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Lyticase: Endoglucanase and Protease Activities That Act Together in Yeast Cell Lysis

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Yeast lytic activity was purified from the culture supernatant of *Oerskovia xanthineolytica* grown on minimal medium with insoluble yeast glucan as the carbon source. The lytic activity was found to consist of two synergistic enzyme activities which copurified on carboxymethyl cellulose and Sephadex G-150, but were resolved on Bio-Gel P-150. The first component was a β-1,3-glucanase with a molecular weight of 55,000. The K_m for yeast glucan was 0.4 mg/ml; that for laminarin was 5.9 mg/ml. Hydrolysis of β-1,3-glucans was endolytic, yielding a mixture of products ranging from glucose to oligomers of 10 or more. The size distribution of products was pH dependent, smaller oligomers predominating at the lower pH. The glucanase was unable to lyse yeast cells without 2-mercaptoethanol or the second lytic component, an alkaline protease. Neither of these agents had any effect on the glucanase activity on polysaccharide substrates. The protease had a molecular weight of 30,000 and hydrolyzed Azocoll and a variety of denatured proteins. The enzyme was unusual in that it had an affinity for Sephadex. Although the activity was insensitive to most protease inhibitors, it was affected by polysaccharides; yeast mannan was a potent inhibitor. The enzyme did not have any mannanase activity, however. Neither pronase nor trypsin could substitute for this protease in promoting yeast cell lysis. A partially purified fraction of the enzymes, easily obtained with a single purification step, had a high lytic specific activity and was superior to commercial preparations in regard to nuclease, protease, and chitinase contamination. Lyticase has been applied in spheroplast, membrane, and nucleic acid isolation, and has proved useful in yeast transformation procedures.

Enzymes capable of degrading the cell wall of yeasts have been isolated from the culture supernatants of numerous microorganisms (13). In general, the yeast lytic activity can be attributed to a β-1,3-glucanase, although lytic β-1,6-glucanases have been characterized (16). Usually, the glucanase activity will not lyse viable yeast cells without either a thiol reducing agent or a second enzyme activity. For some procedures the preparation of yeast spheroplasts requires pure lytic enzymes of known specificity. We have developed a high-yield purification of yeast lytic enzymes ("lyticase") from the culture supernatant of *Oerskovia xanthineolytica* and characterized the β-1,3-glucanase and the protease that are responsible for the lytic activity.

MATERIALS AND METHODS

Materials. The following products were obtained commercially: pustulan and Azocoll (Calbiochem); laminarin (Pierce Chemical Co. or Calbiochem); polyethylene glycol (Carbowax 600 powder, Union Carbide Corp.); CM 52 and DE 52 cellulose (Whatman, Inc.); Sephadex G-150 (Pharmacia Fine Chemicals, Inc.); Bio-Gel P-150, P-30, and P-2 (Bio-Rad Laboratories); yeast extract and peptone (Difco Laboratories); bulk yeast (Red Star); calf thymus DNA, pancreatic DNase I, myoglobin, hemoglobin, cytochrome *c*, lysozyme, and ovalbumin (Sigma Chemical Co.); bovine serum albumin (Miles Laboratories, Inc.); Zymolyase 5000 and 60000 (Kirin Brewery); and Glusulase (Endo Laboratories). A premarket sample of Teichozyme Y was generously provided by Worthington Biochemical Corp. Mannan from *Saccharomyces cerevisiae* X2180-1B and *mnn2* prepared by Fehling's precipitation (12) and the mannan acetolysis fractions were a gift of R. Douglas. *Escherichia coli* alkaline phosphatase was a gift of K. Telander. Rabbit actin was a gift of C. Greer.

Insoluble yeast β-glucan was prepared from Red Star yeast by repeated alkali extractions done by the procedure of Cabib and Bowers (2) through step 1 only; no acid extraction was performed. After each extraction, the glucan was collected by centrifugation in a Sorvall GS3 rotor at 9,000 rpm (14,000 × *g*) for 15 min. Sedimentation in a Sharples centrifuge did not clear the supernatant solution, and the resultant glucan was not an effective inducer, possibly as a result of a lack of small glucan oligomers. The glucan was adjusted to pH 7 with HCl before the final centrifugation and then was washed twice with distilled water. The pellet was then dried by washing once with

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ethanol and twice with ether. The yield was about 15 g of glucan per lb (454 g) of yeast, so approximately 6 lb (2.7 kg) of yeast was required to provide sufficient glucan for a 20-liter culture of *O. xanthineolytica*.

Autoclaved yeast was prepared by mixing Red Star yeast with an equal volume of distilled water and autoclaving for 20 min. The yeast cell wall residue was collected by centrifugation and washed twice with distilled water. The pellet was stored frozen until needed.

Strains and growth conditions. *Arthrobacter luteus* strain 73-14 was obtained from Jeremy Thorner (University of California, Berkeley). This strain was originally obtained from Yashishi Yamamoto (Kirin Brewery, Takasaki, Gunma, Japan). This organism has since been reclassified by Mary Lechevalier (Rutgers University) as *Oerskovia xanthineolytica*.

O. xanthineolytica was grown at 30°C on modified Sistrom's medium (18) designated M63 containing (grams per liter): KH_2PO_4 (13.6), $(NH_4)_2SO_4$ (2.0), KOH (4.2), $MgSO_4 \cdot 7H_2O$ (0.2), and $Fe_2(SO_4)_3 \cdot 6H_2O$ (0.001). This was supplemented with thiamine and biotin (1 μg/ml each). Medium contained one of the following carbon sources: glucose, 0.4%; washed autoclaved yeast, 1.5%; or insoluble yeast glucan, 0.4%.

