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Separation of immunoglobulin G from human serum by pseudobioaffinity chromatography using immobilized L-histidine in hollow fibre membranes

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Abstract

L-Histidine, intended as a pseudobiospecific ligand, was immobilized on poly(ethylenevinyl alcohol) hollow fibre membranes after their activation with epichlorohydrin or butanediol diglycidyl ether. The affinity membranes obtained allowed the one-step separation of immunoglobulin G (IgG) from untreated human serum. Elution was possible under mild conditions with discontinuous pH or salt gradients. IgM was also adsorbed to a certain extent and partially separated from IgG by pH gradient elution. The bound IgG fractions showed p*I* values between 8 and 9.5 and contained IgG_1 and IgG_3. The dissociation constants for IgG on the bisoxirane- and epichlorohydrin-activated membranes coupled with histidine were determined by equilibrium binding analysis to be $2.5 \cdot 10^{-5}$ and $2.0 \cdot 10^{-5}$ *M*, respectively. The maximum binding capacity of the affinity hollow fibre membranes was 80 and 70 mg of IgG per gram of support, respectively. With a cartridge of surface area 1 m^2 (about 19 g of fibres), during a 60-min run, theoretically up to 1.5 g of IgG can be removed from human serum. The histidine affinity membranes are very stable owing to the simple nature of the ligand and the coupling via an ether linkage. Reproducible results were obtained over more than 1 year even with untreated human serum being used regularly.

1. Introduction

Affinity chromatography using particulate materials is a well developed method for the separation of biomolecules from biological broths and blood [1]. In the last decade, pseudobiospecific ligands, e.g., dyes, metal chelates and amino acids, have been increasingly used instead of biospecific ligands in affinity chromatography. They are usually smaller and simpler molecules than biospecific ligands, with higher chemical and physical stability and cheaper [2].

Previous work in our laboratory has shown that the amino acid histidine can be used as a ligand for the purification of proteins [2]. Of special interest is the ability of this ligand to adsorb specifically different immunoglobulins. Thus immunoglobulins G_1 and G_2 (IgG_1 and IgG_2) have been separated from human plasma [3]. Mechanistic, kinetic and thermodynamic studies of protein adsorption on immobilized histidine have been published elsewhere [4,5].

Despite the commercial availability of many

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affinity ligands immobilized on beads for use in column chromatography, there are some drawbacks to the large-scale application of the commonly used soft gels. Flow-rates and thus performance are limited by the compressibility of the gels and pore diffusion. In recent years, microporous membranes were successfully modified and various ligands were coupled for use in affinity chromatography. These new supports are rigid and pore diffusion is negligible (mass transfer being governed mainly by forced convection) [6]. When hollow fibre membranes are used, high surface area/volume ratios can be obtained. The choice of the membrane material may be difficult as a compromise must be found regarding the reactivity of the material, stability in polar solvents, pore size [7] and biocompatibility.

It was shown previously [8] that histidine can be immobilized on different flat sheet membranes to obtain chromatographic supports for purification of IgG from human serum and from other sources. In this work, we tried to combine the specificity of the ligand histidine with hollow fibre membrane technology to create a powerful device for the separation of IgG from human serum and from other sources. We chose poly-(ethylenevinyl alcohol) (PEVA) hollow fibre membranes as the support material. Free hydroxyl groups available on its surface allow easy coupling of primary amines via bifunctional agents. These membranes have been used by other workers for the removal of heparin from blood using immobilized poly(L-lysine) [9].

L-Histidine was immobilized after activation of the membrane by epichlorohydrin or 1,4-butanediol diglycidyl ether and the selectivity and capacity of the pseudobiospecific affinity supports obtained were investigated.

2. Experimental

2.1. Materials

Epichlorohydrin, sodium tetrahydroborate, Coomassie violet and Coomassie brilliant blue were purchased from Merck (Darmstadt, Germany) and 1,4-butanediol diglycidyl ether, low-melting agarose and L-histidine from Sigma (St. Louis, MO, USA).

Prepurified IgG from human plasma (164 g/l) was kindly supplied by Dr. Grandgeorge (Institut Mérieux, Lyon, France).

All other chemicals were of analytical-reagent grade. Ultrapure water was obtained using a Milli-RO/Milli-Q Plus system (Millipore, Bedford, MA, USA).

Poly(ethylenevinyl alcohol) hollow fibre cartridges for serum ultrafiltration were purchased from Kuraray (Japan) (Model Eval 3A, 1.0 m^2 surface area). Each hollow fibre had an effective length of 19 cm, an I.D. of 200 μm, a wall thickness of 20 μm and a nominal molecular mass cut-off of 400 000. One gram (dry mass) would normally consist of 340 hollow fibres; one cartridge contained about 6500 fibres.

