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ACTION OF AMYLOGLUCOSIDASE ON OXIDISED AMYLOSE

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ABSTRACT

The Michaelis constant and maximal velocity of alpha-amylase-free amyloglucosidase decrease with increasing periodate oxidation of amylose. These kinetic features have been explained on the basis of competitive inhibition by the oxidised non-reducing end of the (1→4)-α-D-glucan chain with the active centres of the enzyme. A kinetic model is proposed to demonstrate this special kind of inhibition where the concentration of inhibitor is directly proportional to the substrate concentration. The experimental data fitted this model, and the plots of $1/K_m$ and $1/V$ against the ratio of oxidised/unoxidised non-reducing end-groups were straight lines.

INTRODUCTION

Amyloglucosidase [(1→4)-α-D-glucan glucohydrolase; EC 3.2.1.3] can cleave α-(1→4) bonds at the non-reducing ends of (1→4)-α-D-glucan chains, releasing D-glucose as the sole product. Some amyloglucosidases also exhibit (1→6)-α-D-glucanase activity. In the same way as beta-amylase, amyloglucosidase inverts the configuration of the product released and acts according to an exo-attack pattern.

The Michaelis constant (K_m) and maximal velocity (V) of amyloglucosidase depend[1,2] on the degree of polymerisation (d.p.) of the substrates. The K_m values decrease with the d.p. of the substrate, and k_{+2} ($k_{+2} = V/[E]$; where [E] is enzyme concentration) appears to follow the same pattern[2] up to a certain critical value of d.p. (up to maltoheptaose).

Thoma and Koshland[3] have proposed an explanation for this behaviour of K_m and k_{+2}, based on the possibility of competitive inhibition by the internal residues of the (1→4)-α-D-glucan chains with the active centres of exo-amylases. Inhibition by internal segments of homogeneous (1→4)-α-D-glucan chains cannot be demonstrated with the classical equations for competitive inhibition, since the concentration of inhibitor is directly proportional to the concentration of substrate.

Marshall and Whelan[4] have developed a method based on the use of partially oxidised amyloses to detect traces of alpha-amylase (endo-amylase) in preparations of beta-amylase or amyloglucosidase. Exo-amylase activity is blocked by the presence of these oxidation points, whereas endo-amylase activity can bypass them. Therefore,

in the amyloglucosidase-catalysed hydrolysis of partially oxidised, linear (1→4)-α-D-glucan substrates, there are two possibilities for enzyme–substrate complex formation, namely, an active complex of a terminal non-reducing unit with amyloglucosidase and an inactive complex of a terminal oxidised non-reducing unit with amyloglucosidase. The latter will lead to competitive inhibition, which cannot be demonstrated with the conventional equations since the concentration of inhibitor is also directly proportional to the concentration of substrate.

A kinetic model is now proposed to explain the action of amyloglucosidase on partially periodate-oxidised amylose.

For a linear polymer having n residues of which a proportion p are oxidised, complexed to amyloglucosidase, there will be $(n - np)$ active complexes and np inactive complexes. The concentration of inhibitor is then given by equation *1*.

$$I = \left(\frac{np}{n - np}\right) S = \left(\frac{p}{1 - p}\right) S, \tag{1}$$

where I and S are the concentrations of the inhibitor and substrate, respectively. Thus,

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_m}{S} + \left(\frac{p}{1 - p} \times \frac{K_m}{K_i}\right)\right], \tag{2}$$

where K_i is the dissociation constant of the EI complex.

Equation *2* can be simplified into the linear form of the Michaelis–Menten equation,

$$\frac{1}{v} = \frac{1}{V'}\left(1 + \frac{K'_m}{S}\right), \tag{3}$$

in which V' (observed maximal velocity) and K'_m (observed Michaelis constant) are complex functions given by equations *4* and *5*.

$$V' = V\left(\frac{1}{1 + \frac{p}{1 - p} \times \frac{K_m}{K_i}}\right) \tag{4}$$

$$K'_m = K_m\left(\frac{1}{1 + \frac{p}{1 - p} \times \frac{K_m}{K_i}}\right) \tag{5}$$

Equations *4* and *5* can also be written in the inverse forms *6* and *7*.

