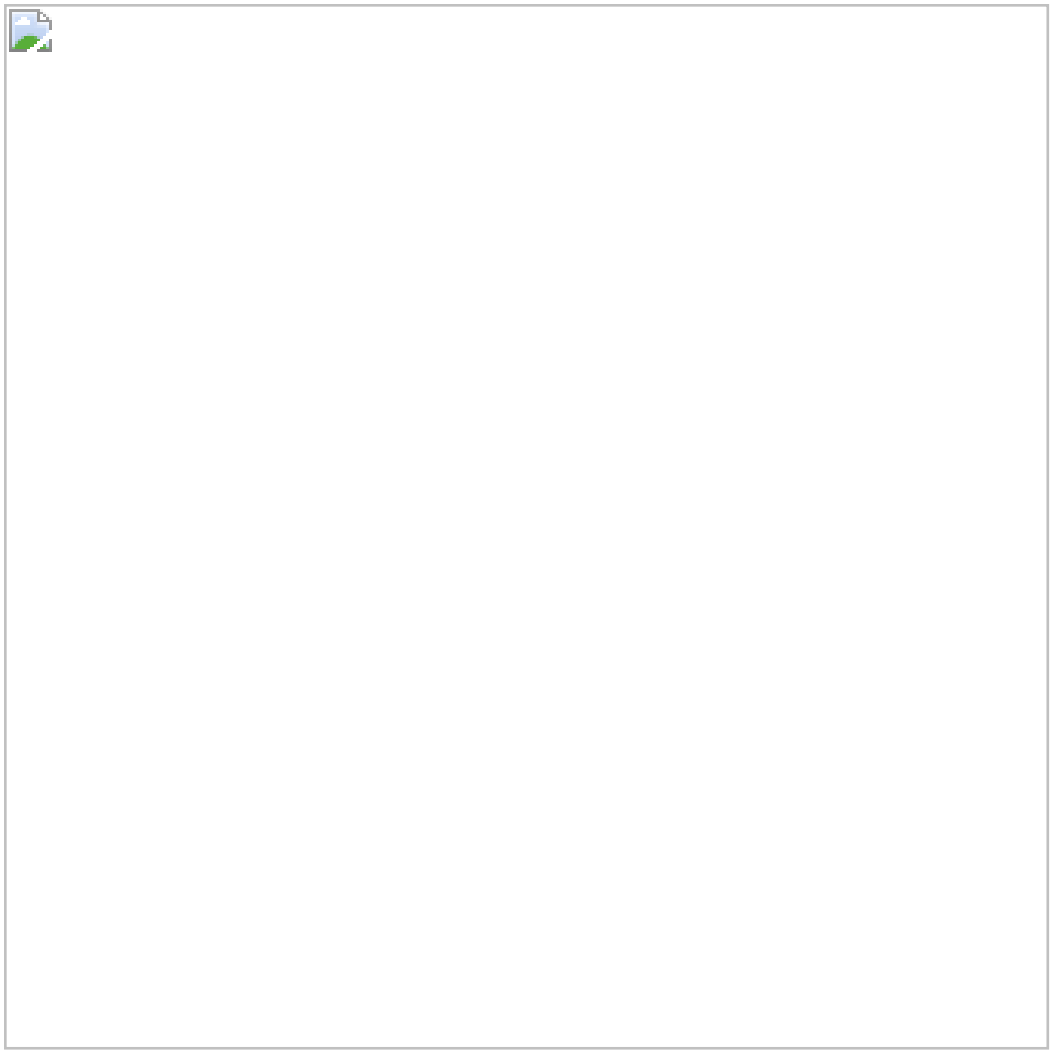

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Cathepsin L-Like Protease from *Xenopus* Embryos That Is Stimulated by Nucleoside Phosphates and Nucleic Acids

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ABSTRACT—An acid thiol protease that was activated at an early stage of embryogenesis was purified from *Xenopus* embryos. The N-terminal amino acid sequence(16 residues) of the heavy chain of the enzyme was similar to that of cathepsin L. The proteolytic activity of the protease was enhanced by ATP. Other nucleoside triphosphates, AMP and nucleic acids also enhanced the proteolytic activity. The possible mechanism and biological significance of the activation of the protease in *Xenopus* embryos are discussed.

