# EndoBind-R™

# Endotoxin Removal from Proteins using EndoBind-R™

*To make protein samples suitable for animal studies and cell culture, contaminating pyrogens, such as lipopolysaccharide (endotoxin) from gram negative bacteria, must be removed. Here we describe the use and optimization of a peptide with high affinity and specificity for endotoxin in column chromatography to produce endotoxin-free samples with high protein recovery.*

- High protein recovery
- High specificity for endotoxin
- Endotoxin removal from low levels of contamination
- Rapid endotoxin removal
- Resistant to a wide range of buffer conditions
- Easy to use


BioDtech's EndoBind-R™

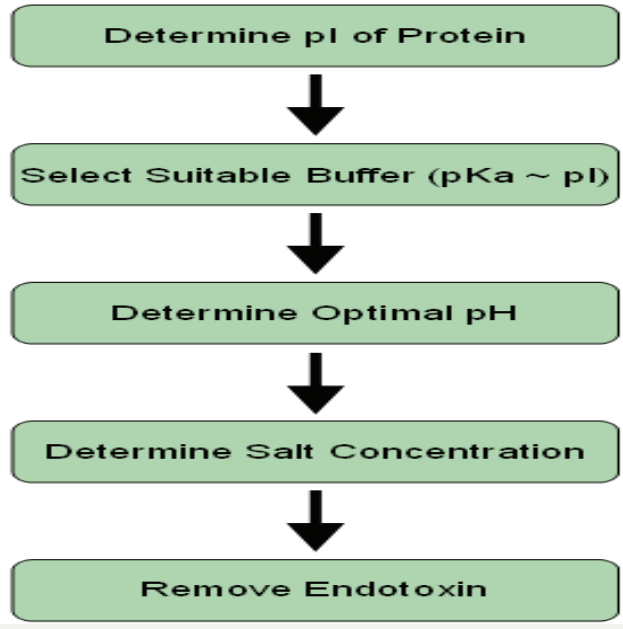
## Introduction

Removal of endotoxin from solutions for animal studies, cell culture, transplantation, stem cell technologies, cell sorting, and other mammalian cell treatments is a priority. The majority of lipid in the outer membrane of gram negative bacteria consists of lipopolysaccharide (LPS), also called endotoxin. Sub-nanogram levels of endotoxin can trigger immune responses and alter the phenotype and function of many cells including monocytes, neutrophils, dendritic cells, hepatocytes, vascular and respiratory epithelium, and arterial smooth muscle cells. For years, the *Limulus* amoebocyte lysate (LAL) test has been the standard for detecting even trace amounts of endotoxin. This test was developed from observations that horseshoe crab amoebocytes aggregate and degranulate in response to LPS as a defense mechanism against gram-negative bacteria [1,2]. This degranulation releases a series of enzymes that include Factor C, the initial activator of a serine protease cascade [3,4]. In the LAL assay, Factor C detects picogram levels of LPS and initiates a clotting reaction. In recent years, the assay has been modified for detection with fluorescence, colorimetry, and turbidity, which made it more quantitative and less open to interpretation. Recently, a 34 amino acid LPS-binding Sushi domain was identified in Factor C. Expression and characterization of this linear peptide showed high binding to ($K_d$ $10^{-6}$-$10^{-8}$) and neutralization of ($ENC_{50}$ 2.25 µM) LPS [5]. BioDtech's **EndoBind-R™** is a DADPA-agarose-conjugated S1 peptide affinity chromatography column. It has been used to remove endotoxin from water, buffers, and cell culture media. Under optimized conditions, it may also be used to produce endotoxin-free protein solutions with high recovery. Additionally, the S1 peptide is highly resistant to a wide range of pH's and ionic strengths making it suitable for many applications in which traditional chromatography methods, such as ion exchangers, affinity ultrafiltration, and immunoaffinity matrices are not applicable [6,7].

