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Sensitive Assay of Glycogen Phosphorylase Activity by Analysing the Chain-Lengthening Action on a Fluologenic Maltooligosaccharide Derivative

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The action of glycogen phosphorylase (GP) is essentially reversible, although GP is generally classified as a glycogen-degrading enzyme. In this study, we developed a highly sensitive and convenient assay for GP activity by analysing its chain-lengthening action on a fluorogenic maltooligosaccharide derivative in a glucose-1-phosphate-rich medium. Characterization of the substrate specificity of GP using pyridylaminated (PA-) maltooligosaccharides of various sizes revealed that a maltotetraosyl (Glc_4) residue comprising the non-reducing-end of a PA-maltooligosaccharide is indispensable for the chain-lengthening action of GP, and PA-maltohexaose is the most suitable substrate for the purpose of this study. By using a high-performance liquid chromatograph equipped with a fluorescence spectrophotometer, PA-maltoheptaose produced by the chain elongation of PA-maltohexaose could be isolated and quantified at 10 fmol. This method was used to measure the GP activities of crude and purified GP preparations, and was demonstrated to have about 1,000 times greater sensitivity than the spectrophotometric orthophosphate assay.

Key words: chain-lengthening action, fluorogenic substrate, glycogen phosphorylase, high-performance liquid chromatography, sensitive assay.

Abbreviations: BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Glc, D-glucose; Glc-1-P, glucose-1-phosphate; GlcPA, 1-deoxy-1-[(2-pyridylamino)-D-glucitol]; GP, glycogen phosphorylase; HPLC, high-performance liquid chromatography; PA, pyridylamino; P_i, inorganic phosphate.

Glycogen, the main carbohydrate storage component in animals, is a branched polysaccharide of D-glucose (Glc) (*1*). Two types of glycosidic linkages exist in the polysaccharide: the α-1,4-linkage, which accounts for 90–95% of all glycosidic linkages, and the α-1,6-branched linkage, which accounts for the remaining 5–10%. When the body requires an energy supply, glycogen is rapidly broken down by the combined activities of glycogen phosphorylase (GP, EC 2.4.1.1) and glycogen debranching enzyme (EC 2.4.1.25 and EC 3.2.1.33) (*1–9*). GP, which catalyses the rate-limiting step of glycogen degradation, removes the α-1,4-glucosyl residues one by one from the outermost chains of the glycogen molecules using inorganic phosphate (P_i). This reaction is called 'phosphorolysis', and results in the release of glucose-1-phosphate (Glc-1-P). The action of GP is essentially reversible, though GP activity in the direction of glycogen synthesis is inhibited in a P_i-rich environment such as an animal body (*10*, *11*).

$$\mathrm{Glc}\alpha 1-4\mathrm{Glc}_{n-1}+\mathrm{P_i}\overset{\mathrm{GP}}{\rightleftarrows}\mathrm{Glc}_{n-1}+\mathrm{Glc\text{-}1\text{-}P}$$

GP exists in two interconvertible forms, GPa (the phosphorylated form, high activity) and GPb (the non-phosphorylated form, low activity). The activity of GP is regulated by the inter-conversion of GPa and GPb, and by allosteric binding of a number of molecules, including AMP, ATP, glucose and glucose-6-phosphate.

Although GP is a key regulatory enzyme in glycogen metabolism, studies on GP have been hampered by the lack of a sensitive and convenient assay for GP activity. Coupled enzyme methods involving phosphoglucomutase and glucose-6-phosphate dehydrogenase (*11–14*), and radioactive methods using ^{3}H-labelled glycogen (*15*) or $^{32}P_i$ (*16*), have been attempted for measuring Glc-1-P formed in the direction of glycogen degradation. However, it is difficult to quantify sub-nanomoles of Glc-1-P in the enzymatic reaction mixture.

