STUDIES ON CARBOHYDRATE-METABOLISING ENZYMES

PART XXIV[1]. THE ACTION OF MALTED-RYE ALPHA-AMYLASE ON AMYLOPECTIN

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ABSTRACT

Amylopectin was digested with a purified alpha-amylase isolated from malted rye-flour. The products of enzyme action were D-glucose, maltose, maltotriose, and a series of branched alpha-limit dextrins having a degree of polymerisation $\geqslant 4$; panose was not formed. The identity of the alpha-limit dextrins has been correlated with the ability of the enzyme to hydolyse some, but not all, of the α-(1→4)-D-glucosidic linkages in the vicinity of the α-(1→6)-D-glucosidic inter-chain linkages.

INTRODUCTION

The action of alpha-amylases (E.C.3.2.1.1.) on amylose results in essentially complete hydrolysis to maltose and D-glucose[2]. The hydrolysis of glycogen and amylopectin, is, however, incomplete because of the presence of the α-(1→6)-D-glucosidic interchain linkages in these polysaccharides. Not only are these linkages resistant to hydrolysis, but they also confer stability on certain adjacent α-D-(1→4) linkages[3]. The number of resistant α-(1→4)-D-glucosidic linkages, and hence the nature of the limit dextrins formed, depends on the source of the alpha-amylase[4]. Whereas salivary and pancreatic amylases yield the tetrasaccharide 6^3-α-D-glucosylmaltotriose as the smallest limit-dextrin[5,6] and *Bacillus subtilis* gives the pentasaccharide 6^2-α-maltosylmaltotriose[7], malted-barley alpha-amylase appears to be unusual in that it was reported to produce the trisaccharide panose as the smallest oligosaccharide containing an α-(1→6)-D-glucosidic linkage[8]. Since it was of interest to see whether this was a property of other cereal alpha-amylases, we have prepared alpha-amylase from malted rye in a highly purified state and examined the nature of the limit dextrins produced by its action on amylopectin. The system of nomenclature for these limit dextrins is that proposed by Whelan[9].

MATERIALS AND METHODS

Malted rye (var. Rheidol) was a gift from Associated British Maltsters Ltd. Amylopectin was starch from waxy maize or waxy sorghum. Glycogen was either

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sweet-corn phytoglycogen A or commercial, shellfish glycogen. Glucoamylase (E.C. 3.2.1.3.) was a gift from Associated British Maltsters Ltd., marketed under the Trade Name Amylozyme M.S. Pullulanase was prepared by a modification[10] of the method of Bender and Wallenfels[11]. D-Glucose contents of polysaccharide or oligosaccharide solutions were determined by the phenol–sulphuric acid method[12] or by enzymic hydrolysis to D-glucose[13] which was then determined by the D-glucose oxidase method of White and Subers[14]. Reducing sugars were determined by the colorimetric adaptation[15] of the Somogyi[16] method. Protein contents of enzyme solutions were determined from the ultraviolet absorption[17].

Alpha-amylase activities were determined by measurement of the reducing sugars liberated from a solution containing 1% of soluble starch. One unit of alpha-amylase activity was defined as the amount which liberated 1 μmole of maltose hydrate per minute at pH 5.0 and 37°.

Descending paper chromatograms were developed in the solvent systems (*A*) ethyl acetate–pyridine–water (10:4:3 v/v) or (*B*) propyl alcohol–ethyl acetate–water (14:2:7 v/v). The former system separated branched and linear oligosaccharides of the same degree of polymerisation (*DP*), whereas the latter separated oligosaccharides strictly on the basis of *DP*. Sugar spots were visualized by using the alkaline silver nitrate reagent of Trevelyan *et al.*[18]. Reference samples of authentic oligosaccharides were available from previous work.

EXPERIMENTAL

A crude enzyme-preparation was obtained by extracting malted rye-flour (1 kg) with 1% aqueous calcium chloride (2 litres) for 10 h at room temperature. After centrifugation to remove grain residues, the supernatant solution was heated in 5.0-ml portions at 75° for 12.5 min to remove beta-amylase[19] and other interfering carbohydrases. Further purification was then carried out by precipitation of the enzyme with glycogen[20]. The purification achieved was generally of the order of 300–400 fold. The purified enzyme used for the alpha-amylolysis of amylopectin had a specific activity of 61 units per milligram of protein and was free from debranching enzyme (limit-dextrinase) activity and maltase activity, as shown by incubation with pullulan and maltose, respectively. Full details of the purification and the properties of the enzyme will be described elsewhere[21].

