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Silica-based metal chelate affinity sorbents II[☆]. Adsorption and elution behaviour of proteins on iminodiacetic acid affinity sorbents prepared via different immobilization techniques

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Abstract

The chromatographic characteristics of some model proteins on silica-based metal chelates run under various experimental conditions are described. The retention of proteins on silica-based iminodiacetic acid (IDA) was compared among the different immobilization methods employed and to chelates bound on soft gels, such as chelating Sepharose and epibromohydrin-activated Sepharose. All Cu(II)-loaded chelators displayed adsorption of proteins in the presence of 0.5–1 *M* NaCl at pH 7–8; however, elution in the pH and imidazole gradient varied with the immobilization chemistry. The chemical structure in the neighbourhood of the metal chelate was of influence on the development of a negative charge with increasing pH. Thus, a negative charge evolves at lower pH with alkyl- than epoxy-immobilized IDA:Cu(II). Glycidoxypropyltrimethoxysilane-immobilized IDA displayed almost identical chromatographic characteristics compared to chelating Sepharose FF. Basic proteins displayed higher retention on butyl-immobilized IDA compared to other chelates; interactions with positively charged amino acid residues seem to be superimposed on the interaction with histidyl residues. Proteins were least retained on 1,1′-carbonyldiimidazole-immobilized IDA:Cu(II); however, the selectivity for human and bovine serum albumins against other proteins employed was highest. The results obtained indicated that chelating interaction with some proteins depend on the spacer length. Thus, less flexible histidyl residues at protein surfaces might not be recognized from chelates immobilized by short spacers.

1. Introduction

Immobilised metal chelators are employed for the reversible immobilization of metal ions, thereby forming metal chelates. However, not the immobilised metal ion alone is responsible for the interaction of a protein with the resulting affinity sorbent. The results from the present investigations rather confirmed that the interaction of the protein with the entire metal chelate is responsible for interaction. The term metal chelate interaction chromatography (MCIC), as proposed by El Rassi and Horváth [1], is therefore preferred against other expressions and abbreviations used.

Many publications on MCIC appeared in the

[☆]For Part I, see ref. 7.

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Table 1
Properties of proteins employed in this study

Protein	Nucleophilic amino acids	His at surface	p*I*	M_r
RNase A (bovine pancreas)	4 His, 0 Trp, 8 Cys as S–S [37]	2 [6]	9.4	13 700
Cyt c (horse heart)	2 His, 1 Trp, 2 Cys [38]	1 [10]	10.6	12 300
Lysozyme (chicken egg white)	1 His, 6 Trp, 9 Cys (8 as S–S) [39]	1 [40]	11.0	14 400
HSA	16 His, 1 Trp, 35 Cys (34 as S–S) [41]	2–3 [15]	4.6	69 000
BSA	16 His, 2 Trp, 35 Cys (34 as S–S) [42]	2–3 [15]	4.8	66 700
DSA	15 His [6]	1 [15]		
OVA (chicken egg)	7 His, 3 Trp, 6 Cys [43]	1 [1]	4.7	45 000

last years concerning the interaction of mostly commercial immobilised metal chelates with proteins [2–4]. Mostly iminodiacetic acid (IDA) is employed as chelator because it combines tight binding of transition metal ions with high accessibility of the resulting metal chelate for amino acid residues at protein surfaces [5]. Today it is widely accepted that the basis for interaction of proteins with IDA chelates is the interaction of nucleophilic groups of amino acids, especially the imidazole group of histidine. It remains questionable, whether other nucleophilic amino acids, such as tryptophan or cysteine, participate in binding [6].

In Part I of this investigation, the covalent immobilization of IDA leading to distinct structures of the chelator is described. The chelates, as obtained through the different methods, displayed a deviate selectivity for amino acids when compared to each other [7].

The impact of the chemical neighbourhood of chelates on the chelate structure was also demonstrated by Chaberek and Martell [8], who developed metal chelates on the basis of carboxymethylated amines with different structure (for chelation of metal ions). Their results clearly demonstrated that small structural changes in the neighbourhood of the chelating group alters the titration curve of metal chelates; titration curves allow directly the calculation of the stability constant of chelates. Among other reasons, the stability constant depended on the ring size of the polydentate complex formed with the central metal ion and on hydroxyalkyl groups located in close proximity to the chelator [9]. These results are very important in view of metal chelates introduced in Part I but also of commercial metal chelate sorbents. Their exact chemical structure is mostly unknown to the user.

In order to gain a more systematic approach, more insight on the interactions of proteins with metal chelates is necessary, such as demonstrated by Porath and co-workers [10,11], using proteins or peptides, respectively. The influence of buffer compositions was demonstrated by El Rassi and Horváth [1] and Porath and Olin [12]. Those results are summarised in the following which should be seen in connection with results from this investigation:

(i) At least one histidyl residue at the protein surface is required for adsorption on immobilised IDA:Cu(II); interactions with cysteine and tryptophan are discussed, but need to be confirmed [6,10].

(ii) At least two histidyl residues are essential for adsorption on immobilised IDA:Ni(II) [4].

(iii) Two histidyl residues in vicinity are needed for adsorption on IDA:Zn(II) or IDA:Co(II), either sequential or conformational [13].

(iv) Ionic interactions can be modulated by buffers of different ionic strength [1].

(v) Adsorption and elution are affected by pH; pH-gradient elution is a common elution technique [14].

(vi) High-affinity metal ion binding sites of proteins may scavenge the metal ion from the immobilised metal chelator without binding [4,15].

Proteins employed in this study were chosen on the basis of their reported behaviour in MCIC; their properties are compiled in Table 1. Serum albumins employed and ovalbumin (OVA) exhibit a net negative charge at most of

the pH values chosen in this study. All other proteins remain positively charged. According to literature, they display a different number of accessible histidyl residues at the protein surface, some at least one, ribonuclease (RNase) A two, also bovine (BSA) and human serum albumin (HSA) at least two [15,16]. It was anticipated that the different properties of these proteins would allow a comparison of their binding properties onto these chelates under various chromatographic conditions. Besides influences of the spacer length, leading to different accessibility of protein surface-located histidine residues, the chemical neighbourhood of the chelate significantly influenced the adsorption of proteins. The appearance of a negative charge at Cu(II) chelates with increasing pH could be linked to the immobilization method employed. From the results of this study more selective adsorption and elution conditions can be predicted for some chromatographic protocols.

The Cu(II)-loaded 1,1′-carbonyldiimidazole (CDI)-immobilised IDA is a specific metal chelate chromatographic sorbent for the purification of some serum albumins.

2. Experimental

2.1. Chemicals and chromatographic materials

Imidazole, BSA, HSA, dog serum albumin (DSA) (all fraction V), OVA grade V from chicken egg and horse heart cytochrome *c* (Cyt c) were obtained from Sigma, Munich, Germany. Lysozyme from chicken egg white and RNase A from bovine pancreas were obtained from Serva, Heidelberg, Germany. All other chemicals were purchased from E. Merck, Darmstadt, Germany or Riedel-de Haen, Seelze, Germany. Analytical grade was used in all cases. Chelating Sepharose FF (CS) was purchased from Pharmacia, Freiburg, Germany.

