Extracellular RNase Produced by *Yarrowia lipolytica*

SUK-CHUN CHENG AND DAVID M. OGRYDZIAK*

Institute of Marine Resources, University of California Davis, Davis, California 95616

Production of extracellular RNase(s) by *Yarrowia lipolytica* CX161-1B was examined in media between pHs 5 and 7. RNase production occurred during the exponential growth phase. High-molecular-weight nitrogen compounds supported the highest levels of RNase production. Several RNases were detected in the supernatant medium. Based on sodium dodecyl sulfate-polyacrylamide gel eletrophoresis, the RNases had estimated molecular weights of 45,000, 43,000, and 34,000. It was found that *Y. lipolytica* secretes only one RNase (the 45,000-molecular-weight RNase) and that the 43,000 and 34,000-molecular-weight RNases are degradation products of this RNase. The alkaline extracellular protease secreted by *Y. lipolytica* was shown to have a major role in the 45,000- to 43,000-molecular-weight conversion, and it was demonstrated that the 45,000-molecular-weight RNase could be purified from a mutant which does not produce the alkaline extracellular protease. Purification of the RNase from a wild-type strain resulted in purification of the 43,000-molecular-weight RNase. This RNase was a glycoprotein with a molecular weight of 44,000 as estimated by gel filtration, an isoelectric point of pH 4.8, and a pH optimum between 6.5 and 7.0.

The yeast *Yarrowia lipolytica* (formerly *Saccharomycopsis lipolytica* or *Candida lipolytica* [33]) provides a model system for the study of protein secretion in eucaryotes. Much has been learned about protein secretion and processing from studies of *Saccharomyces cerevisiae* (7, 28). *Y. lipolytica* is also amenable to genetic (8, 20) and biochemical analyses, and it differs from *S. cerevisiae* in several important respects including dimorphic growth, utilization of hydrocarbons, and the ability to secrete an alkaline protease (22, 24), at least three acid proteases (38) and an RNase(s) (23) into the extracellular medium.

Y. lipolytica has been used industrially (13), and it has potential as a host for secretion of foreign proteins. At high cell densities it produces the alkaline extracellular protease (AEP) at gram-per-liter levels (30), and recently *Y. lipolytica* transformation systems have been developed (5, 9).

In our studies of protein secretion by *Y. lipolytica*, the production of the AEP has been studied most extensively. We have isolated mutations in the structural gene (29) and in other genes (*xpr* mutations) which affect AEP production (23). Some of the *xpr* mutations are pleiotropic and also affect secretion of the acid proteases and RNase(s) (21, 23).

This study of RNase production by *Y. lipolytica* was undertaken to develop another system to complement studies on AEP processing and secretion. In addition, although yeasts in many genera secrete RNases only the RNases secreted by *Rhodotorula glutinis* (18, 19) and *C. lipolytica* (J. Fribourg, M.S. thesis, Massachusetts Institute of Technology, Boston, 1972) have been characterized to any extent.

The major goals of this study were to determine conditions for production of the extracellular RNase(s), to determine how many were produced, to purify and partially characterize the major RNase produced, and to prepare antibody which would be useful in studying intracellular events in processing and secretion of the major RNase. We demonstrated that, although several polypeptides with RNase activity are present in culture broth, *Y. lipolytica* actually secretes only a single RNase which is rapidly partially degraded.

MATERIALS AND METHODS

Strains. *Y. lipolytica* CX161-1B *ade1 A* (ATCC 32338), a strain suitable for genetic studies (20), was obtained from J. Bassel, University of California, Berkeley. *Y. lipolytica* PS6069 *ade1 xpr2-34 A* (ATCC 46028) was isolated by UV mutagenesis of CX161-1B (29).

Media. Cultures were maintained on YM medium (24). Glycerol-Proteose Peptone (GPP medium) was prepared as described previously (24). GPPC2 medium contained (per liter): 20 g of glycerol, 6 g of Proteose Peptone (Difco Laboratories, Detroit, Mich.), 5 g of Difco yeast nitrogen base without amino acids and ammonium sulfate, 60 mg of adenine, and 1.0 ml of antifoam in 0.15 M citrate buffer, pH 5.0. GPPP2 was similar to GPPC2 except that the citrate buffer was replaced with 0.1 M phosphate buffer, pH 6.8.

Skim milk-azide plates were prepared as described previously (24). RNA-azide plates contained (per liter): 2 g of RNA (Sigma; torula yeast RNA, type VI), 0.145 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.15 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of NaCl, 0.625 g of sodium azide, and 20 g of agar in 0.05 M sodium citrate buffer, pH 5.0. Plates contained 15 ml of medium.

Growth of cells and collection of culture broth. Cells were grown in 50 ml of medium in 500-ml baffled flasks or in 500 ml of medium in 2.8-liter baffled Fernbach flasks at 23°C agitated at 300 rpm in a New Brunswick PsycroTherm. For culture volumes above 4 liters, 10-liter fermentations were carried out in a New Brunswick Microferm fermentor at 23°C with an impeller speed of 600 rpm, an air flow rate of 9.5 liters/min, and manual antifoam addition. Cell density was measured with 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 (9,000 × *g* for 10 to 15 min) at 4°C, and the supernatant solution was stored at 4°C.

RNase assay. (i) Agar diffusion. Wells (6-mm diameter) were cut in RNA-azide plates and filled with 35 μl of enzyme solution. Plates were incubated at 40°C for 2 h and then flooded with 0.1% toluidine blue (Sigma Chemical Co., St.

---

* Corresponding author.

Louis, Mo.; type O) dissolved in 50% ethanol. The diameter of the pink halo formed was measured and converted to RNase activity based on a calibration curve of the logarithm of RNase activity (determined by the RNA hydrolysis assay) versus halo diameter.

(ii) **RNA hydrolysis.** A modification of the RNA hydrolysis assay described by Anfinsen et al. (2) was used. Fifty microliters or less of enzyme was combined with 0.1 ml of buffer containing 100 mM sodium citrate (pH 5.0), 10 mM $MgSO_4$, and 1 mM $CaCl_2$, and the solution was made up to 0.45 ml with distilled water. Fifty microliters of 1.5% RNA (Sigma; type XI, Crestfield purified) in the same buffer was added, and the mixture was incubated for 45 min at 40°C. The reaction was stopped by addition of 0.5 ml of a 12% (vol/vol) perchloric acid solution containing 0.4% uranyl acetate. After cooling and centrifugation, the supernatant was diluted 20-fold and the A_{260} was measured. An RNase unit was defined as the amount of enzyme that produces an increase in A_{260} of 0.1/min (before dilution) at 40°C. Samples were assayed in triplicate.

(iii) **2',3'-cUMP hydrolysis.** A modification of the method of Sato et al. (27) was used. A maximum of 0.2 ml of enzyme solution was added to 1.0 ml of 0.25 M citrate buffer (pH 6.0), 0.5 ml of 2',3'-cyclic UMP (2',3'-cUMP; 2.03 mg/ml), and distilled water to a final volume of 2.5 ml. The increase in A_{285} at 30°C was recorded. One unit of RNase activity was defined as the amount of enzyme which produces an increase in absorbance of 1.0/min at 30°C. One unit based on the 2',3'-cUMP hydrolysis assay was equivalent to about 8.6 units based on the RNA hydrolysis assay.

