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Extracellular Acid Proteases Produced by *Saccharomycopsis lipolytica*

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Saccharomycopsis lipolytica CX161-1B produced at least three extracellular acid proteases during exponential growth in medium containing glycerol, Difco Proteose Peptone, and mineral salts at pH 3.4 (Difco Laboratories, Detroit, Mich.). Little extracellular acid protease activity was produced with glutamic acid as the sole nitrogen source, somewhat higher levels were obtained with peptone, and much higher levels were obtained with Difco Proteose Peptone. The relative amounts of the three proteases varied during growth on Difco Proteose Peptone, which suggested that the proteases were not coordinately regulated. The proteases were purified to near homogeneity (as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) by use of ultrafiltration, gel filtration, and DEAE-Sephacel and hydroxylapatite chromatography. Protease I had a molecular weight near 28,000, an isoelectric point of pH 4.9, and a pH optimum of 3.5. Protease II had a molecular weight near 32,000 and a pH optimum of 4.2. Protease III had a molecular weight near 36,000, an isoelectric point of 3.8, and a pH optimum of 3.1. All three proteases were glycoproteins; proteases I, II, and III contained 25, 12, and 1.2% carbohydrate, respectively. The proteases were inhibited by pepstatin and 1,2-epoxy-3-(4-nitrophenoxy) propane and were largely insensitive to diazoacetyl-DL-norleucine methylester and to compounds which inhibit the serine, sulfhydryl, or metallo-proteases.

The yeast *Saccharomycopsis lipolytica* has been used as a model system for the study of secretion in eucaryotes (16–18, 21, 22). *S. lipolytica* CX161-1B, a strain developed for genetic studies (15), has been found to produce one extracellular alkaline protease, no extracellular neutral protease, and at least one extracellular acid protease (19). The production of the single extracellular alkaline protease has been studied in the most detail (17–19, 22), and we have developed a system for investigating the intracellular events in its secretion (unpublished data). Possible secretion mutants (*xpr* mutants) which are affected in the production of the extracellular alkaline protease, RNases, and acid protease(s) have been isolated and investigated (16, 18). At high cell densities, *S. lipolytica* produces extracellular alkaline protease at grams-per-liter levels (24) and has potential as a host for secretion of foreign proteins.

This study of acid protease(s) secretion by *S. lipolytica* CX161-1B was undertaken to determine whether the acid proteases offered another useful model system for the study of secretion in *S. lipolytica* which would complement our alkaline protease secretion work. Results of this study would also be useful in considering *S. lipolytica* as a host for secretion of foreign proteins. In addition, relatively little is known about yeast extracellular acid proteases. Those produced by *Candida albicans* (20) and *Rhodotorula glutinis* K-24 (6) have been purified and characterized in some detail. *Saccharomyces carlsbergensis* has been reported to secrete four different proteases, each of which had a double pH optimum: one acidic between pH 2 and 4 and the other near neutrality between pH 6 and 8 (11). The information on the extracellular acid protease(s) of *S. lipolytica* has been limited to one study which was primarily concerned with an extracellular neutral protease. Abdelal et al. (1) reported that *S. lipolytica* 37-1 when grown with casein at pH 3.2 produced extracellular acid protease(s) active at pH 3.2 but not at pH 8.0, and that when grown with casein at pH 7.0 this strain produced an extracellular neutral protease and no extracellular acid protease.

We include results on the regulation and the number of extracellular acid proteases produced by *S. lipolytica* CX161-1B. We also include procedures for purification of these proteases and data on the molecular weights, carbohydrate

contents, isoelectric points, pH optima, and responses to protease inhibitors of the purified proteases.

MATERIALS AND METHODS

Strain. *S. lipolytica* CX161-1B *ade1* A (ATCC 32338) was obtained from J. Bassel and R. Mortimer from University of California, Berkeley.

Media. YM medium contained 0.3% each of yeast extract and malt extract, 0.5% peptone, 1% glucose; solid medium contained 2% agar. Glycerol-Proteose Peptone (pH 3.4) medium (GPP [pH 3.4]) contained (per liter): 6.7 g of glycerol, 1.6 g of Difco Proteose Peptone (Difco Laboratories, Detroit, Michigan), 1.7 g of Difco yeast nitrogen base without amino acids and ammonium sulfate, 30 mg of adenine, and 1 ml of polypropylene glycol MW2000 (Polysciences, Inc., Warrington, Pa.) in 46 mM citrate buffer (pH 3.4). Other media used for regulation studies were GPP (pH 3.4), containing twice the level of Difco Proteose Peptone (PPX2), or peptone (1.6 g/liter) or glutamic acid (2.4 g per liter) substituted for the Difco Proteose Peptone. The pH was adjusted to 3.4 before the medium was autoclaved.