The lytic assay employed haploid *S. cerevisiae* X2180-1B as substrate. Cells were grown on YPD (1% yeast extract, 2% Difco peptone, 2% glucose) to an absorbance at 600 nm (A_{600}; Zeiss PMQII spectrophotometer, 1-cm cuvette) of 2 or less (they were less sensitive to lysis if grown to a higher density). Cell growth was arrested by the addition of 10 mM sodium azide. Cells were harvested by centrifugation and were resuspended in 50 mM potassium phosphate (pH 7.5) plus 10 mM azide. Yeast suspensions were used within 1 week.

S. cerevisiae 1412-4d (a *MAL3 SUC3*) was used for the invertase release experiment.

Enzymatic assays. Unless otherwise stated, the lytic assay incubation mixture contained: 50 mM potassium phosphate (pH 7.5), 10 mM 2-mercaptoethanol, a yeast cell suspension sufficient to give an A_{800} of 0.8 to 0.9, and enzyme, in a total volume of 2.0 ml. Enzyme was added last, and the A_{800} was read before and after a 30-min incubation at 30°C in a shaking water bath. One lytic unit is defined as a 10% decrease in absorbance in 30 min. The assay was sigmoidal with respect to time and enzyme concentration; activity was determined from the slope between 20% and 70% reduction in A_{800} as provided by increasing enzyme concentrations. Assays for lytic synergy were done in two ways. The first was a standard assay done on combined enzyme fractions. Fold synergy is defined as the activity of the combined fractions divided by the sum of the activity of the two fractions by themselves. The other assay measured lysis in the absence of 2-mercaptoethanol when purified glucanase was mixed with other fractions.

The protease assay mixture contained 10 mg of Azocoll per ml, 100 mM potassium phosphate (pH 7.5), and enzyme, in a total volume of 0.5 ml. After a 30-min incubation at 30°C with shaking, the insoluble Azocoll was removed by centrifugation, and the A_{520} of the supernatant solution was measured against a blank containing no enzyme. One protease unit is defined as one A_{520} unit solubilized in 30 min.

The chitinase assay mixture contained 50 mM potassium phosphate (pH 7.5), 0.5 mg of colloidal chitin (1) per ml, and enzyme, in a total volume of 1 ml. After 1 h at 30°C, the reaction was stopped by boiling for 3 min. The undegraded chitin was removed by centrifugation, and portions of the supernatant solution were treated with Sephadex G-25–filtered Glusulase (2) in a total volume of 0.5 ml for 30 min at 30°C with shaking. (This treatment breaks oligomers of *N*-acetylglucosamine down to monomers.) The reaction tubes were boiled for 3 min and tested for *N*-acetylglucosamine by the Reissig reaction (14). One unit releases 1 nmol of *N*-acetylglucosamine in 1 min.

The DNase assay mixture contained 0.5 ml of DNA solution (0.2 mg of polymerized calf thymus DNA per ml, 8 mM $CaCl_2$, 50 mM Tris, pH 7.5), 50 mM buffer (pH 7.5), and enzyme, in a total volume of 1 ml. The increase in A_{260} was measured over a few minutes. The Kunitz unit (7) was used.

The glucanase assay mixtures contained up to 0.1 ml of enzyme and 0.1 ml of glucan substrate (2.0 mg/ml) or laminarin (10 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.5). The reactions were incubated at 30°C for 0.5 to 4 h and were stopped by the addition of 0.2 ml of alkaline copper tartrate reagent, used for the determination of reducing sugar (19). The substrates for β-1,3- and β-1,6-glucanases were laminarin and pustulan, respectively. When yeast glucan was used as substrate, the insoluble material was removed by centrifugation before the solubilized reducing sugars were assayed. α-Mannanase activity was assayed in an analogous fashion except that the pH of the phosphate buffer was 7.0. One unit releases 1 μmol of reducing sugar equivalent per min.

Invertase was assayed by the method of Goldstein and Lampen (5), a two-step procedure. The A_{540} was read, with glucose used as a standard. One unit equals 1 μmol of glucose released per min.

Protein determinations were done as described by Lowry et al. (9) on samples which had been precipitated with 7% trichloroacetic acid. Bovine serum albumin was used as a standard.

Identification of polysaccharide hydrolysis products. Bio-Gel P-2 (400 mesh) was used to separate oligomers. The column was 1 by 53 cm. Fractions (0.5 ml) were collected at a flow rate of 5 ml/h and assayed for carbohydrate by the phenol-sulfuric acid method of Dubois (3). Positive identification of the sugars was made by descending paper chromatography in ethyl acetate-pyridine-water (5:3:2) as the solvent. Chromatograms were developed for 24 h, and sugars were located with the silver nitrate stain (21). Oligomers of the laminarin series, obtained from R. Cohen, who had made them by partial acid hydrolysis of laminarin, were used as standards.

Gel electrophoresis. Polyacrylamide gels (12%) were electrophoresed in the presence of SDS (sodium dodecyl sulfate) as described by Laemmli (8). Gels were stained for protein with Coomassie blue.

