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KINETIC DIFFERENCE BETWEEN HYDROLYSES OF γ-CYCLODEXTRIN BY HUMAN SALIVARY AND PANCREATIC α-AMYLASES

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γ-Cyclodextrin was found to be hydrolyzed by human salivary and pancreatic α-amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) at appreciable rates. The optimum pH for the enzyme reactions at 37°C in the presence of 0.1 M NaCl was at around pH 5, which was remarkably different from the optimum pH (pH 6.9) of the enzymes for starch. The K_m value (2.9 mg/ml) of pancreatic α-amylase for γ-cyclodextrin was smaller than that (5.3 mg/ml) of salivary α-amylase at pH 5.3, while the V value of the former was 3.7-times larger than that of the latter. The hydrolyses of γ-cyclodextrin by both enzymes took place via the multiple attack mechanism. The degrees of multiple attack by salivary and pancreatic α-amylases for γ-cyclodextrin at pH 5.3 were 2.0 and 1.1, respectively. The distribution of maltodextrins produced by hydrolysis of γ-cyclodextrin by salivary α-amylase was suggested to be independent of the substrate concentration, while that produced by pancreatic α-amylase was presumably dependent on the substrate concentration.

Introduction

α-, β- and γ-cyclodextrins are homologous oligosaccharides composed of six, seven and eight glucose residues forming α-1,4-linked macrocyclic structures. To data there has been only one report [1] on the hydrolyses of cyclodextrins by human salivary α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1). The paper says that γ-cyclodextrin, but not α- and β-cyclodextrins, is slowly hydrolyzed by the enzyme, although detailed data are not presented. Porcine pancreatic α-amylase was reported to be able to hydrolyze γ-cyclodextrin [2], while there have been no reports as to whether human pancreatic α-amylase can hydrolyze cyclodextrins.

Here, we report that both human salivary and pancreatic α-amylases can hydrolyze γ-cyclodextrin at appreciable rates and describe the kinetic differences between hydrolyses of γ-cyclodextrin by the two enzymes.

Materials and Methods

Human salivary and pancreatic α-amylases were prepared from saliva and pancreas tissues by affinity chromatography [3,4] on Sephadex G-100. Fresh human saliva was dialyzed against 10 mM Tris-HCl buffer (pH 7.2)/5 mM $CaCl_2$ and centrifuged at 10000 × g for 15 min. The supernatant (2 ml) was diluted with 4 ml of the same buffer and applied to a column (2.5 × 95 cm) of Sephadex G-100 equili-

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brated with the same buffer. The fractions containing α-amylase activity were pooled, concentrated with a Diaflo ultrafiltration membrane UM-2 (Amicon, Lexington, MA) and dialyzed against 10 mM β-glycerophosphate buffer (pH 6.9)/5 mM $CaCl_2$. Human serum albumin and NaN_3 were added to the impermeate to final concentrations of 5 μg/ml and 0.02%, respectively, in order to stabilize the enzyme and to prevent bacterial growth. Pieces of human pancreas (10 g) obtained at autopsy from four subjects were homogenized in 50 ml of 10 mM Tris-HCl buffer (pH 7.2)/5 mM $CaCl_2$/3 mM phenylmethylsulfonyl fluoride as an inhibitor for proteases, and the homogenate was centrifuged at 100 000 × *g* for 1 h. The supernatant was concentrated to 3.6 ml and filtered through a column (2.5 × 95 cm) of Sephadex G-100. The α-amylase fractions were treated by the same way as described for salivary α-amylase. Activities of salivary and pancreatic α-amylases in the solutions prepared were about 90 and 20 U/ml, respectively. Dilution of the α-amylase solutions was made with 10 mM β-glycerolphosphate buffer (pH 6.9)/5 mM $CaCl_2$/5 μg/ml human serum albumin/ 0.02% NaN_3.

α-Amylase-free glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) was prepared from a culture fluid of *Aspergillus niger* as reported previously [5]. 1 unit glucoamylase activity was the amount that releases 1 μmol glucose/min from soluble starch at pH 4.3 and 37°C.

