Journal of Cellular Biochemistry 66:337–345 (1997)

Characterization of a Metalloproteinase: A Late Stage Specific Gelatinase Activity in the Sea Urchin Embryo

John J. Robinson*

Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada

Abstract We have partially purified and characterized an 87 kDa gelatinase activity expressed in later stage sea urchin embryos. Cleavage activity was specific for gelatin and no cleavage of sea urchin peristome type I collagen, bovine serum albumin or casein was detected. Magnesium and Zn^{2+} inhibited the gelatinase and Ca^{2+} protected against inhibition. Ethylenediamine tetracetic acid, ethylenebisoxyethylenenitriol tetraacetic acid and 1,10-phenanthroline were inhibitory, suggesting that the gelatinase is a Ca^{2+}- and Zn^{2+}-dependent metalloproteinase. No inhibition was detected with serine or cysteine protease inhibitors and the vertebrate matrix metalloproteinase (MMP) inhibitor, Batimastat, was also ineffective. The vertebrate MMP activator p-aminophenylmercuric acetate was without effect. These results allow us to identify both similarities and differences between echinoderm and vertebrate gelatinases. J. Cell. Biochem. 66: 337–345, 1997.

Key words: sea urchin; embryo; gelatinase; metalloproteinase

The structural role of the extracellular matrix (ECM) has long been recognized. More recently additional functional roles for the ECM have been identified and include the control of such cellular properties as shape, growth, migration and differentiation [Adams and Watts, 1993]. Four major classes of ECM molecules have been identified: 1) collagens, 2) elastin, 3) proteoglycans, and 4) structural and cell interactive glycoproteins. These molecules play distinct functional roles which are dependent upon interactions with each other, the cell surface and regulatory molecules in the extracellular space. The relationship between ECM composition and function has been studied in vertebrate systems which has led to the identification of a class of proteases, the matrix metalloproteinases (MMPs), which utilize components of the ECM as substrates. The activities of the MMPs are tightly controlled at both the transcriptional and post-transcriptional levels [Stetler-Stevenson et al., 1993]. Their cell-surface location and substrate specificities suggest that the MMPs are intimately involved in remodelling the ECM.

The sea urchin embryo is characterized by the presence of two ECMs, the hyaline layer on the apical surface and the basal lamina on the basal surface of ectoderm cells. Using antisera prepared against vertebrate ECM molecules, cross-reacting species have been identified in both the hyaline layer and basal lamina [Spiegel et al., 1989; Wessel et al., 1984]. In addition, several collagen encoding genes, expressed in the sea urchin embryo, have been cloned [Exposito et al., 1993, 1994; Wessel et al., 1991]. Collagen has been shown to be essential for gastrulation; disruption of collagen deposition with lathrytic agents resulted in the selective inhibition of gene expression and the arrest of development prior to gastrulation [Wessel et al., 1989; Wessel and McClay, 1987]. However, the extracellular signalling molecules PDGF-BB and TGF-α rescued arrested embryos and restored normal gastrulation [Ramachandran et

Abbreviations: APMA, P-aminophenylmercuric acetate; CMFSW, Ca^{2+}-, Mg^{2+}-free seawater; DTNB, 5,5′-Dithio-bis (2-nitrobenzoic acid); DTT, dithiothreitol; ECM, extracellular matrix; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylenebisoxyethylenenitriol tetraacetic acid; MMP, matrix metalloproteinase; 1,10-Phen, 1,10-phenanthroline; PMSF, phenyl methyl sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

Contract 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@morgan.ucs.mun.ca

Received 7 March 1997; accepted 15 April 1997

al., 1993; Govindarajan et al., 1995]. These results suggest that a properly constituted, collagen containing ECM is required to facilitate the signalling events necessary for gastrulation.

To date, a relatively small number of reports describing metalloproteinase activities in the sea urchin embryo have appeared. The hatching enzyme, responsible for dissolution of the fertilization membrane, has been shown to be a Ca^{2+}- and Zn^{2+}-dependent metalloproteinase [Roe and Lennarz, 1990]. Sequence analysis of the hatching enzyme gene revealed a structure similar to that of vertebrate MMPs; the gene encodes signal- and pro-peptides, a Zn^{2+}-binding catalytic domain and a hemopexin-like C-terminal domain [Ghighlione et al., 1994]. A second Zn^{2+}-dependent secreted metalloproteinase, BP-10, has been identified in the sea urchin embryo [Lhomand et al., 1996]. Several collagen and gelatin degrading metalloproteinase activities have also been identified [Quigley et al., 1993; Karakiulakis et al., 1993; Vafa and Nishioka, 1995; Mayne and Robinson, 1996]. All these species displayed developmentally regulated patterns of activity. In this report we describe the partial purification and characterization of an 87 kDa gelatinase activity which is expressed during the later stages of sea urchin embryonic development.

