Immobilization of *Pseudomonas cepacia* Lipase by Sol-Gel Entrapment and Its Application in the Hydrolysis of Soybean Oil

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ABSTRACT: The immobilization of Lipase PS from *Pseudomonas cepacia* by entrapment within a chemically inert hydrophobic sol-gel support was studied. The gel-entrapped lipase was prepared by the hydrolysis of tetramethoxysilane (TMOS) with methyltrimethoxysilane (MTMS), isobutyltrimethoxysilane (iso-BTMS), and *n*-butyltrimethoxysilane. The immobilized lipase was subsequently used in the hydrolysis of soybean oil to determine its activity, recyclability, and thermostability. The biocatalyst so prepared was equal to or better than the free enzyme in its hydrolytic activity. The catalytic activity of the entrapped lipase strongly depended on the type of precursor that was used in its preparation. The lipase entrapped within TMOS/iso-BTMS showed the highest activity. The catalytic activity of the immobilized lipase was more pronounced during the earlier stages of the reaction. Thermostability of the lipase was significantly improved in the immobilized form. The immobilized lipase was stable up to 70°C, whereas for the free enzyme, moderate to severe loss of activity was observed beyond 40°C. The immobilized lipase was consistently more active and stable than the free enzyme. The immobilized lipase also proved to be very stable, as it retained more than 95% of its initial activity after twelve 1-h reactions.

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KEY WORDS: Entrapment, hydrolysis, immobilization, lipase, sol-gel, soybean oil.

The conversion of fats and oils into value-added products such as fatty acids and their derivatives has been of major commercial interest. The physicochemical synthesis of these products is a well-established industrial process (1). These processes normally involve an inorganic homogeneous catalyst and high temperatures and pressures. These methods are highly energy intensive, and because severe conditions for temperature and pressure result in side reactions, further purification of the final product is normally required (2).

Enzymatic conversion of fats and oils has been suggested as a realistic alternative to the conventional physicochemical methods. The possible use of lipases is a particular interest, as they operate at near-ambient conditions and catalyze specific reactions. However, the high cost of lipase makes the enzymatic processes economically unfavorable. This makes the development of supports for immobilization of lipases an area of high interest. Immobilization of lipase allows for its recycling and reuse in a continuous reactor setting.

Immobilization is the most widely used method to achieve stability in lipases to make them more attractive for industrial use. Common immobilization techniques include physical adsorption onto a solid support, covalent bonding to a solid support, and physical entrapment within a polymer matrix support. Entrapment of lipase entails its capture within the matrix of a cross-linkable resin. This method has several advantages over the other two methods. Unlike the covalent binding method, this method uses a relatively simple procedure that allows the immobilized lipase to maintain its activity and stability. A variety of methods have been used for trapping lipases in a polymer matrix.

In one method lipase is entrapped in a photo-cross-linkable or solution cross-linkable resin. This technique consists of mixing a liquid photo-cross-linkable resin such as polyethyleneglycol dimethacrylate, which contains photosensitive functional groups, an appropriate initiator such as benzoin ethyl ether, and an enzyme solution followed by illumination with near-ultraviolet light for a few minutes or addition of a polymerization accelerator such as dimethylaniline. The immobilized lipase produced by this method has been successfully applied in the hydrolysis of triglycerides (3,4), the esterification of fatty acids (5), and the conversion of other water-soluble (6) and water-insoluble compounds (7–9).

Entrapment of enzymes in an inorganic polymer matrix has received substantial attention in recent years. This method, which was pioneered by Avnir and coworkers (10,11), is based on the sol-gel process. The procedure for the preparation of sol-gel polymers is well documented (12,13). A typical immobilization procedure uses an aqueous solution of enzymes, sodium fluoride (NaF) as catalyst, and alkoxysilane derivatives such as $RSi(OMe)_3$ with R = alkyl, aryl, or alkoxy as gel precursors. Inorganic matrices offer a number of potential advantages; for example, (i) they have a controllable surface area, average pore size, narrow pore size distribution, and fractal dimension; (ii) they are thermally stable; (iii) leakage of enzyme is prevented owing to rigidity of the caging; (iv) they are easily recyclable; (v) they are simple to prepare using inexpensive chemicals; (vi) they retain high enzyme activity; and (vii) they have shown the potential to increase the activity of the enzyme. Reetz and coworkers (14–18) reported a significant increase

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in the activity of the immobilized lipase *Pseudomonas cepacia* by entrapment in a sol-gel matrix using tetramethoxysilane (TMOS), methyltrimeth-oxysilane (MTMS), and other alkyl-modified silane precursors. The esterification activity of the immobilized lipase was significantly increased compared with that of the free lipase; however, the hydrolytic activity of the immobilized lipase was only 41% compared with that of the corresponding free enzyme (15). The relative activity of the immobilized enzyme increased with an increase in the pendant alkyl group of the polymerization precursors. The entrapped lipase also showed good stability when it was subjected to repeated usage. Hsu and coworkers (19) developed a novel procedure for the immobilization of lipase from *P. cepacia* (PS-30) within a phyllosilicate sol-gel matrix. The method was based on cross-linking a polysilicate clay with silicate polymer produced by the controlled hydrolysis of TMOS. The immobilized lipase showed more stability and higher activity compared with free lipase in the esterification of lauric acid with octan-1-ol (19).