2.2. Chromatographic sorbents

Silica-based chelators were prepared as described in Part I [7]. Briefly, GLYMO-IDA is a condensation product of 3-glycidoxypropyltrimethoxysilane (GLYMO) and iminodiacetic acid (IDA). Propyl-IDA and butyl-IDA are synthesised by reaction of 4-aminobutyl- and 3-aminopropyltriethoxysilane with bromoacetic acid, respectively. All α,ω-silano-chelators were bound to non-activated silica. CDI::IDA and Epi::IDA were prepared by reaction of IDA with CDI-activated diol-silica and epibromohydrin-activated Sepharose 4B, respectively. Metal chelates are abbreviated as IDA:M() with M being the metal ion, embracing the oxidation state in parentheses.

2.3. Instruments

The liquid chromatographic system for all chromatographic experiments was the Pharmacia 500 system assembled for zonal and frontal chromatography, respectively.

2.4. Chromatographic conditions

Sepharose 4B-based chelating gels and CS were packed in water. Silica-based chelating gels were dry-packed. All experiments were carried out with 5 mm I.D. columns with bed heights between 20 and 30 mm. Buffers, including water and metal ion solutions, were filtered through disposable filters (0.45 μm) before usage.

2.5. Zonal chromatography

Chromatographic tests were performed using standard fast protein liquid chromatography (FPLC) equipment, operating automatically. The columns were washed first with 30 m*M* EDTA + 0.5 *M* NaCl, pH 6, in order to remove metal ions or contaminants, then water, then 15 m*M* metal ion dissolved in 50 m*M* acetate buffer, pH 5, and finally with 50 m*M* acetate buffer, pH 5, in order to elute those metal ions which are adsorbed due to ionic interactions. The columns were equilibrated with 20–25 column volumes of the starting buffer. All experiments were conducted at 298 K at a flow-rate of 0.5 ml/min, unless stated otherwise.

2.6. Chromatography of proteins

Proteins were dissolved in buffer A at concentrations of 0.3 g/l for albumins and RNase A and 0.1 g/l for lysozyme and Cyt c. A 200-μl volume was applied on a column. Proteins were monitored at 280 nm.

2.7. Elution with salt gradient

Buffer A was a 25 m*M* phosphate buffer, adjusted to pH 5, 6, 7 or 8; buffer B comprised 1 *M* NaCl in addition. Gradients were formed by increasing buffer B concentration from 0 to 50% in 10 min, then to 100% B in 3 min, holding this concentration for 2 min. Then the column was reequilibrated with buffer A. Some investigations were performed in 50 m*M* acetate buffer, pH 5.0 and 50 m*M* Tris · HCl, pH 8.0 as buffer A, respectively. Gradient times were the same as for phosphate-buffered systems.

2.8. Elution with pH gradient

Buffer A was 25 m*M* phosphate + 0.5 *M* NaCl, adjusted to pH 8; buffer B was 100 m*M* phosphate + 0.5 m*M* NaCl, adjusted to pH 2.8. Elution was achieved by increasing the concentration of buffer B in 15 min to 100%, holding for 6 min.

2.9. Elution with 100 mM imidazole gradient

Buffer A was 25 m*M* phosphate + 0.5 *M* NaCl, adjusted to pH 6 or pH 7; buffer B comprised 100 m*M* imidazole in addition. Some experiments were also performed with 0.15 *M* NaCl. For the CDI::IDA chelate only 25 m*M* imidazole was used in buffer B. Gradients were formed by increasing buffer B from 0 to 50% in 20 min, then to 100% in 5 min, holding for 2 min. Then the column was reequilibrated with buffer A.

2.10. Frontal chromatography

Frontal chromatography was performed with automated adsorption, elution and equilibration of up to 7 columns and 5 protein concentrations in one set of experiments. Each run was terminated by the integrator after reaching the plateau region of the frontal breakthrough.

2.11. Adsorption of lysozyme and BSA on Cu(II)- and Fe(III)-loaded chelators

Before the first and after each run the metal chelate columns were washed with 30 m*M* EDTA + 0.5 m*M* NaCl to remove metal ion, adsorbed proteins and contaminants. Then columns were loaded with either Fe(III) or Cu(II) using 15 m*M* $Fe_2(SO_3)_2$ or $CuCl_2$ dissolved in water. For adsorption of lysozyme onto Fe(III)-loaded sorbents, 50 m*M* acetate + 0.5 *M* NaCl, pH 5 was used; for adsorption of BSA the buffer contained only 0.1 *M* NaCl. In case of Cu(II) chelates, 25 m*M* phosphate + 0.5 *M* NaCl, pH 7 was chosen during adsorption. Lysozyme concentrations between 0.04 and 2 g/l were applied. BSA concentrations ranged from 0.3 to 2.5 g/l.

2.12. Contour plots of proteins

Contour plots of the protein surface were achieved using the program BRAGI, developed at the GBF by Schomburg and Reichelt [17]. The program allows a close investigation of the accessibility of amino acids at protein surfaces using X-ray structural analysis data for calculation.

3. Results

3.1. Adsorption at low ionic strength

It is characteristic for IDA metal chelates that the chemical composition changes slightly with pH. Hence, a negative charge develops through deprotonation with increasing pH, as demonstrated in Fig. 1. The titration of metal chelates in solution revealed that the p*K*, corresponding to the development of a negative charge, depends both on the metal ion and the structure of the chelator [8]. Therefore, this p*K* is an important criterion for comparison of IDA

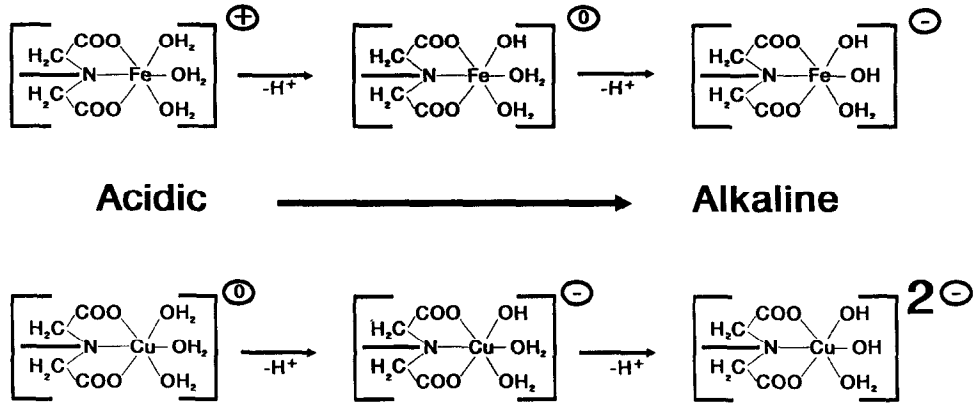

Fig. 1. Appearance of positive or negative charge at Fe(III) chelates and negative charges at Cu(II) chelates depending on pH. Whereas Cu(II) chelates presenting zero charge exhibit truly chelating interactions, charged chelates demonstrate ionic interactions during the adsorption step in addition.

chelators employed in this study. Ionic interactions with proteins, originating from a negative charge, are usually suppressed by the addition of 0.5–1 *M* NaCl. In order to compare the influence of the immobilization chemistry of metal chelates employed in this study on the retention of model proteins, both low and high NaCl concentration were employed.

