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Immobilized metal ion affinity chromatography of synthetic peptides

Binding via the α-amino group

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ABSTRACT

Peptides synthesized by the solid-phase method can be efficiently purified in a single immobilized metal affinity chromatography step based on interaction with the α-amino group if, after coupling of each amino acid residue, unreacted amino groups are irreversibly blocked by acetylation and if no strongly metal-binding amino acids (His, Trp, Cys) are present in the sequence. A difference in basicity for α- and ε-amino functions of *ca.* 2 pH units is sufficiently large to allow selective binding of peptides to immobilized metal ions via the unprotonated α-amino group. The binding is pH-dependent: on Cu^{2+}- and Ni^{2+}-loaded supports most peptides are maximally retarded at pH values around 7.5 and 8.5, respectively. The decreased binding strength at lower pH values is due to protonation of the α-amino function, whereas the reduced affinity at higher pH is caused by metal ion transfer from the matrix to the peptide. The metal ion is captured in a multidentate chelate where, in addition to the α-amino group, up to three adjacent deprotonated amide nitrogens are coordinated to the metal. If the pH is raised further, additional metal ions may be bound in biuret-like structures. Immobilized Ni^{2+}, owing to its higher selectivity and affinity, is the preferred chromatographic support if slightly basic conditions can be tolerated.

INTRODUCTION

Solid-phase peptide synthesis (SPPS) [1], despite improvements in methodology, suffers from the inherent limitation that side-products resulting from incomplete reaction steps cannot be removed from the desired peptide until the synthesis is finished and the product cleaved from the resin. Whereas small peptides can usually be purified adequately by general separation techniques, particularly reversed-phase high-performance liquid chromatography (RP-HPLC), the similarity between the product and the impurities often makes the isolation of large synthetic peptides a more demanding task.

Attempts have been made to facilitate the purification by attachment of affinity handles onto the side-products [2,3] or the target peptide [4–6] in order to allow the use of more specific separation methods. Selective labelling of the desired peptide is accomplished by irreversible blocking (capping) of remaining amino groups after each coupling step by an efficient acylating agent such as acetic anhydride, followed at the end of the synthesis by attachment of the affinity handle to the α-amino group, which

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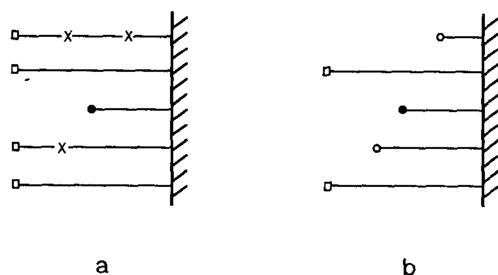

Fig. 1. Schematic composition of (resin-bound) products synthesized (a) without and (b) with the inclusion of a capping protocol. × denotes amino acid deletion, ● truncation and ○ capping, *e.g.* by acetylation. The free α-amino group (□) may be substituted with an affinity handle before removal of the side-chain protecting groups and cleavage of the peptide–resin bond.

will then be present only in the target peptide (Fig. 1). However, in this case the choice of an affinity handle is severely restricted by the requirement that mild conditions must be available for its detachment once the purification is completed. Furthermore, the function of the affinity group may be adversely affected by electrophilic substitution during the cleavage step or by other post-synthetic modifications.

In special cases the presence of certain amino acid residues in the N-terminal part of the peptide may be expoited for affinity purification. Cysteine residues have been used for reversible covalent binding to immobilized mercury derivatives [7] or activated thiols [8], and the use of immobilized metal ion affinity chromatography (IMAC) [9] for the efficient purification of several moderately sized peptides (30–40 amino acid residues) with N-terminal histidine or tryptophan residues was recently described [10]. The simplicity and selectivity of the latter approach prompted the search for more general conditions for its application.

The simplest affinity handle available for metal ion interaction is the α-amino group itself. The participation of unprotonated amino groups in different metal complexes is well established [11], and it has been shown that the α-amino function is of importance in IMAC of peptides [12,13]. Belew and Porath [14] found that peptides lacking His, Trp or Cys were bound to immobilized Cu^{2+} if the α-amino group was free but not if it was blocked and suggested that IMAC could be useful for removing blocked peptides from a crude synthetic mixture. However, this study was limited to pH values below 7. Since pK_a values for α- and ε-amino groups in proteins are higher (*ca.* 8 and 10, respectively) we believed that conditions could be found where this relatively large difference in basicity could be more efficiently exploited for selective binding of a peptide through its α-amino group. This view was further supported by recent findings that α-amino groups in peptides interact strongly with both immobilized Cu^{2+} and Ni^{2+} above pH 7, whereas in proteins they interact only with immobilized Cu^{2+} [15]. We therefore synthesized a number of peptides (Table I) to permit a systematic investigation of the pH dependence and the specificity of the metal binding. Histidine or tryptophan residues were not

TABLE I

STRUCTURE OF PEPTIDES USED IN THIS INVESTIGATION

Peptide	Structure
1	Asp–Ser–Ala–Val–Gly–Tyr–Ala
2	Ac–Asp–Ser–Ala–Val–Gly–Tyr–Ala
3	Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
4	Ac–Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
5	Gly–Ala–Thr–Lys(Ac)–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
6	Ac–Gly–Ala–Thr–Lys(Ac)–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
7	Pro–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
8	Ac–Pro–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
9	β-Ala–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
10	Ac–β-Ala–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
11	Ala–Pro–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
12	Ac–Ala–Pro–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala

included in the peptides in order to simplify interpretation of the data.

