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Journal of Immunological Methods 314 (2006) 67–73

JIM Journal of Immunological Methods

www.elsevier.com/locate/jim

Research paper

Simultaneous metal chelate affinity purification and endotoxin clearance of recombinant antibody fragments

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Received 13 March 2006; received in revised form 5 May 2006; accepted 19 May 2006
Available online 22 June 2006

Abstract

Endotoxins are frequent contaminants of recombinant proteins produced in *Escherichia coli*. Due to their adverse effects, endotoxins have to be removed from recombinant proteins prior their use in cell-based assays or parenteral application. Reduction of endotoxin to less than 10 EU mg^{-1} is, however, one of the most problematic steps during protein purification from *E. coli* and often associated with substantial loss of biological materials. The present paper describes the use of a single step procedure enabling metal chelate affinity purification and endotoxin clearance from antibody fragments produced in *E. coli* using a non-ionic detergent. Endotoxin content was as low as 5 to 9 EU mg^{-1} with a recovery of antibody fragments of over 90%. Non-ionic detergent treatment did not compromise integrity and functionality of these multimeric molecules. Furthermore, recombinant antibody fragments did not stimulate endotoxin-sensitive cell lines confirming the low endotoxin content. In conclusion, this one-step protocol is a rapid, cost effective and automation-compatible procedure suitable for recombinant antibody fragments.

Keywords: Recombinant protein expression; Antibody fragments; Endotoxin removal; Metal chelate affinity chromatography

1. Introduction

The ability to express functional antibody fragments in *Escherichia coli* (Better et al., 1988; Skerra and Pluckthun, 1988) enables the production of significant quantities of material for in vitro experiments, cell-based assays and in vivo applications. Unfortunately, *E. coli* derived products are prone to contamination with endotoxins. Endotoxins are toxic components of the outer membrane of gram-negative bacteria. Exposure to these soluble substances may affect the membrane structure of mammalian cells (Jacobs, 1984; Portoles et al., 1987), inhibit cell growth (Dudley et al., 2003) and decrease tissue culture cell viability (Cotten et al., 1994). Endotoxin induces the activation of monocytes, macrophages (Gao and Tsan, 2003b) and endothelial cells (Munshi et al., 2002). LPS-activated cells release mediators such as pro-inflammatory cytokines, TNF-α and interleukin-1 (Raetz and Whitfield, 2002). Finally, in humans and animals, exposure to endotoxin causes fever and may result in septic shock (Fiuza and Suffredini, 2001; Martich et al., 1993).

The incidence of contaminating endotoxin can complicate the interpretation of experiments or lead to the misinterpretation of results (Dudley et al., 2003; Gao and Tsan, 2003a), highlighting the benefits to eliminate

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doi:10.1016/j.jim.2006.05.012

endotoxin from *E. coli* derived products. Achieving a low level of endotoxin content together with an excellent recovery rate of the target protein is, however, a difficult task and often dependent on the physico-chemical properties of the target molecule (Petsch and Anspach, 2000).

Several methods have been applied to remove endotoxin from biological products (Petsch and Anspach, 2000). Phase separation using non-ionic detergent takes advantage of the hydrophobic properties of endotoxin trapping them in the detergent phase, whereas the hydrophobic proteins remain in the aqueous phase (Aida and Pabst, 1990). It is, however, time consuming and can lead to protein denaturation. Ultrafiltration is an efficient method to eliminate endotoxin from protein solution. It can, however, lead to a substantial loss of the target molecule (Petsch and Anspach, 2000). Anion exchange resins and selective affinity sorbents, which are based on the differential interaction of endotoxin and proteins with functional groups immobilized on the resin, often lead to a loss of biological material as multiple rounds of binding are needed to eliminate substantial amounts of endotoxins (Anspach and Hilbeck, 1995). A method for endotoxin removal from his-tagged monomer recombinant proteins using affinity chromatography in combination with the non-ionic detergent Triton X-114 has been described previously (Reichelt et al., 2006). However, non-ionic detergent can cause change in protein conformation and impair binding properties (Hsu and Youle, 1997; Hsu and Youle, 1998; Tan and Ting, 2000).

We tested whether this one-step protocol using non-ionic detergent could be applied to antibody fragments composed of two non-covalently linked polypeptide chains (Rauchenberger et al., 2003) and to bivalent antibody fragments containing a small homodimerization domain (dHLX) (Pack and Pluckthun, 1992). As case study, we selected a fully human monoclonal antibody MOR102 (#5) targeting the intercellular adhesion molecule 1 (ICAM-1) derived from the Human Combinatorial Antibody Library (HuCAL®) (Boehncke et al., 2005; Knappik et al., 2000). The antibody MOR102 (#5) was expressed in two different antibody formats: (i) non-covalently linked Fab fragments and (ii) bivalent Fab-dHLX fragments as histidine-tagged protein.

We demonstrate that Triton X-114 is effective at eliminating endotoxin using Ni-NTA affinity chromatography from antibody fragments. Protein recovery was at least 90% with endotoxin content as low as 5 to 9 EU mg^{-1} for the two antibody formats tested. Binding activity and functionality were not compromised as assessed in binding and cell-based assays, respectively. Furthermore, antibody fragments purified using Triton-X114 did not activate endothelial cells, which corroborates with low endotoxin content. Therefore, including Triton X-114 washing step in the purification protocol is a straightforward methodology for the removal of endotoxin from recombinant antibody produced in bacteria.

2. Materials and methods

2.1. Strains and plasmids

E. coli TG1 strain was used to express histidine-tagged antibody fragments. Antibody fragments were expressed either as non-covalently linked Fab or as Fab-dHLX format using the pMORPH®×9 plasmid described previously (Rauchenberger et al., 2003).

2.2. Expression

4 ml of overnight culture was used to inoculate 750 ml of 2×YT containing 34 μg/ml chloramphenicol. Cultures were agitated at 200 rpm until the A_{600nm} reached 0.5 and expression was induced by addition of 0.75 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 20 h at 30 °C. Cells were harvested by centrifugation at 5000 rpm for 30 min.

2.3. Purification and endotoxin removal

Cell pellets containing his-tagged proteins were resuspended in 40 ml of IMAC buffer (500 mM NaCl, 200 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.3). Following addition of 0.1% lysozyme and 10 U/ml benzonase, cells were agitated at room temperature for 30 min. Lysates were centrifuged at 20,000×*g*, 4 °C for 30 min and filtered through a 0.45 μm filter (Millipore, MA). A suspension of 1 ml Ni-NTA agarose (Qiagen, Germany) was poured into columns. Cell extracts were split into two equal aliquots and each aliquot was loaded onto one column that was previously equilibrated with 15 column volumes of IMAC buffer. One column was washed with 50 column volumes of respective buffer containing 0.1% (v/v) of TritonX-114 (Sigma-Aldrich, Germany) followed by 20 column volumes of buffer without detergent at 4 °C. The other column was washed with equivalent column volumes of the same buffer but lacking detergent. Elution was achieved using 200 mM imidazole (Sigma-Aldrich, Germany). Elution fractions containing Fab or Fab-dHLX were loaded onto PD10 gel filtration columns (Amersham Bioscience, Sweden)