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A COMPARISON OF β-GLUCANASE AND β-GLUCOSIDASE IN *SACCHAROMYCES LACTIS*

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SUMMARY

Intracellular β-glucanase (β-1,3(4)-glucan glucanohydrolase, EC 3.2.1.6) has been characterized in *Saccharomyces lactis*. A partially purified preparation hydrolyzes laminarin and *p*-nitrophenyl-β-D-glucopyranoside, the classic substrate for measuring β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) activity. β-Glucanase is responsible for only 3.8% of the total *p*-nitrophenyl-β-D-glucopyranoside hydrolysis in crude extracts and its activity is not influenced by the regulatory systems controlling β-glucosidase synthesis in this yeast.

INTRODUCTION

β-Glucanases (β-1,3(4)-glucan glucanohydrolase, EC 3.2.1.6) have recently been described in several genera of yeast[1-3]. The amount of β-glucanase activity is higher in species of yeast whose asci lyse rapidly at maturity. The enzyme has broad substrate specificity hydrolyzing not only β-(1→3)- and β-(1→6)-linkages but also *p*-nitrophenyl-β-D-glucopyranoside, the chromogenic substrate classically employed to determine β-glucosidase activity (β-D-glucoside glucohydrolase, EC 3.2.1.21).

The present work was undertaken to determine the presence and concentration of β-glucanase in another yeast, *Saccharomyces lactis* (*Kluyveromyces lactis*)[4] in which spores are also rapidly released from asci. *S. lactis* is the only yeast in which β-glucosidase synthesis has been extensively examined from biochemical, genetic and regulatory aspects[5,6].

METHODS

Organism

The haploid, heterothallic stocks Y-14 and Y-123 of *Saccharomyces lactis*, previously employed for genetic studies[6] were used. Y-14 has the mating type (a), a medium semiconstitutive level of β-glucosidase (B^m) and is inducible by glucose. Y-123 has the complementary mating type (α), a high semiconstitutive level of β-glucosidase (B^h) and is inducible by methyl-β-D-glucoside.

Cultivation

The cells were grown aerobically on a rotary shaker at 30° in either synthetic succinate medium[7] or in yeast-malt extract broth[8]. Cells were harvested during logarithmic phase and washed with 0.05 M potassium phosphate buffer (pH 5.6). Cell extracts were usually prepared immediately but in some cases the cell paste was stored at −20° without appreciable loss in activity.

Cell extracts

A yeast paste (approximately 1 g wet wt.) was suspended in phosphate buffer and disrupted in a French pressure cell at 22 000 lb/inch^2 at 4°. The broken cell suspension was centrifuged at 10 000 × *g* for 30 min to remove cell debris. Ribosomes were then eliminated by centrifugation at 144 000 × *g* for 1.5 h in a Spinco Model L centrifuge. The supernatant fraction was dialyzed against 500 vol. of phosphate buffer.

Analytical methods

Hydrolysis of the chromogenic substrate *p*-nitrophenyl-β-D-glucopyranoside was determined by measuring the absorbance of liberated *p*-nitrophenol at 400 nm as previously described[9]. For discontinuous assays, enzyme, 0.05 M phosphate buffer and 10^{-3} M *p*-nitrophenyl-β-D-glucopyranoside were incubated in a 1-ml volume at 30°. The reaction was terminated by the addition of 2 ml of a 1 M solution of Na_2CO_3 (pH 10.2). The extinction coefficient for *p*-nitrophenol in 0.67 M Na_2CO_3 is $1.83 \cdot 10^4$ when measured in a 1-cm cuvette. Enzyme units are expressed as nmoles of *p*-nitrophenol formed per min.

Hydrolysis of β-glucans and other natural β-glucosides were determined by measuring the liberated glucose. Enzyme and 0.05 M potassium phosphate buffer (pH 5.6) were incubated in a 0.9-ml volume at 30°. The reaction was started by the addition of 0.1 ml substrate (final concentration 1 mg/ml). At specified times the reaction was stopped by the addition of barium and zinc salts. After centrifugation, the deproteinized supernatant fluids were assayed for liberated glucose with the Glucostat special reagent. The absorbance of the reduced chromogen was read at 400 nm and compared with that of a known concentration of glucose. Incubated substrate controls were employed for each assay. All reactions were carried out in duplicate and at least two different protein concentrations. Enzyme units are expressed as nmoles glucose released per h.

Protein concentration was determined by the method of LOWRY *et al.*[10] using bovine crystalline serum albumin as a standard.

