The purification and properties of yeast proteinase B from *Candida albicans*

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A serine proteinase (ycaB) from the yeast *Candida albicans* A.T.C.C. 10261 was purified to near homogeneity. The enzyme was almost indistinguishable from yeast proteinase B (EC 3.4.21.48), and an M_r of 30000 for the proteinase was determined by SDS/polyacrylamide-gel electrophoresis. The initial site of hydrolysis of the oxidized B-chain of insulin, by the purified proteinase, was the Leu–Tyr peptide bond. The preferential degradation at this site, analysed further with *N*-blocked amino acid ester and amide substrates, demonstrated that the specificity of the proteinase is determined by an extended substrate-binding site, consisting of at least three subsites (S_1 , S_2 and S'_1). The best *p*-nitrophenyl ester substrates were benzyloxycarbonyl-Tyr *p*-nitrophenyl ester (k_{cat}/K_m 3536000 $M^{-1}\cdot s^{-1}$), benzyloxycarbonyl-Leu *p*-nitrophenyl ester (k_{cat}/K_m 2250000 $M^{-1}\cdot s^{-1}$) and benzyloxycarbonyl-Phe *p*-nitrophenyl ester (k_{cat}/K_m 1000000 $M^{-1}\cdot s^{-1}$) consistent with a preference for aliphatic or aromatic amino acids at subsite S_1 . The specificity for benzyloxycarbonyl-Tyr *p*-nitrophenyl ester probably reflects the binding of the *p*-nitrophenyl group in subsite S'_1 . The presence of S_2 was demonstrated by comparison of the proteolytic coefficients (k_{cat}/K_m) for benzyloxycarbonyl-Ala *p*-nitrophenyl ester (825000 $M^{-1}\cdot s^{-1}$) and *t*-butyloxycarbonyl-Ala *p*-nitrophenyl ester (333000 $M^{-1}\cdot s^{-1}$). Cell-free extracts contain a heat-stable inhibitor of the proteinase.

INTRODUCTION

The proteolytic system of the yeast *Saccharomyces cerevisiae* includes four proteinases [*Saccharomyces* aspartic proteinase, commonly proteinase A (EC 3.4.23.6), yeast proteinase B (EC 3.4.21.48), proteinase D and proteinase yscE], two carboxypeptidases [carboxypeptidase Y (EC 3.4.16.1) and carboxypeptidase S (EC 3.4.17.9)] and three aminopeptidases (Wolf, 1980). Recent evidence points to the existence of many additional, as yet uncharacterized, proteolytic enzymes (Achstetter *et al.*, 1984). Carboxypeptidase Y and proteinases A and B are located in the vacuole, and the cytoplasm contains proteins (I^C , I^A , I^B) that specifically inhibit these enzymes. Carboxypeptidase S and two of the aminopeptidases are also located in the vacuole, but no cytoplasmic inhibitors of these enzymes are known. During sporulation proteinase B is required for general protein degradation and spore dispersal (Zubenko & Jones, 1981).

An extracellular aspartic proteinase (EC 3.4.23.6) produced by *Candida albicans* has been extensively studied (Remold *et al.*, 1968; Ruchel, 1981; Negi *et al.*, 1984), but the intracellular proteolytic system of this dimorphic yeast has received little attention. A proteinase with optimum activity at pH 6.6 (Chattaway *et al.*, 1971) and two cytoplasmic enzymes, a metal-ion-dependent aminopeptidase and a dipeptidase (Logan *et al.*, 1983), have been reported. The present paper reports the purification and characterization from *C. albicans* of yeast proteinase B, referred to below as proteinase ycaB to distinguish it from the enzyme isolated from *S. cerevisiae* (proteinase yscB) (Achstetter *et al.*, 1984).

EXPERIMENTAL

Materials

Radiochemicals were from Amersham International, Amersham, Bucks., U.K. All other materials were from Sigma Chemical Co., St. Louis, MO, U.S.A., unless stated otherwise. The organomercurial–agarose (Affi-Gel 501; Bio-Rad Laboratories, Richmond, CA, U.S.A.) bound 4 μ mol of 5-thio-2-nitrobenzoic acid/ml of packed gel (Sluyterman & Wijdenes, 1974). After use the Affi-Gel 501 was regenerated as described by Russell & Pollack (1978).

Triton/toluene/ethanol scintillant was prepared from 700 ml of toluene, 300 ml of Triton X-100, 100 ml of ethanol and 2.5 g of 2,5-diphenyloxazole. For samples containing trichloroacetic acid, the counting efficiency was determined by reference to a quench curve. For samples containing NaOH the counting efficiency was determined by the addition of internal standard to the sample.

