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Protein adsorption on histidyl-aminohexyl-Sepharose 4B II. Application to the negative one-step affinity purification of human β2-microglobulin and Immunoglobulin G

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Abstract

The adsorption of two human proteins, β2-microglobulin and Immunoglobulin G, from uremic patient's blood ultrafiltrate and plasma, respectively, was investigated on the histidyl-aminohexyl-Sepharose 4B adsorbent. Both target proteins could be adsorbed on the gel through a low affinity for immobilized histidine ligand. However, a fine adjustment of the operating conditions (ionic strength, buffer, pH) prevented their adsorption and thus allowed their ''negative affinity'' purification (purity estimated by silver nitrate SDS–PAGE) by the removal of the contaminating proteins. This simple and efficient method provides purification under gentle chromatographic conditions and a further characterization of both molecules.

Keywords: Proteins; Histidyl-aminohexyl-Sepharose 4B; Microglobulins; Immunoglobulins

1. Introduction

Some human diseases involve an accumulation of pathological plasma proteins. Among these diseases, dialysis related amyloidosis (DRA) and autoimmune syndromes (lupus, polyarthritis . . .) are dependent on the increased levels of β2-microglobulin (β2-M) and auto-antibodies, respectively [1,2].

β2-M is an important protein for biomedical diagnostics because its increased serum level is often associated with pathologies (lupus, AIDS, cancer, renal disorders . . .). In the case of renal disorders, β2-M is not removed in a satisfactory manner by glomerular filtration and is increased by dialysis membrane that enhances the β2-M dissociation from HLA-I molecules on the surface of blood nuclear cells. As a consequence, β2-M concentration of uremic patients is raised to 30–50 μg ml^{-1} (instead of 1–2 μg ml^{-1} in healthy individuals). After 5–15 years, dialysis related amyloidosis (DRA) occurs resulting in carpal tunnel syndrome (CTS), lytic bone lesions, destructive arthropathy and spondyloarthropathy. The most distinctive feature of DRA is the deposition of amyloid fibrils, whose principal component is β2-M [1]. The results of analyses of β2-M present in amyloid deposits differ

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greatly. Proteolytic modification (deamination, cleavage), oligomerization of intact molecule, and acidic form due to advanced glycated end products (AGEs) have been reported [3–6]. β2-M analyses are difficult to perform because of its proteolytic sensitivity. Therefore, an efficient purification analytical method should be used for the recovery of the intact protein.

Autoimmune diseases are correlated with increased levels of auto-antibodies. It is a matter of debate if these antibodies are involved in or result from pathological disorders. Nonetheless, their recovery is important for studying the potential role of autoimmune Immunoglobulin G (IgG). As an example, antibodies from the sera of patients with autoimmune diseases were reported to have different catalytic functions [7,8]. Vijayalakshmi recently reviewed the different available methods for their purification and emphasized pseudobiospecific ligand adsorbent, such as histidine, for the high yield purification of autoantibodies under mild elution conditions [9]. These mild methods ensure the recovery of intact (non-denaturated), catalytically functional antibodies.

The recovery of these molecules in their native form is of considerable interest. To this end, the purification method must be efficient in quantitative terms, but even more so in terms of the quality of the recovered products: high purity should be obtained by a minimum of gentle steps to recover molecules with their structural integrity preserved [9].

Chromatography on pseudobiospecific ligands appeared to us as an interesting alternative to low specificity methods (size exclusion, ion-exchange) and to biospecific high affinity ligands (Protein A, Protein G) that invariably involve drastic/denaturing elution conditions, as highlighted by Vijayalakshmi [9,10]. Amino acid ligands, such as Trp, Phe or His, have thus been used for the purification of IgG from different biological sources [11–13], including autoimmune antibodies from human plasma [14]. El kak and Vijayalakshmi showed that histidine-aminohexyl-Sepharose 4B allowed the specific recovery of a monoclonal IgG fraction from the adsorption of all the proteins of mouse ascetic fluids due to discrimination during the elution steps [12]. Because of the low specificity of this adsorbent, Bueno et al. developed histidine grafted hollow fiber membrane, for the highly specific recovery of IgG from human plasma (maximal capacity of 1 g IgG per m^2) [13].