Molecular-weight determinations. (i) Subunit molecular weights were estimated from SDS gels, with bovine serum albumin (68,000), rabbit actin (42,000), pancreatic DNase I (31,000), trypsin (23,800), and lysozyme (14,400) as standards. (ii) Molecular weights

in solution were estimated both by gel filtration on Bio-Gel P-150, with bacterial alkaline phosphatase (80,000), hemoglobin (68,000), ovalbumin (43,000), and myoglobin (17,000) as standards, and by sucrose velocity gradient centrifugation in the manner of Martin and Ames (10), with bacterial alkaline phosphatase (80,000), trypsin (23,800), myoglobin (17,000), cytochrome *c* (12,400), and lysozyme (14,400) as standards.

Enzyme purification: (i) Growth and induction. *O. xanthineolytica* was grown on 20 liters of glucan or yeast medium for 24 to 36 h, starting with a 1% inoculum of a culture grown on glucose medium to an A_{600} of 2 to 3. Cells were grown either in Erlenmeyer flasks using one-fifth the volume of medium or, for large-scale preparations, in a New Brunswick fermentor (two 10-liter cylinders) with maximum aeration at 250 rpm. Samples of the culture supernatant were monitored for lytic activity, and cells and residual substrate were removed by centrifugation (20 min at 14,000 × *g* in a Sorvall GS3 rotor) at the estimated point of highest activity. The turbidity of the medium made the monitoring of growth by absorbance impossible. The use of a steam-driven Sharples centrifuge was not satisfactory for clarifying the supernatant solution. The following procedures were done at 0 to 4°C.

(ii) Concentration and dialysis. The culture supernatant was concentrated to about one-twentieth of its original volume by dialysis against solid polyethylene glycol. The culture supernatant was placed in 2-in. (5-cm)-diameter dialysis tubing which was then covered with Carbowax 6000 powder. The viscous liquid which accumulated outside the tubing was drained off, and new polyethylene glycol was applied several times during the procedure, which took about 24 h. The concentrate (fraction I) was dialyzed against 10 mM sodium succinate (pH 5.0) in the same tubing after collecting the liquid at one end and tying it off.

(iii) Carboxymethyl cellulose chromatography. CM-52 cellulose was equilibrated with 10 mM sodium succinate (pH 5.0). A 200-ml slurry was mixed with fraction I and stirred for 1 h. The mixture was poured into a column (4 by 20 cm) and allowed to settle. The unabsorbed solution was collected, the column was washed with one column volume of buffer, and the activity was eluted with two volumes of buffer containing 0.25 M NaCl. Ten-milliliter fractions were collected, and those containing lytic activity (fraction II) were pooled. Column chromatography with salt gradient elution did not improve the purification achieved.

(iv) Sephadex G-150 chromatography. A portion of fraction II was concentrated 10-fold by dialysis against solid polyethylene glycol, and 1 ml was applied to a 30-ml column (1.5 by 20 cm) of Sephadex G-150 equilibrated with 50 mM succinate buffer (pH 5.0). Flow rate was critical; for best separation, a flow rate of ≤4 ml/h was used. The column was eluted with 120 to 150 ml of 50 mM succinate buffer (pH 5.0), and 2-ml fractions were collected. The second broad peak of protein which contained all the lytic activity (fraction III) was pooled.

(v) Bio-Gel P-150 chromatography. Fraction III was concentrated 10-fold by dialysis against polyethylene glycol and applied to a 70-ml column (1.5 by 40 cm) of Bio-Gel P-150. The column was equilibrated and eluted with 50 mM sodium succinate buffer (pH 5.0). Fractions of 2 ml were collected at a flow rate of 4 ml/h. The two protein peaks (fraction IVa and b) were pooled separately.

(vi) DEAE-cellulose chromatography. Fraction IVb was dialyzed against 10 mM potassium borate (pH 8.5) and applied to a 20-ml column (1.5 by 10 cm) of DE-52 equilibrated with the same buffer. The unabsorbed material (fraction V) was pooled. The remaining protein was eluted with borate buffer containing 0.1 M NaCl.

RESULTS

Induction and purification. When *O. xanthineolytica* was grown with either autoclaved yeast or purified yeast glucan as a carbon source, a large number of hydrolytic enzymes were secreted into the culture medium. These included β-1,3-glucanases, chitinase, and proteases. Induction of lytic and protease activities on yeast and glucan media was compared (Fig. 1). Lytic activity was induced to a comparable level on either carbon source, although induction took 12 to 24 h longer with the glucan medium. The induction of protease, however, was reduced by 80% when glucan medium was used, so these conditions were chosen for purification of the lytic activity.

For large-scale (20-liter) fermentor preparations, it was necessary to concentrate the culture supernatant. This was accomplished by dialysis against solid polyethylene glycol. This concentrate was absorbed onto carboxymethyl cellulose and eluted with 0.25 M NaCl, effecting a 2.7-fold purification.

