THE EFFECT OF DIVALENT METAL CATIONS ON ENDOTOXIN AND PLASMID DNA INTERACTION

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THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2018

UNIVERSITI MALAYSIA SABAH

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IJAZAH: **MASTER OF SCIENCE (BIOTECHNOLOGY)**

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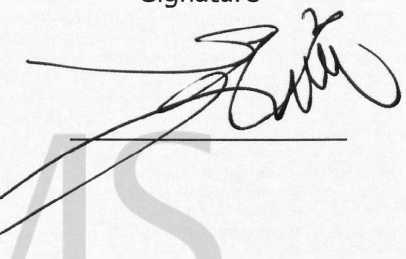

ACKNOWLEDGEMENT

Deepest gratitude and immeasurable appreciation to those who in one way or another have contributed in making this research possible. First and foremost, my deepest gratitude goes to Dr. Clarence M. Ongkudon for his amazing supervision, support, advices, guidance, valuable suggestions and comments throughout my Master's degree journey. I would also like to thank the laboratory assistants of Biotechnology Research Institute, for assisting me and making my works in the laboratory ran smoothly. Thanks to all members of the Bioprocess Engineering Research Group (BERG), for the knowledge they have shared with me and also for the friendship that certainly will make me looking back and reminiscing BRI. Last but not least, deepest appreciation goes to my family, especially my mother, who has been standing by my side and continuously supporting me until this day.

Elvina Clarie Dullah

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ABSTRACT

Endotoxin has the ability to undergo a stable interaction with other biomolecules, especially during the production process. Therefore, the removal of endotoxins from the final product is crucial as endotoxin contamination could pose negative effects on human health, including tissue inflammation and damage, fever, sepsis and eventually death. Moreover, endotoxin-biomolecules interaction is considered to be temperature and pH stable, and thus complicating its removability. Some divalent metal cations, including magnesium chloride (Mg^{2+}), calcium chloride (Ca^{2+}) and zinc sulphate (Zn^{2+}), were believed to have the ability to induce endotoxin aggregation, therefore, these cations might potentially interrupt the stable endotoxin-biomolecule binding thus facilitating endotoxin removal. This project focused on the study of the effects of divalent metal cations on endotoxin, as well as its effect in the presence of plasmid DNA (pDNA). The study was conducted under various physicochemical conditions such as types of divalent metal cation, concentration, pH and incubation time. The experiments were divided into three parts; (1) zeta potential analysis of endotoxin, (2) cation bound analysis by using Arsenazo III, and (3) binding tendency of cations on endotoxin with the presence of pDNA. Transmission electron microscopy (TEM) was done for the cation that exerted the most significant effect towards endotoxin. The analysis of zeta potential at different divalent metal cation concentrations and pH values showed that among the three divalent cations studied (Mg^{2+}, Ca^{2+}, Zn^{2+}), Mg^{2+} had the most significant effect on endotoxin surface charge. The zeta potential of endotoxin reduced from -43.53 mV to 0.02 mV in the presence of Mg^{2+}, while a value of 0.59 mV was observed at lower pH condition. In the cation-endotoxin binding study using Arsenazo III, Zn^{2+} showed the highest theoretical number of bound cation (approximately 0.6) compared to Ca^{2+} and Mg^{2+} (approximately 0.12 and 0.05 respectively). These values were determined based on the Scatchard plot. Part three of this study showed that the tendency of Zn^{2+} to bind with endotoxin was higher even in the presence of pDNA. The result was evident from the low intensity of bands on the agarose gels for both pDNA-Zn^{2+} and endotoxin-pDNA-Zn^{2+}. Ca^{2+} and Mg^{2+} did not show any significant changes in band intensity except for the incubation of pDNA and endotoxin-pDNA complex in pH 1. Apparently, Ca^{2+} and Mg^{2+} neutralized the negative charge of pDNA and the complex as indicated by the low band intensity. Visualization under TEM further verified that even in the presence of pDNA, the aggregated structure of Zn^{2+}-endotoxin remained unchanged. These findings imply that Zn^{2+} is more selective towards endotoxin than pDNA. In summary, among the three types of divalent metal cations studied, Zn^{2+} was found to exert the most significant binding interaction with endotoxin. Cation concentration played a major role in binding with endotoxin, whereas incubation time had little to no effect towards the interaction. These findings could be applied in the downstream processing of pDNA whereby Zn^{2+} has been found to interrupt the stable binding between endotoxin and pDNA, hence allowing the removal of endotoxin and recovery of pDNA.

