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PORCINE-PANCREATIC ALPHA AMYLASE HYDROLYSIS OF SUBSTRATES CONTAINING 6-DEOXY-D-GLUCOSE AND 6-DEOXY-6-FLUORO-D-GLUCOSE AND THE SPECIFICITY OF SUBSITE BINDING*

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ABSTRACT

Hydrolysis of 6-deoxyamylose and mono-6-deoxy-6-fluorocyclomaltoheptaose by porcine-pancreatic alpha amylase produces low-molecular-weight modified products, which have been analyzed by chemical and chromatographic techniques. Results for both substrates show that modified D-glucose and two isomers of modified maltoses are produced in the enzyme reaction. In addition, the formation of maltoses modified in the nonreducing residue is more favored than the formation of maltoses modified in the reducing residue. These results indicate that productive binding of 6-fluoro- and 6-deoxy-D-glucose residues is permitted at subsites 1 through 4 of the amylase-active site but that binding of these modified residues may be less favorable at subsite 3, the subsite at which catalytic attack occurs.

INTRODUCTION

The action of porcine-pancreatic alpha amylase (PPA) on branched and modified substrates has been observed in a number of studies[1–8]. In nearly all instances, these substrates have contained either bulky or charged substituents. Hydrolysis products from these substrates have included modified disaccharides, trisaccharides, and larger products. Bines and Whelan have reported, in a preliminary study on human salivary amylase, that a product with the paper-chromatographic mobility of 6-deoxy-D-glucose is obtained from the enzymolysis of 6-deoxyamylose[9]. Because previous studies on PPA hydrolysis of modified substrates have not observed the formation of modified monomers, we have examined the effects of small substituents on PPA action more closely. Inasmuch as fluorine is smaller than the hydroxyl group, is similar to oxygen in electronegativity, and has

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non-bonded electrons that might participate in hydrogen bonding[10], mono-6-deoxy-6-fluorocyclomaltoheptaose was used as one substrate for the enzyme. Like native cyclomaltoheptaose, the fluorinated derivative is a relatively poor substrate for porcine-pancreatic alpha amylase. Fluorinated cyclomaltoheptaose may be readily synthesized and purified, whereas synthetic difficulties and the formation of side products make the use of fluorinated amylose difficult. The hydrolysis of 6-deoxyamylose by PPA also has been examined. Our results may be interpreted in terms of the ability of PPA to productively bind modified sugars at the five subsites of the active site of the enzyme. Using this approach, it may be possible to assess the importance of the C-6 hydroxyl group for enzyme–substrate interactions. These results may also be compared with those obtained for substrates containing bulky modifying groups (e.g., hydroxyethyl, α-D-glucosyl, and phosphate) at C-6, in order to identify sites where enzyme–substrate interactions are sensitive to steric disruption.

EXPERIMENTAL

Materials. — Amylose (Superlose from Stein–Hall Co.) and PPA (Boehringer–Mannheim, 10,000 U/mL) were commercial samples. The PPA contained no detectable α-glucosidase activity as assayed by using *p*-nitrophenyl α-D-glucopyranoside. Cyclomaltoheptaose was prepared by the method of French *et al.*[11].

Methods. — Ascending and descending paper chromatography were performed on Whatman 3MM paper by using aqueous 70% 1-propanol (solvent *A*) or 8:1:1:1 nitromethane–ethanol–acetic acid–saturated boric acid[12] (solvent *B*). Reducing sugars were detected by the silver nitrate dip method[13]. Total carbohydrate was determined by using the orcinol–sulfuric acid method, as adapted for use with a Technicon AutoAnalyzer[14]. Enzymolysis products were reduced with sodium borohydride by the procedure of Sloneker[15]. Acid hydrolysis was performed in 3M trifluoroacetic acid (TFA) for 2 h at 100° in sealed ampules, unless otherwise indicated. After hydrolysis, samples were cooled, and the acid was removed by repeated evaporation under diminished pressure. Reducing sugars and sugar alcohols were identified by paper or liquid chromatography using authentic materials as standards. Tritiated samples on paper chromatograms were detected by autoradiography after spraying with En^{3}Hance (DuPont–NEN).

