Transesterification of Soy Lecithin by Lipase and Phospholipase

Anna-Marja Aura*, Pirkko Forssell, Annikka Mustranta and Kaisa Poutanen

VTT Biotechnology and Food Research, FIN-02044 VTT, Finland

ABSTRACT: Soy lecithin was modified by enzymatic transesterification in a solvent-free system. 1,3-Specific *Rhizomucor miehei* lipase was found to be efficient in the transesterification with lauric acid and oleic acid, where oleic acid was more incorporated into soy lecithin. Phospholipase A_2 incorporated lauric acid hardly at all, but it hydrolyzed lecithin efficiently. The mixture of lipase and phospholipase A_2 (1:1, w/w) incorporated lauric acid to the same extent as did 1,3-specific lipase alone at the same total enzyme concentration. The main fatty acids replaced were palmitic and linoleic acids by 1,3-specific lipase and its mixture with phospholipase A_2, and linoleic and linolenic acids by phospholipase A_2 alone, suggesting an improved oxidative stability of the resulting product. Hydrolysis could not be prevented, but it could be regulated by incubation time and by enzyme dosage. The minimal water content for significant incorporation of lauric acid into lecithin was below 0.5% of the weight of the reaction mixture.
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KEY WORDS: Lipase, phospholipase, soy lecithin, transesterification.

Most emulsifiers used in the food industry are costly to produce, with the exception of lecithin (1), which is the polar fraction of vegetable oils and contains mainly phospholipids (2). There can be problems caused by the relatively poor oxidative stability of phospholipids and the limitations in their emulsifying properties (1). These limitations potentially can be eliminated by the production of modified lecithins through the use of enzymes under mild conditions.

Lysolecithin is produced commercially by enzymatic hydrolysis of lecithin with phospholipase A_2 (3). It has pronounced oil-in-water emulsifying properties due to its hydrophilicity, and it can prevent the recrystallization of starch (4). Transesterification of polyunsaturated to more saturated fatty acids can improve the oxidative stability and can modify the emulsifying properties of phospholipids (5).

The yield of transesterified phospholipids is diminished by side reactions, hydrolysis, and acyl migration (6,7). Hydrolysis causes a loss of phospholipids, which is enhanced by acyl migration, because this spontaneous nonenzymatic reaction can change the position of the remaining acyl group in the glycerol backbone of a lysophospholipid. The resulting compound becomes more susceptible to further enzymatic hydrolysis, yielding water-soluble components (6) with no emulsifying properties. If specific enzymes are used, incorporation of fatty acids into lysophospholipids also can be a consequence of acyl migration (6).

The extent of side reactions can be regulated by the amount of water in the reaction mixture (6,8,9), by the enzyme dosage (5,6,10), and by the acyl donor concentration (6,7). Free fatty acids are more efficient acyl donors than their methyl or ethyl esters in transesterification of phosphatidylcholine (11). The factors affecting the extent of acyl migration in transesterification of triglycerides have been studied thoroughly. Acyl migration can be minimized by a large quantity of the enzyme, by using optimal incubation time, and by a low amount of water (12).

The specificity of the enzyme also affects transesterification. 1,3-Specific *Rhizomucor miehei* lipase, being selective for the *sn*-1 position of phosphatidylcholine, was efficient in water-saturated toluene (11). Phospholipase A_2, specific for position *sn*-2, did not show transesterification activity, but the enzyme was capable of significantly hydrolyzing phosphatidylcholine (11,13).

The aim of this study was to investigate transesterification of soy lecithin. Two enzymes—1,3-specific *R. miehei* lipase and porcine pancreatic phospholipase A_2—and their mixture (1:1, w/w) were compared, and the possibility of minimizing hydrolysis by varying incubation time, the amount of water, or enzyme dosage was studied. We have studied transesterification of phosphatidylcholine in a solvent-free system and found the 1,3-specific *R. miehei* lipase to be efficient (14). Two acyl donors, lauric and oleic acid, were used. Most of the studies reported earlier concerning enzymatic transesterification of phospholipids have been performed in an organic solvent (5–8,10,11). The present study was conducted in a solvent-free system.

