Covalent-Bonded Immobilization of Lipase on Poly(phenylene sulfide) Dendrimers and Their Hydrolysis Ability

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Covalent-bonded immobilization of lipase from *burkholderia cepacia* onto two poly(phenylene sulfide) (PPS) dendrimers with different generations (two and three) was achieved using carbodiimide as a coupling reagent. The hydrolysis activity of olive oil to fatty acid was studied on enzyme-immobilized PPS dendrimers. Enzyme activity was proportional to the enzyme loading, and highest recovered activity was obtained at the medium enzyme loading for both G2 and G3 dendrimers. The immobilization improved the optimum pH and caused the temperature range to widen. Immobilization of enzyme has enhanced the thermal stability of enzyme activity in comparison with free enzyme. The immobilized enzyme as a biocatalyst for batch hydrolysis of olive oil retained 80∼90% activity even after 20 times of recycling. This retention of activity after recycle is very valuable and powerful in enzyme technology. The present noteworthy and vital availability on enzyme reaction of the covalently bonded immobilized lipase on dendrimer came from the structure of dendrimer with a large number of functional terminal groups, which are easily available for immobilization of many lipases at the situation keeping reactive enzymes on the surface of dendrimer.

Introduction

The use of enzymes as biocatalysts for the synthesis of pharmaceutical products[1] and organic compounds[2] has gained more popularity in recent years, and the number of industrial applications of biocatalysis is growing rapidly.[3] Biocatalysis can be carried out under very mild conditions and with great specificity, leading to increased yield by reducing side products.[4] Lipase enzyme has the ability to catalyze a wide range of reactions such as hydrolysis, alcoholysis, and esterification.[5] Because of their selectivities, lipases are important biocatalysts in several applications,[6] for example, controlled hydrolysis of milk fat,[7] flavor enhancement of butter,[8] manufacturing of cheeselike products,[9] chiral drug intermediates,[10] biosurfactant,[11] and decomposition of racemic mixtures and lipids.[12]

Incidentally, using soluble enzymes as a biocatalyst presents some drawbacks, such as stability under operational conditions,[13] difficulty of product recovery, and impossibility of multiple reuse in industrial process.[14] The most important advantage of the enzyme technology is to maintain the conformational stability of the enzyme so that it can be utilized in industrial processes.[13,14] Immobilization improves stability of a variety of enzymes against several forms of denaturation.[15] Several methods have been reported for the immobilization of lipases on different shapes of supports[15] either by covalent binding,[16] entrapment,[17−19] or adsorption.[20] The properties of supporting matrix and the method of immobilization affect activity of immobilized lipase.[15−20] Hydrophobic polymers have been studied as a support for immobilization of lipase enzyme by entrapment.[21] However, the activity was low because of poor immobilization, that is, enzyme loading. Covalent-bonded immobilization has advantage of no loss of enzyme and long-term stability over adsorption and entrapment methods of immoblization.[16] Polymers, which are hydrogels acting as supports, have a drawback that they swell in organic solvents.[16,22] In addition, some of the functional groups and enzyme molecules may be entrapped within the bulk of the polymer beads, silica gel, alumina, and cellulose support, thus making them unavailable for reaction with the substrate.[16,22]

Dendrimers have unique physical and chemical characteristics with a wide range of applications and have been used in encapsulation of metal nanoparticles, drug molecules, guest molecules, and so forth.[23] Some dendrimers such as poly(amido amine) and poly(propyleneimine) dendrimers are soluble in water and/or in common organic solvents. Their peripheral functional groups are easily available as a support for immobilization of enzyme, keeping reactive enzymes on the surface of dendrimer. However, there has been no report of enzyme immobilization on dendrimers.

In the present paper, the covalent-bonded immobilization of lipase enzyme on poly(phenylene sulfide) (PPS) dendrimer is reported. PPS dendrimer has suitable physical and chemical properties as support for covalent immobilization of enzyme.[24] It is insoluble in water and in most common organic solvents and is thermally stable over a wide range of temperature, and it has functional groups at periphery to attach enzyme. Therefore, it should be assumed that the chemical reaction of this dendrimer with lipase produces the thermostable enzyme-immobilized dendrimer. Then, the use of lipase-immobilized dendrimer for hydrolysis of olive oil as an efficient biocatalyst, which may be suitable for an industrial process to obtain fatty acids using an environmen-

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tally feasible process, is examined in the present work at the viewpoint of efficiency, thermostability, and recyclability.

