ELSEVIER

Journal of Chromatography B, 664 (1995) 55–67

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Retention behaviour of proteins on poly(vinylimidazole)–copper(II) complexes supported on silica: application to the fractionation of desialylated human α_1-acid glycoprotein variants

Marie-Claude Millot[a,*], Françoise Hervé[b], Bernard Sébille[a]

[a]*Laboratoire de Physico-Chimie des Biopolymères, Université Paris XII Val de Marne, Avenue du Général de Gaulle, 94010 Créteil, France*

[b]*Laboratoire Hospitalo-Universitaire de Pharmacologie de Paris XII, Centre Hospitalier Intercommunal de Créteil, 40, Avenue de Verdun, 94010 Créteil Cédex, France*

First received 1 July 1994; revised manuscript received 19 July 1994

Abstract

The retention behaviour of various amino acids, peptides and proteins on poly(vinylimidazole)–Cu(II) complexes supported on silica was investigated. Free amino acids and peptides containing one histidine and in some instances one additional tryptophan residue in their primary structure were found to elute from the supports only after addition of a competing complexing agent to the mobile phase. However, the results obtained with proteins containing metal binding groups suggested that, in addition to the presence of donor–acceptor interactions between the macromolecules and the immobilized metal, other additional (essentially ionic and/or hydrophobic) interactions took place between the proteins and the surrounding of the metal. When donor–acceptor interactions were predominant, proteins were strongly adsorbed on the stationary phase and their elution required the addition of a competing complexing agent in the mobile phase. However, when the binding between the proteins and the supports via donor–acceptor interactions was less favourable, proteins were eluted from the columns without the addition of a competing agent in the mobile phase. With respect to the binding of these proteins, ionic and/or hydrophobic interactions were no longer negligible during the chromatographic process and the retention of the macromolecules by the stationary phase depended on the elution conditions (ionic strength, pH, etc.). These supports were used in the fractionation of the three main genetic variants of desialylated α_1-acid glycoprotein.

1. Introduction

Supports for metal chelate affinity chromatography (IMAC) are generally prepared by incorporating chelatogenic ligands [in this paper, "ligand" denotes the adsorbing moiety (i.e. PVI imidazole rings) bound to the solid matrix], such as carboxymethylated amines [1,2] or hydroxamic acid functions [3] into agarose [4], hydrophilic coated resins [5] or hydrophilized silica [6–9]. After binding of a suitable metal ion, the immobilized polydentate chelates can be used for

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00352-1

peptide [10–13] and protein separation [14]. Separations have been shown to depend strongly on the presence of electron donor groups at the surface of the proteins, such as histidine and tryptophan residues [15,16].

New IMAC supports, consisting of poly(vinylimidazole)–copper(II) complexes supported on silica, have recently been described [17]. The retention of proteins on these supports was found to be enhanced when Cu(II) complexation was performed in the presence of salts, such as $NaNO_3$, [Sil PVI $Cu(NO_3)$], as compared with complexes prepared in pure water, [Sil PVI $Cu(H_2O)$]. This was explained by the presence of a higher number of free coordination sites in [Sil PVI $Cu(NO_3)$] than in [Sil PVI $Cu(H_2O)$] complexes, as suggested by the electron spin resonance (ESR) spectra of both complexes [17].

In this work, the chromatographic properties of poly(vinylimidazole)–copper (II) complexes supported on silica and prepared in the presence of sodium nitrate, [Sil PVI $Cu(NO_3)$], were further investigated. For that purpose, we studied the retention behaviour of various amino acids, peptides and proteins on [Sil PVI $Cu(NO_3)$] complexes. With respect to the proteins studied, different elution conditions were used in order to investigate the interactions that take place between these molecules and immobilized copper(II) during chromatography. In addition, the use of these new supports in the fractionation of the desialylated human α_1-acid glycoprotein (AAG) variants is reported.

2. Experimental

2.1. Preparation of [Sil PVI $Cu(NO_3)$] columns

Homopolymerization of 1-vinylimidazole and coating of the silica surface were carried out as described previously [17]. After extensive washing with 150 ml of a 0.2 *M* $NaNO_3$ solution, the columns (15 × 0.46 cm I.D.) were charged with Cu^{2+} ions by eluting 1–20 ml of a 15 m*M* $Cu(NO_3)_2$ solution in 0.2 *M* $NaNO_3$. The columns were then washed with 0.2 *M* $NaNO_3$ and equilibrated with the elution buffer.

2.2. Liquid chromatography

The HPLC system consisted of Kipp Analytica (Delft, Netherlands) Model 9208 solvent-delivery pumps, a Rheodyne Model 7120 loop injector with a 50-μl sample loop and a Waters (Milford, MA, USA) Model 450 variable-wavelength UV detector.

2.3. Materials and reagents

LiChrospher Si 300 (d_p = 10 μm; porosity 300 Å) was purchased from Merck (Darmstadt, Germany). Cytochrome *c* from horse heart (C), α-chymotrypsinogen A from bovine pancreas (Ch), lysozyme from chicken egg white (Lh) and from turkey egg white (Lt), ubiquitin from bovine red blood cells (U), human serum albumin (HSA), bovine serum albumin (BSA), α-lactalbumin from bovine milk (αL) and all the amino acids were obtained from Sigma (St. Louis, MO, USA). Peptides were kindly supplied by Neosystem (Strasbourg, France). All mineral salts, ethylenediaminetetraacetic acid (EDTA) and imidazole were purchased from Prolabo (Paris, France).

2.4. Purification of human individual α_1-acid glycoprotein (AAG) samples with different phenotypes

Human plasma samples were obtained from healthy donors and frozen at −20°C until used. Selection of the individual plasma samples containing AAG with the F1S/A, F1/A or S/A phenotype was performed after AAG phenotyping in plasma, as described by Eap and Baumann [18]. The purification of individual AAG samples with the F1S/A, F1/A and S/A phenotypes from plasma was carried out by chromatography on Cibacron Blue F3G-A immobilized on Sephadex G-100, as already described [19]. The AAG preparations all appeared to be homogeneous after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoelectrophoresis against anti-whole human plasma.

