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ISOLATION AND CHARACTERIZATION OF YEAST PROTEASE B

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SUMMARY

Protease B was purified from baker's yeast. The final preparation appeared homogeneous by ultracentrifugation and electrophoresis. The $S_{20,\omega}$ value of the enzyme was 3.1 S and its molecular weight was calculated to be 31,000 from the results of sedimentation equilibrium analysis. The amino acid composition of the enzyme was also investigated. The enzyme inactivates phosphogluconate dehydrogenase and uricase, but not malate dehydrogenase, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase or hexokinase.

Three different proteases, usually designated as protease A, B and C have been found in yeast [1-3]. Hata et al. [4] report the high purifications and properties of protease A and C. Protease B has also been partially purified by Hata et al. [1] and Saheki and Holzer [5] and purified to a homogeneous state on acrylamide gel disc electrophoresis by Ulane and Cabib [6]. The present paper describes the large-scale purification and physicochemical properties of protease B.

MATERIALS AND METHODS

Activated CH-Sepharose 4B, SP Sephadex C-50, QAE Sephadex A-25, and Sephadex G-100 were products of Pharmacia Fine Chemicals Inc. p-Toluene sulfonic acid and 3-(2-amino-ethyl) indole were obtained from Pierce Chemical Co. Hide Powder Azure, D-tryptophan methyl ester, and hexokinase (type III) were obtained from Sigma Chemical Co. Phosphogluconate dehydrogenase, uricase, and malate dehydrogenase from yeast were purchased from Oriental Yeast Co., Ltd. Alcohol dehydrogenase and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim.

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Protease B activity was determined as described by Saheki and Holzer [7] using 3% hide powder azure as substrate. One unit of enzyme activity was expressed as the amount producing 1.0 of $A_{590\ nm}$ per min per 1.1 ml. The activities of phosphogluconate dehydrogenase, uricase, malate dehydrogenase, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, and hexokinase were assayed as described by Bergmeyer [8]. One unit of phosphogluconate dehydrogenase or uricase activity was expressed as the amount producing 1 µmole of product per min at 25°C. Protein concentrations were determined by the biuret method [9] or by the method of Kalcker [10]. Sedimentation equilibrium studies were carried out as described by Schachman and Edelstein [11]. Quantitative amino acid analysis was performed by the method of Liu and Chang [12]. The D-tryptophan methyl ester affinity column was prepared using activated CH-Sepharose 4B as described in the manufacturer's manual.

RESULTS AND DISCUSSION

Enzyme Purification —— Two kilograms of commercial baker's yeast was plasmolyzed at pH 7.0 and then autolyzed at pH 5 as described by Hata et al. [1]. The autolysate was dialyzed against tap water overnight and then adjusted to pH 3.8 with 5N HCl and centrifuged at 10,000 rpm for 10 min in a Sorvall RC-5 centrifuge (GS3 rotor). The precipitate was dissolved in 350 ml of 10 mM Na-phosphate buffer, pH 6.0, containing 0.1 M NaCl, adjust to pH 6.0 with 2N NaOH and centrifuged at 15,000 rpm for 5 min in the same centrifuge (SS-34 rotor). The supernatant was decanted and the precipitate was suspended in 50 ml of the same buffer and recentrifuged. The two supernatants were combined, concentrated by ultrafiltration (Amicon XM50) and dialyzed against the same buffer for 4 hours. Then the solution was applied to a QAE Sephadex column (5.5 x 12 cm) equilibrated with 10 mM Na-phosphate buffer, pH 6.0, containing 0.1 M NaCl and material was eluted with the same buffer. The eluate was concentrated by ultrafiltration (Amicon PM10), dialyzed overnight against 50 mM acetate buffer, pH 5.0, and centrifuged at 15,000 rpm for 5 min in the same centrifuge (SS-34 rotor). The resulting supernatant was applied to a SP Sephadex C-50 column (3.5 x 12 cm) equi-

