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Comprehensive study on binding capacity of human immunoglobulin G to Avid AL affinity gel

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Abstract

Avid AL is an affinity chromatographic gel based on a synthetic ligand which has been designed for the purification of immunoglobulins. The binding capacities of the gel for human IgG were investigated under different conditions; various salts; salt concentrations; different buffer systems with pH ranging from 3.8 to 10.0; and, tissue culture supernatant supplemented with 20% serum. At a similar ionic strength, the binding capacity changed according to the salting out effect of the ions used. Kinetic parameters of the affinity adsorption showed that a strong salting out effect significantly decreased the dissociation constant. Avid AL gel purified IgG from human serum with a capacity of 26.2 mg/ml gel using binding buffer containing sodium sulfate. Recombinant Protein A gel was used as a control. In this study it also exhibited enhanced binding capacity with a high salt concentration binding buffer.

1. Introduction

A synthetic ligand, Avid AL, which has been described in earlier studies from our laboratories [1–3], has been found to bind with high affinity to antibodies of many species; including species whose immunoglobulins do not bind well to Protein A or Protein G. Moreover, the advantage of Avid AL gel, with a low-molecular-mass synthetic affinity ligand, compared with Protein A or Protein G is its ability to withstand acid, base, organic solvent, proteolytic enzyme and autoclaving treatment [4]. Due to its action as a biospecific affinity chromatographic material, many protocols have been successfully developed for purification of monoclonal antibodies from serum, tissue culture supernatants, and ascites fluids [5,6]. In many cases, specialized and proprietary buffer systems must be employed in order to increase the binding capacity of the affinity chromatography gel, because the affinity adsorption between ligand and target protein is affected by the type of salt, ionic strength, components of buffer, and pH of solution.

In this report, we describe the effects of different types of salt and pH values on the binding capacity of Avid AL gel, and the results of the binding behavior of the gel using supernatants of cell culture media supplemented with 20% serum.

2. Materials and methods

Avid AL gel was produced by UniSyn Technologies (Tustin, CA, USA). A commercial

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recombinant Protein A (rProtein A) gel was provided by RepliGen (Cambridge, MA, USA). Solutions of purified human IgG containing a concentration of 12.5 mg/ml were purchased from The Binding Site (San Diego, CA, USA). Human serum was obtained from Sigma (St. Louis, MO, USA). Cell culture media (RPMI-1640) and fetal bovine serum (FBS) were obtained from Mediatech (Herndon, VA, USA) and Summit Biotechnology (Greeley, CO, USA), respectively. All other chemicals were of analytical grade.

2.1. Human IgG binding to Avid AL gel

Human IgG was bound to Avid AL gel by placing 18.0 μl of purified human IgG in 972.0 μl phosphate-buffered saline (PBS; 0.02 *M* phosphate, 0.14 *M* sodium chloride, pH 7.0) with 10.0 μl of Avid AL gel taken from a bulk of 20 ml which was washed first with regeneration buffer (20% methanol and 1% acetic acid in water, pH 3.4) and then with PBS for equilibration of the gel before using to bind IgG. The binding adsorption was run in a test tube overnight at 4°C with rotating on a Fisher hematology/chemistry mixer (Fisher Scientific, Pittsburgh, PA, USA). Unbound human IgG in solution, after binding was complete, was determined by using a radial immunodiffusion (RID) assay and a spectrophotometer at 750 nm with Dc protein assay (Bio-Rad, Hercules, CA, USA). The centrifuged supernatant of 5.0 μl was applied to a radial immunodiffusion plate (The Binding Site) following standard operating protocol. The binding capacity of Avid AL gel was determined by calculation of the corresponding amount of IgG for mass balance. The same was done with a rProtein A gel as a reference under the same conditions as used to determine the binding capacity of Avid AL gel.

2.2. pH dependence of human IgG binding

In order to evaluate the effect of buffer systems with different pH values on the binding capacity of Avid AL gel, five buffers were prepared including acetate buffer solution (0.1 *M*, pH 3.8, 4.5 and 5.5), PBS (pH 7.0, 8.0 and 8.5), carbonate buffer solution (0.1 *M*, pH 8.0), borate buffer solution (0.1 *M*, pH 8.0, 9.0 and 10.0), and Tris buffer solution (0.1 *M*, pH 8.0). Pre-equilibrated Avid AL gel (10.0 μl) was added to 972.0 μl of each buffer solution with 18.0 μl of purified human IgG. The binding of IgG to Avid AL gel was performed using the adsorption-equilibrium condition described above.

2.3. Chemical dependence of human IgG binding

Seven solutions, one for each of seven inorganic salts —NaCl, Na_2SO_4, $(NH_4)_2SO_4$, $MgCl_2$, NH_4CH_3COO, NH_4Cl and KCl— dissolved in PBS were prepared. Solution concentrations of 0.25, 0.50 and 0.75 *M* in each salt were prepared by diluting a 1.0 *M* solution of each salt. Since $MgCl_2$ has a solubility of less than 1.0 *M*, concentrations of 0.25, 0.50 and 0.75 *M* were used in the experiment. All of the solutions had a pH value of 7.0. Solutions of both NaN_3, where the concentration was changed from 0.0125 to 0.20% (v/v), and phenol red (from 2.5 to 20 mg/l) were used as factors to illustrate the effect of chemicals commonly used as a preservative and indicator of pH value in the cell culture, respectively, on the binding capacity of Avid AL gel. Conditions identical to those described above have been duplicated for all of the experiments.

