High-productivity capture of α-chymotrypsin on Capto S cation exchanger

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Abstract

This application note describes the capture of α-chymotrypsin from clarified homogenate of *Escherichia coli* using packed bed chromatography with the recently developed strong cation exchanger Capto™ S. This medium has the same physical characteristics as Capto Q, i.e. high capacity and high rigidity, which makes it an excellent choice for high-throughput, and productivity processing in capture and intermediate purification.

Capto S, SP Sepharose™ Fast Flow and SP Sepharose XL were screened for optimal selectivity and recovery conditions prior to further process optimization. The Capto S process was then scaled up 200-fold to an AxiChrom™ 50 column. Productivity and cost calculations show that productivity is 200% higher using Capto S compared with SP Sepharose Fast Flow, and 160% higher compared with SP Sepharose XL. Capto S should allow the capture and recovery of more than 100 kg of target protein in 24 h in a 0.8-m inner diameter (i.d.) column at 20-cm bed height, thus supporting its suitability for high-productivity capture in downstream purification processes.

Introduction

Increasing expression levels of recombinant proteins combined with the need to process larger feed volumes have challenged the concept of agarose-based chromatography media. The Capto family of ion exchange media has been designed to meet such demands. This range comprises the previously introduced Capto Q and Capto MMC plus the more recent strong cation exchanger Capto S. All three media combine the benefits of agarose, i.e. hydrophilicity, low non-specific adsorption and an open pore structure, with a new technology platform that gives high fluid velocities and increased capacity. Capto S thus displays outstanding pressure/flow properties, fast and efficient mass transfer as well as high capacity. These features make it ideal for high-productivity capture and intermediate purification at large scale.

This application demonstrates how Capto S can achieve high-productivity capture in large-scale protein manufacturing. The enzyme α-chymotrypsin spiked into a homogenate of *E. coli* strain BL21(DE3) was used as "real feed". Initial screening at three different pH values was performed on Capto S, SP Sepharose Fast Flow and SP Sepharose XL packed in HiTrap™ columns. Optimizing loading conditions with respect to pH and conductivity is important to best utilize the high capacity of Capto S. This was therefore done prior to a residence time study, which was then performed under the optimal conditions found for each medium. The final purification step was further developed before scaling up, first 20-fold and finally 200-fold to pilot scale in an AxiChrom 50 column. These data were used for scale-up modeling and productivity calculations.

Materials and methods

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

Capto

Model system

The "real feed" consisted of the proteolytic enzyme α-chymotrypsin from bovine pancreas spiked into a clarified homogenate of *E. coli* strain BL21(DE3). This enzyme has a molecular weight of approximately 25 000 and its isoelectric point is 8.7.

Preparation of starting material

E. coli cell paste was either homogenized with a high-pressure homogenizer or by sonication. The latter was used for small-scale homogenization (i.e. less than 100 ml). The homogenate was centrifuged at 25 800 × g for 30 min. The supernatant was filtered with successively smaller pore sizes, the smallest being 0.45 µm. After filtration, α-chymotrypsin was added to 4 mg/ml (approx. 20% of the total protein concentration).

Enzymatic assay

The synthetic peptide Suc-Ala-Ala-Pro-Phe-pNA, is cleaved by α-chymotrypsin at the C-terminal of Phe (1). One of the products, p-Nitroaniline, absorbs at 406 nm. The increase in this absorbance versus time is used to quantify the amount of enzyme in a specific fraction. The accuracy of the assay is approximately ±10%.

Screening

Selectivity screening

HiTrap 1 ml columns prepacked with the three cation exchangers were used to screen for selectivity and recovery at pH 4.8, 5.5 and 6.2.

Screening for loading conditions

Three different pH values and various conductivities were studied with real feed and pure α-chymotrypsin to determine optimal loading conditions regarding conductivity and pH. Tricorn™ 5/100 columns with a column volume (CV) of 2 ml were used.

Determination of dynamic binding capacity

Dynamic binding capacity (DBC) at 10% breakthrough ($QB_{10\%}$) was determined for Capto S, SP Sepharose Fast Flow and SP Sepharose XL with 4 mg/ml α-chymotrypsin spiked into *E. coli* homogenate at residence times of 1.0, 2.5 and 6.0 min in Tricorn 5/100 columns. Loading residence time is defined as packed bed height divided by linear fluid velocity.

