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COMPARISON OF THE ACTION OF GLUCOAMYLASE AND GLUCOSYLTRANSFERASE ON D-GLUCOSE, MALTOSE, AND MALTO-OLIGOSACCHARIDES

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ABSTRACT

The action patterns of glucoamylase (amyloglucosidase) and glucosyltransferase (transglucosylase) on D-[1-^{14}C]glucose, [1-^{14}C]maltose, and [1-^{14}C]malto-oligosaccharides (labeled at position 1 of the D-glucose group at the reducing end) have been investigated by paper-chromatographic and oligosaccharide-mapping techniques. Under the conditions of the experiments, the extent of conversion of D-glucose and of maltose into new oligosaccharides was 2.2 and 1.9% with glucoamylase, and 5.7 and 33% with glucosyltransferase. The major oligosaccharides produced by both enzymes were isomaltose (6-*O*-α-D-glucopyranosyl-α-D-glucose), panose (*O*-α-D-glucopyranosyl (1→6)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucose), and nigerose (3-*O*-α-D-glucopyranosyl-α-D-glucose). The glucosyltransferase also synthesized oligosaccharides from malto-oligosaccharides of higher molecular weight to yield compounds having α-(1→6)-linked D-glucosyl groups at the non-reducing ends. Glucoamylase exhibited little, if any, such activity on malto-oligosaccharides.

INTRODUCTION

The development of the enzymic process for commercial production of crystalline D-glucose and high-glucose syrups from starch is a major technological advance of the past decade in the starch wet-milling industry. This process is based on the use of a fungal enzyme, glucoamylase[1] (amyloglucosidase; EC 3.2.1.3) that converts starch quantitatively into D-glucose[2] because the enzyme is capable of hydrolyzing both the α-(1→4)- and the α-(1→6)-D-glucosidic bonds of starch[3]. Two important findings that were especially pertinent to the development of the enzymic process were first, the demonstration that glucoamylase and glucosyltransferase (transglucosylase; EC 2.4.1.24) also produced by the fungus, are distinct molecular species[4], and second, the successful use of mutation techniques to obtain strains of

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fungi that produce little, if any, glucosyltransferase[5,6]. The glucosyltransferase is undesirable in enzymic conversion of starch as this enzyme effects the synthesis of oligosaccharides having α-D-(1→6) linkages that accumulate in the enzymolyzates[4,7,8]. Glucoamylase itself, while almost devoid of synthetic activity[4], under some conditions may effect the synthesis of α-(1→6)-D-glucosyl oligosaccharides by reversion[9,10] (recombination of free D-glucose) or by glycosylation[11,12] (exchange of D-glucosyl groups) reactions. The action of glucoamylase from *Aspergillus niger* on ^{14}C-labeled D-glucose, maltose, and malto-oligosaccharides has been re-investigated with the view of obtaining quantitative data on the extent of synthesis of oligosaccharides from these substrates. For comparative purposes, parallel experiments have been conducted with the glucosyltransferase from the same organism. The technique of oligosaccharide mapping[13] has been especially valuable in our studies, and new information on the reaction mechanisms of the glucoamylase and the glucosyltransferase has been obtained.

RESULTS AND DISCUSSION

Photographs of radioautograms of digests of [1-^{14}C]maltose with glucosyltransferase and glucoamylase are reproduced in Fig. 1. It may be noted that, under the test conditions, the glucosyltransferase converted maltose rapidly into D-glucose with the concurrent synthesis of large amounts of oligosaccharides, predominantly isomaltose (6-*O*-α-D-glucopyranosyl-α-D-glucose) and panose [*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucose]. The structural characterization of these oligosaccharides had been achieved earlier[14] and, in the current studies, R_t values on paper chromatograms were used for identification. Glucoamylase also converted maltose rapidly into D-glucose, but this enzyme synthesized only small amounts of oligosaccharides (Fig. 1).

