Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 42 (2006) 32–38
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Application of binary immobilized *Candida rugosa* lipase for hydrolysis of soybean oil

W.J. Ting [a], K.Y. Tung [a], R. Giridhar [b], W.T. Wu [b,*]

[a] *Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan*
[b] *Department of Chemical Engineering, National Cheng Kung University, Tainan, Taiwan*

Received 3 March 2006; received in revised form 7 June 2006; accepted 15 June 2006
Available online 21 July 2006

Abstract

Lipase was immobilized to chitosan beads by a binary method and its catalytic efficiency in the hydrolysis of soybean oil was investigated. In the first step, the hydroxyl groups of chitosan were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and lipase molecules were coupled to the active hydroxyl groups. In the second step, more lipase molecules were cross-linked through its amino groups to chitosan by using glutaraldehyde. The effects of temperature, pH and oil to water ratio on the conversion, pH and thermal stability, reusability, storage stability and the kinetic properties were also investigated. Under optimal conditions, 88% of the oil taken initially was hydrolyzed after 5 h. Better thermal stability was exhibited by the immobilized lipase and the pH stability was comparable to that of soluble lipase. Storage for 30 days at 4 °C, showed that the immobilized enzyme did not lose its activity. The relative activity upon six repeated uses was 80%.

Keywords: Immobilization; *Candida rugosa* lipase; Soybean oil; Hydrolysis

1. Introduction

Soybean oil can be hydrolyzed readily like other vegetable oils. Partial hydrolysis of triglycerides will yield mono- and diglycerides and fatty acids. When the hydrolysis is carried to completion, the mono-, di- and triglycerides will hydrolyze to yield fatty acids and glycerol. The produced fatty acids find several applications such as in the manufacture of soaps, surfactants and detergents and in food [1,2]. Free fatty acids are also of considerable importance due to their biomedical properties [3]. The traditional method of hydrolysis involves the use of high temperature, pressure and chemical catalysts [4]. The enzyme-mediated hydrolysis [5,6] is an attractive method. Enzyme-catalyzed hydrolysis is carried out under mild temperatures allowing energy saving and often leads to products of better quality due to high specificity and selectivity of the enzyme [7].

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are the most widely used enzymes in oil hydrolysis [8]. In addition to hydrolysis, they catalyze alcoholysis, acidolysis, amidolysis and inter-esterification [9]. Hence, lipases have tremendous potentials in areas such as food technology, biomedical sciences and chemical industries [10–13]. The unique character of lipase is that it can be activated at oil–water interphase and are capable of preserving their catalytic activity in non-aqueous and biphasic systems and in micellar solutions [14]. However, its low stability and activity or selectivity coupled with the high cost [8,15] prohibits its use in industrial hydrolytic reactions. Several methods of immobilization of lipase on different supports [16–22] have been attempted in the past to improve the stability and reusability of lipase in oil hydrolysis. In all the above studies, immobilized lipase showed improved activity and stability. The stability of the enzyme may also increase by the use of non-conventional media [21,23,24]. Non-conventional media are of special interest in the case of lipases when low water content is desired and the substrates are hydrophobic [24]. Lipase-mediated hydrolysis of vegetable oils or triglyceride in non-conventional media such as super critical fluids [25–27], and organic solvents [28–30] has also been attempted.

In our previous work, *Candida rugosa* lipase was immobilized to chitosan beads by a binary method [31]. In this study, the catalytic ability of the binary immobilized lipase for the hydrolysis of soybean oil was evaluated. The effect of temperature, pH

* Corresponding author. Tel.: +886 6 237 6734; fax: +886 6 275 4228.
E-mail address: wtwu@mail.ncku.edu.tw (W.T. Wu).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.06.009

and oil to water ratio on the conversion, pH and thermal stability, reusability, storage stability and the kinetic properties were also investigated.

2. Materials and methods

2.1. Materials

Soybean oil was obtained from Taiwan Sugar Corporation (Taiwan). Chitosan beads having a deacetylation degree of 92% and a molecular weight of 310 kDa, supplied by Kiotek Corporation (Taiwan) was used as the support for immobilization of *C. rugosa* lipase (Type VII; Sigma, St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, *p*-nitrophenyl palmitate (*p*-NPP) and bovine serum albumin (BSA) were acquired from Sigma (St. Louis, MO). Glutaraldehyde was purchased from Fluka (Milwaukee, WI) and protein assay dye was provided by Bio-Rad laboratories (Hercules, CA). All other chemicals were of analytical grade. All the solutions were prepared in deionized water.

2.2. Formation of chitosan beads

A 3% (w/v) chitosan powder was dissolved in 1% (v/v) acetic acid. Spherical beads of diameter in the range 1–2 mm were produced by adding the chitosan solution dropwise into a coagulant bath consisting of 1N NaOH containing 26% (v/v) ethanol under stirring. After allowing the solution to remain for overnight, the spherical beads were removed by filtration and washed with deionized water until neutrality. The beads were then stored in deionized water at 4 °C until use.

