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Designing new metal affinity peptides by random mutagenesis of a natural metal-binding site

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Abstract

The metal-binding site of a *Helicobacter pylori* ATPase 439 ($heli_{WT}$-tag) was successfully used as a new fusion peptide for immobilized metal ion affinity chromatography (IMAC). It produced higher yields than the frequently used his_6-tag. Due to stronger binding of the peptide to metal ions, harsher elution conditions were, however, necessary. This undesired side-effect was overcome by modifying the $heli_{WT}$-tag by polymerase chain reaction-directed mutagenesis. The modified tags were screened by an automated high-throughput IMAC system, leading to a $heli_{M14}$-tag peptide that could be eluted under conditions similar to those of the his_6-tag but at the same time produced 20% higher yields of the desired protein.

Keywords: Immobilized metal affinity chromatography; *Helicobacter pylori*; Affinity chromatography; Peptides; Proteins

1. Introduction

Immobilized metal ion affinity chromatography (IMAC) [1] is widely used for protein purification. This technique involves adsorbents with and without the desired immobilized metal and usually comprises Ni(II)–NTA (NTA=nitrilotriacetic acid) and Cu(II)–IDA (IDA=iminodiacetic acid) [1,2].

Very few naturally occurring proteins are capable of binding selectively to immobilized metal ions. To achieve this, a his_6-tag (a sequence consisting of six adjacent histidine residues) was tagged to the desired protein and the fusion product subsequently purified via IMAC. IMAC purifications employing his_6-tag-fused proteins have already been successfully used for human interleukin-6 [3], human immunodeficiency virus (HIV)-1 reverse transcriptase [4], and many other proteins [5–8]. Although the his_6-tag allows purification of up to 90% [3,9] and yields of up to 100% recovery [10], other peptides may be engineered that display a higher selectivity in purification processes [1,11]. The availability of additional affinity peptides (other than the his_6-tag) would certainly increase the possibility of selecting for maximum selectivity, smallest impact on gene expression and highest activity of the product. This would enhance the power of IMAC for protein purification significantly. Therefore, the development of new metal affinity tags has been under permanent investigation.

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Metals often play a major role in stability and specific function of the prosthetic group in naturally occurring proteins. A large variety of metal-binding sites exist in organic molecules (e.g., the heme domain in monooxygenases), amino acid residues (e.g., His, Cys, Tyr, etc.) and terminal amino groups, such as the calcium-binding sites in some lipases, for example. In fact, IMAC was first utilized for the separation of serum proteins because many of these proteins have an affinity for metal ions [1,11].

Ni(II)–NTA is frequently applied in IMAC. Therefore, metal-binding sites which are specific for Ni(II) ions are of special interest. However, the number of naturally occurring nickel-binding sites is low compared to sites that are involved in binding other metal ions such as iron and copper. Nickel-binding sites have been described for ureases [12] such as that of *Helicobacter pylori*, a human gastric pathogen associated with chronic gastritis, and cancer [13]. The viability of this microorganism in the hostile environment of the stomach largely depends on the action of its urease. Additionally, an ATPase (ATPase 439) plays a major role in the nickel metabolism of *H. pylori*, i.e., in the detoxification of the cell in cases when Co, Cu, or Ni concentrations rise to fatal concentrations [14]. The metal-binding site of this *H. pylori* enzyme (**His**–Ile–**His**–Asn–Leu–Asp–**Cys**–Pro–Asp–**Cys**) was identified by Melchers et al. [15].

The Cys–x–x–Cys binding motif is well known for its copper-binding specificity (e.g., Cys–Tyr–Ala–Cys in the Cu-ATPase from *H. pylori* [16]) and is generally regarded as important in metal binding. However, the nickel-binding ability of this motif is low. Therefore, it is assumed that additional amino acid residues must be involved in effective nickel binding. The general ability of histidine to bind metal suggested that the two histidines, which are located in the vicinity of the cysteines of the metal binding site of the *H. pylori* ATPase, might be strongly involved in nickel binding [16].

In the present study, the metal-binding site of the *H. pylori* ATPase was used for the purification of heterologously expressed proteins. The enhanced green fluorescent protein (EGFP) [17] was chosen as reporter protein [18,19] because it can easily be detected by visual means and quantified by fluorescent measurements. Furthermore, we describe the development, screening and identification of variants (in the following paragraphs referred to as heli-tag) of the metal binding site of the *H. pylori* ATPase (referred to as $heli_{WT}$-tag) with optimal properties for IMAC.

2. Experimental

2.1. Reagents

Chelating Sepharose Fast-Flow was obtained from Pharmacia (Freiburg, Germany). Ampicillin, imidazole, EDTA and all other reagents were purchased from Fluka (Buchs, Switzerland). All reagents were of analytical grade unless otherwise stated. A Qiaquick Gel Extraction Kit, a QiaprepSpin Mini-Prep Kit and a Qiagen Plasmid Midi Kit were obtained from Qiagen (Hilden, Germany). Restriction enzymes, DNA-modifying enzymes, T4-DNA ligase and Taq polymerase were purchased from MBI Fermentas (St. Leon-Rot, Germany), ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit from Applied Biosystems (Weiterstadt, Germany), and protease Xa from New England Biolabs (Schwalbach, Germany).

