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Note

Ion-exchange chromatography of fluorogenic derivatives of maltooligosaccharides for preparation of α-amylase substrates

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Because human pancreatic α-amylase in serum increases with pancreatic lesions, and salivary α-amylase increases during mumps, the assay of α-amylase activity is very effective in clinical diagnoses. We have proposed a new α-amylase assay in which fluorogenic derivatives of maltooligosaccharides were used as the substrates, the products being separated from the digests and quantitated by high-performance liquid chromatography (HPLC) [1, 2]. The assay is unique in that it is not subjected to interference from endogenous glucose, maltose, or α-glucosidase in the sample. The fluorogenic substrates, O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose (FG5) and O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucitol (FG6R), can be synthesized via many steps from maltopentaose and maltohexaose, but this requires skilful technique and a long time. We have prepared them from dextrin using a relatively simple method including (1) partial introduction of pyridylamino groups into the glucose residues of dextrin, (2) digestion of the modified dextrin to fluorogenic maltooligosaccharides and glucose by α-amylase and glucoamylase, and (3) fractionation of the digest to each oligosaccharide by gel filtration and HPLC. Gel chromatography on Bio-Gel P-4 was not so effective for fractionating the fluorogenic maltooligosaccharides [1]. We tried to separate them by ion-exchange chromatography, because the pyridylamino group has a positive charge at acidic pH and because the hydrophilic properties of the oligomers must vary with the degree of polymerization. This

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paper reports the effective fractionation of pyridylamino derivatives of malto-oligosaccharides and their sugar alcohols by ion-exchange chromatography.

MATERIALS AND METHODS

Materials

Liquefying α-amylase from *Bacillus subtilis* (EC 3.2.1.1) and glucoamylase from *Rhizopus niveus* (EC 3.2.1.3) were purchased from Seikagaku Kogyo (Tokyo, Japan). Amylose EX-1 with an average of seventeen glucose residues was from Hayashibara Biochemical Labs. (Okayama, Japan). Bio-Gel P-4 was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Dowex 50W-X2 was from Dow Chem. (Midland, MI, U.S.A.). All chemicals were of the highest grade available.

Preparation of F-amylose

The partial introduction of pyridylamino groups into some of the C-6 positions of the glucose residues of amylose was carried out in a manner basically similar to that for dextrin [1]. A mixture of 0.4 ml of dichloroacetic acid and 4 ml of dimethylsulphoxide was added to a solution of 2 g of amylose and 3 g of N,N'-dicyclohexylcarbodiimide in 38 ml of dimethylsulphoxide. The mixture was stirred at room temperature for 50 min, and then 1.2 g of oxalic acid in 5 ml of methanol were added. To this reaction mixture, a mixture of 8.5 g of 2-aminopyridine, 3 ml of acetic acid, 3.2 g of sodium cyanoborohydride, and 12 ml of water was added, and the mixture was heated at 90°C for 30 min. Water (300 ml) was added to the reaction mixture, and the precipitate was removed by filtration. The pH of the filtrate was adjusted to 1.0 with 1 *M* hydrochloric acid to decompose excess sodium cyanoborohydride. After the pH was adjusted to 7.0 with 1 *M* sodium hydroxide, the solution was concentrated in vacuo. The residue was dissolved in water and applied to a Bio-Gel P-4 column (90 × 4.5 cm) equilibrated with 0.01 *M* ammonium bicarbonate. Elution was monitored by measuring the absorbance at 320 nm due to pyridylamino groups. The fist peak (F-amylose) was collected and lyophilized. The yield of F-amylose was 1.6 g, and 7.4% of the glucose residues were modified, judging from the absorbance due to pyridylamino groups.

Digestion of F-amylose by glucoamylase and α-amylase

F-Amylose (1.5 g) was dissolved in 160 ml of water and the pH was adjusted to 4.8 with 1 *M* hydrochloric acid. The solution was incubated with *Rhizopus niveus* glucoamylase (10 mg) at 40°C for 5 h. After the pH of the digest was adjusted to 6.0 with 1 *M* sodium hydroxide, 1.6 ml of 0.013% *Bacillus subtilis* liquefying α-amylase in 0.1 *M* calcium acetate buffer of pH 6.0 was added to the digest, and the mixture was incubated at 40°C for 1 h. The digestion was stopped by heating the reaction mixture at 100°C for 10 min. The pH of the digest was adjusted to 4.8 with 1 *M* hydrochloric acid, and then the digest was further incubated with 5 mg of glucoamylase at 40°C for 5 h.

