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Immobilization of lipase within carbon nanotube–silica composites for non-aqueous reaction systems

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ABSTRACT

The immobilization of lipases within sol–gel derived silica, using multi-walled carbon nanotubes (MWNTs) as additives in order to protect the inactivation of lipase during sol–gel process and to enhance the stability of lipase, was investigated. Three sol–gel immobilized lipases (*Candida rugosa*, *Candida antarctica* type B, *Thermomyces lanuginosus*) with 0.33% (w/w) MWNT showed much higher activities than lipase immobilized without MWNT. The influence of MWNT content and MWNT shortened by acid treatment in the sol–gel process on the activity and stability of immobilized *C. rugosa* lipase was also studied. In hydrolysis reaction, immobilized lipase containing 1.1% pristine MWNT showed 7 times higher activity than lipase immobilized without MWNT. The lipase coimmobilized with 2.7% shortened MWNT showed 10 times higher activity in esterification reaction, compared with lipase immobilized without MWNT. The lipase coimmobilized with 2.7% shortened MWNT retained 96% of initial activity after 5 times reuse, while the lipase immobilized without MWNT was fully inactivated under the same condition.

1. Introduction

Sol–gel processes are most popularly used for the immobilization of biomolecules due to their porosity, transparency, chemical stability and convenient preparation [1]. A very large number of enzymes have been immobilized within sol–gel glasses. Although sol–gel immobilized enzymes usually exhibit better activity and stability than free enzymes [2–4], there are some drawbacks in the sol–gel immobilization process. One is the shrinkage of gel during condensation and drying process which may cause denaturation of enzymes. The released alcohols during the hydrolysis of silicon alkoxide can also inactivate enzymes [5]. One way to overcome these drawbacks would be the use of additives to stabilize enzymes within sol–gel matrices. Sugars, amino acids, polyols, and surfactants have been used to increase activity and stability of various enzymes. These additives can increase activity and stability of immobilized enzymes by altering hydration of enzyme and reducing shrinkage of gel [6–9].

Carbon nanotubes (CNTs) have attracted much attention due to their remarkable mechanical, chemical, electrical, and structural properties. High surface area and excellent electronic performance of CNTs also make them to be potential supports for enzyme immobilization and substrates for the development of biosensors [10]. Gavalas et al. prepared CNTs–silica composite materials by using CNTs as additives in the sol–gel process [11]. These materials provided new capabilities for the development of electrochemical devices. For example, CNTs–silica based biosensors containing horseradish peroxidase or glucose oxidase have been successfully developed [10,12].

Several proteins have been immobilized in CNTs–silica composites, but their applications are so far limited to the electron transfer related reactions including oxidoreductases for the development of biosensor. In this work, therefore, we focused on the immobilization of enzymes which can be used for bioconversion in CNTs–silica composites. For this purpose, some lipases were immobilized in these composites. Lipases entrapped in sol–gel silica can be easily released in aqueous solution and sol–gel immobilized lipases usually showed very low activity [13]. Accordingly, CNTs were used as additives to enhance activity and stability of sol–gel immobilized lipase as well.

2. Experimental procedures

2.1. Materials

Commercial *Candida rugosa* lipase (Type VII) and *Mucor javanicus* lipase were purchased from Sigma (St. Louis, USA). *Candida antarctica* lipase B and *Thermomyces lanuginosus* lipase were kindly

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Table 1
Effect of carbon nanotube on the activity of immobilized *Candida rugosa* lipase.

	Hydrolysis activity (μmol/min/g protein)	Esterification activity (μmol/min/g protein)
Control (without MWNT)	103	7.8
1.1% (w/w) MWNT-containing immobilized lipase (Addition of MWNT to TEOS solution)	464	14.4
1.1% MWNT-containing immobilized lipase (Pre-incubation of enzyme with MWNT for 1 h)	740	27.7

gifted by Novo Nordisk (Bagsvaerd, Denmark). Tetraethyl orthosilicate (TEOS), *p*-nitrophenyl butyrate, benzyl alcohol, vinyl acetate, benzyl acetate, and *n*-hexane were provided by Aldrich (Steinheim, Germany). The MWNT (10–15 nm diameter, 95% purity) synthesized by a thermal chemical vapor deposition method was obtained from Iljin Nanotech. (Seoul, Korea). All other chemicals used in this work were of analytical grade and were used without further purification.