MATERIALS

Glucose oxidase (Glucostat) was obtained from Worthington Biochemical Corporation. Pustulan was generously supplied by Dr. T. D. Bock. Laminarin was purchased from K and K Laboratories, Plainview, N.Y. Laminaribose, laminaritriose, gentiotriose and cellotriose were donated by M. Mandels, U.S. Army Natick Laboratory, Natick, Mass., *p*-nitrophenyl-β-D-glucopyranoside and other carbohydrates were obtained from Calbiochem.

TABLE I

SPECIFIC ACTIVITY OF β-GLUCANASE IN CRUDE EXTRACTS

Culture medium	*Specific activity (nmoles glucose released per h per mg protein)*	
	Strain Y-123	*Strain Y-14*
Synthetic succinate	510	760
Synthetic succinate + 1% glucose	340	260

RESULTS

β-Glucanase activity in strains Y-14 and Y-123

Saccharomyces strains, containing either high (Y-123) or medium (Y-14) levels of β-glucosidase, produce high levels of β-glucanase as measured by hydrolytic activity against the β-(1→3)-glucan, laminarin (Table I). In both strains growth on glucose lowers the level of β-glucanase in exponentially growing cultures. It is unlikely that *S. lactis* contains an extracellular β-glucanase since neither Y-14 nor Y-123 can utilize laminarin for growth as the sole carbon source. Both extracellular and intracellular β-glucanase activity have been reported in several species of yeast[2].

Fractionation of β-glucanase and β-glucosidase

In crude extracts, what percentage of *p*-nitrophenyl-β-D-glucopyranoside

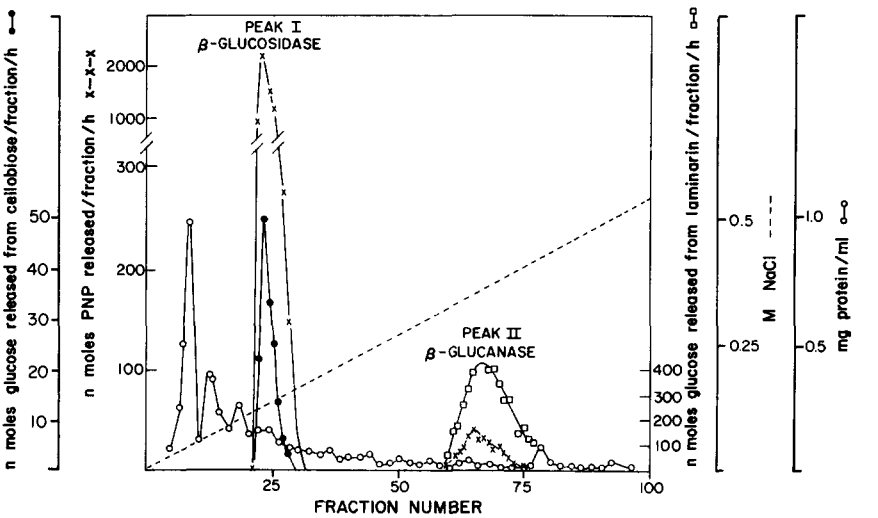

Fig. 1. Elution patterns of β-glucosidase and β-glucanase following chromatography on DEAE-cellulose. A column (20 cm × 1.0 cm) was equilibrated with 0.05 M phosphate buffer (pH 5.7). To this was applied 1.5 ml of a crude extract of Y-14 (12 560 units of *p*-nitrophenyl-β-D-glucopyranoside activity). Enzymes were eluted with a linear NaCl gradinet 0–1.0 M. Fractions of 2.4 ml were collected and assayed for activity against *p*-nitrophenyl-β-D-glucopyranoside, cellobiose and laminarin as described in METHODS. PNP = *p*-nitrophenol.

TABLE II

INDIVIDUAL ACTIVITY OF β-GLUCANASE AND β-GLUCOSIDASE AGAINST *p*-NITROPHENYL-β-D-GLUCOPYRANOSIDE

Activity is expressed as nmoles *p*-nitrophenol released per h per fraction.

Strain	*β-Glucosidase*	*β-Glucanase*	*Total activity**	*% of total activity due to β-glucanase*
Y-14	7 648	305	7 953	3.80
Y-123	41 051	272	41 323	0.60

* % Recoveries of activities against *p*-nitrophenyl-β-D-glucopyranoside and laminarin were identical with extracts from both strains.

hydrolysis is due to β-glucanase activity? To answer this question, extracts from both Y-14 and Y-123 were subjected to chromatography on DEAE-cellulose columns. The protein and enzyme profiles obtained following chromatography of an extract from strain Y-14 are shown in Fig. 1. When fractions were analyzed for activity against *p*-nitrophenyl-β-D-glucopyranoside, two peaks were observed: a major peak (I) eluting at 0.15 M NaCl coincident with the cellobiose hydrolytic activity (β-glucosidase) and a minor peak (II) which eluted from the column at 0.4 M NaCl coincident with the laminarin hydrolytic activity (β-glucanase). Peak I but not Peak II hydrolyzed β-methyl glucoside (not shown). From these experiments and others[18], Peak I has identical properties to the well characterized β-glucosidase in yeast[11–13]. Peak II elutes from DEAE-cellulose at a similar NaCl molarity as the exo-β-glucanase (laminarin hydrolysis) of yeast *Fabospora fragilis*[2] and has been tentatively classified as β-glucanase. Identical elution patterns were obtained when extracts from strain Y-123 were subjected to chromatography.

