

Dermasterias imbricata Trypsin 1: An Enzyme Which Rapidly Hydrolyzes the Reactive-Site Peptide Bonds of Protein Trypsin Inhibitors[†]

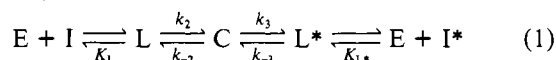
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ABSTRACT: We had surmised that some serine proteinases, which do not appear to be inhibited by protein-proteinase inhibitors, may rapidly hydrolyze the reactive-site peptide bonds of such inhibitors. Therefore, following the report by Camacho and co-workers [Camacho, Z., Brown, J. R., & Kitto, B. (1970) *J. Biol. Chem.* 245, 3964] that a trypsinlike enzyme from the starfish *Dermasterias imbricata* was not inhibited by soybean trypsin inhibitor (Kunitz) (STI), we investigated the trypsin system in this starfish. The major component of this system, trypsin 2, is initially present in the tissue homogenate as a complex with an endogenous inhibitor. Trypsin 2 is similar to bovine trypsin, and to trypsinlike enzymes from other species of starfish, in that it is strongly

inhibited by STI and by several other trypsin inhibitors. The minor component, trypsin 1, differs from trypsin 2 in that it forms rapidly dissociable complexes ($K_1 = 3 \times 10^{-7}$ and 1×10^{-9} M, respectively) with STI and with bovine pancreatic trypsin inhibitor (Kunitz) (PTI), whereas complexes of trypsin 2 with these inhibitors are very long lived. *D. imbricata* trypsin 1 hydrolyzes the reactive-site peptide bonds of these inhibitors approximately 10^5 (STI) and 10^6 (PTI) times more rapidly than does bovine trypsin. The NH_2 -terminal sequences of *D. imbricata* trypsins 1 and 2 show that the two enzymes are members of the class of chymotrypsin-related serine proteinases and that they are products of two distinct genes.

Studies of protein inhibitors of serine proteinases have shown that a very large number (but not all) of these inhibitors interact with their target proteinases according to the general mechanism first elucidated in our laboratory for the interaction of STI¹ with bovine trypsin [for reviews see Laskowski & Sealock (1971) and Finkenshtadt et al. (1971)]. Each inhibitor molecule contains at least one specific peptide bond, called the reactive site, which serves as an especially specific substrate for the target proteinase. Incubation of the inhibitor with catalytic quantities of the target enzyme results in specific hydrolysis of the reactive-site peptide bond, leading to the establishment of an equilibrium between virgin (reactive-site peptide bond intact) inhibitor and modified (reactive-site peptide bond hydrolyzed) inhibitor (Mattis & Laskowski, 1973). If equimolar quantities of enzyme and inhibitor are combined, a stable complex, C, is produced. This complex is clearly one of the intermediates in the hydrolysis of the reactive-site peptide bond by the target enzyme. In two cases, X-ray crystallographic studies suggest that, in the complex, the carbon atom of the reactive-site peptide bond interacts with the γ -oxygen of the catalytic serine residue of the enzyme to form a tetrahedrally distorted adduct (Rühlmann et al., 1973; Huber et al., 1974; Sweet et al., 1974).

The minimal mechanism consistent with what is presently known about serine proteinase-inhibitor interactions (Luthy et al., 1973) is



where E is the proteinase, I and I* are the virgin and modified inhibitor, respectively, and C is the stable complex. L and L* are loose, noncovalent complexes of the enzymes with either virgin or modified inhibitor. L and L* are in very rapid equilibrium with E + I and E + I*, respectively; thus, the

equilibrium constants K_L and K_{L^*} , rather than the individual rate constants, are indicated.

Demonstration of the specific hydrolysis of the reactive-site peptide bond has proven quite difficult for some inhibitors. This is due to the inordinately long times required to produce significant amounts of modified inhibitor when commonly available serine proteinases are used to catalyze the reaction (Tschesche & Kupfer, 1976). Our search for enzymes which could rapidly hydrolyze these resistant reactive sites was initiated by studies in our laboratory on the interaction of human trypsin with STI (Laskowski et al., 1974). Previous reports (Feeney et al., 1969; Travis & Roberts, 1969; Coan & Travis, 1971; Figarella et al., 1975) had found only weak and poorly reproducible inhibition of human cationic trypsin by STI. We found that this weak inhibition was primarily due to a high dissociation rate constant ($k_d = k_{-2} + k_3$ in eq 1) for the human cationic trypsin-STI complex and that both k_{-2} (dissociation to E + I) and k_3 (dissociation to E + I*) were higher for human trypsin than for bovine trypsin. In particular, for the bovine β -trypsin-STI complex, k_3 at pH 8 is $2.5 \times 10^{-6} \text{ s}^{-1}$ (Mattis, 1974), while for the human trypsin-STI complex, k_3 is $2.8 \times 10^{-4} \text{ s}^{-1}$ (Laskowski et al., 1974; Wilson, Sealock, and Laskowski, unpublished experiments); i.e., human trypsin hydrolyzes the reactive site of STI ~100 times faster than does bovine β -trypsin. We therefore surmised that in some (but not all) cases trypsinlike enzymes which are only weakly inhibited by STI might hydrolyze the reactive-site peptide bond especially rapidly. Since the rate of hydrolysis by human trypsin is still quite slow, we started to search for other enzymes which could hydrolyze the reactive-site peptide bond even faster. There were two motivations for such a search. As shown in the following paper (Estell et al., 1980), such an

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¹ Abbreviations used: STI, soybean trypsin inhibitor (Kunitz); STI*, modified STI in which the Arg⁶³-Ile reactive-site peptide bond has been hydrolyzed; PTI, bovine pancreatic trypsin inhibitor (Kunitz); TosArgOMe, *p*-tosyl-L-arginine methyl ester hydrochloride; BzTyrOEt, *N*-benzoyl-L-tyrosine ethyl ester; BAPA, α -*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; GdnBzMum, 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride; Tris, tris(hydroxymethyl)aminomethane; NaDodSO₄, sodium dodecyl sulfate.

enzyme is quite useful in identifying and studying reactive sites of inhibitors where hydrolysis by more common enzymes is quite slow. In addition, finding that the same reactive site can serve as an excellent substrate for one enzyme while it serves as a powerful inhibitor for another, closely homologous, enzyme raises important questions about the nature of the molecular interactions responsible for inhibition.

The report that a trypsinlike enzyme from the starfish *Dermasterias imbricata* was not inhibited by STI (Camacho et al., 1970) led us to investigate this enzyme as one which might rapidly hydrolyze the reactive-site peptide bonds of trypsin inhibitors. Dr. G. B. Kitto kindly sent us this enzyme, as a gift, and preliminary tests indicated that this enzyme hydrolyzed the reactive-site peptide bond of STI even more rapidly than did human trypsin. As the amount of enzyme available to us was too small for further study, we began our own program of isolation. We wish to report here the results of this isolation and some properties of the enzymes so isolated.