EXPERIMENTAL

Chemicals

tert.-Butyloxycarbonyl (Boc) amino acids were purchased from Peninsula Laboratories Europe (St. Helens, UK), Bachem Feinchemikalien (Bubendorff, Switzerland) or Novabiochem (Läufelfingen, Switzerland). Boc-amino acyl resins were synthesized according to Horiki *et al.* [16]. Other chemicals were of analytical grade and used as purchased. Chelating Superose and Chelating Sepharose Fast Flow were obtained from Kabi-Pharmacia (Uppsala, Sweden).

Buffers

Buffers were prepared by dissolving all the salts in Milli-Q water and titrating to the appropriate pH with concentrated sodium hydroxide. The volume was then adjusted to yield a final buffer concentration of 50 m*M* if not otherwise stated. Buffer salts used were sodium dihydrogenphosphate, boric acid and sodium hydrogencarbonate. Sodium chloride (1 *M*) was included in all buffers.

Peptide synthesis

The peptides were synthesized by the solid-phase method using an Applied Biosystems 430A instrument with standard procedures and Boc/benzyl protecting groups. However, capping with acetic anhydride was included after each coupling step. Acetylation of the terminal α-amino group in the target peptides was accomplished by the addition of acetic acid (2 mmol) to an empty glycine cartridge and using the standard conditions for this amino acid. Boc-Lys(Ac)-OH was coupled manually with dicyclohexylcarbodiimide (three equivalents each) in 25% dimethylformamide (DMF)-dichloromethane (DCM) for 2 h.

The peptides were deprotected and cleaved from the resin (*ca.* 500 mg) by treatment with anhydrous hydrogen fluoride (5 ml) in the presence of anisole (500 μl) for 1 h at 0°C. The residue obtained after removal of hydrogen fluoride *in vacuo* was washed thoroughly with chloroform and diethyl ether. The peptide was then extracted into trifluoroacetic acid (TFA) (3 $\times$ 2 ml), and the resulting solution was concentrated under a stream of dry nitrogen to *ca.* 1 ml. Diethyl ether was added to precipitate the product, which was then collected by centrifugation, washed several times with diethyl ether and dried *in vacuo* over potassium hydroxide.

Mass spectrometry

Synthetic products and chromatographic fractions were analysed by plasma desorption mass spectrometry (PDMS) using a BioIon 20 instrument (Applied Biosystems, Uppsala, Sweden). Samples, usually 5–10 μg dissolved in 5–10 μl of 0.1% TFA containing 20–50% ethanol, were applied on nitrocellulose-coated aluminium foils and dried under a stream of nitrogen. Samples with high salt content, *e.g.* IMAC fractions, were usually rinsed with 10–20 μl of 0.1% TFA or distilled water after drying. Positive-ion spectra were collected, normally for 5–30 min.

Reversed-phase HPLC

Synthetic products and IMAC fractions were also analysed by RP-HPLC using a Pharmacia Pep-RPC 5/5 column. The chromatographic equipment included two Constametric pumps (types I and III), a Gradient Master controller and a Spectromonitor III variable-wavelength detector (LDC, Riviera Beach, FL, USA). The gradient was formed by mixing acetonitrile containing 0.1% TFA with 0.1% aqueous TFA (0–40% acetonitrile in 30 min). The flow-rate was 1 ml/min. Solid samples were dissolved in 0.1% TFA. When necessary, IMAC fractions were neutralized prior to application.

Immobilized metal ion affinity chromatography

The fast protein liquid chromatography (FPLC) system (Kabi-Pharmacia) used for IMAC contained two P-500 pumps, a GP-250 gradient programmer and a UV-1 monitor set at 280 nm. The IMAC supports were Chelating Superose (32 μmol/ml Zn^{2+} capacity) prepacked in a 1.5 $\times$ 1 cm I.D. column and Chelating Sepharose Fast Flow packed in a 6.6 $\times$ 0.66 cm I.D. column. The columns were charged with Cu^{2+} or Ni^{2+} and loosely bound metal ions were washed off with 4 ml of 0.1 *M* sodium acetate buffer, pH 4.0. The peptides (50–200 μg) were dissolved in 50–200 μl of the appropriate buffer, applied to the column and eluted isocratically at a flow-rate of 1 ml/min. All buffers contained 1 *M*

sodium chloride in order to suppress undesired ion-exchange effects. After each run the column was washed with 4 ml of sodium acetate buffer, pH 4.0. The peptides were characterized by their capacity factors:

$$k = V_e/V_0 - 1$$

where V_e is the elution volume of the peptide and V_0 the elution volume on the metal-free column.

