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PURIFICATION AND PROPERTIES OF PROTEINASE B FROM YEAST

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Proteinase B (EC 3.4.22.9) was purified from commercial baker's yeast and from wild type strains of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*. For large scale purification a procedure was developed involving hydrophobic chromatography on octyl-Sepharose 4B and gel filtration on Sephadex G-100. A rapid purification of small amounts of proteinase B was achieved by affinity chromatography on the nitrated proteinase B inhibitor, immobilized on CH-Sepharose according to Bünning and Holzer (Bünning, P. and Holzer, H. (1977). J. Biol. Chem. 252, 5316–5323). The enzyme prepared from all three sources appeared to be homogeneous and exhibited a molecular weight of 33 000 in SDS-polyacrylamide gel electrophoresis. Homogeneity and molecular weight were confirmed for the enzyme from baker's yeast by ultracentrifugation studies. Polyacrylamide gel electrophoresis without SDS and electrofocusing however, indicated microheterogeneity of the proteinase B activity. The aminoterminal residue of the enzyme was found to be glycine. Proteinase B turned out to be a glycoprotein, containing 8–9% neutral sugars and 1.5% amino sugars. The enzyme is blocked by *p*-hydroxymercuribenzoate and by the serine proteinase inhibitors DFP and PMSF. Among the proteinase inhibitors from microbial origin, chymostatin and antipain were the most powerful inhibitors of proteinase B.

Introduction

The vacuolar proteinase B (EC 3.4.22.9) is one of the major proteolytic enzymes in yeast [1–5]. It has been partially purified by several groups [6–8] and recently, was purified to homogeneity by Ulane and Cabib [9], Sanada et al. [10] and Looze et al. [11].

There is, however, little knowledge of the properties of this purified enzyme. We have therefore developed a rapid and convenient method for isolation of proteinase B, which is described in the present paper, and use it to demonstrate some properties of the homogeneous enzyme. Proteinase B from commercial baker's yeast is compared with the enzymes purified from wild type strains of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*.

Materials and Methods

Chemicals. Materials were obtained from the following sources: Azocoll was from Calbiochem (La Jolla, CA); *N*-acetyl-DL-phenylalaninenaphthyl ester, DFP, PMSF, TPCK, TLCK, fast garnet GBC, ultrapure guanidine hydrochloride, 5,5′-dithiobis(2-nitrobenzoic acid), dansyl chloride and standard dansyl amino acids were from Serva (Heidelberg); *p*-toluenesulfonic acid and tryptamine were from Merck (Darmstadt); *N*-benzoyl-L-tyrosine-*p*-nitroanilide, *N*-ethylmaleimide, iodoacetamide, soybean trypsin inhibitor and ovomucoid were from Sigma (München). Ficoll, dextran sulfate, DEAE-Dextran, DEAE-Sepharose, octyl-Sepharose-CL-4B, Sephadex

Abbreviations: DFP, diisopropylphosphofluoride; PMSF, phenylmethylsulfonyl fluoride; TLCK, tosyl-L-lysine chloromethylketone; TPCK, tosyl-L-phenylalanine chloromethylketone; SDS, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

G-100 and activated CH-Sepharose were from Pharmacia (Freiburg); helicase was from Industrie Biologique Française S.A. (Villeneuve-La-Garonne); growth media were from Difco (Detroit, MI); chymostatin, antipain, leupeptin, elastatinal and pepstatin were a generous gift from Professor Umezawa. 3-Nitro-Tyr^{41}-proteinase B inhibitor, immobilized on CH-Sepharose 4B, was a generous gift from Dr. P. Bünning. All other chemicals were of reagent grade and were obtained from Roth GmbH.

Yeast strains and growth conditions. Commercial baker's yeast (Pleser Hefe), obtained from BÄKO Gesellschaft eGmbH (Freiburg), was used for large scale preparations of proteinase B. *S. cerevisiae* X2 180 (a) was obtained from R. Mortimer, Berkeley. *S. carlsbergensis* NCYC 74 was obtained from the National Collection of Yeast Cultures, U.K. *S. cerevisiae* LBGH 1 022 was obtained from Dr. J. Schwencke, (Gif-sur-Yvette). This strain was used for the preparation of vacuoles. All strains were grown aerobically at 30°C on minimal medium containing 0.67% yeast nitrogen base (without amino acids) and 2% glucose.

Preparation of vacuoles. Vacuoles were prepared from *S. cerevisiae* LBGH 1 022 according to Schwencke (unpublished data) using procedures developed by Wiemken and Dürr [12], Dürr et al. [13], and Schwencke and de Robichon-Szulmajster [14], as described by Wolf and Ehmann [15]. The spheroplasts obtained by this method were incubated for further derepression of the vacuolar proteinases under starvation conditions at 30°C for 4 h. The vacuoles were broken by mild sonication (three bursts for 5 s) after suspending in distilled water. After centrifugation at 144 000 × *g* for 30 min, aliquots of the supernatant were assayed for proteinase A, proteinase B and carboxypeptidase Y. After addition of pepstatin and glycerol aliquots of the supernatant were subjected to electrofocusing.

