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Purification and Characterization of a Neutral Protease from *Saccharomycopsis lipolytica*

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Saccharomycopsis lipolytica 37-1 produced two inducible extracellular proteases, one under neutral or alkaline growth conditions and the second under acid conditions. Secretion of the neutral protease was repressed in the presence of glycerol or glucose, both of which supported rapid growth of the organism. Ammonium ions also repressed the secretion of the enzyme. The neutral protease activity copurified with esterase activity during ammonium sulfate fractionation, chromatography on diethylaminoethyl-cellulose, and gel filtration on Sephadex G-150. The molecular weight of the enzyme was estimated to be 42,000 by sucrose density gradient centrifugation and 38,500 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The purified enzyme had a pH optimum of 6.8. Phenylmethylsulfonylfluoride inhibited both protease and esterase activities, indicating the presence of a serine residue in the active center. Protease, but not esterase, activity was sensitive to ethylenediaminetetraacetate and was significantly activated by divalent ions. Dithiothreitol inhibited both protease and esterase activities, indicating the presence of a critical disulfide bridge. The enzyme hydrolyzed casein (K_m = 25.6 μM) and hemoglobin as well as the nitrophenyl esters of tyrosine (K_m = 2.4 mM), glycine, tryptophan, and phenylalanine.

Extracellular proteases from molds and bacteria have been studied extensively. In contrast, only limited studies on extracellular proteases from yeasts have been reported (1, 3, 17, 25). In a preliminary report (1), the production of two inducible extracellular proteases by *Saccharomycopsis lipolytica* 37-1 was described. One of the two proteases is produced under neutral or alkaline growth conditions, and the second is produced under acid conditions. The enzymes produced by *S. lipolytica* are of particular interest because of the possible suitability of this organism for the production of single-cell proteins from hydrocarbons and for use in degrading waste oils (1, 2, 11). The present paper reports the purification and characterization of the neutral protease.

MATERIALS AND METHODS

Materials. Casein (Hammersten), *N*-benzyloxycarbonyl nitrophenyl esters, bovine serum albumin, phenylmethylsulfonylfluoride (PMSF), and dithiothreitol were obtained from Sigma. Soybean, ovomucoid, and lima bean inhibitors were purchased from P-L Biochemicals Inc., Milwaukee, Wis. Crystalline yeast alcohol dehydrogenase, ovalbumin, chymotrypsinogen A, and cytochrome *c* were obtained from Boehringer Mannheim Corp., New York. Diethylaminoethyl (DEAE)-cellulose (Whatman DE-52) was obtained from Reeve Angel. Dimethyl suberimidate was prepared from suberonitrile as described by Davies and Stark (10). All other chemicals were of reagent grade.

Organism. *Saccharomycopsis (Candida) lipolytica* 37-1, isolated from frankfurters (3) in this laboratory, was used in all experiments.

Growth of cells and preparation of enzyme solutions. Cells were grown for 18 h at 27°C with agitation on Sabouraud-dextrose broth (Difco). A 20-ml suspension of this culture was used to inoculate the enzyme production medium, yeast nitrogen base (Difco), supplemented with 2% glucose and 1% casein in 0.05 M potassium phosphate buffer, pH 7.6. The cultures were incubated for 5 days with shaking at 27°C.

The basal medium for induction experiments was a defined basal salts medium containing (per liter): $MgSO_4 \cdot 7H_2O$, 0.7 g; $CaCl_2$, 0.4 g; NaCl, 0.5 g in 0.05 M potassium phosphate buffer (pH 7.0 or pH 7.6) or in sodium citrate buffer (pH 3.2) and 1 ml of trace metals solution. One liter of trace metals solution contained: citric acid, 10.0 g; $CaSO_4 \cdot 2H_2O$, 30.4 g; KI, 100 mg; $ZnSO_4 \cdot 7H_2O$, 720 mg; H_3BO_3, 500 mg; $FeCl_3 \cdot 6H_2O$, 5 g; $Na_2MoO_4 \cdot H_2O$, 200 mg, and $MnSO_4 \cdot H_2O$, 302 mg.