Kawakami and co-workers studied the enhancement in thermal stability of immobilized lipases by sol-gel entrapment (20–23). The characteristics of several systems for ester synthesis were examined including hybrid gel-entrapped lipase on an MTMS/TMOS and lipase-entrapped modified silicates on kieselguhr, such as Celite 545 and Hyflo Super-Cel. The prepared sol-gel–entrapped lipases showed an increased thermal stability and higher activity at elevated temperatures. Other studies in this area have also indicated a higher activity and stability for the entrapped lipases within the inorganic matrix (24–26).

In this study, the immobilization of Lipase PS from *P. cepacia* by entrapment within a chemically inert hydrophobic sol-gel support was studied. The gel-entrapped lipase was prepared by hydrolysis of TMOS with MTMS, iso-butyltrimethoxysilane (iso-BTMS), or *n*-butyltrimethoxysilane (*n*-BTMS) in the presence of lipase. The immobilized lipase was subsequently used in the hydrolysis of soybean oil to determine its activity and stability.

EXPERIMENTAL PROCEDURES

Materials. Lipase PS from *P. cepacia* was generously donated by Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). TMOS (95%), iso-BTMS (97%), NaF, polyvinylalcohol (MW, 15,000), and formamide (95%) were purchased from the Aldrich Chemical Company (Milwaukee, WI). *n*-BTMS (95%) was purchased from Geiest, Inc. (Tullytown, PA), and MTMS (95%) was purchased from Fluka (Hauppage, NY). Soybean oil was donated by Archer Daniels Midland Co. (Decatur, IL). Hexane [gas chromatography (GC) grade] was purchased from EM Science (Gibbstown, NJ), bis-(trimethylsilyl)trifluoroacetamide (BSTFA, derivative grade), 1,2,3-tridecanoylglycerol (tricaprin, 95%), and pyridine were purchased from the Sigma Chemical Co. (St. Louis, MO). The materials were used without further purification.

Procedure A—lipase immobilization. A specified amount of Lipase PS from *P. cepacia* was measured into a flask, and 10 mL of water was added. A magnetic stir bar was added, and the mixture was stirred at 150 rpm for about 5 min to make a homogeneous lipase suspension. To this mixture, 1 mL of a 1 M NaF solution and the silica precursor(s) were added. When more than one precursor was used in the experiment, the ratio of the precursors determined the exact amount of each one. Upon addition of the precursors, the reaction occurred almost immediately, and the gel was formed in 2 min. The temperature of the reaction media was monitored and maintained below 40°C during the entire procedure. The flask was then removed from the stirrer and left sealed at room temperature for 24 h. At this point, a liquid phase was present along with the gel. To dry the wet gel, the flask was incubated in a constant-temperature water bath at a temperature of 33°C for about 24 h. Water was circulated into the bath from a Neslab (Portsmouth, NH) TTE-211 Bath/Circulator, which, *via* an external probe, was able to control the temperature of the bath to within ±0.01°C. The resulting ceramic polymer was then broken up and ground in a mortar. The powder was washed with 100 mL of distilled water in a 250-mL flask for 1 h at a mixing speed of 500 rpm. The mixture was then filtered. About 90 mL of the supernatant was collected. This mixture was further examined for its residual activity (see Procedure C for details). The wet paste was dried again at 33°C for 24 h in a constant-temperature water bath. The immobilized lipase was then crushed in a mortar to yield the final product. The fine powder was stored at –4°C until use.

Procedure B—free enzyme reactions. The optimal set of conditions for free enzyme reactions was adopted from a previous study (27). The optimal conditions used were: 50% (w/w) water-to-oil ratio, 360 mg lipase/10 g oil, 40°C, 700 rpm stirring rate, and a 1-h reaction time. The reactions were run in a constant-temperature water bath under which a Thermolyne (Dubuque, IA) Mirak four-place model S73135 magnetic stirrer was placed to agitate the reaction mixture. Water was circulated into the bath from the Neslab TTE-211 Bath/Circulator described in Procedure A. Reactions were run in 50-mL Erlenmeyer flasks with rubber stoppers.

