HPLC Analysis and Optimization of Enzymatic Synthesis of 4'-O-(β-D-Glucopyranosyl)-D-Pantothenic Acid

Maki OKADA, Miho KYOGUCHI, Tofu NAKAYAMA, Rie HIROTA, Teruo AMACHI1 and Takashi UEDA*

Department of Nutritional Physiology, Faculty of Nutrition, Kobe Gakuin University, Arise 518, Ikawadani-cho, Nishi-ku, Kobe, Hyogo 651-2180, Japan
1 Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto, 606-8502, Japan

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Summary We analyzed β-glucosidase-catalyzed transglucosylation to D-pantothenic acid using a reversed-phase HPLC system in order to obtain 4'-O-(β-D-glucopyranosyl)-D-pantothenic acid (PaG) at a higher yield. The HPLC system was simpler and more straightforward for the PaG analysis than the previously employed bioassay method and could also be adopted for efficient isolation of PaG. Penicillium decumbens naringinase showed the highest glucosyl transfer activity to D-pantothenic acid, and the reaction using smaller amounts of naringinase for prolonged periods of reaction time (70 h<) was important to attain higher yields of glucosyl transfer. Maximum overall yields of PaG of 10 and 4% (mol/mol, based on D-pantothenic acid) were obtained using β,β'-trehalose and cellobiose, respectively, as glucosyl donors. The value was 3.6 and 1.4-times higher, respectively, than that obtained by previous synthesis and isolation procedures.

Key Words transglycosylation, 4'-O-(β-D-glucopyranosyl)-D-pantothenic acid, D-pantothenic acid, β-glucosidase

Tomato juice contains a factor, the tomato juice factor (TJF), which promotes the growth of a strain of Leuconostoc oenos, WNB-75, a lactic acid bacterium responsible for malolactic fermentation in wine-making processes (1). The structure of the TJF was identified as 4'-O-(β-D-glucopyranosyl)-D-pantothenic acid (PaG, Fig. 1) (2). The minimum concentration of PaG for the growth promotion of the bacterium is 100 times lower than that of D-pantothenic acid (PaA). Organic synthetic and structure/function relationship studies of a variety of PaA derivatives have shown that the β-glucosylation of the 4'-hydroxyl function of PaA specifically reduces the minimum concentration for growth-promoting activity (2). A biosynthetic pathway of PaG has been found in the dog liver, in which PaG was suggested to be produced by β-transglycosylation to PaA catalyzed by a uridine diphosphate glucose (UDPG)-glucosyltransferase (3). However, the physiological significance of PaG in animals is not yet known.

It has been shown that glycosylation of vitamins and many other bioactive compounds enhances their solubilities, physicochemical stabilities, biological half-lives, membrane permeabilities, and intestinal absorption (4). Thus, in connection with a variety of known biological activities of PaA (5), it is interesting to examine whether or not PaG shows any physiological effects on humans that are altered or improved by glucosylation from those of PaA. To address this problem, an efficient method for the synthesis and isolation of PaG must be established. For this purpose, enzymatic synthesis is of practical advantage because no protection of the starting materials is necessary and the stereochemistry at the newly formed anomeric center is strictly controlled. Although the UDPG-glucosyltransferase (3) is of interest as a catalyst for the β-glucosidic linkage formation, the use of such a system is limited by the restricted availability of the enzyme. An alternative is to use transglucosylation catalyzed by β-glucosidases (6). In this case, the transfer product is also a substrate for the β-glucosidase, so that the success of this procedure as a preparative method depends on the determination of reaction conditions. Although the enzymatic formation of PaG by β-glucosidase-catalyzed transglucosylation to PaA was reported and its potential usefulness for the re-
RESULTS

Giospecific synthesis of PaG was suggested (7), the optimum conditions for enzymatic PaG synthesis remain to be established. In this study, we analyzed the course of enzymatic PaG synthesis to attain the maximum yield of the glucosyl transfer to PaA. We adopted HPLC systems for the analysis and isolation of PaG, and could easily determine the reaction conditions.

Materials and Methods

Materials. Sodium d-pantothenate, cellobiose, salicin, phenyl-β-d-glucopyranoside and almond β-glucosidase (Cat. no., TYB BH201) were purchased from Nacalai Tesque, Kyoto, Japan. β, β'-Trehalose, Penicillium decumbens naringinase (Cat. no., N1385) and Aspergillus niger cellulase (Cat. no., C1184) were from Sigma, St. Louis.

