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INHIBITION, TRANSGALACTOSYLATION AND MECHANISM OF ACTION OF SWEET ALMOND α-GALACTOSIDASE

P. M. DEY

Department of Biochemistry, Royal Holloway College, London University, Englefield Green, Surrey (Great Britain)

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SUMMARY

The presence of essential catalytic groups in sweet almond α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) has been investigated with the use of metal ions and photo-oxidation. Competitive inhibition was shown to occur with both Hg^{2+} and Ag^{+}. From the inhibition data it was postulated that carboxyl and histidine groups in the enzyme active site were responsible for binding the metal ions. The latter group was destroyed by photo-oxidation resulting in inactivation of the enzyme. The enzyme was found to catalyse hydrolytic, transgalactosylation and synthetic reactions on the same active site. All the reactions proceeded yielding products with complete retention of configuration. Two possible mechanisms of action have been suggested and discussed.

INTRODUCTION

Previous studies on sweet almond α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) have indicated that the enzyme is dependent on carboxyl and histidine groups for its activity[1]. Similar conclusions were also drawn from kinetic studies[2,3] with a homogeneous α-galactosidase preparation from broad beans[4]. The present paper deals with the inhibition and transgalactosylation studies on sweet almond α-galactosidase. Possible mechanisms of action are suggested which are based on all the previous results.

MATERIALS AND METHODS

α-Galactosidase from sweet almonds was purified by a method described earlier[5,6]. Enzyme assays were performed spectrophotometrically using *p*-nitrophenyl α-D-galactoside as the substrate[4,7]. Inhibition studies were carried out in Tris–acetic acid or McILVAINE[8] buffer and LINEWEAVER–BURK[9] plots were constructed at each pH value to calculate the K_m and the K_i values.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

In the photo-oxidation experiment, enzyme (75 μg/ml) in McILVAINE[8] buffer (pH 6.0) containing methylene blue (50 μg/ml) was held in a glass-walled thermostat at 30° and exposed to light from a 200-W tungsten lamp kept at 7 cm from the reaction mixture. A similar reaction mixture was kept in the dark at 30° as a control experiment. Aliquots were withdrawn at various time intervals and assayed for enzyme activity. Enzyme samples were similarly treated for estimating thiol, tyrosine and tryptophan groups.

For the transgalactosylation experiments, the incubation mixture contained 160 μg enzyme per ml, 0.05 M *p*-nitrophenyl α-D-galactoside (galactosyl donor) and 1.0 M acceptor sugar; all in sodium acetate buffer (pH 5.5). The incubation was carried out for 2 h at 30° and the reaction stopped by heating at 100° for 2 min. The precipitated protein was centrifuged down and the supernatant was chromatographed on Whatman No. 1 paper using the solvent system, *n*-butanol–pyridine–water (6:4:3, by vol.). The transfer products were detected by 2-aminobiphenyl reagent[10] and identified by using marker spots of authentic samples.

TABLE I

INHIBITION OF SWEET ALMOND α-GALACTOSIDASE BY METAL IONS

The enzyme and the inhibitor were incubated for 20 min at 30° and the residual activity was assayed using *p*-nitrophenyl α-D-galactoside (0.65 mM) as substrate in 0.05 M Tris-acetate buffer (pH 5.5).

Metal ion	*Concn. in assay (M)*	*Residual activity (%)*	*Nature of inhibition*	K_i *(M)*
Ag^+	$5.76 \cdot 10^{-6}$	50	Competitive	$2.46 \cdot 10^{-6}$
Hg^{2+}	$4.67 \cdot 10^{-3}$	50	Competitive	$6.5 \cdot 10^{-4}$
Cu^{2+}	$1.2 \cdot 10^{-3}$	50	Competitive	$1.1 \cdot 10^{-3}$
Cd^{2+}	$1.0 \cdot 10^{-2}$	100	—	—
Zn^{2+}	$1.0 \cdot 10^{-2}$	100	—	—
Pb^{2+}	$1.0 \cdot 10^{-2}$	100	—	—
Be^{2+}	$1.0 \cdot 10^{-2}$	94	—	—
Ca^{2+}	$2.5 \cdot 10^{-1}$	100	—	—
Sr^{2+}	$2.5 \cdot 10^{-1}$	100	—	—
Na^+	1.0	90	—	—

RESULTS AND DISCUSSION

Inhibition by metal ions

The effect of various metal ions on α-galactosidase activity is shown in Table I. Strongest inhibition was observed with Ag^+ and there was weaker inhibition by Hg^{2+} and Cu^{2+}. Other metal ions tested had practically no effect. $AgNO_3$ and Ag_2SO_4 had almost equal inhibitory effects showing that the anions do not alter the enzyme activity. Almond α-galactosidase is, therefore, different to that of *Calvatia cyanthiformis* enzyme which was completely inhibited by 10 mM Hg^{2+} and unaffected by 1 mM Ag^+ or 1 mM Cu^{2+} (ref. 11). This enzyme was, however, considered to have thiol groups in the active site.

