# Immobilization of *Candida rugosa* Lipase by Sol-Gel Entrapment and Its Application in the Hydrolysis of Soybean Oil

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**ABSTRACT:** Immobilization of lipase AY from *Candida rugosa* by entrapment within a chemically inert hydrophobic sol-gel support was studied. The gel-entrapped lipase was prepared by polycondensation of hydrolyzed tetramethoxysilane and isobutyltrimethoxysilane. Certain modifications were incorporated into the conventional immobilization procedure, including the use of glucose as additive and the application of vacuum during the drying and aging stages. The activity and thermostability of immobilized enzyme were subsequently determined in hydrolyzing soybean oil. Hydrolysis results showed more than 95 mol% of the theoretical yield for the formation of FFA after 1 h of reaction at 40°C. The level of FFA was 3.3 times greater than that seen when an immobilized enzyme was prepared by the conventional sol-gel process. The immobilized enzyme retained most of its hydrolytic activity compared to the free enzyme and kept more than 95% activity after 120 h of incubation at 40°C, whereas the free enzyme lost 67% of its activity after 24 h of incubation and almost all of its activity after 96 h of incubation at 40°C. The immobilized enzyme also proved to be very stable, as it retained more than 90% of the initial activity after 16 one-hour reactions. Surface characterization studies suggested that the enzyme-containing sol-gel particles have amorphous morphology and are void of micro/meso pores.

Paper no. J10598 in *JAOCS 80*, 1077–1083 (November 2003).

**KEY WORDS:** *Candida rugosa*, entrapment, hydrolysis, immobilization, lipase, sol-gel, soybean oil.

The conversion of vegetable oils and animal fats into value-added products such as FFA and their derivatives has been of considerable practical interest (1). The established physiochemical methods for this conversion, which normally involve an inorganic homogeneous catalyst and high temperatures and pressures, are highly energy intensive. Enzymatic conversion of fats and oils has been suggested as a realistic alternative to the conventional physiochemical methods. However, the industrial use of biocatalysts has not yet reached an economically significant level owing to the high cost of enzymes and the lack of a means to separate them from the products for reuse. Enzyme immobilization has been suggested as a realistic approach in overcoming these obstacles.

Among the many available immobilization methods, entrapment of enzymes in an inorganic polymer matrix has received a lot of attention in recent years. Reetz and co-workers (2,3) studied the immobilization of *Pseudomonas cepacia* by entrapment in a sol-gel matrix using tetramethoxysilane (TMOS) with several alkyl-modified silane precursors of the type $RSi(OCH_3)_3$. Experimental results revealed a significant increase in the esterification activity of the immobilized enzyme compared with that of the free enzyme. The relative activity of the immobilized enzyme was increased with an increase in the length of the alkyl side chain of the polymerization precursors. The entrapped enzyme also showed good stability when subjected to repeated usage. Hsu and coworkers (4) developed a novel procedure for the immobilization of lipase from *P. cepacia* (PS-30) within a phyllosilicate sol-gel matrix. The immobilized lipase showed more stability and higher activity compared with free enzyme in the esterification of lauric acid with octan-1-ol.

Kawakami and Yoshida (5) studied the immobilization of lipase from *Candida rugosa* by sol-gel entrapment. Several systems, including hybrid gel-entrapped enzyme on a methyltrimethoxysilane (MTMS)/TMOS gel and enzyme entrapped in modified silicates on Celite 545 and Hyflo Super-Cel, were investigated. The prepared sol-gel–entrapped lipases showed increased thermal stability and higher activity at elevated temperatures in the synthesis of the esters of isoamyl alcohol and butyric acid. In another study, Hertzberg and co-workers (6) examined the catalytic activity of an entrapped lipase from *C. rugosa* in calcium alginate in several esterification and transesterification reactions. The immobilized enzyme showed enhanced operational stability compared to the free enzyme.

Other studies on the immobilization of lipase from *C. rugosa* by entrapment include the study by Kimura and co-workers (7) on photo-cross-linkable urethane prepolymers. The enzyme entrapped by this method, when used in the hydrolysis of olive oil, showed good activity and stability. In another study, Yang and Chen (8) examined the hydrolytic activities and specificities of gel-entrapped lipases from *C. rugosa* and *Rhizopus arrhizus* in the hydrolysis of olive oil and tributyrin. Results revealed a much higher activity toward tributyrin. This activity was strongly enhanced by the chain length of the polypropylene glycol-based prepolymer.