2.2. Strains, plasmids and media

Escherichia coli DH5α [F^- end A1 hsdR17 (rk^-, mk^+) sup E44 thi1 λgyrA96 relA1 Δ(argF laczya) U169] was used for cloning. The plasmid containing the EGFP gene (pEGFP) was purchased from Clontech (Palo Alto, CA, USA). *E. coli* was grown at 37°C in Luria–Bertani (LB) medium containing 100 μg/ml ampicillin for positive selection of clones transformed with the heli-pEGFP, his_6-pEGFP or $heli_{wt}$-pEGFP vectors.

2.3. Recombinant DNA techniques and DNA sequencing

Standard recombinant DNA methods were carried out according to the methods described in Refs. [20] and [21]. Fluorescence-based dideoxy DNA cycle sequencing was carried out with a 373A DNA Sequencing System (Applied Biosystems) according to the instructions of the manufacturer.

Table 1
Oligonucleotides used for the fusion of the respective tag-sequence to EGFP

Name	Sequence[a]
Primer 1a	(5′-GCAATACCATGGGG**CATCATCATCATCATCAT**GTGAGGAAGGGCGAG-3′)
Primer 2a	(5′-GCAATACCATGGGG**CATATTCATAATCTTGATTGTCCTGATTGT**GTGAGCAAGGGCGAG-3′)
Primer 3a	(5′-GCAATACCATGGGG**CATNNNCATNNNNNNNNNTGTNNNNNNTGT**GTGAGGAAGGGCGAG-3′)
Primer 4a	(5′-GCAATACCATGGGG**CATAATCATCGTTATGGTTGTGGTTGTTGT***ATAGAAGGACGT*GTGAGCAAGGGC-3′)
Primer 1b	(5′-CAGTTGGAATTCTAGAG-3′)

[a] *Nco*I digestion site is underlined, tag sequences are marked in bold, N represents each of the nucleotides G, C, T and A, respectively), recognition sequence for Factor X_a is in italics.

2.4. Construction of the fusion proteins of random heli-tags (heli-EGFP), wild-type metal-binding site (heli$_{WT}$-EGFP), heli$_{M14Xa}$-tag (heli$_{M14Xa}$-EGFP) and his$_6$-tag (his$_6$-EGFP) with EGFP

The pEGFP plasmid was amplified with polymerase chain reaction (PCR) using the oligonucleotides listed in Table 1. Copies of genes coding for three different fusion proteins (reactions 1, 2 and 4) and one library consisting of genes coding for heli-tag fusion proteins (reaction 3) (Table 2) were amplified.

The PCR products and the pEGFP vector were digested with *Nco*I and *Not*I, respectively (*Not*I is located inside the amplified PCR product). The digested vector [2631 base pairs (bp)] was purified by gel electrophoresis and subsequent extraction. The digested PCR product (approximately 750 bp) was precipitated with ethanol and used directly for ligation. The resulting plasmids (phis$_6$-EGFP, pheli$_{WT}$-EGFP, pheli$_{M14Xa}$-EGFP and pheli$_{XX}$-EGFP) were transformed into *E. coli* using the heat-shock method. Positive colonies were selected on LB agar containing 100 μg/ml ampicillin. The cloning strategy for heli-tag constructs is illustrated in Fig. 1.

2.5. Cultivation and preparation of cell lysates of EGFP fusion proteins

For high-throughput screening (HTS), fluorescent colonies were selected and transferred to sterile microtiter plates containing 250 μl LB medium supplemented with 100 μg/ml ampicillin. The plates were incubated at 37°C for 16 h and 50 μl of each culture was used to prepare a master plate [glycerol stock with 60% (v/v) glycerol]. After centrifugation (2750 *g*, 4°C), the cells were resuspended in 200 μl lysis buffer [50 m*M* sodium phosphate buffer, pH 8.0, 300 m*M* NaCl, 1 mg/ml lysozyme and 1 m*M* phenylmethanesulfonyl fluoride (PMSF)] inside the microtiter plate and were lysed by three freezing and thawing cycles. After a 20-min centrifugation step at 2750 *g*, 200 μl of the supernatant were used for the assay.

Selected clones were grown in 50 ml LB medium containing 100 μg/ml ampicillin. After incubation (16 h, 37°C), the cells were centrifuged, the pellet resuspended in 2 ml of lysis buffer and incubated for 20 min on ice. The cells were sonicated twice for 5 min with a Branson Sonifier 250 (Dietzenbach, Germany). The crude extract was obtained after

Table 2
PCR products obtained by the amplification of the gene coding for EGFP with the respective oligonucleotides

Reaction	Product	Oligonuceotide 1	Oligonucleotide 2	Remarks
1	His$_6$-EGFP	Primer 1a	Primer 1b	Fusion of the his$_6$-tag to EGFP
2	Heli$_{WT}$-EGFP	Primer 2a	Primer 1b	Fusion of native metal-binding site to EGFP
3	Heli$_{XX}$-EGFP	Primer 3a	Primer 1b	Fusion of the heli-tag variant to EGFP
4	Heli$_{M14Xa}$-EGFP	Primer 4a	Primer 1b	Fusion of the heli$_{M14}$-tag and the recognition site for factor X_a to EGFP

