Method optimization and scale-up of the purification of recombinant bovine carbonic anhydrase with IMAC Sepharose 6 Fast Flow

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Application Note 28-4044-80 AA

Affinity chromatography

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Abstract

This application note describes the purification of recombinant bovine carbonic anhydrase II (r-BCA) using IMAC Sepharose™ 6 Fast Flow in the capture step. Immobilized metal ion affinity chromatography (IMAC) is an excellent purification technique for proteins with exposed histidine residues that have affinity for metal ions. r-BCA is an example of such a protein.

IMAC Sepharose 6 Fast Flow is an IMAC medium that the user charges with the metal ion of choice. The medium provides high protein binding capacity for purification of histidine-tagged proteins as well as untagged proteins. As member of the BioProcess™ media family, IMAC Sepharose 6 Fast Flow meets the demands of industrial biotechnology with security of supply, comprehensive regulatory support, and necessary support for process applications.

This study presents results from method optimization and scale-up work. The method was optimized at laboratory scale with HiTrap™ IMAC FF 1 ml column to determine the best choice of metal ion and the preferred elution conditions. Once these parameters were established, they were then used in a scale-up study using HiTrap IMAC FF 5 ml column and HiPrep™ IMAC FF 16/10 20 ml column.

With the r-BCA protein, high purity was obtained with all three metal ions tested (Cu^{2+}, Ni^{2+}, and Zn^{2+}). Binding strength in the order $Zn^{2+} = Ni^{2+} > Cu^{2+}$ was observed. Due to its low toxicity, Zn^{2+} is often the most preferable metal ion to use in process scale, and thus it was chosen for the additional experiments described in this application note.

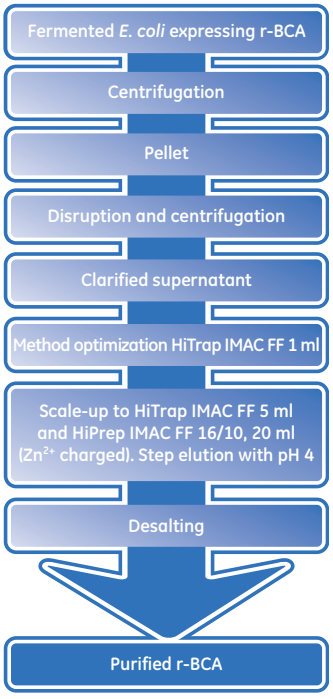


Fig 1. Purification of recombinant bovine carbonic anhydrase II (r-BCA)

Results also showed good recovery and purity in the elution methods tested (imidazole and pH). However, pH elution was chosen for the scale-up studies since it is less expensive than imidazole elution and therefore preferable for work at process-scale.

The scale-up studies gave very good yields with both HiTrap IMAC FF and HiPrep IMAC FF 16/10 columns. No significant change in recovery and purity was seen between the different scales. Moreover, there was only a very low level of leakage of metal ions from the columns, and leaked ions were easily removed by a desalting step.

Introduction

Since its introduction in 1975, IMAC has become one of the most widely used biochemical separation methods for purifying a broad range of biomolecules at the capture, intermediate, or polishing steps.

IMAC is based on the specific interaction between immobilized transitional metal ions and some key amino acid residues exposed on the surface of proteins (mainly histidine and to a lesser extent cysteine and tryptophan). The strength of interaction is primarily dependent on the type, number, and spatial distribution of the relevant amino acid residues and the nature of the immobilized metal ions. The presence of several adjacent histidine residues in histidine-tagged proteins is the most important parameter responsible for the high affinity between immobilized metal ions and such recombinant proteins in crude extracts of *Escherichia coli* or other biological sources. Even denatured proteins that are produced in *E. coli* as inclusion bodies have been purified and refolded using this technology.

Purification of $(histidine)_6$-tagged proteins is at present a widely used technique in laboratories throughout the world. It is also increasingly used for large-scale purification of native, untagged proteins or their recombinant counterparts. IMAC is already being used in several approved biopharmaceutical processes.

