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Purification of a Soluble and a Wall-Bound Form of β-Glucosidase from *Mucor racemosus*

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β-Glucosidase activity in crude extracts of *Mucor racemosus* exists in a soluble form and in a wall-bound form which sediments at 3,500 × *g*. The soluble form and a wall-bound form were purified to homogeneity by ammonium sulfate fractionation, DEAE-Sephadex chromatography, and SP-Sephadex chromatography. Both forms were identical in all parameters measured. Each enzyme is a glycoprotein of 91,000 daltons, with an identical amino acid composition and *N*-terminal amino acid of lysine; both contain about 10% carbohydrate. Both forms catalyze the hydrolysis of cellobiose and *p*-nitrophenyl-β-D-glucoside with identical kinetic constants.

The importance of nutritional factors to morphogenesis in dimorphic species of the fungus *Mucor* has been repeatedly observed. In general, the yeast form develops in an anaerobic atmosphere and requires a hexose-carbon source and an organic nitrogen source. Mycelial development usually occurs aerobically and can utilize ammonium nitrogen and a wide variety of carbon sources, including disaccharides such as maltose and cellobiose, hexoses, and C_2 and C_3 compounds (1).

Several studies have appeared concerning the inability of *Mucor rouxii* to metabolize maltose when in the yeast morphology (6, 7, 25, 27). From uptake experiments, Flores-Carreon et al. (6) proposed that maltose transport is an oxygen-dependent process. Implicit in their argument was a localization of the α-glucosidase within the cellular permeability barrier. Further investigation demonstrated that a large fraction of the α-glucosidase activity is wall bound (7). This necessitated a reexamination of the earlier hypothesis. Instead, it was proposed (25) that the cells are impermeable to maltose under both aerobic and anaerobic conditions. It was suggested that, to be metabolized, maltose must be hydrolyzed external to the cell membrane by the wall-bound α-glucosidase. The authors proposed that either the synthesis or insertion of the α-glucosidase into the wall was inhibited under the anaerobic conditions necessary for yeast development.

Sorrentino et al. (27) examined the α-glucosidase activity of soluble extracts and intact cells of *M. rouxii* generated under a variety of conditions. Their results indicated that the synthesis of α-glucosidase is not impaired in either yeast or mycelia generated under anaerobic conditions. However, since only mycelia generated anaerobically showed high wall-bound activity, they concluded that the enzyme either cannot be incorporated into yeast cell wall or that it is incorporated in a masked or inactive form. The inability of the yeast to grow in a maltose medium was attributed to the necessity for the hydrolysis of maltose to glucose external to the cell membrane, since it was assumed that maltose could not be transported across the membrane. Thus, their model links the inability to grow on maltose to the nature of the yeast cell wall and not to anaerobiosis. It was speculated that the mycelial wall possesses sites necessary for association with the enzyme and that these sites are lacking in the yeast wall.

Our laboratory has been concerned with the inability of *Mucor racemosus* to metabolize or grow on cellobiose, a disaccharide which supports mycelial development. We have shown that exogenous cAMP, an agent which plays a key role in morphogenesis (15), causes a reversible inactivation of preexisting β-glucosidase (2). The inactivation both in vivo and in vitro is consistent with a cAMP-dependent protein phosphorylation. As in the case of the α-glucosidase from *M. rouxii*, the β-glucosidase activity is present in a soluble form and in a wall-bound form. We do not know whether the inactivation is related to the cellular localization of the enzyme.

Thus, the current hypotheses concerning the inability of *Mucor* yeast to utilize disaccharides center on the location and possible modification of the enzyme in vivo. Unambiguous explanations for these phenomena are dependent upon

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purification of the various forms of the glucosidases, an examination of their properties, their identification on two-dimensional gels of crude extracts, and a direct demonstration of their cellular locations. In this report, we present the purification and some of the properties of a soluble and a wall-bound form of β-glucosidase from *M. racemosus*.

