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Monolithic bioreactors: Effect of chymotrypsin immobilization on its biocatalytic properties

E.A. Ponomareva, V.E. Kartuzova, E.G. Vlakh, T.B. Tennikova*

Institute of Macromolecular Compounds of Russian Academy of Sciences, Bolshoy pr. 31, 199004 St. Petersburg, Russia

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ABSTRACT

The effect of different modes of α-chymotrypsin attachment to the surface of methacrylate-based ultrashort monolithic minicolumns on enzyme activity has been studied. The immobilization of protease was carried out via direct covalent binding of chymotrypsin, as well as via its attachment through small and polymer spacers. It was established that the lowest enzyme activity against N-benzoyl-L-tyrosine ethyl ester was found for bioreactor obtained via direct attachment of chymotrypsin to the surface of GMA–EDMA minidisks, whereas the highest parameter close to that determined for dissolved enzyme was found in the case of bioreactor prepared by the introduction of copolymer of 2-deoxy-N-methacryloylamido-D-glucose with N-vinylpyrrolidone and acrolein as a long and flexible polymer spacer. Additionally, the effect of flow rate of substrate recirculation on bioconversion efficiency was examined. Independently on immobilization method, the increase of flow rate led to the raise of biocatalytic efficiency.

1. Introduction

The immobilized enzymes are widely used in biotechnology [1], medical diagnostics [2] and therapy [3], biosensors [4], proteomics [5], etc. Solid phase attachment of enzymes has a number of advantages comparing to application of soluble biocatalysts. Firstly, an attachment of enzyme to a solid support is followed by conformation fixation that, in its turn, leads to the molecule stabilization. Secondly, the immobilization prevents the interaction between individual molecules (aggregation) and, consequently, autolysis probable in the case of proteases. Furthermore, enzyme immobilization facilitates the product removal, makes a biocatalyst reusable and stable for a long time period.

Different enzyme immobilization techniques have been developed within last decades. This process can be performed via physical adsorption of enzymes on a solid support (synthetic and natural polymers, glass, ceramics) [6,7], encapsulating techniques using sol–gel method [8,9], direct covalent binding through the appropriate groups [10–13] or using intermediate bi- or polyfunctional spacers [14,15]. The most popular approach that guarantees the stable enzyme–support linkage is a covalent binding. To prevent the loss of enzymatic activity, the used solid phase has to ensure immobilization procedure without touching the active site of biocatalyst, to provide sterically non-limited enzyme–substrate pair formation, as well as to restrict any diffusion limitations.

In old fashion model of flow-through enzyme reactor, the column packed with porous beads modified with enzyme was used and a substrate molecule predominantly had to diffuse into the pores of packing in order to interact there with the active site of biocatalyst. In such systems, mass transfer is controlled exclusively by molecular diffusion and depends on particle and pore sizes, flow rate and diffusion coefficient of a substrate. In total, the system appears to be quite slow and inefficient. At the same time, widely used in modern chromatography, macroporous monolithic sorbents, characterized by extremely high permeability for a liquid flow, provide the excellent conditions for all processes based on interphase mass exchange with participation of any kinds of molecules, even of large bioobjects (proteins, DNA, and viruses) with low diffusion properties. As most attractive and widely used for dynamic bioaffinity-based processes, solid phases still remain macroporous monoliths based on a copolymer of glycidyl methacrylate and ethylene glycol dimethacrylate (GMA–EDMA). Aside the mentioned properties general for all types of monoliths, this polymer matrix does not provoke any non-specific interactions of biosubstance with a surface, as well as it contains original epoxy

Abbreviations: GMA–EDMA, copolymer of glycidyl methacrylate with ethylene glycol dimethacrylate; VP–Ac, copolymer of vinylpyrrolidone with acrolein; MAG–VP–Ac, copolymer of 2-deoxy-N-methacryloylamido-D-glucose with vinylpyrrolidone and acrolein; ACHT, α-chymotrypsin; BTEE, N-benzoyl-L-tyrosine ethyl ester; K_m, Michaelis-Menten constant; V_{max}, maximal velocity of enzymatic reaction; A, specific activity; $A_{sol.}{}^*/A_{immob}$, ratio of specific activities of soluble and attached enzyme.

* Corresponding author. Tel.: +7 812 323 10 70; fax: +7 812 328 68 69.
E-mail address: tennikova@mail.ru (T.B. Tennikova).

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groups which can be easily converted into others or used for direct immobilization of amino-bearing ligands.

The first attempt to obtain enzymatic bioreactor based on GMA–EDMA monolithic matrix has been described by Abou-Rebyeh et al. [16]. In this work the coupling of carbonic anhydrase to GMA–EDMA monolithic support and kinetic investigations of enzyme reaction were carried out using 4-nitrophenyl acetate and 2-chloro-4-nitrophenyl acetate as substrates. Later, Petro et al. [17] used a lab-made macroporous monolithic column with covalently immobilized trypsin to compare its catalytic properties to a bioreactor based on a column packed with GMA–EDMA polymer beads also modified by trypsin. It was found that improved hydrodynamic characteristics of monolithic solid phase favored the increase of enzymatic efficiency comparatively to the bead-based bioreactor.

