Supporting Information for

Enzyme structure and catalytic properties affected by the surface functional groups of mesoporous silica

Kazuki Murai[a,b], Takayuki Nonoyama[b,c], Takao Saito[a,b] and Katsuya Kato[*,b]

[†]*Department of Applied Chemistry, Graduate School of Engineering, Chubu University*

[‡]*Bio-Integrated Processing Group, Advanced Manufacturing Research Institute, National Institute of Advanced Industrial Science and Technology (AIST)*

[§]*Department of Frontier Materials, Graduate School of Engineering, Nagoya Institute of Technology*

Contents

1. Materials.

Subtilisin (4.1 nm × 7.8 nm × 3.7 nm) from *Bacillus licheniformis*, triblock copolymer poly(ethylene glycol)-block poly(propylene glycol)-block poly(ethylene glycol) (Pluronic P123, $EO_{20}PO_{70}EO_{20}$, *M*n ca. 5800 g/mol) and (3-mercaptopropyl)-trimethoxysilane were purchased from Sigma-Aldrich Co. , St. Louis, MO, USA. *n*-Propyltrimethoxysilane was purchased from Lancaster, England. Tetraethoxysilane (TEOS), methyltrimethoxysilane, ethyltriethoxysilane, phenyltriethoxysilane, and 3-isocyanatepropyltriethoxysilane were purchased from Shin-Etsu Chemical Company, Tokyo, Japan. Cetyltrimethylammonium bromide (CTAB) was purchased from WAKO Pure Chemical Industries, Osaka, Japan. Magnetic nanoparticles were purchased from Ferrotec Corporation, Japan. All other reagents were used as special-grade chemicals.

2. Method.

2.1 Synthesis and functionalization of mesoporous silica material

Mesoporous silica (MPS) materials were prepared according to the procedure described previously.[1] Briefly, CTAB for MPS-1 and MPS-2 and P123 for MPS-3 were selected as organic templates for the preparation of MPS materials.

For MPS-1 and MPS-2, 2.6 g of CTAB was dissolved in 30 g of hydrochloric acid aqueous solution (pH 0.5). For MPS-2, 0.78 g of 1, 3, 5-trimethylbenzene was added to control pore size. To the mixture 3.5 g of TEOS and 3.0 g of 14.7 M ammonia aqueous

solution was added dropwise and the reaction was allowed to proceed at room temperature for 24 h. The resulting white precipitate was filtered and then washed with 50 ml of deionized water and 50 ml of ethanol. After drying at 70 ºC for 3 h, the sample was then heated to 500 ºC (rate 1 ºC/min) in air and kept at this temperature for 4 h.

For MPS-3, 1 g of Pluronic P123 was dissolved in 35 ml of 1.07 M hydrochloric acid aqueous solution, and 7.6 g of *n*-decane was added to control particle size. 2.13 g of TEOS and 1.1 mg of NH_4F was added to the mixture and the reaction was allowed to proceed at 40 ºC for 20 h. The resulting suspension was heated at 100 ºC for 24 h. A white precipitate was collected and the sample was washed with 50 ml of deionized water and 50 ml of ethanol and air-dried at room temperature. The resulting white solid was calcined at 500 ºC for 4 h. Organo-functionalization of the MPS surface was performed using silane coupling reagents by adding 50 mg of MPS to a mixture of 10 ml of toluene and 1 ml of silane coupling reagent. The mixture was refluxed for 6 h and then centrifuged. The resulting solid was washed with acetone, ethanol, and deionized water, and then air-dried at room temperature. Magnetic nanoparticles (MN) were coated onto MPS by ultrasonic irradiation. 3.0 mg of MPS and 3 μl of 19 wt% MN were added to 497 μl of 10 mM phosphate buffer (pH7.0). The mixture was sonicated at room temperature for 10 min. The resulting brown precipitate was and washed with deionized water. Finally, these samples were freeze-dried.