Enzymatic activity in the starting material was determined and set to 100%. An excess of sample was applied to the column. The activity in the fractions during sample loading was determined and used to monitor the breakthrough curve.

Process optimization

Elution was achieved by increasing conductivity. An initial linear gradient of 0-1 M NaCl was transformed to a step elution procedure using 1 M NaCl (see Table 2). This method was optimized to minimize equilibration and wash volumes to save time and minimize buffer consumption without losing recovery or purity.

Scale up

Using the optimized process, the method was verified at three scales for Capto S, all at a constant residence time of 2 min (2). From Tricorn 5/100 (CV 2 ml) to XK 16/40 column, (CV 40 ml) both the fluid velocity and bed height (10 cm to 20 cm) were doubled, thus keeping a constant residence time. Scale up from the XK 16/40 column to AxiChrom 50 (CV 400 ml) was performed by increasing bed diameter and volumetric flow rate. This gave a final scale-up factor of 200. The sample applied at all scales corresponded to 60% of $QB_{10\%}$, i.e. 103 mg α-chymotrypsin/ml medium.

Productivity and cost calculations

The purification cycle specified in Table 2 was used. Equilibration, wash and elution can be carried out at maximum allowed operating velocity. In this study, the operating velocity was 600 cm/h for Capto S and 200 cm/h for SP Sepharose Fast Flow and SP Sepharose XL. Loading velocity corresponded to 2 and 6 minutes residence time, respectively, in a 20 cm bed. Purification yield was assumed to be 95% for all media.

Table 1 states the factors assumed for calculating separation costs. Cost of liquids (including raw materials, preparation and disposal) was estimated at USD 0.5/l. The labor cost is estimated by the equivalent cost of one operator for the same time as the process time. The capital cost assumes a total of USD 500 000 investment for a complete system, including installation of a 1-m i.d. column. The investment for columns scales proportionally to the column cross-sectional area. Capital cost per batch is calculated assuming 10% yearly depreciation and 10% interest and maintenance rate (based on the invested amount) split over 25 batches a year.

Table 1. Factors used to calculate separation costs

Cost factor	Capto S	SP Sepharose Fast Flow	SP Sepharose XL	Unit
Medium cost	1450	700	1250	USD/L
Medium lifetime	100	100	100	Cycles
Buffer cost	0.5	0.5	0.5	USD/L
CIP solution cost	0.5	0.5	0.5	USD/L
Capital cost	4000*	4000*	4000*	USD/batch
Labor cost	100	100	100	USD/h

* One-meter i.d. column.

Results and discussion

Screening

Selectivity screening

Screening on HiTrap columns revealed the pH that gave best recoveries, as well as information about the general selectivity for this sample. Capto S, SP Sepharose Fast Flow and SP Sepharose XL all showed recoveries around 100% at pH 4.8. The selectivities for this particular sample were very similar; all had a small shoulder of contaminants before the major peak containing the enzymatic activity (Fig. 1 A-C). However, when increasing sample load, the small shoulder was not resolved (data not shown). Only minor differences in selectivity were observed between pH 4.8, 5.5 and 6.2.

Screening for loading conditions

A reduction in dynamic binding capacity with conductivity was observed for pure α-chymotrypsin for all three media (Fig 2). However, Capto S may behave differently with other proteins. For example, increasing the conductivity can significantly improve capacity (3). Such behavior makes it important to optimize pH and conductivity to get the best performance from Capto S.

The effect of pH and conductivity on the dynamic binding capacity at three different pH values and various conductivities was investigated using real feed for Capto S (Fig 3). This indicated that while the three pH values gave similar capacities for the low conductivity range using pure enzyme, a significant decrease in capacity was observed for "real feed" at increasing pH. For SP Sepharose Fast Flow and SP Sepharose XL, the capacities at optimal loading conditions (Fig 2B-C) were verified in with real feed. The conductivity in real feed was typically around 5 mS/cm, but since the results for pure α-chymotrypsin (Fig. 2) did not indicate any significant improvement in capacity upon decreasing the conductivity further, dilution of the feed was not considered. None of the three media showed any significant change in capacity when comparing pure α-chymotrypsin with real feed at a constant concentration of the enzyme (Fig 4).

