# Chapter 12

# Chelating Peptide-Immobilized Metal-Ion Affinity Chromatography

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A new method for purifying recombinant proteins called, Chelating Peptide-immobilized Metal Ion Affinity Chromatography, or CP-IMAC is described. This method is based on the hypothesis that a specific chelating peptide (CP) with a high affinity for immobilized metal ions can be used to purify a recombinant protein by extending the gene sequence of the recombinant protein to code for the extra amino acids in the chelating peptide sequence. The resulting fusion protein can then be purified using immobilized metal ion affinity chromatography (IMAC). A four step strategy was followed to develop CP-IMAC: identifying chelating peptides, attaching a CP to a polypeptide to test the feasibility of this concept, purifying a recombinant CP-protein, and lastly purifying a CP-X-protein where X is an enzymatic cleavage site which allows the CP to be removed so as to generate the desired protein.

Recombinant DNA methods have provided the means for obtaining large quantities of interesting proteins whose supply has been limited or nonexistent. The prospects of such a supply and the potential applications of such proteins, have spawned a large biotechnology industry. Recombinant protein purification is a time consuming process critical for obtaining large amounts of protein. In addition, protein purification is the one step in the process that must be continually repeated, compared to the construction of the expression vector, transformation of the host organism, and optimization of fermentation conditions.

The need for more efficient purification methods is clear and presents some unique opportunities for proteins synthesized by recombinant DNA methods. Access to the gene which codes for the protein affords one the opportunity to extend the gene to code for additional amino acids and create a fusion protein which exaggerates a given property that can be exploited for purification purposes. These additional amino acids which provide for a more efficient purification step, can then be removed either chemically or enzymatically after the purification, to generate the desired protein sequence.

The first examples of recombinant proteins with purification handles were reported in 1984 (1-2). Sassenfeld and Brewer (1) efficiently purified a urogastrone

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fusion protein with a C-terminal polyarginine sequence over an anion exchange column. The C-terminal position of the polyarginine sequence allowed it to be removed enzymatically with carboxypeptidase B. Bastia and co-workers (2) fused ß-galactosidase to collagen followed by the protein of interest, the R6K replication initiator. The ß-galactosidase portion allows the fusion protein to be purified over an affinity column, while the collagen sequence which is sensitive to collagenase, provides the cleavage site. More recent examples of recombinant proteins with purification handles, include staphylococcal protein A fusion proteins with specific chemical cleavage sites (3), and chimeric proteins with an eight residue peptide which is recognized by a specific antibody for affinity purification and the enzyme enterokinase for subsequent cleavage (4).

The property we set out to transfer to a recombinant protein to facilitate the purification process is the ability to bind immobilized transition metal ions. We hypothesized that a few extra amino acids which bind metal ions, could be cloned onto N-terminus of the recombinant protein, and allow the expressed protein to be purified using immobilized metal ion affinity chromatography (IMAC). The same amino acid sequence, or chelating peptide (CP) could be cloned on the end of new recombinant proteins and the same purification scheme applied, thus eliminating extensive methods development for each new recombinant protein. This approach, called CP-IMAC, would take advantage of the untapped power of IMAC by making it predictable and therefore a more useful purification method. The relative cost and availability of simple metal salts used to prepare IMAC columns is a significant advantage over the cost of antibodies and other reagents used to prepare affinity columns.

IMAC was introduced in 1975 by Porath who showed that serum proteins could be fractionated over columns containing immobilized metal ions, such as copper, nickel, and zinc (5). A number of proteins have been purified using IMAC since its introduction (6). Figure 1 is an illustration of the immobilized iminodiacetic acid complex formed with metal ions, which leaves up to three open coordination sites for binding to a protein with accessible donor atoms or ligands. The open coordination sites are occupied by rapidly exchanging water molecules which can be displaced by the incoming protein which has a higher affinity for the metal ion than water molecules. The bound protein can be eluted by either lowering the pH of the buffer or by introducing a displacing ligand which competes with the protein for the metal coordination sites and displaces the protein.

Porath attributes protein binding to immobilized metal ions to the presence of cysteine, histidine, and tryptophan residues (5). There is ample precedence for cysteine and histidine binding metals in well characterized metalloproteins, metal peptide complexes, and metal amino acid complexes (7-8). There are no known examples of tryptophan coordinating metal ions through the indole group, either as amino acid complexes, peptide complexes, or in metalloproteins.

