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Biochemical and Genetic Characterization of β-Glucosidase Mutants in *Saccharomyces lactis*

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Mutants with reduced activity for β-glucosidase (β-D-glucoside glucohydrolase EC 3.2.1.21) were isolated from the haploid yeast *Saccharomyces lactis*. Tetrad analysis indicated that in each mutant a single genetic factor, closely linked or allelic to the structural gene for β-glucosidase (B locus), is responsible for the decreased activity. β-Glucosidases produced by wild-type and mutant strains are similar in molecular size and charge but differ in catalytic properties, thermal stability, and serological specificity, indicating that mutants are in the structural gene. All mutants retained their capacity to be induced by either methyl-β-D-glucoside or glucose. In all cases, the mutant phenotype was dominant in heterozygous diploids.

β-Glucosidase synthesis in *Saccharomyces lactis* (*Kluyveromyces lactis*) (15) represents an interesting example of genetic control of enzyme synthesis. The structural gene, the B locus, can exist in at least three allelic forms B^h, B^m, and B^l resulting in high, medium, and low basal levels of β-glucosidase (5). The semiconstitutive level can be altered by addition of methyl-β-D-glucoside (BMG) or glucose. Induction by BMG is controlled by a single unlinked gene, whereas glucose induction is controlled by several unlinked regulatory genes (6). Thus, a single structural gene for β-glucosidase is under the control of two independent regulatory systems.

We have undertaken a search for mutants altering β-glucosidase activity in *S. lactis*. If basal synthesis is controlled intragenically or if the products of the regulatory genes act at the transcriptional level, one might expect to find specific mutations which control the rate of gene expression or response to external inducers.

Since cellobiose has not provided a suitable selective carbon source for β-glucosidase mutants, we have employed a colony plate assay for p-nitro-phenyl-β-D-glucoside (PNPG) hydrolysis (5) to screen for mutants with reduced β-glucosidase activity.

MATERIALS AND METHODS

Organism and cultivation. The parental stocks of *S. lactis* employed, their source, and genotypes are shown in Table 1. Cells were cultured in either a synthetic succinate medium (SSM) (8) or in a yeast-malt extract (YMA) broth (16). The conditions for mating, isolation of spores, and other procedures in tetrad analysis were as previously described (5, 16). Stable diploids were isolated as reported elsewhere (14).

Isolation of mutants. B^h *S. lactis* strains (Y123 and 1009D) were used for isolation of mutants. A 20-ml sample of washed stationary-phase yeast cells (5 × 10 cells/ml) was exposed to ultraviolet irradiation with stirring in a petri dish to 0.01 to 0.1% survival. The irradiated suspension was diluted, plated on YMA agar, and incubated at 30 C. Colonies appearing after 2 to 3 days were replica plated to SSM plates, incubated for 3 days, and screened for β-glucosidase activity by using a plate assay for PNPG hydrolysis (5).

Enzyme assays. Continuous and discontinuous assays of β-glucosidase activity were based on the method of Duerksen and Halvorson (1). Enzyme units are expressed as nanomoles of p-nitrophenol (PNP) formed per minute.

Protein concentration was determined as described by Lowry et al. (7) with bovine serum albumin as a standard.

Enzyme preparations. Crude enzyme solutions were prepared from logarithmically growing yeast cultures in SSM. The washed cells were suspended in 3.0 ml of 0.067 M phosphate buffer, pH 6.8, containing 10^{-3} M mercaptoethanol, and the cell suspension was passed through a French pressure cell at 22,000 psi. Cell debris and ribosomes were removed by centrifugation, and the supernatant fluid was then dialyzed against 500 volumes of 0.067 M phosphate buffer (crude enzyme preparation). All manipulations were performed at 5 C.

Induction of β-glucosidase. The differential rate of enzyme synthesis was determined by a multiple

TABLE 1. *Genotypes of wild-type strains of Saccharomyces lactis employed*

Strain	Source	Mating Type	β-Glucosidase level[a]	Inducibility	
				BMG	Glucose
Y123	NRRL Y1118	α	B^h	+	−
Y14	NRRL Y1140	a	B^m	−	+
1009D	Segregant of Y123 × Y14 cross	α	B^h	+	+

[a] Basal enzyme level was characterized as medium or high as described elsewhere (5).

point assay method previously described (2, 6).

