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PROPERTIES OF A PURIFIED PROTEINASE FROM THE YEAST *CANDIDA ALBICANS*

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Key words Pathogenicity, Carboxyl proteinase, (Candida albicans)

Summary

The proteinase from culture supernatants of *Candida albicans* strain CBS-2730 was purified virtually to homogeneity by ion-exchange chromatography and affinity chromatography. The enzyme consists of a single polypeptide chain with tryptophan at the N- and leucine at the C-terminus. Its molecular weight is approx. 45 000 and the isoelectric point is at pH 4.4. With albumin as a substrate an apparent K_m was determined to be $7 \cdot 10^{-5}$ M. The enzyme is inhibited by pepstatin at equimolar ratio and thus is a carboxyl proteinase (EC 3.4.23.6). Other group-specific inhibitors, though, did not efficiently block the enzyme. Above pH 8.4 the enzyme undergoes alkaline denaturation which is accompanied by dimerization. The enzyme is a glycoprotein. It is stable in presence of non-ionic detergents and can be freeze-dried. The enzyme clots milk at pH 5.5 and has trypsinogen kinase activity. Among several purified proteins that have been tested as a substrate, only horse ferritin was resistant to proteolysis, while myeloma proteins of the A_1- and A_2-type were readily cleaved, as were two proteinase inhibitors of human serum.

Antibodies against purified enzyme did not react with several commercial *Candida* antigen preparations; antibodies against the enzyme, though, have been detected repeatedly in sera from patients with manifest candidiasis.

Introduction

Among the yeasts, *Candida albicans* is, from a medical point of view, of foremost importance as an opportunistic pathogen [1]. Previously, certain strains

Abbreviations EGTA, ethyleneglycoltetraacetic acid, PMSF, phenylmethylsulfonyl fluoride, SDS, sodium dodecyl sulfate

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of the yeast have been shown to hydrolyse serum albumin [2], and a proteolytic enzyme from culture supernatants has been partially characterized [3]. The significance of proteolytic Candida strains as causative agents of severe candidiasis (i.e. thrush, gingivitis and septicemia) has been discussed by several authors [4—6], but detailed investigation has been hampered by the lack of purified enzyme [7]. To permit a search for specific substrates especially with respect to components of the immune system and pathways of physiological regulation that involve proteolysis, we have purified the enzyme and have determined its properties.

Materials

Bovine serum albumin, casein, *N*-ethylmaleimide and other laboratory chemicals were from Merck, Darmstadt, F.R.G. Bovine hemoglobin (crude powder or twice recrystallized), β-lactoglobulin-A, soybean trypsin inhibitor, phenylmethanesulfonyl fluoride (PMSF), 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, pepstatin-A, sodium dodecyl sulfate (SDS), Triton X-100, Tween-80, dithiothreitol, iodoacetamide, benzamidine, human α-2-antitrypsin, bovine trypsinogen, bovine trypsin, *N*-acetylphenylalanyl-L-diiodotyrosine and diazoacetyl-DL-norleucine methyl ester were from Sigma, Taufkirchen, F.R.G. High molecular weight protein standards, DEAE-cellulose (Sephacel), carrier ampholytes, aminohexyl-Sepharose 4-B, Sephadex IEF, agarose-C and Sephacryl S-200 were from Pharmacia, Freiburg, F.R.G.

EDTA, EGTA, DEAE-cellulose, carrier ampholytes, *o*-phenanthroline, azocasein, aprotinin, amido black 10-B, alcian blue, basic fuchsin and all chemicals for acrylamide gel electrophoresis were from Serva, Heidelberg, F.R.G. Yeast carbon base and yeast morphology agar were from Difco, Detroit, U.S.A. Protovita was kindly provided by Roche, Basle, Switzerland. Aquacide 1-A and azocoll were from Calbiochem. Lahn, F.R.G. Human α-2-macroglobulin, human transferrin and Freund's adjuvant were from Behring, Marburg, F.R.G, spheroidal hydroxyapatite was from BDH, Poole, U.K. and Bio-Gel HT was from Bio-Rad, Munich, F.R.G. Nonidet P-40, and *N*-ethylmorpholine were from Fluka, Buchs, Switzerland. 1,2-Epoxy-3-(*p*-nitrophenoxy)-propane was from Eastman, Rochester, U.S.A. and 10 μl glass capillaries (Intraend) were from Brand, Wertheim, F.R.G. Polyamide sheets (A-1700) were from Schleicher and Schull, Dassel, F.R.G.

Recrystallized 4-dimethylamino-azobenzene-4′-isothiocyanate, 4-*N*,*N*-dimethylamino-azobenzene-4′-thiocarbamyl (DABTC)-diethylamine and DABTC-ethanolamine were kindly provided by Dr. M. Engelhard, Dortmund. Purified human A_1- and A_2-myeloma proteins were gifts from Professors N. Hilschmann, Göttingen and F. Skvaril, Berne.

A subcolony of *C albicans* strain CBS-2730 was kindly provided by Professor F. Staib, Berlin. Other strains of *C albicans* were clinical isolates handled in this institute. The kit of the indirect hemagglutination test for the detection of anti-*C albicans* antibodies was obtained from Roche, Basle, two *C albicans* antigen preparations (antigène métabolique, antigène somatique) were from Institut Pasteur, Paris, and *C albicans* antigen preparation for skin tests was obtained from Wulfing/Beecham, Neuss, F.R.G. Cytoplasmatic protein antigens and suitable antisera were provided by Dr. D. Gunesch, Frankfurt, F.R.G.

