Preparation and catalytic evaluation of cytochrome c immobilized on mesoporous silica materials

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Cytochrome c (cyt. c, molecular size of 2.5 nm × 2.5 nm × 3.7 nm) was immobilized on six types of mesoporous silicates (MPS), having pore sizes from 2.8 to 15.0 nm, and their catalytic activities were evaluated by oxidation of thioanisol with H₂O₂. Among the silica materials, cyt. c immobilized on MPS pore size of 3.4 nm) showed the best catalytic activity, and its relative activity was improved 1.7 times over that of cyt. c solution. Enhancement of catalytic activity was obtained by immobilization on MPS having a similar size (mesopore) to the molecular size of cyt. c. Activity stability of cyt. c was also evaluated under the various conditions that caused protein inactivation, like HCl, urea, methanol, and guanidine. As results, stability was improved by the immobilization on silica material having a larger pore size (15.0 nm), offered an environment for encapsulating entire cyt. c molecules, inhibiting cyt. c from unfolding and irreversible decomposition.

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1. Introduction

Ordered mesoporous silica materials (MPSs) templated by surfactant micelles have attracted much attention because of the elegant, predictive synthesis approach and their many potential applications.¹⁾ Among the wide variety of silica mesophases, MCM-41 and SBA-15 have been the most extensively investigated. Though both exhibit two-dimensional hexagonal structures (*p6 mm*), they have some notable differences: (i) SBA-15 is endowed with larger pores and thicker pore walls than MCM-41; (ii) MCM-41 is purely mesoporous in nature, whereas a typical SBA-15 silica contains a significant amount of micropores within the pore walls; and (iii) while the channels of MCM-41 are not connected to each other, those of SBA-15 are interconnected via micropores or secondary mesopores. The pore diameter of MCM-41 can be systematically varied between 2–6 nm. Other interesting physical properties of MCM-41 include a highly specific surface area of up to 1500 m²/g, a specific pore volume of up to 1.3 cm³/g, and high thermal stability, all of which make it suitable for many catalytic applications. The pore diameter of SBA-15 can be tuned from 5 to 20 nm, with surface area and pore volume similar to MCM-41.^{2)–5)}

The issue of pore size tailoring has always captured the attention of research scientists and engineers, because the use of the surfactant micelles as templates opened an opportunity to adjust the pore size through a well-known approach already used for micelle size adjustment. In particular, the increase in the surfactant chain length or the addition of a micelle expander (swelling reagent) were effective in pore size control in MPSs including silicas with two-dimensional hexagonal arrays of cylindrical pores: MCM-41 templated by alkylammonium surfactants (e.g., cetyl trimethylammonium chloride) and SBA-15 silica templated by Pluronic block copolymers [e.g., poly-

(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)]. The use of micelle expanders is particularly convenient, as in an ideal case, one can continuously increase the pore diameter by increasing the amount of the swelling agent used. In the case of MCM-41 and SBA-15, transmission electron microscopy (TEM) images indicated that the use of micelle expander 1,3,5-trimethylbenzene (TMB) allows one to adjust the pore diameter up to 10 and 30 nm, respectively.

In addition to the nature of the pore system, i.e., pore size, shape, and connectivity, depending on the target application, the morphology of the mesophase may be particularly important. Simple morphologies with short, unhindered path length such as small spheres and crystal-like particles, as well as short, straight rods, are beneficial for applications limited by intraparticle diffusion processes such as catalyst separation, guest molecule encapsulation, and internal surface modification.^{6),7)} Thus, not surprisingly, extensive study was devoted to the morphological control of mesoporous silica materials and organosilicates.^{8)–12)}

Biocatalysts such as enzymes and antibodies have been immobilized on supports by several methods including cross linking, covalent attachment, physical entrapment, and physical adsorption. The solid supports used are always polymer resins, natural polymeric derivatives, organic gels, fibers, zeolites, and MPSs. Among these supports, MPSs have attracted particular interest because of the physiological features described above.^{13)–19)} Taking advantage of the structural characteristics of MPSs, enzyme molecules can be directly immobilized through the interaction with the surface silanol group by hydrogen bonding or electrostatic forces. The enzyme immobilized on MPSs supports exhibits higher thermal and pH stability than the native enzyme. The amount of protein adsorbed on MPS depends on the solution pH as well as the specific volume and composition of the adsorbents. The morphologies of MPSs also play an important role in their immobilization performance for proteins.