SDS/polyacrylamide-gel electrophoresis

A modification (Schep *et al.*, 1984) of the method of Laemmli & Favre (1973) for SDS/polyacrylamide-gel electrophoresis was performed with the mini-gel apparatus described by Matsudaira & Burgess (1978). The separating gel was 5–15% (w/v) acrylamide. Samples (up to 15 μ l) mixed with phenylmethanesulphonyl fluoride (80 nmol) and diluted with 20 μ l of sample buffer [62.5 mM-Tris/HCl buffer, pH 6.8, containing 80 mM-2-mercaptoethanol, 3% (w/v) SDS, 2 M-urea (deionized), 10% (v/v) glycerol and 0.001% Bromophenol Blue] were

Abbreviations used: Ac, acetyl; Bz, benzoyl; Boc-, *t*-butyloxycarbonyl-; Cbz-, benzyloxycarbonyl-; -OMe, methyl ester; -OEt, ethyl ester; -ONp, *p*-nitrophenyl ester; -ONb, *p*-nitrobenzyl ester; -Nan, *p*-nitroanilide; -Nna, β -naphthylamide.

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heated at 80 °C for 3 min. Gels were stained with Coomassie Brilliant Blue, scanned, destained and stained with silver (Morrissey, 1981).

H.p.l.c.

Reverse-phase chromatography. Peptides were separated under the conditions described by Murphy *et al.* (1983) except that the solvent was a linear gradient of 0.1% (v/v) trifluoroacetic acid in 0–50% (v/v) acetonitrile.

Gel-permeation chromatography. Proteins were separated on a TSK-gel G3000SW column essentially as described by Templeton *et al.* (1984). Standard proteins used to calibrate the column were ovalbumin (M_r 45000), β -lactoglobulin (M_r 36800), carbonic anhydrase (M_r 29000) and myoglobin (M_r 16700).

Protein and carbohydrate determinations

After precipitation of the protein with trichloroacetic acid (Peterson, 1977) protein was determined by a modified Lowry method (Eggstein & Kreutz, 1967). The protein concentration of the purified proteinase preparation was measured by the method of Bradford (1976). Crystalline bovine serum albumin was used as a standard. Carbohydrate was determined by using the phenol method described by Herbert *et al.* (1971).

Peptide analysis

After dansylation (5-dimethylaminonaphthalene-1-sulphonylation) and acid hydrolysis (Gray, 1972) the dansyl-amino acids were identified as described by Tatemoto (1982). Peptide hydrolysates [110 °C for 24 h in 6 M-HCl containing 1% (w/v) phenol] were analysed as described by Templeton *et al.* (1984).

Preparation of yeast cells

Batch cultures of *Candida albicans* A.T.C.C. 10261 (American Type Culture Collection, Rockville, MD, U.S.A.) were grown aerobically on medium containing the following (per litre): glucose, 15 g; $(\text{NH}_4)_2\text{SO}_4$, 1 g; KH_2PO_4 , 2 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g; biotin, 25 μg ; peptone, 1 g. Fermentor cultures (20 litres) were inoculated with 1 litre of an 18 h batch culture and grown under the following conditions: temperature, 28 °C; aeration, 10 litres/min; stirring, 400 rev./min. Cells (average yield 290 g) were harvested as the culture was entering stationary phase.

Activation of the mercuri-proteinase

The proteinase preparation (50 μl) was incubated for 60 s with 2-mercaptoethanol (400 mM) at 37 °C in acetate buffer [50 mM-sodium acetate buffer, pH 6.0, containing 1 mM-EDTA and 10% (v/v) glycerol] (total volume 51.5 μl). It was then chromatographed on a Sephadex G-25 column (5 mm $\times$ 75 mm) in acetate buffer at a flow rate of 0.13 ml/min. The active proteinase was collected and stored on ice.

Preparation of substrates

Denatured [^{35}S]protein. Batch cultures (200 ml) were grown aerobically on the medium described above except that the $(\text{NH}_4)_2\text{SO}_4$ was replaced by $(\text{NH}_4)_2\text{HPO}_4$ (1 g), the peptone concentration was 0.2 mg/ml, the pH was adjusted to 5.0 and 0.2 mCi of $\text{Na}_2^{35}\text{SO}_4$ (3 $\mu\text{Ci}/\mu\text{mol}$) was added. Cells (about 3 g wet wt.) were harvested,

washed once with water, resuspended in 0.1 M-sodium phosphate buffer, pH 8.0, containing 10 mM-EDTA and 0.02% NaN_3 and disrupted in a Braun homogenizer (Gopal *et al.*, 1982). The debris was removed by centrifuging (1000 g for 10 min). The supernatant and resuspended pellet were re-centrifuged (1000 g for 10 min), and the combined supernatants (10 ml) were centrifuged at 60000 g for 90 min. The supernatant was made 60% (w/v) in urea and stored for at least 18 h at 4 °C. The denatured proteins were fractionated in two batches by gel-permeation chromatography at 4 °C on a Sephadex G-100 column (2.5 cm $\times$ 38 cm) in imidazole buffer (0.1 M-imidazole/HCl buffer, pH 6.6, containing 10 mM-EDTA and 0.02% NaN_3). Protein that was excluded from the column was collected and stored at 4 °C.

Denatured [^3H]acetylated bovine serum albumin. Denatured bovine serum albumin was acetylated at pH 8.0 as described by Levine *et al.* (1976) except that the pH was maintained by means of a pH-stat (end point set at 7.9).

Oxidized insulin B-chain. Insulin B-chain was prepared as described by Schuttler (1979).

