SUPPLEMENTARY INFORMATION

Immobilization and stabilization of subtilisin Carlsberg in magnetically-separable mesoporous silica for the transesterification in an organic solvent

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EXPERIMENTAL METHODS

1. Materials

Subtilisin®Carlsberg (EC 3.4.21.14; alkaline protease from *Bacillus licheniformis*), N-Succinyl Ala-Ala-Pro-Phe *p*-nitroanilide (TP), N-acetyl-*L*-Phenylalanine ethyl ester, glutaraldehyde (GA), N,N-dimethylformamide (DMF), isooctane, n-propanol, t-butanol, sodium silicate solution (10.6% Na_2O, 26.5% SiO_2), 1,3,5-trimethylbenzene (mesitylene, 98%), and P123($(EO)_{20}(PO)_{70}(EO)_{20}$) were purchased from Sigma (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). The other chemicals were purchased from Aldrich (Milwaukee, WI, USA) and used without further purification.

2. Preparation of Mag-MSU-F

The synthesis of Mesocellular silica (MSU-F) was done by following previous protocol with slight modification[1]. Typical synthesis of MSU-F is as follows: 9.7 g of of triblock copolymer pluronic P123($(EO)_{20}(PO)_{70}(EO)_{20}$) and 4.48 mL macetic acid was dissolved in 200 mL water and then 5.9 g of 1,3,5-trimethylbenzne (TMB) was added with heating to 60°C under vigorous stirring for 1 h. 16 mL sodium silicate diluted with 200 mL of water was poured into the solution under vigorous stirring. The solution mixture was heated to 60°C for 20 h, followed by hydrothermal aging at 100°C for 24 h to get white precipitate. The precipitate was filtered and calcined at 550°C in air for 4 h to remove block copolymers. To fabricate mesocellular silica with magnetite (Mag-MSU-F), 1.18 g of

$Fe(NO_3)_3 \cdot 6H_2O$ was dissolved in 40 mL of ethanol, followed by subsequent addition of 1 g MSU-F. The solution was stirred until all the solution was evaporated. Dark brown colors of material were obtained after heat-treatment at 400^oC under flowing of 4 % H_2/Ar.

3. Characterization of Mag-MSU-F

Transmission electron microscopy (TEM, Hitachi H-7600, Tokyo, Japan) was used for high resolution imaging of Mag-MSU-F, imaging was done at 100 keV. BET surface area and pore size analysis were obtained using a Micromeritics (Tristar II 3020, Norcross, GA, USA). Pore-size distributions were calculated using the BJH (Barett–Joyner–Halenda) method. X-ray diffraction (XRD) patterns were obtained with a Rigaku D/Max-2500 diffractometer using Cu Kα radiation (λ = 1.5418 Å) at scanning rate of 4.00°/min. The magnetizations value was measured using a commercial SQUID magnetometer (Quantum Design MPMS 5XL, Quantum Dynamics, San Diego, CA, USA).

4. Immobilization of subtilisin Carlsberg in Mag-MSU-F

Mag-MSU-F (10 mg) was mixed with 1.5 ml of 5 mg/ml free Subtilisin Carlsberg (SC) in a buffer solution (10 mM sodium phosphate buffer, pH 8.0), vortexed for 30 s, sonicated for 3 s, and incubated at room temperature in a shaking condition (200 rpm). After 30 minute incubation for the adsorption of free SC (ADS-SC) into Mag-MSU-F, the samples were washed by buffer solution (100 mM sodium phosphate buffer, pH 8.0), and incubated with 0.5 % glutaraldehyde (GA) solution in phosphate buffer (100 mM sodium phosphate buffer, pH 8.0) without any moving for 30 minute and then shake at 200 rpm for 2 hour. After GA treatment (NER-SC), the samples (ADS-SC and NER-SC) were washed by phosphate buffer (100 mM sodium phosphate buffer, pH 8.0). To cap the un-reacted aldehyde groups, samples were incubated in 100 mM Tris-HCl (pH 8.0) under shaking at 200 rpm for 30 minute. Finally, ADS-SC and NER-SC were washed extensively with buffer solution (10 mM sodium phosphate buffer, pH 8.0), and were stored in the same buffer at 4 ^oC until later use.

