Characterization of Soybean Trypsin Inhibitor Sensitive Protease from Unfertilized Sea Urchin Eggs†

Mark C. Alliegro* and Herbert Schuel

Department of Anatomical Sciences, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14214, and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Received November 13, 1984

ABSTRACT: A serine protease from sea urchin eggs has been isolated by affinity chromatography on soybean trypsin inhibitor–agarose. Benzamidine hydrochloride was included to minimize autodegradation. We present data on the properties of the protease with respect to molecular weight and its interaction with trypsin inhibitors and substrates. The molecular weight of the enzyme is 47 000 by gel filtration under nonreducing conditions and 35 000 by electrophoresis in the presence of sodium dodecyl sulfate and dithiothreitol. The pH optimum and K_m with N^{α}-benzoyl-L-arginine ethyl ester (BAEE) are 8.0 and 75 μM, respectively. The specific activity is comparable to that of bovine pancreatic trypsin. Proteolytic activity was measured by β-casein hydrolysis. The caseinolytic activity is completely inhibited by 1 μmol of soybean trypsin inhibitor (SBTI) per micromole of enzyme. BAEE esterase activity is inhibited competitively by SBTI (K_i = 1.6 nM), lima bean trypsin inhibitor (150 nM), chicken ovomucoid (100 nM), and leupeptin (130 nM). Bowman–Birk inhibitor, benzamidine hydrochloride, and antipain are also inhibitors of the purified enzyme. Inhibition by phenylmethanesulfonyl fluoride and N^{α}-p-tosyl-L-lysine chloromethyl ketone indicates the presence of serine and histidine residues in the active center, respectively. The chymotrypsin inhibitor L-1-(tosylamido)-2-phenylethyl chloromethyl ketone is ineffective. The protease is susceptible to autodegradation which can result in the appearance of a minor 23-kilodalton component. The egg protease appears to be similar in many respects to trypsins and trypsin-like enzymes isolated from a wide variety of sources, including sea urchin and mammalian sperm.

Sea urchin eggs contain a soybean trypsin inhibitor (SBTI)[1]-sensitive protease which has been implicated in several early fertilization events (Schuel, 1985). The protease has been localized to the cortical granule fraction of several different preparations from unfertilized eggs (Schuel et al., 1973; Decker & Kinsey, 1983; Kopf et al., 1983), where it may be stored in an inactive form. Shortly after fertilization (or parthenogenetic activation), the activity can be detected in the ambient seawater, the result of massive exocytosis of the cortical granules (Vacquier et al., 1973; Grossman et al., 1973; Schuel et al., 1976b). Although the exact mechanisms are not yet completely understood, it is evident that sea urchin egg protease plays a major role in the prevention of polyspermy. In this respect, the critical fertilization events which may be protease mediated include the following: (1) cortical granule exocytosis (Lonning, 1967; Longo & Schuel, 1973; Schuel, 1978); (2) hydrolysis of cross-links between the egg vitelline layer and plasma membrane to initiate fertilization envelope elevation (Longo & Schuel, 1973; Schuel et al., 1976a), referred to as vitelline delaminase activity (Carroll & Epel, 1975); (3) removal of sperm receptors from the egg surface (sperm receptor hydrolase activity), which prevents supernumerary sperm binding and detaches those already bound (Carroll & Epel, 1975); (4) generation of H_2O_2 by fertilized eggs, which reduces sperm fertility (Boldt et al., 1981; Coburn et al., 1981). Several other possible functions for egg protease in fertilization and egg activation have been suggested (Schuel, 1978), including dispersal of secretory products in the perivitelline space (Longo & Schuel, 1973).

SBTI-sensitive protease has been purified from sea urchin eggs and characterized biochemically (Fodor et al., 1975; Carroll, 1976; Sawada et al., 1984). However, there are unresolved questions regarding the number of proteases present, their molecular weights and sensitivity to certain active-site inhibitors. A protease has been isolated from unfertilized *Strongylocentrotus purpuratus* eggs and the secretory product collected from fertilized egg cultures by SBTI affinity chromatography (Fodor et al., 1975). The enzymes from both preparations exhibited identical specific activities with BAEE as substrate, had an apparent molecular weight of 23 000 estimated by gel filtration and SDS–PAGE, and showed similar aggregation behavior and similar sensitivities to several trypsin inhibitors, including DFP. DFP is a specific probe for active-site serine residues (Walsh & Wilcox, 1970). On the basis of these criteria, Fodor et al. (1975) concluded that both preparations yield the same enzyme. Similar results have been obtained with *Strongylcentrotus intermedius* eggs by Sawada et al. (1984). These authors report a molecular weight of 28 000 by SDS–PAGE and 30 000 by gel filtration and a kallikrein-like specificity for synthetic peptide substrates. On the other hand, Carroll & Epel (1975) reported the partial separation of two trypsin-like proteases from the fertilization

† This work has been supported by National Science Foundation Grant PCM-82-01561 to H.S.

