Specificity of Sweet-Almond α -Galactosidase

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1. The specificity of purified sweet-almond α -galactosidase has been investigated with 17 substrates. 2. Some of them exhibited inhibition at high substrate concentrations but others did not. Both substrate types were bound and hydrolysed at the same site on the enzyme. 3. The enzyme is specific for α -D-galactosides and β -L-arabinosides. It did not hydrolyse β -D-galactosides or α -D-glucosides. 4. Among galactosides the order of decreasing rates of enzymic hydrolysis was: aryl α -galactosides; sugars; alkyl α -galactosides. 5. All substituents in the aryl moiety of aryl α -galactosides enhanced V_{max} , the electron-releasing ($-\sigma$) groups being more effective than the electron-withdrawing ($+\sigma$) groups. The substituent groups did not alter K_m appreciably. 6. Implications of these results are discussed from a mechanistic viewpoint.

α -Galactosidases have not received much attention in spite of a wide distribution of α -galactosides and these enzymes in Nature (French, 1954; Wallenfels & Malhotra, 1961). An almost 2000-fold purification of an α -galactosidase from sweet almonds has been reported (Malhotra & Dey, 1964, 1967), and the present paper deals with the specificity of this enzyme.

MATERIALS AND METHODS

Enzyme. This was purified as described earlier (Malhotra & Dey, 1967) to a specific activity of 12.7 units/mg. of protein.

Substrates. Melibiose and raffinose were commercial samples and α -D-galactose 1-phosphate was a gift from Dr D. L. McDonald. Alkyl α -D-galactosides were prepared by the method of Dale & Hudson (1930). Crystallization of these compounds presented difficulties, but a prior distillation of a small part of the syrupy product under high vacuum yielded 'seeds' for crystallizing the remainder. Aryl glycosides, except *o*-nitrophenyl β -D-galactoside, were prepared by the procedure given by Montgomery, Richtmyer & Hudson (1942). Deacetylation was carried out at 4° in methanol in the presence of 0.1M-sodium methoxide. The Na^+ ions were then removed by passing through a Dowex 50 (H^+ form) column (2.4 cm. $\times$ 8 cm.) before concentrating the methanolic solution for crystallizing the product. *o*-Nitrophenyl β -D-galactoside was prepared by the method of Seidman & Link (1950). Analyses and physical constants of new compounds are recorded in Table 1.

Assay procedures. All assays were carried out at 30° in

0.1M-acetate buffer, pH 5.5 (i.e. at the optimum pH; Malhotra & Dey, 1964). The liberated aglycone was estimated in the case of nitrophenyl glycosides and melibiose. In other cases, liberated galactose was determined. In each case only the initial steady-state rates, i.e. over the range of zero-order kinetics, were measured.

(a) Nitrophenyl glycosides. Nitrophenol was estimated at 405 m μ . The procedure was the same as described earlier for enzyme assay (Malhotra & Dey, 1967) (see also under Fig. 2).

(b) Melibiose. Glucose liberated during hydrolysis was estimated by the method of Huggett & Nixon (1957) with 'glucose reagent' supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. A stock solution (1M) of melibiose in buffer was kept for 24 hr. at room temperature for mutarotation to proceed to completion. The reaction was started by adding a properly diluted enzyme solution. The test volume was 0.25 ml.; the melibiose concentrations were 0.001–0.5M. The reaction was stopped after 20 min. with 0.1 ml. of 0.3N-trichloroacetic acid. Glucose reagent (5 ml.) was then added and the solution kept at 20° for 30 min. A reddish-brown colour developed which was measured at 436 m μ . Controls containing inactive (boiled) enzyme were run side by side for each melibiose concentration and proper correction was applied. The reagent was calibrated on the same day against several concentrations of glucose.

