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Immobilized metal ion affinity chromatography

Effect of solute structure, ligand density and salt concentration on the retention of peptides

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ABSTRACT

The adsorption characteristics of a variety of synthetic peptide hormones and di-, tri- and tetrapeptides on Cu(II) immobilized on two commercially available high-performance chelating gels run under various experimental conditions are described. Methods for determining the concentration of immobilized Cu(II) *in situ* are also described. The Cu(II)-charged columns exhibit a net negative charge as judged from the significantly higher retention of some basic peptides in the absence of NaCl in the equilibration and elution buffers. At higher NaCl concentrations (2–4 *M*), aromatic interactions seem to be superimposed on the metal ion affinity characteristics of the peptides. The relationship between resolution of peptides and the concentration of immobilized Cu(II) ions has also been established for the Chelating Superose gel where 40 μmol Cu(II) ml^{-1} gel apparently gives the optimum resolution. The nature of the gel matrix also plays a role in the resolution of some peptides, the extent of which is difficult to predict. The results obtained also suggest that peptides containing aromatic and hydroxy amino acids are retarded more than those which lack them. Moreover, these same amino acids apparently strengthen the existing strong binding of peptides containing His, Trp or Cys to a Chelating Superose–Cu(II) column. Dipeptides with C-terminal His (*i.e.*, X-His) are neither bound nor retarded on a column of Chelating Superose–Cu(II) whereas those having the structure His-X are strongly bound. Some tri- and tetrapeptides containing His were also found not to bind to the column. The underlying cause of this anomalous adsorption behaviour is discussed

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and is ascribed to "metal ion transfer" arising from the relatively higher affinity of such peptides towards immobilized Cu(II) ions than the chelator groups (iminodiacetate) which are covalently bound to the gel matrix.

INTRODUCTION

The introduction of commercial high-performance immobilized metal ion affinity (HP-IMA) adsorbents by Toyo Soda (Tokyo, Japan) and recently by Pharmacia LKB (Uppsala, Sweden) has made possible high-performance liquid chromatography (HPLC) applications of this versatile separation method to the high resolution of model proteins[1,2], the iso-forms of human platelet-derived growth factor[3] and synthetic peptide hormones and their analogues[4]. Whereas His, Cys and Trp residues in proteins are postulated to be the primary sites of interaction with immobilized transition metal ions, *i.e.*, 3d-block elements from Co to Zn[5,6], the results of Nakagawa *et al.*[4] suggest that the side-chains of amino acids other than these three can lead to a considerable retardation of some peptides. This might be due to the higher solvent accessibility of most or all of the constituent amino acids in the peptide hormones studied, suggesting that, at least with such low-molecular-weight solutes, their relative retentions might be predicted on the basis of their sequence (composition) and the published retention spectra[7] for α-amino acids on iminodiacetic acid (IDA)–Ni(II). In addition, the data published by Chothia[8] on the relative surface location of amino acid side-chains in a number of proteins might further contribute to the understanding of the relationship between structure and the experimentally found retention times of a series of peptides on an IMA adsorbent.

Preliminary studies on the effects of various salts, temperature and pH on the retention of amino acids[7] and oligopeptides[9] indicated that the coordination of these solutes with immobilized metal ions (IMI) can be significantly modulated by varying the experimental conditions. An extension of this work to proteins[10] indicated that high concentrations of anti-chaotropic salts (*e.g.*, sulphates) promote the retention of proteins on IMA adsorbents by a mechanism that is similar to that operating in hydrophobic interaction chromatography (HIC). However, a recent report by Sulkowski[11] showed that such an effect is variable and dependent on the nature of the sample solute in question, enhancing the retention of acidic proteins and markedly decreasing that of basic proteins. Thus, the chromatographic parameters which can have a bearing on the retention of biomolecules on IMA adsorbents is apparently complex but if the partial contributions of the various parameters could be established with certainty, the versatility of IMA methods could be further appreciated and fully exploited to solve a variety of separation problems. The results presented in this paper are a step in this direction with emphasis on structure–retention aspects in immobilized metal ion affinity chromatography (IMAC) of a series of amino acids and di- and oligopeptides run on Cu(II) ions immobilized on Chelating Superose or TSK gel chelate-5PW. Methods for the determination of the total ligand concentration in IMA adsorbents are outlined, in addition to the relationship between ligand concentration and the extent of retention of a series of peptides. We have also obtained some anomalous adsorption behaviour of His-containing peptides and plausible explanations for our observations are outlined. Finally, the effect of varying the salt

concentration in the eluent buffer on the retention of some selected peptides is reported and compared with previously reported findings.

EXPERIMENTAL

Chelating Superose (bead size *ca.* 10 μm), with a capacity for Zn(II) of 18, 26 and 31 μmol ml^{-1} gel, was kindly provided by Drs. L. Kågedal and M. Sparrman, and TSK gel chelate-5PW (bead size 10 μm and ligand concentration *ca.* 20 μmol ml^{-1} swollen gel) was a generous gift from Eng. Rolf Ehrnström (all of Pharmacia LKB). The chelating gels were provided ready packed in glass columns (75 × 8 mm I.D., bed volume 3.77 ml for the TSK chelate-5PW gel; 21 × 10 mm I.D., bed volume 1.65 ml and three other columns with I.D. 5 mm and lengths of 53, 56 and 60 mm for the Chelating Superose gel) and each is designed for HPLC applications. The chelating groups in Chelating Superose are IDA whereas those in TSK are not specified by the manufacturer but most likely are IDA also.

Individual amino acids and the dipeptide L-carnosine were obtained from Calbiochem (San Diego, CA, U.S.A.). The synthetic peptide hormones and di-, tri- and tetrapeptides were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). Gastrin-related peptide, Leu-enkephalin (sulphated) and neurotensin (residues 3–13) were a generous gift from Dr. Y. Nakagawa (Peptide Institute, Osaka, Japan). With few exceptions, the peptides were of the highest purity as judged from the chemical analysis data provided by the manufacturers. When deemed necessary, the composition of some peptides was checked by amino acid analysis. Analytical-reagent grade imidazole and EDTA (disodium salt) were obtained from E. Merck (Darmstadt, F.R.G.). All other chemicals were of analytical-reagent or reagent grade and were used as received.

The experiments were performed at room temperature (20°C) using a Pharmacia LKB HPLC system consisting of a pump (Model 2150), a variable-wavelength monitor (Model 2151), a controller (Model 2152), a two-channel recorder (Model 2210) and a Helirac fraction collector (Model 2212). Unless stated otherwise, the following buffers were used as eluents and will be referred to in abbreviated form throughout: buffer A (equilibration buffer), 20 m*M* sodium phosphate, 1.0 *M* in NaCl, pH 7.0; and buffer B (final gradient buffer), 0.1 *M* sodium phosphate, 1.0 *M* in NaCl, pH 3.8. Each buffer was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) and deaerated prior to use. However, in those experiments where the TSK gel chelate-5PW column was used, the concentration of NaCl in each of the buffers was reduced to 0.5 *M*.

Immobilization of Cu(II) ions[2,4,12]

Each column was regenerated by washing with three column volumes of a 50 m*M* solution of EDTA dissolved in buffer A (pH adjusted to 7.0) followed by washing with five column volumes of distilled water. Depending on the size of the column, 0.3–1.0 ml of a 0.2 *M* aqueous solution of $CuSO_4 \cdot 5H_2O$ (pH 3.9) was applied to the regenerated column followed by washing sequentially with five column volumes of distilled water, ten column volumes of buffer B [to elute weakly bound Cu(II) ions], five column volumes of distilled water and finally ten column volumes of buffer A for equilibration. The effluent was monitored by continuously recording the absorbance

at 220 nm with the detector set at 0.64 absorbance units full-scale (a.u.f.s.). This procedure resulted in a stable baseline absorbance of the effluent and the column was then ready for use.

Chromatography

Solutions of the individual amino acids or peptides (1 mg per 100 μl) were prepared in glass-distilled water, except in a few instances where small amounts of organic solvents or dilute NaOH were added to effect their dissolution. A mixture of the peptides was prepared by mixing together appropriate aliquots taken from the solutions of each peptide. Usually, about 5–10 μg of each amino acid or peptide (in 1 μl of solution), or an amount ranging from 2 to 12 μg of each peptide in the mixture (in 10 μl of solution), was applied to the equilibrated column for each chromatographic experiment. The flow-rate was maintained at 0.94 ml min^{-1} except for the Chelating Superose (CS) columns with an I.D. of 5 mm, where it was necessary to reduce it to 0.47 ml min^{-1}.

