High-productivity capture of green fluorescent protein on Capto Q

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Abstract

This application note describes the capture of green fluorescent protein (GFP), recovered from homogenized and clarified *Escherichia coli,* using packed bed chromatography on the newly developed strong anion exchanger Capto™ Q. This product is designed to increase speed and throughput in capture and intermediate purification, in order to increase productivity and reduce process cycle times.

Results from screening, process optimization and scale-up are presented. Additional scale-up modeling and productivity calculations based on these experiments indicate that it could be possible to capture and recover more than 100 kg of GFP per 24 h, using Capto Q in a 1.6 m i.d. column at 20 cm bed height. Assuming the same process cycle conditions, the corresponding amount for Q Sepharose™ Fast Flow is 30 kg. The results thus show that Capto Q is well suited for high productivity capture purification.

Introduction

Recent developments in upstream processing have resulted in increased protein expression levels and larger feed volumes. This leads to a need to process larger volumes with higher expression levels in a fast and efficient way, and requires capture media with properties supporting both high flow velocity and high dynamic binding capacity.

Capto Q, a new strong anion exchanger from GE Healthcare, is designed to meet the demands of large-scale manufacturers for fast, efficient, and cost-effective capture and intermediate purification. It is based on a newly developed matrix of highly rigid, chemically modified agarose. Capto Q offers outstanding pressure/flow properties combined with an open pore structure that allows fast mass transfer and gives high dynamic binding capacity.

Planning:

- Properties of starting material

  pI of target protein = 6.2 ⇨ purification on anion exchanger starting at pI + 2 = pH 8.2

  Expression level of target protein in starting material: ~2.5 mg GFP/ml

  Stability of target protein ⇨ high pH could compromise product integrity (e. g. de-amidations)

- Final scale

  Purification of 100 kg target protein in 24 h ⇨ high volume throughput required

  Buffers and cleaning solutions must be compatible with large scale processing

Screening:

- Screening of selectivity on different anion exchangers with pre-packed HiTrap columns
- Screening of working pH on Tricorn 5/100
- Determination of dynamic binding capacity on Tricorn 5/100

Process optimization:

- Transfer of linear salt elution to step elution on Tricorn 5/100
- Optimization of equilibration/re-equilibration/wash volumes on Tricorn 5/100

Scale-up:

- 400 times scale-up from Tricorn 5/100 to FineLINE 70

  residence time was kept constant, bed height and flow velocity was increased

  the scaled-up purification process was verified by comparison of elution profiles, and the measured purification factor, elution volume, and yield between the scales

- Productivity calculations to verify that 100 kg target protein can be recovered in 24 h

Figure 1. Experimental outline

The goal of the work reported was to demonstrate that Capto Q gives high productivity, specified as at least 100 kg of target protein in 24 h, in a capture situation that is representative for large scale protein manufacturing. Recombinant green fluorescent protein (GFP) expressed

Capto™

in *E. coli*, was used as model protein. After initial selectivity screening of several anion exchangers, the capture step was developed, optimized and taken to pilot scale in a FineLINE™ 70 column. This was followed by scale-up calculations to show that the developed capture step would allow production of at least 100 kg of target protein in 24 h.

Materials and Methods

Unless otherwise stated, all equipment and chromatographic media were obtained from GE Healthcare (Uppsala, Sweden) and the chemicals used were of analytical grade.

Experimental procedure

The overall approach for process development and scale-up is outlined in Figure 1.

Model system

Recombinant GFP expressed in *E. coli* has a molecular weight of 28 000 and an isoelectric point (pI) of 6.2. GFP absorbs specifically at 490 nm, and thus can be easily assayed for throughout the purification process. In nature, GFP is found in the pacific jellyfish, Aequorea Victoria.

Preparation of starting material

E. coli cell paste containing GFP was either homogenized with high-pressure homogenization or with sonication, depending on the starting volume. The homogenate was centrifuged in 35 000 g for 40 minutes. Due to high conductivity of the GFP homogenate, the supernatant was diluted with purified, de-ionized water until the conductivity was below 5 mS/cm. The pH was adjusted with NaOH and the sample was filtered through a 0.45 µm filter before loading onto the columns. Total protein content varied between 50 and 70 mg protein/ml, and the amount of GFP was approximately 2.5 mg/ml.

