β-GLUCANASE OF YEAST

Thomas D. Brock[1]

Department of Bacteriology
Indiana University
Bloomington, Ind. 47405

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The basal cell wall structure of yeasts which is responsible for the shape and rigidity of the yeast cell is a β-glucan, containing both β 1,3 and β 1,6 linkages (Phaff, 1963). During conjugation, two yeast cells of opposite mating type fuse by dissolution of cell wall material between them, leading to a typical dumbbell-shaped zygote (Conti and Brock, in preparation). Because this morphogenetic process involves new protein synthesis (Brock, 1961), it seems reasonable that enzymes which attack the basal cell wall structure are involved in the conjugation process, and attention has thus been focused on the β-glucanases of yeast. Although extensive work has been done on β-glucanases of filamentous fungi (Reese and Mandels, 1963), mainly in relation to deterioration phenomena, no work has apparently been done on the yeast enzymes. The present paper will show that yeasts contain intracellular β-glucanase activity of a peculiar nature. In addition, this paper will show that yeast β-glucanase can be mistaken for β-glucosidase, since it hydrolyzes the chromogenic substrate p-nitrophenyl-β-D-glucoside (PNPG), although it does not hydrolyze other simple aryl and alkyl glucosides.

Methods

In most of the work commercial baker's yeast (Fleischman's) was

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used, although certain experiments employed Hansenula wingei strains 5 and 21. The yeast cells were washed twice in water, and disrupted either by: 1, acetone drying followed by extraction of the dried pellet in 0.01 M potassium phosphate pH 5.5 for 1 hour at 30°C.; 2, by sonication in a 10 kc. Raytheon for 1 hour, or 3, by breakage with glass beads in a Nossal disintegrator for 5 minutes. Although extracts prepared in different ways varied in protein content, the specific activity of the β-glucanase remained the same. Extracts were clarified by centrifugation at 39,000 X gravity for 15-30 minutes. In one experiment the extract was centrifuged at 100,000 X gravity for 2 hr. with no diminution in enzyme activity of the supernatant.

The primary assay for β-glucanase activity involved a measurement of the liberation of reducing sugar (glucose) from the glucan substrates laminarin (β-1,3-glucan, water-soluble form, commercially available from K. and K. Laboratories) and pustulan (β-1,6-glucan, a water-soluble purified sample, derived from the lichen Umbilicaria pustulata, a gift of Dr. E.T. Reese). Enzyme and buffer (pH 5.5, 0.01 M potassium phosphate) were incubated with substrate at 1 mg/ml in 1 ml total volume at 30°C. for specified times, the reaction stopped by deproteinizing with zinc hydroxide-barium sulfate, and reducing sugar analyses run by the Nelson-Somogyi procedure (Hawk, Oser and Summerson, 1954) using glucose as a standard. Alternatively, glucose determinations were carried out on the deproteinized sample using the Glucostat reagent (Worthington Biochemicals, Inc.). The unhydrolyzed laminarin was almost devoid of reducing power, but the pustulan had a fairly high reducing power, so that an unincubated blank was run with each experiment and this value deducted from the incubated samples. Protein assays were performed by the Lowry procedure, using crystalline lysozyme as a standard. Specific enzyme activity is expressed as mg glucose released per mg protein per hr. Under the conditions used, the

rate of release of glucose was linear with time throughout the incubation period. When other substrates were used, the assay was performed identically.

With PNPG, the assay was performed either by the release of reducing power, or by the release of p-nitrophenol. In the latter case, deproteinization was not performed, and 0.5 ml of the incubation mixture was added to 4.5 ml of 4% Na_2CO_3, and the optical density read at 420 mμ, and p-nitrophenol was used as a standard.

The enzyme was purified by conventional methods using streptomycin sulfate precipitation of nucleic acids, and ammonium sulfate precipitation of the enzyme using the fraction which precipitated between 50-75% of saturation. The precipitate obtained was suspended in 0.01 M 5.5 potassium phosphate, dialyzed overnight against this same buffer, and chromatographed on a DEAE-cellulose column using a linear gradient from 0-1 M NaCl. The enzyme was located in the column fractions by spot tests using laminarin, pustulan and p-nitrophenyl-β-D-glucoside as substrates. With the first two substrates, color was developed on the spot plates using the Glucostat reagent, whereas with the latter, 4% Na_2CO_3 was added. With one preparation, starch block electrophoresis was run, using 0.05M pH 5.5 potassium phosphate buffer, with 9 volts/cm. After 16.5 hours, the starch was cut in 1 cm segments which were eluted with buffer and assayed.

Paper chromatography for an examination of hydrolysis products was performed on deproteinized samples using Whatman # 3 filter paper with isopropanol: glacial acetic acid: water (54:8:18) in a descending system. The sugars were detected with p-anisidine-phthallic acid spray reagent.