Studies aimed at understanding the mechanism of protein binding to immobilized metals have lead to the hypothesis that the number of exposed histidine and/or cysteine residues determines the strength of the interaction (9). Frontal analysis of model proteins on IMAC columns has been carried out recently, which provides quantitative estimates of the affinity constants of these proteins for immobilized metal ions and binding capacities (10-11). This approach provides a basis for comparing the chromatographic behavior of proteins on IMAC columns and establishes a database to better understand how proteins interact with immobilized metal ions. Spectroscopic structural studies of the complexes formed on the resin, would provide unambiguous answers to questions raised about the binding mechanism of proteins to immobilized metal ions. If the crystal structure of a protein has been determined then the surface exposure of histidine residues can be established and used to design a separation using IMAC. For proteins whose three dimensional structure is

unknown, which includes the vast majority of proteins, trial and error is the best that can be done. Most proteins synthesized by recombinant DNA methods are those that are not readily available and have been studied the least, compared to such classics as myoglobin, cytochrome c, lysozyme, and ribonuclease whose crystal structures have been solved. IMAC would probably be used more often if it were possible to predict which proteins bind immobilized metal ions.

The first step in engineering a recombinant protein to contain an accessible metal binding site, was to identify a chelating peptide sequence (12). With a suitable sequence in hand, its ability to bind immobilized metal ions when attached to a larger polypeptide could be tested (13). The third step was to splice the chelating peptide gene sequence on the front end of a recombinant protein gene sequence and determine whether the metal binding properties were conserved in the expressed CP-protein (13). The last step was to demonstrate that the chelating peptide could be removed chemically or enzymatically when an appropriate cleavage site was included in the expression product. CP-IMAC must be applied to many different systems to become a general method for purifying recombinant proteins. Recently, Hochuli and co-workers have prepared a chelating gel with nitrilotriacetic acid as a tetradentate ligand for immobilizing nickel and have used polyhistidine chelating peptides cloned on the end of dihydrofolate reductase to purify the fusion protein over a nitrilotriacetato Ni(II) column (14).



## Experimental Section

The preparation of IMAC columns and measurement of peptide elution pH values has been described in detail (12). The construction of the expression plasmid for His-Trp-proinsulin and the chromatography of His-Trp-proinsulin on IMAC columns has been described (13).

Peptide Synthesis and Purification. Met-His-Gly-His was prepared by solid phase peptide synthesis using a Beckman 990A Automated Peptide Synthesizer and following the general outline of a previously published protocol (15). The two tripeptide amides, Met-His-Trp-$NH_2$ and Met-His-Tyr-$NH_2$, were prepared and purified using previously published methodology(16-17). The purified peptides gave satisfactory amino acid, mass spectral, and thin layer chromatographic analyses.

Plasmid Construction and Expression of CP-X-protein. The expression plasmid for Met-His-Trp-His-ompA signal peptide-IGF-II, pHS235 was derived from pPRO-IGF-II (Hsiung, H., Eli Lilly & Company, personal communication, 1989.) and contained a lambda PL promoter, an *Escherichia coli* lpp ribosome binding site sequence, and the cI857 repressor gene which was used to regulate the activity of the PL promoter. A synthetic DNA linker shown below was synthesized on an Applied Biosystems model 380B DNA synthesizer and used to modify the 5' end of the IGF-II coding region

*Xba*I

5' CTAGAGGGTATCAT ATG CAT TGG CAT AAA AAG ACA GCT ATC GCT ATT GCC GTG

3'TCCCATAGTA TAC GTA ACC GTA TTT TTC TGT CGA TAG CGA TAA CGG CAC

*Taq*I

GCG CTA GCT GGT TTC GCT ACT GTA GCT CAG GCC GCT TAT 3'

CGC GAT CGA CCA AAC CGA TGA CAT CGA GTC CGG CGA ATA GC 5'

The plasmid was used to transform *E. coli* RV308 cells (18) which were grown overnight at 32°C in 2xTY medium, then diluted into fresh broth (20ml overnight culture/1 L medium) and grown until the O.D. was 0.3. The culture was then switched to a 41°C shaker bath for three additional hours of incubation. The cell pellet was harvested for protein isolation.

