Efficient purification of the pertussis antigens toxin, filamentous haemagglutinin, and pertactin in chromatography workflows

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Efficient purification of the pertussis antigens toxin, filamentous haemagglutinin, and pertactin in chromatography workflows

This application note describes the purification of pertussis toxin (PT), filamentous haemagglutinin (FHA), and pertactin (PRN) in a process based on modern chromatography resins. The described method resulted in higher purity and yield than what can be expected from a purification process based on salt precipitations and sucrose gradient centrifugation.

Introduction

Bordetella pertussis is a Gram-negative bacterium causing whooping cough. There are two types of pertussis vaccines available: whole-cell vaccines and acellular vaccines (purified components). While whole-cell pertussis vaccines are commonly associated with adverse reactions, acellular pertussis vaccines exhibit fewer side effects and, hence, constitute a viable alternative to whole-cell pertussis vaccines for routine use in disease prevention. Acellular vaccines can be based on different immunogens such as detoxified PT, FHA, or PRN. This work aims to demonstrate a purification process for PT, FHA, and PRN with a purity of over 95% for each antigen. The antigens purified using the described method can be used in either acellular pertussis stand-alone vaccines or in combination vaccines.

A method comprising two-fold salt precipitations followed by a sucrose gradient centrifugation step is sometimes used in purification of PT, FHA, and PRN. This purification strategy suffers from poor separation of impurities from the target molecules, resulting in low purity and yield. In addition, the process cannot separate the different targets, resulting in a mixed product without possibility to control antigen ratios. The process is also difficult to scale to meet market demands.

The purification strategy presented here is based on modern, scalable chromatography resins (Fig 1). For both PT and FHA, Capto™ SP ImpRes cation exchange resin was used in an initial capture step. Capto SP ImpRes is a strong cation exchange resin. The resin offers high resolution and excellent pressure-flow properties for fast and efficient purification of biomolecules. For PT, a second polishing step was added in which Capto MMC resin was used. Capto MMC is a multimodal cation exchange resin that can be used to bind proteins at the conductivity of the feed material and is commonly used to solve more difficult purification challenges.

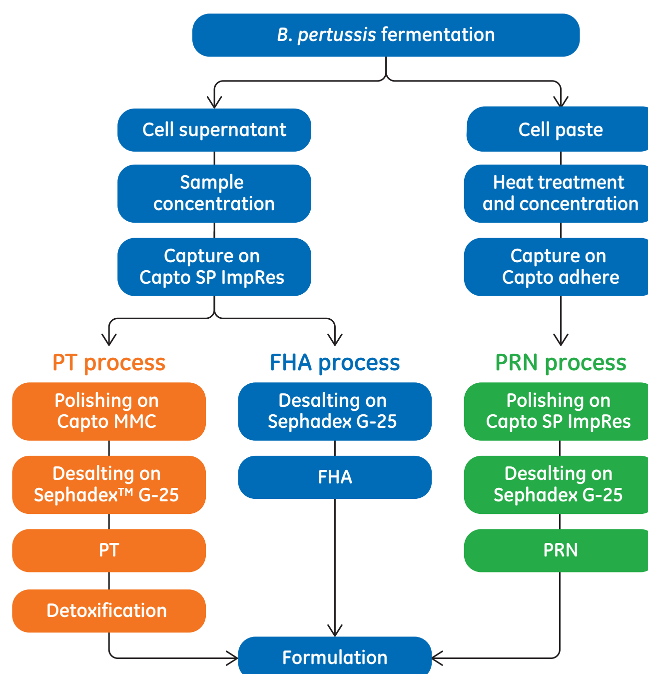


Fig 1. Overview of process design for purification of PT, FHA, and PRN.

For PRN, Capto adhere resin was used in the initial capture step. Capto adhere is a multimodal anion exchanger designed to remove key contaminants such as DNA, host cell proteins, larger aggregates, and viruses. Remaining impurities were removed in a second polishing step using Capto SP ImpRes.

The described process has been previously published by Tian *et al.* (1). The presented work was conducted by GE Healthcare's Fast Trak team in Shanghai, CN.

