Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Magnetism and Magnetic Materials 293 (2005) 349–357
Journal of magnetism and magnetic materials
www.elsevier.com/locate/jmmm

Magnetic enzyme reactors for isolation and study of heterogeneous glycoproteins

Lucie Korecká[a,*], Jana Ježová[a], Zuzana Bílková[b], Milan Beneš[c], Daniel Horák[c], Olga Hradcová[b], Marcela Slováková[b,d], Jean-Louis Viovy[d]

[a]*Department of Analytical Chemistry, University of Pardubice, Námesti Čs. Legií 565, 532 10 Pardubice, Czech Republic*
[b]*Department of Biological and Biochemical Sciences, University of Pardubice, Námesti Čs. Legií 565, 532 10 Pardubice, Czech Republic*
[c]*Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovského Námesti 2, 162 06 Prague, Czech Republic*
[d]*Laboratoire Physicochimie Curie, UMR 168 CNRS/Institute Curie, Paris Cedex 05, France*

Available online 3 March 2005

Abstract

The newly developed magnetic micro- and nanoparticles with defined hydrophobicity and porosity were used for the preparation of magnetic enzyme reactors. Magnetic particles with immobilized proteolytic enzymes trypsin, chymotrypsin and papain and with enzyme neuraminidase were used to study the structure of heterogeneous glycoproteins. Factors such as the type of carrier, immobilization procedure, operational and storage stability, and experimental conditions were optimized.

Keywords: Magnetic enzyme reactor (IMER); Trypsin; Chymotrypsin; Papain; Enzymes; Neuraminidase; Glycoproteins; Magnetic separation

1. Introduction

The high efficiency of many processes in nature and their low energy demand depend on activities of highly effective and specific catalysts—enzymes. The application of enzymes in their native form in biochemical and biomedical fields is not always suitable and optimal [1]. Binding of enzymes on a solid support is an advantageous modification of their application in biosciences and special diagnostic procedures [1,2]. The enhanced stability, rapid and gentle separation of carrier with immobilized enzymes from the reaction media, significantly lower cost provide substantial reasons to utilize these enzyme reactors in clinical practice [1,3].

Immobilization chemistry depends on the type of support, activation method and coupling procedure [1,3]. The choice of the matrix (chemical

*Corresponding author. Tel.: +420 46 603 7709.
E-mail address: lucie.korecka@upce.cz (L. Korecká).

0304-8853/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jmmm.2005.02.030

and magnetic properties, particle size and distribution, porosity) is a key factor influencing quality of the immobilized magnetic enzyme reactors (IMERs) and the scope of final applications and/or process automation [4].

Mostly magnetic microspheres consist of a superparamagnetic core embedded in a polymer shell protecting the enzyme from contact with the metal (mostly iron) oxide [5]. A magnetic colloid (so-called ferrofluid) is an important starting component in the preparation of magnetic polymer nano- and microspheres. Various methods and materials were used for their synthesis. The factors such as the type of carrier (size, porosity, hydrophilic or hydrophobic character, reactive groups on the surface, behavior of particles in magnetic field, paramagnetism, minimal nonspecific sorption of proteins and peptides) and immobilization procedure can be controlled [3,5].

Highly utilized porous particles with high specific surface have one disadvantage: the reaction rate could be very slow mainly due to mass transfer limitations in the pore volume for high molecular-mass substrates. On the other side if nonporous particles are used, only the outer surface of the particles is available for the reaction [6]. Microspheres made from a variety of materials, including polystyrene and other hydrophobic polymers, silica and superparamagnetic polystyrene can sometimes encounter environments that cause unwanted aggregation. To obviate their agglomeration or sedimentation, the colloidal nanoparticles are coated with (encapsulated in) a thin layer of a stabilizer. Stabilizing coating material is a synthetic or natural polymer and may be a polysaccharide, polypeptide, protein, or an antibody or a nucleic acid [5,7].

