



# Rapid method development for native protein purification using ÄKTA avant 25 chromatography system

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# Rapid method development for native protein purification using ÄKTA™ avant 25 chromatography system

ÄKTA avant 25 system controlled by UNICORN™ 6 software was used to develop a three-step chromatography method for purification of native maltodextrin-binding protein from *E. coli*. Anion-exchange chromatography (AIEX) was used for the initial capture step, hydrophobic interaction chromatography (HIC) for intermediate purification and gel filtration (GF) for final polishing. HiScreen™ columns were used in a Design of Experiments (DoE) setup to determine optimal loading conditions for capture. In developing the capture step, salt concentration and pH during equilibration, wash, and elution were controlled automatically by on-line buffer preparation using BufferPro. Screening of chromatography media for the intermediate purification step was performed using HiTrap™ HIC Selection Kit. Finally, polishing by GF ensured removal of remaining impurities resulting in target protein purity of almost 100%. After optimization of the capture step and screening HIC media for the intermediate step, the whole purification process was performed in a single day.

## Introduction

Maltodextrin-binding protein belongs to a large family of periplasmic binding proteins of gram-negative bacteria that act as high-affinity active transporters or serve as receptors for bacterial chemotaxis (1). While maltodextrin-binding protein is frequently used as an affinity tag, purification of the native protein *per se* requires a traditional three-step purification approach to achieve high final purity/yield.

ÄKTA avant 25 is a liquid chromatography system intended for process development and protein purification using rigid, high-resolution chromatography media. The system can be used for media screening, method scouting, and method optimization. Optimization of loading, wash, and elution conditions is facilitated by use of BufferPro automatic on-line buffer preparation, which reduces the time required for buffer

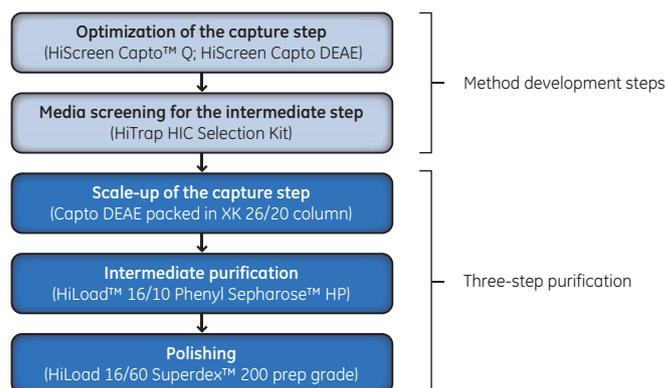


Fig 1. Summary of the different steps and columns used in method development for purification of native maltodextrin-binding protein.

preparation. Column recognition and run data history of individual columns provides traceability and operational security.

UNICORN 6 control software has been specially developed for ÄKTA avant 25 to increase productivity and efficiency. One of the key features of UNICORN 6 software is the Design of Experiments (DoE) functionality. DoE facilitates fast process development by allowing a strategic set up of experimental plans so that parameters affecting the chromatographic results can be tested individually and in combination (interaction effects). The DoE setup allows: 1) Screening to determine which factors were important in the process; 2) determination of optimal factor settings for the process; and 3) minor adjustments to factors in experiments while preventing responses exceeding the set specification limits.

DoE was used in the development of the three-step purification of maltodextrin-binding protein from *E. coli* cell culture described in this Application note. The target protein has a molecular weight ( $M_r$ ) of 41 000 and an isoelectric point (pI) of 5.3. The steps taken for development of a three-step method of purification are shown in Figure 1.



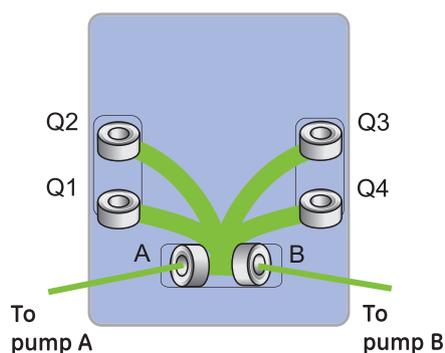
## Materials and methods

### Cell preparation and clarification

The starting material was frozen *E. coli* cell pellet. For every gram of cell pellet thawed, 5 ml of buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl), 50  $\mu$ l of 100 mM phenylmethanesulfonyl fluoride, 5  $\mu$ l of benzonase (20 mg/ml), 5  $\mu$ l of MgCl<sub>2</sub> (1 M), and 100  $\mu$ l of lysozyme (10 mg/ml) were added. The *E. coli* cell solution was fully dissolved by stirring, disrupted by sonication, and clarified by centrifugation at 40 000  $\times$  g for 30 min at 4°C. Supernatant was collected and kept on ice until capture of the target protein by AEX.

