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CRYSTALLIZATION AND AMINO ACID COMPOSITION OF A SERINE PROTEASE FROM RAT SKELETAL MUSCLE

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SUMMARY

A serine protease from rat skeletal muscle was crystallized in good yield, and the homogeneity of the preparation was proved by ultracentrifugical analysis and polyacrylamide disc electrophoresis. The $S_{20,w}$ value of the enzyme was 2.2 S and the molecular weight was calculated to be 22,000-24,000 from the results of sedimentation equilibrium analysis. Analysis showed 87% coincidence in the amino acid composition with that of a serine protease from the small intestine. The apparent Km and $k_{cat}(sec^{-1})$ values for N-acetyl-L-tyrosine ethyl ester were 1.1×10^{-3} M and 9.0, respectively.

Previously, we isolated three different intracellular serine proteases from rat skeletal muscle, liver and small intestine, respectively, [1] and postulated that these proteases regulate the initial degradation of intracellular proteins [2-4]. Recently, Woodbury et al. [5] determined the covalent structure of a serine protease from small intestine and established its homologous relation to chymotrypsin, trypsin and elastase. The present paper reports the isolation, crystallization and some physicochemical properties, including the amino acid composition, of the serine protease from skeletal muscle. The amino acid composition of the enzyme was compared with those of various other serine proteases, such as chymotrypsin, trypsin, elastase and the serine protease from small intestine.

MATERIALS AND METHODS

Male Wistar-strain rats weighing 250 to 300 g were used as a source of serine protease. N-Acetyl-L-tyrosine ethyl ester (ATEE) was obtained from Sigma Chemical Co. p-Toluene sulfonic acid and 3-(2-amino-ethyl) indole were

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obtained from Pierce Chemical Co. All other reagents were commercial products of the highest grade available.

The activities of the serine protease on protein substrate and synthetic substrate were assayed as reported previously [1]. One unit of serine protease was defined as the amount inactivating 50% of the substrate enzyme, apo-ornithine aminotransferase from rat liver, in 30 min or as the amount hydrolyzing 1 μmole of ATEE per min at 37°C. Protein concentrations were determined by the method of Lowry et al. [6] using bovine serum albumin as the standard. Quantitative amino acid analysis was performed by the method of Liu and Chang [7] using 3 N p-toluene sulfonic acid containing 0.2% 3-(2-amino-ethyl) indole.

RESULTS AND DISCUSSION

Purification of the enzyme - Previously we reported [1] small scale purification of the enzyme. In this work we modified the previous purification procedure to obtain sufficient enzyme for crystallization.

Step 1. Rat skeletal muscle (600 g) was homogenized with 5 vol of 0.05 M potassium phosphate buffer, pH 8.0, in a Waring blendor, and the homogenate was centrifuged at 14,000 x g for 20 min. The precipitate was homogenized with 20 vol of 0.5 M potassium phosphate buffer, pH 8.0, in a Waring blendor, and the homogenate was centrifuged at 14,000 x g for 20 min.

Step 2. The resulting supernatant was mixed with salmine sulfate (final concentration 10% w/v) and adjusted to pH 4.5 with concentrated acetic acid. The solution was centrifuged at 14,000 x g for 10 min, solid ammonium sulfate was added to the supernatant to give 75% saturation and the solution was centrifuged at 14,000 x g for 20 min. The precipitate was dissolved in a suitable volume of 0.1 M potassium phosphate buffer, pH 8.0, and dialyzed against the same buffer overnight. Then the solution was centrifuged at 14,000 x g for 20 min to remove insoluble material.

Step 3. The enzyme solution was applied to a column of hydroxylapatite (6 x 20 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 8.0. The column was then eluted with step-wise with 0.25 and 0.5 M potassium phosphate buffer, pH 8.0. The fraction eluted with 0.5 M buffer was concentrated by ultrafiltration and in a collodion bag.

Step 4. The concentrated solution was applied to a Sephadex G-100 column (3 x 100 cm) equilibrated with 0.25 M potassium phosphate buffer, pH 8.0,

and the column was eluted with the same buffer. The fraction of eluate containing the protease was concentrated to over 10 mg protein per ml in a collodion bag.

Step 5. Solid ammonium sulfate was added gradually to the concentrated solution until just before a slight turbidity appeared in the cold (about 40% saturation) and the solution was stood for 40 hours in the cold. The long rod-shaped crystals that formed are shown in Fig. 1. Representative results on the enzyme purification are shown in Table 1. A marked increase in total activity was observed during the purification. A similar increase has been observed during purification of other intracellular serine proteases [1] and has been attributed to removal of inhibitors [8].

Properties of the enzyme - The crystallized enzyme gave a single symmetrical boundary on ultracentrifugation, and the sedimentation constant was calculated to be 2.2 S at a protein concentration of 7.8 mg/ml in 0.25 M potassium phosphate buffer, pH 8.0. The molecular weight was calculated to be 22,000-24,000 from results by the sedimentation equilibrium method. On polyacrylamide gel disc electrophoresis at pH 7.5 and 4.5, the enzyme migrated to the anode as a single protein. The apparent Km and $k_{cat}(sec^{-1})$ values for ATEE were 1.1×10^{-3} M and 9.0, respectively, at 37°C in 0.15 M of potassium phosphate buffer, pH 8.0.

