# Isolation of a Protease from Sea Urchin Eggs before and after Fertilization†

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ABSTRACT: Upon fertilization, sea urchin eggs (*Strongylocentrotus pupuratus*) release a protease into the surrounding sea water. This protease is in a particulate form which can be solubilized. The soluble form was purified by affinity chromatography on columns of immobilized soybean trypsin inhibitor. The purified enzyme is similar to bovine trypsin both in molecular weight (22500) and in susceptibility to inhibitors such as diisopropyl phosphofluoridate and soybean trypsin inhibitor. In contrast, extracts of unfertilized eggs appear to contain an inactive form of the enzyme which can be activated by dialysis at pH 4.6. The enzyme, as purified from extracts activated in this manner, was similar in its properties to that from fertilized eggs.

One of the earliest demonstrable chemical events following the penetration of eggs of sea urchin (*Strongylocentrotus purpuratus*) by sperm is the release of a trypsin-like protease into the sea water (Vacquier et al., 1972). Various studies have indicated that the enzyme is released from cortical granules in response to the entry of sperm (Rünnstrom, 1966; Schuel et al., 1973). Carroll and Epel (1975) have postulated that the enzyme serves two extracellular functions, one of assisting in the elevation of the fertilization membrane and the other of inhibiting polyspermy. Since the enzyme is released before initiation of protein synthesis in the zygote (Epel, 1967), two questions arise. (a) How is this protease stored in the unfertilized eggs? (b) How does the entry of sperm trigger the release of the protease? To establish a basis for exploring the details of these events, we have examined the chemical nature of the protease isolated from both fertilized and unfertilized eggs. Since preliminary studies indicated a similarity of the protease to pancreatic trypsin, we examined methods of affinity chromatography which had been developed for pancreatic enzymes (Robinson et al., 1971; Reeck et al., 1971). This investigation has led to the isolation of a homogeneous enzyme from the exudate released by fertilized eggs. Suitable manipulation of unfertilized eggs yields a similar product.

## Materials and Methods

Sea urchins, *Strongylocentrotus purpuratus,* were collected from the Straits of Juan de Fuca and maintained in sea water aquaria at 7°C. For subsequent experiments sea water was filtered through Millipore membranes (0.2 $\mu$) before use. The urchins were spawned by injection of 0.53 *M* KCl into the body cavity. Sperm was collected in Syracuse watch glasses and then suspended in 100 vol of sea water. Eggs were shed into a beaker of sea water, filtered through cheesecloth, allowed to settle in approximately 40 vol of sea water, and hand-centrifuged.

Suspensions of eggs in water were fertilized by adding sperm suspension in a sperm/egg ratio of 1/100 (v/v). The fertilization was considered to be successful if 90% of the membranes elevated. All handling of gametes was performed at 12°C, using detergent-free glassware.

Benzoyl-L-arginine ethyl ester (Bz-Arg-OEt)[1] and *p*-nitrophenyl *p'*-guanidinobenzoate (NphGdnBzO) were products of Cyclo Chemical Company. Bovine trypsin and (Kunitz) soybean trypsin inhibitor (STI) were obtained from Worthington Biochemicals. New England Nuclear supplied [$^{14}$C]diisopropyl phosphofluoridate. Pancreatic trypsin inhibitor was prepared according to Kassell and Marciniszyn (1971). All other chemicals were of reagent grade. Soybean trypsin inhibitor (Kunitz) was coupled to Sepharose according to Reeck et al. (1971). The derivative (STI–Sepharose) bound 2.5 mg of trypsin per ml of gel.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969); disc gel electrophoresis followed the procedure of Stroupe and Foster (1973) at pH 3.3. Enzymatic activity was followed spectrophotometrically at 253 nm, using as substrate 0.5 m*M* Bz-Arg-OEt in 0.2 *M* Tris (pH 8.0) (Schwert and Takenaka, 1955). One unit of enzyme hydrolyzes 1 $\mu$mol of substrate/min. Protein concentration was calculated from the absorbance at 280 nm, assuming an absorbancy of 1.45 cm$^{-1}$ for a solution containing 1 mg of enzyme/ml. Active-site titrations were carried out with NphGdnBzO (Walsh, 1970). Titrations with soybean trypsin inhibitor were performed by incubating increasing amounts of STI with the enzyme in 0.2 *M* Tris (pH 8.0) for 15 min and measuring the remaining activity (Laskowski and Sealock, 1971). These titrations are somewhat more sensitive than those with NphGdnBzO.