Histidine ligand affinity chromatography exhibits considerable versatility, depending on the high (cumulative) interactions of this amino acid [15]. The specificity of adsorption for a protein depends on both the matrix/ligand system (nature of support, spacer arm, orientation of ligand) and chromatographic conditions (pH, buffer, salt . . .). As an example, histidine coupled poly(ethylene vinyl alcohol) hollow fiber membrane preferentially bound IgG1 and IgG3 in Tris buffer (all the subspecies could be adsorbed in MOPS buffer, pH 6.5) via their Fab fragment [16], while histidine grafted aminohexyl-Sepharose 4B (His-AH-Seph) more efficiently adsorbed Fc fragment of IgG2 subclasses in Tris buffer (and IgG1 with a lower affinity) [17,18].

In a more recent work, we have studied human serum albumin (HSA) adsorption on His-AH-Seph as a function of the chromatographic conditions (pH, buffer, temperature, conductivity) in order to better evaluate the adsorption properties of this pseudo-affinity support [19]. HSA adsorption was quantitatively important (>50 mg HSA per ml of adsorbent) over a broad pH range (6–9) under low ionic strength conditions, suggesting that this adsorbent could be an efficient tool for HSA (and other proteins) removal from biologic solutions. In the present paper, we report the use of His-AH-Seph as ''a negative affinity adsorbent'' for IgG and for β2 microglobulin. When applied to the purification of two proteins of interest, human β2-M and IgGs from ultrafiltrate blood and plasma, respectively, a fine adjustment of the chromatographic conditions leads to optimal removal of other proteins (contaminants).

2. Experimental

2.1. Materials

Aminohexyl-Sepharose 4B (AH-Seph) was obtained from Pharmacia Biotech Fine Chemicals (Uppsala, Sweden). Acrylamide, bovine serum albumin, glycine, methylenbisacrylamide, ammonium persulfate, sodium dodecyl sulphate, tetramethyl ethylene diamine (TEMED) and Tris(hydroxy-

methyl)–aminomethane (Tris–HCl), hydroxyethylpiperazine propanesulfonic acid (EPPS), morpholinoethanesulfonic acid (MES), morpholinopropanesulfonic acid (MOPS), sodium chloride and acetic acid were purchased from Sigma–Aldrich (St Louis, MO, USA). All chemicals used were of analytical grade. Ultra-pure water, obtained with the MilliRO-MilliQ+ System (Millipore, Bedford, MA, USA), was used throughout.

Blood ultrafiltrate was collected from a long-term hemodialysis patient during 5-h hemofiltration sessions (Fresenius F80 polysulfone hemofilter, 50-kDa cut-off, Bad, Hamburg, Germany) by Dr P. Moriniere at Amiens Hospital, France. Ultrafiltrate corresponded to the low molecular weight plasma proteins of uremic patient's blood with β2-M as major components ([β2-M] ~30–50 μg ml^{-1}) and human serum albumin ([HSA] ~0.5–1 mg ml^{-1}).

Blood samples from healthy donors or patients were collected with anticoagulant (sodium citrate, 129 m*M*). Plasma was obtained after blood centrifugation.

2.2. Support derivatization

Histidine was coupled on aminohexy-Sepharose 4B gel via its COOH group using water-soluble carbodiimide at pH 4.5–6 with lateral stirring for 6 h at room temperature as previously described [12].

2.3. Protein determination

Total protein concentrations were determined by the Bradford method [20] using bovine serum albumin as standard. β2-M and IgG concentrations were determined by nephelometry using a Beckman Array Protein System with the Beckman reagents (Beckman Coulter, Gagny, France).

2.4. Routine chromatography

Chromatographic experiments were performed on 2 ml of His-AH-Seph gel (at room temperature, flow-rate: 1 ml min^{-1}). Equilibration of the column was performed by passing three column volumes of the adsorption buffer before injection of a protein solution. In order to minimize the protein loss and the sample dilution, the collection of the unbound fraction was started when the absorbance appeared on the chromatogram (this time corresponding to the dead volume of the column, e.g. ~2 min for a column of 2 ml of gel). The same delay was implemented during the washing step with adsorption buffer before diverting the effluent to waste, so that collected fraction presented the same volume as the injected fraction (no dilution of the non-retained fraction). The column was then washed until the measured absorbance reached the base-line. Elution of proteins was performed by increasing NaCl concentration. After each use, the column was washed with three column volumes of 50 m*M* NaOH, followed by water and finally with the equilibration buffer.