Prior to chromatography on [Sil PVI

$Cu(NO_3)$] supports, the individually purified AAG samples were delipidated by charcoal treatment and then desialylated with neuraminidase, as described previously [20]. The proteins were dialysed against deionized water and finally lyophilized. The microheterogeneity of the three AAG phenotypes samples, F1S/A, F1/A and S/A, and the composition into variants of the peaks obtained after chromatography on [Sil PVI $Cu(No_3)$] support were checked by analytical isoelectric focusing (IEF) on an immobilized pH 4.4–5.4 polyacrylamide gel gradient supplemented with 8 *M* urea and 2% (v/v) 2-mercaptoethanol, as already described [18].

2.5. Chromatographic procedures

Phosphate buffer systems (25–100 m*M*) of pH 6–8, containing 0.2–1 *M* NaBr or Na_2SO_4, were used as eluents. In some experiments, imidazole (0–10 m*M*), histidine (0–1 m*M*), ammonia (0–10 m*M*) or lysine (0–10 m*M*) was added to the mobile phase.

The standard amino acid, peptide and protein solutions were prepared in phosphate buffers and used at concentrations of 0.2, 0.5 and 1 mg/ml, respectively.

The flow-rate was 0.6 ml/min and UV detection was carried out at 280 nm for the proteins and at 225 nm for the amino acids and peptides. The retention times were measured at room temperature.

2.6. Retention coefficient

Chromatography of the different amino acid, peptide and protein samples was performed under similar conditions, before and after metal loading of the columns. The elution volumes of each solute measured with the naked support [V_{PVI} (ml)] and the loaded [Sil PVI $Cu(NO_3)$] support [V_{Cu} (ml)], respectively, were used to calculate the retention coefficient, k'_{Cu}, of the solute, according to the equation

$$k'_{Cu} = \frac{V_{Cu} - V_{PVI}}{V_m} \quad (1)$$

where V_m is the mobile phase volume of the column, as determined by injection of sodium nitrate (V_m was ca. 1.75 ml in our experiments).

3. Results and discussion

3.1. Retention behaviour of amino acids on [Sil PVI $Cu(NO_3)$] supports

The side-chain groups of histidine, tryptophan and cysteine residues in peptides and proteins play an important role in the retention of these molecules on conventional IMAC supports [14]. This was originally suggested by the studies of Porath et al. [4] and subsequently further confirmed by many other studies [8,21,22].

As the studies dealing with the retention behaviour of free amino acids cannot completely depict the interactions between amino acid residues located at the surface of proteins and the immobilized metal, as these interactions are probably influenced by the molecular environment and steric hindrance effects, studies with free amino acids may nevertheless give information on the mechanisms involved in the IMAC of proteins. In this respect, we studied the retention of different amino acids on [Sil PVI $Cu(NO_3)$] supports and the results are shown in Table 1. All the amino acids tested were eluted from the columns only after addition of a competing complexing agent (e.g., lysine) in the mobile phase, whereas their N-acetyl derivatives were eluted in the mobile phase (data not shown). The results in Table 1 showed that tryptophan was strongly retained on [Sil PVI

Table 1
Retention factors of some amino acids on a [Sil PVI $Cu(NO_3)$] support

Amino acid	k'_{Cu}	Amino acid	k'_{Cu}
Glutamic acid	0.5	Phenylalanine	3
Asparagine	3.6	Tryptophan	16.9
Histidine	7.3	Tyrosine	2.7
Methionine	2.4		

Conditions: amount of Cu = 45 μmol; eluent, 25 m*M* phosphate buffer–0.4 *M* NaBr–0.01 *M* (pH 7.2); flow-rate, 0.6 ml/min.

$Cu(NO_3)$] supports. The retention behaviour of tryptophan could be explained by the formation of a π-complex between the amino acid aromatic ring and the metal [21] and also by the presence of stacking interactions between the tryptophan phenyl ring and imidazole rings of the immobilized complex [23]. These possibilities were supported by the low, but significant, affinities of other aromatic amino acids for immobilized Cu(II) (Table 1). Histidine was strongly retained on [Sil PVI $Cu(NO_3)$] supports, but its retention coefficient was approximately half that of tryptophan. The results found with cysteine, which was strongly retained on the stationary phase, are not shown, owing to the poor reproducibility of the peak shape and elution volume obtained with this amino acid (probably because of oxido-reduction reactions between cysteine and the immobilized copper [1]).

Except for tryptophan, the retention behaviour of the different amino acids tested on [Sil PVI $Cu(NO_3)$] complexes was similar to that previously observed on IDA-metal supports [19,24].

3.2. Retention behaviour of peptides

We studied the retention behaviour on [Sil PVI $Cu(NO_3)$] supports of various synthetic peptides that contained one histidine residue and, in some instances, also one tryptophan residue in their primary structures. Lysine was added to the mobile phase in order to desorb these compounds, which were strongly bound to the stationary phase.

As shown in Table 2, the affinities of these peptides (12–15 amino acid residues long) for the immobilized metal complex did not simply depend on the number of strong interacting centres (i.e., histidine and tryptophan residues). For instance, peptide P2, which contained a single histidine residue in its primary structure, was the most retained peptide on these supports. In comparison, peptides P3 and P5, which had an additional tryptophan residue in their structures, were less retained.

From the data in Table 2, it appeared that different parameters, such as the molecular environment and steric hindrance around the electron donor group(s) of the peptides, were important with respect to the retention of these molecules. Notably, the presence of negatively charged amino acid residues in the immediate vicinity of histidine and/or tryptophan residue(s) seemed to have an enhancing effect on peptides retention (peptides P1 and P2). Conversely, other workers [10,12,25] using IDA–metal supports have found that negatively charged amino acid residues had inhibitory effects on peptides retention. These discrepancies could be explained by the respective overall charge of the [Sil PVI $Cu(NO_3)$] and IDA–metal supports under the different experimental conditions used. With respect to the former support, copper ions were coordinated to neutral imidazole rings, the remaining coordination sites being occupied by lysine, when this compound was present in the eluent. Therefore, it could be reasonably assumed that under our experimental conditions (i.e., in the presence of lysine in the mobile phase), the overall charge of [Sil PVI $Cu(NO_3)$] supports was positive. In contrast, IDA–Cu(II) supports carried a negative net charge [22].

