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Journal of Chromatography B, 790 (2003) 153–159

JOURNAL OF CHROMATOGRAPHY B

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Evaluation of chromatographic recycling for imidazole used in the chromatographic purification of His-tag recombinant proteins

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Abstract

The aim of this work was to test a recycling method for imidazole used in immobilized metal affinity chromatography (IMAC) as eluent for recombinant histidine-tag (His-tag) protein. After evaluating two supports, the method was optimized with a mixture of bovine serum albumin, sodium chloride and imidazole. Recycling was performed with an eluate fraction from IMAC of His-tag enhanced green fluorescent protein produced in our laboratory and pure imidazole was recovered in water and was analyzed after being freeze-dried. The imidazole was then reused as eluent in IMAC without any modification in its structure or behavior. This procedure can be used for large-scale chromatography.
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Keywords: Immobilized metal affinity chromatography; Imidazole; Proteins; Enhanced green fluorescent protein

1. Introduction

The development of immobilized metal chelate affinity chromatography (IMAC) [1–6] together with recombinant His-tag protein [7–9] these last 10 years [10] has increased the need for the use of heavy metals for binding and large quantities of imidazole for elution. The use of IMAC with expanded bed adsorption was recently described for native [11] and recombinant His-tag protein [12–14] and allows the development of large-scale IMAC.

The unecological and dangerous nature of heavy metals and the corrosivity of imidazole (irritation of upper respiratory tract by inhalation, severe irritation or burns on skin, gastrointestinal pain if ingested and severe irritations or burns, corneal clouding and corneal edema by contact with eyes, and investigated as a mutagen and reproductive effector) [15] led us to investigate a recycling procedure for imidazole by using chromatographic techniques.

This procedure made it possible to recover proteins in the required buffer and eliminated unnecessary imidazole and salts.

The different parameters were evaluated with recombinant His-Tag protein (enhanced green fluorescent protein: EGFP) produced and purified by IMAC in our laboratory. Since the cloning of its gene starting from the jellyfish *Aequorea victoria* [16], the green fluorescent protein (GFP) has been

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doi:10.1016/S1570-0232(03)00088-6

widely used in cellular biology [17,18]. Its fluorescent properties have been changed by genetic engineering and have led to several mutants, especially EGFP, which have red-shifted excitation spectra (maximal excitation peak at 490 nm) and fluorescence (at 510 nm) 35-fold brighter than wild-type GFP [19]. Therefore, we overexpressed the recombinant His-Tag EGFP in *Escherichia coli* and purified it by IMAC.

When Sephadex was discovered for size exclusion chromatography [20], some interesting adsorption effect were observed. Aromatics such as dintrophenol (DNP-OH), DNP-amino acids, tryptophan and many dyes migrate in the Sephadex columns with retention. Similar finding were also published [21]. Some author [22] explained that adsorption of aromatics to Sephadex was caused by some unknown kind of hydrogen bonding between the aromatic nuclei and the hydroxyls in the matrix. The strength of interaction, as judged from the retention in the columns, depends on the π-electron distribution over the ring system and can be controlled by electron-attracting and electron-withdrawing substituents. We found that imidazole could be retained on desalting matrix. Therefore, we studied two matrices as suitable support to separate proteins, salts and imidazole by using the ability of imidazole to be eluted with delay in comparison with salts. We found that imidazole could be separated from salts and that the fraction could be recovered, analyzed and reused for IMAC without any structural or behavioral change.

2. Experimental

2.1. Instruments

The chromatographic systems used throughout this study were the fast protein liquid chromatography (FPLC) workstation from Amersham Biosciences (Saclay, France). The data were collected and evaluated using the FPLC director data system.

The fluorescence assays were performed with a Versafluor fluorometer from Biorad (Ivry sur Seine, France). The NMR spectrometer was from Bruker (Wissembourg, France) and the SM-Luxpol was from Leitz (Wetzlar, Germany).

