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Trypsin immobilization on mesoporous silica with or without thiol functionalization

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Abstract The effects of pore size, structure, and surface functionalization of mesoporous silica on the catalytic activity of the supported enzyme, trypsin, were investigated. For this purpose, SBA-15 with 1-dimensional pore arrangement and cubic Ia3d mesoporous silica with 3-dimensional pores were prepared and tested as a support. Materials with varying pore diameters in the range 5–10 nm were synthesized using a non-ionic block copolymer by controlling the synthesis temperature. Thiol-group was introduced to the porous materials via siloxypropane tethering either by post synthesis grafting or by direct synthesis. These materials were characterized using XRD, SEM, TEM, N_2 adsorption, and elemental analysis. Trypsin-supported on the solids prepared was active and stable for hydrolysis of N-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA). Without applying thiol-functionalization, cubic Ia3d mesoporous silica with ca. 5.4 nm average pore diameter was found to be superior to SBA-15 for trypsin immobilization and showed a better catalytic performance. However, enzyme immobilized on the 5% thiol-functionalized SBA-15 prepared by directly synthesis was found to be the most promising and was also found recyclable.

Keywords Enzyme immobilization · Trypsin · Mesoporus silica · Cubic Ia3d · SBA-15

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1. Introduction

Mesoporous silica materials have been attracting much interest since they were firstly discovered by Beck et al. [1, 2]. Mesoporous materials possess highly ordered pore structure, large surface area, and large pore diameter (10–300 Å) with a narrow pore size distribution, and therefore have been studied for a wide range of applications in catalysis and separation. In particular, their potential application as enzyme support has been proposed [3–22] due to the tunable pore diameters suitable for immobilizing various enzyme molecules.

Enzymes are known to exhibit high catalytic activity and selectivity under mild reaction conditions, but are difficult to handle and sensitive to inactivation by pH changes, high temperature, and organic solvent. They are prone to denature at harsh reaction conditions and their application as an industrial catalyst is limited due to enzyme separation problems from reactant and product. In this regard, immobilization of enzyme molecules by physical adsorption [10] and covalent bonding methods [12] on various inorganic support materials or on polymers can offer robust physical-chemical environment for enzyme species as well as thermal stability, and enables them to be used in continuous operation [3]. Encapsulation [14] and sol-gel entrapment [15] are also frequently employed as enzyme immobilization techniques.

Initially, MCM-41 had mostly been tested as a support material for enzyme immobilization, but MCM-48 with cubic pore arrangement, which allows better accessibility of substrates from all sides in the pore compared to MCM-41 with one-dimensional pore, was reported as a better support material [18]. However, MCM-48 was not suitable as a support for large enzymes in some cases due to relatively small pore size (25 Å) [16]. Yiu et al. [10] recently conducted a systematic investigation of trypsin immobilization on MCM-41, MCM-48, and SBA-15, and they concluded that trypsin immobilized

on the functionalized SBA-15 was shown to have the highest catalytic activity among the samples compared. These studies established that pore size, pore arrangement, and surface functionalization are the three most important factors that need consideration in enzyme immobilization using mesoporous materials. But, relative contribution among them was not explicitly mentioned so far.

In this work, we have extended the work by Yiu et al. [10] and investigated the trypsin immobilization using a new class of cubic Ia3d large pore mesoporous silica reported by Ryoo's group [23]. For this purpose, SBA-15 with 1-D channel and cubic Ia3d large pore mesoporous silica with 3-D channel were synthesized using non-ionic block copolymer and their thiol-functionalized forms were also prepared using both direct and post-synthesis methods. Cubic Ia3d large mesoporous silica has three-dimensional pore structure and large enough pore size (6–10 nm), combined effect of which can contribute positively towards an enzyme immobilization. Catalytic activity of the trypsin immobilized on the cubic Ia3d large mesoporous silica was evaluated using hydrolysis of BAPNA as a probe reaction and compared with the performance of SBA-15. The effect of pore size was also examined by increasing pore size of the each group of mesoporous materials from 50 Å to 100 Å by controlling the hydrothermal synthesis temperature. Direct thiol group functionalization to the cubic Ia3d mesoporous silica was proved to be a difficult synthesis task, but it was successfully resolved by employing the synthesis route of Che et al. [24] reported recently.