Methods

Cultivation C albicans was grown for 4 days at 26°C in a 10 l fermentor with bovine serum albumin as a nitrogen source [3]. Alternatively, a broth containing 1% bovine hemoglobin, 1.2% yeast carbon base and 0.1% (v/v) Protovita was employed. The broth was sterilized by filtration and was adjusted to pH 5 prior to inoculation of yeast. After 4 days the content of the fermentor was adjusted to pH 6.8 and cells were removed by centrifugation (4000 × *g* for 60 min).

Purification of the enzyme Sodium azide (0.02% w/v) and PMSF were added to the supernatant that was concentrated by poly(ethylene glycol) dialysis down to approx. 1 l. Subsequently the concentrate was dialyzed against citrate buffer (10 mM, pH 6.8) containing 0.02% NaN_3 and 1 mM EDTA and was adsorbed onto DEAE-cellulose. The column was developed stepwise with 0.2 M citrate buffer (pH 6.3). Fractions with enzymatic activity were located by azocoll assay, pooled, dialyzed against 10 mM citrate buffer, pH 6.8, and adsorbed onto a second DEAE-cellulose column that was handled as described above. After subsequent gel filtration through beaded polyacrylamide (Sephacryl S-200), protease was concentrated up to 5 mg/ml by nitrogen pressure dialysis. Homogeneity of the enzyme was tested by gradient gel electrophoresis [8]. The concentrated enzyme was stored at −60°C in presence of 10% glycerol or it was freeze-dried from 0.2 M citrate buffer, pH 6.3, without apparent loss of activity.

Alternatively, an affinity column was employed with pepstatin linked to aminohexyl-Sepharose-4B [9]. The sample was adsorbed to the column (10 × 0.7 cm) in 0.2 M citrate buffer, pH 6.2, and 1 M NaCl and was desorbed with 0.1 M Tris-HCl, pH 8.1, and 1 M HCl.

Enzyme assays The azocoll assay was employed for localization of enzymatic activity [10,11]. Quantitative determination of protease was performed with hemoglobin (1%) as a substrate. After precipitation by trichloroacetic acid (4% final concentration) and centrifugation, soluble peptides remaining in the supernatant were measured directly at 280 nm as well as by the Lowry procedure [12]. One unit of enzymatic activity was defined arbitrarily as the amount of enzyme that released the color equivalent of 1 μM of tyrosine/min from hemoglobin under standard conditions as measured by the Lowry procedure [12] with tyrosine and serum as standards. Hydrolysis of *N*-acetylphenylalanyl-L-diiodotyrosine [13] was measured according to Becker and Rapp [14].

Proteolysis in liquid cultures Yeast carbon base medium was prepared at pH 4.5 and 7 with various proteins as a nitrogen source. Such proteins were human transferrin, human α-1 antitrypsin, human α-2 macroglobulin and horse ferritin. After 4 days at 26 and 37°C, cells were separated by centrifugation and aliquots of the supernatant were analyzed electrophoretically.

Electrophoresis Microelectrophoresis in polyacrylamide gel gradients as well as electrophoresis in polyacrylamide gel slabs was performed as described previously [8,15]. Gels were either stained with Coomassie blue-R [8], or they were stained for glycoproteins with alcian blue [18] and basic fuchsin [17].

Isoelectric focusing Isoelectric focusing in granulated gel was performed according to Radola [18]. After completion of the run and determination of

the pH-profile, fractions were adjusted to neutrality immediately in order to prevent further autodigestion of the enzyme.

Identification of enzyme after electrophoresis Protease samples were separated in polyacrylamide gradient gel slabs at pH 6.7, employing a continuous buffer (25 mM sodium phosphate). Pilot strips were stained directly with Coomassie blue The middle portion of the gel was sliced into fractions of 3 mm width. The fractions were homogenized and were made up to 0.3 ml with substrate solution (1% bovine hemoglobin 0.1 M citrate buffer, pH 3). The samples were placed on a vibrator for 30 min, then the pH was corrected to approx. 3.5 for subsequent proteinase assay.

pH optimum The pH optimum of Candida protease was determined in 0 2 M sodium citrate-HCl buffer of pH 2—6 with bovine serum albumin or hemoglobin as substrate. The pH optimum in presence of a denaturant was tested in the presence of 6 M urea with casein as a substrate [19]. For each pH step, blanks were measured separately.

Stability of Candida protease The effect of basic pH on the stability of Candida protease in solution was tested by exposure of enzyme to 50 mM Tris-HCl buffers (pH 8.12, 8.38, 8.55, 8.72, 8.89, 9.18, 9.3) for 30 min at room temperature prior to proteinase assay at pH 3.5.

Purified enzyme was frozen at —15°C and —60°C. Protection of the enzyme against freeze damage was tried by addition of different concentrations of glycerol, ethylene glycol or Triton X-100.

The stability of Candida proteinase against increasing temperature was tested by 10 min incubation in a water bath in presence of either 0.1 M sodium citrate, pH 6.6 or pH 7.0, or 0.1 M Tris citrate, pH 7.5. Residual enzymatic activity was tested at pH 3.5.

The action of non-ionic detergents (Triton X-100, Tween-80 and Nonidet P-40) as well as the anionic detergent SDS on purified Candida protease has been tested with detergent concentrations ranging from 0.01% to 1% (v/v). The enzyme was incubated with detergent at neutral pH (5 h, 22°C) prior to proteinase assay. The assay was modified according to Retz et al. [20] when SDS was employed.