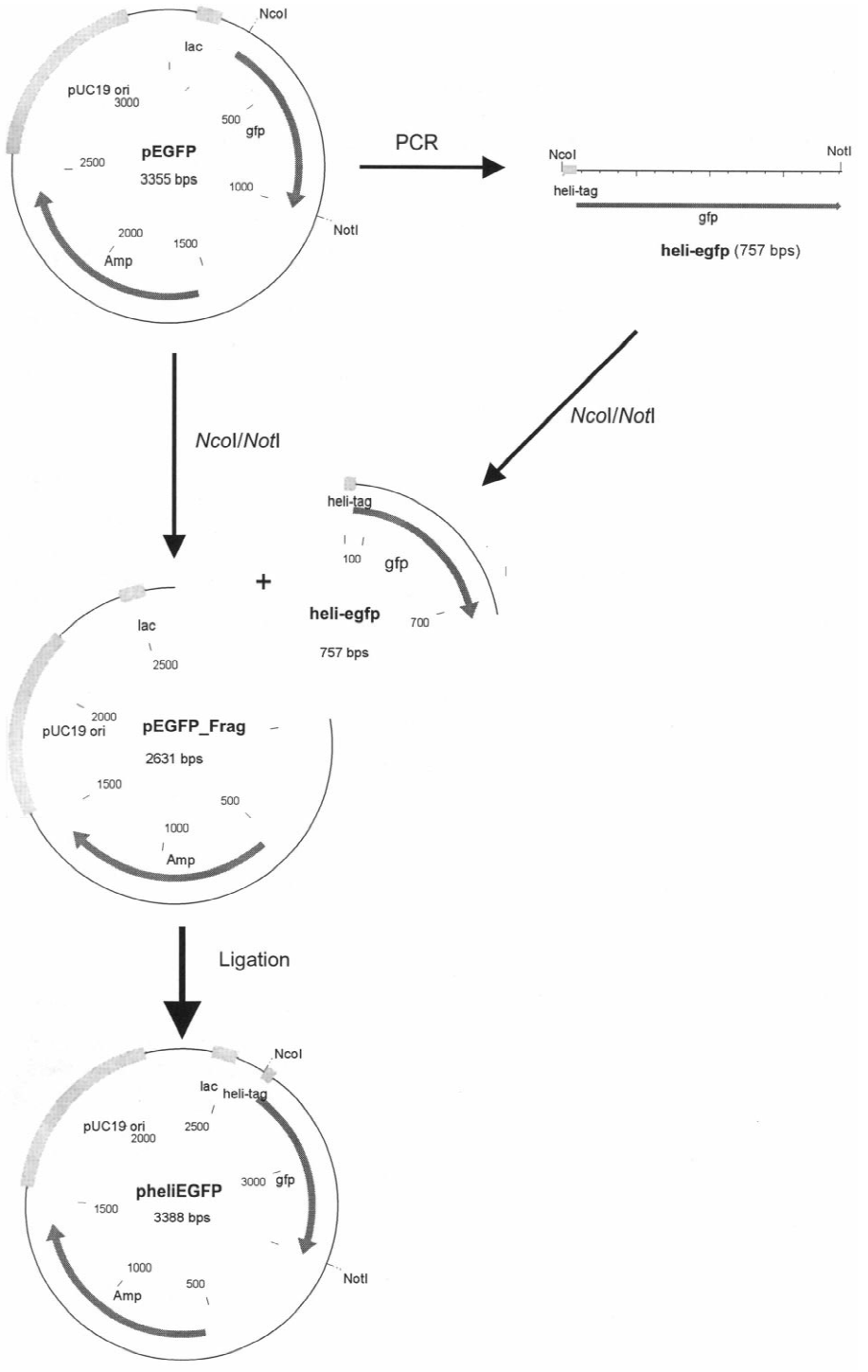

Fig. 1. Cloning strategy for the EGFP fusion proteins. All constructs were prepared in the same way; only one of the oligonucleotides used for PCR differed. In this figure, the heli-tag was chosen as an example.

separation of the cell debris by centrifugation (15 min/2750 *g*).

2.6. High-throughput screening with membrane filter plates

Membrane filter mictrotiter plates (MultiScreen 5 μm; Millipore, Molsheim, Germany) were used for HTS (Fig. 2). A 250-μl volume of a stirred chelating Sepharose suspension was added three times to each well of a 96-well plate, and subsequently centrifuged (16 *g*, 2 min). All subsequent pipetting steps were carried out with a Beckmann Biomek 2000 robot, each followed by a centrifugation step (16 *g*, 2 min). Each mini-column was washed twice with 250 μl water before 250 μl of $NiCl_2$ solution (0.3 *M*) was added, followed by three additions of 200 μl IMAC buffer (50 m*M* sodium phosphate buffer containing 250 m*M* NaCl, pH 7.5). A 200-μl volume of crude cell extract was loaded onto each column and washed twice with 250 μl equilibration buffer. Bound protein was eluted twice with 100 μl of 0.3 *M* or 0.5 *M* imidazole in equilibration buffer. The chelating Sepharose in the filter plates was regenerated with 250 μl 50 m*M* EDTA and 1 *M* NaCl, pH 8.0, in water.