A summary of the total enzyme units recovered from Peaks I and II with *p*-nitrophenyl-β-D-glucopyranoside activity following chromatography of extracts from strains Y-14 and Y-123 is given in Table II. Hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside, resulting specifically from the action of β-glucanase, accounted for only 3.8% of the total enzyme units recovered in eluants from strain Y-14 and 0.6% of the total enzyme units recovered following fractionation of extracts from strain Y-123. The values represent maximal percentages because the assays were carried out at pH 5.6. β-Glucosidase activity is significantly reduced at this pH; while β-glucanase activity is similarly reduced at pH 6.8, the pH at which β-glucosidase activity is normally measured.

Partial purification of β-glucanase

A crude extract from strain Y-123 was prepared as described in MATERIALS AND METHODS. 10 ml of a preparation (containing 62 600 units of *p*-nitrophenyl-β-D-glucopyranoside activity; 9650 units of β-glucanase activity; and 9.4 mg protein per ml) were layered on a preparative DEAE-cellulose column (4.5 cm × 3.5 cm) which had been previously equilibrated with 0.05 M potassium phosphate buffer (pH 5.6). β-Glucosidase activity was fractionated by passing 50 ml of buffer containing increasing concentrations of NaCl through the column. Complete elution of β-glucosidase activity (see Fig. 1) was insured by washing the column successively with 3 vol.

TABLE III

RATE OF HYDROLYSIS OF DIFFERENT SUBSTRATES BY PARTIALLY PURIFIED β-GLUCANASE

Substrate	*Linkage*	*Rate of hydrolysis (nmoles glucose released/ml enzyme per h*
Laminarin	β-1,3	495
Laminaribose	β-1,3	1150
Laminaritriose	β-1,3	2430
Pustulan	β-1,6	53
Gentiobiose	β-1,6	0
Gentiotriose	β-1,6	80
Amygdalin	β-1,6	167
Cellobiose	β-1,4	0
Cellotriose	β-1,4	0
β-Methyl glucoside	—	0
Aesculin	—	0
Salicin	—	0
Arbutin	—	0
p-Nitrophenyl-β-D-glucoside	—	22
β-Methyl xyloside	—	0

of buffer containing 0.3 M NaCl. β-Glucanase activity was then eluted with two times 50 ml buffer containing 0.5 M NaCl. The eluted enzyme was then concentrated by precipitation with saturated $(NH_4)_2SO_4$, freed from salt by dialysis, and refractionated on a second DEAE-cellulose column. Fractions with high β-glucanase activity were pooled, concentrated with $(NH_4)_2SO_4$, dialyzed, and stored at −20°.

Specificity of β-glucanase

The rate of hydrolysis of glucans and glycosides by the partially purified β-glucanase was determined by following the release of glucose (Table III). β-(1→3)-Glucans were hydrolyzed at the fastest rate; highest activity was observed against short chain di- and trisaccharides. β-(1→6)-Linkages were attacked at a reduced rate. Cellobiose, cellotriose and natural alkyl or aryl β-glucosides were not hydrolyzed. Significant hydrolytic activity against the synthetic chromogenic substrate, *p*-nitrophenyl-β-D-glucopyranoside, was observed. The preparation is devoid of detectable β-glucosidase activity as evidenced by the absence of hydrolytic activity against cellobiose and β-methyl glucoside. The specificity reported in Table III is very similar to that reported for β-glucanases in baker's yeast[1] *Saccharomyces cerevisiae*, *Fabospora fragilis*, and *Hansenula anomala*[2], lending further support to the identification of this fraction as β-glucanase.

Specificity of induction period of β-glucosidase

As previously shown several independent regulatory systems govern the induction of β-glucosidase in *Saccharomyces lactis*[6]. In strain Y-123, β-glucosidase activity is specifically induced by methyl-β-D-glucoside, whereas in strain Y-14, glucose induces not only β-glucosidase but several other carbohydrases. Since β-glucanase also hydrolyzes *p*-nitrophenyl-β-D-glucopyranoside (Fig. 1) it was of interest to determine if β-glucanase activity was also elevated by either inducer. To test this possibility, Y-14 and Y-123 were induced with glucose and methyl-β-D-glucoside in synthetic

TABLE IV

EFFECT OF GLUCOSE AND β-METHYL GLUCOSIDE ON ENZYME SYNTHESIS

See METHODS AND MATERIALS for details. β-Glucanase was specifically measured by following the hydrolysis of laminarin. β-Glucosidase activity was determined by hydrolysis of cellobiose (Y-14) or β-methyl glucoside (Y-123).

