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Journal of Chromatography A, 852 (1999) 141–149

JOURNAL OF CHROMATOGRAPHY A

Oriented immobilization of chymotrypsin by use of suitable antibodies coupled to a nonporous solid support

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Abstract

In order to eliminate the kinetic limitation of chymotryptic hydrolysis of proteins due to diffusion, nonporous hydroxyalkyl methacrylate solid support was developed and used for oriented immobilization of chymotrypsin by means of suitable polyclonal antibodies. Nonporous microspheres were prepared by dispersion copolymerization of 2-hydroxyethyl methacrylate and ethylene dimethacrylate in an alcohol–toluene mixture stabilized with cellulose acetate butyrate. The resulting particles were 1.2 μm in diameter and possessed narrow size distribution. After modification with adipic acid dihydrazide they contained 2 μmol of reactive groups available for coupling of anti-chymotrypsin antibodies. Prepared immunosorbent adsorbed 166.7 μg of chymotrypsin per 1 g of dry carrier. Immobilized chymotrypsin retained practically 100% of its native proteolytic activity. Kinetic parameters of catalysis by chymotrypsin immobilized via this way were improved due to the good steric accessibility of the enzyme active site for high-molecular-mass substrates, when digestion of proteins in batch experiments was used.

Keywords: Immosorbents; Stationary phase, LC; Immobilized chymotrypsin; Antibodies

1. Introduction

Enzymatic hydrolysis of a protein molecule followed by separation and detection of the individual peptide fragments in the hydrolysate remains a powerful analytical technique for the identification and characterization of proteins. Numerous advantages of immobilized proteinases, compared with soluble enzymes used for digesting proteins, were described by Amankwa and Kuhr [1]. They immobilized trypsin on the surface of aminoalkylsilane-treated fused-silica capillary via biotin–avidin–biotin technology and used it for on-line digestion of picomole quantities of β-casein. Many examples of good steric accessibilities of active binding sites and increased stability of orientedly immobilized biologically active proteins have been reviewed [2]. Protein inactivation starts with the unfolding of the protein molecule by the contact of water with hydrophobic clusters located on its surface, which results in an ice-like water structure. Dramatically increased activity of amylases in complexes with their antibodies was demonstrated by Shami et al. [3]. Sheriff et al. [4] used X-ray crystallography to determine the three-dimensional structure of antibody–antigen complex by use of anti-lysozyme Fab

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00234-4

and lysozyme. They demonstrated the formation of more than 80 Van der Waals bonds during the antibody–lysozyme interaction. Formation of the complex resulted in exclusion of all water molecules.

The desired efficiency and stability of immobilized chymotrypsin was obtained by the combination of the isolation with the oriented immobilization by adsorption on a suitable immunosorbent [5–7]. Suitable polyclonal antibodies were isolated by biospecific affinity chromatography of a mixed IgG population on a matrix with chymotrypsin immobilized through its active site to natural polyvalent trypsin inhibitor attached to bead cellulose activated with 2,4,6-trichloro-1,3,5-triazine [7]. Stability of the affinity matrix was reached by crosslinking with glutaraldehyde. The failure to detect any chymotryptic activity towards N-succinyl-L-phenylalanine-*p*-nitroanilide showed that the enzyme was immobilized via its active site. The specific immunosorbent for chymotrypsin was prepared by oriented immobilization of anti-chymotrypsin antibodies through their glycosylation sites of the Fc fragments to the hydrazide derivative of bead cellulose [7].

In analogy to nonenzymatic heterogeneous catalysis, in which an important role was played by the rate of diffusion of the reactants to the active surface of the catalyst, the rate of diffusion of the substrate to the binding site of the enzyme significantly affects the kinetic parameters of catalysis by an immobilized enzyme system. In order to eliminate the kinetic limitation of chymotryptic hydrolysis of protein, the porous bead cellulose was replaced by nonporous poly(2-hydroxyethyl methacrylate) microspheres. After digestion of the protein in a batch experiment, immobilized enzyme was removed by centrifugation.

