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Enzyme immobilization in MCM-41 molecular sieve

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Abstract

The immobilization of globular enzymes, cytochrome *c* (bovine heart), papain (papaya latex) and trypsin (bovine pancreas), in the mesoporous molecular sieve MCM-41 was studied. The physical adsorption in the hexagonal 40 Å pure silica phase of MCM-41 showed a clear dependence on enzyme size. The efficiency of papain and trypsin immobilization was pH dependent and favorable at pH values < 7, while immobilization of cytochrome *c* was most efficient at pH > 7 and less susceptible to pH changes. Peroxidase (horseradish) was not significantly retained by the MCM-41 support. Silanation of the mesopore openings after trypsin adsorption eliminated the leaching of enzyme into solution at high pH. The entrapped trypsin enzyme was active for the hydrolysis of *N*-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA). Results for the inhibition of this reaction by poly-L-lysine of various molecular weights are reported. The stability of trypsin was also enhanced by the physical entrapment in MCM-41.

Keywords: Immobilization; MCM-41; Molecular Sieves; Trypsin; Papain

1. Introduction

The fixation of biologically active species onto inorganic materials combines the high selectivity of enzymatic reactions with the chemical and mechanical properties of the support. This blend has brought to light many new applications in the fields of chemical sensing and biocatalysis [1]. Methods of physical immobilization of enzymes include, covalent attachment or adsorption to a support surface, semipermeable membrane or sol–gel entrapment, and microencapsulation, for which comprehensive reviews are available [2]. The immobilization technique should allow the enzyme to maintain its catalytic activity while diminishing other processes that are detrimental to the enzyme, such as autolysis. The support should allow the immobilization of large quantities of enzyme with good enzyme–substrate contact, as well as provide a robust physical and chemical environment. Sol–gel materials have proven versatile as supports in the field of enzyme immobilization. Sol–gels are the product of acid or base catalyzed polymerization of metal alkoxides, where SiO_2 based materials are the most common. For example, the enzyme trypsin has been encapsulated in sol–gel derived amorphous silica [3]. In these studies the hydrolase activity of the entrapped enzyme is shown to be affected by the gel preparation conditions as well as the degree of enzyme loading. Sol–gel entrapment offers many advantages, such as no

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leaching of the entrapped enzymes. A disadvantage of the sol–gel immobilization method is the mass transfer barrier imposed by the narrow pore network, which has an adverse impact on biochemical applications that involve large substrates. Furthermore, control over the pore size distribution of the sol–gel is difficult and only rough substrate size selectivity can be anticipated for the immobilized biocatalysts. Additionally, the enzyme can still aggregate in this type of matrix. A support material with a fixed pore size that precludes aggregation would be an advancement.

Molecular sieves, are porous metal oxides which can discriminate between molecules particularly on the basis of size. As support materials, molecular sieves offer interesting properties, such as high surface areas, hydrophobic or hydrophilic behavior and electrostatic interactions, as well as mechanical and chemical resistance which makes them attractive for enzyme immobilization [4]. However, inclusion of enzymes in the pores of microporous structures (i.e., zeolites) is an impossible task since the pore size of these materials is too small (< 20 Å). A new family of mesoporous molecular sieves referred to as MCM-41 was recently discovered by the Mobil Technology Company [5]. The MCM-41 materials possess a regular array of uniform, unidimensional mesopores with very narrow pore size distribution, which can be systematically varied in size from approximately 20 to ≈ 100 Å. The MCM-41 materials have been used as hosts for photoinduced electron transfer reactions with bulky substrates [6] and conducting polymers [7] as well as metal complexes [8]. The larger pore dimensions of these mesoporous materials offer the possibility of accommodating small enzymes within the channels, which can be several hundred nanometers long [9]. Furthermore, the pore openings of MCM-41 materials can be modified with organosilane groups resulting in a reduction of the channel window diameter [5](a), [10] which could effectively entrap a guest molecule. This approach to enzyme immobilization would resolve some of the disadvantages of other physical entrapment techniques, such as the leaching of adsorbed molecules, the chemical degradation of the anchoring bond of covalently attached enzymes and the barriers to diffusion of substrate and product which are encountered for large poly-