Experimental Procedures

Materials

Frozen pyloric caeca (from the starfish *D. imbricata* and *Pisaster ochraceus*) were obtained from Pacific Biomarine, Venice, CA. Soybean trypsin inhibitor (Kunitz), chicken ovomucoid, lima bean trypsin inhibitor, bovine trypsin, and bovine chymotrypsinogen A were obtained from Worthington Biochemical Corp. Bovine serum albumin was a product of Armour Pharmaceutical Co. Japanese quail ovomucoid was prepared by Dr. I. Kato of this laboratory (Kato et al., 1976), and bovine β -trypsin was prepared by the method of Schroeder & Shaw (1968) as modified by Luthy et al. (1973). Modified soybean trypsin inhibitor (Kunitz) was prepared as described by Mattis & Laskowski (1973). Bovine pancreatic trypsin inhibitor (Kunitz), Trasylol, was a generous gift from Dr. E. Truscheit of Bayer A. G.

The trypsin-specific burst titrant *p*-nitrophenyl *p*-guanidinobenzoate was a product of Nutritional Biochemical Corp. The fluorescent burst titrant 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973) was synthesized in this laboratory by Dr. P. Fankhauser. For use it was dissolved in dimethylformamide (1 mg/mL) and stored on ice. The synthetic substrates TosArgOMe, BzTyrOEt, BAPA, and hippuryl-L-arginine were obtained from Sigma Biochemical Corp. Phenylmethanesulfonyl fluoride was a product of the Eastman Kodak Co. All other chemicals were reagent grade or the best commercially available grade.

Activity Measurements. All spectrophotometric measurements were made by using either a Cary Model 118 or Cary Model 14 spectrophotometer. BAPA assays were made by using a modification of the procedure of Erlanger et al. (1961). Enzyme or enzyme and inhibitor were added to 3 mL of 0.05 M Tris and 0.02 M CaCl₂, pH 8.2, in a 10-mm path length cuvette. After appropriate incubation times, 30 μ L of a 44 mg/mL solution of BAPA in dimethyl sulfoxide was added, and the rate of increase in absorbance at 410 nm was measured. TosArgOMe hydrolysis was monitored at 247 nm by the method of Hummel (1959). Chymotryptic activity was measured in a similar manner, monitoring the hydrolysis of BzTyrOEt at 256 nm (Hummel, 1959). The hydrolysis of hippuryl-L-arginine (Folk et al., 1960) was monitored at 254 nm to measure carboxypeptidase B activity.

Bovine β -trypsin concentrations were measured by active-site titration with GdnBzONp according to the method of Chase & Shaw (1967); the solutions thus assayed were used as reference standards for quantitation of the fluorescence burst

titrations of the starfish trypsins.

All fluorometric measurements were made by using a Perkin-Elmer MPF-2A fluorescence spectrometer with the following settings: excitation wavelength, 360 nm, band-pass, 6 nm; emission wavelength, 450 nm, band-pass, 10 nm. Enzyme solutions were assayed by adding 20 nmol of GdnBzMum in 7 μ L of *N,N*-dimethylformamide to \sim 0.5 nmol of enzyme in 3 mL of 0.1 M veronal and 0.02 M CaCl₂, pH 8.3, in a 10-mm cuvette. After mixing, the increase in fluorescence as compared to that of an enzyme-free blank was measured, and the known concentration of β -trypsin was used to calibrate the signal in terms of nanomoles of enzyme per chart division.

Polyacrylamide Gel Electrophoresis. Analytical gels used to monitor STI \rightarrow STI* conversion were prepared according to the formulation of Davis (1964) as modified by Mattis & Laskowski (1973), and the ratio of [STI] to ([STI] + [STI*]) was determined as described by Mattis & Laskowski (1973). All other gels were obtained precast from Bio-Rad Laboratories, and preelectrophoresed in the various running buffers: 0.188 M Tris and 0.188 M glycine for pH 8.9 gels and 0.205 M Tris, 0.205 M acetic acid, and 0.1% w/v NaDodSO₄, pH 6.6, for NaDodSO₄ gels. Gels were analyzed either by direct scanning at 280 nm and/or by staining with Naphthol Blue Black in 7% acetic acid and then destaining by diffusion or transverse electrophoresis and scanning at 625 nm by using a Gilford spectrophotometer (Model 240). NaDodSO₄ gels were stained with Coomassie Brilliant Blue R-250 and destained by transverse electrophoresis. Tryptic activity was visualized by using the trypsin-specific stain described by Uriel (1963): 5 mg of *N*^α-benzoyl-DL-arginine- β -naphthylamide hydrochloride (Sigma Biochemical Corp.) was dissolved in 2 mL of *N,N*-dimethylformamide and added to 10 mL of a 2 mg/mL solution of Diazo Blue B (practical grade) in 0.05 M KH₂PO₄, pH 7.4. Gels were incubated in this mixture for 30 min at 37 °C and then fixed with 7% acetic acid.

Methods

Amino Acid Analysis. Analyses were performed by using a Durrum Model D-500 analyzer equipped with a ninhydrin detector. Samples ($\sim 2 \times 10^{-9}$ mol of protein) were hydrolyzed under vacuum in 5.7 N glass-distilled HCl at 110 °C for specified periods of time.

Sequence Studies. Sequence studies were carried out on a Beckman sequencer, Model 890C, using a 90-min quadrol program, with polybrene added as extender (Klapper et al., 1978). The phenylthiohydantoin derivatives of the amino acids were analyzed by reverse-phase high-pressure liquid chromatography using the system described by Kohr and Laskowski (unpublished experiments).

Results

The results of the isolation procedures are summarized in Table I. Frozen pyloric caeca from the starfish *D. imbricata* were homogenized in cold 0.1 M Tris buffer, pH 8.5 (\sim 1 g/mL), by using a Waring blender. The pH was monitored throughout the homogenization and kept at 8.5 by the addition of 6 N NaOH. The homogenate was incubated at room temperature for 6 h, then frozen and thawed 13 times, and stored at 4 °C for 3 weeks. This 3-week incubation was used in order to increase the yield of *D. imbricata* trypsin 2 (see below). For preparations in which the object is simply the isolation of *D. imbricata* trypsin 1, we dispense with this incubation. The homogenate was then diluted threefold with 0.1 M Tris, pH 8.5, and centrifuged at 4000g for 1 h in a Beckman Model J-21B centrifuge. The resulting supernatant

Table 1: Purification of *D. imbricata* Trypsins

procedure	protein (mg) ^a	BAPA units ^b	
		trypsin 1	trypsin 2
tissue homogenate ^c	170000	3156	5860
ammonium sulfate precipitation ^c	8663	2934	4350
trypsin 1			
Sephadex G-75	840	2800	
DEAE-Sephadex A-25 (pH 8.5)	244	1134	
DEAE-Sepharose (pH 8.0)	40.8	1100	
DEAE-Sepharose (pH 8.8)	7.8	700	
trypsin 2			
Sephadex G-75	1200		3630
chicken ovomucoid (Sepharose-bound)	620		2430