There has been significant effort to develop an assay for GP activity in the direction of glycogen synthesis. Most assay methods utilizing the chain-lengthening action of GP measure either the incorporation of ^{14}C-labelled Glc residues from ^{14}C-labelled Glc-1-P into glycogen (*17–20*), or the liberation of P_i from Glc-1-P (*21–25*). A spectrophotometric P_i assay has been developed as an alternative to these inconvenient radioisotopic assays. However, although this spectroscopic assay is one of the most sensitive methods reported to date, it cannot detect P_i below 10 pmol in the enzymatic reaction mixture (*25*). Furthermore, it is too cumbersome and time consuming to remove endogenous P_i from each GP preparation prior to activity measurements.

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Most previous GP activity assay methods utilize glycogen as the substrate (*11–25*). However, glycogen is unsuitable as a substrate for precise and sensitive enzyme assays because glycogen molecules do not have a definite structure. In this study, we developed a highly sensitive and convenient method for assaying GP activity by analysing the chain-lengthening action on pyridylaminated (PA-) maltohexaose (Glc_5-GlcPA, where GlcPA =1-deoxy-1-[(2-pyridylamino)-D-glucitol] residue) in a Glc-1-P-rich medium. Pyridylamination had been introduced to obtain stable fluorescent derivatives of oligosaccharides by Hase *et al*. in 1978 (*26*). Molar fluorescent intensities of PA-oligosaccharides are uniform and stable enough for enzyme assays (*27*, *28*). The PA-maltoheptaose (Glc_6-GlcPA) produced was isolated and quantified using a high-performance liquid chromatograph equipped with a fluorescence spectrophotometer.

MATERIALS AND METHODS

Materials—Maltose, α-cyclodextrin, 2-aminopyridine and a Wakosil-II 5C18 HG column (10.0 × 250 mm) were purchased from Wako Pure Chemicals (Osaka, Japan); glucose-1-phosphate disodium salt, rabbit muscle glycogen phosphorylase a (GPa) and b (GPb) were from Sigma (St. Louis, MO, USA). Cyclodextrin glucanotransferase from *Bacillus macerans* was kindly donated by Amano Enzyme (Nagoya, Aichi, Japan).

Preparation of PA-Maltooligosaccharides—PA-maltose was prepared by pyridylamination of maltose as reported previously (*29*). PA-maltooligosaccharides (Glc_{n-1}-GlcPA, where n = degree of polymerization) with a degree of polymerization ranging from three to eight were prepared from α-cyclodextrin and PA-maltose using cyclodextrin glucanotransferase, and were isolated by reversed-phase high-performance liquid chromatography (HPLC) (*30*). Solutions of PA-maltooligosaccharides should be stored at –15°C or below and those of standard PA-maltooligosaccharides for quantitative purposes should be used within 2 years (*27*).

Standard Conditions for the Assay of GP Activity—GP activity was measured in the direction of glycogen synthesis using PA-maltohexaose (Glc_5-GlcPA) and Glc-1-P as the substrates. A mixture (50 μl) containing 100 mM BisTris–HCl buffer (pH 6.8), 40 mM Glc-1-P, 1.6 μM PA-maltohexaose, 1.0 mM β-mercaptoethanol and the enzyme preparation was incubated at 37°C for an appropriate period. To stop the reaction, 50 μl of 500-mM ammonium acetate solution (pH 3.8) was added, and the mixture was heated at 100°C for 5 min. The sample was stored at –15°C or below until HPLC analysis. Then, the chain-lengthened product (PA-maltoheptaose, Glc_6-GlcPA) was isolated and quantified by reversed-phase HPLC on a Wakosil-II 5C18 HG column. The chromatograph used was a Hitachi liquid chromatograph Model L-2130, and the column was eluted with 100 mM ammonium acetate buffer, pH 4.8, at a flow rate of 3.0 ml/min. Elution was monitored by measuring the fluorescence of the 2-pyridylamino residue with a Hitachi fluorescence spectrophotometer Model L-2485. The wavelengths for excitation and emission were 320 and 400 nm, respectively. One unit of GP was defined as the amount of enzyme that produces 1 pmol of PA-maltoheptaose per minute under the conditions employed.

