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THE ACTION PATTERN OF HUMAN SALIVARY ALPHA-AMYLASE IN THE VICINITY OF THE BRANCH POINTS OF AMYLOPECTIN*

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ABSTRACT

Salivary alpha-amylase hydrolyses amylopectin in stages. At the end of the so-called second stage, there are present glucose, maltose, and a series of α-limit dextrins containing (1→4)- and (1→6)-α-D-glucosidic bonds. The structures of the limit dextrins containing a single (1→6)-bond were examined. Six such dextrins were found. Of these, two were capable of being further hydrolysed by alpha-amylase, whereas the remaining four were true, amylase-resistant α-limit dextrins. The structures of the limit dextrins afforded information about those (1→4)-α-D-glucosidic bonds of amylopectin that are capable of being cleaved by salivary alpha-amylase and those that are resistant. In order to define further the action of alpha-amylase, the alpha-amylolytic products of 6-α-maltotriosyl-D-glucose, 6^3-α-maltotriosylmaltotriose, and 6^3-α-maltotriosylmaltotetraose were examined.

INTRODUCTION

Human salivary alpha-amylase (EC 3.2.1.1) is an enzyme specific for catalysing the hydrolysis of (1→4)-α-D-glucosidic bonds in starch and related polymers. Not all such bonds are equally susceptible to its action, and some are completely resistant.

Salivary alpha-amylase is capable of hydrolysing amylose to maltose and maltotriose in the so-called, first stage of alpha-amylolysis. It is now accepted[1,2] that a substantial increase in the concentration of alpha-amylase will bring about the hydrolysis of maltotriose to maltose and D-glucose. The action of salivary alpha-amylase on amylopectin and glycogen proceeds in a manner similar to that for amylose, in that two levels of hydrolysis may also be discerned. Walker and Whelan[2]

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showed that the first stage of hydrolysis of amylopectin and glycogen, equivalent to the release of maltose and maltotriose from amylose, produced maltose, maltotriose, and a series of branched oligosaccharides, the smallest of which was a pentasaccharide. This was characterized by Bines and Whelan[3] as 6^3-α-maltosylmaltotriose (Fig. 1, structure **1**). On increasing the concentration of alpha-amylase, the resulting cleavage of maltotriose to maltose and D-glucose was accompanied by the appearance of a tetrasaccharide as the smallest mixed-link oligosaccharide[2]. This limit dextrin, produced in the so-called, second stage of amylolysis, had already been isolated, and characterized by Nordin and French[4] as 6^3-α-D-glucosylmaltotriose (Fig. 1, structure **2**). In order to account for the formation of a family of branched oligosaccharides of higher molecular weight, Whelan and his co-workers[2,5] advanced the following hypothesis. They regarded 6^3-α-D-glucosylmaltotriose as a true α-limit dextrin, all three of its glycosidic linkages being resistant to the enzyme. The (1→6)-linkage is never hydrolysed by the amylase, and the (1→4)-linkages were presumed to be resistant to the enzyme because of steric factors caused by the proximity of the (1→6)-linkage. They postulated that if, during the course of alpha-amylolysis of amylopectin, a (1→4)-bond was split that was penultimate to a resistant (1→4)- or (1→6)-linkage as defined above, the (1→4)-bond now exposed at a terminal position, and next to the resistant (1→4)- or (1→6)-linkage, would itself be resistant to the enzyme. This hypothesis predicts that the random endo-hydrolytic action of salivary alpha-amylase on amylopectin, taken to the second stage, will give rise to eight α-limit dextrins (Fig. 1, structures **1–8**). Other series of structures of α-limit dextrins have been proposed. French[6] has listed the principal, singly branched dextrins formed by salivary alpha-amylase as **1–3** plus 6^3-α-maltotriosylmaltotetraose (**9**). Brown *et al.*[7] have proposed structures **1–3**, **6**, and **9–12**, but their limit dextrins were

