ELSEVIER

Journal of Chromatography A, 672 (1994) 35–49

JOURNAL OF CHROMATOGRAPHY A

Silica-based metal chelate affinity sorbents I. Preparation and characterization of iminodiacetic acid affinity sorbents prepared via different immobilization techniques

Friedrich Birger Anspach

GBF-National Research Centre for Biotechnology, Biochemical Engineering, Mascheroder Weg 1, D-38124 Braunschweig, Germany

(First received October 12th, 1993; revised manuscript received March 1st, 1994)

Abstract

Iminodiacetic Acid (IDA) was immobilised on silica supports using either activated chromatographic supports or silanes carrying IDA in ω-position to silicium. Through reaction of IDA with glycidoxypropyltrimethoxysilane (GLYMO) before immobilization, a new and simple method is introduced which provides a metal chelate affinity sorbent of both high capacity for Cu(II) (28 μmol/ml) and almost identical chromatographic characteristics to soft gel metal chelate affinity sorbents.

By immobilization of IDA through *n*-alkyl spacers exclusive of an epoxy group a different chemical neighborhood to the chelator is obtained. These metal chelate sorbents demonstrated higher capacities for Cu(II) (39 μmol/ml). The selectivity for lysine was higher, for histidine lower, compared to epoxy-immobilized IDA chelates. It is concluded that the β-hydroxyl group, evolved during reaction with epoxy groups, is integrated with the metal chelate, thus forming a N-(hydroxyethyl)iminodiacetic acid (HEIDA).

The most distinct chromatographic behaviour was observed with CDI-immobilised IDA chelates, which displayed higher selectivity for acidic amino acids than common IDA chelates.

1. Introduction

Immobilised metal chelates are employed as affinity chromatographic sorbents due to their interaction with some proteins. Metal chelate interactions take place with functional groups of amino acids located at the surface of these proteins [1], such as imidazole of histidine, but also tryptophan and cysteine. Following the pioneering work of Porath *et al.*, considering metal chelate-interaction chromatography (MCIC) with proteins, primarily metal chelates based on carboxymethylated amines were investigated, such as iminodiacetic acid (IDA) [2,3] or triscarboxyethylenediamine (TED) [4,5]; recently also nitrilotriacetic acid (NTA) [6] was considered. Particularly IDA is used as a general tool for the purification of proteins from complex mixtures and is introduced today in many laboratories. It has been demonstrated that the replacement of one metal chelator by another, for example IDA with TED, is of great significance for the selectivity of the metal chelate sorbent [4].

0021-9673/94/$07.00
SSDI 0021-9673(94)00210-Z

Changes on selectivity of metal chelate sorbents are also observed by exchanging metal ions, such as Cu(II) against Ni(II) or Zn(II) [7,8,9,10]. Usually, Cu(II) is preferred as metal ion in separation protocols, followed by Zn(II) and Ni(II), in that order. The preference for Cu(II) is due to the high stability of the IDA:Cu(II) chelate and a stronger interaction of this chelate with histidine, compared to most of the other metal ions used in MCIC. The utilization of metal ions, such as Fe(III) [11], Al(III) [12] or Tl(III) [13] is described; however, their use is limited in practice.

The introduction of high-performance immobilized metal chelate sorbents allows a much higher resolution of biopolymers [14]. These high-performance sorbents are intended to display almost identical elution characteristics compared to soft gel sorbents, in order to employ analogous chromatographic protocols. Therefore, immobilization of IDA onto silicas should be accomplished through an epoxy group, as with most soft gels. Unfortunately, this immobilization technique results in low ligand densities when aliphatic amines are bound below pH 8, as required with silica-based matrices [15]. This is a disadvantage, since the interaction strength in MCIC is related to the ligand density [16,17], as is the capacity for proteins.

The object of this study was the introduction of a chemical procedure which allows the synthesis of a silica-immobilised IDA representing almost identical chromatographic characteristics compared to commercial metal chelate sorbents based on soft gels. Apart from that, immobilization techniques involving alkyl-silanes of IDA, were employed on silicas for comparison. Both avenues led to metal chelate affinity sorbents demonstrating high capacities for Cu(II) and good resolution for the amino acids employed. However, immobilization techniques, which either changed the structure or the chemical neighborhood of the chelator IDA, affected significantly the selectivity of the immobilised metal chelates for amino acids, especially histidine.

2. Experimental

2.1. Chemicals and chromatographic materials

Polygosil 500-1525 was obtained from Macherey Nagel, Düren, Germany. Glycine, L-histidine, L-lysine, L-cysteine, L-serine, and L-phenylalanine were obtained from Sigma, München, Germany. L-Arginine, malonic acid, L-tryptophan and L-glutamic acid were obtained from Serva, Heidelberg, Germany. 3-Aminopropyltriethoxysilane, 1,1'-carbonyldiimidazole (CDI), bromoacetic acid, chloroacetic acid, iminodiacetic acid (IDA), epibromohydrin (Epi) and all salts were obtained from E. Merck, Darmstadt, Germany. 3-Glycidoxypropyltrimethoxysilane (GLYMO) was obtained from Aldrich, Steinheim, Germany. 3-Bromopropyltrimethoxysilane and 4-aminobutyltriethoxysilane were obtained from ABCR, Germany. 3-Chloropropyltrimethoxysilane, Sepharose 4B and lanthanum nitrate hexahydrate were obtained from Fluka, Neu-Ulm, Germany. Chelating Sepharose FF (CS) was purchased from Pharmacia, Freiburg, Germany. The adduct of IDA-di-*tert*-butylester and GLYMO was a generous gift from H.-J. Wirth [18] and K.K. Unger of the University of Mainz, Germany.

2.2. Instruments

The liquid chromatographic system used for all chromatographic experiments was the Pharmacia 500 system assembled for zonal and frontal chromatography, respectively. IR analysis was performed on a Perkin-Elmer 881 IR spectrophotometer using potassium bromide pellets. Specific surface area (BET) was determined by N_2-sorption using the micromeritics ASAP 2000 system.

2.3. Elemental analysis

The coverage of the silica surface with silanes was determined with elemental analysis for carbon, nitrogen and hydrogen. The concentration

of immobilised groups was calculated on the basis of the surface area of the native silica support, as described by Kováts *et al.* [19].

2.4. Preparation of activated supports

Polygosil 500-1525 was chemically modified with GLYMO in acetate buffer at pH 5.5, as described by Bogart *et al.* [20]. Either GLYMO-activated silica was employed directly for the immobilization of IDA via method 4 (see below) or converted to diol-silica. To this end the GLYMO-activated silica was suspended in 0.01 *M* HCl at 323 K for 3 h. Lastly activation of the diol-silica with 1,1′-Carbonyldiimidazole (CDI) was performed, as described by Hearn *et al.* [21]. It is essential to use anhydrous acetone, in order to secure the capacity for Cu(II) described in this study.

Sepharose 4B was activated with epibromohydrin in 0.5 *M* NaOH, as described by Hochuli *et al.* [6].