Analysis of terminal amino acids The *N*-terminus of Candida was determined by the method of Chang et al. [21]. Recrystallized 4-*N*,*N*-dimethylamino-azobenzene-4′-isothiocyanate reagent was used for labeling of the *N*-terminal amino acid, which was identified by micro thin-layer chromatography on polyamide foils (3 × 3 cm), 4-*N*,*N*-dimethylaminoazobenzene-4′-thiocarbamyl-(DABTC)-diethylamine and DABTC-ethanolamine were employed as markers [22].

For determination of the C-terminus, cleavage of Candida protease with trypsin-free carboxypeptidase-A was performed [23].

Amino acid analysis was performed in an automatic amino acid analyzer (Biotronik) according to Moore [24].

Inhibitors EGTA (final concentration 1 mM), PMSF (final concentrations 0.1 and 1 mM) and *o*-phenanthroline at a final concentration of 1—4 mM were used according to Sapolski et al. [25]. Pepstatin was added from an alcoholic stock solution (1 mg/ml) at final concentrations ranging from 10^{-4}—10^{-10} M [26]. Dithiothreitol was applied at final concentrations between 1 and 100 mM,

N-ethylmaleimide was used at a final concentration of 5 mM [27] and benzamidine at 10 mM [28].

A pH profile of inhibition was measured employing diazoacetylnorleucine methyl ester (DAN), 1,2-epoxy-3*p*-nitrophenoxy propane (EPNP) and pepstatin as described by Kaehn et al. [29]. The final concentrations of inhibitors during preincubation with the enzyme were. DAN 1 mM, EPNP 10 mg/ml and pepstatin 0.1 mM. Preincubation was performed at room temperature for 10 and 60 min, respectively, in the pH range of 3.2—9.5 with purified proteinase at 10 μg/ml final concentration.

Inhibition of Candida proteinase by human α-2 macroglobulin was tested by incubation at 22°C for 10 h at pH 3.45 and pH 6.0, residual enzymatic activity was tested with albumin as a substrate.

Kinetic experiments Kinetics were measured at pH 3 0 with bovine serum albumin as a substrate at concentrations between 0.8 and 20 mg/ml; the enzyme concentration was constant at approx. 1 μg/ml. Data were plotted according to Lineweaver and Burk [30]. Inhibition kinetics were measured at constant substrate concentration and varying pepstatin concentrations ranging between 10^{-10} and 10^{-5} M. The data were plotted according to Dixon [30].

Stoichiometry of the pepstatin-proteinase bond According to Knight and Barrett [31], the linear portion of a plot of enzymatic activity vs pepstatin concentration was extrapolated and its intercept with the x-axis was taken as the point of equivalence. At a known protein concentration of the enzyme and with its molecular weight as derived from SDS-polyacrylamide gel electrophoresis, the molarity of the enzyme solution was calculated and compared with the molar concentration of inhibitor at the point of equivalence.

Trypsinogen kinase activity Conversion of trypsinogen into trypsin by Candida proteinase was determined essentially as described by Hofmann et al. [32]. The incubation of trypsinogen and Candida proteinase was performed at pH 3.5 and the subsequent casein assay was performed at pH 7.9.

Milk clotting Clotting of milk by different amounts of protease was tested at pH 5.5 [33].

Antisera Antibodies against purified protease were raised in rabbits by repeated subcutaneous injections of active enzyme in Freund's adjuvant at pH 6.6.

Results

Purification of acid proteinase

In a typical experiment the culture supernatant contained 0.047 units/ml of enzymatic activity. After repeated DEAE-cellulose chromatography, fractions contained up to 2.6 and 76 U /ml, respectively. The specific activity was raised accordingly from 0.04 U/mg to 24 U/mg. Residual hemoglobin fragments were removed by affinity-chromatography, thus specific activity was raised to approx. 40 U/mg, entailing a 1000-fold purification.

Due to solubility problems proteolytic activity could not be measured with azocasein [34] or related substrates. Serum albumin, azocoll [11] and hemoglobin [35] proved to be useful, although occasional precipitation of the latter prevented its application in kinetic measurements.

