ELSEVIER

Journal of Chromatography A, 864 (1999) 247–256

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

# Natural poly-histidine affinity tag for purification of recombinant proteins on cobalt(II)-carboxymethylaspartate crosslinked agarose

Grigoriy Chaga*, Dmitry E. Bochkariov, George G. Jokhadze, Jennifer Hopp, Paul Nelson

*Clontech Laboratories, Inc., 1020 E. Meadow Circle, Palo Alto, CA 94303, USA*

Received 30 June 1999; received in revised form 20 September 1999; accepted 20 September 1999

**Abstract**

A natural 19-amino-acid poly-histidine affinity tag was cloned at the N-terminus of three recombinant proteins. The vectors containing the DNA of the fusion proteins were used for transformation of *Escherichia coli* DH5α cells. Each protein was expressed, extracted and purified in one chromatographic step. The purification procedure for each protein can be accomplished in less than 1 h. A new type of immobilized metal ion affinity chromatography adsorbent – $Co^{2+}$-carboxymethylaspartate agarose Superflow – was utilized at linear flow-rates as high as 5 cm/min. The final preparation of each protein is with purity greater than 95% as ascertained by sodium dodecyl sulfate-electrophoresis. Recovery for each purified protein was higher than 77% of the initial loaded amount as judged by biological activity. The operational capacity of $Co^{2+}$-carboxymethylaspartate agarose for each protein was determined. 

*Keywords:* Immobilized metal affinity chromatography; Proteins; Peptides; Cobalt

## 1. Introduction

The natural development from genome oriented to proteome oriented research creates a significant need for novel tools for protein purification. Reduction of time for protein isolation and purification is very advantageous. For research that focuses on protein function and/or interaction, the development of universal purification procedures is relieving the burden to develop unique purification protocols for each individual studied protein.

Recombinant proteins being produced for pharmaceutical purposes impose additional challenges to biochemists. During the development of reproducible purification procedures, especially for labile biomolecules, one must consider as an important factor the time for execution of each purification step.

New agarose based adsorbents with much higher flow resistance and lower back pressure at elevated flow-rates are presently available commercially. With these adsorbents a significant reduction in the time necessary for the completion of purification procedures can be accomplished. The advantage of these novel adsorbents can be fully utilized only if the adsorption principle is based on quick kinetics. Immobilized metal ion affinity chromatography (IMAC) introduced by Porath et al. in 1975 [1] satisfies to a great extent this requirement.

IMAC, introduced as a group separation method, was further developed into one of the most important tools for single step purification of fusion proteins utilizing artificial poly-histidine tags incorporated

*Corresponding author.

0021-9673/99/$ – see front matter 
PII: S0021-9673(99)01008-0

either on the N- or C-terminus of the protein of interest [2,3]. The basic research on the elucidation of the role of the amino acids side chains responsible for binding to immobilized metal ions of the transition group such as $Cu^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ [1,4–7] has found real life application that at present is utilized in more than 50% of all procaryotic recombinant protein purifications. Further research is carried out to isolate and select additional metal ion affinity peptides that would permit easier and reproducible purification of recombinant proteins [8–13].

In a recent paper we reported the identification of a natural polyhistidine peptide sequence with very high affinity towards immobilized ''transition'' metal ions [14]. The conditions under which the purification was performed were near physiological and milder compared to those utilized for purification of 6×His tagged proteins. Consequently, we decided to investigate the possibility of incorporation of this natural poly-histidine affinity tag (HAT) in recombinant proteins and its potential utility for purification of each of these proteins in a single chromatographic step.

This paper reports the results of this investigation along with the study on the utility of a novel crosslinked IMAC adsorbent for rapid purification of HAT tagged proteins.

$Co^{2+}$ ions immobilized on carboxymethylaspartate (CM-Asp) crosslinked agarose (Superflow) were used for rapid one-step purification of recombinant chloramphenicol acetyltransferase (CAT), dihydrofolate reductase (DHFR) and green fluorescent protein – UV-enhanced variant (GFPuv) tagged with the HAT sequence in one chromatographic step. The capacity of the adsorbent for HAT tagged proteins and the recovery of biological activity after the purifications were determined.

## 2. Materials and methods

Aspartic acid (A 8949), bromoacetic acid (B 7130), epichlorohydrin (E 4255), imidazole (I 0250) and sodium borohydride (S 9125) were purchased from Sigma (St. Louis, MO, USA). $CoCl_2 \cdot 6H_2O$ (25,559-9) was purchased from Aldrich (Milwaukee, WI, USA). All buffer substances and salts were of chemical grade or higher.

### 2.1. Synthesis of carboxymethylaspartate Superflow

The synthesis was performed essentially as described in [15]. In short, 20 g of suction-dried Superflow 6 resin was transferred to a 250 ml conical flask, containing 20 ml of 2 *M* NaOH, 1 ml of epichlorohydrin, 40 mg of sodium borohydride and 20 ml of deionized water. The mixture was left on a shaker at ambient temperature. After 3 h 4 ml of epichlorohydrin were added to the mixture and the reaction was allowed to proceed for a total of 24 h.