Protease assay. Protease activity was estimated by the agar diffusion assay done with skim milk plates as described previously (24).

Protein assays. The Lowry procedure as described by Herbert et al. (10) and the dye-binding procedure described by Bradford (3) were used for protein assay with bovine plasma albumin as a standard. A_{280} 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. A_{280}, RNase activity, and in some cases protease activity and salt concentration of the fractions were determined. The conductivities of the samples were measured by a conductivity meter (The London Company, Westlake, Ohio).

(i) **Concentration.** Ultrafiltration of supernatant medium was done with stirred ultrafiltration cells equipped wih Diaflo YM 10 membranes (Amicon Corp., Lexington, Mass.).

(ii) **Gel filtration chromatography.** Concentrated ultrafiltrate (4 to 7 ml) was applied to a Sephadex G-75 column (1.5 by 75 cm) equilibrated with 33 mM phosphate buffer, pH 6.8, containing 1 mM $CaCl_2$.

Concentrated enzyme solution (0.6 ml) was applied to a Bio-Gel P-60 column (0.9 by 60 cm) equilibrated with 50 mM Tris hydrochloride, pH 7.0, containing 10 mM of NaCl and $CaCl_2$.

Concentrated enzyme solution (1.15 ml) was applied to a Sephadex G-100 column (0.9 by 54 cm) equilibrated with 33 mM phosphate buffer, pH 6.8, containing 0.02% sodium azide. The column was pumped at 2.2 ml/h, and 1.1-ml fractions were collected.

(iii) **Ion-exchange chromatography.** Ultrafiltrate concentrated from 2.5 to 10 liters of medium to 100 to 300 ml was dialyzed against 20 mM Tris hydrochloride, pH 7.0, containing 1 mM $CaCl_2$ and 33 mM NaCl and applied to a DEAE-Sephacel column (2.5 by 28.1 cm). The columns were eluted first with 200 to 600 ml of the starting buffer and then with a

1-liter linear gradient of 0 to 1.0 or 1.2 M NaCl in the same buffer. The columns were pumped at 30 to 40 ml/h, and 5-ml fractions were collected.

(iv) **Affinity chromatography.** Procedures for chromatography of RNase on agarose 5'-(p-aminophenylphosphoryl)uridine-2'(3')-phosphate (UMP agarose; Miles-Yeda Ltd.) were modified from those described by Wierenga et al. (35). Samples were dialyzed against 20 mM sodium acetate, pH 5.0, and 30 to 70 ml of enzyme was applied to the column (0.9 by 30 cm). The column was eluted first with about 200 ml of buffer and then with 500 ml of 0.2 M acetate, pH 5.0, containing a linear gradient of 0 to 1.2 M NaCl. The column was pumped at 14 to 17 ml/h, and 3-ml fractions were collected.

(v) **Reactive 2-blue agarose chromatography.** RNase fractions were dialyzed against starting buffer (10 mM Tris hydrochloride [pH 7.0], 5 mM $CaCl_2$) and applied to a Reactive 2-blue agarose column (0.7 by 17.3 cm) (Sigma). The column was eluted with 8 bed volumes of starting buffer and then with 10 bed volumes of a linear gradient of 0 to 0.5 M NaCl in the starting buffer.

(vi) **Electroelution.** Samples were resolved on a 12% polyacrylamide–sodium dodecyl sulfate (SDS) gel. Two vertical strips were cut from the gel, and both the gel and strips were notched to assure alignment. The strips were stained for protein. The RNase band was located, cut out of the gel, and cut into small pieces. Electroelution was done in a pH 8.3 buffer containing 14 g of glycine per ml, 3 g of Trizma base per ml, and 0.05% SDS with an Isco (Lincoln, Nebr.) electrophoretic concentrator (model 1750).

PAGE. Polyacrylamide gel electrophoresis (PAGE) was done in slab gels with a 3% stacking gel. Gels were run at 10 mA per gel during stacking and then at 30 mA. Reagents were obtained from Bio-Rad Laboratories, Richmond, Calif.

(i) **SDS-PAGE.** Samples containing less than 100 μl of protein were mixed with 35 μl of 3% SDS–25% (wt/vol) glycerol–10% mercaptoethanol–50 mM Tris hydrochloride (pH 6.8)–0.001% bromphenol blue and boiled for 2 min. Extracellular proteins from 2.5 ml of supernatant medium were precipitated with 0.625 ml of 50% ice-cold trichloroacetic acid, kept on ice for 1 h, centrifuged at 8,000 × g for 10 to 15 min, washed twice with 5% ice-cold trichloracetic acid, dissolved in 0.1 ml of 50 mM Tris hydrochloride, pH 6.8, and neutralized with about 5 μl of 1 N NaOH. Samples were resolved on 12% polyacrylamide gels (pH 8.3) containing 0.1% SDS at 20°C by the system described by Laemmli (15).

(ii) **Nondenaturing PAGE.** Samples (90 μl) adjusted to contain 10% (wt/vol) glycerol and 0.001% bromphenol blue were resolved on 9% polyacrylamide gels at 5°C by a system similar to the SDS-PAGE system except that SDS was not used.

(iii) **Isoelectric focusing.** Samples were resolved on 7.5% polyacrylamide gels containing 5% (vol/vol) ampholyte, pH 3 to 10 (Bio-Rad), by using chemical polymerization and the gel composition described by Wrigley (37). No stacking gel was used, and the samples were mixed with 50% (wt/vol) sucrose containing 10% (vol/vol) ampholyte before being applied. Gels were focused at 10°C at a constant power of 0.4 W per gel for 6 h and 0.5 W per gel for 2 h. The pH gradient was determined with a flat-head electrode (Beckman Instruments, Inc., Fullerton, Calif.).

(iv) **Protein stains.** Gels were stained for protein with Coomassie brilliant blue R-250 (34) or silver stained as recommended by Bio-Rad (bulletin 1089) based on the method of Merril et al. (17).

(v) **RNase activity stain.** RNase activities on nondenaturing gels were detected by the method of Wilson (36). Gels were incubated for 7.5 min at 40°C in 0.1 M sodium citrate buffer, pH 5.0, containing 0.4% RNA (Sigma; torula yeast RNA, type VI) and stained with 0.2% toluidine blue.

Molecular weight determination. Molecular weight was estimated by gel filtration on a Sephadex G-75 column (1.5 by 86.7 cm) (1) and by SDS-PAGE (28).

