Structure of Lysophosphatidylglyceryl-Remazolbrilliant Blue (LPG-RBB) from the Surface of Dyed Wheat Starch Granules

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ABSTRACT

Wheat starch granule surface was covalently stained with Remazolbrilliant blue-R dye (RBB) and then extracted with 1% SDS containing 1% 2-mercaptoethanol (2-ME) at room temperature for 14.5 hr. The extracted blue-staining material (A_{650}) separated into two fractions. Low molecular weight (LMW) material was further purified by Sephadex G-75 size-exclusion chromatography and thin-layer chromatography. Infrared and nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectroscopy indicated that the structure of the purified LMW material was 18-*O*-(6-lysophosphatidylglyceryl)-RBB.

The importance of the role of the surface of wheat starch granules in determining cake textures (e.g., the degree of springiness and gumminess) has been studied and reported by Seguchi and Matsuki (1977). Surface proteins, lipids, and soluble starch were extracted from prime starch granules of wheat without measurable granule swelling or gelatinization of the starch granules by using 1% SDS solution containing 1% 2-mercaptoethanol (2-ME) at room temperature (Seguchi and Yamada 1989). Wheat starch granules were stained with Remazolbrilliant Blue–R dye (RBB), then extracted with SDS and 2-ME to obtain the surface materials: RBB-soluble starch and a lysophosphatidylglyceryl (LPG)-RBB compound (Seguchi 1995). After removal of these surface materials, the RBB-starch granule was swollen slightly and split into two parts at the equatorial groove by washing with SDS and 2-ME, which suggests that the surface materials were important in maintaining the native form of wheat starch granules (Seguchi and Kanenaga 1997). In this RBB staining and extraction process, it was discovered that the RBB molecule bound not only to soluble starch but also to LPG. Phospholipids such as lysophosphatidylcholine (LPC) (451–734 mg/100 g of flour), lysophoshatidylethanolamine (41–75 mg/100 g of flour), LPG (30–41 mg/l00 g of flour), and other lipids (27–62 mg/l00 g of flour) in wheat starch have been reported (Morrison 1983) and postulated to be templates for the helix structure of amylose (Morrison 1978). Knowledge of the role of these phospholipids in the formation and stability of the starch granule is limited (Fujino et al 1996). Davenport (1968) demonstrated that lysophospholipids can damage the gastric mucosal barrier and cause ulceration. It is quite remarkable that foods may contain much higher concentrations of lysophospholipids than those reported to cause damage. The most likely explanation is that the presence of starch provides protection by inclusion complexing of the lipid (Eliasson and Larsson 1993). The LPG-RBB complex is easily observed on thin-layer chromatography (TLC) plates, and it may be possible to use it in biochemistry and food chemistry research. However, the structure of LPG-RBB compound has not yet been determined. Therefore, the objective of this work was to characterize purified LPG-RBB.

MATERIALS AND METHODS

Materials

Prime starch granules were collected from Western white wheat by the acetic acid (pH 3.5) fractionation technique of Sollars (1958), and washed with ethyl ether. RBB (Rinderknecht et al 1967) and LPG were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

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Preparation and Extraction of RBB-Starch

The procedure for the preparation of the RBB-starch and the extraction of the RBB-stained material was as reported previously (Seguchi 1995). All procedures were performed at room temperature. The starch (12 g) was saturated with 6 mL of water and an RBB solution (1.2 g/120 mL of water) was added to the sample. Na_2SO_4 (24 g) was added, and the solution was stirred 45 min. A solution of Na_2HPO_4 (1.2 g/12 mL of water) was added, and the solution was stirred 75 min. The mixture was centrifuged and the supernatant was discarded. The pellet was resuspended in water and centrifuged, and the supernatant was discarded. Washing in this manner continued until the supernatant was clear. The RBB-starch was freeze-dried, then washed with 20 mL of SDS and 2-ME by agitation for 14.5 hr. After centrifugation (1,700 × *g*, for 20 min), the supernatant was dialyzed against water and freeze-dried.

