Proteolysis of the Major Yolk Glycoproteins Is Regulated by Acidification of the Yolk Platelets in Sea Urchin Embryos

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Abstract. The precise function of the yolk platelets of sea urchin embryos during early development is unknown. We have shown previously that the chemical composition of the yolk platelets remains unchanged in terms of phospholipid, triglyceride, hexose, sialic acid, RNA, and total protein content after fertilization and early development. However, the platelet is not entirely static because the major 160-kD yolk glycoprotein YP-160 undergoes limited, step-wise proteolytic cleavage during early development. Based on previous studies by us and others, it has been postulated that yolk platelets become acidified during development, leading to the activation of a cathepsin B-like yolk proteinase that is believed to be responsible for the degradation of the major yolk glycoprotein. To investigate this possibility, we studied the effect of addition of chloroquine, which prevents acidification of lysosomes. Consistent with the postulated requirement for acidification, it was found that chloroquine blocked YP-160 breakdown but had no effect on embryonic development. To directly test the possibility that acidification of the yolk platelets over the course of development temporally correlated with YP-160 proteolysis, we added 3-(2,4-dinitroanilo)-3-amino-*N*-methyldipropylamine (DAMP) to eggs or embryos. This compound localizes to acidic organelles and can be detected in these organelles by EM. The results of these studies revealed that yolk platelets did, in fact, become transiently acidified during development. This acidification occurred at the same time as yolk protein proteolysis, i.e., at 6 h after fertilization (64-cell stage) in *Strongylocentrotus purpuratus* and at 48 h after fertilization (late gastrula) in *L. pictus*. Furthermore, the pH value at the point of maximal acidification of the yolk platelets in vivo was equal to the pH optimum of the enzyme measured in vitro, indicating that this acidification is sufficient to activate the enzyme. For both *S. purpuratus* and *Lytechinus pictus*, the observed decrease in the pH was ~0.8 U, from 7.0 to 6.2. The trypsin inhibitor benzamidine was found to inhibit the yolk proteinase in vivo. By virtue of the fact that this inhibitor was reversible we established that the activity of the yolk proteinase is developmentally regulated even though the enzyme is present throughout the course of development. These findings indicate that acidification of yolk platelets is a developmentally regulated process that is a prerequisite to initiation of the catabolism of the major yolk glycoprotein.

Approximately one-third of the volume of sea urchin eggs is comprised of organelles called yolk platelets. Because in other embryonic systems there is evidence that yolk platelets provide a source of nutrients for early development, a similar function has been proposed for the yolk platelets in the sea urchin (Williams, 1967). However, there is no evidence to support this theory, since the composition of yolk platelets throughout the course of development from zygote to pluteus remains unchanged in terms of phospholipid, triglycerides, hexose, sialic acid, RNA, and total protein (Armant et al., 1986). On the basis of these observations it was suggested that yolk platelets, rather than being utilized during early development, might serve as an energy reserve to be used in the event that a food source is not immediately available when feeding begins at the late pluteus stage. This proposal was partially substantiated, because in a subsequent study it was found that before metamorphosis, at the feeding pluteus stage, the yolk platelet and its glycoproteins disappear. However, this dramatic change at the late pluteus stage does not occur in response to dietary conditions, since the presence or absence of exogenous food stuffs has no effect (Scott et al., 1990). Gratwohl et al. (1991) proposed that the material stored in the yolk platelets is used for the assembly of new membranes. Using an antiserum prepared against a structure referred to as a toposome, a large hexameric glycoprotein complex, these authors observed labeling of the egg cell surface as well as of cortical granules and yolk platelets. However, our studies, using a monospecific antiserum to a purified preparation of the 90-kD fragment of the major yolk glycoprotein did not support these findings because labeling was found to be restricted to the yolk platelets (Scott and Lennarz, 1989).

The function of the yolk platelet is, therefore, unknown. It is important to note, however, that although yolk platelets are not degraded during earlier development stages, they are not entirely static because their constituent glycoproteins undergo limited, stepwise proteolysis. This phenomenon, reported by Kari and Rottmann (1985) and by Armant et al. (1986), was further studied by Scott and Lennarz (1989) and by Lee et al. (1989). Scott and Lennarz (1989), using immunological tools, found that the major yolk glycoproteins of embryos within the class Echinoidea are conserved, and that they are progressively degraded over the course of development from the 170–160-kD major yolk glycoprotein (YP-160) to glycoproteins of lower mass (115, 108, and 90 kD in *Strongylocentrotus purpuratus* and *Lytechinus pictus*). The yolk platelet has been proposed to be a "lysosome-like" particle, because acid glycosidases and acid phosphatase have been shown to be associated with it (Schuel et al., 1975). In addition, studies using two sea urchin species of the Sea of Japan, *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina* (Yokota and Kato, 1988) and the west coast sea urchin, *S. purpuratus* (Scott et al., 1990) showed that when isolated yolk platelets are incubated in acidified sea water, hydrolysis of the major yolk glycoprotein, YP-160, occurs. Because this process was shown to be blocked by inhibitors of thiol proteinases, it was proposed that the yolk proteinase is a cathepsin B–like enzyme that is active at acidic pH (Yokota and Kato, 1988). A partial purification of a cathepsin B-like enzyme from sea urchin eggs has been recently reported (Okada and Yokota, 1990). In members of another order, the African soft tick, *Ornithodoros moubata*, the presence of a yolk platelet-associated, cathepsin L–like enzyme that is activated by acidification has been reported (Fagotta, 1990*a*, 1990*b*, 1991). All of these observations led us to hypothesize that the yolk platelet of the sea urchin egg becomes acidified during development and that the cathepsin B-like enzyme gets activated.

In the current study, we have directly tested this hypothesis and have studied the properties of the protease that cleaves YP-160. Initially, we found that the agent chloroquine, which can enter acidic organelles and dissipate proton gradients, inhibited YP-160 proteolysis in vivo, suggesting that indeed yolk platelet acidification is required for YP-160 hydrolysis. To prove directly that the yolk platelets become acidified during development, we have used the reagent 3-(2,4-dinitroanilo)-3-amino-*N*-methyldipropylamine (DAMP).[1] DAMP has been shown to enter acidic organelles and to be stable to fixation for EM and subsequent detection with an antibody. DAMP has been used previously to examine acidification of yolk platelets in sea urchins, and the results were reported in abstract form (Valdizan, M. C., and G. L. Decker. 1989. *J. Cell Biol.* 109:203a). We have observed a transient decrease in the internal pH of yolk platelets during development in two sea urchin species studied, *S. purpuratus* and *L. pictus*. This decrease was temporally correlated with cleavage of YP-160 to smaller proteins. A determination of the pH-activity profile of the yolk proteinase in vitro showed that this pH decrease is sufficient to activate the latent enzyme. Because benzamidine was found to be a reversible inhibitor of YP-160 proteolysis we used it to show that the yolk proteinase activity is developmentally regulated and that proteolytic cleavage of YP-160 is not essential for normal development of the embryo to the pluteus stage. By isolating yolk platelets at various stages of development and assaying for yolk proteinase activity in vitro, we have shown that the enzyme is present at all stages from egg to pluteus. Based on this finding we conclude that the activity of the yolk proteinase is regulated by acidification of the yolk platelet.

1. *Abbreviation used in this paper*: DAMP, 3-(2,4-dinitroanilo)-3-amino-*N*-methyldipropylamine.