Inhibition by Ag^+ was competitive (Fig. 1) as was the case with Hg^{2+} and Cu^{2+}. A comparison of the K_i values (Table I) clearly demonstrate a much higher affinity

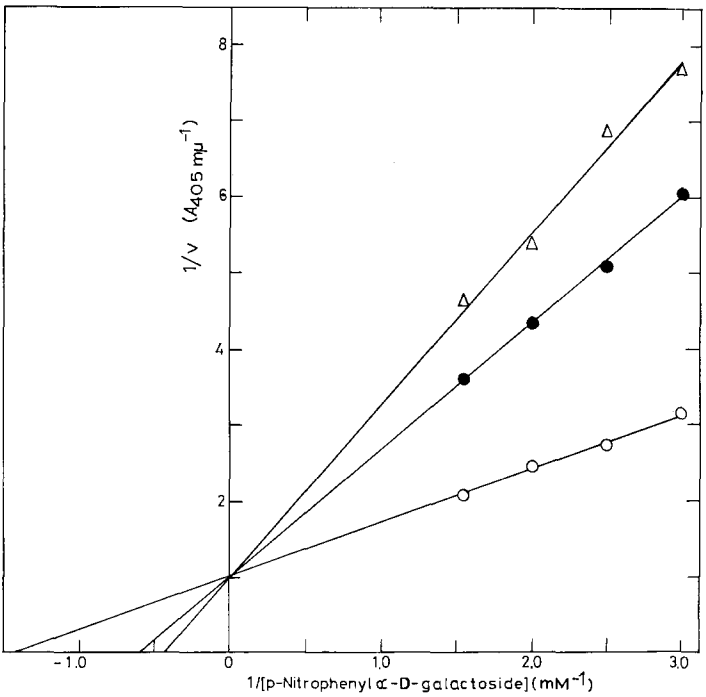
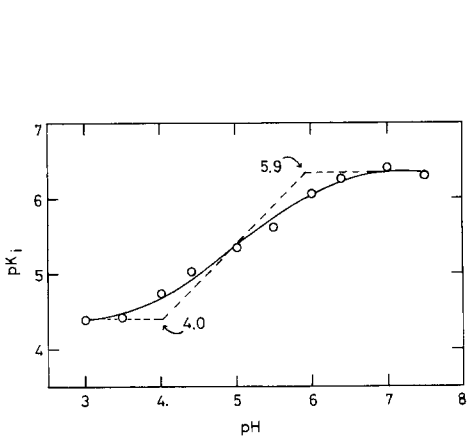


Fig. 1. Inhibition of α-galactosidase-catalysed hydrolysis of *p*-nitrophenyl α-D-galactoside by $AgNO_3$. Conditions of assay and procedure were similar as mentioned in MATERIALS AND METHODS except that the reaction mixture contained various concentrations (○, nil; ●, 3 μM; △, 4 μM) of $AgNO_3$.

of the enzyme for Ag^+. The influence of pH on Ag^+ inhibition has also been studied and as the inhibition is competitive, the groups involved in Ag^+ binding on the enzyme active site must be the same as those involved in substrate binding. The plot of pK_i ($-\log K_i$) against pH showed a S-shaped curve (Fig. 2) which could be divided into three straight line portions (slope, 0, + 1 and 0). The points of intersection of these lines correspond to pH 4.0 and 5.9. Thus, according to DIXON[12], a group of pK 5.9 on the enzyme active site was affected very strongly by the binding of Ag^+ and was shifted to pK 4.0. This group might be the imidazolium group which was shown to be present along with a carboxyl group in the enzyme active site[1]. This formation of Ag^+–enzyme complex may be a case of salt formation with the carboxyl group, stabilized by co-ordination with the tertiary nitrogen of the imidazolium group. The presence of a positively charged atom (*i.e.* Ag^+) in the vicinity will, therefore, strongly favour deprotonation of the imidazolium group and thus lower its pK value.

Ag^+ and Hg^{2+} commonly inhibit enzyme activity by reacting with their essential thiol groups. In the present case, however, the possibility of any thiol group participation in the enzymic catalysis seems to be small because in such cases Ag^+ and Hg^{2+} would be equally effective inhibitors and other heavy metal ions would not be completely ineffective[13,14]. The pK_i would also not be affected in the pH range 3.0–7.5 (ref. 15).



