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Endotoxin reduction in monoclonal antibody preparations using arginine

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Abstract

A monoclonal antibody preparation was found to be contaminated with endotoxin. Several commercial endotoxin removal steps were attempted but failed to produce a significant reduction due to the fact that the endotoxin was associated with the antibody. Here, several methods for endotoxin removal based on immobilizing monoclonal antibodies to chromatographic media have been evaluated. A crucial step in this process was to dissociate the endotoxin from the protein surface for subsequent removal. This was accomplished by introducing different buffer additives in the mobile phase. In agreement with previous reports, non-ionic detergents efficiently removed endotoxin, but it was also found that 0.5 M arginine performed equally well. Since arginine is a non-toxic common amino acid that can be readily removed, it was selected and successfully used in large-scale experiments. With this method, endotoxin could be reduced to <0.2 EU mg^{-1} with recovery of the target protein being >95%. Since this procedure is easily integrated into the existing processes of mAb purification, it offers advantages in speed, cost and effort.

Keywords: Endotoxin removal; Arginine; Affinity chromatography; Antibody purification

1. Introduction

In the last decade, about 20 different monoclonal antibodies (mAbs) have been FDA approved for therapeutic use. An almost identical number of mAbs are available as isotopic labelled diagnostic tools for intra-corporal imaging purposes [1]. Numerous new mAbs for these purposes are currently in clinical trials or under development, of which many are expected to be approved in the coming years.

Antibodies are produced in biological systems, and although well-defined and robust production processes exist, several challenges remain [2]. One particular problem is the removal of endotoxin. Endotoxins are a class of lipopolysaccarides, which form an integral component of the outer cell wall of gram-negative bacteria. They are continuously released into the environment from living and dead bacteria and display high potency for inducing biological effects with a threshold level of 1 ng kg^{-1} body weight and hour [3]. The risk of endotoxin contamination is further increased if transient expression, which relies on the transfection with bacterially produced plasmids, is applied for production.

Many techniques are available today to remove endotoxin from solutions by exploiting certain characteristics of the molecule. Endotoxin molecules tend to form micelles or vesicles in aqueous solution and can thus be removed by filtration [4,5]. Their hydrophobic nature allows a separation in two-phase extraction [6] or by hydrophobic interaction chromatography [7]. Their negative charge can be used for adsorption on anion exchange ligands [8]. Several affinity ligands also show a great potential to selectively bind endotoxins, such as polymyxin B [9], histidine [10], dimethylamine ligands [11], deoxycholic acid [12], polycationic ligands like polyethyleneimine [13] and poly-L-lysine [14] or proteineous affinity ligands derived from a bacteriophage (www.profos.de). However, removal of endotoxin from protein solutions is more difficult. In this case, ultrafiltration will not work and due to electrostatic or hydrophobic interactions, association between endotoxin and the protein might occur, which further complicates the separation. Dissociation of endotoxin from the protein surface can be achieved with detergents, however the removal of the surfactant is then an additional problem in the purification process [15].

Aggregation between protein molecules can be suppressed by arginine, which is therefore a common additive in refolding [16]. Arginine has also been demonstrated to dissociate proteins

Abbreviations: CV, column volume; EDTA, ethylenediaminetetraacetic acid; EU, endotoxin units; mAb, monoclonal antibody; OGP, octyl-β-D-glucopyranoside; TNBP, tri-*n*-butylphosphate; WFI, water for injection

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from protein–protein complexes by significantly facilitating the elution of monomeric antibodies from a Protein A matrix [17]. L-Arginine is a normal metabolite in animals and man and has a low order of toxicity. Low concentrations in the final product are therefore not a problem, thus simplifying downstream applications.

Here we describe an efficient method to reduce protein-associated endotoxin, in which arginine is applied during a wash-step of Protein A bound antibodies. A reduction of endotoxin to a level below 0.2 EU mg^{-1} antibody could be achieved, which meets the criteria for *in vivo* preclinical studies. We have demonstrated that this method is fully scaleable and can thus be applied in large-scale production.

