

# Identification of a Cytotoxic Anti-microbial Preservative Used to Store Ultrafiltration Membranes

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## Abstract

A cytotoxic effect was identified using a CHO cell assay when a recombinant protein was processed from host biomass using ultrafiltration membranes. When ultrafiltration membranes were not utilized in the purification process, the cytotoxic effect was not observed. This led to an investigation of the cytotoxic contaminant identity. Due to limited amounts of various manufactured lots and purification fractions of the protein, a small-scale, high throughput cell suspension growth platform was used to screen fractions and to demonstrate toxicity with a CHO cell line. By combining the capabilities of manufacturing, cell culture and analytical R&D groups, a candidate cytotoxic contaminant was identified and characterized using a combination of small-scale cell culture, HPLC and mass spectrometry. The toxic effect was traced to an anti-microbial preservative used for storage of ultrafiltration membranes used in the purification process. This resulted in development of an LS/MS assay to support manufacturing in eliminating toxicity in the final, purified raw material. Successful identification of such contaminants should lead to more reliable manufacture of recombinant proteins.

## Introduction

### Goal:

- To monitor and identify a toxic substance in a purified recombinant human protein (r-Protein).

### Approach:

- Employ small scale cell culture toxicity assay to determine source(s) of toxic contaminant.
- Develop physical analytical methods to identify and quantify the toxic substance.

## Background

The use of non-animal derived recombinant proteins in bioproduction cell culture media and other biopharmaceutical products and processes has increased due to safety and regulatory concerns. However, toxic substances can become physically associated with such recombinant proteins due to the biological source material or down-stream manufacturing processes.

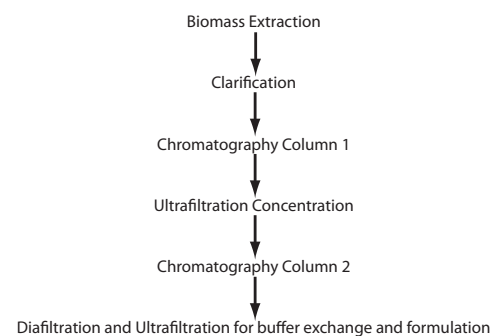
Different lots of a newly manufactured recombinant human protein were found to contain unexpected cytotoxic substances as a result of manufacturing process. The coordinated, combined efforts of manufacturing, cell culture and analytical groups were employed to accelerate the identification and removal the cytotoxic contaminant.

## Materials and Methods

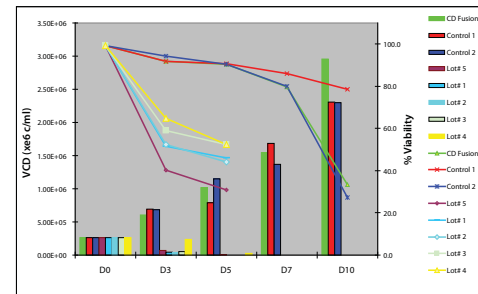
### Cell Culture

CHO-GX2 cells were grown in EX-CELL™ CD CHO Fusion medium (SAFC Biosciences Catalog No. 14365C) at 37 °C with agitation as mini-cultures in 96-well plates for ten days or until culture viability dropped below 60%. Growth and viability were measured with a Guava cell counter. Control and test recombinant proteins were added to the media at a final concentration of 0.25%.

### r-Protein Purification Scheme



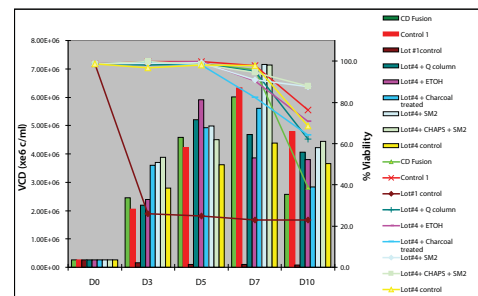
## CHO Mini-Culture Growth and Toxicity Assay



**Figure 1.** Five trial purification lots of r-Protein showed toxicity in a CHO cell mini-culture assay. Lots 1 through 4 were formulated as 20% sterile solutions. Lot 5 was formulated as a lyophilized powder. The bars on the graph represent viable cell density (VCD) and the lines represent % cell viability of the cultures. Media without protein addition and with non-toxic r-Protein derived from plants (Control 1) or from yeast (Control 2) were used as non-toxic controls.