The application of monolithic stationary phases for bioconversion was dramatically extended from the beginning of new century. In general, the papers describing bioreactors obtained via immobilization of trypsin on GMA–EDMA or silica monoliths and intended for protein digestion are presented in the current literature [18–23]. However, there are a significant amount of reports on application of GMA–EDMA monolithic materials for the preparation of flow-through bioreactors based on different enzymes, e.g. immobilized invertase [24], polynucleotide phosphorylase [25], glucose oxidase [26], citrate lyase, malate dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase [27], papain [28], lignin peroxidase [29], deoxyribonuclease [30,31], elastase [32], chymotrypsin [33], beta-secretase [34], pronase [35], pectin lyase [36] glucuronan lyase [37], pepsin [38] and peptide-N-glycosidase F [39].

Direct enzyme attachment (immobilization via epoxy groups of sorbent) or immobilization of enzyme via previously introduced into monolithic surface structure aldehyde or imidazole carbamate groups are usually used. Earlier, it was established that the introduction of any spacers in the preparation of monolithic GMA–EDMA columns for affinity chromatography did not affect the interactions of biocomplements [40]. At the same time, it is well known that enzyme immobilization via spacers is strongly recommended to facilitate the substrate access to the active site of attached enzyme. It is important to emphasize that the introduction of a spacer must not induce any non-specific interactions. It means that selected "arm" should not represent strongly hydrophobic or charged molecule.

The goal of presented research was to investigate the effect of a spacer at covalent attachment of enzyme on its activity. The macroporous monolithic CIM epoxy minidisks prepared from GMA–EDMA copolymer have been used for α-chymotrypsin (ACHT) immobilization. The chosen protease is close to trypsin from the point of view of their molecular structure and mechanism of proteolysis. However, ACHT is more stable and demonstrates different cleavage specificity being active against peptide and ester bonds formed with aromatic amino acids such as tyrosine, phenylalanine and tryptophan. To provide the enzyme flexibility in comparison with direct coupling of enzyme molecule to the matrix, low and high molecular mass spacers bearing highly reactive aldehyde groups were tested. N-benzoyl-L-tyrosine ethyl ester (BTEE) was applied as a substrate to evaluate and compare the efficiency of immobilized protease in terms of enzymatic activity of bioreactors obtained. Additionally, the dependence of enzymatic activity on flow rate of substrate recirculation and the possibility of macromolecular substrate (BSA) hydrolysis using constructed flow-through reactors were examined.

Fig. 1. The image of CIM epoxy minidisk and cartridge.

2. Experimental

2.1. Chemicals and materials

N-benzoyl-L-tyrosin ethyl ester (BTEE), α-chymotrypsin (ACHT) from bovine pancreas (type II), bovine serum albumin (BSA), 25% glutaraldehyde (GA) solution and sodium borohydride were purchased from Sigma–Aldrich GmbH (Taufkirchen, Germany). All salts used for buffer preparation, as well as methanol, 25% aqueous ammonium solution and hydrochloric acid were purchased from Vecton Ltd. (St. Petersburg, Russia) and were of ACS reagent grade. The buffer solutions were prepared by dissolving salts in distilled water and additionally purified by filtration through a 0.45-μm membrane microfilter Milex, Millipore Inc. (Wien, Austria).

Water-soluble copolymer of N-vinylpyrrolidone with acrolein diethyl acetal (VP–DAAc) with 21 mol% DAAc, MW 25,000, and terpolymer of 2-deoxy-N-methacryloylamido-D-glucose with N-vinylpyrrolidone and acrolein diethyl acetal (MAG–VP–DAAc) containing 8 mol% of DAAc, MW 18,000, were synthesized and characterized using previously developed protocols [41,42]. To purify activated polymers from low molecular mass compounds, spin-columns VIVASCIENCE with membrane MWCO-3000 produced by Sartorius Group (Göttingen, Germany) were applied.

CIM epoxy minidisks with a diameter of 5.2 mm and a thickness of 5.0 mm equipped with special stainless steal cartridge were obtained from BIA Separations, d.o.o. (Ljubljana, Slovenia) (Fig. 1).

2.2. Instrumentation

The low pressure chromatographic system LKB (Bromma, Sweden) consisting of 2115 Multiperpex pump and UV detector 2138 Uvicord S was used in all dynamic experiments. The determination of optical density of analyzed solutions was carried out by means of UV–VIS spectrophotometer UVmini-1240 Shimadzu (Kyoto, Japan). Capillary electrophoresis Kapel 105 Lumex (St. Petersburg, Russia) equipped with 60 cm long quartz capillary of 75 μm i.d. was used for the analysis of products obtained at enzymatic hydrolysis of macromolecular substrate (BSA).

2.3. Methods

2.3.1. Direct immobilization of chymotrypsin

A direct immobilization of amino-bearing protein (α-chymotrypsin) was performed by means of coupling to original epoxy groups of GMA–EDMA material at static conditions. The disk previously washed with ethanol, ethanol–water (1:1), water and, finally, 0.1 M sodium borate buffer, pH 9.4, was immersed into 1 mL of a 5 mg/mL of chymotrypsin solution in the same buffer. The binding reaction was allowed to proceed for 18 h at 22 °C.

After the immobilization was completed, unbound chymotrypsin was removed by washing the sorbent with the buffer used for immobilization and then with deionized water. The amount of ligand coupled to the support (q) was calculated as: q_{ACHT} = (protein amount in initial solution) − (protein amount in

solution after immobilization) – (protein amount in washing solution). The protein concentration was measured by Lowry test [43] using ACHT calibration curve.

The prepared enzyme reactor was stored in 0.01 M sodium phosphate buffer, containing 0.15 mol/L NaCl, pH 7.0 (0.01 M PBS), at 4 °C.