The advantage of polymer shell surrounding the magnetic core consists in the possibility of surface functionalization and subsequent immobilization of a target biomolecule [8]. Choosing a suitable method of enzyme immobilization also enables an increase in the stability without negative influence on their catalytic activity. Binding of molecules in areas of active sites or in their vicinity can lead to a decrease or complete loss of activity [1].

Newly developed magnetic particles, alginate-coated magnetite microparticles, cellulose magnetite microparticles, bead cellulose, poly(HEMA-*co*-GMA) were tested for their applicability in the practical use for the preparation of IMERs. The possibility of using them in microfluidic systems was also investigated. Parameters of magnetic particles are described in Table 1. The commercially available particles polyNIPAM (Ademtech, Pessac, France) and polySTYREN (Uptima, France) were used for comparison of particle quality. The aim of our study was to optimize the conditions of employment of newly developed magnetic enzyme reactors (IMERs) namely with direction to accomplish the maximum activity and specificity of the catalyzed reaction. Not only the stage of development and preparation of magnetic reactors with various enzymes [9–11] but also the stage of application of reactors is important for magnetic enzyme reactors. The choice of reaction conditions could influence the quality of the reactors in positive or negative way. In this work, we focused on reaction conditions such as time, temperature, intensity of mixing, environmental pH, rate of enzyme and carrier, necessity of ions, activators and/or surfactants. The distinct consideration was donated to magnetic features of already modificated carriers regarding behavior in the course of changed reaction conditions (pH, necessity of ions and surfactants). Enzymes trypsin, chymotrypsin, papain, neuraminidase) were selected in relation to the possibility of the study of heterogeneous high molecular-mass glycoproteins, especially immunoglobulin G (IgG) [12].

2. Materials and methods

2.1. Chemicals

Chymotrypsin (EC 3.4.21.1.), Trypsin (EC 3.4.22.2.), 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDAC), *N*-α-benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA) (EC 213-011-2), *N*-succinyl-L-phenylalanyl-*p*-nitroanilid (SUPHEPA), 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid ($C_{21}H_{24}NO_{11}Na$, 4-MUNANA), benzamidine, polyoxyethylsorbitane monolaurate (Tween 20), Triton were products

Table 1
Characteristic properties of the carriers used for preparation of IMERs

Type of particles	Type of carrier	Size (μm)	Functional group	Ligand	Application	Developed by	Fig.
Bead cellulose	Hydrophilic Macroporous	80–100	–OH	Papain	Fragmentation and preparation of Fc and Fab of IgG	IMCH AS Czech Republic	1A
				Neuraminidase	Modification of glycosidic chain of IgG		
				Chymotrypsin	Total and partial digestion of IgG		
Alginate coated magnetite	Hydrophilic nonporous	5–10	–COOH	Trypsin	Total and partial digestion of IgG	IMCH AS Czech Republic	1B
Cellulose magnetite	Hydrophilic nonporous	6–8	–OH	Trypsin	Total and partial digestion of IgG	IMCH AS Czech Republic	1C
Poly(HEMA-*co*-GMA) (50/50wt)	Hydrophobic nonporous	3.7	–OH	Trypsin	Total and partial digestion of IgG	IMCH AS Czech Republic	2A
PolyNIPAM	Hydrophobic nonporous	0.29 0.875	–COOH	Trypsin	Total and partial digestion of IgG	Ademtech, France	2B
PolySTYREN	Hydrophobic nonporous	0.86	$-NH_2$	Trypsin	Total and partial digestion of IgG	Uptima, France	—

of Sigma-Aldrich (St. Louis, MO, USA); *N*-Hydroxysulfosuccinimide sodium salt (S-NHS) was obtained from Fluka (Buchs, Switzerland) and Reanal (Budapest, Hungary) was the supplier of sodium periodate; magnetic macroporous bead cellulose L1459-3 (80–100 μm), alginate coated form of ferrite microparticles (5–10 μm), cellulose magnetite microparticles (6–8 μm), poly(HEMA-*co*-GMA) 50/50wt (3.70 μm) were supplied by Institute of Macromolecular Chemistry (Academy of Sciences, Prague, Czech Republic); polyNIPAM particles (0.29–0.875 μm) were from Adem-tech (Pessac, France) and polySTYREN particles were obtained from Uptima (Montlucon Cedex, France). The remaining chemicals were supplied by Lachema (Brno, Czech Republic) and were of analytical reagent grade.

