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Purification of cysteine-containing synthetic peptides via selective binding of the α-amino group to immobilised Cu^{2+} and Ni^{2+} ions

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Abstract

Peptides containing a cysteine residue but lacking histidine and tryptophan were synthesised by the solid-phase method. Their retention behaviour on Cu^{2+}- and Ni^{2+}-loaded immobilised metal ion affinity chromatography (IMAC) supports at pH 5–11 was studied and compared with that observed for the corresponding compounds without the free α-amino group and/or the thiol function.

Unexpectedly, it was found that neither a cysteine side-chain nor a cystine disulphide affects the retention of the peptides. A free α-amino group is required for binding; no retention is observed in its absence. At pH 9 substantial amounts of metal ions were transferred from the chromatographic support to an α-amino-protected cysteine-containing peptide. However, at pH 7 no such transfer occurred. Therefore, the lack of retention observed for peptides with a blocked α-amino function over the entire pH range is not solely caused by metal ion scavenging by the thiol group. Partial dimerisation may occur upon chromatography; the dimers formed are retained strongly due to the presence of two free α-amino groups.

It seems that IMAC on a Cu^{2+}- or Ni^{2+}-loaded support can be used for the purification of cysteine-containing peptides synthesised by the solid-phase method. Inclusion of a capping protocol in the synthesis ensures that a free α-amino group, which can be used as an affinity handle, will be present only on the target peptide.

Keywords: Immobilized metal ion affinity chromatography; Peptides; Cysteine-containing peptides

1. Introduction

Synthetic peptides are widely used in research, for example in epitope mapping, as peptidomimetics and for active site probing. In some cases the peptide purity is not a matter of great concern, for instance in immunisation, but when biological activities are investigated the use of a homogenous product is imperative. Occasionally, impurities formed during synthesis, such as a peptide with a thioanisole-modified tryptophan

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[1] or a benzyl-modified cysteine residue [2], have been shown to exhibit unexpected activity.

RP-HPLC is commonly used for purifying synthetic peptides, but separation of the required peptide from the closely related side products is not always possible. More specific purification methods have been developed where the inclusion of a capping step in the solid-phase synthesis protocol is used to permanently block unreacted amino groups after each coupling step. As a consequence, only the full-length peptide will carry a free α-amino moiety at the end of the synthesis. An affinity handle can be specifically introduced on the target peptide via the free α-amino group to facilitate the purification [3–8]. The limitations of such affinity purification methods, and in particular the problems associated with the introduction and removal of the substituent, made us investigate the use of the free α-amino group as an affinity handle.

We found that we could purify synthetic peptides lacking cysteine [9–11] by immobilised metal ion affinity chromatography (IMAC) [12] based on the interaction of the free α-amino group with immobilised metal ions [13–18]. This method complements standard purification methods in a straightforward way since no modification of the target peptide is required. The interaction between the α-amino group and the immobilised metal ion was found to be strong at neutral pH for Cu^{2+} and at slightly basic pH for Ni^{2+}. Other metal ions, such as Zn^{2+}, Co^{2+} and Mn^{2+}, did not interact with the α-amino group sufficiently to retain the peptides investigated [9]. The ϵ-amino group of lysine binds to Cu^{2+} above pH 9 [9]. Other side-chains considered responsible for the binding of proteins and peptides in IMAC are the imidazole group of histidine, the thiol group of cysteine and the indole group of tryptophan [12,17,19–22]. We have shown that the indole group is not in itself sufficient to cause retention in IMAC (Cu^{2+} and Ni^{2+}) but can contribute to the binding when the peptide is first anchored to the immobilised metal ion, for example, via an α-amino group [10]. Peptides containing a histidine residue are very strongly retained on immobilised Cu^{2+}. However, with Ni^{2+} strong binding is observed only if the α-amino group is free. Blocking of this function reduces the k value by a factor of 5–10. In fact, the α-amino group seems to be as important for the binding to Ni^{2+} as the imidazole moiety [11].

