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Journal of Chromatography A, 690 (1995) 155–159

JOURNAL OF CHROMATOGRAPHY A

Importance of the α-amino group in the selective purification of synthetic histidine peptides by immobilised metal ion affinity chromatography

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First received 14 February 1994; revised manuscript received 27 October 1994; accepted 8 November 1994

Abstract

The retention behaviour of some histidine containing peptides on Cu^{2+}- and Ni^{2+}-loaded immobilised metal ion affinity chromatography (IMAC) supports has been investigated and compared with that observed for the corresponding compounds lacking the free α-amino group and/or the imidazole function.

On immobilised Cu^{2+} all histidine-containing peptides, including those with a blocked α-amino function, were strongly retained above pH 5. The presence of a free α-amino group increased the retention marginally.

On immobilised Ni^{2+} histidine peptides with a free α-amino group were strongly bound with a maximal retention at pH 8.5. Blocking of the amino group or removal of the imidazole moiety reduced the maximal retention by a factor 5 to 10, with no retention observed for peptides lacking both histidine and a free α-amino group. These observations indicate the involvement of two equipotent attachment points in the binding.

It seems that IMAC on a Ni^{2+}-loaded support can be used for the purification of histidine 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.

1. Introduction

We have earlier shown that synthetic peptides lacking histidine can be purified by selective binding via the α-amino group to immobilised Cu^{2+} and Ni^{2+} ions [1–3]. The inclusion of a capping protocol in the solid-phase synthesis ensures that a free α-amino group is present only on the target peptide, whereas failure peptides resulting from incomplete couplings have blocked (acetylated) N-termini. Thus, the α-amino group can be used as a built-in affinity handle.

Tryptophan and cysteine residues are considered the most important, next to histidine, for the retention of peptides and proteins in immobilised metal ion affinity chromatography (IMAC) [4], and so might be expected to impair the selectivity of the purification procedure. However, it was found that the indole group in itself was not sufficient for binding to Cu^{2+}- or Ni^{2+}-loaded IMAC supports although it could contribute to the binding when the peptide was

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SSDI 0021-9673(94)01100-1

first anchored to the immobilised metal ion via the α-amino group [2]. The presence of a thiol group did not affect the retention, but partial oxidation to the dimer was observed [3]. The lack of influence in this case may be ascribed to metal ion transfer (MIT) from the support to the peptide where the metal ion is scavenged by the thiol, alone or in combination with neighbouring amide nitrogens.

For the inclusion of histidine containing peptides in the purification protocol based on interaction of the α-amino group with metal ions, a less favourable situation can be anticipated. The interaction of histidine with transition series metals is well documented [5], and the presence of histidine is a major factor in the binding of proteins [4,6,7] and peptides [8–11] in IMAC. Single N-terminal histidine residues have also been exploited for the purification of synthetic peptides by IMAC [12]. If peptides containing histidine are already tightly bound to the IMAC adsorbent, it is not clear whether additional retention due to the presence of a free α-amino group will be sufficient to achieve adequate separation of the blocked and unblocked peptides. In order to clarify this point in an unambiguous way, a set of histidine-containing peptides with free or protected α-amino groups were synthesised and their retention on Cu^{2+}- and Ni^{2+}-loaded supports investigated. The corresponding peptides without histidine were included as references.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade and used as purchased. *tert.*-Butyloxycarbonyl (Boc) amino acids were obtained from Peninsula Labs. Europe (St. Helens, UK) or Novabiochem (Läufelfingen, Switzerland) and 9-fluorenylmethyloxycarbonyl (Fmoc) amino acids and resins from were Millipore (Sundbyberg, Sweden). Boc-Amino acyl resins were prepared according to Horiki et al. [13]. Chelating Superose was obtained from Pharmacia (Uppsala, Sweden).

2.2. Buffers

The chromatographic buffers (pH range 5–11) contained 50 m*M* sodium dihydrogenphosphate, 50 m*M* boric acid and 1 *M* sodium chloride and were prepared as described previously [1].

2.3. Peptide synthesis

Solid-phase synthesis of peptides was performed on either an Applied Biosystems 430A instrument as described earlier [1] or a Protein Technologies Symphony instrument, using Fmoc chemistry and a capping protocol similar to that previously used [1].

The peptides are listed in Table 1 and will hereafter be referred to in bold numbers with

Table 1
Structure of peptides used in this investigation

Peptide	Structure	Ref.
1A	Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala	[1]
1B	Ac–Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala	[1]
2A	Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–**His**–Ile–Tyr–Ala	
2B	Ac–Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–**His**–Ile–Tyr–Ala	
3A	Leu–Glu–Leu–Arg–Ser–Arg–Tyr–Val–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2	[2]
3B	Ac–Leu–Glu–Leu–Arg–Ser–Arg–Tyr–Val–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2	[2]
4A	Leu–Glu–Leu–Arg–Ser–Arg–Tyr–**His**–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2	
4B	Ac–Leu–Glu–Leu–Arg–Ser–Arg–Tyr–**His**–Ala–Ile–Arg–Thr–Arg–Ser–Gly–NH_2	

indication of a free (**A**) or blocked (**B**) α-amino group.