2.5. SDS–PAGE and isoelectric focusing

SDS–PAGE analyses were performed in a Miniprotean II system (Bio-Rad, Richmond, CA, USA) or using the Phast™ System (Pharmacia Biotech, Uppsala, Sweden), respectively. Proteins of chromatographic fractions were analysed by 10 or 15% (or 4–25% for commercial Phast™system gels) acrylamide gels under non-reducing or under reducing conditions (β-mercaptoethanol) SDS–PAGE according to Laemmli [21]. Isoelectric focusing (IEF) was performed on the commercial Phast™ system gel 3-10. All the presented gels were silver stained as described [22]. Apparent molecular weights and isoelectric points were calculated by linear extrapolation of standard markers (Low or High Molecular Weight and IEF Calibration Kit, Pharmacia Biotech).

2.6. Electrospray ionization mass spectrometry (ESI-MS)

Fractions were analysed by a single quadrupole mass spectrometer (Finnigan SSQ 710, San Jose, CA, USA) with a 100°C drying gas electrospray ionization source (Analytica, Brandford, CT, USA) under 2.5–3.5 kV. Each desalted (using PD-10 column, Pharmacia) sample was diluted in 1 vol. of

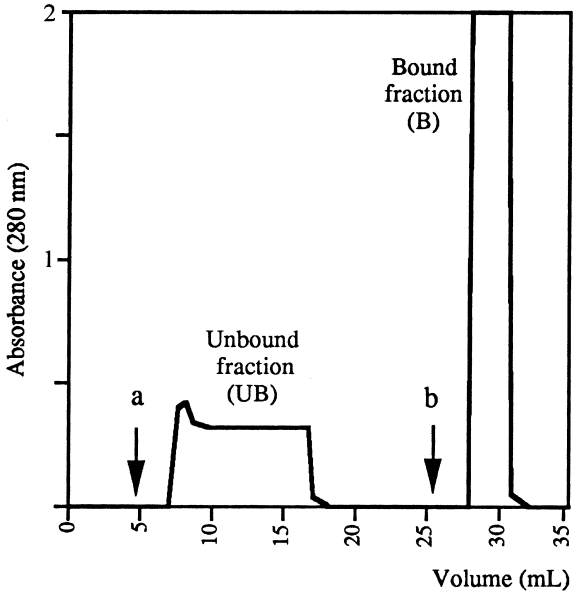

Fig. 1. Separation of proteins from patient's blood ultrafiltrate on His-AH-Seph. Chromatography of ultrafiltrate proteins (5 ml diluted in 1 vol. of 50 m*M* MOPS buffer, pH 7.0) was performed in 25 m*M* MOPS buffer, pH 7.0, at a flow-rate of 1 ml min^{-1}: (a) injection, (b) elution with 25 m*M* MOPS buffer, pH 7.0, 0.5 *M* NaCl.

methanol–acetic acid (100:1, v/v). The flow-rate was set at 1 μl min^{-1} by a syringe pump (Harward, South Natick, MA, USA).

3. Results

3.1. Purification of β2-microglobulin (from blood ultrafiltrate of an uremic patient)

A 5-ml amount of blood ultrafiltrate of a long-term uremic patient (essentially composed of a mixture of HSA and β2-M in a ratio of ~10:1) was added to an equivalent volume of 50 m*M* buffer (acetate, MES or MOPS) at different pHs (5.0–8.0). A typical chromatographic profile on His-AH-Seph is presented in Fig. 1. SDS–PAGE analysis of unbound and bound fractions showed that the adsorption of HSA and β2-M was strongly pH dependent (Fig. 2). At pH 7.0 and 8.0, the separation was very efficient with recovery of β2-M and HSA in the pass-through fraction. The negative purification in the non-retained fraction of all the β2-M of the sample was confirmed by the ESI-MS analysis of chromatographic fractions (Fig. 3). Below pH 6.0, HSA appeared as an important contaminant in the non-retained fraction, while at pH 5.0 both proteins are present in the unbound and bound fractions as shown by the nephelometry assays (Table 1). A more systematic study of pH influence, performed between pH 5.0 and 9.0 (with 0.5 grade increment), confirmed that the negative purification of human β2-M from blood ultrafiltrate could be obtained over a large range near physiologic pH (6.5–8.5). In addition, the

