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Amino acid modified chitosan beads: Improved polymer supports for immobilization of lipase from *Candida rugosa*

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ABSTRACT

Amino acid modified chitosan beads (CBs) for immobilization of lipases from *Candida rugosa* were prepared by activation of a chitosan backbone with epichlorohydrin followed by amino acid coupling. The beads were analyzed by elemental analysis and solid state NMR with coupling yields of the amino acids ranging from 15 to 60%. The immobilized lipase on unmodified chitosan beads showed the highest immobilization yield (92.7%), but its activity was relatively low (10.4%). However, in spite of low immobilization yields (15–50%), the immobilized lipases on the amino acid modified chitosan beads showed activities higher than that of the unmodified chitosan beads, especially on Ala or Leu modified chitosan beads (Ala-CB or Leu-CB) with 49% activity for Ala-CB and 51% for Leu-CB. The immobilized lipases on Ala-CB improved thermal stability at 55 °C, compared to free and immobilized lipases on unmodified chitosan beads and the immobilized lipase on Ala-CB retained 93% of the initial activity when stored at 4 °C for 4 weeks. In addition, the activity of the immobilized lipase on Ala-CB retained 77% of its high initial activity after 10 times of reuse. The kinetic data (k_{cat}/K_m) supports that the immobilized lipase on Ala-CB can give better substrate specificity than the unmodified chitosan beads.

1. Introduction

Enzymes are widely used in food, pharmaceutical, and agrochemical industry given their ability to catalyze specific reactions (enantioselective synthesis), reduce unwanted intermediates, and yield products of high purity [1,2]. Specifically, lipases, which catalyze the hydrolysis of esters such as glyceride, are industrially useful enzyme finding wide use in oil processing, production of surfactants, and preparation of enantioselective pharmaceutics [3]. For their industrial applications, immobilized lipases have been specifically studied due to their enhanced stability, easy separation, and reusability [4]. Various immobilization methods and supports have been developed in order to improve their activity [5,6].

Chitosan is a polysaccharide easily obtained by alkaline hydrolysis of chitin and has been used in pharmaceutical fields, medicines, drug delivery carriers, wound dressing materials, and tissue engineering [7]. It is considered to be a suitable support for enzyme immobilization because it is biocompatible, available in various forms (gel, membrane, fiber, and film), non-toxic, and amenable to chemical modifications [8–10]. Previously, we used chitosan beads (CBs) for the immobilization of ω-transaminase, a useful enzyme in the pharmaceutical field [11].

Many studies have already shown chitosan to be a good support for lipase immobilization [12–14], however, immobilized lipase on chitosan supports usually exhibit lower activity than free lipase. Thus, various kinds of chitosan supports have been developed by modification of the chitosan backbone in order to improve activity of the immobilized lipase, which have both hydrophilic surface and hydrophobic lid near the active site. Itoyama et al. [15] developed chitosan beads with various spacers for lipase immobilization. Ye et al. immobilized lipases on chitosan-tethered poly(acrylonitrile-co-maleic acid) hollow fiber membrane [16] and nanofibrous poly(acrylonitrile-co-maleic acid) membranes functionalized with chitosan [17]. Huang et al. reported nanoporous membrane for lipase immobilization [18] and Tang et al. used chitosan nanoparticles for immobilization support [19]. Also, Chiou et al. immobilized lipase to chitosan beads using natural cross-linker [20] and Rodrigues et al. utilized multipoint covalent immobilization for lipase immobilization on chitosan [21]. Tan et al. immobilized lipase on polyvinyl alcohol (PVA)/chitosan composite [22].

Amino acids, the monomeric units of proteins and bio-origin materials, are chiral molecules with a relatively low molecular weight and have various properties due to their various side chains (acidic, basic, hydrophobic, etc.). They have also been used as pseudobiospecific affinity ligands for proteins [23–25]. Therefore, chitosan beads with various properties could be easily prepared by introducing amino acids onto their polymer backbone. We consider

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that amino acids are suitable materials for the preparation of modified chitosan beads with increased lipase-compatibility. Besides, the preparation process is simple and economic, and some amino acids are expected to improve the catalytic performances of the immobilized lipases due to the increased compatibility with the immobilized lipase. Therefore, it is considered that the development of amino acid modified chitosan bead might be valuable for industrial application of lipase.

In this paper, we modified chitosan beads with various amino acids and screened them for the best activity of the immobilized lipases. The immobilized lipases on the amino acid modified chitosan beads showed good thermal stability, storage stability and reusability and, therefore, are suitable for industrial application.

2. Materials and methods

2.1. Materials

Chitosan (MW 40,000–50,000, Deacetylation 98%) was purchased from Dong Bang Chito Bio Co. (Korea). L-Amino acids were purchased from Sigma (USA). Epichlorohydrin, glutaraldehyde solution, Triton X-100, Gum Arabic and *p*-nitrophenyl palmitate (*p*NPP) were purchased from Aldrich (USA). Commercial *Candida rugosa* lipase (Sigma L-1754 Type VII, Lot 074K0685, 57.3 kDa, nominal activity 1410 units/mg solid, one unit will hydrolyze 1 μmol of fatty acid from olive oil in 1 h at pH 7.2 at 37 °C) and bovine serum albumin (BSA) were purchased Sigma (USA). The protein assay dye was purchase from Bio-Rad Laboratories (USA). All other chemicals used in the study were of analytical grade and used without further purification.

2.2. Preparation of chitosan beads

Chitosan beads were prepared using an air atomization spray nozzle (Model MS 8A from Fuso Seiki Co., Ltd., Japan). A 20 g of chitosan (MW 40,000–50,000) was dissolved in 500 mL 2% (v/v) aqueous acetic acid solution. The chitosan solution (4%, w/v) was sprayed with an air atomization nozzle into 2 L of 1.0N sodium hydroxide solution containing 500 mL ethanol. In order to use the nozzle system, a micro tube pump (Model MP-3 from EYELA Co., Japan) and air compressor was used. As soon as the chitosan solution was sprayed, chitosan beads were formed. The beads were then cured in aqueous NaOH for 12 h and washed ten times with distilled water. The washed beads were then used accordingly with a small portion set aside and dried for analysis.