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Recently, oxidative reactions by biocatalysts (cytochrome c, cytochrome P450, peroxidase, laccase, monooxygenase, and alcohol oxidase) were applied to the degradation, synthesis, and polymerization of organic molecules.^{20)–23)} Cytochrome c (cyt. c) has catalytic ability in the enantioselective sulfoxidation of aryl alkyl sulfides to optically active sulfoxides,²⁴⁾ which are of great pharmaceutical interest as chiral intermediates.²⁵⁾ Moreover, immobilization of oxidative enzymes on MPSs enhances enzyme stability and increases ease of separation and recovery for reuse, while maintaining activity and selectivity. Ono et al.,²⁶⁾ described the immobilization of cyt. c in reversed micelles, and the catalytic activity in reverse micellar solution is 10-fold higher than that in water. Washmon-Kriel et al.²⁷⁾ reported the immobilization of cyt. c in MCM-41 and SBA-15 and the evaluation of the redox activity by cyclic voltammetry. Also, Vinu et al.²⁸⁾ described the adsorption isotherms on various MPSs (MCM-41, SBA-15, and Al-incorporated MPSs). They found that the amount of adsorption on SBA-15 is higher than that on MCM-41. However, little has been reported about the immobilization of cyt. c on different structures of MPSs and the evaluation of catalytic activity and stability of immobilized cyt. c under the various reaction conditions.

In our previous paper, we reported that other type of mesoporous silicate (FMS-16) prepared from alkyltrimethylammonium–kanemite complex,³⁾ with a pore size of 6.2 nm, were good immobilization carriers for lipase from *Candida antarctica* (molecular weight of 33 kDa and dimension of 3.0 nm × 4.0 nm × 5.0 nm). Moreover, these mesoporous material–lipase composites showed enantioselectivities for racemic secondary alcohols similar to those of free enzymes, indicating that the active site of the enzymes immobilized in MPSs remained with no denaturation. Also, our recent communication described that the immobilized aldolase I antibody (monoclonal antibody; molecular weight of 150 kDa and dimension of 14.5 nm × 8.0 nm × 4.0 nm) shows enhanced catalytic activity, with about 1.8 times the average reaction rates compared to that of the native antibody for the mesoporous silica with 16-nm pore size. These results indicate that catalytic properties of enzymes and antibodies are greatly depended on the pore diameters and morphologies of MPSs.

On these bases, we selected two types of MPS (MCM-41, SBA-15, and their derivatives) with various pore (2.8–15.0 nm) and particle (30–1000 nm) sizes for obtaining immobilized cyt. c catalysts with optimized features, because these MPSs were easily controlled their particle characteristics by the procedure described in the many references.¹⁾ The design includes every step of the process: the choice of the solid support (mesoporous, high surface area, and narrow pore size distribution), morphologies of the supports, and characterization of the biocatalyst (protein loading in mesopore, activity, and stability). The oxidation reactions entailed oxidation of thioanisol with H₂O₂ in aqueous solution/organic solvent mixture conditions.

