Purification and Properties of a Trypsin-like Enzyme from the Starfish *Evasterias trochelii**

William P. Winter† and Hans Neurath‡

ABSTRACT: *Evasterias trochelii*, a starfish common to Pacific Northwest intertidal beaches, was selected as a potential source of a trypsin-like enzyme typical of the higher invertebrates. A protease was purified from an extract of the pyloric caeca by chromatography on DEAE-cellulose, DE-52 microgranular cellulose, and gel filtration on Sephadex G-100. The specificity of the enzyme was characteristic of vertebrate trypsin with respect to synthetic substrates and to certain synthetic and natural inhibitors. The enzyme was also capable of activating bovine chymotrypsinogen. The amino acid composition and approximate minimum molecular weight were similar to those of vertebrate trypsins. The enzyme does not appear to exist in zymogen form. It is anionic at neutral pH, unstable below pH 5, and does not require calcium ions for stability. These latter characteristics differentiate starfish trypsin from bovine trypsin. An enzyme resembling carboxypeptidase B was detected but was not further characterized.

Trypsin and trypsin-like enzymes have recently been found in a wide variety of lower animals in addition to the higher chordates which have long served as a source of these enzymes. Prahl and Neurath (1966) reported on the pancreatic proteolytic enzymes of the spiny Pacific dogfish *Squalus acanthias*, a member of one of the most primitive classes of chordates, Elasmobranchii. A report has also appeared describing the occurrence of chymotrypsin-like proteases in the sea anemone *Metridium senile* (Gibson and Dixon, 1969a,b). Recently, Pfleiderer *et al.* (1967) found trypsin in the crayfish *Astaeus spp.* and Kafatos *et al.* (1967) isolated "cocoonase" from the moth *Antheraea*. While the latter enzyme is not a digestive protease in the normal sense, its trypsin-like qualities are evident. In addition, trypsin-like proteases have been found in a marine "worm" of uncertain taxonomic rank (Nilsson and Fange, 1967), black flies (Yang and Davies, 1968), decapod crustaceans (DeVillez and Buschlen, 1967), beetles (Pfleiderer and Zwilling, 1966), butterfly larvae (Lecadet and Chevrier, 1966; Lecadet and Dedonder, 1966), the earthworm (Bewley and DeVillez, 1968), the white shrimp, the horseshoe crab, and the sea pansy (Travis, 1969; Gates and Travis, 1969).

Reports of trypsin among digestive proteases from echinoderms exist in the older literature (Swano, 1936) but in the absence of adequate characterization of specificity, these reports are meaningless in the modern context. Fish (1967) has surveyed the digestive enzyme of the sea cucumber *Cucumaria* and has reported low levels of trypsin. Reports have also been published (Lundblad and Lundblad, 1962) describing the occurrence of proteolytic enzymes in the eggs of various sea urchins. One of these enzymes, presumably trypsin like, may possibly play a role in the early stages of fertilization. The digestive physiology and anatomy of the starfish *Pisaster ochreceus* has also been studied and the occurrence of zymogen granules in the pyloric caeca reported (Mauzey, 1966). We now wish to describe the occurrence, purification, and properties of a trypsin-like protease from a common species of starfish, *Evasterias trochelii*.

Experimental Procedure

Materials and Methods. COLLECTION OF TISSUE. The starfish used in this study were collected on the west side of Camano Island, Island County, Wash. All specimens were obtained at low tide in late spring and early summer immediately following the spawning season. The starfish were dissected by cutting off the rays, slitting the sides of the rays, and removing the pyloric caeca. The caeca were stored frozen for future use.

Preparation of Acetone Powder. The frozen tissue was first passed through a meat grinder, then stirred in 5 volumes of acetone for approximately 4 hr at −5°. After removal of the acetone by filtration with suction, the filter cake was suspended in another 5-volume portion of acetone at the same temperature and stirred overnight. The procedure was repeated once more followed by 5 volumes of ethyl ether–acetone (1:1) and finally 5 volumes of ethyl ether. The preparations were air dried in a thin layer on filter paper or in a vacuum desiccator until no odor of ether persisted.

Activity Measurements. The substrate was 2% Hammarsten casein in 0.05 M phosphate buffer (pH 7.5). Substrate (1 ml) was incubated at 37° with 0.5 ml of sample. After 20 min, 2 ml of 5% trichloroacetic acid was added. Samples were allowed to stand for 30 min and filtered. To 1 ml of the filtrate was added 2 ml of 0.5 N NaOH, followed by 0.6 ml of Folin–Ciocalteau reagent diluted to 1 N in sulfuric acid. Color was allowed to develop for 20 min and the absorbance at 660 nm was read in the spectrophotometer. A standard curve was prepared using bovine trypsin, and re-

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† Present address: Department of Human Genetics, University of Michigan, Ann Arbor, Mich. 48103.