2. Experimental

2.1. Purification of mAb

Typically, recombinant mAbs were expressed in HEK-EBNA293 cells transiently transfected with plasmids carrying the IgG cDNA sequences. Secreted mAbs were then captured from the culture medium by expanded bed adsorption on rProtein A Streamline resin (GE Healthcare, Uppsala, Sweden). A Streamline 100 (GE Healthcare) column was used and the medium was pumped over the column at ∼200 cm h^{-1}, allowing the bed to expand two to three times. This was followed by extensive washing with PBS until no visible cell debris remained in the column. An additional wash-step with Protein A binding buffer (20 mM phosphate buffer, pH 7.3) was then performed to lower the conductivity. The mAbs were eluted in sedimented mode by Protein A elution buffer (100 mM citrate buffer, pH 3.2). The pH of the protein pool was adjusted to 5 with 1 M Tris-Cl, pH 9.0 and diluted with WFI until the conductivity was less than 5 mS cm^{-1}. The final polish step consisted of SP sepharose FF to which the protein pool was batch adsorbed and then eluted with a gradient from 0 to 1 M NaCl in 20 mM sodium acetate, pH 5.0, over 10 column volumes (CV).

2.2. Endotoxin reduction in small scale

Five-milligram aliquots of the purified sample were loaded either onto a SP FF HiTrap (1 mL, GE Healthcare) column or a rProtein A FF column (1 mL, GE Healthcare). When bound to the column, a wash solution containing the binding buffer with different additives (Table 1) was applied to the column over 6 CV with a contact time of 30 min. This was followed by extensive washing with 40 CV binding buffer before the mAb was eluted. All experiments were performed at room temperature.

Before the sample was loaded onto the ion-exchange column, a buffer exchange to SP binding buffer (20 mM sodium acetate, pH 5.0) was first performed by Sephadex G-25 gel filtration (GE Healthcare). Elution was achieved with a gradient to SP elution buffer (20 mM sodium acetate, 1 M NaCl, pH 5.0) over 10 CV.

For the rProtein A FF column, no pre-treatment of the sample was needed. After adsorption, the mAb was washed with Protein A binding buffer and step-eluted with Protein A elution buffer, then the pH in the protein pool was adjusted to 5 as above.

2.3. Endotoxin reduction in large scale

MAbs with a purity of >99% and endotoxin content above 2.5 EU mg^{-1} were loaded onto a MabSelect SuRe column (GE Healthcare) that had been sanitized by three cycles of 0.5 M NaOH over 15 min and equilibrated with Protein A binding buffer. Approximately 12 mg mAb were loaded per milliliter resin. The column was washed with 0.5 M arginine in Protein A binding buffer for 30 min over 6 CV, followed by washing with at least 20 CV of Protein A binding buffer to remove traces of arginine from the mAb. The protein was eluted by lowering the pH with Protein A elution buffer. The eluate was quickly diluted to approximately 2.5 mg mL^{-1} with WFI and adjusted to pH 5 with 1 M Tris-Cl pH 9.0.

2.4. Analysis

The endotoxin content was determined by a LAL assay (Cambrex, Walkersville, MD, USA) with serial dilution of the samples as described by the manufacturer. All buffers were validated with this method prior to use. Protein concentration was estimated by measuring the absorbance at 280 nm and/or with the BCA assay (Pierce, Rockford, IL, USA) and the activity was determined with an in-house ELISA specific to the mAb.

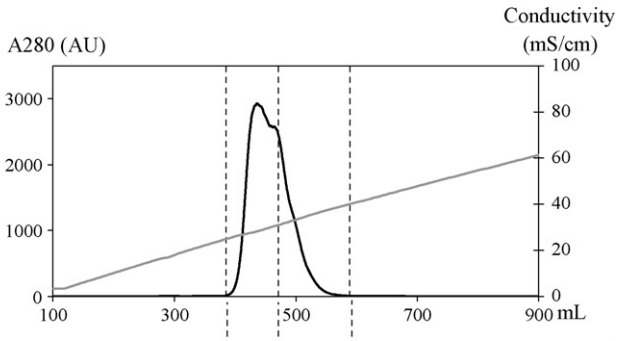
3. Results and discussion

3.1. Association of endotoxin to the mAb

A recombinant mAb was transiently expressed in HEK-EBNA cells and purified using expanded bed adsorption on rProtein A affinity media (GE Healthcare) followed by cation-exchange chromatography. This produced a mAb with a purity

