Isolation and Comparative Properties of Shrimp Trypsin*

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ABSTRACT: An enzyme with proteolytic activity was isolated from the digestive gland (hepatopancreas) of the white shrimp (*Penaeus setiferus*). A trypsin-like specificity was suggested on the basis of assays conducted on synthetic substrates.

The enzyme was isolated by homogenizing the digestive gland with cold acetone and subjecting the resulting powder to several purification steps consisting of salt fractionation, Sephadex G-75 chromatography, and ion-exchange chromatography on DEAE-Sephadex A-50. The preparation was judged to be a single, homogeneous protein by disc electrophoresis and by ultracentrifugation. Characterization of the purified enzyme revealed a number of properties similar to mammalian trypsin. These properties included a molecular weight of 24,000 and inhibition by diisopropylphosphorofluoridate, 1-chloro-3-tosylamido-7-amino-2-heptanone, and soybean trypsin inhibitor, but not by L-tosylamido-2-phenylethylchloromethyl ketone. In contrast, shrimp trypsin has an acidic isoelectric point, no requirement for Ca^{2+} for stability of the enzyme, resistance to autodigestion, and irreversible inactivation below pH 5.0. In addition, the amino acid composition of the molecule suggests some differences in total structure and there seems to be no indication that the enzyme has a zymogen form. Significantly, no chymotrypsin esterase activity is present in hepatopancreas extracts, indicative of a different protein digestion pattern in this species.

Most of our present day knowledge of proteolytic enzymes comes from a few well-characterized proteins from the vertebrates, especially mammals. Very few invertebrate digestive enzymes have been purified; characterization has been based primarily on pH optima under crude conditions of extraction of alimentary tissues (Barrington, 1962; Huggins and Munday, 1968).

A survey study of tryptic digestive enzymes in various species of crustacea has revealed that a trypsin-like proteinase is widely distributed in this class (DeVillez and Buschlen, 1967). More specifically, a number of reports have been published with regard to proteolytic activity in crayfish (DeVillez, 1965; DeVillez and Johnson, 1968). Recently, Pfleiderer *et al.* (1967) purified a trypsin-like proteinase from cardia fluid of the crayfish, *Astacus fluviatilis*, and this group has subsequently shown it to be similar in enzyme specificity to bovine trypsin (Zwilling *et al.*, 1969).

This paper reports the purification to homogeneity of a trypsin-like enzyme from the hepatopancreas (digestive gland) of the white shrimp (*Penaeus setiferus*) which hydrol zes Bz-L-ArgEt and casein and the characterization of its physical and chemical properties as a basis of comparison with the vertebrate trypsins.

Materials

Shrimp heads were obtained from a commercial fishery on the coast of Georgia, quick frozen with Dry Ice, and stored in a freezer. Bz-L-ArgEt and *N*-Ac-L-TyrEt were purchased from the Mann Research Laboratories, 1-chloro-3-tosylamido-7-amino-2-heptanone from Calbiochem, DFP from the Aldrich Chemical Co., and L-tosylamido-2-phenylethyl chloromethyl ketone from the Sigma Chemical Co. Bovine α-chymotrypsin, bovine trypsin, and SBTI[1] were purchased from Worthington Biochemical Corp. Sephadex G-75 and DEAE-Sephadex A-50 were products of Pharmacia Fine Chemicals, Inc.

Methods

Enzyme Assays. Tryptic and chymotryptic activities were measured on Bz-L-ArgEt and *N*-Ac-L-TyrEt, respectively, using the spectrophotometric procedure of Schwert and Takenaka (1955). Assays were performed in 0.1 M sodium borate buffer (pH 8.0) in the absence of $CaCl_2$. A unit of tryptic activity was arbitrarily defined as an absorbance change of 1 optical density unit/min and specific activity as the number of units per optical density unit at 280 mμ. Proteolytic activity was measured by the method of Kunitz (Laskowski, 1955) using casein as the substrate. Protein concentration was determined by the method of Warburg and Christian (1942). All optical density measurements were made on a Zeiss PMQ II spectrophotometer.

Polyacrylamide Electrophoresis. Disc electrophoresis in polyacrylamide gels was performed as described by Davis (1964) using both a 7.5 and a 15% acrylamide gel.

Ultracentrifugation. A Beckman Spinco Model E ultracentrifuge was used to determine the sedimentation coefficient, $s_{20,w}$, by sedimentation velocity (Schachman, 1959) and the molecular weight by the sedimentation equilibrium method of Yphantis (1964). The partial specific volume of the enzyme was calculated from the amino acid composition by the method of McMeekin and Marshall (1952).

