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ORIGINAL PAPER

Preparation of carboxylated magnetic particles for the efficient immobilization of C-terminally lysine-tagged *Bacillus stearothermophilus* aminopeptidase II

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Abstract This article reports the synthesis and use of surface-modified iron oxide particles for the simultaneous purification and immobilization of *Bacillus stearothermophilus* aminopeptidase II (*Bs*APII) tagged C-terminally with either tri- or nona-lysines (*Bs*APII-$Lys_{3/9}$). The carboxylated magnetic particles were prepared by the simple co-precipitation of Fe^{3+}/Fe^{2+} in aqueous medium and then subsequently modified with adipic acid. Transmission electron microscopy (TEM) micrographs showed that the carboxylated magnetic particles remained discrete and had no significant change in size after binding *Bs*APIIs. Wild-type enzyme and *Bs*APII-Lys_3 could be purified to near homogeneity by the carboxylated magnetic particles, but it was not easy to elute the adsorbed *Bs*APII-Lys_9 from the matrix. Free *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9 were active in the temperature range 50–70°C and all had an optimum of 50°C, whereas the optimum temperature and thermal stability of *Bs*APII-Lys_3 and *Bs*APII-Lys_9 were improved as a result of immobilization. The immobilized *Bs*APII-Lys_9 could be recycled ten times without a significant loss of the enzyme activity and had a better stability during storage than *Bs*APII. Owing to its high efficiency and cost-effectiveness, this magnetic adsorbent may be used as a novel purification-immobilization system for the positively charged enzymes.

Keywords *Bacillus stearothermophilus* · Aminopeptidase II · Magnetic particles · Lysine tag · Immobilization

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Introduction

A catalyst enhances the rate of approach toward the equilibrium of a reaction without being substantially consumed during the reaction. Enzymes are a particularly versatile class of catalysts that are very effective and precise in performing and regulating processes in living matter. They often display high regio- and chemoselectivity while operating under mild conditions. In applied bioprocesses, immobilizing enzymes on insert supports is particularly useful since it can facilitate product separation, biocatalyst reutilization, and sometimes help to improve the stability of biocatalysts. However, the conventional methods for enzyme immobilization are usually problematic and laborious. Prior to immobilization, enzyme purification is usually a prerequisite. To solve this problem, the target proteins are virtually fused to affinity tags to endow them with specific binding ability to their unnatural cognate ligands [1]. This strategy is of great interest for simultaneous purification and immobilization of enzymes if the affinity ligands can also serve as the immobilization matrix, and offers several advantages including strong and reversible binding of enzymes to the support, proper exposure of the enzyme

active site, and the lack of substrate diffusion barriers [2]. Immobilization of enzymes on magnetic particles imparts potentially unique properties. The easy dispersibility of the particles facilitates contact with the substrate and therefore alleviates mass-transfer limitations. In this regard, a number of enzymes including glucoamylase [3], chymotrypsin [4], papain [5], D-amino acid oxidase [6], and γ-glutamyltranspeptidase [7] have been successfully immobilized on magnetic particles.

The adsorption of enzymes onto supports can proceed via different types of interactions [8]. The advantage of immobilization via noncovalent adsorption is that the enzyme does not have to be pretreated and crude enzyme preparations can be used directly for these immobilizations. Because it is simple, not expensive, and retains high catalytic activity [9, 10], noncovalent adsorption is a particularly beneficial approach for enzyme immobilization. Essentially, any charged matrix can act as support in immobilization via ionic interaction. Depending on the predominant charge on the enzyme, the ion supports need to be positively (for instance protonated amino groups) or negatively charged (for instance carboxylate) [8]. In this regard, a positively charged chitosan film has been used for the immobilization of tyrosinase [11]. The anionic COOH-terminated monolayer was also subjected to cytochrome *c* immobilization [12]. Additionally, the economical and practical issues like the number of unit operations needed and the time-saving due to a reduction in chromatographic steps make this approach more relevant for industrial applications.

Aminopeptidases are a group of exopeptidases that catalyze the removal of amino acids from amino-termini of proteins and peptides, and are associated with many biological functions such as protein maturation and degradation, hormone level regulation, and plant defense response [13–15]. From the view point of industrial applications, aminopeptidases are useful for improving the bitter off-taste of protein hydrolysates [16] and for transforming L-homophenylalanyl amide to L-homophenylalanine, the versatile intermediate for a class of angiotension I-converting enzyme inhibitors [17]. We previously reported the functional expression of *Bacillus stearothermophilus* aminopeptidase II (*Bs*APII) in recombinant *Escherichia coli* cells [18]. The recombinant enzyme consists of two identical 44.5-kD subunits and shows a marked preference for leucine-*p*-nitroanilide (Leu-*p*-NA). *Bs*APII is sensitive to oxidative damage by hydrogen peroxide, which leads to the disassociation of its dimeric characteristics [19]. To identify residues essential for the catalytic activity of *Bs*APII, we performed site-directed mutagenesis of the conserved glutamate and histidine residues [20, 21]. Since the leucine replacements of Glu250, Glu316, Glu340, His345, and His378 made the enzyme inactive, it is presumed that these five residues are important for the proper function of the enzyme. In the present study, the COOH-terminated magnetic particles were prepared by suspension polymerization of magnetic particles (Fe_3O_4) and adipic acid. The carboxylated magnetic particles were used for single-step purification and immobilization of *Bs*APIIs. The immobilized enzymes, especially *Bs*APII-Lys_9, could be recovered magnetically and used repeatedly. Also, thermal and storage stabilities of the immobilized *Bs*APII-Lys_9 were greatly improved.