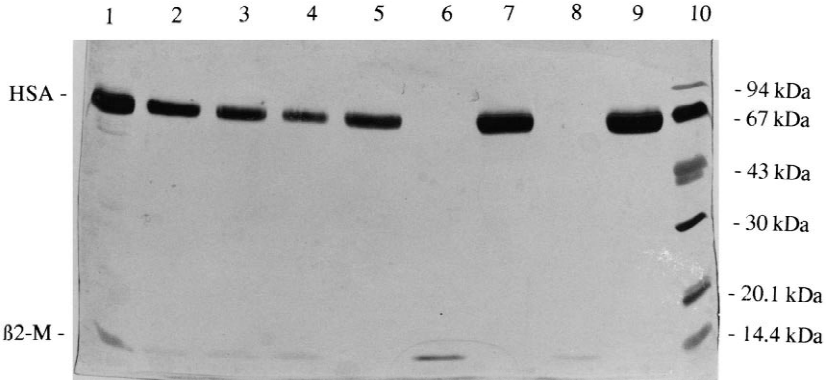

Fig. 2. SDS–PAGE analysis of unbound (UB) and bound (B) fractions from chromatographies on His-AH-Seph of ultrafiltrate proteins as a function of the adsorption buffer pH. 1, Ultrafiltrate proteins; 2, UB fraction at pH 5.0 (25 m*M* MES); 3, B fraction at pH 5.0 (25 m*M* MES); 4, UB fraction at pH 6.0 (25 m*M* MES); 5, B fraction at pH 6.0 (25 m*M* MES); 6, UB fraction at pH 7.0 (25 m*M* MOPS); 7, B fraction at pH 7.0 (25 m*M* MOPS); 8, UB fraction at pH 8.0 (25 m*M* MOPS); 9, B fraction at pH 8.0 (25 m*M* MOPS); 10, low molecular weight standard (Pharmacia Biotech).