2. Materials and methods

2.1 Mesoporous silica materials (MPSs) synthesis

Mesoporous silica material MCM 1 was synthesized according to the modified procedure reported previously.⁸⁾ Cetyl trimethylammonium bromide (1.16 g) was added to deionized water (58.0 g), and the mixture was stirred for 1 h to obtain a clear solution. Nitric acid (HNO₃; 65 wt %, 5.0 g) solution and TEOS (3.0 g) were dissolved into the mixture. After stirring for 11 min, the clear solution was poured into an aqueous solution of 8.12 g of CH₃COONa and 1.75 g of NaOH (pH = 5.5). A white

precipitate was obtained within 1 min; it was filtered and then washed with 50 ml of deionized water and 50 ml of ethanol. After drying overnight at 80°C for 10 h, the sample was heated to 550°C (rate: 1°C/min) in air and kept at this temperature for 4 h.

Other MCM materials (MCM 2 and MCM 3) were prepared according to the previous report.⁹⁾ Cetyl trimethylammonium bromide (CTAB, 2.6 g) was dissolved in a hydrochloric (HCl) solution (30 g, pH 0.5), and the solution was stirred for 1 h at room temperature. Triblock copolymer (Pluronic F127; EO₁₀₆-PO₆₀-EO₁₀₆, number-average molecular weight (*M_n*) ca. 12600 g/mol, BASF) was dissolved in a solution without (MCM 2) or with CTAB (MCM 3) for the material preparation. Tetraethoxysilane (TEOS; 99%, Wako) was added to the solution, and the mixture was stirred for another 2 h. 3.0 g of ammonia (28 wt %, 14.7 M) was added at once. After stirring for 24 h, the white precipitate obtained was filtered and washed with 50 ml of deionized water, and the solid material was treated with the same procedure as that used for MCM 1.

Mesoporous silica material SBA (SBA 1) and its derivatives (SBA 2 and SBA 3) were prepared using the triblock copolymer EO₂₀PO₇₀EO₂₀ (Pluronic P-123; *M_n* ca. 5800 g/mol, Aldrich), TEOS, and *n*-decane as a reagent for controlling the morphology (SBA 2)¹⁰⁾ or trimethylbenzene (TMB, Wako) as a swelling reagent for expanding the pore diameter (SBA 3).¹¹⁾ In a typical synthesis, 2.25 g of TEOS was added dropwise to a solution of P-123 (1 g) in 30 g of 2 M HCl and 7.5 g of H₂O. This mixture was stirred at 40°C for 24 h and then transferred into an autoclave for further reaction at 100°C for 48 h after stirring. The product was filtered, dried at 80°C for 10 h, and calcined at 550°C for 4 h in air (heating rate of 1°C/min). *n*-Decane (7.5 g) or TMB (0.3 g) was added to an HCl solution containing P-123 before combining TEOS.

2.2 Characterization

Wide-angle X-ray diffraction (XRD) spectra were recorded on a Rigaku RINT2100V/PC diffractometer (Fe K α , 40 kV, 30 mA), and small-angle XRD spectra were recorded on a Rigaku RINT-TTR diffractometer (Cu K α , 50 kV, 300 mA). The particle morphologies of the obtained MPSs were observed by field emission scanning electron microscopy (FE-SEM) with a Hitachi S-3000 instrument operated at 20 kV. TEM images were taken by a JEOL JEM 2010, operated at 200 kV. N₂ adsorption–desorption isotherms were measured using a Shimadzu TriStar 3000 system at 77 K after the samples were preheated at 130°C for 4 h under vacuum. Specific surface areas were calculated by the Brunauer–Emmett–Teller (BET) method and the pore size distribution for mesopore was calculated using the Barrett–Joyner–Halenda (BJH) model. To evaluate the secondary structure of the cyt. c protein immobilized on MPS, fourier transform infrared (FT–IR, JOSCO Co., MFT-2000) spectra were obtained in the transparent mode in the wavenumber range 4000–400 cm^{–1} using a pellet made by mixing 99% KBr with 1% sample.