After sample application, the columns were eluted using one of two alternative procedures which are essentially similar to those developed by Nakagawa *et al.*[4]. The CS–Cu(II) columns were eluted for 15 min with buffer A followed by a programmed gradient elution from buffer A to buffer B as follows: 15–30 min, 15% B; 30–40 min, 80% B; and 40–70 min, 100% B. The TSK–Cu(II) column was eluted for 10 min with buffer A followed by a programmed gradient elution from buffer A to buffer B as follows: 10–20 min, 15% B; 20–35 min, 80% B; and 35–60 min, 100% B.

Determination of the concentration of immobilized ligand

Direct method. The Cu(II)-charged column, prepared as described above, is washed with sufficient EDTA solution (50 m*M* dissolved in buffer A, pH 7.0) to elute the immobilized Cu(II) ions. The effluent is collected in a 25- or 50-ml volumetric flask and diluted to the mark with the above EDTA solution. The total amount of Cu(II) ions in the eluate is then determined by atomic absorption spectrometry (AAS) using the EDTA solution as blank.

Frontal analysis[12,13]. Before connecting the column to the system, it is important that the buffer delivery lines and injection loop are filled with the stock solution of Cu(II) or imidazole. A 20 m*M* aqueous solution of $CuSO_4 \cdot 5H_2O$ (pH 3.9) is fed continuously to the regenerated column and the absorbance at 280 nm of the effluent is recorded until a constant plateau level equal to that of the stock solution being fed to the column is reached. The elution volume is then determined from the median bisector of the front of the elution profile. The internal volume of the column is determined from the elution volume of 1 *M* acetic acid (2 μl) injected onto the column. The amount of immobilized Cu(II) ions is calculated according to the empirical equation given under Results and Discussion.

Alternative frontal analysis. An alternative to the previous method is the frontal analysis of a solution of 20 m*M* imidazole dissolved in buffer A on a CS–Cu(II) or TSK–Cu(II) column following the previous procedure. The amount of imidazole bound to the immobilized Cu(II) ions is also calculated as above.

Amino acid analysis

Suitable aliquots of peptides were hydrolysed in evacuated and sealed tubes for

24 h at 110°C with 6.0 *M* HCl. The hydrolysates were analysed according to standard procedures.

Detection of peptides

This was routinely performed by continuously recording the absorbance at 220 nm of the chromatographic effluents or by the ninhydrin method after alkaline hydrolysis[14] of collected fractions. The latter procedure is used occasionally to distinguish peptide peaks from those generated by inorganic salts or impurities which absorb at 220 nm.

Analysis of data

Owing to the continuous development of the Chelating Superose columns during the course of this investigation, two different sizes (with I.D. 5 and 10 mm) were used. The retention of the peptides on the different columns is therefore expressed in terms of V_e/V_t as this treatment will facilitate comparisons of the results obtained on different columns.

RESULTS AND DISCUSSION

Concentration of immobilized ligand

There are four possible methods for determining the ligand concentration in such gels, *viz.*, by elemental nitrogen analysis and by the three procedures outlined under Experimental. The results obtained by the last three methods are presented here. Fig. 1 shows the frontal analysis curves of Cu(II) on CS and TSK columns where the elution fronts obtained are sharp, indicating uniformity of packing of the columns and an even distribution in the size of the small-particle gels. Based on the elution volumes obtained, the capacity of each column for Cu(II) was calculated using several alternative equations. Of these, the following gave results that agreed consistently with those determined by AAS (see Table I):

$$\text{Amount of Cu(II) immobilized } (\mu\text{mol ml}^{-1}) = \frac{(V_e - V_i)C}{V_t}$$

where

V_e = elution volume of the Cu(II) solution;
C = concentration of the Cu(II) solution fed to the column (μmol ml^{-1});
V_i = internal volume of the column [*i.e.*, void volume + gel volume that is available to acetic acid and presumably also to $Cu^{2+}(H_2O)_n$]; and
V_t = total volume of the column.

The values obtained by AAS and frontal analysis differ by about $\pm 10\%$ (Table I), except for the CS gel with the lowest ligand substitution where the variation is about 25%. This large variation might be due to the elution of a high percentage of the immobilized Cu(II) ions by buffer B from the latter gel prior to elution of the tightly bound Cu(II) ions by EDTA for the subsequent determination of its concentration by AAS.

The results obtained by frontal analysis of imidazole on CS–Cu(II) columns are in good agreement with those obtained above but not so on the TSK–Cu(II) column

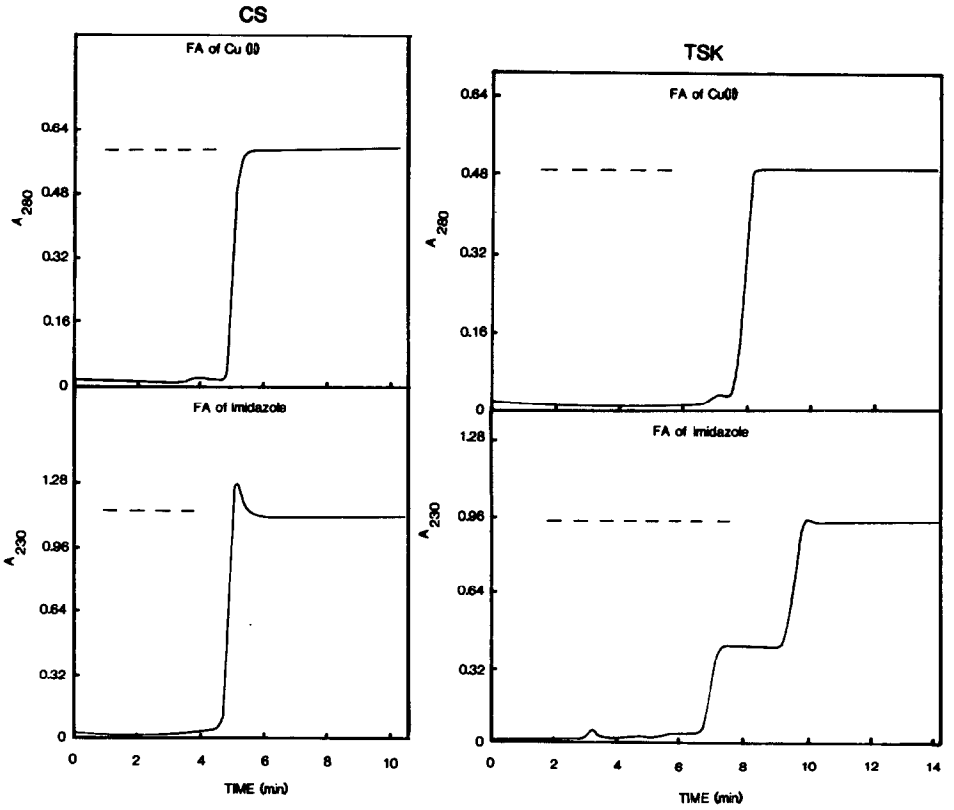


Fig. 1. Frontal analysis of Cu(II) on columns of Chelating Superose [21 × 10 mm I.D.; capacity for Zn(II) = 31 μmol ml^{-1}] and TSK gel chelate-5PW (75 × 8 mm I.D.) and of imidazole on each column after they have been fully charged with Cu(II) ions. The flow-rate in each instance was 0.94 ml min^{-1}. The internal volume (V_i) of the CS column was 1.32 ml and of the TSK column 3.00 ml. For further details, see *Frontal analysis* under Experimental.

(Fig. 1, Table I). In the latter, the imidazole elutes as a double front, indicating heterogeneity in the adsorption sites themselves [*i.e.*, immobilized Cu(II) ions]. Similar results were obtained when two other TSK–Cu(II) columns were tested, although in one of them the boundary between the first and second fronts was not as sharp as that shown in Fig. 1. These results cannot be ascribed to variations in the accessibility of the immobilized Cu(II) ions to such a small molecular sized solute as imidazole (which is also of analytical-reagent grade) but rather to variations in the manner in which the Cu(II) ions are immobilized to the chelator. This in turn implies that there are possibly two species of chelating ligands that are covalently attached to the matrix, one of which chelates the Cu(II) ions by three coordination sites (as in IDA) and the others not. Another plausible explanation can be ascribed to the matrix itself whose structure, whatever its nature, directly affects the uniform availability of the immobilized Cu(II) ions. In either instance, such heterogeneity in the adsorption sites might affect the separation of peptides and proteins and might account for some of the variations in the resolution of peptides we have obtained on the CS- and TSK-based IMA adsorbents.

