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# Functional immobilization of racemase by adsorption on folded-sheet mesoporous silica

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ABSTRACT

Alanine racemase from *Geobacillus stearothermophilus* was physically immobilized on folded-sheet mesoporous silica (FSM) with pore diameters of 4 or 8.5 nm, and the activity, thermal stability, and reusability of the racemase were examined. The FSM with the larger pore diameter showed greater immobilization of the racemase, probably because its pores are large enough to adsorb the enzyme in the inner pore. The immobilized racemase retained 51% activity relative to that of the free enzyme in both the L-alanine (L-Ala) to D-Ala and D-Ala to L-Ala reactions. The racemase also exhibited a predominant thermal stability, and was successfully reused at least six times when immobilized on FSM. These results indicate the potential utility of FSM as an immobilization support for enzymes.



## 1. Introduction

Enzyme immobilization on a solid support is one of the methods to use enzymes in industry because it confers easy product separation and enhanced thermal and pH stability [1,2]. Ideal properties for immobilization support include chemical and mechanical stability, hydrophilicity, inertness toward enzymes, ease of derivatization, biocompatibility, resistance to microbial attack, and a large surface area [1,3,4]. Many organic and inorganic supports have been studied for use in enzyme immobilization [2–5]. There are also several immobilization techniques such as covalent attachment, adsorption, entrapment, etc. [1,2]. Among them, adsorption on solid support is the most convenient, because the procedure is very simple and no modification of both enzymes and supports are required [2]. However, the "ideal" immobilization of functional enzymes is still under investigation.

Mesoporous silica materials (MPs) are a class of inorganic materials with large surface areas (1000 $m^2/g$), high pore volumes, well-ordered pore structures, and narrow pore-size distributions, with relatively large-pore diameters (2–40 nm) that match the sizes of the enzymes. Biomolecules bind to the surfaces of MPs via various forces, such as electrostatic interactions, hydrogen bonding, and van der Waals interactions. The surfaces of MPs can also be functionalized, thus changing their properties [6]. These attractive properties of MPs have led to significant research into their potential utility as immobilizing biomaterials [6–10]. MPs such as MCM-41 (mobile crystalline material), SBA-15 (Santa Barbara amorphous material), and FSM-16 (folded-sheet mesoporous silica) have been investigated for the immobilization of various proteins, including chloroperoxidase [11], cytochrome c [12], papain [13], trypsin [14], lipase [15], and cellulase [16]. Takahashi et al. [17] investigated the immobilization of horseradish peroxidase (HRP) on various MPs and showed that FSM-16 adsorbed large amounts of HRP, which retained remarkable stability and catalytic activity in an organic solvent. Hemoglobin (an oligomeric protein) has also exhibited enhanced thermal stability and resistance to high NaCl and the denaturant guanidine hydrochloride when immobilized on FSM [18]. However, few studies successfully demonstrated the immobilization of industrially important enzymes using MPs.

D-Amino acids are components of the bacterial cell wall and peptide antibiotics, and are also increasingly important as sources of chemical compounds, such as antibiotics, sweeteners, pesticides, and medicines [19]. D-Amino acids can be produced by the chiral resolution of DL-amino acids that are synthesized chemically from the corresponding aldehyde and cyanic acid. However, chemical methods are not suitable for their large-scale production because of their low yields and high costs [19]. Because most L-amino acids are produced cheaply in industrial scale by microbial fermentation, D-amino acid production from their L-isomers by enzymatic racemization and kinetic resolution should be considered [19,20].

*Abbreviations:* MPs, mesoporous silica materials; FSM, folded-sheet mesoporous silica; RacA, alanine racemase.

