Secretory Character of Yeast Chitinase*

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During the conversion of yeast cells to protoplasts, about half of the yeast chitinase was liberated into the medium, an indication that this portion of the enzyme is located in the periplasmic space. At least part of the remaining chitinase appears to be enclosed in vacuoles or in vesicles that co-purify with them, as indicated by a 14-fold enrichment of the enzymatic activity in a vacuole fraction isolated from a protoplast lysate. When protoplasts were incubated in growth medium, part of the chitinase was liberated into the medium. It is concluded that yeast chitinase is a secretory enzyme, like invertase and acid phosphatase. The enzyme appears to be stored in vesicles as a prelude to its secretion into the periplasmic space. The possible function of yeast chitinase in cell division is discussed.

The primary septum of budding yeasts consists of chitin (1). Much has been learned in recent years about the mechanism of synthesis of this polysaccharide (2), but nothing is known about its degradation in yeast. Recently, we have isolated from *Saccharomyces cerevisiae* a chitinase which appears to be a mannan-associated protein (3). To gain some insight into the function of this enzyme, it seemed of interest to determine its subcellular distribution. Other mannan-proteins such as invertase and acid phosphatase, are secreted by the cell into the periplasmic space (4). Our results indicate that chitinase also is a secretory protein, suggesting that it may be involved in cell wall metabolism.

MATERIALS AND METHODS

Most of the materials and methods used are described in the accompanying paper (3). Others are outlined below.

Tunicamycin was obtained from Calbiochem, cycloheximide from Mann, and Zymolyase 60,000 from Kirin Brewery Co., Takasaki, Japan. Contaminating chitinase activity in Zymolyase was removed by adsorption on regenerated chitin (5) for 45 min at 2 °C and pH 7.5. For each mg of Zymolyase, 10.6 mg of chitin was added.

Localization of Chitinase in the Periplasmic Space—*Saccharomyces cerevisiae* prb1122 was grown as described (3) but harvested at $A_{660}^{1cm} = 0.2$ (Coleman junior spectrophotometer, 1-cm light path) corresponding to about 3×10^7 cells/ml. The other strains used, S288C (α *SUC2 mal gal2 CUP1*) and X2180 (homozygous diploid of S288C) were grown in the same way. Cells were incubated for 30 min at 30 °C with 40 mM EDTA, 96 mM β-mercaptoethanol in a total volume of 3.5 ml/g of yeast (wet weight; all subsequent amounts of yeast are also given as wet weight). After centrifuging for 10 min at 12,000 × g, the pellet was washed once with 0.6 M MgSO₄. The

pretreated cells were incubated at 37 °C with 1.4 mg/ml of Zymolase in 0.6 M MgSO₄, 14 mM potassium phosphate, pH 7.5, in a total volume of 3.5 ml/g of yeast. After 15 min, conversion to protoplasts was practically complete. The suspension was centrifuged for 10 min at 1500 × g, and the pellet was washed twice with 10% mannitol containing potassium citrate-phosphate buffer, pH 6.1 (6), at ¹⁄₂₀ of the original (6) concentration. All supernatant fluids were pooled, concentrated with an Amicon pressure cell fitted with YM-10 membrane, dialyzed overnight against 25 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.3, and again concentrated (periplasmic fraction). Protoplasts were lysed in 1 mM EDTA (17 ml/g of original yeast), with the use of a Lourdes Multi-mix homogenizer at half-maximal speed. The lysate was centrifuged at 100,000 × g for 30 min and the supernatant fluid was concentrated in an Amicon cell to a final volume of 1 ml/g of yeast used (cytoplasmic fraction).

Chitinase activity (3) was measured in the periplasmic and cytoplasmic fractions.

To correct the chitinase values in the periplasmic fraction for leakage of intracellular enzyme during protoplast formation, the activity of an internal enzyme, alkaline phosphatase, was measured both in the periplasmic and cytoplasmic fractions (7).

