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Porcine Pancreatic α-Amylase Hydrolysis of Hydroxyethylated Amylose and Specificity of Subsite Binding†

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ABSTRACT: Hydrolysis of partially hydroxyethylated amylose by porcine pancreatic α-amylase gives rise to a number of hydroxyethylated di-, tri-, and tetrasaccharides, as well as larger products. No modified monosaccharides were detected. The structures of the products containing two to four D-glucose residues have been analyzed by chromatographic and enzymatic techniques. In no instance were these oligosaccharides modified in the reducing-end residue. The location of hydroxyethylated glucose residues within the oligosaccharides has been interpreted in terms of the ability of that (hydroxyethyl)glucose to bind productively at each of the five subsites of the enzyme active site. Results indicate that subsite 3, the subsite at which catalytic attack occurs, is especially sensitive to changes in the substrate and that unmodified glucose is required for productive binding at this subsite. Other subsites specifically allow binding of some (hydroxyethyl)glucose isomers, but not others. Hydroxyethylation is permitted at C-2, C-3, and C-6 for residues bound at subsite 1 and is permitted at C-6 and possibly at C-2 and C-3 for residues bound at subsite 5. However, substitution is permitted only at C-3 and C-6 for binding at subsite 2 and at C-2 and C-3 for binding at subsite 4.

Modified polysaccharides have previously been used to study the specificity and binding requirements for porcine pancreatic α-amylase (PPA)[1] and related enzymes. Several factors, however, have limited the use of this experimental approach. Naturally occurring polysaccharides have proved useful (Weill & Bratt, 1967; Saier & Ballou, 1968; Kainuma & French, 1969, 1970; Misaki et al., 1982; Takeda et al., 1983), but substrates containing desired structural features often are not available. Limitations in suitable synthetic procedures have also confined chemical approaches largely to polysaccharides modified at primary positions (Bines & Whelan, 1960; Weill et al., 1975), with a few exceptions (Kainuma & French, 1982). Moreover, analysis of enzymolysis products usually has been limited to trisaccharides or smaller products.

In this report, we describe the hydrolysis of hydroxyethylated amylose by PPA. Hydrolysis products included several modified oligosaccharides which were analyzed by various chemical and enzymatic techniques. Because this substrate contains hydroxyethyl substituents on C-2, C-3, and C-6 hydroxyls, it was possible to examine enzyme–substrate interactions throughout the PPA active site. Although the hydroxyethyl group may be expected to influence enzyme–substrate interactions through steric effects, these effects may be less pronounced than those observed for branched substrates. The hydroxyethyl group retains the hydroxyl moiety, which may help temper disruption of electronic interactions. In addition, the relatively small size of the PPA active site, 5 subsites compared to as many as 9–10 subsites for *Bacillus subtilis* α-amylase (Robyt & French, 1963; Thoma et al., 1971), allows a more complete interpretation of how substrate modifications affect binding at individual subsites.

Materials and Methods

Crystalline PPA (Worthington Biochemicals Corp.) and glucoamylase (*Rhizopus niveus* glucoamylase from Miles Laboratories and *Aspergillus niger* glucoamylase from Takamine Laboratory) were used without further purification. Specificity of both glucoamylases was identical for our uses. Superlose 500, a hydroxyethylated amylose with a degree of substitution of 0.1–0.2 (i.e., 1–2 substituents per 10 glucose residues), was obtained from Stein-Hall Co. Complete acid hydrolysis of this material, followed by gas chromatography, indicated that approximately 50% of the hydroxyethyl groups were located at C-2, 30% at C-6, and 20% at C-3.

Evaporations were conducted below 40 °C under reduced pressure. Total carbohydrate content and reducing sugar content were analyzed by using the orcinol–sulfuric acid and alkaline ferricyanide methods, respectively, as adapted for use

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[1] Abbreviations: PPA, porcine pancreatic α-amylase; Me_3Si, trimethylsilyl; ds, degree of substitution; HE, hydroxyethyl ($HOCH_2CH_2$). Hydroxyethylated oligosaccharides are named by first indicating which hydroxyl group is modified and then by indicating with a superscript numeral which glucose residue holds that group. Residues are numbered from the reducing end. Hence, 3^2-HE-maltotriose is maltotriose containing a hydroxyethyl group on the oxygen at C-3 of the second residue.

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with a Technicon autoanalyzer (Kesler, 1967; Ough & Lloyd, 1965).

Products from amylolysis of (hydroxyethyl)amylose were partially purified on a charcoal column (10 × 52 cm) containing equal weights of Darco G-60 (Atlas Chemical Industries) and Celite 560 (Johns-Manville). A charcoal slurry was washed successively with 1 N HCl, water, and 5% aqueous *tert*-butyl alcohol and again with water before the column was packed. Carbohydrates were eluted by using a *tert*-butyl alcohol gradient generated by connecting a reservoir (filled successively with 4 L of 1%, 2%, 3%, 4%, 5%, 7%, and 9% *tert*-butyl alcohol) to a mixing vessel filled initially with 4 L of water.