INTRODUCTION

The precursor to yolk protein, vitellogenin, is synthesized by the liver and it is selectively taken up by oocytes, where it is condensed and stored as a lipoprotein complex [19]. Much later, during embryonic development, the degradation of the yolk proteins is initiated [15]. Although the contribution to yolk resorption of degradation by lysosomal enzymes has long been recognized, relatively little is known about this process [1, 18]. When *Xenopus* embryos have developed to the gastrula stage, a thiol protease is strongly activated at the post-translational level [9, 11]. We have reported that part of the yolk protein is degraded by the thiol protease and that the protease can be detected around yolk granules in both the cytoplasm of unfertilized eggs and in early embryos during development [12]. These results suggest that the thiol protease might fuse with yolk platelets in the unfertilized egg in an inactive form. When resorption of the vitellus starts during embryogenesis, the protease is activated by an unknown mechanism. In order to characterize this protease and elucidate its activation mechanism, we determined the N-terminal amino acid sequence of the protease and examined the effects of ATP and other phosphorylated compounds on its activity.

MATERIALS AND METHODS

Assay of proteolytic activity

The proteolytic activity was determined in 0.1 M acetate buffer, pH 3.8, that contained 2.5 mM dithiothreitol with [^{3}H] BSA, non-radiolabeled BSA, hemoglobin or histone as substrates. When [^{3}H]BSA was used as the substrate, proteolytic activity was assayed by quantitating the acid-soluble radioactivity released from [^{3}H]BSA [10]. For assays with non-radiolabeled proteins, the proteolytic activity was assayed by monitoring of the liberation of digested products into an acid-soluble fraction using the ninhydrin method [10] and by use of the reagent for quantitation of protein from Bio-Rad.

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Preparation of embryos, purification of the protease and determination of the N-terminal amino acid sequence

Xenopus embryos were allowed to develop to the tailbud stage. The embryos were immersed in cold acetone and homogenized in a glass homogenizer. The homogenate was centrifuged at 3,000 rpm for 10 min at 4°C. The pellet was dehydrated by treatment with several changes of acetone, dried under N_2, and stored at −20°C.

The detailed procedure for purification of the protease has been described previously [10]. Twenty grams of acetone-dried embryos were homogenized in 200 ml of 0.1 M acetate buffer, pH 5.0, which contained 0.1% Triton X-100 and 0.1 mM EDTA, in a glass homogenizer. The homogenate was centrifuged at 7,000×g for 20 min. The proteolytic activity in the supernatant was concentrated by fractionation with acetone (20–50%). The resulting precipitate was dissolved in 0.1 M acetate buffer, pH 5.0, that contained 0.2 M NaCl. The extract was dialyzed against the same buffer and successively chromatographed on columns of Sephadex G-75, CM-cellulose, and hydroxylapatite. The final enzyme preparation represented a 16,000-fold purification and yielded only a single band of protein after electrophoresis on a non-denaturing polyacrylamide gel.

The purified protease consists of two polypeptides with molecular masses of 30 kDa and 13 kDa, respectively [10]. For isolation of the heavy chain of the protein, the purified protein was subjected to electrophoresis on a 0.1% SDS-15% polyacrylamide gel. The band of the heavy chain was cut from the gel. The protein was extracted from the gel and subjected to reverse-phase HPLC (Vydoc C4 column; 0.2 cm×25 cm) with elution with a gradient of acetonitrile from 10% to 80% in 0.1% trifluoroacetic acid. The amino acid sequence of the heavy chain was determined with a model 470A protein sequencer (Applied Biosystems) at the Research Center of Mitsubishi Chemical Industry.

Preparation of DNA

DNA was prepared by the method of Gross *et al.* [3] from *Xenopus* liver.

RESULTS AND DISCUSSION

Analysis of the N-terminal amino acid sequence of the heavy chain of the thiol protease