2.2. Chemical

Bovine serum albumin, imidazole and salts were from Sigma (l'Isle d'Abeau Chesnes, France). All salts were HLPC grade, and the buffers were filtered through a 0.22-μm membrane filter. Chelating Sepharose Fast Flow, G-25 Superfine and the XK 26/20 columns were purchased from Amersham Biosciences. GH-25 Cellufine was from Millipore (Saint Quentin en Yvelines, France).

pET 15b (plasmid for expression) and the *E. coli* Novablue (DE3) which is lysogenic for bacteriophage DE3 were from Novagen (Madison, WI, USA). pEGFP was from Clontech Labs (Palo Alto, CA, USA).

2.3. Preparation of His-tag EGFP

2.3.1. Cloning, expression and cell culture

A 0.72 kilo base pair (kb) fragment corresponding to the entire EGFP coding sequence was obtained by polymerase chain reaction (PCR) using the plasmid pEGFP as template. The 5′ primer contained a 10-nucleotide linker with an *Nde*1 (*Neisseria denitrificans*) restriction site to facilitate the in-frame subcloning with the tag domain of pET 15b and the codons for the first six N-terminal residues. The 3′ primer contained an 8-nucleotide linker with a *Bam*H1 (*Bacillus amyloliquefaciens* H) restriction site, a stop codon and five codons for the C-terminal extremity. The *Nde*I/*Bam*HI digested PCR product was inserted into the *Nde*I/*Bam*HI linearized pET 15b plasmid to create the EGFP His-tag construction. The resulting EGFP His-tag protein was expressed in *E. coli* Novablue (DE3) according to the manufacturer's instructions. The cells of *E. coli* Novablue (DE3) strain transfected by the plasmid pET 15b containing the EGFP His-tag construction were cultured at 37 °C in a bioreactor in 1.5 l of LB medium (yeast extract 5 g/l, bactopeptone 10 g/l, NaCl 5 g/l, glucose 1 g/l) with ampicillin (100 μg/ml) and tetracycline (10 μg/ml). When the absorbance at 600 nm reached 0.6 (1.7×10^{8} cells/ml), IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 0.3 m*M* and the culture temperature was decreased to 30 °C. After 15 h of protein induction, the culture medium was centrifuged and the pellet was treated with an

ultrasonic probe to break the cell walls. The ultrasonic homogenate was centrifuged and the supernatant was used as sample for IMAC.

2.3.2. Purification of His-tag EGFP

IMAC was performed with chelating Sepharose fast flow packed in an XK 26/20 column. Equilibration, feed application and washing were performed with 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8. The clarified feedstock was applied directly onto the column followed by washing with the equilibration buffer until UV baseline was reached. After a run of two column volumes of equilibration buffer, the elution buffer, 500 m*M* imidazole, 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8, was run at 180 cm/h.

2.4. Preparation of desalting gels

The gels were packed in an XK26/20 column. A slurry was prepared with binding buffer in a ratio of 75% settled gel to 25% buffer and was de-gassed.

The column was filled through the outlet with a few centimeters of binding buffer and was closed. The slurry was poured into the column in one continuous motion. The remainder of the column was filled with buffer and the top mounted and connected to a pump. The bottom outlet of the column was opened and the pump set at 10 ml/min (113 cm/h). The packing flow-rate was maintained for 3 bed volumes after a constant bed height was reached (11.4 cm).

2.5. Efficiency testing of packed columns

To check the quality of the column packing, an efficiency test was performed to determine the theoretical plate number. The sample used was acetone (200 μl at 20 mg/ml in distilled water).

2.6. Evaluation of Matrex Cellufine GH-25 and Sephadex G-25 as suitable support to elute imidazole with delay

The two supports were tested in terms of resolution between bovine serum albumin (BSA), sodium chloride and imidazole (5 ml of sample (BSA 1 mg/ml, 1 *M* Nacl, 500 m*M* imidazole) at different flow-rates (1, 5 and 10 ml/min) with a column equilibrated with 150 m*M* NaCl, 50 m*M* sodium phosphate pH 7 buffer. After that, the best gel was tested for imidazole recovery in water without salts.