Amino Acid Analyses. The amino acid composition of shrimp trypsin was determined with a Beckman Model 120C amino acid analyzer according to the chromatographic

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[1] Abbreviation used is: SBTI, soybean trypsin inhibitor.

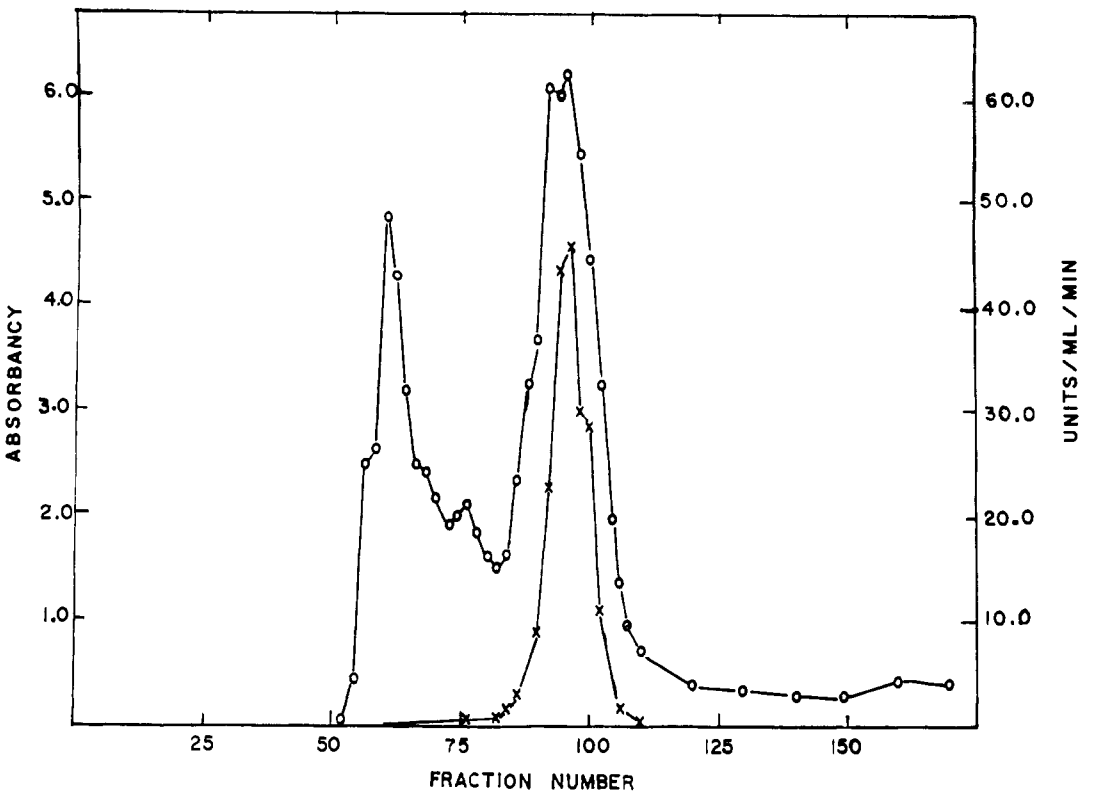

FIGURE 1: Sephadex G-75 chromatography of a 13-ml sample (~50 mg/ml) of 0.4–0.8 saturation $(NH_4)_2SO_4$ salt fraction of crude extract. A column of Sephadex G-75 (3.8 × 66 cm) was equilibrated with 0.01 M Tris-HCl–0.4 M NaCl buffer (pH 8.0). The flow rate was 17 ml/hr and 4.6-ml fractions were collected. Curves are designated as follows: optical density at 280 mμ (O—O), left ordinate; activity against Bz-L-ArgEt (×—×), right ordinate.

