AN ENZYME WHICH DEGRADES THE WALLS OF LIVING YEAST*

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Yeast protoplasts first became available in quantities sufficient for metabolic studies when Eddy and Williamson (1957) demonstrated the effectiveness of snail gut juice in dissolving the wall of intact organisms. Nevertheless, the low potency (and occasional erratic behavior) of the snail preparations and the obvious advantages of purified enzymes have led many investigators to seek microbial sources for the necessary activities. Isolated cell walls and heated cells can readily be lysed by β-1,3-glucanase (Tanaka and Phaff, 1965) or by proteolytic enzymes (Eddy, 1958) but these enzymes (jointly) are ineffective against intact organisms. Thiols have been reported to increase the efficacy of the snail enzyme (a source of β-1,3-glucanases) and this combination will produce protoplasts from stationary-phase organisms which are not usually sensitive (Davies and Elvin, 1964; Anderson and Millbank, 1966).

We have attempted to determine the number of components required for protoplast formation and to purify them using a quantitative

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lytic assay. A new enzyme (termed for the present PR-factor) has been isolated from a strain of Bacillus circulans and partially purified. It will form protoplasts from log phase yeast in combination with a thiol or dilute snail enzyme.

Organisms and methods. Log phase organisms of Saccharomyces cerevisiae, strain LK2G12 were grown as described by Marini et al. (1961). For the quantitative lytic assay, cells were suspended at 2×10^8/ml. in the usual buffer for protoplast formation (0.02 M phosphate buffer, pH 6.0 in 0.6 M KCl) but with Mg^{++} omitted. The enzyme preparations were added and the mixture (2 ml. total volume) shaken at 40° for 1 hr. At this time, the mixtures were lysed by diluting with 5 ml. of water and shaking for 10 min. and the absorbance was measured in the Klett colorimeter using a 660 mμ filter. The decrease in absorbance after dilution (Δ_{660}) was taken as a measure of the removal of wall components necessary for osmotic stability.

Bacillus circulans strain 63-7 was obtained from Prof. K. Arima and grown aerobically for 4 days at 30° on a urea-phosphate-glucose-salts medium. The endo-β-1,3-glucanase described by Horikoshi, Koffler and Arima (1963) was purified from culture filtrates to a specific activity of 1450 units/mg. protein (soluble laminarin from K & K Lab. as substrate). Dr. H. Koffler and Dr. H. Tanaka provided valuable information in advance of publication on the production and properties of the glucanase. PR-factor was also obtained from these filtrates. The reducing sugar formed by various fractions from laminarin, pustulan or yeast glucan was estimated by a modification of the dinitrosalicylic acid procedure suggested by Dr. Tanaka.

Requirement for PR-factor. Although snail enzyme at a high level (10% v/v; 20-30 mg. protein/ml. test mixture) will form protoplasts in 1-2 hrs. from log phase cells of strain LK2G12, it is ineffective at 0.2% (Fig. 1) unless supplemented with a fraction from culture filtrates of

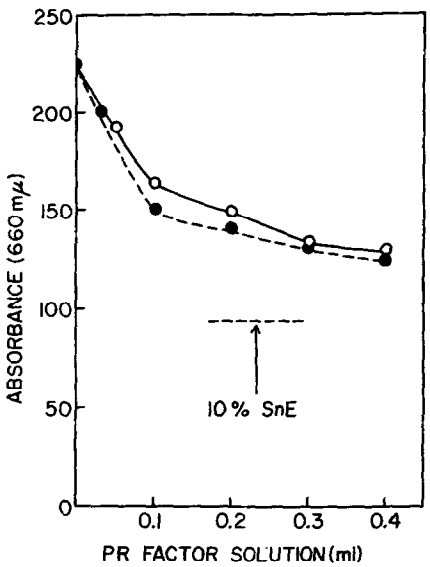

Fig. 1. Requirement of PR-factor for lysis. For general procedure see text. Each mixture contained PR-factor as indicated with (O—O) 0.2% snail enzyme (SnE) or (●---●) 0.02 M β-mercaptoethanol. Neither 0.2% SnE nor the thiol alone caused any decrease in absorbance. The degree of lysis obtained with 10% SnE alone is shown.

B. circulans strain 63-7 (or several streptomycetes). To purify the active material (PR-factor) the filtrates were concentrated to about one-tenth of their original volume, dialyzed and the preparation chromatographed on SE-Sephadex in pH 5.0 acetate buffer with a linear gradient of 0 - 0.5 M NaCl. The active fractions were further purified by gel filtration on Bio-Gel P-10 (Bio-Rad Lab.) or by rechromatography on SE-Sephadex. The amount of enzyme producing a Δ_{660} of -50 in the quantitative lytic assay is defined as one unit.