2.3. Modification of chitosan beads with various amino acids

A schematic illustration for the modification of chitosan beads with various amino acids is shown in Fig. 1. Kiyohara et al. reported a method for easy coupling of amino acids on a glycidyl methacrylate polymer using an epoxide ring opening reaction in aqueous solution [26]. In order to introduce the epoxide ring onto the chitosan beads, they were first treated with excess epichlorohydrin. Zeng et al. reported that epichlorohydrin had greater reactivity toward hydroxyl groups over amine groups when reacted with chitosan under mild basic conditions [27]. Thus, the amine groups on the chitosan beads could be saved for the immobilization of lipase. We have selected eight typical amino acids from the naturally occurring 20 according to their typical properties (Gly, Glu, Lys, Leu, Ala, Phe, Ser, Tyr).

Chitosan beads (3 mL) were treated with excess epichlorohydrin (10 equiv.) in 12 mL of 0.1% aqueous NaOH and 3 mL of ethanol for 2 h at 50 °C [27]. The beads were then washed with distilled water until neutral. The epichlorohydrin activated chitosan beads (3 mL) were then treated with selected amino acids (10 equiv.) for 24 h at 50 °C in aqueous NaOH (pH 13) [26]. The beads were then washed with distilled water until neutral condition. The washed wet beads were used in lipase immobilization and the freeze-dried beads were analyzed by elemental analyzer (Flash EA 1112 series, Thermo Electron Co., USA) and 500 MHz ^{13}C Solid State NMR (Bruker, Avance II, Germany).

2.4. Quantification of epoxide groups on epichlorohydrin activated chitosan beads

The amount of epoxide on the chitosan beads was determined by sulfur analysis after the following treatment [28–29]. Epichlorohydrin activated chitosan beads (1 mL) were mixed with 3 mL of 1 M sodium thiosulfate aqueous solution. The mixture was shaken

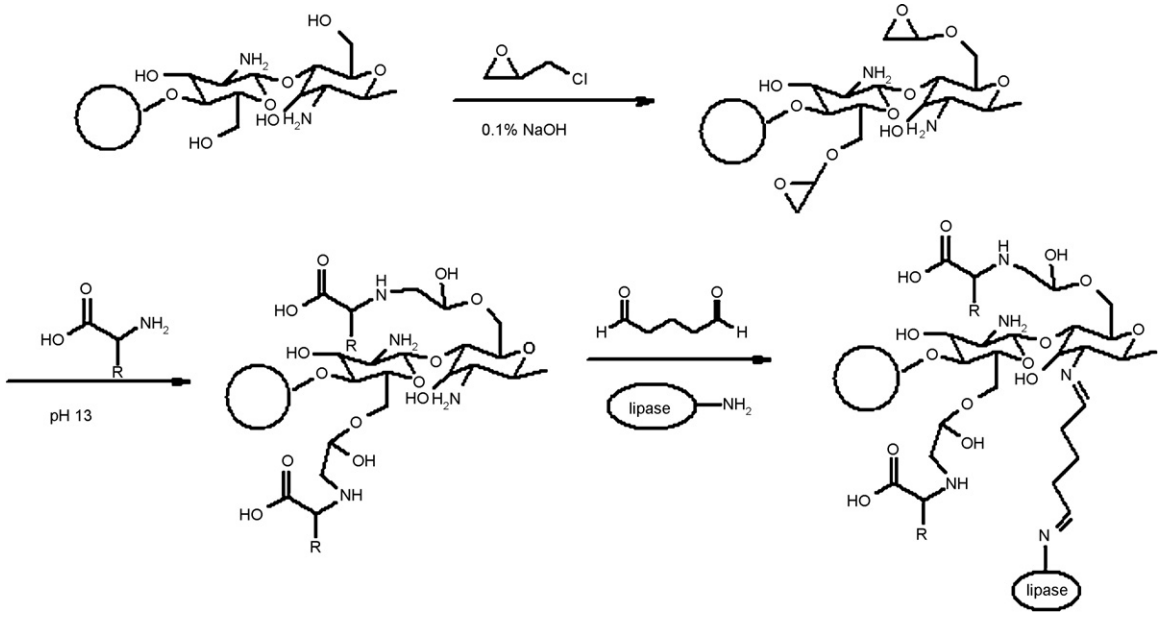

Fig. 1. Schematic illustration for preparation of amino acid modified chitosan beads and immobilization of enzymes.

for 2 h at room temperature. The beads were washed with phosphate buffer solution (PBS) (50 mM, pH 7.0) and reacted with 1 M mercaptoethanol in PBS (50 mM, pH 8.0) for 24 h. After the reaction, the beads were thoroughly washed with buffer and water and freeze-dried. The amount of sulfur on the beads was determined by elemental analysis and converted into the amount of the epoxide.

2.5. Quantification of available primary amine groups on chitosan beads

The amount of the primary amine group on the chitosan beads could be determined by a nitrogen/carbon ratio and the degree of deacetylation. However, the available primary amine groups for chemical reaction are limited due to the diffusion problems of reagents within the beads. Therefore, we have designed the following method to determine the available primary amine groups on chitosan beads. The chitosan beads (3 mL) were treated with of (3-methylthio)propanaldehdye (5 equiv.) in 10 mL of citric acid buffer (50 mM, pH 5.0) at room temperature for 18 h. After the reaction, the beads were washed with buffer (×3), ethanol (×3), and water (×3). Then, the beads were treated with 0.6% $NaBH_4$ solution in 10 mL of boric acid buffer (100 mM, pH 9.0) at room temperature for 18 h. After the reduction step, the beads were thoroughly washed with buffer, ethanol, and water. Finally, the beads were freeze-dried and their sulfur content, which can be converted to the available primary amine groups, was measured by elemental analysis.

