Purification of Yeast Proteinases

Part I. Fractionation and Some Properties of the Proteinases*

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Three types of proteinases contained in baker's yeast were fractionated and partially purified by chromatography on TEAE-cellulose and on DEAE-Sephadex. These enzymes were designated as proteinase A, B and C, and some properties of proteinase A and B were described.

A was similar to pepsin and "acid-proteinase" produced by various fungi, but did not hydrolyze carbobenzoxy-L-glutamyl-L-tyrosine. B had milk-clotting activity and proteolytic activity toward casein, and also esterolytic activity toward N-acetyl-L-tyrosine ethylester and α -N-benzoyl-L-arginine ethylester. This enzyme was inhibited by *p*-mercuribenzoate and diisopropylphosphorofluoridate.

INTRODUCTION

It has long been known that some proteinases and peptidases are contained in yeast cells and can be liberated after the cells are broken by autolysis.¹⁾

Yeast exopeptidases were studied in detail and the characteristics of each enzyme have been clarified by Grassmann et al.,^{2~4)} Johnson⁵⁾ and Felix et al.⁶⁾ On the other hand, yeast proteinases have not been made clear and inconsistent results have been obtained. These enzymes were described first by Dernby⁷⁾

as "Hefetryptase" and "Hefepepsin", with optimum pH at 7.0 and 4.5, respectively. According to Grassmann et al.,^{2~4)} yeast contained only one proteinase similar to papain, which was most active to many proteins at pH 5.0. Thereafter, Hecht and Civin⁸⁾ described that pepsin-like proteinase acting at pH 1.8 was present in the yeast autolysate.

Recently, Lenney⁹⁾ proved that the yeast proteinase consisted of two enzymes, measuring the activity by the method of Kunitz.¹¹⁾ He reported that the two proteinases, A and B, were liberated by the autolysis of four different strains of *Saccharomyces cerevisiae* with chloroform. A exhibited an optimal pH of 3.7 for acid-denatured hemoglobin and was extremely labile in urea solution. On the other hand, B exhibited an optimal pH of 6.2 on urea-denatured proteins and was stable in urea solution and inhibited by sulfhydryl reagents. But purification and further characterization of these enzymes have not yet been performed by any authors.

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In the present series of studies,¹⁰¹ proteinases of yeast have been fractionated by chromatography on TEAE-cellulose and DEAE-Sephadex columns, and separated into three enzymes which were designated as yeast proteinase A, B and C.

MATERIALS AND METHODS

Materials. Compressed baker's yeast was obtained from Oriental Yeast Company. Hammarsten's casein was purchased from E. Merck AG., Darmstadt. Hemoglobin was a product from Difco Laboratories. N-Acetyl-L-tyrosine ethylester (ATEE), α -N-benzoyl-L-arginine ethylester (BAEE) and other synthetic substrates were purchased from Mann Research Laboratories, Inc. and used without any further purification. Diisopropylphosphorofluoridate (DFP) was purchased from Sigma Chemical Company. Soy bean trypsin inhibitor was obtained from Nutritional Biochemical Corporation. *p*-Mercuribenzoate (PCMB) was used after recrystallization.

Measurement of enzyme activity. Proteolytic activity was measured by the method of Kunitz¹¹⁾ and Hagihara et al.¹²⁾ with the modification for micro scale. The incubation mixture contained 2 ml of protein solution and 0.4 ml of enzyme solution. After incubating at 25°C for thirty minutes, 2 ml of 0.44 M trichloroacetic acid was added and the absorbancy at 280 m μ of trichloroacetic acid-soluble products was measured with Shimadzu Model QR-50 spectrophotometer. The proteolytic activity in each purification step was measured by using 0.5% casein solution as the substrate, at pH 6.0, because the optimal conditions of each enzyme were not known before the individual enzyme has been purified. So, after each enzyme was purified, the activity was assayed at optimal conditions of the individual enzyme by using 1% casein or 1% acid-denatured hemoglobin solution (HCl-Hb)¹³⁾ as the substrate. The esterolytic activity was determined by pH-stat method using Radiometer Model SBR2/SBU1/TTT1 titration equipment with 0.01 N NaOH as the titrant. Reaction mixture contained enzyme, 10 mM ATEE or BAEE, and 100 mM

KCl in 2.5 ml of the total solution. It was held at 25°C with circulating water and kept under nitrogen gas stream.