2. Experimental

2.1. Synthesis of mesoporous materials

SBA-15 was synthesized using Pluronic P123 triblock copolymer (EO_{20}-PO_{70}-EO_{20}, BASF) as a structure directing agent and TEOS (tetraethylorthosilicate, Aldrich) as a silica source in acidic conditions. Synthesis of pure SBA-15 was carried out in molar ratio of 1 TEOS: 0.017 surfactant: 6 HCl: 200 H_2O. Pluronic P123 was dissolved in the mixture of HCl and de-ionized water at room temperature. TEOS was added to the surfactant solution and the mixture was magnetically stirred at 35°C for 24 h. Then, it was heated for 24 h at 100°C or at 150°C, followed by filtering, washing with de-ionized water, and dried. After refluxing 1 g sample of as-synthesized mesoporous material in 3.8 g of 37 wt% HCl in 150 ml ethanol for 6 h at 50°C to remove the surfactant, it was filtered with ethanol and dried. Finally, it was calcined at 550°C for 4 h in air.

Cubic Ia3d large pore mesoporous silica was prepared in molar ratio of 1 TEOS: 0.017 surfactant: 1.83 HCl: 195 H_2O: 1.31 butanol. The mixture of 6 g Pluronic P123 and 11.8 g HCl (35wt%, Aldrich) was added to 217 g water in a polypropylene bottle, and was vigorously stirred at 35°C until it becomes clear. 6 g Butanol was added to this clear solution and the mixture was stirred for 1 h at 35°C. After introducing TEOS dropwise to this solution under nitrogen, it was stirred for 24 h at 35°C and heated for 24 h at 100°C or at 130°C. It was filtered, washed with de-ionized water and dried. The white solid was solvent extracted using HCl-ethanol solution and calcined at 550°C for 4 h.

2.2. Thiol-functionalization

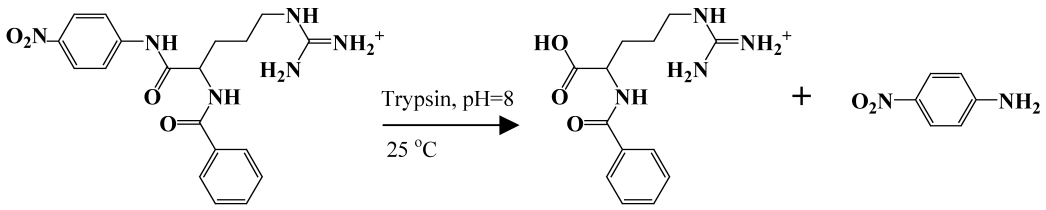
Thiol-containing SBA-15 samples were prepared with either 5 mol% or 10 mol% of 3-mercaptotriethoxysilane ($(EtO)_3SiCH_2CH_2CH_2SH$, (95%, Aldrich)) in the substrate mixture following the same synthesis protocol as in the preparation of pure silica SBA-15. After P123 was dissolved in the HCl-water solution, identical synthesis procedure to SBA-15 was performed with 3-mercaptotriethoxysilane introduced together with TEOS. The samples produced were designated as 5% SH-SBA-15 (D) and 10% SH-SBA-15 (D), respectively. These mixtures were subjected to the hydrothermal heating at 100°C and were filtered, washed, and air-dried. The surfactant template was removed by refluxing with a HCl-ethanol solution (1 g of sample in 150 ml ethanol and 3.8 g HCl) at 50°C for 6 h.

Thiol-containing cubic Ia3d large pore mesoporous silica was prepared as reported by Che et al. [24]. A simple addition of 3-mercaptotriethoxysilane to TEOS during the synthesis procedure for cubic Ia3d large pore mesoporous silica reported [23] was not successful. A mixture of TEOS and 3-mercaptotriethoxysilane was added to a mixture of P123, HCl, and de-ionized water at 40°C. After the mixture was stirred for 24 h, the mesostructured product formed was cured at 100°C for an additional 48 h. The products were filtered without washing and dried at 60°C. The surfactant was removed by solid-liquid extraction using 1 N HCl/ethanol solution at 70°C for 24 h. The samples produced were designated as 5% SH-cubic Ia3d (D) and 9% SH-cubic Ia3d (D) respectively, depending on the mole percentage of thiol group in the synthesis mixture.