EXPERIMENTAL PROCEDURES

Materials. Soy phosphatidylcholine (P-5638) was purchased from Sigma Chemical Company (St. Louis, MO). Free fatty acids for use as transesterification substrates were lauric acid (No. 61620; Fluka Chemie AG, Buchs, Switzerland) and oleic acid (No. 36,452-5; Aldrich-Chemie GmbH & Co. KG,

*To whom correspondence should be addressed at VTT Biotechnology and Food Research, P.O. Box 1500, FIN-02044 VTT, Finland.

Steinheim, Germany). Immobilized *R. miehei* lipase (Lipozyme IM-60; Novo Industri A/S, Bagsvaerd, Denmark) exhibited lipase activity of 700 nkat/g and phospholipase activity of 100 nkat/g. The enzyme was freeze-dried before use, and the dried sample contained 1.5% water (w/w). Phospholipase A_2 was from porcine pancreas (Lecitase 10L; Novo Industri A/S) and was immobilized on Celite No. 535 (Celite Corporation, Lompoc, CA). The phospholipase activity of the immobilized preparation of phospholipase A_2 was 6700 nkat/g, and it contained 0.3% water (w/w). Phospholipase C from *Bacillus cereus* (No. 691 950; Boehringer Mannheim GmbH, Mannheim, Germany) was used for analytical purposes, and the hydrolyzed phospholipids were identified by using monostearoyl-*rac*-glycerol (No. M-2015) and distearin (No. D-9019) from Sigma Chemical Company as standards.

Immobilization. Phospholipase A_2 was immobilized on diatomaceous earth (Celite) by adsorption. Celite was washed with distilled water and dried in an oven before the immobilization. Phospholipase A_2 (0.2 mL, 40 μkat/mL) was diluted in 0.8 mL 20 mM TRIS–HCl buffer (pH 8) containing 20 mM $CaCl_2$, mixed with Celite (1 g) and dried by lyophilization.

Enzyme assays. Phospholipase activity was determined according to Mustranta *et al.* (13), and lipase activity was assayed as described by Mustranta (15).

Moisture content. Moisture content of the enzyme preparations was measured by drying at 105°C overnight, cooling the desiccated preparations, and weighing. Moisture content of the reaction mixtures was measured with a Karl-Fischer titrator (DL 18; Mettler, Zürich, Switzerland).

Transesterification procedure. The water content was adjusted to 1% (vol/wt) of the weight of a substrate mixture (3 g, phosphatidylcholine-fatty acid at a ratio of 1:10, mole/mole) and enzyme, and the mixture was equilibrated with magnetic stirring (150 rpm) at 60°C for 15 min. The substrate mixture was viscous, especially when lauric acid was used as the fatty acid. The total amount of added enzyme was 40% (w/w) of the substrates, if not otherwise stated, and when both enzymes were used, 20% (w/w) of each enzyme was added, making the total enzyme concentration 40% (w/w). Incubation was continued for 6, 16, or 24 h, after which the product was separated from the enzyme by dissolving the mixture in chloroform/methanol (2:1, vol/vol) and by filtration on a sintered-glass funnel. Two replicates were performed.

Fractionation of the lipids. The mixture was evaporated and fractionated in a silicic acid column according to Nyberg (16). The total phospholipid fraction was hydrolyzed with phospholipase C according to Kates (17), and the resulting mono- and diacylglycerols were separated by thin-layer chromatography (TLC) (No. 11798; Merck, Darmstadt, Germany) with petroleum ether/diethyl ether/acetic acid (85:15:1, vol/vol/vol) as the solvent system. The fractions were visualized with primulin (No. P-7522) from Sigma Chemical Company under ultraviolet radiation, identified with authentic standards, and scraped. The fatty acids were saponified and methylated with 20% boron-trifluoride in methanol (No. 801663; Merck). Fatty acid methyl esters were analyzed by gas chromatography, and the total amounts of lysolecithin (monoacylglycerol fraction on the TLC plate) and lecithin (diacylglycerol fraction on the TLC plate) were calculated from the total amount of fatty acid methyl esters of the fractions with methyl heptadecanoate (No. 51640; Fluka Chemie AG) as an internal standard and a weighted average of the molecular weight of the total fatty acids. The total amount of soy phospholipids was taken as the sum of lysolecithin and lecithin fractions.