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Oikawa et al. [21] reported the production of D-glutamate (D-Glu) from L-Glu using immobilized Glu racemase and L-Glu oxidase, which were packed into columns and connected sequentially. In this system, L-Glu is racemized in the first column and L-Glu is then oxidized to α-ketoglutarate in the second column by L-Glu oxidase, so only D-Glu remains. Alanine racemase (EC 5.1.1.1; RacA) is a pyridoxal-5′-phosphate-dependent enzyme that catalyzes the racemization of L-alanine (L-Ala) and D-Ala. It is used in the production of labeled aromatic amino acids, such as L-tyrosine, L-DOPA (3,4-dihydroxy-L-phenylalanine), and L-tryptophan, and in the production of D-Ala from the L-isomer [20]. A four-enzyme-coupled D-amino acid synthesis reaction has also been proposed, in which D-amino acids are produced from α-keto acids by D-amino acid transaminase using D-Ala as the amino donor [22]. In this reaction, the by-products (pyruvate and ammonia) produced by the transaminase are regenerated to L-Ala by the action of an NADH-dependent dehydrogenase, and then D-Ala is produced by RacA. The gene for L-amino acid oxidase with a broad substrate specificity was also cloned from *Rhodococcus opacus* [23], so it is possible that D-Ala is produced by a similar reaction to that producing D-Glu. Therefore, a D-Ala synthesis process based on the racemization of L-Ala should be developed.

In the present study, we investigated the applicability of the mesoporous silica, FSM, to the immobilization of an enzyme that catalyzes racemization reactions. RacA gene from *Geobacillus stearothermophilus* (*GeoRacA*) has been cloned and well characterized [24–27]. The crystal structure of GeoRacA has also been reported [28]. Using GeoRacA as the model enzyme, the influence of FSM pore size on the immobilization of GeoRacA was investigated, and we examined the stability and reusability of the enzyme immobilized on FSM.

## 2. Experimental

### 2.1. Reagents

The bacterial stock of *G. stearothermophilus* (NBRC 12550) was obtained from the National Institute of Technology and Evaluation (Chiba, Japan). Docosyltrimethylammonium chloride ($C_{22}H_{45}N(CH_3)_3Cl$; $C_{22}$-TMA) and kanemite (layered polysilicate) were kindly donated by Lion Corporation (Tokyo, Japan) and Tokuyama Siltech (Yamaguchi, Japan), respectively. A swelling reagent, 1,3,5-triisopropylbenzene (TIPB) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). L-Ala, D-Ala, and β-D-thiogalactopyranoside (IPTG) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All chemicals used in this study were of high-quality analytical grade.

### 2.2. Synthesis of FSM

The method of FSM synthesis has been described by Urabe et al. [18]. Briefly, for FSM-4, kanemite (5.35 g) was dispersed in an aqueous solution (205 mL) containing $C_{22}$-TMA (8.16 g) and stirred vigorously at 70 °C for 2 h. The pH of the suspension was adjusted to 8.5 with 2 N HCl for 40 min, and then incubated for a further 3 h at 70 °C. For the synthesis of FSM with a pore size of 8.5 nm (FSM-8.5), 5.5 mL of swelling reagent (TIPB) was mixed with the $C_{22}$-TMA solution 30 min before the addition of kanemite. The resulting product was washed with deionized water (70 °C), dried overnight at 60 °C, and calcined at 550 °C in air for 6 h to remove the organic compounds.

### 2.3. Cloning and overexpression of GeoRacA

The *GeoRacA* gene was amplified by the polymerase chain reaction (PCR) using the genomic DNA of *G. stearothermophilus* (NBRC 12550) as the template and the primers: forward, 5′-AGAGACATATGAACGACTTTCATCGCGATACGTG-3′ and reverse, 5′-GAGACTCGAGTGAAGCAGATTATGCACTGCTTTCCC-3′. The PCR product was ligated to the pBluescript II (SK+) plasmid (Stratagene, CA, USA), and then sequenced to confirm its veracity using the dideoxynucleotide-chain-termination method. N-Terminally hexa-histidine-tagged GeoRacA was produced by inserting the correct clone into the *Nde*I–*Xho*I sites of the pET28b vector (EMD Chemicals Inc., CA, USA).

*Escherichia coli* strain BL21 Star (DE3; Invitrogen, CA, USA) was transformed with the *GeoRacA* expression vector and grown in Turbo broth at 37 °C. The expression of GeoRacA was induced by the addition of IPTG to a final concentration of 1 mM. After incubation for a further 21 h at 20 °C, the cells were harvested by centrifugation at 7500 × *g* for 5 min. The cell pellet was resuspended in 20 mM Tris–HCl (pH 7.5), 500 mM NaCl containing 10 mM 2-mercaptoethanol, snap frozen in liquid $N_2$, and stored at −80 °C.