In this report protein recovery and endotoxin removal with **EndoBind-R™** were maximized by optimizing buffer conditions. The initial binding between the S1 peptide and LPS is due to electrostatic interactions between the positive residues near the N-terminus of the peptide and the negatively charged phosphoryl head groups of LPS. After this initial binding, hydrophobic interactions between the C-terminal end of S1 and the acyl chains of LPS strengthen the binding [8]. To optimize recovery conditions, buffer pH was adjusted near the isoelectric point (pI) of the protein being purified to minimize electrostatic interactions. Next, the salt concentration required for optimal protein recovery was established. Finally, using optimized buffer conditions, endotoxin removal with **EndoBind-R™** was measured. Since the S1 peptide is unaffected by a wide array of buffer parameters, protein-specific conditions were easily established that provided both high protein recovery and endotoxin removal to levels below 0.01 EU/ml.

## Materials and Methods

Protein elution was performed at room temperature using BioDtech's **EndoBind-R™** with a column volume of 1 ml. Solutions were added to the column in 1 ml aliquots and collected with gravity flow in endotoxin-free glass tubes. The column was equilibrated with buffer before use and was cleaned with washes of 2M sodium chloride and 2% sodium deoxycholate before and after each use and stored in 0.02% sodium azide at 4°C. Salt and pH optimization experiments were done by applying 1 ml of the sample load to the column and collecting the flow-through. Next, the column was washed with two volumes of corresponding buffer with variable salt concentrations. The protein content of the load was compared to the 2 ml wash and recovery was calculated. Endotoxin removal experiments were done by applying 1 ml of the sample load (termed fraction 0) to the column and collecting the flow-through as fraction 1. Next, the column was washed with 4 sequential 1 ml volumes of the corresponding salt-containing buffer and collected as fractions 2-5. The protein and endotoxin content of the load and each fraction were determined and protein recovery and endotoxin removal were calculated.




Endotoxin levels were determined with the PyroGene Recombinant Factor C Endotoxin Detection System (Lonza, Walkersville, MD) according to manufacturer's specifications. Samples were tested at multiple dilutions and the absence of inhibition/enhancement was verified with endotoxin spikes. Protein concentrations were determined by absorbance at $OD_{280}$ as measured by the Synergy II plate reader (BioTek, Winooski, VT). Bovine liver catalase, human apo-transferrin, rabbit IgG, and hemoglobin from bovine erythrocytes were purchased from Calbiochem (La Jolla, CA). Bovine serum albumin was purchased from Sigma (St. Louis, MO). All samples, buffers, and chemicals were prepared using Pyrogen-Free Water (available from BioDtech).

## Results

*The Effect of pH on Recovery of Bovine Serum Albumin*. Several 1 mg/ml samples of bovine serum albumin (BSA) were prepared in solutions of 20 mM sodium acetate at five pH's ranging from 4.0 to 6.0 with 200 mM sodium chloride. Given the importance of electrostatic interactions in endotoxin removal, buffer pH near 4.6, the isoelectric point of the BSA, was targeted. Sodium acetate was chosen as a buffering agent since it has a pKa near the pI of BSA. The protein content of the salt washes and the load were determined and plotted as percent of BSA recovered against the range of pH's tested (Figure 1*A*). At a pH of 4.5 or below, there was poor BSA recovery. In a pH range from 5.0 to 5.5, there was a significant change in protein recovery from below 10% for pH 4.5 to over 70% for pH 5.0. This drastic change in recovery as buffer conditions crossed the pI indicated the importance of electrostatic interactions in chromatography with **EndoBind-R™**. At a pH of 6.0, there is a small decrease in protein recovery as the pH moves further away from the pI.