2.5. Preparation of N-(3-Trisodiumsilanolate-propyl)-N,N-diacetic acid (IDA-propyl-silane)

Procedure A

The silane was synthesised according to a procedure described by Gimpel *et al.* for the chromatography of α-amino acids [22]. Briefly, 22.6 ml 6 *M* NaOH were added to 10 g of aminopropyltriethoxysilane at 273 K. 8.5 g of chloroacetic acid were dissolved in 45 ml 2 *M* NaOH and subsequently added to the silane. The reaction was carried out for one h at 333 K, then the solution was adjusted to pH 12 with 6 *M* HCl and finally the product was precipitated by addition of 9.4 g of barium chloride dissolved in 45 ml of water. The precipitate was filtered, washed with water, suspended in 90 ml 0.5 *M* potassium sulfate and stirred overnight to obtain the potassium salt of the silane. After centrifugation, the supernatant was collected and stored for further use. IR analysis was in concordance with the published data. IR: 3430(–OH); 2920 ($-CH_2-$); 1580($-COO^-$); 1390($-CH_2-$); 1100 ($-Si-O^-$) cm^{-1}.

Procedure B

In a similar reaction 50 ml 3 *M* NaOH and 3.34 g of IDA were added successively to 5 g of 3-chloropropyltrimethoxysilane at 273 K. Reaction conditions and treatment of the product were the same as described for procedure A.

Using 3-bromopropyltrimethoxysilane instead of chlorosilane caused development of bubbles during reaction and did not result in a useful product.

2.6. Preparation of N-(4-trisodiumsilanolate-butyl)-N,N-diacetic acid (IDA-butyl-silane)

This silane was synthesised analogously to IDA-propyl-silane. 6.4 ml 6 *M* NaOH were added to 3 g of 4-aminobutyltriethoxysilane at 273 K. 2.4 g of chloroacetic acid were dissolved in 12.7 ml 2 *M* NaOH and added to the silane. After reaction for 1 h at 333 K and adjusting to pH 12 with 6 *M* HCl, the product was precipitated by addition of 3.1 g of $BaCl_2$ dissolved in 14 ml of water. The filtered cake was suspended in 25.5 ml 0.5 *M* K_2SO_4 and stirred overnight, in order to recrystallize. The supernatant contained the potassium salt of the silane. IR analysis yielded nearly identical results compared to IDA-propyl-silane. IR: 3430(–OH); 2900 ($-CH_2-$); 1620($-COO^-$); 1390($-CH_2-$); 1110 ($-Si-O^-$) cm^{-1}.

2.7. Preparation of GLYMO-IDA-silane

This silane was synthesised as described by Anspach [23] for the immobilization of aliphatic amines. Briefly, 4.24 g of NaOH and 2.82 g of IDA were dissolved in water at 273 K. Then 5 g of GLYMO were slowly added whilst stirring. The reaction mixture was allowed to warm up to room temperature and maintained at this temperature for 4 h. Finally the temperature was raised to 338 K and the reaction mixture stirred

overnight. The reaction mixture forms a stable solution; no precipitation or polymerization of the silane was apparent at the high pH chosen during reaction. It can be stored at room temperature for at least 1 month under these conditions. IR: 3430(–OH); 2930($-CH_2-$); 1570 ($-COO^-$); 1430($-CH_2-$); 1100 ($-Si-O^-$); 1000 (–C–O–) cm^{-1}.

2.8. Preparation of metal chelating sorbents

For the sake of clarity, the different methods for preparation of metal chelate sorbents are differentiated as method 1 to 8.

Method 1: Polygosil 500-1525 propyl-IDA

3 ml of the IDA-propyl-silane solution, as synthesised through procedure B, were adjusted to pH 3.5 with 1 *M* HCl. After adjusting the volume to 10 ml with water, 2 g of Polygosil 500-1525 were added and the suspension evacuated 3 times at 2000 Pa, in order to ensure complete wetting of the pores of the silica with the reaction mixture. The temperature was raised to 368 K over 2 h, while stirring. The chelating sorbent was filtered and resuspended once in water and again filtered. The filtered sorbent was dried overnight in a desiccator and consequently heated to 423 K at 3 Pa, in order to ensure formation of siloxane groups of most of remaining ethoxysilane groups [24]. This procedure improves the stability of the functionalised sorbent in water.

Method 2: Polygosil 500-1525 propyl-IDA

12 ml of the N-(3-trisodiumsilanolatepropyl)-N,N-diacetic acid solution (procedure A) were adjusted to pH 3.5 with 1 *M* HCl. Subsequently, 4 g of Polygosil 500 were added. Reaction conditions and treatment of the modified silica were the same as described in method 1.

Method 3: Polygosil 500-1525 butyl-IDA

20 ml of the IDA-butyl-silane supernatant were adjusted to pH 3.5 and 5 g of Polygosil 500 were added. Reaction conditions and treatment of the sorbent afterwards were exactly the same as described in method 1.

2.9. Epoxy-derived metal chelating sorbents

Method 4: immobilization of IDA onto GLYMO-activated Polygosil 500-1525

0.6 g of NaOH and 2 g of IDA were dissolved in 15 ml of water in that order and adjusted to pH 8.3 with 2.5 *M* NaOH. Then 3 g of GLYMO-activated Polygosil 500 were added to the solution and degassed 3 times, in order to ensure complete wetting of the porous system of the silica. The reaction mixture was heated to 333 K for 24 h with stirring and the product isolated by filtration. The modified silica was washed several times with water. Remaining epoxy groups were converted to diol groups by suspending the sorbent in 0.01 *M* HCl as described above. After washing with water the gel was dried and stored. This product is referred to as GLYMO::IDA to distinguish it from the metal chelate sorbent obtained from GLYMO-IDA-silane (method 6).

Method 5: immobilization of IDA-di-tert-butylester-silane

The immobilization of IDA comprising protected carboxyl groups was carried out according to Wirth *et al.* [17]. In brief, 5 g of Polygosil 500-1525 were heated to 418 K at 3 Pa overnight. This silica was suspended in 30 ml of anhydrous toluene and 0.34 g of IDA-di-*tert*-butylester-silane were consequently added. The suspension was stirred at 383 K for 24 h. Then the sorbent was washed with toluene, 2-propanol, and water. *Tert*-butanol was cleaved from the IDA sorbent by addition of 10 m*M* HCl.

In addition to the reported procedure, the remaining hydroxyl groups at the silica surface were endcapped in 20 ml of a 5% GLYMO solution at pH 3. Reaction conditions were 368 K for 3 h with stirring, as described by Regnier *et al.* [25].

Method 6: preparation of Polygosil 500 GLYMO-IDA

3.3 ml of the GLYMO-IDA-silane solution were adjusted to pH 3.5 with 6 *M* HCl and to 10 ml with water. 3 g of Polygosil 500 were added and the temperature raised to 368 K for 3 h with stirring. The chelating gel was treated as de-

scribed in method 1. This product is referred as to GLYMO-IDA.

Method 7: binding of IDA onto epibromohydrin-activated Sepharose supports

1.5 g of NaOH, 2.5 g of Na_2CO_3 and 3 g of IDA were dissolved in 25 ml of water. Finally, 20 g of suction dried epibromohydrin-activated Sepharose 4B was added and adjusted to pH 11.5. The suspension was shaken overnight at 323 K and the chelating gel filtrated and washed several times with water until the supernatant was neutral. The gel was stored in 0.05% sodium azide solution. It is referred as Epi::IDA.

Method 8: immobilization of IDA onto CDI-activated Polygosil-Diol 500-1525.

