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Production of Galactinol from Sucrose by Plant Enzymes

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Galactinol, 1-*O*-(α-D-galactopyranosyl)-*myo*-inositol, was produced from sucrose as a starting material.

UDP-Glc was prepared with sucrose and UDP using sucrose synthase partially purified from sweet potato roots. Then, the UDP-Glc was converted to UDP-Gal using yeast UDP-Gal 4-epimerase from a commercial source. Finally, galactinol was produced from the UDP-Gal and *myo*-inositol using galactinol synthase partially purified from cucumber leaves. The product was identified as galactinol by the retention times of HPLC, α-galactosidase digestion, and NMR spectrometry.

Key words: galactinol; galactinol synthase; cucumber leaves; UDP-Galactose 4-epimerase; sucrose synthase

Galactinol, 1-*O*-(α-D-galactopyranosyl)-*myo*-inositol, a galactoside of *myo*-inositol, is the precursor in the synthesis of raffinose family oligosaccharides. Raffinose is synthesized through a transfer of the galactosyl moiety of galactinol to sucrose by an enzyme, raffinose synthase. The functions of raffinose family oligosaccharides in plants have been studied extensively.[1)] In cucumbers, raffinose and stachyose are the major sugars transported, although sucrose may also be transported. Recently, it has been reported that galactinol may function as an osmoprotectant in drought-stress tolerance of plants, as well as raffinose (Taji *et al.*).[2)] However, the study on galactinol itself has been limited, because of the commercial unavailability of the reagent. The chemical structure of a combination of galactose and *myo*-inositol suggests that galactinol may have an effect on the metabolism of these compounds, which are known to play important roles in living organisms.

Galactinol was first isolated from sugar-beet by Brown and Serro.[3)] In our previous report, we reported the separation and crystallization of galactinol from sugar-beet molasses.[4)] The molasses contains significant amounts of galactinol, but also higher amounts of raffinose, and further, the sucrose content reaches 40%. Therefore, the molasses was not considered to be a satisfactory source because of laborious processes for galactinol isolation. Then, we studied the method of enzymatic galactinol production.

The synthesis of a glycoside of inositol has been reported. Hart *et al.* described an enzymatic synthesis to prepare inositol-containing oligosaccharides.[5)] By using β-D-galactosidase from *Bacillus circulans*, it was shown that 1D-*chiro*-inositol and 1D-pinitol were galactosylated to give mono- and digalactosylated inositols.

Galactinol is synthesized from UDP-Gal and *myo*-inositol by catalysis of galactinol synthase (GS) in plants. Since UDP-Gal is very expensive as a synthetic material, it is advantageous to start with UDP-Glc, and generate UDP-Gal by catalysis of UDP-Gal 4-epimerase. Further, we propose to synthesize UDP-Glc from sucrose and UDP by catalysis of sucrose synthase (SS), for UDP-Glc is also expensive.

In the overall reaction of galactinol synthesis, sucrose, UDP, and *myo*-inositol are the starting materials, and fructose, UDP, and galactinol are the final products. Then, UDP is recycled in the UDP-Glc synthesis reaction.

Cucumber leaves were used for a GS source. After harvesting cucumber fruits, the leaves have been discarded in agricultural fields, although the leaves are still living, and contain bioactive materials like enzymes. By developing the galactinol synthesizing system, we could use the waste materials as enzymatic sources effectively.

The purpose of this study was to establish the system of galactinol synthesis using partially purified enzymes and inexpensive materials.

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Abbreviations: GS, galactinol synthase; SS, sucrose synthase

Materials and Methods

Chemicals. UDP-D-[U-^{14}C]galactose, lithium salt (10.9 GBq/mmol) was purchased from Amersham Pharmacia Biotech. UDP-Gal 4-epimerase from galactose adopted yeast, and α-galactosidase from green coffee beans were obtained from Sigma, and UDP-Gal was from Calbiochem.

Plant materials. Cucumber plants (*Cucumis sativus* L. cv. Suyo) were grown in an experimental field of the Faculty of Agriculture of Kobe University. The mature leaves were harvested, washed, frozen in liquid nitrogen, and stored at -80°C before use for GS purification. Sweet potato roots were obtained from a farm in Kobe, and stored under the same conditions.

Partial purification of plant enzymes.

