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Participation of Proteasomes in *Xenopus* Embryogenesis

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We examined the effects of various protease substrates on *Xenopus laevis* embryogenesis. Thirty-three peptidyl-MCA substrates were added to the culture medium in which *Xenopus* embryos were developing. Five of the 33 substrates were found to inhibit embryogenesis at the early gastrula stage or much earlier ones. These results suggest that proteases that hydrolyze these substrates are involved in embryonic development. We found that the developmental stage of embryos is crucial for these substrates to inhibit their development. We purified a protease that hydrolyzes Pyr-Arg-Thr-Lys-Arg-MCA, a substrate that inhibits embryogenesis, from *Xenopus* embryos. This protease turned out to be a component of proteasomes. We found that 4 of the 5 substrates that inhibit embryogenesis are among the proteasome substrates. Thus, we concluded that proteasomes play a crucial role in the development of *Xenopus* embryos. Possibly, various catalytic subunits in proteasomes function independently, in stage-specific manners.

Key words: embryogenesis, protease substrate, proteasome, serine protease, *Xenopus laevis*.

Abbreviations: AMC, 7-amino-4-methyl-coumarin; 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.

The ubiquitin/proteasome pathway is responsible for the degradation of many crucial cell regulators (*1*, *2*). The phenotypes of mutants as to the ubiquitin/proteasome system in yeast, *Drosophila*, and *Caenorhabditis elegans* attest to its involvement in a wide variety of cellular processes, such as resistance to stress, damage control, cell cycle progression, signal transduction, regulation of transcription, chromosomal silencing, mitochondrial inheritance, differentiation, and development. In *Drosophila*, Rpn6, a subunit of the proteasome regulatory particle, has been shown to be required for their development (*3*). In *C. elegans*, it has also been shown that the treatment of embryos with RNAi to interfere with the formation of various subunits of the 20S cores and 19S regulatory particles of proteasomes results in embryonic lethal (*4*).

We are interested in the proteases that participate in the embryogenesis of *Xenopus laevis*. Previously, we showed that aprotinin, an inhibitor of serine proteinase, strongly inhibits *Xenopus* embryonic development in a stage-specific manner, when it is added to the culture medium of the embryos. These results suggested that an aprotinin-sensitive secretory serine protease is involved in the development of *Xenopus* embryos (*5*).

To find proteases that participate in embryogenesis, we examined the effects of various peptidyl-MCA substrates on the development of *Xenopus* embryos. Excess synthetic substrate for a specific protease was expected to compete with its natural substrate and to inhibit embryogenesis if the protease is essential for embryogenesis. Of the 33 peptidyl-MCA substrates examined, five were found to inhibit embryogenesis. In particular, Pyr-Arg-Thr-Lys-Arg-MCA and Boc-Arg-Val-Arg-Arg-MCA inhibited the embryonic development at the early gastrula stage. Therefore, we tried to identify the protease that hydrolyzes Pyr-Arg-Thr-Lys-Arg-MCA. It turned out to be a component of proteasomes, suggesting that proteasomes are involved in the embryonic development of *Xenopus laevis*, as found for *Drosophila* and *C. elegans*.

MATERIALS AND METHODS

Embryos and Their Culture—Embryos of *Xenopus laevis* were used throughout the experiments. The methods used for collecting *Xenopus* embryos and their culture were essentially the same as described previously (*6*). Briefly, unfertilized eggs were collected from female frogs after injection of 600 U of a gonadotropic hormone (Gonatoropin; Teikokuzoki), and then 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 a $NaHCO_3$ solution). The fertilized eggs were dejellied by treatment with a 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 morula [Nieuwkoop and Faber stage 7 (*7*)], blastula (stages 8–9), early gastrula (stage 10), mid-gastrula (stage 11), late gastrula (stage 12), and neurula (stage 18) stages in about 4, 7, 10, 13, 16, and 19 h after fertilization, respectively.