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

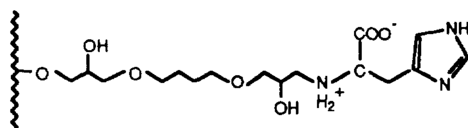
A PEVA hollow fibre cartridge was dismantled. The hollow fibres were finely cut (length about 1 mm), activated in batch mode and coupled with L-histidine. For activation with epichlorohydrin and with 1,4-butanediol diglycidyl ether, the protocols described in Refs. [8] and [10], respectively, were adopted. Reactive oxiranes were introduced into the membrane and subsequently opened and coupled with the primary amino group of histidine. The proposed structures of the immobilized ligands are given in Fig. 1.

2.3. Preparation of human serum

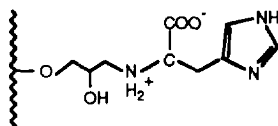
Human blood was taken from a healthy person and kept at room temperature in glass test-tubes for 1 h to allow agglutination. The tubes were then centrifuged for 5 min at 200 *g* and the supernatant (serum) was used without further treatment for chromatography.

2.4. Chromatographic procedures

Finely cut hollow fibres of about 1 mm length (a total of 0.5 g dry mass) were suspended in 25



Histidyl-PEVA membrane, bisoxirane activation (H-B-PEVA)



Histidyl-PEVA membrane, epichlorohydrin activation (H-E-PEVA)

Fig. 1. Structures of histidine coupled to PEVA hollow fibre membrane.

m*M* Tris–HCl buffer (pH 7.4) (equilibration buffer), degassed well and packed into a column (10 cm × 1 cm I.D.) to give a bed volume of ca. 3 ml. All chromatographic procedures were carried out at room temperature at a linear velocity of 20 cm/h with an automated Econo liquid chromatographic system (Bio-Rad Labs., Richmond, CA, USA). Human serum (1.5 ml, about 90 mg of total protein) was diluted 1:5 with the equilibration buffer and injected into the column pre-equilibrated with the equilibration buffer. Elution was performed with the same buffer containing different concentrations of NaCl or using a decreasing pH step gradient with 25 m*M* sodium acetate buffer. The absorbance of the eluate was monitored at 280 nm. After each experiment, the column was washed with 0.05 *M* NaOH, followed by water and finally by the equilibration buffer.

2.5. Protein determination

Protein concentrations were determined by the method of Bradford [11]. Crystalline bovine serum albumin was used as reference protein. In fractions containing pure IgG, the absorbance at 280 nm (A_{280}) was used to determine protein concentration, with a molar absorptivity of 14 for a 1% solution of IgG. The conversion factor from the Bradford value to the value obtained by the A_{280} method was 2.12 ± 0.03.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectrofocusing (IEF)

The chromatographic fractions were analysed by SDS-PAGE (4–15% gradient gels) under non-reducing conditions using a Phast system (Pharmacia, Uppsala, Sweden). The gels were silver stained according to the method provided by the manufacturer. The same apparatus and pH 3–9 gradient gels were used for IEF. The SDS-PAGE gels were scanned using a Shimadzu dual-wavelength flying spot scanning densitometer (Shimadzu, Tokyo, Japan).

2.7. Immunoelectrophoresis and immunodiffusion

Immunoelectrophoresis was carried out in 1% agarose gels (Sebia, Issy-les-Moulineaux, France) according to Grabar and Williams [12] using rabbit–anti-human serum antiserum (polyvalent) and rabbit–anti-human IgG antiserum (monovalent) from Behring Diagnostic (Rueil-Malmaison, France). Radial immunodiffusion was carried out in 1% agarose gels with mouse–anti-human $IgG_{1,2,3,4}$ antisera (Janssen Biochimica, Beerse, Belgium), rabbit–anti-human IgA (Behring Diagnostic) and rabbit–anti-human IgM (Biosys, Compiègne, France) anti-

sera. The gels were stained with Coomassie violet or Coomassie brilliant blue.

2.8. Nephelometric determination of IgG, IgM and human serum albumin

In fractions obtained after chromatography of human serum, the concentrations of IgG, IgM and albumin were determined nephelometrically using a Beckman array protein system with the Beckman reagents for IgG, IgM and serum albumin (Beckman Instruments, Fullerton, CA, USA).

2.9. Equilibrium binding analysis

For these experiments, the retained fraction from the chromatography on a bisoxirane-activated hollow fibre cartridge coupled with histidine was used. Prepurified IgG was injected into the cartridge and the adsorbed protein was then eluted with 0.4 *M* NaCl in the equilibration buffer, desalted and concentrated by ultrafiltration.

The equilibrium binding experiments were carried out in triplicate at 22°C with H-B-PEVA and H-E-PEVA. Finely cut fibres (12.5 mg dry mass) were filled into a 1-ml graduated plastic syringe. To keep the fibres in the syringe, its outlet was covered with a small piece of polyamide tissue before mounting the needle. The fibres were then wetted with thoroughly degassed 25 m*M* Tris-HCl buffer (pH 7.4) 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 (including the needle) and 100 μl of IgG solution were slowly filled into the syringe, the final protein concentration ranging from 0.65 to 14.5 mg/ml. To promote diffusion of the protein into the fibres 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. 120 μl of the solution, the unbound protein concentration was determined and the data were analysed using the Langmuir binding model and the Minim 3.0 non-linear regression computer program based on the Gauss–Newton–Marquardt method (R.D. Purves, University of Otago, New Zealand). After washing with water, followed by 50 m*M* NaOH, water and the equilibration buffer, the membrane-filled syringes could be re-used.