In the current study, immobilization of lipase AY from *C. rugosa* by sol-gel entrapment and the application of the immobilized lipase in the hydrolysis of soybean oil were investigated. The findings from a previous study on lipase PS from *P. cepacia* (9) were instrumental in this investigation. Efforts also were made to modify the immobilization procedure to fit lipase AY from *C. rugosa*.

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## EXPERIMENTAL PROCEDURES

*Materials*. Lipase AY from *C. rugosa* was generously donated by Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). TMOS (95%), iso-butyltrimethoxysilane (iso-BTMS) (97%), sodium fluoride (NaF), and D-glucose (96%) were purchased from the Aldrich Chemical Company (Milwaukee, WI). MTMS (95%) was purchased from Fluka (Buchs, Switzerland). Soybean oil was donated by Archer Daniels Midland Co. (Lincoln, NE). Hexane (GC grade) was purchased from EM Science (Gibbstown, NJ) and bis(trimethylsilyl)trifluoroacetamide (BSTFA, derivative grade), 1,2,3-tridecanoylglycerol (tricaprin, 95%), and pyridine were purchased from the Sigma Chemical Co. (St. Louis, MO). All the materials were used without further purification.

*Immobilization procedure.* Six hundred milligrams of lipase AY from *C. rugosa* and 10 g of water were measured into a widemouthed clear bottle. A magnetic stir bar was added, and the mixture was stirred at 150 rpm for about 5 min to make a homogeneous enzyme solution. To this mixture, 1 mL of a 1 M NaF solution, 9 mL of iso-BTMS, and 1.8 mL of TMOS were added. Upon the addition of the precursors, the reaction occurred almost immediately and the gel state was reached in about 2 min. The temperature of the reaction medium was monitored and maintained below 40°C during the entire procedure. The flask was then left sealed at room temperature for 24 h. The drying procedure was carried out in a constant-temperature water bath at 33°C for about 24 h. The dried polymer was then ground in a mortar. The powder was washed with 100 mL of distilled water while being stirred at 500 rpm for 1 h. About 90 mL of the supernatant was collected from the wash. The supernatant was further examined for determination of the degree of immobilization of the immobilized enzyme (see the *Tests of Degree of immobilization* section). The wet paste was dried again at 33°C for 24 h in a constant-temperature bath. The immobilized enzyme was then crushed in a mortar and stored at 4°C until use. About 6 g of the sol-gel material was produced in this procedure, called procedure 1.

To improve the activity and the stability of the immobilized lipase, certain modifications to procedure 1 were considered. In procedure 2, 20 g of water was used instead of 10 g to dissolve the lipase, and immediately after the formation of the gel the reaction flask was subjected to –0.1 bar of pressure for 6 h to remove methanol and water. The resultant gel was sealed and then kept at room temperature for about 24 h as in procedure 1. In procedure 3, 4 g of D-glucose was added to the enzyme solution prior to the addition of the precursors; otherwise, this procedure was identical to procedure 1. And in procedure 4, the modifications included the addition of 20 g of water, introduction of D-glucose to the enzyme solution, and application of vacuum.

*Free enzyme reactions*. The optimal conditions were: 50% (w/w) water/oil ratio, 300 mg of lipase AY/10 g of soybean oil, 40°C, 700 rpm stirring rate, and 1 h of reaction time (10). Unless otherwise stated, the optimal conditions were used when reference is made to free enzyme reactions. The reactions were run in a constant-temperature water bath while using a Thermolyne (Dubuque, IA) Mirak magnetic stirrer model #S73135. Water was circulated into the bath from a Neslab (Portsmouth, NH) TTE-211 Bath/Circulator that, *via* an external probe, controlled the bath temperature to within ± 0.01°C. The degree of hydrolysis was defined as the mol% of the FFA in the reaction product.

*Tests of degree of immobilization.* The immobilized enzyme was washed with water and, after filtration, about 90 ± 5 mL of supernatant was collected. This supernatant potentially may contain free enzyme, unreacted precursors, siliconols, methanol, and soluble oligomers. To quantify the amount of enzyme in the supernatant, a calibration curve relating the formation of FFA as a function of free enzyme loading for the hydrolysis of soybean oil was constructed. By comparing the supernatant from the immobilization wash procedure with this calibration, the amount of enzyme in the supernatant and the degree of immobilization were determined. To mimic the exact media in the calibration studies, a blank gel with no enzyme was prepared. The supernatant, which was collected from washing this gel, was used as the reaction medium in the calibration experiments. Otherwise, the reaction procedure for the calibration experiments was identical to the free enzyme reactions. For calibration purposes, the enzyme loading was varied from 0.3 to 30 mg lipase per 1 g of soybean oil. The calibration curve showed a monotonically increasing activity as the enzyme loading was increased, which leveled off beyond the upper limit of 30 mg of enzyme per 1 g of soybean oil.