In many instances it is not always possible to predict which metal ion will be the most appropriate for purifying a protein, and there are often other factors to consider when making the choice. For these reasons, uncharged IMAC Sepharose 6 Fast Flow provides flexibility in planning, testing, and optimizing a purification scheme. The medium also has the added advantage of low metal ion leakage and different adsorption specificity compared with other commercial IMAC media.

Key features of IMAC Sepharose 6 Fast Flow include:

- possible to charge with various metal ions for optimized selectivity
- high protein binding capacity
- BioProcess medium designed to match manufacturing needs for security of supply, robust performance, and regulatory support
- available in the convenient and time-saving prepacked HiPrep and HiTrap format

In some cases involving untagged proteins and especially in *E. coli* applications, IMAC may not be applicable for use in the capture step, since unwanted host cell proteins can bind to the medium and displace the target protein. In contrast, the use of IMAC in the intermediate purification steps often gives excellent purity. This study, however, shows the possibility of using IMAC Sepharose 6 Fast Flow in the capture step in the purification of r-BCA from *E. coli* with good results.

The objective of the work was to demonstrate method optimization for purifying r-BCA, focusing on choice of metal ion and elution conditions, and then scale up the method to purify larger amounts of the protein.

Materials and Methods

Unless otherwise stated, all chromatographic media and systems were obtained from GE Healthcare (Uppsala, Sweden). HiTrap IMAC FF 1 ml and 5 ml columns and HiPrep IMAC FF 16/10 20 ml columns were used. The chemicals used were of analytical or reagent grade. All experiments were performed at room temperature (22ºC) on a calibrated ÄKTAexplorer™ 100 system with a 0.2 cm UV cell, controlled by UNICORN™ software.

Preparation of starting material

The molecular weight of r-BCA is M_r 30 000 Da. The enzyme catalyzes esterase activity, which can be measured and followed in a purification process. The active site functions with a single zinc ion that is essential for the catalysis. The amount of protein in solutions containing r-BCA was estimated by absorbance at 280 nm using the factor: $A^{1\ mg/ml}$ at 280 nm = 1.8.

E. coli cell paste containing r-BCA was homogenized in elution buffer by sonication. The crude extract was then clarified by centrifugation at 19 000 rpm for 35 min at 5ºC. The clarified crude extract was adjusted to pH 7.7 and filtered through a 0.45 µm filter and stored at –20ºC until further use.

The extract was thawed and re-filtered through a 0.2 µm filter before purification. The level of expression of the target protein was estimated at 30 mg/g of wet cell paste and 5.2 mg/ml in the clarified extract. The binding capacity of IMAC Sepharose 6 Fast Flow for r-BCA was determined to be 17 mg/ml Zn^{2+}-charged medium, by frontal analysis of the *E. coli* extract.

Choice of metal ion and elution conditions

Initially, three metal ions (Cu^{2+}, Ni^{2+}, and Zn^{2+}) and two elution methods (imidazole and pH) were tested to establish optimal conditions for purifying r-BCA from analytical to process-scale operations. HiTrap IMAC FF 1 ml columns were used to establish the conditions.

Scale-up

When the purification had been optimized, the process was scaled up using Zn^{2+} as the immobilized metal ion and pH step elution to elute the target protein. The scale-up was verified at two scales (HiTrap IMAC FF 5 ml and HiPrep IMAC FF 16/10 20 ml) keeping the residence time constant while bed height and flow velocity were increased.

Leakage of zinc ions

The amount of zinc in the applied sample, flowthrough fraction, and bound fraction (after elution from the column) was determined by atomic absorption spectrophotometry.

Since carbonic anhydrase already contains one mol of zinc per mol of protein, the amount of zinc that leaked from the column is the difference between the zinc content in the sample and the eluted fractions. Desalting (HiPrep 26/10 Desalting) was also performed to remove residual zinc ions from the target protein solutions.

