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Synthesis of Phytosteryl Ester Containing Pinolenic Acid in a Solvent-Free System Using Immobilized *Candida rugosa* Lipase

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ABSTRACT: Phytosteryl ester synthesized with pinolenic acid (PLA) from pine nut oil is expected to have features of both phytosterol and PLA. In this study, lipase from *Candida rugosa* (CRL) was immobilized and then used to optimize conditions for synthesis of phytosteryl ester containing PLA. Lewatit VP OC 1600, a macroporous hydrophobic resin, was selected as the best carrier, and the optimum condition for the immobilization of CRL was established. With immobilized CRL prepared, synthesis of phytosteryl ester with fatty acid from pine nut oil was carried out. Parameters investigated were temperature, molar ratio (phytosterol to fatty acid), enzyme loading, and vacuum. Optimum conditions for synthesis of phytosteryl ester were a temperature of 60 °C, molar ratio of 1:4, enzyme loading of 10% (based on the total weight of the substrate), and pressure of 80 kPa. The maximum conversion of phytosteryl ester was ca. 93 mol % at the optimum condition.

KEYWORDS: *Candida rugosa, enzyme immobilization, phytosteryl ester, pine nut oil, pinolenic acid*

INTRODUCTION

Pine nut oil contains several Δ5-unsaturated polymethylene interrupted fatty acids (Δ5-UPIFAs). Pinolenic acid (PLA), formally designated as *cis*-5,9,12-18:3, is a predominant Δ5-UPIFA of pine nut oil.[1] Several studies have showed that pine nut oil has low density lipoprotein (LDL) cholesterol-lowering potential.[2,3] In addition, it has been shown that pinolenic acid has an appetite-suppressing effect by stimulating the release of cholecystokinin and glucagon like peptide-1, hormones that function as an appetite suppressant.[4,5]

Phytosterols, which comprise plant sterols and stanols, are a group of steroid compounds having a ring structure with carbon side chains. Among this compound family, *β*-sistosterol, campesterol, and stigmasterol are the most common.[6] Because of their chemical structure similarities with cholesterol, phytosterols are known to have a cholesterol-lowering effect by inhibiting cholesterol absorption due to their chemical structure similarities with cholesterol.[7−9] However, despite their health benefits, free phytosterols from vegetable oils are not easily applicable in foods because of their high melting point and low solubility both in water and in oil.[10] In addition, due to their low physical reactivity and solubility, phytosterols must be ingested in excessively high doses.

In comparison with free sterols, phytosteryl esters have higher solubility in the oil phase contributing to the practical application in fat-based food products. Several studies[11−13] have reported on the chemical synthesis of phytosteryl ester with fatty acid. However, chemical method may involve problems such as high energy consumption and formation of side products such as 3,5-diene steroid derivatives.[14] Thus, enzymatic catalysis seems to be a better choice for synthesizing phytosteryl ester because it proceeds efficiently under mild conditions leaving fewer byproducts. The use of immobilized enzymes has advantages over using free enzymes such as improving the control of the reaction, minimizing product contamination by enzymes in food products, and expanding the choice of reactor design.[15] Although lipase-catalyzed synthesis of phytosteryl ester has been previously reported, most of the studies previously mentioned were carried out in the presence of organic solvents.[16,17]

In this study, the immobilization of *Candida rugosa* lipase (CRL) was studied for the synthesis of phytosteryl ester. The synthesis of phytosteryl ester by direct esterification of phytosterol and fatty acid from pine nut oil was carried out in a solvent-free system using the immobilized CRL prepared in this study. The effects of temperature, molar ratio of phytosterol to fatty acid, enzyme loading, and vacuum were explored by monitoring the time course of these reactions.