2 g of CDI-activated Polygosil-Diol 500-1525 were suspended in 70 ml of phosphate buffer, pH 8.3, containing 0.35 g of IDA. The suspension was shaken for 48 h at 328 K. The resulting sorbent was filtered, washed with water and stored in 0.05% sodium azide until used. It is referred to as CDI::IDA.

2.10. Chromatographic conditions

Sepharose 4B based chelating gels were packed in water. Silica based chelating sorbents were dry-packed. All experiments were carried out with 5 mm I.D. columns with bed heights between 20 and 30 mm.

2.11. Zonal chromatography

Chromatographic tests were performed using standard FPLC equipment operating automatically. The columns were washed first with 30 m*M* EDTA + 0.5 *M* NaCl, pH 6, in order to remove any bound metal ion or contaminant, then water, then 15 m*M* metal ion solution and again water. The columns were equilibrated with 20–25 column volumes of the starting buffer. All experiments were carried out at 298 K and a flow-rate of 0.5 ml/min.

2.12. Retention of amino acids

Investigation of the retention behaviour of 7 α-amino acids and malonic acid was performed using 25 m*M* phosphate buffer, pH 6.0. Sample concentrations of 0.2–0.3 mg/ml for serine, glycine, lysine, malonic acid and 0.1 mg/ml for cysteine, tryptophan, phenylalanine and histidine were injected using a 20-μl sample loop. A 206 nm filter was used to monitor the column effluent. Each run was terminated after 10 min.

2.13. Frontal chromatography of Cu(II)

Frontal chromatography was performed with automated adsorption, elution and equilibration. Each run was terminated by the integrator after reaching the plateau region of the frontal breakthrough. Before each run the columns were equilibrated as described for zonal chromatography. To allow adsorption at defined conditions, the metal ion was dissolved in 50 m*M* acetate buffer, pH 5.0. Acetate forms a complex with the metal ion allowing up to 20 m*M* $CuCl_2$ to be dissolved without precipitation of copper hydroxide. Seven concentrations between 0.1 m*M* and 20 m*M* of the metal ion were found adequate to describe the adsorption behaviour.

3. Results

3.1. Chromatography of amino acids

The metal chelators employed in this study are cation exchangers at the chosen pH. For example, the p*K* of one carboxyl group of iminodiacetic acid is reported as 2.65 [26]. Therefore, basic amino acids, such as histidine and lysine, displayed mainly ionic interactions with the carboxylic groups of the chelator. Fig. 1 displays the typical elution behaviour of amino acids on the silica-based GLYMO-IDA chelator under isocratic conditions at pH 6. Charging the chelators with Ca(II) or La(III) did not lead to significant changes of the retention behaviour. Ba(II) displayed almost identical results as Ca(II); therefore, these results are not included

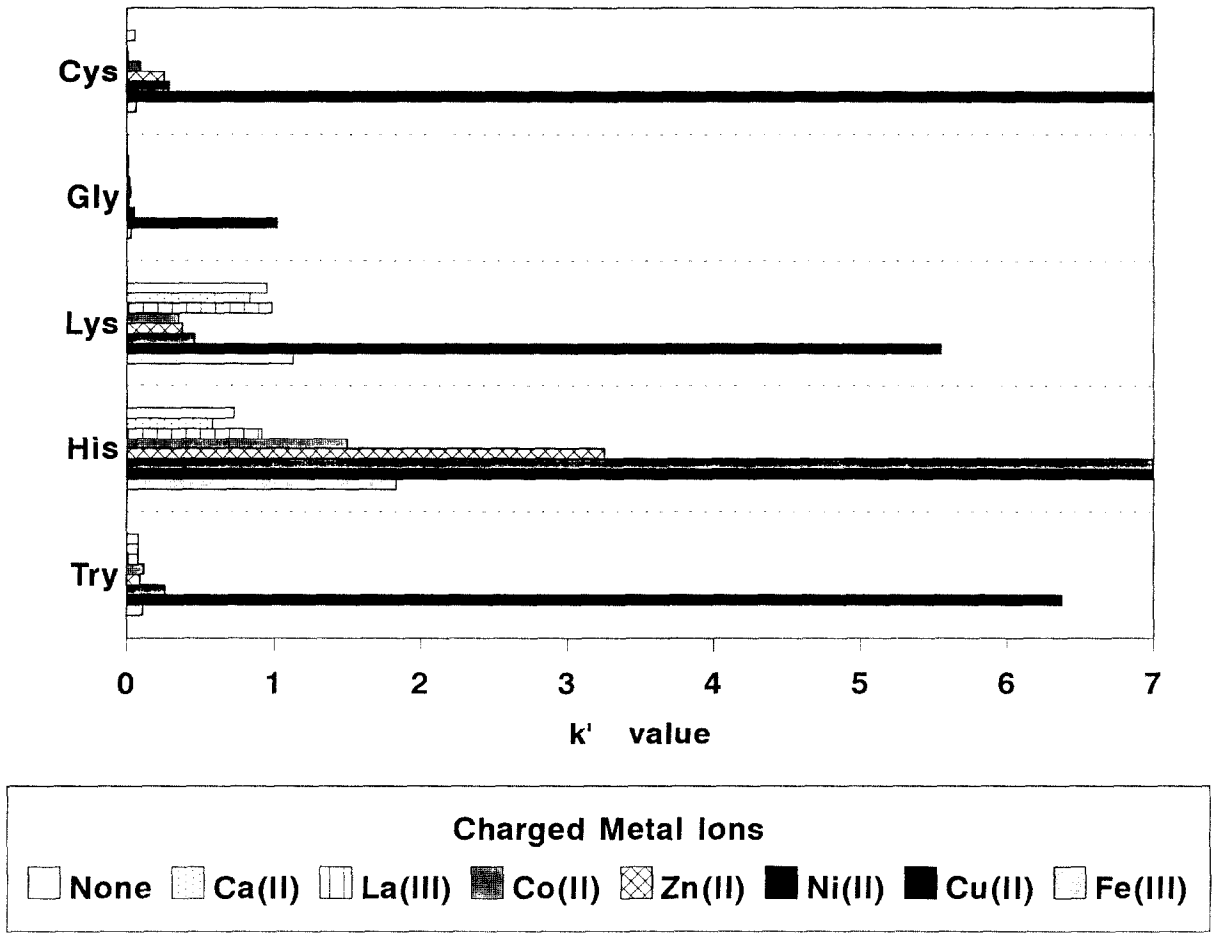

Fig. 1. Retention factors of amino acids on Polygosil 500 GLYMO-IDA charged with hard and borderline metal ions. Column, 30 × 5 mm I.D.; flow-rate, 1.0 ml/min; temperature, 298 K; buffer, 25 m*M* phosphate buffer, pH 6.0. Cys and His were not eluted on the Cu(II)-charged chelate. His eluted at the Ni(II)-chelate at $k' \sim 16$.

in Fig. 1. Co(II)-chelates displayed weak interactions with amino acids. Ionic interactions with carboxylic groups were suppressed after formation of this chelate. The retention time for lysine decreased and evolved closer to other amino acids. The k' value of histidine increased significantly, whereas all other amino acids displayed only small increases in retention time. A further increase in retention was observed when charged with Zn(II) and Ni(II), in that order. Interaction with all amino acids, except lysine, increased. Histidine eluted rather late on high-capacity Ni(II)-charged IDA chelates ($k' \approx 16$ on GLYMO-IDA, $k' > 4$ on butyl-IDA). These results are in concordance with data published by Horváth *et al.* [27].

