ELSEVIER

Journal of Chromatography B, 674 (1995) 13–21

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Interaction of human immunoglobulin G with L-histidine immobilized onto poly(ethylene vinyl alcohol) hollow-fiber membranes

Karsten Haupt, Sonia M.A. Bueno, M.A. Vijayalakshmi*

Laboratoire d'Interaction Moléculaire et Technologie des Séparations (LIMTechS), Université de Technologie de Compiègne, B.P. 649, Compiègne, France

First received 18 April 1995; revised manuscript received 20 June 1995; accepted 20 June 1995

Abstract

L-Histidine as pseudobiospecific ligand was immobilized onto poly(ethylene vinyl alcohol) hollow-fiber membranes to obtain an affinity support for immunoglobulin G (IgG) purification. The interaction of human IgG with the affinity membranes was studied by chromatography and equilibrium binding analysis. Adsorption was possible over a broad pH range and was found to depend strongly on the nature of the buffer ions rather than on ionic strength. With zwitterionic buffers like morpholinopropanesulfonic acid (Mops) and hydroxyethylpiperazineethanesulfonic acid (Hepes), much higher adsorption capacities were obtained than with other buffers like Tris-HCl and phosphate buffers. An inhibition analysis revealed that non-zwitterionic buffers competitively inhibit IgG binding, whereas Mops and Hepes in their zwitterionic form do not. By choosing the appropriate buffer system, it was possible to adsorb specifically different IgG subsets. The IgG molecules were found to adsorb on membrane immobilized histidine via their F_{ab} part. Determination of dissociation constants at different temperatures allowed calculation of thermodynamic adsorption parameters. Decrease in K_D with increasing temperature and a positive entropy value between 20 and 35°C (in Mops buffer) indicated that adsorption is partially governed by hydrophobic forces in that temperature range, whereas at lower temperatures, electrostatic forces are more important for adsorption.

1. Introduction

The amino acid histidine has been used as a general ligand in affinity chromatography of proteins for several years. For example, calf chymosine, myxaline and an acid protease from *Aspergillus niger* [1] as well as catechol-2,3-dioxygenase [2] have been purified on histidine immobilized onto different supports. Of special interest has been the separation of immunoglobulin G from different sources [1]. It was shown that histidine can be coupled to Sepharose in different ways and that the coupling mode and the functional groups remaining free on the ligand largely influence adsorption capacity for IgG [3].

Sepharose-based supports have some disadvantages if the scale of the separation is to be increased. They are compressible and mass transfer is limited by diffusion. Affinity separation

* Corresponding author.

0378-4347/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(95)00282-0

systems with histidine coupled to membrane-based supports for higher scale separation of IgG were therefore developed in our laboratory [4]. The most recent development in this field is the immobilization of histidine onto poly(ethylene vinyl alcohol) (PEVA) hollow-fiber membrane cartridges [5] (the cartridges having been introduced previously by Sakurada et al. [6] as blood purification devices). These affinity membranes were shown to be an efficient tool for IgG purification from untreated human serum on a larger scale, showing at the same time a remarkable selectivity for IgG [5].

This paper describes mechanistic and thermodynamic aspects of IgG adsorption onto these affinity membranes and how a selectivity for specific subsets or subclasses of IgG can be obtained.

2. Experimental

2.1. Materials

Purified IgG from human plasma (164 g/l) was kindly supplied by Dr. Grandgeorge of Institut Mérieux (Lyon, France). Morpholinoethanesulfonic acid (Mes), morpholinopropanesulfonic acid (Mops), hydroxyethylpiperazineethanesulfonic acid (Hepes) were from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. Ultrapure water was obtained using the Millipore Milli RO/Milli Q plus system (Millipore, Bedford, MA, USA).

2.2. Immobilization of L-histidine onto PEVA hollow-fiber membranes

Poly(ethylene vinyl alcohol) hollow-fiber cartridges for blood plasma ultrafiltration were from Kuraray (Osaka, Japan) (Model Eval 3A, 0.4 m^2 surface area, 400 kDa molecular mass cut-off).