Results and discussion

The selection of a suitable metal ion, protein, and chromatographic operating conditions for this application work was made on the basis of well-planned and executed screening experiments, as described in the following sections, with reference to Figures 2 and 3.

Selection of metal ion and elution conditions

The elution profiles after purification of *E. coli* extract on HiTrap IMAC FF 1 ml charged with Zn^{2+}, Ni^{2+}, or Cu^{2+} ions are shown in Figure 2. The results show that r-BCA is bound to the same extent on immobilized Zn^{2+} and Ni^{2+} but distinctly less on immobilized Cu^{2+}. Given these results and the fact that Zn^{2+} is less toxic and more environmentally friendly than either Ni^{2+} or Cu^{2+}, immobilized Zn^{2+} was selected as the metal ion of choice for this application. Lower toxicity and good binding charateristics make Zn^{2+} particularly attractive for process-scale operations.

Figure 3 shows that elution of bound proteins with a linear or stepwise pH gradient (which protonates the amino acid side chains involved in binding) is as effective as elution with imidazole (which is a competitive elution agent).

Column: HiTrap IMAC FF 1 ml
Metal ion: Zn^{2+}, Ni^{2+}, or Cu^{2+}
Sample: 5 ml clarified *E. coli* extract containing 30 mg of r-BCA
Flow rate: 1 ml/min (150 cm/h)
Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, pH 7.4
Elution buffer: 20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4
Elution: Linear gradient (30 CV) from 100% binding buffer to 100% elution buffer

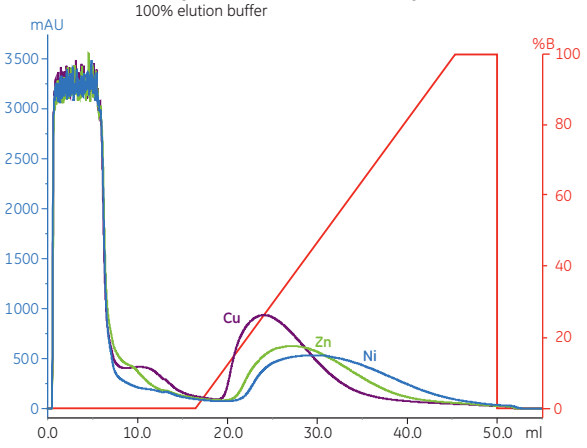
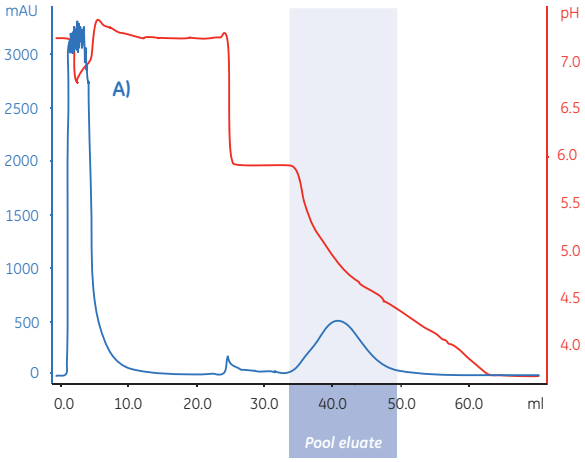


Fig 2. Overlay chromatograms from purification of 5 ml of extract of r-BCA on a 1 ml IMAC Sepharose 6 Fast Flow column charged with Cu^{2+}, Ni^{2+}, or Zn^{2+}. Based on the elution positions of the bound proteins, the affinity of the metal ions for r-BCA was in the order $Zn^{2+} = Ni^{2+} > Cu^{2+}$.