Procedure C—residual activity. Experiments were performed to evaluate the degree of immobilization. As was mentioned in Procedure A, the immobilized lipase was washed with a specified amount of water, and the supernatant was collected and tested for residual activity. After filtration, about 90 ± 5 mL of supernatant was collected. This supernatant potentially contained free enzyme protein, unreacted precursors, siliconols, methanol, and soluble oligomers. To quantify the amount of lipase in the supernatant, a calibration curve was constructed with the formation of free fatty acids (FFA) as a function of free enzyme loading for the hydrolysis of soybean oil. This calibration was based on the activity of the soluble protein part of the enzyme, as it was to quantify the amount of lipase protein in the supernatant wash. By comparing the supernatant from the immobilization wash procedure with this calibration, the amount of lipase in the supernatant and the degree of immobilization were determined. In the calibration procedure, it was assumed that all the soluble

enzyme protein was dissolved in the water. This assumption is accurate at lower enzyme loadings, which is of interest for the residual activity experiments.

To mimic the exact media in the calibration studies, a blank gel with no lipase was prepared. The supernatant, which was collected from washing this gel, was used as the reaction medium in the calibration experiments. The reaction procedure for the calibration experiments was identical to the free enzyme reactions (see Procedure B), except that the supernatant from the blank sol-gel process was used instead of pure water. The enzyme loading was varied in the range of 0.3–12 mg lipase/1 g of soybean oil for this calibration. The calibration curve showed a monotonically increasing activity as the enzyme loading was increased. The activity of the lipase leveled off beyond the upper limit of 12 mg lipase/1 g of soybean oil.

Procedure D—immobilized lipase reactions. Experiments were conducted to examine the stability and reusability of the immobilized lipase in the hydrolysis of soybean oil. The influence of silane precursors, degree of immobilization, immobilized enzyme loading, and reaction kinetics was studied. The following range of conditions was tested: (i) different combinations of TMOS, with MTMS, iso-BTMS, or *n*-BTMS; (ii) enzyme loadings on a support of 15–60 mg of Lipase PS/g of gel; (iii) immobilized enzyme loadings of 1–5 g of gel/10 g of oil; (iv) temperature of 40–80°C; (v) reaction times of 1–48 h; and (vi) up to 12 consecutive lipase reuses.

In a typical reaction procedure, 10 g of oil, 10 g of water, and 1–5 g of the immobolized enzyme were weighed into a 50-mL Erlenmeyer flask, and a magnetic stir bar was added. The flask was placed in a constant-temperature water bath described in procedure B. After 1 h, the stirring was stopped and the reaction flask was removed from the water bath. The reaction mixture was immediately vacuum filtered through grade one filter paper with the aid of a vacuum pump. The resultant paste, which was collected on the filter paper, was carefully scraped off and placed back into the original reaction flask. Fresh soybean oil and water were added, and the procedure was repeated. The supernatant obtained from the vacuum filtration procedure was transferred into a 50 mL flask. This mixture was stirred at 150 rpm in the same water bath for 24 h to examine any potential residual activities that may have resulted from the leakage of lipase through the support.

Sampling and analysis. Excess water from the hydrolysis reactions was removed from the sample by a Virtis (Gardiner, NY) model 10-145MR-BA freezer dryer. In the freeze dryer, sample vials were uncapped and the temperature was reduced to –25°C. Lyophilization proceeded for at least 6 h. When this process was completed, the sample vials were removed from the freeze dryer and stored again in the freezer for GC analysis.

Samples from the reactions were 0.8–1.2 mL in volume and were collected in 5-mL sample vials. Sample vials were kept on a block heater (Lab-Line, Melrose Park, IL) at 120°C before the sample collection. Vials were immediately capped after collection and were kept on the heater for 10 min to ensure lipase denaturation.

Prior to analysis, samples were derived by silylation with BSTFA. The silylating agent reacts with the carboxyl groups of fatty acids and results in trimethylsilylated fatty acids, which are readily separated and quantified with GC methods. The derivatization was done within 6 h of analysis, as the silylated sample decomposes upon standing. The tricaprin internal standard solution (150 μL) and BSTFA (200 μL) were added to the sample vials. The sample vials were then capped using a screw-cap with a Teflon™-faced septum to prevent evaporation. The vials were hand-shaken for 15 s and then heated at 70°C for 15–20 min. Following this heating procedure, the samples were allowed to cool to room temperature before adding 800 mL of hexane. Samples were then stored in the freezer prior to analysis.