Antiserum preparation. A sample containing 250 μg of electroeluted, purified RNase in 0.75 ml was emulsified with 1.75 ml of Freund complete adjuvant (Difco) and injected intradermally into the back of a young, female New Zealand White rabbit. Two weeks later, a booster injection of 100 μg of RNase was given, and the rabbit was bled 35 days after that. A crude immunoglobulin fraction was precipitated and washed twice with ammonium sulfate (50% saturation) at 4°C, dissolved in one-third of the original serum volume in PBS buffer (10 mM phosphate buffer [pH 7.2], 0.9% NaCl), dialyzed overnight against PBS buffer, and stored at −20°C. Ouchterlony double diffusion analysis was performed at 23°C in 1.2% agarose in PBS buffer–0.02% sodium azide. Precipitin lines on the agarose slabs were stained with Coomassie blue R-250 after the slabs were washed with 0.9% NaCl.

Binding of protein to nitrocellulose paper and probing with RNase antiserum. Supernatant samples fractionated by SDS-PAGE were transferred electrophoretically to nitrocellulose paper by a modification of the method described by Towbin et al. (31). The nitrocellulose blots were blocked with 5% bovine serum albumin (Sigma; fraction V) containing 2% (vol/vol) normal swine serum for 30 min. Blots were then incubated for 15 min with a 1:1,000 dilution of rabbit RNase antiserum in 1% bovine serum albumin. After several washings, the blots were incubated for 30 min with a 1:400

FIG. 2. The 34K RNase is a degradation product of the 43K RNase. An RNase preparation (see the text) was further purified by preparative SDS-PAGE followed by electroelution for various times at different temperatures. The samples were resolved by SDS-PAGE on a 12% gel, which was then silver stained. The duration and temperature of electroelution were as follows (lanes): (A) 2 h at room temperature, (B) 3 h at room temperature, (C) 3 h at room temperature (the preparative gel was stained with Coomassie blue before electroelution), (D) 6 h at room temperature, (F) 8 h at room temperature, and (G) 3 h at 10°C. Lanes E and H contained molecular mass markers of 14, 21.5, 31, 45, 66.2, and 92.5 kilodaltons (Kd).

dilution of peroxidase-conjugated anti-rabbit immunoglobulin G swine serum in 1% bovine serum albumin, washed again, and then reacted with a solution containing 0.3 μl of hydrogen peroxide per ml, 0.5 mg of 3′,3′-diaminobenzidine tetrahydrochloride (Sigma; grade II) per ml dissolved in 100 mM Tris hydrochloride, pH 7.4. Swine sera were purchased from Accurate Chemical and Scientific Corp.

pH optimum. The RNA substrate (1.5 mg/ml, final concentration) was dissolved in the following buffers (0.1 M concentration): sodium citrate (pHs 2.0, 3.0, 4.5, 6.0, and 6.5), Tris hydrochloride (pHs 7.3, 8.3, and 9.3), Sorensen phosphate (pHs 5.0, 6.0, 7.0, and 7.45), sodium acetate (pHs 3.6, 4.0, 4.5, 5.0, and 5.5), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (pHs 6.5, 7.0, 7.5, and 8.0), and 2-(N-morpholino)ethanesulfonic acid (MES) (pHs 5.5, 6.0, 6.3, and 6.7). The RNA hydrolysis assay was then run as described above. The initial pH of a reaction was measured at 40°C.

Carbohydrate content. Samples were assayed for carbohydrate content by a modification of the phenol-sulfuric acid method of Dubois et al. (6) with glucose as a standard. The volumes used were reduced to 0.4 ml of the sample, 10 μl of phenol, and 1.0 ml of sulfuric acid.

RESULTS

RNase is produced during exponential growth. In GPP medium there was a lag in RNase production until the cell density reached about 200 Klett units (initial cell density, 50 Klett units). After that, RNase was produced throughout exponential growth. The differential rate of RNase production decreased as cell density increased, and there was little if any RNase produced in the stationary phase. Based on these results, RNase samples were collected early in the stationary phase.
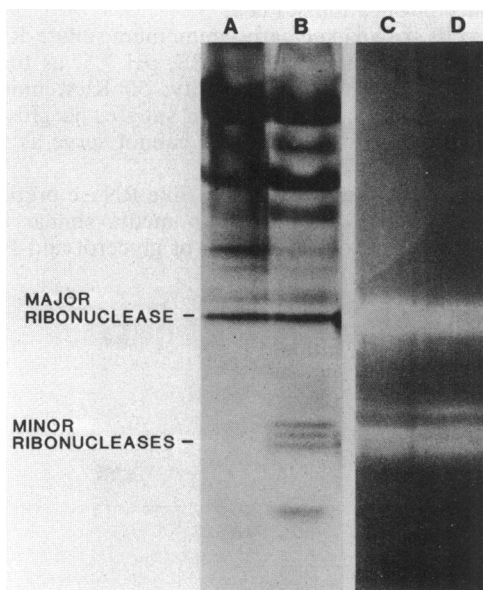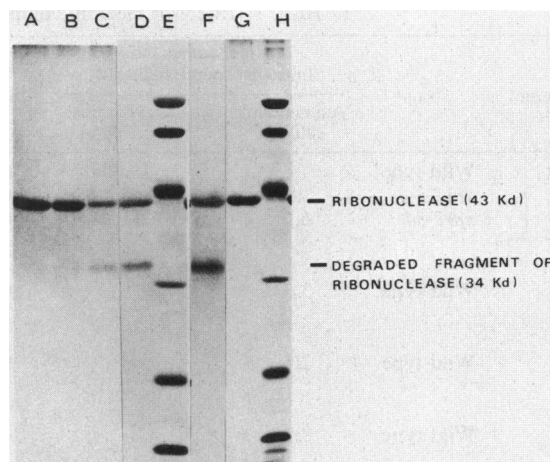

FIG. 1. Several polypeptides with RNase activity were present in the supernatant medium from wild-type cells. The supernatant medium was concentrated by ultrafiltration and chromatographed on G-75 Sephadex. Polypeptides from the two peaks of RNase activity were resolved by nondenaturing electrophoresis on a 9% polyacrylamide gel. Lanes A and C contained material which appeared at the voided volume, and B and D contained material from the major RNase peak which appeared later. Lanes A and B were silver stained, and C and D were stained for RNase activity.

TABLE 1. Effect of medium composition on production of extracellular RN and AEP

Culture no.	Strain	Medium composition (nitrogen source [g/liter])[a]			Buffer (M)[b]	Time (h)	Cell density (Klett units)	Final pH	AEP activity (U/ml)	RNase activity (U/ml)
		Ammonium sulfate	RNA	Proteose Peptone						
1	Wild type			6.0	0.1 (P)	21	3,820	6.5	2,000	196
2	xpr2-34			6.0	0.1 (P)	21	3,550	6.5	0	179
3	xpr2-34	6.0	P[c]		0.1 (P)	21	2,190	6.5	0	93
						28	2,940	6.4	0	107
						32	3,660	6.5	0	118
4	Wild type	3.3			0.1 (P)	21	1,360	6.2	0	7
						28	2,350	5.8	10	26
						32	3,000	5.7	8	39
5	Wild type	2.4			0.1 (P)	21	1,490	6.2	0	13
						28	2,490	6.0	130	39
						32	3,190	6.1	155	49
6	Wild type	3.3	2.0		0.1 (P)	21	1,600	5.9	5	5
						28	2,510	5.6	41	39
						32	2,680	5.7	76	38
7	xpr2-34	3.3	2.0		0.1 (P)	21	1,440	6.0	0	2
						28	2,700	5.7	0	41
						32	3,150	5.8	0	55
8	xpr2-34		2.0	6.0	0.1 (C)	21	3,200	5.9	5	261
9	xpr2-34		2.0	6.0	0.1 (C)	20	3,410	5.9	5	279

[a] All media contained glycerol (20 g/liter), yeast nitrogen base without ammonium sulfate and amino acids (5.0 g/liter), adenine (60 mg/liter), and antifoam (1 ml/liter).
[b] P, Phosphate; C, citrate.
[c] P, Peptone.