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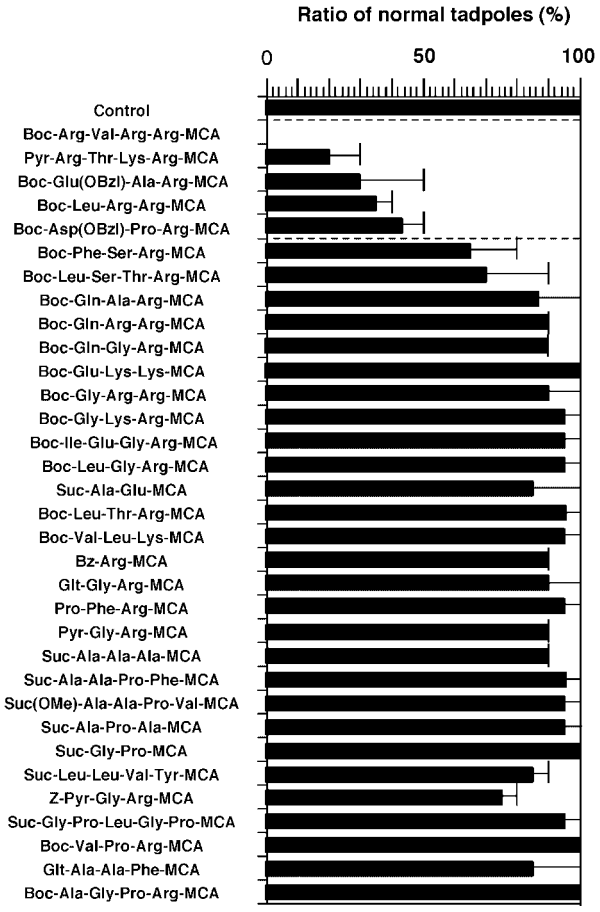

Fig. 1. **Effects of protease substrates on the development of *Xenopus* embryos.** Embryos at the blastula stage were cultured in the presence of 100 μM of various peptidyl-MCA substrates. Control embryos were cultured in the absence of a substrate. In each experiment, about 20 embryos were used. The number of normal tadpoles 2 d after the substrate addition was determined and the percentage of normal tadpoles was calculated.

Protease Assay—The protease assay was performed in 150 μl of 50 mM Tris-HCl, pH 7.0, containing 50 μM Pyr-Arg-Thr-Lys-Arg-MCA (a substrate for serine protease) and the test fraction. After incubation for 60 min at 27°C, the reaction was terminated by adding 50 μl of 40% (v/v) aqueous acetic acid. Fluorescence was measured using excitation and emission wavelengths of 360 and 460 nm, respectively. One unit of protease activity was defined as the amount that hydrolyzed 1 μmol of Pyr-Arg-Thr-Lys-Arg-MCA per min under these conditions.

Purification of a Serine Protease from Xenopus Embryos—Embryos at the gastrula stage (10.2 g) were homogenized in 30 ml of 10 mM Bis-Tris-HCl, pH 6.0, containing 10 μg/ml E-64. Then the homogenate was centrifuged at 750 ×*g* for 5 min at 4°C to remove nuclei and yolk. The supernatant was further centrifuged at 100,000 ×*g* for 1 h at 4°C, and the resulting supernatant was applied to a DE 52 column. After extensive washing, the absorbed material was eluted stepwise with 10 mM Bis-Tris-HCl, pH 6.0, containing 0.05 M, 0.1 M, 0.2 M, 0.5 M, and 1.0 M NaCl. Protease activity was eluted with 0.2 M NaCl.

