Synthesis of Geranyl Acetate by Esterification with Lipase Entrapped in Hybrid Sol-Gel Formed Within Nonwoven Fabric

Jyh-Ping Chen*, Wei-Shin Lin, and Min-Far Chang

Department of Chemical and Materials Engineering, Chang Gung University, Taiwan, Republic of China

ABSTRACT: *Candida cylindracea* lipase was entrapped in organic-inorganic hybrid sol-gel polymers made from tetramethoxysilane (TMOS) and alkyltrimethoxysilanes. By forming the gels within the pores of a nonwoven polyester fabric, a novel immobilized biocatalyst in sheet configuration based on sol-gel entrapment of the enzyme was obtained. Lipases immobilized in sol-gel matrices efficiently catalyzed the direct esterification reaction of geraniol and acetic acid in anhydrous hexane to produce geranyl acetate. The optimal formulation of the sol-gel solution for enzyme immobilization was at a 20:1 molar ratio of water to total silane; a 4:1 molar ratio of propyltrimethoxysilane to TMOS; hydrolysis time at 30 min; and enzyme loading of 200 mg lipase/g gel. Under these conditions, protein immobilization efficiency was 91%, and the specific activity of the immobilized enzyme was 2.6 times that of the free enzyme. Excellent thermal stability was found for the immobilized enzyme in dry form or in hexane solution in the presence of acetic acid, in which case severe inactivation of free enzyme was observed. The immobilized enzyme retained its activity after heating at 70°C for 2 h, whereas the free enzyme lost 80% of its activity.

Paper no. J10038 in *JAOCS 79,* 309–314 (March 2002).

KEY WORDS: Esterification, flavor esters, geranyl acetate, immobilization, lipase, sol-gel.

The production of low M.W. esters as flavor compounds is important to the food industry. Consumers, however, are becoming more concerned and interested in the source and composition of flavorings used in food. The wholesomeness of a food is often associated with the term "natural"; therefore, there is an increased demand for flavors and fragrances that are considered natural. With this increasing demand for natural products, the flavor industry is interested in using biotechnological approaches to produce natural flavors. The large-scale synthesis of natural flavor-active esters using natural raw materials as substrates is possible by using enzymatic techniques (1).

Terpene esters are very important aroma compounds, and because of their organoleptic properties they also are among the important flavor components. Currently, terpene esters are obtained by traditional methods, such as steam distillation from plant material, which imposes a high processing cost and is accompanied by a low yield of the desired flavor components (2). Other developments include their biosynthesis by microbial fermentation. However, difficulties are encountered because of the natural breakdown of esters, which prevents their accumulation (3).

Enzyme reactions in organic solvents offer opportunities for the production of esters of flavor compounds, especially those of geraniol and citronellol. Geranyl and citronellyl esters are essential oils widely used in the food, cosmetics, and pharmaceutical industries. The application of lipase to the synthesis of terpene esters (mainly geraniol esters) is of great interest. Several lipases can catalyze the synthesis of esters either by esterification or interesterification reactions (4); however, the synthesis of terpene acetate by direct esterification usually gives low yields (5).

Many acetate esters (such as those of isoamyl, benzyl, citronellyl, and geranyl alcohols) are components of natural flavors. They can be obtained by lipase-catalyzed esterification in organic solvents, but a major problem with enzymatic acetylations is lipase inactivation by acetic acid. It is more difficult to prepare acetates under conditions similar to those under which higher homologous acids give ester yields that approach 100% (6). Previous reports have also indicated that lipase-catalyzed esterifications with free acetic acid are strongly affected by acid concentration. The presence of acetic acid in the reaction medium can alter either the hydration layer–protein interaction or the overall enzyme structure, which causes enzyme inhibition (7,8). In addition, most lipases have little affinity for two-carbon acids. Synthesis of geranyl acetate can be achieved by substituting free acetic acid with activated acetated esters (9), or by using alternative approaches such as alcoholysis and transesterification (10,11). The immobilization of an enzyme to enhance properties such as activity, thermal stability, and reduced substrate inhibition also can be an alternative way to improve its properties.

