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Semisynthesis of a spin-labeled monogalactosyldiacylglycerol and its application to the assay for galactolipid-transfer activity in spinach leaves *

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A spin-labeled monogalactosyldiacylglycerol with a 12-doxylstearoyl group was newly synthesized from monogalactosyldiacylglycerol isolated from spinach leaves. The spin-labeled monogalactosyldiacylglycerol was incorporated into liposomes and the transfer of monogalactosyldiacylglycerol between liposomes was detected by changes in ESR spectra. In this assay, the activity of the galactolipid-transfer proteins was discriminated from that of galactolipase in spinach leaves. Sephadex G-100 column chromatography of spinach chloroplast protein revealed the presence of galactolipid-transfer protein in the fractions around 30 kDa. From fractionation of the whole leaf protein it proved to be a 28 kDa protein. On the other hand, column chromatographies of the spinach leaf protein by Sephadex G-100, DEAE-Sepharose and CM-Sepharose revealed the occurrence of at least one acidic and two basic galactolipid-transfer proteins in the leaf cell and one of the basic galactolipid-transfer proteins was a 11 kDa protein with the largest activity. Galactolipid-transfer proteins of 11 and 5.1 kDa possessed the phosphatidylcholine-transfer activity detected by a ratio analysis.

Introduction

Both envelope and thylakoid membranes of the chloroplast are characteristic in the occurrence of galactosyldiacylglycerols [1]. Since the synthetic path for galactosyldiacylglycerols is located in the envelope, but not in the thylakoid, the supply of these lipids from the envelope to the thylakoid is needed and the concept of membrane flow [2] and the protein-mediated lipid transportation is proposed as the transfer mechanism [3]. Williams et al. [4] demonstrated the relatively slow transfer of galactolipids from the envelope- to the thylakoid-containing fractions from broad bean leaves. On the other hand, Joyard et al. [5] showed that radioactivity incorporated into the lipids of the envelope after $^{14}CO_2$ labeling of spinach leaves was rapidly transferred to those of thylakoid.

In this article, we examined whether or not a galactolipid transfer protein is present in leaves, by analogy with the occurrence of phospholipid-transfer proteins in plant tissues [6]. For the assay of galactolipid-transfer protein, a method using a spin-labeled galactolipid is developed and the first attempt is made to detect galactolipid-transfer protein in spinach leaves.

* Data supplementary to this article are deposited with, and can be obtained from, Elsevier Science Publishers B.V. (Biomedical Division), BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to BBA/DD/306/72477/813(1985)298. The supplementary information includes the full details of the synthesis steps of spin-labeled monogalactosyldiacylglycerol.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

General. Rhizopus delemer lipase was purchased from Seikagaku Kogyo Co. (600 units/mg). Sephadex G-100, G-25, DEAE-Sepharose CL-6B and CM-Sepharose CL-6B were from Pharmacia Fine Chemicals.

Electron spin resonance (ESR) spectra were recorded on a JEOL JES-PE ESR spectrometer. Shimadzu CS-910 dual-wavelength TLC scanner was used on the analysis of digestive products of *Rhizopus delemer* lipase. A 2 m × 4 mm column of 15% DEGS on Chromosorb W was used at 190°C for gas-liquid partition chromatography (GLC).

Protein content was determined according to Bradford [7] using Bio-Rad protein assay solution. Intactness of chloroplast preparations was determined by measuring photometrically the reduction of ferricyanide according to Lilley et al. [8]. Chlorophyll content was measured according to Mackinney [9].

Semisynthesis of monogalactosyldiacylglycerol. A spin-labeled monogalactosyldiacylglycerol was synthesized as shown in Fig. 1 (V).

Hydrolysis of spin-labeled monogalactosyldiacylglycerol with R. delemer lipase. Regiospecific hydrolysis of spin-labeled monogalactosyldiacylglycerol with *R. delemer* lipase was performed according to Fischer et al. [10]. A $CHCl_3$/methanol (2 : 1, v/v) solution of spin-labeled monogalactosyldiacylglycerol (0.1 mg) and Triton X-100 (0.8 mg) was placed in a 5-ml test-tube. After evaporating the solvents, the residue was dissolved with 0.5 ml of 10 mM Tris-HCl (pH 7.2) buffer and sonicated in a bath-type sonicator for a few seconds. Then the solution was shaken with 10 μl lipase (1 mg) at room temperature for 15 min. The reaction was stopped by adding 40 μl of 0.1 M CH_3CO_2H and the resulting mixture was extracted with $CHCl_3$/methanol (2 : 1, v/v). The chloroform layer was separated, washed and concentrated in vacuo. The residue was dissolved in $CHCl_3$, applied onto TLC plate (Merck 60 F_{254}) and developed with petroleum ether/acetone (3 : 1, v/v) and $CHCl_3$/methanol (85 : 15, v/v), successively. The plate was analyzed with a TLC scanner by illuminating at 254 nm and detecting through a 534 nm W-type interference filter. 32% of spin label was released as free acid and 68% of spin-label remained as lysomonogalactosyldiacylglycerol. When the acid fraction and lysomonogalactosyldiacylglycerol fraction were scraped and subjected to GLC analysis after methanolysis in 5% (w/w) HCl in methanol, it was revealed that the methyl esters released from the *sn*-1 and *sn*-2 positions contained 67 and 33% of oleic acid, respectively.