Preparation of proteinase inhibitor

Stationary-phase *C. albicans* cells suspended in 100 mM-potassium phosphate buffer, pH 7.0 (1 ml/g wet wt. of cells), were boiled for 20 min, and the mixture was cooled and centrifuged (8000 g , 20 min) (J. Kennedy, M. G. Shepherd & P. A. Sullivan, unpublished work). The supernatant, which contained the inhibitor, was stored at 4 °C.

Proteinase assays

Radiolabelled proteins as substrate. Each assay mixture contained proteinase [10–100 μl ; diluted as required in assay buffer containing 10% (v/v) glycerol] and substrate (either 170 μg of [^{35}S]protein or 500 μg of [^3H]acetylated bovine serum albumin) in imidazole buffer (total volume 0.75 ml). Immediately after the addition of the enzyme, and again after 60 min incubation, 0.2 ml portions were mixed with 50 μl of bovine serum albumin (20 mg/ml in water) and 0.25 ml of 15% (w/v) trichloroacetic acid. These samples were stored on ice for about 30 min and then centrifuged at 4000 g for 10 min. Samples of the supernatant (0.1 ml) were taken and their radioactivities counted in duplicate. One unit of enzyme activity is 1 μg of protein degraded per h.

N-Blocked amino acid ester and amide substrates. All assays were carried out at 30 °C. Each assay mixture contained proteinase (5–10 μl), substrate in 10 μl of solvent and acetate buffer (total volume 1.02 ml, except for the fluorescence assays, in which case it was 2 ml). Each reaction was monitored for 5–20 min, except for those substrates that were slowly hydrolysed, in which case the reaction was monitored for up to 60 min. The initial rate of hydrolysis of the *p*-nitrophenyl ester substrates was measured at 348 nm [ϵ_{348} for *p*-nitrophenol 5500 $\text{M}^{-1} \cdot \text{cm}^{-1}$ (Visser & Blout, 1972)] and were corrected for non-enzymic hydrolysis. For other substrates the wavelengths and absorption coefficients were: Ac-L-Tyr-OEt, 237 nm (200 $\text{M}^{-1} \cdot \text{cm}^{-1}$); Ac-L-Tyr-NH₂, 237 nm (220 $\text{M}^{-1} \cdot \text{cm}^{-1}$); Bz-L-Ala-OMe, 255 nm

(870 M⁻¹·cm⁻¹). The hydrolysis of α -Bz-L-Arg-OEt and α -Cbz-L-Arg-Nan was monitored at 253 nm and 410 nm respectively. The hydrolysis of Cbz-L-Leu-Nna and Bz-L-Leu-Nna was monitored by measuring the fluorescence of the free β -naphthylamine (excitation wavelength 340 nm; emission wavelength 410 nm).

Other substrates. Degradation of gelatin was measured by using a gel-plate assay method based on the method of Every (1981). Azocoll degradation was measured in 0.1 M-sodium phosphate buffer, pH 6.6, containing 0.02% NaN₃ as described by Schep *et al.* (1984).

Purification of proteinase ycaB

Step 1. Yeast cells (300 g) were immersed in chloroform (150 ml) for about 12 min and then mixed with water (150 ml). The suspension was adjusted to pH 7.5 with 1 M-NaOH, stored at 4 °C for 24 h and centrifuged at 4000 g for 10 min. The supernatant (270 ml) was adjusted to pH 5.0 with 1 M-HCl and made 0.2% in NaN₃. After 15 h at 28 °C (with stirring at 125 rev./min) the preparation was centrifuged (4000 g, 15 min) and yielded a clear supernatant (286 ml), the crude preparation.

Step 2. The crude preparation was dialysed against water at 4 °C (2 × 9 litres over 3 h). NaCl (4 M) and 12 M-sodium acetate buffer, pH 4.0, were added to give final concentrations of 50 mM-sodium acetate and 130 mM-NaCl. This solution was mixed batchwise (20 min) with pre-equilibrated CM-Sephadex C-50 (20 g), and the gel was washed three times with buffer (400 ml). The proteinase was eluted batchwise (over 1.5 h) with three washes (400 ml each) of 1.5 M-sodium acetate buffer, pH 6.0, containing 1 mM-EDTA and 10% (v/v) glycerol.

Step 3. The preparation was applied to a column of Affi-Gel 501 (1.5 cm × 5.5 cm) at a flow rate of 80 ml/h. The column was then equilibrated with acetate buffer containing 200 mM-NaCl. The proteinase was eluted batchwise (30 min per wash) with eight washes (20 ml each) of 10 mM-HgCl₂ in the equilibration buffer. The pooled supernatants were concentrated in a dialysis sac against poly(ethylene glycol) 20000 at 4 °C and dialysed for 1 h against acetate buffer. When ³H-labelled enzyme was required the concentrated eluate was incubated with [³H]di-isopropyl phosphorofluoridate (160 nCi/nmol; 1.5 nmol/μg of protein) for 7 days at 4 °C. Standard precautions (Cohen *et al.*, 1967) were taken during the handling of di-isopropyl phosphorofluoridate.