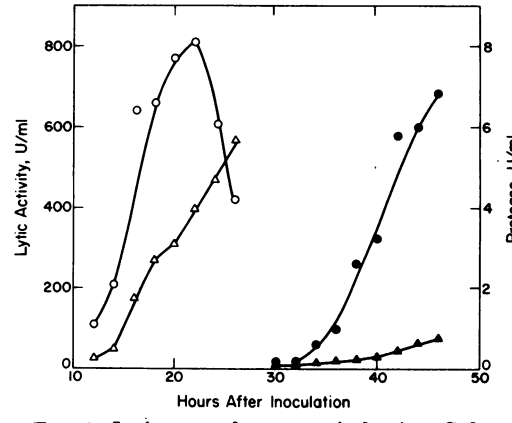

FIG. 1. *Lyticase and protease induction. Cultures (200 ml) of autoclaved yeast (open symbols) or glucan medium (closed symbols) were inoculated with 2 ml of a glucose-grown culture. Samples (5 ml) were taken at intervals, the cells were removed by centrifugation, and the supernatant solution was assayed for lytic (circles) and protease (triangles) activities.*

Early attempts at gel filtration with Sephadex resulted in an apparent total loss of lytic activity. However, it was found that several column volumes of buffer served to elute the activity, suggesting that the lytic enzymes were selectively retained by the dextran matrix. This affinity step completely removed chitinase activity (Fig. 2). The retardation of the lytic activity was unaffected by either salt concentration or pH, but was abolished if the elution buffer contained 1 M glucose.

The lytic activity did not absorb to Bio-Gel P-150. Gel permeation chromatography on this material yielded two peaks, one containing lytic and glucanase activities and the other containing only protease activity (Fig. 3). There was a shoulder of lytic activity where the two peaks overlapped. The material in the first peak from this column, fraction IVa, cochromatographed, with the lytic and glucanase activities and the protein eluting together. The lytic specific activity was constant across the peak. This fraction displayed a single major band on SDS-polyacrylamide gels with a few light bands attributable to proteolysis of the major band (Fig. 4).

When fraction IVa and fraction IVb (the second peak from the Bio-Gel P-150 column) were mixed, pronounced synergy (two- to five fold) for lysis was observed. Fraction IVb contained residual glucanase activity which was separated from the protease activity by DEAE-cellulose chromatography. The protease activity flowed through the column, whereas the glucanase was eluted with 0.1 M NaCl. The protease activity chromatographed as a single species on Bio-Gel P-30 and showed a single major band on SDS-polyacrylamide gels (Fig. 4F), although proteolytic breakdown products were detected after storage for prolonged periods at 0°C or after incubation at elevated temperatures. This was probably due to action of the protease on itself. The protease displayed virtually no lytic activity by itself but stimulated the lytic activity of the glucanase. This was the source of the synergy seen earlier.

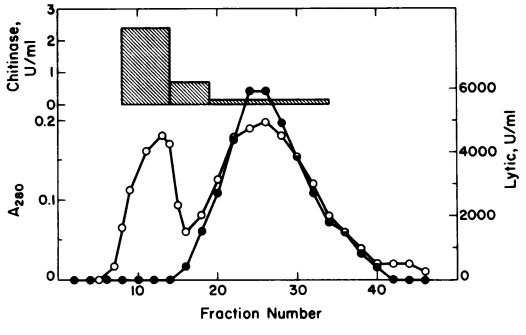

FIG. 2. *Sephadex G-150 chromatography. A concentrated sample of fraction II (1 ml, 7 mg of protein) was applied to a 30-ml Sephadex G-150 column (1.5 by 19 cm) equilibrated with 50 mM succinate buffer, pH 5.0. The column was eluted with the same buffer, and 2-ml fractions were collected. Open circles, A_{280}; closed circles, lytic activity; crossed bars, chitinase activity. Excluded volume is fraction 5; included volume is fraction 16.*

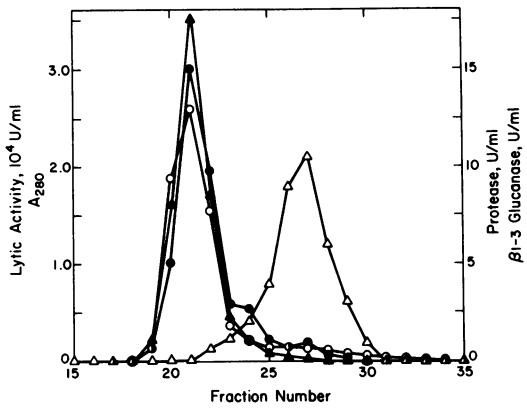

FIG. 3. *Bio-Gel P-150 chromatography. Fraction III was concentrated against polyethylene glycol to ≤1.5 ml and was applied to a 70-ml Bio-Gel column (1.5 by 40 cm) equilibrated with 50 mM sodium succinate buffer, pH 5.0. The column was developed with the same buffer, and 2-ml fractions were collected. The flow rate was 5 ml/h. Symbols ○, A_{280}; ●, lytic activity; △, protease activity; ▲, β-1,3-glucanase activity. Excluded volume is fraction 11; included volume is fraction 35.*

The summary of the purification achieved in a typical preparation starting with culture supernatant from cells grown on yeast glucan is shown in Table 1.

Properties of the glucanase: (i) Electrophoretic properties and molecular weight. When the glucanase was electrophoresed on SDS-polyacrylamide gels, only a single major band appeared, with an apparent molecular weight of 55,000 (Fig. 4D and H). A few light bands with slightly higher mobilities were seen when 5 to 10 μg of protein was applied to the gel, but these bands were seen across the entire Bio-Gel P-150 peak and could be due to cleavage by a contaminating protease in trace amounts. The low-molecular-weight species were enhanced by treatment of the glucanase with purified protease. The molecular weight of the glucanase as calculated from its elution on Bio-Gel P-150 was 65,000, indicating a monomeric structure.