Materials and methods

Chemicals

Iron(II) chloride tetrahydrate, iron(III) chloride tetrahydrate, and adipic acid (AA) were acquired from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). The enzyme assay reagents, Leu-*p*-NA, and *p*-nitroaniline (*p*-NA), were obtained from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). Unless indicated otherwise, all other chemicals were commercial products of analytical or molecular grade.

Preparation of cell-free extracts

To produce *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9, *Escherichia coli* M15 cells harboring pQE-LAPII [18], pQE-LAPII-Lys_3 [22], or pQE-LAPII-Lys_9 [22] were grown at 37°C in 200-ml Luria–Bertani medium supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin to an absorbance at 600 nm of 1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the incubation was continued at 28°C for 12 h. Cells were then harvested by centrifugation and resuspended in 10 ml of 50 mM Tris–HCl buffer (pH 8.0). After homogenization, cells were disrupted on ice by sonication (100 W, 30-s bursts for 5 min) and a centrifugation step (12,000*g*, 30 min) allowed the recovery of a clear supernatant containing the soluble enzymes. The cell-free extract was subsequently filtered on a 0.45-μm membrane (Millipore Corporation, Bedford, MA, USA) and then the enzyme extract was used for one-step purification and immobilization by the carboxylated magnetic particles.

Enzyme assay

Enzyme activity was determined at 50°C as described elsewhere [23] and the formation of *p*-NA was recorded by monitoring the absorbance changes at 405 nm. The reaction mixture (500 μl) contained 2 mM Leu-*p*-NA, 50 mM Tris–HCl (pH 8.0), 1 mM $CoCl_2$, and an appropriate amount of enzyme solution. One unit of aminopeptidase activity is

defined as the amount of enzyme that releases 1 μmol of *p*-NA per min at 50°C.

Synthesis of COOH-terminated magnetic particles

Magnetic particles (Fe_3O_4) were prepared by hydrothermal co-precipitation of ferric and ferrous salts in an alkaline solution followed by washing in hot water [24]. Typically, iron(II) chloride and iron(III) chloride (1:2) were dissolved in nanopure water at a concentration of 0.25 M iron ions and chemically precipitated at room temperature by repeatedly adding 1 M NaOH to maintain a constant pH of 10. The precipitates were heated at 80°C for 35 min under continuous mixing and washed four times in water and several times in ethanol. During washing, the magnetic particles were separated from the washing liquid using a magnetic separator of strength greater than 20 megaoersteds. The particles were finally dried in a vacuum oven at 70°C.

To prepare Fe_3O_4-AA, 5 g Fe_3O_4 was added to 50 ml of 0.1 M AA solution in a round-bottom flask and the contents were then adjusted to pH 2.0 by adding nitric acid. After ultrasonication for 20 min, the suspension was stirred under a nitrogen atmosphere overnight at room temperature. The resulting particles were concentrated by the permanent magnet and washed routinely with deionized water to remove the residual AA.

Adsorption and elution of *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9

Adsorption of *Bs*APII and the lysine-tagged enzymes onto the carboxylated magnetic particles was carried out under native conditions. Briefly, 10 ml of the enzyme extract was incubated with 1.2 g magnetic particles pre-equilibrated with binding buffer (5 mM imidazole, 100 mM NaCl, and 50 mM Tris–HCl; pH 8.0) by slow end-over-end rotation for 1 h at 4°C. The particles were collected magnetically and washed twice with 10 ml of binding buffer. The immobilized enzymes were either stored in the same buffer at 4°C until use or eluted from the matrix using 4 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 300 mM imidazole and 500 mM NaCl.

Characterization of the magnetic particles

The morphology and average size of the magnetic particles were observed by transmission electron microscopy (TEM) using a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan). The sample for TEM analysis was obtained by placing a drop of the solution of magnetic particles dispersed in ethanol onto a Formvar covered copper grid and evaporated in air at ambient temperature. For each sample, over 100 particles from different parts of the grid were used to estimate the mean diameter of the magnetic particles.

The binding of *Bs*APIIs onto the carboxylated magnetic particles was checked by using FTIR spectra with KBr discs in the range of 2,000–500 cm^{-1} on a Shimadzu FTIR-8400 spectrometer (Shimadzu Corporation, Kyoto, Japan).

Gel electrophoresis and determination of protein concentration

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out with 4% polyacrylamide stacking and 12% polyacrylamide separating gels. Prior to electrophoresis, the samples were heated at 100°C for 5 min in dissociating buffer containing 2% SDS and 5% 2-mercaptoethanol. The gels were stained with 0.25% Coomassie brilliant blue dissolved in 50% methanol–10% acetic acid and then destained in a 30% methanol–10% acetic acid solution.