Ascending paper chromatography, used to analyze carbohydrate fractions, employed 6:4:3 1-butanol:pyridine:water (solvent A) or 6:1:2 1-propanol:ethyl acetate:water (solvent B) at 65 °C. Both solvents gave similar results. Descending paper chromatography for preparative work was performed at room temperature by using 9:2:2 ethyl acetate:acetic acid:water (solvent C). Whatman 3MM paper (washed previously with 2% ammonium hydroxide for preparative separations) was used for all chromatography. Reducing sugars were detected by the silver nitrate dip technique (Robyt & French, 1963).

Gel filtration was performed on Sephadex G-15 columns (Pharmacia Fine Chemicals, 1.4 × 100 cm). Carbohydrates were eluted with water at a flow rate of 12 mL/min, collected in 1-mL fractions, and assayed for total carbohydrate.

Oligosaccharide samples were reduced by adding 1% sodium borohydride (0.5–1.0 mL) to a solution of the saccharide (0.4–2.0 mg in 0.1–0.2 mL of water). After 4 h at room temperature, Amberlite IR-120 was added, the solution was filtered and evaporated, and the residue was taken up in 1 mL of methanol and evaporated 3 times. Samples were hydrolyzed in 0.5 M sulfuric acid at 110 °C for 4 h in sealed tubes, neutralized with 0.1 M barium hyroxide, centrifuged, and evaporated.

Gas chromatography was performed on a Packard Model 409 gas chromatograph equipped with flame ionization detectors. Sugars were separated in the form of Me_3Si derivatives on a column (3 mm × 1.8 m) of 3% SE-30 on Gas Chrom Q (Applied Science Laboratories). The chromatograph was operated isothermally at 180 °C for monosaccharides and at 220 °C for disacchardies, with a detector temperature of 235 °C and an injector temperature of 300 °C. Flow rates of 250 and 30 mL/min were maintained for air and H_2, respectively. Products were identified by comparison with authentic standards.

Treatment of HE-amylose with PPA. (Hydroxyethyl)-amylose (20 g) was dissolved in buffer containing 20 mM sodium glycerol phosphate and 10 mM sodium chloride at pH 6.9 (198 mL). The solution was warmed to 39 °C, and PPA (2 mL, 21 000 units) was added. After 3 days, a sample (1.0 mL) of the digest was removed, deionized with Amberlite MB-3 ion-exchange resin, and analyzed by paper chromatography (three ascents, solvent B). Another sample of the digest was reduced with sodium borohydride, acid hydrolyzed, and analyzed by gas chromatography. A large portion (100 mL) of the remaining digest was applied to a charcoal column and separated as described previously. Fractions (20 mL) were collected, and every tenth tube was analyzed by paper chromatography. Samples were pooled into seven major fractions and analyzed for total carbohydrate.

From the results of paper chromatography, fractions 4 and 5 were found to contain substantial amounts of modified oligosaccharides. These were further separated into subfractions (indicated by Roman numerals) by preparative paper chromatography. Modified oligosaccharides were also detected in fractions 6 and 7 but were not further examined because of their size and complexity. Subfractions from preparative paper chromatography were further purified by Sephadex G-15 chromatography for final structural determination.

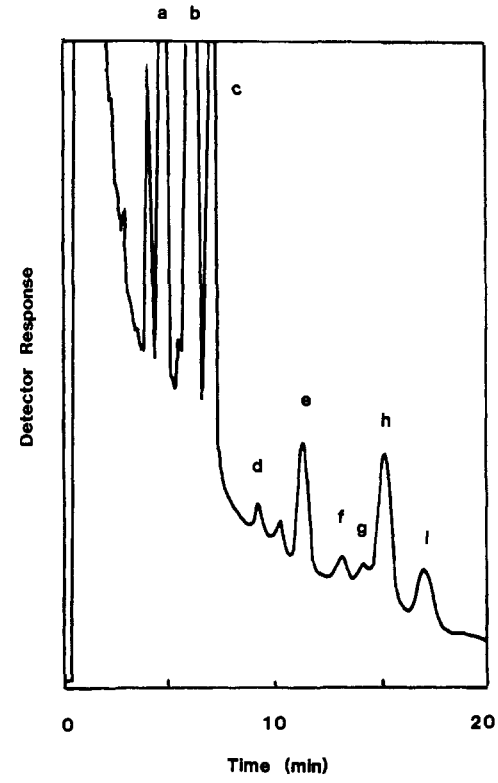

FIGURE 1: Gas chromatography of PPA digest which had been reduced, hydrolyzed with acid, and trimethylsilylated. (a and c) Glucopyranose; (b) sorbitol; (d and g) 3-HE-glucopyranose; (e and h) 2-HE-glucopyranose; (f and i) 6-HE-glucopyranose.