Enzyme assays. Proteinase B was assayed with Azocoll as substrate according to Saheki and Holzer [16]. 1 unit of proteinase B is defined as the amount of enzyme that causes a change in absorbance of 1.0, at 520 nm during 30 min. Proteinase A was measured by the method of Hata et al. [6], as modified by Saheki and Holzer [16]. Carboxypeptidase Y was measured by the method of Aibara et al. [17].

Protein determination. Protein was determined by the method of Lowry et al. [18] using crystalline bovine serum albumin as standard. The concentration of homogeneous proteinase B was determined by measuring its absorbtion at 280 nm (see Results). The spectrophotometrical determination is disturbed by bound $HgCl_2$, which is used for stabilization.

Stabilization of proteinase B. To prevent self-degradation, proteinase B was inhibited by $HgCl_2$. Reactivation of the enzyme was achieved by removal of bound $HgCl_2$, with an excess of mercaptoethanol, followed by gel filtration on a Sephadex G-25 column with 50 mM Tris-HCl buffer, pH 8.0.

Purification of proteinase B. All preparations were conducted at 4°C unless otherwise stated. The standard buffer used for preparation was 0.01 M potassium phosphate, pH 7.0.

1. Homogenization of yeast cells and activation of proteinase B. 2 kg commercial baker's yeast were suspended in 2 l 0.1 M potassium phosphate buffer, pH 7.0 and homogenized by passing the suspension through a Manton-Gaulin homogenizer (Manton-Gaulin Mfg. Co. Everett, MA), five times, at 500 kg/cm^2. The effluent was cooled to 10°C and centrifuged at 27 000 × *g* for 30 min. The pH of the supernatant was adjusted to pH 5.0 with 20% acetic acid. After addition of 100 mg penicillin G and 100 mg streptomycin sulfate the extract was incubated at 25°C for 20 h.

2. $(NH_4)_2SO_4$ precipitation. After adjusting the pH of the extract to 7.0, 10 mM $HgCl_2$ (final concentration) and $(NH_4)_2SO_4$ up to 90% saturation were added. This suspension was stirred for 2 h and then centrifuged at 27 000 × *g* for 60 min. The precipitate was dissolved in standard buffer, pH 7.0, and extensively dialyzed against the same buffer.

3. DEAE-Sephadex Chromatography. DEAE-Sephadex A-50 (30 g dry weight), swollen and equilibrated in standard buffer, was added to the dialysate and stirred for 2 h. Then the suspension was poured into a column (5 × 70 cm), washed with the same buffer and eluted with a linear gradient of 0–0.4 M NaCl in standard buffer, at a flow rate of 100 ml/h. The total volume of the gradient was 3 l. The active fractions were pooled and concentrated in a dialysis bag embedded in solid $(NH_4)_2SO_4$ overnight. After centrifugation at 27 000 × *g*, for 30 min, the precipitate was dissolved in standard buffer

and dialyzed against the same buffer overnight followed by a 2 h dialysis against the equilibrium buffer of the next chromatography.

4. Chromatography on octyl-Sepharose 4B. A column with octyl-Sepharose 4B (1.6 × 25 cm) was equilibrated with standard buffer containing 25% (w/w) $(NH_4)_2SO_4$ (equilibrating buffer). After application of the sample, the column was washed extensively with the equilibrating buffer. Proteinase B was eluted with a linear gradient composed of equilibrating buffer and 10 mM potassium phosphate buffer, pH 7.0/50% ethylene glycol. The proteinase B-containing fractions were pooled and separated from ethylene glycol by gel filtration on a Sephadex G-25 column (5 × 40 cm) equilibrated with standard buffer. The protein was concentrated by adsorbtion on a DEAE-Sepharose column (1.6 × 10 cm) equilibrated with standard buffer and following desorption with 0.5 M NaCl in the same buffer. The active fractions were pooled and further concentrated in a dialysis bag embedded in flake poly(ethylene glycol) (Aquacide II).

5. Gel filtration on Sephadex G-100. The enzyme solution was applied to a column (2.6 × 90 cm) with Sephadex G-100, equilibrated with 0.02 M potassium phosphate buffer, pH 7.0/5% glycerol/0.15 M NaCl. The active fractions were pooled and concentrated in a dialysis bag embedded in flake poly(ethylene glycol).

The purification procedure described above was also used to isolate proteinase B from *S. cerevisiae* and *S. carlsbergensis* of wild type strains, grown on minimal medium. 400–500 g wet weight were used as starting material.

Purification of proteinase B by affinity chromatography. Proteinase B was purified on a smaller scale by the affinity chromatography method of Bünning and Holzer [19,38], In this case the enzyme was purified in the same manner as described above up to Step 3. The enzyme was then applied to a column (2.6 × 2 cm) containing the nitrated proteinase B inhibitor, immobilized on CH-Sepharose. The column was washed with 2 M NaCl in 0.05 M imidazole buffer, pH 7.0, and elution of proteinase B was performed with 6 M urea in 0.05 M imidazole buffer, pH 7.0, at a flow rate of 200 ml/h. The fractions containing proteinase B were diluted immediately after elution with 3 vol. 0.05 M imidazole buffer, pH 7.0/20% glycerol and dialyzed against the same buffer for 5 h. The dialysate was concentrated with flake poly(ethylene glycol).