*Immobilized enzyme reactions*. Experiments were conducted to examine the stability, reusability, effect of temperature, and reaction kinetics for the immobilized enzyme in the hydrolysis of soybean oil. Stability of the immobilized enzyme was determined by its incubation in water prior to use in the hydrolysis reaction and examination of its activity after this procedure. Reusability was determined by subjecting the immobilized enzyme to 16 consecutive 1-h reuses. The reaction kinetics were based on the time course of change of the reactant during the initial 60 min of the reaction, and the effect of temperature in the range of 25–80°C was determined.

In a typical reaction procedure, 10 g of oil, 10 g of water, and 3 g of the immobilized enzyme were weighed into a 50-mL Erlenmeyer flask. The flask was placed in a constant-temperature water bath and subjected to 700 rpm of stirring. After 1 h, stirring was stopped and the reaction mixture was immediately vacuum-filtered through grade one filter paper with the aid of a vacuum pump. The stability experiments differed from the above procedure in that the initial mixture of water and the immobilized enzyme was incubated with mixing at 40°C in a water bath for a period of time (0–120 h) before using the enzyme in the reaction.

For determination of the reusability of the immobilized enzyme, the resultant paste was carefully scraped off the filter and placed back into the original reaction flask. Fresh soybean oil and water were added, and the procedure was repeated. The supernatant from the vacuum filtration procedure was sampled and transferred into a 50-mL flask. This mixture was stirred at 700 rpm in the same water bath for 24 h to

examine any potential residual activities that may have resulted from the leakage of enzyme from the support.

*Sampling and analysis*. Samples from the reactions were first heated to ensure enzyme denaturation, freeze-dried to remove excess water, and finally derivatized with BSTFA. The derivatized samples were then analyzed by GC to determine the concentration of FFA, MG, DG, and TG. A Hewlett-Packard (Wilmington, DE) 6890 Series GC system was used for the chromatography work, and Hewlett-Packard Chemstation software was used for the data analysis. The gas chromatograph was equipped with a Hewlett-Packard (part number 19091J-012) HP-5 column. Sample volumes were 2 μL, the carrier gas was helium, and the gas chromatograph was operated in constant flow mode at a flow rate of 12.0 mL/min. A split injector was used with a split ratio of 15:1 and a temperature of 325°C. The FID 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, and the temperature was then elevated 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, MG, DG, TG, and soybean oil. The standards were derivatized in the same fashion as the reaction samples. Details about the sample preparations and analysis procedures are explained elsewhere (9).

*Characteristics of the immobilized enzyme*. Experiments were performed to investigate the specific surface area (SSA) of enzyme-containing sol-gel materials. Volumetric methods based on the Brunauer, Emmett, and Teller (BET) theory were used (11). The nitrogen adsorption experiments were carried out in an ASAP 2010 (Micromeritics Instrument Corporation, Norcross, GA). About 200 mg of dried sol-gel was weighed into a standard sample tube. The samples were degassed at 50°C for about 48 h prior to the adsorption procedure. A whole-scale adsorption curve was generated. The adsorption data were processed through ASAP 2010 software, which plots the adsorption isotherms, fits the adsorption data into the BET model, and estimates the BET constant, the specific surface area, and the porosity of the tested material (pore volume per gram of solid specimen). Manipulation of the desorption isotherms with this software provides for the fit of the data to other models such as the Barrett–Joyner–Halenda method, which ultimately results in information about pore size, pore size distribution, and pore geometry of the tested material.

*Scanning electron microscopy (SEM).* SEM was performed to study the morphology of the sol-gel enzyme material and to investigate the effect of immobilization procedures on the morphology. SEM images were taken by a JEOL 2000 SEM (JEOL Ltd., Tokyo, Japan). The samples were coated with gold in a customized device prior to the imaging procedures.

*Statistics*. Data shown in tables and figures are mean values and are based on at least three experimental trials. The SD, which are not shown in the figures, were within ±5% of the mean values.

