Exotoxin clearance from mAb samples in a two-step chromatography process

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A heat-stable exotoxin can remain as contaminant of a drug substance throughout the whole manufacturing process, with the risk of adversely affecting patients' health. One such toxin is cereulide secreted by *Bacillus cereus*, a ubiquitous spore former that is of concern during manufacturing of pharmaceuticals. This work demonstrates the clearance of cereulide from monoclonal antibody (mAb) produced in Chinese hamster ovary (CHO) cells, over two purification steps. First, target mAb was captured from cereulide-spiked cell culture supernatant using MabSelect SuRe™ protein A resin. A cleaning investigation was conducted and cereulide carryover between runs were evaluated. Cereulide clearance over the capture step was confirmed with two additional mAbs. Remaining cereulide content after the protein A step could be further reduced to below detectable levels in the following polishing step using the Capto™ adhere multimodal anion exchange resin.

Introduction

Bioburden can be introduced at any time through raw materials or through handling of process materials, and proliferation can occur under favorable conditions such as in upstream processes with controlled temperature and a nutrient-rich environment. Some bacterial strains produce exotoxins that are of concern to humans. *B. cereus*, for example, produces cereulide, a heat-stable peptide-based exotoxin that causes nausea and vomiting when ingested. Once spores germinate and start to grow, exotoxin can be produced and secreted under the appropriate environmental conditions.

Cereulide is a highly heat-, acid-, and protease-stable exotoxin. The hydrophobic nature of its cyclic peptide structure makes it challenging to analyze, thus presenting difficulties in accurately monitoring of its presence in process material. The aim of this study was to demonstrate the clearance of cereulide from mAb-containing cell culture supernatant in a two-step chromatography process. In the first capture step, MabSelect SuRe protein A affinity resin was used. The MabSelect SuRe resin has an alkaline stable recombinant protein A-derived ligand that allows the use of stringent and cost-effective cleaning-in-place (CIP) and sanitization protocols based on up to 0.5 M NaOH. Capto adhere resin was used in the polishing step to remove remaining cereulide. Capto adhere is a multimodal anion exchange resin designed for post-protein A purification of mAbs. The strong anion exchange multimodal ligand gives a different selectivity compared with traditional ion exchange resins. Capto adhere can thus remove key contaminants such as host cell DNA (hcDNA), host cell proteins (HCP), leached protein A, mAb dimers and larger aggregates, as well as viruses in a single polishing step.

A liquid chromatography-mass spectrometry (LC-MS) method was developed to monitor the cereulide reduction over the process steps. The in-house developed analytical assay provided high selectivity and sensitivity in quantification of the cereulide.

Materials and methods

Sample preparation

Three different mAb IgG1 molecules (mAb1, mAb2, and mAb3), expressed in CHO cells, were included in this challenging study. The mAb-containing cell supernatants were each spiked with very high cereulide concentrations, unlikely present in a bioprocess contamination. Cereulide (Chiralix B.V) was delivered as powder in sealed glass vials (2 mg/vial). For spiking of the samples, the cereulide was diluted to a concentration of 50 µg/mL in methanol. The spiked cell supernatant was incubated overnight. As cereulide is challenging to handle due to the hydrophobicity of the cyclic structure, special care was taken during sample preparation, that is, the smallest possible bottle size and magnets were used for mixing to minimize cereulide binding to surfaces.

Capture step

For the capture step, a 4.7 mL HiScreen™ MabSelect Sure column was operated on an ÄKTA™ system controlled through the UNICORN™ software. Process conditions are listed in Table 1. After fraction collection, a sample from the elution pool was mixed with acetonitrile to a final concentration of 80% (v/v) for analysis of cereulide content. The flowthrough, wash, strip and flush was collected directly into acetonitrile to a final concentration of 80% (v/v).