2.4. Column configuration

Approximate 0.5 ml of Avid AL gel was packed into a 1.0-ml column. A Rainin (Woburn, MA, USA) peristaltic pump with an 8-channel head was used for buffer delivery. The column was washed with regeneration buffer until a baseline was obtained according to the absorbance, $A_{280\,nm}$, given by an LKB UV detector. The column was then pre-equilibrated with PBS. A sample of 1.35 ml of filtered human serum dissolved in 10.35 ml of binding buffer

was pumped through the column with a flow-rate of 0.25 ml/min, and 1.0-ml fractions were collected from the start of the loading step. The amount of fed human IgG was about 22 mg. Bound IgG was eluted using neutral elution buffer provided by UniSyn Technologies with a flow-rate of 1.0 ml/min. Protein output was measured and the absorbance of each fraction at 280 nm was used to estimate the protein concentration. Between runs the column was washed with regeneration buffer and PBS with minimal to no protein elution seen. Electrophoretic analyses were performed on 8–25% polyacrylamide gradient gels by using the Phast system from Pharmacia. The amount of purified IgG in fractions was confirmed using radial immunodiffusion.

3. Results and discussion

3.1. Salting-out effect in IgG purification with Avid AL

The effect of different types of salts on the binding capacity of Avid AL gel is summarized in Fig. 1. By adjusting the salt concentration to the same ionic strength in the assay (1.28 *M*), sodium sulfate, ammonium sulfate, ammonium acetate, ammonium chloride and sodium chloride gave an increase of the binding ability up to 44, 38, 33, 30 and 13%, respectively, as compared to potassium chloride. The same ionic strength (1.28 *M*) of buffer containing $MgCl_2$ showed a decreased binding capacity corresponding to 91.2% as compared to the same

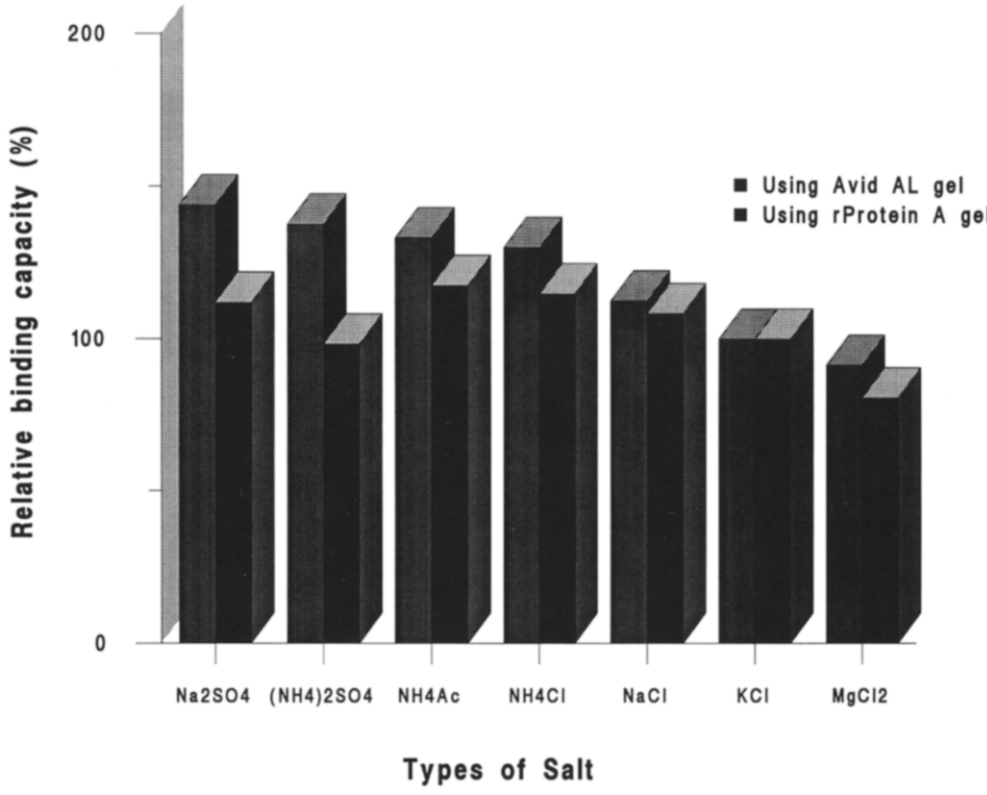

Fig. 1. Effect of various salts on purified human IgG binding to Avid AL and rProtein A gels. The binding capacity is shown as a relative value (percentage of the binding capacity obtained with KCl) and assayed at a total ionic strength of 1.28 *M* in buffer with pH of 7.0.

ionic strength of KCl. From the data, the salt effect on the binding capacity with respect to the various anions and cations can be deduced to be as follows: $SO_4^{2-} > CH_3COO^- > Cl^-$, and $NH_4^+ > Na^+ > K^+ > Mg^{2+}$. This represents the Hofmeister series of the salting out effect of ions (see [7]). For studies of the binding characteristics of Avid AL gel we used rProtein A gel as a reference. The results shown in Fig. 1 indicate that use of the salts, ammonium acetate, ammonium chloride, sodium sulfate and sodium chloride results in an increase of the binding ability to 18, 15, 12 and 8%, respectively, as compared to potassium chloride with the same ionic strength (1.28 *M*) in the buffer. However, for any given salt, no significant relationship has been observed between the binding capacity and the ionic strength for rProtein A gel.