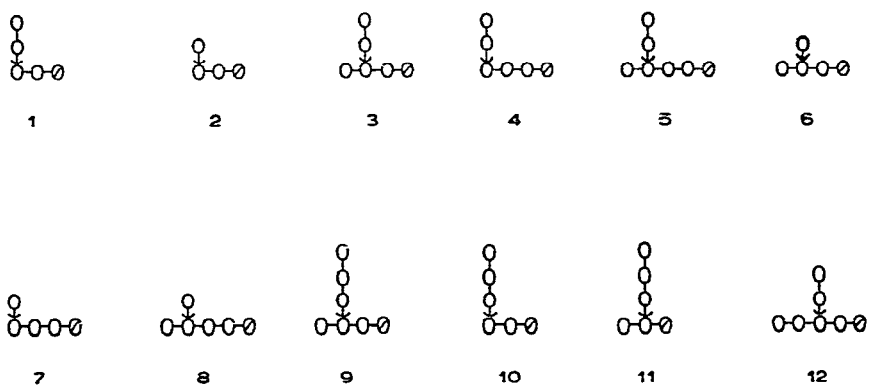


Fig. 1. The structures of the singly branched dextrins present after second-stage, salivary alpha-amylolysis of amylopectin, as proposed by Whelan and his co-workers[2,5] (**1–8**), French[6] (**1–3** and **9**), and Illingworth and Brown[7] (**1–3**, **6**, and **9–12**). The amylolysate products observed in this report are structures **1–3**, **6**, **9**, and **10**. α-(1→4)-D-Glucosidic linkages are represented by ———; α-(1→6)-linkages by ↓; non-reducing D-glucose residues by **0**; and the reducing D-glucose end-group by **0**.

derived from glycogen by the action of porcine pancreatic alpha-amylase and are therefore not directly comparable. Nevertheless, if the structures proposed do describe the limit dextrins produced in this manner, it is seen that the action pattern of porcine pancreatic alpha-amylase is notably different from that of human salivary alpha-amylase.

In the present work, we describe the characterization of the singly branched dextrins present after the second stage of salivary amylolysis of amylopectin. Two of these dextrins were still susceptible to alpha-amylolysis, and were therefore not true limit-dextrins. The observations of what constitutes a structure resistant to further digestion, or one that is still susceptible to cleavage, and at which point, led us to certain conclusions concerning the nature of alpha-amylolytic attack. It is known that both bonds of maltotriose are susceptible to cleavage by alpha-amylase, but not with equal facility. This difference in the susceptibility to attack has been examined by Pazur and Budovich[1] using salivary alpha-amylase, and more recently by Robyt and French[8] in their studies of porcine pancreatic alpha-amylase. Considering two of the isolated dextrins as being substituted maltotrioses, we have examined the susceptibility to cleavage of the two (1→4)-bonds constituting the maltotriose residue in the dextrins. The effect of the substitutions on the hydrolysis of the (1→4)-bonds was thereby measured.

MATERIALS AND METHODS

Crystalline human salivary alpha-amylase was prepared as described by Fischer and Stein[9]. Pullulanase (EC 3.2.1.41) was obtained from *Aerobacter aerogenes* as described by Bender and Wallenfels[11]. Amylopectin was prepared from waxy-maize seed[11], and was hydrolysed with alpha-amylase to the second stage of amylolysis as judged by the disappearance of maltotriose, observed by paper chromatography. Digestion was carried out in α-glycerophosphate buffer (pH 6.8) as described below. The products were fractionated[12,13] on charcoal–Celite 535 (1:1 w/w) at pH 3.0, and the fractions grouped according to their paper-chromatographic behaviour before and after digestion with pullulanase. Digestion of oligosaccharides (2mM) with pullulanase (17 nkat) was carried out at pH 5.0 in 15mM citrate–phosphate buffer at 30° for 18 h. Digestion of oligosaccharides (1mM) with alpha-amylase in 6mM α-glycerophosphate buffer (pH 6.8, and containing 3mM sodium chloride) at 30° was monitored by the increase in reducing power (Nelson's arsenomolybdate reagent[14]), and release of D-glucose (D-glucose oxidase reagent[15]). Sufficient alpha-amylase was present to hydrolyse maltotriose completely in 30 h[2]. Incubations were continued for a further 50 h, and a control digest containing maltose demonstrated the complete absence of any maltase activity in the alpha-amylase. D-Glucose oxidase and peroxidase were purchased from the Boehringer–Mannheim Corporation. All other chemicals were of the highest purity available commercially.

Digest samples for chromatography were inactivated (5 min, 100°), deionized with Biodeminrolit (HCO_3^-) resin[16] (Permutit Co. Ltd.), and applied to Whatman

No. 1 chromatography paper. The papers were irrigated by descent with ethyl acetate–pyridine–water (10:4:3), and the sugar zones located with a silver nitrate–sodium hydroxide dip[17], or the aniline–diphenylamine dip specific for the detection of reducing sugars[18].