2.2. MWNT cutting

To shorten carbon nanotube, 40 mg MWNT was refluxed at 70 °C for 12 h in a 3:1 (v/v) mixture of concentrated H_2SO_4 and HNO_3. The reaction was quenched by adding 20 volume of water. The nanotube/acid mixture was then filtered off using 0.2 μm teflon membranes, followed by washing with deionized water until filtrate reached pH 7.0 and then with ethanol for 10 min [14,15]. The resulting shortened MWNT was dried under vacuum for 60 min and stored at 25 °C to be used for lipase immobilization.

2.3. Procedure for sol–gel immobilization of lipase

For the preparation of lipase solution, about 1 g of enzyme was added in 10 ml of 0.1 M phosphate buffer (pH 7.0) and shaken for 10 min. After centrifugation, the supernatant was used for immobilization experiments. Protein content of lipase solution was determined with Lowry protein Assay Kit. The lipase solution (1 ml) was pre-incubated with various contents of MWNTs for 1 h. For the preparation of hydrolyzed TEOS solution, the mixture of TEOS (1 ml), deionized water (0.5 ml), and 0.1 M HCl (50 μl) was vigorously stirred for 3 h in a 5 ml glass vial. After a clear solution was formed, hydrolyzed TEOS solution was added to the lipase/MWNT solution. The mixture was vigorously shaken for 30 s on a vortex mixer and then gently shaken until gelation. The reaction vessel was left to stand opened and the bulk gel was air-dried at room temperature for 2 days. The bulk gel was crushed in a mortar and then the powder was dried in vacuum oven at 25 °C for 12 h and stored at 25 °C.

2.4. Determination of hydrolysis activity

The 5 mg of immobilized lipase was placed in a conical tube together with 10 ml of 20 mM phosphate buffer (pH 7.0). The reaction was started by adding 0.1 ml of substrate solution (50 mM *p*-nitrophenyl butyrate in DMF) and carried out at 25 °C in a water bath with shaking at 200 rpm. Periodically, 300 μl aliquots were taken and diluted with 300 μl of acetonitrile, and then centrifuged to obtain supernatant. The activity was determined by measuring the increase in absorbance at 400 nm by the *p*-nitrophenol produced during the hydrolysis of *p*-nitrophenyl butyrate [13,16].

2.5. Determination of esterification activity

The immobilized lipase (20 mg) was added to a small magnetically stirred glass vial containing benzyl alcohol (10 mM), vinyl acetate (10 mM), and water-saturated *n*-hexane (1 ml) at 40 °C with continuous shaking. Periodically, 20 μl aliquots were taken and diluted with 40 μl of *n*-hexane to analyze by HPLC. Benzyl alcohol and benzyl acetate were quantified by HPLC equipped with a reverse-phase C18 column (SYMMETRY®, Waters, USA) with UV detector at 250 nm. The mobile phase consisted of acetonitrile/water (50/50, v/v) containing 100 μl phosphoric acid per liter with a flow rate of 1 ml/min. The activity was expressed as μmol of product (benzyl acetate) formed per minute per gram of dry support or protein [13].

To measure the operational stability of immobilized lipase, the lipases were removed after reactions and washed 3 times with *n*-hexane. The subsequent reactions were carried out under the same reaction conditions described above.

3. Results and discussion

3.1. Effect of carbon nanotube as an additive on sol–gel immobilization of lipases

The immobilized lipases produced by sol–gel process using only TEOS usually have displayed extremely low activities. For example, relative activities of less than 5% were obtained in the esterification of lauric acid with octanol in isooctane [2]. In the hydrolysis of *p*-nitrophenyl butyrate, relative activity of immobilized *C. rugosa* lipase was less than 1% of free lipase [13]. In this work, pristine MWNT and shortened MWNT as additives were used to enhance the activity and stability of sol–gel immobilized lipases. Table 1 shows the positive effect of pristine MWNT as an additive in sol–gel immobilization process. Firstly, MWNT was added to the hydrolyzed TEOS solution and sonicated for 10 min to enhance the dispersion of MWNT. The TEOS/MWNT solution was then mixed with enzyme solution and gelated. The MWNT-containing immobilized lipase prepared by using this protocol showed 4.5 and 1.8 times higher activities than immobilized lipase without MWNT in the hydrolysis and esterification reaction, respectively. These results can be partially explained by the protecting effect of MWNT for the shrinkage of gel structure during condensation and drying process which cause partial denaturation of enzymes, because MWNT can be employed as a template in sol–gel process [11,17]. On the other hand, MWNT was added to the enzyme solution and incubated for 1 h in order to induce the adsorption of lipase on MWNT and activate the lid structure of lipase. The enzyme/MWNT solution was then mixed with hydrolyzed TEOS solution and gelated. The immobilized lipase prepared by this method showed 7.2 and 3.6 times higher activities than immobilized lipase without MWNT in the hydrolysis and esterification reaction, respectively. There are some possible explanations for enhanced activities of lipases immobilized after pre-incubation of enzyme solution with MWNT. The hydrolysis and esterification activity of *C. rugosa* lipase can be

Table 2
Influence of carbon nanotubes as additives on the esterification activity of various sol–gel immobilized lipases.