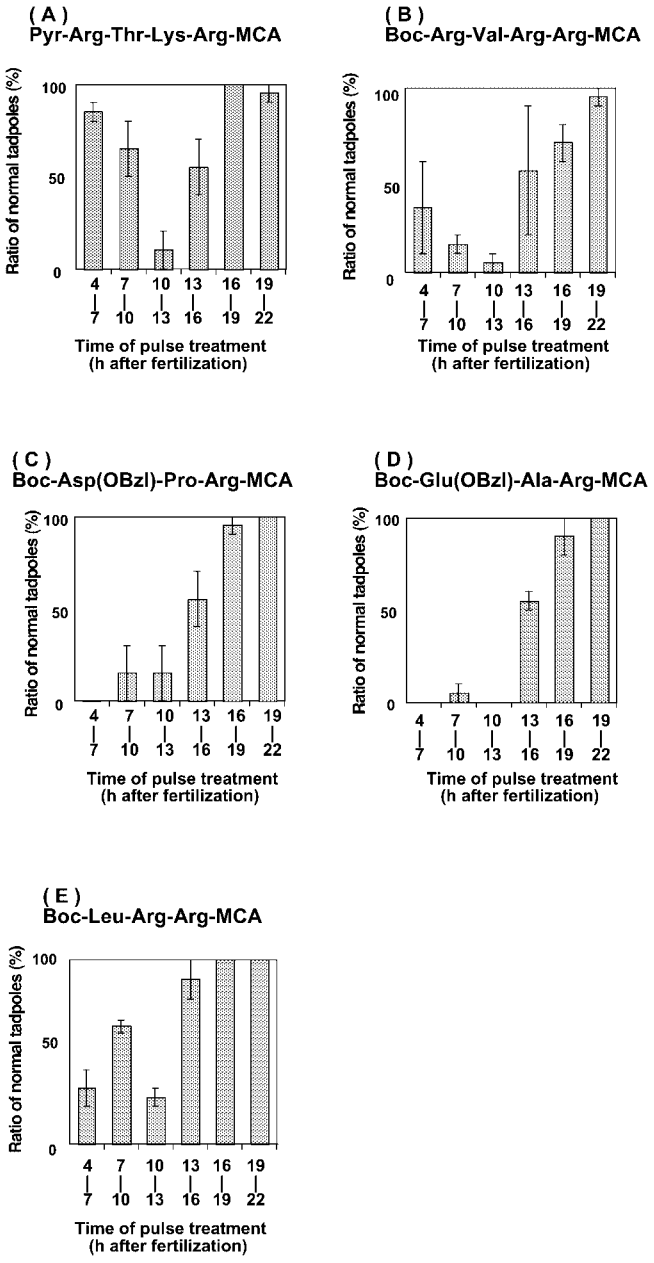

Fig. 2. **Stage specificity of the inhibition of embryonic development by peptidyl-MCA substrates.** Embryos at various developmental stages were temporarily treated with peptidyl-MCA substrates for 3 h, and then washed and cultured in normal medium. The percentage of normal tadpoles 48 h after fertilization were calculated. In each experiment, about 20 embryos were used. The peptidyl-MCA substrates used were: 100 μM Pyr-Arg-Thr-Lys-Arg-MCA (A); 50 μM Boc-Arg-Val-Arg-Arg-MCA (B); 100 μM Boc-Asp(OBzl)-Pro-Arg-MCA (C); 100 μM Boc-Glu(OBzl)-Ala-Arg-MCA (D); and 100 μM Boc-Leu-Arg-Arg-MCA (E). Under our culture conditions, control embryos reached the morula (Nieuwkoop and Faber stage 7), blastula (stages 8–9), early gastrula (stage 10), mid-gastrula (stage 11), late gastrula (stage 12), and neurula (stage 18) stages at about 4, 7, 10, 13, 16, and 19 h after fertilization, respectively.

Non-Denaturing PAGE and Fluorescence Overlay Assay—Native polyacrylamide gel (4%) electrophoresis was performed as described by Mahaffey *et al.* (*8*). To locate proteases in the gels, the gels were soaked for 30 min at 37°C

Table 1. **Summary of purification of the protease hydrolyzes Pyr-Arg-Thr-Lys-Arg-MCA.**

Purification step	Total protein (mg)	Total activity (unit[a])	Specific activity (unit[a]/mg)	Yield (%)	Purification (-fold)
Homogenate	3,710	0.083	2.24×10^{-5}	100	1
750 ×*g* supernatant	1730	0.078	4.51×10^{-5}	94	2
100,000 ×*g* supernatant	252	0.084	3.33×10^{-4}	101	15
DE52	9.53	0.045	4.72×10^{-3}	54	210

[a]One unit was defined as the amount of enzyme that hydrolyzed 1 μmol of Pyr-Arg-Thr-Lys-Arg-MCA/min.

in 10 ml of 50 mM Tris-HCl, pH 7.0, containing 200 μM Pyr-Arg-Thr-Lys-Arg-MCA or Suc-Leu-Leu-Val-Tyr-MCA. The fluorescence produced through hydrolysis of the substrate was detected under UV light.