γ-Cyclodextrin was obtained from Hayashibara Biochemical Laboratories, Inc. (Osaka, Japan) and treated with glucoamylase to remove a trace amount of maltodextrins as follows: 2 g γ-cyclodextrin were incubated with 170 U glucoamylase in 15 ml of 10 mM acetate buffer (pH 4.3)/0.02% NaN_3, for 50 h at 37°C. The reaction mixture was boiled for 7 min to denature glucoamylase and centrifuged for 15 min at 10 000 × *g* at 4°C. After adding *n*-propyl alcohol (50 ml) to the supernatant, the mixture was kept at room temperature for a few hours. The precipitate was filtered, washed twice with 3 ml 80% *n*-propyl alcohol and three times with 5 ml ethyl alcohol, and dried for 10 h at 105–110°C to yield 1.6 g maltodextrin-free γ-cyclodextrin.

Human serum albumin (fraction V), sodium β-glycerophosphate, and α- and β-cyclodextrins were obtained from Sigma (St. Louis, MO). Cyclodextrins were checked to be pure by HPLC using a column of μBondapak C_{18} (Waters, Milford, MA) and 70% acetonitrile as a solvent, and by paper chromatography using *n*-butanol/pyridine/H_2O (6 : 4 : 3, v/v) as a solvent system. Soluble (lintnerized) starch was purchased from J.T. Baker, Phillipsburg, NJ.

All assays were performed at 37°C. The α-amylase activity was standardized by the Nelson method [6] on 0.5% soluble (lintnerized) starch as a substrate, and 1 unit of activity was the amount that produced 1 μmol reducing group per min, at pH 6.9 and 37°C.

Reaction system I for α-amylase assay using γ-cyclodextrin. This α-amylase assay was based on the measurement of glucose produced by hydrolysis of γ-cyclodextrin to maltodextrins with α-amylase followed by hydrolysis of the maltodextrin with glucoamylase. Each reaction mixture (total volume, 0.5 ml) contained the following reagents: an appropriate amount of salivary or pancreatic α-amylase, 10 mg/ml γ-cyclodextrin, 4 U/ml glucoamylase, 100 mM NaCl, 20 μg/ml human serum albumin and 50 mM sodium acetate buffer (pH 5.3)/5 mM $CaCl_2$ unless otherwise stated. After incubation of the reaction mixture for 30 min at 37°C, 1 ml glucose oxidase-peroxidase reagent [7] was added to stop the reaction. 2 ml 5 N HCl were added after incubation for 60 min at 37°C and absorbance was measured at 530 nm.

Reaction system II for α-amylase assay using γ-cyclodextrin. This assay was based on the measurement of reducing power produced by hydrolysis of γ-cyclodextrin with α-amylase in the presence or absence of glucoamylase. The reaction mixture containing glucoamylase was as follows: an appropriate amount of salivary or pancreatic α-amylase, 3–20 mg/ml γ-cyclodextrin, 100 mM NaCl, 20 μg/ml human serum albumin, 4 U/ml (for pH 5.3) or 50 U/ml (for pH 6.9) glucoamylase and 50 mM sodium acetate buffer (pH 5.3)/5 mM $CaCl_2$ or 50 mM β-glycerophosphate buffer (pH 6.9)/5 mM $CaCl_2$. At appropriate intervals, samples of 0.5-ml were taken out and put into 0.5 ml Somogyi alkaline copper reagent to stop the reaction. The reducing power in the samples was measured by the Nelson method [6]. Reaction system II was used for determination of the degrees of multiple attack of salivary and pancreatic α-amylases for γ-cyclodextrin. This system was a modification of the system used by Suetsugu et al. [8].

Results

Determination of appropriate amounts of glucoamylase for α-amylase assay at various pH values. The rates of glucose formation were measured at pH 5.3, 6.0 and 6.9, in the presence of varying amounts of glucoamylase, using reaction system I in order to know the appropriate amounts of glucoamylase at these pH values for complete hydrolysis to maltodextrins produced from γ-cyclodextrin by the action of salivary and pancreatic α-amylases. The glucoamylase concentrations of 4, 10 and 50 U/ml were found to be sufficient for both α-amylases at pH 5.3, 6.0, and 6.9, respectively (Table I).