*The Effect of Salt Concentration on Recovery of Bovine Serum Albumin*. In the previous experiments, 200 mM sodium chloride facilitated recovery of BSA under optimum pH conditions. Next, the effect of various concentrations of sodium chloride on BSA recovery was tested. The previous results indicated that a pH of 5.0 provided efficient protein recovery while maintaining an environment close to the pI of BSA. A 1 ml sample of 1 mg/ml BSA in 20 mM sodium acetate at pH 5.0 was applied to the **EndoBind-R™** column and the flow-through was collected. Next, the column was rinsed with two 1 ml washes of buffer containing varying amounts of sodium chloride. As before, the protein concentration in the washes was compared to the initial load sample and plotted as percent of BSA recovered against the concentration of sodium chloride in the wash (Figure 1*B*). Without salt, less than 1% of BSA was recovered indicating the need to weaken electrostatic interactions or to enhance the hydrophobicity of the protein. As the amount of salt was increased, there was a positive correlation in BSA recovery to a concentration of 150 mM, when the recovery begins to level off at about 95%. This indicates that the minimum optimal amount of sodium chloride to recover BSA in a 20 mM sodium acetate solution at pH 5.0 is 150 mM.

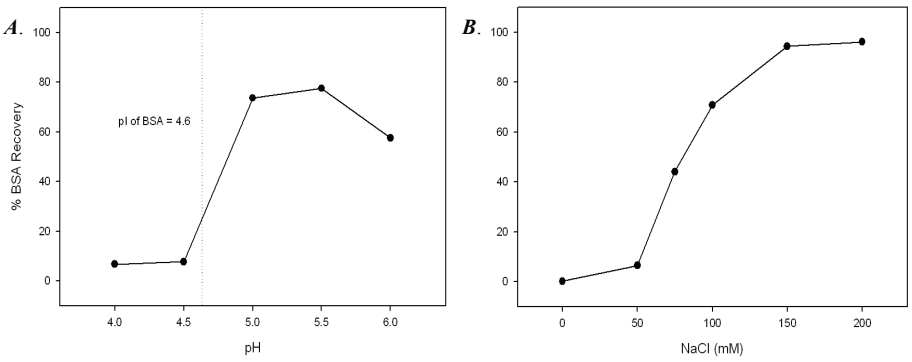



**Figure 1. The Effect of pH and Salt on BSA Recovery.** The effect of pH (*A*) and salt concentration (*B*) was determined using 1 mg/ml samples of BSA in 20 mM sodium acetate. The salt concentration in (*A*) was held constant at 200 mM. The pH in (*B*) was held constant at 5.0

*Removing Endotoxin from Bovine Serum Albumin*. By combining the results of the previous two experiments, suitable sample conditions for BSA recovery were established. Next, endotoxin removal from BSA at these conditions was measured. A 1 mg/ml solution of BSA was prepared in 20 mM sodium acetate at pH 5.0 containing 150 mM sodium chloride. The low endotoxin BSA was tested for contaminating endotoxin and found to be a rather low value of about 0.05 EU/mg. *E. coli O55:B5* endotoxin at a concentration of 50 ng/ml (500 EU/ml) was added to the protein solution and purified with **EndoBind-R™**. The flow-through, fraction 1, contained about 16% of the initial protein (Figure 2). However, the majority of

BSA eluted into the first wash, fraction 2, as a 77% protein peak. Fractions 3 through 5 combined contained less than 6% of the initial BSA load. The LPS content in the sample load measured 506 EU/ml and was reduced to below the detection limit of 0.01 EU/ml in all five column fractions. This represents more than 99.998 % LPS removal and over 99% protein recovery after purification with the **EndoBind-R™** column. Even the 0.05 EU/ml contaminating endotoxin was removed from the starting material. In addition, the low level of residual endotoxin is more than 25-fold below the allowable endotoxin limit of 0.25 EU/ml established by the Food and Drug Administration for injectibles [9].

