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ACTION OF PORCINE-PANCREACTIC AMYLASE ON OXIDIZED–REDUCED AMYLOSE OF LOW DEGREE OF MODIFICATION*

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ABSTRACT

Amylose was oxidized with 0.1–0.2 mol of periodate per glucose residue (G), and then reduced with sodium borohydride or borotritide to give an oxidized–reduced amylose of low degree of modification. Mild acid hydrolysis gave erythritol, 2-*O*-α-D-glucosyl-L-erythritol, higher homologs, and other products. Extensive action of porcine-pancreatic amylase on the polymer gave, besides D-glucose and maltose, oligosaccharides containing one or more oxidized–reduced (modified, M), acyclic residues. The enzymic products containing only one oxidized–reduced residue were identified as a modified tetrasaccharide (MG_3) and a modified pentasaccharide (MG_4). Structures of MG_3 and MG_4 were identified by a combination of enzymic and chemical approaches. With glucoamylase, MG_4 was converted into MG plus D-glucose, whereas MG_3 was totally resistant. On mild acidic hydrolysis, MG_3 was converted into 2-*O*-α-D-glucosyl-D-erythritol plus maltose. These results indicate that MG_3 is G-M-G-G and that MG_4 is G-G-M-G-G. In principle, MG_4 could occupy the five D-glucose residue, substrate-binding site of porcine-pancreatic amylase in such a way that M, the acyclic structure replacing a D-glucose residue, is placed just to the "left" of the catalytic site. The modified structure, being very vulnerable to acidic hydrolysis, might then be expected to be very readily attacked by the amylase, but in fact, it is not.

INTRODUCTION

It is well known and readily appreciated that irregularities or anomalies in the structure of biopolymers may well lead to the failure of enzymes to attack such polymers in the vicinity of these irregularities. In the case of amylase action on starch,

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branching points[2-4], phosphate groups[5], bulky chemical substituents (D. French and Y. C. Chan, unpublished data), or rupture of the glucose ring by periodate oxidation[6] sufficiently distort or prevent enzyme–substrate binding so that productive E-S complexes do not form at the site of the modification. On the other hand, chemical alteration of starch, without introduction of bulky groups, and in such a way as to facilitate acidic cleavage of interresidue linkages might well promote enzymic hydrolysis at the site of modification. To test this general idea, we have carried out a low degree of periodate oxidation of amylose, such that only 10–20% of the D-glucose residues were oxidized. We then reduced the polymer with sodium borohydride (or borotritide) giving a sparsely modified, oxidized-reduced polyol to use as a substrate for porcine-pancreatic amylase. At the sites of oxidation and reduction, the acetal linkages in the polymer are far more sensitive to acid hydrolysis than glycosidic bonds[7]. Thus, to the extent that acid hydrolysis is part of the catalysis mechanism for porcine-pancreatic amylase and that the acyclic structures found in place of D-glucose residues are bound at the catalytic site, these structures might well be especially sensitive by the attack by the enzyme. However, conversely, if there are important components of the reaction mechanism for porcine-pancreatic amylase involving substrate torsion and distortion in the direction of the transition state, such chemical modification of the substrate by cleavage of the carbon chain of the glucose ring could not permit or facilitate distortion of the bond angles, owing to the ease of internal rotation and flexibility of the open-chain molecule. The present paper reports our studies examining this question.

MATERIALS AND METHODS

Materials. — Commercial amylose "Superlose" was a gift of Stein–Hall Co. The amylose was converted into a water-soluble form by dissolving it in dimethyl sulfoxide and then precipitating it with methanol, and drying with acetone. Potassium borotritide was purchased from ICN Chemical & Radioisotope Division, Irvine, CA, and diluted to 0.1M KBT_4 (3Ci/mmol) in 0.1M potassium hydroxide. Twice-crystallized *Rhizopus niveus* glucoamylase was purchased from Seikagaku Kogyo, Tokyo, Japan. Crude yeast maltase was purchased from Mycoferm (now Gist-Brocades N.V.), Delft, Holland. Porcine-pancreatic amylase was a twice-crystallized preparation purchased from Boehringer–Mannheim. Other chemicals were standard reagent grade.