RNase production in different media. Experiments were done to increase RNase production. RNase production increased when 0.1 M citrate replaced phosphate as the buffer in GPP medium. Doubling the concentration of the nutrients approximately doubled the cell yield and the amount of RNase produced. Addition of 1 or 2 g of RNA per liter to complex medium resulted in a relatively minor (4 to 10%) increase in RNase production, but addition of 0.5% Tween 80 caused about a twofold increase. Because the basis for the Tween 80 effect was not known (26) and because the RNA would complicate RNase purification, neither compound was included in the media used for preliminary purification studies.

When cells grown in glycerol-ammonium sulfate-RNA (2.0 g/liter) medium with 0.2 M HEPES, pH 7.5, as the buffer were transferred (inital cell density, 50 Klett units) to a similar medium lacking ammonium sulfate, no growth was obtained. This suggests that RNA cannot serve as the sole nitrogen source for *Y. lipolytica*.

Preliminary purification studies. Five RNase preparations were made. Cells were grown in media similar to GPP containing various concentrations of glycerol and Proteose
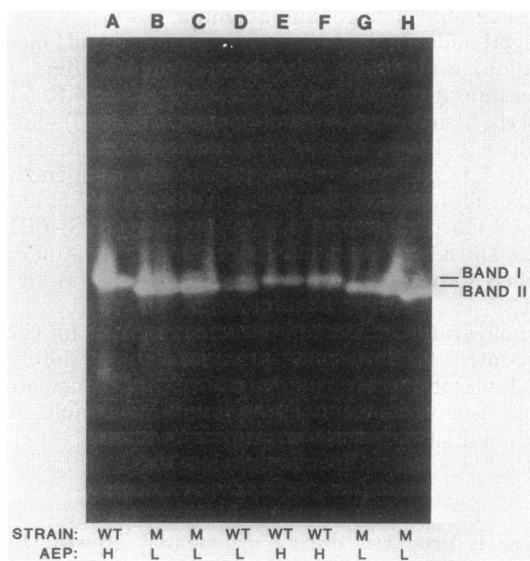

FIG. 3. The form of RNase detected correlated with the amount of AEP produced in the culture medium. Supernatant samples were analyzed by electrophoresis in a nondenaturing 9% polyacrylamide gel which was stained for RNase activity. Lanes A through H correspond to cultures 1 through 9 in Table 1. Bands I and II are the 43K and 45K RNases, respectively. WT and M represent the wild-type and *xpr2-34* mutant strains, and L and H represent low and high levels, respectively, of AEP in the culture medium.
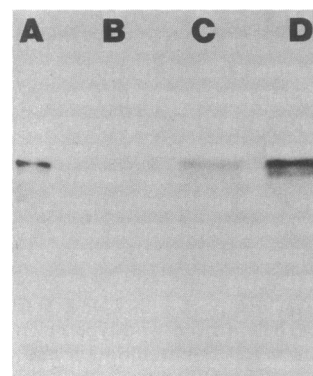

FIG. 4. Protein blot of supernatant medium from a wild-type culture reacted with antiserum against the 43K RNase. Lane A contained the purified 43K RNase standard. Lanes B, C, and D contained 0.1 ml of supernatant medium taken 15, 30, and 45 min after transfer of cells to fresh medium.
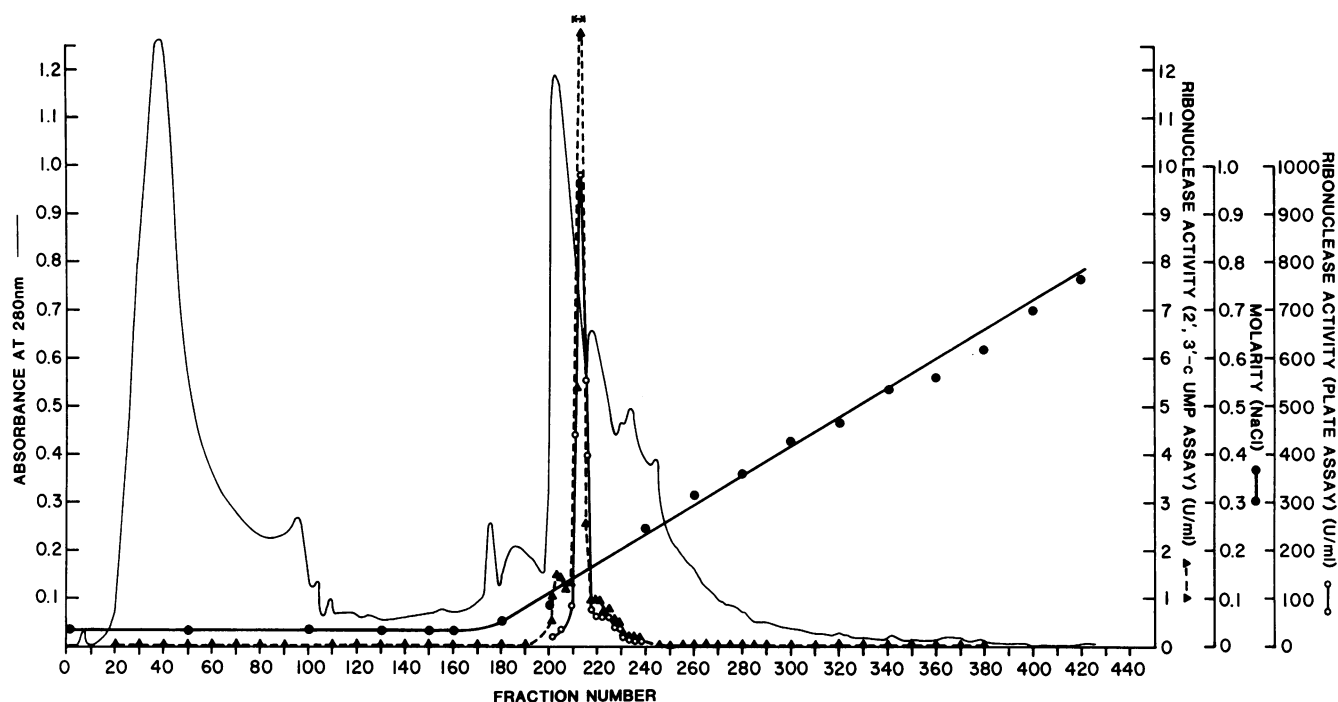
FIG. 5. Purification of RNase activity on DEAE-Sephacel. Ultrafiltered supernatant medium was dialyzed against starting buffer, and 99 ml was applied to the column. Conditions for developing the column are described in Materials and Methods. Fractions 210 to 215 were pooled for further purification.