2.2. Immobilization of trypsin on alginate-coated ferrite microparticles A59

Immobilization was achieved through EDAC and S-NHS activation as described previously [12]. Activity of the enzyme reactor was determined using the low molecular-mass substrate BAPNA (see 2.4).

2.3. Immobilization of papaine on a magnetic macroporous bead cellulose

Immobilization of papain was performed as described previously [12]. Activity of enzyme reactor was determined using the low molecular weight substrate *N*-α-benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA).

2.4. Determination of enzyme activity using low molecular-mass substrate N-α-benzoyl-D, L-arginine-p-nitroanilide (BAPNA) [13]

Soluble or immobilized enzyme (0.1 ml) was added to 1 ml of 0.1 M Tris-HCl buffer pH 7.8 with 0.025 M $CaCl_2$. 0.02 ml of 0.055 M BAPNA in *N*,*N*-dimethylformamide. After incubation for 30 min reaction was stopped with 0.2 ml of 30% (v/v) acetic acid. Using MultiScan RC reader (Labsystems, Helsinki, Finland) absorbance at 405 nm was measured.

2.5. Determination of chymotrypsin activity using low molecular-mass substrate N-succinyl-L-phenylalanyl-p-nitroanilid (SUPHEPA) [9]

Soluble or immobilized enzyme (0.1 ml) with 1.25 ml of 0.1 M Tris-HCl buffer pH 7.8 with 0.025 M $CaCl_2$ was tempered to 37 °C. Substrate of 0.1 ml (0.025 M SUPHEPA) was added. After incubation for 15 min at 37 °C reaction was stopped with 0.2 ml of 30% (v/v) acetic acid. Using MultiScan RC reader (Labsystems, Helsinki, Finland) absorbance at 405 nm was measured.

2.6. Determination of neuraminidase activity using 2-(4-methylumbelliferyl)-α-N-acetylneuraminic acid (4-MU-NANA) [10]

The activity of neuraminidase was measured fluorometrically by determination of the released 4-methylumbelliferyl (4-MU) from the substrate 2-(4-methylumbelliferyl)-α-*N*-acetylneuraminic acid (4-MU-NANA) at 37 °C. Twenty microlitres of 0.1 M potassium acetate buffer pH 4 and 40 μl of 2 mM 4-MU-NANA were added to 50 μl of sedimented carrier. The reaction mixture was gently stirred for 30 min at 37 °C and 1.2 ml stop buffer (0.2 M glycin–NaOH pH 10.6) was added to the separated supernatant. The fluorescence was measured at 365/448 nm. One unit of enzyme hydrolyzes 1.0 μmol of substrate per minute at pH 5.5 and 37 °C.

2.7. Determination of K_M for trypsin IMER using low molecular-mass substrate N-α-benzoyl-D, L-arginine-p-nitroanilide (BAPNA)

BAPNA of 0.1 ml in *N*,*N*-dimethylformamide (concentrations were 0.7, 0.9, 1.1, 1.67 and 2.2 mM) was applied into magnetically active microtitration plate. Six micrograms of trypsin immobilized on magnetic carrier was added. Changes of absorbance at 405 nm during 2 min were measured using MultiScan RC reader (Labsystems, Helsinki, Finland). Total analysis time was 8 min.