Vacuole Preparation—Vacuoles were prepared from *S. cerevisiae* X2180 by metabolic lysis of protoplasts and fractionation of the resulting crude particulate fraction ("mixed particles") on a discontinuous Ficoll gradient (8). Chitinase and proteinase B activities were estimated in the different fractions after light sonication (8).

Secretion of Chitinase by Protoplasts—Protoplasts of prb1122 were prepared with Zymolyase as outlined above, then washed five times with 0.8 M sorbitol, containing citrate-phosphate buffer, pH 6.1, at ¹⁄₂₀ of the original (6) strength, and resuspended in the same solution, in a final volume of 1 ml/g of cells used. The protoplast suspension was diluted in 25 volumes of a medium containing 2% glucose, 0.7% yeast nitrogen base, 20 mg/liter of histidine hydrochloride, 0.8 M sorbitol. The medium was supplemented with 50 μg/ml of aureomycin, to prevent bacterial growth, and incubated at 30 °C. Samples were removed periodically and centrifuged for 10 min at 12,000 × g. Supernatant fluids were saved. The pellets were suspended in 25 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.3 (2.5 ml/g of the original yeast) and subjected to sonic oscillation for two 10-s periods at setting 2 of a Branson Sonifier. After adding digitonin to a final concentration of 0.1%, the extracts were incubated for 2 h at 30 °C and centrifuged for 10 min at 12,000 × g. All supernatant fluids were concentrated in Amicon pressure cells to a final volume of 1 ml/g of yeast used. Chitinase and α-glucosidase activity were measured in all fractions.

Enzymatic Assays—Chitinase (3) and proteinase B (9) activities were determined as described. α-Glucosidase was measured as outlined by Wessels and Niederpruem (10) for β-glucosidase, but with the use of p-nitrophenol-α-D-glucoside as substrate. For alkaline phosphatase, incubation was for 30 min at 30 °C with 1 mM p-nitrophenylphosphate in 50 mM Tris-chloride, pH 8, in a total volume of 1 ml. After stopping the reaction with 0.1 ml of 1 N NaOH, the absorbance at 410 nm was measured.

RESULTS AND DISCUSSION

Because of its facile extractability from intact cells (3), it seemed possible that at least part of the chitinase would be in the periplasmic space. To verify this possibility, the cell wall was digested with lytic enzymes (Zymolyase) in the presence of an osmotic protector, to prevent lysis of the resulting protoplasts, and the amount of enzyme liberated into the

supernatant fluid was measured. With several strains of *S. cerevisiae*, between 30 and 70% of the total chitinase was found in the supernatant fluid (Table I) suggesting that about half of the enzyme was secreted into the periplasmic space during growth.

The recent finding that yeast mutants blocked in secretion accumulate intracellular vesicles containing the secretory products (12), prompted us to ascertain whether the intracellular enzyme might be similarly sequestered. Protoplasts were subjected to metabolic lysis, and vacuoles were isolated by centrifugation in a discontinuous Ficoll gradient (8). In these experiments, protoplasts were prepared from *S. cerevisiae* X2180 with the use of snail gut enzyme (13, 14), because use of *prb* 1122 and Zymolyase treatment resulted in unsatisfactory separation of vacuoles. About half of the recovered chitinase activity was found in the vacuole fraction, with a 14-fold increase in specific activity (Table II). An approximately equal amount of enzyme was found in the gradient pellet (Table II), but its specific activity was even lower than that of the starting material. It cannot be decided at this point whether this portion of enzyme is bound to some other particulate organelle(s) or whether it is in vacuoles that have been trapped within membranes and thereby carried down during centrifugation. A typical vacuole enzyme, proteinase B (8, 9) was also assayed in the various fractions. Its enrichment in the vacuole fraction was similar to that of chitinase but the amount found in the pellet was much lower (Table II). This result may be partially artifactual, due to the presence in yeast of a proteinaceous inhibitor (16) of proteinase B, some of which might have been trapped in the pellet.