GFP activity and recoveries

GFP has an absorbance maximum at 490 nm, therefore GFP was assayed by following the absorbance track at 490 nm in an ÄKTAexplorer™ 100 system. Total protein content in the starting material and eluate was determined (1) and used to calculate recoveries.

Screening

Selectivity

Prepacked 1 ml HiTrap™ columns with different anion exchange media (Capto Q, Q Sepharose Fast Flow, Q Sepharose XL, ANX Sepharose 4 Fast Flow (high sub) and DEAE Sepharose Fast Flow) were screened at pH 8.2.

Working pH

Purification of GFP on Capto Q was run at three different pH values, 7.0, 7.6, and 8.2.

E. coli homogenate is a crude starting material containing numerous cellular components e.g. protein, lipid, and nucleic acid types, many of which are likely to bind strongly at high pH. Moreover, higher pH increases the risk for de-amidation, which could jeopardise product integrity. Thus, pH values higher than 8.2 were not investigated.

Determination of dynamic binding capacity

The dynamic binding capacities for Capto Q and Q Sepharose Fast Flow were evaluated at 10% breakthrough ($Q_{B10\%}$) at residence times between 1 and 6 minutes. GFP-sample was run bypass in the ÄKTAexplorer 100 system and A_{490} maximum was noted. Starting material was loaded onto the columns until the flow through-absorbance was at least 10% of the maximum.

Process optimization

The proteins were eluted by increasing the conductivity of the elution buffer. An initial salt gradient was transferred to a three-stage step elution.

To save time and minimize buffer consumption, the process equilibration/re-equilibration/wash volumes were optimized without sacrificing purity and yield.

Scale-up

When the purification process had been optimized the method was verified at two scales with Tricorn™ 5/100 (column volume (CV) 2 ml) and FineLINE 70 (CV 808 ml) to give a 400-fold scale-up factor. Sample volumes loaded to the columns corresponded to 15 mg GFP/ml medium, and a total of 30 mg and 12 g was loaded onto the Tricorn and FineLINE columns, respectively. During scale-up, the residence time was kept constant while bed height and flow velocity was increased. The residence time is equal to the bed height (cm) divided by the flow velocity (cm/h) applied during sample load.

Results and Discussion

Screening

Selectivity

The HiTrap screening was performed to determine the general selectivity for five anion exchangers. Evaluation of elution profiles and absorbance curves at 280 and 490 nm (Fig 2) reveals differences in selectivity between the media. For Q Sepharose Fast Flow, Q Sepharose XL and ANX Sepharose 4 Fast Flow (high sub), the GFP peak was eluted in a peak containing several other proteins, while for Capto Q and DEAE Sepharose Fast Flow, the target protein seemed more separated from contaminants. In addition, for Capto Q several contaminants were eluted before the target protein, which probably assists the purification of GFP. Thus, Capto Q gave the best elution profile of the five media included in the screening.

Column: HiTrap, 1 ml
Sample: GFP in *E. coli* homogenate
Buffer A: 50 mM Tris/HCl, pH 8.2
Buffer B: 50 mM Tris/HCl, pH 8.2 + 1 M NaCl
Flow: 1 ml/min (156 cm/h)
Gradient: 0 – 100% B, 15 column volumes
System: ÄKTAexplorer 100