L-Aspartic acid (8 g) was dissolved in 50 ml of 1 *M* sodium carbonate and the pH was adjusted to 11.5 with anhydrous pellets of NaOH. The aspartate solution was added to the epoxy-activated Superflow 6 resin and left under stirring overnight. The gel was washed with deionized water, 10% acetic acid, deionized water and 0.2 *M* $NaHCO_3/Na_2CO_3$, pH 10.0.

The adsorbent was transferred to a 250 ml conical flask with 30 ml of 1 *M* $NaHCO_3/Na_2CO_3$, pH 10.0 and the pH was adjusted to 10.0 if necessary. 12.6 g of bromoacetic acid was disolved in 30 ml of 4 *M* NaOH and the pH was adjusted to 10.5 with 10 *M* NaOH. The bromoacetate solution was added to the gel and the suspension was stirred overnight.

The gel was washed with deionized water, followed by 10% acetic acid and deionized water.

The capacity of the adsorbent for $Co^{2+}$ was determined by atomic absorption analyses.

### 2.2. Cloning, expression and isolation of HAT-proteins

All cloning procedures were performed using standard molecular biology techniques.

pHAT cloning vector was constructed from pUC19. Synthetic oligonucleotides containing the HAT sequence along with multiple cloning site (cleavage sites for HindIII, ClaI, SalI, BamHI, SmaI, KpnI, SacI and EcoRI restriction enzymes) were ligated with pUC19 digested with HindIII and EcoRI.

#### 2.2.1. pHAT-CAT

The coding sequence of CAT gene was isolated from plasmid pMAMneo-CAT (Clontech Labs, Palo Alto, CA, USA) and cloned into pHAT vector.

*2.2.2. pHAT-DHFR*

The coding sequence of DHFR gene was isolated from plasmid pQE40w (Qiagen, Carlsbad, CA, USA) and cloned into pHAT plasmid.

*2.2.3. pHAT-GFPuv*

The coding sequence of DHFR gene was isolated from plasmid pGFPuv (Clontech Labs.) and cloned into pHAT plasmid.

All fusion proteins were expressed by the same general method. One liter of LB medium containing 100 μg/ml ampicillin was inoculated with a 5 ml overnight culture of transformed DH5α cells. Cells were grown at 37°C on an orbital shaker to optical density of 0.6–0.8 $A_{650}$. Expression of recombinant proteins was induced by adding isopropyl-1-thio-β-D-galactoside (IPTG) to a 1 m*M* final concentration. The cells were grown for additional 4 h at 37°C after induction. The medium was cooled down and cells were collected by centrifugation at 8000 *g* and 4°C for 30 min.

Lysates for protein purification were prepared according to the commonly used ''Alumina'' method [16]. In general, approximately 0.5 g of cells was transferred to a precooled mortar, 1.5 g of alumina was added and the cells were disrupted by grinding until a paste composition was achieved (approximately 5 min). Five millilters of cold extraction/loading buffer were added and mixed. The suspension was transferred to eppendorf tubes and centrifuged at 6°C for 20 min. The clear supernatant was collected and used as a starting sample after filtration through a 0.45 μm filter.

*2.3. IMAC with native HAT proteins*

IMAC on immobilized metal ions was performed on a column (5.8 cm×0.5 cm I.D.) packed with carboxymethylaspartate Superflow and charged with two column volumes of 100 m*M* $CoCl_2$ in deionized water at a flow-rate of 1 ml/min. Excess metal ions were removed with deionized water. The column was equilibrated with five column volumes of the respective equilibration buffer. The equilibrated sample (2.1 ml) was loaded on the column and the adsorbed material was washed out with 150 m*M* imidazole in the equilibration buffer. Fractions of 1 ml were collected during the chromatography run. A flow-rate of 1 ml/min was utilized during these fast protein liquid chromatogrpahy (FPLC) experiments.

*2.4. IMAC with denatured HAT-proteins*

The extraction and purification was performed in presence of 6 *M* guanidinium·HCl. The purification was performed in a batch/gravity chromatography mode. In short, 1 ml of the adsorbent prepared as described above was equilibrated after charging with $Co^{2+}$ with 5 ml of 50 m*M* sodium phosphate; 0.25 *M* NaCl; 6 *M* guanidinium·HCl pH 7.0 and deposited in a sterile 15 ml tube. Whole cell extract prepared from 0.25 g of cells in the equilibration buffer was added and the adsorbent was mixed for 10 min with the cell extract on an orbital shaker. The non adsorbed material was removed after 1 min centrifugation at 500 *g*. Weakly adsorbed material was removed by washes with 10 ml of the equilibration buffer. Finally the adsorbent was transferred to a 2 ml disposable gravity column and the adsorbed material was eluted with 150 m*M* imidazole in the equilibration buffer. Fractions of 1 ml were collected during the elution.

*2.5. Operational capacity*

The capacity of the adsorbent for each recombinant protein was determined in batch/gravity chromatography mode. In short, 200 mg of the adsorbent prepared as described above was equilibrated, after charging with $Co^{2+}$ ions, with 5 ml of 50 m*M* sodium phosphate; 0.25 *M* NaCl pH 7.0 and deposited in a disposable 2 ml gravity flow column. Three milliliters of the starting sample (obtained from 0.5 to 2 g of cells) was added to the adsorbent and the column was briefly mixed until the adsorbent was dispersed. The suspension was left on ice to settle for 5 min. The non-adsorbed material was collected by opening the outlet of the column. Weakly adsorbed material was removed by washes with 10 ml of the equilibration buffer. Finally the adsorbed material was eluted with 150 m*M* imidazole in the equilibration buffer. Fractions of 0.5 ml were collected during the chromatography. The quantity and purity of the eluted proteins were analysed as described below.