Structural Analysis of Hydrolysis Products. Purified amylolysis products were analyzed by acid hydrolysis both before and after reduction with sodium borohydride. These products were further hydrolyzed with glucoamylase or PPA, and products were purified by paper and gel filtration chromatography. Molecular weights of all oligosaccharide products were estimated from relative elution volumes (elution volume/void volume) on a Sephadex G-15 column, calibrated with a series of maltooligosaccharide standards. Gas chromatographic analysis of all these newly obtained products and their reduced forms was performed as before.

Glucoamylase hydrolysis was performed by incubating *A. niger* glucoamylase (1 unit/1 mg of substrate) in 0.05 M sodium acetate buffer, pH 4.5 (0.5 mL). After 48 h at room temperature, the digest was heated in a boiling water bath, deionized, and concentrated.

Results

Reduction and Acid Hydrolysis of Products from Digestion of HE-amylose with PPA. A sample of the enzyme digest, which had been reduced with sodium borohydride and hydrolyzed with sulfuric acid, was analyzed by gas chromatography. Results show that sorbitol was the only sugar alcohol present (Figure 1).

Separation of Products from Hydrolysis of HE-amylose by PPA. Paper chromatography of seven fractions (indicated by Arabic numerals 1–7) obtained from charcoal column chromatography of hydrolyzed HE-amylose is shown in Figure

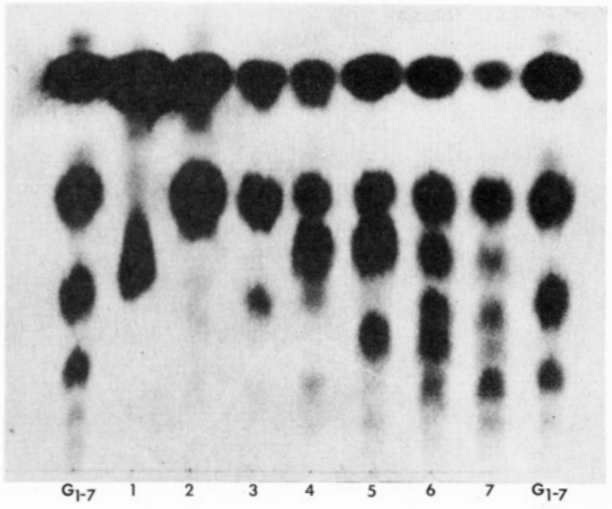

FIGURE 2: Paper chromatography (solvent C) of PPA digest after charcoal column fractionation. G_{1-7}, maltooligosaccharide standards, glucose to maltoheptaose; 1–7, fractions from charcoal chromatography.

Table I: Results of Paper and Gel Filtration Chromatography of Products from Hydrolysis of (Hydroxyethyl)amylose of Porcine Pancreatic α-Amylase

subfraction[a]	R_G[b]	V_e/V_0[c] (±0.03)	M_r[d]
4I-S2	0.96	1.58	350 ± 20
5I-S2	0.96	1.57	360 ± 20
4III	0.57	1.42	580 ± 30
4III-GA1	1.00	1.75	200 ± 10
4III-GA2	0.83	1.53	410 ± 20
4III-GA3	0.53	1.47	500 ± 25
5III	0.58	1.46	510 ± 25
5III-GA1	1.00	1.74	210 ± 10
5III-GA2	0.82	1.53	410 ± 20
5III-GA3	0.52	1.47	500 ± 25
5IV	0.33	1.34	750 ± 30
5IV-GA1	1.00	1.75	200 ± 10
5IV-GA2	0.81	1.51	440 ± 20
5IV-GA3	0.52	1.42	580 ± 30

[a] Charcoal column chromatography fractions (indicated by Arabic numerals) were subfractionated by paper chromatography (subfractions indicated by Roman numerals in order of decreasing chromatographic mobility). Products obtained from further treatment with glucoamylase or by Sephadex G-15 chromatography are indicated by the postscripts GA and S, respectively, and are also numbered in order of decreasing chromatographic mobility. [b] Paper chromatographic mobility of enzymolysis products relative to glucose (solvent B). [c] Relative elution volume (elution volume/void volume) from Sephadex G-15 chromatography. [d] Molecular weights estimated from relative elution volumes, using maltooligosaccharides as standards.

