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Changes in Timing and Site of Appearance of a Protease in *Xenopus* Embryos

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ABSTRACT—A thiol protease was purified from embryos of *Xenopus laevis*. This protease has a relative mass (M_r) of 43 k-44 K. Antiserum raised against the protease was used for analysis by Western blotting of proteins from embryos at various stages and from adult liver and heart. A band corresponding to a protein with an M_r of approxmately 44 k was detected in the unfertilized eggs and embryos with the monospecific antiserum. The size of the protein that reacted with the antiserum in a preparation of proteins from the liver of *Xenopus laevis* was different from that of the proteins from the eggs and embryos (M_r, approximately 70 k). Immunohistochemical localization with the antiserum revealed that the protease was more abundant in the animal hemisphere and in the cells derived from the animal hemisphere than in other cells of the embryo. The protease was highly enriched in the cytoplasm of ectodermal cells than the cytoplasm of endodermal cell. When proteins from *Xenopus* embryos were used as substrate, one protein having M_r of 31 K was mainly digested.

INTRODUCTION

Animal cells contain many different proteases and there are both lysosomal and non-lysosomal pathways of protein degradation [1–3]. We have purified an acidic protease from *Xenopus* embryos. The protease is sensitive to antipain, leupeptin and iodoacetic acid but it is insensitive to phenylmethylsulfonyl fluoride and pepstatin [4]. It has an M_r of 43 k-44 k and can be dissociated into two subunits with M_r of about 30 k and 13 k. The proteolytic activity is activated by nucleic acid. The effect of inhibitors, molecular weight, subunit structure, pH optimum, and phenomenon of activation by nucleic acid seem to be different from those of the proteases described thus far [1–3]. The purpose of the present study was to monitor temporal changes in the level, the distribution and the intracellular localization of the protease protein in *Xenopus* eggs and embryos.

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MATERIALS AND METHODS

Assay of proteolytic activity

The proteolytic activity was determined with [^{3}H]BSA as substrate and was assayed by measuring the acid-soluble radioactivity released from [^{3}H]BSA, as reported previously [4].

Preparation of embryos and purification of the protease

Xenopus eggs were allowed to develop to the tail-bud 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 pellets were dehydrated by treatment with several changes of acetone, dried under N_2, and stored at −20°C.

The procedure for purification of the protease has been described previously [4]. Twenty grams of the 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 succesively chromatographed on columns of Sephadex G-75, CM-cellulose, and hydroxylapatite. The final preparation of enzyme represented a 16,500-fold purification.

Production of antiserum against the protease

New Zealand White rabbits were immunized with the protease. One hundred micrograms of the protease, emulsified in Freund's complete adjuvant (GIBCO), were administered intradermally. Rabbits were given three booster injections intradermally with 100 μg protease without adjuvant. Blood was collected 1 week later. IgG was purified from rabbit serum by chromatography on columns of DEAE-cellulose and Sephadex G-200, with subsequent precipitaion with 33% ammonium sulfate at 4°C.

Immunoprecipitation of the protease

Various amounts of antiserum or control serum were added to 150 μl of PBS that contained purified enzyme (0.22 μg). After incubation for 48 hr at 4°C, the mixture was centrifuged in a microfuge for 10 min. The residual activity in the supernatant was determined with [^{3}H]BSA as substrate.

Western Blot Analysis

Proteins from embryos at various stages were fractionated by electrophoresis on 15% polyacrylamide gels [5] and then transferred to nitrocellulose membranes in a buffer that contained 62.5 mM Tris, 192 mM glycine (pH 8.7) and 20% methanol [6], with a current of 1.2 mA/cm^2 for 90 min at room temperature. The membranes were incubated with blot buffer (5% (mass/vol.) nonfat dry milk in PBS) for 2 hr and then with antiserum diluted 1 : 200 in blot buffer for 1hr. After washing with PBS, each membrane was exposed to anti-rabbit IgG conjugated with peroxidase in blot buffer for 1 hr, washed three times with PBS, and then developed with a solution of the substrate for peroxidase which consisted of azino-di (ethylbenzthiazoline) sulfonic acid in 0.1 M citrate buffer, pH 4.2, supplemented with 0.03% hydrogen peroxide. The enzymatic reaction was terminated by washing with water. Proteins in gels were detected by silver staining [7].