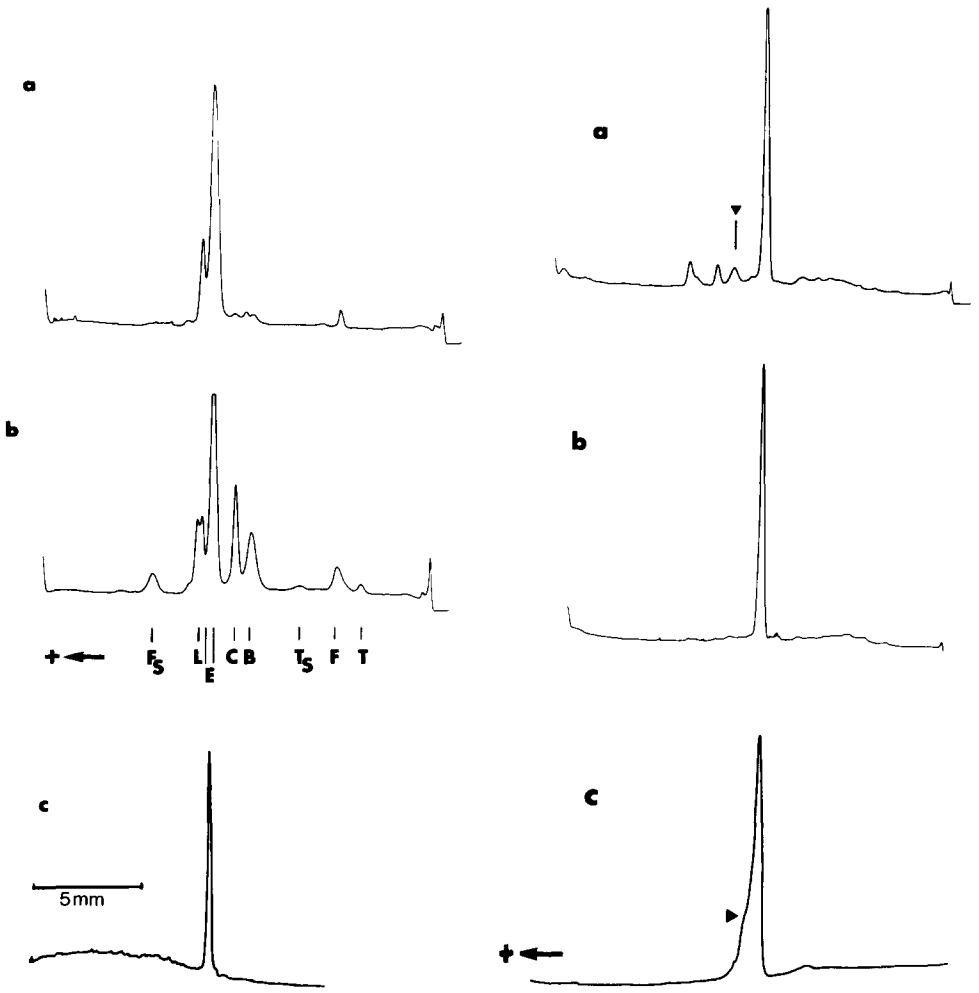

Fig 1. Pherograms of continuous polyacrylamide gradient gels (3—20% T, 2 5% C) after disc gel electrophoresis in presence of 0 1% SDS and subsequent Coomassie blue staining (a) Candida proteinase as desorbed from the second DEAE-cellulose column, the sample was boiled briefly in presence of 1% SDS and 1% (v/v) mercaptoethanol (b) Mixture of the same sample with standard proteins F_S, ferritin subunit, M_r 18 500, L, lactic dehydrogenase subunit, M_r 36 000, C, catalase subunit, M_r 60 000, B, bovine serum albumin, M_r 68 000, T_S, thyroglobulin subunit, M_r 330 000, F, ferritin, M_r 440 000, T, thyroglobulin, M_r 669 000, E, enzyme (proteinase) (c) Pherogram of a 10 μl polyacrylamide gradient gel (1—33% T, 2 5% C) after SDS-disc gel electrophoresis of Candida proteinase that had been further purified by gel filtration through Sephacryl-S 200 The enzyme consists of a single polypeptide chain

Fig 2(a) Pherogram of Candida proteinase after gel gradient electrophoresis in a discontinuous buffer system of pH 8 5 and subsequent staining with Coomassie blue The sample is identical with the sample of Fig 1a The major peak is an inactive aggregate that comigrates with bovine serum albumin (M_r 68 000), it is caused by alkaline denaturation in the course of electrophoresis (compare Fig 2c) A minor peak (▼) is left in the position of the active enzyme, faster polypeptides are possibly due to autolysis (compare 2b) (b) Pherogram of an electrophoresis of Candida proteinase under conditions as in Fig 2a, the sample has been treated with an excess of pepstatin prior to separation The single peak represents the inactive aggregate only (c) Electrophoresis of Candida proteinase under conditions as in Fig a and b. Prior to separation, the sample had been denatured by exposure to pH 9 5 (2 h at room temperature) The major peak is the inactive aggregate, the shoulder (▶) is due to autolysis there is less decay than in Fig 3a, this is possibly due to the increased speed of denaturation

Electrophoresis pattern of Candida proteinase

Under conditions of SDS-polyacrylamide disc gel electrophoresis [15,36] a major peak of approx. M_r 45 000 was revealed (Fig. 1). A comparable position was reached by the enzyme upon electrophoresis under non-denaturing conditions (compare Fig. 3). When active enzyme was submitted to disc gel electrophoresis at pH 8.4 in absence of any detergent, a different pattern was observed. A major peak appeared which comigrated with bovine serum albumin (M_r 68 000). This peak was accompanied by three peaks of lower molecular weight, the largest of them maintaining the approximate position of the active enzyme (Fig. 2a). Due to alkaline denaturation of the enzyme, no proteolytic activity could be assigned to any one of these peaks. Addition of pepstatin to the sample prior to exposure to the pH 8.4 buffer system may prevent the shedding of low molecular weight proteins, although the shift of apparent molecular weight from 45 000 to 68 000 was not affected by presence of the inhibitor (Fig. 2b).

If the active enzyme was preincubated under alkaline conditions, the major peak of bovine serum albumin size was once more revealed and no polypeptides of M_r 45 000 or less could be recognized (Fig. 2c).

Identification of enzyme after gel electrophoresis

Identification of the enzyme after electrophoresis in polyacrylamide gradient gels under non-denaturing conditions required the use of continuous buffers well below the pH of alkaline denaturation. A single peak was obtained that comigrated with ovalbumin (M_r 45 000); slicing and subsequent proteinase assay revealed a corresponding peak of enzymatic activity (Fig. 3).

Isoelectric point

The isoelectric point of Candida proteinase was identified by isoelectric focusing in beaded dextran gel [18], the p*I* was localized at pH 4.45 (Fig. 4). Initial failure of electrofocusing experiments was due to alkaline denaturation; reproducible results can readily be obtained if the sample is applied in the middle of the gel bed, provided wide range ampholytes are employed. Prolonged focusing causes self-digestion of the enzyme, and thus has to be avoided.

pH optimum

Candida proteinase displayed high enzymatic activity between pH 2.5 and 3.9 when tested with hemoglobin as a substrate. The plateau of the pH optimum is narrower with bovine serum albumin [3] and it is shifted to pH 3.7 if the assay is performed in the presence of 6 M urea with casein as a substrate. Under such denaturing conditions, no gross loss of enzymatic activity has been observed.