2. The chromatogram shows a number of subfractions (indicated by Roman numerals in descending order of paper chromatographic mobility). Several subfractions were identified as modified di-, tri-, and tetrasaccharides by their mobility on paper and on gel filtration columns. These subfractions (4I, 4III, 5I, 5III, and 5IV) are shown in Table I, along with their molecular weights estimated from gel filtration chromatography. No isomers of (hydroxyethyl)glucose were detected as products of the digestion.

Structure of 4I and 5I. Subfractions 4I and 5I were purified by Sephadex G-15 chromatography. Each of these subfractions was resolved into two components (designated 4I-SI, 4I-S2, 5I-S1, and 5I-S2). Gas chromatography of 4I-S1 and 5I-S1 identified these components as glucose. Acid hydrolysis and gas chromatography of 4I-S2 and 5I-S2 gave 3-HE-glucose and glucose as products. Reduction followed by acid hydrolysis gave 3-HE-glucose and sorbitol (Figure 3). On

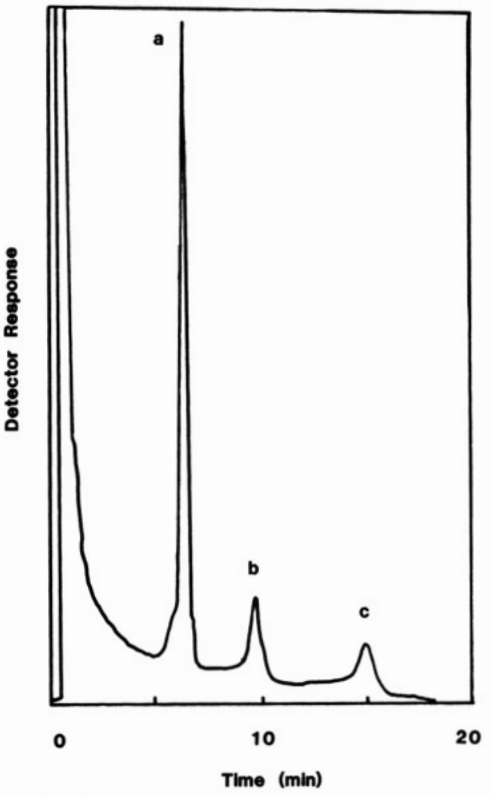

FIGURE 3: Gas chromatography of subfraction 4I-S2 after reduction, acid hydrolysis, and trimethylsilylation. (a) Sorbitol; (b and c) 3-HE-glucopyranose.

the basis of the results, and also on the basis of chromatographic mobility and estimated molecular weight of this compound, the structure of 4I-S2 and 5I-S2 was determined to be 3^2-HE-maltose.

Structure of 4III. Chromatographic analysis had suggested this subfraction consisted of hydroxyethylated trisaccharide(s). Acid hydrolysis and gas chromatography indicated that glucose and 2-HE-glucose were the principal components. Sorbitol was the only sugar alcohol liberated upon hydrolysis of the reduced saccharide (Figure 4).

Treatment of subfraction 4III with glucoamylase gave three products, which were separated by gel filtration. These products were 4III-GA1 (identified as glucose), 4III-GA2 (identified as 2^2-HE-maltose; see Figure 5), and 4III-GA3 (HE-trisaccharide). The ratio of mole percentages of these components, determined by total carbohydrate analysis, was 48:49:4. From these results, we conclude that the major component of 4III is the trisaccharide, 2^2-HE-maltotriose, which is hydrolyzed by glucoamylase to give glucose and 2^2-HE-maltose. This is consistent with our observation that no 2-HE-glucose is liberated when 2-(hydroxyethyl)starch is treated with glucoamylase. These results indicate that glucoamylase cannot hydrolyze a terminal 2-HE-glucose residue.

Acid hydrolysis of 4III-GA3 yielded glucose, 6-HE-glucose, 3-HE-glucose, and small amounts of 2-HE-glucose (Figure 6A). For further analysis, 4III-GA3 was reduced with sodium borohydride and then treated with 0.05 M sulfuric acid for 3 h at 110 °C. This mild acid treatment hydrolyzed only about 15% of the original material and gave monosaccharide and disaccharide products, which were separated by Sephadex G-15 chromatography and examined by gas chromatography. The disaccharide fraction was found to contain maltitol and 3^2-HE-maltitol, as well as other unidentified products (Figure 6B). The monosaccharide fraction contained glucose, sorbitol, 6-HE-glucose, 3-HE-glucose, and 2-HE-glucose (Figure 6C).

