Strategy to develop purification protocols using ÄKTA system

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Strategy to develop purification protocols using ÄKTA™ system

This application note describes a strategy to develop a purification protocol using a Design of Experiment (DoE) approach. DoE is a method to design a study to determine how parameters interact and influence the performance. This method is used to collect more information with higher accuracy in a shorter period of time. The UNICORN™ control system for ÄKTA avant includes DoE functionality that was used to develop the purification protocol. The final three-step purification workflow for purifying recombinant bovine carbonic anhydrase II (Fig 1), including ion exchange (IEX) as the capture step, hydrophobic interaction chromatography (HIC) as a second (intermediate) step, and gel filtration (GF) as the final polishing step, showed purity and yield comparable to a single affinity method[1].

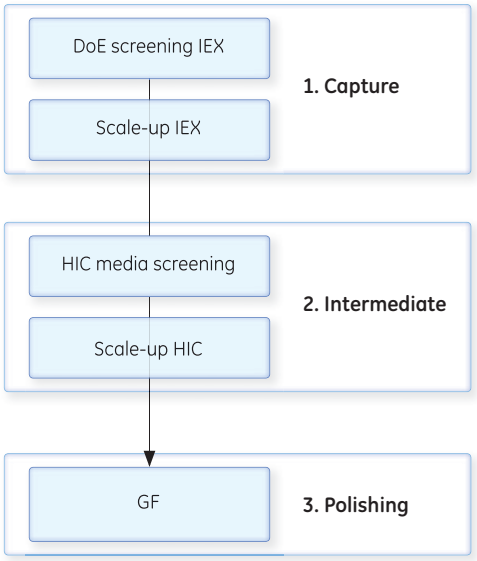


Fig 1. Overview of the workflow.

Introduction

Recombinant bovine carbonic anhydrase II (r-BCA) recovered from homogenized and clarified *Escherichia coli* (*E. coli*) was chosen as a model protein to illustrate a strategy for how to set up a purification protocol with a DoE approach.

Initial tests on ion exchange chromatography (IEX) media in the capture step were performed. Both anion ion exchange (AIEX) and cation ion exchange (CIEX) were tested using binding buffers with a pH 0.5-1 pH unit from the isoelectric point (isoelectric point for r-BCA is 6.4). The results showed that pH 7-9 (AIEX) and pH 4-5.5 (CIEX) gave no satisfactory binding of r-BCA. To minimize time and number of experiments, an initial screening study in a 96-well filter plate was performed to select appropriate pH and IEX media.

Based on the results from the 96-well filter plate study the DoE functionality in UNICORN software was used to set up the IEX screening study on prepacked HiTrap™ columns. The purpose was to investigate how both pH and salt concentration in sample load and salt concentration during wash affect the amount of r-BCA obtained in the capture step. DoE can provide detailed information on which factors influence responses such as purity and yield and on how these factors should be manipulated to give an optimum result.

A HIC media screening was performed with pre-packed columns to select the medium with the highest capacity and selectivity for the r-BCA. The benefits of using pre-packed columns (HiTrap HIC Selection Kit) are ease-of-use and consistent results.

The results of the screening trials were used to scale up both the IEX and the HIC steps.

The resulting three-step purification is an example of an efficient strategy for purification of a challenging protein. It also demonstrates the convenience in using ÄKTA avant 25 chromatography system and UNICORN 6 software in combination with prepacked HiTrap and Tricorn™ columns.

1. BCA can also be purified using sulfanilamide affinity chromatography and IMAC (immobilized metal affinity chromatography).

Materials and methods

All chromatographic steps were performed on ÄKTA avant 25 with UNICORN 6 software. All equipment and chromatographic media were obtained from GE Healthcare. All chemicals used were analytical grade.

E. coli cell paste expressing r-BCA (Mw 29 kDa, pI 6.5) was homogenized and clarified by centrifugation in 35 000 × g for 20 min at 4°C. The pH was adjusted with NaOH and NaCl or ammonium sulfate was added from stock solutions to adjust the conductivity when needed.