In this paper we examine the retention of cysteine-containing peptides on immobilised metal ions. Although this view has been questioned [23], it is commonly assumed that cysteine has a strong affinity towards immobilised metal ions of the transition type [12,17,19–22] causing cysteine peptides to be strongly retarded on IMAC columns. In this case, no separation between the target peptide, with its free α-amino group, and the undesired side products, lacking the α-amino group but containing a cysteine residue, would be possible. To prevent the non-discriminate binding of cysteine peptides to IMAC columns, and also to circumvent oxidation of the thiol group on exposure to metal ions [24,25], we blocked the cysteine thiol with various protecting groups commonly used in peptide synthesis. However, for the peptides studied in this report the thiol group does not affect the retention on IMAC columns. Binding is observed only for peptides having a free α-amino group.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade and used as purchased. *tert.*-Butyloxycarbonyl (Boc) amino acids were obtained from Peninsula Laboratories Europe (St. Helens, UK) or Novabiochem (Läufelfingen, Switzerland) and 9-fluorenylmethyloxycarbonyl (Fmoc) amino acids and resins were from Millipore (Sundbyberg, Sweden). Boc-amino acyl resins were prepared according to Horiki et al. [26]. Chelating Superose was obtained from Pharmacia (Uppsala, Sweden).

2.2. Buffers

The chromatographic buffers (pH range 5–11) contained 50 m*M* sodium dihydrogen phosphate,

50 m*M* boric acid and 1 *M* sodium chloride and were prepared as described previously [9].

2.3. Peptide synthesis

Solid-phase synthesis of peptides was performed on either an Applied Biosystems 430A instrument as described earlier [9] or a Protein Technologies Symphony instrument, using Fmoc chemistry and a capping protocol similar to that previously used [9].

Branched peptides were obtained by the introduction of Boc-Lys(Boc)-OH, allowing both the α- and the ϵ-amino group to react with the next amino acid in the synthesis. Double couplings of each consecutive amino acid were performed after the incorporation of the lysine derivative.

The peptides are listed in Table 1 and will hereafter be referred to in bold numbers with indication of a free (**A**) or blocked (**B**) α-amino group.

2.4. IMAC

All chromatography was performed with an FPLC system (Pharmacia, Uppsala, Sweden) and the conditions for IMAC were as described earlier [9]. The peptides (100 nmoles in 100 μl of the chromatographic buffer) were applied to the column with isocratic elution and the capacity factor (k) determined according to:

$$k = V_e/V_0 - 1$$

where V_e is the retention volume for the peptide on a metal-loaded column and V_0 the retention volume on a metal-free column.

Table 1
Metal analysis of peptide-containing IMAC fractions

pH	Metal	Peptide	Metal/peptide ratio
7	Cu	**1B**	0.0
7	Cu	**2B**	0.0
7	Ni	**1B**	0.1
7	Ni	**2B**	0.1
9	Cu	**1B**	0.0
9	Cu	**2B**	0.3
9	Ni	**1B**	0.0
9	Ni	**2B**	0.2

Samples: 200–400 nmoles of peptide in 500 μl 50 m*M* sodium phosphate–borate buffer containing 1 *M* NaCl. Column: Chelating Superose-Me^{2+} (1.8 × 1 cm I.D.). Elution: sample buffer at 1 ml/min. Detection: UV at 280 nm. Peptide concentrations in applied samples were determined spectrophotometrically at 280 nm and the metal content of column effluents and peptide-containing fractions by atomic absorption spectroscopy.

2.5. Mass spectrometry

Synthetic products and chromatographic fractions were analysed by plasma desorption mass spectrometry (PDMS) using a BioIon 20 instrument (Applied Biosystems, Uppsala, Sweden) as described earlier [9].

2.6. Metal analysis

The content of metal in effluents and peptide-containing fractions was determined by atomic absorption spectroscopy (Mikro-Kemi, Uppsala, Sweden).