### Automatic buffer preparation, integrated fraction collector, and Column Logbook

Automatic buffer preparation increases productivity by minimizing manual work. In this study, pH and salt concentration during equilibration, wash, and elution were varied by use of on-line buffer preparation with BufferPro. The quaternary valve (Fig 2) incorporated in the system was used for this purpose. The valve has four buffer inlets that enable automatic buffer preparation using stock solutions. The following stock solutions were prepared: AEX mix (0.15 M Tris, 0.15 M bis-Tris); 0.2 M HCl, 4 M NaCl; and ultrapure water. The solutions were connected to the quaternary valve through inlet Q1, Q2, Q3, and Q4, respectively.



**Fig 2.** The ports of the quaternary valve were used to create BufferPro gradients: Q1–Q4 = buffer inlets; A = outlet to system pump A via inlet valve A; outlet to system pump B via inlet valve B.

The integrated, temperature-controlled fraction collector was used to prevent sample overheating and/or evaporation and to exclude dust pollution from purified samples. The Column Logbook feature of UNICORN 6 was used to keep track of column and run data and enhance operational security. Individual columns were identified using a 2-D barcode reader and experimental runs were then recorded in the Column Logbook.

### Optimization of the capture step using DoE

DoE was used for optimization of the capture step (Table 1). The sample load for each run was 5 ml of clarified and pH-adjusted cell culture supernatant. From the design, a

scouting protocol was created by the software with the following variables: 1) pH varied from 6.0 to 7.5; 2) gradient slope varied from 10 to 30 column volumes (CV); 3) weak or strong anion exchanger (i.e., Capto DEAE and Capto Q in HiScreen format, respectively). UV absorption was recorded at 280 nm. Flowthrough fractions were collected via the outlet valve and fractions during gradient elution were collected in 96 DeepWell™ plates (4.5 ml/fraction). Pooled fractions from DoE runs were analyzed by SDS PAGE.

**Table 1.** Run scheme for optimization of the capture step

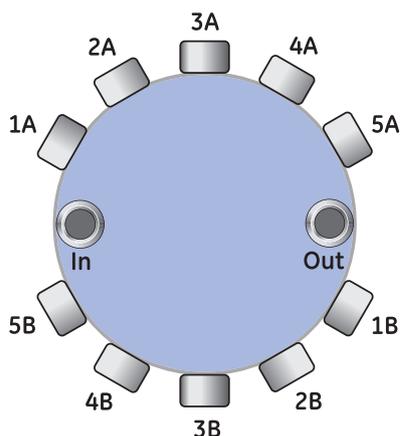
DoE run no.	HiScreen column type	Buffer pH	Length of elution gradient (CV)
1	Capto DEAE	6.0	10
2	Capto DEAE	7.5	10
3	Capto Q	6.75	20
4	Capto Q	6.75	20
5	Capto Q	6.0	10
6	Capto Q	7.5	10
7	Capto DEAE	7.5	30
8	Capto Q	6.0	30
9	Capto DEAE	6.0	30
10	Capto Q	6.75	20
11	Capto Q	7.5	30

Protein for media screening for the intermediate purification step was purified on HiScreen Capto DEAE. A volume of 25 ml of the clarified *E. coli* supernatant was adjusted to pH 7.5 and applied to a prepacked HiScreen Capto DEAE column. After washing out unbound material, elution was performed in a linear gradient (i.e., the same gradient slope as in DoE, run no. 7, Table 1). BufferPro was used for preparation of buffers for the equilibration and wash steps and to vary salt content in the elution gradient.