Peptone. The first step in all cases was ultrafiltration to reduce the sample volume.

When Sephadex G-75 chromatography followed ultrafiltration, two peaks of RNase activity were detected. The first ran near the void volume and contained 23% of the activity. The second peak was included in the large protein peak which was eluted after the void volume. Recovery of RNase activity was near 90%. Fractions from both peaks were examined by nondenaturing PAGE. The gel was stained for RNase activity and protein (Fig. 1). Both peaks contained RNase activity in a major band and several minor bands. The bands from both peaks had similar mobilities, which suggested that the RNases in the peaks were similar and that the RNase activity at the voided volume consisted of aggregates of the RNase in the major peak.

For two preparations, DEAE-Sephacel chromatography was the first step following ultrafiltration. The RNase eluted in a single peak. Purification was about 12-fold, and recovery of RNase activity was about 80%. The RNase was separated from the AEP.

The next step in all five preparations was UMP-agarose affinity chromatography. At least two peaks of RNase activity were obtained in each run. These peaks were examined by nondenaturing PAGE, and both peaks contained a major and several minor RNases with mobilities similar to those shown in Fig. 1. All of the UMP-agarose peaks were examined by SDS-PAGE, and each peak contained a major protein band with an apparent molecular weight of 43,000 (43K protein) and a minor 34K protein band. Rechromatography of samples from a peak in the UMP-agarose column on UMP-agarose still yielded the two polypeptides in each peak.

Chromatography of samples from UMP-agarose on a Reactive 2-blue agarose column resulted in two RNase peaks. Both peaks contained both polypeptides, but the first peak contained mainly the 34K protein and the second contained mainly the 43K protein. Results of nondenaturing PAGE of these peaks established that the 34K protein corresonded to the faster-moving minor RNase and that the 43K protein corresponded to the slower-moving major RNase (Fig. 1). The second peak was rechromatographed on the Reactive 2-blue column, and again two peaks containing some of each polypeptide were obtained.

Chromatography on Bio-Gel P-60 also yielded two peaks, and the first peak contained about 83% of the activity. The fractions were examined by SDS-PAGE. The protein concentration in the second peak was too low for protein bands to be detected by silver staining; however, for the first peak a major 43K band and a minor 34K band were detected.

The chromatography results suggested that the 43K and 34K RNases were not products of different genes but that the 34K RNase was a degradation product of the 43K RNase. This was confirmed by preparative gel electrophoresis of the purified RNase followed by electroelution of the 43K protein. The electroeluted RNase was examined by SDS-PAGE (Fig. 2). For samples electroeluted for a short time (lane A) or at low temperature (lane G), only the 43K protein was detected. For samples electroeluted at room temperature, the amount of 34K protein detected increased as the electroelution time increased (lanes B, D, and F). Therefore, the 34K RNase was derived from degradation of the 43K RNase.

Characterization of the RNases. Based on SDS-PAGE, the two forms of RNase had molecular weights of 43,000 and 34,000. Based on chromatography on Sephadex G-75 of a sample containing predominantly the 43K RNase, the apparent molecular weights of the major and minor RNases were 44,100 and 34,500. The similarity in size obtained by the two methods indicated that the active enzyme was composed of a single polypeptide.
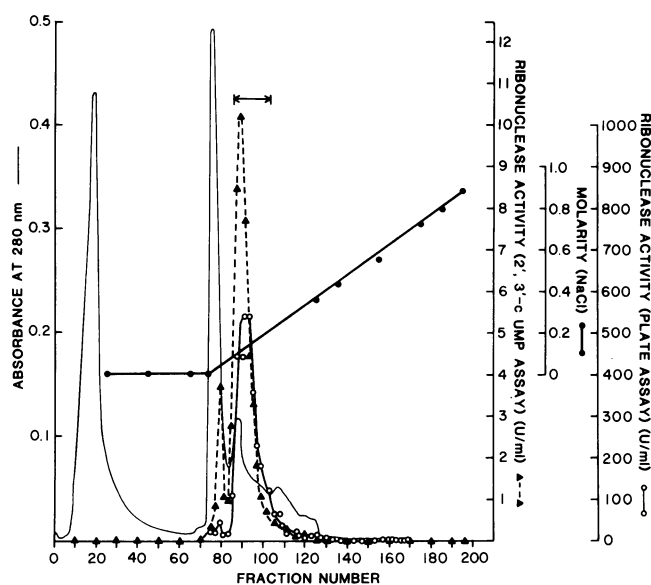
FIG. 6. Purification of RNase activity on UMP-agarose. Fractions 210 to 215 from the DEAE-Sephacel column were dialyzed against starting buffer and applied to the column. Conditions for developing the column are described in Materials and Methods. Fractions 86 to 103 were pooled for further purification.

The pH optimum at 40°C of purified RNase containing predominantly the 43K form was between 6.5 and 7.0 (data not shown). RNase activity fell rapidly above pH 7.0. Highest activities were obtained in the phosphate and MES buffers, which indicated that the enzyme was not inhibited by phosphate.

The isoelectric point of the 43K RNase was estimated to be 4.8 and that of the 34K RNase was estimated to be 4.65.

An RNase sample prepared by ultrafiltration and DEAE-Sephacel and UMP-agarose chromatography was fractionated on a Bio-Gel P-60 column. For the two fractions containing the highest concentration of RNase, the carbohydrate and protein contents (Bradford assay) were assayed. The fractions contained 11 and 12% carbohydrate. The results suggested that the RNase is a glycoprotein.

RNase is secreted as a 45K polypeptide. Conversion of the 43K RNase to the 34K RNase might be caused by very low levels of contaminating protease(s) (25). Therefore, attempts were made to prevent AEP production by developing media in which *xpr* mutants would produce high levels of RNase or media which would repress AEP but not RNase production (Table 1). Strain PS6069, containing the AEP structural gene mutation *xpr2-34*, produced little or no AEP activity and as much (culture 2) or more (culture 9) RNase activity than the wild type (culture 1) in media containing glycerol and Proteose Peptone. Several nitrogen sources were substituted for Proteose Peptone. Peptone supported less RNase production with the mutant (culture 3). Ammonium sulfate repressed AEP production by the wild type, and RNase production was reduced by 75% or more (cultures 4 and 5). Addition of RNA to complex growth medium (culture 9) did not have a major effect on RNase production. Addition of RNA to minimal media (cultures 6 and 7) substantially increased RNase production, but the levels produced were less than in media with Proteose Peptone as the nitrogen source (cultures 1, 2, and 8). Doubling the citrate or phosphate buffer concentrations to 0.2 M resulted in decreased RNase production (data not shown).