2.3.2. Immobilization of chymotrypsin through spacer

2.3.2.1. Amination of epoxy groups of GMA–EDMA material. Prior to aldehyde-bearing spacer introduction, the epoxy groups of GMA–EDMA monolith were converted to amino groups. Amination of epoxy groups of polymer sorbent was carried out by incubation of the disk in 3 mL of 25% aqueous ammonium solution at 40 °C for 5 h. After reaction completion, the disk was washed with distilled water at dynamic conditions for 10 min and then with 0.01 M PBS for 10 min.

2.3.2.2. Immobilization of chymotrypsin through small spacer. Previously, the pore space of the disk was filled by syringe injection with the 0.3 mg/mL solution of glutaraldehyde in 0.01 M PBS, pH 7.0, after that the disk was immersed into 1 mL of the same solution and left for 1.5 h at 22 °C. When the reaction time passed, the disk was installed into chromatographic system and washed with PBS for 10 min at flow rate 1 mL/min that, regarding the same linear flow velocity, corresponded to 5.3 mL/min flow rate applied for standard CIM disk of 12 mm × 3 mm dimensions. The amount of bound glutaraldehyde was calculated as follows: q_{HA} = (amount of glutaraldehyde in initial solution) – (amount of glutaraldehyde in solution after reaction) – (amount of glutaraldehyde in washing solution). For this purpose, the reaction of aldehyde group with specific fuchsin sulfite reagent (Schiff's reagent) was used. The analysis was carried out as follows: 2.5 mL of Schiff's reagent was added to a sample of 0.5 mL of aldehyde containing solution and the absorbance of colored complex was measured in 40 min at λ = 550 nm.

To immobilize ACHT, the disk was washed with 0.01 sodium borate buffer, pH 8.4, and then incubated for 1.5 h in 1 mL of 1 mg/mL ACHT solution in sodium borate buffer, pH 8.4, at 22 °C. Similar to glutaraldehyde immobilization (see above), the pores of the disk were filled with reaction solution by syringe injection and then the disk was immersed into the same solution. The amount of ACHT coupled to the support was calculated in the manner described above (Section 2.3.1). After the disk was washed with 0.01 M PBS, pH 7.0, to quench residual aldehyde groups, as well as to reduce formed Schiff's bases, monolithic sorbent was treated with 1 mg/mL aqueous solution of sodium borohydride for 1 h at 22 °C. Finally, the prepared enzyme reactor was washed by distilled water and stored in 0.01 M PBS at 4 °C.

2.3.2.3. Immobilization of chymotrypsin through macromolecular spacers. Before polymer spacer covalent attachment, 3 mg of polymer was exposed to the 1.5 mL of 0.01 M HCl solution at 80 °C for 1 h to eliminate acetal protection of aldehyde groups. After the reaction was completed, the solution was neutralized with 0.01 M NaOH and dialyzed by the use of spin-column with membrane MWCO-3000.

The procedure of ACHT immobilization through a polymer spacer was generally the same as that developed for glutaraldehyde. Briefly, amino-bearing disk was immersed into 1 mL of polymer solution in 0.01 M PBS containing 1 mg of VP–Ac, or 2 mg of MAG–VP–Ac copolymers. After 1.5 h the disk was washed with PBS for 10 min at a flow rate 1 mL/min and incubated in 5 and 10 mg/mL of ACHT solutions in 0.01 M sodium borate buffer, pH 8.4, for 1.5 h more. The temperature in all cases was kept constant and equal to 22 °C. The disk treatment with sodium borohydride was the same as that used in glutaraldehyde experiment.

The amount of bound polymers was calculated as follows: $q_{polymer}$ = (amount of polymer in initial solution) – (amount of polymer in solution after reaction) – (amount of polymer in washing solution). The polymer quantity was determined using the so-called *iodine test* based on the formation of specific complex of iodine with VP. For that, 3 mL of 3 mM iodine in 0.2 M sodium acetate buffer, pH 4.6, was added to 0.5 mL of polymer solution. The absorbance of colored complex was measured immediately at 460 nm. The concentration of copolymer in solutions was calculated using calibration curve built preliminarily for each polymer.

The amount of ACHT coupled to the support was calculated in the manner described above (Section 2.3.1).

2.3.3. Chymotrypsin column activity determination

The activity of chymotrypsin-immobilized monolithic disks was determined by monitoring N-benzoyl-L-tyrosine (BT) formation, namely, the product of BTEE digestion, at wavelength 256 nm. To compare the activity of immobilized ACHT in four bioreactors obtained, the apparent values of Michaelis constant (K_m) and maximum velocity of enzymatically catalyzed reaction (V_{max}) were calculated using graphical method based on plotting of dependence of hydrolysis velocity on substrate concentration (Michaelis–Menten plot) and its further linearization in inversed coordinates (Lineweaver–Burk plot).

In order to build the Michaelis–Menten plots, in all experiments 2.8 mL of the BTEE solutions with concentrations in the range 0.1–2.0 mmol/L was circulated through the disk for 15 min at 22 °C. BTEE solutions were prepared by dissolving a substrate in the mixture of 0.05 M Tris–HCl buffer, pH 7.8, and 50 mass% aqueous methanol in ratio 1:1. The flow rate in all cases was kept constant and equal to 0.5 mL/min corresponding to the flow rate of 2.7 mL/min recalculated for standard CIM disk. The absorbance of a product was measured by off-line UV-detection. The recirculation system included a small container where the mixing was provided by slight shaking. The evaporation of a liquid was excluded by sealing with a special plug. The BT extinction at 256 nm equal to 964 L/mol cm was used for the calculation of product amount [44]. The Origin 6.0 software was used for plotting of graphs.