Experimental Section

Materials. Second- and third-generation (G2, G3) PPS dendrimers were synthesized by procedures reported elsewhere.[24] Lipase PS Amano (lyophilized powder from *burkholderia cepacia*) was obtained from Amano Pharmaceutical Co. (Nagoya, Japan). 1-Ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC), olive oil, and other chemicals were purchased from either Aldrich Chemical Co. or Tokyo Kasei Kogyo Co. Ltd., Japan.

Activation of Poly(phenylene sulfide) Dendrimer with Carbodiimide. The activation of carboxylic acid terminal groups of PPS dendrimers was achieved by a reaction with EDC: G2 PPS dendrimer (2 g) was added into 50 cm^3 of an aqueous EDC solution (2 mg cm^{-3} EDC), and the pH of the solution was adjusted to 5.0 with 0.1 M HCl. The solution was kept at 25 °C for 24 h under constant stirring. The precipitated EDC-G2 PPS dendrimer was filtered, and the residue was washed several times with methanol and then was dried in a vacuum for 6 h. It was then stored at 4 °C until use. Similarly, G3 PPS dendrimer was activated to obtain EDC-G3 PPS dendrimer.

Immobilization of *burkholderia cepacia* Lipase on PPS Dendrimer. EDC-G2 PPS dendrimer (1 g) was equilibrated in phosphate buffer (0.1 M, pH 7.0) for 2 h, and 1 cm^3 of phosphate buffer, (0.1 M, pH 7.0) containing lipase (2−5 mg cm^{-3}) was added to it. Reaction was carried out at 4 °C for 18 h with continuous stirring of the reaction medium. After the reaction, the lipase-immobilized PPS (lipase-G2 PPS) dendrimer was removed from medium and was washed with 1 M NaCl and with phosphate buffer (0.1 M, pH 7.0). Similarly, EDC-G3 PPS dendrimer was immobilized with lipase enzyme to obtain lipase-immobilized PPS (lipase-G3 PPS) dendrimer.

The amount of lipase immobilized on PPS dendrimer was determined by measuring the initial and final concentrations of protein within the immobilized medium using brilliant blue dye. A calibration curve was constructed with a known concentration (0.05−0.50 mg cm^{-3}) of enzyme using UV−vis spectra and was used in the calculation of enzyme in solutions.[25]

Activity Assay of Free and Immobilized Lipases. The activity of free and immobilized lipases was determined by olive oil hydrolysis.[26] The substrate was prepared by emulsifying 30 cm^3 of olive oil with 70 cm^3 of emulsifier and homogenizing for 3 min. The emulsifier contained NaCl (17.9 g), KH_2PO_4 (0.41 g), glycerol (540 cm^3), gum arabic (10 g), and water (500 cm^3). A reaction mixture consisting of 5 cm^3 of emulsion, 4 cm^3 of 0.2 M sodium phosphate buffer (a constant pH), and 1 cm^3 of enzyme solution was incubated for 30 min at a constant temperature. The reaction was stopped by adding 10 cm^3 of an acetone−ethanol mixture (1:1 v/v). The resultant fatty acids were titrated with a 0.05 M sodium hydroxide solution using a phenolphthalein indicator. These activity assays were carried out by varying the pH (3−8) and temperature (20−70 °C). Release of 1 μmol fatty acid per minute in the assay conditions is expressed as one enzyme (lipase) unit activity (U g^{-1}).[26]

Recycle of Lipase-Immobilized PPS Dendrimer. Activity retention of immobilized lipase was tested at pH 7 and 35 °C by the procedure as described in the Activity Assay section. After activity assay run, the lipase-immobilized PPS dendrimer was washed several times with phosphate buffer (pH 7.0) and was reintroduced into fresh reaction medium. These procedures were repeated up to 20 times.

Measurements. Fourier transform infrared (FTIR) absorption spectra were recorded using a Bio-Rad FTS575C spectrometer equipped with a cryogenic mercury cadmium telluride (MCT) detector. The spectra were collected with 512 scans at 4 cm^{-1} resolution. UV−vis absorption spectra were recorded on a Shimadzu UV 2200 spectrometer. All the measurements were carried out at room temperature.