2.6. Immobilization of lipase on chitosan beads

Chitosan beads (1 mL) were treated with 4 mL of 2.5% glutaraldehyde solution in phosphate buffer (0.1 M, pH 7.0) for 2 h at 25 °C, and washed with distilled water. Then, the activated beads were incubated with 4 mL of lipase (0.25 mg) solution in phosphate buffer (0.1 M, pH 7.0) for 6 h at 25 °C [11]. Finally, the immobilized lipases on the chitosan beads were filtered and washed with distilled water.

The amount of immobilized protein was determined according to Bradford's method [30] using a Bio-Rad protein assay dye reagent concentrate. Bovine serum albumin was used as the standard.

2.7. Activity test of free and immobilized lipase

The activity of free and immobilized lipase was assayed using hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) [14,31,32]. The reaction mixture consisted of 2.7 mL of 50 mM Tris–HCl buffer (pH 7.0) containing 0.4% (w/v) Triton X-100, 0.1% (w/v) Gum Arabic, and 1 mL of lipase immobilized chitosan bead (or 0.25 mg of free lipase). The reaction was initiated by adding 0.3 mL of *p*NPP in 2-propanol (50 mM) followed by mixing for 5 min at 37 °C. The reaction was terminated by adding 100 mM of aqueous Na_2CO_3 solution into the reaction mixture. The terminated solution was then centrifuged at 13,000 rpm for 10 min. The amount of released *p*-nitrophenol was measured by a UV (Mecasys Co. Ltd., Optizen 2120UV, Korea) spectrophotometer at 410 nm. The activity for immobilized lipase was calculated using a standard calibration curve of *p*-nitrophenol.

One unit (U) of lipase activity was defined as the amount of enzyme necessary to produce 1 μmol of *p*-nitrophenol per min under the assay conditions. Specific activity was defined as the number of enzyme unit per mg of protein. Activity retention was defined as the ratio of the activity of the immobilized enzyme on chitosan beads to the activity of the same amount of free enzyme. Bead activity was refined as the activity of the immobilized enzyme per milliliter of chitosan bead.

2.8. Effect of pH and temperature on the activity of the immobilized lipase

The effect of buffer pH on free and immobilized lipase activities was estimated with reaction mixtures containing 50 mM of the Tris–HCl buffer at different pH values in the range of 7.0–8.7 at 37 °C. The effect of temperature on both lipase activities was determined at temperatures from 37 °C to 60 °C at pH 7.0 (50 mM Tris–HCl buffer) under the same assay conditions.

2.9. Stability and reusability of the immobilized lipase

For determination of thermal stability, either free or immobilized lipase on chitosan beads were incubated in Tris–HCl buffer (50 mM, pH 7.0) at different time intervals (30–120 min) at 50 °C. Samples were assayed for relative activity, assuming an unheated control to be 100% active.

The conditions used to measure the reusability were the same as those used for the enzyme activity assay. After each reuse, the immobilized lipase was filtered and washed with 50 mM Tris–HCl buffer (pH 7.0). The storage stability was estimated by measuring the specific activities of the immobilized enzyme stored at 4 °C.

2.10. Kinetic study

The influence of substrate concentration on hydrolytic activities was analyzed for free and immobilized lipases in *p*-nitrophenyl palmitate solutions varying from 200 to 1500 μM at pH 7.0 (50 mM Tris–HCl buffer) and 37 °C. In all cases, enzyme activity was measured as the initial reaction rate (0–5% hydrolysis) to avoid the inhibition that might take place owing to the reaction products.

3. Results and discussion

3.1. Characterization of amino acid modified chitosan beads

Chitosan beads were prepared using an air atomization spray nozzle method and the average size of wet chitosan bead was about 1 mm. Introduction of the epoxide ring onto the beads was achieved through treatment of the bead with excess epichlorohydrin under mild basic conditions, yielding an epoxide group loading of 1.25 mmol/g chitosan. After treatment with epichlorohydrin, the amount of primary amine remaining from the original 2.87 mmol/g, was 2.43 mmol/g. From this, we confirmed that most of the available primary amine groups remained intact under the mild basic conditions. Eight kinds of amino acids were introduced to the epichlorohydrin activated chitosan beads *via* ring opening reactions under basic conditions. The loading amounts of each amino acid and the coupling yields in the amino acid modified chitosan bead were measured by elemental analysis and are summarized in Table 1. Phe showed the lowest amino acid loading (0.16 mmol/g) and coupling yield (15.1%). Other amino acids showed 0.34–0.60 mmol/g of loading and 30–57% of coupling yields. The coupling yields were similar or relatively higher than those previously reported, with exception of Phe and Glu [26]. For further analysis, ^{13}C CP-MAS NMR analyses were performed on the chitosan beads, the epichlorohydrin activated chitosan beads, and the Phe coupled chitosan beads (Phe-CB) (Fig. 2). Fig. 2(A and B) shows the general chitosan peaks of C-1–C-6 while Fig. 2(C) shows a typical aromatic carbon at 128.6 ppm, confirming that the Phe was successfully introduced onto the epichlorohydrin activated chitosan bead.

Table 1
Elemental analysis and amino acid density of modified chitosan beads

	C (%)	H (%)	N (%)	Amino acid density (mmol/g)[b]	Coupling yield (%)[c]
Gly-CB[a]	44.01	6.73	7.26	0.59	51.5
Glu-CB	44.28	6.46	6.82	0.60	56.6
Lys-CB	45.06	7.12	7.78	0.46	43.2
Leu-CB	45.11	7.02	6.90	0.59	55.1
Phe-CB	45.99	6.85	6.92	0.16	15.1
Ser-CB	44.01	6.43	6.95	0.34	30.6
Tyr-CB	45.62	6.34	6.77	0.38	37.2
Ala-CB	44.09	6.70	7.20	0.42	37.5

[a] Amino acid modified chitosan bead (AA-CB).
[b] Calculated from elemental analysis of amino acid modified chitosan bead (AA-CB).
[c] Coupling yield (%) = ((mmol of amino acid/g chitosan bead)/(mmol of epoxide ring (1.25 mmol)/g chitosan bead)) × 100.

