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Purification and Characterization of Yeast β-Glucosidases[1]

LESLIE W. FLEMING[2] AND JACOB D. DUERKSEN
Department of Microbiology, University of Kansas School of Medicine, Kansas City, Kansas

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ABSTRACT

Constitutive β-glucosidases from *Saccharomyces fragilis* (Y-18) and *S. dobzhanskii* (Y-19) precipitated at the same concentration of ammonium sulfate. The partially purified enzymes had similar activation energies, molecular weights, affinities for certain natural and synthetic β-glucosides, and optimal *p*H values for substrate hydrolysis, and they were stable over approximately the same *p*H range. The enzymes, however, could be clearly distinguished by other criteria. Affinities of the synthetic, sulfur-containing β-glucosides for Y-18 enzyme were many times greater than for Y-19 enzyme. The latter enzyme was more resistant to heat. The two enzymes eluted from diethylaminoethyl cellulose at different concentrations of sodium chloride. In precipitin tests, homologous enzyme-antisera systems were highly specific. The β-glucosidase synthesized by a hybrid, *S. fragilis* × *S. dobzhanskii* (Y-42), was unique. Characterization of this enzyme produced values which were intermediate to those for the enzymes from the parental yeast strains. Heat-inactivation slopes and Lineweaver-Burk plots for the Y-42 enzyme were anomalous. It is suggested that hydrolytic activity in Y-42 preparations is due to a spectrum of hybrid enzyme molecules composed of varying amounts of two distinct polypeptides. It is further suggested that these polypeptides may be identical to those synthesized by the parental Y-18 and Y-19 yeast strains.

A few years ago, we reported evidence for the existence of two distinct species of β-glucosidase in a hybrid yeast, *Saccharomyces fragilis* × *Saccharomyces dobzhanskii* (2). The enzymes appeared to be characteristic of those obtained from the parent yeast strains. Additional studies, however, suggested that the hydrolytic activity against β-glucosides in the hybrid yeast might actually result from a population of hybrid molecules with varying compositions of two distinct polypeptides (4). If such were the case, mechanisms which regulate the synthesis of these hybrid enzymes would be unique among the variety of control mechanisms known to exist for other carbohydrase systems in yeasts.

This report deals primarily with procedures used for enzyme purification and characterization of yeast β-glucosidases.

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[2] Present address: Lederle Laboratories, Pearl River, N.Y.

MATERIALS AND METHODS

Organisms. All work was done on enzyme preparations from three strains of *Saccharomyces* yeasts obtained from H. O. Halvorson of the University of Wisconsin. *S. fragilis* was identified as a diploid yeast and *S. dobzhanskii* as haploid by Wickerham and Burton (15, 16). Both yeasts are homothallic. The third strain used was a hybrid, *S. fragilis* × *S. dobzhanskii*. For convenience, the yeasts and associated enzymes will subsequently be referred to as Y-18, Y-19, and Y-42, respectively.

Media. The yeasts were maintained by occasional transfers on slants containing the following ingredients, per liter: succinic acid, 6 g; K_2HPO_4, 8.7 g; $(NH_4)_2SO_4$, 4 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.3 g; yeast extract, 2.5 g; peptone, 5 g; and 1.5% agar. Before being autoclaved, the medium was adjusted to *p*H 5.0. Liquid cultures of yeasts were grown in the same medium, minus the agar.

Large-scale growth. Our studies were made with enzymes extracted from large batches of yeasts grown in an air-circulated, percolator-type fermentor. Starting with a 10% inoculum of logarithmically growing cells, three successive 10-liter quantities of logarith-

mically growing cells were harvested by leaving enough old culture to serve as inoculum for the new culture. Yeast cells were separated by Sharples centrifugation, and the resultant paste was stored at −15 C. Enzyme frozen in this manner was stable for long periods of time.