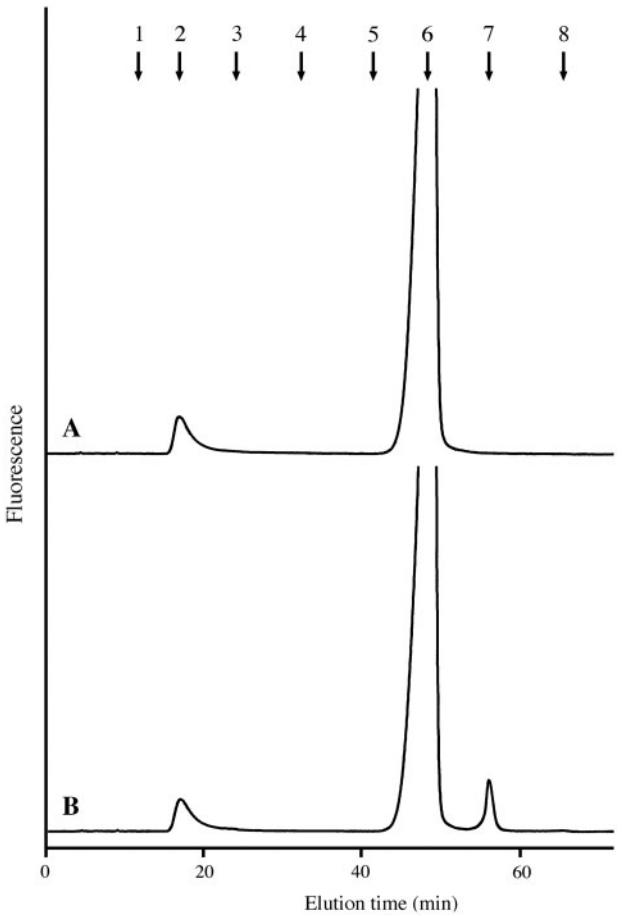

Fig. 1. **HPLC analysis of the chain-lengthening action of rabbit muscle GPb on PA-maltohexaose.** A mixture of PA-maltohexaose (Glc_5-GlcPA) and Glc-1-P was incubated with rabbit muscle GPb, then the enzymatic reaction mixture was analysed by reversed-phase HPLC. (A) Chromatogram of the enzymatic reaction mixture at zero time. (B) Chromatogram of the 80-min incubation mixture. Arrows 1–8 indicate the elution positions of PA-glucose–PA-maltooctaose (GlcPA–Glc_7-GlcPA). The broad peaks appearing around 17 min are due to contaminants.

Preparation of Homogenates of Porcine Liver and Muscle—Porcine liver (1.0 g) was homogenized in 9.0 ml of 100 mM BisTris–HCl buffer, pH 6.8, containing 1.0 mM β-mercaptoethanol using a Potter-Elvehjem homogenizer. After centrifuging the homogenate at 10,000 × g for 25 min, the supernatant was filtered through a Hyflo Super-Cel, and the filtrate was used as the crude GP preparation. Homogenate of porcine skeletal muscle was prepared in the same manner.

RESULTS AND DISCUSSION

Chain-Lengthening Action of Rabbit Muscle GP on PA-Maltohexaose—The chain-lengthening action of rabbit muscle GPb was first examined using PA-maltohexaose (Glc_5-GlcPA). The reaction mixture

