SYNTHESIS OF METHYL α-D-GLUCOOLIGOSACCHARIDES BY ENTRAPPED DEXTRANSUCRASE FROM *Leuconostoc mesenteroides* B-1299

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Running title: Synthesis of methyl glucooligosaccharides by dextransucrase

ABSTRACT

The synthesis of methyl α-D-glucooligosaccharides, using sucrose as glucosyl donor and methyl α-D-glucopyranoside as acceptor, was studied with dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299. The enzyme was immobilized by entrapment in alginate. By NMR and mass spectrometry we identified three homologous series *(S1-S3)* of methyl α-D-glucooligosaccharides. Series *S2* and *S3* were characterized by the presence of α(1→2) linkages, in combination with α(1→6) bonds. Two parameters, sucrose to acceptor concentration ratio (S/A) and the total sugar concentration (TSC) determined the yield of methyl α-D-glucooligosaccharides. The maximum concentration achieved of the first acceptor product, methyl α-D-isomaltoside, was 65 mM using a S/A 1:4 and a TSC of 336 g l^{-1}. When increasing temperature, a shift of selectivity towards compounds containing α(1→2) bonds was observed. The formation of leucrose as a side process was very significant (reaching values of 32 g l^{-1}) at high sucrose concentrations.

Keywords: Glucansucrases, acceptor reaction, methyl polyglucosides, encapsulation, alginate.

INTRODUCTION

Dextransucrases (EC 2.4.1.5) are glucosyltransferases produced by different *Leuconostoc mesenteroides* strains that catalyze the synthesis, from sucrose, of $\alpha(1\rightarrow6)$-linked glucose polymers called dextrans, releasing fructose. Dextransucrases belong to the family of glycansucrases, a group of enzymes that use the free energy of cleavage of sucrose to form new glycosidic bonds (Plou et al., 2002). In the presence of other compounds, generally mono-, di- and short oligosaccharides, the synthesis of acceptor products may occur.

Depending on the acceptor structure, a homologous series –i.e. a mixture of oligosaccharides with an increasing number of glucose moieties - or just a unique acceptor product are synthesized (Robyt and Eklund, 1983). Dextransucrase is able to glucosylate not only carbohydrates, but also other hydroxylated compounds such as phenols (Seo et al., 2005).

The regioselectivity displayed by dextransucrases is highly strain dependent. With dextransucrase from *L. mesenteroides* NRRL B-512F different acceptors have been assayed, resulting in the synthesis of compounds with $\alpha(1\rightarrow6)$ linked glucose moieties (Robyt and Eklund, 1983). However, dextransucrase from the strain B-1299 is also able to form $\alpha(1\rightarrow2)$ linkages (Dols-Lafargue et al., 2001). Glucooligosaccharides containing $\alpha(1\rightarrow2)$ linkages are capable of promoting the development of the beneficial cutaneous flora to the detriment of the undesirable microorganisms, either pathogenic or those associated with infections. Based on the acceptor reaction with maltose, dextransucrase from *L. mesenteroides* B-1299 has been used in the last decade to produce 50 tons per year of glucooligosaccharides containing $\alpha(1\rightarrow2)$ bonds for the dermocosmetic industry (Dols et al., 1998).

In the present work, we have studied the synthesis of methyl α-D-glucooligosaccharides containing $\alpha(1\rightarrow2)$ bonds, using sucrose as glucosyl donor and methyl α-D-glucopyranoside as acceptor, catalyzed by alginate-entrapped dextransucrase B-1299.

MATERIALS AND METHODS

Materials

Dextransucrase from *L. mesenteroides* NRRL B-1299 was produced as described (Dols et al., 1997). The insoluble fraction was used as a lyophilized powder. Alginate SG-300 was provided by System Bio-Industries. Methyl α-D-glucopyranoside and glucose were from Sigma. Merck supplied sucrose and fructose. Leucrose was obtained from Fluka. All other reagents and solvents were of the highest available purity and used as purchased.

Assay of dextransucrase activity

Dextransucrase activity was determined at 30°C measuring the initial rate of fructose production by the dinitrosalicylic acid (DNS) method (Sumner and Howell, 1935) using 100 g l^{-1} sucrose in 20 mM sodium acetate buffer (pH 5.4) containing 0.05 g l^{-1} $CaCl_2$. One unit of dextransucrase activity corresponded to the formation of 1 μmol fructose per min under the above conditions. Experiments were performed in triplicate, with standard deviations lower than 5%.