TABLE I

CAPACITY FOR Zn(II) AND Cu(II) OF CHELATING SUPEROSE (WITH VARYING DEGREES OF LIGAND SUBSTITUTION) AND TSK GEL CHELATE-5PW AS DETERMINED BY SEVERAL ALTERNATIVE PROCEDURES

The Chelating Superose gels are divided into three groups on the basis of their capacity for Zn(II) ions as specified by the manufacturer. The data based on atomic absorption spectrometry and frontal analysis are averages calculated from three independent experiments performed on each gel according to the procedures outlined in detail in the text.

Chelating gel	*Capacity for M(II) (μmol ml^{-1} gel)*			
	Zn(II)[a]	*Cu(II)*[b]	*Cu(II)*[c]	*Imidazole*[c]
Chelating Superose (i)	18	15	19	20
Chelating Superose (ii)	26	30	32	34
Chelating Superose (iii)	31	42	39	41
TSK gel chelate-5PW[d]	–	23	23	20(32)[e]

[a] Determined by the manufacturer (Pharmacia LKB).

[b] Determined by atomic absorption spectrometry.

[c] Determined from frontal analysis data using the empirical equation given in the text.

[d] According to the manufacturer (Toyo Soda) the concentration of chelating groups is about 20 μmol ml^{-1} gel.

[e] Two different values are reported as a result of the two elution fronts obtained on this gel (see Fig. 1).

Of additional interest is the relatively lower capacity for Zn(II) compared with Cu(II) of all three CS gels examined. Smith *et al.*[15] also obtained similar results using Sephadex- and Sepharose-based chelating gels on the basis of which the capacities of the gels for metal ions can be arranged in the order Cu(II) > Ni(II) > Zn(II) > Co(II). These findings agree well with the reported stability constants for complex formation, in free solution, between these metal ions and diaminopropionic acid, amino acids, etc.[16]. The affinities of proteins for immobilized metal ions also follow the order given above, as show previously[2], suggesting that a good correlation exists between the affinities of IMI towards various solutes and their stability constants in free solutions.

Ion-exchange properties

Chelating Superose and TSK gel chelate-5PW behave as cation exchangers at or around neutral pH when free of metal ions. This is illustrated by the results in Fig. 2, where four basic peptide hormones are separated on the basis of their net charge by chromatography on a CS or TSK column equilibrated with 20 m*M* sodium phosphate buffer (pH 7.0). Three of the peptides are separated by isocratic elution whereas the most basic peptide (Met-Lys-bradykinin) can be eluted with 0.5 *M* NaCl in the equilibration buffer. Thus, the metal-free gels have a net negative charge at neutral pH. At lower pH, where the ionization of the two carboxyl groups is sufficiently suppressed, it is possible that the immobilized IDA groups will have a net positive charge and could thus exibit anion-exchange properties. In either instance, the low degree of substitution of these gels limits their use as ion exchangers for peptide separations. Inclusion of 0.5 *M* NaCl in the equilibration buffer abolished such ionic interactions and none of the peptides is bound by either of the gels.

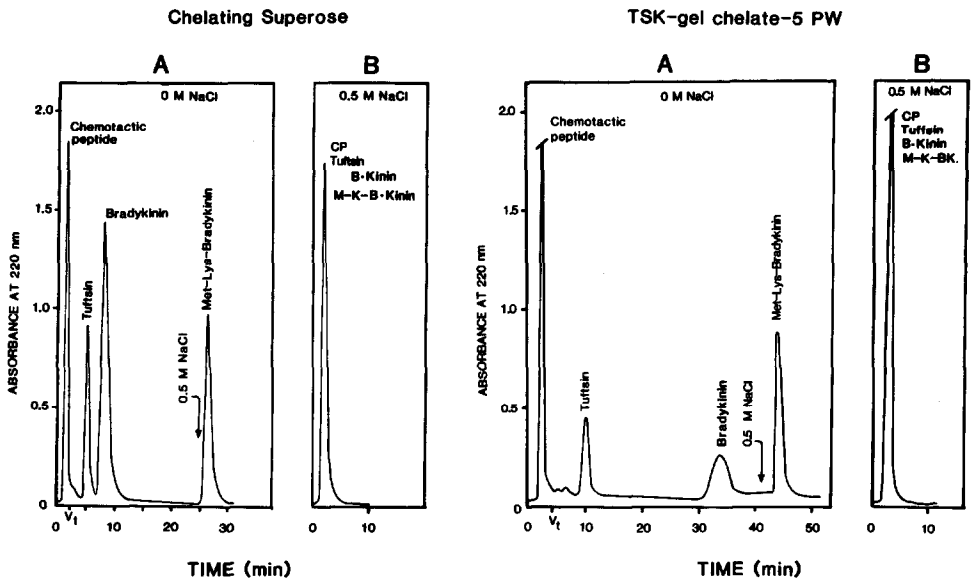


Fig. 2. (A) Cation-exchange characteristics of naked (metal ion-free) columns of Chelating Superose [54 × 5 mm I.D.; V_t = 1.06 ml; capacity for Zn(II) = 31 μmol ml^{-1}] and TSK gel chelate-5PW (75 × 8 mm I.D.; V_t = 3.77 ml). To each column (equilibrated with 20 m*M* sodium phosphate buffer, pH 7.0) were applied 20 μl of the sample solution, containing 100 μg of each of the four peptides, followed by isocratic elution with the equilibration buffer. The flow-rate was 0.47 ml min^{-1} for the CS column and 0.94 ml min^{-1} for the TSK column. The order of elution of the peptides from the two columns was established from individual runs with each peptide. Note that Met-Lys-bradykinin is strongly bound on each column, requiring the use of 0.5 *M* NaCl in the equilibration buffer to elute it. (B) Elution profile obtained when the same mixture of the four peptides was chromatographed on each of the above two columns equilibrated with 20 m*M* sodium phosphate buffer containing 0.5 *M* NaCl (pH 7.0). None of the peptides was bound by either column under these conditions.

Bradykinin is retarded much more on the TSK gel (V_e/V_t = 8.6) than on the CS gel (V_e/V_t = 3.5), which cannot be accounted for by differences in the concentrations of immobilized IDA groups which are, in fact, lower in the TSK gel than in the CS gel (see Table I). Considering the primary structure of bradykinin (see Table III), it is reasonable to propose that the two Phe residues participate in non-ionic interactions (probably of aromatic or hydrophobic character) with the TSK gel chelate-5PW matrix itself.

Effect of varying the salt concentration

Throughout this study, the equilibration and elution buffers contained 0.5 or 1.0 *M* NaCl. It was therefore appropriate to investigate the effects of varying the salt concentration on the resolution of peptides and proteins using the above four peptides as test solutes. Under the standard IMA chromatographic conditions adopted, these peptides are eluted isocratically from both the CS–Cu(II) and TSK–Cu(II) columns because none of them contains His, Trp or Cys residues for strong binding to the immobilized metal ions. Such a study can, of course, be extended to include neutral and acidic peptides and those containing His, Trp or Cys, but we feel that the results obtained here serve as a basis for future explorations in this area.

TABLE II

EFFECT OF VARYING THE CONCENTRATION OF NaCl (0–4.0 *M*) IN THE EQUILIBRATION BUFFER ON THE RELATIVE RETENTION VOLUMES (V_e/V_t) OF THE FOUR PEPTIDE HORMONES ON COLUMNS OF CS–Cu(II) OR TSK–Cu(II)

The data were calculated from the chromatograms shown in Fig. 3.

