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Purification of anti-chymotrypsin antibodies for the preparation of a bioaffinity matrix with oriented chymotrypsin as immobilized ligand

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Abstract

Polyclonal antibodies suitable for the oriented immobilization of chymotrypsin were prepared by chromatography on a bioaffinity matrix which had the enzyme immobilized through its active site to antilysin, covalently linked to bead cellulose. After periodate oxidation of their carbohydrate moieties, the isolated antibodies were coupled to a hydrazide derivative of bead cellulose. The periodate oxidation step, which led to greater efficiency and stability of the immunosorbent, had no deleterious effect on antibody activity as assessed by ELISA. Addition of chymotrypsin to the immunosorbent yielded an enzymically active bioaffinity matrix with the optimum molar enzyme/antibody ratio of 2.

Keywords: Antibodies, anti-chymotrypsin; Chymotrypsin; Enzymes; Polyclonal antibodies

1. Introduction

Procedures for micro-scale analysis of proteins by peptide mapping have been developed which allow peptide maps to be obtained from picomole to femtomole quantities of proteins [1]. Peptide maps, obtained by enzymatic cleavage of proteins using immobilized proteinases, can be employed for a range of purposes: (a) detection of pathological changes of proteins occurring in physiological fluids in submicrogram amounts, (b) detection of post-translational protein modification, (c) identification and localization of genetic variants and (d) quality control and monitoring of genetically engineered protein products. As little as 50 ng of protein may be digested reproducibly by means of chromatography on a small reactor column containing trypsin immobilized on agarose gel [1]. However, the economic feasibility of the process depends upon a combination of the efficiency and stability of the immobilized proteinase matrix. The existence of several complementary sites on the surface of enzymes enables the formation of different biospecific complexes that can be used advantageously, not only for efficient enzyme isolation but also for oriented immobilization of the enzyme on solid supports.

Because the interaction of antibodies with suitable antigenic sites of proteinases does not change their proteolytic activity, the advantages of oriented immobilization of proteinases by reaction with an immunosorbent are (i) the good steric accessibility of the enzyme active site for high-molecular-mass

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substrates and (ii) the increased thermostability of the resulting enzymically active affinity matrix [2–4]. Immobilization of antibodies via the conserved carbohydrate moieties located in the Fc fragment of IgG [5] has been found to increase the steric accessibility of the immunospecific sites on the Fab fragments. Hydrazido-derivatized matrices are the usual solid supports for this immobilization of antibodies to prepare the immunosorbent [6]. By way of illustration, we cite a previous study [7] in which hydrazido-derivatized bead cellulose was used to immobilize anti-ovalbumin antibodies against epitopes on the protein part of ovalbumin, after which ovalbumin was reacted with this immunosorbent to provide an affinity matrix for the isolation of concanavalin A via its interaction with the carbohydrate region of ovalbumin. The fact that concanavalin A interacted with the anti-ovalbumin antibodies in solution, but not with the immunosorbent, may be considered as evidence that the anti-ovalbumin antibodies had been immobilized via their Fc region, thereby rendering their glycosylation sites inaccessible to the lectin.

On the basis of the X-ray crystallographic structure of the complex between lysozyme and the Fab fragment of an elicited antibody [8], it has been concluded that much greater areas of complementary surfaces are brought into contact with each other in immunospecific complex formation involving a protein antigen, rather than a small hapten. The consequent exclusion of water molecules from the contact region leads to greater stability of the antigen–antibody complex by decreasing the destabilizing effect of water contact with nonpolar clusters located on the surface of the protein molecule.

The aim of the present investigation is the preparation of a biospecific affinity matrix with enzymically active chymotrypsin as the immobilized ligand. In order to obtain a matrix with the desired efficiency and stability, the plan of attack has been to isolate anti-chymotrypsin antibodies with affinity for epitopic regions other than the active site of the enzyme; and to couple those antibodies to bead cellulose to form a biospecific immunosorbent for chymotrypsin. For the reasons described above, such oriented complex formation between enzyme and immunosorbent should result in an immobilized proteinase matrix suitable for microscale analysis of proteins by chymotryptic peptide mapping.