2.7. Thiol analysis

The thiol concentration in peptide solutions was determined by spectrophotometric measurement at 343 nm ($\epsilon = 7060$ l mole^{-1} cm^{-1}) after reaction with 2,2′-dipyridyldisulphide [27] at pH 7.0.

3. Results and discussion

Analogues of the model peptides **1** and **4** were synthesised with the Val in position 9 and 8, respectively, replaced by either Cys with a free thiol group (peptides **2** and **5**) or S-acetamidomethyl-cysteine, Cys(Acm), (peptides **3** and **6**). In addition, analogues of peptide **1** with *tert.*-butyl (*t*-Bu) and 3-nitro-2-pyridinesulphenyl (Npys) protected Cys were synthesised.

The effect of the cysteine residue on the retention of peptides on immobilised Cu^{2+} and Ni^{2+} was investigated and the results are shown in Figs. 1 and 2. Except for the lysine-containing

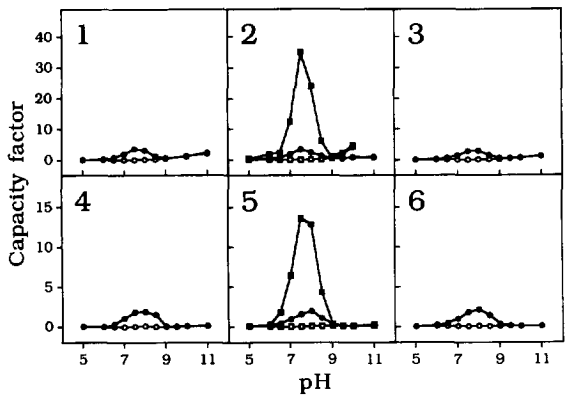

Fig. 1. Capacity factors vs. pH of peptides **1–6** on immobilised Cu^{2+}. The number in each square corresponds to the number of the peptide investigated (Table 2). Peptides with free α-amino groups are indicated with (●, ■) and blocked with (○, □). Monomers of the peptides are marked (●, ○) and dimers (■, □). Column: Chelating Superose (1.8 × 1 cm I.D.) charged with Cu^{2+}. Elution: isocratic with 50 m*M* sodium phosphate–borate, 1 *M* NaCl at 1 ml/min. Sample: 100 nmoles of peptide dissolved in 100 μl of chromatographic buffer. Detection: UV at 280 nm.

peptides **1B–3B** (Cu^{2+}, pH > 9), none of the N-terminally blocked peptides **1B–6B** showed any retention on either Cu^{2+}- or Ni^{2+}-loaded columns. In view of earlier reports [12,17,19–22], these findings were unexpected. That the peptides **2B** and **5B** with free cysteine thiol groups but blocked N-termini were not retained, suggests that the cysteine residue does not contribute to the binding of proteins and peptides in IMAC.

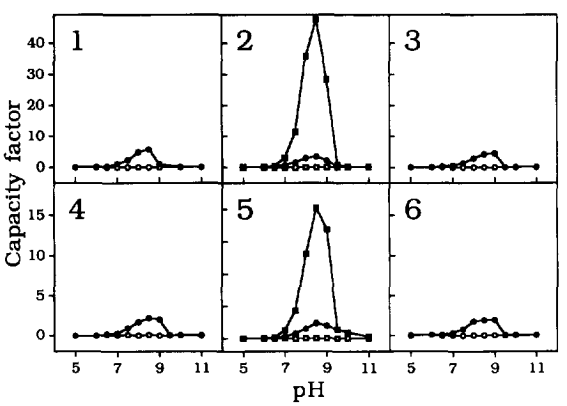

Fig. 2. Capacity factors vs. pH of peptides **1–6** on immobilised Ni^{2+}. Chromatographic conditions as in Fig. 1.