Column:	HiTrap 1 ml (CV=1 ml)
Media:	Capto S, SP Sepharose Fast Flow, SP Sepharose XL
Sample:	10 mg α-chymotrypsin in *E. coli* homogenate, sample volume 4 ml
Equilibration buffer:	50 mM sodium acetate, pH 4.8
Elution buffer:	50 mM sodium acetate, pH 4.8 + 1.0 M NaCl
Fluid velocity:	156 cm/h
Gradient:	Gradient 0-100% (10 CV), 100% (5 CV)
System:	ÄKTAexplorer™ 100 with UNICORN™ software version 5.01

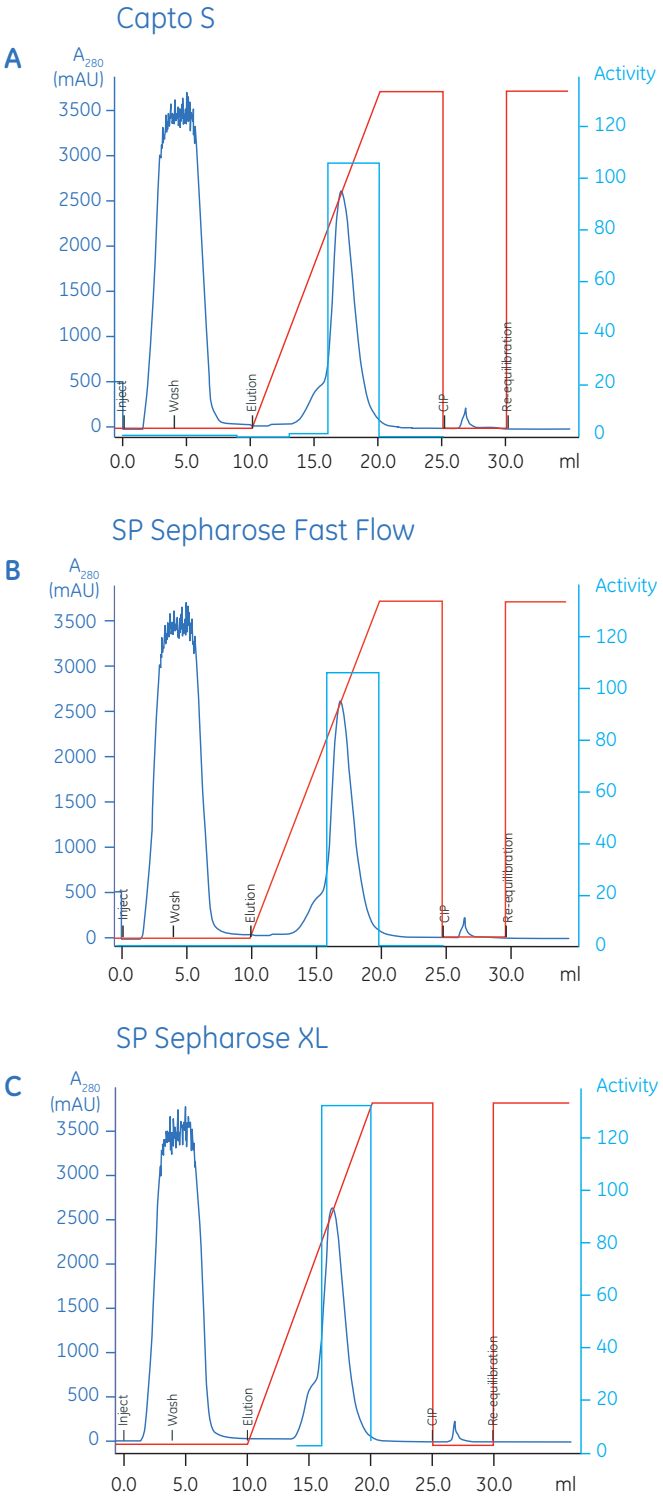

Fig 1. Screening in HiTrap 1 ml columns at pH 4.8 revealed similar selectivities for all three media; a small shoulder of contaminants just before the main peak of activity. **(A)** Capto S; **(B)** SP Sepharose Fast Flow; **(C)** SP Sepharose XL.

