Allantoin as a solid phase adsorbent for removing endotoxins

Vincent Vagenende*, Tim-Jang Ching, Rui-Jing Chua, Pete Gagnon

*Bioprocessing Technology Institute, A*STAR (Agency for Science, Technology and Research), 20 Biopolis Way #06-01 Centros, Singapore 138668, Singapore*

ARTICLE INFO

ABSTRACT

In this study we present a simple and robust method for removing endotoxins from protein solutions by using crystals of the small-molecule compound 2,5-dioxo-4-imidazolidinyl urea (allantoin) as a solid phase adsorbent. Allantoin crystalline powder is added to a protein solution at supersaturated concentrations, endotoxins bind and undissolved allantoin crystals with bound endotoxins are removed by filtration or centrifugation. This method removes an average of 99.98% endotoxin for 20 test proteins. The average protein recovery is ∼80%. Endotoxin binding is largely independent of pH, conductivity, reducing agent and various organic solvents. This is consistent with a hydrogen-bond based binding mechanism. Allantoin does not affect protein activity and stability, and the use of allantoin as a solid phase adsorbent provides better endotoxin removal than anion exchange, polymixin affinity and biological affinity methods for endotoxin clearance.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Endotoxins are of great concern in biological preparations as they interfere with cell-based assays and render biological products unsafe for therapeutic use [1,2]. Endotoxins, also called lipopolysaccharides, originate from the cell surface of Gram-negative bacteria [3]. They are widely present in the environment and human exposure to low levels of endotoxins elicit strong immune responses which can lead to fever, endotoxic shock and even death [4]. Contamination with endotoxins is therefore a common problem and effective methods for removing endotoxins are needed to obtain reliable biological preparations for therapeutic use [2,5,6]. Although several methods and materials have been developed to this end, meeting regulatory or assay-specific threshold levels of endotoxins can be challenging [1,2]. Achieving low endotoxin levels is particularly challenging for proteins, and reduction of endotoxins below 10 endotoxin units (EU) per mg protein is considered one of the most difficult steps during purification of proteins expressed in *Escherichia coli* [7].

Solid phase adsorbents for removing endotoxins from protein solutions include anion-exchange, hydrophobic, biological affinity and mixed mode materials. Anion-exchange materials can strongly bind the negatively charged core polysaccharide region and the lipid A part of endotoxins, but their performance depends on pH and salt concentration and their use is generally limited to neutral and basic proteins [1,8–11]. Hydrophobic materials may bind the fatty acyl chains of the lipid A part of endotoxins, but they are generally less effective and their performance depends on the presence of kosmotropic salts and organic modifiers [1,12]. Biological affinity and mixed-mode chromatographic materials combining hydrophobic and ionic interactions may be less sensitive to buffer conditions [11,13–23]; yet, they generally impose higher costs and they may leak toxic ligands into the final product [1]. Additional limitations of these materials may include limited compatibility with sanitizing agents, low product recoveries and time-consuming equilibration and regeneration steps.

Another established method for removing endotoxins from protein solutions is aqueous two-phase (ATP) micellar extraction with the nonionic detergent Triton X-114 [2,24–28]. Endotoxins preferentially partition to the hydrophobic detergent-rich phase, whereas proteins prefer the aqueous phase. This enables up to 99% endotoxin removal from proteins solutions [24–26]. However, relatively high process costs, difficulty to remove residual detergent and negative effects on protein activity disfavor the use of ATP extraction methods in protein purification processes [2].

Alternative methods for removing endotoxins involve metal affinity interactions. Interactions of endotoxins with free metals enable selective precipitation of endotoxins from plasmid DNA [29,30]. Various metal salts were tested and the best results were obtained with zinc sulfate, which removed more than 80% of endotoxins with about 90% plasmid recovery [30]. The effectiveness of precipitation based techniques for removing endotoxins from protein solutions remains however to be shown. Solid phase adsorbents involving metal interactions include immobilized metal affinity chromatography (IMAC) [7,29,31], hydroxyapatite [32] and

* Corresponding author. Tel.: +65 91802566; fax: +65 64788888.
E-mail addresses: Vincent_Vagenende@bti.a-star.edu.sg,
Vagenende.Vincent@gmail.com (V. Vagenende).

calcium silicate materials [33,34]. IMAC purification of an antibody fragment of *E. coli* lysate provided only limited endotoxin removal with residual endotoxin levels >10^4 EU/mL [7]. An additional on-column washing step with Triton X-114 improved endotoxin clearance by >3 logs [7]. This suggests that endotoxin removal by metal affinity purification is only effective when combined with other interaction mechanisms such as hydrophobic interactions with detergents. Chromatographic purification by hydroxyapatite was able to remove >4 logs of endotoxins of a native IgG pool [32]. Aside from metal affinity interactions, retention of solutes by hydroxyapatite involves ionic interactions [35]. This explains why purification results by hydroxyapatite generally depend on protein isoelectric point and solution conditions including pH and salt concentration [32,35]. Similarly, endotoxin binding by calcium silicate was found to be highly dependent on the presence of salts [33,34].