Sol-gel technology has proved to be a remarkably versatile method for producing solid silica polymers of defined porosity. It is based on the aqueous processing of hydrolytically labile alkylsilane precursors, rather than on high-temperature chemistry (12). The ability to form doped inorganic glasses in aqueous media under room-temperature conditions (where enzymes and cells are active) opens up the possibility of extending sol-gel processing to the encapsulation of enzymes. After pioneering work in the 1980s showed that antibodies and enzymes could be trapped within monolithic silica-poly(3-aminopropylsiloxane) sol-gel polymers (13,14),

*To whom correspondence should be addressed at Department of Chemical and Materials Engineering, Chang Gung University, Kwei-San, Taoyuan 333, Taiwan, Republic of China. E-mail: jpchen@mail.cgu.edu.tw

sol-gel entrapment in SiO_2 has been applied to a variety of enzymes (15). Gel-like materials were obtained that, in optimal cases, had enzyme activities approaching 100% relative to those of the free enzyme (16). These developments spurred a worldwide flurry of activity in the area of sol-gel entrapment of enzymes, including lipases entrapped in sol-gel powders (17–19). Sol-gel matrices appear nontoxic, which has been demonstrated by successful encapsulation of mammalian cells (20) and in an implantable device exhibiting good biocompatibility both *in vitro* and *in vivo* (21).

Although sol-gel immobilized enzymes are readily separated from reaction mixtures by simple filtration, alternative removal methods have been proposed, such as magnetic sol-gel matrices (22). We envisioned a different approach in this study, namely, the specific fixation of lipase-containing organic–inorganic hybrid sol-gel matrices within the pores of nonwoven supports, resulting in a heterogeneous biocatalyst with improved activity, ready reusability, and increased mechanical stability. In this work, parameters influencing the esterification of geraniol and acetic acid with *Candida cylindracea* lipase entrapped in such a supported sol-gel matrix were studied.

MATERIALS AND METHODS

Materials. Candida cylindracea lipase (type VII) was obtained from Sigma (St. Louis, MO) and used without further purification. Tetramethoxysilane (TMOS), methyltrimethoxysilane (MTMS), propyltrimethoxysilane (PTMS), and *n*-octadecyltrimethoxysilane (ODTMS) were obtained from TCI (Tokyo, Japan). *n*-Butyltrimethoxysilane (BTMS) was from United Chemical Technologies, Inc. (Bristol, PA). Octyltrimethoxysilane (OCTMS) was from Aldrich (Milwaukee, WI). The nonwoven polyester fabric sheet was a commercial product from Union Chemical Laboratories (Hsinchu, Taiwan) with an average nominal area density and thickness of 2.66 mg/cm^2 and 200 μm, respectively. All other chemicals used were of reagent grade or higher. Polyvinyl alcohol (PVA; M.W. 22,000) was obtained from Acros (Geel, Belgium). Nonadhesion polyester films treated with fluoropolymer (9956 Medical Heatsealable Film) were from 3M Company (Minneapolis, MN). Hexane was obtained from Acros and was dried on 4 Å molecular sieves to a water content of 30 mg/L.

Immobilization of lipase. TMOS (20 mmol, 3.04 g) or a mixture of TMOS and an organic alkyltrimethoxysilane, 700 μL deionized, distilled water, and 30 μL of 0.04 N HCl were vortexed for 10 s and then shaken at 250 rpm at 4°C for a specific time period (hydrolysis time) to form a homogeneous solution. Lipase (150 mg crude powder) was first dissolved in 2 mL potassium phosphate buffer (0.1 M, pH 7.2) and centrifuged at 5000 × *g* for 5 min to remove insoluble components. The supernatant was shaken with 2 mL PVA solution (4% w/w in water, for coating of the enzyme) and a suitable amount of phosphate buffer (0.1 M, pH 7.2 for adjusting the *R*, the molar ratio of water to silane). This mixture was then mixed with the above hydrolyzed silane solution and shaken for 10 s on a vortex mixer. The resultant mixture was distributed uniformly on the surfaces of three pieces of 5 × 5 cm nonwoven sheets in a Petri dish and shaken at 90 rpm at 4°C and for 30 min to start the gelation process. The nonwoven sheets with gelled silane were transferred to a nonadhesion polyester film, and the viscous solution left in the dish was withdrawn and evenly distributed between the nonwoven sheets, followed by incubating at 4°C for 48 h to complete the gelation process. The immobilized biocatalyst was shaken with 10 mL potassium phosphate buffer for 2 h to remove the nonentrapped enzyme, washed with pentane, and lyophilized. The amount of enzyme immobilized was calculated by subtracting the amount of protein in the wash solution from the original amount of protein used.

Esterification reaction. Ester synthesis was carried out in screw-capped flasks containing 0.15 mmol acetic acid and 0.30 mmol geraniol in 10 mL anhydrous *n*-hexane. The reaction mixture was shaken at 250 rpm and 35°C in a shaking incubator. At regular intervals, 150 μL aliquots were withdrawn from the reaction mixture and analyzed for ester content by GC. The reaction rate was determined from the initial linear portion of the production curve.