A GC method for the analysis of hydrolysis products developed by Wagner (27) in our laboratory was reproduced here. A Hewlett-Packard (Wilmington, DE) 6890 Series GC system was used for chromatography work, and Hewlett-Packard Chemstation software was used for the data analysis. The GC was equipped with a Hewlett-Packard (part number 19091J-012) HP-5 column. Sample volumes of 2 μL were injected into the GC. The carrier gas was helium, and the GC was operated in constant-flow mode with a flow rate of 12.0 mL/min and nominal pressure of 34.0 psi. A split injector was used with a split ratio of 15:1 and a temperature of 325°C. The flame-ionization detector was operated at 350°C and used a helium makeup flow to maintain a constant detector flow of 25.0 mL/min. The oven was initially held at 80.0°C. Immediately after injection, the oven was programmed to elevate the temperature to 180°C at 15.0°C/min, to 250°C at 5.0°C/min, and finally to 325°C at 8.0°C/min. The oven was held at this temperature for 22.95 min before returning to 80.0°C. Total run time for this method was 53.0 min. Calibration of the GC method was carried out by analyzing standard solutions of mixed glycerol, FFA, monoglycerides, diglycerides, triglycerides, and soybean oil to obtain calibration curves. The standards were derived in the same fashion as the reaction samples. Experiments were also performed to determine the precision and repeatability of the analysis. Results of quantitative experimental analyses using this method showed a standard deviation of less than 0.8% in precision and repeatability.

RESULTS AND DISCUSSION

Effect of silane precursors. In the initial phase of our study (27), Lipase PS from *P. cepacia* was immobilized in a sol-gel precursor using varying amounts of TMOS and MTMS. TMOS is a tetrafunctional monomer and a network-forming agent. MTMS is a trifunctional monomer with a pendant CH_3 (R-group). By changing the relative amounts of TMOS and MTMS, the resulting network structure formed from the hydrolysis and polycondensation of these two monomers could potentially be manipulated. The degree of lipase leakage through the network structure could also be controlled by the extent of cross-linking. Gels made with high concentrations of TMOS were expected to be more tightly cross-linked, whereas higher MTMS contents

were expected to form more flexible matrixes. A tightly cross-linked polymer can provide a more stable matrix for the immobilized lipase and minimize lipase leakage. However, a highly cross-linked polymer structure is potentially less porous, with a smaller average pore diameter, and may impose a higher resistance to the diffusion of substrate through the structure and into the active sites. Our optimization efforts focused on identifying the relative amounts of MTMS and TMOS and the resulting gel that would facilitate the flow of substrate into the gel and prevent the leakage of lipase out of the gel.

Experiments were performed to optimize the immobilization parameters. The immobilization procedure is outlined in Procedure A. The molar ratio of MTMS to TMOS was varied from 2.9 to 10.2, with a total precursor amount of about 75 mmol. The catalyst was 1 M NaF and was varied from 1 to 3 mL, whereas the amount of lipase was varied from 250 to 2000 mg in 10 mL of water. This gave a total water-to-silane molar ratio of about 8. After the drying process, about 10 g of immobilized lipase was collected. The optimization experiments were based on the effectiveness of the biocatalyst in the hydrolysis of soybean oil, and formation of FFA was the determining criterion. The procedure used is outlined in Procedure D. The amount of immobilized lipase that was used in these experiments was 3 g/10 g of oil, and the reaction time was 1 h. Table 1 summarizes the results for MTMS/TMOS experiments. Our optimal formulation for the biocatalyst was an MTMS/TMOS ratio of 5.2, 1 mL of NaF catalyst, and 1000 mg of lipase. Under these conditions, the formation of FFA in the hydrolysis of soybean oil was 24%, and the relative activity with respect to the free enzyme was 55% under identical conditions. Reetz and co-workers (15), has reported relative activity of 30–41% for the hydrolysis of emulsified olive oil with immobilized Lipase PS on similar sol-gel–supported material. The stability and reusability of our optimal biocatalyst was also quite remarkable. After five consecutive usages, no loss of activity and no significant residual activity were experienced for this biocatalyst.

In the next stage of our study, the butyl-substituted trimethylsilanes (*n*-BTMS, iso-BTMS) were used individually or as co-precursors with TMOS and MTMS. The main objective in these experiments was to maximize the amount of solid gel formed. In these experiments, the relative amounts of precursors were varied. Also, the amount of water relative to the precursor(s) was changed; however, other parameters, such as NaF catalyst (1 mL of a 1 M solution), enzyme loading (1000 mg/59 mmol of precursors), and type (PS) were kept unchanged. Experimental results are presented in Table 1. Results revealed that as the molar ratio of iso- or *n*-BTMS to TMOS was increased beyond 6, no solid gel was formed under the relatively mild experimental conditions of room temperature and NaF as catalyst. The optimized molar ratio of iso- or *n*-BTMS precursor to TMOS was 3.9. The molar ratio of water to precursors was 10.34:1. No gel was formed when iso- or *n*-BTMS was polymerized individually or in combination with MTMS.