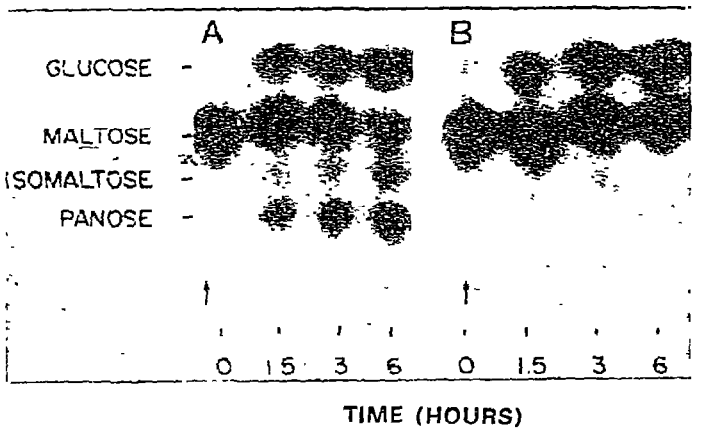

Fig. 1. Radioautograms of digests of [1-^{14}C]maltose with glucosyltransferase (A) and with glucoamylase (B).

Quantitative data on the radioactivities of the D-glucose and oligosaccharides produced from [1-^{14}C]maltose by the two enzymes are presented in Table I. Calculations from these data showed that, under the conditions of the experiments, over 90% of the maltose disappeared during 24 h on incubation of the substrate with either glucosyltransferase or glucoamylase. In the glucosyltransferase digest, 33% of the maltose was converted into new oligosaccharides, but in the glucoamylase digest only 1.9% of the maltose was converted into oligosaccharides. The quantitative data also showed that there were differences in the ratio of the two major oligosaccharides (isomaltose and panose) synthesized by the two enzymes. Thus, in the initial stages of enzymolysis of maltose, the ratio of isomaltose to panose was 0.37 in the glucosyltransferase digest compared to 3.8 in the glucoamylase digest, whereas in the 24-h samples, the ratios were 0.73 and 1.8, respectively. The variation in this ratio and the differences in amounts of oligosaccharides synthesized by the two enzymes reflects differences in reaction mechanisms for the two enzymes. It is possible that the compounds synthesized initially can function at different rates as secondary acceptor-molecules with the enzymes, thereby affecting the ratio of isomaltose to panose.

TABLE I

RADIOACTIVITIES (COUNTS · min^{-1}) OF PRODUCTS IN 50-μl SAMPLES OF DIGESTS OF [1-^{14}C]MALTOSE WITH GLUCOSYLTRANSFERASE AND WITH GLUCOAMYLASE

Time (h)	*Compound*			
	D-Glucose	*Maltose*	*Isomaltose*	*Panose*
			Glucosyltransferase	
0	750	29,800	120	90
1.5	4,900	22,600	920	2,500
3	8,800	16,100	1,700	4,700
6	12,800	11,100	2,500	6,300
12	14,600	6,500	3,500	6,100
24	17,700	3,000	4,300	5,900
			Glucoamylase	
0	130	30,200	60	30
1.5	2,500	27,300	190	50
3	5,300	25,100	210	90
6	9,100	22,400	240	110
12	14,800	15,200	320	240
24	28,100	2,100	380	210

It has been reported that a recombination of D-glucose (reversion reaction) to yield oligosaccharides may occur when high concentrations of D-glucose are incubated with non-purified and purified preparations of glucosyltransferase or glucoamylase[7-11]. The extent of recombination reactions may be calculated from the data on the radioactivity of products in digests of D-[1-^{14}C]glucose with these enzymes. Such data are presented in Table II. Both enzymes do indeed catalyze recombination reactions, yielding isomaltose (R_F value, 0.40) and nigerose[15] (3-*O*-α-D-glyco-

pyranosyl-α-D-glucose; R_F value, 0.54) as the major products. The data in Table II were obtained from experiments in which high concentrations of substrate (30% of D-glucose) and long incubation-periods (96 h) were employed, as is customarily done in commercial starch conversions[1]. Under these conditions, 5.7% of the D-glucose was converted into oligosaccharides by glucosyltransferase, but only 2.2% was converted into oligosaccharides by glucoamylase. Of special note is the observation that the glucosyltransferase produced about five times as much isomaltose as did the glucoamylase.

