Journal of Chromatography, 604 (1992) 29–37
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 195

Interaction of immunoglobulin G with immobilized histidine: mechanistic and kinetic aspects

A. El-Kak, S. Manjini and M. A. Vijayalakshmi

Laboratoire de Technologie des Séparations, Université de Technologie de Compiègne, B.P. 649, 60206 Compiègne (France)

ABSTRACT

A systematic investigation of coupling methods for and the chemistry and chromatographic parameters of immunoglobulin gamma (IgG) adsorption to histidine- and imidazole-coupled Sepharose gels was undertaken in order to elucidate the interactions involved in the mechanism of recognition between IgG and the immobilized histidine. The effects of pH, salt and temperature effects indicated an ion-pairing mechanism, rather than a mechanism based on the net charge of the protein (IgG), but with some localized complementary charges recognizing the unprotonated imidazole nitrogen. The effects of the addition of ethylene glycol and urea indicated the involvement of hydrogen bonding between the ligand and the protein. The immobilized histidine binds to the Fc fragment of IgG with a fairly low affinity, in a way similar to the N-terminum of protein A binding to the Fc fragment of IgG. The kinetic parameters of the chromatographic system indicated a good capacity but a low adsorption rate constant.

INTRODUCTION

Single amino acids have been reported to be fairly selective and efficient immobilized ligands for the purification of a variety of proteins. Fibronectin and plasminogen were specifically retained on and eluted from Arg- and Lys-immobilized columns, respectively [1,2]. Tryptophan, with its predominant aromatic stacking properties, was shown to adsorb selectively proteins rich in aromatic residues [3]. Histidine has been reported to retain a plethora of biomolecules such as proteins, peptides and bioamines, each selectively under given adsorption conditions, particularly at pH values at or around the isoelectric pH of the molecule [4,5]. Of particular interest are the recent publications of immunoglobulin gamma (IgG) purification using histidine-linked insoluble matrices [6–8]. These investigations showed the possibility of recovering the subclasses IgG_1 and IgG_2 from serum extracts containing polyclonal antibodies and monoclonal antibodies from cell culture or ascite fluids.

Although the recovery of subclasses of IgG from different sources, by a very mild desorption using 0.2 *M* NaCl in the adsorption buffer, was demonstrated very clearly, not much is known regarding the mechanism of this selective recognition of IgG subclasses by the polymer-supported histidine ligand. While the ionic properties of both the immobilized ligand and the protein seem to be important in this recognition, the fact that adsorption takes place preferentially at or around the isoelectric pH of the protein is intriguing.

In this work we investigated the possible mechanism(s) of interactions involved in the recognition of IgG by immobilized histidine. Some possibilities of this single amino acid mimicking one of the binding sites of protein A from *Staphylococcus aureus* were evaluated. Moreover, with the aim of eventually scaling up this very simple technique, we studied the kinetic and thermodynamic parameters, such as affinity constants and adsorption rate constants, of this adsorption.

Correspondence to: Dr. M. A. Vijayalakshmi, Laboratoire de Technologie des Séparations, Université de Technologie de Compiègne, B.P. 649, 60206 Compiègne Cedex, France.

0021-9673/92/$05.00

EXPERIMENTAL

AH-Sepharose, CH-Sepharose and Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden), carbodiimide, L-histidine and histamine from Sigma (St. Louis, MO, USA), 1,4-butanediol diglycidyl ether from Aldrich (Milwaukee, WI, USA) and epichlorhydrin and sodium tetrahydroborate from Merck (Darmstadt, Germany). The model peptides used were purchased from Bachem (Bubendorf, Switzerland).

Preparation of adsorbents

For the preparation of histidyl-Sepharose (H-Seph) we adopted the experimental protocol described previously [4]. The gel was prepared by introducing reactive oxirane after activation with epichlorhydrin. Then the active oxirane was opened and coupled to the primary amine of the histidine.

For the preparation of histidyl-AH-Sepharose (H-AH-Seph), AH-Sepharose was used as the starting material and histidine was coupled using water-soluble carbodiimide at pH 4.5–6 with lateral stirring for 6 h at room temperature.

All the other gels were prepared either using oxirane intermediates with or without spacer as indicated or using CH-Sepharose as starting material with carbodiimide chemistry.

The different gels are shown schematically in Fig. 1.