Table 1 summarizes the effect of salt type and concentration on the partition coefficient, α, which is defined as the fraction of the total solute (purified human IgG) adsorbed at any instant because the affinity adsorption of purified human IgG to Avid AL gel represents a partitioning of solute between two phases, liquid and solid. We found that the binding capacity of Avid AL gel improves at higher ionic strengths of Na_2SO_4 and $(NH_4)_2SO_4$ with increasing α. By increasing the ionic strength from 0.75 to 3.0 *M*, a 1.8–2.2-fold increase in α was observed with Na_2SO_4 and $(NH_4)_2SO_4$. However, as expected for cations, only a weak effect on α was observed with increasing ionic strength from 0.25 to 1.0 *M* ($NH_4^+ > Na^+ > K^+ > Mg^{2+}$).

The parameters for the binding of purified human IgG to Avid AL gel, where the 9.4 to 27.5 mg IgG were added per ml gel, were determined using PBS containing different concentrations of Na_2SO_4 (from 0.25 to 1.00 *M*). The corresponding binding capacity is shown in Fig. 2. The apparent dissociation constant, K_a (mg/ml in solution), and the theoretical binding capacity of Avid AL gel, C_t (mg/ml gel), under the given experimental conditions are described by the equation:

$$1/P = C_t/(K_a P_b) - 1/K_a$$

where P is the IgG concentration in free solution at equilibrium with Avid AL gel and P_b is the IgG bound onto the Avid AL gel (mg/ml gel). Both K_a and C_t can be estimated from double reciprocal plots of the respective binding saturation curves and the values are summarized in Table 2. The dissociation constants for purified human IgG in PBS obtained under equilibrium conditions were 0.048, 0.022, 0.0058, 0.0052 and 0.0046 mg/ml, for Na_2SO_4 concentrations of 0, 0.25, 0.50, 0.75 and 1.00 *M*, respectively. It is apparent from Fig. 2 and Table 2 that the value of the dissociation constant estimated for purified human IgG binding to Avid AL gel decreased with increasing concentration of Na_2SO_4 in PBS (pH 7.0). The difference be-

Table 1
Salt effect on the partition coefficient of purified human IgG binding to Avid AL and rProtein A gels

Salt	α^a		α^b		α^c		α^d	
	Avid AL	rProtein A	Avid AL	rProtein A	Avid AL	rProtein	Avid AL	rProtein A
Na_2SO_4	0.45	0.49	0.63	0.47	0.76	1.00	0.83	0.97
$(NH_4)_2SO_4$	0.32	0.45	0.60	0.34	0.69	0.61	0.71	0.56
NH_4Ac	0.42	0.45	0.57	0.55	0.37	0.46	0.49	0.51
NH_4Cl	0.41	0.42	0.40	0.49	0.45	0.55	0.46	0.50
NaCl	0.40	0.48	0.45	0.51	0.41	0.47	0.40	0.47
KCl	0.40	0.42	0.39	0.46	0.36	0.38	0.36	0.43
$MgCl_2$	0.31	0.32	0.35	0.40	0.38	0.40	n.d.	n.d.

n.d. = Not determined.
[a–d] Tested at a salt concentration of 0.25, 0.50, 0.75 and 1.00 *M*, respectively.