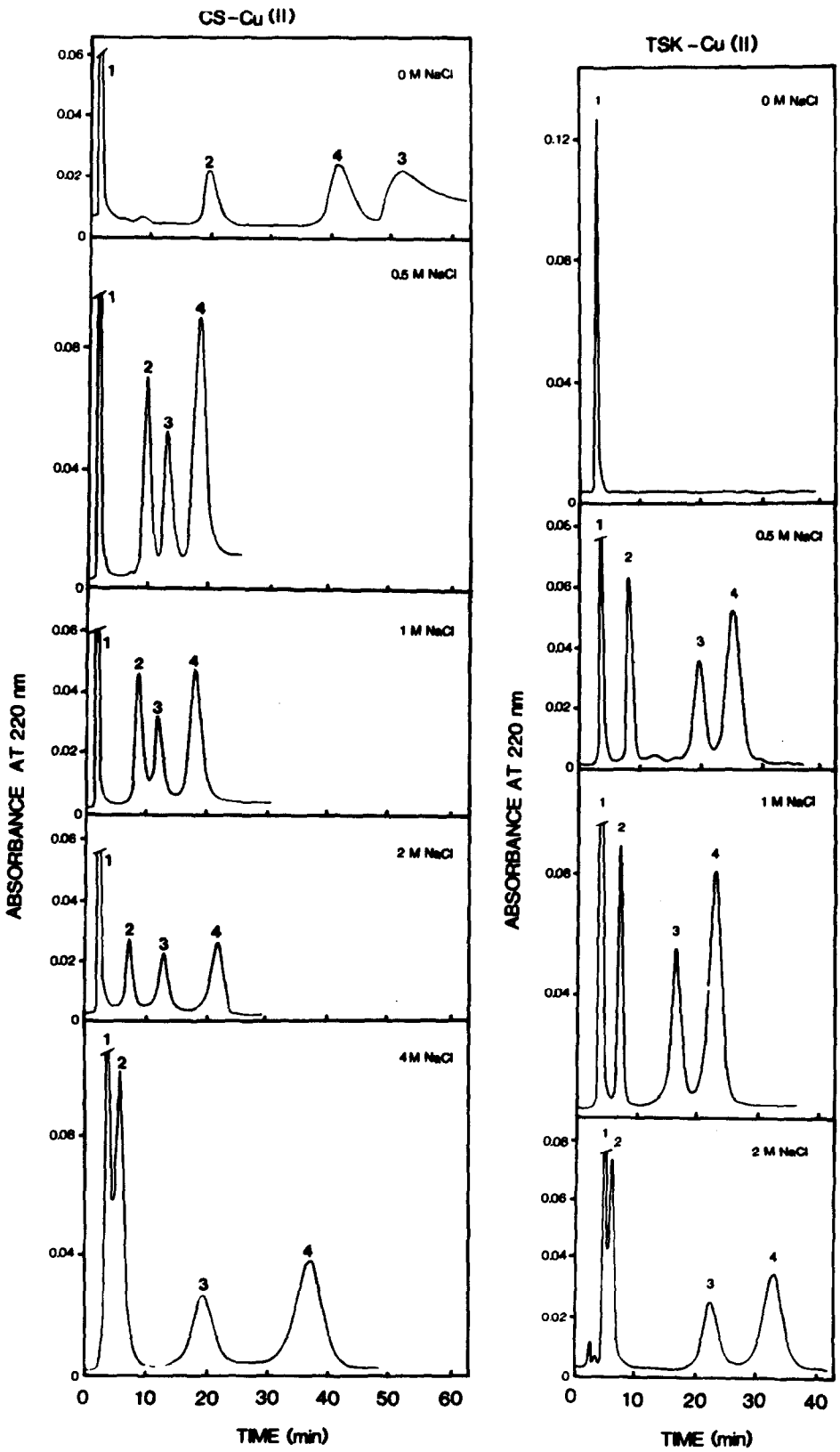
Peptide	*Chelator gel*	V_e/V_t				
		0 M	*0.5 M*	*1.0 M*	*2.0 M*	*4.0 M*
Chemotactic peptide	CS	0.6	0.7	0.7	0.8	1.5
	TSK	0.7	1.0	0.9	1.3	n.d.[a]
Tuftsin	CS	7.5	3.7	3.4	2.7	2.2
	TSK	n.e.[b]	2.2	1.8	1.6	n.d.
Met-Lys-bradykinin	CS	19.2	5.0	4.7	4.8	7.3
	TSK	n.e.	5.0	4.2	5.7	n.d.
Bradykinin	CS	15.5	6.9	6.9	8.2	13.6
	TSK	n.e.	6.4	5.7	8.3	n.d.

[a] n.d. = Not determined.

[b] n.e. = Not eluted after washing with 20 column volumes of the equilibration buffer.

In the absence of NaCl in the equilibration buffer, these peptides are much more retarded on CS–Cu(II) than on the corresponding metal-free gel but they can still be eluted under isocratic conditions (Fig. 3 and Table II). The order of elution of the peptides is also the same as in Fig. 2, indicating that the apparent anion-exchange characteristics of the CS gel are accentuated by the immobilized Cu(II) ions. A different situation arises on the TSK–Cu(II) column, where all except chemotactic peptide are so strongly adsorbed that they are not eluted by the equilibration buffer (Fig. 3 and Table II). Hence, the ion-exchange characteristics of the TSK gel are even more accentuated by the immobilized Cu(II) ions. Both of these results strengthen the hypothesis that the immobilized metal ions have a net negative charge[7,11] at neutral pH, suggesting that the basis for the adsorption of basic peptides and proteins to immobilized metal ions is of a bimodel character and can thus be manipulated by varying the NaCl concentration in the elution buffer to optimize the isolation of a particular solute in a mixture.

In the presence of 0.5 *M* and higher concentrations of NaCl in the equilibration buffer, the apparently predominant ionic interactions are suppressed or essentially eliminated. Thus, Met-Lys-bradykinin elutes before bradykinin from the CS–Cu(II) column, contrary to what was found above, and the separation of the peptides here appears to be governed primarily by their differential affinities towards the immobilized Cu(II) ions. Almost comparable resolutions of the peptides are also obtained on both the CS–Cu(II) and TSK–Cu(II) columns in the presence of 0.5 or 1 *M* NaCl in the elution buffers (see Fig. 3 and Table II). However, at even higher concentrations of NaCl [2 *M* for TSK–Cu(II) and 4 *M* for CS–Cu(II)] a different kind of interaction seems to prevail, leading to a stronger retention of all but one (tuftsin) of the four peptides (see Fig. 3 and Table II). As these three peptides have Phe as a common residue in their side-chains (see Table III for their structure), the additional


CS-Cu (II)
TSK-Cu (II)
0 M NaCl
0.5 M NaCl
1 M NaCl
2 M NaCl
4 M NaCl
ABSORBANCE AT 220 nm
TIME (min)
1
2
3
4

or secondary interactions seem to be of an aromatic/hydrophobic character. That bradykinin is retarded much more than Met-Lys-bradykinin suggests that lysine (a charged hydrophilic amino acid) has a modulating effect on the "hydrophobicity" of this peptide. This hypothesis is consistent with that suggested by Hemdan and Porath[9] based on the adsorption characteristics of di- and tripeptides on an IDA–Ni(II) column. A comparable result has also been obtained by Nakagawa *et al.*[4], where [Leu^5]-enkephalin is eluted much later than sulphated [Leu^5]-enkephalin from a TSK–Cu(II) column, lending further support to the proposed hypothesis.

Effect of ligand concentration

The elution profile of eleven synthetic peptide hormones from three CS–Cu(II) adsorbents with various ligand concentrations is shown in Fig. 4A. The best resolution of the peptides is obtained on the column with the relatively highest ligand concentration [capacity for Zn(II) = 31 μmol ml^{-1} gel], irrespective of whether the peptides are tightly bound or not. With decreasing ligand concentration, the resolution also decreases and in the case of peptides 8 and 9 (angiotensin III and II, respectively) they coelute from the column with the lowest ligand concentration. The pH profiles obtained on the three columns are identical and thus cannot account for the variations in the resolution obtained here. Moreover, it has also been observed[17] that the resolution of proteins was markedly decreased as the capacity of the gel for Zn(II) was increased from 31 to about 40 μmol ml^{-1}. It seems, therefore, that the optimum resolution of peptides and proteins is obtained on the CS–Cu(II) adsorbent with a capacity for Zn(II) of about 31 μmol ml^{-1} [or about 40 μmol ml^{-1} for Cu(II)], as is also evident from the family of curves shown in Fig. 4B. The effect of the column dimensions on the resolution of the peptides was also investigated using two columns with dimensions of 54 × 5 mm I.D. and 21 × 10 mm I.D. The results indicated that the longer the column, the better is the resolution of peptides that elute under isocratic conditions. However, those peptides which are eluted in the gradient are not markedly affected.

For comparison, the same mixture of peptides was chromatographed on a TSK–Cu(II) column (Fig. 5). The resolution of the peptides obtained here is essentially comparable to that obtained on the CS–Cu(II) column [capacity for Zn(II) = 31 μmol ml^{-1}] with the exception that peptides 4, 5, 6 and 9 are markedly

Fig. 3. Effect of varying the concentration of NaCl in the equilibration buffer on the retention times of the four peptide hormones shown in Fig. 2 on a column of CS–Cu(II) (54 × 5 mm I.D.; V_t = 1.06 ml) or TSK–Cu(II) (75 × 8 mm I.D.; V_t = 3.77 ml). In each instance, 2 μl of a solution of the four peptides in distilled water (containing 5 μg of each peptide) were applied to the columns equilibrated with 20 m*M* sodium phosphate buffer containing the indicated concentrations of NaCl (pH 7.0). For the CS–Cu(II) column the flow-rate was 0.47 ml min^{-1}, except when the equilibration buffer contained 4 *M* NaCl, when it was necessary to reduce it to 0.37 ml min^{-1} owing to increased back-pressure. For the TSK–Cu(II) column, the flow-rate was 0.94 ml min^{-1} throughout. The ligand concentration [based on Cu(II)] of the Chelating Superose gel was 42 μmol ml^{-1} and of the TSK gel chelate-5PW 23 μmol ml^{-1}. The peptide mixture was chromatographed under isocratic conditions throughout. 1, Chemotactic peptide; 2, tuftsin; 3, Met-Lys-bradykinin; 4, bradykinin. Note that Met-Lys-bradykinin is much more retarded than bradykinin in the absence of NaCl in the equilibration buffer. Inclusion of 0.5–4 *M* NaCl in the equilibration buffer reverses their elution positions.