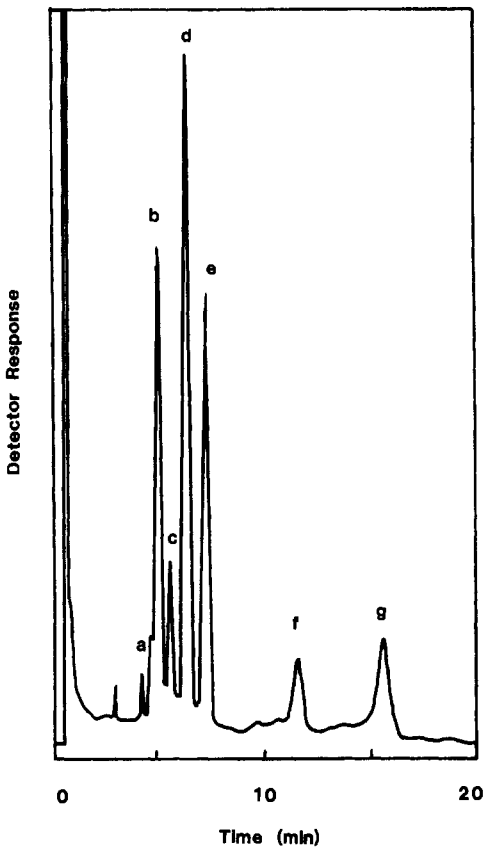

FIGURE 4: Gas chromatography of subfraction 4III after reduction, acid hydrolysis, and trimethylsilylation. (a) 1,2-*O*-Ethylene-α-glucofuranose; (b and e) D-glucopyranose; (c) 1,2-*O*-ethylene-α-glucopyranose; (d) sorbitol; (f and g) 2-HE-glucopyranose.

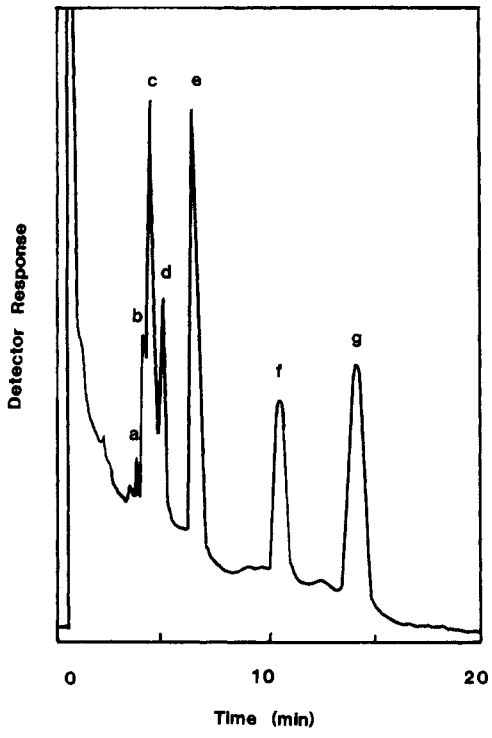

FIGURE 5: Gas chromatography of fraction 4III-GA2 after acid hydrolysis and trimethylsilylation. (a) 1,2-*O*-Ethylene-α-glucofuranose; (b) 1,2-*O*-ethylene-β-glucopyranose; (c and e) glucopyranose; (d) 1,2-ethylene-α-glucopyranose; (f and g) 2-HE-glucopyranose.

These results suggest that 4III-GA3 contains 3^2-HE-maltotriose and trisaccharides modified in their nonreducing termini at carbons 2, 3, and 6. The fact that all three (hydroxyethyl)glucose isomers are released after very limited hydrolysis suggests that any of these isomers may be found in the nonreducing terminus of the mixed trisaccharides.