TABLE II

RADIOACTIVITIES (COUNTS MIN^{-1} OF PRODUCTS IN 50-μl SAMPLES OF DIGESTS OF D [1-^{14}C]GLUCOSE WITH GLUCOSYLTRANSFERASE AND WITH GLUCOAMYLASE

Time (h)	*Compound*		
	D Glucose	*Nigerose*	*Isomaltose*
		Glucosyltransferase	
0	44,300	113	59
6	43,900	402	104
12	43,700	410	312
24	43,400	411	635
48	42,700	500	1,280
96	41,900	524	2,020
		Glucoamylase	
0	38,800	80	119
6	38,600	299	109
12	38,500	348	140
24	38,400	330	152
48	38,200	422	320
96	38,100	463	402

On the basis of the foregoing results, it can be concluded that both glucosyltransferase and glucoamylase do effect reversion reactions and synthesize oligosaccharides, primarily disaccharides, from D-glucose. In this connection, the studies of Hehre *et al.*[11,12] on the action of glucoamylase on highly reactive glucosyl compounds or on pure anomers of glucose should be mentioned. These investigators identified maltose as a product of action of glucoamylase on these substrates. However, in our experiments, maltose was not detectable in digests of a mixture of the two anomers of D-glucose with glucoamylase, perhaps because of differences in the conditions used.

In order to obtain additional information on the reaction mechanisms for glucosyltransferase and glucoamylase, the action of the two enzymes was studied on [1-^{14}C]malto-oligosaccharides by the oligosaccharide mapping procedure[13]. The oligosaccharide maps were obtained by the methods described in the experimental section, and typical maps are reproduced in Fig. 2. The results in this Fgure show that glucoamylase yielded only hydrolytic products (R_F values higher than the R_F values

of the substrates), but that the glucosyltransferase yielded synthetic products as well as hydrolytic products, with some of the synthetic compounds possessing R_F values lower than those of the substrates. It will be noted that isomaltose and panose were produced from maltose, and new oligosaccharides were produced from maltotriose, maltotetraose, maltopentaose, and maltohexaose. Quite clearly, the glucosyltransferase catalyzes transfer-reactions not only with maltose, but also with malto-oligosaccharides of higher molecular weight.

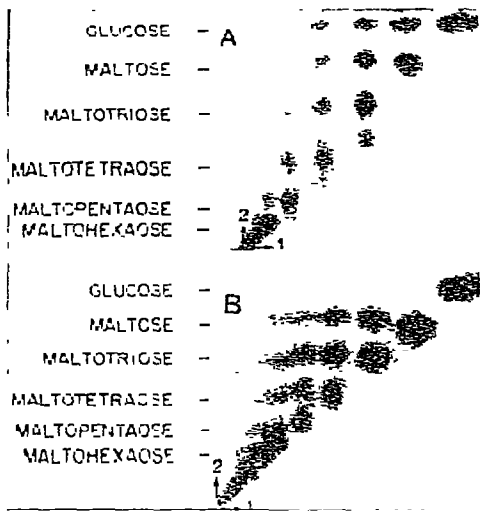

Fig. 2. Radioautograms of the oligosaccharide maps for the action of glucosyltransferase (A) and glucoamylase (B) on [1-^{14}C]malto-oligosaccharides.

The patterns of labeled products produced from [1-^{14}C]malto-oligosaccharides by the two enzymes differ markedly (Fig. 2). The glucoamylase yielded, as the initial product from the individual oligosaccharides, a ^{14}C-labeled oligosaccharide having one less D-glucose group than the substrate, as well as non-labeled glucose, which was detectable on staining the chromatogram for reducing sugars. Glucosyltransferase, on the other hand, yielded hydrolytic products that were about equally labeled. The former result is in accord with a multichain mechanism of action for glucoamylase on linear D-glucose polymers[10,13]. In this mechanism, the enzyme removes one glucose group from the substrate per single encounter of enzyme and substrate, and progressively shortens all of the substrate molecules until complete conversion into D-glucose occurs. The labeling pattern in the products of glucosyltransferase action on oligosaccharides indicates that the glucosyltransferase acts predominantly by a single-chain mechanism.