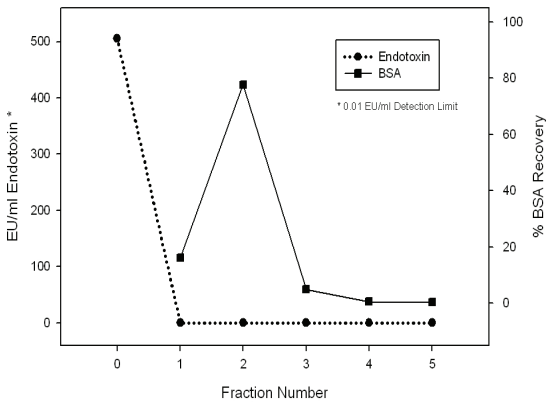



**Figure 2. Endotoxin Removal from BSA.** BSA samples at 1 mg/ml were prepared in 20 mM sodium acetate at pH 5.0 with 150 mM sodium chloride and applied to EndoBind-R™. The protein was recovered in four subsequent 1 ml washes. Protein recovery was determined by absorbance and endotoxin levels were determined by PyroGene (Lonza) assay.

*Purifying Other Common Proteins with **EndoBind-R™***. The methods described above to purify BSA were applied to a variety of proteins to show how optimization of buffer conditions can produce high protein recovery and dramatically reduced endotoxin levels in several commonly used proteins.

<u>*Bovine Liver Catalase*</u>

Catalase is an extremely efficient enzyme that catalyzes the decomposition of hydrogen peroxide into water and gaseous oxygen. It is often used in cell culture applications to act as an antioxidant. Bovine liver catalase (BLC) is a 240 kDa tetramer with a pI of 5.4. Recovery was determined with 1 mg/ml BLC solutions in 20 mM sodium acetate at pH's of 5.0, 5.5, and 6.0. Washes with 0 and 50 mM salt eluted no significant BLC (Data Not Shown). Raising the salt concentration to 100 mM produced only slight protein recovery (Figure 3*A*). However, with 200 mM high levels of BLC were recovered when the pH conditions were greater than the pI. At a pH of 5.0, which is below the pI, almost no protein is removed from the column even with 200 mM salt conditions. When the pH was increased above the pI there was an increase to 70-85% of the initial load.

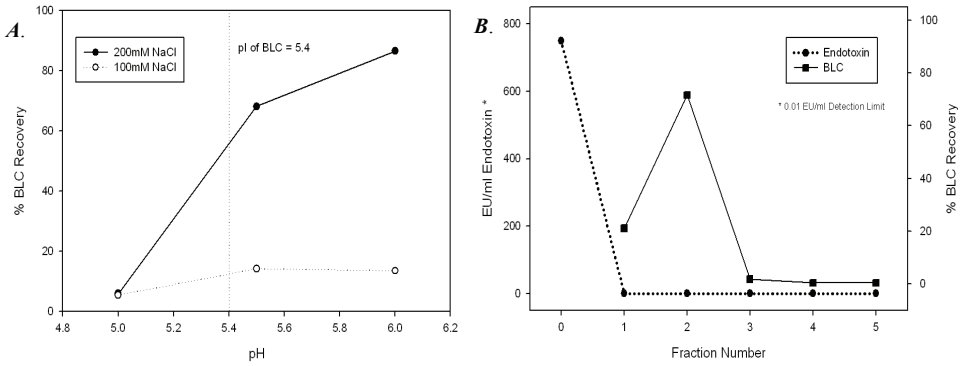



**Figure 3. Recovery Optimization and Endotoxin Removal from BLC.** (*A*) Optimal salt and pH conditions for BLC recovery were determined using 1 mg/ml samples in 20 mM sodium acetate. (*B*) Endotoxin removal was determined in 20 mM sodium acetate at pH 6.0 containing 200 mM sodium chloride.

A sample of 1 mg/ml BLC in 20 mM sodium acetate at pH 6.0 with 200 mM sodium chloride was prepared with 25 ng/ml (250 EU/ml) *E. coli O55:B5* endotoxin. In addition, the BLC stock solution contained nearly 500 EU/mg endotoxin from an unknown source. Correspondingly, the sample load contained 751 EU/ml. After purifying the sample with **EndoBind-R™**, this was reduced to levels below

detection (0.01 EU/ml) in all fractions (Figure 3*B*). Nearly all of the protein eluted in the first two samples with 21% in the first fraction and a peak value of over 70% in the second. In total, more than 95% of the BLC protein load was recovered with removal of over 99.998% of all contaminating endotoxin.

