Surface Staining of Wheat Starch Granules with Remazolbrilliant Blue R Dye and Their Extraction with Aqueous Sodium Dodecyl Sulfate and Mercaptoethanol

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ABSTRACT

Wheat starch granules were stained with Remazolbrilliant blue R (RBB) dye. The RBB-stained starch granules (RBB-starch) were successively extracted with 1% sodium dodecyl sulfate (SDS) solution containing 1% 2-mercaptoethanol (2-ME) at room temperature for 1 day (extraction 1), 5 days (extraction 2), and 15 days (extraction 3). The stained molecules were solubilized first, an indication that the RBB dye was bound to the granule surface. After each extraction with 1% SDS solution containing 1% 2-ME, granules were examined by scanning electron microscopy. The appearance of the residual starch structure changed greatly between extraction 2 and 3. After extraction 3, only a shell-like structure was present. X-ray diffraction analysis of this sample indicated an absence of crystallinity. The SDS extracts were subjected to Sepharose CL-2B gel-filtration chromatography. Two blue peaks were observed for extraction 1: one high and one low molecular size. The low molecular weight peak decreased for extraction 2 and was absent for extraction 3. The high molecular weight blue peak did not disappear, although the ratio of unstained carbohydrate to RBB-stained carbohydrate increased with time. The low molecular weight blue peak was further subjected to the Sephadex G-50 gel-filtration chromatography, and the resulting single peak was analyzed for sugars, proteins and peptides, and lipids. Only lipids were found. The main TLC spot was RBB stained and indicated lysophosphatidylglycerol by Dittmer and periodate spray reagents.

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The effects of surfactants on the starch gelatinization has been reported by many scientists (Harris and Sibblitt 1949, Lord 1950, Leach et al 1959, Bourne et al 1960, Collison et al 1960, Gray and Schoch 1962, Lorenz 1976, Ohashi et al 1980, Hoover and Hadziyev 1981, Ghiasi et al 1982, Eliasson 1985, Eliasson 1986, Doublier et al 1987, Takahashi and Seib 1988, Tester and Morrison 1990, and Hibi 1994). However, the washing effects of the surfactants on the wheat starch granules are poorly known (Seguchi and Yamada 1989). The importance of the role of the surfaces 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). Seguchi (1990) removed the surface proteins from chlorinated and nonchlorinated wheat starch granules and observed the differences in the extracts by sodium dodecyl sulfate (SDS) slab gel electrophoresis and Sephadex G-200 gel-filtration chromatography. When the wheat starch granules were continuously exposed to 1% SDS solution containing 1% 2-mercaptoethanol (2-ME) for 240 hr at room temperature, colorless and transparent granules with a patterned appearance were observed by phase-contrast light microscopy (Seguchi and Yamada 1989). This interesting patterned appearance is indicative of the granule structure. Hall and Sayre (1970), Fannon et al (1992), and Fannon and BeMiller (1993) found surface pores along the equatorial groove of the large wheat starch granules. SDS solution containing 2-ME could dissolve and remove various trace materials from the surface of the native wheat starch granules (Seguchi and Yamada 1989), which could be key components of the structural stability of the starch granule. Removal of certain trace components would gradually loosen the framework of the granule, which would allow the SDS-solution containing 2-ME to penetrate the loosened granules and begin to the dissolution process. Remazolbrilliant blue R dye (RBB) (synonyms; Diamirabrilliant blue R, Ostazin brilliantblue R, Primazinbrilliant blue RL, Remalanbrilliant blue R, Sumifixbrilliant blue R) binds to starch covalently as shown in Figure 1. RBB starch is used for amylase digestion tests (Rinderknecht et al 1967).

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In this work, the surface of wheat starch granules was stained with RBB without granule swelling, and RBB was used as a marker for surface molecules extracted by a 1% SDS solution containing 1% 2-ME. The stained materials were analyzed in an effort to determine the nature of the wheat starch granule surface.

MATERIALS AND METHODS

Materials

Wheat prime starch granules were prepared from western white flour by Sollar's acetic acid (pH 3.5) fractionation technique (Sollars 1958). The protein and moisture contents of the wheat starch granules were 0.06 and 14.0%, respectively. RRB ($C_{22}H_{16}N_2O_{11}S_3Na_2$; MW 626) dye, sudan black dye, lysophosphatidyl glycerol and other standards were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Other reagents were purchased from commercial sources.