(c) Other substrates. Liberated galactose was estimated micro-cuprimetrically (Nelson, 1944) with phosphomolybdate (Hestrin, Feingold & Schramm, 1955) as the colour-developing reagent. Test mixtures (0.4 ml.) contained various concentrations of substrate and suitably diluted enzyme (0.62–12.5 μ g./ml. for aryl galactosides, 8 μ g./ml. for raffinose and 80–320 μ g./ml. for alkyl galactosides). The reaction was started by the addition of enzyme and after incubation for the required time it was stopped with 1.0 ml. of copper reagent. The tubes were then heated in a boiling-water bath for 20 min., cooled and to each was added 1.0 ml. of phosphomolybdate reagent. The total

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Table 1. Physical constants and analyses of some substrates and their tetra-acetates

Name	Melting point	$[\alpha]_D^{20}$	Analysis (%)			
			Found		Calc.	
			C	H	C	H
<i>m</i> -Nitrophenyl tetra- <i>O</i> -acetyl- α -D-galactopyranoside	120–121°	+125° (c 1.0 in CHCl ₃)	51.1	5.0	51.2	4.9
<i>m</i> -Nitrophenyl α -D-galactopyranoside	160–161°	+197° (c 1.0 in water)	48.3	5.1	47.8	5.0
<i>m</i> -Chlorophenyl tetra- <i>O</i> -acetyl- α -D-galactopyranoside	78–79°	+181° (c 1.0 in CHCl ₃)	52.2	5.0	52.3	5.0
<i>m</i> -Chlorophenyl α -D-galactopyranoside	154–155°	198° (c 1.0 in water)	49.8	5.2	49.6	5.2

Fig. 1. Effect of substrate concentration on the initial rate of enzymic hydrolysis of raffinose. Conditions and procedure are described in the Materials and Methods section. The incubation time was 20 min. Inset: Lineweaver-Burk double-reciprocal plot of the same data.

Fig. 2. Effect of substrate concentration on the initial rate of enzymic hydrolysis of *p*-nitrophenyl α -D-galactoside. Substrate and enzyme (1.25 μ g./ml.) in 0.1 M-acetate buffer, pH 5.5 (total volume 0.4 ml.), were incubated at 30°. After 10 min. the reaction was stopped by adding 5 ml. of 0.1 M-Na₂CO₃. E_{405} is plotted against *p*-nitrophenyl α -D-galactoside concentration.

volume was brought to 25 ml. and the blue colour measured at 546 m μ . The reagent was calibrated daily against several concentrations of galactose.

(d) α -D-Galactose 1-phosphate. In this case, estimation of glycone or of aglycone could not be achieved by the available methods. The extent of hydrolysis was therefore compared with some known substrates on paper chromatograms. Test mixtures (0.25 ml.) contained 0.1 M-substrate and 20 μ g. of enzyme protein/ml. The reaction was stopped after 30 min. by heating in a boiling-water bath and a 10 μ l. portion of the solution applied to a Whatman no. 1 filter paper. Descending paper chromatography was performed with butan-1-ol-pyridine-water (6:4:3, by vol.). After drying the chromatogram, it was sprayed with aniline-phthalate reagent (0.97 g. of aniline, 1.66 g. of phthalic acid and 5 g. of trichloroacetic acid in 100 ml. of water-saturated butan-1-ol) and heated at 120° for 3 min. Galactose 1-phosphate gave no trace of galactose.

RESULTS

Influence of substrate concentration. All the substrates tested could be divided into two groups: (i) melibiose, raffinose and *p*-nitrophenyl β -L-arabinoside showed simple Michaelis-Menten kinetics; typical results with raffinose are shown in Fig. 1 (with the corresponding Lineweaver-Burk plot in the inset); (ii) with the remaining substrates, inhibition was observed at higher substrate concentrations; a typical example is shown in Fig. 2. With these compounds the rate increased with rising substrate concentration, passed through a maximum and then decreased as [S] was raised further and finally became constant at a finite value (higher than zero) where it was independent of [S].