Table 1. Purification of Protease B in Yeast

Purification step	Volume	Total protein	Total activity	Specific activity	Recovery
	ml	mg	Unit	Unit/mg	%
1. Plasmolysis	2,800	30,940	196.0	0.006	100
2. pH 5.0 Activation	2,800	23,800	616.0	0.026	314
3. Acid ppt.	544	7,400	761.6	0.103	389
4. QAE Sephadex	2,000	5,100	580.0	0.114	296
5. SP Sephadex	344	1,756	817.2	0.466	417
6. Affinity chromat.	140	48	168.0	3.50	86
7. Sephadex G-100	4	3.3	38.2	11.6	19

librated with 50 mM acetate buffer, pH 5.0. The column was washed with the same buffer and then material was eluted with 0.1 M K-phosphate buffer, pH 7.0. The fraction of eluate containing the protease was concentrated by ultrafiltration (Amicon PM10). Then concentrated NaCl solution was added to a final concentration of 0.5 M NaCl and the solution was applied to a D-tryptophan methyl ester affinity column (2.5 x 5.5 cm) equilibrated with 0.1 M K-phosphate buffer, pH 7.0, containing 0.5 M NaCl. The column was washed first with the same buffer and then with 0.1 M acetate buffer, pH 5.0, and the enzyme was eluted with 0.1 M acetate buffer, pH 3.8. The enzyme solution was concentrated by ultrafiltration (Amicon PM10) and in a collodion bag and applied to a Sephadex G-100 column (3 x 112 cm) equilibrated with 50 mM K-potassium buffer, pH 7.0, and eluted with the same buffer. The fraction of eluate containing the protease was concentrated by ultrafiltration (Amicon PM10) and in a collodion bag. Representative results on enzyme purification are shown in Table 1.

Properties of Protease B —— Figure 1-A shows the ultracentrifugal pattern of mercuri-protease B. The schlieren pattern obtained

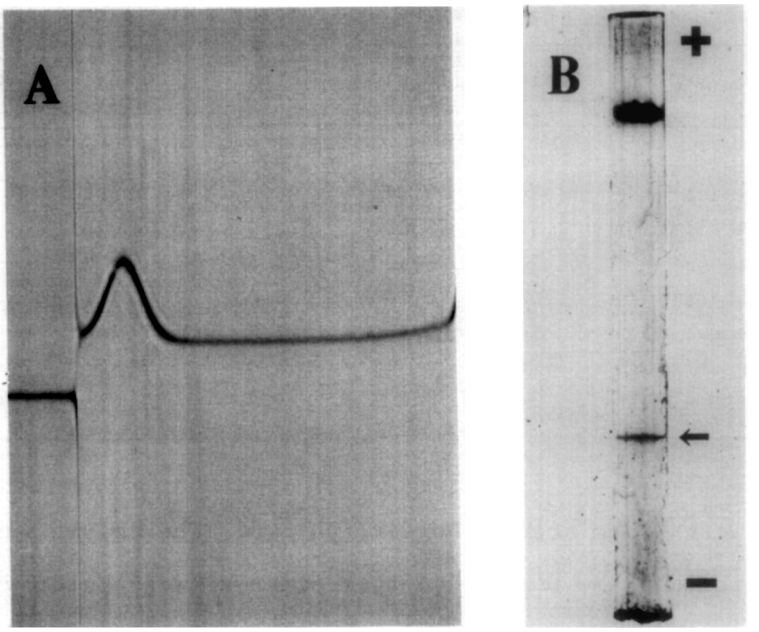

Fig. 1. Ultracentrifugal and polyacrylamide disc electrophoretic patterns of purified protease B. The enzyme was treated with 1 mM mercuric chloride and then passed through Sephadex G-25 to remove excess mercuric chloride. A, Sedimentation velocities were measured at 60,000 rev/min at 11°C. The picture was taken 36 min after attaining maximal speed. The protein concentration was 5.6 mg/ml in 50 mM K-phosphate buffer, pH 7.0. B, Electrophoresis of 50 µg of mercuri-enzyme at pH 4.5 in the presence of 4 M urea. The arrow indicates the position of marker dye.