The chitinase of the vacuole fraction could be easily extracted into the soluble phase by mild sonication in the absence of detergents; therefore, it is probably in solution within the vesicles rather than membrane bound.

The chitinase obtained from intact cells was found to be associated with mannan (3). To determine whether the enzyme is bound to carbohydrate before secretion, chitinase was purified from protoplasts and subjected to disc gel electrophoresis, followed by staining with either Coomassie blue or periodic acid-Schiff reagent. In both cases, the same slow moving band was stained. Furthermore, double diffusion with antiserum against $\alpha(1 \rightarrow 3)$mannosyl residues yielded a precipitin line coinciding with that of enzyme extracted from whole cells (Ref. 3, Fig. 8). It may be concluded that association between enzyme and carbohydrate occurs before crossing the plasma membrane.

Some secretory enzymes have been found to be liberated from protoplasts during incubation in growth medium (4). This is the case with chitinase as well (Fig. 1). The presence of chitinase in the medium was not due to protoplast lysis, because a typically intracellular enzyme, α-glucosidase, was not liberated by the procedure. It remains to be explained why the total chitinase activity, intracellular plus medium, declined during the first hour of incubation. Intracellular α-glucosidase behaved similarly (not shown). Cycloheximide, which blocks secretion of mannan proteins by protoplasts (17) caused only slight inhibition of chitinase liberation at 10μg/ml, and tunicamycin, which inhibits glycosylation (18), was without effect at the same concentration (not shown). These results suggest that the appearance of chitinase in the medium represents secretion of enzyme already present in the cell rather than synthesis of new enzyme.

In contrast to the results with protoplasts, the chitinase secreted by intact cells into the periplasmic space does not reach the medium. No enzyme could be found in spent growth medium, despite the fact that the amount of enzyme extractable from cells did decline at the end of growth (Fig. 2). This decrease does not seem to be due to a variation in extracta-
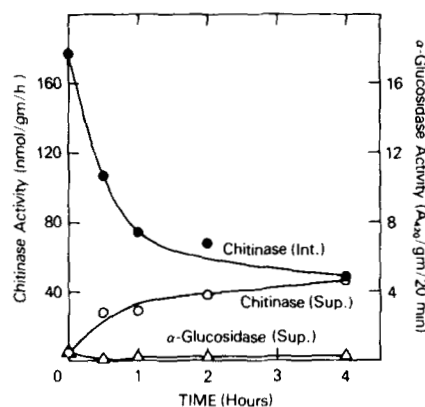
TABLE I

Chitinase distribution in intracellular and periplasmic space

Protoplasts were prepared with chitinase-free Zymolyase. Chitinase was measured in both the solubilized fraction and the protoplasts, as explained under "Materials and Methods."

Strain	Protoplast chitinase (intracellular)[a]	Zymolyase supernatant chitinase (periplasmic)
	%	%
X2180	42 (52.5)	58 (47.5)
X2180 (unbudded cells)[b]	32 (40)	68 (60)
prb 1122	49 (70)	51 (30)
S288C	25 (30)	75 (70)

[a] Sum of protoplast and supernatant chitinase taken as 100%. Values in parentheses were corrected for protoplast lysis by measuring alkaline phosphatase in both fractions (see "Materials and Methods").

[b] Isolated by gradient centrifugation (11).

FIG. 1. **Secretion of chitinase by protoplasts.** For incubation conditions, see "Materials and Methods." *Sup.* refers to enzyme found in the supernatant fluid, *Int.* to enzyme extracted from protoplasts.