An unexpected result was obtained when supernatant samples from the cultures described in Table 1 were resolved on a nondenaturing polyacrylamide gel and stained for RNase activity. There were one or two bands in the region where the major RNase appeared (Fig. 3). Which band(s) was present correlated with the amount of AEP in the supernatant sample. In cultures in which AEP production was high (greater than 76 U/ml), only the upper band appeared (lanes A, E, and F). In cultures in which little AEP was produced, the lower band predominated (lanes B, C, D, H, and I) or was the only band visible (lane G).

Supernatant samples from similar cultures were examined by SDS-PAGE. The presence of the upper band on the nondenaturing gel correlated with the presence of a 43K polypeptide with the same mobility as the purified RNase (data not shown). The presence of the lower band correlated with the presence of a 45K polypeptide. These results strongly suggest that RNase is secreted as a 45K polypeptide which is converted to a 43K polypeptide by the action of AEP.

Detection of extracellular RNase by protein blotting and double antibody enzyme immunoassay. Rabbit antibodies were raised against the purified electroeluted RNase and examined by Ouchterlony double diffusion tests. A single and sometimes double precipitin lines were detected. We suspect that the appearance of the second precipitin line largely depended on the amount of 34K RNase in the sample.

The form of RNase secreted by the wild-type strain was examined by protein blotting. Cells were suspended in glycerol-casein medium at a density of 1,000 Klett units, and supernatant samples were collected after 15, 30, and 45 min. The RNase was detected on protein blots of the supernatant proteins with the RNase antiserum by using a double antibody enzyme immunodetection method (Fig. 4). A major band of 43K which reacted with the RNase antiserum was detected for the purified RNase (lane A). Two closely spaced bands were detected for the wild-type culture. The upper
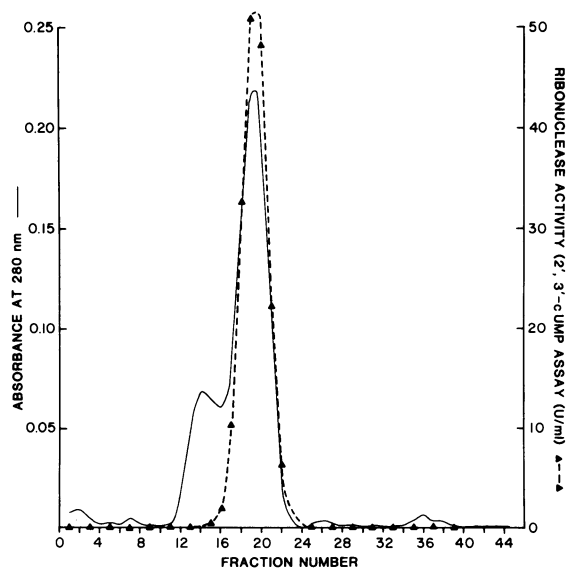

FIG. 7. Purification of RNase activity by gel filtration on Sephadex G-100. Fractions 86 to 103 from the UMP-agarose column were concentrated by ultrafiltration to 1.25 ml, and 1.15 ml was applied to the column.
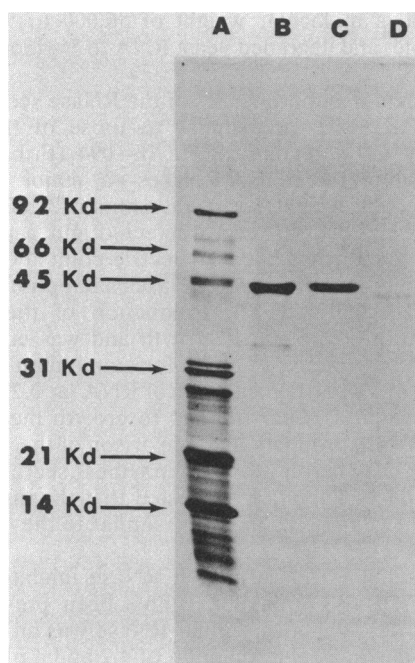
FIG. 8. Purified RNase resolved by SDS-PAGE on a 10 to 15% gradient gel which was silver stained. Lanes: B, 13 μl of combined fractions 86 to 103 from the UMP-agarose column; C, 40 μl of combined fractions 18 to 20 from the Sephadex G-100 column; D, purified 43K RNase; A, the molecular mass standards (indicated in kilodaltons [Kd]) phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin soybean inhibitor, and lysozyme (Bio-Rad).

and lower bands had apparent molecular weights of 45,000 and 43,000, respectively. These results confirmed that wild-type Y. lipolytica secretes a 45K RNase. The presence of the 43K form of the RNase after only 30 min suggests that the 45K to 43K RNase conversion occurs rapidly.

Are other RNases present? The above results suggested that a single RNase was secreted by Y. lipolytica grown in media with a pH between 5.0 and 7.0. In these experiments, staining for RNase activity on gels was done at pH 5.0, and RNases not active at pH 5.0 would not have been detected. Therefore, supernatant media from CX161-1B and PS6069 grown in GPPP2 medium were examined for RNase activity by staining gels at pH 4.0, 5.0, or 6.0. The only forms of RNase detected were those observed in the earlier experiments.

Purification of the 45K RNase. An attempt was made to purify the 45K form of RNase. Strain PS6069 and GPPC2 medium were used to produce the RNase, and the culture supernatant medium was treated with 2 mM (final concentration) phenylmethylsulfonyl fluoride. Ultrafiltration and gel filtration chromatography were performed in the presence of 0.2 and 0.02% sodium azide, respectively, and the RNase preparations were filter sterilized after each step.

The elution profiles from the three columns used are shown in Fig. 5, 6, and 7. Most of the RNase activity eluted from the DEAE-Sephacel column in the major peak (Fig. 5). Fractions 210 to 215 were pooled and applied to a UMP-agarose column. Minor and major peaks of RNase activity were eluted (Fig. 6), and fractions 86 to 103 were concentrated and applied to a Sephadex G-100 column. A single peak of RNase activity was eluted (Fig. 7). In Fig. 8, the results of SDS-PAGE of peak fractions from UMP-agarose

and G-100 columns are shown. By this criterion, the RNase in G-100-purified fractions appeared nearly pure. The position of purified RNase relative to the 43K RNase standard clearly indicated that the 45K RNase was purified. This result proved that AEP has a major role in the 45K to 43K RNase conversion.

The results of purification are summarized in Table 2. The more convenient 2',3'-cUMP hydrolysis assay was used. Two unexpected results were obtained: a 25% increase in yield after DEAE-Sephacel chromatography and only 17% recovery after UMP-agarose chromatography. Reassay by RNA hydrolysis assay still produced an increase in yield (14%) after DEAE-Sephacel chromatography. However, recovery after UMP-agarose chromatography was 65%, similar to values obtained in earlier purifications. The cause of the decrease in 2',3'-cUMP hydrolysis activity was not investigated further.