2. Experimental

2.1. Chemicals

Chymotrypsin (EC 3.4.21.1.), peroxidase–anti-pig IgG for enzyme-linked immunosorbent assay (ELISA), N-succinyl-L-phenylalanyl-*p*-nitroanilide and glutaraldehyde were products of Sigma–Aldrich (St. Louis, MO, USA). Antilysin (bovine pancreatic trypsin inhibitor) was obtained from Léčiva (Prague, Czech Republic), bovine anti-pig IgG for immunoelectrophoresis from EXBIO (Prague, Czech Republic), hemoglobin from Serva (Heidelberg, Germany), and Reanal (Budapest, Hungary) was the supplier of sodium periodate. Hydrazide derivative of macroporous bead cellulose Perloza MT 200 was kindly donated by Dr. M.J. Beneš from the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic. 2-Hydroxyethyl methacrylate (HEMA; Röhm, Germany) and ethylene dimethacrylate (EDMA; Ugilor, France) were purified by distillation under reduced pressure in nitrogen atmosphere. Cellulose acetate butyrate (CAB; acetyl/butyryl groups 35:15; number-average molecular mass (M_n) = 100 000) was a kind gift of Eastman, Kingsport, USA. 2,4,6-Trichloro-1,3,5-triazine (TCT) and adipic acid dihydrazide (AH) were from Fluka (Buchs, Switzerland). All other chemicals were of reagent grade and obtained from Sigma–Aldrich.

2.2. Isolation of IgG from hyperimmune pig serum

The source of anti-chymotrypsin antibodies was serum from pigs that had been immunized according to the standard protocol [8]. After gel filtration on Sephadex G-25, where low-molecular-mass components were removed, pig hyperimmune serum (1.6 ml) was injected on a column (10×1 cm I.D.) packed with DEAE-cellulose (Whatman) and equilibrated with 0.015 *M* phosphate buffer (pH 8) at a flow-rate 2.15 ml/min. The purity of fractions was determined by immunoelectrophoresis [9].

2.3. Purification of anti-chymotrypsin IgG

The IgG fraction eluted from DEAE-cellulose (8 ml) was injected on the column packed with chymotrypsin immobilized through its active site to antilysin-cellulose prepared according to Ref. [7], which had been preequilibrated with 0.1 *M* phosphate buffer, pH 7.0, at a flow-rate of 2.15 ml/min. After washing the column with the equilibration buffer and the same buffer containing 0.5 *M* NaCl to remove other anti-chymotrypsin antibodies, the anti-chymotrypsin IgG (IgG_s) of required specifity was released by an elution with 0.1 *M* glycine buffer (pH 2.3) containing 0.1 *M* NaCl. Eluted fractions were

immediately adjusted to pH 7.0 by titration with 0.5 *M* NaOH.

2.4. Inhibition test

Approximately 20 μl of a solution with adequate quantity of chymotrypsin in 0.1 *M* phosphate buffer, pH 7.2, was added to 100 μl of suitable antibody fraction eluted from affinity matrix–IgG_s for 60 min at 37°C, so that the molar ratio of both reacting components was 1:1. The proteolytic activity of chymotrypsin after the incubation with antibodies was determined from the rate of peptide cleavage in substrates N-succinyl-L-phenylalanyl-*p*-nitroanilide and hemoglobin according to Nagel et al. [10] and Anson and Mirskij [11].

2.5. Oxidation of the carbohydrate moieties on the Fc fragments of specific anti-chymotrypsin antibodies

The isolated immunoglobulins (≈2 mg) were transfered by gel filtration (Sephadex G-25 Fine) in 0.1 *M* acetate buffer, pH 5.5. Sodium periodate (0.01 *M*) was added and the reaction mixture stirred in the dark for 30 min at 4°C. The oxidation was terminated by pouring the mixture in 20 m*M* ethylene glycol under stirring for 10 min. The conditions for the oxidation of IgG molecules were selected according to the results described by Wolfe and Hage [12].

2.6. Estimation of the immunochemical reactivity of anti-chymotrypsin IgG after the periodate oxidation

The effect of the periodate oxidation on the immunochemical reactivity of anti-chymotrypsin IgG molecules was examined by ELISA. Fractions of native and oxidized immunoglobulins were incubated in microtitre wells precoated with chymotrypsin (40 μg/ml). The quantity of chymotrypsin as an immobilized antigen and the concentration of applied labelled antibody were optimized according to the procedure described by Mančal [13]. The amount of bound antibody was then determined by peroxidase–anti-pig IgG conjugate and *o*-phenylenediamine as the chromogen.