^a Determined by the method of Bradford (1976). ^b One unit = 1 μ mol of BAPA hydrolyzed per min. ^c Trypsin 1 activity was determined by completely inhibiting trypsin 2 activity with STI and then assaying with BAPA. Trypsin 2 activity was determined as the difference between the total BAPA activity and the trypsin 1 activity.

was differently precipitated with solid $(\text{NH}_4)_2\text{SO}_4$, and that portion precipitating between 40 and 70% was collected by centrifugation at 4000g for 1 h. During precipitation, the pH was maintained at 8.5 by the addition of 6 N NaOH. Because large amounts of lipid were present in the solutions, approximately 5 mL of 1-butanol per 300 mL of solution was added prior to centrifugation in order to help break the lipid suspension. The $(\text{NH}_4)_2\text{SO}_4$ precipitate was redissolved in 0.01 M Tris and 0.1 M NaCl, pH 8.5. Aliquots (200 mL) of the redissolved precipitate were chromatographed on Sephadex G-75 (equilibrated with 0.01 M Tris and 0.1 M NaCl, pH 8.5) in a Pharmacia K-100/100 (10 \times 95 cm) column at 25 $^\circ\text{C}$ (all columns were run at this temperature).

Two distinct peaks of tryptic activity were obtained by the above chromatographic procedure (part B in Figure 1). The activity of the first peak (*D. imbricata* trypsin 1) could not be inhibited by addition of STI, while STI strongly inhibited the activity of the second peak (*D. imbricata* trypsin 2). Those fractions which contained tryptic activity that could not be inhibited by STI were combined for *D. imbricata* trypsin 1 isolation. At this point, hippuryl-L-arginine assays indicated that this material contained a significant amount of carboxypeptidase B activity. Aliquots (800 mL) of these combined fractions were loaded onto a 2.5 \times 50 cm column of DEAE-Sephadex A-25 in 0.01 M Tris and 0.1 M NaCl, pH 8.5, and eluted with 0.1 M Tris and 0.3 M NaCl, pH 8.5. Under these conditions, the tryptic activity was bound to the column, but the carboxypeptidase B activity passed through in the loading buffer. Fractions from this column showing tryptic activity were combined and dialyzed against 0.1 M Tris, pH 8.0. Hippuryl-L-arginine assays now showed that the carboxypeptidase B activity was less than 0.003 unit/mL (1 unit = 1 μ mol of hippuryl-L-arginine hydrolyzed per min). The dialyzed material was loaded onto a 2.5 \times 15 cm column of DEAE-Sepharose in 0.1 M Tris, pH 8.0, and washed with the same buffer until the wash showed no evidence of hippuryl-L-arginine activity. The column was then developed with a linear gradient of 0–0.3 M NaCl in 0.1 M Tris, pH 8.0 (gradient volume was 1000 mL). The pooled trypsin 1 activity from this column was dialyzed against 0.1 M Tris, pH 8.8, and loaded onto a 0.9 \times 13 M column of DEAE-Sepharose in 0.1 M Tris, pH 8.8. This column was developed with a 200-mL gradient of 0–0.4 M NaCl in 0.1 M Tris, pH 8.8. Polyacrylamide gel electrophoresis at pH 8.9 of the pooled *D. imbricata* trypsin 1 fractions gave a single anionic protein band

which coincided with the tryptic activity as determined by the activity staining procedure given above.

Fractions from the Sephadex G-75 column which contained tryptic activity that could be inhibited by STI were pooled for trypsin 2 isolation. A 2.5 \times 16 cm chicken ovomucoid-Sepharose CL-6B column was prepared as described by Robinson et al. (1971). The column was equilibrated with 0.01 M Tris, pH 8.5. Aliquots (200 mL) of the pooled Sephadex G-75 fractions were loaded onto the column, and trypsin 2 was eluted with 0.2 M benzamidine hydrochloride in 0.01 M Tris and 0.1 M NaCl, pH 8.5. The pooled trypsin 2 fractions from this column were then dialyzed against 0.1 M Tris, pH 8.5. This material also showed a single anionic protein band on polyacrylamide gel electrophoresis, which coincided with the tryptic activity.

As noted above, two peaks of tryptic activity, trypsin 1 and trypsin 2, were obtained by Sephadex G-75 chromatography (Figure 1). However, when isolation procedures were carried out immediately after homogenization, almost no trypsin 2 was found at the expected elution volume of the Sephadex G-75 column. Instead STI-inhibitable tryptic activity was found at a position corresponding to a much higher molecular weight. When the homogenate was stored at 4 $^\circ\text{C}$ prior to isolation, the amount of this high molecular weight species decreased, with a concomitant rise in the amount of *D. imbricata* trypsin 2 (see Figure 1). BAPA assays of the Sephadex G-75 fractions containing the high molecular weight species showed the curvature which is characteristic of enzyme-inhibitor complex dissociations (Hixson, 1970). Operating on the assumption that the high molecular weight component was a complex of *D. imbricata* trypsin 2 with an endogenous trypsin inhibitor, we dropped the pH of an aliquot of this solution to 2, which irreversibly destroyed all tryptic activity. The solution was then tested for its ability to inhibit bovine trypsin and was found to contain a potent trypsin inhibitor. Inactivation of this inhibitor by the action of the endogenous carboxypeptidase B could be the reason for the decrease in complex and the increase in free enzyme when the crude homogenate is allowed to stand at 4 $^\circ\text{C}$. Carboxypeptidase B would remove the newly formed carboxy-terminal Lys or Arg from any modified inhibitor present, thus gradually destroying all inhibitor activity.

Stability. Both starfish trypsins show a remarkable stability in solution at pH 8.5 and above, with little loss of activity on standing at 4 $^\circ\text{C}$ for several months. This stability decreases with decreasing pH, and, at pH 2, total and irreversible loss of activity occurs within minutes. Added Ca^{2+} has no effect on stability.

NaDodSO₄ Gel Electrophoresis. Each starfish enzyme was analyzed by NaDodSO₄ gel electrophoresis using bovine serum albumin, bovine α - and β -trypsin, and both virgin and modified STI for standards. Samples were prepared for electrophoresis by the method of Fairbanks et al. (1971). Each enzyme was inactivated by phenylmethanesulfonyl fluoride prior to reduction of disulfide bonds, and gels were run as described above. *D. imbricata* trypsin 1 ran as a single band corresponding to ~240 amino acid residues, and *D. imbricata* trypsin 2 ran as a single band corresponding to ~200 amino acid residues.

