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Immobilized metal-ion affinity chromatography of recombinant Fab protein OPG C11 in the presence of EDTA–Mg(II)

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Abstract

Undesired adsorption of host cell proteins poses a big challenge for immobilized metal-ion affinity chromatography (IMAC) purification. In this study, by using His_6-tagged protein Fab OPG C11 from *Escherichia coli* fermentation as a model, we found that the presence of low concentrations of EDTA–Mg^{2+} in feed streams weakens the adsorption but makes it more specific towards polyhistidine tag. By combining EDTA–Mg^{2+} treatment and periplasmic extraction, we developed a one-step purification procedure for His_6-tagged recombinant Fab OPG C11 using Ni-IDA (iminodiacetic acid) chromatography. This procedure eliminated the buffer exchange step after periplasmic extraction, which is usually required before IMAC in order to remove EDTA. In addition to savings on time and cost, this procedure eliminates undesired adsorption of most host cell proteins thus significantly improves the purity of polyhistidine-tagged recombinant proteins. The strategy of EDTA–Mg^{2+} treatment may have general application potentials.

Keywords: Immobilized metal-ion affinity chromatography; Proteins

1. Introduction

Immobilized metal-ion affinity chromatography (IMAC) is a widely used technique in the purification of polyhistidine-tagged recombinant proteins [1–3]. The principle is based on the reversible coordinated interaction between immobilized metal ion and side chains of consecutive histidine residues. However, in many cases, this technique alone could not obtain satisfactory purity due to undesired adsorption of protein contaminants containing exposed residues like histidine, cysteine, and tryptophan, whose side chains also show weak interaction with the metal ion [4,5]. In order to reduce such undesired adsorption, a number of factors have been studied. These include selection of different metals and chelators, designing of binding tags with different lengths, residue compositions, and locations [4,6], reducing the ligand density by coating highly activated resins with polymers [5], as well as inclusion of low concentrations of imidazole or high salts. The overall strategy is to introduce weak but more

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selective adsorption for polyhistidine-tagged proteins.

The goal of this study was to develop a quick purification procedure for Fab proteins generated by phage display for initial pharmacological screening studies. To facilitate purification, a His_6 tag was engineered at the carboxyl terminus of each Fab protein to be studied, which was targeted to the periplasmic space of *Escherchia coli* cells. As a start point of this development work, Fab proteins from shake flask cultures were purified by TALON resin (Clontech) in batch mode. Since the Fab proteins were not highly expressed in *E. coli* in our case, in order to meet the quantity requirement on purified Fab, fermentation at 10-l scale was used for cell culture. Host cell protein contamination in TALON adsorption is significant for feed streams from such fermentors, which made it very difficult to meet the purity requirements. Therefore, the goal could not be achieved unless the undesired adsorption of host cell proteins is eliminated. Thus it is necessary to develop a generally applicable strategy to improve the binding selectivity. The raw material of Fab OPG C11 generated from fermentation culture represented the worst-case scenario for host cell protein contamination among the Fab proteins studied, which was chosen as a model Fab protein and used for the development work.

After the failure to get high-purity Fab OPG C11 from whole cell extracts, our focus shifted to periplasmic extracts. Purification from *E. coli* periplasmic space avoided the contamination of cytoplasmic proteins and obviously makes purification of recombinant proteins much easier. However, in order to make periplasmic extraction, EDTA is required to break the peptidoglycan layer on the outer membrane of *E. coli* cells, and Mg^{2+} was added after the extraction to stabilize spheroplasts. Since EDTA is a chelator, it has to be removed from the periplasmic extract before it is loaded onto the IMAC column. As a ''standard'' procedure, a buffer exchange step, e.g. dialysis or ultrafiltration, is a required link between periplasmic extraction and IMAC purification [7]. In order to simplify the purification procedure, we investigated the possibility to load the periplasmic extract directly onto the IMAC column by eliminating the usually required buffer exchange step. Surprisingly we discovered that this approach helped to eliminate the undesired adsorption of most *E. coli* proteins and thus significantly improved the Fab purity. We further examined the impact of EDTA–Mg^{2+} treatment on Ni-IDA (iminodiacetic acid) Sepharose chromatography.

2. Experimental

2.1. Instruments

Chromatography experiments were carried out using an Äkta Explorer 100 system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). An LSL Biolafitte SA fermentor (LSL Biolafitte, Allentown, PA, USA) with Valley instrument control (Valley Instrument, Exton, PA, USA) was used for *E. coli* fermentation. Other major instruments used in this study were: Shimadzu UV-1601 UV–Visible spectrophotometer (Shimadzu, Kyoto, Japan) for UV measurement, Spectra MAX 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) for BCA assays, Sorvall Super T21 (Kendro Laboratory Products, Newton, CT, USA) and Sorvall RC-3B (R&D Service, New Castle, DE, USA) refrigerated centrifuges for centrifugation, and Novex gel electrophoresis system for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Agfa DuoScan T1200 with FotoLook 3.0 software (Agfa, Ridgefield Park, NJ, USA) was used for gel densitometry scanning. Fab purity was estimated using One-Dscan1.33 software (Scanalytics, Fairfax, VA, USA).