Determination of protein concentration and nitrogen content. Protein concentration was determined by the method of Lowry et al.¹⁴⁾ using bovine serum albumin as the standard. Nitrogen content was measured by micro-Kjeldahl method.

RESULTS

1. Fractionation and Purification of the Proteinases.

a) Autolysis and preparation of the crude extract. Autolysis and the extraction of yeast proteinases were carried out as described by Lenney⁹⁾ with a slight modification. Twenty pounds of compressed baker's yeast were plasmolyzed with 4,000 ml of chloroform. After thirty to sixty minutes at room temperature, 4,000 ml of distilled water was added and the pH was adjusted to 7.0 with 1 N NaOH. After the autolysis had been carried out at about 25°C overnight, the suspension was centrifuged at 3,000 r.p.m. for twenty minutes and the supernatant solution was adjusted to pH 5.0, then allowed to stand under toluene at about 25°C overnight. After centrifugation, the supernatant solution (7.4 l, 15.66 mg of protein per ml) was obtained as the crude extract.

b) Fractionation with ammonium sulfate. Since most proteins and proteinases in the crude extract did not precipitate with less than 75% saturation of ammonium sulfate, the crude extract was fractionated by adding ammonium sulfate to 90% saturation at pH 5.0, and was centrifuged at 15,000 r.p.m. for thirty minutes with batch rotor of Spinco Model L ultracentrifuge. The precipitate was dissolved in small amounts of water and dialyzed against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride at 5°C overnight. Dialyzed solution (1,200 ml, 8.25 mg of protein per ml), after centrifuga-

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tion to remove small amounts of precipitates, was passed through TEAE-cellulose column (6.5×24 cm, volume 800 ml) equilibrated with the same buffer as dialyzing solution in order to separate large amounts of nucleic acids and their related compounds. Then, the protein was eluted with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.4 M sodium chloride, 500 ml of the first eluate was discarded and next 1,750 ml of the eluate (5.5 mg of protein per ml) was collected. This eluate was concentrated with carbowax in a cold room and then dialyzed against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride at 5°C for twenty four hours.

c) Preliminary experiments to detect various proteinases. Ten ml of dialyzed solution was applied on TEAE-cellulose column (1×5 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) and eluted by increasing stepwise the concentration of sodium chloride from 0 to 0.4 M in sodium phosphate buffer (pH 7.0). Casein digestion at pH 6.0 with and without 10^{-3} M mercuric chloride was tested for each fraction. The result is shown in Fig. 1.

As the results, two fractions exhibiting proteinase activity, one of which was not inhibited and the other was inhibited by mercuric

FIG. 1. Preliminary Chromatography on TEAE-Cellulose Column (1×5 cm).

(○—○) Protein (●---●) Activity (—HgCl₂)
(△---△) Activity (+HgCl₂) (—) NaCl conc.

chloride, were fractionated. Thus, at least two proteinases were found in this dialyzing solution.

d) Chromatography on TEAE-cellulose. Dialyzed solution (125 ml, 52.5 mg of protein per ml) was applied on TEAE-cellulose column (3.8×32 cm) equilibrated with the same buffer as the dialyzing solution. The elution was performed by increasing logarithmically the concentration of sodium chloride from 0.1 M to 0.4 M in 0.01 M phosphate buffer (pH 7.0). A typical elution pattern is shown in Fig. 2.

FIG. 2. The First Chromatography on TEAE-Cellulose Column (3.8×32 cm).

(—) Protein (---) Activity (----) NaCl conc.