Materials and methods

Sample preparation

The *B. pertussis* fermentation was harvested and cells were separated from supernatant by centrifugation. The PT and FHA containing supernatant was 25× concentrated in an ultrafiltration step using a filter with a M_r 10 000 nominal molecular weight cutoff (NMWCO) pore size membrane. The concentrated sample was diluted 5-fold in 20 mM phosphate buffer, pH 6.0 containing 2 M urea for a conductivity of 7 mS/cm. The diluted sample was filtered using a 0.45 µm membrane prior to chromatography.

To release PRN, the cell pellet was subjected to heat treatment and diluted in an aqueous solution with pH adjusted to 7. The diluted sample was filtered (normal flow filtration) and further concentrated cross-flow filtration using filters with M_r 10 000 NMWCO pore size membranes. The concentrated sample was filtered using a 0.45 µm membrane prior to chromatography.

Purification of PT and FHA

For the capture of PT and FHA, Capto SP ImpRes resin was used. Sample was injected onto the packed column, whereupon the column was washed in two steps for removal of impurities: (1) 20 mM phosphate buffer pH 6.0 containing 2 M urea and (2) 20 mM phosphate buffer, 0.11 M NaCl, pH 6.0 containing 2 M urea. Bound PT was eluted with 20 mM phosphate buffer, 0.19 M NaCl, pH 6.0 containing 2 M urea, whereupon bound FHA was eluted with 20 mM phosphate buffer, 0.35 M NaCl, pH 6.0 containing 2 M urea.

The PT-containing fraction from the initial capture step was directly loaded onto Capto MMC resin. To remove impurities, the packed column was washed with 20 mM phosphate buffer, 1 M NaCl, pH 6.0 containing 2 M urea. Bound PT was eluted with 20 mmol/L Tris buffer containing 1 M NaCl and 2 M urea with pH gradually increasing from 8 to 9.

Purification of PRN

For the capture of PRN, Capto adhere resin was used. Sample was injected onto the packed column, whereupon the column was washed in four steps to remove impurities: (1) 20 mM phosphate buffer pH 7.0, (2) 20 mM phosphate buffer, 1 M NaCl, pH 7.0, (3) 20 mM acetate acid buffer, 1 M NaCl, pH 4.5, and (4) 20 mM acetate acid buffer with gradually increasing salt concentration and pH from 0.25 to 0.35 M NaCl and pH 4 to 5, respectively. Bound PRN was eluted with 20 mM acetate acid buffer with salt concentration increasing from 0.025 to 0.075 M NaCl and pH from 4 to 5.

The PRN-containing fraction was 3-fold diluted with 20 mM acetate acid buffer, pH 5.0 and pH was adjusted to 5.0 before loaded onto a column packed with Capto SP ImpRes resin. The column was washed with 20 mM acetate acid buffer, pH 5.0 before bound PRN was eluted with 20 mM acetate acid buffer, 0.12 M NaCl, pH 5.0.

Desalting

Desalting of the PT-, FHA-, and PRN-containing fractions was performed on Sephadex G-25 resin.

Analysis

Purified PT, FHA, and PRN were identified and analyzed for purity by SDS-PAGE on a 4%–12% NuPAGE™ gel (Life Technologies). Bands were visualized by Coomassie™ staining captured using an ImageQuant™ TL analyzer. Selected bands were recovered and sent to Shanghai Applied Protein Technology Co., Ltd for analysis of protein mass spectrum by liquid chromatography–mass spectrometry (LC-MS) (Thermo LTD Velas).

PT and FHA were analyzed using the Biacore™ T200 system. A direct binding format was used, in which either a mouse anti-PTS1 or anti-FHA monoclonal antibody (NIBSC, UK) was immobilized to a sensor chip (2). Standard curves for respective antigen were constructed using purified antigen of known concentrations. Samples from each purification step were quantified using the standard curve, and the recovery rate was calculated.

