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Note

The action of a bacterial amylase on some modified substrates

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Previous studies have identified some of the oligosaccharides isolated from the amylase hydrolyzates of 6-deoxy-6-iodoamylose[1] and 6-amino-6-deoxyamylose[2]. In this investigation, 6-*O*-methylamylose and 6-deoxyamylose were the substrates for the crystalline, liquefying amylase of *Bacillus subtilis*.

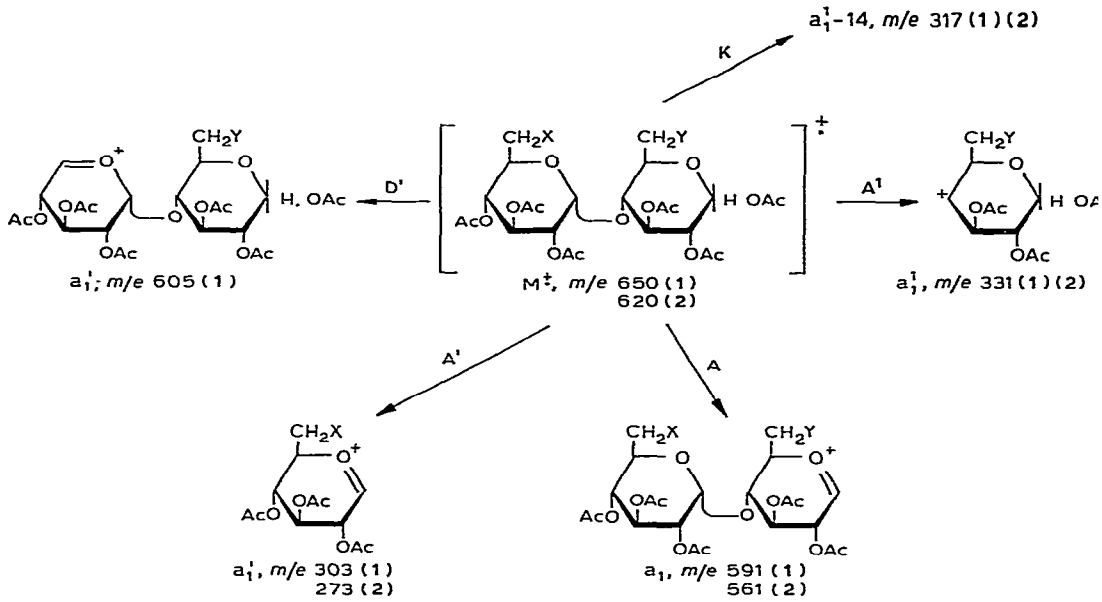
Although 6-*O*-methylamylose was the subject of a previous study[3], no attempt was made to isolate or identify those oligosaccharides which contained 6-*O*-methyl-D-glucopyranosyl residues. 6-Deoxyamylose, first prepared by Bines and Whelan[4], is an interesting substrate, as the reduction of the primary hydroxymethyl group to a methyl group yields a smaller residue. It seemed possible that 6-deoxy-D-glucopyranose itself might be one of the products of the enzymic hydrolysis, especially as Bines and Whelan found it in the hydrolyzates produced by salivary amylase[4].

The two modified amyloses used in this study had degrees of substitution (d.s.) of ~ 0.2, in a range that has been found to be most suitable for isolating oligosaccharides containing modified D-glucopyranosyl residues. Neither 6-*O*-methyl-D-glucose nor 6-deoxy-D-glucose (D-quinovose) was detected in any of the hydrolyzates produced by this bacterial amylase. These results are consistent with those obtained with other chemically modified amyloses in this laboratory; in no case has a chemically modified monomer ever been obtained as a product of the amylase activity.