Activation and ligand coupling were carried out as described in Ref. [5]. Reactive oxirane groups were introduced into the matrix and subsequently opened and coupled with the α-amino group of histidine. The proposed structure of the immobilized ligand is shown in Fig. 1.

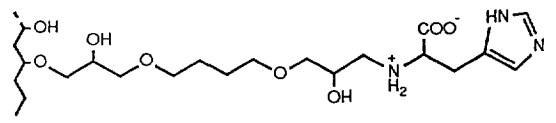

Fig. 1. Proposed structure of histidine coupled to poly-(ethylene vinyl alcohol) membrane after activation with butanediol diglycidyl ether.

2.3. Chromatographic procedures

A histidyl-PEVA hollow-fiber cartridge was disassembled. The hollow fibers were removed and finely cut (length 1 mm). The cut fibers (0.05 g dry weight) were suspended in the equilibration buffer, well degassed and packed into a jacketed and thermostated column (8 cm × 0.5 cm I.D.) to give a bed volume of approximately 0.5 ml (if not otherwise stated). This was done in order to minimize the quantity of IgG to be used. All chromatographic procedures were carried out at 20°C if not otherwise stated, at a linear velocity of 20 cm/h with an automated Econo liquid chromatography system (Bio-Rad Labs., Richmond, CA, USA).

2.4. Protein determination

IgG concentration was determined by measuring the absorbance at 280 nm, with a molar absorptivity of 14.0 for a 1% solution of IgG.

2.5. Isoelectrofocussing (IEF)

The chromatographic fractions were analyzed by isoelectrofocussing (IEF) using the Pharmacia Phast system (Pharmacia, Uppsala, Sweden) and pH 3–9 gradient gels. The gels were silver-stained according to the method provided by the manufacturer.

2.6. Equilibrium binding analysis

Finely cut fibers (12.5 mg dry weight) were filled in a 1-ml graduated plastic syringe. To keep the fibers in the syringe, its outlet was covered with a small piece of polyamide tissue before mounting the needle. The fibers were wetted with thoroughly degassed buffer by drawing the

solution into and pushing it out of the syringe several times. The volume remaining in the syringe was then adjusted to 200 μl and 500 μl of IgG solution was slowly filled into the syringe. To promote diffusion of the protein into the fibers and pores, the solution was slowly pushed out and drawn into the syringe three times, followed by gentle rotation of the syringe for 60 min. At that time, equilibrium was reached and the concentration of free protein in solution did not change any more. After pushing out ca. 500 μl of the solution, the unbound protein concentration was determined. After washing with water, followed by 50 m*M* NaOH, water and the equilibration buffer, the membrane-filled syringes could be reused.

The data were fitted to the Langmuir binding model using non-linear least squares and the Gauss–Newton–Marquardt method with the Minim 3.0 non-linear parameter estimation computer program (R.D. Purves, University of Otago, New Zealand).

3. Results and discussion

3.1. Chromatography of purified human IgG

Fig. 2 shows a typical chromatogram of purified human IgG on the histidyl-PEVA affinity membrane. Adsorption was done in Tris-HCl buffer, pH 7.4 (a) and Mops buffer, pH 6.5 (b) and elution was performed with increasing concentrations of NaCl in the starting buffers. The chromatogram in Tris-HCl buffer shows a large peak representing the unbound IgG (82% of the injected protein) and a smaller second peak eluted with 0.1 *M* NaCl. As a non-saturating amount of IgG was injected into the column, the presence of the large unbound fraction indicates the presence of IgG subsets with different affinity for histidine. In Mops buffer, however, almost 100% of the injected IgG was bound to the column and eluted with 0.1 *M* NaCl. The chromatographic conditions we used in our experiments were the same as those used by El-Kak et al. [3] with histidyl-Sepharose regarding IgG adsorption and elution. Surprisingly, the histidyl-