2.8. Monitoring of magnetic behavior of particles and reactors

Magnetic behavior and tendency of particles for aggregation was subjectively controlled using magnetic separator Dynal MPC-6 (Dynal, Oslo, Norway). In each stage of preparation and application of magnetic reactors the magnetic behavior was controlled using optical microscopy with Olympus BX41, camera 50-50Z, QuickphotoAver.

3. Results and discussion

The newly developed magnetic micro- and nanoparticles with defined hydrophobicity and porosity (see Table 1 and Figs. 1 and 2) were used for the preparation of IMERs. Magnetic particles with controlled tendency to aggregate and controlled level of nonspecific sorption enable highly efficient and gentle separation during immobilization, high specifity and reproducibility of catalyzed reactions without risk of contamination the resulting mixture, elimination of the undesirable dilution and losses of the analyte. Proteolytic enzymes trypsin and chymotrypsin are used for total and partial digestion, papain for fragmentation and preparation of Fc and Fab fragments of IgG molecule and neuraminidase for modification of glycosidic chain localized in Fc part of IgG before isolation of IgG glycoform using affinity chromatography on lectines.

Ferrofluid contains typically particles of 10 nm (or less) in size, which therefore remain disordered in a fluid after the removal of external magnetic field because of Brownian motion [7]. Nevertheless, each colloid nanoparticle has a permanent magnetic dipole moment due to its microdomain nature. Such superparamagnetic particles, unlike

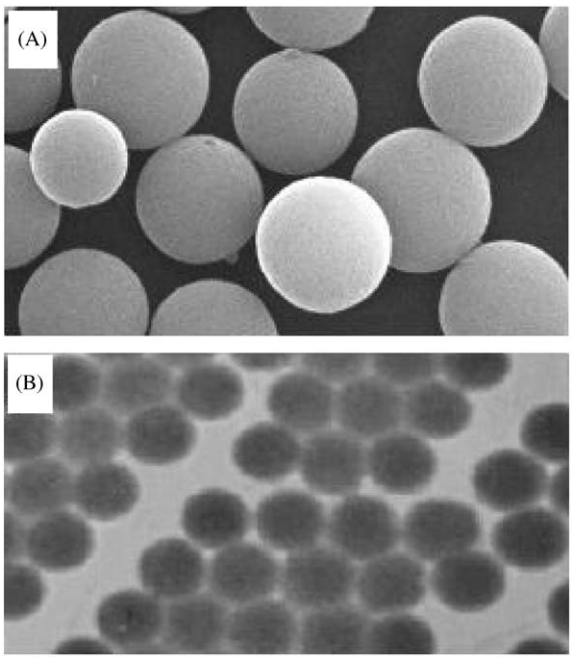

Fig. 2. (A) poly(HEMA-*co*-GMA) particles 50/50 wt (3.70 μm), developed by the Institute of Macromolecular Chemistry, Academy of Sciences, Prague, Czech Republic. (B) polyNIPAM particles (0.29 μm), Ademtech, Pessac, France; electron microscopy.

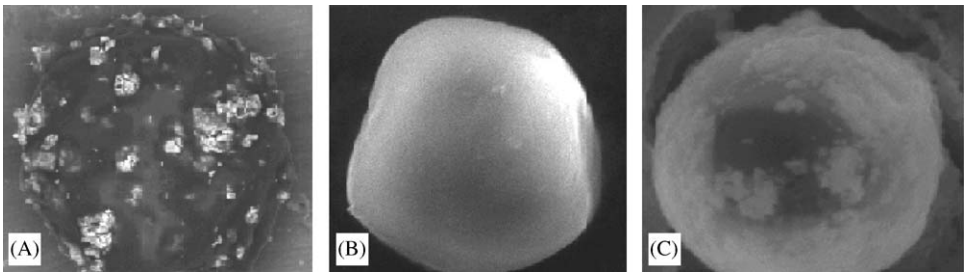

Fig. 1. (A) Bead cellulose (80–100 μm), focused 750 ×, (B) alginate-coated magnetite microparticles (5–10 μm), focused 4000 ×, and (C) cellulose magnetite microparticles (6–8 μm), focused 7000 ×; developed by the Institute of Macromolecular Chemistry, Academy of Sciences, Prague, Czech Republic; electron microscopy photos were kindly provided by the Department of Chemistry of Polymer Materials, University of Pardubice.

ferromagnetic ones, exhibit magnetic behavior only when they are in magnetic field and do not become permanently magnetized (see Fig. 3).