Column:	Tricorn 5/100 (CV=2 ml)
Media:	Capto S, SP Sepharose Fast Flow, SP Sepharose XL
Sample:	4 mg/ml α-chymotrypsin in equilibration buffer
Equilibration buffer:	50 mM sodium acetate, pH 4.8 30 mM sodium acetate, pH 5.1 25 mM sodium acetate, pH 5.5
Elution buffer:	Equilibration buffer + 1.0 M NaCl
Fluid velocity:	600 cm/h (Capto S), 300 cm/h (SP Sepharose Fast Flow, SP Sepharose XL).
Gradient:	Gradient 0-100% (0 CV), 100% (5 CV)
System:	ÄKTAexplorer 100 with UNICORN software version 5.01
Residence time:	1 min (Capto S), 2 min (SP Sepharose Fast Flow, SP Sepharose XL)

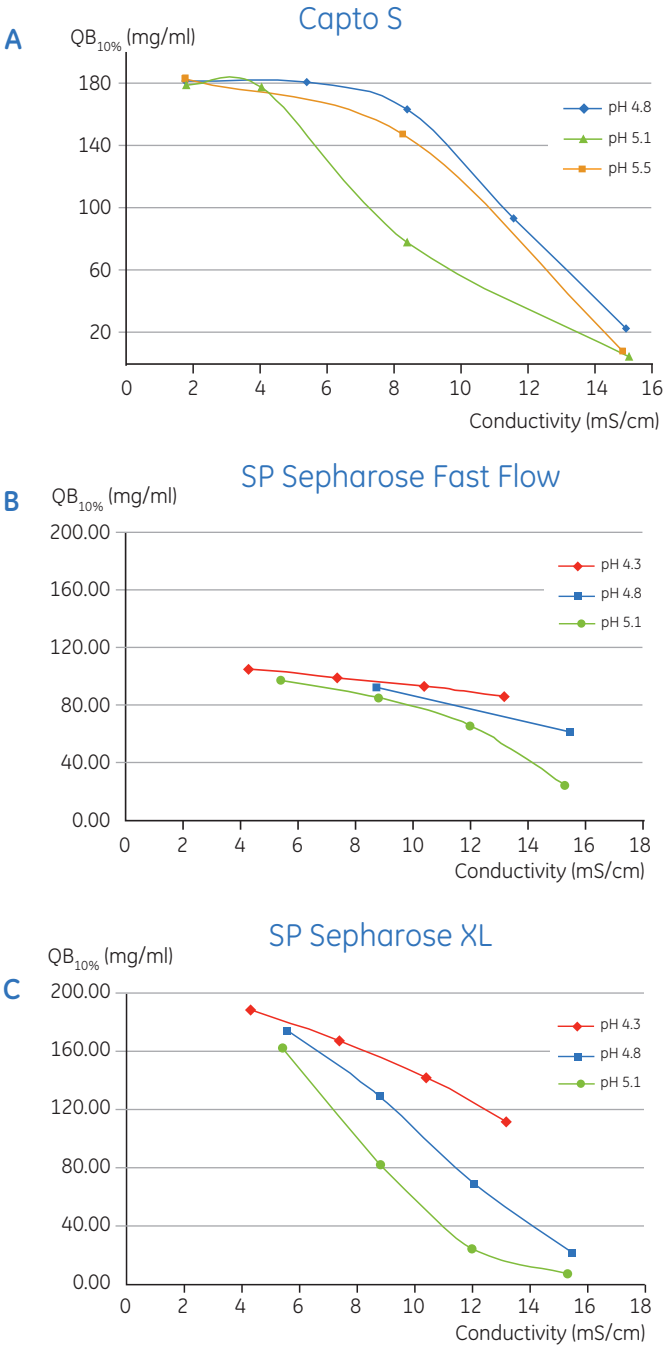

Fig 2. Screening for optimal loading conditions using pure α-chymotrypsin (4 mg/ml) in Tricorn 5/100 columns. **(A)** Capto S; **(B)** SP Sepharose Fast Flow; **(C)** SP Sepharose XL. The lowest conductivities gave the highest capacities.

Column:	Tricorn 5/100 (CV=2 ml)
Medium:	Capto S
Sample:	1 mg/ml α-chymotrypsin in E.coli homogenate in equilibration buffer
Equilibration buffer:	50 mM sodium acetate, pH 4.8 30 mM sodium acetate, pH 5.1 25 mM sodium acetate, pH 5.5
Elution buffer:	Equilibration buffer + 1.0 M NaCl
Fluid velocity:	300 cm/h
Gradient:	Gradient 0-100% (0 CV), 100% (5 CV)
System:	ÄKTAexplorer 100 with UNICORN software version 5.01
Residence time:	2 min