Lipids extraction from thylakoid mebranes and preparation of monogalactosyldiacylglycerol-containing liposomes. Spinach leaves (200 g) were homogenized for 15 s in a Waring blendor with 400 ml of 50 mM potassium phosphate (pH 7.4)/200 mM NaCl/0.1 M sucrose and filtered through two layers of Miracloth (Chicopee Mills, Inc.). The filtrate was centrifuged at 4700 × *g* for 10 min and the precipitate was resuspended in 200 ml of the homogenizing medium. The suspension was centrifuged at 750 × *g* for 5 s and the supernatant was again centrifuged at 4700 × *g* for 10 min. The precipitate recovered was suspended in 20 ml water and stored at −20°C.

A suspension of thylakoid mebranes was extracted by the method of Bligh and Dyer [11]. The lipids were applied onto the TLC plate (Merck silica gel 60, 0.25 mm in thickness, 20 × 20 cm) and developed first with $CHCl_3$/acetone (2 : 1, v/v) up to 18 cm height from the origin and then with acetone/benzene/water (90 : 30 : 8, v/v) upto 12 cm height, on the same plate. Lipids were separated into two fractions A and B: fraction A consisted of monogalactosyldiacylglycerol and more polar lipids and fraction B was of the more polar lipids other than monogalactosyldiacylglycerol.

Donor liposomes were prepared from the spin-labeled monogalactosyldiacylglycerol (1.95 mg) and fraction B (3.05 mg) and acceptor liposomes were from fraction A (15 mg) alone. The lipids were mixed in $CHCl_3$, evaporated and then suspended in 1.0 ml of 50 mM Hepes-NaOH (pH 7.5). After the sonication by a stick-type sonicator (OHTAKE) for 5–20 min at 4°C, the solution was centrifuged at 20000 × *g* for 20 min to remove broken stick residues and the supernatant was used as a liposome solution. The lipid composition of the acceptor liposome was determined by GLC as follows. Lipids (mol%): monogalactosyldiacylglycerol (45), digalactosyldiacylglycerol (27), sulfoquinovosyldiacylglycerol (5), PC (7), PE (5), phos-

phatidylglycerol (13). There was little change in lipid patterns developed on two-dimensional TLC (1st: $CHCl_3/CH_3OH/H_2O$, 65 : 25 : 4 and 2nd: $CHCl_3/CH_3OH/$ conc.$NH_3/$ isopropylamine, 60 : 30 : 4 : 0.5, v/v, respectively) between lipid A and acceptor liposomes.

Spinach chloroplast protein. Spinach chloroplasts (7.8 mg chlorophyll, 38% intactness) were isolated from 200 g of leaves by a method of Cockburn et al. [12] and suspended in 6 ml of 0.33 M sorbitol/50 mM Hepes-NaOH (pH 7.6)/2 mM Na_2EDTA/1 mM $MgCl_2$/1 mM $MnCl_2$. The solution was diluted 4-times with 5 mM dithiothreitol and centrifuged at $59000 \times g$ at 4°C for 30 min. The supernatant was dialyzed against 50 mM Tris-HCl (pH 7.6) and concentrated with Aquacide III (Calbiochem) to give a 1.7 ml solution (54.4 mg protein), which was then applied on Sephadex G-100 column chromatography.

Spinach leaf protein. Spinach leaves (1.1 kg) purchased from a city market were homogenized for 50 s in a Waring blendor with 600 ml of 50 mM potassium phosphate (pH 7.6)/0.2 M NaCl/2 mM dithiothreitol. The homogenate was centrifuged at $16000 \times g$ for 20 min at 4°C. The supernatant was acidified with 1 M HCl to pH 5.1 and stirred for 15 min. After the centrifugation, the supernatant was neutralized with 2 M Tris to pH 7.6 (pH 5.1 supernatant).

Solid ammonium sulfate was added to the pH 5.1 supernatant to 75% saturation and the solution was stirred for 1 h at 4°C. The resulting precipitate was dissolved in a minimal volume of 50 mM Tris-HCl (pH 7.6)/10% glycerol/0.02% NaN_3 and the solution was dialyzed against the same buffer for 4 h. After the centrifugation at $20000 \times g$ for 20 min, the supernatant was recovered.

