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PURIFICATION AND PROPERTIES OF RABBIT TRYPSIN

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Summary

The purification of rabbit pancreatic trypsin (EC 3.4.21.4) by affinity chromatography on Trasylol-Sepharose is presented along with its physical, chemical and immunological relationship to other trypsins. The molecule is a single polypeptide chain, which immunologically cross-reacts with porcine trypsin, but not with rabbit acrosomal proteinase. Sequence homology with other mammalian trypsins is seen at the amino terminus.

Introduction

Proteinases were among the first proteins to be isolated, and therefore have served as a basis for teleological comparisons. Although the rabbit has long been used as a test animal and source of tissue for various biochemical studies, its pancreatic trypsin (EC 3.4.21.4) had never been purified. It has been suggested [1] that rabbit acrosomal proteinase is structurally and immunologically similar to human trypsin. If this is true it should bear even greater similarity to rabbit trypsin. In hopes of clarifying this question, while broadening the data base for teleological comparisons, rabbit trypsin has been purified. This paper describes the purification and properties of the enzyme along with its immunological relationship to other trypsins and rabbit acrosomal proteinase.

Materials and Methods

Trasylol® was a gift from Dr. Ernst Truscheit of Bayer AG. Sepharose 4B was the product of Pharmacia Fine Chemicals. The substrates Bz-L-ArgOEt and NAc-L-TyrOEt were purchased from Sigma Chemical Co. Suc$(Ala)_3$-pNA was purchased from Bachem. Reagents and solvents used in the automatic protein sequencer were obtained from Beckman Instruments. All other chemicals were of reagent grade or equivalent.

Abbreviation: pNA, *p*-nitroanilide.

The Trasylol-Sepharose affinity column was prepared as previously described [2]. Disc gel electrophoresis was performed by the method of Brewer and Ashworth [3]. The esterolytic activity of trypsin and chymotrypsin were measured by the spectrophotometric method of Schwert and Takenaka [4] using Bz-L-ArgOEt and NAc-L-TyrOEt as substrates respectively. Assays were performed at 25° C in 0.1 M Tris · HCl, pH 8.0, containing 0.05 M $CaCl_2$. A unit of activity was defined as a change of one absorbance unit/min. The substrate $Suc(Ala)_3$-pNA was used to assay for elastase activity according to the procedure of Bieth et al. [5].

Amino acid analysis was performed on a Beckman model 120C. Samples were hydrolyzed in vacuo with 6 M HCl for 24 and 72 h. Half-cystine was determined as cysteic acid by the method of Hirs [6]. The sample for tryptophan analysis was hydrolyzed in the presence of 4% thioglycolic acid according to Matsubara and Sasaki [7]. The values for valine and isoleucine were taken from the 72 h analysis. Values for serine and threonine were obtained by extrapolation to zero time.

Ultracentrifugation was performed in a Beckman Model E equipped with absorption optics and scanner. The molecular weight was determined by low speed sedimentation equilibrium at 13 000 rev./min by the method of Edelstein and Schachman [8]. The $E^{1\%}_{280\ nm}$ of the protein was obtained by the method of Babul and Stellwagen [9].

Amino acid sequence analysis was performed with a Beckman 890C protein sequencer using the 0.1 M Quadrol program of Brauer et al. [10]. Residues were identified by amino analysis after back hydrolysis with 6 M HCl, 0.1% $SnCl_2$ at 150° C for 4 h according to Mendez and Lai [11]. We found this method to be much superior to HI hydrolysis.