MATERIALS AND METHODS

Organism. *M. racemosus* (*Mucor lusitanicus*) ATCC 1216B was used throughout.

Media and culture conditions. For β-glucosidase production, 3 liters of yeast extract-peptone-glucose medium (1) in a 6-liter Erlenmeyer flask was inoculated with 10^5 sporangiospores per ml. The cells were grown at 28°C for 24 h in the yeast form by sparging N_2 gas through the culture at 15 liters of N_2 per min. The resulting yeast cells were aseptically centrifuged and distributed among three 14-liter FS-14 fermentors (New Brunswick Scientific Co., New Brunswick, N.J.) containing 10 liters of YNB medium (0.17% yeast nitrogen base [Difco]–0.15% NH_4NO_3–0.15% Na glutamate–1% maltose). The fermentors were operated at 28°C and sparged with sterile air at a rate of 10 liters/min per fermentor. Growth was continued for 17 h. The cells were harvested by filtration on no. 1 paper (Whatman, Inc., Clifton, N.J.). The mycelial pad was washed on the filter with several volumes of 0.02 M Tris-hydrochloride buffer (pH 8.5) and was suspended in a convenient amount of the same buffer. The cells were disrupted by passing them through a French pressure cell twice at 10,000 lb/in^2. The resulting homogenate was centrifuged at 3,500 × *g* for 15 min to remove debris. The pellet was washed twice with Tris buffer, and the washes were combined with the original supernatant as a source of soluble enzyme. For the purification of the wall-bound form, the pellet was washed an additional three times, the washings were discarded, and the pellet was retained as a source of enzyme.

Purification of soluble β-glucosidase. All operations were carried out at 4°C. The crude extract was brought to 70% saturation (455 g/liter) by the addition of solid $(NH_4)_2SO_4$ (Ultra Pure; Schwarz/Mann, Orangeburg, N.Y.). The extract was allowed to stand for 1 h and was then filtered on a sintered glass filter upon which had been laid a pad of Celite (Johns-Manville, Denver, Colo.). The filtrate was retained, brought to saturation (an additional 219 g/liter), and allowed to stand for an additional 12 to 15 h. The extract was centrifuged at 7,000 × *g* for 30 min. The precipitate containing the β-glucosidase was dissolved in about 50 ml of 0.02 M Tris-hydrochloride (pH 8.5) and dialyzed against several changes of 5 liters of the same buffer for 24 h.

The dialyzed extract was applied to a column (2.6 by 40 cm) of DEAE-Sephadex A-25 equilibrated in 0.02 M Tris buffer at a flow rate of 30 ml/h. The column was washed with 1 column volume of buffer, and the enzyme was eluted with a 500-ml gradient of 0.0 to 0.3 M NaCl in the same buffer. Fractions of 5 ml were collected. The enzyme-containing fractions were dialyzed for 24 h against three changes of 1 liter of 0.02 M sodium phosphate buffer (pH 5.8). The extract was applied to a column (2.6 by 40 cm) of SP-Sephadex C-25 equilibrated in phosphate buffer at 30 ml/h. The column was washed with 1 column volume of buffer, and the enzyme was eluted with a 500-ml linear gradient of 0.0 to 0.3 M NaCl in the same buffer. Fractions of 5 ml were collected.

Purification of the wall-bound enzyme. The cell wall fraction (3,500 × *g* washed pellet) was suspended in 500 ml of 0.02 M Tris-hydrochloride buffer (pH 8.5) containing 2.0 M NaCl. The suspension was incubated at 0°C for 12 h with occasional stirring. The suspension was centrifuged at 3,500 × *g*, and the supernatant was retained as a source of wall-bound enzyme. The solubilized wall-bound enzyme was dialyzed for 48 h against several changes of 10 liters of 0.02 M Tris-hydrochloride buffer (pH 8.5). The dialyzed preparation was then purified by DEAE-Sephadex chromatography and SP-Sephadex chromatography as for the soluble enzyme.