The material used in most of the experiments reported here had an activity of 2500 units/mg. protein. It was essentially free of α-mannanase, β-glucosidase, or protease activity. Test of reducing sugar formation with laminarin, yeast β-1, 3-glucan, or pustulan (β-1, 6 linkages) as substrates showed low activities, i. e., 260 units, 11 units and < 1 unit respectively/mg. protein.

Action of PR-factor on yeast. PR factor, like thiols, can act directly on the yeast cell (Table 1) altering the wall in such a way that the snail enzyme (containing β-1, 3-glucanase) can dissolve the remaining wall structure and produce protoplasts. If the yeast is treated first with the snail enzyme and then with PR-factor, the organisms do not become osmotically sensitive. A combination of PR-factor and thiol is also effective (Fig. 1); either of these can be used first.

Table 1

Sequence of action of PR-factor and snail enzyme

Incubation Period		Δ_{660}	Released
0-1 hr.	1-2 hr.		
-		0 (230)*	
Snail enzyme (0.2%)		+8	-
PR-factor (2 units) and snail enzyme		-73	glucan, mannan and invertase
Snail enzyme**	PR-factor	-10	mannan and invertase
PR-factor**	Snail enzyme	-103	
β-mercaptoethanol (0.02 M)		-2	[mannan and invertase]

General test conditions for quantitative lytic assay are described in the text.

*Value in ( ) is A_{660} of control suspension.

**After 1 hr. the reaction mixture was centrifuged and the organisms washed and resuspended in fresh buffer containing enzymes as indicated.

The two stage procedure (as in Table 1) made it possible to determine the optimum pH for the action of PR-factor. The enzyme showed good activity over the pH range of 5 to 8. PR-factor released invertase rapidly from the log phase yeast cell (Fig. 2) along with most of

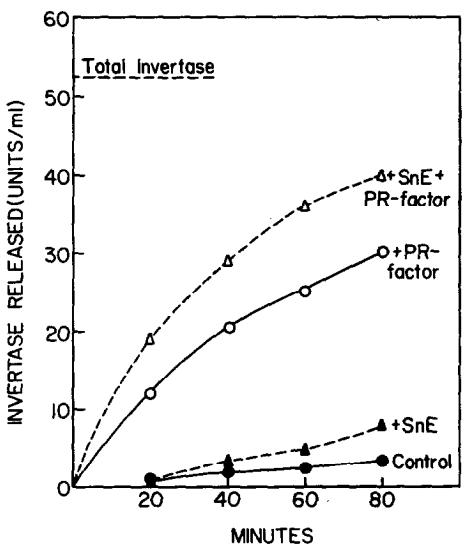

Fig. 2. Release of invertase by PR-factor. Incubation conditions as in the lytic assay. At indicated times samples were centrifuged and the invertase activity present in the supernatants determined. Snail enzyme (SnE) was added at 0.2% and PR-factor at 2 units/ml.

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the mannan (Table 1) but only traces of glucan or free hexose. Snail enzyme (0.2%) had almost no effect. The amount of polysaccharide released by β-mercaptoethanol was only 15% and the invertase was 50% of the quantity liberated by PR-factor under comparable conditions. Thus PR-factor

seems to have a relatively specific ability to remove mannan and the mannan-protein invertase (Neumann and Lampen, 1966) from the wall of the yeast cell.

Components required for protoplast formation. There appear to be several different mechanisms by which the cell wall of log phase yeast can be removed. Three types of agents can be identified: 1) PR-factor releases mannan (and invertase) but negligible amounts of glucan, 2) thiols remove mannan much less effectively but make the wall sensitive to glucanase; and 3) endo-β-1,3-glucanase (as snail enzyme) does not act on intact organisms but will degrade the glucan mesh once PR-factor or a thiol has altered the cell surface.

A combination of any two of these three agents will produce reasonably good (though not complete) conversion of log phase cells to protoplasts. In the presence of thiol, c. 200 units of the purified glucanase (free of PR-factor) were required to form protoplasts, but the amount of PR-factor preparation needed under these conditions (2-4 units) contained only 0.2-0.4 units of β-1,3-glucanase activity. This comparison is still valid if it is based upon glucanase activities determined with yeast glucan as the substrate (3.5 units of purified enzyme required vs. 0.01-0.02 units in the PR-factor preparation). It is possible, therefore, that removal of the yeast wall can occur without rupture of more than a few linkages in the glucan mesh which appears to impart structural strength to the wall.

These findings support the concept (Bacon et al., 1965) that several different lattices provide strength to the yeast wall, but they also reduce the emphasis on disruption of the β-1,3-glucan mesh for conversion of the cells to protoplasts.

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