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Avid AL, a synthetic ligand affinity gel mimicking immobilized bacterial antibody receptor for purification of immunoglobulin G

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ABSTRACT

Avid AL is an affinity gel designed for the purification of immunoglobulin G (IgG). The gel was prepared by first reacting Sepharose with 3,5-dichloro-2,4,6-trifluoropyridine and 4-dimethylaminopyridine and then with 2-mercaptoethanol. The IgG purified by Avid AL is about 95% pure. The binding parameters of Avid AL for the whole IgG, Fab and Fc fragment and the stability of gel were investigated. The IgG bound to Avid AL can be eluted with an acidic buffer or with a novel neutral buffer containing electron donors. The development of such a mild neutral elution buffer is described. Application of Avid AL in a rapid gram-scale IgG purification was demonstrated. The possible mechanism of IgG binding is discussed.

INTRODUCTION

A number of affinity supports for purifying immunoglobulin G (IgG) using synthetic affinity ligands have been prepared [1–11]. Porath and coworkers [1–4] used a thiophilic gel to bind IgG in the presence of high salt and the bound IgG was eluted by lowering the salt concentration. The thiophilic gel was prepared by reaction of 2-mercaptoethanol with divinyl sulfone-activated agarose. The binding capacity of thiophilic gel was 65–70 mg of protein per gram of dry gel. Nopper *et al.* [5] developed a silica gel-based thiophilic (3 S gel) affinity adsorbent for purifying IgG. The 3 S silica gel was prepared by reaction of an epoxide-derivatized silica gel sequentially with sodium hydrosulfide, divinyl sulfone and 2-mercaptoethanol. The 3 S silica gel was able to bind, under high salt conditions, 20 mg of IgG per gram of gel. The bound IgG was eluted with an acidic buffer. An oxirane-activated agarose gel substituted with histidine was shown to bind serum proteins including IgG at low ionic strength. The bound IgG can then be eluted with Tris buffer containing 0.2 *M* NaCl [6,7]. Although this histidyl gel has the advantage of not requiring high salt for binding IgG, its binding capacity, less than 0.3 mg IgG per milliliter of gel, is too low to be practical. Remazol Yellow GCL-coupled Sepharose was shown to bind IgG in addition to its binding to prealbumin [11]. However, the bound IgG was eluted in two broad fractions. Early-eluting fractions were obtained by washing the gel with 20 m*M* phosphate buffer and later fractions were obtained with a 300 m*M* NaCl gradient [11].

The advantage of totally synthetic low-molecular-mass affinity ligands compared with protein A or G is their ability to withstand acid, base, organic solvent, proteolytic enzyme and autoclaving treatments. We have previously described a series of affinity gels made with low-molecular-mass, non-protein affinity ligands capable of the selective purification of IgG [8]. The affinity gels were prepared by reaction of pentafluoropyridine, 4-dimethylaminopyridine and Sepharose Cl-4B under anhydrous conditions and finally reaction of the gel with ethylene glycol or glycine. The postulated structures of the affinity ligands in these affinity gels are shown in Fig. 1A.

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The binding of IgG to these affinity gels required either a low-ionic-strength (0.02 *M* salt) or a high-salt (1.5 *M* salt) buffer [8]. Although affinity gels prepared from these synthetic ligands show good selectivity in IgG purification, there are still some contaminating proteins which elute together with IgG. In an effort to improve the purification selectivity and to allow the binding of IgG to the gel in phosphate-buffered saline (PBS) (0.15 *M* NaCl), we discovered that by first reacting Sepharose with 3,5-dichloro-2,4,6-trifluoropyridine instead of pentafluoropyridine and 4-dimethylaminopyridine and then with 2-mercaptoethanol instead of ethylene glycol or glycine, we obtained a remarkable affinity gel capable of purifying IgG to a high degree of purity. The postulated structure of the affinity binding is shown in Fig. 1B. This gel is named Avid AL. Here we report studies of the binding parameters (dissociation constants and capacities) of Avid AL for the whole IgG molecule and its fragments, *i.e.*, Fab and Fc. We also report the development of a mild neutral IgG elution buffer, the results of a long-term stability study of the gel under various solvent conditions and the scale-up and rapid purification of IgG up to 12 g from serum in a single run. The possible mechanism of IgG binding to Avid AL is discussed.