The action of glucosyltransferase was also tested on pure maltotetraose and maltoheptaose with the objective of obtaining information on the structure of the new products. Chromatograms showing the products from both substrates are

reproduced in Fig. 3. The results in this Figure show that new oligosaccharides having R_F values lower than those of the substrates were indeed produced. In addition, hydrolytic and synthetic products having R_F values higher than those of the substrates also appeared. In the maltoheptaose digest, thirteen new compounds were detectable on the paper chromatogram (Fig. 3), and in the maltotetraose digest, eight new products were present. Structural information on the compounds was obtained by comparison of R_F values of the products to those of reference compounds[14,17] and by the oligosaccharide-mapping method, utilizing beta amylase and glucoamylase as the enzymes[19]. The oligosaccharide maps showed that beta amylase hydrolyzed only one compound in the maltotetraose digest, specifically the unreacted substrate, yielding maltose as the hydrolytic product. All other compounds in this digest were resistant to beta amylolysis. However, of the products in the maltoheptaose digest, several were hydrolyzed by beta amylase: maltotetraose and maltohexaose were converted into maltose, and maltopentaose and maltoheptaose were converted into maltose and maltotriose. The oligosaccharide maps prepared with glucoamylase showed that this enzyme converted all of the oligosaccharides in the two digests into D-glucose. However, as this enzyme hydrolyzes the α-(1→6) linkage at a lower rate than the α-(1→4) linkage[3,18], some compounds having a-(1→6) linkages were not completely hydrolyzed.

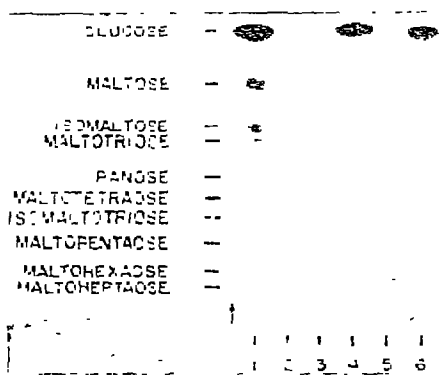

Fig. 3. Paper chromatogram of digests of maltotetraose and maltoheptaose with glucosyltransferase. 1, reference oligosaccharides. 2, enzyme blank. 3, maltotetraose, 4, enzymic digest of maltotetraose; 5, maltoheptaose, and 6, enzymic digest of maltoheptaose.

In view of the action mechanism of beta amylase[19] and glucoamylase[3,18,20] on glucosyl oligosaccharides, the new compound of low R_F values in both digests must contain α-(1→6) linked D-glucosyl groups at the non-reducing ends. In addition, some of the compounds having higher R_F values must also contain α-(1→6)-linked D-glucose residues. Among the latter were isomaltose, panose, and isomaltotriose [*O*-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-α-D-glucose]. The latter products were hydrolyzed slowly by glucoamylase and possessed R_F values identical

to those for reference compounds (Fig. 3). That the new oligosaccharides having low R_F values produced from maltotetraose are indeed terminated by α-(1→6) linked D-glucose residues was verified by methylation analysis. On methylation and g.l.c.–mass spectrometry, the oligosaccharide having the lowest R_F value in the tetraose digest yielded 2,3,4,6-tetra-*O*-methylglucose, 2,3,4-tri-*O*-methylglucose, and 2,3,6-tri-*O*-methylglucose in the molar ratio of 1:1:3, whereas the oligosaccharide having the next lowest R_F value yielded the same derivatives in the ratio of 1:1:2. The original substrate, maltotetraose, yielded only the tetra-*O*-methylglucose and 2,3,6-tri-*O*-methylglucose, in the ratio of 1:3. On the basis of these results, it was concluded that glucosyltransferase can transfer single D-glucosyl groups, not only from maltose but also from malto-oligosaccharides, to the terminal D-glucosyl groups of appropriate acceptors (D-glucose or other malto-oligosaccharides) to yield oligosaccharides having α-(1→6) linked D-glucosyl groups at the non-reducing ends. Such transfer reactions were not effected by the glucoamylase at sufficient rates for new oligosaccharides to accumulate during the enzymolysis of malto-oligosaccharides by this enzyme.

EXPERIMENTAL

Materials — Glucoamylase was isolated from an enzyme extract of *Aspergillus niger* (DIAZYME, Lot F 8150, Miles Laboratories, Inc., Elkhart, ID 46514) in highly pure state by chromatography on DEAE-cellulose[4]. Criteria for purity have been presented earlier[21,22] and consisted of ultracentrifuge patterns, density-gradient sedimentation behavior, electrophoretic migration, and elution patterns from ion-exchange resins. The enzyme solution used in the current experiments contained 900 units of activity per ml of solution, as assayed on starch as the substrate[4].

