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Immobilised metal-ion affinity chromatography purification of histidine-tagged recombinant proteins: a wash step with a low concentration of EDTA

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Abstract

Immobilised metal-ion affinity chromatography (IMAC) is widely used for the purification of recombinant proteins in which a poly-histidine tag is introduced. However, other proteins may also bind to IMAC columns. We describe the use of a washing buffer with a low concentration of EDTA (0.5 m*M*) for the removal of proteins without histidine tag from IMAC columns. Four histidine-tagged recombinant proteins/protein complexes were purified to homogeneity from cell culture medium of insect cells by using an EDTA washing buffer. The presence of a low concentration of EDTA in washing buffers during IMAC may have a general application in the purification of histidine-tagged proteins.

Keywords: Recombinant proteins; EDTA; Histidine

1. Introduction

The heterologous production of therapeutic, diagnostic, and commercially interesting proteins has become increasingly important in the biotechnology field. Most applications demand highly processed recombinant proteins with a high degree of purity. To obtain the required degree of purity, an efficient and selective purification process must be designed. Immobilised metal-ion affinity chromatography (IMAC) has proven to be a powerful tool in the purification of histidine-tagged recombinant proteins [1,2].

IMAC was first reported as a method for the purification of proteins which have a natural affinity for metal ions [3]. The principle of IMAC is that many transition metal ions, e.g., nickel, can form coordination bonds to side chains of amino acids via electron donor groups. The metal ions are bound to a chelating group that is coupled via a linker to a solid-phase matrix. Iminodiacetic acid (IDA) and

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nitrilotriacetic acid (NTA) are the most commonly used chelating groups. Under physiological conditions, the surface accessible side-chains of predominantly histidine, but also of cysteine and tryptophan residues interact with the immobilised transition metal ions.

Nowadays, this technique is most frequently used for the purification of recombinant proteins in which a poly-histidine tail is introduced. IMAC is mostly used as an effective method for the capture of histidine-tagged proteins from complex protein mixtures. However, not only histidine-tagged recombinant proteins can bind to an IMAC column, but also other, contaminating, proteins. These contaminant proteins may contain surface-located patches of histidine residues that are exposed under native conditions. In addition, other protein–metal coordination groups, presumably surface amines, can contribute to binding to the IMAC matrix [4]. Commonly, these proteins are more weakly bound to the metal-ion charged matrix than the recombinant proteins containing five or six consecutive histidine residues. A number of strategies have been developed to separate histidine-tagged proteins from contaminant proteins. A low concentration of imidazole is most frequently used for this purpose [5]. Imidazole has the same structure as a histidine side chain and competes for binding to the metal ions immobilised on the column. Another way to obtain separation between histidine-tagged proteins and contaminant proteins is by adjusting the ionic strength of the washing buffers [6]. In addition, organic solvent washing steps have been reported to remove contaminant proteins [7].

In this study, we show that a washing buffer containing low concentrations of EDTA was effective in removing contaminant proteins from an IDA chelating IMAC column. The EDTA washing buffer was used for the purification of four recombinant histidine-tagged proteins/protein complexes from the cell culture medium of insect cells: gH:gL complex of herpes simplex virus type 1 [$gH_{t(His)}$:gL of HSV-1] [8], gH:gL complex of varicella-zoster virus [$gH_{t(His)}$:gL of VZV] [9], and two non-viral proteins, e.g., epithelial glycoprotein 2 [sEGP-$2_{(His)}$] [10], and protease 3 of neutrophil granulocytes [proPr$3_{(His)}$] [11].

2. Experimental

2.1. IMAC

An Äkta explorer 100 system (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and a 5 ml HiTrap chelating column (Amersham Pharmacia Biotech) were used. The column consisted of IDA groups coupled to highly cross-linked agarose beads via a linker. The column was saturated with Ni^{2+} ions, by using a 0.1 *M* $NiCl_2$ solution. According to the information of the manufacturer the capacity of the column is 23 μmol Ni^{2+} ions per ml of gel. After saturation of the column with Ni^{2+} ions, the column was washed with demineralized water followed by washing with phosphate-buffered saline (PBS), until no Ni^{2+} ions were eluted. Dialysed culture media of recombinant baculovirus infected insect cells, containing the recombinant proteins were applied to the column at a flow-rate of 10 ml/min. The subsequent wash and elution steps were performed at a flow-rate of 5 ml/min. During the wash steps and specific elution, the absorbance at 280 nm (A_{280}) was monitored and fractions of 15 ml and 1 ml, respectively, were collected. Enzyme-linked immunosorbent assay (ELISA) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were used to analyse the polypeptides in the eluted fractions. In addition, fractions were analysed for the presence of Ni^{2+} ions by measuring 615 nm absorbance.