Charcoal chromatography columns were prepared by the method of Chan *et al.*[8]. High-pressure liquid chromatography (l.c.) employed a Waters liquid chromatograph with refractive index detector. Analytical separations were performed on Whatman PXS 10/25 polar amino cyano (PAC) columns, with 85% acetonitrile at a flow rate of 1.0 mL/min as solvent. Fluorinated cyclomaltoheptaose was purified on a Whatman M-9 PAC semi-preparative column operated at 3.0 mL/min with 70:27:3 acetonitrile–water–tetrahydrofuran as solvent. ^{19}F-N.m.r. spectra were obtained with a Bruker 300-MHz instrument.

Substrates. — Samples of 6-deoxyamylose having degrees of substitution (d.s.) of 0.15 and 0.50 were prepared by the method of Weill *et al.*[16] *via* 2,3-di-*O*-acetyl-6-*O*-toluenesulfonylamylose.

Monofluorinated cyclomaltoheptaose was prepared *via* the corresponding mono-*p*-toluenesulfonic (tosyl) ester. Mono-6-*O*-tosylcyclomaltoheptaose was prepared from cyclomaltoheptaose (35 g) by a modification of the procedure of Omichi *et al.*[17]. After treatment with tosyl chloride, the mixture was concentrated to a thick syrup and poured into 2-propanol. The precipitate was filtered off and recrystallized from warm water to give a crude product (20.9 g). A portion of this crystalline material (4.0 g) was dissolved in warm water (3 L) and chromatographed at 40° on a charcoal column (4.8 × 30 cm) by eluting successively with water (4 L), 10% ethanol (2 L), 15% ethanol (2 L), 20% ethanol (4 L), 30% ethanol (4 L), and 25% 1-propanol (3 L). The 30% ethanol fraction was evaporated to give pure mono-6-*O*-tosylcyclomaltoheptaose (2.3 g). Fluorinated cyclomaltoheptaose was prepared by refluxing mono-6-*O*-tosylcyclomaltoheptaose (4.4 g) with anhydrous potassium fluoride (1.5 g) in ethane-1,2-diol (135 mL) for 3 min. The cooled mixture was poured into 2-propanol, and the precipitate was removed by centrifugation. Repeated recrystallization from hot water gave mono-6-deoxy-6-fluorocyclomaltoheptaose (600 mg) containing small amounts of an impurity. Further purification by l.c. yielded pure product, m.p. >200°, having a ^{19}F-n.m.r. spectrum similar to that of 6-deoxy-6-fluoro-D-glucose[18] [D_2O, $CClF_3$ external standard, δ_c 234.3 p.p.m., J_{FH} (geminal) 47.9 Hz, J_{FH} (vicinal) 29.3 Hz]. Acid hydrolysis of this material followed by paper chromatography (solvent *A*, 1 ascent) identified 6-deoxy-6-fluoro-D-glucose, D-glucose, and oligosaccharide products.

Anal. Calc. for $C_{42}H_{69}FO_{34}$: C, 44.37; H, 6.07; F, 1.67. Found: C, 44.28; H, 6.37; F, 1.76.

Enzyme hydrolysis. — 6-Deoxyamylose (d.s. 0.15, 1.0 g) was dissolved in 9:1 dimethyl sulfoxide–water (15 mL), and buffer (15 mL) containing 200mM sodium glycerophosphate, 100mM sodium chloride, and 0.2% sodium azide was added. The pH of the solution was adjusted to 6.9, and diluted with water to a final volume of 150 mL. This solution was filtered and warmed to 37°, and a solution of PPA (15 mL, 650 U/mL) containing 20mM sodium glycerophosphate and 10mM calcium chloride, pH 6.9, was added. After 3 days, a sample was removed and analyzed by paper chromatography (solvent *A*, 2 ascents), which showed D-glucose and maltose as the only major products. The enzyme digest was then applied to a charcoal column (4.8 × 30 cm) and eluted with water (3 L), then with a gradient of 0–8% *tert*-butyl alcohol (7 L). Fractions (25 mL) were collected, and every other fraction was analyzed for total carbohydrate. Every tenth fraction was also analyzed by paper chromatography (solvent *A*, 2 ascents).

Mono-6-deoxy-6-fluorocyclomaltoheptaose (100 mg) was dissolved in 9.0 mL of warm water, and 1 mL of buffer (200mM sodium glycerophosphate, 100mM sodium chloride, 0.2% sodium azide, pH 6.9), was added. An enzyme solution was prepared by centrifuging 1 mL of suspended PPA. The pellet was dissolved in 3 mL

of 20mM sodium glycerophosphate containing 10mM calcium chloride at pH 6.9. This enzyme solution (3 mL) was added to the substrate solution (10 mL) and was allowed to react for 5 days at 37°. Samples (50 μL) were removed daily and examined by paper chromatography (solvent *A*, 2 ascents). An additional sample (400 μL) was removed at the end of the reaction time, and the remaining solution was applied to a charcoal column (2.4 × 30 cm). This column was eluted with water (1 L), and 1, 2, 3, 4, and 7% *tert*-butyl alcohol (500 mL each). Each eluate was collected as a single fraction, evaporated to dryness, and dissolved in 400 μL of water.