RESULTS

Effects of Protease Substrates on the Development of Xenopus Embryos—To identify the proteases participating in *Xenopus* embryogenesis, we examined the effects of various protease substrates on the development of *Xenopus* embryos, assuming that some peptidyl-MCA substrates that enter the cells may compete with a natural substrate for the protease, resulting in the inhibition of embryonic development. For this, we tested 33 peptidyl-MCA substrates by adding each one (100 μM) to culture medium containing embryos at the blastula stage. Two days after the addition of the substrates, we determined the percentages of normally developed tadpoles. As shown in Fig. 1, five of the 33 substrates, Boc-Arg-Val-Arg-Arg-MCA, Pyr-Arg-Thr-Lys-Arg-MCA, Boc-Glu(OBzl)-Ala-Arg-MCA, Boc-Leu-Arg-Arg-MCA, and Boc-Asp(OBzl)-Pro-Arg-MCA were found to markedly inhibit the emergence of tadpoles, the percentage inhibition being more than 50%. In particular, no tadpole developed when blastula stage embryos were treated with Boc-Arg-Val-Arg-Arg-MCA. These results suggest that one or more proteases that hydrolyze these substrates are involved in *Xenopus* embryonic development.

Stage-Specific Inhibition of Xenopus Development by Protease Substrates—To determine the developmental stages of embryos that are sensitive to these peptidyl-MCA substrates, we performed pulse treatment of embryos with these substrates. Neurula emerge from fertilized eggs in about 22 h under our culture conditions. Therefore, we defined six stages, each stage comprising 3 h between 4 h to 22 h after fertilization. Embryos at these stages correspond to those mentioned under Materials and Methods. We cultured the embryos at each stage for 3 h in the medium containing each substrate, and then transferred them to normal medium to monitor their development. As shown in Fig. 2, inhibition of embryogenesis by these substrates showed stage specificity. All five substrates inhibited embryonic development at the early gastrula stage (from 10 to 13 h after fertilization), or at a much earlier stage, but appreciable inhibition was not detected when embryos were treated at later stages (from 16 to 22 h). Moreover, the inhibition patterns obtained were not the same. For instance, Pyr-Arg-Thr-Lys-Arg-MCA (Fig. 2A) inhibited development almost exclusively at the early gastrula stage, and no significant inhibition was detected when embryos were treated with

