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Endotoxin Purification in Recombinant Protein Production using *Escherichia coli*

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Abstract

The host organism *Escherichia coli* (*E. coli*) is preferred when producing recombinant proteins due to its quick growth, well-known genome and cost-effectiveness. A significant challenge associated with the host organism is the presence of lipopolysaccharides (LPS) also known as endotoxins. This potent pyrogen is an inherent part the outer membrane of gram-negative bacteria and trigger severe inflammatory responses in mammals. LPS consists of three parts: core oligosaccharide, O-antigen and lipid A. Lipid A is responsible for the inflammatory response in mammals. The two primary endotoxin detection methods are LAL-test (Limulus Amoebocyte Lysate) and rFC-test (recombinant Factor C). Two main types of purification strategies are reviewed: upstream and downstream. Upstream methods involve preventative measures to minimize endotoxin contamination during cultivation. Downstream methods involve purifying the protein solution after production has been completed.

The upstream method reviewed is the genetically modified *E. coli*-strain KPM404, which produces non-toxic lipid IV_A. Drawbacks of this method include regulatory challenges, as protein solution may yield false positive test results. Downstream methods reviewed include ion-exchange chromatography, affinity chromatography, ultrafiltration and Two-phase extraction with Triton X-114. Ion-exchange chromatography utilizes charge differences between LPS/protein and chromatographic medium. Its disadvantage is that it is ineffective against negatively charged proteins. Affinity chromatography utilizes highly selective polycationic ligands that capture endotoxins. This method struggles with LPS saturation and non-specific bonding of small proteins, requiring continuous washing which increases costs. Ultrafiltration is a size-based filtration method. This method is often used for concentrating protein preparations and is ineffective for solutions containing LPS aggregates and small proteins. Two-phase extraction with Triton X-114 is based on temperature-induced phase separation. This method is effective for hydrophilic proteins but leaves behind detergent residue that require additional purification. This leads to protein loss.

The result of the comparison show that the effectiveness of each method is highly dependent on the physical and chemical properties of the protein. Thus, no single method is optimal. Advancements in protein engineering, genetically modified *E. coli*-strains and endotoxin test methods are required for further progress in the industry.

Keywords Recombinant protein, Endotoxin, Lipopolysaccharides, *Escherichia coli*, Endotoxin purification

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Sammandrag

Värdorganismen *Escherichia coli* (*E. coli*) föredras vid produktion av rekombinanta proteiner på grund av sin snabba tillväxt, välkända genom och kostnadseffektiva odlingsförhållanden. *E. coli* har dock en betydlig nackdel i sitt yttre membran: endotoxiner, som kallas även för lipopolysackarider (LPS). De är potenta pyrogener som inducerar allvarliga inflammatoriska reaktioner hos däggdjur. LPS frigörs vid proteinutvinning och kontaminerar proteinpreparationer. LPS består av tre delar: kärnoligosackarid, O-antigen och lipid A, varav lipid A är ansvarig för den inflammatoriska responsen. För att detektera endotoxiner i proteinpreparat används främst LAL-test (Limulus Amoebocyte Lysate) eller rFC-test (recombinant Factor C). Två huvudkategorier av reningsstrategier analyseras: uppströmsmetoder och nedströmsmetoder. Uppströmsmetoder innebär förebyggande åtgärder som minimerar endotoxiner under proteinproduktionsprocessen. Nedströmsmetoder innebär rening av proteinlösningen efter att produktionen har slutförts.

Uppströmsmetoden som granskas är den genetiskt modifierade *E. coli*-stammen KPM404, som producerar icke-toxiskt lipid IV_A. Nackdelar med metoden inkluderar regulatoriska utmaningar, då proteinlösningen kan ge upphov till falska positiva testresultat. Nedströmsmetoder som granskas inkluderar jonbyteskromatografi, affinitetskromatografi, ultrafiltrering och tvåfasextraktion med Triton X-114. Jonbyteskromatografin utnyttjar laddningsskillnader mellan LPS/proteinet och kromatografibäraren. Nackdelen med metoden är dess ineffektivitet mot negativt laddade proteiner. Affinitetskromatografi använder sig av högt selektiva polykatjoniska ligander som fångar endotoxiner. Metoden kämpar med LPS-mättnad och icke-specifik bindning av små proteiner, och kräver kontinuerlig rengöring vilket höjer kostnaderna. Ultrafiltrering är en storleksbaserad filtreringsmetod. Metoden används även för koncentrering av proteinpreparat, men är ineffektiv för lösningar med LPS-aggregat och mindre proteiner. Tvåfasextraktion med Triton X-114 bygger på temperaturinducerad fasseparering. Metoden är effektiv för hydrofila proteiner, men lämnar kvar detergentrester som kräver ytterligare rening vilket leder till proteinförlust.

Resultaten av jämförelsen visar att metodernas effektivitet påverkas starkt av proteinets fysikaliska och kemiska egenskaper, och därmed är ingen metod optimal. Utvecklingar inom proteinteknik, genetiskt modifierade *E. coli*-stammar och endotoxin-testmetoder krävs för ytterligare framsteg inom branschen.

Nyckelord Rekombinanta proteiner, Endotoxiner, Lipopolysackarider, *Escherichia coli*, Endotoxinborttagning

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Preface and acknowledgements

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Otaniemi, 5th of September 2025
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Abbreviations

Abbreviation	**Full Term**
POI	Protein of Interest
E. coli	*Escherichia coli*
LPS	Lipopolysaccharide
kDa	Kilodalton
OMV	Outer Membrane Vesicle
EU/mL	Endotoxin Unit per millilitre
EU	Endotoxin Unit
LAL	Limulus Amoebocyte Lysate
rFC	Recombinant Factor C
IEC	Ion Exchange Chromatography
pI	Isoelectric Point
AC	Affinity Chromatography

1 Introduction

Recombinant protein production is a fundamental technique in biotechnology and pharmaceuticals, which involves utilizing a host organism to produce the protein of interest (POI). These proteins are not naturally produced by the host organism and are therefore required to be genetically modified.