2.3. Immobilization of lipase

Immobilization of lipase to chitosan beads was carried out by the binary method suggested by Hung et al. [31], with a slight modification. In the first step, the hydroxyl groups of chitosan were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and lipase molecules were coupled to the active hydroxyl groups. In the second step, more lipase molecules were cross-linked through its amino groups to chitosan by using glutaraldehyde. The detailed method is described as follows. One gram of chitosan beads were mixed with 3 ml of 0.25% (w/v) EDC for the activation of its hydroxyl groups. After 15 min, beads were transferred to 3 ml of 0.5% (w/v) lipase at room temperature. After 1 h, the supernatant was decanted and 3 ml of 0.005% (v/v) glutaraldehyde was added to the beads. After 20 min, the supernatant was removed and 3 ml of 0.5% (v/v) lipase in deionized water was added to the beads and allowed to react for 45 min. Finally, the beads were washed thrice in deionized water. The beads were resuspended in deionized water and stored at 4 °C.

2.4. Hydrolysis of soybean oil

Hydrolysis of soybean oil was carried out using different oil–water ratios in a 50 ml flask at room temperature under constant agitation for formation of emulsion of oil and water. The reaction was initiated by the addition of 3 g immobilized lipase to the reaction mixture. After 30 min, the biocatalyst was removed by centrifugation at 10,000 rpm for 10 min at room temperature and the supernatant containing the fatty acids was analyzed.

2.5. Assay of enzyme activity

The activity of free and immobilized lipase was measured using 0.5 g of *p*-nitrophenyl palmitate (*p*-NPP) dissolved in 100 ml of ethanol as the substrate. The increase in absorbance at 410 nm caused by the release of *p*-nitrophenol in the hydrolysis of *p*-NPP was measured spectrophometrically. Free lipase of 0.1 ml (or 0.2 g immobilized lipase) was added to a mixture of 1 ml 0.5% (w/v) *p*-NPP solution and 1 ml 0.05 M PBS buffer (pH 7 for free lipase and pH 9 for immobilized lipase) and incubated for 5 min at 30 °C. The reaction was terminated by adding 2 ml of 0.5 N Na_2CO_3 to the mixture followed by centrifugation (10,000 rpm for 10 min). The supernatant (0.5 ml) was diluted 10 times with deionized water, and the absorbance at 410 nm was measured (Beckman DU 530 spectrophotometer). A molar extinction coefficient (ε_{410}) for *p*-nitrophenol of 15,000 M^{-1} cm^{-1} was used [32]. One unit (U) of lipase activity was defined as the amount of enzyme which catalyzed the production of 1 μmole *p*-nitrophenol per minutes under the experimental conditions.

Specific activity was calculated by dividing total activity (U) by amount of lipase bound to chitosan beads and expressed as U/mg-protein. Relative specific activity (RSA) was calculated by dividing specific activity of lipase in the immobilized preparation by specific activity of soluble lipase.

The total protein originally taken for immobilization and protein present in the supernatants (unbound protein) after immobilization were estimated by the method of Bradford [33] using BSA as a standard. The amount of protein bound on the support was found by subtracting the unbound protein from total protein.

2.6. Analysis of free fatty acids

The formation of free fatty acids was analyzed by a method modified from that of Holcapek et al. [34] using high-performance liquid chromatography (HPLC) equipped with a LichroCART RP-18e column (Merck, Germany) at 35 °C. The samples were diluted 20 times in *n*-hexane before injection. The mobile phase was consisted of solvents A (methanol) and B (hexane/isopropanol; 4/5, v/v) at a flow rate of 1 ml/min. Gradient elution was performed by varying the composition (0% to 50%; 1.67% v/v per min) of B in the mixture after 30 min of injection, then 50% (v/v) of B for 5 min and finally, the composition of B was decreased from 50% to 0% after 0.1 min. The products of hydrolysis were detected at 205 nm in a UV–vis detector (SPD-10A; Shimadzu, Japan). The conversion was determined as the ratio of the amount of triacylglycerol decreased to the amount of triacylglycerol in the soybean oil taken initially in the reaction.

3. Results and discussion

3.1. Immobilization of lipase

The objective of this research was to immobilize lipase to a cheaper support and assess its catalytic ability in soybean oil hydrolysis. The high cost of the popular supports prompted us to use chitosan, which is a biodegradable polymer obtained abundantly from chitin. Chitosan possesses amino and hydroxyl groups and during immobilization the reactive amino groups are cross-linked the amino groups of the enzyme using multifunctional reagents. The hydroxyl groups are generally not utilized in the immobilization of enzymes. The amount of bound protein can be enhanced if the hydroxyl groups are also utilized in the immobilization. Therefore, the binary method was chosen for the immobilization of lipase to chitosan.