For post-synthetic grafting procedure, mesoporous silica support materials were dried at 100°C under vacuum overnight. 1 g each of the dried powder was added to 15 ml of toluene with stirring. 4 mmol of 3-mercaptopropyltrimethoxysilane was then added to the mixture, stirred 4 h in room temperature, and refluxed for 8 h at 110°C. The white solid product was filtered and washed with toluene and dried under vacuum. The samples were each designated as SH-SBA-15 (G) and SH-cubic Ia3d (G).

2.3. Immobilization of trypsin on the mesoporous material

Trypsin from bovine pancreas (Sigma) was used and its immobilization was conducted following the procedure



Scheme 1 Hydrolysis of N-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA) by trypsin (from bovine pancreas, type I)

reported by Yiu et al. [10, 12]. 5 ml Trypsin solution (5.2 μM) in pH = 6.0 buffer (50 mM phosphate) was added to 0.25 g of mesoporous materials in a 20 ml polypropylene bottle. The buffer solution was directly prepared using monobasic sodium phosphate and dibasic sodium phosphate. After the mixture was stirred for 2 h at 4°C, the clear solution was separated from the mixture by centrifugation, and the amount of free enzyme not immobilized was calculated from the differences in UV spectra at 280 nm [13]. Finally, the mesoporous material with immobilized trypsin was washed with pH 6.0 buffer solutions, air-dried and stored at 4°C.

The mesoporous silica with immobilized trypsin was resuspended by stirring in a pH = 8.0 Tris-HCl buffer solution (5 ml, Sigma) for 2 h. The mixture was placed in a UV sample bottle and then the spectrum of the solution was measured by UV spectrometer to calculate the amount of enzyme leached out from the support.

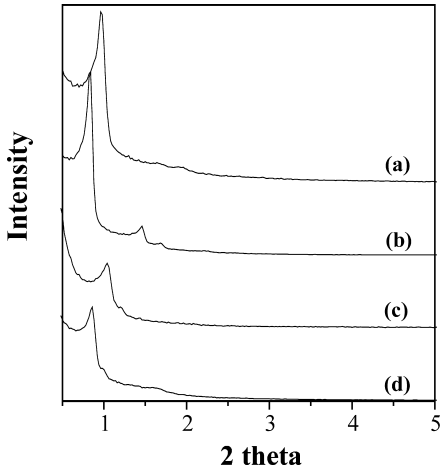


Fig. 1 X-ray diffraction patterns of (a) SBA-15 (100°C)*, (b) SBA-15 (150°C), (c) Cubic Ia3d large mesoporous silica (100°C), (d) Cubic Ia3d large mesoporous silica (130°C): *Synthesis temperature

2.4. Catalytic activity measurements

Hydrolysis of N-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA) [see Scheme 1] was carried out to measure catalytic activity of trypsin-immobilized mesoporous materials. At first, 21.7 mg BAPNA was dissolved in 1 ml DMSO, then diluted with pH = 8 Tris-HCl buffer solution. For 0.25 g trypsin-immobilized samples, 20 ml BAPNA solution was added and stirred at 25°C. Catalytic activities were measured by absorbance of the mixture at 405 nm due to the formation of p-nitroaniline, which imparts yellow color. Samples were taken in regular time intervals (15, 30, 45, 60 and 90 min) and catalytic activities were measured using a UV spectrometer. The catalyst used in the hydrolysis reaction was filtered and washed with a pH = 8 buffer solution for reuse.

3. Results and discussion

Powder XRD patterns of the calcined pure SBA-15 and cubic Ia3d mesoporous silica are shown in Fig. 1. SBA-15 synthesized at 100°C has shown a major peak at 0.8° (100 reflection) and two smaller peaks at ca. 1.6° and 1.8°, which correspond to (110) and (200) reflections, respectively. The corresponding peaks of the SBA-15 sample synthesized at 150°C shifted to the left of the region due to larger channel size. The XRD patterns of cubic Ia3d mesoporous silica were all in accordance with the 3-D symmetry reported previously by Ryoo et al. [23] with the characteristic shoulders to the main peak. Again, the one prepared at 130°C showed the peak shift to left due to the channel expansion. The XRD patterns of the corresponding thiol-functionalized mesoporous materials are shown in Fig. 2. Introduction of additional silane group to TEOS has resulted in diminished peak intensities accompanied by small losses in long- range structural orders [24]. These XRD diffractograms, in general, support that mesoposous silica materials were successfully prepared for enzyme immobilization.