A common host organism used in the production of recombinant protein is the bacteria *Escherichia coli* (*E. coli*). *E. coli* is highly valued because of its quick growth, well-known genome, and its cost-effective nature in large-scale protein production. Despite all its advantages as a host organism, *E. coli* does pose a significant disadvantage: endotoxins. These endotoxins, also known as lipopolysaccharides (LPS), exist in the outer membrane of the *E. coli* cells.

LPS tend to contaminate the protein production batches during the cell lysis phase. Cell lysis is the disintegration of the cell membrane. As a result of cell lysis, both the POI and the unwanted LPS are released into surrounding media and contaminate the protein solution. When LPS enters the human bloodstream, it causes severe inflammatory reactions in humans. Hence, efficient endotoxin detection and purification is essential when intended for human diagnostic use.

Endotoxin purification is of significant role in this thesis. Therefore, the focus in this literature review is: What is the preferred purification method to minimize endotoxins in the production of recombinant proteins in *E. coli*? To answer this, this literature review will establish essential background on recombinant protein production in *E. coli* and structure of endotoxin. Then, the topic of endotoxin contamination and detection will be reviewed before finally presenting and evaluating various endotoxin purification methods.

2 Background and Literature review

This chapter examines all relevant topics of endotoxin purification. Subsection 2.1 covers the recombinant protein production in *E. coli*, as modifications in the production phase minimizes endotoxin release. Subsection 2.2 explores the structure and properties of endotoxin. Understanding these properties is fundamental to develop effective removal strategies for endotoxin. In subsection 2.3, endotoxin detection methods, regulation and contamination will be briefly discussed. In the final subsection 2.4, the knowledge from previous subsections is critical, as it will aid with the evaluation of the methods of endotoxin purification.

2.1 Recombinant protein production in *E. coli*

Recombinant protein production enables mass production of a specific protein by introducing the gene of the protein into a host organism. The host does not naturally possess this gene but can utilize its own cellular transcription and translation to express it. This technique provides considerable advantages over traditional methods, as earlier methods struggled with yield and consistency. This struggle was due to proteins being obtained through the extraction from natural sources or through chemical synthesis. Comparing to the conventional approaches, recombinant technology provides stable and consistent yields with remarkable versatility. This invention has not just improved protein production, it has transformed a multitude of fields, enabling therapeutic development, research and industrial applications [1].

Recombinant protein production improves sectors such as biotechnology, medicine, and industry due to being endlessly customizable and allowing scalable production. In biotechnology, recombinant proteins enable gene cloning, protein engineering and other functional studies such as protein properties. The medical field has transformed to be safer and more effective because of recombinant proteins, with developments made in pharmaceuticals and diagnostics. Industry has also benefited from this revolution, as recombinant enzymes improve manufacturing in food, textiles, and chemicals. Additionally, recombinant proteins play a crucial role in global challenges. They can be applied to environmental remediation efforts such as wastewater treatment and pollution control. They also offer sustainability with bioproducts [2].

2.1.1 *E. coli* as a recombinant protein expression system

Two main types of protein expression systems exist: prokaryotic and eukaryotic. Prokaryotic systems are based on bacteria, and among all prokaryotic host organisms *E. coli* remains one of the most popular [3], [4]. *E. coli* has dominated the market for decades due to its high cell density yields and low cost as its growth media is inexpensive. It is also preferred because of its well-characterized genetics and rapid doubling time of 20 minutes under optimal conditions [2]. Approximately one third of all recombinant proteins and half of all protein products approved are produced using *E. coli* [5]. *E. coli* is suited for both industrial and laboratory-scale production, as it allows adaptable expression of proteins and is compatible with a wide array of vectors and expression systems [3]. Despite the emergence of innovative methods, like cell-free protein synthesis, *E. coli* maintains its dominance. This is because the innovative techniques are either resource-consuming, or their implementation is time-consuming [6].

The recombinant protein production process in *E. coli* begins with vector construction. The gene that codes for the desired protein is isolated and then amplified by polymerase chain reaction or excised from a source using restriction enzyme digestion. The gene is then inserted into a cloning vector, a small self-replicating DNA fragment, and is propagated there. Then, the cloned gene is ligated into an expression vector, which is a type of cloning vector that contains all regulatory elements for gene expression. One of the regulatory elements of the expression vector that *E. coli* requires is a promoter region, that is strong enough to facilitate expression of the protein. A promoter is considered effective if it yields 10-30% of total cellular protein while also balancing low basal activity to halt unwanted transcription [3], [4]. Other regulatory elements are a ribosome binding site, cloning sites, selection marker, and an origin of replication that enables autonomous self-replication. Additionally, a terminator sequence is required to cue the end of transcription [7].

After ligating the gene into the expression vector, the vector is introduced into the host organism through transformation. The host cells that successfully took up the expression vector are then isolated through selection that utilizes the selection markers. As the expression vector carries both the gene of interest and a selection marker, only cells that possess this vector will survive [7]. *E. coli* commonly possesses an ampicillin resistance gene as its selection marker. This ampicillin resisting bla-gene, encodes for β-lactamase. A drawback for this enzyme is that it tends to degrade ampicillin too rapidly, allowing vector-free cells to eventually grow. Some antibiotic-free selection systems have been developed that address this limitation. These antibiotic-free selection systems are valuable for large-scale fermentation where vector stability is essential for high yield protein production [3].

After the selection, the clones that produce the desired protein are then identified through screening methods such as polymerase chain reaction or enzyme assays. After confirming that the proteins contain the expression vector with the gene of interest, the cultivation is initiated. This phase ensures that the conditions for protein expression are optimized by adjusting temperature, pH, and nutrient availability to maximize yield and quality. To further optimize the concentration of the protein, an inducer can be introduced. The inducer interacts with the promoter in the expression vector and triggers a cascade of reactions that enhances protein synthesis [7]. Next, various purification methods are used to isolate the POI from other cellular components and lastly, the protein is characterized to confirm its identity [2].