Capture IEX

Initial capture experiments of r-BCA on AIEX at pH 7-9 and CIEX at pH 4.5-5 were performed (with unsatisfactory results of low or no amount of captured r-BCA, data not shown). Thereafter a screening study was performed using a 96-well filter plate to select appropriate pH and IEX media to capture r-BCA. The pH was set to 5, 8, and 11 and the IEX media were SP Sepharose™ High Performance (CIEX) and Q Sepharose High Performance (HP) (AIEX).

Based on the results from the 96-well filter plate study, a full factorial design was performed to investigate the pH and salt concentration effect on yield and purity of eluted r-BCA. The DoE functionality in UNICORN 6.0 software was used to create the design and to set up a run scheme. See Table 1 and Figure 2. HiTrap Q HP 1 ml columns were used in the screening.

After screening, the optimized conditions were used for a fivefold scale-up step to HiTrap Q HP 5 ml. Buffers were prepared in- line using the UNICORN BufferPro functionality. Gradient elution was performed in both screening and scale-up experiments.

Table 1. Full factorial design IEX capture step

Factors	Low	Center	High
Load pH	10	10.5	11
NaCl load (mM)	0	10	20
NaCl wash (mM)	20	40	60

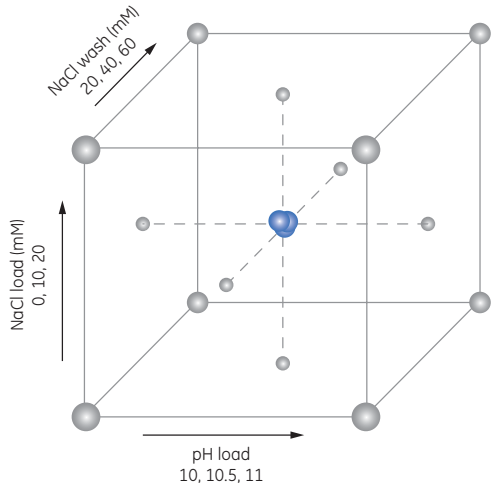


Fig 2. Illustration of the full factorial design used in the IEX screening study.

Intermediate HIC

HIC media screening was performed to find the medium with the highest capacity and selectivity. Columns used can be found in Table 2. The UNICORN software scouting function was used for automation.

In the scale-up from 1 ml to 5 ml, the binding buffer salt concentration was decreased from 2.0 M to 1.7 M ammonium sulfate to ensure that no target protein would precipitate.

Table 2. HIC step: Columns from HiTrap HIC Selection Kit

HIC Screening Columns
HiTrap Butyl HP 1 ml
HiTrap Octyl FF 1 ml
HiTrap Phenyl HP 1 ml
HiTrap Butyl-S FF 1 ml
HiTrap Phenyl FF (low sub) 1 ml

Polishing GF

A pool of the main fractions from the HIC scale-up was loaded on a Superdex™ 200 10/300 GL gel filtration column. Buffer used was 10 mM phosphate, 150 mM NaCl, pH 7.4.

SDS-PAGE analysis

Electrophoresis was used to determine the amount of target protein in flowthrough, wash, and eluate fractions. ExcelGel™ SDS Gradient 8-18 was stained with Deep Purple™ Total Protein Stain and scanned using Typhoon™ FLA 6000 and the results were analyzed with ImageQuant™ TL software.

Results and discussion

Capture IEX

Based on results from the 96-well filter plate study (data not shown) varying two factors (pH and IEX media), favorable binding conditions were obtained with Q Sepharose HP media at high pH.

The binding conditions for the capture IEX step were screened using DoE functionality in UNICORN 6 (Table 1). The amount of target protein in flowthrough and wash was measured and its dependence on pH and conductivity in load and wash was evaluated. Figure 3 shows that pH and conductivity during load and the combined effect between the two affected the recovery of r-BCA during sample loading. The most favorable conditions were found at low conductivity and high pH. The optimal conditions for the wash step can be found in the red area, indicating a low reduction of r-BCA during the wash. An additional study could be of interest to explore an area including even lower conductivity and higher pH to further optimize the process.