The immobilized lipases from our optimal formulations with iso-BTMS/TMOS, *n*-BTMS/TMOS, and MTMS/TMOS were further examined for the hydrolysis of soybean oil. Hydrolysis conditions were: 40°C, 10 g of soybean oil, 10 g of water, a loading of 3.0 g of immobilized lipase/10 g of soybean oil, a stirring rate of 700 rpm, and 1 h of reaction. Enzyme loading was at 1.0 g of crude lipase/8 g of total gel, or 375 mg of enzyme/3.0 g of gel for the immobilized enzyme. Correcting for enzyme losses during washing, the actual loading was at 360 mg/3.0 g of gel. The correction procedure is explained in detail in the next section. Enzyme loading for the free enzyme experiments was 360 mg of lipase/10 g of soybean oil. At these levels, the free and immobilized enzyme experiments were at equal enzyme loading. The hydrolysis procedure was as is outlined in Procedure D. Hydrolysis results are summarized in Figure 1. For the purpose of comparison, free enzyme reaction results are also shown in this figure. As shown, the activity of the immobilized lipase increased on the order of CH_3 (MTMS) < *n*-C_4H_9 (*n*-BTMS) < iso-C_4H_9 (iso-BTMS). The relative activity of the immobilized lipase compared to the free enzyme was 55, 100, and 110% for MTMS/TMOS, *n*-BTMS/TMOS, and iso-BTMS/TMOS, respectively. There was an increase in enzyme activity for gels made from butyl-

TABLE 1
Variations in Sol-Gel Immobilization[a]

Precursors	Precursor molar ratio	H_2O/silane molar ratio	NaF (mL)	Loading (mg)	Gel yield (g)	FFA formation (mol%)
MTMS/TMOS	2.9	8.12:1	1	250	10 ± 0.5	8.0 ± 1.2
MTMS/TMOS	5.2	8.12:1	1	250	10 ± 0.5	11.0 ± 1.5
MTMS/TMOS	10.2	8.12:1	1	250	10 ± 0.5	10.0 ± 3.0
MTMS/TMOS	5.2	8.12:1	2	250	10 ± 0.5	6.5 ± 0.9
MTMS/TMOS	5.2	8.12:1	3	250	10 ± 0.5	7.0 ± 1.1
MTMS/TMOS	5.2	9.56:1	3	250	10 ± 0.5	6.5 ± 1.2
MTMS/TMOS	5.2	8.12:1	1	1000	10 ± 0.5	24.0 ± 1.3
n-BTMS/TMOS	3.9	10.34:1	1	1000	8 ± 0.4	55.9 ± 1.6
iso-BTMS/TMOS	3.9	10.34:1	1	1000	8 ± 0.4	61.2 ± 1.5
iso-BTMS/TMOS	6.2	7.36:1	1	1000	No gel formed	—
iso-BTMS/MTMS	3.7	12.12:1	1	1000	No gel formed	—
iso-BTMS	—	12.82:1	1	1000	No gel formed	—

[a]MTMS, methyltrimethoxysilane; TMOS, tetramethoxysilane; BTMS, butyltrimethoxysilane; FFA, free fatty acid.

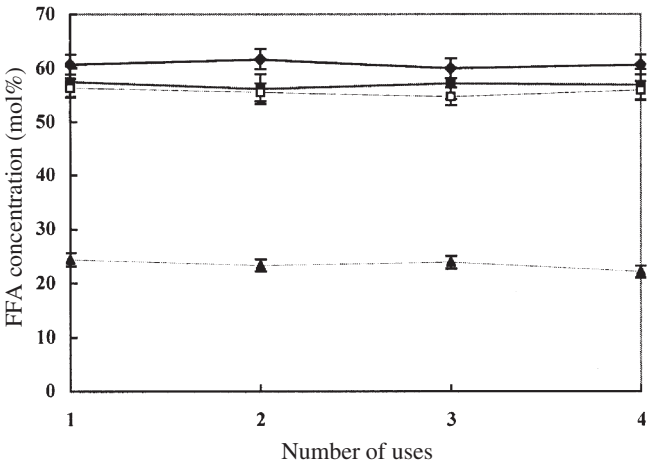

FIG. 1. Effect of different precursors on the immobilized lipase-catalyzed hydrolysis of soybean oil at 40°C, a loading of 3 g of gel/10 g of oil (360 mg free lipase/10 g of oil), 10 g of water, a stirring rate of 700 rpm, and a 1-h reaction. (□) Free lipase; (▲) TMOS/MTMS; (■) TMOS/*n*-BTMS; (◆)TMOS/iso-BTMS. FFA, free fatty acid; TMOS, tetramethoxysilane; BTMS, butyltrimethoxysilane.

