AN ANIONIC TRYPSIN-LIKE ENZYME FROM *STREPTOMYCES EYTHREUS*

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1. Introduction

It had been considered that microbial proteases have very wide ranges of side-chain specificity [1]. Recent investigations, however, have shown the existence of a protease possessing a substrate specificity similar to that of bovine pancreatic trypsin [2, 3]. The protease was found in *Streptomyces fradiae* [2] or in *Streptomyces griseus* [3], and has come to be called trypsin-like enzyme [4]. We have subsequently found a third trypsin-like enzyme, from *Streptomyces erythreus*. It is of interest to isolate enzymes responsible for tryptic activity because of possible structural homologies among trypsin-like enzymes, and particularly in view of the study at present in progress on the tertiary structure of trypsin [5]. This report describes the purification and properties of a trypsin-like enzyme from *St. erythreus* and comparison with the known trypsin-like enzymes and with trypsin.

2. Experimental procedure

Bovine pancreatic trypsin (twice crystallized, salt free) was obtained from Worthington and used without further purification. Trypsin-like enzyme from *St. griseus* was prepared by the method described by Narahashi and Fukunaga [3], and the enzyme from *St. fradiae* was purified by the following procedure. Fraction IV enzyme [6] donated by Dr. Morihara was chromatographed on CM-Sephadex (C-25, 0.05 M acetate, pH 4.0 with a 0.1–0.45 M linear concentration gradient of NaCl), and further purification was performed by SE-Sephadex (C-50) chromatograpy (0.1 M acetate, pH 5.3, 0.1–0.25 M NaCl gradient), giving a single band on polyacrylamide disc electrophoresis (pH 4.0, 7.5% gel). Benzoyl-L-arginine *p*-nitroanilide and benzoyl-L-arginine ethyl ester were the products of the Foundation for Promotion of Protein Research, Osaka University. Soybean trypsin inhibitor (STI) was purchased from Mann Research Laboratories, N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK) from Cyclo Chem., and diisopropyl phosphorofluoridate (DFP) from BDH Chemicals. Calculations of all the kinetic parameters were performed by an IBM 1620 computer according to the program described by Cleland [7].

3. Results and discussion

3.1. *Purification*

Crude extract was prepared as described earlier [8]. The peak before the ribonuclease fraction, shown in fig. 2 of [8], was chromatographed on a QAE-Sephadex (A-50) column equilibrated with 0.1 M tris-HCl buffer containing 0.2 M NaCl. After elution of the column with the same buffer, the preparation was found to be homogeneous by disc electrophoresis (7.5% cross-link, pH 8.0 gel) and ultracentrifugation. The sedimentation constant of the enzyme was found to be 2.40 S (0.05 M phosphate containing 0.1 M NaCl, pH 7.0; 20°). From the values for S, diffusion constant (D_{20}: 10.4×10^{-7}) and partial specific volume calculated from the amino acid composition (0.715), a molecular weight of 20,000 was calculated, which is consistent with values estimated by gel filtration and by a sedimentation equilibrium experiment (N. Yoshida and H. Inoue, unpublished). The isoelectric point of the enzyme was found to be around pH 4.0 by cellulose-acetate film electrophoresis. The enzyme is inhibited by DFP, STI and TLCK, and

Table 1
Kinetic parameters of trypsin and trypsin-like enzymes at pH 8.0.

Enzyme	Benzoyl-L-arginine *p*-nitroanilide (25°)		Benzoyl-L-arginine ethyl ester (30°)	
	$K_m(10^{-5}$ M)	$k_{cat}(sec^{-1})$	$K_m(10^{-5}$ M)	$k_{cat}(sec^{-1})$
Trypsin-like enzyme from				
St. erythreus	3.91 ± 0.33	3.73 ± 0.11	1.81 ± 0.31	50.08 ± 4.42
St. fradiae	6.48 ± 1.16	10.94 ± 0.83	1.53 ± 0.15	66.44 ± 1.57
St. griseus	1.39 ± 0.20	3.68 ± 0.12	0.73 ± 0.11	39.12 ± 1.31
Trypsin	149.3 ± 23.1	0.74 ± 0.08	1.78 ± 0.31	18.96 ± 1.14

splits the peptide bonds of oxidized insulin B-chain in accordance with the cleavage specificity for trypsin.

