Effective Removal of Negatively Charged Interfering Molecules From Proteins

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Abstract

Endotoxins are the most common pyrogenic substances. They are negatively charged lipopolysaccharides that cause fever when injected into animals and humans. Endotoxins are produced by gram negative bacteria such as *E. coli* and they must be removed from purified proteins prior to use in therapeutic applications. Endotoxins have been shown to perturb in vivo and in vitro experiments, even at concentrations of a few ng/ml.

Since the endotoxins are negatively charged, anion exchange chromatography is often employed as a means of removing them. Two factors governing the success of this approach are the affinity of the endotoxins for the particular protein produced, and the affinity of the endotoxin for the particular anion exchange support employed.

This tech note discusses three cases of the removal of endotoxin/B-(1,3)-D-glucans and small negatively charged molecules (SNCM) from proteins. The sequence of cases presented below represent increasingly difficult endotoxin removal scenarios due to increasing interaction between the endotoxins/SNCM and the protein of interest.

Case I illustrates the removal of endotoxin/B-(1,3)-D-glucans using various chromatography supports from an extracellular/recombinant protein produced by yeast fermentation. Macro-Prep® High Q anion exchange support was the most efficient remover of endotoxins; the reduction of endotoxin (EU/ml) was on the order of 2 logs. In case II, removal of SNCM is described. The masking of positive charges on a protein produced in *E. coli* by endotoxins/SNCM is suggested as the cause of poor binding (5% of activity bound) to a cation exchanger compared to the native protein produced in *P. vulgaris*. Treatment of the *E. coli* protein with Macro-Prep High Q anion exchange support to remove endotoxin/SNCM improved binding on a cation exchanger (90% of activity bound).

Case III discusses the removal of endotoxins from a lipophilic protein produced by a recombinant *E. coli* fermentation. The lipopolysaccharides have a very high affinity for the lipophilic protein. Anion exchange chromatography did not remove endotoxin from the protein, however the protein did separate on the Macro-Prep High Q anion exchange support based on the amount of endotoxin associated with the protein. The greater the amount of associated endotoxin, the later the protein eluted in the salt gradient. Thus, a peak which contained 20% of the activity eluted early in the gradient with 99.99% less endotoxin (EU/mg) than the starting material. Subsequent methods development led to a process in which the endotoxins could be removed from the denatured protein followed by refolding into an active state.

Materials and Methods

The level of endotoxin in the column load and collected fractions was measured using the LAL reagent Pyrotell (Associates of Cape Cod). Macro-Prep High Q anion exchange support was obtained from Bio-Rad Laboratories, Inc. Q Sepharose FF, DEAE Sepharose, and DEAE Sephadex were obtained from Pharmacia. A 500 and Cellufine Sulfate were obtained from Amicon. Toyopearl DEAE-650 M was obtained from Toso Hass*. DEAE Trisacryl Plus was obtained from BioSepra Inc*.

All glassware were soaked in 1 M NaOH and all ion exchange supports were soaked in 0.1 N NaOH followed by rinsing to remove any endotoxin contamination. All buffers were prepared from Nanopure water and used fresh.

* This study was conducted in 1997. Currently, BioSepra products are carried by Pall Corporation. Pharmacia products are carried by GE Healthcare. Amicon products do not seem to exist today. Toso Hass products are available with Tosoh Bioscience LLC.

Case I

Endotoxin/B-(1,3)-D-Glucan Removal from Protein X, A Secreted Protein Produced by Yeast

The Limulus amebocyte lysate (LAL) test is extremely sensitive to endotoxin and is considered to be specific for lipopolysaccharide (LPS). The LAL test quantitates the amount of endotoxin in terms of endotoxin units (EU). Yeast does not contain lipopolysaccharides, however, a component of the yeast cell wall, B-(1,3)-D-glucan, does generate a positive signal in the LAL test. In the development of a process for the purification of protein X (MW of 15.4 kD, pI 6.5) from yeast, high endotoxin levels were encountered. Two possible sources responsible for the high endotoxin levels measured were LPS from microbial contamination of the fermentation and B-(1,3)-D-glucan from the yeast cell wall. Microscopic examination of the fermentation indicated no bacterial contamination.