Heat treatment. A 1-ml amount of a crude enzyme preparation containing 600 units of β-glucosidase activity per ml was rapidly heated to 50 C. At 200 second intervals, a 0.1-ml sample was withdrawn and immediately pipetted into 4.9 ml of ice buffer. Residual activity was measured with a continuous assay.

Zone electrophoresis in starch gel. Enzyme samples were electrophoresed by the method of Smithies (13) for 12 hr at 4 C in 3×10^{-2} M tris(hydroxymethyl)aminomethane-borate buffer, pH 7.6. β-Glucosidase activity was revealed by the appearance of a yellow band of released PNP after treatment with PNPG.

Antiserum. A 40-mg amount of immunizing antigen, an enzyme preparation from strain Y123 (72 units/mg), was injected into three female albino rabbits over a 25-day period. The sera were collected, pooled, sterilized by filtration, and stored at −20 C. Nonimmunized rabbits served as controls. Endogenous activity in the sera against PNPG was eliminated by heating at 56 C for 30 min followed by dialysis against buffered saline.

Neutralization test. Enzyme neutralization was determined by antigen titrations. The antibody-antigen mixture was incubated for 48 hr at 4 C, and the sediment was removed by centrifugation. The supernatant fluids were assayed for residual β-glucosidase activity. Control rabbit serum produced no detectable inhibition of β-glucosidase.

Sucrose density gradient centrifugation. Five to 20% (w/v) linear sucrose gradients in 0.067 M potassium buffer, at pH 6.8, were prepared in 18-ml centrifuge tubes by the procedure described by Martin and Ames (12). The sample (0.1 to 0.5 ml) was carefully layered on top and centrifuged at 4 C in a model L-2 preparative centrifuge.

Chemicals. PNPG and BMG were obtained from Calbiochem, Los Angeles, Calif. 1-Thiophenyl-β-D-glucoside (TPG) was synthesized by J. D. Duerksen (1). Chemically pure cellobiose was purchased from Mann Research Laboratories.

RESULTS

Genetic analysis. The mutant survey yielded a large number of mutants from Y123 and 1009D with reduced levels of β-glucosidase. Three of these from Y123 (Y123-2, Y123-9, Y123-40) and two from 1009D (PE25, X33) were chosen for further characterization. To determine whether more than one locus was involved, each of the low activity mutants was crossed with Y14 (B^m). The zygotes were sporulated, the asci were dissected, and only the tetrads in which all four spores were viable were tested for β-glucosidase activity. As seen in Table 2, only medium and low activity levels were detected. In each cross, only 2:2 medium-low segregation ratios were observed, indicating that the reduced activity was the result of a mutation in the B locus or in a gene closely linked to B. Also, in crosses between mutants Y123-2 and Y123-9 (*not shown*), no recombinants were found among 17 tetrads examined.

Induction characteristics. Since the above mutations lowered basal enzyme activity, it was of interest to determine their induction capacity. To test this possibility, the differential rate of enzyme synthesis was measured in mutant and wild-type strains. As shown in Table 3, in response to BMG or glucose, the induction capacity of the mutants was equal to or greater than that in the wild-type strains.

Comparison of β-glucosidases from wild-type and mutant strains: chromatographic, electrophoretic, and sedimentation properties. β-Glucosidases from both wild-type and several mutant strains exhibited identical elution patterns following chromatography of crude extracts on diethylaminoethyl (DEAE) cellulose columns.

To determine the electrophoretic migration rates, crude extracts were subjected to zone electrophoresis in starch gel. One typical activity pattern is shown in Fig. 1. Within the limits of detection, the β-glucosidases from wild-type and mutant strains migrated to the anode at the same rates. A second faster

TABLE 2. *Tetrad analysis of wild type × mutant crosses*

Mutant × wild type cross	No. of tetrads analyzed	Segregation ratio[a]		
		B^h	B^m	B^l
Y123-2 × Y14	17	0	2	2
Y123-9 × Y14	55	0	2	2
Y123-40 × Y14	38	0	2	2
PE25[b] × Y14	4	0	2	2
X23 × Y14	29	0	2	2

[a] β-Glucosidase activity was measured by plate assays as described elsewhere (5).