* Address correspondence to this author at the Department of Anatomical Sciences, State University of New York of Buffalo.

[1] Abbreviations: SBTI, soybean trypsin inhibitor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; PAS, periodic acid–Schiff; TLCK, N^{α}-p-tosyl-L-lysine chloromethyl ketone; L-BAPNA, N^{α}-benzoyl-L-arginine-p-nitroanilide; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; kDa, kilodalton(s); BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/85/0424-3926$01.50/0

product of *S. purpuratus* eggs by affinity chromatography on a column of *p*-aminobenzamidine. One showed sperm receptor hydrolase activity and the other vitelline delaminase activity. These two biological activities appeared to have significantly different specific activities toward BAEE, since the esterase peak from affinity chromatography was slightly offset from the protein peak. Vitelline delaminase activity was found in the low specific activity (leading) fractions, and sperm receptor hydrolase in the high specific activity (trailing) fractions. The esterolytic activity of these proteases was insensitive to PMSF (Carroll, 1976), another specific probe for active-site serine residues (Gold & Fahrney, 1964). The molecular weight of both enzymes was reported to be 47 000 by sucrose gradient centrifugation (Carroll & Epel, 1975). However, more recent molecular weight estimations for the isolated sperm receptor hydrolase have ranged from 53 000 to 69 000 by nondenaturing gel electrophoresis (Carroll et al., 1982). Molecular weight estimates under reducing and denaturing conditions have not been reported for either sperm receptor hydrolase or vitelline delaminase.

In this report, we present further characterization of protease activity isolated from unfertilized eggs by SBTI affinity chromatography. By using unfertilized eggs and including protease inhibitors in our isolation procedure, we have attempted to minimize autoproteolytic modification of the protease or any postsecretional processing which may be inherent in preparations from fertilization product. Preliminary accounts of this study have been reported elsewhere (Alliegro & Schuel, 1983, 1984a).

Experimental Procedures

Materials. Sea urchins (*Strongylocentrotus purpuratus*) were obtained from Pacific Bio-Marine, Venice, CA. Gametes were shed by intracoelomic injection of 0.5 M KCl (Harvey, 1956). Eggs were then rinsed once in 5 volumes of Millipore-filtered artificial seawater (Aquarium Systems, Inc., Wickliffe, OH) by settling and decanting. SBTI–agarose was obtained from Bethesda Research Labs, Rockville, MD. All substrates and inhibitors were purchased from Sigma, except antipain, leupeptin, and Bowman–Birk inhibitor which were gifts of Dr. Walter Troll.

Enzyme Isolation. Protease was isolated from unfertilized eggs by a modification of the procedures of Fodor et al. (1975). Benzamidine hydrochloride (5 mM) was included in buffers at various points in the isolation to minimize autodegradation (Baginski et al., 1982). Approximately 100 mL of settled eggs was lysed for 40 min on a stir plate at room temperature by the addition of 1.5 volumes of cold artificial seawater containing 8% 1-butanol and 33 mM urea. The lysate was centrifuged for 40 min at 16 000 rpm using a Sorvall SS34 rotor (30000*g*). The supernatant was adjusted to pH 4.6 by the dropwise addition of 0.1 N HCl and centrifuged for 45 min at 30000*g*. The resulting supernatant was dialyzed for 24 h at 4 °C in 6 mM sodium acetate, pH 4.55. The precipitate was collected by centrifugation at 30000*g* for 45 min, disrupted by homogenization, and resolubilized in buffer containing 0.2 M Tris, 25 mM EDTA, 2 M KCl, 10% glycerol, 1% 1-butanol, and 5 mM benzamidine hydrochloride, pH 8.0. The preparation at this point is referred to as crude extract.

SBTI affinity chromatography was performed as previously described (Fodor et al., 1975) on a 1.5 × 2.0 cm column of SBTI–agarose. Approximately 250 mL of crude extract was pooled from 7–10 isolates (approximately 1 L of settled eggs starting material) and applied to the column at a flow rate of 10 mL/h. The column was washed exhaustively (100 volumes) with the solubilization buffer and eluted at pH 4.0

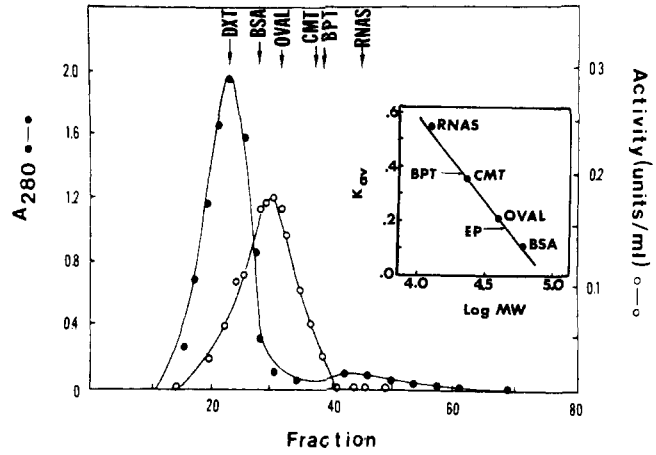

FIGURE 1: Gel filtration of crude extract on a 1.5 × 86 cm column of Sephadex G-75. A 2.5-mL sample was applied to the column, and 2.5-mL fractions were collected. Arrows indicate elution positions of molecular weight standards: DXT, dextran; BSA, bovine serum albumin; OVAL, ovalbumin; CMT, chymotrypsinogen; BPT, bovine pancreatic trypsin; RNAS, ribonuclease A. Inset: standard curve for molecular weight standards. EP, egg protease.

