J. Biochem. 126, 912-916 (1999)

Stage-Specific Inhibition of *Xenopus* Embryogenesis by Aprotinin, a Serine Protease Inhibitor[1]

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Received July 23, 1999; accepted September 1, 1999

We examined the effects of various protease inhibitors on *Xenopus laevis* embryogenesis. Aprotinin, a serine protease inhibitor, was found to inhibit embryogenesis markedly, but other protease inhibitors had virtually no effect. The inhibitory effect of aprotinin was specific for embryos at the blastula or gastrula stage. These results suggest that an aprotinin-sensitive protease involved in embryonic development is secreted from the embryos or appears on the surface of embryonic cells at these stages. We found that various serine proteases are in fact secreted from the embryos with their development and that some of them are sensitive to aprotinin.

Key words: aprotinin, embryogenesis, protease inhibitor, serine protease, *Xenopus laevis*.

Embryogenesis takes place in a three-dimensional space, so spatial interactions between cells and cells and/or cells and extracellular matrix substances are important for subsequent cell fate specification, cell migration, and embryonic morphogenesis. In these interactions, pericellular or extracellular matrix (ECM) proteinases are assumed to play crucial roles because these enzymes can create irreversible changes in the cellular microenvironment (*1*). Matrix metalloproteinases (MMPs) have been shown to be expressed widely during embryonic development of various organisms (*2-5*). As MMPs are known to digest ECM, they are thought to play crucial roles in embryonic development (*6-8*).

Proteinases have been shown to be involved in the development of adult structures in insects. In *Sarcophaga peregrina* (flesh fly), digestion of the basement membrane of imaginal discs by cathepsin L secreted from the discs was found to be essential for their differentiation (*9, 10*), whereas in the imaginal discs of *Drosophila melanogaster*, a membrane-bound serine protease was shown to be implicated in the degradation of ECM, resulting in induction of morphogenesis (*11*).

We have been interested in the proteinases that might participate in the embryogenesis of *Xenopus laevis*, and in this study we examined the effects of various proteinase inhibitors on the development of *Xenopus* embryos. Of these inhibitors, aprotinin, an inhibitor of serine proteinase, was found to be a potent and selective inhibitor of *Xenopus* embryonic development in a stage-specific manner. These results suggest that an aprotinin-sensitive serine protease is involved in the development of *Xenopus* embryos.

MATERIALS AND METHODS

Embryos and Their Culture—Embryos of *X. laevis* were used throughout the experiments. The methods used for collecting *Xenopus* embryos and their culture were essentially the same as described previously (*12*). Briefly, unfertilized eggs were collected from female frogs after injection of 600 U of a gonadotropic hormone (Gonatoropin; Teikokuzoki), and artificially fertilized in 0.5×De Beer solution (1×De Beer solution: 0.11 M NaCl, 1.3 mM KCl, 0.44 mM $CaCl_2$; pH adjusted to 7.4 with $NaHCO_3$ solution). Fertilized eggs were dejellied by treatment with 2% (w/v) cysteine-HCl solution (pH 7.9), and then cultured in 10% (v/v) Steinberg solution (1×Steinberg solution: 60 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 3 mM HEPES-NaOH, pH 7.4). Under our culture conditions, control embryos reached the 4-cell, morula (Nieuwkoop and Faber stage 7), blastula (stage 8-9), gastrula (stage 10-12), neurula (stage 17-20), tailbud (stage 26-34), and tadpole stages in about 2, 5, 8, 15, 30, 48, and 60 h after fertilization, respectively (*13*).

Protease Inhibitors—The protease inhibitors used were: aprotinin (serine proteinase inhibitor, SIGMA); antipain (serine or cysteine proteinase inhibitor, Peptide Institute); leupeptin (serine or cysteine proteinase inhibitor, Peptide Institute); *N*-[*N*-(LO,3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine (E-64) (cysteine proteinase inhibitor, Peptide Institute) and phosphoramidon (metalloproteinase inhibitor, Peptide Institute).

Assay of Protease Activity—Protease activity was assayed with various peptidyl-MCA substrates (Peptide Institute). Reactions were performed in 0.2 ml of 10% (v/v) Steinberg solution, pH 7.4, containing a 50 μM peptidyl-

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Sports, Science and Culture of Japan and by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST).

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Abbreviations: Boc, *t*-butyloxycarbonyl; Bz, benzoyl; Glt, glutaryl; MCA, 4-methyl-coumaryl-7-amide; OBzl, benzyl; Pyr, pyroglutamyl; Suc, succinyl; Suc(OMe), *N*-methoxysuccinyl; Z, carbobenzoxy.