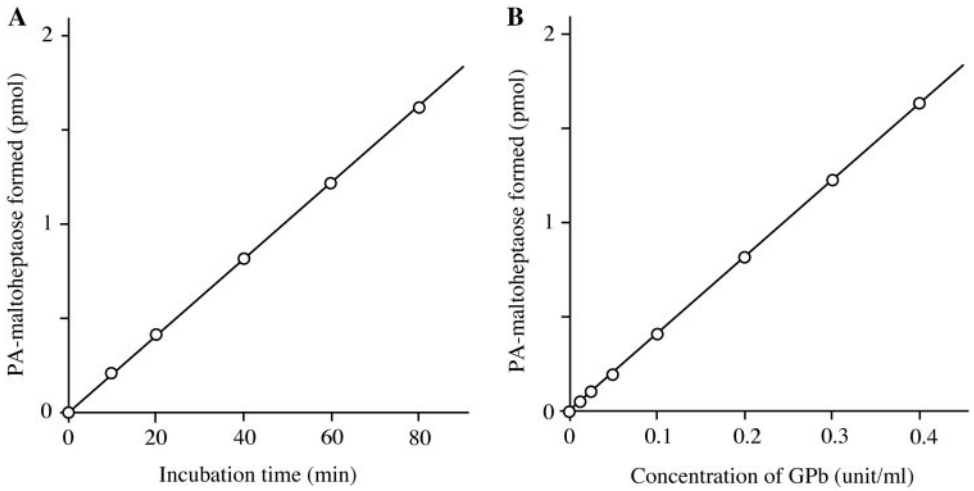

Fig. 2. **Chain-lengthening action of rabbit muscle GPb on PA-maltohexaose.** A mixture of PA-maltohexaose (Glc_5-GlcPA) and Glc-1-P was incubated with rabbit muscle GPb, then the enzymatic reaction mixture was analysed by reversed-phase HPLC. (A) Time course of the formation of PA-maltoheptaose (Glc_6-GlcPA) by the chain-lengthening action of rabbit muscle GPb. (B) Relationships between the enzyme concentration and the amount of PA-maltoheptaose formed by the chain elongation of PA-maltohexaose.

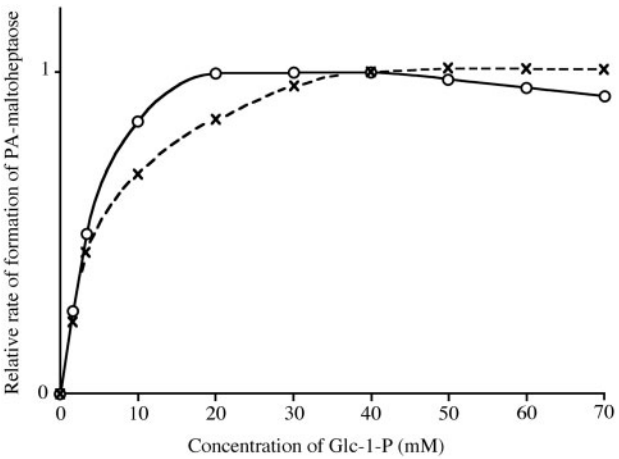

Fig. 3. **Relationship between Glc-1-P concentration and the amount of PA-maltoheptaose formed by the chain-lengthening action of rabbit muscle GP.** Chain-lengthening actions of rabbit muscle GPb (O) and GPa (X) on PA-maltohexaose (Glc_5-GlcPA) were examined at various concentrations of Glc-1-P. PA-maltoheptaose (Glc_6-GlcPA) formed was isolated and quantified by reversed-phase HPLC. In each GPb and GPa series, the rate of formation of PA-maltoheptaose at 40 mM Glc-1-P was taken as unity.

was analysed by reversed-phase HPLC, as shown in Fig. 1. This result demonstrates that excellent separation, and sensitive and convenient detection, can be achieved using a series of PA-maltooligosaccharides. The chain-lengthened product had the same retention time as authentic PA-maltoheptaose (Glc_6-GlcPA), whereas the chain-shortened product (Glc_4-GlcPA) was not detected. These results indicate that the Glc residue of Glc-1-P is transferred to the non-reducing-end Glc residue of Glc_5-GlcPA to provide Glc_6-GlcPA.