Light microscopy

Eggs and embryos were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 10 mM phosphate buffer pH 7.5, for 4 hr at 4°C, washed thrice with PBS, dehydrated in a graded ethanol series, cleared in 0.03% Nonidet p-40 in chloroform, and embedded in Tissue Prep (Fischer Scientific). Thin sections were cut at 4 μm and deparaffinized with xylene. Immunohistochemical staining was carried out by modified version of the procedure of Weinman et al. [8]. The sections were rinsed thrice in PBS, and treated with 0.02 M glycine in PBS and with 4% BSA in PBS. These were first labeled with antiserum against the protease diluted 1 : 20 in 1% BSA in PBS, washed 4 times with PBS, post-labeled with a biotin-labeled anti-rabbit IgG diluted 1 : 50 in 1% BSA in PBS, and washed thrice with PBS. The sections were reacted with avidin-biotin-peroxidase complex, washed thrice with PBS, reacted with 0.02% hydrogen peroxidase and diaminobenzidine tetrahydrochloride in PBS for visualization, and rinsed 4 times in distilled water.

RESULTS

Immunoprecipitation of the protease

To study the characteristics of the antiserum, immunoprecipitation of the protease was carried out. The enzymatic activity remaining in the supernatant was measured with [^{3}H]BSA as substrate after addition of aliquots of antiserum or control serum (Fig. 1). The addition of increasing amounts of antiserum led to the gradual loss of proteolytic activity, demonstrating that the antiserum reacted with the active enzyme protein.

Screening with antiserum against the protease by immunoblotting

Samples of the proteins from embryos at various

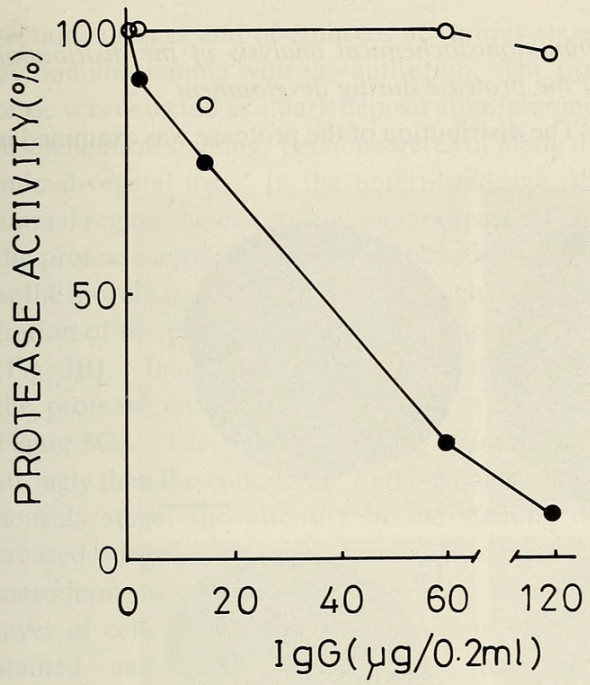

FIG. 1. Immunoprecipitaiton of the protease. Various amounts of antiserum or control serum were addedd to 0.01 M phosphate-buffered saline that contained purified enzyme. Enzymatic activity remaining in the supernatant was measured after centrifugation, by measuring of acid-soluble radioactivity released from [^{3}H]BSA. ●, Antiserum; ○, control serum.