Table 2
Retention factors of some peptides on a [Sil PVI $Cu(NO_3)$] support

Peptide		k'_{Cu}
L-D-L-**W**-I-Y-**H**-T-Q-G-Y-F	P_1	12.8
I-**H**-D-F-R-Y-S-Q-L-A-K-L-G-I-N	P_2	24
S-Q-K-K-S-I-Q-F-**H**-**W**-K-N-S	P_3	1.2
T-P-L-S-P-P-L-R-T-T-**H**-P-Y	P_4	1.4
T-P-P-**H**-G-G-L-L-G-**W**-S-P-Q-G-I	P_5	2.5

Conditions: amount of Cu = 30 μmol; eluent, 25 m*M* phosphate buffer–0.4 *M* NaBr–0.01 *M* lysine (pH 7); flow-rate, 0.6 ml/min. Bold letters in peptide acronyms indicate histidine and tryptophan residues.

3.3. Retention behaviour of proteins

As compared with amino acids and peptides, different chromatographic results were obtained with proteins, probably because of the existence of more complex neighbouring and steric hindrance effects, as suggested previously.

The various proteins studied by chromatography on [Sil PVI $Cu(NO_3)$] supports were classified into two categories on the basis of their retention behaviour. There was a category of proteins that were weakly bound to the supports under our experimental conditions (hen and turkey lysozymes, ubiquitin, α-chymotrypsinogen and cytochrome *c*), i.e., these proteins were retarded by the loaded supports, but not by the naked supports; their elution from the loaded supports was achieved without the addition of a competing ligand in the mobile phase. α-Lactalbumin, human and bovine albumin and α_1-acid glycoprotein belonged to the second category of proteins and were tightly bound to [Sil PVI $Cu(NO_3)$] supports at pH 8.0. Elution of these proteins from the columns required the addition of a competing complexing agent to the mobile phase (i.e., imidazole, ammonia or lysine). The strong interactions observed between the albumins and [Sil PVI $Cu(NO_3)$] supports concurred with the previous finding that these proteins were strongly bound to immobilized metals [26]. The use of the new [Sil PVI $Cu(NO_3)$] supports for the fractionation of human α_1-acid glycoprotein genetic variants is described at the end of the paper.

The mechanisms involved in the retention of the proteins belonging to the first category was investigated further.

Effects of the amounts of immobilized Cu(II) on protein retention

As shown in Fig. 1, the retention coefficients, k'_{Cu}, of hen and turkey lysozymes, ubiquitin, α-chymotrypsinogen and cytochrome *c* increased significantly with increasing amount of Cu(II) immobilized on the stationary phase. This demonstrated the direct involvement of the metal complex in the retention of these proteins on [Sil PVI $Cu(NO_3)$] supports. As the metal complex includes the coordinated metal ion, its surroundings and the bound ligand (i.e., imidazole rings), any of these elements can, however, be involved in protein retention.

The results shown in Fig. 2 demonstrated that interactions between the coordinated metal ion and the electron donor groups of the proteins were involved during the chromatographic process. Indeed, it was found that the addition of imidazole to the mobile phase decreased the retention of the different proteins on the supports. Similar results were obtained when other competing agents, such as histidine, lysine and ammonia, were added to the mobile phase (data not shown).

However, in addition to specific electron donor–acceptor interactions, other interactions between the proteins and the supports seemed to occur. Indeed, a residual retention of the proteins was still observed at the highest imidazole (Fig. 2) or histidine (data not shown) concentrations (i.e., the k'_{Cu} values of the proteins studied were not zero at these concentrations).

Effects of salts and pH on protein retention to [Sil PVI $Cu(NO_3)$]

The retention of the various proteins studied was found to decrease at pH 8 on increasing the concentration of Br^- anions in the mobile phase (Fig. 3a). It has been suggested previously [21] that, at very high salt concentrations, the free coordination sites of the metal can be occupied by anions. Accordingly, our results could be explained by a competitive effect of Br^- anions on binding of the proteins to the metal. However, this explanation did not concur with the low salt concentration range used in our experiments.

More likely, the results shown in Fig. 3a could indicate that, in addition to donor–acceptor attraction effects, ionic interactions between the proteins and the immobilized complex were involved in the retention mechanisms. This possibility was supported by the fact that hen lysozyme, α-chymotrypsinogen and cytochrome *c* carry a positive net charge at pH 8 and could therefore bind to the immobilized metal via ionic interactions (the isoionic point values of these proteins are greater than 9). Accordingly, the overall net charge of the complex must be negative at this pH value. On the other hand, retention of the proteins on [Sil PVI $Cu(NO_3)$] supports was found to decrease on lowering the pH from 8 to 6 (Fig. 3b), indicating that charge-induced interactions between the proteins and

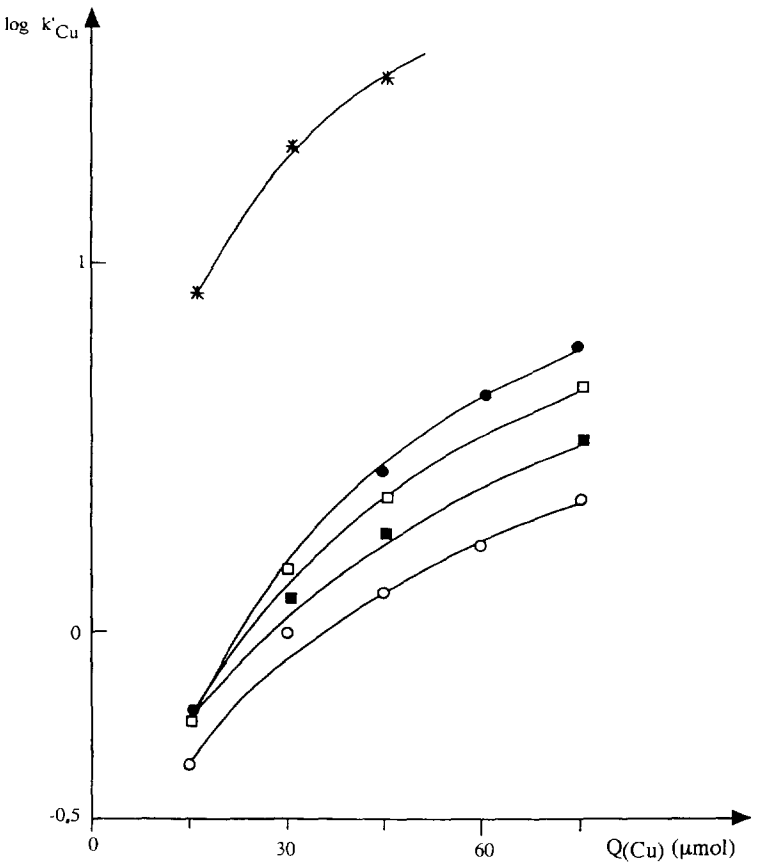

Fig. 1. Plots of logarithm of retention factors of proteins versus the amount of Cu(II) [Q(Cu)] immobilized on the support. Column, 150 × 4.6 mm I.D.; eluent, 25 mM phosphate buffer–0.8 M NaBr (pH 8); flow-rate, 0.6 ml/min. Proteins: (*) Lt; (○) Lh; (□) C; (●) Ch; (■) U.