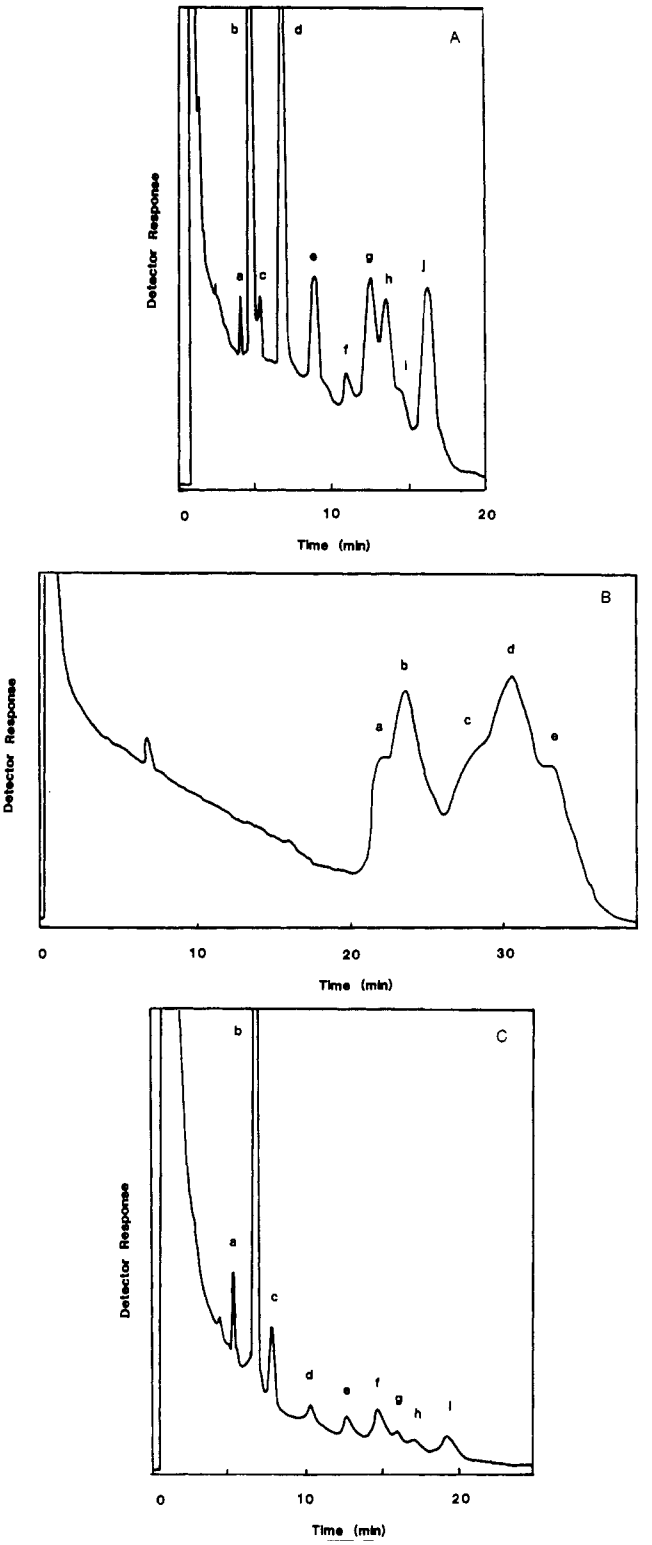

FIGURE 6: (A) Gas chromatography of fraction 4III-GA3 after acid hydrolysis and trimethylsilylation: (a) 1,2-*O*-ethylene-α-glucofuranose; (b and d) glucopyranose; (c) 1,2-*O*-ethylene-α-glucopyranose; (e and h) 3-HE-glucopyranose; (f and i) 2-HE-glucopyranose; (g and j) 6-HE-glucopyranose. (B) Gas chromatography of Me_3Si-disaccharides and Me_3Si-disaccharide alcohols obtained from partial hydrolysis of reduced 4III-GA3 and trimethylsilylation: (a, c, and e) not identified; (b) maltitol; (d) 3^2-HE-maltitol. (C) Gas chromatogram of Me_3Si-monosaccharides and Me_3Si-alditols obtained from acid hydrolysis of reduced 4III-GA3 and trimethylsilylation: (a and c) glucopyranose; (b) sorbitol; (d and g) 3-HE-glucopyranose; (e and h) 2-HE-glucopyranose; (f and i) 6-HE-glucopyranose.

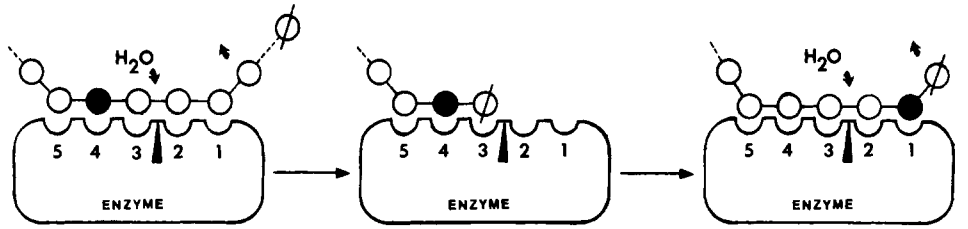

FIGURE 7: Five-subsite model of the porcine pancreatic α-amylase active site showing the hydrolysis of modified substrate to give a modified trisaccharide. The substituted glucose (●) may be bound at subsites 1 and 4. (φ) Reducing glucose; (○) glucose; (▲) point of catalytic attack. The numbers 1–5 refer to individual subsites.