Two-dimensional gel electrophoresis. Two-dimensional analysis of cell extracts and of purified β-glucosidase preparations were performed by the method of O'Farrell (22) as modified by Pedersen et al. (23). The first dimension was a narrow-range (pH 5.0 to 7.0), isoelectric focusing gel; the second dimension was a 7.5 or 10% polyacrylamide sodium dodecyl sulfate (SDS) gel. Molecular-weight determinations of purified β-glucosidase were done on one-dimensional 7.5% polyacrylamide SDS gels. Gels were stained with Coomassie brilliant blue R for protein or with periodate-Schiff reagent for carbohydrate (17).

***N*-Terminus Analysis.** Samples of purified β-glucosidase (100 μg) were dansylated by the method of Gros and Labouesse (11). The dansyl protein was hydrolyzed for 12 h in constant boiling 6 N HCl, and the dansyl amino acids were separated on polyamide thin layers (10).

Amino acid analysis. Purified protein was hydrolyzed at 110°C with constantly boiling 6 N HCl in evacuated and sealed vials for 24, 48, and 72 h. The hydrolysates were analyzed on a MM-50 single-column amino acid analyzer (Glenco Scientific, Inc., Houston, Tex.). Cysteine was determined as cysteic acid by the method of Moore (19). Tryptophan was determined spectrophotometrically by the method of Edelhoch (5). Threonine and serine were determined by extrapolation to zero hydrolysis time.

Enzyme assays, kinetics, and substrate specificity. Enzyme assays were performed at 40°C and were proportional to protein concentration and time. The standard reaction mixture contained 100 μl of enzyme solution in 50 mM sodium acetate buffer (pH 4.5) and 100 μl of 10 mM *p*-nitrophenyl-β-D-glucoside in the same buffer. The reaction was stopped by the addition of 0.8 ml of 0.2 M Na_2CO_3, and the absorbance was read at 400 nm on a Gilford 2500 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). One unit of activity was defined as that amount of enzyme hydrolyzing 1 nmol of *p*-nitrophenyl-β-D-glucoside per min.

For analysis of the kinetic properties and substrate specificity of the enzyme on disaccharides, the conditions were essentially identical to those described above. In these studies, glucose liberation was determined by the glucose oxidase-peroxidase method (Technical Bulletin No. 510; Sigma Chemical Co., St. Louis, Mo.) after boiling to stop the reaction.

Protein determination. Protein concentration was

TABLE 1. Purification of β-glucosidase from *M. racemosus*

Step	Characteristic of β-glucosidase				
	Vol (ml)	U/ml	mg of Protein per ml	Sp act	Recovery (%)
Crude	350	16,300	31.4	519	100
Crude soluble	430	6,720	20.3	331	50.6
70 to 100% $(NH_4)_2SO_4$	56.5	26,100	6.0	4,350	25.6
After DEAE cellulose	72	8,420	0.200	42,100	10.6
After SP Sephadex	49	5,980	0.100	59,800	5.1
Crude wall-bound	500	5,600	4.10	1,370	49.1
NaCl extract	150	7,620	1.81	4,210	20.0
After DEAE cellulose	50	12,800	0.595	21,500	11.2
After SP Sephadex	52	11,500	0.188	61,170	10.4

monitored by the Lowry method (16) with bovine serum albumin as the standard.

Carbohydrate analysis. Total carbohydrate was determined by the phenol H_2SO_4 method (3) with glucose as the standard. Polyacrylamide gels were stained for carbohydrate with periodate-Schiff stain (17).

Immunological methods. Rabbit antiserum was prepared against purified soluble β-glucosidase. A 0.5-mg amount of the protein was emulsified in Freund complete adjuvant and injected intramuscularly. Another 0.5 mg of the protein was injected 30 days later. The animal was bled from an ear vein 7 days after the second injection. Ouchterlony double diffusion was performed on microscope slides in 0.5% agarose–0.85% NaCl.

