Catalytic Activity in Organic Solvents and Stability of Immobilized Enzymes Depend on the Pore Size and Surface Characteristics of Mesoporous Silica

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Received June 14, 2000. Revised Manuscript Received August 23, 2000

An enzyme, horseradish peroxidase (HRP), was adsorbed in the manner of the single immersion method on the silica mesoporous materials FSM-16, MCM-41, and SBA-15 with various pore diameters from 27 to 92 Å, and their enzymatic activities in an organic solvent and the thermal stabilities were studied. FSM-16 and MCM-41 showed a larger amount of adsorption of HRP than SBA-15 or silica gel when the pore sizes were larger than the 50 Å. The increased enzyme adsorption capacity may be due to the surface characteristics of FSM-16 and MCM-41, which would be consistent with the observed larger adsorption capacity of cationic pigment compared with anionic pigment for these materials. The immobilized HRP on FSM-16 and MCM-41 with pore diameter 50 Å showed the highest enzymatic activity in an organic toluene and thermal stability in aqueous solution at the temperature of 70 °C. The immobilized enzymes on the other mesoporous materials including large or small pore sized FSM-16 showed lower enzymatic activity in an organic solvent and thermal stability. Both surface character and size matching between pore sizes and the molecular diameters of HRP were important in achieving high enzymatic activity in organic solvent and high thermal stability.

Introduction

The immobilization of enzymes on inorganic materials is very useful in practical applications, because of the potential to improve the stability of enzymes under extreme conditions.[1] In recent years, periodic mesoporous materials with uniform pore diameters of 15−300 Å have been reported.[2−11] Because these pore diameters approximate molecular diameters of enzymes, their application as useful enzyme supports has been suggested.[12] MCM-41 type mesoporous materials have been investigated as supports for small globular enzymes, such as cytochrome *c* (MW = 12K).[13,14] However, the mechanism underlying enzyme stabilization or adsorption in mesoporous silica has not been explained clearly. Peroxidases have been widely utilized in applications for the decomposition of pollutants, such as lignin or dioxins. In particular, horseradish peroxidase (HRP) has been widely applied as an effective biocatalyst.[15,16] We report here that the combination of two factors, i.e., the surface characteristics of the mesoporous silica and matching of sizes between the enzyme molecule and the pore diameter of the mesoporous silica, are essential for stabilizing the enzymatic activity of HRP.

Experimental Section

Materials. Horseradish peroxidase (HRP) was purchased from Calzyme Laboratories Inc. Three types of ordered mesoporous materials, FSM-16, MCM-41, and SBA-15, were synthesized. A FSM-16 material with a pore diameter of 27 Å was prepared from kanemite by using hexadecyltrimethylammonium chloride, according to the method reported previously.[8] FSM-16 materials with pore diameters of 51 and 89 Å were prepared from kanemite by using hexadecyltrimethylammonium chloride and 1,3,5-triisopropylbenzene (TIPB) in the molar ratio of TIPB/surfactant = 3 and dococyltrimethylam-

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10.1021/cm000487a CCC: $19.00
Published on Web 11/02/2000

Table 1. Physicochemical Properties of Mesoporous Silicas and the Amounts Adsorbed of HRP and Pigments by Mesoporous Silica

porous silica	pore[a] diameter (Å)	BET surface area ($m^2\ g^{-1}$)	total pore volume ($cm^3\ g^{-1}$)	adsorbed amounts HRP[b] ($mg\ g^{-1}$)	MB[c] ($mmol\ g^{-1}$)	ASS[d] ($mmol\ g^{-1}$)
FSM-16/27	27	927	0.84	28	0.29	0.09
FSM-16/51	51	848	0.95	133	0.26	0.09
FSM-16/89	89	770	1.22	183	0.23	0.10
MCM-41/50	50	949	1.01	98	0.20	0.07
MCM-41/66	66	820	1.15	147	0.19	0.09
SBA-15/50	50	695	0.56	10	0.13	0.09
SBA-15/92	92	810	0.94	24	0.12	0.07
silica gel	20−200	451	0.85	49	0.11	0.08

[a] Calculated from the adsorption branch of the N_2 isotherm. [b] Horseradish peroxidase. [c] Methylene blue. [d] Sodium anthraquinone-2-sulfonic acid.