the metal complex were less important at pH 6 than at pH 8. Therefore, the overall net charge carried by the complex at pH 6 must be different from that carried at pH 8. As suggested by the results from previous studies [24,27] and also from an ESR spectroscopy study of [Sil PVI $Cu(NO_3)$] supports [17], it may be that the free coordination sites of the metal are occupied by hydroxyl ions at pH 8, whereas these sites are mainly occupied by water molecules at pH 6. This would explain why the chromatographic behaviour of [Sil PVI $Cu(NO_3)$] supports was different at these two pH values and also why electrostatic interactions between the supports and the (positively charged) proteins were enhanced, and ionic strength effects were more pronounced, at pH 8 than at pH 6.

In addition to donor–acceptor and ionic attraction effects, other effects between proteins and the metal complex can take place. Sigel et al. [23] have shown that the imidazole ring is able to undergo stacking and to form hydrophobic adducts essentially with aromatic rings. Accordingly, salt-promoted hydrophobic interactions between the proteins and the metal complex could be involved in the retention mechanisms. This possibility was supported by the chromatographic results obtained with α-chymotrypsinogen and ubiquitin in the presence of Na_2SO_4 (Fig. 4). It was observed that at a high

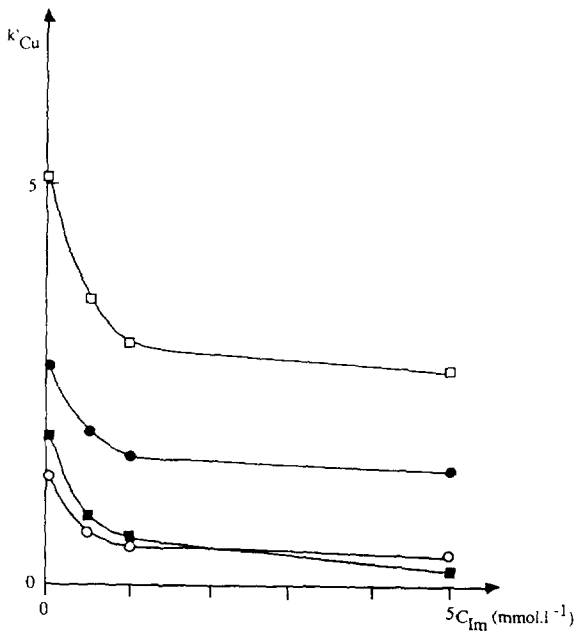

Fig. 2. Effect of imidazole added to the mobile phase on the retention factor of proteins. Column, 150 × 4.6 mm I.D.; amount of Cu, 45 μmol; eluent, 25 m*M* phosphate buffer–0.8 *M* NaBr–imidazole (pH 8); flow-rate, 0.6 ml/min. Proteins: (*) Lt; (○) Lh; (□) C; (●) Ch; (■) U.

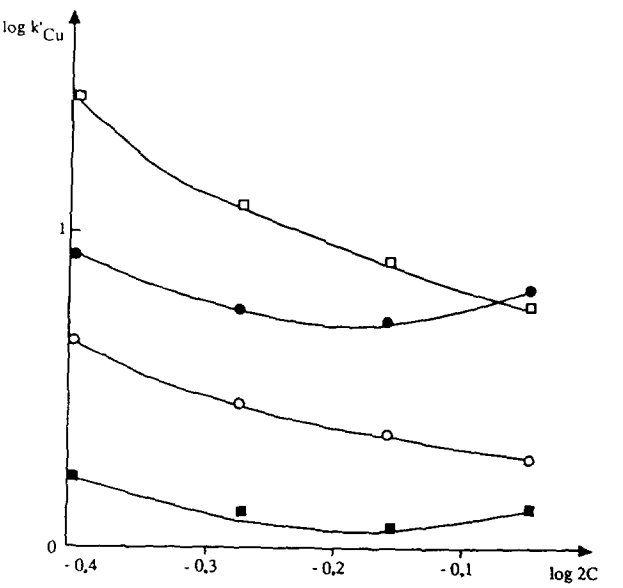

FiG. 4. Plots of logarithm of retention factor of proteins versus logarithm of sodium sulfate molarity (C). Column, 150 × 4.6 mm I.D.; amount of Cu = 72 μmol; eluent, 25 m*M* phosphate buffer–Na_2SO_4 (pH 8); flow-rate, 0.6 ml/min. Proteins: (○) Lh; (□) C; (●) Ch; (■) U.