substituted trimethylsilanes compared to the free enzyme and also compared to the gels made from methyl-substituted trimethylsilanes. This result is consistent with those of other researchers (14,15,18) and has been attributed to the lipophilic nature of the alkyl groups. Free alkyl groups in the monomer create a lipophilic microenvironment that subsequently interacts with the lipase, triggering a phenomenon similar to a classical interfacial interaction. However, unlike the interfacial activation, which is an interactive process, the alkyl effect is believed to be due to a more favorable lipase conformation caused during the sol-gel process. The lipophilic environments are also assumed to facilitate the transport of organic substrates to the biocatalyst sites on the outer surface of the support and possibly in and out of the matrix. Experimental results suggest that as the length of the pendant alkyl chain is increased, the activity of the lipase is enhanced. Moreover, for gels made from the butyl-substituted trimethylsilane chain, the iso-butyl structure results in a higher relative activity compared with the *n*-butyl structure. However, sol-gel formation became practically impossible at room temperature with longer alkyl-substituted precursors.

Precursor screening experiments determined that the Lipase PS was immobilized on iso-BTMS and TMOS at a molar ratio of 3.9:1 and that the water-to-precursor ratio of 10.34 resulted in the highest relative activity of the immobilized lipase compared to the free enzyme. These parameters were kept unchanged throughout the rest of this study, unless stated otherwise.

Degree of immobilization. The immobilization procedure was carried out with a certain nominal amount of lipase. This procedure involved condensation polymerization and formation of the gel, which was followed by subsequent washing and drying. During the immobilization process, lipase was either entrapped within the polymer matrix or loosely adsorbed on the surface of the support. Upon subsequent washing of the gel with water, the adsorbed and loosely entrapped lipase was washed away and only the entrapped lipase remained in the gel. Experiments were performed to quantify the exact amount of entrapped lipase. To accomplish this, a calibration curve was developed for the amount of lipase protein in the supernatant from the wash (see Procedure C for details). Results revealed that at 125, 250, 500, and 1000 mg levels of Lipase PS in 59 mmol of precursor (12.1 mmol of TMOS and 46.9 mmol of iso-BTMS), the total amount of lipase protein that was released into the respective supernatants from the wash corresponded to 9, 12, 20, and 27 mg of crude lipase, respectively. The amount of gel formed in these experiments was about 8 g. Based on 8 g of gel, the enzyme loading for the four levels of lipases tested was calculated at 15, 30, 60, 120 mg/g of gel for an initial loading of 125, 250, 500, and 1000 mg of lipase, respectively. Results showed a better than 92% degree of immobilization in all experiments. Results also revealed that as the initial enzyme loading in the immobilization process was increased, the degree of immobilization also increased. At an enzyme loading of 1000 mg, more than 97% of the lipase was immobilized within the polymer during the sol-gel process. This was particularly interesting, as this level of lipase also resulted in the highest relative activity with respect to the free enzyme.

In the next stage of our study, the amount of immobilized lipase in the hydrolysis of soybean oil was optimized. Entrapped-enzyme loadings were tested in the range of 1–3 g of gel/10 g of oil at 1-g increments. Experimental results showed that as the entrapped-enzyme loading was increased, formation of products also increased. However, no maximal value for loadings was found, because at support levels beyond 3 g, the reaction mixture was quite thick and difficult to process. At this level of loading, the relative activity of the immobilized lipase compared to the free enzyme was 110%.

Effect of temperature. Experiments were performed to examine the thermal stability of Lipase PS entrapped in TMOS/iso-BTMS gel in the hydrolysis of soybean oil. Temperatures in the range of 40–80°C were tested at 10°C increments. Parallel experiments were performed with free enzyme for comparison. Lipases were also subjected to a period of incubation at the reaction temperature prior to the experiments to examine their thermal stability. Other experimental conditions for these reactions were 3 g of immobilized gel, equivalent to 360 mg of free enzyme/10 g of oil, 10 g of water/10 g of oil, a stirring rate of 700 rpm, and 1 h of reaction. Reaction products as a function of the incubation period and reaction temperature are presented in Figure 2. Results showed that as the reaction temperature was increased, the hydrolytic activity of the lipase also increased. The enhancement in activity was much stronger for the immobilized lipase than for the free enzyme. For example, after 3 h of preincubation, the total FFA increased from 61.3 mol% at 40°C to about 74.4 mol% at 80°C for the immobilized lipase. Under similar conditions for the free enzyme, the total FFA was about 53.7 mol% at 40°C, and no consistent upward trend in the total FFA composition was observed as the temperature was raised to 80°C. The