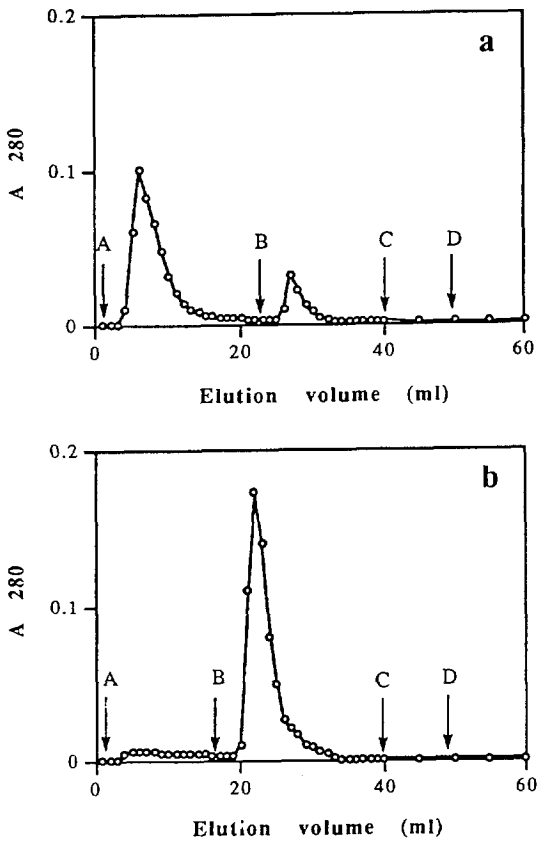

Fig. 2. Chromatography of purified IgG from human plasma on histidyl-PEVA membrane in 25 m*M* Tris-HCl buffer, pH 7.4 (a) and in 25 m*M* Mops buffer, pH 6.5 (b). Injection: 2.5 mg IgG in 1 ml buffer (A). Elution: 0.1 (B), 0.2 (C) and 1 *M* NaCl (D) in the same buffer. Column volume 5 ml.

PEVA hollow-fiber membranes gave a much better adsorption of human IgG than reported for the Sepharose-based support using the same coupling mode of the ligand [3]. This demonstrates the influence of the support material on adsorption of IgG onto immobilized histidine. An explanation could be that in the case of Sepharose, the ligand is surrounded by a large number of hydroxyl groups due to the polysaccharide nature of the material, that may form hydrogen bonds with the ligand and reduce its accessibility for the protein. In the case of PEVA, the number of free hydroxyls is much smaller, and hydrogen bonding should therefore occur to a smaller extent. On the other hand, a part of the ligand molecules may not be accessible for IgG

when coupled onto Sepharose because of diffusion limitation in the gel beads, as activation with bisoxirane may result in cross-linking of the agarose.

3.2. Influence of the buffer system on adsorption

IgG adsorption onto the histidyl affinity membranes seemed to depend on the buffer system used. To clarify this point, adsorption studies were carried out using Tris-HCl, phosphate, acetate, Mes, Mops and Hepes buffers within their respective buffering ranges. Fig. 3 shows IgG adsorption capacity in these buffer systems at different pH. Adsorption of purified IgG from human plasma is possible between pH 5.5 and 9, but the pH-optimum of adsorption is different for each buffer; for example, approximately the same amount of IgG is adsorbed in acetate buffer at pH 6.0 as in Tris-HCl at pH 8.5. In fact, one can distinguish in the figure two different "kinds" of buffers. Acetate, Tris-HCl and phosphate all allow adsorption of similar quantities of IgG (2–4 mg/g support). For these buffers, adsorption does not depend very much on pH. Mes, Mops and Hepes, on the other hand, allow a 3 to 5 times higher adsorption of pure IgG as compared to other buffers (8–17 mg/g support) if they are used at pH below their pK_a (pH 5.4, 6.5 and 7.0, respectively). However, adsorption decreases rapidly with increasing pH to reach similar values as obtained with the other buffers.

From the structure of the buffer ions used it is obvious that acetate, Tris and phosphate carry one or more charges of the same sign —only (+) or only (−)— whereas the zwitterionic buffers Mes, Mops and Hepes carry two charges of opposite signs below their pK_a.