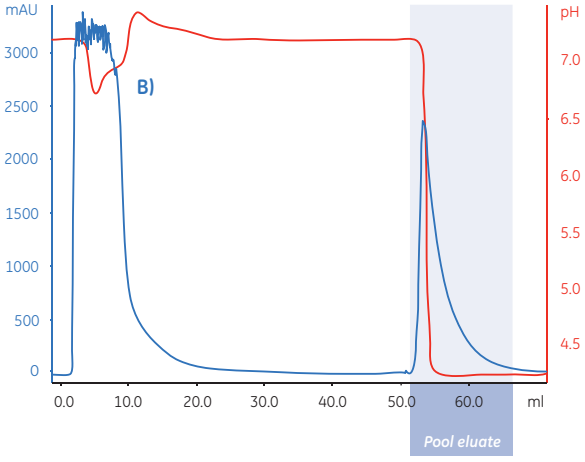
pH gradient elution

Column: HiTrap IMAC FF 1 ml charged with Zn^{2+}
Sample: 2.5 ml clarified cell culture containing 15 mg r-BCA
Binding buffer: 20 mM sodium phopsphate, 0.5 M NaCl, pH 7.4
Elution buffer 1: 20 mM sodium acetate, 0.5M NaCl, pH 6.0
Elution buffer 2: 20 mM sodium acetate, 0.5M NaCl, pH 4.0
Flow rate: 1 ml/min (150 cm/h)
Elution method: Stepwise gradient elution in 15 CV to pH 6 (Elution buffer 1) followed with a linear gradient from pH 6 to pH 4 (Elution buffer 2) in 30 CV



pH step elution

Column: HiTrap IMAC FF 1 ml charged with Zn^{2+}
Sample: 2.5 ml clarified cell culture containing 15 mg r-BCA
Binding buffer: 20 mM sodium phosphate, 0.5M NaCl, pH 7.4
Elution buffer : 20 mM sodium acetate, 0.5 M NaCl, pH 4.5
Flow rate: 1 ml/min (150 cm/h)
Elution method: Stepwise gradient elution from Binding buffer directly to Elution buffer in 15 CV



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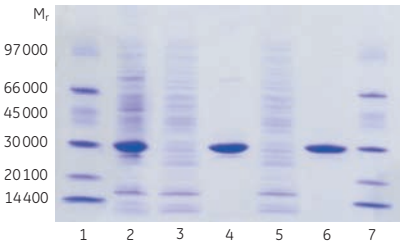


Lanes
1. LMW markers
2. Clarified *E. coli* extract
3. Flowthrough pH gradient elution
4. Eluted pool pH gradient elution
5. Flowthrough pH step elution
6. Eluted pool pH step elution
7. LMW markers

Fig 3. Purification of r-BCA on a 1 ml IMAC Sepharose 6 Fast Flow column charged with Zn^{2+}. The bound fraction was eluted with a linear (A) or stepwise (B) pH gradient. The purity was analyzed by non-reduced SDS-PAGE on PhastGel™ Gradient 8-25 (C). The gel was stained with Coomassie™.

Columns: HiTrap IMAC FF (1 ml and 5 ml) and HiPrep IMAC FF 16/10 (20 ml) charged with Zn^{2+}
Sample: 2.4, 12 and 49 ml of clarified *E. coli* extract containing 12.5, 62.4 and 255 mg r-BCA respectively
Binding buffer: 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4
Elution buffer: 20 mM sodium acetate, 0.5 M NaCl, pH 4.0
Flow rate: 150 cm/h in all cases
Experimental: After sample application, each column was washed with 20 column volumes (CV) Binding buffer followed by stepwise elution with 15 CV 100% Elution buffer.
Detection: Absorbance, 280 nm