2.2 Immobilization of subtilisin on various MPS materials

Facile preparation of immobilized enzyme catalyst was performed as follows. 1.0 mg of subtilisin was dissolved in 900 μl of 10 mM phosphate buffer (pH 7.0). 3 mg of MPS was added to the subtilisin solution and the mixture was stirred at 4 ºC overnight. The obtained mixture was separated by centrifugation at 4 ºC for 10 min at 12000 rpm. The immobilized subtilisin was washed with cold deionized water, and stored at 4 ºC.

2.3 Assay procedure

In the hydrolysis reaction of acetate by subtilisin, alcohol is produced as a product. Hydrolysis reaction was performed by adding 100 μl of substrate solution (R, S-1-phenylethylacetate: acetone = 5 μl:100 μl, v/v) to a mixture of 3 mg of immobilized subtilisin and 900 μl of 10 mM phosphate buffer (pH 7.0). The reaction was allowed to proceed with stirring at room temperature for two days and then terminated by extraction with 1 ml of ethyl acetate. The extracted liquid was dehydrated with anhydrous magnesium sulfate. The rate of conversion and the enantiomer excess of the produced alcohol and remaining acetate were determined by gas chromatography (GC). The GC instrument was equipped with a VARIAN CP-Chirasil-DexCB column (column size was 25 m × 0.25 mm × 0.25 mm) and a flame-ionization detector. The carrier gas was helium (flow rate 50 ml/min). The injector, column, and detector temperatures were 180, 135, and 180 ºC, respectively. The retention times for

(S)-1-phenylethylacetate, (R)-1-phenylethylacetate, (R)-1-phenylethylalcohol, and (S)-1-phenylethylalcohol were 5.4, 5.7, 6.7, and 7.0 min, respectively.

2.4 Studies of subtilisin stability under various conditions with three types of assay

For study of stability during thermal treatment, 1.0 mg of free subtilisin or 3 mg of immobilized subtilisin was added to 900 μl of 10 mM phosphate buffer (pH 7.0). The mixture was incubated at 60 ºC for different times and then cooled to room temperature. The residual activity of each sample was determined by measuring the hydrolysis of 100 μl of substrate solution (R, S-1-phenylethylacetate: acetone = 5 μl:100 μl).

For study of enzyme stability in an organic solvent, 1.0 mg of free subtilisin or 3 mg of immobilized subtilisin was added to a mixture of 900 μl of the same phosphate buffer and 100 μl of the same substrate solution. Subtilisin stability was determined in a reaction solution adjusted to an acetone concentration of 10-50% (v/v).

For study of stability during recycle reaction of the immobilized enzyme catalyst, a magnetic nanoparticle composite MPS (MN-MPS) for industrial application was used. Hydrolysis reaction was performed. Subtilisin immobilized on MP-MPS was then separated by magnetic separation and washed with ethyl acetate and deionized water to remove the residual substrate. The retained activity of washed immobilized subtilisin was measured using a fresh reaction solution.

3. Characterization

Particle morphology was observed by field-emission scanning electron microscopy (FE-SEM: S-4300, Shimadzu, Osaka, Japan) with an accelerating voltage of 10.0 kV. Transmission electron microscopy (TEM) images were taken by a JEOL JEM 2010, operated at 200 kV. Pore diameter distributions were calculated from adsorption isotherms using Barrett-Joyner-Halenda (BJH) method. Specific surface areas were calculated by the Brunauer-Emmett-Teller (BET) method based on desorption isotherms. Wide-angle and small-angle X-ray diffraction (XRD) spectra were recorded on X-ray diffractometers (RINT2100V/PC, Rigaku, Tokyo, Japan with FeKα, 40 kV, 30 mA and RINT-TTR, Rigaku, with CuKα, 50 kV, 300 mA, respectively. The amount of organo-functional group was determined by thermogravimetry differential thermal analysis (TG-DTA, RTG320U, Seiko, Japan). Peaks were collected from room temperature to 1050 ºC (heating rate 10 ºC/min). No-substituted and organo-functionalized MPS were examined by Fourier transform infrared (FT-IR) spectroscopy (MFT-2000, JASCO Co., Japan) in transparent mode in the wavenumber range 4000-400 cm^{-1} using pellets made by mixing 99% KBr with 1% sample. The secondary structure of native subtilisin and subtilisin immobilized on each MPS was determined by circular dichroism (CD) spectrometer (J-820K, JASCO, Japan) in the wavelength range 190-260 nm using a quartz cell (optical path length 0.1 cm) and an integration number of 16; CD spectra were measured for the following concentrations: native subtilisin, 9.1×10^{-6} M subtilisin immobilized on no-substituted,