2.3.4. Hydrolysis of macromolecular substrate (BSA)

Bovine serum albumin (BSA) was used as a macromolecular substrate. To denature protein ternary structure, 3 mL of BSA solution with concentration of 0.3 mg/mL dissolved in 0.1 M sodium phosphate buffer, pH 8.0, was exposed at boiling water bath for 1 min and then incubated at 30 °C for 5 min. After that the solution was circulated through the ACHT-disk at 30 °C for 3 h.

2.3.4.1. Capillary electrophoresis. The CE conditions were as follows: operation buffer 0.01 M sodium borate, pH 9.2, voltage 20 kV, temperature 30 °C, probe concentration 0.3 mg/mL, injection mode during 15 s under pressure 30 mbar, detection at λ = 220 nm, capillary diameter 75 μm, capillary length 60 cm.

3. Results and discussion

3.1. Enzyme immobilization

It is well known that immobilization of enzymes on a solid surface is frequently followed by partial loss of its biological activity. To minimize an effect of solid matrix on enzyme activity and to keep the biomolecule flexibility, that is important regarding an accessibility of its active center, the introduction of a spacer distancing the enzyme from solid phase surface was recommended and usually used in the experiments with bead-based sorbents [45]. In our study the effect of spacer on proteolytic activity of α-chymotrypsin

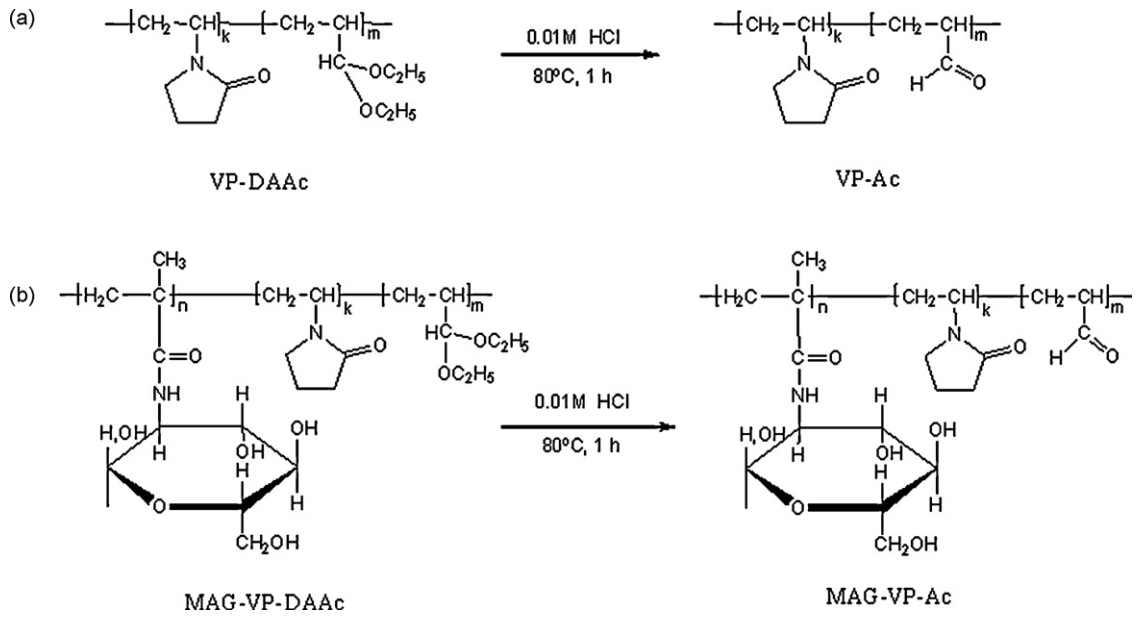

Fig. 2. The structures of polymer spacers and reactions of their activation: (a) copolymer of N-vinylpyrrolidone with acrolein diethyl acetal (VP–DAAc), 21 mol% DAAc, MW 25,000; (b) terpolymer of 2-deoxy-N-methacryloylamido-D-glucose with N-vinylpyrrolidone and acrolein diethyl acetal (MAG–VP–DAAc), 8 mol% DAAc, MW 18,000.

covalently bound to monolithic GMA–EDMA polymer material has been studied.

Covalent immobilization of ACHT on the surface of epoxy-bearing monolithic sorbents was carried out by two ways: (1) by direct one-step reaction of amino groups of enzyme with original epoxy groups of solid matrix; (2) by covalent attachment of enzyme to aldehyde-bearing spacer preliminary introduced into the solid matrix (immobilization through the spacer).

Well-known glutaraldehyde was applied as a short functional spacer. Additionally, two macromolecular spacers, namely, copolymer of N-vinylpyrrolidone with acrolein diethyl acetal, containing 21 mol% of DAAc, MW 25,000, and terpolymer of 2-deoxy-N-methacryloylamido-D-glucose with N-vinylpyrrolidone and acrolein diethyl acetal containing 8 mol% of DAAc, MW 18,000, were selected for investigation. Both of chosen copolymers represent water-soluble and biocompatible macromolecules [46,47]. The introduction of saccharide residues (MAG) into the polymer structure can provoke the formation of enzyme microenvironment close to its natural medium. Moreover, the positive feature of selected molecules is the simplicity of their quantity detection providing easy control of immobilization step. Polymer aldehyde groups formed after activation allow fast covalent binding of amino-bearing ligands at mild conditions with elimination of water as the only side-product. The structures of applied polymers, as