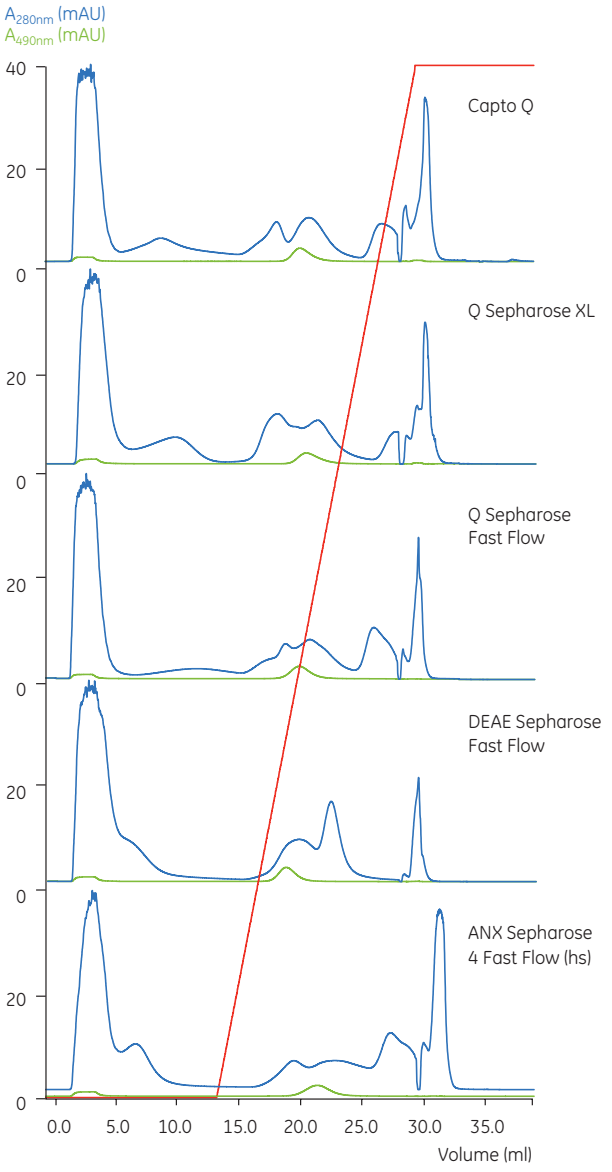

Figure 2. The selectivity and separation ability for GFP, expressed in *E. coli*, on the different media were compared. Based on the combination of the UV curves 280 and 490 Capto Q gave optimal selectivity.

Working pH

The results of the pH screening are shown in Figure 3. At pH 7.0 the absorbance peak from 490 nm was lacking in the eluate which indicates that GFP was not bound to Capto Q at that pH. Chromatograms for pH 7.6 and 8.2 were quite similar, but the elution peak was slightly broader at 7.6. Based on the 280 nm curve it seemed like more contaminants were separated from the GFP eluate at the higher pH. Thus pH 8.2 was established as working pH and used in subsequent experiments.

Column: Tricorn 5/100, 10 cm bed height
Medium: Capto Q
Sample: GFP in *E. coli* homogenate
Buffer A: 50 mM sodium phosphate pH 7.0;
50 mM Tris/HCl, pH 7.6;
50 mM Tris/HCl, pH 8.2
Buffer B: A + 1 M NaCl
Flow: 600 cm/h
Gradient: 0 – 100% B, 15 column volumes
System: ÄKTAexplorer 100