Synthesis of methyl α-D-glucooligosaccharides

The alginate-entrapped dextransucrase (3 U, determined by the DNS assay) was added to 10 ml of 20 mM sodium acetate buffer (pH 5.4) containing 0.1-0.5 M sucrose, 0.02-1.2 M methyl α-D-glucopyranoside and 0.05 g l^{-1} $CaCl_2$. The mixture was incubated at 30°C with magnetic stirring at 200 rpm. At different times, 500 μl aliquots were taken from the reaction mixture, centrifuged 5 min at 6000 rpm using an eppendorf with a 0.45 μm Durapore® membrane (Millipore), and analyzed by HPLC.

HPLC analysis

The concentration of the different products of the homologous series was analyzed by HPLC. The pump (Spectra-Physics Inc., model SP 8810) was coupled to a Nucleosil 100-C18

column (250 x 4.6 mm) (Sugelabor, Spain). The mobile phase was water at 0.5 ml min^{-1}. The column was kept constant at 40°C. A differential refractometer (Waters, model 2410) was used and set to a constant temperature of 45°C. The data obtained were analyzed using the Varian Star Software. Standard deviations were lower than 1%.

Purification of the products

Sucrose (0.5 M) and methyl α-D-glucopyranoside (0.8 M) in 25 ml of 20 mM sodium acetate buffer (pH 5.4) containing 0.05 g l^{-1} $CaCl_2$ were mixed with 2 ml (4 U) of alginate-entrapped dextransucrase. The mixture was incubated at 30°C with orbital shaking (200 rpm) during 64 h. Then, the immobilized biocatalyst was filtered. The oligosaccharides were purified by semipreparative HPLC using a system equipped with a Waters Delta 600 pump, a Nucleosil 100-C18 column (250 x 10 mm) (Sugelabor, Spain) coupled to a precolumn (50 x 10 mm) packed with the same stationary phase. A differential refractometer (Varian, model 9040) set to 35°C and a fraction collector (Waters) were used. Water was the mobile phase (2.4 ml min^{-1}), and the column temperature was kept constant at 40°C. The system was controlled by the software Millenium 32 (Waters). After collection of the different oligosaccharides, water was eliminated by rotary evaporation.

Characterization and identification of the products

NMR spectra were recorded on a Varian Unit (^{1}H-NMR, 500 MHz; ^{13}C-NMR, 125 MHz) spectrometer, with a reverse probe and a gradient unit. The spectra were obtained at 40°C with samples ranging from 6 to 10 mg dissolved in 0.6 ml of deuterated water (D_2O). Proton chemical shifts refer to residual HDO (4.61 ppm). Carbon chemical shifts refer to external acetone (31.07 ppm). 2D-homo- (DQCOSY, TOCSY (HOHAHA), NOESY) and hetero- (HMQC and HMBC) NMR experiments were performed by using the standard software from Varian. Mass spectrometry was determined with a MALDI-TOF system Biflex III (Bruker-

Franzen). The external standards used were angiotensine (m/z 1046.5) and ACTH (m/z 2465.2) with α-cyano-4-hidroxycinnamic acid (m/z 379.0) as the matrix. For the experiments the matrix employed was a saturated solution of 2,5-hydroxybenzoic acid.