The reaction conditions for immobilization of lipase on chitosan beads by the binary method were investigated and optimized in our earlier study [31]. Under optimum conditions, a maximum bound protein of 199 μg/g-chitosan and a specific activity of 15.3 U/mg-protein were obtained by using 0.5% (w/v) lipase initially in the reaction (Table 1). The relative specific activity was 111%. The binary immobilized lipase was used as a catalyst in batch hydrolysis of soybean oil and the influence of pH, temperature, reaction time and substrates molar ratio and the operational stability were evaluated.

3.2. Batch hydrolysis of soybean oil

3.2.1. Effect of oil–water ratio

A suitable microenvironment for efficient oil hydrolysis requires an optimum oil–water ratio in the reaction [35]. Therefore, the effect of soybean oil and water composition in the reaction mixture on the conversion was investigated in 30 min reactions and the results are shown in Table 2. Lipase catalyzed not only hydrolysis but also esterification simultaneously, and a low water activity may shift the thermodynamic equilibrium favoring the esterification [35]. Therefore, large amounts of water are required to shift the equilibrium to hydrolysis. Furthermore, hydrolysis activity of lipase is known to increase with the increasing water content [36] and a similar trend was also exhibited by the binary immobilized lipase in the hydrolysis of soybean oil. The highest conversion (48%) was obtained at an oil–water ratio of 10:5 (w:w) for the binary immobilized lipase (Table 2) and when more water was added, the rate of hydrolysis decreased, resulting in a decreased conversion. At lower water contents (<10:5) the degree of hydrolysis was incomplete. The optimum oil–water ratio for the soluble lipase was found to be 10:7 (w:w) at which the conversion obtained was 45% (w/w). It is evident from these results that the binary immobilized lipase required lower water content as compared to the soluble lipase for nearly the same extent of hydrolysis. A reduction in the optimal water content for the soluble lipase has also been reported when *Burkholderia cepacia* lipase was immobilized in silica aerogels [37]. In general, the availability of water to the biocatalyst to maintain its enzymatic activity varies depending on the water partitioning among all the components of the system [38].

Table 1
Bound protein and activity of lipase immobilized by binary method on chitosan beads

Enzyme used	Activity (U/g-chitosan or ml enzyme solution)	Bound protein (μg/g-chitosan)	Specific activity (U/mg-protein)	Relative SA (%)
0.25%	1.76	122.02	14.42	104.71
0.50%	3.05	198.9	15.33	111.32
0.75%	2.85	269.29	10.58	76.83
1.00%	2.59	282.37	9.17	66.59
Free lipase	1.35	98.00	13.77	100

Table 2
The effect of varying oil to water ratio on the catalytic activity of the binary immobilized lipase in soybean oil hydrolysis

Oil–water ratio (w/w)	Conversion using free enzyme (%)	Conversion using immobilized lipase (%)
10:1	33.10	38.58
10:3	37.71	45.31
10:5	41.40	48.21
10:7	45.08	47.37
10:9	36.04	43.46

3.2.2. Effect of pH and temperature

The catalytic activity of the immobilized lipase in the hydrolysis of soybean oil was investigated at different pH (5–11). The initial pH of reaction medium was adjusted using the following buffers: 0.1 M citric acid and 0.1 M KH_2PO_4 for pH 5, 0.1 M KH_2PO_4 and 0.1 M K_2HPO_4 for pH 6–8 and glycerin and sodium hydroxide for pH 9–11. Generally, an acidic shift in the pH optimum is expected when enzymes are immobilized onto polycationic supports [39]. On the contrary, the optimal pH of lipase for soybean oil hydrolysis exhibited a basic shift from 7 to 8 after immobilization (Fig. 1) to chitosan beads by the

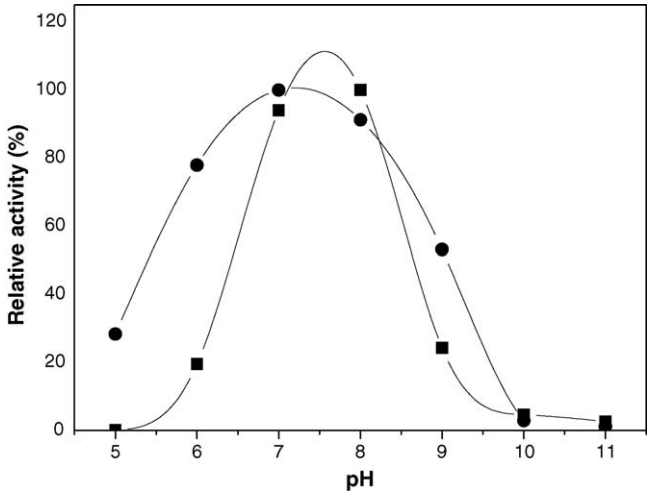

Fig. 1. pH optimum of (●) soluble and (■) binary immobilized lipase in the hydrolysis of soybean oil.