MCA substrate, and concentrated culture medium. After incubation for 22 min at 27°C, the reaction was terminated by adding 0.2 ml of 17% (v/v) aqueous acetic acid. Fluorescence was measured using excitation and emission wavelengths of 380 and 460 nm, respectively. One unit of protease activity was defined as the amount that hydrolyzed 1 μmol of peptidyl-MCA substrate per min under these conditions. To prepare concentrated culture medium, the medium was harvested when embryos reached the gastrula stage and concentrated to 1/1,000 using ultrafiltration and centricon 10 (amicon).

RESULTS

Effect of Protease Inhibitors on the Development of Xenopus Embryos—To investigate the effects of protease inhibitors on the embryogenesis of *X. laevis*, we added various protease inhibitors to the culture medium containing embryos at the morula stage and monitored their development with time. As shown in Table I, aprotinin was found to inhibit embryogenesis markedly, and no embryos reached the neurula stage when 3.2 μM aprotinin was present. No appreciable inhibitory effect was detected with other inhibitors even at much higher concentrations. As shown in Fig. 1, development of embryos was normal until the gastrula stage even in the presence of 3.2 μM aprotinin, but the development suddenly stopped at this stage and could not proceed to the neurula stage. Other inhibitors (leupeptin at 400 μM, and E-64 and phosphoramidon, each at 10 mM) had almost no effect, and tadpoles emerged with almost the same survival ratio as the controls (no addition). Antipain did not inhibit gastrulation at 400 μM and embryos reached the neurula stage. Effect of this inhibitor on the latter developmental stages was not examined, because this experiment was done separately.

Considering that aprotinin is a serine protease inhibitor, one or more serine proteases are likely to be involved in the development of *Xenopus* embryos. However, other serine proteinase inhibitors, leupeptin and antipain, did not inhibit embryogenesis appreciably, suggesting that the target protease is extremely sensitive to aprotinin. As shown in Fig. 2A, yolk plugs of control embryos became smaller 18 h after fertilization and the embryos developed to the late gastrula stage, whereas the size of yolk plugs of aprotinin-treated embryos did not change, indicating that the embryos were still at the early gastrula stage (Fig. 2B). Control embryos reached the neurula stage, and the neural fold appeared 30 h after fertilization (Fig. 2C), whereas the aprotinin-treated embryos stopped developing at the late gastrula stage and no further development was observed (Fig. 2D). These results suggest that aprotinin inhibits gastrulation in *Xenopus* embryogenesis.

Developmental Stage Dependency of Aprotinin Inhibition—It is clear that aprotinin inhibits *Xenopus* embryonic development. To determine the developmental stage of embryos that is sensitive to aprotinin, we collected embryos at the 4-cell stage and morula stage and examined the effect of aprotinin on their development. As shown in Fig. 3, addition of aprotinin at the 4-cell stage arrested the development of embryos at the blastula stage, whereas addition at the morula stage arrested embryonic development at the gastrula stage. These results indicate that aprotinin is effective when embryos are at the blastula and gastrula stages. When embryos were much younger, the effect of aprotinin was not obvious. Aprotinin was not toxic to tadpoles and did not induce any appreciable morphological changes.

As the effect of aprotinin was highly significant compared with those of other serine proteinase inhibitors, there remained a possibility that an unknown substance contaminating aprotinin had interfered with embryonic development. To exclude this possibility, we further fractionated commercial aprotinin using HPLC. As is evident from Fig. 4, a major single peak was eluted from a C_{18} column with a minor shoulder, and the protein in the major peak (aprotinin) was found to show the same inhibitory activity as the original aprotinin, with no appreciable activity detected in other fractions, indicating that aprotinin itself inhibits *Xenopus* embryonic development.

Detection of Protease Activities in the Culture Medium—As aprotinin is a protein with a molecular mass of 6.5 kDa, it is unlikely to be able to penetrate into cells. Therefore,

TABLE I. **Effects of various protease inhibitors on *Xenopus laevis* embryos.**

Protease inhibitor	Concentration	Numbers of embryos reaching the neurula stage
Aprotinin	80 μM	0/25
	16 μM	0/25
	3.2 μM	0/25
	0.64 μM	24/25
E-64	10 mM	27/30
	2 mM	29/30
Phosphoramidon	10 mM	24/25
	2 mM	24/25
Leupeptin	10 mM	19/20
	2 mM	19/20
	0.4 mM	19/20
Antipain	0.4 mM	18/18
Control		29/30

Protease inhibitors were added to the morula stage embryos and cultured for 1 day. Values are numbers of embryos reaching the neurula stage *vs.* numbers of embryos examined.