monium chloride and TIPB in the molar ratio of TIPB/surfactant = 4, respectively. The other conditions were the same as for the synthesis of FSM-16 with the pore diameter of 27 Å.[8] MCM-41 materials with pore diameters of 50 and 66 Å were prepared from water glass (SiO_2/Na_2O = 2:1) by using dococyltrimethylammonium chloride and TIPB according to the method reported by Beck et al.[2] The molar ratios of TIPB/surfactant were 0.7 and 2 for the pore diameters of 50 and 66 Å, respectively. SBA-15 materials with pore diameters of 50 and 92 Å were prepared from tetramethyl orthosilicate (TMOS) by using nonionic triblock copolymer P_{104} [MW 4948, $HO(CH_2CH_2O)_{18}(CH_2CH_2CH_2O)_{58}(CH_2CH_2O)_{18}H$] and TIPB according to the method reported by Stucky et al.[9−12] The structures of the above products were confirmed by means of X-ray powder diffraction (XRD) using a Rigaku RINT-2200 diffractometer equipped with a Cu Kα radiation device.[8] An intense (100) diffraction peak and three other peaks (110, 200, and 210) of highly arranged orders connecting hexagonal structures were observed. The nitrogen adsorption isotherms at 77 K were measured with a Quantachrome Autosorb-1. Specific surface areas were calculated by the BET method using adsorption data ranging from P/P_0 = 0.05 to 0.35, and the pore diameter distribution curve was derived from the adsorption branch by the BJH method. The pore volume was taken as the P/P_0 where the isotherm sharply increased. The pore diameters and specific surface areas of FSM-16/*d*, MCM-41/*d*, and SBA-15/*d* (*d* indicates the pore diameter) are summarized in Table 1. Silica gel (Micro Bead Silica Gel-4B) was used as a reference material, which was purchased from Fuji Silysia Chemical Ltd.

Enzyme Immobilization. The immobilized enzyme was prepared according to the following procedure. FSM-16 or SBA-15 powder (250 mg) was added to 5 mL of a 10 mg mL^{-1} aqueous solution of HRP in a centrifuge tube. The mixture was stirred at 4 °C for 16 h and then centrifuged at 20 000*g* for 10 min at 4 °C, the resulting pellet being washed with deionized water three times and then vacuum-dried. Subtilisin Carlsberg was also immobilized in the mesoporous materials as described above. For the pH profile of enzyme adsorption, 2 mL of 5 mg mL^{-1} HRP was adsorbed to FSM-16 or SBA-15 in 10 mM Na acetate buffer for pH 3.0, 4.0, and 5.0 or 10 mM Na phosphate buffer for pH 6.0, 7.0, 8.0, and 9.0.

Pigment Adsorption. The adsorbed amounts of pigments on the mesoporous materials were measured by means of the as follows. An adsorbent (100 mg) was added to 40 mL of an aqueous solution of a pigment (0.01 mol cm^{-3}) with constant stirring at 60 °C for 7 h. The resulting product was filtered out, washed with deionized water (1000 mL), and then air-dried at room temperature. The adsorbed amounts of pigments were determined as the weight loss at temperatures from 100 to 900 °C under air by thermal gravimetrical analysis (Seiko Thermos plus TG-8210).

Thermal Stability. The thermal stability of HRP immobilized on FSM-16 (HRP/FSM) with various pore sizes was examined by means of phenol degradation.[15] HRP/FSM powder (5 mg) was added to 200 μL of 50 mM Na acetate buffer (pH 4.0) in an Eppendorf tube, followed by heating at 70 °C for 30, 60, 90, or 120 min. After centrifugation separation for 10 min, the precipitate was washed twice with deionized water, and then 200 μL of 50 mM Tris-HCl buffer (pH 7.5) and 4 μL of an aqueous solution of 5000 ppm phenol and 1 μL of 30% H_2O_2 were added to above the precipitate for the enzymatic reaction at 37 °C for 30 min. After centrifugation separation for 10 min, 50 μL of the suspension was added to 50 μL of 1% potassium ferricyanide in 1 M glycine (pH 9.6) and 100 μL of 1% 4-aminotipyrine in 1M glycine (pH 9.6). The residual activity was determined spectroscopically by immediately measuring the absorbance of the reactant at the wavelength of 490 nm.

