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Covalent immobilization of trypsin on to siliceous mesostructured cellular foams to obtain effective biocatalysts

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Abstract

The properties of siliceous mesostructured cellular foams (MCF) with the surface functionalised using different organosilanes to immobilize covalently trypsin have been studied. Four organosilanes were applied: 3-aminopropyltriethoxysilane, 2-aminoethyl-3-aminopropylmethyldimethoxysilane, 2-aminoethyl-3-aminopropyltrimethoxysilane and 3-glicydoxypropyl-triethoxysilane. The samples modified using alkylamines were further activated with glutaraldehyde (GLA), a cross-linker. Commercially available silica gels and Eupergit C were used for comparison. Activity of MCF-based biocatalysts expressed in BAPNA and casein conversion, was significantly higher than of the silica gel- and Eupergit C-based counterparts. In the best systems the determined activity of trypsin was higher than of a free enzyme. The GLA-amino linkages appeared the most effective systems for the covalent immobilization of trypsin. The MCF-based preparations were notably more stable than a native enzyme at 333 K and they also showed good storage stability at 277 K. A unique porous structure of MCF was found to be a critical factor which renders siliceous mesostructured foam a very promising material for immobilization of enzymes.

Keywords: Immobilization; Trypsin; Mesostructured cellular foams; Mesoporous materials

1. Introduction

Immobilization of free enzymes on various water-insoluble supports facilitates product and enzyme separation, improves biocatalysts stability, paves the way for their reuse and application in continuous operations, with a positive effect on the process economy [1]. Trypsin belongs to a group of serine proteases which attracts considerable interest due to a wide variety of possible applications, e.g. in hydrolysis of caseinomecropeptide to obtain a functional [2] and hypoallergenic [3] food, semi-synthesis of human insulin [4], peptide synthesis [5–7], resolution of enantiomeric *O*- or *N*,*O*-derivatized amino acids [8] and transesterification [9].

Numerous techniques had been used to immobilize enzymes, including adsorption and covalent bonding. A host of different supports had also been tested to find that both a suitable matrix and immobilization procedure are key factors to obtain efficient biocatalysts [1]. Synthetic polymers are the most common carriers [1,10–12] but owing to disposal problems, poor solvent stability and often poor reusability alternative supports are highly sought. Mesoporous silicates (MPS) obtained by the templating method demonstrate huge potentials for enzyme immobilization [9,13–27]. They are environmentally acceptable, structurally more stable and resistant to microbial attacks. Their specific surface area is large and pore sizes can be tuned in the range of 4–30 nm, to host comfortably trypsin, a fairly small globular protein (23–24 kDa) the size of which is about 4 nm [19], and also larger enzyme molecules. Moreover, the enzymes show considerable affinity to the MPSs' surface [13–19]. That surface can additionally be modified with various anchor groups to form stronger covalent bonds that reduce protein leaching from the support under most real process conditions [21–27]. Investigations of the MPS-based biocatalysts have clearly indicated that protein load and enzyme activity is related to the pore size and surface structure and they are larger for MPS with larger pore sizes [14,22–24,26,27]. Among various MPSs the more

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recently developed siliceous mesostructured cellular foams (MCF) [28,29] appeared to be particularly efficient in a liquid phase catalysis [30,31] and as carriers for a number of enzymes: α-amylase, glucose oxidase, glucoamylase, invertase, organophosphorus hydrolase and chloroperoxidase [16,23–26]. This is ascribed to their unique texture with ultra large, cage-like, and not rod-like mesopores, up to 40 nm in diameter, connected by windows with diameters up to 20 nm. These mesopores are large enough to host enzymes and allow an easy transport of substrates into its active site, and importantly, create an environment most favourable for the expression of enzyme activity [23,26].

The aim of this work was to investigate the properties of trypsin covalently, directly or via glutaraldehyde or divinyl sulfone, bonded with the siliceous MCFs functionalised with oxirane or amino groups, and to compare these properties with the counterparts prepared similarly but using conventional silica gels and Eupergit C as supports. It may be recalled that the latter is the recognized carrier for the covalent attachment of enzymes in industrial applications [1,32]. Thus, this work is a logical step inasmuch the physical adsorption was so far most frequently applied for trypsin immobilization on MPSs [13,14,19], and the recent studies of its covalent attachment made use of thiol-functionalised SBA-15 carriers [21,27].

2. Experimental

2.1. Materials

Tetraethoxysilane (98%, TEOS), 1,3,5-trimethylbenzene (TMB), 1-chloro-2,3-epoxypropane, chloroacetic acid, 3-aminopropyltriethoxysilane (APTS), 2-aminoethyl-3-aminopropylmethyldimethoxysilane (AEAPMDS), 2-aminoethyl-3-aminopropyltri-methoxysilane (AEAPTS) and 3-glicydoxypropyl-triethoxysilane (GPTS) were from Aldrich (USA). Pluronic PE 9400 was from BASF and *N*-chloroethyl-*N*,*N*-diethylamine was from Serva (Germany). Trihydroxymethylaminomethan (Tris), glutaraldehyde (GLA), divinyl sulfone (DVS), Eupergit C, dimethylsulfoxide (DMSO), *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were from Sigma. Casein (Hammolersten) was from Park (UK), other chemicals were purchased from POCh (Poland). One silica gels was a medium pore size MN Kieselgel 60 from Degussa, the other one was IE Int. Enzymes Ltd. small-porous (0.15–0.3 mmol) type used in chromatography. Trypsin was prepared according to the method described in [33].