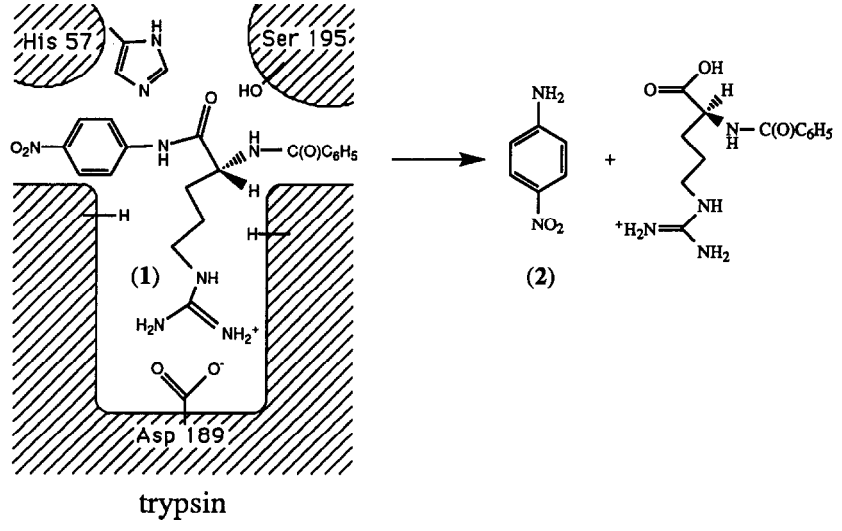

Fig. 1. Reaction scheme for the amide hydrolysis of BAPNA (1) by the protease trypsin. The cleavage of the amide bond in BAPNA to produce *p*-nitroaniline (2) takes place by mediation of a serine side-chain (Ser195) and a histidine group (His57). An aspartic acid residue (Asp189) located at the bottom of the substrate binding pocket is responsible for the selectivity of trypsin towards substrates with positively charged side-chains.

meric substrates in sol–gel preparations [2](a). The MCM-41 hexagonal phase could also be suitable in the evaluation of theoretical models of enzyme immobilization [11] that assume a uniform pore size for the model supports which is in contrast with most amorphous materials that possess a significant pore size distribution [12].

The present work describes the physical immobilization of enzymes in the mesopores of an all silica MCM-41 support. The 40 Å pore size hexagonal phase of these materials was used to immobilize small globular enzymes such as the proteases trypsin and papain, which are important biocatalysts [13], as well as the heme protein cytochrome *c*, which is notable for biological charge transfer reactions [14]. Entrapment of the enzymes through silanation prevented enzyme loss into solution. The accessible silanols in the MCM-41 containing trypsin were reacted with 3-aminopropyltriethoxysilane [1](b). MCM-41 entrapped enzymes could be used in bioreactors and may offer the possibility of substrate size selectivity by choosing the appropriate organosilane chain length or functionality to modify the pore opening. The activity of the MCM-41 entrapped trypsin was evaluated for hydrolysis of BAPNA (**1**) as shown in Fig. 1. The cleavage of the amide bond takes place by mediation of a serine side-chain (Ser195), and other surface residues including a histidine group (His57). An aspartic acid residue (Asp189) located at the bottom of the substrate binding pocket is responsible for the selectivity of trypsin towards substrates with positively charged side-chains such as arginine and lysine.

2. Experimental

2.1. General

X-ray powder diffraction patterns were collected on a Scintag XDS 2000 diffractometer using CuKα monochromatic radiation. Infrared spectra was collected from KBr pellets using a Mattson 2025 FT-IR spectrophotometer. Electronic spectra were recorded using a Hitachi U-2000 UV–Vis spectrophotometer.

2.2. Materials

Cytochrome *c* from bovine heart, papain (EC 3.4.22.2) from papaya latex, trypsin (EC 3.4.21.4) TPCK treated from bovine pancreas, and peroxidase (EC 1.11.1.7) from horseradish were obtained as lyophilized powders from Sigma and used without further purification. Cetyltrimethylammonium bromide, sodium silicate solution (27%) and 3-aminopropyltriethoxysilane were used as received from Aldrich. *N*-α-Benzoyl-DL-arginine-4-nitroanilide (BAPNA) and poly-L-lysine ($\overline{M}_W$ 1000, 25700, 52400 and 123000) were obtained from Sigma.

MCM-41 was prepared from amorphous silica using a cetyltrimethylammonium bromide template according to published procedures [5](a), [15]. The as-synthesized MCM-41 material was calcined in air at 540°C for 10 h. The calcined MCM-41 product was characterized similarly to previously reported samples of the 40 Å mesopore hexagonal phase by X-ray diffraction ($d_{100} = 31.4$ Å).

2.3. Preparation of MCM-41 immobilized cytochrome c

5.0 ml of cytochrome *c* solution (15 μM) in pH 6.0 buffer (50 mM phosphate, Fisher) was added to 0.25 g of MCM-41 in a 15 ml graduated centrifuge tube. The mixture was stirred for 2 h at 4°C. The supernatant was separated from the solid material by centrifugation and the amount of immobilized enzyme was calculated from the difference in supernatant absorbance at 409 nm ($\varepsilon = 92000$) [16] before and after addition of the support. The MCM 41 immobilized cytochrome *c* was washed with deionized water and air dried (cytochrome *c*/MCM41-A). 20 μM solutions of cytochrome *c* were also used for the preparation of MCM-41 immobilized cytochrome *c* in various buffer solutions (pH

6.0, cytochrome *c*/MCM41-B; pH 7.4, cytochrome *c*/MCM41-C; pH 9.0 cytochrome *c*/MCM41-D).