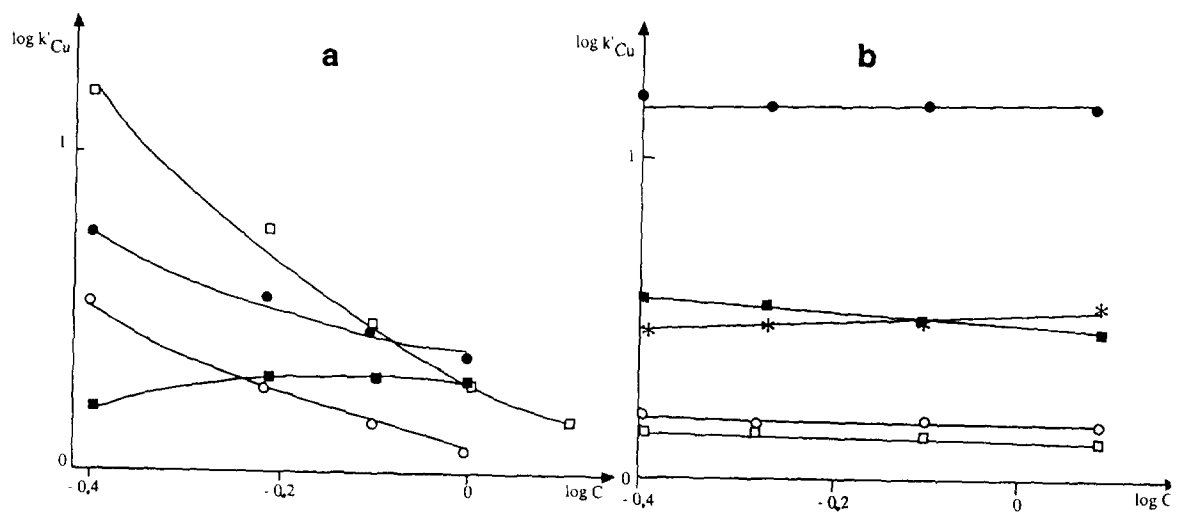

Fig. 3. Plots of logarithm of retention factor of proteins versus logarithm of sodium bromide molarity at (a) pH 8 and (b) pH 6. Column, 150 × 4.6 mm I.D.; amount of Cu, 60 μmol; eluent, 25 m*M* phosphate buffer–NaBr [(a) pH 8; (b) pH 6]; flow-rate, 0.6 ml/min. Proteins: (*) Lt; (○) Lh; (□) C; (●) Ch; (■) U.

concentration of sulfate anions, which are very antichaotropic anions, the retention of α-chymotrypsinogen and ubiquitin to the metal complex was increased. Further, the non-linear relationships which were observed between the retention factor k'_{Cu} of these two proteins and salt concentration also suggested that hydrophobic interactions took place. El Rassi and Horvath [8] have similarly observed the presence of hydrophobic interactions at high salt concentrations. The intensity of additional hydrophobic effects would depend on the presence of suitable residues close to the interaction centre(s) of the proteins.

Finally, the mechanisms of protein retention have to be discussed in terms of donor–acceptor, ionic and hydrophobic interactions between the proteins and the metal complex. With respect to some proteins (human and bovine albumins and human α_1-acid glycoprotein), donor–acceptor interactions seem to be predominant. However, generally, the three types of interactions have to be considered, as it is not always possible to determine their relative contribution to protein retention. For instance, the retention of cytochrome *c* at pH 8 was found to be very sensitive to salt concentration (Fig. 3a), demonstrating the importance of ionic interactions in the retention of this protein on the supports. The retention behaviour of cytochrome *c* in the presence of salts showed some similarities with that of the protein in the presence of an organic solvent (Fig. 5). Indeed, and in contrast to α-chymotrypsinogen, hen lysozyme and ubiquitin, the retention of cytochrome *c* was found to increase on addition of methanol to the mobile phase. A similar behaviour was previously observed by El Rassi and Horvath [8] for cytochrome *c* on chromatography on IDA–Fe(III) supports in the presence of methanol. In both instances, strong ionic interactions between cytochrome *c* and the stationary phase could be involved. Moreover, the peculiar retention behaviour of cytochrome *c* in the presence of methanol was further utilized to separate this protein from its mixture with α-chymotrypsinogen, hen lysozyme and ubiquitin, as shown by the chromatographic results presented in Fig. 6.

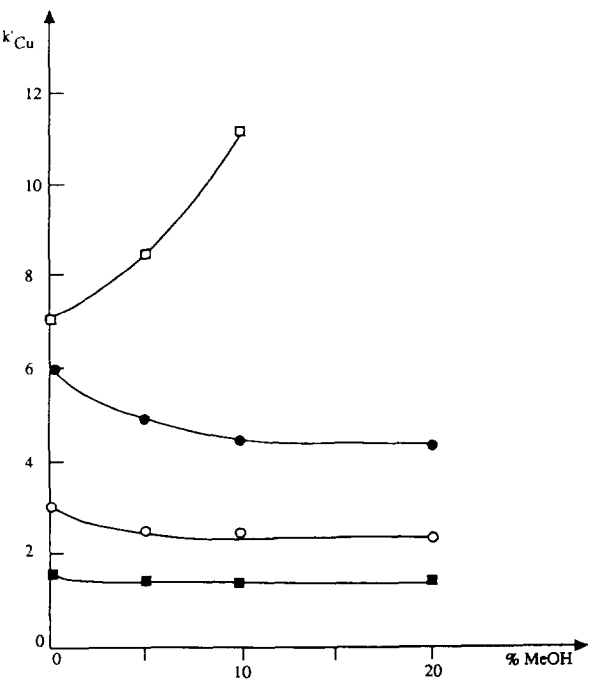

Fig. 5. Effect of methanol added to the mobile phase on the retention factor of proteins. Column, 150 × 4.6 mm I.D.; amount of Cu, 60 μmol; eluent, 25 m*M* phosphate buffer–0.4 *M* NaBr; flow-rate, 0.6 ml/min. Proteins: (○) Lh; (□) C; (●) Ch; (■) U.

The effects of pH on protein retention are more difficult to predict, because of the many pH-dependent factors which have to be simultaneously considered. When the protein metal-binding site is a histidine residue, a decrease in protein retention with decrease in the pH of the eluent should be observed, owing to histidine protonation, which generally occurs between pH 5 and 7. When ionic and hydrophobic interactions are involved in the retention mechanisms, the elution of proteins by varying the pH will depend on the overall net charge and the hydrophobicity of both the proteins and the metal complex at the different pH values. This proposal found some support in the results shown in Fig. 7. It was found that the retention of the various proteins studied was differently influenced by the pH of the mobile phase. The decreased affinities of cytochrome *c* and turkey lysozyme for the immobilized metal complex at pH 6, as compared with the affinities of the two

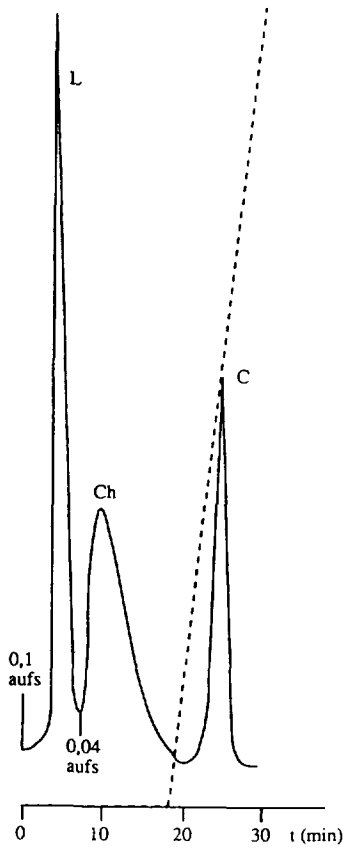

Fig. 6. Separation of a mixture of lysozyme (hen), cytochrome *c* and α-chymotrypsinogen in the presence of methanol. Column, 150 × 4.6 mm I.D.; amount of Cu, 60 μmol; starting eluent, 25 m*M* phosphate buffer–0.8 *M* NaBr–20% (v/v) methanol (pH 6.5); final eluent, 100 m*M* phosphate buffer–0.4 *M* NaBr (pH 6); flow-rate, 0.6 ml/min.