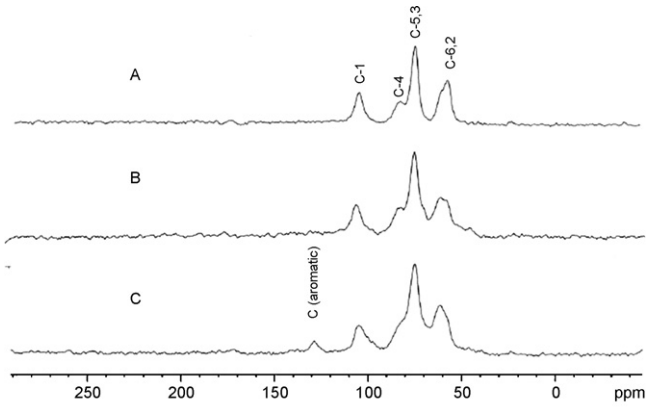

Fig. 2. ^{13}C CP-MAS Solid State NMR spectra of chitosan and modified chitosan: (A) unmodified chitosan bead (CB); (B) epichlorohydrin activated chitosan beads; (C) Phe-CB.

3.2. Lipase immobilization on amino acid modified chitosan beads

The properties of the lipases immobilized on various amino acid modified chitosan beads are summarized in Table 2. Chitosan beads gave the highest loading of lipase (231.9 ± 14.3 μg/mL, 92.7 ± 5.7%) due to the high density of amine groups. Loading yields of lipases on all the amino acid modified chitosan beads were below 53%, very low compared to those of unmodified chitosan beads and most likely due to the fact that the microenvironment of the polymer backbone was altered after the amino acid loadings through the *N*-terminal, even though the amounts of available primary amine groups remained almost the same. Thus, coupling of amino acids to the chitosan beads has introduced negative charges as well as hydrophilicity/hydrophobicity to the microenvironment of the polymer backbone. However, after the amino acid coupling, the formation of ionic complex between the amine groups of chitosan bead and the carboxylic groups of amino acid might prevent effective immobilization of the lipase, even though the amounts of amine groups were increased when Lys was coupled. Among the AA-CBs, Lys-CB showed the highest loading of lipase (130.4 ± 11.3 μg/mL, 52.1 ± 4.5%) as shown in Table 2. It is reasonable from the fact that Lys-CB has more anchoring sites than others due to the amine side chain of lysine. Table 2 also showed that Phe-CB and Tyr-CB gave higher loading of lipase (110.5 ± 3.3 μg/mL for Phe-CB, 114.8 ± 2.6 μg/mL for Tyr-CB) than others. However, the reason for higher loadings is not clear and further study is required to understand the results.

The activity of the immobilized lipase was assayed by the hydrolysis reaction for *p*-nitrophenyl palmitate (*p*NPP). The activities of free and immobilized lipases are summarized in Table 2. In spite of high lipase loading yields, the immobilized lipase on unmodified chitosan beads had the lowest specific activity and activity retention (0.84 ± 0.03 U/mg, 10.4 ± 0.5%), indicating that coupling of the amino acids on chitosan beads provided the immobilized lipase a more compatible microenvironment for its activity. As expected, we confirmed that such a microenvironment could be easily modified for the best of lipase activity through introduction of amino acids as they have various side chains bearing different properties.

The immobilized lipases on Leu-CB and Ala-CB showed activity retentions (51.2 ± 14.8%, 49.6 ± 9.9%) higher than other amino acid modified chitosan beads. Thus, the hydrophobic alkyl side chain of each amino acid improved the activity of the immobilized lipase. It is known that the active site of lipase is surrounded by a hydrophobic region [33,34] and thus the hydrophobic alkyl side chains of the amino acids on the chitosan beads may assist the hydrophobic substrate in accessing the active site of the immobilized lipase as the lipase has hydrophobic lids near its active site. It was reported that the interactions between the hydrophobic group on the poly-

Table 2
Loadings and activities of the immobilized lipase on various chitosan beads

Supports	Protein loading (μg/mL support)	Protein loading yield[b] (%)	Bead activity (U/mL support)	Specific activity (U/mg protein)	Activity retention (%)
Gly-CB[a]	41.1 ± 13.2	16.4 ± 5.3	0.16 ± 0.05	2.91 ± 0.84	35.6 ± 9.7
Glu-CB	39.5 ± 17.2	15.8 ± 6.9	0.14 ± 0.04	2.46 ± 0.72	30.1 ± 8.4
Lys-CB	130.4 ± 11.1	52.1 ± 4.5	0.17 ± 0.01	1.12 ± 0.07	13.7 ± 1.1
Leu-CB	41.5 ± 7.6	16.6 ± 3.0	0.22 ± 0.07	4.19 ± 1.23	51.2 ± 14.8
Phe-CB	110.5 ± 3.3	44.2 ± 1.3	0.16 ± 0.01	1.43 ± 0.25	17.5 ± 2.7
Ser-CB	46.2 ± 20.5	18.5 ± 8.2	0.15 ± 0.06	2.26 ± 0.91	27.6 ± 10.8
Tyr-CB	114.8 ± 2.6	45.9 ± 1.1	0.14 ± 0.02	1.11 ± 0.16	13.6 ± 1.8
Ala-CB	50.4 ± 3.5	20.2 ± 1.4	0.24 ± 0.05	4.10 ± 0.88	49.6 ± 9.9
Chitosan beads (CB)	231.9 ± 14.3	92.7 ± 5.7	0.20 ± 0.01	0.84 ± 0.03	10.4 ± 0.5
Free lipase	–	–	–	8.25 ± 0.27	100

[a] Amino acid modified chitosan bead (AA-CB).
[b] Protein loading yield (%) = (mg of immobilized protein (lipase) on 1 mL chitosan bead)/(mg of added protein in 4 mL protein solution) × 100.

mer support and the hydrophobic lid on the lipase could cause the lid opened-form so that the substrate could have an access to the active site of lipase easily [35]. Several studies have reported that hydrophobic moieties such as the octyl chain on polymer support improved the activity of the immobilized lipase [36,37]. For other hydrophobic amino acid modified (Val, Ile) chitosan beads, the immobilized lipase showed similar activity retentions (54.0 ± 2.1% for Ile-CB, 50.8 ± 12.7% for Val-CB). Other hydrophobic amino acid such as Phe-CB revealed relatively low specific activity and activity retention (1.43 ± 0.25 U/mg, 17.5 ± 2.7%). We considered that such result was primarily due to the low coupling yield of Phe on chitosan beads (15.1%). From Table 2, we confirmed that Ala-CB or Leu CB were the best immobilizing support for lipase, and selected Ala-CB for further tests, because its bead activity and protein loading yield were higher than Leu-CB.