RESULTS

PPA hydrolysis of 6-deoxyamylose. — Two major products were obtained from charcoal chromatography. These were identified as D-glucose, which eluted in the water fraction, and maltose, which eluted near the beginning of the *tert*-butyl alcohol gradient. Several minor products also were detected by paper chromatography. Three of these products (designated 6-DG, 6-DM, and 6,6-DM) were tentatively identified as low-molecular-weight 6-deoxy-oligosaccharides, based on their paper-chromatographic mobilities and longer retention on charcoal columns. The first of these compounds, 6-DG, had a paper chromatographic mobility relative to D-glucose (R_G) of 1.23, identical to that of 6-deoxy-D-glucose. This compound eluted from charcoal at the beginning of the *tert*-butyl alcohol gradient along with maltose and was detected only in small amounts. The second of these components had an R_G value similar to D-glucose ($R_G = 0.99$), but was eluted from the charcoal column after maltose. The paper-chromatographic mobility of this compound was consistent with that predicted for a mono-6-deoxymaltose[19]. Relatively larger amounts of this product were formed. The third product, 6,6-DM, was also detected in fairly small amounts. This compound eluted from the charcoal column after maltose and had an R_G value of 1.33. Fractions from charcoal chromatography that contained 6-deoxy-oligosaccharides were pooled, and the modified products were further purified by descending paper-chromatography (solvent *A*, 8–12 h). Some larger oligosaccharides, which were not analyzed, also were detected in the enzymolysis mixture.

Product 6-DM was resolved into two peaks by l.c., which were designated 6-DM_1 and 6-DM_2. These were purified, and then reduced with sodium borohydride and hydrolyzed with acid to give the results shown in Fig. 1. Hydrolysis of reduced 6-DM_1 yielded 6-deoxy-D-glucose and D-glucitol in 1:1 ratio, indicating that this compound is 4-*O*-(6-deoxy-α-D-glucosyl)-α-D-glucose (6^2-deoxymaltose). Similarly, reduced 6-DM_2 was hydrolyzed to give approximately equal amounts of 6-deoxy-D-glucitol and D-glucose, identifying this compound as 6-deoxy-4-*O*-(α-D-glucosyl)-α-D-glucose (6^1-deoxymaltose).

The products 6-DG and 6,6-DM were purified in amounts too small to be analyzed by this technique. Therefore, a more-highly substituted amylose (d.s.

0.50) was hydrolyzed by PPA, and the products were separated and analyzed by the methods described previously. With these techniques, 6-DG and 6,6-DM were identified as 6-deoxy-D-glucose and 4-*O*-(6-deoxy-α-D-glucosyl)-6-deoxy-α-D-glucose (6^1,6^2-dideoxymaltose), as shown in Fig. 2.

Experiments using the more-highly substituted 6-deoxyamylose also suggested that more 6^2-deoxymaltose was formed than 6^1-deoxymaltose. Estimation from l.c. peak-areas suggested that the ratio of 6^2-deoxymaltose to 6^1-deoxymaltose was ~4:1. To examine this further, a sample of tritium-labelled 6-deoxyamylose (d.s. 0.15) was prepared by reduction of 2,3-di-*O*-acetyl-6-deoxy-6-iodo-amylose with sodium [^{3}H]borohydride. Modified maltose was purified, reduced, and hydrolyzed, and then separated by descending paper-chromatography (solvent *B*, 6 h). The ratio of radioactive 6-deoxy-D-glucose to 6-deoxy-D-glucitol was ~3:1, as determined by autoradiography and liquid scintillation.

