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Immobilization of *Mucor javanicus* lipase on effectively functionalized silica nanoparticles

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Abstract

Mucor javanicus lipase was effectively immobilized on silica nanoparticles which were prepared by Stöber method. Glycidyl methacrylate (GMA), which bears a reactive epoxide group, was incorporated onto the surface of the nanoparticles and the epoxide groups were directly used for multipoint coupling of the enzyme. We also introduced amine residues by coupling ethylene diamine (EDA) to the epoxide group of GMA. *M. javanicus* lipase was covalently immobilized onto the amine-activated silica nanoparticles by using glutaraldehyde (GA) or 1,4 phenylene diisothiocyanate (NCS) as a coupling agent. The lipase loading capacities of the EDA-GA and EDA-NCS nanoparticles (81.3 and 60.9 mg g^{-1}, respectively) were much higher than that of the unmodified GMA nanoparticles (18.9 mg g^{-1}). The relative hydrolytic activities in an aqueous medium of the lipases immobilized on EDA-GA and EDA-NCS attached silica nanoparticles (115% and 107%, respectively) were significantly high and almost in the same range with the free enzyme. This may be due to the improvement of the enzyme–substrate interaction by avoiding the potential aggregation of free lipase molecules. The immobilized lipases were also more resistant to temperature inactivation than the free form. This work demonstrates that the size-controlled silica nanoparticles can be efficiently employed as host materials for enzyme immobilization leading to high activity and stability of the immobilized enzymes.

Keywords: *Mucor javanicus* lipase; Silica nanoparticles; Covalent attachment; High activity; Stability

1. Introduction

Lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) is an efficient enzyme which catalyses the hydrolysis of triacylglycerol to glycerol and fatty acids. The enzyme has been widely used for enzymatic conversion in the various biotechnological applications in the dairy industry, manufacturing of specialty chemicals, organic synthesis, and preparation of enantiomerically pure pharmaceuticals [1]. However, its widespread utilization is often limited by low activity, stability, and inability of reuse. Therefore, there have been many studies to enhance these catalytic behaviors including enzyme immobilization, enzyme modification, and genetic modification [2,3].

Among these methods, immobilization strategy using solid supports has been most extensively researched due to the specific advantages of the immobilization method. First, the immobilized enzymes are easily recovered from the reaction medium for their reuse, which facilitate continuous and large-scale processes. Second, the immobilization of enzymes on a solid matrix limits their conformational variations diminishing unpredictable changes of their characteristic properties, which often occurs in enzyme modification. Moreover, the solid matrix may serve as a shield for harsh environmental conditions like pH and temperature variations. Thus, the immobilized lipase has been widely studied using different solid support materials and immobilization methods to optimize the catalytic features [4,5]. For example, celite [6], silica [7], metals [8], ion exchange resins [9], organic polymers [10], sol–gel materials [11–13], and recently developed nano-structured matrices [14,15] including nanoparticles have been used for the enzyme immobilization as support materials.

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To date, nanoparticles are promising due to their superior features including high surface area with low mass transfer limitation [16]. Several polymeric nanoparticles were demonstrated as suitable host materials for enzyme immobilization. Chen and Su immobilized α-chymotrypsin onto latex nanoparticles with high loadings over 10 wt% with particles smaller than 100 nm [17]. Jia et al. demonstrated the high activity retention of the immobilized α-chymotrypsin using polystyrene nanoparticles [18]. Fe_3O_4 magnetic nanoparticle was also adopted to immobilize yeast alcohol dehydrogenase and high activity up to 62% was retained than the free form [19].

For the efficient immobilization of enzymes to increase operational stability, covalent attachments to activated supports have been frequently used for enzyme immobilization [20,21]. To induce covalent linkage with enzymes, epoxy-activated supports are presumably the most accessible ones to perform very easy immobilization. These activated supports are very stable in aqueous media and able to form covalent linkage with different protein groups including amine, thiol, and phenol ones under mild condition at room temperature. Moreover, the epoxy groups can be easily incorporated into other functional groups by co-polymerization of monomers containing reactive groups or derivatization of the epoxy surface with another activating agent.