### 2.4. Purification of GeoRacA

To the thawed cells were added 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin A, 0.5 mg/mL lysozyme, 1 mM $MgCl_2$, 100 units of DNase1, and 10 mM imidazole. After incubation at 4 °C for 30 min, the cells were disrupted by sonication and centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was then filtered through a 0.45 μm filter and loaded onto a HisTrap HP column (GE Healthcare UK Ltd., Buckinghamshire, England) on the ÄKTA Explorer liquid chromatography system (GE Healthcare UK Ltd.). The protein was eluted with a concentration gradient of 10–500 mM imidazole in 20 mM potassium phosphate (pH 7.5), 500 mM KCl. The peak fractions were collected, concentrated with Amicon® Ultra-15 centrifugal filter units (Millipore Corporation, MA, USA), and dialyzed against 20 mM potassium phosphate (pH 7.5), 50 mM KCl. The purity of the enzyme was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### 2.5. Adsorption of GeoRacA onto FSM

To study the adsorption of GeoRacA onto FSM, an aliquot of FSM (10 mg) was incubated with 1 mL of GeoRacA solution of varying concentrations (0.1–4 mg/mL) at 4 °C for 24 h on a Rotator RT-50 (Taitec Corporation, Saitama, Japan) to establish the adsorption equilibrium. The FSM was removed by centrifugation at 20,800 × *g* for 5 min at 4 °C, and the protein concentration in the supernatant was measured. The amount of protein adsorbed was calculated by subtracting the amount of protein in the supernatant after centrifugation from the amount of GeoRacA before adsorption.

To study the changes in the nitrogen adsorption isotherms and pore-size distributions by GeoRacA adsorption, FSM-8.5 was incubated with GeoRacA (4 mg/mL) or buffer (20 mM potassium phosphate [pH 7.5], 50 mM KCl), washed sequentially with buffer, $ddH_2O$, and ethanol, and then dried at 60 °C.

For the enzyme activity studies, 30 mg of FSM-8.5 was incubated with 1.26 mg of GeoRacA (1 mg/mL) in 20 mM potassium phosphate (pH 7.5), 50 mM KCl for 24 h at 4 °C on a Rotator RT-50. The FSM-immobilized GeoRacA was collected by centrifugation at 10,600 × *g* for 3 min at 4 °C. The amount of immobilized GeoRacA was calculated by measuring the unbound protein in the supernatant. The FSM-immobilized GeoRacA was washed three times, and then resuspended in the buffer to a protein concentration of 0.2 mg/mL.

### 2.6. Characterization

Nitrogen adsorption isotherms were measured at the temperature of liquid $N_2$ (77 K) using NOVA 3000 analyzer (Quantachrome

Instruments, FL, USA). Before measurement, the samples were degassed at 200 °C for 3 h under vacuum. The specific surface area was calculated by the Brunaur–Emmett–Teller (BET) method using adsorption data in the relative pressure range of $P/P_0$ = 0.05–0.30. The total pore volume was estimated from the $N_2$ adsorbed at the maximum relative pressure. The pore-size distribution was determined using the Barrett–Joyner–Halenda (BJH) method from the adsorption branches.

### 2.7. Enzyme activity

GeoRacA activity was measured with a modified circular dichroism assay for glutamate racemase [29]. The molar ellipticity values for L-Ala and D-Ala at 204 nm were taken to be 23.0 and −23.0 mdeg/cm/mM, respectively. The reaction mixture (2.5 mL) containing 3 mM L-Ala, 10 mM potassium phosphate (pH 7.5), and 10 μL of FSM-immobilized or free GeoRacA (2 μg of protein) was incubated at 30 °C for 5 min, and the change in ellipticity with time was monitored with a Jasco J-820 CD Spectropolarimeter (Jasco, Tokyo, Japan). The enzyme activity was calculated using the rate at which 10% of the initial ellipticity changed. One unit of enzyme activity refers to the activity required to convert 1 μmol of one Ala isomer into another per minute.

