Ultrastructural Demonstration of Trypsin-Like Protease in Acrosomes of Sea Urchin Sperm

Abstract. *Two independent cytochemical techniques were used to demonstrate that a trypsin-like protease is bound to the acrosomal tubule of the sea urchin sperm. The enzyme is associated with bindin on the reacted acrosome and presumably functions in the early phases of fertilization.*

Acrosomal proteases have been localized by direct cytochemical methods in only a few vertebrates (mammals and birds) (*1*-*4*). The occurrence of sperm-associated proteases, which may function during fertilization, is widespread and well documented in a variety of animals including annelids (*5*), mollusks (*6*, *6a*), echinoderms (*7*, *8*), and amphibians (*9*). The conclusions in these investigations were based on the fact that acrosomally reacted sperm or sperm extracts dissolved egg coats or artificial gelatin substrates. However, only inferences with respect to an acrosomal location for these proteases can be made. In one species of sea urchin, *Strongylocentrotus purpuratus*, there is good biochemical evidence (hydrolysis of a tritiated substrate) that a protease is indeed present and that the activity of the enzyme correlates with the stimulation of the acrosome reaction by homologous egg jelly (*8*). The evidence suggests an acrosomal location for the enzyme, although no cytochemical data were presented. We now report the localization of a trypsin-like protease on the reacted acrosomes of the spermatozoa of *S. purpuratus*.

We used two unrelated techniques to demonstrate the subcellular location of the protease. In the first method, developed by Kishida *et al.* (*10*) and subsequently modified (*3*, *4*), ferritin was conjugated to soybean trypsin inhibitor (SBTI). In the second method (*1*, *2*) metallic silver was precipitated at the sites of proteolytic activity by hydrolysis of silver proteinate.

The ferritin-soybean trypsin inhibitor conjugate (Fe-SBTI) was prepared as described (*3*) with the following modifications. (i) The conjugate was dialyzed into Millipore-filtered artificial seawater (MSW; Instant Ocean) before use. (ii) To determine inhibitor activity in the conjugate, we omitted the use of ^{125}I-labeled SBTI in favor of titrating known concentrations of trypsin with the Fe-SBTI in the presence of the artificial substrate α-*N*-benzoyl-L-arginine ethyl ester hydrochloride (BAEE; Sigma) and monitoring the hydrolysis of the substrate. The concentration of ferritin in the conjugate was