[$^{14}$C]i$Pr_2$FP was diluted with a 1 *M* solution of i$Pr_2$FP in anhydrous 2-propanol and its specific radioactivity measured according to Robinson et al. (1973). The incorporation of [$^{14}$C]i$Pr_2$FP by enzyme was followed in solutions at pH 8.0 containing 10 m*M* [$^{14}$C]i$Pr_2$FP at 0°C until no detectable enzymatic activity remained (about 1 hr). The labeled enzyme was separated from excess i$Pr_2$FP on a col-

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[1] Abbreviations used are: Bz-Arg-OEt, benzoyl-L-arginine ethyl ester; i$Pr_2$FP, diisopropyl phosphofluoridate; NphGdnBzO, *p*-nitrophenyl *p'*-guanidinobenzoate; STI, soybean trypsin inhibitor (Kunitz).

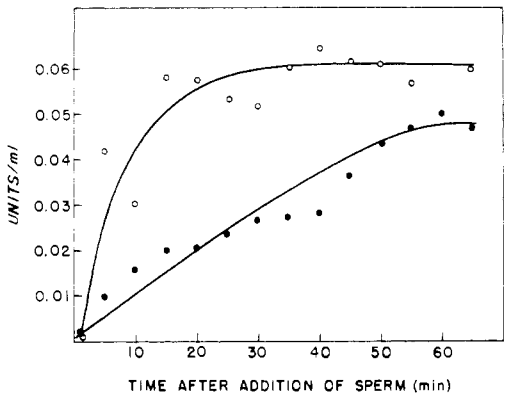


FIGURE 1: Time course of release of the protease. The enzymatic activity of the egg supernatant was assayed before (●) and after (○) a 5-min pre-incubation in 50 m*M* EDTA.

umn of Sephadex G-25 and the radioactivity of the protein measured with a Packard scintillation counter.

## Results

*Preliminary Observations.* As reported by Vacquier et al. (1972) fertilized sea urchin eggs release into the surrounding sea water a protease which hydrolyzes Bz-Arg-OEt. The release appeared to be a result of the fertilization process because this enzymatic activity was not spontaneously released from either gamete in the absence of the other. Vacquier et al. reported that parthenogenic stimulation of eggs also released the enzyme, thus proving that it is derived from the egg rather than the sperm. Moreover, it will be shown later that this trypsin-like enzyme can be isolated from *un*fertilized sea urchin eggs by appropriate activation of extracts in the absence of sperm.

The release of the enzymatic activity from eggs was followed by first removing the eggs by hand centrifugation (approximately 30*g*), then removing the sperm and clarifying the supernatant by centrifugation at 20000*g* for 15 min. Enzymatic activity in the supernatant reached a plateau after approximately 45 min (Figure 1). Treatment of the supernatant with 50 m*M* EDTA (5 min) increased the enzymatic activity, particularly during the early phase of the release (Figure 1). Estimation of the molar concentration of enzyme by titrations with $iPr_2FP$ or STI showed that the increased activity in the presence of EDTA results from an increase in the number of active sites accessible to these titrants, rather than from an increased specific activity of the enzyme. Although activation by EDTA could be due to removal of metal from an essential thiol group in the enzyme, thiol reagents (e.g., 5 m*M* *N*-ethylmaleimide) do not inhibit the enzyme. Since the purified enzyme was not activated by EDTA (see below), it was concluded that this activation of the secreted enzyme was of more complex origin. The enzymatic activity of this crude exudate was also increased approximately threefold by reducing the pH to 4.0 for 5 min, then diluting the solution fivefold in assay buffer at pH 8.0 and assaying it immediately thereafter.