$$\frac{1}{V'} = \frac{1}{V} + \left(\frac{K_m/K_i}{V} \times \frac{p}{1 - p}\right) \tag{6}$$

$$\frac{1}{K'_m} = \frac{1}{K_m} + \left(\frac{1}{K_i} \times \frac{p}{1 - p}\right) \tag{7}$$

Equations *6* and *7* predict that plots of $1/V'$ and $1/K'_m$ *versus* $p/(1-p)$ will be straight lines and that, in the latter case, the slope will be $1/K_i$. Therefore, these observations provide an opportunity to test this theory experimentally. In addition, if p is zero (no oxidation), the observed Michaelis constant K'_m will be reduced to K_m, namely, the Michaelis constant in the absence of oxidation of the substrate. The same considerations apply to the observed maximal velocity. However, if 100% oxidation of substrate occurs, p becomes unity, and K'_m and V' become zero.

EXPERIMENTAL

Amyloglucosidase. — (*a*) *Preparation.* The mould *Rhizopus delemar* (CMC 44,245) was grown in submerged culture in a 14L New Brunswick Fermentor for 36–48 h in 12-litre batches at 32°, with aeration at 10 litres/min and agitation at 400 revs./min. The culture medium contained 3% of malt extract and 0.5% of mycological peptone. The culture was filtered through a coarse Buchner filter to remove fungal material, and concentrated to ~1 litre by rotary evaporation at 40°/~15 mmHg. The concentrate was centrifuged at 12,000 *g* for 10 min, and the supernatant solution was freeze-dried. An aqueous 15% solution (4.0 ml) of the freeze-dried, crude enzyme was mixed with 99% ethanol (16 ml) and centrifuged as above. The precipitate was dissolved to 4.0 ml in 0.02M acetate buffer (pH 4.2), and this solution was applied to a pre-cycled and degassed column (500 × 25 mm) of DEAE-cellulose DE-32 (Whatman Biochemicals Ltd., England) which had been equilibrated with degassed 0.02M acetate buffer (pH 4.2). The column was washed with the same buffer at a flow rate of 3.5 ml/min, and the effluent was analysed for protein with an LKB-Uvicord.

The amyloglucosidase activity was coincident with the first peak, and the combination of this procedure and the ethanolic precipitation gave a purification of ~15-fold. The material obtained showed a specific activity of 1.42 units/mg of protein, and released a maximum of 28% of D-glucose residues from Cibachron Blue Amylose, but no soluble, coloured products were detected after exhaustive hydrolysis, indicating[5] the absence of traces of alpha-amylase.

(*b*) *Measurement of activity.* L-Ascorbic acid (150 nmol), 13 units of D-glucose oxidase [free of (1→4)-α-D-glucanase and catalase activities[6]], 1500 units of peroxidase, and unoxidised/oxidised amylose were dissolved in 2.8 ml of 25mM phosphate/citrate buffer containing 70mM oxalate (pH 5.8). After incubation for 5 min at 40°, 0.2 ml containing 12 munits of amyloglucosidase was introduced into the sample cell and into a control cell that lacked ascorbic acid. The decrease in extinction at 268 nm was recorded at a chart speed of 0.2 cm/min for at least 5 min with a Unicam SP 800 recording spectrophotometer. The change in extinction per min was converted into μmol of D-glucose released per min by the equation: μmol of D-glucose/min = (ΔE/min × 3.0)/18.4, where ΔE/min is the change in extinction per min. One unit of activity is defined as the amount of enzyme required to release 1.0 μmol of product per min under the specified conditions.

Preparation of oxidised amyloses. — Potato amylose type I (Sigma; 500 mg) was dissolved in 40 ml of distilled water, and a solution (10 ml) containing the calculated* amount of sodium metaperiodate, necessary to give 15, 30, 45, and 60% oxidation of amylose, was slowly added with stirring. The mixture was kept in the dark for 4 h, dialysed overnight against water, and freeze-dried. To determine the actual extent of oxidation, each oxidised amylose (10 mg) was dissolved in 4.0 ml of M HCl and incubated at 100° for 3 h. The pH of the solution was adjusted to 7.0 with M NaOH, the volume was made up to 10 ml with distilled water, and the glucose content of each hydrolysate was determined by the method of Lloyd and Whelan[7].