Amylopectin (5.09 g) was wetted with ethanol and then dissolved in M sodium hydroxide (100 ml) with gentle heating. The pH of the solution was adjusted to 5.0 with acetic acid and, after the addition of 145 units of enzyme and sufficient solid calcium chloride to give a final concentration of 50mM, diluted to 500 ml. The digest was incubated at 37°, and the progress of hydrolysis, as measured by the increase in reducing power, was followed with time. Microbial contamination was excluded by layering the digest with toluene. Further additions of enzyme (145 units each) were made after incubation for 1 h, 9 h, 34 h, and 5 days. The total incubation time was 7 days. When the alpha-amylolysis was complete (89.5% conversion of the amylopectin into maltose hydrate), the enzyme was destroyed by heating at 100° for 10 min. The

digest was then filtered and deionized [Amberlite IR-120 (H^+) and IR-45 (OH^-) resins (analytical grade)], and the products were isolated by freeze-drying.

Chromatography of the products of alpha-amylolysis in solvent systems *A* and *B* showed the presence of glucose, maltose, maltotriose, and higher oligosaccharides. The persistence of maltotriose after the extensive alpha-amylolysis shows that the malted-rye enzyme resembles malted-barley alpha-amylase, in so far as it does not have a high affinity for lower maltosaccharides. The chromatograms run in solvent system *A* indicated that the oligosaccharides of $DP \geqslant 4$ all contained α-D-(1→6) linkages as expected, in view of their lower mobility than the corresponding linear maltosaccharides.

A portion (50 mg) of the products was fractionated on the basis of *DP* by chromatography in solvent system *B*, using Whatman No. 1 paper. The total carbohydrate in each fraction was then estimated after elution from the paper with water. In this way, the relative amounts of the products were determined. Further quantities of the various fractions were then obtained by preparative, paper chromatography on Whatman 3MM paper in solvent system *B*.

The homogeneous fractions were then examined by chromatography in solvent system *A* and by degradation with pullulanase and with glucoamylase. Digests containing oligosaccharide (*ca.* 250 μg) and pullulanase (*ca.* 0.06 unit) in a total volume of 30 μl were incubated at 37° for 1.5 days in the presence of toluene vapour. The products were then examined by paper chromatography (solvent system *A*). For glucoamylase hydrolysis, digests containing oligosaccharide (*ca.* 1.25 mg) and enzyme solution (20 μl of a solution containing 0.8 mg of protein/ml) were incubated at room temperature in a total volume of 0.12 ml. Samples (10 μl) were removed after various times of incubation from 0–5 h and examined by paper chromatography (solvent system *A*).

Prior to the large-scale alpha-amylolysis, a preliminary experiment was carried out in which amylopectin (500 mg) was incubated with the purified rye alpha-amylase for 7 days. The apparent percentage conversion into maltose was 90. The products of enzyme action were isolated by preparative, paper chromatography, and the oligosaccharides of *DP* 5, 6, 7, and higher were separately incubated for 1.5 days with a concentration of alpha-amylase which was approximately 25 times that used in the initial alpha-amylolysis. Paper-chromatographic analysis showed that further hydrolysis did not occur. These results confirm the absence of limit-dextrinase impurities in the enzyme preparation, and show that the oligosaccharides are true limit-dextrins.

DISCUSSION

The properties of the products obtained by the action of malted-rye alpha-amylase on amylopectin are shown in Table I, together with the partial formulae of the alpha-limit dextrins in Fig. 1.

Chromatography of the di- and tri-saccharide fractions in solvent system *A* showed that the former consisted of maltose and the latter of maltotriose. Contrary to earlier work[22], it is now generally accepted that isomaltose is not a product of

TABLE I

OLIGOSACCHARIDES PRODUCED BY THE ACTION OF MALTED-RYE ALPHA-AMYLASE ON AMYLOPECTIN

Degree of polymerisation	*Relative amounts* (%)	*Products of enzymic hydrolysis*		*Identity*
		(*a*) *Pullulanase*	(*b*) *Glucoamylase*	
1	9	—	—	Glucose
2	53	—	—	Maltose
3	6	—	—	Maltotriose
4	1	No hydrolysis	Glucose only	6^3-α-D-Glucosyl-maltotriose
5	3	No hydrolysis	Glucose + 6^3-α-D-glucosylmaltotriose	6^3-α-D-Glucosyl-maltotetraose
6	3	Maltose + maltotetraose	Glucose + 6^3-α-D-glucosylmaltotriose	6^3-α-Maltosyl-maltotetraose
7	7	Maltotriose + maltotetraose (major products)	Glucose + 6^3-α-D-glucosylmaltotriose	6^3-α-Maltotriosyl-maltotetraose (major component)
		Maltose + Maltopentaose (minor products)	Glucose + 6^3-α-D-glucosylmaltotriose	6^3-α-Maltosyl-maltopentaose (minor component)
Higher	19	Oligosaccharides of DP 2–8	—	Probably multiply-branched limit-dextrins