The most interacting metal chelates were obtained when charged with Cu(II). Then histidine and cysteine were not eluted on any metal chelate, except CDI::IDA, under the chosen chromatographic conditions. In contrast to histidine, cysteine was not as strongly retained on Ni(II)-charged chelators, indicating a deviate interaction of the Cu(II)-charged IDA chelate with cysteine. In fact, if larger volumes of cysteine- or other mercapto-containing solutions

were passed through the column, a dark precipitate of copper mercaptide formed, insoluble in 15 m*M* EDTA or 1 *M* HCl.

The interaction with amino acids increased with the sequence Ca(II) ≈ Ba(II) ≈ La(III) < Co(II) < Fe(III) < Zn(II) < Ni(II) < Cu(II) when charged onto all IDA chelators and follows the order described elsewhere [28]. The retention factor of amino acids increased with the sequence Glu < Gly < Ser < Phe < Trp < Cys < His for Ni(II)- and Cu(II)-charged IDA.

Non-specific interactions originating from remaining hydroxyl groups of the silica matrix were not apparent in most cases. The main interactions with the IDA chelator and Ca(II)-, La(III)- or Ba(II)-charged IDA were caused by ionic interactions with the carboxylic groups of IDA.

3.2. Capacity of immobilised metal chelators

The equilibrium adsorption isotherms obtained from frontal chromatography of Cu(II) are demonstrated in Fig. 2. The interaction with Cu(II) is nearly irreversible with all chelators examined. Thus, almost rectangular shaped isotherms were obtained. The incline of the curve at elevated Cu(II) concentrations is attributed to interactions of Cu(II) with the Sepharose and silica matrix. Securer data from the rising part of the isotherm were not collected, since lower metal ion concentrations were not detected with the UV spectrophotometer employed in this study.

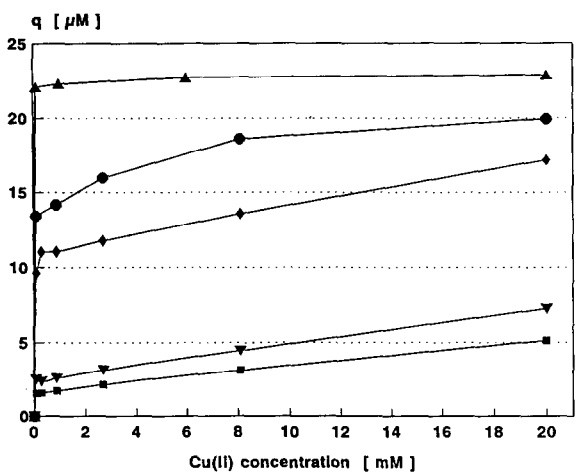

Fig. 2. Equilibrium adsorption isotherms of Cu(II) on metal chelators immobilised on Polygosil 500-1525. Columns, 20-30 × 5 mm I.D.; ■ = CDI::IDA (method 8), ▼ = propyl-IDA (method 1), ◆ = GLYMO-IDA (method 6), ○ = Butyl-IDA (method 3), ▲ = propyl-IDA (method 2). Rectangular shaped isotherms indicate strong interactions of Cu(II) with all metal chelators.

The lowest capacity for Cu(II) was determined for IDA-sorbents synthesised by method 1 (Table 1). Probably side products developed to a large extent under the chosen reaction conditions. This was confirmed by the application of-3-bromo- instead of 3-chloropropyltriethoxysilane. The reaction did not lead to the product of interest under otherwise identical reaction conditions. Instead, the evolution of a gas of undetermined origin was experienced.

Metal chelate sorbents obtained by immobilization of IDA onto activated silica supports (methods 4 and 8) displayed generally lower capacities for Cu(II) compared to sorbents obtained by immobilization of the silanes of the same chelators, employing methods 2, 3 or 6 (Table 1). The low capacities for Cu(II) indicate low ligand densities of the sorbents. This is due to the low pH employed during binding of chelators onto GLYMO- and CDI-activated silicas. Above pH 8.3 considerable amounts of siloxane bonds between silane and silica matrix would be hydrolysed [29]. However, the optimum reaction conditions for the immobilization

Table 1
Capacity of metal chelating sorbents for Cu(II)

Metal chelating sorbent	Method	q_m (μmol/ml)
Chelating Sepharose FF		39
Propyl-IDA	1	4.6
Propyl-IDA	2	39 [a]
Butyl-IDA	3	39.3
GLYMO::IDA	4	7.5
GLYMO-IDA	6	28
CDI::IDA	8	5.1

[a] Capacity decreased with time.

of aliphatic amines onto epoxy-activated supports equals pH 10–12 [4,15]. For CDI-activated matrices contradictory results are reported leading either to highest ligand densities at pH 5–6 [30] or at pH 10–12 [31].

Data from adsorption of Cu(II) and from elemental analysis are compared in Table 2 for sorbents obtained by methods 2, 4 and 6. Although activation of silica with subsequent immobilization of IDA (method 4) led to a higher carbon content compared to the immobilization of the GLYMO-IDA-silane (method 6), a considerably lower capacity was achieved for Cu(II). This result indicates that less than 20% of the epoxy groups of the activated silica react with IDA due to the unfavorable reaction conditions (low pH). The sorbent based on the reaction procedure described by Gimpel *et al.* [32] (procedure A) displayed a higher capacity for Cu(II) at the beginning of the test; however, capacity decreased with time. The instability of this metal chelate sorbent is consistent with the results described by Gimpel. In addition, only 38% of the theoretical capacity of this sorbent was accessible for Cu(II), as calculated from elemental analysis. This is explained by polymerization of silane at the silica surface, occurring either during the immobilization or the preparation of the silane. Consequently, very dense crosslinked structures may develop, not accessible for metal ions. The butyl-IDA sorbent displayed similar capacity for Cu(II) as the propyl-IDA sorbent. The stability of the butyl-IDA sorbent was higher, demonstrating no apparent loss of capacity with time.

A very high accessibility for Cu(II) was obtained through method 6 which displayed a ratio of 0.93 of Cu(II) binding to carbon content. This was the highest ratio obtained in this study, indicating a rather homogeneous metal chelate sorbent. This sorbent displayed also high stability of the linkage between silica surface and IDA. No decrease of ligand density was observed during frontal chromatography of Cu(II).

3.3. Selectivity of immobilised metal chelators

The immobilization methods employed in this study led to different chemical structures in the neighborhood of the metal chelator, as displayed in Table 3. This is due to the type and length of spacers containing at least 3 carbon atoms in case of silica bound IDA (propyl spacer) and either 10 or 12 atoms in case of the bisoxirane spacer, depending on whether the hydroxyl group evolved after formation of the covalent linkage to IDA is considered as part of the chelator or not.