Analytical methods. The concentrations of geraniol and geranyl acetate were determined by GC (Varian CP 3380) equipped with a capillary column (DB-5, 0.32 μm i.d., 30 m long, 1 μm film thickness; J&W Scientific, Folsom, CA) and an FID. The injector, oven, and detector temperatures were at 250, 120, and 260°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 2 mL/min. Protein concentration was determined colorimetrically with bicinchoninic acid protein assay kits from Pierce (Rockford, IL) with the same lipase powder used in immobilization as the standard. All data reported are average values from at least triplicate measurements and an SE <5%. Morphology of the lipase-containing gel was observed by scanning electron microscope (JEOL JSM 5410). Pore size, pore volume, and specific surface area were determined by N_2 gas absorption using a BET surface area and porosimetry system (ASAP 2000; Micromeritics, Norcross, GA) after drying at 100°C for 2 d to remove residual water in the pores and on the surfaces.

RESULTS AND DISCUSSION

Morphology of immobilized lipase. By forming sol-gel materials within the pores of a nonwoven support, a sheet-shape immobilized biocatalyst retaining the benefits of sol-gel entrapment of the lipase but with the added advantages of easy recovery and suitability for use in a bioreactor can be obtained. A composite hydrogel membrane was developed previously following a similar approach, namely, synthesizing a temperature-sensitive hydrogel on nonwoven supports to enhance the mechanical strength and to sustain the high-shear conditions encountered during bioprocessing (23). The concept of supported sol-gel matrices also can be expected to substantially increase the mechanical stability of sol-gel matrices, which up to now have been mainly produced in powder form from the xerogel. These small particles can generate

TABLE 1
Effects of Alkyl Chain Length of Alkyltrimethoxysilane[a] on Enzyme Immobilization

Composition of silane in sol-gel matrices[b]	Degree of protein immobilization (%)	Enzyme-specific activity[c,d] (μmol/h g enzyme)	Specific surface area (m^2/g)	Pore volume (cm^3/g)
TMOS/MTMS	81	6.78	119.5	0.425
TMOS/ETMS	79	11.7	75.1	0.304
TMOS/PTMS	82	13.3	64.6	0.243
TMOS/BTMS	78	1.40	ND	ND
TMOS/OCTMS	75	0.32	ND	ND
TMOS/ODTMS	84	0.11	ND	ND

[a]TMOS, $Si(OCH_3)_4$; MTMS, $CH_3Si(OCH_3)_4$; ETMS, $C_2H_5Si(OCH_3)_4$; PTMS, $C_3H_7Si(OCH_3)_4$; BTMS, $C_4H_9Si(OCH_3)_4$; OCTMS, $C_8H_{17}Si(OCH_3)_4$; ODTMS, $C_{18}H_{37}Si(OCH_3)_4$; ND, not determined.
[b]TMOS/alkyltrimethoxysilane molar ratio = 1:2; hydrolysis time = 20 min; water/silane molar ratio = 21.
[c]Free enzyme activity = 7.98 μmol/h g enzyme.
[d]Enzyme-specific activities are based on weight of original enzyme powder.

a high pressure drop when used in a packed-bed bioreactor; hence, scaleup for production processes is difficult. The distribution of the sol-gel within the pores of the nonwoven fabric was found to be homogeneous. The morphology of the gel was similar to sol-gel powders, where large amorphous regions as well as spherical particles were observed (Fig. 1).

Effects of chain length of alkyl silane. The classical sol-gel process hydrolyzes TMOS in the presence of enzyme by acid or base catalysis. Hydrolysis and condensation of TMOS lead to nano-sized sol-gel particles, which then cross-link to form an insoluble amorphous silica polymer. However, lipases immobilized in sol-gels formed from TMOS often have low enzymatic activity. Since lipases are interphase-active enzymes with hydrophobic domains, alkyl-modified silanes of the type $RSi(OCH_3)_3$, with hydrophobic character and able to form an organic–inorganic hybrid gel matrix, might be expected to be a more suitable environment for lipase entrapment and activity retention. For lipase immobilized on the nonwoven fabric using mixtures of TMOS/alkyl-modified silane, the esterification activity increased with the size of the alkyl group up to the C_3H_7 and then decreased significantly for alkyl groups larger than 3 (Table 1). From BET measurements, pure silica polymer from TMOS had the highest specific surface area and pore volume. The corresponding values decreased in the series TMOS/MTMS > TMOS/ETMS > TMOS/PTMS at a TMOS/alkyltrimethoxysilane molar ratio of 1:2 (Table 1). Although one might intuitively expect enzyme activity to be greater with increasing pore volume (easier accessibility), the opposite was found experimentally, indicating that other factors must be involved, e.g., surface lipophilicity. The preference of lipases for more hydrophobic supports has been demonstrated for alkyl-modified silica polymers. For *Pseudomonas cepacia* lipase entrapped in sol-gel powder formed from TMOS doped with alkyltrimethoxysilanes, the activity increased with alkyl chain length of the alkyltrimethoxysilane (up to *n*-octadecyl) in esterification reactions between lauric acid and octanol in isooctane (24). Considering this correlation between the enzyme activity and alkyl chain length of alkyltrimethoxysilanes, it is important to choose an alkyltrimethoxysilane with suitable hydrophobicity for optimizing immobilized lipase activity.