The time course of the action of rabbit muscle GPb on Glc_5-GlcPA is shown in Fig. 2A, and demonstrates that Glc_6-GlcPA increases linearly with time. The relationship between the enzyme concentration and the amount of Glc_6-GlcPA is also linear under the conditions used (Fig. 2B). The same results were obtained in experiments using rabbit muscle GPa (data not shown).

Effect of Glc-1-P Concentration on Chain-Lengthening Action of Rabbit Muscle GP—The chain-lengthening action of rabbit muscle GPb on PA-maltohexaose (Glc_5-GlcPA) was assayed at various concentrations of Glc-1-P (Fig. 3). The results indicate that the optimal concentration of Glc-1-P is between 20 and 40 mM. Experiments on rabbit muscle GPa showed maximum GPa activity at 40 mM Glc-1-P. Therefore, a Glc-1-P concentration of 40 mM was selected as the standard condition.

Effect of Chain Length of PA-Maltooligosaccharide on Chain-Lengthening Action of Rabbit Muscle GP—Chain-lengthening action of rabbit muscle GPb was examined using PA-maltooligosaccharides (Glc_{n-1}-GlcPA) of various sizes as the maltooligosyl-substrates (Fig. 4). The results indicate that the chain length of PA-maltooligosaccharide significantly influences the rate of the chain-lengthening reaction by GPb. In particular, it has been shown that a maltotetraosyl (Glc_4) residue comprising the nonreducing-end of the PA-maltooligosaccharide is indispensable for the chain-lengthening action of GPb. This result is consistent with a previous report that phosphorolysis (chain-shortening reaction) by GP stops at Glc_4 residues away from the branching points of the outermost chains of the glycogen molecule (*3*). The chain-lengthening action of rabbit muscle GPa was also examined under the same conditions, but no difference was observed between the specificities of GPa and GPb for the chain length of the maltooligosyl-substrates (data not shown). Based on these results, PA-maltohexaose (Glc_5-GlcPA) was selected as the standard maltooligosyl-substrate.

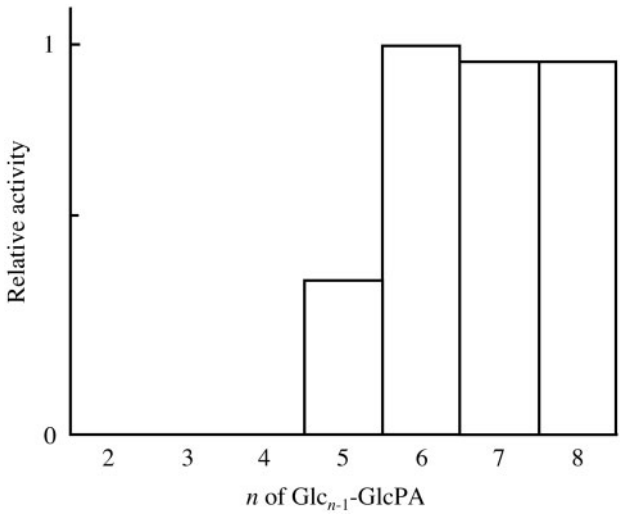

Fig. 4. **Effect of various sizes of PA-maltooligosaccharides on chain-lengthening action of rabbit muscle GPb.** The chain-lengthening action of rabbit muscle GPb was examined using PA-maltooligosaccharides of various sizes as the maltooligosyl-substrates. In this study, PA-maltooligosaccharide is designated as Glc$_{n-1}$-GlcPA, where the reducing-end GlcPA residue cannot form a pyranose-ring (*26*, *35*). Activity towards PA-maltohexaose (Glc$_5$-GlcPA) was taken as unity.