2.2. Synthesis and characterization of MCF carriers

Pristine siliceous MCF were prepared as first reported in [28,29] and later described also in [30,31,34]. In brief, their preparation consisted of the following steps: synthesis of microemulsion (template), formation of pre-condensed silica foams, hydrothermal treatment, filtration, and finally calcination to remove the template. The texture parameters (specific surface area, S_{BET}; pore volume, V_p) of the calcined material were obtained using nitrogen adsorption method at 77 K (Micromeritics ASAP 2000) and BJH algorithm. Diameters of cells, d_p, and of interconnected windows, d_w, were determined from adsorption and desorption isotherms, respectively.

This sample, labelled MCF0, was later treated with water vapour and functionalised in a post-synthetic procedures [34] described below.

2.2.1. Modification of MCF and Z carrier surface

Before grafting MCFs were contacted with water vapour for 5 h and subsequently dried at 473 K for 2 h. The silanols concentration on the MCFs surface determined by thermogravimetry using the method given in [35] was ca. 2.9 OH/nm^2. Eight samples of siliceous foams (labelled MCF1–8) with organic groups prepared by a direct grafting, i.e. reacting under reflux conditions (24 h, 313 K) suitable organosilanes (AEAPMDS, AEAPTS in toluene, and GPTS, APTS in hexane) with silanols present on silica surface to obtain the load of each functional moiety of about 1.0 or 1.5 mmol/g of silica. The procedure of grafting was as follows: 50 cm^3 of solution containing 20 or 30 mmol/dm^3 of organosilanes were stirred under reflux with 1 g of MCFs for 24 h after which the solvent was evaporated at 335 K. Then they were characterised by IR spectroscopy (Zeiss, Specord M 80) using KBr method and by nitrogen adsorption at 77 K. Particle size of MCFs thus prepared was also determined by means of laser diffraction analysis (Fritch, Analysette 22). These samples were later coupled with the enzyme as described below. Sample designation, the load and type of organosilanes applied in the grafting of MCFs is given in Table 1. The surface of silica gels was modified in the same way but the loads were lower, 0.5 or 1 mmol/g.

2.3. Immobilization of enzymes

Functionalised carrier (3–5 cm^3) was rinsed by centrifugation (MCF samples, 7000 rpm, 15 min) or sucking (Z samples) five times with distilled water and the buffer appropriate to the kind of anchor groups. Then their activation was carried out as described in detail before [10,36]. After activation the carrier was filtered off and then suspended in 10–12 mL of trypsin in the same buffer. The slurry was mixed for 2 h at 277 K and stored overnight in the same temperature. The excess protein was removed by washing with a proper buffer (cf. [10,36]) and,

Table 1
Structure parameters of MCF carriers

Carrier	Load (mmol/g)/ organosilane	S_{BET} (m^2/g)	$V_{p\ N2}$ (cm^3/g)	d_p (nm)	d_w (nm)
MCF0	–	620	2.5	25	14
MCF1	1.0/AEAPMDS	422	1.9	22	13
MCF2	1.5/AEAPMDS	372	1.7	22	13
MCF3	1.0/AEAPTS	440	1.9	23	13
MCF4	1.5/AEAPTS	353	1.8	22	13
MCF5	1.0/APTS	441	2.1	24	13
MCF6	1.5/APTS	418	2.0	22	13
MCF7	1.0/GPTS	370	1.7	24	13
MCF8	1.5/GPTS	316	1.6	22	12

finally with distilled water. All the eluates/supernatants were collected and analysed for the presence of protein and activity. The amount of bound protein (activity) was calculated from a difference between the amount used for immobilization and that washed off. In order to block unreacted active groups, the filtered preparation was suspended in 0.5 mol/dm^3 Tris–HCl buffer, pH 7.8 and stored at 277 K. Finally, the preparations obtained were rinsed with 0.1 or 0.05 mol/dm^3 Tris–HCl buffer containing $CaCl_2$ and activities were measured.

2.4. Activity assays

Activity of both native and immobilized trypsin preparation was investigated in two, perhaps the most conventional test reactions involving substrates with small (BAPNA, 435 Da) or very large (casein) molecules. The results obtained are deemed to be representative, respectively, for the case of low molecular weight substrates and those with very large molecules.

2.4.1. Activity of native trypsin

Amidase activity was assayed by the addition of 50 mm^3 of BAPNA (80 mmol/dm^3 in DMSO) to 1 cm^3 of trypsin in 0.1 mol/dm^3 Tris–HCl buffer, pH 7.8, containing 10 mmol/dm^3 $CaCl_2$. Samples were pre-incubated and incubated in water bath at 310 K. After exactly 5 and 10 min of incubation, reaction was stopped by addition of 2 cm^3 of 30 % acetic acid solution. The absorbance (405 nm, spectrophotometer Heliosα, Unicam) was measured and the enzyme activity calculated. The enzyme activity unit (U) was defined as the amount of the enzyme that cause absorbance increase of one unit per minute under the assay conditions.