MATERIALS AND METHODS

Growth of Embryos

Stronglyocentrotus purpuratus (*Sp*) 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-seawater (MFSW; 0.45 µm) and fertilized with a 100-fold numerical excess 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).

Substrate Gel Zymography

Substrate gel zymography was performed essentially as described previously [Heussen and Dowdle, 1980]. Sodium dodecyl sulfate containing gels were prepared by copolymerizing acrylamide and gelatin or a test substrate at a final concentration of 0.1% (w/v). Samples of eggs, embryos, or the purified gelatinase were dispersed for 30 min at room temperature in Laemmli solubilizing solution from which both dithiothreitol (DTT) and bromophenol blue had been omitted [Laemmli, 1970]. Electrophoresis was performed at 10 mA and 4°C for 4.5 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.

Sucrose Density Gradient Ultracentrifugation

Gastrula or pluteus stage embryos were suspended to 10% (v/v) in Ca^{2+}- and Mg^{2+}-free seawater (CMFSW; 500 mM NaCl, 10 mM KCl, 2 mM $NaHCO_3$, and 3 mM Na_2SO_4), containing 50 mM Tris-HCl, pH 8.0 and 20 mM benzamidine-HCl, and were homogenized by 20 strokes in a hand-held Dounce homogenizer (type A) at 0°C. The homogenate was centrifuged at 2,000*g* for 8 min at 4°C, and the supernatant was loaded onto 10–40% (w/v) sucrose step gradients. After centrifugation at 160,000*g* for 18 h at 4°C, the gradients were fractionated and the fractions containing the 87 kDa gelatinase identified by gelatin substrate gel zymography.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [1970].

Quantitative Gelatinase Assay

This assay was based on quantitating the gelatinase-dependent cleavage of gelatin into trichloracetic acid (TCA) soluble peptides. One unit of gelatinase activity was defined as the amount of gelatinase required to cleave 0.1 µg of gelatin/min into TCA soluble peptides at 37°C. Typically, 1- to 5-µL aliquots of gelatinase were added to a reaction volume of 100 µL containing the following: 0.1% (w/v) gelatin, 50 mM Tris-HCl, pH 8.0 and 10 mM $CaCl_2$. After various periods of time at 37°C, an equal volume of ice-cold 20% (w/v) TCA was added and the pellets harvested by centrifugation and assayed for protein [Lowry et al., 1951]. In all assays, a reaction mixture consisting of the gelatinase, gelatin, Tris-HCl, and $CaCl_2$ was added to an equal volume of ice-cold 20% (w/v) TCA at zero time. The resultant pellet was used

to establish a zero time value for protein content. Percentage cleavage was determined using the formula

$$\frac{\text{OD zero time} - \text{OD 60 min}}{\text{OD zero time}} \times 100.$$

The number of units of gelatinase activity was calculated from the values of percentage cleavage. In all assays, the assay time point was within the linear range of gelatinase activity vs. time and gelatinase concentrations were within the linear range of gelatinase activity vs. gelatinase concentration.

Preparation of Collagen

Peristomes were dissected from animals, the epidermis removed by scraping and the tissue cut into small pieces. Rat tails were a gift from Dr. Sean Brosnan, Department of Biochemistry, Memorial University. The tail tendons were removed by dissection and cut into small pieces. Collagen was extracted as described by Burke et al. [1989]. Tissue pieces were homogenized and the homogenate extracted with distilled water for 16 h at 4°C. The pellet was harvested by centrifugation at 10,000*g* for 15 min at 4°C and extracted twice more with distilled water. The final pellet was suspended in 20 mL of 0.5 M acetic acid containing 0.5 mg pepsin (Worthington Biochemical Co., Freehold, NJ) and digestion allowed to proceed for 16 h at 4°C. The pH of the digestion mixture was adjusted to 7.6 with NaOH and insoluble material removed by centrifugation at 15,000*g* for 1 h at 4°C. The supernatant was made 5 M in NaCl and rotated overnight at 4°C. Protease inhibitors, benzamadine hydrochloride, phenyl methyl sulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA) were present throughout the procedure at concentrations of 20 mM, 50 µg/ml and 5 mM respectively. After salting-out with 5 M NaCl, collagen was stored in distilled water containing protease inhibitors at the final concentrations indicated above. Protein concentrations were determined using bovine serum albumin as a standard [Lowry, et al., 1951].

Quantitation of Collagen Cleavage Activity

Following SDS-PAGE fractionation of collagen the Coomassie Brilliant Blue stained α chains were excised and the dye eluted in 25% (v/v) pyridine. The eluted dye was 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 used to correct for background binding of Coomassie Brilliant Blue. Values obtained for α chains incubated in the absence of the 87 kDa species were normalised to 100%.