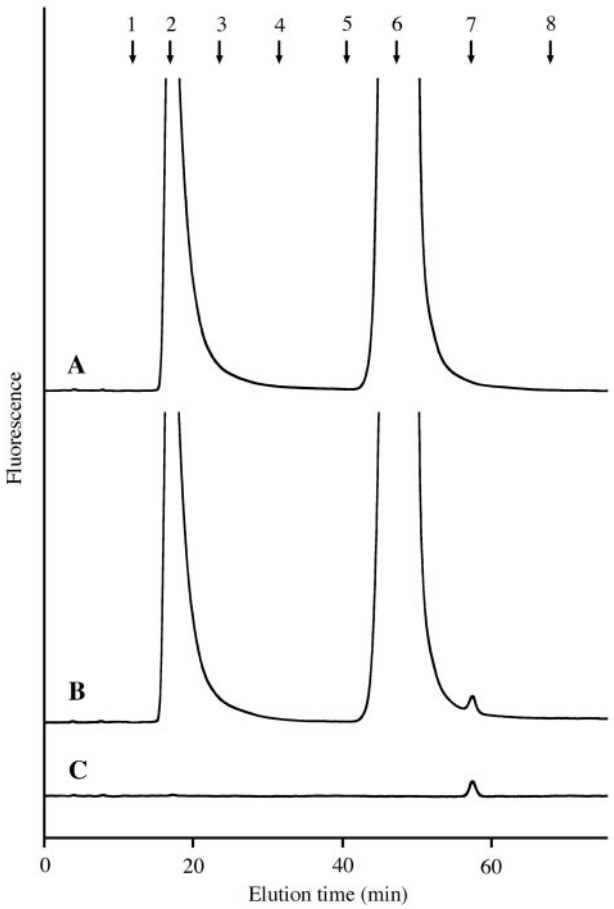

Fig. 5. **Isolation and quantification of PA-maltoheptaose at the 10-fmol level.** A mixture of PA-maltohexaose (Glc$_5$-GlcPA) and Glc-1-P was incubated with rabbit muscle GPb, then the enzymatic reaction mixture was analysed by reversed-phase HPLC. (A) Chromatogram of the enzymatic reaction mixture at zero time. (B) Chromatogram of the 10-min incubation mixture. (C) Chromatogram of 10 fmol of authentic PA-maltoheptaose (Glc$_6$-GlcPA). Arrows 1–8 indicate the elution positions of PA-glucose–PA-maltooctaose (GlcPA–Glc$_7$-GlcPA). The broad peaks appearing around 17 min are due to contaminants.

Sensitivity of the Present Method—A mixture (50 µl) containing 40 mM Glc-1-P, 1.6 µM PA-maltohexaose (Glc$_5$-GlcPA), 100 mM BisTris–HCl buffer (pH 6.8) and 1 mM β-mercaptoethanol was incubated with 1 mU of rabbit muscle GPb at 37°C for 10 min. To stop the reaction, 50 µl of 500-mM ammonium acetate solution (pH 3.8) was added, and the mixture was heated at 100°C for 5 min. The reaction product was analysed by reversed-phase HPLC; the salient parts of the chromatograms are magnified in Fig. 5. Ten femtomoles of the chain-lengthened product could be detected at the elution position of authentic PA-maltoheptaose (Glc$_6$-GlcPA).

Although the spectrophotometric P$_i$ assay is one of the most sensitive methods reported to date, it cannot detect <10 pmol of P$_i$ in the enzymatic reaction mixture (*25*). In contrast, the fluorescent assay described in this study can detect 10 fmol of the reaction product. The sensitivity of the present method is therefore about 1,000 times greater than that of the spectrophotometric P$_i$ assay.