Table 1
Effect of different buffer additives on endotoxin removal from mAbs

Additive	Column	Start endotoxin (EU mg^{-1})	Final endotoxin (EU mg^{-1})	Endotoxin reduction (%)	Protein recovery (%)
1% Triton X-100 0.3% TNBP	HiTrap SP FF	0.9	<0.4	>56	99
1% Triton X-114	HiTrap SP FF	0.9	<0.4	>56	90
3% OGP	HiTrap SP FF	0.9	<0.5	>44	78
3 mM EDTA	HiTrap SP FF	0.9	0.8	11	98
0.5 M Arginine	HiTrap ProtA	2.5	<0.6	>76	88



	Pre-peak	F 1	F 2	Post-peak	
Protein	-	2.6	1.2	-	*mg/mL*
Endotoxin	<0.25	10	5	<0.25	*EU/mL*
Endotoxin	-	3.8	4.2	-	*EU/mg*

Fig. 1. Cation-exchange chromatography on purified mAbs showing co-elution of endotoxin with the mAb in the NaCl gradient. The table shows protein and endotoxin concentration, the endotoxin concentration is also recalculated into EU mg^{-1} to give a more comparable unit.

of >99% but with endotoxin above the acceptable level for the intended use.

Initially, reduction of the endotoxin level was attempted with several chromatographic methods known to remove endotoxin from solutions, i.e. anion exchange (Q sepharose, GE healthcare), Detoxigel (Pierce), EndoTrap (Profos, Regensburg, Germany) and Mustang E (Sartorius, Goettingen, Germany). Despite the endotoxin binding properties of these adsorbers, there seemed to be a limit at around 2 EU mg^{-1} that could not be passed (data not shown). These results indicate an association between the endotoxin molecules and the mAb.

Evidence for this association was given during cation-exchange chromatography where co-elution of the endotoxin with the mAb in the NaCl gradient was observed (Fig. 1). In contrast to the mAb, endotoxins are negatively charged and should not bind to the resin at pH 5.0. However, binding mediated by the mAb would explain this behavior. No endotoxin was detected in the buffer that has passed the column neither before nor after the protein peak. A strong correlation between endotoxin levels and protein concentration was also seen in the two fractions in which the eluate was collected. According to analytical size-exclusion chromatography, both fractions contained a non-aggregated form of the mAb (data not shown).

Associations between endotoxin and proteins have been proposed previously [15]. Due to the nature of the endotoxin molecule, both electrostatic and hydrophobic interactions can take place. In addition, it has also been suggested that stable Ca^{2+} bridges between the phosphoric acid groups of the endotoxin and carboxylic groups of the protein could be responsible for protein–endotoxin association [18].

3.2. Effect of additives on the mAb–endotoxin interaction

In order to reduce the endotoxin level, the proposed interactions between the endotoxin and the antibody must be broken and the dissociated endotoxin then separated from the protein. In a parallel set of experiments in small scale, the effect of different additives targeting the various modes of interaction was investigated. The test set consisted of Triton X-100/tri-*n*-butylphosphate (TNBP), Triton X-114, octyl-β-D-glucopyranoside (OGP), ethylenediaminetetraacetic acid (EDTA) and arginine (Table 1). The wash procedure was performed on-column with the additive in the mobile phase. The mAb was adsorbed onto a cation-exchange column for all additives except for arginine where rProtein A affinity resin was used due to the high conductivity of the 0.5 M arginine solution.

All non-ionic detergents tested in our study showed positive results regarding recovery and retained activity of the mAb. The endotoxin levels were reduced from 0.9 to values between <0.4 and <0.5 EU mg^{-1} (Table 1). This is in agreement with previous reports where Triton X-114 was successfully used to remove endotoxin from a protein solution by phase separation [6] and recent methods combining affinity chromatography with a detergent wash [19,20]. Adsorption of endotoxins onto polymyxin B has also been shown to be facilitated by addition of OGP [15]. It is proposed that through addition of detergents, endotoxins can be extracted from hydrophobic surfaces on the protein into the solution by forming mixed micelles with the detergent [6]. Using Triton X-100/TNBP even allows the combination of a well-established method for virus inactivation [21] with the ability to remove endotoxin as demonstrated here.

Chelating agents like EDTA disturb the bridging effect of Ca^{2+}, thus preventing the aggregation of lipopolysaccharides [22]. It has been observed that even low concentrations of Ca^{2+} can reduce the endotoxin removal efficiency of certain affinity resins [23]. However, the wash with EDTA only resulted in a minor endotoxin reduction, indicating that the protein–endotoxin interaction is not primarily Ca^{2+} dependent.