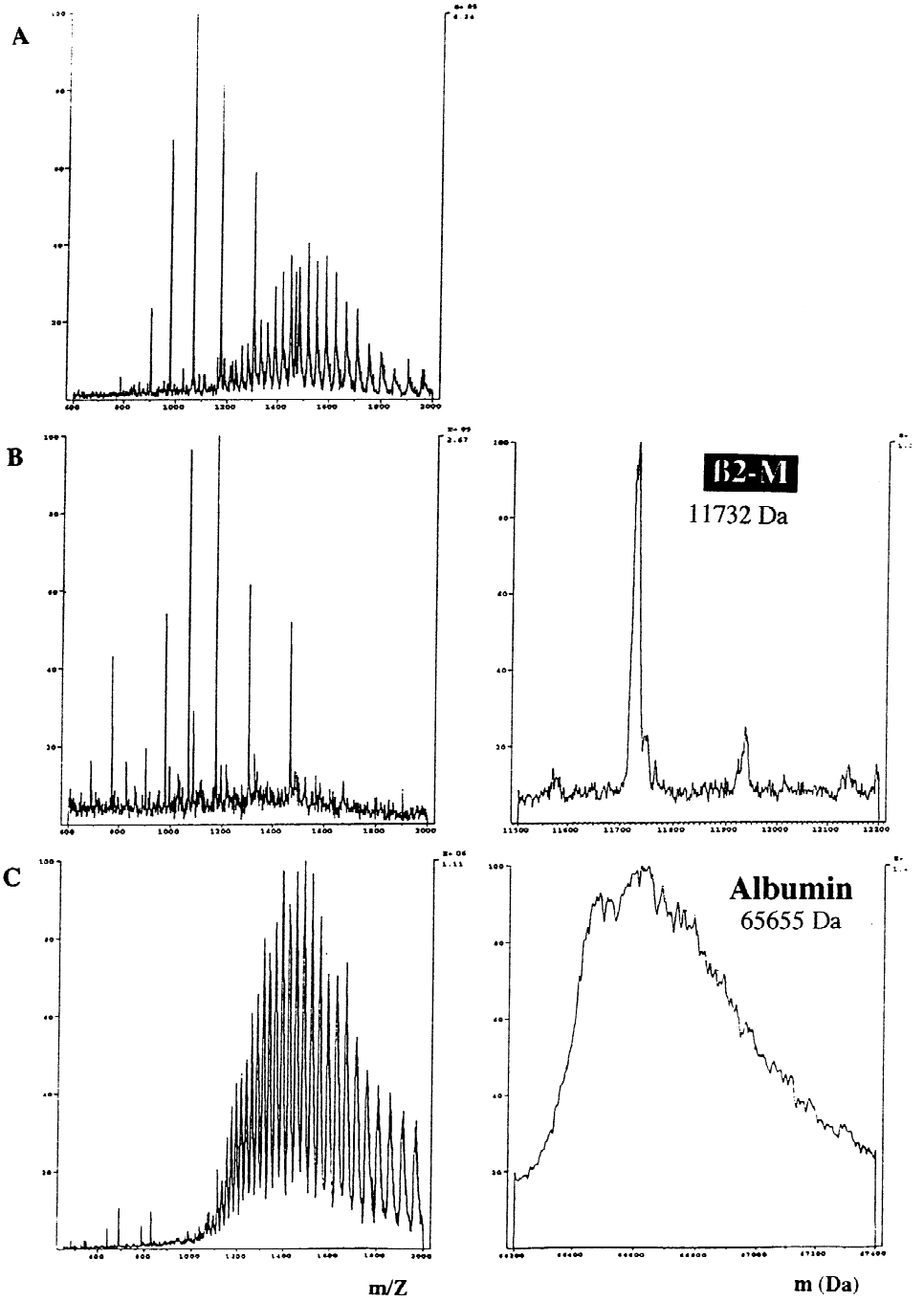

Fig. 3. Electrospray ionization mass spectrometry (ESI-MS) analyses of the separation of ultrafiltrate proteins on His-AH-Seph (Fig. 1). (A) Analysis by ESI-MS of ultrafiltrate composed of a mixture of β2-M and HSA; (B) ESI-MS analysis of the unbound fraction (pure β2-M); (C) ESI-MS analysis of the bound fraction (pure HSA). The left and right panels represent original spectra before deconvolution and protein mass determination (obtained by computer deconvolution of spectra), respectively. Details of sample preparation for ESI-MS are described in Experimental section.

Table 1
Protein determination of HSA and β2-M during chromatography of patient's blood ultrafiltrate on His-AH-Seph as a function of adsorption pH

Adsorption pH	Unbound fraction				Bound fraction			
	HSA		β2-M		HSA		β2-M	
	mg	(%)	μg	(%)	mg	(%)	μg	(%)
5.0	1.43	44.3	63.4	37.1	1.62	50.2	72.7	42.5
6.0	0.82	25.3	150.6	88.1	2.26	69.8	0	0
7.0	0	0	174.6	102.1	3.12	98.6	0	0
8.0	0	0	136.5	79.8	3.41	105.2	0	0

[HSA] and [β2-M] were determined by Bradford method and nephelometry, respectively.

separation of untreated (no buffer dilution) patient's ultrafiltrate could even be performed with the recovery of purified β2-M in the unbound fraction.

3.2. Purification of IgG (from human plasma)

3.2.1. Influence of plasma dilution

The same His-AH-Seph gel was used to process plasma proteins (1.2 ml of plasma) 20-fold diluted in 25 m*M* MOPS buffer, pH 7.2. The purity of all non-retained fractions (4 ml each one) was analysed by SDS–PAGE (Fig. 4). Electrophoresis showed the real efficiency of the presented method for negative IgG purification in the unbound fraction, since all the other plasma proteins were adsorbed on the gel. Approximately 10% of the IgG was adsorbed on the gel.

In order to determine the influence of the ionic strength on the IgG adsorption, we processed different dilutions of 1 ml of plasma through the chromatographic column under the same operating conditions (25 m*M* MOPS buffer, pH 7.2). Both the non-retained and the adsorbed fractions were further analysed by rate nephelometry to determine IgG and HSA (Table 2). The same purity of the non-retained IgG fraction was observed by SDS–PAGE for the 50-fold diluted plasma (data not shown), but due to the lower ionic strength, a larger amount of anti-

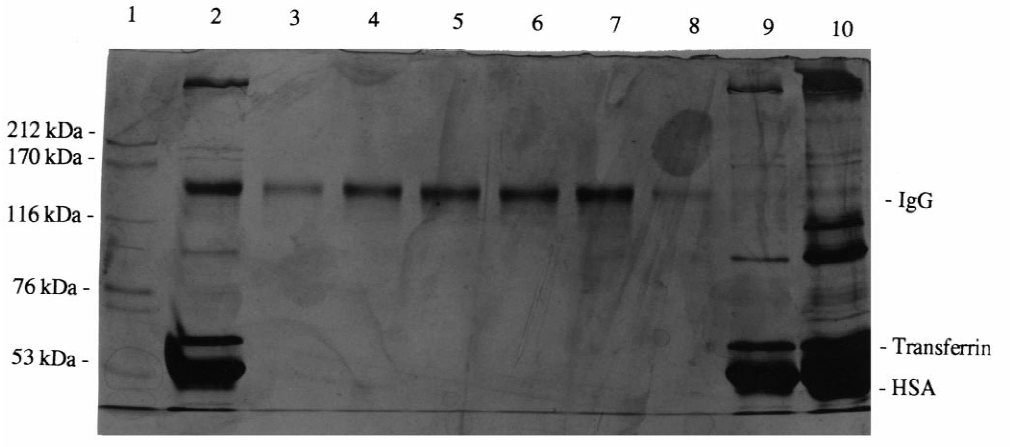

Fig. 4. Non reducing SDS–PAGE analysis of separated proteins from 20-fold diluted plasma proteins on His-AH-Seph chromatography (Fig. 5). 1, High molecular weight standard (Pharmacia Biotech); 2, injected plasma proteins; 3–8, non-retained fractions; 9, 3-fold diluted eluted fraction (the same proteins concentration as 2); 10, eluted fraction.