In this article, we prepared one-sized silica nanoparticles using Stöber method [22] as support materials for enzyme immobilization. To utilize covalent attachment of enzyme onto silica nanoparticles, glycidyl methacrylate (GMA) was attached onto the surface of the nanoparticles and its epoxide functional groups were used directly or used after suitable linker molecules were attached. As a coupling linker agent, glutaraldehyde (GA) or 1,4 phenylene diisothiocyanate (NCS) was employed. Thermal deactivation of the free and immobilized enzymes at various temperatures was also investigated.

2. Experimental

2.1. Materials

Mucor javanicus lipase was purchased from Fluka (Milwaukee, WI). 4-Nitrophenyl butyrate (4-NB), glutaraldehyde (GA), and 1,4 phenylene diisothiocyanate (NCS) were purchased from Sigma Chemical (St. Louis, MO). All other reagents and solvents were purchased from Aldrich Chemical (Milwaukee, WI) and were of the highest grade commercially available.

2.2. Synthesis of the silica sphere

The monodisperse silicas sphere in the range of 300 nm were prepared on the basis of a reported method [22,23]. Forty millilitres of aqueous ammonia (28 wt%) was added into a solution containing 1.01 of ethanol and 80 ml of deinoized water. Sixty millilitres of tetraethoxysilane (TEOS) was added into the above-prepared mixture at room temperature under vigorous stirring and the reaction mixture was kept stirring for 6 h to yield uniform silica spheres.

A mixture solution containing 3.85 ml of glycidyl methacrylate (GMA), 50 mg of 2,2′-azo-bis(isobutyronitrile) (AIBN), and 3.0 g of cetyltrimethylammoniumbromide (C_{16}TAB) was added into 400 ml of colloidal solution and further reacted at 70 °C for 2 days. The resulting silica with epoxy group nanocomposite was centrifuged and dried at 80 °C for 12 h.

2.3. Amination of silica sphere with epoxy group

The epoxy group modified silica sphere was reacted for amination as reported previously [24]. Two grams of silica with epoxide was added into 200 ml ethylenediamine (EDA) and refluxed at 80 °C for 7 h. After the reaction was finished, the solution was washed several times with ethanol and deionized water. The formed powder was dried in air for overnight.

2.4. Preparation of GA or NCS-activated silica nanoparticles

Aminated silica nanoparticles were treated with a coupling agent, GA or NCS, to increase enzyme loading capacity and activity. For the preparation of GA-activated silica nanoparticles, the aminated nanoparticles (50 mg) were washed twice with an aqueous buffer (100 mM sodium phosphate buffer, pH 8.0) and gradually immersed in the same phosphate buffer containing 0.1% (w/w) GA for 2 h at room temperature in a shaking condition (200 rpm). Slow and steady adding of silica nanoparticles is important for minimizing the aggregation among the silica nanoparticles. After the reaction, the excess GA was removed by washing twice with 100 mM phosphate buffer (pH 8.0) and another twice with 10 mM phosphate buffer (pH 6.5), and stored at 4 °C.

For the preparation of NCS-activated silica nanoparticles, the aminated silica nanoparticles (50 mg) were incubated in a dichloromethane (19 ml) solution of DIEA (*N*,*N*′-diisopropylethyleneamine, 1 ml) at room temperature in a shaking condition (200 rpm). The resulting substrates were thoroughly washed with dichloromethane three times and dried in a vacuum oven for 20 min. Then, the nanoparticles were gradually immersed in a shaking condition (200 rpm) in an acetonitrile (15 ml) solution containing 15 mg of NCS activating agents (1,4-phenylene diisothiocyanate) overnight at room temperature. After the reaction period, the nanoparticles were washed with dichloromethane three times, air dried, and stored at 4 °C in a vacuum desiccator containing anhydrous calcium chloride until use.

2.5. Immobilization of M. javanicus lipase onto functionalized silica nanoparticles

The enzyme was covalently attached directly onto the epoxy-activated silica nanoparticles, or after GA or NCS activation onto aminated silica nanoparticles. For the direct attachment of the enzyme onto the epoxy group attached silica nanoparticles, the GMA-silica nanocomposites (10 mg) were washed three times with 100 mM carbonate buffer (pH 9.0). Then, the nanoparticles were mixed with 1.5 ml of the free lipase (5 mg ml^{-1}) in a buffer solution (100 mM carbonate buffer, pH 9.0), vortexed for

30 s, sonicated for 3 s, and incubated for 20 h at room temperature in a shaking condition (250 rpm). After the immobilization period, the samples were washed five times with phosphate buffer (10 mM, pH 6.5) and stored at 4 °C.