MATERIALS AND METHODS

Materials. Pine nuts (*Pinus koraiensis*) were purchased from the Agricultural Marketing Center (Seoul, Korea). Oil from the pine nuts was extracted with *n*-hexane, and the oil was turned into fatty acid by saponification method according to our previous study.[18] Fatty acid from pine nut oil used in this study was composed of *cis*-9,12-octadecadienoic acid (46 mol %), *cis*-9-octadecenoic acid (27 mol %), *cis*-5,9,12-octadecatrienoic acid (13 mol %, pinolenic acid), octadecanoic acid (5 mol %), hexadecanoic acid (2 mol %), *cis*-5,9-octadecadienoic acid (2 mol %), *cis*-11-docosenoic acid (1 mol %), and *cis*-5,11,14-eicosatrienoic acid (1 mol %). *β*-Sitosterol (from soybean, ≥40%) was purchased from Sigma Aldrich Co. (Seoul, Korea). The *β*-sitosterol was analyzed by gas chromathography and found to contain three major phytosterol analogues (*β*-sitosterol, 42.7 mol %; campesterol, 27.1 mol %; dihydrobrassicasterol, 25.2 mol %; total, 95.0 mol %).[18] CRL was purchased from Meito Sangyo Co., Ltd. (Tokyo, Japan). Nine carriers were tested for the immobilization of

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CRL. Lewatit VP OC 1600 (Lewatit), Accrurel MP 1000 (Accurel), and Duolite A568 (Duolite) were purchased from Lanxess Energizing Chemistry (Leverkusen, Germany), Membrana GmbH Accurel system (Obernburg, Germany), and Rohm and Haas (Chauny, France), respectively. Amberlite XAD 7HP (XAD7), Celite 545 (Celite), Zeolite, Dowex 50w x8 (Dowex), Amberlite XAD4 (XAD4), and octyl silica were purchased from Sigma Aldrich Co. (Seoul, Korea). Phytosteryl ester (purity >98.0%) used as a standard was supplied by ADM (Decatur, IL). Other chemicals used in this study were of analytical grade unless otherwise noted.

Enzyme Immobilization. For carrier screening, 1.5 g of powder type CRL was suspended in 15 mL of sodium phosphate buffer solution (100 mM, pH 7.0). Enzyme suspension was stirred for 3 h at 400 rpm and then centrifuged for 30 min at 3100*g* at 25 °C. Supernatant was taken, and its protein concentration was determined according to the method of Lowry et al.[19] The supernatant (15 mL) was added to flasks containing 1.5 g of individual carriers. All of the carriers and their abbreviations are listed in Table 1. Hydrophobic carriers were prewetted in ethanol and washed with 60 mL of buffer solution before the enzyme solution was added. The enzyme solutions with carriers were shaken at 200 rpm and incubated in an orbital shaker at 30 °C for 15 h. Aliquots of supernatant were withdrawn from each flask, and the amount of protein in the supernatant was determined to estimate the protein fixation level (%) and the amount of protein bound at the particle (mg g^{-1}). Subsequently, the carriers were separated from the enzyme solution by filtration and washed immediately with 75 mL of buffer solution to remove unbound enzyme. The fixation level and the amount of protein in the immobilized enzyme were estimated by subtracting the protein remaining in the enzyme solution after the binding from the initial protein concentration. The carriers with the immobilized enzyme were dried overnight at room temperature and then in a vacuum drying oven for 12 h at 40 °C. The immobilized enzymes were stored at 4 °C prior to use.

Table 1. Carriers for Immobilization of CRL and Their Characteristics[a]

	full name	abbreviation	characteristics
hydrophobic	Lewatit VP OC 1600	Lewatit	DVB-cross-linked methacrylate polymer
	Accurel MP 1000	Accurel	macroporous polypropylene
	Amberlite XAD4	XAD4	polystyren DVB
	octyl silica	octyl silica	octyl-functionalized hydrophobic silica
hydrophilic	Amberlite XAD 7HP	XAD7	nononionic weakly polar macroreticular acylic ester
	Celite 545	Celite	Kieselghr soda ash flux calcined
	Zeolite	Zeolite	microporous, aluminosilicate minerals
	Dowex 50w x8	Dowex	cation-exchange styrene DVB
	Duolite A568	Duolite	polymerized phenol-formaldehyde ion-exchange resin

[a]All of the carriers were used for the immobilization of CRL in this study.

The fixation level (%) and the amount of protein adsorbed in the immobilized enzyme particle (mg g^{-1}) were calculated by the following equations:

$$\text{fixation level (\%)} = \frac{a-b}{a} \times 100$$

$$\text{protein amount in the particle (mg g}^{-1}\text{)} = \frac{a-b}{c+a-b} \times 100$$

where a is the amount of initial protein in the enzyme solution (mg), b is the amount of unbound protein in enzyme solution after immobilization (mg), and c is the amount of carrier used for the immobilization (g).