3.3. Effect of pH and temperature on the activity of the free and the immobilized lipase

The effect of pH on the activity of the free and the immobilized lipases on Ala-modified chitosan beads (Ala-CB) was investigated in the pH range of 7.0–8.7 (Tris–HCl buffer range) at 37 °C. The result is shown in Fig. 3. For free enzyme, the maximum activity was observed at pH 7.0, while it was shifted to pH 7.2 after immobilization on unmodified chitosan beads and pH 7.5 after immobilization on Ala-CB. It has been reported that free lipases from *C. rugosa* have maximum activity at between pH 7.0 and 8.0 in several literatures [14,16,38]. The shift of optimal pH of the immobilized lipase has been reported for different lipase sources and support types [5,39]. Generally, immobilized lipases on polycationic supports shift its pH optimum to an acidic side [16,39,40]. In our case, both the immobilized lipases shifted it to a basic side. For the unmodified chitosan beads, the result might be ascribed to the fact that glutaraldehyde crosslinking during immobilization step reduces positive charges of chitosan and enzyme. Similar results were reported for immobilization of lipase [41,42]. The immobilized lipase on Ala-CB gave more basic shift (pH 7.5) than unmodified chitosan beads as shown in Fig. 3. These results may be attributed to the carboxyl groups of amino acids on Ala-CB, which has led to the pH optimum shifted towards more basic side. These data support that Ala-CB has more negatively charge than unmodified chitosan beads. Similar cases on cationic supports have been reported [14,15,38]. Furthermore, we found that the immobilized lipases retained a relatively higher activity in a basic pH range than free lipase (Fig. 3), suggesting that the stability of the immobilized lipase was higher than that of free lipase at a basic pH.

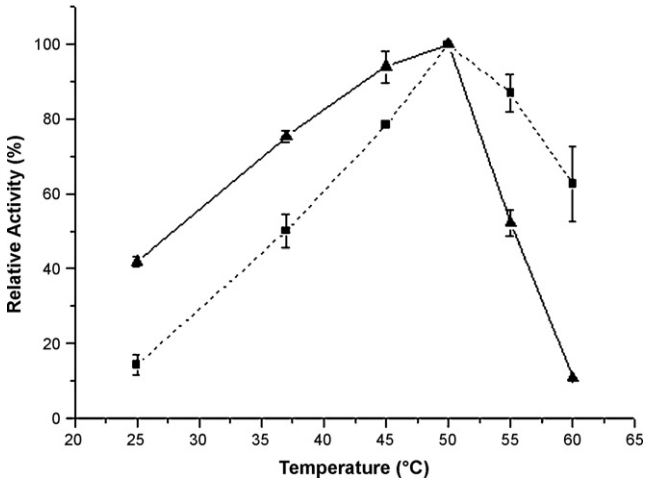

Fig. 4. Effect of temperature on the activity of free and immobilized lipase on Ala-CB: free lipase (▲), the immobilized lipase on Ala-CB (■).

The effect of temperature on the activity of the free and immobilized lipases on Ala-CB was investigated in the temperature range of 25–60 °C at pH 7.0 (Fig. 4). It was found that both free and immobilized lipase showed the highest relative activity at 50 °C. However, the immobilized lipase on Ala-CB exhibited higher relative activities above 50 °C than the free one, indicating that the immobilized lipase on Ala-CB was more stable at higher temperatures than the free lipase. Several, similar cases have also been previously reported [43,44].

3.4. Thermal stability of the free and the immobilized lipase

The thermal stabilities of free and immobilized lipase are shown in Fig. 5. At 55 °C, the relative activity of the free lipase decreased 43% from its initial activity (Fig. 5C), while the immobilized lipase on chitosan beads maintained 67% of its initial activity after 120 min (Fig. 5B). The immobilized lipase on Ala-modified chitosan beads retained 82% of the initial activity after 120 min (Fig. 5A). Generally, the immobilized enzymes exert higher thermal stability than the free enzyme due to the slow denaturizing process. The immobilized lipase on Ala-CB was likely less denaturized than that on

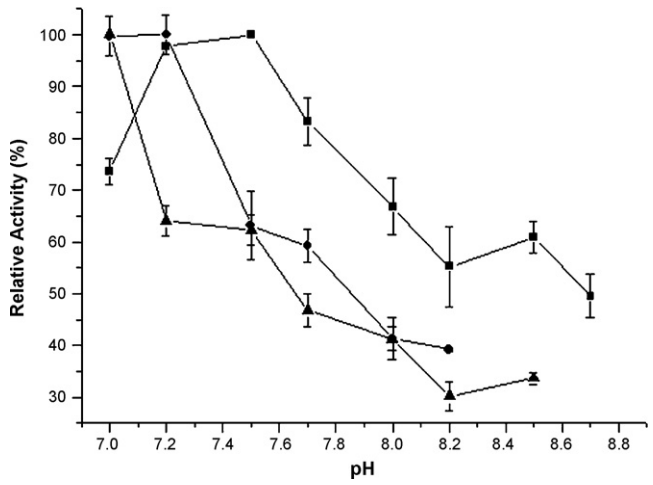

Fig. 3. Effect of pH on the activity of free and immobilized lipases on chitosan beads: free lipase (▲), immobilized lipase on Ala-CB (■), immobilized lipase on CB (●).