Glucosyltransferase was also isolated from the same strain of *Aspergillus niger* by a chromatographic procedure, as described in an earlier publication[7]. Ultracentrifuge patterns and paper-electrophoretic strips indicated homogeneity in the enzyme preparation. The sample employed in the current experiments contained 30 units of activity per ml of solution[7]. Beta amylase was purchased from the Wallerstein Laboratories, New York, N.Y.

D-[1-^{14}C]Glucose and [1-^{14}C]maltose (having the label at position 1 of the D-glucose residue at the reducing end) were purchased from commercial suppliers. Solutions of the labeled compounds, when examined by paper chromatography and radioautography, yielded a single, radioactive component having R_F values of authentic D-glucose and maltose. [1-^{14}C]Malto-oligosaccharides labeled at C-1 of the D-glucose residue at the reducing end were prepared from D-[1-^{14}C]glucose and cyclohexaamylose (cyclomaltohexaose) by utilizing *Bacillus macerans* amylase[13]. Typical radioactivities of these compounds, as measured by a Geiger–Muller end-window counter, were: 39,900 c.p.m. for D-glucose, 20,200 c.p.m. for maltose, 13,800 c.p.m. for maltotriose, and 8,100 c.p.m. for maltotetraose. Maltoheptaose was prepared from cycloheptaamylose (cyclomaltoheptaose) as previously described[23],

and purified by preparative, paper chromatography[14]. Isomaltose and panose were available in this laboratory[14]. Isomaltotriose was provided by H. Erbetova, Academy of Science, Prague, Czechoslovakia. Maltotetraose was purchased from the Pierce Chemical Co., Rockford IL 61105. Other chemicals were reagent-grade compounds purchased from various suppliers.

Enzymolysis of [1-^{14}C]maltose — In a typical experiment, a sample of 0.1 ml of 60% [1-^{14}C]maltose solution (total radioactivity 1.2×10^6 c.p.m.) in 0.1M citrate buffer of pH 4.8 was mixed with 0.1 ml of the glucoamylase solution. A sample of 50 μl was removed immediately after addition of the enzyme, placed on a paper chromatogram, and dried in a stream of hot air to inactivate the enzyme. Subsequent samples were placed on the chromatogram after incubation of the mixture for periods of 1.5, 3, 6, 12, and 24 h at room temperature. The chromatograms were developed in butyl alcohol–pyridine–water (6:4:3 by volume) by three ascents of the solvent. A radioautogram of the chromatogram was obtained by placing the dried chromatogram in contact with X-ray film for 24- to 48-h periods and subsequently developing the film. A similar experiment was performed by using [1-^{14}C]maltose in 0.1M acetate buffer of pH 3.5 and 0.1 ml of glucosyltransferase solution as the enzyme. The concentrations of enzymes were such that 90% of the maltose disappeared in a 24-h incubation period. Photographs of the radioautograms of the two digests are reproduced in Fig. 1.

As the products were sufficiently separated from each other on the paper chromatogram, it was possible to measure radioactivities by placing the G.-M. tube on the area of the chromatogram corresponding to the desired compound. Values for the radioactive products formed from maltose by glucoamylase and glucosyltransferase are recorded in Table I. Identification of the radioactive products is based on R_F values in comparison to reference compounds. The structural characterization of isomaltose and panose as products of glucosyltransferase action on maltose had been reported earlier[15].

Enzymolysis of D-[1-^{14}C]*glucose.* — A sample of 0.1 ml of a 60% solution of D-[1-^{14}C]glucose (total radioactivity 1.6×10^6 c.p.m.) in citrate buffer of pH 3.5 was mixed with an equal volume of the glucoamylase or the glucosyltransferase solution. Analyses of the digests at various time-intervals were performed as described in the preceding section. The incubation was conducted for a longer period (96 h) than with the [1-^{14}C]maltose digests. The R_F values of the new oligosaccharides in the D-glucose digests were 0.54 for nigerose and 0.40 for the isomaltose. Under comparable conditions, the R_F value for D-glucose was 0.67, and for maltose 0.46. Radioactivity values for the products in the digests of D-[1-^{14}C]glucose were obtained as already indicated, and these values are recorded in Table II. The isolation and characterization of nigerose as a product of enzyme action on maltose has been described earlier[14].