Enzymatic Activity in an Organic Solvent. For evaluation of the catalytic activity of HRP immobilized in mesoporous silica, the oxidative reaction of 1,2-diaminobenzene in toluene was selected, and *tert*-butylhydroperoxide was used as an oxidant. A portion (20 mL) of 50 mM 1,2-diaminobenzene in anhydrous toluene and 5 mL of 1.1 M *tert*-butylhydroperoxide in decane were mixed in a triangular flask. The reaction was initiated by adding a complex of mesoporous solid immobilized HRP containing 0.5 mg of protein with constant stirring at 37 °C. Conversion at 37 °C for 7 h via the oxidative reaction was determined spectroscopically by measuring the absorbance of the supernatant at 470 nm. The same weights of porous silicas were also used to measure the catalytic activity as the background value.

Results and Discussion

Adsorption of Enzyme for Mesoporous Silicas. Table 1 lists the adsorbed amounts of HRP for various mesoporous materials and silica gel. The FSM-16/51, FSM-16/89, MCM-41/50, and MCM-41/66 (each of the average pore diameters were 51, 89, 50, and 66 Å) materials showed the adsorption of large amounts of HRP, 98−183 mg g^{-1}, while FSM-16/27 (pore diameter was 27 Å) showed the adsorption of a small amount, 28 mg g^{-1}. However, SBA-15/50 and SBA-15/92 (pore diameters were 50 and 92 Å) showed the adsorption of very small amounts of HRP, 10 and 24 mg g^{-1}, even though they had larger pore diameters than the HRP molecular diameter. Silica gel with a broad pore size distribution of between 20 and 200 Å also adsorbed a small amount, 49 mg g^{-1}.

The adsorption isotherms of nitrogen and the pore size distribution curve for the FSM-16 materials before and after HRP loading are shown in Figure 1. The pore volume of FSM-16/27 was not changed before and after HRP loading (Figure 1a). This result indicates that HRP molecules do not occupy the mesopore space of FSM-16/27 and exist on the outer surface of particles. The pore volume of FSM-16/51 and FSM-16/89 decreased after the immobilization treatment. The pore volume of the FSM-16/51 and FSM-16/89 materials after loading enzyme were ca. 52 and ca. 27% of those of the FSM-16

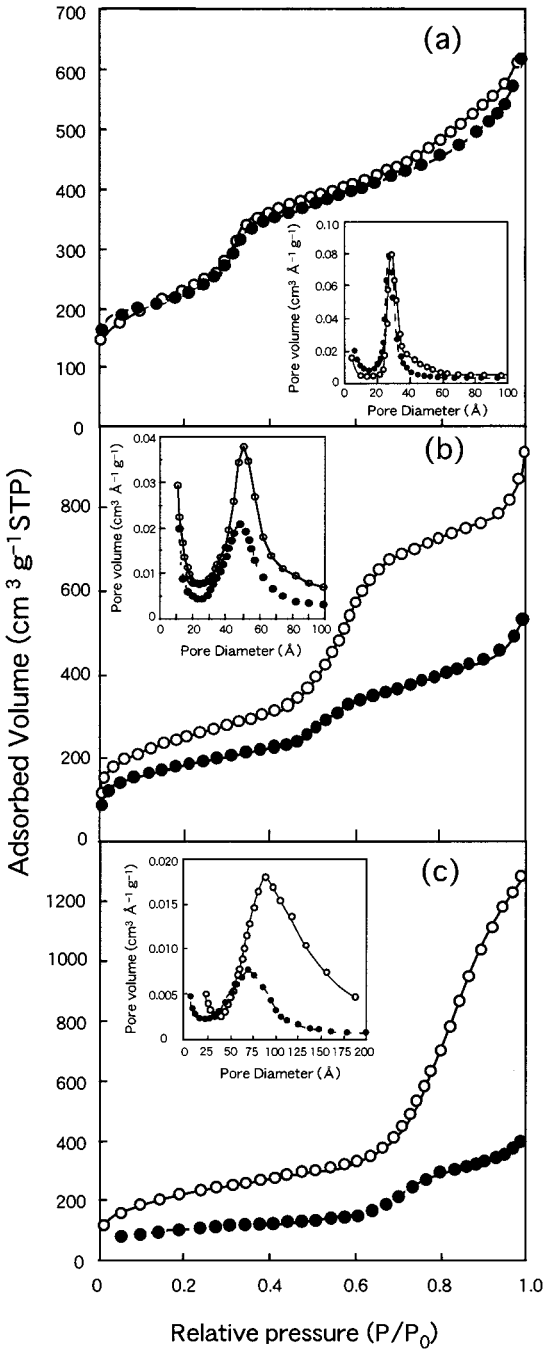

Figure 1. Changes of nitrogen adsorption isotherms plots and pore-size distribution curves from the adsorption branch before (○) and after (●) loading HRP in mesoporous silica FSM-16 supports with the average pore size of 27 Å (a), 51 Å (b) and 89 Å (c), respectively.