If IgG adsorption capacities are plotted against the conductivities of the buffer solutions (Fig. 4), one can clearly observe that the best adsorption is obtained with Mes, Mops and Hepes in their zwitterionic form (low conductivity), whereas at higher pH, where the ring nitrogens are deprotonated (only the negative charge is left), conductivities and obtained IgG adsorption capacities are in the same range as with the other buffers. On the other hand, adsorption did not depend very much on the ionic strength of the buffers. Table 1 shows the maximum adsorption capacity at the optimum pH of each buffer and the ionic strength of the solution. For example, Mes, Mops and Hepes allowed the highest adsorption of IgG despite the fact that the ionic strengths of these

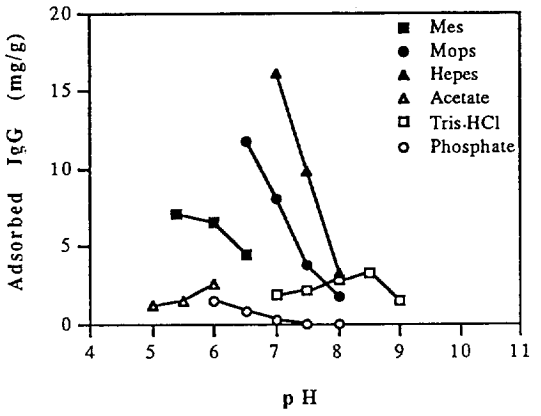

Fig. 3. Adsorption capacity of histidyl-PEVA membrane for human IgG (mg/g support) in different buffers as a function of pH. Buffer concentration was 25 m*M*. A saturating amount of IgG at a concentration of 1 mg/ml was injected and elution performed with 25 m*M* acetate buffer, pH 4.0, containing 1 *M* NaCl.

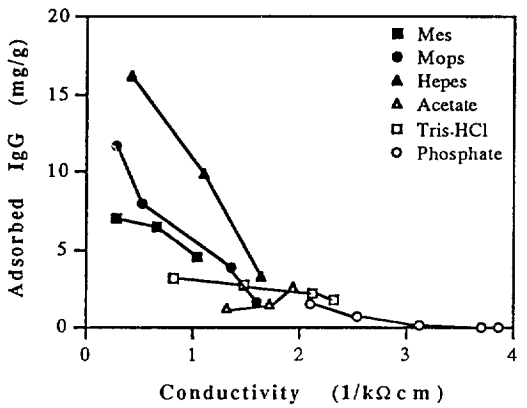

Fig. 4. Plot of adsorption capacity of histidyl-PEVA for human IgG (mg/g support) in different buffers as a function of conductivity of the buffer solution. For conditions, see Fig. 3.

Table 1
Ionic strength and maximum adsorption capacity at optimum pH in the different buffers used (buffer concentration was 25 m*M*)

Buffer	Ionic strength	Maximum adsorbed IgG (mg/g support)
Mops	0.046	11.9
Hepes	0.045	16.2
Mes	0.046	7.0
Tris-HCl	0.010	3.2
Phosphate	0.028	1.5
Acetate	0.024	2.6

solutions were higher than these of acetate, Tris-HCl and phosphate. This is also confirmed by the fact that elution of the retained IgG was not possible with the zwitterionic salt glycine-betaine at a concentration as high as 1 *M* (result not shown).

A possible explanation to this phenomenon could be that all buffer ions and the respective counter ions interact with the protein molecules via charge–charge interactions and mask the binding site for histidine, but Mes, Mops and Hepes in their zwitterionic form do not. The fact that in Ches (cyclohexylaminoethanesulfonic acid) buffer (pH 8.5–9.5) this effect was not observed (results not shown), though this is a zwitterionic buffer, indicates that at pH 8 is the upper pH limit of the adsorption. The protonation states of the charged groups on the IgG molecule involved in the interaction may hinder adsorption at this pH.