One possible explanation for the beneficial effect of incorporating hydrophobic alkyl groups in sol-gel matrices is the activation in the microenvironment of the enzyme based on the interaction of the long-chain alkyl group with the hydrophobic or amphiphilic domains of the lipase. This interpretation is akin to the classical interfacial activation of lipases, which occurs at the water–oil interface of lipophilic substrates such as TG (25). Lipases are enzymes with

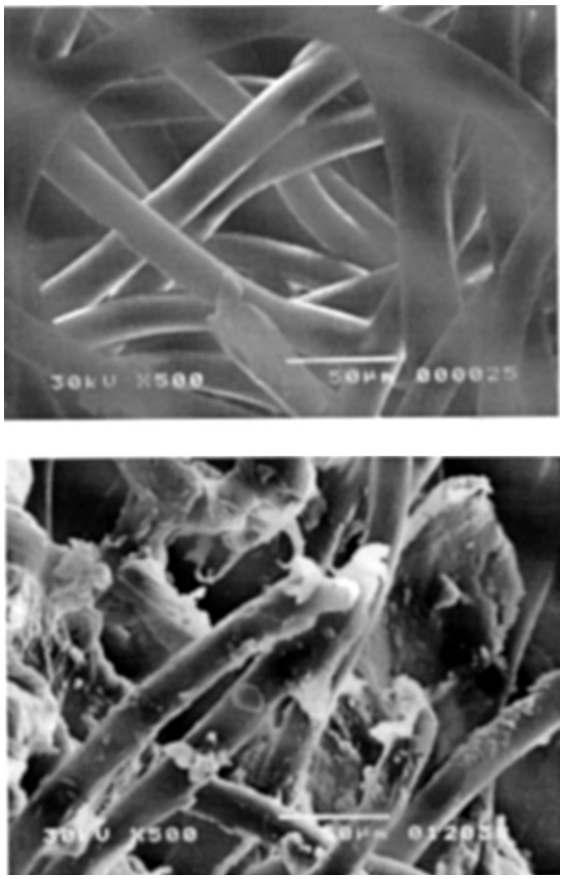

FIG. 1. Photomicrographs of nonwoven fabric and lipase-containing hybrid sol-gel formed within nonwoven fabric. The bar represents 50 μm.

lipophilic domains that interact with substrate molecules, which induce or stabilize enzyme conformational changes, especially the movement of the α-helical loop of the lipases. Opening of this so-called lid uncovers the active sites, which results in the active form of the lipase (26). This effect may come about in the case of sol-gel–entrapped lipase when hydrophobic alkyl groups are in the matrix. It is also possible that lipophilic interactions between the hydrophobic parts of the gel and the lipase occur during the sol-gel process, which leads to the fixation of the active lipase conformation when entrapped in the sol-gel matrix.

Effects of silane composition, hydrolysis time, and R *ratio during sol-gel formation.* Figure 2 shows optimization of the molar ratio of PTMS to TMOS, where maximal activity was obtained at >80% PTMS. It should be noted, however, that 100% PTMS gel had poor gelation behavior and lacked the network-forming ability of TMOS; hence, 80% PTMS seemed to be the best composition.

The influence of hydrolysis time during gel formation on enzyme activity also was studied. Hydrolysis times of less than 30 min produced immobilized lipases with low activity. For hydrolysis times less than this, the mixtures were heterogeneous when the lipase solution was added. Free water in the heterogeneous mixture may cause lipase denaturation and decrease activity yield. The highest activity occurred when the hydrolysis time was 30 min, and lipase activity decreased rapidly when the hydrolysis time was 60 min. The morphology of the sol-gel was different when the hydrolysis time was 60 min, being gel-like instead of powder-like. This could be responsible for the low enzyme activity observed. Efficiency of protein immobilization remained unchanged at 93% in those cases.