‡ To whom correspondence should be addressed.

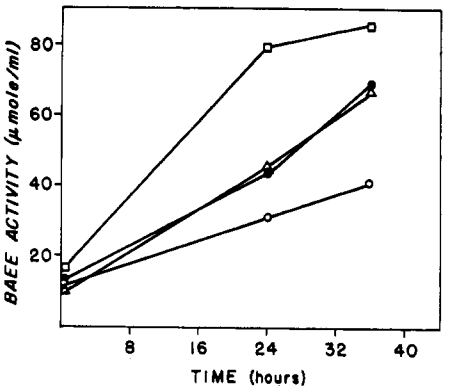

FIGURE 1: Pseudoactivation of starfish trypsin. Pyloric caeca were pulped in a meat grinder and allowed to autolyze under toluene at room temperature. Samples were removed, weighed, and extracted with 4 parts (v/w) of solvent. pH was adjusted with NaOH: (O-O) pH 7.0; (●-●) pH 8.3; (△-△) pH 9.2; (□-□) 6 M urea. The amount of protein extracted (Folin–Ciocalteau reagent) remained practically constant during the extraction period.

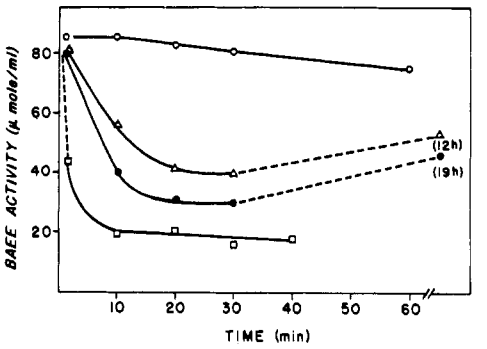

FIGURE 2: Activity loss during early autolysis periods. Caeca were homogenized in 4 volumes (v/w) of solvent. Samples of the homogenate were used immediately without centrifugation. Extraction conditions were as follows: (O-O) water 4°; (●-●) water 22°, 0.2 M $CaCl_2$, 22°; (□-□) pH 7.2, 22°. The pH of the aqueous extracts was raised from 6.5 to 7.2 by the addition of NaOH.

sults reported in terms of trypsin equivalents. Activity toward BAEE[1] (10^{-2} M in 10^{-2} M Tris buffer, pH 7.9, containing 0.02 M calcium chloride) was assayed in a Radiometer TTT1 pH stat. The hydrolysis of TAME (10^{-3} M in 0.04 M Tris buffer, pH 8.1, containing 0.01 M calcium chloride) was followed in a Gilford Model 2000 recording spectrophotometer at 247 nm. Chymotrypsin and carboxypeptidase B were assayed using ATEE and BGA in a similar manner to the BAEE assay at 266 nm and 220 nm, respectively. Activities of crude extracts are expressed in units of μmoles of substrate hydrolyzed per min per ml.

Amino Acid Analyses. Analyses were performed with the Beckman Model 120-B amino acid analyzer following the procedure of Spackman *et al.* (1958). Samples were hydrolyzed in 5.7 N glass-distilled HCl, sealed under vacuum, at 110° for specified periods of time. Tryptophan was determined by the method of Barman and Koshland (1967) using 2-hydroxy-5-nitrobenzyl bromide. The method was modified to remove urea and excess reagent by dialysis, followed by lyophilization. This modification was necessitated by the failure of the modified protein to precipitate in trichloroacetic acid. Cysteine was determined as cysteic acid by the method of Hirs (1956).

Electrophoresis. Disc gel electrophoresis was carried out according to the method of Davis (1964). The trypsin-specific stain described by Uriel (1963) was used to detect the enzyme as follows. *N*-Benzoylarginine-β-naphthylamide (10 mg) dissolved in 1 ml of dimethylformamide was added to 9 ml of distilled water containing 10 mg of diazo blue B. This solution was mixed with 9 ml of Tris buffer, 0.05 M (pH 7.4). The stain was developed by incubating the gels at 37° for 2–4 hr. The gels were then washed with 2% acetic acid and photographed. Electrophoresis on cellulose acetate was performed using the Beckman microzone electrophoresis equipment. Strips were strained for protein with ponceau S and for activity be the conventional Uriel procedure (Uriel, 1963).

