Effect of Phospholipids Transesterified Enzymatically with Polyunsaturated Fatty Acids on Gelatinization and Retrogradation of Starch

Tri Agus Siswoyo and Naofumi Morita*

Department of Applied Biological Chemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, 1-1, Gakuen-cho, Sakai 599–8531, Japan

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Summary The effects of phospholipids (PLs) transesterified with polyunsaturated fatty acids (PUFAs) with lipase (Aspergillus niger) on gelatinization and retrogradation of starch during storage were studied by differential scanning calorimetry (DSC). The resulting transesterified PLs were rich in PUFAs and linoleic acid, while the total percentage of PUFAs incorporated was 20.2%. The addition of PLs or PLs enzymatically transesterified with PUFAs (PUFA-PLs) to the starch sample decreased the gelatinization enthalpy of starch (\(\Delta h_g\)) slightly, but clearly increased the starch-lipid complexes (\(\Delta h_s\)). After 21 days of storage, the percent of retrogradation of starch became lower by the addition of 4% PLs or 4% PUFA-PLs to the starch sample when compared with the control. These results suggest that PLs retard retrogradation of starch during storage, whereas PUFA-PLs retard it greatly. The addition of PLs or PUFA-PLs increased the amount of \(\Delta h_{l,p}\), while re-gelatinization enthalpy decreased during storage, which suggests that PLs or PUFA-PLs could retard the retrogradation of starch.

Key Words gelatinization, retrogradation, transesterification, lipase, polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) of the n-3 series play a key role in the prevention and treatment of a wide range of human diseases and have been recognized as important dietary compounds. Imbalance of these compounds in the body is believed to cause a variety of diseases such as cardiovascular, hypertension, inflammatory and autoimmune disorders, depression and certain disrupted neurological functions (1–3). Long-chain PUFAs such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) are mainly obtained from seafoods which have originated from linolenic acid by a series of chain elongation and desaturation steps (3). Therefore, high consumption of marine lipids faithfully reflects sufficient dietary PUFA intake.

Though PUFAs can be emulsified with the aid of emulsifiers, their application to processed foods is considerably restricted because of their low solubility in water. Recently, phospholipids (PLs) enzymatically transesterified with PUFAs (PUFA-PLs) have been reported (4). Since PLs have been extensively used as an emulsifier, it can be expected that such esterified PUFA-PLs may serve to improve the nutritive quality of food-stuffs and to supply people who dislike fish with PUFAs for good health.

PLs are used throughout the world to improve food processing, such as clarifying starch pastes (5), enhancing the viscosity of starch, increasing dough tolerance, and improving baking properties (6–7). For this reason, the present study was focused on the preparation of PUFA-PLs by lipase-catalyzed transesterification between purified egg PLs and concentrated PUFAs from menhaden oil, and on their usefulness for starch processing in relation to gelatinization or retrogradation.

MATERIALS AND METHODS

Materials. Wheat starch (type “Hermes”, provided by Okumoto Flour Milling Co., Ltd., Osaka) was defatted with a hot butanol and water (3:1, v/v) mixture, and re-extracted three times in a screw-capped tube. To remove the remaining butanol, the starch sample was washed repeatedly with deionized water and then freeze-dried. PLs of egg lecithin origin (Wako Pure Chem. Ind., Osaka) were purified according to the method described by Sridhar and Lakshminarayana (8), and the concentrate of PUFAs was obtained from menhaden fish oil (Sigma Chemical Co., USA) according to the urea-complex method described by Wanasundara and Shahidi (9).

Immobilized lipase. Crude lipase from Aspergillus niger (Amano Pharmaceutical Co., Ltd., Nagoya) was immobilized with diatomaceous earth (Celite). Briefly, crude lipase (0.5 g) was suspended in ice-cold 20 mM acetate buffer, pH 6.5, containing 20 mM CaCl₂ (20 mL), to which chilled acetone (2 mL) and celite (2 g) were added. The mixture was then stirred for 3 h. Lipase-immobilized supports were filtered, washed several times

* To whom correspondence should be addressed.
E-mail: morita@biochem.osakafu-u.ac.jp
with chilled acetone and then dried in a desiccator for 2 h. The activity of lipase was measured at 410 nm by the spectrophotometric method with lauric acid 4-nitrophenyl ester as the substrate (10). One unit of activity was defined as the quantity of enzyme which liberates 1 μmol p-nitrophenol/min under routine assay conditions.