2.3 Immobilization of cyt. c on MPSs and measurement of their oxidation activities

Cyt. c (from horse heart, molecular weight = 12 kDa, isoelectric point = 10.7, dimension = 2.6 nm × 3.2 nm × 3.3 nm, SIGMA, St. Louis, MO) was used. Solid support (3.0 mg) was suspended in 20 μ l of cyt. c solution (12.5 mg/ml) in 480 μ l of 10 mM phosphate buffer (pH = 7.0) for 24 h at 4°C with stirring, which was confirmed to be long enough to reach the adsorption equilibrium. The supernatant was first separated by centrifugation after the solid materials were washed three times with cold

water. The amount of immobilized cyt. c was calculated from the concentration difference in supernatant determined according to the Bradford method.²⁹⁾ The activity assays on immobilized and free cyt. c were determined by oxidation of thioanisol with H_2O_2 . The reaction conditions and the determination method of substrate conversion are as follows: the solution of thioanisol for enzymatic reaction was prepared by dissolving 125 mg thioanisol in 1.0 ml methanol. For a 3.0 mg immobilized enzyme sample, 2.0 μl of thioanisol solution, 2.0 μl of 1 M H_2O_2 , 500 μl of methanol, and 500 μl of 10 mM phosphate buffer at pH = 7.0 were added and stirred for 30 min at 25°C. Also, the catalytic activity of free enzyme (20 μl of cyt. c solution) before immobilization is expressed as 100% (the same as used originally in each immobilized enzyme preparation). After the reaction terminated, the supernatant obtained by centrifugation was subjected to high-performance liquid chromatography (HPLC) to determine the conversion. HPLC was performed on a Shimadzu LC 10-AD with UV detection at 242 nm and Wakosil-II 5C18HG (Wako Pure Chemicals, Osaka) with a mobile phase of acetonitrile/water (8.0/2.0; v/v) at a flow rate of 0.5 ml/min. The retention times of thioanisol and oxidation product methyl phenyl sulfoxide were 7.4 and 4.2 min, respectively. No oxidation reactions occurred without cyt. c under the abovementioned reaction conditions. Each reported value was the mean of at least three experiments.

3. Results and discussion

3.1 Characterization of mesoporous silica materials (MPs)

MCM 1, MCM 2, and MCM 3 mesoporous silica materials were synthesized in an oil-in-water microemulsion using alkyl trimethylammonium as a template (MCM 1 and MCM 2) and triblock copolymer F127 as a morphology control of the particle (MCM 3). Also, SBA 1, SBA 2, and SBA 3 were synthesized using triblock copolymer P123 as a template and *n*-decane (SBA 2) or trimethylbenzene as a swelling reagent (SBA 3). These materials were characterized for the morphologies of the particles by FE-SEM and TEM images, their mesoporous phase identification, BET surface area, BJH pore size distribution and sharpness of BJH pores.

The particle morphologies and pore structures of MPS materials are shown in Fig. 1 (FE-SEM) and Fig. 2 (TEM). The particles MCM 1, MCM 2, and MCM 3 were composed of small particles below 100 nm, which can be roughly measured from the SEM images. Slight morphological differences were detected for the MCM materials with (MCM 2) and without F127 (MCM 3); that is, the addition of F127 reduced the particle size below 50 nm, and the grains had a relatively sharp distribution. Elongated particles measuring ~ 800 nm in length and ~ 500 nm in width were obtained from the conventional procedure of SBA-15-type mesoporous silica (SBA 1). Addition of *n*-decane produced a silica particle with a homogeneous rod-like morphology. The diameter of the nanorod is less than 200 nm and the rod length is around 300 nm. Thus, a large amount of *n*-decane used in the micelle preparation can be regarded as a morphology controller as well as a swelling reagent. SBA 3, synthesized from a mixture of two organic templates, P123 and TMB, displayed a larger size (~ 1000 nm), which was detected from the SEM image. Schmidt et al.¹²⁾ reported that siliceous mesostructure cellular forms prepared with a procedure similar to that used for SBA 2 had large particles measuring approximately 15 μm . With an addition of TMB, the morphology turns from cylindrical (SBA 1) to granular structures.