To estimate the effect of the initial protein concentration of CRL solution, varying amount of free type CRL was suspended in the buffer solution at concentrations of 5, 10, 20, 30, 40, 75, 100, 150, and 200 mg mL^{-1}(free CRL (mg)·buffer solution $(mL)^{-1}$). The rest of the immobilization process was carried out in the same manner as for the carrier screening described above.

Activity Test for the Immobilized Enzyme. Enzymatic esterification of phytosterol and fatty acid was carried out as activity test. Fatty acid from pine nut oil (3.614 g, 13.66 mmol) and phytosterols (1.386 g, 3.41 mmol) were placed in a 50 mL water-jacketed glass vessel and mixed at 300 rpm. The vessel reactor was preheated to 50 °C, and then 0.25 g of enzyme (5% of the total weight of the substrates) was added. Samples (50 μL) were withdrawn periodically and mixed with 950 μL of chloroform. Subsequently, the sample was filtrated with a 0.45 μm GHP Acrodisc syringe filter (Pall Corp., Port Washington, NY) to eliminate the enzyme and used for gas chromatography (GC) analysis.

The apparent activity was defined as the initial reaction rate divided by the amount of immobilized enzyme. The specific activity was defined as the initial reaction rate divided by the amount of protein. The apparent activity and the specific activity were calculated as follows:

$$\text{apparent activity } (\mu\text{mol g}^{-1}\text{particle min}^{-1}) = \frac{d}{e \times f}$$

$$\text{specific activity } (\mu\text{mol mg}^{-1}\text{protein min}^{-1}) = \frac{d}{g \times f}$$

where d is mole of synthesized phytosteryl ester during the initial reaction time (μmol), e is the amount of immobilized enzyme particles used in the reaction (g), f is the initial reaction time (min), and g is the protein amount in the immobilized enzyme used in the reaction (mg).

Optimization of the Enzymatic Esterification of Phytosterol and Fatty Acid from Pine Nut Oil. Reactions were performed in a 50 mL water-jacketed glass vessel. A total of 5 g of phytosterol and fatty acid from pine nut oil at various molar ratios was placed in a reactor preheated to the desired temperature using a water circulator. The reaction was initiated by adding immobilized enzyme to the substrate mixture and subsequently stirred at 300 rpm. The vacuum was controlled by a micrometering valve (Swagelok, Solon, OH) and monitored by a digital vacuum gauge (Teledyne, Thousand Oaks, CA). Samples (50 μL) were withdrawn periodically for GC analysis.

Analysis of Products. Samples (10 mg) were withdrawn at appropriate time intervals during the enzymatic reaction and dissolved in chloroform (1 mL). A gas chromatograph (model 3800; Varian, Palo Alto, CA) equipped with a fused silica capillary column (DB-1ht, 15 m × 0.25 mm i.d. × 0.15 μm film thickness, J&W Scientific, Folsom, CA) and a flame ionization detector (FID) were used for analysis. The aliquot (1 μL) was injected in split mode (split ratio of 50:1). Initially, the column was held at 120 °C for 3 min and programmed to rise to 370 °C and the flow rate of 20 °C min^{-1}. The column was then held at 370 °C for 5 min. The carrier gas was helium at a flow rate of 2.0 mL min^{-1}. The injector and detector temperatures were maintained at 370 °C. Phytosteryl ester synthesized in the present study was identified using phytosteryl ester standard (purity >98.0%).

The degree of conversion refers to the percentage of phytosteryl ester converted from fatty acid from pine nut oil and can be calculated as follows:

$$\text{degree of conversion (mol\%)} = \frac{h}{h+i} \times 100$$

where h is mole of phytosteryl ester, and i is mole of remaining fatty acid from pine nut oil.