2.4. Preparation of MCM-41 immobilized papain

5.0 ml of papain solution (15 μM) in pH 6.0 buffer (50 mM phosphate, Fisher) was added to 0.25 g of MCM-41 in a 15 ml graduated centrifuge tube. The mixture was stirred for 2 h at 4°C. The supernatant was separated from the solid material by centrifugation and the amount of immobilized enzyme was calculated from the difference in supernatant absorbance at 278 nm ($\varepsilon = 52000$) [16] before and after addition of the support. The MCM-41 immobilized papain was washed with deionized water and air dried (papain/MCM41-A). MCM-41 immobilized papain was also prepared using 7.6 μM (papain/MCM41-B) and 3.3 μM solutions of papain in various buffer solutions (pH 6.0, papain/MCM41-C; pH 7.4, papain/ MCM41-D; pH 9.0 papain/ MCM41-E).

2.5. Preparation of MCM-41 immobilized peroxidase

5.0 ml of peroxidase solution (15 μM) in pH 6.0 buffer (50 mM phosphate, Fisher) was added to 0.25 g of MCM-41 in a 15 ml graduated centrifuge tube. The mixture was stirred for 2 h at 4°C. The supernatant was separated from the solid material by centrifugation and the amount of immobilized enzyme was calculated from the difference in supernatant absorbance at 403 nm ($\varepsilon = 91000$) [16] before and after addition of the support. The MCM-41 immobilized peroxidase was washed with deionized water and air dried.

2.6. Preparation of MCM-41 immobilized trypsin

5.0 ml of trypsin solution (15 μM) in pH 6.0 buffer (50 mM phosphate, Fisher) was added to 0.25 g of MCM-41 in a 15 ml graduated centrifuge tube. The mixture was stirred for 2 h at 4°C. The supernatant was separated from the solid material by centrifugation and the amount of immobilized enzyme was calculated from the difference in supernatant absorbance at 280 nm ($\varepsilon = 33700$) before and after addition of the support. The MCM-41 immobilized trypsin was washed with deionized water and air dried (trypsin/MCM41-A).

2.7. Preparation of MCM-41 entrapped trypsin in toluene

0.1 g of trypsin/MCM41-A and 15 ml of dry toluene were stirred together in a 20 ml screw-cap glass vial. ≈ 2 ml of 3-aminopropyltriethoxysilane (APS) were added and the capped solution was stirred overnight at room temperature. The organic phase was separated from the solid by centrifugation. The product was washed with dry toluene, ethanol and deionized water, air dried and weighed.

2.8. Preparation of MCM-41 entrapped trypsin in methylene chloride

A batch of MCM-41 immobilized trypsin was prepared by adding 50.0 ml of trypsin solution (8.7 μM) in pH 6.0 buffer (50 mM phosphate, Fisher) to 1.5 g of calcined MCM-41 in a round bottom flask. The suspension was stirred overnight at 4°C. The supernatant was removed from the trypsin immobilized MCM-41 by filtration. The product was washed with cold pH 6.0 buffer and deionized water. The MCM41 immobilized trypsin material was air dried and weighed (trypsin/MCM41-B). 0.75 g of trypsin/MCM41-B, 50.0 ml of methylene chloride and 2.5 ml of 3-aminopropyltriethoxysilane (APS) were combined in a 100 ml round bottom flask. The mixture was stirred overnight at 0°C in a refrigerated methanol/water bath. The silanated MCM41 immobilized trypsin product (trypsin/ M41APS) was separated from the solution by filtration, washed with methylene

chloride and air dried. The dry product was washed with pH 6.0 buffer and deionized water, air dried and weighed.

2.9. Determination of trypsin activity

The activity of the immobilized trypsin was determined at 25°C by monitoring the catalytic hydrolysis of BAPNA (**1**), as shown in Fig. 1. The immobilized trypsin (0.1–0.2 g) was mixed with a pH 8.0 buffer solution (5 ml, 50 mM Tris–HCl, 10 mM $CaCl_2$) containing BAPNA (5 mM). The rate of *p*-nitroaniline (**2**) formation was determined from the supernatant solution absorbance at 405 nm after separation of the solids by centrifugation. Poly-L-lysines (10 g/l) of various average molecular weights (1000, 25700, 52400 and 123000) were used as trypsin inhibitors by combining with MCM-41 entrapped trypsin (0.1 g)/BAPNA (1.0 ml, 1 mM)/buffer (2.0 ml, 50 mM Tris–HCl, 10 mM $CaCl_2$) samples at 25°C.