Amino Acid Analysis. Results of amino acid analyses on the starfish proteinases are presented in Table II. Cysteine was determined as cysteic acid from 24-h hydrolysates of the performic acid oxidized enzymes (Hirs, 1967). Tryptophan was not determined. The remaining amino acids were determined from 24- and 48-h hydrolyses of the native proteins. Serine and threonine were estimated by extrapolation to zero

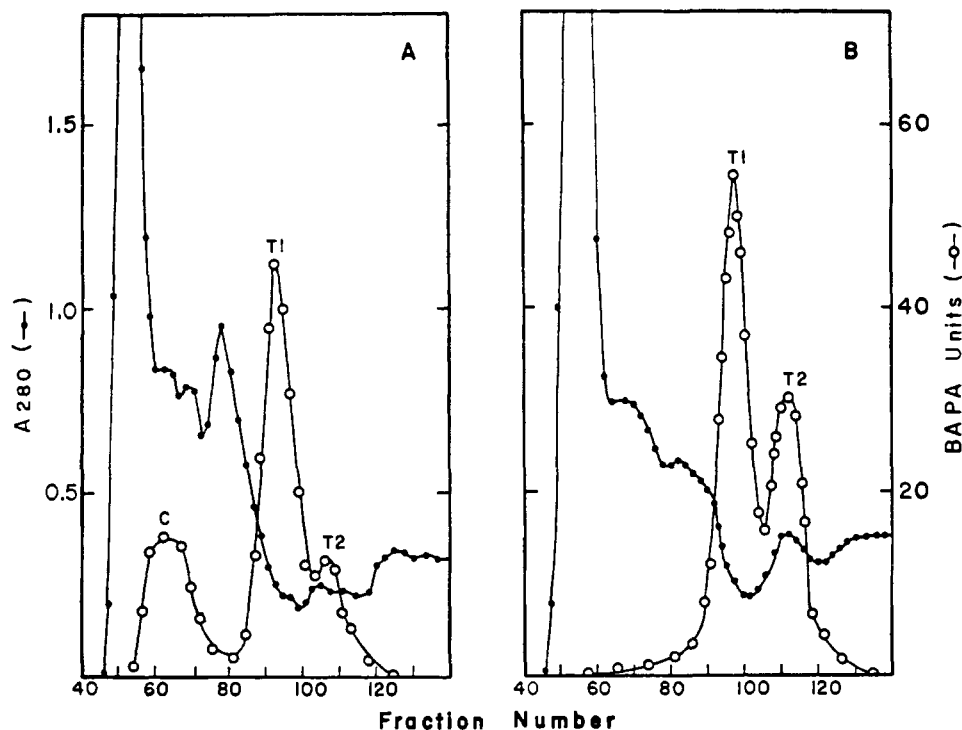


FIGURE 1: Sephadex G-75 chromatography of *D. imbricata* trypsins. T1 is trypsin 1, T2 is trypsin 2, and C is the trypsin 2-endogenous inhibitor complex. In (A), isolation was carried out immediately after homogenization, while in (B) the crude homogenate was stored at 4 °C for 3 weeks prior to isolation.

Table II: Amino Acid Compositions of *D. imbricata* Trypsins

AA	composition	
	trypsin 1	trypsin 2
Asp	24.1 (24)	17.9 (18)
Thr	16.3 (16)	11.8 (12)
Ser	24.9 (25)	30.0 (30)
Glu	15.8 (16)	14.8 (15)
Pro	12.3 (12)	12.1 (12)
Gly	27.8 (28)	27.2 (27)
Ala	21.1 (21)	17.1 (17)
Cys/2	7.7 (8)	6.2 (6)
Val	18.9 (19)	13.9 (14)
Met	2.3 (2)	1.2 (1)
Ile	12.3 (12)	13.0 (13)
Leu	14.9 (15)	9.7 (10)
Tyr	7.1 (7)	9.8 (10)
Phe	3.2 (3)	5.2 (5)
Trp	ND ^b	ND ^b
His	6.5 (7)	7.9 (8)
Lys	6.9 (7)	7.9 (8)
Arg	8.0 (8)	5.3 (5)
total: ^a	230	211
<i>M_r</i> : ^a	23 600	21 700

^a Does not include tryptophan. ^b ND, not determined.

time; valine and isoleucine were determined from the 48-h hydrolyses. All other amino acids were averages of the values for each hydrolysis. The data were converted to number of residues per molecule by minimizing the sum of the squares of the deviations from integer values (Hoy et al., 1974).

NH₂-Terminal Sequence Studies. The NH₂-terminal amino acid sequences of the starfish trypsins were determined by

automated sequence analysis, with the Beckman sequencer, using ~0.2 mg of *D. imbricata* trypsin 1 and ~0.5 mg of *D. imbricata* trypsin 2. Polybrene (4.5 mg) was added to each sample, and one cycle was run with no phenyl isothiocyanate in order to remove possible low molecular weight contaminants. The yield of the first amino acid residues, isoleucine in both cases, was ~90% with an average yield for each successive step of 95%. The first 17 amino acid residues of each proteinase were presented in comparison with bovine trypsin in Table III. These sequences clearly indicate that *D. imbricata* trypsins 1 and 2 belong to the family of chymotrypsin-related serine proteinases. The differences in sequence between trypsin 1 and trypsin 2 (at positions 5, 8, 9, 10, and 16) show that these enzymes are products of different genes and do not arise from autolysis of a single starting component.

Activity toward Synthetic Substrates. Both starfish enzymes hydrolyze the synthetic trypsin substrates BAPA and TosArgOMe, and they produce bursts with the trypsin-specific titrants GdnBzONp and GdnBzMum. For each enzyme, activity toward the chymotrypsin substrate BzTyrOEt was found to be less than 1% of the TosArgOMe activity.

The rate of TosArgOMe hydrolysis by *D. imbricata* trypsin 1 was measured over the concentration range from 2.5×10^{-5} to 4×10^{-3} M TosArgOMe in 0.05 M Tris and 0.01 M CaCl₂, pH 8.1; no evidence for substrate activation was seen in this concentration range (Figure 2). The data were analyzed by means of a nonlinear least-squares computer fit to the Michaelis-Menten equation. From this fit, *K_m* and *k₃* were calculated to be $7.6 \times 10^{-5} \pm 0.5 \times 10^{-5}$ M and $3.3 \times 10^2 \pm 0.04 \times 10^2$ s⁻¹, respectively. These values compare with *K_m*

Table III: Comparison of the NH₂-Terminal Sequences for Trypsin 1, Trypsin 2, and Bovine

	1	10
trypsin 1 (<i>D. imbricata</i>):	Ile-Val-Gly-Gly-His-Glu-Ser-Val-Arg-Gly-Ser-Arg-Pro-Tyr-Gln-Ala-Ala...	
trypsin 2 (<i>D. imbricata</i>):	Ile-Val-Gly-Gly-Lys-Glu-Ser-Thr-Ala-His-Ser-Arg-Pro-Tyr-Gln-Val-Ala...	
trypsin (bovine): ^a	Ile-Val-Gly-Gly-Tyr-Thr-Cys-Gly-Ala-Asn-Thr-Val-Pro-Tyr-Gln-Val-Ser...	

^a Mikes et al. (1966).