Amino acid composition of the enzyme - Dayhoff and Hunt [9] classified amino acids into four groups and compared the contents of these groups in various proteins, including trypsinogen, chymotrypsinogen and elastase. Using their method, we calculated the individual amino acid compositions and the contents of these groups of amino acids in the serine proteases from skeletal muscle and small intestine, and compared the values with those for trypsinogen, chymotrypsinogen and elastase. As shown in Table 2, the contents of basic amino acids in the two serine proteases are double those in trypsinogen, chymotrypsinogen and elastase. We did not measure the asparagine and glutamine contents of the serine protease from skeletal

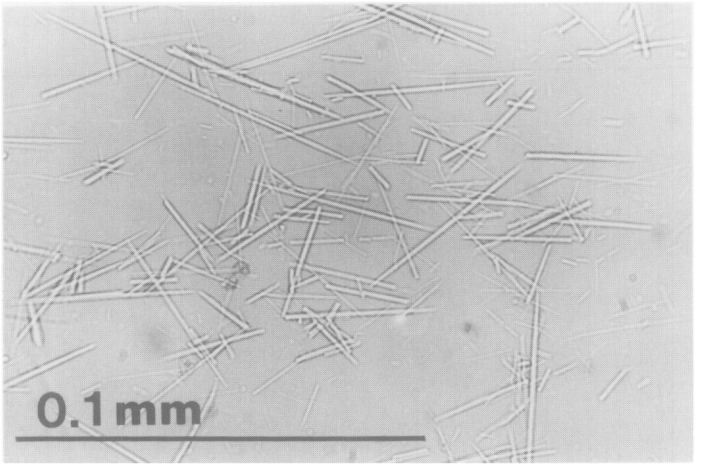

Figure 1. Photomicrograph of crystals of the serine protease from rat skeletal muscle.

Table 1. Purification of the serine protease from rat skeletal muscle

Purification step	Volume	Total protein	Total activity	Specific activity		Recovery
	ml	mg	U[a]	U/mg[a]	U/mg[b]	%
1. Crude extract	15,000	174,000	55,200	3.2	2.3	
2. Ammonium sulfate	2,000	5,500	126,340	23.0	7.5	100
3. Hydroxylapatite	2.5	24.4	75,700	3,101	553.2	60
4. Sephadex G-100	1.0	13.2	73,170	5,526	1,280	58
5. Crystallization	1.0	7.5	56,604	7,577	1,762	45

a. Units of apo-ornithine aminotransferase inactivating activity.
b. Units of ATEE hydrolyzing activity.

muscle, but its contents of aspartic acid and glutamic acid are similar to those of asparagine plus aspartic acid and glutamine plus glutamic acid, respectively, in the serine protease from small intestine. The contents of individual amino acids in these two serine proteases show a similarity of approximately 45 and 87%, within the limits of 1 and 1.5% variation, respectively. In contrast, the similarities in the amino acid compositions of serine protease from small intestine and those of trypsinogen, chymo-

Table 2. Amino acid compositions of various serine proteases[a]

		TRYPSINOGEN	CHYMOTRYPSINOGEN	ELASTASE	SERINE PROTEASE from SMALL INTESTINE[b]	SERINE PROTEASE from SKELETAL MUSCLE
Percent of Each Amino Acid	Ala	6.1	9.2	7.1	7.1	6.9
	Arg	0.9	1.8	5.0	5.4	4.7
	Asn	7.4	4.3	7.5	2.2	-
	Asp	3.9	4.5	2.5	4.0	6.9
	Cys	5.2	4.1	3.3	2.7	3.4
	Gln	5.2	4.1	6.3	2.2	-
	Glu	0.9	2.7	1.7	5.4	7.7
	Gly	10.9	9.4	10.4	8.0	9.4
	His	1.3	0.8	2.5	4.0	2.6
	Ile	6.6	3.9	4.2	8.0	4.3
	Leu	6.1	7.8	7.5	7.1	6.0
	Lys	6.6	5.1	1.2	5.8	9.4
	Met	0.9	1.2	0.8	2.2	3.4
	Phe	1.3	2.7	1.2	2.7	3.4
	Pro	3.5	4.5	2.9	6.7	7.1
	Ser	14.8	10.2	9.2	5.8	4.3
	Thr	4.4	9.4	7.9	5.8	6.9
	Trp	1.7	3.3	2.9	0.9	0.9
	Tyr	4.4	1.4	4.6	4.0	4.3
	Val	7.9	9.8	11.2	9.8	8.6
Average percent composition of amino acid groups[c]						
	Acidic	4.8	7.1	4.2	9.4	-
	Basic	8.7	7.8	8.7	15.2	16.7
	Aromatic	7.4	7.3	8.7	7.6	8.6
	Hydrophobic	22.7	25.3	25.0	29.8	25.7

a. Data on trypsinogen, chymotrypsinogen, and elastase were taken from Dayhoff and Hunt [9].
b. Calculated from the data of Woodbury et al. [5].
c. Classified as reported by Dayhoff and Hunt [9]: Acidic, aspartate plus glutamate; Basic, lysine plus arginine plus histidine; Aromatic, phenylalanine plus tyrosine plus tryptophan; Hydrophobic, leucine plus valine plus isoleucine plus phenylalanine plus methionine.

trypsinogen and elastase, respectively, were approximately 29 and 45%, 31 and 43%, and 26 and 47%, within the same limits of variation.

Recently, Woodbury et al. [5] determined the complete covalent structure of the serine protease from small intestine and found that the degrees of sequence identity of this enzyme with bovine chymotrypsin, trypsin, and porcine elastase are 33, 34, and 35%, respectively; the

homology of sequence structure was mainly found in the region of the active site structures and in the terminal sequences and Woodbury et al. [5] postulated that the serine protease from small intestine diverged from a common ancestor before chymotrypsin, but after trypsin [5]. Our results suggest that these two intracellular serine proteases may contain many homologous sequences besides those in the active site. Furthermore, the close similarity of the two intracellular proteases indicates that they are a different type of serine proteases from extracellular serine proteases, such as trypsin and chymotrypsin.

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