Preparation of RBB-Starch

All procedures were performed at room temperature. Following the method of Rinderknecht et al (1967), starch (2 g) was suspended in 1.0 ml of water and stirred vigorously. A solution (20 ml) of 10% aqueous RBB solution was added to the suspension. During the following 45 min, sodium sulfate (4 g) was added in several portions. The reaction mixture was then treated with a solution of 200 mg of trisodium phosphate in 2 ml of water, and stirring was continued for another 75 min. The mixture was cen-

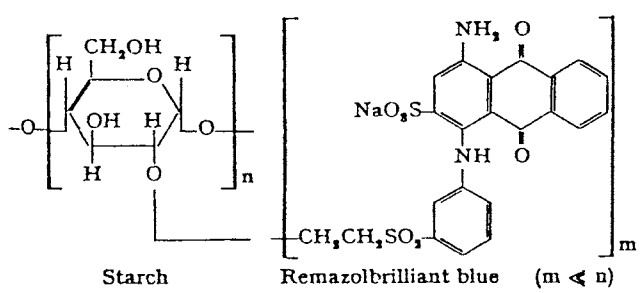

Fig. 1. Starch labeled covalently with Remazolbrilliant blue R (RBB) (Rinderknecht et al 1967).

trifuged and the supernatant discarded. The dark blue RBB-starch was resuspended in water and the supernatant discarded. Washing in this manner was continued until the supernatant was completely colorless. The RBB-starch was freeze-dried.

Extraction of RBB-Starch with SDS and 1% 2-ME

RBB-starch (2 g) was mixed with 40 ml of 1% SDS solution containing 1% 2-ME and stirred one day (extraction 1) at room temperature. The mixed solution was then centrifuged, and the pelleted starch was mixed with fresh 1% SDS solution containing 1% 2-ME and stirred for an additional four days (extraction 2). This process was repeated once more with stirring for an additional 10 days (extraction 3). All three supernatants were dialyzed against a large volume of water and freeze-dried (SDS-extracts). The starch was washed with a large volume of water, collected by centrifugation (SDS-starch), and freeze-dried.

Scanning Electron Microscopy

SDS-starch was coated with gold and viewed with a Hitachi S-430 scanning electron microscope operated at 20 kV.

Microscopic Observation of I_2-Stained Starch Granules

SDS extracted starch granules were stained with iodine (Krisman 1962) and heated in boiling water for a few minutes. One drop of the starch suspension was put between a slide and cover glass, and subjected to light microscopic observation after the cover glass was pressed to various degrees with a thumb.

Sepharose CL-2B and Sephadex G-50 Gel-Filtration Chromatography of the SDS-Extracts

Each SDS-extract (5.3 mg) was placed in 1.0 ml of 90% dimethyl sulfoxide (DMSO) solution, boiled for 3 min, and subjected to Sepharose CL-2B gel-filtration chromatography (1.0 × 95 cm). The eluting solvent was DMSO and water (9:1, v/v) and flow rate was 0.05 ml/min, or one column volume per 24 hr. The total carbohydrate (Dubois et al 1956) and the blue color (A_{650}) of each fraction were determined. Conditions for Sephadex G-50 gel-filtration chromatography were the same.

X-ray Diffraction Analysis

X-ray diffraction analysis was performed with a JEX-7E diffractometer (Japan Electron Optics Laboratory LTD). Starch (300 mg) was suspended in water and removed by filtration, then subjected to x-ray diffraction analysis while wet (Sugimoto et al 1982). Operating conditions were: 30 kV and 15 mA and the diffractograms of the samples were obtained at 2θ angles from 4° to 30°.

Determination of Reducing Power

Starch (10 mg) was suspended in 1 ml of 90% DMSO solution, boiled for 3 min, and dialyzed against 90% DMSO solution. The reducing power was then determined by the method of Hizukuri et al (1981). The total sugar of the same samples was measured by phenol sulfuric acid method (Dubois et al 1956).

Sudan Black Staining

Starch (500 mg) was suspended in 0.5 ml of 0.1% aqueous sudan black solution for several hours at room temperature (Seguchi and Yamada 1989). The mixture was centrifuged and the supernatant discarded. The starch pellet was washed with water until the supernatant was completely colorless. SDS-starch after the extraction 1 was also stained with sudan black in the same manner, and freeze-dried.