The column was regenerated by washing with 500 m*M* EDTA in PBS, which removed all Ni^{2+} ions. After washing with demineralized water the column was reloaded with Ni^{2+} ions and re-used. This re-use had no effect on the reproducibility of the experiments.

2.2. ELISA

A catching ELISA was used to determine $gH_{t(His)}$:gL concentrations. Monoclonal antibody 52S, directed against authentic gH, was coated in a 96-well plate overnight at 4°C (in 50 m*M* $NaHCO_3$ buffer, pH 9.6). Serial dilutions of the fractions were added and incubated for 1 h at 30°C. After washing with PBS containing 0.3% Tween-20, polyclonal antibody anti-gH1 R83 [12] was added and incubated

for 1 h at 30°C. After washing, horseradish peroxidase-conjugated swine anti-rabbit antibodies were added. *O*-Phenylenediamine was used as substrate and the absorbance was measured at 492 nm.

2.3. Recombinant proteins

Four recombinant proteins/protein complexes were produced in the baculovirus expression system. These four were the recombinant histidine-tagged gH:gL complex of herpes simplex virus type 1 [$gH_{t(His)}$:gL of HSV-1] [8], the recombinant histidine-tagged gH:gL complex of varicella-zoster virus [$gH_{t(His)}$:gL of VZV] [9], and two non-viral proteins, e.g., histidine-tagged epithelial glycoprotein 2 which lacks the transmembrane region [sEGP-$2_{(His)}$] [10], and histidine-tagged protease 3 of neutrophil granulocytes including its two amino acid propeptide [proPr$3_{(His)}$] [11].

High Five insect cells (Invitrogen, Life Technologies Breda, The Netherlands) were grown in Insect-Xpress (BioWhittaker, Verviers, Belgium) as monolayer cultures. Insect cells were infected with recombinant baculoviruses at a multiplicity of infection of 1. The recombinant baculovirus designated Bacg$H_{t(His)}$:gL contained the open reading frames of herpes simplex virus type 1 (HSV-1) glycoprotein L (gL) and of histidine-tagged, truncated, HSV-1 glycoprotein H [$gH_{t(His)}$] under control of polyhedrin promoters [8]. Recombinant baculovirus BacVZVg$H_{t(His)}$:gL which contained both the gL and $gH_{t(His)}$ open reading frames of varicella-zoster virus under control of polyhedrin promoters was obtained from L. Maresova (Department of Experimental Virology, Prague, Czech Republic). The recombinant baculovirus BacsEGP-$2_{(His)}$ contained the gene sEGP-$2_{(His)}$ under control of a polyhedrin promoter and was obtained from W. Helfrich [10]. The recombinant baculovirus BacproPr$3_{(His)}$ was obtained from Y. van der Geld (Department of Clinical Immunology, University of Groningen, The Netherlands). This virus contained the open reading frame of proPr$3_{(His)}$ under control of the polyhedrin promoter.

The culture medium of the infected insect cells was harvested 3 days after infection, clarified by low-speed centrifugation, and dialysed against 20 m*M* sodium phosphate, pH 6.0 containing 80 m*M* NaCl. Dialysis tubing with molecular mass cut-off of M_r 12 000–14 000 (Serva, Heidelberg, Germany) was used. After dialysis, 1/100 volume of 5.0 *M* sodium chloride was added to the culture medium before application to the column.

3. Results

3.1. Purification of histidine-tagged $gH_{t(His)}$:gL of herpes simplex virus type 1

IMAC purifications were performed using insect cell culture medium containing histidine-tagged recombinant $gH_{t(His)}$:gL originating from herpes simplex virus type 1 [8]. The recombinant $gH_{t(His)}$:gL complex is a heterodimer which consists of gL and a truncated form of glycoprotein H [$gH_{t(His)}$] [13]. The truncated gH contains six histidine residues at the C-terminus, which allows purification by IMAC.