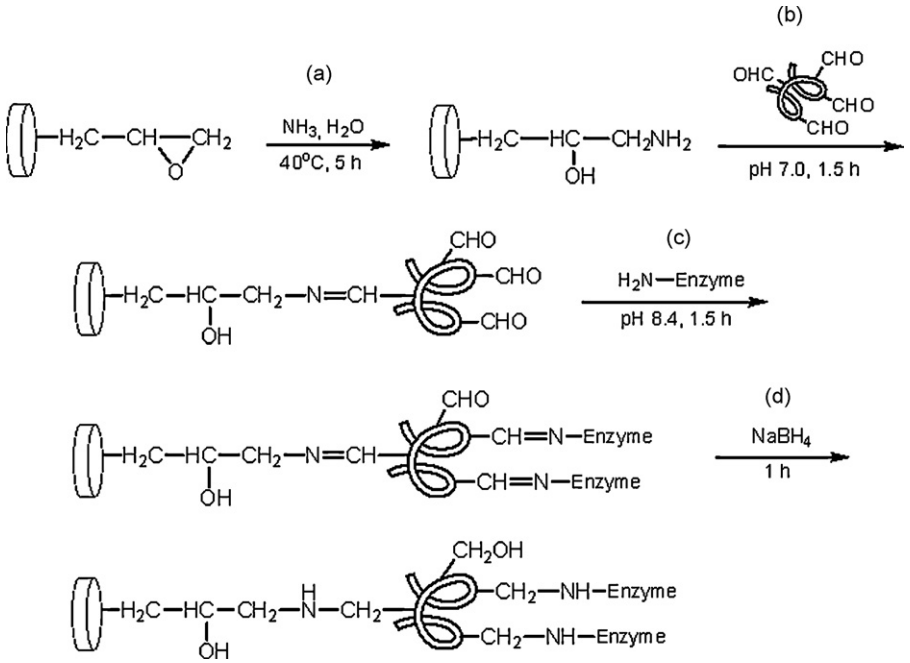

Fig. 3. The scheme of chymotrypsin immobilization via aldehyde-bearing polymer spacer: (a) aminolysis of epoxy groups of GMA–EDMA material; (b) covalent binding of aldehyde-bearing spacer; (c) enzyme immobilization; (d) reduction of formed Schiff's base and unreacted aldehyde groups. The scheme of enzyme immobilization using glutaraldehyde as a short spacer was similar.

Table 1
Summarized results on α-chymotrypsin immobilization on CIM minidisks.

Bioreactor	Enzyme immobilization way	Concentration of solutions applied for immobilization				Amounts of immobilized ligands	
		Spacer		ACHT		Spacer mg/mL	ACHT mg/mL
		mg/mL	μmol of aldehyde groups/mL	mg/mL	μmol of ACHT/mL		
I	Direct immobilization	–	–	5.0	0.21	–	0.30 ± 0.01
II	Via glutaraldehyde	0.3	21.0	1.0	0.04	0.08	0.34 ± 0.03
III	Via VP–Ac	1.0	2.1	5.0	0.21	0.06	0.34 ± 0.02
IV	Via MAG–VP–Ac	2.0	0.5	10.0	0.42	0.04	0.31 ± 0.02

Characteristics of solid phase: Design: disk (short columns); material: GMA–EDMA; pore size:1.6 μm; disk thickness: 5 mm; disk diameter: 5.2 mm; disk volume: 0.1 mL; void disk volume: 0.06 mL. *Immobilization conditions*: Solution volume: 1 mL; buffers and reaction time—direct enzyme attachment: 0.01 M sodium borate buffer (pH 9.4) for 18 h; spacer attachment: 0.01 M PBS (pH 7.0) for 1.5 h, enzyme binding through a spacer: 0.01 M sodium borate buffer (pH 8.4) for 1.5 h; temperature: 22 °C.

well as the reaction schemes of their activation are presented in Fig. 2.

Contrary to direct protein immobilization via epoxy groups of monolithic sorbent, the other two ways of ACHT attachment include several steps (Fig. 3): (a) amination of epoxy groups of GMA–EDMA material; (b) spacer covalent binding; (c) enzyme immobilization; (d) reduction of formed Schiff's bases and the excess of aldehyde groups with sodium borohydride.

According to elemental analysis data, sorbent's amination at developed conditions provided 26% conversion of epoxy groups that corresponded to approximately 1 mmol amino groups per gram sorbent. Due to high content of amino groups at the surface, as well as high reactivity of aldehyde groups, the reaction of spacer binding was carried out at room temperature (22 °C) for 1.5 h using 0.01 M PBS, pH 7.0. The neutral pH value was chosen to prevent total depletion of aldehyde groups that would take place at slightly alkaline pH conditions usually used for such reactions. Furthermore, too high spacer density, especially macromolecular one, can negatively affect enzyme functioning because of possible steric limitations. At the same time, after aldehyde-bearing spacer attachment the content of free aldehyde groups on polymer chain is distinctly reduced. Therefore, to provide maximal enzyme immobilization capacity for followed reaction of ACHT coupling usually recommended pH 8.4 was applied.

Obviously, to compare correctly the activity of ACHT immobilized by different ways, the amounts of enzyme bound to the sorbent unit have to be equal. Direct immobilization of ACHT at pH 9.4 on CIM material allowed obtaining bioreactor with 0.30 mg of ACHT per minidisk. To construct bioreactor with enzyme immobilized on solid surface via intermediate spacer, it was necessary to find out some optimal ratio between initial amounts of spacer and enzyme that would provide desirable biocatalyst immobilization capacity (about 0.3 mg/disk) using minimal surface density of introduced spacer. From preliminary investigation of dependence of enzyme immobilization capacity on spacer's concentration it was concluded that the most optimal mass ratio between glutaraldehyde and enzyme was equal to 1:3 whereas for polymers it was found to be equal to 1:5 (data are not presented).