Purification of Low Molecular Weight Material

The freeze-dried extract was dissolved in 1 mL of 90% dimethyl sulfoxide (DMSO). After boiling for 3 min, the solution was subjected to Sepharose CL-2B (1.0 × 95 cm) size-exclusion chromatography using 90% DMSO as the eluent. A profile was obtained by determining absorbance at 650 nm. The peak fraction of low molecular weight (LMW) material was separated, dialyzed against water, and freeze-dried. The dried sample was suspended in 1 mL of SDS and 2-ME and was completely dissolved by boiling for 3 min. The solution was subjected to Sephadex G-75 (1.0 × 95 cm) size-exclusion chromatography using SDS and 2-ME as the eluent. The blue-colored fractions (A_{650}) were dialyzed against water and freeze-dried. The sample was dissolved in chloroform and TLC was performed in a TLC plate (Silicagel 70 plate, Wako Chemical Industries, Ltd., Japan) using $CHC1_3$-methanol-water (65:25:4, v/v) (Seguchi 1995) for development. The TLC pattern was recorded with a Shimadzu high-speed TLC scanner CS-920 (Shimadzu, Japan). The major fraction was collected by scraping the TLC plate with a spatula, then extracting the powder with $CHC1_3$. The extracted material was subjected to TLC again to obtain purified LPG-RBB.

Analysis of LPG-RBB

The infrared spectrum of the LMW material (LPG-RBB) was recorded using a Nippon Denshi JIR 5500 spectrometer (KBr disc method). ^{1}H (300 MHz) and ^{13}C (75 and 125 MHz) nuclear magnetic resonance spectra were recorded using Varian XL-300 and Unity-500 (125 MHz, Nano probe) spectrometers.

Measurements were performed in CD_3OD at room temperature. With the Unity-500, 40 µL of sample was used for the Nano probe. Because it was possible that the isolation procedure might have altered the structure of the LMW material, isolation was performed three times.

RESULTS AND DISCUSSION

Purification of RBB-LPG

Figure 1 shows the profile of the Sepharose CL-2B size-exclusion chromatography of the extract from the RBB-starch with SDS and 2-ME (Seguchi 1995). The high molecular weight (HMW) material was soluble starch (Seguchi and Kanenaga 1997). The structure of RBB-starch was reported by Rinderknecht et al (1967). The LMW peak (fractions 33–47) was subjected to Sephadex G-75 size-exclusion chromatography. A single peak (A_{650}) was obtained.

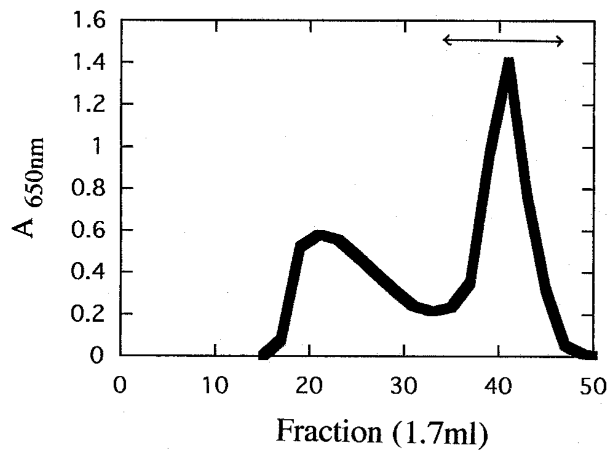

Fig. 1. Sepharose CL-2B gel-filtration chromatography profile of wheat starch granule surface stained with Remazolbrilliant blue-R dye extracted with 1% SDS containing 1% 2-mercaptoethanol. Arrows indicate the area of the low molecular weight fraction.