Analytical gel electrophoresis and gel electrofocusing. Polyacrylamide gel electrophoresis was performed according to Davis [20] at pH 7.0. The gels were stained for protein with Coomassie brillant blue R-250. Proteinase B activity was detected in the gel with *N*-acetyl-DL-phenylalanine-β-naphthyl ester as substrate as described by Wolf and Fink [21]. For activity staining of proteinase B inhibited by $HgCl_2$, activation prior to electrophoresis was performed with mercaptoethanol. SDS-polyacrylamide gel electrophoresis was carried out with 12% gels according to Manson et al. [22]. Gel electrofocusing was carried out as described by Wrigley [23]. Polyacrylamide gels (7.5%) containing 1% carrier ampholyte, pH range 5–8, were run at 400 V for 4 h. After electrofocusing the gels were cut into 2-mm slices which were extracted with 0.2 ml distilled water or 0.1 M potassium phosphate buffer, pH 7.0/10% glycerol.

Molecular weight determination. The molecular weight of proteinase B was determined by SDS-polyacrylamide gel electrophoresis. Bovine serum albumin, ovalbumin, bovine heart lactic dehydrogenase, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin were used as references. From the data a linear regression line was calculated for the standard curve. Molecular weight determination by the sedimentation equilibrium method was performed in a Beckman model E analytical ultracentrifuge according to Yphantis [24], for 48 h at 16 000 rev./min and 5°C. Prior to ultracentrifugation the $HgCl_2$-inactivated proteinase (0.8 mg/ml) was dialyzed overnight against 0.02 M potassium phosphate buffer, pH 7.0/5% glycerol/0.15 M NaCl. The partial specific volume $\bar{v}$ was calculated from the amino acid composition as described by Schachmann [25] and Gottschalk [26].

Amino acid analysis. Purified proteinase B was dialyzed against distilled water. Aliquots of the dialyzed sample, each containing 0.2 mg protein, were placed in glass tubes and freeze-dried. After sealing the tubes under vacuum, the samples were hydrolyzed in 6 M HCl for 24, 48 and 72 h, respectively, at 105°C. Amino acid analysis was carried out with a Biotronik LC-6 000 amino acid

analyzer. Tryptophan was determined with the amino acid analyzer after hydrolysis in 2 M toluenesulfonic acid for 22 h and spectrophotometrically by the method of Edelhoch [27]. Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively, after oxidation with performic acid as described by Moore [28]. Cysteine was also determined spectrophotometrically in the presence of denaturant according to Ellmann [29]. Titration of thiol residues was performed in 50 mM Tris-HCl buffer, pH 8/0.5% SDS. *O*-Phosphoserine was estimated by hydrolysis in 6 M HCl under vacuum for 4 h at 110°C [30]. Correction was made for 65% loss based on the loss of *O*-phosphoserine in a control experiment.

Determination of NH_2-terminal group. The NH_2-terminal amino acid was determined by the dansylation method according to Gray [31].

Carbohydrate analysis. The neutral sugar content of proteinase B, free from bound $HgCl_2$, was determined by the phenol sulfuric acid method of Dubois et al. [32] with glucose as reference. The monosaccharides were identified after hydrolysis with 0.5 M HCl for 2 h, at 100°C, by gas liquid chromatography according to Lehnhardt and Winzler [33]. Total hexosamine was determined after hydrolysis with 4 M HCl for 4 h at 100°C with D-glucosamine as standard according to Gatt and Berman [34] and Spiro [35]. The hexosamines were identified with the amino acid analyzer. Sialic acid was determined by the method of Jourdian et al. [36] with *N*-acetylneuraminic acid as reference.

Results

Purification of proteinase B

Previous attempts to purify proteinase B on a large scale were limited by the instability of the enzyme. This difficulty was overcome by Bünning who introduced the reversible inhibition of proteinase B by $HgCl_2$ [19,37,38]. Immediate and complete reactivation of proteinase B could be achieved by reacting the inhibited enzyme with an excess of mercaptoethanol. During the purification procedure described here, proteinase B was stabilized by inhibiting with 10 mM $HgCl_2$ (final concentration) at the $(NH_4)_2SO_4$ precipitation step (Step 2 in Table I). This amount of $HgCl_2$ was usually sufficient to inhibit the proteinase completely up to the last step of purification. If, however, the enzyme still exhibited some activity, $HgCl_2$ was added again to final concentration of 1 mM.

The chromatography of proteinase B on octyl-Sepharose 4B (Step 4) and Sephadex G-100 (Step 5) is shown in Figs. 1 and 2, respectively. A summary of a representative purification of proteinase B on a large scale and by affinity chromatography is summarized in Table I and Table II, respectively. The purified enzyme could be stored at −20°C in the presence of 20% glycerol and 10^{-4} M $HgCl_2$ without loss of enzyme activity over 2 months. Freeze-thawing in the absence of glycerol resulted in total loss of activity as mentioned by Ulane and Cabib [9].

