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Effect of maltotriitol on the action pattern of porcine pancreatic α-amylase using amylose as a substrate

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ABSTRACT

The effect of the oligosaccharide analog maltotriitol (G_3OH) on the action pattern of porcine pancreatic α-amylase (PPA) was examined using amylose as a substrate. Fluorescence titration indicated that two molecules of G_3OH can bind to one molecule of PPA. The slope in the blue value *versus* extent-of-reaction plot was shifted by G_3OH from that for multiple attack in the direction of that for random attack as the G_3OH concentration increased. From these it is inferred that at least one molecule of G_3OH can bind at the active site of the enzyme so as to inhibit the sliding of the retained-product fragment after the initial cleavage of an amylose molecule.

INTRODUCTION

In the porcine pancreatic α-amylase (PPA)-catalyzed hydrolysis of polymeric substrates such as amylose, it has been proposed that the reaction proceeds through multiple attack at the optimum pH, 6.9, and through random attack at pH 10.5 (refs. 1–6). The multiple attack mechanism implies that on forming an enzyme–substrate complex the enzyme can successively cleave the substrate molecule several times before the enzyme–product complex dissociates. In random attack the enzyme hydrolyzes only one bond of the substrate molecule per encounter, and subsequently attacks another substrate molecule.

The difference between these action mechanisms has been detected mainly by measuring the rate of decrease in the blue value[3,5], the fluorescence value[6], or the viscosity[7], and by paper chromatography of the hydrolyzate[1,2,4]. The blue-value method is the most convenient and useful for analyzing the effect of low molecular weight compounds on the action pattern of α-amylases hydrolyzing long-chain substrates.

Although it is known that the action pattern of PPA is affected by changes in pH and by the addition of methyl α-glucoside[5,6], information concerning the molecular mechanism of these changes has been lacking. However, maltotriitol (G_3OH), having its reducing-end residue in the alditol form, appeared to be a good candidate for use in further studies on the action pattern of PPA; it has no reducing power yet can bind to the

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active site of the enzyme. In this paper, we aimed to examine the relationship between the action pattern of PPA and the mode of binding of G_3OH to PPA, using spectroscopic titration.

EXPERIMENTAL

Materials. — PPA[6], glucoamylase[8], and amylose EX-I (ref. 6), having a number average degree of polymerization ($\overline{d.p.}_n$) of 17, were the same as those described in the previous papers. Amylose from potato was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and its $\overline{d.p.}_n$ was determined to be ~300 from reducing power measurements by the modified Somogyi–Nelson method[9]. G_3OH was purchased from Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and used without further treatment. Its purity was estimated as 99% by high performance liquid chromatography (h.p.l.c.). Other chemicals were of reagent grade and were used as purchased.

Methods. — Fluorescence measurement was performed at 25° with a Union Giken fluorescence spectrophotometer FS-401, on samples dissolved in a 50mM sodium glycerophosphate buffer (pH 6.9) containing 25mM NaCl.

The excitation wavelength was fixed at 295 nm. The fluorescence titration of PPA with G_3OH was carried out by the successive addition of concentrated G_3OH solution using a microsyringe. The initial PPA sample was used as a standard to correct for instrumental drift. Correction was also applied as needed for the small absorbance of G_3OH at 295 nm.

The PPA-catalyzed hydrolysis of amylose having $\overline{d.p.}_n$ values of 17 and 300 was carried out at 25° in 50mM buffers containing 25 mM NaCl. The buffers used were: pH 6.9, sodium glycerophosphate; pH 10.5, Na_2CO_3–$NaHCO_3$. At appropriate intervals, 1.5 mL of reaction mixture was withdrawn and put into 1.5 mL of 0.08M NaOH to stop the reaction. The solution was then divided into two parts for, respectively, measurement of the blue color of the amylose–I_3 complex or a quantitative product analysis, and determination of the reducing power. In the case of amylose of $\overline{d.p.}_n$ 300, blue-value measurement was conducted by adding 1 mL of NaOH-quenched solution to 6 mL of I_2–KI solution (2mM I_2 and 20mM KI) and, after dilution up to 20 mL, reading the absorbance at 630 nm. The blue value was expressed as a percentage ratio of the absorbance after a given hydrolysis time to that observed at time zero. In the case of amylose having $\overline{d.p.}_n$ 17, h.p.l.c. was used for the separation and the quantitative analysis of individual oligosaccharides in the hydrolyzate. The instrument used was a Toyo Soda high speed liquid chromatograph HLC-802UR. The conditions for separation were the same as those described in the previous paper[8], except for the column temperature of 54°.