### 2.6. Protein analyses

The purity of the eluted proteins was checked by Sodium-dodecyl sulfate (SDS)-electrophoresis on a Phast Gel gradient 8-25 using Phast gel SDS buffer strips and PhastSystem (Pharmacia Biotech). The gels were stained with Coomassie Brilliant Blue.

The protein concentration was determined by UV spectrophotometry and by the method reported by Bradford [17] using albumin as a standard.

Enzyme activity was determined as follows:

Chloramphenicol acetyltransferase activity was determined as described by Hash [18], and dihydrofolate reductase activity was determined according to Osborn and Huennekens [19]. All activity assays were performed on pools of the various chromatographic peaks after overnight dialysis at 4°C against the respective assay buffer.

GFPuv was followed by fluorescence. In short, 100 μl of the fractions collected during the chromatography runs were deposited in microplate wells and the fluorescence was detected in Perceptive Bio-Systems Cytofluor–2 fluorescence multiwell plate reader.

## 3. Results

### 3.1. Carboxymethylaspartate superflow

After synthesis [15], the adsorbent was charged with $Co^{2+}$ ions and the excess metal ions were removed by washing with deionized water. The immobilized metal ions were extracted with 0.2 *M* EDTA pH 7.0 and the adsorbent capacity was determined to be 17 μmol $Co^{2+}$ per ml packed gel by atomic absorption analyses.

### 3.2. Cloning and expression of HAT tagged proteins

Fig. 1 is a schematic presentation of a vector used for expression of the HAT tagged protein. The pHAT-GFPuv vector was prepared in such manner that it can be further utilized for cloning of a second partner recombinant protein – it contained a multiple cloning site after the cDNA encoding GFPuv protein. In this case GFPuv fluorescence could be used for choosing proper transformants, for cell localization

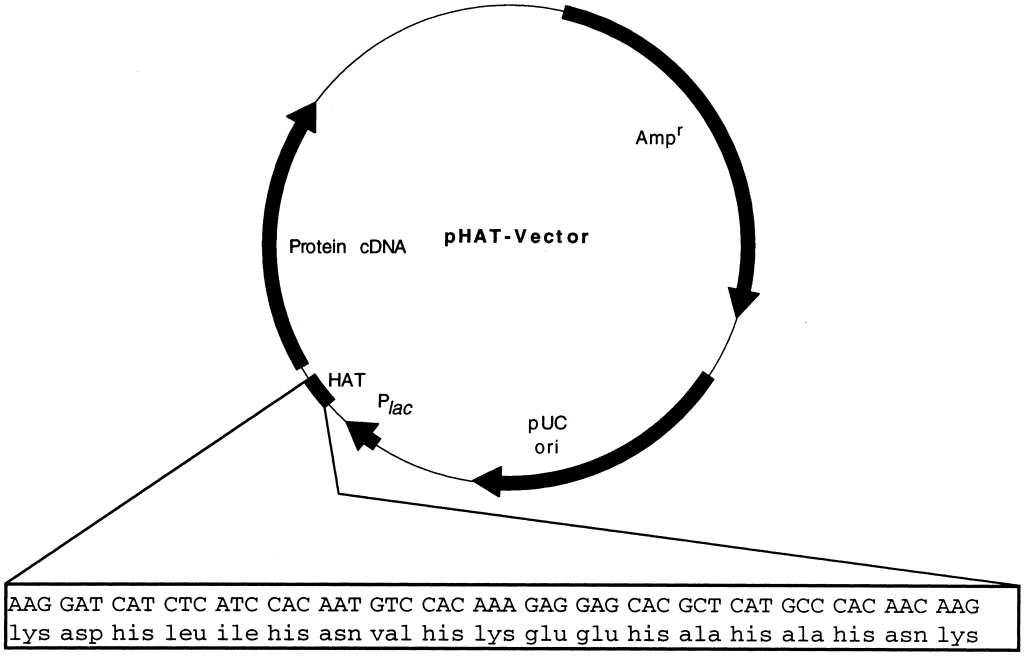


Fig. 1. Schematic presentation of a vector used for expression of HAT tagged proteins.

studies as well as for quick assay of the partner protein during and after purification.