3. Results and discussion

Enzyme immobilization may combine the physical properties of the support material with the basic biochemical activity of the enzyme and consequently offers an operational advantage over free enzymes. Adsorption is one of the most simple methods of physical immobilization of enzymes [2](c) with the added advantage of being inexpensive and 'mild' towards the enzyme. This method profits from interactions between the support surface and the outer shell of the enzyme. These interactions can be altered by solution conditions such as pH which might make the adsorption process reversible. Silanols located on the pore walls of MCM-41 can promote adsorption through hydrogen bonding interactions with hydrophilic residues of the enzyme. Therefore, enzyme adsorption studies were conducted to determine the optimum pH at which the enzyme–support interactions are favored.

Four enzymes were included in the present study, which range in size in the order peroxidase > trypsin ≈ papain > cytochrome *c*. Table 1 summarizes some physical properties of these enzymes. The enzyme cytochrome *c*, is a globular shaped heme protein of relative molecular mass, M_r [17] of 12300 Da, with an approximate radius of 16.6 Å [18]. Only 4% of the heme group is exposed to the protein surface, which possesses nearly equal amounts of hydrophobic and hydrophilic surface residues [14]. Immobilization of cytochrome *c* by adsorption was easily achieved by stirring buffered enzyme solutions with the MCM-41 support at 4°C. Fig. 2 displays the UV spectra of the supernatant before addition of the MCM-41 support and after adsorption of the enzyme. The difference in solution concentration, calculated from the band at 409 nm, yielded the enzyme loading of the support (mg of immobilized enzyme per gram of MCM-41), as shown in Table 2. No apparent change in enzyme solution concentra-

Table 1
Physical properties of free enzymes used for MCM-41 immobilization

Enzyme	Relative molecular mass (M_r)	Isoelectric point [a], pH	Partial specific volume [b], cm^3/g	Spherical molecular diameter [c], Å
cytochrome *c*	12300	10.0	0.72	30
papain	20700	8.75	0.724	36
trypsin	23400	10.5	0.73	38
peroxidase	44000	–	0.699	46

[a] References [14] and [1].
[b] Reference [25].
[c] Calculated from the partial specific volume.

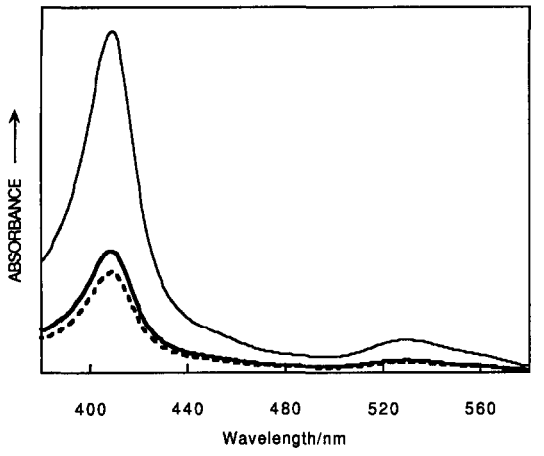

Fig. 2. UV–Vis spectra of cytochrome *c* solution (pH 6.0) (a) before (thin line); (b) after adsorption to MCM-41, 2 h contact (bold line); and (c) after 24 h contact with the MCM-41 support (dashed line).

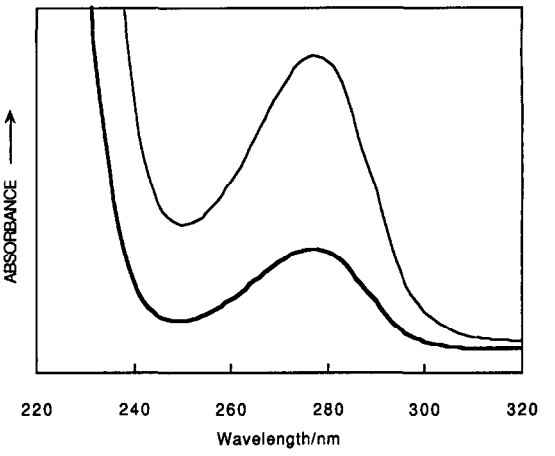

Fig. 3. UV–Vis spectra of papain solution (pH 6.0) (a) before (thin line); and (b) after adsorption to MCM-41, 2 and 24 h contact (bold line).

tion was observed after 2 h. Under similar conditions the protease papain (M_r 20700) was immobilized in MCM-41.