Methyl α-D-glucopyranosyl-(1→2)-bis[-O-α-D-glucopyranosyl-(1→6)]-O-α-D-glucopyranoside, P_4^{S2}. **^{1}H-NMR** (δ, ppm): 5.18 (H_1^{III}; $J_{1,2}$ = 3.5 Hz), 5.12 (H_1^{IV}; $J_{1,2}$ = 3.8 Hz), 4.98 (H_1^{II}; $J_{1,2}$ = 3.9 Hz), 4.84 (H_1^{I}; $J_{1,2}$ = 3.7 Hz), 3.99 (H_{6a}^{I}), 3.98 (2H: H_{6a}^{II} + H_5^{IV}), 3.88 (H_5^{II}), 3.86 (H_{6a}^{III}), 3.85 (2H: H_{6a}^{IV} + H_3^{III}), 3.83 (H_5^{I}), 3.79 (H_{6b}^{II}), 3.78 (H_{6b}^{IV}), 3.77 (2H: H_{6b}^{III} + H_3^{IV}), 3.76 (H_{6b}^{I}), 3.75 (H_5^{III}), 3.73 (H_3^{II}), 3.68 (H_2^{III}), 3.67 (H_3^{I}), 3.58 (H_2^{II}), 3.57 (H_2^{I}), 3.55 (2H: H_2^{IV} + H_4^{II}), 3.52 (H_4^{I}), 3.48 (H_4^{III}), 3.45 (H_4^{IV}). **^{13}C-NMR** (δ, ppm): 100.4 (C_1^{I}), 98.9 (C_1^{II}), 97.2 (C_1^{IV}), 96.4 (C_1^{III}), 76.6 (C_2^{III}), 74.5 (C_3^{I}), 74.3 (C_3^{II}), 73.9 (C_3^{IV}), 72.9 (C_5^{IV}), 72.7 (C_5^{III}), 72.4 + 72.2 (3C: $C_2^{I+II+IV}$), 72.3 (C_3^{III}), 71.4 (C_5^{II}), 71.0 (C_5^{I}), 70.5 + 70.4 (4C: $C_4^{I+II+III+IV}$), 66.9 (C_6^{II}), 66.6 (C_6^{I}), 61.5 + 61.4 (2C: $C_6^{III + IV}$), 56.1 (CH_3-O).

Methyl α-D-glucopyranosyl-(1→6)[-O-α-D-glucopyranosyl-(1→2)]-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranoside, P_4^{S3}. **^{1}H-NMR** (δ, ppm): 5.18 (H_1^{II}; $J_{1,2}$ = 3.7 Hz), 5.10 (H_1^{III}; $J_{1,2}$ = 3.8 Hz), 4.97 (H_1^{IV}; $J_{1,2}$ = 3.4 Hz), 4.82 (H_1^{I}; $J_{1,2}$ = 3.8 Hz), 4.04 (H_{6a}^{I}), 3.97 (H_{6a}^{II}), 3.94 (H_5^{II}), 3.92 (2H: H_5^{III+IV}), 3.86 (H_{6a}^{IV}), 3.85 (H_{6a}^{III}), 3.84 (H_3^{II}), 3.82 (H_5^{I}), 3.80 (H_{6b}^{I}), 3.78 (H_{6b}^{IV}), 3.77 (2H: H_{6b}^{II} + H_3^{III}), 3.76 (H_{6b}^{III}), 3.74 (H_3^{IV}), 3.70 (H_2^{II}), 3.67 (H_3^{I}), 3.58 (2H: H_2^{I} + H_4^{II}), 3.57 (H_2^{IV}), 3.56 (H_2^{III}), 3.54 (H_4^{I}), 3.46 (H_4^{III}), 3.43 (H_4^{IV}). **^{13}C-NMR** (δ, ppm): 100.2 (C_1^{I}), 98.6 (C_1^{IV}), 97.2 (C_1^{III}), 96.4 (C_1^{II}), 76.4 (C_2^{II}), 74.2 (C_3^{I}), 74.0 (C_3^{III}), 73.8 (C_3^{IV}), 72.8 + 72.7 (2C: $C_5^{III + IV}$), 72.5 (C_3^{II}), 72.3 + 72.2 + 72.0 (3C: $C_2^{I+III+IV}$), 71.0 (C_5^{II}), 70.4 + 70.3 (4C: $C_4^{I+II+III+IV}$), 66.8 (C_6^{I}), 66.4 (C_6^{II}), 61.4 + 61.3 (2C: $C_6^{III + IV}$), 56.0 (CH_3-O).

RESULTS AND DISCUSSION

Acceptor reaction with methyl α-D-glucopyranoside

The acceptor reaction using 0.5 M sucrose as glucosyl donor and 0.8 M methyl α-D-glucopyranoside as acceptor was studied with dextransucrase from *L. mesenteroides* B-1299. We observed that methyl α-D-glucopyranoside was a better acceptor than its corresponding β-linked isomer. The enzyme was immobilized by entrapment in alginate beads as described (Gómez de Segura et al., 2004). The immobilization yield was 57%, and the diameter of the beads was around 2 mm. Fig. 1 shows the HPLC chromatogram obtained at the end of reaction, i.e. when all the starting sucrose was exhausted. The oligosaccharides were eluted in order of increasing polymerization degree. We purified by semipreparative HPLC and characterized by NMR most of the oligosaccharides produced in the reaction. They were classified in three different homologous series.