Strain	*Inducer*	*Specific activity (nmoles glucose released per h per mg protein)*	
		β-Glucanase	*β-Glucosidase*
Y-14	None	249	1
	$1 \cdot 10^{-3}$ M glucose	213	64
Y-123	None	274	202
	$2 \cdot 10^{-3}$ M methyl-β-D-glucoside	234	378

succinate medium. Extracts were prepared and assayed for β-glucosidase and β-glucanase. As shown in Table IV in strain Y-14 β-glucosidase activity was increased over 60-fold in extracts by glucose whereas β-glucanase activity was not increased under these conditions. Similarly, in an extract prepared from a methyl-β-D-glucoside induced culture of Y-123, β-glucosidase activity was increased 88% while β-glucanase activity was less than in uninduced cells. Therefore, the presence of either glucose or methyl-β-D-glucoside inducers of β-glucosidase, did not increase β-glucanase activity in these strains.

DISCUSSION

The results reported here in *Saccharomyces lactis* support the conclusion in other organisms[1,14,15] that β-glucanase and β-glucosidase are separate enzymes. They can be separated by chromatography on DEAE-cellulose. When β-glucanase (Peak II, Fig. 1) was purified by chromatography from β-glucosidase, it was devoid of activity against cellobiose, β-methyl glucosidase, aesculin, salicin and arbutin, typical substrates of β-glucosidase in *S. lactis*[6,13] and other yeasts[11,12]. On the other hand β-glucanase was active against laminarin, laminaribose, laminaritriose and amydalin which are not substrates of β-glucosidase. β-Glucosidase, but not β-glucanase is induced by glucose and methyl-β-D-glucoside. Both enzymes hydrolyze *p*-nitrophenyl-β-D-glucopyranoside. At the optimum pH of 5.7 β-glucanase activity in crude extracts can account for less than 3.8% of the total *p*-nitrophenyl-β-D-glucopyranoside hydrolysis. Thus although both enzymes have an overlapping specificity for *p*-nitrophenyl-β-D-glucopyranoside, the chromogenic substrate may be used in *S. lactis* to measure β-glucosidase synthesis (pH 6.8) without significant interference by β-glucosidase activity.

Several lines of evidence imply that β-glucanases may play a key role in several morphogenetic events in the life cycle of yeast. For example, β-glucanase may be active in the lysis of ascus walls. *Fabospora fragilis*, a yeast in which spores are liberated from asci at maturity, has a 7-fold higher level of β-glucanase than *Saccharomyces cerevisiae*, a yeast where the ascus wall remains intact until the spores germinate[2]. ABD-EL-AL AND PHAFF[2] purified the β-glucanases from both of these yeasts as well as

Hansenula anomala. The β-glucanases are exo-splitting enzymes which sequentially cleave glucosyl units from non-reducing ends of glucans. In each yeast, a single protein is believed to be responsible for hydrolysis not only of β-(1→3)- but also β-(1→6)-linkages. Thus the yeast exo-β-glucanases differ in specificity from those described in other fungi. Exo-β-glucanases from *Sporotricium pruinosum* and a *Basidomycete* sp. specifically act on β-(1→3)-linkages with little or no action on β-(1→4)- or β-(1→6)-bonds[15].

β-Glucanases may also be involved in the budding process. MATILE *et al.*[3] have found glucanase containing particles in *Saccharomyces cerevisiae*. The vesicles containing β-glucanase are secreted into the wall at the sites of new buds. In this regard it is interesting to note that in the fission yeast *Schizosaccharomyces pombe* BARRAS[16] has found evidence for a β-(1→3)-glucanase which has no activity on β-(1→6)-linkages. In contrast to other yeast enzymes the enzyme shows only endo hydrolytic action. This may reflect a difference in the growth process or in the cell wall or both.

BROCK was the first to study β-glucanase activity in *Hansenula wingei*[17] and was the first to suggest that the enzyme may be involved in the dissociation of the cell wall. In this yeast β-glucanase activity increases during cell fusion which precedes the conjugation process. A 100-fold purified preparation of β-glucanase hydrolyzes β-(1→3)- and β-(1→6)-linkages as well as *p*-nitrophenyl-β-D-glucopyranoside. If β-glucanase plays such a crucial role in budding, conjugation or cell wall growth, one can easily understand the inability thus far to obtain mutants deficient in this enzyme.

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