Growth of cells and collection of culture broth. Cells grown overnight in YM broth at 23°C in a roller drum were inoculated into 500-ml baffled flasks containing 100 ml of medium for regulation studies or into 2,800-ml baffled Fernbach flasks containing 500-ml of medium for purification and characterization studies. Cultures were grown at 23°C with aeration, and samples were collected at various cell densities. Cell density was measured by using a Klett-Summerson photoelectric colorimeter with a green filter; 250 Klett units corresponded to 1 mg (dry weight) of cells per ml. The cells were removed by centrifugation (5,000 × *g* for 10 min) at 4°C, and the supernatant solution was stored at 4°C.

Acid protease assay. The acid protease assay was derived from the hemoglobin hydrolysis assay described by Larson and Whitaker (9). The substrate contained acid-denatured bovine hemoglobin (type II; Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 5 g/liter in 0.05 M acetate–0.05 M phosphate–5×10^{-6} M EDTA buffer adjusted to the desired pH (9). Enzyme (0.4 ml) and substrate (3.0 ml) were combined and incubated for 1 h at 25°C. The reaction was stopped with 3.0 ml of 10% trichloroacetic acid, the precipitate was filtered, and 1.0 ml of the filtrate was assayed by the Lowry procedure (5). A protease unit was the amount of protease causing an increase in absorbance of 0.1 at 750 nm after 1 h. Assays were done in duplicate.

Protein assays. The Lowry procedure as described by Herbert et al. (5) was used for protein assay with bovine serum albumin as the standard. Absorbance at 280 nm was used to monitor the protein content of column fractions.

Enzyme purification. All enzyme purification steps were done at 0 to 4°C unless otherwise noted. The columns were pumped at a rate of 24 ml/h, and 4.8-ml fractions were collected.

(i) Concentration. Ultrafiltration of supernatant fractions was done by using stirred ultrafiltration cells equipped with a Diaflo YM 10 membrane (Amicon Corp., Lexington, Mass.).

(ii) Gel filtration chromatography. Two milliliters of concentrated ultrafiltrate was applied to a Sephadex G-75 column (1.5 by 86 cm) equilibrated with 56 mM citrate buffer (pH 4.0). Absorbance at 280 nm and protease activity of the fractions were determined, and the active fractions were pooled.

(iii) Ion-exchange chromatography. The pooled active fractions were dialyzed (Spectrapor, 6,000 to 8,000 molecular weight cutoff; Spectrum Medical Industries Inc., Los Angeles, Calif.) overnight against 3 liters of the ion-exchange column starting buffer (10 mM disodium citrate [pH 6.0]). The dialyzed sample was applied to a DEAE-Sephacel column (2.5 by 20 cm) equilibrated with the starting buffer, and the column was eluted with a 400-ml linear gradient of 0.0 to 1.0 M NaCl in the starting buffer. Absorbance at 280 nm and protease activity of the fractions were determined.

(iv) Hydroxylapatite chromatography. For each peak of activity from the DEAE-Sephacel chromatography, the active fractions were pooled and dialyzed overnight against 3 liters of the hydroxylapatite column starting buffer (10 mM phosphate [pH 6.8]). The dialyzed sample was applied to a hydroxylapatite (Bio-Gel HT) column 0.9 by 15 cm) equilibrated with starting buffer, and the column was eluted with a 400-ml linear gradient of 10 to 400 mM phosphate buffer. Absorbance at 280 nm and protease activity were determined, and the active fractions were pooled.

PAGE. Polyacrylamide gel electrophoresis (PAGE) was done as described below.

(i) Sample preparation. Pooled fractions from hydroxylapatite chromatography were dialyzed against distilled deionized water overnight, and then concentrated either by lyophilization, by precipitation with 10% trichloroacetic acid, or by packing the dialysis membrane with an excess of polyethylene glycol (Carbowax PEG 8000; Fisher Scientific Co., Pittsburgh, Pa.).

(ii) SDS-PAGE. Protease preparations were boiled for 5 min in 2% sodium dodecyl sulfate (SDS)–8 M urea – 1% mercaptoethanol and were resolved on 9 or 10% polyacrylamide (0.375 M Tris [pH 8.8]) slab gels (1.5 mm; 10 by 14 cm) containing SDS by using the system described by Laemmli (8). A 3% stacking gel (0.125 M Tris [pH 6.8]) was used, and the gels were run at 20°C at 15 mA per gel during stacking and then at 35 mA per gel. The gel slabs were stained with Coomassie brilliant blue R.

(iii) Isoelectric focusing. Active protease preparations were resolved on 7.5% polyacrylamide slab gels (1.5 mm; 10 by 14 cm) containing 5% Pharmalyte (pH 2.5 to 5.0), by using chemical polymerization and the gel composition described by Wrigley (26). Gels were focused at 10°C at a constant power of 0.4 W per gel for 24 h and at 0.6 W per gel for 1 h. The gels were stained with Coomassie brilliant blue R. The pH gradient was determined by using an isoelectric focusing calibration kit (range, pH 2.5 to 6.5; Pharmacia Fine Chemicals, Piscataway, N.J.).