In order to verify the hypothesis raised above, the binding of IgG to immobilized histidine was studied at different buffer concentrations using an equilibrium binding analysis. Mops and Tris-HCl buffer were chosen as representatives for the two different groups of buffers. The data were analyzed in a similar way as used for enzyme inhibition analysis. Adsorption is described by the Langmuir adsorption isotherm:

$$Q_a = Q_x C/(K_{Dapp} + C)$$

where Q_a is the amount of adsorbed protein, Q_x the maximum adsorption capacity, C the equilibrium unbound protein concentration and K_{Dapp} the apparent dissociation constant of the complex. If we assume competitive inhibition of adsorption by the buffer, the apparent dissociation constant contains the inhibitory function ϕ ($K_{Dapp} = K_D \cdot \phi$, were $\phi = 1 + I/K_I$; K_I is the inhibition constant and I the buffer concentration), whereas the maximum adsorption capacity is not affected. The inhibition constant K_I can be determined by plotting K_{Dapp} as a function of I which yields the straight line:

$$K_{Dapp} = K_D + K_D \cdot I/K_I$$

with the ordinate intercept K_D and the abscissa intercept $-K_I$.

Fig. 5 shows the Langmuir isotherms for Tris-HCl pH 7.4 (a) and Mops pH 6.5 (b) and the plot K_{Dapp} as a function of I (c). Binding in Tris-HCl is strongly inhibited by the buffer with a $K_I = 3$ m*M*, whereas Mops has almost no inhibitory effect, which proves the hypothesis raised above.

3.3. Isoelectric point distribution and subclass composition in the retained and non-retained fractions of IgG

The isoelectric points of the retained and non-retained IgG fractions have been determined by isoelectrofocussing using purified IgG from human plasma. The results are shown in Table 2. The isoelectric point distribution in the adsorbed fraction depended upon the buffer system used. As can be seen, with Mops buffer, the whole IgG fraction with p*I* ranging from 6.6 to 9.3 was adsorbed, whereas with the other buffers, only IgG within a given range of p*I* values were retained. A very good selectivity was obtained using Tris-HCl pH 8.5 (p*I* 8.2–9.3) or Hepes buffer, pH 7.0 (p*I* 7.0–9.3).

IgG subclass composition of the retained and non-retained fractions was determined by Ouchterlony immunodiffusion. It also depended on the buffer system used. For instance, in Mops buffer, IgG_1, IgG_2 and IgG_3 were bound, whereas in Tris-HCl, only IgG_3 and a part of the IgG_1 fraction was retained (figures not shown).