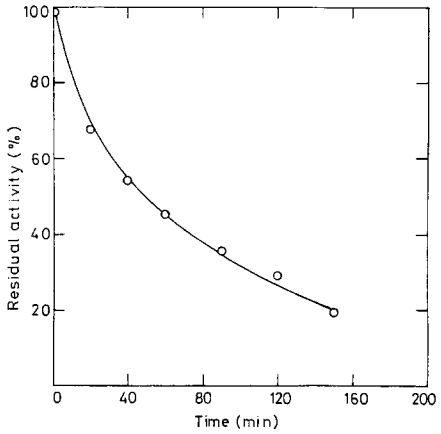


Fig. 2. The pK_i values as a function of pH at 30° for the inhibition of α-galactosidase-catalysed hydrolysis of *p*-nitrophenyl α-D-galactoside by $AgNO_3$.

Fig. 3. Inactivation of α-galactosidase by photo-oxidation in the presence of methylene blue. The experimental conditions are described in MATERIALS AND METHODS.

Inhibition by SH reagents

N-Ethyl maleimide had no effect on α-galactosidase activity up to a concentration of 10 mM. A weak inhibition (29%) was shown by 5 mM iodoacetamide. With *p*-chloromercuribenzoate (PCMB) the inhibition (27%) was found at low concentration (1 μM) but increased very slowly with increasing PCMB concentration (40% inhibition at 10 μM). This behaviour was qualitatively similar to that of competitive inhibition by Hg^{2+} and this could be possibly attributed to the reaction of PCMB with the histidine group. The chance of reaction with any thiol group is excluded on the grounds that estimation of the group by the method of BOYER[16] gave negative results.

Effect of photo-oxidation

Photo-oxidation in the presence of methylene blue brought about inactivation of the α-galactosidase (Fig. 3). This inactivation is known to be caused by the destruction of histidine, tryptophan, tyrosine or thiol groups in several enzymes[17–21]. Tryptophan and tyrosine residues were estimated by the method of GOODWIN AND MORTON[22] and thiol groups, after BOYER[16], both before and after photo-oxidation. The results presented in Table II show that these residues were not affected by photo-oxidation. It is therefore apparent that the histidine groups are destroyed during inactivation of the enzyme by photo-oxidation.

Effect of sugars and their derivatives

The results of inhibition studies by sugars and their derivatives are listed in Table III. Strongest competitive inhibition was produced by D-galactose and 1:4-galactonolactone followed by a weaker inhibition by L-arabinose and D-glucose. The inhibition by L-arabinose was expected because of its structural similarity to D-galactose and also that *p*-nitrophenyl β-L-arabinoside was hydrolysed by the enzyme[23]. It is remarkable that whereas a β-galactoside had no inhibitory effect, the α-glucoside

TABLE II

EFFECT OF PHOTO-OXIDATION ON TYROSINE, TRYPTOPHAN AND THIOL CONTENT OF SWEET ALMOND α-GALACTOSIDASE

The aminoacid residues are expressed in moles present in one mole of enzyme (mol. wt. 33 000).[6]

Aminoacid residue	*Untreated enzyme*	*Photo-oxidised enzyme*
Tyrosine	16.9 ± 1.2	17.0 ± 1.3
Tryptophan	9.2 ± 1.0	9.4 ± 1.2
Free thiol (native enzyme)	Nil	Nil
Masked thiol (urea denatured)	7.5 ± 0.2	7.5

inhibited the enzyme. The configuration of the anomeric carbon atom, therefore, seems to be important. α-D-Galactose 1-phosphate was neither a substrate[23] nor an inhibitor; the lack of binding could be due to steric effects (large aglycon) or due to electronic effects (the phosphate group will be negatively charged at the pH of assays. The non-inhibitory nature of sodium galactonate shows that open chain derivatives of galactose have no affinity for the enzyme. The inhibition by 1:4-galactonolactone was in accordance with the generalization of LEVVY[24] and CONCHIE[25] that glycosidases are strongly inhibited by the aldonolactones of identical configuration.

TABLE III

INHIBITION OF SWEET ALMOND α-GALACTOSIDASE BY SUGARS AND THEIR DERIVATIVES

Experiments were carried out in McILVAINE[8] buffer (pH 5.5) keeping the assay conditions similar as for metal-ion inhibition (see Table I).