DISCUSSION

A major finding of this study was that, despite the fact that several polypeptides with RNase activity can be detected in supernatant culture medium, Y. lipolytica, when grown in media between pHs 5 and 7, primarily secretes a single RNase (45K). This conclusion is based on three different results. First, electrophoretic examination on nondenaturing gels of RNases secreted by a wild-type and a mutant strain in different media revealed both the 45K and 43K RNases. We assume that the 34K RNase was not detected because it was a minor species and because the gels were stained for only 7.5 min. Staining for longer times increased the intensity of the bands, but the 45K and 43K RNases could no longer be distinguished. The mutant, when grown in certain media, produced only the 45K RNase, and in fact it was possible to purify the 45K RNase from the mutant. The relative amount of 43K RNase correlated quite well with the amount of AEP produced in a culture. These results demonstrated that the 43K RNase was derived from the 45K RNase mainly by AEP action. Second, during purification a minor 34K RNase copurified with the 43K RNase, and no other RNases were detected. The 34K species was shown to be a degradation product of the 43K species. We assume that multiple bands detected in the higher-mobility region of the nondenaturing gels are degradation products and that the 34K polypeptide is the major species in this region. The possibility that quite low levels of an RNase with mobility similar to that of the 34K species are secreted was not totally excluded. Third, when protein blots of extracellular culture medium were reacted with antiserum against the 43K RNase, both the 45K and 43K RNases were detected. This established that the

TABLE 2. Purification of extracellular RNase produced by
Y. lipolytica PS6069

Fraction	Vol (ml)	Total activity (U)[a]	Total protein (mg)[b]	Sp act (U/mg)	Yield (%)
Culture broth	2,190	510	82	6.2	100
Ultrafiltrate	85	563	36	16	110
DEAE-Sephacel (210–215)	99	698	11	63	137
UMP-agarose (86–103)	55	119	0.39	305	23
Ultrafiltrate	1.25	150	0.30	500	29
Sephadex G-100 (18–20)	3.2	110	0.13	845	22

[a] 2',3'-cUMP hydrolysis assay.
[b] Bradford protein assay.

wild type could produce the 45K RNase. When the same RNase antiserum was used to immunoprecipitate labeled polypeptides from supernatant medium and cell extracts, a 45K polypeptide was detected (S.-C. Cheng and D. M. Ogrydziak, submitted for publication). The fact that the antiserum raised against the 43K RNase reacted with and immunoprecipitated the 45K protein proved that the two RNases were antigenically related and strongly supports a precursor-product relationship.

AEP has a major role in the 45K to 43K RNase conversion, but in some cases some of the 43K RNase was formed in the absence of detectable AEP activity (Fig. 3, lanes B and C). The degradation might have been caused by the acid proteases known to be produced by Y. lipolytica (38). There is less evidence that AEP has a role in the 43K to 34K conversion. The 43K and 34K degraded forms of RNase retained RNase activity. Since the RNase and AEP are often produced at the same time, this probably makes teleological sense.

The studies on RNase production in different media yielded some insights into the regulation of RNase production. The type of nitrogen source is a factor regulating RNase production. Proteose Peptone was a better nitrogen source for RNase production than was peptone, and peptone was better than ammonium sulfate (Table 1; unpublished data). It seems that more RNase is produced in media with higher-molecular-weight nitrogen sources. Regulation of AEP is quite similar (22).

Phosphate represses RNase production in several microorganisms. This does not seem to be true for Y. lipolytica. Fairly high levels of RNase were made in media with 0.1 M phosphate buffer (Table 1). Higher levels were made in 0.1 M citrate buffer, but this probably was not caused by a release of phosphate repression because a similar increase was not obtained when 0.1 M MES or HEPES was substituted for phosphate (data not shown).

Addition of RNA to media with Proteose Peptone resulted in only slight increases in RNase production. Addition of RNA to media with ammonium sulfate as a nitrogen source resulted in a greater percentage of increase, but the amount of RNase produced was less than with Proteose Peptone alone. The importance of protein in regulation of RNase production has also been demonstrated by Lindberg and Drucker (16) for RNase N of Neurospora crassa. Perhaps there is a good chance that most protein substrates found in nature also contain RNA but little chance that RNA substrates do not contain protein.

Characterization of the catalytic activity of the RNase was not a goal of this study. The enzyme hydrolyzes RNA but not DNA and for this reason is called an RNase. Its large size and ability to cleave 2',3'-cUMP suggest that it acts by producing nucleoside 2',3'-cyclic phosphates as intermediates and that it differs from the small, guanine-specific RNases produced by several fungi (32).

The 45K RNase contains no N-linked carbohydrate based on the results of immunoprecipitation experiments done with tunicamycin or endoglycosidase H (Chen and Ogrydziak, submitted). This suggests that the carbohydrate detected on the RNase was O linked. The 11 to 12% carbohydrate content is probably a substantial overestimate due to the anomolous response of bovine plasma albumin in the Bradford assay (Bio-Rad Protein Assay Manual).

Many yeasts secrete RNases (4, 14), but only those secreted by R. glutinis and C. lipolytica have been characterized in any detail. Nakao and Ogata (18) purified an RNase from the culture broth of R. glutinis. The purified

enzyme had a molecular weight of 56,000 to 58,900, was thermostable, and degraded yeast RNA to 3'-ribonucleotides (17).

The regulation and properties of the RNase secreted by Y. lipolytica CX161-1B are similar to those of the RNases secreted by C. lypolytica NRRL Y-1094 (Fribourg, M.S. thesis). Two extracellular RNases—a major (45,000 to 50,000 molecular weight) form and a minor (71,000 to 74,000 molecular weight) form—were detected and partially purified. The two enzymes have isoelectric points between pHs 4.0 and 4.25. A mixture of the RNases had optimum activity between pHs 6.5 and 7.0. Production of the RNase(s) occurred during exponential growth and was enhanced by substitution of Proteose Peptone for ammonium sulfate and by addition of Tween 80. Addition of RNA (at 0.2 to 2 g/liter) or phosphate (at 1 g/liter or less) to growth media slightly increased RNase production. Comparison of the sizes, properties, and regulation of the enzymes secreted by C. lipolytica and Y. lipolytica suggested that the major RNase secreted by C. lipolytica might be similar to the 45K RNase secreted by CX161-1B.

An intracellular RNase and an RNase inhibitor from C. lipolytica UCD FST no. 60-26 have been previously described (11, 12). The intracellular RNase was an acidic 32K protein having an isoelectric point of 4.2 and a pH optimum of 6.0. The procedures used to extract the enzyme were quite harsh, and it is possible that the intracellular 32K RNase found in UCD FST 60-26 is in fact a proteolytic product (like the 34K RNase) of a larger enzyme similar to the 45K RNase produced by Y. lipolytica.

