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Chemistry and preparation of affinity ligands useful in immunoglobulin isolation and serum protein separation

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ABSTRACT

A number of synthetic affinity gels having high affinity for immunoglobulins and albumin have been prepared by first reacting hydroxyl groups of a polymer with pentafluoropyridine and 4-dimethylaminopyridine in an anhydrous polar organic solvent and then reacting the gel further with nucleophiles such as ethyleneglycol or glycine in basic aqueous solutions. Immunoglobulins can be adsorbed to the gel in either high-salt or low-salt buffers, while albumin can only be adsorbed under low-salt conditions. The identity of the eluted proteins was analyzed by gradient polyacrylamide gel electrophoresis and enzyme-linked immunosorbent assay techniques. Human, goat, mouse and rabbit serum proteins were fractionated on these gels by using different adsorption and desorption conditions. The possible structures of the ligand are discussed. The results showed that the chromatographic behavior of these new gels with synthetic, low-molecular-weight ligands was remarkably similar to that of the more complex immunoglobulin binding gel such as immobilized Protein A or Protein G.

INTRODUCTION

The interaction between specific sites of a protein and a solid matrix forms the basis of protien separation by adsorption chromatography. Known mechanisms for interaction between sites on a protein molecule and ligand sites of the chromatographic matrix may involve ionic, hydrophobic, hydrogen bonding, Van der Waals, charge-transfer, covalent or salt-promoted interaction[1,2]. These interactions may operate singly or, alternatively, two or more of them may operate simultaneously to give rise to a so-called mixed-mode chromatography[3]. Highly specific binding between a specific protein and a bio-affinity ligand has been amply demonstrated in protein purification by affinity chromatography[4–6]. The mechanism for such a specific binding may involve one or more of the above listed interactions. Affinity ligands of non-biological origin have also been used successfully as group-selective protein adsorbent. For example, dye ligand chromatography that uses Cibacron Blue F3GA as the affinity ligand has been effective in purifying a large number of nucleotide-

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binding and other proteins[7]. Recently Porath and co-workers[8–16] developd a novel and rapid method for selectively purifying antibody by salt-promoted chromatography on a "thiophilic" adsorbent. This adsorbent was prepared by reacting a hydroxyl group carrying polymer such as Sepharose sequentially with divinylsulfone and mercaptoethanol. In view of the structural simplicity of the ligand in thiophilic gel with a molecular weight of less than 400, the selectivity of thiophilic gel toward antibody is indeed remarkable. This is in contrast to the much more complex structure of other antibody binding ligands such as Protein A which has a molecular weight of about 42 000. The exciting results from Porath's group have given us much impetus to search for low-molecular-weight ligands capable of performing or mimicking the selectivity of such complex immunoglobulin binding proteins as Protein A or Protein G.

We wish to describe a class of affinity gels prepared by using synthetic low-molecular-weight ligands capable of selectively binding immunoglobulins in either low- or high-salt buffers and albumin under low-salt conditions. These gels were able to bind immunoglobulins from different animal species with high degree of selectivity and avidity. Their binding selectivities are very reminiscent of those of Protein A or Protein G. The affinity gels were obtained by first reacting Sepharose Cl-4B or other hydroxyl carrying polymers with pentafluoropyridine (PFP) and 4-dimethylaminopyridine (DMAP) in an anhydrous polar organic solvent such N,N′-dimethylformamide (DMF) or acetonitrile and then reacting the intermediate gel formed with nucleophiles in aqueous solution. Patent applications dealing with processes described herein have been filed.

MATERIALS AND METHODS

Chemicals

Sepharose Cl-4B was from Pharmacia (Uppsala, Sweden) and Fractogel TSK HW 75F from Toyo Soda (Tokyo, Japan). PFP and DMAP were purchased from Aldrich (Milwaukee, WI, U.S.A.), potassium sulfate, glycine, ethylene glycol, diethanolamine, citric acid, Tween 20, hydrogen peroxide, human immunoglobulin G (IgG), human albumin, goat serum and *p*-nitrophenyl phosphate were from Sigma (St. Louis, MO, U.S.A.), DMF and acetone from J. T. Baker (Phillipsburg, NJ, U.S.A.), human serum was from Irvine Scientific (Santa Ana, CA, U.S.A.), mouse serum and rabbit serum were from Pel-Freez (Roger, AK, U.S.A.), rabbit anti-human albumin, rabbit anti-human albumin HRP conjugate and *o*-phenylenediamine from Dako (Carpenteria, CA, U.S.A.), goat anti-human IgG and goat anti-human IgG alkaline phosphatase conjugate were from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Instrumentation

The effluent from the chromatographic column was monitored continuously at 280 nm with an LKB 2238 Uvicord SII and the pH of the effluent with an LKB 2195 pH/ion monitor. Fractions were collected with an LKB 2070 Ultrorac II fraction collector. Electrophoretic analyses were performed on 10–15% polyacrylamide gradient gels by using Phast system from Pharmacia. Vmax Kinetic microplate reader (Molecular Devices) was used for serum proteins enzyme-linked immunosorbent assay (ELISA).