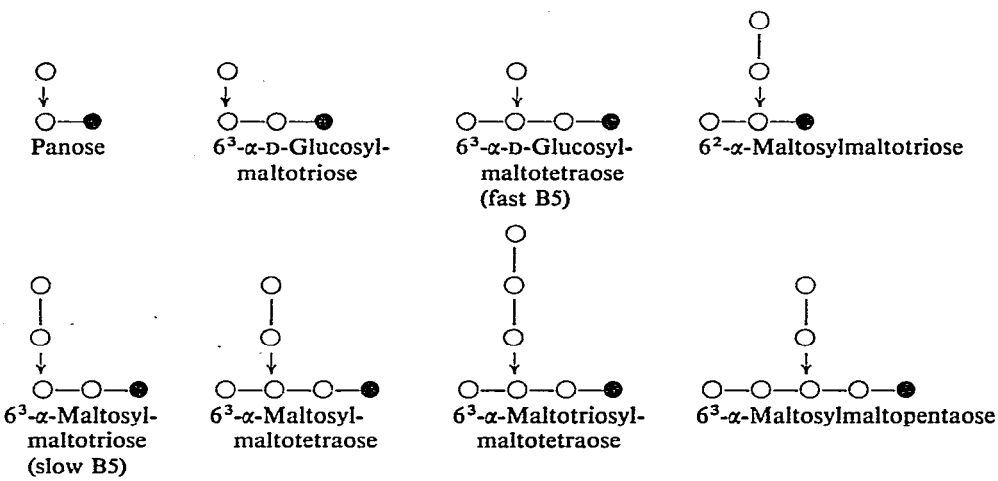

Fig. 1. Partial formulae of alpha-limit dextrins. Key: ○ represents a non-reducing D-glucose residue; ● represents a reducing D-glucose residue; ↓ represents an α-(1→6)-D-glucosidic interchain linkage; — represents an α-(1→4)-D-glucosidic linkage.

alpha-amylolysis[4]; this latter view is therefore confirmed by the present results. The absence of panose (6^2-α-D-glucosylmaltose) in the trisaccharide fraction shows that malted-rye alpha-amylase resembles most other alpha-amylases in its inability to hydrolyse linkage H (see Fig. 2). The reported[8] production of panose by malted-barley alpha-amylase, which was isolated in a yield of 0.45%, would indicate that this amylase could cleave at linkage H.

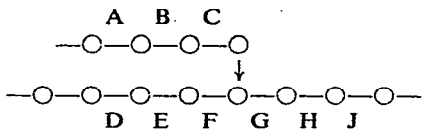

Fig. 2. Segment of amylopectin near an interchain linkage. Key: as in Fig. 1. Linkages A, B, and C are in the side chain; linkages D, E, and F are in the outer part of the main chain.

This fact might suggest that the two cereal alpha-amylases are different, although this would appear to be unlikely since the two cereals are members of the same botanical tribe *Hordeae*, and their carbohydrase complements are very similar[23]. In view of the difficulty in preparing, by conventional methods, even the crystalline form of malted-barley alpha-amylase free from other carbohydrases[24], it is possible that the formation of panose was due to the presence of an enzymic impurity in the amorphous, malted-barley alpha-amylase preparation used by Bines[8]. This possibility is favoured by the results of a preliminary examination of the products of hydrolysis of amylopectin by malted-barley alpha amylase, purified by the method used for the rye alpha-amylase, which indicated the absence of panose[23].

Chromatography of the tetrasaccharide fraction in solvent system *A* showed that this oligosaccharide was apparently homogeneous and had the mobility of authentic 6^3-α-D-glucosylmaltotriose isolated from a salivary alpha-amylolysate of amylopectin. This identity was supported by the lack of hydrolysis by bacterial pullulanase, which has been shown[25] to be unable to remove single α-(1→6)-linked D-glucose residues, and by hydrolysis with glucoamylase to give glucose as the only hydrolysis product. The inability to detect maltotriose in the glucoamylase digest is explained by the relative rates of hydrolysis of the α-(1→4)- and α-(1→6)-D-glucosidic linkages[26]. The tetrasaccharide produced by the action of malted-barley alpha-amylase on amylopectin was also tentatively identified as 6^3-α-D-glucosylmaltotriose[8].

The pentasaccharide fraction on chromatography in solvent system *A* migrated with the faster-moving component of the pentasaccharide fraction of a salivary alpha-amylolysate, 6^3-α-D-glucosylmaltotetraose (fast B5). Slow B5 (6^3-α-maltosylmaltotriose) was absent. The homogeneity of this fraction was confirmed by the inability of pullulanase to cause any hydrolysis. The action of glucoamylase yielded glucose and 6^3-α-D-glucosylmaltotriose, as expected.