Preparation of amylose partially oxidized by sodium metaperiodate. — Amylose 5.67 g) was dispersed in 100mM sodium metaperiodate (70 mL) and allowed to react in the dark at room temperature. The consumption of periodate was monitored by determining the 290-nm absorption[8] of diluted supernatant solution during the reaction. After 72 h, the amount of periodate consumed was 0.2 mol. per D-glucose residue, and the reaction was stopped by adding an excess of ethylene glycol. The partially oxidized amylose was precipitated by adding 140 mL of acetone, washed several times with 65% acetone, and then dehydrated with 100% acetone. Other preparations having a degree of modification (d.m.) of 0.1–0.5 were made similarly (d.m. = the fraction of original D-glucose residues converted into M residues).

Reduction of oxidized amylose. — The 0.2-d.m. oxidized amylose (4 g) was suspended in 50 mL of distilled water, and sodium borohydride (400 mg) was added. After 24 h at room temperature, the reaction was stopped by adding acetic acid to pH 5.0. The oxidized–reduced amylose was precipitated by adding 100 mL of acetone, washed three times with 65% acetone, and then dried with acetone.

To prepare tritium-labeled, oxidized–reduced amylose, 5 mg of periodate-oxidized amylose was dissolved in 1 mL of distilled water and reduced by 0.1M KBT_4 (10 μL) dissolved in 0.1M potassium hydroxide for 2 h at room temperature. After reduction, the excess of KBT_4 was decomposed by adding acetic acid to pH 5.0. The mixture was dried in a vacuum desiccator overnight, and then dissolved in 250 μL of distilled water and kept in a freezer.

Chromatography and radioautography. — Ascending and descending paper chromatography were performed with Whatman 3 MM paper with 3:2:2 (v/v) butyl alcohol–pyridine–water. After irrigation, chromatograms were treated by the glucoamylase dip-method[4] to convert the weakly reducing oligosaccharides into D-glucose, the D-glucose then being revealed by the silver nitrate dip-method.

To survey enzyme action on various modified oligosaccharides, the two-dimensional paper-chromatographic technique[9] was used. High-performance liquid chromatography (l.c.) was conducted with a Waters Liquid Chromatograph model ALC/GPC 201 using a μBondapak Carbohydrate Analysis column.

For radioautography of tritium-labeled oligosaccharides, the irrigated paper chromatogram was treated with a 7% solution of PPO (2,5-diphenyloxazole)[10] in diethyl ether and then dried. The PPO-treated paper chromatogram was kept in contact with Kodak x-Omat S film XS-5 at —80° for one week. Then the film was developed with Kodak liquid X-ray developer.