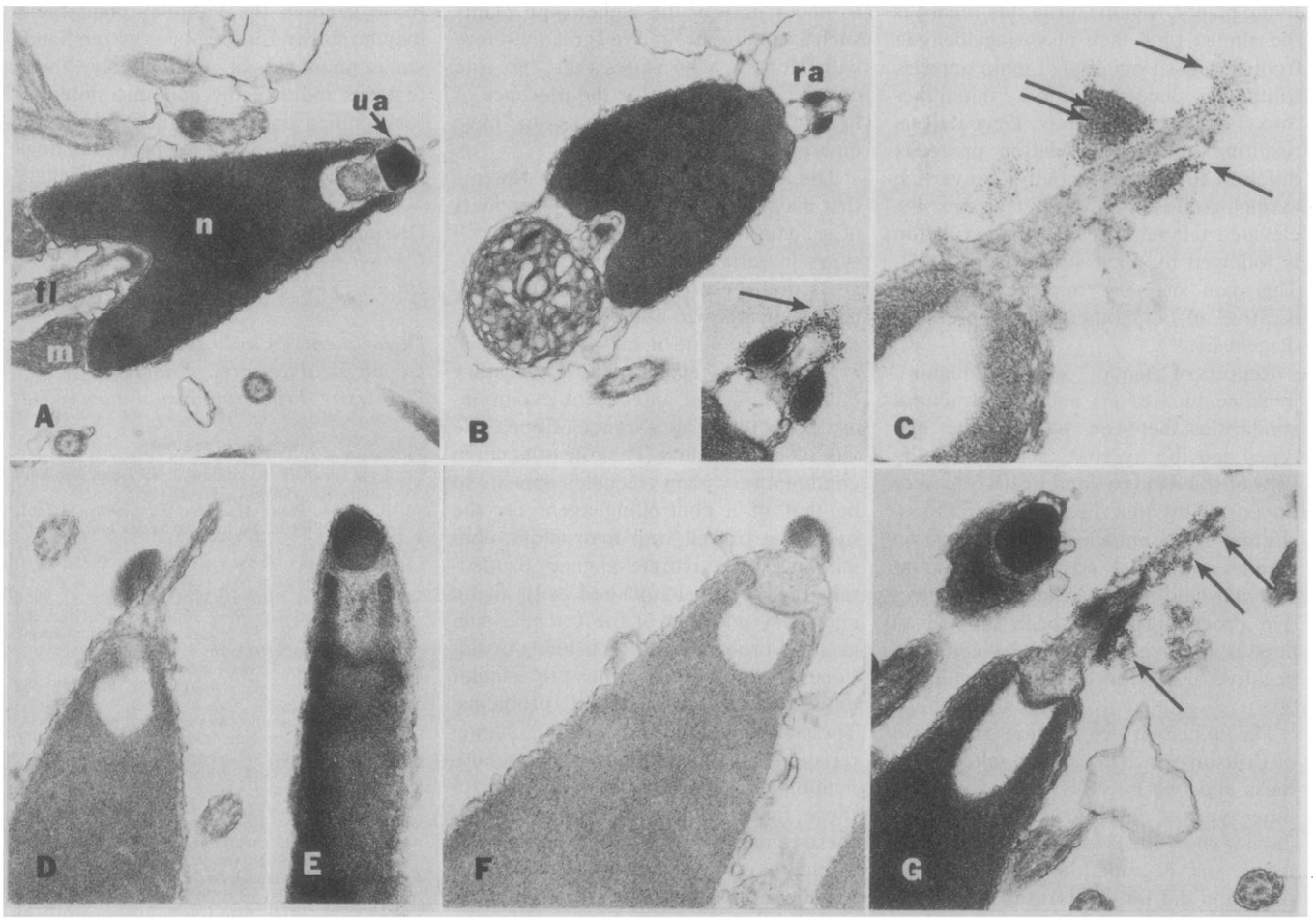

Fig. 1. Electron micrographs of ferritin-SBTI-treated sperm [except (D)] of *S. purpuratus* showing specific binding of Fe-SBTI to the acrosomal tubule and associated material, prevention of Fe-SBTI labeling by SBTI preliminary treatment, and lack of "sticking" of unconjugated ferritin. (A) Acrosomally unreacted sperm. There is no ferritin labeling on any sperm component (× 21,000). Abbreviations: *ua*, unreacted acrosome; *fl*, flagellum; *m*, mitochondrion; *n*, nucleus. (B) Sperm with reacted acrosome (*ra*) showing Fe-SBTI labeling restricted to the acrosomal tubule (inset, arrow) (× 21,000; inset, × 46,000). (C) Another Fe-SBTI-treated, reacted sperm with ferritin (single arrows) and bindin clump (twin arrows) (× 68,000). (D) Reacted sperm treated with unconjugated ferritin. There is no evidence of ferritin binding (× 34,000). (E) Sperm treated with 30 m*M* Ca^{2+} before Fe-SBTI. The acrosome is unreacted, and there is no ferritin bound (× 34,000). (F) Preliminary treatment of sperm with SBTI. Ferritin is not present (× 34,000). (G) Ionophore-treated sperm. There is an unreacted acrosome (upper left) with no bound ferritin. The reacted acrosome (center) binds many ferritin molecules (arrows) (× 34,000).