We previously reported on the purification and some properties of a protease that was activated in *Xenopus* embryos at an early stage [9, 10]. The purified protease resembled a thiol protease in terms of its substrate specificity and the effects of specific inhibitors of thiol proteases. It had a molecular mass of 43 kDa-44 kDa and was composed of two subunits with molecular masses of 30 kDa and 13 kDa, respectively. It has been reported that cathepsin L has a molecular mass of 23 kDa-24 kDa [5]. However, it has also been reported that cathepsin L has a molecular mass of 30 kDa and consists of a heavy chain of 25 kDa and a light chain of 5 kDa [6]. It is not yet clear whether cathepsin L occurs in a two-chain or a single-chain form *in vivo* [6]. Although our protease resembled cathepsin L, its identity to this enzyme remains to be proven. We determined the N-terminal amino acids of the purified thiol protease. The N-terminal amino acid sequence determined with the heavy chain was homologous to the sequences of N-terminal regions of members of the superfamily of thiol proteases (Fig. 1). Of the 16 amino acids in the sequence, 76% were identical to those in the N-terminal sequence of rat cathepsin L [4] and human cathepsin L [7]. The residues in the N-terminal sequence were 70% homologous to those in the N-terminal region of chicken cathepsin L [2], 47% homologous to those of rat cathepsin H and 40% homologous to those of rat cathepsin B [17]. Thus, it appears that our protease, which is found around yolk granules and which digests some of the yolk proteins, is cathepsin L. Fusion of yolk platelets with cathepsin L-like protease seems to be one mechanism for digestion of yolk proteins.

Xenopus embryo	A P K T V D L R E K G - - Y V K - - NDV K
Rat cathepsin L	I P K T V D W R E K G - - C V T - - P V K
Human cathepsin L	A P R S V D W R E K G - - Y V T - - P V K
Chicken cathepsin L	A P R S V D W R E K G - - Y V T - - P V K
Rat cathepsin H	Y P S S M D W R K K G - N V V S - - P V K
Rat cathepsin B	L P E S F D A R E Q W S N C P T I A Q I R

FIG. 1. N-terminal amino acid sequence of the protease from *Xenopus* embryos. Homologies between the protease from *Xenopus* embryos and other members of the thiol protease superfamily are indicated. The determined sequence is shown and is compared with the N-terminal amino acid sequences of cathepsin L from rat, human and chicken and cathepsins H and B from rat (ND, Not determined).

Effects of nucleotides on the activity of the protease

We found that the proteolytic activity of the purified protease against[^{3}H] BSA was enhanced by the addition of ATP to the reaction mixture used for assays of the protease (Table 1). Activity was unchanged upon addition of $MgCl_2$ to the reaction mixture and the proteolytic activity was unchanged upon addition of $MgCl_2$ to a reaction mixture that contained ATP, even though Mg^{2+} ions are usually required as a cofactor in ATP-dependent phosphotransferase reactions. Thus, there was no apparent involvement of MgATP in the reaction. This result resembles the effect of ATP on the activities of cathepsins D, E and L that were reported previously [8, 13, 14, 16, 20]. Addition of the chloride of another divalent cation, $CaCl_2$, also had no effect on the proteolytic activity (data not shown), and EDTA too was without any effect. These results indicate that heavy metal

TABLE 1. Effects of ATP, $MgCl_2$ and EDTA on the proteolytic activity

Addition			Proteolytic activity (cpm)
ATP	$MgCl_2$	EDTA	
−	−	−	2,650
−	+	−	2,320
−	−	+	2,570
+	−	−	4,890
+	+	−	4,680

The proteolytic activity was assayed in the presence of 0.1% [^{3}H] BSA and 0.02 μg of enzyme with either no additions or addition of 1 mM ATP, 1 mM $MgCl_2$ and/or 1 mM EDTa. The activity is given in terms of acid-soluble radioactivity (cpm) released from [^{3}H] BSA.

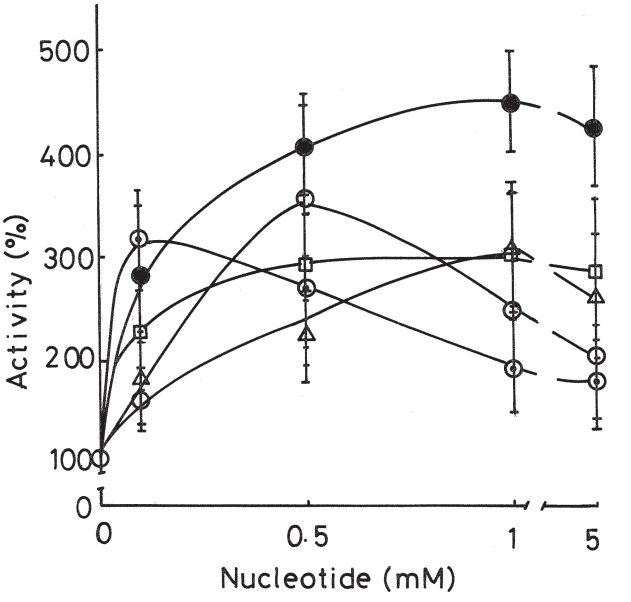

FIG. 2. Effects of nucleotides on proteolytic activity. Nucleotides at various concentrations were added to a 0.2 ml-aliquot of 0.1 M acetate buffer, pH 3.8, that contained 2.5 mM dithiothreitol plus enzyme (0.02 μg/0.2 ml), with 0.1% [^{3}H] BSA as the substrate. The activity is expressed in terms of acid-soluble radioactivity released from the substrate , which is given as a percentage of the radioactivity released in the absence of nucleotides from [^{3}H] BSA. (○), ATP; (□), AMP; (●), GTP; (○), UTP; (△), CTP.