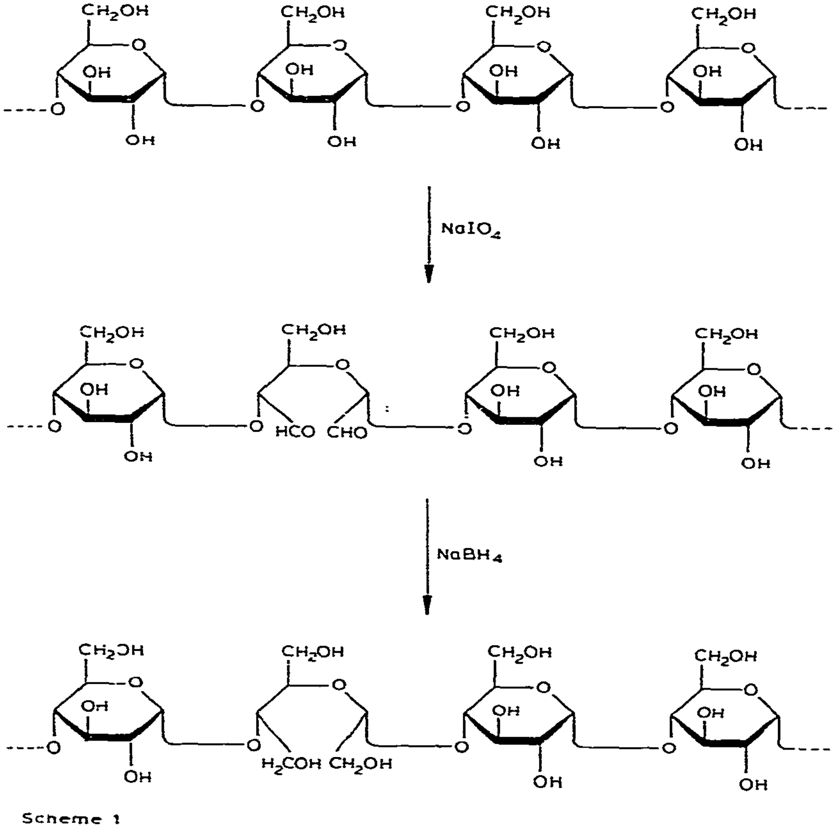
Digestion of oxidized-reduced amylose with porcine-pancreatic amylase. — The 0.2-d.m. oxidized–reduced amylose (500 mg) was dissolved in 2 mL of dimethyl sulfoxide at room temperature. Distilled water (20 mL) was then added and 750 IU of porcine-pancreatic amylase dissolved in 5 mL of 20mM sodium glycerophosphate buffer (pH 6.9) was added to the oxidized–reduced amylose solution. The reaction proceeded for 72 h at 38°. Under these conditions, the amylase action reached the "second stage"[11] where all unmodified regions of the amylose chain were converted into D-glucose (G_1), maltose (G_2), and a trace of maltotriose (G_3). At this point, the reducing value of the digest was 79% of the theoretical maltose. Liquid chromatography revealed G_1, G_2, G_3, a tetramer having one modified residue (MG_3), and a pentamer having one modified residue (MG_4). MG_3 and MG_4 were purified by separation on a column[12] of Biogel P-2, followed by preparative paper-chromatography. Reducing values were determined by the Somogyi–Nelson method[13], and total carbohydrate by the phenol–sulfuric acid method[14].

Reaction of tritium-labeled, oxidized–reduced amylose was performed similarly on a small scale. The products were used directly for two-dimensional chromatography or for separation of the inidividual components by preparative paper-chromatography. With radioactive substrates, the digests were the same except for the concentration (unmeasured) of substrate.

Mild acid degradation of oxidized-reduced amylose, MG_3, and MG_4. — Oxidized-reduced amylose (1 mg in 0.5 mL of water) was treated with 0.5M trifluoroacetic acid (0.5 mL) for 18 h at room temperature. In control experiments, these conditions were shown to cleave all oxidized-reduced (M) units with no appreciable hydrolysis of D-glucosidic bonds. MG_3 and MG_4 were similarly treated. Following the hydrolysis, the trifluoroacetic acid was removed by evaporation at 35–40° under diminished pressure (water aspirator). After the first evaporation, distilled water was added to the sample, and evaporation was repeated two or three times to effect complete removal of the acid.

RESULTS AND DISCUSSION

Structure of oxidized-reduced amylose. — The chemical reactions used in the preparation of low-d.m. oxidized-reduced amylose are shown in Scheme 1. To test



Scheme 1. Preparation of oxidized-reduced amylose of low degree of modification.