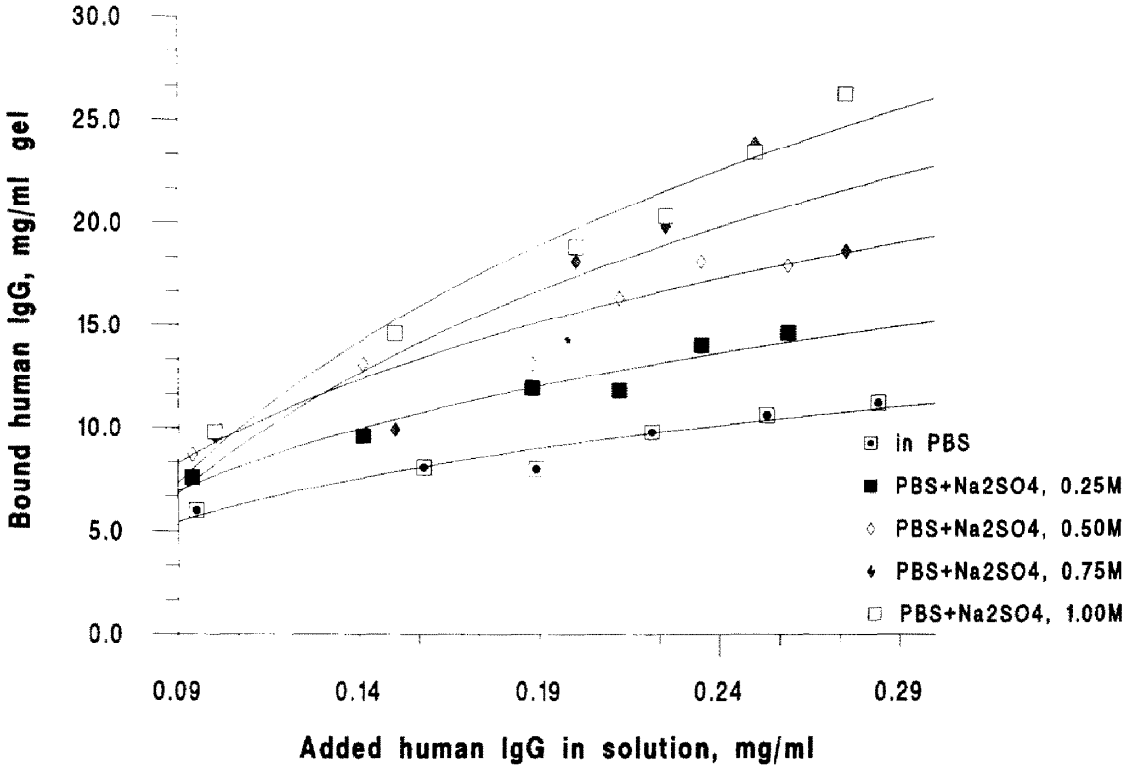

Fig. 2. Relationship between the concentration of purified human IgG and the amount of IgG bound in PBS containing different concentrations of Na_2SO_4 (the saturation curves).

tween K_a values for purified human IgG in PBS with no added Na_2SO_4 and in PBS plus 1.00 *M* of Na_2SO_4 was as much as ten-fold. The theoretical binding capacity of Avid AL gel for purified human IgG was 13.5 mg/ml gel in PBS, which is similar to the results reported by Ngo and Khatter [4], who used a packed Avid AL column (0.4 ml) and tested the binding capacity under equilibrium conditions.

The salting out effect of anions, such as sulfate, rather than the ionic strength is the major factor responsible for the observed increase in the binding capacity of Avid AL gel. It is assumed that this is made possible by a lowering of the dissociation constant for the affinity adsorption of purified human IgG onto the gel. This suggests that the high ionic strength causes aggregation by strengthening hydrophobic

Table 2
Binding parameters of Avid AL gel for purified human IgG in PBS containing different concentrations of Na_2SO_4

Na_2SO_4 concentration (*M*)	Dissociation constant K_a (mg/ml)	Binding ability C_t (mg/ml gel)
PBS	0.0480	13.5
PBS + Na_2SO_4, 0.25	0.0220	16.1
PBS + Na_2SO_4, 0.50	0.0058	22.7
PBS + Na_2SO_4, 0.75	0.0052	27.1
PBS + Na_2SO_4, 1.00	0.0046	29.9

interaction between proteins. Consequently, in the presence of sulfate the specific affinity of IgG for Avid AL may be improved by reducing the repulsion between IgG molecules. In general, the repulsion of bound IgG molecule on the gel prevents the affinity attraction of additional unbound IgG molecules to an available adjacent ligand. For rProtein A gel, the effect of salting out increases the hydrophobic attraction between rProtein A and IgG. Therefore, the opportunity for enhancing IgG binding to functional sites of rProtein A is realized.

These initial results were confirmed by performing column chromatography experiments. 0.5 ml of Avid AL gel was packed in a 1.0-ml column. The chromatograms shown in Fig. 3 are for such columns, wherein human serum was diluted by either PBS (regular binding buffer) or PBS containing 0.75 *M* of Na_2SO_4 as an improved binding buffer. The bound IgG, then, was eluted using neutral elution buffer with pH of 7.4. Between runs the column was washed with regeneration buffer and PBS with minimal to no protein elution seen. This operation yielded 8.0 mg and 14.6 mg bound IgG on the column, respectively. The binding capacity for Avid AL in the high salt buffer was calculated at 26.2 mg IgG/ml gel based on the amount of IgG eluted, which was 2.1-fold higher than those of the run using PBS as a binding buffer. A comparison of the characteristics for IgG purification using Avid AL gel has been listed in Table 3 for the two binding buffer systems. In both cases, the IgG was recovered at greater than 90% purity estimated by using the ratio of eluted IgG amount determined by RID to eluted protein amount determined by spectrophotometry at 280 nm. This result was confirmed by use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8–25%) analysis, as shown in Fig. 4. In particular, albumin and possibly transferrin in human serum cannot be strongly adsorbed onto the Avid AL gel in presence of high salt buffer solution when the amount of IgG being isolated is more concentrated than other proteins in solution [1]. Therefore, non-specific adsorption, as a major source of contamination in IgG purification by Avid AL gel, has not been significantly observed when sodium sulfate was added to increase the ability to isolate IgG from human serum (see lane 8 in Fig. 4).