**Transesterification.** Transesterification of PLs with immobilized lipase was carried out at 40°C and with shaking on a rotary shaker at 150 rpm in the optimized reaction mixture consisting of PLs (0.6 g), PUFA concentrates (2 g), hexane (18 mL) and immobilized lipase (21 × 10^3 units/g) according to the method described by Totani and Hara (4). A molecular sieve (1 g) was added 24 h after incubation and the reaction was stopped by the addition of an acetone/ethanol (1:1 v/v) mixture. The transesterified PUFA-PLs were recovered from the reaction mixture by the method of Sridhar and Lakshminarayana (8).

Gas chromatography (GLC). Methylation of fatty acids incorporated in the PLs was done according to the method described by Jham et al. (11). GLC was carried out to characterize the amount of transesterified PLs. Analytical conditions were as follows: apparatus, Yanaco-GC 3800 gas-chromatograph equipped with a flame ionization detector; column, glass capillary column (0.3 cm i.d. × 200 cm) packed with 10% DEGS on 80–100 mesh chromosorb WAW (Nihon Gasukuro Kogyo); carrier gas, N2 at a flow rate of 40 mL/min; column temperature, programmed from 80 to 222°C at a rate of 3°C/min; injector temperature, 250°C. The respective fatty acid methyl esters were identified by comparing their retention times with those of authentic compounds, and quantified on the basis of their peak areas corrected by absolute response factor (12).

Preparation of starch-lipid complex. Starch (2 g) was dissolved in 3.4 mL of water to which various dispersion systems of PLs dispersed in water were added. These system were stirred at 360 rpm for 1 h at room temperature and completed with heating for 1 h at 60°C while stirring at 360 rpm; all of the samples were immediately cooled to room temperature in a water bath. The iodine-binding capacity of starch or starch-lipid complex was determined by potentiometric titration as described by Colburn and Schoch (13) using a Tao IM-1B ion meter in conjunction with a bright platinum electrode (TAO Electronics Ltd., Tokyo).

Differential scanning calorimetry (DSC). The DSC measurement was done using a Shimadzu-DSC-60 (Kyoto) as reported by Zhang et al. (14) and Morita et al. (15) with liquid paraffin as a reference. Starch-lipid complexes (10–11 mg) in aluminum DSC pans were weighed. The pans were sealed and scanned at a rate of 5°C/min from 30 to 120°C under nitrogen gas. The gelatinization temperature of onset temperature (Tg), peak temperature (Tp) and recovery temperature (Tr) of starch and starch-lipid complex, and enthalpy values for starch (ΔHg) and starch-lipid complex (ΔHg−c) were measured to characterize the thermal properties of starch. The samples were placed in aluminum capsules, which were hermetically sealed. Gelatinized samples were analyzed for starch retrogradation after storage for several days at 21°C. The temperature was raised from room temperature to 120°C at a rate of 5°C/min. Retrogradation of starch during storage was estimated from a DSC thermogram. The expressed value of retrogradation as a percentage was defined as the ratio of the regelatinization enthalpy (ΔHt) in the second run DSC heating to gelatinization enthalpy (ΔHg) in the first run DSC heating (16).

Statistical analysis. Values were obtained as the means ±SD of 3 determination, and analyzed by ANOVA followed by Duncan’s multiple range test. Differences among samples were considered significant at p < 0.05.

**RESULTS AND DISCUSSION**

Transesterification efficiency of PUFA-PLs

Fatty acid compositions after saponification or its additional urea treatment from menhaden oil are compared in Table 1. The DHA and EPA contents in the PUFAs concentrate were approximately 26.1 and 37.9%, respectively. The fraction rich in EPA and DHA prepared by the urea-complex method has been reported to serve as a good substrate for transesterification (17). Table 2 shows the fatty acid compositions of purified PLs per se and PLs transesterified by the use of immobilized lipase. Though the arachidonic acid (AA) content was 6.6% in the purified egg PLs, it increased about 2-fold after transesterification with lipase. Likewise, EPA and DHA, increased in their contents from 1.5 and 4.1% (before transesterification) to 3.8 and 11.8% (after transesterification), respectively. Although the transesterification efficiency was only 20.2%, the existence ratio of polyunsaturated fatty acids became apparently higher as reported by Totani and Hara (4).