EXPERIMENTAL

Materials

Avid AL gel, a prepacked radial flow Avid AL gel (Avid AL AvidPak) 1.5-l column and neutral elution buffer were obtained from BioProbe International (Tustin, CA, USA). Avid AL was prepared by first reacting 3,5-dichloro-2,4,6-trifluoropyridine with 4-dimethylaminopyridine and then with 2-mercaptoethanol according to a procedure similar to that described previously [8]. A protein G mini-column was obtained from Life Technologies (Gaithersburg, MD, USA). Goat serum, rabbit IgG, monoclonal antiperoxidase mouse IgG1, horseradish peroxidase, monobasic sodium phosphate, sodium chloride, sodium azide, acetic acid, tris(hydroxymethyl)aminomethane (Tris), triethylamine and sodium acetate were purchased from Sigma (St. Louis, MO, USA), methanol from VWR Scientific (Cerritos, CA, USA) and TMB peroxidase substrate from Kirkegaard & Perry Labs. (Gaithersburg, MD, USA).

Fab and Fc fragments of rabbit IgG were prepared enzymatically by using a Fab kit from BioProbe International.

The effluent from the chromatographic column was monitored continuously at 280 nm with an LKB 2238 Uvicord SII and the pH of the effluent was continuously monitored with an LKB 2195 pH/ion monitor. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10–15% gradient polyacrylamide gel in a Phast system from Pharmacia. Absorbance at 280 nm was measured with a Hewlett-Packard Model 8452A diode-array spectrophotometer. A Molecular Device microtiter plate reader was used in enzyme-linked immunosorbent assay (ELISA) experiments.

Binding of IgG, Fab and Fc fragments of IgG to Avid AL

Binding under equilibrium conditions. The Avid AL gel (0.4 ml) was packed in small columns. Each column was washed first with 5 ml of 20% methanol–1% acetic acid and then with 10 ml of PBS. Either IgG, Fab or Fc fragments, at various concentrations, were dissolved in PBS and 2 ml of these solutions were each applied to the gel and one column volume of PBS was added to remove unbound IgG or the fragments. The unbound fractions were collected and quantified. The amount of bound IgG or its fragments was calculated from the difference between the amount applied and the amount unbound.

Binding under pseudo-equilibrium conditions. The Avid AL gel (0.4 ml) was packed in small columns. Each column was washed first with 5 ml of 20% methanol–1% acetic acid and then with 10 ml of PBS. Different concentrations of IgG, Fab or Fc fragments dissolved in PBS (2 ml) were applied to the gel. The gel was washed with PBS until the absorbance at 280 nm reached the baseline, then the bound IgG, Fab or Fc was eluted with 50 m*M* sodium acetate (pH 2.8) containing 20% glycerol. The amount of IgG, Fab or Fc was measured in the applied sample, flow-through sample and the eluted samples by bicinchoninic acid (BCA) protein assay.

Development of a neutral elution buffer

Elution of goat IgG with different concentrations of triethylamine at pH 7.4. For this experiment Avid AL gel (1 ml) was packed in a small column. Four columns were prepared. Each column was washed with 10 ml 20% methanol–1% acetic acid and then each column was equilibrated with PBS. Goat serum (3 ml), filtered through a 0.45-μm filter, was diluted with 12 ml of PBS and applied to each column. Each column was washed with 15 ml of PBS. Bound goat IgG was eluted from columns with different concentrations of triethylamine dissolved in PBS with the pH maintained at pH 7.4. The amounts of IgG eluted with different concentrations of triethylamine at same pH were measured.

Elution of goat IgG with 0.4 M triethylamine at different pH values. Avid AL gel was packed in five columns. Each column containing 1 ml of Avid AL was washed with 10 ml of 20% methanol–1% acetic acid, then equilibrated with PBS and goat serum (3 ml) diluted with 12 ml of PBS was applied to each column. Each column was washed with 15 ml of PBS and bound IgG was eluted with 0.4 *M* triethylamine at different pH values. The amount of IgG eluted was measured.