TABLE I

LARGE SCALE PURIFICATION OF PROTEINASE B FROM BAKER'S YEAST INCLUDING CHROMATOGRAPHY ON OCTYL-SEPHAROSE

Step	Total protein (mg)	Total activity (units)	Specific activity (U/mg protein)	Yield (%)	Purification factor (-fold)
Crude extract	48 800	6 348	0.13	100	1
$(NH_4)_2SO_4$	6 100	5 555	0.91	88	7
DEAE-Sephadex A-50	871	3 255	3.7	51	29
Octyl-Sepharose CL-4B	64	3 152	33	34	256
Sephadex G-100	33	1 331	40	21	310

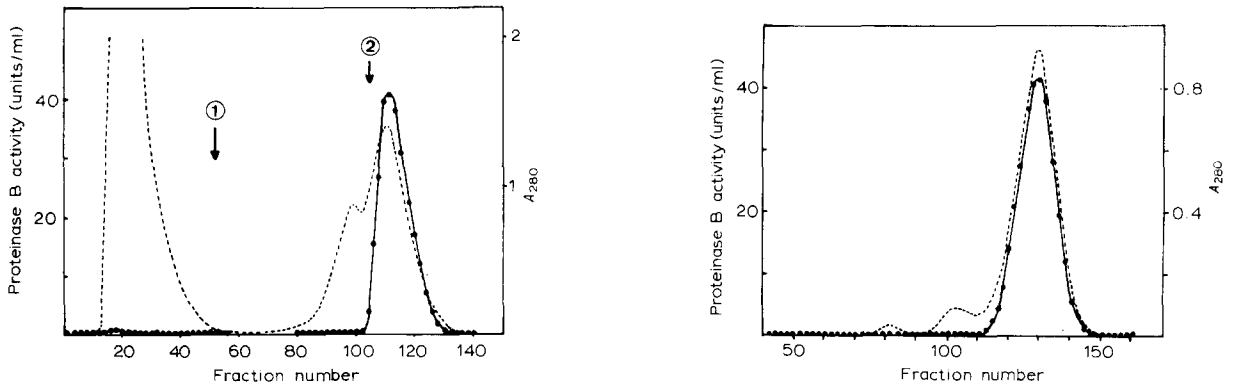

Fig. 1. Octyl-Sepharose CL-4B chromatography of proteinase B. The enzyme solution was applied on a column (1.6 X 25 cm) of octyl-Sepharose CL-4B equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, saturated to 25% with $(NH_4)_2SO_4$. After exhaustive washing, the enzyme was eluted with a linear gradient of 220 ml of the equilibrating buffer and of 0.01 M potassium phosphate buffer, pH 7.0/50% ethylene glycol. Elution was initiated at the point indicated by the arrow (1). The flow rate was 28 ml/h and fractions of 4 ml were collected. After the gradient was finished the remaining activity was completely eluted by washing the column further with 100 ml of 0.01 M potassium phosphate buffer, pH 7.0/50% ethylene glycol starting at the point indicated by the arrow (2). •———•, proteinase B activity; · · · · · ·, protein.

Fig. 2. Gel filtration on Sephadex G-100. The enzyme was applied on a Sephadex G-100 column (2.6 X 90 cm), equilibrated with 20 mM potassium phosphate buffer, pH 7.0, containing 5% glycerol and 0.15 M NaCl. Elution was performed with the same buffer at a flow rate of 20 ml/h and fractions of 2 ml were collected. •———•, proteinase B activity; · · · · · ·, protein.

Homogeneity and molecular weight of proteinase B

Purified proteinase B from commercial baker's yeast, from *S. cerevisiae* X 2180 and from *S. carlsbergensis* NCYC 74 was subjected to polyacrylamide gel electrophoresis in the presence of SDS. The enzymes from all three sources migrate as a single band with the same mobility, as shown in Fig. 3. From SDS-polyacrylamide gel electrophoresis a molecular weight of 33 000 was calculated for these enzymes on the basis of standard marker proteins. Homogeneity of the proteinase B preparation from baker's yeast was also demonstrated by the linear relationship between log A_{280} and r^2 in sedimentation equilibrium ultracentrifugation experiments. A molecular weight of 33 700 ± 900 was calculated for proteinase B, which is in good agreement with the value calculated from the SDS-polyacrylamide disc gel electrophoresis.

Amino acid composition and NH_2-terminus of proteinase B

The amino acid composition of proteinase B from

TABLE II

PURIFICATION OF PROTEINASE B FROM BAKER'S YEAST USING AFFINITY CHROMATOGRAPHY ON IMMOBILIZED NITRATED PROTEINASE B INHIBITOR

Step	Total protein (mg)	Total activity (units)	Specific activity (U/mg protein)	Yield (%)	Purification factor (-fold)
Crude extract	1940	252	0.13	100	1
$(NH_4)_2SO_4$	179	159	0.89	63	6.8
DEAE-Sephadex A-50	49.7	104	2.1	41	16.1
3-Nitro Tyr^{41} $I^{B}2$-CH-Sepharose 4B	1.1	43	39	17	308