ABSTRAK

KESAN LOGAM KATION DWIVALEN TERHADAP ENDOTOKSIN DAN INTERAKSI DENGAN DNA PLASMID

Endotoksin berupaya membentuk interaksi yang stabil dengan biomolekul terutamanya semasa proses pembuatan. Penyingkiran endotoksin dari produk akhir adalah penting kerana pencemaran endotoksin menyebabkan kesan negatif kepada kesihatan manusia seperti keradangan dan kerosakan tisu, demam dan akhirnya kematian. Interaksi endotoksin-biomolekul terjadi pada suhu dan pH yang stabil, dan perkara ini merumitkan proses penyingkiran endotoksin. Beberapa logam kation dwivalen seperti magnesium klorida (Mg^{2+}), kalsium klorida (Ca^{2+}) dan zink sulfat (Zn^{2+}), berupaya untuk memisahkan endotoksin. Kation ini mempunyai pontensi untuk mengganggu ikatan stabil endotoksin-biomolekul dan seterusnya memudahkan penyingkiran endotoksin. Projek ini mengkaji kesan kation ke atas endotoksin, serta interaksi endotoksin dengan kehadiran DNA plasmid (pDNA). Kajian ini melibatkan pelbagai parameter seperti penggunaan pelbagai jenis kation, kepekatan, pH dan waktu pengeraman. Eksperimen ini terbahagi kepada tiga; (1) analisis keupayaan zeta endotoksin, (2) analisis kation terikat menggunakan Arsenazo III, dan (3) kecenderungan pengikatan kation terhadap endotoksin dengan kehadiran pDNA. Pengamatan di bawah elektron mikroskop juga telah dilakukan untuk kation yang menunjukkan kesan paling ketara terhadap endotoksin. Keputusan keupayaan zeta menunjukkan bahawa antara tiga kation yang dikaji (Mg^{2+}, Ca^{2+}, Zn^{2+}), Mg^{2+} memberi kesan yang tertinggi terhadap keupayaan zeta endotoksin bagi kedua-dua jenis parameter yang dikaji iaitu kepekatan kation dan pH yang berbeza. Dengan peningkatan kepekatan Mg^{2+}, keupayaan zeta endotoksin telah berkurang daripada -43.53 mV kepada 0.02 mV, manakala dengan berkurangnya pH Mg^{2+}, keupayaan zeta endotoxin berkurang kepada 0.59 mV. Dalam kajian pengikatan kation, Zn^{2+} menunjukkan nombor pengikatan yang tertinggi (dianggarkan 0.6) berbanding Ca^{2+} dan Mg^{2+} (masing-masing dianggarkan 0.12 dan 0.05). Nilai-nilai ini adalah berdasarkan plot Scatchard yang dihasilkan. Bahagian ketiga kajian menunjukkan kecenderungan Zn^{2+} untuk bercantum dengan endotoksin adalah lebih tinggi walaupun dengan kehadiran pDNA. Ini kerana keamatan jalur pDNA-Zn^{2+} yang terhasil di gel agarosa adalah kurang berbanding keamatan jalur endotoksin-pDNA-Zn^{2+}. Manakala Ca^{2+} dan Mg^{2+} tidak menunjukkan perubahan keamatan jalur yang ketara kecuali selepas pengeraman dengan pDNA dan kompleks endotoksin-pDNA pada pH 1. Ca^{2+} dan Mg^{2+} meneutralkan cas negatif pDNA dan kompleks berdasarkan keamatan jalur yang rendah yang diperhatikan pada gel. Pengamatan melalui mikroskop elektron mengesahkan walaupun dengan kehadiran pDNA, bentuk agregat Zn^{2+}-endotoksin tidak berubah. Ini menunjukkan Zn^{2+} lebih tertarik kepada endotoksin daripada pDNA. Ringkasnya, dengan membandingkan tiga jenis kation, Zn^{2+} cenderung mengagregat endotoksin. Manakala kepekatan kation memainkan peranan penting dalam pengikatan cation-endotoksin, dimana tempoh pengeraman kation dan endotoksin menunjukkan sedikit kesan terhadap interaksi tersebut. Penemuan ini boleh digunakan untuk proses hiliran dalam pembuatan pDNA kerana pengikatan terpilih oleh Zn^{2+} terhadap endotoksin boleh menggangu ikatan stabil diantara endotoksin dan pDNA, seterusnya membolehkan pemisahan pDNA daripada endotoksin dengan kemungkinan untuk mendapatkan hasil pDNA yang tinggi.

