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An Assay Method for Glycogen Debranching Enzyme Using New Fluorogenic Substrates and Its Application to Detection of the Enzyme in Mouse Brain

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An assay method for glycogen debranching enzyme involving fluorogenic dextrins as substrates was developed. Two dextrins were prepared from 6-*O*-α-D-glucosyl-α-cyclodextrin and glucose by taking advantage of the action of *Bacillus macerans* cyclodextrin glucanotransferase, and converted by pyridylamination to fluorogenic derivatives. Structural analysis of the fluorogenic dextrins by FAB-MS, partial acid hydrolysis, and glucoamylase digestion revealed that they were Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glc-PA (FD6) and Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glc-PA (FD7). Using the glycogen debranching enzyme from rabbit muscle, FD6 and FD7 were, respectively, hydrolyzed to PA-maltopentaose and PA-maltohexaose, in addition to glucose, showing that these two fluorogenic dextrins are suitable substrates for assaying the glycogen debranching enzyme. An assay method involving the separation and quantification by HPLC of the characteristic fluorogenic products was successfully applied to determination of the distribution of the enzyme activity in mouse cerebrum.

Key words: assay methods, fluorogenic substrate, glycogen debranching enzyme, HPLC.

Glycogen debranching enzyme is known to be involved in the degradation of glycogen into glucose-1-phosphate and glucose in concert with phosphorylase (*1*). A deficiency of the enzyme causes Type III glycogen storage disease (*2*). Several glycogen debranching enzymes have been purified from mammalian tissues (*3-8*) and yeast (*9*), and the gene encoding the human muscle enzyme has been cloned and expressed in insect cells (*10, 11*). Glycogen debranching enzyme is unique in that it is multi-catalytic, with two distinct active sites on a single polypeptide chain (*12-14*) exhibiting maltooligosaccharide transferase (1,4-α-glucan: 1,4-α-glucan 4-α-glycosyltransferase, EC 2.4.1.25) and amylo-1,6-glucosidase (dextrin 6-α-glucosidase, EC 3.2.1.33) activities.

The enzyme is usually assayed by measuring either the activity needed to release glucose from 6-*O*-α-D-glucosyl-α-cyclodextrin (*15, 16*), glycogen phosphorylase limit dextrin (*16, 17*), or 6^3-α-glucosylmaltotetraose (*16*), or that needed to incorporate ^{14}C-glucose into glycogen (*18, 19*). 6-*O*-α-D-Glucosyl-α-cyclodextrin is the most specific of the substrates, but its susceptibility is lower than those of glycogen phosphorylase limit dextrin and 6^3-α-glucosylmaltotetraose. These more susceptible substrates are, however, also hydrolyzed to produce glucose by other enzymes such as α-amylase [EC 3.2.1.1] and α-glucosidase [EC 3.2.1.20]. Thus, if an enzyme preparation contains one or more of these enzymes, measurement of the liberated glucose using the more susceptible substrates will result in the activity of the debranching enzyme being overestimated. With regard to the other assay method, *i.e.* the measurement of ^{14}C-glucose incorporated into glycogen, the debranching enzyme activity is also overestimated because radioactive glycogen of smaller molecular weight is formed from the mother glycogen through the transglycosylation action of endo-hydrolytic enzymes. To solve the problem of activity overestimation when assaying glycogen debranching enzyme, a dextrin with a definite structure should be used as the substrate, and a product specifically characteristic of the debranching enzyme should be measured. HPLC is suitable for product analysis of an enzymatic reaction mixture. If a substrate is tagged with fluorescence, a specific fluorogenic product can be determined very sensitively. We report here on the preparation of fluorogenic substrates with definite structures for the glycogen debranching enzyme and a sensitive assay method involving HPLC.

MATERIALS AND METHODS

Materials—6-*O*-α-D-Glucosyl-α-cyclodextrin and maltoheptaose were purchased from Wako Pure Chemicals (Osaka). Pyridylaminated maltoheptaose (PA-maltoheptaose) was prepared by pyridylamination of maltoheptaose as reported previously (*20*). PA-glucose, PA-maltose, PA-maltotriose, PA-maltotetraose, PA-maltopentaose, and PA-maltohexaose were obtained by partial acid hydrolysis of PA-maltoheptaose.

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Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; FD6, Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glc-PA; FD7, Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glc-PA; Glc, D-glucose; PA-, pyridylamino-; X, Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glc-PA.