Purification of IgG from serum using Avid AL and acidic elution buffer

An Avid AL AvidPak radial flow column (1.5 l) was washed with five column volumes of 20% methanol–1% acetic acid at a flow-rate of 150 ml/min. The column was then equilibrated with PBS at the same flow-rate. Goat serum (750 ml) was filtered and diluted with 3 l of PBS and this diluted serum was applied to the column at a flow-rate of 75 ml/min. The column was washed with ten column volumes of PBS at a flow-rate of 150 ml/min. Bound proteins were eluted with 7 l of 50 m*M* sodium acetate (pH 2.8) at a flow-rate of 75 ml/min. The eluted antibodies were collected in a beaker containing 500 ml of 1 *M* Tris and 1 *M* NaCl with the pH adjusted to 7.5. This allowed immediate neutralization of the eluted fractions, which prevented precipitation of the eluted IgG. The column was regenerated with four column volumes of 20% methanol–1% acetic acid at a flow-rate of 150 ml/min. The column was equilibrated with PBS and stored at 4°C.

Purification of IgG from serum using Avid AL and a newly developed neutral elution buffer

An Avid AL AvidPak radial flow column (1.5 l) was washed with five column volumes of 20% methanol–1% acetic acid at a flow-rate of 150 ml/min. The column was then equilibrated with PBS at the same flow-rate. Goat serum (750 ml) was filtered and diluted with 3 l of PBS and this diluted serum was passed through the column at a flow-rate of 75 ml/min. The column was washed with 18 l of PBS at a flow-rate of 150 ml/min. The bound IgG was eluted with the neutral elution buffer at a flow-rate of 75 ml/min. The column was regenerated by washing with 7 l of 20% methanol–1% acetic acid at a flow-rate of 150 ml/min. The column was then equilibrated with PBS.

Comparison of the purity of various affinity-purified IgGs

The purity of goat IgG purified by using immobilized protein G, Avid AL with acid elution and Avid AL with neutral elution was analysed by SDS-PAGE using 10–15% gradient polyacrylamide gel in a Pharmacia Phast system.

Comparison of the activity of the antibody eluted with neutral and acidic elution buffer

Mouse ascites fluid (0.1 ml) containing anti-peroxidase (clone p 6-36 mouse IgG1 isotype) from Sigma was used as an example of monoclonal antibody for comparing the effects of acidic and neutral elution buffers on the activity of the purified antibody. Avid AL gel (0.5 ml) was washed three times with 5 ml of PBS. The monoclonal antibody in ascites fluid was diluted with 0.8 ml of PBS and the diluted ascites fluid (1 ml) was mixed with 0.5 ml of Avid AL. The gel suspension was incubated at room temperature for 30 min with continuous shaking, then the gel was washed three times with 2.5 ml of PBS and was divided into two equal parts in two Eppendorf tubes. In the first Eppendorf tube, bound IgG was eluted with 0.5 ml of 50 m*M* sodium acetate (pH 2.8) containing 20% of glycerol. In the second tube, the bound IgG was eluted with 0.5 ml of the neutral elution buffer. The eluted antibody was diluted with Tris-buffered saline (TBS) to give an antibody concentration range of 0.1–100 μg/ml. Antibody solutions, 0.1 ml each, were placed in individual wells of a microwell plate. The antibody

solution was incubated overnight. The wells were then washed three times with 0.1 *M* Tris containing 0.01% of Tween 20. Possible sites of the microwell not covered by antibodies were blocked by incubating with 1% BSA solution in the washing buffer. A 0.1-ml volume of peroxidase solution (10 μg/ml) was added to each well. After incubation for 2 h, the wells were washed with the washing solution, then TMB peroxidase substrate was added. After incubation the reaction was stopped for 5 min with 0.1 ml of 0.5 *M* sulfuric acid. The absorbance at 450 nm was measured using a Molecular Devices microtiter plate reader.

Stability of Avid AL gel under different conditions

Avid Al gel (40 ml) was washed three times with 200 ml of water and divided into four equal parts (10 ml each) in four 50-ml test-tubes. In the first tube the gel (10 ml) was washed with 30 ml of 0.1 *M* NaOH and soaked in 20 ml of 0.1 *M* NaOH. In the second tube the gel (10 ml) was washed with 0.1 *M* acetic acid and soaked in 20 ml 0.1 *M* acetic acid. In the third tube the gel (10 ml) was washed with 20% aqueous ethanol and soaked in 20 ml of the same solution. The remaining 10 ml of water-washed gel was washed with PBS and soaked in 20 ml of PBS. Each gel was stored at room temperature for 45 days. The binding capacity of each gel was determined at different intervals of time. For measuring the effect of different storing solutions on the binding capacity, 1 ml of the treated gel was removed from the suspension, packed in a small column and washed with 10 ml of 20% methanol–1% acetic acid and finally equilibrated with PBS. Filtered goat serum (3 ml), diluted with 12 ml of PBS, was passed through the column. The column was then washed with 15 ml of PBS. Bound IgG was eluted with 15 ml of 50 m*M* sodium acetate (pH 2.8). The absorbance of the eluted samples at 280 nm was measured and the IgG eluted was calculated and expressed in terms of milligrams of IgG bound per milliliter of gel.