Hydrogen-bonding is an important interaction mechanism in nature where it imparts selectivity to biomolecular recognition processes that are mainly driven by ionic, hydrophobic and metal-based interactions [36–38]. Although the role of hydrogen-bonding as a supplementary binding mechanism has been implied in mixed mode anion-exchange chromatography [39–43] and hydrophilic interaction chromatography [44–46], its use as a primary binding mechanism for bioseparations remains largely unexplored. Recently we discovered that undissolved crystals in supersaturated solutions of the purine-derived compound allantoin adsorb endotoxins through hydrogen-bonding [47]. When allantoin crystalline powder is added to an aqueous solution at concentrations above its solubility limit, a suspension of undissolved allantoin crystals is obtained. Undissolved allantoin crystals – which we refer to as supersaturated allantoin – adsorb endotoxins through hydrogen-bonding with amide-groups on the crystal surface, and endotoxin adsorption on supersaturated allantoin results in layer-like clusters of endotoxin molecules that cover a major fraction of the crystal surface [47]. The majority of the undissolved allantoin crystals appear as 500 nm crystals and the corresponding specific surface area results in a remarkably high endotoxin binding capacity (3×10^7 EU/g) [47].

In this study we test the use of supersaturated allantoin as a solid phase adsorbent for removing endotoxins for 20 proteins. Endotoxin removal results are evaluated over a wide range of endotoxin concentrations and buffer conditions, and the effects of allantoin on protein activity and stability are measured by flow cytometry and differential scanning calorimetry. The process time, cost and robustness of our method are compared with available endotoxin removal methods and the use of supersaturated allantoin as a general method for removing endotoxins from protein solutions is proposed.

2. Materials and methods

2.1. Materials and sample preparation

Allantoin, dithiothreitol (DTT), calcium chloride (CaCl$_2$), glycerol, lysozyme, carbonic anhydrase II, ovalbumin, bovine serum albumin (BSA), thyroglobulin, protein L, deoxyribonuclease I (DNase), γ-globulin from bovine blood, insulin, protein A, trypsin inhibitor, β-lactoglobulin, concanavalin A type IV from Canavalia Ensiformis (jack bean) and fetuin were purchased from Sigma–Aldrich. 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium chloride (NaCl), imidazole and dimethyl sulfoxide (DMSO) were purchased from Merck Chemicals. RNase and chymotrypsinogen A were purchased from GE Healthcare (LMW gel filtration calibration kit). Chorionic gonadotropin from human urine and human transferrin (Holo) were purchased from Calbiochem.

The extracellular domain of mDectin-1 (NCBI Reference Sequence NP_064392.2 amino acids 73–244) with a 6× histidine tag at the C-terminus was produced from Rosetta™(DE3) *E. coli* cells and purified by Nickel-affinity chromatography. Biosimilar trastuzumab (IgG) was expressed in CHO cells and purified by protein A affinity chromatography. The Fab fragment of trastuzumab was obtained by papain digestion of the IgG followed by purification by calcium-modified hydroxyapatite [48].

Endotoxins are lipopolysaccharides (LPS) produced by Gram-negative bacteria. LPS from *E. coli* O55:B5 purified by ion-exchange chromatography were purchased from Sigma–Aldrich. LPS were dissolved in endotoxin free water to a final concentration of 1 mg/mL and filtered with a 0.22 μm Millex®-GV syringe filter (PVDF, 13 mm; Merck-Millipore). The LPS solution was subsequently diluted to 100,000 EU/mL by adding standard buffer (20 mM HEPES, 150 mM NaCl, pH 7.5). The diluted LPS solution was used for spiking at different endotoxin concentrations (10, 100, 1000 and 10,000 EU/mL, respectively).

2.2. Endotoxin removal by allantoin

Allantoin crystalline powder was added to the protein solution (1 mL) at the indicated concentration and the solution was mixed by shaking. The solution was then incubated on a rotary disc at 30 rpm for 15 min at room temperature. An additional set of experiments was performed using different incubation times ranging from 0 to 6 h. After incubation, undissolved allantoin was removed by filtration through a 0.22 μm filter. At higher allantoin concentration (≥50 mg/mL), filtration was preceded by centrifugation to remove undissolved allantoin. The endotoxin level of each sample was measured before adding allantoin and after removal of undissolved allantoin, and endotoxin reduction factors were determined by dividing the respective values. Endotoxin concentrations were measured by a standard kinetic chromogenic Limulus Amebocyte Lysate (LAL) assay using LAL reagent (Endosafe Endochrome-K™, Charles River Laboratories Inc.). Protein recovery was determined by UV absorbance at 280 nm. Values of endotoxin removal and protein recovery reported in the study are the average of minimum two independent endotoxin removal experiments.

2.3. Endotoxin removal by commercially available materials

Endotoxin removal by allantoin (300 mg/mL) was compared with the following commercially available endotoxin-binding chromatographic adsorbents: an anion-exchange membrane (Sartobind® Q SingleSep nano, Sartorius Stedim), a pre-packed column with polymixin B (Detoxi-Gel™ 1 mL column, Pierce), and pre-packed 1 mL columns with EndoTrap® red and EndoTrap blue (Hyglos GmbH), respectively. All chromatographic adsorbents were equilibrated and regenerated following the specifications of the manufacturer. Buffer used for equilibration consisted of 20 mM HEPES, pH 7.5 and 150 mM NaCl, and the equilibration buffer used for EndoTrap blue was enriched with 0.1 mM CaCl$_2$ following the manufacturer's specification. Proteins (lysozyme and BSA) were dissolved in the respective equilibration buffers to a final concentration of 1 mg/mL and endotoxin was added to a final concentration of 1000 EU/mL. Endotoxin removal was conducted by passing 10 mL of sample through the membrane/columns and the flow-through was collected for further analysis.

2.4. Enzymatic activity of carbonic anhydrase

The enzymatic activity of carbonic anhydrase before and after incubation with 300 mg/mL allantoin was determined by measuring the time required for a saturated CO$_2$ solution to lower the pH of 12 mM Tris–buffer from 8.3 to 6.3 [49]. Five independent time