Immobilization of lipase on GA-activated silica nanoparticles was carried out at room temperature in a shaking condition. The GA-activated silica nanoparticles (10 mg) were washed twice with 100 mM sodium phosphate buffer (pH 8.0) and another twice with 10 mM phosphate buffer (pH 6.5). Then, the nanoparticles were mixed with 1.5 ml of the free lipase (5 mg ml^{-1}) in a buffer solution (10 mM phosphate buffer, pH 6.5), vortexed for 30 s, sonicated for 3 s, and incubated for 20 h at room temperature in a shaking condition (250 rpm). After the incubation, the samples were incubated with 100 mM Tris–HCl buffer (pH 8.0). The capping of unreacted aldehyde groups was performed in a fresh Tris–HCl buffer (100 mM Tris, pH 8.0) at 200 rpm for 30 min. After Tris-capping, the samples were washed twice by Tris–HCl buffer (100 mM, pH 8.0) and another twice by phosphate buffer (10 mM sodium phosphate buffer, pH 6.5), and stored at 4 °C.

Immobilization of lipase on NCS-activated silica nanoparticles was carried out at room temperature in a shaking condition. The NCS-activated silica nanoparticles (10 mg) were washed three times with 100 mM carbonate buffer (pH 9.0). Then, the nanoparticles were mixed with 1.5 ml of the free lipase (5 mg ml^{-1}) in a buffer solution (100 mM carbonate buffer, pH 9.0), vortexed for 30 s, sonicated for 3 s, and incubated for 20 h at room temperature in a shaking condition (250 rpm). After the immobilization, the samples were washed five times by phosphate buffer (10 mM sodium phosphate buffer, pH 6.5), and stored at 4 °C.

The protein leaching amount from the silica nanoparticles into the supernatant was measured by Lowry (Bio-Rad, Hercules, CA) [25] method. The final enzyme loading on the nanoparticles was calculated from the differences between the initial lipase amount and the leached amount of lipase into the washing solutions.

2.6. Activity of free and immobilized lipase

The activities of free and immobilized lipase were measured by the hydrolysis of 4-nitrophenyl butyrate in 10 mM sodium phosphate buffer at room temperature. The absorbance increase at 400 nm was monitored using the spectrophotometer (Model Cary 5G UV–vis–NIR spectrophotometer from Varian Inc., Palo Alto, CA). For the activity measurement of the immobilized enzyme systems, a small aliquot of samples was mixed with 4-nitrophenyl butyrate (500 μM) and shaken at 250 rpm at room temperature. The increase of absorbance at 400 nm in the supernatant was measured time-dependently after centrifugation at 5000 × *g*.

2.7. Thermal stability measurement of free and immobilized lipase

Thermal stabilities of the free and immobilized lipase were checked in an aqueous buffer (10 mM sodium phosphate, pH 6.5) at two different temperatures (50 and 60 °C) for 120 min. After each time point, the residual activity of each sample was determined by measuring hydrolytic enzyme activity of substrates as described above. The relative activity (%) was calculated by the ratio of the residual activity to the initial activity of each sample.

2.8. Characterization of silica nanoparticles

Field emission scanning electron microscopy (FESEM) was used to determine the size and surface morphology of the silica colloids and epoxy- and amine-group modified silica sphere. The measurements were performed with a Hitachi (S-4700FE-SEM, Japan) scanning electron microscope (SEM) operated at an accelerating voltage of 20 kV. The microscopic features of the sample were observed with a transmission electron microscope (TEM, EM 912 Omega) operated at 200 kV. The elemental analysis (EA) (Elementar Analysensysteme, Elementar Vario EL, Germany) of the epoxy- and amine-group modified silica sphere was also performed.