Determination of peptide/Cu^{2+} ratios

Peptide **5** was purified by preparative RP-HPLC and dissolved in water. The peptide concentration (1.27 m*M*) was determined by amino acid analysis. The solution was then diluted with an equal volume of 100 m*M* phosphate–borate–2 *M* sodium chloride at pH 7–11 and 100 μl (63.5 nmol) chromatographed on Cu^{2+}-charged Chelating Superose (1.5 × 1 cm I.D.) as described above. In each case the eluate corresponding to the peptide peak was collected in a preweighed tube and the copper content determined by atomic absorption spectroscopy (Mikro-Kemi, Uppsala, Sweden).

Spectroscopy

Absorption maxima of peptide–Me^{2+} complexes. Peptides **5** and **6** (1 μmol) were each dissolved in 1 ml of 50 m*M* phosphate–borate containing 1 *M* sodium chloride in the pH range 7–11. To each solution was added 100 μl of either 10 m*M* copper sulphate or 10 m*M* nickel nitrate. The resulting mixture was centrifuged and the absorption spectrum in the range 300–700 nm was recorded with the aid of a Pye Unicam SP8-100 spectrophotometer.

Absorption maxima of peptide eluates. Chelating Sepharose (*ca.* 75 μl) was packed in a Pasteur pipette and charged with Cu^{2+} or Ni^{2+}. The peptides **5** and **6** were each dissolved in phosphate–borate–sodium chloride buffers over a pH range of 7–11 at a concentration of 2 m*M*. The column was pre-equilibrated with the appropriate buffer, then 500 μl of the peptide solution were applied and the column was eluted with 1 ml of the same buffer. The absorption maximum of the eluate in the range 300–700 nm was determined.

RESULTS AND DISCUSSION

Influence of buffer concentration

The effect of phosphate and borate concentration on the retention of the peptides **1**–**4** on Chelating Sepharose–Cu^{2+} at different pH values is shown in Table II. The peptides with blocked α-amino groups (**2** and **4**) are largely unaffected by the buffer concentration and show very low overall retention, whereas those with free α-amino groups (**1** and **3**) are generally more strongly bound although less so at higher buffer concentrations. As 1 *M* sodium chloride is present in all buffers this effect can not be ascribed to ion-exchange properties of the matrix but is most likely due to competition by phosphate and borate ions for the available coordination sites of the immobilized metal ions. However, in borate buffer at pH 8.5 and 9 the effect is reversed and the peptides **1** and **3** are more retarded as the buffer concentration is increased. The negligible influence on the retention of the acetylated peptides rules out the possibility that this might be caused by a general salting-out effect. We favour the interpretation that at this concentration borate ions are so efficiently competing for the coordination sites that, although the peptide may still bind via the α-amino group, chelation by further metal ion coordination to deprotonated amide nitrogens and subsequent release of the peptide as a peptide–metal complex is prevented. This mechanism is discussed in further detail in the following section. At pH 10 the competition by borate may interfere with the binding of the ε-amino group, hence the decreased retention of peptides **3** and **4** in 0.5 *M* buffer.

Influence of pH

The data given in Table II clearly show that the retention of several peptides on immobilized Cu^{2+} is pH-dependent. These effects are also observed in Fig. 2, where mixed phosphate–borate buffers with high buffering capacity over the entire region pH 5–11 were used. Most of the peptides with a free α-amino group (**1**, **3**, **5** and **7**) have a clear retention maximum at pH 7–8, whereas their acetylated counterparts (**2**, **4**, **6** and **8**) are not retained at pH$\leqslant$9. The peptides **3**, **4**, **7**–**10** and **12**, which all carry free ε-amino functions, show increased retention at pH$>$9. The latter effect is related to binding via the ε-amino group, which gradually becomes de-

TABLE II

CAPACITY FACTORS OF MODEL PEPTIDES CHROMATOGRAPHED ON IMMOBILIZED Cu^{2+} AT DIFFERENT BUFFER CONCENTRATIONS

Sample: 100 μg of peptide dissolved in 100 μl of sodium phosphate–1 *M* sodium chloride or sodium borate–1 *M* sodium chloride. Column: Chelating Sepharose–Cu^{2+} (6.6 × 0.66 cm I.D.). Elution: sample buffer at 1 ml/min. Detection: UV at 280 nm. The α-amino groups of **2** and **4** are acetylated.

Peptide	Buffer concentration (m*M*)	Capacity factor						
		Phosphate			Borate			
		pH 6	pH 7	pH 8	pH 8	pH 8.5	pH 9	pH 10
1	5	0.7	5.4	5.6	6.2	0.1	0.1	0.0
	50	1.0	3.8	3.4	4.6	0.1	0.1	0.0
	500	0.3	3.4	3.0	3.0	0.9	0.5	0.0
2	5	0.0	0.1	0.0	0.1	0.0	0.0	0.0
	50	0.0	0.0	0.0	0.1	0.0	0.0	0.0
	500	0.0	0.0	0.0	0.1	0.0	0.0	0.0
3	5	0.2	2.1	4.0	6.8	0.4	0.5	0.9
	50	0.2	1.4	3.3	5.9	0.4	0.4	0.9
	500	0.1	0.3	2.6	4.8	2.3	1.1	0.6
4	5	0.0	0.1	0.0	0.1	0.2	0.3	0.9
	50	0.0	0.0	0.0	0.1	0.2	0.3	1.0
	500	0.0	0.1	0.0	0.1	0.1	0.2	0.7

protonated in this pH region since peptides without lysine (**1** and **2**) or with a blocked ε-amino function (**5** and **6**) are not retained at pH≥9. Correspondingly, the rise in retention as the pH is increased from 5 to 7–8 reflects deprotonation and binding of the free α-amino group.