Paper chromatography (PC), SDS-Slab Gel Electrophoresis, and Thin-Layer Chromatography (TLC)

PC was performed by the double ascending method using a solvent system of pyridine, *n*-BuOH, and water (4:6:3, v/v) and components were visualized by aniline hydrogen phthalate spray (Partidge 1949). SDS-PAGE was performed as reported previously (Seguchi and Yamada 1989). TLC was performed using a solvent system of chloroform, methanol, and water (65:25:4, v/v) and visualization was achieved by the periodate-Schiff reagent (Warner and Lands 1963) and Dittmer reagent sprays (Dittmer and Lester 1964).

RESULTS AND DISCUSSION

Preparation of RBB-Starch and Extraction with 1% SDS and 1% 2-Me

Wheat starch granules were stained with RBB at room temperature without apparent damage to the granule and judged by their size (Fig. 2). SDS-starch extracted for 1, 5, and 15 days was observed by SEM (Fig. 3B–D). After 1 day (extraction 1) (Fig. 3B), virtually no changes in the granules were found when compared to control starch granules (Fig. 3A). However, a small central depression was observed in large granules (Fig. 3B). After 5 days (extraction 2), the central hollow of the granules was clearer, and granules had become flatter and more lenticular (Fig. 3C). When the SDS-extracted starch granule was stained with I_2 followed by boiling for several minutes, a small crevice (arrow) in the center and surrounding portions of the granule was observed by light microscopy (Fig. 4A). When pressure was applied to the I_2 stained starch granules between a slide and cover glass, the granules appeared to undergo peeling where an outer shell was removed easily (Fig. 4B) as evidenced by broken pieces of outer shell and hole-like structures (Fig. 4E arrow). The inside center portion appeared separated from the surrounding portion of the granules (Fig. 4B arrow). When even stronger pressure was applied to the cover glass, smaller debris could be observed (Fig. 4C and D). But non-SDS extracted starch granules, after the same I_2 treatment, did not break apart even after applying high pressure. Instead, they maintained their original round granular shape, which suggested that the structure in the starch granules was changed from a packed structure to a hollow structure by SDS extraction. First, the SDS solution containing 2-ME dissolved the starch granule surface polymers that had been stained with RBB, then the aqueous KI-I_2 solution could easily penetrate into the granule to produce a hollow structure. Using phase-contrast microscopy, Seguchi and Yamada (1989) observed concentric circles in both flat and round granules of the same SDS-starch, indicating a contrast in refractive indices between crystalline and amorphous regions. After 15 days (extraction 3), native granular

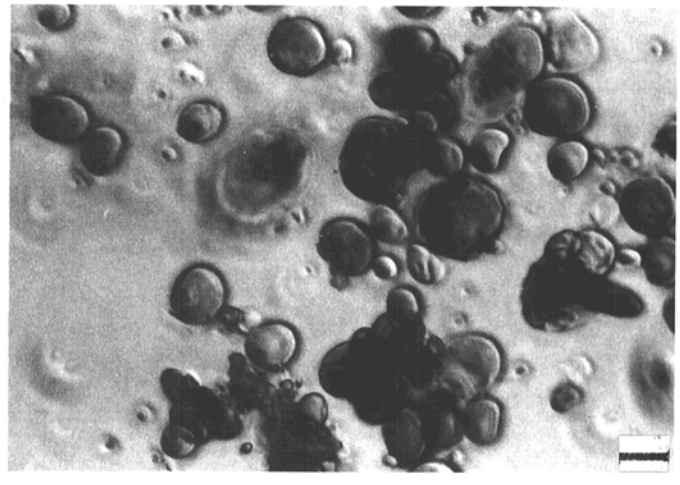

Fig. 2. Light micrograph of Remazolbrilliant blue R (RBB) stained starch granules (RBB-starch). Bar = 10 μm.

structure could not be observed, and almost all of the granules were eroded away to give only shell-like structures (Fig. 3D).