Table 2
Nephelometric determination of HSA and IgG during chromatography of human plasma on His-AH-Seph as a function of dilution factor

Dilution factor	Unbound fraction				Bound fraction				
	HSA		IgG		HSA		IgG		IgG capacity (mg per ml of adsorbent)
	mg	(%)	mg	(%)	mg	(%)	mg	(%)	
50	BT	0	5.64	63.4	38.8	102.1	2.24	25.2	1.12
20	BT	0	6.46	72.6	36.4	95.9	1.40	12.4	0.71
10	BT	0	7.24	81.4	36.9	97.2	0.75	8.4	0.38
5	BT	0	8.02	90.2	40.3	106.2	0.45	5.1	0.23

Dilution factor consisted of the dilution of 1 ml of plasma in 25 m*M* MOPS buffer, pH 7.2, before chromatography (performed in the same buffer). BT, below threshold of detection.

bodies bound to the adsorbent (25.2% as compared with 12.2% for the 20-fold diluted plasma). When lower plasma dilution was applied (e.g. 5- or 10-fold), IgG recovery in the unbound fraction increased, but other contaminant proteins (especially transferrin) were observed in the pass-through fraction.

In such a case, contaminants could be removed by re-processing of the non-retained fraction. SDS–PAGE analysis showed that pure IgG fractions could be obtained after the second run (Fig. 5). The other chromatographic runs of the non-retained fractions showed that polyclonal IgG fractionation could go on even after four runs of the non-retained fractions on the column.

3.2.2. Influence of adsorption pH

The adsorption pH is a critical point in the histidine ligand chromatography of proteins. Its effect on pure IgG and pure HSA adsorption on His-AH-Seph was previously described [18,19]. In the present work, we studied the concomitant adsorption of both the molecules present in plasma proteins. A 200-μl aliquot of human plasma, diluted in 10 ml of the chosen equilibrium buffer (25 m*M* MES, MOPS or EPPS at different pH) was chro-

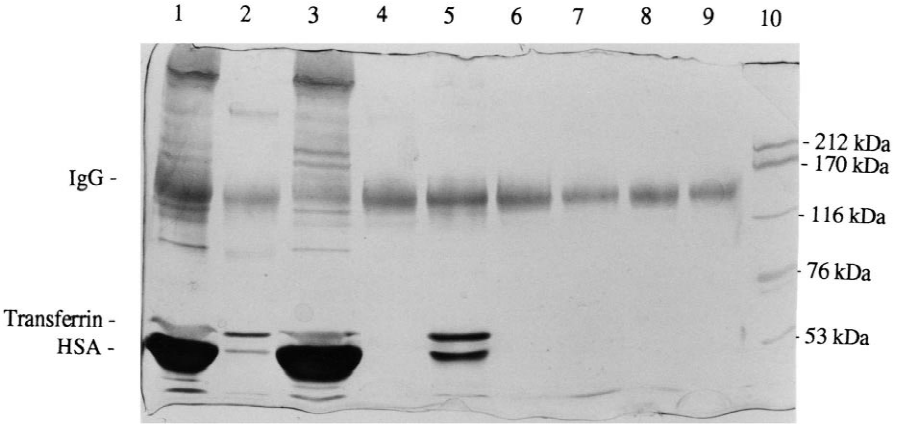

Fig. 5. Non reducing SDS–PAGE of separated plasma proteins during four chromatographic runs on His-AH-Seph (Fig. 8). 1, Injected plasma proteins (10-fold diluted); 2, unbound (UB) fraction (run 1); 3, bound (B) fraction (run 1); 4, UB fraction (run 2); 5, B fraction (run 2); 6, UB fraction (run 3); 7, B fraction (run 3); 8, UB fraction (run 4); 9, B fraction (run 4); 10, high molecular weight standard (Pharmacia Biotech).