At pH values below 7–8 the peptide is bound via the α-amino group or as a bidentate chelate in which the carbonyl c ygen of the N-terminal amino acid is also coordinated to the metal ion in the equatorial plane (Fig. 3a). This is consistent with the formation of ternary complexes in solution, in which case it has been found [17] that the initial step involves coordination of the metal ion to the terminal amino group and the adjacent amide oxygen.

A free amino group is sufficient for anchoring a peptide to immobilized cupric ions, as can be seen for some of the lysine-containing compounds (**4**, **8**, **10** and **12**). Tyramine, which has no amide oxygen or nitrogen that can participate in the metal ion coordination, is also retarded above pH 7.5 (Fig. 4). However, the retention is considerably lower than that for peptide **11**, suggesting that a neighbouring peptide oxygen contributes significantly to the metal binding (see below).

The decrease in retention as the pH is raised further, from 7–8 to 9, is probably not caused by buffer ion competition since the same behaviour is observed in borate, carbonate and mixed phosphate–carbonate buffer (not shown). The coordination of hydroxide ions resulting in a decreased retention seems to be unlikely since these would be expected to also prevent binding of the ε-amino group at pH >9, at which the hydroxide ion concentration is even higher. Further investigations demonstrated that the lowered retention is caused by metal ion transfer (MIT) from the chelating support to the peptide.

When an excess of peptide with a free α-amino group (**5**) was passed through a column of Cu^{2+}-loaded Chelating Sepharose the process could be observed visually. At pH≥8 the blue colour of the chelated copper at the top of the column faded or disappeared completely while the eluate turned violet. The visible spectra of the eluates in the range 300–700 nm were identical to those obtained by

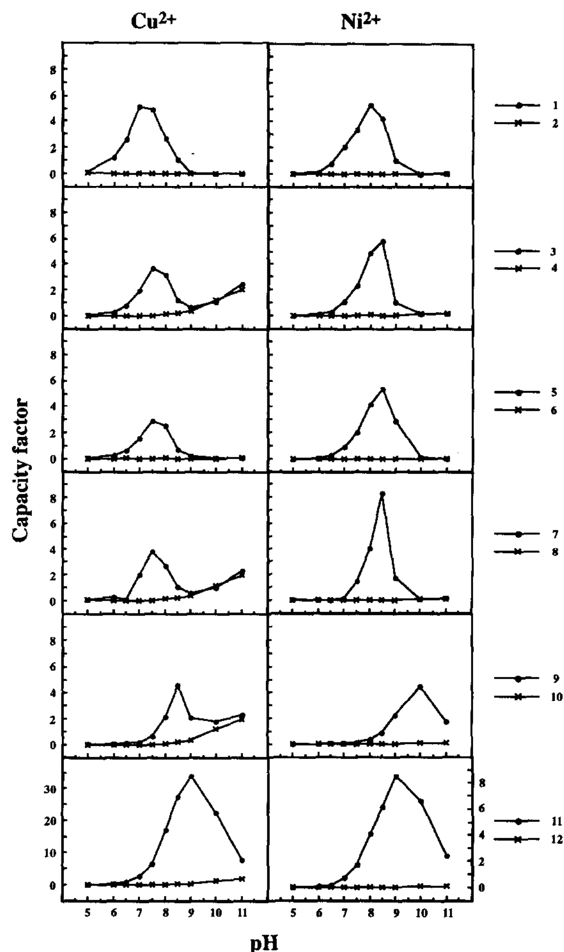

Fig. 2. Retention of peptides **1–12** on immobilized Cu^{2+} and Ni^{2+} at different pH values. Column: Chelating Superose (1.5 × 1 cm I.D.) charged with Cu^{2+} or Ni^{2+}. Sample: 70–130 μg of peptide dissolved in 100 μl of 50 m*M* sodium phosphate–borate–1 *M* sodium chloride. Elution: isocratic with sample buffer at 1 ml/min. Detection: UV at 280 nm. Note change of scale in the lower left-hand corner.