2.2. Chemicals

HiTrap chelating Sepharose HP 1 and 5 ml columns were purchased from Amersham Pharmacia Biotech. Ni-NTA (nitrilotriacetic acid) and TALON were from Qiagen (Valencia, CA, USA) and Clontech (Palo Alto, CA, USA), respectively. Imidazole was from USB (Cleveland, OH, USA). Metals, namely nickel sulfate, cobalt chloride, ferric chloride, and copper chloride, were from Spectrum (New Brunswick, NJ, USA). PBS without Mg^{2+}–Ca^{2+} 10× stock solution and 0.5 *M* EDTA, pH 8.0 were purchased from Gibco BRL (currently Invitrogen,

Grand Island, NY, USA). Antibody His-13 against the His_6 tag was developed by DuPont Pharmaceuticals. Peroxidase-conjugated goat anti-human λ and κ light chain (bound and free) antibodies were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Fab constructs

Phage-displayed Fab antibodies were isolated from the Human Combinatorial Antibody Library (HuCAL-Fab) (MorphoSys, Martinsried/Munich, Germany). Procedures have been previously described [8]. Briefly, three rounds of selection were performed on specific antigens coated directly on Maxi-Sorb plates (NUNC, Rochester, NY, USA). Polyclonal pools of selected Fabs were subcloned into expression vectors containing Myc- and His_6-tags. Ligated expression vectors were electroporated into electrocompetent TG1 *E. coli* (Stratagene, La Jolla, CA, USA) and were plated for colony isolation. Colonies were robotically picked into 384-well plates, replicated for expression, and screened against specific antigens by an enzyme-linked immunosorbent assay (ELISA). Positive clones were rescreened for confirmation against specific and non-specific antigens.

2.4. E. coli fermentation

A 10-ml inoculum was prepared by introducing a single colony of *E. coli* TG1/Fab from a chloramphenicol selection plate to a shake flask containing supplemented M9 medium (K_2HPO_4 1.95 g/l, KH_2PO_4 0.95 g/l, $(NH_4)_2SO_4$ 0.5 g/l, $MgSO_4$ 0.45 g/l, casamino acids 20 g/l, yeast extract 5 g/l, glycerol 30 g/l, CAM 50 mg/l and trace elements). The inoculum was aseptically fed into a LSL Biolafitte SA fermentor containing 10 l from the same medium. The growth conditions for the culture were maintained for the duration of the process at the following set points, temperature at 30 °C, pH at 7.0, the D.O. (dissolved oxygen) at 30%. Once the A_{600} value reached 20, protein expression was induced by the injection of IPTG to final concentration of 1 m*M*. The culture was harvested 3 h post-induction at a final A_{600} of 40 and at 45 g/l of wet cell paste.

2.5. Feed preparation

Typically 10-l fermentation gave approximately 300 g (wet mass) of cell paste. For whole cell extract, each gram of cell paste was resuspended in 9 ml of 2× phosphate-buffered saline (PBS) (without Mg^{2+}–Ca^{2+}) to make a ten-fold dilution. The cell suspension is homogenized with a single pass through a Microfluidizer, model M-110EH (Microfluidics, Newton, MA, USA) at an inlet pressure of 21 000 p.s.i. (1 p.s.i.=6894.76 Pa). The cell lysate is centrifuged at 5000 *g* for 15 min to remove cellular debris. The extract was further clarified by centrifuging at 60 000 *g* for 1 h at 4 °C. The periplasmic extract was prepared by resuspending each gram of *E. coli* cell paste in 9 ml of BBS buffer (200 m*M* boric acid, 150 m*M* NaCl, 2 m*M* EDTA, pH 8.0). The cell suspension was stirred gently at 4 °C overnight. $MgSO_4$ was added to a final concentration of 5 m*M* in order to stabilize spheroplasts. The periplasmic extract was obtained by centrifuging at 60 000 *g* for 1 h at 4 °C. The extracts, either from whole cell or periplasm, were filtered through a 0.45-μm cellulose acetate filter (Corning) before loading onto the Ni-IDA column.

2.6. Purification procedures

IMAC purification was performed using HiTrap chelating columns (Amersham Pharmacia Biotech), either 1 ml (diameter 0.7 cm, bed height 2.5 cm) or 5 ml (diameter 1.6 cm, bed height 2.5 cm), charged with Ni^{2+}, on an Äkta Explorer 100 system. Typically, 2.5 ml whole cell extract or 5 ml periplasmic extract per ml bed was loaded onto the column previously equilibrated with 5–10 CVs (column volumes) of 2× PBS (without Mg^{2+}–Ca^{2+}). After washing with 20 CVs of 2× PBS (without Mg^{2+}–Ca^{2+}), the elution was carried out with linear gradient of 0–250 m*M* imidazole in 20 CVs. The column was sanitized and regenerated each time after purification. The sanitization procedure was as the following: 3 CVs of 1 *M* NaOH with contact time of 30 min, 3 CVs of 1% Tween 20 in PBS (without Mg^{2+}–Ca^{2+}), 3 CVs of 6 *M* urea, 3 CVs of de-ionized water, 3 CVs of 0.5 *M* EDTA, pH 8.0, 5 CVs of de-ionized water. After sanitization, the column

was re-charged with 4 CVs of 0.05 *M* $NiSO_4$, followed by extensive wash with 5 CVs of de-ionized water.

2.7. Analysis

Antigen-binding ELISA was performed as described [8]. The coated antigen was recombinant mouse osteoprotegerin–Fc chimera (Fc–MOPG) (R&D Systems, Minneapolis, MN, USA), which was incubated with serial dilutions of Fab OPG C11 from 0 to10 μg/ml. The plate was detected with anti-human Fab–alkaline phosphatase conjugate (Jackson Laboratory, Bar Harbor, ME, USA) at a dilution of 1:2500, which was developed with ATTO Phos (Roche Diagnostics, Mannheim, Germany) and detected in the fluorescence mode at an excitation wavelength of 430 nm and emission wavelength of 535 nm.

UV and BCA (Pierce) were routinely used to measure the total protein concentration. SDS–PAGE with 4–20% Tris–glycine Novex gels (Invitrogen) was used to evaluate the purity. Western blotting was used occasionally in order to identify the Fab bands. Anti-His_6 tag antibody, His-13, as well as antibodies against human λ and κ light chains, were used for Fab detection.