SBA-15 was made of uniform spaghetti-shaped particles according to the scanning electron microscopy (SEM) images (not shown), and regular mesopore arrangement was clearly visible in the transmission electron microscopy (TEM) images. No clear particle morphology, on the other hand, was observed for cubic Ia3d materials. As shown in Fig. 3, TEM images of all the cubic Ia3d mesoporous materials confirm clear arrangement of pores with uniform size. 5% Thiol containing sample, 5% SH-cubic Ia3d (D), showed characteristics of a physical mixture of both 1-D and 3-D

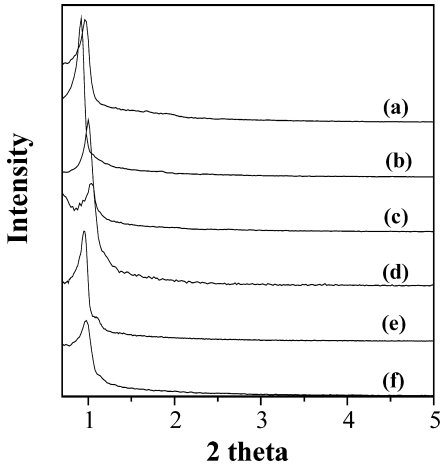


Fig. 2 X-ray diffraction patterns of (a) 5% SH-SBA-15 (grafting), (b) 5% SH-SBA-15 (direct), (c) 10% SH-SBA-15 (direct), (c) 5% SH-cubic Ia3d (grafting), (e) 5% SH-cubic Ia3d (direct), (f) 9% SH-cubic Ia3d (direct)

channels in the product, whilst 9% SH-cubic Ia3d (D) has 3-D pore arrangement.

Nitrogen adsorption-desorption isotherms of the mesoporous materials prepared without surface functionalization are shown in Fig. 4. The N_2 isotherms of these samples are of the type IV with clear hysteresis loop at relative pressures from 0.6 to 0.8. The pore diameter of samples prepared at 100°C was ca. 6 nm with narrow pore size distribution, and at higher synthesis temperature the pore diameter significantly increased to 10 nm. BET surface area, BJH average pore diameter, and pore volume of all the mesoporous materials prepared in this work are summarized in Table 1. The pore size distribution and textual properties of functionalized mesoporous materials indicated that their mesopore structures were maintained even after introduction of the or-

Table 1 Textual properties of mesoporous materials prepared

Materials	Pore size (nm)	Pore vol. (cc/g)	Surface area (m^2/g)
SBA-15 (100°C)	6.4	0.87	747
SBA-15 (150°C)	10.8	1.13	513
Cubic Ia3d (100°C)	5.4	0.69	693
Cubic Ia3d (130°C)	10.8	1.12	620
5% SH-SBA-15 (grafting)	5.0	0.74	620
5% SH-SBA-15 (direct)	4.8	0.63	551
10% SH-SBA-15 (direct)	4.2	0.44	453
5% SH-cubic Ia3d (grafting)	4.4	0.43	470
5% SH-cubic Ia3d (direct)	6.4	0.77	740
9% SH-cubic Ia3d (direct)	5.0	0.71	631