*Human Transferrin*

Transferrin (Tf) is an iron-transporting glycoprotein which exists in several different forms depending on how many of its iron-binding sites are filled. It is a common component of mammalian cell cultures and is required for proper growth. It is also commonly used to chelate metal ions in culture. When it is in the iron-free form, it is termed apo-Tf and has a pI of 5.9. When the enzyme is iron-saturated it is called holo-Tf and has a pI of 5.2. Human apo-Tf was purified from endotoxin using **EndoBind-R™** and therefore buffer conditions around a pH of 6.0 were targeted. However, because any iron-containing Tf would lower the pI, a range of pH from 4.5 to 6.0 was employed to broaden the focus. Surprisingly lower pH conditions lended to better Tf recovery (Figure 4*A*). At a pH of 5.0 or below, significant Tf was recovered even in the absence of salt. At pH levels above 5.0, Tf was only recovered with 100 mM or more of sodium chloride. These results may indicate that the protein preparation was contaminated with holo-Tf or the media had significant free iron ions to convert a majority of the apo-Tf to the holo-Tf form. Even with the complications in Tf, high levels (>99%) could be recovered under the right buffer conditions.

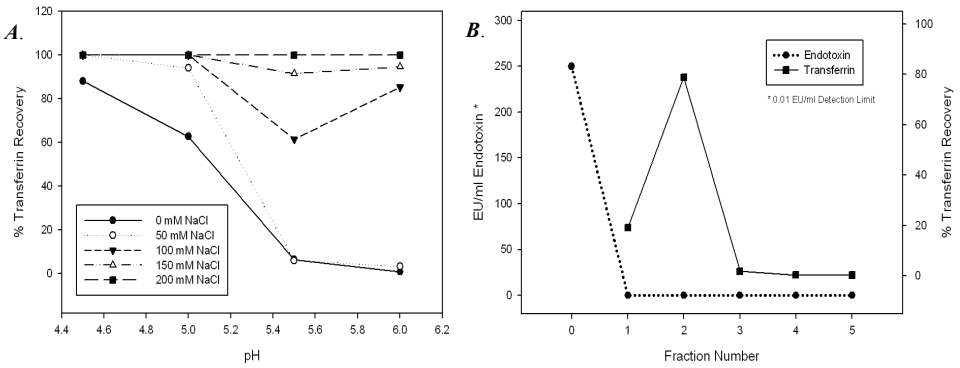



**Figure 4. Recovery Optimization and Endotoxin Removal from Human Transferrin.** (*A*) Optimal salt and pH conditions for transferrin recovery were determined using 1 mg/ml samples in 20 mM sodium acetate. (*B*) Endotoxin removal was determined in 20 mM sodium acetate at pH 5.0 containing 100 mM sodium chloride.

Using conditions of 20 mM sodium acetate at pH 5.0 with 100 mM sodium chloride, Tf recovery and endotoxin removal were determined. The contaminating endotoxin level of Tf was about 0.5 EU/mg, two orders of magnitude better than BLC. A 1 ml sample of 1 mg/ml Tf with 25 ng/ml *E. coli O55:B5* endotoxin was applied to **EndoBind-R™** and washed. The sample load contained 244 EU/ml but was reduced to levels below the assay detection limit (0.01 EU/ml) in all fractions after purification with **EndoBind-R™** (Figure 4*B*). As with the other proteins tested, Tf eluted into the first two samples with about 19% in fraction 1 and a peak value of nearly 80% in fraction 2. In all fractions combined, over 99% of the protein was recovered with removal of over 99.995% endotoxin, well below the endogenous endotoxin levels.

*Rabbit IgG*

IgG is the predominant immunoglobulin present in mammalian serum and is involved in many assays. The hypervariable region of polyclonal IgG causes it to have the most heterologous pI of any known protein with a reported range of 6.1 to 8.5. Samples of 0.5 mg/ml rabbit plasma IgG were prepared in 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) at pH's of 6.5, 7.0, and 7.5 with sodium chloride concentrations from 0 to 200 mM. Over 60% of the protein was recovered in all conditions, but higher recovery correlated with higher pH (Figure 5*A*). In addition, there was a marked advantage to using higher levels of salt. Antibody recovery increased to over 90% when using 100 mM sodium chloride and approached 100% when the salt was increased to 200 mM.