After autoclaving (121°C, 20 min), 10 ml of a vitamin solution was added to the medium. The vitamin solution contained (per liter): D,L-Ca pantothenate, 200 mg; biotin, 250 μg; nicotinic acid, 200 mg; thiamine, 100 mg; riboflavin, 100 mg; inositol, 500 μg; *p*-aminobenzoic acid, 50 mg; pyridoxine, 100 mg; and folic acid, 400 μg (sterilized separately by

filtration). The induction medium was supplemented with carbon and nitrogen sources. Sugars were used at concentrations of 2%; $(NH_4)_2SO_4$ was at a concentration of 0.2%; and casein was at a concentration of 1%. The inoculum for these cultures was 25 ml of cell suspension per liter of medium. In all cases, the crude enzyme solution was obtained by centrifugation at 20,000 × *g* for 15 min at 4°C.

Protein was determined by the method of Lowry et al. (16) with crystallized bovine serum albumin as a standard.

Protease assay. Proteolytic activity of the enzyme was measured at 30°C by a modification of the method of Hayashi et al. (14). The reaction mixture (final volume, 1 ml) contained: 100 μmol of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.0; 5.0 mg of Hammersten casein (Sigma); and 0.2 ml of enzyme solution. After approximately 10 min, 1 ml of 6% trichloroacetic acid was added, and the suspension was centrifuged for 20 min at 10,000 × *g*. Trichloroacetic acid-soluble hydrolysis products were assayed with the phenol reagent (16). Control experiments lacking enzyme or casein were routinely included. All determinations were made in the range in which reaction rates were constant and proportional to enzyme concentration. One enzyme unit was defined as the amount of enzyme that catalyzes the release of one nanomole of tyrosine equivalent per minute.

Esterase assay. Esterase activity was measured employing *N*-benzyloxycarbonyl tyrosine *p*-nitrophenyl ester (ZTNE) as a substrate (20). The reaction mixture contained 0.5 ml of methanolic buffer (0.06 M Tris-hydrochloride in methanol), pH 8.0, 50 μl of 1.2×10^{-3} M ZTNE in dioxane, and deionized water to 0.9 ml total volume. The rate of spontaneous hydrolysis of the ester was measured for 30 s, and then 100 μl of appropriately diluted enzyme was added and quickly mixed. The rate of enzymatic hydrolysis was measured at 400 nm in a Hitachi-Perkin-Elmer model 139 recording spectrophotometer. Esterase units were determined from the initial rate of reaction by drawing a tangent at the origin. One unit of enzyme activity is defined as the amount of enzyme catalyzing the release of 1 μmol of nitrophenol per min.

Sucrose gradient ultracentrifugation. Density gradient centrifugation was carried out as described by Martin and Ames (21) using the IEC rotor in an IEC B60 preparative centrifuge. Samples (0.2 ml) of enzyme preparation were layered on 13-ml linear sucrose gradients (5 to 20%, wt/vol, in 0.1 M potassium phosphate buffer, pH 7.6) and centrifuged at 38,000 rpm for 16 h at 3°C.

RESULTS

Induction of the enzyme. *S. lipolytica* produced two enzymes induced under different pH conditions. Cultures grown at pH 3.2 in a medium containing casein produced a protease active at pH 3.2 but not active at pH 8.0. Cultures grown in medium containing casein at pH 7.0 or 7.6 produced an enzyme active at pH 8.0 but not active at pH 3.2 (Table 1).

The enzyme produced under neutral or alkaline conditions was an inducible extracellular enzyme with highest activity observed in cultures grown on casein as the sole source of carbon and nitrogen (Table 2). Cultures without casein produced negligible protease activity, whereas cultures containing both casein and other carbon or nitrogen sources showed intermediate enzyme production. Glycerol was a more effective catabolite repressor than glucose. The presence of $(NH_4)_2SO_4$ also reduced protease activity.