3. Results

3.1. The problem of host cell protein contamination

As shown in Fig. 1, a number of *E. coli* proteins from fermentation culture adsorbed unspecifically to TALON resin. The Fab was not highly expressed and the Fab band could barely be seen in the microfluidized whole cell extract. Fab OPG C11 in the TALON eluate had a purity less than 10%, estimated by gel densitometry scanning. This represented the worst case scenario for host cell contamination among Fab proteins used in this study, thus Fab OPG C11 was chosen as a model Fab protein for the development work.

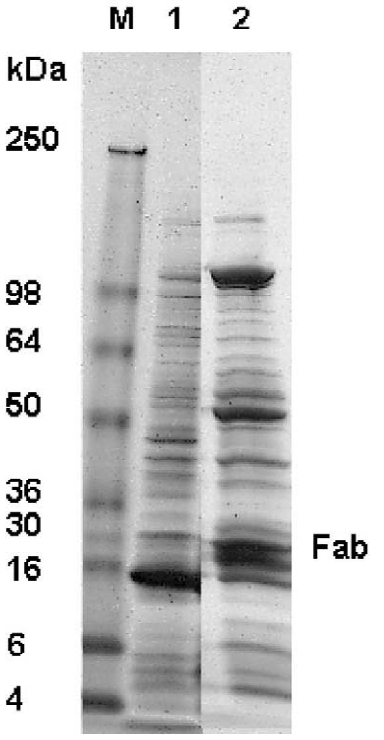

Fig. 1. SDS–PAGE analysis of TALON-purified Fab OPG C11 on 4–20% Tris–glycine gel. M: molecular mass marker. Lanes 1=OPG C11 whole cell extract, the feed stream of TALON batch binding; 2=Fab OPG C11 eluted from TALON binding.

3.2. Metal and chelator selection

A number of metals were scouted for their ability for protein binding and for Fab separation, the results of which were summarized in Table 1. Fresh HiTrap chelating Sepharose HP (IDA) 1 ml column was charged with different metal ions. The columns were loaded with 25 ml of OPG C11 microfluidized whole cell extract (equivalent to 2.5 g cell paste). After washing, bound proteins were eluted with linear gradient of imidazole. Mg^{2+}, Mn^{2+}, and Fe^{2+} showed no protein binding at all. Ni^{2+} and Cu^{2+} bound moderately and gave the best separation of Fab peak from contaminant peak, which was roughly equivalent to each other. The selectivity of Co^{2+} column for His_6-tagged Fab was not as good as Ni^{2+} or Cu^{2+} in this case, despite of weak protein binding.

Three widely used chelators, IDA (iminodiacetic acid), NTA (nitrilotriacetic acid), and TALON (car-

Table 1
Scouting of different metals for Fab binding and separation

Resin	Binding metal	Protein binding[a]	Fab separation[b]
TALON (start point)	Co^{2+}	+	++
Mg-IDA	Mg^{2+}	−	N/A
Zn-IDA	Zn^{2+}	++++	+
Co-IDA	Co^{2+}	+	++
Mn-IDA	Mn^{2+}	−	N/A
Fe-IDA	Fe^{2+}	−	N/A
Ni-IDA	Ni^{2+}	+++	+++
Cu-IDA	Cu^{2+}	+++	+++

[a] From + to ++++: stronger protein binding; −: no protein binding.

[b] From + to +++: better Fab separation; N/A: not applicable.

boxymethylated aspartic acid, or CM-Asp), were charged with Ni^{2+} and scouted for Fab separation. It was found that the tridentate chelator, IDA, gave the best separation (Fig. 2). Fab bound stronger on Ni-IDA, thus was separated farther apart from the contaminant peak, than the other two chelators. Probably this tridentate chelator provides three binding sites, which favors that multi-point binding of His_6-tagged proteins. Ni-TALON could not separate Fab from contaminants at all, though it is thought that Fab may come out at the tailing part of the peak. As tetradentate chelators, Ni-NTA and Ni-TALON could only provide two protein-binding sites, which might explain slightly less protein binding (smaller total eluting peak area compared to Ni-IDA). However, significant difference exists between these two chelators for Fab separation. Based on the scouting results, Ni-IDA Sepharose HP column was selected for the development work. Despite of this, the purity of Fab OPG C11 eluted from Ni-IDA column was still poor, typically in the range of 35–45%. Ni^{2+} was chosen over Cu^{2+} because it is most widely used and is compatible with phosphate buffer.

3.3. The effects of EDTA–Mg^{2+} treatment

The effect of EDTA–Mg^{2+} treatment on Ni-IDA Sepharose chromatography was studied by adding EDTA and $MgSO_4$ to clarified whole cell extract to final concentrations of 2 and 5 m*M*, respectively, which are the same as in periplasmic extraction, before column loading. It was found that the giant contaminant peak almost completely disappeared, as shown in Fig. 3A. The Fab peak shifted forward slightly, suggesting that the binding was slightly weakened.