To show endotoxin removal, a 0.5 mg/ml IgG sample was prepared in 50 mM PIPES at pH 7.5 with 100 mM sodium chloride. The contaminating endotoxin level of IgG was less than 2 EU/mg. 25 ng/ml *E. coli O55:B5* endotoxin was added to the protein sample and a 1 ml aliquot was applied to **EndoBind-R™**. The initial load contained 278 EU/ml endotoxin and was reduced to levels below assay detection limit (0.01 EU/ml) after passage through the column (Figure 5*B*). Though recovery was slightly broader than the previous proteins due to the highly heterologous pI, nearly 70% eluted into fraction 2 and contained less than 0.01 EU/ml endotoxin. Combining all 5 fractions, the recovery was over 97% of the initial antibody load.

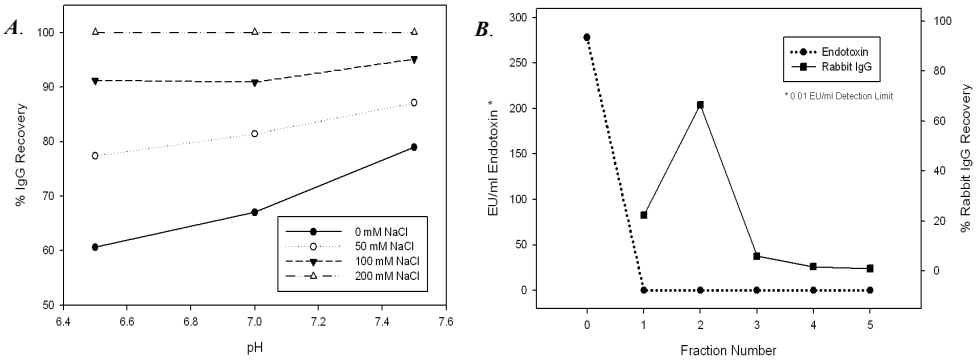



**Figure 5. Recovery Optimization and Endotoxin Removal from Rabbit IgG.** (*A*) Optimal salt and pH conditions for IgG recovery were determined using 0.5 mg/ml samples in 50 mM PIPES. (*B*) Endotoxin removal was determined in 50 mM PIPES at pH 7.5 containing 100 mM sodium chloride.

*Hemoglobin from Bovine Erythrocytes*

Hemoglobin is the major oxygen-transporting component of red blood cells. It is also used as a nitric oxide scavenger in many applications. In addition, advancements in blood replacement therapies underline the heightened need for the removal of pyrogens in blood constituents [10]. The 64 kDa functional protein exists as a homotetramer composed of four globin polypeptide chains each with a heme group. The pI of bovine hemoglobin has been reported in the range of 6.8-7.0 and therefore 50 mM PIPES buffer was used for purification. A series of 1 mg/ml preparations of hemoglobin from bovine erythrocytes was prepared in 50 mM PIPES buffer at pH's of 6.5, 7.0, and 7.5 with various sodium chloride concentrations. There was no dramatic difference in protein recovery over the pH range tested (Figure 6*A*). However, the optimum conditions were at a pH of 7.0, where hemoglobin carries no net charge. There was a heightened need for salt in hemoglobin purification, with 200 mM sodium chloride needed to achieve recovery above 80%.