Purification of the neutral protease. The crude enzyme solution was fractionated with ammonium sulfate. The fraction precipitating at 0°C between 40 and 60% saturation which contained 85% of protease activity was dissolved in 0.02 M Tris-hydrochloride buffer (pH 8.5); this solution was dialyzed against the same buffer. The dialyzed solution was pumped at a rate of 60 ml/h onto a column (40 by 1.6 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with 0.02 M Tris-hydrochloride buffer, pH 8.5. After washing with 100 ml of the equilibrating buffer, protein was eluted with a linear gradient from 0.02 to 0.5 M Tris-hydrochloride buffer, pH 8.5 (total volume, 400 ml). Both protease and esterase activities showed similar elution patterns. The two activities eluted be-

TABLE 1. *Effect of pH on enzyme induction*

Growth with casein at pH:	Assay pH	Sp act[a]
3.2	3.2	226
3.2	8.0	0
7.0	3.2	0
7.0	8.0	160

[a] Enzyme was assayed in culture fluids after 48 h of growth with casein as a substrate as described in the text, except that sodium citrate buffer was used for assay at pH 3.2. Specific activity is expressed as nanomoles of tyrosine equivalents released per minute per milligram of protein.

TABLE 2. *Effects of various carbon and nitrogen sources on neutral protease production by S. lipolytica*

Additions to basal medium	Differential activity[a]
Casein	1,650
Casein, glucose	200
Casein, glycerol	72
Casein, ammonium sulfate	1,100
Glucose, ammonium sulfate	10

[a] Differential activity was measured after 42 h of growth at pH 7.0. Differential activity is defined as esterase units per optical density of the culture at 460 nm.

tween 0.22 and 0.30 M Tris-hydrochloride, with the peaks at 0.25 M. Fractions containing enzyme activities were combined and concentrated by ultrafiltration. The concentrated protein solution was applied to a Sephadex G-150 column (110 by 1.6 cm; void volume, 79 ml) which was eluted at a rate of 8 ml/h with 0.05 M potassium phosphate buffer, pH 7.6. Representative fractions were examined for enzymatic activities towards casein and ZTNE. Fractions tested had the same ratio of activities toward the two substrates (Fig. 1).

The results of the purification are summarized in Table 3. The final enzyme preparation appeared homogeneous upon electrophoresis on 7.5% polyacrylamide gels at pH 8.3 (13).

Molecular weight. The molecular weight of the purified protease, measured by sucrose gradient ultracentrifugation with yeast alcohol dehydrogenase as a marker, was estimated to be 42,000. The molecular size of the protease was also estimated from a standard calibration curve on Sephadex G-150 (4) prepared with yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin, chymotrypsinogen A, and cytochrome *c*. A value of 38,000 was obtained. A sample of protease, which yielded a single band after polyacrylamide gel electrophoresis, was treated with sodium dodecyl sulfate and mercaptoethanol prior to electrophoresis in 0.05 M sodium phosphate buffer, pH 7.0 (28). Only one band was obtained; the molecular weight of the band determined from a plot of electrophoretic mobilities against the logarithm of molecular weights (28) of the bands obtained from cross-linked yeast alcohol dehydrogenase was 38,500. This value is in agreement with those obtained for the native enzyme by sucrose gradient ultracentrifugation and by gel filtration, showing that the enzyme is made of a single polypeptide chain.

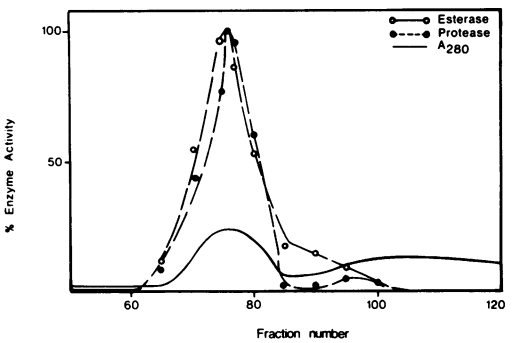

FIG. 1. *Elution pattern of protease and esterase from a Sephadex G-150 column. Protease sample containing 18 mg/ml in 0.1 M phosphate buffer, pH 7.6, was applied to a Sephadex G-150 column (120 by 1.6 cm) at a rate of 8 ml/h.*