Enzyme extraction and purification. Cell paste was thawed in either 0.05 M phosphate buffer or Tris-succinate-magnesium (TSM) at neutral *p*H. TSM buffer consisted of 10 mmoles of tris[2-amino-2-(hydroxymethyl)-1,3-propanediol], 4 mmoles of succinic acid, and 5 mmoles of magenesium acetate. The suspended cells were ruptured with an MSE ultrasonic oscillator. Operating at a maximal output of 2 amps, two 30- or 45-sec exposures were sufficient for maximal release of enzyme. A concentration of 10^{-4} M 2-mercaptoethanol was maintained in the enzyme preparations at all times. Sonically treated yeast suspensions were centrifuged at 1,000 × *g* for 25 min to obtain crude, cell-free β-glucosidase. Yeast cells which had been frozen for several months apparently underwent considerable autolysis, since most of the enzyme could be recovered in a single buffer wash. It was also found that a few drops of toluene accelerated the release of enzyme from thawed cell paste. Some of our crude β-glucosidase was obtained in this manner.

Enzyme preparations were kept cool throughout the purification procedures. Nucleic acids were first precipitated with 1% streptomycin sulfate (E. R. Squibb & Sons, New York, N.Y.) at *p*H 6.3, and, after 6 hr at 6 C, the precipitate was eliminated by centrifugation at 20,000 × *g* for 15 min. Pulverized ammonium sulfate (Mallinckrodt Chemical Works, St. Louis, Mo.) was then added slowly to the supernatant fluid with constant stirring. During this manipulation, the *p*H was maintained at approximately 6.8. Material which precipitated between 0.45 and 0.65 saturation after overnight refrigeration contained most of the β-glucosidase activity. Amounts of ammonium sulfate required were determined by the Kunitz equation (7).

After ammonium sulfate fractionation, enzyme pellets were resuspended in buffer and filtered through high-porosity gels to de-salt the preparations and achieve further purification. Sephadex G-100 or G-200 (Pharmacia Inc., New Market, N.J.) was used. Columns were built from thin slurries of deaerated gel which had been washed with glass-distilled, deionized water and swelled for 3 days.

Optimal conditions for adsorption and elution of β-glucosidase from tricalcium phosphate gel (6) were determined empirically. With the enzyme in 0.1 M phosphate buffer (*p*H 7.4), small portions of gel were added until spot-testing demonstrated no activity in the supernatant fluid of centrifuged samples. The whole preparation was then centrifuged at 800 × *g* for 15 min. The supernatant fluid was poured off, and the gel was resuspended in 0.15 M citrate-phosphate buffer (*p*H 6.2). Elution was carried out for several hours at 6 C with intermittent shaking.

The final enzyme purification procedure was ion-exchange chromatography. Type 40 diethylaminoethyl (DEAE) cellulose (Schleicher and Schuell Co., Keene, N.H.) was prepared as described by Sober et al. (14), and columns were built as described previously for Sephadex. Large volumes of buffer were percolated slowly through the packed column to equilibrate the cellulose to buffer conditions. After applying the enzyme, elution was achieved by a linear sodium chloride gradient, generated by two interconnected reservoirs.

Enzyme and protein assays. β-Glucosidase activity was quantitated by either of two procedures. The continuous method consisted of mixing 0.1 ml of enzyme with 0.3 ml of a 10^{-2} M solution of the chromogenic substrate *p*-nitrophenyl-β-D-glucoside (PNPG; Calbiochem) and 2.6 ml of 0.05 M phosphate buffer (*p*H 6.8). Optical density of the liberated *p*-nitrophenol was read periodically at 400 mμ in a Bausch & Lomb colorimeter. For the discontinuous assay method, 0.1 ml of enzyme was mixed with 0.1 ml of 10^{-2} M PNPG, and 0.8 ml of the same buffer was used in the continuous assay procedure. After the yellow color of the *p*-nitrophenol had developed sufficiently, the reaction was stopped by 2 ml of a 1 M solution of sodium carbonate. These assays were read at 400 mμ in a Beckman spectrophotometer. The extinction coefficients of *p*-nitrophenol at the *p*H values prevailing in the continuous and discontinuous assay procedures are 9.6 × 10^3 and 1.83 × 10^4, respectively, when measured in 1-cm cuvettes (3). By either assay method, a unit of β-glucosidase was defined as that amount of enzyme necessary to cause an optical density change at 400 mμ of 0.001 per minute per 3 ml of reaction mixture at 30 C.