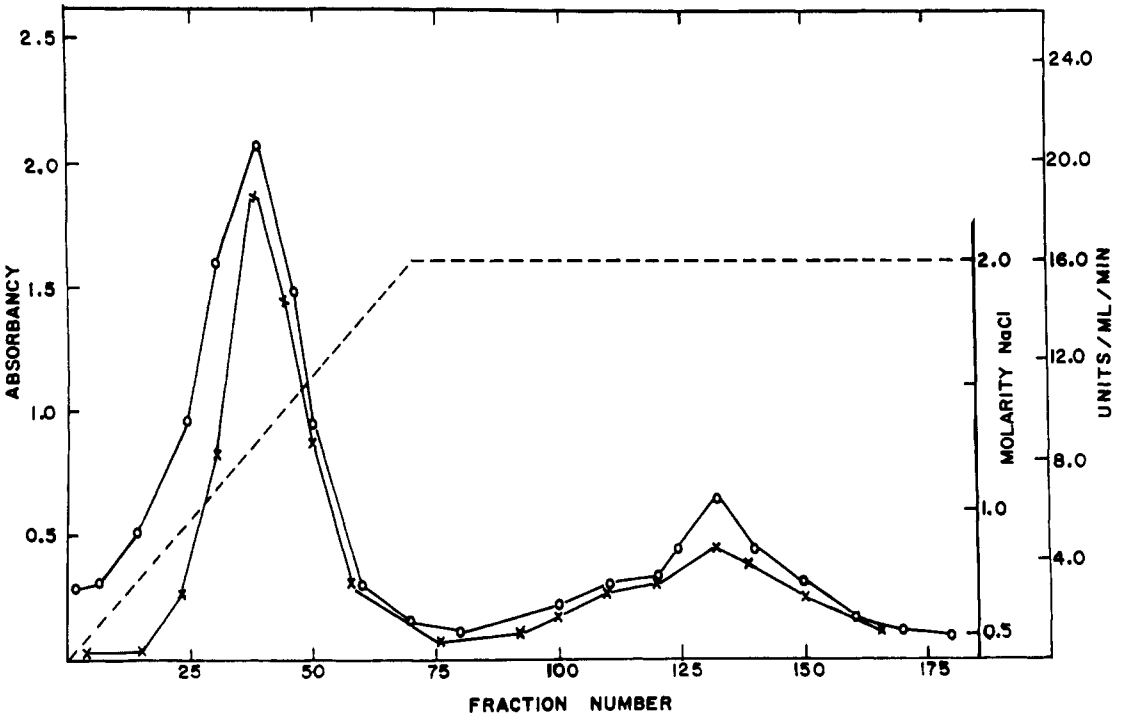

FIGURE 2: DEAE-Sephadex A-50 chromatography of active fraction obtained from Sephadex G-75 column. The column (1.8 × 14 cm) was equilibrated with 0.01 M Tris-HCl–0.4 M NaCl buffer (pH 8.0) and a linear salt gradient of 0.4–2 M NaCl (200 ml) was applied (as shown by dashed line). The flow rate was 30 ml/hr and 3-ml fractions were collected. Curves are designated as follows: optical density at 280 mμ (O—O), left ordinate; activity against Bz-L-ArgEt (×—×), right ordinate.