Gas-chromatographic analysis. Fatty acid methyl esters were determined by gas chromatography on an HP-5890 (Hewlett Packard Company, Little Falls, DE) equipped with a flame-ionization detector. An HP-FFAP (free fatty acid phase) column (25 m, 0.30 μm film thickness and 0.32 mm diameter) was used with helium (35 cm/s) as a carrier gas. The injector temperature was 250°C, detector temperature was 260°C, and the temperature program during the analysis went from 50 to 240°C (7°C/min), after which the temperature was constant (240°C) for 30 min.

RESULTS AND DISCUSSION

Comparison of the enzyme preparations. The enzyme preparations compared were 40% (w/w) 1,3-specific *R. miehei* lipase, 40% (w/w) immobilized phospholipase A_2, and the mixture (1:1, w/w) of these two enzymes at a total enzyme concentration of 40% (w/w). When the lauric acid-to-soy lecithin molar ratio was 10:1, 1,3-specific *R. miehei* lipase could incorporate up to 27% lauric acid in the total soy phospholipids in 24 h, whereas only 7% incorporation could be achieved with immobilized phospholipase A_2 (Fig. 1A). When these enzyme preparations were added together, the incorporation of lauric acid was almost equal (23%) to the incorporation with lipase alone, even though the overall enzyme dosage remained the same (40%, w/w of the reactants). The reason for the small difference in the incorporation was probably the use of diatomaceous earth as a carrier for phospholipase A_2, and not the enzymes themselves enhancing each other. The carrier could have modified the availability of water so that water was brought to the site of the 1,3-specific lipase, and thus the incorporation by this enzyme was enhanced. Alternatively, the reaction may be saturated for catalyst at the 20% level of enzyme.

In earlier studies, when the fatty acid-to-phosphatidylcholine ratio was 10:1, only 12% incorporation of heptadecanoic acid into egg phosphatidylcholine by 1,3-specific *R. miehei* lipase was found after 24 h at 40°C (11). However, Svensson *et al.* (6) reported 30–40% incorporation of heptadecanoic acid into egg phosphatidylcholine with *R. miehei* lipase in toluene after 24 h at 40°C and a fatty acid-to-phosphatidylcholine ratio of 40:1. A higher degree of incorporation as compared to the present study may be due to the higher fatty acid-to-phosphatidylcholine ratio and the organic solvent environment, which allows better accessibility of enzymes to substrates and better availability of water at the site of the enzyme.