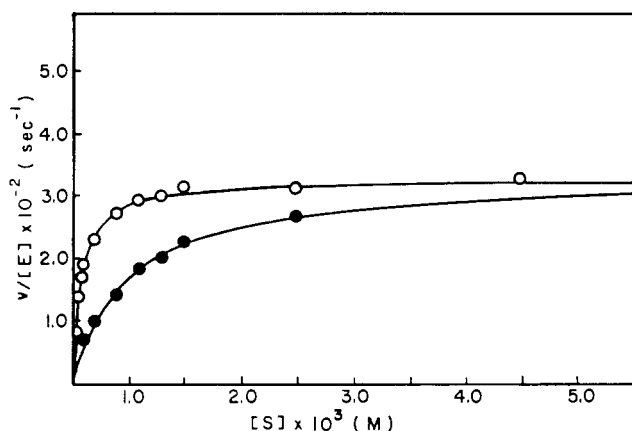


FIGURE 2: Plot of initial reaction velocity (moles of substrate hydrolyzed per second per mol of enzyme) vs. substrate concentration for trypsin 1 with TosArgOMe (O) and for the same reaction in the presence of 1.6×10^{-6} M STI (●). The curves shown are computer-generated fits of the data to the Michaelis-Menten equation.

$= 1.25 \times 10^{-5}$ M and $k_3 = 9.8 \times 10^1 \text{ s}^{-1}$ for bovine trypsin (Trowbridge et al., 1963).

The rates of hydrolyses of 1×10^{-3} M BAPA by both *D. imbricata* trypsin 1 and *D. imbricata* trypsin 2 were found to vary linearly with enzyme concentration with enzyme concentrations up to 1×10^{-6} M. A detailed study of this system was not made, due to the presence of both D and L isomers in the substrate. Assuming that the enzymes were saturated with respect to substrate under assay conditions, the molar specific activities (turnover numbers) for each enzyme were 25.5 s^{-1} for *D. imbricata* trypsin 1 and 1.3 s^{-1} for *D. imbricata* 2. The turnover number determined for bovine trypsin was 0.4 s^{-1} , which is in agreement with the data of Erlanger et al. (1961) and Nakata & Ishii (1970). The unexpectedly high turnover number for *D. imbricata* trypsin 1 (~60 times that for bovine trypsin) caused operational difficulties in early work on this enzyme by leading to overestimation of the amount of trypsin 1 on hand.

Inhibition Studies. Incubation of *D. imbricata* trypsin 1 or of *D. imbricata* trypsin 2 with a 100-fold molar excess of the serine proteinase specific reagent phenylmethanesulfonyl fluoride for 1 h leads to greater than 98% loss of enzymatic activity. This finding is consistent with numerous other indications that both enzymes are serine proteinases.

The following protein inhibitors of bovine trypsin were tested for inhibition of *D. imbricata* trypsins 1 and 2: PTI, STI, lima bean trypsin inhibitor, chicken ovomucoid, and Japanese quail ovomucoid. Each enzyme was incubated with varying amounts of inhibitor (from equimolar to 10-fold molar excess) for periods of time ranging from 15 min to several hours in order to ensure that sufficient time was available for association. Aliquots (100 μL) of these solutions were then withdrawn and transferred to 3 mL of 1×10^{-3} M BAPA. After thorough mixing, the rate of BAPA hydrolysis was determined spectrophotometrically. Trypsin 2 was inhibited by all the inhibitors employed; within experimental accuracy equimolar quantities of PTI, STI, and lima bean trypsin inhibitor inhibited trypsin 2 completely, while Japanese quail ovomucoid and chicken ovomucoid inhibited it partially. In contrast, the rate of BAPA hydrolysis by trypsin 1 was unaffected by preincubation with any of the above-listed inhibitors. The inhibition assay used here closely corresponds to that used by most laboratories engaged in the study of protein inhibitors of serine proteinases. If this were to be the sole criterion for inhibition, we would conclude that *D. imbricata* trypsin 1 is not inhibited by any of the inhibitors tested.

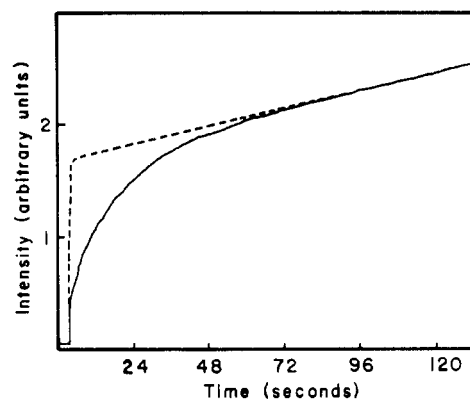


FIGURE 3: Fluorescence burst titrations of *D. imbricata* trypsin 1 (---) and an equimolar mixture of trypsin 1 with PTI (—), using the substrate GdnBzMum. To the enzyme or enzyme-inhibitor mixture (1.3×10^{-7} M in 0.1 M sodium barbitol and 0.02 M CaCl_2 , pH 8.3), after suitable preincubation, was added 7 μL of a 1 mg/mL solution of GdnBzMum in *N,N*-dimethylformamide. The increase in fluorescence resulting from the hydrolysis of substrate was monitored as a function of time. The difference between the curves at a given time is proportional to the amount of enzyme-inhibitor complex present at that time.

The experiment described above has two possible explanations. One is that during preincubation there was no significant formation of an inactive enzyme-inhibitor complex. The other is that a significant quantity of such a complex was formed but, upon transfer to the BAPA solution, dissociated so rapidly that it escaped detection. We estimate that the longest dissociation half-life we would fail to detect in this experiment would be $t_{1/2} = 30 \text{ s}$.

In order to check further the possibility of a rapidly dissociating complex, we repeated incubations of trypsin 1 with STI and with PTI, using the fluorogenic burst substrate GdnBzMum to monitor free enzyme, in a variation of the procedure of Zahnley & Davis (1970). This method allows the detection of complexes having a dissociation half-life greater than 3 s. For the mixture of trypsin 1 with STI, the fluorescence burst was immediate; i.e., there was either no complex or the complex dissociated with a half-life shorter than 3 s. For the mixture of trypsin 1 with PTI, however, the burst was a first-order reaction with $t_{1/2} = 11 \text{ s}$, indicating a dissociation half-life for the trypsin 1-PTI complex of 11 s (Figure 3). This half-life is dramatically shorter than the value of $t_{1/2} = 10^7 \text{ s}$ reported for the bovine trypsin-PTI complex by Vincent & Lazdunski (1972).

The textbook method for determining an enzyme-inhibitor equilibrium constant (K_i) is by measurement of enzyme activity upon the addition of enzyme to a substrate/inhibitor mixture, with the inhibitor concentration held constant and the substrate concentration varied. This is widely applied to small, competitive inhibitors with K_i values in millimolar or micromolar range. As first pointed out by Green & Work (1953) and later by many others, among them Laskowski & Sealock (1971), this is generally a poor method for determining K_i values for protein proteinase inhibitors. For most such systems, K_i values are very low (10^{-8} – 10^{-13} M); therefore, it is common to use concentrations of inhibitor that are closely comparable to the concentrations of enzyme. Yet, the equations that are used to analyze the data require that the inhibitor be present in a large molar excess over the enzyme. An even more important objection is that since the half-time for complex dissociation is often long (for many serine proteinase-protein inhibitor systems, $t_{1/2}$ ranges from hours to weeks) attainment of the enzyme-substrate-inhibitor steady state usually requires a very long time, frequently much longer time

than the time allowed for the assay. Thus, a number of workers reach the erroneous conclusion that many of the proteinase-protein proteinase inhibitor systems are not competitive with substrate. For these reasons, the classical K_i determination has been avoided by most careful researchers in the proteinase inhibitor field. This method is quite valid, however, provided that care is taken to ensure that a significant excess of inhibitor over enzyme is present and that the dissociation half-life of the enzyme-inhibitor complex is much shorter than the time required for the assay procedure used.