When evaluating the capacity factors for peptides **1A–6A** with free α-amino groups, we found that both the cysteine peptides **2A** and **5A** and the Acm-protected peptides **3A** and **6A** were retained to almost exactly the same extent as the model peptides **1A** and **4A**. The capacity factors increased from pH 5 to 7.5 on immobilised Cu^{2+} (Fig. 1) and from pH 5 to 8.5 on immobilised Ni^{2+} (Fig. 2) and then decreased as the pH was further raised to 9 (Cu^{2+}) or 9.5 (Ni^{2+}). Only the lysine-containing peptides **1A–3A** showed increasing capacity factors from pH 9 to 11 and only on immobilised Cu^{2+}. These findings suggest that the binding occurs by the same mechanism as proposed earlier [9], where the peptide is initially bound via the α-amino group and the neighbouring amide oxygen. The thiol group of cysteine does not seem to contribute to the binding significantly, as indicated by the similarities in retention of the free cysteine peptides, the S-protected peptides and those lacking cysteine.

The lack of influence by the thiol function on the elution behaviour, observed both for peptides with a free and with a protected α-amino group, may be a result of very low metal affinity. However, high affinity leading to the transfer of the metal ion from the matrix to the thiol group (and neighbouring amide nitrogens) provides an alternative explanation [21,23]. In order to eliminate this ambiguity we decided to examine the thiol–metal interaction for the peptide **2B** in some detail. A peptide without a free α-amino group was chosen since this was expected to reduce the complexity of the analysis.

The peptides **1B** and **2B** were chromatographed on Cu^{2+}- or Ni^{2+}-loaded Chelating Superose at pH 7.0 and 9.0. The buffers were carefully deaerated by air suction and then purged with oxygen-free helium during the chromatographic runs. The metal content (Cu or Ni) of the peptide-containing fractions and column effluents was assessed by atomic absorption spectroscopy. The results are presented in Table 1. At pH 7 no scavenging of Cu^{2+} was observed. In the case of Ni^{2+}, some metal appeared in all

Table 2
Structure of peptides used in this investigation

Peptide	Structure	Reference
1A	Gly-Ala-Thr-Lys-Gly-Pro-Gly-Arg-Val-Ile-Tyr-Ala	Ref. [9]
1B	Ac-Gly-Ala-Thr-Lys-Gly-Pro-Gly-Arg-Val-Ile-Tyr-Ala	Ref. [9]
2A	Gly-Ala-Thr-Lys-Gly-Pro-Gly-Arg-**Cys**-Ile-Tyr-Ala	
2B	Ac-Gly-Ala-Thr-Lys-Gly-Pro-Gly-Arg-**Cys**-Ile-Tyr-Ala	
3A	Gly-Ala-Thr-Lys-Gly-Pro-Gly-Arg-**Cys**(Acm)-Ile-Tyr-Ala	
3B	Ac-Gly-Ala-Thr-Lys-Gly-Pro-Gly-Arg-**Cys**(Acm)-Ile-Tyr-Ala	
4A	Leu-Glu-Leu-Arg-Ser-Arg-Tyr-Val-Ala-Ile-Arg-Thr-Arg-Ser-Gly-Gly-NH_2	Ref. [10]
4B	Ac-Leu-Glu-Leu-Arg-Ser-Arg-Tyr-Val-Ala-Ile-Arg-Thr-Arg-Ser-Gly-Gly-NH_2	Ref. [10]
5A	Leu-Glu-Leu-Arg-Ser-Arg-Tyr-**Cys**-Ala-Ile-Arg-Thr-Arg-Ser-Gly-Gly-NH_2	
5B	Ac-Leu-Glu-Leu-Arg-Ser-Arg-Tyr-**Cys**-Ala-Ile-Arg-Thr-Arg-Ser-Gly-Gly-NH_2	
6A	Leu-Glu-Leu-Arg-Ser-Arg-Tyr-**Cys**(Acm)-Ala-Ile-Arg-Thr-Arg-Ser-Gly-Gly-NH_2	
6B	Ac-Leu-Glu-Leu-Arg-Ser-Arg-Tyr-**Cys**(Acm)-Ala-Ile-Arg-Thr-Arg-Ser-Gly-Gly-NH_2	
7A	(Gly-Ala-Thr-)$_2$Lys-Gly-Pro-Gly-Arg-Val-Ile-Tyr-Ala	
7B	(Ac-Gly-Ala-Thr-)$_2$Lys-Gly-Pro-Gly-Arg-Val-Ile-Tyr-Ala	