2. Experimental

2.1. Chemicals

Chymotrypsin (EC 3.4.21.1), anti-pig IgG, 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 from EXBIO (Prague, Czech Republic), and hemoglobin from Serva (Heidelberg, Germany). North Bohemian Chemical Works (Lovosice, Czech Republic) provided the macroporous bead cellulose Perloza MT 200 (particle size 80–100 μm); and the hydrazide derivative thereof was kindly donated by Dr. M.J. Beneš from the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic. Fluka (Buchs, Switzerland) was the source of cyanuric chloride (2,4,6-trichloro-1,3,5-triazine), and Reanal (Budapest, Hungary) the supplier of sodium periodate. Other chemicals were of reagent grade.

2.2. Isolation of antibodies

The source of anti-chymotrypsin antibodies was serum from pigs that had been immunized according to standard protocol [9]. After gel filtration on Sephadex G-25, pig antiserum (2 ml) was applied to a column (10×1 cm I.D.) of DEAE–cellulose (Whatman) equilibrated at 0.6 ml/min with 0.015 *M* potassium phosphate buffer (pH 8). Elution of the column with this medium removed the immunoglobulins (IgG), after which 0.3 *M* phosphate buffer (pH 8) was applied to elute the remaining serum proteins (Fig. 1).

2.3. Identification of antibodies

The presence and purity of antibodies were tested by double immunodiffusion [10] and by immunoelectrophoresis [11]. Turbidometric analysis with poly(ethylene glycol) [12] was used to assay the IgG

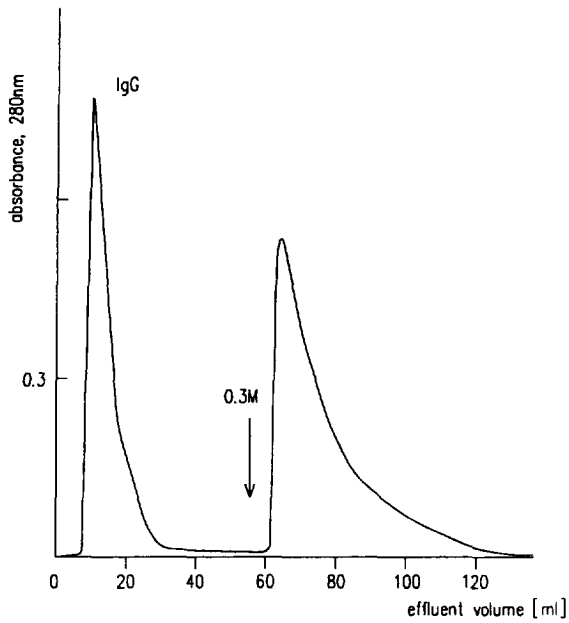

Fig. 1. Isolation of immunoglobulins from pig antiserum by ion-exchange chromatography. Antiserum (2 ml) was applied to a column (10×1 cm I.D.) of DEAE–cellulose and the IgG fraction eluted with 0.015 *M* potassium phosphate buffer (pH 8.0). The arrow indicates the stages at which more concentrated phosphate buffer (0.3 *M*) was applied to elute the remaining serum proteins. Flow-rate: 0.6 ml/min.

contents of eluted fractions from the chromatographic procedures.

2.4. Preparation of affinity sorbent for the isolation of antibodies

Antilysin was attached to Perloza MT 200 that had been activated with 2,4,6-trichloro-1,3,5-triazine [13]. The remaining active groups were blocked with 0.1 *M* 2-aminoethanol. The amount of coupled antilysin was 6 μmol/g dry cellulose. After equilibration of a column (10×0.7 cm I.D.) of antilysin–cellulose with 0.1 *M* phosphate buffer (pH 7.2), chymotrypsin (40 mg) was applied in the same buffer. After washing, the sorbent was crosslinked by glutaraldehyde (0.25% in 0.1 *M* phosphate buffer, pH 7.0) for 8 h. The remaining active groups were blocked with 0.2 *M* Tris–HCl buffer (pH 8.3).

2.5. Purification of anti-chymotrypsin IgG

The IgG fraction eluted from DEAE–cellulose (8 ml) was applied to the above column of chymotrypsin immobilized through its active site to antilysin–cellulose, which had been pre-equilibrated at 0.6 ml/min with 0.1 *M* phosphate buffer, pH 7.0. After washing the column with the equilibration buffer and the same buffer containing 0.5 *M* NaCl to remove other antibodies, elution with 0.1 *M* glycine buffer (pH 2.3) containing 0.1 *M* NaCl was used to release the anti-chymotrypsin IgG. Eluted fractions were adjusted immediately to pH 7 by titration with 0.1 *M* NaOH.