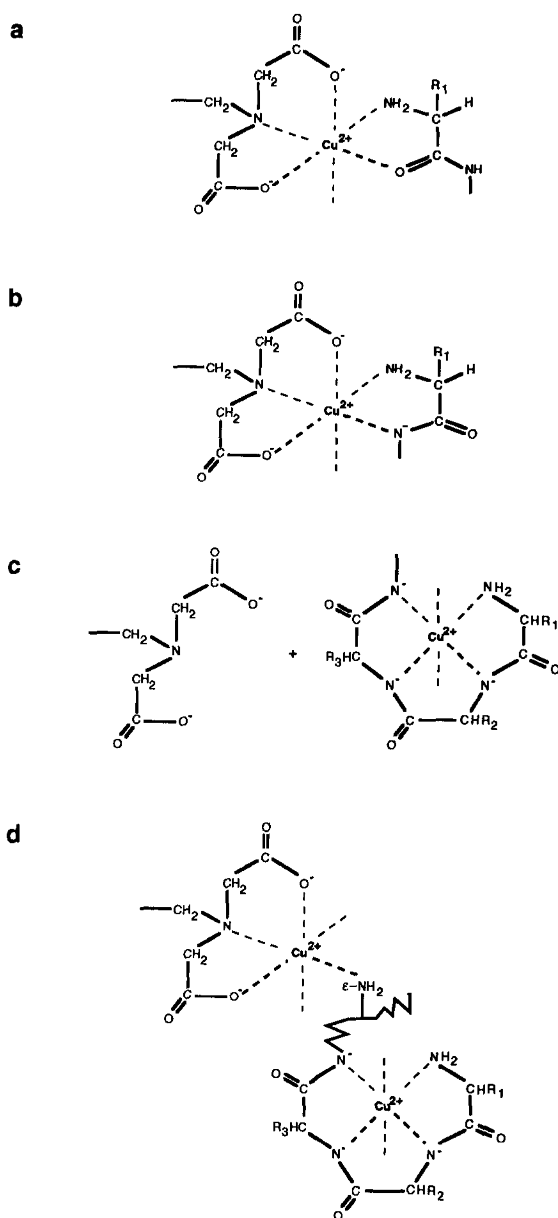

Fig. 3. Proposed mechanism for metal ion transfer from iminodiacetate–Cu^{2+} support to peptide. (a) The peptide is initially bound via the α-amino group or as an N,O complex. (b) At pH > 7 the adjacent amide proton is lost and the peptide reoriented to form an N,N complex. (c) At slightly higher pH values further amide nitrogens are deprotonated and the metal ion captured in a quadridentate chelate. Above pH 10 biuret-like complexes may be formed. (d) At pH > 9 the peptide can bind via the deprotonated ε-amino group.

mixing the peptide and copper sulphate in equimolar amounts in the appropriate buffer. At pH ≥ 9 the absorption maximum occurred at 515 nm, which is characteristic for a quadridentate complex [18]. For the corresponding acetylated peptide (**6**) the loss of copper from the column was observed only at pH ≥ 10 and the absorption maximum was shifted to 560 nm, indicating that three deprotonated amide nitrogens are coordinated to the metal ion.

Metal analysis confirmed that copper was re-

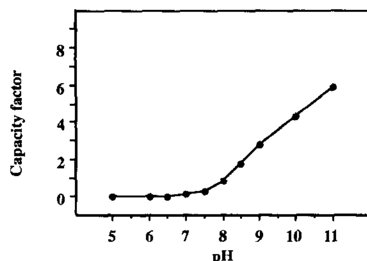

Fig. 4. Retention of tyramine on Cu^{2+}-loaded Chelating Superose at different pH values. Column dimensions: 1.5 × 1 cm I.D. Sample: 35 μg of tyramine dissolved in 100 μl of 50 m*M* sodium phosphate–borate–1 *M* sodium chloride. Elution: isocratic with sample buffer at 1 ml/min. Detection: UV at 280 nm.

moved from the column. When a known amount of peptide **5** was chromatographed on Chelating Superose–Cu^{2+}, the amount of copper transferred to the eluate increased as the pH was raised from 7 to 11 (Table III). The 2:1 Cu^{2+}:peptide ratio at pH 11 is consistent with chelation of a second copper ion in the C-terminal part of the peptide where deprotonated amide nitrogens are coordinated to the metal in a biuret-like fashion.

A possible mechanism of MIT is illustrated in Fig. 3. Amide deprotonation is reinforced by the metalation of the carbonyl oxygen (Fig. 3a). The pK_a value has been shown to drop from about 15 to typically 5–6 for binary and to 6–8 for ternary

TABLE III

AMOUNT OF COPPER TRANSFERRED FROM CHROMATOGRAPHIC SUPPORT TO ELUATE BY PEPTIDE 5 AT DIFFERENT pH VALUES

Sample: 63.5 nmol of **5** dissolved in 100 μl of 50 m*M* sodium phosphate/borate–1 *M* sodium chloride. Column: Chelating Superose–Cu^{2+} (1.5 × 1 cm I.D.). Elution: sample buffer at 1 ml/min. Detection: UV at 280 nm. The copper content in the peptide-containing fraction was determined by atomic absorption spectroscopy.

pH	Cu (nmol)	Cu/peptide ratio
7.0	0	0
8.0	17	0.3
9.0	80	1.2
10.0	69	1.1
11.0	118	1.9

Cu^{2+}-complexes [11]. The change from O- to N-coordination (Fig. 3b) places the deprotonated nitrogen of the neighbouring amide bond in a favourable position for interaction with the adjacent equatorial binding site of the metal ion. Formation of a new bond will displace one of the carboxyls of the iminodiacetate and weaken the interaction between the metal ion and the chromatographic support. As a further consequence of the peptide bond planarity, interaction with the third amide nitrogen will thereby be facilitated, eventually yielding the quadridentate chelate shown in Fig. 3c.