[b] Poor viability of spores precluded extensive analysis.

TABLE 3. *β-Glucosidase induction in wild-type and mutant strains*[a]

Strain	Differential rate			Inducibility factor	
	Basal	BMG	Glucose	BMG	Glucose
Y123 (wild type)	23.5	52		2.2	
Y123-2	0.7	1.8		2.6	
Y123-9	0.8	2.45		3.0	
Y123-40	7.0	16.5		2.4	
1009D (wild type)	12	24	36	2.0	3.0
PE25	2.5	10.5	21.5	4.2	8.6
X33	5.0	21	13.5	4.2	2.7

[a] Exponentially growing cultures were induced with methyl-β-D-glucoside (BMG, 2×10^{-2} M) and glucose (5×10^{-3} M) as described in Materials and Methods, Y123 is inducible by BMG, and 1009D is inducible by both BMG and glucose (6). From the linear increase in enzyme synthesis over a two-generation period, the differential rate of enzyme synthesis (Δ enzyme per generation) was calculated. The inducibility factor is the ratio of the differential rate of induced-to-basal enzyme synthesis.

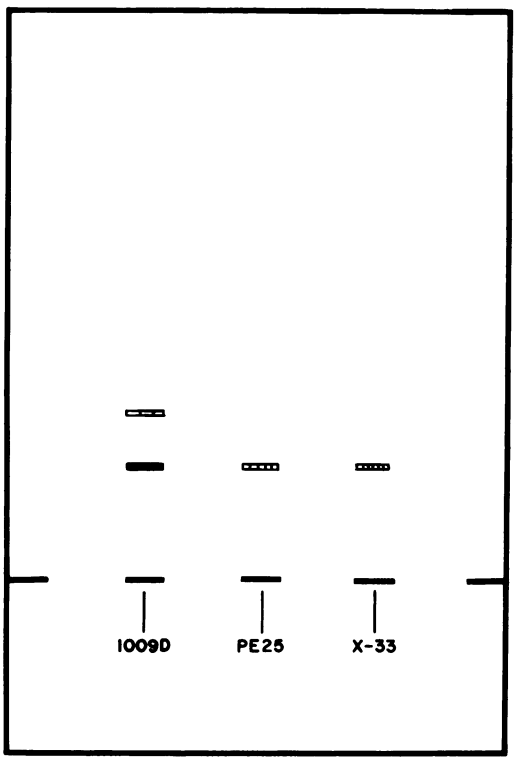

FIG. 1. *β-Glucosidase activity pattern following vertical starch gel electrophoresis. See Materials and Methods. Sample plots contained 0.05 ml (1.25 mg of protein) of each enzyme preparation. Left to right: 1009D (1,153 units/ml), PE25 (198 units/ml), and X33 (41.5 units/ml).*

moving band with lower activity against PNPG was often noted from extracts of wild-type strains.

Further the β-glucosidase activities in extracts from wild-type and mutant strains were indistinguishable by sucrose gradient centrifugation (Fig. 2). Thus, no gross differences in size or charge, or both, between β-glucosidases from mutant or wild-type strains were observed.

Enzyme substrates and complexants. Employing PNPG as substrate, typical Michaelis-Menten saturation curves were observed for all β-glucosidases. The apparent K_m values computed from Lineweaver-Burk plots are summarized in Table 4. In general, the β-glucosidases from mutant strains have an apparent K_m value significantly different from the wild-type. The mutant enzymes have higher affinities for TPG and lower affinities for glucose than the wild-type enzyme. Similar differences in affinity constants (*not shown*) were observed with BMG and cellobiose; however, these may not be significant, due to the low affinity of the enzymes for these alkyl-β-glucosides.

Heat inactivation. When enzyme preparations from wild-type and mutant strains were assayed for residual enzyme activity after incubation at 50 C, the β-glucosidases produced by mutant strains were significantly more heat sensitive than the wild-type enzyme (Fig. 3). The times required to inactivate 50% of the heat labile activity were 640 sec for the wild-type, 220 sec for Y123-2 and PE25, and 160 sec for Y123-9 and X33.