In each case, the total reducing power after termination of the PPA reaction and then after complete hydrolysis of the amylose by glucoamylase was determined as glucose equivalent by the modified Somogyi–Nelson method[9]. The values were corrected for the reducing power of the initial amylose solution, then the extent of reaction was expressed as the percentage of bonds split by PPA.

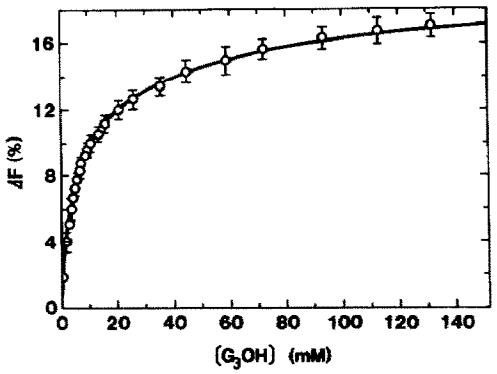

Fig. 1. Fluorescence titration of PPA with G_3OH at pH 6.9 and 25°. PPA concentration, 1.46μM. The excitation and emission wavelengths were fixed at 295 and 340 nm, respectively. A circle (○) and vertical bar represent the mean value of three runs and its standard deviation, respectively. The solid line represents the theoretical curve according to equation *3*.

RESULTS

Binding of G_3OH to PPA. — The emission maximum in the fluorescence of PPA was found to be about 345 nm at the excitation wavelength of 295 nm. The fluorescence intensity was decreased by the addition of G_3OH, but the maximum was hardly shifted. The same phenomenon was seen with the substrate maltotriose (not shown in this paper). The extent of decrease in the fluorescence intensity accompanying the binding of G_3OH to PPA was expressed as a percentage, based on the fluorescence intensity of the free enzyme, and defined as ΔF. The dependence of ΔF on the concentration of G_3OH is shown in Fig. 1.

A characteristic of the binding-saturation curve is its biphasic nature. The result can be reasonably explained by assuming that G_3OH forms first a 1:1 complex and then a 2:1 complex with PPA as represented in the following equation,

$$\mathrm{PPA} \underset{K_{d1}}{\overset{+G_3OH}{\rightleftharpoons}} \mathrm{PPA}\cdot G_3OH \underset{K_{d2}}{\overset{+G_3OH}{\rightleftharpoons}} \mathrm{PPA}\cdot(G_3OH)_2$$

where K_{d1} and K_{d2} are the dissociation constants of the PPA·G_3OH and PPA·$(G_3OH)_2$ complexes, respectively. Provided the fluorescence intensity decrease is proportional to the concentration of each complex and the changes are additive, the observed ΔF can be expressed as follows,

$$\Delta F = \Delta F_1 + \Delta F_2 \qquad (2)$$

where ΔF_1 and ΔF_2 are the fluorescence intensity decreases corresponding to the PPA·G_3OH and PPA·$(G_3OH)_2$ complexes, respectively. Equation *2* can then be expanded to give equation *3* by using K_{d1} and K_{d2}.

$$\Delta F = \frac{\Delta F_{max\,1}}{1 + \frac{K_{d1}}{S_0} + \frac{S_0}{K_{d2}}} + \frac{\Delta F_{max\,2}}{1 + \frac{K_{d2}}{S_0} + \frac{K_{d1} \cdot K_{d2}}{S_0^2}} \quad (3)$$