Protein was determined by the colorimetric procedure of Lowry et al. (9) with the Folin-Ciocalteau phenol reagent. Standard curves were established with crystallized bovine serum albumin (Pentex Inc., Kankakee, Ill.)

Activation energies. The effect of temperature on the initial velocities of PNPG hydrolysis was determined by continuous assays. Data were collected at 5-degree increments over the temperature range of 10 to 40 C and plotted in the conventional Arrhenius manner: log k versus $1/T$, wherein k is the initial velocity and T is the absolute temperature. Activation energies (E) for the enzymes were calculated from the slope of the resulting line: $-(\Delta H + RT)/2.303\ R$.

Michaelis constants. K_m values for the enzymes were determined by the double-reciprocal plot method of Lineweaver and Burk (8). By plotting the initial velocities of hydrolysis (as apparent enzyme units) for a wide range of PNPG concentrations, straight lines were obtained. The substrate concentration at which the enzymes functioned at one-half maximal velocity was calculated from the point of intercept of the straight line on the ordinate ($1/V$) and from the slope of the line (K_m/V).

Enzyme-inhibitor dissociation constants. Specificity of the β-glucosidases was studied with regard to their ability to form complexes with several β-glucosides and related compounds. This was done by determining the inhibition of enzymatic hydrolysis of PNPG by a fixed concentration of the test compound over a wide range of substrate concentrations. Initial velocities of substrate hydrolysis were plotted against the various substrate concentrations in Lineweaver-Burk

fashion to obtain apparent K_m values for the inhibiting compounds. Enzyme-inhibitor dissociation constants, or affinity constants (K_i), were then calculated from the equation: $K_i = i/(K_p - 1/K_m)$, wherein i is the concentration of the inhibitor and K_p is the apparent K_m.

β-Glucosides used in determining K_i values were obtained from various sources. The natural β-glucosides amygdalin, esculin, and salicin were supplied by Calbiochem, as were cellobiose and methyl-β-D-glucoside. Thioethyl-β-D-glucoside was synthesized by the method of Schneider et al. (13). The compound slowly crystallized as the monohydrate with a melting point of 40.5 to 42 C. Desiccation over P_2O_5 removed the water to give the anhydrous form with a melting point of 95.5 to 97.5 C. Thiophenyl-β-D-glucoside was made by essentially the same procedure by G. Marchin of this laboratory.

RESULTS

Table 1 shows the various treatments used for partial purification of the yeast β-glucosidases. The excessive loss of activity following treatment with streptomycin was unusual, but otherwise the figures are typical. Regardless of the yeast strain source, the enzyme preparations displayed essentially the same characteristics throughout the purification procedures. An exception was the different gradient molarities at which the enzymes eluted from DEAE cellulose. This point will be discussed later. The data in Table 1 were obtained with Y-18 (*S. fragilis*) enzyme and show a 55-fold increase in specific activity. Most of our studies were made with enzymes which had been purified 50- to 70-fold.

Partially purified enzymes could be stored at 6 C for weeks with minimal loss of activity. When stored at −20 C or in a lyophilized state, the enzymes were fully active after 3 months. Stability of the enzyme activity was dependent on the nature of the buffer system. Figure 1 shows a precipitous inactivation of Y-19 (*S. dobzhanskii*) enzyme in 0.15 M citrate-phosphate buffer at

TABLE 1. *Purification of yeast β-glucosidase*

Treatment[a]	Total enzyme units (× 10^{-3})	Total protein	Specific activity	Enzyme yield
		mg	*units/mg*	%
Supernatant fluid	511.9	1,735	295	100
Streptomycin	422.4	1,765	239	83
$(NH_4)_2SO_4$	316.8	1,125	282	62
Sephadex	149.6	285	525	29
$Ca_3(PO_4)_2$	127.1	95	1,338	25
DEAE	73.5	4.5	16,333	14

[a] Each treatment is described fully in the Materials and Methods section.