2.6. Oxidation of the carbohydrate moieties of antibodies

The isolated immunoglobulin (12 mg) was dissolved in 0.1 *M* acetate buffer, pH 5.5 (20 ml), after which sodium periodate (0.1 *M*, 2 ml) was added and the reaction mixture stirred in the dark at 4°C for 30 min. The oxidation was terminated by rendering the mixture 20 m*M* in ethylene glycol and stirring for 10 min [14]. Low-molecular-mass components were then removed by gel permeation on a column (10×1.5 cm I.D.) of Sephadex G-25 Fine equilibrated with 0.1 *M* acetate buffer (pH 4.8) containing 0.5 *M* NaCl, after which the oxidized antibodies were immobilized on bead cellulose with adipic acid dihydrazide.

2.7. Preparation of the immunosorbent matrix with oriented chymotrypsin as immobilized ligand

Periodate-oxidized immunoglobulins (0.6 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 bead cellulose [15]. After being stirred for 24 h at 4°C, the sorbent slurry was washed with acetate buffer to remove unbound immunoglobulins, this washing procedure being continued until the absorbance of the supernatant at 280 nm had decreased to zero. These immobilized antibodies were then used for biospecific adsorption of chymotrypsin (1 mg/ml) in 0.1 *M* phosphate buffer (pH 7.2).

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

The effect of periodate oxidation on the immunochemical reactivity of anti-chymotrypsin IgG was examined by ELISA [16]. Fractions of the native and oxidized immunoglobins were incubated in microtitre wells precoated with chymotrypsin (25 mg/ml), after which, bound antibody was measured using a peroxidase-anti-pig IgG conjugate and *o*-phenylenediamine as the enzyme substrate.

2.9. Determination of chymotryptic activity

Activity towards N-succinyl-L-phenylalanyl-*p*-nitroanilide, a low-molecular-mass substrate, was determined according to Nagel et al. [16], whereas protease activity was assessed by the Anson and Mirskij procedure [17], with hemoglobin as substrate.

2.10. Determination of the amount of immobilized protein

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

3. Results and discussion

An affinity matrix, with enzymatically active chymotrypsin as the immobilized ligand, is easily prepared by the use of an immunosorbent in which the anti-chymotrypsin antibodies are directed towards epitopic regions of the enzyme that do not include its active site. Such antibodies may be isolated by biospecific affinity chromatography of a mixed IgG population on a matrix with chymotrypsin immobilized in a single orientation by virtue of its covalent attachment to the support (bead cellulose) through its active site. The upper panel of Fig. 2 illustrates such immobilization of chymotrypsin by means of antilysin (pancreatic trypsin inhibitor) and subsequent stabilization of the affinity matrix by crosslinking with glutaraldehyde. The lower panel summarizes the isolation of the required anti-chymotrypsin antibodies by chromatography on the resulting column. Preparation of the proposed affinity matrix by the suggested protocol yielded a column