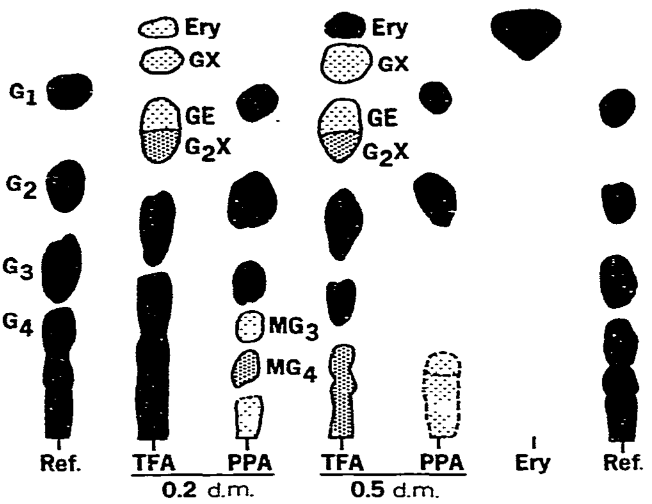
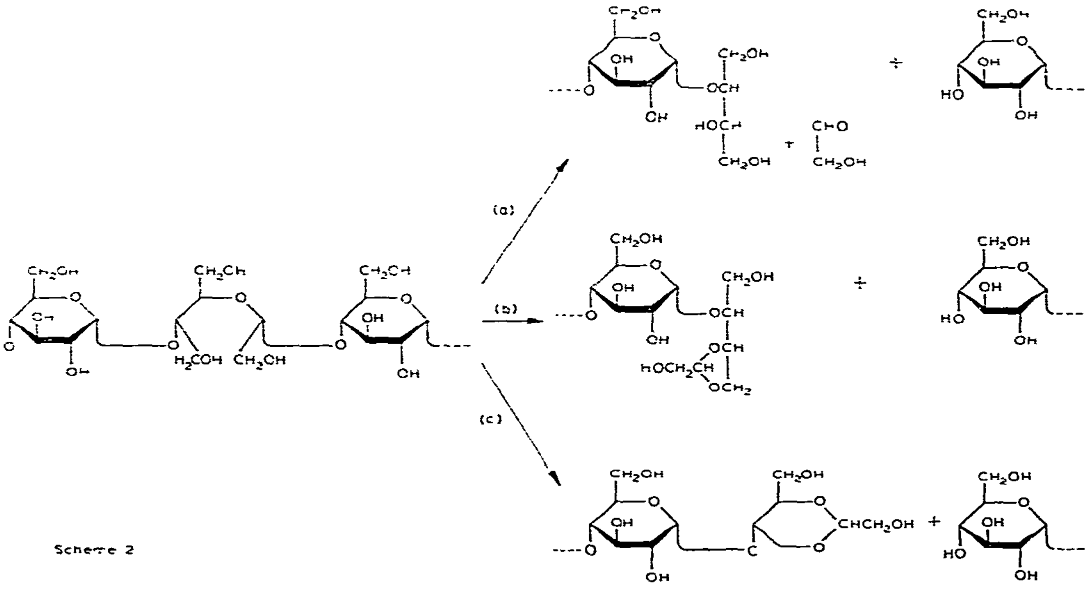


Fig. 1. Paper chromatogram showing degradation products of oxidized–reduced amylose. Ref.: D-glucose and reference malto-oligosaccharides (G_1, G_2, G_3, and so on); MG_3 and MG_4 are "tetrasaccharide" and "pentasaccharide" containing one modified residue; TFA: products of action of 0.25M trifluoroacetic acid at room temperature; PPA: products of extensive action of porcine-pancreatic alpha-amylase; Ery: erythritol; GX: α-D-glucosyl-X; GE: *O*-α-D-glucosylerythritol; G_2X: α-maltosyl-X. The intensity of the spots or areas on the chromatogram is: solid black, intense or very intense; heavily stippled, medium intensity; lightly stippled, weak intensity; lightly stippled with dashed line border, very weak intensity.

the nature of our oxidized–reduced amylose, we submitted it to mild, partial acid hydrolysis by using 0.25M trifluoroacetic acid overnight at room temperature. These conditions are similar to those used in the "mild" Smith degradation[7] and do not give chromatographically detectable cleavage of glycosidic bonds in amylose or starch oligosaccharides. The partially degraded polysaccharides showed paper-chromatographic series obviously different in R_F values from the reference series of D-glucose and malto-oligosaccharides (Fig. 1). Each spot appeared to consist of at least two compounds. Paper chromatograms (not shown) of higher resolving power split the spots into two distinct series. With the 0.1-d.m. material (not shown), erythritol could not be detected. With the 0.2-d.m. material, erythritol was barely visible, but with the 0.5-d.m. sample, the erythritol spot was fairly intense. This result is in accord with the non-random attack of periodate as discussed by Painter and Larsen[15]. There is a spot of R_F value between D-glucose and erythritol that, on reaction with the silver reagent, gave only a weak brown stain. This "brown spot" subsequently turned out to be α-D-glucosyl-X, where X is a cyclic acetal of erythritol and glycolaldehyde[7,16]. Compound X itself has a very high R_F value, reacts very weakly with the silver reagent, and is almost invisible on the chromatograms. By contrast, erythritol, X, and the various glycosides or modified oligosaccharides containing these moieties or M give intense spots on the radioautographs. In the nonradioactive hydrolyzate, the two partly resolved compounds migrating between D-glucose and maltose are 2-*O*-α-D-glucopyranosyl-L-erythritol (higher mobility, weakly staining) and maltosyl-X (lower mobility, more strongly staining). Other compounds of lower mobility stain more intensely owing to the treatment on the chromatogram with the glucoamylase dip reagent, which converts maltose, higher oligosaccharides, and their glycosides in part into D-glucose, which is strongly staining. Incidentally, glycerol and erythritol give rather strong stains with the silver reagent, although these alditols are nonreducing to the ordinary alkaline reagents. The chemistry of the mild acidic cleavage reactions[7-16] is shown in Scheme 2.