The experimental conditions for immobilized enzyme reactors (time, temperature, intensity of mixing, environmental pH, rate of enzyme and carrier, necessity of ions, activators and/or surfactants) should be optimized. Trypsin immobilized on alginate-coated magnetite microparticles was used for estimation the optimal parameters (pH, buffer, temperature, detergents) compared with soluble form of enzyme.

The optimal pH values are approximately at the same level for both free and immobilized enzyme, but activity of immobilized trypsin is significantly higher. Only in the range of extreme values of pH (4 and 11) the higher activity of soluble enzyme was proved (Fig. 4).

Various kinds of buffers (0.1 M carbonate buffer pH 9.0, 0.1 M Tris-HCl buffer pH 9.0, 0.1 M phosphate buffer pH 9.0, universal Britton–Robinson buffer pH 9.0) were utilized for the determination of the most suitable reaction buffer for efficient digestion of highly heterogeneous high molecular-mass analytes. The best enzyme activities were obtained with carbonate buffer (0.1 M, pH 9.0) and Tris-HCl buffer (0.1 M, pH 9.0) in comparison with 0.1 M phosphate buffer pH 9.0 and universal Britton–Robinson buffer pH 9.0, where the activities were only about 60% of buffers mentioned above. Due to the tendency of particles to aggregate Tris-HCl is not recommended.

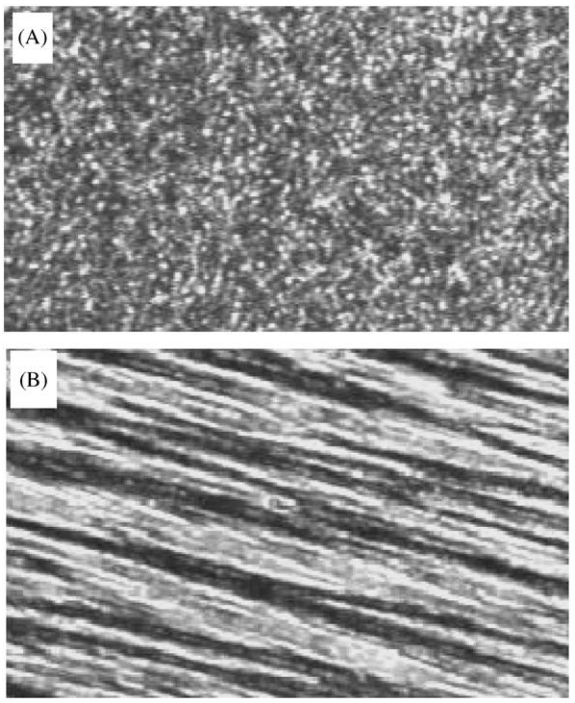

Fig. 3. Behavior of magnetic particles (A) without magnetic field and (B) in external magnetic field (polyNIPAM particles 0.875 μm, Ademtech, Pessac, France); optical microscopy (250 ×).

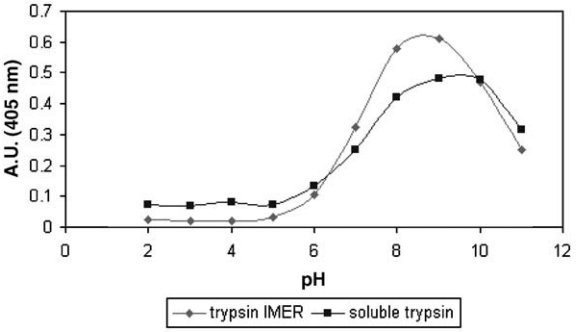

Fig. 4. Comparison of pH values for free trypsin and trypsin IMER; trypsin immobilized on alginate-coated magnetite microparticles. Enzyme activity was measured from pH 2 to 11.