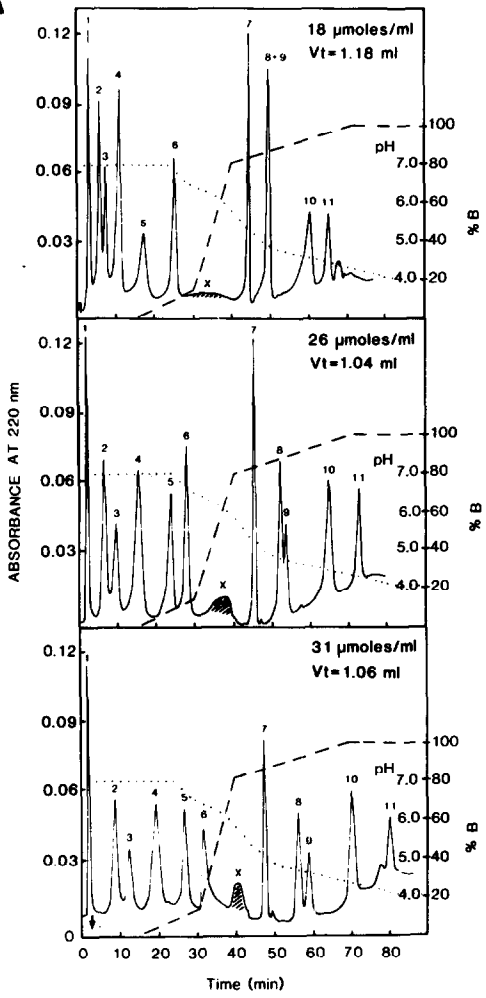
TABLE III

RELATIVE RETENTION VOLUMES (V_e/V_t) AND ELUTION pH FOR A SERIES OF SYNTHETIC PEPTIDE HORMONES AFTER HP-IMAC ON CS–Cu(II) (21 × 10 mm I.D.; V_t = 1.65 ml) AND TSK–Cu(II) (75 × 8 mm I.D.; V_t = 3.77 ml) COLUMNS

For experimental details see text. In calculating the V_e/V_t ratios, appropriate corrections were made for the "dead volume" in the connecting tubes. With few exceptions, the purity of each peptide is given as 95% or better by the manufacturers. The elution profile of peptides 1–11 are shown in Figs. 4A and 5.

No.	*Peptide*	*Primary structure*[a]	*CS–Cu(II)*		*TSK–Cu(II)*	
			V_e/V_t	*pH*	V_e/V_t	*pH*
1	Chemotactic peptide	For-Met-Leu-Phe	0.9	7.0	0.8	7.0
2	Tuftsin	Thr-Lys-Pro-Arg	3.5	7.0	1.8	7.0
3	Met-Lys-bradykinin	Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	4.4	7.0	4.7	6.9
4	Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	6.8	7.0	5.5	6.5
5	Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	11.7	6.8	7.3	6.0
6	Somatostatin	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys	14.4	6.5	8.3	5.6
7	Thyrotropin-releasing hormone (TRH)	PyroGlu-His-Pro-$NH_2 \cdot H_2O$	25.2	5.0	10.8	4.9
8	Angiotensin III (human)	Arg-Val-Tyr-Ile-His-Pro-Phe-Ac-OH $\cdot 4H_2O$	29.3	4.7	12.1	4.8
9	Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	30.4	4.7	14.4	4.3
10	Luteinizing hormone-releasing hormone (LH-RH)	PyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	35.1	4.4	14.7	4.3
11	Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	38.9	4.2	17.2	3.8
12	[Tyr[8]]-substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Tyr-Gly-Leu-Met-NH_2	11.7	6.8		
13	Physaelamine	PyroGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2	0.9	7.0		
14	Neurotensin (bovine)	PyroGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	0.9	7.0		
15	Neurotensin (residues 3–13)	Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	2.5	7.0		
16	Liver cel growth factor	Gly-His-Lys	1.5	7.0		
17	Eledoisine-related peptide	Lys-Phe-Ile-Gly-Leu-Met-NH_2	4.5, 6.5	7.0		
18	Gastrin-related peptide	AoC-Trp-Met-Asp-Phe-NH_2	3.0, 7.0	7.0		
19	MSH-release inhibiting factor (MIF)[b]	Pro-Leu-Gly-$NH_2 \cdot \frac{1}{2}H_2O$	5.3			
20	Delta sleep-inducing peptide (DSIP)	Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu	2.7, 3.8, 6.2	7.0		
21	Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	5.3	7.0		
22	[Leu[5]]-enkephalin	Tyr-Gly-Gly-Phe-Leu	6.4	7.0		
23	Tyr-bradykinin	Tyr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	8.2	7.0		
24	Des-Arg[9]-bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	7.3	7.0		

[a] For = Formyl; PyroGlu = pyroglutamic acid; AoC = *tert.*-amyloxy-carbonyl; Ac = acetyl.
[b] MSH = melanocyte-stimulating hormone.



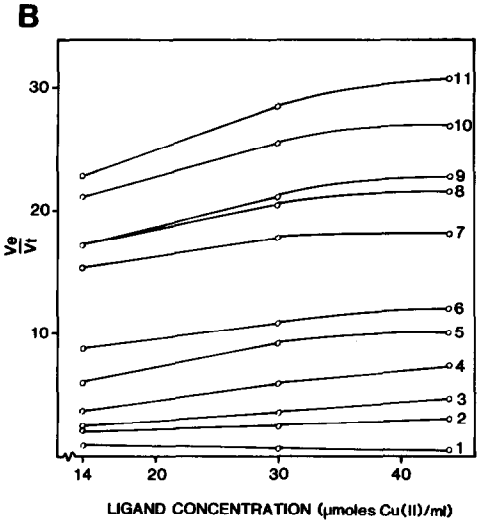


Fig. 4. (A) Effect of varying the concentration of immobilized ligand [Cu(II)] on the resolution of a mixture of eleven synthetic peptide hormones on columns of CS–Cu(II). The total volume and capacities for Zn(II) of each column (I.D. 5 mm) are shown. The actual concentration of immobilized Cu(II) ions on each of the columns is given in Table I and the names and structures of the peptides are given in Table III. The flow-rate was maintained at 0.47 ml min^{-1} and the chromatogram was developed by a programmed gradient elution procedure as described. (B) Effect of varying the concentration of immobilized ligand [Cu(II)] on the retention (expressed as V_e/V_t) of the eleven peptides shown in (A). Note that the retention volumes and the resolution of the peptides increase with increase in the concentration of immobilized ligand.

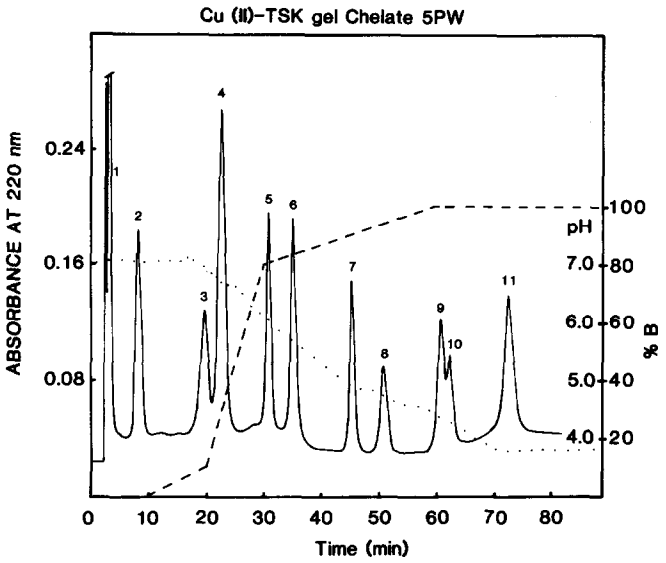


Fig. 5. Chromatography of the eleven peptide hormones shown in Table III and Fig. 4A on a column (75 × 8 mm I.D.; V_t = 3.77 ml) of TSK–Cu(II). The chromatogram was developed by a programmed gradient elution procedure as described at a flow-rate of 0.94 ml min^{-1}. Note that (i) the order of elution of the peptides is the same as on the CS–Cu(II) column shown in Fig. 4A; (ii) peptides 4, 5, 6 and 9 are more retarded on this column than on the corresponding CS–Cu(II) column (*cf.*, Fig. 4A and Table III), reflecting the influence of the polymer matrix; and (iii) peptides 9 and 10 elute close to each other, in contrast to the situation in Fig. 4A.

retarded on this column as judged from a comparison of the pH at which they elute from the two columns (see Table III). This might be due to a significant contribution from secondary interactions between the TSK–Cu(II) adsorbent and these solutes, probably arising from their higher content of aromatic amino acids.