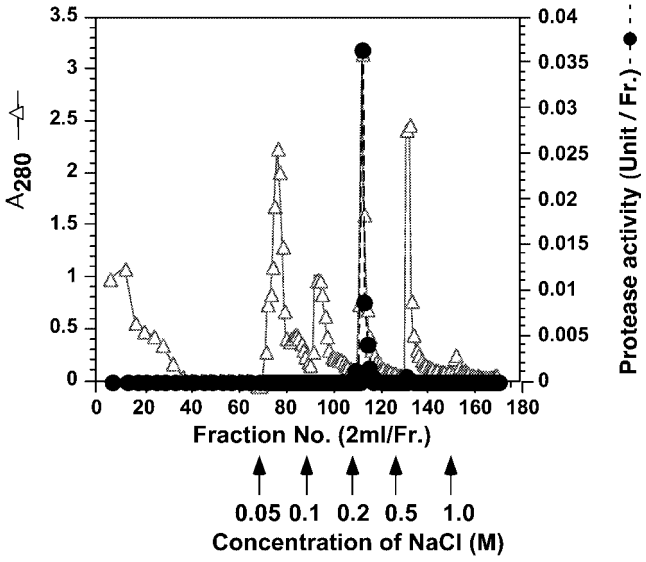

Fig. 3. **Elution profile of the Pyr-Arg-Thr-Lys-Arg-MCA degrading protease from a DE 52 column.** The clear supernatant (20 ml) obtained on centrifugation at 100,000g was applied to a DE 52 column. The absorbed material was eluted stepwise with 10 mM Bis-Tris-HCl, pH 6.0, containing 0.05 M, 0.1 M, 0.2 M, 0.5 M, and 1.0 M NaCl. The active fraction was eluted with 0.2 M NaCl. The flow rate was 1.2 ml/min. Open triangles, A_{280}; Solid circles, Pyr-Arg-Thr-Lys-Arg-MCA degrading activity. One unit was defined as the amount of enzyme that hydrolyzed 1 μmol of Pyr-Arg-Thr-Lys-Arg-MCA/min.

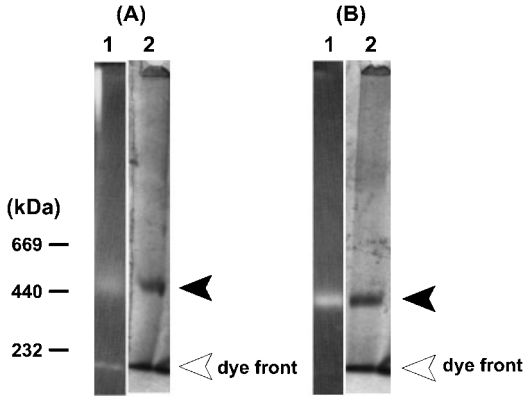

Fig. 4. **Detection of proteasomes by non-denaturing PAGE and fluorescence overlay assaying.** The active fraction from DE 52 was electrophoresed on native 4% gels, and then the gels were treated with a 200 μM Pyr-Arg-Thr-Lys-Arg-MCA (A, lane 1) or Suc-Leu-Leu-Val-Tyr-MCA (B, lane 1) solution. The fluorescent proteolytic product was visualized by illumination with UV light. Lane 2 is the same gel after staining with Coomassie Brilliant Blue, respectively. The solid arrowhead indicates the fluorescent band, and the open arrowhead indicates the dye front.