Chromatography. Ion-exchange chromatography was carried out on both DEAE-cellulose (Schleicher and Schuell, capacity of 0.85 mequiv/g) and DE-52 microgranular cellulose (Reeve Angel Co.). Prior to chromatography the absorbant was suspended in water and the fines removed. The absorbant was then equilibrated in 10^{-3} M ammediol buffer, pH 8.8 (Sigma Chemical Co.). Sephadex G-100 (Pharmacia) was equilibrated in 10^{-3} M ammediol buffer (pH 8.8) containing 0.05 M sodium chloride. G-100 columns were developed under a constant pressure head of 40 cm of water. All chromatographies were carried out at 4°.

Protein Analysis. Total protein was determined either by the Folin–Ciocalteau reaction, using casein as a standard, or by the method of Warburg and Christian (1941) based on the absorbance at 280 and 260 nm.

Chemicals. Other compounds used in this work were obtained as follows. TAME, BAEE, and other esterase substrates were obtained from Cyclo Chemical Corp. Hammarsten casein was a product of Mann Chemical Co. Elastin, bovine trypsin, and chymotrypsin, and soybean and lima bean trypsin inhibitors were obtained from the Worthington Biochemical Corp. Chicken and turkey ovomucoid were purified by Dr. Royce Haynes of this laboratory. TLCK and TPCK were purchased from Cyclo Chemical Co. Nagarse was a product of Nagarse and Co., Osaka, Japan.

Results

Search for Zymogen. In view of the widespread occurrence of trypsin in zymogen form in vertebrate pancreas, experiments were first undertaken to determine whether the starfish enzyme would also occur as an inactive enzyme precursor. Acetone powders of the caecum tissue were prepared as described in Experimental Procedure. Unless otherwise noted, the powder was extracted with 10 volumes of water per weight of powder. The extraction mixture was stirred for 4 hr at 4°, centrifuged, and the pale brown, slightly turbid supernatant tested for activation.

Trypsin was added to a final concentration of 0.20 mg/ml

[1] Abbreviations used are: BAEE, α-*N*-benzoyl-L-arginine ethyl ester; TAME, α-*N*-toluenesulfonyl-L-arginine methyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; TLCK, tosyllysyl chloromethyl ketone; TPCK, tosylphenylalanyl chloromethyl ketone; BGA, α-*N*-benzoylglycylarginine; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate.

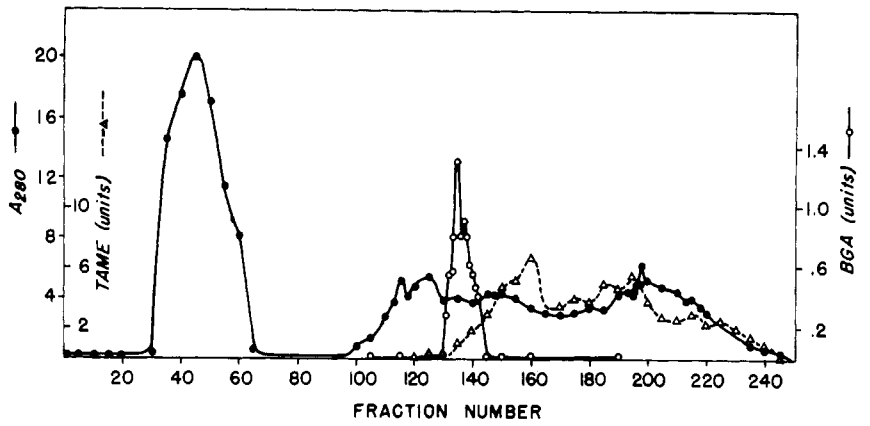

FIGURE 3: Chromatography of extracts of starfish caeca on DEAE-cellulose column (5 × 40 cm). At fraction 60 a linear gradient was established from 5 × 10^{-3} M sodium phosphate, pH 6.5 (2 l.), to 0.5 M NaCl in the same buffer (2 l.) at a flow rate of 180 ml/hr. Fraction volume 20 ml.

and 1-ml portions dialyzed against 0.1 M Tris, pH 7.8, in the presence of 0.02 M $CaCl_2$ at 4°. No net activation was observed when digests were tested after 1, 3, 8, and 20 hr. In another attempt to demonstrate activation, the extracts were subjected to gel filtration on either Bio-Gel P-30 or Sephadex G-50 in order to separate any inhibitors which might interfere with trypsin-catalyzed activation. Activation was also attempted by treating 1 ml of extract with 0.1 mg/ml of subtilisin or with crude starfish trypsin. The latter was added as 10 μl of the fluid which was pressed from the caeca in the course of autolysis at room temperature.