Protein concentration was determined with Bio-Rad protein assay reagent and bovine serum albumin as the standard.

Effects of pH and temperature on the immobilized enzymes

The effect of temperature on the immobilized enzymes was also evaluated by incubating *Bs*APII-, *Bs*APII-Lys_3-, and *Bs*APII-Lys_9-conjugated magnetic particles (1.2 g; wet weight) with 10 ml of 50 mM Tris–HCl buffer (pH 8.0) at various temperatures (30–80°C) for 10 min. The amount of aminopeptidase activity was determined according to the assay method described above. The thermal stability of the immobilized enzyme was performed in 10 ml of 50 mM Tris–HCl buffer (pH 8.0) at different temperatures (30–70°C). After 30 min incubation, the residual activity was determined under the standard assay conditions. The experiments were performed in triplicate and the data were expressed as mean values.

To investigate the pH effect on the immobilized enzyme, *Bs*APII-, *Bs*APII-Lys_3-, and *Bs*APII-Lys_9-conjugated magnetic particles (1.2 g; wet weight) were incubated with 10 ml of 50 mM Tris–maleate buffer (pH 4.5–6), 50 mM potassium phosphate buffer (pH 6–8), 50 mM Tris–HCl buffer (pH 8–9), or 50 mM glycine–NaOH buffer (pH 9–11) for 30 min at room temperature, and the amount of aminopeptidase activity was determined according to the assay conditions.

Reusability of the immobilized enzymes

The immobilized enzymes were repeatedly used to hydrolyze Leu-*p*-NA in batch reactions. Enzyme-conjugated magnetic particles (0.12 mg) in 1 ml of 50 mM Tris–HCl

buffer (pH 8.0) containing 2 mM Leu-*p*-NA and 1 mM $CoCl_2$ were shaken (100 rpm) at 50°C for 10 min each time. The activity was determined under the standard assay conditions. After each cycle of reaction, the enzyme–matrix complex was washed with 1 ml of 50 mM Tris–HCl buffer (pH 8.0) and reused for another run. The experiments were performed in triplicate and the data were expressed as mean values.

Storage stability

The operational stability was assessed for the immobilized enzymes (approximately 6 mg for each enzyme; wet weight) during storage in 50 ml of 20 mM Tris–HCl at 4°C. At a specific time interval, aliquots were withdrawn to determine the aminopeptidase activity under the standard assay conditions. The experiments were performed in triplicate and the data were expressed as mean values.

Results and discussion

Adsorption of the engineered enzymes

In our previous studies, three expression plasmids (pQE-LAPII, pQE-LAPII-Lys_3, and pQE-LAPII-Lys_9) were constructed for the heterologous production of *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9 in *E. coli* [18, 22]. The mRNAs of these constructs encode a His_6 tag at their N-termini. Except for that, there is a lysine tag at the C-terminal ends of *Bs*APII-Lys_3 and *Bs*APII-Lys_9. In this study, *E. coli* M15 cells harboring each of the expression plasmids were induced under the conditions described in the "Materials and methods" to overproduce these three enzymes. Analysis of the cell-free extracts of the recombinant *E. coli* cells revealed a predominant protein with apparent M_r of approximately 44.5 kDa in each extract (Fig. 1). The specific activity for the cell-free extracts of IPTG-induced *E. coli* (pQE-LAPII), *E. coli* (pQE-LAP-Lys_3), and *E. coli* (pQE-LAPII-Lys_9) was 16.42, 21.71, and 18.22 U/mg protein, respectively.

To ascertain the time required to reach adsorption equilibrium of the *Bs*APII enzymes, binding experiments were performed at 4°C and the adsorption efficiency was defined as the percentage of the adsorbed activity relative to that added initially. The results showed that more than 61% of the enzyme activity had been adsorbed onto the magnetic particles after 10-min incubation (Fig. 2). The time necessary to achieve the adsorption equilibrium was around 60 min, which was mostly attributed to the rapid diffusion of *Bs*APIIs to the matrix and interactions between the enzymes and the carboxylated magnetic particles. After 60-min incubation, about 15–17% of the protein molecules in the crude extracts were bound to the carboxylated magnetic particles. Based on the adsorbed activity and the wet weight of the matrix, the specific activities for Fe_3O_4-AA/*Bs*APII, Fe_3O_4-AA/*Bs*APII-Lys_3, and Fe_3O_4-AA/*Bs*APII-Lys_3 were estimated to be 15.39 ± 1.24, 27.72 ± 3.45, and 41 ± 5.63 U/g adsorbent, respectively.