Results

Separation of PT from FHA was performed in an initial capture step using Capto SP ImpRes resin (Fig 2). Remaining impurities in the PT-containing fraction were efficiently removed in a second polishing step using Capto MMC resin (Fig 3). Purification of PRN was performed in a two-step process where Capto adhere resin was used in the initial capture step and Capto SP ImpRes resin was used in the second polishing step (Fig 4 and 5). Fractions containing PT, FHA, or PRN were desalted on Sephadex G-25 resin (Fig 6). The final purity of PT and FHA was calculated to 99.1% and 96.6%, respectively (Fig 7). For PRN, the final purity was calculated to 99.1% (Fig 8).

Resin: Capto SP ImpRes
Sample: Pretreated sample containing PT and FHA
Wash 1: 20 mM phosphate buffer, 2 M urea, pH 6.0 (B0)
Wash 2: 11% B1
Elution 1: 19% B1
Elution 2: 35% B1

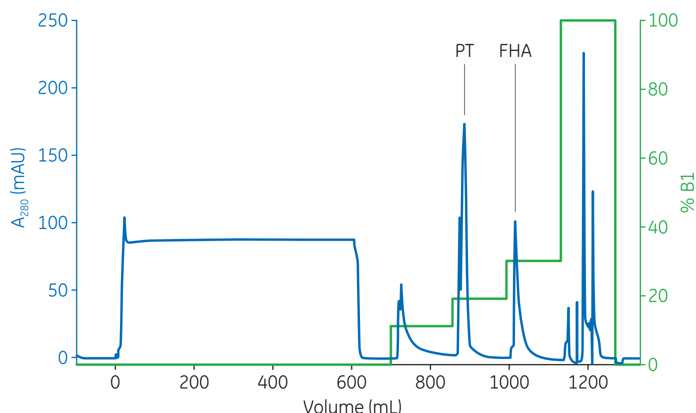


Fig 2. Chromatogram from initial capture of PT and FHA from pretreated sample. B1 = 20 mM phosphate buffer, 1 M NaCl, 2 M urea, pH 6.0.

Resin: Capto MMC
Sample: PT-containing fraction from initial capture step
Wash: 20 mM phosphate buffer, 1 M NaCl, 2 M urea, pH 6.0
Elution: 20 mmol/L Tris buffer, 1 M NaCl, 2 M urea, pH 8–9

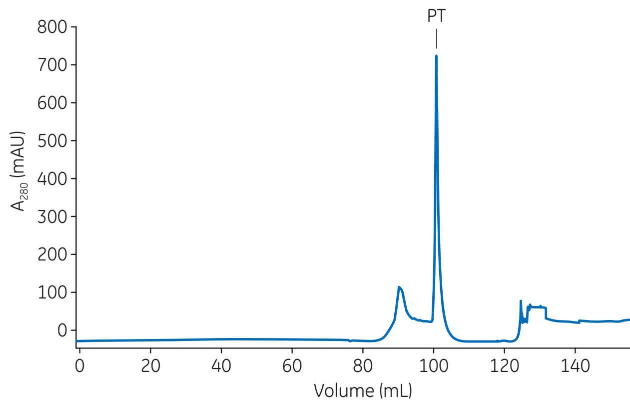


Fig 3. Chromatogram from second polishing step for PT.

Resin: Capto adhere
Sample: Pretreated sample containing PRN
Wash 1: 20 mM phosphate buffer pH 7.0 (B0)
Wash 2: 20 mM phosphate buffer, 1 M NaCl, pH 7.0 (B1)
Wash 3: 20 mM acetate acid buffer, 1 M NaCl, pH 4.5 (B2)
Wash 4: 20 mM acetate acid buffer, 0.25–0.35 M NaCl, pH 4–5 (35% to 25% B2)
Elution: 20 mM acetate acid buffer, 0.025–0.075 M NaCl, pH 4–5 (7.5% to 2.5% B2)