The summarized results of ligand immobilization on CIM minidisks, as well as applied immobilization conditions are presented in

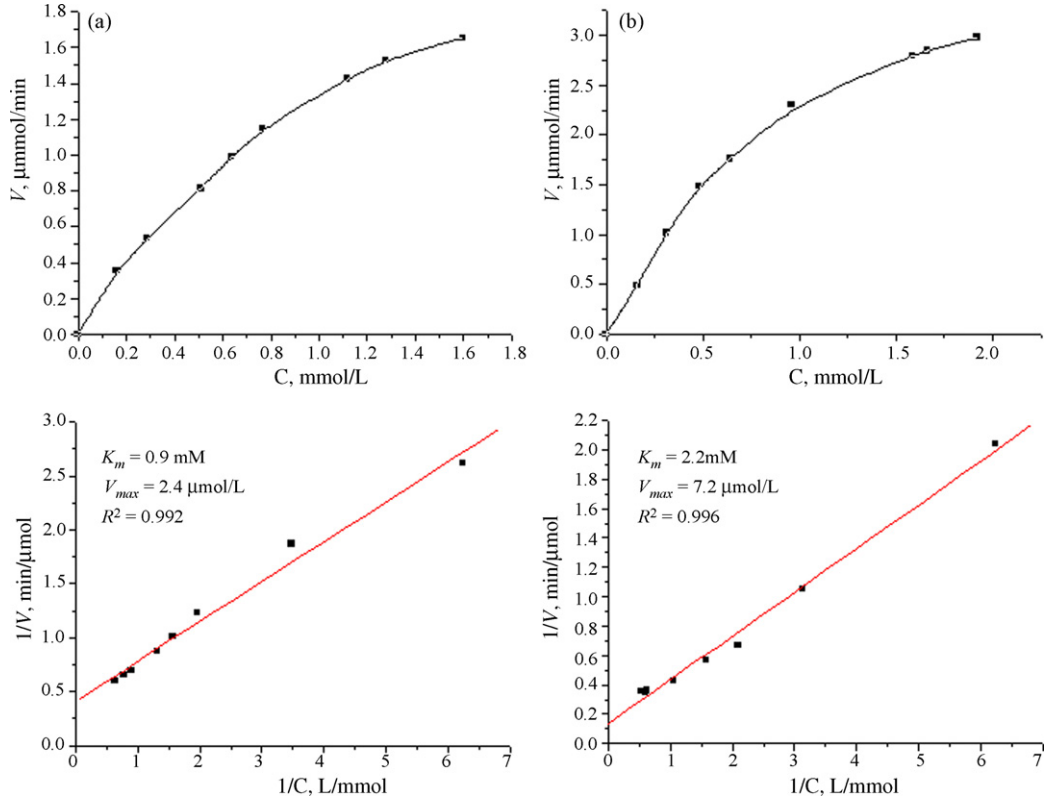

Fig. 4. Michaelis–Menten and Lineweaver–Burk plots: (a) direct enzyme immobilization (bioreactor I); (b) enzyme immobilization via MAG–VP–Ac terpolymer (bioreactor IV).

Table 2
Effect of different immobilization methods on the activity of bound α-chymotrypsin.

Bioreactor	Immobilization approach	K_m[a] (mM)	V_{max}[b] (μmol/min)	A_{immob}[c] (U/mg)	$A_{sol.}$[d]/A_{immob}
I	Direct immobilization	0.9 ± 0.2	2.4 ± 0.2	8.3	3.4
II	Via glutaraldehyde	0.5 ± 0.3	4.0 ± 0.2	11.8	2.4
III	Via VP–Ac	1.5 ± 0.2	5.8 ± 0.3	17.1	1.7
IV	Via MAG–VP–Ac	2.2 ± 0.1	7.2 ± 0.3	23.2	1.2

[a] K_m: Michaelis–Menten constant.
[b] V_{max}: maximum velocity of enzymatically catalyzed reaction.
[c] Specific activity of immobilized ACHT.
[d] Specific activity of ACHT in the solution was equal to 28.6 ± 0.4 U/mg, K_m = 0.3 ± 0.2 mM.

Table 1. In the case of glutaraldehyde, the concentrations of spacer and enzyme necessary to get the ACHT immobilization capacity close to that established for the direct attachment (0.3 mg/disk), were found as 0.3 and 1.0 mg/mL, respectively. In turn, to reach the same ACHT amount bound to CIM minidisk using macromolecular spacers containing different molar content of aldehyde groups, namely, 21 and 8 mol%, polymer and enzyme concentrations were increased up to 1.0 and 5.0 for VP–Ac, and 2.0 and 10.0 mg/mL for MAG-VP–Ac.