The chromatogram comparison of three different

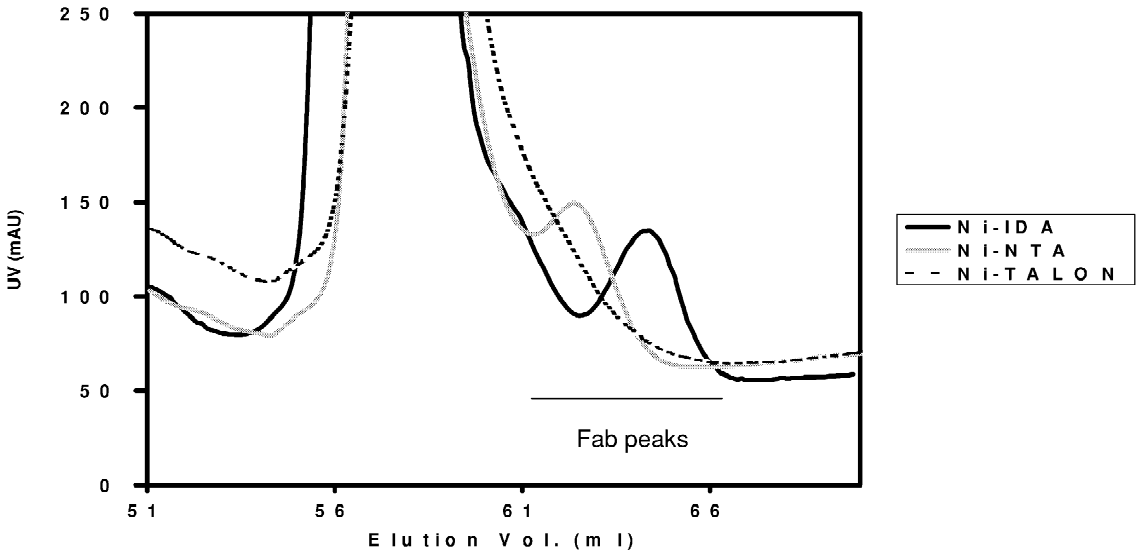

Fig. 2. Scouting of different chelators for Fab separation: the elution profiles of Fab OPG C11 on Ni-IDA (solid black line), Ni-NTA (solid gray line), and Ni-TALON (dashed black line). Experimental conditions: 25 ml of OPG C11 whole cell extract (equals to 2.5 g cell paste) was loaded onto 1 ml column, Fab was eluted with linear gradient of 0–250 m*M* imidazole in 20 CVs.

