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Selection of optimum affinity tags from a phage-displayed peptide library
Application to immobilized copper(II) affinity chromatography

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Abstract

Immobilized metal affinity chromatography (IMAC) is a versatile tool for the purification of proteins with affinity for immobilized metals. Moreover, this technique has also been used for the separation of proteins that do not exhibit significant metal affinity in the native form, by their fusion to a short metal-binding peptide (a tail), most commonly, a sequence consisting of six adjacent histidine residues (His_6). A phage-displayed random hexamer library is used to select for peptides with affinity for immobilized copper. The study follows our previous investigation in which a stringent selection protocol led to the selection of only one copper-binding peptide containing two histidines. The less stringent conditions employed in this work resulted in the selection of a more diverse population of peptides, but again, dominated by peptides containing two histidines (13 out of 19). The prevalence of peptides with two histidines, in contrast to peptides with a higher number of histidines (e.g. His_6 or HHHMVH), is explained based on the differences in the pH dependence of their affinity for copper. As discussed, the selected peptides with two histidines will be superior affinity tails than peptides with a higher histidine content (e.g. His_6). Moreover, a peptide with a single histidine but with a very high copper affinity, is also identified. Its high copper affinity is related to the presence of several hydrophobic residues in the neighborhood of histidine. Chromatography of human interleukin-1β (hIL-1β) and several other proteins containing a single surface-exposed histidine surrounded by several hydrophobic residues confirmed that such a sequence could also serve as a very effective metal binding domain for protein purification using immobilized copper(II) columns. © 1997 Elsevier Science B.V.

Keywords: Immobilized metal affinity chromatography; Phage display library; Affinity tags; Peptides; Proteins; Interleukin

1. Introduction

Immobilized metal affinity chromatography (IMAC), introduced by Porath et al. [1], is a versatile tool used for protein separation. IMAC utilizes chelated metal ions such as Cu(II)–IDA (IDA, iminodiacetic acid) and Ni(II)–NTA (NTA, nitrilotriacetic acid) as affinity ligands for protein binding [1–3]. Factors such as the accessibility, micro-environment of the binding residue (i.e. histidine, cysteine and tryptophan), co-operation between neighboring amino acid side groups and local conformations play important roles in protein retention [4]. The low cost of metals and the ease of regenera-

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tion of the stationary phase, in contrast to other methods of biospecific affinity chromatography, such as antigen–antibody [5], are the attractive features of IMAC.

Proteins that display insufficient metal binding to be purified using IMAC can usually be endowed with metal affinity by their fusion to a metal-binding peptide (as an affinity tail). One commonly used affinity tail is the sequence comprising six adjacent histidine residues (His_6). Some examples of IMAC separations using His_6 fusions include the purification of HIV-1 reverse transcriptase [6], human interleukin-6 [7], malaria transmission blocking vaccine candidate [8] and many others [9–12].

While His_6 fusions offer purification factors corresponding to up to 90% purity [7,13] for a great variety of target proteins expressed in relatively large amounts, other peptides may be found that impart a higher selectivity to the process. That is, proteins fused to such peptide(s) may be purified, in a single chromatography step, to a significantly higher extent than those fused to His_6. In addition to the potential for attaining higher selectivity, the availability of a larger number of affinity tails, displaying the desired level of metal affinity, will enable the protein engineer to select a tail with minimal impact on the expression and activity of the fusion product. Although Ni(II)–NTA is the ligand of choice in most IMAC experiments using affinity tails [9–12], a recent report indicates that Cu(II)–IDA could also serve as an efficient alternative and, in some cases, may even lead to an increased resolution of the target protein as compared to that obtained with Ni(II)–NTA [14].

Recently, we screened a phage-displayed random hexamer library for the selection of peptides with affinity for immobilized copper [15]. However, only one metal binding peptide, Ser–Pro–His–His–Gly–Gly (SPHHGG) was selected. This lack of diversity was attributed to the high selection pressure imposed on the population during the protocol. In this investigation, conditions were chosen such that the selected peptides would exhibit sufficiently high copper affinity to retain a peptide–phage fusion complex on Cu(II)–IDA but would not bind too strongly to require harsh elution conditions or to cause any undesirable effects, such as metal ion transfer (MIT) from the column [3]. The less stringent protocol used in this investigation resulted in the selection of a more diverse population of copper-binding peptides than in the previous case. However, in this case also, most of the selected peptides contained two histidines. The domination of the selected populations, in both cases, by peptides containing two histidines as opposed to peptides with a higher histidine content (e.g. His_6), is explained based on the hypothesis that the copper affinity of the two types of peptides may vary with pH in different fashions. Peptides such as His_6 may retain their copper affinity until low pH values are reached, probably due to the presence of multiple copper-binding groups. At high pH values, the selected peptides with two histidines may exhibit a stronger copper affinity, potentially due to the presence of a single strong copper-binding site. Thus, the selected peptides may serve as highly efficient affinity tags (i.e. higher loading capacity, milder elution conditions and higher purity) for protein purification using Cu(II)–IDA.