Chromatographic supports that had been successful in removing endotoxin from other protein preparations failed to provide satisfactory endotoxin removal from protein X produced in yeast. Therefore, a panel of supports was evaluated to identify the top performing support to determine which would be used for endotoxin polishing. Twenty supports were initially screened. Six of these supports were selected for further evaluation. These supports were equilibrated in PBS, pH 7.4, for endotoxin polishing of 350 ml of protein X which had been previously passed through a Q Sepharose FF gel column operating in the flowthrough mode. Five ml of sample was passed over each column. The eluates were collected and assayed for EU. Figure 1 shows these results.

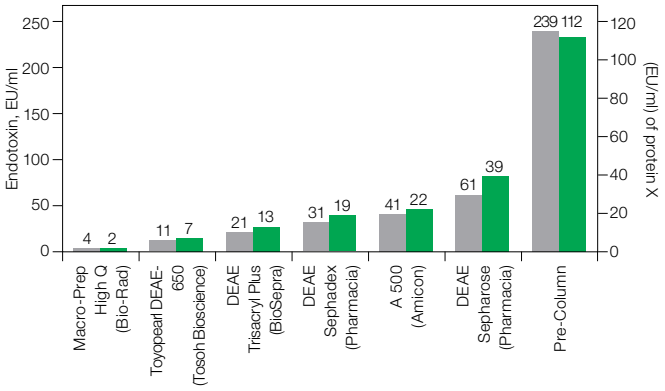


Fig. 1. Results from the evaluation of six different anion exchange supports for endotoxin removal from protein X. ■, endotoxin (EU/ml), %, ■, (EU/ml) of protein X. Note: This study was conducted in 1997. For the availability of these products refer to the note in Materials and Methods section.

Macro-Prep High Q strong anion exchange support was chosen for the endotoxin polishing step because it resulted in the lowest ratio of endotoxin per mg of protein X. The polishing step was performed on a 100 ml column that had been washed with 0.1 N NaOH and then equilibrated with PBS, pH 7.2. The concentrated eluate from the previous anion exchange step was diluted to 3.2 mg/ml and loaded at 5 ml/min. The flowthrough fraction, containing protein X, was collected.

Figure 2 summarizes the results of the Macro-Prep High Q anion exchange chromatography which reduced the endotoxin level from 650 EU/ml to 1.29 EU/ml with a product yield of 84%.

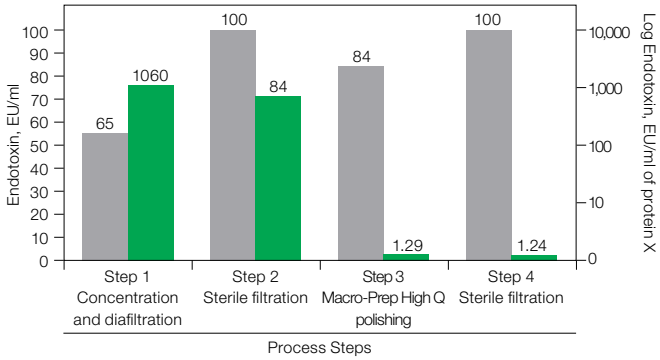


Fig. 2. Summary of results from endotoxin polishing from protein X. ■, step yield, %, ■, log endotoxin, EU/ml of protein X.

Case II

Removal of Small Negatively Charged Molecules From Protein Y, A Bacterial Enzyme Expressed in *E. coli*

This case illustrates the removal of small negatively charged molecules (SNCM) from protein Y (MW 112 kD, pI 8.4). The substances responsible for the anomalous chromatographic behavior discussed below are referred to as SNCM because no work was performed to determine their identity, although the most likely candidates are either endotoxins or small fragments of DNA.

In the development of a recombinant production method for protein Y in *E. coli*, a significant difference between the chromatographic behavior of protein Y produced in recombinant *E. coli* and the native host was observed. Ninety-five percent of protein Y produced by the native host bound to Cellufine Sulfate in 20 mM phosphate, pH 7.2. However, only 10% of protein Y produced by *E. coli* was bound under these conditions and it appeared as though protein Y produced in *E. coli* was less basic than the protein produced in the native host.