Typical chromatography

Purification was performed using a column (6.3 × 1.0 cm I.D.) containing the gels prepared as described above. The bed volume was 5 ml. A linear flow-rate of 45 cm h^{-1} was used throughout. The temperature was kept at 4°C unless specified otherwise. A 184-μl aliquot containing 30 mg of human placental IgG was injected into the column, pre-equilibrated with 25 m*M* Tris–HCl buffer (pH 7.4). Elution was carried out successively using the same buffer containing 0.2 and 1 *M* NaCl. The absorbance of the eluate was measured at 280 nm. Fractions of 3 ml were collected. After each use the column was washed with three column volumes of 50 m*M* NaOH solution followed by washing with water and finally with the equilibrating buffer 25 m*M* Tris–HCl (pH 7.4).

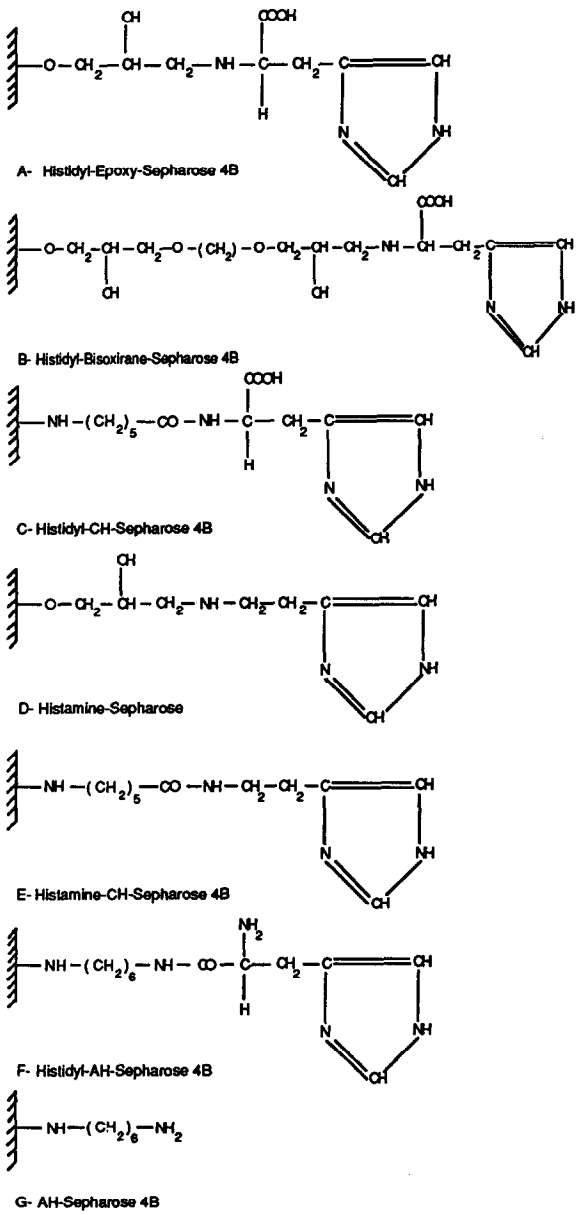

Fig. 1. Schematic structures of the adsorbents.

Study of thermodynamic and kinetic parameters

Determination of dissociation constant, K_D. For this study, a column (1.3 × 1.0 cm I.D.) was filled with H-AH-Seph gel and a preparation of human placental IgG containing 157 mg ml^{-1} of IgG (*ca.* 95% in IgG) was used.

To the column, equilibrated with 25 m*M* Tris–HCl (pH 7.4) a protein solution (dissolved in the same buffer) with different concentrations ranging from 0.5 to 25 mg/ml^{-1} of IgG was fed. The absorbance of the effluent at 280 nm was monitored continuously. The injection was continued in a frontal mode until the absorbance of the effluent reached a plateau. The column was then washed with the same buffer until the absorbance of the effluent at 280 nm reached the baseline. The adsorbed IgG was then eluted successively with the same buffer containing 0.2 and 1.0 *M* NaCl.

For each concentration the capacity of the column was determined as the amount of IgG retained in milligrams per millilitre of the gel. Protein concentrations were determined using a molar absorptivity of 212 000 l mol^{-1} cm^{-1} for IgG. The data obtained were fitted to the Langmuir model as shown below.

$$Q_a = \frac{Q_x K_a [C]}{1 + K_a [C]} \quad (1)$$

where C is the concentration of protein solution (mg ml^{-1} of IgG or M), K_a is the equilibrium association constant (ml mg^{-1} or l mol^{-1}), Q_a is the amount of adsorbed protein (mg IgG ml^{-1} gel) and Q_x is the maximum adsorption capacity of the gel (mg IgG ml^{-1} gel).