Another interesting feature of the investigation was that one member of the selected population contained a single histidine residue in its sequence and yet, it demonstrated very high copper affinity, based on the elution behavior of its phage fusion. This high copper affinity was related to the presence of several hydrophobic residues in the neighborhood of histidine. Chromatography of human interleukin-1β (hIL-1β), a protein containing a single surface-exposed histidine surrounded by several hydrophobic residues, confirmed that such a sequence could also serve as a very effective metal-binding domain for protein purification from Cu(II)–IDA columns.

2. Experimental

2.1. Reagents

Epoxy-activated Sepharose 6B and Chelating Sepharose Fast-Flow were obtained from Pharmacia (Piscataway, NJ, USA). DNA Sequenase version 2.0 and other reagents used for sequencing were obtained from US Biochemicals (Cleveland, OH, USA).

2.2. Random combinatorial peptide libraries

We are currently maintaining a hexamer library in phage fuse5, a derivative of coliphage fd. The library was obtained from Dr. G. Smith (Washington University, St. Louis, MO, USA) and was constructed as described by Scott and Smith [16]. The *E. coli* strain (K91kan) needed to propagate the phage was provided with the library. Procedures involving the propagation, amplification and titering of the phage and the procedures for preparing the phage DNA for sequence analysis are described in detail by Scott and Smith [16].

2.3. Immobilization of Cu(II)

For use in the screening experiments, we synthesized agarose gel with an IDA concentration that was 10% of that of the commercially available gel (Chelating Sepharose Fast Flow 1:1), as described by the manufacturer (Pharmacia). This step was taken in order to minimize the background binding of the phage particles to immobilized copper due to high IDA concentration in commercial gel. Note that these gels were only used for screening purposes. The chromatography experiments were conducted using the commercial gels.

2.4. Selection of peptide ligands from the library

A schematic of the selection process is shown in Fig. 1. The process performed for selection of copper-binding peptides was: (1) 50 μl of bead-immobilized copper, equilibrated with 20 m*M* sodium phosphate buffer (0.5 *M* NaCl, pH 7.0) was mixed with $1.3 \cdot 10^{12}$ phage by continuously vortex-mixing the mixture for 30 min; (2) the beads were then centrifuged for 10 min at 3000 *g* in a bench-top centrifuge and the supernatant, containing phage that had detached from the beads or did not attach, was discarded. The beads were resuspended in fresh buffer; (3) the vortex-mixing and centrifugation steps were repeated five times; (4) after the last wash, a buffer containing 0.1 *M* sodium acetate (0.5 *M* NaCl, pH 5.5) was added to the beads and the washing steps were continued for four more rounds,