5. Activity and stability of ADS-SC and NER-SC

The activity of immobilized SC was determined by the hydrolysis of 160 μM N-Succinyl Ala-Ala-Pro-Phe *p*-nitroanilide (TP) in aqueous buffer (10 mM sodium phosphate, pH 8.0), and the amount of hydrolysed *p*-nitroaniline was calculated by the time-dependent increase of the absorbance at 410 nm (A_{410}) using a UV-Vis spectrophotometer (UV, Shimadzu, Kyoto, Japan)[2]. In detail, ADS-SC and NER-SC were transferred into a glass vial containing 2 mL of buffer (10 mM sodium phosphate buffer, pH 8.0) and 20 μL of TP (10 mg/mL in DMF), which initiates the enzymatic hydrolysis of TP. After incubation under shaking at 200 rpm for a specific time, the samples (ADS-SC and NER-SC) were separated by magnet capturing

method, and the absorbance of reacted substrate solution was measured. Immediately after each activity measurement, the samples were washed three times with a fresh buffer solution (10 mM sodium phosphate buffer, pH 8.0) to remove all residual substrate and products. The samples were incubated at room temperature while shaking (200 rpm) until the next use. The relative activity was measured at same condition and calculated as the ratio of residual activity over initial activity (t = 0) each time point.

6. Transesterification and analysis

For the transesterification reaction, the SC samples with 10 mM phosphate buffer (pH 8.0) (free SC, ADS-SC and NER-SC) were prepared. To dehydrate the samples, the solution of free SC or immobilized SC was frozen by liquid nitrogen, and subsequently lyophilized for overnight. To dehydrate isooctane and n-propanol, they were mixed with molecular sieves, and incubated for more than one day. The transesterification of N-acetyl-*L*-Phenylalanine ethyl ester with n-propanol was catalyzed by lyophilized samples (free SC , ADS-SC and NER-SC) and performed at 250 rpm at 30°C. In brief, N-acetyl-*L*-Phenylalanine ethyl ester (10 mM) was dissolved in 6 mL of isooctane containing 1 M n-propanol, and then free SC and the immobilized SC was dispersed in above substrate solution and was sonicated for 30 sec respectively. The reaction was initiated by shaking at 250 rpm and at 30°C[3]. After enzyme reaction, for recycling use, they were washed with 3 mL of tert-butanol for three times under 200 rpm at 30°C, and then tert-butanol was washed off with isooctane three times in the same method. The initial reaction rate of transesterification was determined by the time-dependent yield of N-acetyl-*L*-Phenylalanine ethyl ester and used to represent SC activity at each time point. The conversion was calculated from the total amount of N-acetyl-*L*-Phenylalanine propyl ester. The initial reaction rate was calculated from the linear increase of N-acetyl-*L*-Phenylalanine ethyl ester conversion yield during the 10 hr. The SC recycling stability was evaluated by the relative activity. The initial activity of the immobilized SC is assumed to be 100%, as the relative activity of immobilized SC was defined as the ratio of the activity to that of the initial enzyme activity.

N-acetyl-*L*-Phenylalanine propyl ester in the reaction mixture was quantified by using GC-A7890 gas chromatography (Agilent Technologies, CA, USA) equipped with a flame ionization detector (FID) and HP-1 capillary column (60 m × 0.25 mm × 0.25 μm, Agilent Technologies, CA, USA). The injector temperature was maintained at 250°C, the detector temperature at 250°C, and the oven temperature at 150°C. The carrier gas used was high

purity nitrogen with an airflow rate of 30 mL/min. Oven temperature was initially set at 150°C, increased up to 200°C for 2 min, finally reached to 250°C, and then maintained at this final temperature for 2 min. The sample (150 µl) was collected at each time point, and diluted in isooctane containing 0.1% formic acid that stops the enzyme reaction. For the GC analysis, 1.67 mM of methyl heptadecanoate was used as an internal standard for each injection.