with 0.1 M Tris, 25 mM EDTA, 5% glycerol, 0.5% butanol, 1.0 M KCl, 2.0 M urea, and 5.0 mM benzamidine. Addition of benzamidine to the elution buffer was required to preserve the enzymatic activity of the affinity-purified protease (see Results).

Assay Procedures. Esterase activity was monitored with BAEE as substrate as previously described (Schwert & Takenaka, 1955). The assay mixture contained 0.5 mM BAEE and 0.2 M Tris, pH 8, and the reaction was initiated by addition of 20 μL of enzyme preparation (50 μL for kinetic studies). The total volume was 2.5 mL. One unit of enzyme activity hydrolyzes 1 μmol of BAEE per minute. Activity toward L-BAPNA was assayed according to Ehrlanger (1961). Protease activity was determined by hydrolysis of β-casein according to Reimerdes & Klostermeyer (1976). Protein was monitored by the dye binding technique of Sedmack & Grossberg (1977) or by the absorbance at 280 nm (Layne, 1957).

Molecular Weight. SDS–PAGE was carried out according to Laemmli (1970) in the presence of dithiothreitol, with a 5% stacking gel and a 10% or 12% running gel. Protein was stained with Coomassie blue or silver stain (Merril et al., 1981). The PAS staining technique of Kapitany & Zebrowski (1973) was used for detection of carbohydrate. Isolated chicken erythrocyte ghosts were used as an internal standard for glycoprotein staining. Molecular weight standards (Sigma) used were BSA (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), and bovine pancreatic trypsin (23 000). Gel filtration was performed on calibrated columns of Sephadex G-75 (1.5 × 86 cm) and G-100 (1.5 × 112 cm) equilibrated with the solubilization buffer described above. Molecular weight standards (Pharmacia) were BSA (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000), and ribonuclease A (13 700).

Results

Enzyme Isolation. Following the protocol of Fodor et al. (1975), BAEE esterase activity was first observed in egg lysates upon dialysis vs. acetate buffer. Gel filtration of these crude extracts on columns of Sephadex G-75 or G-100 yielded a single peak of BAEE esterase activity with an apparent molecular weight of 45 000–48 000 (Figure 1). Whether benzamidine was included from the first step (egg lysis) and throughout the isolation or completely omitted, this result was

Table I: Purification of Protease from Unfertilized *S. purpuratus* Eggs

	protein (mg/mL)	act. (units/mL)	sp act. (units/mg of protein)	purification (*x*-fold)	yield (%)	*n*
crude extract	5.80 ± 0.360	0.75 ± 0.056	0.13 ± 0.009	1.0	100.0	42
Sephadex G-75, crude extract	0.22 ± 0.040	0.25 ± 0.020	1.23 ± 0.010	9.5	94.9	7
SBTI–agarose	0.07 ± 0.010	7.80 ± 2.42	96.0 ± 16.81	738.5	91.4	5
Sephadex G-100, purified protease	0.005[a]	0.39 ± 0.162	78.0		77.8	3

[a] This figure represents the sensitivity level of the assay and is probably an overestimate for protein. The specific activity indicated is probably an underestimate.