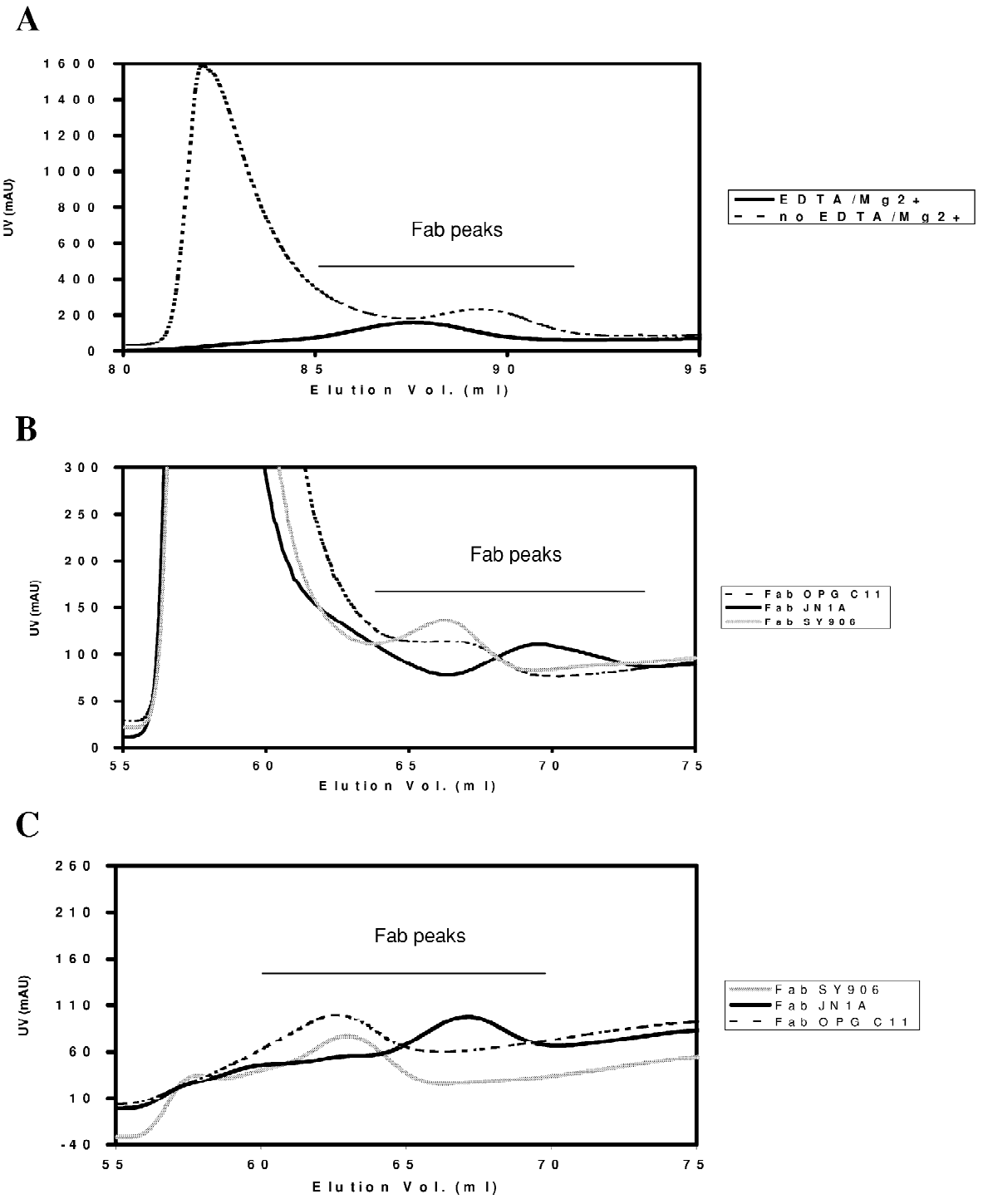

Fig. 3. The effect of EDTA–Mg^{2+} treatment on Fab elution profile. (A) Comparison of Fab OPG C11 elution profiles before and after EDTA–Mg^{2+} treatment; (B) comparison of elution profiles of three Fab proteins, OPG C11, JN1A, and SY906 before EDTA–Mg^{2+} treatment; (C) comparison of elution profiles of three Fab proteins, OPG C11, JN1A, and SY906 after EDTA–Mg^{2+} treatment. Experimental conditions: 50 ml (A) or 25 ml (B, C) of whole cell extracts (equal to 5 or 2.5 g cell pastes, respectively) were loaded onto 1 ml Ni-IDA Sepharose HP columns, Fabs were eluted with linear gradient of 0–250 m*M* imidazole in 20 CVs.

Fab proteins on Ni-IDA, either in the presence or in the absence of EDTA–Mg^{2+}, was shown in Fig. 3B and C, respectively. Interestingly, Fab JN1A-1, with a κ light chain, came out later in the imidazole gradient elution, indicating stronger binding to the Ni-IDA column and thus better separation from contaminants. Indeed, this Fab protein yielded good purity even without EDTA–Mg^{2+} (>85%) treatment. Fab proteins SY906-1 and OPG C11, both with λ light chains, showed slightly weaker binding to the column. The difference in elution volume may reflect heterogeneity among different Fab proteins. And such heterogeneity was recognized by the Ni-IDA column in the presence of EDTA–Mg^{2+}. In other words, the binding of EDTA–Mg^{2+} treated feed stream was not due to non-specific adsorption or artifacts. The Fab peaks shifted forward and contaminant peaks almost disappeared, which indicated that binding was weakened but more selective to His_6 tag, which bound stronger than contaminants. Despite of the appearance of single-peak pattern, Fab OPG C11 eluted from column in the presence of EDTA–Mg^{2+} still had minor contamination from host cell proteins, which is shown later in Figs. 4A and 5B(lane 3).

3.4. Purification from periplasmic extract

Though EDTA–Mg^{2+} treatment reduced the host cell contamination to a very minor extent, it is still difficult to resolve Fab proteins from contaminants with very close binding affinity to His_6 tag. Fig. 4A, for example, compares EDTA–Mg^{2+} treated whole cell extract of Fab OPG C11 and non-histidine tagged Fab fNS2-H9. The non-histidine tagged Fab fNS2-H9 was used as a control, because it does not bind to Ni-IDA column due to lack of His_6 tag. Therefore, the bound proteins are solely *E. coli* proteins. It is clear that the two frontal shoulders of the Fab peak are host cell protein peaks and the tailing of the second shoulder overlaps the Fab peak. This may explain the remaining contaminant bands on SDS–PAGE (Fig. 5B, lane 3). However, when periplasmic extracts were prepared and loaded directly onto the column (Fig. 4B), The Fab peak was better separated from contaminant peaks. The host cell protein binding control, fNS2-H9, gave a clean baseline in the region where Fab OPG C11 came out. The host cell protein peak (Fig. 4B) was much higher than the counterpart of Fab OPG C11, which seemed like nonspecific low-affinity binding of host cell proteins in the absence of Fab OPG C11. The result of the periplasmic extract of Fab OPG C11 from frozen paste fell in between the whole cell extract and the periplasmic extract from fresh paste (data not shown). This is expected, as some cells were broken during the processes of freezing and thawing.

3.5. Purity, yield, and antigen-binding activity of purified Fab OPG C11

The SDS–PAGE of Fab OPG C11 purified under different conditions is shown in Fig. 5. The major host cell contaminants were the two bands just below the Fab band (Fig. 5B), which were observed in all the Fab eluates under normal conditions. Several strategies, such as high salt wash, shallower gradient, and low concentration imidazole wash, did not succeed in removing these bands (data not shown). Overloading was also used in expectation that higher affinity His_6-tagged Fab would compete and displace weakly bound contaminating proteins. Indeed, the contaminant peak became smaller, but these contaminants remain in the Fab peak fractions (Fig. 5B, lane 2). These contaminants were reduced significantly by EDTA–Mg^{2+} treatment (Fig. 5B, lane 3), however, a band with high molecular mass increased slightly under this condition. Thus the overall purity did not improve significantly (Table 2). Periplasmic extraction avoided the contamination of most cytoplasmic proteins and thus gave very clean Fab after Ni-IDA chromatography in the presence of EDTA–Mg^{2+} (Fig. 5A, lane 4).

Table 2 summarizes the purity and yield of Fab OPG C11 purified under different conditions. It is clear that direct loading of periplasmic extract gave much better purity as well as good yield. Surprisingly, the whole cell extract under normal purification condition gave lower yield. The possible reason for this is that under normal purification conditions (no EDTA–Mg^{2+} treatment) the separation was not good so that some Fab OPG C11 was lost in the contaminant peak. When purified directly from periplasmic extract, the contaminant peak was reduced to minimal level, therefore more Fab was recovered.