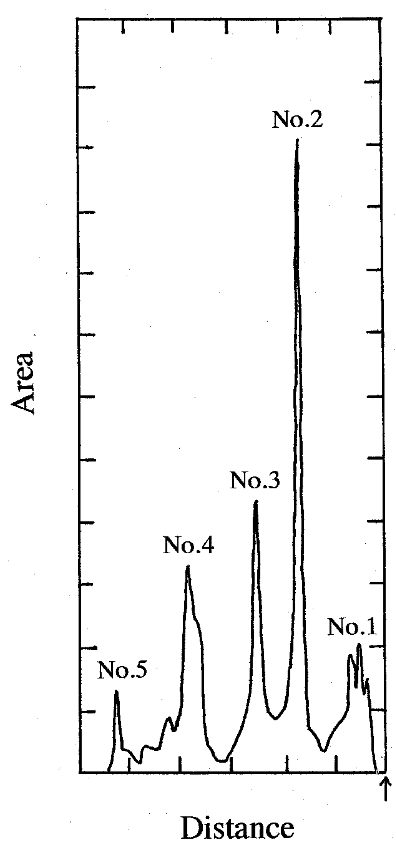

Fig. 2. Thin-layer chromatography profile of purified low molecular weight materials from the Sephadex G-75 gel-filtration chromatography. Arrow indicates application.

The purified LMW material was also subjected to TLC (Fig. 2). Peak No. 2, which was 36.0 % of the total blue material, was collected from the TLC plate by scraping it with a spatula and extracting with chloroform. TLC was then performed again to obtain the LMW material (3.7 mg). This sample produced a positive Dittmer blue color (Dittmer and Lester 1964) and a positive red-purple color with the periodate-Schiff reagent (Warner and Lands 1963), which indicates the presence of an LPG moiety in this LMW material (Seguchi 1995) and suggests an LPG-RBB structure. Other peaks (No. 1, 3, and 5) were not tested.

Structural Analysis of LPG-RBB

The infrared spectrum of the RBB-LPG revealed absorbances at 3,400 and 1,600 cm^{-1} due to an OH group, and strong absorption at 2,800–3,000 cm^{-1} due to its fatty acid moiety, which indicates the presence of lipids in LPG-RBB. The ^{1}H-NMR (300 MHz, CD_3OD) spectrum of LPG-RBB showed strong chemical shifts (1.8–2.2 ppm) due to the fatty acid moiety. Signal peaks at 7.2-8.2 ppm showed RBB moiety in LPG-RBB. The ^{13}C-NMR (75 MHz) spectrum of LPG-RBB also showed clear signal peaks due to fatty acid moiety. However, additional information could not be obtained because of the low level of the signal peaks. A sensitive measurement, using ^{13}C-NMR was performed with the Unity-500 instrument (125 MHz, Nano probe). The ^{13}C-NMR spectrum of RBB, LPG, and LPG-RBB is shown in Fig. 3 and Table I. The ^{13}C-NMR spectrum of the RBB moiety and the LPG moiety in LPG-RBB showed almost all the same signal peaks as did free RBB and free LPG. However, the C-6 component at 63.85 ppm of the glycerol moiety in the LPG changed to 69.30 ppm in the LPG-RBB, and the C-18 component at 56.40 ppm of the hydroxylethylsulfonyl group of RBB changed to 74.76 ppm, which suggests that