Table 2. *Specificity of sweet-almond α -galactosidase in 0.1M-acetate buffer, pH 5.5, at 30°*

Substrate	$10^3 \times K_m$ (M)	V_{max} (μ moles min. ⁻¹ mg. ⁻¹)	[S] for onset of inhibition (mM)
Melibiose	2.24	1.61	No inhibition
Raffinose	12.5	11.8	No inhibition
Methyl α -D-galactoside	10.9	0.59	200
Ethyl α -D-galactoside	6.25	0.62	62
<i>n</i> -Propyl α -D-galactoside	6.25	1.08	50
Phenyl α -D-galactoside	5.0	22.7	7.5
<i>o</i> -Cresyl α -D-galactoside	4.54	32.2	1.2
<i>m</i> -Cresyl α -D-galactoside	8.33	32.7	1.2
<i>p</i> -Cresyl α -D-galactoside	4.76	40.0	7.5
<i>m</i> -Chlorophenyl α -D-galactoside	8.33	32.7	8.0
<i>o</i> -Nitrophenyl α -D-galactoside	0.33	43.0	7.5
<i>m</i> -Nitrophenyl α -D-galactoside	1.57	23.6	7.5
<i>p</i> -Nitrophenyl α -D-galactoside	0.53	27.0	7.5
<i>p</i> -Nitrophenyl β -L-arabinoside	33.3	5.0	No inhibition
α -D-Galactose 1-phosphate		Not hydrolysed	
<i>o</i> -Nitrophenyl β -D-galactoside		Not hydrolysed	
<i>p</i> -Nitrophenyl α -D-glucoside		Not hydrolysed

Fig. 3. Lineweaver-Burk double-reciprocal plot of data of Fig. 2.

If only the initial rising part of the curve was considered, the corresponding Lineweaver-Burk plot was linear (Fig. 3), indicating that substrate inhibition was negligible under these conditions. Such plots were used to obtain K_m and V_{max} values for this type of substrate. The K_m and V_{max} values for the compounds that were found to be hydrolysed are shown in Table 2, together with the substrate concentration at which inhibition set in for the second type of substrates. Both substrate types, however, seem to be bound to the same active site on the enzyme molecule, as is indicated by competitive inhibition of enzymic hydrolysis of *p*-nitrophenyl α -D-galactoside by melibiose (Fig. 4).

Fig. 4. Inhibition of *p*-nitrophenyl α -D-galactoside hydrolysis by melibiose. Conditions of assay and procedure were as given in Fig. 2, except that the reaction mixture contained various concentrations (●, nil; ○, 2.5mM; ▲, 5mM; △, 10mM) of melibiose.

The competitive inhibitor constant for melibiose (K_i 1.95×10^{-3} M) was almost equal to its Michaelis constant (K_m 2.24×10^{-3} M).

Glycone specificity. *o*-Nitrophenyl β -D-galactoside and both the *p*-nitrophenyl glucosides (α as well as β) were not hydrolysed by the enzyme. *p*-Nitrophenyl β -L-arabinoside was hydrolysed at about one-fifth the velocity for the corresponding α -galactoside. Its K_m was about 60 times that of galactoside. A similar relationship has been reported with *Escherichia coli* β -galactosidase (Wallenfels, Lehmann & Malhotra, 1960).

Aglycone specificity. The values in Table 2 indicate that the substrate-dependence of K_m and V_{max} are unrelated to each other. A substrate with a lower K_m is not necessarily more rapidly hydrolysed. Similarly, certain substrates with approximately equal K_m (e.g. phenyl α -D-galactoside and *p*-cresyl α -D-galactoside) were hydrolysed at different rates. This, coupled with the fact that pH and temperature also affect K_m and V_{max} differently (O. P. Malhotra & P. M. Dey, unpublished work), might suggest that K_m is more or less equal to the dissociation constant of the enzyme-substrate complex.

The enzyme had a higher affinity for melibiose (lower K_m) than for raffinose. On the other hand, the trisaccharide was hydrolysed at a more rapid rate than the disaccharide, in agreement with the results of earlier workers (Courtois, 1958; Percheron & Gullieux, 1964). Among the three alkyl galactosides, ethyl and *n*-propyl derivatives had almost the same K_m values, which were somewhat lower than that of the methyl compound. The alkyl galactosides were the least reactive substrates.