The number-average degree of polymerisation ($\overline{d.p.}$) of the amylose employed in this work was estimated by determining the reducing end-groups of the molecule by the periodate-oxidation method[8]; a value of 800 was found.

RESULTS AND DISCUSSION

The data in Table I show that the amount of glucose recovered after the acid hydrolysis of each oxidised amylose does not significantly differ from that theoretically expected. Drummond *et al.*[9] have demonstrated that, for an oxidised pullulan, oxidation takes place randomly. Probably, the same is true for amylose, so that the chance of having oxidation points located in the reducing ends of the (1→4)-α-D-glucans is equal to the degree of oxidation.

Fig. 1 shows the D-glucose released from unoxidised and oxidised amyloses by the action of amyloglucosidase at 40° and pH 5.8, when a substrate concentration of 0.166% (w/v) was employed. The rate of the hydrolysis decreases as the degree of oxidation increases. Initial velocities can be estimated directly from the initial

TABEL I

AMOUNTS OF D-GLUCOSE OBTAINED ON ACID HYDROLYSIS OF UNOXIDISED AND OXIDISED AMYLOSES[a]

Theoretical oxidation (%)	D-*Glucose in the samples*[b] *(mg/ml)*	D-*Glucose in the hydrolysates (mg/ml)*	*Recovered* D-*glucose (%)*	*Oxidised* D-*glucose (%)*
0	1.11	1.01	100.0	0.0
15	1.11	0.83	82.1	17.9
30	1.11	0.70	69.3	30.7
45	1.11	0.54	53.4	46.6
60	1.11	0.34	34.8	65.2
glucose	1.11	1.00	[c]	—

[a]As determined by the enzymic method of Lloyd and Whelan[7]. [b]Calculated by the formula: mg of D-glucose/ml = mg of amylose × (180/162). [c]10% of D-glucose was lost during the acid hydrolysis.

*Weight (mg) of $NaIO_4$ = (mg of amylose × % × 214)/16,200.

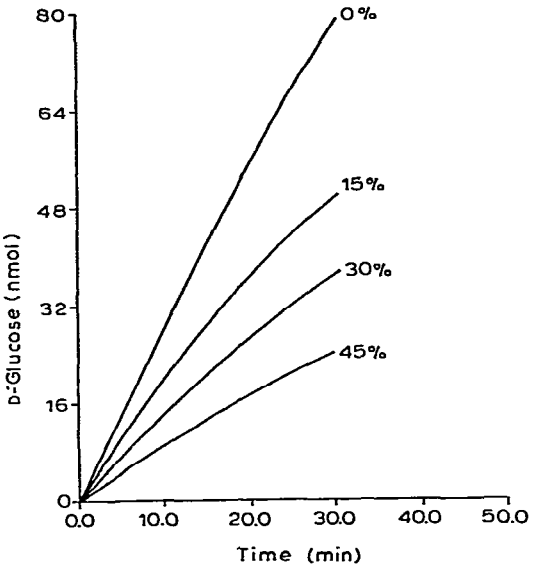

Fig. 1. The release of D-glucose from unoxidised and oxidised amylose by the action of amyloglucosidase. For details, see Experimental.