Scheme 2. Alternative pathways for degradation of oxidized–reduced amylose of low degree of modification or modified oligosaccharides during mild acid treatment. (*a*) Classical hydrolysis with formation of substituted erythritol and glycolaldehyde plus the right-hand fragment of the polymer or oligosaccharide chain; (*b*) formation of a 5-membered (dioxolane) ring plus right-hand fragment through acetal transfer; (*c*) formation of a 6-membered (1,3-dioxane) ring plus right-hand fragment through acetal transfer. The stereochemistry at the ring methylidene C atom is not known. For any of the pathways, borotritide-labeled reactant would give radioactive erythritol, glycolaldehyde, or products containing these radioactive moieties.

After mild acid hydrolysis of the 0.2-d.m. material, paper chromatography (Fig. 1) showed an abundance of low-mobility material in the range d.p. >5. By contrast, under similar treatment, the 0.5-d.m. material gave only a small amount of product in the pentasaccharide and higher range. This result indicated that the 0.2-d.m. material contains "runs" of D-glucose residues in which the space between the M units is long enough to accommodate the substrate-binding site of porcine-pancreatic amylase. This, in turn, ensures that, when the modified amylose is used as a substrate for the amylase, at least some of the M residues will be found in oligosaccharides containing a single M residue.

Products of action of porcine-pancreatic amylase on oxidized-reduced amylose. — Very extensive (Fig. 1) or exhaustive (Fig. 2) action of porcine-pancreatic amylase converts the oxidized–reduced amylose mainly into D-glucose, maltose, and a trace of maltotriose (these products originating from runs of D-glucose residues in the substrate), two modified oligosaccharides that we designate MG_3 and MG_4, and unresolved products of higher molecular weight. The chromatographic and biochemical properties of MG_3 and MG_4 indicate that they are a modified tetrasaccharide (MG_3) and a modified pentasaccharide (MG_4), respectively. The paper-chromatographic mobilities of MG_3 and MG_4 are considerably higher than those of maltotetraose and maltopentaose, respectively, as is appropriate for compounds containing C-H bonds in addition to those of the comparable unmodified oligosaccharides. MG4 is rapidly and quantitatively converted into MG_3 plus D-glucose by glucoamylase, but MG_3 is totally resistant (Fig. 3 and 4). It is our experience and that of others[17] that α-D-glucopyranosides containing aglycons other than D-glucose are resistant to glucoamylase. Specifically, 1-*O*-α-D-glucopyranosyl-D-glycerol, 2-*O*-α-D-glucopyranosyl-L-erythritol, and 4-*O*-α-D-glucopyranosyl-D-glucitol (maltitol) are essentially inert as substrates. On the other hand, maltose is slowly attacked, and higher malto-oligosaccharides are good substrates. From our understanding of the specificity of glucoamylase, this observation suggests that the structure of MG_4 is

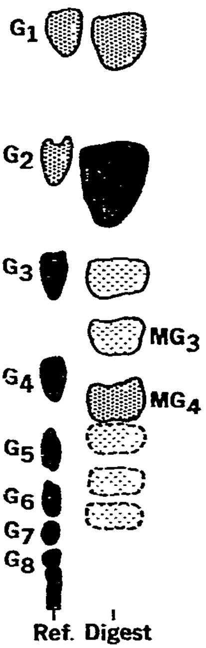


Fig. 2. Exhaustive action of porcine-pancreatic alpha-amylase on 0.2-d.m. oxidized–reduced amylose. Tracing of descending paper-chromatogram. Symbols as in Fig. 1. The three unidentified zones in the digest in the chromatographic range of G_5–G_7 are probably oligosaccharides of higher molecular weight containing two oxidized–reduced D-glucose residues.