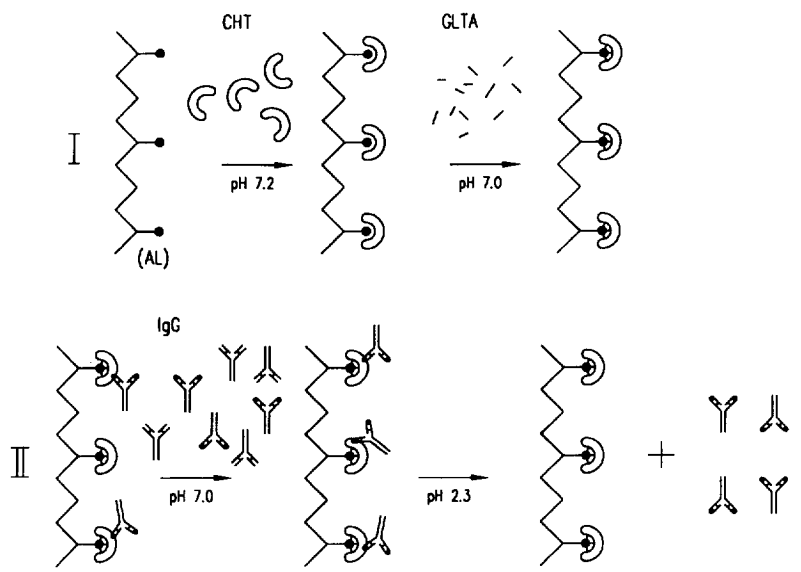

Fig. 2. Schematic representations of (I) the oriented immobilization of chymotrypsin by covalent coupling of its active site to antilysin–cellulose and subsequent crosslinking of the affinity matrix with glutaraldehyde; and (II) the use of this chromatographic matrix for the isolation of anti-chymotrypsin IgG with affinity for epitopic regions away from the enzyme active site.

from which no chymotrypsin could be eluted, thereby confirming the success of its covalent attachment to the antilysin–cellulose. Furthermore, the failure to detect any chymotryptic activity towards N-succinyl-L-phenylalanyl-*p*-nitroanilide showed that the enzyme was immobilized via its active site.

To confirm the feasibility of the second stage of the proposed protocol (Fig. 2), the immunoglobulin fraction obtained by DEAE–cellulose chromatography of pig antiserum reflecting chymotrypsin immunization of the animal (Fig. 1), was subjected to affinity chromatography (Fig. 3). Most of the applied material was eluted either by the buffer used for its application (0.1 *M* phosphate buffer, pH 7, the first peak) or by the same buffer supplemented with 0.5 *M* NaCl (the second peak). Elution with 0.1 *M* glycine buffer (pH 2.3) containing 0.1 *M* NaCl was used to elute the anti-chymotrypsin IgG antibodies. The failure of these antibodies to interfere with the proteolytic activity of chymotrypsin was established by examining the rate of peptide cleavage in hemoglobin [17] by an enzyme that had been incubated with the anti-chymotrypsin antibody fraction for 30 min. Essentially, no decrease in proteolytic activity resulted from incubation of chymotrypsin with specific anti-chymotrypsin antibodies IgG in a 1:1 molar ratio (Table 1). These isolated antibodies are thus suited for the preparation of a biospecific affinity matrix bearing immobilized chymotrypsin in orientations that allow protein molecules ready access to its active site.

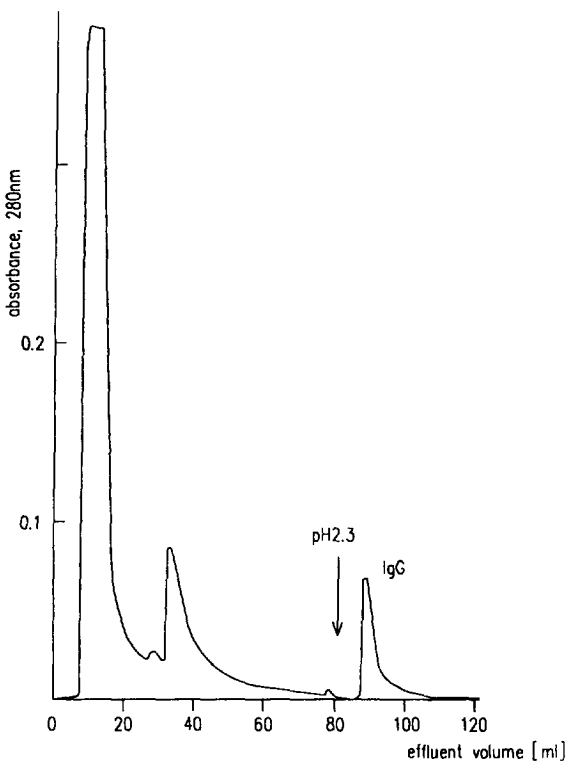

Fig. 3. Isolation of anti-chymotrypsin antibodies by chromatography on a matrix comprosed of chymotrypsin immobilized via its active site to antilysin–cellulose. The IgG fraction obtained by ion-exchange chromatography (Fig. 1) was applied to the column (10×0.7 cm I.D.) in 0.1 *M* phosphate buffer (pH 7), and the anti-chymotrypsin antibodies were eluted with 0.1 *M* glycine buffer (pH 2.3) containing 0.1 *M* NaCl. Flow-rate: 0.6 ml/min.