3. Results and discussion

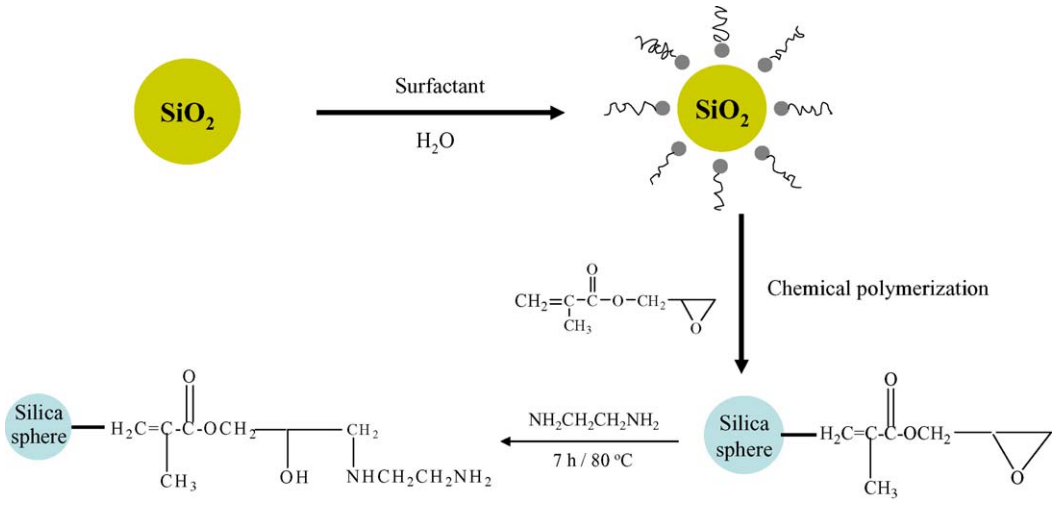
3.1. Nanoparticle preparation

In the present study, size and morphology controlled silica nanoparticles were prepared by Stöber method [22]. These nanoparticles were functionalized with epoxide group containing GMA by chemical polymerization [26]. And then, the GMA attached nanoparticles were incorporated with amine group containing EDA to demonstrate the linkage effect of the covalent attachment of enzymes. The whole procedures were summarized in Scheme 1.

The structural features of the silica nanospheres were shown in Fig. 1. SEM and TEM images (Fig. 1) indicate that the size of silica nanoparticles is around 300 nm and uniform spheres were shown.

To induce covalent attachment with lipases, epoxide group containing GMA was incorporated on the silica surface via chemical polymerization in the presence of an initiator, AIBN. The details of the polymerization were described in Section 2. TEM image revealed that the GMA attachment onto the surface of the silica nanoparticles (Fig. 2). The elevations of the carbon (C), hydrogen (H) in EA analysis also confirmed the GMA attachment onto the silica nanoparticles.

Ethylene diamine (EDA) was also introduced onto the GMA attached silica nanoparticles to introduce amine groups on the surface of the nanoparticles. SEM images visualize the EDA introduced silica nanoparticles (Fig. 3). Because the EDA structure had two reactive amines, EDA attachment tended to connect the silica nanoparticles by coupling with the epoxide groups on the silica nanoparticles. Therefore, the silica nanoparticles seemed to be aggregated. The introducing of the EDA was also confirmed by the elevating nitrogen (N) in the EA analysis.



Scheme 1. Schematic representation for the preparation of GMA attached SiO_2 nanosphere and EDA attached SiO_2 nanosphere.

3.2. Immobilization of lipase on functionalized silica nanoparticles

For high loading capacity and activity of the immobilized enzymes onto the surface of the silica nanoparticles, three different immobilization methods were utilized. First, direct multipoint covalent attachments of enzymes to the epoxide groups of GMA-activated silica nanoparticles were used. Second, lipase was covalently linked via a coupling agent, GA, using EDA-activated silica nanoparticles (EDA-GA). Because the GA has two aldehydes, it can connect the surface amine residues of the lipases with the amines of the silica surface. Third, NCS was chosen as a coupling agent, and the covalent immobilization between the amine residues of the enzymes and the amines on the surface of the silica nanoparticles was experimented (EDA-NCS). We expect that the alterations in the linkage chemistry can enhance the enzyme loading capacity and activity.

The lipase loading amount was measured via the incubation time (1, 4, 8, 12, 16, and 20 h) to study the effects of enzyme coupling time on the immobilization amount using the three immobilization strategies. As a result, the loading amount did not increase much as time went on up to 20 h. The loading amounts at time 1 h was already about 90% of that of 20 h. This demonstrates that the immobilization was nearly finished in a very short time about 1 h. For a tight immobilization, 20 h incubation was chosen for measuring loading capacities and activities.

