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KINETIC BEHAVIOUR OF SWEET ALMOND α-GALACTOSIDASE

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SUMMARY

The effect of temperature and pH on the activity of sweet almond α-galactosidase (α-galactoside galactohydrolase, EC 3 2 1 22) has been studied with melibiose and *p*-nitrophenyl α-D-galactoside (PNPG) Activation energies for the two substrates were found to be 12 4 and 19 0 kcal/mole, respectively The pK_m *vs* $1/T$ plots were linear for both substrates, and the enthalpies of binding were −7 3 and −10 2 kcal/mole for PNPG and melibiose, respectively Changes in K_m and v_{max} of melibiose with pH suggested the participation of a group with pK 5 75 in the enzyme–substrate binding and of another group with pK around 3 in the catalytic step Similar studies with PNPG also revealed the presence of two groups (pK 3 4 or above and 6 7 or below), the pK values of which were strongly influenced by the substrate concentration The group dissociating in the lower pH range appears to play a minor part only in the PNPG hydrolysis In spite of apparently marked differences in the effect of temperature and pH, all the substrates appear to be hydrolysed at the same site on the enzyme molecule The differences probably arose from differences in the mechanisms of the reaction or in the nature of the rate-limiting step in the two cases

INTRODUCTION

Studies on the effect of temperature and pH yield some useful information about the enzyme-catalysed reactions Such studies have not been reported in any detail on α-galactosidase (α-galactoside galactohydrolase, EC 3 2 1 22) probably because the enzyme was not available in a sufficiently purified form[1] We have already described an intensive purification of this enzyme from sweet almonds[2,3] as well as its specificity[4] The present paper deals with the effect of temperature and pH on the activity of this enzyme Earlier we showed that substrates could be divided into two main groups, namely those which followed simple Michaelis–Menten kinetics and others with which inhibition was observed at high substrate concentrations Both types of substrates, however, appear to be hydrolysed at the same site in the enzyme molecule[4] In the present studies, parallel experiments were carried out with each type

Abbreviation PNPG, *p*-nitrophenyl α-D-galactoside

of substrate The results seem to suggest a change in the mechanism of the reaction or in the nature of the rate-limiting step when the substrate is changed

MATERIALS AND METHODS

Enzyme preparation

α-Galactosidase was purified as described earlier[2] to a specific activity of 12 7 units/mg protein

Substrate

Melibiose was obtained from British Drug Houses Alkyl galactosides were prepared according to DALE AND HUDSON[5], and *p*-nitrophenyl α-D-galactoside (PNPG) was prepared as described earlier[4,6]

Assay procedure

The assay procedures for the enzymatic hydrolysis of PNPG and melibiose were the same as described earlier[4]. The K_m and the v_{max} were calculated from Lineweaver–Burk plots

Nonenzymatic hydrolysis

PNPG (0 197 mM for alkaline hydrolysis and 0 79 mM for acid hydrolysis) was kept in acid or alkali of required strengths at the desired temperature. Aliquots (5 ml) were withdrawn at regular intervals, were chilled and were neutralized with calculated amounts of alkali or acid The yellow colour developed by adding Na_2CO_3 was estimated at 405 mμ A similar procedure was adopted for melibiose (0.05 M) except that the aliquots (1 0 ml) after the chilling and neutralization were analysed for liberated glucose by the method of HUGGETT AND NIXON[7] using the glucose reagent (Boehringer und Soehne, Mannheim) which was calibrated daily against several concentrations of glucose.

Whereas both acid and alkaline hydrolyses were studied with PNPG, only the acid hydrolysis could be investigated with melibiose because under the conditions required for hydrolysis (65–75° and 0 1 M NaOH), alkali imparted a brown colour to the sugar and thus interfered with the final glucose estimation

TABLE I

EFFECT OF TEMPERATURE ON THE ENZYMATIC HYDROLYSIS OF MELIBIOSE AND PNPG IN 0 05 M PHOSPHATE BUFFER (pH 6 0)

v_{max} is expressed as μmoles of substrate hydrolysed per min per mg enzyme

Temp	*Melibiose*		*PNPG*	
	K_m *(mM)*	v_{max}	K_m *(mM)*	v_{max}
20°	2 9	0 92	0 43	12 2
30°	5 26	1 84	0 68	37 5
40°	8 69	3 51	1 25	106 6
45°	—	—	1 05	164 5
50°	—	—	1 40	250 0