X-ray Diffraction Analysis of the SDS-Starch and Measurement of Reducing Power

The SDS-starch was subjected to x-ray diffraction analysis (Fig. 5). The unextracted control starch showed the typical A-type pattern of wheat starch, in which separation of peaks 4a and 4b is typically observed. After the extraction 1, the A-type structure was maintained. However, the intensity of the diffraction was lower, indicating a decreased crystallinity. When the SDS-extraction was continued for five or more days, the SDS-starch lost all identifiable or measurable crystallinity, probably depending on penetration of SDS-solution into crystalline portion. Lack of observation of any V-type structure indicated that lipid-amylose complexes are not present. The reducing power of SDS-starch preparations were measured by the method of Hizukuri et al (1981). The reducing power of the preparations (Table I) gradually increased with increasing extraction time, which may mean that oxidation is occurring by the shear force (Seguchi et al 1994).

Sepharose CL-2B Gel-Filtration Chromatography of SDS-Extracts

The color (A_{650}) and carbohydrate contents (CH) in the SDS extracts from RBB-starch were measured (Table II). The concentration of blue color in the extracts decreased with each extraction period. These data indicates that 1% SDS solution containing 1% 2-ME solubilized the starch polymers from the outside to the inside of the granules, assuming that the RBB dye was mainly bound to the granule surface. Figures 6A–C show the profiles of Sepharose CL-2B gel-filtration chromatography of the SDS extracts of extractions 1, 2, and 3, respectively. Fig. 6A shows two clear peaks (fractions 20 and 37) in both carbohydrate and A_{650}, fractions 20 and 37 were same the positions of V_o (the void volume) and V_{Total}, respectively, in the column. The concentration of color in fractions 20 and 37 were 6.11 and 8.33, respectively. In extractions 2 and 3 (Fig. 6B and C), the high molecular weight peak was present, while the low molecular weight peak gradually decreased. At the same time, the concentration of color in the high molecular weight peaks decreased from 6.11 to 0.85 to 0.47, respectively, in extractions 1, 2 and 3. The Sepharose CL-2B gel-filtration chromatography profile of the SDS extracts of extraction