3.2. Comparison of kinetic parameters of immobilized chymotrypsin

To characterize and compare the properties of immobilized ACHT in all bioreactors obtained, BTEE was used as specific substrate. The Michaelis–Menten plots were built in a range of its concentrations from 0.16 to 1.92 mmol/L (pH 7.8, 22 °C). The double-reciprocal Lineweaver–Burk plots were used to calculate K_m and V_{max}. One example demonstrating the graphs built for two bioreactors obtained for direct and through MAG–VP–Ac spacer ACHT immobilization methods are presented in Fig. 4. The data of Table 2 reflect the effect of enzyme coupling approach on apparent kinetic parameters. K_m values determined for BTEE hydrolysis by immobilized ACHT were found to be in the range of 0.5–2.2 mM depending on immobilization method. As seen from Table 2, the lowest values of K_m, 0.5 and 0.9 mM, were established for ACHT immobilization using short glutaraldehyde spacer and direct coupling procedure, respectively. The values obtained appeared to be very close to that determined for the same enzyme reaction carried out in a solution (0.3 mM). The introduction of long polymer spacers led to the increase of K_m to 1.5 for VP–Ac polymer spacer and to 2.2 mM for terpolymer.

In contrast to K_m, another effect was observed for V_{max} and, consequently, for specific activity values established for all enzymatic states. As it was expected, the specific activity of bound ACHT was found to be lower than that measured for free enzyme (28.6 U/mg). The lowest specific activity, 8.3 U/mg, was established for ACHT directly immobilized on CIM monolithic minidisk. The introduction of even short spacer increased activity of immobilized enzyme approximately 1.5 times. In turn, comparatively to bioreactor obtained via direct ACHT attachment, the immobilization of enzyme through polymer spacers resulted in enzyme activity grown up to 17.1 and 23.2 U/mg for VP–Ac and MAG–VP–Ac, respectively. Furthermore, in the case of terpolymer spacer, ACHT specific activity seemed to be close to the value mentioned above for free enzyme. Obviously, the result observed for bioreactors prepared by immobilization of enzyme via spacer cannot be related to different orientations of the enzyme molecule at solid surface because of identical chemistry of binding reaction touching the same amino acids residues of ACHT. Therefore, the catalytic activity of immobilized enzyme is defined only by length and nature of used spacer.

The covalent immobilization of biomolecules via macromolecular spacer was found to be favorable in comparison with small intermediate molecule. In this case, the high value of ACHT activity can be related to solution-like spacious molecular behavior providing facilitated access of a substrate to the active site of enzyme, as well as to minimized effect of solid matrix. In turn, the difference in resulting activity values found for VP–Ac and MAG–VP–Ac spacers can be explained by different reactive (aldehyde) group densities along polymer chain. Thus, reactive groups in MAG–VP–Ac are separated each from other by longer oligomer blocks that allows formation of some kind of loop-like polymer conformation permeable for the substrate small molecules. Such spacious model also assumes the lower number of Schiff's bonds on enzyme molecule that results in more stable conformation of ACHT globule. The high specific activity of the ACHT immobilized via MAG–VP–Ac macromolecule can also belong to a positive influence of D-glucose residues.

The bioreactors obtained were characterized by high enzymatic stability that means that specific activity detected during 4 months stayed practically constant.

3.3. Effect of flow rate on bioreactor efficiency

To study the effect of flow rate on enzyme reaction efficiency, the bioreactors with minimum (bioreactor I) and maximum (bioreactor IV) specific activity were tested. In these experiments BTEE hydrolysis was investigated at constant substrate concentration equal to 1.3 mmol/L and different recirculation flow rates, e.g. 0.2, 0.4, 1.0 and 1.5 mL/min. The applied flow rates correspond to 1.1, 2.1, 5.3 and 8.0 mL/min regarding standard CIM disk with thickness of 3 mm and diameter of 12 mm or to 1.6, 3.1, 7.8 and 11.8 cm/min of linear flow velocity, respectively.

Contrary to the data published for enzymes covalently bound to sorbent beads [16], in our case, the increase of flow rate at recirculation of reaction mixture through the disk led to significant raise of substrate conversion (Fig. 5). This extraordinary result cannot be related to be very important for flow-through monoliths value as residence time, or retention time of dissolved molecules within porous space. In recirculation model, the gradient of composition of liquid phase permanently flowing through thin monolithic layer is insignificant taking into consideration very small changes in substrate concentration and, consequently, the concentration of reaction products. The latter is confirmed by the absence of inhibiting effect of the products releasing from enzyme active sites. Obviously, the probable change in liquid phase composition is minimized by the increase of recirculation flow rate. The most reasonable explanation of result obtained is another well known phenomenon, namely, enhanced diffusivity of substrate molecules being in a mobile phase that is common for all interphase processes realized on monoliths where mass transfer is controlled by convection rather than diffusion. In turn, the enhanced flow rate increasing diffusivity leads to the elevation of a number of efficient contacts between molecules of dissolved substrate and immobilized enzyme (enzyme–substrate complex formation). In fact, taking into account microsecond time scale of biocatalyzed reaction, the formation of complex under constant liquid flow conditions seems to be time-

