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Rapid purification of immunoglobulin G using aza-arenophilic chromatography: novel mode of protein–solid phase interactions

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Abstract

A derivative of aza-arenophilic gel having a dichlorosubstituent and an hydroxy ion as a capping nucleophile has been prepared. The properties of this gel in relation to IgG purification have been investigated in details. In the presence of high salt (1.5 *M*), albumin and some other serum proteins did not bind to the gel. IgG and some other minor proteins, however, were bound to the gel. The bound proteins can be eluted with an acidic buffer. SDS-PAGE showed that the fraction eluted with 0.1 *M* sodium acetate pH 4.2 consisted mainly of IgGs.

1. Introduction

The purification of antibodies for both therapeutic and diagnostic applications remains one of the major down-stream bioprocessing activities in biotechnology. Protein A, a bacterial surface protein having high selectivity and affinity for the conserved regions in the Fc part of some antibodies has been extensively used in the affinity chromatographic purification of antibodies [1]. However the weak binding affinity of Protein A toward IgGs from goat, sheep, rat and mouse makes it impractical as an affinity chromatographic ligand [1–3]. The desire to have an affinity ligand with a selectivity broader than that of Protein A, with a lower cost and a greater stability has stimulated the development of a number of synthetic affinity ligands useful for affinity purification of IgGs [4–11].

Porath et al. [4,5] discovered that an immobilized nonionic sulfone-thioether ligand of the structure $CH_2CH_2SO_2CH_2CH_2SCH_2CH_2OH$ in the presence of "water-structuring" salts exhibits unusually high selectivity and affinity in binding IgGs but shows practically no affinity toward albumin. The bound IgGs were removed from the gel by decreasing the concentration of the "water-structuring" salts. The term "thiophilic" was used to describe such an interaction. A one-step procedure for the purification of monoclonal antibodies using "thiophilic" gel has been described [12].

Vijayalakshmi [6,7] showed that immobilized histidyl residues can be used as universal pseudobiospecific affinity ligands for the purification of IgGs. In general the antibodies must be in a low ionic strength buffer at a pH near the isoelectric point of the antibodies for binding to the im-

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mobilized histidine gel. The bound IgGs were eluted by using a salt gradient of increasing ionic strength. Different subclasses of IgGs were eluted at different salt concentrations. In addition to its use in IgG purification, histidyl gel was also used in the purification of factor VIII and the removal of pyrogen [7,13,14].

Ngo et al. [8–11] described the purification of IgG from a variety of sera and ascites fluids by a pseudo-affinity chromatography on an immobilized pyridinium gel. The exquisite selectivity and high affinity of the interaction between the immobilized pyridinium ligands and proteins, particularly with IgGs, are rather unexpected. This newly recognized protein–ligand interaction is termed "aza-arenophilic", where aza stands for nitrogen and areno for aromatic compounds [11]. Proteins can be adsorbed onto aza-arenophilic gel under a wide range of conditions and the bound proteins can be eluted with high salt, low pH or buffer containing electron rich compounds [10]. Immunoglobulins from several animal species have been purified to a high degree of homogeneity. In this communication the purification of IgG from goat serum using a derivative of aza-arenophilic gel having a hydroxyl substituent (Fig. 1) will be presented. A major objective of this work is to point out the versatility and utility of an affinity ligand having two pyridine rings with the proposed structure shown in Fig. 1 in protein purification. A proposed mechanism for the protein–immobilized pyridinium ligands interaction is presented and discussed.