To estimate the global experimental error for this method, all syringes used in these experiments were loaded with the same amount and concentration of protein. The differences in the unbound protein concentrations between the syringes and between two series of experiments did not exceed 5%.

3. Results and discussion

3.1. Immobilization of histidine on PEVA hollow fibres

Membrane supports for histidine immobilization were selected to meet the following requirements: functional groups available on the polymeric materials for coupling of the ligand; stability of the material during the coupling procedure; materials currently available for extracorporeal devices (good biocompatibility); hydrophilicity, low non-specific adsorption of protein; and large surface area.

According to these criteria and after screening other membrane materials, namely cellulose acetate and polysulfone, PEVA hollow fibres were chosen for further studies with immobilized histidine. The hollow fibre cartridge employed in the present work is actually in clinical use for serum ultrafiltration. The material has free hydroxyl groups that can be activated with bifunctional agents, e.g., epichlorohydrin and bisoxiranes. Histidine can then be coupled to the reactive epoxy function via its amino group; the carboxy group and imidazole ring remain free. Coupling via activation with 1,4-butanediol diglycidyl ether provides a ten carbon atom spacer arm containing two ether functions. Epichlorohydrin activation results in a three carbon atom spacer. We used both activation modes to

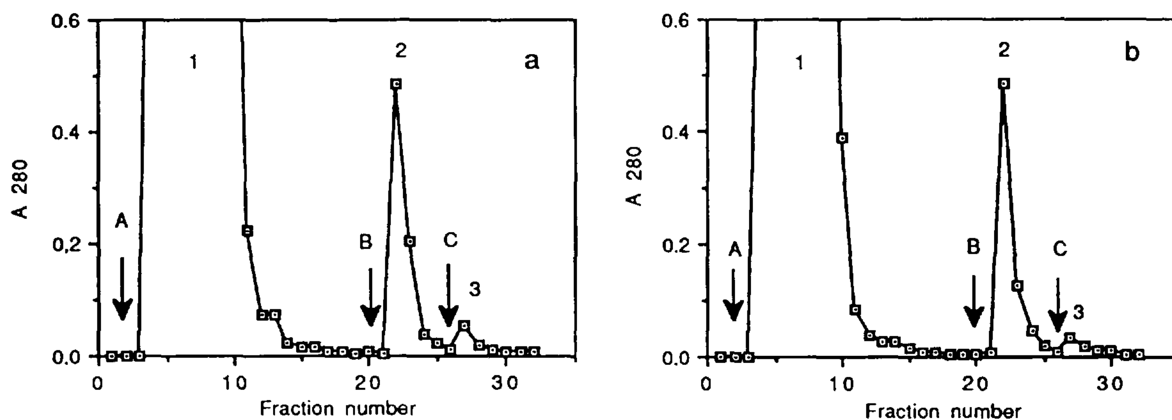


Fig. 2. Separation of IgG from untreated human serum on (a) H-B-PEVA and (b) H-E-PEVA hollow fibre membranes. Conditions: (A) 0.025 *M* Tris–HCl buffer (pH 7.4); (B) 0.025 *M* Tris–HCl buffer (pH 7.4) + 0.1 *M* sodium chloride; (C) 0.025 *M* Tris–HCl buffer (pH 7.4) + 0.2 *M* sodium chloride; injection, 1.5 ml of human serum; fraction volume, 6.0 ml. The numbers 1–3 indicate the protein peaks obtained.

see if a longer spacer improves IgG separation or capacity. These coupling methods have also been used in previous work in our laboratory to immobilize histidine on Sepharose and nylon–methacrylate flat membranes [4,8].

3.2. Separation of IgG from human serum

Untreated human serum was chromatographed on both affinity membranes coupled with histidine after activation with epichlorohydrin and bisoxirane. Elution was performed either with different concentrations of NaCl in the equilibration buffer or by a step pH gradient.

Fig. 2a shows the chromatogram on the bisoxirane-activated support (H-B-PEVA) using salt elution and Fig. 3a shows the SDS-PAGE of the eluted fractions under non-reducing conditions. Peak 1 represents the non-retained protein and contains albumin, immunoglobulins and other serum proteins. The second and third peaks eluted consecutively with 0.1 and 0.2 *M* NaCl contain immunoglobulins with albumin and other proteins as minor contaminants. The high-molecular-mass protein bands in the eluted peaks that do not, or poorly, penetrate the gel probably correspond to IgM. When SDS-PAGE was carried out under reducing conditions, these bands disappeared (not shown).