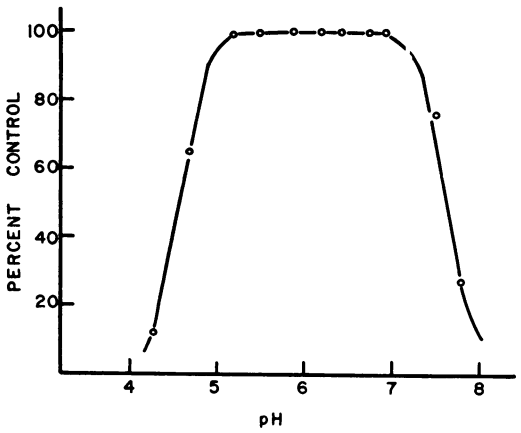

FIG. 1. *The pH stability of Y-19 enzyme. Small portions of enzyme were diluted in citrate-phosphate buffer at various pH values and stored at 6 C. Residual enzyme activity was assayed by the discontinuous method after 60 hr of storage. A control dilution was stored under similar conditions at pH 6.8.*

*p*H values above 7.3. In TSM buffer (*p*H 8.5), the enzymes lost approximately half of their initial activity after 12 hr at room temperature.

Curves for the hydrolysis of PNPG by all the β-glucosidases appeared to obey zero-order kinetics (Fig. 2). Moreover, the velocity of the reactions was directly proportional to enzyme concentration, indicating an absence of inhibiting substances or dependence on cofactors. At an adequate substrate concentration (10^{-3} M), enzymatic hydrolysis was linear over the usual

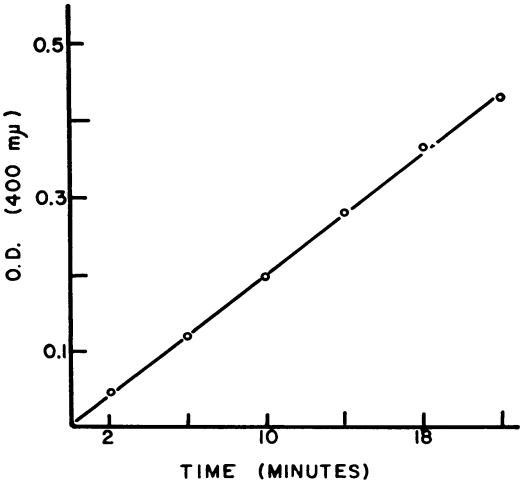

FIG. 2. *Rate of release of p-nitrophenol from PNPG by Y-18 enzyme. The system was run at 30 C in 0.05 M phosphate buffer (pH 6.8).*

assay period. Duerksen and Halvorson (3) reported that an inducible β-glucosidase from *Rhodotorula minute* liberated 1 mole of glucose and 1 mole of *p*-nitrophenol from each mole of PNPG.

Figure 3 shows small differences in the *p*H values at which the three yeast enzymes hydrolyze PNPG at maximal velocity: 5.7 to 5.8 for Y-18, 6.1 to 6.2 for Y-19, and 5.9 to 6.1 for Y-42. Since the molar absorptivity of *p*-nitrophenol is *p*H-dependent, these data were obtained by discontinuous assays.

The effect of temperature on initial velocities of substrate hydrolysis was studied over the range of 10 to 40 C at 5-degree increments. Figure 4 shows a conventional Arrhenius plot of the data for Y-18 enzyme. An activation energy of 15,800 calories per mole was calculated from the slope of the line. The Y-19 enzyme required 16,800 calories for activation. Hu et al. (5) reported a value of 16,000 calories for the enzyme from the hybrid yeast, Y-42.