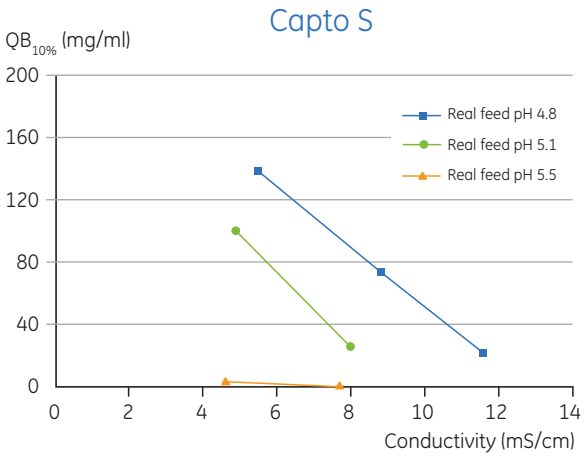

Fig 3. Screening for optimal loading conditions on Capto S using "real feed" spiked to 1 mg/ml α-chymotrypsin in *E. coli* homogenate, in Tricorn 5/100 columns. The best capacity was found at pH 4.8.

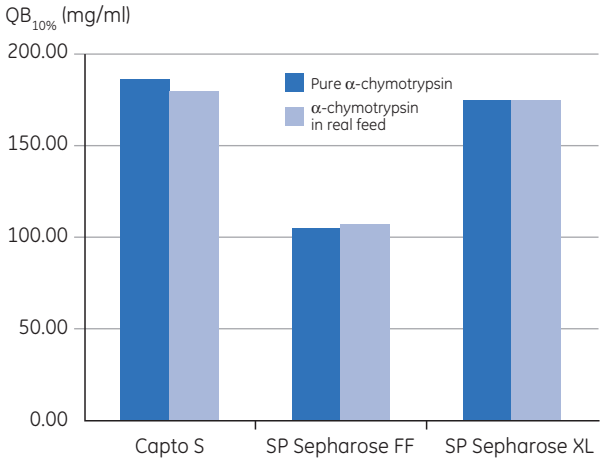

Fig 4. Comparing $QB_{10\%}$ for pure α-chymotrypsin and real feed at a constant concentration of the enzyme (4 mg/ml) showed no significant change in capacity. Conductivity was approx. 6 mS/cm. Residence time 2 minutes.

Determination of dynamic binding capacity

The residence time study was performed at optimal conditions for each ion exchanger. Highest capacity was obtained for Capto S. Capacity versus residence time (Fig 5) shows that in practice, equilibrium capacity is reached almost instantaneously for Capto S and SP Sepharose XL, suggesting very fast and efficient mass transfer.

Process optimization

The linear gradient initially used for elution was converted to step elution. Buffer volumes for equilibration, wash and re-equilibration were optimized to minimize process time and buffer consumption. Attempts were made to see if additional material could be eluted with 3 M NaCl, 50 mM Tris, pH 9.0. Since these conditions did not elute any further material, the step was omitted and cleaning with 1.0 M NaOH was done immediately after elution. Table 2 summarizes the final process used for scale-up.

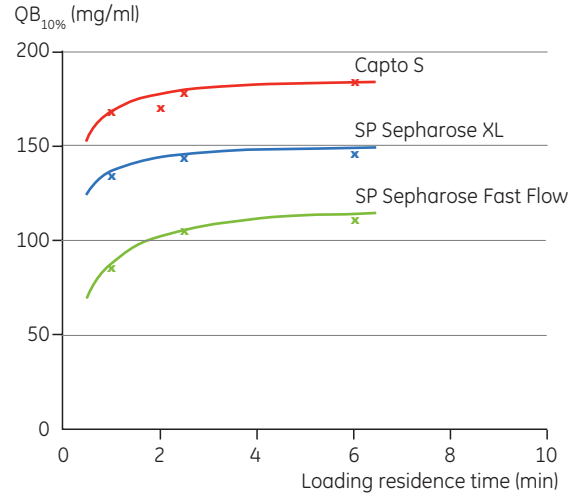

Fig 5. Dynamic binding capacity ($QB_{10\%}$) at different loading residence times in Tricorn 5/100 columns. Sample was α-chymotrypsin (4 mg/ml) in *E. coli* homogenate in equilibration buffer.