Molecular weight determination. Molecular weights were estimated by gel filtration on a Sephadex G-75 (3) column. The column was eluted at 20 ml/h with citric acid (0.4 mM)-phosphate (9.2 mM) buffer at pH 6.8, and 2.0-ml fractions were collected. The molecular weight standards (Pharmacia) were RNase A, chymotrypsinogen A, ovalbumin, and bovine serum albu-

TABLE 1. Extracellular acid protease production with various levels and types of nitrogen sources

Nitrogen source	Time[a] (h)	Cell density (mg [dry wt]/ml)	Acid protease (U/mg [dry wt] of cells)
PPX1	24	3.4	40
PPX2	24	2.9	61
Peptone	24	3.1	6
Glutamic acid	24	1.0	1.9
Glutamic acid	37	1.6	1.9

[a] After inoculation, cultures were inoculated at a cell density of 0.02 mg/ml.

min. Molecular weights were also estimated by SDS-PAGE (25). Phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin soybean inhibitor, and lysozyme were used as molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.).

Carbohydrate content. For each protease the pooled active fractions from hydroxylapatite chromatography were concentrated by ultrafiltration and applied to a Bio-Gel P-60 column (1.0 by 21 cm) and eluted with 50 mM phosphate buffer (pH 6.8). The column was pumped at 12 ml/h, and 3-ml fractions were collected and assayed for acid protease activity. The one or two most active fractions were then assayed for carbohydrate content by the phenol-sulfuric acid method of Dubois et al. (4) with mannose as a standard. In some cases, the fractions were further concentrated by using a stream of nitrogen gas before the carbohydrate content was determined.

pH optima. Protease samples obtained from the DEAE-Sephacel column were dialyzed overnight at 4°C against 10 mM Sorensen citrate buffer (pH 4.0). The hemoglobin substrate was adjusted to the desired pH, and the acid protease assay was run as described above. The pH reported was that value measured immediately after the addition of the protease to the substrate.

Inhibition studies. Pooled active fractions of each protease from the hydroxylapatite column were dialyzed against 0.1 M acetate–0.1 M phosphate buffer adjusted to the pH optimum of each protease. The concentrated inhibitor solutions were diluted with water and mixed with an equal volume of enzyme solution. Phenylmethylsulfonyl fluoride (Calbiochem La Jolla, Calif.) was dissolved in 95% ethanol. The 4-hydroxy-mercuribenzoic acid (Calbiochem) was dissolved in dilute alkali. Pepstatin A (Sigma) was dissolved in 50% methanol. The 1,2-epoxy-3-(*p*-nitrophenoxy) propane (Sigma) was dissolved in methanol with dilute alkali. Diazoacetyl-DL-norleucine methylester (Sigma) was dissolved in methanol. EDTA (Eastman Organic Chemicals Rochester, N.Y.) was dissolved in water. The enzyme-inhibitor mixture was preincubated for 60 min at 25°C, and then protease activity was determined at the pH optimum of each protease. Cupric acetate was included in the diazoacetyl-DL-norleucine methylester and 1,2-epoxy-3-(*p*-nitrophenoxy) preincubation mixtures. For 1,2-epoxy-3-(*p*-nitrophenoxy) a preincubation at 4°C for 20 h preceded the standard preincubation. The protease assays were done as described except that EDTA was omitted from the assay buffer.

RESULTS

Regulation of extracellular acid protease(s) production. The relative amounts of alkaline and acid extracellular protease(s) produced by *S. lipolytica* CX161-1B depended on the pH of the medium. For growth at pH 3.4, substantial protease activity was found at pH 3.0, but no protease activity was detected when the assay was done at pH 9.0 (19). In early experiments, cells were grown in GPP (pH 3.4), and cell-free culture broth was collected at a cell density of 3 mg (dry weight) of cells per ml. The pH optimum for the proteolytic activity of the supernatant fractions was pH 3.1, and this pH was used in the regulation experiments for assaying the acid protease(s).

In GPP (pH 3.4), extracellular acid protease activity was produced during the growth phase (Fig. 1). The initial rate of acid protease production (differential rate of 10 U/mg [dry weight of cells]) rapidly increased six- to sevenfold after the cell density exceeded 1 mg (dry weight) of cells per ml. The rate gradually declined as the culture approached the stationary phase.

