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Optimization of Lipase Pretreatment Prior to Lipase Immobilization to Prevent Loss of Activity

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Abstract In our previous work, a method of pretreating lipase was developed to prevent loss of its activity during covalent immobilization. In this study, *Rhizopus oryzae* lipase was pretreated before immobilization and then immobilized on a silica gel surface. The effects of the various materials and conditions used in the pretreatment stage on the activity of immobilized lipase were investigated. Immobilized lipase pretreated with 0.1% of soybean oil had better activity than those pretreated with other materials. The optimal temperature, agitation speed, and pretreating time for lipase pretreatment were determined to be 40°C, 200 rpm, and 45 min, respectively. The activity of immobilized soybean oil pretreated lipase was 630 U/g matrix, which is 20 times higher than that of immobilized non-pretreated lipase. In addition, immobilized lipase activity was maintained at levels exceeding 90% of its original activity after 10 reuses.

Keywords: Pretreatment, immobilization, lipase, soybean oil

Lipase is an enzyme that hydrolyzes fat or oil to glycerol and fatty acids [2]. It also catalyzes various other reactions, such as esterification, interesterification, and transesterification [5–7, 16]. Lipase also has an enantioselectivity, and thus it can be used to synthesize optically active compounds [12]. Thus, lipase is a versatile enzyme with many industrial applications.

However, lipase is too expensive to be discarded after a single usage. Thus, it should be immobilized to allow reuse after batch reactions. This immobilized form of lipase can also be used in continuous reaction systems [1, 14]. Furthermore, many investigators have reported that the thermal, pH, and mechanical stabilities of lipase can be enhanced by immobilization [2, 10], and thus, allow lipase to be used under the severe conditions that are often required industrially.

Various methods of immobilizing enzymes have been reported, and of these, covalent bonding using glutaraldehyde as a cross-linker has been widely studied [8, 9, 13]. Because of the high bonding strength produced between enzyme and carrier, the activities of covalently immobilized enzymes can be retained for extended periods. However, the immobilization procedures used are complicated and enzyme activities tend to decrease markedly as a result of the immobilization process [11, 13, 15]. In our previous work, we developed a pretreatment method that prevents loss of lipase activity during covalent immobilization [4]. When lipase was pretreated with soybean oil for 30 min prior to immobilization, immobilized lipase activity was 530 U/g matrix, which is 16 times higher than that of the immobilized non-pretreated lipase. However, pretreatment conditions, such as temperature and agitation speed, were not optimized. Moreover, the effects of pretreating materials, concentrations, and pretreatment times were not investigated.

In this study, *Rhizopus oryzae* lipases were pretreated with various materials and then immobilized on an activated silica gel surface. The effects of pretreating materials, concentrations, and pretreating times were investigated with a view toward increasing immobilized lipase activity. Pretreatment temperatures and agitation speeds were also optimized.

MATERIALS AND METHODS

Materials

Rhizopus oryzae lipase and glutaraldehyde were purchased from the Fluka Co. (Switzerland). 3-Aminopropyltriethoxysilane was purchased from the Sigma Co. (U.S.A.). Silica gel was kindly provided by Chong Kun Dang Pharmaceutical Co. (Korea). All other chemicals were of reagent grade.

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Preparation of Lipase

One g of lipase was suspended in 100 ml of a 1 mM phosphate buffer (pH 7) and centrifuged at 4,000 ×*g* for 15 min at 4°C. Supernatant was stored at 4°C for immobilization studies.

Pretreatment of Lipase

Various amounts of pretreating materials were added to 20 ml of the lipase solution. Mixtures were incubated at various temperatures and agitation speeds ranging from 30 to 120 min. Subsequently, 10 ml of the lipase solutions produced were used for immobilization.

Preparation of Activated Silica Gel for Lipase Immobilization

One g of dry silica gel was mixed with 10% 3-aminopropyltriethoxysilane in 20 ml of acetone and incubated at 50°C for 2 h with constant stirring. The silica gel was then washed with water, dried at 60°C for 2 h, and suspended in 20 ml of a 1 mM phosphate buffer solution (pH 7). Two ml of 25% (w/v) glutaraldehyde was then added and incubated at 20°C for 2 h to activate the silica gel. The activated silica gel was then washed with water and dried at 60°C for 2 h.