In the equation S_0 is the initial concentration of G_3OH and ΔF_{max1} and ΔF_{max2} denote the fluorescence intensity decreases that should be observed when all PPA molecules have formed PPA·G_3OH complex, and when all PPA·G_3OH complex has formed PPA·$(G_3OH)_2$ complex, respectively. The be values of K_{d1} and K_{d2} were estimated by a least-squares fit between the calculated and observed fluorescence intensity changes according to equation *3* (ref. 10). The results thus obtained for K_{d1}, K_{d2}, ΔF_{max1}, and ΔF_{max2} were 3.20 mM, 47.0mM, 11.6%, and 7.53%, respectively.

Effect of G_3OH on the kinetics of amylose hydrolysis by PPA. — It has been shown that the action pattern of PPA depends on pH. In the hydrolysis of amylose of $\overline{d.p.}_n$ 17, catalyzed by PPA, the product distribution was quantitatively monitored at intervals by h.p.l.c. at the optimum pH 6.9 (where multiple attack occurs) and at the alkaline pH 10.5 (where mainly random attack occurs). The results are shown in Fig. 2 as plots of the concentrations of oligosaccharides from glucose to maltoheptaose against the extent of reaction. Comparing Fig. 2A and Fig. 2B, one can see that glucose, maltose, and maltotriose are produced in larger amounts at pH 6.9 than at pH 10.5, and conversely that maltotetraose and the longer oligosaccharides are produced in smaller amounts at pH 6.9 than at pH 10.5. It can be inferred from these findings and the previous observations[4,6] that sliding by glucosyl and maltosyl units of the retained product-fragment is actually occurring on the active site of the enzyme in the PPA- catalyzed hydrolysis of amylose, to give multiple attack.

The blue value is an index of the random hydrolysis of long-chain amylose substrates, and the extent of reaction measured by reducing value is an index of the total

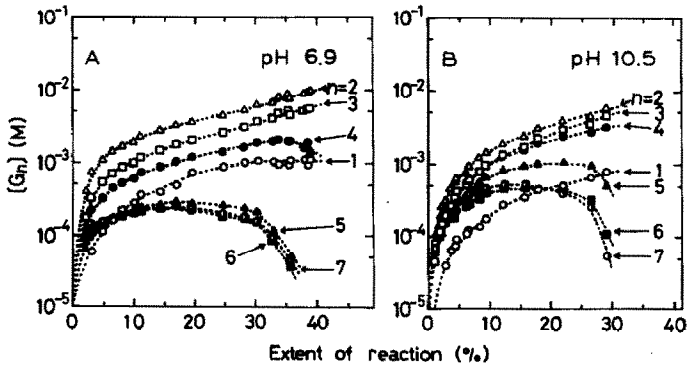

Fig. 2. Product distribution curves in the hydrolysis of amylose having a $\overline{d.p.}_n$ of 17, catalyzed by PPA at pH 6.9 (A) and pH 10.5 (B). PPA concentration, 18.9nM at pH 6.9 and 113nM at pH 10.5; amylose concentration, 1.0%. Symbols: ○, glucose; △, maltose; □, maltotriose; ●, maltotetraose; ▲, maltopentaose; ■, maltohexaose; ○, maltoheptaose; n = 2, etc. indicate d.p.