Three fractions of the hydrolyzate of 6-*O*-methylamylose were removed from paper chromatograms for identification. Although the hydrolyzate of 6-deoxyamylose was subjected to even more extensive chromatography, only four of the fractions isolated are identified in this communication. All of these oligosaccharides were acetylated, and analyzed by electron-impact, mass spectrometry (e.i.m.s). The results of the mass-spectral analyses of the peracetylated disaccharides are shown in Scheme 1.

The peracetates of disaccharides usually show a molecular ion, M^+, even though it is often small. The a_1 ion ($M^+ - 59$), corresponding to the loss of acetoxyl, is often larger than M^+. $M^+ - 43$, corresponding to the loss of acetyl, is usually present, even though it is not shown in Scheme 1. The d_1 or d_1' ion is also frequently present, depending on the nature of CH_2X and CH_2Y.

The determination of the location of the modified residue usually depends on

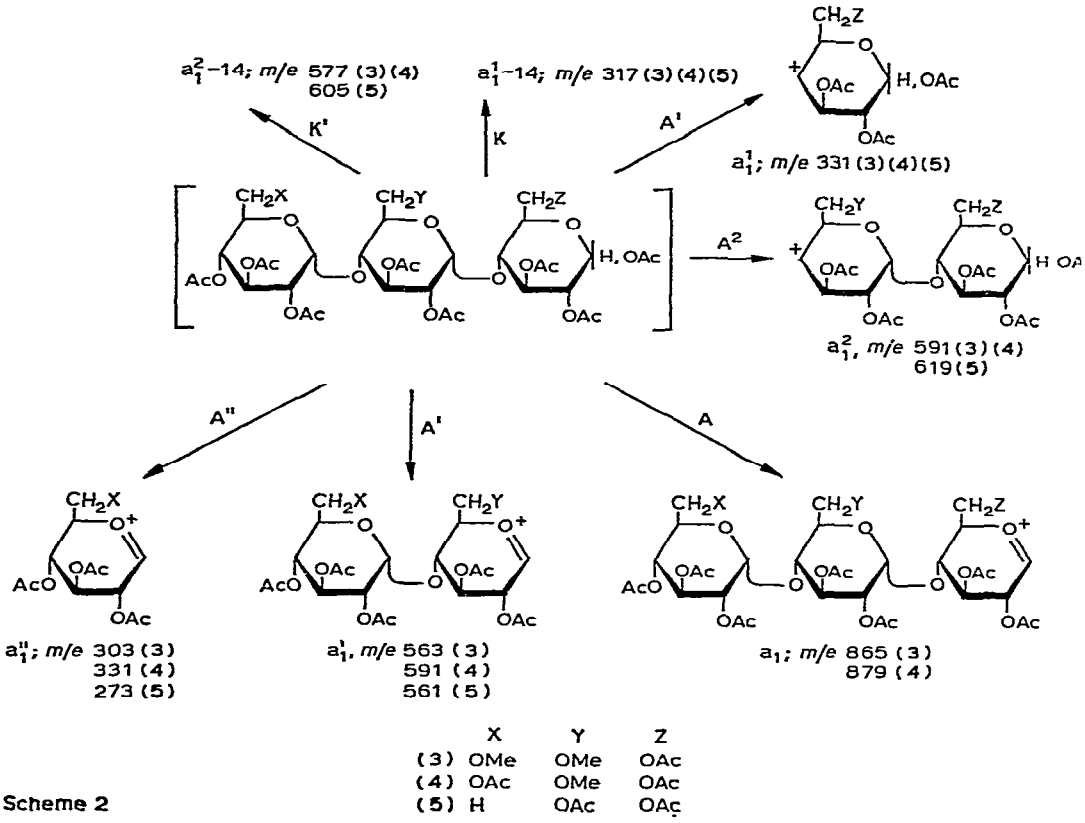


Scheme 1

the intensities of the a_1' and a_1^1 peaks. For compound **1** (see Scheme 1), the peak at *m/e* 303 is twice the size of that at *m/e* 331. For compound **2**, the peak at *m/e* 273 is seven times that at *m/e* 331. It has also been observed[5] that the a_1^1 ion is accompanied by an ion at $a_1^1 - 14$, the intensity of which is often greater than that of a_1^1 itself. In both cases in Scheme 1, the *m/e* 331 was given the a_1^1 assignment, because the $a_1^1 - 14$ ion was present and large. Compounds **1** and **2** have thus been assigned the structures 6^2-*O*-methylmaltose, and 6^2-deoxymaltose, respectively.

