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PURIFICATION AND CHARACTERIZATION OF A PROTEOLYTIC ENZYME FROM *CANDIDA ALBICANS*

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SUMMARY

In media containing proteins as the nitrogen source some strains of *Candida albicans* excrete proteolytic enzymes with an optimum of activity at pH 3.2. A 140-fold purification of the enzyme from one of these strains was achieved by gel filtration and chromatography on DEAE-Sephadex. The molecular weight of the protein was found to be 40 000; the frictional ratio, 1.3. The proteolytic activity was not influenced by the addition of heavy metal ions, SH-blocking reagents, *p*-bromophenacyl bromide, *N*-diazoacetylnorleucine methyl ester, or various inorganic ions. Cleavage of oxidized insulin β-chain and albumin demonstrated a low side-chain specificity; a preferential attack, on hydrophobic amino acid residues was observed after incubation for short periods of time.

INTRODUCTION

A proteolytic effect due to an excreted enzyme could be demonstrated with certain strains of *Candida albicans*[1,2,3] by protein staining of serum-protein–agar containing 0.12% albumin. Substitution of albumin by low molecular weight nitrogen sources, *e.g.* urea or ammonium salts, did not elicit the formation of proteolytic activity. The action of the enzyme was restricted to pH values between 3 and 4. In liquid media the microorganisms produce and maintain this pH. An enzyme was purified and characterized from the culture medium of strain CBS 2730.

METHODS

Culture medium

To increase protease formation the microorganism was grown in a solution of 0.5 g $MgSO_4$, 1 g KH_2PO_4, 20 g glucose, 2 g human or bovine serum albumin (Behring Werke, Marburg), and 1.25 ml Protovita (polyvitamin preparation, Hoffmann La-Roche Co.) in 1 l of water, adjusted to pH 4 with HCl, instead of the usual Sabouraud

liquid medium. After sterilization by filtration the solution was inoculated to 500 viable cells/ml and incubated with shaking at 26° for 3–5 days. The pH usually decreased to 3.0–3.2 during this time. The cells were removed by filtration.

Concentration of dilute enzyme

After filtration the culture broth contained approx. 600 mg of protein per l. Since lyophilization of the dialyzed or native medium partly destroyed the enzyme activity, an apparatus for the concentration of large volumes was constructed. A dialysis membrane was held in place by a compressed rubber O-ring between two rings fitted over a central stem and clamped together. One of the two outlets served as an air valve during the filling of the apparatus, after which it was closed; the open second outlet connected to the reservoir of culture medium standing above the apparatus permitted the solution to flow into the dialysis sac at the rate it evaporated. A stream of air was funneled past the dialysis membrane. The apparatus was set up in the cold room. The distance between the centerpiece and the dialysis tubing was 2 mm, thus it was possible to achieve a final volume of 20–50 ml.

Enzyme assay

0.5-ml samples of the enzyme solution were incubated with 2 ml of a 1% soln of bovine serum albumin in 0.05 M sodium citrate buffer, pH 3.2 for 60 min at 37°. The reaction was stopped by the addition of 5 ml of a 5% solution of trichloroacetic acid and the albumin removed by centrifugation. The amount of proteolytic products was determined in 2 ml of the supernatant solution according to the method of LOWRY *et al.*[4]. Control samples were treated with trichloroacetic acid before incubation or incubated without albumin.

Density-gradient centrifugation

Enzyme samples were placed on top of linear sucrose gradients between 5 and 20% soln prepared in an automatic mixing device[5] in 4.5-ml tubes. Centrifugation in a SW 39 rotor (Beckman spinco) was carried out for 20 h at 39 800 rev./min. The tubes were emptied into a fraction collector with the aid of a peristaltic pump and the position of the enzyme was determined by the assay for proteolytic activity. The time for acceleration and deceleration was corrected[6], while the density and viscosity in the fractions were determined according to DEDUVE, BERTHET AND BEAUFAY[7]. Bovine serum albumin served as the control.

Digestion of insulin

Performic acid-oxidized insulin[8] was separated into its components by continuous paper electrophoresis (Cp apparatus, Beckman, wick moistener position 9, overflow position 7) in 0.02 M acetic and formic acid buffer, pH 2.0, 600 V and 45 mA. After concentration by rotatory evaporation and lyophilization, 10 mg of the β-chain were incubated with 0.1 mg of enzyme in 10 ml of 0.01 M acetic acid for various periods of time. The reaction was stopped by the addition of dil. ammonia. The product was lyophilized and separated on paper or cellulose thin-layer plates[9] using the fingerprint technique. The first dimension was electrophoresis in 0.01 M pyridine–acetic acid buffer (pH 6.5), at 200 V/cm; the second dimension, chromatography in *n*-butanol–pyridine–acetic acid–water (30:20:6:24, by vol.).