As the results, two fractions exhibiting strong proteolytic activity were obtained. One of them, which was eluted in the "break-through" fraction, showed strong activity on casein digestion at pH 6.0 and also milk-clotting activity. However, these activities were lost mostly in standing at 5°C or even in freezing at -17°C overnight. As described later, only proteinase B showed these activities, but further purification was impossible owing to the instability of the enzyme. The other fraction exhibiting proteolytic activity was pooled as indicated with an arrow in Fig. 2 and further purification was performed. The collected solution (720 ml, 0.82 mg of protein per ml) was precipitated by adding ammonium sulfate to 90% saturation at pH 5.0. After the precipitate was dissolved in a small amount of water, the solution was dialyzed in the same

way as described before the chromatography. The dialyzed solution (55 ml, 10.2 mg of protein per ml) was applied to the TEAE-cellulose column (2.1×25.8 cm) and rechromatographed by the same method. As shown in Fig. 3, a single component of protein was obtained on this chromatography. The fraction (380 ml, 1.1 mg of protein per ml), pooled

as indicated with an arrow, was precipitated with 90% saturation of ammonium sulfate. After the precipitate was dissolved in a small amount of water, the solution was dialyzed against 0.01 M sodium phosphate buffer (pH 7.0).

This dialyzed fraction showed proteolytic activity on casein from pH 5.2 to pH 6.0, and also exhibited strong esterolytic activity on ATEE at pH 8.0. The proteolytic activity was partially inhibited by both 10^{-3} M PCMB and 10^{-3} M DFP, while esterolytic activity was completely inhibited with these reagents. These facts suggest that this fraction still contained at least two proteinases. So, further fractionation was conducted.

e) Chromatography on DEAE-Sephadex A-50. The dialyzed solution (9 ml, 14.3 mg of protein per ml) was applied on DEAE-Sephadex A-50 column (0.8×32 cm) equilibrated with the same buffer as the dialyzing solution, and developed by increasing linearly the concentration of sodium chloride from 0.1 M to 0.45 M in 0.01 M phosphate buffer (pH 7.0).

FIG. 3. The Second Chromatography on TEAE-Cellulose Column (2.1×26 cm).

(—) Protein (○---○) Activity (---) NaCl conc.

FIG. 4. The Third Chromatography on DEAE-Sephadex A-50 Column (0.8×32 cm)

(●—●) Protein, (○---○) Proteolytic activity, (□---□) Esterolytic activity (---) NaCl conc.

An elution pattern is shown in Fig. 4. At least three peaks of protein were obtained, the last two showing proteolytic activity on casein, and only the last one showed estero-lytic activity on ATEE. We designated pro-teinase A for the enzyme without estero-lytic activity and proteinase C for the enzyme with estero-lytic activity.

After the two fractions were pooled as indicated with an arrow in Fig. 4, respectively, some characteristics of each enzyme were tested.

2. Characteristics of proteinase A and B

Enzymatic characteristics of proteinase A were studied with the fraction obtained by DEAE-Sephadex chromatography (see Fig. 4). This fraction exhibited a typical absorption spectrum of protein in the ultra-violet region. Absorbing maximum was at $276\text{ m}\mu$ and ratio of absorbancy at $280\text{ m}\mu$ to $260\text{ m}\mu$ was 1.41. On the other hand, characteristics of prote-inase B were investigated with the "break-through" fraction of the first chromatography on TEAE-cellulose (see Fig. 2). Because of the unstable nature of B, the above fraction was prepared in the day of the experiment.

a) **Effect of pH on activity.** The pH activity curves of A and B for hydrolysis of casein or acid-denatured hemoglobin were presented in Fig. 5 and Fig. 6, respectively. A showed an optimal pH at 2 for the hydrolysis of casein

FIG. 5. Activity-pH Curves of Proteinase A

Enzyme ($11.8\text{ }\mu\text{g}$ for casein hydrolysis; $5.9\text{ }\mu\text{g}$ acid-denatured hemoglobin hydrolysis) was assayed for 1% substrate in 0.05 M buffer as follows; glycine buffer, from pH 1.0 to 3.5; acetate buffer, from pH 4.0 to 5.5, or phosphate buffer, from pH 6.0 to 7.5.