Table 2. The optimized process used for Capto S scale-up

Step	Column volumes	Buffer composition	Residence time (min)	Step time (min)
Equilibration	4	50 mM sodium acetate, pH 4.8	2	8
Loading	25	4 mg/ml α-chymotrypsin in *E. coli* homogenate in 50 mM sodium acetate, pH 4.8.	2	50
Wash	5	50 mM sodium acetate, pH 4.8	2	10
Elution	5	50 mM sodium acetate, pH 4.8 + 1.0 M NaCl	2	10
Cleaning (CIP)	5	1.0 M NaOH	6	30
Re-equilibration	2	50 mM sodium acetate, pH 4.8	2	4
Total	46			112

Scale-up

Figure 6 shows chromatograms from the three-step Capto S scale up. Very similar profiles were obtained. Figure 7 compares the purity of the eluted material at each scale. These chromatograms reveal one main peak comprising approximately 90% of the total peak area for all three chromatograms.

Table 3 summarizes the purification data. The high recoveries obtained could be due to underestimating the activity in the starting material, since storage of this material led to a decrease in enzymatic activity whereas the eluates retained their activity. Flow-through and wash fractions did not contain any activity.

Column:	Tricorn 5/100 (bed height 9.7 cm, CV=1.9 ml), XK 16/40 (bed height 20.7 cm, CV=41.5 ml) AxiChrom 50 (bed height 22 cm, CV=431 ml)
Medium:	Capto S
Sample:	4 mg/ml α-chymotrypsin in *E. coli* homogenate in equilibration buffer
Equilibration buffer:	50 mM sodium acetate, pH 4.8
Elution buffer:	50 mM sodium acetate, pH 4.8 + 1.0 M NaCl
Fluid velocity:	285 cm/h (Tricorn 5/100) 624 cm/h (XK 16/40) 645 cm/h (AxiChrom 50)
Gradient:	Gradient 0-100% (0 CV), 100% (5 CV)
System:	ÄKTAexplorer 100 (Tricorn 5/100 and XK 16/40) ÄKTApilot™ (AxiChrom 50)
Residence time:	2 min