PPA hydrolysis of mono-6-deoxy-6-fluorocyclomaltoheptaose. — Because cyclomaltoheptaoses are poor substrates for alpha amylase and because of the low water solubility of the fluorinated derivative, only small amounts of modified amylolysis products could be conveniently produced and analyzed. For this reason, products of PPA hydrolysis were reduced with sodium [^{3}H]borohydride and

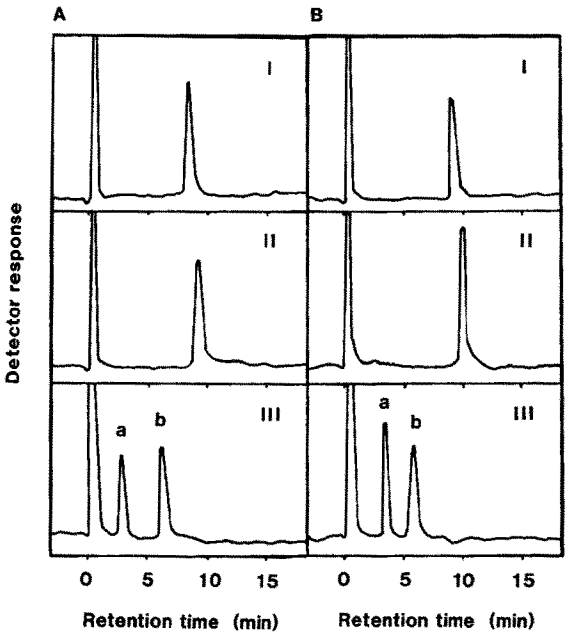

Fig. 1. A. Analysis of 6-DM_1 by l.c. (I) 6-DM_1; (II) 6-DM_1 after reduction with sodium borohydride; (III) Acid hydrolysis products of reduced 6-DM_1; (*a*) 6-deoxy-D-glucose; (*b*) D-glucitol. B. Analysis of 6-DM_2 by l.c. (I) 6-DM_2; (II) Reduced 6-DM_2; (III) Acid hydrolysis products of reduced 6-DM_2; (*a*) 6-deoxy-D-glucitol; (*b*) D-glucose. Conditions for chromatography are as described in the Experimental section.

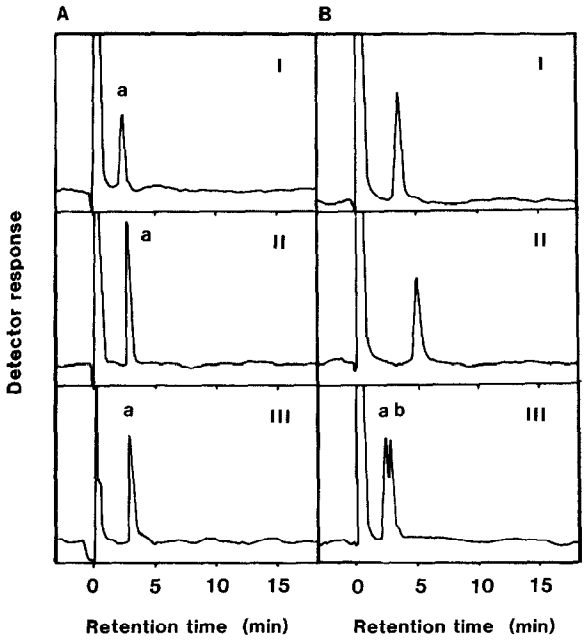

Fig. 2. A Analysis of 6-DG by l.c. (I) 6-DG; (*a*) 6-deoxy-D-glucose; (II) Reduced 6-DG; (*a*) 6-deoxy-D-glucitol; (III) Acid hydrolysis products from reduced 6-DG; (*a*) 6-deoxy-D-glucitol. B Analysis of 6,6-DM by l.c. (I) 6,6-DM; (II) Reduced 6,6-DM; (III) Acid hydrolysis products of reduced 6,6-DM; (*a*) 6-deoxy-D-glucose; (*b*) 6-deoxy-D-glucitol.

analyzed by radiotracer techniques instead of methods used for analysis of 6-deoxysugars.

Paper chromatography (2 ascents, solvent *A*) of the final enzymolysis mixture showed the presence of small amounts of 6-deoxy-6-fluoro-D-glucose (R_G 1.19). Paper chromatography of *tert*-butyl alcohol fractions from charcoal chromatography showed 6-deoxy-6-fluoro-D-glucose eluting in the 1% *tert*-butyl alcohol fraction (compared with D-glucose, which eluted with water). Another product appeared in the 2–3% *tert*-butyl alcohol fractions. The product had paper-chromatographic mobility similar to D-glucose (R_G = 0.97), but eluted from charcoal after maltose. A portion of each of the charcoal chromatography fractions, and of the final enzyme-hydrolysis solution (100 μL of each) was reduced with 1% sodium borohydride solution containing 0.2 mCi of tritiated sodium borohydride. Descending paper chromatography (solvent *B*, 5 h) followed by autoradiography again showed two modified products. These components had mobilities relative to D-glucitol equal to 1.72 and 0.31, compared with values of 1.70, 1.00, and 0.11 for 6-deoxy-6-fluoro-D-glucitol, D-glucitol, and maltitol, respectively. On this basis, these compounds were identified as the reduced forms of 6-deoxy-6-fluoro-D-glucose and 6-deoxy-6-fluoromaltose.