Results

Both baker's yeast and H. wingei possess a non-particulate intracellular enzyme which hydrolyzes laminarin. Since these yeasts will

not grow on laminarin as a sole source of carbon and energy, it follows that the enzyme must be intracellular *in vivo*. Assays with whole cells reveal that these unbroken yeasts contain negligible laminarinase activity, and no activity can be detected in supernatants of young cultures. If PNPG is used as substrate, the situation is more complex. *H. wingei* strains possess both a laminarinase and a β-glucosidase, and both enzymes are able to act on PNPG. These two enzymes can be separated on DEAE chromatography, and it can be clearly shown that the β-glucosidase does not act on laminarin. Thus assays using laminarin as substrate reveal only the β-glucanase activity, whereas assays using PNPG reveal the summation of two activities. This finding suggests that PNPG should be used with caution when studying the genetics or control of β-glucosidase synthesis in yeast. The role of the *H. wingei* laminarinase in conjugation is under study (Brock, 1964).

The rest of the work in this paper will deal with the enzyme from baker's yeast, which has unusual specificities. The strain of baker's yeast used possesses no detectable β-glucosidase, so that all of the activity against PNPG in whole cells, extracts, or purified preparations is due to β-glucanase. From a DEAE column, one peak is obtained which contains hydrolyzing activity against laminarin, pustulan and PNPG, and during the 100-fold purification from the crude extract, these three activities remain together, and the ratio of their activities is unchanged. The three activities remain together during starch block electrophoresis, and the ratio of their activities is also unchanged after electrophoresis. All three activities are equally inhibited by 1 mg/ml delta-gluconolactone, a potent inhibitor of many glycosidases (Levvy et al., 1964).

From either laminarin or pustulan, only glucose is released, as shown by paper chromatography, and this is true even if only very short incubation times are used. If gluconolactone is present during the

reaction, no glucose is released, but no intermediate products are detected on the paper. Since the purified enzyme is devoid of conventional β-glucosidase activity, these results show that the enzyme itself releases only glucose from either substrate.

Using the purified enzyme from the DEAE-cellulose column, a variety of substrates have been tested, and the results are shown in Table 1. It can be seen that against β-1,3 linkages, the enzyme acts like a conventional exohydrolase, relasing only glucose units, and acting better

Table 1

Activities of 100-fold purified baker's yeast β-glucanase against various substrates

Substrate	Linkage	Specific activity, mg glucose/mg protein/hr. Reducing power release	Glucose release
Laminarin	β -1,3	17	17
Pustulan	β -1,6	24	23
p-nitrophenyl-β-D-glucoside	-	-	21
Laminaritriose	β -1,3	-	51
Laminaribiose	β -1,3	-	56
Gentiobiose	β -1,6	-	7.3
Gentiotriose	β -1,6	-	11
Lichenin	Mixed β -1,4; 1,3	0	-
Cellobiose	β -1,4	-	0
Cellotriose	β -1,4	-	1.9
β-phenyl glucoside	-	0	-
β-methyl glucoside	-	0	-
β-methyl xyloside	-	0	-
salicin (salicyl alcohol-β-D-glucoside)	-	0	-

Laminaribiose, laminaritriose, gentiotriose and cellotriose were gifts of E.T. Reese. All other substrates were commercial products. All substrates at 1 mg/ml concentration.

on short chains (e.g. laminaribiose and laminaritriose) than on long chains. These results are confirmed by the inactivity of the enzyme against lichenin, a mixed β-1,4; β-1,3 polymer, which is attacked readily by endo-β-1,3-hydrolases, but not by exo-hydrolases (Reese and Mandels, 1963). However, the action of the enzyme against β-1,6 linkages is anomalous, since the enzyme is almost devoid of activity against the short chains (e.g. gentiobiose and gentiotriose), which it would be expected to hydrolyze if it were a conventional exo-hydrolase, as the chromatographic studies with pustulan had suggested.

These results can best be interpreted as: 1) a single enzyme possesses both β-1,3 and β-1,6-glucanase activity; 2) the β-1,3 activity is a typical exohydrolase, working best on small chains; 3) the β-1,6 activity is atypical, acting best on long chains, but still releasing only glucose. The enzyme is devoid of conventional β-glucosidase activity, since it is inactive against various aryl- and alkyl-glucosides as well as cellobiose, although it is quite active against PNPG. The basis for this anomalous enzyme behavior will have to await further work. As noted above, whole cells of baker's yeast lack laminarinase activity. They do possess strong activity against laminaritriose and PNPG, which activity is increased if the cells are treated with toluene. Toluene-treated cells are still low in laminarinase activity, suggesting that the laminarin substrate cannot pass through the yeast cell wall.

Many commercially available enzymes used as biochemical reagents are derived from baker's yeast, and they may be contaminated with β-glucanase. I have found significant laminarinase activity in purified yeast hexokinase, for instance.

The β-glucosidases of Saccharomyces dobzhanskii and of the hybrid S. dobzhanskii X S. fragilis, studied by Halvorson's group (Gorman et al., 1964) are not β-glucanases, since highly purified preparations of these enzymes (kindly supplied by Dr. J. Duerksen) have no significant activity on either laminarin or pustulan.

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