In order to explore the possibility that the acetone treatment itself was responsible for the apparent lack of activatable zymogen, similar activation attempts were made on extracts prepared from fresh or frozen tissue. No activation resulted by incubation with bovine trypsin, subtilisin, or concentrated starfish caecum extract. An apparent activation was observed when finely mashed tissue was allowed to autolyze and the soluble proteins extracted at various pH by the addition of 6 M urea (Figure 1). However, when the caeca were homogenized in 4 volumes of water rather than 10, and the milky homogenate was assayed for tryptic activity as soon as possible after homogenization, enzymatic activity declined (Figure 2) and approached a level which is identical with the initial level observed in the preceding series of experiments (Figure 1). No net increase in enzymatic activity therefore resulted during the overall extraction.

Purification Procedure. Two slightly different purification schemes were employed in the course of this work. In the earlier work, 0.1 M sodium acetate buffer (pH 5.0) containing 0.25 M sucrose was used in the initial homogenization; in later preparations, this was replaced by 70% saturated ammonium sulfate containing 0.01 M sodium phosphate (pH 6.5) and 5 × 10^{-3} M benzamidine. In both cases, the enzyme extract consisted of a cloudy layer floating on the aqueous extract. A typical preparation started with 400 g of frozen tissue which was homogenized in a blender with 1200 ml of extractant, either buffered sucrose or ammonium sulfate. The homogenate was allowed to stand for 2–3 hr at 4° and then was centrifuged at 27,000*g*. The floating layer was recovered and homogenized with 1.5 volumes (about 600 ml) of 0.1 M sodium phosphate (pH 6.5) containing 5 × 10^{-3} M benzamidine. The enzyme was then solubilized by adding the nonionic detergent Brij-35 at the ratio of 1 ml of 40% aqueous detergent per 10 g fresh weight of tissue. This mixture was stirred overnight at 4° and then centrifuged at 50,000*g* in a Sorvall RC2-B centrifuge. The supernatant liquid was filtered through a bed of glass wool to remove finely divided floating precipitate and the filtrate was dialyzed against 5 × 10^{-3} M sodium phosphate (pH 6.5) containing 5 × 10^{-4} M benzamidine. The final volume was about 325 ml.

The dialyzed sample was pumped at a rate of 180 ml/hr on a 5 × 40–50 cm column of DEAE-cellulose which was precycled and equilibrated with the above phosphate–benzamidine buffer. The column was washed with 900 ml of starting buffer and eluted with a linear gradient of NaCl (see Figure 3). Fractions containing activity toward TAME were pooled, dialyzed against 5 × 10^{-3} M phosphate (pH 6.5), and lyophilized.

The lyophilized product of the DEAE-cellulose column was then subjected to gel filtration. It was first dissolved

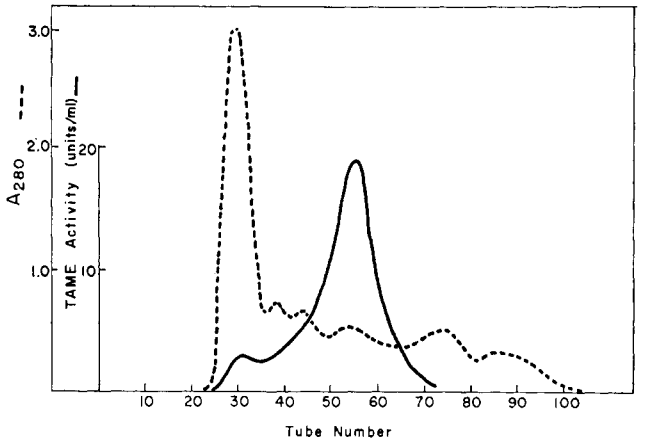

FIGURE 4: Gel filtration on Sephadex G-100. A 7 × 90 cm column was used with upward flow. A sample load of 80 mg of enzyme was applied in a volume of 100 ml. Flow rate was 40 ml/hr. Fraction volume 15 ml. Solvent was 0.001 M ammediol buffer, pH 8.8, containing 0.05 M NaCl.

TABLE I: Purification of Starfish Trypsin.

