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The Removal of Endotoxins in the Actual Production Process

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ABSTRACT

Endotoxins, also called lipopolysaccharides (LPS), are mostly found in the outer membrane of Gram-negative bacteria, which are widely used as a common expression system to produce some biological products. The presence of small amounts of endotoxin can cause side effects in host organism such as endotoxin shock, tissue injury, and even death. Due to these reactions, it is essential to remove endotoxins in the final products. In the actual production process, endotoxin contamination can occur at any process unit. In this article, we review the structure and characteristics of endotoxins and the effects that it causes in vivo. The endotoxin removal strategies are also elaborated according to the relevant mechanism. Besides, the endotoxin removal also faces enormous challenges in the actual production process.

KEYWORDS

Endotoxin also known as lipopolysaccharide (LPS); Gram-negative bacteria; Toxin

1. INTRODUCTION

With the development of synthetic biology, Gram-negative bacteria, especially, *Escherichia coli*, are widely used as a common expression system to produce some biological products, which has been demonstrated to possess good safety, high efficacy, inexpensive culturing, easy scale-up and short turnaround time [1, 2, 3]. From the statistics of the food and drug administration (USA) and European medicines agency, 29.8% of protein-based recombinant drugs are produced using products derived from *E. coli* [4]. It can release endotoxins during the growth and division of bacteria or even cell death. Endotoxins need to be removed to control the limitation of the product during the production processes or in the final product to avoid any adverse side The selection of a suitable endotoxin removal system is based on the properties of the bio-products being purified. This article gives a short overview of the properties of endotoxins, then highlights different methods of endotoxin removal in theory and in actual production process. Additionally, the difficulty and the challenges associated with removing endotoxin will also be discussed. Perhaps this article will provide new ideas for endotoxin removal in actual production.

2. ORIGIN OF ENDOTOXINS AND CLINICAL ASPECTS

Endotoxins are mostly found in the outer membrane of Gram-negative bacteria [5]. They not only are responsible for the bacterial organization and stability [6], but also participate in the interaction of the bacterial cell with its environment and possible hosts. Although endotoxins are firmly anchored within the bacterial cell wall [7], they are continuously liberated into the environment throughout the entire lifecycle of cell growth. This results in a high concentration of endotoxins when the bacteria are used in industrial production. Besides, bacteria can grow in nutrient-poor media, such as water,

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saline, and buffers, resulting in contamination of the product by bacterial cells and subsequent endotoxin contamination during the purification process.

The principle of endotoxin toxicity is that endotoxins directly activate the immune system, especially monocytes and macrophages, to release mediators, such as tumor necrosis factor, several interleukins, and free radicals, which have potent biological activity and are responsible for the adverse effects seen upon endotoxin exposure [8]. The harms caused by endotoxins include alterations in the structure and function of organs and cells, changes in metabolic functions, increased body temperature, activation of the coagulation cascade, modification of hemodynamics and induction of shock. Many attempts have been made to prevent or treat the deleterious effects of endotoxins on immune cells, such as the use of anti-endotoxin antibodies, and endotoxin partial structures for blocking endotoxin receptor antagonists. Nevertheless, the interaction of endotoxins with immune cells is not only mediated by specific receptors. Cell priming may also occur by non-specific intercalation of endotoxin molecules into the membranes of the target cells [9]. In small quantities, the symptoms are mild. But, in severe cases, the residual endotoxin in the biological agents could seriously threat to health [10, 11].

Finally, it should be mentioned that endotoxins may also have beneficial effects. They have been used in artificial fever therapy, to destroy tumors and to improve, non-specifically, the immune defense. But, it still has the uncertainty for the human health [12]. Besides, any superfluous endotoxin exposure must be strictly avoided to prevent complications. This is especially true for intravenously-administered medicines [5].

3. STRUCTURE OF ENDOTOXINS AND CHARACTERISTICS

Endotoxins are also known as lipopolysaccharides (LPS). The general structure of all endotoxins is a polar heteropolysaccharide chain, including three distinct domains: the O-antigen region, a core oligosaccharide part and a lipid A part that it is the toxic part of endotoxins (Figure 1).