Results

The rabbit pancreas, which is a "diffuse brownish mass lying in the mesoduodenum" [12], was excised from animals killed for other organs, and stored

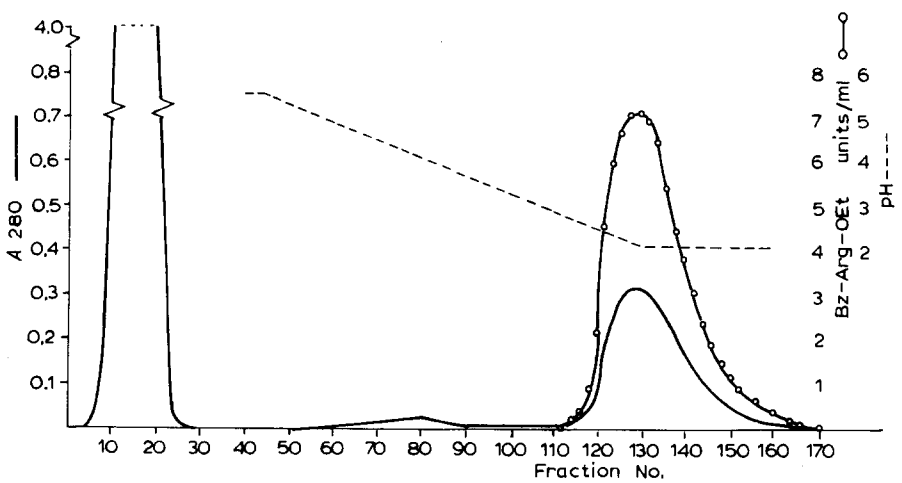

Fig. 1. Chromatography of crude, activated rabbit pancreatic extract on Trasylol-Sepharose affinity column (3.5 × 12 cm) developed with a linear gradient of decreasing pH.

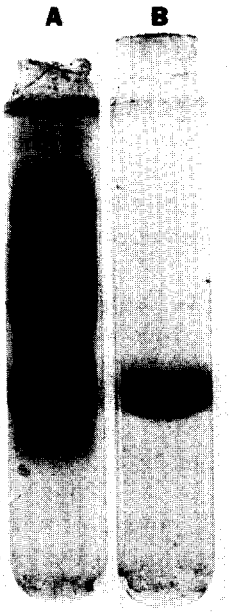

Fig. 2. Polyacrylamide disc gel electrophoresis at pH 4.3 of (A) crude extract and (B) pure rabbit trypsin.

at −15°C until needed. The frozen glands (156 g) were cut into small pieces and homogenized in a Waring blender with 0.1 M Tris · HCl, pH 8.0, 0.05 M $CaCl_2$; final volume 450 ml of homogenate. The pH of the homogenate was adjusted to 8.0 and stirred overnight at room temperature. Centrifugation at 25 000 × *g* for 15 min yielded three phases; a thick lipid phase on top of a brown liquid phase with a pellet of connective tissue at the bottom. The liquid phase was removed from between the two solid phases and was found to contain activity against Bz-L-ArgOEt, NAc-L-TyrOEt, and $Suc(Ala)_3$-pNA indicating the presence of trypsin, chymotrypsin and elastase. In preliminary experiments the NAc-L-TyrOEt activity, although adsorbed to the affinity column was unstable when eluted at acid pH and was not well separated from the trypsin. Therefore the chymotrypsin activity was abolished prior to chromatography by acidification to pH 2 for 1 h, followed by re-adjustment to pH 8.0 for application to the Trasylol column.

This crude extract containing 500 units of Bz-L-ArgOEt activity was applied to a Trasylol-Sepharose column (3.5 × 12 cm) equilibrated with 0.05 M Tris · HCl pH 8.0. The column was washed with 0.05 M sodium citrate pH 5.5, 0.5 M NaCl until the 280 nm absorbance of the effluent was zero. This fall through contained only $Suc(Ala)_3$-pNA activity. The column was then developed with a 200 ml linear pH gradient from 0.05 sodium citrate pH 5.5, 0.5 M NaCl to 0.05 M citric acid pH 2.1, 0.5 M NaCl, followed by washing with the second buffer. As can be seen in Fig. 1, all of the trypsin activity was eluted under the pH gradient. The specific activity across the peak was 22 units per 280 nm absorbance unit. In the absence of Ca^{2+} the specific activity decreased to approx. 15. The purified protein gave only one band on disc gel electrophoresis at pH 4.3 (Fig. 2).