RESULTS AND DISCUSSION

We have previously developed a series of fluoropyridine-derived affinity gels for the selective purification of IgG [8]. Using these affinity gels, one can obtaine fairly pure IgG. The mode of operation, however, was not entirely convenient, *i.e.*, the sample needed to be loaded with either a low-salt buffer (20 m*M*) followed by a high-salt wash (1.5 *M* NaCl) or with a buffer containing high salt (1.5 *M*). Further, an increase in the selectivity of these affinity gels for IgG will make them more useful. With the aims of improving IgG binding conditions and selectivity, we succeeded in developing a better affinity gel for IgG purification, Avid AL. In Avid AL, the affinity ligand is based on dichloropyridine, not difluoropyridine, and the pyridyl moiety is substituted at the 2-position with a hydroxyethylthiol rather than a hydroxyethoxyl group (Fig. 1A and B). Unlike previously prepared affinity gels for IgG purification [8], which required high salt (1.5 *M* NaCl) in the sample loading and wash buffer, Avid AL binds IgG, but not albumin, under physiological saline conditions (*e.g.*, 150 m*M* NaCl and 10 m*M* sodium phosphate, pH 7.4). Further, IgG purified from serum showed a much higher degree of purity, *i.e.*, more than 95% pure. The electrophoresis of the purified IgG is shown in Fig. 2.

The binding parameters of Avid AL for IgG, Fab and Fc fragments were investigated under equilib-

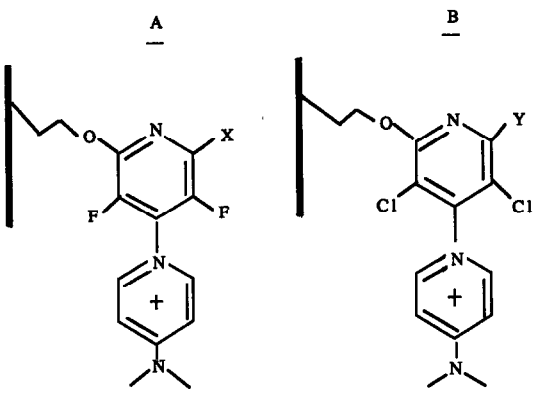

Fig. 1. Postulated structures of the affinity ligand of the adsorbents used for IgG purification.

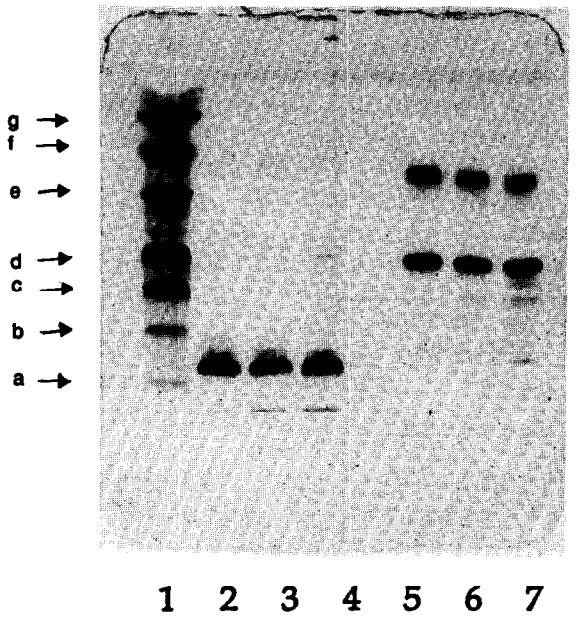

Fig. 2. SDS-PAGE (10–15% gradient) of IgG purified from goat serum using a 1.5-l Avid AL AvidPak radial flow column and immobilized protein G gel. Lanes: 1 = molecular mass markers: (a) myosin, (b) phorphorylase, (c) bovine serum albumin, (d) ovalbumin, (e) α-chymotrypsinogen, (f) lactoglobulin and (g) lysozyme; 2 = IgG purified using immobilized protein G gel, run under non-reducing conditions; 3 = IgG purified using an Avid AL AvidPak column, eluted with 50 m*M* sodium acetate (pH 2.8), run under with non-reducing conditions; 4 = IgG purified using an Avid AL AvidPak column, eluted with Avid AL neutral elution buffer, run under non-reducing conditions; 5, 6 and 7 = samples in lanes 2, 3 and 4, respectively, run under reducing conditions.