The first construct (not shown) included in its entirety the 32-mer peptide that we identified in a previous study [14] with the following primary sequence as a purification tag:

**SLKDHLIHNVHKEEHAHAH-NKISVVGVGAVGM**

The results obtained from this construct containing GFPuv at the C-terminus of the peptide were indistinguishable from the construct containing a truncated 19-mer HAT peptide (Fig. 1). The utility of the shorter peptide which was more convenient for future cloning and purification applications was further investigated. For example it is possible to utilize synthetic oligopeptides for the incorporation of the tag in already existing vectors with cloned recombinant proteins. All results reported in this paper are based on this 19-mer HAT peptide cloned at the N-terminus of CAT, DHFR or GFPuv with the following primary amino acid sequence:

**KDHLIHNVHKEEHAHAHNK**

Cloning of the CAT sequence from pMAMneo-CAT into pHAT was straightforward since a SalI site was conveniently located in both plasmids. The same restriction site was used to identify the right transformant with 779 base pair (bp) insert. As both ends of the fragment contained the same SalI restriction site, it was necessary to confirm the orientation of the insert to be in the same direction as the lacZ gene. The initial pHAT plasmid contained a unique EcoRI restriction site. The CAT gene also contains an EcoRI site 247 nucleotides from the 5′-end of SalI site. Restriction with EcoRI generated a 571 bp fragment as expected for the correct orientation of CAT cDNA. A clone containing both a 779 bp SalI fragment and a 571 bp EcoRI fragment (pHAT-CAT) was chosen for the expression of HAT-CAT fusion protein.

The DHFR sequence in pQE40w does not contain convenient cloning sites adjacent to the coding region. Cloning sites compatible with pHAT were introduced by polymerase chain reaction (PCR) amplification of the coding region of DHFR using pQE40w as a template. Primers for the PCR were designed to eliminate the initiator ATG codon of the native DHFR cDNA and to introduce a KpnI restriction site. Two PCRs yielded fragments of expected length (599 bp) that were inserted into the KpnI site of pHAT, transformants were screened for a 578 bp KpnI fragment as well as for a 326 bp SacI fragment to confirm the orientation of the insert. One individual clone from each PCR was selected for the sequence analyses. For each clone four independent sequencing reactions were performed. It was found that the entire sequence of the insert was identical to the DHFR sequence from pQE40W minus the initiation codon. One of the clones containing pHAT-DHFR was chosen and used for expression and purification studies.

Preparation of a construct for expression of HAT-GFPuv fusion protein was not as straightforward as for the other two proteins since the coding sequence of GFPuv contains many of the restriction sites that constitute the multiple cloning site of pHAT plasmid. So, it was necessary to modify the pHAT plasmid in order to introduce a new unique restriction site into the multiple cloning site. Two complimentary oligonucleotides with ClaI and EcoRI compatible ends, enterokinase cleavage site and the new multiple cloning site containing a unique HpaI site were used. As ClaI enzyme does not digest methylated DNA efficiently, the initial pHAT vector was first produced and isolated from DM1 cells in order to have sufficient ClaI digestion. The modified pHAT was produced by ligation of the annealed synthetic fragments into pHAT digested with ClaI and EcoRI, followed by transformation into DH5α cells and screening transformants for the presence of the HpaI site. One of the linearized clones was sequenced to confirm the presence and the sequence of the insert. This clone was used for the construction of pHAT-GFPuv.

The fragment containing GFPuv coding sequence was amplified from pGFPuv with primers designed to amplify the sequence from the first base after the ATG start codon to the last base just before the stop codon flanked with ClaI sites on both ends. A fragment corresponding to the expected 827 bp was a major PCR product as analyzed by agarose gel electrophoresis. This fragment was digested with ClaI and ligated into ClaI digested pHAT followed by transformation into DH5α cells. Transformants with the unique HpaI restriction site that was intro-

duced with the new multiple cloning site were additionally screened for a 252 bp EcoRI/BamHI fragment to confirm the correct orientation of the insert. One of the clones that gave the right size fragment was further analysed by sequencing. The sequence of the region containing PCR insert matched exactly the expected sequence.

### *3.3. Purification of HAT tagged proteins*

Fig. 2a–c present single step purifications of HAT-CAT, HAT-DHFR and HAT-GFPuv, respectively. The linear flow-rate during the chromatography runs was 5 cm/min. All chromatography steps were completed within 40–50 min. 5 m*M* imidazole in the equilibration/loading buffer was utilized to eliminate adsorption of weakly bound proteins and decrease the purification time by completing it in only two steps. After washing out the non adsorbed or weakly adsorbed proteins, the imidazole concentration was increased to 150 m*M* to elute the adsorbed HAT-CAT, HAT-DHFR or HAT-GFPuv, respectively. The recovery of CAT activity when applied at 5 m*M* imidazole in the equilibration buffer was about 50% (i.e. about 50% of the total loaded activity was detected in the non adsorbed material). Due to the low yield the experiment was repeated in absence of imidazole in the equilibration/loading buffer with a washing step at 5 m*M* imidazole (Fig. 2a) which did not result in desorption of any detectable protein or any significant loss of CAT activity. The imidazole used for elution had an inhibitory effect on the enzymatic activity of CAT. After dialysis for removal of imidazole the total recovery (from non adsorbed and desorbed material) was close to 100%. Coupled with the low strength of initial binding in presence of imidazole, we can speculate that imidazole may have a structural effect on CAT that affects the accessibility of the HAT tag and this effect is reversible. It also appears that once adsorbed to immobilized $Co^{2+}$ ions in absence of imidazole, the affinity tag of HAT-CAT forms a strong enough complex with the immobilized metal ions and that the intermediate washing step at 5 m*M* imidazole is not disrupting coordination bonds.