Neutralization with anti-β-glucosidase antiserum. If the β-glucosidases from mutant and wild-type strains are antigenically identical, the same amount of antiserum should neutralize equal amounts of enzyme units from wild-type and mutant extracts. To test this, neutralization tests were performed by adding increasing amounts of antibody to a constant amount of β-glucosidase (Table 5). The neutralization with homologous antigen (Y123 and 1009D) proceeded linearly until all of the enzyme was neutralized. In the heterologous case, with mutant enzymes, the neutralization of enzyme by antiserum was significantly less efficient. For example, 40 μliters of anti-β-glucosidase antiserum completely neutralized enzyme from wild-type cells (Y123 and 1009D) but neutralized only 44 and 65% of the enzyme from mutants Y123-2 and X33, respectively. These results clearly show immunological differences between wild-type and mutant enzymes which further supports the conclusion that the mutant enzymes are altered proteins.

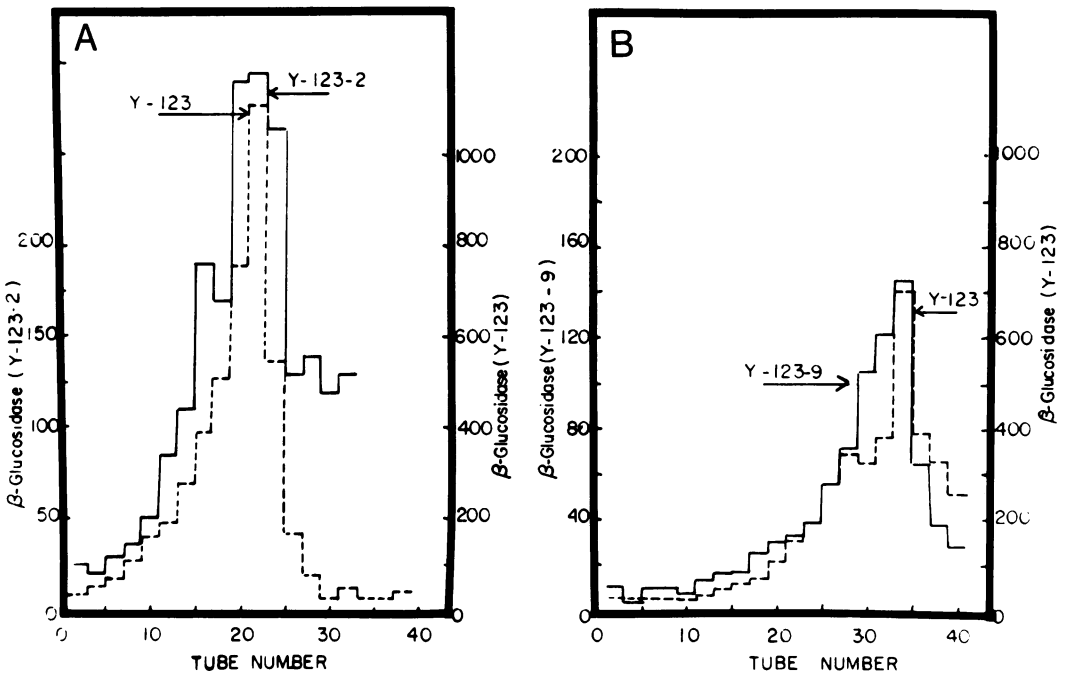

FIG. 2. *Centrifugation patterns of β-glucosidases from wild-type and mutant strains. (A) Extracts from strain Y123 (0.83 mg of protein in 0.1 ml) and mutant Y123-2 (1.19 mg of protein in 0.1 ml) were sedimented in separate gradients for 20.5 hr at 25,000 rev/min at 5 C. Fractions of 10 drops were collected. Alternate tubes were assayed for β-glucosidase activity by measuring PNP released after 1 hr as described in Materials and Methods. β-Glucosidase activity is plotted as the optical density reading at 400 nm × 1,000. (B) Extracts from wild-type strain Y123 (as above) and mutant Y123-9 (1.5 mg of protein in 0.5 ml) were layered on separate sucrose gradients. The rotor was run for 9 hr at 25,000 rev/min at 5 C. β-Glucosidase activity was determined as described above, with the exception that Y123 fractions were incubated with PNPG for 40 min and Y123-9 fractions for 90 min.*