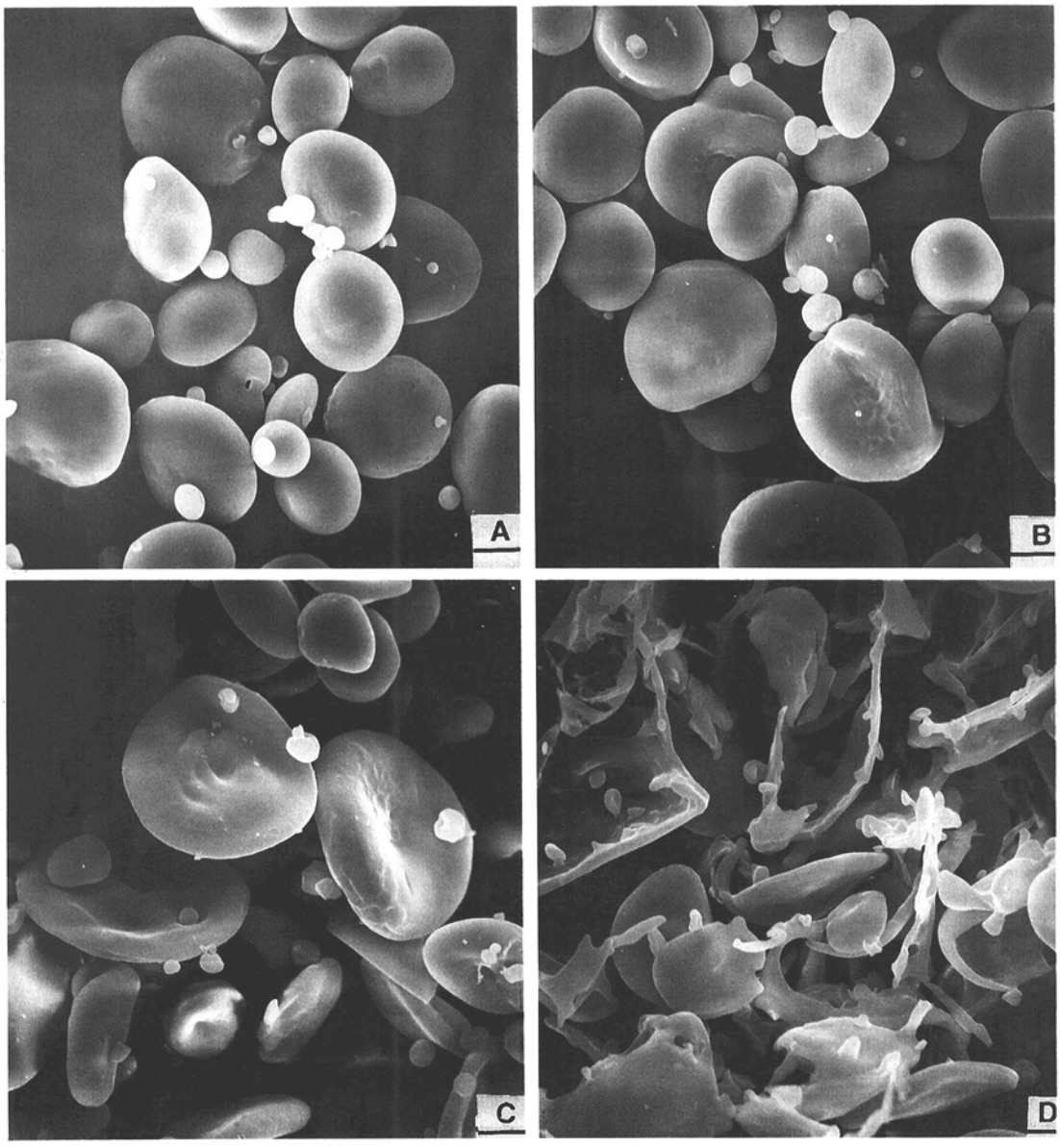

Fig. 3. Scanning electron micrographs. **A:** unextracted starch; **B, C,** and **D:** sodium dodecyl sulfate (SDS) starch after extractions 1, 2, and 3, respectively. Bar = 10 μm.

3 (Fig. 6C) showed virtually no stained low molecular weight peak, and the high molecular weight peak had a color concentration of only 0.47. When the high molecular weight peak was treated with KI-I_2, its λ_{max} showed 510–520 nm, indicating almost all amylopectin. These results suggest that low molecular weight materials which are mainly extracted in extraction 1 are located at the starch granule surface, along with some high molecular weight carbohydrates. RBB stained high molecular weight carbohydrates did dissolve in the first SDS extracts, followed by unstained high molecular weight carbohydrates gradually dissolving afterward. Morrison and Gadan (1987) reported that there should be gradients of amylose and lysophospholipid composition along the outer radii of wheat starch granule, and some of these would be easily extracted with SDS solution. With the removal of these starch granule surface materials, the SDS solution could gradually penetrate the granules and make a hollow structure (Fig.4E).

Analysis of the Low Molecular Weight Materials

During extraction 3, the low molecular weight materials virtually disappeared in the SDS-extracts and the appearance of the starch granules changed dramatically (Fig. 3D). Perhaps then, the low molecular weight materials are important in maintaining granule form. The low molecular weight materials (fractions 35–43) from Sepharose CL-2B gel-filtration chromatography (Fig. 6A) were pooled, concentrated, and subjected to Sephadex G-50 gel-filtration chromatography. One sharp peak was obtained. This peak was concentrated and analyzed by PC for sugars, by SDS-PAGE for proteins and peptides, and by TLC for lipids. Neither sugars, proteins, or peptides were detected by PC and SDS-PAGE