Materials and Methods

Sea Urchins and Other Materials

S. purpuratus and *L. pictus* were obtained from Marinus (Long Beach, CA) and embryos were prepared and cultured at 14°C as described (Heifetz and Lennarz, 1979). Antipain, benzamidine, chloroquine, chymostatin, elastatinal, leupeptin, PCMB, PMSF, pepstatin, TLCK, and TPCK were purchased from Sigma Chemical Co. (St. Louis, MO). Z-Phe-Ala-CH_2F was purchased from Enzyme Systems Products (Dublin, CA). ^{125}I-labeled donkey antirabbit second antibody was purchased from Amersham Corp. (Arlington Heights, IL). DAMP and antibody to dinitrophenol were obtained from Molecular Probes, Inc. (Eugene, OR). Goat anti-rabbit IgG-gold conjugate (GAR G_{10}) was purchased from Amersham Corp. Lowicryl K_4M was obtained from Polysciences. Artificial sea water (Instant Ocean) was obtained from Aquarium Systems (Mentor, OH). Bicinchoninic Acid (BCA) was purchased from Pierce Chemical Co. (Rockford, IL).

Protein Determination, SDS-PAGE, and Western Analyses

The embryos were harvested and proteins extracted as described (Scott and Lennarz, 1989). Protein estimation was done using the BCA method (Smith et al., 1985). The proteins were visualized by SDS-PAGE (5% polyacrylamide; Laemmli, 1970) and Western blot analysis as described (Towbin et al., 1979) with the antiserum generated using the 90-kD fragment of major yolk protein as the antigen (Scott and Lennarz, 1989).

Enzyme Assays

The assays for cathepsin B were carried out by using the substrate Z-Arg-Arg-AMC (Bachem, Philadelphia, PA) as described by Okada and Yokota (1990). Briefly, an aliquot of enzyme was added to 200 mM KH_2PO_4, 4 mM cysteine, 2 mM EDTA, pH 5.5, in a final volume of 235 μl. The assay was started by addition of 15 μl of a 5-mM stock substrate dissolved in DMSO. The fluorescence at 460 nm, upon excitation at 380 nm, was continuously recorded using a Perkin Elmer LS3 fluorometer. The rate of hydrolysis was calculated and the percent inhibition determined for various inhibitors. In all assays the amount of substrate hydrolyzed was less than 10% of the total added.

DAMP Treatment and Immunoelectron Microscopy

Sea urchin eggs and embryos were prepared as described and incubated in 30 μM DAMP in ASW for 60 min at 14°C, a modification of the method of Anderson (1989). After incubation, the specimens were washed three times in ASW or calcium-free ASW. Duplicate specimens were processed without the addition of DAMP, as controls. Fixation was in freshly prepared 1% paraformaldehyde with 1% glutaraldehyde in ASW at 4°C for 60 min. After additional rinses in sea water, the specimens were dehydrated in dimethylformamide and embedded in Lowicryl K_4M by the rapid method of Altman et al. (Altman, L. G., B. G. Schneider, and D. S. Papermaster. 1983. *J. Cell Biol.* 97:309a) with the exception that all steps were carried out at 4°C. For immunogold localization, 70–90-nm sections were cut with a diamond knife on a Reichert Ultracut ultramicrotome and mounted on Formvar–carbon–coated 300 mesh nickel grids. Labeling was done by the method of Anderson (1989). Briefly, after washing with buffer for 30 min the grids were incubated for 16 h at 4°C with anti-DNP IgG diluted 1:200. Consecutive grids were incubated with nonimmune serum or without primary antibody, to control for nonspecific labeling. They were again washed and incubated on droplets of GAR G_{10} diluted 1:20 for 2 h at room temperature. The grids were thoroughly washed with distilled water, poststained

with 2% aqueous uranyl acetate for 10 min, washed, and air dried. Electron microscopic examination was carried out using a JEOL 1200EX Electron Microscope, at 80 kV. Micrographs were taken at 15,000× and 30,000× magnification. Quantification of gold label and calculation of pH were as described by Anderson (1989). Quantification of gold particles per square micrometer of yolk platelets and nuclei was done by visual inspection. Since the uptake of DAMP results in swelling of acidic organelles, it was necessary to normalize the surface area of platelets in DAMP-treated specimens. This was accomplished by determining average yolk platelet surface area in specimens not treated with DAMP but similarly fixed and embedded, and using this average surface area for calculation of estimated pH, using the formula of Orci et al. (1986), and assuming that the pH of the nucleus is neutral.

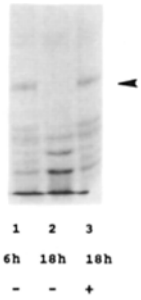


Figure 1. Chloroquine (40 μM in ASW) inhibits the in vivo proteolysis of YP-160 in *S. purpuratus*. SDS-PAGE of total proteins stained with Coomassie blue. YP-160 is indicated by the arrowhead.

Results

Chloroquine Inhibits the In Vivo Hydrolysis of YP-160

In view of the in vivo observation of degradation of yolk platelet glycoprotein during development and in vitro findings that upon acidification isolated yolk platelets catabolize their constituent glycoproteins (Yokota and Kato, 1988; Scott et al., 1990), it seemed reasonable to postulate that yolk platelets become acidified in vivo. To test this hypothesis initially we examined the effect of chloroquine on YP-160 hydrolysis in developing embryos, because chloroquine has been shown to be effective in dissipating proton gradients in a variety of biological systems (Allison and Young, 1964; DeDuve et al., 1974). Chloroquine was added to *S. purpuratus* embryos 6 h after fertilization and at 18 h after fertilization, aliquots were withdrawn, processed as described in Materials and Methods, and analyzed by SDS-PAGE. The results shown in Fig. 1 reveal that in control embryos the level of YP-160 is unchanged at 6 h after fertilization, whereas by the time of hatching this form of the yolk glycoprotein is virtually absent (lanes *1* and *2*). In contrast, although embryos cultured in the presence of chloroquine developed normally to the blastula stage, they exhibited little or no proteolysis of YP-160 (lane *3*). Addition of ammonium chloride also has the same effect (data not shown). This result provides strong indirect support for the idea that acidification of yolk platelets is important for the proteolysis of the YP-160 glycoprotein.