Retention spectra of amino acids

The results obtained are compiled in Table IV, and show that all the amino acids are strongly retarded or bound by CS–Cu(II), although there are large variations in the strength of their binding. Of particular interest is the non-binding of β-alanine to CS–Cu(II) (Table V), indicating that the α-amino nitrogen of amino acids is of primary importance for their binding to IMAC adsorbents. It is therefore apparent that, for all the amino acids, their α-NH_2 nitrogens and their carboxyl groups participate to form a bidentate coordination bond with IDA–Cu(II) whereas His, Cys and Trp are bound by an extra coordination bond to form a stronger tridentate complex. The aliphatic basic and acidic amino acids including Pro and Ser are significantly retarded on the column but elute at the beginning of the pH gradient (in the pH range 7.0–6.5). The remaining amino acids are more strongly bound and elute in the pH range 6.5–4.5. Of these, the hydroxy-containing (Thr, Pro-OH) and aromatic (Phe, Tyr) amino acids and also Asn and Met are eluted earlier than the most strongly bound amino acids of all, *viz.*, Trp, Cys and His. These results differ markedly from those reported for IDA–Ni(II)[7], where all except Trp, Cys and His had V_e/V_t values less than 10. Of

TABLE IV

RELATIVE RETENTION VOLUMES OF THE NATURALLY OCCURRING AMINO ACIDS OBTAINED AFTER HP-IMAC ON A CHELATING SUPEROSE–Cu(II) COLUMN

About 5–10 μg of each amino acid in 1 μl of buffer were applied to the equilibrated column (21 × 10 mm I.D.; V_t = 1.65 ml) and elution was performed as described under Experimental. After application of each amino acid, they were eluted by a decreasing pH gradient identical with that shown in Fig. 4A. The flow-rate was maintained at 0.94 ml min^{-1} and the effluent was continuously monitored at 220 nm with the detector output set at 0.16–0.64 a.u.f.s. The Chelating Superose gel has a capacity for Zn(II) of 31 μmol ml^{-1} gel (according to the manufacturer) and for Cu(II) of 42 μmol ml^{-1} gel as determined by us (see Table I).

Amino acid	V_e/V_t	*Amino acid*	V_e/V_t
Gly	12	Cys-Cys	26
Ala	11	Phe	17
Ser	14	Tyr	19
Thr	17	Trp	24
Val	11	His	31
Leu	13	Arg	15
Ile	12	Lys	13
Pro	14	Asp	14
Pro-OH	20	Glu	11
Met	16	Asn	17
Cys	26	Gln	14

TABLE V

RELATIVE RETENTION VOLUMES OF SOME SELECTED DI- AND TRIPEPTIDES, CONTAINING Phe, Tyr, Cys AND His RESIDUES OBTAINED AFTER HP-IMAC OF EACH ON A CHELATING SUPEROSE–Cu(II) COLUMN (21 × 10 mm I.D.; V_t = 1.65 ml).

For experimental details, see text and Table IV.

No.	*Amino acid or peptide*	V_e/V_t	*No.*	*Amino acid or peptide*	V_e/V_t
1	Phe	17.0	21	Histamine	30.2
2	Phe-Phe	2.6	22	His-His	39.0, 41.0
3	Phe-Phe-Phe	7.4	23	His-Leu	27.0
4	Pre-Asp	1.1	24	His-Pro	26.0
5	Tyr	19.0	25	Pro-His-Asp	24.0
6	Tyramine	1.4	26	Asp-His	3.1
7	Tyr-Tyr	4.3	27	His-Phe	29.6
8	Tyr-Tyr-Tyr	14.5	28	His-Trp	36.5
9	Trp	24.0	29	His-Tyr	31.3
10	Trp-Trp	24.5	30	Tyr-His	1.5
11	Trp-Asp	2.0	31	His-Gly-Gly	28.0
12	Arg-Trp	3.7	32	Gly-Gly-His	1.5
13	Gly-Trp	7.1	33	Cyclo(Gly)-His	26.8
14	Trp-Tyr	10.3	34	L-Carnosine (β-Ala-L-His)	23.0
15	Tyr-Trp	10.8	35	L-Anserine (β-Ala-L-1-methyl-His)	23.4
16	Cys	26.0	36	β-Ala	1.5
17	Cys-Cys	26.0	37	Gly-His-Lys	1.5
18	Cys-bis-Phe	9.4		(liver cell growth factor)	
19	Cys-bis-His	8.3	38	Asp-Ala-His-Lys	1.5
20	His	31.0	39	ACTH (residues 3–10) (Tyr-Met-Glu-His-Phe-Arg-Trp-Gly)	1.5

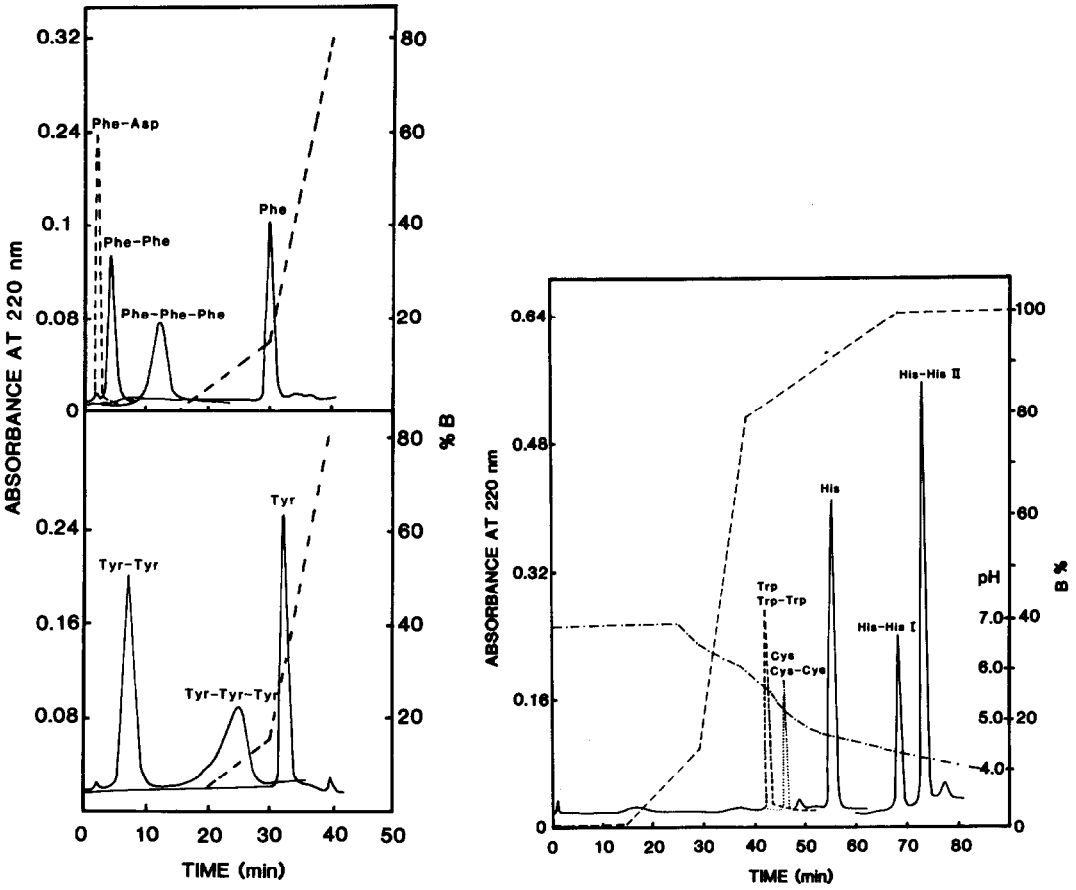


Fig. 6. Composite chromatograms showing the elution profiles of Phe, Tyr and their respective di- and tripeptides on a column (21 × 10 mm I.D.; V_t = 1.65 ml) of CS–Cu(II) with a capacity for Cu(II) of 42 μmol ml^{-1} [31 μmol ml^{-1} for Zn(II)]. Each amino acid or peptide was run separately on the column at a flow-rate of 0.94 ml min^{-1}. The adsorbed solutes (Phe, Tyr and Tyr-Tyr-Tyr) were eluted by a programmed gradient elution procedure as described. The quantitative elution data are compiled in Table V.

