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The adsorption characteristics, activity and stability of trypsin onto mesoporous silicates

D. Goradia, J. Cooney, B.K. Hodnett, E. Magner*

Materials and Surface Science Institute, Department of Chemical and Environmental Sciences, University of Limerick, Limerick, Ireland

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Abstract

The immobilization of the hydrolytic enzyme trypsin onto various mesoporous silicates (MPS) was studied. MPS were prepared using cationic or non-ionic surfactants (average pore diameters were in the range of 28–300 Å). All MPS were characterised by X-ray diffraction, scanning electron microscopy and nitrogen porosimetry. Enzyme purity strongly influenced loading. Trypsin adsorbed on MPS was found to be desorbed more readily by polyethylene glycol than by ammonium sulphate, suggesting that hydrophobic–hydrophilic interactions were important. Immobilized trypsin showed 10–20 times higher activity than the free trypsin and was stable for 4–6 weeks when stored at 4 or 25 °C. The trypsin–MPS catalyst was successfully reused for up to 6 cycles

Keywords: Adsorption; Trypsin; Mesoporous silicates; Immobilization; Stability; Desorption

1. Introduction

Research in the last decade on mesoporous silicates (MPS) has demonstrated the attractive features, such as large surface areas up to 1000 $m^2\ g^{-1}$ [1,2], chemical and mechanical stability and resistance to microbial attack. Their large pore dimensions 2–10 nm can accommodate enzymes within their long channels [3]. Furthermore, the pore openings of MPS can be modified with organosilane groups, entrapping a guest molecule [4]. The immobilization of biomolecules by adsorption in MPS offers many advantages over other methods of immobilization, such as membrane encapsulation, cross-linking, covalent binding and sol–gel entrapment. For example, during the synthesis of sol–gels, tetramethylorthosilicate or tetraethylorthosilicate is hydrolysed first and then condenses to make SiO_2-based materials [5]. However, the formation of pores and channels is not controlled and dimensions range from 0.1 to 500 nm; often interconnected micropores and channels are formed, allowing only small substrates to penetrate, while bigger substrates clog the channels, slowing the rate of reaction. Mesoporous molecular sieves can be prepared with one-dimensional channel systems with uniform pore diameters in the range 20–300 Å [3,6]. As a result, many efforts have been directed towards developing techniques for the efficient immobilization of biomolecules such as proteins and enzymes [7,8]. Immobilization of enzymes onto inorganic materials such as MPS has been studied due to their potential applications in separation technologies [9], biocatalysis [10,11] and biosensors. The adsorption of enzymes onto inorganic materials such as MPS, under mild conditions, may have very useful practical applications [12–14].

There have been a number of reports describing the use of MPS to immobilise proteins [15,16]. Diaz and Balkus [17] have immobilized cyt *c*, papain, trypsin onto MCM-41, SBA-15 and a layered niobium oxide NB-TMS4, and compared their stabilities with those of the native proteins. An increase in stability was observed for MPS-immobilized cytochrome *c* (cyt *c*).

Deere et al. [18,19] have studied the immobilization of cyt *c* onto MPS in detail and have observed that, for significant adsorption of cyt *c* to occur the pore diameter of MPS should

* Corresponding author. Tel.: +353 61 202629; fax: +353 61 202568.
E-mail address: edmond.magner@ul.ie (E. Magner).

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be larger than that of protein and the surface charges of the protein and of the MPS should be complementary. The peroxidative activity of the adsorbed protein was up to 10 times higher than that of the aqueous analogue [20].

Yiu et al. [21] studied the adsorption of trypsin onto MCM-41, MCM-48, SBA-15 and on MPS with functionalized surfaces. SBA-15-supported trypsin showed a higher activity than trypsin adsorbed on to the smaller pore MCM-41. Other studies using SBA-15-functionalized surfaces have shown that tailoring of MPS surface with functional groups is also important in enhancing the interactions of protein surfaces and the MPS.

Stevens and co-workers [22] examined the use of MPS in the separation of biological molecules, based on size selectivity. Lysozyme, trypsin and riboflavin were adsorbed onto siliceous MCM-41. The rate of adsorption depended on the size of the adsorbing molecule and the pore size of the MPS; the fastest rate of adsorption was observed for the smallest molecule, riboflavin, suggesting that the rate of adsorption and the capacity of the mesoporous materials depended on the pore to solute size ratio.

Recovery and reusability of immobilized enzymes are two important aspects from an application standpoint. He et al. found that in comparison to enzymes in solution, the activity of MCM-41-immobilized penicillin acylase remained constant at 250 U min^{-1} g^{-1} even after a number of uses (6×), indicating that the immobilized enzyme was stable after several uses and did not leach from the support [23].

In the last decade, enzymatic bioreactors have been widely employed in various industrial processes [24,25]. For example, trypsin is commonly used in the hydrolysis of casinomacropeptide [24]. The hydrolytic product (peptides) is of immense importance in the food industry and is recognised as a functional food. The design and development of processes for the continuous production of the peptide on a large-scale at a competitive cost is of interest. Another important application of immobilized hydrolytic enzymes like trypsin is in the manufacture of hypoallergenic infant food [25]. This product contains peptides obtained from natural proteins and is treated with proteolytic enzymes to limit protein hydrolysis thereby destroying the allergenic epitopes of natural protein. To manufacture these peptides, bioreactors with enzymes such as trypsin can be successfully used.