binary method, in spite of the polycationic nature of chitosan. It is well known that the procedure of enzyme immobilization on insoluble supports has a variety of effects on the state of ionization and dissociation of the enzyme and its environment [40]. The EDC reagent used for the activation of the hydroxyl groups of chitosan in this study would make it polyanionic and when the enzyme was coupled with a polyanionic support the pH optimum would shift in the alkaline direction. A similar observation on pH optimum has also been made in the case of lipase immobilized on chitin, which is closely related to chitosan [41].

It is worthwhile to discuss the pH effect on the hydrolytic activity after the immobilization process. Garcia et al. [42] obtained an optimum pH of 7 when a lipase from *C. rugosa* was immobilized by adsorption on flat sheets made of microporous polypropylene and used for the hydrolysis of milk fat triglycerides. Similarly, an optimum pH of 7 was reported by Kang and Rhee [43] when using *C. rugosa* lipase immobilized by adsorption on swollen Sephadex for the hydrolysis of olive oil. Recently, Liu et al. [44] used micron-sized magnetic beads for lipase immobilization and the optimum pH of free enzyme during olive oil hydrolysis shifted from 7 to 8 after immobilization. The optimum pH of the free lipase decreased from 10 to 9 when it was immobilized by entrapment into carrageenan beads and used in the hydrolysis of olive oil in biphasic system [19]. On the contrary, the pH of the free as well as the immobilized lipase in the hydrolysis of olive oil remained unchanged when it was immobilized on polyphenylene sulfide dendrimers [16]. In the present study, the optimum pH of the free lipase shifted from 7 to 8 when it was immobilized by the binary method. Therefore, the pH shift depends mainly on the method of immobilization and the interaction of enzyme and support.

The temperature dependence of the soybean oil hydrolysis reaction catalyzed by free and immobilized lipase was studied in the interval from 25 °C to 45 °C and the results are shown in Fig. 2. A 5° rise in the optimum temperature for soybean oil hydrolysis exhibited by the immobilized lipase indicated that lipase had an improved resistance to heat induced inactivation when immobilized onto chitosan. The improved stabilization might have resulted from multipoint attachment of the enzyme molecules to the support or by acquiring higher hydrophobicity [41]. The effect of temperature on free and immobilized lipase activities in the hydrolysis of oil has been investigated in the past. The optimum temperature of free lipase in the hydrolysis of olive oil shifted from 37 °C to 50 °C when it was immobilized onto micron-size magnetic beads [44]. The optimum temperature of free lipase in the hydrolysis of olive oil shifted from 35 °C to 60 °C when it was entrapped in chitosan with multi-point attachment [41]. Similarly, the apparent temperature optimum for the soluble enzyme was increased by about 7 °C when it was immobilized by cross-linking to polyacrylamide beads [45]. In the present study, the temperature optimum of soluble lipase increased by about 5 °C when it was immobilized by the binary method. This would help in carrying out the hydrolysis reaction at a lower temperature, thereby reducing the energy consumption considerably.

3.2.3. Time course of soybean oil hydrolysis

Fig. 3 displays the time course of soybean oil hydrolysis using 3 g immobilized lipase prepared using varying amounts (0.25–1 g) of lipase in 100 ml deionized water. The reactions were carried out using 10 g oil in 5 ml of buffer (pH 8) and at 40 °C for 24 h. The rate of hydrolysis was increased when the amount of lipase in the immobilized enzyme preparation was varied. As is evident from Fig. 3, the hydrolysis rate was highest when immobilized enzyme prepared by using 0.5% (w/v) lipase was used in the reaction. When the amount of lipase in the immobilized enzyme was increased to 0.75% (w/v), the rate of hydrolysis was found to decrease, which was due to the lower activity (2.85 U/g-chitosan, Table 1). The immobilized lipase containing 0.5% (w/v) showed the highest activity (3.05 U/g-chitosan, Table 1) in hydrolysis. Almost 88% of the oil taken initially was hydrolyzed after 5 h; giving an indication that the lipase immobilized by the binary method exhibited a good catalytic activity in oil hydrolysis.