Immobilization of Lipase

The activated silica gel (500 mg) was mixed with 10 ml of a lipase solution and incubated at 20°C for 12 h. The immobilized lipase was recovered by filtration, washing with water, and drying overnight at room temperature.

Assay of Immobilized Lipase Activity

Activity was determined using the method developed by Kwon and Rhee [3]. Free fatty acids produced by lipase from soybean oil were determined by observing color development using cupric acetate-pyridine as a color-developing reagent.

RESULTS AND DISCUSSION

Effect of Pretreating Materials on Lipase Pretreament

The effects of the various oils and fatty acids on lipase pretreatment were investigated. These materials (200 mg) were added to 20 ml of lipase solution. Pretreatment mixtures were incubated at 37°C for 30 min with constant stirring. Subsequently, 1 ml of pretreated lipase solution was used for lipase immobilization. Table 1 shows the activity of immobilized lipases pretreated with these different materials. In our previous work, we suggested that the effects of lipase pretreatment before immobilization are attributable to the steric effect of the fatty acid at the active site [4]. This steric effect in the vicinity of the active site prevents covalent bond formation near the active site during immobilization, and thus, enzymatic regions far from the active site would then react with the silica gel surface. Therefore the activity of immobilized lipase can be retained. If this rationale is correct, then longer fatty acids should be more effective at protecting the lipase active site during immobilization. Fatty acids with carbon numbers over 16, such as linoleic acid, oleic acid, stearic acid, and palmitic acid, were found to be effective in pretreating lipase. The activities of immobilized lipase pretreated with these materials were over 10 times higher than that of immobilized non-pretreated lipase. Moreover, we believe that unsaturated fatty acids are likely to be more effective than saturated fatty acids, because immobilized lipases pretreated with linoleic acid and oleic acid had higher activities than stearic acid pretreated lipase. This aspect needs further study but the curved structure of unsaturated fatty acid seems to protect the active site of lipase more than the linear structure of saturated fatty acid. Fatty acids with a low carbon number were not effective for lipase pretreatment. Dodecanoic and octanoic acids enhanced the activities of immobilized lipase by only 5 and 3 times, respectively. Furthermore, acetic acid, propionic acid, and butyric acid, which have 2, 3, and 4 carbons, respectively, were not effective for lipase pretreatment. Moreover, acetic acid and butyric acid decreased immobilized lipase activity. Because the result matches well with our theory, we suggested that this work confirmed the steric effect of the fatty acid at the lipase active site on lipase pretreatment.

Table 1. Effect of pretreating materials on immobilized lipase activity.

Material	Activity (U/g matrix)	Material	Activity (U/g matrix)
Linoleic acid	397.7±7.3	Acetic acid	23.3±3.7
Oleic acid	367.7±4.3	Soybean oil	530.7±6.3
Stearic acid	308.0±5.0	Olive oil	513.3±5.7
Palmitic acid	343.3±2.7	Palm oil	350.0±10.0
Dodecanoic acid	163.3±1.7	Tributyrin	32.7±2.3
Octanoic acid	89.3±5.7	Triacetin	33.0±3.0
Butyric acid	27.7±2.3	Glycerol	31.7±1.3
Propionic acid	42.0±2.0	No pretreatment	32.3±1.7

Vegetable oils were also found to be effective for lipase pretreatment, and the activities of immobilized lipase pretreated with these materials were over 10 times higher than that of immobilized non-pretreated lipase. In particular, soybean oil was found to be highly effective, and lipase pretreated with soybean oil showed an activity of 537 U/g matrix, which was over 17 times higher than that of non-pretreated lipase. However, triacetin and tributyrin were not effective at pretreating lipase. The activities of immobilized lipases pretreated with these materials were below 40 U/g matrix, which was similar to that of

immobilized non-pretreated lipase. Whereas vegetable oils are mainly composed of fatty acids with carbon numbers of 16, triacetin and tributyrin are composed of acetic and butyric acids, respectively. During the pretreatment stage, they are hydrolyzed to glycerol, acetic acid, and butyric acid. Because these fatty acids have 2 or 3 carbons and showed no effect on lipase pretreatment, it is thought that triacetin and tributyrin are not effective for lipase pretreatment.