Although the enzyme in the exudate did not sediment during 60 min of centrifugation at 30000*g*, particulate character was indicated by the behavior of the enzyme during filtration and by microscopic examination of the gel remaining on the filter. Enzymatic activity in the exudate did not pass through Millipore filters (0.2 $\mu$), columns of Bio-Gel A, 1.5m, or Sephadex G-75 equilibrated with various salt solutions and detergents (e.g., 1% BRIJ-35). When at-

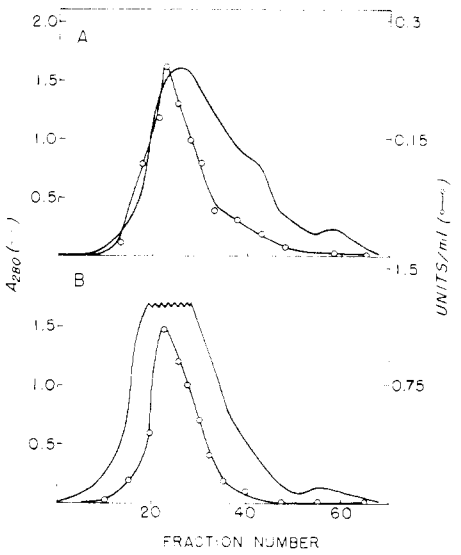


FIGURE 2: Gel filtration of the partially solubilized enzyme from (A) fertilized egg supernatants and (B) unfertilized eggs. Columns of Bio-Gel A 1.5m (1.5 × 90 cm) were equilibrated in 3 *M* urea, 1% butanol, 10 m*M* EDTA, and 0.2 *M* Tris (pH 8.0). Fractions of 2 ml were collected.

tempts were made to concentrate the enzyme by ultrafiltration through an Amicon UM-10 membrane, the activity was found in a gel-like layer directly on top of the membrane. The material in this gel appeared to be in the form of fragmented, uniform sheets (as seen at 100x magnification). These particles stained positively for carbohydrate, and positively with a staining technique specific for trypsin-like enzymes using benzoyl-L-argininenaphthylamide (Garner et al., 1971). The particulate nature of the enzyme precluded conventional methods of purification; hence, solubilization was attempted.

*Solubilization.* Various mildly denaturing or dissociating conditions were applied to solubilize the enzyme in exudates of fertilized eggs and to facilitate its purification. Two parameters were monitored in these solubilization experiments: stability and passage of the activity through small columns of agarose (Bio-Gel A, 1.5m). After various trials, it was found that activity could be recovered quantitatively when the enzyme was dissolved in 0.2 *M* Tris (pH 8.0), containing 3 *M* urea, 1% butanol, and 50 m*M* EDTA. The enzymatic activity passed directly through an agarose column pre-equilibrated with that solvent, indicating an apparent molecular weight in excess of 1500000 (Figure 2A). While this behavior stood in marked contrast to that in nondissociating solvent, it still represented only a partial solubilization since the inclusion of 2 *M* KCl and 10% glycerol led to a further decrease in apparent molecular weight. Finally, satisfactory conditions of solubilization comprised 2 *M* KCl, 10% glycerol, 1% butanol, 50 m*M* EDTA, and 0.2 *M* Tris at pH 8.0. In this solvent the molecular weight of the enzyme approximated 23000, as judged on a calibrated column of Sephadex G-75 (Figure 3A). The product of this separation did not reaggregate when the dissociating solvent was replaced by nondissociating solvents.

*Isolation from Fertilized Egg Supernatants.* Once solubilization was achieved, the trypsin-like enzyme was readily purified by affinity chromatography on immobilized STI by the following procedure. Approximately 30 min after fertilization, the eggs were removed by gentle, hand-centrifugation (approximately 100 ml of eggs from five sea urchins).