Purification Step	Number of Runs[a]	Total TAME Units	Equivalent Wt of Trypsin	Total Protein (mg)	A_{280}/A_{260}	Relative Purification (mg of trypsin/mg of protein)
Extraction of 6850 g of frozen tissue	14	191,750	1330	N.D.		
DEAE-cellulose	16	151,440	1050	75,000	0.9	0.014
G-100	21	122,800	853	7,700	0.8	0.11
DE-52 (1)	8	85,630	595	818	0.6–0.8	0.73
DE-52 (2)	4	28,460	197	160	0.8–1.0	1.2

[a] See text for details.

in 10^{-3} M ammediol buffer (pH 8.8) containing 5×10^{-2} M NaCl. The concentrate was adjusted to yield an activity toward TAME of 60 μmoles/min per ml or the equivalent of 1 mg of bovine trypsin/l. Samples of 70–100 ml of this solution were pumped through a 7 × 90 cm Sephadex G-100 column which had been equipped for upward flow and equilibrated with the same ammediol buffer (Figure 4).

The active fractions were pooled and either lyophilized or concentrated by ultrafiltration in a Diaflow cell using the UM-1 membrane. The partially purified enzyme was adjusted to a concentration of 120–240 TAME units per ml (equivalent to 2–4 mg/ml of bovine trypsin) and the sample pumped onto a 2.5 × 30–40 cm column of DE-52 microgranular cellulose equilibrated with 10^{-2} M ammediol buffer (pH 8.8). The column was washed with starting buffer and then eluted with a linear gradient from zero to 0.3 M NaCl in 10^{-3} M ammediol buffer (pH 8.8) as described in Figure 5.

Two active enzyme fractions were obtained from the DE-52 column. Fraction B was rechromatographed on a similar column equilibrated with 0.25 M NaCl in 10^{-3} M ammediol buffer. The gradient used was 0.25 M–0.31 M NaCl (Figure 6). Fraction A was not further characterized.

Because of the relatively low yield of enzyme at each step, it was advantageous to repeat each particular step, pooling the products rather than carrying one batch of tissue through the entire procedure. Hence yields and purifications are summed over a series of runs starting with 14 separate extractions and involving in the final step 4 final chromatograms on DE-52, as shown in Table I.

Properties of Trypsin (Peak B). Figure 7 shows disc gel electrophoresis of trypsin from peak B at pH 8.9. The strongly anionic character of the enzyme is evidenced by its high mobility, nearly equivalent to that of the dye marker. Although the enzyme was free of other proteins, all preparations contained a small amount of nonproteinaceous component which could not be removed by several methods tried. The impurity imparted a pale brown color on the solution and contributed to the absorbance usually attributed to nucleic acids. The contaminant was not RNA since treatment with ribonuclease followed by dialysis failed to increase the 280/260 ratio. Protamine or streptomycin, at various concentrations, failed to precipitate the 260-nm-absorbing component except at levels which also precipitated the enzyme.

The purified trypsin was stable at high pH (pH 8–10) but

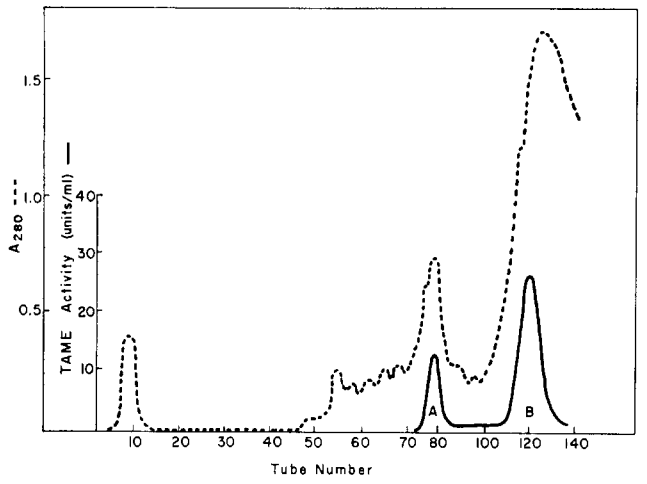

FIGURE 5: A typical chromatography on DE-52 microgranular cellulose (2.5 × 33 cm). Elution was carried out with a linear NaCl gradient from 0 to 0.3 M. Total gradient volume was 5 l. Solvent was 0.001 M ammediol buffer, pH 8.8. Sample represented 50 mg of enzyme in a volume of 200 ml. Flow rate was 100 ml/hr. Fraction volume 15 ml.