RESULTS

We have previously shown that the sea urchin embryo exhibits a dynamic pattern of gelatinase activity [Mayne and Robinson, 1996]; representative data are reproduced in Figure 1A. While the egg (lane 1) possesses a 41 kDa gelatinase activity which predominates during early development, later development is characterized by the emergence of a species of approximately 87 kDa (lanes 4 and 5). The dominance of this activity in the gastrula and pluteus stage embryos has prompted us to partially purify and characterize this species.

Pluteus stage embryos (lane 5, Fig. 1A) were homogenized and fractionated in a 10–40% (w/v) sucrose density gradient and the fractions analysed by gelation substrate gel zymography. One fraction contained a single gelatinase activity (Fig. 1B) which on analysis was found to comigrate with the 87 kDa gelatinase (data not shown). This fraction was further characterized. To determine the specificity of the gelatinase activity we performed substrate gel zymography utilizing gelatin, casein and bovine serum albumin as potential substrates (Fig. 2A). Cleavage activity towards gelatin was manifest (lane 1) while no activity toward casein (lane 2) or bovine serum albumin (lane 3) was detected. In quantitative assays in which gelatin cleavage activity was normalized to 100% we measured 0 and 5.2% cleavage activity with casein and bovine serum albumin, respectively. These results suggest that the 87 kDa gelatinase possesses a substrate specific cleavage activity. We have extended this work and tested two collagen species as substrates for the gelatinase. Collagen was prepared from sea urchin peristome and rat tail tendon and used to test the 87 kDa species for gelatinase and collagenase activities (Fig. 2B and 2E). In contrast to rat tail tendon type I collagen we have found that sea urchin peristome type I collagen exists as gelatin at a temperature of 37°C [Robinson, 1997]; this result is not unexpected since the sea urchin, *Strongylocentrotus purpuratus,* lives at temperatures of 12–15°C. When peristome collagen was incubated at 37°C (Fig. 2B, lanes 1

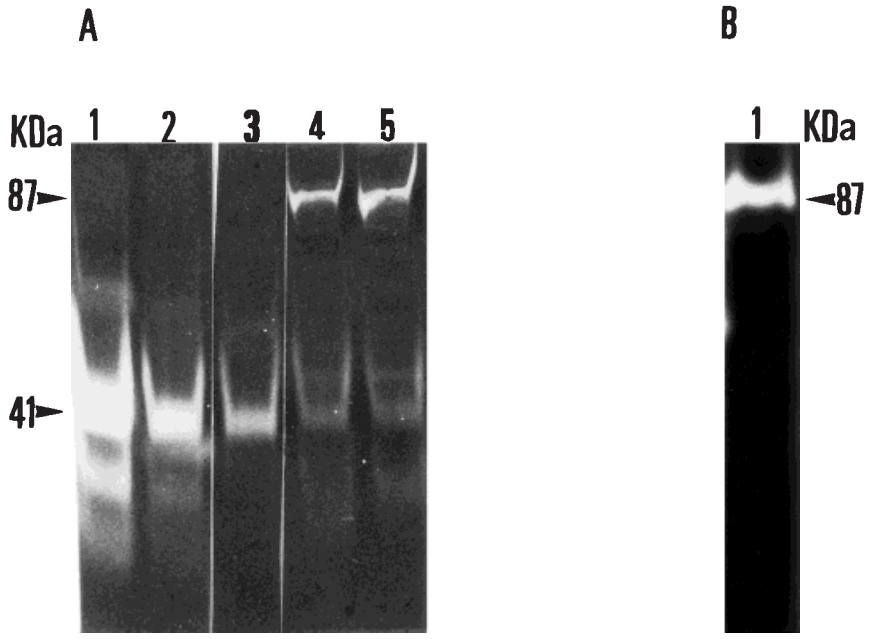

Fig. 1. Gelatin substrate gel zymographic analysis of eggs and various stage embryos (**A**) or the partially purified 87 kDa gelatinase (**B**). Aliquots (15 µg) of eggs or various stage embryos (A) or an aliquot (5 µg) of a fraction obtained from a 10–40% (w/v) sucrose density gradient, loaded with a gastrula stage embryo homogenate (B), were fractionated in a 10% (w/v) polyacrylamide gel [Laemmli, 1970] containing 0.1% (w/v) gelation. Lanes 1, 2, 3, 4 and 5 (A) represent unfertilized eggs and 1-, 21.5-, 53- and 70.5-h-old embryos respectively.