IDA carries a negative charge at each carboxyl group and a positive charge at the nitrogen with one exception; CDI-immobilised IDA does not accommodate a positive charge due to the amidic character of the nitrogen. As a consequence, model proteins bearing a net negative charge at elevated pH values, such as albumins and OVA, were expelled from IDA at low ionic strength and eluted in the void volume. Proteins with net positive charge adsorbed at low and eluted at elevated NaCl concentration, similar to the retention on a cation exchanger.

The different IDA:Cu(II) chelates demonstrated a distinct adsorption behaviour at pH 8 and low ionic strength, depending on the immobilization method employed. Whereas all proteins adsorbed on chelates bound through an epoxy group, butyl-IDA:Cu(II) and CDI::IDA:Cu(II) did not retain serum albumins and OVA at low salt concentration. It appeared that the chemical neighbourhood of IDA, as obtained through the different immobilization methods [7], affected the p*K* corresponding to the development of a negative charge on IDA:Cu(II) chelates. A negative charge at copper chelates is discussed by other groups as well [11,18].

3.2. Elution with increasing salt gradient

Chromatographic results from silica-based GLYMO-IDA, butyl-IDA and CDI::IDA at pH 7 are illustrated in Figs. 2, 3, and 4, respectively. On all naked IDA sorbents albumins and OVA were expelled due to their net negative charge. The chromatographic behaviour of the basic proteins Cyt c, RNase A, and lysozyme corresponded on all the chelators employed to their retention on a cation exchanger.

Metal ion

When Ca(II) or La(III) were coordinated with IDA, proteins demonstrated nearly identical retention times as observed with naked IDA. Although adsorption of Ca(II) onto IDA sorbents was confirmed by atomic absorption spectroscopic measurements, the resulting IDA:Ca(II) chelate is weak, as were IDA chelates with other alkaline-earth metals, too. Consequently, these chelates demonstrated mainly the properties of naked IDA.

Results from Fe(III)-loaded GLYMO-IDA and butyl-IDA are indicative for a mixed interaction mechanism. IDA:Fe(III) behaved similar to naked IDA at pH 7. However, retention times

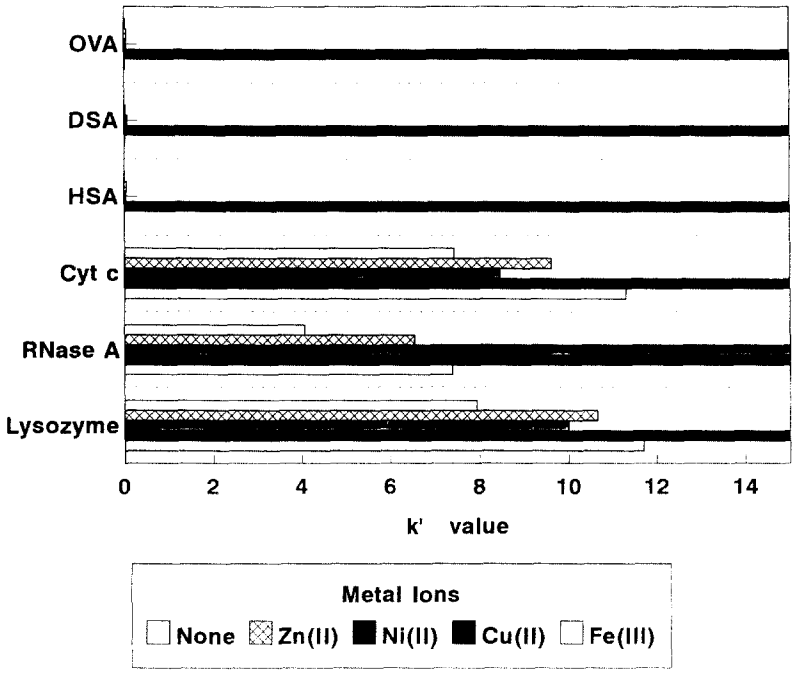

Fig. 2. Elution of proteins with increasing ionic strength on Polygosil 500 GLYMO-IDA charged with and without metal ions. Column, 30 × 5 mm I.D.; flow-rate, 0.5 ml/min; temperature, 298 K; buffer, 25 m*M* phosphate, pH 7.0; linear gradient in 10 min from 0 to 0.5 *M* NaCl, then in 3 min to 1.0 *M* NaCl. None of the proteins eluted on the GLYMO-IDA:Cu(II), parts of RNase A did not elute on the Ni(II)-loaded chelate, too.

of lysozyme and Cyt c were slightly increased, but not as high as with IDA:Cu(II). Serum albumins did not bind at pH 7 or 8, but at pH 6 and 5. Therefore, a positive charge on Fe(III)-chelates is questionable, as could be assumed from the complexation reaction of Fe(III) and IDA in water (Fig. 1). However, the model proteins and experimental conditions employed in this study do not allow a statement to be drawn on the actual interaction mechanism. Using other model proteins and chromatographic conditions, Sulkowski [19] was able to demonstrate more clearly the mixed interaction mechanism of IDA:Fe(III) and proteins.

On IDA:Zn(II) and IDA:Ni(II), basic proteins were retained more strongly than on naked IDA and alkaline-earth metal chelates, indicating the presence of chelating interactions. On GLYMO-IDA:Zn(II), Cyt c and lysozyme displayed slightly higher interaction than on the Ni(II) chelate. RNase A demonstrated higher interaction with chelated Ni(II) than Zn(II). In case of Ni(II)-loaded CS and GLYMO-IDA, RNase A did not elute in the salt gradient (data of CS not shown). It is important to mention that RNase A did not adsorb on GLYMO-IDA:Ni(II) at high salt concentration. Ni(II)-loaded Epi::IDA and GLYMO-IDA always exhibited broad and flat elution peaks of RNase A, indicating kinetic effects during desorption. It appeared that desorption of this protein from the latter chelates was not complete at elevated NaCl concentration and that parts of the total protein mass was adsorbed strongly.

The strongest chelating interactions were observed with all Cu(II)-loaded chelators. Proteins were not eluted in the salt gradient at pH 7 on GLYMO-IDA:Cu(II). DSA was eluted from butyl-IDA:Cu(II) with low mass recovery and BSA, HSA and RNase A did not elute on CDI::IDA:Cu(II). This is consistent with data obtained from the retention of amino acids [7] and results published by El Rassi and Horváth [1] and other groups [20,21]. Thus, all proteins