Analytical HPLC. The analytical HPLC was performed using a Gilson 305 system which was equipped on-line with a Rainin auto-sample injector (model AI-3). The programmed HPLC and data acquisition were controlled by a computer program. The established HPLC conditions for PaG analysis were as follows: column, YMC J'sphere ODS M80 (YMC Co. Ltd., Kyoto, Japan; 4.6×150 mm); flow rate, 0.7 mL/min; detection, absorbance at 213 nm; solvent A, 0.1% (v/v) trifluoroacetic acid in H2O; and solvent B, 0.1% trifluoroacetic acid in a 9:1 (v/v) mixture of acetonitrile and H2O. After injection (100 μL) onto a column equilibrated with 6%B (v/v), the column was initially developed isocratically with 6%B for 3 min followed by successive linear gradients from 6%B to 11%B in 5 min, and then from 11%B to 100%B in 1 min. The column was then washed isocratically with 100%B for 5 min followed by a linear gradient of 100%B to 6%B in 1 min. PaA and PaG were eluted at 11.0 min and 12.4 min, respectively, under these conditions. The amounts of PaG and PaA were determined from peak integrals using authentic samples which were used for calibration.

Screening of catalyst for PaG synthesis. The reaction mixture consisted of 23 μmol of sodium d-pantothenate, varying amounts of a glucosyl donor [cellobiose (up to 300 μmol), salicin or phenyl-β-d-glucopyranoside (up to 10 μmol), or β, β'-trehalose (up to 1 mmol)], 100 μmol of sodium acetate buffer, pH 5.0, and enzyme (final protein concentration, 0.1–10 mg/mL) in a final volume of 1.0 mL. The reaction was started by the addition of enzyme, and the mixture was incubated at 37°C for 10 min followed by heating at 100°C for 3 min to stop the reaction. After centrifugation (10,000 rpm for 5 min), the supernatant was analyzed for PaG by HPLC as described above.

The yield (%) of PaG production was calculated as:

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\text{mole of PaG produced} \times 100 / \text{mole of PaA used} \times \text{as a substrate}
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β-Glucosidase assay. The amount of enzyme used for PaG synthesis was expressed in terms of cellobiose hydrolysis activity, which was assayed as follows. The reaction mixture consisted of 5 μmol of cellobiose, 5 μmol of sodium phosphate buffer, pH 7.0, and enzyme in a final volume of 0.5 mL. The reaction was started by the addition of enzyme, and the mixture was incubated at 37°C for 10 min followed by heating at 100°C for 3 min to stop the reaction. Glucose formed in the reaction mixture was found according to Putter and Becker (8) using a kit. One unit (U) of cellobiose hydrolysis activity is defined as the amount of enzyme catalyzing glucose (1 μmol/min) under these conditions.

Partial purification of naringinase. To perform PaG synthesis efficiently, we partially purified P. decumbens naringinase to concentrate it. The P. decumbens naringinase, which was supplied as a powder (5 g), was dissolved in the minimum volume of 0.01 M sodium phosphate buffer, pH 7.0 (buffer A). After centrifugation, the supernatant was put on a Sephadex G-25 (coarse) column (2.8×60 cm) equilibrated with buffer A and eluted for desalting. Active fractions were combined and put on a DEAE-Sepharose CL6B column (2.6×19 cm) that was equilibrated with buffer A. The column was washed with 3 column volumes of buffer A. The β-glucosidase activity, which was coeluted with glucosyl transfer activity to PaA, was eluted with a linear gradient of NaCl (0–1.0 M) in buffer A (400 mL each). Active fractions were combined and concentrated to the minimum volume with a PM10 membrane using an Amicon 8200 ultrafiltration unit.

Enzymatic synthesis of PaG. The reaction mixture consisted of 23 μmol of sodium d-pantothenate, 1 mmol of β, β'-trehalose, 10 μmol of sodium acetate buffer, pH 5.0, and partially-purified naringinase (final concentration, 0.08 U/mL; for definition of enzyme unit, see above) in a final volume of 1.0 mL. Alternatively, the reaction mixture consisted of 460 μmol of sodium d-pantothenate, 6 mmol of cellobiose, 200 μmol of sodium acetate buffer, pH 5.0, and partially-purified naringinase (final concentration, 0.08 U/mL) in a final volume of 20 mL. The reaction was started by the addition of enzyme. The mixture was filtered through a 0.22-μm Millipore filter (Millipore) and kept aseptic at 37°C for 7 d during the reaction. After incubation, the mixture was heated at 100°C for 3 min to stop the reaction followed by centrifugation (8,000 rpm for 10 min). Aliquots of the supernatant were subjected to preparative HPLC on a YMC D-ODS-5 S-5 120 A column (2.5×30 cm). After sample injection, the column was isocratically developed with a 900:70:1 (v/v) mixture of H2O: acetonitrile: trifluoroacetic acid at a flow rate of 4.5 mL/min, and the changes in absorbance at 213 nm were monitored. The PaG fractions were pooled, concentrated, and lyophilized. Elemental analysis of the PaG thus obtained was as follows: Anal. Found: C, 47.33; H, 7.10; N, 3.70. Calcd. for C13H27O15N: C, 47.24; H, 7.14; N, 3.67%.