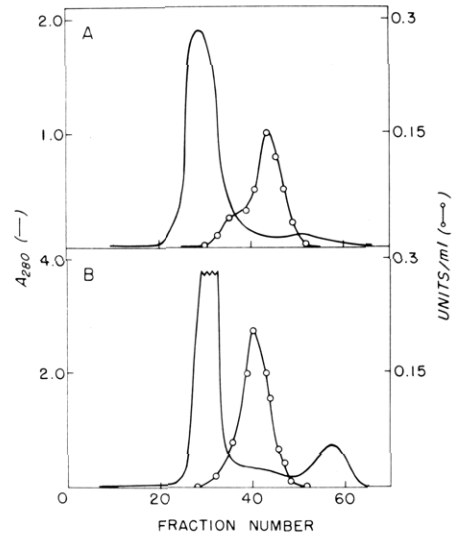


FIGURE 3: Gel filtration of the fully solubilized enzymes from (A) fertilized egg supernatants and (B) unfertilized eggs. Columns of Sephadex G-75 (1.5 × 90 cm) were equilibrated in 2 *M* KCl, 10% glycerol, 1% butanol, 50 m*M* EDTA, 0.2 *M* Tris (pH 8.0), and eluted at 15 ml/hr. Fractions of 2.5 ml were collected.

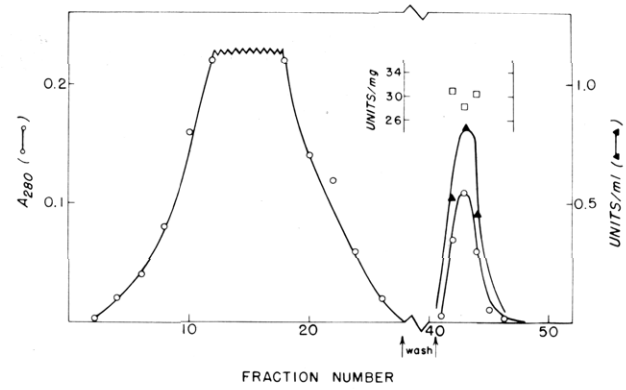


FIGURE 4: Affinity chromatography of the enzyme from unfertilized eggs on a column (1.5 × 2 cm) of STI–Sepharose. Details of the eluting solvents are given in the text. Fractions of 5 ml were collected. Similar results were observed with extracts of fertilized eggs.

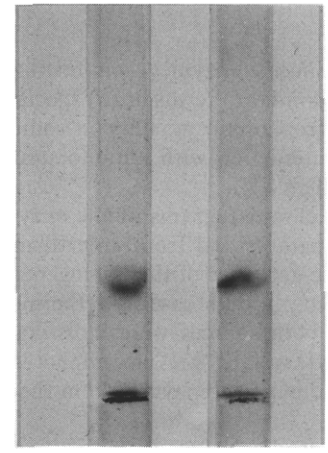

FIGURE 5: Sodium dodecyl sulfate gels of the protease purified from fertilized egg supernatants (left) and unfertilized eggs (right). The proteins migrated from the top toward the anode.

The supernatant, which contained the trypsin-like enzyme, was centrifuged for 50 min at 30000*g* to remove the sperm. The enzyme in the supernatant was concentrated by ultrafiltration through an Amicon UM-10 membrane and recovered in a gel on top of the membrane. In this way, approximately 2 ml of gel was obtained from 100 ml of eggs. The enzyme was released from the gel by incubation in 15 vol of a solubilizing buffer (0.2 *M* Tris (pH 9.0), containing 50 m*M* EDTA, 1% butanol, 10% glycerol, and 2 *M* KCl) for 30 min. The soluble extract was adjusted to pH 8.0 and applied to a column (1.5 × 2 cm) of STI–Sepharose, equilibrated with the same buffer. The column was washed with buffer until protein elution was complete (Figure 4). The enzyme was then eluted at pH 4.0 with 2 *M* urea in 0.1 *M* Tris, 25 m*M* EDTA, 0.5% butanol, 5% glycerol, and 1 *M* KCl. Homogeneity was indicated by the constant specific activity across the eluted fractions. Only one protein component was detected by either sodium dodecyl sulfate gel electrophoresis (Figure 5) or disc gel electrophoresis. The overall yield of the trypsin-like enzyme from fertilized egg supernatants was approximately 90%. Typically, about 0.25 mg of enzyme could be obtained from 100 ml of settled eggs.