CP-IMAC Purification of Met-His-Trp-His-ompA signal peptide-IGF-II. Granules containing the expression product from pHS235 were collected and the protein sulfitolysed as described previously for IGF-II (19). The sulfitolysis reaction mixture was applied to a 1 X 8 cm Cu(II) IMAC column equilibrated in 0.1M $Na_2HPO_4$, 0.1M NaCl, 7M urea, pH 7.7 buffer. The bound material was eluted from the column with a pH gradient generated with 0.1M acetic acid, 0.1M NaCl, 7M urea, pH 3.6 buffer on an FPLC as described previously (9). The bound pool of protein was desalted into 0.1M $NH_4HCO_3$ pH 7.9 buffer on a Sephadex G-25 column and then lyophilized. The cleavage reaction with signal peptidase using lyophilized CP-X-protein was carried out as previously described (20). After 1.3 hours the reaction mixture was applied directly to the Cu(II) IMAC column as described above. Samples of the crude sulfitolysis reaction mixture and pools from the IMAC steps were analyzed by SDS-PAGE. The gel consisted of 15% acrylamide and 18% glycerol in 0.1M Tris-phosphate buffer, pH 6.8. Electrophoresis was carried out at room temperature with a constant current of 35 mA. The proteins in the gel were then fixed with TCA and stained with Coomasie.

## Results and Discussion

### Identify a Chelating Peptide

A chelating peptide intended to serve as a purification handle for recombinant proteins, must have certain thermodynamic and kinetic properties. It must have a high affinity for immobilized metal ions and the kinetics of complex formation must be fast. A slow rate of complex formation would defeat the purpose of using a chelating peptide as a purification handle, since the complex must form during the time of the chromatographic separation. First row transition metals, Fe(II), Co(II), Ni(II), Cu(II), and Zn(II) were chosen as candidates for immobilized metal ions, since they have fast water exchange rates and ligand exchange rates in general (7). The affinity of a peptide for a given metal will be largely determined by the amino acid composition of the peptide. Certain amino acids bind metals in metalloproteins, form complexes with metals either as amino acids or as part of a peptide, and include, histidine, cysteine, aspartate, glutamate, methionine, lysine, and tyrosine (7). Structural studies have been carried out on a limited number or metal peptide complexes, which show that a dipeptide contains a sufficient number of donor atoms to occupy three coordination sites on a metal (21). Since only three coordination sites are available for binding immobilized metal ions, a dipeptide should be sufficient.

Potential chelating peptides were obtained commercially and included a series of di- and tripeptides which contained either histidine, aspartic acid or lysine in at least one position (12). A convenient method for measuring the relative affinity of these peptides for immobilized first row transition metals was to carry out an IMAC separation and elute the peptides from the column by lowering the pH of the buffer. The elution pH, or pH required for elution, is a relative measure of affinity for the immobilized metal ion on a particular column. In other words, the lower the elution pH, the higher the affinity for the metal ion, since more protons are required to compete with the metal ion to protonate the donor atoms. A survey of approximately fifty peptides on Fe(II), Co(II), Ni(II), Cu(II), and Zn(II) IMAC columns revealed

that histidine containing peptides had the highest affinity for these immobilized metal ions (12). None of the peptides bound the Fe(II) or Zn(II) columns in phosphate buffer. The aspartic acid and lysine containing peptides were retained only on the Ni(II) and Cu(II) columns but eluted at pH 7.5 with various retention times. Of the twenty one histidine containing peptides studied, a little over half bound Cu(II) and Ni(II) with various affinities, but three had unusually high affinities for Co(II), Ni(II), and Cu(II). Figure 2 shows the separation that can be achieved between histidine containing peptides on a Ni(II) IMAC column and one of these peptides, His-Trp. These three peptides, His-Trp, His-Tyr-$NH_2$, and His-Gly-His, are therefore suitable candidates for chelating peptide purification handles.