The dissociation constant, K_D, which is equal to $1/K_a$, was calculated from the linearized plot of the equation:

$$\frac{[C]}{Q_a} = \frac{[C]}{Q_x} + \frac{1}{Q_x K_a} \quad (2)$$

Determination of adsorption rate constants. This was done by using the split-peak approach as described elsewhere [9]. A solution of IgG (2 mg ml^{-1}) was prepared in 15 ml of equilibrating buffer (Tris–HCl, 25 m*M*, pH 7.4). Aliquots of 1 ml were injected consecutively into the equilibrated column. Unadsorbed protein was collected as fraction 1 (peak 1) and the adsorbed protein was later recovered as fraction 2 (peak 2). In this manner fifteen fractions representing peak 1 were collected before proceeding with the recovery of the retained protein. In each peak, amounts of protein were determined by multiplying the absorbance at 280 nm by the volume of the fraction collected and dividing by 1.4 (assuming $A^{1\%}_{280\,nm} = 14$ for IgG). From the available information, the rate constant of adsorption could be calculated from the equation [9]

$$f = \exp(-k_a Q_x / \delta) \quad (3)$$

where f is the ratio of unretained to injected protein, Q_x is the maximum loading capacity of the column (mol) and δ is the flow-rate (l s^{-1}). Q_x was obtained from the previous experiment (adsorption isotherm) as 10.5 mg.

RESULTS

In a previous paper [7], it was shown that residual charges on immobilized histidine resulting from the coupling chemistry used and the functional group of the histidine implied in the coupling, and also the presence of a spacer arm, play important roles in the efficiency of the IgG purification. Moreover, the selectivity for the IgG_1 subclass was unimpaired in all instances whereas the introduction of the spacer, aminohexyl group, allowed the recovery of a second subclass, IgG_2 [7].

Fig. 2 shows the elution of IgG from a placental serum extract (containing >95% IgG) at pH 7.4 on the different gels (see Fig. 1). It is clear that the absence of free COOH groups improves the efficiency of IgG adsorption and recovery. This systematic investigation of adsorbents with histidine, with or without a spacer, and histamine instead of histidine has shown that those adsorbents with free COOH groups exhibited lower IgG retention capac-

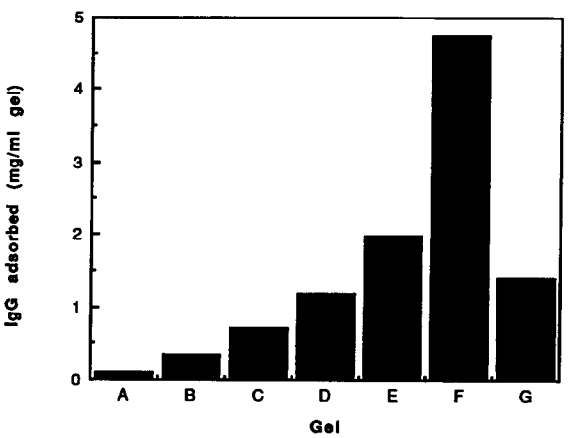

Fig. 2. IgG retention capacity in mg IgG ml^{-1} adsorbent. For A–G, see Fig. 1.

ities when compared with those without the free COOH of the histidine ligand (compare adsorbents A, B and C with D, E and F).

Further investigations reported here were limited to two adsorbents, H-AH-Seph and H-Seph, in order to evaluate the influence of free COOH groups and the presence of a spacer between the matrix and the ligand.

Influence of pH, ionic strength and adsorption temperature on IgG retention

Fig. 3a and b show the efficiency of the purification of IgG subclasses using H-AH-Seph and H-Seph columns, respectively. Although the ionization of the ligand is different in these two adsorbents, the optimum pH of the adsorption was found to be 7.4