Preparation of GS enzyme solution.[6,7] Cucumber leaves (400 g) were homogenized in 50 mM Hepes-NaOH (pH 7.0) buffer containing 1 mM dithiothreitol mixed with 1 mM phenylmethanesulphonyl fluoride. After centrifugation of the homogenate, the supernatant solution was fractionated with solid $(NH_4)_2SO_4$. The fraction precipitated in 35–55% saturation was dissolved in the Hepes buffer, dialyzed against the buffer overnight, and the solution was clarified by centrifugation.

The supernatant was chromatographed on a DEAE-cellulose column (DE-52, Whatman), eluting stepwise with the Hepes buffer containing 110 mM and 175 mM NaCl. The active fractions of GS were collected. The activity was stable at -10°C for at least two months.

Preparation of SS enzyme solution. The partial purification of SS was done by the method of Murata.[8] Briefly, peeled sweet potato roots (800 g) were homogenized in 50 mM Tris-acetate buffer. After centrifugation, the supernatant solution was precipitated by addition of solid $(NH_4)_2SO_4$ to 50% saturation, followed by dialysis. The dialysate was applied on a DE-52 column. The column was washed with 5 mM Tris-acetate (pH7.2) buffer containing 0.1 M Na acetate, and then eluted with a linear Na acetate gradient ranging from 0.1 to 0.6 M. Active fractions were collected, and precipitated by $(NH_4)_2SO_4$ again. The dialyzed solution was used as SS enzyme solution for UDP-Glc synthesis.

Enzyme assay.

Assay for GS activity. The activity of GS was measured by the method of Smith *et al.*[6] with a modification. The reaction mixture contained GS enzyme solution (protein concentration, 27.5 mg/ml), 50 mM Hepes-NaOH buffer (pH 7.5), 10 mM UDP-Gal, 20 mM *myo*-inositol, 5 mM $MnCl_2$, 3 mM dithiothreitol, and 1.16 kBq UDP-[U-^{14}C]-galactose in a total volume of 55 μl. The mixture was incubated at 40°C for 20 min. The reaction was started by addition of *myo*-inositol, and stopped by addition of cold ethanol (200 μl). One ml of DE-52 suspension (DE-52:H_2O = 0.3:1.0, w/v) was added, and the mixture was shaken for 20 min at room temperature. After centrifugation, 500 μl of the supernatant was transferred to a vial with scintillator cocktail (Scintisol EX-H, Dojindo Laboratories), and the radioactivity was measured with a liquid scintillation counter (LSC-5100, Aloka). The same volume of water in place of *myo*-inositol was used as a control. One unit of enzyme activity was defined as 1 μmol of galactinol formed per min.

Assay for SS activity. The activity of SS was measured by the method of Murata,[8] and Delmer[9] with a modification. The reaction mixture contained SS enzyme solution, 50 mM Tris-acetate buffer (pH 6.0), 50 mM sucrose, and 25 mM UDP in a total volume of 200 μl. The mixture was incubated at 37°C for 120 min. The reaction was stopped by heating in boiling water. The mixture was clarified by centrifugation, and chromatographed on Whatman 17CHR paper with 1 M ammonium acetate-95% ethanol (3:7), descendingly. Spots were detected under a UV (252 nm) lamp. Standards of UDP-Glc and UDP were run simultaneously. A strip containing UDP-Glc was eluted with water. The UDP-Glc content was estimated by the absorbance at 260 nm. One unit of the enzyme activity was defined as the amount of enzyme required to form 1 μmol of UDP-Glc from UDP for 1 min.

Production of galactinol. Using the GS enzyme solution, galactinol was produced from UDP-Gal and *myo*-inositol. The production was investigated under various conditions to identify the optimal conditions. According to the results, the GS enzyme assay mixture was modified, and the reaction mixture contained GS enzyme solution, 50 mM Hepes-NaOH buffer (pH 7.5), 10 mM UDP-Gal, 50 mM *myo*-inositol, 3 mM dithiothreitol, and 2 mM $MnCl_2$ in a total volume of 200 μl. The mixture was incubated at 40°C for 120 min. The reaction was stopped by addition of cold ethanol (1 ml). The solution was evaporated *in vacuo* to dryness, and analyzed by HPLC using a Sugar-Pak column.

To produce galactinol from UDP-Glc as a starting material, UDP-Gal 4-epimerase and UDP-Glc were added to the GS reaction mixture in place of UDP-Gal. After reaction, the mixture was analyzed by HPLC.