ethyl-group-modified and 3-mercaptpropyl-group-modified MPS were prepared at, 9.1 × 10^{-6} M, 9.1 × 10^{-6} M, and 5.8 × 10^{-6} M, respectively. Corrected steady-state emission spectra for subtilisin were recorded by spectrofluorometry (F-4500, HITACHI Co., Japan) with a xenon arc lamp.

To evaluate the tertiary structure of native subtilisin and subtilisin immobilized on several MPS-2, Trp fluorescence emission spectra were collected in the range 250-400 nm by exciting directly each Trp residue at 280 nm; the excitation and emission slit widths were 1 and 2.5 nm, respectively. Background fluorescence from the solvents was recorded and subtracted from the enzyme spectra. Steady-state measurements were performed in a fluorescence cuvette (3.0 ml^2) with 10 mm excitation/emission path lengths (emission collected through a perpendicular face). All measurements were performed at room temperature. The concentration of all enzyme samples was 1.0 × 10^{-5} M.

The denaturation temperature of subtilisin was determined by differential scanning calorimetry (DSC, DSC120U, Seiko, Japan). Peaks were collected from room temperature to 85 ºC (heating rate 1 ºC/min). Magnetization of the magnetite/silica composite sample was measured by vibrating sample magnetometry (BHV-35H, Riken, Japan) at room temperature.

REFERENCE

(1) Kato, K.; Suzuki, M.; Tanemura, M.; Saito, T. *J. Ceram. Soc. Jpn* **2010**, *118*, 410-6.

Additional Data:

Table S1. Structural properties of the synthesized MPS materials.

Sample name	Pore diameter (nm)	Surface area (m^2/g)	Pore volume (cm^3/g)
MPS-1	2.7	963	1.4
MPS-2	5.3	829	1.9
MPS-3	12.3	501	1.8