Step 4. The [³H]di-isopropyl phosphorofluoridate-treated preparation was fractionated by gel-permeation chromatography on a column (7.5 mm × 60 mm) of TSK-gel G3000SW in 25 mM-imidazole/HCl buffer, pH 6.6, containing 10% (v/v) glycerol at a flow rate of 0.7 ml/min. Fractions (0.7 ml) that contained the radiolabelled proteinase were pooled (12 ml) and concentrated by partial freeze-drying.

Step 5. The preparation (3.4 ml) was applied to a column (4.5 mm × 70 mm) of Polybuffer exchanger (PBE 94 chromatofocusing kit; Pharmacia, Uppsala, Sweden) equilibrated with 25 mM-imidazole/HCl buffer, pH 6.6, containing 10% (v/v) glycerol. The radiolabelled

proteinase was eluted with Polybuffer/HCl, pH 4.0, containing 10% (v/v) glycerol. Radioactive fractions were pooled (3 ml) and partially freeze-dried.

Step 6. The concentrated eluate (0.9 ml) was fractionated on a TSK-gel G3000SW column (7.5 mm × 1200 mm) in 25 mM-imidazole/HCl buffer, pH 6.6, containing 1 mM-EDTA and 10% (v/v) glycerol at a flow rate of 0.4 ml/min. The radioactive fractions were pooled (6.4 ml), concentrated to 0.7 ml and stored at -80 °C.

RESULTS

Measurement of proteinase activity in crude preparations

The release of trichloroacetic acid-soluble radioactivity from denatured [³⁵S]protein was due to proteolytic cleavage because it was accompanied by a concomitant increase in the number of free amino groups in the total incubation mixture (results not shown). Proteinase activity had a broad pH optimum centred around pH 6.6, and the K_m for denatured [³⁵S]protein was 400 μg/ml as determined by the method of Eisenthal & Cornish-Bowden (1974). Denatured [³H]acetylated bovine serum albumin was a suitable alternative substrate for the enzyme provided that there was less than 3 mol of acetyl groups/mol of protein. No deacetylase activity was present in the crude proteinase preparation (results not shown). Gelatin and Azocoll were also degraded by the proteinase, but neither substrate was suitable for routine assays. The coefficients of variation for the radioassay and the Azocoll assay were 0.076 and 0.125 respectively. The difference between the coefficients of variation, tested as described by Sachs (1982), is statistically significant at the 5% level.

Inhibitors of proteinase activity

Proteinase activity was inhibited approx. 95% by phenylmethanesulphonyl fluoride (2 mM), *p*-chloromercuribenzoate (12 nmol/μg of protein), HgCl₂ (13 nmol/μg of protein) and chymostatin (8 μg/ml). Di-isopropyl phosphorofluoridate (12 mM) and leupeptin (240 μg/ml) also inhibited the enzyme (by 80% and 60% respectively). Enzyme activity was not affected by *p*-aminobenzamidine (5 mM), nitrilotriacetic acid (10 mM) or pepstatin (27 μg/ml). EDTA (9 mM) increased the proteinase activity (19%). The inhibition by *p*-chloromercuribenzoate and HgCl₂ was reversed by the addition of thiol reagents (e.g. cysteine and mercaptoacetate) to the assay mixture. Mercaptoacetate (550 mM) gave 80% recovery of activity from the mercuri-proteinase preparation and had negligible effect on the native proteinase.

Purification of proteinase ycaB

Typical results of the purification are given in Table 1. The crude preparations contained 20 mg of carbohydrate/ml and 0.2 mg of protein/ml, but more than 90% of the carbohydrate was removed by the CM-Sephadex step. The enzyme was eluted from Affi-Gel 501 with HgCl₂ to prevent self-degradation (Kominami *et al.*, 1981a). Preliminary experiments showed that the partially purified mercuri-proteinase was stabilized by 10% (v/v) glycerol. To facilitate the detection of the proteinase in subsequent steps the mercuri-proteinase was labelled with [³H]di-isopropyl phosphorofluoridate. The extent of the inactivation was less than 10%. The elution volumes for the [³H]di-isopropyl phosphorofluoridate-labelled protein and untreated enzyme on the TSK-gel G3000SW

Table 1. Purification of proteinase ycaB

Enzyme activity was not measured after the chromatofocusing column (see the text). Abbreviation: N.D., not determined.

Step	Volume (ml)	$10^{-3} \times$ Total activity (units)	A_{280}	Total protein (mg)	$10^{-3} \times$ Specific activity (units/mg)	Purification (fold)
Crude preparation	286	3600	37	60	60	1
Eluate from CM-Sephadex	1290	2770	0.4	N.D.	N.D.	N.D.
Concentrated eluate from Affi-Gel 501	6.45	1290	2.2	3	430	6
Concentrated eluate from TSK-gel column	3.4	615	0.56	0.8	800	13
Final preparation	0.7	50	0.14	0.03	1700	30

column were identical. The proteinase was recovered from step 5 as a single peak (80% recovery of radioactivity), and subsequent gel-permeation chromatography (step 6) gave one peak of radiolabelled protein (95% recovery) (results not shown). The overall purification (30-fold) omits the 100-fold increase in specific activity during step 1. The purification was conveniently monitored by the absorbance at 280 nm (Table 1); however, the purification based on these data (1500-fold) is misleading because the crude preparation contained nucleic acid.