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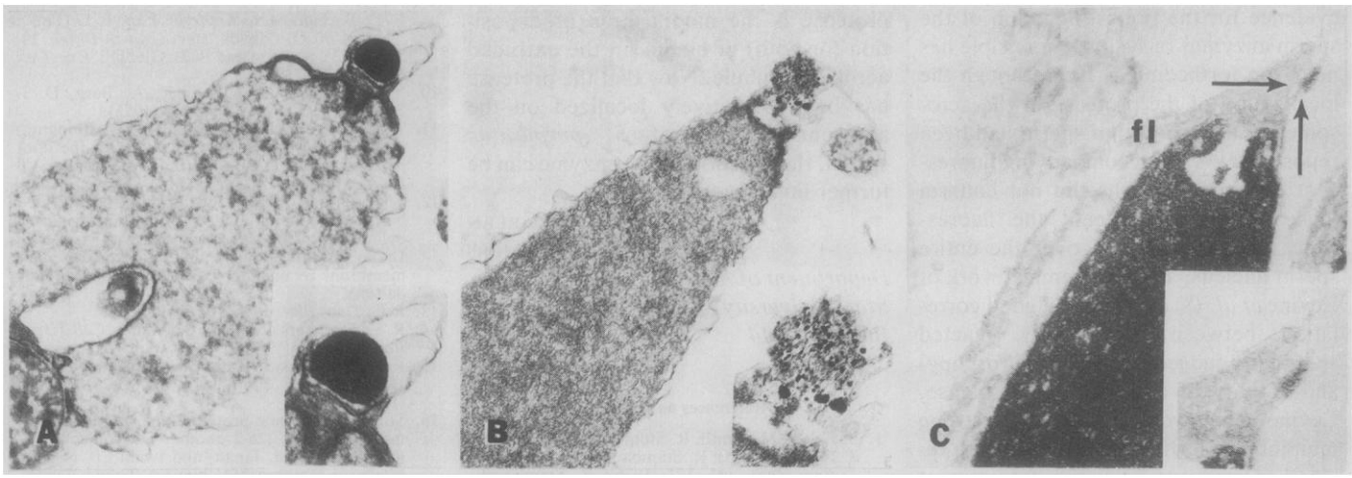

Fig. 2. Electron micrographs of sperm treated with silver proteinate, showing that the silver reaction occurs only on reacted sperm acrosomes and that preliminary treatment with SBTI prevents the silver reaction. (A) Unreacted sperm with no silver reaction (× 25,000; inset, × 42,000). (B) Reacted sperm with silver precipitate only on the acrosome (× 29,000; inset, × 58,000). (C) Preliminary treatment of sperm with SBTI, showing no silver reaction. The acrosomal tubule (between the arrows) is adherent to the flagellum (*fl*) (× 34,000; inset, × 48,000).

estimated by its absorbance at 440 nm (*11*). (iii) The Fe-SBTI was either concentrated (*12*) or left unconcentrated and used at concentrations ranging from 0.72 to 6.63 μM ferritin, with the lower concentrations providing the best results.

Sea urchin sperm were obtained by injection of 0.53*M* KCl. Acrosome reactions were stimulated with alkaline MSW (*p*H 9.4) (*13*), and the sperm were centrifuged and resuspended in MSW (*p*H 8.2) at room temperature. To each portion (0.1 ml) of sperm either (i) 0.2 ml of Fe-SBTI, (ii) 0.1 ml of SBTI (50 mg/ml), or (iii) 0.2 ml of unconjugated ferritin (same concentration as the conjugate) was added. All solutions were made up in MSW. As a control, the sperm were incubated with the SBTI for 15 minutes before the addition of 0.4 ml of Fe-SBTI for 15 minutes. After the incubation period, all samples were processed for electron microscopy (*14*). In several experiments, acrosome reactions were induced by 7.5 μM calcium ionophore A23187 (*15*) in the presence of Fe-SBTI.