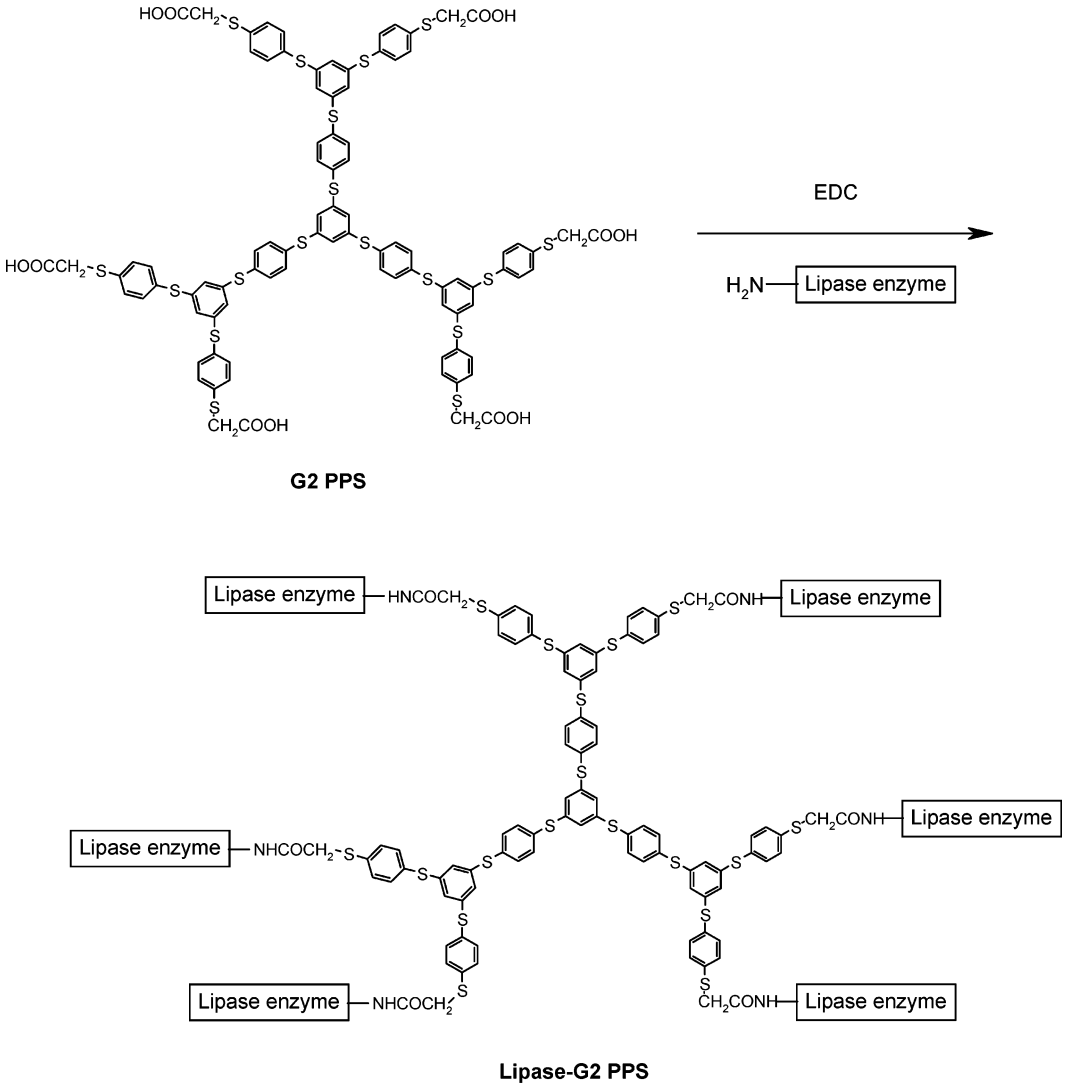
Results and Discussion

Immobilization and Hydrolytic Activity of Lipase onto PPS Dendrimer. The covalent-bonded immobilization of lipase on G2 PPS dendrimer was carried out as presented in Scheme 1. The carboxylic acid groups of G2 PPS dendrimer were activated with EDC, followed by condensation of amino groups from lipase enzyme to activated carboxylic acid groups of G2 PPS dendrimer. The condensation formed an amide bond between the enzyme and the support (PPS dendrimer). Similarly, covalent-bonded immobilization of lipase on G3 PPS dendrimer was carried out. Figure 1 presents comparative FT-IR spectra of carboxylic acid terminated G2 PPS dendrimer, lipase enzyme, and lipase-immobilized G2 PPS (lipase-G2 PPS) dendrimer. As can be seen from the FTIR spectra, amide bond formation between the support and the enzyme has taken place. The IR bands responsible for covalent-bonded immobilization in lipase-G2 PPS dendrimer are seen at 1642 cm^{-1} for amide I, at 1566 cm^{-1} for amide II, and at 1446 cm^{-1} for amide III. They are present prominently, different from those for lipase. The amount of carboxylic acid groups in a lipase-immobilized PPS dendrimer may be negligibly small, as estimated from the disappearance of C═O stretching band at 1756 cm^{-3}, which was observed for carboxylic acid terminated G2 PPS dendrimer.