2.7. Preparation of poly(HEMA–co-EDMA) microspheres by dispersion polymerization

Polymerization was carried out in a thermostated 100-ml reaction vessel equipped with an anchor-type stirrer. A 3.2 g amount of cellulose acetate butyrate was dissolved in 68 g of toluene–2-methylpropan-1-ol mixture (weight ratio of toluene to 2-methylpropan-1-ol, 0.9) and 0.24 g of dibenzoyl peroxide dissolved in 11.04 g of HEMA was added. The reaction mixture was stirred for 5 min, and then heated at 70°C under stirring at 500 rpm. A 0.96-g amount of EDMA was added 2 h after the start of the polymerization; the overall polymerization time was 8 h. The resulting microspheres were washed six times with toluene and dried. Particle size was determined by measuring at least 300 particles from photographs taken on scanning electron microscope (JFM 6400, Joel). Two types of mean particle size were calculated: number-average (d_n) and weight-average (d_w), ($d_n = \Sigma d_i / N$, $d_w = \Sigma d_i^4 / \Sigma d_i^3$, where N is the number of particles). Particle size distribution was characterized by polydispersity index (PDI) calculated as a ratio of weight- to number-average particle diameter.

2.8. Preparation of hydrazide-functionalized poly(HEMA–co-EDMA) microspheres

Hydrazidation of particles consisted of two steps. In the first step, poly(HEMA–co-EDMA) microspheres were activated with TCT. To 1 g of poly-(HEMA–co-EDMA) microspheres, 35 ml of acetone (24.5 ml)–water (10.5 ml) mixture were added and the suspension stirred overnight. A 0.145-g amount of 10% NaOH was added and stirring continued at laboratory temperature for 1 h. On addition of acetone solution of TCT (NaOH–TCT 1:1 molar ratio) to the suspension at 0°C, the mixture was allowed to react for 45 min at 0°C. The product was washed three times by centrifugation with 10 ml of acetone cooled to 0°C and three times with 10 ml of ice-cold water.

In the second step, adipic acid dihydrazide (1.15 mol excess relative to TCT used for activation) was reacted with the carrier. Immediately after the activation, the suction-filtered activated poly(HEMA–co-

EDMA) was dispersed in a solution of adipic acid dihydrazide in 20 ml of 0.05 *M* borate buffer (pH 9) and stirred at laboratory temperature for 4 h, while adding 1 *M* NaOH to maintain pH 9. The product was washed with water (eight times, 10 ml each) and freeze-dried. The amount of bound dihydrazide was determined from the nitrogen analysis prior to and after the reaction.

2.9. Preparation of the immunosorbent matrix with oriented chymotryspin

Periodate-oxidized immunoglobulins (0.3 mg/ml) in 0.1 *M* acetate buffer (pH 4.0) containing 0.5 *M* NaCl were stirred with an equal volume of hydrazide-derivatized sorbent (1–1.5 ml of sedimented sorbent). After being stirred for 24 h at 4°C, the sorbent slurry was washed with acetate buffer, 0.1 *M* phosphate buffer (pH 7.2) and finally with phosphate buffer enriched with 0.5 *M* NaCl to remove unbound immunoglobulins. Effectivity of the immobilization of IgG molecules was approximately determined from the difference in absorbance at 280 nm before and after the immobilization and by ELISA. These immobilized antibodies were then used for biospecific adsorption of chymotrypsin. Chymotrypsin (1 mg/ml) in 0.1 *M* phosphate buffer (pH 7.2) was added to the immunosorbent, which was equilibrated with this buffer, and incubated for 1 h. The sorbent slurry was washed with phosphate buffer and phosphate buffer containing 2 *M* NaCl to remove unbound nonspecifically sorbed chymotrypsin.

2.10. Determination of chymotryptic activity

Activity towards N-succinyl-L-phenylalanyl-*p*-nitroanilide, a low-molecular-mass substrate, was determined according to Nagel et al. [10], protease activity with hemoglobin as substrate was assessed by Anson and Mirskij [11].

2.11. Determination of the amount of immobilized protein

The amount of immobilized protein was determined approximately from the difference in absorbance at 280 nm before and after the immobilization. Amino acid analysis in a Durrum D-500 amino acid analyzer was used for precise determination.

3. Results and discussion

With the aim of developing an optimal proteolytical reactor for preparing peptides by chymotryptic cleavage of proteins, oriented immobilization of chymotrypsin by means of suitable antibodies was studied.