Bacillus macerans cyclodextrin glucanotransferase was purified from a crude culture filtrate (Amano Pharmaceuticals, Nagoya) (*21*). One unit of the enzyme was defined as the amount of the enzyme that produced 1 μmol of FG4P from 2 mM FG5P and 15 mM *p*-nitrophenyl α-glucoside per minute at 37°C and pH 6.9 (*21*). Glucoamylase from *Rhizopus delemar* (38 units/mg) and phosphorylase *b* (containing glycogen debranching enzyme) from rabbit muscle were from Toyobo (Osaka) and Sigma, respectively.

Toyopearl HW 40F was obtained from Tosoh (Tokyo), Wakosil Z-ODS-10 and Wakosil-II 5C18 HG columns (2× 25 cm and 4.6×250 mm, respectively) from Wako Pure Chemicals, and a Cosmosil 5C18P column (1×25 cm) from Nacalai Tesque (Kyoto).

Preparation of Fluorogenic Dextrins—A mixture of 6-*O*-α-D-glucosyl-α-cyclodextrin (250 mg) and glucose (590 mg) in 38 ml of 50 mM sodium acetate buffer, pH 6.9, containing 3 mM calcium chloride was incubated with 2 ml cyclodextrin glucanotransferase (9.4 units/ml) at 37°C for 2.5 h. After inactivation of the enzyme by heating at 100°C for 10 min, the solution was subjected to ultra-filtration through an Amicon Diaflo membrane YC 05 (cut-off molecular weight, 500) to remove glucose. Pyridylamination (*20*) of the reaction mixture was carried out as follows. The lyophilized sample was dissolved in a mixture of 3 g 2-aminopyridine and 1.1 ml acetic acid, and then the resulting solution was heated at 90°C for 60 min. To the mixture, 13.2 ml of a reducing reagent (prepared by dissolving 10 g borane-dimethylamine complex in 4 ml acetic acid and 2.5 ml water) was added, and then reduction was carried out at 80°C for 50 min. One hundred milliliters of 3 M ammonium hydroxide was added to the reaction mixture, and excess reagents were extracted five times with chloroform (100 ml each time). The pH of the water phase was adjusted with acetic acid to 6.0 and then the solution was concentrated to a small volume under reduced pressure to remove residual chloroform. After adding 100 ml water to the solution, desalting was carried out by reversed-phase HPLC as follows. A quarter of the solution was applied to a Wakosil Z-ODS-10 column equilibrated with water. After washing of the column with 300 ml water, pyridylaminated dextrins were eluted with a 0.1% acetic acid-2% 1-butanol solution at the flow rate of 10 ml/min. The elution was monitored by measuring the absorbance at 320 nm. The fraction eluted with the 0.1% acetic acid-2% 1-butanol solution was concentrated to dryness under reduced pressure. The residue was dissolved in a small volume of water, and then chromatographed on a Toyopearl HW 40F column (1.8×195 cm) equilibrated with 50 mM ammonium acetate buffer, pH 6.0. The two major peaks obtained were further purified by HPLC on a Cosmosil 5C18P column as peak A (FD7) and peak B (FD6), respectively.

HPLC—For the purification of FD6 and FD7, a Cosmosil 5C18P column was used. The eluent was 50 mM ammonium acetate buffer, pH 4.5, containing 0.1% 1-butanol, at the flow rate of 3.5 ml/min. The elution was monitored by measuring the fluorescence (the excitation and emission wavelengths were 320 and 400 nm, respectively). For analytical purposes, a Wakosil-II 5C18 HG column was used. The eluent was 50 mM ammonium acetate buffer, pH 4.5, containing 0.05% 1-butanol, at the flow rate of 1 ml/min. The elution was monitored by measuring the fluorescence as described above.

Fast Atom Bombardment Mass Spectrometry (FAB-MS)—The molecular mass was measured by FAB-MS using a JEOL JMS-HX100 mass spectrometer equipped with an FAB ion source and a DA-5000 data processor. A sample (1 μg) mixed with glycerol (0.5 μl) was bombarded with a neutral atom beam accelerated at a potential of 10 keV.

Partial Acid Hydrolysis of Fluorogenic Dextrins—FD7 (32 nmol) or FD6 (32 nmol) was hydrolyzed with 200 μl of 0.5 M hydrochloric acid at 100°C for 9 min. After neutralization with 0.5 M sodium hydroxide, the hydrolysate was analyzed by HPLC on a Wakosil-II 5C18 HG column.