A small amount of another disaccharide was obtained from the hydrolyzate of the 6-deoxyamylose; it is not shown in Scheme 1, because its structure does not fit the diagram. Peaks at *m/e* 533 and *m/e* 517 represent $M^{\pm} - 43$ and $M^{\pm} - 59$, corresponding to a molecular weight of 576 for the peracetylated disaccharide. This suggested that the oligosaccharide isolated was (*O*-3,6-anhydro-α-D-glucopyranosyl)-(1 → 4)-D-glucopyranose. There was a peak at *m/e* 331, and at *m/e* 317, identifying the D-glucopyranose ion as a_1^1. There was a very large peak at *m/e* 229, identifying the ion of the 3,6-anhydro-D-glucopyranosyl residue as the a_1'. The presence of 3,6-anhydro-D-glucopyranosyl residues was not unexpected. Subsequent paper-chromatographic analyses showed the presence of small proportions of the corresponding anhydro sugar in the acid hydrolyzates of almost all of the 6-deoxyamyloses.

The molecular ion of a peracetylated trisaccharide may often be absent, but the a_1 and d series of ions provide the information necessary to determination of the molecular weight; this may be seen in Scheme 2. The a_1 ions at *m/e* 865 for compound **3**, and at *m/e* 879 for **4**, identify the original fraction 3 as a 6,6-di-*O*-methylmaltotriose,



Scheme 2

and 4 as a 6-*O*-methylmaltotriose, but the location of the primary methyl ethers was complicated.

The peak for the a'' ion is usually quite large, suggesting, for the two examples in Scheme 2, that X is OMe for compound **3**, and OAc for compound **4**. Other evidence for the assignment of structure rests with the *m/e* values for a_1', a_1^1 $a_1^{1'}$ — 14, a_1^2, and a_1^2 — 14. All of the values led to the assignment of the structure $6^{2,3}$di-*O*-methylmaltotriose for compound **3**, and 6^2-*O*-methylmaltotriose for compound **4**.

A trisaccharide, compound **5**, was also found in the hydrolyzate of 6-deoxyamylose. All of the analytical data indicated the presence of one 6-deoxy-D-glucopyranosyl residue. There was a large peak at *m/e* 273, but none at *m/e* 259, suggesting that 273 is a_1''. There was an even larger peak at *m/e* 331, and a sizable one at *m/e* 317, leading to the assignment a_1^1 for *m/e* 331. The peaks at *m/e* 561 and 619 were assigned as the a_1^1 and a_1^2 fragment ions, respectively. All of the mass-spectral data were consistent with the structure 6^3-deoxymaltotriose for compound **5**.

A maltotetraose containing two 6-deoxy-D-glucopyranosyl residues was isolated, acetylated, and analyzed by e.i.m.s. The molecular ion was not detected, but $M^+ - CH_3CO$ was present at *m/e* 1095. Although other useful ions were present in the spectrum, it was not possible to determine the sequence of the residues.

The structures of the oligosaccharides obtained from the hydrolyzates (catalyzed by the liquefying amylase of *Bacillus subtilis*) are consistent with those obtained from the hydrolyzates of other modified substrates[1,2]. The presence of $6^{2,3}$-di-*O*-methylmaltotriose suggests some clustering of the modified residues, but the proportion of this fraction was small, and some substitution on adjacent D-glucopyranosyl residues was to be expected, even with completely random reaction. A 6^2-deoxymaltotriose would have been in better agreement with previous results[1,2] than 6^3-deoxymaltotriose, but the latter is an entirely plausible structure.