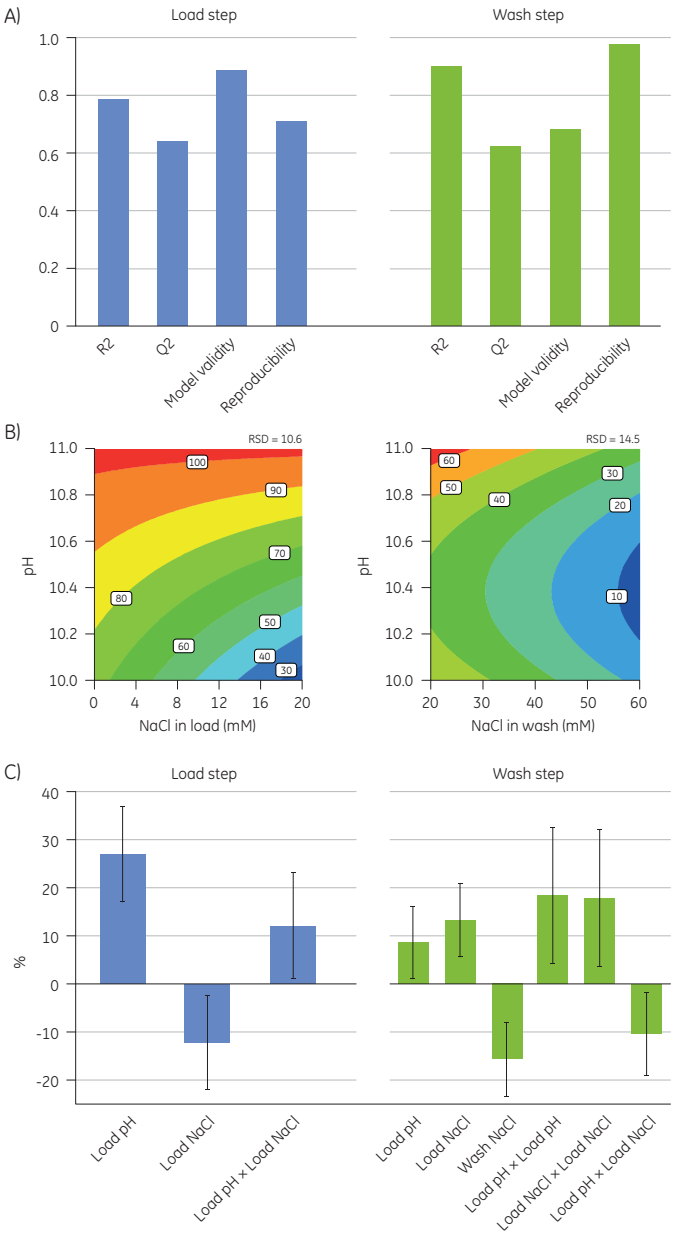


Fig 3. Result plots for the DoE evaluation. (A) The summary of fit plot describes the quality of the obtained models, in this case indicating good quality of the model describing the loss of r-BCA in load and wash step. (B) The response surface shows that lower conductivity and high pH will give a higher amount of bound r-BCA in the load and wash step, visualized by the red areas. (C) Parameters that affect the loss of r-BCA in load and wash steps are shown in the coefficient plots. The plots confirm that high pH and low conductivity results in a higher amount of bound r-BCA during load and wash steps. The coefficient plot for the r-BCA wash (right) indicates that the conditions used in the loading will have a significant impact on amount of bound r-BCA during wash. The interaction effects are also significant for the amount of bound r-BCA obtained during sample loading.

In some results the amount of eluted protein was low, in some cases below the detection limit (data not shown), resulting in a non-significant DoE model for elution. The purity and the amount of eluted protein were instead analyzed with comparative evaluation by SDS-page (Fig 4). The analysis indicated that high pH in load and wash resulted in high yield of r-BCA.