At first, commonly used protocols for IMAC purification of His-tagged proteins were used, with washing steps using different concentrations of imidazole, NaCl and final elution of the purified protein by 50 m*M* EDTA. Wash buffers containing a relatively low imidazole concentration (10 m*M*) resulted in elution of contaminating proteins and in high losses of $gH_{t(His)}$/gL during wash steps. The recombinant $gH_{t(His)}$:gL complex eluted by 50 m*M* EDTA in the final purification step was still contaminated with a large number of contaminating proteins (data not shown). Commercial culture medium of insect cells, in which the recombinant proteins are secreted, contains a non-removable detergent, and this very well could interfere with generally used IMAC procedures [1,14].This prompted us to look for another procedure to purify recombinant proteins from culture medium of infected insect cells.

A 5 ml column which contained iminodiacetic acid chelating groups charged with nickel ions (an Ni-IDA column) was used to purify recombinant $gH_{t(His)}$:gL complex from the culture medium. The column was loaded with 850 ml dialysed culture medium containing recombinant $gH_{t(His)}$:gL complex. After loading the sample, the column was washed with PBS until the A_{280} reached baseline level (wash step I). Next, the column was washed with a washing buffer consisting of PBS sup-

plemented with 0.5 m*M* EDTA and 5 m*M* imidazole for 20 column volumes (wash step II). In the A_{280} elution profile, a high peak rapidly appeared after the start of wash step II and it was followed by a broad absorbance peak from 6 to 20 column volumes after the start of wash step II (Fig. 1A). Analysis by SDS–PAGE with subsequent silver staining showed that several contaminating proteins were present in fractions 2 to 8 (Fig. 1B, lanes 2 to 8). In addition, a small amount of recombinant $gH_{t(His)}$:gL was demonstrated in fraction 8. During wash step II, the column became fainter in colour, most likely due to the elution of nickel ions from the column. Approximately, 37% of the total amount of Ni^{2+} ions initially bound to the column material was eluted during this wash step.

After wash step II, the column was washed with three column volumes PBS with 0.2 *M* NaCl (wash step III) followed by three column volumes PBS (wash step IV). During wash step III, a protein was eluted with a molecular mass of approximately 33 kD, next to recombinant $gH_{t(His)}$:gL (Fig. 1B, lane 9). This protein was not eluted by wash steps I and II and was not related to gH or gL, as assayed with antibodies specific for recombinant $gH_{t(His)}$:gL (data not shown). During wash step IV (PBS), no proteins were detected in the corresponding fraction 10 (data not shown).