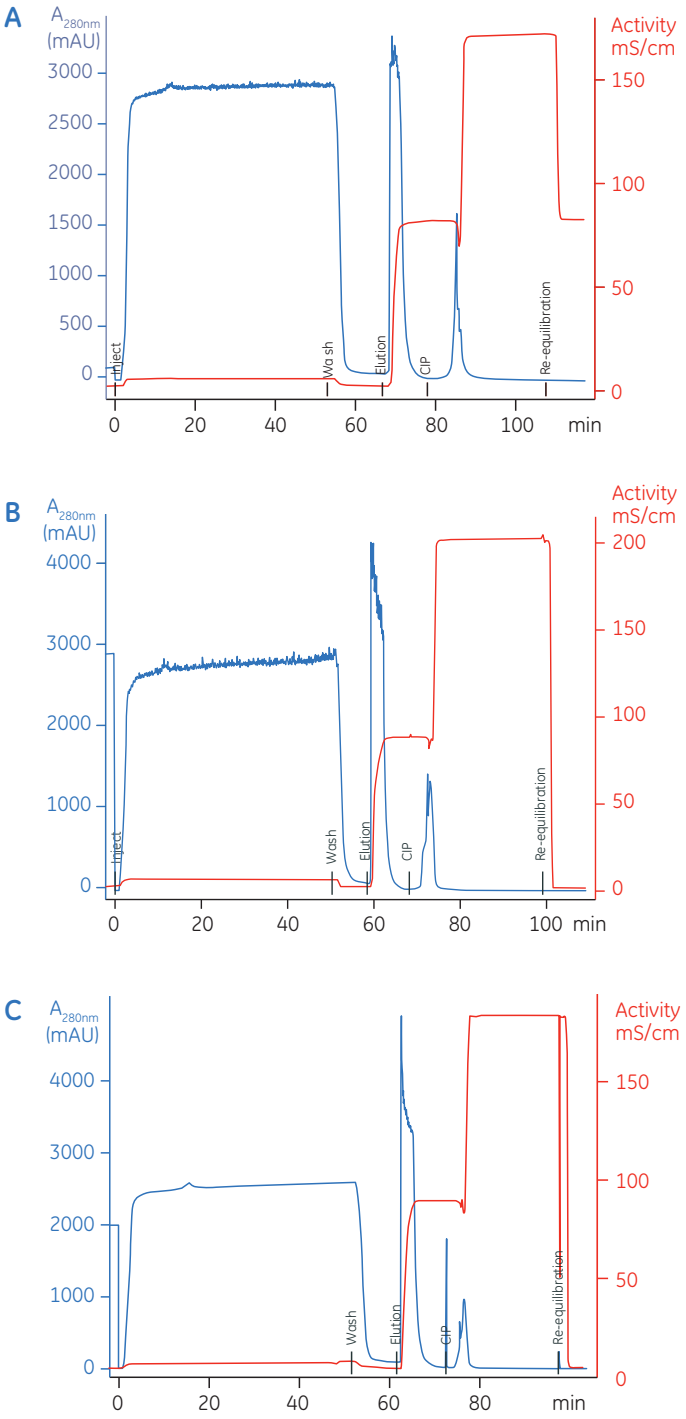

Fig 6. Three-step scale-up. **(A)** Tricorn 5/100; **(B)** XK 16/40; **(C)** AxiChrom 50. Very similar profiles were obtained for all three. Buffers and method are according to Table 2.

Column:	Superdex™ 75 10/300 GL
Sample:	50 µl of the elution pool from Figure 6
Buffer:	10 mM sodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4
Fluid velocity:	38 cm/h
System:	ÄKTAexplorer 10 with UNICORN software version 5.01

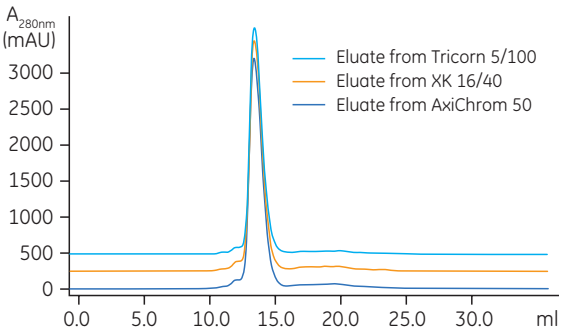

Fig 7. Gel filtration of eluted material from the chromatograms shown in Figure 6. Each main peak comprises approximately 90% of the total peak area.

Table 3. Purification factors, elution volumes and recoveries from Capto S runs on Tricorn 5/100, XK 16/40 and AxiChrom 50

Column	Column volume (ml)	Purification factor	Elution volume (CV)	Recovery* (%)
Tricorn 5/100	1.9	4	3	114
XK 16/40	41.5	3	3.5	102
AxiChrom 50	431	4	3	116

* The high recovery is probably due to underestimation of the enzymatic activity in the starting material. The activity decreased in storage.

Productivity and cost calculations

Productivity is defined as mass of target protein recovered per unit volume and time. The productivity at different loading residence times shown in Figure 8 was obtained using the optimized process sequence and cycle times from Table 2, The yield for all three media was assumed to be 95%, which is realistic for Capo S considering experimental data. Due to high equilibrium capacity in combination with rapid mass transfer, maximal productivity is obtained at minimal loading residence time during the loading step. The operational window is also significantly larger for Capto S than for the other two media since they cannot withstand fluid velocities higher than 200 cm/h in large columns, i.e. residence times less than 6 minutes cannot be obtained for SP Sepharose XL and SP Sepharose Fast Flow.

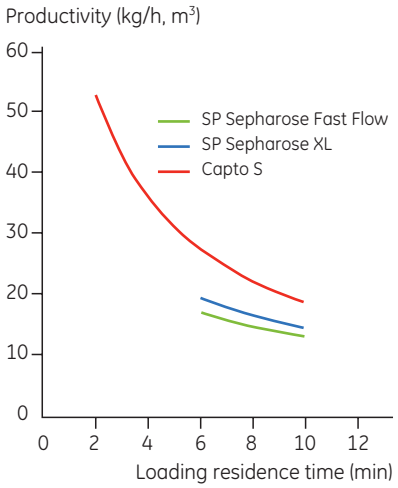

Fig 8. Productivity versus loading residence time. The superior performance and wider operating window of Capto S are clearly evident.

Separation cost is defined as total cost per unit mass of target protein recovered from the step. Using the cost factors stated in Table 1, the results in Figure 9 (for a 1 m i.d. column) were obtained.