Effect of Temperature on Lipase Pretreatment

Lipase solutions (20 ml) were pretreated with 200 mg of soybean oil, olive oil, linoleic acid and, oleic acid, which showed better effect on lipase pretreatment than the other materials, at various temperatures ranging from 25 to 45°C with stirring at 150 rpm for 30 min, and then immobilized on activated silica gels. Fig. 1 shows that the optimal temperature for lipase pretreatment was 40°C. Immobilized lipases pretreated with soybean oil and olive oil showed similar activities at 35°C to 40°C. However, lipase activities were markedly decreased at 25°C. Pretreatment with fatty acids had similar effects at 25°C to 45°C. The effect of oils on lipase pretreatment was more dependent on pretreatment temperature than that of fatty acids. In previous work, we suggested that fatty acids from oil hydrolysis play an important role in lipase pretreatment [4]. In other words, oil hydrolysis is important when oil is used to pretreat lipase. Therefore, lipase pretreatment using oil depends on temperature. On the other hand, lipase pretreatment using fatty acid does not need the hydrolysis reaction, and thus pretreatment temperatures are less important.

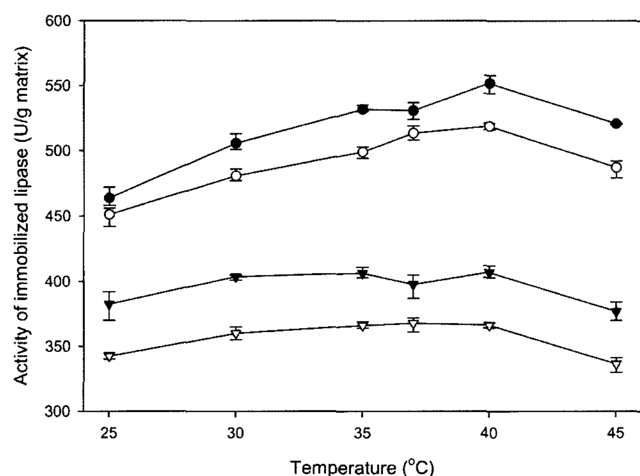

Fig. 1. Effect of pretreatment temperature on immobilized lipase activity.
Lipase solutions (20 ml) were pretreated with 200 mg of soybean oil (●), olive oil (○), linoleic acid (▼), and oleic acid (▽) at various temperatures with stirring at 150 rpm in 100-ml shake flasks for 30 min. The pretreated lipases so produced were then immobilized on activated silica gels. The activity of immobilized non-pretreated lipase was 31.5 U/g matrix.

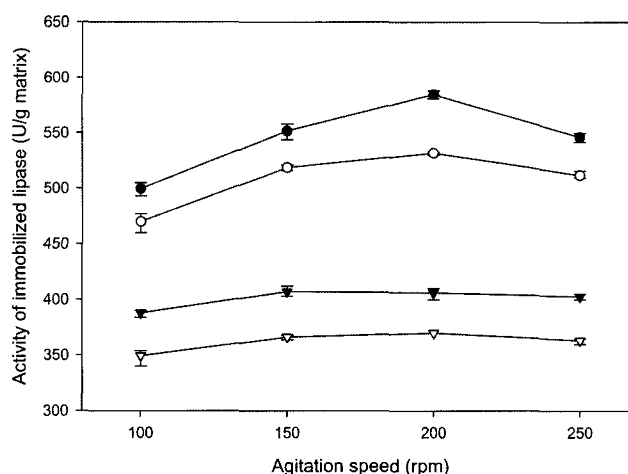

Fig. 2. Effect of agitation speed on immobilized lipase activity.
Lipase solutions (20 ml) were pretreated with 200 mg of soybean oil (●), olive oil (○), linoleic acid (▼), and oleic acid (▽) at 40°C with stirring at various agitation speeds in 100 ml shake flasks for 30 min. The pretreated lipases so produced were then immobilized on activated silica gels. The activity of immobilized non-pretreated lipase was 33.2 U/g matrix.