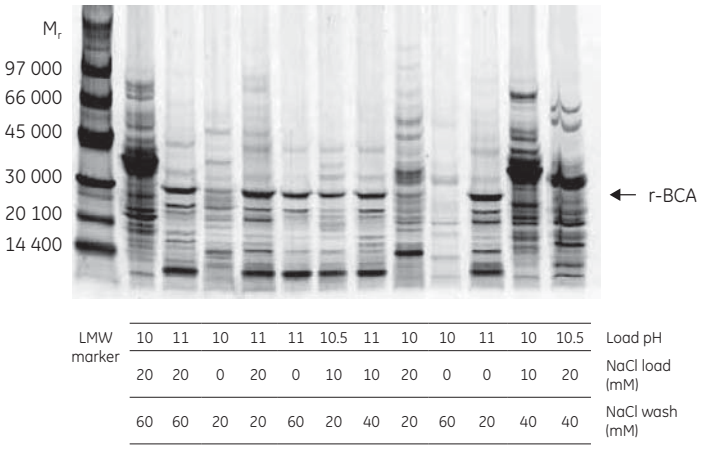


Fig 4. No significant DoE model was obtained for the elution because some results showed too low amounts for detection. The purity and the amount of eluted protein were analyzed by comparative SDS-PAGE. The target protein has a relative molecular mass (M_r) of 29100. LMW: low molecular weight marker.

The most favorable conditions obtained in the IEX capture screening were applied in a scale-up experiment (Fig 5). The overall results (Fig 10) show that a relatively high purity and yield could be obtained in the very first capture step.

Column: HiTrap Q HP 5 ml
Sample: 50 ml clarified *E. coli* supernatant expressing r-BCA
Buffers: Buffer Pro, Stock solutions: 0.2 M piperazine, 4 M NaCl, 0.2 M HCl, high quality water
Flow rate: 5 ml/min
Gradient: 0% to 50% B (30 column volumes [CV]), 50% to 100% B (10 CV)

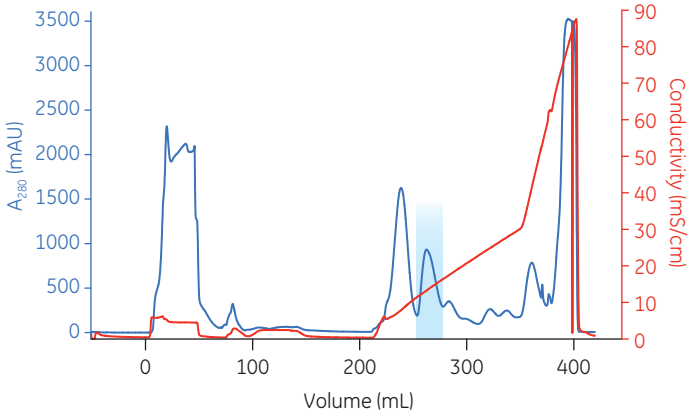


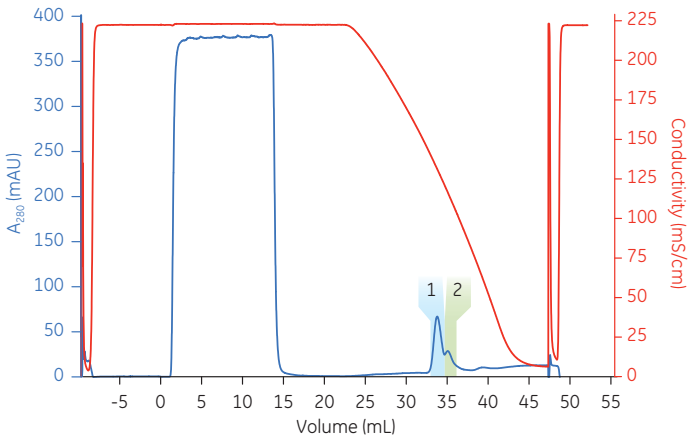
Fig 5. Scale-up experiment of the Capture IEX step on HiTrap Q HP 5 ml using optimized conditions from the IEX screening study. Recombinant BCA is indicated in the figure.