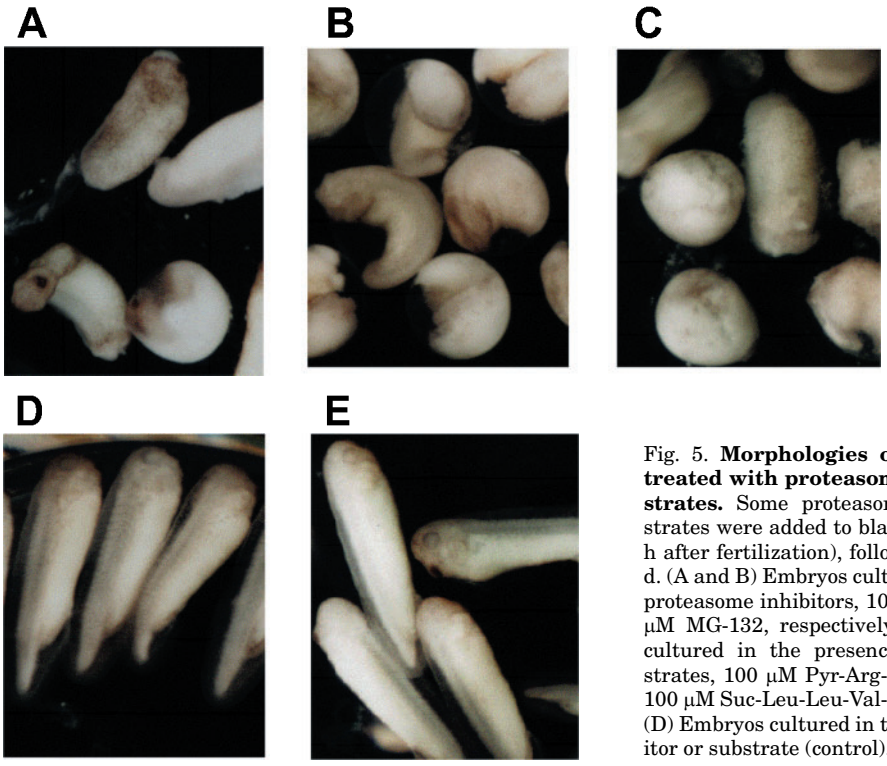

Fig. 5. **Morphologies of *Xenopus* embryos treated with proteasome inhibitors or substrates.** Some proteasome inhibitors or substrates were added to blastula stage embryos (9 h after fertilization), followed by culturing for 2 d. (A and B) Embryos cultured in the presence of proteasome inhibitors, 100 μM MG-115 and 200 μM MG-132, respectively. (C and E) Embryos cultured in the presence of proteasome substrates, 100 μM Pyr-Arg-Thr-Lys-Arg-MCA and 100 μM Suc-Leu-Leu-Val-Tyr-MCA, respectively. (D) Embryos cultured in the absence of an inhibitor or substrate (control).

it at the morula stage (from 4 to 7 h). Whereas, Boc-Asp(OBzl)-Pro-Arg-MCA was effective throughout the gastrula to morula stages, and clear inhibition was detected when embryos were treated with it at the morula stage (Fig. 2C). These results suggest that multiple proteases participate in the development of *Xenopus* embryos in stage-specific manners.

Purification of a Protease That Hydrolyzes Pyr-Arg-Thr-Lys-Arg-MCA from Xenopus Embryos—As Pyr-Arg-Thr-Lys-Arg-MCA was almost selectively effective at the early gastrula stage, the protease that hydrolyzes this substrate was assumed to play a crucial role in the development of *Xenopus* embryos only at this stage. To characterize this protease, we tried to purify it from *Xenopus* embryos. The specific activity of the protease increased by 15-fold with successive two centrifugations of the embryonic homogenate. The final step of purification comprised chromatography on a column of DEAE-cellulose (DE52). The protease activity was eluted from the column with 0.2 M NaCl (Fig. 3), and the specific activity had increased by 210-fold compared with the level for the initial homogenate (Table 1). The recovery of the protease activity in this fraction was 54%.

The active fraction was electrophoresed on a native 4% polyacrylamide gel and protease activity was localized by fluorescence substrate overlay assaying with Pyr-Arg-Thr-Lys-Arg-MCA. The protease activity was found to coincide with a major protein band stained with Coomassie Brilliant Blue (Fig. 4A). This protein had an extremely high molecular size, being more than 500 kDa, suggesting that this band contains proteasomes comprising multiple subunits.

It is known that proteasomes can be separated on a native polyacrylamide gel and their protease activity is detectable by overlaying the gel with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-MCA (*9*). This substrate is generally used to detect proteasomes (*10*). As shown in Fig. 4B, the protease activity detected with Suc-Leu-Leu-Val-Tyr-MCA again coincided with the major protein band. Therefore, we concluded that the major protein purified in this way is proteasomes.

To confirm the conclusion further, we examined whether a proteasomal inhibitor, MG-115 (carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal), inhibited Pyr-Arg-Thr-Lys-Arg-MCA degrading activity, and found that 100 μM of MG-115 inhibited 86% of Pyr-Arg-Thr-Lys-Arg-MCA degrading activity (data not shown).

Using the fluorescence substrate overlay assay, we examined whether or not purified proteasomes hydrolyze the other substrates that inhibit embryonic development, and found that Boc-Arg-Val-Arg-Arg-MCA, Boc-Glu(OBzl)-Ala-Arg-MCA, and Boc-Leu-Arg-Arg-MCA were hydrolyzed by proteasomes like Pyr-Arg-Thr-Lys-Arg-MCA, but no appreciable hydrolysis was detected with Boc-Asp(OBzl)-Pro-Arg-MCA (data not shown). These results suggest that at least four of the five protease substrates that inhibit *Xenopus* development are proteasome substrates.