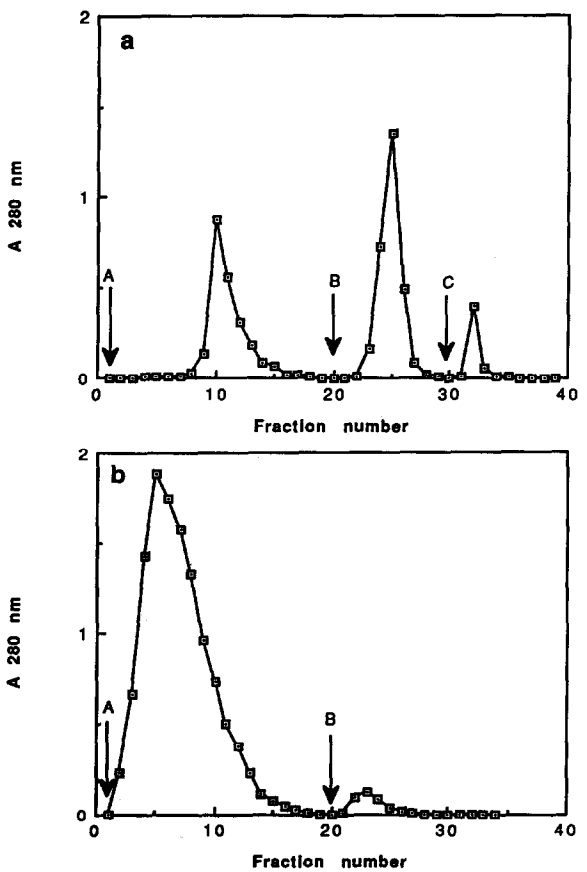

Fig. 3. Typical elution profiles of IgG chromatographed on (a) H-AH-Seph and (b) H-Seph (see text for details). (A) 25 m*M* Tris–HCl buffer (pH 7.4.); (B) A + 0.2 *M* NaCl; (C) A + 0.4 *M* NaCl.

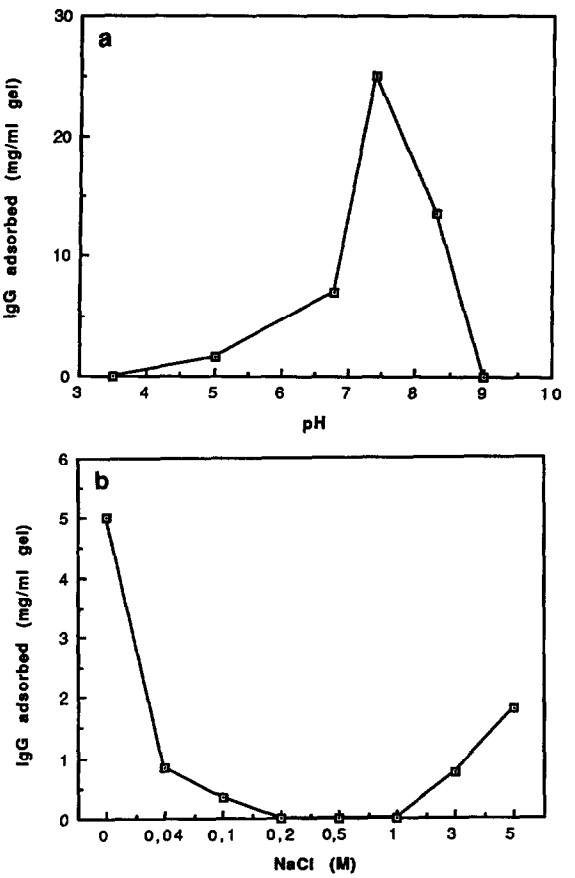

Fig. 4. Influence of adsorption parameters on the retention of IgG on H-AH-Seph: (a) pH; (b) added NaCl concentration.

(Fig. 4a). The difference in their ionization was reflected only in the amount of IgG_1 adsorbed, 5 mg ml^{-1} for H-AH-Seph compared with *ca.* 0.5 mg ml^{-1} for H-Seph. Moreover, both adsorbents retained IgG_1 at low salt concentrations in the adsorption buffer.

This indicates a mechanism of recognition based on ion-pair formation between the ligand and IgG_1. However, secondary interactions such as hydrogen bonding and mild hydrophobicity cannot be excluded. In fact, as shown in Fig. 4b, the efficiency of adsorption is maximum in the absence of any added NaCl in the buffer whereas it passes through a minimum (no adsorption) from 0.2 to 1.0 *M* added NaCl in the buffer. There was a slight increase in the amount of retained IgG at NaCl concentrations >3.0 *M*.

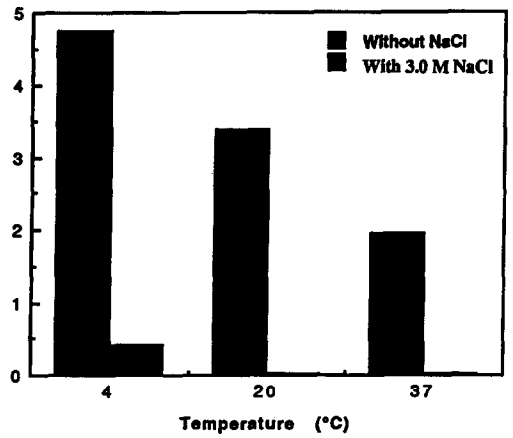

Fig. 5. Effect of temperature and NaCl concentration on IgG adsorption on H-AH-Seph.