The precipitate recovered between 35 and 75% saturation of ammonium sulfate from the pH 5.1 supernatant solution was also dissolved, dialyzed and centrifuged as described above and the resulting solution was recovered (35–75% precipitate).

Assay of transfer activity. The reaction was started by adding 10 μl of the donor solution (19.5 μg of spin-labeled monogalactosyldiacylglycerol, 22.1 nmol) to a 70 μl solution of acceptor liposomes (30 or 40 μl) and a protein fraction. The solution was transferred to the ESR cell and the spectrum was observed after 15 min at room temperature under the following conditions: radiofrequency 5 mW, field 3250 ± 50 G, modulation 100 KHz 2 G, sweep-time 4 min, response 1.0 and amplitude 4×100. Peak height designated h in Fig. 2B was measured in cm.

The protein solution possessing higher ionic strength than 0.1 M NaCl or containing sulfhydryl reagents is not suitable for our assay system, because aggregation of liposome or reduction of nitroxide occurred, respectively. The eluates from DEAE- and CM-Sepharose columns were passed through Sephadex G-25 (fine) equilibrated with 50 mM Tris-HCl (pH 7.6)/10% glycerol/0.02% NaN_3 before assays.

Phosphatidylcholine-transfer activity was assayed according to Kader et al. [13].

The relation between the peak height h (cm) and exchange rate a (%). Fig. 3A gives the observed peak height of the spectra of spin-labeled monogalactosyldiacylglycerol/fraction A liposome (0.625 mg lipid/ml) at various concentrations of spin-labeled monogalactosyldiacylglycerol (%, w/w of the total lipid).

When donor and acceptor liposomes were incubated together with protein solution, the peak height h in the ESR spectrum gradually increased (Fig. 2C1). Because ESR spectrum is a superimposition of donor and acceptor spectra, h is expressed as:

$$h = h_d + h_a$$

where h_d and h_a designate peak height of donor and acceptor spectra, respectively.

If a% of spin-labeled monogalactosyldiacylglycerol is transferred from donor to acceptor liposomes by exchange mechanism, the concentration of spin-labeled monogalactosyldiacylglycerol in donor liposome decrease from 39 to $39(1 - 0.01a)$%, while that in acceptor liposome increase from 0 to $a/12$%. Since the mass of acceptor liposome (7.5 mg/ml) is 12-times that of donor liposome (0.625 mg/ml) in our assay system, h is expressed as:

$$h = h_d + h_a$$

$$= h(39 - 0.39a) + 12h(a/12)$$

where $h(x)$ is the reading from Fig. 3A at x(%,

w/v) on the abscissa. The relationship between h and exchange rate a is shown in Fig. 3B.

Although h is not proportional to a, the increase of peak height is nearly proportional to the transferred amount of spin-labeled monogalactosyldiacylglycerol for the initial short time of the reaction. The galactolipid-transfer protein activities were expressed as peak height h in cm.

Results

Semisynthesis of monogalactosyldiacylglycerol

A spin-labeled monogalactosyldiacylglycerol was newly prepared by a slight modification of procedure of Heinz and Tulloch [14] and the product was the mixture of regioisomer concerning the position of acyl groups attached to the *sn*-glycerol moiety. As shown in Fig. 1, principal steps of the reaction are (1) stepwise introduction of the oleoyl group and the 12-doxylstearoyl group at *sn*-1 and *sn*-2 positions of glycerol moiety, respectively and (2) selective removal of protective groups of galactosyl moiety.

In the first acylation of II (Fig. 1) by oleoyl chloride at $-70°C$ for 1 h, a trace amount of dioleoyl product was detected by TLC, but the main product was III. Further column chromatography gave pure III in good yield (64%). Then, the second acyl group was introduced by the acyl imidazolide method [15,16] under neutral condition. The reaction condition was optimized and after 24 h at 50°C, IV was formed in 68% yield.

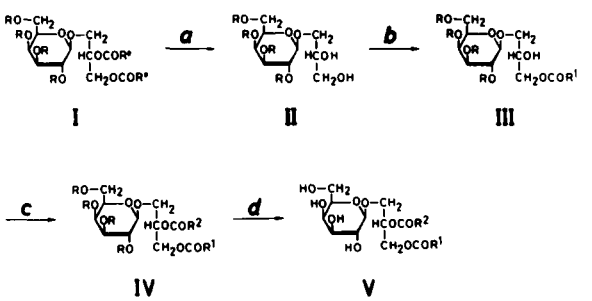

Fig. 1. Synthesis of the spin-labeled monogalactosyldiacylglycerol (V). R = $CH(OC_2H_5)CH_3$, R′ = alkyl, COR^1 = oleoyl, and COR^2 = 12-doxylstearoyl. (a) Sodium methoxide/methanol; (b) oleoyl chloride/pyridine/CH_2Cl_2; (c) 12-doxylstearoyl imidazolide; (d) Dowex 50W-X8 resins (H^+ form)/methanol.