Hydrolyses catalyzed by the crystalline liquefying amylase of *Bacillus subtilis* have now been conducted with a sufficient number of modified substrates to permit attempting an explanation in terms of the model introduced by Robyt and French[5], and extended by Thoma *et al.*[6].

A modified D-glucose monomer was not found in any of the hydrolyzates, suggesting that a D-glucopyranosyl residue that has a modified hydroxymethyl group is not hydrolyzed if the modified residue is alone on the reducing side of the cleavage site. Disaccharides containing one modified D-glucopyranosyl residue, and trisaccharides having one or two such residues, are formed during the reaction, indicating that the modified units can occupy sites to the "right" of the cleavage site as long as there is also at least one unmodified D-glucopyranose residue present.

The Robyt and French[5] model shows D-glucose, maltose, and maltotriose as being primarily produced by the enzymic hydrolysis of some of the larger malto-oligosaccharides formed in the early stages of the reaction. Table I (column 2) shows

TABLE I

COMPARISON OF YIELDS OF PRODUCTS OF LOW MOLECULAR WEIGHT FROM AMYLOSE AND FROM MODIFIED AMYLOSES

Product	*Amylose*[a]	*6-Deoxyamylose (d.s. 0.20)*	*6-Amino-6-deoxyamylose (d.s. 0.33)*
D-Glucose	0.24	0.33	3.03
Maltose	0.56	0.99	2.70
Maltotriose	**1.00**	**1.00**	**1.00**

[a]Results of Robyt and French[5].

that D-glucose itself is not a favored product in the reaction with native amylose; maltotriose is the major oligosaccharide of low molecular weight present in this hydrolyzate. The other two columns in Table I show that the relative amounts of D-glucose and maltose increase with increase in the d.s. value. It is possible that the modified D-glucopyranosyl residues do not fit some of the other binding sites on the enzyme; the increased relative yield of D-glucose could then be the result of movement of the malto-oligosaccharide over the enzyme until a favorable fit for reaction is obtained.

EXPERIMENTAL

General. — Corn amylose (A. E. Staley Co.) and crystalline *Bacillus subtilis* amylase (Enzyme Development Corp.) were commercial samples. The preparation of 6-*O*-methylamylose has been described[3]. 6-Deoxyamyloses were prepared by the method of Bines and Whelan[4], and by conversion of 2,3-di-*O*-acetyl-6-*O*-*p*-tolylsulfonylamylose into 2,3-di-*O*-acetyl-6-deoxy-6-iodoamylose with sodium iodide in either 2,5-hexanedione or *N*,*N*-dimethylformamide, followed by reduction with sodium borohydride in dimethyl sulfoxide.

Methods. — Descending paper-chromatograms were developed with 6:4:3 (v/v) 1-butanol–pyridine–water, and 4:1:1 (v/v) 1-butanol–ethanol–water, on Whatman 3MM paper, with detection by the alkaline silver nitrate method of Trevelyan *et al.*[7]. Mass spectra were recorded with an AEI MS 30 instrument having a direct-insertion probe. The source temperature was 200°, and the ionizing potential was 70 eV.

Enzymic hydrolyses. — These were conducted at 25°, with a ratio of 0.2 mg of enzyme/mg of substrate; this caused rapid and extensive hydrolysis. Experiments were conducted at pH 5.7, 0.01M with respect to acetate, and in the absence of buffer. The 6-*O*-methylamylose was dissolved in dimethyl sulfoxide, and the solution diluted with water until the concentration of dimethyl sulfoxide was 20%. The 6-deoxyamylose was dissolved in hot water, and the solution was subjected to the action of the enzyme, and then concentrated before chromatography. Peracetates of the isolated fractions were prepared by means of acetic anhydride–pyridine in the usual way.

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