2.7. Purification with Ni–NTA spin columns

Ni–NTA SpinPrep Columns were purchased from Qiagen. Cell lysates of 50 ml cultures were prepared as described above. IMAC buffer and IMAC buffer supplemented with 0.3 *M* or 0.5 *M* imidazole for elution were used. The procedure was performed according to the manufacturer's instructions.

2.8. Purification by fast protein liquid chromatography (FPLC)

The experimental set-up consisted of a glass column (16 mm I.D.) packed with chelating Sepharose Fast-Flow gel, two peristaltic pumps to supply the solutions to the column at the desired flow-rates and concentrations, a UV detector (LKB UV-MII) connected to a printer (LKB RIC 102) and a fraction collector (LKB FRAC-200, all purchased from Pharmacia). The column was equilibrated with three bed volumes of water and was loaded with three bed volumes of 0.3 *M* $NiCl_2$ solution followed by a washing and equilibration step with three bed volumes of IMAC buffer. A 1-ml volume of crude cell extract was applied to the column with a flow-rate of 1.5 ml/min, followed by four bed volumes of IMAC buffer. Elution of bound protein was achieved by an increasing gradient (0.5% of 0.5 *M* imidazole in water per ml mixed elution solution), and finally three bed volumes of 0.5 *M* imidazole in water. The column was cleaned and regenerated with 50 m*M* EDTA and 1 *M* NaCl, pH 8.0, in water to release the metal, cell debris and bound proteins. The eluted

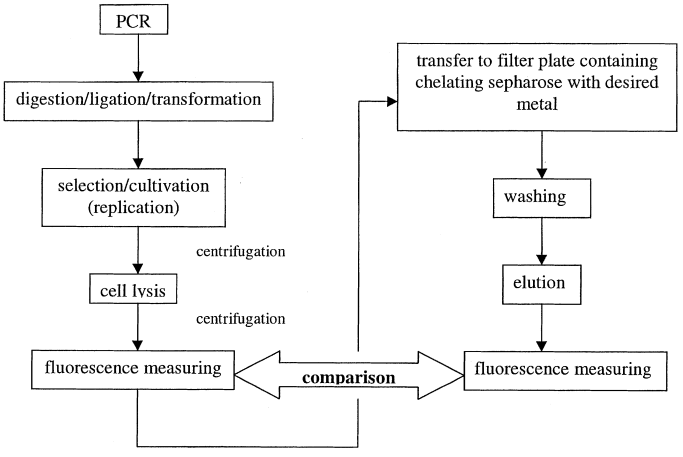

Fig. 2. Schematic overview of the single steps during the HTS for new metal affinity tags. After the selection of the clones, the subsequent steps could be performed semi-automatically using a Beckmann 2000 pipetting robot.

fractions were tested both optically and spectrometrically.

2.9. Digestion of the fusion protein heli$_{M14Xa}$-EGFP with factor X_a

The heli$_{M14Xa}$-EGFP solution was purified with the IMAC procedure described above and then dialyzed (Spectra/Por membrane M_r cut-off: 6000–8000; Roth, Karlsruhe, Germany) against IMAC buffer for 24 h to remove imidazole. Subsequently, the protein was incubated with 1 μl factor X_a (1 mg/ml) for 4 h at room temperature. Finally, the solution was purified once again by IMAC.

2.10. Determination of fluorescence

Fluorescence was measured in black 96-wells microtiter plates (FluoroNunc plates, Nunc, Rochester, USA) with a FLUOstar (BMG, Offenburg, Germany) using a 485 nm filter for excitation and a 538 nm emission filter in the range of the respected maxima of EGFP (488 nm for excitation, 507 nm for emission). Diluted samples of the cell supernatant were used to prove linearity and determination ranges (not shown).

3. Results and discussion

3.1. IMAC of EGFP fused to the heli$_{WT}$-tag and the his$_6$-tag

The purification of proteins by IMAC using their natural metal binding sites was described previously [22]. However, very few studies on the general usage of such metal binding sites with regard to the purification of heterologous proteins by IMAC have so far been published.

To compare the metal binding properties of the commercially available (and most frequently used) his$_6$-tag with the heli$_{WT}$-tag, a naturally occurring binding site of a *H. pylori* ATPase 439, the two peptides were fused to EGFP as reporter gene. Plasmids isolated from positive transformants were sequenced and one clone of each construct, with the desired tag fused to the N-terminus of EGFP, was used for subsequent binding studies.

For this purpose, crude cell extracts of transformed *E. coli* DH5α cells were loaded onto an IMAC column. The EGFP allowed a visual control during the purification process. It revealed that both the his$_6$-EGFP and the heli$_{WT}$-EGFP were bound to the matrix loaded with Ni(II). Native EGFP (without any fused tag), left the column during the subsequent washing steps. The imidazole gradient elution released the tag-fused proteins. The amount of imidazole used, elution profiles and protein yields differed significantly (Fig. 3). The his$_6$-tag-fused EGFP was eluted with 0.05 *M* imidazole; the elution of the heli$_{WT}$-EGFP required 0.25 *M* imidazole solution. But, the elution profile for heli$_{WT}$-EGFP was sharper than for his$_6$-EGFP. This minimized the required downstream processing such as the concentration of the sample, for example. The purified heli$_{WT}$-EGFP revealed a 20% higher fluorescence than the his$_6$-EGFP. The purified heli$_{WT}$-EGFP yielded 68% of the initial fluorescence in a single-step purification of the cell lysate. The effective recovery rate of the purified heli$_{WT}$-EGFP was expected to be higher because cellular and media components, which also contributed to the initial fluorescence, were removed by IMAC.