TABLE II

ACID AND ALKALINE HYDROLYSIS OF MELIBIOSE AND PNPG

Substrate	*Catalyst*	*Temp*	*Pseudo first-order rate constant* $\times 10^4$ *(min*$^{-1}$*)*
PNPG	0 1 M NaOH	55°	354 ± 16
	0 1 M NaOH	65°	968 ± 36
	0 1 M HCl	55°	9 53 ± 0 07
	0 1 M HCl	65°	31 0 ± 6
Melibiose	1 0 M HCl	65°	14 2 ± 0 9
	1 0 M HCl	75°	34 8 ± 1 0

RESULTS

Effect of temperature

The K_m and v_{max} values of PNPG and melibiose at different temperatures are shown in Table I In both cases, v_{max} increased with temperature Arrhenius plots were linear in both cases and gave activation energies of 19 0 and 12 4 kcal/mole for the enzymatic hydrolysis of PNPG and melibiose, respectively.

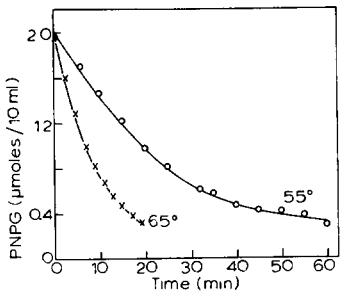
Nonenzymatic hydrolyses of PNPG catalysed by HCl and NaOH and of melibiose catalysed by HCl were also studied at different temperatures. Typical results are shown in Fig 1 The pseudo first-order rate constants obtained are given in Table II Activation energies obtained from these data are compared with those of the enzymatic reaction given in Table III It is interesting to note that the difference in the activation energies of nonenzymatic and enzymatic hydrolyses is much larger with melibiose than with PNPG

K_m also increased with temperature but to a different extent than did v_{max} Thus whereas v_{max} for PNPG increased from 12 2 to 250 μmoles of PNPG hydrolysed per min per mg enzyme between 20 and 50°, K_m rose from 0 43 mM to 1 4 mM Plots of pK_m (−log K_m) against $1/T$ gave straight lines for both substrates Their slopes gave enthalpy values of −7 3 and −10 2 kcal/mole substrate bound for a complex formation of the enzyme with PNPG and melibiose, respectively

TABLE III

ACTIVATION ENERGIES FOR THE ENZYMATIC, ACID AND ALKALINE HYDROLYSIS OF PNPG AND MELIBIOSE

Catalyst	*Temp range*	*Activation energy (kcal/mole)*	
		PNPG	*Melibiose*
0 1 M HCl	55–65°	26 1	—
0 1 M NaOH	55–65°	22 9	—
1 0 M HCl	65–75°	—	21 1
α-Galactosidase	20–40°	19 0 (up to 50°)	12 4



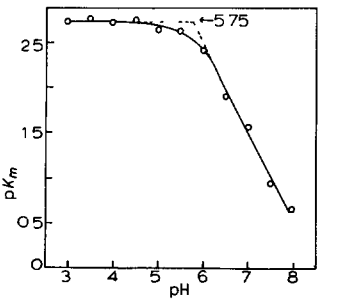


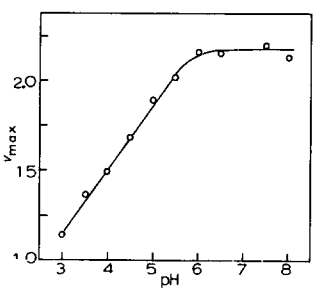
Fig 1 Hydrolysis of PNPG with 0 1 M NaOH at 55° and 65° Procedure is given in MATERIALS AND METHODS

Fig 2 Effect of pH on K_m for the enzymatic hydrolysis of melibiose Citrate–phosphate buffers were used, and experiments were carried out at 30° K_m is expressed as M