Glucoamylase Digestion of Fluorogenic Dextrins—FD6 (32 nmol) or FD7 (32 nmol) was incubated with 0.3 or 0.07 units of glucoamylase at 37°C in 215 μl of 50 mM ammonium acetate buffer, pH 4.8, for 15 min. To stop the enzymatic reaction, 200 μl of 1 M acetic acid was added to the reaction mixture and then the solution was heated at 100°C for 5 min. The digest was analyzed by HPLC on a Wakosil-II 5C18 HG column.

Glycogen Debranching Enzyme Assay—A rabbit muscle phosphorylase *b* preparation (5 mg) containing glycogen debranching enzyme was dissolved in 0.5 ml of 50 mM sodium maleate buffer, pH 6.0, containing 0.05% gelatin, 5 mM EDTA, and 10 mM β-mercaptoethanol, and the resulting solution was used as 1% glycogen debranching enzyme. A reaction mixture (200 μl) comprising 0.15 mM FD7 (or 0.55 mM FD6), 50 mM sodium maleate buffer consisting of 0.05% gelatin, 5 mM EDTA, and 10 mM β-mercaptoethanol, pH 6.0, and 15 μl of the enzyme solution was incubated at 37°C for an appropriate period. The enzymatic reaction was terminated by adding 135 μl of 1 M acetic acid, followed by heating at 100°C for 5 min. The digest was analyzed by HPLC on a Wakosil-II 5C18 HG column. One unit of the glycogen debranching enzyme was defined as the amount of the enzyme that produced 1 nmol of PA-maltopentaose or PA-maltohexaose from FD6 or FD7 per minute under the conditions used.

Cell Fractionation of Mouse Cerebrum—ICR mouse cerebrum (1.0 g) was homogenized in 9 ml of 5 mM Tris-HCl buffer, pH 7.3, containing 0.32 M sucrose with a Potter-Elvehjem-type Teflon homogenizer. The homogenate was centrifuged at 900×*g* for 10 min. The resulting precipitate was suspended in the same buffer to make a 6 ml solution (P1 fraction), and then the supernatant was centrifuged at 7,000×*g* for 10 min. The resulting precipitate was suspended in the same buffer to make a 6 ml solution (P2 fraction). The supernatant was centrifuged at 105,000×*g* for 1 h. The resulting precipitate was suspended in the same buffer to make a 6 ml solution (P3 fraction), and the supernatant (6 ml) was used as the cytosolic fraction.

RESULTS AND DISCUSSION

Preparation of Fluorogenic Dextrins—Two branched dextrins were prepared through the action of *Bacillus macerans* cyclodextrin glucanotransferase on 6-*O*-α-D-glucosyl-α-cyclodextrin and glucose. The products were converted to fluorogenic compounds by pyridylamination of their reducing-end residues. Two fractions, F1 and F2, were isolated on gel-filtration (Fig. 1), and then further

purified by HPLC as peaks A and B (Fig. 2), with respective yields of 24 and 23 mg.

Structural Assignment of Fluorogenic Dextrins—The molecular weight of peak B was 1,068.4, as determined by FAB-MS (1,068.4 is the calculated molecular weight for $C_{41}H_{68}O_{30}N_2$), indicating that it was a pyridylaminated glucose hexamer. The partial acid hydrolysate of peak B contained PA-glucose, PA-maltose, PA-maltotriose, PA-maltotetraose, PA-maltopentaose, an unknown product, X, and the remaining peak B; no PA-maltohexaose was detected (Fig. 3B). Considering that peak B was derived from 6-*O*-α-D-glucosyl-α-cyclodextrin, it was thought to be a PA-6-α-glucosylmaltopentaose. In the partial hydrolysate of PA-maltoheptaose, the amounts of the PA-maltooligosaccharides decreased gradually from PA-maltose to PA-maltohexaose (Fig. 3A). In contrast, there was a marked difference between the amounts of PA-maltotriose and PA-maltotetraose produced in the partial acid hydrolysate of peak B (Fig. 3B). This suggests that the branching occurred at the fourth glucose residue of PA-maltopentaose from the reducing-end, because PA-maltotetraose is formed through successive cleavage of the α-1,4- and α-1,6-glucosidic linkages, while PA-glucose, PA-maltose, and PA-maltotriose are formed through only one cleavage of the corresponding α-1,4-glucosidic linkage. Peak B was, therefore, Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glc-PA (FD6). The unknown product, X, was thought to be Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glc-PA, formed through the cleavage of the α-1,4-glucosidic linkage of the non-reducing-end glucose. The structure of peak B was further confirmed by glucoamylase digestion. Glucoamylase hydrolyzes not only α-1,4-glucosidic linkages, but also α-1,6-glucosidic linkages in starch and glycogen, although the rate of hydrolysis of α-1,4-glucosidic linkages is faster than that of α-1,6-glucosidic linkages (*22*). In the course of digestion, the α-1,4-linked glucose residues in a PA-6-α-glucosylmaltooligosaccharide should be liberated preferentially and successively from the non-reducing-end up to the branching point, resulting in the accumulation of a PA-branched oligosaccharide whose non-reducing-end structure is Glcα1-6Glcα1-4-. A large amount of X was detected in the glucoamylase digest of peak B (Fig. 3D), which was collected and partially hydrolyzed. The hydrolysate contained PA-glucose, PA-maltose, PA-maltotriose, PA-maltotetraose, and remaining X, indicating that X was Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glc-PA (Fig. 3F). Thus, in FD6, branch-