(●—●) Hydrolysis of casein
(○—○) Hydrolysis of HCl-Hb

FIG. 6. Activity-pH Curves of Proteinase B.

Enzyme ($540\text{ }\mu\text{g}$) was assayed for 1% casein in 0.05 M borate buffer from pH 8.0 to 11.0. Other buffers are the same as in Fig. 5.

FIG. 7. Effect of Enzyme Concentration of Proteinase A and B on the Hydrolysis of Proteins

Each enzyme was assayed at the optimum pH, using 1% of casein or acid-denatured hemoglobin as the substrate. The experimental details are described in the text.

(○—○) Hydrolysis of HCl-Hb
(●—●) Hydrolysis of casein

and at 3 for the hydrolysis of acid-denatured hemoglobin. Optimal pH of B was at about 9 for the hydrolysis of casein. As shown in Fig. 7 relationships between the activity at optimum pH of each enzyme and the enzyme concentration showed linearity until optical density of trichloroacetic acid-soluble products exhibited about 0.3 for both enzymes. Proteinase A showed stronger activity on acid-denatured hemoglobin than on casein.

b) Effect of pH and temperature on the stability of the enzymes. Stability-pH curves of proteinase A and B at various temperature were illustrated in Fig. 8 and Fig. 9, respectively. A was stable from pH 5 to 6 after fifteen hours incubation at 25°C, and about 90% of the initial activity remained at pH 5 after thirty minutes incubation at 50°C. Furthermore, this enzyme was stable under freezing at least for six months. On the other hand, partially purified B was very unstable enzyme. As shown in Fig. 9 and Fig. 10, only 40% of initial activity of B remained at the most, after preincubation at 37°C for thirty minutes. Although stabilizing effects of many metals (Ca^{+2} , Mg^{+2} , Ba^{+2} , Mn^{+2} , Fe^{+2} and Co^{+2}), EDTA and L-cysteine, which were known to stabilize

FIG. 8. Stability-pH Curves of Proteinase A.

Proteinase A (5.8 μg) was preincubated in 0.4 ml of 0.05 M buffer solution at various conditions, and then remaining activity was assayed as described in the text.

- (○—○) Preincubation at 25°C for 30 min.
- (●—●) Preincubation at 25°C for 15 hr.
- (△---△) Preincubation at 45°C for 30 min.

FIG. 9. Stability-pH Curves of Proteinase B

Proteinase B (580 μg) was preincubated and assayed at the same conditions as in Fig. 8.

- (○—○) Preincubation at 25°C for 30 min.
- (●—●) Preincubation at 37°C for 30 min.

FIG. 10. Rate on Inactivation of Proteinase B at Different Temperatures.

Proteinase B (624 μg) was incubated in 0.05 M phosphate buffer (pH 6.0), at various temperatures and then remaining activity was assayed as described in the text.

- (○—○) Preincubation at 25°C
- (●—●) Preincubation at 37°C
- (△---△) Preincubation at 45°C

some enzymes, were tested at various concentrations, these materials showed no effect on the stability of B.

c) Effect of various inhibitors and activators on proteinase A and B. After the preincubation of A or B with various inhibitors and activators at pH 6 for thirty minutes, remaining activities of these enzymes were determin-

TABLE I. EFFECT OF VARIOUS INHIBITORS ON PROTEINASE A AND B

Inhibitors	Final Concentration (M)	Remaining Activity (%)	
		Proteinase A	Proteinase B
None	—	100	100
DFP	10^{-3}	95	0
	10^{-4}	100	5
PCMB	10^{-3}	102	0
	10^{-4}	101	0
Iodoacetoamide	10^{-3}	98	98
	10^{-4}	95	—
HgCl ₂	10^{-3}	97	9
Soy-bean trypsin inhibitor	0.02%	101	93

Proteinase A (5.9 μ g) or proteinase B (488 μ g) was preincubated with and without inhibitor in 0.05 M phosphate buffer (pH 6.0) at room temperature for 30 minutes and then remaining activities were assayed by the method of protein digestion as described in the text.