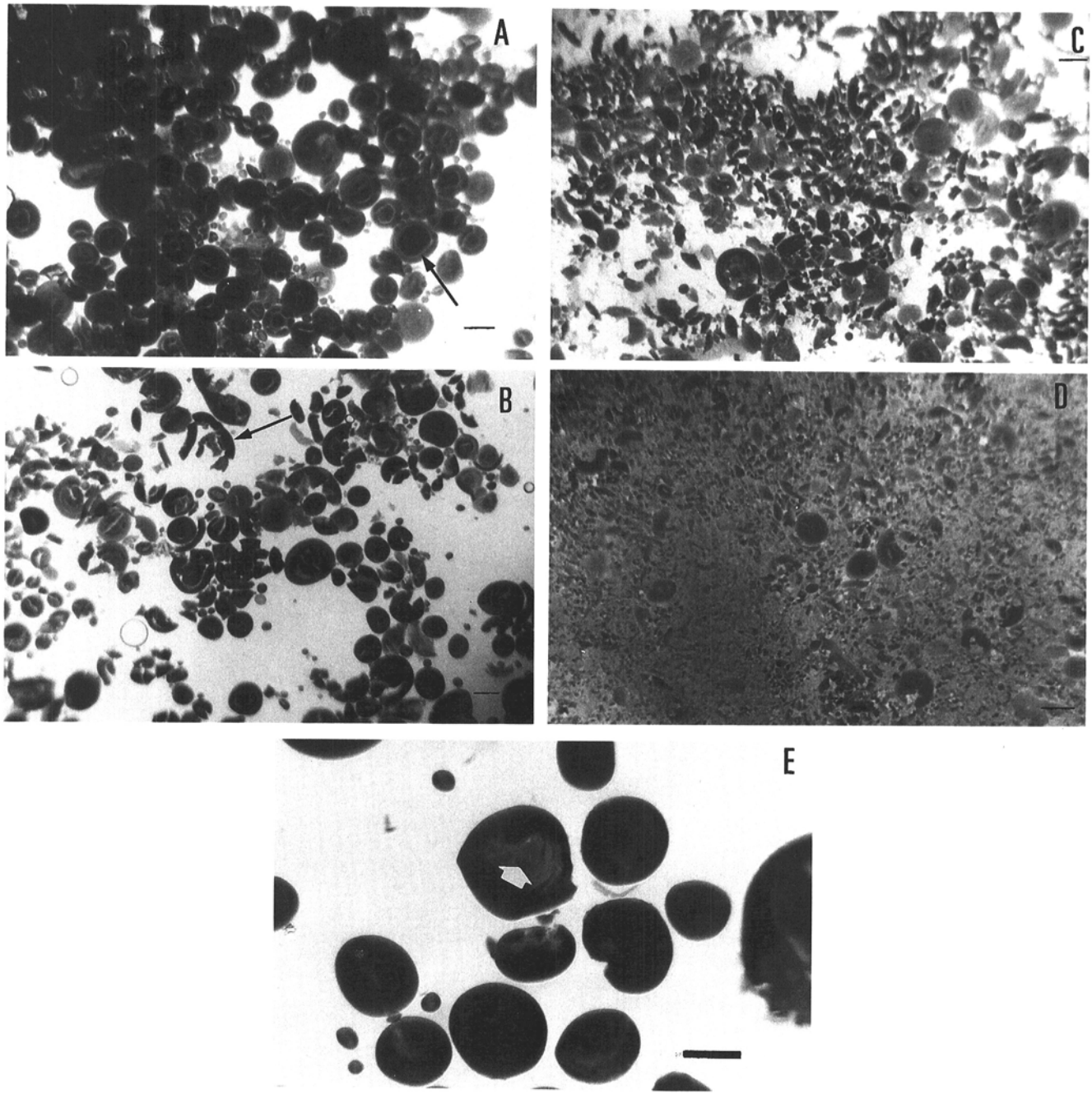

Fig. 4. Light micrographs of sodium dodecyl sulfate (SDS) extracted (5 days) starch granules after staining with I_2 and boiling in water for several minutes. **A:** glass cover slip lightly pressed; **B, C,** and **D:** glass cover slip gradually pressed harder; **E:** glass cover slip heavily pressed. Note hollow structure of crushed granules. Bar = 10 μm.

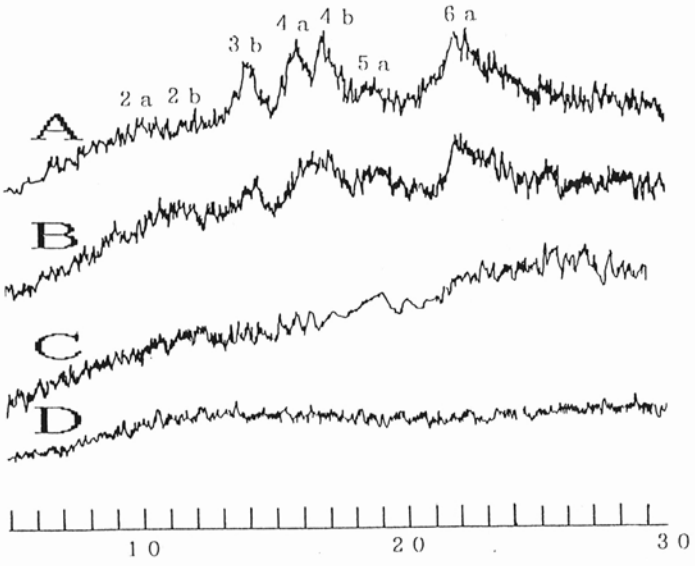

Fig. 5. X-ray diffraction analysis of sodium dodecyl sulfate (SDS) starch. **A:** unextracted starch; **B, C,** and **D:** starch after extractions 1, 2, and 3, respectively.