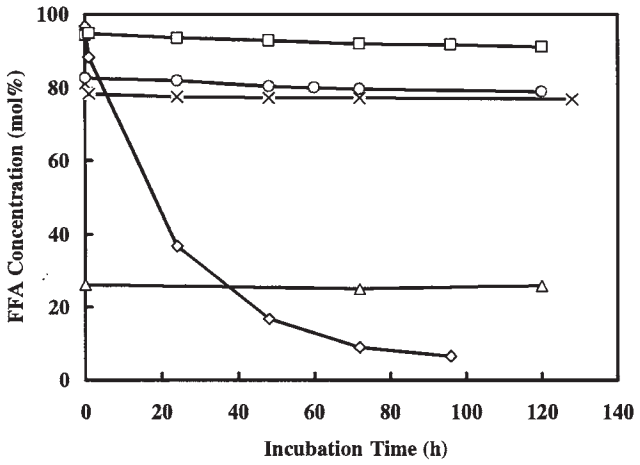


**FIG. 1.** Effect of immobilization procedures on the thermostability of immobilized lipase in the hydrolysis of soybean oil at 40°C, a loading of 3 g of gel per 10 g of oil (300 mg free lipase per 10 g of oil), 10 g of water, a stirring rate of 700 rpm, and 1 h of reaction. Enzyme/water mixtures were incubated at 40°C prior to the reactions. (◇) Free lipase AY (from Amano Pharmaceutical Co. Ltd., Nagoya, Japan), (△) immobilized lipase AY prepared *via* procedure 1, (○) immobilized lipase AY prepared *via* procedure 2, (×) immobilized lipase AY prepared *via* procedure 3, (□) immobilized lipase AY prepared *via* procedure 4.

## RESULTS AND DISCUSSION

*Precursors and immobilization procedure*. Experiments were performed to evaluate the activity and stability of the immobilized lipase. In procedure 1, the immobilized lipase AY was prepared according to the formulation presented in the Experimental Procedures section. The soybean oil was subjected to the hydrolysis reaction in the presence of the immobilized enzyme (Fig. 1, at zero incubation time). For comparison, free enzyme reaction results also are shown in this figure. The enzyme immobilized by procedure 1 resulted in 25 mol% of FFA after 1 h of reaction without preincubation in water, whereas about 95 mol% of FFA was formed when the same amount of free enzyme was used. The tests of degree of immobilization for this procedure show that more than 95% of the free enzyme was immobilized. The lower activity of the immobilized enzyme by this procedure may be due to the denaturation of enzyme during the immobilization procedure or inaccessibility of the immobilized enzyme to the substrates. Accordingly, improvement of the immobilization procedure was attempted.

Methanol is a co-product of the sol-gel procedure and is believed to inhibit the activity of certain lipases including lipase AY (12). This possibility was further evaluated by simulating the effect of methanol in the following experiment. Six hundred milligrams of lipase AY was incubated in 10 g of water and 6 g of methanol for 1 h (this is equal to the theoretical amount of methanol released by the hydrolysis of silanes when 600 mg of lipase AY is immobilized). Methanol and water were then removed by freeze-drying. The recovered lipase AY was subjected to the hydrolysis reaction with 20 g of soybean oil and 20 g of water at 40°C and a mixing intensity of 700 rpm.

After 1 h of reaction, less than 5 mol% of FFA was formed, whereas more than 95 mol% of the theoretical amount of FFA was formed when the same amount of lipase was used without incubation in the methanol/water solution. The results indicate that the removal of methanol during the formation and aging of the gel may increase the activity of the immobilized lipase. Results further suggest that the sol-gel matrix may physically play a role as a protective shield for the lipase, as the activity of the immobilized enzyme (about 25% FFA) is more than five times that of the free enzyme in the methanol environment (less than 5%).

Based on the these findings and on methanol's potential role in deactivation of lipase AY, procedure 2, which included application of a vacuum to remove methanol, was used during the immobilization procedure. About 6 g of the sol-gel material was formed in this procedure. Tests of the degree of immobilization showed that more than 95% of the enzyme was immobilized in this procedure. Based on 95% immobilization of the enzyme, the actual enzyme loading was determined at 285 mg of lipase AY per 3 g of gel. The immobilized lipase AY was then used in the hydrolysis of soybean oil (Fig. 1, procedure 2, at zero incubation time). As shown, the removal of methanol during the aging process had a significant effect on the activity and stability of the immobilized enzyme. The level of FFA formed was about 81 mol% after 1 h of hydrolysis.

Low activity of the immobilized enzyme also may have been caused by the inaccessibility of the enzyme to the substrates. To make the immobilized enzyme more available to the substrate, a third procedure was devised to manipulate the porosity of the lipase-containing sol-gel particles through the use of nonstructural pore-forming agents. Particles with meso/macro pore structure and increased specific surface area could make more active enzyme sites accessible to the substrates. The role played by chemical additives in the porosity of enzyme-free sol-gel material is documented in the literature (13). Preliminary investigations concentrated on the use of solvents such as formamide, ethyl glycol, and *N*,*N*,-dimethylformamide, which are known to induce an increase in the specific area of the enzyme-free sol-gels. However, when enzyme was included in the sol-gel process and temperature limitations were imposed to the aging and drying procedures, no significant improvement in the activity of the immobilized lipase was observed.