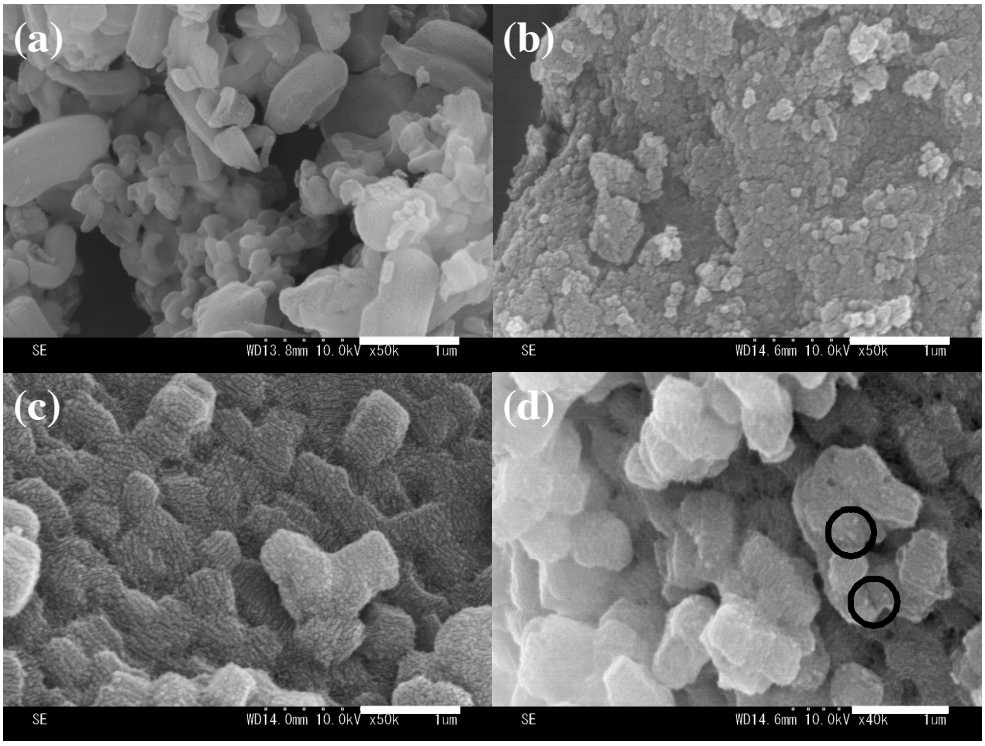

Figure S1. Field-emission scanning electron microscopy images; (a) MPS-1; (b) MPS-2; (c) MPS-3; (d) MN-MPS-2. Black circles denoted magnetic nanoparticles coated on the MPS-2 surface. Scale bar = 500 nm.

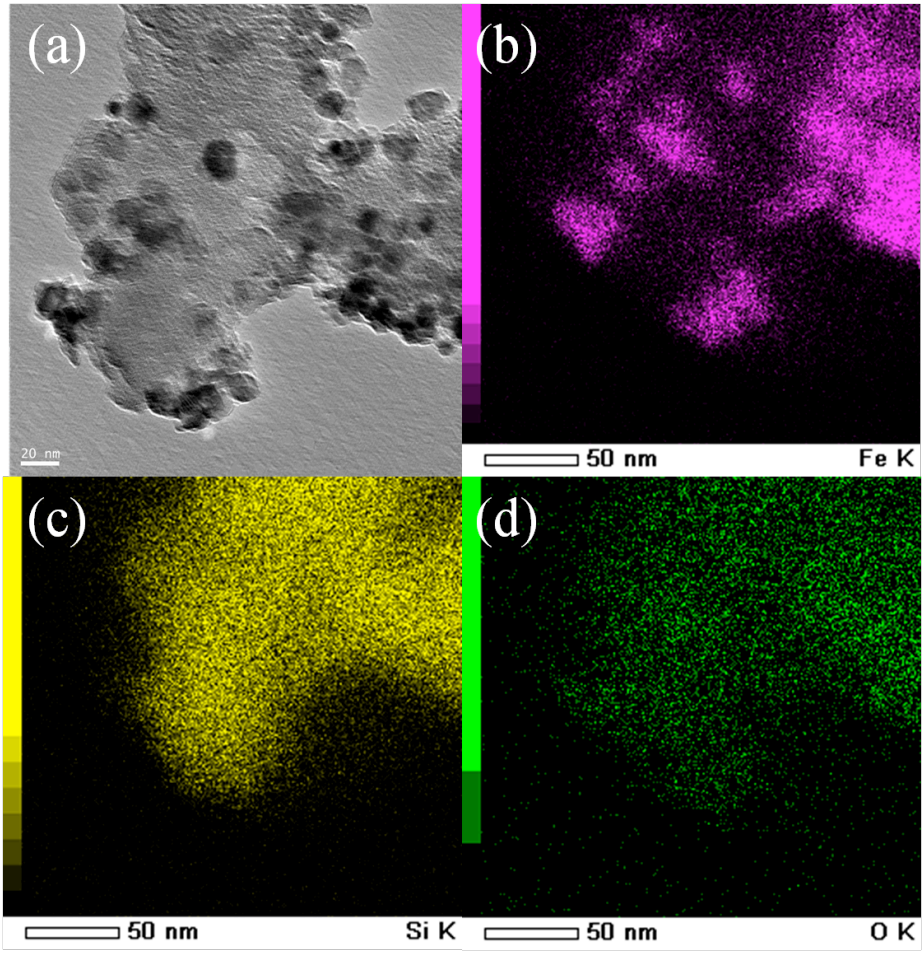

Figure S2. Transmission electron microscopy (TEM) image and energy dispersive X-ray analysis (EDX) mapping results of MN-MPS-2; (a) TEM image; (b) Fe-mapping; (c) Si-mapping; (d) O-mapping.