### Table 1. Fatty acid compositions of saponified oil and its PUFA concentrate from menhaden (Brevoortia tyrannus).

| Fatty acids | Menhaden oila | PUFAsb |
|------------|---------------|--------|
| C14:0      | 8.6           |        |
| C15:0      | 0.7           | 0.3    |
| C16:0      | 29.0          | 12.7   |
| C16:1      | 2.2           | 5.0    |
| C18:0; C18:1 | 16.4       |        |
| C18:2      | 5.0           | 15.2   |
| C20:4      | 1.5           | 1.4    |
| C20:5      | 15.6          | 37.9   |
| C22:0      | 1.1           |        |
| C22:1      | 2.1           |        |
| C22:6      | 15.4          | 26.1   |
| Other      | 2.4           | 1.4    |

a Saponified for 1 h at 60°C with 1.3% KOH.
b Urea/fatty acids ratio, 4; crystalization temperature, 4°C; solvent, methanol.
Table 2. Fatty acid compositions of purified PLs per se and its further transesterified PLs with Aspergillus niger lipase

| Fatty acids | Purified PLs (%) | Transesterified PLs (%) |
|------------|------------------|------------------------|
| C20:4      | 6.6              | 13.3                   |
| C20:5      | 1.5              | 3.8                    |
| C22:6      | 4.1              | 11.8                   |
| Other      | 87.8             | 70.1                   |

Transesterification efficiency (%) 20.2
Recovery of PLs (%) 59.0

Table 3. Thermal properties of defatted starch in the presence of PLs and PUFA-PLs.

| PLs     | $T_o$ (°C) | $T_p$ (°C) | $T_r$ (°C) | $\Delta h$ (J/g) |
|---------|------------|------------|------------|------------------|
| Control | 65.0±0.3a | 68.9±0.2a  | 74.2±0.9a  | 7.8±0.7c         |
| 0.5%    | 64.2±0.5a | 68.6±0.3a  | 74.5±1.0ab | 7.2±0.8bc        |
| 1.0%    | 67.6±0.7a | 71.6±0.6a  | 78.6±0.9bc | 6.1±0.8ab        |
| 2.0%    | 69.4±0.4a | 72.1±0.5a  | 80.2±1.8c  | 6.1±0.4ab        |
| 4.0%    | 66.5±0.3a | 71.8±0.9b  | 76.1±1.9abc| 5.3±0.2a         |

Values followed by the same letter in the same column are not significantly different at $p<0.05$ using Duncan’s multiple test. Average±SD, n=3.

Abbreviations: $\Delta h_p$, enthalpy value of gelatinization of starch; $T_o$, onset temperature; $T_p$, peak temperature; $T_r$, recovery temperature.

Physicochemical properties of starch-lipid

The effects of PLs and PUFA-PLs on the thermal properties of defatted starch are summarized in Table 3. Addition of PLs or PUFA-PLs caused a significant increase in starch gelatinization onset ($T_o$) and peak temperatures ($T_p$), and a significant decrease in gelatinization enthalpies ($\Delta h_p$) in both treatments. The increased amount of PLs added to starch tended to decrease the area of the first endothermic peak in DSC thermograms due to the enthalpy of gelatinization of starch granules and tended to increase the second peak due to the enthalpy of gelatinization of starch-lipid complexes ($\Delta h_{sl}$), as reported by Eliasson and Ljunger (18). As shown in Fig. 1, the melting peak of the starch-lipid complexes disappeared completely in the case where the starch was defatted with hot aqueous butanol. Table 4 compares the $\Delta h_{sl}$ of a complex between defatted starch and PLs or PUFA-PLs in different amounts. When PLs or PUFA-PLs were added to defatted starch, these starch-lipid complexes were higher in enthalpy value than the control. The increase in the amount of PLs indicates the formation of starch-lipid complex, probably being caused by splitting of the bound lipid chains during delipidation of starch. Furthermore, the addition of 4% PUFA-PLs increased the $\Delta h_{sl}$ by 1.06 relative to that of 4% PLs, suggesting that PUFA-PLs rich in long-chain fatty acids promote the formation of starch-lipid complex rather than PLs. It is likely that PUFA-PLs have a more favorable configuration for forming starch-lipid complexes than PLs with short-chain fatty acids.