The steady-state kinetic constants were calculated by measuring the enzyme activity at different concentrations of L-Ala or D-Ala (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10 mM) in 10 mM potassium phosphate (pH 7.5). FSM-immobilized or free GeoRacA samples (2 μg of protein) were incubated with 2.5 mL of substrate at 30 °C. The $V_{max}$ and $K_m$ values were calculated by extrapolation of the curves using Prism 4 software (GraphPad Software Inc., CA, USA). It is noted that the optimal temperature of GeoRacA was reported to be 50 °C [21]. We assayed the enzyme activity at 30 °C instead of 50 °C, because enzyme activity is too high to analyze the kinetic constant in our system. Relatively high amount of FSM-immobilized GeoRacA is required to reduce errors and obtain reliable results.

### 2.8. Thermal stability experiment

To investigate the thermal stability of the enzyme, a 50 μL aliquot of 0.2 mg/mL FSM-immobilized or free GeoRacA sample was treated at 80 °C for the indicated times using iCycler (Bio-Rad Laboratories, K.K., Tokyo, Japan), then 10 μL of the sample was incubated with 2.5 mL of 3 mM L-Ala in 10 mM potassium phosphate (pH 7.5) at 30 °C.

### 2.9. Recycling experiment

The recyclability of FSM-immobilized GeoRacA was evaluated. For each cycle, a reaction mixture containing 4 μg of FSM-immobilized GeoRacA and 3.5 mL of 3 mM L-Ala in 10 mM potassium phosphate (pH 7.5) was incubated for 1 min at 30 °C with shaking. The supernatant was then removed by centrifugation at 2150 × *g* for 5 min at 4 °C and the concentration of D-Ala was measured.

## 3. Results and discussion

### 3.1. Characterization of FSMs

FSMs with different pore diameters (4 and 8.5 nm; designated FSM-4 and FSM-8.5, respectively) were synthesized with or without swelling reagent. Their nitrogen adsorption isotherms and pore-size distributions are shown in Fig. 1A and B, respectively. The structure parameters of the FSMs are listed in Table 1.

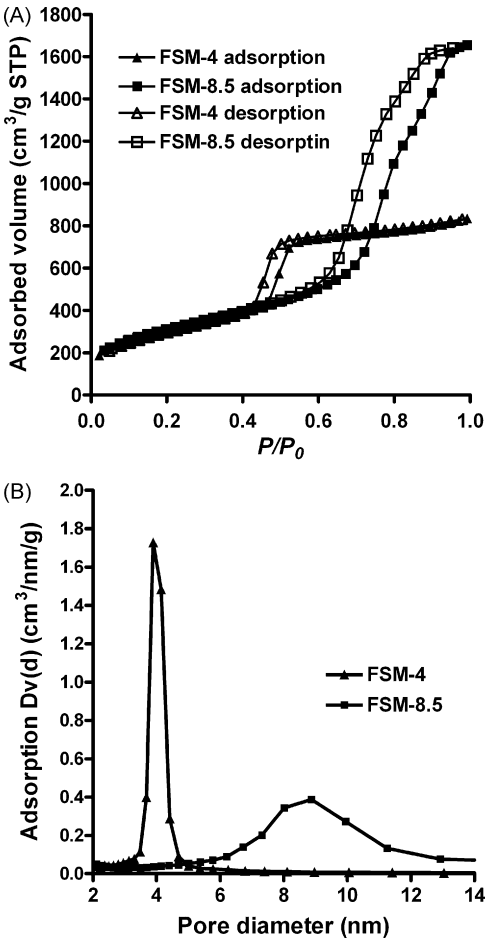


**Fig. 1.** Nitrogen adsorption/desorption isotherms and pore-size distributions for the FSMs. (A) Nitrogen adsorption/desorption isotherms: FSM-4 adsorption (▲), FSM-4 desorption (△), FSM-8.5 adsorption (■), FSM-8.5 desorption (□). (B) Pore-size distributions: FSM-4 (▲), FSM-8.5 (■). The isotherms were measured using a NOVA 3000 analyzer. The pore-size distributions were determined using the BJH method from the adsorption branches. The samples were pretreated at 200 °C for 3 h under vacuum.