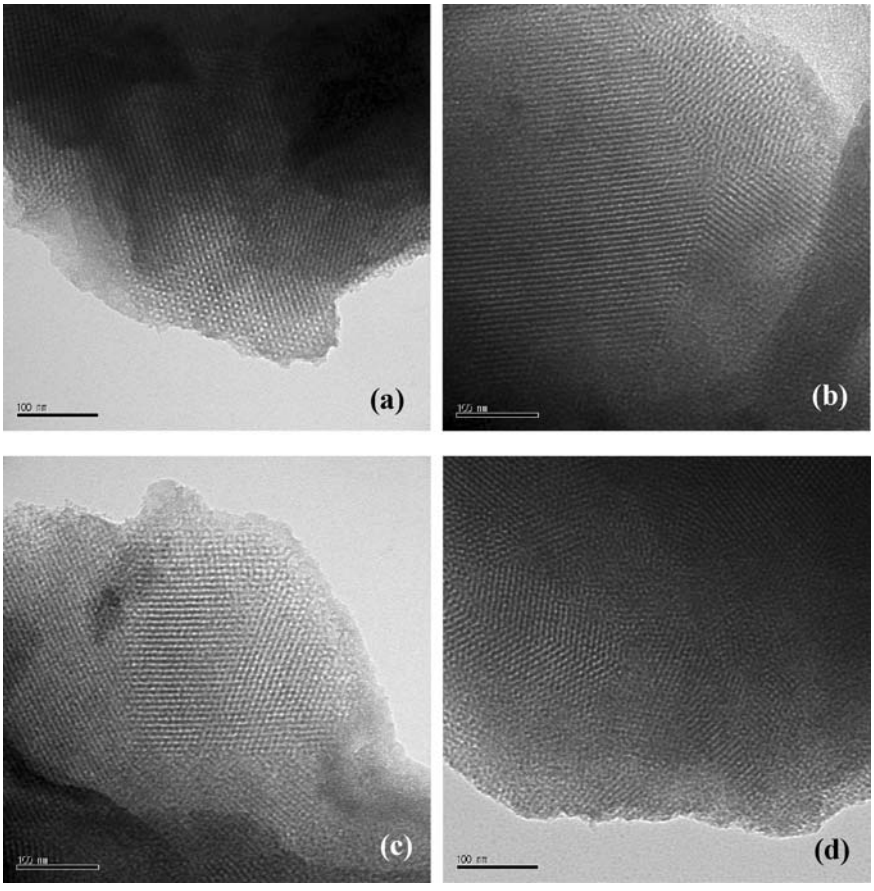


Fig. 3 TEM images of (a) Cubic Ia3d large mesoporous silica (100°C), (b) Cubic Ia3d large mesoporous silica (130°C), (c) 5% SH-cubic Ia3d (direct), (d) 9% SH-cubic Ia3d (direct)