Results from previous investigations indicated that the chemical neighborhood of immobilised chelators affects the elution behaviour of amino acids [33]. The deviation in selectivity is a result of changes of the recognition mechanism. In Fig. 3a and 3b the separation factors of some amino acids for glycine of sorbents obtained through methods 4, 6 and 7 are compared against Chelating Sepharose FF (CS). For clarity the logarithms of the separation factors are considered as

$$\Delta \lg\alpha = \lg\alpha_2 - \lg\alpha_1$$

at which, for example, α_1 and α_2 are the separation factors of CS and other metal chelate sorbents, respectively. $\Delta \lg\alpha$ has no direct link to

Table 2
Comparison of ligand densities from elemental analysis and adsorption of Cu(II) for silica-based metal chelating sorbents

Method	Sorbent	Carbon content (%C)	Ligand density ($\mu M/m^2$)	Capacity for Cu(II) ($\mu M/m^2$)	Ratio
4	GLYMO::IDA	1.84	3.1	0.38	0.12
2	Propyl-IDA	2.33	5.73	2.2	0.38
6	GLYMO-IDA	1.18	1.95	1.82	0.93

The ratio is calculated as capacity:ligand density. A low ratio displays an non-homogeneous sorbent.

Table 3
Chemical structure of metal chelators. The distinct structures are a result of the chemistry employed during immobilization onto silicas and soft gels

Method	Chemistry	Spacer	Structure of metal chelator
1	Propyl-Cl	$-(CH_2)_3-$	$-N(CH_2-COOH)_2$
2	Propyl-NH_2	$-(CH_2)_3-$	$-N(CH_2-COOH)_2$
3	Butyl-NH_2	$-(CH_2)_4-$	$-N(CH_2-COOH)_2$
4,5,6	GLYMO	$-(CH_2)_3-O-CH_2-$	$-CH(OH)-CH_2-N(CH_2-COOH)_2$
7	Epibromohydrin	$-CH_2-$	$-CH(OH)-CH_2-N(CH_2-COOH)_2$
CS	Bisoxirane	$-CH_2-CHOH-CH_2-O(CH_2)_4-O-CH_2-$	$-CH(OH)-CH_2-N(CH_2-COOH)_2$
8,9	CDI	$-(CH_2)_3-O-CH_2-CHOH-CH_2-$	$-O-C(=O)-N(CH_2-COOH)_2$

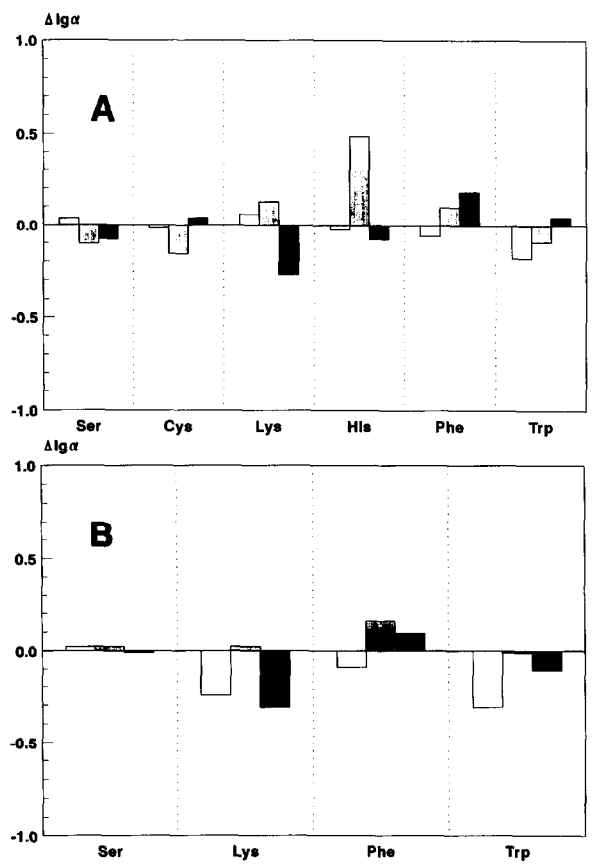

Fig. 3. Variation of selectivity for amino acids of metal chelates depending on chromatographic support and ligand density in comparison to CS. (A) Ni(II)-chelates, (B) Cu(II)-chelates; for each amino acid the left bar represents Sepharose 4B Epi::IDA, the centre bar Polygosil 500 GLYMO-IDA and the right bar Polygosil 500 GLYMO::IDA.

physicochemical parameters. It is utilised for graphic demonstration of selectivity differences. Positive and negative values indicate higher or lower selectivity for amino acids under consideration, respectively.

The separation factors are obtained from the capacity factors (k') of the amino acid under consideration and glycine. The hold-up time (t_0) was determined from solvent peaks.

$$\alpha = \frac{k'(\text{aminoacid})}{k'(\text{glycine})}$$

Histidine and cysteine were not included in figures of Cu(II)-charged chelators due to irreversible adsorption of these amino acids under the chosen chromatographic conditions.

$\Delta\lg\alpha$ values close to zero are considered not significant in this study due to data fluctuations caused by the short columns and dispersion effects. High-performance columns should have been used instead to confirm such small $\Delta\lg\alpha$ values. For the same reason Zn(II)- or Co(II)-charged chelators were not considered for comparison in this study. However, large differences are significant as observed for histidine on the Ni(II)-charged sorbent obtained through method 6. The higher selectivity of this high capacity silica-based metal chelate for histidine might be due to the interaction of neighboring chelates with imidazole and α-aminocarboxylic acid of the same histidine molecule leading to a tempor-

ary bridge between both metal chelate molecules. Sorbents with lower ligand densities, such as the GLYMO::IDA or the Epi::IDA, demonstrated lower selectivity for histidine.

Hydrophobic interactions with the spacer of the Epi::IDA sorbent are low; thus lower selectivities for tryptophan and phenylalanine were observed with this sorbent. The different $\Delta\lg\alpha$ values of lysine for GLYMO-IDA and GLYMO::IDA are due to the contribution of ionic charges, originating from the chelator and/or the chromatographic matrix. Apparently, remaining hydroxyl groups of the silica matrix are more effectively shielded by application of method 4, which led to a higher carbon content. Unfortunately, this method led to a low coverage with IDA and consequently low capacity for Cu(II), as described above.

In Fig. 4 the differences in selectivity for chelators obtained through method 5 is compared against Polygosil 500-1525 GLYMO-IDA. After removal of the large *tert*-butyl protection groups from the sorbent obtained through method 5, some charged hydroxyl groups were exposed at the silica surface, leading to additional ionic interactions with lysine. The higher retention of hydrophobic amino acids might be due to non-specific interactions with uncleaved protection groups. After this sorbent was endcapped with GLYMO, it displayed only minor differences in selectivity compared to the sorbent obtained by method 6. Endcapping seems to cleave remaining protection groups, as is evident from the decrease of selectivity for hydrophobic amino acids.

Changing the linkage between metal chelator and the chromatographic matrix led to large differences in selectivity of silica based sorbents for amino acids (Fig. 5). The immobilization of IDA onto CDI-activated silica was associated with a loss of selectivity for lysine when charged

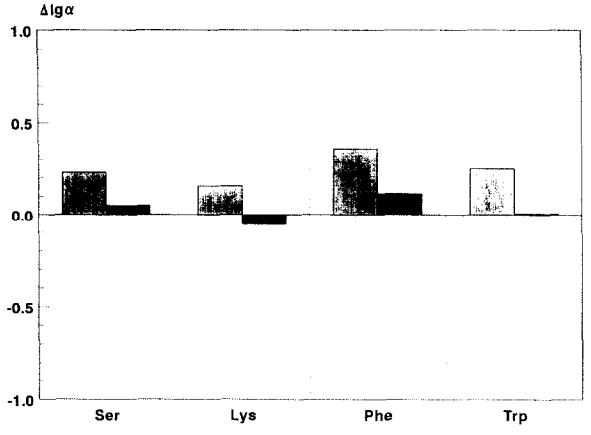

Fig. 4. Variation of selectivity for amino acids of Cu(II)-chelate sorbents obtained through method 5, before and after endcapping in comparison to GLYMO-IDA (method 6); left bars without endcapping, right bars endcapped with GLYMO.