A maximum enzyme loading was observed at 3.14 mg g^{-1} dendrimer for lipase-G2 PPS dendrimer and at 4.09 mg g^{-1} dendrimer for lipase-G3 PPS dendrimer (see Table 1). In a case of lipase-G2 PPS dendrimer, increasing the lipase content from 1.17 to 3.14 mg g^{-1} dendrimer increased its specific enzyme activity from 770 to 2072 U g^{-1} dendrimer, as listed in Table 1. Similarly, for lipase-G3 PPS dendrimer, enzyme activity increased from 1176 to 2700 U g^{-1} dendrimer with enzyme loading ranging from 1.75 to 4.09 mg g^{-1} dendrimer. These results indicate that the enzyme activity per loaded enzyme, that is, enzyme activity divided by enzyme loading, was almost independent of enzyme loading and dendrimer generation with a range of 636∼674 U g^{-1} dendrimer/mg g^{-1} dendrimer. In other words, enzyme activity is proportional to the enzyme loading.

The highest recovered activity (91 and 98%) after 20 times of recycling was obtained with the medium enzyme content

Scheme 1. Covalent-Bonded Immobilization of Lipase Enzyme on G2 PPS Dendrimer to Form Lipase-G2 PPS Dendrimer



(2.12 and 2.59 mg g^{-1} dendrimer) for both G2 and G3 dendrimers, respectively (see Table 1). A high enzyme loading on the support generally leads to a low recovered activity. This is either by overpopulation of the space of the support by the enzyme, bringing the diffusion limitation of relatively large substrate (olive oil), or by the presence of lipase−lipase interactions, becoming more important and hindering the substrate conversion.[27,28] Generally lipase activity is dominant at the oil/water interface. Lipases have inherent affinity toward hydrophobic media.[28] They have a high level of hydrophobicity as compared to conventional proteins. The decrease in recovered activity of immobilized enzyme at low enzyme loading can be attributed to inefficient dispersion of the enzyme in the hydrophobic environment of dendrimer because of a low amount of enzyme. The interior of PPS dendrimer provides a proper hydrophobic microenvironment for lipase, and thus a reasonable recovered activity of immobilized lipase is obtained at higher enzyme loading. The highest recovered activity at medium enzyme content results from the compensation of the two factors described above.

The covalent-bonded immobilization of lipase on two different supports, silica[16] and agarose,[27] was studied, and the recovered lipase activity was found to vary between 33% and 82%. These activities were dependent on the type of support. This activity of lipase immobilized on polymer membrane[28] was 78%. However, the activity of lipase immobilized on PPS dendrimer is comparable to or higher (98%) than values reported on different supports (silica, agarose, polymer membrane/beads, and cellulose).[15,16,19,27,28]

pH and Temperature Effects on Hydrolysis Activity of Immobilized Enzyme. The effect of pH on the hydrolysis activity of free and immobilized enzymes on olive oil hydrolysis was assayed in the pH range 3.0−8.0 (Figure 2). Maximum activity has been obtained at pH = 7.0−7.5 for immobilized enzymes as well as for free lipase. Many research groups have reported similar optimum pH range on hydrolysis activity after immobilization. It depends on the method of immobilization as well as on the interaction of enzyme and support.[20,29,30] At 90% hydrolysis activity (as shown by the dotted horizontal line in Figure 2), while free enzyme is active in the pH range 6.8−7.1, lipase-G2 PPS dendrimer is in the pH range 5.9−7.5 and lipase-G3 PPS dendrimer is in the pH range 5.9−7.7. An immobilized enzyme has a wider pH range of hydrolysis activity than a free enzyme. This result can be explained by the reason that, during hydrolysis of olive oil, the formed fatty acids may form a film on the outer surface of the free enzyme. The