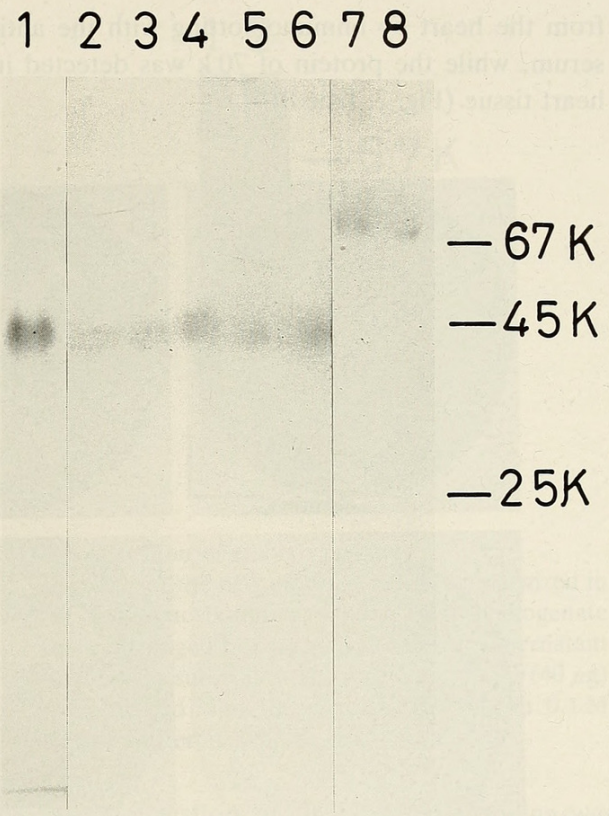

FIG. 2. Screening with antiserum for protease in embryos by immunoblotting.
Purified enzyme protein was subjected to electrophoresis in a 15% polyacrylamide gel and detected by silver staining (Lane 1). Proteins (20 μg) from unfertilized eggs, and from embryos at the morula, gastrula, neurula and tail bud stages (Lanes 2–6) and proteins (20 μg) from liver (lane 7) and heart (lane 8) of *Xenopus laevis* were fractionated in a 15% polyacrylamide gel and transferred to a nitrocellulose membrane. Protein was visualized by treatment with antiserum. lane 2, unfertilized egg; lane 3, embryo at morula stage; lane 4, embryo at gastrula stage; lane 5, embryo at neurula stage; lane 6; embryo at tail bud stage; lane 7, liver of Xenopus; lane 8, heart of Xenopus. Standard proteins used were bovine serum albumin (67 k), hen egg albumin (45 k) and chymotrypsinogen A (25 k).

stages and from the liver of adult frogs were fractionated by gel electrophoresis, transferred to nitrocellulose membranes, and subjected to immunoblotting with antiserum (Fig. 2). The purified enzyme gave a single band after electrophoresis in a 15% polyacrylamide gel (Fig. 2, lane 1). The relative mass (M_r) of the enzyme was estimated to be about 44 k. On screening with antiserum of proteins from unfertilized eggs and from embryos at the morula, gastrula, neurula and tail-bud stages, a band corresponding to an M_r of about 44 k was visualized from embryos at all stages (Fig. 2, lanes 2–6). When control serum was employed no bands were detected from embryos at any stage or from the adult liver (data not shown). The band corresponding to an M_r of 44 k coincided in terms of size with the purified protease detected by silver staining. The resulted indicate that the antiserum was monospecific for the protease protein from embryos. The relative intensity of the bands was almost constant among the proteins from unfertilized eggs and from embryos at the morula, gastrula, neurula and tail-bud stages. The protease in early embryos was already present as a maternal proteins in unfertilized eggs.

When a sample of the proteins from the liver of *Xenopus laevis* was immunoblotted, a protein with M_r of 70 k was visualized by treatment with antiserum, but the band corresponding to an M_r of 44 k was not observed (Fig. 2, lane 7). Also, the protein of 44 k was not detected in the proteins

from the heart by immunoblotting with the antiserum, while the protein of 70 k was detected in heart tissue (Fig. 2, lane 8).

Immunohistochemical analysis of the distribution of the protease during development