The effects of various nitrogen sources on the production of extracellular acid protease activity were investigated (Table 1). The samples in Table 1 were all taken during active growth of the cultures. The cell densities in single-strength Difco Proteose Peptone (PPX1), PPX2, and peptone medium after 48 h were 5 to 6 mg (dry weight) of cells per ml. The cell densities after 72 h in glutamic acid medium were slightly over 2 mg (dry weight) of cells per ml. A slight decrease in pH to near 3.0 occurred in each of these media

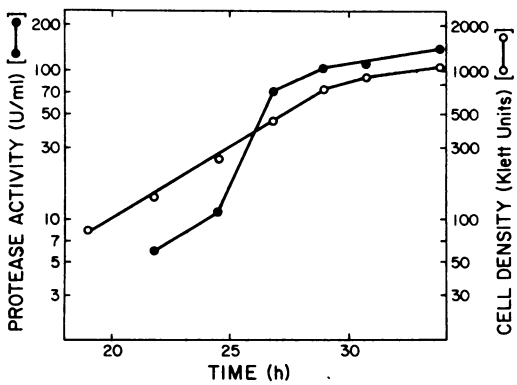

FIG. 1. Extracellular acid protease activity (measured at pH 3.1) during growth of *S. lipolytica* CX161-1B in GPP (pH 3.4). The culture was inoculated at a Klett value of 5. Symbols: ○, Klett reading; ●, Protease activity of cell-free broth.

TABLE 2. Purification of acid extracellular proteases produced by *S. lipolytica* CX161-1B

Fraction	Vol (ml)	Total activity (U)	Total protein[a] (mg)	Sp act (U/mg)
Culture broth	563	63,600	550	116
Ultrafiltrate	2	57,400	160	359
Sephadex G-75 (pooled fractions)	24	43,300	45.7	947
DEAE-Sephacel[b] (pooled fractions)				
Peak I	24	3,230	19.6	165
Peak II	24	7,090	2.4	2,950
Peak III	24	23,300	3.3	7,060
Hydroxylapatite[b,c]				
Protease I	72	3,110	5.3	587
Protease II	67	3,100	0.8	3,880
Protease III	67	18,600	1.5	12,400

[a] Determined by the Lowry method.
[b] Amounts indicated are corrected for the fact that only a portion of the previous fraction was taken through this step.
[c] Specific activity reported is that of the midpeak fraction. Total protein was calculated by dividing total activity by specific activity.

during cell growth. The cells grew at similar rates when peptone or PPX2 was substituted for PPX1 in GPP (pH 3.4). They grew more slowly and acid protease production was greatly reduced when glutamic acid was substituted as the sole nitrogen source. Activity was somewhat higher with peptone but still much lower than with Difco Proteose Peptone. In PPX2 medium there was a slight lag in growth and a definite lag in acid protease production early in the fermentation compared with those in PPX1 medium. However, with PPX2 both final cell density and acid protease production per cell were higher, so PPX2 medium was used for producing acid proteases for purification and characterization studies. In the experiment shown in Table 1, the maximum acid protease activity for PPX2 was 285 U/ml (at 30 h) and for PPX1 was 139 U/ml (also at 30 h).

The low initial rate of acid protease production in GPP (pH 3.4) and the fact that the rapid increase in the rate of production was delayed in PPX2 medium (i.e., the increase occurred at a higher cell density) suggested that acid protease production was repressed when the low-molecular-weight nitrogen components of Difco Proteose Peptone were readily available. Acid protease production would be derepressed after these components were used. To test this hypothesis, a concentrated solution of Difco Proteose Peptone was dialyzed (Spectrapor, 6,000 to 8,000 molecular weight cutoff) to remove low-molecular-weight components. The dialyzed Difco Proteose Peptone was used to replace Proteose Peptone at an equivalent nitrogen concentration in GPP (pH 3.4). In this medium, the lag time for cell growth was reduced significantly and the rapid increase in acid protease production occurred much earlier than in standard GPP (pH 3.4) (data not shown).

Isolation and purification. Cell-free culture broths were collected at 2.8, 3.3, and 5.5 mg (dry weight) of cells per ml, concentrated by ultrafiltration, and run separately on a Sephadex G-75 column. For each column, the active fractions were pooled and run on a DEAE-Sephacel ion-exchange column. Three peaks of activity eluted from each of the DEAE-Sephacel columns. The peaks eluted at similar positions, but the relative amount of activity in each peak differed (see below). These results demonstrated that *S. lipolytica* produces at least three extracellular enzymes with proteolytic activity at pH 3.1.

Based on these results, purification and characterization studies were done on culture broth collected at a cell density of 2.9 mg (dry weight) of cells per ml (Table 2). The supernatant fraction was concentrated about 300-fold, and 2.0 ml was run on a Sephadex G-75 column. One peak of protease activity was found (Fig. 2). The five most active fractions were pooled and run on a DEAE-Sephacel ion-exchange column. Three clearly separated peaks of activity (designated acid proteases I, II, and III based on their order of elution) were found (Fig. 3).