Gel preparation

Preparation of PFP-substituted intermediate gel. Sepharose Cl-4B gel (100 ml) was washed with 5 × 100 ml distilled water. The washed gel was suspended in 100 ml distilled water in a 2-l beaker mounted on a shaker rotating at 100 rpm. To the gel 1 l dry acetone was added over 30 min. The gel was filtered and resuspended in 1 l dry acetone and was tumbled at room temperature for 15 min. The gel was filtered and 300 ml dry DMF were added to the gel and the gel was tumbled for 5 min. After filtering the gel was suspended in 100 ml of DMF containing 27.5 mmol DMAP. To the gel suspension were further added 250 ml DMF containing 25 mmol PFP. The gel was tumbled at room temperature for 2 h. Then the gel was washed with 1 l DMF and 2 × 1 l acetone. The washed, substituted gel can be stored in 200 ml acetone at 4°C for several weeks.

Preparation of PFP-O gel. A 10-ml volume of PFP-substituted intermediate gel was washed with 100 ml distilled water and 100 ml 0.1 *M* sodium hydrogencarbonate, pH 9.0. The gel was suspended in an equal volume of 0.1 *M* sodium hydrogencarbonate, pH 9.0 containing 10% ethylene glycol and tumbled at room temperature for 24 h. The gel was washed with 100 ml of 0.1 *M* sodium hydrogencarbonate, pH 9.0 and resuspended in twice its volume of 0.1 *M* sodium hydroxide and tumbled for 14 h at room temperature. The gel was washed with 100 ml distilled water, 100 ml 1 *M* sodium chloride, 100 ml distilled water and then 100 ml phosphate-buffered saline (PBS). When not in use the gel was stored in PBS at 4°C.

Preparation of PFP-glycine gel. A 10-ml volume of PFP-substituted intermediate gel was washed with 100 ml distilled water and 100 ml 0.1 *M* sodium hydrogencarbonate, pH 9.0. The gel was suspended in an equal volume of 1 *M* glycine in 0.1 *M* sodium hydrogencarbonate, pH 9.0 and the gel was tumbled at room temperature for 24 h. The gel was washed with 100 ml 0.1 *M* sodium hydrogencarbonate, pH 9.0 and resuspended in twice its volume of 0.1 *M* sodium hydroxide and tumbled for 14 h at room temperature. The gel was washed with 100 ml distilled water, 100 ml 1 *M* sodium chloride, 100 ml distilled water and then 100 ml PBS. When not in use the gel was stored in PS at 4°C.

Chromatographic procedures

Binding in presence of low salt concentration. A 4-ml volume of the gel packed in a disposable column (9 × 0.8 cm) was washed with about 20 ml of 20 m*M* sodium phosphate, pH 7.4. Filtered serum (1 ml) was appropriately diluted with 20 m*M* sodium phosphate buffer, pH 7.4 and passed through the column at a flow-rate of 0.25 ml/min. The column was washed with the same buffer at a flow-rate of 1 ml/min and then bound proteins were eluted first with 10 m*M* sodium phosphate, pH 7.4 containing 0.5 *M* potassium sulfate and then with 0.1 *M* glycine buffer, pH 5.0, 4 and at last 2.8 at the same flow-rate of 1 ml/min. Fractions of 3 ml were collected for each wash and the absorbance at 280 nm was read for each fraction. For PFP-O gel, the concentration of albumin and IgG in some selected fractions was determined by using the specific sandwich ELISA method.

