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In vitro action of human and porcine α-amylases on cyclomalto-oligosaccharides

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ABSTRACT

The vitro action of human and porcine pancreatic α-amylases on cyclomalto-oligosaccharides (cyclodextrins) was investigated both by a high-performance liquid chromatographic analysis and a quantitative analysis of the reducing power of cyclodextrin hydrolyzates. Cyclomalto-octaose (γ-cyclodextrin) was hydrolyzed to produce mainly maltose, but cyclomalto-hexaose and -heptaose were little affected both by human and porcine α-amylases. Quantitative analysis of reducing power revealed that the ring-opening rate of γ-cyclodextrin catalyzed by human pancreatic α-amylase was 2.8 times slower than that catalyzed by the porcine enzyme. The number of multiple attacks on γ-cyclodextrin and its inhibitor constants for human pancreatic α-amylase and porcine pancreatic α-amylase were almost the same.

INTRODUCTION

Cyclomalto-oligosaccharides (cyclodextrins) are cyclic oligosaccharides produced from starch by certain bacterial enzymes[1–3]. They have the ability to form inclusion compounds with smaller molecules that fit into their cavities[1–6]. This property can be used for the improvement of foodstuffs, cosmetics, pharmaceuticals, agricultural chemicals, *etc.*[3,7].

Because cyclodextrins are used as food additives to aid in emulsification, the stabilization of flavors, and the masking of undesirable tastes, it is important to examine the digestibility and the toxicity of these products. It has been reported that cyclomalto-heptaose (β-cyclodextrin) has no toxic action in rats[8]. However, the susceptibility of cyclodextrins to enzymic digestion has hardly been investigated. In this paper, we report the *in vitro* action of human and porcine α-amylases on cyclodextrins as studied both by high performance liquid chromatography (h.p.l.c.) and by measurement of the reducing power of cyclodextrin hydrolyzates.

EXPERIMENTAL

Materials. — Porcine pancreatic α-amylase (PPA)[9] and glucoamylase[10] were the same as those described in the previous papers. Human salivary α-amylase (HSA) was

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purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and used without further purification. Activity of HSA (international units/liter) was determined using 2-chloro-4-nitrophenyl β-maltopentaoside as a substrate (Diacolor AMY Rate, purchased from Ono Pharmaceutical Co., Ltd., Osaka, Japan). Human pancreatic α-amylase (HPA), which was purified according to the method of Matsuura *et al.*[11], was a generous gift from Professor T. Yamamoto. Cyclomaltohexaose (α-cyclodextrin) and β-cyclodextrin were the same as those described in the previous paper[12]. Cyclomalto-octaose (γ-cyclodextrin) and linear maltopentaose (G_5) were purchased from Nakarai Chemicals Ltd., Kyoto, Japan and used without further purification. Other chemicals were of reagent grade and used without further purification.

Methods. — The PPA-catalyzed hydrolysis of cyclodextrins was carried out at 37° in a 50mM sodium glycerophosphate buffer, pH 6.9, containing 25mM NaCl. The HPA- and HSA-catalyzed hydrolyses were done at 37° in a 20mM sodium glycerophosphate buffer, pH 7.0, containing 10mM NaCl and 10mM calcium acetate. At appropriate intervals, 1 mL of reaction mixture was withdrawn and put into 1 mL of 0.1M NaOH solution to stop the reaction. After this solution was filtered through a Millipore filter the filtrate was subjected to h.p.l.c. for the separation and quantitative measurement of the individual oligosaccharide products. 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[10], except that the column temperature was 56°.