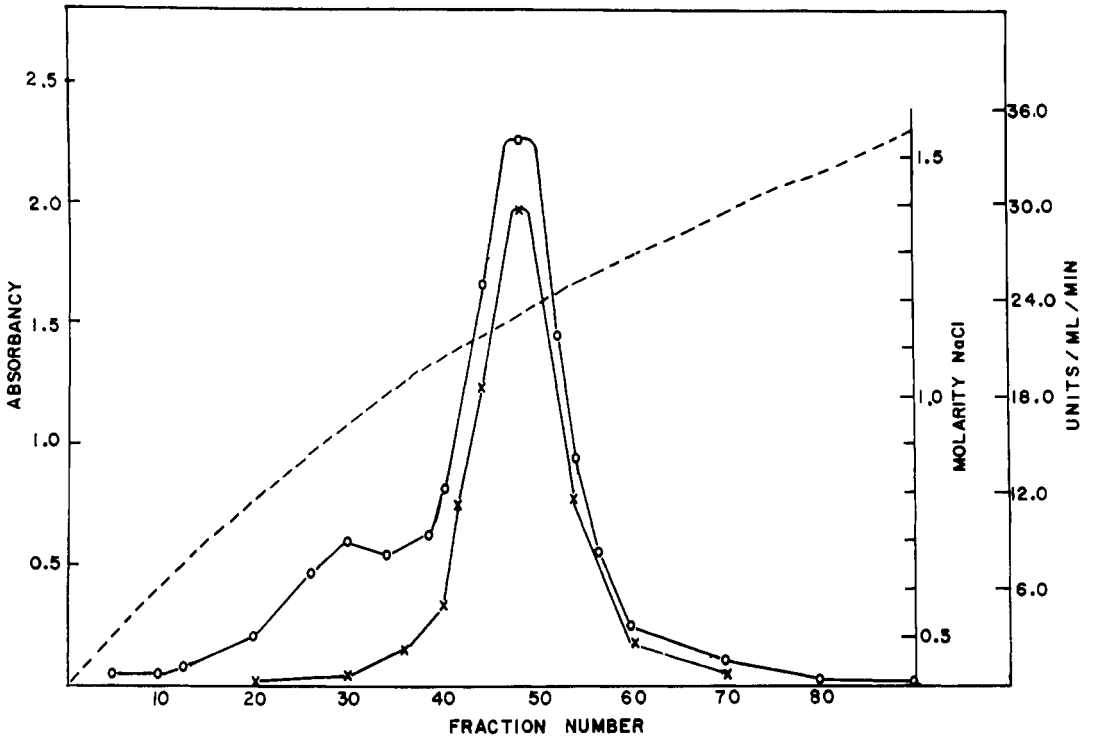

FIGURE 3: Rechromatography of major peak (from Figure 2) on DEAE-Sephadex A-50. The column (1.8 × 14 cm) was equilibrated with 0.01 M Tris-HCl–0.4 M NaCl (pH 8.0) and an exponential gradient (shown by dashed line) applied through a 220-ml mixing chamber. The limiting buffer was 0.01 M Tris-HCl–2.0 M NaCl (pH 8.0). The flow rate was 20 ml/hr and 3-ml fractions were collected. Curves are designated as follows: optical density at 280 mμ (O—O), left ordinate; activity against Bz-L-ArgEt (×—×), right ordinate.

procedure of Spackman *et al.* (1958), following hydrolysis with 6 N HCl in evacuated tubes. Hydrolysis times of 6, 12, 22, 76, and 96 hr were used in the standard determination. Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, from performic acid oxidized samples (Hirs, 1956). Tryptophan was determined by the Spies and Chambers (1949) colorimetric method.

Results

Enzyme Purification. STEP 1. PREPARATION OF EXTRACT. Frozen shrimp heads were partially thawed and longitudinally sliced into halves. This facilitated the simple removal of the digestive glands which were scooped out and homogenized in a Waring Blendor with cold acetone (−15°). The acetone powder formed was stored in the freezer until used. Approximately 10 g of acetone powder were obtained from the digestive glands of 200–300 shrimp.

The acetone powder obtained above was stirred with 250 ml of 0.1 M sodium borate buffer (pH 8.0) overnight at 4°. The insoluble material was separated from the extract by centrifuging at 7970*g* for 15 min and reextracted with another 250-ml portion of borate buffer. After centrifugation the extracts were pooled and the precipitate was discarded. The total volume of extract was 505 ml.

STEP 2. AMMONIUM SULFATE PRECIPITATION. Solid ammonium sulfate was slowly added to the extract obtained in step 1 with stirring to 40% saturation and stirring continued for 30 min after final addition of salt. The small amount of precipitate which formed was removed by centrifugation (10,400*g* for 30 min) and discarded. The supernatant was brought to 80% saturation by the further addition of solid ammonium sulfate. The precipitate was collected by centrifugation as above and dissolved in the least possible amount (23 ml) of 0.01 M Tris-HCl buffer (pH 8.0). At this stage it was imperative to either freeze the solution or apply it immediately to a Sephadex G-75 column as there was a loss of activity of the concentrated material upon standing which was probably attributable to autolysis of the enzyme.

STEP 3. SEPHADEX G-75 COLUMN CHROMATOGRAPHY. The enzyme solution was split into two samples and alternately applied to a column of Sephadex G-75 equilibrated with 0.01 M Tris-HCl–0.4 M NaCl buffer (pH 8.0). The Sephadex G-75 elution profile of a 13-ml sample (~50 mg/ml) is shown in Figure 1. The active material was eluted coincidentally with a green fraction.

STEP 4. DEAE-SEPHADEX A-50 COLUMN CHROMATOGRAPHY. The most active fractions from the G-75 columns were pooled (304 ml) and applied directly to a DEAE-Sephadex A-50 column equilibrated with a starting buffer made up of 0.01 M Tris-HCl–0.4 M NaCl (pH 8.0). After passage of an inactive protein fraction through the column the active enzyme was eluted by a linear salt gradient (200 ml) from 0.4 to 2 M NaCl as shown in Figure 2. The esterase active material eluted in two peaks, the major one at approximately 0.75 M NaCl, and a minor peak at 2 M NaCl. The major component was pooled, dialyzed *vs.* the starting buffer, and applied to a second DEAE-Sephadex column. The active material was eluted by applying an exponential gradient from 0.4 to 2 M NaCl. The elution profile from the second DEAE-Sephadex column is shown in Figure 3. The specific activity of the

TABLE 1: Purification of Shrimp Trypsin.

Step	Vol. (ml)	Total Protein (OD_{280})	Total Act. (units)	Sp Act. (units/ OD_{280})	Recov (%)	Purifcn
Crude extract	505	8200	3940	0.48	100	1
$(NH_4)_2SO_4$, 40–80%	23	1752	2392	1.36	60.7	2.8
Sephadex G-75 column	304	623	1888	3.0	47.9	6.3
1st DEAE-Sephadex A-50 column	144	164	994	6.1	25.2	12.7
2nd DEAE-Sephadex A-50 column	66	81	845	10.4	21.4	21.6

enzyme was increased from 6.2 to 10.4 by the use of this procedure.