The IgG fraction obtained by DEAE-cellulose-based ion-exchange chromatography of pig antiserum after immunization of the animal was subjected to orientedly immobilized chymotrypsin. This biospecific sorbent was prepared by the immobilization of chymotrypsin via its active site to antilysin–cellulose [7] and was used for repeated isolation of required anti-chymotrypsin antibodies. Glycine buffer (0.1 *M*, pH 2.3) containing 0.1 *M* NaCl was used for the elution of these antibodies (Fig. 1).

To confirm that these antibodies do not deactivate chymotrypsin, an inhibition test was carried out. The enzyme was incubated with specific anti-chymotrypsin antibodies IgG_S in 1:1 molar ratio for 60 min at 37°C. The protein content of the solution was calculated from the absorbance at 280 nm measured by a direct spectrophotometric technique. According to the results presented in Table 1, no decrease of proteolytic activity occurred after the incubation of IgG with chymotrypsin. An increased activity of chymotrypsin in complex with its antibodies was even detected, which is in agreement with the literature [3]. With respect to the data cited in Ref. [14], we had to verify in advance that the oxidation by sodium periodate has no influence on binding activity of specific antibodies that should be used for oriented immobilization of chymotrypsin. The antigenic affinity of oxidized anti-chymotrypsin antibodies according to the ELISA test was essentially unchanged by the periodate oxidation of their carbohydrate moieties [7]. These facts confirmed the suitability of these antibodies for preparation of immunosorbent.

In order to eliminate the kinetic limitation of chymotryptic protein hydrolysis, nonporous poly(2-hydroxyethyl methacrylate) microspheres were prepared. Dispersion polymerization is a suitable tech-