The above conditions could be met in the *D. imbricata* trypsin 1-STI system. We therefore determined the rate of TosArgOMe hydrolysis by 2×10^{-9} M trypsin 1 in the presence of 1.6×10^{-6} M STI for the substrate concentration range of 1×10^{-4} to 2×10^{-3} M. These results are shown in Figure 2. Note that STI acts as a strictly competitive inhibitor of *D. imbricata* trypsin 1—the value of V_{\max} is unaffected, but K_m is greatly increased. Least-squares computer fitting of the data in Figure 2 yields $K_i = 3 \times 10^{-7}$ M for the *D. imbricata* trypsin 1-STI system. By comparison with simple inhibitors of trypsin, such as benzamidine, STI is a very powerful inhibitor of trypsin 1. It is therefore the short lifetime of the trypsin 1-STI complex that leads to the lack of inhibition in the conventional assay procedure. The same analysis of competitive inhibition was applied to the trypsin 1-PTI system. In these experiments, 2×10^{-8} M PTI and 1×10^{-9} M trypsin 1 were used, ensuring an excess of inhibitor over enzyme. The dissociation half-time for the trypsin 1-PTI complex (11 s) was sufficiently short that it did not interfere with the assay procedure, which required ~ 8 min. From these data, K_i for the trypsin 1-PTI system was determined to be 1×10^{-9} M.

Hydrolysis of Peptide Bonds in Proteins by *D. imbricata* Trypsin 1. The results presented above show that *D. imbricata* trypsin 1 and STI form a moderately strong complex ($K_i = 3 \times 10^{-7}$ M) but that this complex dissociates very rapidly compared to the complex of STI with bovine trypsin. In the introduction, we pointed out that in some cases fast dissociation (high k_{-2} in eq 1) is correlated with rapid reactive-site bond hydrolysis (high k_3 in eq 1). It is therefore reasonable to expect that *D. imbricata* trypsin 1 would be capable of rapidly catalyzing the hydrolysis of the Arg⁶³-Ile reactive site in STI. STI (3.4×10^{-7}) was incubated with 6.6×10^{-11} mol of trypsin 1 in 2 mL of 0.5 M KCl, 0.05 M CaCl₂, and 0.05 M Tris, pH 8.0, at 21 °C. Aliquots were withdrawn at various times and analyzed by polyacrylamide gel electrophoresis. Rapid but partial conversion of STI to a new electrophoretic species, corresponding to STI* (Mattis & Laskowski, 1973), was observed (Figure 4). The conversion reached a steady state in which $78 \pm 2\%$ of the original STI was converted to STI*. Mattis & Laskowski (1973) report that 77% of STI is converted to STI* under the same conditions, when bovine trypsin is used to catalyze the reaction. To confirm that hydrolysis is at the Arg⁶³-Ile reactive site of STI, we subjected the hydrolysis product to 13 cycles of Edman degradation in the Beckman sequencer. Two residues were found at each position, exactly corresponding to residues 1-13 and 64-76 of the sequence of STI as determined by Koide et al. (1972).

The data shown in Figure 4 allow us to evaluate k_{cat} (which is to be identified with k_2 in eq 1) as $1.2 \times 10^{-1} \text{ s}^{-1}$. An equivalent number reported by Mattis (1974) for the reaction of STI and bovine β -trypsin is 2.5×10^{-6} [see also Finkenstadt et al. (1974)]. Thus, *D. imbricata* trypsin 1 hydrolyzes the reactive-site peptide bond of STI some 50 000 times faster than does bovine β -trypsin.

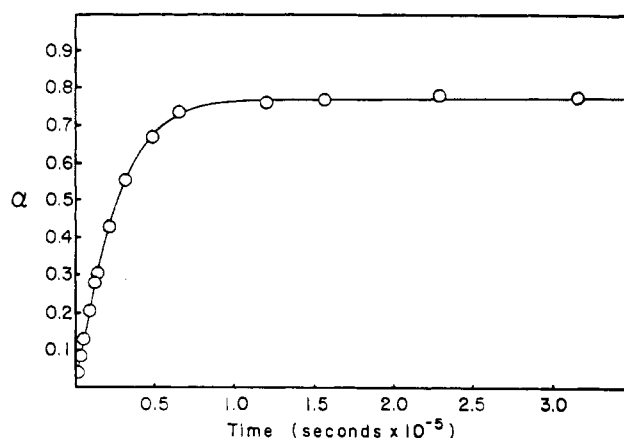


FIGURE 4: Change in composition [$\alpha = [\text{STI}^*]/([\text{STI}] + [\text{STI}^*])$] with respect to time for STI incubated with *D. imbricata* trypsin 1 (0.02 mol %). α was measured for each aliquot from the 625-nm scan of Amido Blue Black stained polyacrylamide disc gel electrophoretogram (Mattis & Laskowski, 1973).

From the determination of K_i for STI as an inhibitor of TosArgOMe hydrolysis by *D. imbricata* trypsin 1, we would expect K_m for the $\text{STI} \rightarrow \text{STI}^*$ conversion by this enzyme to be 3×10^{-7} . Unfortunately, the polyacrylamide gel experiments become very difficult when the concentration of STI in the initial solution is lower than 1×10^{-6} M, preventing the direct measurement of K_m . As would be expected, however, the data in Figure 4 were not affected by starting STI concentrations in the range from 3.6×10^{-4} to 3.6×10^{-6} M.

The dramatically rapid rate of hydrolysis of the Lys¹⁵-Ala reactive site in PTI by *D. imbricata* trypsin 1 (as compared to bovine trypsin) is discussed in detail in the following paper (Estell et al., 1980) along with a somewhat more detailed analysis of the kinetics of the STI reactive-site hydrolysis.

Since the reactive-site peptide bonds of two trypsin inhibitors were rapidly and specifically hydrolyzed, the question arose as to whether or not *D. imbricata* trypsin 1 was capable of hydrolyzing the reactive-site peptide bonds in inhibitors of serine proteinases other than trypsin. To test this possibility, we employed the carbohydrate-free third domain of turkey ovomucoid (Kato et al., 1976, 1977, 1978). This domain strongly inhibits chymotrypsin, elastase, and subtilisin at a Leu-Glu reactive site. It does not inhibit trypsin. *D. imbricata* trypsin 1 (2.6×10^{-10} M) was incubated with 7×10^{-7} mol of turkey ovomucoid third domain in 2 mL of 0.1 M Tris, pH 8.2, for 24 h at 21 °C. Analysis on the Beckman sequencer indicated no new amino termini. Trypsin 1, therefore, does not hydrolyze all reactive-site peptide bonds but is probably limited to the reactive sites of trypsin-specific inhibitors, although many more tests will be required to make this statement general.