The hydrolysis of γ-cyclodextrin by HPA and PPA was quantitatively monitored by a procedure based on the method of Suetsugu *et al.*[13]. At appropriate intervals 1.5 mL of reaction mixture was discharged into 1.5 mL of 0.01M NaOH, and the solution was immidiately boiled for 4 min to completely inactivate HPA and PPA. The reducing power in one half of this solution was determined as glucose equivalent by the modified Somogyi-Nelson method[14]. The pH of the other half was adjusted to 4.3 for HPA and 4.8 for PPA with 0.02M HCl solution and 100mM acetate buffer, pH 4.5, and subsequently subjected to treatment with glucoamylase (1.1 μM) for 10–24 hr at 25° in order to completely hydrolyze oligosaccharide products into glucose. The hydrolysis was verified by h.p.l.c., and reducing power of the glucose produced was measured by the modified Somogyi-Nelson method[14]. During this treatment renaturation of the HPA and PPA did not occur, judging from the invariant reducing power.

The inhibitor constant of cyclodextrins for HPA and PPA were determined by measurement of reducing power by the modified Somogyi-Nelson method[14], using G_5 as a substrate.

RESULTS AND DISCUSSION

Comparison of oligosaccharide products in the HPA-, HSA-, and PPA-catalyzed hydrolysis of cyclodextrins. — Figure 1 shows the time course of changes in the concentrations of oligosaccharides produced from γ-cyclodextrin by the action of HPA, HSA, and PPA at 37°. It was found that the three α-amylases hydrolyze γ-cyclodextrin

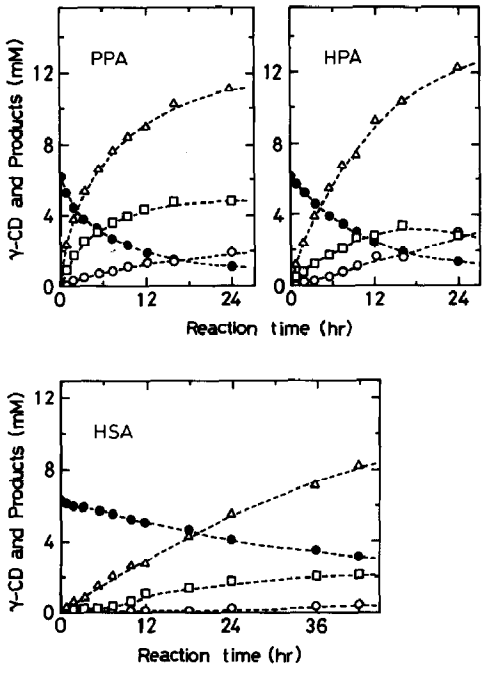

Fig. 1. Product distribution in the hydrolysis of γ-cyclodextrin catalyzed by α-amylases: ○, glucose; △, maltose; □, maltotriose; ●, γ-cyclodextrin (γ-CD). γ-Cyclodextrin concentration, 6.18mM; other experimental details are given in the text.

mainly to maltose. They do not produce maltotetraose or longer oligosaccharides in detectable amounts. After 24 hr at 37° about 80% of the γ-cyclodextrin was hydrolyzed by HPA and PPA at enzyme concentrations of 50nM, and about 40% of γ-cyclodextrin was hydrolyzed by HSA at 1570 $U.L^{-1}$. α- And β-cyclodextrins were not hydrolyzed by any of the three α-amylases under the same experimental conditions. However, PPA hydrolyzed about 20% of β-cyclodextrin on incubation for 24 hr at an enzyme concentration of 8.7μM. Based on the above findings, the rate of PPA-catalyzed hydrolysis of γ-cyclodextrin is estimated to be about 3000 times faster than that of β-cyclodextrin. On the other hand, HSA did not hydrolyze β-cyclodextrin at an enzyme concentration of ~13 750 $U.L^{-1}$, even after 42 hr of incubation.