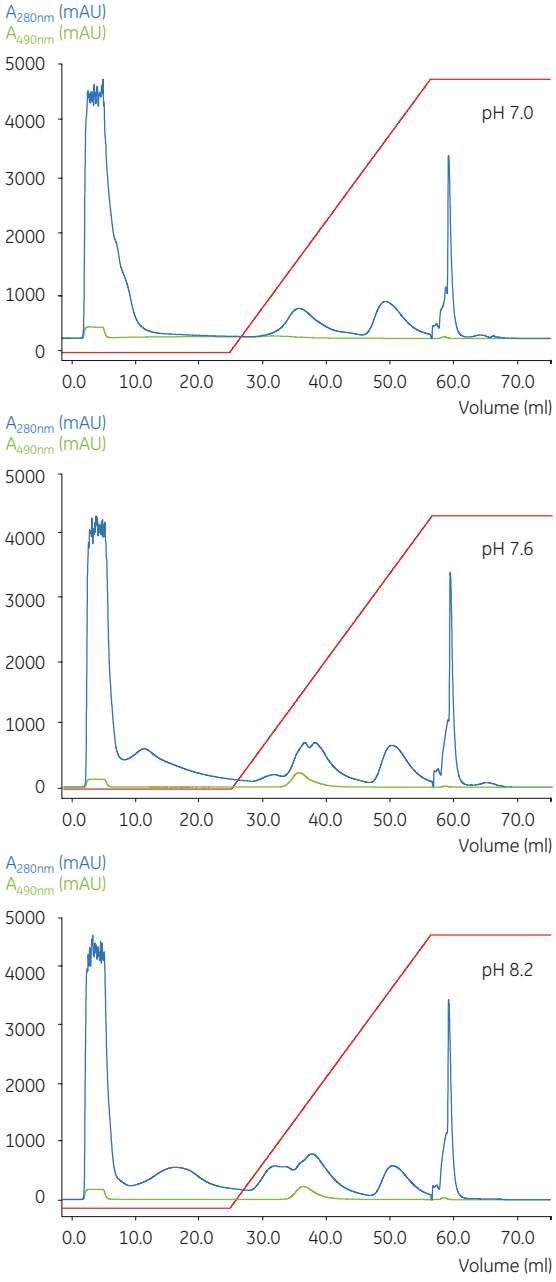

Figure 3. The result from the pH optimization on Capto Q

Dynamic binding capacities

The dynamic binding capacities for Capto Q and Q Sepharose Fast Flow are shown in Table 1.

Table 1. Dynamic binding capacities at different residence times for the media investigated

Column	Residence time (min)*	$Q_{B10\%}$ (mg GFP/ml medium)
Capto Q	1	16
Capto Q	3	28
Capto Q	4	31
Capto Q	6	34
Q Sepharose Fast Flow	1	10
Q Sepharose Fast Flow	3	15
Q Sepharose Fast Flow	6	16

* residence time equals bed height divided by flow velocity during sample load

Process optimization

Based on the results from the initial gradient elution, a step elution protocol consisting of three steps was established. To save time and minimize buffer consumption, the process equilibration/re-equilibration/wash volumes were optimized.

The optimized method is described in Table 2. The first elution step also functioned as a wash step for components that elute prior to GFP. The GFP target protein was collected in step 2, and step 3 was included before cleaning in place (CIP) to avoid precipitation of highly charged species in the column. The whole process time could be shortened considerably by excluding the wash step. In addition, the optimization also resulted in a shortening of the equilibration and re-equilibration steps.

Table 2. The optimized steps that were used for scale-up

Step	Volume (CV)	Residence time (min)	Step time (min)
Equlibration	6	2	12
Loading	*	*	*
Step 1 (0.1 M NaCl)	6	2	12
Step 2 (0.33 M NaCl)	6	2	12
Step 3 (1 M NaCl)	4	2	8
Cleaning	5	6	30
Re-equilibration	3	2	6
Total	30 + loading		80 + loading

* Loading volume and residence time were varied

Scale-up

Figure 4 shows the chromatograms from Tricorn 5/100 and FineLINE 70 runs using the optimized method described in Table 2. Scale-up was verified by comparison of elution profiles, and the measured purification factor, elution volume and yield that were found to be similar between the scales. Data are summarized in Table 3.

Table 3. Purification factor, elution volume and yield from Tricorn 5/100 and FineLINE70 runs

Run	Purification factor	Elution volume (CV)	Yield (%)
Tricorn	5.8	4	94 ± 3
FineLINE	4.9	2.6	93 ± 3