Reduction with sodium borohydride was carried out as described by Abdel-Akher *et al.*[19]. After treatment with sodium borohydride overnight at room temperature, excess of reagent was decomposed with 3M acetic acid, and the solution was deionized with Biodeminrolit (HCO_3^-) resin[16] and evaporated. The remaining boric acid was removed as methyl borate by successive evaporations to dryness with methanol. Periodate over-oxidation was carried out as described by Parrish and Whelan[20]. Oligosaccharides (5 mg) in 0.1M acetate–HCl buffer (pH 1.1, 12 ml) were treated with 0.3M sodium metaperiodate (3.0 ml) at 50° in the dark. Liberated formaldehyde was measured after 24 and 48 h, by using the chromotropic acid reagent[20]. Oligosaccharide concentrations were determined by acid hydrolysis in 0.75M sulphuric acid at 100° for 6 h, by the method of Pirt and Whelan[21]. Liberated D-glucose was determined, after neutralization of the hydrolysate, by the D-glucose oxidase method[15].

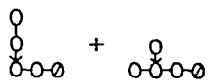
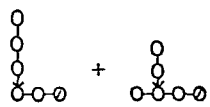
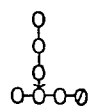
RESULTS AND DISCUSSION

The amylopectin amylolysate was fractionated on charcoal–Celite. D-Glucose and maltose emerged first, followed by the α-limit dextrins. The fractions containing the oligosaccharides were chromatographed on paper before and after treatment with pullulanase. The first fraction (Table I, No. 1) to emerge migrated slightly slower than maltotetraose, was not attacked by pullulanase (as determined by paper chromatography), and yielded 2 mol of formaldehyde per mol of tetrasaccharide when over-oxidized with periodate. In the over-oxidation reaction, the molecule is eroded from the reducing end, and one mol of formaldehyde is produced for each glucose residue destroyed[20]. Erosion ceases when a (1→6)-linkage is encountered. Therefore, the position of a (1→6)-D-glucosidic linkage, relative to the reducing end of the molecule, can be determined[3]. The lower chromatographic mobility of the α-limit tetrasaccharide, relative to maltotetraose, indicated that it contained a (1→6)-bond, and the absence of action of pullulanase was indicative of a single α-D-glucosyl group joined to the rest of the molecule by the (1→6)-linkage. Pullulanase will not hydrolyse such a linkage, but will do so when maltose or a larger maltosaccharide is joined to another maltosaccharide by a (1→6)-α-D-glucosidic linkage[22]. Based on the collected evidence, the oligosaccharide was identified as the already known[4] 6^3-α-D-glucosyl-maltotriose (Fig. 1, 2).

Similar examination of later fractions from the charcoal column was made, with, in some cases, an additional test. The oligosaccharide was reduced with borohydride, and again treated with pullulanase, and the products were fractionated on paper. The borohydride reduces the C-chain of the oligosaccharide (that maltosaccharide chain carrying the reducing group[23]), so that this no longer reacts on the

TABLE I

PERIODATE OVER-OXIDATION AND HYDROLYSIS BY PULLULANASE OF THE α-LIMIT DEXTRINS OF AMYLOPECTIN

Fraction[a]	*Molar proportions of formaldehyde released by over-oxidation*	*Products of debranching the dextrin with pullulanase*[b]	*Products of debranching reduced dextrin*[b]	*Proposed structure*[c]
1	2.0	Nil	Nil	
2	1.9	M_2, M_3	—[d]	
3	—[d]	M_2, M_3, B_5	—[d]	
4	1.9	M_2, M_4	M_2	
5	2.1	M_2, M_3, M_4	M_2, M_3	
6	1.9	M_3, M_4	M_3	

[a]The dextrins are listed in the order of their elution from the charcoal–Celite column. [b]Key: M_2, M_3, etc., represent maltose, maltriose, and the maltosaccharide series; B_5 represents a pentasaccharide containing an α-(1→6)-linked side-chain. [c]For key, see Fig. 1. [d]Not done.

paper chromatogram with aniline–diphenylamine[18]. The A-chain[23] liberated by pullulanase [that maltosaccharide joined to the C chain by a (1→6)-linkage] was able to reduce aniline–diphenylamine.

The fractions following the tetrasaccharide contained oligosaccharides that migrated on paper slightly slower than maltopentaose. The first such fraction to emerge (Table I, No. 2), when treated with pullulanase, yielded sugars that migrated on paper with maltose and maltotriose. The pentasaccharide yielded 2 molar proportions of formaldehyde when subjected to periodate over-oxidation. These two properties identify the compound as 6^3-α-maltosylmaltotriose[3] (Fig. 1, **1**).