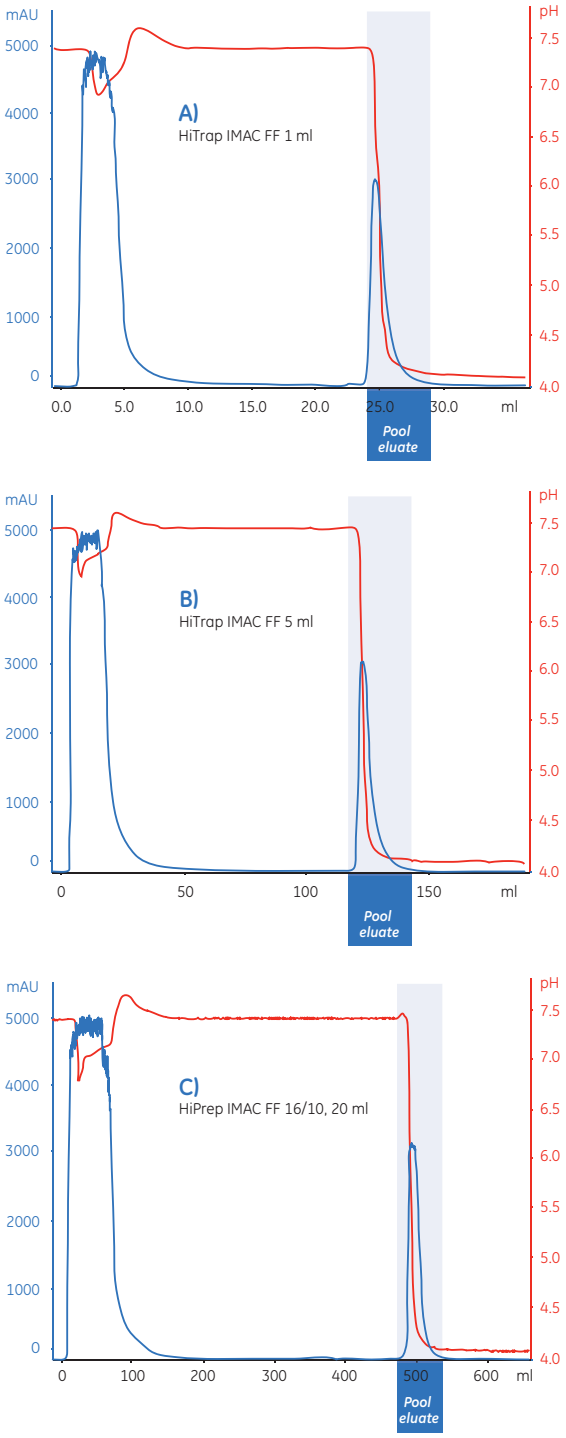


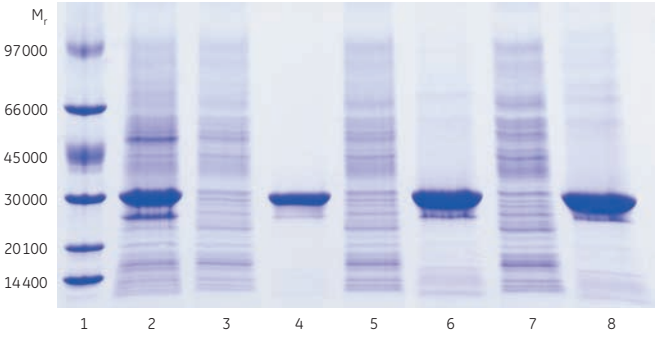
Fig 4. Scaling up the purification of r-BCA from 1 ml to 5 ml to 20 ml columns of IMAC Sepharose 6 Fast Flow charged with Zn^{2+} ions.

Considering the much lower costs of buffer salts compared with imidazole, elution of bound proteins by lowering the pH is the most preferred elution method for process-scale operations. Therefore pH elution was chosen for the scale-up studies.

Scale-up

The results show trouble free scale-up of the purification from 1 ml to 5 ml to 20 ml columns with yields of 12, 56, and 235 mg of r-BCA respectively. Figure 4 shows the chromatograms and Figure 5 shows the SDS-PAGE analysis of the flowthrough fractions and eluted pools from the different runs. Table 1 compares yields and recoveries, showing the scalability of the application.

More than 90% of the protein was recovered in each instance at a load of 74% of binding capacity (i.e. 17 mg/ml medium). The recovery of applied activity was approximately 90%, and no significant change in recovery and purity was seen between the different scales of operation.