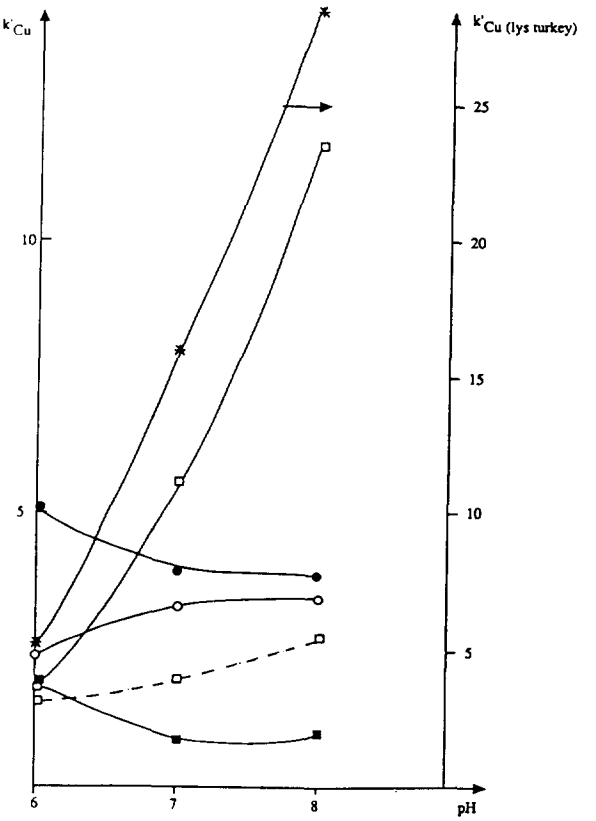

Fig. 7. Plots of the retention factor of proteins versus pH. Column, 150 × 4.6 mm I.D.; amount of Cu, 45 μmol; eluent, 25 m*M* phosphate buffer–0.4 or 0.8 *M* (dashed line) NaBr; flow-rate, 0.6 ml/min. Proteins: (*) Lt; (○) Lh; (□) C; (●) Ch; (■) U.

proteins at the other pH values, could be explained by the protonation of the histidine residues and the existence of weaker ionic interactions between cytochrome *c* or turkey lysozyme and the metal complex at pH 6. Further, the effects of pH on cytochrome *c* retention were significantly lowered at high ionic strength (dashed line in Fig. 7). These results further supported the important role played by ionic interactions in the retention mechanisms of cytochrome *c*. In contrast, the retention of ubiquitin and α-chymotrypsinogen on the supports was found to increase on decreasing the pH from 8 to 6 (Fig. 7). The lower retention of ubiquitin at pH 8 than pH 6 was explained by mutual repulsion between this protein and the support, which were both negatively charged at the alkaline pH value (the isoionic point value of ubiquitin is 6.7) [28]. However, the results obtained with α-chymotrypsinogen have not yet been explained.

3.4. Fractionation of desialylated human α_1-acid glycoprotein genetic variants by chromatography on [Sil PVI Cu(NO_3)] support

Human plasma AAG is a heterogeneous protein, consisting of a mixture of at least two genetic variants, the A variant and the F1 and/or S variants, that differ in several amino acid

substitutions in the peptide chain [29,30]. The AAG variants are revealed when the desialylated form of AAG is analysed on isoelectrofocusing [18]. The F1, S and A variants are distinguished by their electrophoretic migration, as one "fast" and two "slow" bands, respectively. Three main phenotypes are observed for AAG in the human population, F1S/A, F1/A and S/A, depending on the presence of two or three of the variants F1, S and A in plasma. The AAG phenotypes are genetically determined [29].

A fractionation method for the desialylated AAG variants has recently been developed, by chromatography on an IDA–Cu(II) adsorbent [20]. The A variant was found to be strongly bound to the immobilized metal complex at pH 7 and was eluted only after the addition of a competing complexing agent (imidazole) to the eluent. In contrast, the F1 and S variants had no significant binding affinity for the support and were eluted in the flow-through volume.

In this work, the retention behaviour of the desialylated F1, S and A variants of human AAG on [Sil PVI $Cu(NO_3)$] was investigated. For this purpose, the three AAG phenotypes, F1S/A, F1/A and S/A, were separately purified from individual human plasma, as described previously [19]. Prior to chromatography, the purified AAG samples were desialylated with neuraminidase.

In preliminary experiments, we found that the desialylated AAG samples were all totally adsorbed on [Sil PVI $Cu(NO_3)$] supports at pH 7, thus indicating that the three variants F1, S and A were bound to the immobilized metal. The addition of a competing complexing agent to the mobile phase was necessary to desorb the proteins from the supports.

The fractionation of the desialylated AAG variants was achieved using a lysine gradient for elution. Fig. 8 illustrates the chromatographic profile obtained with desialylated F1S/A AAG on [Sil PVI $Cu(NO_3)$] at pH 7. The chromatography of F1S/A AAG resolved two major peaks, peaks 1 and 2 (the minor peak shown in Fig. 8 was found to contain non-protein impurities). Peak 1 eluted with 10^{-3} *M* lysine, whereas peak 2 eluted with lysine concentrations up to $8 \cdot 10^{-3}$ *M*. The same fractionation results were achieved with the F1/A and S/A AAG samples, indicating that the affinity of each respective variant for [Sil PVI $Cu(NO_3)$] supports was not modified by the presence of the other variant(s).