The typical TEM micrographs for the magnetic particles without and with bound *Bs*APIIs are shown in Fig. 3. It was clear that the magnetic particles were essentially monodisperse and had a mean diameter of 29.3 ± 6.8 nm. After the binding of *Bs*APIIs, the particles remained discrete and had a mean diameter (30.6 ± 5.6 nm for Fe_3O_4-AA/*Bs*APII-Lys_3 and 31.2 ± 6.7 nm for Fe_3O_4-AA/*Bs*APII-Lys_9) similar to that of the unbound ones. These observations reveal that the adsorption process did not significantly change the size of the magnetic particles. The binding of *Bs*APIIs to the matrix was also confirmed by FTIR analysis. Figure 4 shows the FTIR spectra of naked Fe_3O_4, Fe_3O_4-AA, and Fe_3O_4-AA-bound *Bs*APIIs. For the naked Fe_3O_4, the peak at 569 cm^{-1} relates to Fe–O group. The peak at 1,694 cm^{-1} in the IR spectrum of Fe_3O_4-AA is the typical stretching vibration of the C=O bond from AA. However, the corresponding vibration of the C=O bond completely disappears

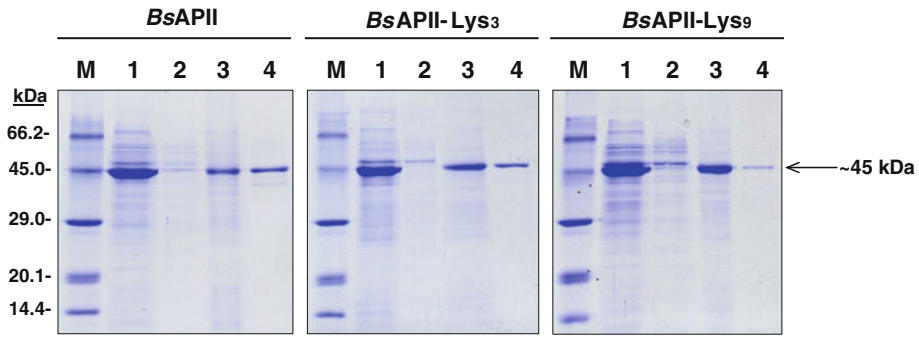

Fig. 1 SDS–PAGE analysis of *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9 tethered on the carboxylated magnetic particles before and after the elution by 50 mM Tris–HCl buffer (pH 8.0) containing 300 mM imidazole and 500 mM NaCl. *Lanes*: *1* the tagged enzymes in the crude extract state, *2* the filtrate, *3* the pellets of *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9 after the elution, *4* the eluted enzymes

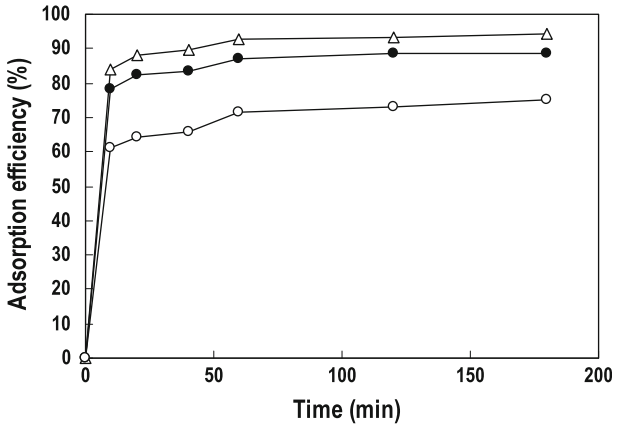

Fig. 2 Time course for the adsorption of *Bs*APII (*open circles*), *Bs*APII-Lys_3 (*closed circles*), and *Bs*APII-Lys_9 (*open triangles*) onto the carboxylated magnetic particles. The adsorption was carried out in a reaction mixture (30 ml) containing 50 mM Tris–HCl buffer (pH 8.0), 3.6 g Fe_3O_4-AA particles and each of the crude extracts at a protein concentration of 450 μg/ml. Results are reported as means ($n = 3$) and the standard deviations were lower than ±3.5%

in the IR spectra of Fe_3O_4-AA-bound *Bs*APIIs. The reason for this disappearance remains to be elucidated. Compared with the spectrum of Fe_3O_4-AA, the characteristic bands appearing at 1,527 and 1,658.93 cm^{-1} in Fe_3O_4-AA-bound *Bs*APIIs can be assigned to NH and NH_2 bending vibrations, respectively. These results indicate that some proteins in the crude extract were successfully coated onto the carboxylated magnetic particles.

Purification of *Bs*APIIs

*Bs*APII and poly-lysine-tagged enzymes in the crude extracts were purified by adsorption-elution on the carboxylated magnetic particles. As shown in Fig. 1, the adsorbed *Bs*APII and *Bs*APII-Lys_3 could be eluted from the matrix by 50 mM Tris–HCl buffer (pH 8.0) containing 300 mM imidazole and 500 mM NaCl. However, *Bs*APII-Lys_9 adsorbed strongly to the matrix and was not easily recovered by the elution buffer. From 10 ml of the crude extract, the purification procedure resulted in a protein yield of 0.21–1.81 mg (Table 1). The specific activity of the purified *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9 was 25.51, 60.71, and 76.82 U/mg protein, respectively. As compared with the crude extract, the significant increase in specific activity indicates that the enzyme purity of *Bs*APII-Lys_3 and *Bs*APII-Lys_9 was greatly improved due to the presence of poly-lysine tag at their C-termini. Principally, the added positive charge, in the form of lysine residues, located at the C-termini of *Bs*APII-Lys_3 and *Bs*APII-Lys_9 allows them to adsorb onto the negatively charged magnetic particles through electrostatic interaction. However, the low selectivity of the prepared matrix did not exclude the adsorption of *Bs*APII and other contaminated proteins (Fig. 1, lane 3), indicating that many proteins in the crude extract are positively charged at the working pH. This reflects the fact that the lysine-tagged enzymes can only be partially purified by the carboxylated magnetic particles.