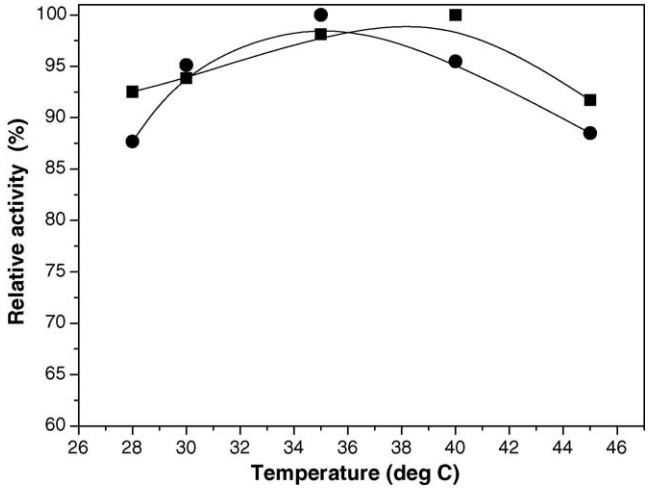

Fig. 2. Temperature optimum of (●) soluble and (■) binary immobilized lipase in the hydrolysis of soybean oil.

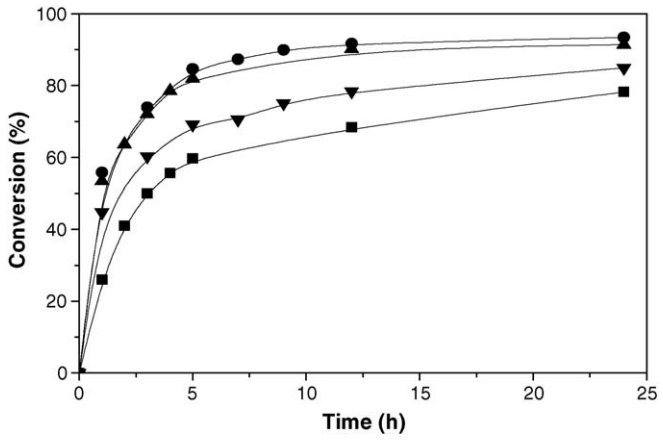

Fig. 3. Time course of soybean oil hydrolysis catalyzed by the binary immobilized lipase prepared by using varying amount (g) of lipase in 100 ml deionized water: (■) 0.25%, (●) 0.5%, (▲) 0.75% and (▼) 1%.

3.2.4. Thermal inactivation

The thermal inactivation of lipase immobilized by the binary method was evaluated by incubating the immobilized enzyme in buffer (pH 8) at temperatures varying from 25 °C to 55 °C for 1 h. After 1 h, 10 g of soybean oil was added for hydrolysis at 40 °C for 30 min. As shown in Fig. 4, there was no significant loss of activity of the immobilized lipase from 25 °C to 40 °C. However, the thermal stability of binary immobilized lipase decreased at higher temperatures. Free lipase was stable only up to 35 °C, after which the catalytic activity decreased. At 55 °C, the hydrolysis activity retained by the binary immobilized lipase was 75%, whereas the free lipase retained only 60% of the activity.

The energy of activation (E_a) of the immobilized lipase was evaluated by Arrhenius equation. The binary immobilized lipase exhibited lower activation energy (0.56 kcal/g mole) as compared to the soluble lipase (1.39 kcal/g mole). The lower activation energy indicated a lower sensitivity to temperature.

3.2.5. pH stability

The pH stability of the binary immobilized lipase was investigated by incubating the immobilized enzyme in buffers of varying (5–11) pH for 1 h at 25 °C and then determining the catalytic activity in soybean oil hydrolysis at the optimum pH and temperature. As seen in Fig. 5, the binary immobilized lipase and the soluble lipase exhibited similar relative activities in the pH range of 6–7. However, the relative activity was decreased at higher pH. Enzyme desorption studies revealed higher leakage of the protein from the support when the pH was increased. The immobilized lipase exhibited more than 90% of relative activity in the pH range of 5–9. Scanning electron microscope (SCM) images of the lipase immobilized chitosan beads incubated in buffers of higher pH (10–11) revealed that the surface of the beads have become irregular in shape. Considerable dimensional deformation could often lead to structural collapse and rupture of the beads. The loss of enzyme activity at higher pH might be attributed to the release of enzyme from the surface of the bead due to structural deformations.

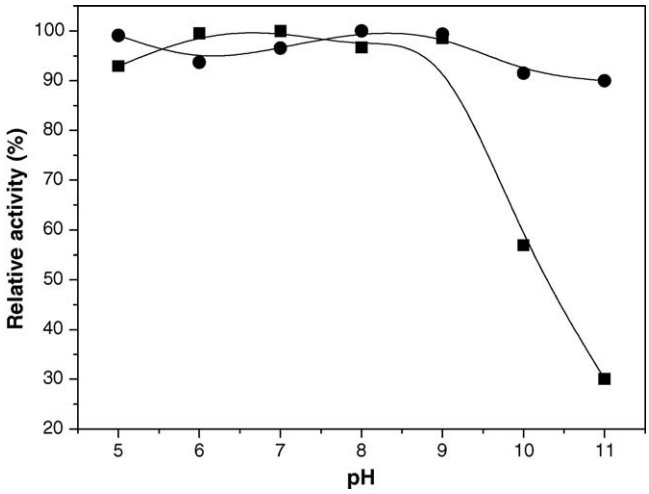

Fig. 5. pH stability of (●) soluble and (■) binary immobilized lipase in the range 5.0–11.0.