Binding in presence of high salt concentration. A 4-ml volume of the gel packed in a disposable column was washed with about 20 ml of 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride. A 1-ml volume of filtered serum was diluted 10-fold in 10 m*M* phosphate buffer, pH 7.4 containing 1.5 *M* sodium chloride and

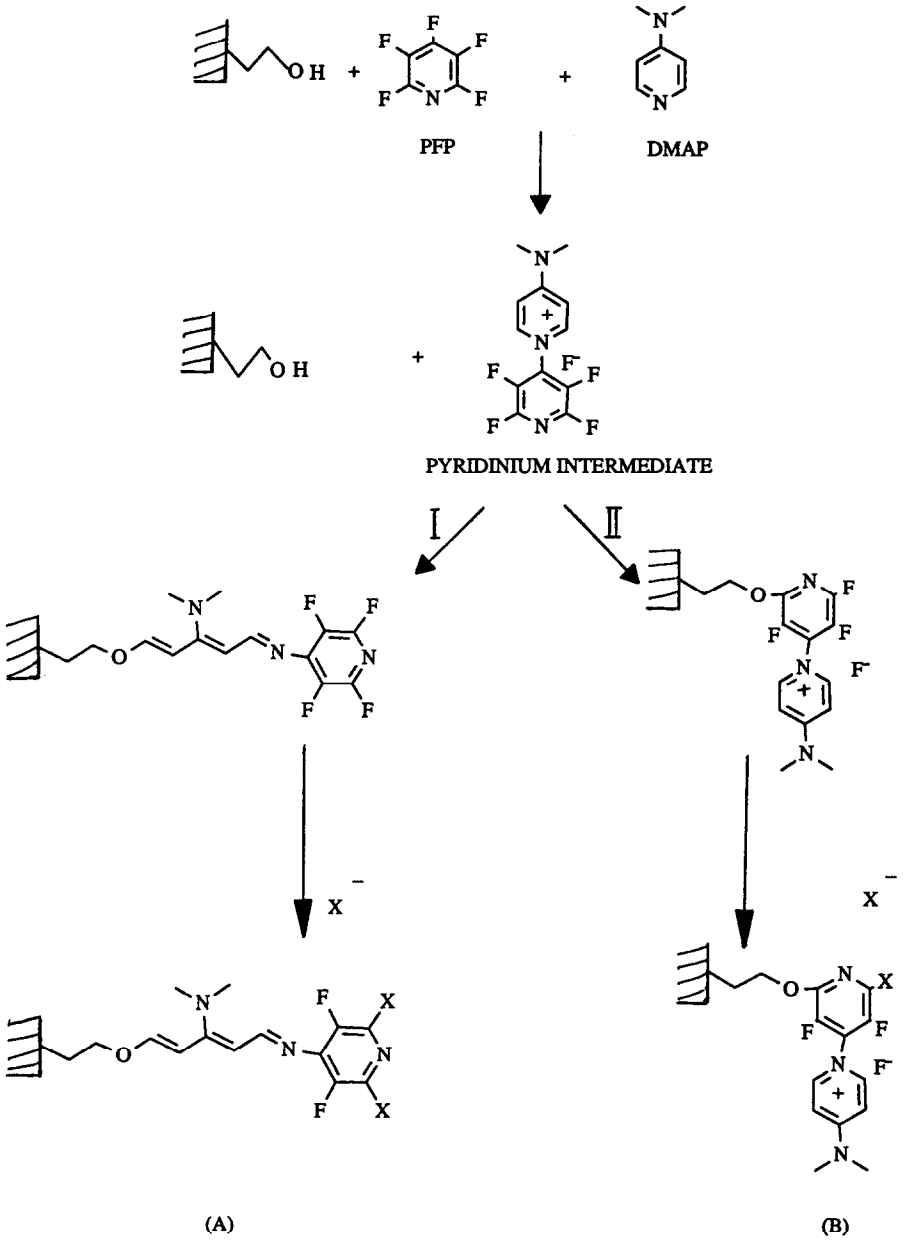

Fig. 1. Postulated synthetic steps for the preparation of the affinity gel.

passed through the column at a flow-rate of 0.25 ml/min. The column was washed with the same buffer at a flow-rate of 1 ml/min and bound proteins were eluted by washing the gel with 0.1 *M* glycine buffer pH 3.5 at the same flow-rate. Fractions of 3 ml were collected and the absorbance at 280 nm was read for each fraction.

RESULTS

The reaction of Sepharose Cl-4B with PFP and DMAP in organic solvent gave an intermediate product which upon further reaction in aqueous solution with nucleophiles (ethylene glycol or glycine) resulted in gels with unique selectivity toward immunoglobulins and albumins from several animal species. The synthetic routes and possible structures of the ligand of immunoglobulin-binding gels are shown in Fig. 1. Elemental analysis of the gel gave an N:F ratio of 3:2 which is consistent with both structures. The ligand density of the gel was calculated to be 1–13 mmol per g dry gel.