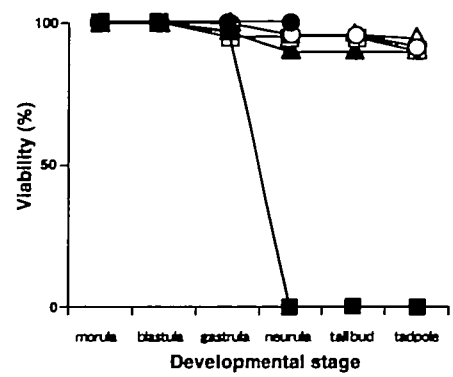

Fig. 1. **Effect of protease inhibitors on the development of *Xenopus* embryos.** Embryos at the morula stage were cultured in the presence of protease inhibitors. The protease inhibitors used were: 3.2 μM aprotinin (■), 400 μM leupeptin (□), 400 μM antipain (●), 10 mM E-64 (▲), and 10 mM phosphoramidon (○). Control embryos (△) were cultured in the absence of inhibitors. The numbers of embryos reaching the indicated stages were counted to calculate the percentage viability. In each experiment, 20–30 embryos were used.

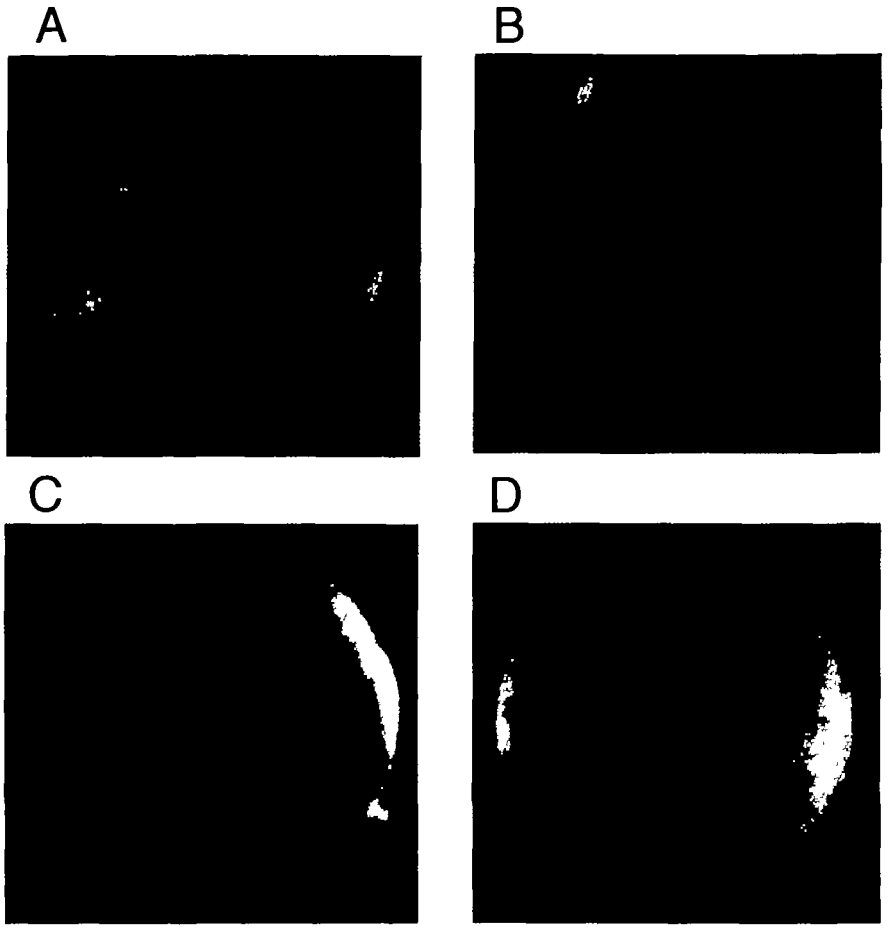

Fig. 2. **Morphologies of aprotinin-treated *Xenopus* embryos.** Aprotinin at a final concentration of 3.2 μM was added to morula stage embryos (5 h after fertilization) and cultured at 21°C. (A) and (B) are embryos 18 h after fertilization, cultured in the absence and presence of aprotinin, respectively. (C) and (D) are embryos 30 h after fertilization, cultured in the absence and presence of aprotinin, respectively.