The material was concentrated by either ultrafiltration or by elution from a short DEAE-Sephadex column using 2 M NaCl as eluent. All further experiments to be described were performed using this preparation.

Table I gives a summary of the purification scheme followed.

Criteria of Homogeneity and Molecular Weight Studies. Because of the strong adsorption properties of the trypsin-like enzyme on DEAE-Sephadex it seemed logical to assume that the protein was of a very acidic nature. Disc electrophoresis (50–200 μg) of the enzyme preparation in a 7.5% acrylamide system confirmed this, the protein (single band) tending to migrate with the tracking dye. For this reason a 15% gel was utilized in order to ensure that no impurities would be undetected. The results, shown in Figure 4, indicate the presence of a single anionic protein band. In a very acidic system (pH 2.3), the protein precipitated on the gel could not be made to move (see below).

The purified enzyme was homogeneous in the ultracentrifuge at pH 8 in 0.01 M Tris-HCl–0.1 M NaCl at a concentration of 7 mg/ml. A sedimentation constant ($s_{20,w}$) of 2.4 was calculated from the schlieren peaks in Figure 5. By comparison, the $s_{20,w}$ value for bovine trypsin extrapolated to zero concentration is 2.5 (Desnuelle, 1960).

Sedimentation equilibrium experiments were performed on material inhibited with DFP (see below). The results of the boundary depletion equilibrium run is shown in Figure 6. There was no significant deviation from linearity over the

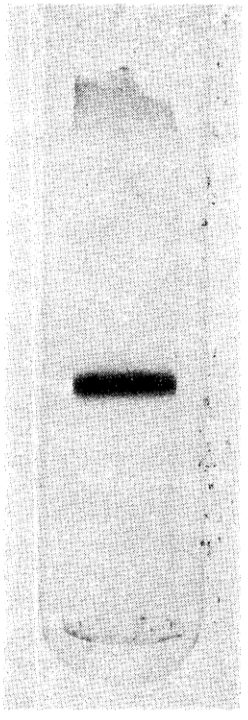

FIGURE 4: Disc electrophoresis of shrimp trypsin (100 μg). Running time 2 hr at 1.5 mA/tube at pH 9.5; gel is 15% acrylamide. Cathode is at the top and anode is at the bottom. Direction of migration is from top to bottom. Patterns were stained with 1% Amido Schwartz in 7.5% acetic acid.

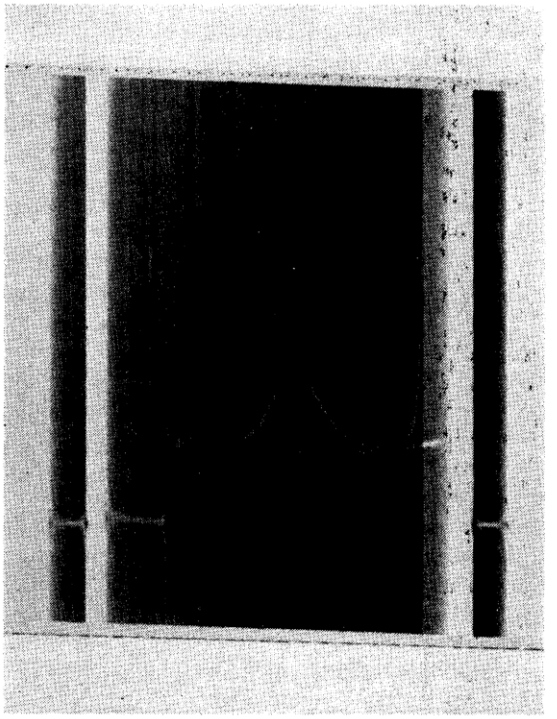

FIGURE 5: Sedimentation velocity pattern of shrimp trypsin (7 mg/ml) in 0.01 M Tris-HCl–0.1 M NaCl (pH 8) taken 152 min after obtaining maximum speed of 60,000 rpm; bar angle 60°. Sedimentation is from left to right.

TABLE II: Amino Acid Composition of Shrimp Trypsin.