Sodium borohydride reduction

To one-third of the above digest, 200 mg of sodium borohydride were added, and the mixture was kept at room temperature for 3 h. By adding 1 *M* hydrochloric acid to the solution, the remaining sodium borohydride was decomposed, and the pH of the solution was adjusted to 4.0 with 1 *M* sodium hydroxide.

Ion-exchange chromatography on Dowex 50W-X2

The sample was applied on a column of Dowex 50W-X2 (124 × 1.5 cm) equilibrated with 0.1 *M* pyridine—acetic acid buffer of pH 5.6. After washing with 350 ml of the same buffer, the column was developed by a linear gradient generated by mixing 1 l of this buffer with 1 l of 0.35 *M* pyridine—acetic acid buffer, also of pH 5.6. The elution was monitored by measuring absorbance at 310 nm due to pyridylamino group.

High-performance liquid chromatography

The HPLC apparatus used was a Waters Assoc. Model M-45. A column (150 × 4.6 mm) packed with Cosmosil $5C_{18}$ (Nakarai Chemicals, Kyoto, Japan) was used. The elution was carried out with 0.1 *M* ammonium acetate buffer of pH 4.0 containing 0.05% 1-butanol at a flow-rate of 1.8 ml/min. The detector was a Hitachi fluorescence spectrophotometer, Model 650-10M. The wavelength of excitation was 320 nm and that of emission was 400 nm. O-6-Deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose (FG4), FG5, and O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose (FG6) and their sugar alcohols (FG4R, FG5R, and FG6R), of which the structures had been confirmed [1, 2], were used as the standard compounds for HPLC analysis in this study.

RESULTS AND DISCUSSION

Pyridylamino derivatives of maltooligosaccharides were prepared from amylose as summarized in Fig. 1. Glucoamylase was used to liberate non-reducing-end glucose from F-amylose one by one, and to produce the limit-dextrins of which the non-reducing-end glucose residues are modified. The digest of F-amylose or its reduction product with sodium borohydride was applied on the Dowex 50W-X2 column and the elution was carried out as described under Materials and methods. The elution patterns are shown in Figs. 2 and 3. The peaks in the figures were identified by comparing their elution positions on HPLC with the standards as illustrated in Fig. 4A—D. Peaks X and Y seemed to be heptamers from their elution positions, but they are not, because they were eluted faster than the corresponding hexamers on HPLC (Fig. 4E and F). They may be products formed by a side-reaction on the introduction of pyridylamino group into amylose. It was estimated that a higher oligomer would be eluted faster than a lower one on ion-exchange chromatography from analogy with the high-mannose-type glycopeptides obtained from Taka-amylase A or ovalbumin [3, 4]. The fractionations of the fluorogenic

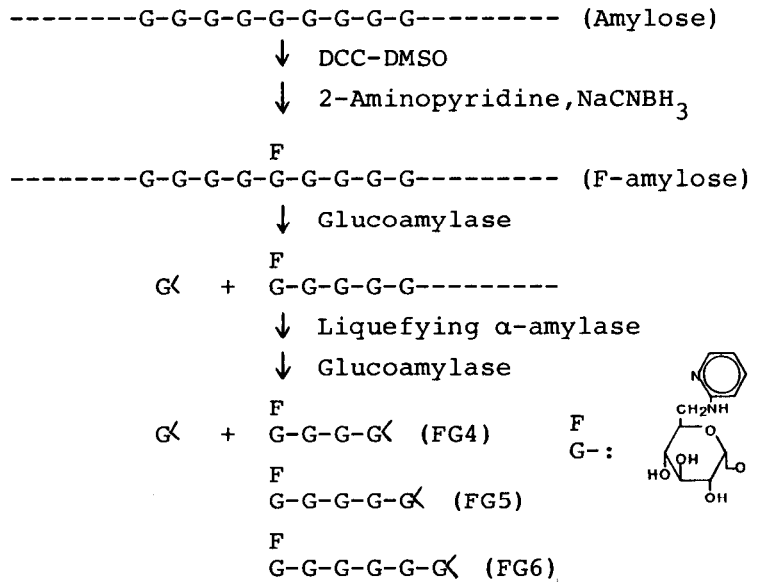

Fig. 1. Preparation of pyridylamino derivatives of maltooligosaccharides. G, Glucose residue; $^{F}_{G}$, 6-deoxy-6-[(2-pyridyl)amino]-D-glucose residue; G<, reducing-end glucose residue; —, α-1,4-glucosidic bond.