TABLE II

Distribution of chitinase and proteinase B in subcellular fractions

Crude particulate material ("mixed particles") was fractionated with a discontinuous Ficoll gradient and analyzed as outlined under "Materials and Methods." Band A represents material found at the interface between 6 and 7.5% Ficoll, Band B between 7.5 and 10% Ficoll. Protein was measured according to Lowry *et al.* (15).

| Fraction	Protein	Chitinase			Proteinase B		
		Activity	Specific activity	Recovery	Activity	Specific activity	Recovery
	mg/g yeast	*milliunit/g yeast*	*milliunit/mg protein*	%	*units/g yeast*	*units/mg protein*	%
Mixed particles	0.97	0.98	1.01	100	61	63	100
Vacuole fraction (meniscus)	0.024	0.33	13.8	34	34	1416	56
Band A	0.005	0.03	6	3	0.8	160	1.3
Band B	0.03	0.03	1	3	2.8	93	4.6
Pellet	0.66	0.31	0.47	32	8.9	13	14.6
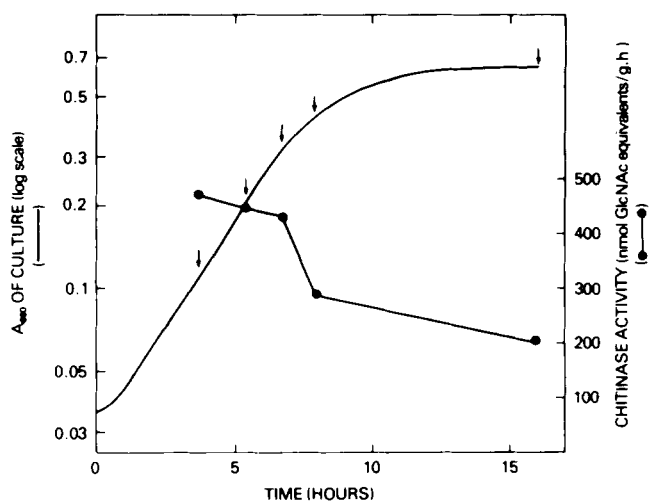
FIG. 2. **Chitinase activity as a function of yeast growth.** Samples of the culture were harvested at the times indicated by *arrows* and chitinase was extracted with digitonin as in the first step of enzyme purification.

bility of the chitinase. Although less protein was obtained with digitonin and β-mercaptoethanol from stationary phase than from logarithmic phase cells, the amount of chitinase in the extract was not increased by cell disruption with glass beads (not shown).

In conclusion, the results show clearly that yeast chitinase is a secretory protein. From its subcellular localization, it seems probable that the enzyme is sequestered in vesicles or vacuoles as a prelude to secretion. In this respect, it differs somewhat from another periplasmic enzyme, acid phosphatase, which only appears in substantial amounts in cytoplasmic compartments when its secretion is blocked (12). As for invertase, in addition to the major periplasmic glycoprotein, an internal form devoid of carbohydrate was isolated (19). Recent reports, however, indicate the presence of glycosylated enzyme in intracellular vesicles (20, 21). β-Glucanase has also been found both in the periplasmic space and in intracellular vesicles (22), but the enzyme has not been characterized and it is not known whether the activities measured in the two compartments correspond to the same protein.

It is not clear what retains the chitinase in the periplasmic space. Hydrophobic forces (binding to plasma membrane?) seem to be involved, as evidenced by the need for digitonin to extract the enzyme from intact cells. This result contrasts, however, with the liberation of periplasmic chitinase when the cell wall is digested by lytic enzymes in the absence of digitonin.

With regard to the physiological function of yeast chitinase,

its localization excludes the possibility of action on an extracellular substrate because of the insolubility of chitin. Its strategic position in the periplasmic space, however, would allow the enzyme to act on chitin secreted into that space by the cell during septum formation. A function of the enzyme in cell division is suggested by its higher level during logarithmic growth (Fig. 2), as compared to the stationary phase. Perhaps chitinase plays a role in the final fission of septa which leads to cell separation. It is not yet clear how the temporal and spatial regulation required for such a function would be achieved.

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