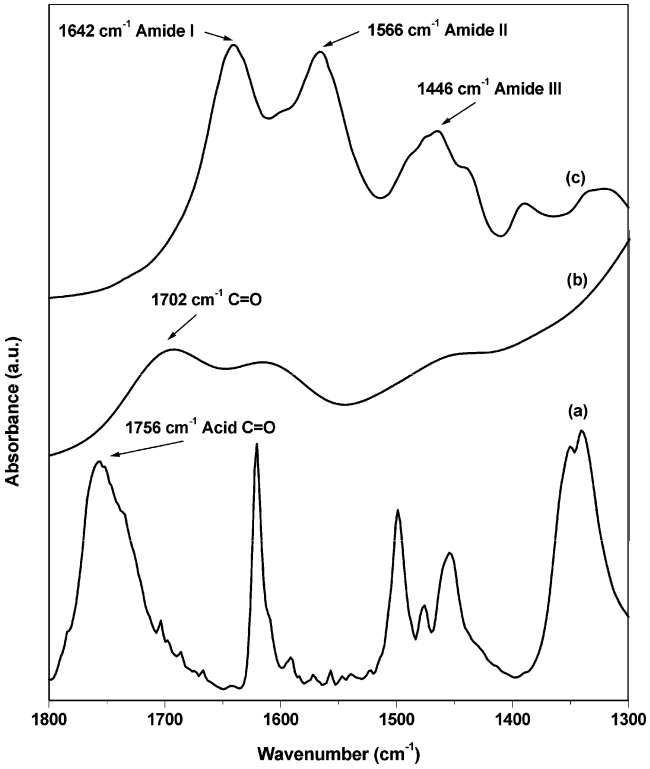


Figure 1. Comparative FT-IR spectra of (a) carboxylic acid terminated G2 PPS dendrimer, (b) lipase enzyme, and (c) lipase-G2 PPS dendrimer (2.12 mg g^{-1} dendrimer).

Table 1. Enzyme Loading in Lipase-G2, G3 PPS Dendrimers and Their Activities on hydrolysis of olive oil

enzyme loading (mg g^{-1} dendrimer)[a]	recovered activity (%)	enzyme activity (U g^{-1} dendrimer)[b]
	Lipase-G2 PPS (6 Terminal Groups)	
1.17	78	770
2.12	91	1428
3.14	87	2072
	Lipase-G3 PPS (12 Terminal Groups)	
1.75	85	1176
2.59	98	1646
4.09	62	2700

[a] Amount of enzyme immobilized on PPS dendrimer was calculated using Brilliant Blue dye (*25*). [b] Olive oil hydrolysis activity of immobilized enzyme was determined by Sigma procedure by evaluating formed fatty acids with a 0.05 N NaOH solution (*26*).

fatty acid film acts as a barrier for olive oil to enter the active site of the enzyme by protecting the enzyme.[28] Such an effect may be less on an immobilized enzyme.

The temperature profile on hydrolysis activity of free and immobilized enzymes was investigated in the temperature range 20−70 °C. The plot of hydrolysis activity versus temperature for free enzyme and lipase-G2 and G3 PPS dendrimers has been presented in Figure 3. Whereas the optimum temperature for activity of free enzyme was about 40 °C, the temperature for lipase-G2 and G3 PPS dendrimers was in the range of 27−45 °C. Immobilization of the enzyme has enhanced the thermal stability of enzyme activity as compared with the free enzyme. At 90% hydrolysis activity (as shown by the dotted horizontal line in Figure 3), while the free enzyme is active in the temperature range 34−45

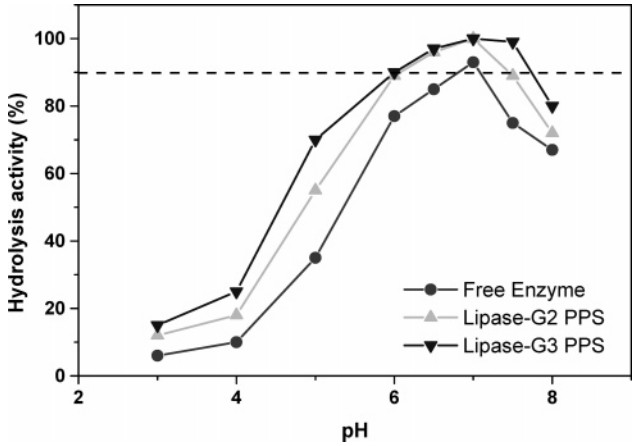


Figure 2. Effect of pH on hydrolysis activity of olive oil at 35 °C. ● free enzyme (0.5 cm^3, 1 mg cm^{-3}); ▲ lipase-G2 PPS dendrimer (0.5 g, 2.12 mg g^{-1} dendrimer); ▼ lipase-G3 PPS dendrimer (0.5 g, 2.59 mg g^{-1} dendrimer).