HAT-DHFR had a lower affinity than HAT-CAT and it was necessary to load the lysate containing the protein in the absence of imidazole. After extensive washing the protein was eluted with 150 m*M* imidazole (Fig. 2b). HAT-GFPuv was adsorbed efficiently in presence of 5 m*M* imidazole and eluted with 150 m*M* imidazole (Fig. 2c).

The material balance of these experiments as well as the operational capacity determined in batch/gravity mode is presented in Table 1. The operational capacity follows the observed trend – HAT-DHFR having the lowest and HAT-GFPuv having the highest affinity for immobilized $Co^{2+}$ ions.

Elution of all three HAT proteins was possible also by decreasing the pH of the system – approximately 80% of the adsorbed tagged proteins were eluted at pH 6.0 with a complete elution at pH 5.0 utilizing sodium phosphate and sodium acetate buffers respectively (not shown).

A significant decrease in the capacity of the adsorbent was observed when a column chromatography was performed at the reported elevated flow-rate of 5 cm/min compared with the batch adsorption. On average the capacity dropped approximately two times. The influence of imidazole on the adsorption strength was studied. Since the binding of GFPuv on the column can be visualized by UV light, an additional experiment was performed with the protein loaded in absence of imidazole. Under these conditions the protein was adsorbed tightly as a strong green band on the top of the column (at the inlet of the column). Upon introduction of 5 m*M* imidazole in the equilibration/loading buffer the green band started smearing down the column. The protein did not migrate as a solid band which indicates that at this concentration imidazole is close to equilibrium with the protein for competitive binding to the immobilized metal ions. In the case of HAT-DHFR we did not perform an intermediate wash and the eluted protein had a similar purity as that of the eluted materials from the other two HAT-proteins for the purification with an imidazole wash (either as a separate washing step or as a competitor in the loading buffer).

The purity of the eluted HAT proteins was analysed by SDS-gel electrophoresis. The results are presented in Fig. 3.

In order to determine if the HAT tag can be utilized under denaturing conditions, we purified all three fusion proteins in the presence of denaturants. Initial attempts to purify HAT proteins in presence of