The stoichiometric *R* during the immobilization step could have influenced the specific activity of immobilized lipase, which increased with increasing *R* values up to 20 and then decreased at higher *R* values. Polymerization under low water content conditions will result in aggregation of lipase floc, and gels grow around the "floc" rather than the individual lipase molecules, which leads to low enzyme activities. Also, at low water concentration, alcohol condensation will likely occur instead of water condensation during growth of the sol-gel network, and the alcohol liberated may damage the lipase. Hence, changes in gelation behavior, with an increasing amount of lipase remaining in the aqueous supernatant, were observed at high *R* values.

Effects of enzyme loading. The effect of the amount of enzyme added during the immobilization step was studied (Fig. 3). Lipase activity increased with increasing enzyme loading up to 200 mg and decreased thereafter. Enzyme denaturation by support material at low loading and mass transfer limitation at high loading could explain the optimal enzyme loading observed. Since high enzyme loading is possible with this method, we can postulate that the major part of the enzyme is immobilized near the surface of the gel, which is readily accessible to substrates. In addition, aggregation phenomena of the enzyme might occur at very high concentrations, resulting in low dispersion in the gel matrix.

Properties of immobilized lipase. The optimal conditions found for preparing the immobilized lipase were a TMOS/PTMS molar ratio = 1:4, hydrolysis time 30 min, $R = 20$, and enzyme loading = 200 mg. Under these conditions, the specific activity was 20.9 μmol/h g enzyme (2.62 times that of the free lipase) with a protein immobilization efficiency of 91%. The physical properties of the matrices from BET measurements were 49.7 m^2/g, 0.193 cm^3/g, and 15.5 nm for

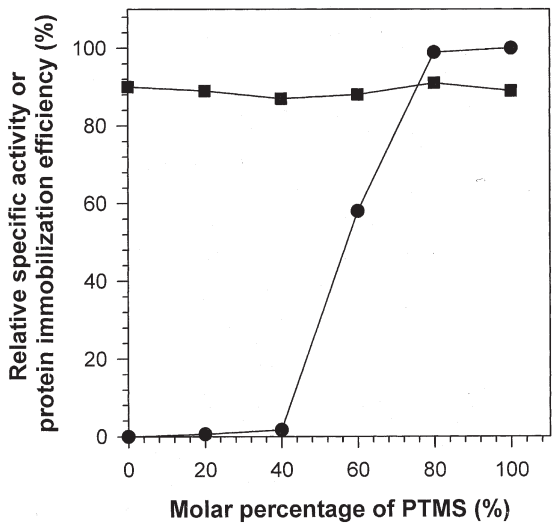

FIG. 2. Influence of the composition of tetramethoxysilane (TMOS)/propyltrimethoxysilane (PTMS) sol-gel materials formed within nonwoven fabric on the specific activity (●) and protein immobilization efficiency (■) of immobilized lipase. The specific activity of 100% PTMS (21.2 μmol/h g enzyme) was taken as 100%. Protein immobilization efficiency is the amount of protein immobilized compared to the original amount of protein (200 mg) used.

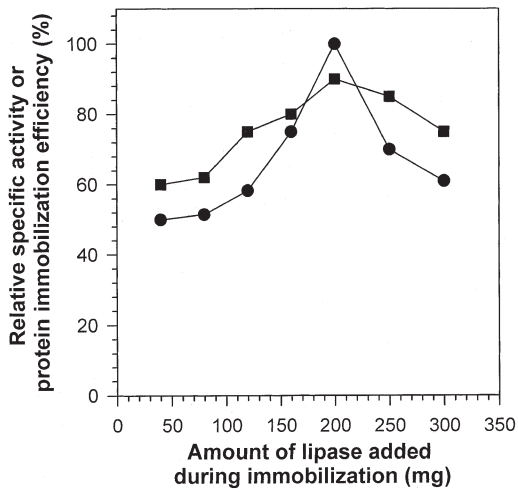

FIG. 3. Influence of enzyme loading during the immobilization step on the specific activity (●) and protein immobilization efficiency (■) of immobilized lipase. TMOS/PTMS (1:4) sol-gel materials formed within nonwoven fabric were used. The specific activity with 200 mg enzyme loading (20.9 μmol/h g enzyme) was taken as 100%. Protein immobilization efficiency is the amount of protein immobilized compared to the original amount of protein used. See Figure 2 for abbreviations.

surface area, pore volume, and pore size, respectively. These values are within the range of values reported for similar sol-gel powders, indicating that efficient sol-gel matrices can be formed within the pores of nonwoven fabric (16). The thickness and nominal area density of the nonwoven fabric were increased 2.4-fold to 480 ± 40 μm and 4.5-fold to 12.1 ± 1.3 mg/cm^2 after sol-gel formation.