*Purification from Unfertilized Eggs.* Since the protease is released prior to new protein synthesis (Epel, 1967), it was reasonable to expect that the enzyme must preexist in the egg, possibly either in an enzyme–inhibitor complex or as a zymogen. With this working assumption, extracts of unfertilized eggs were examined for enzymatic activity. None was found until extracts from unfertilized eggs were dialyzed against acidic solutions. The following procedure releases an amount of enzymatic activity somewhat greater than that released upon sperm entry.

The eggs were lysed at 10°C by adding 150 ml of sea water (containing 8% butanol and 33 m*M* urea) to 100 ml of washed, packed eggs. The enzyme was extracted using a rotary shaker at 150 rpm for 30 min. The extract was centrifuged for 40 min at 30000*g*. Little or no esterase activity could be detected at this stage in either the supernatant or the pellet, but enzymatic activity appeared when the supernatant was first adjusted to pH 4.6, clarified by centrifugation, and dialyzed for 20 hr at 4°C against 10 m*M* sodium acetate (pH 4.6). A precipitate was collected by centrifugation, disrupted in a Potter-Elvehjem homogenizer, and solubilized by stirring for 2 hr in 100 vol of 0.2 *M* Tris (pH 9.0), 1% butanol, 50 m*M* EDTA, 10% glycerol, and 2 *M* KCl at 0°C. This solution contained enzymatic activity which, upon gel filtration, behaved like that from fertilized eggs (Figure 3B).

After solubilization, the solution was adjusted to pH 8.0, filtered, and purified by affinity chromatography on STI–Sepharose (Figure 4). As in the case of the fertilized eggs, the extract was applied to the column in Tris–butanol–EDTA–glycerol–KCl buffer at pH 8.0 and eluted with urea–Tris–butanol–EDTA–glycerol–KCl buffer at pH 4.0. The enzyme was homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 5) and by disc gel electrophoresis. A constant specific activity toward Bz-Arg-OEt was seen in the various fractions eluted from the affinity column at pH 4.0. The yield from the affinity column was approximately 90%. Approximately 1.25 mg of enzyme was obtained from 100 ml of settled eggs, which is approximately 5 times as much enzyme as is released into the sea water by fertilized eggs.

*Comparison of the Enzymes Purified from Fertilized Egg Supernatants and from Unfertilized Eggs.* The proper-

Table I: Characteristics of Sea Urchin Egg Protease Derived from Fertilized and Unfertilized Eggs.

| | Fertilized Eggs | Unfertilized Eggs | Bovine Trypsin[a] |
|---|---|---|---|
| Specific act. toward Bz-Arg-OEt (units/mg) | 30 | 30 | 70 |
| Apparent mol wt | | | 23300 |
| (a) Purified enzyme | | | |
| 1. Na dodecyl sulfate gel | 22500 | 22500 | |
| 2. Gel filtration | | 23000 | |
| (b) Crude exudate | | | |
| 1. Gel filtration in urea–butanol–EDTA (Fig. 2) | >1500000 | >1500000 | |
| 2. Gel filtration in butanol–EDTA–KCl–glycerol (Fig. 3) | 23000 | 23000 | |

[a] Walsh (1970).

ties of the trypsin-like enzymes from fertilized and unfertilized eggs were similar in terms of (a) specific enzymatic activity; (b) electrophoretic mobility in sodium dodecyl sulfate gels; (c) interaction with inhibitors; and (d) aggregation behavior (Table I).