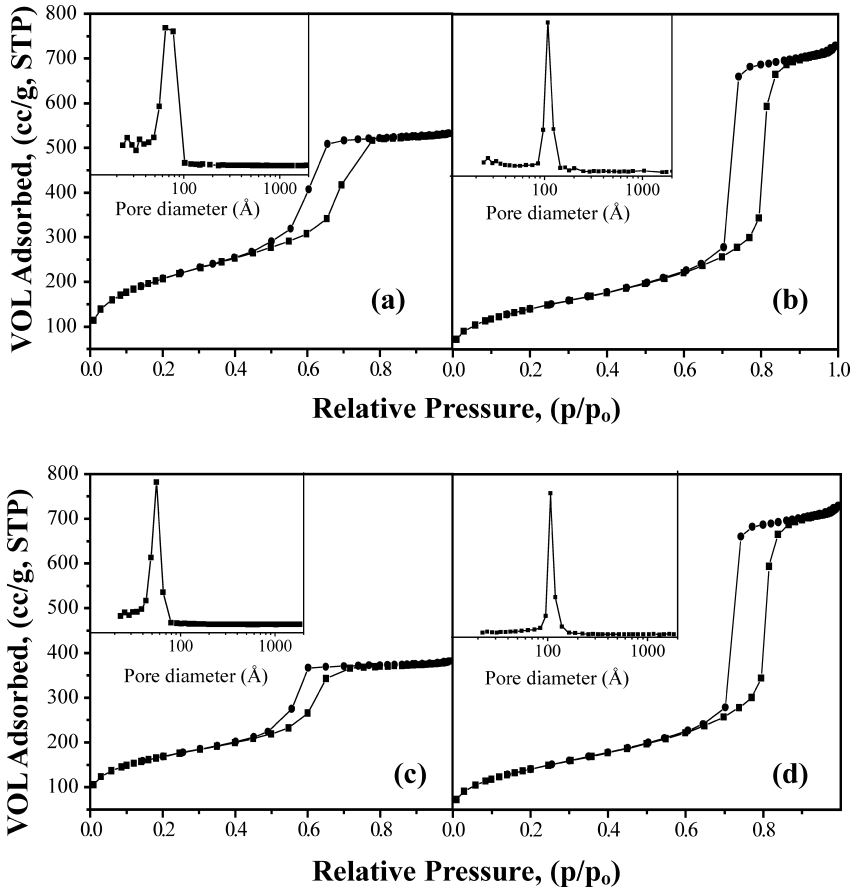


Fig. 4 N_2 adsorption-desorption isotherm of (a) SBA-15 (100°C), (b) SBA-15 (150°C), (c) Cubic Ia3d large mesoporous silica (100°C), (d) Cubic Ia3d large mesoporous silica (130°C)

ganic species. The amount of thiol loading in the functionalized mesoporous materials was also measured. According to EA analysis, 5% SH-SBA-15 (D) contained 2.2% w/w (equivalent to 4.1 mol%) of S and 10% SH-SBA-15 (D) contained 3.6% w/w (7 mol%) of S in the products obtained, respectively. On the other hand for thiol-grafted samples, SH-SBA-15 (G) contained 2.5% w/w (4.7 mol%) of S, and 2.8% w/w (5.2 mol%) of S for SH-cubic Ia3d (G). Thus organic-containing samples prepared by direct synthesis had higher thiol loadings than those prepared by grafting method, even though thiol-group in the grafting solution was substantially higher (24 mol%).

Trypsin was immobilized on the surface of the mesoporous silica samples both by physical adsorption (on pure silica) and covalent bonding (on functionalized silica). The amount of the enzyme adsorbed and the amount of enzyme leached out from the adsorbed support were measured by a UV spectrometer at 280 nm, and these are summarized in Table 2. All the un-functionalized pure silica mesoporous molecular sieves adsorbed more than 88% of the trypsin introduced. However, a significant portion of the trypsin adsorbed was found to be leached out from the support during stirring in a pH = 8 buffer for 2 h, as was reported previously [10]. This is due to weak interaction between trypsin and surface of the purely siliceous support materials. On

Table 2 The percentage of trypsin adsorbed on the mesoporous materials and the percentage leached out after adsorption

Materials	Adsorbed (%)	Leached from support (%)
SBA-15 (100°C)	100	33
SBA-15 (150°C)	88	53
Cubic Ia3d (100°C)	97	20
Cubic Ia3d (130°C)	97	28
5% SH-SBA-15 (grafting)	85	8
5% SH-SBA-15 (direct)	93	5
10% SH-SBA-15 (direct)	78	9
5% SH-cubic Ia3d (grafting)	89	8
5% SH-cubic Ia3d (direct)	92	5
9% SH-cubic Ia3d (direct)	81	7

average, cubic Ia3d mesoporous silica with 3-D symmetry had adsorbed more enzyme than SBA-15 with 1-D pore structure, but less was removed from the support via leaching. Apparently, 3 D channels with adequate pore size would induce faster and more efficient influx of enzyme molecules to a porous material, whilst those enzyme molecules entrapped inside the 3 D pores would find it more difficult to diffuse out to the bulk solution than in I D channels. In addition, solid

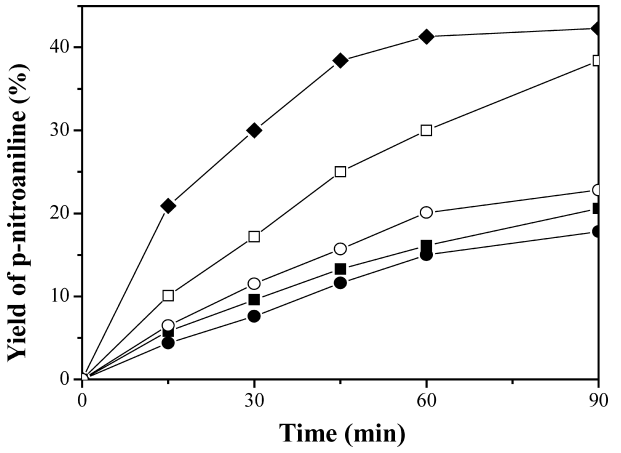


Fig. 5 BAPNA hydrolysis activity catalyzed by trypsin immobilized on the mesoporous materials; Free enzyme, ♦ SBA-15 (100°C), ● SBA-15 (150°C), □ Cubic Ia3d large mesoporous silica (100°C), ○ Cubic Ia3d large mesoporous silica (130°C)