ed and the results are shown in Table I. A was not inhibited by DFP, sulfhydryl reagents and soybean trypsin inhibitor. EDTA and urea (final concentration was 6 M) did not show any effect on the activity of A. On the other hand, B was strongly inhibited by both

DFP and sulfhydryl blocking reagents. However, L-cysteine (10^{-3} M) and 2,3-dimercaptoethanol (10^{-3} M), which are known to activate sulfhydryl enzyme like papain, did not activate this enzyme.

d) Action of proteinase A and B on various synthetic substrates. Hydrolyzing activities of A and B on various synthetic substrates were examined by detecting the products by thin-layer chromatography or titration method. A did not hydrolyze glycylglycine, L-leucylglycine, L-leucylglycylglycine, L-alanyl-glycylglycine, BAEE, N-acetyl-L-tyrosine amide, and carbobenzoxyglycyl-L-leucine. However, ATEE, carbobenzoxy-L-glutamyl-L-tyrosine, and carbobenzoxyglycyl-L-phenylalanine were slowly hydrolyzed by this enzyme and these hydrolyzing activities were completely inhibited by DFP and PCMB. So, these activities were explained as due to the contamination of proteinase C as described in the next paper.¹⁰⁾ As shown in Fig. 11, B showed weakly esterolytic activity on ATEE and BAEE at pH 8.0. K_m values for BAEE and ATEE were 6.5 mM and 30~40 mM, respectively, while specific activity on both substrates showed

mostly the same value ($0.21 \sim 0.23 \mu\text{moles/minute/ml}$ of enzyme solution). These estero-lytic activities of B were completely inhibited by DFP and PCMB.

e) Milk-clotting activity of proteinase B.

Milk-clotting activity was the marked characteristics of B which was not found in the cases of A and C. Usually, this activity is determined by measuring the time until particles of casein are formed on the wall of the test tube, using skim milk or dry milk as the substrate.¹⁵⁾ However, such activity of B was so weak and long time was needed to produce particles of casein. Thus the clotting-activity

of B was determined according to the method as described next, which was based on the fact that this enzyme produces turbidity in casein solution at pH 6.0. Assay mixture, which contained 0.4 ml of the enzyme solution and 2 ml of 0.6% casein solution in 0.1 M sodium acetate buffer (pH 6.0) containing 10^{-3} M CaCl_2 , was incubated at room temperature ($24 \sim 25^\circ\text{C}$), and then optical density at $610 \text{ m}\mu$ was determined with the Hirama photo-electric colorimeter. Activity was expressed as the reciprocal of the time ($1/T$) required for 0.5 of optical density at $610 \text{ m}\mu$. Although increase of absorbancy at $610 \text{ m}\mu$ was not proportional

FIG. 12. Casein-clotting Activity of Proteinase B

(A) Time courses of casein-clotting activity of proteinase B determined from optical density difference at $610 \text{ m}\mu$ for various enzyme concentration.

(B) Relationship between enzyme concentration and reciprocal of time ($1/T$) when optical density had reached to 0.5.

Protease B; (1) 0.051 (2) 0.128 (3) 0.255 (4) 0.510 (5) 0.766 (6) 1.020 mg.

TABLE II. COMPARISON OF VARIOUS ACTIVITIES OF PROTEINASE B

Preincubation	Remaining Activity (%)			
	Casein digestion	Milk-clotting activity	Esterolytic activity toward	
			BAEE	ATEE
None	100	100	100	100
At 37°C for 30 min.	38	32	15.4	16.9
With 10^{-4} M PCMB	0	7.2	1.5	3.1
With 10^{-3} M DFP	0	2.4	2.2	0

Experimental conditions are the same as in Table I and Fig. 9.