For galactinol production from sucrose as a starting material, SS, sucrose, and UDP were added to the epimerase-GS reaction mixture in place of UDP-Glc. However, galactinol production was not detected on an HPLC chromatogram.

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Then, UDP-Glc was separated from the SS reaction mixture by preparative paper chromatography, as described in 'Assay for SS activity'. The UDP-Glc eluted from the paper was used for the epimerase-GS reaction mixture.

Protein measurement. The amount of protein was estimated using the procedure of Bradford, modified by Read and Northcote.[10] Bovine serum albumin was used as the standard. The eluted protein in column chromatography was monitored by UV absorbance at 280 nm.

HPLC. HPLC analysis for sugars in galactinol preparations was done on two systems. One system was equipped with a Cosmosil $5NH_2$-MS column (4.6 × 150 mm, Nacalai tesque), a pump (L-6000, Hitachi), an RI monitor (L-3300, Hitachi), and a chromato-integrator (D-2500, Hitachi). The column temperature was ambient. The eluent was a mixture of acetonitrile and water (77:23). The flow rate was 1 ml/min.

The other was equipped with a Sugar-Pak Na column (7.8 × 300 mm, Waters) and Shimadzu HPLC LC-10 series. The column temperature was 50°C. The eluent was water. The flow rate was 1 ml/min. To the eluate, NaOH was added to a concentration of 0.1 N with a pump (LC-10AD, Shimadzu), and the mixture was monitored with an electrochemical detector (Coulochem II, ESA, Inc, MA, USA). Pulsed amperometric detection was done with the following pulse settings: E1 = 200 mV, T1 = 500 msec, E2 = 700 mV, T2 = 100 msec, E3 = −900 mV, T3 = 100 msec, and AD = 300 msec.

For nucleotide analysis, the HPLC system was equipped with a Cosmosil $5NH_2$-MS column described above, and Hitachi HPLC L-7000 series. According to Brown *et al.*,[11] 5 mM KH_2PO_4 (pH 3) and 500 mM (pH 4) were used as the initial eluent (Buffer A) and as the final (Buffer B) respectively. In the first 30 min, 10% of Buffer B was run isocratically, followed by a linear gradient of 10–100% B in the next 30 min. The flow rate was 1 ml/min. Sample volume was 5 μl. The UV absorbance at 254 nm of the column effluent was monitored. Peaks were identified by cochromatography with individual, authentic samples of reference nucleotides.

Identification of galactinol preparation. Galactinol preparations were identified using an HPLC chromatographic method, α-galactosidase digestion, and NMR analysis. HPLC was done using Cosmosil $5NH_2$ and Sugar-Pak columns, as described above.

The digestion mixture of a galactinol preparation contained 1% galactinol, and 5 units/ml α-galactosidase in a total volume of 100 μl. The mixture was incubated at 37°C for 120 min. The reaction was stopped by addition of ethanol (500 μl). The solution was concentrated to 10 μl by evaporation *in vacuo*. The sugar components were analyzed by HPLC using a Cosmosil $5NH_2$ column, eluted with a mixture of acetonitrile and water (77:23) at 1 ml/min.

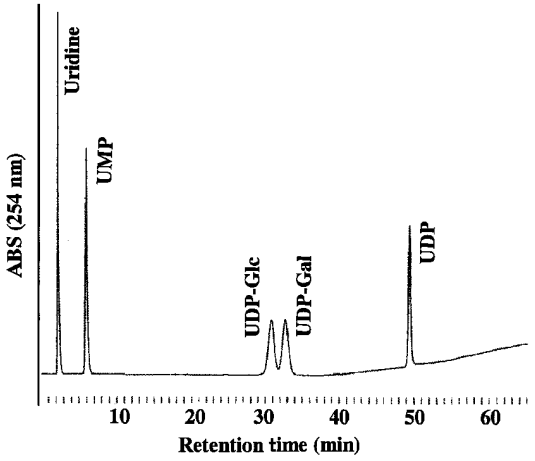

Fig. 1. HPLC Separation of a Reference Mixture of Uridine Nucleotides.

Chromatographic conditions: anion exchange on a Cosmosil $5NH_2$-MS column, with isocratic elution for the first 30 min, and with elution in a linear gradient of KH_2PO_4 for the next 30 min. For details, see text.