The histogram in Fig. 5 shows the influence of temperature and high salt concentration on the adsorption efficiency and selectivity of retention of IgG subclasses on H-AH-Seph. Whereas 4 and 20°C favour the retention of 23.7 and 16.9 mg of IgG_1 and IgG_2, at 37°C no IgG_2 was retained on the column and only 9.8 mg of IgG_1 was retained and eluted when 0.2 *M* NaCl was added to the buffer. The presence of 3.0 *M* NaCl in the adsorption buffer showed a moderate retention of IgG at 4°C (2.1 mg ml^{-1}) which is almost ten times lower than that in the absence of NaCl. The retention at 20 and 37°C was almost negligible. This indicates the involvement of electrostatic and hydrogen bond interactions rather than hydrophobic interactions between the protein and the ligand.

Effect of water structure-modifying additives in the adsorption buffer

In order to elucidate better the water structure-mediated interactions, we studied the influence of added urea, ethylene glycol and sorbitol on the retention of IgG. Urea concentrations >3.0 *M* decreased the IgG retention almost to zero. The graph describing the effect of ethylene glycol was somewhat bell-shaped. The adsorption decreased with increasing concentration of ethylene glycol from 0 to 30%, and then increased with higher concentrations of ethylene glycol from 30 to 50%. Moreover, when the ethylene glycol concentration was increased to 60% the adsorption dropped to the minimum level (Fig. 6). This can be attributed to the fact that ethylene glycol at concentrations higher than 50% will totally destroy the hydrogen bonds and the structured water molecules, which in turn can result in denaturation of the protein. These effects of the addition of urea or ethylene glycol suggest strongly the involvement of hydrogen bonds in the mechanism of recognition between the IgG and the immobilized histidine. Moreover, when 1.0 *M* sorbitol was included in the adsorption buffer the IgG retention decreased by up to 60% (data not shown).

Identification of the ligand binding site on IgG

It was important to determine whether IgG was bound to the immobilized histidine at Fc or Fab. We therefore chromatographed separately the Fc and Fab fragments, obtained by papain hydrolysis, under the same conditions as used for IgG retention. It was found that the Fc fragment but not the Fab bound to the adsorbent.

However, we could not probe the specific amino acid residues of Fc implied in this binding mechanism. On the other hand, the data obtained for the adsorption parameters indicated the importance of ion-pairing and hydrogen-bonding mechanisms in IgG binding to the immobilized histidine (Figs. 3–5). In order to pinpoint the specific amino acid residues or peptide sequences involved in this recognition, we used seven synthetic peptides containing N-terminal aspartic acid, based on the fact that the ionic

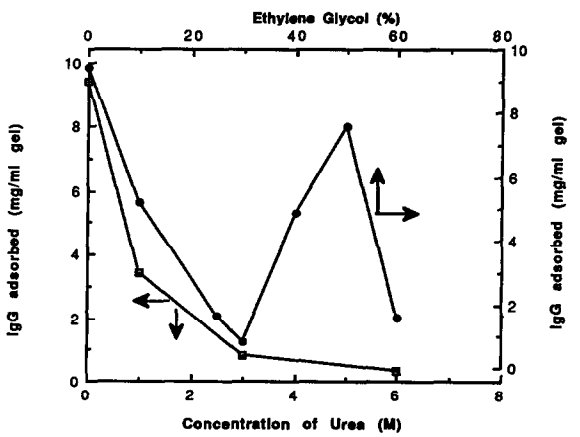

Fig. 6. Influence of water structure-modifying additives on IgG adsorption on H-AH-Seph. □ = Urea; ● = ethylene glycol.

interaction seemed the most predominant. We studied their retention behaviour on both H-Seph and H-AH-Seph at pH 7.4. All the peptides were eluted almost unretarded and without discrimination on the H-Seph column (data not shown). H-AH-Seph showed differences (Fig. 7). In fact, peptide 1 was very strongly retained and eluted from the column only with the addition of 1.0 *M* NaCl to the adsorption buffer. It was also interesting that peptides 7 and 4, differing only by a sulphate in a tyrosyl residue, showed distinct differences in their retention. Peptide 4 was not adsorbed to the column whereas peptide 7 was totally retained and eluted on addition of 0.2 *M* NaCl to the adsorption buffer. Another striking feature was that peptide 6 with a C-terminal lysine residue was partially retained and eluted with the addition of 0.2 *M* NaCl to the initial buffer.

Evaluation of the thermodynamic and kinetic parameters of IgG adsorption to H-AH-Seph

The high efficiency of specifically purifying IgG_1 and IgG_2 from different sources of IgG using H-AH-Seph adsorbent prompted us to study the kinetic aspects of this adsorption for an eventual scale-up of the separation of IgG from either placental serum or plasma.