The optimal values of temperature were established. The shift to higher values for the soluble form of enzyme was observed. The optimal temperature for immobilized enzyme is about 35 °C following by rapid decrease of enzyme activity at higher temperatures (Fig. 5).

The nature of the carrier (hydrophobic polySTYREN, polyNIPAM, polyHEMA) and the size of particles of about 10 μm and less influence the tendency to aggregate in a large extent [5–7]. Surfactants make the surface of the microspheres less hydrophobic, results in lowering of aggregation and consequently increasing of binding efficiency. Several characteristics of surfactants such as critical micelle concentration (CMC), should be considered in relation to applied concentration, size and surface nature of particules [14,15].

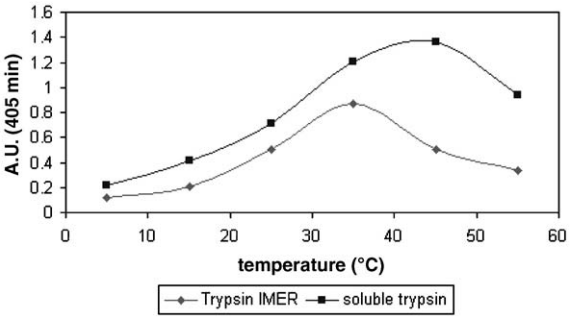

Fig. 5. Optimization of temperature for free trypsin and trypsin immobilized on alginate-coated magnetite microparticles. Enzyme activity was measured using BAPNA at reaction temperatures from 5 to 55 °C.

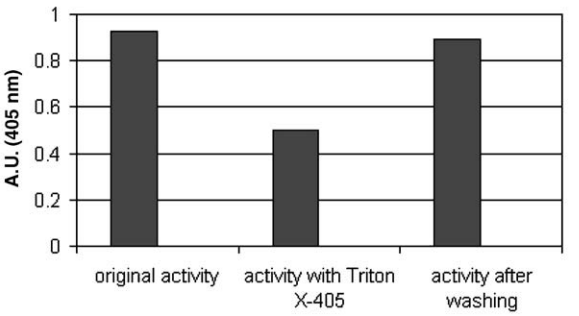

Fig. 6. Negative effect of Triton X-405 in the substrate buffer to the proteolytic activity of trypsin IMER; trypsin immobilized on polyNIPAM particles (hydrophobic, nonporous, 0.875 μm, Ademtech, France). Enzyme activity was measured using BAPNA.

Qualitatively different influence of surfactants to the binding efficiency and to the proteolytic activity was observed. The surfactant molecules adsorbed on the particle surface block their aggregation and zero interference with the coupling of trypsin molecules to the surface was provided [4]. On the other hand the proteolytic activity of immobilized trypsin is reduced in the presence of surfactant in the substrate buffer (Fig. 6).

The affinity of the enzyme for substrate is characterized by the constant of Michaelis–Menten (K_M). K_M for the soluble form and immobilized trypsin were determined in batch-wise mode using BAPNA (see 2.7). In comparison with K_M of free trypsin (K_M 1.625 mmol/l) K_M of immobilized trypsin has decreased to the value of 1.183 mmol/l (Fig. 7). We assume that decrease of K_M could be originated by preconcentration of substrate in proximity of particle surface. During immobilization, the stearic accessibility of active sites and conformation of them were not negatively influenced.