The synthesized galactinol was purified by HPLC using a Cosmosil $5NH_2$ column, as described above. The galactinol fractions were collected, evaporated *in vacuo* to dryness, and dehydrated. The sample was dissolved in D_2O containing dioxane as an internal standard, and measured the ^{13}C-NMR (DPX-250, Bruker) spectrum.

Results and Discussion

The nucleotides were analyzed using HPLC with an anion exchange Cosmosil $5NH_2$-MS column to monitor galactinol production. The resolution of a reference mixture of uridine, UMP, UDP-Glc, UDP-Gal, and UDP was first checked. Under the elution conditions of Brown *et al.*,[11] the sugar nucleotides were not separated from one another. The elution gradient was modified to the first 30 min isocratic, and the next 30 min gradient elution. The retention times were 30.76 min for UDP-Glc and 32.68 min for UDP-Gal. The results (Fig. 1) confirmed that separation was attainable.

The GS enzyme solution was obtained from cucumber leaves through partial purification by $(NH_4)_2SO_4$ precipitation and DEAE anion exchange chromatography. The activity was eluted with 50 mM Hepes-NaOH (pH 7.0) buffer containing 175 mM NaCl (Fig. 2). Active fractions (50 units/250 ml) were collected, and stored at −30°C before use.

The SS enzyme solution was obtained from sweet potato roots through partial purification by the precipitation and chromatography as GS (Fig. 3).

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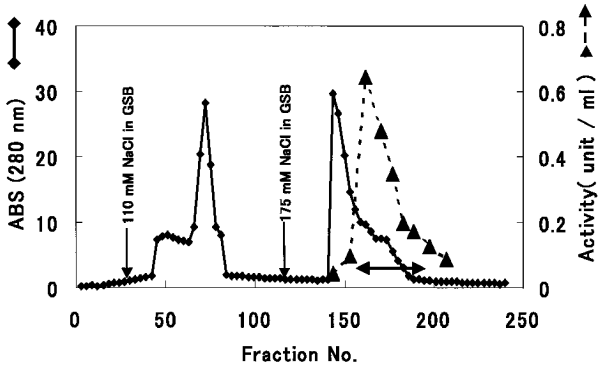

Fig. 2. Elution Profile of Cucumber Leaf Galactinol Synthase and Protein from a DE-52 Anion Exchange Column.

The enzyme activity was eluted from the column (3.5×27 cm) with 50 mM Hepes-NaOH (pH 7.0) buffer (GSB) containing 175 mM NaCl. The enzyme assay was done with UDP-[U-^{14}C]galactose. Pooled fractions are indicated by the arrow.

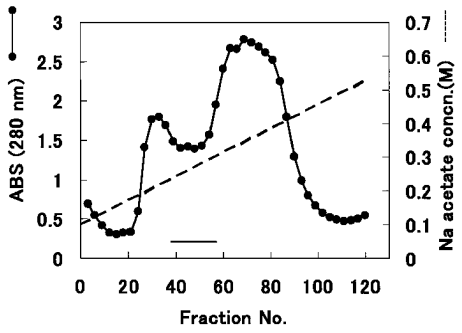

Fig. 3. Elution Profile of Sweet Potato Root Sucrose Synthase and Protein from a DE-52 Column.

The enzyme activity was eluted from the column (3.5×25 cm) with a linear Na acetate gradient ranging from 0.1 to 0.6 M. Active fractions, indicated by the bar, were collected.

The column was eluted with a linear gradient of 0.1 M to 0.6 M Na acetate. The active fractions were collected, concentrated by $(NH_4)_2SO_4$ precipitation (0.4 units/10 ml), and stored at −30°C before use.

Using the GS enzyme solution, galactinol was synthesized from UDP-Gal and *myo*-inositol. The reaction mixture was analyzed with HPLC on a Sugar-Pak column, as shown in the Fig. 4. Galactinol formed (t_R 6.754 min) was eluted at the retention time of authentic galactinol. The contiguous, large peak is *myo*-inositol added as a substrate. UDP-Gal was included in the peak of t_R 4.761 min.