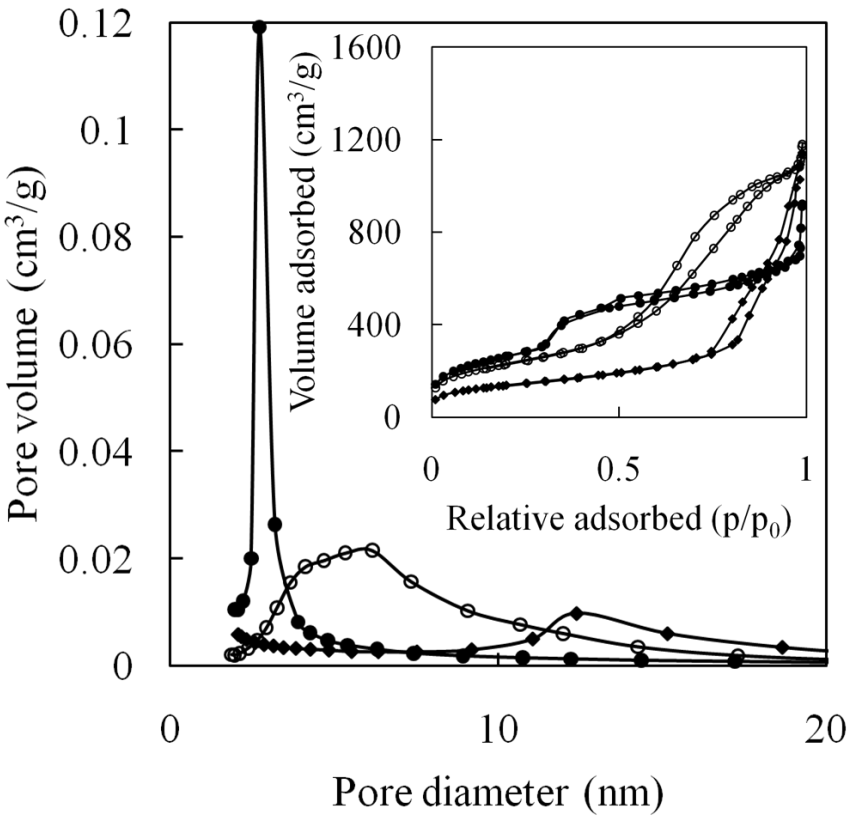

Figure S3. Pore-size distributions obtained from adsorption isotherms by the BJH method. N_2 adsorption-desorption isotherms for mesoporous silica materials. (inset) ●= MPS-1; ○= MPS-2; ♦= MPS-3.