### Screening of HIC media for intermediate purification

HIC media screening<sup>1</sup> was performed using HiTrap HIC Selection Kit, which includes seven different HIC media (Table 2). To enable screening with seven different columns, an extra column valve (Fig 3) was connected to the system. Sample preparation was performed by pooling selected fractions from the capture step and adding an equal amount of 3 M ammonium sulfate. Prepared sample (2 ml) was loaded to each column via a capillary loop. The columns were then washed with 2 CV of binding buffer (20 mM Tris-HCl, 1.5 M ammonium sulfate, pH 7.5) followed by elution in a linear gradient of 10 column volumes, from binding buffer to 20 mM Tris-HCl pH 7.5. Fractions of 1 ml were collected in 96 DeepWell plates using the fraction collector. The temperature in the fraction collector was maintained at 20°C.

<sup>1</sup> As the purity after the HIC step was relatively high (i.e., 96% from Phenyl Sepharose High Performance) no further optimization of the intermediate step was performed.



**Fig 3.** The column valve is used to select the column that is being used. Up to five columns can be connected to one valve. In = inlet from injection valve via a built in pressure monitor; 1A–5A = ports for connection to tops of columns; 1B–5B ports for connection to bottoms of columns; Out = outlet to UV monitor via built-in pressure monitor.

**Table 2.** Scouting scheme for HIC media using the seven columns included HiTrap HIC Selection Kit

Run no.	Column position	HiTrap column
1	1	HiTrap Phenyl FF (high sub)
2	2	HiTrap Phenyl FF (low sub)
3	3	HiTrap Phenyl HP
4	4	HiTrap Butyl FF
5	5	HiTrap Butyl-S FF
6	6	HiTrap Butyl HP
7	7	HiTrap Octyl FF

### Three-step purification

After optimization of the capture step and screening HIC media for the intermediate step, the whole purification process, including cell preparation and clarification, capture, intermediate purification, and polishing was performed in a single day. For the capture step, 24 DeepWell plates were used, while 96 DeepWell plates were used for the intermediate and polishing steps.

#### Capture

Cell paste (53 g) was prepared and clarified according to above. A volume of 250 ml clarified supernatant was applied onto the Capto DEAE packed in an XK 26/20 column via the sample pump at a flow rate of 25 ml/min (i.e., maximum flow rate for the sample pump). After washing out unbound material, elution was performed in a linear gradient for 25 CV. BufferPro was used for preparation of equilibration and wash buffers and to vary salt concentration in the gradient. Twenty-one 10 ml fractions were pooled in 24 DeepWell plates giving a total volume of 210 ml<sup>2</sup>.

<sup>2</sup> To utilize the maximum flow rate of the sample pump, the capture step was scaled up to an XK 26/20 column. However, only 75 ml (36%) of the pooled material was further purified by hydrophobic interaction chromatography.

#### Intermediate purification

The pooled material from the capture step was adjusted with 20 mM Tris, 3 M ammonium sulfate, pH 7.5 to a concentration of 1 M ammonium sulfate<sup>3</sup>. A volume of 75 ml of this material was further purified on HiLoad 16/10 Phenyl Sepharose HP. Sample was loaded to the column via the sample pump at a flow rate of 2.5 ml/min. After washing out of unbound material with binding buffer (i.e., 20 mM Tris, 1 M ammonium sulfate, pH 7.5), elution was performed in a gradient from binding buffer to 20 mM Tris-HCl, pH 7.5. Thirteen 2 ml fractions were pooled giving a total volume of 26 ml.

<sup>3</sup> A linear gradient starting from 1 M ammonium sulfate produced a shallower gradient compared with the gradient used for the HIC-screening experiments, while the target protein was still adsorbed to the column.

#### Polishing

A HiLoad 16/60 Superdex 200 pg column was equilibrated with elution buffer (i.e., PBS buffer, pH 7.4). Sample (5 ml) from the HIC step was loaded onto the column via a capillary loop. Elution was performed for 1.2 CV at a flow rate of 0.87 ml/min. Nine 1 ml fractions were pooled giving a total volume of 9 ml. As the purity after the polishing step was close to 100%, no further optimization was performed.

### Analysis

#### Electrophoresis (SDS PAGE) and mass spectrometry

Samples were adjusted to pH 8.5 with 1 M NaOH. A working solution of a CyDye™, Cy™3, was prepared in dimethylformamide (5 nmol to 12.5 µl DMF). Cy3 solution (1 µl) was added to 50 µg of protein followed by incubation for 30 min in an ice bath in the dark. The reaction was stopped with 1 µl of 10 mM lysine. Samples were reduced and run on a Novex™ 4–20% Tris-Glycine gel at 70 V for approximately 4 h. The gels were scanned using Ettan™ DIGE Imager. This was performed at several different PMT settings due to the large difference in amount of target protein and contaminants. The same gels were then Coomassie™ stained and scanned with ImageScanner™ II.