Proteolytic activity was determined by mixing 1 cm^3 of 1.0% casein in 0.2 mol/dm^3 Tris–HCl buffer, pH 7.8, and 1 cm^3 of enzyme in 0.05 mol/dm^3 Tris–HCl buffer, pH 7.8, containing 20 mmol/dm^3 $CaCl_2$. Samples were pre-incubated and incubated at 310 K for 5 and 10 min. Reaction was stopped by the addition of 3 cm^3 solution of trichloroacetic acid (0.11 mol/dm^3), acetic acid (0.33 mol/dm^3) and sodium acetate (0.2 mol/dm^3), and after 20 min the suspension was centrifuged (10,000 rpm, 15 min). Absorbance of digested products, which are soluble in stopping reagent, was measured spectrophotometrically at 280 nm against water. In the control samples, the enzyme was added after stopping reagent. One unit of proteolytic activity (U) was defined as the amount of the enzyme that gave a rise in absorbance of one unit per minute.

Protein concentration was determined by UV–vis spectrometry at $\lambda = 280$ nm and/or by Lowry's method (Sigma procedure P 5656).

2.4.2. Activity of immobilized trypsin

Immobilized enzyme preparation (0.2–1.2 cm^3) in 10 or 20 cm^3 of 0.1 or 0.05 mol/dm^3 Tris–HCl buffer, pH 7.8, containing 10 or 20 mmol/dm^3 $CaCl_2$ was placed into a batch reactor (200 rpm, 310 K). Then 0.5 or 1.0 cm^3 of preheated substrate (BAPNA) was added. In the case of proteolytic activity, 10 or 20 cm^3 of preheated casein was added. After 1, 3, 5, and 10 min of mixing, 1 cm^3 (BAPNA test) or 2 cm^3 (casein test) of sample was taken and it was added to the appropriate stopping reagent. In the blank the buffer was added instead of the enzyme.

2.5. Substrate hydrolysis in a batch reactor

Preparation of MCF-5 bound trypsin (0.15 cm^3) in 20 cm^3 of 0.05 mol/dm^3 Tris–HCl buffer, pH 7.8, containing 10 mmol/dm^3 $CaCl_2$ was placed into batch reactor (200 rpm, 310 K). Then 1 cm^3 of preheated BAPNA in DMSO was added (final concentration 3 mmol/dm^3). After various elapsed time intervals 100 mm^3 sample was taken, immediately diluted 10 times and added to the stopping solution. Then the released *p*-nitroanilide was determined by UV–vis spectrometry.

Caseinolytic process was carried out in batch reactor (200 rpm, 310 K) by mixing 0.2 cm^3 of MCF5 based catalyst in 10 cm^3 of 0.05 mol/dm^3 Tris–HCl buffer, pH 7.8, containing 20 mmol/dm^3 $CaCl_2$, with 10 cm^3 of casein in the buffer. At predetermined times 500 mm^3 sample was taken, immediately diluted four times and added to stopping solution. After that the released oligopeptides were determined by UV–vis spectrometry (280 nm).

Similar procedure was also applied to the native enzyme. To compare the performance of a free and immobilized enzyme under process conditions, product concentrations, seen by the values of respective absorbencies, were plotted against time for the same initial enzyme activities (during first 5 min).

2.6. Procedure for an inactivation experiment

Inactivation of a native and MCF5-bound trypsin was investigated in a stirred reactor of 100 cm^3 volume at 333 K using the 0.05 mol/dm^3 Tris–HCl buffer and10 mmol/dm^3 $CaCl_2$ (pH 7.8). First 0.5 cm^3 of native trypsin (1.0 mg/cm^3) or 1 cm^3 of MCF5 was added to preincubated buffer and then, after 10 s of vigorous mixing, the first sample of 2 cm^3 was taken and cooled rapidly to 273 K in an iced-water bath. At certain times aliquots were taken, cooled and stored in an ice water until the activity measurement, which was conducted after 1 h of storage. The activity was assayed using BAPNA and referred to the initial values.

2.7. Estimation of kinetic parameters

Kinetic studies were carried out in a batch mode (310 K, 200 rpm) using MCF5 preparation and BAPNA concentrations in the range of 0.5–5.0 mmol/dm^3 in 0.1 mol/dm^3 Tris–HCl buffer with 10 mmol/dm^3 $CaCl_2$ (pH 7.8). For each concentration of substrate the absorbance was monitored (405 nm, $\varepsilon = 10{,}500\ M^{-1}\ cm^{-1}$) similarly as in [19] and initial reaction rate was calculated for substrate conversion lower than 7%. For comparative study, experiments were also carried out using a native enzyme. In the case of casein similar procedure was applied with the following modifications: MCF5 preparation, casein concentrations 0.1–1.0%; process monitoring at 280 nm; for each sample a blank was prepared in which only a buffer was added into the substrate solution. The progress of reaction

was calculated from a difference of absorbencies in a sample and in a blank. Finally, the reaction mixtures were left till next day to check whether the reaction came to the end (no increase in absorbance during three consecutive analyses with 2 h intervals).

Kinetic parameters were calculated from Lineweaver–Burk plots using a linear regression analysis.

3. Results and discussion

3.1. Properties of modified carriers

As shown in Table 1 the modification of pristine MCF with various organosilanes resulted in a small decrease in values of all texture parameters, more for the MCFs treated with organosilanes with two amino groups and glycidyl entity than a single NH_2 group. But despite this decrease the size of both caverns and windows was still large enough to allow the effective transport of both trypsin and less bulky substrates. On the whole the texture parameters of the modified silica gels were considerably less attractive than those of MCF samples (data not given here). The size of pores in Kieselgel-based catalysts (Z1–Z6) was, in principle, still large enough (4.0–4.6 nm) to host trypsin, unlike in other silica gel-based samples (Z7–Z12), where it was far too small (2.1–2.3 nm). Thus, in those samples only external surface of the particles could be effective (Table 2).