Most of the pH profiles presented in Fig. 2 can be interpreted to be a result of two counteracting effects: binding and metal ion transfer. The profiles of **1–8** might represent the behaviour of many peptides. Peptide **11**, which has a proline as the second amino acid and consequently carries no removable amide proton in this position, is very strongly bound (k >30 at pH 9). The reduced binding at higher pH values for this peptide is again caused by metal ion transfer from the matrix to the peptide, but here the terminal carboxyl rather than the amino group may be involved in the chelate formation. Peptide **9** with an N-terminal β-alanine also behaves anomalously in so far as the retention maximum is shifted *ca.* 1 pH unit upfield. This change may be due to the more basic β-amino group but may also reflect a reduced tendency for amide deprotonation in the less favourable six-membered ring.

Influence of metal ion

When Cu^{2+} is replaced by Ni^{2+} as the immobilized metal ion the general chromatographic behaviour remains the same (Fig. 2). However, maximal adsorption occurs at higher pH values, usually around pH 8.5, which is in agreement with observations on the formation of complexes in solution [11].

Capacity factors are usually at least as high as for immobilized Cu^{2+}. However, for peptide **11**, which has proline as the second residue, the maximal k value is reduced from 30 to 9 upon replacement of Cu^{2+} with Ni^{2+}, which might reflect differences in coordination characteristics for the two metals.

The selectivity for binding via the α- *vs.* the ε-amino group seems to be higher for the Ni^{2+}-loaded support since none of the α-acetylated peptides is

retarded at pH ⩽ 11. For purification of synthetic peptides according to the concepts outlined above, an iminodiacetate–Ni^{2+} support would be the medium of choice, at least in cases where slightly basic conditions can be tolerated.

It can be shown that nickel ions are also transferred from the iminodiacetate to the peptide. At pH 10–11 elution of Chelating Sepharose–Ni^{2+} with peptide **5** produces a yellow colour. The absorption maximum at 420 nm of the eluate is consistent with a square-planar coordination to one amino and three depronated amide nitrogens [11].

Attempts to use other immobilized metal ions (Zn^{2+}, Co^{2+} and Mn^{2+}) were unsuccessful.

*IMAC purification of Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala (**3**)*

The usefulness of IMAC based on metal ion interaction with the α-amino group for isolation of synthetic peptides is demonstrated in the purification procedure for peptide **3** which, in addition to the α-amino group, also carries a free ε-amino function.

The crude product was applied on Cu^{2+}- and

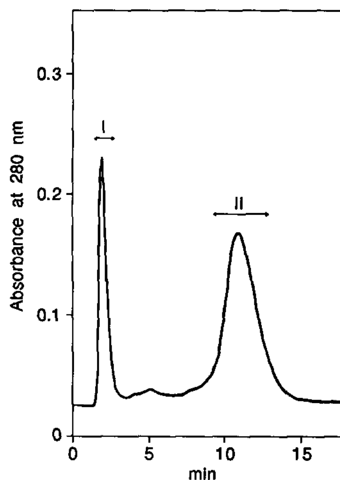

Fig. 6. Chromatography of crude peptide **3** on Chelating Superose–Ni^{2+}. The conditions were as in Fig.5 but the sample and elution buffer was 50 m*M* sodium phosphate–borate–1 *M* sodium chloride at pH 8.5.

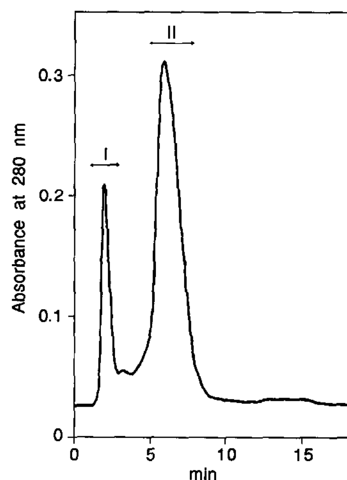

Fig. 5. Chromatography of crude peptide **3** on Chelating Superose–Cu^{2+}. Column dimensions: 1.5 × 1 cm I.D. Sample: 1.25 mg dissolved in 250 μl of 50 m*M* sodium phosphate–borate–1 *M* sodium chloride, pH 7.5. Elution: isocratic with sample buffer at 1 ml/min. For mass spectrometry the fractions indicated were concentrated and desalted on a 5 × 0.5 cm I.D. Pep-RPC column. After adsorption, the column was rinsed with 0.1% TFA and the peptides eluted with a steep acetonitrile gradient (0–60% in 1 min).