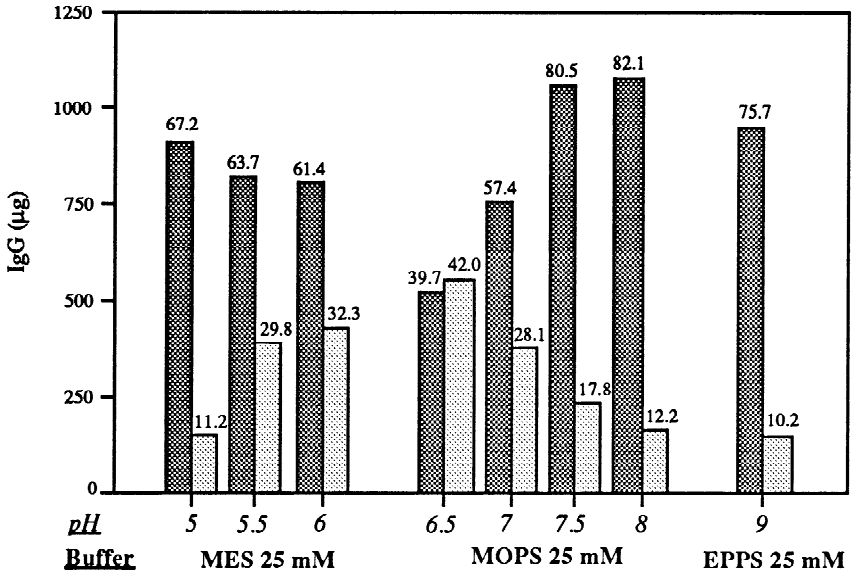

Fig. 6. Nephelometry determination of IgG after chromatography on His-AH-Seph of plasma proteins as a function of pH of the adsorption buffer.

matographed on His-AH-Seph. Nephelometric determination of unbound and bound IgG as a function of the adsorption buffer pH are presented in Fig. 6. Maximal IgG adsorption was observed at pH 6.5 (25 m*M* MOPS buffer) in agreement with the work of Haupt et al. [16]. Under low ionic strength equivalent to a salt concentration of 5 m*M*, almost complete albumin adsorption (>95%) was achieved at pHs from 7.0 to 9.0.

From these results, it could be shown that total IgG recovery in the non-retained fractions was possible by adjusting the pH of plasma. To study this, 500 µl of plasma, 10-fold diluted in 25 m*M* MES, pH 5.0, or in 25 m*M* MOPS, pH 7.0, were injected on the gel, respectively. At pH 7.0, ~20% of IgG was adsorbed on the gel. When the pH 5.0 buffer was used for the plasma protein adsorption, >95% of injected IgG could be recovered in the non-retained fractions (Fig. 7). This agrees well with the established data on the pH optimum for IgG adsorption [16].

3.3. Separation of plasma proteins of a uremic patient

In this case, a significant amount of β2-M was present in plasma in addition to the other proteins. A 10-fold diluted uremic patient's plasma (100 µl) was applied on the His-AH-Seph gel with 25 m*M* MOPS, pH 7.2, as an equilibrium buffer. SDS–PAGE analysis of the unbound and bound fractions revealed that the non-retained fraction contained a mixture of human IgG and β2-M (Fig. 8).

4. Discussion

4.1. Purification of β2-M

The present work reports a simple and efficient method, allowing one-step purification of β2-M from patient's blood ultrafiltrate under very mild conditions, as shown in the Results section. As already shown [19], HSA present in the ultrafiltrate was adsorbed on the His-AH-Seph gel over a broad range of pH, mainly through electrostatic interactions between this protein and NH_3^+ residual charged group of grafted histidine. As far as β2-M was concerned, the interactions between the gel and this protein seemed to be more complex. Despite a low isoelectric point (p*I* 5.7), β2-M adsorption through a ion-exchange phenomenon was not observed near neutral pH in these ionic strength conditions (salt concentration equivalent to ~75 m*M* [NaCl]; Fig. 1),