D-Glucose has been used as a nonstructural template in the sol-gel reaction and is believed to have the potential to create mesopores upon its extraction (by water) from the structure of the sol-gel materials (14). This idea was incorporated into immobilization procedure 3 by the addition of 4 g of D-glucose to the enzyme solution prior to the sol-gel procedure. About 6 g of final product was yielded in this procedure. Based on 95% immobilization of the enzyme, the enzyme loading was determined as 285 mg of lipase AY per 3 g of the gel. The resultant gel was then used to hydrolyze soybean oil (Fig. 1, procedure 3, at zero incubation time). The addition of D-glucose to the enzyme solution prior to gelation resulted in about 80 mol% FFA formation after 1 h. However, contrary to our initial hypothesis, structural analysis of the matrix showed no increase in micro- and mesoporosity and no increase in BET specific surface area of the matrix (discussed below). Nevertheless, the improvement in the activity of the immobilized enzyme mirrored that of procedure 2, in which methanol was removed from the gel environment. This behavior may be due in part to the fact that strong associations exist between lipase and glucose molecules, and structural changes, particularly around the lid covering the active site of the lipase, are reported in the literature (15). These structural changes can inhibit hydrolytic activity by some lipases and enhance that of other lipases. Lipase AY is in the latter group. The association between D-glucose and lipase AY occurs as they are mixed together and prior to the formation of methanol, which occurs during the immobilization process. This association may have nullified the association between methanol and enzyme molecules and therefore offset methanol inhibitions.

Procedure 4, which combined the effects of procedures 2 and 3, was devised to maximize the activity of the immobilized enzyme. In this procedure, glucose was added to the precursors prior to the polymerization, and methanol was removed from the sol-gel reaction medium as in procedure 2. Similarly, 6 g of immobilized enzyme was prepared in this procedure. Based on 95% immobilization of the enzyme, the enzyme loading was determined to be 285 mg of lipase AY per 3 g sol-gel material. The hydrolysis reaction results for the immobilized enzyme are included in Figure 1 (procedure 4, zero incubation time). There was some improvement over the use of vacuum alone or of D-glucose alone in the immobilization procedures. The activity of the immobilized enzyme was comparable with that of the free enzyme and resulted in more than 95 mol% of FFA after 1 h of hydrolysis. The synergy among the modifications used in the immobilization procedure, which ultimately results in either the removal of methanol or counterbalance of its inhibitory effects, appears to have more than compensated for the presence of methanol. The apparent equal reactivity of the free enzyme and the immobilized enzyme by procedure 4 does not take into account the inaccessibility of a portion of the immobilized enzyme. It is well within reason to assume that the specific activity of the immobilized enzyme is higher than that of the free enzyme, because the nominal concentration of free enzyme was 300 mg per 10 g of oil and that of the immobilized enzyme, including the inaccessible enzymes, was 285 mg per 10 g of oil. The enhancement in the activity of the immobilized enzyme could in part be due to the phenomenon known as alkyl effect (9) and also to favorable conformations of lipase AY when entrapped in the sol-gel material. Lipase PS immobilized *via* procedures 2, 3, and 4 did not demonstrate any activity enhancement beyond the immobilized PS *via* procedure 1 (9). The results for lipase PS also support our initial hypothesis about methanol inhibition because lipase PS is believed to be resistant to methanol and its inhibitory effects (12).

*Effect of temperature*. Experiments were performed to examine the thermal stability of the immobilized and the free lipase AY at 40°C. The free (300 mg) and immobilized enzymes (3 g) were initially incubated at 40°C in 10 g of water for a period of time prior to hydrolysis of soybean oil (Fig. 1). Results showed