The column was equilibrated with 150 m*M* NaCl, 50 m*M* sodium phosphate pH 7 buffer. Five ml of sample [bovine serum albumin (BSA) 1 mg/ml, 1 *M* NaCl, 500 m*M* imidazole] were injected onto the column and different times of water injection were tested in order to recover the imidazole delayed in the water.

2.7. Validation of procedure with His-tag EGFP as sample

The eluate of IMAC was injected onto the desalting column with the procedure developed above.

2.8. Recovery, validation of recycling imidazole and characterization

The imidazole fraction recovered from desalting chromatography was evaporated under reduced pressure or freeze-dried. The imidazole thus obtained was re-used as eluent for IMAC after validation. IMAC was performed with chelating Sepharose fast flow packed in XK 16/20 column at 2 ml/min. Equilibration, feed application and washing were performed with 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8. A semi-purified His-tag EGFP was applied directly onto the column followed by washing with the equilibration buffer until UV baseline was reached. After a run of two column volumes of equilibration buffer, a linear gradient of 400 m*M* recycling imidazole was performed. Recovery of EGFP was determined by fluorescence and was compared with that obtained by native imidazole used in the same conditions.

After 10 cycles, the imidazole was characterized by NMR and melting point determination.

2.9. Analytical procedure

2.9.1. Fluorimetric assay

Throughout the experiments, the EGFP was estimated by monitoring fluorescence at 510 nm after excitation at 490 nm. A curve of reference fluores-

cence (RFU)/quantity of protein pure EGFP indicated the quantity of EGFP recovered.

2.9.2. NMR analysis and melting point determination

^{1}H NMR spectra were recorded on a Bruker AC200 spectrometer at 200.13 MHz in {[$^2H_6$]dimethyl sulfoxide (DMSO-d_6)} at 303 K. Chemical shifts were recorded in δ (parts per million, ppm) units relative to tetramethylsilane as an internal standard. Melting points were determined with a SM-Luxpol Leitz hot-stage microscope and were not corrected.

3. Results and discussion

3.1. Efficiency of packed columns

Cellufine had a lower efficiency (2600 plates) than Sephadex (3600 plates) according to the difference in bead size. However, our application was different from the usual one and we needed to verify the behavior of imidazole with Matrex GH-25. Therefore, to adjust the ability of Matrex Cellufine GH-25 for better resolution, we increased the height of the bed gel until the same theoretical plate number was obtained. The increase in height was 4.6 cm, i.e. a 43% increase in terms of height and volume.

3.2. Evaluation of Matrex Cellufine GH-25 and Sephadex G-25 as suitable support to elute imidazole with delay

Table 1 shows that Sephadex G-25 allowed better resolution than Matrex GH-25 between BSA and NaCl but also between NaCl and imidazole, i.e. our objective. In the latter conditions, Sephadex G-25 gave a resolution of 1.5 at 1 ml/min but only a 1.4-fold resolution at 10 ml/min. The optimized column was therefore not sufficient to separate salts from imidazole, so to work at 10 ml/min for fast recycling, it was necessary to increase the gel volume to obtain 1.5-fold resolution. Therefore, we increased the volume of the gel to 23% to obtain a resolution of 1.5 between salt and imidazole at 10 ml/min.