In common with other glycosidases, sweet-almond α -galactosidase also hydrolysed aryl α -D-galactosides more rapidly than alkyl or sugar galactosides. All substituents on the aryl moiety increased V_{max} , some being relatively more effective

than others. A plot of $\log V_{max}$ against Hammett's substituent constant (σ) is shown in Fig. 5. The points for electron-releasing groups ($-\sigma$) lie on a straight line with slope (ρ) equal to -1.5 . The points for electron-withdrawing groups ($+\sigma$), on the other hand, do not lie on a straight line, indicating that the results cannot be quantitatively explained by their respective Hammett's constant alone. With the exception of the nitro group in *ortho* and *para* positions, the substituents did not bring about any major changes in K_m .

α -D-Galactose 1-phosphate was not hydrolysed. It is, in fact, the only case found so far in which a change in the aglycone part made the compound refractory to the action of the enzyme. A 5mM concentration of this compound did not inhibit the enzymic hydrolysis of *p*-nitrophenyl α -D-galactoside (0.65mM), indicating that it did not possess any affinity for the enzyme. To what extent this is due to the negative charges on the phosphate group is unknown.

DISCUSSION

The inhibitory action of high substrate concentrations on sweet-almond α -galactosidase is somewhat different from the 'uncompetitive inhibition' that is usually observed in the cases of inhibition by an excess of substrate where the activity falls continuously and tends to become zero at very high [S]. The latter type of inhibition is explained by presuming that, at higher substrate concentrations, a second substrate molecule becomes attached to the enzyme to give an inactive complex (Laidler, 1958). This may be represented as:

(where E, S, P, ES and ESS have their usual significance). The difference between 'uncompetitive inhibition' and the inhibition observed in the present case is that the rate does not tend to become zero. It becomes constant at a value higher than zero. It would appear that in this case the complex ESS is not completely inactive; it probably reacts at a lower rate than ES. The scheme may then be written as:

in which $k'_{+2} < k_{+2}$. The observed limiting velocity at very high substrate concentration would be equal to $k'_{+2}[E]_0$ where $[E]_0$ is the total enzyme concentration. The value of V_{max} calculated from

Fig. 5. Effect of substituents on the enzymic hydrolysis of aryl α -D-galactosides. σ , Hammett's substituent constant. For *meta*- and *para*-substituted compounds the σ values were taken from Hammett (1940) and for *ortho*-substituted compounds they were taken from Mamalis & Rydon (1950) and Nath & Rydon (1954).

the rising part as described above would be proportional to k_{+2} and represents the velocity that would have been obtained at high [S] if there were no inhibition with an excess of substrate.

Removal of C-6 of the galactose moiety (as in β -L-arabinoside) lowered the affinity for the enzyme as well as the $V_{\max}$, suggesting that one point of specific binding between enzyme and a galactoside might be through the $\text{CH}_2(\text{OH})$ group at position 6 of the galactose residue. In the absence of this, the enzyme efficiency is decreased. However, this binding is not absolutely essential. Inversion of configuration at C-1 or C-4 resulted in a complete loss of hydrolysability by the enzyme. Our unpublished results further showed that, whereas an α -glucoside was a competitive inhibitor, a β -galactoside was not, suggesting a relatively greater importance of proper configuration at the anomeric carbon atom, at least for binding to the enzyme.

Nath & Rydon (1954) studied the rates of acid- and alkali-catalysed hydrolysis of various aryl β -glucosides and found that increasing Hammett's substituent constant (σ) enhanced the rate of alkali-catalysed reaction and lowered that of the acid hydrolysis. It is reasonable to presume that the non-enzymic hydrolysis of α -galactosides should not be different from that of β -glucosides. Qualitatively it would appear that both types of situations exist on the active site of the enzyme. The predominant mechanism depends on the peculiar structure of the enzyme-substrate complex. From Fig. 5, the effect (and possibly contribution to the overall enzymic activity) of the basic group appears to be less pronounced.

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