Series *S1* was formed by methyl α-D-glucooligosaccharides containing only α(1→6) bonds (Fig. 2), which is identical to that obtained with dextransucrase from *L. mesenteroides* B-512F (Jones et al., 1956). We purified and characterized the oligosaccharides from P_2^{S1} to P_6^{S1}. The nomenclature used for peak assignation was the following: P_n^{Sp}, where *n* indicates the polymerization degree of the compound, and *Sp* indicates the homologous series at which it belongs (*p* from 1 to 3).

Series *S2* and *S3* presented α(1→2) linkages (Fig. 2). The oligosaccharides that belong to series *S2* show an α(1→2) linkage at the non-methylated end of the molecule. Oligosaccharides that belong to series *S3* present an α(1→2) branch linkage, in the next-to-last glucose moiety, starting from the non-methylated end. We purified and characterized the compounds P_3^{S2}, P_4^{S2} and P_4^{S3}. The compound P_3^{S2} was previously described by Smith et al. (1998), and was obtained using a dextransucrase from a mutant strain of *L. mesenteroides* B-1355. The tetrasaccharides P_4^{S2} and P_4^{S3} are new compounds that had not been described before. The logarithmic representation of retention time versus polymerization degree for

each series gave an acceptable regression (data not shown), which allowed us to assign the unidentified peaks above 40 min in Fig. 1 to P_5^{S3}, P_5^{S2} and P_5^{S1}, respectively.

Dols et al. (1998) also reported the formation of three equivalent homologous series with dextransucrase B-1299 using maltose as acceptor. It is noteworthy that, in contrast with our results, the smallest molecule in series *S3* that they obtained was an hexasaccharide. The use of alkyl glucosides with longer fatty alcohol chains has also been explored (Richard et al., 2003).

Effect of donor and acceptor concentrations

A complete study of the conditions for the synthesis of methyl α-D-glucooligosaccharides with dextransucrase B-1299 was performed varying the initial concentration of the substrates and the molar ratio between them.

Fig. 3 shows the effect of sucrose and methyl α-D-glucopyranoside concentrations on the formation of the first acceptor product, methyl α-D-isomaltoside (P_2^{S1}). The values correspond to the end of the reaction. When we increased the amount of methyl α-D-glucopyranoside, maintaining the concentration of sucrose, there was an enhancement in the yield of methyl α-D-isomaltoside. The maximum concentration achieved for this acceptor product was 50.4 mM (18.0 g l^{-1}). However, when increasing the amount of sucrose, maintaining the acceptor concentration, the production of methyl α-D-isomaltoside only improved slightly.

Table 1 summarizes the concentration of the different products obtained at the end of the reaction, using 0.5 M sucrose and 0.8 M methyl α-D-glucopyranoside. Dextransucrase B-512F was also assayed as a reference. It is important to point out that the percentage of the initial sucrose that is used for the synthesis of oligosaccharides is 91.4% for dextransucrase B-512F and only 44.7% for B-1299. The rest of sucrose is basically consumed in two processes: (1) the formation of dextran and (2) the synthesis of leucrose.

We also investigated the effect of total sugar concentration (TSC, sucrose + acceptor) on oligosaccharides production. A linear increase in the concentration of methyl α-D-isomaltoside when rising TSC was observed (data not shown). The higher the ratio acceptor:sucrose, the higher the slope of this line. Using 0.3 M sucrose and 1.2 M methyl α-D-glucopyranoside (336 g l^{-1} TSC), the concentration of methyl α-D-isomaltoside achieved was 64.8 mM (23 g l^{-1}).