Effect of detergents

The effect of non-ionic detergents (Triton X-100, Tween-80, Nonidet P-40) and of SDS on purified Candida protease was tested at detergent concentrations between 0.01 and 1% by incubation at 37°C in phosphate-buffered saline. According to the azocoll and serum albumin assay, non-ionic detergents at 0.1% (v/v) caused a slight improvement of activity, while SDS was tolerated at

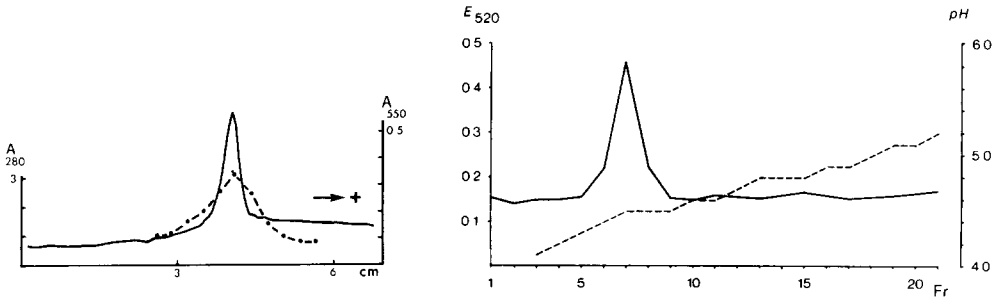

Fig 3 Section of the densitometric profile after Coomassie blue staining of a continuous polyacrylamide gel gradient (3—30% T, 2 5% C) after electrophoresis of Candida proteinase in presence of a continuous buffer system (25 mM sodium phosphate pH 6 7), the perpendicular line ( ) indicates the relative position of ovalbumin (M_r 44 000) A parallel channel of the gel has been sliced, proteolytic activity within the slices as measured by hemoglobin assay is indicated by the dashed curve (— —)

Fig 4 Profile of proteolytic activity as measured by the azocoll assay of Candida proteinase after electrofocusing in granulated gel employing narrow-range carrier ampholytes (pH 4—6), the dashed line represents the pH gradient Crude culture supernatant after concentration and dialysis against 10 mM citrate buffer (pH 6 5) was used as a sample A single peak of enzymatic activity was detected at pH 4 5, an identical result has been obtained after focusing in a pH gradient of 2—11

0.01% (w/v), it caused a decrease of 25% of activity at 0.1% (w/v) and it destroyed all activity at 1% w/v within a few minutes

Thermal stability

After 10 min of incubation at various temperatures, the course of denaturation of Candida proteinase at pH 6.6, 7.0 and 7.5 was monitored. The result indicates an increased thermal sensitivity above pH 7.0 when the pH approaches the value where alkaline denaturation takes place at room temperature (pH 8.45). Thus, at 37°C only 15% of the initial activity was left at pH 7.5 as compared to 95% at pH 6.6 and 7.0. At 45°C virtually all activity was destroyed at any pH tested. Electrophoretic analysis of such thermally denatured proteinase revealed a significant degree of autolysis following the conformational change associated with alkaline denaturation.

Alkaline denaturation

Like other proteinases of the carboxyl type, Candida proteinase was found to denature irreversibly at alkaline pH. There seems to exist a well-defined pH step (8.4—8.5) at which the enzyme at room temperature undergoes such denaturation, which is a fast process being complete within a few minutes. Denaturation results in aggregation of the enzyme, the single molecular species formed possibly represents an elongated dimer (Fig. 2). If the inhibitor pepstatin was present in excess during the process of aggregation, such dimers virtually appeared as a single peak upon gel gradient electrophoresis, while accompanying polypeptides of lower molecular weight were always present to a varying extent if the inhibitor was absent (Fig 2a, b). Thus alkaline denaturation includes formation of an enzymatically inactive dimer that undergoes autolytic degradation.