Effect of Agitation Speed on Lipase Pretreatment

Lipase solutions (20 ml) were pretreated with 200 mg of soybean oil, olive oil, linoleic acid, and oleic acid at 40°C with stirring at various agitation speeds for 30 min, and then immobilized on activated silica gels. Fig. 2 shows that the effect of oils on lipase pretreatment was best at 200 rpm and decreased slightly at 150 rpm and 250 rpm. In particular, the effects of oil pretreatment on activity were markedly reduced at 100 rpm. On the other hand, the effects of fatty acid pretreatments on activity were similar at 150 rpm to 250 rpm and did not reduce markedly at 100 rpm. Agitation accelerates oil hydrolysis. Thus, the effect of oils on lipase pretreatment is more dependent on agitation speed than that of fatty acids.

Effect of Pretreatment Concentration on Lipase Pretreatment

Lipase solutions were pretreated with various amounts of the pretreatments at 40°C with stirring at 200 rpm for 30 min, and then immobilized on activated silica gels. Results are shown in Fig. 3. When 0.05% of soybean oil was used to pretreat lipase, the immobilized lipase activity was reduced by 30% versus the activity of immobilized lipase, which was pretreated with 0.1% of soybean oil. Moreover, the activities of immobilized lipases pretreated with 0.1% to 2% of soybean oil were similar, but when 3 and 4% soybean oil was used, activities were reduced slightly. When from 0.1% to 3% of olive oil was used as a pretreatment, activities were similar, and when the concentration was reduced to 0.05%, the activity was reduced by 25% versus the activity at 0.1%. However, when linoleic acid and oleic acid were added to lipase solution at 0.1% to 4%, activities were

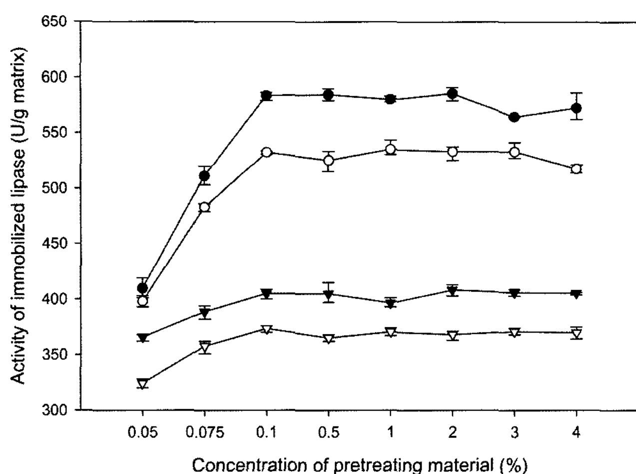

Fig. 3. Effect of pretreatment concentrations on immobilized lipase activity.
Lipase solutions (20 ml) were pretreated with various amounts of soybean oil (●), olive oil (○), linoleic acid (▼), and oleic acid (▽) at 40°C with stirring at 200 rpm in 100-ml shake flasks for 30 min. The pretreated lipases so produced were then immobilized on activated silica gels. The activity of immobilized non-pretreated lipase was 30.2 U/g matrix.

unchanged over this concentration range. Even when 0.05% of linoleic acid and oleic acid were used to pretreat lipase, activities were only reduced by 10% versus the activity at 0.1%. According to these findings, the optimal pretreatment concentration was determined to be 0.1%.