Sugars and their derivatives	*Concn. in assay (mM)*	*Residual activity (%)*	*Nature of inhibition*	K_i *(mM)*
L-Arabinose	100	60	Competitive	11.7
D-Glucose	100	86	Competitive	452.0
D-Galactose	2.5	63	Competitive	4.45
D-Fructose	1000	90	—	
Maltose	1000	78	—	—
Lactose	100	84	—	—
Sucrose	1000	100	—	—
o-Nitrophenyl β-D--galactoside	40	100	—	—
p-Nitrophenyl α-D--glucoside	40	89	—	—
α-D-Galactose 1--phosphate	5	100	—	—
1:4-Galactonolactone	6.2	55	Competitive	1.61
Sodium galactonate	6.2	100	—	—

Transfer and synthetic activities

When glucose and sucrose were used as galactosyl acceptors the resulting products were melibiose and raffinose, respectively. These compounds were isolated in crystalline forms after chromatographic separation on a charcoal column. They were further characterised as shown in Table IV. When galactose was used as an

TABLE IV

ALAC-CHARACTERIZATION OF TRANSGALACTOSYLATION PRODUCTS FORMED BY SWEET ALMOND α-GALACTOSIDASE

Property	*Transfer product Acceptor: glucose*	*Melibiose (standard)*	*Transfer product Acceptor: sucrose*	*Raffinose (standard)*
Melting point	181–182°	182°	77–78°	78°
$[\alpha]_D^{30}$ (*c*, 0.5% in water)	+129°	+130°	+106°	+105.2°
R_{Glc}	0.38	0.38	0.36	0.36
Melting point of the acetate	177.5°	177°	99°	99–101°

acceptor, three new oligosaccharides (R_{Glc}, 0.30, 0.37 and 0.49) were produced which, when treated separately with excess α-galactosidase, yielded only galactose. The chromatographic mobilities of the compounds were typical of a homologous series[26,27] and a comparison with the standard compounds showed that they were galactobiose, galactotriose and galactotetrose. The galactose residues in all these compounds were all linked by α(1 → 6) linkages. When maltose was used as a galactosyl acceptor, two new oligosaccharides (R_{Glc}, 0.19 and 0.33) were produced. The product with a R_{Glc} of 0.33 on incubation with an α-glucosidase preparation[28] yielded glucose and melibiose whereas incubation with excess α-galactosidase yielded galactose and maltose. The possible structure of the sugar may therefore, be written as, (I) Gal α 1 → 6 Glc α 1 → 4 Glc or (II) Gal α 1 → 6 Glc 4 ← 1 α Glc. Normally reactions of glycosidases start from the non-reducing end of substrate molecules and therefore the structure (II) would be preferred. On incubating α-galactosidase with galactose alone, galactobiose and galactotriose were formed.

From these results it would appear that the enzyme catalyses transfer as well as synthetic reactions. The primary alcoholic group of the acceptor sugar was preferred for both transfer and synthetic reactions and the configuration of the transferred galactosyl residue was retained. If a similar mechanism operates for the enzyme-catalysed hydrolysis of an α-galactoside, one would then expect the liberation of free galactose with an α-configuration. The results summarised in Table V show that it is in fact true. It is also significant that all the three reactions (*i.e.* hydrolysis, transfer

TABLE V

ANOMERIC NATURE OF THE GALACTOSE MOIETY LIBERATED DURING ENZYMIC HYDROLYSIS

p-Cresyl α-D-galactoside (0.15%, w/v) and purified sweet almond α-galactosidase[6] (125 μg/ml) were incubated at 30° in 0.1 M sodium acetate buffer (pH 5.5) and the optical rotation was noted at various time intervals. Complete hydrolysis of the substrate occurred in the first 3 min of incubation.

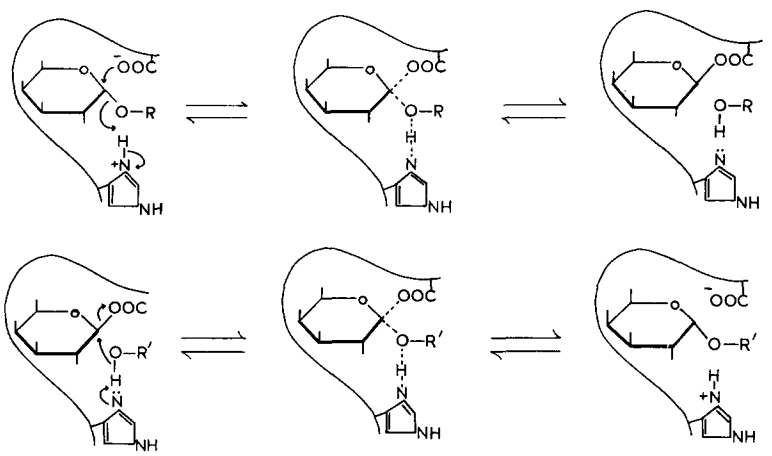
Time (min)	$[\alpha]_D^{30}$
3	+150°
30	+100°
60	+ 85°
120	+ 85°

and synthesis) catalysed by the enzyme are inhibited to the same extent by Ag^+ suggesting that a common active site is involved.