With respect to generating the proposed chromatographic matrix for preparing peptides by chymotryptic cleavage of proteins, the way to create the specific immunosorbent for the enzyme is to prepare a chromatographic support with the above antibodies covalently attached via the glycosylation sites of the Fc fragment, because such immobilization allows greater steric accessibility of the immunospecific Fab sites [5]. Periodate oxidation of these glycosylation sites results in the generation of reactive aldehydes which can interact with nucleophils such as hydrazides. On the grounds that the antigenic affinity of the anti-chymotrypsin antibodies was essentially unchanged by periodate oxidation of their carbohydrate moieties (Fig. 4), this procedure was used to covalently attach the isolated anti-chymotrypsin antibodies to the hydrazide derivative of bead cellulose.

Periodate-oxidized anti-chymotrypsin antibodies were therefore immobilized on bead cellulose by this means to give an immunosorbent with 2.06 μmol/ml of sedimented cellulose. The immunoglobulin content was determined by amino acid analysis. The

Table 1
Comparison of the activities of chymotrypsin after incubation with antibodies (molar ratio 1:1)

Relative activity of the enzyme	Substrate[a] (%)	Hemoglobin (%)
After incubation with IgG (1st and 2nd peak in Fig. 3)	106	74
After incubation with specific anti-chymotrypsin IgG antibodies	115	101

[a] N-succinyl-L-phenylalanyl-*p*-nitroanilide.

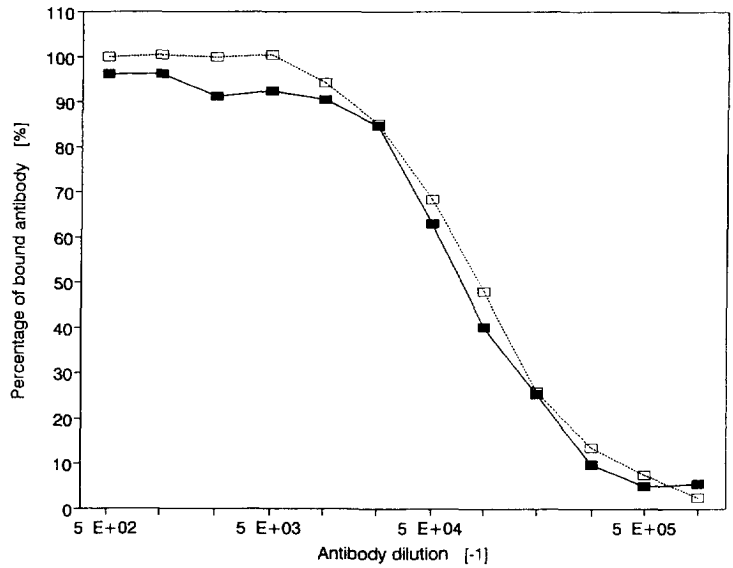

Fig. 4. Comparative ELISA study of the interactions of chymotrypsin with native (□) and periodate-oxidized (■) anti-chymotrypsin IgG. The extent of binding is expressed relative to the maximal immunoglobulin capacity for chymotrypsin.

biospecific affinity matrix resulting from biospecific adsorption of chymotrypsin, exhibited hydrolytic activity towards hemoglobin as well as low-molecular-mass substrates. Quantitative assessment of the hydrolysis of N-succinyl-L-phenylalanyl-*p*-nitroanilide yielded a value of 4.05 μmol/ml of sedimented cellulose for the concentration of immobilized chymotrypsin. The molar ratio of biospecifically adsorbed chymotrypsin molecules to immobilized antibody is thus 2:1, the value predicted for enzyme occupancy of the site of each of the two Fab fragments in IgG.

4. Conclusion

The selective isolation of anti-chymotrypsin antibodies with affinity for epitopic regions of the enzyme, other than the active site by means of a chromatographic support with chymotrypsin immobilized via its active site, has made possible the production of an immunosorbent for biospecific adsorption of chymotrypsin in such a manner that its enzymatic activity is retained. This development of a chromatographic matrix, involving oriented complex formation between enzyme and immunosorbent, is important in the context of microscale analysis of proteins by chymotryptic peptide mapping.

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