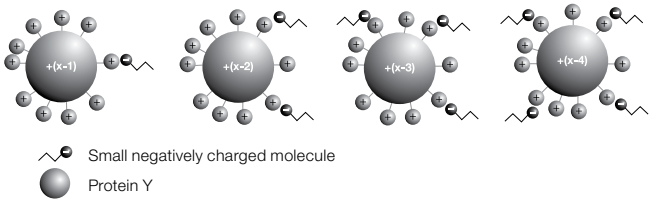


Fig. 3. Schematic representation of the hypothetical masking of positive charges on protein Y by small negatively charged molecules.

Protein Y, from the native host, is basic because it possesses a net excess of positive charges at neutral pH. Protein Y produced in *E. coli* is less basic because some of the positive charges were masked by SNCM that bind with high enough affinity to copurify. Figure 3 and Table 1 illustrate the theoretical masking of positive charges by SNCM, resulting in populations of protein Y carrying different charges. Furthermore, the copurification of SNCM with protein Y produced in *E. coli* alters binding and elution behavior.

Table 1. Relationship between hypothetical masking of positive charges on protein Y by small negatively charged molecules and binding to a cation exchanger. Assume X positive charges on unmasked *E. coli* protein Y.

# of Positive Charges	Binding	Chromatographic Behavior
X	Strongest	Complete binding even at pH 7.2, 20 mM phosphate
X-1	–	–
X-2	–	–
X-3	–	Binding at pH 6.8, 5 mM phosphate, but no binding at pH 7.2, 20 mM phosphate
X-X	No binding	

This hypothesis was tested by incubating protein Y prepared by two different processes with Macro-Prep High Q support. Macro-Prep High Q support was expected to remove the negative charged molecules that masked the positive charges of protein Y, thus restoring its basicity. As a result of treating the samples with Macro-Prep High Q anion exchange support, binding for protein Y prepared by the process which involved acid precipitation increased from 5% binding to 100% and binding for the other process increased from 1% binding to >95% binding. Figures 4 and 5 show the dramatic improvement in the percent of activity binding to the column and the improved peak shape for *E. coli* protein Y when run on a cation exchanger before and after treatment with Macro-Prep High Q anion exchange support.

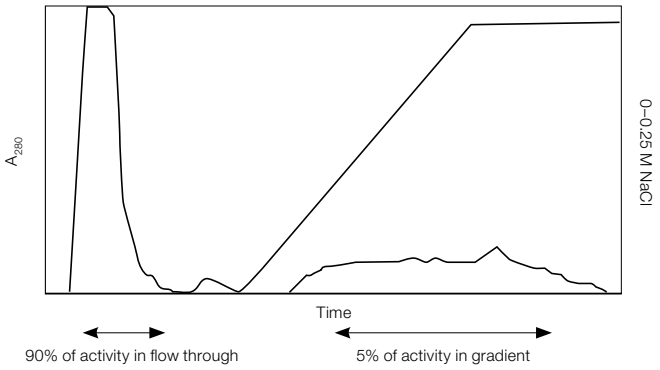


Fig. 4. Cation exchange chromatography in 20 mM phosphate, pH 7.2 of *E. coli* protein Y before treatment with Macro-Prep High Q anion exchange support.

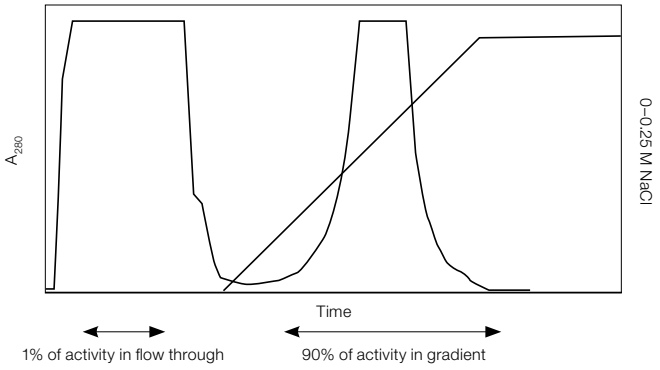


Fig. 5. Cation exchange chromatography in 20 mM phosphate, pH 7.2 of *E. coli* protein Y after treatment with Macro-Prep High Q anion exchange support.