The reduced 6-fluoromaltose mixture was purified by paper chromatography

(solvent *B*, 22 h), and samples were hydrolyzed for either 2 or 4 h in 4.5M TFA. Under these conditions, both samples contained <4% unhydrolyzed disaccharide. Paper chromatography (solvent *B*, 5 h descent) and autoradiography showed the presence of tritium-labeled 6-deoxy-6-fluoro-D-glucitol and D-glucitol. Results of liquid scintillation of these compounds estimated that the ratio of D-glucitol to 6-deoxy-6-fluoro-D-glucitol was 15:1 and 11:1 for the 2 and 4 h hydrolyzates, respectively. These results indicate that both 4-*O*-(6-deoxy-6-fluoro-α-D-glucosyl)-α-D-glucose (6^2-fluoromaltose) and 6-deoxy-6-fluoro-4-*O*-(α-D-glucosyl)-D-glucose (6^1-fluoromaltose) are produced during PPA hydrolysis but that the formation of the isomer modified in the nonreducing residue is greatly favored over the formation of the isomer modified in the reducing residue.

DISCUSSION

Substrates containing 6-deoxy-D-glucose and 6-deoxy-6-fluoro-D-glucose gave similar results when hydrolyzed with porcine-pancreatic alpha amylase. In both instances, products included modified D-glucose and both possible isomers of maltose modified at C-6 of the D-glucose residues. In addition, for each of these substrates, formation of maltose modified in the nonreducing residue was favored over formation of maltose modified in the reducing residue.

The active site of PPA has been shown to consists of five subsites, which bind a series of five D-glucose residues, with hydrolysis occurring between the second and third subsite from the reducing end[20]. From analysis of the products obtained from amylolysis of modified substrates, it is possible to determine whether productive binding of a particular substitution occurs at individual subsites (Fig. 3). Several amylose substrates containing bulky modifications at C-6 have been observed previously[1–8]. In no instances were modified D-glucose or maltose obtained as products of PPA hydrolysis. Substrate-binding specificities for PPA substrates containing 6-*O*-hydroxyethyl-, 6-*O*-D-glucosyl-, and 6-phosphate groups are summarized in Table I. In contrast to these modifications, which are not productively bound at subsites 3 or 4, both 6-deoxy-D-glucose and 6-deoxy-6-fluoro-D-glucose

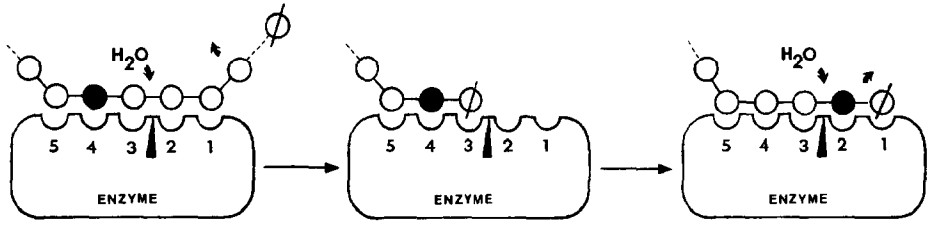

Fig. 3. Five-subsite model for the active site of porcine-pancreatic alpha amylase, showing the formation of a modified disaccharide from a modified substrate. Formation of the modified product requires productive binding of the modified residue at subsites 2 and 4. —○— = D-glucose, —∅— = reducing residue, —●— = modified D-glucose. The point of catalytic attack is indicated by an arrow. The numbers 1–5 refer to individual subsites.