Effect of temperature

The temperature of the reaction was modified between 0 and 40°C, using 0.2 M sucrose and 0.2 M methyl α-D-glucopyranoside (Table 2). The percentage of sucrose transformed into oligosaccharides with a polymerization degree ≤ 4 only varied in the range 18-24%. As expected, the reaction rate increased with temperature up to 30°C; as a result, the end-of-reaction fell from 120 h (0°C) to 24 h (30°C). The reaction rate diminished at 40ºC, probably due to enzyme inactivation. In fact, when rising temperature to 50°C, the acceptor reaction did not occur.

A modification of the selectivity was observed with the variation of temperature. At higher temperatures, the production of compounds with α(1→2) linkages increased. The shift in reaction selectivity towards α(1→2) linkages when rising temperature was also reported in the reaction with maltose (Dols-Lafargue et al., 2001).

Synthesis of leucrose

As a consequence of all reactions catalysed by dextransucrase, fructose is liberated as a side-product. When its concentration is high enough, it competes with the remaining acceptor (and with the acceptor products) for the glucopyranosyl residues of sucrose. As a result, the synthesis of the ketodisaccharide leucrose [D-glucopyranosyl-α-(1→5)-D-fructopyranoside], used as a non-cariogenic sweetener, takes place (Buchholz et al., 1998). We determined the production of leucrose at the end of the acceptor reaction, varying the

concentration of sucrose and methyl α-D-glucopyranoside. When the acceptor concentration was low (≤ 0.1 M), the formation of leucrose was significant, reaching values of 93 mM (32 g l^{-1}) at 0.5 M sucrose and 0.02 M methyl α-D-glucopyranoside. An increment in the acceptor concentration to 0.8 M produced a 10-fold decrease in the leucrose yield (3 g l^{-1}).

Buchholz et al. (1998) stated that the initial fructose concentration influenced substantially the yield of leucrose. Thus, they used a high acceptor concentration (2.2-2.5 M fructose) to achieve 75% yield with dextransucrase B-512F. In our work, without the addition of exogenous fructose, the formation of leucrose was really significant, which implies that dextransucrase B-1299 may become an alternative to dextransucrase B-512F for leucrose synthesis.

Operational stability of immobilized dextransucrase

An acceptor reaction using 0.2 M sucrose, 0.2 M methyl α-D-glucopyranoside and 0.3 U ml^{-1} dextransucrase entrapped in alginate beads was carried out at 30°C. When the reaction was finished (confirming by HPLC the total exhaustion of sucrose) the biocatalyst was filtered and rinsed with water (50 ml). The recycled biocatalyst was added to a fresh solution containing the substrates, and the acceptor reaction was followed again by HPLC. The procedure was repeated 5 times. The percentage of sucrose that was converted into oligosaccharides remained almost constant in the five reaction cycles, although the time needed for total sucrose consumption slightly increased for successive cycles. No significant effect on reaction selectivity was found.

CONCLUSIONS

The enzymatic method for the synthesis of methyl α-D-glucooligosaccharides requires mild conditions and the linkage selectivity can be modulated varying the dextransucrase-producing strain of *L. mesenteroides*. Glucooligosaccharides containing α(1→2) bonds are being currently produced for the dermocosmetic industry. The presence of a methyl group in the

anomeric carbon may confer new properties to these compounds, especially in terms of absorption, bioavailability, stability, etc. However, some experiments to determine their potential use in dermocosmetics are required.

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Table 1.- Production of methyl α-D-glucooligosaccharides catalyzed by dextransucrases from *L. mesenteroides* B-512F and B-1299. Experimental conditions: 0.5 M sucrose, 0.8 M methyl α-D-glucopyranoside, 0.16 U ml^{-1} dextransucrase immobilized in alginate, 20 mM sodium acetate buffer (pH 5.4), 0.05 g l^{-1} $CaCl_2$, 30°C. Reaction time: 64 h.

***L. mesenteroides* strain**	**Production of oligosaccharides (mmol l^{-1})**						**Conversion (%)[a]**
	P_2^{S1}	P_3^{S1}	P_4^{S1}	P_3^{S2}	P_4^{S2}	P_4^{S3}	
B-512F	167	87.5	38.2	——	——	——	91.4
B-1299	50.4	15.3	13.1	27.1	9.5	6.8	44.7

[a] Percentage of the initial sucrose that is converted into oligosaccharides with polymerization degree ≤ 4.