By utilizing its own cellular machinery, *E. coli* can express proteins in large quantities. However, prokaryotic systems fail at posttranslational modifications such as glycosylation and phosphorylation leaving the proteins simplistic [2]. There are other challenges encountered when using gram-negative bacteria like *E. coli*, especially when expressing eukaryotic proteins. The absence of post-translational modification machinery like glycosylation hinders the production of stable eukaryotic proteins [2]. Other common issues include protein misfolding, often leading to the formation of inclusion bodies [8] due to *E. coli* attempting to express complex or membrane-bound proteins [2]. Additional challenges are low yields of complex or disulfide rich proteins and cellular stress. One significant problem when creating proteins for therapeutic use, which is also the focus of this thesis, is endotoxin contamination. Endotoxin is a potent pyrogen and is therefore required to be removed from protein solutions. It is important to note that endotoxin contamination is not a problem for all applications. If the proteins are to be used as industrial enzymes or materials, where they are not introduced into the body, endotoxin removal is not necessary.

2.2 Endotoxin structure and properties

Endotoxin, also known as lipopolysaccharide (LPS), is found in the outer membrane of gram-negative bacteria. LPS is a large, amphipathic molecule with a molecular weight ranging from 10 to 20 kilodaltons (kDa). It is composed of three covalently bonded components: lipid A, the core oligosaccharide, and the O-antigen [9]. LPS plays a fundamental part of the bacterial membrane as it provides both structural integrity and a permeability barrier against small, hydrophobic molecules. This makes gram-negative bacteria resistant to multiple antimicrobial compounds. Additionally, LPS is heat stable which allows it to stay active even in feverish environments [10].

In contrast to the typical phospholipid bilayers in cell membranes, gram-negative bacteria such as *E. coli* possess an asymmetric bilayer where the LPS faces the extracellular environment. LPS is amphipathic, as it consists of both hydrophobic and hydrophilic regions [11]. This amphipathic nature results in aggregation of LPS in water. This trait of self-assembling into micelles is induced by hydrophobic interactions between lipid A molecules that make the lipids cluster together [12]. Additional electrostatic interactions of divalent cations stabilize phosphate groups [9]. In LPS, the hydrophobic lipid A is embedded in the membrane, and the hydrophilic O-antigen faces outwards, as seen in Figure 1. This arrangement ensures that the LPS contributes to host pathogen interactions and immune system activation when released during cell lysis [13], [14].

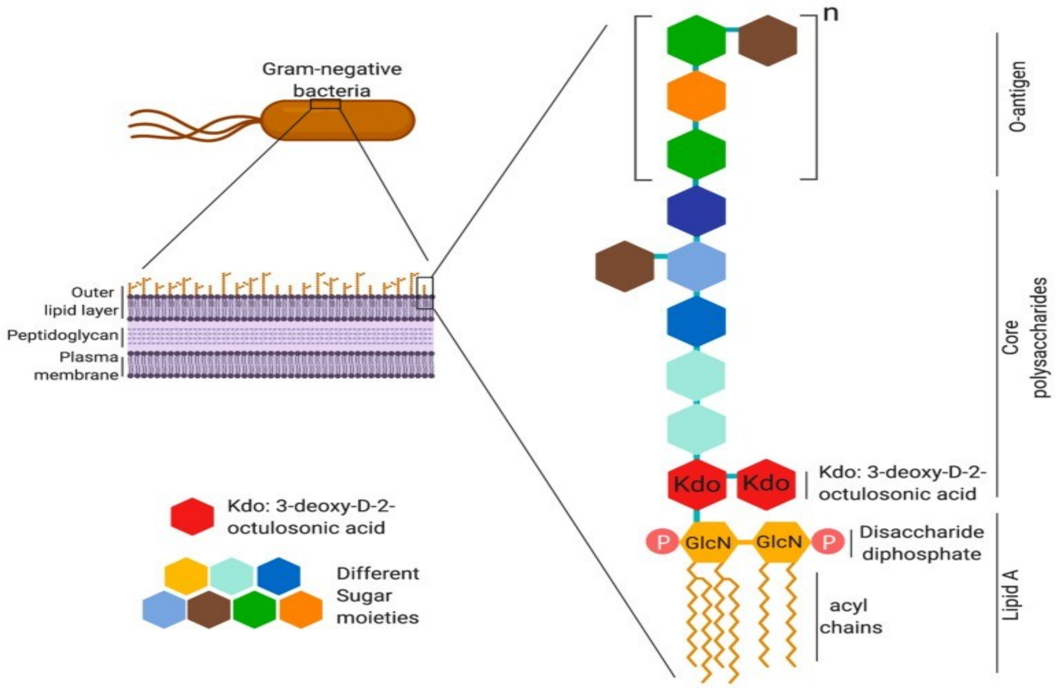

Figure 1. Structural details of LPS from gram-negative bacterium (adapted from [10]).

The structure of LPS is conserved; it does not change drastically between strains or species of gram-negative bacteria. Among the three LPS components, the hydrophobic lipid A is the most conserved part. It is also the main virulence factor responsible for endotoxin toxicity in LPS. Lipid A consists of a phosphorylated glucosamine disaccharide spine which links to two different components. The first component is an 8-carbon sugar acid called Kdo, which is essential in most strains of *E. coli*. Lipid A is linked to two of these and without it LPS cannot be assembled. The second component lipid A is connected to is fatty acid (acyl) chains. The amount of acyl chains determines the immunostimulatory capacity of LPS [5]. *E. coli* comprises of a lipid A with six fatty acid chains, which is highly immunostimulatory [10]. Although conserved, subtle variations in lipid A among bacterial species, such as the number of phosphate groups or the position of esterified acyl chains, affect immunogenicity and toxicity [10].

The core oligosaccharide is connected to lipid A through Kdo. The core oligosaccharide is also essential in connecting lipid A to the O-antigen maintaining membrane integrity. Its structure has low variability, although it may vary among some strains of species. The O-antigen is in direct contact to the outer environment and therefore necessary when adhering to tissues. It is the most diverse component, and some gram-negative bacteria do not synthesize this component [11], [12], [13], [15]. The O-antigen is highly variable in length and has a significant impact on the molecular weight of LPS. Extreme cases can vary from as little as 2.5 kDa to as much as 70 kDa [9].