At the outset it was very important to know the strength of binding and the maximum capacity. It was also important to know the rate of adsorption for automated operation.

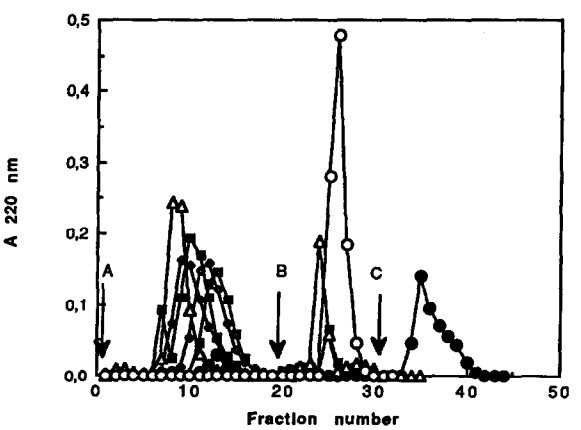

Fig. 7. Elution of model peptides on H-AH-Seph with 25 m*M* Tris–HCl buffer (pH 7.4) at 4°C. ● = DASGE (1); ◆ = DRVYIHPF (2); □ = DYM (3); ◇ = DYMG (4); ■ = DY (5); △ = DAHK (6); ○ = DY(SO_3)MG (7).

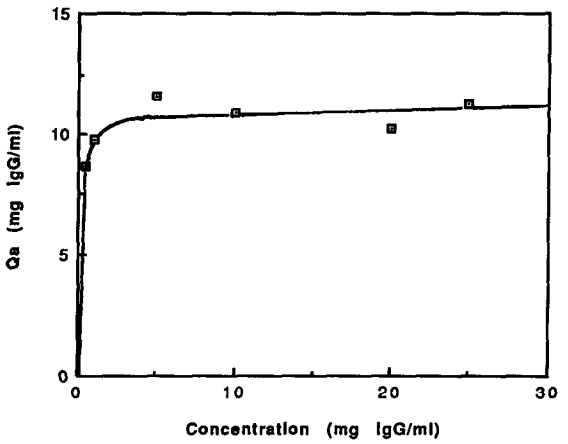

Fig. 8. Adsorption isotherm of human IgG on H-AH-Seph.

Determination of the dissociation constant (K_D). This was done by studying the adsorption isotherm using the frontal elution mode as described under Experimental. The adsorption isotherm, as shown in Fig. 8, follows a Langmuir pattern. From the linearized plot (Fig. 9), K_D was determined as $1.4 \cdot 10^{-6}$ *M* and the maximum capacity of the gel was 10.5 mg IgG ml^{-1} gel.

Determination of the adsorption rate constant. This was done by using the split peak method [9] as described under Experimental. The cumulative

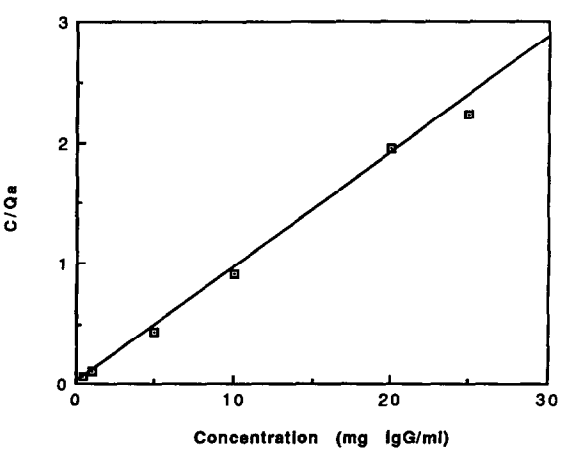

Fig. 9. Linearized plot of Fig. 8 using eqn. 2 (see text for more details). $y = 2.0182 \cdot 10^{-2} + 9.4796 \cdot 10^{-2}x$; $R^2 = 0.981$.