Compared to the his$_6$-tag, the main disadvantage of the heli$_{WT}$-tag was that five-times more imidazole was required to elute the protein from the column. This reduces the usefulness of this method because it brings about higher expenditures for downstream processing and may also lead to the denaturation of sensitive proteins.

To our knowledge, the metal binding site of the *H. pylori* ATPase 439 is, except for the his$_6$-tag, the shortest peptide with selective affinity for nickel under IMAC conditions. Hisactophilin, an M_r 10 000 histidine-rich protein, was successfully fused with EGFP to act as a control during the *E. coli* fermentation process and was later used for protein purification. Flaschel and co-workers reported that the affinity of hisactophilin fusion proteins for Ni(II)–NTA is twice as high compared to the his$_6$-tag [23,24]. The applicability of this method is limited because of the high molecular mass of the hisactophilin fusion protein. Another study dealt with the HAT epitope (LD**H**LI**H**NV**H**KEE**H**A**H**A**H**NK) derived from a chicken lactate dehydrogenase [25,26]. This tag is also quite large in comparison to

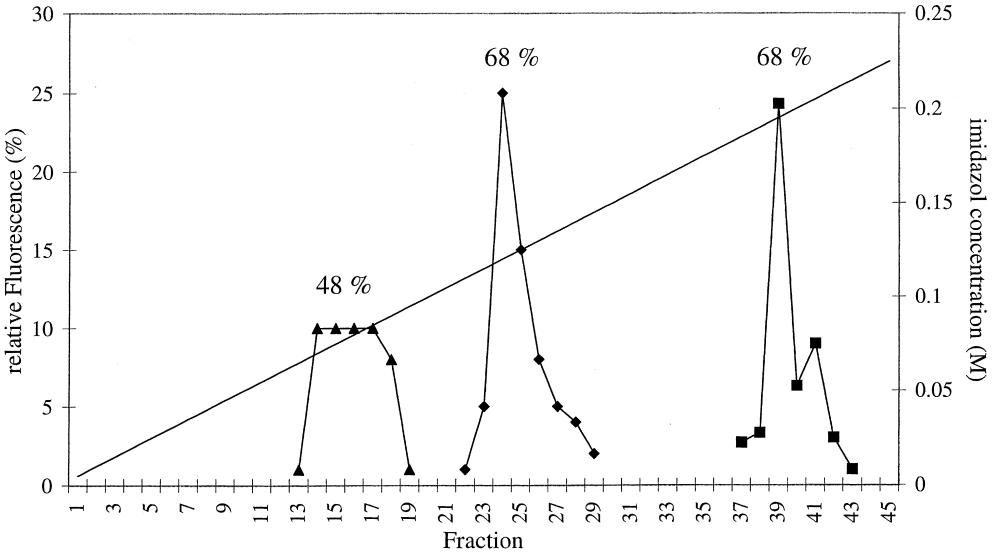

Fig. 3. Performance of the his_6-tag (▲), $heli_{WT}$-tag (■), $heli_{M14}$-tag (♦) for the purification of EGFP via IMAC. The solid line represents the imidazole concentration during the elution process. Each fraction has a volume of 2 ml. The total yields of relative fluorescence are marked at the top of the peaks.

the his_6-tag and therefore an alteration of the properties of the purified protein might be possible. Nevertheless, it is useful because the HAT epitope has a higher affinity for Co-charged Talon material than the his_6-tag. The small size and the higher yield of the $heli_{WT}$-tag compared to the his_6-tag render it very attractive for IMAC. We decided to vary this metal-binding site by focusing on the reduction of required amount of imidazole for elution and on retaining the enhanced binding capacity.

3.2. Selection of conserved regions inside the metal binding site

The binding motif of the ATPase 439 from *H. pylori* [15] (**His**–Ile–**His**–Asn–Leu–Asp–**Cys**–Pro–Asp–**Cys**) was used as a query for a BLAST search [27]. Four other putative metal transporting enzymes were retrieved, two from *H. pylori*, one from *H. felis* and one from *Deinococcus radiodurans* (Table 3). The **H**x**H**xxx**C**xx**C** consensus sequence was deduced and chosen for the formation of random heli-tags. This supports the hypothesis that the histidines and cysteines are mainly responsible for binding. Interestingly, the second histidine is replaced by a tyrosine in the *H. felis* sequence which may lead to a putative cadmium specificity [28].