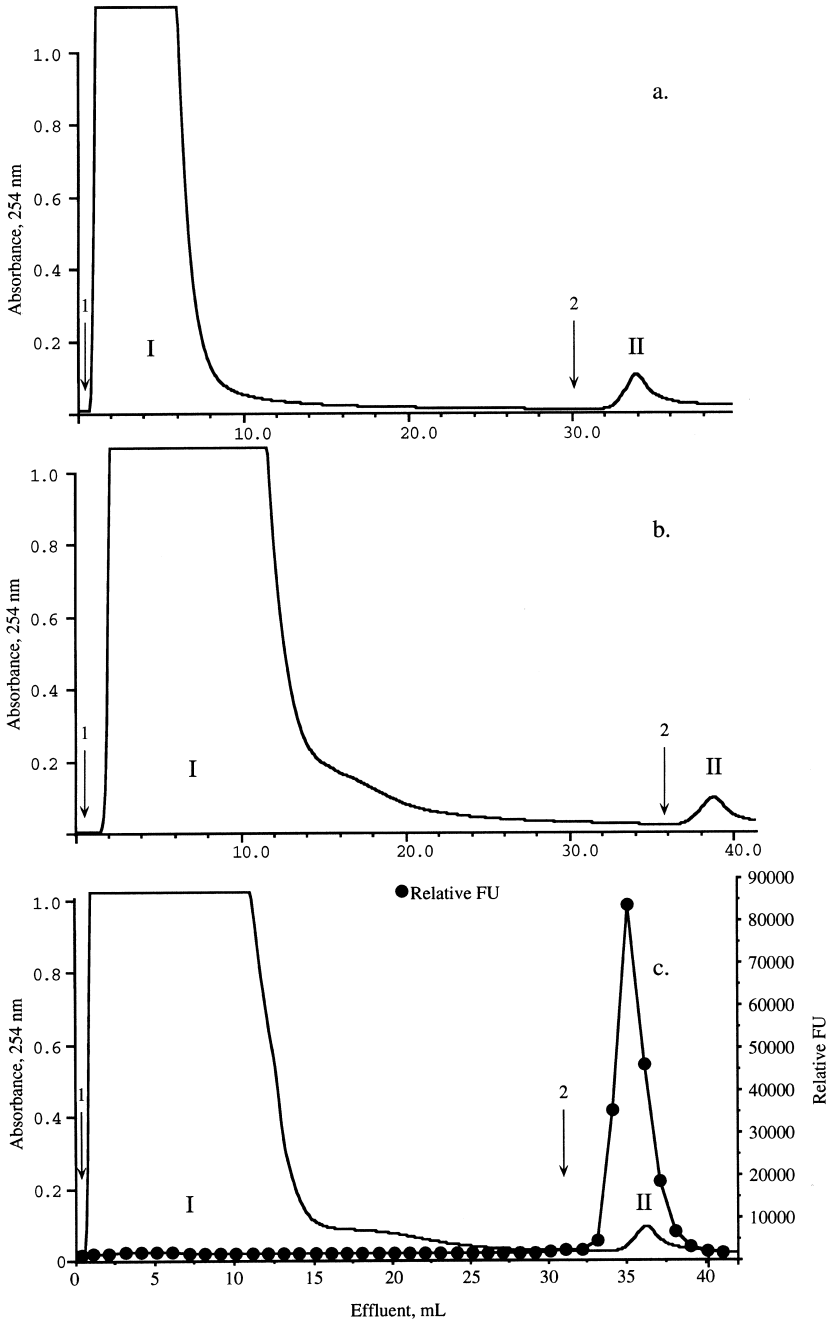


Fig. 2. Single step IMAC purification of HAT proteins on $Co^{2+}$-carboxymethylaspartate agarose. (a) IMAC of HAT-CAT. The arrows are as follows: 1 – loading and wash of non adsorbed material with 50 m*M* sodium phosphate; 0.25 *M* NaCl pH 7.0; 2 – elution of HAT-CAT with 50 m*M* sodium phosphate; 0.25 *M* NaCl; 150 m*M* imidazole pH 7.0. (b) IMAC of HAT-DHFR. The arrows are as follows: 1 – loading and wash of non adsorbed material with 50 m*M* sodium phosphate; 0.25 *M* NaCl pH 7.0; 2 – elution of HAT-DHFR with 50 m*M* sodium phosphate; 0.25 *M* NaCl; 150 m*M* imidazole pH 7.0. (c) IMAC of HAT-GFPuv. The arrows are as follows: 1 – loading and wash of non adsorbed material with 50 m*M* sodium phosphate; 0.25 *M* NaCl; 5 m*M* imidazole pH 7.0; 2 – elution of HAT-DHFR with 50 m*M* sodium phosphate; 0.25 *M* NaCl; 150 m*M* imidazole pH 7.0.

Table 1
Material balances and capacities obtained from IMAC purification of three HAT tagged proteins

| Recombinant protein | Initial protein (mg total) | Pure protein (mg total) | Purification factor | Recovery (%) | Operational capacity (mg/ml) |
|---|---|---|---|---|---|
| HAT-CAT | 15.24 | 1.47 | 10 | 94 | 11.5 |
| HAT-DHFR | 11.56 | 0.84 | 14 | 77 | 4.9 |
| HAT-GFPuv | 10.31 | 0.97 | 11 | 95 | 15.8 |

8 *M* Urea were not successful. Upon equilibration of the IMAC column with urea a significant change in color was observed, which is an indication of its complexation with immobilized cobalt ions. In the presence of 6 *M* guanidinium·HCl as described in the Materials and Methods section we obtained significantly better results. The purity of the eluted HAT proteins was analysed by SDS-gel electrophoresis after overnight dialysis of the fractions against 8 *M* urea in 50 m*M* sodium phosphate; 0.25 *M* NaCl pH 7.0 and is presented in Fig. 4. The presence of guanidinium·HCl has a lesser negative effect on the binding strength of the adsorbent, but the loading of all HAT-proteins in absence of imidazole was necessary in order to ensure stable adsorption.

## 4. Discussion

This study reports the utility of a naturally existing HAT from lactate dehydrogenase in the successful single chromatographic step purification of recombinant proteins. The tag appears to be readily exposed in all three of the example proteins that we used in this study. All three proteins were purified to near homogeneity utilizing similar conditions. The op-