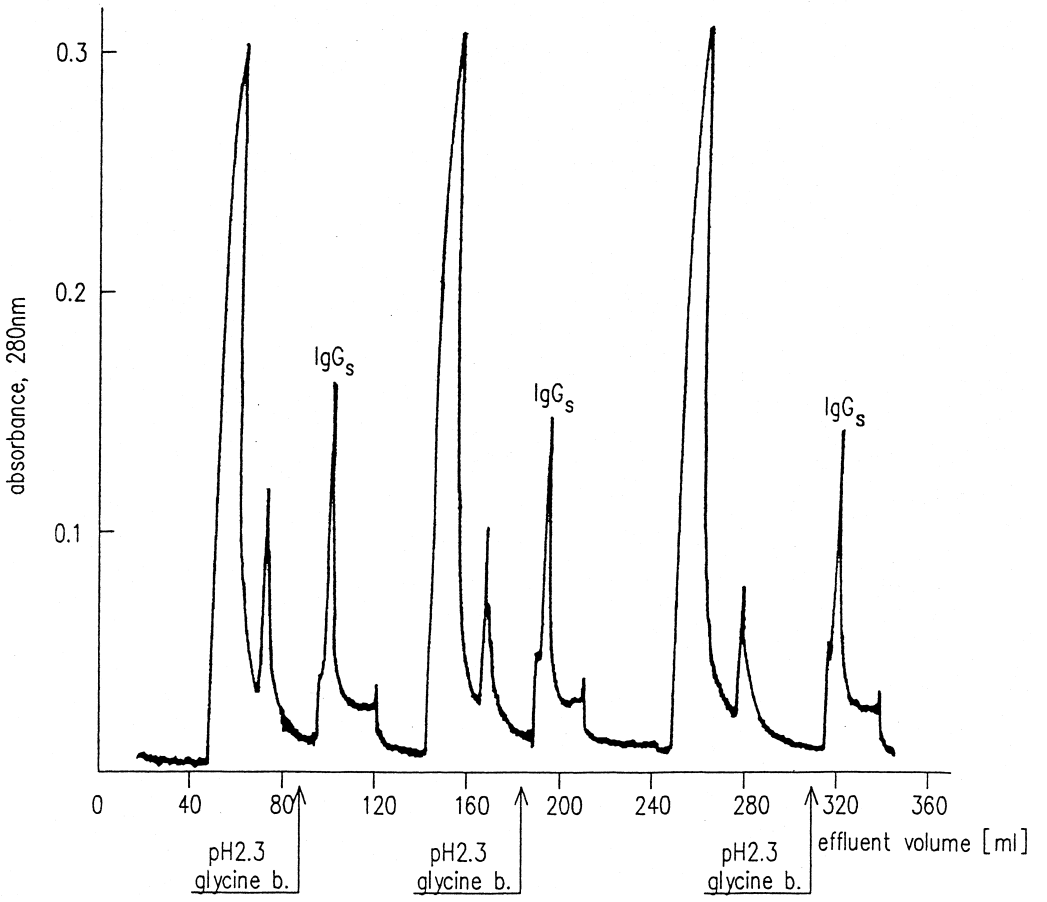

Fig. 1. Isolation of anti-chymotrypsin antibodies by chromatography on a matrix with chymotrypsin immobilized via its active site to antilysin-cellulose. The IgG fraction was injected on the column (10×0.7 cm I.D.) in 0.1 *M* phosphate buffer (pH 7), the non-specifically sorbed molecules of immunoglobulins were removed by phosphate buffer enriched with 0.5 *M* NaCl. Glycine buffer (0.1 *M*, pH 2.3) containing 0.1 *M* NaCl was injected to elute the anti-chymotrypsin IgG_s.

nique to obtain spherical particles of relatively narrow distribution in a micrometer size range [15]. The preparation of hydrophilic polymeric supports, the advantage of which consists in low non-specific adsorption of biologically active compounds, by the dispersion method has been, however, difficult [16]. The design of both the solvent composition and steric stabilizer of the polymerization is very important for achieving narrow particle size distribution. In this report, dispersion polymerization of HEMA in a mixture of toluene and 2-methylpropan-1-ol of a given composition was stabilized using

Table 1
Comparison of the proteolytic activities of chymotrypsin after incubation with various fractions of the anti-chymotrypsin antibodies (molar ratio 1:1)

Relative activity of chymotrypsin (%) after incubation with	Substrate	
	N-Suc-L-Phe-*p*-NA	Hemoglobin
Other anti-chymotrypsin antibodies	98	79
Specific anti-chymotrypsin antibodies (IgG_s)	121	112

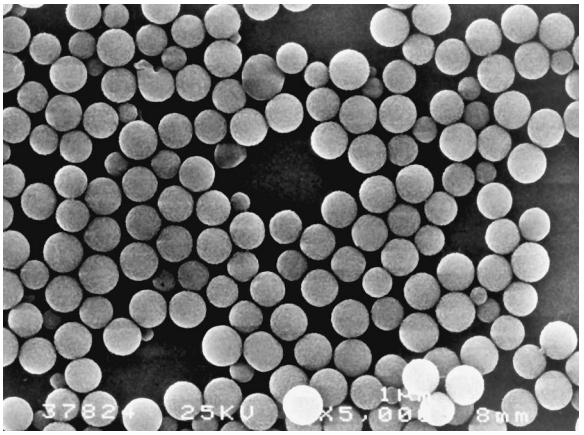

Fig. 2. Scanning electron micrograph of poly(HEMA–co-EDMA) microspheres (8 wt.% EDMA).

cellulose acetate butyrate. Dibenzoyl peroxide was used as an initiator of polymerization. Particles were crosslinked with ethylene dimethacrylate added at a later stage after the start of polymerization in order to prevent an agglomeration. Resulting poly(HEMA–co-EDMA) microspheres (8 wt.% EDMA) had a d_n of 1.2 μm and PDI of 1.1 (Fig. 2). Swellability of the particles was characterized in terms of toluene and water regain [17]. Both media are known to be thermodynamically bad solvents for poly(HEMA). However, regain of water (≈1.5 ml water per gram of polymer) was larger than that of toluene (≈0.7 ml/g) because of solvation of poly(HEMA) chains by water.

In order to obtain a support for immobilization of glycoproteins, poly(HEMA–co-EDMA) particles were modified by adipic acid dihydrazide (AH). The procedure of synthesizing the dihydrazide support is shown in Fig. 3.

The first step of the synthesis involved conversion of poly(HEMA–co-EDMA) into a highly reactive chlorotriazine intermediate. The binding via TCT has been popular since it is hydrolytically more stable in comparison with commercially available sorbents containing amino acids, where activation with cyanogen bromide is used [18]. Activation of poly(HEMA–co-EDMA) microspheres proceeded in an aqueous acetone, where both NaOH and TCT were quite soluble. The resulting intermediate was washed, a small sample withdrawn for elemental analysis, and the rest immediately proceeded to further reaction.