Intermediate HIC

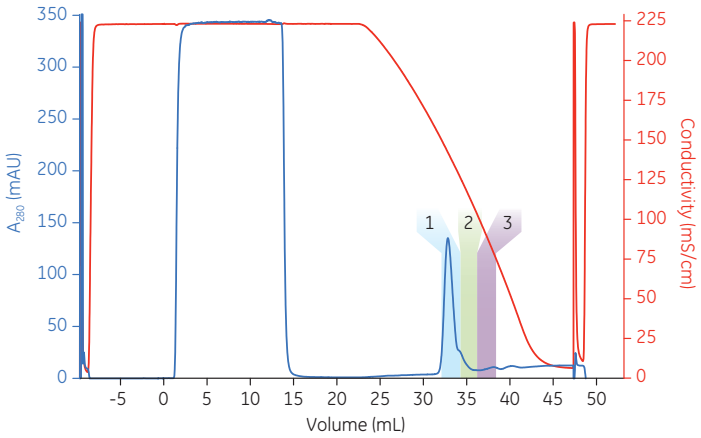
When purifying the sample obtained from the initial IEX capture, the Butyl HP medium was found to give the highest yield and selectivity in the HIC media screening (Fig 6 and Fig 7). The results were similar for all media and show that no target protein was obtained in the flowthrough. However, the highest yield was obtained for Butyl HP medium.

Column:	HiTrap 1 ml; Phenyl HP, Butyl HP, Octyl FF, Butyl-S FF, and Phenyl FF (low sub)
Sample:	15 ml eluate pool of r-BCA from HiTrap Q Sepharose HP (1ml)
Binding buffer:	50 mM TrisHCl pH 7.0, 2 M ammonium sulfate
Elution Buffer:	50 mM TrisHCl pH 7.0
Flow rate:	1 ml/min
Gradient:	100% to 0% B (20 CV)

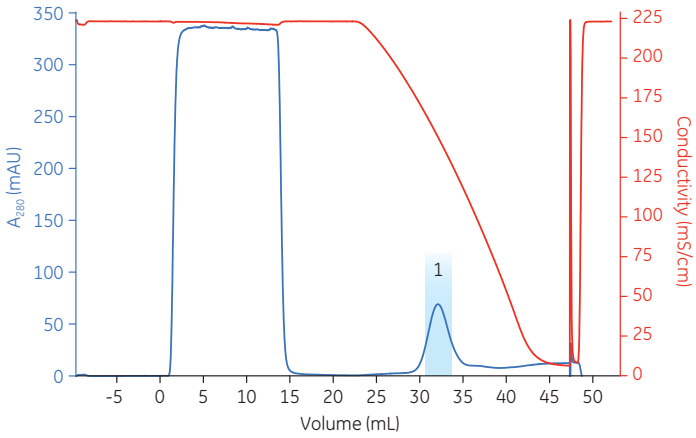
A) Phenyl HP



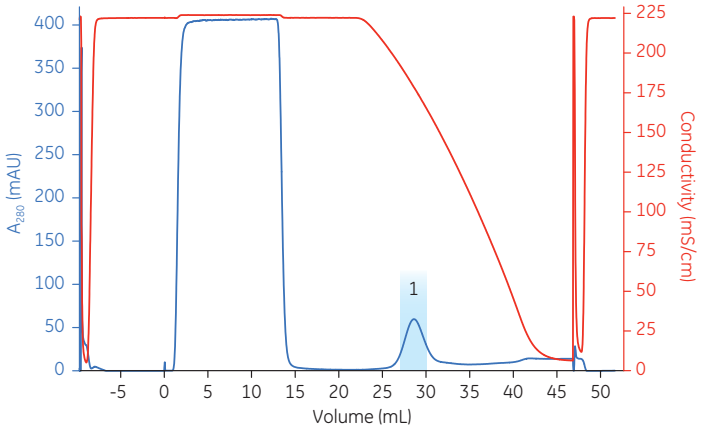
B) Butyl HP



C) Pheny FF (low sub)



D) Butyl-S FF



E) Octyl FF

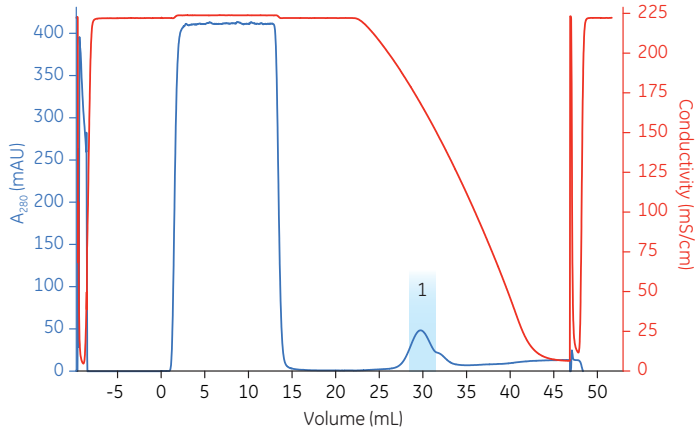


Fig 6. Intermediate HIC media screening study, fractions (indicated by number) were analyzed by SDS-PAGE to find the medium with the highest capacity and selectivity, see Figure 7.