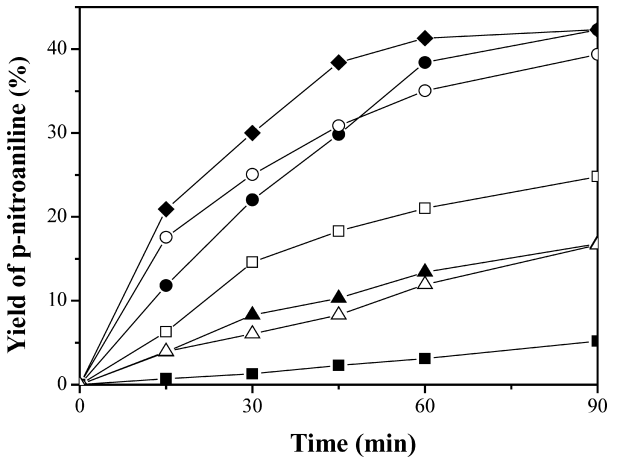


Fig. 6 BAPNA hydrolysis catalyzed by trypsin immobilized on the thiol-containing mesoporous materials; Free enzyme, ♦ 5% SH-SBA-15 (grafting), ● 5% SH-SBA-15 (direct), ▲ 10% SH-SBA-15 (direct), □ 5% SH-cubic Ia3d (grafting), ○ 5% SH-cubic Ia3d (direct), △ 9% SH-cubic Ia3d (direct)

samples with larger pore size (prepared at higher synthesis temperature) were proved inadequate for physical adsorption for immobilizing enzymes due to excessive leaching.

Trypsin contains S-S groups in its structure that can interact with a thiol group [12], and thus stronger chemical bonding between the thiol-functionalized mesoporous silica and trypsin is expected than in physical adsorption with pure silica mesoporous supports. In accordance with this expectation, trypsin from the thiol functionalized materials leached out from the solid phase noticeably decreased to less than 10% of the immobilized enzyme. For 10% SH-SBA-15 (D) and 9% SH-cubic Ia3d (D) samples, the access of trypsin to pores must be hindered significantly due to smaller pore sizes of the support caused by the presence of the large amount of organic group. But, the rest of the thiol-functionalized samples demonstrated acceptable level of enzyme loadings. In addition, thiol-group introduced by direct method proved better than those prepared by grafting at the similar thiol level of 5%.

The catalytic activity of the mesoporous materials immobilized with trypsin was monitored at 25°C in a pH = 8 buffer using N-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA) as a reactant for hydrolysis. The catalytic activities of purely siliceous SBA-15 and cubic Ia3d mesoporous silica with immobilized trypsin were compared in Fig. 5. One thing clearly emerging from the comparison is that three-dimensional channel structure of cubic Ia3d mesoporous silica is advantageous to SBA-15 for catalytic reaction. Trypsin immobilized MCM-48 with 3-D channels was reported earlier [10], but small pore size of MCM-48 interrupted free movement among substrate, product, and buffer ions and its catalytic performance was found inferior to SBA-15. Cubic Ia3d mesoporous silica in our experiment possesses the same 3-D pores but large enough pores to accommodate trypsin (3.8 nm) as well as the substrates effectively, and resulted in substantially improved performance over SBA-15. On increasing the pore size of cubic Ia3d mesoporous silica, however, catalytic activity of the mesoporous material declined due to lower amount of the enzyme immobilized (see Table 2). Therefore, mesoporous silica support with 3-D pore structure with proper pore size, which can accommodate enzyme species and allowing free movement of substrates, would be promising for enzyme immobilization. Trypsin immobilized on the mesoporous materials by physical adsorption, however, generally showed poor catalytic activities compared with free enzyme case except for cubic Ia3d (D) prepared at 100°C.