The second step in the synthesis involved a reaction of the intermediate with AH in borate buffer at pH 9. After thorough washing, the product was freeze-dried and the total amount 2 μmol of the hydrazide attached to 1 g of poly(HEMA–co-

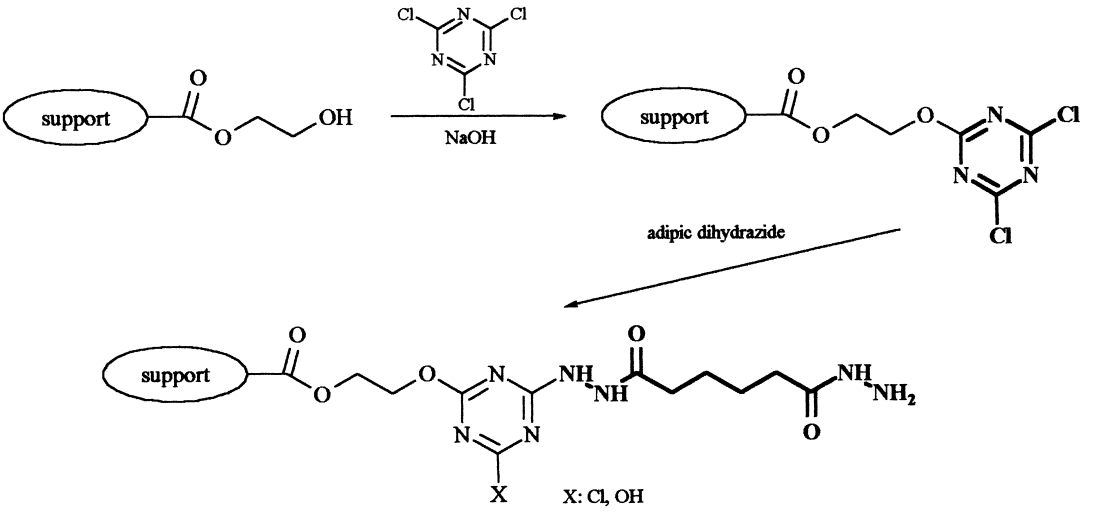

Fig. 3. Synthesis of the dihydrazine support.

EDMA) determined by nitrogen analysis. We suppose that hydrazide groups are exposed predominantly on the surface of microspheres, since it is known that macroporous cellulose beads 100–250 μm in size activated by the same procedures contained much more hydrazide – 12.7 μmol/ml of swollen material, which corresponds to 85 μmol per g of dry product [19]. However, in the latter case, the hydrazide groups were located mainly inside the porous cellulose matrix.

The resulting carrier was used for the attachment of anti-chymotrypsin antibodies. The IgG molecules were coupled through their carbohydrate moieties after the periodate oxidation. Only this way of antibody immobilization ensures steric accessibility of their binding sites. The immobilization procedure, the advantage of which was described by O'Shannessy [20] in 1990, is shown in Fig. 4.

The amount of anti-chymotrypsin amount antibodies used for the immobilization (see Section 2) is sufficient. An increase in the quantity of IgG molecules has no influence on the total amount of the molecules immobilized to the sorbent. The immunoglobulin content was determined by amino acid analysis (4.1 nmol IgG/g of dry carrier). The second amino acid analysis performed after the sorption of chymotrypsin molecules (7.9 nmol chymotrypsin/g of dry carrier) confirmed the molar ratio 1:2 in agreement with the results cited in our previous study [7]. The same molar ratio of immobilized antibody to purified antigen of 1:2 was also observed by Domen et al. [21].

The low-molecular-mass substrate (N-succinyl-L-phenylalanyl-*p*-nitroanilide) and the high-molecular-mass substrate (hemoglobin) were used for the determination of the proteolytic activity of the immobilized chymotrypsin in a batch experiment. The found activity – 166.7 μg of chymotrypsin per 1 g of dry carrier – corresponded to the amount of immobilized chymotrypsin detected by amino acid analysis. It can be therefore concluded that the enzymatic activity of chymotrypsin remained unchanged after the immobilization.