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AMPs	-	Antimicrobial peptides
BET	-	Bacterial endotoxin test
BPI	-	Bactericidal/permeability – increasing
CA	-	Cluster analysis
$Cacl_2$	-	Calcium chloride
DLS	-	Dynamic light scattering
DNA	-	Deoxyribonucleic acid
DPV	-	Differential plus voltammetry
ECP	-	Eosinophile cationic protein
EDTA	-	Ethylenediaminetriaceticacid
EIS	-	Electrochemical impedance spectroscopy
ENP	-	Endotoxin – neutralizing protein
EtBr	-	Ethidium bromide
FPLC	-	Fast protein liquid chromatography
FRET	-	Fluorescence resonance energy transfer
GFP	-	Green fluorescent protein
HCl	-	Hydrochloric acid
kDa	-	Kilodalton
KDO	-	Keto – deoxyoctulosonate
LAL	-	Limulus amebocyte lysate

LBP	-	Lipopolysaccharide – binding protein
Lf	-	Lactoferrin
LPS	-	Lipopolysaccharide
Lpt	-	Lipopolysaccharide transport
LTA	-	Lipoteichoic acid
MAC	-	Minimal agglutination concentration
MAT	-	Monocyte activation test
MDA	-	Multivariate discriminant analysis
$MgCl_2$	-	Magnesium chloride
MNC	-	Mononuclear cells
MSD	-	Minimum sample dilution
PCA	-	Principal component analysis
pDNA	-	Plasmid DNA
RPT	-	Rabbit pyrogen test
SAM	-	Self assembled monolayer
SEM	-	Scanning electron microscopy
TEM	-	Transmission electron microscope
TP1	-	Tachyplesin I
TR-FRET	-	Time – resolved fluorescence resonance energy transfer
$ZnSO_4$	-	Zinc sulphate
TAE	-	Tris acetate

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Biotechnology industry has grown exponentially and it could be the most promising approach in finding solutions pertaining to pollution, conservation and cost reduction especially in biomanufacturing. Moreover, a lot of techniques have been developed in order to produce biopharmaceutical products in a very convenient way, i.e; cost and time effective. For instance, Gram-negative bacteria such as *Escherichia coli* has been well characterized and is currently being widely used in recombinant DNA production. However, one of the drawbacks in using Gram-negative bacteria is the release of endotoxins and contamination of final products.

Endotoxins or lipopolysaccharide can be found in the outer layer of Gram-negative bacteria. It is often released during the cell growth or death (Buttenschoen *et al.,* 2010). There are three major components of endotoxins which are the o-specific antigen, core oligosaccharide, and lipid A (Cho *et al.,* 2012), by which the lipid A is the most conserved part that causing the toxicity of endotoxins. Small amounts of endotoxin contaminants in human body could cause endotoxin shock, inflammation, tissue injury and even lead to fatality (Magalhaes *et al.,* 2007). Thus, any drugs, particularly injectable drugs for human have to be screened for endotoxin contamination so that it did not exceeded the specified quantity of endotoxin according to the dosing protocol (Trivedi *et al.,* 2003).