(ii) pH optimum. The glucanase activity measured with laminarin had a pH optimum of 6.0 (Fig. 5). The lytic activity, however, showed a pH optimum at 8.0 with a steep drop between pH 7.5 and 7.0. At pH 9 and above, yeast cells

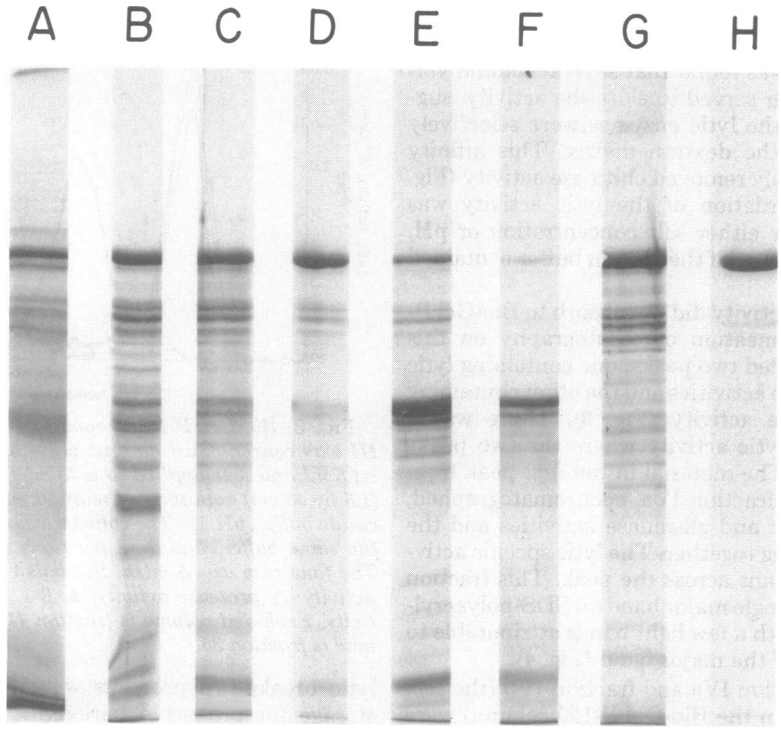

FIG. 4. *SDS-polyacrylamide gel electrophoresis. Samples were applied to a 12% polyacrylamide gel. (A) Dialyzed concentrate of culture fluid (fraction I), 15 μg; (B) carboxymethyl cellulose pool (fraction II), 10 μg; (C) Sephadex G-150 pool (fraction III), 10 μg; (D) Bio-Gel P-150 peak I (fraction IVa), 10 μg; (E) Bio-Gel P-150 peak II (fraction IVb), 10 μg; (F) DEAE-cellulose, unabsorbed material (fraction V), 5 μg; (G) DEAE-cellulose, salt wash, 10 μg; (H) fraction IVa rechromatographed on Bio-Gel P-150, 10 μg.*

TABLE 1. *Purification of lytic activity*

Fraction	Lytic activity			Protease activity		
	U/mg	Fold purification	Yield (%)	U/mg	Fold purification	Yield (%)
Culture supernatant	11,600	1	100	9.9	1	100
Fraction I	11,400	0.99	85	18.7	1.91	163[b]
Fraction II	25,000	2.7	57	26.3	2.67	57
Fraction III	48,000	4.2	44	18.1	1.84	19[b]
Fraction IVa	42,000[c]	3.6	25	—	—	—
Fraction IVb	24,000	2.07	5	79.9	8.1	20
Fraction V	<900[d]	—	—	98.9	10.0	11

[a] The greater than 100% yield may have been due to the instability of the protease in the culture supernatant.
[b] The low yield of protease at this step was due to the separation from another nonlytic protease.
[c] The specific activity goes down as a result of separation from the synergistic protease.
[d] Some lysis was seen with large amounts of pure protease.

became sensitive to lysis in the absence of enzyme, and the assay was invalid.

(iii) Substrate specificity and action pattern. The glucanase was active on both laminarin and insoluble yeast glucan, both predominantly β-1,3-linked glucose polymers, but showed no activity on pustulan, a β-1,6-linked glucan, or mannan. The enzyme released no more than 5% of the potential reducing ends of either laminarin or yeast glucan. Approximately 50% of the glucan as measured by the phenol-sulfuric acid assay was converted to soluble oligomers after exhaustive hydrolysis; the addition of new enzyme and buffer to the insoluble residue did not result in the release of any more carbohydrate to the supernatant solution. All of the laminarin was converted to small oligomers after exhaustive hydrolysis.

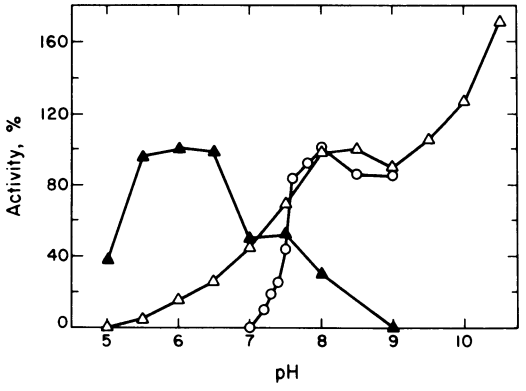

FIG. 5. *pH optima. Assays were performed in the following buffers: pH 5.0 to 6.5, sodium succinate; pH 6.5 to 8.0, potassium phosphate; pH 8.0 to 9.0, potassium borate; pH 9.0 to 10.5, sodium carbonate. Symbols: ▲, laminarinase activity of purified glucanase (fraction IVa); ○, lytic activity of pure glucanase; △, proteolytic activity of pure protease (fraction V).*