The active fractions in acid protease peak I from the DEAE-Sephacel column were pooled and chromatographed on hydroxylapatite. A single major peak of acid protease activity with a possible shoulder of activity was obtained (Fig. 4A). Similarly, single major peaks with shoulders were obtained for hydroxylapatite chroma-

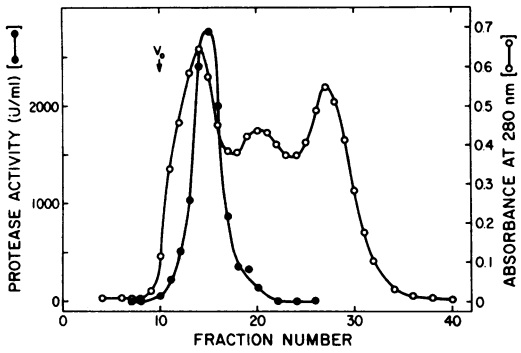

FIG. 2. Purification of protease activity by gel filtration on Sephadex G-75. Ultrafiltered (2 ml; 300-fold-concentrated) supernatant liquid was applied to a column (1.5 by 86 cm) of Sephadex G-75, and 4.8-ml fractions were collected. Fractions were pooled for further purification. V_o, Position of the void volume. Symbols: ○, absorbance at 280 nm; ●, protease activity at pH 3.1.

tography of acid proteases II and III (Fig. 4B and C).

The active fractions from the hydroxylapatite columns were dialyzed, and portions were lyophilized and examined by SDS-PAGE. Single protein bands were obtained for proteases I (Fig. 5, lane A) and III (Fig. 5, lane C), but none could be seen for protease II. The remaining portion of protease II was concentrated by using polyethylene glycol and examined by SDS-PAGE, and a single band appeared (Fig. 5, lane B).

The SDS-PAGE results indicate that the peaks of proteolytic activity in each case contained primarily one protein. To exclude the possibility that these proteins were not the proteases but that the proteases were minor undetected proteins, purified samples were examined by isoelectric focusing. Half of the gel was stained for protein, and pieces (1.0 by 1.5 cm) were cut from the other half and assayed for protease activity at pH 3.1. For these assays, the gel was crushed and added to 4.0 ml of substrate and the assay mixture was incubated for 24 h at 25°C. The gel pieces from areas corresponding to the protein bands for proteases I and III contained substantial levels of proteolytic activity as compared with gel pieces from areas which corresponded to regions lacking protein bands. For protease II, two protein bands were detected, both of which were proteolytically active. One had barely migrated into the gel, and the other had migrated to a point between protease I and III.

Molecular weight. Based on gel filtration chromatography on Sephadex G-75, the molecular weights of acid proteases I, II, and III were estimated to be 28,300 ± 300 (n = 3), 32,400 ± 750 (n = 3), and 36,100 ± 1,700 (n = 3),

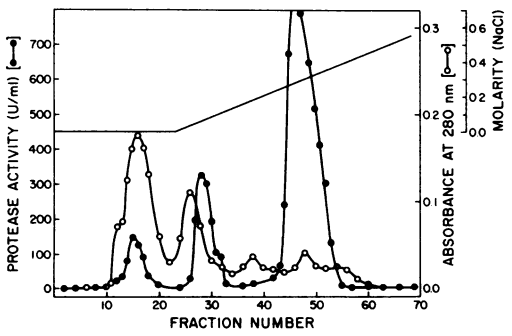

FIG. 3. Purification of protease activity on DEAE-Sephacel. Fractions 13 to 17 from Sephadex G-75 column were dialyzed overnight against starting buffer and applied to the column. Conditions for developing the column are described in the text. The flow rate was 24 ml/h, and 4.8-ml fractions were collected. Symbols: ○, absorbance at 280 nm; ●, protease activity at pH 3.1.

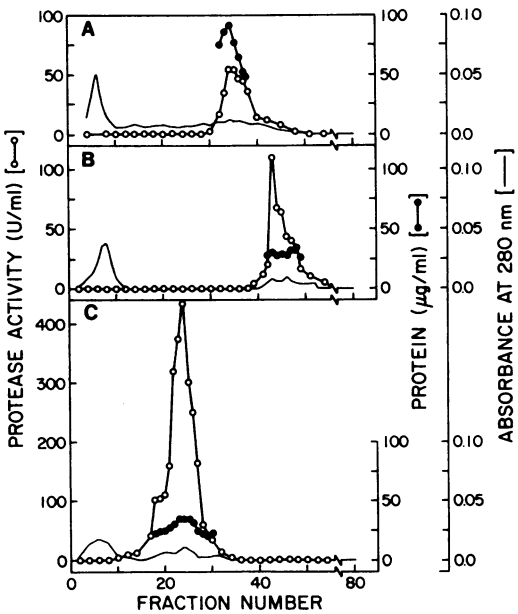

FIG. 4. Purification of protease activities on hydroxylapatite (Bio-Gel HT). Fractions from DEAE-Sephacel were dialyzed overnight against starting buffer and applied to the column. Conditions for developing the column are described in the text. The flow rate was 24 ml/h, and 4.8-ml fractions were collected. (A) Fractions 13 to 17 of extracellular acid protease I. (B) Fractions 28 to 32 of extracellular acid protease II. (C) Fractions 45 to 51 of extracellular acid protease III. Symbols: ●, protein concentration by the Lowry method; ○, protease activity at pH 3.1.