## Attach a CP to a Polypeptide and Purify using IMAC

Luteinizing Hormone Releasing Hormone (LHRH) analogs were used to test the ability of a CP, His-Trp, to purify a large polypeptide through binding immobilized metal ions (13).The sequences of the LHRH analogs used is shown below:

| | |
|---|---|
| 2-10 LHRH | His-Trp-Ser-Tyr-Gly-Leu Arg-Pro-Gly-$NH_2$ |
| 3-10 LHRH | Trp-Ser-Tyr-Gly-Leu Arg-Pro-Gly-$NH_2$ |
| 4-10 LHRH | Ser-Tyr-Gly-Leu Arg-Pro-Gly-$NH_2$ |

The 2-10 LHRH analog contains an N-terminal dipeptide which corresponds to the chelating peptide sequence, His-Trp. 4-10 LHRH has the same sequence as 2-10 LHRH but lacks the N-terminal CP. A mixture of these LHRH analogs was applied to a Ni(II) IMAC column and the analogs without the CP eluted in the wash at pH 7.5, while the CP containing 2-10 LHRH analog required a pH of 4.4 for elution, as shown in Figure 3. This experiment demonstrated that the ability to bind immobilized Ni(II) could be transferred to a polypeptide which otherwise has no affinity for Ni(II) by simply extending the sequence to include a CP, His-Trp.

## Purify a Recombinant CP-Protein

His-Trp-proinsulin was used as a model system for a recombinant protein to determine whether such a small chelating peptide could be used to immobilize and purify a larger recombinant protein on an IMAC column (13). The S-sulfonates of His-Trp-proinsulin and proinsulin were isolated from *Escherichia coli* engineered to overproduce these proteins as trpLE' fusion proteins. A mixture of these two proteins was applied to a Ni(II) IMAC column and the proteins were eluted with a pH step gradient. Figure 4 shows the separation of His-Trp-proinsulin from proinsulin which can be achieved by virtue of the CP, His-Trp, on the N-terminus of His-Trp-proinsulin. Proinsulin was a good test case, since it has an endogenous transition metal binding site and binds IMAC columns. The affinity of His-Trp-proinsulin for immobilized Ni(II) is greatly increased over that for proinsulin, as a much lower pH is required for elution. This increase in binding affinity can only be attributed to the presence of the CP, His-Trp, which therefore has a higher affinity for Ni(II) than the B10 histidine in proinsulin.

The examples presented have been used to prove the feasibility of CP-IMAC as a purification method for recombinant proteins. In an actual practice, most foreign proteins expressed in host organisms such as *E. coli* contain an N-terminal methionine whose presence might prevent an N-terminal chelating peptide from binding immobilized metal ions. In fact the histidine containing peptides with the highest affinity for immobilized metal ions, all contain an N-terminal histidine residues (12). Those with histidine in the second position only bound immobilized

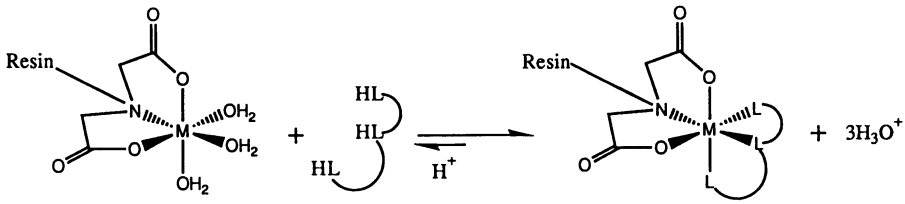


Figure 1. Schematic representation of an immobilized iminodiacetic acid (IDA) complex with a six coordinate metal ion. Three open coordination sites are occupied by water and are displaced by the incoming protein, represented as $H_3L_3$, where L is the ligand or donor atom on the protein. Bound protein can be eluted by lowering the pH of the buffer, thereby protonating the donor atoms so they can no longer coordinate the metal ion.