TABLE I

AMINO ACID COMPOSITION

Amino acid	Rabbit		Human	Bovine
	Value	Integer	Human	Bovine
Lysine	11.1	11	11	14
Histidine	4.1	4	3	3
Arginine	3.5	4	6	2
Aspartic acid	20.5	21	21	22
Threonine	9.7	10	10	10
Serine	31.9	32	24	33
Glutamic acid	13.7	14	21	14
Proline	8.6	9	9	9
Glycine	19.9	20	20	25
Alanine	13	13	13	14
Half-cystine	9.8	10	8	12
Valine	15.4	15	16	17
Methionine	1.8	2	1	2
Isoleucine	11.3	11	12	15
Leucine	15.4	15	12	14
Tyrosine	7.8	8	7	10
Phenylalanine	4.6	5	4	3
Tryptophan	3.6	4	3	4

The homogeneity of the preparation was further evidenced by a linear log conc. vs. R^2 plot of sedimentation equilibrium data. The molecular weight calculated from these data is 21 700, which is only slightly less than that of other mammalian trypsins. The $E^{1\%}_{280\ nm}$ was determined to be 9.9. The amino acid composition is given in Table I along with that of bovine [13] and human trypsin [14] for comparison. The amino acid content does not vary markedly from that of other trypsins and closely agrees with that of human except for serine and glutamic acid, which more closely approximates that of bovine trypsin. The molecular weight calculated from the amino acid analysis is 22 000 which is in good agreement with sedimentation equilibrium data.

Automated sequence analysis was performed on 7 mg of protein (300 nmol). The initial yield was 50% with a repetitive yield of 96%. No amino acid was detected on analysis of residue seven, but this residue is cysteine in porcine trypsin and is probably the same for rabbit. Back hydrolysis of the cysteine thiazolinone apparently does not yield an amino acid, as no unusual peaks were seen. The amino terminal sequence is quite homologous with that of other mammalian pancreatic proteases. The sequence of the first ten residues in comparison with the sequence of porcine trypsin [15] is shown in Table II. The only sequence difference is seen at residue number eight, where Glx was found relative to Ala in porcine and Gly in bovine.

TABLE II

RABBIT TRYPSIN SEQUENCE

	1	2	3	4	5	6	7	8	9	10
Rabbit	Ile	Val	Gly	Gly	Tyr	Thr	(Cys)	Glx	Ala	Asx
Porcine	Ile	Val	Gly	Gly	Tyr	Thr	Cys	Ala	Ala	Asn

As an additional indication of purity, only isoleucine was observed in the analysis of residue number one. This is evidence that the purified rabbit trypsin is a β-trypsin (a single polypeptide chain). With antisera to rabbit trypsin prepared in guinea pigs, the Ouchterlony double diffusion technique [16] showed no cross-reactivity against either bovine, or human trypsin. However, a cross-reaction was seen against porcine trypsin, but no cross-reaction could be detected with either crude or purified rabbit acrosomal proteinase [17].

Discussion

The pancreatic endopeptidase system of the rabbit is apparently very similar to that found in other mammalian species. Elastolytic and chymotryptic activities were found in addition to trypsin, which was purified. Rabbit chymotrypsin displays an unusual lack of stability at low pH. However, the trypsin is quite similar to other cationic mammalian trypsins. Although it differs slightly in molecular weight and amino acid content, the amino terminal sequence is homologous with that of other mammalian trypsins.

Stambaugh and Smith [1] have reported that rabbit acrosomal proteinase immunologically cross-reacts with bovine trypsin by the Ouchterlony technique, but no cross-reaction was found between rabbit acrosomal proteinase and rabbit trypsin anti-sera. Neurath et al. [18] reported that anti-sera to bovine trypsin precipitated only 30% of porcine trypsin, and 15% of dog fish trypsin. Therefore the data presented here suggests that rabbit trypsin bears more homology to other trypsins than to rabbit acrosomal proteinase. However, a positive immunological cross-reaction requires considerable homology, while the absence of a cross-reaction does not infer the absence of homology. The only solution to the question lies in amino acid sequence analysis.

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