Recently, Gómez et al. [26] successfully demonstrated the use of a bioreactor using encapsulated trypsin and found trypsin to be a stable catalyst for the transesterification of *N*-acetyl-L-tyrosine ethyl ester with propan-1-ol. The immobilized trypsin showed no leaching or loss of activity, even after reuse for five separate reactions. In this paper, we characterise this reactor system in detail. To date, there is very little known about the interaction of a hydrolytic enzyme such as trypsin with MPS. Trypsin has been extensively characterised and was chosen as an enzyme in the present study mainly due to its small size, ease of spectophotometric quantification and its well-defined activity and selectivity. Trypsin is a globular enzyme with spherical molecular diameter (38 Å) [17] [molecular mass ∼23,800 Da] [27]. It belongs to group of serine proteases and is one of the three principle digestive proteinases. It has an active site consisting of aspartic acid, histidine and serine residues and the surface of trypsin possesses –S–S–, amine and thiol groups [21].

This study examines the adsorption, desorption, activity and stability of MPS-immobilized trypsin. The adsorption of trypsin onto MPS materials such as MCM-41 and SBA-15 has been reported previously [7,21]. However, storage stability, reusability or influences of impurities in trypsin on uptake of enzyme have not been reported. Enzyme purity, storage stability and in particular reusability, are important if MPS-immobilized trypsin is to be used for bioreactors and biocatalysis.

2. Experimental

2.1. Reagents

Two sources of trypsin were used; namely purified trypsin (bovine pancreas 95% pure, TPCK treated, minimum 10,000 BAEE units/mg protein, # T-1426) and impure trypsin (porcine pancreas, 1000–2000 BAEE units/mg solid, # T-7409), cetyltrimethyl ammonium bromide (CTAB, 99%), ammonia (37%, w/v), polyethylene glycol (PEG, average molecular weight 1000 Da), brilliant blue G, DMSO (99%), BAPNA, BSA (fraction V), methanol (HPLC grade), potassium phosphate buffer and ammonium sulphate buffer were obtained from Sigma–Aldrich. Commercial kieselgel silica (COS) was obtained from Fluka (Reidal de Häen, product number 31607). Tetraethoxysilane (TEOS, 98%) and 2-cyanoethyltriethoxysilane (CEOS, 98%) were purchased from Lancaster. Pluronic-F127 ($EO_{106}PO_{70}EO_{106}$) was obtained from BASF. Water was purified (18.2 MΩ) using an Elgastat spectrum system.

2.2. Synthesis and characterisation of MPS materials

CEOS is a phase modifier, depending on the amounts added during synthesis MCM-48 or MCM-41 can be produced [28]. When NH_3 and CEOS is used, a disordered, mesoporous material with large pore diameters (CNS) is formed [28]. CNS was synthesised by mixing 50 g CTAB with 450 g of water. This solution was heated to 30 °C until all of the surfactant was dissolved, after which 50 ml TEOS, 6.25 ml CEOS, 2.5 ml NH_3 were added. The gel mixture was heated to 105 °C for 24 h. The surfactant was removed by refluxing in a methanol solution containing 1.5% HCl and 2.5% H_2O. The CNS material was refluxed until no change in mass was observed. CNS–Cal was formed by calcination of CNS (ramp rate 1 °C min^{-1}) and held at 650 °C for 6 h under a stream of air (100 cm^3 min^{-1}).

MCM-41/28 was synthesised by mixing 10 g CTAB, 1 g NaOH and 90 g water. On dissolution of the CTAB, 9 ml of

TEOS and 2.5 ml of CEOS were added and stirred at 35 °C for 30 min. The mixture was then heated to 150 °C for 24 h. The product was filtered and added to 200 ml H_2O. This was then stirred and heated to 70 °C for 10 min. The product was then filtered. The surfactant template was removed by calcination at 650 °C for 6 h (ramp rate 1 °C min^{-1}).

For the synthesis of MCM-41/33, 45 g TMAOH and 136 g CTAB were dissolved in 927 g H_2O by continuous stirring at 35 °C until completely dissolved. Fumed silica (50 g) was then added to the mixture. The reaction mixture was stirred for another hour until a gel was formed. The resultant mixture was then left to age at room temperature for 20 h, then heated to 150 °C for 48 h. The remaining material was filtered and dried at room temperature. Finally, the surfactant template was removed by calcination at 550 °C for 8 h (ramp rate 1 °C min^{-1}).

MPS-F 127 was synthesised using the non-ionic tri-block copolymer Pluronic-F127 as the structure directing agent [29]. The silicate was prepared by stirring 1 g of Pluronic-F127 surfactant, 7.5 g of H_2O and 30 g of 2 M HCl at room temperature. When the surfactant was dissolved, 2.28 ml of TEOS was added and the solution was stirred for 24 h at room temperature. The gel, which formed was then aged at 100 °C for 20 h in a Teflon-lined stainless steel reactor. The product was washed and calcined at 500 °C for 6 h (ramp rate 1 °C min^{-1}).