Fig. 7. Composite chromatogram showing the elution profiles of His, Trp, Cys and their respective dipeptides on a column (21 × 10 mm I.D.; V_t = 1.65 ml) of CS–Cu(II). See the legend to Fig. 6 for further details and Table V for the quantitative elution data. His-His gives rise to two distinct fractions reflecting the presence of a closely related impurity in the commercial dipeptide used.

interest in the present connection is the relatively high V_e/V_t values obtained for Asn, Met and the aromatic and hydroxy-containing amino acids, indicating that peptides containing these amino acids but no His, Trp or Cys residues would be retarded to varying extents on a CS–Cu(II) column provided that the amino-terminal residue is not blocked. With some exceptions, the results in Table III support this hypothesis.

Effect of solute structure

The foregoing results indicate that there is an apparent relationship between the retention of the peptides and their primary structures. Accordingly, the peptides

shown in Figs. 4A and 5 and Table III can be grouped into three categories, *viz.*, peptides 1–4, which elute isocratically, peptides 5 and 6 (containing Trp and Cys), which are bound moderately, and peptides 7–11 (containing His), which are bound tightly. On the basis of an extended series of experiments using a variety of peptides other than those shown in Fig. 4A or 5 and the retention spectra of some selected amino acids and di- or tripeptides (see Table V and Figs. 6 and 7), an attempt will be made here to rationalize the differential retentions of peptides on CS–Cu(II) adsorbent. By virtue of the relatively small molecular size of the peptides used here, it is assumed that most or all of their constituent amino acids are exposed to the solvent and are thus available for interaction with the immobilized Cu(II) ions.

Role of the α-amino and carboxyl groups

Peptides that lack His, Trp or Cys and whose amino terminal residues are blocked do not bind to CS–Cu(II) (Table III, peptides 1, 13 and 14). Removal of the blocked amino terminal residue results in a noticeable increase in retention, *e.g.*, neurotensin (residues 3–13) and neurotensin (Table III). The α-amino group of peptides is thus of primary importance for coordination with immobilized Cu(II) ions. However, this is not the case for the carboxyl terminal group, as peptides with blocked C-terminals are significantly retarded on the CS–Cu(II) column (Table III, peptides 5, 12, 17 and 19). These findings are in agreement with, and give support to, the recent proposal by Porath[18] that transition metal ions preferentially coordinate with nitrogen atoms. They also indicate that IMAC could be useful for separating blocked peptides from the mixture obtained after peptide synthesis. The ε-amino group of lysine and the guanidino group of arginine do not seem to coordinate with CS–Cu(II) since their V_e/V_t values are not markedly different from those of the aliphatic amino acids (Table IV). A comparable result has also been reported by Brookes and Pettit[16] for the interaction of these basic amino acids with Cu(II) ions in free solution.

Role of the aromatic amino acids and His, Cys and Trp

The significantly high retention on CS–Cu(II) of Phe and Tyr (Table V and Fig. 6) or of peptides containing these two residues, *e.g.*, angiotensin II and III (1 His, 1 Phe, and 1 Tyr) compared with TRH (1 His), indiates that they strengthen the binding of peptides, and possibly even proteins, to such adsorbents. However, the presence of charged amino acids in the immediate vicinity of Phe or Tyr markedly decreases the retention of the peptides, *e.g.*, Phe-Asp (Table V) and Met-Lys-bradykinin (Table III). Moreover, the spacing out of these residues in the peptide chain should also be taken into account, as our results show that the V_e/V_t of Phe-Phe or Tyr-Tyr is much lower than for the corresponding tripeptides or single amino acids. It is thus apparent that secondary and higher structures of peptides and proteins should be considered in order to assess with certainty the partial contributions of the aromatic amino acids to their overall retention on IDA–Cu(II) adsorbents.

His, Cys and Trp are by far the most important amino acids involved in strong coordination with metal ions of Groups IB, IIB and VIII. The results in this paper provide ample support as far as immobilized Cu(II) ions are concerned, where the strength of their binding follows the order: His-His > Cys = Cys-Cys > Trp = Trp-Trp (Table V). This relationship is also reflected in the retention of peptides containing these residues, *e.g.*, TRH (1 His and a blocked α-amino group) is bound

more strongly than somatostatin (1 Trp, 2 Cys, involved in a disulphide and 3 Phe). Likewise, angiotensin I (2 His) is bound more strongly than LH-RH (1 His and 1 Trp). The spacing out of these three residues in a peptide chain also influences their strength of binding. Our data thus support and extend the proposal by Sulkowski[6] in this respect. The conformation of the peptide must also be taken into consideration as it has a direct bearing on the availability of these residues for interaction. A case in point is somatostatin, whose structure is reminiscent of the "zinc fingers" in DNA-binding proteins[19,20], where its Trp residue is exposed to the surface and thus accessible for binding to immobilized Cu(II) ions.

Anomalous adsorption behaviour

In the course of this study, we came across some His-containing peptides that did not bind to CS–Cu(II), *viz.* Gly-His-Lys (GHK[a]) and Tyr-His (Table V, peptides 37 and 30, respectively). Smith *et al.*[15] also obtained such unexpected adsorption behaviour. Their results showed, with few exceptions, that peptides having the structure X-His did not bind to (Sephadex G-25)–IDA–Cu(II) whereas those with the structure His-X were bound irrespective of the nature of the amino acids represented by X. Despite the high affinity constants in solution for GHK, which they quoted (log K = 19.95), their explanation for its apparent inability to bind to immobilized IDA–Cu(II) was ascribed to the formation of different kinds of metal–solute complexes in solution and on the matrix-bound metal ion. We differ in this last interpretation and instead outline below an alternative hypothesis which is consistent with solution or column data obtained by others and with our own results on columns.

GHK is a so-called "liver cell growth factor"[21] normally found in human plasma in association with human serum albumin (HSA) and α-globulin fractions. The GHK–Cu(II) complex is also reported to have diverse biological action such as wound healing and tissue repair as well as significant superoxide dismutase activity[22]. GHK is able to remove Cu(II) from the HSA–Cu(II) complex very effectively at neutral pH[23,24], indicating that its affinity for Cu(II) (pK = 16.2, see refs. 24 and 25) is equal to or higher than that of HSA. The X-ray structure of the GHK–Cu(II) complex shows that the Cu(II) ion is coordinated to three nitrogens of GHK, *viz.*, the α-amino, imidazole and one peptide nitrogen as well as two oxygens of water[22]. Although GHK is postulated to form a ternary complex with Cu(II) that is already bound to HSA to form [HSA–Cu(II)–GHK] *in solution*, a similar situation, *i.e.*, the formation of [CS–Cu(II)–GHK], does not seem to occur on a CS–Cu(II) column, probably as a result of steric hindrances or the instability of such a ternary complex on the column. Its lack of binding to CS–Cu(II) can thus be ascribed to the removal of Cu(II) ions from the column to form a GHK–Cu(II) complex which, of necessity, will not be retarded on or bound by the CS–Cu(II) column. We have also considered an alternative explanation for the non-binding of GHK to CS–Cu(II), *i.e.*, the C-terminal Lys affects the pK of its adjacent His residue[26] to such an extent that the peptide as a whole does not bind to the CS–Cu(II) column. However, as its neutral analogue (Gly-His-Gly) also does not bind[15], this proposal was rejected in favour of that outlined above.

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[a] GHK is erroneously abbreviated as GHL[22] in the literature. As K, and not L, is the one-letter code for lysine we have used it consistently in this paper.