TABLE 4. *Kinetic properties of β-glucosidase produced by mutant strains*[a]

Strain	PNPG $K_m \times 10^{-3}$	TPG $K_i \times 10^{-3}$	Glucose $K_i \times 10^{-3}$
Y123 (wild type)	0.10	1.71	2.0
Y123-2	0.037	0.63	3.3
Y123-9	0.031	0.55	3.2
Y123-40	0.15	2.67	2.7
1009D (wild type)	0.11	2.14	2.6
PE25	0.043	0.70	3.0
X33	0.075	1.62	7.5

[a] 1-Thiophenyl-β-D-glucoside (TPG) and glucose were employed at a final concentration of 5×10^{-3} M. From Lineweaver-Burk plots with each enzyme preparation, the kinetics of inhibition of *p*-nitro-phenyl-β-D-glucoside (PNPG) hydrolysis by TPG and glucose were strictly competitive. K_m and K_i values were determined from these Lineweaver-Burk plots. Activity was measured by the continuous method.

Dominance tests. The expression of the above mutations was examined in heterozygous diploids. If the mutations were in adjacent loci which controlled the potential for

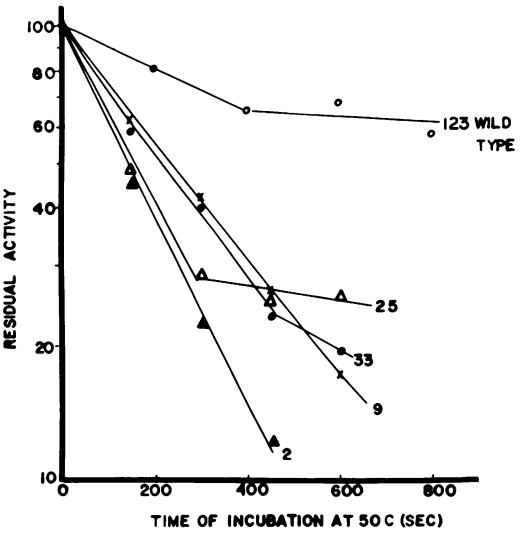

FIG. 3. *Heat inactivation of β-glucosidases from wild-type and mutant strains at 50 C. All cell extracts contained 600 units of β-glucosidase activity per ml. Enzyme was assayed by the continuous method.*

TABLE 5. *Neutralization of β-glucosidase activity from wild-type and mutant strains by anti-β-glucosidase antibody*

Amt of serum added (μliters)	Total β-glucosidase remaining in supernatant fluid after centrifugation				
	Wild type		Mutants		
	Y123	1009D	Y123-2	X33	PE25
Normal					
100	8.0	12.5			
Anti-β-glucosidase					
0	8.2	13.0	6.6	4.3	9.0
5	7.0				
10	1.6	9.5	5.3	4.8	8.6
20	0	1.1	4.7	3.7	8.4
30	0	0.1	4.0	2.6	7.7
40	0	0	3.7	1.5	0
50	0	0			0

[a] Enzyme neutralization was determined by antigen titrations as described in Materials and Methods. Continuous assays were employed.

expression of the B locus (promoter or operator type genes), mutations at these sites should not effect the expression of the B locus on another chromosome (*cis*-dominance). In this case, the specific activity of β-glucosidase in a diploid should approach the arithmetic mean of the input genes. Similarly, an intermediate specific activity might be expected if the mutation resulted in a structural mutation at this B locus. On the other hand, dominance of the mutant characteristic would imply presence of a cytoplasmic character which controlled expression of the B locus on both chromosomes. Alternatively, dominance of the mutant characteristic might reflect destruction of enzyme activity resulting from in vivo hybridization between functional and nonfunctional subunits of a multimeric enzyme.

To determine the expression of this mutation, heterozygous diploids were prepared by mating each mutant with Y14, a haploid strain of opposite mating type.