Properties of the purified proteinase

At -80°C the purified mercuri-proteinase lost activity at the rate of 7% per month. The preparation showed one band after SDS/polyacrylamide-gel electrophoresis and staining with Coomassie Blue. Silver staining revealed up to nine other bands, which represented less than 10% of the total protein. More than 80% of the [^3H]di-isopropyl phosphorofluoridate label was coincident with the main band (referred to below as band P). The remainder of the radioactivity migrated with a minor protein band, referred to below as band F (M_r 15000).

Incubation (30°C for 10 h) of activated [^3H]di-isopropyl phosphorofluoridate-labelled proteinase resulted in the loss of over 75% of the enzyme activity. The amount of protein in band P decreased during the incubation, with a concomitant loss of radioactivity. No radioactivity was detected elsewhere in the gel.

Values obtained for the minimal M_r were 30000 ± 2000 (s.d.) from SDS/polyacrylamide-gel electrophoresis, 34000 by TSK-gel-permeation chromatography and 21000 with a Sephadex G-100 column.

The time course of degradation of the oxidized insulin B-chain (20 nmol) by the purified proteinase (0.2 pmol) was monitored by using reverse-phase chromatography. Two peptides were produced in equal amounts as the B-chain disappeared. These accounted for all of the B-chain in the incubation mixture. Other degradation products (less than 1%) were not detected until 95% of the B-chain had been degraded. *N*-Terminal and amino acid analyses showed that primary site of cleavage by proteinase ycaB was the Leu-Tyr peptide bond. At a

higher proteinase/B-chain ratio (1:400) more extensive degradation was observed (eight peaks by reverse-phase chromatography) after 1 h incubation at 37°C . These peptides were not analysed.

N-Blocked amino acid ester and amide substrates

The kinetics of hydrolysis of a series of *p*-nitrophenyl ester substrates was examined with partially purified proteinase (concentrated eluate from Affi-Gel 501). Cbz-Lys-ONp (0.66 pmol/s per μg of protein at $100\ \mu\text{M}$), Cbz-Trp-ONp (2.3 pmol/s per μg of protein at $30\ \mu\text{M}$), Cbz-Ile-ONp (3.3 pmol/s per μg of protein at $100\ \mu\text{M}$) and Cbz-Val-ONp (6.6 pmol/s per μg of protein at $150\ \mu\text{M}$) were hydrolysed only slowly by the proteinase and were not examined further. It was not possible to investigate the hydrolysis of Cbz-Trp-ONp beyond $30\ \mu\text{M}$ because of its low solubility in water. Cbz-Leu-ONp, Cbz-Tyr-ONp, Cbz-Phe-ONp, Cbz-Gly-ONp, Cbz-Asn-ONp, Cbz-Ala-ONp and Boc-Ala-ONp were readily hydrolysed by the proteinase.

Progress curves for the hydrolysis of Cbz-Leu-ONp, Cbz-Phe-ONp and Cbz-Tyr-ONp showed a lag phase at substrate concentrations above $30\ \mu\text{M}$, $80\ \mu\text{M}$ and $75\ \mu\text{M}$ respectively. The lag phase was due to impurities in these substrates that formed finely suspended precipitates under the assay conditions. Removal of the precipitate did not affect the steady-state velocity values.

The results with Cbz-Tyr-ONp and Cbz-Leu-ONp are presented as half-reciprocal plots ($[S]/v$ against $[S]$) in Fig. 1. K_m and k_{cat} values determined for these and other substrates are summarized in Table 2. No hydrolysis of Bz-Arg-OEt ($95\ \mu\text{M}$), Cbz-Arg-Nan ($97\ \mu\text{M}$), Cbz-Leu-Nna ($50\ \mu\text{M}$), Bz-Leu-Nna ($58\ \mu\text{M}$) or Ac-Tyr-NH₂ ($225\ \mu\text{M}$) was detected. Bz-Ala-OMe (1 mM) and Ac-Tyr-OEt ($970\ \mu\text{M}$) were only hydrolysed slowly (8.3 pmol/s per μg of protein and 46 pmol/s per μg of protein respectively). Proteinase activities on two *p*-nitrophenyl ester substrates and denatured [^{35}S]protein were compared and the following values obtained: 1.72 nmol of *p*-nitrophenol/s per ml with Cbz-Leu-ONp ($50\ \mu\text{M}$), 11.30 nmol of *p*-nitrophenol/s per ml with Cbz-Tyr-ONp ($107\ \mu\text{M}$) and 1.97 mg of protein degraded/h per ml with denatured [^{35}S]protein ($172\ \mu\text{g}$).

Fig. 1. Plots of $[S]/v$ against $[S]$ for Cbz-Tyr-ONp (○) and Cbz-Leu-ONp (●)

Assay mixtures contained proteinase (10 μ l for Cbz-Leu-ONp and 5 μ l for Cbz-Tyr-ONp) and substrate, as indicated (10 μ l in acetonitrile), in 50 mM-sodium acetate buffer, pH 6.0, containing 1 mM-EDTA and 10% (v/v) glycerol (total volume 1.02 ml). The proteinase preparations used in this experiment contained 19 ng of protein/ μ l. Each point is the average for at least triplicate determinations. The straight line is that obtained by analysis of the data by the method of Eisenthal & Cornish-Bowden (1974) for K_m and V .