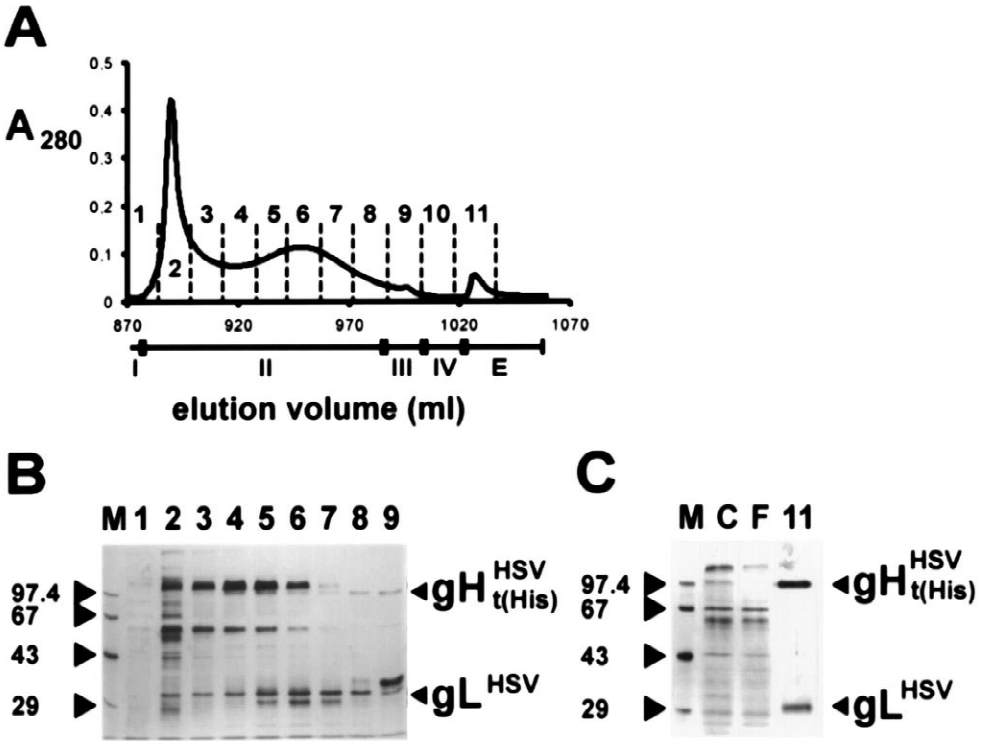

Fig. 1. (A) Elution pattern monitored at 280 nm of the purification of recombinant $gH_{t(His)}$:gL by IMAC. A 5 ml Ni-IDA column was loaded with 850 ml of dialysed culture medium containing HSV $gH_{t(His)}$:gL. After loading, the column was washed with PBS (wash step I) until A_{280} reached baseline level. Next, the column was washed with 20 column volumes PBS supplemented with 5 m*M* imidazole plus 0.5 m*M* EDTA (wash step II). The column was washed with PBS containing 0.2 *M* NaCl (wash step III), and PBS (wash step IV), subsequently. The proteins were specifically eluted with PBS containing 50 m*M* EDTA (wash step E). (B) and (C) SDS–PAGE (12.5% gels) analyses with subsequent silver staining of the fractions collected during the purification of $gH_{t(His)}$:gL complex from the culture medium. The lanes indicated in panels B and C correspond to the respective fraction numbers indicated in the elution pattern (A). The fractions 1 to 9, the culture medium (indicated with C), the flow-through (indicated with F), and fraction 11 were analysed. The position of $gH_{t(His)}$ and gL and the molecular masses (kDa) of the marker proteins are indicated.

With buffer E (PBS with 50 m*M* EDTA) all nickel ions are eluted from the column. Recombinant $gH_{t(His)}$:gL complex was eluted together with 51% of the initially bound Ni^{2+} ions, immediately after applying buffer E. The residual 12% of Ni^{2+} ions eluted thereafter by the same buffer. Recombinant $gH_{t(His)}$:gL complex with a high degree of purity was obtained, as assayed by SDS–PAGE (Fig. 2C). The recombinant $gH_{t(His)}$:gL complex in the eluate had a molecular mass of 130 kDa when analysed by gel filtration on a Superose 6 column (data not shown). This is in agreement with the expected size of 127 kDa for a heterodimer between $gH_{t(His)}$ and gL since the molecular masses of $gH_{t(His)}$ and gL produced in insect cells are approximately 97 and 30 kDa, respectively [8].