## Toxin Removal Trial

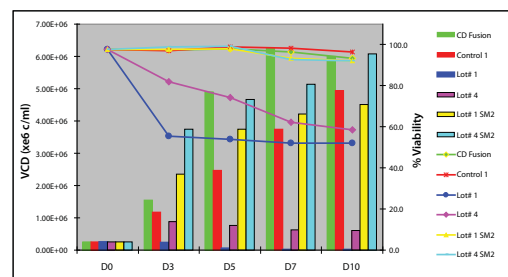
Preliminary analysis of reagents, buffers and trial purification fractions did not reveal the source of the toxin. Therefore, various methods were tested to remove the toxin from toxin-containing r-Protein.



**Figure 2:** Comparison of different methods for removal of toxin from purified r-Protein. Purified r-Protein Lot 4 from above was treated by various methods (Q Sepharose batch extraction, ethanol extraction, charcoal treatment, batch extraction with SM2 hydrophobic interaction chromatography (HIC) beads, and detergent plus SM beads extraction) to try to remove the toxic material. The r-Protein from these treatments was tested as above. The bars on the graph represent viable cell density (VCD) and the lines represent % cell viability of the cultures.

## Toxin Removal with SM2 Beads

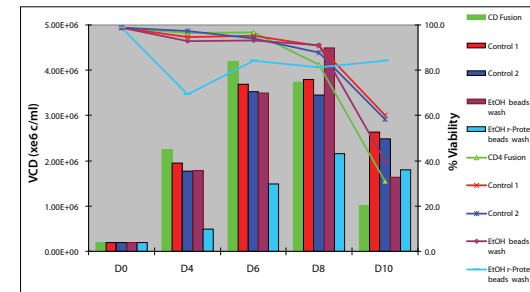
The results in Figure 2 indicated that the toxin could be removed most effectively from the purified r-Protein by treatment with SM2 beads. This was confirmed with all five toxin-containing lots of purified r-Protein.



**Figure 3:** The SM2 HIC beads removed the toxic substance from all five lots of purified r-Protein. The results of SM2 beads treatment of two of the purified r-Protein lots is shown. The bars on the graph represent viable cell density (VCD) and the lines represent % cell viability of the cultures.

## Extraction of Toxin from SM2 Beads

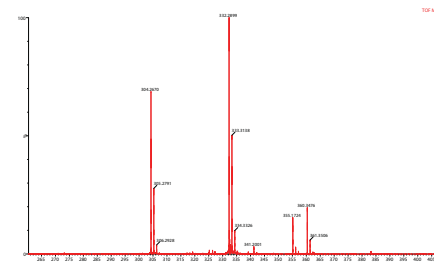
The SM2 HIC beads that had been used to remove the toxin from the purified r-Protein were subsequently extracted with ethanol in order to obtain toxic material for analysis and possible identification.



**Figure 4.** Toxic material was successfully extracted from the SM2 beads used to remove the toxin from the purified r-Protein. The bars on the graph represent viable cell density (VCD) and the lines represent % cell viability of the cultures. On days 4 through 8 the ethanol extract of the SM2 beads from the r-Protein treatment showed a negative effect on both cell growth and viability that was not seen with the ethanol extract of naive SM2 beads.

## Identification of a Candidate Substance by Mass Spectrometry

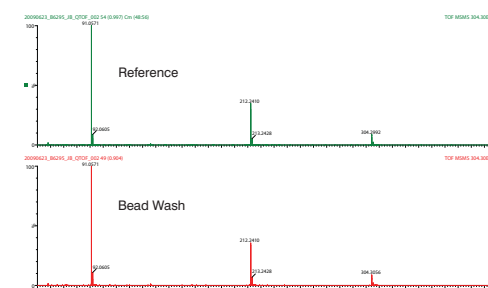
Comparison of the MS spectra of the ethanol extracts of naive SM2 beads versus r-Protein treated SM2 beads allowed the identification of a peak with an accurate mass corresponding to the molecular mass for benzalkonium chloride (BAC).