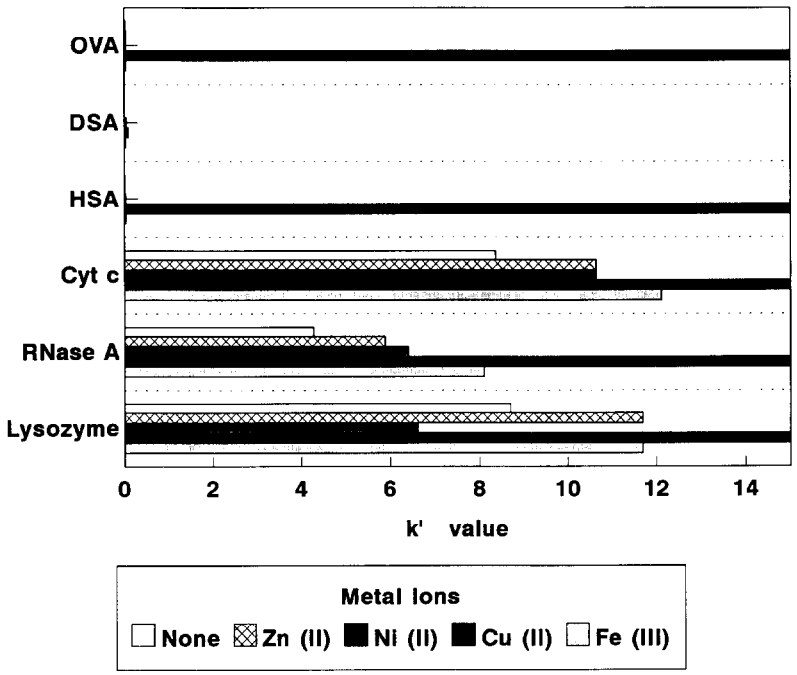

Fig. 3. Elution of proteins with increasing ionic strength on Polygosil 500 butyl-IDA charged with and without metal ions. Chromatographic conditions as in Fig. 2. Only DSA eluted on the Cu(II)-loaded chelate. Ionic interactions are more apparent than with the GLYMO-IDA chelator.

bear at least one accessible histidyl residue which is recognised by all Cu(II)-loaded chelators employed. The high-affinity binding site in BSA and HSA at the N-terminus, involving His 3 [22], does not take part in binding of these proteins onto chelates [15]. Therefore, binding must be ascribed to other histidyl residues present in BSA, HSA and DSA.

Immobilization chemistry

CDI::IDA behaved the most different compared to other chelates. If other metal ions than Cu(II) were loaded onto CDI::IDA, all proteins eluted at low salt content (Fig. 4); Fe(III) was not investigated.

BSA, HSA and RNase A did not elute from Cu(II)-loaded CDI::IDA. If 1 *M* NaCl was applied in the equilibration buffer all serum albumins adsorbed at pH 7 and 8. This indicates chelating interactions of CDI::IDA:Cu(II) with proteins. On the contrary, Cyt c and lysozyme eluted in the salt gradient. These results led to the conclusion that the interactions are ionic rather than chelating. At pH 6, serum albumins were not eluted without exception, but basic proteins less retained than at pH 7. At pH 8 the opposite was true; serum albumins were expelled at low ionic strength. The latter result corresponds well to results from GLYMO-IDA and butyl-IDA.

Non-specific ionic interactions

Considering non-specific ionic interactions, most silica-based sorbents displayed only slightly increased retention of basic proteins compared to Sepharose-based sorbents, except propyl-IDA. Compared with other silica-based affinity sorbents prepared in previous studies [23,24], the impact of these ionic interactions was tolerable; irreversible adsorption of proteins never occurred. However, in comparison to butyl-IDA and other silica-based chelators, on propyl-IDA non-specific interactions were most expressed. Native Sepharose 4B displayed also weak non-

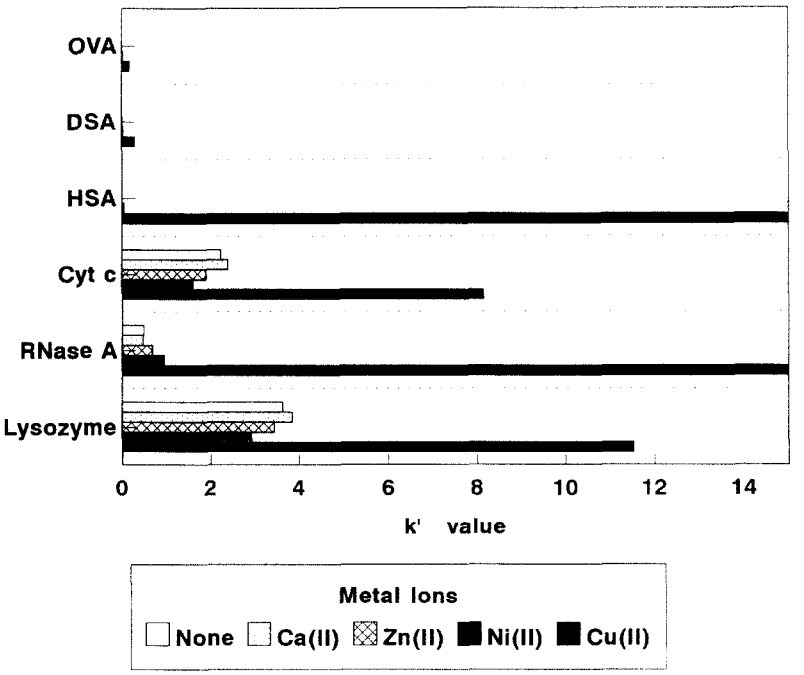

Fig. 4. Elution of proteins with increasing ionic strength on Polygosil 500 CDI::IDA charged with and without metal ions. Chromatographic conditions as in Fig. 2. RNase A and HSA did not elute on the Cu(II)-loaded chelate.

specific interactions with basic proteins. However, with both Sepharose- and silica-based chelators the majority of ionic interactions originated from the carboxyl group of IDA or metal chelates exhibiting a net negative charge, but neither from hydroxyl groups located at the silica surface nor the Sepharose matrix.

pH effects

The development of a negative charge at metal chelates does not occur in a narrow pH range but changes gradually in a range of approximately two pH units [8]. With all Cu(II)-loaded IDA sorbents the change of the interaction mechanism was most apparent, leading to non-binding of proteins at elevated pH. Comparing the adsorption behaviour of negatively charged serum albumins and OVA in Table 2, the development of a negative charge occurs at rather low pH (pH 5–7) on propyl-IDA and butyl-IDA but at elevated pH ($\geq$ pH 8) on epoxy-immobilised IDA. The exact pH cannot be provided at which the negative charge becomes effective; yet, the charge density on the metal chelate affinity sorbent increases with increasing pH. The present results indicated kinetic effects at the transition pH. Kinetic effects either led to partial breakthrough of negatively charged proteins or to apparent low protein recoveries in the salt gradient.

Histidyl residues coordinate to IDA chelates in their unprotonated form only [18]. Therefore, at pH 6 proteins were less retained on IDA:Ni(II) than at pH 7 or 8. At pH 6, all proteins, including RNase A, eluted in the salt gradient; thus interactions were mainly ionic. On IDA:Cu(II), proteins demonstrated significant changes in the elution behaviour at pH 5 only (Table 2). However, at this pH only basic proteins eluted at high salt concentration.