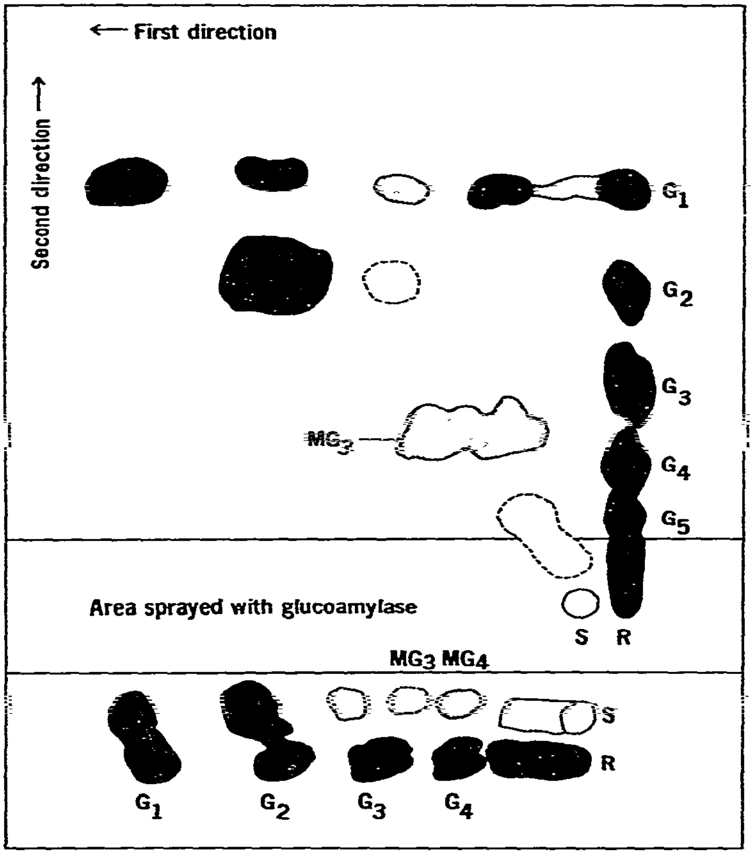


Fig. 3. Tracing of two-dimensional paper chromatogram[9] showing the action of glucoamylase on a porcine-pancreatic alpha-amylase digest of 0.2-d.m. oxidized-reduced amylose. The two R channels are reference series for the first and second direction of the chromatogram. Samples were applied at the two S positions. After irrigation in the first direction, the bottom section of the chromatogram (as shown), containing the first R channel and one S channel was cut off for reference. The remaining S channel was sprayed with glucoamylase solution. After allowing enzyme action on the paper, the chromatogram was dried, the second reference was applied, and the chromatogram was irrigated in the second direction.

most probably G-G-M-G-G so that there is only a single glucoamylase-susceptible D-glucose residue at the nonreducing end of the oligosaccharide. Inasmuch as MG_4 is quantitatively converted by glucoamylase into D-glucose plus MG_3, this means that MG_3 is G-M-G-G. Partial acid hydrolysis under very mild conditions (Fig. 4) leads to maltose plus D-glucosylerythritol from MG_3 and to maltose plus maltosylerythritol and maltosyl-X from MG_4, plus other products of low molecular weight, including glycolaldehyde (not visible in Fig. 4). Fig. 3 shows that, under the conditions of two-dimensional chromatography, MG_4 is completely hydrolyzed by glucoamylase to give D-glucose plus MG_3. However, the higher oligosaccharides produced by exhaustive action of porcine-pancreatic amylase on oxidized-reduced amylose are