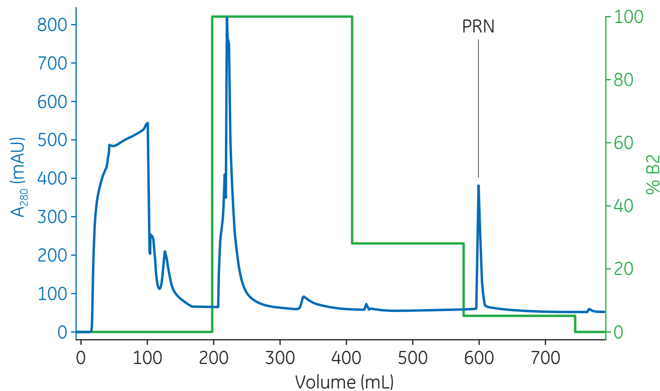


Fig 4. Chromatogram from initial capture of PRN from pretreated sample.

Resin: Capto SP ImpRes
Sample: 3-fold diluted PRN-containing fraction from initial capture step
Wash: 20 mM acetate acid buffer, pH 5.0 (B0)
Elution: 20 mM acetate acid buffer, 1 M NaCl, pH 5.0 (B1)

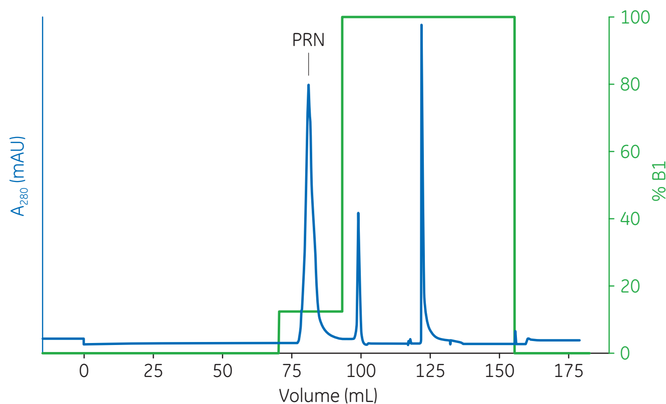
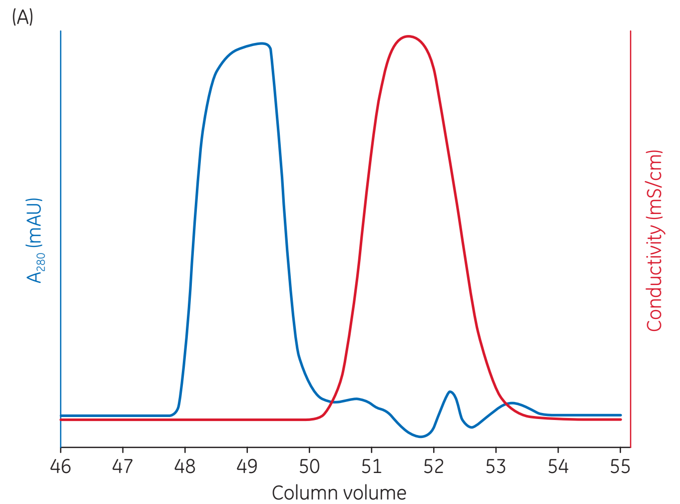
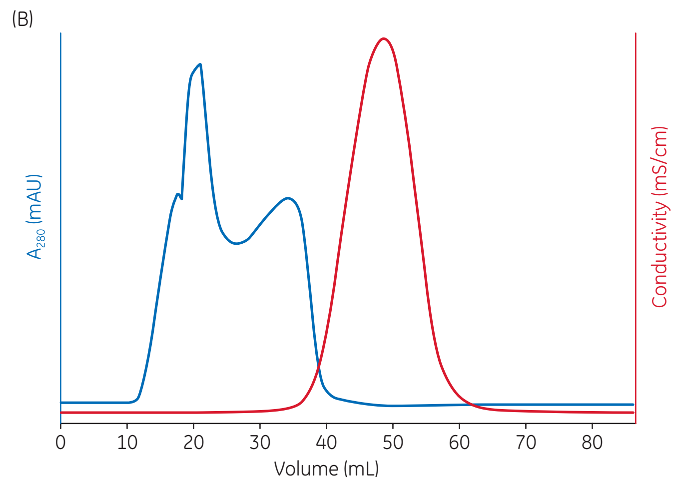


Fig 5. Chromatogram from second polishing step for PRN.