The results of fractionating human serum which has been diluted with 20 m*M* phosphate buffer pH 7.4 on PFP-O gel are shown in Fig. 2. Almost all of the UV absorbing materials from the diluted serum were adsorbed on the gel. Six major fractions were obtained by eluting the adsorbed materials sequentially with 10 m*M* phosphate, pH 7.4 containing 0.5 *M* potassium sulfate, 0.1 *M* glycine buffer, pH 5, 4 and 2.8. From the electrophoresis of the eluted fractions under either reducing or non-reducing conditions (Fig. 3A and B), it was observed that fractions in peaks I and II that were eluted by a buffer solution with 0.5 *M* K_2SO_4 contained mostly albumin and possibly transferrin. Fractions in peak III, eluted by lower ionic strength buffer of pH 5, contained IgG. Using the same buffer, a smaller quantity of IgG was also eluted in fractions of peaks IV. Fractions in peaks V and VI, elued respectively with buffers of pH 4 and 2.8 were shown to contain a number of other serum proteins. The identity of albumin and IgG was further confirmed by ELISA for specific protein determinations

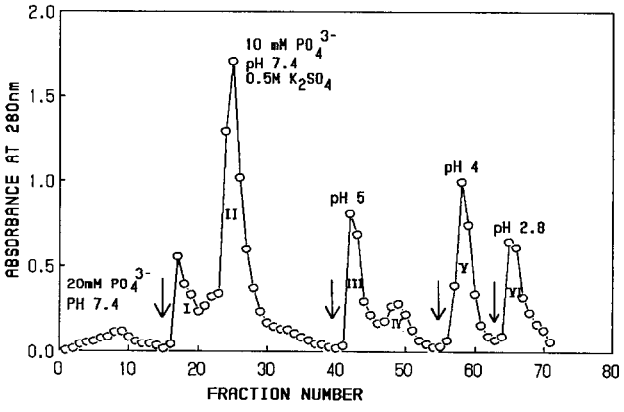

Fig. 2. Fractionation of human serum on PFP-O gel. Gel volume was 4 ml. Serum was diluted 10-fold with 20 m*M* sodium phosphate, pH 7.4 and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elutions were carried out with different buffers at the fraction indicated by arrows. I and II were fractions eluted with 10 m*M* sodium phosphate, pH 7.4 containing 0.5 *M* potassium sulfate. III and IV contained fractions eluted with 0.1 *M* glycine, pH 5. V contained fractions eluted with 0.1 *M* glycine, pH 4 and VI contained fractions eluted with 0.1 *M* glycine, pH 2.8.

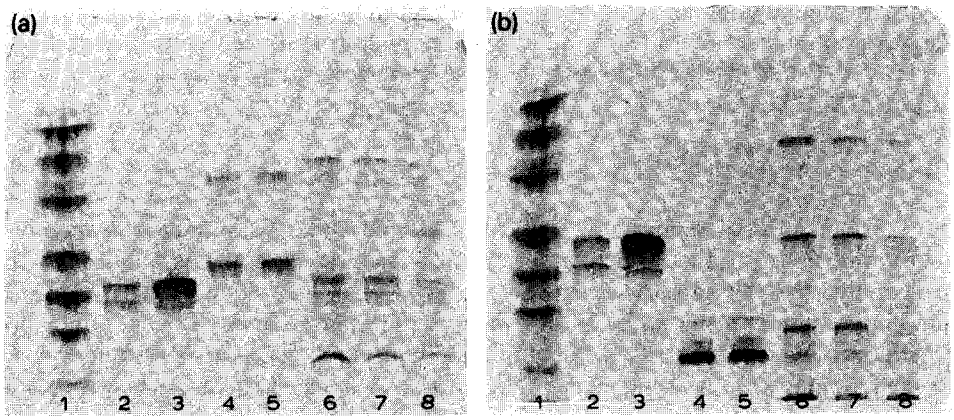

Fig. 3. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (A) and non-reducing (B) conditions of fractions shown in Fig. 2. Lanes: 1 = molecular weight marker reference proteins; 2 = peak I of Fig. 2; 3 = peak II of Fig. 2; 4 and 5 = peak III of Fig. 2; 6 and 7 = peak V of Fig. 2; 8 = peak VI of Fig. 2.