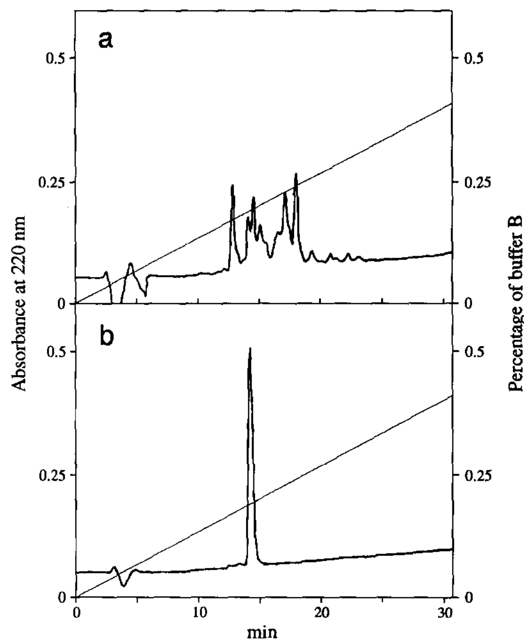

Fig. 7. RP-HPLC of (a) 750 μl (50%) of fraction I and (b) 100 μl (10%) of fraction II from IMAC of peptide **3** on Chelating Superose–Cu^{2+} (Fig. 5). Column: Pep-RPC, 5 × 0.5 cm I.D. Flow-rate: 1 ml/min. Solvent: A: 0.1% aqueous TFA. Solvent B: 0.1% TFA in acetonitrile.

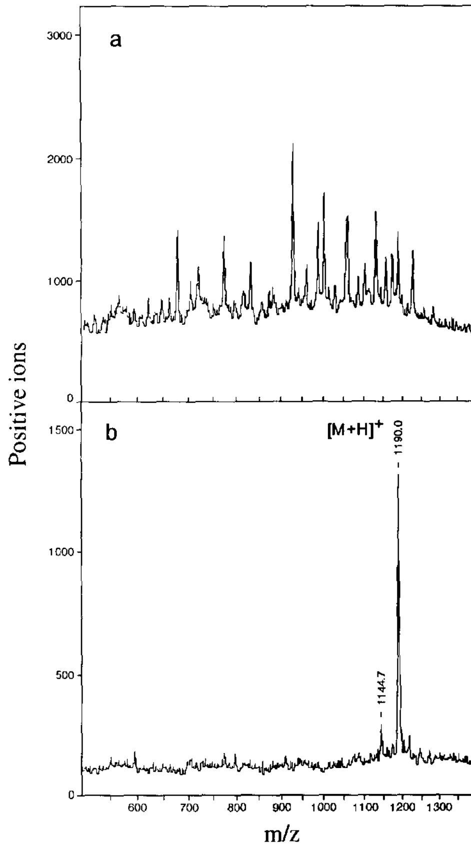

Fig. 8. PDMS of (a) fraction I and (b) fraction II from IMAC of peptide 3 on Cu^{2+}-loaded Chelating Superose (Fig. 5). The desalted samples (5 μl) were mixed with ethanol (2 μl) on nitrocellulose-coated aluminium foils, dried and rinsed with distilled water (20 μl). Most peaks in (a) can be attributed to various acetylated peptides and their fragment ions. The peak at 1144.7 mass units in (b) is due to decarboxylation. The calculated molecular weight for peptide 3 is 1189.4.

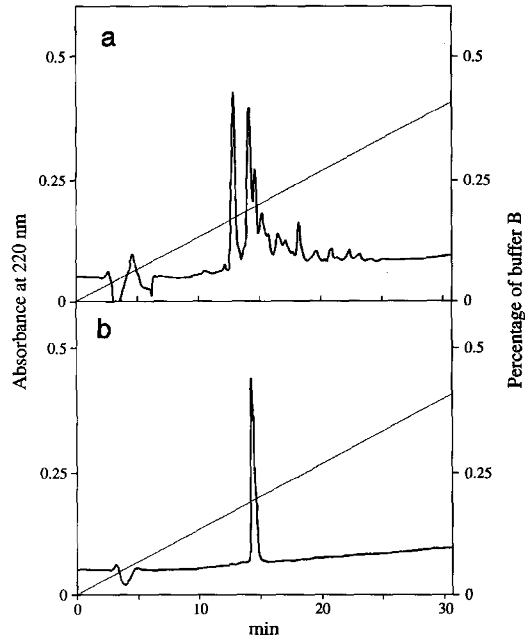

Fig. 9. RP-HPLC of (a) 750 μl (40%) of fraction I and (b) 100 μl (3%) of fraction II from IMAC of peptide 3 on Chelating Superose–Ni^{2+} (Fig. 6). Conditions are as in Fig. 7.

Ni^{2+}-loaded Superose and eluted isocratically at pH 7.5 and 8.5, respectively (Figs. 5 and 6). In both cases two major fractions were obtained, which were further analysed by mass spectrometry and RP-HPLC (Figs. 7–10). As expected, the non-retarded material (I) is a mixture of small amounts of truncated peptides which have been acetylated in the capping steps, whereas in the main fraction (II) only peptide **3** was detected.