Buffer salt

Exchange of phosphate against acetate, pH 5 or Tris · HCl, pH 8, provided no benefits. Using acetate resulted in nearly identical retention of proteins as observed with phosphate buffers. Tris

Table 2
Retention behaviour of proteins on Cu(II)-loaded chelators

Sorbent	HSA				OVA				DSA				Cyt C				RNase A				Lysozyme			
	8	7	6	5	8	7	6	5	8	7	6	5	8	7	6	5	8	7	6	5	8	7	6	5
CS	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	+	○	○	○	○	○	○	○	+
Epi::IDA	/	○	○	/	/	○	○	/	/	○	○	/	/	○	○	/	/	○	○	/	/	○	○	/
GLYMO-IDA	○	○	○	○	–	○	○	○	–	○	○	○	○	○	+	+	○	○	○	+	○	○	○	+
CDI::IDA	□	○	○	○	□	–	▲	▲	□	–	○	○	+	+	+	+	○	○	▲	▲	○	+	▲	▲
Propyl-IDA	–	○	○	○	–	–	□	○	–	–	□	○	○	○	○	○	○	○	○	○	○	○	○	○
Butyl-IDA	–	○	○	○	–	○	○	○	–	□	○	○	○	○	○	+	○	○	○	+	○	○	○	+

Chromatographic conditions: 25 m*M* phosphate; linear gradient in 10 min from 0 to 0.5 *M* NaCl, in 3 min to 1 *M* NaCl, pH as indicated below proteins. Symbols: – = not retained; + = retained and eluted; ○ = not eluted; □ = low mass recovery; ▲ = broad peak; / = no data

caused a higher displacement of metal ions than phosphate. However, chromatographic properties did not change significantly. The displacement of Cu(II) by Tris is due to the complexation of this metal ion by Tris, resulting in a competition of IDA and Tris for it. This phenomenon was most apparent at low salt concentration.

In another study [25] the displacement of concanavalin A by Tris from Cu(II)-loaded IDA was observed over a long period of time. Concanavalin A is considered to bind very strongly onto this chelate [26]. However, in the presence of Tris displacement of the metal ion took place and consequently a decrease in capacity was evident over a long time scale.

3.3. Adsorption at high ionic strength

At high ionic strength ionic interactions on metal chelates are suppressed; thus, specific interactions with proteins are the dominating force under these conditions.

Spacer length

Considering Ni(II)-loaded IDA, complete adsorption of RNase A was evident on both CS and GLYMO-IDA in the presence of 0.5 or 1 *M* NaCl and pH 7 (data not shown). On Ni(II)-loaded Epi::IDA, butyl- and propyl-IDA, RNase A was not adsorbed. RNase A exposes 2 histidyl residues at the protein surface. It is discussed in literature that at least 2 accessible histidyl residues are required for adsorption on IDA:Ni(II) chelates [5]. The result mentioned above indicate that one of the two histidyl residues might only be reached by a long spacer, as provided by CS and GLYMO-IDA.

Propyl-IDA:Cu(II) did not display chelating interactions with OVA and DSA in contrast to epoxy-bound chelates (data not shown). Butyl-IDA displayed kinetic effects during adsorption of DSA and OVA, resulting in the breakthrough of minor amounts of these proteins (see also results at low salt concentrations). These results can be explained by the short spacers of these metal chelate sorbents. Short spacers restrict the flexibility of immobilised chelates. Consequently, the imidazole group of a histidyl residue cannot be reached if not exposed at the protein surface. It is plausible that also protein surface-located histidyl residues differ in their degree of flexibility, depending on the environment. Therefore, histidyl residues demonstrating low flexibility may only be attached to chelates immobilised through longer spacers, such as GLYMO or bisoxirane.

Elution with decreasing salt gradient

All proteins adsorbed on Cu(II)-loaded GLYMO-IDA, CDI::IDA, and butyl-IDA at pH 8 and 1 *M* NaCl (see also Fig. 6 for comparison).

It was of interest whether serum albumins would be expelled and consequently eluted at this pH and low salt concentration after being adsorbed at high salt content. In practice, elution at low ionic strength seemed to be an extremely slow process on the time scale of the experiment (25–30 column volumes in 10 min). Only on butyl-IDA:Cu(II) elution was apparent without doubt; also partial breakthrough of DSA and OVA took place. On CDI::IDA:Cu(II) elution did in fact not occur. On GLYMO-IDA, OVA eluted in a broad peak; the elution of serum albumins was uncertain. Likely, once the chelate–protein complex is formed at 1 *M* NaCl, desorption depends mainly on the dissociation rate at low ionic strength, which appears to be slow. Desorption seems not to be enhanced by repulsion of both negatively charged serum albumins or OVA and chelates at low ionic strength.

Elution with imidazole gradient

Imidazole is a competitive substrate, displacing adsorbed proteins at pH 7 or 8 through formation of a chelate–imidazole complex. Such elution conditions are important for sensitive proteins which may lose biological activity at low pH. In practice, best reproduction of chromatographic conditions is obtained by adding 1 m*M* imidazole in the equilibration buffer [6], thereby forming the chelate–imidazole complex before adsorption of proteins. However, some proteins, such as Cyt c, were found not to adsorb under this condition [10]. Imidazole was therefore not used in the equilibration buffer, in order to avoid misinterpretation of results. Thus, imidazole elution from this study cannot be compared directly with data from other groups.

Best binding conditions were found at pH 7 for Cu(II)- and Ni(II)-loaded IDA (Fig. 5). Below pH 6, basic proteins did not adsorb even

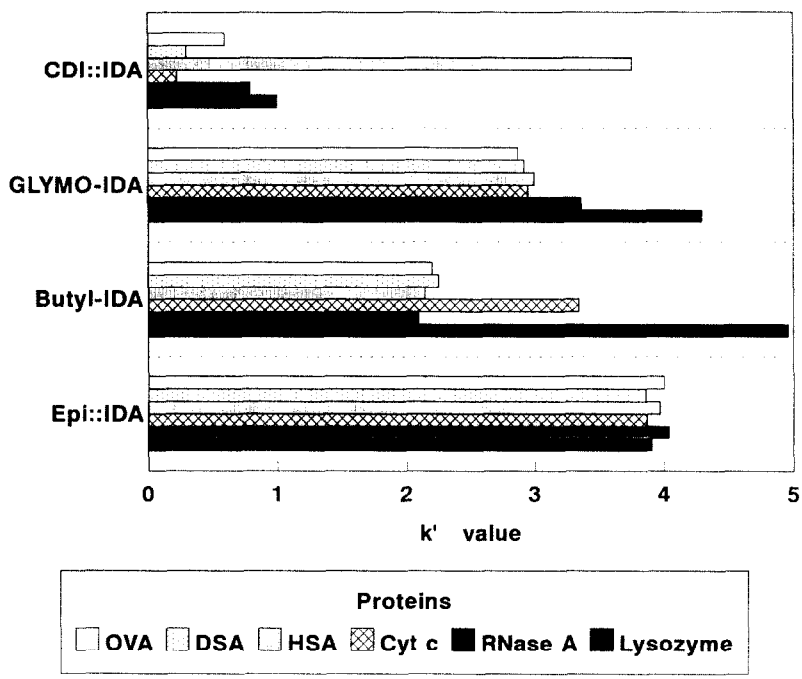

Fig. 5. Elution of proteins with imidazole gradient on Cu(II)-loaded chelators with different immobilization chemistry and spacer length. Column, 30 × 5 mm I.D.; flow-rate, 0.5 ml/min; temperature, 298 K; buffer, 25 m*M* phosphate + 0.5 *M* NaCl, pH 7; gradient in 20 min to 50 m*M* imidazole, then in 5 min to 100 m*M* imidazole; for the CDI::IDA chelate concentrations of imidazole of 12.5 and 25 m*M* were realised, respectively.

on Cu(II)-loaded butyl-IDA or epoxy-immobilised IDA. These results confirmed that interactions at pH 6 and low ionic strength were mainly ionic with these chelates. Consistently, BSA, HSA and RNase A eluted at higher retention times (≈30%) from Ni(II)-loaded than from Cu(II)-loaded CS and GLYMO-IDA. This seems to be contrary to the chromatography of human serum transferrin from Ni(II)- and Cu(II)-loaded CS which was eluted later from the latter chelate [13]. Perhaps the absence of imidazole in the adsorption buffer effects protein elution. However, an explanation for this behaviour has not been found yet.