rium and pseudo-equilibrium conditions. The binding of either whole IgG molecules or its fragments (Fab of Fc) appears to follow simple Langmuir adsorption isotherms (Fig. 3A, C and E). The dissociation constants and binding capacities were determined from double reciprocal plots of the respective binding saturation curves (Fig. 3B, D and F) and the values are summarized in Table I. The dissociation constants obtained under pseudo-equilibrium conditions were 17.4, 23.9 and 8.8 μM, respectively, for IgG, Fab and Fc fragments. The binding capacities were 14.0, 5.3 and 1.8 mg/ml gel for IgG, Fab and Fc, respectively. These values are not significantly different from those obtained under equilibrium conditions, which give dissociation constants

TABLE I

DISSOCIATION CONSTANTS AND BINDING CAPACITIES OF Avid AL FOR BINDING IgG, Fab AND Fc FRAGMENTS OF IgG

Compound bound	Dissociation constant (μM)	Binding capacity (mg/ml gel)
Whole IgG	17.4	14.0
Fab fragments of IgG	23.9	5.3
Fc fragments of IgG	8.8	1.8

of 23.9, 30 and 10.7 μM for IgG, Fab and Fc fragments, respectively. The corresponding binding capacities are 18, 5.1 and 2.1 mg/ml gel for IgG, Fab and Fc fragments, respectively. It is apparent from Fig. 3 and Table I that the dissociation constants for IgG Fab and Fc are similar, with Fc fragment having the highest and Fab fragment the lowest affinity for Avid AL, while the whole IgG molecule has a binding constant between those of the Fab and Fc fragments. However, the binding capacities differ by as much as eight fold between the binding of IgG and the Fc fragments. The simultaneous presence of two Fab and one Fc fragments in the whole IgG appears to have synergistic effects in increasing the binding capacity of whole IgG to Avid AL. The binding constant of Avid AL for IgG is of the same order of magnitude as that of a triazine dye gel for binding of dehydrogenase [12].

During our studies on affinity purification of antibody, we have encountered antibodies, particularly of monoclonal origin, that are not stable under acidic conditions. For the purification of these antibodies, it was necessary to develop alternative, neutral, milder elution conditions. From the structure of the affinity ligand (Fig. 1B), one can envisage it as an electron acceptor. If this were the case than the complementary binding site(s) on IgG would necessarily be electron donors. Therefore, one could expect that compounds capable of donating electrons such as an amine with its lone pair of electrons or other electron donors such as thiols would be able to compete successfully with IgG in complexing with the affinity ligand and consequently to displace IgG. We indeed demonstrated that triethylamine of pH 7.4 in the concentration range 0.4–0.9