The catalytic activities of trypsin immobilized on the thiol-functionalized mesoporous materials were compared in Fig. 6. Free enzyme solution showed the highest catalytic activity among the samples compared because of the larger amount of the enzyme involved in the reaction than the immobilized cases, but the absence of significant mass transfer step to the active sites could also have been a contributing factor. The catalytic activities of the thiol functionalized samples were generally higher than those of the pure silica samples prepared using physical adsorption method. In addition, the activities measured for the directly prepared thiol containing silica samples were substantially higher than those prepared by grafting: 5% SH-cubic Ia3d (G) with the three-dimensional pore structure, however, showed significantly improved catalytic activity profile over 5% SH-SBA-15 (G).

5% SH-SBA-15 (D) and 5% SH-cubic Ia3d (D) showed good catalytic activities, which approach that of the free enzyme. Apparently, when the thiol loading is in the optimum range in the mesoporous materials, pore arrangement seems to have less important influence in catalytic performances. Interestingly, thiol functioalization showed little improvement in catalytic activity over the pure silica cubic Ia3d material,

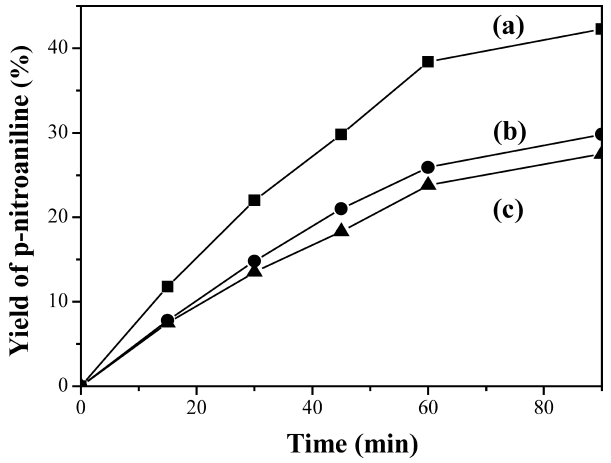


Fig. 7 Recovery and reusability of trypsin immobilized on the thiol-containing mesoporous materials; (a) Fresh catalyst, (b) 1st recycle, (c) 2nd recycle (used catalysts was stored at 4°C for three days)

except at the beginning of the reaction. Cubic Ia3d with thiol functioalization by direct method was a challenging task, and it is believed 5% SH- cubic Ia3d (D) prepared is in fact a mixture of 1 D and 3 D channel systems. This explains the indifference of the thiol addition on catalytic activity, which may have been masked by the change in pore arrangement from 3 D to a mixture of 1 D and 3 D. Higher loadings of the thiol was not desirable due to lower amount of enzyme immobilized and hindered diffusion of the substrates.

Enzyme molecules are not easy to recover and re-use, and thus the testing of recycling a support- immobilized enzymes is important. After the first catalytic reaction, used 5% SH-SBA-15 (D) sample was stored in a pH = 8 Tris-buffer at 4°C for 3 days. The catalytic activity of this material was compared with the fresh catalyst as shown in Fig. 7. After each experiment, used catalyst was filtered and washed several times with a pH = 8 buffer solution, and the same hydrolysis of BAPNA experiment was repeated. This result shows that the initial activity of the 5% SH-SBA-15 (D) with supported trypsin decreased to ca. 70% in the second run, and it leveled off to ca. 65% activity in the third run.

4. Conclusions

Two representative classes of mesoporous silica, SBA-15 and cubic Ia3d large pore mesoporous silica, were selected as a trypsin support, based on their differences in pore dimensions and structures. The cubic Ia3d large mesoporous silica with three-dimensional pore structure was confirmed to be an excellent support for enzyme immobilization due to the enhanced accessibility of the substrate to the active site of enzyme molecules. The catalytic activities of the thiol-functionalized mesoporous molecular sieves by covalently bonding were superior to those samples prepared by physical adsorption since strong interacting between thiol group and trypsin molecule enables them to retain the higher amount of the enzyme in the solid support. Direct synthesis was a better method to introduce thiol group than by post-synthetic grafting. 5%-SH-SBA 15 (D) and 5%-SH-cubic Ia3d (D) showed the highest catalytic activity among the samples tested approaching to higher than ca. 85% of the catalytic activity corresponding to the free enzyme molecules. Re-use of the trypsin immobilized 5%-SH-SBA 15 (D) was demonstrated, which maintained ca. 65–70% of the activity of the fresh catalyst.

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