Analysis of the identity by mass spectrometry (MS) was performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) and liquid chromatography-mass spectrometry (LC-MS).

## Results and discussion

### Method development

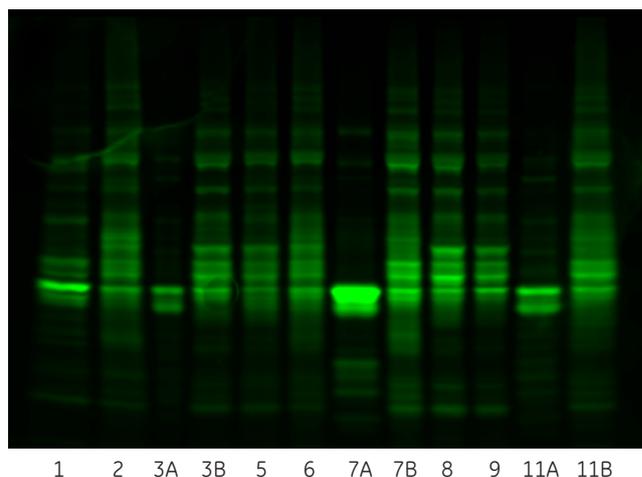
#### Capture step

DoE was used for optimization of the capture step. Chromatograms from the design are shown in Figure 4. Resolution of the protein peak/peaks increased at higher pH and shallower gradient. The protein peaks from the different runs were pooled, if possible in two separate pools, otherwise in one. Analysis was then performed by SDS-PAGE (Fig 5). All pooled samples contained one band corresponding to maltodextrin-binding protein ( $M_r$  41 000). Where two separate pools were collected, the highest purity was found in the first eluting peak. The highest purity of the target protein was obtained in DoE run 7, that is, on Capto DEAE at pH 7.5 and gradient slope of 30 CV.

#### Intermediate purification

Results from the HIC-media screening are shown in Figure 6. The different Fast Flow media resulted in broad elution peaks with relatively low resolution, while sharper peaks and higher resolution were obtained on both Phenyl Sepharose High Performance and Butyl Sepharose High

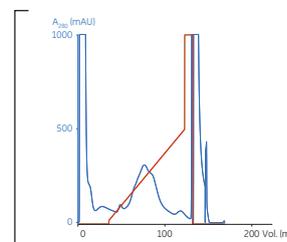
Performance. Phenyl Sepharose High Performance gave the best resolution and was selected for the three-step purification process.