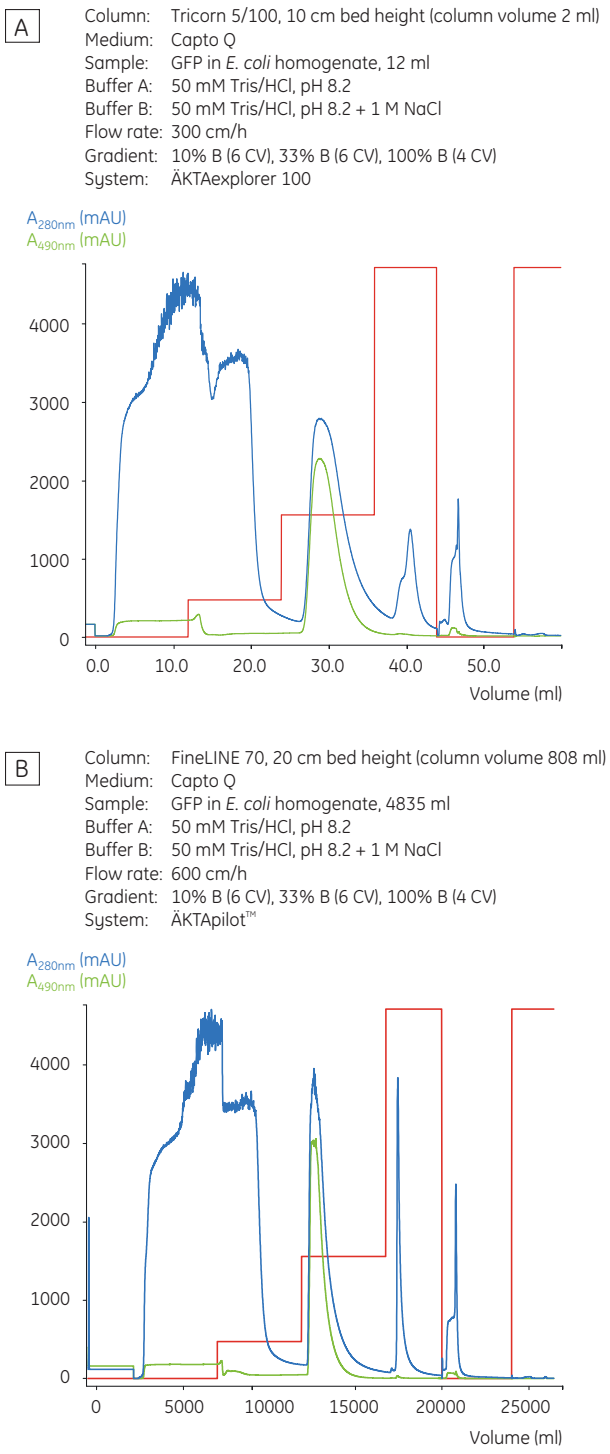

Figure 4. 400 times scale-up. Comparison of GFP capture on two scales; A. Tricorn 5/100 and B. FineLINE 70. Conditions as described in Table 2; note that the residence time is identical at the two scales.

Table 4. Optimized process for GFP on Capto Q and Q Sepharose Fast Flow

	Capto Q			Q Sepharose Fast Flow		
Step	Volume (CV)	Residence time (min)	Step time (min)	Volume (CV)	Residence time (min)	Step time (min)
Equilibration	6	2	12	6	6	36
Loading	3.1	3.6	11.3	1.7	6	10.4
Step 1 (0.1 M NaCl)	6	2	12	6	6	36
Step 2 (0.33 M NaCl)	6	2	12	6	6	36
Step 3 (1 M NaCl)	4	2	8	4	6	24
Cleaning	5	6	30	5	6	30
Re-equilibration	3	2	6	3	6	18
Total	33.1		91.3	31.7		190.4

Conclusion on experimental data

A capture step was developed that gave high yield of target protein as well as removal of bulk impurities in less than two h process time. Recoveries were above 90% at all scales, which is important when working with high value products. The purification factor was approximately 5, indicating that the majority of contaminants had been removed. Not only were the recoveries high but the product eluted in a small volume (2.6 column volumes in FineLINE 70) which is important for the following intermediate chromatography purification step. Finally, a short overall process time means improved product integrity by reducing exposure time to proteases and other contaminants detrimental to product quality and yield. Altogether the results show that Capto Q is well suited for capture purification.

Scale-up modeling and productivity calculations

A theoretical scale-up of Capto Q and Q Sepharose Fast Flow was performed using the experimental data.