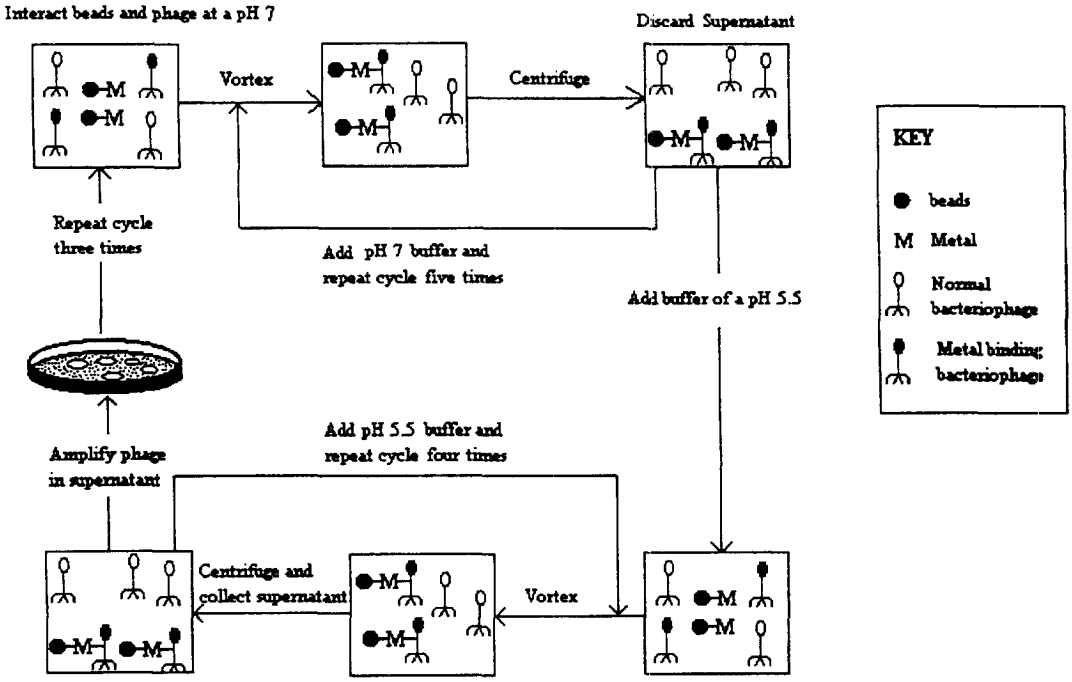

Fig. 1. Schematic of the protocol used in this investigation for the selection of a more diverse population of copper-binding peptides from the library.

this time collecting the supernatant in each wash; (5) at the end of four washes, the supernatant of the four washes was titered to determine the phage concentration; (6) the collected phage were amplified by passage through *E. coli* K91kan and either used to produce colonies for sequencing or were subjected to another round of selection.

2.5. Analysis of selected phage (phage DNA analysis)

Phage that were selected for their ability to bind metals were isolated and the region of the phage genome that contained the cloned peptide sequence was analyzed by DNA sequencing analysis. Phage DNA was isolated and sequenced using commercially available kits (Sequenase, Amersham, Arlington Heights, IL, USA).

2.6. Construction of the His_6 phage

The His_6 phage was constructed essentially as described for the library [17]. Briefly, the replicative form of fuse5 was restricted with Sfi I. Two complementary oligonucleotides (GGGCTCATCATCATCATCATCATGGGGCCGCTG and CGGCCCCATGATGATGATGATGATGAGCCCCGT) were synthesized at the University of Pittsburgh's DNA synthesis facility. The oligonucleotides were annealed and ligated to the cut vector. The ligated DNA was used to transform *E. coli* DH5α. The transformation reaction was grown overnight in 5 ml of LB-broth containing 25 mg/ml tetracycline. The clarified supernatant from this culture was used as a phage stock to infect *E. coli* K91kan cells. Transductant colonies were selected, phage were purified from each colony and the resultant phage were sequenced. All selected phage had the correct insert coding for His_6.

2.7. IMAC experiments

The experimental set-up consisted of a glass column (1 cm I.D.), a peristaltic pump (Pharmacia) to supply the solutions to the column at a desirable flow-rate and a fraction collector, FRAC-100 (Pharmacia).

The column was packed (bed volume of 3 ml) with chelating Sepharose Fast-Flow gel (Pharmacia). The gel was washed with deionized water, degassed, washed with seven column volumes of 0.1 *M* sodium acetate buffer (0.5 *M* NaCl, pH 4.0) and was loaded with copper using seven column volumes of 50 m*M* $CuSO_4$ solution in the same buffer. The column was then washed with ten column volumes of 0.1 *M* sodium acetate buffer (0.5 *M* NaCl, pH 4.0) to remove the unbound copper and was equilibrated with seven column volumes of 20 m*M* sodium phosphate buffer (0.5 *M* NaCl, pH 7.0).

The input samples consisted of phage solution (approximately 10^{12} phage particles/ml) or hIL-1β with the raw cell extract of *E. coli*. The sample preparation of native hIL-1β has been described in detail elsewhere [20]. Sample was applied to the column at a flow-rate of 0.4 ml/min. Elution of the bound phage or protein was achieved by developing a decreasing step gradient in pH from pH 7.0 to 4.2 or from pH 7.0 to 4.7, respectively (seven column volumes at each pH). After the pH gradient, the column was washed with EDTA to release the metal and remove any bound phage/protein. The eluted fractions for phage chromatography experiments were analyzed by standard titering procedures [17], while the analysis of hIL-1β was done using the Quantikine immunoassay kit (R & D Systems, Minneapolis, MN, USA). The technique employs the 'sandwich' enzyme immunoassay using antibodies raised specifically against hIL-1β. Proper dilution of the samples was made in order to ensure operation in the linear range of the assay.