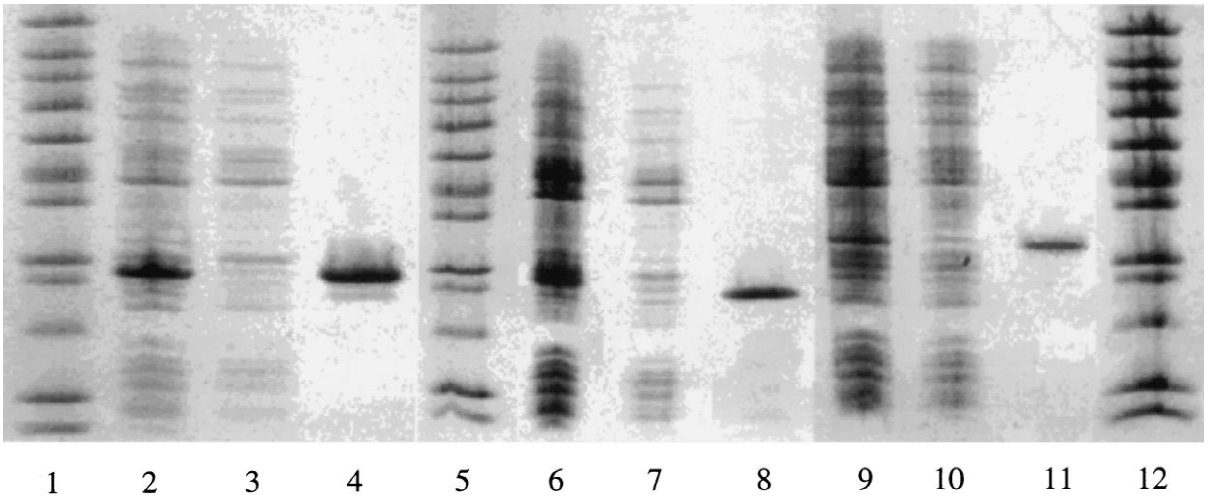


Fig. 3. Composite picture of SDS-electrophoretic analyses of fractions obtained from FPLC IMAC purification of HAT-CAT, HAT-DHFR and HAT-GFPuv on $Co^{2+}$-carboxymethylaspartate agarose Superflow. The lanes are as follows: 1=Sigma Marker Wide Molecular Weight Range (M-4038) – bands from top to bottom correspond to $M_r$ 116 000, 97 000, 84 000, 66 000, 55 000, 45 000, 36 000, 29 000, 24 000, 20 000, 14 200, and 6500; 2=whole cell extract from pHAT-CAT transformed *E. coli* – starting sample for HAT-CAT purification; 3=peak I (Fig. 2a), non adsorbed material; 4=peak II (Fig. 2a), purified HAT-CAT; 5=Sigma Marker Wide Molecular Weight Range; 6=whole cell extract from pHAT-DHFR transformed *E. coli* – starting sample for HAT-DHFR purification; 7=peak I (Fig. 2b), non adsorbed material; 8=Peak II (Fig. 2b), purified HAT-DHFR; 9=whole cell extract from pHAT-GFPuv transformed *E. coli* – starting sample for HAT-GFPuv purification; 10=peak I (Fig. 2c), non adsorbed material; 11=peak II (Fig. 2c), purified HAT-GFPuv; 12=Sigma Marker Wide Molecular Weight Range.

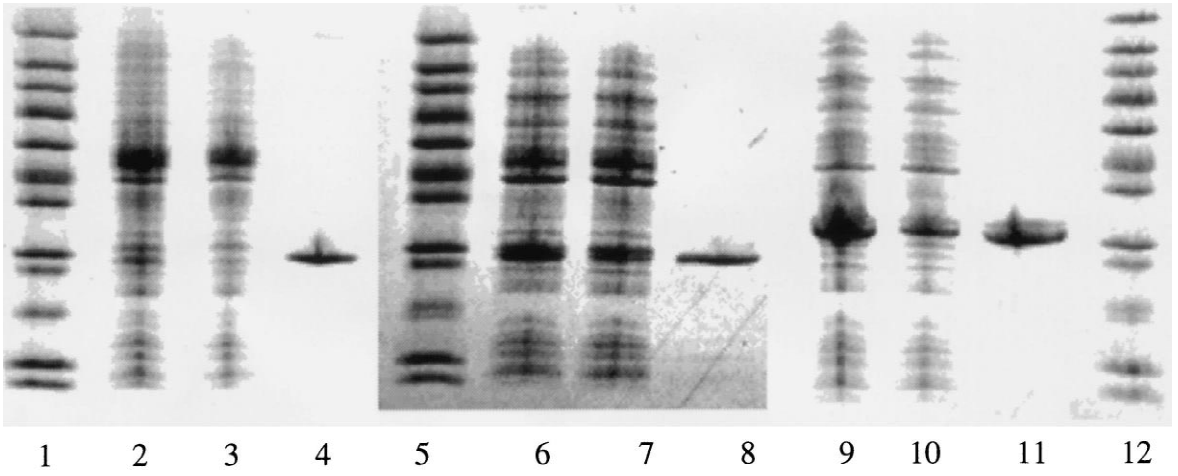


Fig. 4. Composite picture of SDS-electrophoretic analyses of fractions obtained from IMAC purification under denaturing conditions of HAT-CAT, HAT-DHFR and HAT-GFPuv on $Co^{2+}$-carboxymethylaspartate agarose Superflow. 1=Sigma Marker Wide Molecular Weight Range (M-4038) – bands from top to bottom correspond to $M_r$ 116 000, 97 000, 84 000, 66 000, 55 000, 45 000, 36 000, 29 000, 24 000, 20 000, 14 200 and 6500; 2=whole cell extract from pHAT-CAT transformed *E. coli* – starting sample for HAT-CAT purification; 3=non adsorbed material; 4=purified HAT-CAT; 5=Sigma Marker Wide Molecular Weight Range; 6=Whole cell extract from pHAT-DHFR transformed *E. coli* – starting sample for HAT-DHFR purification; 7=non adsorbed material; 8=Purified HAT-DHFR; 9=Whole cell extract from pHAT-GFPuv transformed *E. coli* – starting sample for HAT-GFPuv purification; 10=non adsorbed material; 11=purified HAT-GFPuv; 12=Sigma Marker Wide Molecular Weight Range.

timization of the purification conditions was a quick and simple process.

The loading conditions reported here for purification of HAT tagged proteins are the same or even more selective than the conditions for removal of non specifically adsorbed proteins (after adsorption at elevated pH) for hexa-histidine tagged proteins [20]. This is a good indication that the affinity of the HAT-tagged proteins for $Co^{2+}$ ions immobilized on CM-Asp is high. The conditions for purification are also very mild (neutral pH, low salt) and good recoveries were observed with all three example proteins (both enzymatic activity and fluorescence).