Effects of Proteasome Inhibitors on the Development of Xenopus Embryos—Proteasome-specific inhibitors are commercially available. To confirm the participation of proteasomes in embryonic development, we examined the effects of three inhibitors, MG-115, MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) (*11*) and *clasto*-lactacystin-beta-lactone on the development of *Xenopus* embryos, and found that MG115 and MG132 inhibited the emergence of tadpoles. On treatment of morula stage embryos with these inhibitors, their morphology of the

embryos became abnormal (Fig. 5A, B) like on treatment with Pyr-Arg-Thr-Lys-Arg-MCA (Fig. 5C). Normal embryos at this stage were almost all tadpoles with broadened fin and distinct eye protrusions (Fig. 5D). No appreciable inhibitory effect was detected with 50 μM of *clasto*-lactacystin-beta-lactone (data not shown). Interestingly, Suc-Leu-Leu-Val-Tyr-MCA, a general substrate for proteasomes, had almost no inhibitory effect on the development of *Xenopus* embryos (Fig. 5E), suggesting that the catalytic subunit that hydrolyzes this substrate is not involved in embryonic development, although it is a proteasome subunit.

DISCUSSION

We demonstrated that five of the 33 MCA-substrates examined severely interfered with the development of *Xenopus* embryos, the percentage emergence of tadpoles being less than 50%. We interpreted this phenomenon as follows. Each MCA-substrate may enter embryos and compete with a natural substrate to be hydrolyzed by a specific protease, resulting in inhibition of normal development. If this interpretation is correct, all effective MCA-substrates have to enter embryos. Therefore, among the 28 non-effective substrates, there might be potentially effective ones that cannot enter embryos.

In the course of purification of a protease from *Xenopus* embryos that hydrolyzes Pyr-Arg-Thr-Lys-Arg-MCA, we came to the conclusion that this protease is an intrinsic component of proteasomes. A proteasome is a huge protein complex consisting of multiple subunits. Therefore, it was possible to purify proteasomes from embryonic lysates through two sequential centrifugations and one DE52 column chromatography. The purified protein gave a major single band on native gel electrophoresis. We employed the fluorescence substrate overlay assay to identify proteasomes. This is a convenient method for detecting proteasomes in native gels using Suc-Leu-Leu-Val-Tyr-MCA, which is a general substrate for proteasomes (*10*). According to Tsubuki *et al.* (*12*), it is possible to roughly estimate the molecular size of proteasomes from their relative mobility, and the molecular size of the purified proteasomes was more than 500 kDa, in agreement with those of known proteasomes.

We found that proteases that hydrolyze four of the five inhibitory substrates are included in proteasomes. We assume that, in *Xenopus* embryos, a proteasome-specific substrate is sequentially hydrolyzed in a developmental stage-specific manner, and that this process is indispensable for normal embryonic development. As the participation of proteasomes in embryogenesis has been suggested for *Drosophila* (*3*) and *C. elegance* (*4*), the same may be true for *Xenopus laevis*.

We found that the morphology of embryos treated with Pyr-Arg-Thr-Lys-Arg-MCA becomes abnormal. Similar morphological abnormality was also induced when embryos were treated with other inhibitory substrates, but not with non-inhibitory ones. To confirm that the inhibition of proteasomes induces morphological abnormality of embryos, we examined the effects of three potent proteasome inhibitors, MG-115, MG-132 and *clasto*-lactacystin-beta-lactone. As MG-115 and MG-132 induced morphological abnormality of embryos, we concluded that proteasomes play an essential role in embryogenesis. The relation between proteases inhibited by the five inhibitory MCA-substrates, and those inhibited by MG-115 and MG-132 remains to be clarified.

In our experiment, embryonic inhibitory effect was not detected with 50 μM of *clasto*-lactacystin-beta-lactone, the active chemical species of lactacystin (*13*), covalently modifying the active site threonine residues of the beta-subunit (*14*). The reason for this is not clear. Possibly it may not enter the embryos or may be decomposed in the medium, as it is known to be unstable in aqueous solutions (*14*).

It is noteworthy that Boc-Asp(OBzl)-Pro-Arg-MCA was not hydrolyzed by proteasomes, although it inhibited embryonic development. Possibly, other than proteasome proteases, ones located elsewhere are also involved in embryonic development. The protease that hydrolyzes this substrate may be one of these proteases, like aprotinin-sensitive serine protease we reported previously (*5*).

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