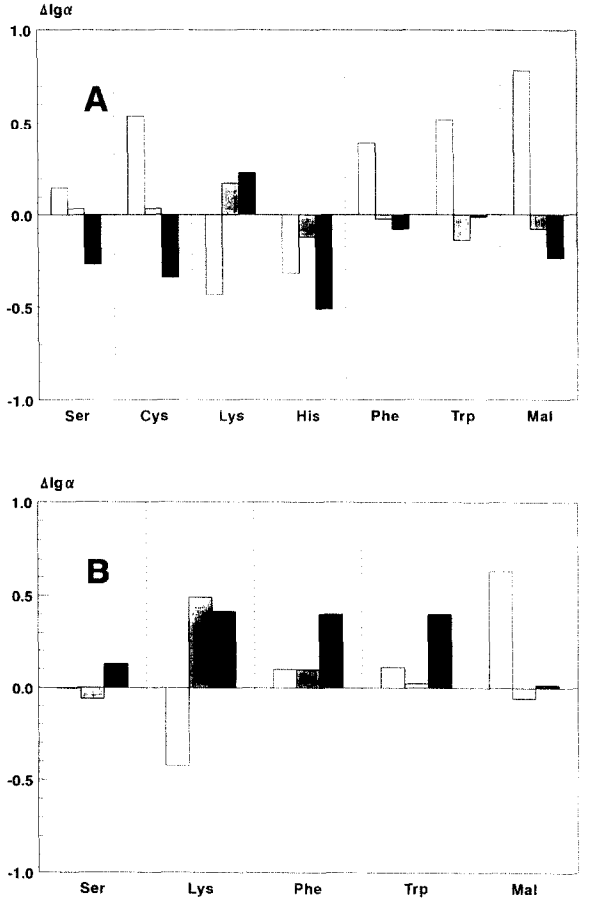

Fig. 5. Variation in selectivity for amino acids of silica-based metal chelates depending on immobilization chemistry in comparison to GLYMO-IDA (method 6). (A) Ni(II)-chelates, (B) Cu(II)-chelates; for each amino acid the left bar represents CDI::IDA, the centre bar propyl-IDA and the right bar butyl-IDA.

with Ni(II) or Cu(II). The selectivity for hydrophobic amino acids, cysteine and malonic acid increased. In contrast to epoxy- and alkyl-bound IDA, cysteine eluted from the Cu(II)-charged CDI::IDA sorbent.

It is remarkable that interactions of amino acids with CDI::IDA chelates followed the same sequence of metal ions observed with other chelators. Hence, the type of interaction is probably mainly chelating and not ionic. Unfortunately, the capacity of this type of metal chelate was low compared to the other chelates (Table 1).

IDA-sorbents bound via propyl or butyl spacers (methods 2 and 3) displayed significantly higher selectivities for lysine but lower selectivities for histidine and cysteine (Fig. 5a). Zn(II)- or Co(II)-charged chelators of this type as well as the chelator itself did not show higher selectivity for lysine (data not shown). Therefore, the higher retention of lysine cannot be explained on the basis of ionic interactions with remaining silanol groups only. Cu(II)-charged butyl-IDA displayed higher selectivities for tryptophan and phenylalanine (Fig. 5b). Probably synergistic interactions took place of both the chelate and the hydrophobic spacer. Such effects were found with chiral ligand exchangers on the basis of amino acid derivatives as well [34].

4. Discussion

4.1. Properties of metal chelates

Metal chelates are characterised by the interaction of a metal ion with a polydentate ligand, such as IDA or EDTA. Metal ions, belonging to the 3d-block elements, may reach the noble gas shell through attachment of ligands. The stabilization of transition metal chelates is enhanced by inductive charge compensation of the central metal ion by the ligands. This compensation ensues a dispersion of any charge, either localised at the metal ion or the chelator, over the entire structure of the metal chelate, the extent depending on the electronegativity of central metal ion and ligands. With Ca(II), Ba(II) and La(III) such a charge compensation is not possible. As a consequence, these metal chelates displayed different characteristics compared to metal chelates involving transition metal ions. For example, although adsorption of Ca(II) onto IDA-sorbents was confirmed by AAS measurements (data not shown), the IDA:Ca(II)-complex displayed almost identical elution behaviour as IDA without metal ions.

At strong metal chelates the retention of amino acids is explained as follows. Metal ions form chelates with different structures, depending on oxidation state and position in the periodic system. All transition metal ions employed form distorted octahedral structures in water. The distortion depends on both the type of metal ion and the chelator. For example, Cu(II) exhibits quadratic planar structure in a symmetric ligand field, such as plain water; however, it changes to a distorted octahedral structure when complexed by a chelator (Fig. 6), such as IDA (Jahn–Teller distortion [35]). The coordination sites of the metal chelate octaeder are occupied by the polydentate chelator and water molecules. Since water is a weak ligand it can be exchanged by stronger ligands, such as α-amino or carboxyl groups of amino acids and their functional

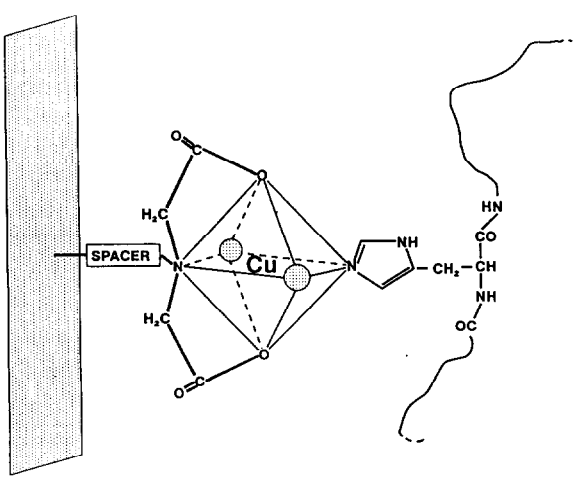

Fig. 6. Octahedral structure of immobilised IDA:Cu(II). Spheres symbolise water ligands. Binding of proteins occurs by exchange of water against imidazole of protein surface located histidines.

groups, ensuing further stabilization of the metal chelate.

After charging with the metal ion a charge might be left at the chelate (Fig. 7), depending on the type of chelator, the oxidation state of the metal ion, and the environment the metal ion is solved in (pH, buffer ions, etc.). Water molecules can be exchanged by OH^- with increasing pH. Therefore, at high pH a neutral or negative charge may develop with IDA when charged with a metal ion. This negative charge might be the reason for stronger interaction with lysine at Cu(II)-charged butyl-IDA and propyl-IDA chelates.