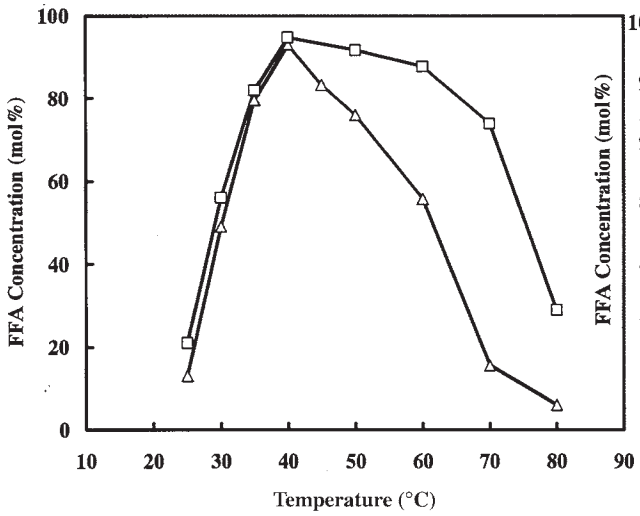


**FIG. 2.** Effect of temperature on the activity of the immobilized and free lipase AY in the hydrolysis of soybean oil at a stirring rate of 700 rpm for 1 h of reaction. (□) Immobilized enzyme prepared *via* procedure 4 with a loading of 285 mg of AY per 10 g of oil and 10 g of water; (△) free enzyme loading of 300 mg of AY per 10 g of oil and 10 g of water.

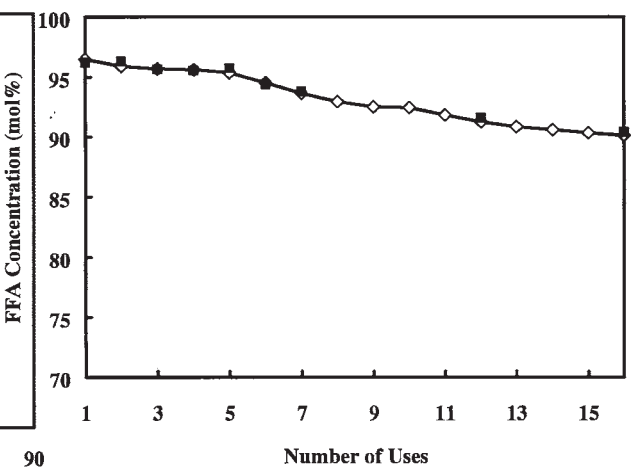


**FIG. 3.** Reuse of immobilized lipase and residual activity at 40°C, a loading of 3 g of gel per 10 g of oil, 10 g of water, and a stirring rate of 700 rpm. (◇) One-hour reaction, immobilized lipase AY loading of 285 mg of lipase/3 g of gel, (■) residue reaction.

that free lipase AY lost 67% of its activity after 24 h of incubation and almost all of its activity after 96 h of incubation at 40°C, whereas the immobilized enzymes retained most of their activity after up to 120 h of preincubation. The observed enhancement in thermostability of the immobilized enzymes was consistent with that of immobilized lipase PS (9) and also the work of Kawakami and Yoshida (5) and may be attributed to the degree of cross-linking of the sol-gel matrix. Apparently, stable conformations similar to the interphase activation occur as a result of interaction of enzyme 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 enzyme (3).

Experiments were also performed to examine the effect of temperature on the hydrolytic activity of free lipase AY and immobilized lipase AY prepared *via* procedure 4. Temperatures in the range of 25–80°C were tested (Fig. 2). Free and immobilized lipase AY demonstrated similar hydrolytic activities at lower temperatures, and both reached their maximal activity at 40°C of about 95 mol% of FFA. At temperatures above 40°C, however, the free enzyme appeared to be much more sensitive to temperature than the immobilized enzyme. The FFA formation for the free enzyme was as follows (mol%, °C): 70, 50, 55, 60, and <5, 80. The immobilized enzyme was more resistant to temperatures above 40°C and retained most of its activity up to 60°C. FFA formation was (mol%, °C) 88, 60; 76, 70; and ~30, 80.

*Enzyme reusability and stability.* One of the most important characteristics of an immobilized enzyme is its stability and reusability over an extended period of time. Experiments were performed to examine the recyclability and the stability of the immobilized lipase AY prepared *via* procedure 4. After each standard 1-h hydrolysis, lipase-containing gel was recovered by filtration and then reused. This procedure was repeated 16 times. The supernatant, which contained both water and the organic phases, was stirred at 40°C for 12 h to determine the activity released from the immobilized support. Examination of Figure 3 shows a slight decreasing trend in the formation of the products. Overall production of FFA dropped from 95 to about 89% over the 16 consecutive runs. In general, the decrease in the production is attributed to several factors: (i) immobilized enzyme losses during the recovery, (ii) entrapped-enzyme leakage during the reaction, (iii) enzyme denaturation during the reaction, and (iv) losses of adsorbed enzymes during the reaction. However, there was no significant increase in the level of FFA in the supernatant upon further incubation (Fig. 3). This is indicative of very little leakage of enzyme from the support over the course of the reaction. Some immobilized enzyme losses are inevitable during the processing and could be blamed for some of the decline in the production rate of FFA. However, because there was only a 6% decrease in FFA production in 16 consecutive 1-h reactions and because the immobilized enzyme was very stable after 120 h of incubation (Fig. 1), one may conclude that the immobilized enzyme prepared by the described sol-gel procedure (procedure 4) is suitable for repeated applications.