3.2. Effect of sodium azide on IgG–Avid AL binding

In contrast to the effect of salts studied on binding capacity, a significant decrease in the binding capacity of either Avid AL gel or rProtein A gel can result when sodium azide, commonly used as a bacteriostatic, is present in PBS as a binding buffer. But the proposed structure of the affinity ligand is consistent with its functioning as an electron acceptor. Therefore, it is possible that the sodium azide, as an electron donor, competes with IgG molecules for binding the affinity ligand and consequently displaces the IgG from the functional site. In this case, the relative binding capacity of Avid AL with increasing sodium azide from 0.0125 to 0.20% (v/v) showed a corresponding decrease of 97.2 to 70.8%, respectively, as compared to the binding capacity of Avid AL in PBS only (see Fig. 5). Under the same conditions, rProtein A gel exhibits a similar corresponding decrease of 94.5 to 52.0%, respectively, as compared to the binding capacity of rProtein A gel obtained in PBS.

3.3. Effect of phenol red on IgG–Avid AL binding

The same reason can be used to describe the decrease in the binding capacity of Avid AL gel when phenol red, which is usually used as a pH indicator in cell culture medium, is present in binding buffer (PBS). Fig. 6 shows the binding behavior of Avid AL gel and rProtein A gel for human IgG in PBS containing phenol red where the concentration was varied from 2.5 mg/ml to 20.0 mg/l. Defining the interaction between the ligand and IgG in PBS as 100%, a significant decline (to 50.9%) was observed for the binding capacity of Avid AL in the presence of 20.0 mg/l of phenol red in binding buffer. A similar decrease (to 78.7%) in binding capacity is observed

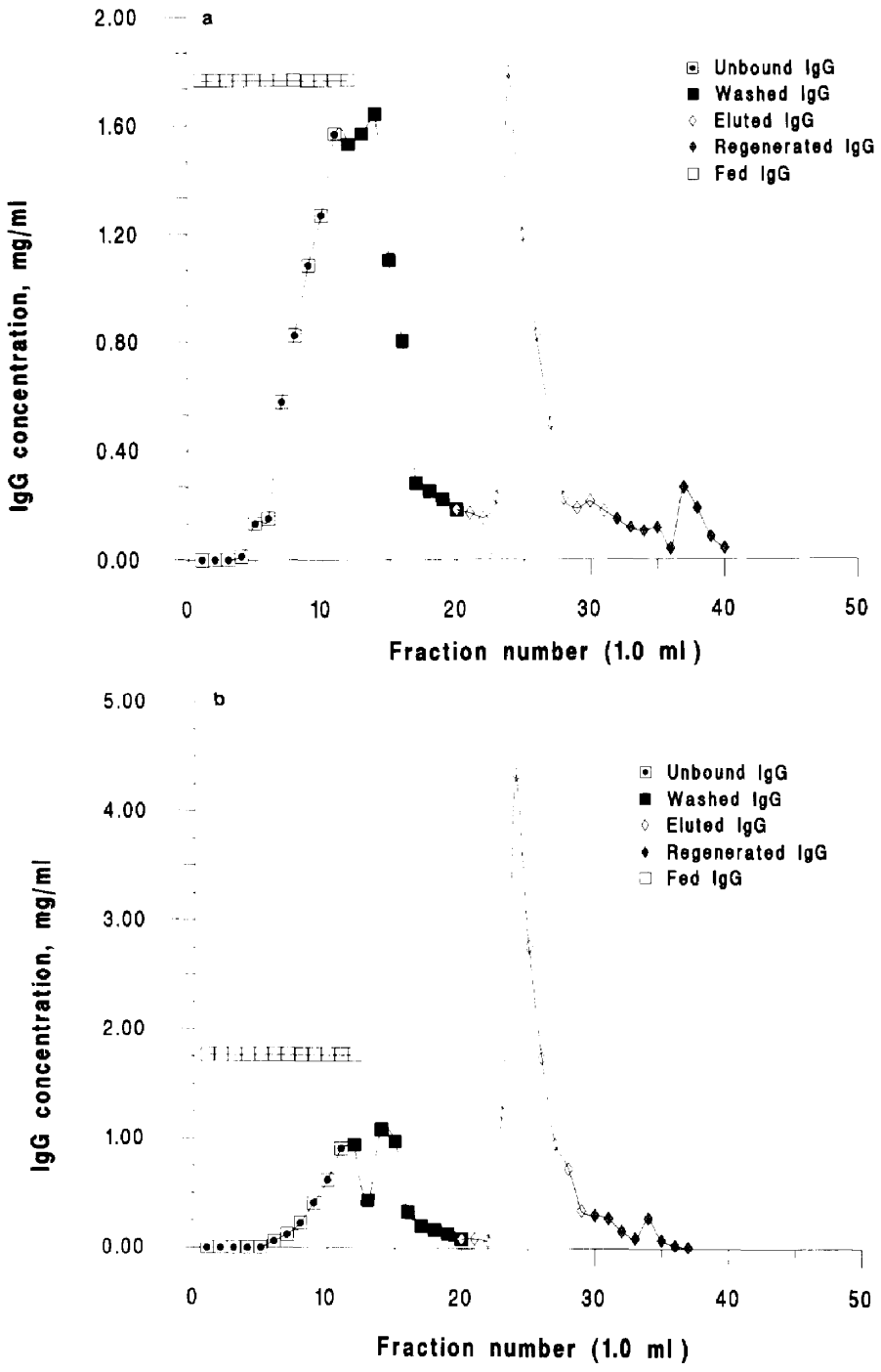

Fig. 3. (a) Isolation of IgG from human serum by Avid AL gel with PBS loading and washing conditions. (b) Isolation of IgG from human serum by Avid AL gel with PBS–0.75 *M* of sodium sulfate loading and washing conditions. For both of experiments, gel volume was 0.5 ml. Human serum was diluted 8.9-fold with binding buffer to make a IgG concentration of about 2 mg/ml in solution and applied at the flow-rate of 0.25 ml/min at room temperature. Fractions of 1.0 ml were collected. Elution was carried out with neutral elution buffer, pH 7.4.