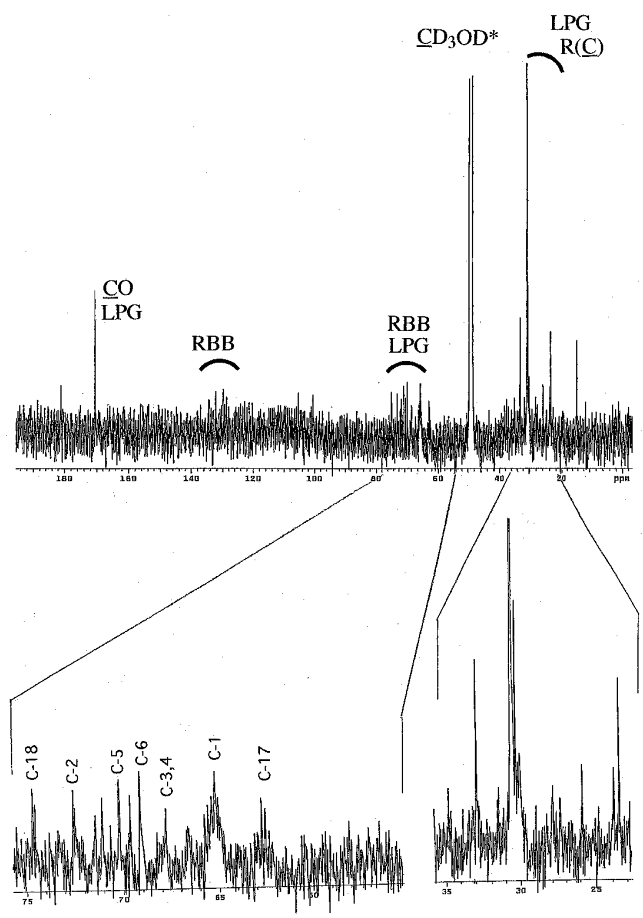

Fig. 3. ^{13}C-NMR spectroscopy of purified low molecular weight of lysophosphatidylglyceryl-Remazolbrilliant blue (LPG-RBB).

TABLE I
^{13}C-Nuclear Magnetic Resonance Chemical Shifts (ppm) of Lysophosphatidylglyceryl (LPG), Remazolbrilliant Blue (RBB), and LPG-RBB

Carbon	LPG	RBB	LPG-RBB
C-1	66.22		65.40
C-2	72.65		72.80
C-3	67.55		67.80
C-4	67.55		67.80
C-5	69.90		70.60
C- 6	63.85		69.30
C-1		139–141	
C-2		121–125	
C-3		132–135	
C-4		132–135	
C-4a		121–125	
C-5		126–130	
C-6		126–130	
C-7		126–130	
C-8		126–130	
C-8a		126–130	
C-9		180–190	
C-9a		121–125	
C-10		180–190	
C-10a		126–130	
C-11		139–141	
C-12		121–125	
C-13		132–135	
C-14		121–125	
C-15		126–130	
C-16		126–130	
C-17		62.00	62.80
C-18		56.40	74.76

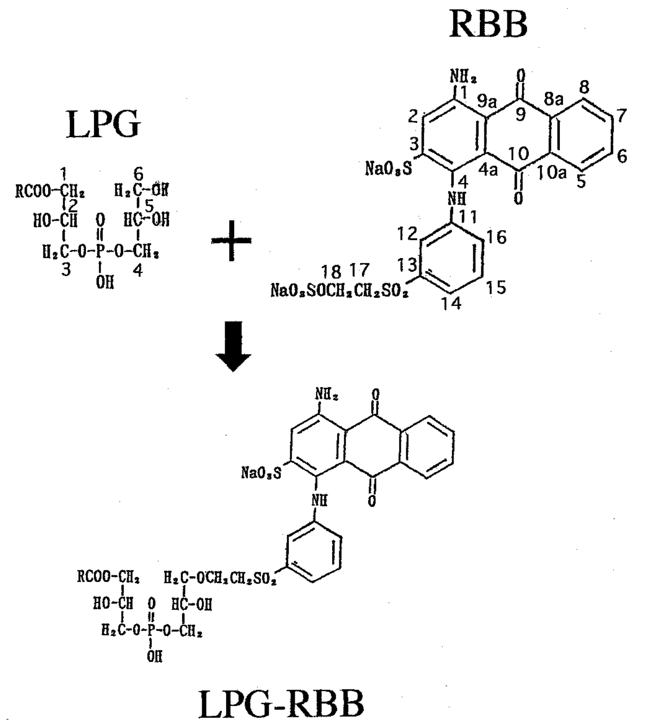

Fig. 4. Structure of low molecular weight of lysophosphatidylglyceryl-Remazolbrilliant blue (LPG-RBB).

the C-6 end of the glycerol moiety in the LPG binds covalently to the C-18 end of ethylsulfonyl group in RBB. From this analytical data, the structure of LPG-RBB was determined to be 18-*O*-(6-lysophosphatidylglyceryl)-RBB as shown in Fig. 4.

CONCLUSIONS

When wheat starch granule was stained with RBB, the extracted material showed HMW and LMW peaks when profiled by Sepharose CL-2B size-exclusion chromatography. The LMW material was further purified by Sephadex G-75 and double TLC, and was examined by infrared, ^{1}H-NMR, and ^{13}C-NMR spectroscopy. The structure of the LMW material was identified as 18-*O*-(6-lysophosphatidylglyceryl)-RBB.

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