The distribution of the protease was examined in

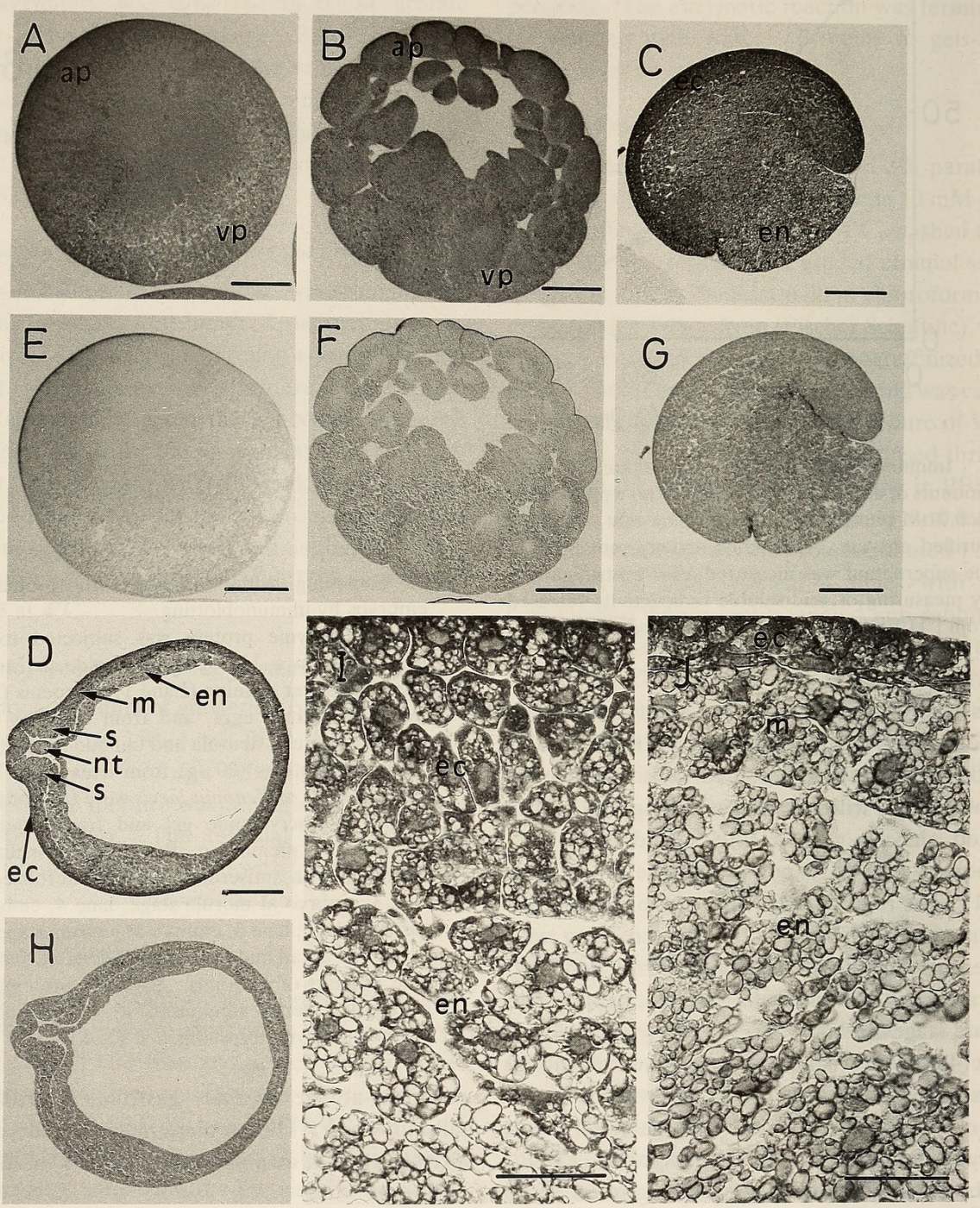

Fig. 3. Immunohistochemical localization of the protease in dissected eggs and embryos.
All sections were cut along the animal-vegetal axis and stained with antiserum (A-D, I and J) or control serum (E-H). A and E, Unfertilized egg; B and F, embryo at morula stage; C and G, embryo at gastrula stage; D and H, embryo at neurula stage; I, higher magnification of embryo at gastrula stage; J, higer magnification of embryo at neurula stage.
Abbreviations are as follows: ap, animal pole; ec, ectoderm; en, endoderm; m, mesoderm; nt, notochord; s, somites; vp, vegetal pole. Bar length: A-H, 0.25 mm; I and J, 0.025 mm.

sections of eggs and of embryos at various stages by immunostaining with the antiserum. The protease was detected as a dark deposit after immunohistochemical staining. Sections were cut along the animal-vegetal axis. In the unfertilized egg, the animal region showed strong immunoreactivity for the protease antigen (Fig. 3A). The animal region at the morula stage also contained a higher concentration of the protease than did the vegetal region (Fig. 3B). Immunohistochemical localization of the protease antigen in the gastrula is shown in Figure 3C. The ectoderm was stained more strongly than the endoderm. In the embryos at the neurula stage, the intensity of the staining decreased from the ectoderm, through the mesoderm, to the endoderm (Fig. 3D). The outer layer of cells of the ectoderm was most strongly stained and cells derived from the dorsal mesoderm, such as the notochord and somites, also stained with higher intensity than the endoderm. The animal portion of the egg develops into ectodermal tissue, while an explant of the vegetal portion forms endodermal tissue. Furthermore, when animal pole cells are put in contact with vegetal pole cells, the fate of the animal pole cells changes and they form mesoderm tissue [9, 10]. Thus, in our experiments, the ectoderm and mesoderm stain more heavity than the endoderm as a result of the fact that the animal region stains more heavily stronger than the vegetal region. In sections treated with control serum, no differences among the regions were detected (Fig. 3E-3H).