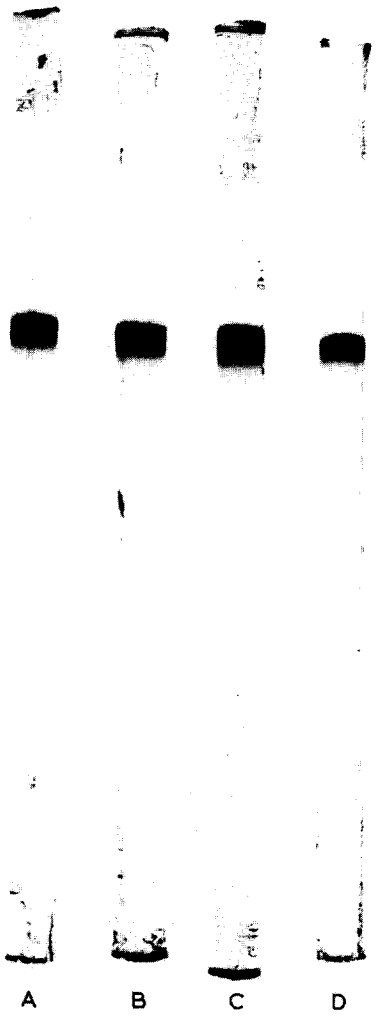

Fig. 3. SDS-polyacrylamide gel electrophoresis of proteinase B from different sources. The enzyme protein (10 μg protein each) was subjected to polyacrylamide gel electrophoresis in the presence of SDS after denaturation at 100°C, for 3 min, in 1% SDS without mercaptoethanol. Electrophoresis was carried out in 12.0% gels containing 0.1% SDS. Gels were stained with Coomassie brillant blue. A; Proteinase B from baker's yeast. B: Proteinase B from *S. cerevisiae* X2180. C: Proteinase B from *S. carlsbergensis* NCYC74. D: Mixture of equal amounts of A, B and C.

baker's yeast, from *S. cerevisiae* and from *S. carlsbergensis* is shown in Table III. After performic acid oxidation of the enzyme from baker's yeast, 1.3 mol cysteic acid/mol proteinase B were found. This could indicate 2 mol half-cystine/mol proteinase B. However, if one takes into consideration the value of 0.85 mol half-cystine, determined by titration with DTNB in the presence of 0.5% SDS, the existence of 1 half-cystine residue is more probable.

The tryptophan content of proteinase B from baker's yeast, being evaluated spectrophotometrically according to Edelhoch [27], was in good agreement with the value obtained from amino acid analysis after hydrolysis with *p*-toluenesulfonic acid (Table III). The partial specific volume of the enzyme calculated from the amino acid composition according to Schachmann [25] and Gottschalk [26]

TABLE III

AMINO ACID COMPOSITION OF PROTEINASE B FROM DIFFERENT SOURCES

The data correspond to the average of values obtained for at least duplicate acid hydrolysates of the enzyme for 24, 48 and 72 h at 105°C. (n.d., not determined.)

Amino acid	Residues/mol enzyme [a]		
	Baker's yeast	*S. cerevisiae*	*S. carlsbergensis*
Aspartic acid	44.1	44.3	40.6
Threonine [b]	21.1	20.1	18.7
Serine	23.7	23.2	24.3
Glutamic acid	18.2	19.5	19.7
Proline	10.2	10.4	10.1
Glycine	27.0	27.3	26.4
Alanine	28.0	29.4	28.2
Half-cystine [c]	1.3	n.d.	n.d.
Valine [d]	17.5	19.3	19.4
Methionine [c]	1.8	n.d.	n.d.
Isoleucine [d]	11.4	12.8	12.9
Leucine	21.1	22.0	21.5
Tyrosine	8.0	7.8	8.4
Phenylalanine	9.1	9.6	10.5
Lysine	20.4	17.3	19.8
Histidine	8.4	7.5	7.5
Arginine	6.0	5.7	6.2
Tryptophan [e]	2.3	n.d.	n.d.

[a] On the basis of a molecular weight of 33000.
[b] Values obtained from extrapolation to zero time of hydrolysis.
[c] Values determined as cysteic acid or methionine sulfone after performic acid oxidation and hydrolysis of the enzyme.
[d] Values of 72 h hydrolysis were used.
[e] Values determined after 22 h of hydrolysis with 3 N *p*-toluenesulfonic acid.

TABLE IV

CARBOHYDRATE COMPOSITION OF PROTEINASE B

n.d., not determined.

Carbohydrate moiety	Baker's yeast		*S. cerevisiae*	*S. carlbergensis*
	By weight (g/100 g)	Molar ratio (mol/mol) [a]	By weight (g/100 g)	By weight (g/100 g)
Neutral sugar	8.0	15	8.8	9.1
Mannose		15	n.d.	n.d.
Amino sugar	1.5	2.5	1.5	1.5
Glucosamine		2.1	n.d.	n.d.
Galactosamine		0.4	n.d.	n.d.
Neuraminic acid	<0.1		<0.1	<0.1

[a] On the basis of a molecular weight of 33 000.

was 0.713 $cm^3 \cdot g^{-1}$. End group analysis of proteinase B from all three sources by the dansylation method revealed glycine as the amino-terminal residue.

Carbohydrate composition of proteinase B

The carbohydrate content of proteinase B from baker's yeast, from *S. cerevisiae* and from *S. carlsbergensis* was analyzed colorimetrically. Proteinase B contained, dependent on from which source, 8.0–9.1% (w/w) neutral sugars, 1.5% (w/w) amino sugars and less than 0.1% sialic acid in all cases (Table IV). Quantitative analysis of the carbohydrate composition by gas liquid chromatography and on the amino acid analyzer revealed mannose, glucosamine and galactosamine to be present at a molar ratio of 15 : 2.1 : 0.4 in the enzyme from baker's yeast.