Particle size analysis (results not shown here) indicated that the size of beads of pristine MCFs was notably reduced owing to stirring the mixture during a post-synthesis modification. The mean size of particles decreased from about 30 μm, in MCF0, to about 18 μm after modification. But the size of the largest particles was reduced about twice, from ca. 100 μm to less than 50 μm.

Examination of IR spectra from the samples showed the presence of organosilanes applied in the modification of MCFs, and hence the corresponding functional groups bound with silica materials (Fig. 1). The broad band at 3600–3000 cm^{-1} seen in MCF0 and assigned to hydrogen bonded silanol species [37,38] was notably reduced in the modified samples. The organosilane presence was identified by the methylene stretching bands of the propyl chain in the region of 2950–2850 cm^{-1}, seen in IR spectra of both MCF4 and MCF8, and their deformation bands at 1455–1410 cm^{-1} [37]. The N–H absorption bands overlapped with O–H bands at 3300–3500 cm^{-1} [39].

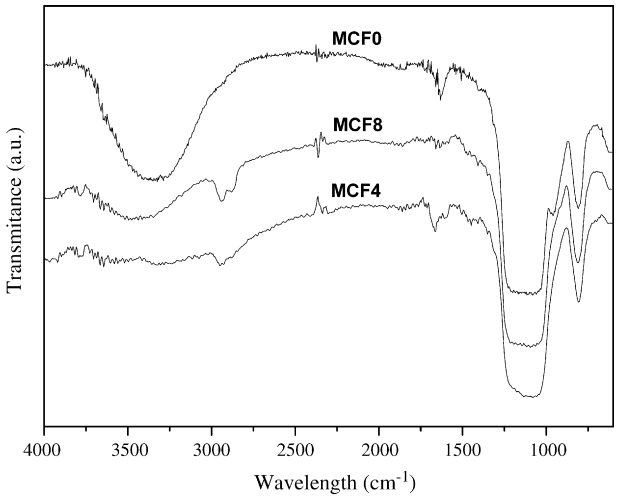

Fig. 1. IR spectra from as obtained and modified MCFs.

Table 2
Structure parameters of Z carriers

Carrier	Load (mmol/g)/ organosilane	S_{BET} (m^2/g)	$V_{p\ N2}$ (cm^3/g)	d_m (nm)
MN–Kieselgel 60 [0.2–0.5 mm]				
Pristine	–	343	0.7	5.1
Z1	0.5/GPTS	304	0.58	4.5
Z2	1.0/GPTS	237	0.45	4.3
Z3	0.5/APTS	304	0.59	4.6
Z4	1.0/APTS	299	0.55	4.4
Z5	0.5/AEAPMDS	299	0.58	4.5
Z6	1.0/AEAPMDS	267	0.49	4.0
IE Int. Enzymes silica gel [0.15–0.3 mm]				
Pristine	–	627	0.36	2.3
Z7	0.5/GPTS	385	0.22	2.2
Z8	1.0/GPTS	182	0.11	2.2
Z9	0.5/APTS	404	0.23	2.2
Z10	1.0/APTS	310	0.17	2.2
Z11	0.5/AEAPMDS	413	0.23	2.1
Z12	1.0/AEAPMDS	158	0.09	2.1

3.2. Activity of biocatalysts

From practical point of view the effective (measured) activity of biocatalysts is the most important property and their stability follows suit. The former is determined by enzyme loading, activity of immobilized enzymes and accessibility of their active sites to substrates. In the following we discuss each of these factors separately to formulate a more general final conclusion.

The results of activity measurements and protein load for MCF series and Eupergit are collected in Table 3 and those from selected samples of silica gel-based families (Z) in Table 4. As expected, the MCF catalysts appeared to be significantly more active in the proteolysis of casein than Z samples and also Eupergit C. Moreover, activity of some of the best samples was considerably higher than of trypsin anchored to acrylic and cellulosic carriers [40,41]. The protein loading of MCF catalysts was also notably larger than of Z preparations, but not so much as activity. MCF catalysts were also very active in the conversion of BAPNA, unlike Z-family samples, which strangely enough, were entirely inactive in that case (BAPNA tended to precipitate from the mixture immediately after the addition of silica gel-based catalysts and the reason for this is not clear). Surprisingly, these huge differences in proteolytic activities occurred despite still quite considerable protein loading of all silica gel-based catalysts. The protein loading of all small mesopore gels was similar, over 2 mg/cm^3, and roughly the same value was determined for Z6 (Kieselgel series), the pores of which were ca. 4 nm, i.e. of trypsin size.

Table 3
Immobilization of trypsin on MCF carriers (30 mg of protein per carrier)

Carrier	Bound protein (mg/cm^3)	Yield (protein) (%)	Expected activity[a] (U/cm^3)	Measured activity (U/cm^3)	Immobilization efficiency[b] (%)	Yield (activity) (%)
Glutaraldehyde activation						
MCF1	5.47	27.9	336/37.0	321/6.5	95.4/17.6	29.6/5.2
MCF2	3.34	34.2	185/19.4	168/4.2	91.0/21.6	32.6/7.2
MCF3	5.74	35.9	328/37.8	298/6.2	90.8/16.4	36.1/5.7
MCF4	nd	nd	nd/nd	46/1.4	nd/nd	3.7/1.1
MCF5	17.97	71.1	532/61.4	390/14.7	73.3/23.9	19.7/4.3
MCF6	12.17	24.1	583/67.3	592/10.0	101.5/14.9	20.7/3.3
Immobilization via oxirane rings						
MCF7	6.18	15.3	561/64.8	160/3.5	28.5/5.4	7.5/1.4
MCF8	5.61	32.2	368/42.4	59/1.8	16.1/4.2	6.6/1.7
Eupergit	0.27	5.4	53.1/6.12	4.1/0.4	7.7/6.5	1.6/1.3
Divinylsulfone activation						
MCF0	8.17	72.5	612/70.6	177/3.0	29.0/4.2	18.7/3.4

Activity was measured in presence of BAPNA/casein as substrate.
[a] Activity amount in the coupling mixture minus that in eluate then recalculated for 1 cm^3 of the carrier (activity balance).
[b] Ratio of measured activity to expected activity, it quantifies the expressed activity of the bound enzyme molecules.