The storage and operational stability of the reactors as important factors ensuring proper and efficient application of enzymatic reactors should be verified. Storage stabilities of IMERs with trypsin, papain, chymotrypsin and neuraminidase were determined. Activities of trypsin and papain remained high for ten weeks with only gradual decrease (Fig. 8). On the other side the activities of

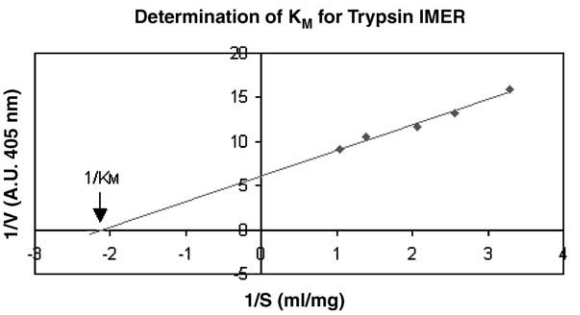

Fig. 7. Determination of K_M of immobilized enzyme; trypsin immobilized on alginate-coated magnetite microparticles. Enzyme activity was measured using BAPNA on MultiScan RC reader (Labsystems, Helsinki, Finland).

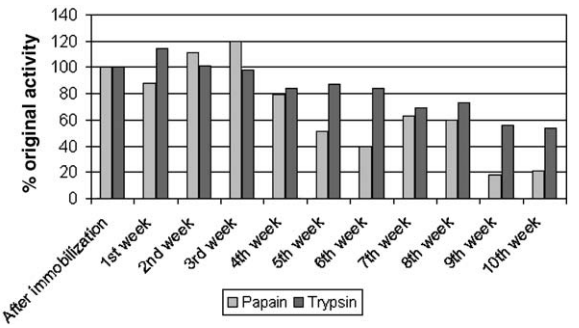

Fig. 8. Storage stability of papain and trypsin IMERs; papain immobilized on magnetic bead cellulose, trypsin immobilized on alginate-coated magnetite microparticles. Enzyme activity was measured using BAPNA during 10 weeks.

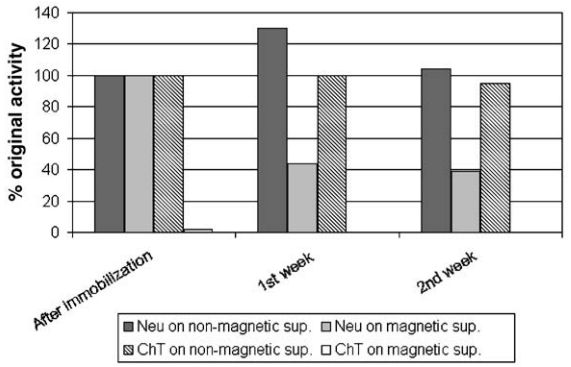

Fig. 9. Comparison of storage stability of neuraminidase (Neu) and chymotrypsin (ChT) immobilized on nonmagnetic and magnetic supports (magnetic bead cellulose). Enzyme activity was measured using 4-MU-NANA for neuraminidase and SUPHEPA for chymotrypsin during 2 weeks of storage.

chymotrypsin and neuraminidase immobilized on magnetic supports significantly decreased during a few days. Simultaneously with magnetic carriers the nonmagnetic forms of microspheres prepared by the same technology were utilized for immobilization of above-mentioned enzymes (Fig. 9).

Distinct decrease of activity was recorded both for neuraminidase (to 40% of original activity) and chymotrypsin, where the activity of enzyme immobilized on magnetic bead cellulose was only 1% of original activity immediately after immobilization in comparison with nonmagnetic form of macroporous bead cellulose.

Recorded decrease of these enzymes corresponds with already published details concerning the effect of Fe^{3+} ions to the enzyme activity [16,17].

Acquired results have confirmed the importance of optimalization both the preparation of reactors and conditions for application of prepared reactors. Factors such as nature of carrier (size, porosity, hydrophilic or hydrophobic character) and modification of surface (alginic acid etc.) influence the resulting enzymatic activity. Optimalization of the reaction conditions (pH, temperature, necessity of surfactants, E:S ratio, initial concentration of substrate) is necessary for efficient application of prepared enzymatic reactors. Selected highly active magnetic enzyme reactors with proper parameters (size, homogeneity) could be applied to magnetically active microfluidic devices [4]. Only particles of 1 μm and less could be focused in a channel of magnetically active microfluidic analytical device directly coupled to mass spectrometric analysis. This work is in progress.