Endotoxin could interact with various biomolecules such as a cationic protein, lipopolysaccharide-binding protein (LBP), an amyloid P component, bactericidal/permeability-increasing protein (BPI), a lactoferrin, enzymes used in the biological endotoxin assay (anti-LPS) and a lysozyme (Magalhaes *et al.,* 2007).

The types of interactions between endotoxin and biomolecule have been hypothesized to be influenced by hydrophobic interaction, affinity interaction or ionic binding. Disaggregation or aggregation of the biomolecules usually occurs after the biomolecule and endotoxin have interacted. Moreover, toxic activities *in vivo* were triggered due to the physicochemical change of endotoxin aggregate (Andra *et al.,* 2004). It is believed that these interactions lead to endotoxin masking hence the difficulties in its removal during downstream processes. According to Petsch *et al.* (1998), the endotoxin masking effect could also alter the Limulus coagulation cascade in Limulus Amebocye Lysate (LAL)-based endotoxin detection method thus causing the results to be false-positive (Petsch *et al.,* 1998). In addition, it has been presented at the Parenteral Drug Association Annual Meeting that by using quantitative analysis, endotoxin contents in several biopharmaceutical products are usually underestimated and this is due to the endotoxin masking effect (Dawson, 2014).

The separation of small proteins from large endotoxin aggregates often involves ultrafiltration technique. However, this technique is not applicable when the proteins are larger than endotoxins. This is due to the probability of endotoxin monomers to infuse with proteins and pass through the filtration membrane (Petsch and Anspach, 2000), as well as causing damage to the proteins by physical forces (Pyo *et al.,* 2001). Moreover, when it is associated with labile biomolecules, especially proteins, endotoxin removal becomes one of the most difficult tasks in the downstream processes as endotoxin is temperature and pH stable (Reichelt *et al.,* 2006). Anion exchangers have been extensively used for endotoxin adsorption. However, when the interaction occurred between endotoxins and negatively charged biomolecules, the biomolecules may co-adsorb onto the matrix causing a significant loss of biological material during decontamination. Positively charged biomolecules could form complexes with endotoxins causing the biomolecules to co-elute with endotoxins thus reducing the endotoxin removal efficiency (Anspach and Hilbeck, 1995).

Other types of pyrogen also contribute to the challenges in biopharmaceuticals production where it could cause endotoxin-like negative effects on human. However, to trigger pyrogenic responses similar to endotoxin, higher

concentrations of the non-endotoxin pyrogens are required (Brunson and Watson, 1976). Endotoxin detection methods, with the exception of LAL-based assay (Fennrich *et al.,* 1999) could be used to detect non-endotoxin pyrogens such as peptidoglycan, enterotoxins, lipoteichoic acid (LTA) and exotoxins (Sandle, 2015).

Endotoxin detection method can be divided into conventional methods and biosensor-based methods. Some of the conventional methods are still being used until today despite of the development of advanced detection methods. However, the usage of conventional methods in endotoxin detection depends on the type of biopharmaceuticals production method employed. The most well-known conventional methods in endotoxin detection are rabbit pyrogen test, Limulus amebocyte lysate (LAL) test, monocyte activation test and bovine whole blood assay, with LAL test still being the widely used method to date (Magalhaes *et al.,* 2007). There are three major types of biosensor-based endotoxin detection method which are the electrochemical biosensor, optical biosensor and mass-based biosensor. Each of the biosensors varies in terms of its principle and efficiency of detection. Some of the biosensors may be combined with the conventional methods to improve endotoxin detection. The choice and suitability of the endotoxin detection as well as the endotoxin removal methods depend on the biomolecules that it interacts with.