Immobilized lipase	Protein content (%, g protein/g gel)	Specific activity (μmol/min/g protein)
Without MWNT		
Candida rugosa	2.9	7.8
Mucor javanicus	1.9	35.1
Candida antarctica type B	3.2	12.5
Thermomyces lanuginosus	3.9	22.4
With 0.33% MWNT		
Candida rugosa	2.9	30.7
Mucor javanicus	1.8	34.2
Candida antarctica type B	3.2	25.4
Thermomyces lanuginosus	3.8	60.7

increased by pretreatment of suitable organic solvents or adsorption on hydrophobic matrix. The opening of the lid covering active site of *C. rugosa* lipase is proposed as the reason for the activity enhancement, both in aqueous and anhydrous organic media [18,19]. The highly hydrophobic nature of MWNT can also induce the opening of the lid structure and activate *C. rugosa* lipase by the interaction with the hydrophobic domains of lipase. Protection of gel shrinking by MWNTs can also contribute enhanced lipase activity in sol–gel process. Besides, lipases adsorbed on carbon nanotube during pre-incubation can retain activity in sol–gel process, because *C. rugosa* lipase immobilized on MWNTs showed high activity and stability [20]. Therefore, pre-incubation of enzyme solution with MWNTs and then gelation with hydrolyzed TEOS solution was used to investigate various effects of MWNTs on immobilized lipase for the following study.

Table 2 shows the effect of MWNTs as additives on esterification activity of various immobilized lipases. With the exception of *M. javanicus* lipase, three immobilized lipases (*C. rugosa*, *C. antarctica* type B, *T. lanuginosus*) with 0.33% MWNT showed higher activities than lipase immobilized without MWNT. Although the activation of lid-containing lipase by hydrophobic MWNTs was predicted [20], lid-containing *M. javanicus* lipase did not show the enhancement of activity while *C. antarctica* lipase B which does not have lid structure showed 2 times higher activity. Therefore, increased activity of immobilized lipase by adding MWNT in sol–gel process should be understood by the multiple effects of MWNT such as protection of gel shrinkage, stabilization of lipase by adsorption, and role as a template during gelation.

3.2. Hydrolysis and esterification activity of immobilized lipases

The contents of carbon nanotube can influence on the specific activity of immobilized lipase. Figs. 1 and 2 show the effect of pristine MWNT contents on the hydrolysis and esterification activity of immobilized lipases, respectively. The specific activities of lipases coimmobilized with pristine MWNT for hydrolysis reaction showed the highest activity at 1.1% MWNT content. Over 1.1% of pristine MWNT decreased the activity of immobilized lipases. In the esterification reaction, maximal activity was obtained with 0.23% pristine MWNT and over 1.1% of pristine MWNT decreased the activity of immobilized lipases. Activity decrease of immobilized lipase with increasing pristine MWNT content may be caused by the impurities of MWNT, because commercial MWNTs can contain metal/metal oxide impurities which cannot be removed by general washing procedure. Therefore, purified and shorten MWNTs by strong acid treatment were also used as additives in sol–gel process. Shortened MWNT showed higher dispersity in buffer and entangled structures of MWNTs may be reduced. In the hydrolysis reaction, the

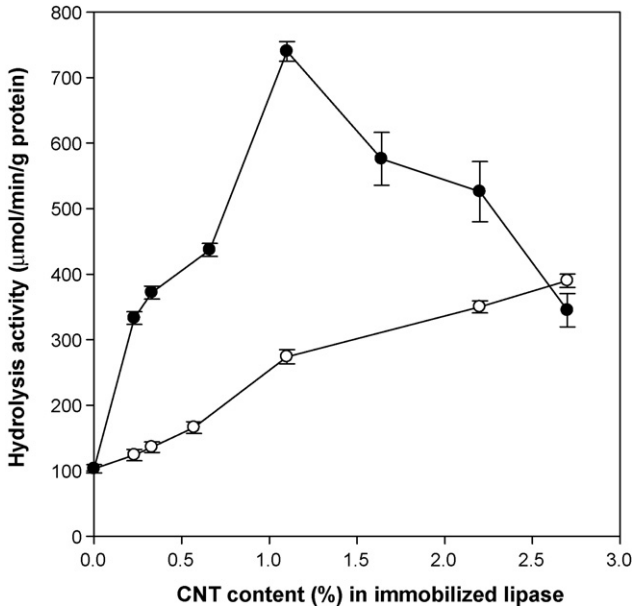

Fig. 1. Influence of carbon nanotube content on the hydrolysis activity of immobilized *C. rugosa* lipase. Hydrolysis conditions: 5 mg immobilized lipase, 0.5 mM *p*-nitrophenyl butyrate, 10 ml 20 mM phosphate buffer (pH 7.0), 25 °C. (●) Pristine MWNT and (○) shortened MWNT.