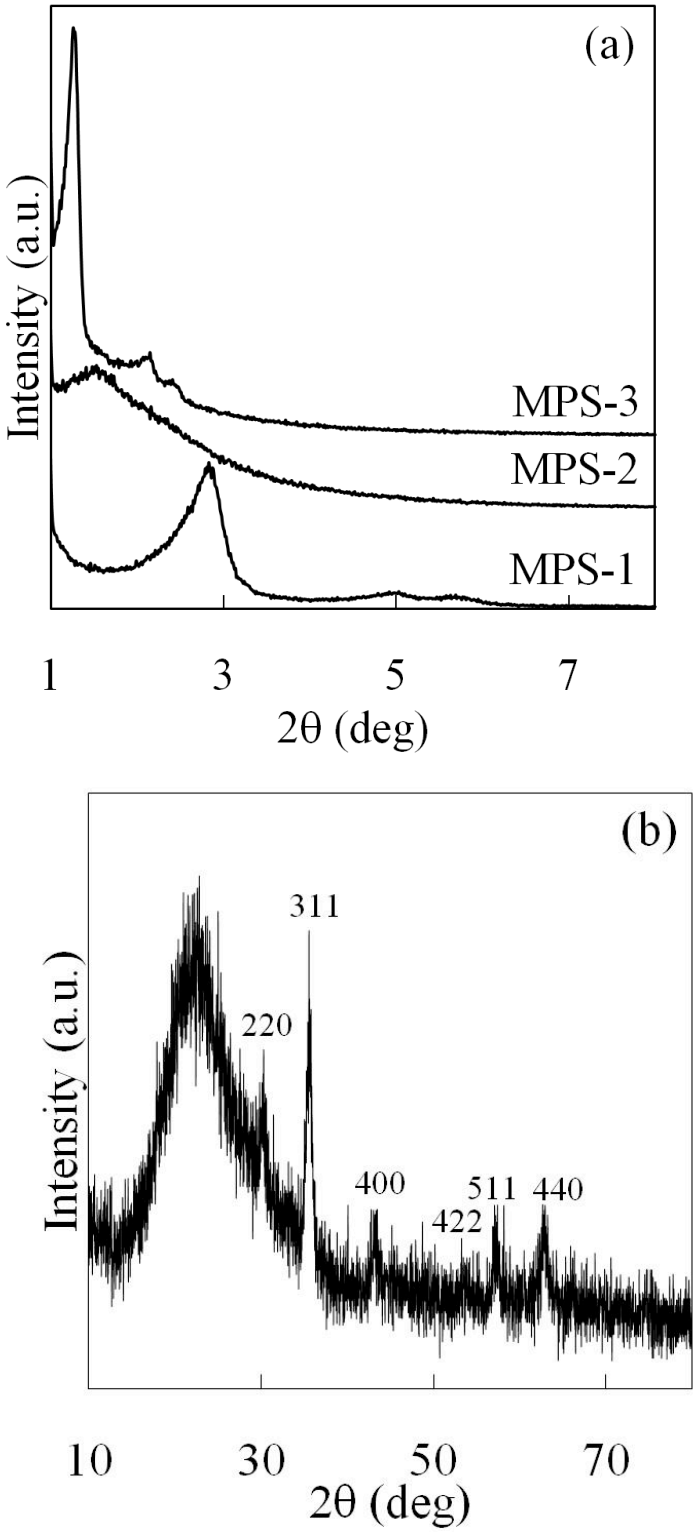

Figure S4. X-ray diffraction patterns: (a) Small-angle patterns for MPS-1, MPS-2, and MPS-3; (b) Wide-angle pattern for MN-MPS-2.