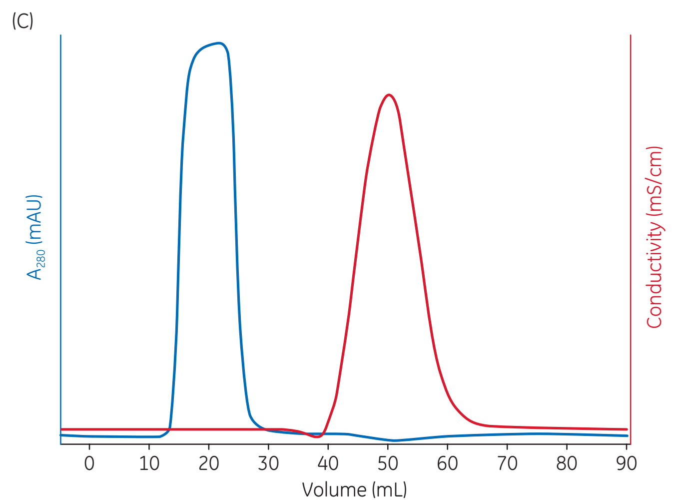


Fig 6. Desalting of fractions containing (A) PT, (B), FHA, and (C) PRN on a Sephadex G-25 resin.

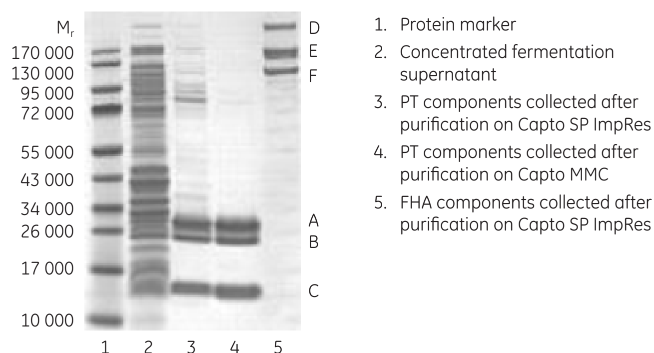


Fig 7. SDS-PAGE profile of purified PT and FHA. Bands A to F, identified by LC-MS, show high homology to data for *B. pertussis* from the UniProt™ database (www.uniprot.org).

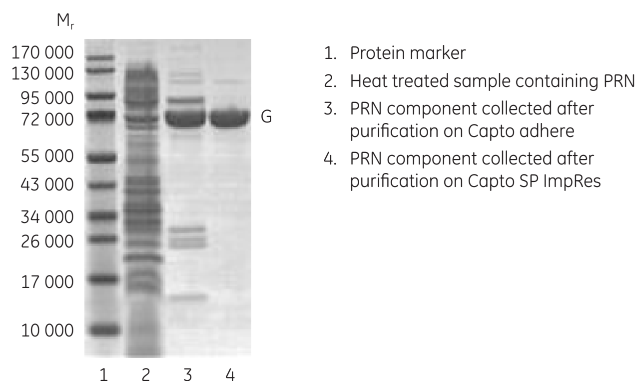


Fig 8. SDS-PAGE profile of purified PRN. Band G, identified by LC-MS, show high homology to data for *B. pertussis* from the UniProt™ database (www.uniprot.org).

Antigen analysis using the Biacore system was found to constitute a sensitive and stable platform for quantification of the target molecules and, hence, was used to calculate recovery after each step (Table 1). In this study, a rapid, robust, and label-free antigen quantification method was developed using Biacore T200, allowing efficient monitoring and optimization of the purification process.

Overall purification recovery of PT after the Capto SP ImpRes and Capto MMS steps was determined to 31%. Recovery of FHA after the Capto SP ImpRes step was 28%. The overall purification recovery for PRN was not analyzed.