All the silicates used were characterised by nitrogen gas adsorption/desorption isotherms at 77 K measured using a Micrometrics Gemini ASAP 2000 system. Samples were pre-heated at 150 °C under vacuum for 16 h (to remove bound H_2O). The pore size data were analyzed by the thermodynamically based Barrett–Joyner–Halenda (BJH) method [30] using the desorption branch of the isotherm. Surface areas were measured using the Brunauer–Emmett–Teller (BET) method [31].

2.3. XRD analysis

XRD analysis was performed on a Philips X'Pert, PRO MPD 3050/60 model using a (Cu Kα source), wavelength (λ = 1.54 Å). The scanning range of 2θ was set between 1° and 45° with a step size of 0.02; generator settings were 40 kV, 35 mA.

2.4. Enzyme adsorption

Enzyme adsorption isotherms were generated by mixing enzyme (0.1–42 μM) with an MPS suspension (final concentration of 1 mg ml^{-1}) in 1.5 ml eppendorff tubes at 25 °C for 16–18 h. The amount of trypsin adsorbed was measured by a difference method with enzyme concentrations determined before and after adsorption by UV absorption at 280 nm (trypsin, ε = 14,300 M^{-1} cm^{-1}) [32]. The effect of ionic strength on the adsorption process was examined using a buffer solution (5 mM potassium phosphate, pH 6.5) with varying concentrations of NaCl.

2.5. Desorption

To study desorption of enzyme from MPS, the adsorbed MPS was washed with buffer (25 mM potassium phosphate, pH 6.5), centrifuged (micro centrifuge) for 1 min (13,000 rpm) and the buffer removed. This was repeated four times for each sample. Desorption solutions (1 ml) were then added to each eppendorff tube. The desorption solutions used were 10% (w/v) polyethylene glycol (PEG), 1 M $(NH_4)_2SO_4$ and 10% (w/v) methanol (all solution were made in 25 mM phosphate, pH 6.5). Desorption was carried out at 5 °C, for 16–18 h. After each desorption period had elapsed at least 0.6 ml of supernatant was removed and centrifuged, and the absorbance was then read at 280 nm. Other experiments involved changing parameters such as buffer, ionic strength, temperature and addition of PEG/1 M $(NH_4)_2SO_4$ combinations.

2.6. Determination of protein concentration in 10% methanol

Protein concentration in methanol was determined using the Bradford assay [33].

2.7. Amidolytic activity assay

The activity of immobilized trypsin was determined at 25 °C, by a modification of the method of Erlanger et al. [34] by monitoring the catalytic hydrolysis of *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). A range of substrate concentrations (0.1–10 mM) was used in 25 mM phosphate buffer and pH 6.5. Measurements were also made using free trypsin. MPS without adsorbed protein was tested for activity and was found to exhibit no amidolytic activity towards BAPNA (ε_{molar} = 10,500 M^{-1} cm^{-1}) [32,34]. Kinetic parameters were calculated from Lineweaver–Burk plots. The data were also fitted to the Michaelis–Menten equation using non-linear regression analysis (Prism 3.0). Data were in good agreement between the two models and average values are reported.

2.8. Storage stability measurements

The storage stability of the enzymes was studied by measuring the residual activity after incubation of free and immobilized enzyme (concentration 0.005 mg/ml) in 25 mM phosphate buffer, pH 6.5 in the absence of substrate at 4 and 25 °C for up to 10 weeks. Assays for amidolytic activity using BAPNA was performed in triplicate at various time intervals to determine the percentage remaining activity.

2.9. Reusability measurements

The reusability of MPS-immobilized trypsin was studied by measuring the residual activity. Each time, immobilized trypsin (concentration 0.005 mg/ml) was washed four times

with 25 mM phosphate buffer, pH 6.5 at 25 °C. The assay for amidolytic activity using BAPNA was performed in triplicate to determine the percentage of activity remaining.

The reusability of MPS was also studied. Trypsin-loaded MPS was regenerated by calcination (ramp rate 1 °C min^{-1}) at 650 °C for 6 h in a stream of air (100 cm^3 min^{-1}).

2.10. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE)

A sample of 20 μg of pure trypsin and 75 μg of impure material were combined with sample buffer containing 62.5 mM Tris–HCl buffer, pH 6.8, 2% SDS, 0.7 M β-mercaptoethanol, 10% (v/v) glycerol and 0.1% (w/v) bromophenol blue, and boiled prior to electrophoresis. Samples were loaded on a 15% polyacrylamide gel slab containing 0.1% SDS [35]. The gels were run at a constant current of 20 mA at room temperature. The protein bands were visualised using Coomassie blue R-250 stain and compared with standard molecular weight markers (Biorad Prestained Broad range markers).