Application of the Present Method to Assays of Crude GP Preparations—In order to be widely applicable, a GP assay method must be compatible with crude GP preparations such as liver and muscle homogenates, as well as purified preparations. The present method was applied to the assay of porcine liver and muscle homogenates. GP activities were assayed according to the standard conditions, except that the substrates were incubated with 3.0 µl of the homogenate at 37°C for 10 min. Reversed-phase HPLC results of the enzymatic reaction mixtures are shown in Fig. 6. GP activities in the homogenates were calculated to be 15.8 U/ml and 9.3 U/ml, respectively. The formation of PA-maltotriose (Glc$_2$-GlcPA) from PA-maltohexaose (Glc$_5$-GlcPA) indicated the existence of α-amylase, and PA-maltotriose detected in the chromatograms shown in Fig. 6C and E were 0.9% and 0.7% of the total PA-maltooligosaccharides, respectively. Formation of Glc$_4$-GlcPA, due to the activity of contaminating α-glucosidase, was <0.1%. These results indicate that the activities of α-amylases and α-glucosidases have negligible effect on the GP assay developed in this study. Although the liver homogenate included several fluorescent compounds, their elution positions on reversed-phase HPLC were significantly different from that of PA-maltooligosaccharides.

Supersensitive Assay of GP Activity in Normal Human Serum—It is well known that GP in human serum is a sensitive marker for the diagnosis of acute myocardial infarction and acute coronary syndrome (*31*, *32*). Although an enzyme-linked immunosorbent assay (ELISA) indicated that a small amount of GP protein exists in healthy human serum (*33*, *34*), it has been difficult to detect its low activity without purification

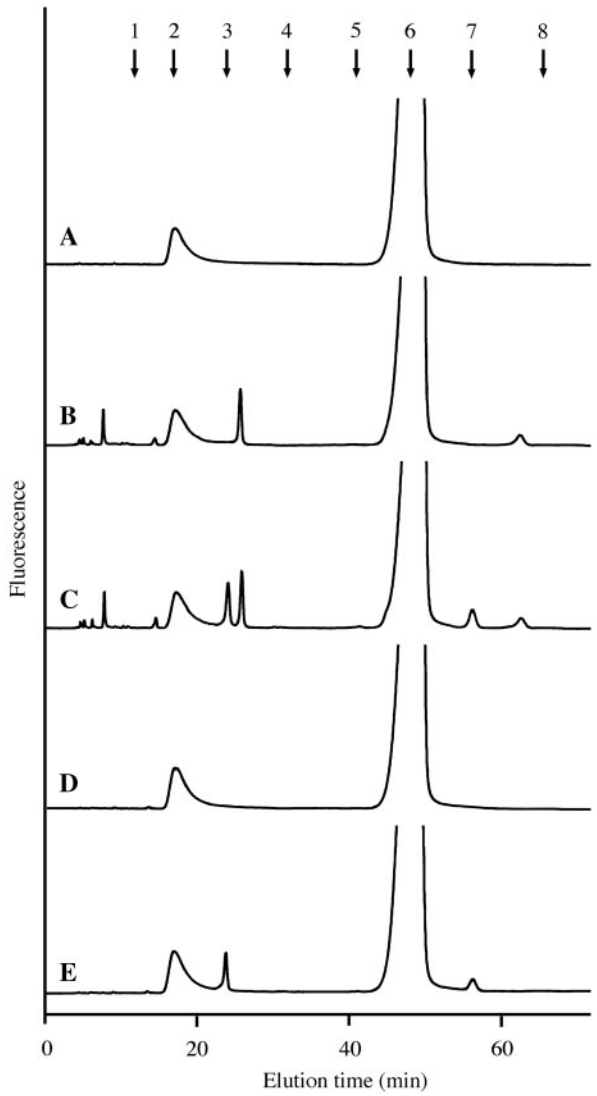

Fig. 6. **HPLC analysis of the products formed by GP activities of crude homogenates.** The present method was applied to GP assays of crude porcine liver and muscle homogenates. The enzymatic reaction mixtures were analysed by reversed-phase HPLC. Elution profiles: (A) substrates only; (B) mixture of substrates and porcine liver homogenate without incubation; (C) B with 10-min incubation; (D) mixture of substrates and porcine muscle homogenate without incubation; (E) D with 10-min incubation. Arrows 1–8 indicate the elution positions of PA-glucose–PA-maltooctaose (GlcPA–Glc_7-GlcPA). PA-maltotriose (Glc_2-GlcPA) produced from PA-maltohexaose (Glc_5-GlcPA) were due to the action of α-amylases. The broad peaks appearing around 17 min are due to contaminants.