*Kinetics of the hydrolysis of soybean oil*. Reaction kinetics ultimately determine the reaction residence time and are of particular interest in process scaleup and design. The initial first-hour kinetics for the hydrolysis reaction was investigated for both the free and the immobilized lipase AY prepared *via* procedure 4. The enzyme loading for the free enzyme experiments was 300 mg of lipase AY per 10 g of soybean oil. The enzyme loading for the immobilized enzyme experiments was 285 mg of lipase AY per 3.0 g of gel and per 10 g of soybean oil (Fig. 4). The trend for both experiments was an initial surge in FFA formation followed by a slower rate as the reaction progressed.

*Characterization of the immobilized enzyme.* Lipase-containing sol-gel particles were characterized for particle size, specific surface area, and porosity, which are fundamental to the immobilized enzyme's catalytic and operational properties.

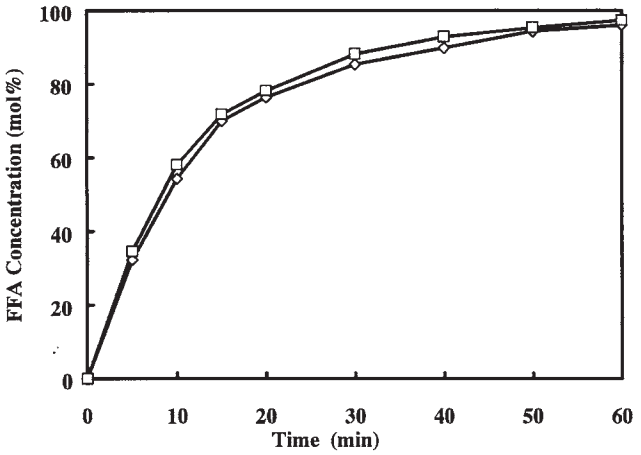


**FIG. 4.** One-hour time course of the hydrolysis of soybean oil at 40°C, 10 g of oil, 10 g of water, and a stirring rate of 700 rpm. (□) Immobilized lipase with a loading of 285 mg of lipase AY per 10 g of oil and 10 g of water; (◇) free enzyme with a loading of 300 mg lipase AY per 10 g of oil and 10 g of water.

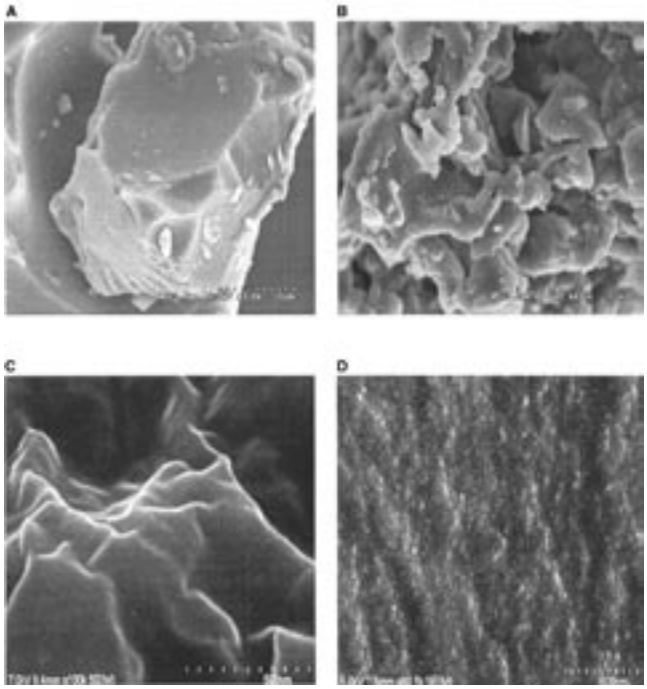


**FIG. 5.** Scanning electron microscopy images. (A) and (C), immobilized enzyme prepared *via* procedure 1; (B) and (D) immobilized enzyme prepared *via* procedure 4. Magnifications: (A) 3K; (B) 4.5K; (C) 100K; (D) 60K.

The characterization studies provided critical feedback on the enzyme immobilization procedure modifications and will be helpful in understanding the mechanism of enzyme immobilization.