The study of endotoxin and bio-product interactions is important as it could be used to invent the most effective endotoxin detection and removal methods. It is important to note that the study of endotoxin interaction depends heavily on the method of endotoxin detection. Therefore, the selection of a suitable endotoxin detection method would ensure reliable analysis of endotoxin interaction with other biomolecules. In this study, a number of endotoxin detection methods were gathered and compared from literatures. Other analytical methods such as dynamic light scattering (DLS), docking programs, electron microscopy and fluorescence resonance energy transfer (FRET) can be used to observe the physicochemical changes occurring during the interaction process.

Previous study has shown that divalent metal cations could affect and act as selective precipitants for endotoxins. These cations include nickel ion, magnesium

ion, zinc ion, calcium ion and copper ion. These cations are considered to have high potential in removing endotoxins by means of selective aggregation (Ongkudon and Danquah, 2011a). In this study, three divalent metal cations namely calcium, magnesium and zinc ions were selected to study their effects on endotoxins, as well as on endotoxin-plasmid DNA (pDNA) complex under various parameters such as concentration, pH and incubation time. The properties of endotoxin-cation interaction such as zeta potential and cation bound per endotoxin molecule were studied. Gel electrophoresis was employed to study the effects of cations on endotoxin-pDNA interactions.

1.2 Problem Statement

Plasmid DNA has become a promising DNA vaccines and gene therapies for various acquired, infectious and genetic disease (Lara *et al.,* 2012). The production of recombinant pDNA using Gram negative bacteria is more favourable nowadays as it is less expensive and saves time. However, the existence of endotoxin lipopolysaccharide in Gram-negative bacteria has become an issue for pDNA production. Endotoxin carry over will occur during the extraction of pDNA which decreases the purity of pDNA and hence, further purification is needed so that it meets the clinical safety standards of endotoxin level (Young, 2016). To purify pDNA from endotoxin contamination seems to be complicated as endotoxin could form a stable binding with pDNA and it also has several unique characteristics such as heat and pH stable. In response to this problem, this study intended to investigate the effects of selected divalent metal cations on endotoxin as previous study found that cations have better interaction with endotoxin compared to pDNA. Moreover, the study was done in various parameters such as concentration, pH and incubation time in order to find the most effective conditions that the cations-endotoxin interactions could occur. The ability of cations to bind with endotoxin will interrupt the stable binding of endotoxin-pDNA which leads to the high recovery of pDNA.

1.3 Aims of Study

1) To study the effect of divalent metal cations on endotoxin surface charge under different cation concentrations and pH values.

2) To determine the maximum theoretical number of divalent metal cations bound per endotoxin molecule under different cation concentrations and incubation times.

3) To study the effects of divalent metal cations on endotoxin under different cation concentrations, pH values and incubation times in the presence of pDNA.

1.4 Significance of Study

This study provides an understanding of how divalent metal cation affects endotoxin behaviour such as surface charge and cations binding ability under various physicochemical conditions. As stated in the introduction, some divalent metal cations have the potential to be used as a selective precipitant for endotoxin removal. Thus, the data obtained from this study provides information regarding to which divalent metal cation has the highest tendency to bind and precipitate endotoxin molecules. Moreover, since endotoxin also has a binding affinity towards other biomolecules, pDNA was chosen in this study to form a complex with endotoxin molecules. The ability of divalent metal cations in altering pDNA-endotoxin interaction is potentially useful in minimising endotoxin carry-over during bioproducts manufacturing.

CHAPTER 2

LITERATURE REVIEW

2.1 Endotoxin (lipopolysaccharide)

In 1892, endotoxin was recognized by a German physician and bacteriologist named Richard Friedrich Johannes Pfeiffer (Holst, 2011). Endotoxin, also termed lipopolysaccharide (LPS) can be found in most Gram-negative bacteria which are responsible in making up 75% of the bacterial outer membrane (Ramachandran, 2014). As endotoxin forms the permeability barrier of Gram-negative bacteria, it basically keeps these bacteria from harmful materials surrounding them. The structure of an endotoxin molecule can be divided in three major parts which are; (1) O antigen, (2) core polysaccharide, and (3) lipid A (Figure 2.1).