Endotoxin removal from a 1 mg/ml hemoglobin preparation was performed as before in 50 mM PIPES at pH 7.0 with 200 mM sodium chloride. The hemoglobin stock contained over 300 EU/mg. However, since

the origin and nature of this contaminating endotoxin was unknown, 25 ng/ml (250 EU/ml) *E. coli O55:B5* endotoxin was added to the sample and purified with **EndoBind-R™**. Nearly 85% of the hemoglobin was recovered even though it has a high affinity for endotoxin [11]. The protein peak in fraction 2 contained about 60% of the initial load and, like all the fractions, contained less than 0.01 EU/ml of endotoxin (Figure 6*B*). The load measured 540 EU/ml owing to the 250 EU/ml from *E. coli O55:B5* and the 300 EU/ml from contaminating endotoxin. This reduction in endotoxin levels is more than 99.998% and is below the detection limit. In particular, the hemoglobin results highlight the very efficient removal of endotoxin by **EndoBind-R™**. This is due to two unique properties of hemoglobin: its high binding affinity for endotoxin, as mentioned previously, and its capacity to increase the biological activity of LPS 8- to 27-fold, as measured by LAL-based assays [11,12].

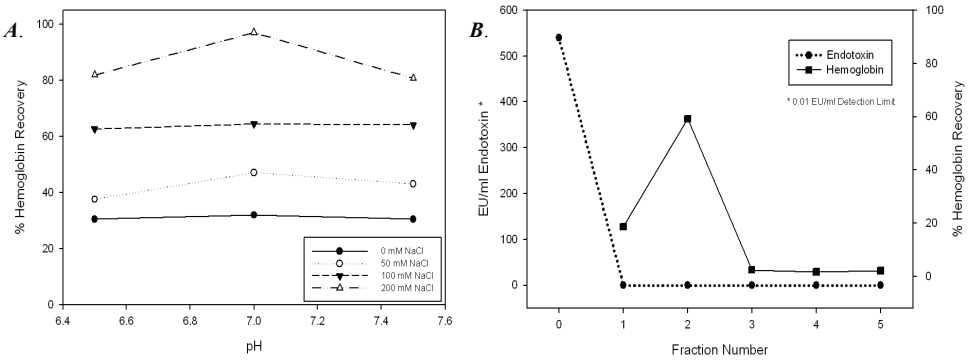



**Figure 6. Recovery Optimization and Endotoxin Removal from Hemoglobin.** (*A*) Optimal salt and pH conditions for hemoglobin recovery were determined using 1 mg/ml samples in 50 mM PIPES. (*B*) Endotoxin removal was determined in 50 mM PIPES at pH 7.0 containing 200 mM sodium chloride.

## Discussion

Protein purification with BioDtech's **EndoBind-R™** produced samples with high protein recovery and removal of endotoxin to sub-picogram levels. Efficient endotoxin removal owes to the high affinity of the S1 peptide on the column to the lipid A portion of LPS. This binding occurs via initial electrostatic interactions followed by hydrophobic interactions that reinforce the association [8]. Because of the nature of this binding scheme, the pI of the protein being purified and the salt and pH conditions of the buffer were critical. In the examples given, after optimal purification conditions were determined, peak fraction pro-

| Protein | Recovery (%) | Initial Endotoxin (EU/ml) | Endotoxin After EndoBind-R™ (EU/ml) |
|---|---|---|---|
| **BSA** | 99.7 | 506 | <0.01 |
| **Catalase** | 95.3 | 751 | <0.01 |
| **Transferrin** | 99.9 | 244 | <0.01 |
| **IgG** | 97.5 | 278 | <0.01 |
| **Hemoglobin** | 83.9 | 540 | <0.01 |

**Table 1. Summary of Protein Recovery and Endotoxin Removal with EndoBind-R™**
Protein recovery was determined by adding protein levels from all 5 column fractions compared to the initial load. Endotoxin levels were determined by PyroGene (Lonza) assays using multiple dilutions of all samples. Initial endotoxin levels often vary due to contaminating endotoxin from purchased proteins. Endotoxin levels after purification with EndoBind-R™ were below the detection level of 0.01 EU/ml.