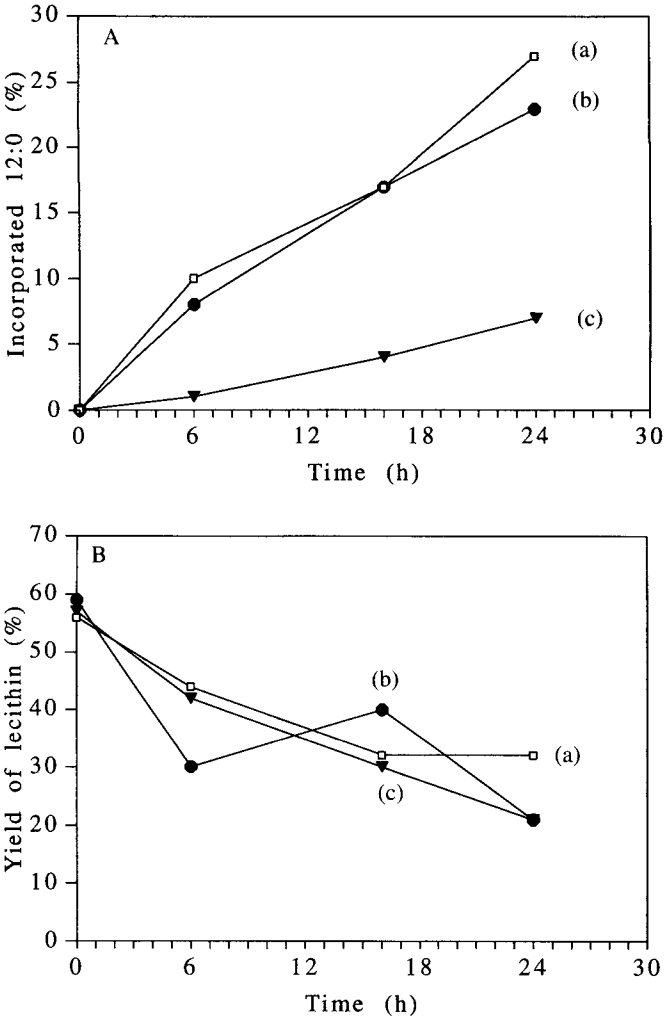

FIG. 1. Comparison of enzyme preparations in the transesterification of soy phospholipids at 60°C, with 150 rpm magnetic stirring and 1% water content in the system. A) Incorporated lauric acid in the total phospholipids; B) the yield of unhydrolyzed lecithin in the total phospholipids. Curves: (a) 1,3-specific *Rhizomucor miehei* lipase; (b) mixture of 1,3-specific lipase and phospholipase A_2 (1:1, w/w); (c) phospholipase A_2. The total enzyme dosage was 40% (w/w of the substrate) in all cases.

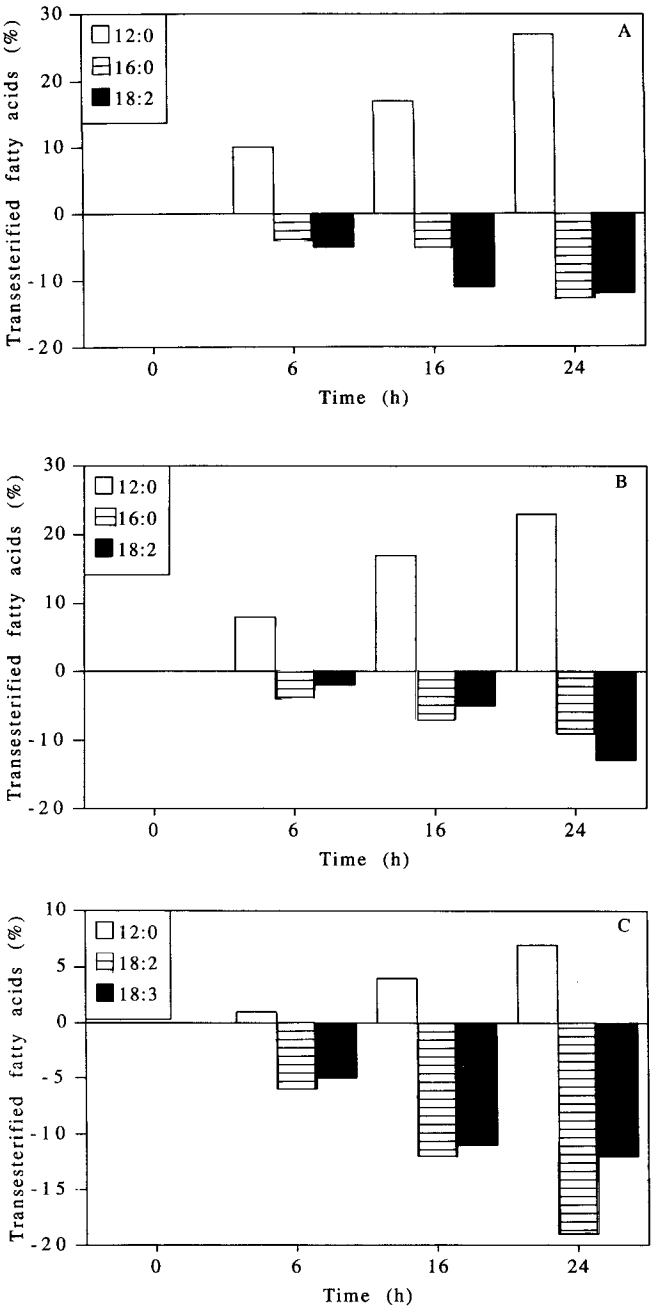

FIG. 2. The replaced fatty acids in transesterification of soy phospholipids with different enzyme preparations. Reaction conditions were as described in Figure 1. A) 1,3-Specific *Rhizomucor miehei* lipase; B) mixture of 1,3-specific lipase and phospholipase A_2 (1:1, w/w); C) phospholipase A_2.