The reactions were done under various conditions to find the optimal conditions for galactinol formation. In the substrate saturation experiments, the reaction was saturated at 10 mM UDP-Gal (Fig. 5-a) and 50 mM *myo*-inositol (Fig. 5-b). The activity reached a maximum at 2 mM Mn^{2+} (Fig. 5-c). Handley and Pharr[12] also reported that Mn^{2+} stimulated the activity of GS from cucumber leaves, and the optimal concentration was 1 mM. The reason for this

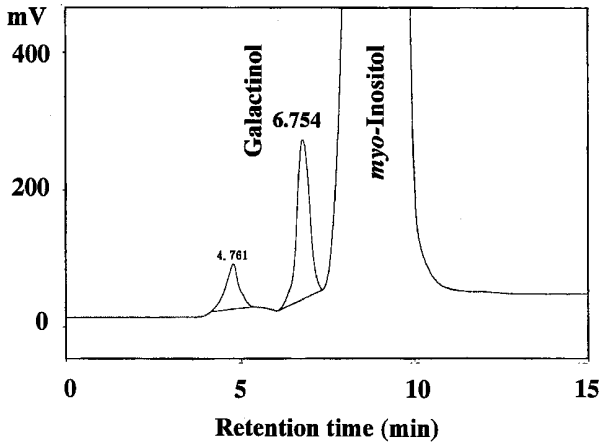

Fig. 4. HPLC Profile of Galactinol Synthase Reaction Mixture Using a Sugar-pak Column.

The eluate was monitored with an electrochemical detector. The reaction mixture contained UDP-Gal and *myo*-inositol as substrates. A peak at 4.761 min is unidentified, but includes UDP-Gal.

discrepancy is not clear. Optimal pH and temperature were 7 to 8 (Fig. 5-d) and 40°C (Fig. 5-e), respectively. Treatment of 40°C for 2 hr decreased the activity of the GS enzyme solution by 50% (Fig. 5-f). The optimal conditions for the GS reaction were chosen from these results, and applied to the following experiments.

Using UDP-Gal 4-epimerase from yeast in addition to the GS enzyme solution, galactinol was also formed from UDP-Glc and *myo*-inositol. The results of HPLC are very similar to those of the GS reaction (data not shown). The optimum temperature and pH of the two-enzyme reaction system were 40°C and 7.5, respectively. The values were also the same as those of the GS activity, suggesting that the reaction was rate-limited by the GS catalytic process.

Finally, galactinol synthesis from the three substrates (sucrose, UDP, and *myo*-inositol) by the three enzymes (SS, UDP-Gal 4-epimerase, and GS) was investigated. However, the synthesis was not detected on the HPLC chromatogram with various concentrations of UDP. By sugar nucleotide analysis with a Cosmosil 5NH_2 column, the syntheses of UDP-Glc and -Gal were confirmed (data not shown). The reasons why galactinol was not detected may be that the retention time of galactinol is similar to one of a large amount of *myo*-inositol, and/or that GS was inhibited by UDP. It is known that GS activity is inhibited by UDP competitively.[12] The activity of the GS enzyme solution was also inhibited at 5 mM of UDP by 70% (Fig. 6). We think that the production of galactinol at a lower concentration of UDP was too low to detect by HPLC, and at a higher one, GS was inhibited.

The UDP-Glc synthesized from sucrose and UDP using SS enzyme solution was separated by paper

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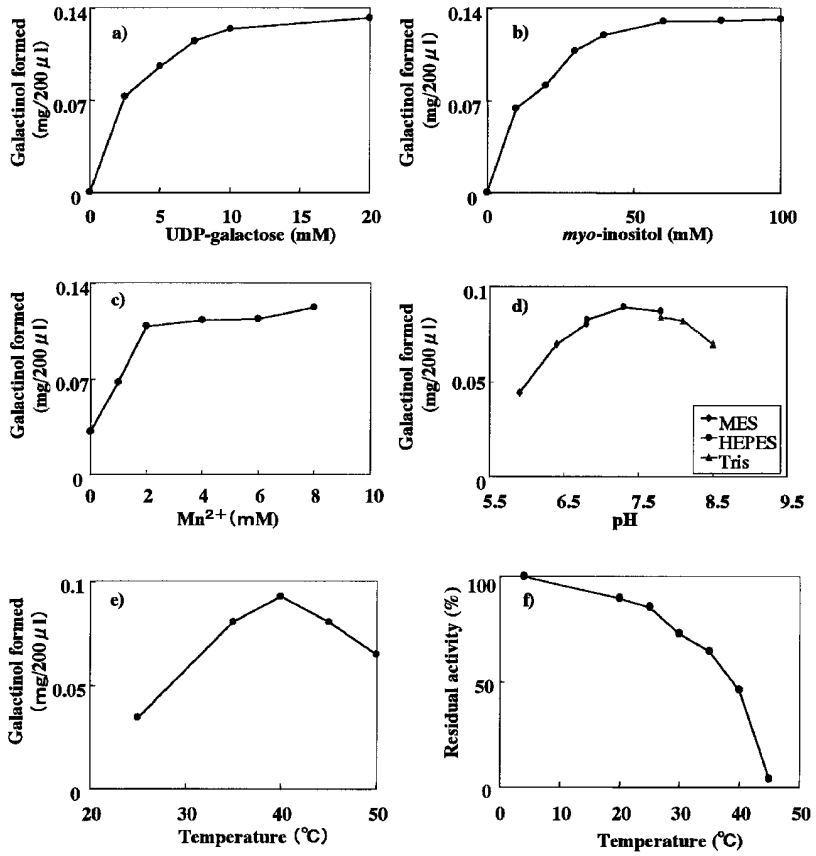