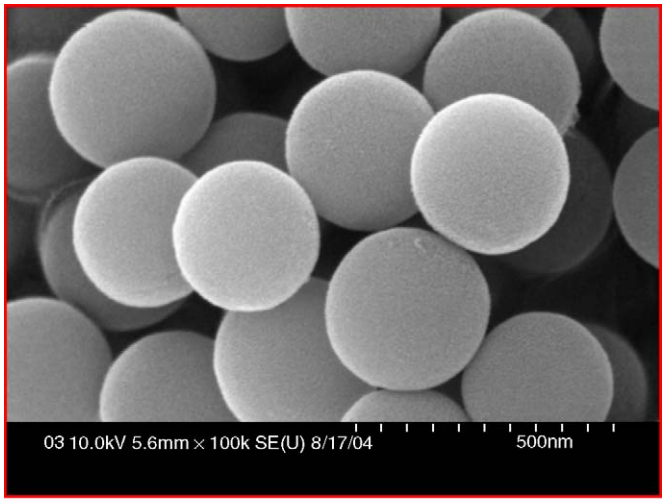
After 20 h incubations, the enzyme loading capacities of the EDA-GA and EDA-NCS nanoparticles (81.3 and 60.9 mg g^{-1}) were much enhanced, about three to five times, than that of the unmodified GMA nanoparticles (18.9 mg g^{-1}). The EDA-GA activated nanoparticles showed the highest loading capacity and it reached up to 7.52 wt% of the support material. When the silica nanoparticles were directly used without any chemical modification, there was no significant amount of enzyme loading observed indicating nonspecific adsorption did not contribute to the overall enzyme loading. The specific activity was also enhanced about five times by using GA or NCS linker to the amine-activated silica nanoparticles (0.95 and 0.88 A400/min per mg/ml lipase) than that of direct covalent attachment of enzymes with GMA-activated silica nanoparticles (0.17 A400/min per mg/ml lipase). Interestingly, the relative activity of the lipases immobilized on EDA-GA and EDA-NCS attached silica nanoparticles were significantly high (115% and 107%, respectively) and almost in the same range with the free enzyme (Table 1).

The enhanced loading capacities and activities of the nanoparticles with additional coupling groups like EDA-GA and EDA-NCS are considered to be due to the spacer-arm effect [26]. The coupling agent, GA or NCS, can move the enzyme away from the surface of nanoparticles, and prevent undesirable side attachment between large enzyme molecules and support. In this way, EDA-GA or EDA-NCS activated nanoparticles can give more accessible surface areas for the immobilization of enzymes. The increased loading on EDA-GA may also be partly ascribed to the enhanced hydrophobicity of the silica surface caused by those chemical treatments, leading to more

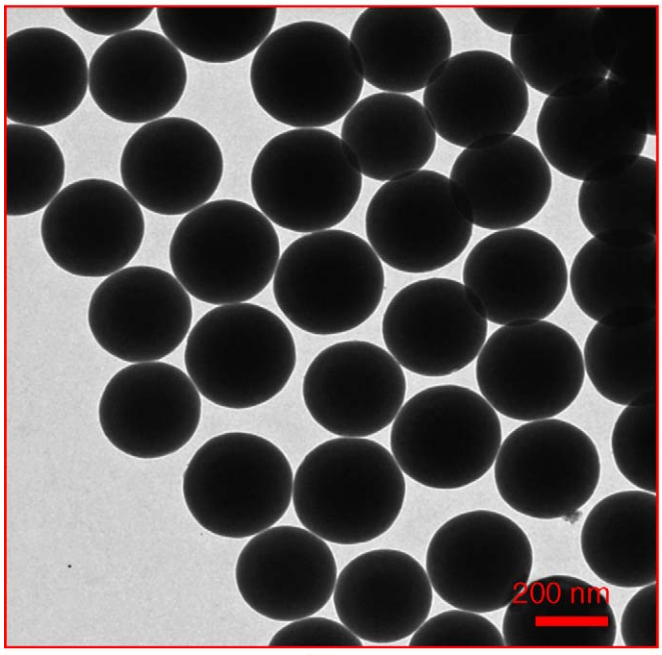
Table 1
Lipase (LP) loading capacity and activity

Sample	LP loading (wt%)	LP loading (mg LP per g silica)	Specific activity (A400/min per g silica)	Specific activity (A400/min per mg immobilized LP)	Relative activity (%)
GMA-LP	1.86	18.9	0.0024	0.11	21
EDA-GA-LP	7.52	81.3	0.0643	0.63	115
EDA-NCS-LP	5.74	60.9	0.0414	0.59	107
Free LP	–	–	–	0.55[a]	100

[a] Instead of immobilized lipase, free lipase was used for the determination of the activity.