Washing the mAb with arginine reduced endotoxin more than fourfold from 2.5 to <0.6 EU mg^{-1} with only minor losses in material and activity. The interaction of arginine with proteins is not fully understood, but it is proposed that arginine interacts with aromatic side chains of the protein, thus locally loosening the structure around these residues [24,25]. This interaction might be sufficient to break the weak interactions and dissociate the endotoxin. Arginine does also carry a positive charge that could have an effect by neutralizing the negatively charged phosphate groups of the endotoxin lipid A moiety, thus abolishing ionic interactions with the protein. An additional benefit of using arginine is its potential to increase the recovery of monomeric mAb in the eluate from Protein A columns [17,26].

The principle of adsorbing proteins to a solid support and performing a subsequent dissociation and removal of endotoxin by extensive washing with a variety of substances has been described previously thus supporting our approach. Recently, a combination of ion-exchange chromatography and alkanediols was evaluated for model proteins [27]. A non-ionic detergent has also been used to reduce endotoxin from a His-tagged proteins, including antibody Fab fragments, bound to an immobilized metal affinity chromatography column [19,20]. The design also enables removal of the endotoxin reducing agent from the final product by washing the mAb with buffer prior to elution. With

Table 2
Reduction of endotoxin from mAbs with arginine in large scale

Batch/run	Start endotoxin (EU mg^{-1})	Final endotoxin (EU mg^{-1})	Endotoxin reduction (%)	Protein recovery (%)
1/–	3.1	<0.3	90	98
2/1	22	2.4	89	92
2/2	2.4	<0.2	90	99

this procedure, a more than 1000-fold decrease of Triton X-100 was observed in our experiments by measuring the absorbance at 280 nm (data not shown).

3.3. On-column reduction of endotoxin from mAb in large scale using arginine

Particularly in the context of *in vivo* studies or biopharmaceuticals, arginine is more suitable than detergents because it is non-immunogenic and non-toxic. In addition, it does not form aggregates or micelles and does not interfere with endotoxin assays. Arginine was therefore selected and applied to a larger batch of approximately 500 mg of the same mAb.

By performing a 30 min wash, the endotoxin was reduced by 90% to <0.3 EU mg^{-1} (Table 2). The procedure was successfully repeated with a second batch where a repetitive run of the arginine wash was performed. A final endotoxin level of <0.2 EU mg^{-1} antibody was reached with an overall protein recovery above 95%. Each run gave a 10-fold decrease of the endotoxin, which demonstrates good reproducibility of the process. When analyzing the endotoxin content of the protein and wash fractions, it was found that the endotoxin was enriched in the arginine wash fraction (Fig. 2).

The performance of the method is here shown to be equally efficient for endotoxin removal from 5 and 500 mg mAb, demonstrating good scalability. It is therefore believed that the method is also suitable for production scale processes. The procedure can easily be integrated into existing procedures, since only one additional washing step during capture needs to be introduced without intermediate elution of the sample.

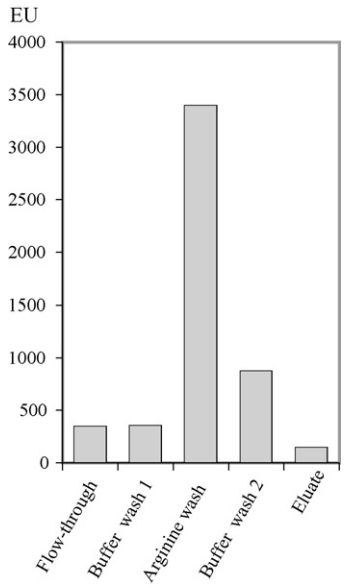


Fig. 2. Distribution of endotoxin into the different pools during the described arginine wash of mAb. The *y*-axis shows total endotoxin units.

4. Conclusion

Here we demonstrate that washing of a mAb bound to an affinity resin with 0.5 M arginine is an effective way for specific removal of protein-bound endotoxin. This method is reproducible at different scales and is easy to perform and monitor. It also allows good recovery and does not affect the activity of the protein.

We believe that this method for endotoxin reduction can be used in the preparation of other antibodies, as well as different proteins.

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