(Fig. 4). Albumin was found mainly in the high-salt, flow-through fractions, *i.e.* in peak II and only a small amount in peak I, while IgG was found almost exclusively in peaks III and IV with a much smaller quantity in peak V. The recovery of isolated IgG was estimated to be 55%. Attempts were made to simplify the isolation of immunoglobulins from the serum by applying serum which has been diluted with a high-salt buffer. Fig. 5 showed results of such an experiment. Two major peak fractions were obtained, the first peak was the high-salt (1.5 *M*), flow-through fractions which contained mostly albumin and some minor other serum proteins. The second peak, eluted with 0.1 *M* glycine, pH 3.5, contained the major portion of the IgG and some other serum proteins. The recovery of IgG was greater than 80%. From the

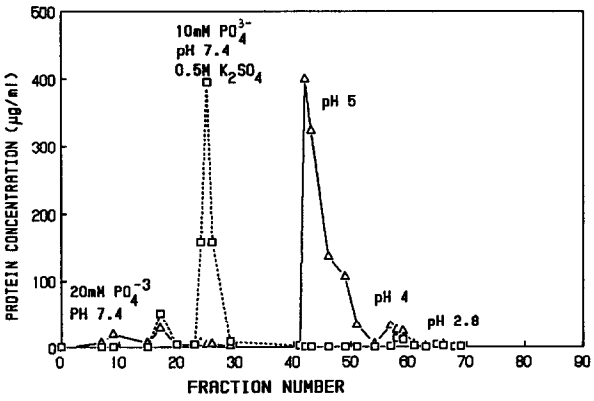

Fig. 4. Sandwich ELISA for human serum albumin and IgG in fractions obtained from chromatography of human serum on PFP-O gel (Fig. 2). □ = Albumin; △ = IgG.

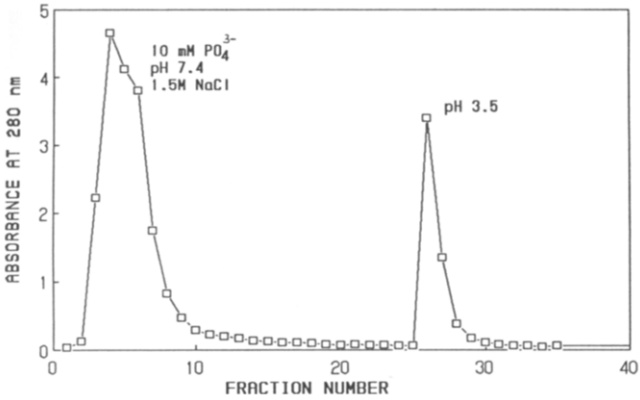

Fig. 5. Fractionation of human serum on PFP-O gel under high salt loading condition. Gel volume was 4 ml. Serum was diluted 10-fold with 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elution was carried out with 0.1 *M* glycine, pH 3.5.

electrophoresis (Fig. 6) of fractions shown in Fig. 5, it was clear that IgG isolated by using this high-salt procedure was not as pure as that obtained by the procedure used in Fig. 2. Goat, mouse and rabbit sera, all prediluted in high-salt buffer (10 m*M* phosphate, pH 7.4 containing 1.5 *M* sodium chloride) have been individually chromatographed on a column of PFP-O gel and were fractionated into two major fractions. The recovery of IgG isolated from sera was greater than 60%. In every case, the second fraction was the IgG-rich fraction. The chromatograms are shown in Figs. 7, 9 and 11, respectively. The electrophoretic patterns of these results are respectively shown in Figs. 8, 10 and 12. A similar chromatogram (Fig. 13) was obtained when rabbit serum was fractionated on a glycine-substituted gel (PFP-glycine gel). The first peak fraction was rich in albumin and the second was rich in IgG.

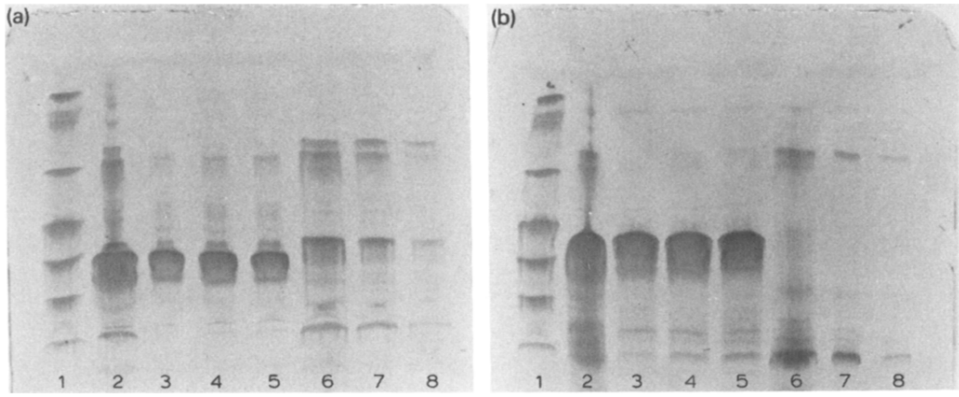

Fig. 6. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (a) and non-reducing (b) conditions of fractions shown in Fig. 5. Lanes 1 = molecular weight marker reference proteins; 2 = unfractionated whole serum; 3, 4 and 5 = unbound, flow-through fractions; 6, 7 and 8 = fractions eluted with 0.1 *M* glycine, pH 3.5.