3. Results and discussion

An ideal peptide library is a collection of all possible amino acid sequences for a peptide of a given length (for example, 20^6 possible sequences for a hexamer). Typically, phage-displayed libraries contain 55 to 70% of all possible sequences [21]. The peptides are produced by the insertion of random oligonucleotides into the genome of the phage, near the 5′-end of gene pIII, which codes for a coat protein. The inserted peptides are thus displayed to the aqueous environment at the amino terminus of the coat protein. Typically, for the selection of phage-displaying peptides with specific

affinity for the ligand, the phage library and the ligand are first mixed. Following the interaction between the peptide sequences on the phage and the ligand in the surrounding medium, the bound phage are eluted and amplified by passage through their host, *E. coli*. The amplified phage is used as the starting material in the next round of selection and amplification. After several rounds of selection and amplification, phage-displaying peptides with the desired affinity for the ligands may be isolated. The sequence of displayed peptides can be easily obtained by sequencing the DNA cloned in the isolated phage. In this manner, individual binding peptides can be isolated from a mixture that may contain tens of millions of different sequences.

In our first attempt, we used this approach at pH 6 to select for peptides with high copper affinity that will elute from a Cu(II)–IDA column at pH values below six [15]. This procedure led to the selection of the sequence Ser–Pro–His–His–Gly–Gly (SPHHGG). Indeed, all the histidine-containing peptides (seventeen out of twenty clones sequenced) had the same sequence. To compare the elution behavior of the phage carrying the selected peptide (SPHHGG) with that of His_6 (the most commonly used affinity tail), a His_6–phage fusion was constructed. Chromatography of the selected peptide and the peptide–phage fusion revealed that both the SPHHGG peptide and SPHHGG–phage fusion eluted from the column at a higher pH (pH 5.5) than that for the corresponding His_6 peptide and the His_6–phage fusion (see Fig. 2 for the phage fusion). The chromatography of *E. coli* cell extract shows that the higher pH region of the elution profile, where the SPHHGG–phage fusion elutes, is substantially less contaminated than the EDTA fraction where the His_6–phage fusion elutes (See Fig. 3). Thus, fusion of the peptide SPHHGG may lead to a higher purification factor of the fusion product and elution under milder conditions as compared to the fusion of His_6. Although a successful outcome was achieved, the limited diversity (selection of only one peptide) among the selected colonies was unexpected. The finding was also counterintuitive, as the

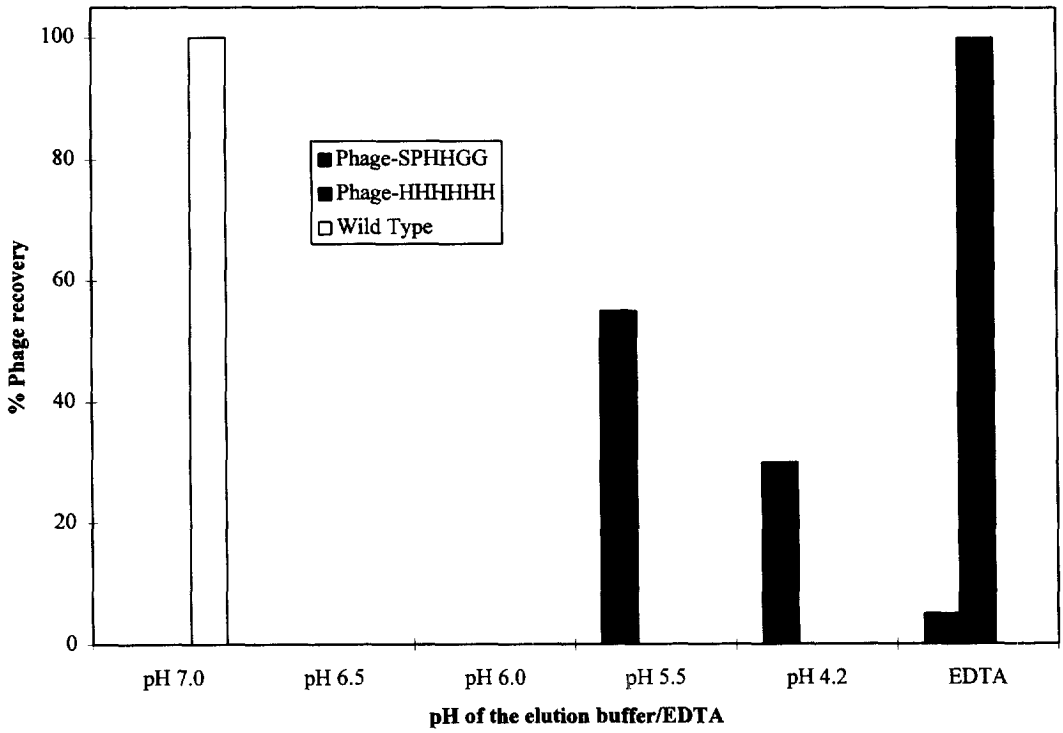

Fig. 2. Chromatography of the wild type phage (control) and phage fused to peptides SPHHGG and His_6, from a Cu(II)–IDA column.