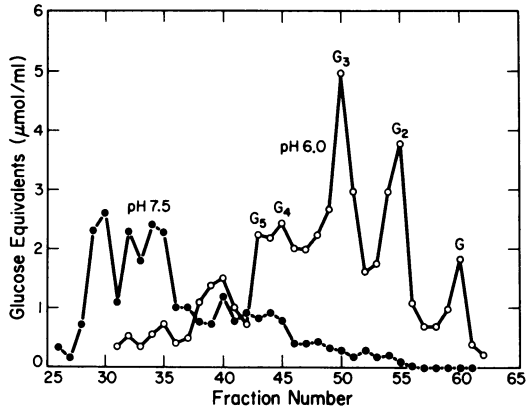

FIG. 6. *Glucanase digestion products of laminarin. Digestion mixtures (1-ml total volume) contained 20 mg of laminarin, 10 mM sodium succinate buffer (pH 6.0) or 10 mM potassium phosphate (pH 7.5), and 6 U of purified glucanase (as measured on laminarin at pH 6.0). Digestions were carried out for 24 h at 30°C. Samples were loaded on a Bio-Gel P2 column and analyzed as described in Materials and Methods. Symbols: ●, pH 7.5; ○, pH 6.0. Excluded volume is fraction 29; included volume is fraction 65.*

The products of exhaustive glucanase digestion of laminarin are shown in Fig. 6. The enzyme cleaved endolytically and released a variety of oligomers. The final products were pH dependent. At pH 6.0, the pH optimum for laminarinase activity, smaller oligomers predominated, with major amounts of laminaritriose, laminaribiose, and glucose. At pH 7.5, the optimal pH for the lytic reaction, larger oligomers, pentasaccharides or higher, predominated. The hydrolysis of yeast glucan showed a similar pH dependence, with trisaccharides and pentasaccharides the major product at pH 6.0 and 7.5, respectively.

(iv) Kinetics. Glucanase activity was measured at different substrate concentrations of both yeast glucan and laminarin. The results were markedly different. The apparent K_m for glucan was 0.4 mg/ml, whereas the apparent K_m for laminarin was 5.9 mg/ml, revealing a 10-fold higher affinity of the enzyme for its natural substrate. The maximum velocity was calculated to be 1.05 nanoequivalents per min for glucan and 5.63 nanoequivalents per min for laminarin. The turnover numbers calculated were 120 min^{-1} $molecule^{-1}$ for glucan and 620 min^{-1} $molecule^{-1}$ for laminarin.

Stability. The purified glucanase was quite stable. Fifty percent of the activity remained after 3 weeks at room temperature. Seventy percent of the activity remained after 3 weeks at 2 to 4°C or frozen at −18°C, with or without 20% glycerol. For long-term storage, freezing was used, but for periods of less than 1 month, storage at 2 to 4°C in the presence of sodium azide was satisfactory.

(vi) Activation and inhibition. As mentioned above, the Bio-Gel P-150 chromatography yielded two peaks of activity, one of glucanase and one of protease, which, when assayed together, gave up to five times the expected activity. Further purification of the second peak led to the separation of the protease from residual glucanase activity. The synergy was due to the protease activity. When 2-mercaptoethanol was removed from the lytic assay mixture, the glucanase lost its lytic activity entirely (Fig. 7A). The addition of either protease or 2-mercaptoethanol restored the lytic activity (Fig. 7B). Neither the protease nor the 2-mercaptoethanol had any effect on the glucanase activity on polysaccharide substrates.

Inhibition of the lytic activity of the glucanase by various sugars was also examined. Glucan was a potent inhibitor (50% inhibition at 0.03 mg/ml); laminarin inhibited at 10 times the concentration for glucan. Glucose was inhibitory at low concentrations (50% at 0.8 mg/ml), but mannan inhibited only at high concentrations (50% at 3.1 mg/ml).

(vii) Invertase release. The extent of solubilization of invertase, a cell wall mannoprotein, was used as a measure of lytic enzyme digestion of the cell wall. After a 1-h preincubation with 2-mercaptoethanol, either the glucanase or the protease or both released essentially all the invertase, even at low concentrations of enzyme (Fig. 8A). Preincubation was required because

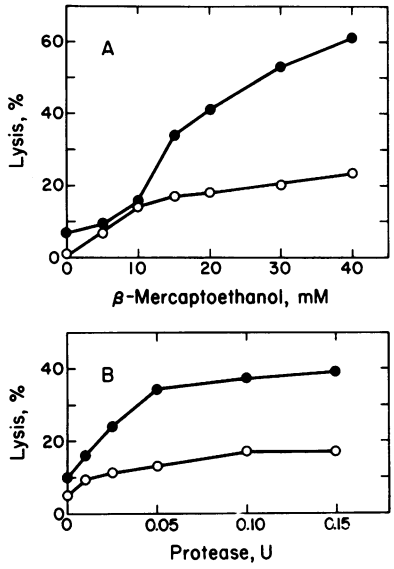

FIG. 7. *Stimulation of the lytic activity of the glucanase by the protease and 2-mercaptoethanol. (A) Standard lytic assays were performed except that the amount of 2-mercaptoethanol was varied from 0 to 40 mM. The glucanase (fraction IVa) was held constant at 0.8 U per assay. Symbols: ●, 2-mercaptoethanol and glucanase; ○, 2-mercaptoethanol alone. (B) As above except that no 2-mercaptoethanol was added and protease (fraction V) was added in amounts ranging from 0.01 to 0.15 U per assay. Symbols: ●, glucanase and protease; ○, protease alone.*