4. Conclusions

The magnetic carriers and their utilization in the proteomics and genomics are in an extraordinary growth phase. Investigators and biotechnologic companies are taking an active interest in their practical application even in molecular genetics, cytology, immunology, microbiology and clinical biochemistry. Magnetic particles of various parameters, size 0.150–150 μm, hydrophilic or hydrophobic, porous or nonporous, with defined quantity of surface function groups or modified with various ligands (antibodies, lectins, streptavidine etc.) are already commercially available. Not at all times these particles suit the purpose of requirements (zero nespecific sorption, stability, minimal aggregation) essential for binding procedure and/or proper application. In conjunction with Institute of Macromolecular Chemistry, Academy of Sciences, Prague, we tested particles developed at this Institute. Newly developed cellulose particles and particles modified with alginic acid, with direction to minimalize nonspecific sorption of proteins and peptides, measured up these requirements. IMERs consisted of enzymes trypsin, chymotrypsin, papain and neuraminidase immobilized on these particles could be successfully used for study of heterogenous proteins and/or glycoproteins.

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic 203/02/0023, Ministry of Education Czech Republic VZ 253100002 and by EU project "Microproteomics" (Contract no. QLG2-CT-2001-01903).

References

[1] Z. Bílková, M. Slováková, A. Lyčka, et al., J. Chromatogr. B 770 (2002) 25.
[2] A. Španová, B. Rittich, D. Horák, et al., J. Chromatogr. A 1009 (2003) 215.
[3] W. Tischer, V. Kasche, Tibtech 17 (1999) 326.
[4] Z. Bílková, N. Minc, R. Stefanescu, M. Beneš, M. Gaboyard, D. Horak, M. Slovakova, J. -L. Viovy, M. Przybylski, Microreactors for total and limited proteolysis based on magnetic nanoparticles developed for μ-chip application, Proteomics, 2005, submitted.
[5] D. Tanyolac, A.R. Özdural, React. Funct. Polym. 45 (2000) 235.
[6] D. Horák, M. Karpíšek, J. Turková, M. Beneš, Biotechnol. Prog. 15 (1999) 208.
[7] B.M. Berkovsky, V.F. Medvedev, M.S. Krakov, Magnetic Fluids. Engineering Applications, Oxford University Press, Oxford, 1993.
[8] K.S. Cudjoe, Immunomagnetic Particle-Based Techniques, in: R.K. Robinson, C.A. Batt, P.D. Patel, (Eds.), Encyclopaedia of Food Microbiology. Academic Press, London, UK, pp. 1088–1094.
[9] Z. Bilkova, J. Mazurova, J. Churacek, et al., J. Chromatogr. A 852 (1999) 141.
[10] Z. Bílkova, M. Slovakova, D. Horak, et al., J. Chromatogr. 770 (2002) 177.
[11] Z. Bilkova, M. Slovakova, A. Lycka, et al., J. Chromatogr. B 770 (2002) 25.
[12] L. Korecka, Z. Bilkova, M. Holcapek, et al., J. Chromatogr. B 808 (2004) 15.
[13] H.F. Gaertner, A.J. Puigserver, Enzyme Microb. Technol. 14 (1992) 150.
[14] E.S. Ahuja, J.P. Foley, Anal. Chem. 67 (1995) 2315.
[15] T. Le Saux, A. Varenne, P. Gareil, J. Chromatogr. A 1038 (2004) 275.
[16] P.A. Munro, P. Dunnill, M.D. Lilly, Biotechnol. Bioeng. 19 (1) (1977) 101.
[17] R.W. Myers, Y.Ch. Lee, G.H. Thomas, et al., Anal. Biochem. 101 (1980) 166.