The second pentasaccharide fraction (No. 3), when treated with pullulanase, gave (paper chromatography) maltose, maltotriose, and a residual sugar having the same mobility as the original pentasaccharide. This fraction was presumed to be a mixture of the already identified 6^3-α-maltosylmaltotriose and a dextrin containing a pullulanase-resistant α-(1→6)-linkage. This second pentasaccharide therefore contained a D-glucosyl A-chain. Although the oligosaccharide was not subjected to

periodate over-oxidation, the structure may be presumed to be 6^3-α-D-glucosylmaltotetraose (Fig. 1, **6**), as all of the other α-limit dextrins produced can be regarded as molecules of maltotriose variously substituted at the non-reducing end-group (Table I). The structure 6^3-α-D-glucosylmaltotetraose conforms to this pattern.

The next two fractions contained hexasaccharide limit-dextrins. The first one (Table I, No. 4) was completely hydrolysed to maltose and maltotetraose by pullulanase, but, following borohydride reduction of the dextrin prior to cleavage of the (1→6)-linkage, the only reducing sugar found was maltose. Periodate over-oxidation yielded 2 mol. proportions of formaldehyde per hexasaccharide, and the structure was therefore 6^3-α-maltosylmaltotetraose (Fig. 1, **3**).

The second hexasaccharide fraction (Table I, No. 5) seemed to be a mixture of 6^3-α-maltosylmaltotetraose (**3**) and 6^3-α-maltotriosylmaltotriose (**10**) in roughly equal amounts, as judged by paper chromatography. Thus, pullulanolysis produced maltose, maltotriose, and maltotetraose, and, with prior reduction, maltose and maltotriose. Again, 2 mol. proportions of formaldehyde were produced per hexasaccharide during periodate over-oxidation.

The action of pullulanase on the heptasaccharide fraction (Table I, No. 6), before and after reduction, indicated that the A- and C-chains were maltotriose and maltotetraose, respectively, and the liberation of 2 mol. proportions of formaldehyde confirmed that the structure was 6^3-α-maltotriosylmaltotetraose (Fig. 1, **9**).

The data on formaldehyde release and pullulanolysis are recorded in Table I.

Further treatment of each fraction with alpha-amylase revealed that two of the dextrins, 6^3-α-maltotriosylmaltotriose (**10**) and 6^3-α-maltotriosylmaltotetraose (**9**) were capable of hydrolysis to 6^3-α-D-glucosylmaltotriose (**2**) and 6^3-α-D-glucosylmaltotetraose (**6**), respectively. The four remaining dextrins (**1**, **2**, **3**, and **6**) therefore represent the true α-limit dextrins of amylopectin, and are compared in Fig. 1 with those proposed by Whelan and Roberts[5] and by French[6].

Interest in the hydrolysis of the hexa- and hepta-saccharides lay not only in their susceptibility to further amylolysis, but in the position of the α-(1→4)-bond, or bonds, so cleaved. Pazur and Budovich[1] have shown that both bonds in maltotriose are cleaved by alpha-amylase, but not with equal facility. Considering the A-chains of 6^3-α-maltotriosylmaltotriose (**10**) and 6^3-α-maltotriosylmaltotetraose (**9**) as maltotrioses substituted at their reducing ends by maltotriose and maltotetraose, respectively, it might be expected that both the α-(1→4)-bonds of the maltotriose A-chain would be cleaved. However, no D-glucose was released during the course of this amylolysis, and no maltosylmaltotriose or maltosylmaltotetraose was detected. Cleavage must have occurred exclusively at the bond nearer to the α-(1→6)-linkage. Moreover, the rate of hydrolysis was considerably lower than that of free maltotriose.

The constraint on alpha-amylase, such that it hydrolyses only one bond in maltotriose substituted in this fashion, was further examined by a study of the amylolysis of 6-α-maltotriosyl-D-glucose (Fig. 2, **13**). The conditions of digestion were the same, and hydrolysis was again monitored by measuring both increase in reducing

power and release of D-glucose. Again, the glucosidic bond cleaved was that nearer to the α-(1→6)-linkage. Traces of D-glucose released during hydrolysis indicated that only ~5% of the alternative α-(1→4)-bond had been broken.