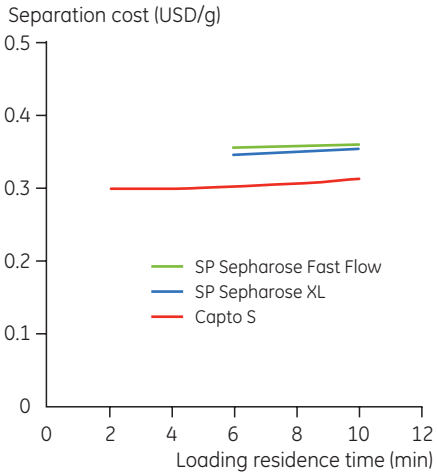

Fig 9. Separation costs versus loading residence time. The figures are lowest for Capto S. Note that the cost for SP Sepharose Fast Flow and SP Sepharose XL is almost identical as the effects of the differences in dynamic capacity and medium cost cancel each other out.

Because of rapid mass transport of Capto S, the dynamic capacity is essentially independent of loading residence times above 2 minutes (Fig 5). The amount of product recovered in a cycle is proportional to dynamic capacity, which is almost constant when varying residence time. Therefore, the cost of running a cycle is essentially constant regardless of the loading residence time. The minimum loading residence time for the three media, shown in both the productivity and the separation costs (Figs 8 and 9) reflects the maximum allowed velocity for each medium (20-cm bed, column i.d. 1 m).

Figure 10 shows the cost breakdown per medium at the point of minimum separation cost. Costs for media, buffers and solvents dominate, whereas the costs for capital and labor are a small fraction of the total separation cost.

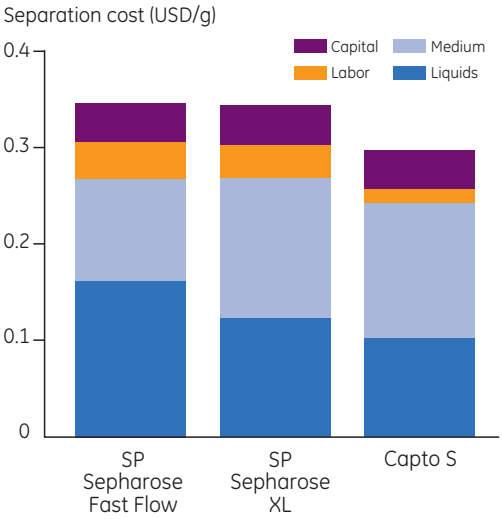

Fig 10. Cost distribution per medium. Total separation costs are lowest for Capto S.

To show the effect of high productivity in a practical situation, the column size and number of cycles required to capture and recover 100 kg of α-chymotrypsin per 24 h was calculated. For example, only a third of the medium volume was required to purify the same amount of target protein as shown in Table 4. Separation cost are also indicated.

Table 4. Required column size (bed vol.) and number of cycles to capture 100 kg α-chymotrypsin in 24 h

Ion exchanger	Bed volume (L)	Number of cycles	Separation cost (USD/g)
Capto S	82	13	0.29
SP Sepharose XL	215	6	0.35
SP Sepharose Fast Flow	246	7	0.35

The high-flow capabilities of Capto S means that smaller columns and shorter cycle times can be used compared to Sepharose based media.

Capto S has the highest operating velocity of the tested media. In the present application, it also has the highest binding capacity. The resulting productivity is therefore 160-200% higher than SP Sepharose XL and SP Sepharose Fast Flow. In addition, the total separation cost is 10-20% lower.

Conclusions

A high-recovery capture step was developed using the strong cation exchanger Capto S. Cycle time was less than 2 h, which is important for a capture step since proteolytic enzymes need to be removed quickly. In comparison, the same process would take almost 5 h for SP Sepharose FF and SP Sepharose XL. The purification factor varied between 3 and 4 for the different scales, and the product was almost 90% pure as indicated by analytical gel filtration.

Capto S proved to be very suitable for this type of application. The medium provides very high capacity in real feed plus high purity of the eluted fraction. The conductivity profile for this application made optimization rather straightforward. However, to take advantage of the full potential for Capto S, screening for optimal loading conditions is always recommended.

Results from the 200-fold scale up demonstrate that Capto S is well suited for high-throughput, large-scale protein manufacturing. Productivity calculations show that the excellent binding capacity of Capto S results in the lowest separation cost compared with the other two media. In addition, high maximum fluid velocity and rapid mass transfer give the highest productivity.

The high mechanical stability of Capto S results in good flow properties. Combined with its high dynamic capacity, Capto S has been proven to deliver high productivity and short process times. These attributes give the users greater freedom in choosing column dimensions as well as other operating conditions.

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