peptide-containing fractions. It was found that nickel on this gel, despite extensive washing with various buffers, displayed some unspecific leakage at pH 7. However, it is obvious that the withdrawal of Ni^{2+} from the column was not caused by the thiol group. At pH 9 substantial amounts of metal were detected in the fractions containing the cysteine peptide indicating a strong interaction between the thiol and the immobilised metals. In view of the high pH, a simple model for the scavenging mechanism would be the formation of a complex between the metal and the thiolate (S^-) moiety.

MS and thiol analysis confirmed that the samples applied onto the column were essentially free from dimers. However, the analysis also revealed the presence of dimer as well as monomer in all chromatographic fractions containing the peptide **2B**. Attempts to determine the thiol recovery by reaction with 2,2′-dipyridyldisulphide were complicated by the presence of dimer and small amounts of metal ions which interfered with the analysis. There was no doubt, however, that substantial amounts of thiol were present in all examined peptide fractions. At pH 7 more than 35% of the thiol was recovered from the Cu^{2+}-loaded column, and more than 80% from the Ni^{2+}-loaded column. At pH 9 reliable estimates could not be made because of extensive peak-broadening and concomitant sample dilution. It seems that the single exposed cysteine thiol group does not coordinate to immobilised Cu^{2+} or Ni^{2+} at neutral pH. It remains to be studied whether such a coordination can take place in the presence of a second electron donor like the unprotonated amino or imidazole group. However, it is clear that the low retention observed for the α-amino-protected cysteine peptides over the entire pH range investigated is not solely due to scavenging of the metal ions.

When peptides **2A** and **5A** were chromatographed without helium purging of the buffers an additional peak was observed eluting very late in the run. PDMS analysis showed that these peaks contained dimers of the peptides. Dimerisation occurred also with the N-terminally blocked peptides **2B** and **5B** but in these cases the dimers were not retained on the IMAC adsorbent and were only detected by PDMS of the breakthrough material. Dimers of peptides **2A**, **2B**, **5A** and **5B** were formed over the entire pH range, albeit to a small extent, and their capacity factors (indicated by closed (**A**) and open (**B**) squares) are included in Figs. 1.2, 1.5, 2.2 and 2.5.

The capacity factors of the dimers of peptides **2A** and **5A** were high, up to seven times higher than for the corresponding monomers. The increased retention is presumably not caused by

interactions of the disulphide bridge with the metal ion since the dimers of **2B** and **5B** are not bound. It seemed that a more likely explanation for the retention was the presence of two α-amino groups in the dimeric material. To examine the effect of the number of α-amino groups on dimer binding we constructed a set of dimers by air-oxidising an equimolar mixture of peptides **2A** and **2B** in the chromatographic buffer for 24 h. The dimers formed were: (a) **2A2A** with two free α-amino groups; (b) **2B2B** with two blocked α-amino groups; and (c) **2A2B** with one free and one blocked α-amino group. The dimer mixture was chromatographed on immobilised Cu^{2+} and Ni^{2+} and the fractions were analysed by PDMS (Fig. 3). Peptide **2B2B** appeared in the void volume (I), **2A2B** eluted approximately where peptide **2A** normally appeared (II) and **2A2A** had the very high retention observed previously for the unblocked dimers (III). These results suggest that it is the number of free α-amino groups and not the presence of a disulphide bridge that determines the elution order in IMAC. This was confirmed by studying branched peptides. As shown in Fig. 4, the branched peptide **7A** has higher capacity factors than the model peptide **1A** (Figs. 1.1 and 2.1) and the corresponding acetylated branched peptide **7B**.