Effect of temperature on the immobilized enzymes

The effect of temperature on the immobilized enzymes was investigated by using Leu-*p*-NA as substrate (Fig. 5a). The maximal activity of free *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9 appeared at 50°C, but the optimum temperature of the immobilized *Bs*APIIs was shifted to 55°C. Additionally, the immobilized *Bs*APIIs were less sensitive to the change of temperature than the free enzyme when the reaction temperature ranged from 40 to 60°C.

The thermal stability of the free and immobilized *Bs*APIIs was determined by incubating the enzymes in a temperature range of 30–70°C for 30 min and subsequently measuring their residual activities. Figure 5b shows that both free and immobilized *Bs*APII shared a similar profile of thermal stability below 50°C. However, the residual activity of immobilized enzyme was consistently higher than that of the free enzyme when the incubation temperatures were above 50°C. It is believed that surface immobilization of the enzyme on magnetic particles can somehow increase the thermal stability, probably by increasing its molecular rigidity [25], thus preventing any undesirable change in the tertiary molecular structure due to heating. Stability enhancement of immobilized enzymes as a function of temperature has also been observed for other enzymes immobilized on different supports [26–28].

Effect of pH on the immobilized enzymes

The pH dependence of the free and immobilized enzymes in the pH range of 4.5–10.5 was studied at 30°C. As shown in Fig. 6a, the free and immobilized enzymes showed a similar behavior of pH dependence. The maximum activity for free *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9 and their immobilized forms was found at around pH 7.5. A high activity for the immobilized enzymes was kept in the pH range of 6.5–9.5, whereas the native enzymes were more sensitive than the immobilized forms in the same pH range (Fig. 6b). This phenomenon was also reported for the immobilization of *Saccharomyces cerevisiae* alcohol dehydrogenase onto magnetic particles [29]. It was presumed that the configuration of *Bs*APIIs was fixed on the surface of supports and led to an increase in the enzymes' tolerability to pH variance in surroundings. Practically, it would be ideal if the immobilized enzymes could be functional over a wide range of pH values.