Table 1
Resolution BSA/NaCl and NaCl/imidazole for Matrex GH-25 and Sephadex G-25

	Resolution	
	BSA/NaCl	NaCl/imidazole
Matrex GH-25		
1 ml/min	2.40	1.20
5 ml/min	1.95	1.03
10 ml/min	1.83	0.90
Sephadex G-25		
1 ml/min	3.00	1.50
5 ml/min	2.80	1.45
10 ml/min	2.60	1.40
Sephadex G-25+23% of column volume		
10 ml/min	4.20	1.50

3.3. Evaluation of chromatographic procedure to recover imidazole

With the optimized Sephadex column, several experiments were performed on a sample assay (BSA 1 mg/ml, 1 *M* NaCl, 0.5 *M* imidazole) to determine the ideal time of water injection onto the column to recover imidazole in water without salts.

Time of injection just after elution of the BSA allowed only a decrease in salts at the end of the peak elution of imidazole. If water injection was performed at the elution volume of the BSA, the decrease in salts occurred at the elution volume of imidazole. If water was injected at the beginning of the BSA elution peak, the decrease in salts overlapped the start of the imidazole peak. At the end of the sample injection, water was injected and the imidazole peak was recovered in the water without salt. The same result was obtained when the sample was displaced by water.

3.4. Validation of the procedure with His-tag EGFP eluted from IMAC as sample

Following the experiments described above, several experiments were performed with a fraction of IMAC elution (5 ml) to determine the ideal moment of water injection onto the column to recover imidazole in water without salts. If water was injected 5 ml just after the injection of EGFP, this led to a decrease in salts at the start of peak imidazole