Acylation using acid/dicyclohexylcarbodiimide/4-dimethylamiopyridine [17] resulted in recovering the corresponding acid anhydride.

The final step of the synthesis was the hydrolysis of IV (Fig. 1) by Dowex 50W-X8 resins [18]. The reaction proceeded rapidly and selectively affected the ethoxyethyl group. However, the product contained unfavorable contaminants presumably derived from resins with similar polarity on the TLC development. A further purification method was devised: the reaction product was silylated with chlorotrimethylsilane/hexamethyldisilazane [19] and then applied to a column chromatography to separate the silylated monogalactosyldiacylglycerol, which was subsequently desilylated by tetrabutylammonium fluoride in tetrahydrofurane [20,21]. The reaction was quantitative (95% recovery). The molar ultraviolet absorption coefficient of the product was 2280 $l \cdot mol^{-1} \cdot cm^{-1}$. TLC behavior of V (Fig. 1) showed the identity with monogalactosyldiacylglycerol, though microanalysis of V was unsatisfactory, presumably because of the difficulty of removing water from the analytical sample. When V was subjected to *R. delemer* lipase hydrolysis, 32% of spin-labeled fatty acid was released and 68% of spin-labeled fatty acid was recovered as lysomonogalactosyldiacylglycerol. GLC analysis of fatty acid and lysomonogalactosyldiacylglycerol revealed that 67% of oleic acid was released and 33% of oleic acid was recovered as lysomonogalactosyldiacylglycerol. Since this enzyme releases the fatty acid bound at the 1-position of glycerol moiety of monogalactosyldiacylglycerol [10], acyl migration reaction seems to occur during the isolation of III (Fig. 1) or under the reaction condition of introducing the second acyl group to III.

Assay of monogalactosyldiacylglycerol-transfer activity

The best is to use the thylakoid membrane as the actual acceptor of spin-labeled galactolipid, but this cannot be applied because the nitroxide radical of spin-labeled monogalactosyldiacylglycerol was rapidly reduced by thylakoid membrane (Nishida, I. and Yamada, M., unpublished data). Thus, liposomes were used as donor and acceptor membranes.

Since the binary system of water and mono-

galactosyldiacylglycerol cannot form the dispersion of liposomes [22], donor liposomes were prepared by mixing the spin-labeled monogalactosyldiacylglycerol with the polar lipids of spinach leaf thylakoids (fraction B) and acceptor liposomes were also prepared from thylakoidal polar lipids (fraction A).

Fig. 2B shows an ESR spectrum of the donor liposome solution (0.625 mg lipids/ml) when the concentration of the spin-labeled monogalactosyldiacylglycerol in donor liposomes was 39% (w/w) of the total lipids. The spectrum of the solution showed a characteristic spin-spin interacted broad signal with a small shoulder which was caused by the dilution of spin-labeled monogalactosyldiacylglycerol with the polar lipids. There was no change in the spectrum for 24 h at room temperature. When the acceptor liposomes were added to the solution of donor liposomes, the small shoulder slightly increased its peak height (designated *h* in Figs. 2B and 2C1) with time, indicating that spin-labeled monogalactosyldiacylglycerols were transferred gradually from donor liposomes to acceptor liposomes in the absence of a protein. But, an advanced increase of the peak height was observed in the presence of a protein (0.64 mg) from spinach chloroplast stroma, but no increase in the presence of bovine serum albumin (10 μg). In the assay of galactolipid-transfer activities, the reaction was started by adding donor liposomes to the solution containing the acceptor liposomes and the spinach protein. After 15 min incubation at room temperature, the peak height (*h*) was measured in cm. The increase in the peak height was nearly proportional to the added amount of the protein.