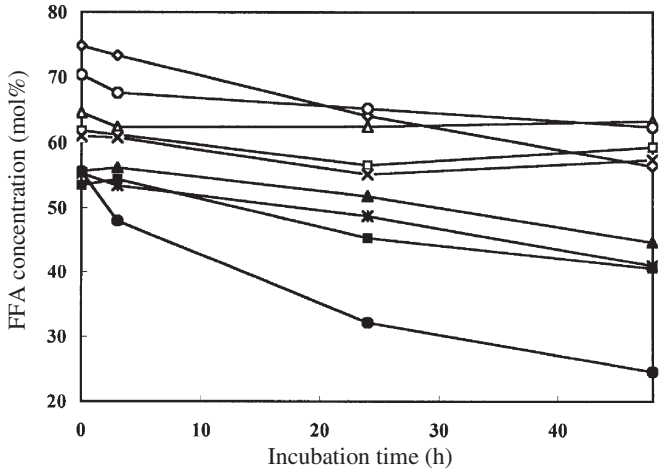

FIG. 2. Effect of temperature and incubation on lipase activity, subject to a loading of 3 g of gel/10 g of oil, 10 g of water, a stirring rate of 700 rpm, and a 1-h reaction. Free lipase reaction: (*) 40°C; (●) 50°C; (▲) 60°C; (■) 70°C. Immobilized lipase reaction: (×) 40°C; (○) 50°C; (△) 60°C; (□) 70°C; (◆) 80°C. For abbreviation see Figure 1.

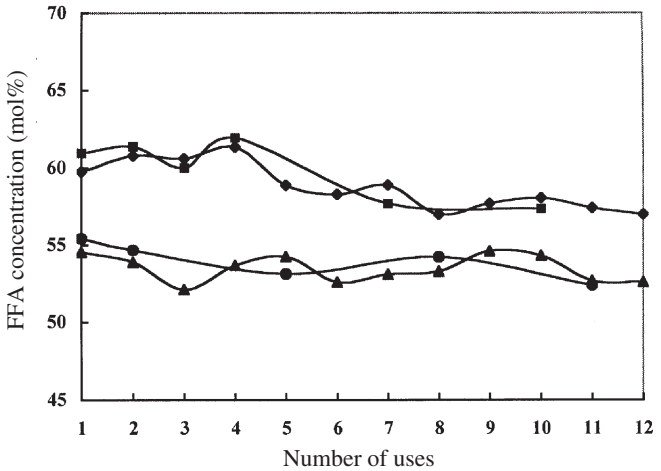

FIG. 3. Reuse of immobilized lipase and residual activity at 40°C, a loading of 3 g of gel/10 g of oil, 10 g of water, and a stirring rate of 700 rpm. (◆) 1-h reaction, immobilized lipase with a loading of 120 mg of lipase/g of gel; (■) residual reaction; (▲) 1-h reaction, immobilized lipase with a loading of 60 mg of lipase/g of gel; (●) residual reaction. For abbreviation see Figure 1.

optimal temperature was based on both the activity and the stability of the lipase. As Figure 2 shows, the immobilized lipase was stable up to 70°C at all incubation periods. Some loss of activity was observed at 80°C for this lipase. For the free enzyme, all temperatures above 40°C were detrimental to enzyme activity, and a moderate to severe loss of activity was observed beyond 40°C. The observed enhancement in thermostability of the immobilized lipase is consistent with the work of Kawakami (21) and has been attributed to the conformation change of the lipase. It appears that stable conformations similar to the interphase activation occur due to the interaction of lipase with the polymer surface. Both physical and chemical interactions such as hydrogen bonding and ionic interactions are believed to be responsible for the enhanced thermostability of the immobilized lipase (15).

Residual activity. One of the most important characteristics of an immobilized lipase is its stability and reusability over an extended period of time. Experiments were performed to examine the recyclability and stability of the used gel after storage. In the first set of experiments, reusability and degree of leakage of lipase from the support were examined. Enzyme loadings of 60 and 120 mg/g of gel were tested in this set of experiments. After each standard 1-h reaction, lipase-containing gel was separated from the supernatant by centrifugation and filtration. The lipase-containing gel was then reused by the addition of fresh soybean oil and water (see Procedure D). The supernatant, which contained both water and organic phases, was subjected to a residual activity test. In this test, the supernatant was simply incubated for 24 h at the reaction temperature. The purpose of this test was to quantify any further reaction that might have occurred after the removal of the immobilized lipase. Occurrence of an additional reaction beyond the initial 1-h reaction time was attributed to the leakage of lipase from the support. This procedure was repeated 12 times.

Results summarized in Figure 3 show a decrease in the formation of products. However, this decrease was slight and was more pronounced over the first six runs for the 120-mg enzyme loading; no significant decrease in the activity of the enzyme was observed over the last six runs. This suggests some loss of lipase during the first six experiments caused by lipase leakage from the gel. However, since the degree of activity leveled off for later runs, this leakage may well have been from the adsorbed and weakly entrapped lipase on the surface of the support. Results also showed no significant increase in the amount of FFA in the supernatant upon additional incubation after removal of the immobilized enzyme, which is also an indication of very little leakage of lipase during the reaction.