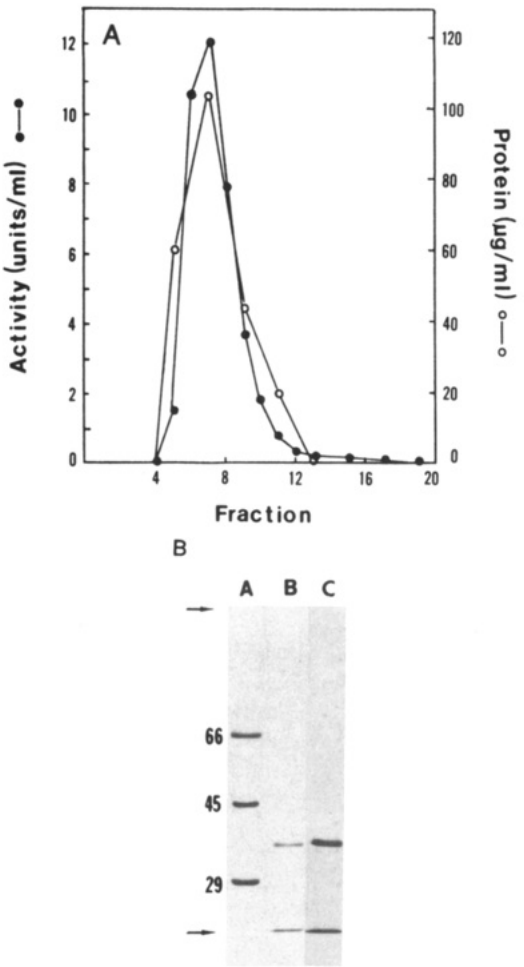

FIGURE 2: (A) Affinity chromatography of egg protease activity on SBTI–agarose. Fractions (4.0 mL) are numbered from the initiation of elution with pH 4 buffer. No activity was eluted above pH 4. (B) SDS–PAGE of the pooled, 10-fold-concentrated activity peak from (A) stained with Coomassie blue. The upper arrow indicates the stack-running gel interface and the lower arrow the dye front. Numbers indicate molecular weights of standards ($\times 10^{-3}$). Lane A, standards; lane B, protease preparation at 5 μg of protein; lane C, protease at 20 μg of protein.

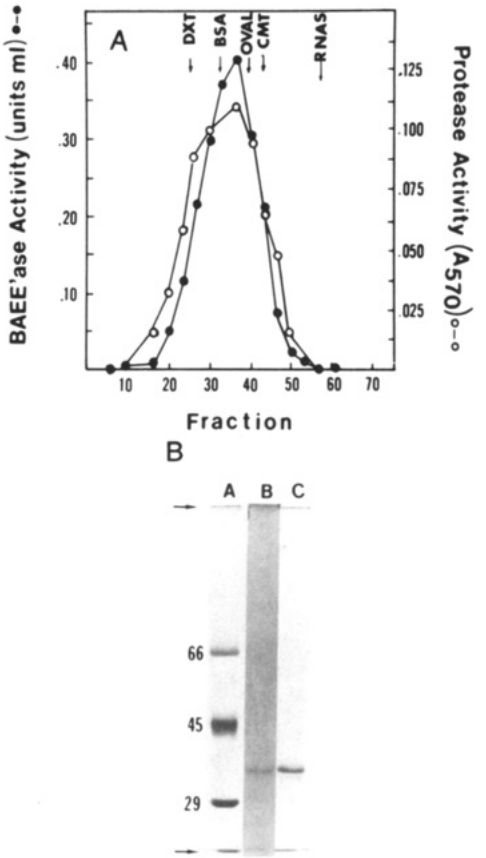

FIGURE 3: (A) Sephadex G-100 gel filtration of the affinity-purified protease. Fraction size = 2.8 mL. Protein was immeasurable in these fractions. Arrows indicate elution positions of molecular weight standards. (B) SDS–PAGE of pooled, 100-folded-concentrated activity fractions from (A). Lane A, silver-stained molecular weight standards; lane B, Coomassie blue stained protease preparation; lane C, same gel as in lane B, destained and restained with silver.

highly consistent. Bovine pancreatic trypsin was used as an added standard and eluted with an apparent molecular weight of 21 000.

Affinity chromatography of extract pooled from 1 L of settled eggs (7–10 isolates) yielded approximately 2.1 mg of protein. The esterase activity was recovered as a single, sharp peak that was superimposable with the protein peak (Figure 2A). An enrichment of 740-fold was obtained in this step (Table I). Deletion of benzamidine from the elution buffer resulted in a rapid loss of activity (within hours). This extreme instability of the affinity-purified protease has been observed by other investigators (Sawada et al., 1984; K. A. Walsh, personal communication). In the presence of benzamidine, the enzyme could be kept for several weeks at 0 °C or lyophilized and stored.

The purity and molecular weight of the protease recovered from the affinity column were assessed by SDS–PAGE and gel filtration. SDS–PAGE analysis of the pooled, lyophilized affinity fractions resulted in the appearance of a single, major protein band at 35 kDa (Figure 2B). In some cases, several faint bands at lower molecular weights could be observed. We suspect these were degradation products, since they always appeared more prominently as a function of storage time. The protein lacks a significant carbohydrate component as judged by the absence of PAS staining. Application of the affinity-purified protease to a Sephadex (G-100) column again yielded a single activity peak at 47 kDa (Figure 3A). The esterase exhibited high proteolytic activity using β-casein as substrate. Casein hydrolysis was completely inhibited by 1 μmol of SBTI per micromole of enzyme. Finally, when the 47-kDa esterase peak from gel filtration was subjected to SDS–PAGE, the

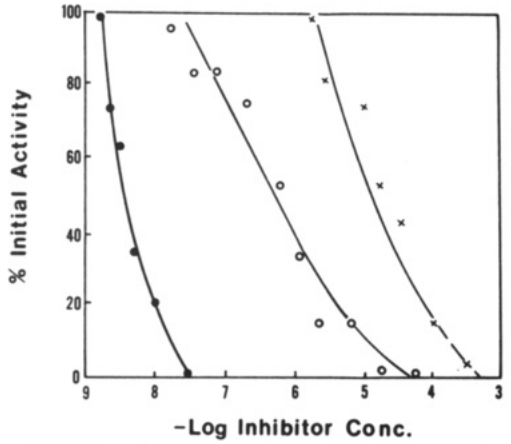

FIGURE 4: Inhibition of affinity-purified protease by SBTI (●), chicken ovomucoid (○), and antipain (×). Each point represents the mean of three trials. Reactions were started by addition of 50 μL (approximately 0.5 unit) of the affinity-purified enzyme. In other experiments, the reaction was started by substrate addition after a 10-min preincubation of enzyme + substrate. The results were similar.