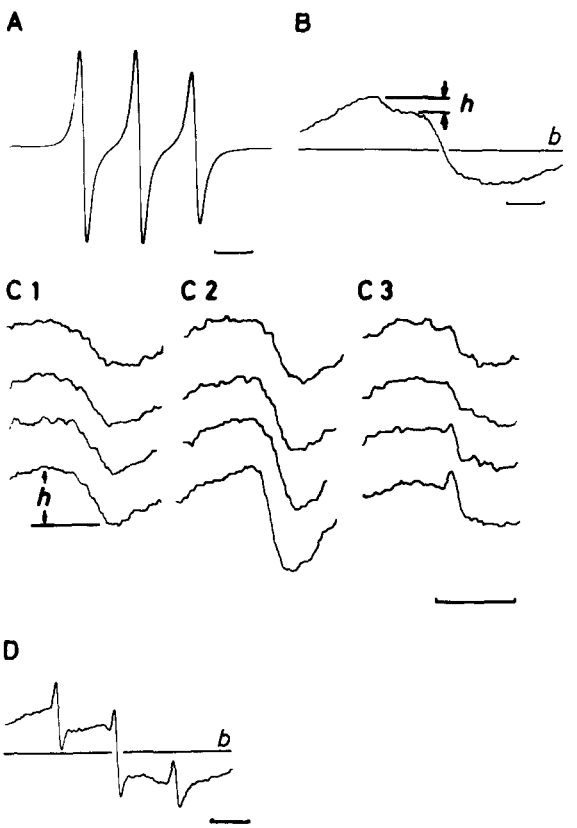

Fig. 2. ESR spectra. (A) V (Fig. 1) in ethanol ($3.22 \cdot 10^{-4}$ M). (B) A solution of donor liposome (39% spin-labeled monogalactosyldiacylglycerol, 0.624 mg lipids/ml). Peak height was designated as *h*. (C) ESR spectra after 2.5-, 5-, 10- and 15-min incubation from the top, respectively. (C1) donor liposome (0.625 mg lipid/ml) plus acceptor liposome (7.5 mg lipid/ml). (C2) The same conditions as C1 except that spinach stroma protein (0.64 mg) was added. (C3) The same conditions as C2 except that acceptor liposome was omitted. (D) Donor liposome (0.625 mg lipids) plus spinach leaf lipase (fraction number 38 in Fig. 6A, 30 μl), after 107-min incubation. Bars indicate 10 G. Mark (b) in the spectra (B) and (D) indicates the base line.

However, there was some activity increasing *h*, independent of the transfer activity of spin-labeled monogalactosyldiacylglycerol in the spinach protein. This activity was easily distinguished from galactolipid-transfer activity. When protein solutions were incubated with donor liposomes alone, the increase in peak height was recognized for galactolipase (Fig. 2C3) but not for the galactolipid-transfer protein. Furthermore, prolonged incubation of donor liposomes with lipase released a appreciable amount of spin-labeled fatty acid into the reaction medium and this was observed as a sharp triplet as shown in Fig. 2D.

Galactolipid-transfer protein in spinach leaves

Galactolipid-transfer protein activities in the chloroplast stroma protein and their fractionation by Sephadex G-100 column chromatography. When the stroma protein (20 μl, 0.64 mg) was incubated with 10 μl of donor, 40 μl of acceptor liposome solution and 10 μl of 50 mM Hepes-NaOH (pH 7.6), the peak height increased with time: 2.8 cm (2.5 min), 4.6 cm (15 min) and 6.2 cm (30 min),

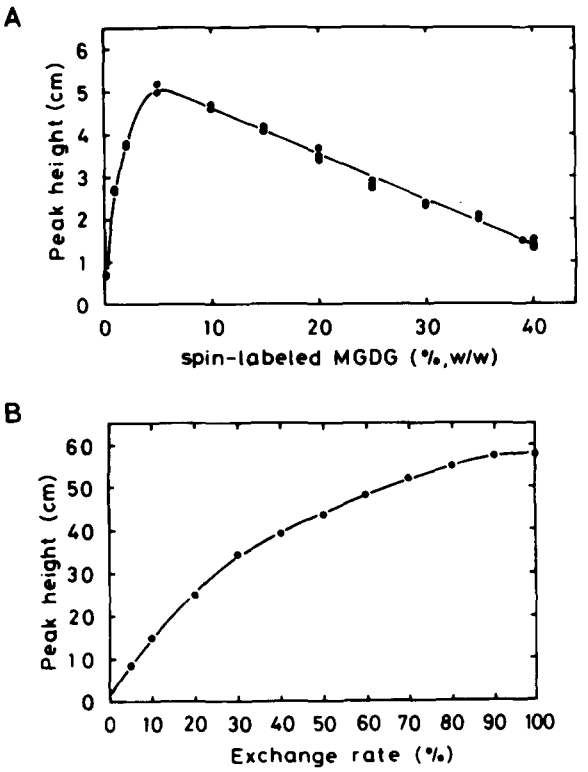

Fig. 3. Relation between peak height h and exchange rate a. (A) Observed peak height at various concentrations of spin-labeled monogalactosyldiacylglycerol (MGDG). Total lipid concentration were adjusted to 0.675 mg/ml. (B) Calculated peak height (cm) at various exchange rates (%).