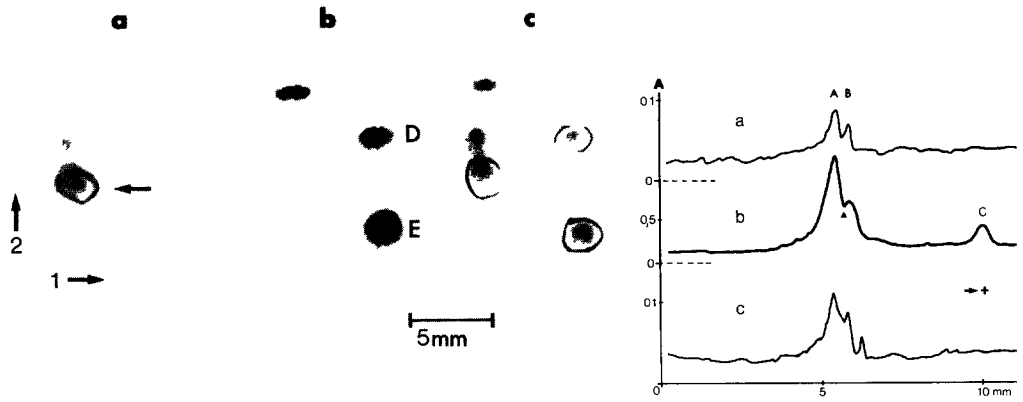

Fig 5 Determination of the N-terminal amino acid of Candida proteinase. (a) Chromatographic separation of the 4-*N,N*-dimethylaminoazobenzene-4′-isothiocyanate labeled reaction product on 30 × 30 mm micro polyamide foil (section), acetic acid (33% in H_2O) was used as a solvent in the first dimension and toluene hexane acetic acid (2 + 1 + 1) was employed in the second dimension, the start was at the lower left corner A single red spot was obtained (←), the accompanying spot is a blue 4-*N,N*-dimethylamino-azobenzene-4′-thiocarbamyl (DABTC) by-product). (b) Separation under identical conditions of the blue compounds DABTC-ethanolamine (E) and DABTC-diethylamine (D), the spot at the top is unreacted 4-*N,N*-dimethylaminoazobene-4′-isothiocyanate (c) Separation under identical conditions of a mixture of the markers (D, E) and the N-terminal amino acid, which is DABTC-tryptophan (Ref 22)

Fig 6 Glycoprotein-specific staining of Candida proteinase after disc electrophoresis in continuous polyacrylamide gradient micro-gels (1—33% T, 2 5% C) The sample underwent alkaline denaturation in the course of electrophoresis at pH 8 8 (a) Scan after periodic acid-Schiff-staining (b) Scan after subsequent staining of gel a with Coomassie blue The gel had been cut (▲) between both periodic acid-Schiff-positive peaks (A, B) prior to the second staining to allow for identification, both peaks reappeared at a comparable ratio of intensities, additionally the peak of a smaller polypeptide (C) appeared Peaks B and C represent products of autolysis, apparently peak B carries the sugar moiety of the enzyme (A) (c) Scan after alcian blue staining In this instance, enzyme had been treated with 0 1% SDS prior to electrophoresis The alignment of gel (C) is only tentative No alcian blue stained peak was detected if the SDS treatment was omitted

C- and N-terminus

Enzyme samples that were electrophoretically pure were submitted to the method of Chang [21] for determination of the N-terminal amino acid. By comparison with the standard pattern of Chang, the N-terminus has been identified as tryptophan (Fig. 5). The determination of the C-terminal amino acid was performed according to Nirata et al. [23]. After two different incubations with carboxypeptidase *a*, the following amino acids were identified with the amino acid analyzer.

	Leu	Tyr	Ile	Lys	Phe
10 min	37 63 mM	8 0	7 08	3.71	2 17
50 min	53 9 mM	20 23	16 85	8 07	3 62

Glycoprotein staining

Candida protease is a glycoprotein as has been proven by staining with sugar-specific dyes [16,17]. Comparison of such patterns with Coomassie blue stained peaks revealed a specific distribution of sugar residues among the autolytic fragments of the enzyme (Fig. 6).

Kinetics and inhibition

Kinetic properties of Candida proteinase have been tested with bovine serum albumin as a substrate at pH 3.0, when plotted according to Lineweaver and Burk [30], a linear function was obtained with an x-intercept indicating an apparent K_m in the range of $7 \cdot 10^{-5}$ M. The kinetics in presence of the inhibitor pepstatin were tested at pH 3.0. Inhibition of Candida proteinase was tested by titration with pepstatin at pH 3.2. The results were plotted according to Dixon [30] and yielded a biphasic curve. When plotted according to Knight and Barrett [31], extrapolation of the initial linear portion of the curve yielded an x-intercept in the proximity of 20 nM of pepstatin (Fig. 7). On the assumption that both enzyme and inhibitor were pure and at a known concentration of the enzyme (0.91 μg/ml), the molecular weight of the enzyme (45 000) was confirmed and equimolar binding of the enzyme and the inhibitor may be assumed.

Neither diazoacetyl-DL-norleucine methylester nor 1,2-epoxy-3-*p*-nitrophenoxypropane [29] effectively inhibited the enzyme, although their mode of binding to either of the two critical aspartic acid residues of acidic proteases is considered to be covalent [37].

Treatment of Candida protease by a commercial preparation of human α-2 macroglobulin at pH 3.45 and 6.0 did not cause any significant degree of inhibition at various ratios of concentration ranging from equimolar conditions