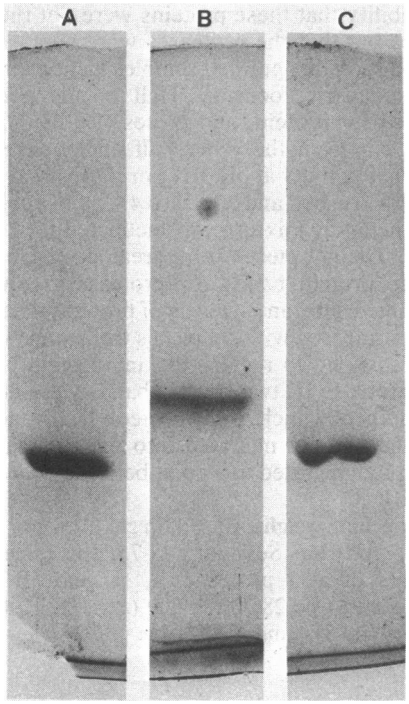

FIG. 5. SDS-PAGE of purified extracellular acid proteases. Lanes are as follows: (A) 14 μg of acid protease I; (B) 16 μg of acid protease II; and (C) 21 μg of acid protease III. Lanes (A) and (C) were nonadjacent lanes from the same gel, and lane (B) was from a separate gel.

respectively. (Values are the means ± standard deviation; n is the number of determinations.) The molecular weights estimated by SDS-PAGE for acid proteases I, II, and III were 40,300 (n = 2), 40,500 (n = 1), and 40,700 (n = 2), respectively. The reasons for the differences in relative and absolute molecular weights compared with those obtained by gel filtration are not known. However, the acid proteases are glycoproteins (see below) for which abnormally high molecular weight estimates based on SDS-PAGE are often obtained (10).

Carbohydrate content. The three acid proteases are glycoproteins with quite different carbohydrate contents (Table 3). Based on the percentage of carbohydrate in Table 3 and the molecular weights determined by gel filtration, the molecular weights of the polypeptide portions of acid proteases I, II, and III are 21,200, 28,500, and 35,700, respectively. The molecular weights of the carbohydrate portions are 7,100, 3,900, and 400, respectively.

Isoelectric point. Based on their positions in the isoelectric focusing gels in comparison to the calibration standards, the isoelectric points for acid proteases I and III were estimated to be pH 4.9 and 3.8, respectively.

TABLE 3. Carbohydrate content of the extracellular acid proteases of *S. lipolytica*

Acid protease	Concn (μg/ml)[a]		% Carbohydrate
	Protein	Carbohydrate	
I	250 (2)	82 ± 4.7 (4)	25
II	72 (2)	10 ± 1.5 (3)	12
III	430 ± 3 (4)	5.3 ± 1.0 (5)	1.2

[a] Scaled down fivefold from the standard procedures. Values are the means ± standard deviations. The numbers in the parentheses are the number of determinations.

pH optima. The pH optima for the acid proteases with acid-denatured hemoglobin as a substrate are shown in Fig. 6. The optimum for acid protease I was ca. pH 3.5; for acid protease II, ca. pH 4.2; and for acid protease III, ca. pH 3.1. At pH 6.0 and 7.0, the acid proteases were less active against casein than against hemoglobin, and no activity against casein was found at pH 8.0.

Effects of inhibitors. The activities of the acid proteases were largely unaffected by diazoacetyl-DL-norleucine methylester, phenylmethylsulfonyl fluoride, 4-hydroxy-mercuribenzoic acid, and EDTA (Table 4). Inhibition was obtained with pepstatin and 1,2,-epoxy-3-(*p*-nitrophenoxy).

Growth phase dependence. Preliminary experiments had suggested that the relative amounts (percentage of total activity) of the three extracellular acid proteases varied throughout the growth cycle. To confirm this result, cells were grown in PPX2 medium, and supernatant fractions were collected at 1.5, 3.4, and 5.1 mg (dry weight) of cells per ml. The cell-free culture

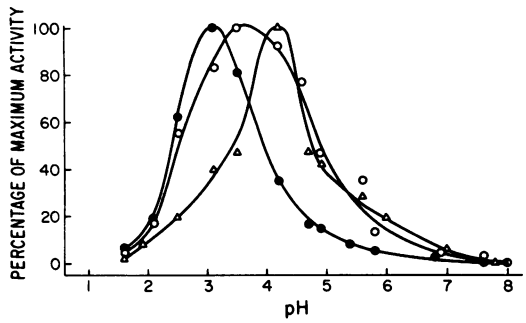

FIG. 6. Effect of pH on extracellular acid protease activities of *S. lipolytica* CX161-1B. Acetate-phosphate-EDTA buffer adjusted to the desired pH was used for the pH control. Symbols: ●, protease III; ○, protease I; △, protease II.