The immunostimulatory LPS is released during bacterial lysis when recombinant proteins are extracted. When administered to humans, this contaminating LPS circulates as micelles in the bloodstream. This is due to their amphipathic nature, where they aggregate in the form of outer membrane vesicles (OMVs). In the bloodstream an LPS-binding host protein will extract individual, monomeric LPS molecules from the OMV aggregate and transport it to a co-receptor called CD14. As CD14 is a co-receptor, it will present the LPS to the main signalling receptor complex, that is made up of two parts: the Toll-like receptor 4 (TLR4) and an essential small protein called MD-2. This receptor complex is located on the surface of immune cells and is called (TLR4)/MD-2. The small protein MD-2 interacts with the acyl chains in lipid A, and this triggers two parallel reactions [10]. These two reactions activate the immune response with the release of tumour necrosis factor and pro-inflammatory cytokinesis. These are toxic components that may lead to septic shock [16]. Even amounts of 1 to 2 mg of LPS intravenously can lead to severe effects or death [12].

While all the parts of LPS are immunogenetic, only lipid A is highly bioactive. Due to its innate ability to activate an immune response in mammals, LPS can be used as a biomarker for early detection of gram-negative infections. LPS also contributes to the innate antibiotic resistance that gram-negative bacteria possess. Due to its hydrophobic nature, the outer membrane of the gram-negative bacteria hinders the penetration of antimicrobial agents. Additionally, many gram-negative bacteria are shown to form biofilms, which provide both stability and resilience to antimicrobial agents by increasing the hydrophobicity of the molecule [12]. LPS enter the human body through infection, food, supplements, and pharmaceutical drugs. Therefore, endotoxin detection and removal are critical in biotechnology and pharmaceutical preparations, especially in the production of recombinant proteins using gram-negative bacteria such as *E. coli*.

2.3 Endotoxin detection, regulation, and contamination

An excessive amount of LPS can activate a severe inflammatory response which can lead to organ damage and sepsis. Therefore, endotoxin detection and purification are crucial in recombinant protein production. It is important to recognize that other bacterial products produced by *E. coli*, such as lipoproteins, may induce similar adverse effects. These bacterial products may act alone or synergize with LPS, enhancing immune responses. Thus, all bacterial contaminants should be considered when handling and purifying recombinant proteins. This section will focus on endotoxin regulation, as well as established and emerging detection methods in recombinant protein preparations [17].

2.3.1 Standards

When selecting a suitable endotoxin detection method, regulatory guidelines must be taken into consideration. The European Pharmacopoeia chapter 2.6.14, United States Pharmacopoeia chapter <85> and Japanese Pharmacopoeia chapter 4.01 are interchangeable chapters when implementing endotoxin regulation as all are calibrated against World Health Organization (WHO) International Standard for Endotoxin [18]. These chapters are harmonized for international regulatory use and are interchangeable across ICH (International Council for Harmonisation) regions. As described in European Pharmacopoeia General chapter 2.6.32 [19], the Limulus Amoebocyte Lysate assay and recombinant Factor C test are universally accepted.

Endotoxin is measured in endotoxin units per millilitres (EU/mL). 1 EU equals 0.1-0.2 nanogram of endotoxin per millilitre of solution. In general, an endotoxin level of 0.1 EU/mL is considered acceptable for in vitro studies. The maximum limit of endotoxin is set to 5 EU per kilogram of body weight per hour by the pharmacopoeias [9]. As a result of this, the effects of endotoxins are dose-dependent, and the acceptable endotoxin limit for a specific product depends on the amount administered to a patient [20][21].

2.3.2 Limulus Amoebocyte Lysate

The most common test is the Limulus Amoebocyte Lysate (LAL) test, which utilizes horseshoe crab blood cells, called amoebocytes. An innate enzyme in the amoebocytes called Factor C causes a clotting reaction when exposed to LPS. Gel-clot, turbidimetric and chromogenic assays are all LAL methods where the standard endotoxin reference varies from 0.03 EU/mL to 0.001 EU/mL [17].

The gel-clot assay is a qualitative end-point method where LPS triggers an enzymatic cascade, which leads to gel clots forming. It involves a one-time reading taken after a fixed incubation period. The method is simple and cost-effective, with a detection sensitivity of 0.03 EU/mL. However, it is limited by low sensitivity and low throughput [17] [22].

Turbidimetric and chromogenic assays are more precise than gel-clot, with a sensitivity of 0.001 EU/mL. Additionally, both methods offer real-time data which is more accurate. Turbidimetric assay measures the turbidity (optical density) of clotting when endotoxins react with amoebocytes. In contrast to the turbidimetric method, which uses the natural coagulogen protein in LAL as a detection substrate, chromogenic method uses a synthesized peptide which leads to a colour change in the reaction mixture when reacting with LPS. The downside of this method lies in its colour-changing property, as a substrate with colour will interfere with this assay. This method is preferred for low endotoxin samples, although it is more expensive as it requires a synthetic substrate. As a result of the lower cost and no need for a synthetic substrate, turbidimetric method is more widely used in routine screening [17] [22].

Despite the sensitivity of LAL assay, it has limitations. Amoebocytes rely on a multitude on enzyme reactions that are sensitive to pH, salt, and temperature. If any of these factors are thrown out of balance, the enzyme activity and optical readings can be inhibited. Also, substances that interfere at any stage, such as other proteins, will result in the amoebocytes switching onto an alternate coagulation pathway prompting false positive test [23]. LPS also has a strong binding affinity to recombinant proteins and other bacterial components, masking its existence to LAL assays [17]. Lastly, the importance of the horseshoe crab blood has caused the population to drop alarmingly. It is necessary to use an alternate method to the LAL-assay which does not rely on harvesting blood from the endangered species [22].