Papain is a thiol hydrolase of industrial importance as a meat tenderizer and as an agent for clarifying protein cloudiness in beer. From a crystal structure [19], papain possesses an ellipsoidal shape with rough dimensions 37 × 37 × 50 Å. Its hydrophilic residues are generally at the surface, but the limited solubility of papain in water is attributed to some hydrophobic surface groups. The UV spectra in Fig. 3 illustrate the adsorption of papain in the mesoporous support. The band at 280 nm was used to monitor the adsorption process, as well as in later experiments, the leakage of enzyme into solution. The effect of pH on the MCM-41 immobilization of cytochrome *c* and papain, was studied at different pH values using several buffered solutions, as shown in Table 2. The immobilization of papain was favored at lower pH values, with significant loading differences between adsorption at pH 6 and 9. The amount of enzyme retained by the support was also dependent on solution concentration. At pH 6.0

Table 2
Immobilization efficiency at different solution pH

Immobilized enzyme	Solution conc.	Enzyme loading (mg/g_{MCM-41})		
	(μM)	pH 6.0	pH 7.4	pH 9.0
cytochrome *c*/MCM41-A	15	3.8		
cytochrome *c*/MCM41-B	20	5.0		
cytochrome *c*/MCM41-C	20		5.8	
cytochrome *c*/MCM41-D	20			5.7
papain/MCM41-A	15	4.9		
papain/MCM41-B	7.6	4.1		
papain/MCM41-C	3.3	1.7		
papain/MCM41-D	3.3		1.66	
papain/MCM41-E	3.3			0.50
trypsin/MCM41-A	12	3.8		
trypsin/MCM41-B	8.7	4.7		
peroxidase/MCM41-A	17	0.4		

increasing the enzyme concentration to 15 μM, rendered an enzyme loading of 4.9 mg/g, compared to 1.7 mg/g from a 3.3 μM solution. Aging of the adsorbed material in the papain solutions for 24 h did not improve the loading. In fact, no significant change in loading was observed after stirring for the first two hours. Cytochrome *c* adsorption was less susceptible to the pH of the solution. The loading values obtained suggest that mild basic solutions are favorable to immobilization. This distinct adsorption behavior between cytochrome *c* and papain could be attributed in part to the difference in isoelectric points (see Table 1). In the range of pH studied, cytochrome *c* is below its isoelectric point, thus a positive charge in the surface of the enzyme would favor immobilization to MCM-41, by means of accessible silanols in the host. At pH 9, papain is past its isoelectric point, consequently the nature of the surface residue charge has changed producing a very significant decrease in the loading capacity of the enzyme into the MCM-41 support. In contrast with papain adsorption, the cytochrome *c* loading increased by 10% after aging the adsorbed material 24 h in enzyme solution (Fig. 2) at pH 6.0. This difference may be attributed to the enzyme size. The rough dimensions of papain are close to the pore size of the support (≈ 40 Å) which makes diffusion into the MCM-41 channels difficult. The occlusion of the enzyme may block the pore openings and inhibit further adsorption of enzyme from solution. However, cytochrome *c* is a smaller enzyme which can probably migrate deeper into the pore system. Contact with the pore walls will probably make the diffusion process slow, but further adsorption of enzyme would be possible with time.

Leakage of adsorbed enzyme into solution is a common disadvantage of the adsorption immobilization technique [2](c). The desorption of immobilized papain from MCM-41 was noted at high pH. For example at pH 9.0 approximately 70% of the immobilized enzyme leaks into solution after 24 h compared to only 15% in pH 6.0

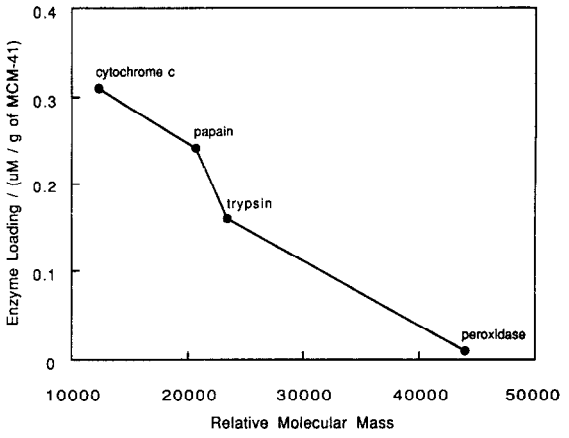

Fig. 4. Effect of enzyme size on the immobilization efficiency of the MCM-41 host.

buffer. No significant leakage was detected for immobilized cytochrome *c* at pH 6.0, 7.4 and 9.0. This is consistent with restricted cytochrome *c* diffusion out of the pore due to the contact with the pore walls of the MCM-41 host, which improves the retention of the enzyme [1](b). These results suggest that the larger papain may be plugging the mesopore openings.