The imidazole concentration utilized for intermediate wash is somewhat lower than the one we used to purify successfully lactate dehydrogenase from chicken breast muscle, the N-terminal peptide from which was used to derive the HAT purification tag [14]. Instead of 9 m*M* imidazole in the starting buffer for purification of LDH, the loading of the HAT proteins was performed in absence of imidazole and an intermediate 5 m*M* imidazole wash was performed when necessary. In the case of HAT-DHFR we did not perform intermediate wash. The eluted protein had a similar purity to that of the eluted materials from the other two HAT tagged proteins for the purification of which we incorporated imidazole wash (either as a separate washing step or as a competitor in the loading buffer). We can attribute this finding to three factors:

1. $Co^{2+}$ ions immobilized to carboxymethylaspartate adsorbents have very high selectivity (i.e. very few unwanted proteins have amino acid clusters that can effectively bind to the adsorbent).
2. The conditions for loading are an additional factor for successful selection of the HAT tagged proteins (i.e. neutral pH at which only polyhistidine clusters are able to coordinate with the adsorbent).
3. The cell host line does not produce significant amounts of unwanted polyhistidine proteins.

Efficient binding was observed at a linear flow-rate of 5 cm/min. Elevated flow-rates decrease the time for purification and increase the yields of the target protein. Capacities might decrease significantly as a result of such high flow-rates. An interesting question to study is the comparative purity of the eluted HAT tagged proteins at different protein/

adsorbent ratios. Our initial observations point out that at sample loads that are close to the operational capacity of the adsorbent the purity of the target protein is higher (data not shown). It will be interesting to determine if a protein/protein displacement takes place under these conditions and develop a procedure for optimization of sample/adsorbent ratios.

Another interesting study would be to determine to what extent the use of $Co^{2+}$ metal ions contribute to the selectivity/capacity of carboxymethyl aspartate ligand by substituting them with $Zn^{2+}$ or $Ni^{2+}$ metal ions. In a previous paper we determined that cobalt appears to have the highest capacity for the protein containing the HAT sequence as a natural peptide at its N-terminus, namely lactate dehydrogenase from chicken muscle [14]. Whether the same behavior will be observed with recombinant proteins containing the HAT-peptide remains to be determined.

It was determined that urea has a much stronger negative effect on the binding of HAT tagged proteins than guanidinium·HCl. It appears that carbamide coordination is sufficiently strong to result in a significant decrease of the binding of HAT tagged proteins. Upon closer inspection of the HAT tag one can see that there is pair of histidine and asparagine (the amide of aspartic acid). This observation prompted us to build an artificial tag consisting of interleaving histidine and asparagine residues. Our study on this novel tag is still under way, but the first results are very promising indeed and we plan to report the results in the near future.

## References

[1] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.
[2] E. Hochuli, H. Döbeli, A. Schacher, J. Chromatogr. 411 (1987) 177.
[3] E. Hochuli, W. Bannwarth, H. Döbeli, R. Gentz, D. Stueber, Bio/Technology 6 (1988) 1321.
[4] E. Sulkowski, Trends Biotechnol. 3 (1985) 1.
[5] E. Sulkowski, K. Vastola, D. Oleszek, W. Von Muenchhausen, in: T.C.J. Gribnau, J. Visser, R.J.F. Nivard (Eds.), Affinity Chromatography and Related Techniques and Theoretical Aspects/Industrial and Biomedical Applications, Analytical Chemistry Symposium Series, Vol. 9, Elsevier, Amsterdam, 1982, p. 313.
[6] E.S. Hemdan, J. Porath, J. Chromatogr. 323 (1985) 255.
[7] J. Porath, Protein Expression Purif. 3 (1992) 263.
[8] M.C. Smith, T.C. Furman, C. Pidgeon, Inorg. Chem. 26 (1987) 1965.
[9] D.B. Smith, K.S. Johnson, Gene 67 (1988) 31.
[10] M.C. Smith, T.C. Furman, T.D. Ingolia, C. Pidgeon, J. Biol. Chem. 263 (1988) 7211.
[11] M.C. Smith, J.A. Cook, T.C. Furman, P.D. Gesellchen, D.P. Smith, H. Hsiung, ACS Symp. Ser. 427 (1990) 168.
[12] A.V. Patwardhan, G.N. Goud, R.R. Koepsel, M.M. Ataai, J. Chromatogr. 787 (1997) 91.
[13] A.V. Patwardhan, G.N. Goud, R.S. Pasquinelli, R.R. Koepsel, M.M. Ataai, Biotechnol. Tech. 12 (1998) 421.
[14] G.S. Chaga, J. Hopp, P. Nelson, Biotechnol. App. Biochem. 29 (1999) 19.
[15] T. Mantovaara, H. Pertoft, J. Porath, Biotechnol. Appl. Biochem. 13 (1991) 315.
[16] J.F. Eikenberry, T.A. Bickle, R.R. Traut, C.A. Price, Eur. J. Biochem. 12 (1970) 113.
[17] M. Bradford, Anal. Biochem. 72 (1976) 248.
[18] J.H. Hash, Methods Enzymol. 43 (1975) 737.
[19] M.J. Osborn, F.M. Huennekens, J. Biol. Chem. 233 (1958) 969.
[20] T.-T. Yang, P.S. Nelson, G.L. Bush, D.I. Meyer, S.R. Kain, Am. Biotechnol. Lab. 1 (1997) 12.