Table II: Inhibition of Egg Protease by PMSF and TLCK[a]

inhibitor	concn (mM)	preincubation time (min) 15	30	60
TLCK	1.0		32.8 ± 3.90	
TLCK	10.0	77.1 ± 9.70	86.7 ± 8.08	100.0 ± 0.00
PMSF	1.0		12.6 ± 5.40	
PMSF	5.0	43.1 ± 7.16	55.1 ± 4.44	50.0 ± 5.32

[a] Figures are percent inhibition ± SEM (n = 3). Fifty microliters of inhibitor at the designated concentrations was preincubated with an equal volume of crude extract. Residual enzymatic activity was measured after addition of 0.5 mM BAEE in 0.2 M Tris, pH 8.

resultant molecular weight estimation was again 35000 (Figure 3B). In summary, the native molecular weight of the protease by gel filtration is 47000; under reducing and denaturing conditions, it is 35000.

Kinetics. The pH optimum of the purified protease with BAEE was 8.0, and the K_m was found to be 7.5×10^{-5} M. A plot of the data as $V_0/[s]$ vs. V_0 was linear, suggesting that only one esterolytic activity is present in this preparation. The enzyme did not hydrolyze L-BAPNA under a variety of assay conditions employing Tris or veronal buffers, even over prolonged incubation periods. Bovine trypsin was found to be fully active against L-BAPNA under the same conditions.

The esterolytic activity of the egg protease is strongly inhibited by soybean and lima bean trypsin inhibitors, chicken ovomucoid, antipain, leupeptin, and Bowman–Birk inhibitor (Figure 4 and Table III below). Benzamidine hydrochloride is a competitive inhibitor of bovine trypsin (Shaw et al., 1965). In certain experiments (e.g., under conditions of low substrate concentration as in K_m and K_i determinations, and for PMSF and TLCK inhibition), benzamidine was excluded to avoid interference. Since the affinity-purified protease is highly unstable in the absence of benzamidine, we used crude extracts in these experiments. Alternatively, crude extracts were fractionated by ammonium sulfate precipitation between 25% and 40% saturation. The results were similar in either case. Inhibition by PMSF and TLCK (Table II) indicates the presence of serine and histidine residues in the active center, respectively (Gold & Fahrney, 1963; Shaw et al., 1965). Inhibition is competitive with soybean and lima bean trypsin inhibitors, ovomucoid, and leupeptin (Figure 5 and Table III). Consistent with results obtained by Sawada et al. (1984), the chymotrypsin inhibitor TPCK (10 mM, 30 min) was without effect.

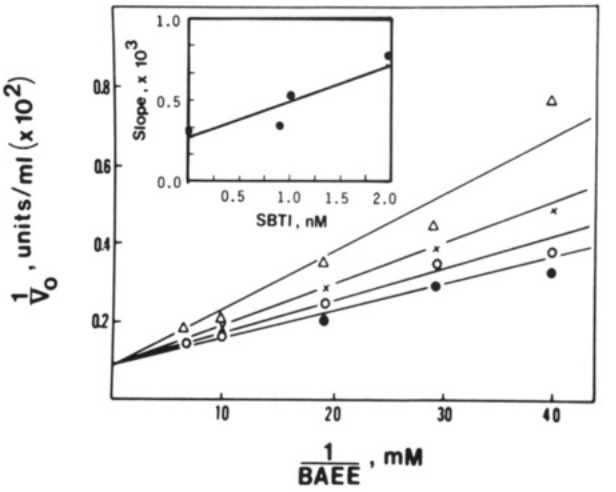

FIGURE 5: Inhibition of egg protease by SBTI at varying substrate concentrations. Each point represents the mean of three trials. Inset: slopes vs. inhibitor concentrations for K_i determination. K_i = −x-axis intercept (Segel, 1975): (●) 0 nM SBTI; (○) 0.9 nM SBTI; (×) 1.0 nM SBTI; (△) 2.0 nM SBTI.

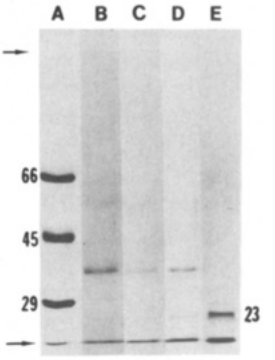

FIGURE 6: Molecular weight determination of egg protease by SDS–PAGE in the presence and absence of protease inhibitors. Lane A, molecular weight standards; lane B, protease with 5 mM benzamidine; lane C, TLCK–protease; lane D, protease in the absnece of TLCK or benzamidine; lane E, bovine pancreatic trypsin. Stained with Coomassie blue. Approximately 2 μg of protein was loaded in lanes B–D.