The effect of enzyme size on enzyme immobilization capacity of MCM-41 was evaluated further using ≈ 15 μM solutions (pH 6.0, 50 mM phosphate) of peroxidase (M_r 44000) and trypsin (M_r 23400) as well as papain and cytochrome *c*. Fig. 4 shows the relation between relative molecular mass and moles of adsorbed enzyme at 4°C. A clear correlation between enzyme size and molar loading is observed. The large enzyme peroxidase is not significantly retained by the support when compared to the other enzymes. Nonetheless the loading capacity of peroxidase (0.4 mg/g) was similar to that of acid phosphatase (M_r 68000) adsorbed onto zeolite 13X, where the relative pore and enzyme dimensions preclude diffusion into the micropores [4]. This suggests that peroxidase is adsorbed on the outer surface of the MCM-41 particles while the other enzymes do indeed interact with the mesopore system of the support since the loading capacity for trypsin, papain and cytochrome *c* is much higher. Enzyme

hydrophobicity may also be considered as a contributing interaction that affects the immobilization. The average hydrophobicity ($H\Phi_{ave}$) [16] of trypsin, for which most hydrophilic residues are located at the enzyme surface, is much lower than that of cytochrome *c*, and the $H\Phi_{ave}$ value is similar for both papain and cytochrome *c*. Since no clear correlation between the enzyme loading efficiency and $H\Phi_{ave}$, or the isoelectric point is observed, it can be inferred that the enzyme size factor outweighs hydrophobic interactions at low pH.

Papain and trypsin belong to the family of enzymes known as proteases, or peptidases, which hydrolyze peptide bonds in proteins and peptides. The active site of papain is a cysteine side chain, which can easily become blocked [19]. This is not the case for trypsin, where the active site involves a serine group (Fig. 1). Furthermore, the nature of the substrate binding pocket of trypsin makes the enzyme activity specific for amides with positively charged side-chains, such as arginine and lysine. These differences make trypsin a more suitable enzyme for activity evaluations. The activity of MCM-41 immobilized trypsin (trypsin/MCM41-B) was monitored at 25°C in pH 8 buffer, using the substrate *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), according to the reaction scheme shown in Fig. 1.

Assuming a similar rate for the BAPNA (**1**) hydrolysis by free and adsorbed trypsin, the MCM-41 immobilized trypsin showed activity recovery corresponding to 87% of the immobilized enzyme. However, by removing the solid from the supernatant substrate solution and monitoring *p*-nitroaniline (**2**) production of the solution, it was estimated that 52% of the immobilized enzyme had leaked into solution, which is consistent with the desorption of enzyme expected at higher pH, and previously observed for papain (vide supra). Although ≈ 40% of the observed activity appears to be attributed to trypsin immobilized in the MCM-41 host, more enzyme leaks out when the supernatant is replaced by new buffer solution, such that one could anticipate the complete removal of the enzyme with continued use of the biocatalyst. If the channel openings can be chemically reduced in size, then the enzyme would become physically entrapped. These restricted apertures can be generated by silanation of MCM-41 using alkoxyorganosilanes which have previously been proven to be effective in the reduction of mesopore dimensions [5](a), [10]. Although silanation is usually carried out in dry organic solvents, such as toluene, which might denature an enzyme, it has been successfully accomplished under a variety of conditions [1](b). Reduction of the pore size through silanation would restrict enzyme movement and avoid diffusion into the bulk solution. A preliminary attempt to entrap immobilized trypsin was made using 3-aminopropyltriethoxysilane (APS) in a toluene solution. APS has been used as a precursor for the covalent immobilization of enzymes, and offers the possibility of improving the immobilization of the enzyme not only by physical restriction of the pore openings, but also by Van der Waals interactions with hydrophilic residues. The ≈ 6 Å long APS chain was expected to reduce the pore window by ≈ 12 Å [10]. The siloxide portion of APS is not expected to react with the enzyme since encapsulation of enzymes by the sol–gel process employs similar alkoxides without a problem [2](a). The modification of MCM-41 with APS in toluene was successful in stopping leakage of the enzyme in basic solution, however, the activity recovery of the entrapped enzymes was only ≈ 1% of that expected from the enzyme loading value. This loss in activity could be attributed to enzyme denaturing in the toluene solvent.

Methylene chloride has previously been used in combination with trypsin encapsulation in polypyrrole microcapsules (400 nm in diameter) [20]. The encapsulated enzyme in this study remained active after exposure to the methylene chloride solvent. Therefore, the silanation of MCM-41 immobilized trypsin was performed using APS in methylene chloride solvent at 0°C.