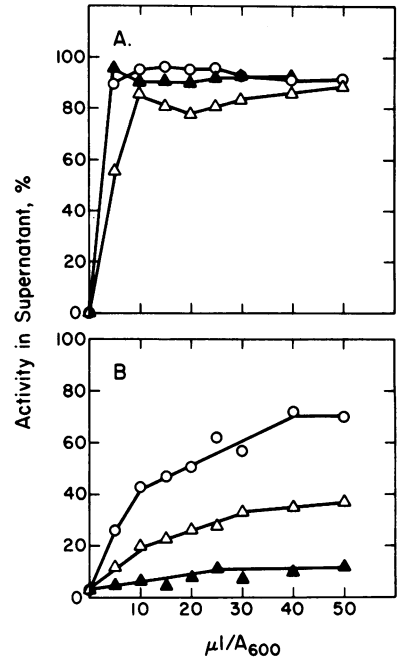

FIG. 8. *Invertase release by the lytic enzymes. (A) S. cerevisiae 1412-4D was grown on yeast extract-peptone-maltose (2%) overnight to an A_{600} of 2. Cells were centrifuged, washed, and resuspended in 50 mM potassium phosphate (pH 7.5). 2-Mercaptoethanol was added to give a final concentration of 10 mM, and the cells were incubated at 30°C for 1 h. The cells were again centrifuged, washed, and resuspended in phosphate buffer. To 0.5 ml of cells at an A_{600} of 2, 5 to 50 μl of enzyme(s) was added, and incubation was conducted for 30 min at 30°C. Cells were centrifuged and resuspended in 0.5 ml of phosphate buffer. Samples of both the supernatant solution and the cell suspension were assayed for invertase activity. Percent release is the amount in the supernatant solution divided by the sum of the amounts in both the supernatant solution and the cell suspension. (B) Same as the above except that the incubation with 2-mercaptoethanol was omitted. Symbols: ▲, glucanase alone (stock solution, 1,000 U/ml); △, protease alone (stock solution, 10 U/ml); ○, both enzymes.*

2-mercaptoethanol interfered with the invertase assay. In the absence of the 2-mercaptoethanol pretreatment (Fig. 8B), the glucanase did not release any invertase; the protease alone released a maximum of 30%, but the combination released up to 70% of the invertase to the supernatant solution. Invertase activity was unaffected by these levels of protease.

Properties of the protease. The synergistic protease and glucanase activities copurified through the Sephadex G-150 step. Separation was achieved on Bio-Gel P-150, and complete purification was accomplished with DEAE ion-exchange chromatography. This protease displayed several unique properties. First, it restored lytic activity to the glucanase in the absence of 2-mercaptoethanol. Neither trypsin nor pronase was able to substitute for the native protease, even when added at up to 20,000 times the proteolytic activity as measured on Azocoll. Second, the protease was retarded on Sephadex and eluted after the first column volume. This retention, also seen with the purified protease, indicated that the protease had an affinity for the matrix itself and was not indirectly bound through the glucanase. Third, the protease, though unaffected by most protease inhibitors, was inhibited by polysaccharides, in particular by bulk mannan (Table 2). The protease did not, however, display any α-mannanase activity. A molecular weight of 30,000 for the protease was determined by SDS-gel electrophoresis and one of 25,000 was determined by sucrose velocity sedimentation, suggesting a monomeric structure. The protease displayed an alkaline pH optimum (Fig. 5). The purified protease was stable at −20°C at pH 8.5 (potassium borate buffer, 10 mM). The addition of glycerol destabilized the enzyme. Approximately 50% of the

activity was lost in 1 week at room temperature.

Comparison to commercial preparations. Fraction II and the purified glucanase were compared with several commercial lytic enzyme preparations: Glusulase, Zymolyase 5000 and 60,000, and Teichozyme Y. Glusulase is a concentrate of snail digestive juice; Zymolyase and Teichozyme Y are lyophilized powders from culture supernatants of *A. luteus* and *O. xanthineolytica*, respectively. Both the Zymolyase 5000 and Teichozyme Y powders contained only about 10% protein by weight. Zymolyase 60,000 is a more purified form of Zymolyase 5000. The content of lytic, chitinase, DNase, and protease activities indicated considerable contamination of the commercial preparations (Table 3). Fraction II was significantly freer from the contaminating activities and had a high lytic specific activity. Contaminating activities were not detected in the purified glucanase.

Fraction II, our standard laboratory reagent for making spheroplasts, had a pH optimum of 7.5, required reducing reagent for full activity, and was inhibited significantly by glucose. The enzyme preparation was very stable. At 2 to 4°C no loss of activity was detected over several months when stored with sodium azide as an antimicrobial agent. At 22°C the enzyme decayed 50% in 1 week, but could be stabilized by saturated ammonium sulfate or 20% glycerol. The preparation lost 50% of its activity when frozen.

TABLE 2. *Protease inhibition by carbohydrates and glycoproteins*

Compound	Concn (mg/ml) giving 50% inhibition
Mannan, X2180-1B	0.18
Mannan, *mnn2*	0.24
Acetolysate I[a]	2.0
Acetolysate II[a]	5.0
Mannotetraose	>10.0
Laminarin	>10.0
Mannose	200
Glucose	200
Ovalbumin	25
Bovine serum albumin	>100

[a] Acetolysates I and II are mannan acetolysis products with an average size of 100 and 10 mannose residues, respectively.