Thus, in that sample the proteins were also predominantly immobilized on the external surface. But the activity of Z6 was higher than of Z7–Z12 catalysts and similar to that showed by other Kieselgel-based samples (Z1–Z5), with larger pores and hence higher protein loading, and also Eupergit C, in which the protein content was about tenfold less. We may, thus, infer that the surface of Z7–Z12 was considerably overpacked with proteins (trypsin and ballast). But beyond that the data presented clearly evidence the fact that catalytic activity of immobilized enzymes depends not only on the protein loading but the other factors already aforementioned.

In typical enzyme preparations applied in the immobilization, ballast proteins are always present in addition to enzyme molecules and the support may show a preference toward one or the other. Moreover, enzymes are subject to stress during immobilization and hence they denature, and a part of immobilized enzymes cannot be reached by more bulky substrates. A quantitative insight into these processes can be gained by balancing the amount of activity of the native preparation used in immobilization with the activity amounts of both immobilized enzyme preparations and eluates. The calculated values of expected catalysts activity and obtained from the measurements, as well as their ratio, called immobilization efficiency (IE) are listed in Tables 3 and 4. The data indicate that immobilization was notably more effective in the case of MCF carriers than Z carriers and also Eupergit C. The values of IE expressed in amidolytic activity (BAPNA) approached and even exceeded 100%, and in proteolytic (casein) reached 20%, which is quite remarkable result [40,41], bearing in mind the hampered accessibility of very large molecules of casein to trypsin embedded in the pores of MCFs. This might indicate that nearly all immobilized trypsin molecules were involved in the BAPNA conversion, but owing to the limited accessibility and a hindered transport, perhaps only about 20% in the conversion of casein. For silica gel-based catalysts, Eupergit C and MCF0 the value of IE was

Table 4
Immobilization of trypsin on Z carriers (33 mg of protein per carrier)

Carrier	Bound protein (mg/cm^3)	Yield (protein) (%)	Expected activity (U/cm^3)	Measured activity (U/cm^3)	Immobilization efficiency (%)	Yield (activity) (%)
Glutaraldehyde activation						
Z3	4.85	70.7	206/27.6	0.0/0.2	0.0/0.7	0.0/0.5
Z4	3.57	52.1	79/8.2	0.0/0.4	0.0/4.9	0.0/0.9
Z5	3.38	53.4	103/15.1	0.0/0.4	0.0/2.6	0.0/1.0
Z6	2.63	39.3	18/2.1	0.0/0.4	0.0/19.0	0.0/0.9
Z9	2.63	42.4	17/2.3	0.0/∼0.0	0.0/∼0.0	0.0/∼0.0
Z10	2.60	40.3	11/1.3	0.0/∼0.0	0.0/∼0.0	0.0/0.1
Z11	2.33	37.6	14/1.7	0.0/0.1	0.0/5.9	0.0/0.2
Z12	2.1	33.8	14/1.6	0.0/0.1	0.0/6.25	0.0/0.2
Immobilization via oxirane rings						
Z1	5.24	74.7	272/37.4	0.0/0.2	0.0/0.5	0.0/0.5
Z2	3.71	54.0	139/16.4	0.0/0.1	0.0/0.6	0.0/0.3
Z7	2.40	36.4	37/4.3	0.0/0.1	0.0/2.3	0.0/0.3
Z8	2.29	36.1	23/2.7	0.0/0.1	0.0/3.7	0.0/0.2

Activity was measured in presence of BAPNA/casein as substrate.

not larger than a few percent. As both MCFs and silica gels were modified similarly we can conclude that an open, cage-like structure of MCFs, modified with suitable entities, is the most likely explanation of this behaviour.

Eupergit C appeared to be not as effective carrier for trypsin as expected; somewhat better than the most conventional silica gels but notably inferior to MCFs (cf. Table 3). Affinity between the surface of Eupergit and proteins was low, so as the activity and immobilization efficiency. With the value of protein yield (ratio of protein effectively adsorbed to that used in immobilization) of over 70% the pristine siliceous cellular foam (MCF0, cf. Table 3) appeared to be very efficient protein adsorbent, similarly as earlier observed for glucoamylase [26]. Yet the activity expressed in both reactions was notably lower than of the modified MCF-based catalysts. Thus, these observations underscore the importance of both open structure and microenvironment necessary for the expression of high enzymatic activity and created by a suitable surface topology additionally modified with appropriate entities.

On the whole, trypsin anchored to supports via GLA-amino entity, and especially using GLA-APTS system, appeared to be considerably more active in the both test reactions than when it was attached using glycidyl group, and the same trend was observed before for the MCF-bound invertase [26]. The tentative explanation is that this system created a dimeric or trimeric structure following the creation of a longer spacer between the matrix and protein surface. Such microenvironment was not observed in the sol gel samples, MCFs with glycidyl residue, MCF0 and also Eupergit C, in which protein amine groups were almost directly attached to the carriers' surface, and that could adversely affect their superstructure.