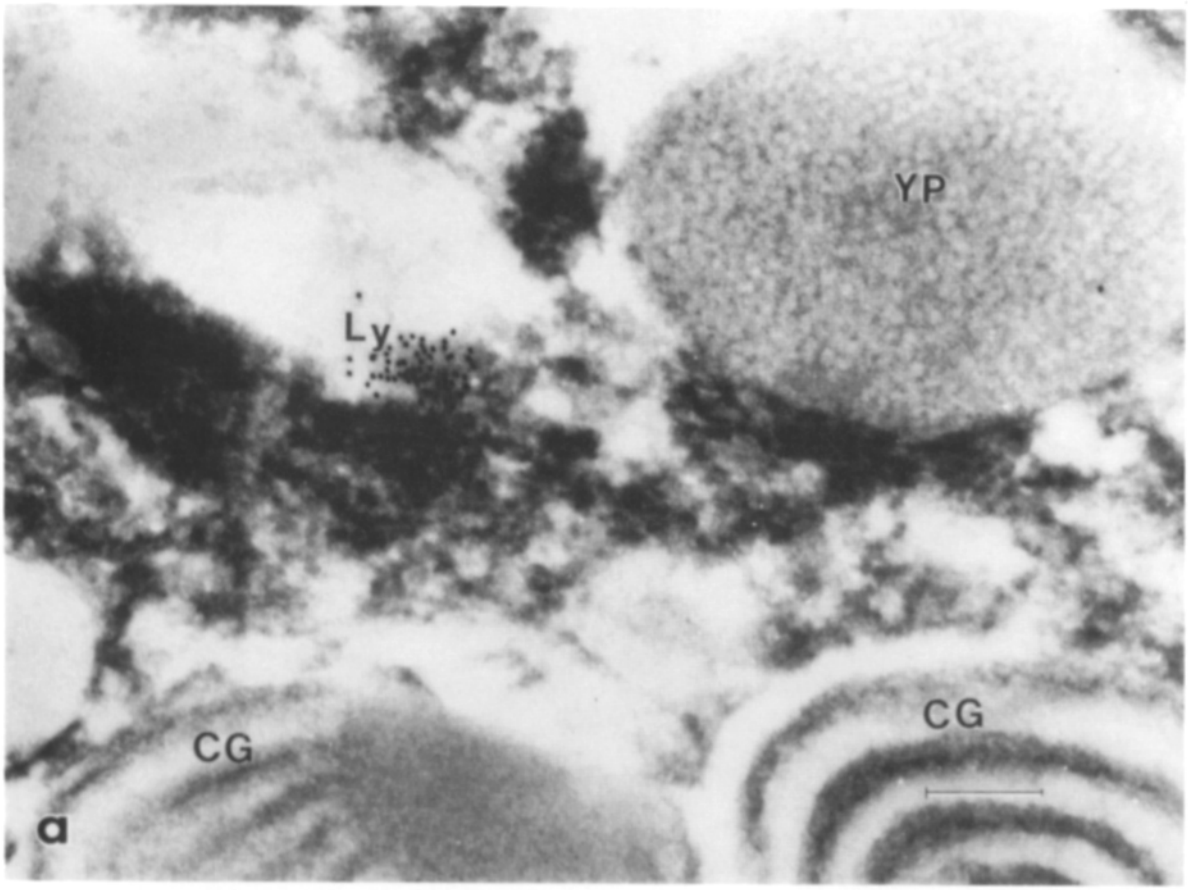
Yolk Platelets Become Acidified during Development In Vivo

To directly test the hypothesis that yolk platelets become acidified we used DAMP, which has been shown to enter cells and become localized to acidic organelles (Anderson et al., 1984). DAMP is a weak base which diffuses freely throughout the cell, but in acidic compartments becomes protonated and impermeant to membranes. Subsequently, it can be cross-linked to proteins within the organelle by fixation with glutaraldehyde. The compartments containing DAMP are then identified by using an antibody that recognizes the dinitrophenyl moiety, which in turn can be visualized using gold-labeled second antibody.

Earlier experiments by us and others (Scott and Lennarz, 1989; Lee et al., 1989) have shown that in *S. purpuratus* the proteolysis of YP-160 is initiated at 6 h after fertilization and is essentially complete by 18 h. We used DAMP in order to test whether this proteolysis correlated with acidification. Aliquots of a culture of *S. purpuratus* embryos were treated with DAMP at various stages as described in Materials and Methods. The results in Fig. 2 reveal that there is virtually no gold label in the platelets of the egg and that there is a large increase of label in the yolk platelets of the embryo at 12 and 24 h after fertilization. The values for the average number of gold particles detected per yolk platelet are shown in Table I. There is a statistically significant difference ($P < 0.001$ by Student's t test) in the number of gold particles in egg yolk platelets as compared to those in 12- and 24-h embryos. It is notable that in both *S. purpuratus* (Fig. 2) and *L. pictus* (see Fig. 5), the gold label over the acidic yolk platelets is unevenly distributed and patchy. It is possible that this patchy labeling is the result of clustering of sites of disulfide-linked aggregates of yolk glycoproteins (Scott et al., 1990), to which the DAMP has been crosslinked by aldehyde fixation. However, despite this uneven distribution, the total number of particles observed was very reproducible. The results in Table I reveal that in *S. purpuratus* eggs the pH of yolk platelets in the egg was 6.8 and that there was a transient drop of 0.7 U (to pH 6.1) at blastula (12 h). After this point, the pH slowly rose almost to its original value.

To test whether the observed drop in pH was sufficient to activate the yolk proteinase, the pH dependence of the yolk proteinase was studied in vitro. Yolk platelets were prepared from *S. purpuratus* eggs as described (Scott et al., 1990) and then sonicated and centrifuged at 100,000 *g*. The resulting supernatant was assayed for proteinase activity using the synthetic peptide Z-Arg-Arg-AMC. The pH-activity profile shown in Fig. 3 reveals that the enzyme is inactive at pH 6.8 and that maximal activity is observed at pH 6.1, which is the pH of the yolk platelets at blastula stage. At pH 4.2, the pH at which most lysosomal enzymes are optimally active, the proteinase shows only 30% of maximum activity. Thus, the drop in pH of 0.7 U observed in vivo would be expected to afford optimum conditions for yolk proteinase activity. The results in Fig. 3 also show that, as expected, leupeptin fully inhibits the yolk proteinase. The pH-activity profile is in good agreement with that reported for a partially purified yolk proteinase (Okada and Yokota, 1990). Thus, yolk platelets become acidified during development, and this acidification correlates with the time that YP-160 proteolysis is initiated in *S. purpuratus* embryos.

In the species *L. pictus*, proteolysis of YP-160 is not initiated until gastrulation and is complete only at the prism stage (Scott and Lennarz, 1989). Thus, it was of interest to determine whether in *L. pictus* the yolk platelets are acidified, and if this acidification correlated temporally with the proteolysis of YP-160 that is initiated at gastrulation. Aliquots of a culture taken at various stages of development were divided into two portions. One portion was treated with DAMP and processed for EM as described above. The other portion was