To avoid the formation of dimers during the chromatography of cysteine peptides, we blocked the thiol function with various protecting groups commonly used in peptide synthesis. Peptides **3A** and **6A**, protected with an Acm group, did not show the additional peaks in IMAC, and PDMS analysis of the chromatographic fractions revealed that the molecular mass of the peptides was unchanged. The capacity factors are in the same range as for the model peptides **1A** and **4A** indicating that the protected thiol group does not contribute to the binding. Similar results were obtained with the *t*-Bu-protected analogue of peptide **2A** (data not shown). Npys as a protecting group for cysteine, on the other hand, was found to be unstable on exposure to immobilised metal ions above pH 7 and the cleavage product

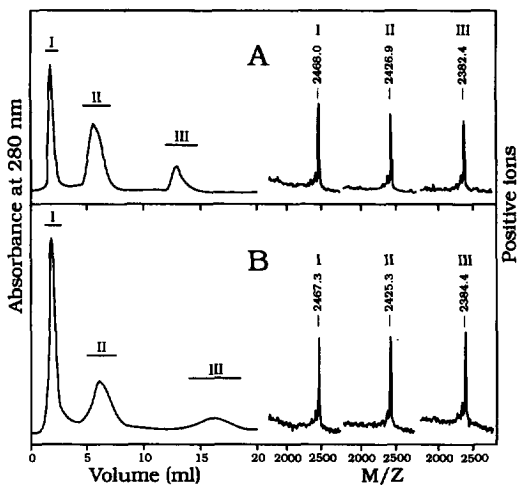

Fig. 3. Chromatography of the dimers **2A2A**, **2A2B** and **2B2B** on immobilised Cu^{2+} (A) and Ni^{2+} (B). Column: Chelating Superose (1.8 × 1 cm I.D.). Sample: resulting solution after air-oxidation of **2A** and **2B** (200 nmoles each) in 200 μl of 50 m*M* sodium phosphate–borate, 1 *M* NaCl, pH 7.5 (A) or pH 8.5 (B). Elution: 30-min gradient from pH 7.5 to 5.0 (A) or from 0 to 0.5 *M* NH_4Cl (B) in the application 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 washed with 0.1% aqueous TFA and the peptides eluted with a steep acetonitrile gradient (0–60% in 1 min). The desalted samples (5 μl) were mixed with ethanol (2 μl) on nitrocellulose-coated aluminium foils, dried and rinsed with distilled water (20 μl). The calculated molecular mass for dimer **2A2A** is 2384.8, for **2A2B** 2426.8 and for **2B2B** 2468.8.

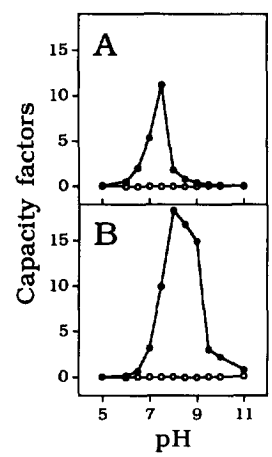

Fig. 4. Capacity factors vs. pH of the branched peptides **7A** (●) and **7B** (○) on immobilised Cu^{2+} (A) and Ni^{2+} (B). Chromatographic conditions as in Fig. 1.

could be observed visually as a yellow band migrating slowly through the column. Apart from the expected monomer, the material after the chromatographic run also contained a small amount of the dimer. In addition, monomer with the molecular mass increased by ca. 32 amu, presumably due to oxidation of the cysteine thiol to the corresponding sulphinic acid [24] was observed as a minor component. In many cases, there is no need for a protecting group on the cysteine residue since the dimers that occur after IMAC purification can readily be reduced to monomers. However, where protecting groups are required, as in the unambiguous formation of specific disulphide bridges, care must be taken in their selection.