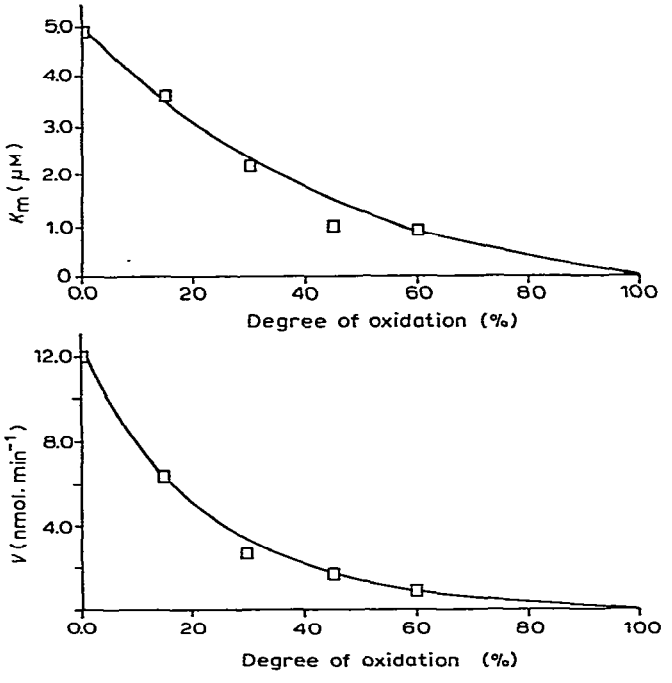

Fig. 2. Dependence of the apparent K_m and V of amyloglucosidase on the degree of oxidation of amylose. The kinetic parameters were calculated by using the Lineweaver–Burk plot of the initial velocities of the hydrolysis of oxidised and unoxidised amyloses catalysed by amyloglucosidase free of alpha-amylase: enzyme, 40nM; pH 5.8; 40°.

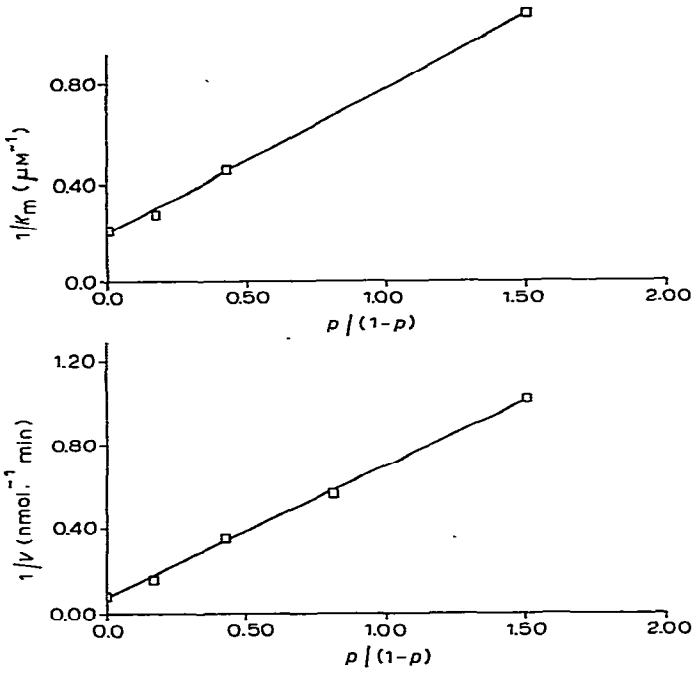

Fig. 3. Hydrolysis of unoxidised and oxidised amyloses catalysed by amyloglucosidase. The values of K_m and V are those displayed in Fig. 2; p denotes the proportion of the residues of the amylose molecule which are oxidised.

slopes obtained from such reaction–time curves as the typical examples shown in Fig. 1.

The values of K_m and V_{max} decrease with increasing oxidation, as shown in Fig. 2. These kinetic features, obtained with unoxidised and oxidised amyloses, are consistent with those predicted theoretically. Thus, the plots of $1/K_m$ and $1/V$ against $p/(1 - p)$ are straight lines, as shown in Fig. 3. As stated above and demonstrated here, the observed K'_m and V' should attain the value of zero (unmeasurable) when $p = 1$ (100% of residues oxidised). Equally, as p approaches zero, K'_m and V' tend to K_m and V.

The inhibition constant K_i was calculated from the inverse of the slope $1/K_m$ against $p/(n - p)$ (Fig. 3a) and was 1.02 μM. The alternative way to calculate this constant would be to use as inhibitor an oxidised amylose-limit-dextrin, prepared by exhaustive treatment[4] of the modified amylose with alpha-amylase-free amyloglucosidase. This approach should provide more information about the kinetics of amyloglucosidase action on oxidised amylose.

Although further studies will be necessary to confirm the formation of an inactive complex between the oxidised non-reducing end of (1→4)-α-D-glucans and amyloglucosidase, the theory discussed here, which is based on such an event and is supported by the experimental results, suggests that this kind of competitive in-

hibition might occur during the action of amyloglucosidase on partially oxidised amyloses.

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