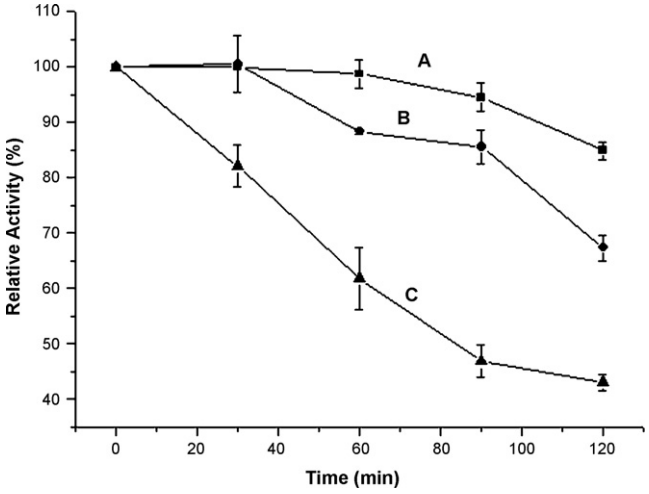

Fig. 5. Thermal stability of free and immobilized lipase at 55 °C; immobilized lipase on Ala-CB (A, ■), immobilized lipase on CB (B, ●), free lipase (C, ▲).

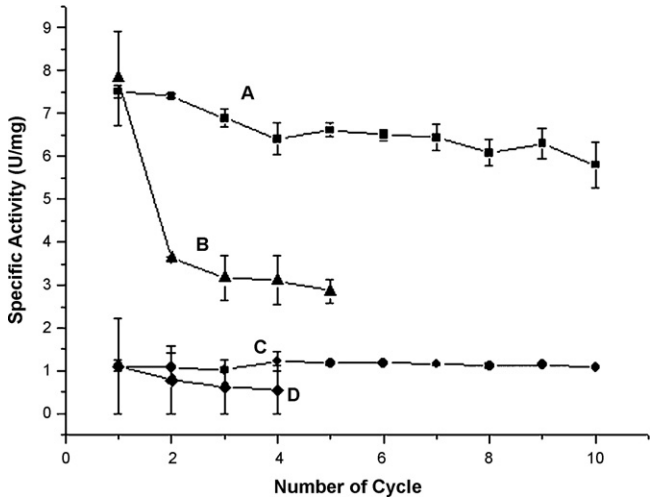

Fig. 6. Reusability of the immobilized lipases: on Ala-CB washed with 1% Triton X-100 and 10% 2-propanol in Tris–HCl buffer in each reuse step (A, ■), on Ala-CB washed with only Tris–HCl buffer (B, ▲), on CB beads washed with 1% Triton X-100 and 10% 2-propanol in Tris–HCl buffer in every reuse step (C, ●), on CB washed with only Tris–HCl buffer (D, ♦).

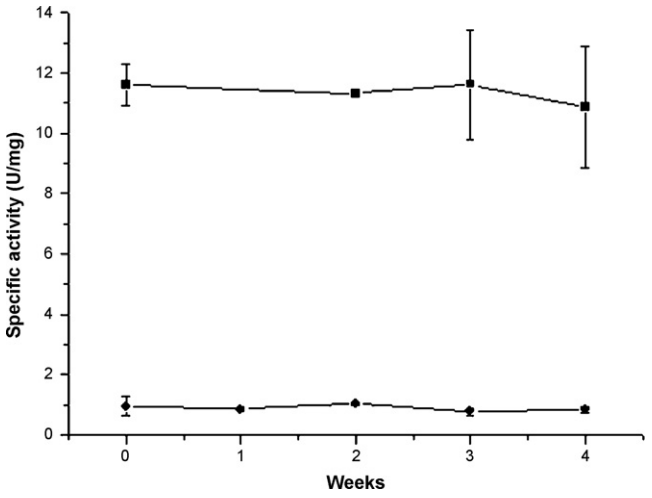

Fig. 7. Storage stability of the immobilized lipases at 4 °C: on Ala-CB (■), on CB (●).

unmodified chitosan beads at high temperatures, owing to a more compatible microenvironment of the polymer backbone (Fig. 5).

3.5. Reusability and storage stability of the immobilized lipase

The reusability of the immobilized lipase was tested up to 10 cycles. Two kinds of chitosan beads, Ala-CB and unmodified chitosan beads were used. The immobilized lipases on each chitosan beads were washed with 50 mM of Tris–HCl buffer (pH 7.0) after each run. During the experiments, all the immobilized lipases showed a steep decline in activity after each cycle (Fig. 6B and D). For CB, the activity of the immobilized lipase decreased up to 50% of the initial activity after 4 uses. For Ala-CB, the activity decreased up to 37% after 5 uses. We assumed that when only buffer solution was used for washing, the product and the unreacted substrates were not thoroughly removed from the chitosan beads and thus inhibited the enzyme reaction. Therefore, in order to improve the deactivation problem, the chitosan beads were thoroughly washed with 1% Triton X-100 and 10% 2-propanol in 50 mM of Tris–HCl buffer (pH 7.0) after each run because it was considered that the surfactant and 2-propanol might help the removal of the hydrophobic substrate (*p*NPP) and the product (palmitic acid) from the beads. Additionally, it has been reported that Triton X-100 is a compatible surfactant for lipase [45]. The results after changing washing solvent are excellent and are shown in Fig. 6. The immobilized lipase on Ala-CB retained 77% of its high initial activity after 10 uses (Fig. 6A). For CB, the immobilized lipase retained 90% of its initial activity after 10 uses (Fig. 6C). Therefore, we confirmed that the washing procedure was an important factor for maintaining the activity of immobilized lipase.

The storage stability of the immobilized lipase was measured at 4 °C for 4 weeks (Fig. 7). After 4 weeks, the specific activity of the immobilized lipase on Ala-CB retained 93% of its initial activity, while that of the immobilized lipase on CB retained 83% of its initial activity. The immobilized lipase on Ala-CB showed better storage stability maintaining high specific activity.