(a)



(b)

Fig. 1. (a) SEM image of colloidal silica nanoparticles. (b) TEM image of colloidal silica nanoparticles.

favorable environment for the hydrophobic adsorption of the enzyme protein. Moreover, the attachment of the proper linker can improve the enzyme–substrate interaction by avoiding the potential aggregation of free lipase molecules [18]. Increased hydrophobicity resulting from the additional coupling agents can be a reason for increased enzyme loading. Therefore, it is possible to enhance the lipase loading capacity and activity by using a proper coupling agent.

3.3. pH effect on the activity of free and immobilized lipase

Free and variously immobilized lipase activities were investigated in terms of pH. The effect of pH on the lipase activity was studied in the pH range from 6 to 10 and the results were described in Fig. 4. The optimum pH of free lipase was 8.0, and that of immobilized lipase on the GMA, EDA-GA, and EDA-NCS activated silica nanoparticles was shifted by 1.0 pH unit to the alkaline region. This shift in pH optimum is consistent with the previous report [27]. Moreover, the pH profiles of the immobilized lipase were broader than that of the free form indicating that the immobilized methods preserved the enzyme activity over a wide pH range. These results may be attributed to the stabilization of immobilized lipase via multipoint attachment on the surface of the silica nanoparticles.

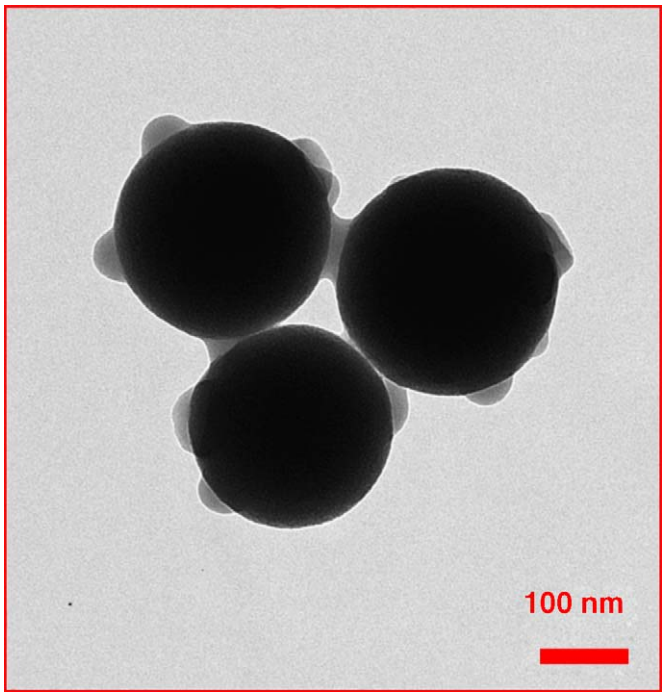


Fig. 2. TEM image of the silica nanoparticles with epoxy group.

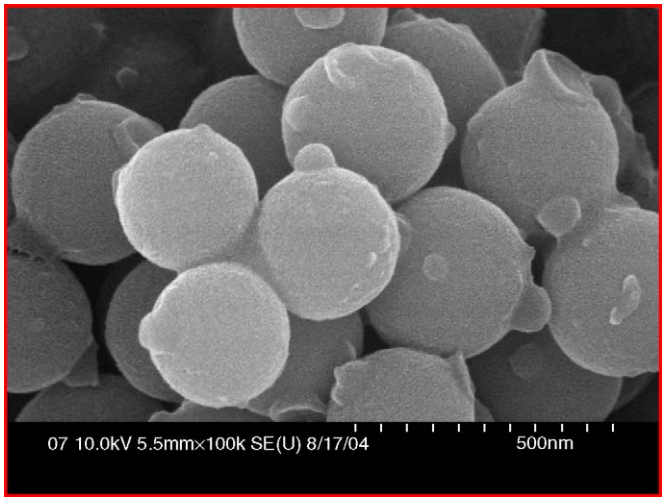


Fig. 3. SEM image of the silica nanoparticles with amine group.