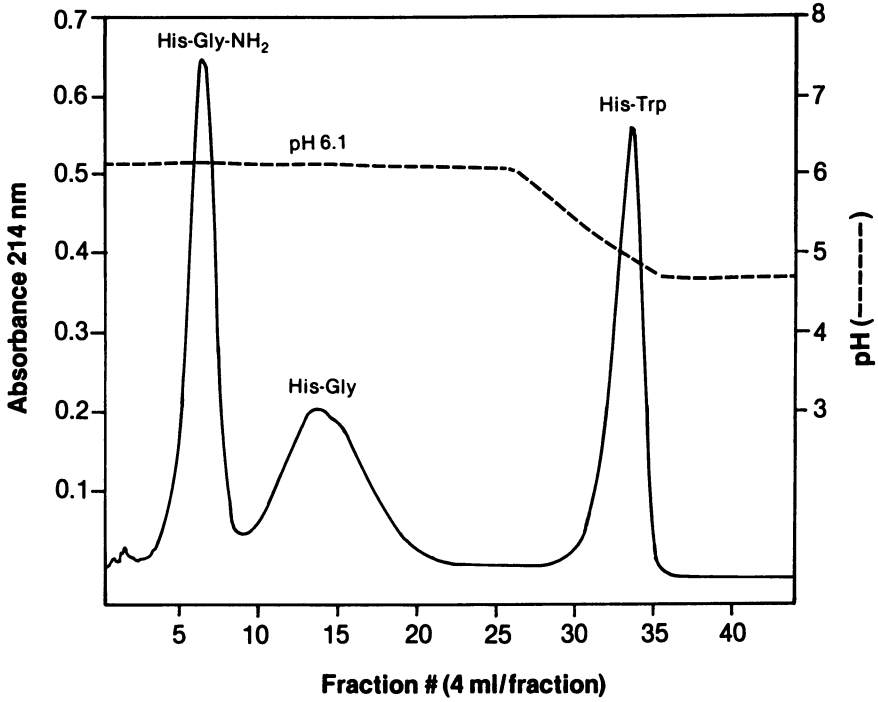


Figure 2. Separation of histidine containing peptides on a Ni(II) IDA Sephadex G-25 column. Peptides were eluted with the pH gradient shown. (Reproduced with permission from Ref. 12. Copyright 1987 ACS)

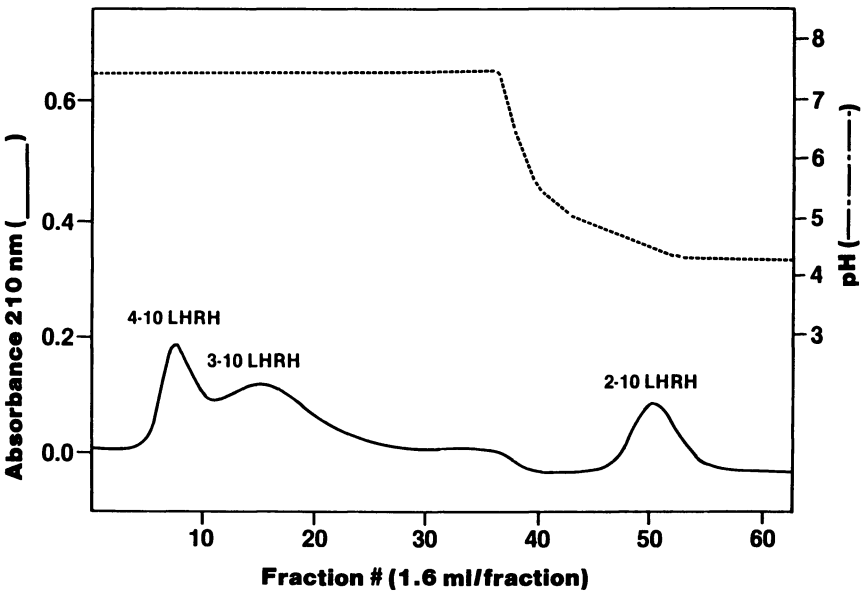


Figure 3. Separation of LHRH analogs on a Ni(II) IDA Sephadex G-25 column. The analogs were eluted with the pH gradient shown. (Reproduced with permission from Ref. 13. Copyright 1988 The American Society for Biochemistry and Molecular Biology)