3.3. Variation of the $heli_{WT}$-tag by PCR-directed mutagenesis

Starting from the **H**x**H**xxx**C**xx**C** consensus sequence, an exhaustive library of potential metal-binding motifs would contain 20^6 different se-

Table 3
Result of the BLAST search for putative metal-binding peptide homologues to the $heli_{WT}$-tag

Protein	Microorganism	Sequence	Ref.
P-type ATPase 429	*Helicobacter pylori*	**H**I**H**NLD**C**PD**C**	[15]
Putative heavy-metal cation transporter	*Helicobacter pylori*	**H**I**H**NLD**C**PD**C**	[15]
Putative heavy-metal cation transporter	*Helicobacter pylori*	**H**I**H**NLD**C**PD**C**	[30]
Hypothetical protein	*Deinococcus radiodurans*	**H**I**H**DLT**C**PD**C**	[29]
Putative cadmium-exporting P-type ATPase	*Helicobacter felis*	**H**FTGLD**C**PD**C**	[28]

quences which are impossible to obtain using conventional peptide synthesis. Therefore, partially degenerated oligonucleotides with four conserved positions were used to establish a heli-tag. Using the same strategy as before, the tags were fused to EGFP by PCR, resulting in a heli-tag library (heli$_{XX}$-tags).

Colonies that expressed EGFP could easily be identified under UV light. Approximately 5% of the colonies on the plates were non-fluorescent because they had stop-codons within the heli-tags.

About 100 clones (fluorescent and non-fluorescent) were sequenced to examine the variation of the randomly synthesized heli-tags (Fig. 4). All amino acids could be found at least once in one of the heli-tags. Because of the degeneration of the genetic code it was suspected that some of the amino acids were incorporated more frequently than others. The calculated values represent the distribution of amino acids under the simplified assumption that all codons were used in equal amounts (Fig. 3). However, the distribution of amino acids was purely random and no significant accumulation of special amino acids occurred.

3.4. High-throughput screening

As mentioned above, an exhaustive library of variants would contain 20^6 clones. This is impossible to achieve by conventional FPLC or by commercially available Ni–NTA columns (e.g., Qiagen). Therefore, a parallel screening method using microtiter plates was developed.

A 96-well membrane filter plate was filled with chelating Sepharose resulting in 96 mini-columns for IMAC. The throughput was enhanced by the use of a pipetting robot. The experimental set-up was tested by loading a homogeneous solution of heli$_{WT}$-tag-EGFP into all 96 wells. The wells in the outer rows generally produced lower yields. Thus, these rows were not used for screening, only 56 clones were therefore screened in parallel. The test proved the reliability of the screening method; i.e., all wells provided the same yield (within an error range of 10%; data not shown). However, the amount of imidazole (0.5 *M* for heli$_{WT}$-EGFP and 0.25 *M* for his$_6$-EGFP) required for the elution of the respective fusion proteins from the columns was about five-times higher than would be expected from conventional FPLC (0.25 *M* for heli$_{WT}$-EGFP and 0.05 *M* for his$_6$-EGFP).

Fluorescent clones from the heli-tag library were selected and transferred to microtiter plates. After cell lysis and separation of the cell debris, the EGFP in the supernatant was quantified by fluorescence

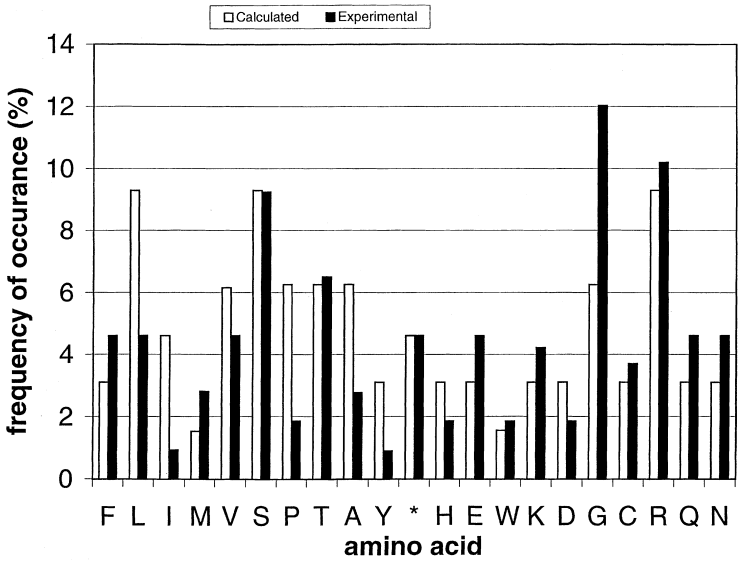

Fig. 4. Distribution of amino acids in the partially randomized heli$_{XX}$-tags. To prove the quality of the partially randomized oligonucleotides obtained by PCR-directed mutagenesis, 100 clones were sequenced and the occurrence of each amino acid (including the stop codons) was counted and compared to the calculated number of amino acids.

measurements and applied onto the mini-columns. The initial fluorescence of the sample was set as 100%.

We hoped to find heli-tags with excellent yields but with an elution behavior that was as good as that of the his_6-tag. Fig. 5 shows the distribution of the relative fluorescence from different clones that were eluted with 0.3 *M* imidazole. The recovery of relative fluorescence varied between 0 and 50%. Of 3000 clones screened, 47% showed medium performance, with a yield of 15–30% relative fluorescence after elution. These values corresponded to the $heli_{WT}$-tag (30%), which can only be partially eluted under these assay conditions. This low yield could result from either weak binding or inefficient elution with 0.3 *M* imidazole. Some clones (10%) showed hardly any fluorescence in the eluted fractions. A relative high number of around 40% of heli-tags displayed relative fluorescence yields between 30 and 50%. To reduce expenditures of a second screening round, only tags which displayed yields higher than 45% were chosen for screening with Ni–NTA spin columns. $Heli_{XX}$-tags were named according to their position on the 96-well plate (A–Z; numbered from the upper left corner to the bottom right corner).