Table III: Sea Urchin Egg Protease: Summary of Inhibitor Kinetics

inhibitor	inhibition	K_i (nM)	ID_{50} (μM)[a]	ID_{50} (μM)[b]
soybean trypsin inhibitor	competitive	1.6	0.006	0.012
chicken ovomucoid	competitive	100	1.1	0.8
lima bean trypsin inhibitor	competitive	150	3.5	1.2
leupeptin	competitive	130	4.2	1.0
antipain			32.0	
benzamidine hydrochloride			1300.0	
Bowman–Birk inhibitor			6.0	

[a] Inhibitor concentration yielding 50% initial activity, experimentally determined. [b] Calculated from the equation $ID_{50} = (1 + [s]/K_m)K_i$, where K_m and K_i have been experimentally determined.

Autodegradation. The possible effects of autoproteolysis on the molecular weight of the protease were examined in the presence and absence of inhibitors. Samples of the affinity-purified enzyme were either (1) added to an equal volume of SDS sample buffer immediately upon elution from the affinity column, (2) immediately preincubated with TLCK for 15 min at 4 °C and then added to sample buffer, or (3) dialyzed for 24 h at 4 °C in the presence or absence of benzamidine. In

each case, a single major band was observed at 35 kDa (Figure 6). However, when benzamidine was removed by dialysis, an additional protein band was observed which comigrated precisely with bovine pancreatic trypsin at 23 kDa. Complete conversion from 35 to 23 kDa was not observed even after 90 days of storage at 4 °C or 5 h at 37 °C (less than 2% activity remaining). Boiling in SDS sample buffer for up to 10 min did not result in the appearance of the 23-kDa band. Trace levels of esterolytic and proteolytic activity were associated with the 23-kDa protein which were completely abolished by 10^{-6} M SBTI. However, these were detectable only when purified protease was stored for several days at 4 °C and a large sample (approximately 3-fold more total activity) was subjected to gel filtration. Neither the 23-kDa band nor the associated activity was detected in routine preparations. The 23-kDa protein appears to be a minor component of this preparation resulting from autodegradation of the 35-kDa protease.

Discussion

This report describes the characterization of a protease with trypsin-like specificity that has been isolated from eggs of the sea urchin *Strongylocentrotus purpuratus*. Isolation of the protease from unfertilized eggs minimizes the chances of any postsecretional processing. We have also taken precautions against autoproteolysis by the inclusion of such inhibitors as benzamidine and TLCK. Our results indicate that the sea urchin egg protease is similar in many respects to bovine and other trypsins. The pH optimum and K_m of the protease with the synthetic trypsin substrate BAEE are comparable to those for bovine trypsin (Barman, 1969). Inhibition by PMSF and TLCK confirms the presence of serine and histidine residues in the active site, respectively (Fodor et al., 1975; Sawada et al., 1984). Previously reported insensitivity of sea urchin egg protease to PMSF (Carroll, 1976) may be due to the use of a lower inhibitor concentration coupled with the instability of PMSF in aqueous solution (James, 1978). Soybean trypsin inhibitor, chicken ovomucoid, lima bean trypsin inhibitor, and leupeptin are competitive inhibitors of the protease, and with relative potencies in that order. Thus, the egg protease exhibits similar inhibitor kinetics to pancreatic trypsin as well [for examples and other references, see Umezawa (1982) and Kassell (1970)]. All of these inhibitors except PMSF have been used previously to experimentally induce polyspermy in a variety of urchin species (Hagstrom, 1956; Lonning, 1967; Longo & Schuel, 1973; Schuel et al., 1973, 1976a,b; Schuel & Schuel, 1981; Vacquier et al., 1972b). In fact, the *relative* potencies of these inhibitors with the egg protease have been statistically correlated with their ability to promote polyspermy (Alliegro & Schuel, 1984b). Sawada et al. (1984) found that a variety of peptidylargininal derivatives (which are potent inhibitors of bovine trypsin and the egg protease from *S. intermedius*) inhibit fertilization envelope elevation. Finally, the protease substrate gelatin has been shown to induce low levels of polyspermy in fertilization cultures of *Arbacia* eggs (Dunham et al., 1982). These combined observations provide strong support for the role of sea urchin egg protease in fertilization envelope elevation and the prevention of polyspermy. We are currently raising an antibody to the 35-kDa enzyme to be used in future studies (Alliegro & Schuel, 1984a), including a subcellular immunolocalization.