Water is a co-product during esterification and an important factor in esterification reactions catalyzed by lipases. From Figure 4 it may be seen that the free enzyme lost its activity with increasing water content in the reaction medium. When the water content in the reaction medium was increased to 0.2% (addition of 20 μL water during reaction), the activity of the free enzyme decreased about 50%, but the activity of the immobilized lipase remained >90%. Water bound near or at the enzyme's active site is considered essential, but accumulation of water in the reaction medium will decrease lipase activity and affect the long-term stability of the lipase. On the other hand, the activity of the lipase entrapped in sol-gel matrices is rather insensitive to the water content in the reaction medium, which is an added advantage for long-term operation of the immobilized lipase. Two factors may contribute to this: the water-absorbing ability and enzyme structure maintenance capability of the sol-gel matrices.

To test the thermal and storage stability of the immobilized lipase, we measured the residual activity of the free and immobilized lipases in dry conditions and in hexane with or without acetic acid. The thermal stability of lipase entrapped in sol-gel matrices is much better than that of the free enzyme. Immobilized lipase could be heated at 70°C for 2 h with full retention of activity, but the free enzyme lost about 80% of its activity under the same conditions (Fig. 5). For long-term storage, the immobilized enzyme left at room temperature retained 92% of initial enzyme activity after 2 mon. In contrast,

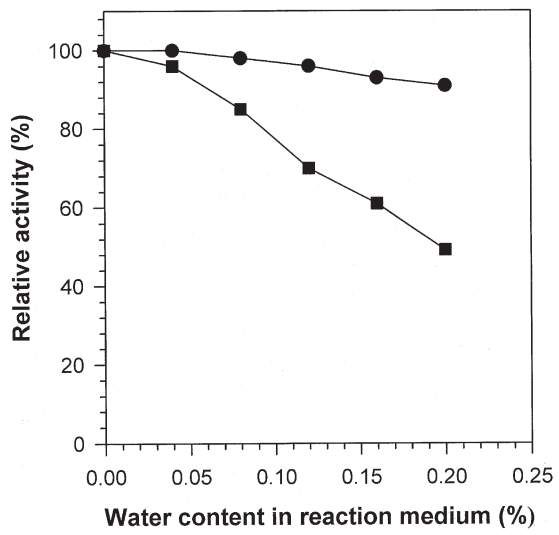

FIG. 4. Influence of water content during the reaction on the activity of free lipase (■) and lipase entrapped in sol-gel materials formed within nonwoven fabric (●). TMOS/PTMS (1:4) sol-gel materials were used. The activity with no added water was taken as 100%. See Figure 2 for abbreviations.

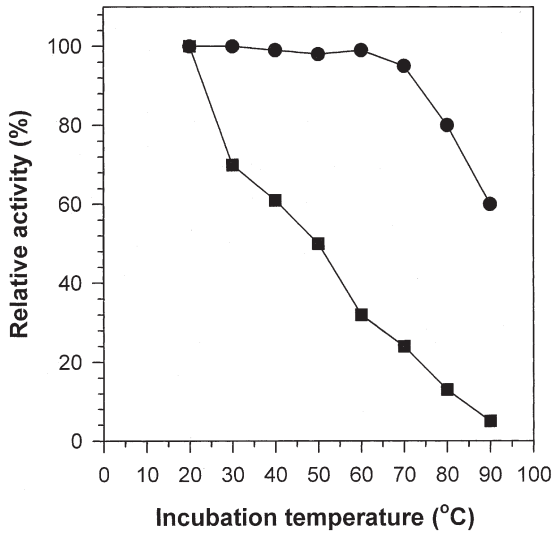

FIG. 5. Thermal stabilities of free lipase (■) and lipase entrapped in sol-gel materials formed within nonwoven fabric (●) in dry forms. TMOS/PTMS (1:4) sol-gel materials were used. Residual enzyme activity was determined at 35°C by following the standard assay method after incubating the enzyme at the temperature indicated for 2 h. Enzyme activities without heat treatment were taken as 100%. See Figure 2 for abbreviations.

under the same condition, the free lipase powder lost all its activity within a week. As shown in Figure 6, enzyme immobilization also reduced the effect of acetic acid on lipase stability in hexane, presumably by inhibiting the effect of acetic