The lipase-containing gels recovered from the twelve 1-h runs were then subjected to 2 wk of storage at room temperature. Four immobilized lipases with enzyme loadings of 15, 30, 60, and 120 mg of lipase/g of gel were used in this set of

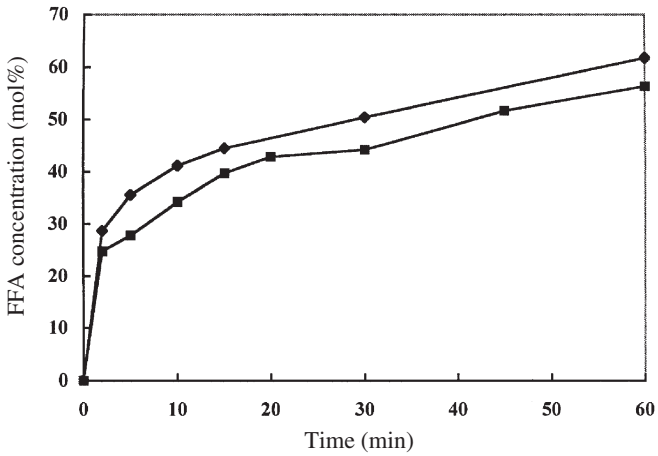

FIG. 4. One-hour time course of the hydrolysis of soybean oil at 40°C, 10 g of oil, 10 g of water, and a stirring rate of 700 rpm. (◆) Immobilized lipase: 3 g of gel/10 g of soybean oil; (■) free lipase: 360 mg of Lipase PS/10 g of oil. For abbreviation see Figure 1.

experiments. After 2 wk of storage, the stored lipases were subjected to a routine 1-h reaction (see Procedure D). The results showed that the immobilized lipase maintained its catalytic activity at or above 95% of the average formation of FFA of the first 12 uses. This is indicative of very little or no lipase denaturation during storage time.

Kinetics of the hydrolysis of soybean oil. Two factors of particular interest in the scaled-up process are reaction kinetics and reactor residence time. It was emphasized earlier that the sol-gel–immobilized lipase made from iso-BTMS and TMOS resulted in the highest concentration of FFA. The level of FFA for this system was slightly higher than that formed with free enzyme.

The hydrolysis reaction was further investigated for both free and immobilized enzyme during the first hour of reaction. The enzyme loading for the free enzyme experiments was 360 mg of Lipase PS/10 g of soybean oil. The enzyme loading for the immobilized enzyme experiments was equal to the free enzyme experiments, at 360 mg of Lipase PS/3.0 g of gel or 10 g of soybean oil. The rate of reaction was compared for the entrapped and free enzyme. Reaction results for the formation of FFA during the first hour of the reaction are presented in Figure 4. As this figure illustrates, the entrapped and free enzyme showed a sudden surge in the formation of FFA at the beginning of the reaction, followed by a slower rate as the reaction progressed. The trend was quite similar in the two cases examined.

To investigate the kinetics of the reaction at longer reaction times, the supported and free enzymes were subjected to 18 and 48 h of reaction, respectively. As Figure 5 shows, in using gels that were made by the immobilization of Lipase PS in iso-BTMS/TMOS precursors, FFA formation reached beyond 96 mol% after 18 h of reaction, compared to the free enzyme, which reached the same level of FFA after 42 h of reaction. In general, activity of the lipase was somewhat enhanced due to the immobilization over the course of the hydrolysis reaction.

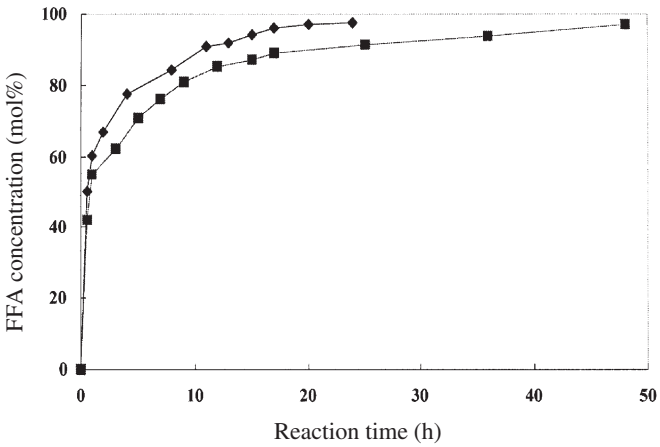

FIG. 5. Time course of the hydrolysis of soybean oil at 40°C, 10 g of oil, 10 g of water, and a stirring rate of 700 rpm. (◆) Immobilized lipase: 3 g of gel/10 g of soybean oil; (■) free lipase: 360 mg of Lipase PS/10 g of oil. For abbreviation see Figure 1.

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