Fig. 1. SEM micrographs of the mesoporous silica materials. (A) MCM 1, (B) MCM 2, (C) MCM 3, (D) SBA 1, (E) SBA 2, and (F) SBA 3. Scale bar: 500 nm.

Fig. 2. TEM micrographs of the mesoporous silica materials. (A) MCM 2 and (B) SBA 3. Scale bar: 20 nm.

It can be observed from the TEM images (Fig. 2) that MCM 2 is formed of uniform and small domains of cylindrical small pores (~ 3 nm) and the SBA 3 obtained had homogenous, large pores (~ 15 nm).

Structural properties of the MPS materials prepared in the present case derived from nitrogen sorption measurements are summarized in Table 1. All MCM samples synthesized had a very high surface area of 765–1131 m^2/g and a pore diameter between 2.8–3.2 nm with a narrow pore size distribution. The average pore diameter of the SBA samples gradually increased from 9.5 to 15.0 nm with a swelling reagent, *n*-decane or TMB, in the organic template. Also, the samples exhibited similar large mean pore diameters (9.5–15.0 nm), high BET surface areas of 605–689 m^2/g , and pore volumes of 1.2–1.6 cm^3/g .

Table 1. Structural properties of mesoporous silicates prepared in this manuscript

Mesoporous silica	Pore diameter ^a (nm)	Surface area ^b (m ² /g)	Pore volume ^a (cm ³ /g)	Average particle size ^c (nm × nm)
MCM 1	2.8	765	1.2	40 × 40
MCM 2	3.4	1131	1.3	80 × 80
MCM 3	2.8	860	1.7	30 × 30
SBA 1	9.5	656	1.2	800 × 500
SBA 2	11.3	689	1.4	300 × 200
SBA 3	15.0	605	1.6	1000 × 800

^aCalculated according to BJH analysis (adsorption branch between 1.7 and 300 nm diameters). ^bSpecific surface area according to BET. ^cAverage particle size is determined by SEM measurements.

Fig. 3. (A) Wide-angle and (B) small-angle XRD of the mesoporous silica materials: MCM 1, MCM 2, MCM 3, SBA 1, SBA 2, and SBA 3.

The adsorption and desorption isotherms of nitrogen on MCM 1, MCM 2, and MCM 3 showed the typical type IV isotherm. At the adsorption branch, the adsorption amount increased gradually with an increase in the relative pressure (P/P_0) between 0.3 and 0.4 caused by capillary condensation of nitrogen in the mesopores. The desorption branch of the isotherm coincides with the adsorption branch. Nitrogen isotherms on SBA 1, SBA 2, and SBA 3 also behave like representative type IV curves, with a sharp capillary condensation step in the relative pressure range of $0.7 < P/P_0 < 0.9$ in the isotherms of three SBA samples, associated with large pores of $> \sim 8.0$ nm.

The mesoporous structure of MPS materials was investigated using powder XRD (**Fig. 3**). MCM 2 displayed three well-resolved diffraction peaks corresponding to (100), (110), and (200) reflections, characteristic of mesoporous materials with ordered two-dimensional (2D)-hexagonal mesostructure. MCM 1 exhibited a weak and broad diffraction peak in the low-angle diffraction region, showing that the mesostructure of the material synthesized at low pH = 5.5 was not well ordered. No sharp peak could be obtained for MCM 3, in which F127 triblock copolymer was mixed with cetyltrimethylammonium for reducing the particle size below 50 nm; this result can be attributed to the disordered mesostructure. The XRD results indicate that an

Fig. 4. Effect of pore size of MPSs on equilibrium adsorption isotherms for cyt. c.

additive (F127) or an acidic condition for preparation of MCM is unfavorable for the formation of an ordered mesostructure.