Michaelis constants (K_m) were determined by the method of Lineweaver and Burk (8). Values of 11×10^{-5} and 6.9×10^{-5} M PNPG were obtained for Y-18 and Y-19 enzyme, respectively. Plots for the Y-42 enzyme produced anomalous slopes (Fig. 5). The anomaly was not due to analytical errors, but represents a true biphasic slope. An average K_m of 8.6×10^{-5} M PNPG was calculated from the lesser slopes and 1.25×10^{-3} M from the greater slopes. Hu et al. did not observe this biphasic nature in their inverse plot for the Y-42 enzyme. This difference

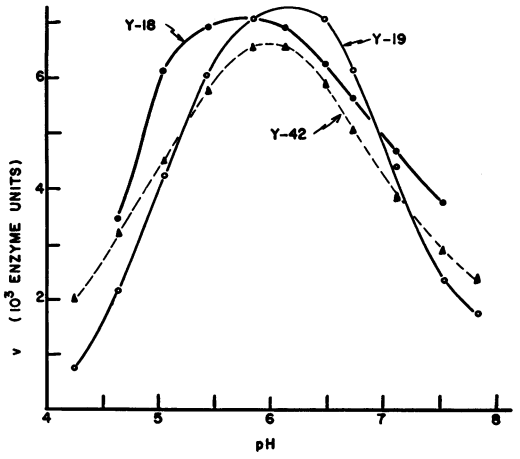

FIG. 3. *Optimal pH values for hydrolysis of PNPG by β-glucosidase from various yeast strains. Reaction mixtures contained (in addition to enzyme and substrate) 0.8 ml of 0.15 M citrate-phosphate buffer at the desired pH instead of the usual phosphate buffer. Initial velocities are expressed as apparent enzyme units.*

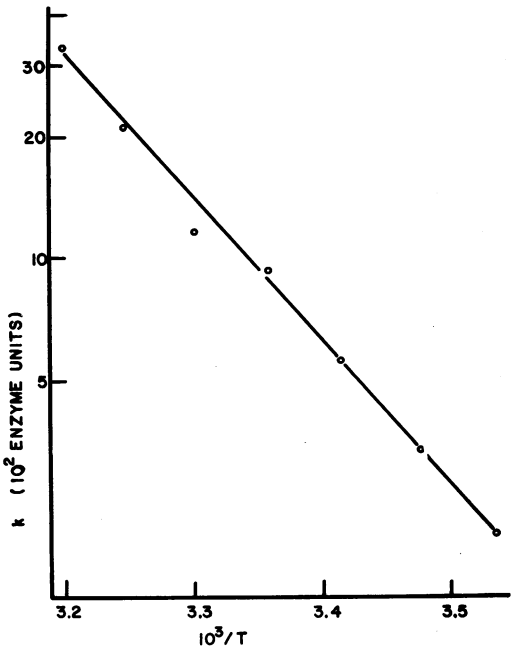

FIG. 4. *Arrhenius plot of PNPG hydrolysis by Y-18 enzyme. The effect of different temperatures on the rate of substrate hydrolysis was determined by continuous assays. Activation energy (E) was calculated from the slope of the line.*

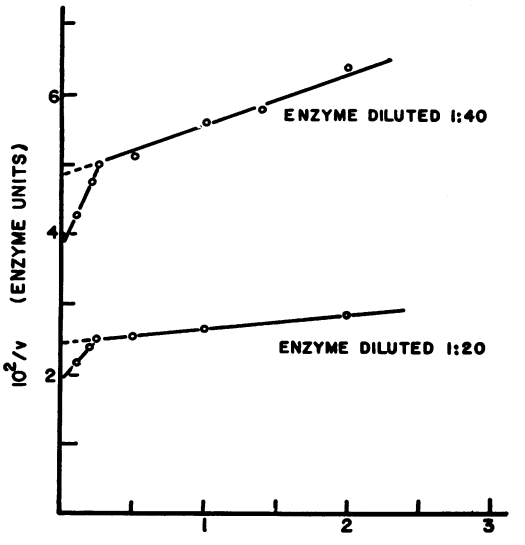

FIG. 5. *Lineweaver-Burk plots of PNPG hydrolysis by Y-42 enzyme. Differences in the initial velocity of hydrolysis at various substrate concentrations were determined by discontinuous assays and expressed as apparent enzyme units. Two K_m values could be calculated from plots obtained by using the enzyme at different concentrations.*