Amino Acid	Found[a]	Nearest Integer
Lysine	4.6 ± 0.5	5
Histidine	4.9 ± 0.5	5
Arginine	3.3 ± 0.4	3
Aspartic acid	29.5 ± 0.3	30
Threonine	10.0[b]	10
Serine	23.6[b]	24
Glutamic acid	23.7 ± 0.4	24
Proline	11.3 ± 0.5	11
Glycine	27.5 ± 0.5	28
Alanine	16.2 ± 0.3	16
Half-cystine	7.3 ± 0.5[c]	8
Valine	18.0[d]	18
Methionine	1.7 ± 0.1[c]	2
Isoleucine	13.6[d]	14
Leucine	9.9 ± 0.1	10
Tyrosine	9.7 ± 0.3	10
Phenylalanine	6.4 ± 0.3	6
Tryptophan	3.0 ± 0.5[e]	3
	Total number of residues	227

[a] Data are expressed as amino acid residues per molecule, assuming 10 and 16 residues of leucine and alanine, respectively. [b] Threonine and serine values are based on a first-order approximation to zero time. [c] Averages of 22-hr hydrolysates of oxidized samples. [d] Isoleucine and valine values are from 96-hr hydrolysates. [e] Determined colorimetrically.

length of the plot, indicative of the homogeneity of the enzyme preparation. Using the slope from Figure 6 and a calculated partial specific volume of 0.714 from the amino acid composition, a molecular weight of 24,000 was obtained.

Amino Acid Composition. The results of the amino acid analysis of shrimp trypsin are shown in Table II. The number of residues have been based on values of 10 and 16 for leucine and alanine, respectively. From the average and extrapolated values rounded to the nearest whole numbers the molecular weight was calculated to be 23,881 which is consistent with the physically estimated value of 24,000. In Table III a comparison of this composition is made with that of several other trypsin-like enzymes whose compositions have been determined.

Effect of pH and Temperature on Enzyme Activity. Shrimp trypsin was assayed with Bz-L-ArgEt at various pH values over a range of 3.5–9.5. The buffers used were the following: 0.05 M sodium acetate (pH 3.5–6.0), 0.05 M sodium phosphate (pH 6.0–8.0), and 0.05 M Tris-HCl (pH 7.8–9.5). The optimum pH was found to be rather broad, occurring over the range of pH 7.0–9.5.

The effect of pH on the stability of the enzyme was determined by incubation of samples of the extract (80 μg/ml) at several pH values from 3.3 to 10.4 at 4°. The samples were incubated for periods of 2 hr to 38 days and then assayed at pH 8 with Bz-L-ArgEt. The results of the experiment are shown in Figure 7. The enzyme was found to maintain

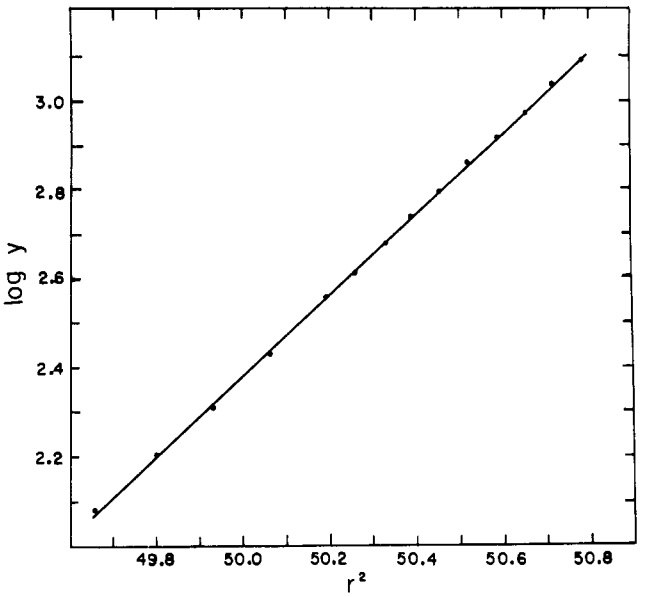

FIGURE 6: Plot of log y *vs.* r^2 from data obtained from the sedimentation equilibrium run of a solution of DIP–shrimp trypsin (0.35 mg/ml) in 0.01 M sodium phosphate–0.1 M NaCl (pH 6.6) at 4°, with a rotor velocity of 36,000 rpm.

maximum activity for as long as a month if the pH was 6.2 or above. However, below this pH, rapid, irreversible enzyme inactivation occurred. It should be noted that in early experiments, 0.05 M $CaCl_2$ was routinely present in our assay mixtures. It was subsequently found that, unlike bovine trypsin, there was no requirement for this ion for enzyme stability or activity.