Mechanism of action

SWAIN AND BROWN[29] demonstrated that a multifunctional catalyst was much more efficient than a mixture of compounds possessing the same catalytic groups. The idea of enzymes functioning as multifunctional catalysts has since been put forward for a number of cases *e.g.* fumarase[30], maltase[31], yeast invertase[32,33], β-galactosidase[13] *etc.*

The specificity studies[23] of sweet almond α-galactosidase especially with aryl α-D-galactosides, showed that the electronic nature of the aglycon had a profound influence on the rate of enzymic hydrolysis. Two sets of straight lines ($\varrho = +0.054$ and -1.5) were obtained by plotting log v_{max} against Hammett substituent constants (σ) which met at a point corresponding to phenyl α-D-galactoside ($\sigma = 0$). This resembles alkaline and acid hydrolysis of aryl glycosides[34] and may therefore, be attributed to the presence of a basic and an acidic group on the enzyme active site. These groups were identified by kinetic studies as carboxyl (deprotonated) and histidine (protonated), respectively[1]. Photo-oxidation and inhibition by Ag^+ discussed in the present paper led to similar postulations. The results of specificity experiments[23] also indicated that the enzyme was more specific for the glycon configuration of the substrate. This, according to the empirical rule formulated by KOSHLAND[35], would suggest that the glycon–oxygen cleavage is most likely to occur during the enzymic hydrolysis of the substrate. A two-step mechanism of action may be postulated from these results, as follows:

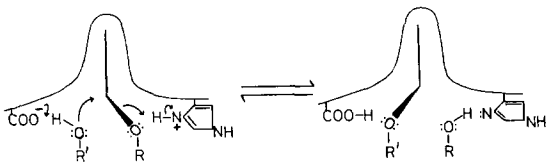


Two-step mechanism

The aglycon is split by a concerted action of the carboxyl and the imidazolium group in the enzyme active site. This is followed by the intake of the acceptor molecule (R'OH), which may be water or a sugar, and formation of the final product *via* a transition state. It has been shown that for *p*-nitrophenyl α-D-galactoside, the reaction rate does not attain zero at pH values lower than 4.5 (ref. 1). Thus the deprotonated carboxyl group in the enzyme active site seems to be less important than the protonated imida-

zolium group. It is therefore likely that the electrophilic attack of imidazolium group is sufficient to cleave the glycosyl–oxygen bond with the formation of a carbonium ion at C-1 of the galactose moiety. The carbonium-ion intermediate need not lead to racemization in an enzyme-catalysed reaction because its configuration might be retained by its specific mode of binding to the enzyme active site (*cf.* refs. 36 and 37). On the other hand it is more likely that a Walden inversion might take place at each step leading to retention of configuration in the final product as shown in the above scheme.

An alternative one-step mechanism may also be suggested as follows:



One-step mechanism

It would involve the formation of a ternary complex of the enzyme, substrate and the acceptor. The carboxyl and the imidazolium groups would play a similar role as in the two-step mechanism. This mechanism would involve a "front-side attack" (*cf.* ref. 35) on the anomeric carbon atom of the galactose moiety which would lead to a product with retention of configuration. An examination of a space-filling atomic model of phenyl α-D-galactopyranoside will show that the stable C1 chair conformation of REEVES[38] would not allow a "front-side attack" (*cf.* refs. 13 and 39). A similar steric hindrance could be observed in the less stable 1C conformation. Such a hindrance will disappear in the B2 and 3B boat conformations. The 3B conformation should be preferred because it is free from the interaction between the two "flag-pole" bonds which is very strong in B2. Thus, it can be assumed that during the complex formation between the enzyme and the substrate, the latter undergoes a change in the galactose conformation from C1 to 3B. The less important role of the carboxyl group could be explained more readily by the one-step mechanism because this group serves only to remove a proton from the acceptor (R'OH). If the carboxyl group (deprotonated) is not available for participation in the reaction, this proton will have to be transferred to a water molecule which is not as efficient a base and therefore the reaction will proceed at a reduced rate.

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