The CIP fraction was analyzed for cereulide content without dilution with acetonitrile. For evaluation of post-protein A clearance of cereulide, mAb-containing eluates from two process cycles were pooled. For conditioning of the sample for loading onto the Capto adhere column, pH of the pooled fractions was adjusted to pH 5 with 2 M Tris base.

Evaluation of cereulide carryover

An investigation of cereulide carryover was conducted, in which three consecutive runs on the same MabSelect SuRe column was performed. In the first run, the MabSelect SuRe step was conducted according to Table 1, where the column was loaded with cereulide-spiked mAb cell culture supernatant at 2.5 mg cereulide/L resin. The standard CIP procedure, 0.1 M NaOH at a contact time of 15 min, was used. No cereulide-containing material was further loaded in the following two runs. The second run was a blank (mock) run performed using conditions in Table 1, except for the loading phase that was omitted. In the third run, the process was conducted with mAb-containing cell culture supernatant, but without cereulide. The mock eluate from run two and the product pool from run three were collected and mixed with acetonitrile to a final concentration of 80% (v/v) for analysis of cereulide carryover between cycles.

Evaluation of different CIP protocols

Four different CIP protocols were evaluated, in which four different MabSelect SuRe columns were subjected to either 0.1 or 0.5 M NaOH for 15 or 60 min. Again, the column was loaded with cereulide-spiked mAb-containing cell culture supernatant at 2.5 mg cereulide/L resin using the process conditions in Table 1. After CIP and re-equilibration, the resin was removed from the column and residual cereulide was extracted from the resin with 80% (v/v) acetonitrile under agitation for approximately 16 h.

Polishing step

For the polishing step, a 1 mL HiTrap™ Capto adhere column was operated in flow-through mode on an ÄKTA system controlled through the UNICORN software. Process conditions are listed in Table 2. After fraction collection, a sample from the flowthrough was mixed with acetonitrile to a final concentration of 80% (v/v) for analysis of cereulide content. The rinse solution was collected directly into acetonitrile to a final concentration of 80% (v/v). The CIP fraction was analyzed for cereulide content without acetonitrile addition.

Table 1. Conditions adopted from the customer process for the mAb capture step on the 4.7 mL HiScreen MabSelect SuRe column

Phase	Volume	Buffer	Residence time (RT) (min)
Equilibration	4 CV	25 mM Tris, pH 7.4 + 100 mM NaCl	4.7
Load	30 g mAb/L resin (2.5 mg cereulide/L resin)	Harvested cell culture fluid	4.7
Wash	5 CV	25 mM Tris, pH 7.4 + 100 mM NaCl	4.7
Elution	3 CV	50 mM acetate, pH 3.7	6.0
Strip	3 CV	100 mM phosphoric acid	4.7
Flush	1 CV	25 mM Tris, pH 7.4 + 100 mM NaCl	4.7
CIP	3 CV	0.1 M NaOH	4.7 (15 min contact time)
Re-equilibration	10 CV*	25 mM Tris, pH 7.4 + 100 mM NaCl	4.7

CV = column volumes.

* In a large-scale process, a smaller re-equilibration volume, such as 4 CV, would typically be used.

Table 2. Conditions adopted from the customer process for the mAb polishing step on the 1 mL HiTrap Capto adhere column

Phase	Volume	Buffer	Residence time (min)
Equilibration†	7 CV	100 mM sodium acetate, pH 5.0 + 130 mM NaCl	4.0
Load	250 g mAb /L resin	Pooled fractions from capture step adjusted to pH 5.0 by addition of 2.0 M Tris base	4.0
Wash	7 CV*	100 mM sodium acetate, pH 5.0 + 130 mM NaCl	4.0
Rinse	3 CV	Water	8.0
CIP	3 CV	1.0 M NaOH	8.0
Re-equilibration†	10 CV	100 mM sodium acetate, pH 5.0 + 130 mM NaCl	4.0

CV = column volumes.

* Fraction collection was stopped at 100 mAU and the process was continued with rinse.