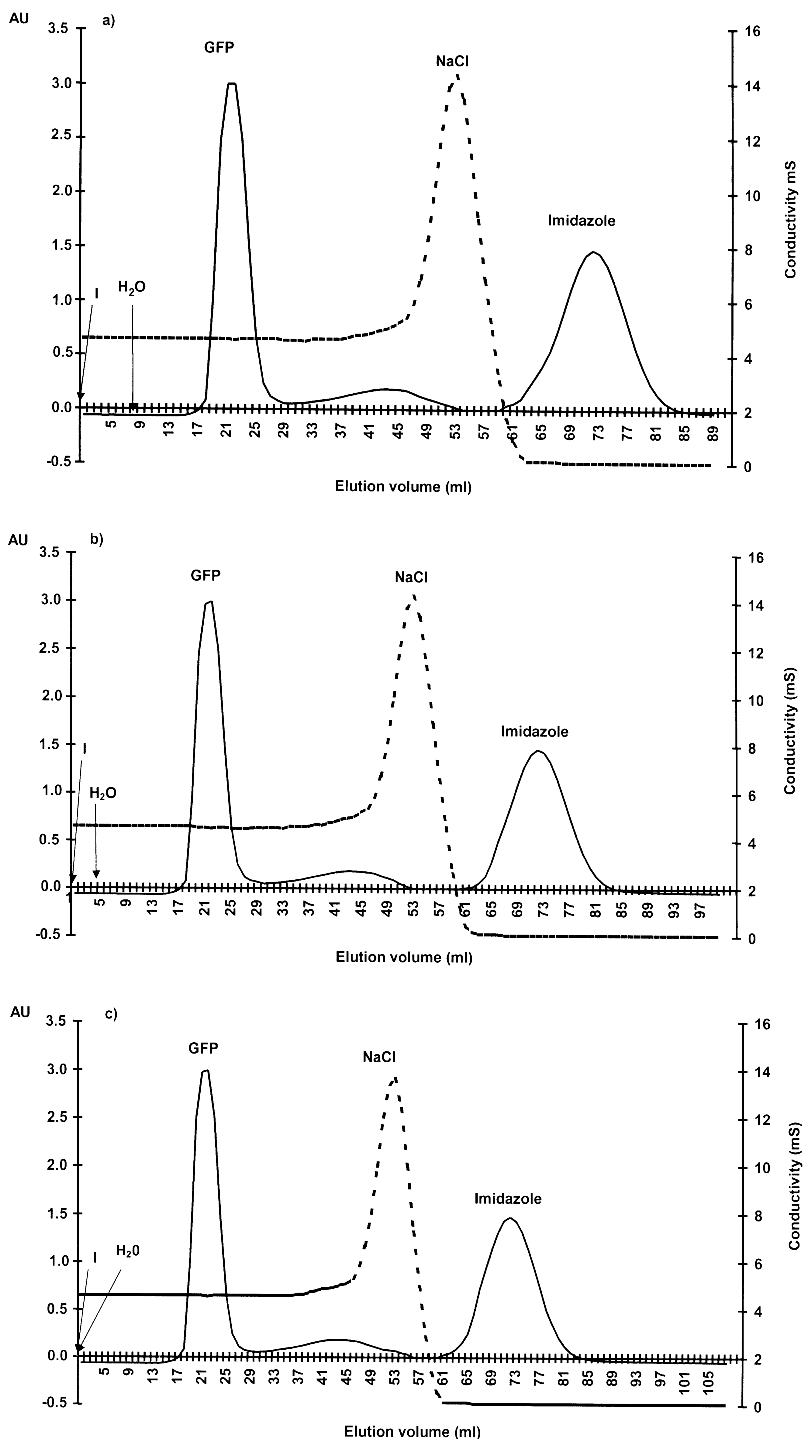

Fig. 1. Group size-exclusion chromatography of eluate fraction from IMAC. Column: XK 26/20 (85 ml of Sephadex G-25 Superfine). Sample: His-tag EGFP eluate from IMAC by imidazole. Buffer: 150 m*M* NaCl, 50 m*M* sodium phosphate, pH 7; flow-rate: 10 ml/min; detection at 280 nm: (a) injection of water at one sample volume at the end of injection of the sample, (b) injection of water at the end of injection of the sample, (c) sample displaced by the water.

elution. If water was injected at the end of EGFP injection, the decrease in salts was observed a few ml before the elution of imidazole and imidazole was recovered in the water without salt. When the sample was displaced with water, imidazole was also recovered in the water. The three possibilities are represented in Fig. 1.

3.5. Recovery and characterization of imidazole

To confirm imidazole purity, melting points were measured on a hot-stage apparatus. Melting points for native imidazole used in these experiments were 86.4 versus 85.6 °C for recovered imidazole after concentration to dryness (rotary evaporator), and