RESULTS AND DISCUSSION

Immobilization of CRL. *Carrier Screening for Immobilization of CRL.* In this study, enzyme immobilization was carried out by physical adsorption to the carrier. Physical adsorption is the most straightforward immobilization process, which leaves substrate specificity unchanged.[20] The choice of the carrier is one of the crucial factors for the immobilization process. Prewetting of hydrophobic carriers with a polar solvent is the costumed procedure to avoid enzyme adsorption only on the outer shell of the carrier.[21,22] Polar solvents lower the surface tension of the carrier and presumably facilitate the contact of the lipase solution with the carrier, resulting in increased adsorption rate and improved immobilization efficiency.[23,24] In this study, ethanol prewetting of hydrophobic carriers was employed as previously reported.[21,25−27] Nine different hydrophilic and hydrophobic carriers were compared for the CRL immobilization. XAD 7 showed the highest fixation level (72.6%), followed by Lewatit (71.5%), and Duolite (52.8%) (Figure 1a). On the basis of the same amount of immobilized enzyme used, their activity was measured, and only Lewatit and Accurel showed esterification activity (Figure 1b). From the results, the fixation level seems to be irrelevant to the activity of immobilized enzyme. Several studies reported that the chemical and geometrical characteristics of the carriers are important factors for interaction with the immobilized enzyme.[25,27,28] In this study, CRL immobilized on hydrophilic carriers had low enzymatic activity. The high losses of activity of the enzyme immobilized on hydrophilic carriers seem to be due to a change in the conformation of lipase upon adsorption to a form that has reduced activity or the existence of steric hindrance imposed by the carrier matrix, which constrains flexibility of the lipase molecule.[25,29] The active site of CRL is composed of hydrophilic amino acids (i.e., Ser 209, His 449, and Glu 341), which form a catalytic triad. This active site is covered by surface loops, so-called, lid, and upon change in the external conditions, lipase goes through conformational rearrangements of these loops, leading to the "open state" conformation. In the open state of CRL, the nucleophilic serine of the catalytic triad is positioned in the center of a hydrophobic patch on the L-shaped substrate binding site. These structural features are suited to substrates, such as a triglyceride and a fatty acid.[30−33] Thus, when the hydrophobic carrier is used, the hydrophobic surroundings of the carrier cause higher local concentration of substrates, which are hydrophobic, resulting in stimulation of lipase activity. Furthermore, when the hydrophobic carrier is used, the open form of the enzyme becomes adsorbed to the carrier, and the equilibrium of the enzyme state shifts toward the open form.[25,28,29] Hence, Lewatit, which showed the highest activity, was selected as a carrier for CRL immobilization to study the effects of the process variables.

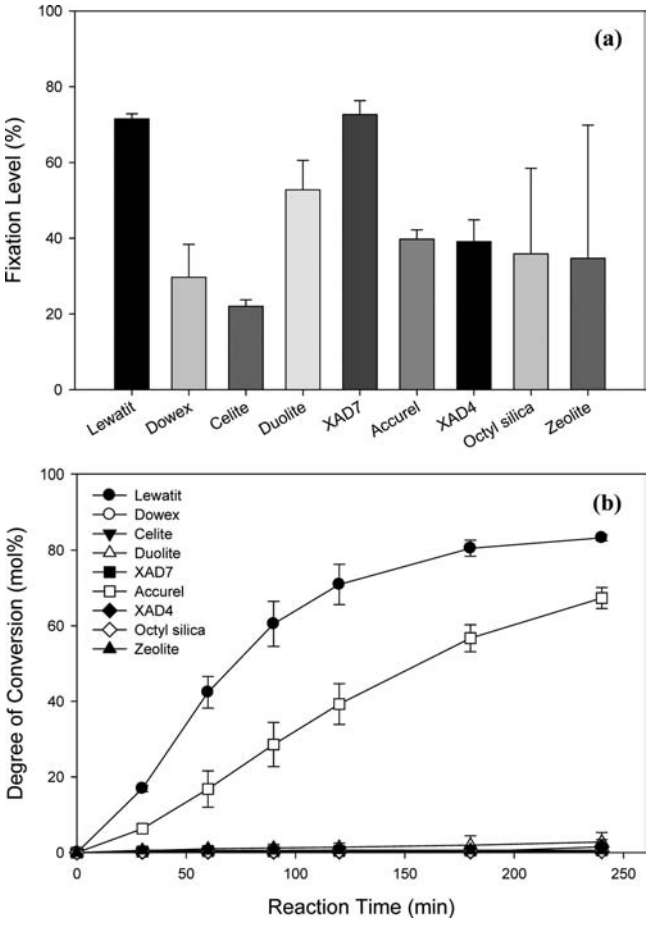

Figure 1. Carrier screening for immobilization of CRL: effect of carriers on the fixation level (a) and degree of conversion in the esterification of phytosterol and fatty acid from pine nut oil (b). Detailed protocols are indicated in the Materials and Methods.