4.2. Immobilization methods

Various methods for the immobilization of IDA and other chelators onto silicas are described in literature [27,36]. Most methods proceed from glymo-activated silicas which allow the binding of IDA through an epoxy group in a similar reaction as described for soft gels [4,37]. Since commonly used silicas are hydrolysed at ph >8, either excess of aliphatic amines are required due to their high pK value or special reaction conditions [36] are employed for reaction with the epoxy group of glymo. However, such reaction conditions may not be disadvantageous from an economic point of view only, but also ecologically, if large quantities of used reagents have to be disposed of. In addition, results from this work demonstrated that even with a large excess of iminodiacetic acid only low ligand densities were obtained, attributed to low reaction rates at the chosen pH (method 4).

On silica-based supports highest ligand densities of low molecular weight ligands are obtained by utilising the silanes of these ligands [38]; but often the chemistry is time consuming employing protection groups [17], as described in this study for the sake of comparison (method 5). In contrast to this, method 6 allows synthesis of the IDA-silane and successive binding onto silica without any further step of isolation. At the chosen pH no condensation of silanol groups takes place in water due to the three negative charges left at the silanol molecule [24]. Accordingly, reactions of primary or secondary amines to the epoxy group of GLYMO can be performed in water under these conditions, as described for other silanes as well [32]. The resulting ligand-silane may be stored in the reaction solution, whereby the time scale depends mainly on the stability of the ligand at high pH. This chemistry is (i) simple and cheap, since no activation of the silica is needed and hydrophilic or ionic silanes are obtained without complicated derivatisation chemistry of the silane, (ii) it is ecologically feasible since very few by-products are obtained which do not need to be separated, (iii) no organic solvents are used, and (iv) high capacities and stable affinity sorbents are obtained by carefully choosing reaction conditions, such as concentration of the silane and pH. This reaction is particularly important for the immobilization of aliphatic amines, demanding high pH during reaction with epoxy groups due to their high pK values. The method allows the formation of homogeneous affinity sorbents as evident from elemental analysis and capacity for Cu(II) on immobilised IDA (Table 2). In addition, silica based metal chelate sorbents displayed negligible non-specific ionic

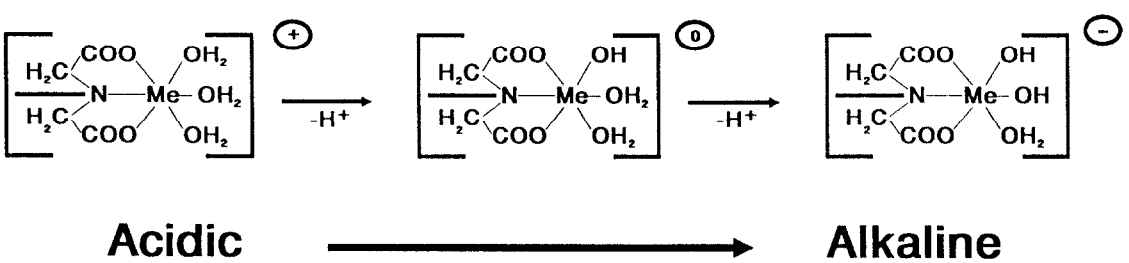

Fig. 7. Appearance of positive and negative charges at metal chelates depending on oxidation state of metal ions and pH. The character of the metal chelate sorbent may shift from an anion exchanger to a cation exchanger.

interaction, comparable to sorbents based on soft gels.

4.3. Chemical neighborhood

The application of GLYMO, epibromohydrin and bisoxirane for the immobilization of IDA involves the reaction of the nitrogen of IDA with the epoxy group of these reagents. As a consequence a hydroxyl group in β position to the tertiary amine evolves at the spacer (Table 3). This β-hydroxyl group is in proper distance to the nitrogen atom to be integrated with the metal chelator (method 4–7 and CS). A similar compound, N-(hydroxymethyl)iminodiacetic acid comprising α-hydroxyl group, is described in literature [2]. The stability constant (lg K) of the Cu(II) complex of this compound is 11.7, compared to 11.1 for the IDA:Cu(II) complex. The integration of a β-hydroxyl group in the chelating complex should be more likely than with α-hydroxyl group, because the distance between the nitrogen and oxygen of the β-hydroxyl group is almost identical to the distance between nitrogen and oxygen of the carboxyl group. The influence of a hydroxyl group located in close proximity to iminodiacetic acid (α-, β- or γ-position to nitrogen) in solution was investigated by Chabarek *et al.* [39]. They concluded that the hydroxyl group is incorporated in the metal chelate, consequently leading to stronger interaction with metal ions. If the β-hydroxyl group of the immobilised IDA is part of the Cu(II) chelate also, the spacer length of epoxy-derived metal chelates is 2 carbon atoms shorter than the immobilized chelator itself. This implies that the spacer of the metal chelate obtained from epibromohydrin-activated Sepharose 4B may contain one carbon atom only. The proposed chemical structure of the Cu(II)-charged chelate is illustrated in Fig. 8. Thus, the name of this type of immobilised chelator should be N-(hydroxyethyl)iminodiacetic acid (HEIDA) rather than iminodiacetic acid (IDA). In contrast, the chelator consists of the immobilised IDA only, when methods 2 and 3 are employed for the immobilization on silicas.

The largest differences in the retention behaviour were observed on the CDI::IDA sorbent. Especially the high selectivity for malonic acid should be considered. It appears that Cu(II) and Ni(II) ions are harder with CDI::IDA than with other chelates, therefore demonstrating higher interactions with carboxylic groups. Probably, the remaining hydroxyl group of the diol spacer is to far away to get involved into the metal chelate. Moreover, through the reaction of the amine with CDI-activated supports a carbamate group is formed. The nitrogen gets amidic character and consequently less nucleophilic than the amine. Due the decrease in electron density at the nitrogen atom the stability constant of the metal chelate should be affected. The structure of this metal chelate cannot be determined from these experiments; therefore, the presumed structure in Fig. 9 should be considered with care.

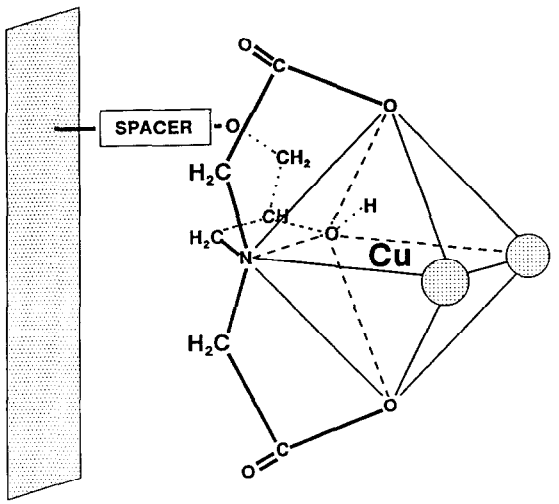

Fig. 8. Proposed octahedral structure of Cu(II)-charged N-(hydroxyethyl)iminodiacetic acid (HEIDA). The β-hydroxyl group at the spacer is in proper distance to the nitrogen for integration in the metal chelate.