In the presence of 0.15 *M* NaCl, silica-based IDA:Cu(II) and IDA:Ni(II) demonstrated non-specific ionic interactions in addition, especially with Cyt c and lysozyme (data not shown). Non-specific ionic interactions were most apparent with propyl-IDA. Presumably, the close proximity of hydroxyl groups at the silica surface resulted in ionic interactions with positively charged proteins. In the presence of 0.5 *M* NaCl, as represented in Fig. 5, all proteins eluted between 10 and 20 m*M* imidazole at very close retention times for individual chelating gels, except on CDI::IDA. Discrimination of surface properties of different proteins did not take place on GLYMO-IDA and Epi::IDA. This is a major disadvantage of this particular elution method.

Protein interactions with the CDI::IDA chelate were rather weak. Therefore, a shallower imidazole gradient was applied for this chelate. Most proteins were recovered from the column at approximately 2–3 m*M* imidazole (Fig. 5). The interaction with HSA was stronger; elution took place between 5 and 7 m*M* imidazole.

The interaction of basic proteins with epoxy- and butyl-immobilised IDA seems to be different, because the elution order of proteins changed (Fig. 5). Elution order is Cyt c < RNase A < lysozyme on GLYMO-IDA:Cu(II) whereas RNase A < Cyt c < lysozyme was found on the butyl-IDA:Cu(II) at pH 7. Retention of amino acids indicated lower selectivity for histidines but higher selectivity for lysine with butyl-IDA:Ni(II) compared to other chelates [7]. In view of these results, the interaction of RNase A seems to occur mainly with histidyl residues while Cyt c and lysozyme interact with basic amino acid residues, such as lysine and/or arginine, in addition. Both proteins are rich in the latter amino acids.

Elution with pH gradient on Cu(II)-loaded chelators

By decreasing the pH, histidyl residues are protonated, consequently leading to elution of adsorbed proteins. This represents a common elution protocol [16,27,28]. In a first set of experiments, a steep pH gradient was chosen. As a result, eluted proteins displayed similar retention times. With a shallower pH gradient better discrimination of proteins was achieved (Fig. 6).

CDI::IDA allows the separation of some proteins in the pH gradient. The peak of OVA was relatively broad compared to other proteins and the mass recovery of DSA relatively low. Perhaps DSA does not completely elute under these chromatographic conditions.

On epoxy-immobilised chelates, serum albumins and OVA were most strongly retained. Results varied with variation of the spacer length. On GLYMO-IDA and CS, HSA did not elute even on a prolonged time scale. Andersson *et al.* [15] observed the same chromatographic behaviour of HSA on CS:Cu(II) under these conditions. On the contrary, on Epi::IDA all proteins eluted at low pH.

Obviously, a shallow pH gradient is necessary to discriminate between surface properties of different proteins. These results are in concordance with results published by Nakagawa *et al.* [14], who applied very shallow pH gradients over a period of 80 min for the isolation of various peptides.

3.4. Bleeding of Cu(II)

Bleeding of Cu(II) was apparent with all immobilised metal chelates at elevated imidazole concentration or low pH. It was most evident with butyl-IDA and propyl-IDA, increasing in that order. With propyl-IDA the first 2 mm of

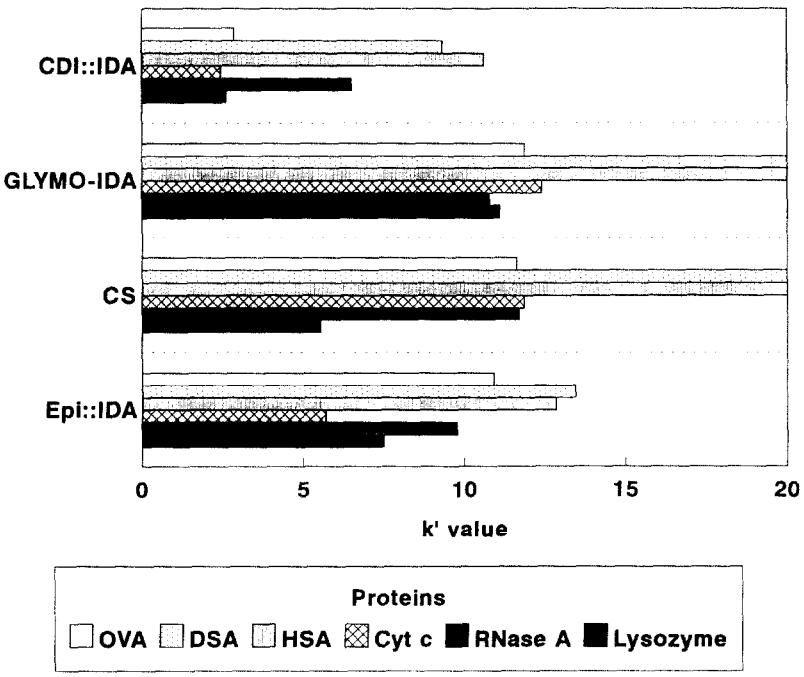

Fig. 6. Elution of proteins with decreasing pH on Cu(II)-loaded chelators with different immobilization chemistry and spacer length. Buffer, 25 m*M* phosphate + 0.5 *M* NaCl, pH 8; linear gradient in 15 min to pH 2.8 and holding at this pH for 10 min. Other chromatographic conditions as in Fig. 5

the column bed were virtually uncharged after running 8 times the pH gradient.

3.5. Capacity of affinity sorbents

IDA:Fe(III) chelates

During adsorption of lysozyme in the frontal chromatographic mode significant amounts of Cu(II) were displaced from propyl-IDA:Cu(II) at pH 7. Thus, determination of the protein capacity was not possible. Loading propyl-IDA with Fe(III) provided reproducible chromatographic conditions. Apparently, bleeding of Fe(III) from this chelate did not occur at pH 5. For the sake of comparison, also the other chelators were charged with Fe(III) and analyzed at this pH. Protein capacities and apparent dissociation constants (K_d) are displayed in Table 3. Comparing the capacities of the chelators for these proteins represent more or

Table 3
Adsorption of lysozyme and BSA on Fe(III) chelates

	Capacity (mmol/l)		Capacity (g/l)		Apparent K_d (*M*)	
	Lys	BSA	Lys	BSA	Lys	BSA
GLYMO-IDA	1.35	0.37	19.4	25.2	$7.9 \cdot 10^{-5}$	$3 \cdot 10^{-6}$
Propyl-IDA	1.53	0.59	22	40	$5.6 \cdot 10^{-5}$	$6.6 \cdot 10^{-6}$
CDI::IDA	0.09	0.05	1.3	3.6	$1.9 \cdot 10^{-4}$	$1.1 \cdot 10^{-5}$
CS	1.39	1.1	20	75	$8.3 \cdot 10^{-5}$	$2.9 \cdot 10^{-6}$

less a mirror of the Cu(II) capacities on these chelators, as described in Part I [7]. The CDI-immobilised chelate yielded the lowest capacity, as a consequence of the lower ligand densities. Except for CDI::IDA:Fe(III), dissociation constants of different metal chelates to a particular protein were close. CDI::IDA:Fe(III) displayed higher dissociation constants for lysozyme and BSA, indicating lower interactions with the chelate.