Table 2.- Effect of temperature on the production of methyl α-D-glucooligosaccharides catalyzed by dextransucrase from *L. mesenteroides* B-1299. Experimental conditions: 0.2 M sucrose, 0.2 M methyl α-D-glucopyranoside, 0.3 U ml^{-1} dextransucrase entrapped in alginate, 20 mM sodium acetate buffer (pH 5.4), 0.05 g l^{-1} $CaCl_2$.

Temperature (ºC)	**Reaction time (h)**	**Production of oligosaccharides (mmol l^{-1})**						**Conversion (%)**[a]
		P_2^{S1}	P_3^{S1}	P_4^{S1}	P_3^{S2}	P_4^{S2}	P_4^{S3}	
0	120	11.2	5.0	3.6	1.7	1.2	< 1	19.5
10	50	10.7	3.8	2.8	2.8	1.7	< 1	18.7
30	24	10.7	2.6	2.7	6.2	2.7	1.9	25.1
40	50	7.8	1.4	3.1	8.3	2.0	2.1	23.4

[a] Percentage of the initial sucrose that is converted into oligosaccharides with polymerization degree ≤ 4.

FIGURE LEGENDS

Fig. 1.- HPLC analysis of the methyl α-D-glucooligosaccharides produced by dextransucrase from *L. mesenteroides* B-1299 using 0.5 M sucrose and 0.8 M methyl α-D-glucopyranoside. Experimental conditions: 0.16 U ml^{-1} dextransucrase entrapped in alginate, 20 mM sodium acetate buffer (pH 5.4), 0.05 g l^{-1} $CaCl_2$, 30°C, 64 h.

Fig. 2.- Representation of the structures of the oligosaccharides belonging to the homologous series *S1*, *S2* and *S3*.

Fig. 3.- Synthesis of methyl α-D-isomaltoside (P_2^{S1}) with variable concentrations of sucrose and methyl α-D-glucoside. Experimental conditions: 0.3 U ml^{-1} dextransucrase B-1299 encapsulated in alginate, 20 mM sodium acetate buffer (pH 5.4), 0.05 g l^{-1} $CaCl_2$, 30°C.

Fig. 1.- Gómez de Segura et al.