Relative positions of XRD peaks for SBA 1 prepared according to the procedure for conventional SBA-15 mesoporous silica corresponded to the 2D-hexagonal structure characteristics. SBA 2 was synthesized from a mixture of P123 and *n*-decane for changing the morphology of the particle. The XRD pattern of SBA 2 indicated three resolved peaks that were indexed as (100), (110), and (200) diffractions associated with a 2D-hexagonal symmetry, but the patterns were somewhat less well resolved for SBA 2 compared to that for SBA 1. SBA 3 was synthesized using the organic auxiliary chemical TMB as a micelle expander, mixed with the silica source (TEOS) and simultaneously added to the surfactant solution under mechanical stirring. The XRD data showed that SBA 3 exhibited a mesoporous structure pattern; however, TMB led to phase formation from a highly ordered hexagonal mesostructure to mesostructure cellular form.

3.2 Effect of pore size of MPS for the adsorption amount of cyt. c

Figure 4 shows adsorption isotherm for MCM 3 and SBA 2/cyt. c systems; 1, 2, 3, 4, and 5 ml of 250 or 125 μ g/ml were stirred with 3 mg of MCM 3 or SBA 2, respectively, overnight at 4°C, then the absorbance of the supernatant was measured and the equilibrium concentration and adsorption amount were calculated. The maximum equilibrium adsorption amount of cyt. c onto MCM 3 and SBA 2 was 0.22 and 0.38 mg protein/mg silica, respectively. The dimension of cyt. c was 2.6 nm × 3.2 nm × 3.3 nm. Since the dimension of cyt. c was similar to the pore size of MCM 3 (2.8 nm), cyt. c can be expected to be fitted into the pores. In contrast, since the larger pore size of SBA 2 (11.3 nm) is approximately three times the dimension of cyt. c, the latter was absorbed with very high capacity. The pore size-dependent potential of the mesoporous materials is a very important factor in biocatalyst and biosensor applications, and studies are ongoing in this regard.

Fig. 5. (A) Changes in nitrogen adsorption isotherms of cyt. c-MCM 3 composites and (B) corresponding pore size distribution obtained from the adsorption branch by the BJH method.

To confirm the structural stability of cyt. c after adsorption on the mesoporous silicates, FT-IR spectra, especially in amide I and amide II regions, were recorded for all free cyt. c and the cyt. c-immobilized SBA 2 adsorbents (0.4 mg cyt. c/mg SBA 2) (data not shown). The amide band I because of the C=O stretching mode and the amide II band because of the N-H deformation were assigned to 1670 and 1555 cm^{-1} , respectively. These values obtained for SBA 2 were very close to those observed for free cyt. c (1675 and 1560 cm^{-1} , respectively). The intensity ratio between these two bands was virtually unaffected by adsorption of the protein onto SBA 2. These results indicated that no serious denaturation accompanied the change in the secondary structure (α -helix and β -sheet) during the adsorption process. Further studies about the stabilization of proteins onto mesoporous materials by circular dichroism and differential scanning calorimetry as well as FT-IR are in progress in our laboratory.^{30,31)}

3.3 Cyt. c loading onto MPS

Nitrogen adsorption isotherms and the pore size distribution curve for the mesoporous silica material before and after cyt. c adsorption is shown in **Fig. 5** (for MCM 3) and **Fig. 6** (for SBA 2). Also, pore characteristics of the silica materials before and after immobilization are shown in **Table 2**. The reduction in pore volume after cyt. c immobilization for two MPSs was most likely caused by the adsorption of protein inside the pores. For MCM 3, only a small amount of cyt. c (0.1 mg protein/mg MCM 3) could occupy the mesoporous space because of the relatively small main entrance size of the pore (2.8 nm) in comparison to cyt. c molecular size (2.6 nm $\times$ 3.2 nm $\times$ 3.3 nm). Large decrease in pore volume of MCM 3 (22.7%) was observed after cyt. c immobilization. Therefore, most of the protein molecules were expected to be adsorbed on the pore entrance. The pore volume (23.3%) did not change for the adsorption with higher concentration of protein (0.2 mg/mg). In contrast, SBA 2, which had a larger main pore diameter (11.3 nm) than MCM 3, undoubtedly allowed comfortable entrapment of protein in the