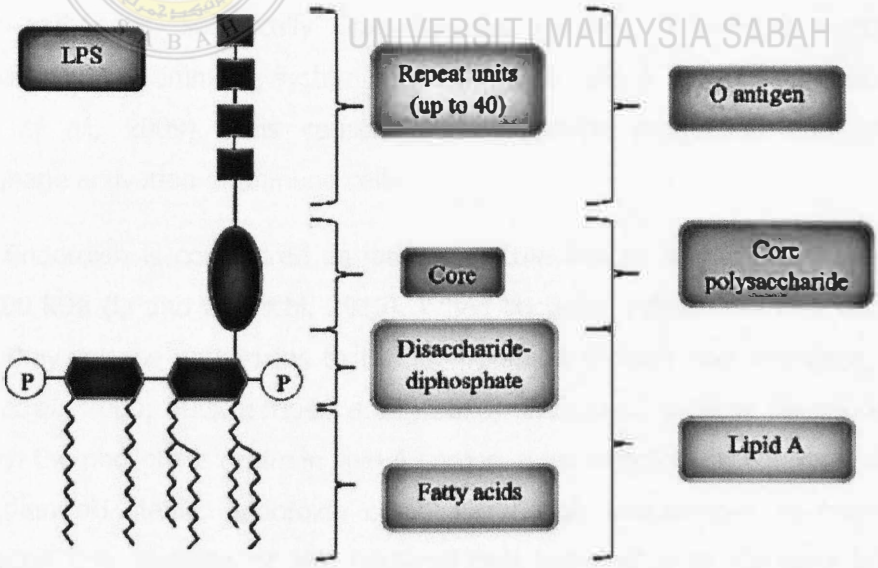

Figure 2.1 : The major parts of endotoxin structure.

Source : Petsch *et al.,* 2000

The outermost part of the endotoxin vesicles in Gram-negative bacteria is made up of O antigen polysaccharide. It is identified as polymers composed of repeating sequences of three to six sugar residues known as O-unit. The repeating unit varies among strains hence leads to the serological specificity of the bacteria (Erridge *et al.,* 2002). O antigen acts as a crucial component of the bacterial survival toolkit, working in the aggressive regions of mammalian tissues (Kalynych *et al.,* 2014).

A non-repeating core polysaccharide is divided into the outer core and inner core which connect to O antigen and lipid A respectively. Since the outer core is well exposed to the selective pressures of host responses, environmental stress and bacteriophages, it is mostly structurally diversified. The inner core is highly conserved with sugars such as heptose and keto-deoxyoctulosonate (KDO) (Raetz and Whitfield, 2002).

Lipid A is a hydrophobic membrane anchor of endotoxin which is highly conserved and responsible in the immunostimulatory activity of endotoxin (Matsuura, 2013). Basically, lipid A is made up of two glucosamine units with fatty acid attached to it and one phosphate group on each glucosamine unit. In addition, Raetz and co-workers mentioned that due to the lipid A conserved structure in various pathogens, specifically Gram-negative bacteria, TLR4/MD2 receptor of mammalian innate immune system has recognized lipid A as a foreign substance (Raetz *et al.,* 2009). This causes robust cytokine production and prompts macrophage activation of immune cells.

Endotoxin is considered as large molecule due to its molecular weight of 200-1000 kDa (Li and Boraschi, 2016). When bacteria undergo cell division or cell death, they release endotoxins to the environment (Petsch and Anaspach, 2000; Yaron *et al.,* 2000; Buttenschoen *et al.,* 2010). Endotoxin is partly phosphorylated by which the phosphate group in lipid A confers a net negative charge on endotoxin. Other than pH stable, endotoxin could stand high temperature as heat-based sterilization only removes or kills bacterial cells but unable to eliminate bacterial endotoxin. However, endotoxin is still removable when exposed for an extended period of time at a raised temperature although this method is not applicable to

highly heat-sensitive biomolecules such as proteins (Bononi *et al.,* 2008). Endotoxin carries out several functions in the immune system such as inflammatory response development, indicator for bacterial pathogen invasion detection, and septic shock (Rosenfeld and Shai, 2006). Thus, based on the above mentioned characteristics of endotoxin, it is important to study the interaction of endotoxin with other biomolecules such as proteins and its effect on endotoxin pathogenic activity.