Kinetic analysis of the hydrolysis of γ-cyclodextrin catalyzed by HPA and PPA. — HPA and PPA hydrolyze linear substrates by a multiple attack mechanism[15-21], and it can now be shown that this mechanism operates when γ-cyclodextrin is cleaved by HPA and PPA. According to this mechanism, an encounter between α-amylase and cyclodextrin which results in a ring-opening reaction is followed by a sliding of the linear oligosaccharide product on the active site of the enzyme and subsequent cleavage of the initial product to produce smaller oligosaccharides before the enzyme-oligosaccharide product-complex dissociates. A novel method for evaluating the degree of multiple attack on the linear oligosaccharides produced when Taka-amylase A digests cyclodextrins was developed by Suetsugu *et al.*[13], who measured two kinds of reducing power

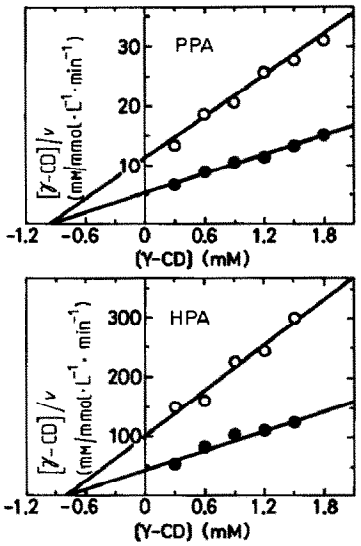

Fig. 2. Plots of $[\gamma\text{-CD}]/v$ *versus* $[\gamma\text{-CD}]$ for hydrolysis catalyzed by α-amylases: ○, initial velocity (v) determined from the reducing power of the α-amylase hydrolyzate; ●, v determined from reducing power after glucomylase treatment. PPA concentration, 65.4nM; HPA, 17.6 nM; other conditions as described under METHODS.

concurrently. One was the reducing power of the α-amylase hydrolyzate, which contains linear oligosaccharides produced both from the ring-opening of the cyclodextrin and from multiple attack; the other was the reducing power after glucoamylase treatment of the hydrolyzate. Glucoamylase hydrolyzed the product oligosaccharides completely into glucose. The ring-opening rate of cyclodextrin and the number of multiple attacks can be quantitatively evaluated from the two kinds of reducing power measurement.

Figure 2 shows the relationship between the γ-cyclodextrin concentration and the initial velocity determined by measuring the reducing power before and after glucoamylase treatment of the hydrolyzate. As mentioned above, hydrolytic activity before glucoamylase digestion is a combination of the ring-opening of cyclodextrin and repetitive attack on the product, and this apparent molucular activity is designated as $\tilde{k}_O$. The molecular activity obtained after glucoamylase digestion is an apparent molecular activity $\tilde{k}_G$, proportional to the number of ring-openings. The fact that the two pairs of straight-line initial-velocity plots in Fig. 2 intersect on the horizontal axes suggests that the distribution of oligosaccharides produced by HPA- and PPA-catalyzed hydrolyses of γ-cyclodextrin is independent of the cyclodextrin concentration. As shown in Fig. 1, the h.p.l.c. analysis of HPA and PPA hydrolyzates of γ-cyclodextrin did not reveal maltotetraose or longer oligosaccharides, indicating that the cleavage of the ring is the rate-limiting step in the HPA- and PPA-catalyzed hydrolyses of this cyclodextrin. Thus the molecular activity k_O for the ring-opening rate is $\tilde{k}_G$ divided by eight, the number of glucose residues in γ-cyclodextrin. The Michaelis constant K_m and the rate parameters k_O, $\tilde{k}_O$, and $\tilde{k}_G$ for HPA and PPA are summarized in Table I.

TABLE I

Kinetic parameters of the hydrolysis of γ-cyclodextrin and G_5 catalyzed by HPA and PPA at 37°[a]

Enzyme	*Substrate*	K_m (mM)	$\tilde{k}_O$[b] (min^{-1})	$\tilde{k}_G$[c] (min^{-1})	k_O[d] (min^{-1})	*Total number of attacks*	*Number of multiple attacks*
HPA (pH 7.0)	γ-CD[e]	0.80 ± 0.24	440 ± 50	1030 ± 140	129 ± 18	3.4	2.4
	G_5	0.45 ± 0.04			(2.10 ± 0.07) × 10^4		
PPA (pH 6.9)	γ-CD[e]	0.97 ± 0.13	1350 ± 80	2880 ± 140	360 ± 18	3.8	2.8
	G_5	0.50 ± 0.05			(5.25 ± 0.22) × 10^4		