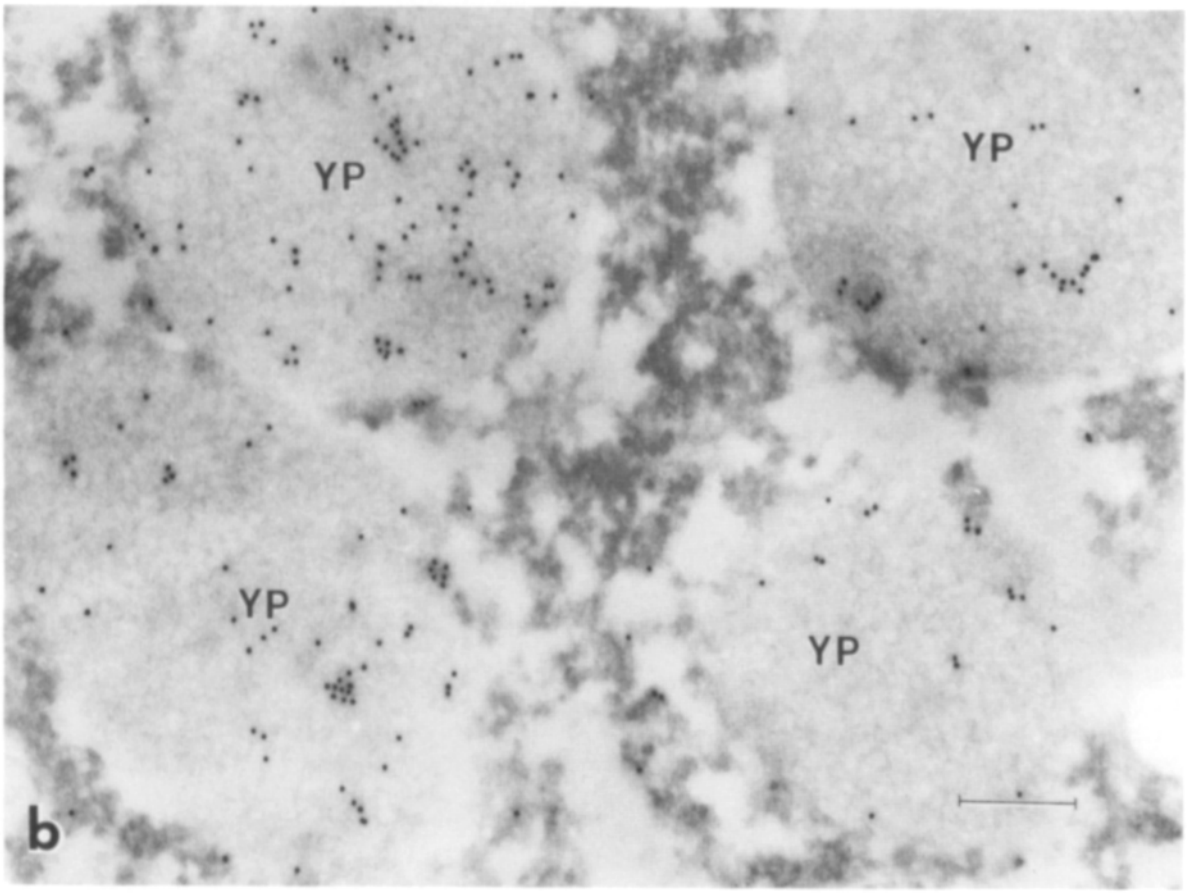
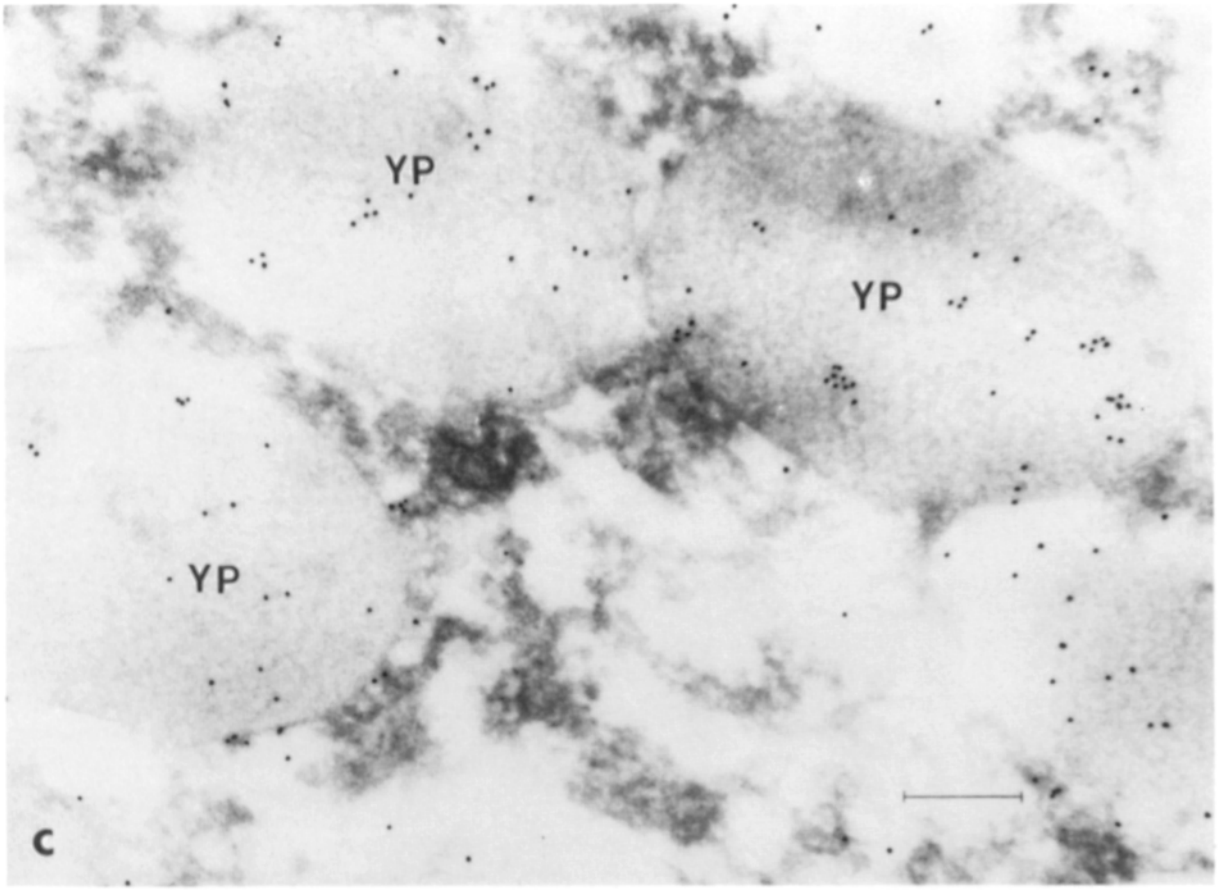