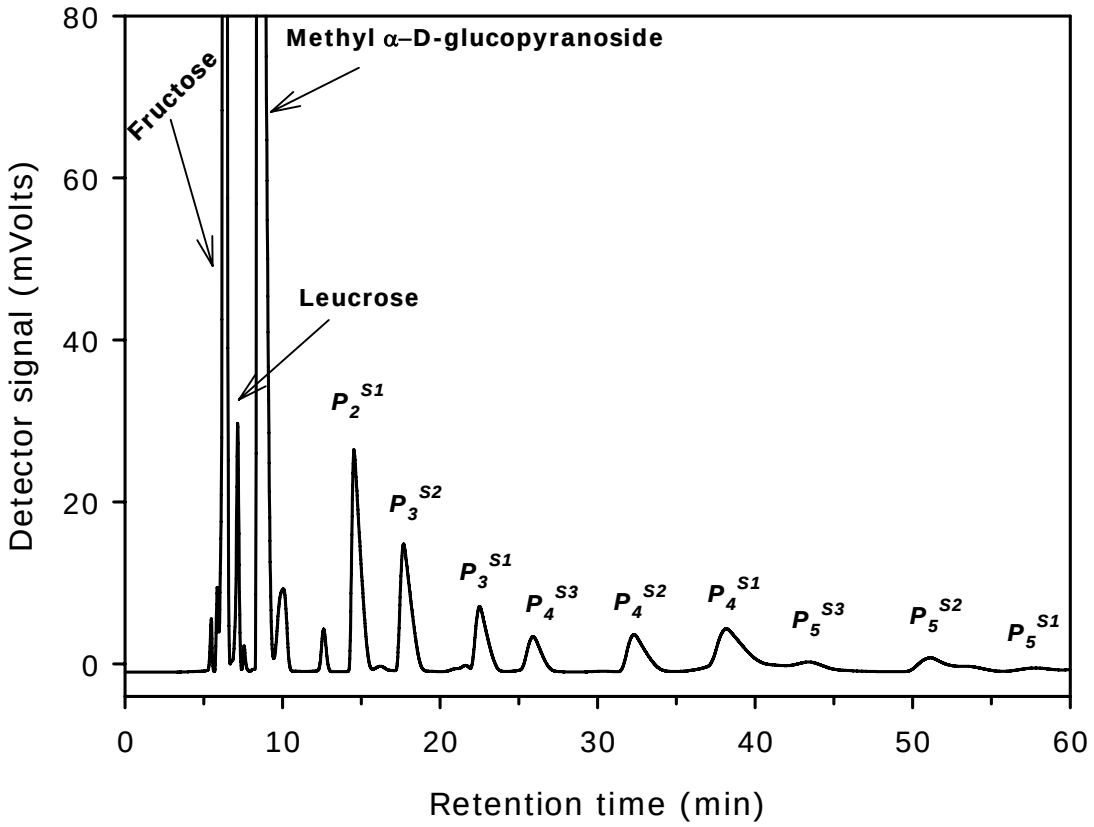

Fig. 2.- Gómez de Segura et al.

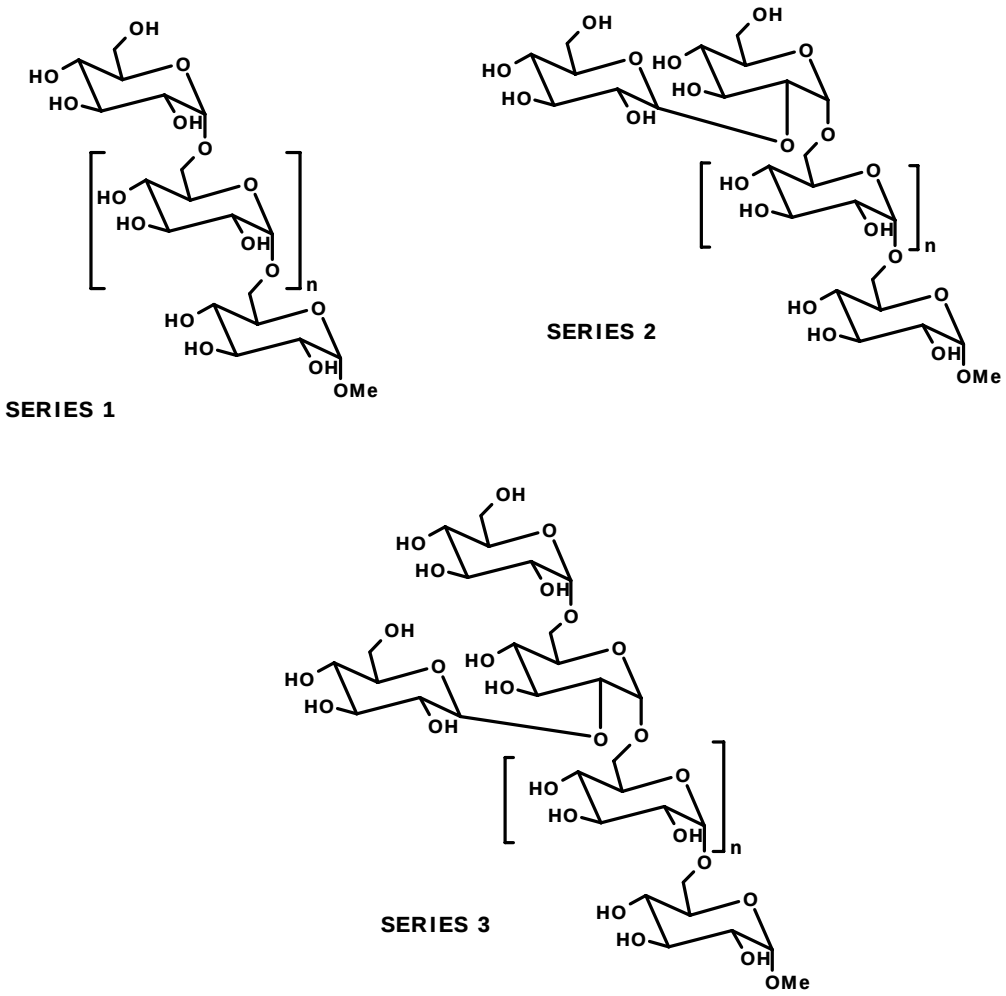

Fig. 3.- Gómez de Segura et al.