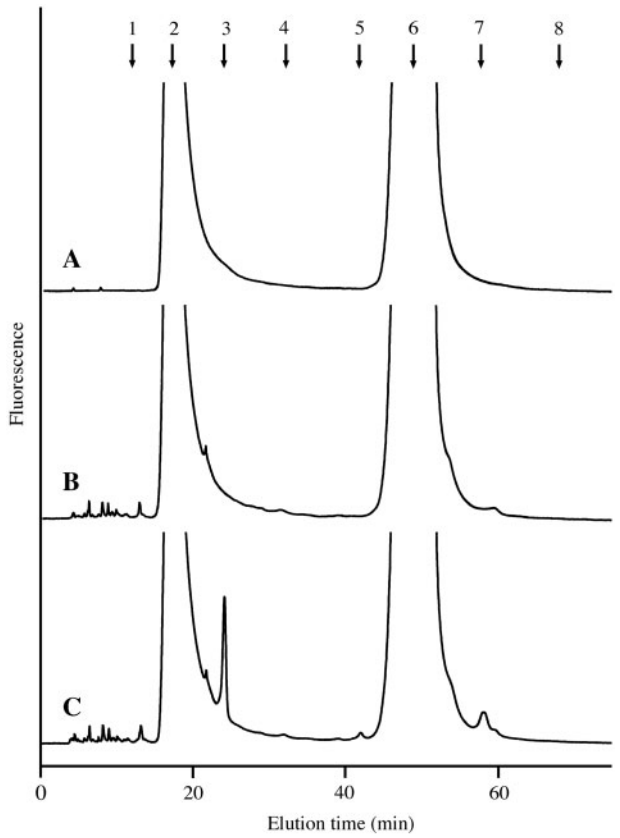

Fig. 7. **HPLC analysis of the product formed by GP activity in normal human serum.** PA-maltohexaose (Glc_5-GlcPA) and Glc-1-P were incubated with normal human serum, and the reaction mixture was analysed by reversed-phase HPLC. Magnified elution profiles: (A) substrates only; (B) mixture of substrates and normal human serum without incubation; (C) B with incubation. Arrows 1–8 indicate the elution positions of PA-glucose–PA-maltooctaose (GlcPA–Glc_7-GlcPA). PA-maltotriose (Glc_2-GlcPA) produced from PA-maltohexaose was due to the action of α-amylase. The broad peaks at around 17 min are due to contaminants.

and/or concentration (*25*, *31*). Here, the present method was applied to detect the low activity directly. For the supersensitive assay of GP activity, the volume of the crude enzyme solution used in the assay should be reduced in order to suppress background peaks on reversed-phase HPLC. This assay was performed according to the standard conditions, except that the substrates were incubated with 1.0 µl of the serum at 37°C for 60 min. Magnified reversed-phase HPLC chromatograms of the enzymatic reaction mixtures are shown in Fig. 7. As shown in Fig. 7C, Glc_6-GlcPA produced by the chain elongation of Glc_5-GlcPA was detected. From this result, GP activity in this serum was calculated at 0.18 U/ml.

In conclusion, we have developed a highly sensitive and convenient assay of GP activity by analysing the chain-lengthening action on PA-maltohexaose in a Glc-1-P-rich medium. All the substrates used in this assay have a defined structure, which is indispensable for achieving reproducible and reliable results. The present method can be applied to both crude and purified GP preparations, and its sensitivity is about 1,000 times greater than that of the standard spectrophotometric P_i assay. The method described here is highly sensitive and convenient for assaying GP activity, and thus will have a wide range of applications.

CONFLICT OF INTEREST

None declared.

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