and 2) or 60°C (lanes 3 and 4) for 30 min followed by incubation at 37°C in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of the 87 kDa gelatinase, cleavage of both α chains occurred when the gelatinase was present. In contrast, incubation of the rat tail tendon collagen at 37°C in the presence (lane 5) or absence (lane 6) of the gelatinase did not result in cleavage. However, when the rat tail tendon collagen was incubated at 60°C for 30 min followed by incubation at 37°C in the presence (lane 7) or absence (lane 8) of the gelatinase, cleavage occurred when the gelatinase was present. Clearly, the 87 kDa gelatinase can utilize both peristome and rat tail tendon gelatins as substrates but has no cleavage activity towards rat tail tendon collagen. When peristome collagen was preincubated at 15°C (Fig. 2E, lanes 1 and 2) or 60°C (lane 3) followed by incubation at 15°C in the presence (lanes 2 and 3) or absence (lane 1) of the 87 kDa gelatinase no collagen cleavage activity was detected. In quantitative assays approximately 50% cleavage of the peristome collagen α chains occurred upon preincubation at 60°C while preincubation at 15°C did not result in any measurable cleavage. These results suggest that the 87 kDa gelatinase does not possess collagenase activity.

To determine if the metal ion requirements of the 87 kDa species were similar to those of vertebrate MMPs we examined the effects of Ca^{2+} and Zn^{2+} on gelatinase activity. The differences in recorded activities in the presence and absence of exogenously added Ca^{2+} were minimal (Table I). However, Mg^{2+}, a quantitatively important cation in seawater, caused partial inhibition. In the presence of equimolar amounts of Ca^{2+} and Mg^{2+} the loss of activity was significantly reduced, suggesting a protective role for Ca^{2+}. Concentrations of Zn^{2+} as low as 10 µM were inhibitory and again Ca^{2+} effectively protected the gelatinase from inhibition. To further explore the metal ion requirements for activity we assayed the gelatinase in the presence and absence of either the divalent metal ion chelator EDTA or the Zn^{2+}-specific chelator 1, 10-phenanthroline (Table II). Upon preincubation and assay in the presence of 5 mM EDTA, gelatinase activity was reduced to 31.8%. If both Ca^{2+} (10 mM) and EDTA (5 mM) were present in the assay mixture activity was restored to 72.6% of the control value. If both Ca^{2+} and EDTA were omitted from the assay mixture, preincubation with EDTA suppressed gelatinase activity to 36.8% of the control value, suggesting that the trace amounts of Ca^{2+} pres-