In comparison with directly immobilized proteolytic enzyme on the carrier, a biospecific matrix involving a complex between enzyme and specific antibodies, which was developed in this study, has certain advantages. Chymotrypsin in a complex with some specific antibodies displays a higher proteolytic activity and stability (Table 1). The active sites of all chymotrypsin molecules are highly sterically accessible even for the high-molecular-mass protein. This is due to chymotrypsin orientedly immobilized by specific antibodies with affinity for epitopic regions other than the active site of the enzyme. In addition, it is due to the ability of IgG molecules to operate as spacer arms (Fig. 5). However, to achieve such behaviour, digestion of proteins

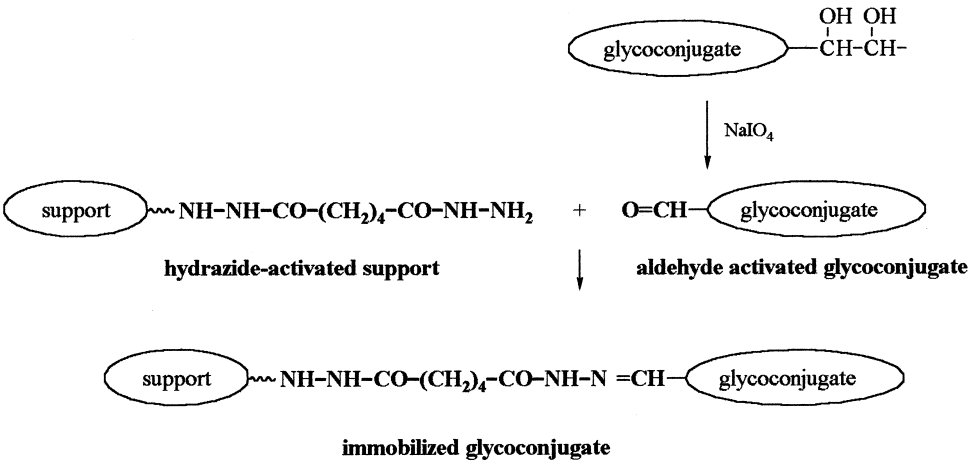

Fig. 4. Immobilization procedure.

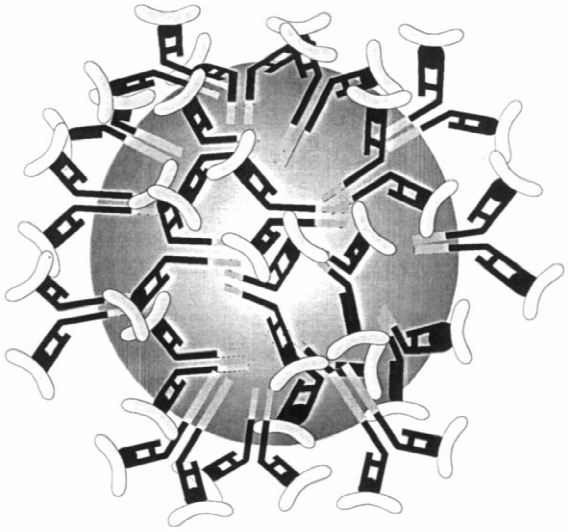

Fig. 5. Schematic representation of the orientedly immobilized chymotrypsin on the immunosorbent.

must be performed in a batch experiment. Thanks to the specific interaction between the immunosorbent and chymotrypsin molecules, there are no high demands on the purity of the applied enzyme. The oriented immobilization and isolation can be thus carried out in a single step.

4. Conclusion

Existing sorbents for enzyme immobilization are primarily prepared by the suspension polymerization methods. Newly developed dispersion polymerization technique offers a cheap alternative, the main advantage of which consists in the possibility to obtain narrow size distribution of polymeric particles in the micrometer size range in a single step. Proteolytic reactor with a good steric accessibility of active sites of chymotrypsin molecules, especially for high-molecular-mass substrates, an increased stability, and a minimal kinetic limitation was prepared. For these purposes, the polyclonal antibodies were isolated on the biospecific sorbent prepared by the attachment of chymotrypsin through its active site to its immobilized natural inhibitor. In order to eliminate the kinetic limitation of catalysis, nonporous poly(2-hydroxyethyl methacrylate) microspheres prepared by dispersion polymerization were used. Because digestion of proteins is usually done in batch experiments, steric accessibility of active sites of immobilized chymotrypsin is practically the same as that of non-immobilized enzymes in solution. Chromatographic matrix containing oriented complex between enzyme and immunosorbent can be applied in reproducible hydrolysis of analysed protein by peptide mapping.

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic 203/96/0124, 203/98/0885, 203/98/1231 and by VS-96058 MŠMT ČR.

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