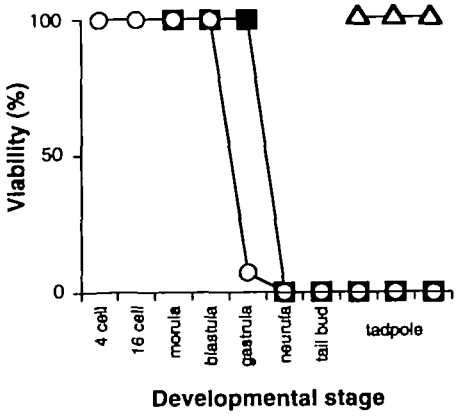

Fig. 3. **Stage specificity of the inhibition of embryonic development by aprotinin.** Aprotinin at a final concentration of 3.2 μM was added to culture medium when embryos were at the 4-cell (○), morula (■), and tadpole (△) stages, respectively, and culture was continued. The embryos and tadpoles were examined for their development in comparison with controls. In each experiment, 6–15 embryos were used.

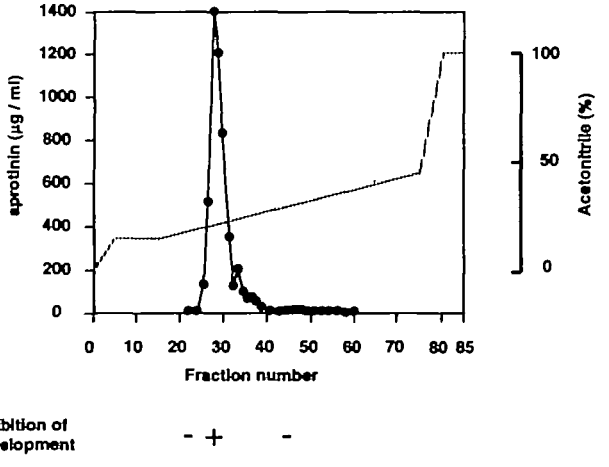

Fig. 4. **Analysis of commercial aprotinin by HPLC.** Commercial aprotinin was applied to a reverse-phase HPLC column (C_{18}) and eluted with a linear gradient of 15–45% solution B (0.1% trifluoroacetic acid in acetonitrile) in solution A (0.1% trifluoroacetic acid in H_2O). Flow rate was 1 ml/min. The amount of aprotinin in each fraction was estimated from the intensity of the band on SDS polyacrylamide gel electrophoresis. Effects on embryonic development were assayed using a fixed amount of fractions 23, 30, and 45 and expressed as effective (+) or non-effective (−). The dashed line indicates the concentration of acetonitrile (%).

any aprotinin-specific proteinase would be extra-cellular or membrane-bound. To examine whether proteinases are secreted from embryos during their development, we assayed proteinase activity in the culture medium using various substrates for serine proteinase. For this, we collected culture medium when embryos reached the

TABLE II. **Protease activities in the culture medium.**

Substrate	Activity (unit[a]/mg)	Activity in the presence of 3 μM aprotinin relative to control (%)
Suc-Gly-Pro-MCA	2.1×10^{-3}	97±5
Boc-Glu(OBzl)-Ala-Arg-MCA	1.1×10^{-3}	24±0
Boc-Gln-Ala-Arg-MCA	9.2×10^{-4}	11±0
Pyr-Arg-Thr-Lys-Arg-MCA	8.2×10^{-4}	18±1
Pro-Phe-Arg-MCA	7.5×10^{-4}	37±7
Boc-Arg-Val-Arg-Arg-MCA	5.0×10^{-4}	61±4
Boc-Leu-Ser-Thr-Arg-MCA	4.9×10^{-4}	62±4
Suc-Ala-Ala-Pro-Phe-MCA	4.7×10^{-4}	79±1
Boc-Leu-Arg-Arg-MCA	3.9×10^{-4}	52±1
Boc-Gln-Arg-Arg-MCA	2.9×10^{-4}	4±0
Suc-Ala-Ala-Ala-MCA	2.5×10^{-4}	104±2
Boc-Phe-Ser-Arg-MCA	2.3×10^{-4}	56±2
Boc-Asp(OBzl)-Pro-Arg-MCA	1.7×10^{-4}	
Suc-Ala-Pro-Ala-MCA	1.6×10^{-4}	
Boc-Leu-Thr-Arg-MCA	1.2×10^{-4}	
Boc-Leu-Gly-Arg-MCA	N.D.[b]	
Boc-Ala-Gly-Pro-Arg-MCA	N.D.	
Boc-Gln-Gly-Arg-MCA	N.D.	
Boc-Glu-Lys-Lys-MCA	N.D.	
Boc-Ile-Glu-Gly-Arg-MCA	N.D.	
Suc-Ala-Glu-MCA	N.D.	
Boc-Val-Leu-Lys-MCA	N.D.	
Bz-Arg-MCA	N.D.	
Glt-Gly-Arg-MCA	N.D.	
Pyr-Gly-Arg-MCA	N.D.	
Suc(OMe)-Ala-Ala-Pro-Val-MCA	N.D.	
Suc-Leu-Leu-Val-Tyr-MCA	N.D.	
Z-Phe-Arg-MCA	N.D.	
Z-Pyr-Gly-Arg-MCA	N.D.	