Sephadex G-150 chromatography. The molecular weight of β-glucosidase was estimated on a column (2.6 by 40 cm) of Sephadex G-150 operated at 22°C at a flow rate of 10 ml/h in 0.1 M sodium phosphate buffer (pH 6.8). Fractions of 0.88 ml were collected.

RESULTS

Purification. Extracts of derepressed *M. racemosus* contained β-glucosidase activity which could be separated into two components by low-speed (3,500 × *g*) centrifugation. Presumably, the sedimenting form was bound to the wall fraction in some manner. About 50% of the activity was in each fraction. We have purified the soluble form and a form which can be solubilized from the wall fraction by washing with 2.0 M salt at 0°C (Table 1). Both forms were purified 100- to 200-fold in routine purifications, depending upon the starting specific activity. The specific activity of either form after SP-Sephadex chromatography was always about 60,000 in several independent purifications.

Figure 1 shows the column profile for the soluble enzyme from DEAE-Sephadex chroma-

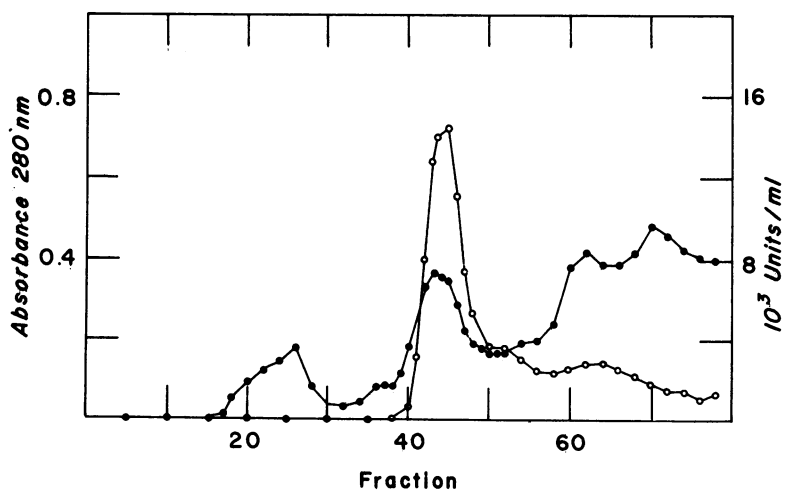

FIG. 1. Chromatography of β-glucosidase of DEAE-Sephadex. The column (2.6 by 40 cm) was equilibrated in 0.02 M Tris-hydrochloride (pH 8.5) at a flow rate of 30 ml/h. After sample application, about 1 column volume of buffer was passed through the column. The enzyme was eluted with a 500-ml linear gradient from 0 to 0.3 M NaCl in Tris-hydrochloride buffer. Fractions of 5.0 ml were collected. ●, Absorbance at 280 nm; ○, β-glucosidase activity on *p*-nitrophenyl-β-D-glucoside.

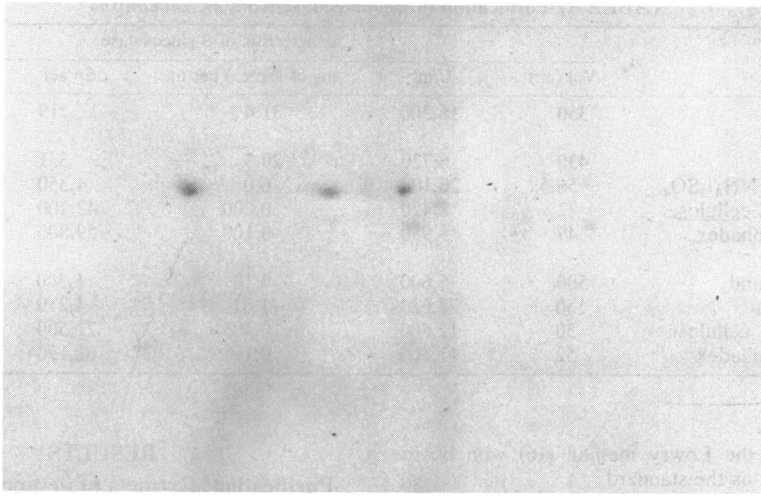

FIG. 2. Two-dimensional gel of the proteins solubilized from washed walls by 2.0 M NaCl treatment at 0°C. The first dimension is a pH 5.0 to 7.0 isoelectric focusing gel; the second dimension is a 7.5% polyacrylamide SDS gel. The arrow marks the position of β-glucosidase.