The cysteine residues in a peptide with an internal disulphide bridge are already protected and will not alter during the chromatography. We have used [Arg^8]-vasopressin (AVP, Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2; S–S bridge between Cys and Cys) and its deamino analogue (dAVP, Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2, Mpa = β-mercaptopropionic acid; S–S bridge between Mpa and Cys) to illustrate the retention behaviour of a disulphide-containing peptide on immobilised Cu^{2+} and Ni^{2+} (Fig. 5). The effects are similar to those described above. dAVP lacking an N-terminal amino group is not retained, confirming that the presence of a disulphide bridge does not affect the retention. AVP which has a free α-amino function is retained, although the retention maximum is lower than expected on both metals. These results are supported by Yip et al. [16] who studied the adsorption properties of several synthetic, biologically active peptides in IMAC. Three of these are of interest here: [Asu^{1,6}]-oxytocin (Tyr-Ile-Gln-Asn-Asu-Pro-Leu-Gly-NH_2, Asu = L-α-aminosuberic acid; ω-carboxyl of Asu linked to α-amino group of Tyr) was not retained at pH 7 presumably due to the absence of an N-terminal amino group. In oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2; S–S bridge between Cys and Cys) (also studied by Belew et al. [17]) and [Phe^4]-oxytocin (Cys-Tyr-Ile-Phe-Asn-Cys-Pro-Leu-Gly-NH_2; S–S bridge between S and S) both of which were retained, though to differing degrees, a free α-amino group is present. Furthermore, PDMS analysis of IMAC fractions of AVP and dAVP showed that no dimerisation had occurred.

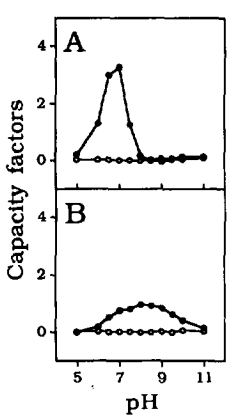

Fig. 5. Capacity factors vs. pH of [Arg^8]-vasopressin (●) and its deamino analogue [Mpa^1, Arg^8]-vasopressin (○) on immobilised Cu^{2+} (A) and Ni^{2+} (B). Chromatographic conditions as in Fig. 1.

The usefulness of IMAC for the isolation of synthetic cysteine peptides via interaction of the α-amino group with an immobilised metal ion is demonstrated in the purification of the peptide **2A**. The crude peptide was applied on Ni^{2+}-loaded Superose and eluted with an increasing gradient of NH_4Cl at pH 8.5 (Fig. 6A). Three major fractions were obtained, whereby fractions IIa and IIb were combined (fraction II). Dithiothreitol (DTT) was added to reduce the dimers formed during the chromatography. The reduced fractions were further analysed by HPLC and mass spectrometry (Figs. 6B–E). As expected, peptide **2A** was detected in the bound material (II) together with a minor impurity corresponding to a *t*-Bu-modified peptide **2A**. Truncated peptides that had been acetylated in the capping steps were present only in the non-retarded fraction (I).

However, the formation of dimers may affect the selectivity in an unfavourable way. It is quite likely that the acetylated truncated peptides participate in the dimerisation, thereby forming both homodimers and heterodimers. The resulting mixture will consist of peptides with two free α-amino groups (full-length homodimers),