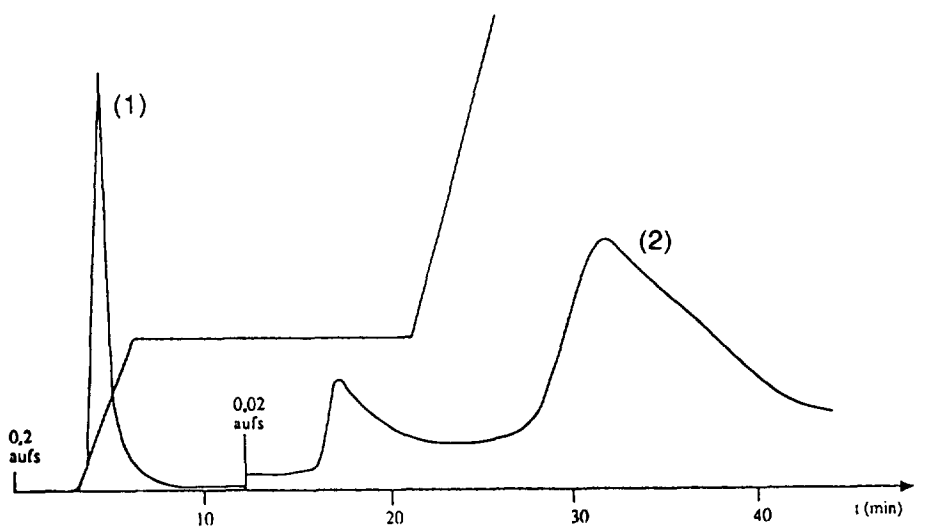

Fig. 8. Elution of a desialylated F_1S/A AAG sample on a [Sil PVI $Cu(NO_3)$] support. Column, 150 × 4.6 mm I.D.; amount of Cu, 37 μmol; starting eluent, 25 m*M* phosphate buffer–0.4 *M* NaBr (pH 7); final eluent, 25 m*M* phosphate buffer–0.4 *M* NaBr–0.01 *M* lysine (pH 7); flow-rate, 0.6 ml/min.

After completion of chromatography, the variants of the different peaks were characterized by analytical IEF on an immobilized pH 4.4–5.4 polyacrylamide gel gradient. The electrophoretic patterns are shown in Fig. 9, which also shows the patterns of the different AAG samples used in chromatography. Depending on the desialylated AAG sample used in chromatography, peak 1 was found to consist of only the F1 (F1/A AAG), or the S (S/A AAG) or of a mixture of the F1 and S variants (F1S/A AAG). However, in all AAG samples, peak 2 signified the A variant in a pure form. The elution order of the desialylated AAG variants on [Sil PVI $Cu(NO_3)$] supports was similar to that previously observed on IDA–Cu(II) adsorbents [20].

The total recovery of desialylated AAG measured in peaks 1 and 2 was between 70 and 80%. The recoveries near 95% which were obtained in other experiments by eluting the variants in an isocratic mode with 10 m*M* lysine in the mobile phase suggested that the A variant was not totally recovered when a lysine gradient was used for the elution. However, the use of high and constant lysine concentrations to elute totally the A variant from [Sil PVI $Cu(NO_3)$] can result in a small leakage of metal from the support.

The discrepancies that were observed between the retention behaviour of the F1 and S variants on [Sil PVI $Cu(NO_3)$] supports and on IDA–Cu(II) adsorbents, respectively, are difficult to explain. The F1 and S variants were retained on the former support, whereas they showed no significant binding to the latter adsorbent [20]. Among other possibilities, it can be assumed that the retention of the desialylated F1 and S variants on [Sil PVI $Cu(NO_3)$] involved the presence of additional (ionic and/or hydrophobic) interactions that did not exist with IDA–Cu(II) adsorbents. Moreover, the conformation of the desialylated variants seems to be slightly modified as compared with that of the native (sialylated) variants [31], and this could favour the presence of additional interactions with the [Sil PVI $Cu(NO_3)$] supports.

As discussed previously [20], the higher affinity of the A variant for the immobilized metal, as compared with the F1 and S variants, cannot be accounted for by differences in the number of

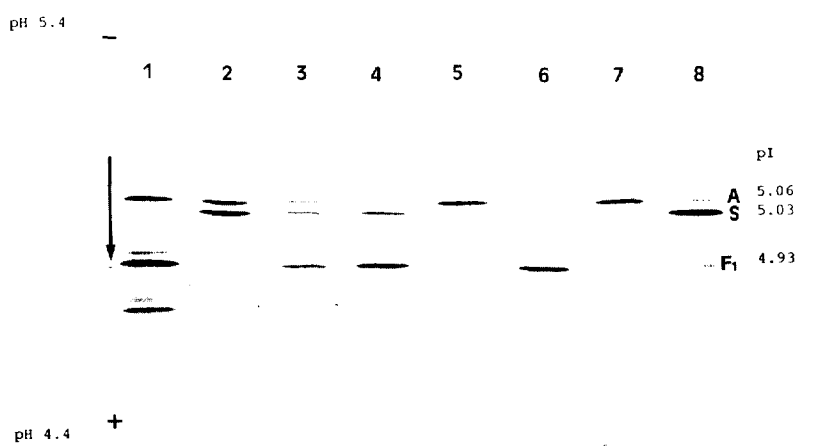

Fig. 9. Electrophoretic patterns on immobilized pH 4.4–5.4 polyacrylamide gel gradient with 8 *M* urea and 2% 2-mercaptoethanol of the isolated peaks 1 and 2 (Fig. 8) after chromatography on [Sil PVI $Cu(NO_3)$] supports of different desialylated AAG samples. Lanes: 1, 2 and 3, individually purified AAG samples with the F_1/A, S/A and F_1S/A phenotypes, respectively; 4, peak 1 (variants F_1 and S) isolated from desialylated F_1S/A AAG; 6, peak 1 (variant F_1) isolated from desialylated F_1/A AAG; 8, peak 1 (variant S) isolated from desialylated S/A AAG; 5 and 7, peak 2 (variant A) isolated from desialylated F_1S/A, F_1/A or S/A AAG. The amounts of the various samples loaded on the gel were 20 μg. The desialylated variants were detected in the gel by staining with Coomassie Brilliant Blue R-250. The pH scale and the approximate isoelectric point (p*I*) for each variant are indicated. The faint, more anodic bands, also observed after staining with Coomassie Brilliant Blue, indicated the presence of a small proportion (less than 5%) of incompletely desialylated protein.

histidine and tryptothan residues between the variants. Indeed, three histidine and three tryptophan residues are found in the primary structure of the different variants [29,30]. The A variant contains a single cysteinyl residue in its primary sequence, which appears to be substituted by an arginyl residue in the F1 and S variants [30]. However, the A variant cysteinyl residue was found to be neither accessible nor free, owing to covalent binding [20]. Hence a possible contribution of this residue in binding of the A variant to [Sil PVI $Cu(NO_3)$] seemed unlikely, unless a conformational change of the A variant occurred during the chromatographic process. On the other hand, the A variant and the F1S variants, which are encoded by two different genes, differ by at least 22 amino acid substitutions in their primary sequence [29,30]. Therefore, the heterogeneity between the variants for binding to the immobilized metal could be due to differences in the accessibility and microenvironment of the metal binding groups (histidine and tryptophan side-chain groups) likely to be present at the surface of the variants.