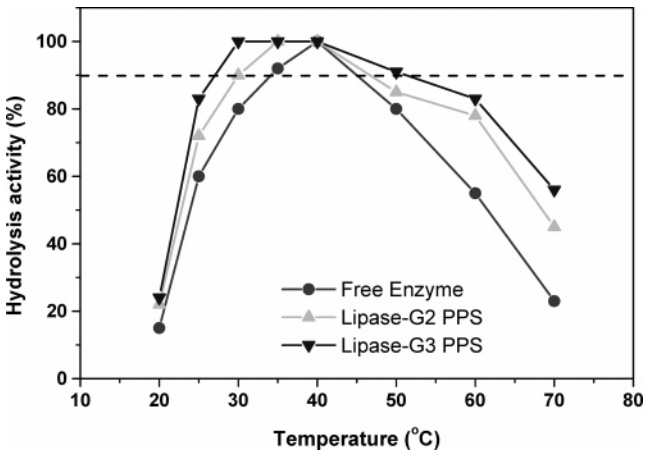


Figure 3. Effect of temperature on hydrolysis activity of olive oil at pH = 7. Symbols represent the same meaning as in Figure 2.

°C, lipase-G2 PPS dendrimer is in the range of 30−47 °C and lipase-G3 PPS dendrimer is in the range of 27−51 °C. This could be due to multiple point attachment of enzyme on dendrimer limiting the conformational changes and movements under various temperatures as well as the pH conditions.[20,28] It has been reported that the thermal stability of immobilized lipase was enhanced by using a hydrophobic substrate.[31] PPS dendrimer is a hydrophobic substrate which might effectively maintain the thermal stabilization of lipase.

Time Effect on Hydrolysis Activity of Immobilized Enzyme at High Temperatures. The time profile on hydrolysis activity of free and immobilized enzymes at two different temperatures, 50 and 70 °C, was investigated. The comparative plot is seen in Figure 4. It has been observed that there is a smaller amount of deactivation of the immobilized enzyme than that of the free enzyme. At 50 °C, while the free enzyme showed only 30% activity, the immobilized enzyme presented 70% activity after 210 min. Whereas the free enzyme lost almost all of its hydrolysis activity after heat treatment for 210 min at 70 °C, the immobilized enzyme afforded resistance to thermal deactivation and maintained 50% activity. These results suggest that the hydrolysis activity of the immobilized lipase becomes significantly lower at higher temperature. However, the time-dependent thermal deactivation of the immobilized lipase is

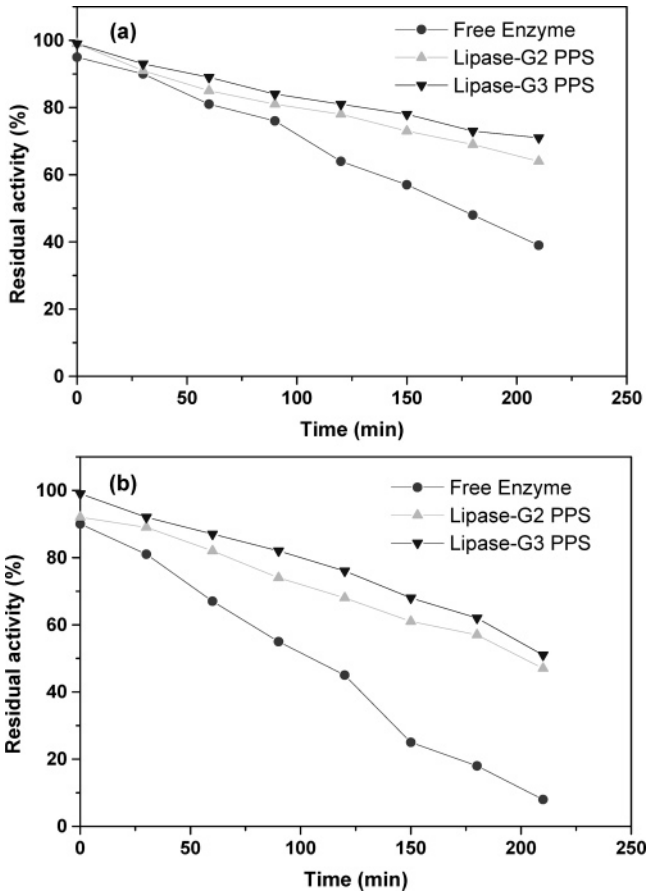


Figure 4. Effect of time on hydrolysis activity of olive oil at pH = 7. (a) At 50 °C, (b) at 70 °C. Symbols represent the same meaning as in Figure 2.

less than that of the free enzyme, and then the immobilized enzyme has greater applications in industry. It has been reported by some researchers that the thermal stability increased by immobilization.[16,28] One of the possible reasons for thermal stability on the hydrolysis activity of immobilized enzyme could be that since the enzyme molecule is attached with a covalent bond to support matrix, it avoids the denaturation of enzyme molecule at high temperature.[16] In addition, PPS dendrimer, which is used as a support matrix in the present study, is thermally stable over a wide range of temperatures. Moreover, it is possible that enzyme attached to PPS dendrimer by covalent bonding preserves the ternary structure of enzyme from denaturation.