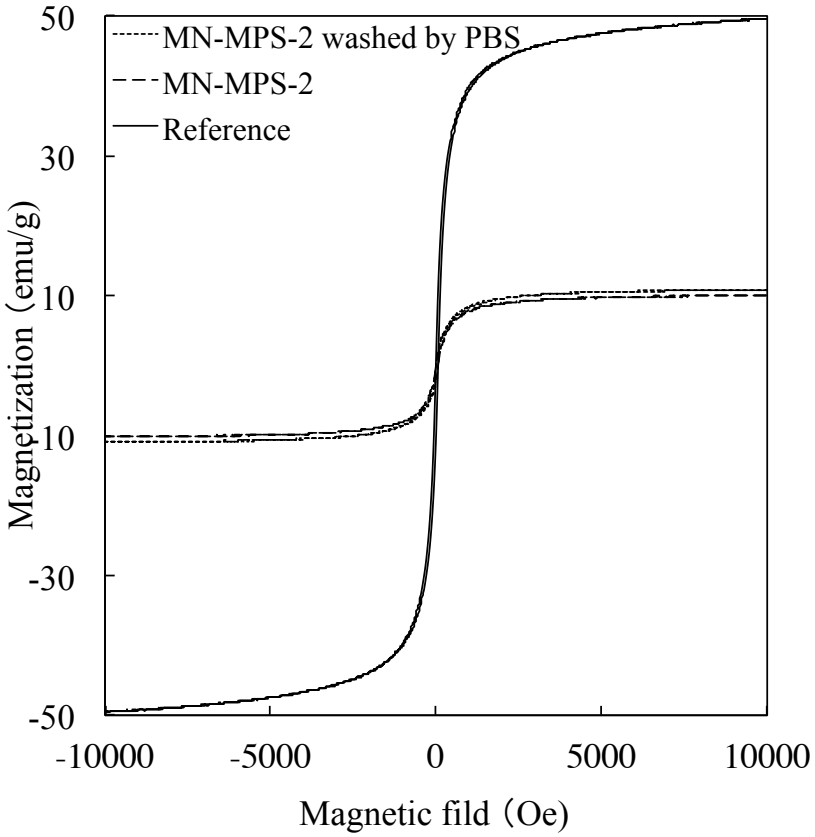

Figure S5. Susceptibility curves for the reference, MN-MPS-2, and washed MN-MPS-2.

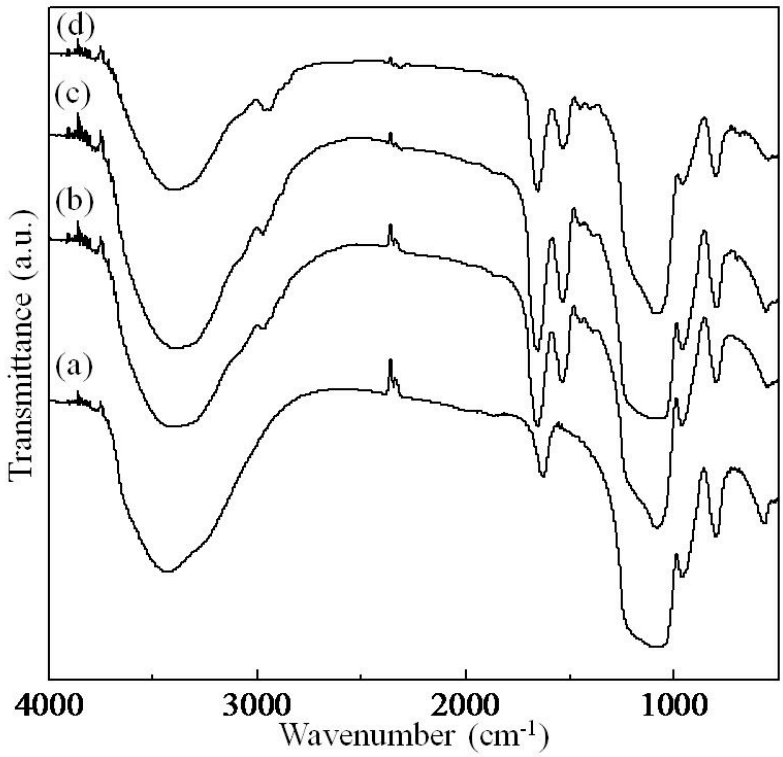

Figure S6. Fourier-transform infrared spectra: (a) MPS-2 material; (b) Subtilisin immobilized on no-substituted MPS-2; (c) Subtilisin immobilized on ethyl-group-modified MPS-2; (d) Subtilisin immobilized on mercaptopropyl group-modified MPS-2.