TABLE 4. Effects of inhibitors on activity of the extracellular acid proteases of *S. lipolytica*

Inhibitor	Concn[a]	Acid protease activity remaining (%)		
		I	II	III
Control[b]		100	100	100
PMSF[c]	1 mM	94	115	99
	10 mM	103	113	99
PCMB[c]	1 mM	107	113	96
EDTA[c]	1 mM	98	111	99
	10 mM	107	113	99
DAN[c]	1 mM + 1 mM Cu^{2+}	119	113	92
	10 mM + 4 mM Cu^{2+}	112	111	99
EPNP[c]	1 mM + 1 mM Cu^{2+}	124	113	102
	10 mM + 4 mM Cu^{2+}	17	78	50
Pepstatin[d]	1 μM	52	63	48
	10 μM	31	46	28
	100 μM	5	25	6

[a] Concentration of inhibitor in the enzyme-inhibitor preincubation mixture.

[b] Methanol and ethanol at the levels present in the preincubation mixtures did not affect activity.

[c] PMSF, Phenylmethylsulfonyl fluoride; PCMB, 4-hydroxy-mercuribenzoic acid; DAN, diazoacetyl-DL-norleucine methylester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy). Concentrations of acid proteases I, II, and III in the preincubation mixtures were 2.4, 0.52, and 0.75 μM, respectively, as estimated from their protease activity.

[d] Concentrations of acid proteases I, II, and III in the preincubation mixtures were 0.19, 0.03, and 0.29 μM, respectively, as estimated from their protease activity.

broths were concentrated by ultrafiltration, run on a DEAE-Sephacel column, and protease activity of the column fractions was measured at pH 3.1. As expected, three peaks of protease activity were detected for each column. The absolute amount of each of the three proteases increased as the cell density increased (Table 5). The relative amounts of proteases I and II decreased about twofold, and the relative amount of protease III increased about threefold as the cell density increased from 1.5 to 5.1 mg (dry weight) of cells per ml. If the stabilities in the supernatant fractions and the recoveries after ultrafiltration and ion-exchange chromatography are at all similar for the three proteases at the different cell densities, then the three proteases are clearly not coordinately regulated.

DISCUSSION

This study demonstrated that *S. lipolytica* CX161-1B produces at least three proteases with acidic pH optima. The fact that these proteases were found in the extracellular medium during exponential growth suggests that they are secreted and not present due to cell lysis. Abdelal et al. (1) found that production of extracellular acid protease activity by *S. lipolytica* 37-1 occurs in casein medium at pH 3.2 but not at pH 7.0. The relative amounts of acid and alkaline extracellular protease activity produced by *S. lipolytica* CX161-1B also depends on the pH of the medium (19). At pH 3.4, CX161-1B produces no neutral or alkaline extracellular proteases (19).

In addition to regulation by the pH of the medium, acid extracellular protease production was greatly affected by the nitrogen source in medium (Table 1). As is true for the alkaline extracellular protease of *Candida lipolytica* NRRL Y-1094 (17), at least some of the acid extracellular proteases seem to play a nutritional role. If low-molecular-weight nitrogen sources are present, then acid protease production is low. It greatly increases only if higher-molecular-weight nitrogen sources, which must first be hydrolyzed before they can be assimilated by the cells, are present in the medium.

The facts that the polypeptide portions of each protease are of quite different sizes and that the smallest protease contains substantially more carbohydrate than the next largest protease, which contains more than the largest protease, strongly suggest that the three proteases are not derived from a single gene product by differential modification. Also, the amounts of the three proteases at different cell densities do not suggest that any of the proteases are digestion products of the other proteases. If the three proteases are products of three different genes, then the fact that the relative amount of each protease varies throughout growth in GPP (pH 3.4) suggests that the proteases are not coordinately regulated.