Figure 2. Use of DAMP (30 μM) to detect acidification of yolk platelets in vivo. Electron micrographs of thin sections of *S. purpuratus* eggs (*a*), 12 h (early blastula, *b*), and 24 h (hatched blastula, *c*) embryos treated as described in Materials and Methods reveal the distribution of gold particles in yolk platelets during development. *d* shows a section of 12-h embryos cultured in the presence of benzamidine. YP, yolk platelet; cg, cortical granule; L, lipid droplet; Ly in *a* is presumed to be a lysosome. The micron maker equals 200 nm.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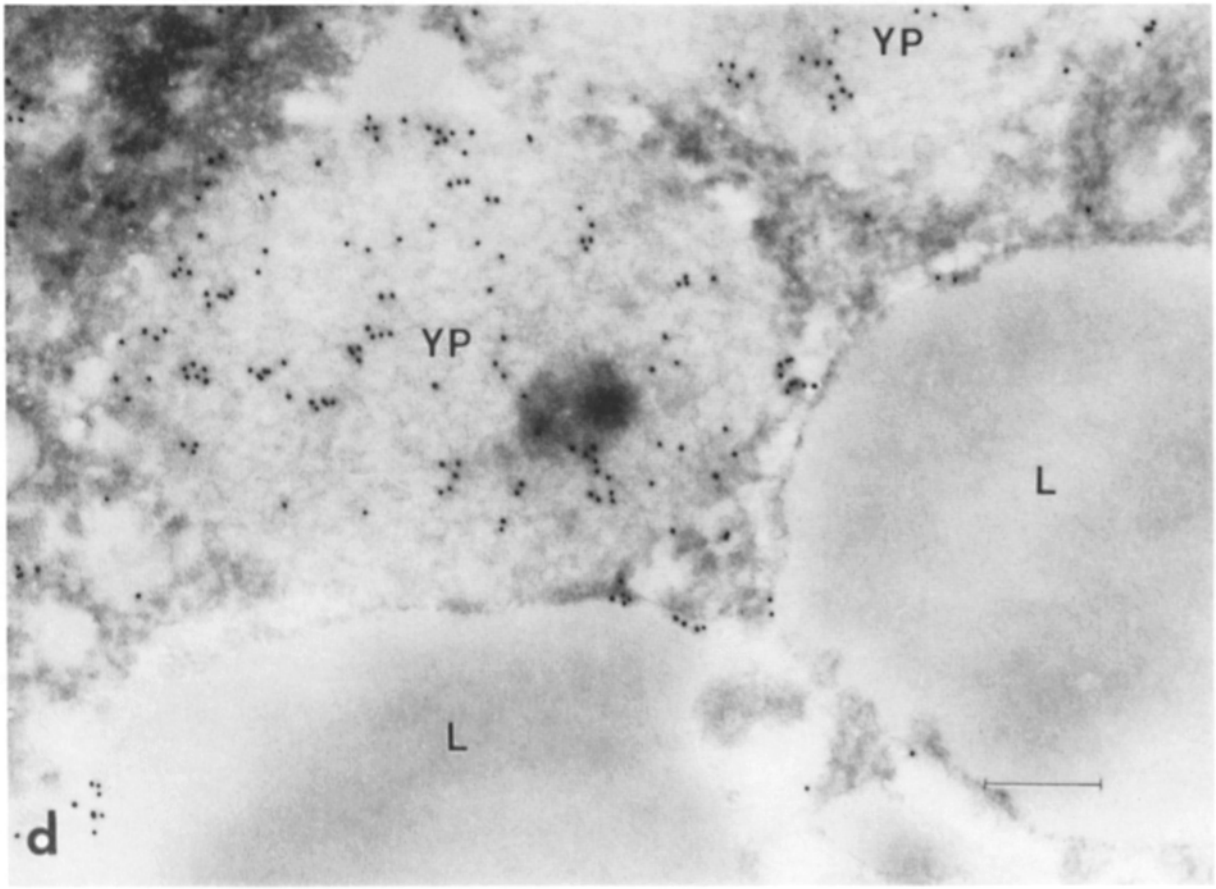
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Table I. Distribution of DAMP (30 μM) in Yolk Platelets over the Course of Development of S. Purpuratus Embryos

Time of Harvest	*n*	Average number of gold grains per square micron of yolk platelet	Calculated pH of yolk platelets	Calculated pH change
Egg	50	5 ± 3.6*	6.8	0
12 h (blastula)	50	55 ± 30	6.1	−0.7
24 h (hatched blastula)	35	41 ± 24	6.6	−0.2
12 h (+ benzamidine)	50	39 ± 21	6.4	−0.4

n = the number of yolk platelets counted.
* Values shown are ± SD. The *P* values were <0.001. See text.

analyzed for YP-160 proteolysis by SDS-PAGE. The results shown in Fig. 4 *a* show that YP-160 proteolysis was initiated at gastrulation and was complete by the prism stage (72 h). These observations were confirmed by Western blot analysis of the level of YP-160 (Fig. 4 *b*). Analysis of the distribution of DAMP by EM (Fig. 5) revealed an increase in the number of gold particles bound per yolk platelet during mid to late gastrula stage. Embryos treated with chloroquine for 30 min before addition of DAMP contained fewer gold particles at 72 h than control embryos at 72 h (Table II), indicating that chloroquine blocked the acidification process. There is a statistically significant difference ($P < 0.001$) between the numbers of gold particles found in the platelets of embryos at late gastrula and prism stages as compared to platelets of early embryonic stages and eggs. The finding that the average number of gold grains labeling acidic yolk platelets in *S. purpuratus* differs from that in *L. pictus* cannot be explained by variation from one experiment to another (Griffith, 1988) because the experiments with *L. pictus* and *S. purpuratus* embryos were done at the same time. One possible explanation for this difference may be the presence of fewer protein sites for crosslinking DAMP in the *L. pictus* embryos. Nevertheless, in both species of sea urchin embryos the time of yolk platelet glycoprotein hydrolysis correlates with the time of acidification of the organelle.

Control specimens exhibited no labeling above background with second antibody gold alone. In a number of experiments heavy gold labeling of lysosome-like organelles, distinct from yolk platelets, mitochondria, or other organelles was observed (see Fig. 2 *A*). When it was possible to determine a surface area for these organelles, the calculated pH was found to be 4.8, a value within the range of that found for lysosomes. Occasionally, light labeling of small vesicles was observed, but this binding was not quantified and these vesicles remain unidentified.

Effect of Various Enzyme Inhibitors on YP-160 Hydrolysis

It has been proposed that the yolk proteinase is a cathepsin B-like enzyme that is activated by acidification of the yolk platelets (Yokota and Kato, 1988; Scott et al., 1990). This idea is consistent with the fact that a cathepsin B has been detected and partially purified from sea urchin eggs (Okada and Yokota, 1990). Alternatively, it has been suggested, on the basis of comparison of peptides generated by breakdown of the 160-kD glycoprotein in vivo with those generated by trypsin digestion of the major yolk protein, that a trypsin-like proteinase may be involved (Giga and Ikai, 1985; Lee et al., 1989). Although such a comparison clearly is not a compelling reason to invoke the involvement of a trypsin-like enzyme, we undertook a survey of the possible effect of a variety of protease inhibitors on yolk platelet glycoprotein catabolism. Table III lists the inhibitors tested in *S. purpuratus*, along with the class of enzyme they are known to inhibit and their effect on YP-160 breakdown in vivo. The inhibitors were added after fertilization, and the embryos were harvested after hatching and analyzed for 160-kD major yolk glycoprotein content by SDS-PAGE followed by Coomassie blue staining. None of the inhibitors tested were lethal, because in all cases the embryos hatched normally in their presence. The results of this survey of inhibitors, shown in Fig. 6, indicate that antipain and leupeptin (lanes *8* and *4*) inhibit YP-160 hydrolysis in vivo. This supports the idea that the yolk proteinase is a thiol proteinase. The inhibition by Z-Phe-Ala-CH_2F (lane *1*), a novel specific cathepsin B inhibitor, confirms that the yolk proteinase is a cathepsin B-like enzyme. In view of the in vivo acidification observed we tested the aspartate proteinase inhibitor pepstatin (lane *3*). This inhibitor had no effect, showing that a pepsin-like enzyme acting at acidic pH is not involved in YP-160 proteol-

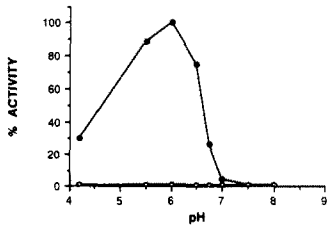

Figure 3. The pH-activity profile of the *S. purpuratus* yolk proteinase in vitro. The percent activity (●) at various pH is calculated by assuming the maximum rate to be 100%. The activity in presence of leupeptin (50 μg/ml) is shown by (○).