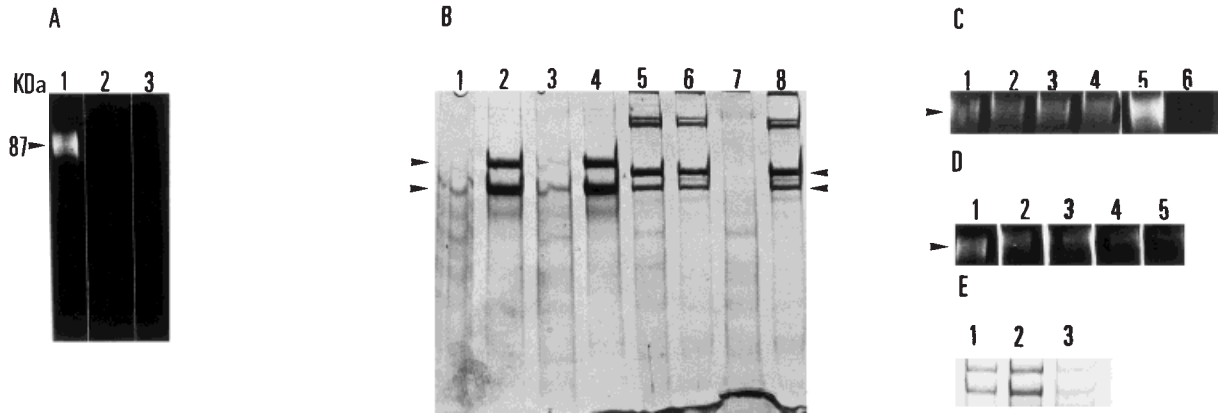

Fig. 2. Substrate specificity (A), gelatinase and collagenase activities (B and E), and inhibition of the gelatinase activity (C and D) of the 87 kDa species. **A:** Aliquots (2 µg) of the partially purified gelatinase were analysed by substrate gel zymography in gels containing gelatin (lane 1), casein (lane 2) or bovine serum albumin (lane 3). **B:** Aliquots (15 µg) of peristome (lanes 1–4) or rat tail tendon (lanes 5–8) collagens in 50 mM Tris-HCl, pH 8.0, were incubated at 37°C (lanes 1, 2, 5 and 6) or 60°C (lanes 3, 4, 7 and 8) for 30 min followed by incubation at 37°C for 16 h in the presence (lanes 1, 3, 5 and 7) or absence (lanes 2, 4, 6 and 8) of 1 µg of gelatinase. Reactions were stopped by the addition of an equal volume of ice-cold 20% (w/v) TCA and the samples fractionated in an 8% (w/v) polyacrylamide gel [Laemmli, 1970]. **E:** Aliquots (15 µg) of peristome collagen in 50 mM Tris-HCl, pH 8.0, were incubated at 15°C (lanes 1 and 2) or 60°C (lane 3) followed by incubation at 15°C for 16 h in the presence (lanes 2 and 3) or absence (lane 1) of 1 µg of gelatinase. Reactions were stopped by the addition of an equal volume of ice-cold 20% (w/v) TCA and the samples fractionated in an 8% (w/v) polyacrylamide gel. **C:** Aliquots (2 µg) of gelatinase were solubilized for gelatin substrate gel zymography as described in Materials Methods. During solubilization DTNB, Batimastat or DTT was present (lanes 1, 3 and 6) at 0.5 mM, 0.1 mM or 10 mM. Following electrophoresis the gels were processed as described in Materials Methods. **D:** Aliquots (2 µg) of gelatinase were analyzed by gelatin substrate gel zymography. Following electrophoresis individual lanes were excised and processed as described in Materials and Methods except that $CaCl_2$ was omitted and EDTA was present at 0 (lane 1), 5 mM (lane 2), 10 mM (lane 3), 20 mM (lane 4) or 30 mM (lane 5).

ent in our solutions were insufficient to restore the lost activity. The inhibitory effect of EDTA was also seen in our qualitative gelatin substrate gel zymogram (Fig. 2D). Inhibition was observed as the EDTA concentration was increased between 0 (lane 1) and 30 mM (lane 5). Following preincubation and assay in the presence of 2 mM 1,10-phenanthroline gelatinase activity was reduced to 35.7% of the control value and this loss of activity could be partially reversed by Ca^{2+}. When the chelator was added directly to the assay mixture, without preincubation with the gelatinase, less inhibition was observed; 44.6% with EDTA and 44.7% with 1,10-phenanthroline. Again the presence of exogenously added Ca^{2+} significantly protected the gelatinase from inhibition. Collectively, these results suggest that the 87 kDa gelatinase requires both Ca^{2+} and Zn^{2+} for activity.

To further explore the role of Ca^{2+} we quantitated reconstitution of the ethylenebisoxyethylenenitriol tetraacetic acid (EGTA)-inhibited gelatinase in the presence of increasing concentrations of this cation (Fig. 3). The inhibited enzyme retained approximately 18% activity and the lost activity was progressively restored to 85% of the uninhibited value upon incubation with increasing concentrations of Ca^{2+}. The apparent dissociation constant for reactivation was 95 µM. This value is well below the calcium concentration present in seawater (10 mM). These data clearly define a functional role for Ca^{2+} but do not distinguish between structural and catalytic mechanisms of action.

TABLE I. Effects of Ca^{2+}, Mg^{2+}, and Zn^{2+} on the 87 kDa Gelatinase Activity

$CaCl_2$	$MgCl_2$		$ZnCl_2$		%
10 mM	10 mM	10 µM	50 µM	100 µM	Activity
+	−	−	−	−	100
−	−	−	−	−	95.6 ± 7.7
−	+	−	−	−	53.3 ± 4.5
+	+	−	−	−	82.1 ± 6.2
−	−	+	−	−	66.0 ± 5.2
−	−	−	+	−	46.4 ± 3.7
−	−	−	−	+	11.3 ± 4.7
+	−	−	+	−	87.4 ± 4.2

The quantitative gelatinase assay was used. Values represent means ± SEM (n = 5 for assays of gelatinase activity ± $CaCl_2$ and n = 3 for all other assays).

TABLE II. Effects of Metal-Ion Chelators on Gelatinase Activity

	Preincubated with chelator		No preincubation with chelator		
$CaCl_2$	EDTA	1,10-Phen	EDTA	1,10-Phen	% Activity
+	−				72.8 ± 6.0
−	−				36.8 ± 5.8
+	+				72.6 ± 5.2
−	+				31.8 ± 4.2
+		−			65.5 ± 6.5
−		−			39.2 ± 5.1
+		+			62.5 ± 1.5
−		+			35.7 ± 5.0
+			+		86.2 ± 7.5
−			+		55.4 ± 4.2
+				+	88.5 ± 5.5
−				+	55.3 ± 6.0

Values represent means ± SEM (n = 4).
In some experiments the gelatinase was preincubated with the chelator for 15 min at room temperature and then added to the assay mixture which contained $CaCl_2$ (10 mM) and/or chelator (5 mM) as indicated above. When assays were performed without preincubation, the chelator and/or $CaCl_2$ were added directly to the assay mixture. All values are calculated relative to controls which are normalized to 100% activity and did not contain chelators.