The lag times and growth patterns on the various Difco Proteose Peptone-containing media were somewhat unexpected. One might expect to find the highest growth rates and shortest lag times when the concentration in the medium

TABLE 5. Production of extracellular acid proteases at different cell densities

Cell density (mg [dry wt] of cells per ml)	Acid protease activity[a] (U per peak)		
	I	II	III
1.5	7,610 (57)	3,220 (24)	2,420 (18)
3.4	10,500 (32)	8,020 (24)	14,800 (44)
5.1	21,600 (31)	9,120 (13)	38,900 (56)

[a] Activity in each peak was assayed at pH 3.1 and normalized to a starting volume of 500 ml of culture broth. The volumes used were 1,390, 990, and 450 ml for cell densities of 1.5, 3.4, and 5.1 mg/ml, respectively. Values in parentheses represent the percentages of total activity.

of the low-molecular-weight components of proteose-peptone is greatest. However, the growth rate did not decrease after the low-molecular-weight nutrients had been used (this occurs at a cell density of about 1.2 to 1.6 mg [dry weight] of cells per ml; unpublished data). Also, the lag time increased as the amount of low-molecular-weight nutrients increased; the lag time was longest in PPX2 medium and shortest in dialyzed proteose-peptone medium.

Although acid protease II ran as a single band on SDS-PAGE, peak II from the DEAE-Sephacel column may contain more than one acid protease. Two adjacent peaks of protease activity were originally found in the DEAE-Sephacel peak II fractions, but upon reassay only one was found. There was a double protein peak and a possible shoulder on the protease activity peak from the hydroxylapatite chromatography of acid protease II. For the acid protease II fractions, no activity was recovered after lyophilization, and protein bands were difficult to detect after isoelectric focusing and SDS-PAGE. These results are consistent with peak II containing two acid proteases: one extremely unstable and the other less stable than acid proteases I and III. The elution position of acid protease II from the DEAE-Sephacel column suggested that it had an isoelectric point between that of acid proteases I and III. In the one isoelectric focusing gel where bands were detected for protease II, two bands of protease activity were found. One had the mobility expected for a protein with an isoelectric point between that of acid proteases I and III, but the position of the second band near the origin is difficult to explain unless perhaps it represents active aggregated material.

Acid proteases I and III also ran as single bands on SDS-PAGE. The proteases in the shoulders of activity adjacent to the major peaks of the hydroxylapatite columns either must have mobility similar to that of acid protease I or III or must be present at low levels undetectable at the loadings used on the SDS-polyacrylamide gels. The shoulders might contain separate gene products, but quite probably they contain slightly modified products from the acid protease I or III genes. The polypeptide portion might be partially degraded or the carbohydrate content might be somewhat heterogeneous or both.

Many yeasts have been found to produce acid extracellular proteases (2, 7). However, few have been characterized to any extent. *C. albicans* produces an acid extracellular protease with an optimum of activity at pH 3.2 and a molecular weight of 40,000 (20). The protease is not inhibited by diazoacetyl-DL-norleucine methylester which was unexpected for a pepsin-like carboxyl-protease (23). *Rhodotorula glutinis* produces an extracellular protease with optimum activity at pH 2.0 to 2.5 which is inactivated by pepsin inhibitor SP-1 (6). It is not known whether the *C. albicans* or *R. glutinis* acid extracellular proteases are glycoproteins. *S. carlsbergensis* has been reported to secrete four proteases which have double pH optima against casein: pH 3.0 and 7.0 (II). The four proteases are glycoproteins with the carbohydrate content ranging from 2 to 38%. The acidic intracellular protease (proteinase A) from *Saccharomyces cerevisiae* has been studied in much greater detail than any of the yeast acid extracellular proteases. It has a molecular weight of 41,500, a carbohydrate content of 8.5%, and a pH optimum against acid-denatured hemoglobin of 3.0, and it is inhibited by pepstatin, DAN, and EPNP, which suggests that it is a carboxyl protease (13).

The *S. lipolytica* extracellular acid proteases have many properties similar to those of other carboxyl(acid) proteases (23). The *S. lipolytica* acid proteases have acid pH optima in the range 2.0 to 5.0, and they are largely insensitive to compounds which inhibit the serine, sulfhydryl, or metallo proteases (12). They are inhibited by pepstatin and 1,2-epoxy-3-(p-nitrophenoxy) but not by diazoacetyl-DL-norleucine methylester (22). They have molecular weights from 28,000 to 36,000, and their isoelectric points are between pH 3.0 and 5.0, the range found for other microbial extracellular acid proteases (14).

Several pleiotropic *S. lipolytica* mutants which produce reduced levels of alkaline extracellular protease (*xpr* mutants) and extracellular RNase(s) also produce less than 10% of wild-type levels of acid extracellular protease activity (16). Based on the results of this study, the low level of acid protease activity in these mutants has to be due to reduced levels of at least proteases II and III (Table 2). If the pleiotropic *xpr* mutants affect secretion, then these results demonstrate that some component or step of the secretion process is shared by the extracellular alkaline protease, RNases, and acid proteases.

In conclusion, production of extracellular acid proteases by *S. lipolytica* will be more complicated to study than production of the single extracellular alkaline protease. At least three acid proteases are produced, and they seem to be regulated independently. The information in this paper on the regulation, pH optima, and relative amounts produced during cell growth of the acid proteases will be useful in future secretion studies with *S. lipolytica* CX161-1B.

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