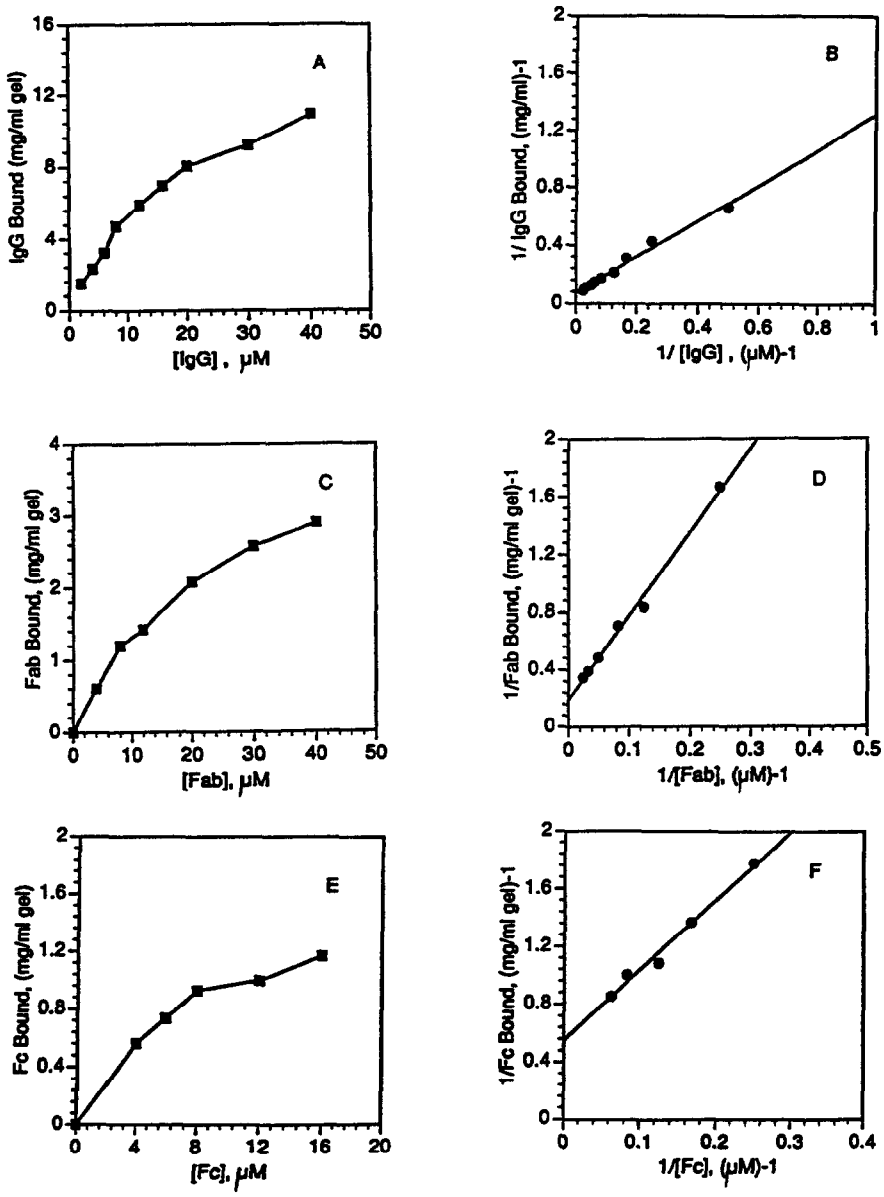

Fig. 3. Determination of binding constants and binding capacities for whole IgG, Fab and Fc fragments by Avid AL. (A) Relationship between the concentration of IgG and the amount of IgG bound (the saturation curve); (B) double reciprocal plot of data in (A) for the graphical determination of IgG binding constant and binding capacity; (C) relationship between the concentration of Fab and the amount of Fab bound (the saturation curve); (D) double reciprocal plot of data in (C) for the graphical determination of Fab binding constant and binding capacity; (E) relationship between the concentration of Fc and the amount of Fc bound (the saturation curve); (F) double reciprocal plot of data in (E) for the graphical determination of Fc binding constant and binding capacity. Experimental conditions are described in the text.

M was able to elute IgG from Avid AL (Fig. 4A). In fact, the pH of the eluting buffer has no significant effect on the ability of triethylamine to elute IgG from Avid AL in the pH range 4–10 (Fig. 4B). The possibility of an amine displacing IgG bound to Avid AL gel via an ion-exchange rather than an

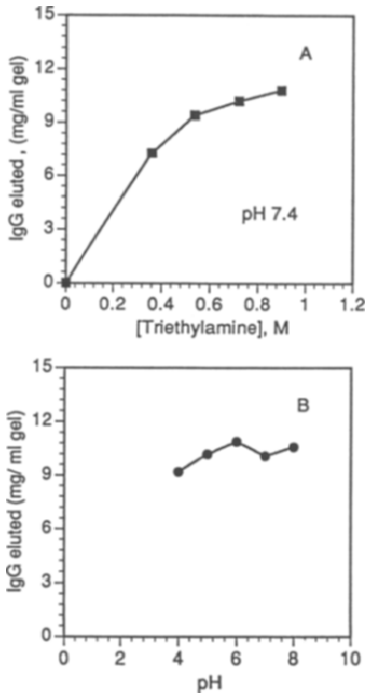

Fig. 4. Elution of goat IgG bound to Avid AL gel with different concentrations of triethylamine at different pH. (A) Goat IgG eluted from Avid AL gel with different concentrations of triethylamine. Goat serum (3 ml) was diluted fourfold with PBS and applied to 1 ml of gel. IgG was eluted with different concentrations of triethylamine at pH 7.4. (B) Goat IgG eluted from Avid AL gel with 0.4 *M* triethylamine at different pH values. Goat serum (3 ml) was diluted fourfold with PBS and applied to 1 ml of gel. IgG was eluted with 0.4 *M* triethylamine at different pH values.