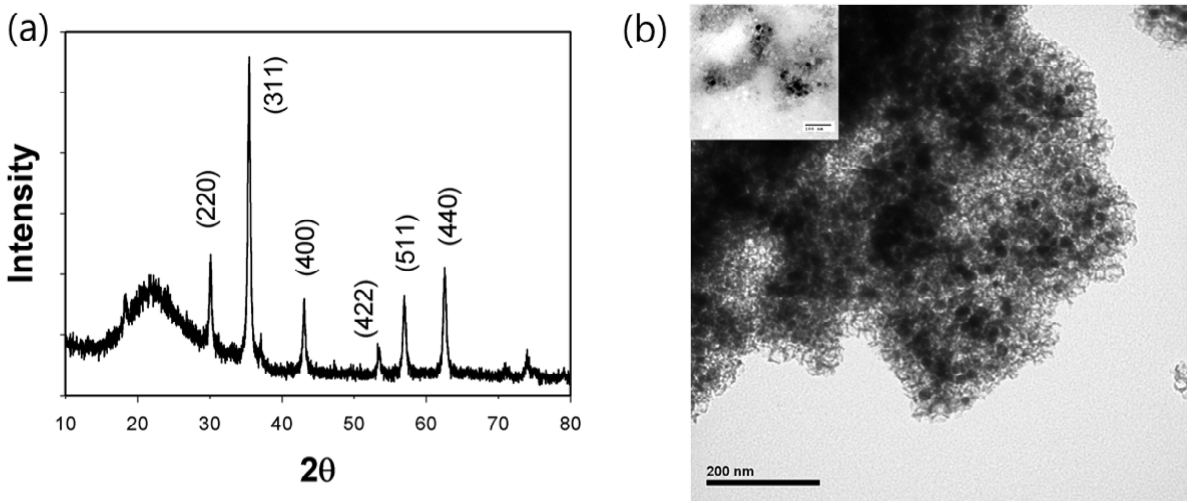
SUPPORTING DATA

Table S1. Physical properties of Mag-MSU-F

S_{BET} (BET surface area)	300 m^2/g
V (total pore volume)	1.45 cm^3/g
d_{ad} (main pore size)	38.3 nm
d_{de} (window pore size)	17.5 nm
M (magnetization value)	46.03 emu/g

d_{ad} and d_{de} were calculated using the BJH (Barett-Joyner-Halenda) method; and M is the magnetizations value was measured using a commercial SQUIC magnetometer.

Fig. S1 Characterization of Mag-MSU-F, (a) X-ray diffraction (XRD) pattern and (b) TEM image



Calculation of theoretically maximum Subtilisin® Carlsberg (SC, EC 3.4.21.14; alkaline protease from *Bacillus licheniformis*) loading amount in Mag-MSU-F.

Specification:

Pore volume of Mag-MSU-F	1.45 cm^3/g (= 1.45 x 10^{21} nm^3)
Size of SC[4]	7.72 x 5.58 x 5.37 nm^3

Number of SC molecules in 10 mg of Mag-MSU-F

= Pore volume of Mag-MSU-F / Size of SC

$$= \frac{1.45 \times 10^{19} nm^3}{7.72 \times 5.58 \times 5.37 nm^3} = 6.27 \times 10^{16}$$

Moles of SC molecules

= Number of SC molecules / *Avogadro* constant

$$= \frac{6.27 \times 10^{16}}{6.02 \times 10^{23}/mol} = 1.04 \times 10^{-7} mol$$

Weight of SC molecules

= Moles of SC molecules / MW of SC molecule

$$= \frac{1.04 \times 10^{-7} mol}{27600\, g/mol} = 2.87 \times 10^{-3} g = 2.87\, mg$$

Weight percent of SC loading amount

= SC weight / (SC + Mag-MSU-F) weight

$$= \frac{2.87\, mg}{(2.87 + 10.0) mg} = 22.32\, wt\%$$

Supporting information reference

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