3.1. *Kinetics*

Rate assay of the trypsin-like enzyme from *St. erythreus* for esterase and amidase activity has been made and compared with those for the other trypsin-like enzymes and for bovine trypsin, using benzoyl-L-arginine derivatives as specific substrates. The kinetic parameters are summarized in table 1. Assuming that a normality of trypsin solution is approximately 60% [9, 10], a k_{cat} value of 40 sec^{-1} may be given for benzoyl-L-arginine ethyl ester. Thus, in apparent catalytic efficiency toward the esterase substrate there is little difference among trypsin-like enzymes. In contrast to this situation, it will be noted that trypsin hydrolyzes benzoyl-L-arginine *p*-nitroanilide at a relative catalytic efficiency (k_{cat}/K_m) one-hundredth as fast as trypsin-like enzymes. Here the value of k_{cat} drops significantly, but most of the effect on k_{cat}/K_m is a consequence of the marked increase in K_m. If we assume a three step mechanism for all the enzymes [11], the available data may be accounted for in terms of equal binding of a specific esterase substrate to trypsin and the trypsin-like enzymes and equal rate for the deacylation of the acyl enzymes, but with a difference between the affinity of a specific amidase substrate for trypsin and for the other enzymes, and with distinguishable different rates for the formation of the acyl enzymes. Although a more detailed discussion must await the estimation of dissociation constants and rate constants for the acylation and deacylation of benzoyl-L-arginine ethyl ester, these findings are relevant to a consideration of the mechanism of trypsin-like enzyme action.

It is noteworthy that in the inactivation process by TLCK there is a remarkable difference in efficiency, as shown in fig. 1. The preliminary kinetics for the inactivation according to the method described by Kitz and Wilson [12] show that dissociation constants for trypsin and that for the enzyme from *St. erythreus* are 3×10^{-4} M and 4×10^{-5} M respectively, and that trypsin is inhibited with a rate constant of alkylation one-fifteenth of that for the trypsin-like enzyme.

3.3. *Amino acid composition*

Amino acid analysis of the three trypsin-like enzymes was done on the 24 hr acid hydrolysates, but tryptophan content was determined by the method of Spies and Chambers [13]. The results are presented in table 2 and compared with that for bovine trypsin [14].

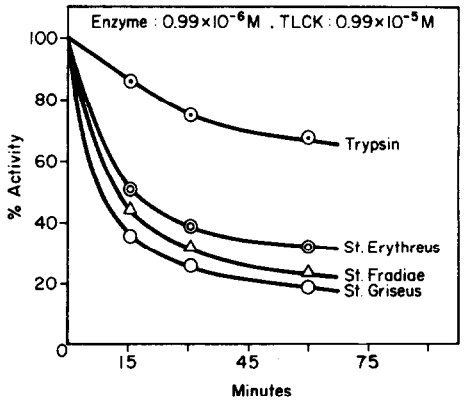

Fig. 1. Inactivation of trypsin and trypsin-like enzymes by TLCK at pH 8.0, 0.1 M tris-HCl buffer, 25°.

Table 2
Amino acid composition of trypsin-like enzymes and trypsin.

Amino acid	No. of residues per enzyme molecule			
	Trypsin-like enzyme			Trypsin
	St. erythreus	*St. fradiae*	*St. griseus*	
Lysine	8	4	5	14
Histidine	3	1	1	3
Arginine	3	8	6	2
Aspartic acid	31	13	18	22
Threonine	15	19	16	10
Serine	9	18	14	33
Glutamic acid	23	15	14	14
Proline	11	10	9	9
Glycine	31	31	29	25
Alanine	27	24	26	14
Half-cystine	4	4	6	12
Valine	24	11	13	17
Methionine	3	3	2	2
Isoleucine	5	7	7	15
Leucine	8	12	10	14
Tyrosine	5	7	7	10
Phenylalanine	3	4	5	3
Tryptophan	2	4	3	4
No. of residues	215	195	191	223

The enzyme from *St. erythreus* was shown to have 215 amino acid residues, which account for a molecular weight of 21,800. The anionic enzyme and trypsin both have three histidine residues, whereas the enzymes from *St. fradiae* and *griseus* contain only a single histidine which must be the TLCK-reactive residues. Although it is reasonable that the enzyme from *St. erythreus* has a high acidic amino acid content, consistent with its anionic character, it is interesting to find that the enzyme has a high valine value, not shared by the two other trypsin-like enzyme nor even by trypsin. It is noteworthy that the trypsin-like enzymes have two or three disulfide linkages, while bovine trypsin has six. These findings suggest that there are considerable differences between the secondary and tertiary structures of the enzymes from the bacterium and those of pancreatic trypsin. The data of circular dichroism of the enzymes seem to support the structure differences (H. Inoue and N. Yoshida, unpublished).

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