5. Conclusions

The different chemistries employed for the immobilization of IDA led to distinct chemical structures in the neighborhood of IDA. As a consequence, the selectivity of formed metal

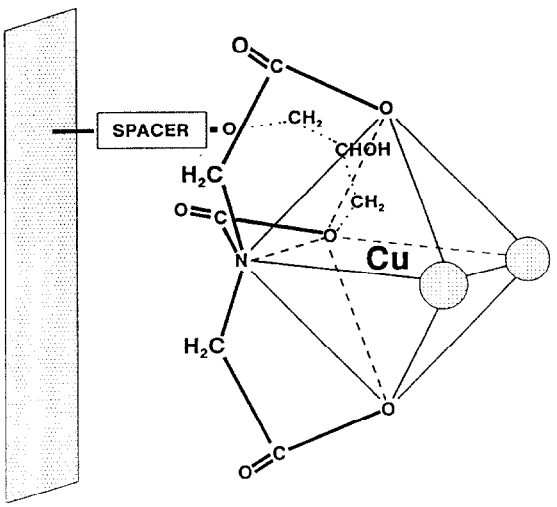

Fig. 9. Presumed structure of Cu(II)-charged CDI::IDA. Maybe other oxygen atoms take part with the metal chelate instead of or in addition to the carbamate oxygen.

chelates for amino acids was influenced. Probably, the changes in selectivity are related to a different structure of these IDA chelates, as proposed in Fig. 8 and Fig. 9.

The different chemical methods yielded the following:

(i) The introduction of reaction method 6 resulted in immobilised metal chelates with high capacity and almost identical chromatographic characteristics compared to soft gel metal chelate sorbents.

(ii) Reaction method 3 results in a butyl-IDA chelator demonstrating high capacity for Cu(II). The Cu(II) chelate demonstrated less selectivity for histidine but increased selectivity for lysine compared to the GLYMO-IDA chelate. Thus, the butyl-IDA chelate may show higher interaction with proteins rich in lysine than common epoxy-derived IDA chelates.

(iii) Reaction method 8 yields a CDI::IDA chelator demonstrating the largest deviation in selectivity compared to CS or GLYMO-IDA. The Cu(II) chelate displayed increased selectivity for acidic amino acids; thus it might be useful for the purification of proteins rich in acidic amino acids.

Most chromatographic protocols obtained from soft gel metal chelate sorbents should be applicable for the silica-based GLYMO-IDA sorbent. The butyl-IDA and CDI::IDA chelates may address specific properties of certain proteins; however, practical chromatographic protocols for the purification of those proteins need to be developed.

Acknowledgments

Financial support was provided by the "Hochschulnachwuchsförderungsprogramm" of the German federal ministry of education and research (Möllemann II).

References

[1] E. Sulkowski, *Trends Biotechnol.*, 3 (1985) 1.
[2] L. Kågedal, in J.-C. Janson and L. Rydén (Editors), *Protein Purification*, VCH Publishers, New York, NY, 1989, p. 231.
[3] G. Lindeberg, H. Bennich and Å. Engström, *Int. J. Peptide Protein Res.*, 38 (1991) 253.
[4] J. Porath and B. Olin, *Biochemistry*, 22 (1983) 1621.
[5] P. Samaraweera, J. Porath and J.H. Law, *Arch. Insect Biochem. Physiol.*, 20 (1992) 243.
[6] E. Hochuli, H. Döbeli and A. Schacher, *J. Chromatogr.*, 411 (1987) 177.
[7] P. Jungblut, H. Baumeister and J. Klose, *Electrophoresis*, 14 (1993) 638.
[8] L. Andersson, E. Sulkowski and J. Porath, *J. Chromatogr.*, 421 (1987) 141.
[9] B.-L. Yang and S. Goto, *Sep. Sci. Technol.*, 26 (1991) 637.
[10] O. Rossetto, G. Schiavo, P.P. Delaureto, S. Fabbiani and C. Montecucco, *Biochem. J.*, 285 (1992) 9.
[11] P. Scanff, M. Yvon and J.P. Pelissier, *J. Chromatogr.*, 539 (1991) 425.
[12] L. Andersson, *J. Chromatogr.*, 539 (1991) 327.
[13] J. Porath, B. Olin and B. Granstrand, *Arch. Biochem. Biophys.*, 225 (1983), 543.
[14] Y. Nakagawa, T.T. Yip, M. Belew and J. Porath, *Anal. Biochem.*, 168 (1988) 75.
[15] P.-O. Larsson, M. Glad, L. Hansson, M.-O. Mansson and K. Mosbach, *Adv. Chromatogr.*, 21 (1982) 41.
[16] M. Belew and J. Porath, *J. Chromatogr.*, 516 (1990) 333.
[17] H.-J. Wirth, K.K. Unger and M.T.W. Hearn, *Anal. Biochem.*, 208 (1993) 16.
[18] H.-J. Wirth, *PhD Thesis*, University of Mainz, Mainz, 1990.

[19] J. Gobet and E.sz. Kováts, *Adv. Sci. Technol.*, 1 (1984) 77.
[20] G.R. Bogart, D.E. Leyden, T.M. Wade, W. Schafer and P.W. Carr, *J. Chromatogr.*, 483 (1989) 209.
[21] G.S. Bethell, J.S. Ayers, M.T.W. Hearn and W.S. Hancock, *J. Chromatogr.*, 219 (1981) 361.
[22] M. Gimpel and K. Unger, *Chromatographia*, 17 (1983) 200.
[23] GBF, *Deutsche Patentanmeldung*, 1993, DE 4217101A1.
[24] C.J. Brinker and G.W. Scherer, *Sol-Gel Science*, Academic Press, London, 1990, pp. 97.
[25] S.H. Chang, K.M. Gooding and F.E. Regnier, *J. Chromatogr.*, 125 (1976) 103.
[26] A.E. Martell and R.M. Smith, *Critical Stability Constants*, Vol. 1, Plenum Press, New York, NY, 1974, p. 1.
[27] Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 359 (1986) 241.
[28] Cs. Horváth and G. Nagydiosi, *J. Inorg. Nucl. Chem.*, 37 (1975) 767.
[29] B. Anspach, K.K. Unger, P. Stanton and M.T.W. Hearn, *Anal. Biochem.*, 179 (1989) 171.
[30] K. Ernst-Cabrera and M. Wilchek, *J. Chromatogr.*, 397 (1987) 187.
[31] G.S. Bethell, J.S. Ayers, W.S. Hancock and M.T.W. Hearn, *J. Biol. Chem.*, 254 (1979) 2572.
[32] M. Gimpel and K. Unger, *Chromatographia*, 16 (1982) 117.
[33] F.B. Anspach and J. Klein, *X. International Symposium on HPLC of Proteins, Peptides, and Polynucleotides, 1990*, Poster No. 119.
[34] P. Roumeliotis, K.K. Unger, A.A. Kurganov and V.A. Davankov, *J. Chromatogr.*, 255 (1983) 51.
[35] H.L. Schläfer and G. Gliemann, *Einführung in die Ligandenfeldtheorie*, Akademische Verlagsgesellschaft, Frankfurt, 1967.
[36] L. Fanou-Ayi and M. Vijayalakshmi, *Ann. N.Y. Acad. Sci.*, 413 (1983) 300.
[37] P.D.G. Dean, W.S. Johnson and F.A. Middle, *Affinity Chromatography: A practical approach*, IRL Press, Oxford, 1985, p. 34.
[38] J.N. Kinkel, B. Anspach, K.K. Unger, R. Wieser and G. Brunner, *J. Chromatogr.*, 297 (1984) 167.
[39] S. Chaberek and A.E. Martell, *J. Am. Chem. Soc.*, 76 (1954), 215.