15) J. R. Whitaker, *Food Tech.*, **13**, 86 (1958).

to the time, $1/T$ was proportional to the amounts of the enzyme present, as shown in Fig. 12. According to this method, the effect of inhibitors and stability of B were tested and the results are shown in Table II.

f) Comparison of various activities of proteinase B. The stability at 37°C and the effect of DFP or PCMB on B were compared with caseinolytic activity, esterolytic activity and milk-clotting activity. As shown in Table II, the incubation at 37°C and the treatment with DFP or PCMB caused almost the same effects toward all of these activities. This fact shows that single enzyme, that is, proteinase B exhibited those three activities.

3. Characteristics of Proteinase C

The fraction of proteinase C, which was obtained from final DEAE-Sephadex chromatography (see Fig. 4), contained 0.89 mg of protein per ml and nitrogen content of this protein was 13.4%. This fraction showed strong activity on ATEE at pH 8.0 (specific activity was 66.3 μ g ATEE/minute/mg of protein). Furthermore, this enzyme hydrolyzed carbobenzoxy-L-glutamyl-L-tyrosine and carbobenzoxyglycyl-L-phenylalanine at pH 5.0. All of these activities were strongly inhibited by both DFP and PCMB as in the case of proteinase B. Detailed characteristics of this enzyme will be reported in the next paper.¹⁰⁾

DISCUSSION

According to the earlier studies on yeast proteinases, it was assumed that yeast contained two different proteinases.^{1,9)} One of them was most active at acidic pH region and the other at slightly acidic or at neutral pH region, and the latter contained essential sulfhydryl groups like papain. However, their studies had presented confused results concerning to characteristics of each proteinase, because properties of each enzyme had been discussed on yeast autolysate itself without any fractionation or purification.

In the present paper, we fractionated three types of proteinases, designated yeast pro-

teinase A, B and C, respectively, from the autolysate of baker's yeast by ion-exchange chromatography, and clarified some properties of A and B.

A had an optimal pH of 2 for casein and 3 for acid-denatured hemoglobin digestion, and was not inhibited by sulfhydryl blocking reagents like PCMB and mercuric chloride. These properties are fairly resembled to those of "proteinase A" described by Lenney.⁹⁾ However, A in the present study was stable in 6 M urea solution, whereas Lenney's proteinase A was extremely labile in urea solution. On the other hand, enzymatic properties of A were similar to pepsin¹⁶⁾ and "acid-protease"¹⁷⁾ produced by various molds, but A in the present paper could not hydrolyze carbobenzoxy-L-glutamyl-L-tyrosine.

B was very unstable and its activity was rapidly lost even at 5°C. Therefore, highly purification could not be carried out, but the comparison of stability at 37°C and inhibition by PCMB and DFP proved that proteolytic activity at pH 9, milk-clotting activity at pH 6, and esterolytic activity for ATEE and BAEE at pH 8, were shown by a single enzyme, B, which was presented in the "break-through" fraction of TEAE-cellulose chromatography. B was inhibited not only by PCMB, which generally inhibit sulfhydryl enzyme, but also by DFP, which is specific inhibitor for serine enzyme. This strange characteristics will be discussed in the next paper.¹⁰⁾ Hydrolytic properties of B toward BAEE and susceptibility to sulfhydryl blocking reagents resembled to those of proteinases of plant origin¹⁸⁾ and cathepsin B,¹⁹⁾ but similarity of specific activities toward ATEE and BAEE seems to be unique to yeast proteinase B. Furthermore, milk-clotting activity of proteinase B were

16) F. A. Bovey and S. S. Yanari, "The Enzymes", edited by P. D. Boyer et al., second ed., Academic Press, 1960, Vol. 4, p. 63.

17) B. Hagihara, *ibid.*, p. 193.

18) E. L. Smith and J. R. Kimmel, *ibid.*, p. 133.

19) J. S. Fruton, *ibid.*, p. 233.

milar to those of papain, ficin and bromelain.¹⁵⁾

Chromatographic behaviors of proteinase A and C were very similar to one another and could not be separated clearly on TEAE-

cellulose chromatography, whereas these two enzymes were fractionated on DEAE-Sephadex chromatography. The final purification and some physical properties of both enzymes will be described in the succeeding paper.