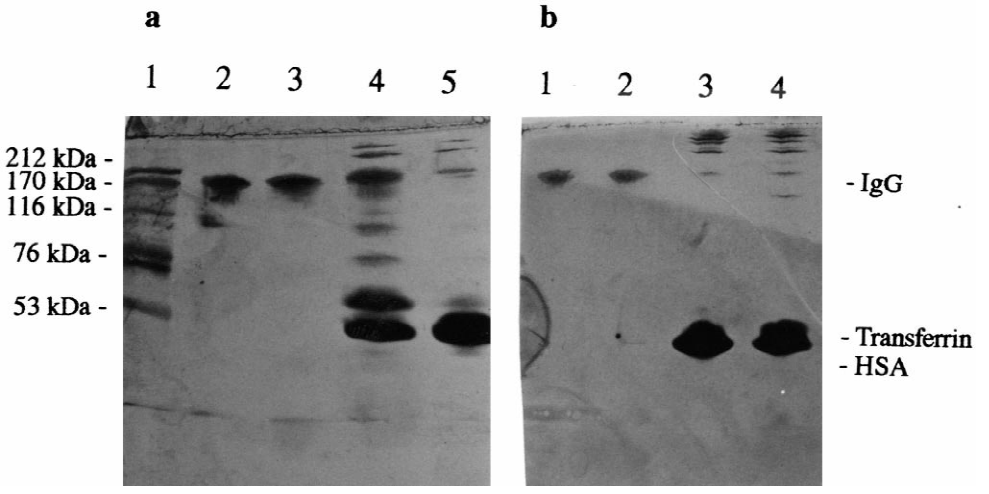

Fig. 7. Non-reducing SDS–PAGE analysis of unbound (UB) and bound (B) fraction of human plasma proteins (10-fold diluted) separated on His-AH-Seph at two adsorption pHs: pH 7.0 and pH 5.0. (a) 1, High molecular weight standard (Pharmacia Biotech); 2–3, UB fractions (pH 7.0); 4–5, B fractions (pH 7.0); (b) 1–2, UB fractions (pH 5.0); 3–4, B fraction (pH 5.0).

even if under lower ionic strength conditions (~15 m*M* [NaCl]) β2-M could be partially adsorbed on the gel (Fig. 8). Adsorption occurred near isoelectric point of β2-M (Fig. 1), as happened for the other proteins purified on immobilized histidine (not including albumin) [10]. The adsorption mechanism combined non-covalent molecular interactions (electrostatic, hydrogen bond, hydrophobic, charge transfer, van der Waals . . .) through a dipole (grafted ligand)-dipole (protein) recognition. The resulting association is of medium strength (~10^{-5} to 10^{-6} *M*), which could be easily weakened by adding salt to the adsorption buffer (for a recent review, see Ref. [23]).

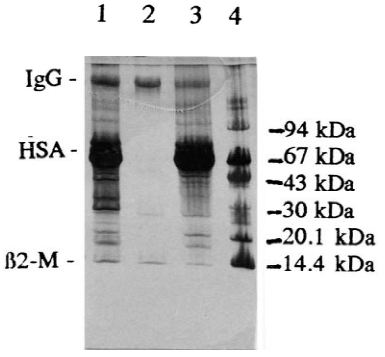

Fig. 8. Non-reducing SDS–PAGE analysis of uremic patient's plasma proteins after His-AH-Seph chromatography. 1, 10-Fold diluted plasma; 2, unbound fraction; 3, bound fraction, 4, low molecular weight proteins standard (Pharmacia Biotech).

In the present study, neutral pH and ionic strength conditions prevented the adsorption of β2-M by ion-exchange or by histidine ligand affinity. Since all the other proteins were adsorbed on the His-AH-Seph under the same conditions, the ''negative'' affinity purification of β2-M in the non-retained fraction was achieved.

4.2. IgG recovery

We have equally studied the use of a histidine adsorbent (His-AH-Seph) for the negative purification of autoimmune IgG directly from human plasma. The results described in Section 3.2 confirmed the high capacity of the gel for protein adsorption already described in Refs. [12,19]. IgG adsorption was in accordance with the gel capacity determined by El kak et al. [18]. In other words, it was very low when salt concentration increased. The gel's adsorption capacity towards IgG was much lower in the presence of other plasma proteins such as HSA (Table 2). Thus, it seemed better to purify IgG from human plasma by negative affinity, in the non-retained fraction, as was optimized in the present work. pH and ionic strength were demonstrated to significantly participate in the (non-)adsorption capacity for IgG of the His-AH-Seph gel. Finally, a

20-fold dilution of plasma proteins in 25 m*M* MOPS buffer at pH 7.2 resulted in a non-retained fraction of high purity for IgG (Fig. 4). Even though a pH 5.0 buffer gave a higher concentration of IgG in the non-retained fractions, it tended to be somewhat less pure. These mild chromatographic conditions allowed the recovery of IgG with native conformation as compared with Protein A/Protein G methods, as recently demonstrated by the example of natural catalytic antibodies [24].

In conclusion, the versatility of histidine ligand chromatography was exploited for the ''negative'' affinity purification of two important biomedical proteins (β2-M and IgG) under mild conditions (neutral pH). In principle, ''negative adsorption'' chromatography should give excellent recoveries of native proteins, and the pure protein could be further concentrated using chromatographic or other approaches, if desired.

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