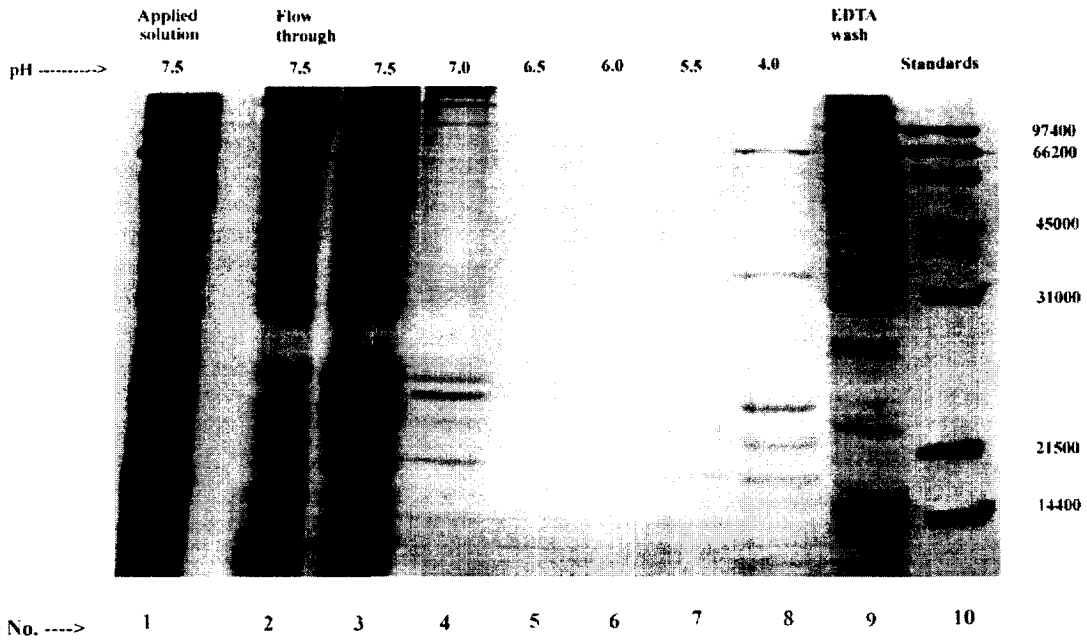

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the Cu(II)–IDA chromatography of *E. coli* cell extract. The gel (15% polyacrylamide) was silver-stained and overexposed to show minor contaminants. A 200-μl volume of *E. coli* cell extract (pH 7.5), dissolved in 10 ml of 20 m*M* sodium phosphate buffer (0.5 *M* NaCl, pH 7.5), served as the input sample (Lane 1). Lane 2 is the unbound proteins eluted during loading. A step gradient of pH (seven column volumes at each pH) was used to elute the bound proteins. Lanes 3 to 8 represent the fractions eluted at pH values of 7.5, 7, 6.5, 6, 5.5 and 4.0, respectively. At the end of the pH gradient, a solution of EDTA was applied (seven column volumes) to elute the remaining proteins (Lane 9). Lane 10 shows the molecular mass standards (from top): phosphorylase B (97 400), bovine serum albumin (66 200), ovalbumin (45 000), bovine carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400).

procedure used in the first protocol [15] should have favored the selection of peptides such as His_6 that elute from the Cu(II)–IDA column at a lower pH than a peptide such as SPHHGG that elutes at pH 5.5.

We initially thought that the library may not contain representative numbers of higher histidine-containing peptides or that the expression and the infectivity of the phage carrying such peptides may be low. The results of the selection experiments under less stringent conditions, however, were inconsistent with this initial hypothesis, as discussed next.

The less stringent protocol used in this investigation is outlined in Fig. 1. The selection pressure imposed on the library is considerably less in this protocol compared to that in the previous one [15]. The number of washing and re-equilibration steps are reduced from fourteen in the previous case to five in Fig. 1. The procedure is also modified to eliminate the peptide–phage fusions with very high affinity (pH 5.5 elution), but select those that exhibit sufficient affinity for immobilized copper (pH 7.0 binding). Using this procedure, twenty random colonies, after the third round of selection, were picked and the relevant portion of the phage genome was subjected to DNA sequencing analysis. Due to the difficulty of reading the sequence in one of the lanes of the sequencing gel, nineteen sequences are reported.