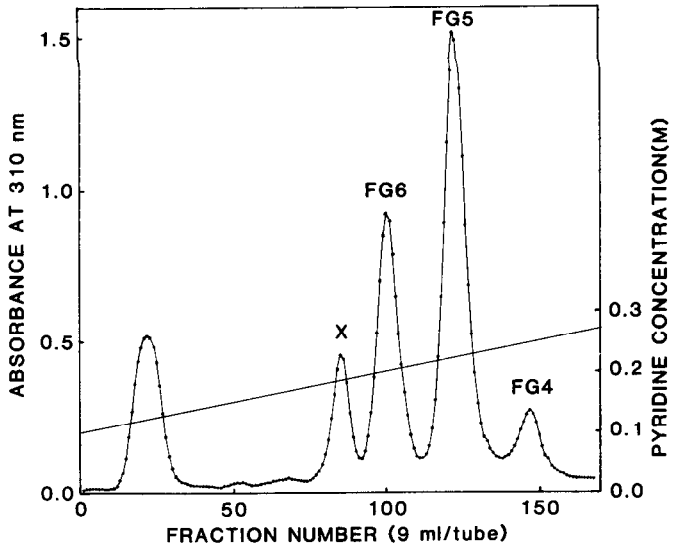

Fig. 2. Ion-exchange chromatography of the digest of F-amylose on Dowex 50W-X2. A digest of F-amylose (500 mg) was diluted with water to 100 ml, and then applied on the column. The elution was carried out as described in Materials and methods.

derivatives of the maltooligomers were satisfactorily performed, taking advantage of their ionic features. By this method, it will be easy to scale up the production of the substrates for α-amylase assay.

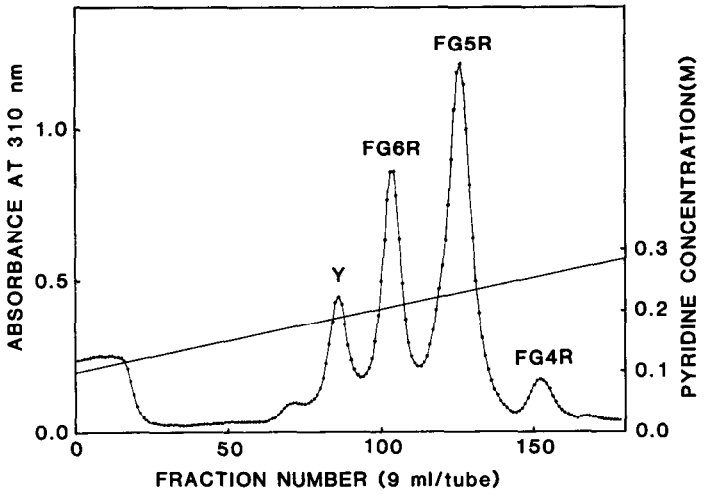

Fig. 3. Ion-exchange chromatography of the reduction product of the digest of F-amylose on Dowex 50W-X2. The reduction product of the digest of F-amylose (500 mg) was diluted with water to 250 ml, and then applied on the column. The elution was carried out as described in Materials and methods.

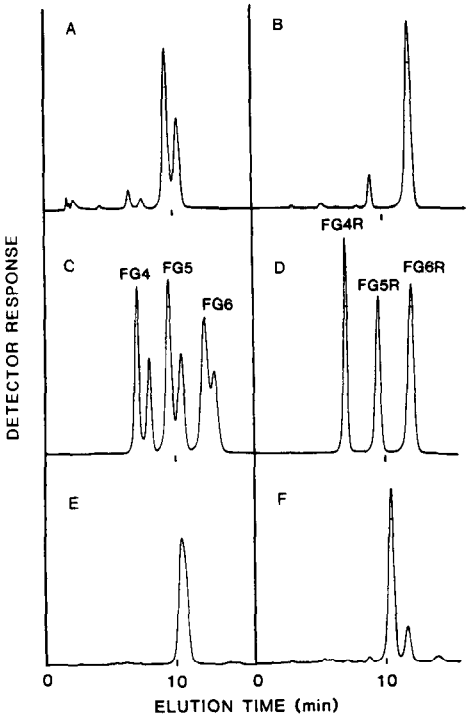

Fig. 4. HPLC analyses of the peaks isolated by ion-exchange chromatography. Analyses were carried out as described in Materials and methods. (A) FG5 in Fig. 2; (B) FG6R in Fig. 3; (C) standard mixtures (FG4, FG5, and FG6); (D) standard mixtures (FG4R, FG5R, and FG6R); (E) peak X in Fig. 2; (F) peak Y in Fig. 3. FG4, FG5, and FG6 were detected as the two peaks of their anomeric forms (α, β) on HPLC analysis as reported previously [1].

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