Microheterogeneity of proteinase B

When purified proteinase B was subjected to polyacrylamide gel electrophoresis, at pH 7.0, in the absence of denaturing agents at 4°C, at least four distinct protein bands were observed, all exhibiting *N*-acetyl-DL-phenylalanine-β-naphthyl ester hydrolyzing activity. This microheterogeneity was also observed on electrophoresis at pH 8.9 and 4.5. Moreover, electrofocusing of the homogeneous proteinase B in the pH range 5–8, displayed several active bands with esterase activity (Fig. 4B). Since in the presence of SDS only a single band appeared after electrophoresis of the enzyme, these bands are derived from enzyme species with the same molecular weight but different charges. This is confirmed by results obtained from a Ferguson plot. The spectrum of active proteinase B bands was not the same for enzyme preparations from different sources. Proteinase B purified from commercial baker's yeast and from *S. cerevisiae* X 2 180 showed similar mobilities after electrophoresis at pH 7, which could be clearly distinguished from the faster moving bands of proteinase B from *S. carlsbergensis* NCYC 74. In order to find out whether these different species arose from artefacts caused by autolysis during the preparation or existed originally in the yeast we also purified proteinase B from vacuoles.

Proteinase B from vacuoles

Earlier studies have indicated that proteinase B is loacted in the vacuoles [39]. Therefore, analysis of the purified enzyme from isolated vacuoles should show whether the enzyme exists in multiple forms as well in this subcellular organelle. The isolated vacuoles exhibited high specific activities of proteinase B (0.4 U/mg), proteinase A (11.2 U/mg) and carboxypeptidase Y (0.028 U/mg), indicating an 4–6-fold enrichment of these enzymes compared to the crude cell extract, activated at pH 5.0. The vacuolar extracts were concentrated in the presence of glycerol and pepstatin, in order to inhibit proteinase A. Without activation at pH 5.0, the extracts were then subjected to electrofocusing in polyacrylamide gels, pH range 5–8. The gels were sliced and the pro-

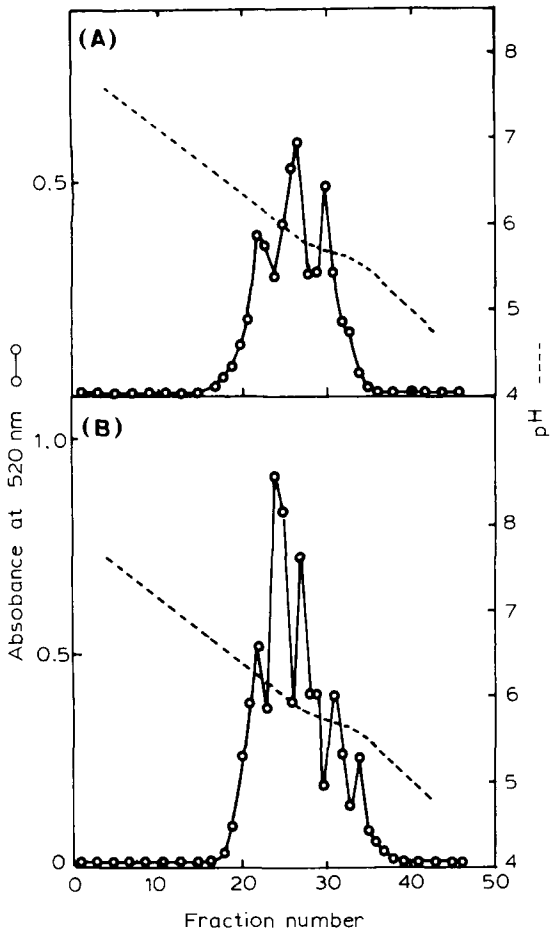

Fig. 4. Polyacrylamide gel electrofocusing, pH range 5–8, of proteinase B. A. Proteinase B purified from isolated vacuoles. Vacuoles were prepared as described in Materials and Methods, suspended in water and disrupted with mild sonication. After centrifugation of the homogenate at 14 400 × *g* for 30 min pepstatin (20 μg/ml) and glycerol (10%, w/w) were added to the supernatant. The solution was concentrated and then subjected to electrofocusing. B. Proteinase B purified from a yeast homogenate as summarized in Table I including the pH 5-activation step. In both cases gels were sliced after electrofocusing and the slices were eluted for determination of pH and activity.

teinase B activity was measured in single fractions. Distribution of proteinase B activity is shown in Fig. 4A. For comparison, the activity bands obtained after electrofocusing of proteinase B which was purified from an extract activated at pH 5.0, (see Table I) are shown in Fig. 4B. After direct purification from vacuoles activity peaks were observed at pH 6.15, 5.80 and 5.65, while the enzyme purified after activation at pH 5 exhibited peaks of proteinase activity at pH 6.15, 6.0, 5.8, 5.6 and 5.5, indicating that proteinase B also exists in multiple forms in the vacuoles.

Inhibitor studies

The results of inhibitor studies are summarized in Table V. Proteinase B was inhibited by low concentrations of chymostatin and antipain. In the case of elastatinal high concentrations were required for complete inhibition of proteinase B. Leupeptin and pepstatin showed no effect on the activity of proteinase B.