Effect of pH on hydrolyses of γ-cyclodextrin by salivary and pancreatic α-amylases. The rates of glucose formation in reaction system I were measured at various pH values. The optimum pH was at around 5.0 for both salivary and pancreatic α-amylases (Fig. 1), while the optimum pH of the enzymes for starch has been known to be at 6.9 [9]. The activities at the pH below 4.7 could not be precisely measured because of instability of the α-amylases.

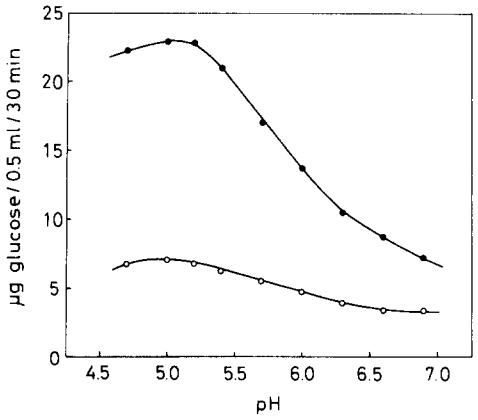

Fig. 1. Effect of pH on human salivary (○) and pancreatic (●) α-amylase activities. The concentrations of γ-cyclodextrin and both enzymes were 10 mg/ml and 60 mU/ml, respectively. 4 U/ml glucoamylase were used at the pH below 5.4, 10 U/ml glucoamylase at pH 5.7 and 6.0, 30 U/ml glucoamylase at pH 6.3 and 6.6, and 50 U/ml glucoamylase at pH 6.9. 50 mM sodium acetate/5 mM $CaCl_2$ buffer was used for the pH below 6.0 and 50 mM β-glycerophosphate/5 mM $CaCl_2$ buffer for the pH above 6.3.

Comparison among rates of hydrolyses of α-, β- and γ-cyclodextrins by salivary and pancreatic α-amylases. The rates of α-amylolysis of three cyclodextrins were measured with reaction system I. α-Cyclodextrin was hardly hydrolyzed by both α-amylases. The hydrolysis of β-cyclodextrin was extremely slow compared

TABLE I

EFFECT OF GLUCOAMYLASE CONCENTRATION ON THE RATE OF GLUCOSE FORMATION IN REACTION SYSTEM I

50 mM sodium acetate/5 mM $CaCl_2$ buffer were used for pH 5.3 and 6.0, and 50 mM β-glycerophosphate/5 mM $CaCl_2$ buffer for pH 6.9. The concentration of both α-amylases was about 60 mU/ml.

Glucoamylase (mg/ml)	Glucose formed (μg/0.5 ml per 30 min)					
	Salivary α-amylase (pH)			Pancreatic α-amylase (pH)		
	5.3	6.0	6.9	5.3	6.0	6.9
1	5.8			20.4		
2	6.3	3.9		20.7	12.4	
4	6.6	4.2		22.0	13.0	
10	6.6	4.4	3.0	21.9	13.4	6.3
20		4.4	3.3		13.3	6.8
50			3.6			7.2
100			3.6			7.2

TABLE II

RATES OF HYDROLYSES OF α-, β- AND γ-CYCLODEXTRINS BY HUMAN SALIVARY AND PANCREATIC α-AMYLASES

The concentrations of salivary and pancreatic α-amylases were 300 and 140 mU/ml, respectively, in the case of hydrolyses of α- and β-cyclodextrins, and that of both α-amylases was 80 mU/ml for the hydrolyses of γ-cyclodextrin.