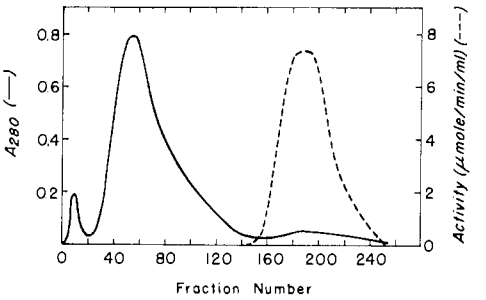

FIGURE 6: Rechromatography of trypsin B. Conditions were the same as those in Figure 5 except that a linear salt gradient from 0.25 to 0.30 M NaCl was applied. Gradient volume was 4 l. Sample contained 100 mg of enzyme in about 35 ml of solvent. Fraction volume 15 ml.

TABLE II: Amino Acid Composition of Starfish Trypsin.

Amino Acid	Residue per 10^5 g of Protein	Min Mol Wt	Residues per 24,400 g of Protein	Nearest Integer per 24,400 g of Protein	Nearest Integer × Min Mol Wt
Aspartic acid	128.6	778	31.4	31	24,118
Threonine	61.8	1,618	15.1	15	24,270
Serine	74.5	1,342	18.1	18	24,156
Glutamic acid	80.6	1,241	19.7	20	24,820
Proline	54.1	1,848	13.2	13	24,024
Glycine	117.0	885	28.5	28	23,940
Alanine	64.0	1,562	15.6	16	24,992
Cystine/2[a]	33.4	2,994	8.1	8	23,952
Valine	76.7	1,304	18.7	19	24,776
Methionine	8.8	11,364	2.1	2	22,728
Isoleucine	46.4	2,155	11.3	11	23,705
Leucine	51.9	1,927	12.7	13	25,051
Tyrosine	33.1	3,021	8.1	8	24,198
Phenylalanine	18.8	5,319	4.6	5	26,595
Tryptophan[b]	22.2		5.1	5	
Lysine	36.4	2,747	8.9	9	24,723
Histidine	16.0	6,250	3.9	4	26,000
Arginine	16.5	6,060	4.0	4	24,240
				229	24,429

[a] Determined as cysteic acid by the method of Hirs (1956). [b] Determined by the method of Barman and Koshland (1967).

unstable below pH 5. Dialysis against 0.05 M acetate buffer (pH 4.5) at 4° resulted in the irreversible loss of 80% of the activity. At pH 3.0 in citrate buffer or in dilute (10^{-3} M) HCl, the protein precipitated and the loss of activity was nearly quantitative (>95%).

Amino Acid Composition. Amino acid analyses were carried out as described in Experimental Procedure. Due to the limited amounts of purified enzyme available, only two hydrolysates (20 and 70 hr) were analyzed. Serine and threonine values were estimated by linear extrapolation to zero time. The 70-hr values were used for isoleucine and valine, and means were used for all other amino acids. The results are presented in Table II.

Specificity Toward Substrates. Starfish trypsin exhibited esterase activity toward BAEE and TAME. Activity toward the chymotryptic substrate ATEE was 2% of the TAME activity in all preparations tested. Figure 8 shows the dependence of hydrolysis rate of TAME on substrate concentration in 0.01 M Tris buffer (pH 8.1) containing 10^{-2} M $CaCl_2$. From these data, $K_{m(app)}$ was calculated as 4.4×10^{-5} M and $k_{3(app)}$ as 83.3 sec^{-1} at 25°. These values compare with $K_{m(app)}$ and $k_{3(app)}$ for bovine trypsin of 6.4×10^{-6} M and 75.0 sec^{-1}, respectively, determined at 25° in the presence of 10^{-2} M $CaCl_2$–10^{-1} M NaCl, pH 8.4 (Baines *et al.*, 1964). The effect of pH on the hydrolysis of 10^{-2} M TAME in 0.1 M KCl is illustrated in Figure 9. Rates were corrected for the

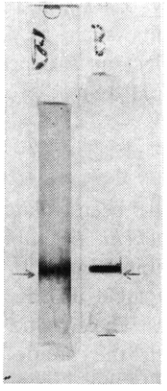

FIGURE 7: Disc gel electrophoresis of trypsin B. The right-hand gel was stained with Amido Schwarz for protein. The left-hand gel was stained for trypsin by the method described in the text. The position of the dye marker, not visible after staining, is marked by the arrows.