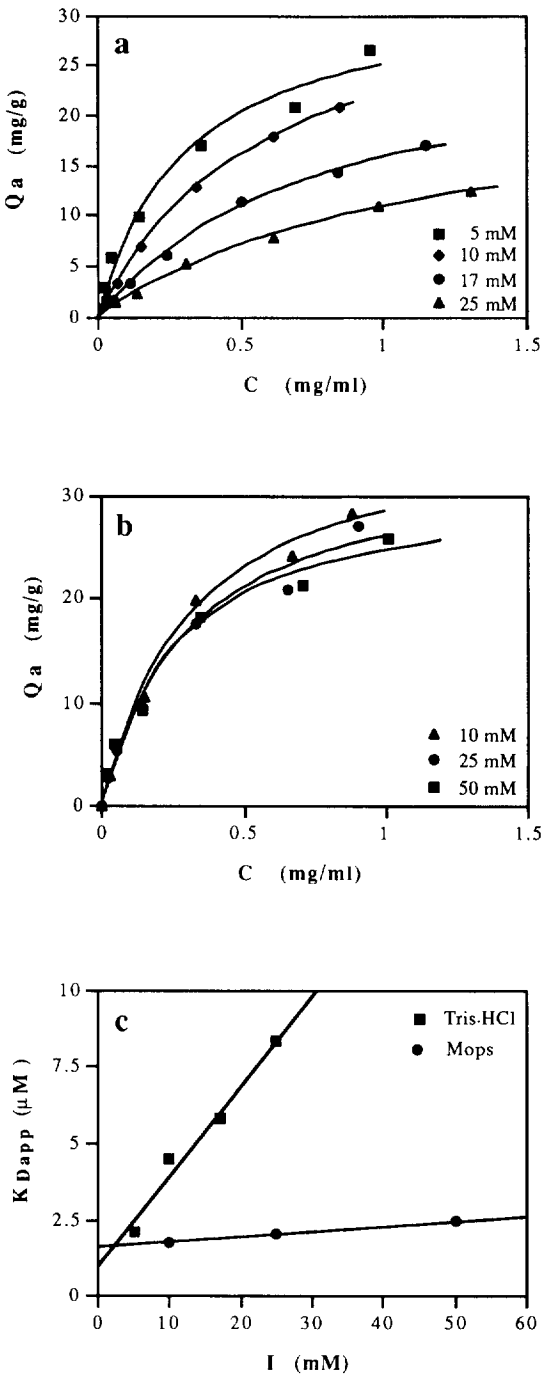

Fig. 5. Langmuir isotherms for IgG adsorption on histidyl-PEVA membrane in Tris-HCl, pH 7.4 (a), and Mops, pH 6.5 (b), at different buffer concentrations. (c) Secondary plots of the apparent dissociation constants from (a) and (b) as a function of buffer concentration.

3.4. Identification of the binding site

In order to know whether the histidine ligand adsorbs human IgG on the F_{ab} or on the F_c part, IgG was digested with pepsin and with papain using the protocols described in Ref. [7]. Papain digestion of IgG yields one F_c and two F_{ab} fragments. Pepsin digestion yields one $(F'_{ab})_2$ fragment (two F'_{ab} connected by disulfide bridges) containing the Hinge region, whereas the F_c part is further degraded to several small fragments. The $(F'_{ab})_2$ can be reduced using ß-mercaptoethanol which yields two F'_{ab}. For pepsin digestion, the immobilized enzyme was used. After a gel permeation step to remove the small fragments and to exchange the buffer, the $(F'_{ab})_2$ fragments were injected into a column containing histidyl-PEVA hollow fibers in 25 m*M* Mops buffer pH 6.5 and in 25 m*M* Tris-HCl buffer pH 7.4. The retained and non-retained fractions were collected and concentrated. A part of the $(F'_{ab})_2$ was reduced to F'_{ab} by ß-mercaptoethanol and, after buffer exchange by gel permeation, chromatographed on histidyl-PEVA hollow fibers.

For papain digestion, the soluble enzyme was used. After digestion, the F_{ab} and F_c fragments were separated from intact IgG and papain by gel permeation on Sephadex G-75. The fraction containing F_{ab} and F_c was injected into a protein A Sepharose column to separate F_{ab} from F_c (F_c is retained on protein A and subsequently eluted, whereas F_{ab} comes out in the non-retained peak). Both purified fragments were then injected separately in 25 m*M* Mops pH 6.5 and in 25 m*M* Tris-HCl pH 7.4 into a column containing histidyl-PEVA hollow fibers.

All chromatographic fractions were analyzed by SDS-PAGE (not shown).

The results of these experiments are summarized in Table 3. The F_c fragments are not retained in either buffer. The F_{ab}, F'_{ab} and $(F'_{ab})_2$ fragments are completely retained in Mops buffer, whereas in Tris-HCl, about the same ratio of retained to non-retained fragments was obtained as with the intact IgG molecules. The fact that F'_{ab} as well as F_{ab} are retained indicates that the binding site is not situated in the Hinge region. This result may seem contradictory to that re-

Table 2
Isoelectric point distribution of IgG in the retained and non-retained fractions with the different buffer systems used

		pI non-retained IgG	pI retained IgG
Acetate	25 m*M* pH 6.0	6.6–8.0	7.0–9.3
Phosphate	10 m*M* pH 6.5	6.6–7.4	7.0–9.3
Tris-HCl	25 m*M* pH 8.5	6.6–8.2	8.2–9.3
Mes	25 m*M* pH 5.4	6.6–7.0[a]	7.0–9.3
Hepes	25 m*M* pH 7.0	6.6–7.0[a]	7.0–9.3
Mops	25 m*M* pH 6.5	no peak	6.6–9.3

[a] Very small peak.

ported by El-Kak et al. [3], but we have to note that the source of IgG used in this work as well as the affinity support were different.