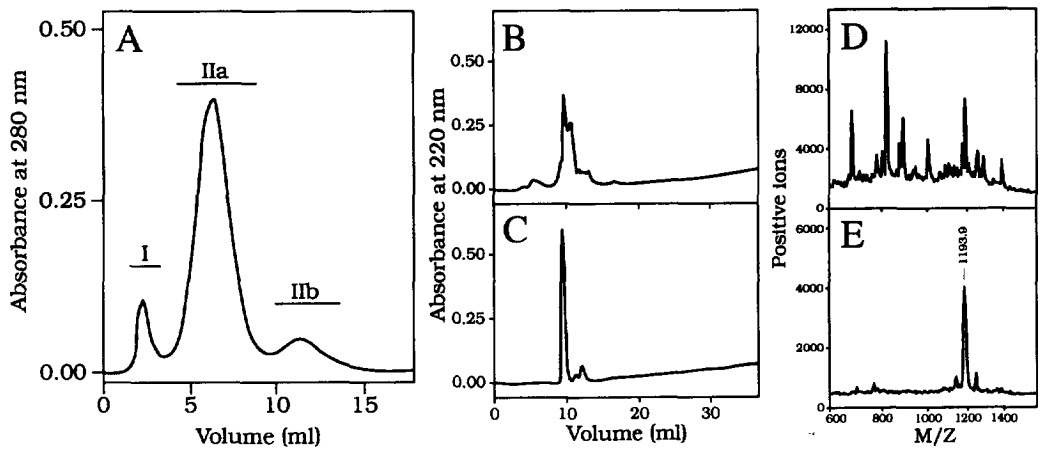

Fig. 6. (A) Chromatography of crude peptide **2A** on immobilised Ni^{2+}. Column: Chelating Superose (1.8 × 1 cm I.D.). Sample: 1.25 mg dissolved in 250 μl of 50 m*M* sodium phosphate–borate, 1 *M* NaCl, pH 8.5. Elution: 30-min gradient from 0 to 0.5 *M* NH_4Cl in the same buffer at 1 ml/min. The fractions I and II (IIa + IIb) were reduced to monomers by adding DTT to a final concentration of 1 mg/ml before further analysis. For mass spectrometry the chromatographic fractions were treated as in Fig. 3. (B) RP-HPLC of 1.2 ml (80%) of DTT-reduced fraction I (part A). 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. Gradient: 0–60% B in 30 min. (C) RP-HPLC of 0.55 ml (5%) of DTT reduced fraction II (part A). Chromatographic conditions as in part B. (D) PDMS of fraction I (part A). Conditions as in Fig. 3. Most peaks can be attributed to various acetylated peptides and their fragment ions. (E) PDMS of fraction II (part A). Conditions as in Fig. 3. The calculated molecular mass for peptide **2A** is 1193.4.

with one free (heterodimers and full-length monomers) and with only blocked α-amino groups (truncated monomers and dimers). Separation of the heterodimers and the full-length monomers with this method is not possible (cf. Fig. 3). A more complex mixture can be expected if more than one cysteine residue is present in the sequence.

When using this purification method for Cys-peptides it is advisable to work as quickly as possible and in a non-oxidising environment, or to block the cysteine residue with a suitable protecting group. An alternative, though elaborate way to isolate the desired peptide in pure form would be to perform a complete oxidation, e.g. by air or by potassium ferricyanide, before the purification. Thus the strongly retained homodimers can be isolated and reduced. If desired, additional material may be recovered by rechromatography of the reduced and reoxidised heterodimeric fraction.

Scavenging of the metal ion by the thiol group alone or in combination with proximal amide nitrogens is not expected to affect the elution characteristics of the peptide. If, however, the α-amino group is also involved in the metal ion transfer, the selectivity will be lost since binding of the peptide to the chromatographic support via the α-amino function will not be possible. In such cases, generating and purifying dimers according to the procedure described above should enable the separation of the target peptide provided the N-termini can be properly positioned on the metal ions.

4. Conclusion

We have shown that IMAC can be used for the selective purification of cysteine-containing synthetic peptides. The target peptide with its free α-amino group will be well separated from the truncated peptides. A particular advantage of this affinity method is that no modification of the target peptide is necessary. However, in some cases protection of the cysteine residue may be required.

It seems that neither a cysteine residue nor a

disulphide bridge will affect the retention on immobilised Cu^{2+} or Ni^{2+} ions. The strong binding observed for dimers can be ascribed to the presence of two α-amino groups in the molecule. We may also conclude that a thiol group is not in itself able to scavenge the metal ions at neutral pH. However, at pH 9 the presence of a cysteine residue in the sequence leads to substantial transfer of metal ions from the chromatographic support to the peptide even though the α-amino group is blocked.

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