As expected, unlike the initial procedure [15], a more diverse population of histidine-containing peptides was observed (Table 1). Table 1 shows that the selected peptides also contained sequences with no histidine (three of nineteen). The selection of such peptides is similar to the result of our previous work [15]. The chromatography experiments revealed that such peptides elute from the column in the high pH wash and thus exhibit insignificant affinity towards

Table 1
List of peptides with affinity for Cu(II)–IDA (selected using protocols described in Fig. 1 and Ref. [15])

Protocol used	Histidine content	Selected sequences and the frequency of selection
(See Ref. [15])	0	Leu–Arg–Trp–Thr–Ala–Asp (3/20)
	2	Ser–Pro–His–His–Gly–Gly (17/20)
	1, 3, 4, 6	–
(See Fig. 1.)	0	Met–Ala–Leu–Leu–Trp–Asp (1/19)
		Ile–Arg–Ile–Arg–Ser–Leu (1/19)
		Ile–Phe–Ser–Leu–Leu–Asn (1/19)
	1	Ala–Met–Leu–Lys–Leu–His (1/19)
	2	Ser–Ser–His–Tyr–Val–His (2/19)
		Gly–His–Ser–Glu–His–Pro (1/19)
		Phe–Leu–Glu–His–Glu–His (1/19)
		Val–Arg–Ser–His–Leu–His (2/19)
		Arg–Ser–His–Val–His–Arg (1/19)
		Ser–Ala–Gly–Gln–His–His (1/19)
		Ser–Val–Gly–Gln–His–His (1/19)
		Ser–Pro–Gly–Gln–His–His (1/19)
		Leu–Thr–Met–His–His–Pro (1/19)
		Thr–Gly–Arg–His–His–Val (1/19)
		Ala–Ser–His–His–Val–Lys (1/19)
	4	His–His–His–Met–Val–His (2/19)
	3, 5, 6	–

Cu(II)–IDA [15]. The selection of these peptides was explained based on their potential affinity, within the context of the phage, to the metal or, more likely, to the spacer arm or the gel matrix itself.

Amongst the histidine-containing peptides, similar to the previous case, most contained two histidines (thirteen of nineteen). However, this time, due to a lower selection pressure, peptides with one (one of nineteen) and four (two of nineteen) histidines were also selected. Fig. 4 shows the chromatography of the phage fusion carrying a four-histidine-containing peptide, HHHMVH, and the wild-type phage (containing no peptide fusion) as a control.

The wild type phage eluted in pH 7.0 wash from the column. The fusion phage of the selected sequence containing four histidines (HHHMVH), on the other hand, exhibited tight binding and was eluted only upon treatment with EDTA (similar to the elution behavior of His_6–phage fusion; Fig. 2). Peptides with high histidine content, such as HHHMVH, are, thus, not only present in the population but also, similar to His_6 fusion, elute from the column in the very last fraction, presumably indicating the highest copper affinity. However, in spite of elution at a lower pH than that required for the two histidine-containing peptides (e.g. SPHHGG), peptides such as HHHMVH or His_6 are not the prevalent members of the population. In fact, it seems likely that if slightly more stringent conditions were employed during selection (such as those in the previous work [15]), we would not see such peptides and the population would again be completely dominated by peptides such as SPHHGG, containing two histidines.

We postulate that the higher frequency of selection for peptides with two histidines compared to peptides with a higher histidine content may be the result of the differences in the pH dependence of their copper affinities. The selected peptides with two histidines may contain a single strong copper-binding site, which, at high pH, could potentially outweigh the collective strength of multiple histidines in peptides such as His_6 or HHHMVH. At lower pH values, however, peptides such as His_6 or HHHMVH may exhibit a higher overall binding affinity than the selected two-histidine-containing peptides. Thus, in selection protocols that are performed at high pH (e.g. pH 7 in Fig. 1 and pH 6 in the previous investigation [15]), we observe domination by peptides with two histidines. The results demonstrate