3.5. Determination of the thermodynamic parameters of adsorption

Dissociation constants for IgG adsorption on histidyl-PEVA were determined by frontal chromatography in Mops buffer at 4, 15, 20, 27, and 35°C. Purified human IgG was injected in concentrations ranging from 0.1 to 5 mg/ml in 25 m*M* Mops buffer, pH 6.5, until the concentration of the non-retained protein reached a plateau. After washing, bound IgG was eluted with 1 *M* NaCl in 25 m*M* acetate buffer, pH 4, and protein concentration determined. Fig. 6a shows the corresponding Langmuir isotherms. The thermodynamic parameters of adsorption were determined as described in [2] and are shown in Table 4. From the van't Hoff reaction isotherm

$$\Delta G = \Delta G^\circ - RT \cdot \ln K_D$$

at equilibrium when $\Delta G = 0$, the equation

$$\Delta G^\circ = RT \cdot \ln K_D$$

is obtained that allows ΔG° to be calculated at a given temperature from the dissociation constant. The temperature dependence of K_D is given by the van't Hoff reaction isobar. In its integrated form:

$$\ln K_D = \Delta H^\circ / RT + J$$

(J is an integration constant), when plotting ln K_D as a function of $1/T$, ΔH° is given by the slope if a straight line is obtained (in that case, ΔH° is temperature-independent in this temperature range). From the Gibbs–Helmholtz relationship:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

Table 3
Adsorption of the different IgG fragments obtained by pepsin or papain digestion on histidyl-PEVA hollow fibers

Digestion by	Fragments	Retention in 25 m*M*	
		Mops pH 6.5	Tris-HCl pH 7.4
Pepsin	$(F'_{ab})_2$	yes	yes[b]
+ β-ME[a]	F'_{ab}	yes	yes[b]
Papain	F_{ab}	yes	yes
	F_c	no	no

[a] β-mercaptoethanol.
[b] Partially.

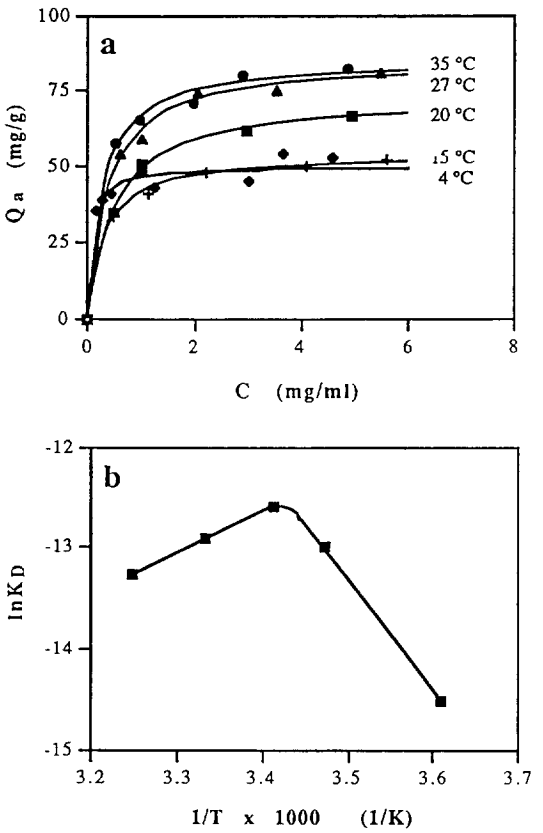

Fig. 6. (a) Langmuir isotherms of IgG adsorption on histidyl-PEVA membrane in 25 m*M* Mops buffer, pH 6.5, at 4, 15, 20, 27, and 37°C. IgG was injected at concentrations ranging from 0.25 to 5 mg/ml until the pass-through protein concentration *C* did not change any more. After washing, elution was performed with 25 m*M* acetate buffer, pH 4.0, containing 1 *M* NaCl. (b) Plot of ln K_D as a function of $1/T$.

the standard entropy change $\Delta S°$ can be obtained.