Effect of Batch Operation on Hydrolysis Activity of Immobilized Enzyme. From an industrial point of view, operational effect on hydrolysis activity of immobilized enzyme must be examined. The immobilized enzyme can be repeatedly used many times, different from the free enzyme that is used only once. Hydrolysis activity of the immobilized enzyme over a wider range of pH and temperature, described above, gives economically a great advantage over the free enzyme. The effect of the reuse of the immobilized enzymes (lipase-G2, G3 PPS dendrimers) at pH 7.0 and 35 °C has been plotted in Figure 5 as remaining activity in % against the number of multiple batch uses. The activity of both lipase-G2 and G3 PPS dendrimer has remained 85∼90% even after 20 times of recycling. This hydrolysis activity is high in comparison with lipase-

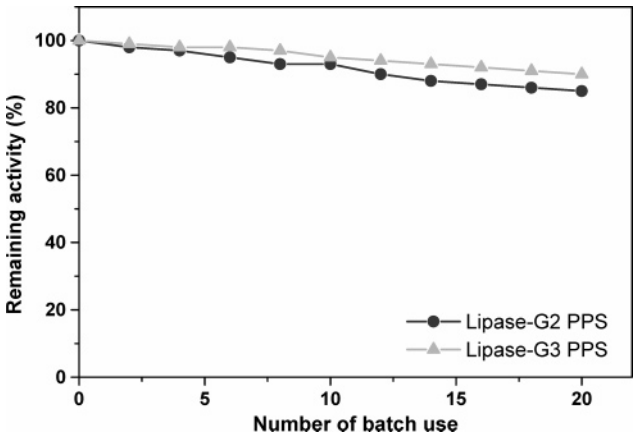


Figure 5. Effect of reuse on hydrolysis activity of olive oil at 35 °C and pH = 7. ● lipase-G2 PPS dendrimer (0.5 g, 2.12 mg g^{-1} dendrimer); ▼ lipase-G3 PPS dendrimer (0.5 g, 2.59 mg g^{-1} dendrimer).

immobilized polymer membrane, which presented 37% activity after 20 times of recycling of multiple batch operation.[28] It was also observed that lipase immobilized on silica lost entire activity after 20 times of recycling because of poor binding.[32] Storage of the immobilized enzyme at 4 °C for 3 months still retained 90% activity for olive oil hydrolysis, indicating their stability for ages.

Conclusions

This is the first report of enzyme immobilization on dendrimer. Lipase enzyme was immobilized onto PPS dendrimers by coupling reaction. The lipase-immobilized PPS dendrimer exhibited high activity and stability on the hydrolysis of olive oil. Optimum activity of immobilized enzyme had wider pH and temperature ranges than that of free enzyme. Multiple batch utilization of immobilized enzyme retained 80% activity even after recycling 20 times. The maximum recovered hydrolysis activity (98%) is the highest among data obtained until now with various other supports. It was confirmed that immobilization of lipase enzyme on PPS dendrimer is useful for an industrial process to obtain fatty acids from oils (olive oil) under mild conditions. The high availability of the lipase-immobilized PPS dendrimer on the hydrolysis reaction originates in the covalent-bonding immobilization of lipases on many terminal groups in the periphery of dendrimer. Then, all lipases are available for the reaction with the substrate without embedding or entrapping within the dendrimer, different from enzyme-immobilized supports so far reported.

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References and Notes

(1) Faber, K. In *Biotransformation in Organic Chemistry;* Schneider, M. P., Ed.; Springer: Berlin, 1995; pp 69−80.
(2) Boland, W.; Frossl, C.; Lorenz, M. *Synthesis* **1991**, 1049−1072.
(3) Brookes, I. K.; Lilly, M. D.; Drozd, J. W. *Enzyme Microb. Technol.* **1986**, *8*, 53.
(4) Pandey, A.; Benjamin, S.; Soccol, C. R.; Nigam, P.; Krieger, N.; Soccol, V. T. *Biotechnol. Appl. Biochem.* **1999**, *29*, 119−131.
(5) Mukherjee, K. D. *Biocatalysis* **1990**, *3*, 277−293.