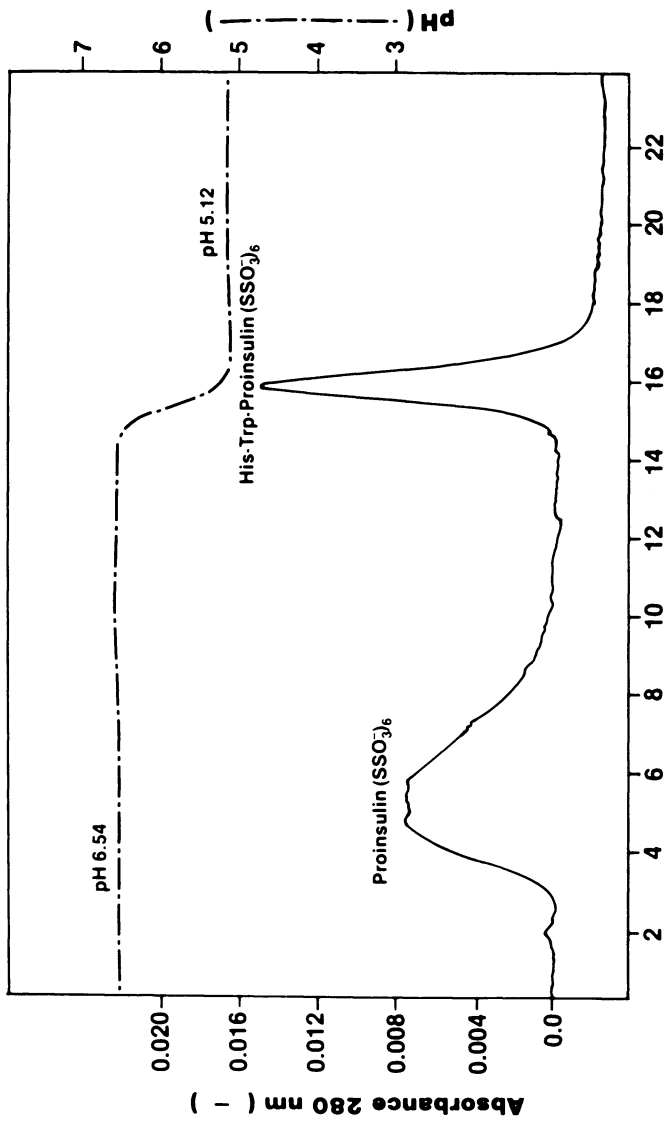


Figure 4. Separation of the S-sulfonates of His-Trp-proinsulin and proinsulin on a Ni(II) IDA 4% agarose column. The proteins were eluted with the pH gradient shown. (Reproduced with permission from Ref. 13. Copyright 1988 The American Society for Biochemistry and Molecular Biology)

Ni(II) and less strongly than peptides with N-terminal histidines. Methionyl analogs of the three chelating peptides identified were therefore synthesized for further study.

The chromatographic behavior of these peptides on Co(II), Ni(II), and Cu(II) IMAC columns was studied as described previously for the histidine containing peptides (12). A summary of the elution pH values for these chelating peptides is given in Table I.

Table I. Comparison of Chelating Peptides to Methionyl Chelating Peptides

| | Elution pH | | |
|---|---|---|---|
| Peptide | Co(II) | Ni(II) | Cu(II) |
| His-Gly-His | 5.9 | 4.8 | 4.4 |
| Met-His-Gly-His | 7.5 | 5.3 | 4.7 |
| His-Tyr-$NH_2$ | 6.4 | 4.7 | 4.4 |
| Met-His-Tyr-$NH_2$ | 7.5 | 4.9 | 7.5 |
| His-Trp | 6.4 | 4.9 | 4.4 |
| Met-His-Trp-$NH_2$ | 7.5 | 4.8 | 7.5 |



The presence of an N-terminal methionine abolished the ability of Co(II) to bind any of the chelating peptides. All three methionyl chelating peptides retained their affinity for immobilized Ni(II) although the relative affinities are somewhat lower as seen by the slightly higher elution pH values. Only one methionyl chelating peptide, Met-His-Gly-His, had a high affinity for immobilized Cu(II), whereas the others eluted from the column during the wash step. The requirement for an N-terminal histidine in single histidine containing peptides is apparently quite strict for immobilized Cu(II). These results indicate that methionine in the N-terminal position of a recombinant protein should not affect the ability of these peptides to bind immobilized Ni(II) and that the presence of more than one histidine will allow immobilized Cu(II) to be used as well. The generality of this method, CP-IMAC, is therefore extended by these findings, since the majority of recombinant proteins contain an N-terminal methionine.