The affinity chromatograms of the enzymes from fertilized egg supernatants and from unfertilized eggs revealed that the specific activities of the homogeneous preparations were 30 units/mg in both cases. Furthermore, the purified enzymes from both sources were indistinguishable during sodium dodecyl sulfate gel electrophoresis. These data suggest that the purified enzymes from the two sources are identical.

As with the crude exudate from fertilized eggs, solubilization of the acid-dialyzed preparations of unfertilized eggs in Tris–EDTA–butanol–urea buffer yielded enzymatic activity in the breakthrough fractions during gel filtration on Bio-Gel A 1.5m (Figure 2B). When KCl and glycerol were added to the above buffer, activities from both fertilized and unfertilized eggs chromatographed with apparent molecular weights of 23000 (Figure 3B). These results suggest that prior to purification the enzymatic activities from both fertilized and unfertilized eggs exhibit similar aggregation behavior. In contrast no aggregation could be detected on Sephadex G-75 with the purified enzyme from either source, even in nondissociating solvents (e.g., 0.1 *M* Tris, pH 8.0).

Both enzymes, at all stages of purity, were completely inhibited by 10 m*M* $iPr_2FP$ and 10 m*M* NphGdnBzO. Furthermore, addition of a molar excess of either soybean trypsin inhibitor (Kunitz) or pancreatic trypsin inhibitor (Kunitz) completely inactivated the enzymes.

Taken together, the similarities in aggregation properties, response to inhibitors, mobilities on sodium dodecyl sulfate gels, and specific activities toward Bz-Arg-OEt suggest that these two enzymes are identical.

*Comparison with Bovine Trypsin.* The similarity between bovine pancreatic trypsin and sea urchin egg protease is indicated in Table I. The molecular weights of the bovine and echinoderm enzymes were found to be quite similar (23300 and 22500, respectively), as determined by sodium dodecyl sulfate gel electrophoresis. Neither enzyme contained any detectable carbohydrate. The susceptibility to various inhibitors ($iPr_2FP$, NphGdnBzO, STI, and pancreatic trypsin inhibitor) is also similar for bovine trypsin and for the sea urchin protease. However, the egg protease was less sensitive than trypsin to trypsin inhibitors from lima beans or egg white (ovomucoid). Like bovine trypsin (Chase and Shaw, 1967), the sea urchin protease releases a stoichiometric "burst" of nitrophenol from NphGdnBzO indicating titration of 1.04 active sites per 22500 daltons. The sea urchin enzyme incorporated approximately 0.8–1.0 equiv of [$^{14}$C]$iPr_2FP$ and bound 1 mol of STI per mol of enzyme (the concentration of the enzyme was determined both by the size of the burst with NphGdnBzO and by amino acid analysis, assuming a mol wt of 22500). Preliminary experiments[2] demonstrate that *S*-carboxymethyllysozyme is preferentially hydrolyzed at arginyl residues by the enzyme isolated from unfertilized eggs. Under similar conditions trypsin specificity is directed toward both lysyl and arginyl residues of that substrate (Canfield, 1963).

## Discussion

A trypsin-like enzyme has been isolated from the exudate released by sea urchin eggs in response to fertilization and purified to apparent homogeneity. The protease has a mol wt of 22500 and is inactivated by the incorporation of 1 mol of $iPr_2FP$ per mol of active enzyme. It resembles pancreatic trypsin also in reacting stoichiometrically with the active-site titrant NphGdnBzO and with naturally occurring trypsin inhibitors from the soybean or the pancreas. Thus, the sea urchin egg enzyme has characteristics of the family of serine proteases which includes trypsin, thrombin, cocoonase, and acrosin (Walsh, 1975; Neurath, 1975). It is probable that the isolated enzyme is responsible for the protease activity designated EII by Lundblad (Lundblad, 1954; Lundblad et al., 1972).