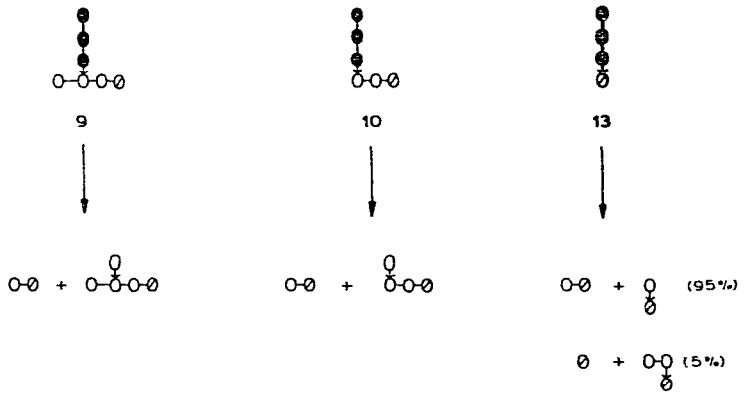


Fig. 2. The further hydrolysis of dextrins **9** and **10** (isolated at the second stage of salivary amylolysis of amylopectin, Fig. 1), and of 6-α-maltotriosyl-D-glucose (**13**). The dextrins are depicted as derivatives of maltotriose (filled circles). Other symbols as in Fig. 1.

Substrates with the structure of a maltotriose, modified at the reducing residue by substitution at the primary hydroxyl group, *e.g.*, 6^1-α-D-glucosylmaltotriose, were not available. However, the absence of the α-limit dextrin 6^3-α-maltosylmaltopentaose (Fig. 3, **12**) from the hydrolysate of amylopectin infers that maltotriose (that portion of structure **12** marked in solid circles), so modified, possesses α-(1→4)-linkages readily susceptible to second-stage alpha-amylolysis.

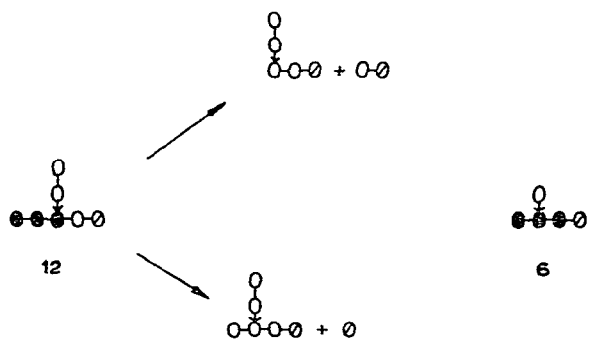


Fig. 3. Substituted maltotriosyl units (filled circles) occurring within dextrins presumed to be susceptible (**12**), or known to be resistant (**6**), to salivary alpha-amylolysis (as judged from the structure of true α-limit dextrins). Symbols as in Fig. 1.

Modification of the non-reducing end of maltotriose by, say, a (1→6)-bonded α-D-glucosyl residue, to produce the limit dextrin 6^3-α-D-glucosylmaltotriose (**2**), completely inhibits alpha-amylolysis.

The attempt to elucidate the action pattern of salivary alpha-amylase around the branch point, by examining its attack on maltotriose (the minimum linear substrate for the enzyme) modified by (1→6)-substitution at any of the three residues, leads to the following conclusions. Participation of the reducing residue of maltotriose in an α-(1→6)-bond by way of C-1 appears to direct the site of amylolytic attack almost exclusively to one of the α-(1→4)-bonds of the maltotriose, that nearest the point of substitution. This behaviour contrasts with the susceptibilities, albeit unequal, of both these bonds in free maltotriose[1]. However, both bonds would appear to be available for cleavage when the reducing residue of maltotriose is participating in an α-(1→6)-bond by way of C-6. Substitution of the primary hydroxyl group located on the non-reducing-end D-glucosyl residue completely prevents hydrolysis. From the formation of α-limit dextrins such as 6^3-α-D-glucosylmaltotetraose (**6**), it may be concluded that substitution of the primary hydroxyl group located on the central residue of maltotriose prevents hydrolysis of either of the α-(1→4)-bonds. These conclusions are summarized in Fig. 4.

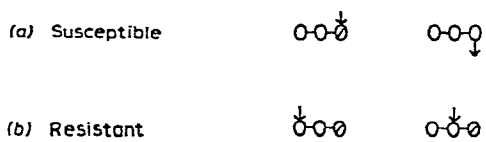


Fig. 4. A summary of substituted maltotriosyl structures that are (*a*) susceptible to, and (*b*) resistant to, salivary alpha-amylolysis. Symbols as in Fig. 1.

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