† In a large-scale process, a smaller equilibration/re-equilibration volume, such as 4 CV, would typically be used.

Analytical method

As cereulide is depleted over time, most likely due to adhesion to surfaces and proteins, the chromatography fractions were analyzed within 1 day by LC-MS using an AQUITY™ H-class UPLC system coupled to a Xevo™ G2 Q-TOF MS instrument (Waters, Milford, MA, US). All samples were analyzed as received, apart from the CIP fractions that were neutralized to pH 7 by addition of concentrated formic acid (3–5 µL added to 100 µL sample) before analysis. A 25 µL sample was injected onto an AQUITY UPLC CSH C18 1.7 µm 2.1 × 50 mm reversed phase column (Waters) operated at 40°C. The column was equilibrated with Buffer A (0.02% trifluoroacetic acid + 10 mM ammonium acetate in ultrapure LC-MS grade water). Elution was performed using Buffer B (0.02% trifluoroacetic acid in 70:30 acetonitrile:2-propanol): 50% (at 0–2 min), 50%–80% (at 2–3 min), 80%–100% (at 3–12 min), 100% (at 12.1–13.9 min), 100%–50% (at 13.9–14 min), and 50% (at 14–15 min) at a flow rate of 0.5 mL/min. MS data was collected in positive polarity and sensitivity mode at a m/z range of 500–1500, scan rate of 2 spectra/s and an electrospray ionization (ESI) potential set at 3 kV. Extracted ion chromatograms (XIC) for m/z 1170.7, corresponding to the cereulide peptide signal, were used for quantification against an external standard curve (0.1–200 ng/mL). LC-MS standard solutions were prepared by serial dilution of the cereulide stock solution (50 µg/mL), the same as used for spiking the mAb supernatant, in 90% acetonitrile, 0.02% trifluoroacetic acid. The limit of detection for the cereulide was determined to be 0.01 ng/mL using the cereulide standard.

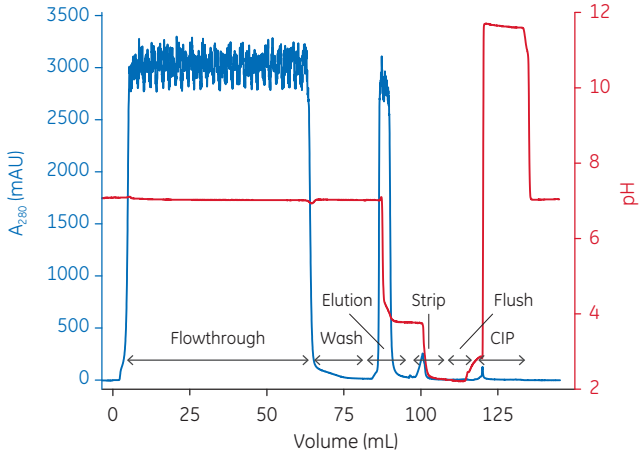
Results and discussion

Cereulide clearance in a two-step chromatography process

Chromatograms from mAb capture and polishing are shown in Figure 1. Analysis of collected fractions from the mAb capture step showed that most of the cereulide peptide (96%) was removed in the flowthrough. A small amount (1.4%) of cereulide co-eluted with the mAb in the product pool. The remaining cereulide content was reduced to below limit of detection (LOD) (0.01 ng/mL) in the subsequent polishing step. Results are summarized in Table 3. The cereulide log reduction value (LRV) was 1.9 over the MabSelect SuRe step and > 2.9 over the Capto adhere step based on LOD. Using spiked process material also for the Capto adhere step, the LRV was confirmed to be 3.1 (data not shown) resulting in a total LRV of 5 over the two-step process. The mAb recoveries over the MabSelect SuRe and Capto adhere steps were > 95% and > 90%, respectively, resulting in a total mAb recovery of close to 90% over the process.