The results for the epichlorohydrin activated support (H-E-PEVA) are shown in Figs. 2b and 3b. As can be seen, the chromatographic pattern and specificity are similar for both supports.

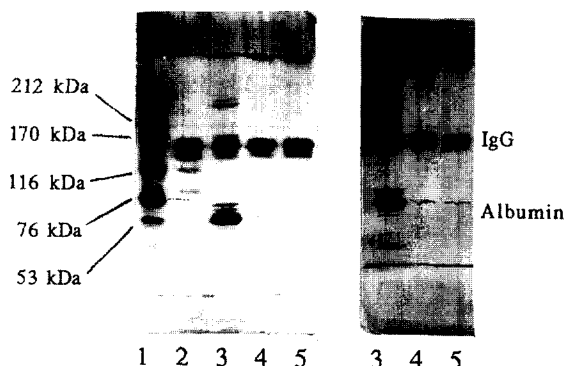


Fig. 3. SDS-PAGE under non-reducing conditions of the fractions from chromatography (salt elution) on (left) H-B-PEVA and (right) H-E-PEVA hollow fibre membrane. Lanes: 1 = molecular mass markers (myosine, 212 000; α_2-macroglobulin, 170 000; β-galactosidase, 116 000, transferrin, 76 000, glutamic dehydrogenase, 53 000); 2 = prepurified IgG from human plasma (Institut Mérieux); 3 = peak 1; 4 = peak 2; 5 = peak 3.

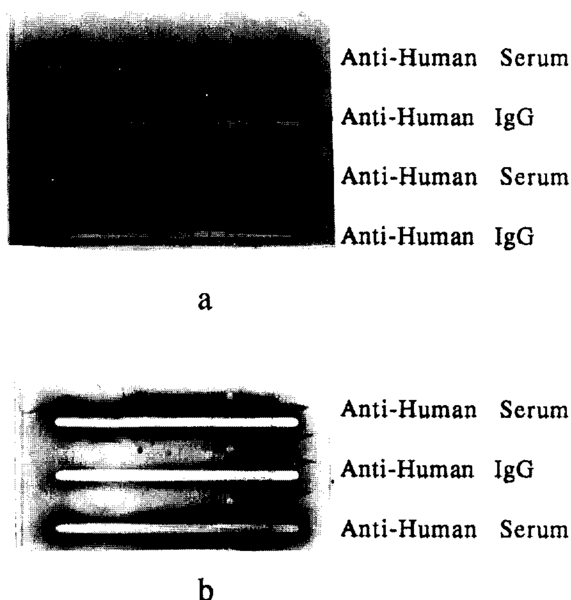


Fig. 4. Immunoelectrophoretic analysis of the fractions from chromatography (salt elution) on (a) H-B-PEVA and (b) H-E-PEVA hollow fibre membrane. Wells: 1 = peak 1; 2 = peak 2; 3 = peak 3.

Immunoelectrophoresis with rabbit–anti-human serum antiserum and with rabbit–anti-human IgG antiserum revealed that, for both membranes, the main bands in peaks 2 and 3 corresponded to IgG (Fig. 4a and b). Retained and non-retained fractions were analysed for IgA and IgM by immunodiffusion. IgA was detected only in the non-retained fraction, whereas IgM was present in both the retained and non-retained fractions (not shown).

Among the proteins eluted with 0.1 *M* NaCl in H-B-PEVA, IgG and IgM were estimated to represent 82% and 14.7%, respectively, of the total protein by scanning the SDS-PAGE gels.

The results obtained are similar to those reported by El-Kak and Vijayalakshmi [3] for histidyl-Sepharose regarding IgG adsorption and elution. The mild conditions for elution using 0.2 *M* NaCl is an advantage of the histidine affinity system as they help to preserve the native conformation of the IgG molecules.

In order to improve the separation of IgG, elution was performed with a discontinuous decreasing pH gradient. The results for H-B-PEVA are shown in Fig. 5a. Peak 1 representing the non-retained fraction contained albumin and other serum proteins, whereas IgG was eluted at pH 5, pH 4 and pH 4 + 1 *M* NaCl (peaks 2, 3 and 4). The peaks were analysed by SDS-PAGE (Fig. 6a). In peak 2, eluted at pH 5, IgG was estimated to be about 91.5% pure by scanning the gel on a densitometer. Contamination by serum proteins other than immunoglobulins was detected in the IgG fractions eluted at pH 4 (76% IgG) and pH 4 + 1 *M* NaCl (48% IgG). The high-molecular-mass protein eluted in these