TABLE II

COMPARISON OF DIFFERENT ELECTRON DONORS FOR ELUTING IgG BOUND TO Avid AL AT NEUTRAL pH

Electron donor (ED)	Concentration of ED (*M*)	Amount of IgG eluted (mg eluted from 1 ml of Avid AL presaturated with IgG)
Triethylamine	0.36	7.3
Mercaptoglycerol	0.68	14.7
Dithiothreitol	0.33	14.1
Acetonitrile	0.96	8.0

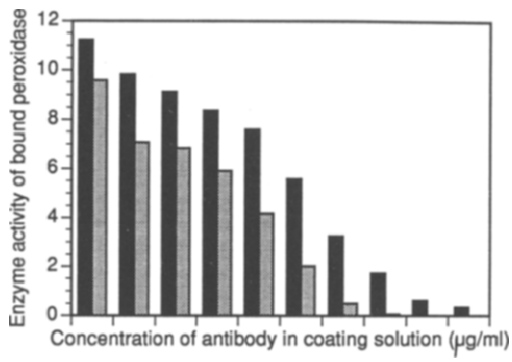

Fig. 5. Comparison of binding activity of mouse IgG1 anti-horseradish peroxidase (HRP) eluted from Avid AL with 50 m*M* sodium acetate, pH adjusted to 2.8 with concentrated HCl (shaded bars), with that eluted with a neutral elution buffer (solid bars). The eluted anti-HRP was coated on a microwell plate at various concentrations indicated. After incubating with HRP and washing off unbound, excess enzyme, the bound HRP activity was measured using TMB. The solid bars are for neutral elution buffer and the shaded bars are for acidic elution buffer. For experimental conditions, see the text.

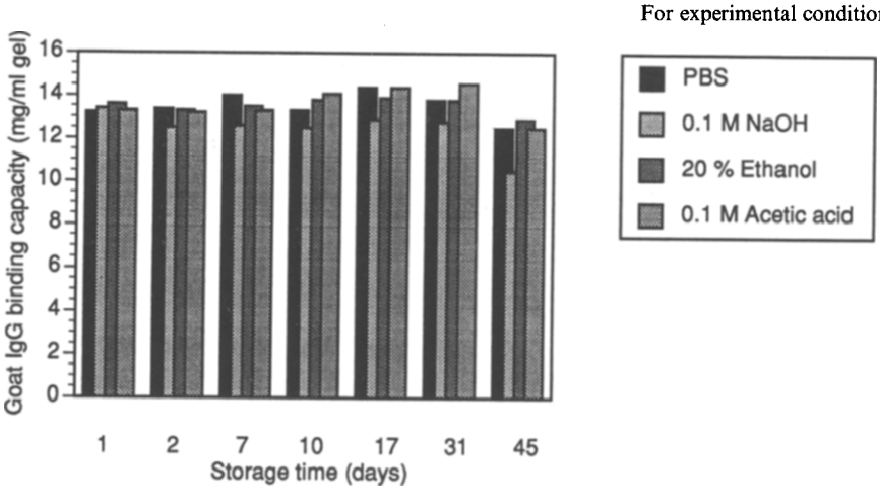

Fig. 6. Stability of Avid AL gel stored at room temperature under conditions commonly used for depyrogenation and cleaning (PBS, 20% ethanol, 0.1 *M* acetic acid and 0.1 *M* NaOH). For determination of the binding capacity, 3 ml of goat serum were diluted fourfold with PBS and applied to 1 ml of gel from the stock suspension. IgG was eluted with 50 m*M* sodium acetate, pH 2.8 adjusted with concentrated HCl.