(A)

Column:	4.7 mL HiScreen MabSelect Sure column
Equilibration:	4 CV 25 mM Tris, pH 7.4 + 100 mM NaCl
Sample:	mAb-containing cell supernatant spiked with cereulide
Load:	30 g mAb/L resin (2.5 mg cereulide/L resin) , 4.7 min RT
Wash:	5 CV 25 mM Tris, pH 7.4 + 100 mM NaCl, 4.7 min RT
Elution:	3 CV 50 mM acetate, pH 3.7, 5 min RT
Strip:	3 CV 100 mM phosphoric acid
Flush:	1 CV 25 mM Tris, pH 7.4 + 100 mM NaCl
CIP:	3 CV 0.1 M NaOH, 15 min contact time
Equilibration:	10 CV 25 mM Tris, pH 7.4 + 100 mM NaCl



(B)

Column:	1 mL HiTrap Capto adhere column
Equilibration:	7 CV 100 mM sodium acetate, pH 5.0 + 130 mM NaCl, 4 min RT
Sample:	mAb pool from MabSelect SuRe capture step
Load:	250 g mAb/L resin, 4 min RT
Wash:	7 CV 100 mM sodium acetate, pH 5.0 + 130 mM NaCl, 4 min RT
Rinse:	3 CV of water
CIP:	3 CV 1 M NaOH, 24 min contact time
Equilibration:	10 CV 100 mM sodium acetate, pH 5.0 + 130 mM NaCl

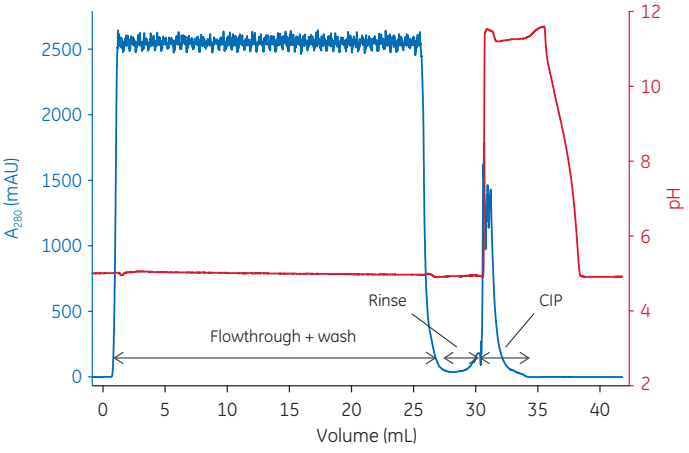


Fig 1. Purification of mAb1. (A) Capture using MabSelect SuRe resin. Samples from the elution pool, flowthrough, wash, strip, flush, and CIP were collected for determination of cereulide content. (B) Polishing using Capto adhere resin. Samples from the flowthrough, rinse, and CIP were collected for determination of cereulide content.

Table 3. Cereulide content in collected fractions from two parallel runs

Collected fractions Run 1/Run 2	Buffer	Cereulide conc. (ng/mL)	Amount cereulide (ng)	Amount (ng cereulide/ mg mAb)	Remaining cereulide (%)
MabSelect SuRe capture step					
Sample feed	N/A	200	11 750	83	100
Flowthrough	N/A	178/178	11 239/ 11 225	N/A	95.7/95.5
Wash	25 mM Tris, pH 7.4 + 100 mM NaCl	6/4	91/68	N/A	0.8/0.6
Elution	50 mM acetate, pH 3.7	12/11	161/159	1.2 / 1.1	1.4/1.4
Strip	100 mM phosphoric acid	6/6	76/85	N/A	0.6/0.7
Flush	25 mM Tris, pH 7.4 + 100 mM NaCl	2/2	16/12	N/A	< 0.1/< 0.1
CIP	0.1 M NaOH	< 0.01/< 0.01*	< 0.153/< 0.153	N/A	$< 1.3 \times 10^{-3}$/ $< 1.3 \times 10^{-3}$
Capto adhere polishing					
MabSelect SuRe elution pool	N/A	9.1	228	0.9	1.9
Flowthrough + wash	100 mM sodium acetate, pH 5.0 + 130 mM NaCl	< 0.01	< 0.262	< 0.001	$< 1.1 \times 10^{-3}$
Rinse	Water	< 0.01	< 0.262	N/A	$< 1.1 \times 10^{-3}$
CIP	1 M NaOH	< 0.01	< 0.262	N/A	$< 1.1 \times 10^{-3}$