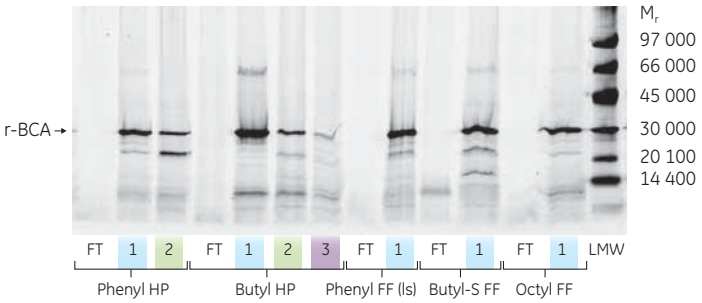


Fig 7. SDS-PAGE analysis of eluted fractions from the HIC media screening. Butyl HP medium showed the highest capacity and sufficient selectivity. LMW: Low molecular weight marker. FT: flowthrough.

The intermediate scale-up was performed on HiTrap Butyl HP 5 ml column (Fig 8). The results show an equally high yield and purity as the small-scale purification using HiTrap Butyl HP 1 ml column (Fig 10).

Column: HiTrap Butyl HP 5 ml
Sample: 50 ml eluate pool of r-BCA from HiTrap Q HP (5ml)
Binding buffer: 50 mM TrisHCl pH 7.0, 1.7 M ammonium sulfate
Elution Buffer: 50 mM TrisHCl pH 7.0
Flow rate: 5 ml/min
Gradient: 100% to 0% B (15 CV)

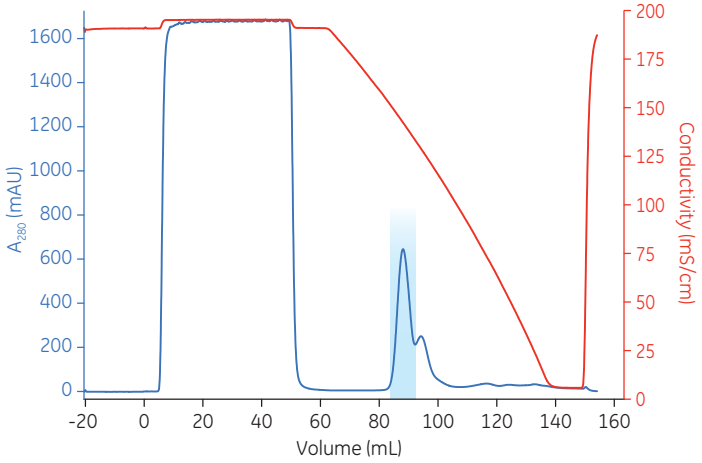


Fig 8. Scale-up experiment of the intermediate HIC step using HiTrap Butyl HP 5 ml. The selected r-BCA pool is indicated in the figure.

Polishing GF

The eluate from the HIC scale experiment was purified in a final polishing step using Superdex 200 10/300 GL (Fig 9). The fractions obtained were analyzed by SDS-PAGE (Fig 10). The results showed that the purified target protein was homogeneous and free from contaminants.