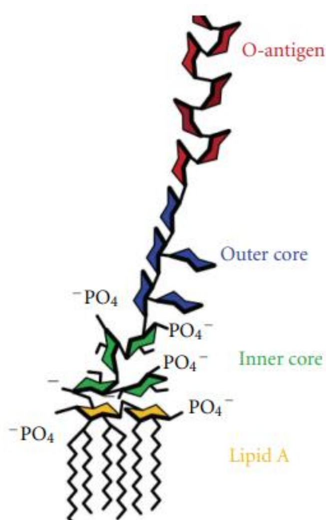

Figure 1. Structure of lipopolysaccharides (Source: http: //en.wikipedia. org/wiki/Lipopolysaccharide, accessed on 10 Jan 2012)

The Lipid A structures were first studied based on *Enterobacteria* [5]. The common architecture of Lipid A is a disaccharide with glucosamine monomer. The two glucosamine monomers are linked between position 1 and 6, and both of them are phosphorylated to produce bisphosphorylated β-(1-6)- linked glucosamine disaccharide [13]. Furthermore, there are fatty acids ester-linked at positions 3 and 3′and amide linked at positions 2 and 2′ [14]. The position 6′ is attached to the oligosaccharide region [14] The core unit of LPS is oligosaccharide part that consists of 8-12 sugar units [14]. It includes hexose region outer core and heptose region inner core where the heptose may be substituted

by a phosphate, pyrophosphate, or phosphorylethanolamine group [15] The phosphate groups and charged sugar residues in the inner core and Lipid A are responsible for the stability of LPS by interactions with cations [13]. The O-antigen region is composed of repetitive subunits with eight sugar units. One O-chain unit contains 50 identical subunits and the frequent components in O-chain structures are deoxysugars [13]. Meanwhile, it has diversity, including g linear or branched backbones which are substituted by many kinds of aglycones, such as O-acetyl phosphate, N-acetyl phosphate and phosphorylethanolamine [15].

Due to the unique structure of endotoxins, they have many characteristics. Firstly, the molecular mass of endotoxins exhibits diversity. The oligosaccharide part of lipopolysaccharides decide that endotoxins have a hydrophilic feature, meanwhile, the variability of the oligosaccharide chain make the molar mass of an endotoxin monomer vary from 10 to 20kDa, even extreme masses of 2.5 (O-antigen-deficient) and 70 (very long O-antigen) kDa can also be found [5]. There are monomers, micelles and vesicles in aqueous solutions Secondly, endotoxins have both hydrophobicity and hydrophilicity. Owing to the lipid A part, endotoxins have a hydrophobic property. The presence of a large number of sugar groups leads to the hydrophilicity of endotoxins. Because of their amphipathic structures, endotoxins easily form various supra-molecular aggregates in aqueous solutions. These aggregates result from non-polar interactions between lipid chains as well as of bridges generated among phosphate groups by divalent cations [6]. Thirdly, the core region close to lipid A and lipid A itself are partially phosphorylated ($pK1=1.3$, $pK2 = 8.2$ of phosphate groups at lipid A), thus endotoxin molecules exhibit a net negative charge [16, 17]. In addition to these, endotoxins have thermal stability and acid-base stability. Endotoxin molecules are highly heat-stable and are not destroyed under regular sterilizing conditions. Endotoxins can be inactivated when exposed at temperature of 250º C for more than 30 minutes or 180º C for more than 3 hours [18, 19]. Acids or alkalis of at least 0.1M strength can also be used to destroy endotoxin in laboratory scale [20], but it needs to be soaked for 4 hours. Finally, endotoxins are soluble in water. During production, endotoxins can be removed by rinsing with water.

4. METHODS OF ENDOTOXIN REMOVAL

4.1. Adsorption Method

Adsorption is the most commonly used method. The first one is adsorption by activated carbon. Due to its large surface area, strong adsorption capacity, low price and easy operation, activated carbon is widely used for endotoxin removal. It is suitable for removing low molecular weight of hydrophobic endotoxins, and the removal effect under the slightly acidic condition is the best. In the actual production process, adding 1% activated carbon and stirring for 1 hour at room temperature can achieve a removal rate of 93.5% for endotoxins. The endotoxin limitation of the final product accord with quality standards. If changing the addition amount of activated carbon, stirring time, and temperature, the endotoxin limitation of product can get to 0.5EU/mg (the limitation of Class II medical devices) and even get to 0.05 EU/mg (the limitation of Class III medical devices). This method is the cheapest and simplest. But due to the non-selectivity of activated carbon, this method not only makes excessive loss of the target production but also is difficult to remove the residual activated carbon, which seriously affects the quality of products.

The other adsorption method is affinity adsorption by a wide variety of adsorbents, which have high specificity and selectivity for endotoxins in formulations. The function of removing endotoxins of adsorbents is mainly ruled by electrostatic interaction forces [21]. Another results showed that the property and structure of amino acid ligands also have great influence on endotoxin adsorption capacities [22]. According to the common understanding, an endotoxin-selective affinity sorbent should guarantee both the clearance of endotoxins and a recovery of other molecules of close to 100%. There are many different chemical structures for endotoxins, which is leaded by the variability of the

O-antigen and the core oligosaccharide. This places high demands on the structure and function of affinity ligands that should be of diffuse recognition or should be group-selective for the small structure elements existing in various endotoxins Because of the low concentration to be cleared, strong interactions are mandatory The most common affinity ligands are Polymyxin B, Histamine and histidine, Deoxycholic acid, Polycationic ligands, Polymeric matrices with cationic functional groups, and Immunoaffinity ligands and so on [23]. The other forefront technology is membrane adsorbers which combines membrane and adsorbent. It is not only an alternative to particulate sorbents but may improve endotoxin clearance significantly [24, 25]. The affinity adsorption method has a high removal rate for endotoxins, and results in minimal loss of target products but relatively, it is very expensive, which limits the application of affinity adsorption method to high value-added fields, such as protein , RNA and DNA.