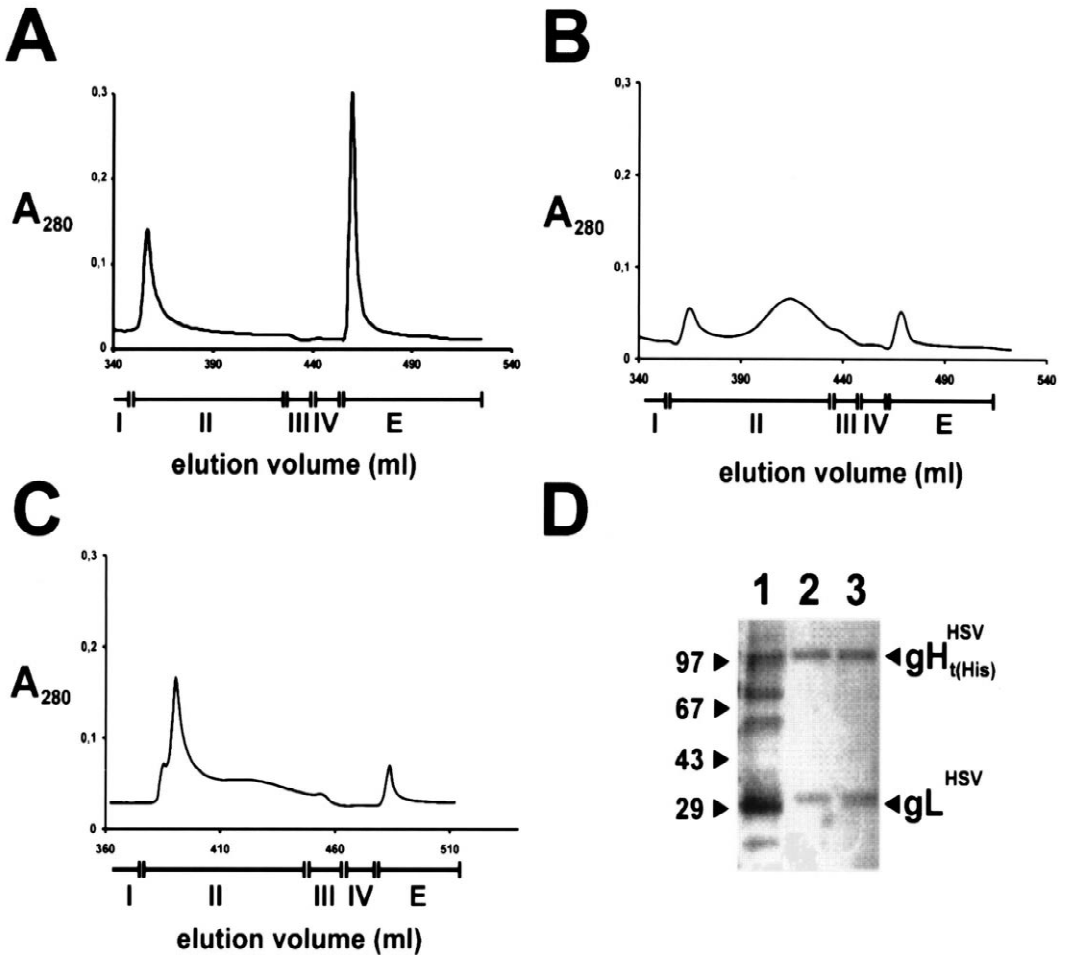

Fig. 2. (A) to (C) Elution profiles monitored at 280 nm of the purification of recombinant $gH_{t(His)}$:gL by IMAC. A 5 ml Ni-IDA column was loaded with 350 ml of dialysed culture medium containing HSV $gH_{t(His)}$:gL. The column was washed with PBS (wash step I) until A_{280} reached baseline level. Next, the column was washed with approximately 15 column volumes PBS supplemented with either 5 m*M* imidazole (A), 0.5 m*M* EDTA (B), or 5 m*M* imidazole plus 0.5 m*M* EDTA (C) (wash step II). Next, the column was washed with PBS containing 0.2 *M* NaCl (wash step III), and PBS (wash step IV), subsequently. The proteins were eluted with PBS containing 50 m*M* EDTA (elution E). (D) SDS–PAGE analysis of the elution fractions corresponding to the elution patterns shown in panels A to C. The proteins were separated on 12.5% gels and were visualised by silver staining. The molecular masses (kDa) of the marker proteins are indicated.

3.2. EDTA and imidazole in the washing buffers

In the purification procedure described above, contaminant proteins were efficiently removed from the column using a washing buffer containing a low concentration of both EDTA and imidazole. Next, it was investigated whether this efficient removal of the contaminant proteins was due to the addition of EDTA, of imidazole, or of a combination of EDTA and imidazole.

To this end, a 5 ml Ni-IDA column was loaded with 300 ml dialysed culture medium containing recombinant $gH_{t(His)}$:gL complex. After loading, the column was washed with PBS until the A_{280} reached baseline level (wash step I). To compare the effect of EDTA and imidazole, the column was washed (wash step II) with approximately 15 column volumes of PBS supplemented either with 5 m*M* imidazole, or with 0.5 m*M* EDTA, or with 5 m*M* imidazole and 0.5 m*M* EDTA.

The A_{280} elution profiles are shown in Fig. 2A–C. During wash step II, an A_{280} peak rapidly eluted from the column, independent of whether the washing buffer contained a low concentration of EDTA, imidazole, or EDTA plus imidazole. However, this peak was larger when the washing buffer contained 5 m*M* imidazole (Fig. 2A and C). A broad peak from six to 15 column volumes after the start of wash step II was seen when the washing buffer contained a low concentration of EDTA (Fig. 2B and C). This was not found when the column was washed with PBS containing only 5 m*M* imidazole (Fig. 2A).