3.2.6. Reuse and storage stability

The reusability of binary immobilized lipase is important for economical use of the enzyme in repeated batch or continuous oil hydrolysis. In the reusability studies, it was found that the relative activity of the immobilized lipase decreased after the second usage (Fig. 6). The relative activity was decreased to 82% after 6 uses and 60% after 10 uses. The leakage of protein from support's surface during stirring may be a main reason for the loss of activity. Activity retention of 11% after three reuses in olive oil hydrolysis [46], 65% after four reuses in sunflower oil hydrolysis [47], and 10.5% after seven reuses in palm oil hydrolysis [20] has been reported for lipases immobilized to various supports. In comparison, the activity retention of binary immobilized lipase was higher.

To determine the change in activity of the binary immobilized lipase with time, immobilized lipase was stored at 4 °C and at room temperature for a period of 30 days and the result is shown in Fig. 7. It can be seen from the results that the relative activity

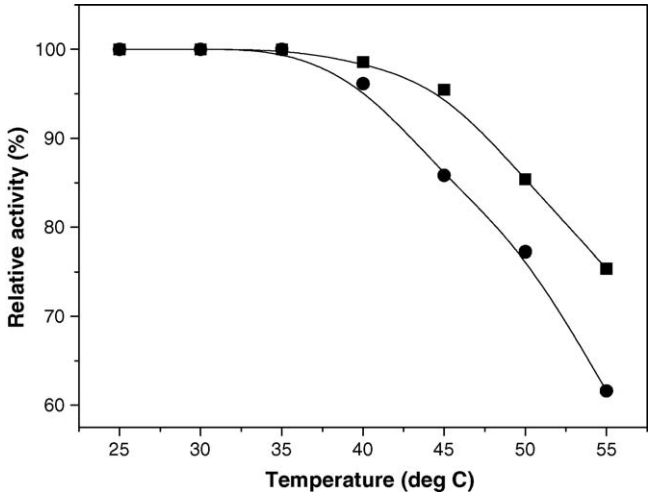

Fig. 4. Thermal stability of (●) soluble and (■) binary immobilized lipase in the range of 25–55 °C.

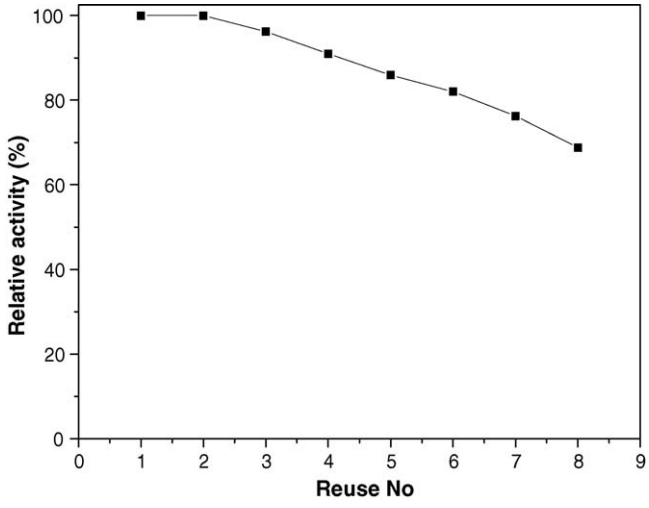

Fig. 6. Reusability of binary immobilized lipase in soybean oil hydrolysis.

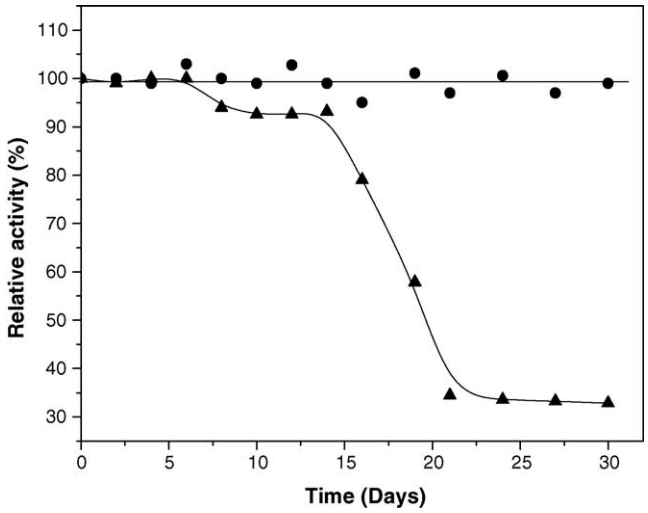

Fig. 7. Storage stability of binary immobilized (●) at 4 °C and (▲) at room temperature in deionized water for 30 days.

of the binary immobilized lipase stored at 4 °C did not change significantly after 30 days, which is an advantage for industrial usage. The relative activity of the binary immobilized lipase stored at room temperature dropped to 30% after 30 days. The enhanced stability of the binary immobilized lipase makes it an excellent biocatalyst for oil hydrolysis.