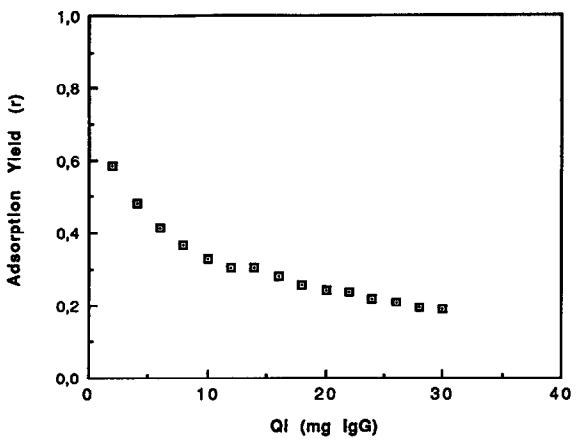

Fig. 10. Effect on adsorption yield of cumulative amounts of IgG injected.

amount of injected protein and the cumulative amount of unadsorbed protein were calculated for fifteen adsorptions. The ratio of the unadsorbed to injected protein was expressed as f, hence $1 - f$ gives the adsorption yield. From Fig. 10 it is clear that the adsorption yield decreases with increasing cumulative amount of injected protein. From eqn. 3, the rate constant was determined to be 56 l mol^{-1} s^{-1} using the values in the linear region of the graph.

DISCUSSION

The adsorption parameters indicated a charge–charge interaction. However, this is not based on the net charge of the protein, but on some localized charges forming ion pairs with the residual charge of the immobilized histidine.

At pH 7.4 IgG has a net positive charge whereas the adsorbent will have a positive charge on the α-NH_2 group and the imidazole nitrogen is non-protonated. At lower pH (*e.g.*, pH 5.0) both nitrogens are protonated and IgG also has a net positive charge. Hence there is no retention. Moreover, at pH 6.8, where the imidazole N is non-protonated, the retention was not significant (Fig. 4a), perhaps owing to the lack of perfect charge balancing with a localized protonation in the IgG.

Moreover, the isoelectric point distribution of the total IgG from placenta and that of the retained and non-retained fractions on the histidine adsorbents were determined according Righetti *et al.* [10]. The placental IgG had a distribution of pI from 4.36 to 10.11 whereas the fractions (IgG_1) retained and consequently eluted with 0.2 M NaCl from H-Seph or H-AH-Seph had pI values from 7.9 to 8.9. Moreover, those eluted with 1.0 M NaCl only from the H-AH-Seph column had pI values only in the range 6.8–7.2. This means that only the isoelectric forms of IgGs within a narrow range of pH were selectively retained on the adsorbents. Moreover, the subsets of IgG were retained more or less strongly on the adsorbent. This again shows the predominance of charge–charge interactions between the localized charges of the protein and the imidazole nitrogen. Nevertheless, the hydrogen-bonding interactions do play a role, as shown by the influence of additives such as urea and ethylene glycol in the elution buffer (Fig. 6).

In order to determine whether the immobilized histidine binds to the Fc or the Fab domain of the IgG, we studied the retention of Fc and Fab fragments, resulting from papain hydrolysis of the IgG as described by Goding [11]. We found that both the H-Seph and H-AH-Seph adsorbents retained only Fc whereas the Fab was unretarded on the columns (data not shown). It is known that protein A from *Staphylococcus aureus* binds to the Fc region of the IgG [12].

Moreover, the binding of IgG to immobilized histidine seems to be weak. The amino acid residues of protein A binding to Fc of IgG are commonly reported to be FQQNFYLNICK (the B domain) [12]. There are no histidine residues involved here. However, Moks *et al.* [13] reported the existence of five different domains in protein A binding to Fc region of IgG with differences in their binding strength. In fact the N-terminal sequence, called the E domain, with histidine at position 3 (AQHDEA) has the weakest affinity and binds to immobilized IgG. This weak affinity binding and the presence of histidine, reported as playing an important role, can be compared with the weak affinity of IgG, bound through its Fc region, to the immobilized histidine.

Nevertheless, in another independent investigation, we used some IgG variants differing only in their amino acid sequences at the CDR regions (parts of the Fab domain) for their retention on to the H-AH-Seph column. We found that whereas all six IgG variants studied were retained and eluted with 0.2 M NaCl, the elution volumes were different

for different variants (unpublished data). This indicates that in the whole IgG molecule whereas the primary binding site could be in the Fc region, there could be a minor cooperative effect due to secondary binding sites with long-range forces of interaction with the amino acid residues at the CDR regions.

In an attempt to identify the particular amino acids involved in the ion pairing with the histidine, we studied the retention of a few synthetic model peptides. These data did not show any preferential binding of N-terminal aspartic acid-containing peptides. Peptides with C-terminal E or K showed improved retention, with C-terminal E peptide being strongly retained. This again indicates that the ionic interactions are not the only forces responsible for the selective retention of IgG subclasses on the adsorbent. Further, in a more recent study, we equally successfully eluted the retained IgG_1 by using either E or K at 25 m*M* concentration, added to the same adsorption buffer, instead of using 0.2 *M* NaCl as in this study (unpublished data). Moreover, the column was able to distinguish between the sulphated and the non-sulphated Y residue in the peptide sequence. To our knowledge, nothing has been reported about the presence of sulphated tyrosine residues in the IgG.