3. Results and discussion

The five MPS materials used in these studies exhibited quite different pore sizes and surface areas. The physicochemical properties of these MPS materials are presented in Table 1. The isotherms are all of type IV. Each shows a strong sharp increase in nitrogen adsorption at P/P_0 in the range 0.3–0.5, behaviour typical of mesoporous solids [30,31]. As the relative pressure increases, the isotherms exhibited a sharp inflection characteristic of capillary condensation within mesopores. The sharpness of this step suggests a uniform size pore system. A large hysteresis effect was observed at relative pressures above 0.7 for COS and CNS. In MCM-41/33, MCM-41/28 and MPS-F127, this was not the case. CNS had the largest and MPS-F127 had the smallest pore volume, at 1.26 and 0.35 cm^3 g^{-1}, respectively. All MPS materials were characterised using XRD. MCM-41/33 and MCM-41/28 exhibited hexagonal structures. CNS, MPS-F127 and COS did not exhibit any XRD ordering.

Trypsin readily adsorbed onto all the MPS materials used in this study. Maximum adsorption was achieved with CNS after a contact time of ∼14 h (Fig. 1). By comparison, the rate of adsorption of trypsin for all other MPS material was similar with a contact time of ∼16–18 h required to reach steady state levels.

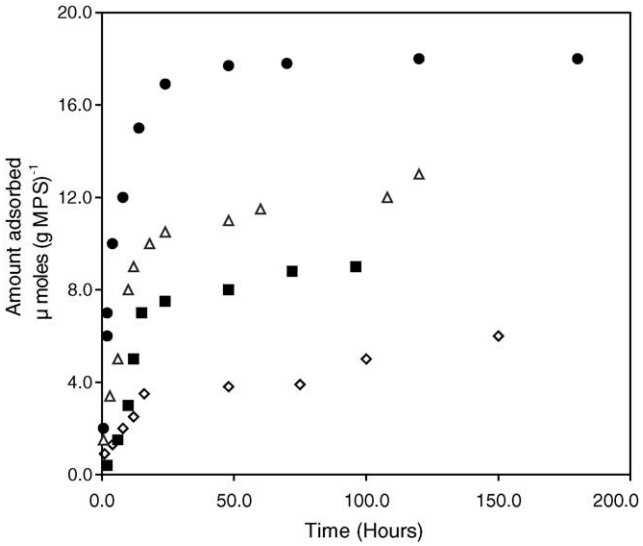

Fig. 1. Adsorption of purified trypsin onto CNS (●), COS (△), MCM-41/33 (■), MCM-41/28 (◇) (25 °C, 25 mM phosphate buffer, pH 6.5) as a function of time.

3.1. Adsorption isotherm of purified trypsin

The adsorption isotherms (Fig. 2) demonstrate that purified trypsin has the highest affinity for CNS with 18 μmol (g PS)$^{-1}$ being adsorbed. MCM-41/33 exhibited a plateau value of 7 μmol (g MPS)$^{-1}$. The maximum amount of trypsin adsorbed on to MCM-41/28 was 3.5 μmol (g MPS)$^{-1}$. MCM-41/28 material has an average pore size of 28 Å, too small for trypsin to penetrate and indicating that multi-layer adsorption occurs with MCM-41/28 and is largely confined to the external surface of the silicate. A previous study by Deere et al. demonstrated that in circumstances where the protein/enzyme (cyt *c*) is too large to enter the mesopores, multi-layer adsorption occurs [36]. Accordingly, multi-layer adsorption is also likely to occur with trypsin on MCM-41/28. The pores in the COS material with an average diameter of 60 Å are large enough to allow penetration of trypsin into the pore with a maximum adsorption of 10 μmol (g MPS)$^{-1}$. MPS-F 127 shows a lower loading of 5 μmol (g MPS)$^{-1}$,

Table 1
Physicochemical properties of MPS materials

Mesopore material	Average pore size (Å)	BET surface area (m^2 (g MPS)$^{-1}$)	Total pore volume (cm^3 (g MPS)$^{-1}$)	Trypsin maximum load (μmol (g MPS)$^{-1}$)		Trypsin volume adsorbed (cm^3 (g MPS)$^{-1}$)[a]	
				Pure	Impure	Pure	Impure
CNS	180	448	1.26	18	3	0.31	0.05
COS	60	489	0.84	10	2.3	0.17	0.04
MCM-41/33	33	966	1.06	7	2.1	0.12	0.03
MPS-F127	51	610	0.35	5	1.32	0.08	0.02
MCM-41/28	28	981	0.86	3.5	1.03	0.06	0.02

[a] Calculated on the basis of the volume of one trypsin molecule by using a spherical molecular diameter of 38 Å [17].

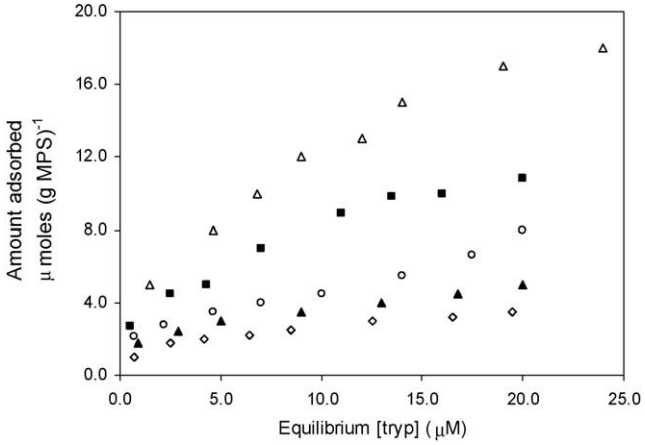

Fig. 2. Adsorption isotherms for purified trypsin onto different MPS (25 °C, 25 mM phosphate buffer, pH 6.5): CNS (△), COS (■), MCM-41/33 (◯), MPS-F127 (▲), MCM-41/28 (◊).