To define the intracellurar localization of the protease, magnified photographs of sections at the gastrula and neurula stages were examined (Fig. 3I and J). The cytoplasm without yolk granules or particles in the ectodermal cells at the gastrula stage was stained more darkly than the cytoplasm in the endodermal cell (Fig. 3I). The cytoplasm without particles in the ectodermal cells at the neurula stage was also stained as a dark deposit (Fig. 3J). But the endodermal cells were weakly stained.

Digestion of embryo proteins

The activity of the protease with embryo proteins as substrate is shown in Figure 4. Embryo proteins were treated with the enzyme, then the

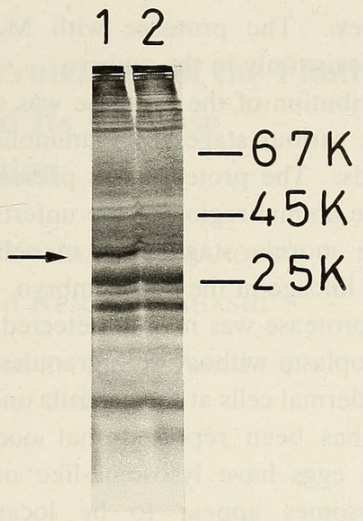

Fig. 4. Digestion of embryo proteins
Embryos at the neurula stage were homogenized in 0.1 M acetate buffer at pH 5.0. The homogenate was centrifuged in a microfuge and the supernatant was used as substrate. The embryo proteins (40 μg) were treated with the enzyme (0.5 μg), in 0.1 M acetate buffer at pH 3.8.

extent of digestion of the embryo proteins was assessed by SDS-polyacrylamide gel electrophoresis. When embryo proteins were digested in the 0.1 M acetate buffer at pH 3.8, a protein with M_r of 31 k was the most susceptible to digestion (Fig. 4, lane 2) (Fig. 4, lane 1, enzyme-free).

DISCUSSION

The developmental profile of a novel thiol protease in *Xenopus* was monitored using and antiserum raised against the protease. On screening with antiserum by immunoblotting, the protein that reacted with the antiserum was detected among the proteins from the unfertilized egg. The concentration of protease was almost the same among the proteins from unfertilized eggs as it was among proteins from embryos at the morula, gastrula, neurula and tail-bud stages. However, we have already reported that the proteolytic activity is barely detectable in the unfertilized egg, when assays of thiol protease sensitive to antipain is performed in the presence of DTT [11]. Thus, the proteolytic activity seems to be inhibited by some unidentified mechanism in the unfertilized egg.

The protease with M_r of 44 k was not detected in adult tissues, and a protein of 70 k was detected in

these tissues. The protease with M_r of 44 k appears to exist only in the embryo.

The distribution of the protease was studied in embryos at various stages by immunohistochemical methods. The protease was present at high levels in the animal region of the unfertilized egg and at the morula stage, and in cells of the ectodermal lineage in the early embryo. Furthermore, the protease was mainly detected throughout the cytoplasm without yolk granules or particles in ectodermal cells at the gastrula and neurula stage. It has been reported that oocytes and unfertilized eggs have lysosome-like organelles. These lysosomes appear to be located in a peripheral zone of the cytoplasm [12, 13]. Thus, this protease seems not to be localized in the lysosome.

Many non-lysosomal proteases have been purified and characterized [1–3]. We reported previously that the proteolytic activity which is sensitive to the protease inhibition, antipain increases during early development and antipain inhibits the incorporation of [^{3}H] uridine into RAN in *Xenopus* embryos [11, 14]. This study was carried out by antiserum raised against the antipain sensitive protease. Following fertilization, the synthesis of proteins, DNA replication and cell division begin [15, 16]. Synthesis of RNA becomes detectable at the midblastula stage [16–18]. This protease may function as one of components required for maintenance of high rates of the synthesis of RNA, proteins or DNA in the early embryo.

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