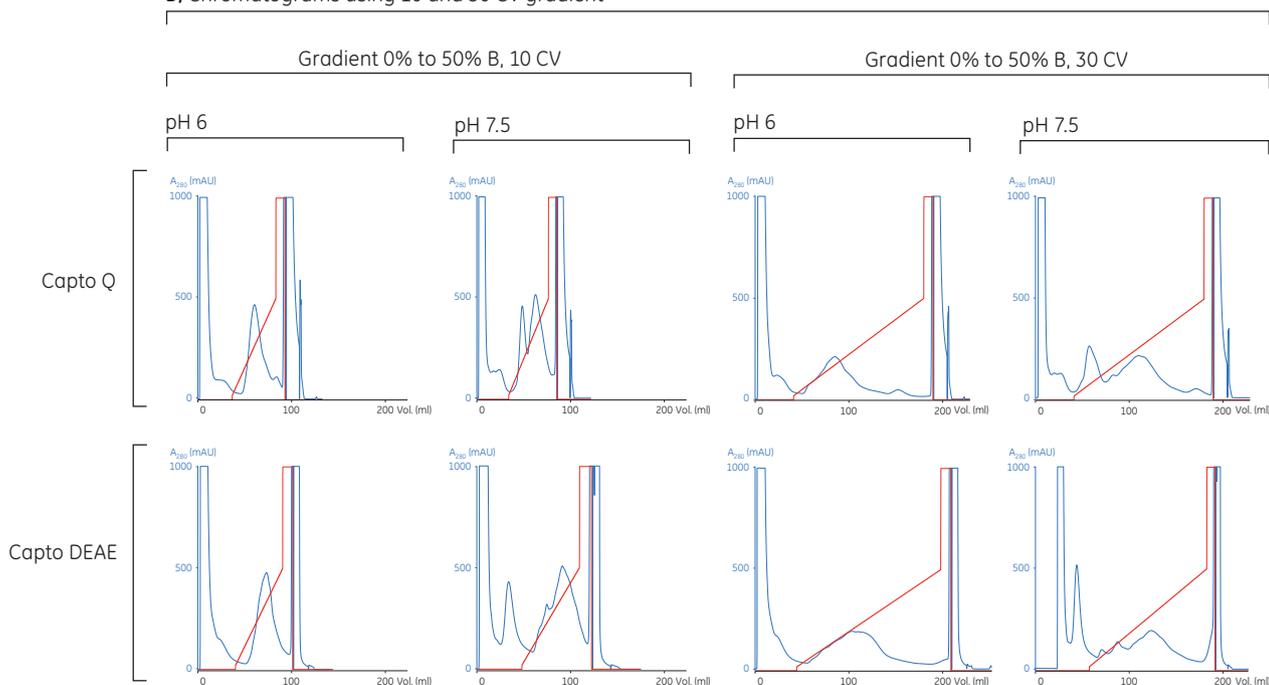
**Fig 5.** SDS-PAGE of pooled fractions collected in the optimization of the capture step showing DoE runs 1–11 according to Table 1. Lanes 3, 7, and 11 are from DoE runs where separation occurred in two peaks. DoE runs 4 and 10 were identical to run 3 (center points, data not shown).

Column: HiScreen Capto Q and HiScreen Capto DEAE  
Sample: 5 ml of clarified and pH-adjusted cell culture supernatant  
On-line buffer preparation: BufferPro on-line gradient formation using stock solutions: 0.15 M Tris, 0.15 M bis-Tris (inlet Q1); 0.2 M HCl (inlet Q2); 4 M NaCl (inlet Q3); and ultrapure water (inlet Q4) pH varied from 6.0 to 7.5  
Gradient: 0% to 50% B in 10 to 30 CV  
Flow rate: 4 ml/min  
System: ÄKTA avant 25