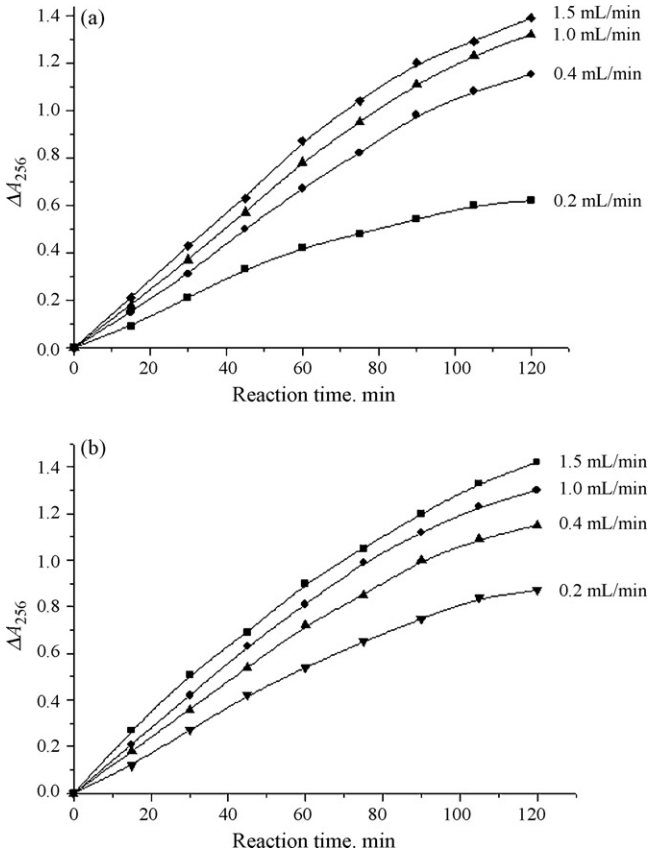

Fig. 5. Effect of flow rate on the efficiency of BTEE hydrolysis using monolithic α-chymotrypsin reactors: (a) direct enzyme immobilization (bioreactor I); (b) enzyme immobilization via MAG–VP–Ac (bioreactor IV). *Conditions*: BTEE concentration was 1.3 mmol/L; 22 °C; pH 7.8.

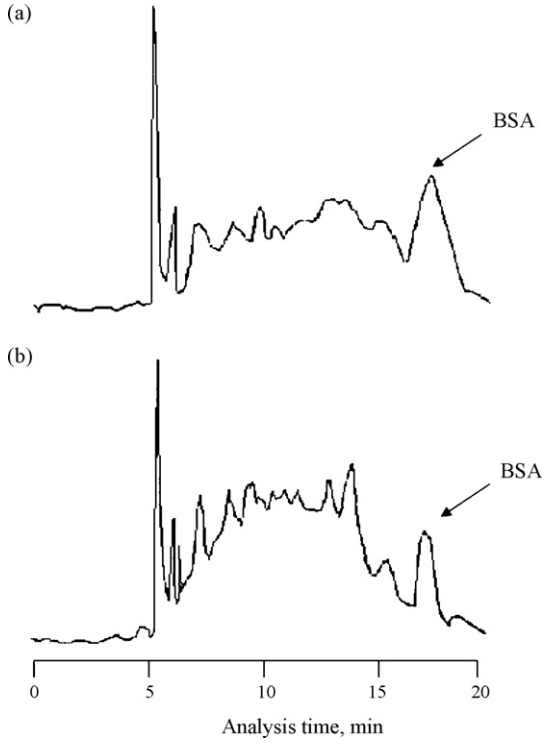

Fig. 6. Capillary electrophoresis of BSA digest: (a) direct ACHT immobilization (bioreactor I); (b) enzyme immobilization via MAG–VP–Ac (bioreactor IV). *Conditions*: pH 9.2, voltage 20 kV, 30 °C, probe concentration 0.3 mg/mL.

limiting step of such a process. Additionally, the observed result can be also explained by quick and non-restricted removal of reaction product that, in turn, favors to the shift of reaction balance to the side of complex formation.

3.4. Hydrolysis of macromolecular substrate

The opportunity to use enzyme immobilized on monolithic solid phase via terpolymer spacer (bioreactor IV) to cleave a macromolecular substrate (BSA) was studied. To compare the efficiency of protein digestion, the disk with directly immobilized enzyme (bioreactor I) was used as a standard. The hydrolysis of BSA was performed by circulation of thermally denatured substrate through the disks with bound ACHT. The digests obtained were analyzed by capillary electrophoresis provided with spectrophotometric detector (Fig. 6). It was shown that both types of monolithic reactors enabled bioconversion of protein. However, in the case of ACHT immobilized via MAG–VP–Ac spacer the efficiency of digestion was obviously higher. This important result can be commented from two positions. First, the positive influence of spacer length really increases a flexibility of enzyme molecule that allows non-limiting enzyme–substrate pair formation. And second, despite long macromolecular spacer chains and their loop-like conformation, it does not sterically restrict the interaction between two big protein molecules.

4. Conclusions

A few original results are discussed in present paper. First, the application of monolithic materials as highly permeable solid phases for flow-through enzyme reactors is still not deeply studied. Second, the demonstration of positive effect of polymer long-chain spacer introduced as an intermediate between monolith surface and biocatalyst molecule can also be related to the novel approaches of enzyme immobilization. And third, the established privilege of using of high operative flow rates for enzymatic hydrolysis of both small and macromolecular substrates looks as appropriate result regarding the convection mechanism of interphase mass transfer in porous monolithic media. Though, for conventional conception of enzyme kinetics these data are quite unusual and can be practically important.

In this paper the influence of enzyme immobilization methods on its activity was studied. Four bioreactors with the same α-chymotrypsin capacity were prepared using direct immobilization of enzyme on monolithic solid phase, as well as its coupling with the same matrix via introduction of short and long spacers. It was established that ACHT activity depended on immobilization mode, as well as length and nature of applied spacer. The highest specific activity was revealed for α-chymotrypsin immobilized on CIM minidisk via MAG–VP–Ac spacer, whereas the lowest activity was determined for directly immobilized enzyme. Moreover, the bioreactor prepared via covalent attachment of ACHT through terpolymer provided better digestion of protein substrate. Finally, the effect of flow rate on bioconversion efficiency was examined. In both cases of enzyme immobilization, e.g. with or without spacers, the increase of flow rate led to the raise of biocatalytic efficiency.

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