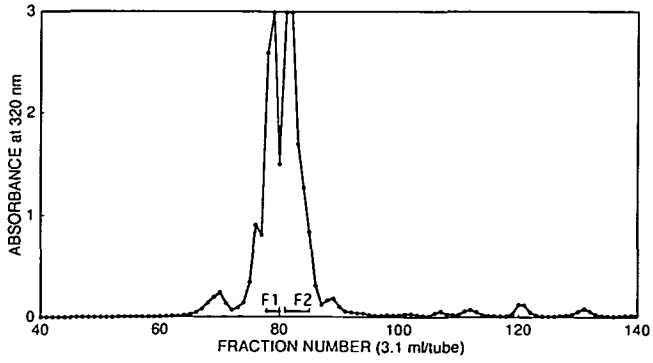

Fig. 1. **Gel-filtration of a pyridylaminated dextrin reaction mixture.** A mixture of pyridylaminated dextrins prepared with cyclodextrin glucanotransferase was applied onto a Toyopearl HW 40F column equilibrated with 50 mM ammonium acetate buffer, pH 6.0. Two fractions, F1 and F2 (indicated by bars), were collected.

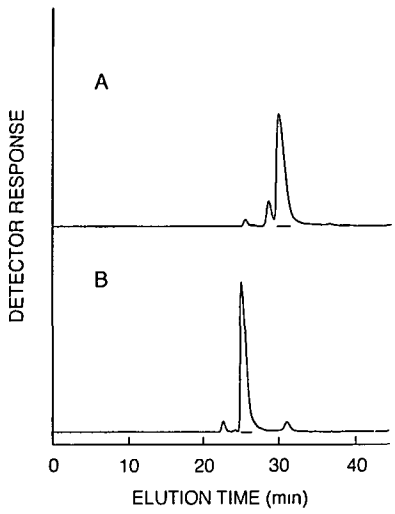

Fig. 2. **Purification of fluorogenic dextrins by HPLC.** Fractions F1 and F2 obtained on gel-filtration (Fig. 1) were separately purified by HPLC as described under "MATERIALS AND METHODS." A, HPLC of F1; B, HPLC of F2. The fractions indicated by bars were used as peak A (from F1) and peak B (from F2).

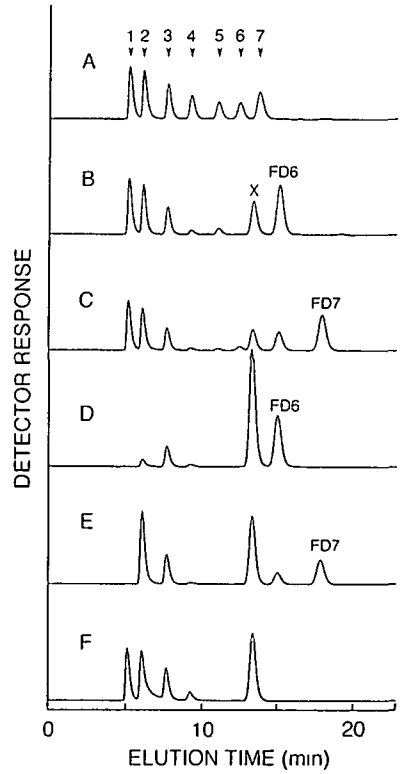

Fig. 3. **HPLC of partial acid hydrolysates and glucoamylase digests of fluorogenic dextrins.** A, partial acid hydrolysate of PA-maltoheptaose; B, partial acid hydrolysate of FD6; C, partial acid hydrolysate of FD7; D, glucoamylase digest of FD6; E, glucoamylase digest of FD7; F, partial acid hydrolysate of X. The arrowheads indicate the following elution positions: 1, PA-glucose; 2, PA-maltose; 3, PA-maltotriose; 4, PA-maltotetraose; 5, PA-maltopentaose; 6, PA-maltohexaose; 7, PA-maltoheptaose.

ing occurred at the second glucose residue of PA-maltopentaose from the non-reducing end.