3.3. Kinetics of oil hydrolysis

The reaction kinetics of the binary immobilized lipase was analyzed by using varying initial weights of soybean oil at 40 °C and using 0.5 g of the immobilized enzyme. The Michaelis–Menten equation was used to fit the kinetic parameters and the double reciprocal plot of the reaction rate and the initial weight of oil were used to evaluate Michaelis constant, K_m and maximum reaction velocity, V_{max}. The data in Table 3 shows the comparison of the kinetic parameters of immobilized lipase with that of the soluble lipase. The observed difference in the kinetic parameters can be explained in terms of the structural changes in the enzyme molecule caused by the binary immobilization procedure or lower affinity between active sites of lipase and oil. The V_{max} of immobilized lipase (254 U/mg-protein) was about 12-fold higher than that of free lipase (21.6 U/mg-protein). The V_{max} value indicates the actual hydrolytic activity of free and immobilized lipase under substrate non-limited conditions. Comparison of free lipase, the maximum activity yield of immobilized lipase could be calculated to be 1176%. K_m of immobilized lipase (1841 mg) was about 4-fold higher than that of free lipase (469 mg).

Table 3
Kinetic parameters for the hydrolysis of soybean oil by the binary immobilized lipase

	V_{max} (U/mg-protein)	K_m (mg)
Immobilized lipase	254	1841
Free lipase	21.6	469

4. Conclusions

Lipase immobilized to chitosan beads by the binary method showed a higher catalytic activity in soybean oil hydrolysis compared to soluble lipase. Eighty eight percent of the oil taken initially was hydrolyzed after 5 h under optimal conditions. The thermal stability of the immobilized lipase was better than that of the free lipase. The pH stability of the immobilized lipase was comparable to that of the free lipase at lower pH, however the stability decreased on account of release of enzyme due to structural deformations on the surface of the chitosan beads at higher pH. Decrease in enzyme activity found in the repeated use might be due to leakage of protein from support's surface possibly due to higher stirring speed while the reaction was being carried out. Taken together, the binary immobilized lipase appears to be highly favorable for the hydrolysis of soybean oil compared to the soluble form. In addition, the use of chitosan, which is a cheap and abundant biodegradable biopolymer, as a support for immobilization would result in overall cost reduction and make the process environment friendly.

Acknowledgement

This research was supported by grants (NSC 92-2214-E-007-012 and NSC 93-2214-E-007-006) from National Science Council of Taiwan.

References

[1] K. Hill, Pure Appl. Chem. 72 (2000) 1255.
[2] N.O.V. Sonntag, J. Am. Oil Chem. Soc. 61 (1984) 229.
[3] M. Habulin, Z. Knez, Eur. J. Lipid Sci. Technol. 104 (2002) 381.
[4] Y.J. Wang, J.Y. Sheu, F.F. Wang, J.F. Shaw, Biotechnol. Bioeng. 31 (1988) 628.
[5] Y. Kimura, A. Tanaka, K. Sonomoto, T. Nihira, S. Fukui, Eur. J. Appl. Microbiol. Biotechnol. 17 (1983) 107.
[6] W.M. Linfield, R.A. Barauskas, L. Sivieri, S. Serota, R.W. Stevenson, J. Am. Oil Chem. Soc. 61 (1984) 191.
[7] C. Albasi, J.P. Riba, I. Sokolovska, V. Bales, J. Chem. Technol. Biotechnol. 69 (1997) 329.
[8] P. Villeneuve, J.M. Muderhwa, J. Graille, M.J. Haas, J. Mol. Catal. B Enzym. 9 (2000) 113.
[9] A. Pandey, S. Benjamin, C.R. Soccol, P. Nigam, N. Krieger, V.T. Soccol, Biotechnol. Appl. Biochem. 29 (1999) 119.
[10] G. Langrand, N. Rondot, C. Triantaphylides, J. Baratti, Biotechnol. Lett. 12 (1990) 581.
[11] R. Talon, M.C. Montel, J.L. Berdague, Enzyme Microb. Technol. 19 (1996) 620.
[12] T. Izumi, F. Tamura, M. Akutsu, R. Katou, S. Murakami, J. Chem. Technol. Biotechnol. 68 (1997) 57.
[13] S.W. Tsai, B.Y. Liu, C.S. Chang, J. Chem. Technol. Biotechnol. 65 (1996) 156.
[14] S. Harikrishna, N.G. Karanth, Cat. Rev. 44 (2002) 499.
[15] R. Sharma, Y. Chisti, U.C. Banerjee, Biotechnol. Adv. 19 (2001) 627.
[16] O. Yemul, T. Imae, Biomacromolecules 6 (2005) 2809.
[17] J.M. Moreno, M.J. Hernaiz, J.M. Sanchez-Montero, J.V. Sinisterra, M.T. Bustos, M.E. Sanchez, J.F. Bello, J. Mol. Catal. B Enzym. 2 (1997) 177.
[18] M.T. Reetz, A. Zonta, J. Simpelkamp, Biotechnol. Bioeng. 49 (1996) 527.
[19] P.D. Desai, A.M. Dave, S. Devi, J. Mol. Catal. B Enzym. 31 (2004) 143.
[20] Z. Knezevic, L. Mojovic, B. Adnadjevic, Enzyme Microb. Technol. 22 (1998) 275.