Effect of Initial Protein Concentration of CRL Solution for Immobilization. The initial concentration of enzyme solution employed for the immobilization is another important factor for the immobilization process. It affects not only the adsorption of the enzyme on carrier but also the activity of immobilized enzyme. Several studies showed that once the equilibrium is reached, the amount of lipase adsorbed is correlated in terms of a Langmuir isotherm, which shows a finite initial slope and a well-defined plateau value at the end.[29,34,35] In this study, the fixation level and protein amount in particle were measured to investigate the effect of initial protein concentration of CRL solution on the adsorption (Figure 2a). There was a high correlation coefficient between the protein amount in the particle and the initial protein concentration of CRL solution. Moreover, the fixation level stayed constant as the initial protein concentration increased to 8 mg mL^{-1}, and the level slightly decreased as the initial protein concentration further increased. These results indicate that the amount of carrier used was sufficient for the enzyme protein amount employed for the immobilization.

The activity of the immobilized enzyme tends to reach an asymptotic value as the amount of lipase adsorbed increases.[29,36] The effect of the protein amount fixed in the carrier on the apparent activity and specific activity is illustrated by Figure 2b. The apparent activity of the immobilized enzyme increased as the protein amount in the carrier increased to 96.09 mg g^{-1}. However, the apparent activity reached the equilibrium as the protein amount further increased. Furthermore, a similar trend was observed for the specific activity when the protein amount in the carrier was increased to 96.09 mg g^{-1}. However, the specific activity significantly decreased as the protein amount in the carrier further increased. This result is consistent with the results of Koops at el. (1999) and Bosley and Peilow (1997).[26,37] The suppression of the specific activity is a result of the conformational changes in the enzyme. At low protein amount in particle, in virtue of the excess surface area, one molecule of enzyme is able to interact

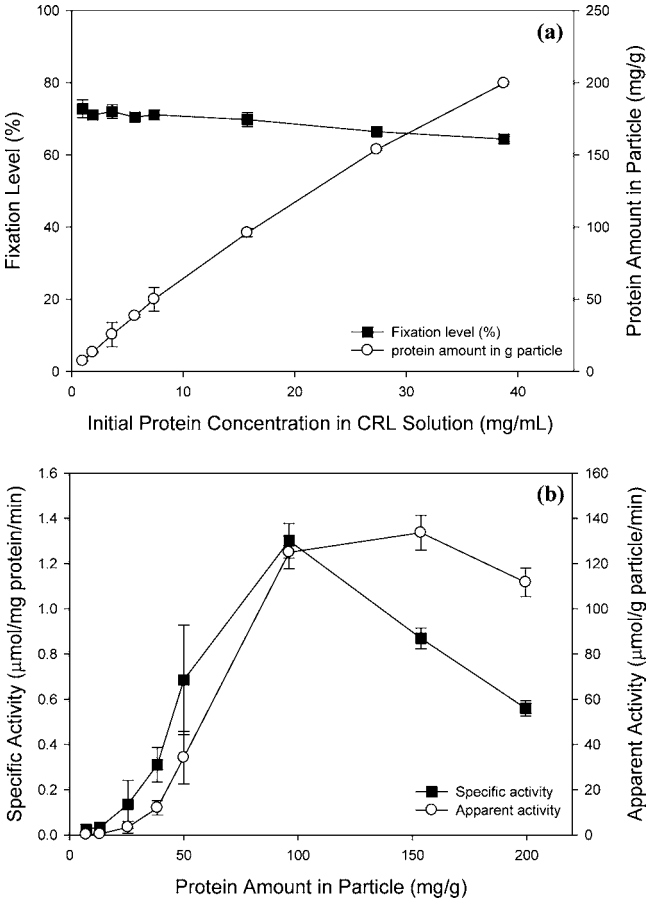

Figure 2. Effect of initial protein concentration in CRL solution on fixation level and protein amount in the immobilized enzyme particle (a) and specific activity and apparent activity of the immobilized enzyme (b). Detailed protocols are indicated in the Materials and Methods.

with a larger surface area of the carrier and "spread over" the surface, leading to inactivation. On the other hand, at high protein amount in the carrier, mass-transfer limitations lead to reduced activity.[26,37] Moreover, due to lack of surface area, the enzyme tends to be packed by forming more than monolayer on the surface of the carrier, causing the decrease in the activity.[35] Therefore, for the initial protein concentration in CRL solution, 15.75 mg mL^{-1}, which corresponds to 96.09 mg g^{-1} of protein amount in particle, was selected as optimum condition.