3.6. Kinetics parameters

The kinetic parameters of the free and the immobilized lipases (K_m^{app} and k_{cat}^{app}) on chitosan beads (Ala-CB, CB) were calculated from Lineweaver–Burk plots (Fig. 8) and compared (Table 3). In this case, the bulk concentration of Triton X-100 was fixed (0.4%), but the concentration of the substrate and the mole fraction of substrate in the micelle were varied [46]. From Table 3, the value of k_{cat}^{app} (8.0×10^3 min^{-1}) for free lipase was higher than that of the immobilized form, but the immobilized lipase on Ala-CB showed a higher k_{cat}^{app} (4.5×10^3 min^{-1}) value than that of unmodified chitosan beads (2.0×10^2 min^{-1}). Considering that the activity of the immobilized lipase on Ala-chitosan at pH 7.0 was lower by about 25% than its pH optimum at 7.5, the k_{cat}^{app} value for Ala-CB at pH 7.5 is expected to be higher. The k_{cat}^{app} values of enzymes are related to the biocompatible environment of enzyme on the beads [16]. Therefore, these data suggest that the Ala-CB allowed a more compatible environment for lipase than the unmodified form. Also, comparing with others [43] of similar conditions, our V_{max}^{app} value for Ala-CB was 4 times higher. The immobilized lipases on Ala-CB and CB revealed 0.61 and 0.66 mM of K_m^{app} values, respectively. These values were slightly lower than that of the free lipase (0.94 mM). A similar case has been reported before by Perez et al. [43] whereby they used the immobilized lipase on cellulignin under the same conditions as ours and have explained this result by suggesting that reagents like glutaraldehyde can modify the interactions between the lipase and the support, causing alterations in the reaction

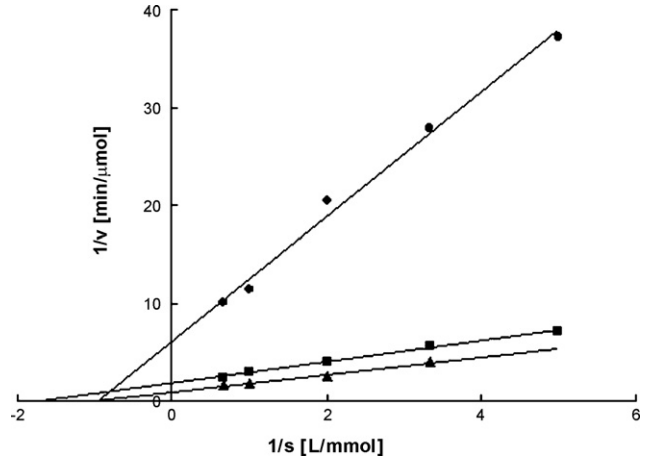

Fig. 8. Lineweaver–Burk plots of free (▲), immobilized lipase on Ala-CB (■) and CB (●).

Table 3
Kinetic parameters of free and immobilized lipases

	k_{cat}^{app} (min^{-1})	K_m^{app} (mM)	k_{cat}^{app}/K_m^{app} (min^{-1} mM^{-1})
Free lipase	8.0×10^3	0.94	8.5×10^3
The immobilized lipase on Ala-modified chitosan beads	4.5×10^3	0.61	7.4×10^3
The immobilized lipase on unmodified chitosan beads	2.0×10^2	0.66	3.3×10^2

rate [43]. In the enzyme reactions requiring surfactant, enzymes have to be activated within a micelle, firstly. Paiva et al. have suggested that the lipase can be activated by the immobilization support, as well as the water/lipid interface [47]. Therefore, we have considered that chitosan or chitosan-glutaraldehyde aided activation of lipase can change the K_m^{app} values of the lipases on chitosan bead. Specificity constant (k_{cat}^{app}/K_m^{app}) of an enzyme is an important parameter for comparing the enzyme catalytic efficiencies for a substrate. Table 3 shows that the specificity constant of the immobilized lipase on Ala-CB (7.4×10^3 min^{-1} mM^{-1}) exhibited twenty times higher than that (3.3×10^2 min^{-1} mM^{-1}) of the immobilize lipase on CB, but slightly lower than that of free lipase (8.5×10^3 min^{-1} mM^{-1}). Thus, the immobilized lipase on Ala-CB revealed lower specificity than free lipase, but higher specificity for substrate (*p*NPP) than the unmodified chitosan beads.

4. Conclusions

Amino acid modified chitosan beads were developed in order to improve the activity of immobilized lipases on chitosan beads. The amino acids were introduced onto the chitosan beads after activation with epichlorohydrin with loading levels confirmed by elemental analysis and structural analysis by solid state NMR. Lipase *from C. rugosa* was immobilized onto the amino acid modified chitosan beads and its activities were assayed. Even though unmodified chitosan beads showed the highest immobilization yield, the activity of the immobilized lipase was low. However, in spite of low immobilization yields, the immobilized lipase on amino acid modified chitosan beads showed higher activities than that of unmodified chitosan beads. Especially the immobilized lipase on Ala coupled chitosan beads (Ala-CB) and Leu coupled chitosan bead (Leu-CB) revealed excellent activity due to the hydrophobic nature of the side chains. The immobilized lipase on Ala-CB showed optimal conditions at 50 °C and pH 7.5 and a higher thermal stability than the free lipase or the immobilized lipase on unmodified chitosan bead at 55 °C. The reusability of the immobilized lipase was tested and it was confirmed that the washing procedure was important. The immobilized lipase showed good storage stability and kinetic properties as well. From the kinetic data, we can conclude that the immobilized lipase on Ala-CB reveal lower specificity than free lipase, but higher specificity for substrate (*p*NPP) than unmodified chitosan beads. We also confirmed that the amino acid modified chitosan beads were good a support for the immobilization of lipase and that they could be applied for immobilization of other useful enzymes.