Endogenous proteinase inhibitor

Degradation of Azocoll by proteinase ycaB is inhibited by a heat-stable factor obtained from yeast cells of *C. albicans*. The amount of proteinase inhibitor in crude preparations was measured with the use of Cbz-Leu-ONp (10 μ M) as substrate. One unit of proteinase inhibitor was defined as the amount of inhibitor preparation required to decrease the rate of hydrolysis of Cbz-Leu-ONp to 50% of that obtained in the absence of the inhibitor preparation. The crude inhibitor preparations contained between 0.04 and 0.1 unit of inhibitor/ μ g of protein (early-stationary-phase and late-stationary-phase yeast cells grown on low-sulphate medium respectively). To establish that the inhibition of proteinase activity was not due to competition by a better substrate than that used to measure proteinase activity, the rate of degradation of a [35 S]inhibitor preparation (2000 d.p.m./ μ g) was compared with the rate of degradation of denatured [35 S]protein (500 d.p.m./ μ g). The inhibitor/proteinase

Table 2. Kinetic parameters for the hydrolysis of *p*-nitrophenyl ester substrates

Enzyme activity was measured as described in the Experimental section with a partially purified proteinase preparation (concentrated eluate from Affi-Gel 501). Each preparation of active enzyme (see the Experimental section) contained less than 10 μ g of protein, and the protein concentration was therefore calculated from the specific activity on 50 μ M-Cbz-Leu-ONp (0.1 pmol/s per ng). The active enzyme preparation was stored on ice, and under these conditions the loss of enzyme activity during the experiment (1%/h) was negligible. At the concentration used in these experiments the solvent, acetonitrile (1%), did not affect proteinase activity. Kinetic parameters were derived by the direct linear plot (Eisenthal & Cornish-Bowden, 1974) by using a program written for the Apple II computer. Calculated k_{cat} values are based on an M_r of 30000, one active site per enzyme molecule and an estimated purity of 20% after the Affi-Gel 501 step. Assays with Cbz-Asn-ONp, Cbz-Gly-ONp and Cbz-Ala-ONp were limited to substrate concentrations less than K_m .

Substrate	K_m (μ M)	k_{cat} (s $^{-1}$)	k_{cat}/K_m (M $^{-1}$ ·s $^{-1}$)
Cbz-Gly-ONp	200	65	325 000
Cbz-Asn-ONp	700	230	322 000
Cbz-Tyr-ONp	48	170	3 536 000
Cbz-Ala-ONp	300	250	825 000
Boc-Ala-ONp	90	30	333 000
Cbz-Leu-ONp	8	18	2 250 000
Cbz-Phe-ONp	10	10	1 000 000

ratio used in this incubation was sufficient to inhibit Cbz-Leu-ONp hydrolysis by 95%. No degradation of the [35 S]inhibitor protein was detected, whereas the denatured [35 S]protein was rapidly degraded (60 μ g of protein degraded/h).

DISCUSSION

The present work has established a sensitive and reproducible radioassay method for proteinase ycaB and shown that it is to be preferred over the Azocoll assay method used for proteinase yscB (Saheki & Holzer, 1975; Kominami *et al.*, 1981a). On the basis of the physical and enzymic properties reported in the present work proteinase ycaB is almost indistinguishable from proteinase yscB. Proteinase activity in crude preparations was inhibited by typical inhibitors of yeast proteinase B (phenylmethanesulphonyl fluoride, di-isopropyl phosphorofluoridate, *p*-chloromercuribenzoate, HgCl $_2$ and chymostatin). It was also partially inhibited by leupeptin, whereas Kominami *et al.* (1981a) found no inhibition of proteinase yscB. Proteinase activity was not inhibited by pepstatin, an inhibitor of the *C. albicans* extracellular aspartic proteinase (proteinase ycaA) (Ruchel, 1981). Kominami *et al.* (1981a) inactivated proteinase yscB during the purification with HgCl $_2$ and subsequently re-activated the enzyme by treatment with 2-mercaptoethanol and chromatography. In the present work the procedure was simplified by adding mercaptoacetate directly to the assay mixture to activate the mercuri-proteinase. The increase in proteinase specific activity (approx. 100-fold) during inactivation of the inhibitor

* No information was provided on the relative rate of hydrolysis of these two peptide bonds.

Fig. 2. Primary specificity of various serine proteinases on the oxidized B-chain of insulin

Peptide bonds marked with solid arrows are hydrolysed more rapidly than those marked with broken arrows.

(step 1) is similar to that reported by Saheki & Holzer (1975). Labelling partially purified proteinase with [^3H]di-isopropyl phosphorofluoridate facilitated not only further purification but also the subsequent characterization. Under the conditions used the extent of inactivation of the mercuri-proteinase was less than 10%, and the values for extent of incorporation (results not shown) were consistent with this enzymic analysis. Similar results have been obtained with other serine proteinases, including proteinase yscB, which has a reactive cysteine residue in or close to the active site (Kominami *et al.*, 1981a).