2.4. IMAC

All chromatography was performed with a fast protein liquid chromatography (FPLC) system (Pharmacia) and the conditions for IMAC were as described earlier [1]. The peptides (100 nmol 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.

2.5. Mass spectrometry

Synthetic products and chromatographic fractions were analysed by plasma desorption mass spectrometry (PD-MS) using a BioIon 20 instrument (Applied Biosystems, Uppsala, Sweden) as described earlier [1].

3. Results and discussion

Peptides **2** and **4** were derived from the reference peptides **1** and **3** by a substitution of His for Val in position 9 and 8, respectively.

The retention behaviour of the peptides on a Cu^{2+}-loaded IMAC column was investigated (Fig. 1). The reference peptides **1A** and **3A** have been described earlier [1,2] and show increasing retention as the pH is raised due to deprotonation of the free α-amino group. Increasing the pH above 7.5 results in decreased retention caused by metal ion transfer from the chromatographic support to the peptide. The lysine-containing peptides **1A** and **1B** are retained above pH 9 as a result of the deprotonation of the ϵ-amino group. Peptides **1B** and **3B** with blocked N-termini do not show any retention between

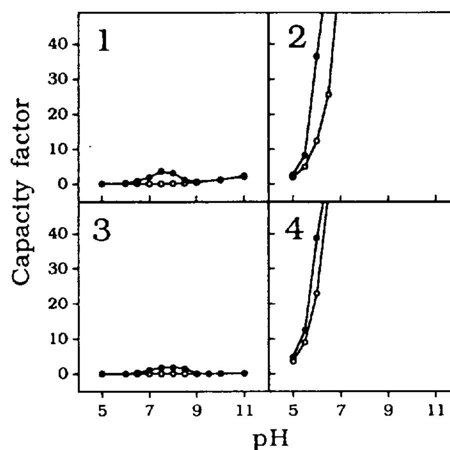

Fig. 1. Capacity factors vs. pH of peptides **1–4** on immobilised Cu^{2+}. The number in each square corresponds to the number of the peptide investigated (Table 1). Peptides with free α-amino groups are indicated with ● and blocked with ○. Column: Chelating Superose (1.8 × 1 cm I.D.) charged with Cu^{2+}. Elution: isocratic with 50 mM sodium phosphate–borate, 1 M NaCl at 1 ml/min. Sample: 100 nmol of peptide dissolved in 100 μl of chromatographic buffer. Detection: UV at 280 nm.

pH 5 and 11, other than the lysine effect observed with peptide **1B**.

The imidazole group of the histidine residue has a lower pK_a value (ca. 6.5) than the α-amino group (ca. 8). Consequently, deprotonation and binding of histidine peptides is expected to occur at lower pH values than for peptides without histidine. Indeed, both the N-protected and free peptides **2** and **4** bind strongly even at pH 5.5, and above pH 6.5 they all have capacity factors greater than 50 (Fig. 1). Obviously, the α-amino group contributes to the binding. The capacity factors for the unblocked peptides are higher than for the acetylated ones. However, because of the strong interaction between histidine and Cu^{2+}, the relative importance of this contribution is small.

The peptides were also analysed on a Ni^{2+}-loaded column (Fig. 2). The behaviour of the reference peptides **1** and **3** is similar to that observed on Cu^{2+}, but maximal retention occurs at pH ≈ 8.5 and binding via the lysine ϵ-amino

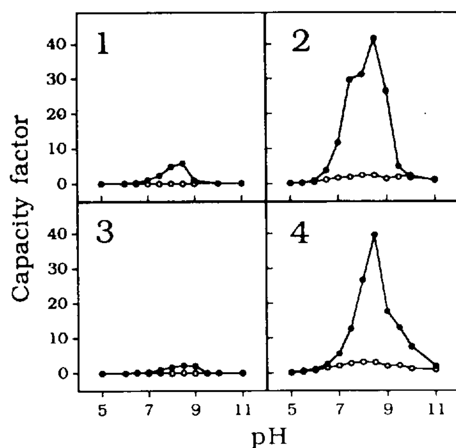

Fig. 2. Capacity factors vs. pH of peptides **1–4** on immobilised Ni^{2+}. Indications and chromatographic conditions as in Fig. 1.

group is not observed [1,2]. However, the influence of the histidine residue, as seen in the binding of peptides **2** and **4**, is considerably less on Ni^{2+} than on Cu^{2+}. The N-terminally blocked histidine peptides **2B** and **4B** are only weakly bound, having even lower capacity factors than the unblocked reference peptides **1A** and **3A**. An entirely different behaviour is observed for the histidine peptides with free α-amino groups (**2A** and **4A**) which are strongly retained. The capacity factors are 5–10 times higher than for the corresponding peptides lacking the α-amino or the imidazole group suggesting the involvement of two equipotent attachment points in the binding.