Structural analysis. 1H-NMR spectra of the purified PaG were recorded using a JEOL DMX500 spectrometer in D2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate monohydrate as the internal standard.
Results and Discussion

We adopted reversed-phase HPLC systems for efficient analysis and isolation of PaG. These systems show a good separation of PaG and PaA, which could be monitored by absorbance changes at 213 nm. The HPLC analysis is simpler and more straightforward than the previously-employed bioassay method (7); the bioassay of PaG required hydrolytic conversion of PaG to PaA prior to the assay, whereas the HPLC analysis does not require such treatment but permits simultaneous determination of PaG and PaA. It should also be noted that the present HPLC uses a volatile solvent system instead of a non-volatile system (9), and thus is also beneficial for preparative purposes.

One of the aims of this study is to attain higher yields in enzymatic PaG synthesis, which provides a basis for exploring the unique physiological activity of PaG. Although the potential usefulness of the enzymatic method for preparing PaG has been reported (7), details of the optimum reaction conditions remain to be established. We therefore reexamined the glucosyl transfer activity to PaA from several β-glucosides using β-glucosidases that are currently commercially available, as described in Materials and Methods. The results showed that, among the enzymes examined, the enzyme naringinase of P. decumbens showed the highest glucosyl transfer activity to PaA when cellobiose or β,β'-trehalose was used as the glucosyl donor. Salicin and phenyl-β-D-glucopyranoside served only as poor glucosyl donors (less than 1% of cellobiose and β,β'-trehalose), and the glucosyl transfer activities of Aspergillus niger cellulase and almond β-glucosidase were much lower (less than 1%) than that of P. decumbens naringinase with any of the glucosyl donors. The naringinase-catalyzed transglucosylation proceeded regiospecifically. The structure of the transfer product was confirmed by 1H-NMR to be 4'-O-(β-D-glucopyranosyl)-pantothenic acid: the coupling constant of the anomeric proton (4 ppm) of the glucopyranosyl moiety was 7 Hz, which indicates that the anemic carbon of the glucopyranosyl group is in a β-configuration (2, 10, 11). The chemical shift of a methine proton in the D-pantothenic acid moiety, 4.1 ppm, was evidence for the glucopyranosyl group being attached to the 4'-hydroxyl function of PaA (7, 10, 11). Thus, we selected P. decumbens naringinase as the catalyst for further optimization studies. The yield of PaG increased with increasing initial concentrations of these glucosyl donors (data not shown). Therefore, the initial concentrations of cellobiose and β,β'-trehalose were set near saturation levels (e.g., 0.3 M and 1.0 M, respectively) for further studies (see below). Because cellobiose is available at less expense than β,β'-trehalose, we then examined the effects of the amount of enzyme and the time of the reaction on the yield of PaG, mainly using cellobiose as the glucosyl donor (Fig. 2A). With large amounts of enzyme (e.g., 1.6 U/mL, final concentration), the PaG once produced decomposed immediately. The maximum yield was obtained in reactions using smaller amounts of enzyme (0.08-0.16 U/mL) for longer periods of time (more than 70 h). Thus, when β,β'-trehalose was reacted with PaA using 0.08 U/mL naringinase, the amount of PaG formed remained constant over 200 h, during which an equilibrium between enzymatic formation and decomposition of PaG may be maintained (Fig. 2B).

After the enzymatic reaction under the established optimum conditions, followed by improved isolation procedures (see Materials and Methods), we obtained 2.3 μmol (0.93 mg) of PaG from a 1-mL reaction mixture containing 1 mmol of β,β'-trehalose and 23 μmol of PaA, and 19 μmol (7.4 mg) of PaG from a 20-mL reaction mixture containing 6 mmol of cellobiose and 460 μmol of PaA. These values respectively correspond to overall yields of 10% and 4% and were 3.6- and 1.4-times higher than the calculated values reported in a previous study (7).
REFERENCES