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References

[1] P. Polaina, A.P. MacCabe, Industrial Enzymes: Structure, Function and Applications, Springer, Dordrecht, The Netherland, 2007.
[2] K. Buchholz, V. Kasche, U.T. Bornscheuer, Biocatalysts and Enzyme Technology, Wiley-VCH Verlag GmbH, Weinheim, 2005.
[3] P. Woolley, S.B. Petersen, Lipases: their Structure Biochemistry and Application, Cambridge University Press, 1994.
[4] J.M. Woodley, Solid Supports Catal. Org. Synth. 71 (1992) 254–271.
[5] V.M. Balcâo, A.L. Paiva, F.X. Malcata, Enzyme Microb. Technol. 18 (1996) 392–416.
[6] K.M. de Lathouder, D.T.J. van Benthem, S.A. Wallin, C. Mateo, F. Fernandez Lafuente, J.M. Guisan, F. Kapteijn, J.A. Moulijn, J. Mol. Catal. B-Enzym. 50 (2008) 20–27.
[7] M.N.V. Ravi Kumar, R.A.A. Muzzarelli, C. Muzzarelli, H. Sashiwa, A.J. Domb, Chem. Rev. 104 (2004) 6017–6084.
[8] B. Krajewska, Enzyme Microb. Technol. 35 (2004) 126–139.
[9] A. Dinçer, A. Telefoncu, J. Mol. Catal. B-Enzym. 45 (2007) 10–14.
[10] G.D. Altun, S.A. Centinus, Food Chem. 100 (2007) 964–971.
[11] S.S. Yi, C. Lee, J. Kim, D. Kyung, B.G. Kim, Y.S. Lee, Process Biochem. 42 (2007) 895–898.
[12] S.H. Chiou, W.T. Wu, Biomaterials 25 (2004) 197–204.
[13] A. Kilinç, M. Teke, S. Önal, A. Telefoncu, Prep. Biochem. Biotechnol. 36 (2006) 153–163.
[14] T.C. Hung, R. Giridhar, S.H. Chiou, W.T. Wu, J. Mol. Catal. B-Enzym. 26 (2003) 69–78.
[15] K. Itoyama, S. Tokura, T. Hayashi, Biotechnol. Prog. 10 (1994) 225–229.
[16] P. Ye, J.K. Xu, A.F. Che, J. Wu, P. Seta, Biomaterials 26 (2005) 6394–6403.
[17] P. Ye, J.K. Xu, J. Wu, C. Innocent, P. Seta, Biomaterials 27 (2006) 4169–4176.
[18] X.J. Huang, D. Ge, Z.K. Xu, Eur. Polym. J. 51 (2007) 3710–3718.
[19] Z.X. Tang, J.Q. Qian, L.E. Shi, Mater. Lett. 61 (2007) 37–40.
[20] S.H. Chiou, T.C. Hung, R. Giridhar, W.T. Wu, Prep. Biochem. Biotech. 37 (2007) 265–275.
[21] D.S. Rodrigues, A.A. Mendes, W.S. Adriano, L.R.B. Gonçalves, R.L.C. Giordano, J. Mol. Catal. B-Enzym. 51 (2008) 100–109.
[22] T. Tan, F. Wang, H. Zhang, J. Mol. Catal. B-Enzym. 18 (2002) 325–331.
[23] G.J. Doellgast, A.G. Plaut, Immunochemistry 13 (1976) 135–139.
[24] M. Kim, S. Furusaki, T. Sato, T. Sugo, I. Ishigaki, K. Saito, J. Chromatogr. 585 (1991) 45–51.
[25] M. Kim, S. Furusaki, T. Sugo, I. Ishigaki, K. Saito, J. Chromatogr. 586 (1991) 27–33.
[26] S. Kiyohara, M. Sasaki, K. Saito, K. Sugita, T. Sugo, J. Membr. Sci. 109 (1996) 87–92.
[27] X. Zeng, E. Ruckesteine, Ind. Eng. Chem. Res. 35 (1996) 4169–4175.
[28] A. Subramanian, A.V. Rau, H. Kaligotla, Carbohydr. Polym. 66 (2006) 321–332.
[29] J.A. Scoble, R.K. Scopes, J. Chromatogr. A 752 (1996) 67–76.
[30] M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[31] U.K. Winkler, M. Stuckmann, J. Bacteriol. 138 (1979) 663–670.
[32] N. Gupta, P. Rathi, R. Gupta, Anal. Biochem. 311 (2002) 98–99.
[33] L. Brady, A.M. Brzozowski, Z.S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J.P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, L. Thim, U. Menge, Nature 343 (1990) 767–770.
[34] U. Derewenda, A.M. Brzozowski, D.M. Lawson, Z.S. Derewenda, Biochemistry 31 (1992) 1532–1541.
[35] R. Ferandez-Lafuente, P. Aemisén, P. Sabuquillo, G. Fernández-Lorente, J.M. Guisán, Chem. Phys. Lipids 93 (1998) 185–197.
[36] J.M. Palomo, G. Muñoz, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente, J.M. Guisán, J. Mol. Catal. B-Enzym. 279 (2002) 19–20.
[37] A. Bastida, P. Sabuquillo, P. Armisen, R. Fernández-Lafuente, J. Huguet, J.M. Guisán, Biotechnol. Bioeng. 58 (1998) 486–493.
[38] F.M. Gomes, E.B. Pereira, H.F. de Castro, Biomacromolecules 5 (2004) 17–23.
[39] B. Krajewska, Z. Piwowarska, Biocatal. Biotransform. 23 (2005) 225–232.
[40] F. Vaillant, A. Millan, P. Millan, M. Dornier, M. Decloux, M. Reynes, Process Biochem. 35 (2000) 989–996.
[41] A. Wyss, U. von Stockar, I.W. Marison, Biotechnol. Bioeng. 93 (2006) 28–39.
[42] J.F. Shaw, R.C. Chang, F.F. Wang, Y.J. Wang, Biotechnol. Bioeng. 35 (1990) 132–137.
[43] V.H. Perez, G.S. da Silva, F.M. Gomes, H.F. de Castro, Biochem. Eng. J. 34 (2007) 13–19.
[44] P. Ye, J. Jiang, Z.K. Xu, Colloid Surf. B-Biointerfaces 60 (2007) 62–67.
[45] J.N. dos Prazeres, J.A.B. Cruz, G.M. Pastore, Braz. J. Microbiol. 37 (2006) 505–509.
[46] J.C. Martin, J.F. Bello, F.J. Burguillo, M.G. Roig, J. Mol. Catal. 93 (1994) 37–52.
[47] A.L. Paiva, V.N. Balcão, F.X. Malcata, Enzyme Microb. Technol. 27 (2000) 187–204.