The preferential binding of selected proteins with carriers was evaluated from measurements of the specific activities of trypsin in eluates. As can be seen from Fig. 2, the values for MCF-bound trypsin were slightly lower than observed for a native enzyme (reference value), except for MCF5 and MCF0. This signifies some sort of preferential sorption of trypsin proteins on the modified matrices of MCFs and slightly higher wash-out of ballast proteins. It is likely that amino groups, and also glycidyl residues residing in a cage-like pores, created microenvironment more favourable for the enzyme than ballast molecules, with a very positive effect for MCFs' activity. This is in accord with the opposite trend of notably higher affinity towards ballast proteins, and not trypsin, revealed by the unmodified MCF0, which also contributed to its lower specific activity (cf. Fig. 2). The catalysts of Z family revealed even

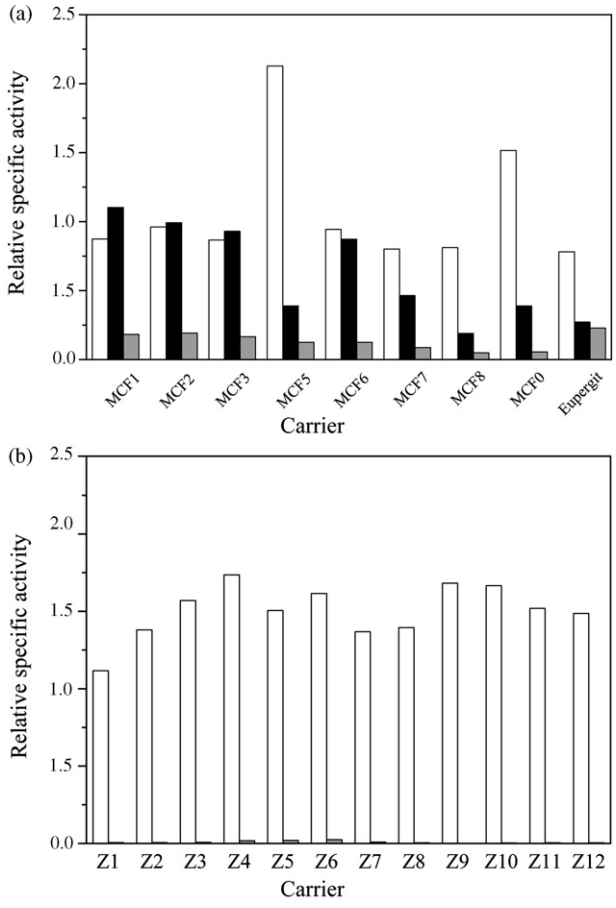

Fig. 2. Relative specific activities of trypsin in eluates (white) and bound to MCF (a) or silica gel carriers (b), measured by amidolytic (BAPNA, black) and caseinolytic (grey) assays. Specific activities of a native trypsin: 6.5 U/mg (casein), 57 U/mg (BAPNA).

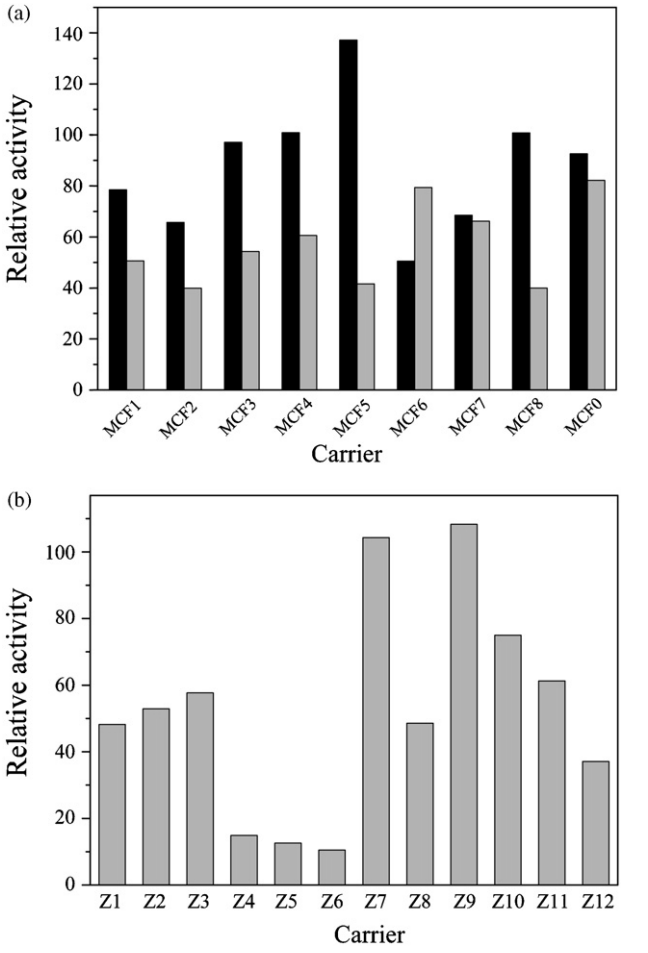

Fig. 3. Storage stability of trypsin at 277 K (0.05 M Tris–HCl buffer, pH 7.8) immobilized on MCF (a) and silica gel (b) carriers. Activity was determined in amidolytic (BAPNA, grey) and caseinolytic (black) assays.

stronger preferential bonding of ballast proteins than MCF0. It may be recalled that they were chemically modified in the same way as MCFs, yet lacked a unique pore structure of cellular foams. Again, this can be seen as one of the main reasons of low activity of the silica gel-based catalysts.