TABLE I
Changes of Reducing Power of SDS[a]-Extracted Wheat Prime Starch Granules

Extraction Time (days)	Reducing Power (μmoles/g × 100)
0	1,130
1	772
5	2,950
15	9,940

[a] 1% sodium dodecyl sulfate solution containing 1% 2-mercaptoethanol.

TABLE II
Color (A_{650}) and Total Soluble Carbohydrate from Wheat Starch Granules Extracted with 1% Sodium Dodecyl Sulfate Solution Containing 1% 2-Mercaptoethanol[a]

Extraction	Soaking Time (days)	Solubilized Carbohydrates (μg)/g[b]	A_{650} of Soluble Carbohydrates (mg)
1	1	388	3.71
2	5	412	0.55
3	15	962	0.23

[a] Three replicates. Standard deviation ±5.
[b] Solubilized carbohydrate determined by phenol sulfuric acid method (Dubois et al 1956).

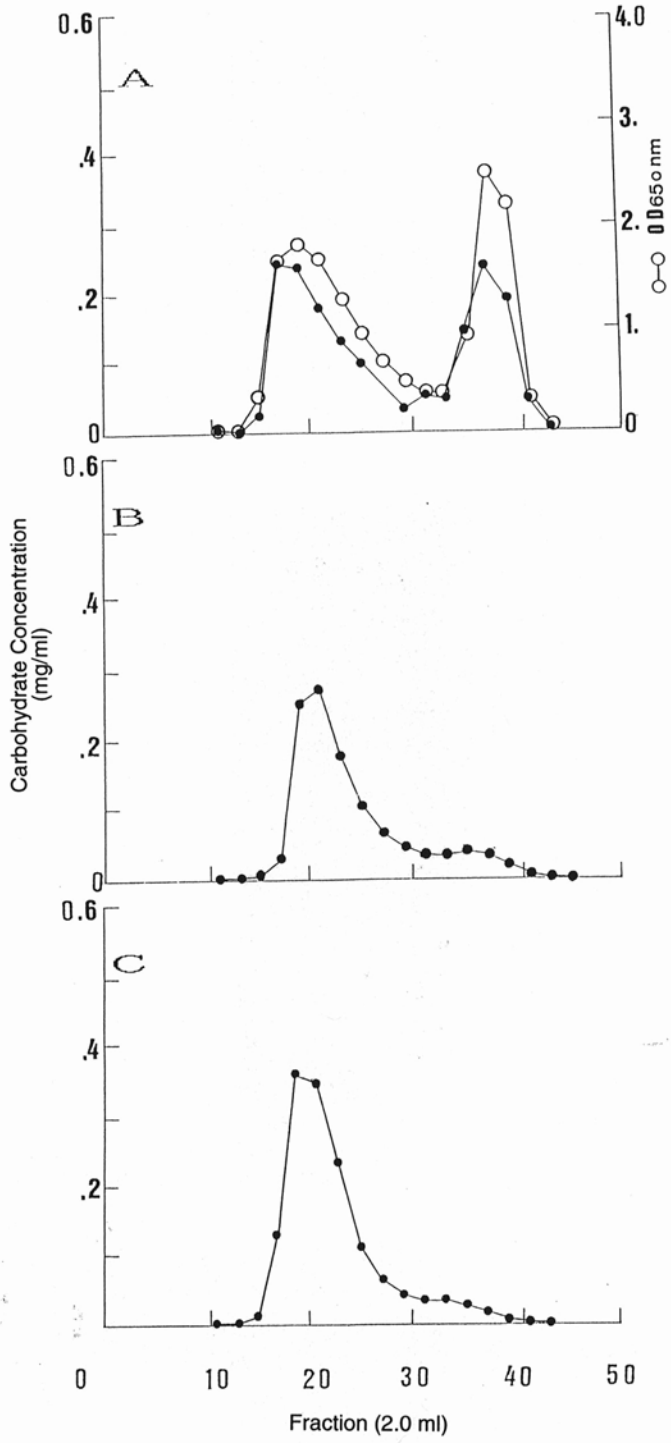

Fig. 6. Profiles of Sepharose CL-2B gel-filtration chromatography of sodium dodecyl sulfate (SDS) extracts. **A–C:** extractions 1, 2, and 3, respectively.