Substrate	Glucose formed (μg/0.5 ml per 30 min)	
	Salivary α-amylase	Pancreatic α-amylase
α-Cyclodextrin	negligible	negligible
β-Cyclodextrin	<1	<1
γ-Cyclodextrin	9.0	29.8

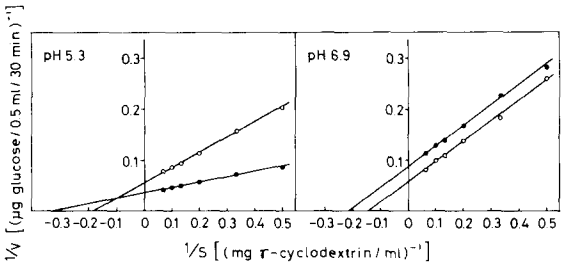

Fig. 2. Lineweaver-Burk plots for hydrolyses of γ-cyclodextrin by human salivary (○) and pancreatic (●) α-amylases. α-Amylase concentration: 114 mU/ml salivary and 58 mU/ml pancreatic α-amylase at pH 5.3, and 166 mU/ml salivary and 72 mU/ml pancreatic at pH 6.9. Buffer: 50 mM sodium acetate/5 mM $CaCl_2$ for pH 5.3 and 50 mM β-glycerophosphate/5 mM $CaCl_2$ for pH 6.9.

with that of γ-cyclodextrin (Table II). Therefore, only γ-cyclodextrin was used as a substrate through this study.

Lineweaver-Burk plots for hydrolyses of γ-cyclodextrin by salivary and pancreatic α-amylases. The rates of glucose formation in reaction system I were measured at pH 5.3 (an alkaline border of the optimum pH of human salivary and pancreatic α-amylases for γ-cyclodextrin) and at pH 6.9 (the optimum pH for starch). Lineweaver-Burk plots

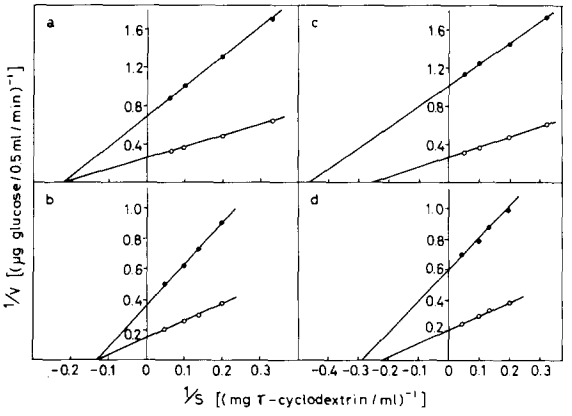

Fig. 3. Lineweaver-Burk plots for hydrolyses of γ-cyclodextrin by human salivary and pancreatic α-amylases in the presence (○) and absence (●) of glucoamylase at pH 5.3 and 6.9. (a) Salivary (0.78 U/ml), pH 5.3; (b) salivary (2.2 U/ml), pH 6.9; (c) pancreatic (0.22 U/ml), pH 5.3; (d) pancreatic (0.92 U/ml), pH 6.9. The rate was expressed as the glucose equivalents formed/min per 0.5 ml.

depicted on the basis of the values obtained showed that the V value (1.1 μg glucose/30 min per mU) of pancreatic α-amylase was 3.7 times larger than that (0.30 μg glucose/30 min/mU) of salivary α-amylase at pH 5.3, while there was not such a large difference at pH 6.9 (0.21 and 0.32 μg glucose/30 min per mU) for salivary and pancreatic α-amylases (Fig. 2). The K_m values (5.3 and 7.1 mg/ml) at pH 5.3 and 6.9) of salivary α-amylase were a little larger than those (2.9 and 4.7 mg/ml at pH 5.3 and 6.9) of pancreatic α-amylase at both pH values.

Determination of degrees of multiple attack of salivary and pancreatic α-amylases for γ-cyclodextrin.