Structure of 5III. Acid hydrolysis of 5III gave glucose, 6-HE-glucose, and 2-HE-glucose as products. Sorbitol was the only sugar alcohol released upon acid hydrolysis of reduced 5III. Reaction of 5III with glucoamylase gave products 5III-GA1, 5III-GA2, and 5III-GA3, which had the same chromatographic properties as 4III-GA1 (glucose), 4III-GA2 (2^2-HE-maltose), and 4III-GA3 (mixed modified trisaccharides). The relative mole percentages of these components were found to be 42%, 45%, and 12%, respectively. Analysis of 5III-GA1 and 5III-GA2 by previously described methods identified these compounds as glucose and 2^2-HE-maltose, formed in a 1:1 ratio from hydrolysis of 2^2-HE-maltotriose. Acid hydrolysis of reduced 5III-GA3 gave glucose, sorbitol, and 6-HE-glucose as products. On the basis of the previous structural analysis of 4III-GA3, and the reported observation that glucoamylase may cleave α1→4 linkages immediately adjacent to α1→6 branch linkages (Kainuma & French, 1970), the structure of 5III-GA3 has been assigned as 6^3-HE-maltotriose.

Structure of 5IV. Chromatographic results indicated that subfraction 5IV consisted of modified tetrasaccharides. Acid hydrolysis released glucose, 2-HE-glucose, and 6-HE-glucose, and small amounts of 3-HE-glucose. Sorbitol was the only reduced product obtained from hydrolysis of reduced 5IV. Glucoamylase treatment of 5IV gave three products with chromatographic properties similar to those of glucose, modified disaccharide, and modified trisaccharide, as observed for glucoamylase digestion of 4III and 5III. Further analysis (as previously described) identified these compounds as glucose (5IV–GA1), 2^2-HE-maltose (5IV-GA2), and trisaccharide (5IV-GA3), with relative mole percentages of 62%, 28%, and 11%, respectively. These glucoamylase products have been interpreted as arising from hydrolysis of 2^2-HE-maltotetraose (with formation of 2 mol of glucose per mol of 2^2-HE-maltose formed), plus hydrolysis of other tetrasaccharides (with formation of 1 mol of glucose per mol of trisaccharide formed). Analysis of the trisaccharide 5IV-GA3 showed it to be composed of glucose, 3-HE-glucose, and traces of 6-HE-glucose, with sorbitol being the only reduced product detected after hydrolysis of the reduced trisaccharide. Earlier results had shown that 3^2-HE-maltotriose and 6^3-HE-maltotriose are not further degraded by glucoamylase (see analysis of 4III). Therefore, we propose that the tetrasaccharides 3^2-HE-maltotetraose and 6^3-HE-maltotetraose are hydrolyzed by glucoamylase to give the two trisaccharides in 5IV-GA3, 3^2-HE-maltotriose and 6^3-HE-maltotriose. The specificity of bond cleavage observed here for *A. niger* glucoamylase is identical with that observed by Takeda et al. (1983) for substrates phosphorylated at C-3 and C-6.

The original tetrasaccharide mixture (5IV) was further treated with PPA to verify these structural assignments. Results (data not shown) were consistent with a scheme in which 2^2-HE-maltotetraose is hydrolyzed to form glucose and 2^2-HE-maltotriose, 6^3-HE-maltotetraose is hydrolyzed to glucose and 6^3-HE-maltotriose, and 3^2-HE-maltotetraose is hydrolyzed to form either glucose plus 3^2-HE-maltotriose or maltose plus 3^2-HE-maltose.

Table II: Binding of Hydroxyethylated Glucose to Subsites of Porcine Pancreatic α-Amylase

position of HE group in glucose	porcine pancreatic α-amylase subsite[a]				
	1	2	3	4	5
C-2	+	−	−	+	*
C-3	+	+	−	+	*
C-6	+	+	−	−	+

[a] Subsite numbering as in Figure 7. Plus signs indicate that productive binding of substituted glucose is allowed. Minus signs indicate that productive binding is not allowed. Asterisks indicate that binding seems to occur but has not been definitely demonstrated.

Discussion

The active site of PPA has been shown to consist of five subsites that can bind consecutive glucose residues, with cleavage occurring between subsites 2 and 3 (Robyt & French, 1970). By observing the products obtained from amylolysis of modified substrates, it is possible to determine which subsites will allow productive binding of the modified glucose residues (Figure 7). This has been done by using partially hydroxyethylated amylose modified at the C-2, C-3, and C-6 hydroxyls. Results have led to several conclusions about binding of (hydroxyethyl)glucose (summarized below and in Table II) that are consistent with action observed on naturally occurring substrates containing α1→6 branches (Kainuma & French, 1969, 1970) and α1→3 branches (Misaki et al., 1982). It should be noted that these results only reflect productive binding and that they do not exclude the possibilty of nonproductive binding of modified residues, where the modified residue may be bound but hydrolysis does not occur.

(1) No modified monosaccharides were obtained as products, and no modified sugar alcohols were obtained upon reduction and acid hydrolysis either of the crude enzyme digest or of any of the purified products. Therefore, no (hydroxyethyl)glucose isomers are productively bound at subsite 3.