which indicates no amylose and surface and internal protein in the stained low molecular weight peak. Starch granule surface protein (Seguchi and Yamada 1989) would be excluded by the Sephadex G-50 gel-filtration step. TLC analysis revealed several RBB stained blue spots (Fig. 7). These were identified as phospholipids by the Dittmer reagent. Components 3, 4, and 5 in Figure 7 gave a positive Dittmer blue color. Components 3 and 4 in Figure 7 gave a positive red-purple color with the periodate-Schiff reagent, an indication of lysophosphatidylglycerol. A known sample of lysophosphatidylglycerol showed a different Rf value than the RBB-stained material. Lysophosphatidyl serine and lysophosphatidyl choline did not stain with RBB, but appeared on the same TLC plate (Seguchi and Yamada 1989). The presence of lysophospholipids in the wheat starch granule is widely known (Eliasson and Larsson 1993). However, the role of these lipids in the starch granule structure is poorly understood.

The starch granule surface also stained with sudan black dye (Fig. 8A) that stains lipids. After extraction, the granules with 1% SDS solution containing 1% 2-ME (extraction 1), sudan black did not stain the starch granule surface (Fig. 8B), indicating removal of surface lipids.

From those results, it is reasonable to think that some lysophospholipids such as lysophosphatidylglycerol prohibit the penetration of the SDS-solution into starch granules. The lysophospholipids are complexed with amylose and would be present on or near the surface of the granules (Morrison and Gadan 1987) and would keep the form of the starch granule from

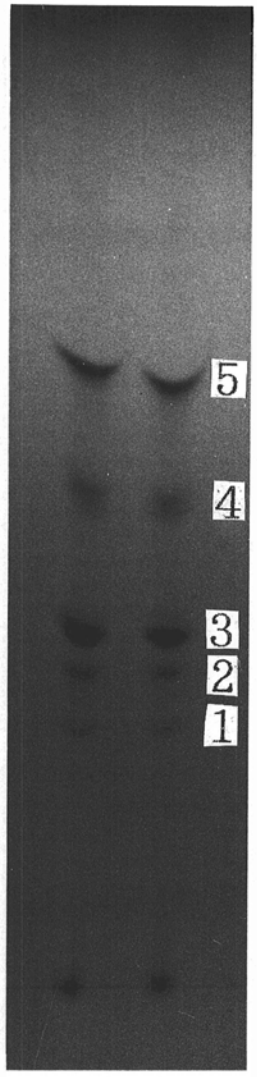

Fig. 7. Thin-layer chromatograph of sodium dodecyl sulfate (SDS) extract of Remazolbrilliant blue R-stained starch granule. Two columns are the same.

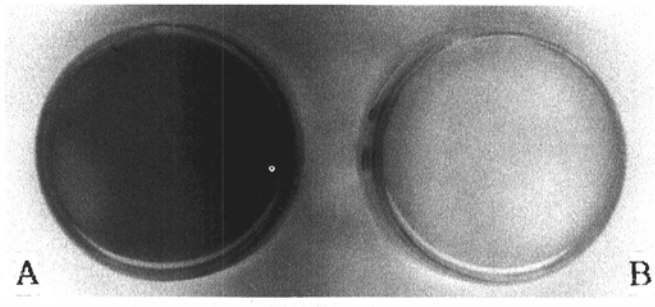

Fig. 8. Sudan black stained starch **(A)** and starch from extraction 1 **(B)** dried in petri dishes and photographed.

collapsing. The SDS-solution initially dissolves the starch granule surface lysophospholipids and reduces the surface tension of the granule or the SDS complexes with amylose. At the same time, surface high molecular weight carbohydrate is gradually dissolved, which could change the nature of the starch granule surface and allow the solution to penetrate the granule and form the hollow structure (Fig. 4E).

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