The molecular weight of the protease in our hands was 47 000 under nondenaturing conditions. When the 47-kDa protein recovered from gel filtration was subjected to SDS–PAGE, it appeared as a single band at 35 kDa. We did not observe a lower molecular weight peptide which could account for the 12-kDa difference between gel filtration and SDS gels. Carroll & Epel (1975) have also reported a native molecular weight of 47 000 for the sea urchin egg protease. Furthermore, our gel filtration elution profile is in very close agreement with that of Fodor et al. (1975). However, these authors report a molecular weight approximately half of our estimate.[2] This difference may be at least partially due to autoproteolysis, since we have observed the appearance of a 23-kDa protein under conditions permitting proteolytic degradation (i.e., removal of inhibitors or during prolonged storage). The appearance of this minor, active proteolytic component is of interest, since trypsins serving specialized functions are often composed of a subunit similar in molecular weight and amino acid content to bovine trypsin, plus an additional regulatory component (Neurath, 1984). The question of how this relates to sea urchin egg trypsin will require additional study.

In contrast to the conclusions of Carroll & Epel (1975), but in agreement with Fodor et al. (1975) and Sawada et al. (1984), the results of our affinity purification and subsequent kinetic analysis suggest the presence of only one protease with BAEE esterase activity. We base our conclusion on the following criteria: (1) The esterase peak recovered from SBTI affinity chromatography was superimposable with the protein peak. (2) Gel filtration of the affinity-purified enzyme resulted in the appearance of a single, sharp peak of esterase activity. (3) Electrophoresis under reducing and denaturing conditions yielded a single discrete band of protein. (4) $V_0/[s]$ vs. V_0 replots of our kinetic data resulted in a straight line, whereas a deviation from linearity is expected with a heterogeneous preparation (Segel, 1975). Specific activities of the hypothesized sperm receptor hydrolase and vitelline delaminase with BAEE were reported to be widely divergent (Carroll & Epel, 1975). If these two activities were present in our preparation, we should expect a biphasic curve. It is possible that modification of the protease after fertilization could lead to the appearance of two separate biological activities. In this regard, the active 23-kDa proteolytic fragment may be of extreme interest.

Trypsin-like proteases have also been identified in sperm. The molecular weights of the sperm proteases under reducing and denaturing conditions have been reported as 34 000 for sea urchins (Levine & Walsh, 1981) and 35 000 for mammals (Muller-Esterl & Fritz, 1981). It is interesting but not surprising that the sperm and egg proteases bear a resemblance in this respect as well as by other criteria such as substrate and inhibitor specificities. Just how closely they are related awaits a more detailed comparison. In situ, they may serve significantly different purposes. For instance, the sperm protease may be involved in the acrosome reaction and ultimately in penetration of the egg (Levine & Walsh, 1979). On the other hand, the egg protease is apparently involved in the cortical reaction with one of the results being the block to polyspermy (Schuel, 1984). Their respective roles in fertilization could conceivably be a function of subcellular localization, accessibility of physiological substrates, or subtle differences in structure and amino acid composition.

Whereas a good deal is known about the molecular nature and functions of bacterial and mammalian trypsins, relatively little information is available for the invertebrate groups (Zwilling & Neurath, 1981; Neurath 1984). A 35-kDa trypsin-like enzyme has also been demonstrated in the digestive

[2] According to their Figure 3, the column void volume is approximately twice that expected for a column of the reported dimensions (Copper, 1977). This could lead to a 2-fold overestimate of V_e and a 2-fold underestimate of molecular weight. Under these circumstances, the molecular weight would be 46 000.

tract of the annelid *Sabellaria* by SDS–PAGE (Peaucellier, 1983) and from ascidian sperm by gel filtration (Sawada et al., 1982). A better understanding of the molecular nature of sea urchin egg trypsin should be of help in more thoroughly defining its functional role in fertilization and in our understanding of the evolutionary relationship between trypsins of diverse origin.

ACKNOWLEDGMENTS

We thank Drs. Barry Eckert, Sabina Sobel, and Kenneth Edds for use of their laboratory equipment, Drs. Walter Troll and Kenneth Walsh for reading and commenting on the manuscript, and Mary Anne Alliegro for help with graphics.

Registry No. BAEE, 971-21-1; SBTI, 9078-38-0; trypsin inhibitor, 9035-81-8; trypsin, 9002-07-7; *Strongylocentrotus purpuratus* serine proteinase, 96758-73-5.