3.3. Behaviour and performance of MCF-based trypsin biocatalysts

As shown in Fig. 3, the MCF-based preparations preserved higher amidolytic activity (BAPNA conversion) after 1-month storage. Since the most stable preparations appeared to be MCF3–5 activated with GLA, MCF8 with oxirane ring and MCF0, we can hardly specify factors responsible for this behaviour. We can observe a weak tendency that higher amine group contents on the carrier result in lower stabilities. Thus, in probability terms, the attachment of trypsin molecules via a few covalent bonds might have a negative effect on its stability due to the change of conformation. We also observed that preparations with a lower initial activity appeared to be more stable. Indeed, the least active catalysts (small pore silica gel-based) appeared to be more stable than the Kieselgel-based counterparts and equally stable to the samples from MCFs family. The increase in activity of immobilized enzymes during initial few days of storage seen for a few samples in Fig. 3 is not quite unusual. It was already observed before [42,43] and a tentative explanation for this phenomena has been offered.

To compare the performance of a native enzyme and MCF-based preparations in the most real situation of BAPNA and casein conversion batch tests were carried out under conditions that initial rates of reactions were the same and the recorded variations in absorbances are displayed in Fig. 4. As can be seen, the performance of both native and immobilized enzyme is quite similar during the whole period, even in the presence of casein (Fig. 4b). This demonstrates and evidences two important properties of the catalysts prepared: (i) no deactivation of immobilized enzyme due to pore blockage, or for other reason, occurred during the run, (ii) mass transport limitations could be effectively eliminated by a suitable choice of reaction conditions. Some discrepancies will be discussed later.

Although higher stability of the enzyme after covalent attachment to the carrier is not an inherent feature of the immobilization, it is usually expected. To check whether it was the case thermal (333 K) stability of the free and support-bound trypsin was evaluated. As it is seen from Fig. 5, the enzyme covalently bound with MCF via GLA-amino entity shows significantly slower activity decay. While the free enzyme lost half of its activity in 3 min, immobilized enzyme needed for this four times more. Generally, thermal inactivation of proteases is a complex process due to the simultaneous autolysis effect and thermal denaturation. When trypsin is covalently anchored to the carrier, autodigestion can be omitted. Thus, the markedly higher stability of MCF-enzyme preparation can partly be caused by the limitation of autolysis and partly by the protein conformational stabilization. The extent of autolysis in the inactivation of trypsin is difficult to estimate in the native enzyme. For low trypsin concentration the autolysis was shown to be negligible [44] and this applies to the

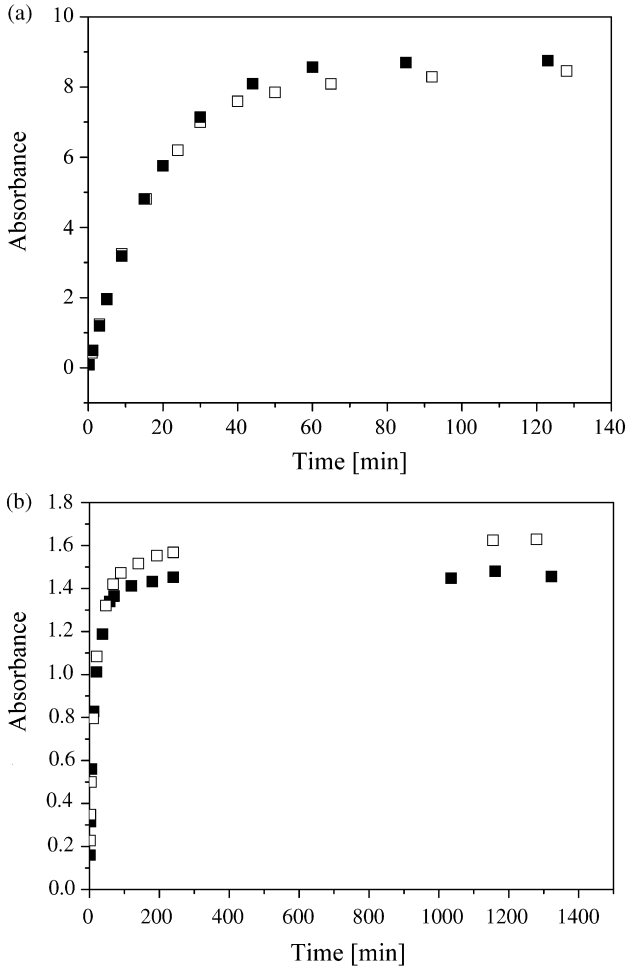

Fig. 4. Variation of *p*-nitroanilide concentration (BAPNA) (a) or absorbance of products (casein) (b) with elapsed reaction time using: a native (□) and MCF-bound (■) trypsin. Reaction conditions: 310 K, 200 rpm, BAPNA concentration 2.5 mmol/dm^3, casein concentration 0.5%, 0.1 M Tris–HCl buffer, pH 7.8 with 10 mmol/dm^3 $CaCl_2$. Enzyme activities—BAPNA: 0.384 U/cm^3 (native) and 0.384 U/cm^3 (immobilized on MCF5); casein: 0.088 U/cm^3 (native) and 0.084 U/cm^3 (immobilized on MCF5).