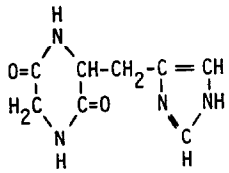
To our knowledge, only two such events are unequivocally documented in the IMAC literature[26,27]. Thus Muszyńska *et al.*[26] have shown that Zn(II)-depleted and inactive carboxypeptidase A strips off Zn(II) ions from an IDA–Zn(II) column and elutes from it in its enzymatically active form, fully loaded with Zn(II) ions. Likewise, Andersson *et al.*[27] have shown that HSA strips off Ni(II) ions from an IDA–Ni(II) column, eluting from it in the form of an HSA–Ni(II) complex. Clearly, these results illustrate the occurrence of "metal ion transfer"[29] on IMAC of some specific proteins and peptides. For such an event to take place, the following conditions seem to be necessary: (i) the association constant solute–M(II) > C–M(II), where C = chelator and M(II) = metal ion, and (ii) the ternary complex C–M(II)–solute is unstable and dissociates in favour of a solute–M(II) complex, leading to a "metal ion transfer" event. If, on the other hand, the association constant C–M(II) > S–M(II), then a stable, ternary complex can be formed and the solute could be bound by or, retarded on, the IMA adsorbent.

The question thus arises: can this hypothesis explain the other results we obtained in Table V? We believe it does, for reasons outlined below.

Of the His-containing dipeptides shown in Table V, those having the structure X-His do not bind to CS–Cu(II) whereas those having the structure His-X are bound strongly with V_e/V_t values in excess of 25. The only exception is His-His, which is the most strongly bound dipeptide to CS–Cu(II) that we have found during this investigation. The underlying principle for the results obtained is apparently "metal ion transfer" from the CS–Cu(II) to those dipeptides having X-His as their structure simply because such dipeptides form a strong tridentate coordination bond with the immobilized Cu(II) ions via the free α-amino group of the amino terminal residue X, the intervening peptide nitrogen and the imidazole of His, as in the case of GHK. Owing to such strong binding, their affinity towards the immobilized Cu(II) ions is apparently much stronger than that of Cu(II) ions to the IDA of Chelating Superose, with the result that such dipeptides strip off the Cu(II) ions from the CS–Cu(II) and subsequently pass through the column unretarded. When the order is reversed, as in His-X, the α-amino nitrogen of the X residue is blocked and will in effect not coordinate with the immobilized Cu(II) ions. Under such circumstances, the overall interaction is weakened as the peptide nitrogen does not form such a strong coordination bond with CS–Cu(II) as does the α-amino nitrogen. Consequently, such peptides will bind to CS–Cu(II) through their His residues and their overall retention will be about the same as that of a single His residue. The carboxyl group does not contribute significantly to the overall binding of His-containing di- or tripeptides, as can be deduced from the finding that histamine binds as strongly as His to CS–Cu(II) (see Table V).

Further support for the above hypothesis is provided by the retention of L-carnosine and L-anserine (peptides 34 and 35, respectively, in Table V). These two peptides are strongly bound to CS–Cu(II) despite the fact that they also have X-His as their general structure. However, in β-Ala its NH_2 group is not in the α-position and apparently does not form a coordination bond with immobilized Cu(II) ions as does the α-amino group of the common amino acids (see Table V, peptide 36). These two

peptides are thus bound to immobilized Cu(II) ions entirely through their His residues. The same holds for cyclo-Gly-His:



where the α-amino group of Gly is blocked, showing that this dipeptide is bound to CS–Cu(II) entirely through its His residue. These results are consistent with the proposition that a free α-amino group in combination with a His residue at the carboxyl terminus of dipeptides results in "metal ion transfer" from CS–Cu(II) to the dipeptide in question.

The discussion outlined above also applies to the His-containing tripeptides shown in Table V. As would be expected, His-Gly-Gly is bound but not Gly-Gly-His. The latter tripeptide is a well studied synthetic molecule which mimics the Cu(II) transport site of human serum albumin[23]. It binds Cu(II) in free solution as a 1:1 complex in the pH range 6.5–11 and the dissociation constant of the peptide–Cu(II) complex is $1.18 \cdot 10^{-16}$, compared with $6.61 \cdot 10^{-17}$ for albumin–Cu(II)[23]. The Cu(II)-binding site of human serum albumin is attributed entirely to the tetrapeptide Asp-Ala-His-Lys situated at the amino terminus of the molecule[30]. Despite its very high affinity towards Cu(II), it does not bind to immobilized Cu(II) either (Table V, peptide 38). In view of what has been discussed earlier, it is both consistent and reasonable to propose that the lack of binding of these peptides to CS–Cu(II) is also due to "metal ion transfer" from CS–Cu(II) to these peptides. On the basis of these findings we can postulate that the corresponding Cu(II)-binding sites of bovine (Asp-Thr-His-Lys) and rat (Glu-Ala-His-Lys) serum albumins[25] would also strip off Cu(II) from a CS–Cu(II) column and thus not bind either. The corresponding site for dog serum albumin (Glu-Ala-Tyr-Lys)[25] would not be expected to bind to CS–Cu(II), nor would it strip off Cu(II) ions from the column owing to replacement of the important His residue by Tyr at position 3 of its sequence.

Direct proof to the proposed hypothesis would be metal ion analysis of those peptides [X-His, GHK and the Cu(II)–binding tetrapeptides of the various serum albumins] after their passage through a CS–Cu(II) column. Unfortunately, these peptides were not available for such experiments. However, some orienting experiments are in progress aimed at adapting modern mass spectrometric methods to establish the Cu(II) content in small amounts of such peptides. Moreover, our results suggest that the passage of such peptides through a CS–Cu(II) column would be a very elegant and simple method for optimally loading them with Cu(II) ions for further analysis, *e.g*, to load Gly-His-Lys in order to use it as a therapeutic agent for wound-healing purposes[22].

Practical implications

The results presented here serve as guidelines when one is investigating the optimum conditions necessary for the separation of peptides and proteins on IMA adsorbents through a systematic variation of the relevant chromatographic param-

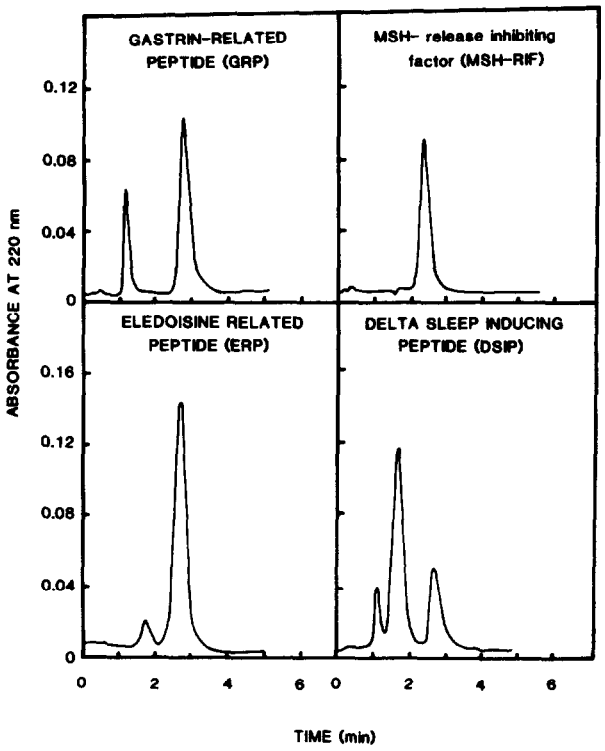


Fig. 8. Test of the purity of some commercially available synthetic peptide hormones by IMAC on a column (21 × 10 mm I.D.; bed volume 1.65 ml) of CS–Cu(II) equilibrated with buffer A. Each peptide was eluted under isocratic conditions at a flow-rate of 0.94 ml min^{-1}. The primary structures of the peptides are shown in Table III.

eters. It is envisaged that IMAC will be of special interest for researchers in the area of peptide synthesis as concerns both the purification of a specific peptide or an independent check of its purity by a separation mechanism which is different from that of the commonly used reversed-phase chromatography (RPC). An example of this is presented in Fig. 8, where the purity of four commercially available synthetic peptide hormones was determined by HP-IMAC on a CS–Cu(II) column. With one exception, the preparations contained one or more impurities.

A distinct advantage of IMA-based adsorbents over RPC is the use of entirely aqueous buffers as eluents in the former, a consideration which is important for safety in the laboratory working environment. IMAC is also a versatile method in that a range of different metal ions can be immobilized on the same chelating gel, making it possible to choose the most suitable immobilized metal ion for a particular application. An additional parameter that can be varied is the salt concentration in the equilibration buffer. Our results indicate that at low concentrations of salt in the equilibration buffer, ionic interactions are superimposed on metal ion recognition, which could be exploited to magnify small differences in the surface properties of peptides or proteins and thus lead to their optimum separation. We are convinced that this point will receive increasing attention in the future use of IMAC. We would also

like to point out that, as far as we know, denaturation of proteins or peptides on IMA adsorbents is a rare phenomenon.

ACKNOWLEDGEMENTS

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