We intended to determine the degree of multiple attack with reaction system II by comparing the rates of production of reducing power by the hydrolysis of γ-cyclodextrin in the presence and absence of glucoamylase. Lineweaver-Burk plots for the hydrolyses of γ-cyclodextrin by salivary and pancreatic α-amylases at pH 5.3 and at 6.9 are shown in Fig. 3. The values of K_m and V obtained in the presence of glucoamylase were almost the same, at both pH values, as those obtained using reaction system I. The degree of polymerization (DP_n) of hydrolysis products should correspond to a ratio of V in the presence of and absence of glucoamylase. The degree of total attack was obtained by dividing the number of glucose residues in γ-cyclodextrin molecule by DP_n, and the degree of multiple attack was given by subtracting 1 (a degree of first attack by α-amylase) from the degree of total attack. The values

TABLE III

DEGREES OF POLYMERIZATION (DP_n) OF PRODUCTS AND OF MULTIPLE ATTACK IN THE HYDROLYSES OF γ-CYCLODEXTRIN BY HUMAN SALIVARY AND PANCREATIC α-AMYLASES AT pH 5.3 AND 6.9

See Fig. 3 for enzyme concentrations used.

Enzyme	pH	DP_n of products	Degree of total attack	Degree of multiple attack
Salivary α-amylase	5.3	2.7	3.0	2.0
	6.9	2.4	3.4	2.4
Pancreatic α-amylase	5.3	3.9	2.1	1.1
	6.9	2.9	2.7	1.7

of degrees of DP_n, total attack and multiple attack thus obtained are shown in Table III. Both salivary and pancreatic α-amylases were found to hydrolyze γ-cyclodextrin via the multiple attack mechanism and the degree of multiple attack of salivary α-amylase was shown to be a little larger than that of pancreatic α-amylase at both pH values (pH 5.3 and 6.9).

Discussion

Some bacterial and fungal α-amylases have been reported to hydrolyze cyclodextrins [10–13], while only a few papers [1,2] have dealt with the hydrolyses of cyclodextrins by mammalian α-amylases. To our knowledge this is a first report on the comparative study of human salivary and pancreatic α-amylases on the hydrolyses of cyclodextrins.

The appropriate concentrations of glucoamylase in the α-amylase assay using γ-cyclodextrin were 4, 10 and 50 U/ml at pH 5.3, 6.0, and 6.9, respectively (Table I). This result was reasonable because the optimum pH of the glucoamylase used in this study was around 4.5. Since the specific activity of glucoamylase was about 30 U/mg, we needed to use 1.7 mg glucoamylase/ml, at pH 6.9, in reaction systems I and III. This amount of enzyme protein did not interfere with reaction system I, but made blank values increase by about 0.1 units compared with those observed at pH 5.3 in reaction system II. However, the absorbance (around 0.12) of a blank (at a γ-cyclodextrin concentration of 10 mg/ml, pH 6.9) in reaction system II was not so great that there was no trouble in this system either.

The optimum pH of both salivary and pancreatic α-amylases for the action on γ-cyclodextrin was at around 5.0 as shown in Fig. 1. This remarkable shift of the optimum pH from pH 6.9 for the hydrolysis of starch is not so peculiar: Walker and Whelan [14], for instance, reported that the optimum pH of human salivary α-amylase for the hydrolysis of maltotriose was pH 5.5 in the presence of 0.1 M chloride. A big difference in the rate of glucose formation between pH 5.0 and 6.9 was observed for pancreatic α-amylase compared with that for salivary α-amylase. This may reflect the differences in the structure of the active site between the two enzymes. α-Amylase activity fluctuated markedly at the acid pH (below 5.5) without human serum albumin probably because of instability of α-amylase under acidic circumstances and the activity was increased by introducing 0.1 M NaCl to the reaction mixture at pH 5.3, though not much (about 1.3-times). Hence, we used the reaction systems containing human serum albumin and NaCl throughout this study.

We obtained almost the same result as described by French [1] on the rates of hydrolyses of cyclodextrins by salivary α-amylase (Table II). A similar result was also found with pancreatic α-amylase. Supposing that the degrees of multiple attack of salivary and pancreatic α-amylases for γ-cyclodextrin at pH 5.3 are close to that of both enzymes for starch at pH 6.9 because that of salivary α-amylase for amylose has been reported to be 2.0 [15], the hydrolyses of γ-cyclodextrin by salivary and pancreatic α-amylases at pH 5.3 were calculated to be about 2 and 5%, as rapid as that of starch at pH 6.9.