[21] Z. Knezevic, S. Bobic, A. Milutinovic, B. Obradovic, L. Mojovic, B. Bugarski, Process Biochem. 38 (2002) 313.
[22] L. Mojovic, Z. Knezevic, R. Popadic, S. Jovanovic, Appl. Microbiol. Biotechnol. 50 (1998) 676.
[23] Y.Y. Linko, M. Lamsa, A. Huhtala, O. Rantanen, J. Am. Oil Chem. Soc. 72 (1995) 1293.
[24] N. Krieger, T. Bhatnagar, J.C. Baratti, A.M. Baron, V.M. de Lima, D. Mitchell, Food Technol. Biotechnol. 42 (2004) 279.
[25] H. Sovova, M. Zarevucka, Chem. Eng. Sci. 58 (2003) 2339.
[26] J.W. Hampson, T.A. Foglia, J. Am. Oil Chem. Soc. 76 (1999) 777.
[27] K. Rezaei, F. Temelli, J. Am. Oil Chem. Soc. 77 (2000) 903.
[28] K. Naoe, S. Awatsu, Y. Yamada, M. Kawagoe, K. Nagayama, M. Imai, Biochem. Eng. J. 18 (2004) 49.
[29] S.Y. Huang, C.F. Chen, J. Chin. Inst. Chem. Eng. 32 (2001) 205.
[30] Z.M. He, J.C. Wu, C.Y. Yao, K.T. Yu, Biotechnol. Lett. 23 (2001) 1257.
[31] T.C. Hung, R. Giridhar, S.H. Chiou, W.T. Wu, J. Mol. Catal. B Enzym. 26 (2003) 69.
[32] P. Lotrakul, S. Dharmsthiti, World J. Microbiol. Biotechnol. 13 (1997) 163.
[33] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[34] M. Holcapek, P. Jandera, J. Fischer, B. Prokes, J. Chromatogr. A 858 (1999) 13.
[35] S. Colombie, R.J. Tweddell, J.S. Condoret, A. Marty, Biotechnol. Bioeng. 60 (1998) 362.
[36] H. Yan, K. Nagahama, J. Chem. Eng. Jpn. 36 (2003) 557.
[37] S. Maury, P. Buisson, A. Perrard, A.C. Pierre, J. Mol. Catal. B Enzym. 32 (2005) 193.
[38] R.H. Valivety, P.J. Halling, A.D. Peilow, A.R. Macrae, Eur. J. Biochem. 222 (1994) 461.
[39] L. Goldstein, Y. Levin, E. Katchalski, Biochemistry 3 (1964) 1913.
[40] E. Akertek, L. Tarhan, Appl. Biochem. Biotechnol. 50 (1995) 291.
[41] J.F. Shaw, R.C. Chang, F.F. Wang, Y.J. Wang, Biotechnol. Bioeng. 35 (1990) 132.
[42] H.S. Garcia, F.X. Malcata, C.G. Hill Jr., C.H. Amundson, Enzym. Microb. Technol. 14 (1992) 535.
[43] S.T. Kang, J.S. Rhee, Biotechnol. Bioeng. 33 (1989) 1469.
[44] X. Liu, Y. Guan, R. Shen, H. Liu, J. Chromatogr. B 822 (2005) 91.
[45] K. Bagi, L.M. Simon, B. Szajani, Enzyme Microb. Technol. 20 (1997) 531.
[46] R. Fernandez-Lafuente, P. Armisen, P. Sabuquillo, S. Fernandez-Fadiloglu, Z. Soylemez, J. Agric. Food. Chem. 46 (1998) 3411.
[47] M. Murray, D. Rooney, M. Van Neikerk, A. Monyenegro, L.R. Weatherley, Process Biochem. 32 (1997) 479.