Assumptions

The residence time was kept constant at the different scales. The bed height was fixed to 20 cm, and only loading velocity was optimized. All other steps were conducted at 600 cm/h (Capto Q) or 200 cm/h (Q Sepharose Fast Flow) except for the cleaning step, where the total time was maintained at 30 minutes. This gave estimated cycle times of 180 minutes for Q Sepharose Fast Flow and 80 minutes for Capto Q (Table 4), exclusive of the loading step.

Column size was not defined but the smallest size that allows productivity of 100 kg of target protein in 24 h was determined from the results of the calculations.

Measured dynamic capacities (Table 1) were assumed to be independent of scale.

The loading volume was calculated from 70% of the dynamic binding capacity at 10% breakthrough for both media. Yield was assumed to be a constant 93.5%, regardless of conditions.

It was also assumed that the column and the packing could withstand the flow velocities without prohibitively large pressure drops. Often, the feed is the most viscous liquid, so a lower feed velocity may have to be considered in future scale-up runs. This would certainly affect Q Sepharose Fast Flow but have a marginal affect on Capto Q. This is an effect of the high rigidity of the new base matrix, which makes it possible to use high flow velocities also when viscous samples are applied.

Productivity calculations

The results of productivity calculations for varying the loading velocity and keeping the other steps constant are shown in Figure 5. Maximal productivity for Capto Q and Q Sepharose Fast Flow are achieved at residence times of 3.6 and 6 minutes, respectively. For Sepharose Fast Flow based media, 6 minutes corresponds to the shortest possible residence time based on the flow restrictions of the media. The optimized large-scale processes are shown in Table 4, and the resulting amount of protein, assuming a 1.6 i.d. column with 20 cm bed height is used, is summarized in Table 5.

Table 5. Summary productivity calculations expressed as kg produced during 24 h in constant cyclic operation

Stationary phase	Productivity (kg/24h)	Loading residence time (min)
Capto Q	103	3.6
Q Sepharose Fast Flow	30	6

In summary, scale-up modeling and productivity calculations based on the experiments described above indicate that it could be possible to capture and recover 103 kg of GFP per 24 h, using Capto Q in a 1.6 m i.d. column at 20 cm bed height. Assuming the same process cycle conditions, the corresponding amount for Q Sepharose Fast Flow is 30 kg.

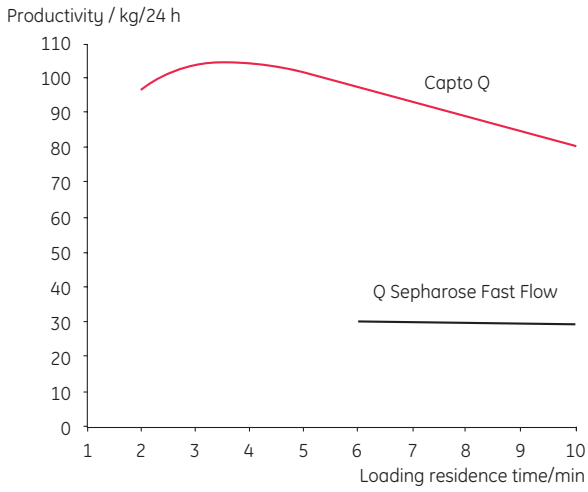

Figure 5. Comparison of calculated productivity on Capto Q and Q Sepharose Fast Flow

Overall conclusions

The work reported here shows that Capto Q gives high productivity in a capture situation that is representative for large scale protein manufacturing. The combination of high volume throughput and high capacity makes Capto Q the optimal choice for processing large amount of protein in a fast and efficient way, with unit operations of reasonable size. Data on yield, purification factor and elution volume also show that Capto Q is well suited for capture purification.

References

1. Wiedenmann J. et al. Identification of GFP-like Proteins in Nonbioluminescent, Azooxanthellate Anthozoa Opens New Perspectives for Bioprospecting. *Marine Biotechnology* **6**, 270-277 (2004).

Product Information

Contact your local GE Healthcare representative for further information about any of the products used in this application

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