Fig. 5. The Activity of Cucumber Galactinol Synthase under Various Conditions.
a) UDP-Gal, b) *myo*-inositol, c) Mn^{2+}, d) pH, e) temperature, f) thermostability. The enzyme activities were assayed under various conditions to find the optimum.

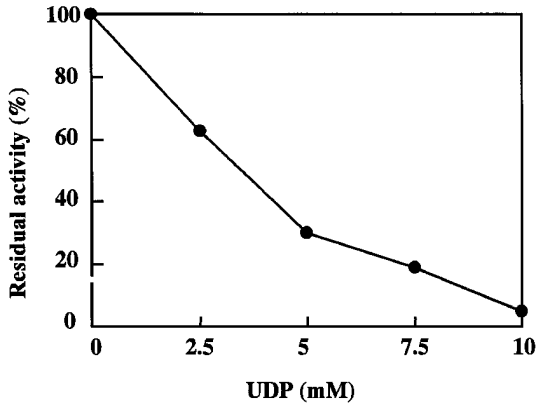

Fig. 6. UDP Inhibition of Galactinol Synthase.
The activity of GS was assayed in the reaction mixture of UDP-Gal as a substrate with increasing concentrations of UDP.

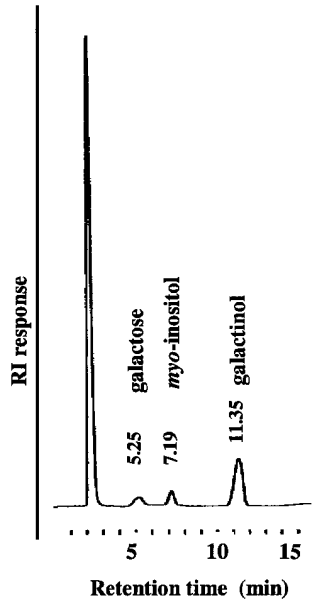

Fig. 7. HPLC Profile of α-Galactosidase Digestion Mixture of Galactinol Preparation Using a Cosmosil $5NH_2$ Column.
The galactinol preparation was synthesized using GS, SS, and UDP-Gal 4-epimerase, and digested with α-galactosidase for galactinol identification. Sugar components were analyzed with Cosmosil $5NH_2$ column, eluted with a mixture of acetonitrile and water (77:23) at 1 ml/min.