Hydrolysis of lecithin occurred concomitantly with the incorporation of the fatty acid into phospholipids (Fig. 1B), as has also been reported previously (11,13). The amount of lysolecithin was high (40–45%) even before the enzyme addition, which may have been caused by deterioration of the lecithin. The possibility of endogenous phospholipolytic activity in the substrate cannot be ruled out. Phospholipase A_2 could not incorporate lauric acid significantly (Fig. 1A), which was in accordance with the findings of Svensson *et al.* (11). However, it hydrolyzed lecithin to the same extent as 1,3-specific lipase did (Fig. 1B), even though its nominal phospholipolytic activity was 67 times greater than that of 1,3-specific lipase. The difference between the performance and the nominal activity of phospholipase A_2 could be due to different enzyme–substrate ratios or to different stirring efficiencies in the reaction mixture and in the enzyme assay. Also, the amount of enzyme used in the study was excessive.

The main fatty acids replaced in the resulting product with 1,3-specific *R. miehei* lipase and its mixture with phospholipase A_2 were palmitic and linoleic acids (Fig. 2A,B). It seems that phospholipase A_2 acted on different fatty acids than 1,3-specific lipase did because the polyunsaturated fatty acids—linoleic and linolenic acids—were removed efficiently by phospholipase A_2 alone (Fig. 2C).

Somewhat surprising was the observation that use of the enzyme mixture did not affect the pattern of fatty acids even half as much as the use of phospholipase A_2 alone. After 24 h of incubation, only palmitic acid was replaced, to a somewhat smaller extent, by the enzyme mixture than it was by 1,3-specific lipase alone, and it was not replaced at all by phospholipase A_2. This is probably caused by the predominant distribution of saturated fatty acids at the position *sn*-1 in plant phospholipids (18) and by the phospholipase A_2 specificity to the position *sn*-2 of phospholipids. There were not enough differences between the fatty acid changes caused by 1,3-specific lipase alone and its mixture with phospholipase A_2 to verify that the lipase could enhance phospholipase A_2 activity.

Effect of enzyme concentration. A high enzyme dosage (40% of 1,3-specific lipase, w/w of reactants) was needed for effective incorporation of lauric acid into soy phospholipids (Fig. 3). Further additions were not possible in the solvent-free system because the reaction mixture was gummy and already difficult to mix. The next step would have been a continuous column reactor, containing immobilized enzyme, to use a higher enzyme-to-substrate ratio. Incorporation of lauric acid increased almost linearly with the enzyme dosage used in this study. Hydrolysis of lecithin depended on the enzyme concentration in another way. The increase of enzyme concentration did not result in a linear increase in hydrolysis of lecithin, but even a 10% (w/w) enzyme dosage significantly hydrolyzed lecithin (Fig. 3). However, differences between the enzyme dosages were quite small as stirring efficiency was higher with the lower enzyme dosages, which was beneficial for the reaction. When operated in hexane, the optimal enzyme concentration was lower (15%, w/w of reactants) in a study where nonimmobilized *R. miehei* lipase was used to catalyze the incorporation of eicosapentaenoic acid into phosphatidylcholine (10).