Table 3
Comparison of properties of purifying IgG from human serum using Avid AL gel in two different binding buffer systems

Item	Experiment	
	1	2
Volume of column (ml)	0.5	0.5
Binding buffer	PBS	PBS + 0.75 *M* Na_2SO_4
Fed IgG (mg)	21.2	21.2
Bound IgG (mg)	8.0	14.6
Eluted IgG (mg)	6.2	13.1
Regenerated IgG (mg)	1.0	0.9
Recovery yield (%): IgG bound/IgG eluted	77.5	89.7
Binding capacity (mg/ml gel): IgG eluted/volume of column	12.4	26.2

Each IgG amount shown was calculated based on the data determined by RID.

for the rProtein A gel in the presence of 20.0 mg/l of phenol red in PBS.

Addition of FBS to the binding buffer results in an even more pronounced decrease in the binding capacity of Avid AL for human IgG. With respect to the affinity adsorption in PBS at pH 7.0, the use of PBS containing 20% FBS resulted in a decrease of the binding capacity of Avid AL to 42.5%. The determined binding capacities of Avid AL gel and rProtein A gel have been listed in Table 4. Use of the cell-culture medium, RPMI, in place of PBS appears to result in little or no significant change in the IgG binding capacity of Avid AL gel or rProtein A gel. At a concentration of 20% FBS in either PBS or RPMI, the binding capacity of Avid AL decreased to 42.5 and 46.2%, respectively (see Table 4). However, no significant influence on the binding capacity of rProtein A gel was observed under the same conditions. On the other hand, as can be seen in Table 4, increasing the concentration of Na_2SO_4 in this system decreases the amount of affinity adsorption on Avid AL gel. In contrast, rProtein A gel showed an increase in IgG binding capacity with the increase in Na_2SO_4 concentration. In order to avoid disturbance by undesired proteins during purification of the target protein, dilution of undesired proteins, such as those in FBS, in solution may be necessary before using this affinity gel. It is commonly understood that high concentration of undesired proteins can lower the affinity adsorption between target protein and affinity ligand.

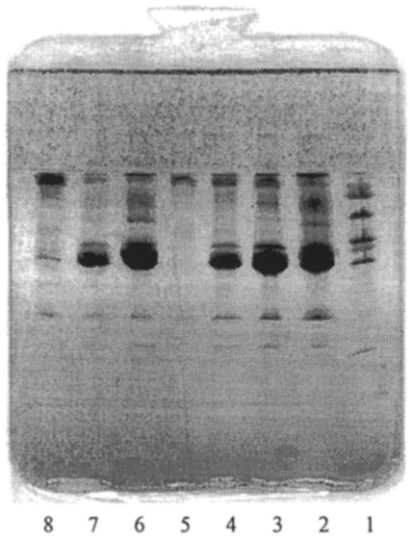

Fig. 4. Sodium dodecyl sulfate gradient (8–25%)–polyacrylamide gel electrophoresis in non-reducing conditions of fractions obtained from human serum in Fig. 3. Lanes: 1 = Molecular mass marker reference proteins; 2 = unfractionated human serum; 3, 6 = unbound, flow-through fractions; 4, 7 = unbound, after column wash; 5, 8 = fractions eluted with neutral elution buffer, pH 7.4. Lanes 3–5 are corresponding to Fig. 3a, lanes 6–8 to Fig. 3b.