tography. The major peak of activity eluted from the column at about 0.1 M NaCl. Two smaller, more acidic activity peaks eluting at higher salt concentrations were evident. The major peak eluting from the DEAE column was further purified on an SP-Sephadex column. The glucosidase activity was eluted from the SP-Sephadex at about 0.1 M NaCl (data not shown).

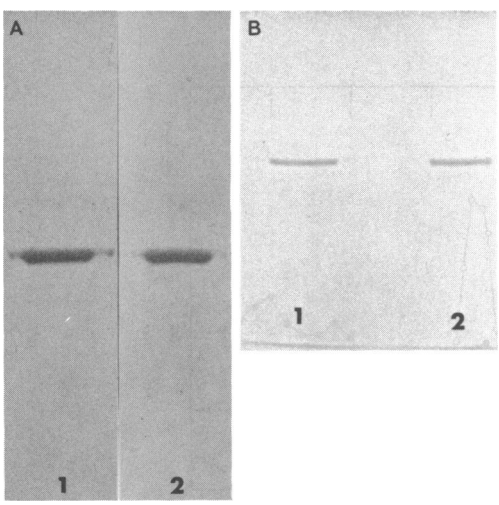

FIG. 3. One-dimensional 7.5% polyacrylamide SDS gels of purified soluble and purified wall-bound β-glucosidase. (A) Lanes 1 and 2, 3.0 μg of the soluble and wall-bound enzyme, respectively, stained with Coomassie blue R; (B) lanes 1 and 2, 10 μg of the soluble and wall bound enzyme, respectively, stained with periodate-Schiff reagent.

About 40% of the wall-bound activity is released by incubation with 2.0 M salt. This suggests that at least some of the enzyme is bound to the wall by noncovalent forces. Two-dimensional analysis of the proteins liberated from the wall fraction (Fig. 2) indicates that only four or five polypeptides are released. At least three of these have a molecular weight of about 91,000 but differ in charge. One of the major 91K spots comigrates with purified soluble β-glucosidase.

The fraction of bound β-glucosidase activity which cannot be released with salt might represent a distinct class of enzyme bound by covalent interactions. The solubilized wall-bound form behaves identically to the soluble enzyme on DEAE and SP-Sephadex chromatography, and the specific activity of the purified protein is essentially identical to that of the soluble enzyme (Table 1).

Gel electrophoresis and molecular weight. Figure 3 shows one-dimensional gels of the purified soluble and wall-bound enzymes. In each case, a single polypeptide with an apparent molecular weight of 91,000 is present. Both forms stained with periodate-Schiff reagent, indicating that they are glycoproteins. Since we can detect as little as 0.1 μg of protein on these gels, they suggest that each form is greater than 95% purity. Reaction of each purified enzyme form with phenol-sulfuric acid indicated that both forms possess 0.1 mg of carbohydrate per mg of protein (as glucose equivalents).

Since SDS gels sometimes give anomolous molecular weights with glycoproteins, we estimated the molecular weight of the soluble enzyme on a calibrated Sephadex G-150 column.