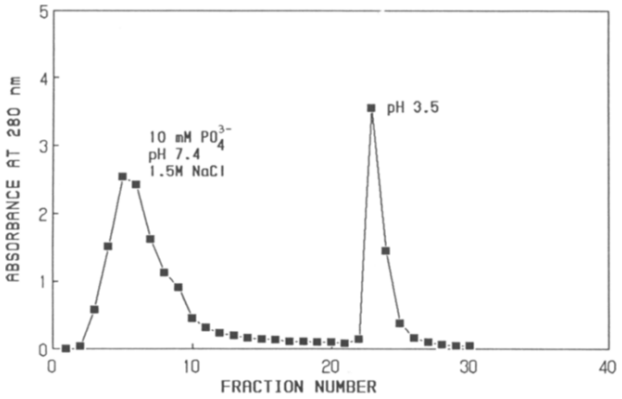

Fig. 7. Fractionation of goat serum on PFP-O gel under high salt loading condition. Gel volume was 4 ml. Serum was diluted 10-fold with 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elution was carried out with 0.1 *M* glycine, pH 3.5.

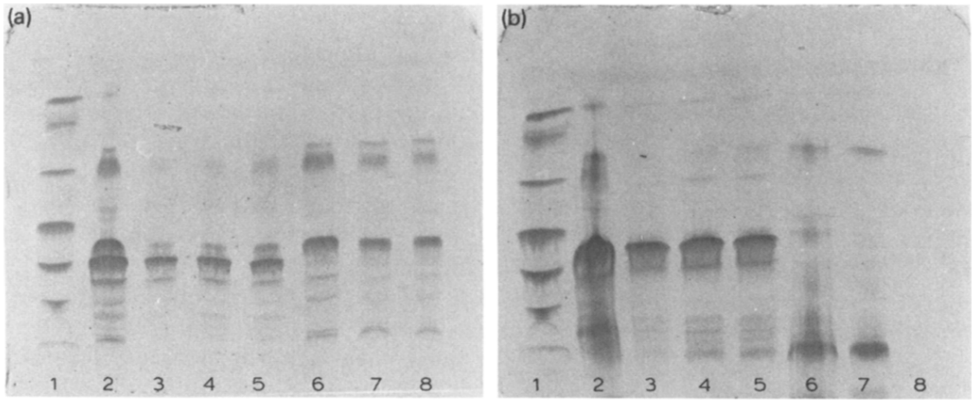

Fig. 8. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (a) and non-reducing (b) conditions of fractions shown in Fig. 7. Lanes: 1 = molecular weight marker reference proteins; 2 = unfractionated whole serum; 3, 4 and 5 = unbound, flow-through fractions; 6, 7 and 8 = fractions eluted with 0.1 *M* glycine, pH 3.5.

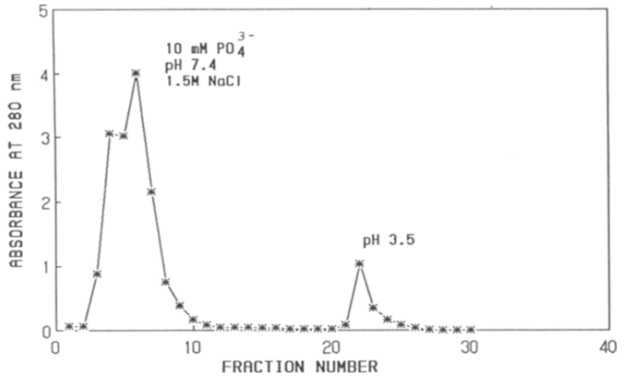

Fig. 9. Fractionation of mouse serum on PFP-O gel under high salt loading condition. Gel volume was 4 ml. Serum was diluted 10-fold with 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elution was carried out with 0.1 *M* glycine, pH 3.5.