The effect of temperature on the enzyme activity of shrimp trypsin was determined by the caseinolytic method of Kunitz (Laskowski, 1955). The enzyme was found to have a maximum proteolytic activity at 49°. In separate experiments

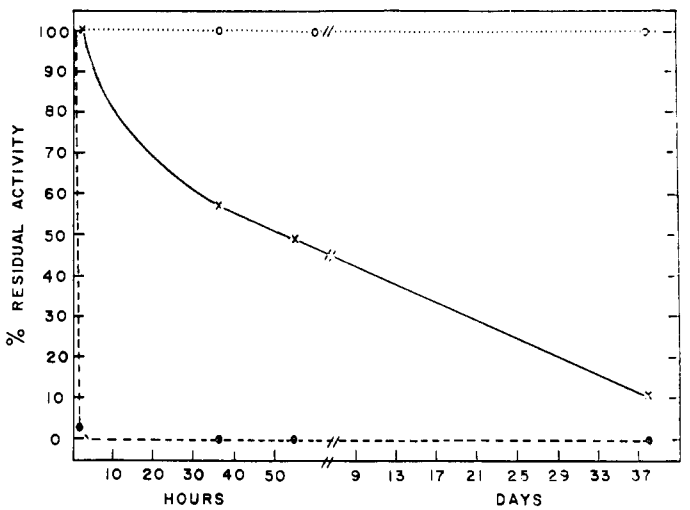

FIGURE 7: pH stability of shrimp trypsin. Samples of the enzyme (8×10^{-2} mg/ml) were incubated in universal buffer at various pH values at 4°. Residual activity was calculated from the esterolytic activity at pH 8.0. (O···O) pH 6.24–10.4, (X—X) pH 4.85, and (●---●) pH 3.32.

TABLE III: Amino Acid Composition of Trypsin-Like Enzymes.

Amino Acid	Residues/Molecule					
	Shrimp[a]	Coco-onase[b]	Human[c]	Beef[d]	Pork[e]	Sheep[f]
Lysine	5	13	11	14	10	12
Histidine	5	4	3	3	4	3
Arginine	3	6	6	2	4	4
Aspartic acid	30	26	21	22	18	20
Threonine	10	16	10	10	11	15
Serine	24	23	24	33	24	26
Glutamic acid	24	15	21	14	17	14
Proline	11	9	9	9	10	9
Glycine	28	22	20	25	26	19
Alanine	16	16	13	14	16	17
Half-cystine	8	4	8	12	12	12
Valine	18	20	16	17	16	17
Methionine	2	1-2	1	2	2	2
Isoleucine	14	12	12	15	15	10
Leucine	10	12	12	14	16	14
Tyrosine	10	9	7	10	8	6
Phenyl-alanine	6	5	4	3	4	5
Tryptophan	3	3	3	4	6	

[a] From Table II. [c] Kafatos *et al.* (1967a). [c] Travis and Roberts (1969). [d] Walsh and Neurath (1964). [e] Travis and Liener (1965). [f] Travis (1968).

using esterolytic activity as the assay, it was possible to incubate the extract at 37° at concentrations of 1 mg/ml for at least 24 hr without loss of activity.

Effect of Inhibitors on Enzyme Activity. It is a well-known fact that 1-chloro-3-tosylamido-7-amino-2-heptanone (Shaw *et al.*, 1965) and DFP (Desnuelle, 1960) are potent inhibitors of mammalian trypsins reacting with histidine and serine residues, respectively. In order to determine whether this relationship could be extended to the shrimp enzyme, the effect of these two compounds as well as SBTI and the chymotrypsin inhibitor, L-tosylamido-2-phenylethyl chloromethyl ketone (Schoellmann and Shaw, 1963), were determined.

In the presence of a 10-fold molar excess of DFP or a 1000-fold molar excess of 1-chloro-3-tosylamido-7-amino-2-heptanone at 22° in 0.05 M Tris-HCl (pH 7.5), rapid inhibition of esterase activity occurred, being essentially complete within 20 min. L-Tosylamido-2-phenylethyl chloromethyl ketone, as expected, was without effect at 100-fold molar excess. Significantly, SBTI at an inhibitor–enzyme molar ratio of 1.5:1 completely inactivated the enzyme within 15 min.

Discussion

An interesting aspect of the purification of the shrimp trypsin was the elution of two distinct, active peaks from the first DEAE-Sephadex column. The material which eluted by addition of 2 M NaCl had the same migration rate in disc electrophoresis and a comparable amino acid composition as the major component which eluted at a lower salt concentration. However, there must be some physicochemical difference between the two active fractions since rechromatography of the 2 M peak on a second DEAE-Sephadex column did not change the elution profile, eliminating the possibility of an artifact.[2] These results differ somewhat from those of Pfleiderer *et al.* (1969) who found that in the crayfish a single peak of activity from the DEAE-Sephadex A-50 column gave two distinct, active bands on disc electrophoresis. It is difficult to interpret their results; however, it should be noted that the patterns which they showed were prepared by electrophoresis of lyophilized material. Lyophilization of our preparation caused a 20% decrease in the specific activity of the enzyme, and could cause artifacts to be formed.