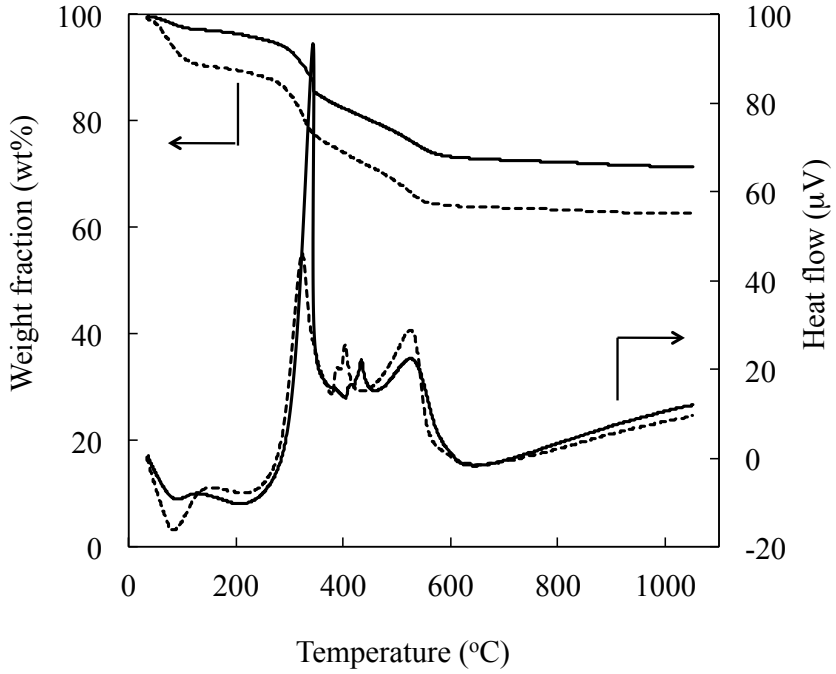

Figure S7. Thermogravimetry- and differential thermal analysis curves for subtilisin immobilized on SH-MPS-2 (solid line) and Et-MPS-2 (dotted line).

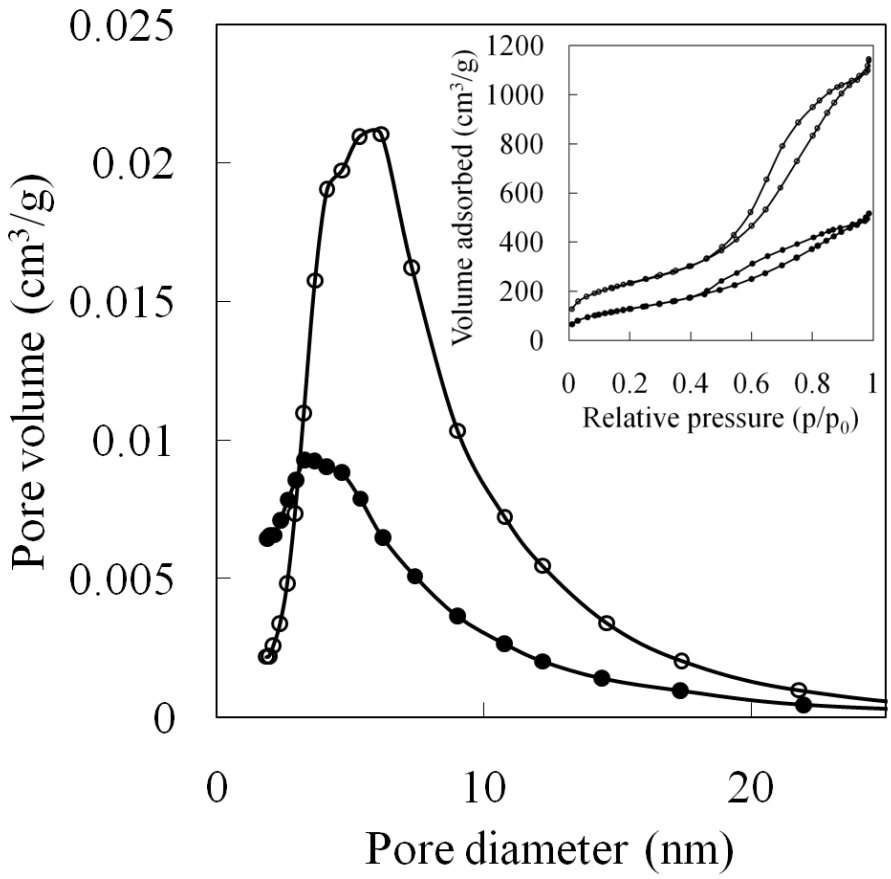

Figure S8. Pore-size distributions obtained from adsorption isotherms by the BJH method. N_2 adsorption-desorption isotherms before and after immobilization onto MPS-2. (inset) ●= MPS-2 immobilized subtilisin ; ○= MPS-2 before immobilization of subtilisin.