Column: Superdex 200 10/300 GL
Sample: 0.5 ml eluate pool from HiTrap Butyl HP (5 ml)
Buffer: PBS, pH 7.4
Flow rate: 0.5 ml/min

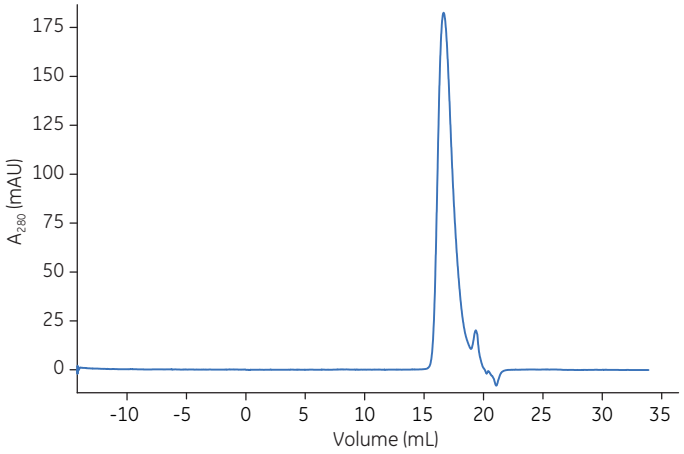


Fig 9. A gel filtration run using a Superdex 200 10/300 GL column was performed as the final polishing step.

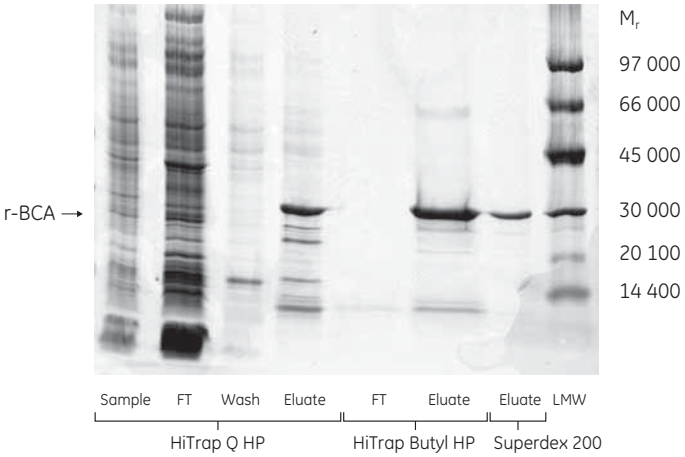


Fig 10. Selected fractions from steps of the three-step purification were analyzed by SDS-PAGE. The chromatography results from the selected fractions can be found in Figure 5, Figure 8, and Figure 9. LMW: Low molecular weight marker. FT: flowthrough.

Conclusions

This study demonstrates the successful development of a three-step purification protocol for purifying r-BCA from *E. coli*. The use of pre-packed columns and the automation and DoE functionality built into ÄKTA avant 25 and UNICORN 6 provided an efficient, simple, and generic approach for setting up the IEX capture and HIC media screening and evaluation. The resulting purification is an example of how to set up a purification of a protein and the strategy can be applied on various proteins, from the easily purified to the more challenging ones.

Ordering information

Product	Code number
ÄKTA avant 25	28-9308-42
HiTrap HIC Selection Kit*	28-4110-07
HiTrap Butyl HP, 5 × 1 ml	28-4110-01
HiTrap Butyl HP, 5 × 1 ml	28-4110-05
HiTrap Q HP, 5 × 1 ml	17-1153-01
HiTrap Q HP, 5 × 5 ml	17-1154-01
Q Sepharose High Performance, 75 ml	17-1014-01
SP Sepharose High Performance, 75 ml	17-1087-01
Superdex 200 10/300 GL	17-5175-01
UNICORN 6	28-9589-95

* Kit includes HiTrap 1 ml columns containing the following media: Phenyl Sepharose High Performance, Butyl Sepharose High Performance, Phenyl Sepharose 6 Fast Flow (low sub), Phenyl Sepharose 6 Fast Flow (high sub), Butyl-S Sepharose 6 Fast Flow, Butyl Sepharose 4 Fast Flow, and Octyl Sepharose 4 Fast Flow

Related literature	Code number
ÄKTA avant data file	28-9573-45
ÄKTA laboratory-scale chromatography systems handbook	29-0108-31
Strategies for Protein Purification handbook	28-9833-31
Purifying Challenging Proteins – Principles and Methods	28-9095-31
Hydrophobic Interaction and Reversed Phase Chromatography – Principles and Methods	11-0012-69
Ion Exchange Chromatography and Chromatofocusing – Principles and Methods	11-0004-21