Lanes
1. LMW markers
2. Clarified *E. coli* extract, diluted 1:33
3. Flowthrough HiTrap IMAC FF 1 ml, diluted 1:4
4. Eluted pool HiTrap IMAC FF 1 ml, diluted 1:4
5. Flowthrough HiTrap IMAC FF 5 ml, diluted 1:4
6. Eluted pool HiTrap IMAC FF 5 ml, diluted 1:5
7. Flowthrough HiPrep IMAC FF 16/10 20 ml, diluted 1:4
8. Eluted pool HiPrep IMAC FF 16/10 20 ml, diluted 1:4

Fig 5. Non-reduced SDS-PAGE analysis on ExcelGel™ Gradient 8-18 of the main fractions from the scale-up experiments. The gel was stained with Coomassie.

Zinc leakage

The leakage of zinc ions was evaluated (Table 2) and show that total leakage of Zn^{2+} from IMAC Sepharose 6 Fast Flow was less than 5% at all scales. Desalting effectively removed Zn^{2+} that was mixed with the target protein. However, desalting does not remove the zinc ion (1mol/mol protein) that is anchored at the active site of r-BCA. These results demonstrate that IMAC Sepharose 6 Fast Flow medium is well adapted for process applications.

Table 1. Data and results from the scale-up purification of r-BCA on IMAC Sepharose 6 Fast Flow. Comparisons of r-BCA yields and recoveries for the different runs show scalability of the application. The loading was 74% of the binding capacity of the medium.

Column	Fraction	Amount applied (mg)	Amount eluted (mg)	Recovery of protein	Recovery of r-BCA activity
HiTrap IMAC FF 1 ml	Clarified *E. coli* extract	12.5	—	—	—
	Eluted pool	—	11.7	94%	93%
HiTrap IMAC FF 5 ml	Clarified *E. coli* extract	62.4	—	—	—
	Eluted pool	—	56.1	90%	84%
HiPrep IMAC FF 16/10 (20 ml)	Clarified *E. coli* extract	255	—	—	—
	Eluted pool	—	235	92%	90%

Table 2. Amount of Zn^{2+} desorbed from the column at the different scales of operation, and amount of Zn^{2+} in the eluate as well as in the desalted fractions.

Sample	% desorbed Zn^{2+} from column	Molar ratio before desalting (mol Zn^{2+}/mol protein)	Molar ratio after desalting (mol Zn^{2+}/mol protein)
Eluted pool HiTrap IMAC FF 1 ml	2.9%	1.7	0.9
Eluted pool HiTrap IMAC FF 5 ml	4.4%	2.7	1.3
Eluted pool HiPrep IMAC FF 16/10 (20 ml)	2.7%	1.6	1.4

Conclusions

This study demonstrates that IMAC Sepharose 6 Fast Flow is well adapted for laboratory- to process-scale purification of untagged proteins with good selectivity and recovery of the target protein. No problems where encountered in the scale-up of the capture step for purification of r-BCA from *E. coli* extract.

The elution of the target protein was conveniently performed with a simple, stepwise pH gradient resulting in a concentrated fraction upon elution. This elution method is also economically sound and environmentally friendly. For some proteins that are sensitive to exposure to low pH, elution with a linear or stepwise gradient of imidazole is a good alternative.

Zn^{2+} was found to be the best metal ion for use in this application at process-scale since it is less toxic and more environmentally friendly than Ni^{2+} and Cu^{2+}. The amount of Zn^{2+} that co-eluted with the target protein was very low, less than 5%. The Zn^{2+} ions that leaked from the column were easily removed from the target protein solution by a desalting step, leaving only the Zn^{2+} ion anchored at the active site of the protein.

The scale at which the chromatographic separation was performed did not have any significant effect on the selectivity, recovery, and purity of the target protein. In all instances, the recovery of enzymatic activity of the target protein was about 90%.

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