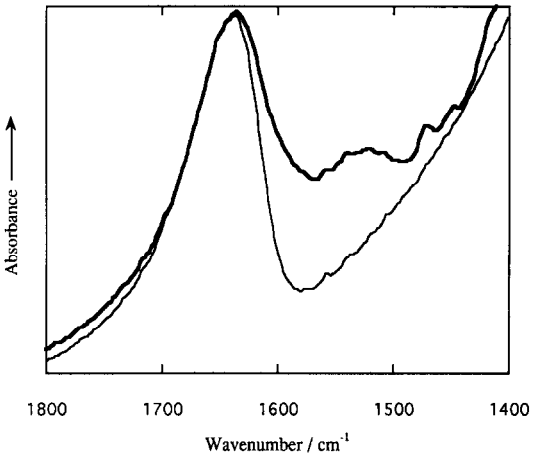

Fig. 5. FT-IR spectrum of MCM-41 host (a) before (thin line); and (b) after silanation (bold line) in methylene chloride at 0°C.

Assessment of the effect methylene chloride has on the hydrolase activity of MCM-41 adsorbed trypsin was difficult to measure since the enzyme is desorbed during the wash of methylene chloride treated samples without silanation. To verify the surface modification with the silane, a sample of MCM-41 was treated with APS under similar conditions used for enzyme entrapment. Fig. 5 displays the FT-IR spectrum of the silanated material. Bands corresponding to $-NH_2$ and $-CH_2$ deformation vibrations are observed at 1540 and 1470 cm^{-1} respectively. The MCM-41 entrapped trypsin enzyme did not leak into solution when the material was immersed in pH 9.0 buffer, which would normally release $\approx 90\%$ of the enzyme after 24 h. The activity of entrapped trypsin was 13% of that expected from the enzyme loading. This activity recovery value is low compared to other trypsin immobilization techniques such as covalent attachment to artificial membranes (41.5%) [21] and copolymers (65%) [22] as well as sol–gel entrapment (45%) [2](a). This level of trypsin

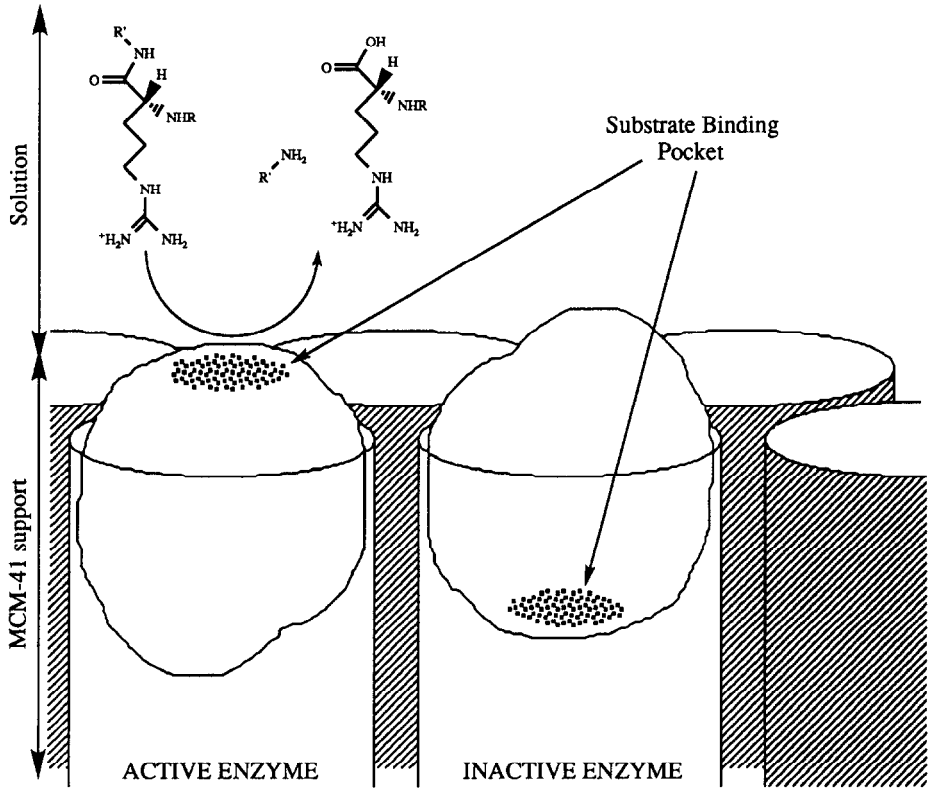

Fig. 6. Schematic diagram illustrating the effect of adsorbed enzyme orientation on the activity of MCM-41 immobilized trypsin.

activity may reflect partial denaturing from the silanation process, however, the activity of the adsorbed enzyme before silanation is unknown because of interference from enzyme leaching. A second possibility is that the tight fit of this enzyme in the channels of MCM-41 may preclude access to the catalytic site. Trypsin has a specific side-chain binding pocket located on the surface of the enzyme close to the active site. At the bottom of this pocket is a negatively charged aspartic acid residue (see Fig. 1), which favors binding with amino acids that possess positively charged side-chains, such as arginine and lysine [23]. The binding pocket, as well as other residues, participate in the alignment of the substrate on the enzyme surface so hydrolysis of the susceptible peptide bond could take place. Consequently, the accessibility to the substrate binding pocket of trypsin, and hence catalytic activity, would depend on the orientation of the enzyme when it was originally adsorbed, such as depicted in Fig. 6. The evaluation of enzymatic activity using larger mesoporous structures might resolve this issue by allowing better enzyme mobility within the channels, or permitting enzyme–substrate interactions regardless of enzyme orientation.