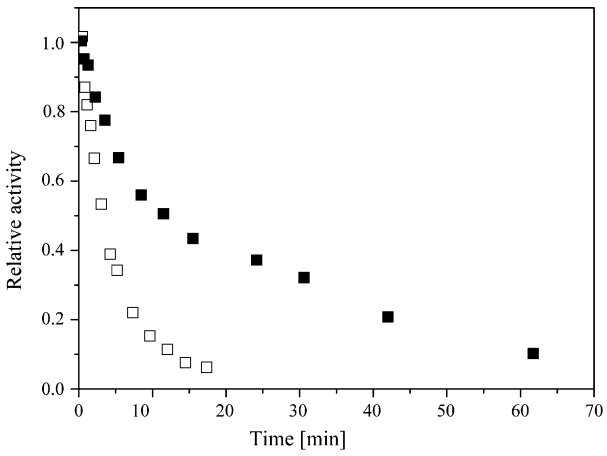

Fig. 5. Relative activity of a native (□) and MCF5-bound (■) trypsin vs. time of incubation at 333 K in 0.1 M Tris–HCl buffer (pH 7.8) with 10 mmol/dm^3 $CaCl_2$ (200 rpm).

Table 5
Kinetic constants for amidolytic and caseinolytic activity of free trypsin and immobilized on to MCF6

Substrate	Kinetic parameter	Native enzyme	Immobilized
BAPNA	K_m (mM)	12.92 ± 0.95	6.47 ± 0.32
	k_{cat} (min^{-1})	5.716 ± 0.296	3.380 ± 0.122
	k_{cat}/K_m (mM^{-1} min^{-1})	0.44	0.52
Casein[a]	K_m (A_{280})	1.383 ± 0.089	1.893 ± 0.137
	k_{cat} (min^{-1})	0.0153 ± 0.0013	0.0250 ± 0.0017
	k_{cat}/K_m ($1/A_{280}$ min)	0.011	0.013

[a] Substrate concentration was expressed as absorbance measured at the end of the reaction, usually after 30 h. This value was assumed to correspond to substrate concentration.

case under study. Moreover, in the experiment calcium ions were added to buffers to limit autodestruction of trypsin. Thus, we conclude that inactivation of trypsin displayed in Fig. 5 was prevalently caused by thermal denaturation.

The parameters of Michaelis–Menten equation were determined for both test reactions using a native and MCF6-bound trypsin to obtain the values given in Table 5. For amidolytic reaction catalyzed by immobilized trypsin the value of saturation constant K_m was half of the value determined for a native enzyme, a clear sign of higher ''apparent'' affinity of BAPNA to the bound enzyme. Most likely it was caused by the larger affinity to the surface of modified MCF that might have resulted in the higher concentration of substrate in boundary layer than in the bulk solution. This observation is in good agreement with the data presented in Fig. 4a where BAPNA conversion appeared to be slightly higher after 30 min of reaction catalyzed by immobilized trypsin. On the other hand, reaction rate constant was also lower for MCF6 sample, and hence the value of ratio k_{cat}/K_m was similar to that of the native enzyme.

In the case of casein conversion the estimation of kinetic parameters was more difficult due to the poorly defined concentration of both substrate and product. The reaction progress was recorded by monitoring the increase in absorbance at 280 nm, caused by the increasing content of small polypeptides soluble in trichloroacetic acid. The final concentration/absorbance of products stabilized after ca. 30 h, and this value was assumed to correspond to the substrate concentration. As our goal was not to obtain a precise kinetics of proteolysis of macromolecular substrate, this seemed acceptable. Unlike in the case of amidolysis, somewhat higher values of both k_{cat} and K_m were obtained and the value of k_{cat}/K_m was also slightly higher. It was expected that a hampered access of big substrate molecules to the active sites of catalyst, and concomitant diffusional limitations, would cause a dramatic change in kinetic parameters. Yet, the only appreciable result appeared to be slightly smaller values of reaction rate and conversion, after prolonged time of the reaction (Fig. 4b). It can be explained by differences in the amount and molecular weight arrays of various products when the reaction is carried out with the native and immobilized trypsin. Generally, somewhat larger value of K_m reflects the effect of diffusion, but the lack of larger difference in the values of kinetic parameters is caused by the presence of large mesopores in MCFs, which enable the substrate to reach active sites of the enzyme, and also to the small size of MCF particles, with all positive consequences of that for mass transport.

4. Conclusions

Mesostructured cellular foams with trypsin covalently anchored to their surface by glutaraldehyde-amino linkage show very attractive biocatalytic properties, far superior to the conventional silica gel- and Eupergit C -based counterparts and also acrylic-based supports previously tested. Activity of trypsin immobilized on MCFs can even be superior to the native enzyme, but it depends on a type and density of the functional group attached and also the organosilane compound used in the modification of MCFs' surface. The GLA-amino linkage, in general and GLA-APTS in particular, appeared to be the very efficient system for the covalent immobilization of trypsin. Results of test reactions indicate that a vast majority of immobilized trypsin was effectively involved in the conversion of a low molecular substrate (BAPNA), and perhaps about 20%, or a bit more in the proteolysis of the bulky molecules of casein. The MCF-based preparations were notably more stable than a native enzyme at 333 K and they also showed good storage stability at 277 K. A unique porous structure of modified MCFs is a critical factor which renders siliceous mesostructured foam a very promising material for immobilization of enzymes.

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