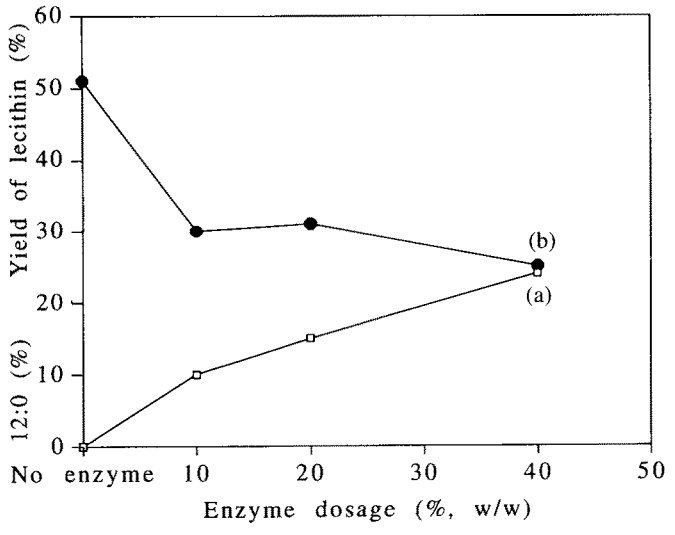

FIG. 3. Effect of enzyme dosage on the transesterification of soy phospholipids by 1,3-specific *Rhizomucor miehei* lipase. Incubation time was 24 h. Other reaction conditions were as described in Figure 1. Curves: (a) incorporated lauric acid in the total phospholipids; (b) the yield of unhydrolyzed lecithin in the total phospholipids.

Effect of incorporated fatty acids. Oleic acid was incorporated into the total soy phospholipids more efficiently than was lauric acid (44 and 27% in 24 h, respectively; Fig. 4A). Hydrolysis of lecithin also proceeded faster in the reaction mixture when oleic acid was used instead of lauric acid (Fig. 4B). The yield of lecithin after 24 h of incubation was 15 and 32% for oleic and lauric acids, respectively. The reaction mixtures of the same molar ratios contain different amounts of free fatty acid on a weight basis. The higher the molecular weight, the more fatty acid there was per lecithin, and the easier the mixture was to stir. The molecular weight of oleic acid is higher than that of lauric acid. Thus, the higher mass ratio in the substrate mixture and the increased stirring efficiency may have contributed to the result.

Effect of water content. Water content is crucial for the optimization of the yield of transesterified phospholipids. It seemed that the minimal water content of the reaction mixture for the significant incorporation of lauric acid into soy phospholipids by 1,3-specific *R. miehei* lipase was below 0.5% (w/w) (Fig. 5). The dependence of the degree of hydrolysis was not greatly influenced by the amount of water in the range tested (Fig. 5). This may have been due to an inhomo-

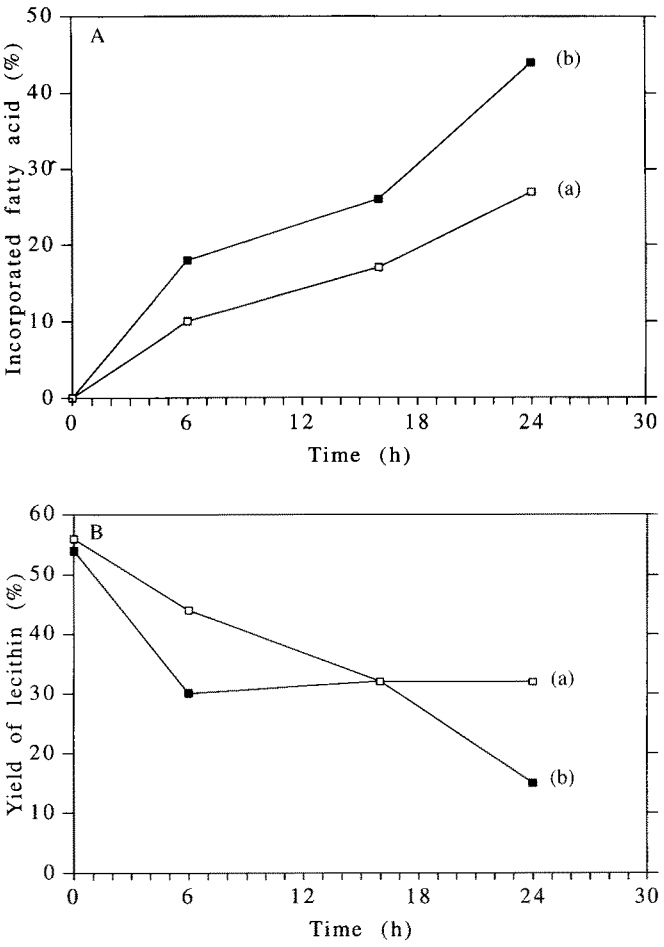

FIG. 4. Incorporation of lauric (a) and oleic (b) acids in soy phospholipids by 1,3-specific *Rhizomucor miehei* lipase. Reaction conditions were as described in Figure 1. A) Incorporated fatty acids in the total phospholipids; B) unhydrolyzed lecithin in the total phospholipids.