The specific activities of β-glucosidase in extracts from the parental and diploid strains are shown in Table 6. In each case, β-glucosidase activity in the diploid extract was less than the arithmetic mean predicted for equal expression of structural genes or *cis*-dominance characteristics. In all cases, the specific activity for β-glucosidase was less than that observed in haploid mutant strains.

DISCUSSION

In this paper, five independently isolated mutants with reduced β-glucosidase activity are reported. These can all be grouped in one class that, for the following reasons, is concluded to be in the structural gene (B) for β-glucosidase. (i) All mutants map at the B locus or in a closely linked gene. (ii) All mutants retain the same induction capacity to BMG or glucose, making unlikely the possibility that they are mutations in regulatory genes. (iii) β-Glucosidase produced by mutant strains differs in a number of properties from the wild-type enzyme. The mutant enzymes are more heat sensitive at 54 C than the wild-type enzyme. Mutant enzymes have lower K_m values for PNPG, higher affinities (K_i) for TPG, and lower affinities (K_i) for glucose as competitive inhibitors than are characteristic of the wild-type enzyme. (iv) The mutant and wild-type enzymes have different neutralization equivalents with antisera against the wild-type enzyme.

The parental strains of *S. lactis* Y123 (B^h) and Y14 (B^m) were isolated independently from nature (17). Strain 1057A (B^l) was a spontaneous mutant arising as a segregant from a parental cross (5). B^h, B^m, and B^l map at a single locus (5). Marchin and Duerksen have recently isolated the β-glucosidases produced in each of these strains (9, 10). The β-glucosidases can be distinguished from each other by *p*H optima, K_m values, affinities for aryl β-glucosides, and activation energies (11). Sero-

TABLE 6. *Test for dominance of β-glucosidase alleles in Saccharomyces lactis*[a]

Mutant strain	Specific activity β-glucosidase		Per cent arithmetic mean
	Mutant haploid	Heterozygous diploid	
Expt I			
Y123-2	5.75	1.74	32
Y123-9	2.92	1.61	40
Expt II			
PE25	6.96	4.42	60
X33	4.15	2.98	50

[a] The specific activity of β-glucosidase (nanomoles of *p*-nitrophenyl-β-D-glucoside per mg of protein per min) was determined by continuous method as described in Materials and Methods. Diploids were constructed by crossing each mutant with a strain of opposite mating type (Y14). This strain carries the B^m allele of the B locus and has a specific activity in experiments I and II of 5.07 and 7.76, respectively. In a control diploid (Y14 × Y123), the specific activity obtained was essentially that expected for the independent expression of both β-glucosidase alleles (83 to 100% of the expected mean value).

logical studies showed that, since the amount of CRM was the same in each strain, the structural modification also caused either a reduced turnover number or affected the aggregation of newly synthesized subunits. Therefore, whether β-glucosidase mutants arise naturally or are induced, all mutants with reduced levels of β-glucosidase described thus far are the result of mutations in the structural gene.

The specific activity of β-glucosidase in extracts of diploids derived from crosses of wild-type and mutant strains does not approach the arithmetic mean predicted on the assumption of equal expression of each B gene. The dominance of the mutant B allele presents an extremely interesting phenomenon. A similar observation was recently reported in *Neurospora crassa* where a mutation in the gluc-1 gene results in reduced β-glucosidase activity. This mutation is also dominant over gluc-1^+ mutations in heterokaryons (3). In *S. lactis*, the evidence that the reduced activity for β-glucosidase results from a mutation in the structural gene largely rules out the possibility that the dominance of the mutant phenotype is caused by positive or negative cytoplasmic gene products. A more likely possibility is suggested by the experiments on β-glucosidase in the hybrid yeast *S. fragilis* × *S. dobzhanskii* (4). The β-glucosidase produced by the hybrid is not characteristic of the enzymes produced by each parental strain but is a broad spectrum of hybrid enzyme molecules composed of varying amounts of two distinct polypeptides. In this yeast, the parental subunits are randomly polymerized in the cell cytoplasm producing a binomial distribution of hybrid enzyme molecules. Clearly, if a similar mechanism for random mixing of subunits was operative in *S. lactis*, the low specific activity in heterozygous diploids could be explained as unequal mixing of mutant and wild-type polypeptides. Further proof must await characterization of the β-glucosidase from heterozygous diploids.

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