could be explained by the fact that they used an initial heating step in their purification procedure. The similarity between their K_m and our K_m from the lesser slope (9×10^{-5} M versus 8.6×10^{-5} M) would indicate that these curves are the same. It would seen reasonable also, on the strength of this similarity of the K_m values, to use the data on the Y-42 enzyme of Hu et al. for comparative purposes.

Table 2 compares the affinity constants of several compounds for the Y-18 and Y-19 enzymes. Values obtained by Hu et al. (5) for the Y-42 enzyme are included for comparison. Although the enzymes from the two parental yeast strains displayed similar K_i values for some of the natural and synthetic β-glucosides, affinities of the sulfur-containing glucosides for the Y-18 enzyme were found to be several times greater than for the Y-19 enzyme. The nature of the inhibition exerted on enzymatic hydrolysis of PNPG by the various compounds was competitive. The low affinity of the Y-18 and Y-19 preparations for cellobiose indicated the absence of cellobiase activity.

Heat-inactivation studies showed the Y-18 and Y-19 enzymes to be different. By bringing small portions of enzyme up to desired temperatures quickly in a water bath, and periodically assaying for residual enzyme activity, it was demonstrated that the Y-18 enzyme was considerably less resistant to heat. Figure 6 shows that this enzyme has a half-life of 4 min at 48 C, whereas a comparably purified Y-19 preparation has a half-life of approximately 18 min at 53 C. The most interesting aspect of these studies was the consistent biphasic nature of the inactivation slope for the partially purified Y-42 enzyme. Such slopes were not obtained with crude Y-42 enzyme. Inactivation by 4.5 M urea also demonstrated some difference between Y-18 and Y-19 enzyme, though these differences may possibly be more apparent than real. In Fig. 7, the initial slopes of inactivation are similar, but the secondary slopes and the amounts of residual activity for each are dissimilar. The behavior of Y-42 enzyme towards urea treatment, as we shall discuss later, is different from that of Y-18 and Y-19 enzyme.

TABLE 2. *Affinity constants (K_i) of various compounds for β-glucosidases*

Substrate	K_i(M × 10^5)		
	Y-18	Y-19	Y-42[a]
p-Nitrophenyl-β-D-glucoside	11[b]	6.9[b]	9[b]
Esculin	2	12	6
Amygdalin	44	142	100
Salicin	65	97	32
Thiophenyl-β-D-glucoside	177	917	330
Glucose	344	847	880
Thioethyl-β-D-glucoside	1,400	8,330	—
Methyl-β-D-glucoside	1,920	2,160	—
Cellobiose	14,300	17,200	—

[a] Data from Hu et al. (5).
[b] K_m.