**Table 1**
Physiochemical properties of FSMs.

| Sample | BET surface area ($m^2$/g) | Total pore volume ($cm^3$/g) | Pore diameter (nm) |
|---|---|---|---|
| FSM-4 | 1081 | 1.30 | 4 |
| FSM-8.5 | 1108 | 2.57 | 8.5 |

As shown in Fig. 1A, the nitrogen adsorption isotherms of FSM-4 and FSM-8.5 were both type IV by the International Union of Pure and Applied Chemistry (IUPAC) classification, which is characteristic of mesoporous materials [30]. The pore diameters of FSM-4 and FSM-8.5 were 4 and 8.5 nm, respectively (Fig. 1B). The total pore volumes of FSM-4 and FSM-8.5 were 1.30 and 2.57 $cm^3$/g, respectively, and their surface areas were both around 1100 $m^2$/g (Table 1). These values are very high compared with those of other MPs with similar pore diameters, such as MCM-41 and SBA [17,31–33].

It is noteworthy that the pore-size distribution of FSM-8.5 is broader than that of FSM-4 (Fig. 1B). We noted that the pore diameter of the FSM increased with increasing amounts of swelling agent. However, the distribution of the pore size also became broader.

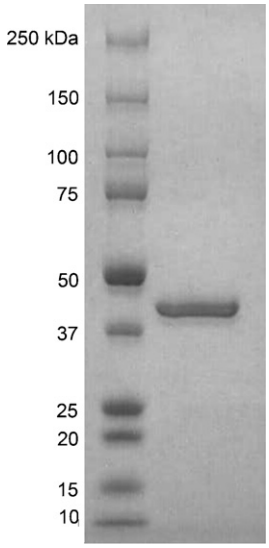


**Fig. 2.** SDS-PAGE of purified GeoRacA. The sample (1 μg) was separated electrophoretically on a 5–20% polyacrylamide gradient gel. The gel was stained with Bio-Safe Coomassie Stain.

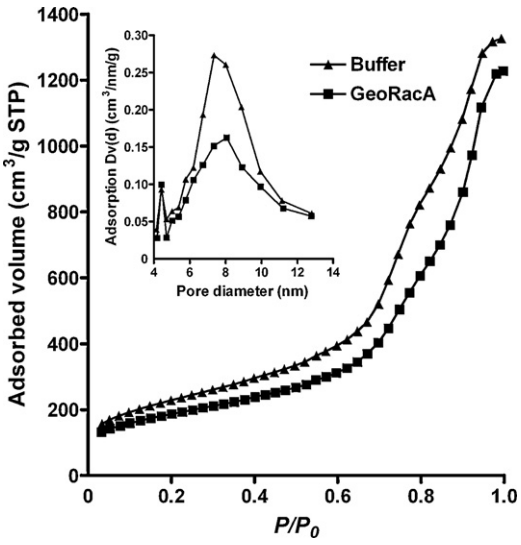


**Fig. 4.** Changes in the nitrogen adsorption isotherms and pore-size distributions of FSM-8.5 before and after GeoRacA was immobilized. Buffer (▲) or GeoRacA (■) were adsorbed onto FSM-8.5 and degassed at 60 °C for 24 h under vacuum. The isotherms were then measured with a NOVA 3000 analyzer. The pore-size distributions were determined using the BJH method from the adsorption branches.

### 3.2. Isolation and purification of GeoRacA

The cDNA clone of *GeoRacA* was subcloned into the bacterial expression vector pET28b to express an N-terminally hexa-histidine-tagged protein. The sequenced gene had some discrepancies from that reported by Tanizawa et al. [25], but was identical to the gene reported by Shaw et al. [28]. The construct was introduced into *E. coli* strain BL21 Star (DE3), and the expression of GeoRacA was induced. The protein was purified through a HisTrap HP column, and the final yield of purified GeoRacA was ~300 mg/L of culture. As shown in Fig. 2, the protein was purified to near homogeneity, suitable for subsequent studies. The specific activity of purified GeoRacA was 239 units/mg. We note that this value is lower than the 940 units/mg reported by Watanabe et al. [26]. This discrepancy may be attributable to the different assay conditions used, and/or to the N-terminal hexa-histidine tag on the GeoRacA used in our study, which may have suppressed its activity.