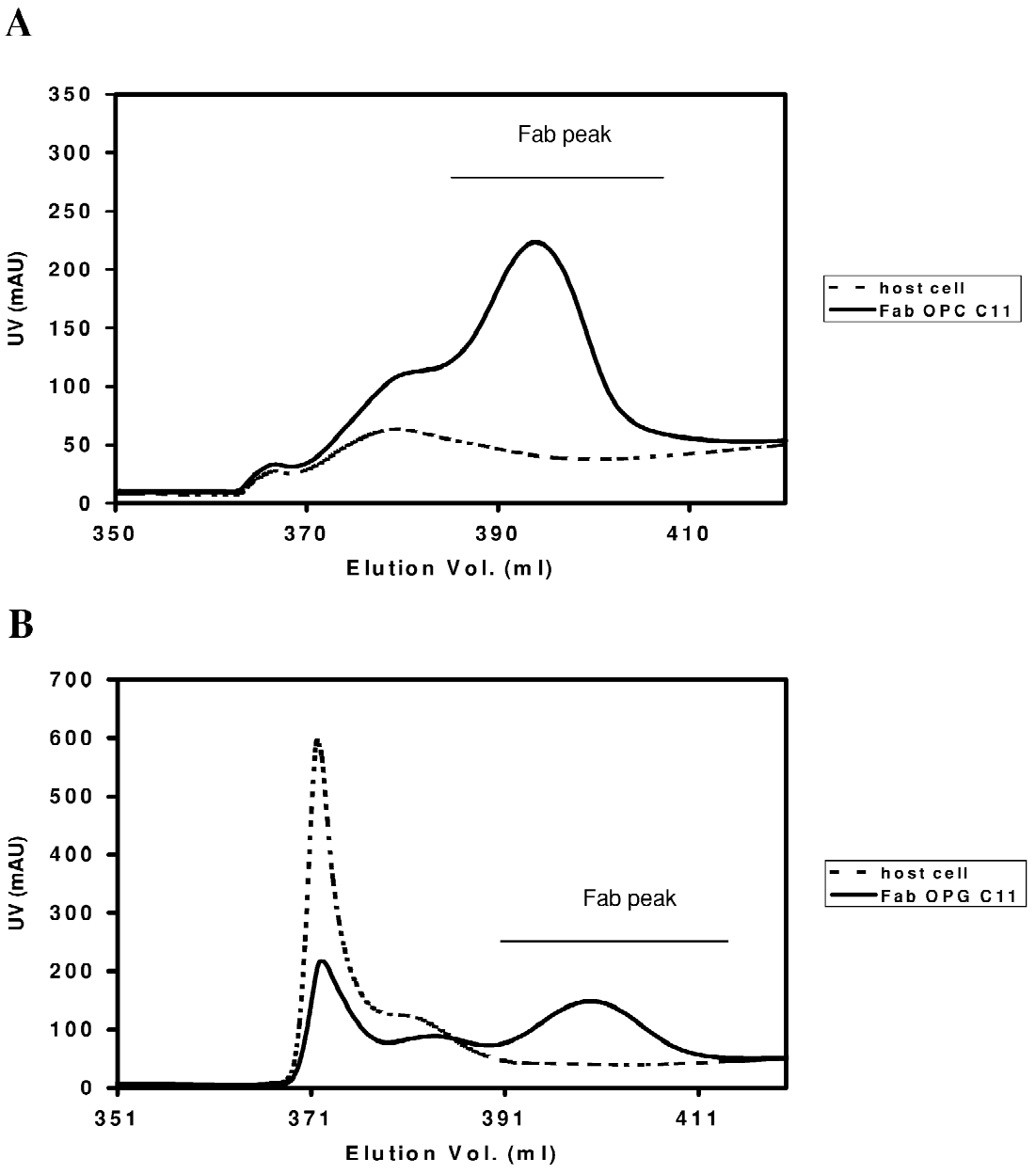

Fig. 4. The comparison of purification from periplasmic extract vs. whole cell extract. (A) The elution profile comparison of Fab OPG C11 and host cell protein control in whole cell extract, EDTA–Mg^{2+} treated; (B) the elution profile comparison of Fab OPG C11 and host cell protein control in periplasmic extract, prepared from fresh cell paste. Experimental conditions: 250 ml of whole cell extracts (equal to 25 g cell pastes) or 500 ml of periplasmic extracts (equal to 50 g cell pastes) were loaded onto 5 ml Ni-IDA Sepharose HP columns, bound proteins were eluted with linear gradient of 0–250 m*M* imidazole in 20 CVs.

The antigen-binding activity of Fab OPG C11 purified from periplasmic extract in the presence of EDTA–Mg^{2+} was determined by ELISA (Fig. 6) and was compared to a reference Fab OPG C11, which was from shake flask culture and purified from whole cell extract under normal conditions by