Results of SSA experiments for the gels made from different precursors are presented in Table 1. All of the sol-gel samples prepared from TMOS and iso-BTMS exhibited very low SSA, and the adsorption isotherms for these samples were all-type II, which is known to be characteristic of nonporous material (11). The sol-gels made from TMOS and MTMS exhibited larger SSA values than for TMDS/iso-BTMS sol-gels. The SSA values of the tested material may have been primarily a function of the silane precursors used in their formulations. The presence of the enzyme did not have a significant effect on the SSA of the gels. All the gels of TMOS and iso-BTM, made with four different procedures, had relatively small SSA, and the modification efforts, which involved glucose addition and/or vacuum application, did not have a significant effect on the SSA of these gels, in contrast to the enzyme activity results. For example, gels made from TMOS and MTMS, which had the highest SSA, showed the lowest activity when used in the hydrolysis of soybean oil (about 10 mol% FFA was formed after 1 h of hydrolysis). The gels made from TMOS and iso-BTMS had the lowest SSA and the highest activity (about 95 mol% of FFA was formed under otherwise identical conditions).

Intuitively, the specific activity of an immobilized enzyme is expected to be higher for supports with larger SSA. However, experimental results did not concur, which suggests that the enhancement in the activity of our sol-gel immobilized samples should be attributed to something other than the SSA and porosity. SEM studies revealed that the immobilized enzyme prepared *via* procedure 4 had a significantly larger porosity (on a μm scale). The porosity of the structure may be responsible for some of the activity enhancement. However, the free alkyl groups of the precursors (e.g., iso-BTMS) are believed to be responsible for the bulk of the activity enhancement (2). The free alkyl group of the precursors creates 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 fixation of the lipase into a more favorable conformation during the sol-gel process. The lipophilic environments are also expected to facilitate the transport of organic substrates to the biocatalyst active 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 enhancement in the activity of the enzyme is increased (9). However, sol-gel formations become practically impossible at or below 40°C with longer alkyl-substituted precursors.

**TABLE 1**
**Specific Surface Area of the Immobilized Sol-Gel Material[a]**

| Sample # | Sample description | Specific surface area ($m^2/g$) |
|---|---|---|
| 1 | TMOS/MTMS, procedure 1 with no enzyme | 250.5 ± 8.3 |
| 2 | TMOS/MTMS, procedure 1 with lipase AY | 220.3 ± 4.2 |
| 3 | TMOS/iso-BTMS, procedure 1 with no enzyme | 8.6 ± 0.8 |
| 4 | TMOS/iso-BTMS, procedure 1 with lipase AY | 5.4 ± 0.6 |
| 5 | TMOS/iso-BTMS, procedure 2 with lipase AY | 5.5 ± 0.4 |
| 6 | TMOS/iso-BTMS, procedure 3 with lipase AY | 4.5 ± 0.6 |
| 7 | TMOS/iso-BTMS, procedure 4 with lipase AY | 6.1 ± 0.5 |

[a]TMOS, tetramethoxysilane; MTMS, methyltrimethoxysilane; iso-BTMS, isobutyltrimethoxysilane; lipase AY, derived from *Candida rugosa* (Amano Pharaceutical Co. Ltd., Nagoya, Japan).

Some representative images of the immobilized enzymes from procedures 1 and 4 at two different magnifications are presented in Figure 5. The images in this figure were chosen after thorough analysis of a large number of specimens in the samples are representative of the whole sample. The particle size analysis of the samples showed a normal distribution with an arithmetic mean size of about 8.4 μm for the enzyme-containing sol-gel particles. Therefore, the images shown in Figures 5A and 5B could be representative of a single particle. The immobilized enzyme prepared *via* procedure 4 (Fig. 5B) is more porous (on a μm scale) than the immobilized enzyme prepared by procedure 1 (Fig. 5A). However, porosity in this scale is not reflected in the SSA measurements (Table 1). That the hydrolysis reaction experiments (Fig. 1) showed a much higher activity for the gels made by procedure 4 further suggests that modifications by procedure 4 and the resultant structure (at a μm level) may be responsible for some of the activity enhancements of the immobilized enzyme. At a greater magnification (Figs. 5C, D), both structures suggest amorphous morphologies. The lack of micro/meso porosity in these particles is consistent with earlier results on SSA experiments.

## ACKNOWLEDGMENTS

The authors express their gratitude to the Nebraska Research Initiative and the Nebraska Soybean Board and the Biotechnology Core Facility at the School of Biological Science of the University of Nebraska for their support of this work.

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[Received March 24, 2003; accepted August 15, 2003]