### 3.3. Adsorption of GeoRacA to FSM

Fig. 3 shows the adsorption isotherms for GeoRacA on FSM-4 and FSM-8.5. The capacity was higher for the large-pore FSM-8.5 than for FSM-4. GeoRacA consists of two identical subunits of 43 kDa,

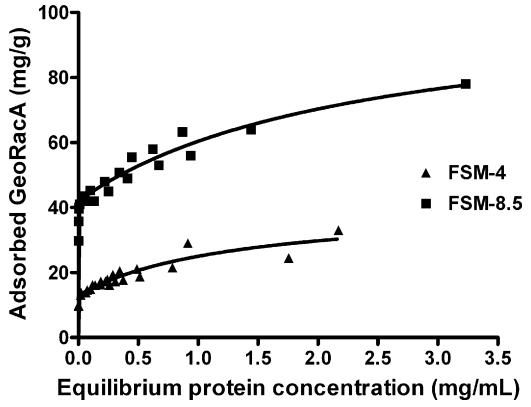


**Fig. 3.** Adsorption isotherms of GeoRacA on FSM-4 (▲) and FSM-8.5 (■). Various concentrations of GeoRacA (1 mL) were adsorbed onto 10 mg of FSM for 24 h at 4 °C, and the protein concentration in the supernatant was measured.

and the unit cell dimensions are 88 Å × 52 Å × 42 Å [24,28]. Because the dimensions of GeoRacA are larger than the pore diameter of FSM-4, the smaller amount adsorbed is expected to be located at the pore entrance and/or on the external surface of the particle. The surface areas of FSM-4 and FSM-8.5 are similar (1081 and 1108 $m^2/g$, respectively), whereas the total pore volume of FSM-8.5 is about twice as great as that of FSM-4 (Table 1), and its pore diameter is also larger than GeoRacA, suggesting that GeoRacA is immobilized within the pores of FSM-8.5. To confirm that GeoRacA is present within the pores of FSM-8.5, the nitrogen adsorption before and after GeoRacA loading was also investigated. As shown in Fig. 4, the nitrogen adsorption and the peak height of the pore-size distribution curve decreased after the full adsorption of GeoRacA. The surface area and total pore volume also decreased (from 828 to 671 $m^2/g$ and from 2.06 to 1.90 $cm^3/g$, respectively). These data show that the pores of FSM-8.5 were occupied by GeoRacA. We noted that the surface area and total pore volume also decreased in some degree after incubation with buffer only (compare with Table 1), but the shape of the nitrogen adsorption curve did not change (Figs. 1A and 4). These results indicate that the pore structure of FSM was not disturbed by the buffer. The cause of the reductions in the surface area and total pore volume may be attributable to the adsorption of the buffer components.

The amount of GeoRacA adsorbed onto FSM was compared with the adsorption of other proteins, and was lower than that of HRP (183 mg/g) [17] and hemoglobin (212 mg/g) [18]. This lower adsorption may be attributable to the buffer pH used (higher than the pI of GeoRacA). The adsorption of enzymes onto silica supports is maximized when the buffer pH is just below the pI of the protein and decreases above its pI [11,34].

The buffer pH used in this study was 7.5, which is higher than the pI of GeoRacA (6.7, estimated from the amino acid sequence), because the purified GeoRacA aggregated and precipitated in the buffer in a pH range of 5–7 (data not shown). Since the silica support surface is negatively charged at pH > 2 [35], both the silica surface and GeoRacA are negatively charged, and low loading was consequently achieved. The modification of the pI of the enzyme using molecular biology techniques, such as molecular evolution, may help to resolve this problem.

**Table 2**
Kinetic constants of free and FSM-immobilized GeoRacA.

| | Direction | $K_m$ (mM) | $V_{max}$ (μmol/min/mg enzyme) | $V_{max}/K_m$ |
|---|---|---|---|---|
| Free | L-Ala to D-Ala | 3.70 | 527 | 142 |
| | D-Ala to L-Ala | 4.38 | 316 | 72.2 |
| FSM-immobilized | L-Ala to D-Ala | 2.41 | 315 | 131 |
| | D-Ala to L-Ala | 3.70 | 255 | 69.0 |

The steady-state kinetic constants were calculated by extrapolation of the curves using GraphPad Prism 4 software.