**A)** Chromatogram at pH 6.75 and gradient 20 CV, Capto Q (center point in the design)

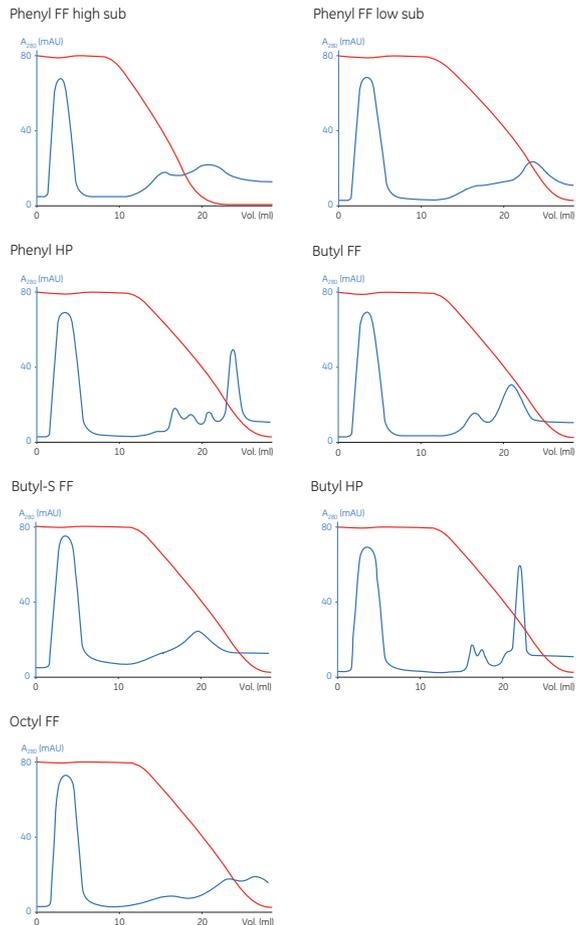


**B)** Chromatograms using 10 and 30 CV gradient



**Fig 4.** Optimization of the capture step using HiScreen Capto Q and HiScreen Capto DEAE.

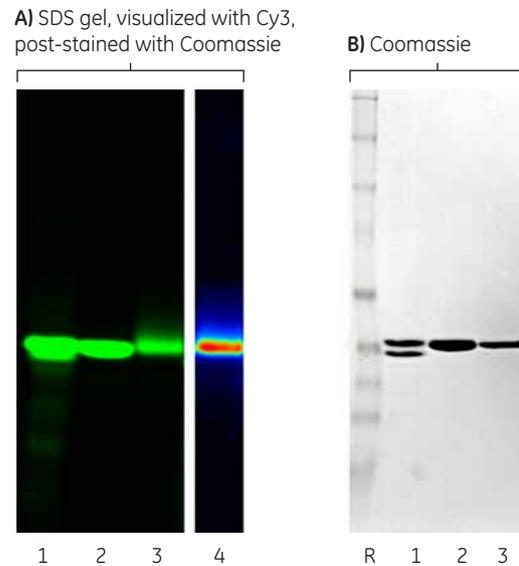
Columns: HiTrap HIC Selection Kit  
 Sample: 2 ml pool from the capture step was applied via a sample loop  
 Buffer A: 20 mM Tris-HCl, 1.5 M ammonium sulfate, pH 7.5  
 Buffer B: 20 mM Tris-HCl, pH 7.5  
 Gradient: 0% to 100% B in 10 CV  
 Flow rate: 1 ml/min  
 System: ÄKTA avant 25



**Fig 6.** Screening for HIC media using HiTrap HIC Selection Kit.

### Three-step purification

The whole purification process, including cell preparation and clarification, capture, intermediate purification, and polishing was performed in a single day. The purification is summarized in Table 3. Chromatograms from the three purification steps are shown in Figure 7 and results from electrophoresis in Figure 8.



**Fig 8.** SDS-PAGE of eluted pools from the capture, intermediate purification, and polishing steps of the purification of maltodextrin-binding protein. A) Visualization with Cy3 and post-stained with Coomassie. Lane 1: Pool 1 from Capto DEAE; lane 2: Pool from HiLoad 16/10 Phenyl Sepharose HP; Lane 3: Pool from HiLoad 16/60 Superdex 200 pg; lane 4: Pool from HiLoad Superdex 200 pg (enhanced image). B) Coomassie stained gel. R: Rainbow™ molecular weight markers; lane 1: Pool 1 from Capto DEAE; lane 2: Pool from HiLoad 16/10 Phenyl Sepharose HP; lane 3: Pool from HiLoad 16/60 Superdex 200 pg with Coomassie only.

#### A) Capture on Capto DEAE packed in XK 26/20 (bed height 10.6 cm, bed volume 52.6 ml)

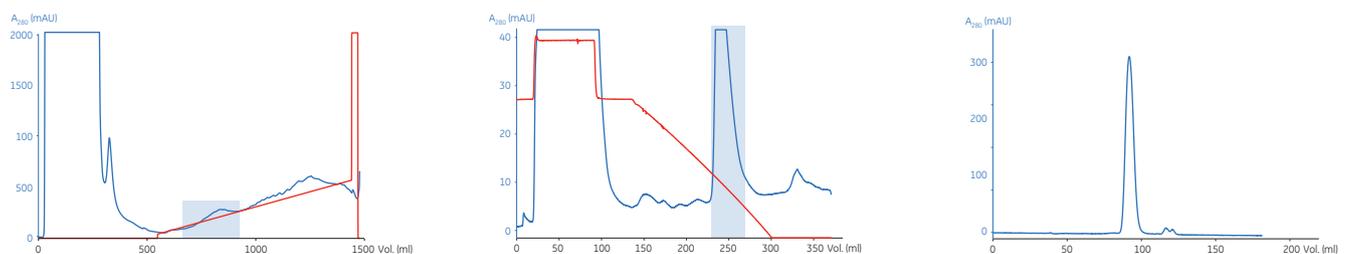
Sample: 250 ml of clarified and pH-adjusted *E. coli* supernatant  
 Buffer: BufferPro, pH 7.5 (AIEX mix consisting of 0.15 M Tris, 0.15 M bis-Tris; inlet Q1), 0.2 M HCl (inlet Q2), 4 M NaCl (inlet Q3), and ultrapure water (inlet Q4).  
 Gradient: 0% to 40% B in 25 CV  
 Flow rate: 25 ml/min  
 System: ÄKTA avant 25