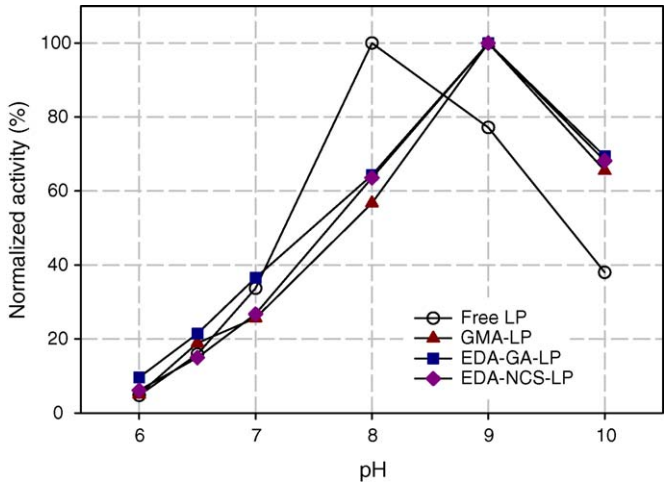


Fig. 4. pH profiles of the free and immobilized lipase.

3.4. Thermal stability

Multipoint attachment of enzymes is supposed to be accompanied by the improved enzyme stability and these effects were investigated at high temperatures of 50 and 60 °C. The thermal stabilities of free and immobilized lipase onto EDA-GA and EDA-NCS activated silica nanoparticles were evaluated by incubating them in aqueous buffer at two different temperatures, 50 and 60 °C, without substrate.

Fig. 5 shows the thermal stability of the free and immobilized lipase. At 50 °C, the immobilized lipase seemed to be stable for 120 min with 95% residual activity for EDA-GA, and 90% for EDA-NCS, respectively. The free lipase activity remained 67% at this temperature after 120 min incubation. At 60 °C, the free lipase retained its activity about levels of 19%, whereas the immobilized lipase onto the EDA-GA or EDA-NCS activated nanoparticles retained 62% or 68% to the initial activity, respectively. This apparent increase in the thermal stability of the immobilized lipase indicates that the multipoint attachment can efficiently resist the thermal deactivation. It was reported that the thermal stability enhancement was general advantages of the immobilized enzymes [28].

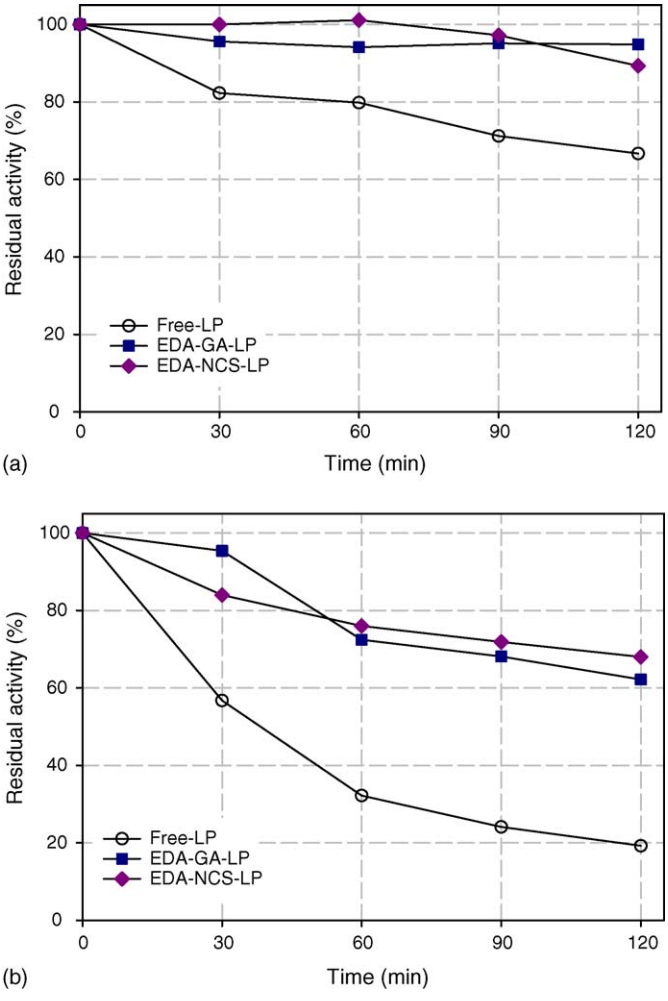


Fig. 5. Thermal stabilities of free and immobilized lipase at 50 °C (a) and 60 °C (b).