Digestion of bovine serum albumin

100-mg amounts of the protein were dissolved in 50 ml of 0.01 M acetic and 1–2 mg of protease, dissolved in 0.1 ml of 0.1 M Na-citrate–HCl buffer, pH 3.1, were added. Aliquots of 0.25 ml were withdrawn after 5, 10, 15, 30, 60, 90, 120, and 180 min, immediately diluted with 1 ml of 0.005 M NaOH and assayed with the aid of the ninhydrin reagent of MOORE AND STEIN[10]. For control determinations 10-mg samples of serum albumin were hydrolyzed in 0.5 ml of 6 M HCl in a glass tube sealed under vacuum for 22.5 and 40 h at 110°. The samples were diluted with water to 5 ml, and aliquots between 0.1 and 0.25 ml were again used as above for ninhydrin values of 100% hydrolysis.

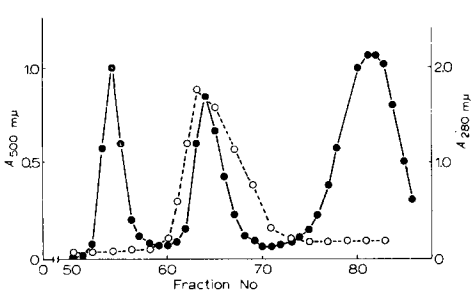
Gel electrophoresis

The methods of ORNSTEIN[11] and SMITHIES[12] were used for disc and starch gel electrophoresis. The buffer for disc electrophoresis at pH 4.5 was 0.05 M sodiumcitrate, adjusted with conc. HCl. The lower and upper gel contained 4.5 and 7% of acrylamide, respectively.

RESULTS

Purification of the enzyme

After filtration of the culture medium and concentration of the filtrate in a cold air stream the solution was chromatographed on Sephadex G-75. Fig. 1 shows the



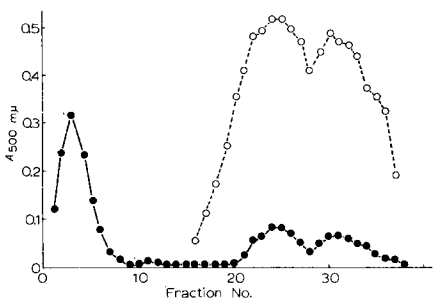


Fig. 1. Gel filtration upon Sephadex G-75. The column (3 cm × 100 cm) was equilibrated with 0.05 M citrate buffer (pH 4.0). Protein concentration was measured at 280 mμ (right hand scale, continuous line). The enzymatic activity was measured by the albumin-digestion test described in METHODS (left hand scale, broken lines). Fractions contained 7 ml of eluate.

Fig. 2. Ion-exchange chromatography on DEAE-Sephadex A-25. The dimensions of the column were 0.8 cm × 10 cm. After 10 fractions (arrow) a linear gradient from 0.01 M to 0.2 M sodium citrate buffer (pH 6.5) was started. Protein concentration was measured by the method of LOWRY *et al.*[4] (continuous line); activity was measured by the albumin-digestion test described in METHODS (broken line). Fractions contained 1.5 ml. Total gradient volume was 100 ml.

distribution of the enzyme activity in the eluate. The active fractions were combined, dialyzed against 0.01 M sodium citrate buffer (pH 6.5), and applied to a DEAE-Sephadex A-25 column equilibrated with the same solution. The enzyme was eluted as two partially separated peaks (Fig. 2). Under the same conditions, rechromatography of the material from the center of the two peaks showed, in each of two runs, an isolated

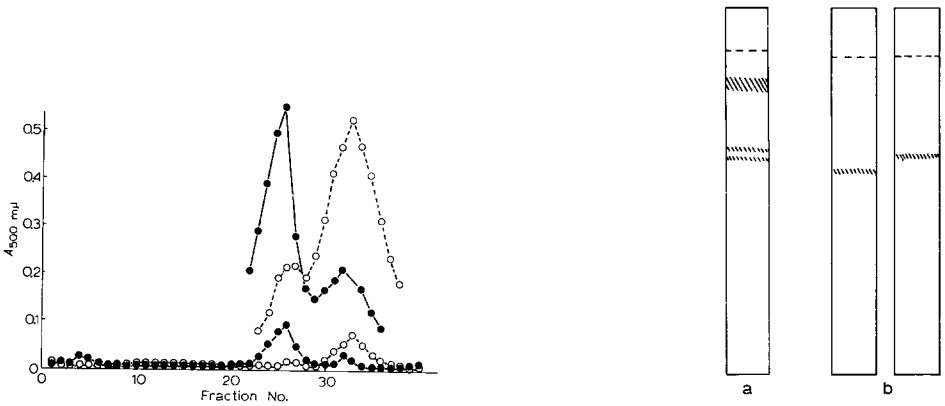


Fig. 3. Rechromatography of the material from Fractions 22–27 (continuous lines) and 31–36 (broken lines) on columns of the same dimensions and by the same method as described in the legend of Fig. 2.