## Purify a Recombinant CP-X-Protein and Remove the CP to Generate the Desired Protein

The last step in developing CP-IMAC as a general technique for purifying recombinant proteins involves removing the CP to generate the desired protein sequence. Cleavage of the CP sequence can either be accomplished chemically or enzymatically. Chemical cleavage reactions are usually dependent on the sequence of the protein or amino acid composition. For example, cyanogen bromide has been used to remove leader sequences from fusion proteins, but requires that the protein of interest contain no internal methionine residues. Tryptophan oxidative cleavage reactions could be carried out to remove His-Trp, provided the protein of interest contains no internal tryptophan residues (22). Enzymatic cleavage sites have been

used to produce human growth hormone (23), lymphokines (4), urogastrone (1), R6K replication initiator (2), as well as others from fusion proteins (3).

A general enzymatic cleavage site, the ompA signal peptide which is specifically recognized by the enzyme signal peptidase (20), was cloned into the expression product following a CP to show that a CP can be removed to generate the desired protein. The protein in this case was human insulin-like growth factor-II or IGF-II and the CP was Met-His-Trp-His. The N-terminal sequence of the expression product is shown below using the one letter abbreviations for amino acids.

CP ompA signal peptide IGF-II

MHWH-KKTAIAIAVALAGFATVQA-AYRPSE....



The CP-IMAC purification scheme for the model CP-X-protein, involved passing a crude sulfitolysed lysate of *E. coli* over an IMAC column to bind the CP-X-protein and provide purified protein, cleaving the CP and ompA signal peptide with signal peptidase, and then passing the cleavage reaction mixture over the IMAC column to retain contaminating *E.coli* proteins which bound the immobilized metal ion in the first step.

Figure 5 shows the SDS-PAGE analysis of the steps in the purification scheme for Met-His-Trp-His-ompA signal peptide-IGF-II from *E. coli* using CP-IMAC. A band at approximately 11 kDa in the crude sulfitolysis mixture of granules (lane 2) is the expression product and the one below 9.4 kDa is IGF-II present in the granule preparation. Both bands cross reacted with antibodies against IGF-II in a Western blot (data not shown) and the band below 9.4 kDa also migrated in the same position as an authentic sample of IGF-II (lane 1). The presence of IGF-II in the crude lysate, indicated that some cleavage of the expression product had occurred. Preliminary Western blot analysis of the periplasmic fraction indicated that the IGF-II in the granule preparation was not a contaminant of the periplasmic fraction. This result was somewhat surprizing, as cleavage of the ompA signal peptide fusion proteins is reported to occur only in the membrane when the protein is translocated into the periplasmic space (24). The fact that the majority of the expression product was not processed nor secreted into the periplasm may be due to the presence of the chelating peptide sequence, since other recombinant fusion proteins lacking a CP are secreted with the signal peptide removed (24).

Lanes 3 and 4 in Figure 5 show the results of the first Cu(II) IMAC step on crude Met-His-Trp-His-ompA signal peptide IGF-II, from which two pools were made. The proteins with no affinity for immobilized Cu(II) represent the unbound pool (lane 3) and included the processed IGF-II. Lane 4 shows the pool of bound material which was eluted by lowering the pH of the buffer. The expression product, Met-His-Trp-His-ompA signal peptide IGF-II, was the major component while some higher molecular weight *E. coli* proteins were also found in this pool. The pool of bound material was then reacted with signal peptidase. The cleavage reaction products shown in lane 6 illustrate that the expression product has been completely consumed to generate IGF-II. The reaction mixture was then passed over the Cu(II) IMAC column again so as to retain the *E. coli* proteins present in the sample and collect the processed IGF-II. The last two lanes in Figure 5 are fractions from the column flow through which contained the processed and purified IGF-II. The single band in the gel indicated that the protein had been purified to a homogeneous state.