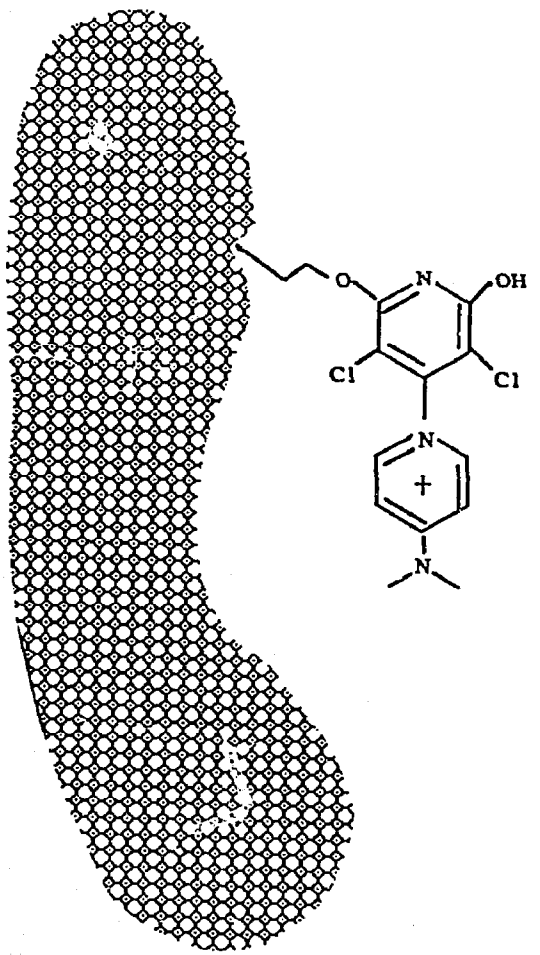

Fig. 1. Proposed structure for an aza-arenophilic gel having a dichloro-substituent and hydroxy ion as a capping nucleophile.

2. Experimental

2.1. Chemicals

3,5-Dichloro-2,4,6-trifluoropyridine (DCTFP) and 4-dimethylanimopyridine (DMAP) were from Aldrich (Milwaukee, WI, USA). Sepharose Cl-4B and gradient polyacrylamide gels for electrophoretic analysis were from Pharmacia (Piscataway, NJ, USA). HPLC-grade solvents, N,N'-dimethylformamide and acetone were from J.T. Baker (Phillipsburg, NJ, USA); human serum and goat serum were from Sigma (St. Louis, MO, USA).

2.2. Preparation of the affinity gel (DCTFP-OH Gel)

Sepharose Cl-4B (50 ml) was washed with 5 × 100 ml of distilled water and then suspended in 50 ml of distilled water in a large 1-l beaker mounted on a shaker rotating at *ca.* 70 rpm. To the gel suspension 750 ml of dry acetone was added gradually over 30 min. The gel was filtered and resuspended in 750 ml of dry acetone and was agitated for 15 min. The gel was filtered

and resuspended in 300 ml of dry N,N'-dimethylformamide (DMF) and after shaking the gel for 5 min the gel was filtered and suspended in 50 ml of DMF containing 14 mmol DMAP. To the gel suspension was immediately added 150 ml of DMF containing 13 mmol of DCTFP. The gel was tumbled at room temperature for 2 h. Then the gel was washed with 1 l of DMF, 2 × 1 l of acetone, 1 l of distilled water and 1 l of 0.1 *M* sodium bicarbonate. The washed activated gel was suspended in an equal volume of 0.1 *M* NaOH and tumbled at room temperature for 24 h. The gel was then washed with 1 l of 0.1 *M* sodium bicarbonate and resuspended in 0.1 *M* NaOH and tumbled for an additional 4 h at room temperature. The gel was finally washed with 1 l of distilled water, 1 l of 1 *M* NaCl, 1 l of distilled water and 1 l of PBS. The gel was stored in phosphate buffer saline (PBS) at 4°C.

2.3. Chromatographic procedures

A volume (3 ml) of packed gel in a 0.8 × 9 cm column was washed with 20 ml containing 1.5 *M* NaCl. A 5-fold diluted goat serum (1 ml serum and 4 ml PBS containing 1.5 *M* NaCl) was applied to the column and a flow-rate of 0.25 ml/min was maintained by means of a peristaltic pump. After all the diluted serum has passed through the packed gel, the column was washed with the same buffer at a higher flow-rate of 1.0 ml/min until the background absorbance was low. Most of the bound proteins, mainly antibodies, were eluted with 0.1 *M* sodium acetate pH 4.2 (adjusted with conc. HCl). The column was cleansed with 0.1 *M* glycine pH 3 and 10% methanol solution.

2.4. SDS-gradient polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic analyses of various protein fractions were performed on 10–15% polyacrylamide gradient gels on a Pharmacia Phast system. The gels were stained with Coomassie brilliant blue.

2.5. Elemental analysis

The elemental analysis of the N and Cl contents of the gel was performed by Galbraith Laboratory (Knoxville, TN, USA).