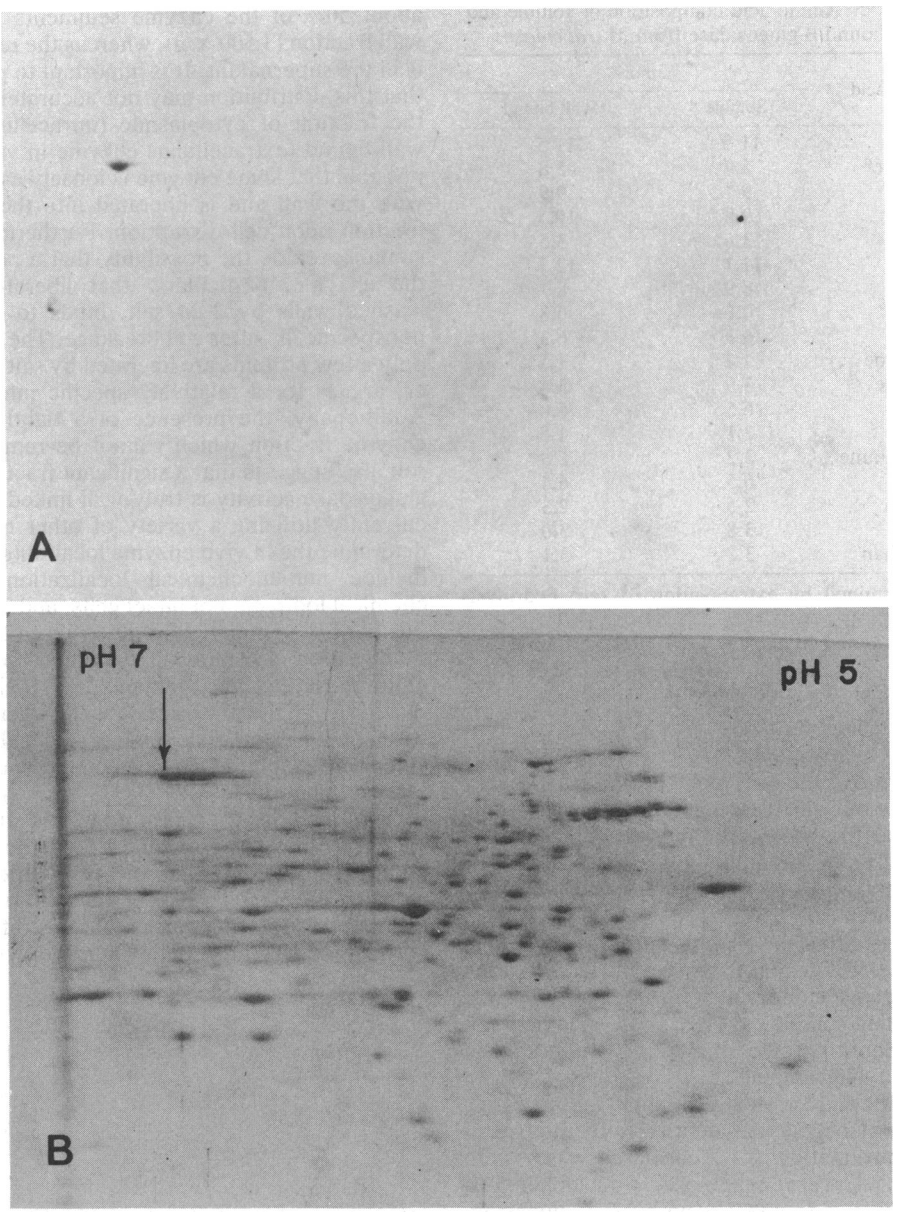

FIG. 4. Two-dimensional gel analysis of *Mucor* β-glucosidase. (A) A 1-μg amount of purified soluble enzyme; (B) 100 μg of the soluble proteins from derepressed cells. The electrophoresis conditions in A and B were similar to those described in the legend to Fig. 2, except the second dimension was a 10% polyacrylamide SDS gel.

The apparent molecular weight by this method is also 91,000 (data not shown).