The movement of trypsin, which causes autolysis in solution, is restricted by immobilization. No significant activity loss was noted after incubation of MCM-41 entrapped trypsin at 25°C for a week, compared to the total deactivation of free trypsin in solution, after 24 h. This increased stability is similar to that of other immobilization techniques that preclude the contact between individual protease enzymes. In order to better evaluate the accessibility of the entrapped enzyme, poly-L-lysine, a known trypsin inhibitor [16,24], was added to the BAPNA substrate solution. Using the partial specific volume of poly-L-lysine ($\bar{v}$ = 0.78 cm^3/g) [3](b) approximate values of the spherical molecular diameter were calculated for samples of the polyamino acid of various average molecular weights, as summarized in Table 3. The relative inhibition of the enzymatic BAPNA hydrolysis by the poly-L-lysines is shown in Table 4. BAPNA hydrolysis by free trypsin (2 mM) is greatly inhibited by the low molecular weight poly-L-lysine ($\overline{M}_W \approx 1000$) with respect to inhibition by the larger polyamino acids. Inhibition of the specific activity of MCM-41 entrapped trypsin shows a similar trend as observed in solution. The inhibition efficiency appears to decrease with increase in inhibitor size. However, the relative inhibition by the larger polyamino acids appears enhanced with respect to soluble trypsin. This increased BAPNA hydrolysis inhibition relative to solution enzyme by the large poly-L-lysines could be attributed to coating of the pore surface by the polymer, which would hinder the diffusion of BAPNA, as previously proposed for trypsin entrapped in sol–gel samples [3](b). If this is the case, the results suggest that the biocatalyst is indeed

Table 3
Estimated size of poly-L-lysines used as inhibitors of BAPNA hydrolysis by MCM-41 entrapped trypsin

Average molecular weight ($\overline{M}_W$)	Spherical molecular diameter [a], Å
1000	14
25700	40
52400	51
123900	68

[a] Calculated from the partial specific volume of poly-L-lysine ($\bar{v}$ = 0.78 cm^3/g) [3](b).

Table 4
Relative inhibition by poly-L-lysine of the *N*-α-benzoyl-DL-arginine-4-nitroanilide hydrolysis by trypsin

Inhibitor $\overline{M}_W$	Relative percent inhibition of		
	Free trypsin	MCM-41 entrapped trypsin	Sol–gel entrapped trypsin [a]
1000	100	100 [b]	–
3800	–	–	100
25700	31	80	–
29600	–	–	9
52400	11	77	–
123900	15	68	–

[a] Reference [3](b).
[b] The specific acivity of trypsin/M41APS was 5.5×10^{-4} U/mg.

located in the pore system and that obstruction of the pore opening inhibits activity. If the inhibition is due to competitive hydrolysis by poly-L-lysine, which is the case for the small inhibitor, then the relative dimensions of the larger inhibitor (≈ 60 Å) and the pore size of the MCM-41 host (≈ 40 Å) suggest that interaction between trypsin and this poly-L-lysine can only take place at the pore opening. This would suggest, in combination with the leakage experiments, that the MCM-41 entrapment method is effective in restricting the enzyme mobility, but does not hinder interaction with bulky substrates. Additionally, the active enzyme in MCM-41 probably has a preferred orientation dictated by the size of the channel. This would be an important advantage over sol–gel entrapment, for which the average pore size precludes interaction with large polymeric substrates, and the enzymes could be randomly oriented with respect to the pore openings. Indeed, inhibition values obtained from Ref. [3](b) exemplify the dramatic change in inhibition efficiency between the small poly-L-lysine ($\overline{M}_W \approx 4000$) and poly-L-lysine of moderate size ($\overline{M}_W \approx 30000$) for L-BAPNA hydrolysis by sol–gel entrapped trypsin.

4. Conclusions

The physical immobilization of small enzymes in the mesopore structure of MCM-41 has been demonstrated. The loading efficiency of the immobilized enzymes show a clear correlation with the enzyme size, suggesting that the mesopores participate in the immobilization process. Silanation of the support after immobilization was effective in stopping enzyme leakage from the support without hindering interaction with large substrates. The present results require that we examine the immobilization of enzymes in larger pore (> 40 Å) MCM-41 structures in order to better evaluate the effects of an order silica support. Additionally, the nature of the silane used to modify the pore openings is an important variable that will be studied further.

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