2.3.3 Recombinant Factor C

Recombinant Factor C (rFC) test was introduced a decade ago, as a synthetic, animal free option for LAL-based endotoxin detection methods. This newer method uses the enzyme recombinant Factor C similarly to how LAL assay uses Factor C. The full length of this animal derived enzyme was cloned and synthetically produced in *E. coli*. It was then cloned again and expressed as rFC, a recombinant protein. This newer method has a high sensitivity of 0.001 EU/mL, on par with LAL assays. This method has multiple advantages, one of them being it is solely sensitive to LPS and does not react to glucan, which LAL does [22]. Additionally, rFC can bind strongly to LPS that is both free in the protein batch and LPS that is bound to other protein compounds. This makes rFC a remarkable option for endotoxin removal [23]. Compared to traditional LAL assays, rFC is more accurate and sensitive to endotoxins under the same lab conditions [24].

2.3.4 Endotoxin contamination in *E. coli* systems

Endotoxins are an inherent part of the gram-negative bacterial cell membrane, and they pose significant safety risks in pharmaceutical applications, such as septic shock. During the protein production, the endotoxins in the membrane of *E. coli* bond strongly to proteins, especially those of hydrophobic or of basic nature [6]. These endotoxins are released into the surrounding media simultaneously as the extraction of the recombinant protein occurs.

There are multiple options for protein extraction methods: mechanical methods, physical methods, chemical methods, and enzymatic methods [27]. Since all these methods are host dependent, the characteristics of the host should be taken into consideration [25], [26].

Endotoxins contaminate the protein solution through various phases during production. The first contamination phase is during fermentation, where *E. coli* naturally sheds OMVs that contain LPS, phospholipids and periplasmic proteins. Additionally, free LPS aggregates are released during fermentation due to cell wall turnover or stress. The second stage of contamination occurs during the protein extraction phase. The physical extraction of proteins disrupts the outer membrane, releasing LPS. The chemical extraction method solubilizes the outer membrane and frees LPS micelles. Enzymatic extraction methods degrade peptidoglycan, a crucial polymer that provides structural support to the cell wall. This destabilizes the outer membrane, releasing LPS [25].

Endotoxins tend to interact with proteins. As LPS forms aggregate structures, multiple proteins can interact with them to form protective complexes that may hide endotoxins. These complexes complicate removal, hence the need for different removal methods. These methods are reviewed in the next subsection 2.4 [9].

2.4 Endotoxin purification and their challenges

The dangers of endotoxin contamination in therapeutic application necessitates purification of the protein product. This process of endotoxin removal is called depyrogenation. Depyrogenation is essential for patient safety and for obtaining the target protein in a highly concentrated form, which simplifies downstream applications. The first step of purification is cell lysis. The host cell lysate is then clarified by centrifugation, and the protein is purified from waste and toxins. The purification step uses various downstream and upstream methods, such as ultrafiltration, ion exchange chromatography and engineered protein hosts [2]. The ideal method is dependent on the properties of the cell culture and protein.

Challenges with purifying the POI are vast, as each protein have their own properties, therefore one method of endotoxin purification may not work for another recombinant protein [17]. Often, the purifiable samples contain multiple heterogenous proteins with comparable properties, making isolation of a specific protein harder. If the target protein at low abundance, they are susceptible to contamination from abundant proteins and require both specific isolation and purification methods. Complex biological samples like cell lysates interfere with purification processes due to their diverse biomolecule content. Other interfering factors are the stability of the protein as some are sensitive to changes in pH, salt concentration or temperature [2].

There are multiple upstream and downstream approaches when purifying and recombinant proteins produced with *E. coli*. When choosing the appropriate method, the following parameters should be taken into consideration: protein size, solubility, stability, and affinity to form specific ligands. This implies the characteristics of the POI should be known prior to selecting the method. For the case of complex biological samples or proteins, multiple methods can be utilized to maximize protein purity and concentration.

2.4.1 Downstream approaches

Downstream methods are at the end-stage of protein production, where the product is purified with methods such as chromatography and ultrafiltration. There are a multitude of different downstream approaches to utilize when purifying endotoxins from recombinant protein solutions. To achieve a selective removal, it is important to consider the chemical and physical structure of both the endotoxin as well as of the adsorbents, proteins, and solution conditions.

One of the most versatile and widely used methods for endotoxin removal, are adsorption techniques. These methods depend on multiple properties of both endotoxins and adsorbent materials. A few of these properties considered in the following methods are chemical composition, hydrophobicity and electrostatic nature of endotoxin and ligands. Two types of adsorption techniques are discussed in subsequent subsection: the ion exchange chromatography and the affinity chromatography.

Ion exchange chromatography

One type of adsorption method is ion exchange chromatography (IEC). In this method, the protein solution will flow through a column packed with a resin with adsorbent materials. This method utilizes the different charges of both the endotoxins, proteins, and adsorbent materials. IEC includes the usage of both anion exchange resins and cation exchange resins [25] and is commercially and widely available [29].

Since endotoxins are negatively charged due to the phosphate groups in lipid A, they naturally bind to positively charged groups on anion resins [25]. One issue with anion resins is that the effectiveness lowers when in presence of acidic proteins. Acidic proteins are negatively charged and therefore compete with endotoxins for binding sites in the anion resin adsorbent. As the adsorbent has a limited capacity, some endotoxin ends up slipping through. Another approach to IEC is using cation resins [28]. Cation resins are embedded with negatively charged particles that bind to positively charged proteins. This allows LPS to flow through. Cation resin has issues with negatively charged basic proteins, as they do not bind to the resin. Instead, the proteins flow through with the LPS. Therefore, both methods allow purification if the protein is acidic and are not suitable for basic protein solutions [30].

Common drawbacks for IEC include low flow rates and fouling. Another drawback is that IEC requires high buffer consumption, optimization of parameters and long regeneration times. This inflates the cost of this method, making the cost moderate [31]. Because of these drawbacks, a study was conducted on device Sartobind® Lab Q and S membrane absorber [30]. This was demonstrated to be an effective choice for standard protein purification, as it can utilize both anionic and cationic resins. Lab Q and Lab S are effective resins as they are reusable when washed with NaOH/KCl. Both Lab Q and Lab S allow a faster flow through of protein solution, as they are not packed in the traditional columns. Instead, they are stacked in flat membrane sheets with pore sizes ranging from 0.45 µm to 0.3 µm [30].