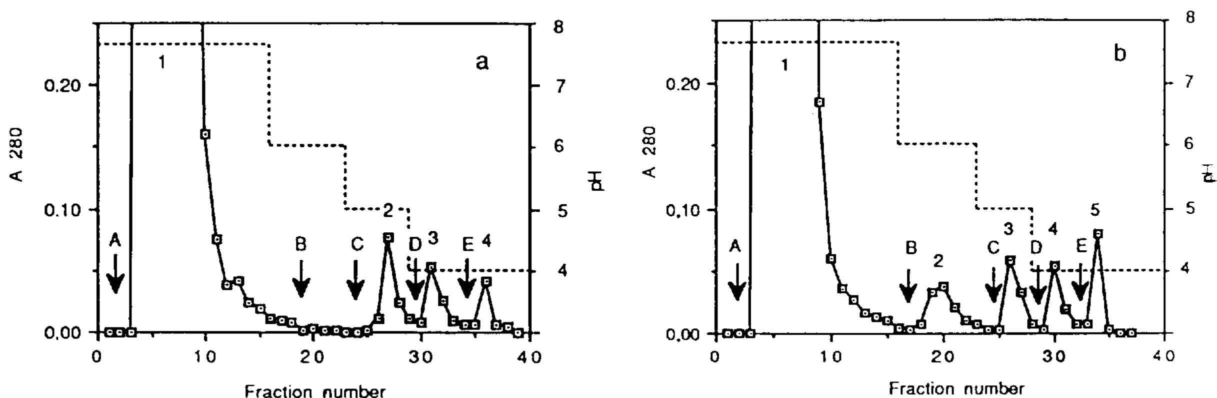


Fig. 5. Separation of IgG from untreated human serum on (a) H-B-PEVA and (b) H-E-PEVA hollow fibre membranes. Conditions: (A) 0.025 *M* Tris–HCl buffer (pH 7.4); (B) 0.025 *M* acetate buffer (pH 6); (C) 0.025 *M* acetate buffer (pH 5); (D) 0.025 *M* acetate buffer (pH 4); (E) 0.025 *M* acetate buffer (pH 4) + 1 *M* sodium chloride; injection, 1.5 ml of human serum; fraction volume, 6.0 ml. The numbers 1–5 indicate the protein peaks obtained.

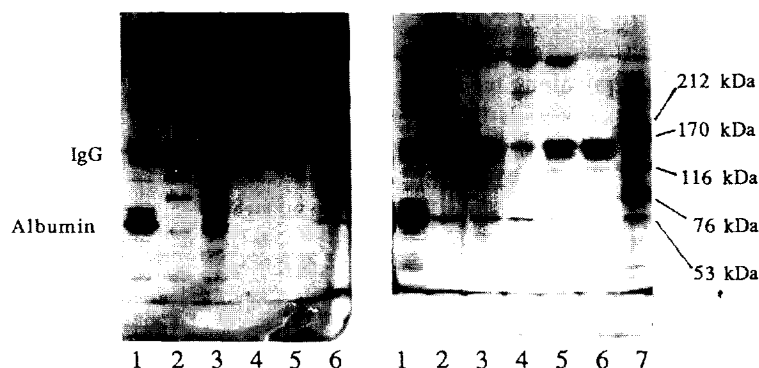


Fig. 6. (left) SDS-PAGE under non-reducing conditions of the fractions from chromatography (pH elution) on H-B-PEVA hollow fibre membrane. Lanes: 1 = peak 1; 2 = prepurified IgG from human plasma (Institut Mérieux); 3 = molecular mass markers; 4 = peak 2; 5 = peak 3; 6 = peak 4. (right) As (left) but on H-E-PEVA hollow fibre membrane. Lanes: 1 = peak 1; 2 = peak 2; 3 = peak 3; 4 = peak 4; 5 = peak 5; 6 = prepurified IgG from human plasma (Institut Mérieux); 7 = molecular mass markers.

last two peaks corresponded to IgM (17% for peak 3 and 40.4% for peak 4).

Both the non-retained and the pooled retained fractions of human serum chromatographed on H-B-PEVA with salt gradient elution were further analysed by rate nephelometry to determine IgG, IgM and albumin. The results are given in Table 1. As can be seen, 18% of the IgG and 19% of the IgM present in the injected sample were adsorbed. The albumin concentration in the retained fraction was below the detection limit of the method.

Similar results were obtained with H-E-PEVA (Figs. 5b and 6b), except that an additional peak containing mainly IgG appeared at pH 6. This indicates a slightly weaker binding of IgG on this support than on H-B-PEVA. The purity of the IgG eluted at pH 6, 5, 4 and 4 + 1 M NaCl was slightly inferior to that obtained with H-B-PEVA.

Concluding this part of the study, we can say that a high purity of the separated IgG fractions was achieved using H-B-PEVA and pH gradient elution, even with the untreated human serum used as a starting material. It seems, however, that not all IgG fraction is adsorbed, a phenomenon that was also reported by El-Kak and Vijayalakshmi [3].

In a control experiment, human serum was injected into a column containing an underivatized PEVA membrane in the same manner as for H-B-PEVA. Elution was performed with 0.1,

Table 1
Nephelometric determination of IgG, IgM and albumin during chromatography of human serum on H-B-PEVA

Fraction	IgG		IgM		Albumin	
	mg	%	mg	%	mg	%
Injected	14.8	100	2.6	100	60.7	100
Non-retained	10.9	74	1.8	69	61.8	100
Pooled retained fractions	2.6	18	0.5	19	<D.L.[a]	–

Injection, 1.5 ml of human serum; elution, 0.1 and 0.2 M NaCl.
[a] Below detection limit.