TABLE 3. *Purification of the neutral protease from S. lipolytica 37-1*

Fraction	Protein (mg/ml)	Volume (ml)	Total[a] activity units	Sp act (U/mg of protein)
Crude extract	0.45	3,800	62,712	36.6
Ammonium sulfate	23.90	30	58,700	81.6
DEAE-cellulose	4.10	36	35,200	245.0
Sephadex G-150	1.90	40	22,500	293.3

[a] Enzyme was assayed with casein as a substrate.

Optimal pH. The pH optimum for the purified protease was at pH 6.8 in potassium phosphate buffer (Fig. 2). The activity declined sharply on the acidic side of the curve, with no detectable activity at pH 3.2.

Specificity of the purified enzyme. The purified enzyme was tested for its ability to hydrolyze various proteins and nitrophenyl esters (Table 4). Hemoglobin and bovine serum albumin were hydrolyzed, but to a lower extent than was casein. Esterase activity was highest against the tyrosine ester, but significant activity was also obtained with esters of glycine, tryptophan, phenylalanine, and leucine.

Kinetic studies. Hyperbolic saturation curves were obtained when the velocities of the protease and esterase activities were plotted against different concentrations of casein and ZTNE, respectively. The K_m and V_{max} values for protease and esterase activities were obtained from Lineweaver-Burke plots for casein and ZTNE. The values obtained were 25.6 μM

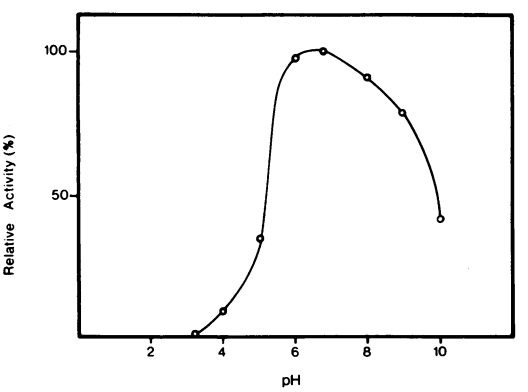

FIG. 2. *Effect of pH on protease activity. Activity at the pH optimum was taken as 100%. Buffers used: citrate, pH 3.2 to 6.0; potassium phosphate, pH 6.0 to 8.0; Tris-hydrochloride, pH 8.0 to 9.0; and glycine-KOH, pH 9.0 to 10.0.*

TABLE 4. *Substrate specificity*

Substrate	Sp act (× 10^4)	
	Protease[a]	Esterase[b]
Casein	282 (100)[c]	
Hemoglobin	118 (42)	
Bovine serum albumin	13.3 (5)	
Z-tyrosine[b]		17.11 (100)
Z-glycine		3.02 (17.6)
Z-tryptophan		2.58 (15)
Z-phenylalanine		1.67 (10)
Z-leucine		1.36 (8)
Z-valine		0.57 (4)
Z-proline		0.18 (1)
Z-cysteine		0.0 (0)

[a] Protease activity was assayed with 0.5% substrate.

[b] Esterase activity was assayed with ZTNE (Z) of the amino acids listed. The activity was determined as described in the text at a substrate concentration of 6×10^{-5} M.

[c] Numbers in parentheses designate percentages.

for casein and 2.40 mM for ZTNE and 800 U/mg of protein for the protease and 3.1×10^5 U/mg for the esterase.

Effect of inhibitors and salts. Of the protease protein inhibitors tested, only ovomucoid inhibitor gave marked inhibition (Table 5). The enzyme was activated by lima bean inhibitor and was not affected by soybean inhibitor. The proteolytic function of the enzyme was inhibited by both PMSF and ethylenediaminetetraacetate (EDTA) at concentrations of 5 mM. Esterase activity was completely inhibited by PMSF but was not significantly affected by EDTA. The effects of PMSF and EDTA indicated that both protease and esterase activities involved a serine residue in the active center and that the proteolytic activity required a metal ion for action. The divalent ions Ca^{2+}, Mn^{2+}, and Ba^{2+} activated the protease, but the same ions had little or no effect on the esterase activity. Mercury and copper had a slight inhibitory effect on the protease activity (Table 5). Dithiothreitol inhibited both protease and esterase activities, indicating the presence of a critical disulfide linkage.