4.2. Ultrafiltration Method

With the rapid development of membrane separation technology, the ultrafiltration method for removing endotoxins is increasingly studied. Ultrafiltration method is based on the size of molecular weight to remove endotoxins. As we know, the molar mass of an endotoxin varies from 2.5KDa to70KDa, this means that the removal efficiency of endotoxins by ultrafiltration membranes with different pore sizes is different. Actually, there are monomers, micelles, and vesicles in aqueous solutions [26]. If choosing small pore ultrafiltration membrane, maybe the effect of the endotoxin removal is very good, but the product recovery rate is not satisfied, vice versa. So it is necessary to balance the endotoxin removal effect and product recovery rate, and then choose the most suitable ultrafiltration membrane. This situation partly applies to protein-free solutions. For protein-containing solutions, ultrafiltration would fail if interactions between endotoxins and proteins cause endotoxin monomers to permeate with proteins pick-a-pick through the membrane [5]. In addition, proteins can be damaged by physical forces [27]. Some literature shows that the removal rate of endotoxins by ultrafiltration can reach over 95%, but in our actual production process, the removal rate of endotoxins using 1kDa ultrafiltration membranes is only about 87%, which is much lower than expected. The reason for this result is the introduction of endotoxins due to inadequate cleaning of the dead corners of the equipment. So the key of using ultrafiltration to remove endotoxins is equipment cleaning, which is the key to achieving the desired effect.

4.3. Ion Exchange Method

Phosphate groups exist in the structure of endotoxins, which cause endotoxins to exhibit negative charges above their isoelectric point. The interaction between the anionic phosphate in LPS and the cationic ligands on the sorbents are mostly utilized as the mechanism of endotoxin removal [28] One of the most currently used chromatographic techniques for endotoxin removal is anion-exchange chromatography (AEC), which has rapid separation, wide selection of AEC media, sodium hydroxide (NaOH) sanitization and does not require any solvents [29]. Salt gradient is vital in AEC as it is used for different nucleic acids elution according to charge density [5]. High salt concentration (high ionic strength) is maintained to avoid low charge density impurities adsorption [5] Competing interactions at the binding sites may occur during protein purification. The negatively charged proteins compete with endotoxins for binding sites which eventually exhaust the ligand binding capacity. Presence of only net positively charged proteins will cause protein molecules to experience repulsion from the ligands and compete with ligands to capture endotoxin, which can lead endotoxins to be dragged out of the column and diminish the endotoxin removal efficiency of the ligand [13]. It is potentially useful for the decontamination of positively-charged proteins, such as urokinase [30]. However, decontamination of negatively-charged proteins would be accompanied by a substantial loss of the product due to adsorption [6,17]. For pDNA purification, negatively charged phosphate group on DNA interacts with positively charged ligands, which cause adverse effects. Fractogel DEAE has recently been identified as potentially the best commercial media for therapeutic pDNA production

attributing to its excellent pDNA purification, recovery, reproducibility, robustness and dynamic capacity [31]. Through optimizing various chromatographic conditions, the use poly (GMA-EDMA)-based AEC column for the direct purification of plasmid DNA from lysed bacterial cell lysates obtained high endotoxin removal and plasmid recovery [32]. Expanded bed AEC is another alternative media which has no other distinct advantages but the capability of purifying highly viscous solutions with ease [31].

Cation-exchange chromatography (CEC) is lesser known compared to AEC. However, it is proved to be more efficient than anionic exchanger in terms of endotoxin removal by many researchers [5]. Poly-cationic ligands offer extremely strong attraction/binding for endotoxins, which is proven by the fact that desorption of endotoxins is still low even at high-salt conditions [33]. Known agents for cationic exchanger are PEI, which as a hydrophilic polymer has superior biocompatibility and exhibits hydrophobic interactions with endotoxin, zirconia-immobilised PLH, which is not viable as it is expensive and unstable under alkaline conditions [6] and Poly-L-lysine (PLL), which works well for protein recovery and still usable after binding capacity exhaustion [23].