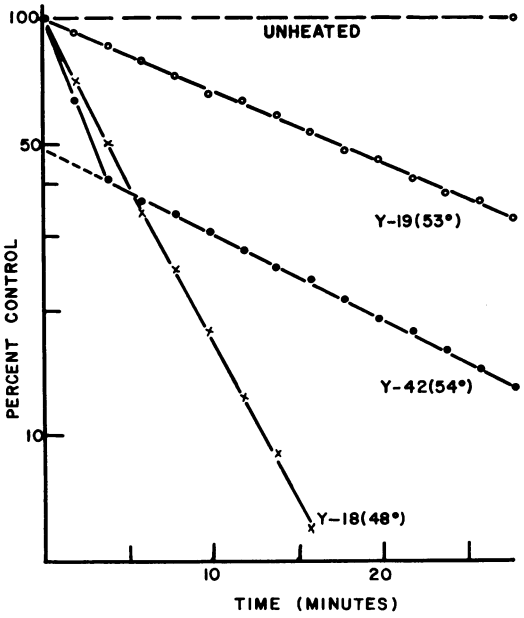

FIG. 6. *Heat inactivation of yeast β-glucosidases. Enzyme pools were quickly heated to indicated temperatures. At 2-min intervals, small samples were withdrawn and added to cold 0.05 M phosphate buffer (pH 6.8). Continuous assays were used to determine residual enzyme activity. Controls were samples not subjected to heat, but otherwise treated in a similar manner.*

DISCUSSION

It should not be surprising to find that functionally similar enzymes are alike in many other respects. The constitutive β-glucosidases synthesized by *S. fragilis*, *S. dobzhanskii*, and a hybrid, *S. fragilis* × *S. dobzhanskii*, are all precipitable by ammonium sulfate between 0.45 and 0.65 saturation, and are stable over approximately the same *p*H range. Differences in activation energies and optimal *p*H values for substrate hydrolysis are apparently minimal. In connection with studies to be reported later (4), molecular weights of these enzymes were determined by the method of Martin and Ames (10). Although the method is

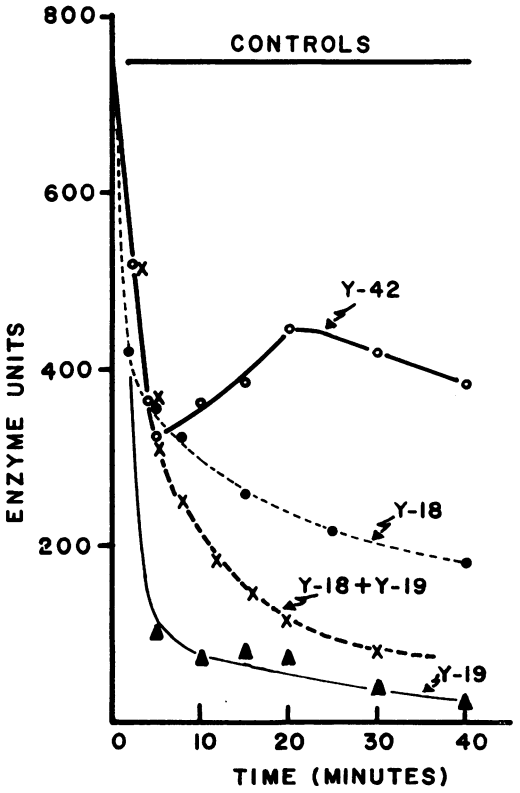

FIG. 7. *Inactivation of various yeast β-glucosidases by urea. The enzymes were diluted to the same unitage in TSM buffer (pH 6.7) and then mixed with urea at 30 C to produce a final concentration of 4.5 M. Samples were withdrawn periodically and diluted in 0.05 M phosphate buffer (pH 6.8). Residual enzyme activity was assayed by the discontinuous method. Controls consisted of enzymes in buffer solution and were assayed at the beginning and end of the test period.*

crude, the native enzymes were calculated to have similar weights—in the range of 325,000 to 335,000. Moreover, the enzymes are alike in their affinities for certain natural and synthetic β-glucosides.

Notwithstanding their similarities, the Y-18 and Y-19 enzymes can be clearly distinguished from each other. Affinities of the synthetic, sulfur-containing glucosides (thiophenyl- and thioethyl-β-D-glucoside) for the Y-18 enzyme are many times greater than for the Y-19 enzyme. Hu et al. (5) ascribed differences in the avidity of complexes formed between glucosides and β-glucosidases (either constitutive or inducible) to differences in the nature of groups in close proximity to the catalytic site of the enzymes. Enzymes from the two parental yeast strains also differ markedly as regards their stability at elevated temperatures.