Optimization of Lipase-Catalyzed Esterification of Phytosteryl Ester with Fatty Acid from Pine Nut Oil. *Temperature.* The reaction temperature affects not only the reaction rate, but also the stability and the activity of the enzyme. The effect of the temperature on the synthesis of phytosteryl ester was investigated as a function of the reaction time (Figure 3). The range of temperatures tested was between 30 and 70 °C. For these trials, the enzyme loading and molar ratio of phytosterol to fatty acid were kept at 5% (based on the total weight of the substrate) and 1:4, respectively. As temperature increased from 30 to 60 °C, the initial rate of the reaction as well as the degree of conversion value increased. In general, the increase in temperature can improve solubility of a compound and reduce viscosity, resulting in enhancement of the reaction rate, by making interactions between the enzyme and substrates easier. Even though high temperatures enhance

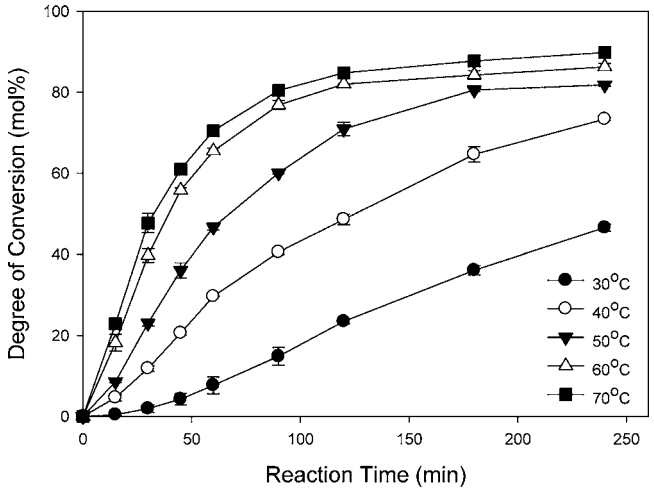

Figure 3. Effect of the temperature on the synthesis of phytosteryl ester as a function of the reaction time. For these trials, enzyme loading and molar ratio of phytosterol to fatty acid were kept at 5% (based on the total weight of the substrate) and 1:4, respectively.

the mixing of the reaction mixture, temperatures higher than 60 °C were reported to result in a significant decrease in esterification rate due to the deactivation of CRL.[14,38,39] However, in this study, the deactivation of the enzyme did not occur at temperatures as high as 60 and 70 °C. These results indicate the thermal stability of the immobilized CRL prepared in this study is superior to free type CRL or immobilized CRL used in the previous studies.[14,38,39] Although there are several reports on the synthesis of phytosteryl ester via enzymatic reaction at low temperatures in the range of 35 and 45 °C, all of these studies were carried out in the presence of solvent.[17,39,40] Use of solvent-free system is more desirable both from an environmental and an economic perspective.[41,42] Therefore, in this study, 60 °C was selected as an optimal temperature for the reaction, because there was no significant difference between 60 and 70 °C.

Molar Ratio. The effect of the molar ratio of the substrates on the synthesis of phytosteryl ester was investigated as a function of the reaction time (Figure 4). The range of molar ratios of phytosterol to fatty acid tested was between 1:1 and 1:5. For these trials, the enzyme loading and the temperature were kept at 5% (based on the total weight of the substrate) and 60 °C, respectively. In terms of stoichiometry, use of an amount of phytosterol equal to the number of fatty acid residues is sufficient to obtain the complete conversion of the fatty acid residues to their corresponding phytosteryl esters. Although an equimolar ratio of substrates should be ideal in terms of economic aspects, here, it appeared to be not advantageous for the reaction rate and the maximum yield of phytosteryl ester (Figure 4). Both the initial rate and the maximum conversion increased as the molar ratio of phytosterol to fatty acid increased from 1:1 to 1:4. The maximum conversion at 1:4 was over 86 mol %. However, there was no significant difference in both the initial rate and the maximum conversion value when the molar ratio was further increased to 1:5.