**Figure 5.** A series of unique ions were identified by MS in the ethanol extract of the SM2 beads used to remove the toxin from the purified r-Protein. The ethanol extracts for naive SM2 beads and SM2 beads exposed to the toxic, purified r-Protein were analyzed by direct infusion ESI-MS. The exposed bead extract spectrum shows prominent ions at m/z 304, 332, and 360.

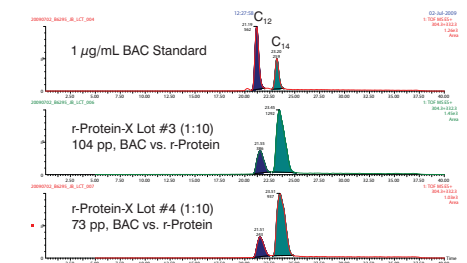
## MS-MS Identified Benzalkonium Chloride

The ions at m/z 304, 332, and 360 in the r-Protein SM2 bead wash were selected for MS-MS analysis, revealing a series of related structures. In combination with exact mass of the parent ions, the peaks were identified as C<sub>12</sub>/C<sub>14</sub>/C<sub>16</sub> homologs of Benzalkonium Chloride (BAC). Mass spectral data matched well with authentic BAC reference material, typically supplied as C<sub>12</sub>-C<sub>16</sub> homologs.



**Figure 6.** MS-MS analysis revealed that the spectra for the m/z 304 peak from the r-Protein SM2 bead wash and authentic benzalkonium chloride were identical. Samples were analyzed by direct infusion, as above.

## Quantitation of BAC in Samples



**Figure 7.** LC-MS analysis allowed for determination of the amount of BAC in purified r-Protein samples. Extracted ion chromatograms were generated for C<sub>12</sub> and C<sub>14</sub> homologs of BAC. Summed sample peak areas vs. reference were used to quantify BAC.

## Confirmation of BAC Toxicity with Control 1

A series of different concentrations of BAC were incubated with non-toxic r-Protein Control 1. The BAC-loaded samples were analyzed in the CHO mini-culture growth assay. The samples showed a dose dependent toxicity effect.

Viability Average %	Day 0	Day 3	Day 5
CD Fusion Medium Control	99.8	94.8	92.2
r-Protein Control 1	99.8	97.1	95.4
r-Protein Control 2	99.8	95.0	94.3
r-Protein Lot 3	99.8	63.0	26.8
r-Protein Lot 4	99.8	79.8	62.1
Control 1 + BAC 3ug/L	99.8	87.6	87.8
Control 1 + BAC 12ug/L	99.8	83.3	79.8
Control 1 + BAC 50ug/L	99.8	62.0	57.2
Control 1 + BAC 100ug/L	99.8	59.3	43.3
Control 1 + BAC 200ug/L	99.8	33.4	34.7
Control 1 + BAC 400ug/L	99.8	38.3	31.9
Control 1 + BAC 600ug/L	99.8	32.3	27.8
Control 1 + BAC 800ug/L	99.8	30.0	20.4

## Source of Toxin

It was subsequently determined that BAC is used as an anti-microbial preservative by the manufacturer of the diafiltration devices used in the last step of the r-Protein purification procedure.<sup>1</sup>

## Conclusions

- A CHO cell mini-culture assay was developed for analysis of limited amounts of purified recombinant proteins and purification fractions for cell toxicity. This enabled the analysis of r-Protein purification fractions and toxin removal strategies.
- The toxin could be removed from the contaminated r-Protein with SM2 hydrophobic interaction chromatography beads and could be eluted with ethanol from SM2 beads exposed to toxic r-Protein.
- Benzalkonium chloride (BAC) was identified in the ethanol eluates by MS-MS analysis and confirmed by comparison with purified BAC. An LC-MS method was developed that allowed quantitation of BAC in r-Protein.
- BAC was confirmed as the toxic substance by loading onto non-toxic r-Protein and subsequent CHO mini-culture growth analysis.
- The identification of BAC as the toxic contaminant and the assays developed during this work will allow analysis and manufacture of non-toxic r-Protein for cell culture and other biopharmaceutical applications.
- This work demonstrates the power of coordinated efforts by a cross-functional team to solve an unanticipated manufacturing problem.

## Acknowledgements

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## References

Vendor MSDS Sheet #M113288 and #M113290.