Figure 4A shows a two-dimensional gel of 1 μg of purified soluble β-glucosidase. The purified wall-bound form also gave a single spot which comigrated with the soluble form. Figure 4B shows the spectrum of polypeptides from derepressed cells grown in a medium containing 1% cellobiose. The position of the β-glucosidase is indicated by the arrow. Several polypeptides with the identical molecular weight and a charge slightly more acidic than the purified enzyme are evident in the crude extracts.

Amino acid analysis and *N*-terminus. Both forms of the enzyme showed a single amino terminus of lysine in the dansyl-chlorine procedure.

Table 2 shows the amino acid composition for

TABLE 2. Amino acid composition of soluble and wall-bound β-glucosidase from *M. racemosus*

Amino acid	mol%	
	Soluble	Wall bound
Aspartic	11.9	11.5
Threonine[a]	5.7	5.8
Serine[a]	9.1	9.9
Glutamic	9.8	10.5
Proline	3.6	3.5
Glycine	11.1	12.7
Alanine	8.9	8.7
Cysteine[b]	0.8	0.8
Valine	6.3	6.3
Methionine	1.1	1.5
Isoleucine	3.9	3.4
Leucine	6.5	6.0
Tyrosine	2.1	1.8
Phenylalanine	3.2	2.8
Lysine	6.5	6.4
Histidine	2.5	2.3
Arginine	3.8	3.0
Tryptophan[c]	3.2	3.1

[a] Determined by extrapolation to zero hydrolysis time.

[b] Determined as cysteic acid after performic acid oxidation.

[c] Determined spectrophotometrically.

both forms of the enzyme. The weight recovery for the two forms were 95.5 and 97% after being corrected for 10% carbohydrate content. It is apparent from the data shown in the table that the composition of the two forms is virtually identical.

Immunodiffusion. Figure 5 shows the results of immunodiffusion of the two purified enzyme forms against rabbit antiserum prepared against the purified soluble enzyme. From the results, it is apparent that the enzymes share identical antigenic determinants.

Kinetics and substrate specificity. Both forms of the enzyme showed identical substrate specificity and kinetics. β-Glucosidases which were hydrolyzed were cellobiose, *p*-nitrophenyl-β-D-glucoside, 6-bromo-2-naphthyl-β-D-glucoside, and 5-bromo-4-chloro-3-indolyl-β-D-glucoside. No hydrolysis of maltose, sucrose, trehalose, melibiose, lactose, raffinose, melizitose, cellulose, or carboxymethylcellulose could be detected. The K_m for cellobiose was 0.22 mM, and the V_{max} was 6.1 nmol/min per μg of protein. For *p*-nitrophenyl-β-D-glucoside, the K_m was 0.31 mM, and the V_{max} was 8.7 nmol/min per μg of protein.

DISCUSSION

In this paper, we report the purification of a soluble and a wall-bound form of β-glucosidase from *M. racemosus*. In crude cell-free extracts, about 50% of the enzyme sediments with the wall fraction (3,500 × *g*), whereas the remainder is in the supernatant. It is important to point out that this distribution may not accurately reflect the fraction of cytoplasmic (intracellular) and wall-bound (extracellular) enzyme in vivo. It is possible that some enzyme is loosely associated with the wall and is liberated into the soluble fraction upon cell disruption. Furthermore, we cannot exclude the possibility that a portion of the enzyme, particularly that liberated from washed walls by 2 M salt, binds to the wall nonspecifically after cell breakage. The fact that only a few proteins are liberated by salt, however, argues for a relatively specific interaction. Additionally, the presence of a tightly bound enzyme fraction which cannot be removed by salt also suggests that a significant fraction of β-glucosidase activity is truly wall linked. We are currently utilizing a variety of other means to determine the in vivo enzyme location(s). These include immunochemical localization, radiochemical iodination of intact cells, and measurements of the amount of β-glucosidase liberated by digestion of osmotically stabilized cells with chitosanase, a lytic enzyme (8). Preliminary results from the chitosanase experiments indicate that greater than 80% of the cellular β-glucosidase content is located external to the plasma membrane.