The molecular weight of peak A was 1,230.7 (1,230.4 is the calculated molecular weight for $C_{47}H_{78}O_{35}N_2$). In a similar manner to that described for peak B, partial acid hydrolysis (Fig. 3C) and glucoamylase digestion (Fig. 3E) led to the conclusion that peak A was Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glc-PA (FD7).

It is likely that a transglycosylation reaction took place at least two times, FD6 and FD7 being formed from 6-*O*-α-D-glucosyl-α-cyclodextrin and glucose. The first transglycosylation reaction would be the ring-opening of 6-*O*-α-D-glucosyl-α-cyclodextrin, resulting in the formation of 6-α-glucosylmaltoheptaose(s); the product(s) would then be changed through a second transglycosylation to the dextrins from which FD6 and FD7 were derived.

Glycogen Debranching Enzyme Assay Involving Fluorogenic Dextrins—Fluorogenic dextrins FD6 and FD7 have one α-1,6-linked glucose residue and, as depicted in Fig. 3, all of their fluorogenic hydrolytic products can be separated and quantified by HPLC, indicating that they are suitable as substrates for a glycogen debranching enzyme assay involving HPLC. The feasibility of the proposed assay method was examined using a rabbit muscle phosphorylase *b* preparation containing glycogen debranching enzyme. As shown in Fig. 4, the enzyme acted on FD6 and FD7 to give PA-maltopentaose and PA-maltohexaose, respectively. However, FD7 was much more susceptible to the enzyme than FD6. The time courses of the reactions are shown in Fig. 5, from which it can be seen that both PA-maltopentaose and PA-maltohexaose increased linearly with time. The relation between the enzyme concentration and the amount of product produced was also linear with each substrate under the conditions used (Fig. 6).

Although FD7 is a more susceptible substrate than FD6 with respect to glycogen debranching enzyme, its susceptibility to endo-hydrolytic enzyme(s) will also be greater than that of FD6. Hence, if an enzyme preparation to be assayed contains a large amount of endo-hydrolytic enzyme(s), FD6 would be the preferred substrate.

Application of the Assay Method to Detection of Glycogen Debranching Enzyme Activity in Mouse Cerebrum—Amylo-1,6-glucosidase activity has been reported in a homogenate of rat brain (*23*), and activity associated with the incorporation of glucose into glycogen has also been detected in the 16,000×*g* supernatant of a rat brain homogenate (*24*), although in both cases the activity was very low. We therefore decided to test the effectiveness of our glycogen debranching enzyme assay method by employing it to examine the intracellular distribution of the activity of the enzyme in mouse cerebrum. FD7 was incubated with each subcellular fraction (fractions P1, P2, and P3, and the cytosolic fraction) for 1 h, and the digests

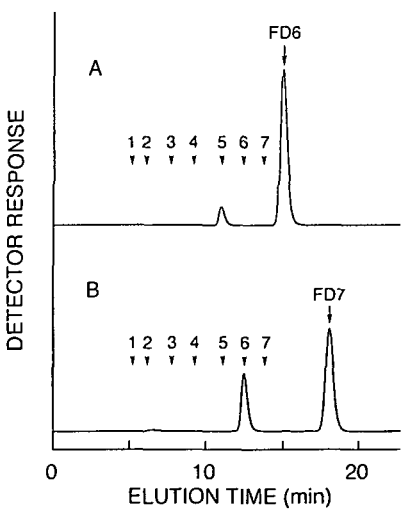

Fig. 4. **HPLC of FD6 and FD7 digests with glycogen debranching enzyme.** The fluorogenic dextrins, FD6 (0.15 mM) and FD7 (0.15 mM), were separately digested with 0.07% glycogen debranching enzyme for 3 and 1 h, respectively, and then each digest was analyzed by HPLC as described under "MATERIALS AND METHODS." A, digest of FD6; B, digest of FD7. The arrowheads indicate the following elution positions: 1, PA-glucose; 2, PA-maltose; 3, PA-maltotriose; 4, PA-maltotetraose; 5, PA-maltopentaose; 6, PA-maltohexaose; 7, PA-maltoheptaose.