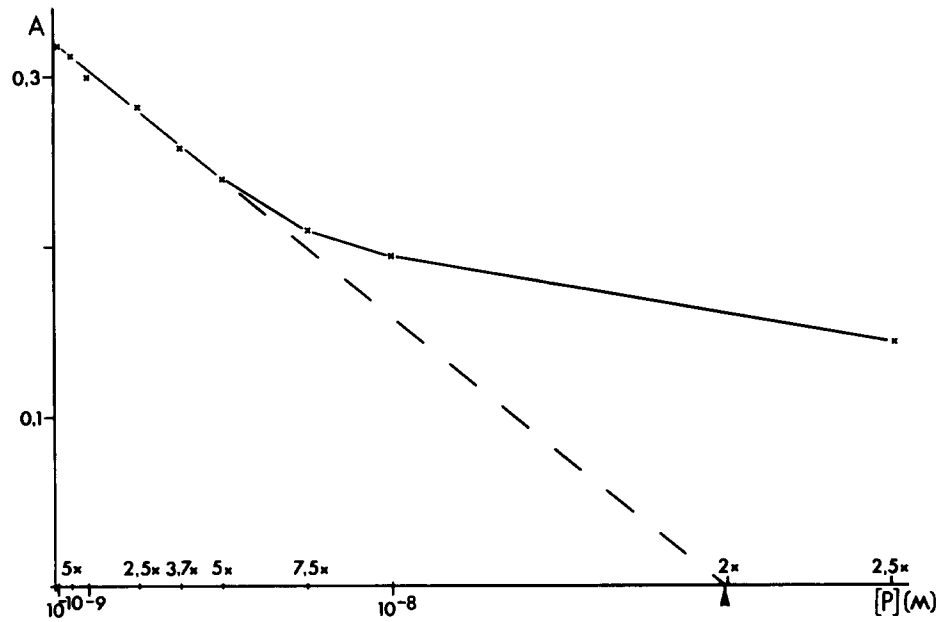

Fig 7 Inhibition of Candida proteinase by pepstatin Hydrolysis of serum albumin (10 mg/ml) by Candida proteinase (0 91 μg/ml) was measured at pH 3,2 and 37°C The assay was started by addition of enzyme to a mixture of substrate and pepstatin at various concentrations of the inhibitor (P) Trichloroacetic acid-soluble products of proteolysis were measured at 280 nm (A) Each point represents the mean of four determinations The linear regression of six points at the left was calculated, the intercept of this line with the abscissa (▲) indicates the point of equivalence

to 6-fold molar excess of macroglobulin. Electrophoresis under non-denaturing conditions revealed total hydrolysis of the inhibitor at pH 3.45 and partial cleavage at pH 6.0.

Clotting

After 5 min of incubation at 35°C, Candida proteinase at a concentration of 0.01 mg/ml was found to clot fresh milk at pH 5.5, thus in our hands it is approximately as efficient as porcine pepsin.

Trypsinogen kinase activity

Bovine trypsinogen was preincubated at 35°C and pH 3.5 for different time intervals from zero to 30 min with Candida proteinase. Aliquots of this digest (first stage) were mixed with casein substrate and were incubated at pH 7.9 for different time intervals in the second stage. At approx. 10 μg/ml of Candida protease and a 50-fold molar excess of trypsinogen, tryptic activity was raised approximately 40-times by 10 min incubation in the first stage of the experiment

N-Acetylphenylalanyl-L-diiodotyrosine

Hydrolysis by Candida proteinase of the synthetic pepsin substrate *N*-acetyl-phenylalanyl-L-diiodotyrosine (ADP) was tested at pH 3.2. For comparison, equimolar samples of porcine pepsin were employed with the same substrate at pH 2.0 [13,14]. According to these experiments, the molar catalytic activity of pepsin is at least 20-times higher as compared with Candida proteinase.

Proteolysis of some mammalian proteins

Candida protease cleaved human transferrin, α-1 antitrypsin, α-2 macroglobulin and both IgA_1 and IgA_2-myeloma proteins; the light chains, though, of these immunoglobulins, were not cleaved. So far only horse ferritin has been found to be fully resistent against Candida proteinase at pH 3.5.

Immunology

Antisera against purified Candida proteinase were elicited in rabbits. Such antisera produced precipitates with active proteinase as well as with proteinase after alkaline denaturation. The antisera did not react with a commercial polysaccharide antigen from Candida cell walls (Candida HA-test, Roche, Basle) [38], nor did they react with both the metabolic and the somatic Candida antigen of the Pasteur Institute. Negative reaction was also observed with the Candida antigen of Beecham, Neuss. Antisera against Candida proteinase did not react with two protein antigens from *C albicans* that had been isolated previously from lysed yeast cells [39].

Discussion

Carboxyl proteinases (EC 3.4.23.6) have been isolated from various fungal species preferentially belonging to the genus *Aspergillus* and *Penicillium* [40]. From the medical point of view, the acid proteinase of the yeast *C albicans* is of special interest, since it may be related to the pathogenicity of certain strains

of this 'opportunist' [4—6]. Although a proteinase of *C albicans* has already been partially characterized [3], we have re-investigated its properties employing methods that were not available for the previous study. Purification of Candida proteinase has been improved in the light of the determination of its isoelectric point, by recognition of its inhibition by the group-specific peptide pepstatin [26], and by the detection of its limited stability in the alkaline pH range.

Electrophoretic analysis of enzymatically active enzyme has been performed at neutral pH. A single protein species of such preparations has been identified to bear enzymatic activity (Fig. 3).

Apparently, tryptophan and leucine are the N- and C-terminal residues, respectively. Tryptophan is not present in the N-terminal sequence of cathepsin-D from hog and beef, nor does it occur in the N-terminal sequences of bovine and porcine pepsin and of some microbial carboxyl proteinases [41]. Upon SDS-polyacrylamide gel electrophoresis the single peptide chain of the enzyme revealed a molecular weight in the range of 45 000. This is in the range of the different variants of *Aspergillus oryzae* proteinases [42].

The isoelectric point of Candida proteinase has been determined in the pH range of 4.4—4.5, thus, it is located between the variants of cathepsin-D (pH 5—7) [43] and the Aspergillus proteases (pH 3.7—4.3) [44]. The substrate specificity and the pH optimum of Candida proteinase have been investigated previously [3]. In addition, we found an extended pH optimum of the enzyme vs. hemoglobin; this is possibly due to the poor solubility of bovine serum albumin in the vicinity of its isoelectric point (pH 4.8). As has been shown with cathepsin-D (Ref. 43), Candida proteinase is quite stable in 6 M urea and cleavage of casein under such conditions is observed up to pH 5.5; such shift has been ascribed to destabilization of the enzyme structure [43].

Candida proteinase and its denaturation products are glycoproteins as has been demonstrated by staining with the periodic acid-Schiff-procedure and by alcian blue [16,17]. This corresponds with data from *Aspergillus oryzae* proteinases [44] and from cathepsin-D [43].