The high viscosity of the reaction mixture caused by the high melting point of phytosterol accounts for the decreased reaction rate and maximum conversion at molar ratios lower than 1:4. To overcome this problem at smaller molar ratios, solvent was used in the previous studies.[14,17,40] In those cases,

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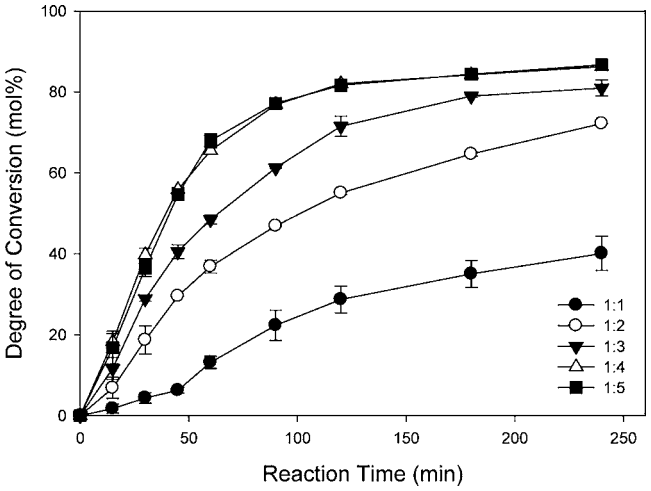

Figure 4. Effect of the molar ratio of phytosterol to fatty acid on the synthesis of phytosteryl ester as a function of the reaction time. For these trials, enzyme loading and temperature were kept at 5% (based on the total weight of the substrate) and 60 °C, respectively.

the optimum molar ratio ranged between 1:1 and 1:3 (phytosterol to fatty acid), which was smaller than in the case of the solvent-free system.[43,44] Moreover, the trials using stepwise addition of phytosterol into the reaction mixture had been attempted, but the time consumed to reach the equilibrium was significantly longer than our results.[45,46] Hence, in this study, 1:4 (phytosterol to fatty acid) was selected as an optimum molar ratio to study the effects of the other process variables.

Enzyme Loading. The effect of the enzyme loading on the synthesis of phytosteryl ester as a function of the reaction time was investigated (Figure 5). The range of enzyme loading tested was between 2.5% and 15% (based on the total weight of the substrate). For these trials, the molar ratio of phytosterol to fatty acid and temperature were kept at 1:4 and 60 °C, respectively.

Torrelo et al. (2008) reported that addition of lipase could be another negative factor for increasing the viscosity of the reaction mixture because the free type of CRL is powder form.[46] To solve this problem, they suggested using a solution form of the lipase. However, because immobilized CRL was used in this study, the addition of enzyme affected neither viscosity of reaction mixture nor the esterification rate even at high enzyme loading. From our result, high enzyme loading appeared to be preferable for the esterification reaction. By increasing the enzyme loading, the initial rate also enhanced. However, although the time consumed until equilibrium varied, similar maximum conversion values of ca. 86 mol % were obtained with all of the enzyme loading tested, except for the 2.5% trial. In particular, at both 10% and 15% enzyme loadings, the esterification reaction reached the equilibrium within 60 min, and the maximum conversion was similar at both conditions. Thus, 10% enzyme loading was selected as optimum, when the cost of the operation was considered.

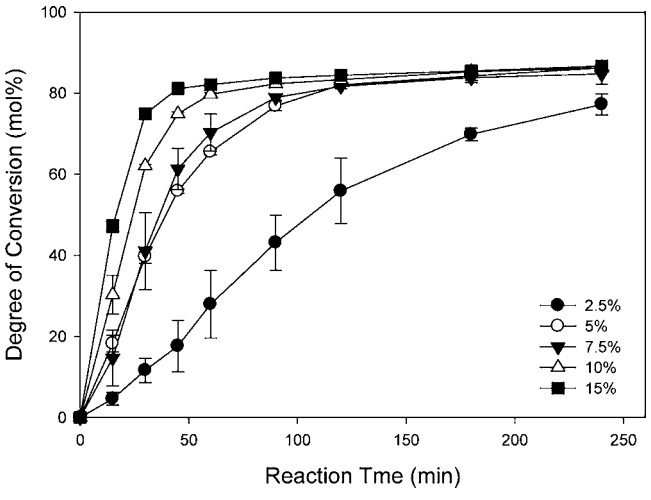

Figure 5. Effect of the enzyme loadings on the synthesis of phytosteryl ester as a function of the reaction time. For these trials, the molar ratio of phytosterol to fatty acid and temperature were kept at 1:4 and 60 °C, respectively.