Table 4. Enthalpy melting for starch-lipid complexes in defatted starch.

| Additive | Added amount (%) | $T_p$ (°C) | $\Delta h_{sl}$ (J/g) |
|----------|-----------------|------------|----------------------|
| None     | 0               | 98.9±1.0   | 2.2±0.4              |
| PLs      | 4               | 99.7±1.6   | 3.4±0.8              |
| PUFA-PLs | 2               | 109.3±2.1  | 2.8±0.3              |
|          | 4               | 111.0±2.8  | 3.6±0.3              |

Average±SD, n=3. Abbreviations: $\Delta h_{sl}$, enthalpy value of starch-lipid complex; $T_p$, peak temperature.

4% PUFA-PLs increased the $\Delta h_{sl}$ by 1.06 relative to that of 4% PLs, suggesting that PUFA-PLs rich in long-chain fatty acids promote the formation of starch-lipid complex rather than PLs. It is likely that PUFA-PLs have a more favorable configuration for forming starch-lipid complexes than PLs with short-chain fatty acids.

To clarify the nature of the starch-lipid complex, the iodine affinity for the complex was measured. Figure 2 exemplifies potentiometric titration curves of three typical systems. The titration curve of defatted starch (the
control) raised after 12 mL of 0.001 N iodine solution. On the other hand, both starch+PLs and starch+PUFA-PLs were sigmoidal in the titration curve until about 4 and 2 mL of iodine solution, respectively. This result suggests that the presence of PLs and PUFA-PLs caused a great reduction in iodine affinity for starch. This view is supported by a considerable change in the color of solutions during titration. When amylose form a complex with iodine, the blue color of the amylose-iodine complex will appear clearly. However, a purple color of the solution indicates the absence of amylose-iodine complex. Therefore, the purple color thus observed suggests that these lipids might occupy the iodine binding sites of amylose. The failure in the appearance of blue color could be attributed to no interaction between iodine and lipid because amylose reacted with iodine to form a blue complex even in the presence of lipids (19). Furthermore, iodine titration of starch-lipid systems showed sigmoidal characteristics and the system became blue upon addition of the iodine solution.

**Retrogradation of starch**

The DSC curves of the control and two complexes between starch and PLs or PUFA-PLs stored for up to 21 d are shown in Fig. 3. The $\Delta h_t$ values of all the samples tested were very small after the first heating because starch was completely gelatinized during the heating. However, PLs tended to increase the $\Delta h_t$ as compared with the control, whereas PUFA-PLs increased it more distinctly.

The complex with starch and 4% PLs showed a reduction in the $\Delta h_t$ when compared with the control without PLs after 21 d storage. In the course of storage, the $\Delta h_t$ also decreased gradually. Furthermore, PUFA-PLs brought about a greater change in the peak area. This result suggests that the retrogradation of starch was retarded by the coexistence of PUFA-PLs. Figure 4 shows the percent of retrogradation ($\Delta h_t/\Delta h_g$) for control, starch+4% PLs, and starch+4% PUFA-PLs as a function of storage time. As is obvious from the figure, the percent of retrogradation became lower by the addition of 4% PLs or 4% PUFA-PLs to the starch sample when compared with the control. This result indicates that PLs or PUFA-PLs play an important role in starch retrogradation.

In conclusion, this research has referred to the phenomenology of the effects of PLs or PUFA-PLs on starch gelatinization and retrogradation. The results indicate that PLs retard the retrogradation of starch and that PUFA-PLs are more effective in retardation of the return.
rogradation. Alternatively, the delipidation of starch may enable its OH group to form new hydrogen bonds and result in an increase in retrogradation. However, further study is necessary to clarify the structural characteristics of amyllose- or amylopectin-lipid complexes, especially the interaction between starch and PLs different in chain lengths.

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