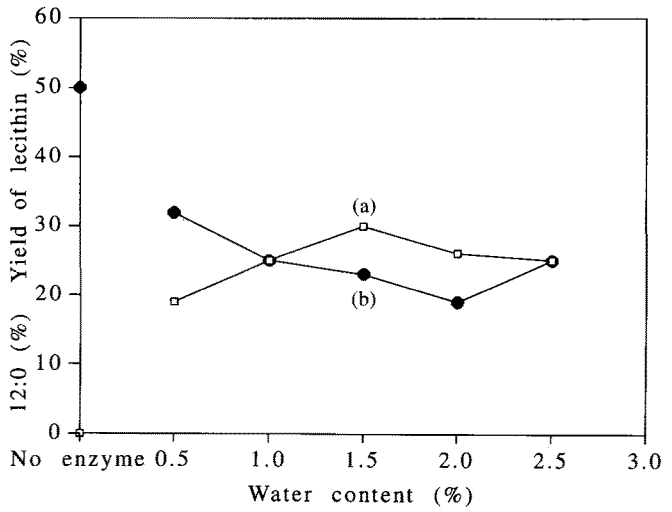

FIG. 5. Effect of water content of the system on transesterification of soy phospholipids by 1,3-specific *Rhizomucor miehei* lipase. Reaction conditions were as described in Figure 1. Incubation time was 24 h. Curves: (a) incorporated lauric acid in the total phospholipids; (b) the yield of unhydrolyzed lecithin in the total phospholipids.

geneous distribution of water in the viscous substrate mixture incubated at a rather high temperature (60°C). Svensson *et al.* (6) adjusted the water activity by equilibrating the reaction mixture at different humidities, and they found a linear correlation between water activity and hydrolysis at 40°C, when 1,3-specific *R. miehei* lipase-catalyzed transesterification of phosphatidylcholine in toluene was studied.

In another study concerning the hydrolysis of soy phosphatidylcholine by immobilized *R. miehei* lipase in an organic solvent, the predicted degree of hydrolysis was dependent on both the amount of catalyst and the amount of water so that, at higher enzyme concentrations, hydrolysis was retarded by the increase of water content (8). These results were in accordance with the results presented here, because the amount of the enzyme used was high.

The degree of hydrolysis increased with water content more strongly when tallow was interesterified with 1,3-specific *R. miehei* lipase in a solvent-free reaction mixture, containing water from 0 to 2.2% (19), than when phospholipids were transesterified in this study. Perhaps the environment surrounding the enzyme was less polar in the reaction mixture containing tallow than in the mixture used in the present study that contained phospholipids. This could affect the influence of water on the hydrolysis.

Over half of the incorporated fatty acids, lauric, and oleic acids were found in the lysolecithin fraction throughout the study. This incorporation was higher with phospholipase A_2 alone than with 1,3-specific *R. miehei* lipase. The amount of lauric acid in lysolecithin also increased when the enzyme dosage was low (results not shown).

In conclusion, soy lecithin could be transesterified enzymatically by immobilized 1,3-specific *R. miehei* lipase and its mixture with immobilized phospholipase A_2. Phospholipase A_2 alone was active in hydrolysis but incorporated hardly any lauric acid by transesterification. The main fatty acids replaced with phospholipase A_2 were polyunsaturated acids, whereas palmitic acid was also significantly replaced with *R. miehei* lipase alone or the mixture of the enzymes. A long incubation time (24 h), a high enzyme concentration (40% w/w of reactants), and a water content above 1% (w/w) were required for the highest incorporation of lauric acid into soy phospholipids. Hydrolysis could not be avoided. Both incorporation and hydrolysis were enhanced by the use of oleic acid instead of lauric acid as the reactant. Further studies are needed to verify the changes in the functional properties, oxidative stability, and emulsifying activity caused by the enzymatic transesterification of soy lecithin.

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