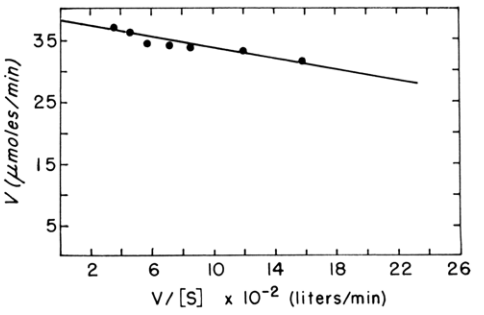

FIGURE 8: Effect of substrate concentration on reaction velocity: substrate, TAME; temperature, 25°, pH 8.1, in the presence of 10^{-2} M $CaCl_2$; substrate concentration, 10^{-3}–10^{-4} M.

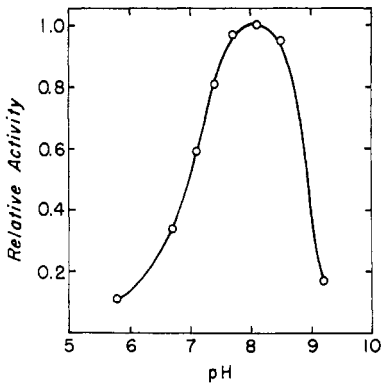

FIGURE 9: Effect of pH on TAME hydrolysis by trypsin B. For assay conditions, see the text.

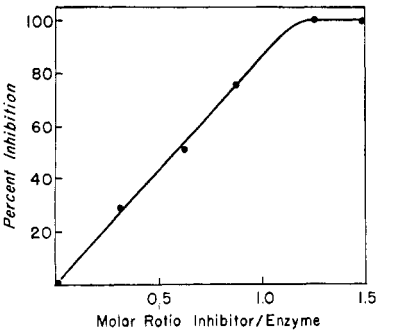

FIGURE 11: Titration of starfish trypsin with soybean trypsin inhibitor in 0.1 M Tris, pH 8.0, at 0°. The reaction was allowed to proceed at 0° for 15 min before assaying for residual activity.

spontaneous hydrolysis at each pH. On this basis, pH 8.1 was selected for all routine assays.

The molar specific activities (turnover numbers) for BAEE (10^{-2} M) were 5.78×10^3 min^{-1} and for TAME (10^{-3} M) 3.53×10^3 min^{-1}. Calculation of the turnover number of BAEE was based on the a ssumption that at chosen substrate concentrations, the enzyme was saturated with respect to substrate. Enzyme concentration was determined by active-site titration with NPGB (Chase and Shaw, 1967).

Starfish trypsin was tested for its ability to activate bovine chymotrypsinogen and compared in this reaction to bovine trypsin. The zymogen (1 mg/ml) to enzyme ratio was 100:1 based, for bovine trypsin, on the specific activity toward BAEE (57 μmoles/min per mg) and, for starfish trypsin, on the specific TAME activity (144 μmoles/min per mg). Chymotrypsin was assayed by the hydrolysis of ATEE followed in the pH-Stat. The activity which resulted from activation by bovine trypsin (zymogen:enzyme = 10:1) was taken as 100% (Figure 10).

Starfish trypsin exhibited no activity toward *N*-acetylglycine ethyl ester or toward methyl esters of L-phenylalanine or L-lysine.

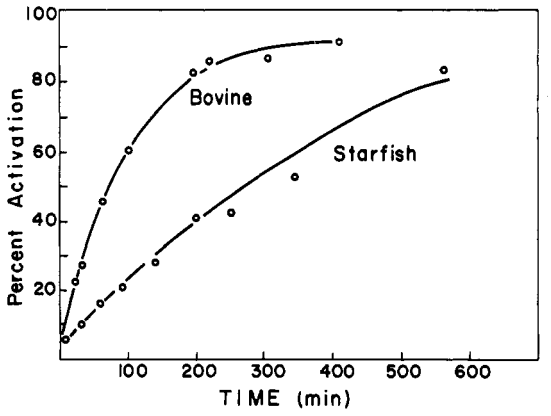

FIGURE 10: Activation of bovine chymotrypsinogen by bovine and starfish trypsins. Zymogen to enzyme ratio was 100:1; temperature, 30°, pH 8.1. Calcium concentration: 10^{-2} M.

Inhibitors. Starfish trypsin was inhibited by DFP, losing 95% of its initial activity within 60 min in the presence of 10^4 molar excess of DFP at 0° (pH 8.8). The enzyme was also inactivated by TLCK. A 5.0×10^{-5} M solution of trypsin was treated with TLCK at a final concentration of 3.0×10^{-4} M in 0.1 M Tris buffer (pH 8.0) at 0°. The progress of the reaction was approximately second order and yielded a second-order rate constant of 1.2 M^{-1} sec^{-1}, as compared with 5.6 M^{-1} sec^{-1} for bovine trypsin under similar conditions (Shaw *et al.*, 1965).