Because *D. imbricata* trypsin 1 hydrolyzes the peptide bond in BAPA, the Arg⁶³-Ile bond in STI, and the Lys¹⁵-Ala bond in PTI much more rapidly than does bovine trypsin, it was of interest to determine whether or not *D. imbricata* trypsin 1 is simply a hyperactive trypsin. To test this, we measured the rates of activation of bovine chymotrypsinogen A by bovine trypsin and by *D. imbricata* trypsin 1 at pH 8.3. Under these conditions, *D. imbricata* trypsin 1 activates bovine chymotrypsinogen A ~ 16 times more slowly than does bovine trypsin—thus, the surmise that trypsin 1 is hyperactive with respect to all substrates is not correct.

Discussion

This paper describes the isolation of *D. imbricata* trypsins 1 and 2. Of these, trypsin 2 shares several properties with

trypsinlike enzymes from many other sources, while trypsin 1 differs strikingly. Its complex with PTI dissociates 1 000 000 times more rapidly than that of bovine trypsin. The rate of reactive-site peptide bond hydrolysis in PTI by this enzyme is also about 1 000 000 times faster (Estell et al., 1980). In the case of STI, the rates for both dissociation of the complex and reactive-site peptide bond hydrolysis are approximately 10^5 times faster for *D. imbricata* trypsin than for bovine trypsin. These truly dramatic differences in the kinetics of enzyme-inhibitor interactions are not reflected in equally dramatic differences for the kinetics of interaction with small substrates and ordinary protein substrates. The rate of hydrolysis of BAPA is ~ 60 times greater, but, for TosArgOMe, the rate is only 3 times greater, and the rate of hydrolysis for the Arg¹⁵-Ile bond in bovine chymotrypsinogen A is 16 times slower for *D. imbricata* trypsin 1 than for bovine trypsin. The striking differences in the kinetics of enzyme-inhibitor complex dissociation (to yield the enzyme and virgin as well as modified inhibitor) between homologous enzymes may shed considerable light upon the molecular events responsible for rapid reactive-site hydrolysis in one case and strong inhibition in the other. This point is discussed somewhat more quantitatively in the following paper (Estell et al., 1980).

Since *D. imbricata* trypsin 1 rapidly and specifically hydrolyzes the reactive-site peptide bonds of trypsin inhibitors, and since it is quite stable at high pH, this proteinase is very useful for preparing large quantities of modified inhibitors. The isolation of larger amounts of this enzyme should allow for broader utilization of the semisynthetic inhibitor modification technique developed in this laboratory (Sealock & Laskowski, 1969; Leary & Laskowski, 1973; Kowalski & Laskowski, 1976a,b). Given large amounts of inhibitor with the reactive-site peptide bond hydrolyzed, we find that this system allows for the replacement of the newly formed carboxy- and amino-terminal amino acids with other amino acids. This permits tailoring of inhibitor specificity and studies on the role of the reactive-site amino acids in inhibition. This enzyme may also provide a method for identifying reactive-site peptide bonds in trypsin inhibitors which have heretofore proven difficult to identify.

D. imbricata appears to be the only species of starfish examined, so far, in which there is any suggestion that an enzyme with the properties of trypsin 1 is present. Studies with *Evasterias trocheli* (Winter & Neurath, 1970), *Pisaster giganteus* (Bundy & Gustafson, 1973), *Lysastrosoma antiochica* (Kozlovskaya & Elyakova, 1974), and *Pisaster brevispinus* (Gilliam & Kitto, 1976), and studies in this laboratory with *P. ochraceus*, have failed to detect a trypsinlike enzyme that cannot be inhibited by STI. *D. imbricata* trypsin 2, which is similar to the enzymes isolated from the above sources (it can be inhibited by STI), is present in *D. imbricata* pyloric caeca homogenates at amounts ~ 40 times those of trypsin 1. The physiological significance of trypsin 1 is as yet unclear, but we wish to point out that the combination of trypsin 1 with endogenous carboxypeptidase B activity would provide *D. imbricata* with a powerful system for inactivating ingested trypsin inhibitors.

We believe that *D. imbricata* trypsin 1 is the same enzyme identified by Camacho et al. (1970) as proteinase B. The sample of proteinase B sent to us showed the same rate of STI reactive-site hydrolysis, and titration with GdnBzMum, followed by BAPA assay, showed the same turnover number (per mole of enzyme active site) for BAPA. The major difference in the two preparations is that trypsin 1 has a specific activity for BAPA of $64.8 \mu\text{mol}/(\text{min mg of protein})$, whereas pro-

teinase B has a specific activity of $1.8 \mu\text{mol}/(\text{min mg of protein})$ (Camacho et al., 1970), indicating a 36-fold greater purification of trypsin 1. Although Camacho et al. (1970) reported that their other trypsinlike enzyme, proteinase A, was also not inhibited by STI, we find no evidence of any additional trypsinlike enzyme which could not be inhibited by STI. All other tryptic activity found by us arises from *D. imbricata* trypsin 2 and is strongly inhibited by STI.

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References

- Bradford, M. M. (1976) *Anal. Biochem.* 72, 248.
- Bundy, H. F., & Gustafson, J. (1973) *Comp. Biochem. Physiol. B* 44, 241.
- Camacho, Z., Brown, J. R., & Kitto, B. (1970) *J. Biol. Chem.* 245, 3964.
- Chase, T., Jr., & Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* 29, 508.
- Coan, M. H., & Travis, J. (1971) *Proc. Int. Res. Conf. Proteinase Inhibitors, 1st*, 1970, 294.
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404.
- Erlanger, B. F., Kolowsky, N., & Cohen, W. (1961) *Arch. Biochem. Biophys.* 95, 271.
- Estell, D. A., Wilson, K. A., & Laskowski, M., Jr. (1980) *Biochemistry* (following paper in this issue).
- Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry* 10, 2606.
- Feeney, R. E., Means, G. E., & Bigler, J. C. (1969) *J. Biol. Chem.* 244, 1957.
- Figarella, C., Negri, G. A., & Guy, O. (1975) *Eur. J. Biochem.* 53, 457.
- Finkenstadt, W. R., Hamid, M. A., Mattis, J. A., Schrode, J., Sealock, R. W., Wang, D., & Laskowski, M., Jr. (1974) *Proc. Int. Conf. Proteinase Inhibitors, 2nd*, 389.
- Folk, J. E., Piez, K. A., Carroll, W. R., & Gladner, J. (1960) *J. Biol. Chem.* 235, 2272.
- Gilliam, E. B., & Kitto, G. B. (1976) *Comp. Biochem. Physiol. B* 54, 21.
- Green, N. M., & Work, E. (1953) *Biochem. J.* 54, 347.
- Hirs, C. H. W. (1967) *Methods Enzymol.* 11, 59.
- Hixson, H. F. (1970) Ph.D. Thesis, Purdue University, West Lafayette, IN, p 73.
- Hoy, T. G., Ferdinand, W., & Harrison, P. M. (1974) *Int. J. Pept. Protein Res.* 6, 121.
- Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., & Steigemann, W. (1974) *J. Mol. Biol.* 89, 73.
- Hummel, B. C. W. (1959) *Can. J. Biochem. Physiol.* 37, 1393.
- Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, S. A., & Elmore, D. T. (1973) *Biochem. J.* 131, 107.
- Kato, I., Schrode, J., Wilson, K. A., & Laskowski, M., Jr. (1976) *Protides Biol. Fluids, Proc. Colloq.* 23, 235.
- Kato, I., Kohr, W. J., & Laskowski, M., Jr. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 36, 2592.
- Kato, I., Kohr, W. J., & Laskowski, M., Jr. (1978) in *Regulatory Proteolytic Enzymes and Their Inhibitors* (Magnusson, S., Ottelson, M., Foltmann, B., Dano, K., & Neurath, H., Eds.) p 197, Pergamon Press, Oxford.
- Klapper, D. G., Wilde, C. E., III, & Capra, J. D. (1978) *Anal. Biochem.* 85, 126.