3. Results

A reaction sequence for the formation of aza-arenophilic gels has previously been proposed [8,11]. By analogy, the aza-arenophilic gel described here was formed by the reaction of the hydroxyl groups of the gel with the condensation product of DCTFP and DMAP and hydroxy ions. The proposed structure of the resulting gel is shown in Fig. 1. The elemental analysis performed on the gel gave a N:Cl ratio of approximately 3:2. This ratio is consistent with the N and Cl contents of the proposed ligand structure. From the nitrogen contents of the gel, the ligand density was calculated to be *ca.* 1.1 mmol/g dry gel.

The chromatographic profile of goat serum diluted with PBS containing 1.5 *M* NaCl on a column of the aza-arenophilic gel is shown in Fig. 2. The profile is typical of affinity chromatography. A large quantity of proteins (e.g. serum albumin) which did not bind to the gel in spite of the presence of a high concentration of salt (1.5 *M* NaCl) was collected in the first peak. The second major protein fraction was obtained by eluting with an acidic buffer (0.1 *M* sodium acetate pH 4.2). This fraction contained mainly immunoglobulins. The gel was regenerated by treating the gel with 0.1 *M* glycine-HCl buffer pH 3. A small amount of proteins was eluted by this treatment.

The identity of proteins contained in selected fractions of the first and second peaks were analyzed by SDS-PAGE (Fig. 3). The electrophoresis of fractions in the first major peak, i.e. the flow-through fraction, indicated that the major protein was albumin (see lanes 3 and 4 in Fig. 3). This observation was rather unexpected because in the presence of a high concentration of salt one would expect a tendency of albumin to bind to the gel via a hydrophobic type of

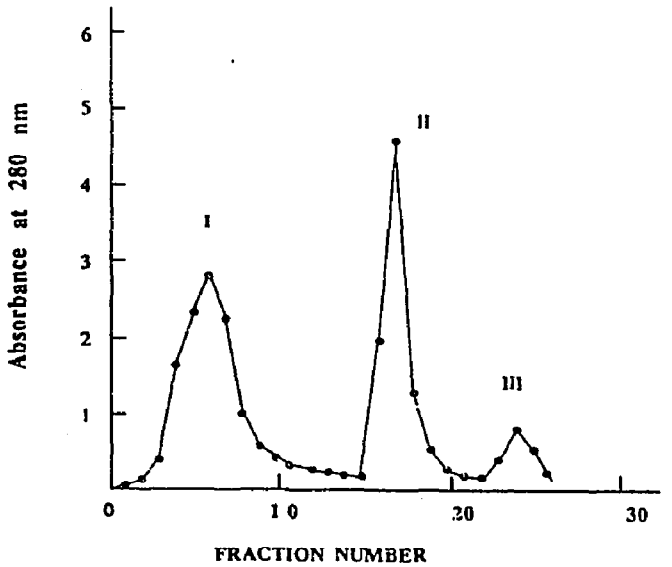

Fig. 2. Purification of goat IgG using an aza-arenophilic gel. Goat serum was diluted with 20 m*M* phosphate buffer pH 7.4 containing 1.5 *M* NaCl. The diluted serum was applied at a flow-rate of 0.25 ml/min at room temperature and fractions of 3 ml were collected. *Peaks*: I = non-adsorbed proteins, II = proteins desorbed by 0.1 *M* sodium acetate pH 4.2, III = proteins desorbed by 0.1 *M* glycine, pH 3.

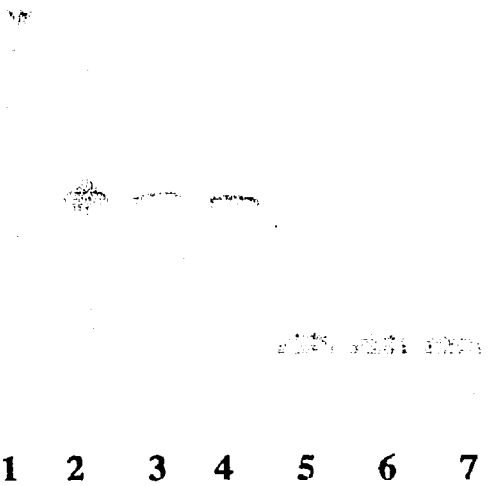

Fig. 3. Non-reducing SDS-polyacrylamide gradient gel (10–15%) electrophoretic analysis of serum proteins fractionated on aza-arenophilic gel. Lane 1 = molecular mass markers; lane 2 = unfractionated diluted serum; lanes 3–4 = un-adsorbed, flow-through fractions, and lanes 5–7 = fractions eluted with acidic buffer (mainly IgGs). Proteins at the top of the figure are of smaller molecular mass. IgG (molecular mass of 160 000 Da) remains close to the origin at the bottom of the figure.

interaction. The electrophoresis of the second fraction showed that immunoglobulins were the major protein components.