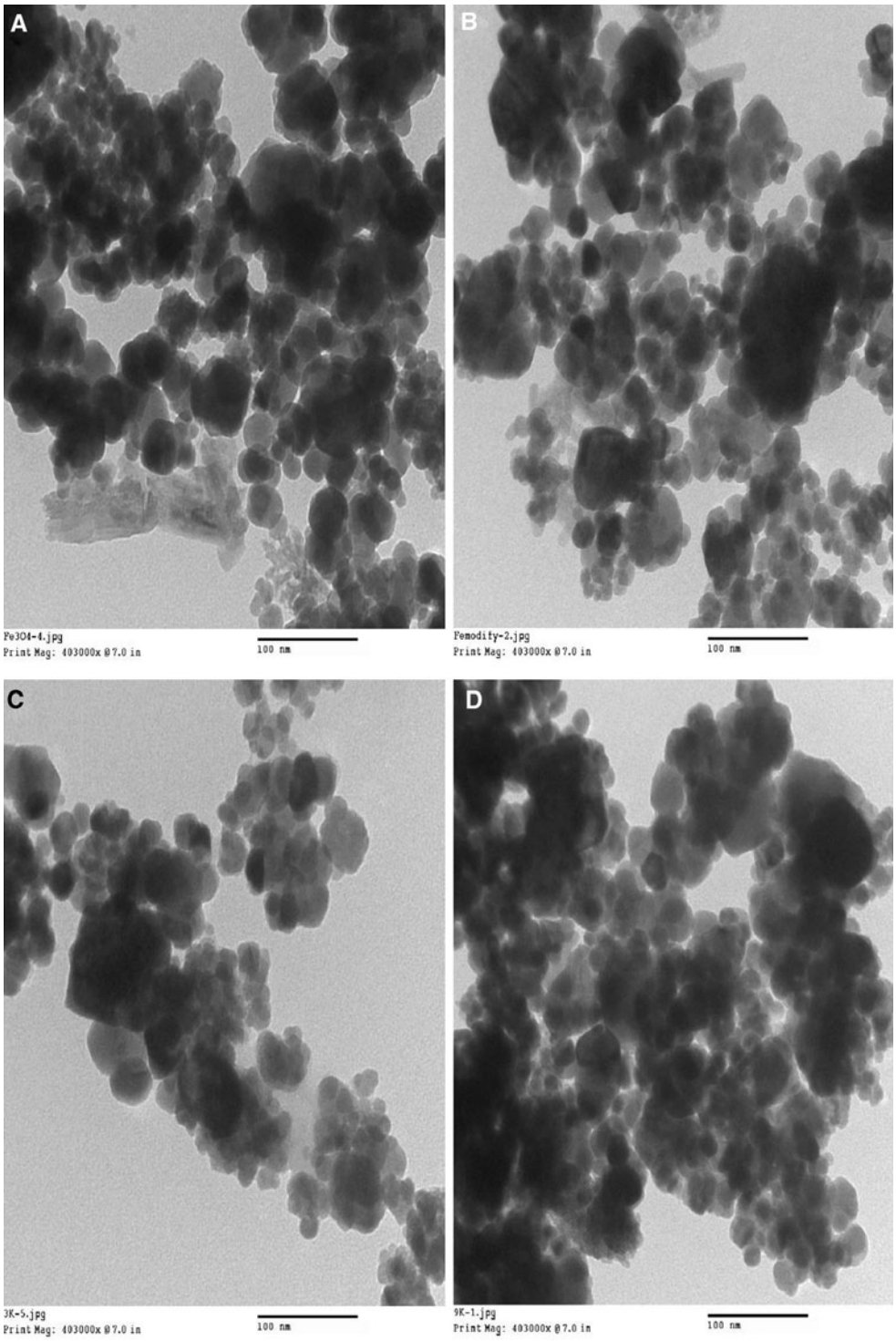

Fig. 3 TEM micrographs of Fe_3O_4, Fe_3O_4-AA, and Fe_3O_4-AA-bound *Bs*APIIs. **a** Naked Fe_3O_4; **b** Fe_3O_4-AA; **c** Fe_3O_4-AA/*Bs*APII-Lys_3; **d** Fe_3O_4-AA/*Bs*APII-Lys_9

Kinetic parameters

In enzyme catalysis, it is important to quantify the efficiency of an enzyme in terms of a number of kinetic parameters. Typically, V_{max} represents the maximum rate of a reaction with enzyme active sites saturated with substrate, and Michaelis–Menten constant, K_M, is equivalent to the substrate concentration at which the rate of conversion is half of V_{max}. Comparison of kinetic parameters will reflect the efficiency and kinetics under different environments. In this regard, kinetic analysis of the free and immobilized enzymes was carried out. The apparent kinetic constants for the free and immobilized enzymes were determined from double-reciprocal plots. As shown in Table 2, V_{max} and K_M

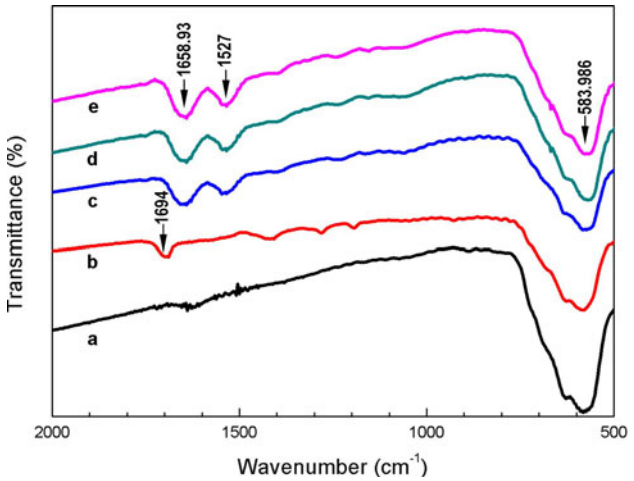

Fig. 4 FTIR spectra of Fe_3O_4, Fe_3O_4-AA, and Fe_3O_4-AA-bound *Bs*APIIs. **a** Naked Fe_3O_4; **b** Fe_3O_4-AA; **c** Fe_3O_4-AA/*Bs*APII; **d** Fe_3O_4-AA/*Bs*APII-Lys_3; **e** Fe_3O_4-AA/*Bs*APII-Lys_9

Table 1 Purification scheme of the engineered enzymes

Step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)
Crude extract					
*Bs*APII	10	4.33	71.09	10.41	1
*Bs*APII-Lys_3	10	4.16	90.36	21.71	1
*Bs*APII-Lys_9	10	4.60	83.81	18.22	1
Fe_3O_4-AA					
*Bs*APII	4	1.81	46.17	15.86	1.5
*Bs*APII-Lys_3	4	1.24	75.31	60.73	2.8
*Bs*APII-Lys_9	4	0.21	16.92	76.91	4.2

values were 0.92–1.02 $nmol^{-1}$ and 127.3–294.1 nmol min^{-1} for free *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9, and 1.23–1.51 $nmol^{-1}$ and 112.8–246.7 nmol min^{-1} for the immobilized enzymes, respectively. The V_{max} value of immobilized enzymes was more than 11.4% lower than that of the free enzymes, indicating that the immobilization process might hinder the substrates' accessibility to the enzyme. These results are consistent with those of other enzymes immobilized onto the magnetic particles [29–31].