3.5. Secondary screening with Ni–NTA SpinPrep columns

Several heli-tags which were obtained in primary screens and differed in their binding and elution properties were chosen for purification with Ni–NTA columns. It should be mentioned that the results obtained with Ni–NTA columns were not identical with those obtained with the FPLC purification [i.e., a concentration of 0.3 *M* imidazole was required for Ni(II)–NTA columns whereas only 0.05 *M* imidazole was required for FPLC]. Nevertheless, the system was easy to handle and fast. The untagged

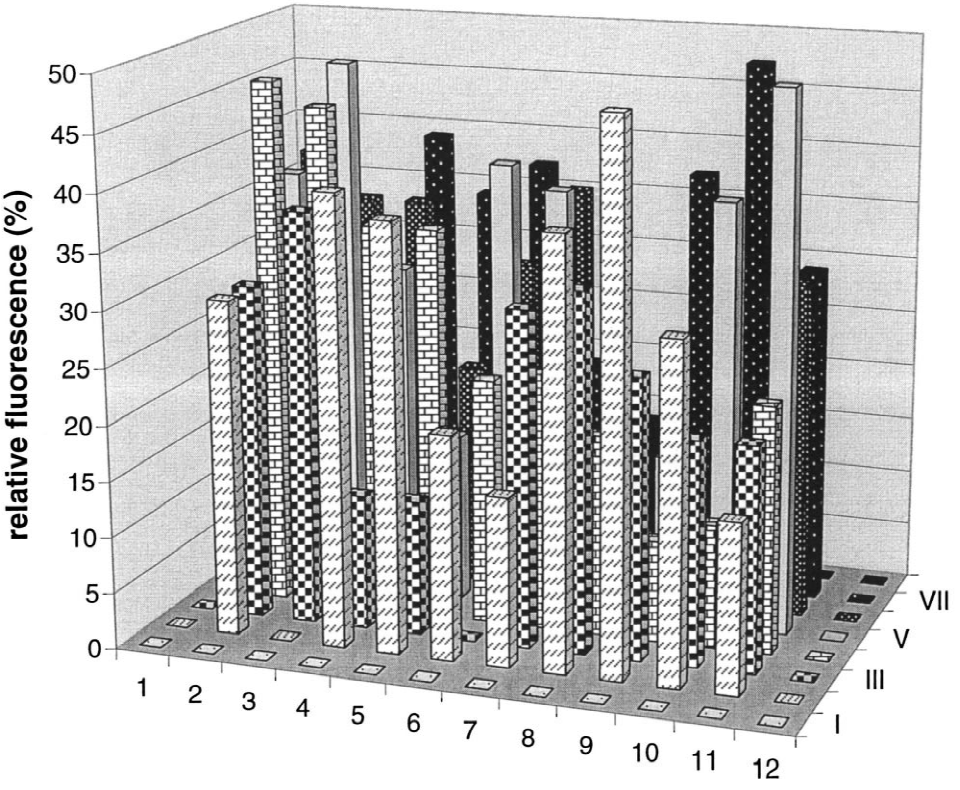

Fig. 5. Fluorescence yield obtained in mini-columns in the HTS. The outer rows were not loaded because they had already previously revealed lower yields. Each column represents the relative fluorescence in the elution step obtained by one $heli_{XX}$-EGFP clone.

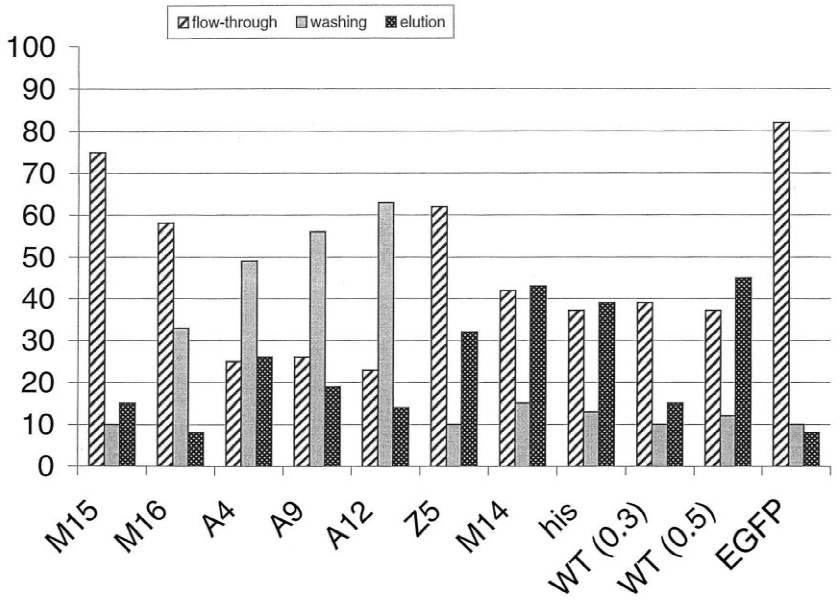

Fig. 6. Purification of several fusion proteins for the purification of EGFP with Ni–NTA. Different clones from several screening rounds were further tested with Ni–NTA spin columns. The flow-through and the washing solution is composed of fluorescent cells, components of the media used, as well as untagged EGFP. Letters and numbers refer to different heli$_{XX}$-EGFP clones [letters indicate the screening round, numbers the position in the plate (from 1–56)], ''his'' stands for his$_6$-EGFP, ''WT'' for heli$_{WT}$-EGFP eluted with 0.3 and 0.5 *M* imidazole and ''EGFP'' for the protein with no fusion-tag.