A neutral isoelectric point (pI) is the pH value at which a molecule does not carry net electrical charge [32]. If the pH of the solution is above the pI, the molecule loses protons and becomes negatively charged. The opposite happens if the pH is below the pI of the molecule. When the pI of proteins is higher than the pH of the solution, Q-membranes can be utilized to bind negatively charged endotoxins. This entails that Q-membranes are an anionic exchange method. When the protein pI is lower than the solution pH, the cationic exchange S-membrane is used to bind positively charged proteins. Both Lab Q and Lab S are scalable from lab (15 mL) to industrial (5 L) scale. This IEC method is efficient, with a reduction of 99.4% in endotoxins while 95% of proteins were retained [30]. Although a promising method, one drawback is its sensitivity to high salt concentration, as high salt concentration affects the electrostatic interactions between the resins and the protein/endotoxin. Additionally, the issue with basic proteins still stands.

Affinity chromatography

Another adsorbent method is the affinity chromatography (AC). This method is commercially available and utilizes a solid matrix that is attached to ligands [33]. These ligands are immobilized molecules that are chosen based on specific and reversible interactions with endotoxin [25]. This ensures, that solely endotoxins bind to the ligands, while the proteins flow past. For protein solutions, the most effective approach for AC are adsorbers with polymetric matrices with cationic functioning groups [34].

One commonly used solid matrix is the polymeric matrix, coated with cationic groups. The polymeric matrices are solid supports with pores that are coated with amine rich polymers, such as polyethyleneimine. The cationic groups on the polymers bind to the negatively charged phosphate groups on the LPS. The pore sizes are large enough for endotoxin molecules to enter, yet too small for proteins. As the proteins are too large to enter the pores, they have less access to binding sites and pass through with minimal retention. This minimizes protein loss. One significant strength of the polymer matrix is its strong selectivity to LPS with removal of up to 99% and remarkably high protein recovery of >99%. This method is also reusable, as the LPS can be removed with high-salt or alkaline washes. These benefits make this method a semi scalable, robust LPS removal for large batches. One inherent weakness is that high loads of LPS can saturate the bonding sites, requiring frequent washes. Another issue is the leaching of the ligands, where over time the ligands detach and contaminate the protein solution. This heightens the cost of the method, as the column must be replaced. Additionally, if the protein is small or positively charged, non-specific binding can occur [28].

Ultrafiltration

Ultrafiltration (UF) is a widely used method for protein concentration and partial endotoxin removal. UF utilizes a size-exclusion membrane with the molecular weight cut off ranging from 10-100 kDa. Endotoxins are heterogenous molecules found in a wide range of conformations and aggregation states, ranging from 1 to 1000 kDa and even up to 8000 kDa [35]. With UF, the protein solution is pushed through the membrane, resulting in a separation of the smaller endotoxins and proteins. As a result of the simplicity of this method, UF is readily available, easy to incorporate and is therefore cost-effective.

One issue with UF is protein size. UF is ineffective for smaller proteins, as they will co-permeate with endotoxins. Additionally, the concentration of the protein solution has an impact on the endotoxin removal capacity. A higher protein concentration reduces the endotoxin removal, as they may bind to each other. To battle this, the solution can be diluted, and the endotoxin removal capacity rises to 99% while keeping the protein yield at 94% [36], [37]. Unfortunately, the protein size or concentration is not the only limitation. Elevated temperatures of 100°C or acidity of 3.5 pH to 4.5 pH may degrade the endotoxin aggregates into lipid A which are small and range from 2 to 4 kDa. Additionally, when in the presence of proteins, endotoxins can dissociate into smaller subunits. Therefore, the smallest of endotoxins cannot be filtered with UF as they will pass through the membrane [35]. To enhance the efficiency of UF, the issue of endotoxins passing through the membrane can be avoided with calcium ions to promote endotoxin aggregation. Unfortunately, this method is limited to those proteins that are not affected by calcium ions [28].

Two-phase extraction

Another effective endotoxin removal method is the Two-phase extraction that utilizes a cost-effective and readily available detergent Triton X-114 [38]. The non-ionic Triton x-114 forms micelles that incorporate endotoxins via hydrophobic interactions. When heated to above 22°C, Triton x-114 reaches its cloud-point [35]. Cloud-point is the temperature at which a liquid becomes turbid (cloudy) due to phase separation. This phase separation includes a detergent rich phase that isolates endotoxins, and a protein rich aqueous phase [28]. The phases are then separated by centrifugation.

Two-phase extraction method works well for soluble hydrophilic proteins, such as enzymes or antibodies. When purifying recombinant proteins from endotoxins, removal efficiencies reached 98-99%. However, the drawback of this method is that a certain amount of Triton x-114 will always remain in the solution, requiring additional purification steps such as adsorption or filtration [35]. This implies a time-consuming process and a product loss of 10-20%. Another drawback is the temperature sensitivity of Triton x-114, which limits its usage with heat-labile proteins [28].

2.4.2 Upstream approaches

Moving on to upstream approaches, they minimize endotoxin contamination already in the transcription phase by utilizing endotoxin-free expression systems. Genetically engineered *E. coli* is an example of production hosts that are LPS-deficient. Implementing upstream approaches minimizes the reliance on downstream purification methods.

Endotoxin-free E. *coli strains*

Genetically modified *E. coli* is used to eliminate endotoxin contamination. According to [5], the novel method of the engineered endotoxin-free *E. coli* strain KPM22 demonstrated the existence of a stable strain that minimizes endotoxin response in humans. KPM22 is engineered from its parental K-12 strain, which is a wild-type *E. coli*. Wild-type *E. coli* is the unmodified strain of *E. coli*. K-12 is comprised of a lipid A with six acyl chains that are highly immunostimulatory. Lipid A is also attached to two Kdo sugars, which until now, were believed to be required for the stability of *E. coli*. This was demonstrated to be incorrect by the KPM22 strain [5].