DISCUSSION

Induction of the proteases of *S. lipolytica* 37-1 occurred in media with pH values compatible with enzyme function. The alkaline protease of *Neurospora crassa* was produced also only in alkaline culture conditions, whereas the acid protease was produced independently of pH (12). Neutral protease production by *S. lipolytica* occurred during the initial stages of growth in the presence of casein, even during times of little growth, suggesting that the enzyme is truly extracellular. These results are in agreement with the preliminary report by Ahearn et al. (1). Extracellular protease release for other yeasts was reported also for *Rhodotorula* (17) and for *Candida albicans* (25). These enzymes were characterized from culture media, but no induction studies have been published. The reduction in protease secretion was controlled by a mechanism analogous to catabolite repression. Regulation of extracellular protease secretion by readily assimilable carbohydrates is well documented for several bacterial species (7, 15, 19) and a fungus (27). In all cases, enzyme secretion was repressed by the presence of a preferred carbon source, as was the enzyme of *S. lipolytica*. The repression of enzyme secretion by ammonia reported here has been reported also for proteases from *Aspergillus nidulans* (8) and *N. crassa* (12).

The *S. lipolytica* protease has a narrow substrate specificity compared with other proteases that hydrolyze casein, hemoglobin, serum albumin, gelatin, and an array of other proteinaceous substrates. The esterase activity of the enzyme also exhibits more specificity than some other microbial enzymes since the enzymes from *Bacillus subtilis*, *Streptomyces fradiae*, and *A. oryzae* hydrolyze the esters of tyrosine, phenylalanine, and tryptophan with

TABLE 5. *Effect of inhibitors and salts on enzyme activity*

Inhibitor[a]	Relative sp act (%)	
	Protease[b]	Esterase[c]
None	100	100
PMSF	0	0
EDTA	0	95
Dithiothreitol	35	58
Soybean inhibitor	98	
Ovomucoid inhibitor	56	
Lima bean inhibitor	250	
$CaCl_2$	372	125
$MgCl_2$	276	98
$MnCl_2$	334	81
$BaCl_2$	208	82
$HgCl_2$	89	
$CuSO_4$	76	

[a] PMSF, EDTA, and dithiothreitol were added at a final concentration of 5 mM. Protease protein inhibitors were tested at 50-fold the enzyme concentration. The effect of salts was examined at a final concentration of 1 mM and in the presence of 0.5 mM EDTA.

[b] Protease activity was assayed with casein as a substrate.

[c] Esterase activity was assayed with ZTNE as a substrate.

equal frequency (23). The enzyme from *S. lipolytica* can be classified as a serine protease based on its sensitivity to PMSF. The EDTA sensitivity of the enzyme as well as its neutral pH optimum further classifies the enzyme as a metalloprotease. The dual sensitivity to EDTA and PMSF, though uncommon, has been reported for certain other bacterial and fungal proteases (5, 6, 9, 22). Studies with inhibitors from natural sources indicate that the enzyme from *S. lipolytica* is similar to other microbial proteases (24, 26) in the lack of sensitivity to the soybean inhibitor and the sensitivity to ovomucoid inhibitor. However, the activation by the lima bean inhibitor has been reported for other proteases.

The molecular weight of the neutral protease from *S. lipolytica* is similar to that reported for the acid protease from *C. albicans* (25). However, the two enzymes differ in several important properties; the activity of the enzyme from *C. albicans* is not affected by EDTA, mercaptoethanol, or Ca^{2+}, all of which were shown to affect protease activity from *S. lipolytica*. It will be of interest to compare the properties of the acid proteases from the two yeasts.

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