DISCUSSION

The purification of lytic activity described in this paper has two critical steps. First, the use of purified glucan as the carbon source during enzyme induction results in a much lower induction of protease. A partially purified fraction is stable and highly effective in the formation of spheroplasts. The second critical step is the Sephadex affinity column. Lytic enzymes bind to the column and are separated from other hydrolytic enzymes which elute in the column volume. This step is similar to the glucan absorption step employed by both Rombouts and Phaff (15) and Jeffries (T. Jeffries, Ph.D. thesis, Rutgers University, New Brunswick, N.J., 1975). In our hands, the Sephadex affinity step was more reliable.

The yeast lytic enzyme system ("lyticase") produced by *O. xanthineolytica* consists of a β-1,3-glucanase and an alkaline protease. The glucanase attacks β-1,3-glucans in a random endolytic fashion, releasing oligosaccharides, in a pH-dependent fashion. At pH 7.5, the optimum for cell lysis, oligomers of five or more predominated, whereas at pH 6.0, the optimum for laminarinase activity, smaller oligomers and glucose predominated. This effect, which was seen with both yeast glucan and laminarin, probably is due to a pH effect on the enzyme. This action pattern was different from that seen for other lytic glucanases, with which laminaripentaose is the major or sole hydrolysis product (6, 15, 23). The enzyme showed a 10-fold higher affinity for glucan than for laminarin. Consistent with this was

TABLE 3. *Comparison of activities found in various lytic enzyme preparations*

Lytic enzyme source	Activity (U/mg)				Ratio		
	Lytic	Protease	Chitinase	DNase	Lytic/protease	Lytic/chitinase	Lytic/DNase
Zymolyase 500	3,200	120	1.9	4,900	28	1,700	0.66
Zymolyase 60000	4,700	50	0.4	14,000	94	12,000	0.34
Glusulase	15[a]	0.2	1.2	—[b]	66	12	—[b]
Teichozyme Y	240	30	6.1	51,000	8	38	4.6×10^{-3}
Fraction II	32,000	29	22	<100	1,100	1,400	>320
Fraction III	22,000	13	1.4	<100	1,800	16,000	>220
Fraction IVa	31,000	$<1 \times 10^{-2}$	<0.5	<100	$>3 \times 10^{6}$	>62,000	>310

[a] The published pH optimum of this preparation (6.0) gave an even lower value.
[b] Intrinsic absorbance of glusulase made the assay impossible.

the observation that 10 times as much laminarin as glucan was required to inhibit the lytic activity. A high affinity for yeast glucan may account for the lytic capacity of the glucanase. Glucanase-mediated lysis required either 2-mercaptoethanol or the alkaline protease, neither of which affects the activity on laminarin or glucan. The reducing agent and the protease probably acted directly on the yeast cell surface.

The alkaline protease was quite specific. Although the proteolytic activity measured on the Azocoll substrates was low, small amounts were effective in lytic synergy with the glucanase. Neither trypsin nor pronase (or other proteases present in the culture fluid) could be substituted for the protease in the lytic reaction. The protease was unaffected by a variety of protease inhibitors, but it was strongly inhibited by mannan. An affinity for polysaccharides may explain the retention of the protease by the Sephadex matrix. The purified protease was free from detectable glucanase and α-mannanase activity; thus, lysis was promoted by attack on the protein portion of the mannoprotein, not on the carbohydrate portion. Mercaptoethanol may substitute for the protease by reducing disulfide bonds in the mannoprotein.

Villanueva (22) has proposed that the glucan layer of the cell wall, which is responsible for structural integrity and rigidity, is covered by a layer of mannoprotein which must be modified in some way before a lytic glucanase can reach its substrate and promote cell lysis. This theory is supported by the synergistic effect of glucanase and protease activities in cell lysis and in invertase solubilization. The results of the latter experiment suggest that some invertase is exposed at the cell surface and some is sequestered beneath the glucan layer, accounting for the extra invertase released by the combination of both enzymes.

The enzyme system described here is similar to that isolated from *Arthrobacter* GJM by Vrsanká et al. (23, 24). Here also, cell lysis requires the combined action of a β-1,3-glucanase and an alkaline protease or 2-mercaptoethanol. The characteristics of the individual proteins, however, are different. The GJM glucanase is smaller (20,000 daltons) and specifically releases laminaripentaose. Pronase substitutes for the alkaline protease in their system, but this could be due to a difference in the cell wall of the substrate yeast, CCY 21-4-13. Although Zymolyase is the only β-1,3-glucanase which is claimed to be active against yeast cells by itself, the recent isolation from crude Zymolyase of a protease which promotes yeast cell lysis by the glucanase (4) suggests that the glucanase may not be pure. Assays which use glucan or cell walls as substrate (such as the cup-plate assay employed by Rombouts and Phaff [15, 16] for the *Bacillus circulans* enzymes) show no requirement for thiols or protease in the assay but do not establish the effectiveness of the enzyme against yeast cells.

The partially purified fraction II lyticase was easily obtained in large quantities with a single purification step. It had a high lytic specific activity and was purer than any commercial preparation with regard to nuclease, protease, and chitinase contamination. This fraction has been used successfully for spheroplast formation (11), membrane isolation (17), and nucleic acid isolation (20), and has proved useful in yeast transformation procedures.

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ADDENDUM IN PROOF

We found recently that one alkaline extraction of yeast cells (instead of three) produces a glucan fraction that is satisfactory in the induction of lyticase with low protease content.

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