The serine inhibitors diisopropylphosphofluoride and phenylmethylsulfonyl fluoride inhibited proteinase B. Reagents that modify histidine residues such as tosyl-L-phenylalanine chloromethylketone and tosyl-L-lysine chloromethylketone had only a small effect on proteinase B, although incubation of the enzyme with these reagents was continued for 48 h at 4°C. Thiol reagents such as *p*-hydroxymercuribenzoate or $HgCl_2$ strongly depressed the activity of proteinase B, even at low concentrations. However, none of the other thiol reagents tested, *N*-ethylmaleimide and iodoacetamide, inhibited proteinase B. Proteinase B was not inhibited by the chelating agents ethylenediaminetetraacetate and *o*-phenanthroline. Among neutral proteinase inhibitors tested, ovomucoid was the most potent inhibitor depressing 80% of the original proteinase B activity at a concentration of 0.1 mg/ml. Trasylol® and soybean trypsin inhibitor were only weak inhibitors of proteinase B.

Modification of the only SH residue in proteinase B with *p*-hydroxymercuribenzoate causes total loss of activity (Table V). Thus, proteinase B requires not only a reactive serine hydroxyl group but also a sulfhydryl group. The active enzyme was incubated with $5 \cdot 10^{-4}$ M DFP in 0.05 M Tris-HCl, pH 8.0, for 30 min at room temperature. After incubation, the solution was dialyzed against water overnight and lyophilized. The *O*-phosphoserine content in the enzyme was found to be about 1 mol/mol enzyme. After DFP treatment of proteinase B 0.82 mol thiol residue/mol enzyme were titrable with DTNB in 0.5% SDS. This value is identical to that obtained with the untreated enzyme.

If, however, proteinase B was incubated first

TABLE V

EFFECTS OF VARIOUS COMPOUNDS ON PROTEINASE B ACTIVITY

Prior to treatment with different reagents, the mercury inhibited pure enzyme (cf. Table I) was activated by the addition of 5% (v/v) mercaptoethanol and passed through a column of Sephadex G-25 equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. Incubation of proteinase B with reagents was done at room temperature for 30 min. Relative activities refer to controls with solvent only.

Added compound	Concentration	Percent inhibition
	(μg/ml)	(%)
Chymostatin	0.1	29
	0.3	96
Antipain	1.0	46
	3.0	86
Elastatinal	50	72
	200	91
Leupeptin	200	0
Pepstatin	20	0
	(mM)	(%)
Phenylmethylsulfonyl fluoride	1.0	100
Diisopropylphosphofluoride	1.0	100
Tosyl-L-phenylalanine chloromethylketone [a]	1.0	34
Tosyl-L-lysine chloromethylketone [a]	1.0	2
p-Hydroxymercuribenzoate	0.1	100
Iodoacetamide	2.0	0
N-Ethylmaleimide	2.0	0
Mercury chloride	0.1	100
Ethylenediamine tetraacetate	1.0	0
o-Phenanthroline	1.0	0
	(μg/ml)	(%)
Bovine pancreas kallikrein inhibitor (Trasylol®)	200	2
	500	12
Soybean trypsin inhibitor	10	0
	100	36
Lima bean trypsin inhibitor	10	0
	100	65
Chick egg white ovomucoid	10	19
	100	79

[a] Incubation overnight at 4°C.

with $2 \cdot 10^{-4}$ M *p*-hydroxymercuribenzoate, the serine residue was no longer reactive towards DFP. The observed interferences of the DFP and *p*-hydroxymercuribenzoate reactions are noteworthy and will be discussed later.

Discussion

Until recently the instability of proteinase B, mainly caused by autodigestion, prevented its isolation on a large scale. Therefore incubation of proteinase B with $HgCl_2$ was introduced by Bünning to prevent self-degradation [37,38]. This method enabled the development of a purification procedure, yielding homogenous proteinase B in larger amounts (Table I). Proteinase B was also purified by affinity chromatography on the immobilized, nitrated proteinase B inhibitor according to Bünning and Holzer [19,37,38]. Both procedures resulted in the same specific activity of proteinase B. The affinity chromatography makes possible a rapid purification of the enzyme, but is limited by the

time-consuming preparation of the modified inhibitor and the low binding capacity of the affinity column (about 18 mg nitrated inhibitor binds about 3–4 mg proteinase B). In addition, the affinity column could be used only three times.

Proteinase B obtained by either method appeared homogeneous in SDS-polyacrylamide disc gel electrophoresis (Fig. 3) and in the ultracentrifugation experiments. Ulane and Cabib [19] reported that they could not detect any protein band after SDS-polyacrylamide electrophoresis of purified proteinase B. One explanation for this finding is, that the authors boiled the proteinase B in the presence of mercaptoethanol. Since mercaptoethanol activates the $HgCl_2$-inhibited proteinase B immediately, it is likely that the enzyme is autodigested during the heat treatment with mercaptoethanol in the SDS-containing solution. In our experiments, incubation of the enzyme in the presence of mercaptoethanol and SDS led to several bands in SDS-polyacrylamide gels, corresponding to molecular weights of less than 33 000, whereas proteinase B boiled in the absence of mercaptoethanol showed a single band corresponding to a molecular weight of 33 000 (Fig. 3). From the ultracentrifugation experiments with proteinase B a very similar molecular weight of 33 700 was calculated. The value obtained by both methods is in good agreement with the molecular weight of proteinase B as reported by Lenney and Dalbec [7], Bünning and Holzer [19] and Sanada et al. [10]. Ulane and Cabib [9] however, reported values of 43 000–44 000. These authors purified the enzyme from isolated vacuoles, while all other groups, including us, purified proteinase B from yeast extract after activation at pH 5. However, we have obtained evidence that proteinase B purified from the activated extract of vacuoles is not subjected to proteolytic degradation. At present we have no explanation for this discrepancy in the reports on the molecular weight of proteinase B.