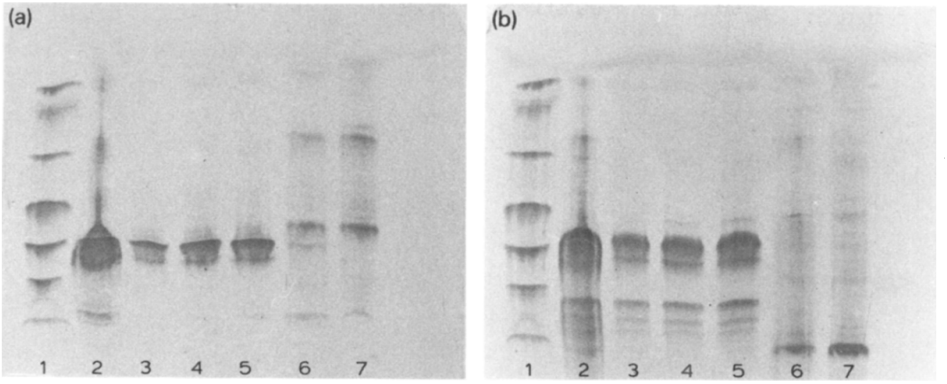

Fig. 10. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (a) and non-reducing (b) conditions of fractions shown in Fig. 9. Lanes: 1 = molecular weight marker reference proteins; 2 = unfractionated whole serum; 3, 4 and 5 = unbound, flow-through fractions; 6 and 7 = fractions eluted with 0.1 *M* glycine, pH 3.5.

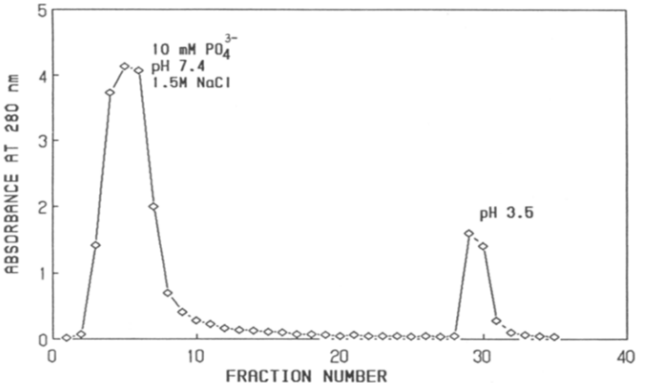

Fig. 11. Fractionation of rabbit serum on PFP-O gel under high salt loading condition. Gel volume was 4 ml. Serum was diluted 10-fold with 10 m*M* sodium phosphate, pH 7.4 containing 1.5 *M* sodium chloride and was applied at a flow-rate of 0.25 ml/min at room temperature. Fractions of 3 ml were collected. Elution was carried out with 0.1 *M* glycine, pH 3.5.

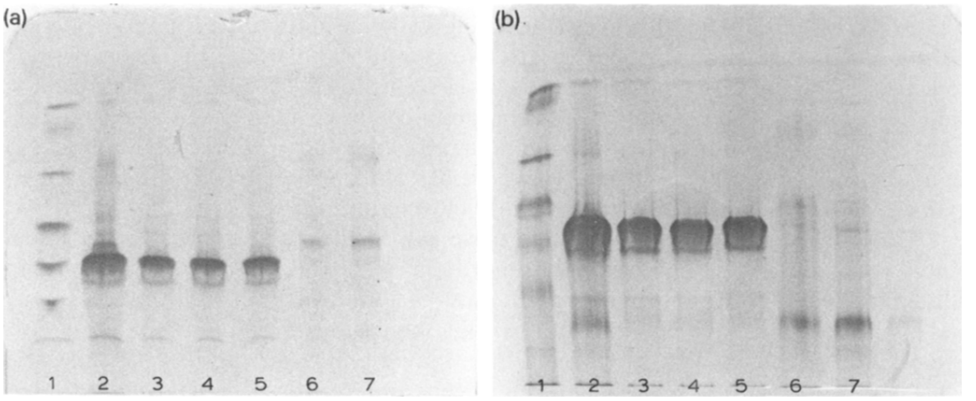

Fig. 12. Sodium dodecyl sulfate gradient (10–15%) polyacrylamide gel electrophoresis under reducing (a) and non-reducing (b) conditions of fractions shown in Fig. 11. Lanes: 1 = molecular weight marker reference proteins; 2 = unfractionated whole serum; 3, 4 and 5 = unbound, flow-through fractions; 6, 7 and 8 = fractions eluted with 0.1 *M* glycine, pH 3.5.