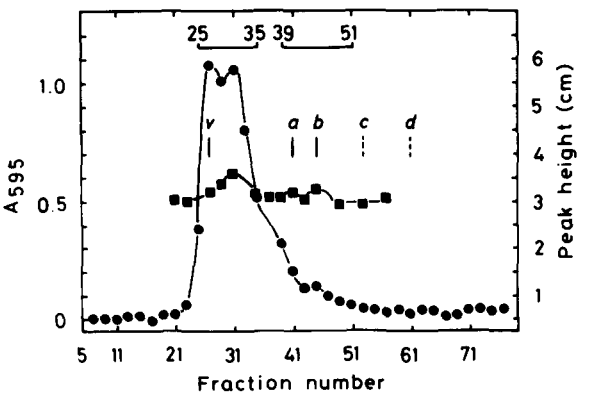

Fig. 4. Fractionation of spinach stroma protein by Sephadex G-100 column chromatography. Spinach stroma protein was applied to a 1.6×31.5 cm Sephadex G-100 column which had been previously equilibrated with 50 mM Tris-HCl (pH 7.6). The column was calibrated by elution positions of Dextran blue 2000 and NaCl. Marks (a), (b), (c) and (d) indicate the expected elution positions of 31, 24, 11 and 5.1 kDa proteins, respectively. Mark (v) indicates the position of void volume. Protein was eluted with the same buffer at a flow-rate of 31 ml/h. Fraction volumes were 1 ml. Aliquots of fractions (20 μl) were assayed for protein assay [7] and subjected to ESR analysis. Absorbance at 595 nm and peak height after 15-min incubation are denoted by ● and ■, respectively.

whereas 2.1 cm (2.5 min), 2.6 cm (15 min) and 2.8 cm (30 min) in the absence of the protein. However, from the result of the incubation of donor liposome alone with the stroma protein, a part of the peak height increase was due to the signal of the spin-labeled fatty acid released from the liposomes into the medium by the chloroplast lipase.

The stroma protein (1.3 ml, 41.6 mg) was further fractionated by the Sephadex G-100 column. As shown in Fig. 4, a lipase activity was eluted in void volume and there was a low activity in the fraction of about 30 kDa. Fractions 25–35 and 39–51 were combined and precipitated by 95% saturation of ammonium sulfate, respectively. The precipitates were dissolved in 50 mM Tris-HCl (pH 7.6) and dialyzed against the same buffer. As shown in Fig. 5, fractions 39–51 increased h with time only when incubated with both donor and acceptor liposomes, indicating galactolipid-transfer activity in these fractions.

Fractionation of the spinach leaf proteins by Sep-

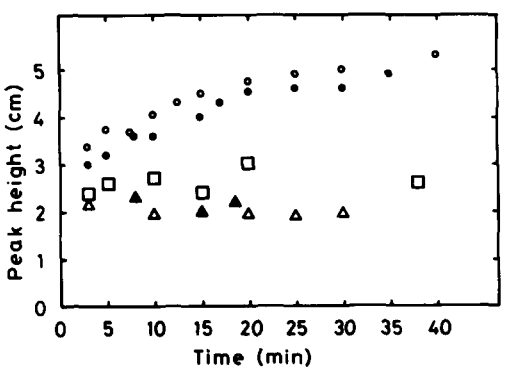

Fig. 5. Changes of peak height with time. Active fractions (39–51 in Fig. 4) were concentrated as described in the text and aliquots of the concentrate (179 μg protein) was incubated with donor (50 μg lipid) and acceptor (600 μg lipid) liposomes in 80 μl of 50 mM Tris-HCl (pH 7.6). Peak height increases with time (○ and ●, independent experiments). □, In the absence of protein; △, in the absence of acceptor liposome; ▲, donor liposome alone.

hadex G-100 and ion-exchange column chromatographies. Spinach leaves (1.1 kg) were homogenized and the pH 5.1 supernatant of the homogenate was salted out by the 75% saturation of ammonium sulfate. The resulting precipitate was then applied on a Sephadex G-100 column (4.4 × 100 cm) and eluted with 50 mM Tris-HCl (pH 7.6)/10% glycerol/0.02% NaN_3. The elution profile is shown in Fig. 6A. The lipase activity was again eluted in void volume, whereas galactolipid-transfer activities were eluted behind fraction 54 as a broad peak with a small shoulder around 28 kDa. Fractions 54–80 were collected and used for further fractionation by ion-exchange column chromatography. Fractions behind 80 were discarded because brown pigments were eluted together.