Fig. 4. Disc electrophoreses. (a) Protein eluted from Sephadex G-75 (Fig. 1, Fract. 60–70). (b) Protein from the rechromatographies shown in Fig. 3: Fract. 25–26 (left hand column); Fract. 32–33 (right hand column). 0.05 to 0.2 mg of protein dissolved in the buffer (see METHODS) were layered onto the top of the micro-column and covered with a slurry of starch granules. The run was terminated after 3 h and the columns were stained with amido black.

fraction and a much smaller amount of the second component (Fig. 3). Table I gives a summary of these steps. Disc electrophoresis of the active fractions after Sephadex G-75 filtration revealed three protein bands at pH 4.5 (Fig. 4a); starch gel separation and disc electrophoresis at pH 8.0 showed only one band. After ion-exchange chromatography only two bands remained in disc electrophoresis. The electrophoreses of the material from Fractions 25–26 and 32–33 of the two rechromatographies shown in

TABLE I

PURIFICATION OF *C. albicans* PROTEINASE IN A TYPICAL RUN

Fraction	*Protein concn. (mg/ml)*	*Vol. (ml)*	*Specific activity (arbitrary units)*
Culture medium	0.6	1000	1
Conc. medium	8.8	39	1.4
Sephadex G-75 eluate	0.93	102	4.1
DEAE-Sephadex eluate	0.27	7.9	140

Fig. 3 finally showed only a single band each (Fig. 4b); the lower one corresponding to the first, and the upper one, to the second peak of the ion-exchange chromatographies. The specific activities and the pH optima of the DEAE-Sephadex fractions were identical.

Molecular weight

Using columns of Sephadex G-75 and G-100 prepared as described by CRESTFIELD, STEIN AND MOORE[13], the elution volumes of several proteins of known molecular weight and of the protease were compared. The evaluation gave a molecular weight of approx. 40 000 (Fig. 5). From the K_D values of the proteins on the G-75 columns the Stokes radius of the proteinase could be calculated[14] (a = 30 Å). This value to-

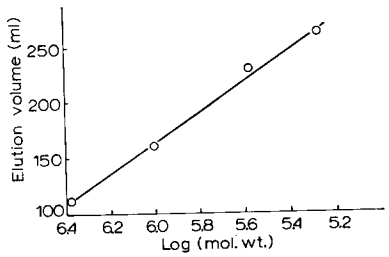


Fig. 5. Determination of the molecular weight of the proteinase on a 3 cm × 100 cm Sephadex G-75 column in 0.05 M sodium citrate buffer (pH 4.0). Proteins in the mixture applied to the column in the order of their elution: bovine γ-globulin, bovine serum albumin, *C. albicans* proteinase, chymotrypsinogen, cytochrome *c*.

gether with the $s_{20,w}$ value calculated from the results of density-gradient centrifugation and a sedimentation run in the synthetic boundary cell (Fig. 6) (s = 3.5 and 3.2), permitted the calculation of a molecular weight of 42 000. The frictional ratio was found to be 1.3 (see ref. 15, 16).

Side-chain specificity

When 0.1 mg of the enzyme had digested 10 mg of performic acid-oxidized insulin β-chain, a fingerprint of the products showed about 20 amino acids and peptides. Ninhydrin determinations on a digest of bovine serum albumin by 1% enzyme

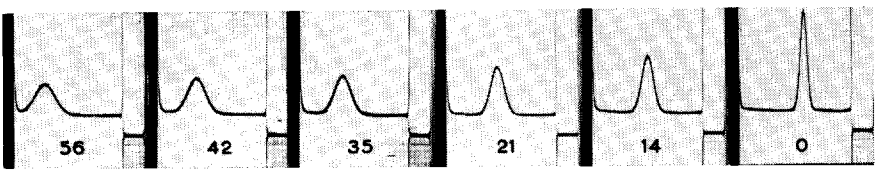


Fig. 6. Ultracentrifugation of a lyophilized sample of the proteinase after DEAE-Sephadex chromatography in 0.05 M sodium citrate buffer (pH 4.5) in an artificial boundary cell (Beckman, spinco div., model E centrifuge), times in min after reaching 59 800 rev./min are indicated in the figure.