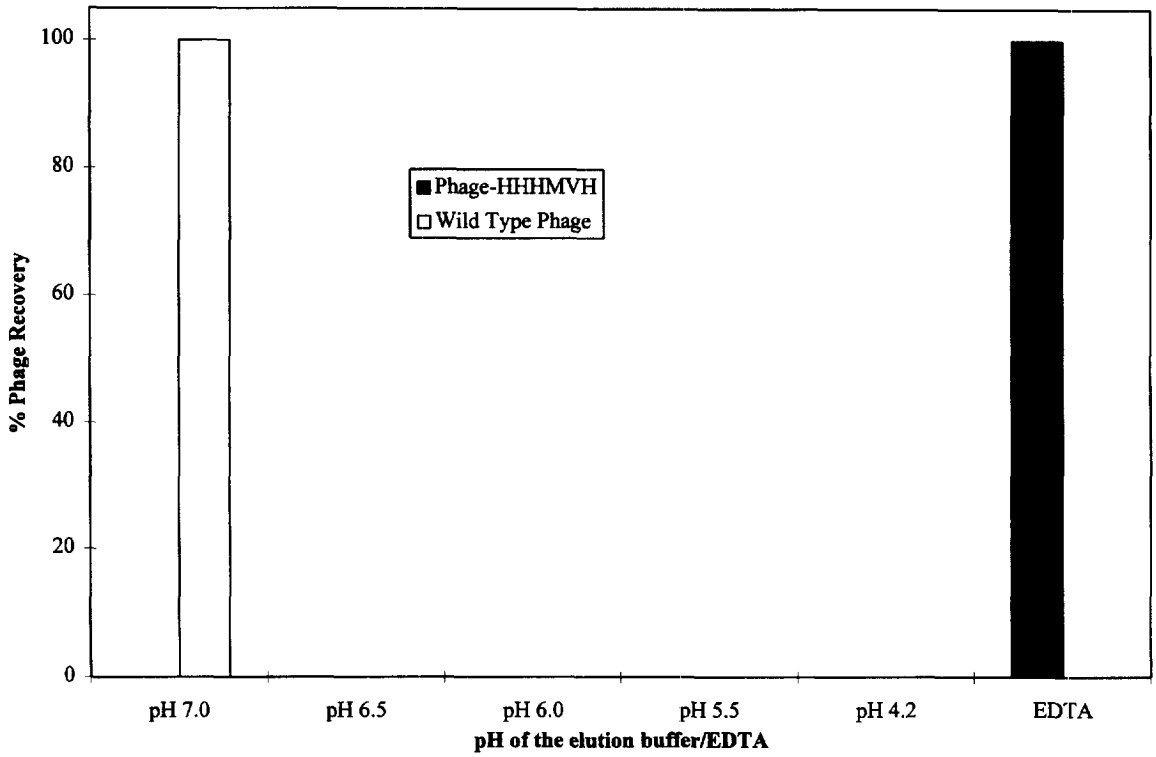

Fig. 4. Chromatography of the wild type phage (control) and phage fused to peptides HHHMVH and His_6, from a Cu(II)–IDA column.

that conformations other than helical His–X_3–His [22], may also provide sufficient affinity for Cu(II)–IDA for IMAC applications.

The two-histidine-containing peptides, such as SPHHGG and those in Table 1, may serve as very efficient affinity tails for protein purification using Cu(II)–IDA. In IMAC experiments, where input mixtures are typically loaded at high pH (e.g. pH 7–8), the fusion proteins of these peptides would show a higher capacity, elution at a milder pH and in a region of the elution profile where the elution of *E. coli* proteins is minimal (Fig. 2).

Finally, we discuss the peptide AMLKLH, with one histidine. The elution behavior of the fusion phage of this peptide (Fig. 5) indicated that it retained the fusion phage strongly on the column (i.e. elution in pH 4.2 fraction) but, unlike His_6 or HHHMVH, without resulting in excessively tight metal binding that would require EDTA for elution. The elution pH of the fusion phage (pH 4.2) is significantly lower than the normally observed pK_a value of a single surface-exposed histidyl residue, generally ranging from 7 to 6. The observed reduction in the pK_a of histidine may have been caused by the presence of four hydrophobic residues in the sequence of the peptide (Ala, Met, Leu, Leu). Hemdan et al. [23] have observed that bovine ubiquitin, bovine calmodulin and chicken egg white lysozyme elute from Cu(II)–IDA columns at pH 5.0. We searched for the sequences of these proteins and found that all three proteins contain a single surface-exposed histidine surrounded by a large number of hydrophobic residues (e.g. Leu–His–Leu–Val for ubiquitin [24], Leu–Gly–His–Arg for lysozyme [25] and Leu–Arg–His–Val for calmodulin [26]). Furthermore, we have also shown previously that hIL-1β, a protein with a single surface-exposed histidine in the neighborhood of several hydrophobic residues (Ala–Leu–His–Leu, [27,28]) elutes from a Cu(II)–IDA column in the pH 5.5 fraction [20]. Although