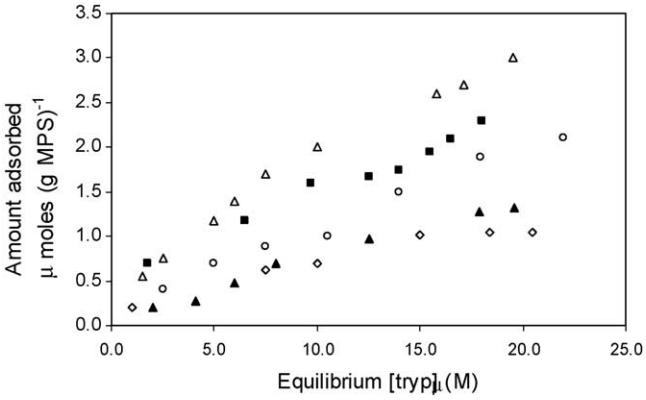

Fig. 3. Adsorption isotherms for impure trypsin onto different MPS (25 °C, 25 mM phosphate buffer, pH 6.5): CNS (△), COS (■), MCM-41/33 (◯), MPS-F127 (▲), MCM-41/28 (◊).

despite its larger average pore size distribution; the reason for this low amount of adsorption must lie in differences in the structure of MPS and the different materials used in their synthesis (i.e. silica source, structure directing agents) [37]. Recently, Takahashi et al. [7] have shown that larger levels of protein adsorption occurred on MPS (MCM-41, FSM-16) templated with cationic surfactants and suggested that this was due to the higher level of negatively charged groups on the surface of these materials compared with MPS templated with non-ionic surfactants (SBA-15).

3.2. Adsorption isotherms for impure trypsin

In order to determine the effect of impurities on the amount of adsorption of trypsin onto various MPS, impure trypsin was adsorbed onto all MPS materials. The results suggest a similar trend in adsorption as with pure trypsin. The conditions used for the immobilization of impure trypsin onto MPS materials were similar to those used for pure trypsin (phosphate buffer, pH 6.5, 25 °C). The results clearly indicate that the purity of the enzyme plays a vital role in the immobilization process. The total volume of impure trypsin adsorbed onto CNS was 0.05 cm^3 (g MPS)$^{-1}$. The maximum amount of impure trypsin adsorbed on CNS was 3 μmol (g MPS)$^{-1}$, a five-fold reduction over that observed with purified trypsin (see Fig. 3). A possible explanation for the reduced amount of adsorption is that the impure material could contain impurities which could block the pore openings such that, trypsin is unable to get into the pores. In addition, such impurities might have higher affinities for MPS materials, preventing trypsin from being adsorbed. To investigate possible contaminants, this sample and purified trypsin were examined using SDS–PAGE and it was found that the former material contained a significant amount of small peptide and protein impurities. This impurity in the sample may have had a higher affinity for the MPS materials, inhibiting uptake of trypsin. Note that the level of loading of trypsin (monitored at 280 nm) from the impure sample is only an estimate as proteins other than trypsin may be adsorbed on to MPS.

Pure trypsin did not show the same affinity for MPS materials, on which impure trypsin had been previously adsorbed (Fig. 4). A sample of CNS was exposed to impure trypsin followed by purified trypsin. The amount of trypsin adsorbed from the impure source at 360 min was 0.8 μmol (g MPS)$^{-1}$ and a further 0.8 μmol (g MPS)$^{-1}$ was adsorbed over the next 360 min when purified trypsin was added. At larger contact times, 960 min trypsin adsorbed more strongly, but never reached the values (typically 16 μmol (g MPS)$^{-1}$ observed for the MPS material not exposed to impure trypsin. These studies show that, for adsorption to occur, the purity of protein/enzyme must also be taken into account [19].

3.3. Desorption from MPS

Adsorbed trypsin was found to be stable on the MPS materials on repeated washing with adsorption buffer (25 mM phosphate, pH 6.5). The enzyme was washed repeatedly (five

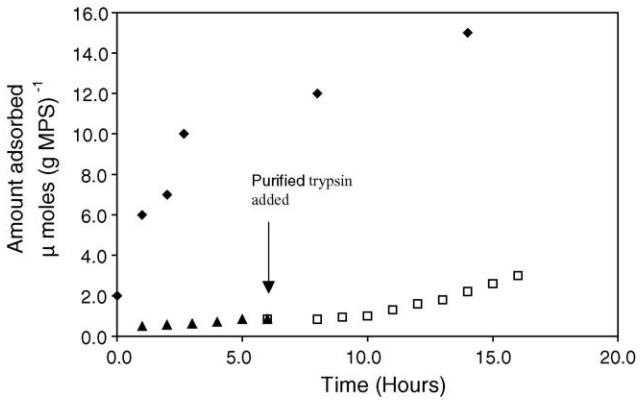

Fig. 4. Adsorption of purified trypsin onto CNS: (♦) adsorption of purified trypsin onto CNS, (▲) adsorption of impure trypsin onto CNS and (□) adsorption of purified trypsin onto CNS previously exposed to impure trypsin (25 °C, 25 mM phosphate buffer, pH 6.5).