Effect of Pretreating Time on Lipase Pretreatment

The effect of pretreating times on immobilized lipase activity was also examined using 20 mg of the pretreating materials in 20 ml of lipase solution at 40°C and 200 rpm. As shown in Fig. 4, the optimal pretreatment times for lipase pretreatment with soybean oil and olive oil were both 45 min. The activity of immobilized lipase pretreated with soybean oil for 45 min was 630 U/g matrix, but when pretreatment with soybean oil was performed for longer than 45 min, immobilized lipase activity markedly decreased. In particular, when lipase was pretreated with soybean oil for 120 min, the activity decreased by 20% versus that of immobilized lipase pretreated for 45 min. However, when lipase was pretreated with linoleic acid and oleic acid, the pretreating times were found to have no effect on activity. Although further study is required to explain this difference of the effects of oils and fatty acids on lipase pretreatment, we believe that the glycerol produced by oil hydrolysis has an effect.

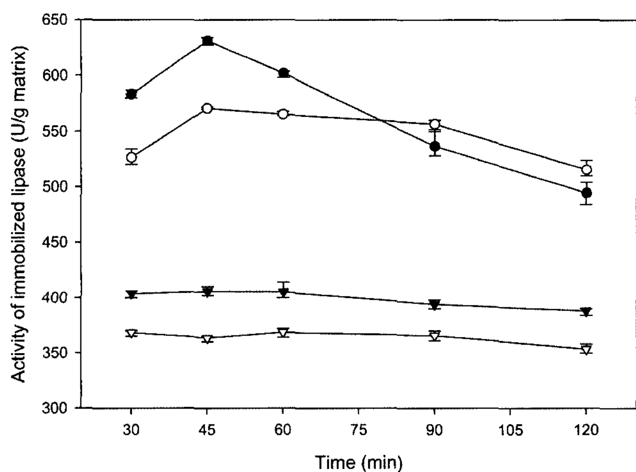

Fig. 4. Effect of pretreating time on immobilized lipase activity.
Lipase solutions (20 ml) were pretreated with 20 mg of soybean oil (●), olive oil (○), linoleic acid (▼), and oleic acid (▽) at 40°C with stirring at 200 rpm in 100-ml shake flasks for various times. The pretreated lipases so produced were then immobilized on activated silica gels. The activity of immobilized non-pretreated lipase was 34.5 U/g matrix.

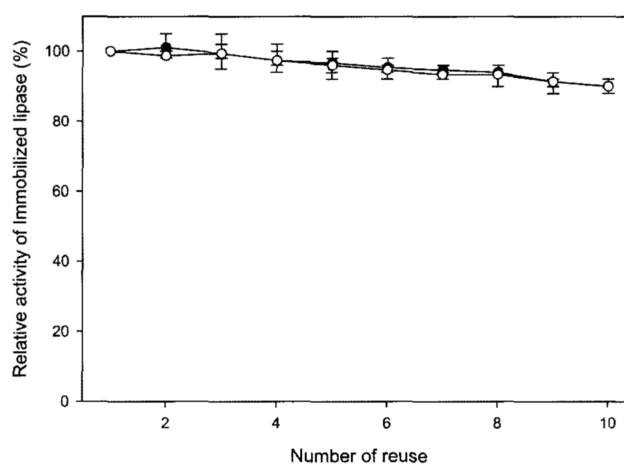

Fig. 5. Reusability of immobilized lipases.
Lipase solutions (20 ml) were pretreated with 20 mg of soybean oil (●) and linoleic acid (○) at 40°C with stirring at 200 rpm in 100-ml shake flasks for 45 min. Pretreated lipases were then immobilized on activated silica gels, and used to hydrolyze soybean oil in repeated batch reactions. Soybean oil hydrolysis was performed for 30 min at 37°C in a shaking water bath. Immobilized lipases were filtered and washed before reuse. The 100% relative activities are 630 U/g matrix for immobilized soybean oil pretreated lipase and 405 U/g matrix for immobilized linoleic acid pretreated lipase, respectively.

Reusability of Immobilized Lipase

The immobilized soybean oil and linoleic acid pretreated lipases were used in a consecutive series of hydrolysis reactions. Fig. 5 shows that the activities of both immobilized lipases were maintained at levels exceeding 90% of their original activities after being used 10 times. Lipase pretreatments with oil and fatty acid had no effect on immobilized lipase reusability.

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