This dramatic improvement in binding and peak shape following treatment with Macro-Prep High Q anion exchange support to remove SNCM supports the hypothesis that the copurification of protein Y with SNCM is the cause of poor binding in the untreated sample.

Case III

Removal of Endotoxin from Protein Z, A Serum Protein Involved in Lipid Transport

This case represents a high level of difficulty in removing endotoxins because of the high affinity between the lipophilic protein Z (MW 28.3 kD, pI ~6) and endotoxin. Traditional organic solvent extraction methods for removal of lipids bound to lipoproteins reduced the endotoxin by 97%, which was not sufficient. When a protein Z preparation produced in *E. coli* was separated on the Macro-Prep High Q anion exchange support, the endotoxins were not removed from protein Z and the protein was fractionated based on the amount of bound endotoxin. Protein Z molecules containing low levels of endotoxin elute at low salt concentrations, while protein containing more bound endotoxin elutes later in the gradient. Figure 6 shows protein Z run on a 15 ml Macro-Prep High Q anion exchange support column equilibrated at pH 7.2 with 20 mM sodium phosphate and eluted with a 0–400 mM NaCl gradient. The bar graph overlaid on the chromatogram represents the ratio of endotoxin per mg of protein Z in various fractions. The fractions indicated by the last three bars contained 51,000, 923,000, and 1,000,000 EU/mg. Peak 1 contained only 0.014% of the starting endotoxin. The protein recovered from this peak contained 20% of the protein Z in the load.

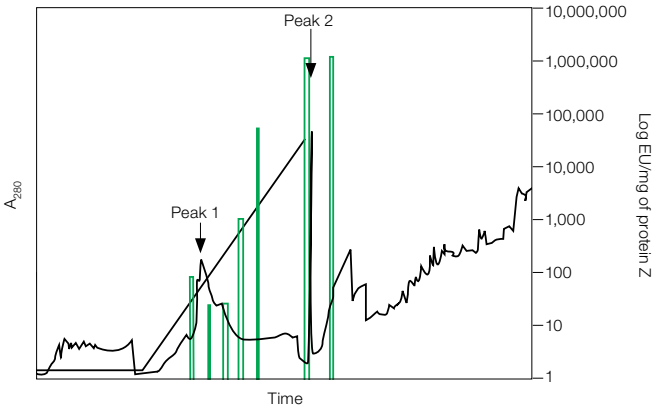
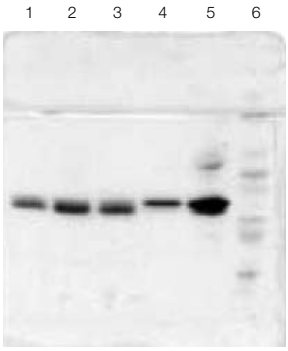


Fig. 6. Protein Z chromatographed on Macro-Prep High Q with overlay of endotoxin per mg of protein Z in pooled fractions. Note increasing levels of endotoxin associated with late eluting peaks.

Bands on the SDS-PAGE analysis of the fractions show protein Z eluted throughout the gradient (Figure 7). Since protein Z migrated to the same position in fractions containing different amounts of endotoxin, it appeared that denaturation with SDS or other denaturants might be a viable means of stripping endotoxin from the protein. Therefore, denaturation of protein Z was investigated as a method of removing endotoxin from the protein.



Lane 1 Pooled Peak 1
Lane 2 Pooled region between Peak 1 and Peak 2
Lane 3 Pooled Peak 2
Lane 4 Protein Z Standard
Lane 5 Load on column before chromatography
Lane 6 MW Standard

Fig. 7. SDS-PAGE analysis of pooled peaks from protein Z separated on Macro-Prep High Q Support. (See Figure 6.)