Polyacrylamide gel electrophoresis of homogeneous proteinase B at pH 7.0 and 8.9 as well as electrofocusing in polyacrylamide gel (pH range 5–8) revealed several protein bands, all exhibiting *N*-acetyl-DL-phenylalanine-β-naphthyl ester hydrolyzing activity. Three possible mechanism may be considered to cause this multiplicity: (1) proteolytic modification of the enzyme during preparation; (2) formation of complexes of proteinase B with proteinase B inhibitors and its degradation products originating from proteolytic modification of the inhibitor during the pH 5-activation step; (3) variations in the carbohydrate composition of the enzyme. The first possibility could be excluded. Proteinase B purified without proteolytic activation from isolated vacuoles showed a similar heterogeneity after electrofocusing in polyacrylamide gels as the enzyme purified after activation at pH 5.0 (Fig. 4). The second possibility arose as a result of the report of Huse [40] who showed that activation of the inactive proteinase B-inhibitor complex with proteinase A resulted in complexes of proteinase B with various fragments of partially digested inhibitor, regaining up to 90% of proteinase B activity. Since proteinase A is known to be a major factor for the activation of proteinase B at pH 5 [4], it seems to be possible that several catalytic active complexes of proteinase B with inhibitor fragments are formed. However, the following results indicate that this is unlikely. Firstly, proteinase B purified from vacuoles showed the same heterogeneity as the enzyme purified after pH 5 activation. Since the proteinase B inhibitor is localized in the cytoplasm [42], no inhibitor was present in the vacuolar proteinase fraction, used in this experiment. Moreover, proteinase A was inhibited in this experiment by pepstatin. Secondly, recent work from this laboratory [43] and from Bünning et al. (unpublished data), showed that proteinase A splits the proteinase B inhibitor into two peptides, which are inactive towards proteinase B. From this limited fragmentation of the proteinase B inhibitor no more than two different complexes of proteinase B with inhibitor fragments would be expected. Only a single amino-terminal residue was detected in the purified enzyme, suggesting the microheterogeneity of proteinase B may result from differences in its carbohydrate content. It is well-known that even in a homogeneous state glycoproteins show multiple forms in electrofocusing experiments [44,45]. Proteinase B is a glycoprotein as discussed (Table IV). Each form found after electrophoresis at pH 7 in the absence of SDS contains a carbohydrate moiety, since gels stained with periodic acid-Schiff reagent exhibited the same pattern of bands as gels stained with Coomassie blue (data not shown). However, no direct evidence could be

obtained that the multiplicity of the enzyme is a result of the heterogeneity of the carbohydrate structure of the enzyme. Amino acid analysis revealed that proteinase B from all three sources has a quite similar composition.

The difference index (*DI*) according to Metzger et al. [46] was calculated for the different enzymes. Values between 2.45 and 2.94 were found if the amino acids methionine, cysteine and tryptophan are not considered in the calculations. A *DI* value of 0 indicates identical compositions, whereas a *DI* value of 100 is representative for two proteins without any common residue. The $S\Delta Q$ value for the different enzymes according to Marchalonis and Weltman [47] was calculated to be between 2.83 and 4.02, indicating a strong relationship of the enzymes. Results from performic acid oxidation and from DTNB-titration of the enzyme from baker's yeast give evidence that only 1 mol cysteine occurs per mol proteinase B and that the enzyme encloses no disulfide bridges (Table III). These findings agree with the amino acid composition recently reported for the enzyme from baker's yeast by Sanada et al. [10] and by Looze et al. [11]. The single SH residue is essential for catalysis, as shown by inhibition studies with *p*-hydroxymercuribenzoate (Table V). On the other hand, the catalytical activity is not affected by iodoacetic amide and *N*-ethylmaleimide (Table V). Similar observations are reported for carboxypeptidase Y [48]. The inhibition of proteinase B by DFP or PMSF indicates that in addition to the sulfhydryl residue a serine residue is essential for the catalysis. The reaction of the serine residue with DFP is blocked in the presence of *p*-hydroxymercuribenzoate, suggesting that the serine and cysteine residues are located close together in the enzyme. A similar interference of the DFP and the *p*-hydroxymercuribenzoate reaction was also observed in carboxypeptidase Y [48] and in alkaline proteinase from streptomyces [49]. Depending on the source, proteinase B contains 8–9.1% (w/w) neutral carbohydrate and additionally 1.5% (w/w) amino sugars (Table IV). In the enzyme isolated from baker's yeast, mannose, glucosamine and galactosamine occur in a molecular ration of 15 : 2.1 : 0.4. The non-stoichiometry in the last residue may reflect some heterogeneity in the carbohydrate moiety. Two other vacuolar proteolytic yeast enzymes, namely proteinase A and carboxypeptidase Y [6,50] are also glycoproteins. Proteinase A contains like proteinase B both glucosamine and galactosamine [51]. Carboxypeptidase Y differs from the proteinases A and B by the lack of galactosamine [52].

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