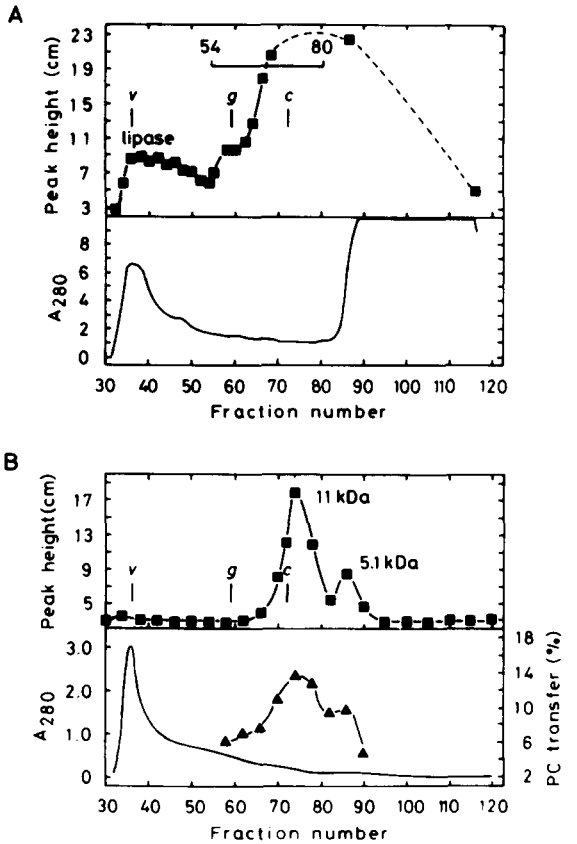

Fig. 6. Fractionation of spinach leaf protein by Sephadex G-100 column. The sample solution was applied to a 4.4×100 cm column of Sephadex G-100 which had been previously equilibrated with 50 mM Tris-HCl (pH 7.6)/10% glycerol/0.02% NaN_3. The column was calibrated by elution positions of Dextran blue 2000 and cytochrome *c* (horse heart, 12.5 kDa). Marks (v), (g) and (c) indicate the positions of void volume, 28 kDa and 12.5 kDa, respectively. Proteins were eluted with the same buffer at a flow-rate of 46 ml/h. Fraction volumes were 14.5 ml. Peak height after 15-min incubation and PC-transfer rate (%) after 20-min incubation are denoted by ■ and ▲, respectively. Reaction mixture for the assay of galactolipid-transfer protein contained 10 μl of donor, 30 μl of acceptor and 30 μl of fractions in a total volume of 80 μl. (A) 75% precipitate. (B) 35–75% precipitate.

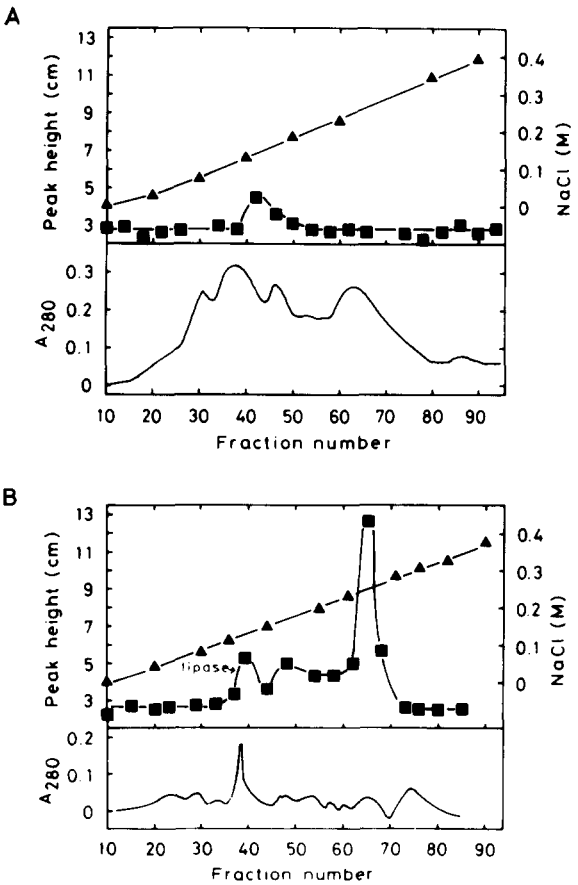

Fig. 7. Fractionation of spinach leaf protein by ion-exchange column. Active fractions (54–80) from Fig. 6A were dialyzed and applied, as described in the text, to 2.5×25 cm and 2.5×23 cm columns of DEAE- (A) and CM-Sepharose CL-6B (B), respectively, which had been previously equilibrated with 10 mM Tris-HCl (pH 7.6)/10% glycerol/0.02% NaN_3. Each column was eluted first with the same buffer and second with NaCl containing buffer at a flow-rate of 50 ml/h. Fraction volumes were 10 ml. Reaction mixture contained 10 μl of donor, 30 μl of acceptor and 40 μl of protein solution. Peak height after 15-min incubation and concentration of NaCl are denoted by ■ and ▲, respectively.

However, when the pH 5.1 supernatant of the leaf extract was salted out by the 35–75% saturation of ammonium sulfate, brown pigments were removed. Fractionation of the 35–75% precipitate by Sephadex G-100 column gave two new peaks of 11 and 5.1 kDa (Fig. 6B). The lipase activity and the small shoulder of 28 kDa were removed by this treatment.

Galactolipid-transfer protein activities of fractions 54–80 from the Sephadex G-100 were applied on a DEAE-Sepharose column joined to a CM-Sepharose column [13] equilibrated with 50 mM Tris-HCl (pH 7.5)/10% glycerol/0.02% NaN_3. Both columns were separated and eluted with the same buffer containing NaCl gradient, respectively. One acidic galactolipid-transfer protein activity was eluted at 0.15 M NaCl from the DEAE column (Fig. 7A), while one lipase activity (0.13 M) and two basic galactolipid-transfer protein activities (0.17 and 0.25 M) eluted from the CM column (Fig. 7B). The 11 kDa galactolipid-transfer protein corresponds to the basic one eluted at 0.25 M NaCl in the CM column as judged from the relative activity.