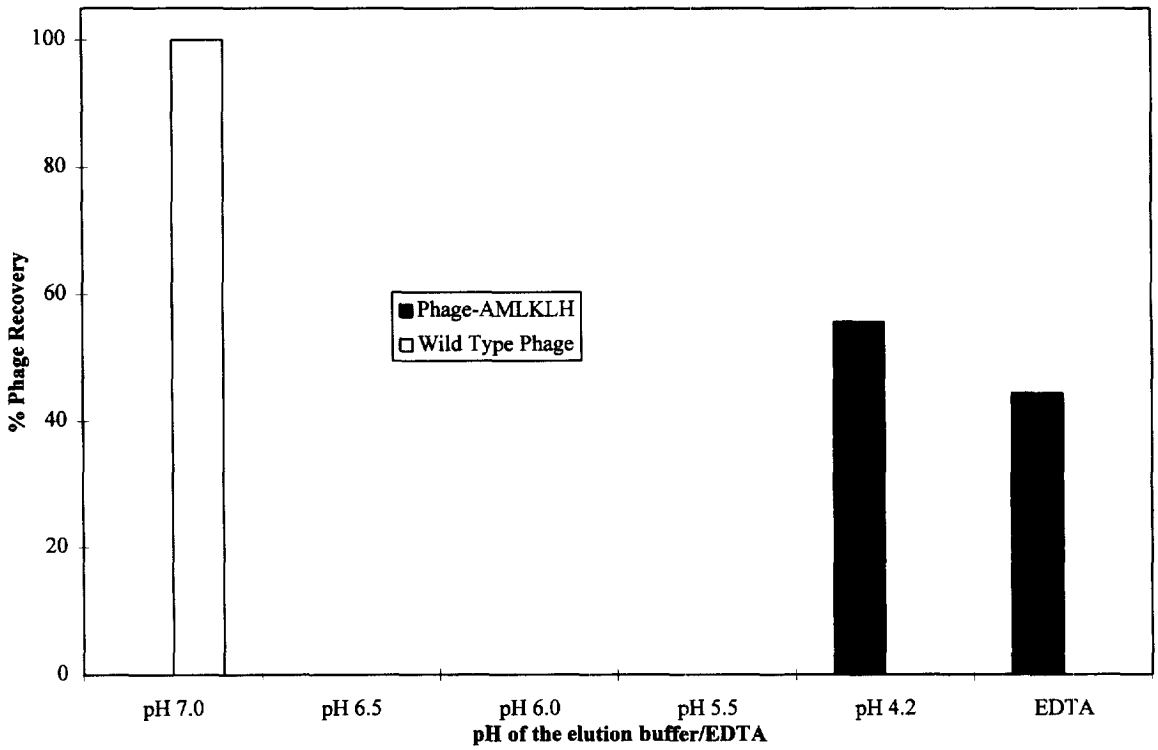

Fig. 5. Chromatography of the wild type phage (control) and phage fused to the peptide AMLKLH, from a Cu(II)–IDA column.

several factors (e.g. accessibility of the binding residue, flexibility of the binding site) are important for protein retention in IMAC [4,22], we feel that the potential reduction in the pK_a of histidine, caused by the presence of several hydrophobic residues in the vicinity of histidine in the examples discussed above, is responsible for the strong binding of these proteins with Cu(II)–IDA.

HIL-1β can be effectively purified from *E. coli* cell extract, since the pH 5.5 fraction is essentially free of cellular proteins (Fig. 3) [20]. Thus, a sequence comprising a single histidine in the vicinity of several hydrophobic residues may also serve as a very efficient tail for purification of fusion proteins from Cu(II)–IDA. Moreover, the use of sequences with one and two histidines, as opposed to peptides such as His_6 or HHHMVH, may offer the potential for reduced biosynthetic pressure on the host cell and may result in increased expression levels for the fusion proteins [29].

4. Summary

A phage-displayed random hexamer library was used in this investigation to select for peptides with affinity for immobilized copper. The study followed our previous investigation [15] in which a stringent selection protocol led to the selection of only one copper-binding peptide containing two histidines. The less stringent procedure used in this investigation led to the selection of a more diverse population of peptides, but again, peptides with two histidines were the most prevalent [13–19]. The domination of the peptides with two histidines as opposed to peptides with a higher number of histidines (e.g. His_6 or HHHMVH) was explained based on the hypothesis that the copper affinity of the two types of peptides changes with pH in different fashions. As discussed, the selected peptides with two histidines will be superior affinity tails than peptides with a higher histidine content (e.g. His_6

and HHHMVH). The results also featured the selection of a peptide with a single histidine but with a very high copper affinity. The high copper affinity of this peptide was related to the presence of several hydrophobic residues in the neighborhood of histidine. Chromatography of hIL-1β and several other proteins containing a single surface-exposed histidine surrounded by several hydrophobic residues confirmed that such a sequence could also serve as a very effective metal-binding domain for protein purification using Cu(II)–IDA.

Acknowledgments

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