Stiefel and Keller [16] reported that highly purified human salivary α-amylase has a higher specific activity on soluble starch, while purified human pancreatic α-amylase has relatively more activity on insoluble starch [16]. They attributed this phenomenon to the configurational differences between the two enzymes, that is, they proposed the concept that since pancreatic α-amylase has a less compact structure than salivary α-amylase steric hindrance in the binding of pancreatic α-amylase with insoluble starch may be less than that of salivary α-amylase. Our results showing a greater V value and a smaller K_m value for pancreatic α-amylase than for salivary α-amylase might be explained by this concept, because γ-cyclodextrin has glucosidic bonds which are less flexible compared with those of soluble starch. Saito et al. [17] reported that human pancreatic α-amylase hydrolyzes maltotriose at a higher rate than human salivary α-amylase, while maltopentose is hydrolyzed at almost the same rate by the two enzymes and both maltohexose and maltoheptose are hydrolyzed at lower rates by the former than the latter enzyme. Their results also might be accounted for by Stiefel and Keller's concept [16], because the shorter maltodextrins have greater rigidity.

Each pair of straight lines shown in Fig. 3c and d did not meet on the abscissa in contrast to each pair in Fig. 3a and b. This may indicate that the distribu-

tion of maltodextrins produced by the action of pancreatic α-amylase on γ-cyclodextrin was dependent on the concentration of the substrate, while that of salivary α-amylase was probably independent of the substrate concentration. An alternative concept is that pancreatic α-amylase has a lower binding affinity for the maltodextrins as compared with γ-cyclodextrin, whereas with salivary α-amylase the binding affinities appear to be similar. However, this seemed unlikely because the K_m values (2–4 mg/ml) of salivary α-amylase for maltotetrose, maltopentose, and maltohexose at pH 7.0 and at 37°C are known to be almost the same as those of pancreatic α-amylase [17], and the K_m values of both enzymes for the matodextrins are of the same order as those (7.1 and 4.7 mg/ml for salivary and pancreatic α-amylases, respectively) for γ-cyclodextrin at pH 6.9 and at 37°C.

We found that γ-cyclodextrin was hydrolyzed by both human salivary and pancreatic α-amylases via the multiple attack mechanism (Table III). Abdullah et al. [2] also showed, by using a different method, that porcine pancreatic α-amylase hydrolyzes γ-cyclodextrin via the multiple attack mechanism, but did not determine the degree of multiple attack. The values of degree of multiple attack determined by us were not so different from the value (1.6) obtained on the action of a fungal α-amylase Taka-amylase A (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) for cyclodextrins by Suetsugu et al. [8]. The degrees (2.0 and 2.4) of multiple attack by human salivary α-amylase for γ-cyclodextrin at pH 5.3 and 6.9 were quite similar to that (2.0) for amylose at pH 6.9 [15]. It seemed from this fact that the process of multiple attack after the first cleavage of α-1,4-bond in γ-cyclodextrin by the enzyme is probably similar to that in amylose. The difference in degree of multiple attack by salivary and pancreatic α-amylases for γ-cyclodextrin between pH 5.3 and 6.9 was not so much as seen in the paper of Robyt and French [15], in which they reported that the degree of multiple attack of porcine pancreatic α-amylase for amylose at pH 6.9 (an optimum pH) and 10.5 were 6.0 and 0.7, respectively. This discrepancy between our findings and those of Robyt and French may be due to the difference in enzyme source, namely, our enzymes were from man and theirs from hog.

The amount of glucose produced in reaction system I was directly proportional to the amount of salivary or pancreatic α-amylase. Furthermore, coexistence of both α-amylases in a reaction mixture did not interfere with the action of each enzyme (unpublished data). We can, therefore, suggest that γ-cyclodextron may be usable as a defined substrate for the assay of the activity of each α-amylase.

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