Recombinant therapeutic proteins expressed in Gram-negative bacteria have the potential risk of carrying endotoxins. Downstream processes are becoming more challenging as the released endotoxins from the Gram-negative cell wall will bind to the protein of interest. Moreover, endotoxin contamination can also occur at any stage of a bioproduction process. The impact of endotoxin contamination leads to a considerably low product yield as well as product loss resulted from the separation steps employed to remove endotoxins (Salema *et al.,* 2009). Moreover, endotoxin has become a major concern in the biopharmaceutical manufacturing processes as it is the only impurity with a specification limit required by the United States Pharmacopeia (USP) (DeCrane, 2015). The measurement unit of endotoxin is in endotoxin unit, EU where one endotoxin unit is equal to 120 pg of endotoxin (Magalhaes & Pessoa, 2009). According to a study by Petsch and Anaspach, the target protein at the final stage of purification was found in the range of g/L while endotoxins were present in µg/L range. This low concentration of endotoxin creates a problem as routine analysis in picograms amount is not possible by direct detection method (Petsch and Anaspach, 2000). In addition, as endotoxin is one of the microbial by-products, this pyrogen is able to alter the impurity profiles in the downstream process (Clontz *et al.,* 2013). The occurrence of this phenomenon after the final step of impurities removal leads to the excessive impurity levels that may affect the acceptance of the final product or worst, go undetected. This will greatly affect the quality and safety of the product.

2.2 The Occurrence of Endotoxin and Biomolecules Interactions

The behaviour of endotoxin towards multiple types of protein is varied when it is under fixed physicochemical environment (e.g. viscosity, pH and temperature). The interaction of endotoxin with an important component of the innate immune system such as lysozyme is initially determined by the hydrophobic interaction (Ohno and Morrison, 1989). The study found that the lysozyme enzymatic activity was lost when interaction with endotoxin occurs and this is due to a noncompetitive inhibition which changes the biological activity of endotoxin. In addition, endotoxin can be highly aggregated thus hindering the lysozyme activity (Liping, 1999). When a complex of lysozyme-endotoxin is analysed using LAL test and ultrafiltration, an absence of monomeric endotoxin molecules will be obtained which considered as negative result (Liping, 1999). Monomeric endotoxins are usually correlated with the inactivity of endotoxins.

Endotoxin also interacts with endotoxin-neutralizing protein (ENP) where the interaction is dose dependent (Andra *et al.,* 2004). A partially completed neutralization can be obtained at an ENP/Endotoxin molar ratio of 20:1 which can be observed from the TNFα production in human mononuclear cells (MNC) and the LAL assay. ENP can alter active endotoxin into an inactive form by changing the three-dimensional structure of endotoxin, specifically the lipid A structure from a cubic to a multilamellar phase. The stability of the endotoxin-ENP binding is dependent on the aggregate structure of endotoxin although the dependency trend is still uncertain.

Eosinophil cationic protein (ECP) is another type of protein which shows a high affinity towards endotoxin. ECP is a type of human-secreted protein which is often used as a readout for the active inflammatory disease analysis. Pulido and co-workers studied the endotoxin-agglutinating activity of ECP by discovering the minimal agglutination concentration (MAC) or the minimal peptide concentration that could trigger bacterial agglutination (Pulido *et al.,* 2012). There were five *Escherichia coli* strains (D21, D21e7, D21e19, D21f1, D21f2) with various lengths of endotoxin were utilized, and the agglutination was analysed with ECP and peptides: [1-45] and [6-17]-Ahx-[23-36]. The results showed no agglutination from the D21f2 strain with the shortest endotoxin even after 12 hours of incubation with 5 μM ECP. Similar results were also observed for the incubation with peptides