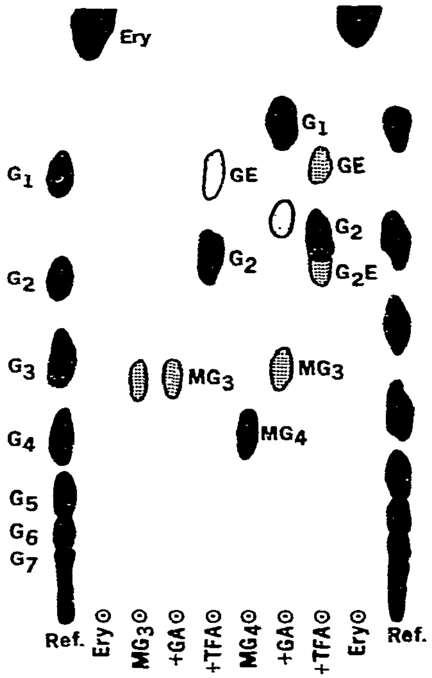


Fig. 4. Paper chromatogram of the action of glucoamylase and trifluoroacetic acid on MG_3 and MG_4. Symbols as in Fig. 1.

not converted by glucoamylase into MG_3. This fact, together with their chromatographic properties, indicates that they are such multiply-modified oligosaccharides as G-M-G-M-G-G, G-G-M-G-M-G-G, and more complicated structures. We have not investigated these further.

Modified amylose and the active site of porcine-pancreatic amylase. — Under the extensive conditions of enzymolysis used, unmodified amylose and its oligosaccharides are totally converted into D-glucose and maltose plus a trace of maltotriose[11]. The formation of MG_3 and MG_4 as stable end-products and the failure to form such smaller modified structures as D-glucosylerythritol, D-glucosyl-X, maltosylerythritol, or maltosyl-X shows that the oxidized–reduced glucose residues are totally unaffected by the enzyme treatment. These results are best interpreted according to the scheme in Fig. 5. For formation of MG_3, the modified residue M must have occupied subsites I and V during the cleavage at the nonreducing and reducing end, respectively. The sequence of these cleavages is unknown and is probably of no significance. Similarly, for formation of MG_4, M must have occupied, in turn, both subsite V and subsite Zero; that is, a virtual subsite just to the right of the binding site. It can be rigorously concluded that M does not occupy subsites II, III, or IV to give productive complexes, as otherwise, we would find singly modified oligosaccharides other than MG_3 and MG_4. In particular, we consider it highly significant that M cannot bind at subsite III, although, by binding MG_4 in this way,

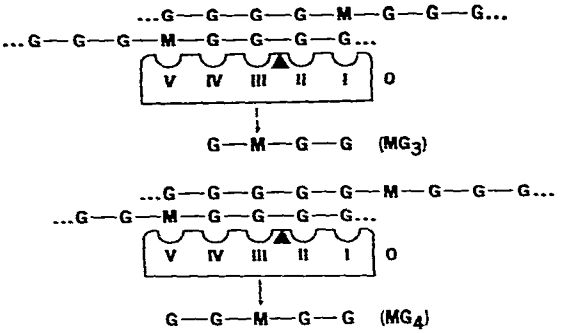


Fig. 5. Schematic binding-modes of oxidized–reduced amylose that give rise to MG_3 and MG_4. Roman numerals I–V indicate the substrate-binding subsites of porcine-pancreatic amylase, starting at the reducing end of the substrate. Subsite zero (0) is a virtual subsite off the right-hand edge of the substrate-binding site. To produce MG_3 (G-M-G-G), the M (modified D-glucose residue) must have been alternately at subsites I and V (the sequence is not known and probably is of no significance). To produce MG_4 (G-G-M-G-G), M must have been alternately at subsites zero and V. The catalytic groups in the enzyme are represented by the black wedge.

there could be total occupancy of the porcine-pancreatic amylase binding-site. Such hypothetical binding of MG_4 to the enzyme would give either a productive complex, contrary to our results, or an inhibitor complex.

Conversion of a D-glucose residue into an M residue makes it impossible for it to assume the shape of a G residue. The C-2–C-3 bond distance in the original D-glucose residue is only 1.5 Å, whereas the minimum van der Waals distance between 2-CH_2 and 3-OH_2 in the M residue is ~4 Å.

This conformational change in going from G to M may be a major or the sole factor in the inability of the M residues to bind at subsites II, III, or IV with formation of productive complexes.

While attractive arguments may be advanced as to how the M residues could participate in such binding, the fact remains that they do not.

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