(2) Productive binding of 3-HE-glucose seems to be prohibited only at subsite 3. Formation of 3^2-HE-maltose indicates that 3-HE-glucose may bind at subsites 2 and 4, and formation of 3^2-HE-maltotriose provides evidence for binding at subsite 1. A modified trisaccharide, 3^3-HE-maltotriose, has been tentatively identified in fraction 4III-GA3. The presence of this product would suggest that 3-HE-glucose may bind productively at subsite 5.

(3) Degradation of 2^2-HE-maltotetraose by PPA to produce only glucose and 2^2-HE-maltotriose indicates that 2-HE-glucose may be bound at subsite 1 but not at subsite 2. The presence of 2^2-HE-maltotriose as a product also points to binding at subsite 4. Some evidence for the formation of trace amounts of 2^3-HE-maltotriose was obtained. Formation of

this product could be taken as evidence for binding at subsites 2 and 5. However, on the basis of evidence just described, we feel that this does not reflect binding at subsite 2. A small amount of this product could arise from chains modified at the nonreducing terminus, which would require binding only at subsite 5. The formation of only small amounts of this material, moreover, might further suggest that this modification is poorly tolerated at subsite 5.

(4) Formation of 6^3-HE-maltotetraose indicates that binding of 6-HE-glucose is permitted at subsites 1 and 5. Hydrolysis of this saccharide by PPA to form 6^3-HE-maltotriose indicates that C-6 hydroxyethylation is allowed at subsite 2 but not at subsites 3 or 4. The identification of 6^3-HE-maltotriose in fractions 4III-GA3 and 5III-GA3 also suggests that 6-HE-glucose may be bound at subsites 2 and 5.

Low-resolution X-ray diffraction studies (Payan et al., 1980) have located the PPA active site in a deep cleft running down the side of the enzyme. Our results suggest that the hydroxyethyl modification disrupts productive enzyme–substrate complex formation either through steric effects by preventing proper alignment of the substrate in the active site or through disruption of critical electronic interactions, such as hydrogen bonding. In either instance, the effect of hydroxyethylation at C-2, C-3, and C-6 is particularly critical at the catalytic subsite 3. We are presently studying PPA hydrolysis of a number of modified substrates. Results (in preparation) indicate that substrates with small hydrophobic substituents are hydrolyzed by PPA more readily than bulky or charged groups. These trends are supported by published observations. Bines & Whelan (1960) have reported (without supporting data) that 6-deoxyglucose is obtained as a product from hydrolysis of 6-deoxyamylose (ds = 0.82), whereas Maley et al. (1966) have reported that glycogen containing 2-amino-2-deoxyglucose is digested by PPA to give two major products that seem to be modified tri- and tetrasaccharides. In addition, the data of Takeda et al. (1983) indicate that binding of glucose phosphorylated at C-6 is allowed only at subsites 1, 2, and 5 and that glucose phosphorylated at C-3 may be bound only at subsite 4.

The reaction catalyzed by amylases and related enzymes is suggested to proceed at some point through a half-chair or sofa oxycarbonium ion transition state (Thoma, 1968; French, 1981) similar to the transition state proposed for acid-catalyzed hydrolysis of glycosides (BeMiller, 1967) and lysozyme (Blake et al., 1967). Enzyme assistance in forming or stabilizing this transition state is thought to be instrumental for enzyme catalysis. Several factors have been proposed to account for this assistance, including acid–base catalysis (Vernon, 1967), induction of steric strain (Blake et al., 1967), electrostatic stabilization (Warshel & Levitt, 1976), and binding site complementarity (Chipman & Schindler, 1981). Wolfenden (1983) has recently reiterated the suggestion that well-orchestrated electronic interactions between substrate and enzyme are crucial to the formation of charged intermediates in a nonaqueous enzyme active site. For carbohydrases, such interactions could include stabilization through formation of enzyme–substrate covalent intermediates (French, 1981) or through electrostatic effects (Warshel & Levitt, 1976).

The PPA active site, observed in X-ray studies, may resemble the hydrophobic clefts found for many enzyme active sites, including that of lysozyme. From studies involving modified PPA substrates, it seems that enzyme–substrate interactions are seriously disrupted at the catalytic subsite by bulky or charged modifications but are disrupted to a much lesser extent by small hydrophobic modifications. Our results, summarized in Table II, also indicate that the effect of substrate alteration is particularly important at the catalytic subsite but that certain specific modifications may critically affect binding at other subsites as well.

Registry No. α-amylase, 9000-90-2; (hydroxyethyl)amylose, 9050-73-1; 2-*O*-ethyl-D-glucose, 10230-16-7; 3-*O*-ethyl-D-glucose, 40879-13-8; 6-*O*-ethyl-D-glucose, 52598-05-7.

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