(6) Nagao, A.; Kito, M. *Biocatalysis* **1990**, *3*, 295−305.
(7) Balcao, V. M.; Paiva, A. L.; Malcata, F. X. *Enzyme Microb. Technol.* **1996**, *18*, 392−416.
(8) Gandhi, N. N.; Sawant, S. B.; Joshi, J. B. *Biotechnol. Bioeng.* **1995**, *46*, 1−12.
(9) Paiva, A. L.; Balcao, V. M.; Malcata, F. X. *Enzyme Microb. Technol.* **2000**, *27*, 187−204.
(10) Hernáiz, M. J.; Sánchez-Montero, J. M.; Sinisterra, J. V. *J. Mol. Catal. A: Chem.* **1995**, *96*, 317.
(11) Chopineau, J.; McCafferty, F. D.; Therisod, M.; Klibanow, A. M. *Biotechnol. Bioeng.* **1988**, *31*, 208−214.
(12) Lokotsch, W.; Fitsche, C.; Syldatk, C. *Appl. Microbiol. Biotechnol.* **1989**, *31*, 467−472.
(13) Buckland, B. C.; Robinson, D. K.; Chartrain, M. *Metab. Eng.* **2000**, *2*, 42.
(14) Oliveira, A. C.; Rosa, M. F.; Aires-Barros, M. R.; Cabrel, J. M. S. *Enzyme Microb. Technol.* **2000**, *26*, 446−450.
(15) Brady, C.; Netcalfe, L.; Slaboszewski, D.; Frank, D. *J. Am. Oil Chem. Soc.* **1988**, *65*, 917−921.
(16) Moreno, J. M.; Hernaiz, J. H.; Sanchez-Montero, J. M.; Sinestra, J. V.; Bustos, M. T.; Sanchez, M. E.; Bello, J. F. *J. Mol. Catal. B: Enzymol.* **1997**, *2*, 177−184.
(17) Wang, X.; Ruckenstein, E. *Biotechnol. Prog.* **1993**, *9*, 661−665.
(18) Reetz, M. T.; Zonta, A.; Impelkapt, J. *Biotechnol. Bioeng.* **1996**, *49*, 527−534.
(19) Hsu, A.; Foglia, T. A.; Shen, S. *Biotechnol. Appl. Biochem.* **2000**, *31*, 179−183.
(20) Basri, M.; Yunus, W. M. Z. W.; Yong, W. S.; Ampon, K.; Razak, C. N A.; Salleh, A. B. *J. Chem. Technol. Biotechnol.* **1996**, *66*, 169−173.
(21) Yokozeki, K.; Yamanaka, S.; Takinami, K.; Hirose, Y.; Tanaka, A.; Sonomoto, K.; Fukui, S. *Eur. J. Appl. Microbiol. Biotechnol.* **1982**, *14*, 1−5.
(22) Mustranta, A.; Forssell, P.; Poutanen, K. *Enzyme Microb. Technol.* **1993**, *15*, 133.
(23) Imae, T. In *Encyclopedia of Nanoscience and nanotechnology*; Nalwa, H. S., Ed.; American Scientific Publishers: 2004; Vol. 3, pp 685−701.
(24) Yemul, O. S.; Ujihara, M.; Maki, N.; Imae, T. *Polym. J.* **2005**, *37*, 1−12.
(25) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−252.
(26) Sigma Diagnostics LIPASE (Procedure No. 800).
(27) Moreno, J. M.; Arroyo, M.; Hernáiz, M.; Sinisterra, J. V. *Enzyme Microb. Technol.* **1997**, *21*, 553.
(28) Bayramoğlu, G.; Kaçar, Y.; Denizli, A.; Arika, M. *J. Food Eng.* **2002**, *52*, 367−374.
(29) Arika, M. Y.; Denizli, A.; Salih, B.; Pi_kin, E.; Hasirci, V. *J. Membr. Sci.* **1997**, *129*, 65−76.
(30) Bryjak, J.; Bachmann, P. B.; Melizewska, I.; Trochimczuk, A.; Kolarz, M. N. *Chem. Eng. J.* **1997**, *65*, 249−256.
(31) Arika, M. Y. *J. Appl. Polym. Sci.* **2000**, *77*, 2000−2008.
(32) Dosanjh, N. S.; Kaur, J. *Biotechnol. Appl. Biochem.* **2002**, *36*, 7−12.

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