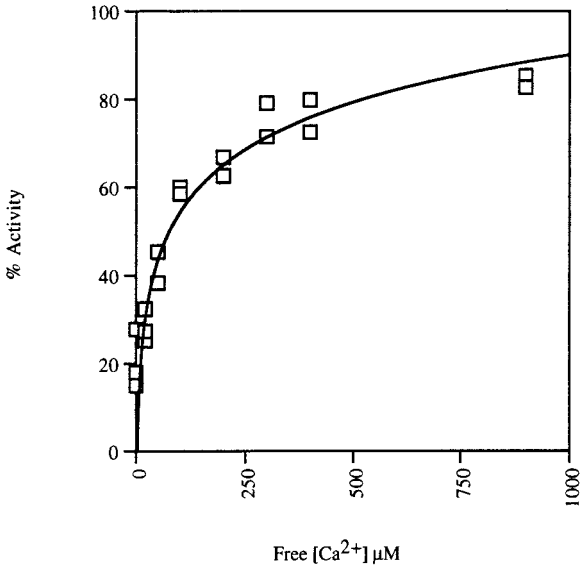

Fig. 3. Reactivation of the EGTA-inhibited gelatinase. Partially purified gelatinase was dialysed against 50 mM Tris-HCl, pH 8.0 containing 0.1 mM EGTA for 24 h at 4°C. After dialysis aliquots of the gelatinase were diluted to 80 µl in 50 mM Tris-HCl, pH 8.0 containing 0.1 mM EGTA and varying amounts of $CaCl_2$ were added to give the free Ca^{2+} concentrations indicated. After 2 h incubation on ice gelatinase activity was determined using the quantitative assay. As a control an aliquot of the gelatinase was dialysed and incubated in the absence of EGTA, assayed in the presence of 10 mM $CaCl_2$ and this activity normalized to 100%. Free Ca^{2+} concentrations were determined using the computer program EQCAL from BIOSOFT, Cambridge, England.

The biochemical nature of the gelatin cleavage activity was examined by treating the 87 kDa species with serine and cysteine protease inhibitors (Table III). The cysteine protease inhibitor 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) had no effect on gelatin cleavage activity while the serine protease inhibitors, Benzamidine and PMSF, were also ineffective; in fact PMSF appeared to activate the gelatinase activity about 1.26-fold. To compare the echinoderm 87 kDa gelatinase with vertebrate MMPs we incubated the gelatinase with p-aminophenylmercuric acetate (APMA), a known activator, or Batimastat, a known inhibitor, of the Zn^{2+}-requiring vertebrate MMPs. Batimastat, at a concentration of 0.1 mM, had no effect on gelatinase activity. Batimastat is known to inhibit vertebrate MMPs by chelating the catalytic Zn^{2+} ion at the active site. APMA activates vertebrate MMPs by inducing autoproteolytic activity in the latent MMP; this activation may involve a displacement of the active site Zn^{2+} from a ligand interaction with a cysteine residue in the cleaved prodomain. Preincubation of the gelatinase with APMA had no effect on activity. In additional experiments in which eggs and various stage embryos were incubated in the presence or absence of 2 mM APMA and analysed by gelatin substrate gel zymography no differences in the activity of the 87 kDa species were observed (data not shown). In gelatin substrate gel zymographic analysis both DTNB and Batimastat were ineffective at inhibiting activity (Fig. 2C, lanes 1–4) while the reducing agent dithiothreitol completely abolished activity (lanes 5 and 6).

DISCUSSION

Studies on vertebrate MMPs have identified a broad range of proteolytic activities which utilize a variety of native or denatured extracellular matrix components as substrates. MMPs are involved in controlled, connective tissue remodelling, an event important to several physiological process including embryonic development, wound healing and pregnancy [Chen, 1992; Matrisian, 1992]. The physiological importance of the MMPs is reflected in the multiple level controls exerted on their activities. Many

TABLE III. Effects of Various Reagents on the Activity of the 87 kDa Gelatinase

Reagent	% Activity
Control	100
DTNB	101.3 ± 10.5
Benzamidine	103.8 ± 6.2
PMSF	126.5 ± 8.6
APMA	97.6 ± 5.3
Batimastat	92.7 ± 4.3

Values represent means ± SEM (n = 3).
Aliquots of the 87 kDa gelatinase were incubated at room temperature for 30 min in the presence or absence (control) of DTNB (1 mM), Benzamidine (5 mM), PMSF (0.5 mM), APMA (2 mM) or Batimastat (0.1 mM). Following incubation the aliquots of gelatinase were added to the standard, quantitative assay mixture containing 10 mM $CaCl_2$. In the case of Benzamidine, PMSF and Batimastat the assay mixtures also contained these reagents at the final concentrations indicated above. All values are normalized to controls which were incubated and assayed in the absence of added reagents.