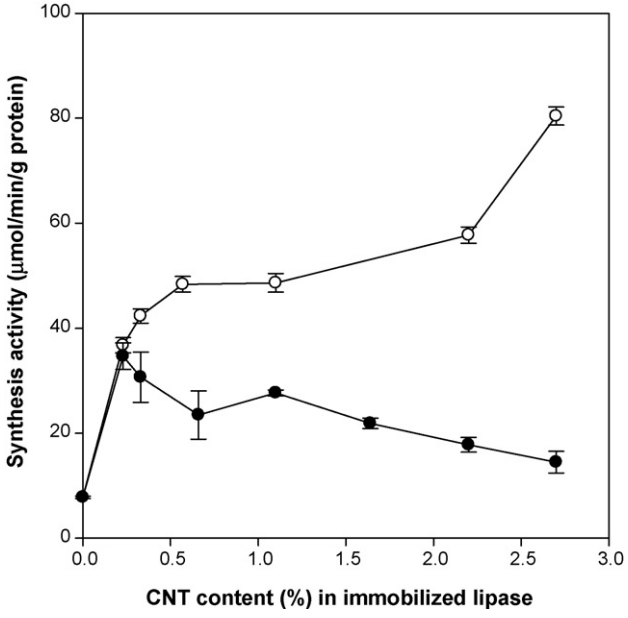

Fig. 2. Influence of carbon nanotube content on the esterification activity of immobilized *C. rugosa* lipase. Esterification conditions: 20 mg immobilized lipase, 10 mM benzyl alcohol, 10 mM vinyl acetate, 1 ml water-saturated *n*-hexane, 40 °C. (●) Pristine MWNT and (○) shortened MWNT.

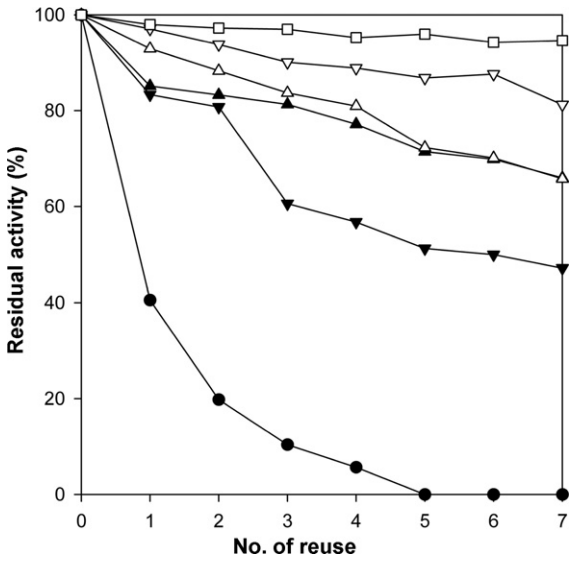

Fig. 3. Effect of carbon nanotube content on the operational stability of immobilized *C. rugosa* lipase in the esterification reaction. Esterification conditions: 20 mg immobilized lipase, 10 mM benzyl alcohol, 10 mM vinyl acetate, 1 ml water-saturated *n*-hexane, 40 °C. (●) Control (without MWNT), (▲) 0.23% pristine MWNT, (▼) 1.1% pristine MWNT, (△) 0.23% shortened MWNT, (▽) 1.1% shortened MWNT, and (□) 2.7% shortened MWNT.

specific activities of lipases coimmobilized with shortened MWNT increased with increasing CNT content, although the activities were less than pristine MWNT-containing immobilized lipases. Lower activity in the lipases coimmobilized with shortened MWNT may be caused by the activity loss due to higher adsorption efficiency of lipase which can induce more undesirable adsorptions and conformational changes, compared with pristine MWNT-containing immobilized lipases. However, more study is needed to understand the lower specific activity of immobilized lipase with shortened MWNT. In the case of esterification reaction, the specific activities of lipases coimmobilized with shortened MWNT increased with increasing CNT content. Besides, lipases coimmobilized with shortened MWNT showed much higher activity than immobilized lipases containing pristine MWNT. It may be caused by the stabilization effect in organic media due to high adsorption efficiency of lipase onto shortened MWNT.