We also tested an SBA-type mesoporous silica. However, the amount of GeoRacA adsorbed was about half the amount of FSM-8.5 adsorbed, even though their pore sizes (9.8 nm) and surface areas (955 $m^2/g$) were similar (data not shown). The $^{29}Si$ NMR spectra [36,37] revealed that the surface composition of FSM is unique compared with those of other amorphous silicas, including SBA. For example, amorphous silicas contain both Q2 (20%) and Q3 (80%) silanol groups on their surfaces [38], whereas all the silanol groups of FSM are Q3 [37]. It has also been reported that the surface silanol groups of FSM are more acidic than those of amorphous silicas [37,38]. It is possible that these unique surface properties of FSM make it more highly adsorbent for proteins than SBA.

In general, a higher enzyme capacity is an important economic consideration for immobilized enzyme supports [3], and enzymes should be adsorbed in the pores of the mesoporous silica to maximize the benefits of immobilization [10,39]. Therefore, we used FSM-8.5 in subsequent experiments.

### 3.4. Kinetic constants for FSM-immobilized and free GeoRacA

Table 2 shows the kinetic constants for free and FSM-immobilized GeoRacA. The $K_m$ values for the L- to D-Ala and D- to L-Ala reactions of FSM-immobilized GeoRacA were 4.38 and 3.70 mM, respectively, slightly higher than those of free GeoRacA (3.70 and 2.41 mM, respectively). The $V_{max}$ value was also reduced by immobilization. The $V_{max}/K_m$ ratios (which reflect the catalytic efficiency of an enzyme for its substrate) of FSM-immobilized and free GeoRacA were 72.1 and 142, respectively, in L- to D-Ala direction (Table 2). Although the catalytic efficiency was reduced by immobilizing the enzyme on FSM, 51% of its efficiency was retained relative to that of the free enzyme. The higher $K_m$ and lower $V_{max}$ of FSM-immobilized GeoRacA comparing with the free enzyme suggests that the immobilized enzyme does not easily interact with its substrate [40]. It is also probable that some enzyme conformational changes and/or active-site block is induced by the enzyme–support interaction [41]. GeoRacA is a homodimer and the interaction of each subunit is important in maintaining the active site [28]. Therefore, it is possible that the activity of the dimeric enzymes is more sensitive to conformational changes than that of monomeric enzymes. Ozyilmaz et al. [42] reported that the elevated $K_m$ and reduced $V_{max}$ of immobilized enzymes relative to those of the free forms are attributable to limited substrate diffusion and steric hindrance.

### 3.5. Thermal stability of FSM-immobilized and free GeoRacA

The thermal stability of enzymes in industrial applications is important because reactors are sometimes operated at high temperatures to improve productivity and to avoid microbial contamination [1]. It has also been expected that the thermal stability is correlated with enzyme longevity.

To investigate the thermal stability, free or FSM-immobilized GeoRacA was incubated at 80 °C and its residual activity was measured. As shown in Fig. 5, the activity of the free enzyme was dramatically reduced to 60% after incubation for 10 min, after which its activity continued to decrease. In contrast, FSM-immobilized GeoRacA retained 100% of its activity after 10 min, and retained 50% activity after 60 min. This result clearly indicates that the enzymatic thermal stability is improved by immobilization in the pores of FSM.

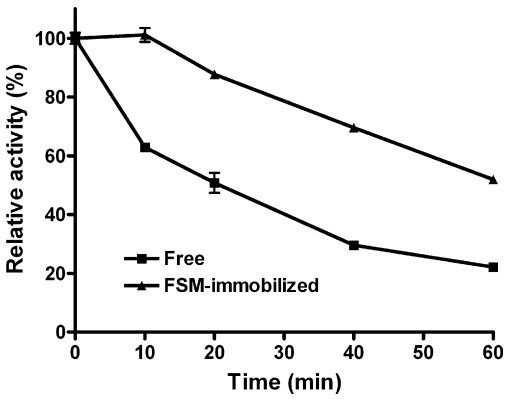


**Fig. 5.** Thermal stability of free and FSM-immobilized GeoRacA. Free (■) or FSM-immobilized (▲) GeoRacA (0.2 mg/mL) was incubated at 80 °C for the indicated times, and then its residual activity was measured. The relative activity was calculated with respect to the activity of samples not treated with heat.