materials before loading enzyme. The total molecular volumes of HRP loaded to FSM-16/51 and FSM-16/89 were 8.6 and 9.2% of the intact pore volume the FSM-16 materials. These values were too small against the decrease in free pore volume of FSM-16/51 and FSM-16/89, 48 and 73%. This would be attributed to blocking the pore space of mesoporous material with some enzyme molecules. The larger decrease in pore volume for FSM-16/89 than FSM-16/51 is due to the large loading amount of HRP molecules for FSM-16/89 as shown in Table 1.

XRD measurements confirmed that the structure of FSM-16 was maintained after immobilization treatment. These results suggested that HRP molecules were adsorbed in the pore spaces of the FSM-16 materials with pore diameters of 51 and 89 Å.

The HRP molecule is an elongated object, and the long and short axes of the HRP molecule were ca. 64 and ca. 37 Å[17] as shown in Figure 2a. The geometrical dimensions also suggest it is reasonable to suppose that HRP molecules are immobilized in mesopore space of FSM-16 with pore diameters 51 and 89 Å.

The image models of immobilized HRP in the FSM-16 materials with various pore sizes were constructed using a computer schematic models (Figure 2b−d).

Effects of Surface Characteristics of Mesoporous Silica. To clarify why the adsorbed amounts of enzymes for FSM-16 and MCM-41 are larger than for SBA-15, the adsorbed amounts of cationic and anionic pigments were measured for the mesoporous materials and shown in Table 1.The adsorbed amounts of cationic methylene blue (MB) for the FSM-16 and MCM-41 materials were larger than those for the SBA-15 materials and silica gel. The adsorbed amounts of anionic sodium anthraquinone-2-sulfonic acid (ASS) were approximately equal for the three types of mesoporous materials. The adsorbed amounts of MB for FSM-16 and MCM-41 were 2−3 times greater than those of ASS, while significant differences in the adsorbed amounts were not observed between MB and ASS for SBA-15 and silica gel (Table 1). The results indicate that FSM-16 and MCM-41 materials tend to adsorb large amounts of cationic molecules. This adsorption property may be related to the methods used for their synthesis, in which cationic alkyltrimethylammonium salts were used as template for the synthesis, while SBA-15 materials were prepared by using a nonionic surfactant.

To further investigate the ionic effect of enzyme adsorption to mesoporous silicas, the pH profiles of the adsorbed amounts of HRP for FSM-16 and SBA-15 were examined (Figure 3). The large adsorbed amount of HRP in the lower pH region decreased steeply with increasing pH from 3 to 6 and decreased gradually in the higher pH region from 6 to 9. The titration curve for HRP[18] showed that HRP molecules have a positive charge at lower than pH 6 and that the charge density decreases with increasing pH. These results explain why FSM-16 and MCM-41 are favorable for the adsorption of cationic pigment molecules. In the case of SBA-15, a significant difference in the adsorption amount with pH was not observed. The higher HRP adsorption ability of FSM-16 or MCM-41 than SBA-15 suggests that the interactions between the enzyme and the mesopore surface are significant.

Enzymatic Activity in Organic Solvent and Thermal Stability. Figure 4 shows conversion yield of the 1,2-diaminobenzene oxidation reaction catalyzed by HRP immobilized in various mesoporous silicas and native HRP. Each immobilized enzyme used for the reaction had the same enzyme content, 0.5 mg of HRP, in comparing the activities of the enzymes. The immobilized HRP in mesoporous materials exhibited relatively high enzymatic activities compared to the back-