The purification procedure was continued by washing the column with PBS supplemented with 0.2 *M* NaCl (wash step III) and PBS (wash step IV). Finally, the proteins were specifically eluted with PBS containing 50 m*M* EDTA (specific elution ''E''). The fractions corresponding to the specific elution were analysed by silver stained polyacrylamide gels. This analysis showed that when the column was washed with PBS containing 5 m*M* imidazole, the specifically eluted fraction contained several contaminant proteins in addition to recombinant $gH_{t(His)}$:gL (Fig. 2D, lane 1). When the column was washed with PBS containing 0.5 m*M* EDTA or with PBS containing 0.5 m*M* EDTA and imidazole, the specifically eluted fraction contained recombinant $gH_{t(His)}$:gL to near homogeneity (Fig. 2D, lanes 2 and 3).

The yields from the purification using PBS containing 5 m*M* imidazole and 0.5 m*M* EDTA as wash step II were determined (elution profile shown in Fig. 2C). The fractions collected during elution with PBS supplemented with 50 m*M* EDTA (wash step E) contained 51% of the amount of recombinant $gH_{t(His)}$:gL present in the starting material. The flow-through fraction contained less than 2% of the recombinant $gH_{t(His)}$:gL applied to the column. Respectively, 9% and 4% of recombinant $gH_{t(His)}$:gL of the starting material was found in the fractions collected during wash steps II and III.

3.3. General applicability of a washing buffer with a low concentration of EDTA

To investigate whether the above-described purification procedure could be used as a general strategy, three other histidine-tagged recombinant proteins were purified from the culture medium of insect cells by using this method. For wash step II, PBS supplemented with 0.5 m*M* EDTA plus 5 m*M* imidazole was used. Recombinant $gH_{t(His)}$:gL complex from varicella-zoster virus (VZV), recombinant protease 3 of neutrophil granulocytes including its two amino acid propeptide [$proPr3_{(His)}$], and recombinant secreted epithelial glycoprotein-2 [$sEGP\text{-}2_{(His)}$] were purified. Elution profiles were obtained that were largely similar to that shown in Fig. 1A. By using the purification protocol, $gH_{t(His)}$:gL of VZV and $proPr3_{(His)}$ were purified to near homogeneity (Fig. 3A and B) and in yields comparable to those obtained for recombinant $gH_{t(His)}$:gL of HSV (approximately 50%). This was estimated by using silver-stained polyacrylamide gels. Recombinant $sEGF\text{-}2_{(His)}$ was also purified at a high degree of purity (Fig. 3C). However, the yield (based on the silver-stained gel was approximately 5–10% of the starting material) was lower than that found for the other three histidine-tagged proteins.

4. Discussion

IMAC has been shown to be a powerful technique

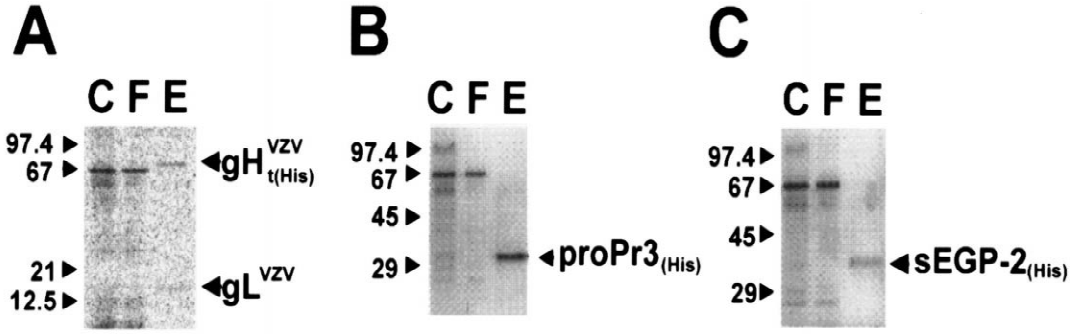

Fig. 3. SDS–PAGE (12.5% gels) analyses of the fractions collected during purification of three histidine-tagged recombinant proteins/protein complexes from insect cell culture medium by IMAC. The polypeptides were visualised by silver staining. The culture medium (indicated with C), the flow-through (indicated with F), and the eluate (indicated with E) were analysed. (A) $gH_{t(His)}$:gL complex of VZV, (B) $proPr3_{(His)}$, and (C) $sEGP\text{-}2_{(His)}$. The molecular masses (kDa) of the marker proteins are indicated together with the recombinant proteins.