0.2 and 1 *M* NaCl, followed by 50 m*M* NaOH. The adsorbed protein which was completely eluted with 0.1 *M* NaCl was equivalent to 12% of the total adsorbed protein on H-B-PEVA under the same conditions. The peak contained mainly IgG and some albumin (results not shown).

3.3. Adsorption studies with prepurified human IgG

During chromatography of human serum on the affinity supports with immobilized histidine, IgG is not only separated from human serum but also fractionated into "subsets". Adsorption studies were therefore carried out using prepurified IgG from human plasma in order to study this phenomenon further.

Prepurified IgG from human plasma was chromatographed on both the histidyl affinity membranes produced by epichlorohydrin and bisoxirane activation. We show here only the results obtained with H-B-PEVA as the results obtained with H-E-PEVA are very similar. Fig. 7 shows the elution pattern using salt elution. The first large peak represents the unretained protein and the second and third peaks contain proteins eluted with 0.1 and 0.2 *M* NaCl, respectively.

Table 2
Isoelectric points and sub-class composition in the fractions from chromatography on H-B-PEVA using salt or pH gradient elution (IgG_4 was not detected)

Peak	p*I*	IgG_1	IgG_2	IgG_3
Pass-through	6–8	+	+	–
0.1 *M* NaCl	8–9.5	+	–	+
0.2 *M* NaCl	8–9.5	+	–	+
pH 5	8–9.5	+	–	+
pH 4	8–9.5	+	–	+
pH 4 + 1 *M* NaCl	8–9.5	+	–	+

The peaks were first analysed by isoelectrofocusing. The non-retained peak contained IgG with a p*I* between 6 and 8. All retained fractions contained IgG with pI between 8 and 9.5. The subclass composition in the different peaks was determined qualitatively by immunodiffusion with sheep-anti-human IgG subclass antisera and the results are given in Table 2. The non-retained peak contained IgG_1 and IgG_2 and the retained fractions IgG_1 and IgG_3.

The elution pattern of prepurified IgG using pH gradient elution is shown in Fig. 8. The first peak represents the unretained protein and

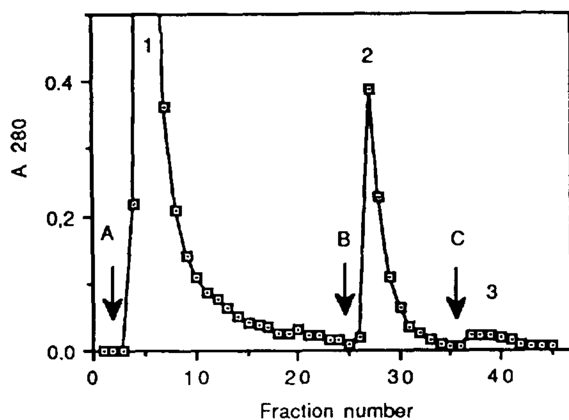


Fig. 7. Chromatography of prepurified IgG from human plasma on H-B-PEVA hollow fibre membrane. Conditions: (A) 0.025 *M* Tris-HCl buffer (pH 7.4); (B) 0.025 *M* Tris–HCl buffer (pH 7.4) + 0.1 *M* sodium chloride; (C) 0.025 *M* Tris–HCl buffer (pH 7.4) + 0.2 *M* sodium chloride; injection, 12 mg of IgG; fraction volume, 3.0 ml. The numbers 1–3 indicate the protein peaks obtained.

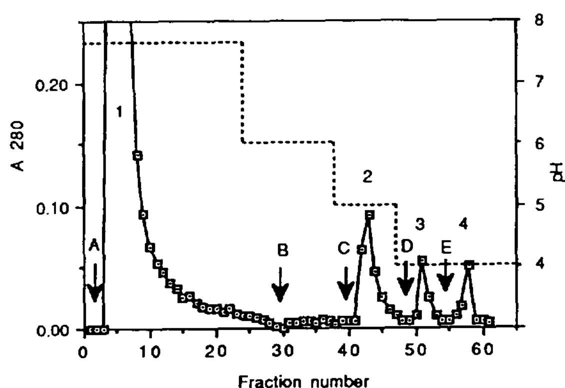


Fig. 8. Chromatography of prepurified IgG from human plasma on H-B-PEVA hollow fibre membrane. Conditions: (A) 0.025 *M* Tris–HCl buffer (pH 7.4); (B) 0.025 *M* acetate buffer (pH 6); (C) 0.025 *M* acetate buffer (pH 5); (D) 0.025 *M* acetate buffer (pH 4); (E) 0.025 *M* acetate buffer (pH 4) + 1 *M* sodium chloride; injection, 12 mg of IgG; fraction volume, 3.0 ml. The numbers 1–4 indicate the protein peaks obtained.

peaks 2, 3 and 4 were obtained by elution at pH 5, 4 and 4 + 1 M NaCl, respectively.