Discussion

Maeda and Ohnishi [23] developed a convenient assay system of phospholipid-transfer protein using spin-labeled PC. Similarly, attempt was made to develop an assay system of galactolipid-transfer protein using spin-labeled monogalactosyldiacylglycerol. Thus, a novel synthesis of a spin-labeled monogalactosyldiacylglycerol was required.

Semisynthesis of the spin-labeled monogalactosyldiacylglycerol

Heinz and Tulloch [14] reported earlier semisynthesis of monogalactosyldiacylglycerol, in which the protective groups of galactosyl moiety was hydrolyzed by trimethyl borate. This method resulted in the complete decomposition of the spin-labeled moiety, as indicated by the disappearance of the fluorescence-quenching spot on TLC (Merck F_{254}). When IV (Fig.1) was treated with 0.01 M HCl in tetrahydrofurane at room temperature, a large amount of IV was recovered even after 24 h. The other deprotecting methods [24,25] caused incomplete hydrolysis or decomposition of the spin-labeled moiety. The present method succeeded in the selective hydrolysis of protective groups of galactosyl moiety without affecting the doxyl group.

If *R. delemer* lipase would hydrolyze compound I to release fatty acid from the C-1 position, spin-labeled monogalactosyldiacylglycerol would be synthesized by a convenient process in which compound I is hydrolyzed by this lipase and then acylated by the doxyl acid. However, we cannot apply this method, because compound I was not attacked by this lipase.

As protective groups other than acetyl, methylthiomethyl ether [26,27] was unsuccessful to be introduced. Trimethylsilyl ether could be easily introduced and selectively removed by the fluoride ion, but it would be hydrolyzed under such a strong basic condition as used in the subsequent deacylation.

Fractionation of galactolipid-transfer protein extracted from the plant tissue

In the assay using spin-labeled lipid the presence of galactolipase confuses the detection of galactolipid-transfer protein activity.

It has so far been demonstrated that galactolipase was contained in young spinach leaves [28] and runner bean leaves [29]. In the presence study, galactolipase was recognized in the eluate from the Sephadex G-100 column but excluded in the void volume in the fractionation of both whole-leaf and chloroplast stroma extracts.

One acidic and two basic galactolipid-transfer proteins were separated from spinach whole-leaf extracts by ion-exchange chromatography (Figs. 7A and 7B). Among those galactolipid-transfer proteins only the major basic one was determined as 11 kDa protein in the present study (Figs. 6B and 7B).

On the other hand, a 30 kDa galactolipid-transfer protein was separated from the chloroplast extract by Sephadex G-100 column chromatography (Fig. 4). This would correspond to the 28 kDa galactolipid-transfer protein in the whole-leaf extract (Fig. 6A). This galactolipid-transfer protein would not be a contaminant from the cytosol, since no 11-kDa galactolipid-transfer protein was observed in the chloroplast stroma protein fractionation (Fig. 4), although the 11 kDa galacto-

lipid-transfer protein possessed higher activity than the lipase and 28 kDa shoulder (Fig. 6A).

Further experiments are required to show the localization of 5.1 kDa galactolipid-transfer protein.

On the role of galactolipid-transfer protein in the plant tissue

The mechanism of lipid transfer was proposed to be carried out by membrane flow [2] and the galactolipid-transfer protein.

Douce and Joyard [30] presented the invagination of the inner membrane of barley etioplast envelope during greening and they proposed that those vesicles were involved in the transportation of lipids as well as lamellar proteins synthesized outside the chloroplast. However, there was no evidence for such invagination in the chloroplast during normal developing in the light [30].

In addition, the present study demonstrated existence of galactolipid-transfer proteins in spinach leaves. Galactolipid-transfer protein with molecular weight of about 30 kDa isolated from chloroplasts would be functioning as a truck carrying the galactolipids between the envelope and thylakoid membranes in mature and young chloroplast, and may play an important role in metabolic turnover of those lipids, though further purification is required to confirm the operation of this galactolipid-transfer protein in this organelle.

Recently Kader et al. [13] reported the occrrence of a 9 kDa phospholipid-transfer protein in spinach leaves. This protein possessed a broad capacity for transferring phospholipids. Our study showed that a basic protein of 11 kDa from spinach leaf cytosol possessed not only galactolipid-transfer activity, but also phospholipid-transfer activity. One expectation is that this protein corresponds to the basic protein isolated by Kader et al. The role of galactolipid-transfer proteins in cytosol and the relationship between cytosol galactolipid-transfer proteins and phospholipid-transfer protein [13] remains to be clarified.

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