- Koide, T., Tsunazawa, S., & Ikenaka, T. (1972) *J. Biochem. (Tokyo)* 71, 165.
- Kowalski, D., & Laskowski, M., Jr. (1976a) *Biochemistry* 15, 1300.
- Kowalski, D., & Laskowski, M., Jr. (1976b) *Biochemistry* 15, 1309.
- Kozlovskaya, E. P., & Elyakova, L. A. (1974) *Biochim. Biophys. Acta* 371, 63.
- Laskowski, M., Jr., & Sealock, R. W. (1971) *Enzymes*, 3rd Ed. 3, 375.
- Laskowski, M., Jr., Kato, I., Leary, T. R., Schrode, J., & Sealock, R. W. (1974) *Proc. Int. Res. Conf. Proteinase Inhibitors*, 2nd, 597.
- Leary, T. R., & Laskowski, M., Jr. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 32, 465.
- Luthy, J. A., Praissman, M., Finkenshtadt, W. R., & Laskowski, M., Jr. (1973) *J. Biol. Chem.* 248, 1760.
- Mattis, J. A. (1974) Ph.D. Thesis, Purdue University, West Lafayette, IN.
- Mattis, J. A., & Laskowski, M., Jr. (1973) *Biochemistry* 12, 2239.
- Mikes, O., Holeysovsky, V., Tomasek, V., & Sorm, F. (1966) *Biochem. Biophys. Res. Commun.* 24, 346.
- Nakata, H., & Ishii, S. (1970) *Biochem. Biophys. Res. Commun.* 41, 393.
- Robinson, N. C., Tye, R. W., Neurath, H., & Walsh, K. A. (1971) *Biochemistry* 10, 2743.
- Rühlmann, A., Kukla, D., Schwager, P., Bartels, K., & Huber, R. (1973) *J. Mol. Biol.* 77, 417.
- Schroeder, D. D., & Shaw, E. (1968) *J. Biol. Chem.* 243, 2943.
- Sealock, R. W., & Laskowski, M., Jr. (1969) *Biochemistry* 8, 3703.
- Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., & Blow, D. M. (1974) *Biochemistry* 13, 4212.
- Travis, J., & Roberts, R. C. (1969) *Biochemistry* 8, 2884.
- Trowbridge, C. G., Krehbiel, A., & Laskowski, M., Jr. (1963) *Biochemistry* 2, 843.
- Tschesche, H., & Kupfer, S. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* 357, 769.
- Uriel, J. (1963) *Ann. N.Y. Acad. Sci.* 103, 956.
- Vincent, J. P., & Lazdunski, M. (1972) *Biochemistry* 11, 2967.
- Winter, W. P., & Neurath, H. (1970) *Biochemistry* 9, 4673.
- Zahnley, J. C., & Davis, J. G. (1970) *Biochemistry* 9, 1428.

Thermodynamics and Kinetics of the Hydrolysis of the Reactive-Site Peptide Bond in Pancreatic Trypsin Inhibitor (Kunitz) by *Dermasterias imbricata* Trypsin 1[†]

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ABSTRACT: Incubation of pancreatic trypsin inhibitor (Kunitz) (PTI) with catalytic amounts of *Dermasterias imbricata* trypsin 1 leads to rapid yet highly specific hydrolysis of the Lys¹⁵-Ala reactive-site peptide bond. Aside from providing a facile means for the preparation of modified inhibitor (PTI*), this finding also allows rapid measurement of the peptide bond hydrolysis equilibrium constant, K_{hyd} . The pH dependence of this constant over the pH 6.2-8.8 range shows that peptide bond hydrolysis does not perturb the pK values of any preexistent ionizable groups (in PTI) which ionize in this pH range. K_{hyd}^0 is 0.95, and the pK of the α -amino group of Ala¹⁶ in PTI* is 8.3. From the rate of equilibration, we calculate that k_{cat}/K_m is quite similar to k_{cat}/K_m for the hydrolysis of the same bond in PTI by bovine trypsin, while k_{cat} for the *D.*

imbricata trypsin 1 catalyzed reaction is 10⁶ times greater than the value of k_{cat} for the bovine trypsin-PTI reaction. The ratio of the dissociation rate constants for the enzyme-inhibitor complex (k_{-2}/k_3)—one to form virgin inhibitor and free enzyme (k_{-2}) and the other (k_3) to form modified inhibitor and free enzyme—is about 50 for the *D. imbricata* trypsin 1-PTI complex and about 90 for the bovine trypsin-PTI complex. These values are very similar even though the individual rate constants for the two enzymes differ by a factor of 10⁶. The values of k_{cat}/K_m and k_{-2}/k_3 for the hydrolysis of the Arg⁶³-Ile reactive-site peptide bond in STI are also quite similar for *D. imbricata* trypsin 1 and bovine trypsin, although k_{cat} is 50 000 times greater for the starfish enzyme.

Niekamp et al. (1969) and Mattis & Laskowski (1973) have shown that hydrolysis of the reactive-site peptide bond in soybean trypsin inhibitor (Kunitz), STI¹, does not proceed to completion but that an equilibrium between virgin (re-

active-site peptide bond intact) and modified (reactive-site peptide bond hydrolyzed) inhibitors, governed by the equilibrium constant K_{hyd} , becomes established. K_{hyd} is given by

$$K_{\text{hyd}} = [\text{I}^*]/[\text{I}] \quad (1)$$

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¹ Abbreviations used: STI, soybean trypsin inhibitor (Kunitz); STI*, modified STI in which the Arg⁶³-Ile reactive-site peptide bond has been hydrolyzed; PTI, bovine pancreatic trypsin inhibitor (Kunitz); PTI*, modified PTI in which the Lys¹⁵-Ala reactive-site peptide bond has been hydrolyzed; GdnBzMum, 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride; MeSAcNHBzMum, 4-methylumbelliferyl *p*-(dimethylsulfonio)acetamido]benzoate bromide; Tris, tris(hydroxymethyl)aminomethane.