pore. The pore volume of SBA 2 decreased by 4.3% when a low concentration of protein (0.1 mg protein/mg SBA 2) was used for immobilization. The slight decrease in SBA 2 pore volume was probably due to its initial larger pore volume compared to MCM 3. In the case of higher protein concentration (0.4 mg/mg) for the adsorption experiment, a large decrease of SBA pore volume was observed (29.5%). The decreases in pore diameter and pore volume of both materials after cyt. c adsorption suggested that some cyt. c was immobilized on the pore mouths of the supports.

3.4 Oxidation activity of thioanisole to phenyl-methylsulfoxide by cyt. c immobilized on mesoporous silica materials

Immobilization experiments were performed by contacting 3 mg of mesoporous silica with a protein concentration of 12.5 mg/ml. Mesoporous silicate (3 mg) was suspended in 20 μl cyt. c solution (including a protein amount of 0.25 mg) in 1 ml of 100 mM phosphate buffer (pH = 7.0) overnight at 4°C with stirring. The supernatant was first separated from the solid materials by centrifugation, and the protein content in the supernatant was determined according to the Bradford method. The adsorption experiments demonstrated that all of 0.25 mg of the cyt. c protein was immobilized on 3 mg of MPS.

Oxidation of thioanisole was used to assess the activity of the immobilized and free cyt. c. All amounts of MPS-immobilized cyt. c prepared from MPS and cyt. c solution were used for the catalytic experiments (**Fig. 7**). For an approximately 3.2 mg immobilized protein sample, 1004 μl of a mixture of thioanisole, H_2O_2 , methanol, and phosphate buffer was added and stirred for 30 min at 25°C. The rate of conversion from thioanisole to the oxidation product phenylmethylsulfoxide was determined by HPLC. The catalytic activity of free cyt. c (0.25 mg) before immobilization is expressed as 100% under the same reaction conditions. No oxidation reactions occurred without cyt. c or with only MPS, which has no cyt. c, under the above mentioned reaction conditions. As shown in **Fig. 8** immobilization of cyt. c

Fig. 6. (A) Changes in nitrogen adsorption isotherms of cyt. c-SBA 2 composites and (B) corresponding pore size distribution obtained from the adsorption branch by the BJH method.

Table 2. Physicochemical properties of the cyt. c-MPSs composites

mg(Cyt.c)/ mg(MPS)	Pore volume (cm³/g)		Pore size (nm)	
	MCM 3	SBA 2	MCM 3	SBA 2
0	1.72	1.39	2.7	11.3
0.1	1.33	1.33	2.5	10.9
0.2	1.32	—	2.4	—
0.4	—	0.98	—	8.7

Fig. 7. Oxidative reaction of thioanisole with cyt. c immobilized on MPS.

resulted in enhancement of catalytic activity on all MPSs except SBA 1; that is, the MPS provided an environment where the adsorbed cyt. c was catalytically more active than aqueous cyt. c, with MCM 2 exhibiting the largest relative activity. Relative rates of 171 and 100% were obtained for MCM 2 immobilized- and free-cyt. c, respectively. Also, cyt. c adsorbed on MCM 1 and MCM 3 (122 and 113%) showed less activity compared to that on MCM 2. Relative catalytic activities of 137 and 127% were obtained for SBA 2 and SBA 3, respectively, by oxidation reaction; however, an extremely low activity rate (77%) was observed for SBA 1. Both SBA 1 (800 nm × 500 nm) and SBA 3 (1000 nm × 800 nm) had especially large particle size; the long cylindrical structure of SBA 1 was not effective as a protein carrier for the catalytic reaction.