The generality of this approach has been demonstrated with the expression of a removable chelating peptide which was used to purify IGF-II. This same sequence, Met-His-Trp-His-omp A signal peptide, can now be cloned onto the end of

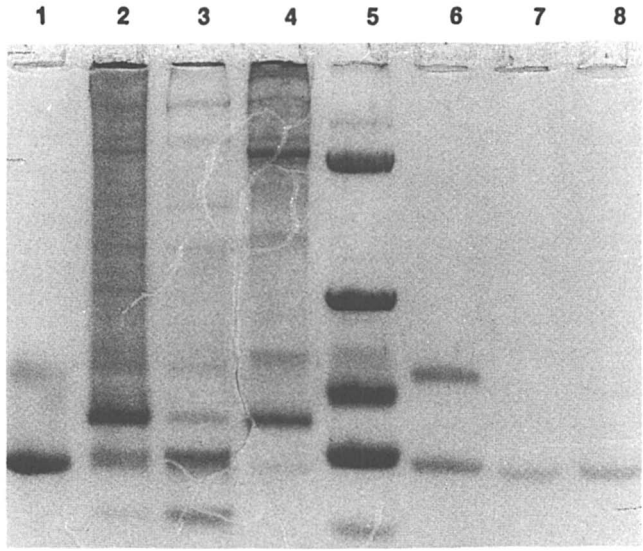


Figure 5. SDS-PAGE analysis of the CP-IMAC purification of Met-His-Trp-His-ompA signal peptide-IGF-II S-sulfonate as described in the text. Lane1, sample of IGF-II S-sulfonate; lane 2, sulfitolysed granules; lane 3, pool of unbound protein from first Cu(II) IMAC step; lane 4, pool of bound protein from first Cu(II) IMAC step; lane 5, molecular weight markers of 43 kDA, 22 kDa, 12.5 kDa, 9.4 kDa, and 3.5 kDa; lane 6, products of signal peptidase cleavage of material in lane 4; lane 7 and 8, fractions of unbound material from the second Cu(II) IMAC step.

recombinant proteins, and the same purification scheme followed to yield pure protein. The capacity of IMAC columns for proteins is very large (10), and should therefore allow large amounts of recombinant proteins to be purified using CP-IMAC.

The design of optimal chelating peptide sequences will depend on our understanding of the mechanism of protein or peptide binding to immobilized metal ions. The chelating peptides we have used were discovered through screening numerous peptides for their ability to bind immobilized transition metal ions. For metals with coordination numbers of six, three open sites are left after the metal has been immobilized with iminodiacetic acid. A peptide with three strong donor atoms in the correct orientation may be the ideal chelating peptide. Whether a simple trihistidine sequence with three imidazole groups, could fulfill the orientation requirement for strong binding is not clear. The presence of more than three histidine residues in a chelating peptide may provide greater opportunity for the metal to encounter a complete set of donor atoms with the correct orientation, and therefore tighter binding. Conversely, a peptide containing three histidine residues separated by other amino acids, which has the proper orientation of donor atoms, may have as high an affinity for immobilized metal ions as peptides containing an excess of histidine residues.

Immobilization of metal ions with tetradentate or pentadentate ligands reduces the number of open coordination sites on the metal ion available for protein or peptide binding. The effect of reducing the number of open coordination sites is to lower the affinity of proteins for that immobilized metal ion (25). Hochuli and co-workers used a tetradentate ligand, nitrilotriacetic acid, to immobilize Ni(II) and polyhistidine peptides with two to six histidine residues as purification handles(14) using the CP-IMAC concept. The apparent affinity of Ni(II) for fusion proteins containing these peptides increases with the number of histidine residues. Stability constants for the complexes formed were not measured but the amount of fusion protein retained on the columns seemed to increase with each successive histidine residue. This may be due to increasing the probability of the immobilized metal ion seeing two imidazole groups with the proper spatial arrangement necessary for optimal binding.

Metal ions with a preferred coordination number of four, such as Cu(II) or Zn(II) have even fewer open coordination sites available after immobilization. The requirements for spatial orientation of the donor atoms in a chelating peptide may be different for metals with square planar or tetrahedral geometries compared to metals which form octahedral complexes. The increase in crystal field stabilization energy for Cu(II) complexes compared to analogous Ni(II) complexes often results in higher affinities, and may therefore be more desirable (7).

The studies described here have demonstrated the feasibility of CP-IMAC and its utility in purifying recombinant proteins. The readily available reagents for performing CP-IMAC on a recombinant CP-protein, makes it an ideal technique for obtaining large amounts of protein for further study with only one or two chromatographic steps.



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