Hence it is difficult to correlate these observations with the retention of IgG on the histidine-coupled adsorbents.

Kinetic parameters

The capacity of H-AH-Seph was calculated to be 10–12 mg IgG ml^{-1} gel, comparable to that reported for protein A–Sepharose [14] and protein A–Ultrogel [15].

The dissociation constant, K_D, as determined from the adsorption isotherm was 1.4×10^{-6} *M*. This indicates a weak affinity which is consistent with the easy and non-denaturing desorption of the bound IgG. Moreover, in another study in which we compared membrane-based histidine adsorbents with that of protein A coupled to a nylon membrane, we found K_D values in the region of 10^{-5} *M* [8].

The adsorption rate constant determined by the split peak method [9] gave a value of 56 l mol^{-1} s^{-1}. This indicates slow adsorption kinetics and implies that the flow-rates will influence the IgG adsorption pattern. In fact, a previous study using an ethanol precipitate of human placental serum containing about 90% IgG did show differences in the retention with varying flow-rate [6]. However, the suitability of this model [9] for the study of the resolution of IgG subclasses using total IgG cannot be clearly demonstrated. Therefore, we are pursuing on the one hand the same study using the prepurified IgG_1 and on the other a different approach based on the variation in flow-rates using both soft (Sepharose) and hard (silica) gels as support matrices to validate these data and the mathematical model. The results will be published elsewhere.

These parameters depend on the porosity and the nature of the supporting polymer matrices. In one of our studies using a low-porosity, small-particle-size support matrix we observed a much higher capacity (unpublished data), indicating diffusion-controlled limitations. Moreover, the systematically observed positive effect of a spacer arm supports this fact.

CONCLUSIONS

This study has shown that a single amino acid residue, histidine, can be very effective in purifying two different subclasses of human IgG from polyclonal sera. The weak affinity interactions are very advantageous for a high recovery of native proteins compared with the protein A-immobilized gels. The high capacity and the reproducibility are favourable features in using this system for scaling-up operations.

ACKNOWLEDGEMENTS

Valuable discussions with Dr. Vazquez and Dr. Marchalonis (USA) and Dr. Domurado (France) are gratefully acknowledged. Our sincere thanks are due to Professor P. G. Righetti for the short stay of A.E. in his laboratory (Milan) to carry out the isoelectric point determinations.

REFERENCES

1 M. Vuento and A. Vaheri, *Biochem. J.*, 183 (1979) 331.
2 L. Suamri, F. Spitz and L. Arzadon, *J. Biol. Chem.*, 251 (1976) 3693.
3 M. A. Vijayalakshmi and J. Porath, *J. Chromatogr.*, 177 (1979) 201.
4 S. Kanoun, L. Amourache, S. Krishnan and M. A. Vijayalakshmi, *J. Chromatogr.*, 376 (1986) 259.
5 M. A. Vijayalakshmi, *Ph. D. Thesis*, University of Compiègne, Compiègne, 1980, p. 108.

6 A. Mbida, S. Kanoun and M. A. Vijayalakshmi, in J. F. Stoltz and C. Rivat (Editors), *Biotechnology of Plasma Proteins*, INSERM Publication No. 175, Paris, 1988, p. 237.
7 A. El-Kak and M. A. Vijayalakshmi, *J. Chromatogr.*, 570 (1991) 29.
8 S. Manjini and M. A. Vijayalakshmi, *Biotechnol. Bioeng.*, (1991) in press.
9 H. Place, B. Sebille and C. Vidal-Madjar, *Anal. Chem.*, (1991) in press.
10 P. G. Righetti, E. Gianazza and B. Bjellqvist, *J. Biochem. Biophys. Methods*, 8 (1983) 89.
11 J. W. Goding, *J. Immunol. Methods*, 13 (1976) 215.
12 S. Johnsson and G. Kronvall, *Eur. J. Immunol.*, 4 (1974) 29.
13 T. Moks, L. Abrahmsen, B. Nilsson, U. Hellman, J. Sjöqvist and M. Uhlen, *Eur. J. Biochem.*, 156 (1986) 637.
14 *Pharmacia Handbook of Affinity Chromatography, Principles and Methods*, Pharmacia, Uppsala, 1986, p. 48.
15 *Practical Guide for Use in Affinity Chromatography and Related Techniques*, IBF, Villeneuve la Garenne, 2nd ed., 1983, p. 79.