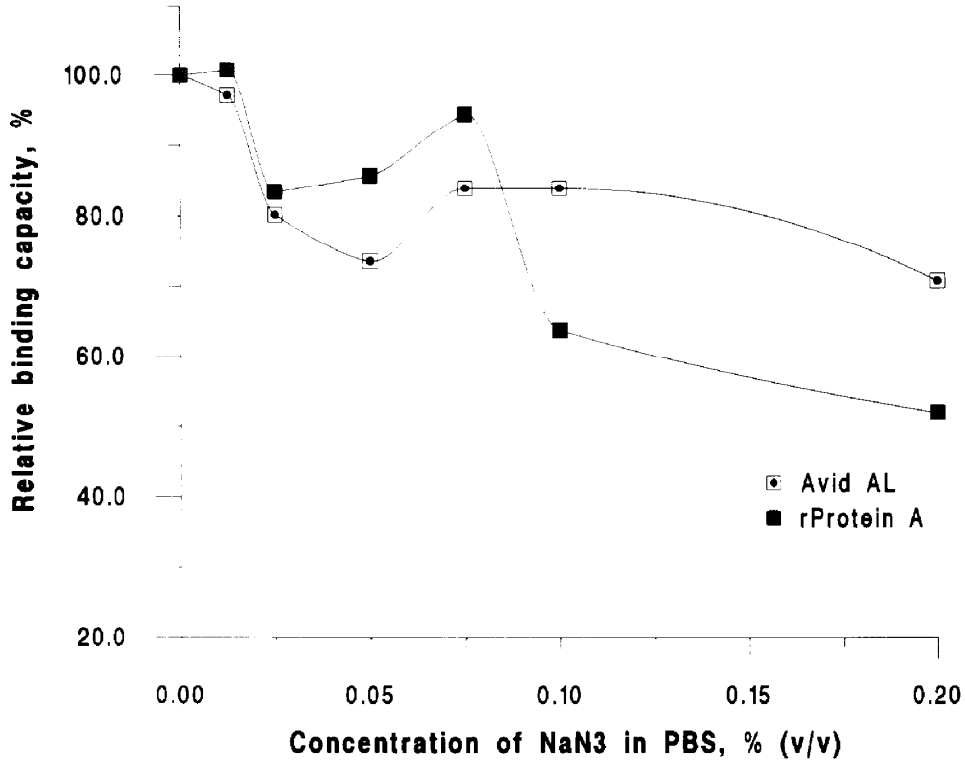

Fig. 5. Effect of NaN_3 in PBS on the binding capacity of Avid AL and rProtein A gels. The binding capacity is shown as a relative value corresponding to that obtained in PBS without NaN_3.

3.4. Effect of pH on IgG–Avid AL binding

Previous studies indicated that Avid AL was chemically stable to acid and base treatments. For further characterization of Avid AL, a wide range of buffers with different pH values has been used to evaluate the binding capacity. Five buffers ranging in pH from 3.8 to 10.0 were tested to determine the effect of pH on IgG binding to this gel. Fig. 7 shows the binding capacity of each gel at each pH relative to the binding capacity in PBS at pH 7.0. Obviously, Avid AL continues to work well for binding IgG, when pH of the binding buffer has an acidic value. Using other buffers with pH higher than 7.0, less binding of purified human IgG to Avid AL relative to rProtein A gel was observed. Unfortunately, at pH 3.8, neither Avid AL nor rProtein A gels can adsorb any measurable quantity of IgG. From the data shown in Fig. 7, it has been realized that in the range of pH 7.0–8.5, the Avid AL gel has similar binding ability in PBS, carbonate or borate buffers. However, use of primary amine buffering system, such as Tris buffer, allows the primary amine to compete with the IgG for binding to either Avid AL or rProtein A gel. Therefore, decreased binding capacities of both gels have been obtained.

4. Conclusions

In general, the affinity chromatographic gel Avid AL displays a significant increase in binding capacity for human immunoglobulin with increasing concentration of Na_2SO_4. Approximately 13.1 mg of human IgG has been recovered from a column with packed 0.5 ml of Avid AL gel. This binding capacity of 26.2 mg/

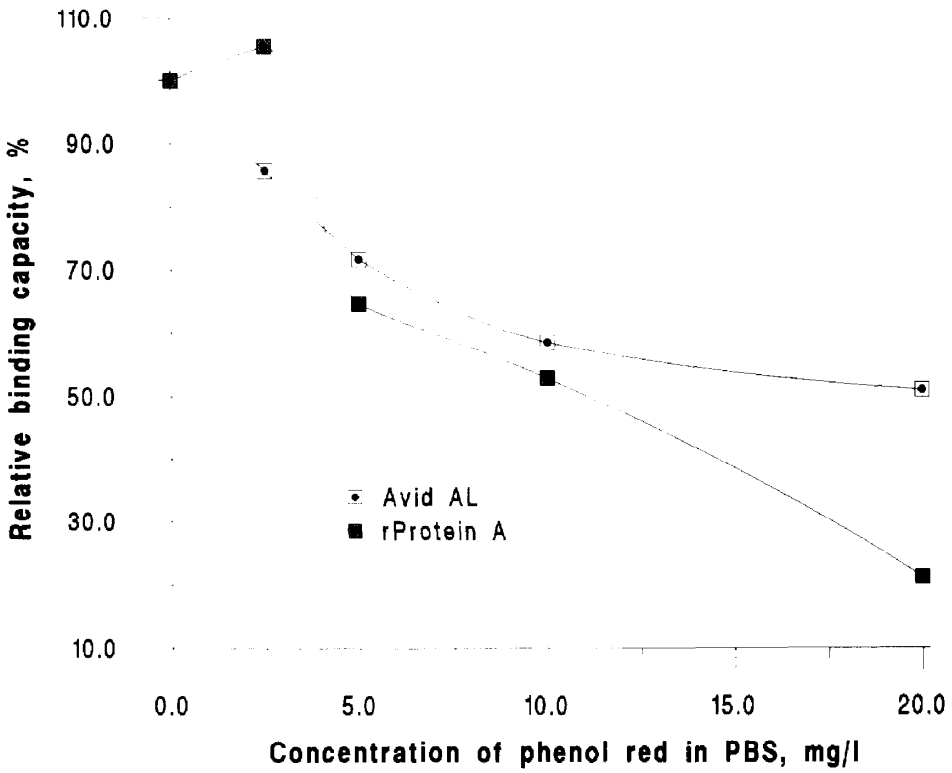

Fig. 6. Effect of phenol red in PBS on the binding capacity of Avid AL and rProtein A gels. The binding capacity is shown as a relative value corresponding to that obtained in PBS without phenol red.