A lint-like precipitate of the free form of GeoRacA was seen after thermal treatment, indicating that the free enzyme was denatured and aggregated. In contrast, the denaturation of the protein was prevented by its immobilization on FSM. It is possible that the structure of the enzyme becomes rigid with the association between GeoRacA and the pore walls of FSM, thereby improving the thermal stability of GeoRacA. Ravindra et al. [43] also proposed that the thermal stability of the enzyme is conferred by the hydration of the molecule in the narrow silica pores, which is induced by the special water-structuring properties of the silanol groups at the silica surface. Further study should be required to elucidate the enzyme stabilizing mechanism by FSM.

We also noted that FSM-immobilized GeoRacA retained 94% of its activity after storage for 4 months at 4 °C (135 μmol/min/mg at 0 months versus 127 μmol/min/mg at 4 months), whereas free GeoRacA retained only 14% of its activity (239 μmol/min/mg at 0 months versus 34.3 μmol/min/mg at 4 months). This result clearly indicates that the immobilization of the enzyme on FSM can help to maintain its activity. This feature may also be useful for industrial purposes, because enzymes can be stored in the liquid phase, making them easy to prepare when required. Commercial enzymes are usually in a powdered form, which can be difficult to disperse, can contaminate industrial plants, or are hazardous inhalants for the workers who must dissolve the enzymes.

### 3.6. Recycling FSM-immobilized GeoRacA

In general, the reusability of an immobilized enzyme is important for economic reasons. Therefore, we conducted an experiment to assess the recyclability of FSM-immobilized GeoRacA.

As shown in Fig. 6, when the initial activity of FSM-immobilized GeoRacA in the first cycle was defined as 100%, the immobilized enzyme was still active and about 50% of its original activity was retained after five cycles of reuse. Its activity decreased thereafter and reached a plateau after 10 cycles. This result demonstrates the high potentiality of FSM as a suitable immobilization support.

When the ellipticity of the supernatant of the first-cycle reaction was remeasured after incubation at 30 °C for several hours, no change in ellipticity was observed. This means that no active race-

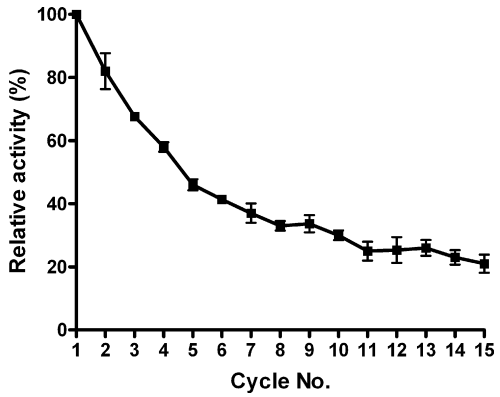


**Fig. 6.** Recycling FSM-immobilized GeoRacA. For each cycle, FSM-immobilized GeoRacA (4 μg of protein) was incubated with 3 mM L-Ala (3.5 mL) for 1 min at 30 °C with shaking, and then the concentration of D-Ala was measured. The values are shown as residual activities relative to the specific activity of the first cycle, deemed to be 100%.

mase dissociated from the FSM (data not shown). The observed reduction in activity may be the result of disruption of the FSM when the immobilized enzyme was collected by centrifugation at each cycle. It is also possible that the substrate (L-Ala) is adsorbed onto FSM and affects the activity of the enzyme, because the silanol groups on the silica surface may interact with the amino group of Ala. Tortajada et al. [41] also reported that the activity of arabinofuranosidase, which was electrostatically immobilized on a hierarchical porous nanosized organosilica, was reduced by glucose, and suggested that the silica surface was coated by glucose, thereby preventing substrate access to the enzyme active site.

## 4. Conclusion

The mesoporous silica, FSM was synthesized and investigated as a potential immobilization support for GeoRacA. The enzyme can be immobilized by simple adsorption on FSM. FSM with a pore size of 8.5 nm (larger than the enzyme) showed a greater enzyme loading capacity, probably because the enzyme was adsorbed within the pores of the FSM. The activity of the immobilized enzyme was retained well, and the enzyme exhibited superior thermal and storage stability relative to that of the free enzyme. These results indicate that FSM protects enzymes from denaturation. In addition, the FSM-immobilized GeoRacA could be reused at least six times. These findings prove the usefulness of FSM as an immobilization support for GeoRacA.

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