Table 2
Summary of amount of desorption of MPS-immobilized trypsin by various materials

MPS	Amountof trypsin adsorbed (μmol (g MPS)$^{-1}$)	Amount of MPS-immobilized trypsin desorbed (% desorbed)			
		10% PEG	10% Methanol	1 M $(NH_4)_2SO_4$	Buffer
MCM-41/28	3.5	2.80 (80)	0.14 (4)	1.05 (30)	0.17 (5)
MCM-41/33	5	3.00 (60)	0.27 (5)	1.00 (20)	0.21 (4)
COS	10	4.00 (40)	0.4 (4)	1.70 (17)	0.30 (3)
CNS	18	3.24 (18)	0.90 (5)	1.98 (11)	0.40 (2)

times) and small amounts of enzyme were detected in the supernatant solution.

The stability of the adsorbed enzyme was investigated in the presence of other materials. Polyethylene glycol (PEG) of average molecular weight 1000 Da and 1 M ammonium sulphate $(NH_4)_2SO_4$ was used. PEG was found to desorb the enzyme from the MPS materials. PEG (10%) desorbed 17–18% of the enzyme immobilized on CNS and 1 M $(NH_4)_2$ (SO_4)) desorbed 11%. Methanol desorbed only 4–5% of the enzyme over the same adsorbed enzyme range refer to Table 2.

Greater amounts of desorption were observed with PEG by comparison with methanol, indicating that the larger size of PEG was not important in the desorption process. The reason for greater amounts of desorption by PEG could be due to the presence of more amphiphilic groups than in methanol. Desorption from MCM-41/33 was similarly investigated with 10% PEG and 1 M $(NH_4)_2SO_4$. PEG alone desorbed 50–65% of the adsorbed trypsin. This is approximately three times the amount desorbed from CNS. This suggests that trypsin is more tightly bound to the CNS material by comparison with MCM-41/33.

Desorption from COS and MCM-41/28 was also investigated. Adsorbed trypsin (40%) was removed by PEG from COS, whereas the corresponding value with 1 M $(NH_4)_2SO_4$ was 17%, suggesting that adsorption of trypsin was dominated by hydrophobic–hydrophilic properties rather than electrostatic interactions with COS. The amount of trypsin desorbed by PEG from the MCM-41/28 material was 80% suggesting that interactions between the trypsin and MCM-41/28 are weaker than with other MPS. Also, since there is multi-layer adsorption most of the interactions could be trypsin–trypsin rather than MPS–trypsin. Thirty percent of adsorbed trypsin was desorbed with 1 M $(NH_4)_2SO_4$ and 5–10% after washing with adsorption buffer (25 mM phosphate, pH 6.5) (Table 2), This is in contrast with the findings reported by Yiu et al. [4], who reported 35–72% desorption after washing with buffer (50 mM Tris–HCl, pH 8.0). This difference could be explained by the higher pH used. Diaz and Balkus [17] reported a consistent increase in desorption for trypsin and papain at higher pH.

In general, PEG desorbs more trypsin from MPS materials than ammonium sulphate suggesting that hydrophilic/hydrophobic properties are more prominent than electrostatic interactions.

3.4. Competitive adsorption isotherms for pure trypsin

In these experiments, adsorption of trypsin was initiated in the presence of PEG and ammonium sulphate and the competitive inhibition of trypsin adsorption by these materials was studied.

For example, in the absence of PEG and ammonium sulphate, the amount of adsorption achieved was 18 μmol (g MPS)$^{-1}$. Adsorption in the presence of 10% PEG only allowed for the adsorption of 0.6 μmol (g MPS)$^{-1}$ CNS (refer Table 3) over the same equilibrium concentration range suggesting that adsorption and desorption occur via different mechanisms. Ammonium sulphate was less effective in inhibiting the adsorption of trypsin $\sim$10 μmol (g MPS)$^{-1}$. Therefore as expected from the desorption results, the presence of PEG inhibited adsorption of trypsin more readily than ammonium sulphate indicating that hydrophilic–hydrophobic interactions dominate in the trypsin–CNS system.

The competitive inhibition of trypsin adsorption onto MCM-41/33 was studied and displays the same adsorption characteristics as CNS. PEG (10%) alone inhibited adsorption and a plateau level of $\sim$1 μmol g^{-1} of MCM-41/33 was reached, as against 7 μmol g^{-1} of MCM-41/33 adsorption in the absence of PEG. Thus, in comparison to ammonium sulphate, PEG was the strongest at inhibiting adsorption onto CNS and MCM-41/33 materials, suggesting that the adsorption of trypsin with MCM-41/33 and CNS is hydrophobically dominated.