[a] Kinetic parameters were determined by non-linear least-squares fit of observed initial-velocity data to calculated values[22]. [b] $\tilde{k}_O$, apparent molecular activity in the hydrolysis of γ-cyclodextrin (combination of the ring-opening of γ-cyclodextrin and repetitive attack on the product). [c] $\tilde{k}_G$, apparent molecular activity determined after glucoamylase treatment of the hydrolyzate of γ-cyclodextrin. [d] k_O, molecular activity in the ring-opening of γ-cyclodextrin and the hydrolysis of G_5. [e] γ-Cyclodextrin.

The $\tilde{k}_G/\tilde{k}_O$ value, which is equivalent to the mean degree of polymerization of oligosaccharide products, is quite useful for evaluating the number of multiple attacks. The total number of attacks, including the initial cleavage of the cyclodextrin ring, is equal to the number of glucose residues in γ-cyclodextrin (8) divided by $\tilde{k}_G/\tilde{k}_O$. Therefore the number of multiple attacks is the total number of attacks minus one. These values were summarized in Table I.

It was found that the rate of ring-opening of γ-cyclodextrin (k_O value) was about 150 times smaller than the molecular activity for G_5 in both HPA- and PPA-catalyzed reactions. This finding supports the assumption that ring-opening is the rate-limiting step in the HPA- and PPA-catalyzed hydrolyses of γ-cyclodextrin. Table I also shows that the rate of ring-opening of γ-cyclodextrin by HPA is 2.8 times slower than that by PPA. Thus a multiple-attack mechanism for both HPA- and PPA-catalyzed reactions was confirmed by using γ-cyclodextrin as a substrate in the present study.

Inhibition by cyclodextrins of HPA- and PPA-catalyzed reactions. — The linear oligosaccharide G_5 is a suitable substrate for analyzing the type of inhibition shown by cyclodextrins, and for evaluating the inhibitor constants, because G_5 has only one cleavage site[23]. The effect of clyclodextrins is shown in Fig. 3, where $[G_5]/v$ *versus* $[G_5]$ is ploted for the PPA-catalyzed hydrolysis of G_5 in the presence and absence of α-cyclodextrin. All three kinds of cyclodextrins act as competitive inhibitors of the HPA- and PPA-catalyzed hydrolyses of G_5, since the plots of the velocities in the presence and absence of cyclodextrins are parallel. It was found that in the case of PPA the inhibition by α-cyclodextrin (K_i 1.24mM) was slightly weaker than that by β-cyclodextrin (K_i 0.46mM) and γ-cyclodextrin (K_i 0.44mM). The K_i value for γ-cyclodextrin with HPA (0.65mM) was found to be nearly the same as that with PPA. Furthermore, the K_i values for γ-cyclodextrin with both HPA and PPA approximate the K_m values (see Table I).

These findings show that only γ-cyclodextrin is hydrolyzed *in vitro* by HPA and PPA, and that cyclodextrins might be inhibitory when the natural substrates of these enzymes are present. Further experiments will be done to obtain more information on the digestibility of cyclodextrins by human α-amylases.

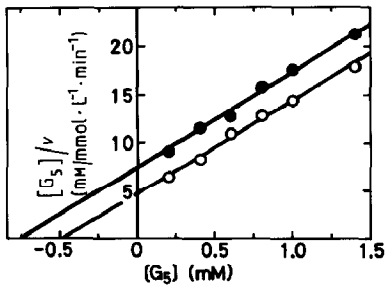

Fig. 3. Plots of $[G_5]/v$ *versus* $[G_5]$ for the PPA-catalyzed hydrolysis of G_5 with 0.62 mM α-cyclodextrin (●) and without α-cyclodextrin (O). PPA concentration, 1.99 nM; pH, 6.9; temperature, 37°.

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