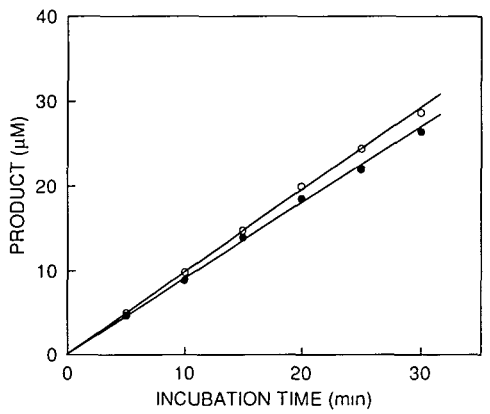

Fig. 5. **Time courses of hydrolysis of FD6 and FD7 by glycogen debranching enzyme.** The fluorogenic dextrins, FD6 and FD7, were digested and then the amounts of the products were determined as described in the text. ●, PA-maltopentaose in the digest of FD6. The concentrations of the enzyme and FD6 were 0.23% and 0.55 mM, respectively. ○, PA-maltohexaose in the digest of FD7. The concentrations of the enzyme and FD7 were 0.07% and 0.15 mM, respectively.

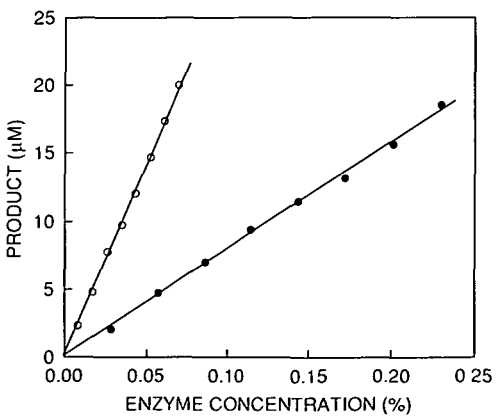

Fig. 6. **Relation between the enzyme concentration and the product amount.** The fluorogenic dextrins were digested with various concentrations of rabbit muscle glycogen debranching enzyme for 20 min. ●, PA-maltopentaose in the digest of FD6 (0.55 mM); ○, PA-maltohexaose in the digest of FD7 (0.15 mM).

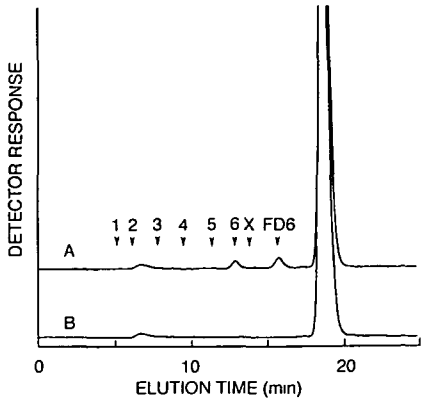

Fig. 7. **HPLC of a FD7 digest with the cytosolic fraction from mouse cerebrum.** FD7 was incubated with the cytosolic fraction obtained from mouse cerebrum for 1 h, and the digest was analyzed by HPLC as described under "MATERIALS AND METHODS." A, digest with the cytosolic fraction; B, FD7 only. The arrowheads indicate the following elution positions: 1, PA-glucose; 2, PA-maltose; 3, PA-maltotriose; 4, PA-maltotetraose; 5, PA-maltopentaose; 6, PA-maltohexaose; 7, PA-maltoheptaose.

were analyzed by HPLC. As a typical result, a chromatogram of the digest with the cytosolic fraction is shown in Fig. 7. PA-maltohexaose and FD6 were detected in all the fractions, indicating the presence of glycogen debranching enzyme and α-glucosidase, respectively. The activities of glycogen debranching enzyme in fractions P1, P2, and P3, and the cytosolic fraction were 0.26, 0.24, 0.09, and 0.39 unit/ml, respectively. Glycogen debranching enzyme thus appears to be involved in the degradation of glycogen with glycogen phosphorylase in mouse cerebrum.

Concomitant α-glucosidase hydrolyzing FD7 did not interfere with the measurement of glycogen debranching enzyme activity as the products, PA-maltohexaose and FD6, could be well separated by HPLC. These results indicate that the proposed assay method is practicable, even with crude enzyme preparations. In addition, quantification of the fluorogenic product at the picomole level demonstrates that this enzyme assay is highly sensitive.

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