LITERATURE CITED

1. **Andrew, P.** 1969. Estimation of molecular size and molecular weights of biological compounds by gel filtration. Methods Biochem. Anal. **18**:1–53.
2. **Anfinsen, C. B., R. R. Redfield, W. L. Choate, J. Page, and W. R. Carroll.** 1953. Studies on the gross structure, cross-linkages, and terminal sequences in ribonuclease. J. Biol. Chem. **207**:201–211.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Burt, W. R., and J. Cazin, Jr.** 1976. Production of extracellular ribonuclease by yeasts and yeastlike fungi, and its repression by orthophosphate in species of Cryptococcus and Tremella. J. Bacteriol. **125**:955–960.
5. **Davidow, L. S., D. Apostolakos, M. M. O'Donnell, A. R. Proctor, D. M. Ogrydziak, R. A. Wing, I. Stasko, and J. R. DeZeeuw.** 1985. Integrative transformation of the yeast Yarrowia lipolytica. Curr. Genet. **10**:39–48.
6. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Hamilton, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
7. **Ferro-Novick, S., W. Hansen, I. Schauer, and R. Schekman.** 1984. Genes required for completion of import of proteins into the endoplasmic reticulum in yeast. J. Cell Biol. **98**:44–53.
8. **Gaillardin, C. M., V. Charoy, and H. Heslot.** 1973. A study of copulation, sporulation and meiotic segregation in Candida lipolytica. Arch. Mikrobiol. **92**:69–83.
9. **Gaillardin, C., A. M. Ribet, and H. Heslot.** 1985. Integrative

transformation in the yeast *Yarrowia lipolytica*. Curr. Genet. **10:**49–58.

10. **Herbert, D., P. J. Phipps, and E. R. Strange.** 1971. Chemical analysis of microbial cells. Methods Microbiol. **5B:**209–344.

11. **Imada, A., J. W. Hunt, H. V. De Sande, A. J. Sinskey, and S. R. Tannenbaum.** 1975. Purification and properties of an intracellular ribonuclease from *Candida lipolytica*. Biochim. Biophys. Acta **395:**490–500.

12. **Imada, A., A. J. Sinskey, and S. R. Tannenbaum.** 1972. A new macromolecular inhibitor of yeast ribonuclease mediated through purine nucleotides. Biochim. Biophys. Acta **268:**674–679.

13. **Kapoor, K. K., K. Chaudhary, and P. Tauro.** 1982. Citric acid, p. 709–747. *In* G. Reed (ed.), Prescott Dunn's industrial microbiology, 4th ed. AVI Publishing Co., Inc. Westport, Conn.

14. **Kockova-Kratochvilova, A.** 1982. Urease and extracellular nucleases of yeasts. Folia Microbiol. **27:**404–412.

15. **Laemmli, U. K.** 1970. Cleavage of structure proteins during the assembly of the head of the bacteriophage T4. Nature (London) **227:**680–685.

16. **Lindberg, R. A., and H. Drucker.** 1984. Regulation of a *Neurospora crassa* extracellular RNase by phosphorus, nitrogen, and carbon depressions. J. Bacteriol. **157:**380–384.

17. **Merril, C. R., D. Goldman, and M. L. V. Keuren.** 1983. Silver staining methods for polyacrylamide gel electrophoresis. Methods Enzymol. **96:**230–239.

18. **Nakao, Y., and K. Ogata.** 1963. Degradation of nucleic acids and their related compounds by microbial enzymes. VIII. Degradation of ribonucleic acid by extracellular enzymes of *Rhodotorula glutinis*. Agric. Biol. Chem. **27:**499–506.

19. **Ogata, K., S.-D. Song, and H. Yamada.** 1971. Studies on extracellular ribonuclease from yeast. Part I. Purification and physico-chemical properties of RNase from *Rhodotorula glutinis*. Agric. Biol. Chem. **35:**58–64.

20. **Ogrydziak, D., J. Bassel, R. Contopoulou, and R. Mortimer.** 1978. Development of genetic techniques and the genetic map of the yeast *Saccharomycopsis lipolytica*. Mol. Gen. Genet. **163:**229–239.

21. **Ogrydziak, D. M., S. C. Cheng, and S. J. Scharf.** 1982. Characterization of *Saccharomycopsis lipolytica* mutants producing lowered levels of alkaline extracellular protease. J. Gen. Microbiol. **128:**2271–2280.

22. **Ogrydziak, D. M., A. L. Demain, and S. R. Tannenbaum.** 1977. Regulation of extracellular protease production in *Candida lipolytica*. Biochim. Biophys. Acta **497:**525–538.

23. **Ogrydziak, D. M., and R. K. Mortimer.** 1977. Genetics of extracellular protease production in *Saccharomycopsis lipolytica*. Genetics **87:**621–632.

24. **Ogrydziak, D. M., and S. J. Scharf.** 1982. Alkaline extracellular protease produced by *Saccharomycopsis lipolytica* CX161-1B. J. Gen. Microbiol. **128:**1225–1234.

25. **Pringle, J. R.** 1975. Methods for avoiding proteolytic artifacts in studies of enzymes and other proteins from yeasts. Methods Cell Biol. **12:**149–185.

26. **Reese, E. T., and A. Maguire.** 1969. Surfactants as stimulants of enzyme production by microorganisms. Appl. Microbiol. **17:**242–245.

27. **Sato, S., T. Uchida, and F. Egami.** 1966. Action of ribonuclease T2 on 2′,3′-cyclic nucleotides and related compounds. Arch. Biochem. Biophys. **115:**48–52.

28. **Schekman, R., and P. Novick.** 1982. The secretory process and yeast cell-surface assembly, p. 361–396. *In* J. N. Strathern, E. N. Jones, and J. R. Broach (ed.), Molecular biology of the yeast *Saccharomyces*. Metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

29. **Simms, P. C., and D. M. Ogrydziak.** 1981. Structural gene for the alkaline extracellular protease of *Saccharomycopsis lipolytica*. J. Bacteriol. **145:**404–409.

30. **Tobe, S., T. Takami, S. Ikeda, and K. Mitsugi.** 1976. Production and some enzymatic properties of alkaline protease of *Candida lipolytica*. Agric. Biol. Chem. **40:**1087–1092.

31. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.

32. **Uchida, T., and F. Egami.** 1970. Microbial ribonucleases with special reference to RNases T1, T2, N1, and U2. Enzymes **4:**205–250.

33. **Van Der Walt, J. P., and J. A. Von Arx.** 1980. The yeast genus *Yarrowia* gen. nov. Antonie van Leeuwenhoek J. Microbiol. **46:**517–521.

34. **Weber, K., J. R. Pringle, and M. Osborn.** 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. Methods Enzymol. **26:**3–27.

35. **Wierenga, R. K., J. D. Huizinga, W. Gaastra, G. W. Welling, and J. J. Beintema.** 1973. Affinity chromatography of porcine pancreatic ribonuclease and reinvestigation of the N-terminal amino acid sequence. FEBS Lett. **31:**181–185.

36. **Wilson, C. W.** 1969. A rapid staining technique for detection of RNase after polyacrylamide gel electrophoresis. Anal. Biochem. **31:**506–511.

37. **Wrigley, C. W.** 1971. Gel electrofocusing. Methods Enzymol. **22:**559–564.

38. **Yamada, T., and D. M. Ogrydziak.** 1983. Extracellular acid proteases produced by *Saccharomycopsis lipolytica*. J. Bacteriol. **154:**23–31.