The engineered KPM22 survives with a lipid IV_A, which is a precursor to lipid A. Lipid IV_A only has four acyl chains that make it bind weakly to the TLR4/MD-2 complex. In fact, it blocks TLR4 by competing with toxic LPS making it a receptor antagonist. As a result of these properties, lipid IV_A is non-endotoxic. Despite this innovative method, the downside of KPM22 is its temperature sensitivity, as it fails to grow at over forty degrees Celsius. This poses as an issue as industrial fermentations run at 37 - 42°C. Hence, a temperature resistant strain was engineered: KPM404. This strain is derived from an industrial expression strain BL21(DE3). This strain is widely used in recombinant protein productions for its effectiveness [5] and is commercially available as ClearColi® BL21(DE3) [40].

To comprehend how KPM404 is engineered, the assemblance of the lipid A needs to be discussed. Lipid A is assembled in these steps. First, lipid IV_A is synthesized. Next, two Kdo are synthesized according to the genes *kdsD* and *gutQ* in the *E. coli* and attached to IV_A. Finally, two more fatty acids are attached, forming the hexa-acylated lipid A that is highly toxic. The engineered KPM404 contains a deletion of the genes *kdsD* and *gutQ*, which blocks Kdo synthesis. The deletion of genes lpcL and lpxM also inhibits the attachment of the two fatty acids, leaving the LPS assembly to stall at the precursor lipid IV_A. To ensure that lipid IV_A does not acquire variable modification which can reintroduce endotoxicity, the genes pagP, lpxP and eptA are deleted. An additional mutation at the gene msbA148 allows LPS to be transported to the outer membrane and stabilize it despite not having lipid A [5].

Endotoxin-free *E. coli* strains, such as KPM404, offer significant advantages by including stable genetic deletions that minimize the immunostimulatory endotoxin. These endotoxin-free strains reduce cost and complexity significantly, as no further purification methods are required [39]. To prove the reduced immunological effect of the engineered strains, a recombinant protein called HSP70 was produced in both KPM404 and its non-engineered parental strain BL21(DE3). Both products were then evaluated with LAL. The protein produced in KPM404 elicited 97.2 ± 0.5% lower response compared to its parental strain. However, a key limitation for endotoxin-free *E. coli* strains arises because of the LAL assay. LAL assay reacts to the conserved glucosamine backbone from lipid IV_A [41] and may elicit false positives. This is a challenge, especially as LAL is the standard testing method according to European Pharmacopoeia.

Another limitation for endotoxin-free strains such as KPM404 is their slow growth rates. Conventional strains have a 50% higher growth rate compared to endotoxin-free *E. coli* strains [42]. This reduced growth rate is a major drawback, as the quick growth rate is one of the key-reason *E. coli* is used as an expression host. Nevertheless, the elimination of additional downstream application, offered by endotoxin-free *E. coli* strains, can compensate for this limitation. Additionally, when KPM404 cultures reach sufficient densities, the amount of protein expressed is of the same level as wild-type *E. coli* [39].

3 Discussion

3.1 Summary of key findings

When producing recombinant proteins in *E. coli*, endotoxins are an inherent part of the process as they occupy the cell membrane of the bacteria. Therefore, depyrogenation is essential in therapeutic applications as endotoxins trigger immune responses.

The purification process can be implemented upstream when *E. coli* is translating proteins. These solutions often utilize engineered *E. coli* hosts. The upstream approach discussed is the endotoxin-free *E. coli* strains. When genetically modifying *E. coli* by gene deletion, the lipid A synthesis can be blocked to prevent endotoxin formation. This method is quite advantageous, as it results in about a 97.2% lowered LAL-test result compared to wild-type *E. coli*. By utilizing engineered *E. coli*, the need for downstream purification methods is reduced. This method has its limitations, with the most notable being false positives with the LAL assay.

If endotoxins are present after cell lysis, downstream methods can be applied. The downstream process begins the clarification of the protein batch with centrifugation. The batch is then then purified with multiple different methods: Ultrafiltration, two-phase extraction, and adsorption-based endotoxin removal. The choice of purification method depends on protein and endotoxin properties as well as culture conditions.

The first downstream method discussed is the adsorption-based method IEC. This method utilizes either anionic resins that bind negatively charged endotoxins letting proteins flow through, or cationic resins that bind positively charged proteins and lets LPS flow through. IEC works solely for basic proteins, as acidic proteins either bind to anionic resins or flow through the cationic resins with LPS. This method is efficient, with a >99% LPS removal and 95% protein retention, although performance drops drastically in high salt conditions.

Another adsorption-based method is the AC method that utilizes specified ligands. The polycationic ligands have a high endotoxin removal of >98% and a high protein recovery of >98%. AC can also utilize polymeric matrices, which include porous resins that exclude proteins. These proteins flow through the matrices while trapping endotoxins. This method is prone to LPS saturation and requires frequent washing. This makes the method reusable.

The third downstream approach for endotoxin removal is UF, that utilizes size-exclusion membranes. Since endotoxins vary widely in size, smaller ones can pass through this membrane and contaminate the protein batch. An improvement for this is the addition of calcium ions that stabilizes endotoxin aggregates. When optimized, this method can improve endotoxin removal up to 99% with a 94% protein yield.

The last approach is two-phase extraction with Triton X-114. This method utilizes the detergent Triton X-114 that adsorbs endotoxin. When heating the solution above the cloud point (22 degrees Celsius) the solution separates into a protein-phase and a detergent-rich phase that includes the endotoxin. The protein is then purified with centrifugation. This method is highly effective for hydrophilic proteins solutions, as 98-99% of endotoxins are removed. The downside of this method is the residual detergent that requires additional cleanup, resulting in a 10 – 20% product loss. Additionally, this method is not scalable for heat-sensitive proteins.