Scale-up to manufacturing scale

The described process can easily be scaled to commercial scale using the components listed in Table 2. Based on the results from the small-scale process, a large-scale process for purification of PT, FHA, and PRN was determined (Table 3). Considering a productivity of 10 million doses per year produced in 40 batches, each batch needs to produce 250 000 doses. With the requirement of 20 µg target molecule per dose, each batch needs to produce 5 g purified target.

Based on a purification recovery for PT of 31%, a total process recovery after fill and finish of 24% can be expected. Hence, 20 g PT in crude sample is needed for each batch. For FHA, a total process recovery after fill and finish of 24% can be expected based on a purification recovery of 28%. Hence, 21 g FHA in crude sample will be needed for each batch.

Table 1. Recovery rates of purified PT and FHA

Sample	Step	Injection concentration (µg/mL)	Injection volume (mL)	Eluate concentration (µg/mL)	Eluate volume (mL)	Recovery rate (%)
PT	Capto SP ImpRes	226	300	639	60	57
	Capto MMC	639	47	2760	6	55
						31 (total)
FHA	Capto SP ImpRes	230	300	217	90	28

Table 2. Determined component requirements for purification of PT and FHA at manufacturing scale

	PT			FHA		PRN		
	Step1	Step2	Desalt (3 ×)	Step1	Desalt (3 ×)	Step1	Step2	Desalt
Resin type	Capto SP ImpRes	Capto MMC	Sephadex G-25	Capto SP ImpRes	Sephadex G-25	Capto adhere	Capto SP ImpRes	Sephadex G-25
Column volume	12.6 L	3.14 L	7 L	12.6 L	27.6 L	2 L	0.5 L	3.8 L
Column type	Chromaflow™ 400	BPG 200	BPG 300	Chromaflow 400	Chromaflow 600	BPG 140	AxiChrom™ 70	BPG 200
Column bed height	10 cm	10 cm	10 cm	10 cm	10 cm	13 cm	12 cm	12 cm

Table 3. Requirements for production of PT and FHA at manufacturing scale

	PT	FHA	PRN
Dose/year	10 000 000	10 000 000	10 000 000
Batch/year	40	40	40
Dose/batch	250 000	250 000	250 000
Amount/dose	20 mg	20 mg	20 mg
Pure sample/batch	5 g	5 g	5 g
Total recovery	24%*	24%†	ND‡
Amount in crude sample	20 g	21 g	ND‡

*After fill and finish, based on a purification recovery of 31% (95% purity)

†After fill and finish, based on a purification recovery of 28% (95% purity)

‡Not determined

Conclusions

Here, we describe the purification of PT, FHA, and PRN in an easily scalable process based on modern chromatography resins. Using a traditional purification approach, based on salt precipitations and sucrose gradient centrifugation, a recovery of 10% at a purity of 85% can be expected. With the described method, recovery of PT and FHA could be increased to about 30% at a purity of more than 95%. The purity achieved for PRN was calculated to above 90%. Using the described process, the target molecules were successfully purified to high purity and yield in an environmentally friendly way.

Acknowledgement

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Reference

1. Tian, Y., Shi, Q., Sui, L. Purification of pertussis toxin, filamentous haemagglutinin and pertactin. *Chin J Biologicals*, **26**, 1186–1189 (2013).
2. Frostell, Å., Mattsson, A., Eriksson, Å., Wallby, E., Kärnhall, J., Illarionova, N.B., Estmer Nilsson, C. Nine surface plasmon resonance assays for specific protein quantitation during cell culture and process development. *Analytical Biochemistry* **477**, 1–9 (2015).

Ordering information

Product	Description	Product code
Capto SP ImpRes	Cation exchange resin, 5 L	17546804
Capto MMC	Multimodal cation exchange resin, 5 L	17531704
Capto adhere	Multimodal anion exchange resin, 5 L	17544404
Sephadex G-25 resin	Size exclusion chromatography resin, 5 kg	17003303
Biacore T200	Label-free detection system	28975001
Series S Sensor Chip CM5	Biacore sensor chip	BR100530

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