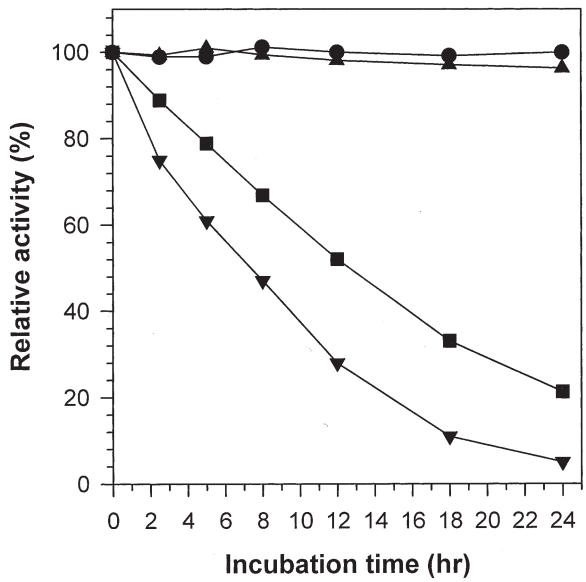

FIG. 6. Thermal stabilities of free lipase and lipase immobilized in sol-gel materials formed within nonwoven fabric in wet forms. TMOS/PTMS (1:4) sol-gel materials were used. Residual enzyme activity was determined at 35°C by following the standard assay method at the times indicated after incubating the enzyme in hexane with or without acetic acid at 40°C. Initial enzyme activities were taken as 100%. (■), free enzyme in hexane. (▼), free enzyme in hexane containing 15 mM acetic acid. (●), immobilized enzyme in hexane. (▲), immobilized enzyme in hexane containing 15 mM acetic acid. See Figure 2 for abbreviations.

acid on enzyme structure (7,8). Facile synthesis of geranyl acetate by direct esterification in hexane could be carried out with the immobilized biocatalyst prepared here. It is likely that the lipase not only is physically entrapped but also forms additional interactions with the matrix through hydrogen, ionic, or hydrophobic interactions. This postulated enzyme stabilization by multipoint attachment to the support can account for the catalytically active lipase.

Ester synthesis was carried out with free and immobilized lipase. The conversion using free enzyme (100 mg) was 15% after 24 h in contrast to 95% when using the immobilized enzyme (immobilized biocatalyst containing 100 mg enzyme). This immobilized enzyme can thus be used for high-yield synthesis of geranyl acetate. For repeated batch use, the immobilized enzyme was used in 24-h batch incubations and removed from the reaction mixture simply by decanting the whole mixture. The recovered immobilized enzyme was washed with hexane to remove residual substrates and products, followed by acetone to remove water in the gel matrix. No loss of sol-gel materials was noted after repeated use of the immobilized enzyme, indicating a stable formation and strong adherence of the sol-gels to the nonwoven fabric. An initial loss of 14–18% activity was observed after three or four cycles, and thereafter the activity remained fairly constant at 82 to 86% of initial activity for up to 10 cycles. The initial loss of activity may be due to the presence of a small amount of adsorbed lipase that is removed after the first few runs.

ACKNOWLEDGMENTS

Financial support from the National Science Council of ROC (NSC89-2214-E182-017) and Chang Gung Memorial Hospital is highly appreciated.