3.3. Operational stability of immobilized lipases

Fig. 3 shows the operational stability of immobilized lipase in the esterification at 40 °C. The lipase coimmobilized with MWNT had extremely high stability after reuse. After 5 times reuse, residual activity of lipase coimmobilized with 2.7% shortened MWNT was 96% of initial activity, while the lipase immobilized without MWNT was fully inactivated. Both activity and stability of the lipases coimmobilized with shortened MWNT were higher than those of pristine MWNT-containing immobilized lipases. Although lipase coimmobilized with 1.1% pristine MWNT showed the highest activity in the hydrolysis reaction, the activity and stability in the esterification reaction were lower than others. With increasing shortened MWNT content in the immobilized lipase, higher activity and stability could be obtained in the esterification reaction. It means that shortened MWNT is very useful additive to enhance the activity and stability of immobilized lipase prepared by sol–gel process. For further study, the effect of MNWT length and functionalization on the sol–gel immobilized lipase will be investigated in detail.

4. Conclusions

The activity and stability of immobilized lipase in hydrolysis and esterification reaction can be increased by using MWNTs as additives. Especially, purified and shorten MWNT treated by strong acid was very useful additive in sol–gel immobilization to be applied for the esterification using organic solvents as reaction media. Enhanced activity and stability of lipase immobilized with shortened MWNT may be induced by the protection for the denaturation of lipase and activation of lipase. However, further studies may be needed to understand the effect of carbon nanotube on immobilized lipase.

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References

[1] Y. Liu, M. Wang, J. Li, Z. Li, P. He, H. Liu, J. Li, Chem. Commun. (2005) 1778.
[2] M.T. Reetz, A. Zonta, J. Simpelkamp, Angew. Chem. Int. Ed. Engl. 34 (1995) 301.
[3] P. Vidinha, V. Augusto, M. Almeida, I. Fonseca, A. Fidalgo, L. Ilharco, J.M.S. Cabral, S. Barreiros, J. Biotechnol. 121 (2006) 23.
[4] A.C. Pierre, Biocatal. Biotransform. 22 (2004) 145.
[5] D. Avnir, T. Coradin, O. Lev, J. Livage, J. Mater. Chem. 16 (2006) 1013.
[6] I. Gill, A. Ballesteos, J. Am. Chem. Soc. 120 (1998) 8587.
[7] M.T. Reetz, P. Tielmann, W. WiesenhÖfer, W. KÖnen, A. Zonta, Adv. Synth. Catal. 345 (2003) 717.
[8] F. Péter, L. Poppe, C. Kiss, E. Szoc-Bíró, G. Preda, C. Zarcula, A. Olteanu, Biocatal. Biotransform. 23 (2005) 251.
[9] J.D. Brennan, D. Benjamin, E. DiBattista, M.D. Gulcev, Chem. Mater. 15 (2003) 737.
[10] J. Wang, M. Gu, J. Di, Y. Gao, Y. Wu, Y. Tu, Bioprocess Biosyst. Eng. 30 (2007) 289.
[11] V.G. Gavalas, R. Andrews, D. Bhattacharyya, L.G. Bachas, Nano Lett. 1 (2001) 719.
[12] J. Lim, P. Malati, F. Bonet, B. Dunn, J. Electrochem. Soc. 154 (2007) A140.
[13] S.H. Lee, T.T.N. Doan, S.H. Ha, Y.-M. Koo, J. Mol. Catal. B: Enzyme 45 (2007) 57.
[14] J. Li, Y. Zhang, Appl. Surf. Sci. 252 (2006) 2944.
[15] Y. Wang, L. Gao, J. Sun, Y. Liu, S. Zheng, H. Kajiura, Y. Li, K. Noda, Chem. Phys. Lett. 432 (2006) 205.
[16] M. Abramic, I. Lescic, T. Korica, L. Vitale, W. Saenger, J. Pigac, Enzyme Microb. Technol. 25 (1999) 522.
[17] V.G. Gavalas, S.A. Law, J.C. Ball, R. Andrews, L.G. Bachas, Anal. Biochem. 329 (2004) 247.
[18] C. Torres, C. Otero, Enzyme Microb. Technol. 19 (1996) 594.
[19] S. Chamorro, J.M. Sánchez-Montero, A.R. Alcántara, J.V. Sinisterra, Biotechnol. Lett. 20 (1998) 499.
[20] S. Shah, K. Solanki, M.N. Gupta, Chem. Cent. J. 1 (2007) 30.