In the silver proteinate experiments, alkaline MSW-reacted sperm (0.1 ml) were incubated in 2 ml of MSW (*p*H 7.5) containing 2.1 mg of silver proteinate (*16*) and 0.85 mg of potassium bromide for 2 to 4 hours at 37°C in the dark. After the incubation period, the silver was developed in 10 percent D-76 (Kodak) in calcium-free MSW for 5 minutes, and the preparation was fixed in 2 percent sodium thiosulfate in MSW and washed in MSW before the sperm were prepared for electron microscopy (*14*).

Several morphological events occur in the fraction of a second in which the acrosome reaction takes place (*14*), including the fusion and dehiscence of the sperm plasma membrane and the acrosomal vesicle membrane, the alteration in appearance of the acrosomal vesicle contents, and most striking, the eversion of the basal portion of the acrosomal vesicle to form the acrosomal tubule. The tubule rapidly elongates, probably through the polymerization of actin filaments (*17*), and the material previously occupying the vesicle now coats the tubule (Fig. 1C) and is thereby exposed to the external milieu. This material contains bindin (*18*), which is thought to be responsible for the species-specific binding of sperm to eggs. We now report that the acrosomal vesicle also contains a protease with some characteristics of trypsin.

As evidenced by the Fe-SBTI binding sites, the enzyme appears to be intimately associated with the limiting membrane of the acrosomal tubule and the electron-opaque clumps of bindin coating the tubule (Fig. 1C). Ferritin was not bound to any other component of the reacted sperm (Fig. 1B), nor was any ferritin observed on intact, unreacted sperm (Fig. 1A). Furthermore, unconjugated ferritin did not adhere to sperm (Fig. 1D), and when reacted sperm were given the preliminary incubation with SBTI, ferritin grains were only rarely seen (Fig. 1F). The localization of ferritin was the same whether sperm were reacted with alkaline MSW or with ionophore (Fig. 1G). In Fig. 1G both reacted and unreacted acrosomes are shown; ferritin labeled only the reacted sperm.

Since it has been demonstrated biochemically that sperm treated with 30 mM Ca^{2+} have detectable protease activity (*8*), yet have not undergone acrosomal tubule formation (as assessed by light microscopy), we also treated sperm with 30 mM Ca^{2+} before exposing them to Fe-SBTI. The electron micrographs of these sperm (Fig. 1E) show an acrosome morphologically indistinct from untreated, unreacted acrosomes (Fig. 1, A and G) and there is no trace of ferritin labeling in these sperm. If there is enzyme activity in similarly treated sperm (*8*), the exposure of the enzyme is apparently not sufficient to accommodate the binding of the large Fe-SBTI (~ 440,000 daltons) conjugate in our investigation.

The silver proteinate experiments provide further evidence for the presence and location of the sperm protease. Although the preservation of this material is not optimal owing to the harsh treatment of this procedure, it is clear that the reaction product is present only on the reacted acrosome and not on any other sperm component (Fig. 2B). Furthermore, unreacted acrosomes, as indicated by the compact, intact, membrane-bounded acrosomal granule, do not show reaction product (Fig. 2A). Also, reacted sperm given preliminary treatment in SBTI are not labeled by the silver proteinate procedure (Fig. 2C). The demonstration of intact, normal looking acrosomal tubules in this material is difficult, but many sections through cytolyzed acrosomal tubules do demonstrate the silver precipitate, while those sections through unreacted acrosomes do not. For both the ferritin and silver proteinate experiments, unstained sections were also observed. The cytochemical localizations were identical to those in stained specimens, demonstrating that the electron-opaque stains did not obscure the visualization of the cytochemical products.