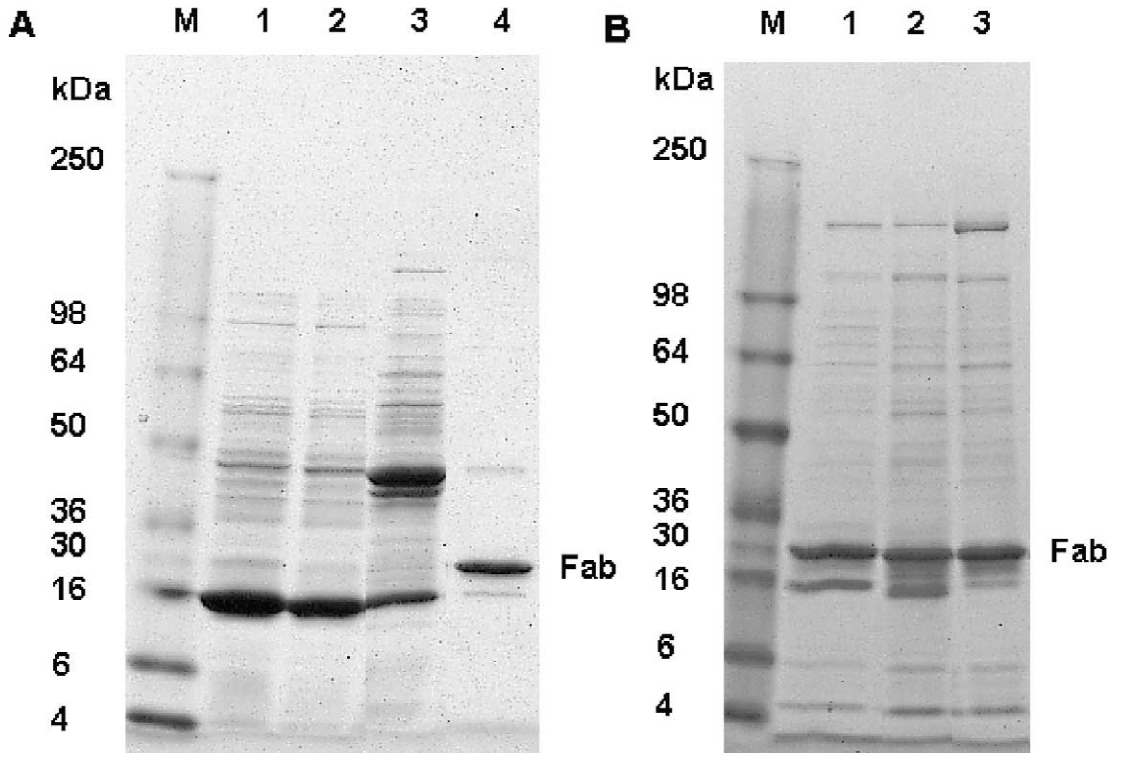

Fig. 5. SDS–PAGE analysis of Fab OPG C11 purified by Ni-IDA chromatography under different conditions. (A) From undialyzed periplasmic extract (load at 50 ml or 5 g paste per ml bed): lanes 1=periplasmic extract feed without buffer exchange, 2=flow through, 3=contaminant peak, 4=Fab eluate. (B) Eluted Fab OPG C11 from whole cell extract: lanes 1=load at 25 ml (2.5 g paste) per ml bed, no EDTA–Mg^{2+} treatment, 2=load at 50 ml (5 g paste) per ml bed, no EDTA–Mg^{2+} treatment, 3=load at 25 ml (2.5 g paste) per ml bed, after EDTA–Mg^{2+} treatment. M: molecular mass marker. Fab was eluted with linear gradient of 0–250 m*M* imidazole in 20 CVs.

TALON resin. It was apparent that the activity of Fab OPG C11 from these two sources was very similar.

4. Discussion

Host cell protein contamination is a major challenge to IMAC purification. To reduce or eliminate such contaminants has been a major focus of IMAC study. The overall strategy is to introduce weak but more selective adsorption for polyhistidine-tagged proteins. It was found that the high density of chelating ligand contributes to undesired multi-point weak adsorption of contaminating proteins. Reduction of ligand density by using lowly activated

Table 2
Yield and purity of Fab OPG C11 purified under different conditions

Feed	Yield[a] (mg/g)	Fab purity[b] (%)
Whole cell extract	0.12	41.2
Whole cell extract, treated with EDTA–Mg^{2+}	0.19	48.0
Periplasmic extract without buffer exchange step	0.16	90.0

[a] Yield is defined as mg pure Fab obtained from 1 g cell paste. The amount of pure Fab was estimated by BCA assay and gel densitometry scanning.

[b] Fab purity was estimated by gel densitometry scanning.

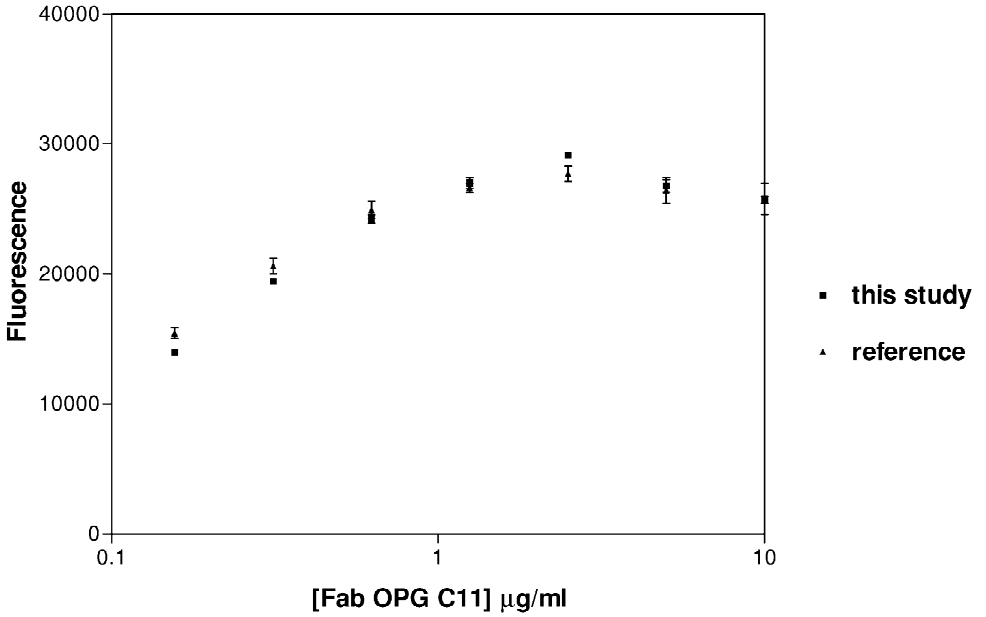

Fig. 6. The antigen-binding ELISA of: (1) Fab OPG C11 purified in this study, and (2) a reference Fab OPG C11, which was from shake flask culture and purified from whole cell extract by TALON resin.

matrices [9] or by coating the high activated matrices with dextran [5] resulted in dramatic reduction of undesired adsorptions. Recently, Westra et al. [10] reported that a wash step with low concentration EDTA (0.5 m*M*) helps to remove weakly bound contaminants from insect cells. This strategy also reduced the ligand density because low concentration of EDTA strips Ni^{2+} off the column. However, this could cause product leakage during the EDTA washing step, which may reduce product recovery.