In addition to the above, commonly used cationic and anionic resins can also effectively remove endotoxins. Laboratory data shows that the endotoxin removal rate can reach 81.7%. This result may be better in actual production process that because high concentrations of acid and alkali can degrade endotoxins. This method is cheaper than chromatography. Lastly, Electrodialysis method (EM) is also can remove endotoxins that endotoxins exhibit negative charges. From our laboratory data, the removal rate of endotoxins can get to 93.6% at 1.3h, and 97.2% at 2.3h. Afterwards, although it increased, the increase was minimal. Ultimately, the removal rate of endotoxins by electrodialysis can reach 97.7%. This method not only has a good removal effect on endotoxins, but also is quite easy to operate.

4.4. Hydrophobic Interaction Chromatography(HIC)

Hydrophobic interactions between the lipid A portion and sorbent are also considered to be important attributes that removal techniques can take advantage of [28]. Immobilized hydrophobic ligands interact with nonpolar protein surfaces through van der Waals forces for high endotoxin removal [34]. Protein and endotoxin are adsorbed onto the ligands and later separated using salt addition based on gradient elution [35]. Other chromatographic method that is dominated by hydrophobic interactions is reverse-phase chromatography (RPC). RPC is divided into liquid (RPLC) and ion-pair (RPIPC) [36]. For RPLC, hydrophobic interaction occurs between the ligands and bases while hydrophobic ligands in RPIPC interact with hydroorganic eluents. Retention time in RPLC depends on the molecular structure and size of nucleic acids. RPIPC is reproducible and effective for commercial production of therapeutic pDNA. However, RPC is toxic and requires organic solvents for operation [37]. At present, the technology is not yet mature and further research is needed. In actual production, it is relatively less commonly used than other methods.

5. CHALLENGES OF ENDOTOXIN REMOVAL

Although there are many methods for removing endotoxins, there are still many challenges in the actual production process. The first one, gram-negative bacteria can grow in nutrient-poor media, this means that even though the endotoxin is completely removed during the purification process, the endotoxin is still growing rapidly in actual production process. The whole purification process is exposed to the air, at the same time, it need long time to complete each purification unit in mass-produce. According to our experimental results, the fermentation broth, which only had our production and underwent sterile filtration, was left at room temperature for 13 hours, and then the endotoxin increased by 2×10^2 times. For 43 hours, and the endotoxin increased by 9×10^3times. This requires us to quickly remove endotoxins and use multiple methods for removal in different purification unit. The second one, endotoxins have both monomers and aggregates, resulting in

uneven properties, so it's difficult to remove them through a single method. Choosing a combination of two or more methods can achieve the goal of removing endotoxins. The above is not the most difficult in protein-free solutions, the most challenging part that endotoxins and proteins can interact with each other is in protein-containing solutions. Many studies have reported that lipopolysaccharide-binding protein (LBP), bactericidal/permeability-increasing protein (BPI), amyloid P component, cationic protein (38, 39), or the enzyme employed in the biological endotoxin assay (anti-LPS) factor from Limulus amebocyte lysate (LAL) [40] show interactions with endotoxins. These proteins are directly involved in the reaction of many different species upon administration of endotoxin [41, 42]. The interactions of other proteins with endotoxins even have no strong links to a biological mechanism, which is generally assumed that electrostatic interactions are the main driving force. The other interaction mechanism is hydrophobic interactions with proteins, even though there is no strong evidence [5]. Besides above, more probable mechanism is dynamically stable calcium bridges between proteins and endotoxins which are resulted by the competition of protein-bound carboxylic groups and endotoxin-bound phosphoric acid groups for $Ca2^{+}$ [3]. Regardless of the mechanism, these interactions not only result in hiding endotoxin molecules, which are not removed in the removal procedures, but also lead to significant loss of target proteins. Among existing methods, affinity chromatography can be used to remove endotoxins, but much time is needed to screen out suitable fillers that generate strong interactions with endotoxins or have a specific dissociation of protein-endotoxin complexes This is much more difficult than we imagined. Finally, equipment cleaning should be given the highest attention in the actual production. Strict cleaning procedures must be established, which is the cornerstone of the success for the entire experiment. It is also a step that people often overlook.

6. CONCLUSION

The endotoxin removal methods reviewed in this paper include laboratory scale and actual production process. Each method is not perfect, the choice of endotoxin removal method depends on the level of purity, rapidness, difficulty of operation, availabilities of chemicals and the cost. In the actual production process, due to the existing difficulties of endotoxin removal in reality, a single method cannot be implemented in one step, two or more methods are needed to achieve the goal. Besides, special attention should also be paid to the cleanliness of the method itself to avoid introducing new endotoxin contamination. Despite the many challenges in endotoxin removal, with the advancement of science and technology, efficient and cost-effective novel removal methods of endotoxins will no longer be a dream.

AUTHOR CONTRIBUTION STATEMENT

All authors listed have significantly contributed to the writing of this article.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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