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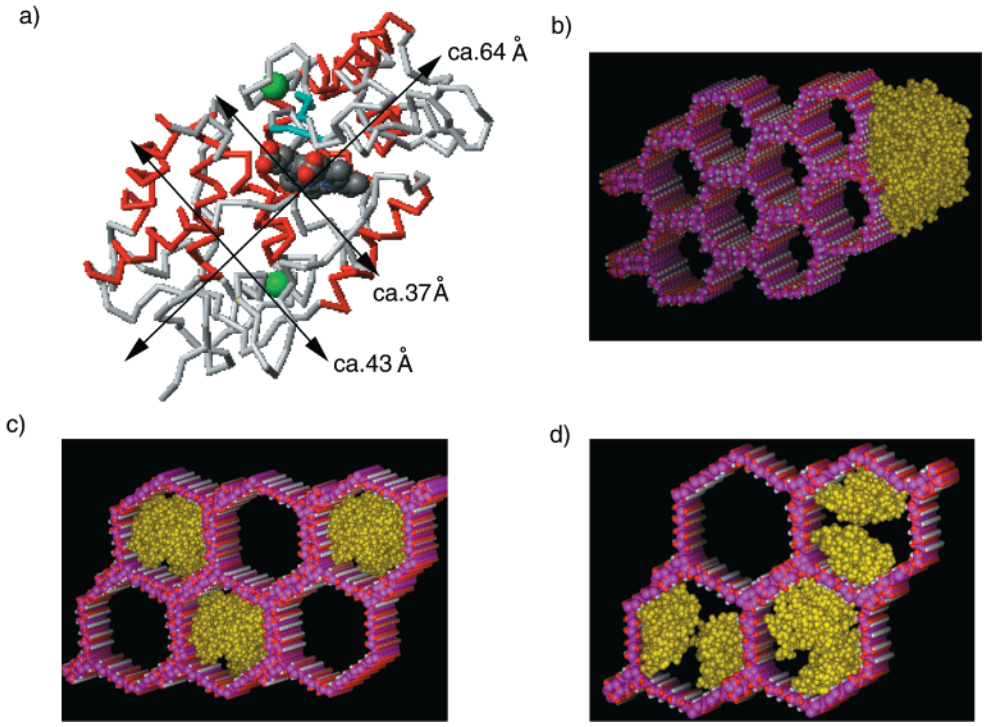

Figure 2. Structural model of an HRP molecule (a**)** and image models of immobilized HRP in FSM-16 with various sizes using a computer schematic model. The FSM-16 model was constructed in accordance with the folded sheet formation mechanism. The pore diameters of the FSM-16 model selected were 30, 50, and 90 Å for models b−d**,** respectively.

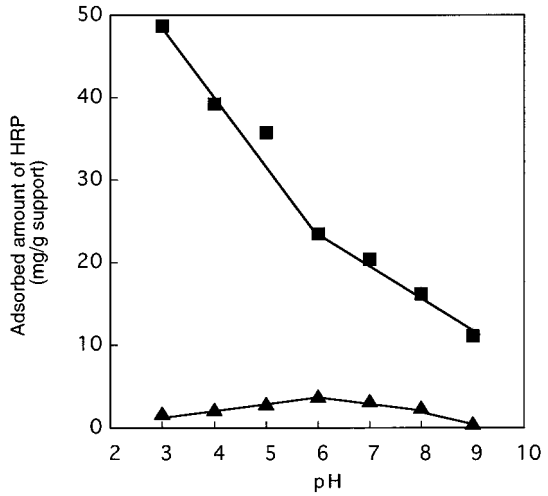

Figure 3. pH profiles of HRP adsorption to FSM-16/51 (■) and SBA-15/50 (▲).

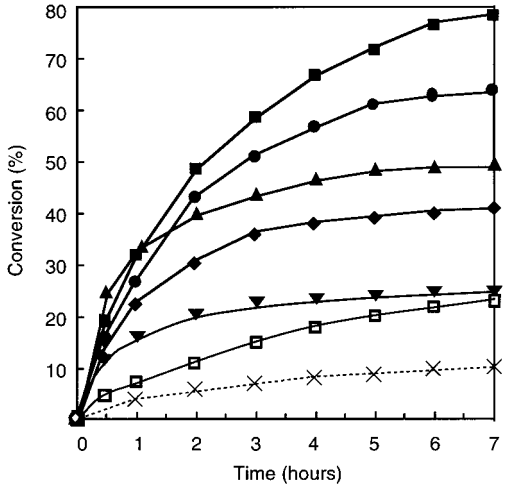

Figure 4. Catalytic activity of various porous silica with immobilized HRP (0.5 mg each) in an organic solvent: (■) FSM16/51; (▲) FSM-16/89; (▼) FSM-16/27; (●) MCM-41/50; (◆) silica gel; (□) HRP free FSM-16/51; (◇) native HRP.