to purify recombinant histidine-tagged proteins from complex mixtures. However, not only histidine-tagged proteins bind to IMAC columns, but also contaminant proteins. Several strategies have been developed to remove contaminant proteins from the column. In this study, we examined whether a washing buffer containing a low concentration of EDTA (0.5 m*M*) can be used for this purpose. This does not seem to be a logical choice since EDTA is a chelating agent and therefore strips the metal ions from the column without specificity for the type of protein bound to the metal ion. Surprisingly, we found that by using a washing buffer that contained a low concentration of EDTA, proteins with and without a histidine-tag were fractionated. Although, our method may have general applicability for the purification of histidine-tagged proteins produced in the baculovirus expression system, it will be limited to those proteins that do not need metallic co-factors for native activity.

By using a washing buffer containing a low concentration of EDTA (0.5 m*M*), four recombinant histidine-tagged proteins/protein complexes, $gH_{t(His)}$:gL of HSV, $gH_{t(His)}$:gL of VZV, $sEGP\text{-}2_{t(His)}$, and $proPr3_{(His)}$, were purified to near homogeneity in this study. During the wash step with a low concentration of EDTA, the column became fainter in colour, indicating that nickel ions eluted from the column. In addition, increasing amounts of the histidine-tagged proteins were found in the fractions collected near the end of this wash step, as assayed by silver-stained gels. This indicated that the histidine-tagged proteins eventually would be eluted from the column by the washing buffer containing 0.5 m*M* EDTA. Supplements of low concentrations of imidazole (5 m*M* imidazole) and 0.2 *M* NaCl added to the wash buffer of 0.5 m*M* EDTA in PBS did not have any effect on the final result. For $gH_{t(His)}$:gL of HSV, $gH_{t(His)}$:gL of VZV, and $proPr3_{(His)}$, the amount of protein eluted from the column during this phase was usually relatively small. However, a large portion of recombinant $sEGP\text{-}2_{t(His)}$ was eluted from the column at the end of this wash step. A possible explanation for this may be that histidine-tagged $sEGP\text{-}2_{t(His)}$ interacts with less nickel ions than the other histidine-tagged proteins used in this study. Although a six-histidine tag was introduced in this protein, a number of these histidines may not be accessible for interaction with the matrix. We only used a concentration of 0.5 m*M* EDTA in PBS as washing buffer. It may be worthwhile, particularly for the recovery of $sEGP\text{-}2_{t(His)}$ to investigate washing buffers with lower EDTA concentrations than 0.5 m*M*.

From our results, it can be concluded that proteins without a histidine tag were easier eluted by 0.5 m*M* EDTA than proteins with a histidine tag. There are several explanations for this result. The most likely explanation is that the immobilised nickel preferentially binds to histidine-tagged proteins since it acts as either a tridentate or a bidentate ligand. The non-histidine-tagged proteins act most likely as an

unidentate ligand. Thus, the equilibrium constant of formation is much higher for the Ni-IDA–histidine-tagged protein complex relative to the Ni-IDA–non-histidine-tagged protein complexes. This could explain why the non-histidine tagged proteins are eluted easier using the hexadentate EDTA. Another explanation could be that proteins with five or six consecutive histidines of the histidine tag interact with several nickel ions of the matrix and that proteins without a histidine tag interact with less nickel ions. Therefore, proteins without a histidine tag are eluted easier from the column when nickel ions are removed by the chelating effect of the low concentration of EDTA in the mobile phase. A study using engineered histidine-containing cytochrome *c* molecules showed that proteins containing multiple accessible histidines have an apparent binding affinity that is as much as a factor of 1000 higher than the binding affinity of a single-histidine protein [4,15]. This indicates that proteins that interact with the matrix via multiple-site binding are far better retained on an IMAC column.

In conclusion, a washing buffer that contained a low concentration of EDTA was effective in the purification of histidine-tagged recombinant proteins $gH_{t(His)}$:gL of HSV, $gH_{t(His)}$:gL of VZV, sEGP-$2_{t(His)}$, and proPr$3_{(His)}$ from the culture medium of insect cells. We obtained these histidine-tagged recombinant proteins at a high level of purity. The described procedure could also be extended to work for recombinant histidine-tagged proteins produced by other expression systems.

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