Next, we examined the stability of the catalytic activities immobilized on four MPSs (MCM 2, MCM 3, SBA 2, and SBA 3) under protein denaturation conditions. The stability experiments were performed as follows: the MPS-immobilized cyt. c (3 mg) was heated at 100°C for 10 min or was soaked overnight at 4°C in 0.01 M HCl, 5 M urea, 5 M guanidine

Fig. 8. Oxidation activities of immobilized cyt. c immobilized on mesoporous silica materials. Relative activity was calculated as follows: $1 - (\text{oxidation activity in the supernatant solution after immobilization}) / (\text{oxidation activity before immobilization}) \times 100\%$. The immobilization method is described in the text. The catalytic activity of free enzyme before immobilization is expressed as 100%. Free means the cyt. c solution before immobilization.

hydrochloride, or 50% methanol solution. Then, the remaining oxidation activity was checked by the above-described procedure. As a control, cyt. c solution (free cyt. c) was treated under the same conditions. The catalytic activity of the untreated free cyt. c is expressed as 100%. **Figure 9** shows the results of immobilized cyt. c's oxidation activity stabilization under the above mentioned conditions. It is evident that cyt. c immobilized onto SBA 3, with the largest pore size of 15.0 nm, had the highest stability, similar to the oxidation activity of SBA 3-immobilized cyt. c without the treatment (Fig. 8). In contrast, cyt. c adsorbed on MCM-type silicates showed lower stability than those on SBA-type silicates, especially MCM 3, with the smallest particle size of 30 nm × 30 nm, which was less active after the treatment of the denaturation conditions. The activity stability of cyt. c on MCM 1 showed a similar tendencies to that

Fig. 9. Stability of oxidation activity of immobilized cyt. c under various conditions. Relative activity was calculated as follows: $1 - (\text{oxidation activity of immobilized or free cyt. c after thermal and solvent treatment}) / (\text{oxidation activity of free cyt. c before thermal and solvent treatment}) \times 100\%$. The catalytic activity of free enzyme before thermal and solvent treatment is expressed as 100%. Free means the cyt. c solution before immobilization.

on MCM 3. The support pore characters were important, because the activity stability of the immobilized cyt. c onto SBA 2 and SBA 3 was greater than that onto MCM 2 and MCM 3. The above results shows that the encapsulation of whole molecule of cyt. c inside the pore of MPS enhanced its stability, however, the stability effect for an each reagent is not clear. Supports with larger main pore diameters appeared to be better for protein storage stability than those with smaller pores. In addition, particle morphologies of mesoporous silica materials were one of the key parameters influencing storage stability of the protein.

4. Conclusion

Six types of mesoporous silica materials (MPS) were synthesized for the immobilization of cyt. c. The immobilized cyt. c activities were checked by the oxidation of thioanisole to phenylmethylsulfoxide with H_2O_2 as an oxidant. The catalytic activity and stability depended on the morphologies of the particles and pore characteristics. Maximum cyt. c activity was obtained when the protein was immobilized onto MCM 2 with the smallest pore size and small particle size (80 nm $\times$ 80 nm). The long cylindrical structure of SBA 1 indicated the lowest catalytic activity. SBA 3-immobilized cyt. c retained the highest activity after heating and solvent treatment, which was caused by protein denaturation, because SBA 3, with the largest pore size (15.0 nm), offered an environment for encapsulating entire cyt. c molecules, inhibiting cyt. c from unfolding and irreversible decomposition. Since the pore size of MCM-type materials is relatively small (~ 3.0 nm) and cyt. c molecules can only attach to the entrance of the pore, cyt. c molecules supported on MCM-type materials are less stable. Capacity measurements with two materials, MCM 3 and SBA 2, showed different capacities, and SBA 2, with a larger pore size (11.3 nm), had approximately 1.6 times higher saturation capacity than MCM 3 (2.8 nm). The conclusion of this study may be significant for understanding the protein immobilization in mesoporous silica materials and will undoubtedly be useful for their applications in the fields of biocatalysts and biosensors.

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