ground data. The immobilized HRP in FSM-16/51, of which pore size was matched to the molecular size of HRP, exhibited the highest activity, 78% conversion, after 7 h. The immobilized enzyme in MCM-41/50 also showed high activity, 63% conversion, after 7 h. The immobilized enzyme in FSM-16/89 and silica gel, which have pore sizes larger than the molecular diameters of HRP, showed medium activity, 47% and 40% after 7 h, respectively. FSM-16/27 with the enzyme immobilized on the outer surface showed low activity, 21% conversion after 7 h. The oxidative activities of the same weight of porous silica materials were also measured as the background value. FSM-16/51 showed lower oxidative activity than HRP immobilized in FSM-16/27 (Figure 4). Other mesoporous silica materials also showed nearly the same background activities (data not shown). Silica is a known catalyst for some oxidation reactions. To measure the real background values, ovalbumin (OVA, MW = 45K) or subtilisin Carlsberg (MW = 27.5K) immobilized in mesoporous silica materials were prepared. These samples showed very low activities, lower than 5% conversion after 7 h (data not shown).

Next, the immobilized enzymes were treated at 70 °C in the buffer solution and then their catalysis of the phenol polymerization reaction was measured[15] to investigate their thermal stability (Figure 5). The immobilized enzyme on FSM-16/51 exhibited the highest thermal stability, 80% residual activity after heat treatment for 120 min. The thermal stability showed the order of FSM-16/51, FSM-16/89, FSM-16/27, and

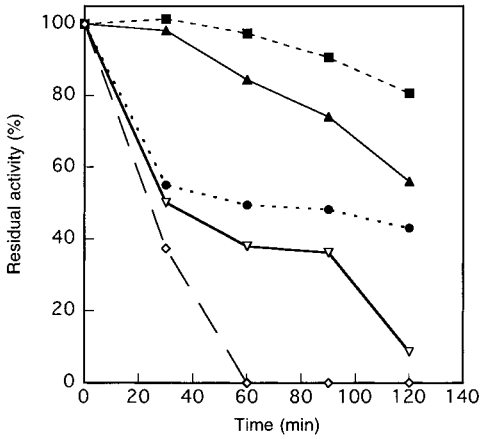

Figure 5. Effect of thermal incubation of immobilized HRP on enzymatic activity for phenol polymerization at 37 °C: (■) FSM16/51; (▲) FSM-16/89; (●) FSM-16/27; (▽) silica gel; (◇) native HRP.

native HRP, which was the same as the order of activity in the organic solvents. Similar results were obtained for another enzyme, subtilisin Carlsberg with the long and short axes of ca. 46 and ca. 39 Å, respectively.

These findings suggest that FSM-16 or MCM-41 is favorable for stabilizing enzymes or for repetitive application. Immobilized HRP showed high activity in organic solvents and heat treatment when the enzyme molecules were adsorbed to FSM-16 and MCM-41, which have average mesopore sizes that matched the molecular diameters of the enzyme. These matching pores of mesoporous materials would prevent increased conformational flexibility of enzyme. It is important to clarify the best fitting distance of the gap between the enzyme and the pore wall using FSM-16 and MCM-41 with other pore sizes. If the pore diameter is much larger than the diameters of a enzyme, the organic solvent would be incorporated into the mesopore easily, inducing environmental change in the pores. This may account for the lower activity of HRP immobilized in FSM-16/89 than FSM-16/51 despite the larger amount of immobilized enzyme in FSM-16/89. When the pore diameter is smaller than the spherical molecular size of the enzyme, the enzyme is adsorbed on the outer surface of particles, resulting in rapid inactivation due to direct exposure to the environmental changes.

Conclusions

The loading efficiency in this case exhibits a clear correlation with the pore characteristics of mesoporous materials on which the enzyme is immobilized; i.e., the adsorbed amount of HRP for FSM-16/51 or MCM-41/50 prepared by a cationic surfactant was much higher than that for SBA-15/50 prepared by a nonionic surfactant. The interaction between enzyme and mesoporous silica was suggested to be a critical factor for immobilization of the enzyme molecule. When the average mesopore size of FSM-16 just matches the molecular diameters of the enzyme, immobilized HRP exhibits the peak activity in an organic solvent and the best stability. Enzyme immobilization with FSM-16 or MCM-41 having a suitable mesopore size would be useful for and applicable to industrial processes and other applications, especially certain environmentally useful enzymatic reactions such as the decomposition of lignin or dioxins.

CM000487A