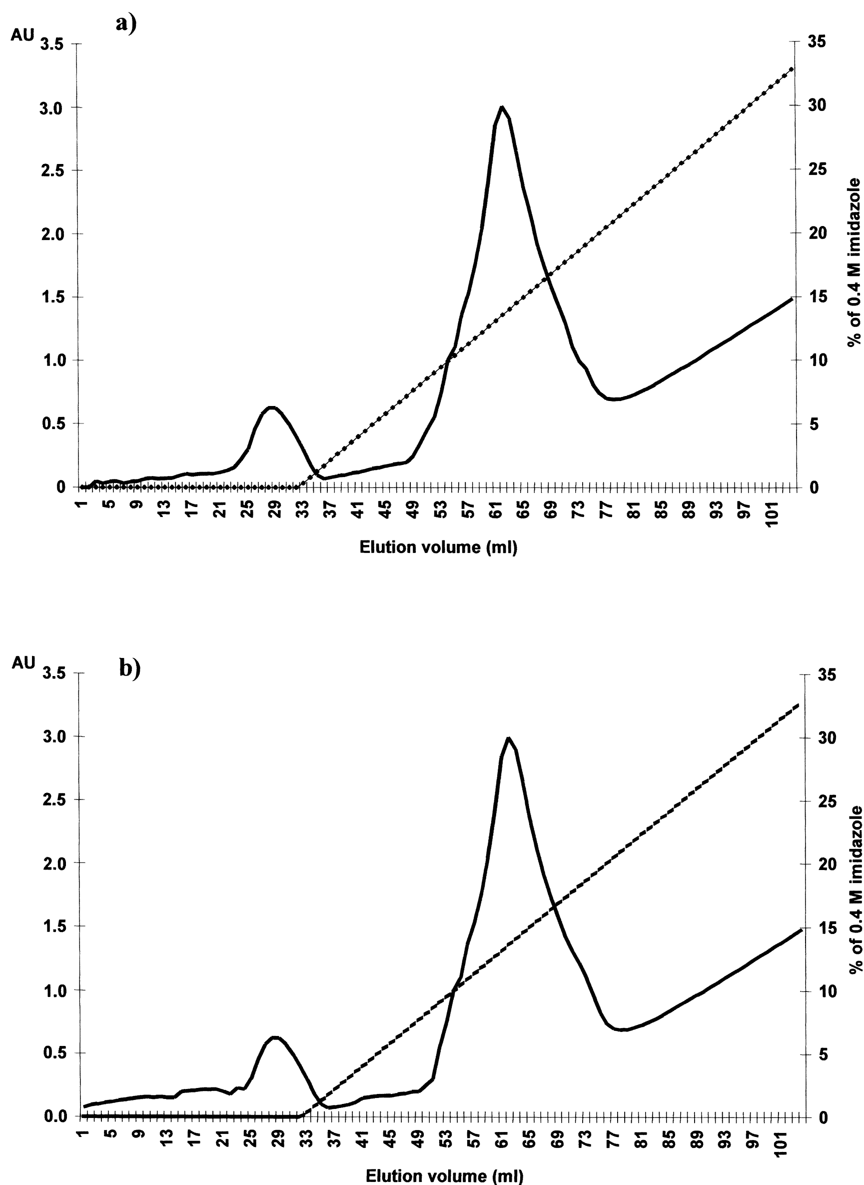

Fig. 2. Group size exclusion chromatography of eluate fraction from IMAC. Column: XK 16/20 (4 ml of chelating Sepharose fast flow). Sample: semi-purified recombinant His-tag EGFFP. Equilibration buffer: 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8. Elution buffer: (a) equilibration buffer with 400 m*M* recycled imidazole, (b) equilibration buffer with 400 m*M* native imidazole.

85.7 °C for recovered imidazole after freeze-drying. There was a slight difference (0.8 °C maximum) attributable to the absence of recrystallization after recovery. Subsequently, ^{1}H NMR analysis was performed. Native imidazole in deuterated dimethyl sulfoxide exhibited three single absorption lines (singlets, s) at δ=7.01 ppm (s, 2H, H-4 and H-5), 7.63 ppm (s, 1H, H-2), 12.03 ppm (large s, 1H, NH). Except for the recovered product after evaporation which showed a residual water peak around 3.5 ppm (probably resulting from incomplete water evaporation), all three imidazole samples exhibited the same NMR spectrum.

3.6. Validation of recycled imidazole

The recycled imidazole was used as eluent for IMAC. The level of EGFP recovered from IMAC with the two types of recycled imidazole was similar to that obtained with the native imidazole. The level of EGFP measured by fluorescence was 9.05×10^{7} RFU for recycled imidazole versus 9.07×10^{7} RFU for native imidazole. The EGFP was eluted by the same concentration of imidazole as shown in Fig. 2. The baseline drift was due to the imidazole gradient because imidazole absorbs at 280 nm.

4. Conclusion

This paper demonstrates the feasibility of recycling the imidazole used when purifying a recombinant His-tag protein with IMAC, without any modification to its structure or behavior. Two supports were tested for imidazole recovery in water based on the ability of imidazole to be eluted later than salts in these supports. The fractions were evaporated under reduced pressure or freeze-dried. The imidazole thus obtained was characterized by NMR and melting point determination and was re-used successfully as eluent in IMAC. This recycling procedure can be used on a large scale for preparative chromatography.

Acknowledgements

This work was supported by the Université V. Segalen Bordeaux 2 and the Conseil Regional d'Aquitaine. Moreover, we thank Ray Cooke for linguistic help.

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