It can be seen in Fig. 6b and Table 4 that K_D decreases with increasing temperature from 3.4 μM at 20°C to 1.8 μM at 35°C. $\Delta H°$ being positive, adsorption should therefore be entropy-favoured due to the positive entropy term. This is surprising as adsorption seemed to be governed by electrostatic forces (elution was possible with low salt concentrations) though hydrophobic interactions seem to be the only ones that increase with temperature [8]. At temperatures lower than 18°C, the slope of the curve is of opposite sign (negative $\Delta H°$), indicating that the nature of the interaction changes with temperature. At lower temperature, electrostatic interactions, e.g., charge–charge or charge–dipole, are the most important factor for adsorption. A similar behaviour has been reported by Gill et al. [9] for cytochrome b_5 adsorption onto an anion exchanger. It has also been shown by Ståhlberg et al. [10] that adsorption of a protein onto a charged surface is governed by electrostatic and van der Waals interactions. Interactions involving hydrogen bonding might also play a role. As interaction of IgG with the ligand is of medium strength (K_D about 10^{-6} *M*), masking electrostatic interaction points by adding salt (during elution) could weaken it to an extent that adsorption is not possible any more.

Table 4
Dissociation constants and thermodynamic parameters for IgG adsorption onto histidyl-PEVA in 25 m*M* Mops buffer, pH 6.5

Temperature (°C)	K_D (μM)	$\Delta G°$ (kJ/mol)	$\Delta H°$ (kJ/mol)[a]	$\Delta S°$ (J/mol K^{-1})[a]
4	0.5	−33.5	ca. −13	ca. −238
15	2.0	−31.4		
20	3.4	−30.7		
27	2.5	−32.2	ca. +33	ca. +216
35	1.8	−33.8		

[a] Given the non-linearity of the plot ln K_D as a function of $1/T$ in the temperature range studied, it was not possible to calculate exactly $\Delta H°$ and $\Delta S°$. The represented values were estimated from the slopes of the linear parts of the curve.

4. Conclusion

We showed that IgG adsorption onto histidine immobilized on PEVA hollow-fiber membranes is strongly influenced by the nature and protonation state of the buffer ions. This indicates that the interaction is governed by electrostatic forces, but thermodynamic analysis of binding revealed that hydrophobic forces also play a role, depending on the temperature range studied. By choosing the appropriate buffer, the system can be fine-tuned if only a special subset or subclass of IgG is to be adsorbed. Histidine was found to bind to the F_{ab} part of IgG. This gives a further potential possibility of adjusting the system for purifying specific paratopes, in rather gentle chromatographic conditions.

Acknowledgements

The authors thank Dr. Grandgeorge from the Institut Mérieux, France, for supplying the purified human IgG. The PEVA hollow-fiber cartridges were a kind gift from Dr. Cécile Legallais of the University of Compiègne (Compiègne, France). Mrs. Sonia Bueno acknowledges the doctoral fellowship from CNPq, Brasília, Brazil and the leave of absence from the University of Campinas, Faculty of Chemical Engineering (Campinas, SP, Brazil).

References

[1] M.A. Vijayalakshmi, Trends Biotechnol., 7 (1989) 71.
[2] K. Haupt and M.A. Vijayalakshmi, J. Chromatogr., 644 (1993) 289.
[3] A. El-Kak, S. Manjini and M.A. Vijayalakshmi, J. Chromatogr., 604 (1992) 29.
[4] S. Mandjiny and M.A. Vijayalakshmi, in C. Rivat and J.-F. Stoltz (Editors), Biotechnology of Blood Proteins, Colloque INSERM, John Libbey Eurotext, 227 (1993) 189.
[5] S.M.A. Bueno, K. Haupt and M.A. Vijayalakshmi, J. Chromatogr., 667 (1995) 57.
[6] Y. Sakurada, A. Sueoka and M. Kawahashi, Polymer J., 19 (1987) 501.
[7] P. Tijssen, Laboratory Techniques: Practice and Theory of Enzyme Immunoassays, Elsevier, Amsterdam, 1985, p. 117.
[8] V.V. Mozhaev and K. Martinek, Enzyme Microbiol. Technol., 6 (1984) 50.
[9] D.S. Gill, D.J. Roush and R.C. Willson, J. Colloid Interface Sci., 167 (1994) 1.
[10] J. Ståhlberg, B. Jönsson and C. Horváth, Anal. Chem., 64 (1992) 3118.