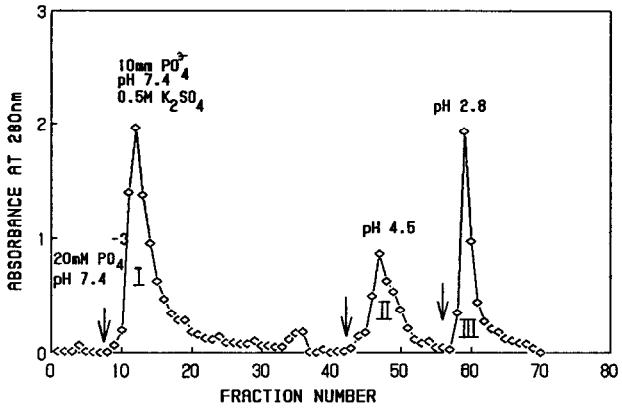

Fig. 13. Fractionation of rabbit serum on PFP-glycine gel. Gel volume was 4 ml. Serum was diluted 100-fold with 20 m*M* sodium phosphate, pH 7.4 and was applied at a flow-rate of 1.25 ml/min at room temperature. Fractions of 3 ml were collected. Elutions were carried out with different buffers at the fraction indicated by arrows. I contained fractions eluted with 10 m*M* sodium phosphate, pH 7.4 containing 0.5 *M* potassium sulfate. II contained fractions eluted with 0.1 *M* glycine, pH 4.5. III were fractions eluted with 0.1 *M* glycine, pH 2.8.

DISCUSSION

A number of chromatographic matrices capable of selectively binding immunoglobulin and albumin from serum have been prepared by first reacting the hydroxyl groups of a polymeric matrix with PFP and DMAP and then with nucleophiles. The postulated synthetic steps leading to the formation of the affinity gels are shown in Fig. 1. The first step involved the reaction of PFP and DMAP to form a pyridinium intermediate. The second step involved the reaction of the hydroxyl group of the gel with the pyridinium intermediate. There are two possible points of attack by the hydroxyl group on the pyridinium ion. One possible point of nucleophilic attack (route I) would be on the 2′ position of the pyridinium ring with the consequence of forming an open-chain, extended conjugated system. Such a gel will have the ligand structure depicted in Fig. 1A. The second attack (route II) by the hydroxide ion can occur at position 2 of the fluoropyridine ring with the resultant formation of a gel having a structure as shown in Fig. 1B. We cannot at this time make a definitive conclusion from these two possible alternatives regarding the chemical structure of the ligand on the gel. The use of pyridine compounds allows us to have some degree of control on the ionization state of the ligand by changing the pH of the buffer. It is also an extension of our previous work on the use of 2-fluoro-1-methylpyridinium salts as hydroxyl group activating agent in affinity chromatography[17,18].

The most noticeable property of these gels is their remarkably high protein binding capacity. Almost all of the serum proteins were bound to the gel when serum diluted by 20 m*M* phosphate buffer, pH 7.4 was passed through the gel column (Figs. 2 and 13). Another unique property of the gel is the adsorption of albumin at low-salt and its desorption at high-salt buffer. Immunoglobulins on the other hand were adsorbed at either low or high salt concentrations and were desorbed only by lowering the pH (Figs. 2, 5, 7, 9, 11 and 13). High degree of selectivity in the binding of serum

albumin and IgG by the gel was demonstrated by gradient polyacrylamide gel electrophoresis and ELISA (Figs. 3, 4, 6, 8 and 10). Further work is in progress to fine-tune the binding selectivity of the gel toward albumin and IgG by selecting optimal buffers for the binding and elution conditions.

The chromatographic behavior of both PFP-O and PFP-glycine gels differs from the thiophilic gel described by Porath and co-workers[8–16] in that (i) the structure of the ligand contains no element of sulfur, (ii) high salt concentration is not a necessary condition for protein adsorption (in fact albumin was desorbed at high salt concentration) and (iii) the desorption of IgG is obtained by lowering the pH rather than reducing the salt concentration in the eluting buffer.

The mechanism of protein adsorption to PFP-O or PFP-glycine gel is not known yet. The adsorption mechanism is not consistent with anyone of the known interaction alone, *i.e.* pure ion-exchange, pure hydrophobic or other interactions. It is, however, possible that more than one interaction might be operative as in the case of mixed-mode chromatography[3]. By looking at the two possible structures for the ligand of either PFP-O or PFP-glycine gel, it is not difficult to imagine the operationof more than one interaction in the adsorption of proteins to the gel. It is likely that ion-exchange, hydrophobic and charge-transfer types of interaction are simultaneously involved.

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