ions did not inhibit the activity of the protease by reacting with sulfhydryl groups on the protease. We studied the effects of ATP at various concentrations (Fig. 2). The proteolytic activity was most stimulated by ATP at 0.5 mM and was lower at higher concentrations of ATP. Addition of AMP to the assay system gave results similar to those obtained with ATP. Other nucleoside triphosphates (GTP, CTP, TTP) were tested in the assay system. The addition of GTP at 1 mM had the greatest stimulatory effect on the proteolytic activity (approximately 4-fold stimulation).

Effects of DNA on the activity of the protease against BSA, hemoglobin, histone and embryonic proteins from Xenopus.

The rate of proteolysis of non-radiolabeled BSA was enhanced approximately 4-fold by the addition of DNA from *Xenopus* liver at a concentration of 2.5 μg per ml (Fig. 3). The extent of hydrolysis of hemoglobin was increased approximately 3.5-fold by DNA at 5 μg per ml. The proteolysis of embryonic proteins using the protease was increased approximately 1.8-fold by the addition of DNA from *Xenopus* liver at 5 μg per ml. However, when histone was used as the substrate, no such stimulation was observed. Among the tested substrates, non-radiolabeled BSA was the substrate whose cleavage was most effectively enhanced by the addition of DNA.

We reported previously that degradation of BSA by the protease was activated by RNA and polyribonucleotides [10]. In this study, the degradation of histone by the protease was unaffected by the addition of DNA, and the effects of DNA on the proteolytic activity varied among substrates. The nature of the stimulation of the protease does not seem straightforward, and our results do not lend themselves to a simple interpretation. These results indicate that the effects of nucleic acids might be due to the interaction of phosphorylated compounds with the enzyme-substrate complex and might not involve direct effects on the enzyme.

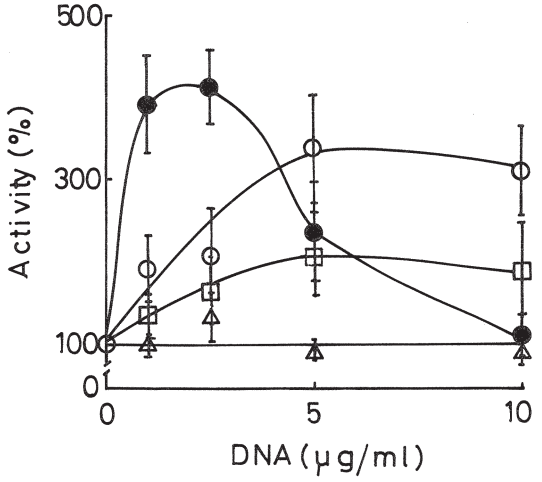

FIG. 3. Effects of DNA on proteolytic activity against protein substrates. Proteolytic activity was measured in 0.5 ml of 0.1 M acetate buffer, pH 3.8, that contained 2.5 mM dithiothreitol, 1% (w/v) protein substrate and enzyme (0.32 μg). The products released from the protein substrates were quantitated by the ninhydrin method. The activity is expressed in terms of the amount of product released from the substrate, which is given as a percentage of the amount of the product released in the absence of DNA. (□), Embryonic proteins; (●), BSA; (○), hemoglobin; (△), histone.

It has been reported that anionic compounds, such as nucleotides and polynucleotides, might have biological significance in the regulation of proteolysis in lysosomes [20]. The results of this study suggest the possibility that phosphorylated compounds might have anionic effects in the cytoplasm that simulate the effects of polyanionic substances in the lysosome. Yolk proteins in *Xenopus* are complex lipoglycophosphoproteins that contain phosphorylated compounds. Further studies of the stimulation of cathepsin L-like protease by phosphorylated compounds might contribute to our understanding of the mechanism of digestion of yolk proteins in the cytoplasm of *Xenopus* embryos at early stages of development.

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