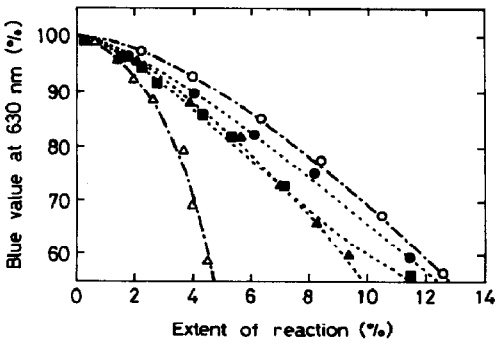

Fig. 3. Effect of G_3OH on the blue value *versus* extent-of-reaction plot. The PPA-catalyzed hydrolysis of amylose of $\overline{d.p.}_n \sim 300$, concentration 0.098%, was carried out at 25°; pH 6.9 (○) and pH 10.5 (△) in the absence of G_3OH, and at pH 6.9 in the presence of G_3OH (●, 3.79mM; ▲, 50.0mM; ■, 202mM). The PPA concentrations were: ○, 3.27nM; ●, 3.28nM; △, ▲, and ■, 13.1nM.

number of bonds hydrolyzed. Therefore, when the long-chain amylose was used as a substrate, the slope of the blue value *versus* extent-of-reaction plot at pH 10.5, where mainly random attack occurs, must be steeper than that at pH 6.9, where mainly multiple attack occurs. This was confirmed as shown in Fig. 3. The effect of various concentrations of G_3OH is also shown in the figure, where only the early stage of reaction is traced, so as to exclude interference from secondary attack by PPA on the oligosaccharide products. It is obvious that the slope of the plot was shifted to that obtained at pH 10.5 as the G_3OH concentration was increased.

DISCUSSION

The relationship between the action pattern of PPA and the mode of binding of G_3OH will be discussed on the basis of the known features of the structure of the enzyme and its mechanism[4,11]. The active site of PPA consists of five subsites, each of which interacts with a successive glucose residue of the substrate with an inherent affinity, the subsite affinity. The catalytic site is located between the third and fourth subsites, numbered from the subsite proximal to the nonreducing end of the substrate (Fig. 4).

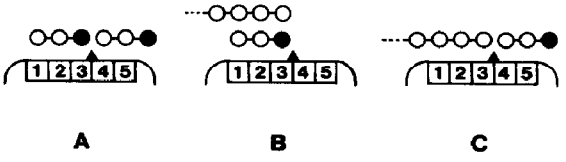

Fig. 4. Schematic representation of the modes of binding of G_3OH to PPA: A, the most probable binding mode in the PPA·$(G_3OH)_2$ complex; B, a competitive-inhibitory binding mode in which G_3OH competes with the substrate; C, the multiple attack-blocking binding mode, in which G_3OH competes with the substrate or inhibits the sliding of the retained product-fragment. The active site of PPA is indicated by the numbers 1–5 and the catalytic site by the symbol ▲; ○ and ● represent the glucosyl and glucitol residues, respectively.

From the results of the fluorescence titration of PPA with G_3OH it was concluded that two molecules of G_3OH bind to the active site of one molecule of PPA. Considering the subsite structure and the sliding polarity of PPA[4,11], and the dissociation constants of the PPA·G_3OH complexes, it can be inferred that one molecule of the two G_3OH's is bound in the active site to the left of the catalytic site, and the other one to the right, as shown in Fig. 4A.

The presence of one G_3OH molecule in the region to the left of the catalytic site (Fig. 4B) would lead to competitive inhibition, but it would not affect the mechanism of action. Binding of the substrate, hydrolysis, and sliding of the retained product-fragment (thought to be bound in subsites 1, 2, and 3) would occur during intervals when the inhibitor is dissociated.

The binding of one G_3OH molecule in the right-hand region (subsites 4 and 5) of the unoccupied enzyme would again cause competitive inhibition. However, binding in this region could also occur after the release of the first product-fragment, while the retained product-fragment occupied subsites 1–3 (Fig. 4C). In this situation the sliding of the product-fragment would be blocked, and the action pattern would be shifted in the direction of random attack. The shift should become more pronounced with increase in the concentration of G_3OH, as was actually observed.

Although the possibility may exist that G_3OH can bind also to a secondary site, removed from the active site, the effect of the compound on the action pattern shows that at least one molecule of G_3OH binds to subsites 4 and 5, preventing multiple attack while it is bound.

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