#### B) Intermediate purification on HiLoad 16/10 Phenyl Sepharose HP

Sample: 75 ml of pooled material from the capture step. Ammonium sulfate concentration adjusted to 1 M  
 Buffer A: 0.2 M Tris, 1 M ammonium sulfate, pH 7.5  
 Buffer B: 0.2 M Tris-HCl, pH 7.5  
 Gradient: 0% to 100% B in 10 CV  
 Flow rate: 2.5 ml/min  
 System: ÄKTA avant 25

#### C) Polishing on HiLoad 16/60 Superdex 200 pg

Sample: 5 ml pooled fractions  
 Flow rate: 0.87 ml/min  
 System: ÄKTA avant 25



**Fig 7.** Three-step, optimized purification method used for purification of native maltodextrin-binding protein from *E. coli*. A) Capture step, B) Intermediate purification and C) polishing. The blue-shaded areas show fractions collected from the capture and intermediate steps.

**Table 3.** Summary of sample volumes, volumes of collected elution pools, and purity of maltodextrin-binding protein obtained from a three-step purification

Medium	Sample applied (ml)	Eluted pool volume (ml)	Purity <sup>a</sup> (%)
Capto DEAE	250	210	55
Phenyl Sepharose High Performance	50	26	96
Superdex 200 prep grade	5	9	~100

<sup>a</sup> Purity, i.e. percent of peak area determined from gel filtration and/or electrophoresis  
As no quantitative assay for maltodextrin-binding protein was available, mass balance could not be calculated

The capture step on Capto DEAE was very efficient. However one main and some minor contaminants were still present resulting in a purity of ~ 55%. Most of these contaminants could be removed in the intermediate step on Phenyl Sepharose High Performance, resulting in a purity of 96%. Finally, by gel filtration on Superdex 200 prep grade, the remaining impurities could be removed, resulting in a purity of close to 100%. The identity of the maltodextrin-binding protein was confirmed by MS and MS/MS. The molecular mass ( $M_r$ ) of the intact maltodextrin-binding protein was confirmed by MALDI-ToF MS as  $M_r$  40 802.

## Conclusions

ÅKTA avant 25 and UNICORN 6 were used for fast method development for the three-step purification of maltodextrin-binding protein. DoE enabled fast optimization of the capture step. On-line buffer preparation was used to vary pH and salt concentration during equilibration, load, wash, and elution. HIC media screening for the intermediate purification step was performed with an extra column selection valve connected to the system. Finally, polishing was performed by gel filtration.

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After optimization of the capture step and screening HIC-media for the intermediate step, the whole purification process was performed in a single day. Final purification resulted in a purity of the target protein close to 100%.

## References

1. Quijoch, F. A and Ledvina, P. S. Atomic structure and specificity of bacterial periplasmic receptors for active transport and chemotaxis: variation of common themes. *Mol. Microbiol.*, **20** (1), 17–25 (1996).

## Ordering information

Product	Code no.
ÅKTA avant 25 chromatography system	28-9308-42
UNICORN 6 local and remote workstation license with DVD	28-9589-93
UNICORN 6 local and remote workstation license without DVD (download only)	28-9589-95
HiScreen Capto Q, 1 × 4.7 ml	28-9269-78
HiScreen Capto DEAE, 1 × 4.7 ml	28-9269-82
XK 26/20 column	18-1000-72
HiTrap HIC Selection Kit, 7 × 1 ml	28-4110-07
HiTrap Phenyl FF (low sub), 5 × 1 ml	17-1353-01
HiTrap Phenyl FF (high sub), 5 × 1 ml	17-1355-01
HiTrap Phenyl HP, 5 × 1 ml	17-1351-01
HiTrap Butyl FF, 5 × 1 ml	17-1357-01
HiTrap Butyl-S FF, 5 × 1 ml	17-0978-13
HiTrap Butyl HP, 5 × 1 ml	28-4110-01
HiTrap Octyl FF, 5 × 1 ml	17-1359-01
HiLoad 16/10 Phenyl Sepharose HP, 1 × 20 ml	17-1085-01
HiLoad 16/60 Superdex 200 pg, 1 × 120 ml	17-1069-01
Full-Range Rainbow Molecular Weight Markers, 250 µl	RPN800E

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