Although proteolytic activity in invertebrate sperm is well known (*6a*),

evidence for the precise location of the sperm enzymes or lysins responsible has not been forthcoming. Even though the localization of the protease in the acrosomes of the sea urchin sperm had been reported (*19*), the accompanying fluorescent photomicrographs did not confirm this observation. Indeed, the fluorescence can be observed over the entire sperm nucleus. The biochemical work of Levine *et al.* (*8*) does show a good correlation between acrosomally reacted sperm (as judged by light microscopy) and activity of a trypsin-like protease. Levine *et al.* were able to show strong inhibition of fertilization of eggs with reacted sperm given preliminary treatment with either PMSF (phenylmethanesulfonyl fluoride) or DFP (diisopropyl fluorophosphate), two serine protease inhibitors. Their use of the well-known trypsin substrate BAEE, and our ferritin labeling with soybean trypsin inhibitor indicate that the protease in question is related to trypsin. Hoshi *et al.* (*20*) have shown inhibition of fertilization in sea urchins by chymostatin and TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone), which are chymotrypsin-specific inhibitors, and NPGB (*p*-nitrophenyl-*p'*-guanidino-benzoate) which inhibits both trypsin and chymotrypsin; but trypsin-specific inhibitors were ineffective. Hoshi *et al.* believe that a chymotrypsin-like enzyme is involved in sperm penetration of the vitelline coat. However, since their work consisted of inseminating eggs in the presence of the inhibitors, it is difficult to evaluate whether the inhibitors were acting on the sperm, the eggs, or both. At present, both trypsin- and chymotrypsin-like enzymes in the acrosomes cannot be ruled out.

Our study leaves open the question of the role of such sperm enzymes in fertilization. The protease appears to be located at the tip of the acrosomal tubule indicating a possible role in the dissolution of the vitelline coat (*21*), but numerous experiments with SBTI [see (*22*) for review] show that fertilization does occur in the presence of some trypsin inhibitors. Another possibility, as previously pointed out (*8*), is that the enzyme may function in the acrosome reaction itself. This would not be surprising, as phospholipase activity has been reported in sea urchin sperm (*23*). If phospholipase mediates gamete membrane fusion as suggested (*23*), or acrosomal vesicle dehiscence in the initial stages of the acrosome reaction, a trypsin-like enzyme may activate the phospholipase (*22*), as can occur with mammalian pancreatic trypsin in the digestive tract (*24*). Another possibility for the role of the protease is the modification or deposition (or both) of bindin on the extruded acrosomal tubule. Now that the protease has been definitively localized on the acrosomal tubule of *S. purpuratus* sperm, the function of the enzyme can be further investigated.

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Intrafloral Pollen Differentiation in the New World Lecythidaceae, Subfamily Lecythidoideae

Abstract. *Floral biology, pollen germination, and scanning electron microscopy studies have indicated different types of pollen within the same flower in at least two species of New World Lecythidaceae, subfamily Lecythidoideae. Two types of pollen are produced in different parts of the androecium and serve different functions. One type attracts pollinators by providing a reward, and the other functions in fertilization. The former type has lost its ability to germinate, at least under in vitro conditions.*

The Lecythidaceae or Brazil nut family is pantropical in distribution (*1*) and consists of four subfamilies, 20 genera, and 278 species. However, in the New World subfamily Lecythidoideae, consisting of ten genera and about 206 species (*1*), an adaptive radiation of the androecium has occurred in response to animal pollen vectors.

In the Lecythidoideae, the structure of the androecia ranges from symmetrical with many stamens and no nectar to asymmetrical with fewer stamens and with nectar. The least complicated androecial type is found in *Gustavia*, *Grias*, and *Allantoma*, which have symmetrical androecia with many stamens that are fused at their bases into a ring (Fig. 1A). In *Couroupita*, *Lecythis*, *Corythophora*, *Bertholletia*, *Eschweilera*, and *Couratari*, the ring is prolonged on one side into a straplike structure that arches over the summit of the ovary (Fig. 1, B to E). This structure consists of the ligule, a stamen-free area adjacent to the staminal ring, and the hood, a distal portion with variously modified appendages. The hood may be open or tightly appressed to the summit of the ovary.

The intricate hood-ligule structure of asymmetrically flowered Lecythidoideae encourages pollination in the following ways: (i) nectar or pollen, which attract

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