4. Conclusions

The present study demonstrated the successful immobilization of *M. javanicus* lipase onto variously functionalized silica nanoparticles. Besides, the direct correlation between several surface modifications of nanoparticles and enzyme loading capacity, activity, and stability was also verified. The attachment of GA and NCS onto the EDA-activated silica nanoparticles resulted in a remarkable increase in the enzyme loading capacity and activity. Immobilized lipase retained high level of activity over a wider range of pH than that of free from. Thermal stability was considerably enhanced by the immobilization. Since this nanoparticle-based enzyme immobilization preserves high enzyme loading and activity with enhanced stability, it can be applied to various enzymatic applications, such as biosensors and bioremediations.

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References

[1] R.D. Schmid, R. Verger, Angew. Chem. Int. Ed. 37 (1998) 1608.
[2] G. DeSantis, J.B. Jones, Curr. Opin. Biotechnol. 10 (1999) 324.
[3] V.V. Mozhaev, Trends Biotechnol. 11 (1993) 88.
[4] B. Al-Duri, Y.P. Yong, J. Biochem. Eng. 4 (2000) 207.
[5] M. Reslow, P. Adlercreutz, B. Mattiason, Enzyme Microb. Technol. 73 (1999) 384.
[6] A. Sagiroglu, A. Kilinc, A. Telefoncu, Artif. Cell Blood Substit. 32 (2004) 625.
[7] C.M.F. Soares, H.F. de Castro, F.F. de Mareas, G.M. Zanin, Appl. Biochem. Biotechnol. 77 (2000) 745.
[8] F. Bellezza, A. Cipiciani, U. Costantino, J. Mol. Catal. B: Enzym. 26 (2003) 47.
[9] N.N. Gandhi, V. Vijayalakshmi, B. Sawant, J.B. Joshi, Chem. Eng. J. 61 (1996) 149.
[10] J. Bryjak, B. Bachmann Pawlow, I. Melizewska, A. Trochimczuk, M.N. Kolarz, Chem. Eng. J. 65 (1997) 249.

[11] M.T. Reetz, A. Zonta, J. Simpelkamp, Biotechnol. Bioeng. 49 (1996) 527.
[12] I. Gill, A. Ballesteros, J. Am. Chem. Soc. 120 (1998) 8587.
[13] A.C. Pierre, Biocatal. Biotransform. 22 (2004) 145.
[14] R.M. Blanco, P. Terreros, M. Fernandez-Perez, C. Otero, G. Diaz-Gonzalez, J. Mol. Catal. B: Enzym. 30 (2004) 83.
[15] M.B.F. Martins, S.I.D. Simoes, M.E.M. Cruz, R. Gaspar, J. Mater. Sci.: Mater. Med. 7 (1996) 413.
[16] H. Jia, G. Zhu, B. Vugrinovich, W. Kataphinan, D.H. Reneker, P. Wang, Biotechnol. Prog. 18 (2002) 1027.
[17] J.-P. Chen, D.-R. Su, Biotechnol. Prog. 17 (2001) 369.
[18] H. Jia, G. Zhu, P. Wang, Biotechnol. Bioeng. 84 (2003) 406.
[19] M.-H. Liao, D.-H. Chen, Biotechnol. Lett. 23 (2001) 1723.
[20] J. He, X. Li, D.G. Evans, X. Duan, C. Li, J. Mol. Catal. B: Enzym. 11 (2000) 45.
[21] P. Wang, S. Dai, S.D. Waezsada, A.Y. Tsao, B.H. Davison, Biotechnol. Bioeng. 74 (2001) 249.
[22] W. Stober, A. Fink, E. Bohn, J. Colloid Interface Sci. 26 (1968) 62.
[23] J.-S. Yu, S.B. Yoon, G.S. Chai, Carbon 139 (2000) 1442.
[24] S.-H. Choi, G.T. Kim, Y.C. Nho, J. Appl. Polym. Sci. 71 (1999) 643.
[25] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[26] G. Bayramoglu, B. Kaya, M.Y. Arica, Food Chem. 92 (2005) 261.
[27] J.F. Shaw, R.C. Chang, F.F. Wang, Y.J. Wang, Biotechnol. Bioeng. 35 (1991) 132.
[28] M.N. Gupta, Eur. J. Biochem. 203 (1992) 25.