The M_r value of proteinase ycaB (30000–34000) from SDS/polyacrylamide-gel electrophoresis and gel-permeation chromatography (TSK-gel) suggested that the native proteinase is a single polypeptide. This is consistent with the value (32000–33000) obtained for proteinase yscB (Kominami *et al.*, 1981a). The lower value obtained on Sephadex G-100 (M_r 21000) probably results from interaction of the proteinase and the gel matrix. The M_r of proteinase ycaA is 45000 (Ruchel, 1981). Band F (M_r 15000), which contained about 20% of the [^3H]di-isopropyl phosphorofluoridate label, may be a di-isopropyl phosphorofluoridate-sensitive esterase that co-purified with the proteinase. Alternatively, specific but limited proteolytic cleavage of the native proteinase during the early stages of the purification could have generated nicked protein molecules (20% of the total preparation) that retained a near-native conformation (Magni *et al.*, 1982). The latter alternative is more likely, since a single peak of radioactivity was obtained on the final TSK-gel column (an esterase of M_r 15000 comprising 20% of the preparation should have been resolved from the proteinase and detected under these conditions).

The specificity of proteinase ycaB on the oxidized B-chain of insulin (Fig. 2) is distinctly different from those of trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), elastase (EC 3.4.21.11) and proteinase ycaA (Remold *et al.*, 1968). Proteinase yscB (at a substrate/enzyme ratio of 500:1) hydrolysed both Leu-15-Tyr-16 and Phe-25-Phe-26 peptide bonds (Kominami *et al.*, 1981b). The extracellular microbial serine proteinases (EC 3.4.21.14) thermomycin and subtilisin specifically hydrolyse the Leu-15-Tyr-16 peptide bond. The specificity (at low enzyme/substrate ratios) of proteinase ycaB for the Leu-15-Tyr-16 peptide bond demonstrates that the enzyme has an extended substrate-binding site.

Kinetic analysis of the *p*-nitrophenyl ester substrates shows that the extended substrate-binding site involves at least three subsites (S_2 , S_1 and S'_1 ; see Fig. 3). The results obtained with Cbz-Ala-ONp ($k_{\text{cat.}}/K_m$ 825000 $\text{M}^{-1}\cdot\text{s}^{-1}$) and Boc-Ala-ONp ($k_{\text{cat.}}/K_m$ 333000 $\text{M}^{-1}\cdot\text{s}^{-1}$) show that subsite S_2 is important in determining the specificity of proteinase ycaB. With subtilisin the most specific substrates have an alanine residue in subsite S_2 (Moriyama, 1974). For the insulin B-chain an alanine residue is in the S_2 subsite during hydrolysis of the Leu-15-Tyr-16 peptide bond but not for the peptide bonds involving the other leucine residues.

Cbz-Tyr-ONp ($k_{\text{cat.}}/K_m$ 3536000 $\text{M}^{-1}\cdot\text{s}^{-1}$), Cbz-Leu-ONp ($k_{\text{cat.}}/K_m$ 2250000 $\text{M}^{-1}\cdot\text{s}^{-1}$) and Cbz-Phe-ONp ($k_{\text{cat.}}/K_m$ 1000000 $\text{M}^{-1}\cdot\text{s}^{-1}$) were the most specific *N*-blocked amino acid ester substrates for proteinase ycaB. The specificity for Cbz-Leu-ONp was expected from the results of the insulin B-chain experiment. The

Fig. 3. Schematic representation of an extended substrate-binding site [from Schechter & Berger (1967)]

Amino acid residues in the substrate are represented by P_1 – P_4 , P'_1 and P'_2 , binding sites on the enzyme are represented by S_1 – S_4 , S'_1 and S'_2 , and the catalytic site is represented by C.

2-fold-higher specificity for Cbz-Tyr-ONp may be due to strong binding of the nitrophenyl group in the subsite S'_1 . The presence of a strongly bound nitrobenzyl leaving group in subsite S'_1 is known to modify the specificity of elastase so that Ala-Ala-Ala-Leu-ONb is a better substrate than is Ala-Ala-Ala-Ala-ONb, whereas in the absence of the nitrobenzyl group alanine is the preferred amino acid residue for subsite S_1 (Atlas & Berger, 1972). The observation that Cbz-Leu-ONp is readily hydrolysed whereas Cbz-Leu-Nna is not hydrolysed provides further evidence that the size and nature of the leaving group (in the acylation step) is critical to the specificity of proteinase ycaB. That subsite S'_1 might also have a critical role in the specificity of proteinase ycaB with respect to peptide substrates is suggested by an examination of the amino acid sequence of the oxidized insulin B-chain. If the B-chain is aligned with a tyrosine residue in position P_1 , then P'_1 is either a leucine or a threonine residue. With a leucine residue in position P_1 then P'_1 is either a valine or a *S*-sulphocysteine residue. Only with Leu-15 in P_1 is an aromatic amino acid residue (tyrosine) in P'_1 .

We conclude that an aliphatic amino acid residue (e.g. leucine) is preferred at position P_1 and that an aromatic amino acid residue (e.g. tyrosine) is preferred at position P'_1 . The specificity of proteinase ycaB could be further characterized from the kinetics of hydrolysis of defined peptide substrates based on the sequence Ala-Leu-Tyr.