* LOD = 0.01 ng/mL. N/A = not applicable.

Evaluation of cereulide carryover

As the initial capture step is exposed to high impurity levels and the protein A column is intended to be used for over hundred cycles, it is important to ensure that no carryover occurs between process cycles. To evaluate carryover, cereulide spiked mAb-containing cell culture supernatant was loaded on the MabSelect SuRe column, followed by elution and CIP using 0.1 M NaOH at a contact time of 15 min. In two subsequent runs, one blank run followed by a run with mAb-containing cell culture supernatant (no cereulide spiking), no detectable carryover between runs was observed, with no measurable amounts of cereulide in the mock eluate (run 2) or in the mAb-containing product pool (run 3) (data not shown).

Evaluation of different CIP protocols

Even though no carryover was observed using the standard CIP protocol, a low amount of cereulide (0.4%) could be extracted from the resin removed from the column (Fig 2). To prevent cereulide buildup on the column when used over several cycles, more stringent CIP protocols were evaluated. After running four MabSelect SuRe columns with cereulide-spiked mAb-containing harvest material at a load of 2.5 mg cereulide/L resin, the columns were subjected to different CIP protocols, using either 0.1 or 0.5 M NaOH for 15 and 60 min.

The results in Figure 2 show that most efficient cleaning was achieved with 0.5 M NaOH for 1 h, with 0.07% of initial cereulide content extracted from the resin. When excluding the CIP procedure, as much as 1.5% of the initial cereulide content was found on the resin. Using 0.5 M NaOH for 1 h, 0.03% remaining cereulide peptide could be detected upon extraction and sampling of column parts, whereas when excluding CIP, as much as 4% of the initial cereulide content was found on column parts. Although materials used in the laboratory-scale columns included in this study are not the same as in equipment used in larger scales, the results clearly show the importance of an efficient CIP protocol to prevent carryover of cereulide that might accumulate on the column over cycles. The later-generation MabSelect™ PrismA protein A resin allows for cleaning with up to 1.0 M NaOH, which would most probably enable even more efficient removal of trace amounts of cereluide to prevent buildup over cycles.

Study on repeatability and generality of capture step

To demonstrate consistency of the process, the capture step was repeated with the same antibody (mAb1). Generality of the capture step was confirmed with two additional mAb molecules (mAb2, and mAb3). Independent of mAb, the larger part of the cereulide content was removed in the flowthrough, with only residual amounts remaining in the elution pool (Fig 3). Although varying slightly between mAbs, the cereulide content was low in all product pools (Fig 4).

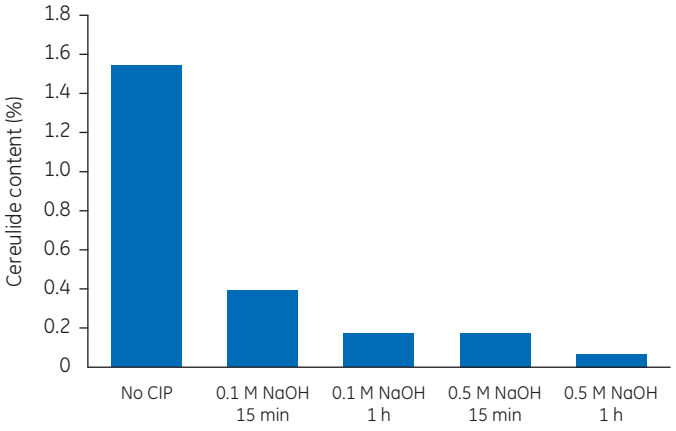


Fig 2. Evaluation of CIP strategies for the MabSelect SuRe column loaded at 2.5 mg cereulide/L resin.

