402.

Alliegro MC, McClay DR. 1988. Storage and mobilization of extracellular matrix proteins during sea urchin development. Dev Biol 125:208–216.

Alliegro MC, Ettensohn CA, Burdsal CA, Erickson HP, McClay DR. 1988. Echinonectin: A new embryonic substrate adhesion protein. Journal of Cellular Biology 107:2319–2327.

Bode W, Grams F, Reinemer P, Franz-Xaver G, Ulrich B, McKay DB, Stocker W. 1995/ 1996. The metzincins: A superfamily of structurally related metalloproteinases. Zoology 99:237–246.

Coussens LM, Fingelton B, Matrisian LM. 2002. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 295:2387–2392.

Del Rosso M, Fibbi G, Pucci M, D'Allessio S, Del Rosso A, Magnelli L, Chiarugi V. 2002. Multiple pathways of cell invasion are regulated by multiple families of serine proteases. Clinical Envir Metastasis 19:193–207.

Epel D, Weaver AM, Mazia D. 1970. Methods for the removal of the vitelline membrane of sea urchin eggs. Expl Cell Res 61:64–68.

Garcia-Touchard A, Henry TD, Sangiorgi G, Spagnoli LG, Mauriello A, Conover C, Schwartz RS. 2005. Extracellular proteases in atherosclerosis and restenosis. Arterioscler Thromb Vasc Biol 25:1119–1125.

Govindarajan V, Ramachandran RK, George JM, Shakes DC, Tomlinson C. 1995. An ECM-bound, PDGF-like growth factor or a TGF-α-like growth factor are required for gastrulation and spiculogenesis in the *Lytechinus* embryo. Dev Biol 172:541–551.

Huessen C, Dowdle EB. 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal Biochem 102:196–202.

Ingersoll EP, Wilt FH. 1998. Matrix metalloproteinase inhibitors disrupt spicule formation by primary mesenchyme cells in the sea urchin embryo. Dev Biol 196:95–106.

Karakiulakis G, Papakonstantinou E, Maragoudakis ME, Misevic GN. 1993. Expression of type IV collagen degrading activity during early embryonal development in the sea urchin and the arresting effects of collagen synthesis inhibitors on embryogenesis. J Cell Biochem 52:92–106.

Laemmli UK. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) 272:680–685.

Lowry OA, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Mayne J, Robinson JJ. 1998. Calcium-protein interactions in the extracellular environment: Calcium binding, activation and immunolocalization of a collagenase/gelatinase activity expressed in the sea urchin embryo. J Cell Biochem 71:546–558.

Mayne J, Robinson JJ. 2002. Localization and functional role of a 41 kDa collagenase/gelatinase activity expressed in the sea urchin embryo. Dev Growth Differ 44:345–356.

Nomura K, Shimizu T, Kinoh H, Sendai Y, Inomata M, Suzuki N. 1997. Sea urchin hatching enzyme (envelysin): cDNA cloning and deprivation of protein substrate specificity by autolytic degradation. Biochemistry 36: 7225–7238.

Quigley JP, Braithwaite RS, Armstrong PB. 1993. Matrix metalloproteases of the developing sea urchin embryo. Differentiation 54:19–23.

Ramachandran RK, Seid CA, Lee H, Tomlinson CR. 1993. PDGF-BB and TGF-β rescue gastrulation, spiculogenesis and LpS1 expression in collagen-disrupted embryos of the sea urchin genus *Lytechinus*. Mech Dev 44:33–40.

Ranganathan L, Rimsay R, Robinson JJ. 2004. Zymogen activation and characterization of a major gelatin-cleavage activity localized to the sea urchin extraembryonic matrix. J Cell Biochem 93:1075–1083.

Robinson JJ. 1991. Protein-protein interacting and structural entities within the sea urchin extraembryonic matrix, the hyaline layer. Arch Biochem Biophys 291:126–131.

Robinson JJ. 1997. Comparative biochemical analysis of sea urchin peristome and rat tail tendon collagen. Comp Biochem Physiol 117B:307–313.

Robinson JJ. 2000. Effects of calcium and magnesium on a 41 kDa serine-dependent protease possessing collagen-cleavage activity. J Cell Biochem 80:139–145.

Robinson JJ, Sharpe C, Calloway C. 2003. Identification and partial characterization of two inducible gelatin-cleavage activities localized to the sea urchin extraembryonic matrix, the hyaline layer. Biochim Biophys Acta 1621:67–75.

Roe JL, Lennarz WJ. 1990. Biosynthesis and secretion of the hatching enzyme during sea urchin embryogenesis. J Biol Chem 265:8704–8711.

Somerville RPT, Oblander SA, Apte SS. 2003. Matrix metalloproteinases: Old dogs with new tricks. Genome Biology 4:216–216.11.

Spiegel E, Howard L, Spiegel M. 1989. Extracellular matrix of sea urchin and other marine invertebrates. J Morphol 199:71–92.

Vafa O, Nishioka D. 1995. Developmentally regulated protease expression during sea urchin embryogenesis. Mol Reprod Dev 40:36–47.

Vu TH, Werb Z. 2000. Matrix metalloproteinases: Effectors of development and normal physiology. Genes Dev 14: 2123–2133.

Wessel GM, McClay DR. 1987. Gastrulation in the sea urchin embryo requires the deposition of cross-linked collagen within the extracellular matrix. Dev Biol 121:149–165.

Wessel GM, Marchase RB, McClay DR. 1984. Ontogeny of the basal lamina in the sea urchin embryo. Dev Biol 103: 235–245.