We referred earlier to the fact that similar characteristics were displayed by the yeast β-glucosidases throughout most of the purification procedure. An exception was that Y-19 enzyme eluted from columns of DEAE cellulose at an applied salt concentration of 0.215 M, whereas Y-18 enzyme eluted at 0.230 (4). Perhaps the most decisive difference between the Y-18 and Y-19 enzymes is their serological specificity. In another paper (2), we reported that homologous enzyme-antisera systems were highly specific in precipitin tests.

In most comparisons of the yeast enzymes, comments on the Y-42 enzyme were intentionally omitted. It was apparent after preliminary studies that this enzyme was unique. If one looks carefully at the optimal *p*H values for PNPG hydrolysis, the value for Y-42 enzyme appears to fall between those for the Y-18 and Y-19 enzymes. Although the affiinity constants presented for Y-42 enzyme were determined in another laboratory, it is interesting to note that some of these values also fall between those for the enzymes from the parental yeast strains. Y-42 enzyme was found to react curiously in the presence of strong urea solutions. At some point on the inactivation slope, we consistently observed a transient, but significant, rise in enzymatic activity (Fig. 7). It can be seen that the slope for Y-42 enzyme (to the inflection point) is exactly superimposable on that obtained from a mixture of the Y-18 and Y-19 enzymes. This slope, in turn, fell between those for the individual enzyme preparations from the parental yeasts.

Two additional observations attest to the uniqueness of the Y-42 enzyme, but are difficult to explain satisfactorily. It was pointed out earlier that this enzyme produced anomalous Lineweaver-Burk plots. Neilands and Stumpf (6) suggested that this type of anomaly might indicate an enzyme that requires two substrate molecules, and that the attachment of one facilitates the rate of reaction of the second molecule With some reservations, however, it may be more plausible to interpret these results in terms of current concepts of molecular, or allosteric, transition (11). One could envisage that a natural equilibrium exists between two quaternary structures in Y-42 enzyme preparations and that the axis of symmetry is conserved in either structure. If the two structures displayed different affinities for PNPG, it is conceivable that excess substrate could cause a conformational change to the higher-affinity structure. Such a change would obviously result in an enhanced rate of substrate hydrolysis. It should be pointed out, however, in examples of allosteric effects that the transitions in kinetic be-

havior do not occur so abruptly. It is entirely possible that the observed transition may merely reflect heterogeneity among the combinations of the Y-18 and Y-19 subunits. Differences in conformation would be expected and are indicated in the heat-inactivation kinetics.

Heat-inactivation slopes for the Y-42 enzyme were biphasic. If we invoked again the presence of two different quaternary structures for this enzyme, such biphasic slopes would be expected if the two structures possessed different heat stabilities. Moreover, the fact that the lesser slopes could be extrapolated to approximately 50% on the ordinate suggests that the two quaternary structures are present in approximately equimolar quantities. Cline and Hu (1) reported rates of heat inactivation for sugar dehydrogenases which were biphasic and characteristic of two separate first-order reactions. These investigators felt that their results were reflecting conformational differences in the enzymes. It is interesting to note in Fig. 7 that the inflection point on the Y-42 inactivation slope occurs after approximately 50% of the enzyme has been inactivated. Urea, like excess substrate, may have caused a molecular transition to an enzyme structure with greater affinity for PNPG.

A disquieting aspect of molecular transition which relates to explaining some of our results is the fact that the model, as proposed by Monod et al. (11), implies subunit homogeneity in allosteric proteins. The bulk of our data seems to support the interpretation that hydrolytic activity in Y-42 preparations results from a spectrum of hybrid enzyme molecules composed of varying amounts of two distinct polypeptides. These polypeptides, in turn, are considered to be identical to those synthesized by the parental Y-18 and Y-19 yeast strains. Some reconciliation would be possible if we hypothesized that the ratio of Y-18 and Y-19 polypeptides in any given Y-42 enzyme molecule dictated which of the two quaternary configurations the molecule would assume.

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