The inhibition of Candida proteinase by the group-specific inhibitory pentapeptide pepstatin [26], as well as its resistance to inhibitory agents that are specific for other types of proteinase allows for classification, thus the enzyme belongs to the group of carboxyl proteinases (EC 2 4.23.6) [35]

The analysis of the binding kinetics of pepstatin to Candida proteinase is hampered by the strong enzyme-inhibitor bond [45]. Plots of enzyme activity vs. pepstatin concentration at constant substrate concentration according to Dixon [30] yielded bent curves, biphasic plots have also been demonstrated with other carboxyl proteinases [29,31].

The point of equivalence between the enzyme and pepstatin was estimated according to Knight and Barrett [31]. An equimolar ratio of binding was found (Fig. 7), which is in agreement with findings from related enzymes [26,29,31].

Diazoacetylnorleucine methyl ester and epoxy-nitrophenoxypropane [37] did not inhibit Candida proteinase. This confirms previous results with Candida protease [3] and related proteases of microbial origin [46].

Alkaline denaturation of Candida proteinase

A peculiar feature of carboxyl proteinases is their irreversible denaturation in

the neutral to moderately alkaline pH range [14]. Candida proteinase denatures at pH 8.4—8.5, the initial step according to electrophoretic analysis seems to be dimerization; no higher oligomer has been observed. The dimer is enzymatically inactive: it undergoes autolysis, though, that can be inhibited by pepstatin Possibly, homologous sites of the monomers are involved in the aggregation, these sites may include the active site of the enzyme. The dimer does not migrate like a globular particle of M_r 90 000, rather it behaves like a particle of M_r 70 000 upon gel electrophoresis. This discrepancy may be explained by presence of an elongated dimer, that undergoes alignment upon sieving in the gel matrix.

Attempts to prevent dimerization and to recover enzymatic activity from the dimer by dissociation have failed so far. Autolysis that causes release of polypeptides smaller than M_r 40 000 occurs in the course of alkaline denaturation, it can be prevented by addition of pepstatin (compare Figs. 2a and b). Our purified enzyme proved quite stable upon freezing or freeze-drying, loss of activity that has been ascribed to such treatment of the enzyme [3] was rather due to alkaline denaturation of the enzyme. CBS-proteinase was stable upon exposure to non-ionic detergents; the anionic surfactant SDS, though, caused rapid inactivation at a concentration of approx. 0.1% (w/v).

Hydrolysis of purified proteins

Among those serum proteins that proved to be susceptible to proteolysis by Candida proteinase, there are two proteinase inhibitors, namely α-1 antitrypsin, which by concentration is the most important (serine) proteinase inhibitor in serum [47] and α-2 macroglobulin. The latter is of particular interest since it is considered to be an inhibitor of all four types of proteinase [48], hence it might be the only non-antibody inhibitor of Candida proteinase in human serum. Employing purified macroglobulin, though, no significant inhibition of Candida proteinase was found. While other microbial pathogens with preference for mucous membranes produce a proteinase that is specific to IgA_1 only [49,50], *C albicans* proteinase can cope with the structurally different IgA_2 molecule as well [51].

Recently hydrolysis of salivary proteins by Candida proteinase at acidic pH has been communicated [6]. With IgA_2 myeloma protein we recognized proteolysis even at neutrality. This finding appears to be significant, since the IgA_1- and IgA_2-subtypes are distributed at a 1 1 ratio in secretory IgA fractions [50]; it possibly explains the data of Budtz-Jorgensen [4], who found a positive relationship between incidence of gingival stomatitis and infection with proteolytic Candida.

Candida proteinase did not digest horse ferritin nor did the yeast liquify gelatin. The inability to hydrolyze gelatin is a feature of mammalian cathepsin-D that is not shared by the pepsins [46].

Candida proteinase cleaves the pepsin substrate *N*-acetylphenylalanyl-L-diiodotyrosine at a very low rate, it thus resembles the porcine pepsin subtype C the denaturation of which by alkali takes places above pH 8.5 [13]; this is close to the denaturation point of Candida proteinase (pH 8.4) and differs from the other pepsins that denature at neutrality.

Trypsinogen kinase activity

Trypsinogen kinase activity has been demonstrated with carboxyl protein-

ases from different fungi such as *Penicillium janthinellum* [32], *Penicillium roqueforti* [52] and *Aspergillus oryzae* [42]. The trypsinogen kinase activity of Candida proteinase fits well into this pattern. Whether such activity can interfere with the regulation of serine proteinase activity in the host organism such as the clotting system and the complement system or whether such activity may be important for the intracellular persistence of *C albicans* in macrophages (Ref. 53), remains to be investigated

Immunology

Cross reaction between four commercial Candida antigens and anti-proteinase sera has been tested; there was no reaction in any of these experiments nor did the sera react with two cytoplasmic proteins of *Candida* that had been isolated recently from lysed yeast cells [39].

Candida proteinase is only inhibited by a several-fold excess of specific antibodies, this agrees with previous findings from cathepsin-D [43].

Antibodies in patients with manifest mycosis as demonstrated by the hemagglutination test (Roche, Basle) in some instances reacted with the proteinase antigen In one case presence of the enzyme in a patient's serum prior to appearance of specific antibodies could be demonstrated immunologically as well as by proteolysis assay. Whether presence of Candida proteinase itself can be interpreted as an early sign of serious yeast infection is presently under investigation.

A paper on the diagnostic value of purified Candida proteinase has very recently been published by Macdonald and Odds [54].

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