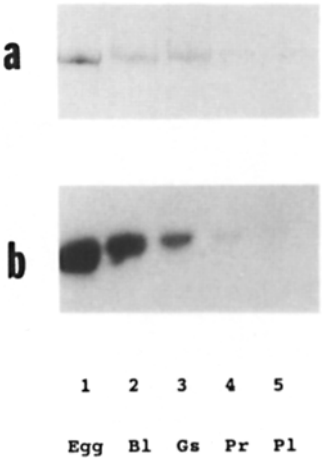

Figure 4. Yolk platelet acidification correlates with YP-160 proteolysis in *L. pictus*. In *a*, a Coomassie blue-stained gel of embryo proteins over the course of *L. pictus* development is shown. Note that YP-160 is present in the egg (lane *1*), blastula (lane *2*), and gastrula (lane *3*) but markedly decreases at the prism (lane *4*) and pluteus (lane *5*) stages. In *b*, the proteins at the same stages were examined by Western blot analysis.

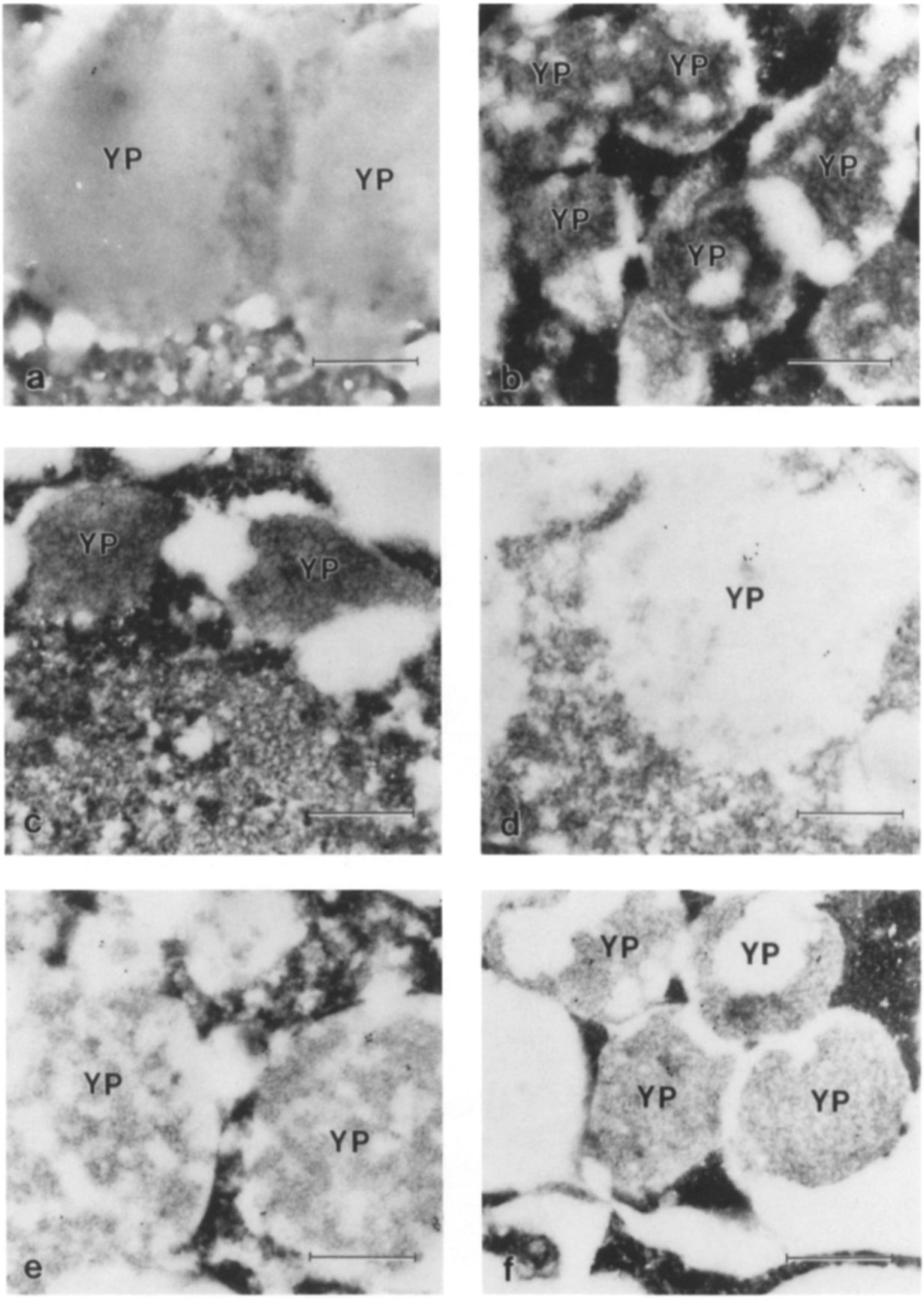

Figure 5. Electron micrographs of thin sections of *L. pictus* eggs and embryos treated with 30 μM DAMP as described in Materials and Methods are shown. The egg (*a*), blastula (*b*), gastrula (*c*), late gastrula (*d*), and prism (*e*) stages are shown. In *f* the effect of treatment of the prism stage embryos with 300 μM chloroquine for 30 min before addition of DAMP is shown. The micron marker equals 500 nm.

Table II. Distribution of DAMP (30 μM) in Yolk Platelets over the Course of Development of L. Pictus Embryos

Time of Harvest	*n*	Average number of gold particles per square micron of yolk platelet	Calculated pH of yolk platelets	Calculated pH change
Egg	50	1.5 ± 1.1*	7.0	0
24 h (hatched blastula)	50	1.6 ± 1.4	7.2	+0.2
42 h (mid gastrula)	50	2.5 ± 1.5	7.2	+0.2
48 h (late gastrula)	50	10 ± 5.0	6.2	−0.8
72 h (prism)	50	10 ± 4.0	6.5	−0.5
72 h (+ chloroquine)	33	5.0 ± 2.2	7.2	+0.2

n = the number of yolk platelets counted.
* Values shown are ± SD. The *P* values were <0.001. See text.

ysis. Among the various serine proteinase inhibitors tested, only two inhibitors, benzamidine (lane *7*) and TLCK (lane *2*), both of which block the action of trypsin-like enzymes, inhibited YP-160 hydrolysis. The two other serine proteinase inhibitors tested, chymostatin (lane *6*) and elastatinal (lane *5*), had no effect. The serine proteinase inhibitor PMSF could not be used in vivo since it is toxic to the embryos.

Benzamidine Is a Weak Inhibitor of the Yolk Proteinase

Given the strong evidence that a thiol proteinase operating at acid pH is functional in YP-160 degradation, the finding of the inhibitory effect of TLCK and benzamidine, both of which are serine protease inhibitors that act optimally on trypsin-like enzymes, initially was puzzling. However, because TLCK has been shown to block cathepsin B (Thie and Houseman, 1990), we focused on trying to understand the inhibitory effect of benzamidine.

Three possible explanations for the inhibition of YP-160 hydrolysis by benzamidine were considered. (*a*) Since benzamidine is a weak base it might have inhibited by virtue of its ability to prevent acidification of the yolk platelets. However, this possibility was excluded by measurement of the acidification of yolk platelets in the presence of benzamidine. As shown in Fig. 2 and Table I, benzamidine did not block acidification in *S. purpuratus*. The remaining possibilities considered were that: (*b*) benzamidine did, in fact, inhibit the yolk glycoprotein proteinase per se; or (*c*) benzamidine exerted its effect by inhibiting a trypsin-like enzyme in the yolk platelet that is also involved in proteolysis.