The capacity for lysozyme was lower than for BSA with all chelates. At first, this seems to be inconsistent with results obtained from other porous affinity sorbents. The capacities for larger proteins is usually lower than for smaller proteins due to pore size restrictions. However, the reason for the low capacity for lysozyme in this study is the higher NaCl concentration employed (0.5 *M* NaCl for lysozyme, 0.1 *M* was required for adsorption of BSA). The dependence on salt concentration is unusual for metal chelate interactions and indicative for ionic interactions [19]. Probably, the iron chelate exhibits a negative charge at pH 5. Therefore, ionic interactions may play a key role in the adsorption of proteins at IDA:Fe(III). Hence, the dissociation constant is an apparent thermodynamic value, the interaction is taking place at least partial via several charged groups at the protein surface.

IDA:Cu(II) chelates

Cu(II)-loaded butyl-IDA and GLYMO-IDA were stable during the application of proteins. Double reciprocal plots of experimental data revealed that the chelators displayed Langmuir-type adsorption behaviour, characterised by a linear relationship of transformed adsorbate concentration in solution and the stationary phase. Both lysozyme and BSA displayed lower interaction with Cu(II)-loaded butyl-IDA than GLYMO-IDA (Table 4) at pH 7. Furthermore, protein capacities were lower with butyl-IDA.

Both metal chelate sorbents demonstrated a lower capacity for BSA than for lysozyme. Thus, the copper chelate demonstrates rather chelating interaction. BSA requires more space in the porous system due to its size; therefore, the accessibility of the affinity ligands is lower [29,30].

The stability of the covalent linkage of GLYMO-IDA is high. In 10 consecutive runs no apparent loss in protein capacity took place (Fig. 7). Data fluctuations in Fig. 7 demonstrate the error in the determination of the breakthrough time, as calculated automatically by the integrator. The results confirm that the capacity of this copper chelate is nearly independent of the salt concentration, as typical for metal chelate interactions.

In contrast, the propyl-IDA sorbent demonstrated a loss of capacity with time. In zonal chromatography, such a decline in capacity is usually not evident because only parts of the total capacity of chromatographic sorbents are utilised. Of course, the loss in protein capacity restricts the life time of the propyl-IDA sorbent.

4. Discussion

It is apparent that chelators bound through an epoxy group onto silica, such as the glycidoxy group in GLYMO, displayed the most comparable results with CS. With CS, IDA is also bound

Table 4
Adsorption of lysozyme and BSA on Cu(II) chelates

	Capacity (mmol/l)		Capacity (g/l)		Apparent K_d (*M*)	
	Lys	BSA	Lys	BSA	Lys	BSA
Butyl-IDA	1.67	0.176	24	12	$1.6 \cdot 10^{-5}$	$3 \cdot 10^{-6}$
GLYMO-IDA	3.06	0.346	44	23.5	$5.9 \cdot 10^{-6}$	$1.7 \cdot 10^{-6}$

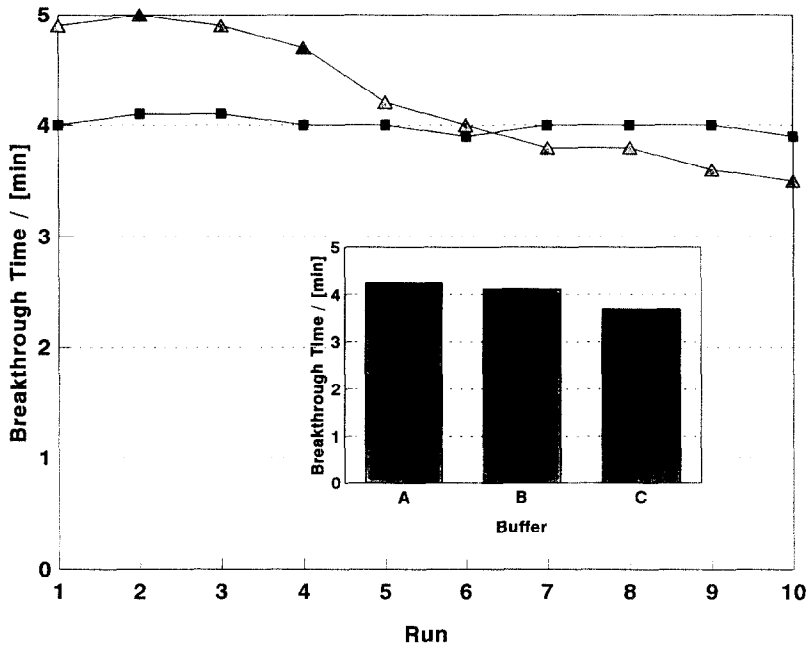

Fig. 7. Capacity and hydrolytic stability of Cu(II)-loaded silica-based sorbents during frontal chromatography of lysozyme. Column 25 × 4 mm I.D.; flow-rate, 0.5 ml/min; temperature, 298 K; buffer, 25 m*M* phosphate + 0.5 *M* NaCl, pH 7.0, lysozyme concentration 1 g/l. The capacity of Polygosil 500 butyl-IDA (▲) decreases from run to run, whereas the capacity of the GLYMO-IDA chelator (■) is almost constant. Inset: dependence of capacity on salt concentration on the GLYMO-IDA chelate; (A) no NaCl, (B) 0.5 *M* NaCl, (C) 1.2 *M* NaCl in phosphate buffer, pH 7.0.

through an epoxy group [6,28]. The adsorption and elution behaviour of GLYMO-IDA chelates was almost identical to CS under the chosen chromatographic conditions. This is in concordance with results obtained from the chromatography of amino acids [7]. Furthermore, Cu(II)- and Fe(III)-loaded GLYMO-IDA demonstrated high capacity for lysozyme and BSA. The capacity is similar to silica-based ion exchangers. Therefore, GLYMO-IDA is recommended in case published chromatographic protocols shall be applied for a silica-based metal chelate sorbent.

4.1. Spacer length

It is a general observation in affinity chromatography that long spacers lead to improved flexibility of affinity ligands [31,32]. Likely, this is true also for metal chelate affinity sorbents. Likely, also the mobility of amino acid residues located at the protein surface is of influence for binding.

Considering X-ray structural analysis data of OVA [33], most of the histidyl residues are not accessible. The adsorption of OVA onto IDA:Cu(II) occurs through His 44, which is situated in a hydrophobic environment at the protein surface. Due to the short spacer of propyl-IDA this histidyl residue may not be reached; therefore, no adsorption of OVA is observed on this chelate. Butyl- or GLYMO-spacers may facilitate the correct orientation of IDA in the encounter with this histidyl residue. In the experiment, OVA was indeed retained on both Cu(II)-loaded chelators. However, with the butyl-IDA partial breakthrough of OVA occurred. This indicates that steric restrictions at close encounter of protein and ligand take place. With GLYMO-IDA:Cu(II), no breakthrough of OVA took place.