4. Conclusions

The retention behaviour of a variety of amino acids, peptides and proteins was studied by chromatography on new immobilized metal affinity adsorbents, the [Sil PVI $Cu(NO_3)$] supports. The results have shown that small compounds, such as amino acids and peptides, that contained histidyl and tryptophanyl residues were strongly bound to the supports through electron donor–acceptor interactions, since the recovery of these compounds required the addition of a competing complexing agent to the mobile phase. In comparison, proteins containing metal binding groups showed a more heterogeneous retention behaviour. Some proteins (albumin, α_1-acid glycoprotein, etc.) were strongly adsorbed to the supports in a fashion similar to that observed with small compounds. In contrast, the elution of cytochrome *c*, hen and turkey lysozymes, α-chymotrypsinogen and ubiquitin from [Sil PVI $Cu(NO_3)$] supports was only retarded and the delay in elution progressively increased with increasing metal content of the stationary phase. These discrepancies could be accounted for by differences in the accessibility of the immobilized metal complex to the proteins, given that this complex is in close proximity to the polymer backbone of the support. Only proteins with well exposed metal binding centres, situated in a favourable environment, would be able to bind strongly to the stationary phase through electron donor–acceptor interactions. With respect to the other proteins, labile interactions between the immobilized metal itself and the proteins would occur and secondary effects, such as ionic and hydrophobic interactions, would no longer be negligible during the elution.

References

[1] J.W. Wong, R.L. Albright and N.H.L. Wang, *Sep. Purif. Methods*, 20 (1991) 49.
[2] Z. El Rassi and C. Horvath, in K.M. Gooding and F. Regnier (Editors), *HPLC of Biological Macromolecules: Methods and Applications*, Marcel Dekker, New York, 1990, p. 179.
[3] N. Ramadan and J. Porath, *J. Chromatogr.*, 321 (1985) 81.
[4] J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature*, 258 (1975) 598.
[5] Y. Kato, K. Nakamura and T. Hashimoto, *J. Chromatogr.*, 354 (1986) 511.
[6] L. Fanou-Ayi and M. Vijayalakshmi, *Ann. N.Y. Acad. Sci.*, 413 (1983) 300.
[7] D.A.P. Small, T. Atkinson and C.R. Lowe, in I.M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition, Proceedings of 5th International Symposium*, Academic Press, Orlando, FL, 1983, p. 267.
[8] Z. El Rassi and C. Horvath, *J. Chromatogr.*, 359 (1986) 241.
[9] A. Figueroa, C. Corradini, B. Feibush and B.L. Karger, *J. Chromatogr.*, 371 (1986) 335.
[10] J. Gauthier, J. Amiot and M.A. Vijayalakshmi, *Prep. Biochem.*, 20 (1990) 23.
[11] Y. Nakagawa, T.T. Yip, M. Belew and J. Porath, *Anal. Biochem.*, 168 (1988) 75.
[12] T.T. Yip, Y. Nakagawa and J. Porath, *Anal. Biochem.*, 183 (1989) 159.
[13] G. Lindeberg, H. Bennich and A. Engström, *Int. J. Pept. Protein Res.*, 38 (1991) 259.
[14] J. Porath, *Protein Expression Purif.* 3 (1992) 263.

[15] E.S. Hemdan, Y.J. Zhao, E. Sulkowski and J. Porath, *Proc. Natl. Acad. Sci., U.S.A.*, 86 (1989) 1811.
[16] E. Sulkowski, *BioEssays*, 10 (1989) 170.
[17] M.C. Millot, B. Sébille, A. Halli, H. Hommel and A.P. Legrand, *Chromatographia*, 37 (1993) 584.
[18] C.B. Eap and P. Baumann, *Electrophoresis*, 9 (1988) 650.
[19] F. Hervé, J.C. Duché, N. Sportes and J.P. Tillement, *J. Chromatogr.*, 539 (1991) 401.
[20] F. Hervé, J.C. Duché, J. Barré, M.C. Millot and J.P. Tillement, *J. Chromatogr.*, 577 (1992) 43.
[21] J. Porath, *J. Mol. Recog.*, 3 (1990) 123.
[22] E. Sulkowski, in R. Burger (Editor), *Protein Purification: Micro to Macro*, Alan R. Liss, New York, 1987, p. 149.
[23] H. Sigel, R. Tribolet and O. Yamauchi, *Comments Inorg. Chem.*, 9 (1990) 305.
[24] E.S. Hemdan and J. Porath, *J. Chromatogr.*, 323 (1985) 255.
[25] E.S. Hemdan and J. Porath, *J. Chromatogr.*, 323 (1985) 265.
[26] L. Andersson, E. Sulkowski and J. Porath, *Bioseparation*, 2 (1991) 15.
[27] B. Vulliez, G. Picard and B. Sébille, *Hydrometallurgy*, 11 (1983) 47.
[28] E.S. Hemdan, Y.J. Zhao, E. Sulkowski and J. Porath, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 1811.
[29] K. Schmid, in F.W. Putnam (Editor), *The Plasma Proteins, Structure, Function and Genetic Control*, Vol. 1(4). Academic Press, New York, 2nd ed., 1975, p. 183.
[30] L. Dente, M.G. Pizza, A. Metspalu and R. Cortes, *EMBO J.*, 6 (1987) 2289.
[31] F. Hervé, E. Gomas, J.C. Duché and J.P. Tillement, *J. Chromatogr.*, 615 (1993) 47.