Oligosaccharide maps. — The oligosaccharide-mapping procedure is especially suited for investigating the action patterns of various amylases and transferases on malto-oligosaccharides[13,16]. In this procedure, a sample of 50 μl of [1-^{14}C]malto-

oligosaccharide was placed at one corner of a chromatogram (27 × 25 cm), and the chromatogram was developed in one direction by five ascents of 6:4:3 (by volume) butyl alcohol–pyridine–water. The area of the dried chromatogram containing the oligosaccharides was then sprayed lightly and uniformly with a solution of the enzymes under study. The sprayed chromatogram was maintained for 30 min at room temperature, during which time the paper strips had dried and the enzyme had become inactivated. The chromatogram was rolled in a cylinder, stapled, and developed in the second direction in the foregoing solvent system. In some instances it was desirable to place a sample of reference compounds (malto-oligosaccharides) in line with the other compounds at the bottom of the chromatogram to be developed in the second direction. After five ascents of the solvent, the chromatogram was dried and used to obtain a radioautogram as described in a preceding section. The chromatogram was then stained by the silver nitrate method[24] to reveal the reducing sugars. In most experiments, several dilutions of the individual enzymes were employed in order to obtain oligosaccharide maps at several stages of enzymolysis. Satisfactory results were obtained with enzyme solutions that hydrolyzed about 25% of the glucosidic bonds of the oligosaccharides. Photographs of radioautograms of oligosaccharide maps for the two enzymes are reproduced in Fig. 2.

Glucosyltransferase action on maltotetraose and maltoheptaose. — Samples of 0.1 ml of a 10% solution of the oligosaccharide were mixed with 0.1 ml of 0.2M acetate buffer of pH 3.5 and 0.1 ml of glucosyltransferase solution. The mixtures were incubated for 4 h at room temperature and then heated for 15 min in a boiling water-bath in order to inactivate the enzyme. A chromatogram of the digests, appropriate reference compounds, and blanks was prepared, developed by 5 ascents of the solvent system (6:4:3 butyl alcohol–pyridine–water), and stained by the silver nitrate procedure[24]. A photograph of a typical chromatogram is reproduced in Fig. 3.

Samples of 50 µl of the digest were also used for oligosaccharide-mapping experiments. After development of the chromatogram in one direction, the area of the chromatogram containing the compounds was sprayed with 5 ml of 0.1% beta amylase solution in 0.05M acetate buffer of pH 5.0 or with 5 ml of 0.05% glucoamylase solution in 0.05M acetate buffer of pH 5.0. A reference mixture of malto-oligosaccharides was placed on the chromatogram in line with the products in the maltotetraose or maltoheptaose digests. The chromatogram was developed in the second direction by three ascents of the solvent system. The finished chromatograms were stained with silver nitrate reagent[24] to locate the reducing products. Of the compounds in the maltotetraose digest, only maltotetraose was hydrolyzed by the beta amylase to yield maltose. In the maltoheptaose digest, maltotetraose and maltohexaose were hydrolyzed to maltose, whereas maltopentaose and maltoheptaose were hydrolyzed to maltose and maltotriose by the beta amylase. In both digests, all of the compounds were hydrolyzed to D-glucose by the glucoamylase. However, as the α-(1→6) D-glucosidic linkage is hydrolyzed at a lower rate than the α-(1→4) linkage by the glucoamylase[21], oligosaccharides having α-(1→6) linkages were not completely hydrolyzed to D-glucose.

The two oligosaccharides having lowest R_F values in the maltotetraose digest (Fig. 3) were isolated by preparative, paper chromatography[25]. Each oligosaccharide was methylated by the Hakomori method[26], following the procedure described in a recent publication[27]. The partially methylated alditol acetates from each compound were identified by g.l.c. and mass-spectrometric methods[27,28] and were: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol. The ratio of these products was 1:1:3 for the oligosaccharide having the lowest R_F value and 1:1:2 for the oligosaccharide having the second-lowest R_F value. Methylation of the substrate, maltotetraose, yielded only the tetra-*O*-methyl derivative and the 2,3,6-tri-*O*-methyl derivative, in the ratio of 1:3.

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