Vacuum. It is well-known that lipase-catalyzed esterification in nonaqueous media is critically sensitive to the amount of water in the system. When 1 mole of phytosterol reacts with 1 mole of fatty acid in the esterification, 1 mole of water is formed along with 1 mole of phytosteryl ester as products. There is a critical lower limit on the water content below which enzymes will not be able to maintain their catalytic activities.[47,48] This critical water content is referred to as the "essential water". This water is needed to maintain the three-dimensional configuration necessary for the catalytic activity. However, too much water can lead to undesired lipase-catalyzed side reactions such as hydrolysis. Therefore, the effect of vacuum on the synthesis of phytosteryl ester as a function of the reaction time was investigated (Figure 6). The range of

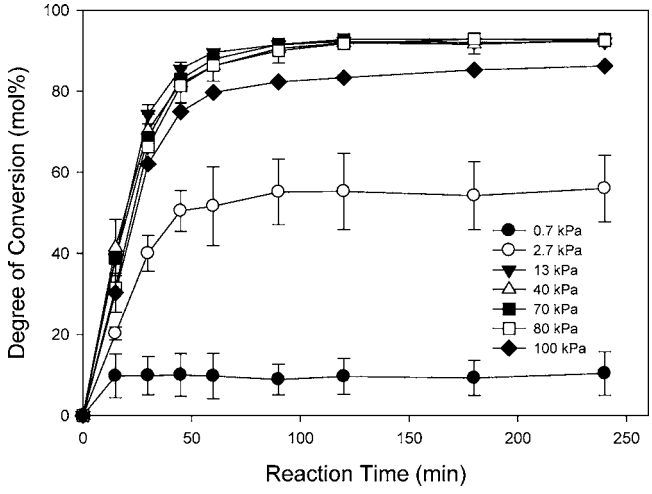

Figure 6. Effect of the pressures on the synthesis of phytosteryl ester as a function of the reaction time. For these trials, the molar ratio of phytosterol to fatty acid, temperature, and enzyme loading were kept at 1:4, 60 °C, and 10% (based on the total weight of the substrate), respectively.

vacuum tested was between 0.7 and 100 kPa. For these trials, the molar ratio of phytosterol to fatty acid, temperature, and enzyme loading were kept at 1:4, 60 °C, and 10% (based on the total weight of the substrate), respectively. As expected, vacuum appeared to be very effective to increase the maximum conversion. With slight decrease in the pressure from 100 to 80 kPa, the maximum conversion increased from 86 to 93 mol %. However, when the pressure was decreased from 80 to 13 kPa, there was no significant difference in either the initial rate

or the maximum conversion. On the other hand, with further decrease in pressure, both the reaction rate and the maximum conversion decreased drastically. This indicates that immobilized CRL needs essential water to act efficiently on the esterification of phytosteryl ester. Most lipases require low water content for esterification,[49] and the reaction equilibrium may be shifted toward the synthesis reaction by the removal of water.[50] However, too high vacuum may lead to a decrease of enzyme activity due to the lack of essential water for the catalytic activity of the enzyme.[47,50] A similar trend was observed in the glycerolysis of docosahexaenoic acid ethyl ester and glycerol under a vacuum system.[51] Consequently, pressure lower than 13 kPa was not appropriate in this study, because the extremely high vacuum stripped off the essential water for the enzyme activity leading to the reduction in the reaction rate. Therefore, 80 kPa was selected to be an optimum when the cost of the operation was considered.

In conclusion, CRL was successfully immobilized with Lewatit as a carrier, and the immobilized CRL was utilized to produce phytosteryl ester of fatty acid from pine nut oil. High proportion of phytosterol in the reaction mixture led to the inhibition of esterification for the synthesis of phytosteryl ester. The lowest degree of conversion was obtained when the highest vacuum was applied. The maximum conversion of ca. 93% was achieved after 90 min under optimum condition.

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Notes

The authors declare no competing financial interest.

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