TABLE I

PRODUCTIVE BINDING OF SUBSTRATES MODIFIED AT C-6 TO SUBSITES OF PORCINE-PANCREATIC ALPHA AMYLASE

C-6 Modification	*Porcine-pancreatic alpha amylase subsite*[a]					*Ref.*
	1	*2*	*3*	*4*	*5*	
6-*O*-hydroxyethyl	+	+	−	−	+	8
6-*O*-α-D-glucosyl	+	+	−	−	+	3
6-phosphate	+	+	−	−	+	7
6-deoxy	+	+	+[b]	+	n.d.	This study
6-deoxy-6-fluoro	+	+	+[b]	+	n.d	This study

[a]Substite numbering as in Fig. 3. Plus signs indicate that productive binding is allowed. Minus signs indicate that productive binding is not allowed. N.d. indicates that binding has not been determined. [b]Results suggest that although productive binding at subsite 3 is allowed, it is not as favorable as binding at other subsites.

may be productively bound at subsites 1 through 4. By comparison, it seems that bulky substituents such as hydroxyethyl groups and (1→6)-α-D-glucosyl branches affect enzyme–substrate interactions mainly through steric disruption. Similar effects are also noted for the bulky, charged phosphate group.

Our results suggest that the deoxy- and deoxyfluoro-substituents are tolerated less well at the subsite where catalytic attack occurs (subsite 3) because formation of maltose modified in the reducing residue is less favored than formation of maltose modified in the nonreducing residue. Subsite 3 has been shown to be especially sensitive to substrate modification in experiments involving hydroxyethyl-[8] and 2-deoxy-derivatives[21]. The ratio of disaccharides modified in the nonreducing residue to disaccharides modified in the reducing residue has also been shown to be greater for fluoromaltoses (11–15:1) than for deoxymaltoses (3–4:1). This difference may indicate that the 6-fluoro- group disrupts productive binding at subsite 3 to a greater extent than the 6-deoxy modification.

Fluorine has often been considered a useful analog for hydroxyl groups, because it is about the same size, is highly electronegative, and contains nonbonded electron pairs similar to oxygen, which might allow it to act as a hydrogen-bond acceptor (but not a hydrogen-bond donor). Hydrogen-bonding properties of fluorinated carbohydrates have not been thoroughly studied. Spectroscopic studies on such compounds as 2-fluoroethanol and 1-fluoro-2-propanol have shown them to exist primarily in conformations favorable for intramolecular hydrogen-bond formation[22,23]. N.m.r. studies of Abraham and Monasterios[24] have shown, however, that the predominant rotational conformer for 2-fluoroethyl trichloroacetate is the same as that for 2-fluoroethanol, even though the added trichloroacetic ester would not be expected to participate in strong intramolecular hydrogen bonds. Further n.m.r. studies on 2-fluoroethanol by Griffith and Roberts[25] also gave no evidence for strong intramolecular hydrogen-bond formation, and recent spectro-

scopic studies on 3-fluoro-1-propanol have concluded that hydrogen bonds in this compound must be weak compared with those of 3-amino-1-propanol[26]. We have observed the behavior of 6-deoxy-6-fluoro-D-glucose on a variety of chromatographic media (l.c., t.l.c., paper chromatography, and charcoal chromatography). This compound consistently resembles 6-deoxy-D-glucose rather than D-glucose in its chromatographic mobility. Whether or not this observation is related to hydrogen-bonding capabilities, it seems that fluoro sugars do not closely resemble unmodified sugars with respect to their interactions with chromatographic media and solvents. Interactions with biomolecules, such as enzymes, may be similarly affected.

Other factors, associated with either substrate binding or hydrolysis, may contribute to the observed distribution of products as well. The rate of acid-catalyzed hydrolysis of methyl 6-deoxy-α-D-glucopyranoside is about 5 times greater than for methyl α-D-glucopyranoside, which is, in turn, 6–7 times greater than those for the corresponding 6-halo derivatives[27]. However, we have observed[21] that glucosidic linkages of 2-deoxy-D-*arabino*-hexose residues, which are very acid-labile, are not readily hydrolyzed by PPA. unfavorable interactions between enzyme and modified D-glucose residues at subsites other than subsite 3 may also contribute to producing the observed product distributions. These might include less-favorable interactions with 6-deoxy-D-glucose at subsites 2 or 4, or with 6-deoxy-6-fluoro-D-glucose at subsite 1. Alternatively, such results might conceivably arise from unexpectedly favorable interactions between enzyme and modified substrate, such as highly favorable interactions with 6-deoxy-6-fluoro-D-glucose at subsite 2 or 4.

In any event, our results suggest that, although porcine pancreatic alpha amylase activity is influenced by the presence of 6-deoxy-D-glucose or 6-deoxy-6-fluoro-D-glucose in the substrate, this effect is not crucial. The effects noted for larger modifying groups seem to originate largely from steric disruption, rather than from disruption of critical electronic or hydrogen-bond interactions.

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