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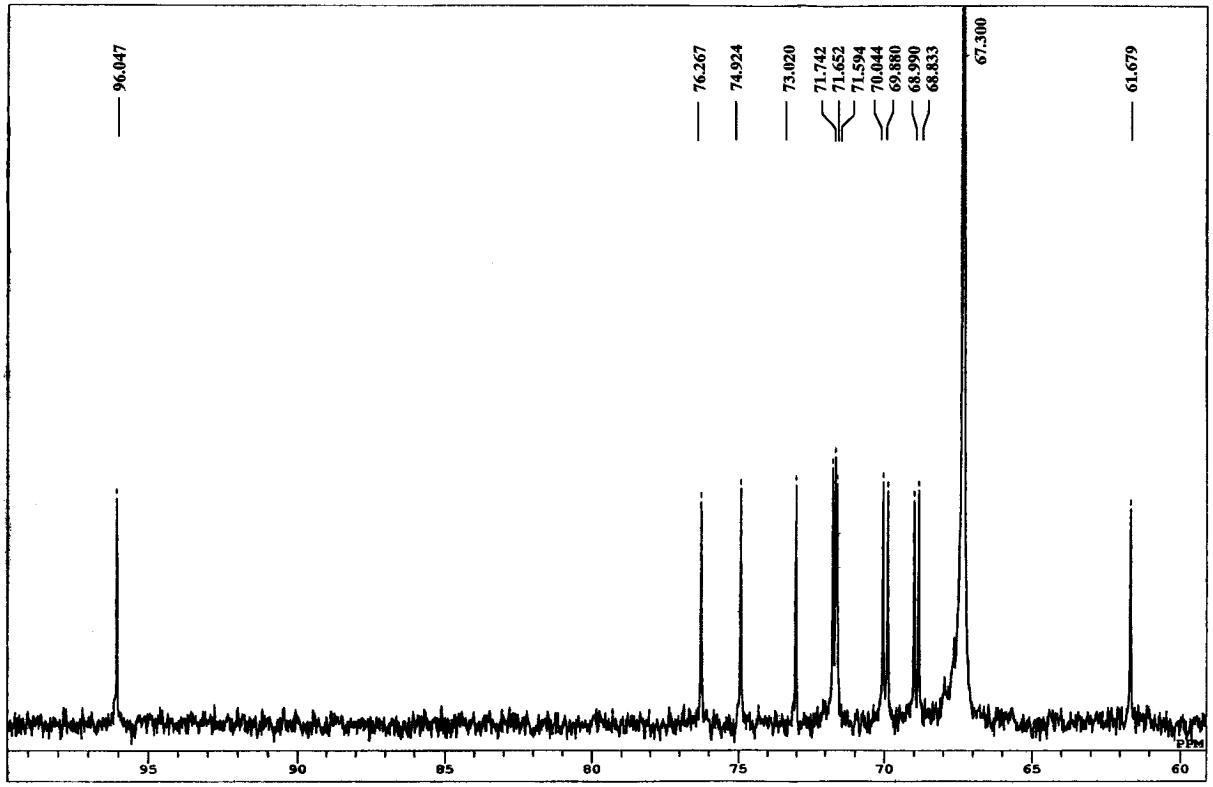

Fig. 8. ^{13}C-NMR Spectrum of Galactinol Preparation.

Galactinol was prepared from sucrose, UDP, and *myo*-inositol using SS, GS, and UDP-Gal 4-epimerase. The synthesized galactinol was purified by HPLC. The galactinol preparation was dehydrated, and dissolved in D_2O containing dioxane as an internal standard.

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chromatography, and recovered. When the UDP-Glc preparation was used in the three-enzyme system without UDP, galactinol was synthesized. The three-enzyme reaction system was thus required to improve separation of GS from UDP.

The synthesized galactinol fractions were collected to identify them as galactinol using retention times on HPLC, α-galactosidase digestion, and NMR spectra. Purified galactinol was analyzed on two HPLC columns (Cosmosil 5NH_2-MS and Sugar-Pak Na), and showed a single and symmetrical peak. The retention times were identical to the authentic ones. After a preparation of galactinol was digested by α-galactosidase, sugar components of the products were also analyzed by HPLC (Fig. 7). The retention times of two peaks indicated the presence of galactose and *myo*-inositol. The molar ratio was 1:1, and no other major product was detected on the chromatogram. Furthermore, the synthesized galactinol dissolved in D_2O containing dioxane was used to measure the ^{13}C-NMR spectrum (Chemical shifts (ppm) α-galactose moiety: C-1′, 96.1; C-2′, 69.0; C-3′, 70.0; C-4′, 69.9; C-5′, 71.7; C-6′, 61.7; *myo*-inositol moiety: C-1, 76.3; C-2, 68.8; C-3, 73.0; C-4, 71.7 or 71.6; C-5, 74.9; C-6, 71.7 or 71.6), as shown in Fig. 8. The spectrum coincided with that of authentic galactinol, and with that reported by Schweizer and Horman.[13] These results indicated that the preparation was identical to galactinol.

In conclusion, galactinol was synthesized from sucrose with UDP and *myo*-inositol by catalysis of SS, GS, and UDP-Gal 4-epimerase. However, the separation of UDP-Glc from UDP was required, following the SS reaction. The yield (10% of UDP-Gal added) was rather low. Some modifications of the method for higher yield are in progress.

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