Reusability

The duration of a biocatalyst is an important feature when considering its potential application in industry. To investigate the reusability of the immobilized enzymes, several repetitive cycles of the immobilized *Bs*APIIs were operated. At each of the repeated cycles, the immobilized enzymes were recovered by magnetic separation and recycled for the hydrolysis of Leu-*p*-NA. As shown in Fig. 7, the activity of the immobilized enzymes did not decrease significantly during the repeated use, especially the immobilized *Bs*APII-Lys_9. After 10 cycles of usage, the remaining activity of Fe_3O_4-AA/*Bs*APII-Lys_9 was approximately 75% of the first use. These results indicate that the immobilized *Bs*APII-Lys_9 had a good durability and magnetic recovery.

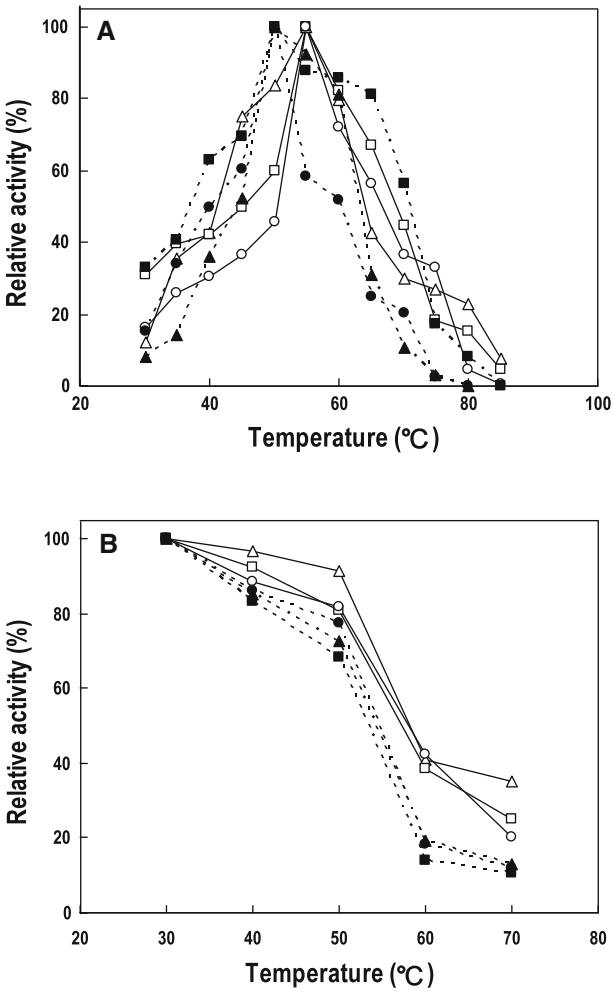

Fig. 5 Effect of temperature on activity (**a**) and stability (**b**) of free and immobilized enzymes. Results are reported as means ($n = 3$) and the standard deviations were lower than ±4.1%. *Closed triangles* free *Bs*APII, *open triangles* immobilized *Bs*APII, *closed squares* free *Bs*APII-Lys_3, *open squares* immobilized *Bs*APII-Lys_3, *closed circles* free *Bs*APII-Lys_9, *open circles* immobilized *Bs*APII-Lys_9

Storage stability

The storage stability of an enzyme plays an important role in biocatalyst-mediated bioprocesses since the economics of industrial bioprocesses are influenced by the enzyme production cost, which accounts for more than half of the overall production cost. Therefore, an enzyme that has long

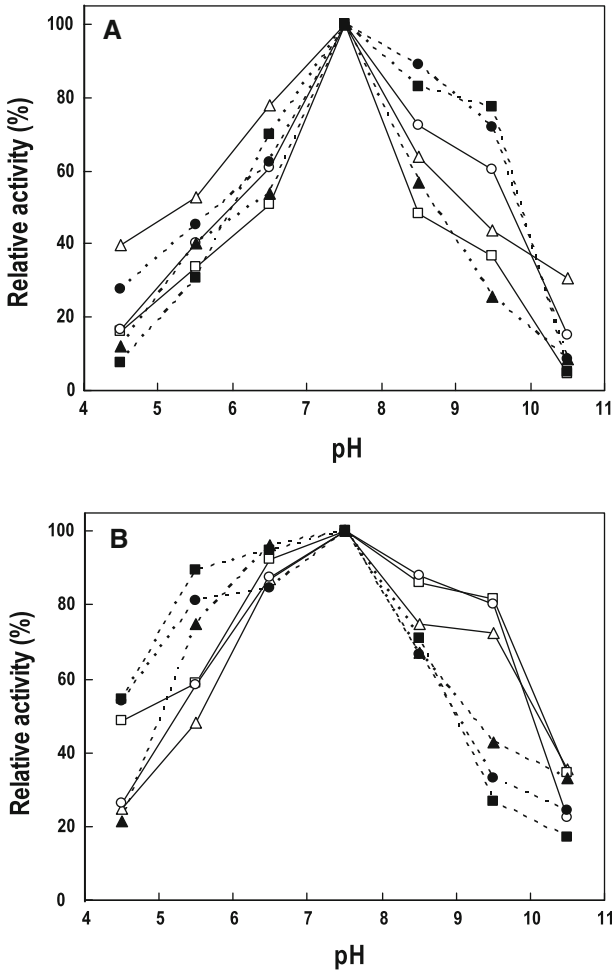

Fig. 6 Effect of pH on activity (**a**) and stability (**b**) of free and immobilized enzymes. Results are reported as means ($n = 3$) and the standard deviations were lower than ±5.9%. *Closed triangles* free *Bs*APII, *open triangles* immobilized *Bs*APII, *closed squares* free *Bs*APII-Lys_3, *open squares* immobilized *Bs*APII-Lys_3, *closed circles* free *Bs*APII-Lys_9, *open circles* immobilized *Bs*APII-Lys_9