Soybean trypsin inhibitor reacted with starfish trypsin completely. Titration of the enzyme with successive additions of soybean trypsin inhibitor yielded an equivalence point very near 1.0 (Figure 11). The molarity of the enzyme was determined by reaction with NPGB (Chase and Shaw, 1967). The complex did not appear to dissociate below pH 5. With lima bean trypsin inhibitor, both the reaction stoichiometry and extent of maximum inactivation differed. Approximately a tenfold molar excess was required to achieve maximum inhibition and about 70% of the activity was lost under these conditions. Under similar conditions, turkey and chicken ovomucoid were both without effect in molar excesses up to tenfold.

Other compounds that were without inhibitory effect included EDTA, *o*-phenanthroline, and *p*-mercuribenzoate. Calcium, which stabilizes bovine trypsin, was without effect on starfish trypsin at low concentrations. At higher concentrations (approximately 0.2 M), 5–10% inhibition was observed. Reducing agents such as 2-mercaptoethanol and cysteine showed no effect at a concentration of 10^{-3} M in 0.01 ammediol buffer (pH 8.8).

Discussion

The protease which has been isolated in this work from the starfish *Evasterias trochelii* resembles enzymatically pancreatic trypsin. It is inhibited by DFP, by the active site titrant NPGB, by the affinity label TLCK as well as by soybean and lima bean inhibitors. The enzyme hydrolyzes three typical trypsin substrates, *i.e.*, the amide BAA and the esters BAEE and TAME. Unlike bovine trypsin, the starfish, enzyme is unstable at acidic pH and does not require calcium for stabilization at neutral or alkaline pH (pH 7–10). The sensitivity to pH is similar to that observed for the analogous enzymes isolated from the crayfish (Zwilling *et al.*, 1969). In

contrast to the bovine enzyme, starfish trypsin is not inhibited by turkey or chicken ovomucoid.

Although the isoelectric point of the starfish protease has not been determined, the strong binding to DEAE-cellulose suggests that it is below neutrality and probably lower than pH 5. In this regard, the enzyme resembles many if not all invertebrate trypsins and also corresponding enzymes of rat, human, and dogfish pancreas.

All attempts to demonstrate a true zymogen precursor form of the starfish trypsin were without success. Absence of a soluble zymogen was similarly noted in the white shrimp (Gates and Travis, 1969) and may be a general characteristic of invertebrate systems. Gibson and Dixon (1969b) have reported that in the sea anemone *Metridium*, treatment of prepared tissue with trypsin results in the release of additional enzyme. However, since these preparations were not soluble, such a reaction does not necessarily fall into the category of zymogen activation.[2]

The isolation and characterization of trypsin from an invertebrate presents an opportunity to inquire further into the evolutionary relationships among the phyla. At the present time, the echinodermata are one of the few classes of invertebrates for which there exists a postulated evolutionary relationship to the chordates. Evidence for this kinship derives from the comparative anatomy of the dipleurula larvae of the echinoderms and the immature forms of the hemichordates (Romer, 1967). Detailed study of related proteins now makes possible valid comparison at the molecular level of organization. While the present work is too limited to establish any detailed relationship, it nevertheless has demonstrated that a protein of similar size, composition, and function to bovine trypsin exists in the starfish. In addition, it has recently been shown (Arnon and Neurath, 1969) that antibodies toward bovine trypsin cross-react with starfish trypsin and *vice versa*. These findings are suggestive of homology at the level of amino acid sequence, and support for this probability (Arnon and Neurath, 1969; Neurath *et al.*, 1967; Winter *et al.*, 1968; Boyden, 1969) is found in the amino acid composition of starfish trypsin which is not greatly different from that of other species (Walsh, 1970). The only rigorous probe of homology is sequence analysis and this requires larger amounts of pure enzyme than are yet available.

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[2] While this manuscript was in preparation, reports by Camacho (1970) and Camacho *et al.* (1970) of a zymogen of a trypsin-like protease in the starfish *Demasterias* came to our attention. However, as in the case of the chymotrypsin-like protease of the sea anemone (Gibson and Dixon, 1969a,b), activation wasobs erved in whole tissue and could, therefore, be the result of digestion of an inhibitor or of solubilization of enzyme. We are obliged to Dr. Kitto for making the manuscript of the paper by Camacho *et al.* (1970) available to us prior to publication.

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