*IMAC purification of Ala–Pro–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala (**11**)*

Isocratic elution at pH values which yield maximal retardation, *i.e.* pH ≈ 7.5 for Cu^{2+}- and pH ≈ 8.5 for Ni^{2+}-loaded supports, is adequate for the chromatography of most of the examined peptides. However, for strongly bound compounds such as **11** gradient elution will produce sharper peaks and reduce the time required for the separation (Fig. 11). Since strongly basic conditions should normally be avoided in the handling of peptides, elution by a gradient of decreasing, rather than increasing, pH or by inclusion of a complexing agent such as imidazole or ammonium chloride in the buffer is recommended. As can be seen in the chromatograms the elution profile is largely independent of the type of gradient used, although it seems that imidazole, at least in this case, gives the least satisfactory result. Imidazole, having a high affinity for the Cu^{2+}, displaces the relatively weakly bound peptides which are eluted in a sharp zone ahead of the imidazole. If 1 m*M* imidazole is included in the starting buffer the desired peptide binds weakly to the column and is only partially resolved from the acetylated side-products.

Analysis by RP-HPLC and PDMS (not shown)

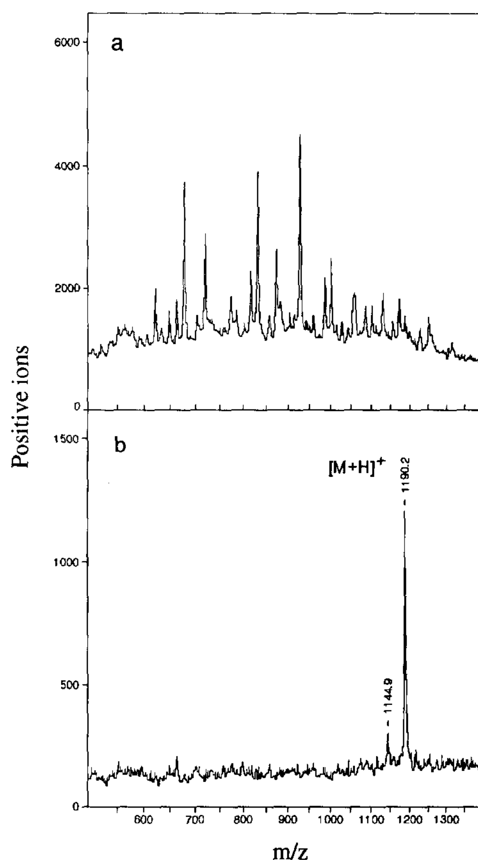

Fig. 10. PDMS analysis of (a) fraction I and (b) fraction II from IMAC of peptide 3 on Ni^{2+}-loaded Chelating Superose (Fig. 6). Conditions are as in Fig. 8.

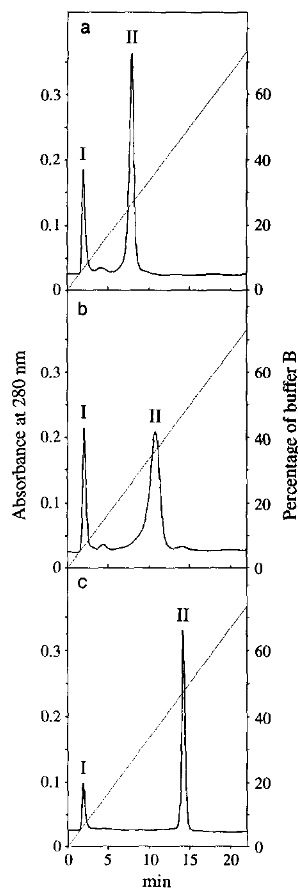

Fig. 11. Chromatography of crude peptide 11 on Chelating Superose–Cu^{2+} (1.5 × 1 cm I.D.). (a) Buffer A: 50 m*M* sodium phosphate–1 *M* sodium chloride, pH 8.0. Buffer B: 50 m*M* sodium phosphate–1 *M* sodium chloride, pH 5.0. Flow-rate: 1 ml/min. Sample: 1.25 mg dissolved in 250 μl of buffer A. (b) Buffer A: 50 m*M* sodium phosphate–1 *M* sodium chloride, pH 8.0. Buffer B: 50 m*M* sodium phosphate–1 *M* sodium chloride–0.1 *M* ammonium chloride, pH 8.0. Flow-rate: 1 ml/min. Sample: 1.25 mg dissolved in 250 μl of buffer A. (c) Buffer A: 50 m*M* sodium phosphate–1 *M* sodium chloride, pH 8.0. Buffer B: 50 m*M* sodium phosphate–1 *M* sodum chloride–20 m*M* imidazole, pH 8.0. Flow-rate: 1 ml/min. Sample: 0.3 mg dissolved in 60 μl of buffer A.

in all cases gives the same general pattern as that observed for peptide **3**: fraction I contains various acetylated peptides, whereas only peptide **11** is detected in fraction II.

CONCLUSIONS

Experimental evidence given in this report suggests that immobilized Cu^{2+} or Ni^{2+} supports can be used for simple and effective separation of side-products from peptides synthesized by the solid-phase method. Future work will clarify whether this mode of affinity purification can be applied to larger peptides and to those containing strongly metal-binding amino acids (His, Trp and Cys) in a suitably protected form.

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