Table 3
Competitive adsorption of trypsin onto MPS in the presence of PEG or ammonium sulphate

MPS	Amount of trypsin adsorbed (μmol (g MPS)$^{-1}$)	10% PEG (μmol (g MPS)$^{-1}$)	1 M $(NH_4)_2SO_4$ (μmol (g MPS)$^{-1}$)
CNS	18	0.6	10
MCM-41/33	7	1	3.6

3.5. Influence of ionic strength

The significance of ionic strength on adsorption of trypsin onto COS was investigated initially by monitoring the adsorption process at different phosphate buffer concentrations. A range of concentrations was investigated from 25 to 200 mM, at pH 6.5. The amount of enzyme adsorbed was found to be strongly dependent on the buffer concentration with the amount adsorbed decreasing significantly with increasing buffer concentration.

The ionic strength was subsequently examined using a low concentration of buffer (5 mM phosphate, pH 6.5) and different concentrations of NaCl. As expected, higher NaCl concentrations inhibited adsorption of the enzyme, with the amount of enzyme adsorbed decreasing with increasing ionic strength. In the presence of 2 M NaCl solution ($I = 2.0$) 5.6 μmol g^{-1} COS was adsorbed at an equilibrium trypsin concentration of 9.6 μM. This amount is considerably higher than that observed for cyt *c* and shows that the ionic strength does not significantly affect the adsorption of trypsin on to MPS as reported by Deere et al. [19] Adsorption of cyt *c* was performed in presence of 2 M NaCl and only ∼0.8 μmol g^{-1} of cyt *c* was adsorbed. This indicates that different adsorption behaviour can occur for proteins of similar size and IEP.

3.6. Amidolytic activity

Table 4 presents the kinetic rate constants obtained for each of the MPS–trypsin systems. The values are compared to those of aqueous trypsin under the same conditions.

The k_{cat}/K_m values reported here are of a similar magnitude to reported values for unmodified trypsin using BAPNA as a substrate [38]. All of the silicates provide an environment in which the adsorbed trypsin was more catalytically active than aqueous trypsin with CNS–trypsin exhibiting the largest relative rates. Relative rates of 2 and 11 were obtained for MCM-41/33 and COS–trypsin, respectively. Similar results have been reported for chloroperoxidase, penicillin acylase, and lipase immobilized on a mesoporous silicate material where up to 30-fold rate enhancements were observed [11,23,39].

From Table 4, the larger pore size material (CNS) provides a greater enhancement effect than the smaller pore size MCM-41/33. The possible reason for this difference may be as previously reported by Deere et al. [19] that, there may be substantial multi-layers of trypsin on the outside surface area of MCM-41/33, thus taking into account multi-layered stacking of the enzyme molecules on the carrier surface, hence the internal layers of enzyme are less accessible for the substrate molecules and therefore, significant amount of trypsin may not be readily available to the substrate.

COS material gave a lower enhancement rate compared to the CNS material. This could be due to the larger pores of CNS allowing for faster substrate access to the active site of the enzyme. The adsorbed enzyme had displayed a higher activity (viz. CNS and COS) in comparison to the aqueous enzyme indicating that the diffusion of the substrate to the adsorbed trypsin (on the external surface and within the pores of COS as well as CNS) was not rate limiting. The higher values obtained with CNS and COS are in agreement to Deere et al., who showed MPS-immobilized cyt *c* had a higher enhancement rate than free enzyme [20]. While the reasons for these enhancements are not understood but such enhancements are attractive properties for use of these MPS system as biocatalytic supports in bioreactors and in biosensor.

3.7. Storage stability test

The storage stability of immobilized and free enzyme was investigated at 4 and 25 °C for 8 weeks. As seen in Fig. 5, a 70% loss of activity was observed for free enzyme after storage for 30 days at 4 °C. This might be due to protein–protein interaction (autolysis of the trypsin) [40]. MPS-immobilized trypsin lost only 20% of its activity at the same temperature in 30 days. This enhanced stability is probably a result of the prevention of autolysis by immobilization.

At room temperature (25 °C), a 80% loss was seen after 30 days for free enzyme, whereas the immobilized enzyme retained 60% of its activity over the same time period (refer Fig. 5). The results mentioned above indicated that the immobilized enzyme had a much better storage stability than free enzyme. In conclusion, the enhanced stability of the MPS-immobilized trypsin system offers an attractive platform for biocatalysis.

3.8. Reusability test

The reusability of immobilized enzymes is important for economical use of an enzyme, because of the importance for repeated applications in a batch or continuous reactor. Immobilized trypsin was active for all six reuses and retained 40% of activity after six uses (see Fig. 6). The observed decrease in activity is likely a result of desorption of the enzyme. Also some loss of activity of the immobilized preparation in repeated use is a common phenomenon [41]. Since the MPS–enzyme system is washed four times before every cy-

Table 4
Table of kinetic constants for amidolytic activity of MPS-immobilized trypsin and aqueous trypsin

MPS	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	Relative rates (k_{cat}/K_m)
MCM-41/33	0.65 ± 0.07	4 ± 0.6	6	2
COS	0.58 ± 0.1	16 ± 3.9	27	11
CNS	0.57 ± 0.13	42 ± 6.0	73	29
Aqueous trypsin	0.77 ± 0.07	1.8 ± 0.2	2.5	1