To determine whether benzamidine inhibits the yolk proteinase in vitro we used the cathepsin B substrate, Z-Arg-Arg-AMC, that has been shown to be a good substrate for the yolk proteinase (Okada and Yokota, 1990). Yolk platelets were prepared (Scott et al., 1990), sonicated for 15 s using a Branson probe sonicator and centrifuged at 100,000 *g* for 1 h. The supernatant was assayed for cathepsin B activity. The effect of various inhibitors on the cleavage of Z-Arg-Arg-AMC is shown in Table IV. As expected, the thiol proteinase inhibitors leupeptin and iodoacetamide inhibited hydrolysis of this substrate, as did benzamidine and TLCK. As noted above, the inhibition of yolk protein hydrolysis in vitro by TLCK has been documented (Yokota and Kato, 1988). The IC_{50} for inhibition by benzamidine was determined to be 640 μM. When this value is compared to the IC_{50} for leupeptin (10 μM) it is obvious that benzamidine is a very poor inhibitor of the yolk proteinase. Because leupeptin and iodoacetamide caused 100% inhibition, suggesting that the proteinase activity was solely due to a thiol proteinase, and the fact that the assay was carried out at pH 5.5, a condition in which trypsin is virtually inactive and the serine proteinase inhibitor PMSF that blocks trypsin-like enzymes did not inhibit substrate hydrolysis even at 1000 μM, we conclude that benzamidine exerts its effect by acting directly on the yolk glycoprotein proteinase. However, it is a poor inhibitor of this process, because the IC_{50} of 640 μM is much

Table III. Summary of the Effect of Inhibitors on YP-160 Proteolysis In Vivo and the Class of Proteinase They Inhibit

Inhibitor	YP-160 Proteolysis inhibited	Class of enzyme inhibited
Antipain	Yes	Thiol proteinase
Leupeptin	Yes	Thiol/Serine proteinases
Z-Phe-Ala-CH_2F	Yes	Thiol proteinase (cathepsin B)
Pepstatin	No	Aspartic proteinase (pepsin)
Chymostatin	No	Serine proteinase (chymotrypsin)
Elastatinal	No	Serine proteinase (human neutrophil elastase)
TLCK	Yes	Serine proteinase (trypsin)
Benzamidine	Yes	Serine proteinase (trypsin-like)

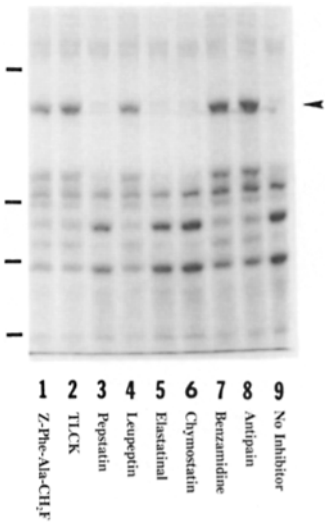

Figure 6. The effect of various inhibitors on the in vivo proteolysis of YP-160 in *S. purpuratus*. The embryos were harvested at hatched blastula stage, and proteins were extracted and analyzed by SDS-PAGE. The Coomassie blue-stained gel shows the protein profile in the absence of any inhibitor (lane *9*) and in the presence of antipain or leupeptin (100 μg/ml each, lanes *8* and *4*), Z-Phe-Ala-CH_2F (5 μg/ml, lane *1*), benzamidine (500 μg/ml, lane *7*), TLCK (50 μg/ml, lane *2*), chymostatin (100 μg/ml, lane *6*), elastatinal (20 μg/ml, lane *5*), and pepstatin (100 μg/ml, lane *3*). The molecular mass markers represent 200, 116, 97, and 68 kD.

Table IV. Effect of Various Potential Inhibitors on Cathepsin Activity in Yolk Platelet Supernatant

Inhibitor	Percent activity*
None	100
Iodoacetamide (200 μg/ml)	0
Leupeptin (50 μg/ml)	0
TLCK (25 μg/ml)	0
Benzamidine (500 μg/ml)	0
PMSF (20 μg/ml)	89
TPCK (25 μg/ml)	42

* Activity was measured by hydrolysis of Z-Arg-Arg-AMC. The percent activity is expressed relative to the control as being 100%.

higher than the reported value of 18 μM for the inhibition of trypsin by benzamidine (Mares-Guia and Shaw, 1965).

The Yolk Proteinase Activity Is Developmentally Regulated

Because benzamidine is known to be a reversible inhibitor in other systems and since it inhibited the yolk proteinase activity in vitro and YP-160 catabolism in vivo, we used it to study the developmental expression of the yolk proteinase by determining the effect of removal of this inhibitor on the subsequent breakdown of YP-160. One batch of *S. purpuratus* embryos was cultured in the presence of benzamidine; a control batch of embryos from eggs of the same female received no drug. At 6 h (8-cell stage) and 24 h (hatched blastula) aliquots were withdrawn from each culture and the embryos were washed twice to remove benzamidine. A portion of each washed culture was allowed to continue growth; in all these cases the washed embryos gastrulated and developed to prism stage normally. Aliquots from all three cultures (control, benzamidine-treated, and washed) were recovered at various stages and analyzed. The results in Fig. 7 show the protein profile in the egg (lane *1*) and in the embryo over the course of development (lanes *2–6*). It was clear that most of the YP-160 was proteolyzed by 12 h and proteolysis was essentially complete by 18 h. In contrast, YP-160 was still present at 12, 28, and 70 h (lanes *6–8*) when the benzamidine was present. Note that at 70 h the embryos had still not gastrulated in the presence of benzamidine, but they were viable as evidenced by their ability to swim. Washing out the benzamidine at 6 h, followed by continued cultivation, showed that much of the YP-160 was proteolyzed by 12 h (lane *9*) and was not detectable by 28 and 70 h (lanes *10* and *11*). However, washing out benzamidine later, at 24 h after fertilization, followed by continued cultivation revealed that significant amounts of YP-160 were still present at 28 and 70 h (lanes *12* and *13*). It is important to note that under these conditions the embryos gastrulated and developed normally to the pluteus stage after the removal of the benzamidine.

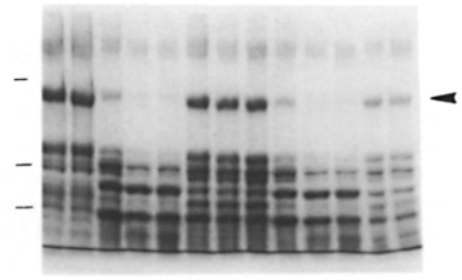

Figure 7. Yolk proteinase activity is developmentally regulated. The protein profile in the egg (lane *1*), 6 h (lane *2*), 12 h (lane *3*), 28 h (lane *4*), and 70 h (lane *5*) embryos is shown. Lanes *6–8* show the protein profile at 12, 28, and 70 h in the presence of benzamidine (500 μg/ml). Lanes *9–11* show the protein profile at 12, 28, and 70 h, in the case where benzamidine is washed out at 6 h. The protein profile at 28 and 70 h, upon washing out the benzamidine at 24 h is shown in lanes 12 and 13. The molecular mass markers represent 200, 116, and 97 kD.