Interestingly enough, no evidence of activation of our enzyme could be found in the crude extract nor in the purified preparation. This would seem to indicate that the shrimp enzyme does not have a zymogen form as are found in mammalian trypsins and also in the invertebrate enzymes isolated from the silk moth (Kafatos *et al.*, 1967a) and the sea pansy (Coan and Travis, 1969). However, because of the inherently high activity of the original extract the possibility still exists that the enzyme had had sufficient time to activate completely before assays were made. If indeed the shrimp trypsin is not secreted in zymogen form, the greater stability of the shrimp trypsin is of a certain adaptive value.

The most striking observation noted during purification of the shrimp protease was the complete absence of chymotrypsin-like esterase activity. Whether this reflects the coprophagous nature of the shrimp or whether other methods of protein digestion are, indeed, available is not known at this time. However, further digestion by catheptic-like enzymes is possible. A low molecular weight (11,000) protease has been found in the digestive juice of crayfish by both Pfleiderer *et al.* (1967) and DeVillez and Lau (1969). The latter has found his enzyme to have a broad endopeptidase specificity with a preference for leucine peptide bonds. Enzymes of this type may also exist in the shrimp.

Trypsin-like enzymes are generally characterized by a molecular weight in the range of 25,000, a high isoelectric point, unusual acid stability, and by inhibition by DFP (Desnuelle, 1960) and 1-chloro-3-tosylamido-7-amino-2-heptanone (Shaw *et al.*, 1965). To this can also be added, in many examples, a requirement for Ca^{2+} for enzyme stability (Desnuelle, 1960). In shrimp trypsin, one encounters an enzyme with the proper structural characteristics, but with physical parameters vastly different from its mammalian counterparts. Thus, the enzyme is inhibited by DFP, 1-chloro-3-tosylamido-7-amino-2-heptanone, and SBTI (all inhibitors of bovine trypsin) and has a molecular weight near 25,000. In contrast, shrimp trypsin is acidic in nature, unstable at low pH, but exceedingly stable at alkaline pH in the absence of Ca^{2+}, even at moderately high temperatures.

A comparison of the amino acid content of several trypsin-like enzymes from different species is shown in Table III.

[2] B. J. Gates and J. Travis, unpublished results.

The most prominent difference between the amino acid composition of our enzyme and others isolated is in the content of charged amino acids. The shrimp enzyme has a greater number of potentially acidic residues and fewer basic residues than the other proteases listed, although such an anionic character has also been reported for trypsin-like enzymes in several other species (Neurath *et al.*, 1967). The greater number of acidic residues give the molecule a net negative charge and would tend to decrease its solubility at acidic pH values. This could also result in an irreversible change in conformation causing inactivation of the enzyme below pH 5.0. The shrimp enzyme has a total of 8 arginine and lysine residues per molecule in comparison to 4–19 residues for the other proteinases listed in Table III. The greater stability of the shrimp enzyme could be attributed to the fewer number of trypsin-labile bonds; this is based on the assumption that the enzyme from the shrimp has a specificity for bonds involving the carboxyl group of lysine and arginine residues.

Both shrimp and human trypsin have been found to have 8 half-cystine residues whereas the other mammalian trypsins have 12 and cocoonase, 4, only 2 of which appear to be disulfide linked (Kafatos *et al.*, 1967a). A correlation was made by Travis and Roberts (1969) with regard to human trypsin, suggesting that a decrease in the number of disulfide bonds was directly responsible for both difference in tertiary structure and lack of reactivity toward macromolecular inhibitors such as SBTI. The finding that the shrimp enzyme is inhibited by SBTI, as is cocoonase (Kafatos *et al.*, 1967b), whereas human and sea pansy trypsins are not (Coan and Travis, 1969), is indicative of the importance of other types of bonds in such interactions. Although there is no apparent theme attached to the inhibitory prowess of SBTI, it is most important to note that the very fact that inhibition does occur with both the invertebrate and vertebrate trypsins would suggest an overall similarity in a binding site for SBTI at both levels and, therefore, a common evolutionary origin for trypsin-like proteolytic enzymes.

Speculation by Mitchell (1969) that a lower isoelectric point might explain the difference in specificity in chlostridiopeptidase B. and trypsin is also unwarranted, the shrimp isoelectric point being much lower than that of the bacterial enzyme.

Although we have not studied the specificity in any detail, we feel safe to call the enzyme a trypsin because of its ability to hydrolyze Bz-L-ArgEt and the inhibitory effect of 1-chloro-3-tosylamido-7-amino-2-heptanone and DFP. Zwilling *et al.* (1969) in their characterization of a similar enzyme from the crayfish have examined its enzymatic properties and have found it specific for arginine and lysine peptide bonds. If the two enzymes did come from a common ancestor, shrimp must be on a branch point because it has adapted to the absence of chymotrypsin.

Investigations are continuing as to the mechanism of this and other digestive enzymes from the shrimp hepatopancreas.

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