In this study, the task to obtain Fab with satisfactory purity in one-step purification is very challenging. The expression level of Fab proteins was very low, and contamination from fermentation material was severe. Several commonly used strategies, including low concentration imidazole in feed stream and washing buffer, shallow gradient elution, high salt wash, overloading, were applied but failed to reduce the undesired host cell contamination. The contamination in Fab OPG C11 strain is the worst among the Fabs studied, typically only ~40% purity could be obtained after Ni-IDA column. Therefore, Fab OPG C11 was chosen as a model Fab protein in our work of purification development. Our results demonstrated that EDTA–Mg^{2+} treatment helps to remove the undesired contaminant adsorptions by weakening the adsorption and make it more selective to His_6-tag. By combining the EDTA–Mg^{2+} treatment and periplasmic extraction, good purity was achieved after one-step IMAC purification (Fig. 5A(lane 4) and Table 2). Fab proteins purified under this condition retain full antigen-binding activity as compared to Fab purified under normal conditions, as determined by antigen-binding ELISA (Fig. 6). The success of this approach enables direct loading of periplasmic extract onto IMAC columns, making it possible to eliminate the buffer exchange step that is usually required to link periplasmic extraction to immobilized metal-ion affinity chromatography.

This strategy was applied successfully to another Fab protein Fab OPG I20. The purity of Fab OPG I20 was improved to 92.4% from direct loading of periplasmic extract prepared even from frozen cell paste, compared to 71.0% from the microfluidized whole cell extract, while the yields of pure Fab from periplasmic extract and whole cell extract were 0.35

and 0.42 mg/g paste, respectively, at the loading of 50 ml periplasmic extract or 5 g paste per ml bed. Fab SY906-1 was not secreted efficiently (less than 20%) into the periplasmic space, therefore, purification from whole cell extract seems more practical, which could give a purity up to 86.6% in the presence of EDTA–Mg^{2+} with the yield of 0.21 mg/g paste, compared to a purity of 77.6% with a yield of 0.23 mg/g paste in the absence of EDTA–Mg^{2+}. Fab JN1A-1 was excluded from this study, since satisfactory purity (>85%) was obtained under normal purification conditions (no EDTA–Mg^{2+} treatment) from microfluidized whole cell extract. While EDTA–Mg^{2+} treatment may have general application potentials, it may be necessary to optimize the concentration of EDTA to be used for specific feed streams and IMAC resins, in order to achieve the best separation.

In order to explain why EDTA–Mg^{2+} treatment helped to make binding more specific to the polyhistidine tag, we speculated two possible impacts of EDTA–Mg^{2+} on Ni-IDA chromatography: (1) formation of Mg-EDTA–Ni-IDA complex, and (2) lower ligand density achieved by stripping Ni^{2+} from the column. Mg^{2+} forms chelating complex with EDTA, but not with IDA [2,11], nor does it bind to His_6-tagged Fab proteins (Table 1). EDTA can provide six co-ordination sites, if four of them is occupied by Mg^{2+} at pH 7.4, then there are still one or two sites left (depending on buffering conditions) for Ni-IDA. Therefore, the Mg-EDTA–Ni-IDA complex acts like tetradentate or pentadentate chelator for the His_6-tag, the binding is weaker but more specific to the polyhistidine tag. If this is true, then Ni-NTA or Ni-TALON would show no binding (or very little) to His_6-tag in EDTA–Mg^{2+} treated feed, since Mg-EDTA–Ni-NTA (or TALON) would be pentadentate or hexadentate (six sites are all occupied, no site left available for His6-tag). In other words, it might be likely that the EDTA–Mg^{2+} treatment under current conditions only works for IDA resin. The more widely proposed possibility is that EDTA strips some Ni^{2+} from the column, so the ligand density is lower than normal thus binding is weaker but more specific to His_6-tag (like dextran-coated resin). If this is true, then there would be less difference for Ni-NTA for feeds with and without EDTA–Mg^{2+} treatment, because it is harder for EDTA to compete with NTA (tetradentate) than IDA (tridentate) for stripping Ni^{2+}. Also, in the presence of excess amount of Mg^{2+}, Ni^{2+} has to compete with Mg^{2+} for EDTA as well. Upon saturation with Mg^{2+}, EDTA would be less capable of stripping Ni^{2+} off the column, which may contribute to less protein loss in the presence of 2 m*M* EDTA. After chromatography, the faint blue color of Ni-EDTA was observed on the column, which might suggest the complex formation between EDTA and Ni^{2+}. However, a preliminary study showed that the presence of EDTA–Mg^{2+} has less impact on protein binding to the tetradentate chelator Ni-NTA and very little impact to another tetradentate chelator Ni-TALON, compared to the tridentate chelator Ni-IDA (data not shown), which indicates that metal stripping might be the dominant effect over formation of Mg-EDTA–Ni-IDA complex. Apparently, further study is required to elucidate the interaction of EDTA–Mg^{2+} to IMAC resins.

In conclusion, by using His_6-tagged Fab OPG C11 as model Fab protein, we developed a purification procedure by combining EDTA–Mg^{2+} treatment and periplasmic extraction, which made it possible to eliminate the buffer exchange step that is usually required to link periplasmic extraction and IMAC purification. In addition to savings on time and cost, this approach successfully removed host cell contamination in one-step IMAC purification, which could have general application potentials.

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