By IEF analysis, pI values between 8 and 9.5 were found for the IgG subsets in the eluted peaks. Immunodiffusion showed that the non-retained peak contained IgG_1 and IgG_2 and the retained fractions contained IgG_1 and IgG_3 (Table 2). IgG_4 could not be detected in either of the fractions, probably because this subclass is present in the serum only in a very low concentration [13].

In contrast to already existing affinity membranes for IgG purification with protein A as ligand (Sepracor, USA), which does not adsorb IgG_3 [13], our membranes with immobilized histidine are able to adsorb IgG_3 but not IgG_2. We therefore believe that this system is a good complement to the existing means for IgG purification.

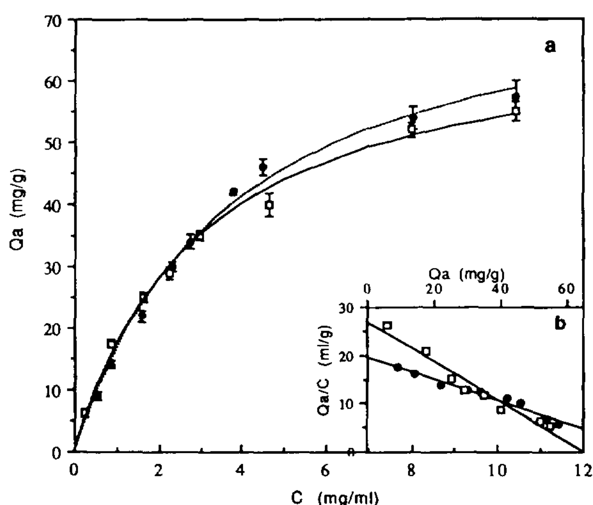


Fig. 9. (a) Adsorption isotherms of human plasma IgG (retained fraction) and (b) Scatchard plots. ● = H-B-PEVA; □ = H-E-PEVA. Experimental procedures are described in detail under Experimental.

3.4. Determination of the dissociation constant for IgG and capacity of the affinity membranes

The dissociation constant, K_D, and maximum binding capacity, Q_x, for IgG (retained fraction) were determined in an equilibrium binding analysis. The technique we employed for this analysis (see Experimental) had some considerable advantages, namely low sample and time requirements and the fact that convection is forced into two directions so that the protein will easily reach the inner surface and the pores of the hollow fibres.

Fig. 9 shows the adsorption isotherms for H-B-PEVA and H-E-PEVA. The shapes of the isotherms indicate Langmuir-type adsorption, which can be described, at equilibrium, by the equation $Q_a = Q_x C/(K_D + C)$, where K_D is the dissociation constant of the system, C is the unbound protein concentration at equilibrium, Q_a is the amount of adsorbed protein and Q_x the maximum binding capacity of the adsorbent [14].

For H-B-PEVA, K_D and Q_x were $2.5 \cdot 10^{-5}$ M and 80 mg/g support, respectively, and for H-E-PEVA, K_D was $2.0 \cdot 10^{-5}$ M and Q_x 70 mg/g support, respectively (Table 3).

The values obtained for K_D indicate a weak affinity, which is consistent with the easy and non-denaturing desorption of IgG and advantageous for the good recovery of non-denatured proteins. The K_D for human IgG reported previously [8] for different flat sheet membranes with immobilized histidine were slightly higher but of the same order of magnitude [$(3.3–14.0) \cdot 10^{-5}$ M]. The K_D for human IgG obtained previously [8] on a commercial protein A membrane (Nygene, New York, USA) was $5.0 \cdot 10^{-5}$ M and thus in the same range.

The capacity of 80 and 70 mg IgG/g support for our affinity membranes is in the same range as the values reported previously for flat sheet membranes [8]. On the other hand, it is certainly possible to optimize the performance of our system by increasing the ligand density on the

Table 3
Ligand concentration, dissociation constant (K_D) for IgG and maximum binding capacity (Q_x) of H-B-PEVA and H-E-PEVA determined in batch mode

Support	Ligand density[a] (μmol/g support)	K_D (10^{-5} M)	Q_x (mg/g)
H-B-PEVA	60	2.5 ± 0.2	79.8 ± 2.9
H-E-PEVA	126	2.0 ± 0.2	70.0 ± 2.8

[a] Determined by elementary analysis of nitrogen.

membrane. The fact that H-B-PEVA had a 14% higher capacity than H-E-PEVA (despite the higher ligand density of the latter; see Table 3) indicates that a longer spacer arm might render the ligand more accessible and facilitate adsorption.