tein recovery was usually in excess of 70% and total protein recovery approached 100% in all but one example (Table 1). In all protein solutions tested, endotoxin was removed to below the detection limit of 0.01 EU/ml (1 picogram/ml). This demonstrates that **EndoBind-R™** can remove endotoxin efficiently in a wide range of buffers, pH's, and salt concentrations as has been previously reported [6,7]. These results include loads in excess of 750 EU/ml which contain endotoxin from two distinct sources. Similar purification has been achieved using samples with endotoxin levels ranging from 10 to 10,000 EU/ml and protein concentrations as high as 40 mg/ml (Data Not Shown). The methods used here are also applicable to "ramp-up" experiments for larger scale preparations. In addition to the parameters addressed here, other variables such as chelating agents, detergents, specific buffer and salt ions, and protein concentrations can be adjusted to meet specific protein criteria. Protein purification with **EndoBind-R™** will prove invaluable in mammalian cell and tissue culture, animal studies, transplantations, and stem cell technologies.

## References


1. **Levin, J. and F.B. Bang.** 1964. The role of endotoxin in the extracellular coagulation of *Limulus blood*. Bull. Johns Hopkins Hosp. **115**: 265-274.
2. **Levin, J. and F.B. Bang.** 1964. A description of cellular coagulation in the *Limulus*. Bull. Johns Hopkins Hosp. **115**: 337-345.
3. **Nakamura, T., T. Morita, and S. Iwanaga.** 1986. Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in *Limulus* hemocytes. Eur. J. Biochem. **154**: 511-521.
4. **Muta, T., T. Miyata, Y. Misumi, F. Tokunaga, T. Nakamura, Y. Toh, Y. Ikehara, and S. Iwanaga.** 1991. *Limulus* Factor C: an endotoxin-sensitive serine protease zymogen with a mosaic structure of complement-like, epidermal growth factor-like, and lectin-like domains. J. Biol. Chem. **266**: 6554-6561.
5. **Tan, N.S., M.L.P. Ng, Y.H. Yau, P.K.W. Chong, B. Ho, and J.L. Ding.** 2000. Definition of endotoxin binding sites in horseshoe crab Factor C sushi proteins and neutralization of endotoxin by sushi peptides. FASEB J. **14**: 1801-1813.
6. **Yau, Y.H., B. Ho, N.S. Tan, M.L. Ng, and J.L. Ding.** 2001. High therapeutic index of Factor C Sushi peptides: potent antimicrobials against *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **45**: 2820-2825.
7. **Ding, J.L, Y. Zhu, and B. Ho**. 2001. High-performance affinity capture-removal of bacterial pyrogen from solutions. J. Chromatog. **759**: 237-246.
8. **Li, P, M. Sun, B. Ho, and J.L. Ding**. 2006. The specificity of Sushi peptides for endotoxin and anionic phospholipids: potential applications of POPG as an adjuvant for anti-LPS strategies. Biochem. Soc. Trans. **34**: 270-272.
9. **U.S. Department of Health and Human Service, Food and Drug Administration**. 1985. Bacterial Endotoxins/Pyrogens.
10. **Griffiths, E., A. Cortes, N. Gilbert, P. Stevenson, S. MacDonald, and D. Pepper**. 1995. Haemoglobin-based blood substitutes and sepsis. Lancet. **345**: 158-160.
11. **Kaca, W., R.I. Roth, and J. Levin**. 1994. Hemoglobin: a newly recognized lipopolysaccharide (LPS) binding protein which enhances biological activity. J. Biol. Chem. **269**: 25078-25084
12. **Kaca, W., R.I. Roth, A. Ziolkowski, and J. Levin**. 1994. Human hemoglobin increases the biological activity of bacterial lipopolysaccharides in activation of Limulus amoebocyte lysate and stimulation of tissue factor production by endothelial cells. J. Endotoxin Res. **1**: 243-252.


bdti™

*BioDtech, Inc. was organized in 2003 to develop and market products for detection, removal and neutralization of biological toxins.*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*Endotoxin removal products:*

| | | |
|---|---|---|
| *EndoBind-R™* | *1 ml column* | *EBR-3001.01* |
| *EndoBind-R™* | *5 ml column* | *EBR-3005.01* |
| *EndoBind-R™* | *Bulk resin* | *Inquire* |

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