[a]One unit was defined as the amount of enzyme that hydrolyzed 1 μmol of peptidyl-MCA substrate/min. [b]N.D., not detected ($<1.0\times10^{-4}$ unit/mg).

gastrula stage, concentrated it, and used it for proteinase assay. As shown in Table II, 15 of 29 peptidyl-MCA substrates tested were hydrolyzed, indicating that significant amounts of proteinases were secreted into the medium under our culture conditions. In particular, Suc-Gly-Pro-MCA, Boc-Glu(OBzl)-Ala-Arg-MCA, and Boc-Gln-Ala-Arg-MCA were efficiently hydrolyzed. Hydrolysis of Boc-Glu(OBzl)-Ala-Arg-MCA, Boc-Gln-Ala-Arg-MCA, Pyr-Arg-Thr-Lys-Arg-MCA, Pro-Phe-Arg-MCA, and Boc-Gln-Arg-Arg-MCA were inhibited in the presence of 3 μM aprotinin to give less than 50% of maximum activity, suggesting that some proteinases are sensitive to aprotinin. The proteinase that hydrolyzed Boc-Gln-Arg-Arg-MCA seemed to be extremely sensitive to aprotinin, although its total activity was not so high.

DISCUSSION

The results we obtained in this study suggested that a serine protease extremely sensitive to aprotinin participates in embryogenesis of *X. laevis*, because only aprotinin was shown to interfere with the development of embryos. The inhibitory effect of aprotinin was limited to embryos at the blastula or gastrula stages. Judging from the stage specificity of aprotinin inhibition, we assume that the aprotinin-sensitive protease plays a role in gastrulation. During gastrulation, cells are known to show marked migration (*14, 15*). Possibly, the aprotinin-sensitive protease makes it easier for the cells to migrate by proteolysis of ECM and/or cell surface proteins. Recently, Vallet *et al.* reported that the reduction of sodium transport in A6 cells by aprotinin was due to inhibition of the channel activating protease (CAP1) that belongs to serine protease family (*16*). If activation of the sodium channels is needed for gastrulation, CAP1-like protease is thought to participate in this process, and the aprotinin-sensitive proteinase could be the CAP1-like proteinase itself.

We demonstrated that *Xenopus* embryos secrete various proteinases during their development, and that some of them are inhibited by aprotinin at a concentration of 3 μM, which is the same concentration that inhibits embryonic development. These proteases may be candidates for the aprotinin-sensitive proteinase. However, it is uncertain whether the aprotinin-sensitive proteinase is secreted into the medium or whether it stays on the surface of embryonic cells at this stage.

It has been reported that MMPs are implicated in degradation of the ECM during mammalian or amphibian embryogenesis (*6, 7, 17*). However, phosphoramidon, a metalloproteinase inhibitor, did not inhibit *Xenopus* embryogenesis in our system. This may be simply because phosphoramidon is not as effective against *Xenopus* MMPs. It is known that phosphoramidon is a potent inhibitor of prokaryotic metalloproteases (*18*) and neutral endopeptidase (*19*), but not as effective against MMPs (*20, 21*).

Some serine proteases involved in hatching have been detected and characterized in the culture medium of embryos of several animal species using a strategy similar to ours (*22, 23*). Although the developmental stages of embryos at which these hatching proteases act are clearly later than the blastula or gastrula stages, comparison between these proteinases and the aprotinin-sensitive proteinase would be intriguing.

It is still open to discussion whether the inhibition of embryonic development by aprotinin is really due to inhibition of the aprotinin-sensitive proteinase or to inhibition of another unknown biochemical reaction. It is known that aprotinin shows antibacterial activity (*24, 25*) and binds to acidic glycoproteins or mucopolysaccharides, including heparin (*26*). Therefore, there remains a possibility that aprotinin exerts a fatal effect on *Xenopus* embryos through an unknown mechanism other than inhibition of serine proteinases. Purification of aprotinin-sensitive proteinase from the culture medium of embryos may provide a clue to clarifying the molecular mechanism of aprotinin's effect on *Xenopus* embryonic development.

We thank Dr. K. Shiokawa for helpful discussion.

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