In order to determine whether there is any protein interaction cooperativity during the adsorption process, the data were also analysed in a Hill plot using the transformed Langmuir equation $\ln(Q_a/L) = \ln K_a + n \ln C$ (Fig. 10), where $L = Q_x - Q_a$ is the equilibrium free ligand concentration and n the cooperativity coefficient ($n = 1$ means no cooperativity) [14]. By plotting $\ln(Q_a/L)$ versus $\ln C$, the n value obtained from the slope of the straight line was found to be 0.94 for H-E-PEVA and 1.05 for H-B-PEVA, which proves that there is no cooperativity due to protein–protein interaction.

3.5. Separation of IgG from human serum using an intact hollow fibre cartridge derivatized with histidine

In order to verify that activation, ligand coupling and IgG separation are possible using the intact hollow fibre cartridge, the latter was activated with bisoxirane and histidine was coupled in a continuous mode. Activation and coupling solutions were recirculated through the cartridge using a peristaltic pump. The histidine immobilization on the membrane was verified by its ability to bind Cu^{2+} ions.

A 65-ml volume of human serum (containing about 600 mg of IgG) was diluted 1:10 with the equilibration buffer and injected with an inlet flow-rate of 58 ml/min (the filtrate/inlet flow-rate ratio was 0.86) and elution was performed with 0.1, 0.2 and 1.0 *M* NaCl. Fractions of 30 ml were collected and the absorbance at 280 nm was determined. A schematic diagram of the cartridge and its mounting during the separation is given in Fig. 11. The elution pattern obtained was similar to the chromatographic pattern obtained with cut fibres. The protein was totally eluted with 0.1 *M* NaCl. SDS-PAGE and immunoelectrophoresis revealed that the peak contained IgG of the same purity as obtained with cut fibres (not shown). For regeneration, 0.3 *M* NaOH was pumped through the cartridge for 30 min at a flow-rate of 100 ml/min.

3.6. Stability of the affinity membranes

For practical applications, the stability of an affinity membrane is very important. Repeated injections of prepurified IgG and human serum over 2 years (more than 100 injections) always

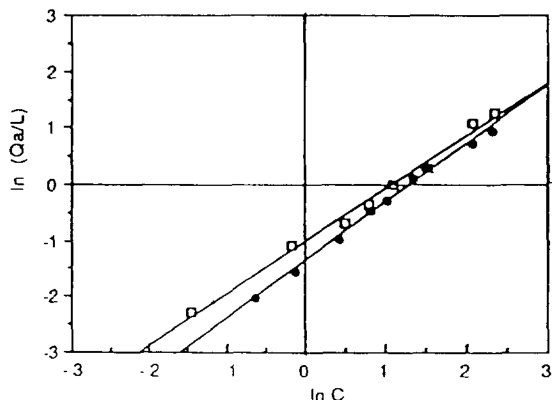


Fig. 10. Determination of the protein interaction cooperativity (Hill plot of the data in Fig. 9). ● = H-B-PEVA; □ = H-E-PEVA.

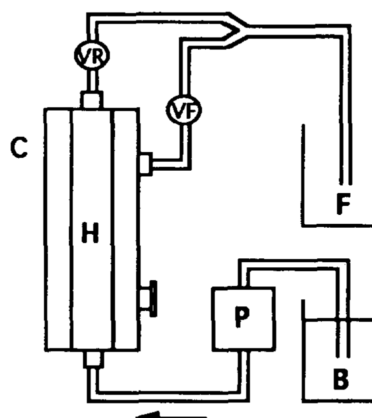


Fig. 11. Mounting scheme of the hollow fibre cartridge during separation of IgG from human serum. B = reservoir; P = peristaltic pump; C = cartridge; H = hollow fibre bundle; VR = retentate valve; VF = filtrate valve; F = fraction collector.

gave the same elution pattern and no changes in selectivity and capacity were observed. For regeneration of the membrane, up to 0.5 *M* NaOH could be used. We can therefore state that the affinity membranes produced are perfectly stable for at least 2 years. In contrast to protein A or G, the pseudobiospecific ligand histidine is not, or to a much lesser extent, subjected to physical, chemical or microbial degradation. This facilitates handling and storage of the cartridge and increases its lifetime considerably. Another advantage is that even if ligand leakage were to occur, this would be much less problematic than with protein A or G if the purified IgG is destined for clinical or analytical application.

In conclusion, we can claim that the histidyl-PEVA hollow fibre membranes produced are a versatile and effective tool for IgG separation from human serum. The moderate affinity allowed the elution of the adsorbed IgG under mild conditions, with salt or a pH step gradient. IgM is also adsorbed to a certain extent. If elution is effected by pH gradient, IgM (in contrast to IgG) is eluted only at pH 4 and pH 4 + 1 *M* NaCl. An affinity hollow fibre cartridge with 1 m^2 surface area can theoretically isolate 1.5 g of IgG per hour and the results are reproducible over at least 2 years. The affinity support is selective for IgG_1 and IgG_3, the latter being separated quantitatively from serum. The adsorbed IgGs have p*I* values between 8 and 9. We consider that these histidyl affinity hollow fibre membranes are a good alternative or a complement to existing tools for large-scale IgG purification.

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