REFERENCES

Alliegro, M. C., & Schuel, H. (1983) *J. Cell Biol. 97*, 180a.
Alliegro, M. C., & Schuel, H. (1984a) *J. Cell Biol. 99*, 85a.
Alliegro, M. C., & Schuel, H. (1984b) *Biol. Bull. (Woods Hole, Mass.) 166*, 473–481.
Baginski, R. M., McBlaine, P. J., & Carroll, E. J., Jr. (1982) *Gamete Res. 6*, 39–52.
Barman, T. E. (1969) *Enzyme Handbook*, Vol. 2, p 618, Springer Verlag, Heidelberg.
Boldt, J., Schuel, H., Schuel, R., Dandekar, P., & Troll, W. (1981) *Gamete Res. 4*, 365–377.
Carroll, E. J., Jr. (1976) *Methods Enzymol. 45*, 343–353.
Carroll, E. J., Jr., & Epel, D. (1975) *Dev. Biol. 44*, 22–32.
Carroll, E. J., Jr., Lois, A. F., & Lackey, D. A. (1982) *J. Cell Biol. 95*, 154a.
Coburn, M., Schuel, H. & Troll, W. (1981) *Dev. Biol. 84*, 235–238.
Cooper, T. (1977) *The Tools of Biochemistry*, p 171, Wiley, New York.
Decker, S., & Kinsey, W. H. (1983) *Dev. Biol. 96*, 37–45.
Dunham, P., Nelson, L., Vosshall, L., & Weissmann, G. (1982) *Biol. Bull. (Woods Hole, Mass.) 163*, 420–430.
Ehrlanger, B. F. (1961) *Arch. Biochem. Biophys. 95*, 271–274.
Fodor, E. J. B., Ako, H., & Walsh, K. A. (1975) *Biochemistry 14*, 4923–4927.
Gold, A. M., & Fahrney, D. E. (1964) *Biochemistry 3*, 783–791.
Grossman, A., Levy, M., Troll, W., & Weissmann, G. (1973) *Nature (London), New Biol. 243*, 277–278.
Hagstrom, B. E. (1956) *Ark. Zool. 10*, 307–315.
Harvey, E. B. (1956) *The American Arbacia and Other Sea Urchins*, Princeton University Press, Princeton, NJ.
James, G. T. (1978) *Anal. Biochem. 86*, 574–579.
Kapitany, R. A., & Zebrowski, E. J. (1973) *Anal. Biochem. 56*, 361–369.
Kassell, B. (1970) *Methods Enzymol. 19*, 853–862.
Kopf, G. S., Moy, G. W., & Vacquier, V. D. (1983) *J. Cell Biol. 95*, 924–932.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Layne, E. (1957) *Methods Enzymol. 3*, 447–450.
Levine, A. E., & Walsh, K. A. (1979) *Dev. Biol. 72*, 126–137.
Levine, A. E., & Walsh, K. A. (1981) *J. Biol. Chem. 255*, 4814–4820.
Longo, F. J., & Schuel, H. (1973) *Dev. Biol. 34*, 187–199.
Lonning, S. (1967) *Sarsia 30*, 107–116.
Merril, C. R., Dunau, M. L., & Goldman, D. (1981) *Anal. Biochem. 110*, 201–207.
Muller-Esterl, W., & Fritz, H. (1981) *Methods Enzymol. 80*, 621–632.
Neurath, H. (1984) *Science (Washington, D.C.) 224*, 350–357.
Peaucellier, G. (1983) *Eur. J. Biochem. 136*, 435–445.
Reimerdes, E. H., & Klostermeyer, H. (1976) *Methods Enzymol. 45*, 26–28.
Sawada, H., Yokosawa, H., Hoshi, M., & Ishii, S. (1982) *Gamete Res. 5*, 291–301.
Sawada, H., Miura, M., Yokosawa, H., & Ishii, S. (1984) *Biochem. Biophys. Res. Commun. 121*, 598–604.
Schuel, H. (1978) *Gamete Res. 1*, 299–383.
Schuel, H. (1984) *Biol. Bull. (Woods Hole, Mass.) 167*, 271–309.
Schuel, H. (1985) in *Biology of Fertilization* (Metz, C. B., & Monroy, A., Eds.) Vol. 3 (in press).
Schuel, H., & Schuel, R. (1981) *Dev. Biol. 87*, 249–258.
Schuel, H., Wilson, W. L., Chen, K., & Lorand, L. (1973) *Dev. Biol. 34*, 175–186.
Schuel, H., Longo, F. J., Wilson, W. L., & Troll, W. L. (1976a) *Dev. Biol. 49*, 178–184.
Schuel, H., Troll, W., & Lorand, L. (1976b) *Exp. Cell Res. 103*, 442–447.
Schwert, G. W., & Takenaka, Y. (1955) *Biochim. Biophys. Acta 16*, 570–575.
Sedmack, J. J., & Grossberg, S. E. (1977) *Anal. Biochem. 79*, 544–552.
Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Shaw, E., Mares-Guia, M., & Cohen, W. (1965) *Biochemistry 4*, 2219–2224.
Umezawa, H. (1982) *Annu. Rev. Microbiol. 36*, 75–99.
Vacquier, V. D., Epel, D., & Douglas, L. A. (1972a) *Nature (London) 240*, 34–36.
Vacquier, V. D., Tegner, M. J., & Epel, D. (1972b) *Nature (London) 240*, 352–353.
Vacquier, V. D., Tegner, M. J., & Epel, D. (1973) *Exp. Cell Res. 80*, 111–119.
Walsh, K. A., & Wilcox, P. E. (1970) *Methods Enzymol. 19*, 31–41.
Zwilling, R., & Neurath, H. (1981) *Methods Enzymol. 80*, 633–664.