REFERENCES

1. Langrand, G., C. Triantaphylides, and J. Baratti, Lipase Catalyzed Formation of Flavour Esters, *Biotech. Lett. 9*:647–650 (1988).
2. Claon, P.A., and C.C. Akoh, Effect of Reaction Parameters on SP435 Lipase-Catalyzed Synthesis of Citronellyl Acetate in Organic Solvents, *Enzyme Microb. Technol. 16*:835–838 (1994).
3. Lee, K.K.B., L.H. Poppenborg, and D.C. Stuckey, Terpene Ester Production in a Solvent Phase Using a Reverse Micelle-Encapsulated Lipase, *Ibid. 23*:253–260 (1998).
4. Welsh, F.W., R.E. Williams, and K.H. Dawson, Lipase Mediated Synthesis of Low Molecular Weight Flavor Esters, *J. Food Sci. 55*:1679–1682 (1990).
5. Claon, P.A., and C.C. Akoh, Enzymatic Synthesis of Geraniol and Citronellol Esters by Direct Esterification in *n*-Hexane, *Biotechnol. Lett. 15*:1211–1216 (1993).
6. Langrand, G., N. Randot, C. Triantaphylides, and J. Baratti, Short-Chain Flavour Esters Synthesis by Microbial Lipases, *Ibid. 12*:581–586 (1990).
7. Okumura, S., M. Iwai, and A. Tsujisaka, Synthesis of Various Kinds of Esters by Four Microbial Lipases, *Biochim. Biophys. Acta 575*:156–165 (1979).
8. Laboret, F., and R. Perraud, Lipase-Catalyzed Production of Short-Chain Terpenyl Esters of Interest to the Food Industry, *Appl. Biochem. Biotechnol. 82*:185–198 (1999).
9. Akoh, C.C., and L.N. Yee, Lipase-Catalyzed Transesterification of Primary Terpene Alcohols with Vinyl Esters in Organic Media, *J. Mol. Catal. B: Enzym. 4*:149–153 (1998).
10. Yee, L.N., C.C. Akoh, and R.S. Philips, Terpene Ester Synthesis by Lipase-Catalyzed Transesterification, *Biotechnol. Lett. 17*:65–70 (1995).
11. de Castro, H.F., P.C. de Oliveira, and E.B. Pereire, Evaluation of Different Approaches for Lipase-Catalysed Synthesis of Citronellyl Acetate, *Ibid. 19*:229–232 (1997).
12. Hench, L.L., and J.K. West, The Sol-Gel Process, *Chem. Rev. 90*:33–79 (1990).
13. Venton, D.L., K.L. Cheeseman, R.T. Chatterton and T.L. Anderson, Entrapment of Highly Specific Antiprogesterone Antiserum Using Polysiloxane Copolymers, *Biochim. Biophys. Acta 797*:343–347 (1984).
14. Glad, M., B. Nörrlow, B. Sellergren, N. Siegbahn, and N. Mosbach, Use of Silane Monomers for Molecular Imprinting and Enzyme Entrapment in Polysiloxane-Coated Porous Silica, *J. Chromatogr. 347*:11–23 (1985).
15. Avnir, D., S. Braun, O. Lev, and M. Ottolenghi, Enzyme and Other Proteins Entrapped in Sol-Gel Materials, *Chem. Mater. 6*:1605–1614 (1994).
16. Gill, I., and A. Ballesteros, Bioencapsulation Within Synthetic Polymers: Sol-Gel Encapsulated Biologicals, *Trends Biotechnol. 18*:282–296 (2000).
17. Kawakami, K., and S. Yoshida, Entrapment of Lipase in Silica Glass by the Sol-Gel Methods and Its Esterification Activity in Organic Media, *Biotechnol. Tech. 8*:441–446 (1994).
18. Kuncova, G., and M. Sivel, Lipase Immobilized in Organic–Inorganic Matrices, *J. Sol-Gel Sci. Technol. 8*:667–671 (1997).
19. Hsu, A.F., T.A. Foglia, and S. Shen, Immobilization of *Pseudomonas cepacia* Lipase in a Phyllosilicate Sol-Gel Matrix: Effectiveness as a Biocatalyst, *Biotechnol. Appl. Biochem. 31*:179–183 (2000).
20. Peterson, K.P., C. Peterson, and E.J. Pope, Silica Sol-Gel Encapsulation of Pancreatic Islets, *Proc. Soc. Exp. Biol. Med. 218*:365–369 (1998).
21. Gerritsen, M.G., A. Kros, V. Sprakel, J.A. Lutterman, R.J.M. Nolte, and J.A. Jansen, Biocompatibility Evaluation of Sol-Gel Coatings for Subcutaneously Implantable Glucose Sensors, *Biomaterials 21*:71–78 (2000).
22. Reetz, M.T., A. Zonta, V. Vijayakrishnan, and K. Schimossek, Entrapment of Lipases in Hydrophobic Magnetite-Containing Sol-Gel Materials: Magnetic Separation of Heterogeneous Biocatalysts, *J. Mol. Catal. A: Chem. 134*:251–258 (1998).
23. Chen, J.P., and S.H. Chiu, A Poly(*N*-isopropylacrylamide-*co*-*N*-acryloxysuccinimide-*co*-2-hydroxyethyl methacrylate) Composite Hydrogel Membrane for Urease Immobilization to Enhance Urea Hydrolysis Rate by Temperature Swing, *Enzyme Microb. Technol. 26*:359–367 (2000).
24. Reetz, M.T., A. Zonta, and J. Simpelkamp, Efficient Heterogeneous Biocatalysts by Entrapment of Lipases in Hydrophobic Sol-Gel Materials, *Angew. Chem. Int. Ed. Engl. 34*:301–303 (1995).
25. Gonzalez-Navarro, H., and L. Braco, Lipase-Enhanced Activity in Flavour Ester Reactions by Trapping Enzyme Conformers in the Presence of Interfaces, *Biotechnol. Bioeng. 59*:122–127 (1998).
26. Chamorro, S., J.M. Sánchez-Montero, A.R. Alcántara, and J.V. Sinisterra, Treatment of *Candida rugosa* Lipase with Short-Chain Polar Organic Solvents Enhances Its Hydrolytic and Synthetic Activities, *Biotechnol. Lett. 20*:499–505 (1998).

[Received July 18, 2001; accepted December 7, 2001]