EGFP product revealed a relative background fluorescence of 8% (Fig. 6). This trace fluorescence was most likely due to insufficient washing of the material. Yields obtained with the his$_6$-tag and the heli$_{WT}$-tag on the other hand were lower than the yields obtained by FPLC (not shown).

One clone, M14, was identified and seemed to possess the desired properties. The yield was similar to that of the his$_6$-tag, but a lower imidazole concentration was necessary for elution. The tag of this clone was termed heli$_{M14}$-tag. M14 was further investigated by FPLC to allow for adequate comparison with the his$_6$-tag and heli$_{WT}$-tag. The sequence of M14 was determined as **His**–Asn–**His**–Arg–Tyr–Gly–**Cys**–Gly–Cys–**Cys**.

3.6. Comparison of heli$_{M14}$-tag with the his$_6$- and heli$_{WT}$-tags

Fig. 3 shows the elution profiles of the heli$_{WT}$-tag, heli$_{M14}$-tag and his$_6$-tag. As mentioned above, the heli$_{WT}$-tag was eluted at significant higher imidazole concentrations but in a smaller volume than the his$_6$-tag. The novel heli$_{M14}$-tag could be eluted under milder conditions (i.e., lower imidazole concentration) than the heli$_{WT}$-tag, but still maintained the high yield and the sharp elution profile of the heli$_{WT}$-tag.

3.7. Removal of the heli$_{M14}$-tag from recombinant proteins

Compared to other methods of protein purification, IMAC is quite expensive. Consequently, its industrial use is mainly restricted to expensive, pharmaceutical proteins. In particular, when dealing with pharmaceutical proteins, the requirements for retention of biological activity and antigenic properties are essential. The complete removal of a tag-peptide from the protein might be required to allow the usage of the heli-tags for this purpose.

An established method to accomplish this involves the insertion of a protease cleavage site between the desired protein and the fusion peptide. For this purpose, proteases from the blood coagulation sys-

tem were used, because their recognition sites are well defined and they are strongly selective.

Using the same cloning strategy as described above, we introduced the cleavage site of factor X_a between the $heli_{M14}$-tag and EGFP (_IEGR, location of cutting is indicated by an underscore), which is not present in the EGFP sequence. The fusion protein with the factor X_a cleavage site ($heli_{M14Xa}$-EGFP) was subsequently purified as described for $heli_{M14}$-EGFP. It revealed a similar yield of 63% (not shown). The purified protein was dialyzed against IMAC buffer (yield 86%) to remove imidazole and divided into two aliquots. Half of the solution was incubated with factor X_a. Afterwards, the protein was separated from the cleaved $heli_{M14Xa}$-tag by another IMAC. The un-cleaved protein and the cleaved $heli_{M14Xa}$-tag peptides were expected to bind to the column and cleaved EGFP to flow through. Only 2% residual fluorescence was detected in the elution step. After concentration of the flow-through and the washing solution, a total of 42% relative fluorescence (compared to the applied amount) was recovered. As a control, the other half of the solution was purified by IMAC without prior incubation with factor X_a. This led to 90% recovery of the initial fluorescence. This demonstrates that the loss of fluorescence in the first IMAC can mainly be attributed to the elimination of fluorescent cells and components of the media used.

4. Conclusion

The wild-type metal binding site of the ATPase 439 from *H. pylori* could be used successfully for the purification of recombinant EGFP. This metal affinity tag revealed an 20% enhanced yield compared to the frequently used his_6-tag. However, a high imidazole concentration was necessary to release the protein from the adsorbent. This elevated imidazole concentrations might however lead to denatured proteins. Therefore, the natural metal-binding site of the *H. pylori* ATPase 439 cannot be regarded as a real alternative to the his_6-tag.

To further improve the properties of the $heli_{WT}$-tag for IMAC applications, a library of $heli_{WT}$-tag variants was developed by directed evolution employing partially randomized oligonucleotides. The high number of possible variants made the development of a high-throughput assay system necessary. The use of 96-well membrane filter plates, leading to 96 mini-columns for IMAC when filled with chelating Sepharose, provided a flexible assay system that could easily be modified to fit the desired properties of the tags under study: the elution with pH gradients or the optimization of binding to or elution from columns charged with different metals could be varied. Using the high-throughput system, a new $heli_{M14}$-tag could be identified which combined the high yield of the $heli_{WT}$-tag with the low imidazole concentrations required for his_6-tag elution. Hence, $heli_{M14}$-tag is a promising new tool for IMAC.

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