Although GLYMO-IDA is immobilised through an epoxy group onto silica, it appears

that the spacer between the silica matrix and the chelator is somewhat shorter than with CS. This led to two exceptions of the compatibility of silica-based and commercial chelator with the proteins employed. HSA and BSA were adsorbed only on Ni(II)-loaded CS. Thus, the binding histidyl residues of these proteins (at least two [15]) are available only for interaction with the long spacer of CS:Ni(II) but not with GLYMO-IDA.

With RNase A, X-ray structural data revealed that the two histidyl residues with highest probability for interaction are located at different positions on the surface of the protein. RNase A displays a kidney-like shape [34]. While His 105 is highly accessible at the protein surface, His 119 is located in the active site at a less accessible position. This might be an explanation that RNase A adsorbed on CS:Ni(II) only.

Chelates bound via long spacers are able to penetrate more deeply into cavities or metal binding sites of proteins. After binding onto a histidyl residue they might be able to interact with amino acid residues located close to the chelate, in addition. Consequently, chelate–protein complexes might be formed which are more stable at low pH than a typical metal chelate. Although BSA and HSA display a change in protein conformation at pH 4, consequently leading to exposition of more histidyl residues, it seems to be unlikely that they would be uncharged at pH 4. The former interpretation would explain the apparent irreversible binding of HSA and BSA, as observed during pH gradient elution with Cu(II)-loaded GLYMO-IDA and CS. Such behaviour was found with HSA [15] and human serum transferrin [13], as well. With chelates immobilised by short spacers such strong interactions are unlikely, since only single amino acid residues at exposed positions can interact.

4.2. Flexibility of the chromatographic matrix

Comparing results from affinity sorbents based on either inorganic and polymeric chromatographic matrices is a complex task. Besides differences of the chemical composition also structural deviations of the matrices exist. Silica gel is a very rigid matrix which does not allow any structural changes of the surface [35]. In contrast, polymer gels consist of polymer chains, demonstrating a certain degree of mobility [32]. This often leads to compression of a column bed. However, it also allows some flexibility of those polymer chains which are not cross-linked [36].

Differences in the adsorption behaviour on different types of matrices will be more pronounced in case the active site is located in a cavity of the protein. It might be reached by a sufficiently long spacer, as noticed with OVA. In other cases an extremely long spacer or a flexible matrix is required to either extend to the bottom of a cavity or to adapt to a certain extent to the shape of a protein. This might have been required for His 119 of RNase A, which is located at the bottom of the kidney-like structure of this protein.

4.3. Adsorption and elution

Although differences between the metal chelate sorbents were most apparent employing an increasing salt gradient, in practice this elution method plays a minor role. Also, elution at low pH is not always possible, as was experienced with HSA. An imidazole gradient leads to smallest peak volumes compared to other elution models. In case imidazole is absent in the equilibration buffer, as was done in this study, retention times of different proteins were nearly identical, *i.e.* no discrimination of surface properties of proteins took place. Therefore, this method is not recommended in situations where adsorbed proteins shall be separated during elution.

It can be assumed that initially the formation of the metal chelate–protein complex is controlled by ionic interaction. Therefore, interaction of both negatively charged proteins and chelates is possible only at elevated salt content. After formation of the metal chelate–protein complex elution at low ionic strength is controlled mainly by the reverse reaction rate, which appeared to be slow. After dissociation, low ionic strength prevents renewed binding of pro-

teins; however, low ionic strength seemed not to promote the desorption process. Probably, adsorption of serum albumins on GLYMO-IDA and CDI::IDA-Cu(II) chelates is caused by an extremely slow reverse reaction rate which on the other hand indicates that the stabilities of each chelate–protein complex are high.

4.4. Recommendations

Beside chelating interactions, ionic interactions play a key role in MCIC. Primarily, this seems to be a negative property of these group-specific affinity sorbents, thus should be suppressed by addition of salt. However, with a given separation problem, comprising negatively charged contaminants, low salt concentration and high pH may expel negatively charged proteins from a negatively charged chelate which otherwise would have a chance to bind. This is particularly true for butyl-IDA:Cu(II). In case of Ni(II)- or Zn(II)-loaded chelators protein interaction increases at low salt concentration.

The spacer length should be seen as a variable by manufacturers of chelating chromatographic supports. No doubt, highest flexibility of an affinity ligand is achieved with a long spacer; however, high flexibility is not always needed. For those separation problems where the product of interest exposes a histidyl residue at the protein surface but the contaminant's histidyl residue is not as accessible and cannot be reached, a higher selectivity should be possible with a short spacer.

The immobilization chemistry of a commercial chelating gel is usually not known to the user. The present investigation revealed that chromatographic protocols might not be interchangeable without changing chromatographic conditions. This implies that the exact chemical structure of the chelate and the linkage of commercial chelating sorbents should be made available to the user, in order to avoid inconveniences.

5. Conclusions

All the silica-based metal chelate sorbents employed in this study displayed chelating interactions with histidyl residues at protein surfaces. The propyl-IDA however displayed highest non-specific interactions and lowest stability. Thus, it will be disregarded in future applications.

The protein capacities of Cu(II)-loaded GLYMO-IDA and butyl-IDA were comparable to silica-based ion exchangers. The capacity of CDI::IDA:Cu(II) was lowest but can be improved. All three metal chelators displayed low non-specific interactions and good stability of the chemical linkage.

GLYMO-IDA:Cu(II) displayed almost identical chromatographic characteristics compared to commercial CS.

The selectivity of metal chelate affinity sorbents is changed by alteration of the spacer length and/or binding chemistry. Hence, the butyl-IDA chelator and CDI::IDA chelator demonstrated the most different elution behaviour compared to epoxy-immobilised metal chelate sorbents. It seems that butyl-IDA:Cu(II) recognises also lysine and/or arginine residues. CDI::IDA:Cu(II) displayed tight interactions only with serum albumins; thus, the selectivity for these proteins was highest compared to other chelates employed.

The current state of investigation revealed that further optimization in view of pH, ionic strength and composition of the adsorption buffer might be advantageous. Coming investigations will demonstrate whether these new metal chelators represent attractive alternatives for the isolation of proteins from various sources.

Abbreviations

Butyl-IDA	IDA immobilised by butyl-spacer
Butyl-IDA:Cu(II)	Cu(II)-loaded butyl-IDA
CDI	1,1′-Carbonyldiimidazole
CDI::IDA	CDI-immobilised IDA
CDI::IDA:Cu(II)	Cu(II)-loaded CDI::IDA
CS	Chelating Sepharose FF
CS:Cu(II)	Cu(II)-loaded CS
Cyt c	Cytochrome *c*

DSA	Dog serum albumin
Epi::IDA	Epibromohydrin-immobilized IDA
FPLC	Fast protein liquid chromatography
GLYMO	3-Glycidoxypropyltrimethoxysilane
GLYMO-IDA	GLYMO-immobilised IDA
GLYMO-IDA:Cu(II)	Cu(II)-loaded GLYMO-IDA
HSA	Human serum albumin
IDA	Iminodiacetic acid
IDA:Cu(II)	Cu(II)-loaded IDA
MCIC	Metal chelate interaction chromatography
OVA	Ovalbumin
Propyl-IDA	IDA immobilised through propyl spacer
RNase	Ribonuclease

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