In our isolation procedure, the final step in the purification consists of affinity chromatography, similar to that developed previously for the isolation of pancreatic trypsins from other species (Robinson et al., 1971). Prior to this step, it was necessary to solubilize the enzyme. While upon fertilization the enzyme is released in a form which is easily separated from the eggs (Vacquier et al., 1972), it is apparently aggregated but nonsedimentable and will neither pass through Millipore filters nor enter Dextran gels. Both Mano (1966) and Grossman et al. (1973a) describe the particulate nature of this product of fertilization.

In contrast to the product of fertilization, the purified protease does not self-aggregate. Thus, in the form secreted into the sea water from intact fertilized eggs, the enzyme must be bound to other components. Carroll and Epel (1975) have partially purified proteases released from membraneless sea urchin eggs on fertilization. They report molecular weights of 47000 by sucrose gradient centrifugation and higher aggregates in 8% sucrose at pH 5.6. Furthermore, they distinguish two species of enzyme, one which functions in the elevation of the fertilization membrane, and the other which hydrolyzes sperm receptor sites. Although these particular assays have not been performed in the present work, the isolated enzyme may have been separated from other egg components which modulate its physiological function.

The time course of release of protease in the present experiments is similar to that observed by Grossman et al.

[2] Unpublished observations of J. Lau, E. J. B. Fodor, and K. A. Walsh.

(1973b) but much slower than that reported by Vacquier et al. (1973) from membraneless eggs. The faster release observed by Vacquier et al. is probably accounted for by the absence of the perivitelline space, the fertilization membrane, and the outer jelly coat in their experiments.

Since Lundblad (1954), Epel (1967), and others have reported that the activity of the enzyme is not apparent in unfertilized eggs, but does appear in the time interval between sperm entry and protein synthesis (Epel, 1967), the enzyme must exist in an inactive form prior to fertilization. Indeed, our extracts of unfertilized eggs did not contain detectable enzyme activity until these extracts were incubated at low pH. When the product of acid treatment was purified by techniques similar to those applied to the enzyme from fertilized egg supernatants, the final homogeneous product resembled in all respects that purified from fertilized eggs.

It seems likely that the fertilization product is derived from the particulate fraction of Grossman et al. (1973a) or the cortical granules of Schuel et al. (1973). These authors and Mano (1966) have suggested *intra*cellular roles for this enzyme, whereas both Vacquier et al. (1972, 1973) and Carroll and Epel (1975) provided evidence for *extra*cellular roles. Present findings indicate that only $\frac{1}{5}$ of the enzyme is released from the egg into the sea water upon fertilization, but do not establish whether the remaining $\frac{4}{5}$ of the enzyme is in the cytoplasm, the perivitelline space, the jelly coat, or bound to a membrane. It is therefore possible that the enzyme could play a dual role, both intracellular and extracellular. Whereas it is usual to consider the role of proteases as degradative in nature, serving nutritional, solubilizing, or penetrating purposes, it is important to consider the possibility that a protease may serve as a control agent in the initiation of metabolic or developmental processes. In other systems, limited proteolysis mediates the activation of zymogens, the release of hormones, and the assembly of fibers in response to physiological signals (Walsh, 1975; Neurath, 1975). Common characteristics of these processes are the "poised" nature of the zymogens, the irreversibility of the proteolytic events, and the catalytic amplification of physiological signals. One such process serves a morphogenic role in initiating septum formation by chitin synthetase in budding yeast (Cabib and Farkas, 1971); another serves a developmental role in releasing a moth (*Antheraea polyphemus*) from its cocoon during metamorphosis (Felsted et al., 1973). Since an unfertilized egg is also "poised" in its chemical nature, and since sperm entry triggers qualitative changes in its metabolism (Epel et al., 1969), the role of proteases in initiating these changes should be examined.

The physiological mechanism of activation and release of the protease is not clear. Current studies are directed toward the characterization of the inactive precursor of the enzyme and the mechanism by which components of sperm trigger the activation process.

Acknowledgments

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