The use of IMAC for separation of histidine-containing peptides with free α-amino groups from those with blocked N-termini is illustrated in Fig. 3. On a Cu^{2+}-loaded IMAC column, separation of the peptides **2A** and **2B** can be accomplished by elution with a decreasing pH gradient (Fig. 3A), but with peptides **4A** and **4B** baseline separation is not achieved (Fig. 3B). As can be expected from the results in Fig. 1, the contribution by the α-amino group to the binding of the peptide, although evident, is not always sufficient to allow separation on this support. However, on a Ni^{2+}-loaded column separation is easily achieved either by elution

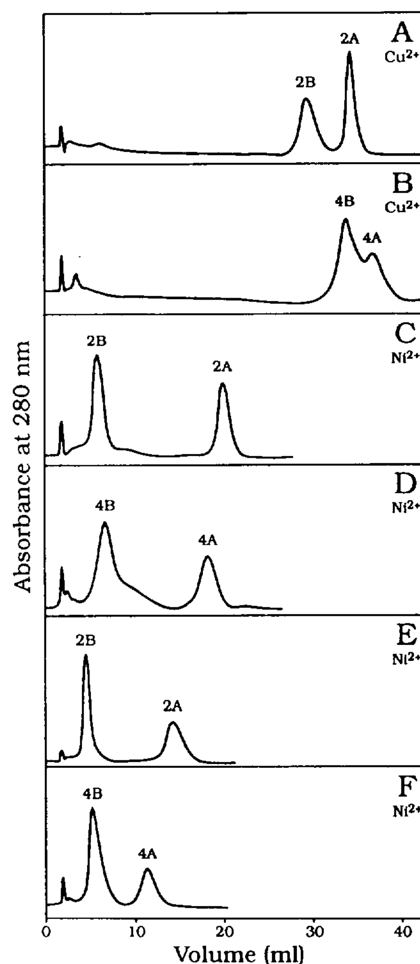

Fig. 3. Chromatography of mixtures of peptides with and without free N-termini on immobilised Cu^{2+} (A and B) and Ni^{2+} (C–F). One mixture contained the peptides **2A** and **2B** (A, C and E) and the other **4A** and **4B** (B, D and F). Column: Chelating Superose (1.8 × 1 cm I.D.). Sample: 100 nmol of each peptide dissolved in 200 μl of 50 mM sodium phosphate–borate, 1 M NaCl, pH 7.5 (Cu^{2+}) or pH 8.5 (Ni^{2+}). Elution: 30 min linear gradient from pH 7.5 to 5.0 (A and B), pH 8.5 to 5.0 (C and D) or 0 to 0.5 M NH_4Cl in the pH 8.5 buffer (E and F) at 1 ml/min.

with a decreasing pH gradient or with an increasing NH_4Cl gradient (Fig. 3C–F).

In other affinity methods designed for purification of synthetic peptides, special affinity handles are introduced on the α-amino group of the target peptide while this is still being attached to the resin (e.g. Refs. [14–19]). The decision of whether or not to use an affinity handle has to be

made prior to cleavage. After the peptide has been cleaved from the resin, there is no easy way to selectively introduce an N-terminal affinity handle since functional groups in the side chains are no longer protected from attack by the derivatisation agent. On the other hand, it might be an unnecessary effort to introduce an affinity handle in cases where standard purification methods would suffice. In this method the separation is based on the interaction of the free α-amino group with immobilised metal ions and such considerations need not be taken in account. In addition, material losses associated with the introduction and removal of an additional affinity handle can be avoided and the solubility of the peptide is not affected. Since no modification of the target peptide is required, the method complements standard purification procedures such as RP-HPLC and ion-exchange chromatography in a straightforward way.

There may be cases where selectivity is not achievable. Truncated peptides with two or more histidines may be retained too strongly to allow their separation from the target peptide. Combinations of histidine and tryptophan may also cause problems. As on previous occasions, impurities resulting from post-synthetic modifications (alkylation, oxidation etc.) or incomplete deprotection are not amenable to separation by this method. However, their removal in a subsequent RP-HPLC step should be facilitated.

4. Conclusions

Earlier work [1–3] has established that selective purification based on the affinity of the α-amino group for Cu^{2+} and Ni^{2+} ions can be achieved for peptides lacking histidine provided a capping procedure is included in their solid-phase synthesis. We can now conclude that the method is also useful for the purification of synthetic peptides containing histidine. On a Ni^{2+}-loaded IMAC support, the target peptide with its free α-amino group will be strongly retained whereas truncated peptides with blocked α-amino functions will show very low or, if they contain histidine, moderate retention. A free α-amino group seems to be at least equally important for the binding to Ni^{2+} as an imidazole function. On immobilised Cu^{2+}, baseline separation is not always achievable.

Acknowledgement

We are grateful to Dr. Lennart Andersson for helpful suggestions and valuable discussions and to Ms. Judith Scoble for linguistic revision of the manuscript. This project was supported by the Swedish National Board for Industrial and Technical Development (NUTEK).

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