4. Discussion

A series of pseudo-biospecific affinity gels called aza-arenophilic gels have been prepared by reacting a hydrophilic gel such as Sepharose with an adduct of pentahalopyridine and 4-dimethylaminopyridine and subsequently capping the remaining reactive halogen with nucleophiles such as mercaptans, amines or hydroxyl ions [8–11]. All of these aza-arenophilic gels have a high affinity for protein binding. The selectivity of the gels can be altered by changing the nature of the halogen or the capping nucleophiles. Aza-arenophilic gels with fluoro-substituents and ethylene glycol as the capping nucleophile adsorb almost all serum proteins under low ionic conditions. Under high salt conditions only immunoglobulins and some minor proteins are retained [9]. Another aza-arenophilic gel with chloro-substituents and mercaptoethanol as the capping nucleophile was shown to be an effective affinity gel for the purification of IgG from serum diluted with PBS (0.15 *M* NaCl) [10]. The bound IgG can be eluted with an acidic buffer or with a neutral buffer (pH around 7) containing "electron-rich" compounds such as triethylamine, acetonitrile, mercaptoglycerol or dithiothreitol [10,11].

In this communication it is shown that another derivative of aza-arenophilic gels, i.e. one with dichloro-substituents and simple hydroxyl ions as the capping nucleophiles (Fig. 1), can be used for purifying IgG from goat serum in an affinity chromatographic mode provided the serum is diluted approximately 5-fold with PBS containing 1.5 *M* NaCl (Fig. 2). The purity of the resulting IgG preparation was high as judged by SDS-PAGE (Fig. 3).

The mechanism of protein adsorption to aza-

arenophilic gels has not been fully understood. Since some proteins are bound to the gel at high salt concentration, e.g. IgG, and others are bound only at low salt concentration, e.g. albumin, it is clear that ionic and conventional hydrophobic interactions are not likely to be the dominant forces in the binding of proteins to an aza-arenophilic gel [12]. The absence of a sulfur atom in the gel used in this study negates the operation of a "thiophilic" interaction as proposed by Porath et al. [4,5]. It is known, however, that pyridinium serves as an electron acceptor in charge-transfer interaction [15–20]. The "electron donor" candidates in proteins are tryptophan, tyrosine and phenylalanine [30]. 1-Benzyl-3-carbozamide-pyridinium chloride readily forms charge transfer complexes with tryptophan in an aqueous system [15–18]. The quaternary nitrogen of a pyridinium can potentially form an ion–dipole interaction between the positively charged quaternary nitrogen of the pyridinium ring and the electron-rich aromatic rings of the protein. This kind of interaction was demonstrated in a model system using ethenoanthracene based macrocyclic cyclophane and an ammonium ion [22,23]. In biological systems, the receptors of quaternary ammonium ligands such as acetylcholinesterase and antibody to phosphocholine were shown to consist primarily of aromatic amino acid residues [24–27]. The tendency of the positively charged amino groups of lysine, arginine, asparagine, glutamine and histidine to make Van der Waals contacts with the π-electrons of the amino acid residues is well documented [28]. It appears that the mechanism of protein binding to the aza-arenophilic gel with a structure shown in Fig. 1 may involve cation–π interaction as suggested by Dougherty and Stauffer [23] in their model studies and their analysis of binding site structure of natural quaternary amine receptors. Furthermore the pyridinium moiety of the affinity ligands can serve as excellent electron acceptor in the formation of charge transfer complexes with aromatic amino acid residues of proteins acting as electron donors. Protein adsorption by charge transfer interactions has been proposed and discussed extensively by Porath as a tool for protein purification [29,30]. Further studies on the behavior of protein adsorption on aza-arenophilic gel with different proteins having well defined structures and with synthetic peptides should provide more information on the mechanism of protein binding to this uniquely interesting matrix. It is highly desirable to synthesize and fully characterize the proposed affinity ligand first and then attach the ligand to a solid phase. The affinity gel described here is suitable for protein and IgG purification in the presence of high salt. If PBS is to be used in the IgG purification, then the aza-arenophilic gel with 2-mercaptoethanol as the capping nucleophilic substituent should be used.

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