MMPs have been found to be Zn^{2+}- and Ca^{2+}-dependent endopeptidases, the Zn^{2+} being chelated at the active site and fulfilling a catalytic role while the Ca^{2+} may be involved in stabilizing the structure of the protease and/or its proteinaceous substrate. The Ca^{2+} and Zn^{2+} dependencies of these enzymes have been established by their susceptibility to inhibition by EDTA and 1,10-phenanthroline and in addition a conserved Zn^{2+}-binding site has been identified from primary sequence data.

Several laboratories have utilized substrate gel zymography and reported species specific and developmentally regulated patterns of gelatinase activity during sea urchin embryonic development [Quigley et al., 1993; Vafa and Nishioka, 1995; Mayne and Robinson 1996]. Using methodology similar to that employed in studies of vertebrate MMPs the metalloprotein nature of the sea urchin gelatinases has been established; thus most of the gelatinases expressed in the sea urchin embryo have been shown to be inhibited by both EDTA and 1,10-phenanthroline and therefore classified as Ca^{2+}- and Zn^{2+}-dependent proteases. In addition, we have purified a 41 kDa gelatinase, present in the sea urchin egg, and found it to be Ca^{2+}- but not Zn^{2+}-dependent [Mayne and Robinson, 1996]. In the present study we have examined the metal ion requirements of the late stage specific 87 kDa gelatinase. Sensitivity to both EDTA and 1,10-phenanthroline suggests requirements for Ca^{2+} and Zn^{2+} although concentrations of exogeneously added Zn^{2+} as low as 10 μM were inhibitory.

The inhibitory effect of Zn^{2+} is probably due to its binding at non-specific metal ion binding sites containing N or S ligands rather than to a Ca^{2+}-specific binding site. Assays in the presence and absence of exogenous Ca^{2+} did not result in any significant difference in gelatinase activity, indicating that activating Ca^{2+} is tightly bound to the enzyme. Since Ca^{2+} can rescue the 1,10-phenanthroline-inhibited gelatinase it seems likely that the Zn^{2+}-binding site can also accommodate Ca^{2+}. Based on ionic radii and hydration energies Ca^{2+} and Zn^{2+} would not be expected to bind to the same site(s) on the gelatinase [Vyas et al., 1989]. The results reported here suggest that in the presence of 10 mM Ca^{2+} the unoccupied Zn^{2+}-binding site on the 1,10-phenanthroline-inhibited gelatinase can accommodate Ca^{2+} and that Ca^{2+} bound at this site(s) can duplicate the functional role played by Zn^{2+}. However, since the gelatinase, isolated from gastrula or pluteus stage embryos, is 1,10-phenanthroline sensitive the high concentration of Ca^{2+} (10 mM) present in sea water cannot displace the bound Zn^{2+}.

At present primary structural data for invertebrate MMPs are scant. The sea urchin hatching enzyme gene has been sequenced and found to have considerable sequence and organizational homologies with the vertebrate MMPs [Ghighlione et al., 1994]. A second Zn^{2+} metalloproteinase, BP-10, secreted by the blastula stage embryo contains a conserved active site domain but is otherwise unrelated to vertebrate MMPs [Lhomand et al., 1996]. It seems likely that echinoderms contain at least two classes of metalloproteinases of which only one is closely related to the vertebrate MMPs. In this context while the 87 kDa gelatinase appears to be a Ca^{2+}- and Zn^{2+}-dependent metalloproteinase it is not inhibited by Batimastat, a known inhibitor of vertebrate Zn^{2+}-dependent metalloproteinases [Grams et al., 1995]. The inhibitory action of Batimastat is due to its chelation with the active site Zn^{2+}; in the case of the 87 kDa gelatinase, this interaction appears to be precluded. In addition, when early stage embryos or the partially purified gelatinase were incubated with the organomercurial APMA, no activation of this enzyme occurred. Similar results have been reported from other laboratories [Quigley et al., 1993; Karakiulakis et al., 1993].

Collectively, these results suggest that while echinoderms express Ca^{2+}- and Zn^{2+}-dependent, matrix degrading proteases at least some of these activities are not likely activated by the cysteine switch mechanism common to vertebrate MMPs. Further delineation of the similarities and differences between vertebrate and echinoderm metalloproteinases awaits more detailed biochemical analysis.