These results clearly indicated that the yolk proteinase activity is developmentally regulated since very little yolk glycoprotein breakdown occurs subsequent to the washout of benzamidine at 24 h. Thus, the proteinase activity apparently is present as early as 6 h but it is markedly reduced by 24 h. This decline of the activity may be due to the increase in pH in the yolk platelet by 24 h detected by DAMP (Table II), and a concomitant decrease in proteinase activity. If so, it seems possible that the enzyme is present at all stages. To test this, we isolated yolk platelets from *S. purpuratus* embryos at various stages in development. After sonication and centrifugation at 100,000 *g*, the supernatant was assayed at pH 6.1 using Z-Arg-Arg-AMC, and the specific activity of the enzyme was calculated. The results shown in Fig. 8 clearly demonstrate that the yolk proteinase is present in the platelets at all stages across development. This observation strongly supports the idea that in vivo the enzyme activity is controlled by the pH in the yolk platelets.

In other experiments we found that benzamidine inhibits gastrulation in *S. purpuratus* if it is present at the early gastrula stage. However, the results in Fig. 7 show that YP-160 hydrolysis is not necessary for development through the gastrula stage to the pluteus stage. Thus, inhibition of YP-160 proteolysis and the inhibition of gastrulation by benzamidine are two unrelated events.

Discussion

Although the precise role of the yolk platelets in embryonic

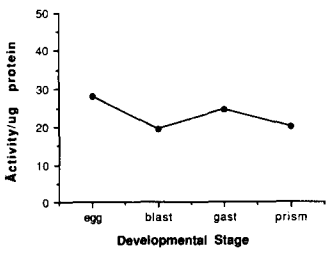

Figure 8. The yolk proteinase in *S. purpuratus* is present at all stages of development. Aliquots of a culture were harvested at blastula, gastrula and prism and yolk platelets were made, sonicated and centrifuged at 100,000 *g*. The yolk proteinase activity in the supernatant was measured at pH 6.1 using Z-Arg-Arg-AMC as substrate. The activity per unit protein remains relatively constant across development.

development in the sea urchin remains to be defined, the results of earlier studies (see Introduction), demonstrating stepwise proteolysis of the major glycoproteins, indicate that even early in development this organelle is not static. The current study has shown directly that the yolk platelet undergoes transient acidification and that it is at the stage of maximal acidification that proteolysis of YP-160 occurs. This correlation with peak acidification holds both for *S. purpuratus*, in which proteolysis is initiated at the early blastula stage (12 h), and *L. pictus*, in which it occurs at the gastrula stage (48 h). The acidification was measured using the probe DAMP, as first described by Anderson et al. (1984). Assuming that the nucleus has a neutral pH, the formula devised by Orci et al. (1986) may be used to estimate the pH of the compartments under study, in this case, the yolk platelet. This formula is not appropriate for estimation of pH in all circumstances; for example, in specific zones of the Golgi (Anderson and Pathak, 1985). Also, since DAMP, as well as other weak bases, causes swelling of acidic compartments, its use to calculate compartmental pH may not be possible in many cases. The fact that the surface area of yolk platelets in *S. purpuratus* and *L. pictus* remains relatively constant throughout the developmental stages studied here, i.e., about 0.8 square micron, allowed us to correct the measured surface area of these acidic yolk platelets back to that of control platelets, and thereby obtain an estimate of the internal pH of the yolk platelets at each stage of development. In doing this, we have made the assumption that the nucleus is neutral in the egg and in the embryo. Confirmation of approximate pH values was obtained by counting gold grains over organelles presumed to be lysosomes (see Fig. 2 *a*). The pH in these putative lysosomes was calculated to be 4.8, a value within the range of lysosomal pH found by others (for reviews see Mellman et al., 1986; Anderson and Orci, 1988).

The relative increase in the number of gold grains in the platelets of eggs to the platelets in later stage embryos is independent evidence of an increase in the concentration of H^+ in the organelle even if the calculated pH is in error by some fixed value. In the case of both *S. purpuratus* and *L. pictus* embryos, acidification to a pH of ~6.1–6.2 was observed, with the overall pH decrease being about 0.75 pH U. As noted above, in both of these species the time of the maximal change in pH correlated with the time of onset of catabolism of YP-160. However, in both species the change was transient, and the measured pH values returned to near neutrality. A determination of the pH optimum of the enzyme from *S. purpuratus*, using the synthetic cathepsin-B substrate Z-Arg-Arg-AMC, showed that this drop in pH was sufficient to change the enzyme from being essentially inactive to being maximally active. This profile was similar to that obtained by Okada and Yokota (1990), using the partially purified yolk proteinase from another species of sea urchin. Since in vitro assays revealed that the enzyme is present at all stages from egg to pluteus, it seems likely the enzyme activity is regulated by the observed transient acidification, rather than by some activation and subsequent inactivation processes.

It is of interest that the maximal pH decrease that occurs in the yolk platelet is less than that described for acidification of lysosomes, endosomes, *trans*-Golgi cisternae, transport vesicles, or secretory granules (Mellman et al., 1986). Although Schuel et al. (1975) showed that the yolk platelet contains several hydrolases with acidic pH optima, our findings of a relatively modest decline in pH that is transient argue that the yolk platelet is not a lysosomal particle. Rather, it may be a unique organelle with a proton pump, possibly of the electrogenic vacuolar type, such as has been described in early and late endosomes, some secretory vesicles, *trans*-Golgi cisternae, *trans*-Golgi network, and certain other classes of vesicles (Mellman et al., 1986; Anderson, 1989). This proton pump may be developmentally regulated and thereby control the timing of proteolytic processing of the major yolk glycoprotein.

Prior work done using isolated yolk platelets in vitro suggested that the yolk proteinase is a cathepsin B–like enzyme (Yokota and Kato, 1988; Scott et al., 1990) and recently such an enzyme has been partially purified from sea urchin eggs (Okada and Yokota, 1990). However, based on the observation that tryptic digests of yolk protein are similar to the degradation products, in vivo, it was suggested that the yolk proteinase is a trypsin-like enzyme (Giga and Ikai, 1985; Lee et al., 1989). To better understand the specificity of this enzyme, we examined the effects of a variety of thiol and serine proteinase inhibitors on YP-160 proteolysis in vivo. The analysis of YP-160 proteolysis by SDS-PAGE showed that the thiol proteinase inhibitors antipain and leupeptin inhibited proteolysis. This result, along with the finding that a newly reported cathepsin B inhibitor, Z-Phe-Ala-CH_2F (Van Noorden and Everts, 1991), blocked YP-160 degradation, confirmed the conclusion that the yolk proteinase is a cathepsin B–like enzyme. We were surprised to find that benzamidine and TLCK inhibited proteolysis. Several lines of evidence presented in the Results led us to conclude that benzamidine is a weak inhibitor of the cathepsin B–like yolk proteinase. The use of this compound in vivo as a reversible proteinase inhibitor allowed us to carry out inhibitor treatment and wash-out experiments so as to determine the temporal expression of enzyme activity towards YP-160. The results of such inhibitor studies revealed that, in excellent agreement with the observed transient pH change in the yolk platelet, the enzyme activity in vivo is also transient. Because the in vitro experiments revealed that the enzyme (measured at pH 6.1) is present at all stages of development, it seems clear that pH regulation is a key element in controlling proteolysis. It is also clear that the yolk platelet is not simply a lysosome, but a novel membrane-rich organelle that has the ability to regulate proton fluxes. The molecular basis of this pH regulation obviously is an important aspect of further studies on the cell biology of the yolk platelet.

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