We have detected Cbz-Leu-ONp-hydrolysing activity in the vacuole fraction from *C. albicans* (P. C. Farley, M. G. Shepherd & P. A. Sullivan, unpublished work) and a heat-stable inhibitor of Cbz-Leu-ONp-hydrolysing activity in cell-free extracts of *C. albicans*. In *S. cerevisiae* proteinase yscB is located in the vacuole and the endogenous inhibitor in the cytoplasm of the cell. The vacuolar proteinase functions in general protein degradation rather than in specific proteolytic modification of proteins. A similar role is proposed for proteinase ycaB.

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REFERENCES

- Achstetter, T., Emter, O., Ehmann, C. & Wolf, D. H. (1984) *J. Biol. Chem.* **259**, 13334–13343
- Atlas, D. & Berger, A. (1972) *Biochemistry* **11**, 4719–4722
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Chattaway, F. W., Odds, F. C. & Barlow, A. J. E. (1971) *J. Gen. Microbiol.* **67**, 255–263
- Cohen, J. A., Oosterbaan, R. A. & Berends, F. (1967) *Methods Enzymol.* **11**, 686–702
- Eggstein, M. & Kreutz, F. H. (1967) in *Techniques in Protein Chemistry* (Bailey, J. L., ed.), p. 340, Elsevier, London
- Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720
- Every, D. (1981) *Anal. Biochem.* **116**, 519–523
- Gaucher, G. M. & Stevenson, K. J. (1976) *Methods Enzymol.* **45**, 415–433
- Gopal, P., Sullivan, P. A. & Shepherd, M. G. (1982) *J. Gen. Microbiol.* **128**, 2319–2326
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
- Herbert, D., Phipps, P. J. & Strange, R. E. (1971) *Methods Microbiol.* **5B**, 209–344
- Kominami, E., Hoffschulte, H. & Holzer, H. (1981a) *Biochim. Biophys. Acta* **661**, 124–135
- Kominami, E., Hoffschulte, H., Leuschel, L., Maier, K. & Holzer, H. (1981b) *Biochim. Biophys. Acta* **661**, 135–141
- Laemmli, U. K. & Favre, M. (1973) *J. Mol. Biol.* **80**, 575–599
- Levine, N., Hatcher, V. B. & Lazarus, G. S. (1976) *Biochim. Biophys. Acta* **452**, 458–467
- Logan, D. A., Naider, F. & Becker, J. M. (1983) *Exp. Mycol.* **7**, 116–126
- Magni, G., Natalini, P., Santarelli, I. & Vita, A. (1982) *Arch. Biochem. Biophys.* **213**, 426–433
- Matsudaira, P. T. & Burgess, D. R. (1978) *Anal. Biochem.* **87**, 386–396
- Morihara, K. (1974) *Adv. Enzymol. Relat. Areas. Mol. Biol.* **41**, 179–243
- Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310
- Murphy, C. J., Shepherd, M. G. & Sullivan, P. A. (1983) *Biochemistry* **22**, 1665–1669
- Narayanan, A. S. & Anwar, R. A. (1969) *Biochem. J.* **114**, 11–17
- Negi, M., Tsuboi, R., Matsui, T. & Ogawa, H. (1984) *J. Invest. Dermatol.* **83**, 32–36
- Ottesen, M. & Svendsen, I. (1970) *Methods Enzymol.* **19**, 199–215
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Remold, H., Fasold, H. & Staib, F. (1968) *Biochim. Biophys. Acta* **167**, 399–406
- Ruchel, R. (1981) *Biochim. Biophys. Acta* **659**, 99–113
- Russell, C. S. & Pollack, S. E. (1978) *J. Chromatogr.* **166**, 632–636
- Sachs, T. (1982) *Applied Statistics: A Handbook of Techniques* (Reynarowych, Z., translator), p. 275, Springer-Verlag, New York
- Saheki, T. & Holzer, H. (1975) *Biochim. Biophys. Acta* **384**, 203–214
- Sanger, F. & Tuppy, H. (1951) *Biochem. J.* **49**, 481–490
- Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
- Schep, G. P., Shepherd, M. G. & Sullivan, P. A. (1984) *Biochem. J.* **223**, 707–714
- Schuttler, A. (1979) *Doktors der Naturwissenschaften Dissertation, Mathematisch-Naturwissenschaftlichen Fakultät, Rheinisch-Westfälischen Technischen Hochschule*
- Sluyterman, L. A. Æ. & Wijdenes, J. (1974) *Methods Enzymol.* **34**, 544–547
- Tatemoto, K. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5485–5489
- Templeton, M. D., Sullivan, P. A. & Shepherd, M. G. (1984) *Biochem. J.* **224**, 379–388
- Visser, L. & Blout, E. R. (1972) *Biochim. Biophys. Acta* **268**, 257–260
- Wang, S.-S. & Carpenter, F. H. (1967) *Biochemistry* **6**, 215–224
- Wolf, D. H. (1980) *Adv. Microb. Physiol.* **21**, 267–338
- Zubenko, G. S. & Jones, E. W. (1981) *Genetics* **97**, 45–64

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