Effect of pH

The effect of pH on K_m and v_{max} of melibiose is shown in Figs 2 and 3 The pK_m–pH plot (Fig 2) shows the presence of a dissociable group on the active site of the free enzyme with a pK of 5 75 (*cf* ref 8) The protonated (or undissociated) form of this group is required for enzyme activity because the enzyme–substrate affinity appears to decrease as the pH is raised The v_{max} rises gradually as the pH is raised from 3 to 6 and becomes more or less pH-independent beyond that point (Fig 3) The rate of change of v_{max} with pH 3–6 is small The pH corresponding to half the optimal velocity is found to be about 3

The K_m and v_{max} of PNPG varied with pH in a more complex manner K_m is



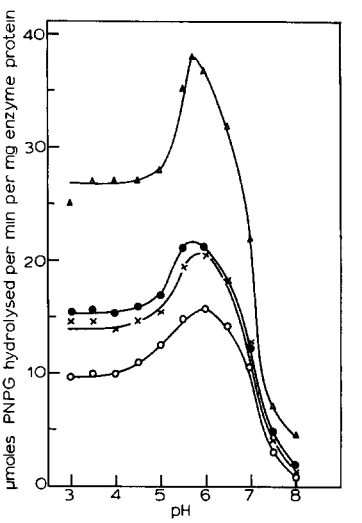


Fig 3 Effect of pH on v_{max} for the enzymatic hydrolysis of melibiose Citrate–phosphate buffers were used, and experiments were carried out at 30° v_{max} is expressed as μmoles of substrate hydrolysed per min per mg enzyme protein

Fig 4 Effect of pH on the enzymatic hydrolysis of PNPG Citrate–phosphate buffers were used, and experiments were carried out at 30° PNPG concentrations were 0 5 mM (○—○), 1 0 mM (×—×), 1 5 mM (●—●), and ∞ (*i e* v_{max})(△—△)

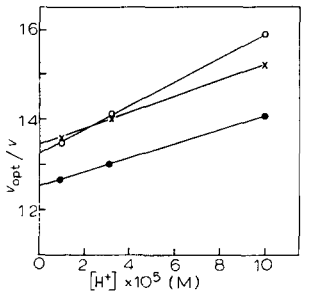


Fig 5 Plots of v_{opt}/v *versus* H^+ concentration at different PNPG concentrations 0 5 mM (○—○), 1 0 mM (×—×), and 1 5 mM (●—●) Data of Fig 4

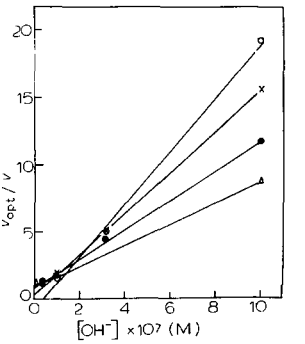


Fig 6 Plots of v_{opt}/v *versus* OH^- concentration at different PNPG concentrations 0 5 mM (○—○), 1 0 mM (×—×), 1 5 mM (●—●), and ∞ (*i e* v_{max}) (△—△) Data of Fig 4

practically independent of pH (0 57–0 87 mM, pH 3 0–7 5) except between pH 7 5 and 8 0 (K_m = 2 1 mM) The behaviour of v_{max} is shown in Fig 4 Optimal velocity is observed at pH 5 7 There is a continuous fall in v_{max} as the pH is raised above 5 7 The half-optimal velocity is observed at pH 7 1 On the lower pH side, there is a small fall only to pH 5 0, and the v_{max} is unchanged in the pH range 3 0–5 0 This last effect seems to be a function of the substrate concentration, and the falling part of the curve can be extended to lower pH's if the substrate concentration is lowered (Fig 4) As the half-optimal velocity could not be achieved on the acidic side of the pH optimum, the pH-sensitive part of this data was analysed according to the WALEY equation[9,10] The results are shown in Figs 5 and 6 The pK values of groups dissociating on both sides of pH optima at different substrate concentrations, as obtained from slopes of plots of Figs 5 and 6, are shown in Table IV The pK of the group dissociating on the acidic side seems to fall while that of the group dissociating on the alkaline side appears to increase as the substrate concentration is increased

Hydrolysis of different substrates

Earlier results suggested that melibiose and PNPG were hydrolysed at the same site in the enzyme molecule[4] Their behaviour with temperature and pH, however,