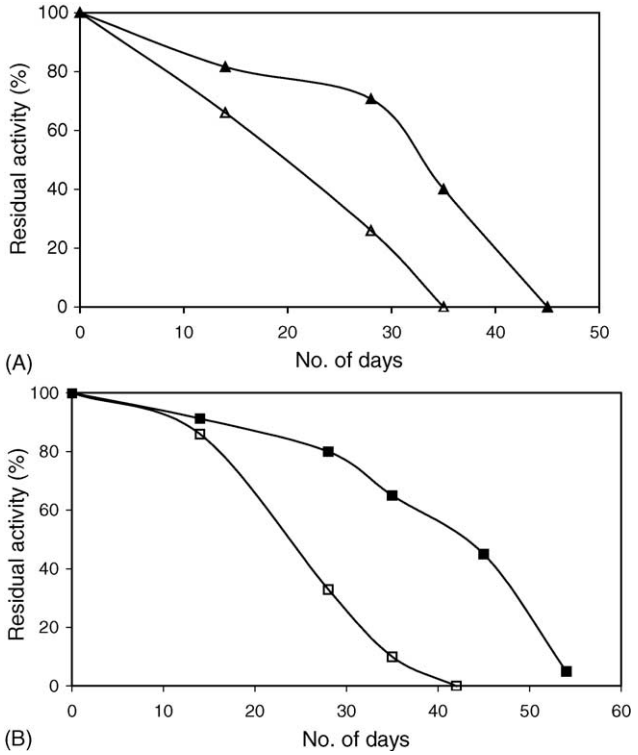

Fig. 5. Storage stability profile of trypsin at 4 and 25 °C (25 mM phosphate buffer, pH 6.5). (A) CNS-immobilized-trypsin at 25 °C (▲), free trypsin at 25 °C (△) and (B) CNS-immobilized-trypsin at 4 °C (■), free trypsin at 4 °C (□).

cle (which is about 24× for six cycles) approximately another about 10–15% of material is lost in the process of washing and decanting.

The reusability of CNS was investigated by successive trypsin adsorption. The loaded trypsin was removed by calcination. CNS was characterised for its pore size and pore volume. The physicochemical properties of CNS after each use are presented in Table 5. As can be seen from Table 5, after the second and third reuse pore size and pore volume

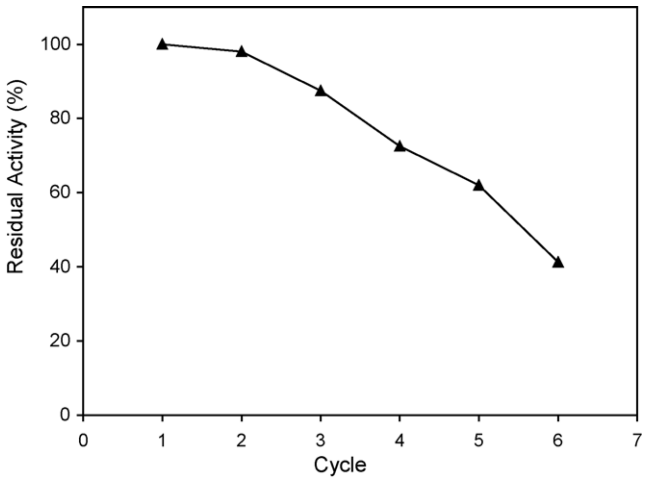

Fig. 6. Reusability of CNS-immobilized trypsin (25 °C, 25 mM phosphate buffer, pH 6.5).

Table 5
Physicochemical properties of CNS after reuse

Characteristics	First use	Second use	Third use
Pore size (Å)	269	202	187
Pore volume (cm^3 (g MPS)$^{-1}$)	1.26	1.07	0.87
BET surface area (m^2 (g MPS)$^{-1}$)	650	470	210
Volume of trypsin adsorbed (cm^3 (g MPS)$^{-1}$)	0.36	0.33	0.29
Trysin maximum load (μmol (g MPS)$^{-1}$)	21	19	17
Initial concentration of trypsin (μM)	0.1–42	0.1–42	0.1–42

were reduced to 65–70% of the original values. The reason for the gradual reduction in pore size and pore volume could be that it is possible that upon re-calcination MPS-immobilized trypsin is burned off causing deposition of carbon species inside the pores of CNS. Even after the third reuse, pore size of CNS (19 nm) was sufficiently large, and a considerable amounts of trypsin was adsorbed onto MPS, indicating that the MPS materials can be recycled.

4. Conclusions

Trypsin was readily adsorbed by all MPS materials used in this study. An interesting feature of this study is the influence of enzyme purity on the adsorption of the enzyme. Desorption of the enzyme in the presence of PEG indicates hydrophobic–hydrophilic interactions are important with MPS. Adsorption of trypsin onto MPS was inhibited by PEG. This in agreement with the desorption results. The activity of the immobilized enzyme was higher than that of free trypsin; thus, showing the suitability of such MPS materials for use as immobilization matrices. The higher storage stability and good reusability of MPS-immobilized trypsin system shows that the MPS holds promise as biocatalytic support. The physico-chemical properties of immobilized trypsin of practical importance were improved after immobilization. This suggests it has significant potential applications, especially, as it can also be reused several times. These findings prove the usefulness of the examined MPS–trypsin system for practical application in biocatalysis and bioreactors.

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