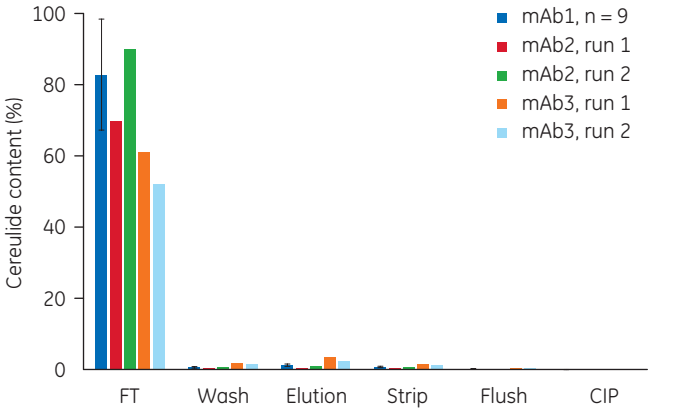


Fig 3. Cereulide content in fractions collected from the MabSelect SuRe step. Cereulide clearance was demonstrated in repeated runs (n = 9) using the same mAb1 molecule as well as in duplicate runs with mAb2 and mAb3.

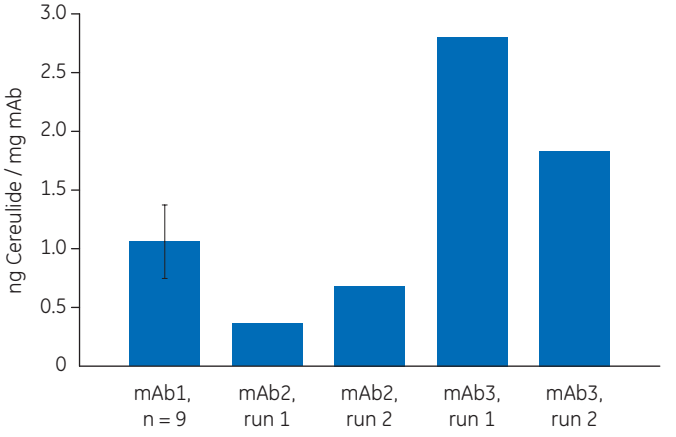


Fig 4. Cereulide content in MabSelect SuRe product pool from three different mAb molecules.

Conclusions

Here, we demonstrated clearance of the *B. cereus* exotoxin cereulide from mAb produced in CHO cells in a two-step chromatography process. The bulk of the cereulide content was removed in the initial MabSelect SuRe capture step. Residual cereulide levels could be reduced to below LOD in the subsequent polishing step using Capto adhere multimodal anion exchange resin. Even though no carryover was detected between two subsequent MabSelect SuRe cycles, trace amount of cereulide was extracted from the used resin after standard CIP using 0.1 M NaOH at a contact time of 15 min. Harsher CIP with increased NaOH concentration and contact time further reduced the amount of cereulide extracted from the MabSelect SuRe resin. Therefore, cleaning the column using a more stringent CIP protocol after a known exposure to *B. cereus* cereulide can be recommended. The MabSelect PrismA protein A resin allows for even more stringent CIP procedure, using up to 1.0 M NaOH, which would minimize the risk of cereulide buildup on the column over cycles. The in-house developed analytical assay, based on LC-MS, offered high selectivity and sensitivity in monitoring cereulide clearance over the process steps. Although a third chromatography step would potentially provide additional cereulide reduction, enabling increased process robustness, MabSelect SuRe and Capto adhere resins allow for the design of a two-step chromatography process for good cereulide clearance in the purification of mAbs.

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