(w/w) for 3 h showed that more than 90% of the peptide bonds could be cleaved. With the β-chain, the amount of enzyme and the incubation time were gradually reduced until at 0.01 mg of protease and digestion for 30 min, only 7 spots were found; 6 of these were formed in sufficient amounts to permit isolation by elution from paper. Analysis[17] revealed the compositions presented in Table II. The first peptide bonds split by the enzyme, therefore, involved the carboxyl groups of amino acids with hydrophobic side chains and, in one case, tyrosine:

TABLE II

AMINO ACID COMPOSITION OF THE PEPTIDES ISOLATED FROM A DIGEST OF INSULIN β-CHAIN BY *C. albicans* PROTEASE

Peptides were hydrolyzed for 22.5 h in 6 M HCl at 105° in a tube sealed under vacuum.

Amino acid	*Quantity (μmoles) found in peptide No.*					
	1	*2*	*3*	*4*	*5*	*6*
Lys	—	0.005	0.82	—	—	—
His	—	0.68	trace	—	—	—
Arg	0.59	—	—	—	—	—
$CysSO_3H$	0.63	0.27	—	—	—	—
Asp	0.01	0.34	—	0.010	0.005	—
Thr	—	trace	0.85	—	—	0.10
Ser	—	0.31	—	trace	—	0.008
Glu	0.67	0.32	—	0.015	0.30	0.97
Pro	0.005	—	0.89	—	—	—
Gly	1.42	0.39	—	—	—	—
Ala	trace	trace	0.90	trace	0.31	0.91
Val	—	—	—	0.45	0.24	0.10
Ileu	—	—	0.005	—	—	0.005
Leu	—	0.75	—	1.02	trace	trace
Phe	1.48	—	—	—	—	—
Tyr	0.71	—	—	0.52	—	—

NH_2-Phe.Val.↓Asn.Glu.His.Leu.Cys.Gly.Ser.His.Leu.↓Val.↓Glu.Ala.↓Leu.Tyr.Leu.Val.↓Cys.Gly.Glu.Arg.Gly.Phe.Phe.↑Tyr.Thr.Pro.Lys.Ala-COOH.

pH Optimum, inhibition and stability

The pH optimum of the enzyme in the culture medium, as well as in all of the enriched fractions, is pH 3.2 (Fig. 7). With a thoroughly dialyzed preparation from DEAE-Sephadex the following substances were tested for their ability to enhance or inhibit enzyme activity. The figures in parentheses denote the final molar concentrations in the enzyme–substrate solution: EDTA (0.005; 0.075; 0.01), mercaptoethanol (0.05; 0.1), Na_2HPO_4 (0.01; 0.1; 0.5), KH_2PO_4 (0.01; 0.1; 0.5), $CaCl_2$ (0.005; 0.01; 0.1), $MgCl_2$ (0.01; 0.1; 0.5), $MnCl_2$ (0.01; 0.1; 0.2), $(NH_4)_2SO_4$ (0.01; 0.11; 0.5), *p*-bromophenacyl bromide (0.001; 0.005), *N*-diazoacetylnorleucine methyl ester

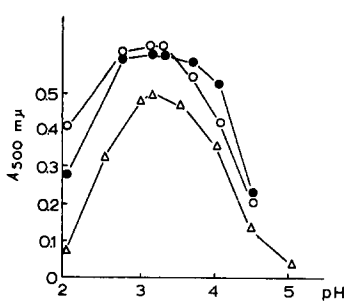


Fig. 7. pH-optimum of the proteinase determined by the albumin digestion test described in METHODS: △, starting material and fractions from Sephadex G-75 filtration; ●, Fractions 20–28 of Fig. 3 (singly or batch); ○, Fractions 30–35 of Fig. 3.

(0.001 ; 0.005), *p*-chloromercuribenzoate (0.001 ; 0.05), salyrgan (0.001 ; 0.05), kallikrein (equal weight to the enzyme). None of the additions effected a change in the observed activity.

The enzyme gradually loses activity by standing in solution in the cold or in the frozen state. Repeated freezing and thawing, as well as lyophilization, rapidly causes the activity to diminish.

DISCUSSION

The *C. albicans* proteinase, like most of the bacterial enzymes of this group, shows very little side-chain specifity[18–24], although a certain preference for hydrophobic residues could be demonstrated. There is a possibility, however, that the extensive degradation of serum albumin and the insulin β chain could be caused by contaminating amino- or carboxypeptidases. The pH optimum of approximately 3 has been found in only a small group of proteinases[25–28], while most of the pH optima center around the values of 2, 4 to 6, and 7 to 9 (ref. 28–36), however.

The lack of inhibition by *p*-bromophenacylbromide and *N*-diazoacetylnorleucine methyl ester[37] does not suggest a close relationship between the active center and the pepsin catalytic site.

A number of *C. albicans* strains have been tested for proteolytic activity. It was found that fungi possessing these proteinases can be isolated on serum-protein agar of pH 5. When injected into white mice, only the proteolizing strains cause extensive infection. Perhaps other experiments will elucidate whether these extracellular proteinases are directly involved in the pathogeneses of candidamycosis.

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