TABLE IV

DISSOCIATION CONSTANTS OF THE GROUPS ON THE ACTIVE SITE OF SWEET ALMOND α-GALACTOSIDASE AT DIFFERENT PNPG CONCENTRATIONS

PNPG concn (mM)	pK^*_4	pK_B**
0 5	3 4	6 7
1 0	3 3	6 8
1 5	3 2	6 9
∞ (*i e* for v_{max})	—	7 1

* Dissociating on the acidic side of pH optimum
** Dissociating on the alkaline side of pH optimum

TABLE V

COMPARISON OF K_m AND K_i VALUES FOR CERTAIN α-GALACTOSIDES

In 0 1 M acetate buffer (pH 5 5), 30°, K_i was determined using PNPG as the substrate

Compound	K_m *(mM)*	K_i *(mM)*
Methyl-α-galactoside	10 9	12 5 ± 0 5
Ethyl-α-galactoside	6 25	5 83 ± 0 14
Melibiose	2 24	1 95 ± 0 1
Melibiose (citrate–phosphate buffer, pH 6 0)	4 25	3 76 ± 0 26

were widely different Therefore, the competitive inhibition of PNPG hydrolysis by melibiose and alkyl galactosides was examined again Table V compares K_i values of these compounds with the respective K_m values and shows clearly that alkyl galactosides, melibiose and PNPG (and presumably other aryl galactosides and raffinose) are all bound and hydrolysed at the same site

DISCUSSION

Variations of K_m and v_{max} with the nature of the substrate[4], temperature and pH bear no resemblance to each other This suggests that in the present case, $K_m \approx K_s$ (*i e* dissociation constant of the enzyme–substrate complex into enzyme and substrate) The strictly linear relationship between pK_m and $1/T$ also suggests that K_m, in this case, is a simple constant rather than a complex function of several velocity constants

The pK 5 75 for a group on the active site as obtained from the pK_m–pH plot for melibiose (Fig 2) must refer to the group as it exists in the free enzyme and not in the enzyme–substrate complex because the curve concaves downward and the substrate (melibiose) does not have any dissociable group in this pH range (*cf* ref 8) This group might be an imidazolium group (literature pK value 5 6–7 0 (ref 11)) The group showing a pK of 7 1 in the enzyme–PNPG complex (pH at v_{max} (opt)/2 in Fig 4) is probably the same group, and the discrepancy in the two values may be due to the peculiar effect of the substrate binding A rising trend in the pK of this group as a result of increased saturation with the substrate is indicated in Table IV

The presence of another group, dissociating at a lower pH, is clearly indicated by Figs 3 and 4 Its pK in the free enzyme must be 3 3 or higher (see Table IV), but the same is lowered to about 3 as a result of substrate binding This group might be a carboxyl group (literature pK value 3 0–4 7 (ref 11) required as COO^-) in the active form of the enzyme The role of this group is less pronounced in the fully saturated enzyme–PNPG complex (v_{max} is pH-independent below pH 5) than in an enzyme–melibiose complex (continuous fall in v_{max} below pH 6)

It is interesting to note that the substrate (PNPG) binding lowers the pK of the group dissociating on the acidic side and raises that of the group dissociating on the alkaline side of the pH optimum A similar shifting of the two pK values was observed when chitotriose was bound to lysozyme[12] In other words the changes (presumably conformational) induced by the substrate in an enzyme molecule enlarge the effective pH range of the enzyme, *i e* the induced changes favour the catalytic reaction[13]

It has been shown that all the substrates are being bound and hydrolysed at the same site Therefore, the differences observed in the effect of temperature and pH with different substrates must be attributed to differences in the mechanism of reaction or in the nature of the rate-limiting step If it is presumed that COO^- has the function of a base in the enzyme-catalysed reaction and imidazolium that of an acid, the enzymatic hydrolysis of aryl galactosides would be somewhat similar to the acid-catalysed reaction (*e g* a higher energy of activation and a decrease in the rate of hydrolysis with increasing Hammett's substituent constant[4]) while that of melibiose might be similar to a concerted action mechanism (*e g* large decrease in the energy of activation)

It is noteworthy that similar conclusions, namely the existence of acidic and basic groups and changes taking place in the mechanism or possibly in the nature of a rate-limiting step, were also drawn from specificity studies[4]

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