indicates the presence of a single component. The sedimentation coefficient of the protein peak, calculated for water at 20°C, was 3.1 S. The homogeneity of the mercuri-enzyme was also confirmed by polyacrylamide gel disc electrophoresis in the presence of 4 M urea as shown in Fig. 1-B. The molecular weight of the enzyme was determined to be 31,000 by sedimentation equilibrium analysis. This value was consistent with the values determined by two other methods: the molecular weight of native protease B was estimated as 30,700 by Sephadex G-100 column chromatography and that of the mercuri-enzyme as 32,000 by sodium dodecylsulfate polyacrylamide gel electrophoresis. These values

Table 2. Amino Acid Composition of Protease B

Amino acids	g	No. of residues
	100g protein	31,000g protein
Lysine	7.09	15.0
Histidine	2.80	5.6
Arginine	3.16	5.6
Tryptophan	1.06	1.7
Aspartic acid	12.65	29.5
Threonine[a]	6.85	17.9
Serine[a]	7.41	21.9
Glutamic acid	6.71	14.2
Proline	3.20	8.6
Glycine	2.12	8.8
Alanine	7.63	26.6
Half-cystine	0.76	0.9
Valine	4.14	11.0
Methionine	0.94	2.0
Isoleucine	3.61	8.6
Leucine	7.35	17.4
Tyrosine	4.19	7.1
Phenylalanine	3.84	7.2

The values are the average of the 24, 48, and 72 hours hydrolyses.
[a]Extrapolated to zero hour hydrolysis.

were much less than that reported by Ulane and Cabib [6]. Table 2 shows the amino acid content of the enzyme. In addition it contained relatively high content of glucosamine and galactosamine.

Inactivation of Yeast Enzymes —— Previously, Cabib and Ulane [13], and Hasilik and Holzer [14] reported that the physiological function of protease B was the activation of pre-chitin synthase by limited proteolysis. The purified protease does not contain either protease A or C activity. Therefore, in an attempt to investigate the catabolic role of protease B in yeast, we examined the actions of the preparation on various commercially available yeast enzymes. Figure 2 showed its actions on phosphogluconate dehydrogenase and uricase from yeast. The purified protease B was very unstable and lost 50% of its activity autocatalytically in 15 min at 37°C. However, it did not lose any activity in 30 min at 25°C. Therefore, all experiments were carried out at 25°C. Under these conditions, one unit of

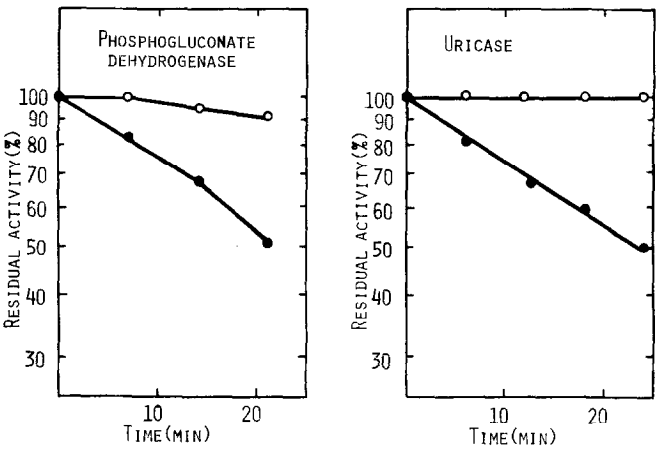

Fig. 2. Inactivation of phosphogluconate dehydrogenase and uricase by protease B. Phosphogluconate dehydrogenase (0.69 units) and uricase (0.24 units) were incubated with (●) or without (○) 0.12 and 0.15 units, respectively, of protease B in 0.6 ml of K-phosphate buffer, pH 7.0, at 25°C.

protease B inactivated 0.04 units of phosphogluconate dehydrogenase and 0.12 units of uricase per minute. It did not inactivate malate dehydrogenase, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, or hexokinase. These results suggest that protease B has a rather specific action on intact protein substrates, and thus that it participates in intracellular particular protein regulation in yeast.

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