3.2 Comparison of methods

All endotoxin purification methods possess various challenges, with one issue being protein heterogeneity as different proteins require specifically tailored purification methods. Additionally, cell lysates that contain multiple compounds such as lipids and other contaminants hinder purification, making the process more complex. Another issue is the stability of the protein, as pH, salt and temperature sensitivity will limit the purification method choice. This literature review will evaluate each endotoxin purification method under optimal conditions. Therefore, the results shown below in Table 1 are derived when each method is performing at its highest efficiency. However, when choosing a suitable purification method, other practical consideration such as weakness, strength, cost and availability should be considered. These parameters are detailed in Table 2.

Table 1. Comparison of endotoxin removal methods when parameters are optimized.

Method	LPS purification	Protein recovery	Best for
Anionic IEC	>99%	95%	Acidic proteins
AC	>98%	>98%	High-value proteins
UF	99%	94%	Large proteins
Two-phase extraction	<99%	80-90%	Hydrophilic heat-stable proteins
Endotoxin-free strains	~97%	100%	Industrial recombinant proteins

Table 2. Weakness, strength, cost and availability of each endotoxin removal method.

Method	Weakness	Strength	Cost and availability
Anionic IEC	Requires high salt concentrations that affects protein stability	High specificity and scalable	Moderate cost and highly available
AC	Acidic proteins. Saturated and leaching ligands	High capacity and highly selective	High cost and moderately available
UF	Small proteins	Simple and concentrates proteins	Low cost and highly available
Two-phase extraction	Protein loss and detergent residues	High efficiency and easy to scale	Very low cost and highly available
Endotoxin-free strains	Risk of false positives in LAL assay. Slower growth rate	Eliminates downstream endotoxin removal	Low cost and limited availability

As Table 1 and Table 2 illustrate, each purification method possesses distinct strengths and weaknesses. The key takeaway is that no single method is the best, as different methods work for different proteins. The choice of method is context-based and dependent on the properties of the target protein. Properties that are taken into consideration are size, acidity, hydrophobicity, and complexity of the protein. Additionally, the availability and cost of each method is to be taken into consideration.

For larger and more complex proteins, UF is well-suited. It is a simple and scalable method making it cost-effective and highly available. However, its success is solely dependent on ensuring LPS is smaller than the target protein. This indicates that characteristics such as electrical charge and hydrophobicity are less relevant. As LPS tends to form aggregates, UF is not preferred for protein purification, and is mainly used for concentrating protein solutions.

If the POI is acidic (has a lower pI than the solution pH) anionic IEC is a highly effective option and cost-efficient. This method is also highly scalable although it is more complex and expensive compared to UF. This method is inefficient for basic proteins, and the performance is sensitive to buffer conditions and salt concentrations, as changes in these may alter the pI of the protein. For both laboratory scale and industrial scale research and production, both UF and IEC are the preferred options. As for IEC, this is due to the membranes being cost-effective and readily available from many suppliers. UF is also widely used because of its robustness, cost-effectiveness and high availability.

AC is the preferred method for purifying high-value proteins that require extreme purity, minimal endotoxin contamination and minimal protein-loss. This method is expensive, as the specialized resins are saturated quickly by LPS and require frequent washing. As a result of its inflated cost, this method is mostly used for laboratory-scale research and is not preferred for industrial-scale production. However, AC is still employed in industrial productions because of its high specificity, when other methods are not sufficient.

For stable, hydrophilic proteins that are thermostable, the two-phase extraction with Triton X-114 is effective. However, this method is not as scalable as the other methods, as detergent residue requires purification. Therefore, it is not preferred for industrial production as it adds complexity to downstream processing which reduces efficiency and appeal for purification applications. Despite this, as two-phase extraction with Triton X-114 is widely available, sold commercially and cost-effective it is still utilized for laboratory scale production, where the additional purification steps are easy to apply.

Endotoxin-free strains like KPM404 are a promising method as they remove all endotoxin in the cultivation phase. This moves the burden from purification of endotoxin to the prevention of it, simplifying downstream purification. However, its limitation of the regulatory LAL assay and slower growth rates currently restricts its adoption. Due to this, endotoxin-free strains are less adopted industrially and in research even though they are commercially available and relatively cost-effective. That is why downstream methods remain the current standard, and the chosen method depends on protein property.

4 Conclusions

The goal of this literature review was to identify the preferred method for endotoxin purification in recombinant protein production using *E. coli*. This goal was reached by establishing foundation by explaining production process, structure of LPS and methods for its detection. This review confirmed the importance and complexity of endotoxin purification, as multiple factors should be considered. As the traits of recombinant proteins vary drastically from each other, no method alone is perfect.

A strong approach to purifying protein solutions is by combining different methods. For instance, an initial purification with IEC can remove the bulk of endotoxins, and a follow up polishing step with specialized AC can be applied to achieve maximal purity. The most significant insight is the development from reactive purification to proactive prevention, where upstream engineered strains (such as KPM404) can tackle the problem at the source. While downstream methods are necessary and effective, the engineered strains show a potentially economical and long-term solution if developed.

As the scope is a bachelor's thesis, only a handful of purification methods were reviewed. Therefore, there are many other options not included in this thesis that might work better for a specific protein, as every protein have their own specific trait that should be considered. As for the trends found across sources, the most notable was that the sources presented the same methods for endotoxin purification. This also shows the stall in new accepted methods in endotoxin purification methods. A reason for this may be the slow approval of new methods, as United States Pharmacopoeia and European Pharmacopoeia has preferences. As a result of this stall, most of the articles found upon this topic were published ten years ago or more.

From this analysis, several promising future research possibilities emerged. As AC is highly specific and expensive, research into a new, cheaper ligand can make this method more accessible for a wider range of proteins beyond-high-value proteins. As for genetically modified strains, they eliminate endotoxins well yet react with LAL-assay. Either, a new assay, or a more specific assay needs to be worked on, or preferred: strain development should take place, where the residual lipid IV_A should be removed to eliminate reactivity with the LAL-assay. Additionally, development into a method where a combination of endotoxin-free strains and an optimized downstream purification step should be done. This could become the new gold standard, as it may offer high efficiency and safety. By combining novel upstream techniques and refined downstream methods, the field of endotoxin purification may develop safely and provide proteins that are needed in modern medicine and biotechnology.

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