Gastrula and pluteus stage sea urchin embryos undergo considerable shape changes driven by realignments of cell-cell and cell-ECM contacts. The compositions of both the hyaline layer and basal lamina are dynamic throughout embryonic development [Wessel et al., 1984; Robinson, 1990; Brennan and Robinson, 1994] and remodelling of these ECMs should generate changing cell surface-ECM interactions. The timing of appearance of the 87 kDa gelatinase is consistent with an involvement in the regulation of ECM composition in the gastrula and pluteus stage embryos. In contrast with the 41 kDa gelatinase/collagenase present in the sea urchin [Robinson, 1997] the 87 kDa species did not possess cleavage activity with peristome type I collagen. This result is similar to that seen with some vertebrate MMPs: the 72 kDa gelatinase has been shown to possess collagenolytic activity with interstitial type I collagen while the 92 kDa gelatinase was unable to cleave type I collagen [Aimes and Quigley, 1995]. The 87 kDa gelatinase probably regulates collagen composition in concert with one or more collagenases by cleaving collagen-derived peptide fragments generated by the collagenase(s).

ACKNOWLEDGMENTS

I thank Jackie Dalton and Betty Ann Lewis for typing the manuscript and Suresh Belkhode, Karl Bruff and Melanie Green for performing some preliminary experiments. Batimastat was a gift from British Biotech Pharmaceuticals Limited, Oxford, England.

REFERENCES

Adams JC, Watts FM (1993): Regulation of development and differentiation by the extracellular matrix. Development 117:1183–1198.

Aimes RT, Quigley JP (1995): Matrix metallproteinase-2 is an interstitial collagenase. J Biol Chem 270:5872–5876.

Brennan C, Robinson JJ (1994): Cloning and characterization of HLC-32, a 32 kDa protein component of the sea urchin extraembryonic matrix, the hyaline layer. Dev Biol 165:556–565.

Burke RD, Bouland C, Sanderson AI (1989): Collagen diversity in the sea urchin *Strongylocentrotus purpuratus*. Comp Biochem Physiol 94B:41–44.

Chen WT (1992): Membrane proteases: Role in tissue remodelling and tumor invasion. Cur Opin Cell Biol 4:802–809.

Exposito J-Y, D'Allesio M, DiLiberto M, Ramirez F (1993): Complete primary structure of a sea urchin type IV collagen α chain and analysis of the 5′ end of its gene. J Biol Chem 268:5249–5254.

Exposito J-Y, Suzuki H, Geourjon C, Garrone R, Solursh M, Ramirez F (1994): Identification of a cell lineage-specific gene coding for a sea urchin α 2(IV)-like collagen chain. J Biol Chem 269:13,167–13,171.

Ghighlione C, Lhomand G, LePage T, Gache C (1994): Structure of the sea urchin hatching enzyme gene. Eur J Biochem 219:845–854.

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.

Grams F, Crimmin M, Hines L, Huxley P, Pieper M, Tschesche H, Bode W (1995): Structure determination and analysis of human neutrophil collagenase complexed with a hydroxamate inhibitor. Biochemistry 34:14,012–14,020.

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

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 272: 680–685.

Lhomand G, Ghighlione C, LePage T, Gache C (1996): Structure of the gene encoding the sea urchin blastula protease 10 (BP10), a member of the ostacin family of Zn^{2+}-metalloproteases. Eur J Biochem 238:747–751.

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

Matrisian LM (1992): The matrix degrading metalloproteinases. BioEssays 14:455–462.

Mayne J, Robinson JJ (1996): Purification and metal ion requirements of a candidate matrix metalloproteinase: A 41 KDa gelatinase activity in the sea urchin embryo. Biochem Cell Biol 74:211–218.

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 TFG-α rescue gastrulation, spiculogenesis and LpSl expression in collagen—disrupted embryos of the sea urchin genus *Lytechinus*. Mech Dev 44:33–40.

Robinson JJ (1990): Polypeptide composition and organisation of the sea urchin extraembryonic hyaline layer. Biochem Cell Biol 68:1083–1089.

Robinson JJ (1997): Comparative biochemical analysis of sea urchin peristome and rat tail tendon collagen. Comp Biochem Physiol (in press).

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

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

Stetler-Stevenson WG, Liotta LA, Kleiner DE (1993): Extracellular matrix 6: Role of matrix metalloproteinases in tumor invasion and metastasis. FASEB J 7:1434–1441.

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

Vyas MN, Jacobson BL, Quiocho FA (1989): The calcium binding site in the galactose chemoreceptor protein. J Biol Chem 264:20,817–20,821.

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.

Wessel GM, Zhang W, Tomlinson CR, Lennarz WJ, Klein WH (1989): Transcription of the spec I-like gene of *Lytechinus* is selectively inhibited in response to disruption of the extracellular matrix. Development 106:355–365.

Wessel GM, Etkin M, Benson S (1991): Primary mesenchyme cells of the sea urchin embryo require an autonomously produced nonfibrillar collagen for speculogenesis. Dev Biol 148:261–272.