The hexasaccharide fraction on treatment with pullulanase yielded maltose and maltotetraose, whereas the action of glucoamylase gave glucose and 6^3-α-D-glucosylmaltotriose. These observations therefore identify it as 6^3-α-maltosylmaltotetraose. The absence of 6^3-α-maltotriosylmaltotriose in this fraction is hardly surprising since (*a*) linkage C (Fig. 2) in this oligosaccharide is readily hydrolysed and (*b*) it is unlikely that hydrolysis of linkage F can take place when the side chain consists of more than a single D-glucose residue (see below).

The heptasaccharide fraction, when incubated with pullulanase, gave approximately equal amounts of maltose and maltopentaose, together with maltotriose and maltotetraose, also in approximately equal amounts. The maltotriose and maltotetra-

ose predominated. This suggests that the heptasaccharide fraction consisted of a mixture of two components, one being a maltosylmaltopentaose and the other a maltotriosylmaltotetraose. The identity of these as 6^3-α-maltosylmaltopentaose and 6^3-α-maltotriosylmaltotetraose, respectively, was confirmed by glucoamylase action which yielded 6^3-α-D-glucosylmaltotriose and glucose.

A portion of the amylopectin molecule in the region of a branch point is shown in Fig. 2. It can be seen by reference to Table I that only two of the α-(1→4)-D-glucosidic linkages are resistant *per se* to alpha-amylolysis, namely, those marked G and H, since all the lower alpha-limit dextrins are 6^3-α-substituted maltosaccharides. The formation of these oligosaccharides and the apparent resistance of some of the other linkages to hydrolysis may be considered in terms of the ability of the enzyme to attack the side and main chains. On the assumption that a side chain is degraded before a main chain, it is clear that cleavage of linkage C must take place, for a glucosyl substituted maltosaccharide to be produced. This may be followed by the hydrolysis of linkages D, E, or F. Since 6^3-α-D-glucosylmaltopentaose is not formed, it is apparent that a cleavage at D must be followed by a cleavage at F. It has recently been shown that this takes place with porcine, pancreatic alpha-amylase[27]. Hence, after hydrolysis of linkage C, we need consider only two possibilities, namely, hydrolysis at linkages E or F. This would result in 6^3-α-D-glucosylmaltotetraose and 6^3-α-D-glucosylmaltotriose, respectively, which are the two glucosyl-maltosaccharides that have been identified.

If cleavage of the side chain takes place at B, it is apparent that the product must be a maltosyl-oligosaccharide. Such a cleavage may be followed by the hydrolysis of linkages D or E to yield either 6^3-α-maltosylmaltopentaose or 6^3-α-maltosylmaltotetraose. The significant point to note is that when cleavage of the side chain takes place at B, hydrolysis cannot then take place at F, presumably because of steric hindrance by the maltosyl side-chain which is not observed when the side chain is a single D-glucose residue. This would explain the complete absence of 6^3-α-maltosylmaltotriose from the pentasaccharide fraction. The malted-rye enzyme therefore differs in this respect from the salivary, pancreatic, and bacterial alpha-amylases[4].

Should hydrolysis of the side chain in the region of the α-(1→6)-D-glucosidic linkage take place at A, then several possibilities arise. Further attack on the side chain, *viz.* hydrolysis of linkage C, could then take place, followed by hydrolysis of linkages E or F as described above. However, other possibilities are the hydrolysis of linkages D, E, and F. Hydrolysis at D would yield 6^3-α-maltotriosylmaltopentaose which could either persist as one of the higher alpha-limit dextrins or else be slowly further hydrolysed. If, after hydrolysis at A, the next cleavage takes place at E, the resulting product is 6^3-α-maltotriosylmaltotetraose which is resistant to further alpha-amylolysis. Cleavage of linkage F can be considered to be unlikely because of the steric hindrance by the maltotriosyl side-chain, similar to that discussed above for the maltosyl-substituted oligosaccharides.

It is interesting to note the effect that the presence of the outer part of the main chain has on the hydrolysis of the side chain. The resistance of 6^3-α-maltotriosyl-

maltotetraose to further alpha-amylolysis shows that a single D-glucose residue attached by linkage F to the D-glucose residue involved in the branch point virtually stops hydrolysis at linkage C, assuming that, in this case, the main chain is degraded before the side chain. The corresponding linkage in 6^3-α-maltotriosylmaltotriose is rapidly hydrolysed. This presumably reflects the different conformation of that part of the molecule consisting of the D-glucose residues joined by linkages B, C and the α-D-(1→6) linkage when an outer part of a main chain is present, with consequent resistance to the formation of an enzyme–substrate complex so that hydrolysis of linkage C is hindered. Similar observations have been made with salivary alpha-amylase[28]. It is also possible that the resistance to hydrolysis of 6^3-α-maltotriosyl-maltotetraose may be ascribed, in part, to the lack of sufficient D-glucose residues for adequate binding to the alpha-amylase in the vicinity of the active centre.

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