electron donor–acceptor type of mechanism cannot be ruled out. An amine can act as a cation which competes with the ligand of Avid AL for the cation-binding sites on IgG. We therefore tested several charge-neutral, electron-rich compounds such as mercaptoglycerol, dithiothreitol and acetonitrile as the active ingredients in the formulation of neutral elution buffer. Indeed, all these charge-neutral, electron-rich compounds were very effective in displacing IgG bound to Avid AL gel (Table II). These results reinforce our notion that an electron donor–acceptor type of interaction plays a pivotal role in the mechanism of IgG binding to Avid AL. Further, we also demonstrated that IgG purified by using Avid AL and a neutral elution buffer has a consistently higher binding activity than that purified by using Avid AL and acidic elution buffer (Fig. 5). The purity of the IgG eluted with a neutral elution buffer is also comparable to that purified by other methods (Fig. 2). The stability of Avid AL under conditions commonly used for depyrogenation and storage is of paramount importance if the affinity gel is to be used in large-scale preparative work. The results of our study showed that Avid AL is stable in PBS, 0.1 *M* NaOH, 20% ethanol and 0.1 *M* acetic acid for up to 45 days (Fig. 6).

To demonstrate the utility of Avid AL in purifying multi-gram amounts of antibody, we packed Avid AL in a 1.5-l radial flow column. As shown in Fig. 7, 750 ml of goat serum can be processed, without prior treatment of the serum, within 5 h. This operation yielded 12 g of highly purified IgG (Fig. 2). The antibody can be eluted using either acidic or neutral buffer (Fig. 7a and b).

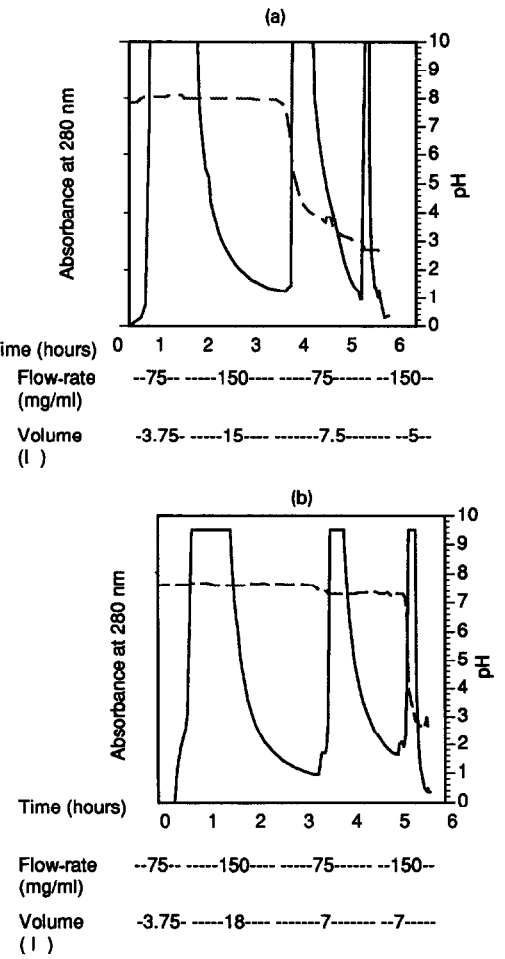

Fig. 7. Purification of IgG from goat serum using 1.5-l Avid AL AvidPak radial flow column. Solid lines refer to the absorbance profile (280 nm) and dashed lines to the pH scale as indicated by arrows. (a) Goat serum (750 ml) was diluted fourfold with PBS and passed through the column at a flow-rate of 75 ml/min. The column was then washed with PBS at a flow-rate of 150 ml/min. Elution was carried out with 50 m*M* sodium acetate, pH adjusted to 2.8 with concentrated HCl, at a flow-rate of 75 ml/min. The column was regenerated with a solution containing 20% methanol–1% acetic acid at a flow-rate of 150 ml/min. (b) Conditions as in (a), except that elution was performed with a neutral elution buffer at a flow-rate of 75 ml/min.

CONCLUSIONS

We have demonstrated that a novel synthetic affinity support, Avid AL, designed specifically for purifying IgG, was able to purify goat IgG directly from PBS-diluted serum. The affinity support, prepared with low-molecular-mass, non-protein, synthetic ligand unlike other IgG mimetic supports, does noet require a high concentration of salt for binding IgG to the adsorbent. In contrast to immobilized protein A or G gels, which require an acidic buffer to elute the bound IgG, a neutral buffer can be used to elute IgG bound to Avid AL. IgG eluted with neutral buffer consistently showed higher binding activity. It was demonstrated that electron donors were able to dissociate IgG from Avid AL. Further, we have shown that Avid AL is stable under conditions commonly used in depyrogenation and storage conditions. These conditions are known to render immobilized protein A and G gels inactive.

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