Table 4
Effect of cell culture medium supplemented 20% FBS with and without Na_2SO_4 on the binding capacities of both Avid AL and rProtein A gels in batch assay at pH of 7.0

Binding buffer	Avid AL[a] (%)	rProtein A[a] (%)
PBS	100	100
RPMI	97.2	90.6
PBS + 20% FBS	42.5	90.6
RPMI + 20% FBS	46.2	86.6
RPMI + 20% FBS + 0.25 *M* Na_2SO_4	49.1	70.9
RPMI + 20% FBS + 0.50 *M* Na_2SO_4	45.3	89.8
RPMI + 20% FBS + 0.75 *M* Na_2SO_4	41.5	93.7
RPMI + 20% FBS + 1.00 *M* Na_2SO_4	36.8	131.5

[a] The binding capacity is shown as a relative value (percentage of the binding capacity obtained in PBS).

ml gel is far superior to the binding capacities reported previously [3]. It is important to note that binding buffer containing sodium azide will harshly decrease the affinity adsorption of IgG to Avid AL. In addition, diluting the tissue culture supernatants supplemented 20% FBS is necessary to reduce either the concentration of other mixed proteins or the percentage of phenol red presented for successful IgG purification using Avid AL affinity adsorption chromatography. Considering the above results in the presence of 20% FBS supplemented in tissue culture supernatants, rProtein A gel appears to be the most suitable material for human IgG purification without significant decrease in binding capacity. As a synthetic ligand, Avid AL maintains functional activity in acidic conditions in which rProtein A gel can lose its affinity binding capacities, even through with elevating pH over 7.0 rProtein A gel appears to be preferable in binding capaci-

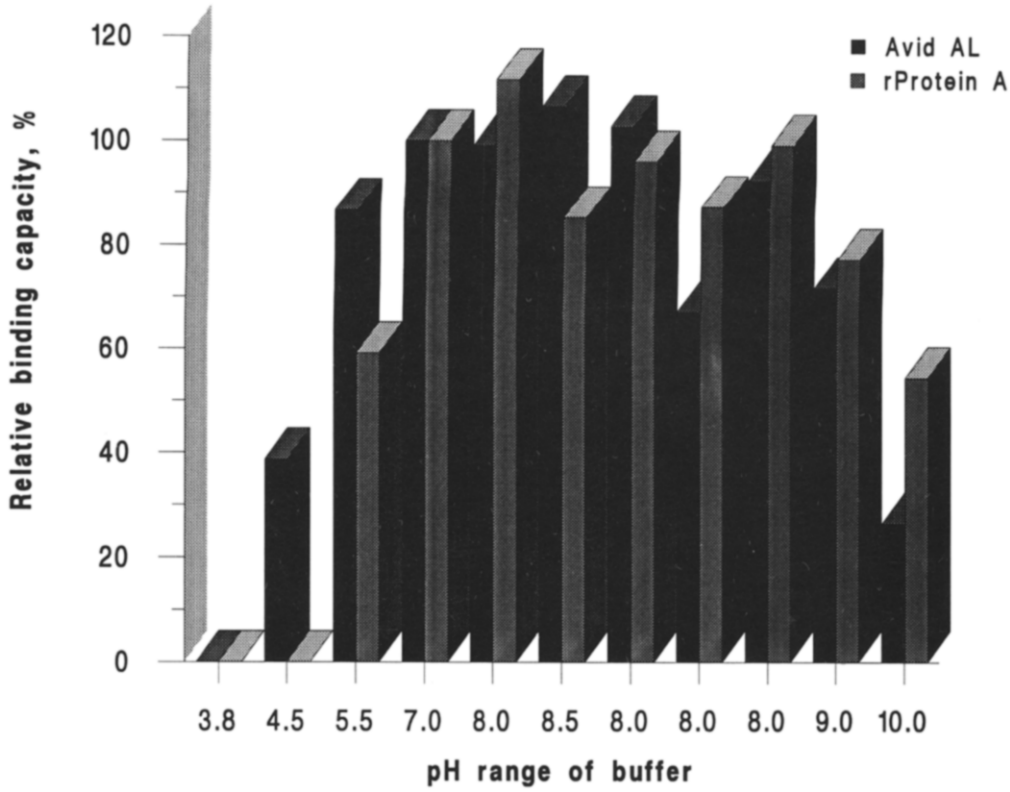

Fig. 7. Effect of pH on binding capacity of Avid AL and rProtein A gels. The relative binding capacity is shown corresponding to that obtained in PBS, pH 7.0. pH 3.8 = sodium acetate; pH 4.5 = sodium acetate; pH 5.5 = sodium acetate; pH 7.0 = PBS; pH 8.0 = PBS; pH 8.5 = PBS; pH 8.0 = sodium carbonate, Tris and sodium borate (from left to right); pH 9.0 = sodium borate; pH 10.0 = sodium borate.

ty relative to that observed with Avid AL gel. This unique ability to efficiently bind immunoglobulins at low pH may be exploited in GMP purification protocols. Therefore, the combination between virus inactivation and the column loading step, to reduce overall processing time, will be investigated in future studies.

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