Endotoxin contaminated (41,000 EU/mg) protein Z (857 mg) was denatured in to 90 ml of 20 mM sodium phosphate, 7.5 M urea pH 7.4, centrifuged, and loaded onto a column packed with 420 ml Macro-Prep High Q strong anion exchange support. Figure 8 shows the chromatogram from the NaCl gradient elution. The flowthrough fraction contained highly pure protein (99.9% endotoxin removal and 88% recovery) based on UV spectra obtained from scans between 220 nm and 320 nm and SDS-PAGE. The low salt fraction contained small amounts of protein Z, along with other contaminating proteins. The small shoulder of the peak which was eluted in the high salt contained protein, but the larger, main peak had a spectra that corresponded to large amounts of nonprotein material. This peak also contained high levels (>1.3 million EU/mg protein) of endotoxin. The biological activity of protein Z was restored by removal of urea by dialysis. Table 2 summarizes the protein recovery and endotoxin reduction data for the process in which 77% of the protein was recovered and 99.99 % of the endotoxin was removed.

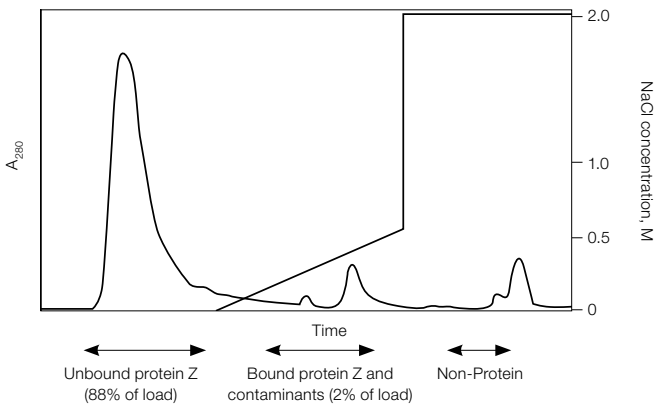


Fig. 8. Denatured protein Z purified on Macro-Prep High Q under nonbinding conditions. See Table 2 for endotoxin reduction data.

Table 2. Results of endotoxin reduction for protein Z run on Macro-Prep High Q support under denaturing conditions.

Sample	Protein Z, mg	EU x 10^3	EU/mg Protein Z
Load	857	35,100	40,960
Unbound pool	758	29	38.3
Gradient pool	~20	<1	–
2 M NaCl strip	<5	6,680	>1.3 x 10^6
Final lyophilized preparation	662	3.5	5.3

Summary

Three cases of increasingly difficult endotoxin/SNCM removal were studied. In the case of protein X, there was little to no interaction between endotoxin/SNCM and the protein Macro-Prep High Q anion exchange support proved superior to other anion exchangers in its ability to remove endotoxin/SNCM. In one experiment, Macro-Prep High Q anion exchange media reduced the endotoxin/SNCM level from 650 to 1.3 EU/ml.

In the second case, the affinity between protein Y produced in *E. coli* and endotoxin/SNCM was strong enough that gel filtration and cation exchange chromatography could not remove the endotoxin. Prior to treatment with Macro-Prep High Q anion exchange support, only 5% of protein Y from *E. coli* bound to a cation exchanger. Following treatment with Macro-Prep High Q anion exchange support to strip contaminants from the protein, 90% of the protein Y activity bound and eluted in a sharp peak from a cation exchange support. In the third example, no chromatographic techniques were found that could remove endotoxin from protein Z. Analysis of fractions from Macro-Prep High Q anion exchange showed the later a fraction eluted, the more endotoxin was associated with protein Z. SDS-PAGE revealed that all fractions eluting throughout the gradient contained protein Z. Peak 1 contained protein Z with an endotoxin content of 25.2 EU/mg. This represents a 99.99% removal of the initial endotoxin and a 20% yield. Protein Z eluting later in the gradient had endotoxin levels up to 1,000,000 EU/mg. Chromatography of protein Z which had been denatured with urea and refolded resulted in a 77% protein Z recovery and a 99.99% reduction in endotoxin.

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