Table 2 The kinetic parameters of free and immobilized enzymes

Enzyme	K_M ($nmol^{-1}$)	V_{max} (nmol min^{-1})	V_{max}/K_M
Free			
*Bs*APII	1.02	294.1	288.3
*Bs*APII-Lys_3	0.97	169.9	175.2
*Bs*APII-Lys_9	0.92	127.3	138.4
Immobilized			
*Bs*APII	1.51	246.7	163.3
*Bs*APII-Lys_3	1.33	130.1	97.8
*Bs*APII-Lys_9	1.23	112.8	91.7

storage stability without a decrease in its biological activity has been sought in the last decade. Figure 8 shows the storage stability of the free and bound enzymes at the indicated

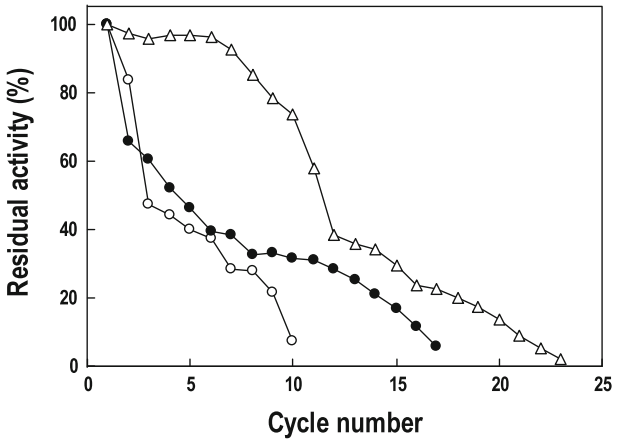

Fig. 7 Reuseablity of the immobilized enzymes. Results are reported as means ($n = 3$) and the standard deviations were lower than ±3.7%. *Open circles* immobilized *Bs*APII, *closed circles* immobilized *Bs*APII-Lys_3, *open triangles* immobilized *Bs*APII-Lys_9

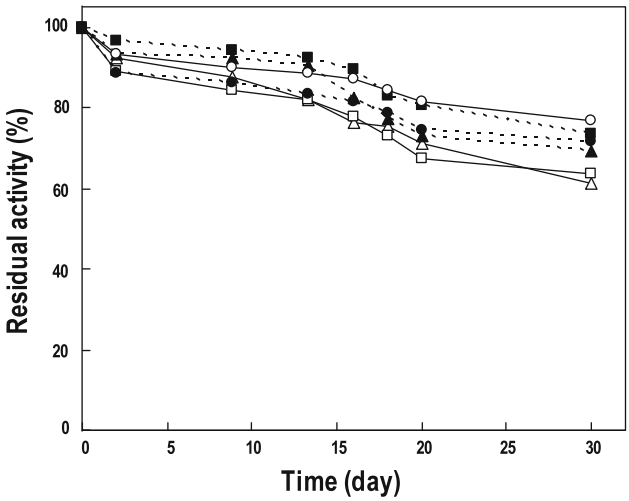

Fig. 8 Storage stability of free and immobilized enzymes. Results are reported as means ($n = 3$) and the standard deviations were lower than ±6.7%. *Closed triangles* free *Bs*APII, *open triangles* immobilized *Bs*APII, *closed squares* free *Bs*APII-Lys_3, *open squares* immobilized *Bs*APII-Lys_3, *closed circles* free *Bs*APII-Lys_9, *open circles* immobilized *Bs*APII-Lys_9

temperatures. After 30-day incubation, the residual activity of free *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9 at 4°C was 69.4, 74.5, and 71.7%, respectively. The bound enzymes also retained more than 61.2% of the original activity at 4°C. These results suggested that the storage stability of *Bs*APII, *Bs*APII-Lys_3, and *Bs*APII-Lys_9 was kept after being bound to magnetic particles. Additionally, the linear relationship revealed that both the deactivation of free and bound enzymes followed a first-order rate equation. The corresponding first-order deactivation rate constants for free and immobilized enzymes were calculated to be 0.0015–0.0029 h^{-1} at 4°C.

Conclusion

In this study, the carboxylated magnetic particles were prepared and used for immobilization of C-terminally lysine-tagged *Bs*APIIs. The immobilized enzymes had an activity comparable to the free *Bs*APIIs. Also, the immobilized enzymes showed enhanced stability towards more harsh temperature conditions, exhibited good durability, and could be readily recovered by magnetic separation. On the basis of these advantages, the *Bs*APII-conjugated magnetic particles are expected to be useful as catalytic devices for constructing reactors for the practical application of the enzyme.

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