By all parameters measured in this study, the soluble form of β-glucosidase and the loosely bound (salt-extracted) form are identical. The molecular weight is 91,000; the *N*-terminus is lysine; each form has identical substrate specificity, kinetics, and amino acid analysis; and

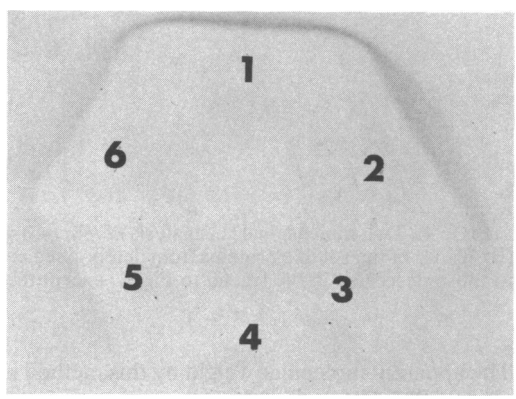

FIG. 5. Ouchterlony double-diffusion analysis of soluble and wall-bound β-glucosidase. The center well contained antiserum against purified soluble β-glucosidase. The outer wells contained: 1, 1 μg of soluble enzyme; 2, 0.5 μg of soluble enzyme; 3, 0.25 μg of soluble enzyme; 4, 0.15 μg of wall-bound enzyme; 5, 0.3 μg of wall-bound enzyme; 6, 0.6 μg of wall-bound enzyme.

each possesses about 10% carbohydrate. The finding that the purified protein is glycosylated may be a clue to its in vivo location. Several examples of wall-bound fungal glycosidases are known. These include invertase from *Saccharomyces* (14) and *Neurospora* (13, 16), β-glucosidase (4), laminarinase (9), and trehalase (13) from *Neurospora*. The invertase of *Saccharomyces* is probably the best characterized of these. It exists in two forms, an intracellular (light) form (20) and a wall-bound mannoprotein form (14, 26). If the soluble β-glucosidase reported in this study represents an intracellular precursor to the wall-bound enzyme, it is already glycosylated, unlike the intracellular precursor of *Saccharomyces* invertase. Alternatively, the soluble enzyme we have purified might be a fraction of wall-bound β-glucosidase which is liberated into the soluble fraction by cell disruption.

Several experiments presented in this paper suggest that charge heterogeneity of β-glucosidase exists in *M. racemosus*. Ion-exchange chromatography usually indicates multiple activity peaks (Fig. 1). In two-dimensional gels of crude extracts, a series of proteins differing only in charge (more acidic) from the purified protein is observed (Fig. 4B). In addition, salt liberates at least three proteins of 91,000 molecular weight from washed walls (Fig. 2). One of these has a pI identical to that of the purified soluble protein, whereas the other two are more acidic. We are currently investigating whether the species in question are immunologically related and result from the proposed enzyme phosphorylation (2).

Sorrentino et al. (27) have proposed that *M. rouxii* cannot grow in the yeast morphology on maltose owing to the inability of the cells to incorporate α-glucosidase into the yeast wall or that it is incorporated in an inactive form. They speculated that specific sites which are necessary for enzyme binding might be present in mycelial walls but absent in yeast walls. An alternative explanation for their results might be that the secretory process for the enzyme shares common steps with mycelial wall synthesis and that these steps are absent in yeast wall synthesis. Novick and Schekman (21) have shown that, in *Saccharomyces*, conditional invertase secretory mutants also fail to synthesize cell wall under nonpermissive conditions.

The inability of *Mucor* yeast to metabolize disaccharides is a unique property of *Mucor* yeast. An unambiguous explanation for this phenomenon requires the purification of the various forms of the glucosidases involved in disaccharide metabolism and an examination of the regulation of their synthesis, activity, and cellular localization. It is our expectation that such experiments will provide insights into the molecular aspects of dimorphism.

ACKNOWLEDGMENT

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