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ABSTRACT

PROTEIN AND ENDOTOXIN INTERACTIONS AND ENDOTOXIN REMOVAL FROM PROTEIN SOLUTIONS

by
Liping Li

The investigation objectives of this study include: endotoxin disaggregation by proteins; protein concentration effect on protein-endotoxin binding and endotoxin removal; size exclusion high performance liquid chromatography (SE-HPLC) studies on endotoxin dissociation by bovin serum albumin (BSA); use of Ca^{+2} to re-aggregate endotoxin in hemoglobin solutions and the subsequent removal of endotoxin by ultrafiltration; quantitative determination of Ca^{+2} effects on endotoxin removal and protein yield in a two-stage ultrafiltration.

It is known that some proteins can disaggregate endotoxins and form complexes with lipopolysaccharides (LPS). It is also known that Ca^{+2} can act as "bridges" to aggregate LPS in aqueous solutions. However many questions remain unanswered. What are the effects of protein concentration on such LPS disaggregation? Can Ca^{+2} act as "bridges" to re-aggregate LPS subunits in protein solution where LPS vesicles have been disaggregated by protein molecules into small fragments? How will the presence of Ca^{+2} affect the endotoxin removal and protein recovery? This study will investigate these problems.

The effects of protein concentration on endotoxin disaggregation, protein-LPS binding, and endotoxin removal have been investigated. It was found that hemoglobin A_o

and albumin could disaggregate LPS and their concentrations had significant effects on protein-LPS interaction. In contrast, lysozyme and cytochrome C did not disaggregate LPS.

In the study of size exclusion high performance liquid chromatography (SE-HPLC) studies on endotoxin dissociation by bovine serum albumin (BSA), it was found that BSA could disaggregate LPS. BSA concentration had a significant effect on the LPS disaggregation. Pre-incubation of the BSA-LPS mixture could enhance the LPS disaggregation.

Ca^{+2} was used to re-aggregate endotoxin in hemoglobin solutions and to subsequently remove endotoxin by ultrafiltration. It was found that Ca^{+2} could re-aggregate LPS in protein solutions where LPS had been disaggregated by hemoglobin A_o. Such re-aggregation improved the endotoxin removal efficiency by ultrafiltration.

In the study of quantitative determination of Ca^{+2} effects on endotoxin removal and protein yield in a two-stage ultrafiltration, it was found Ca^{+2} would re-aggregate LPS from protein solution. In the presence of Ca^{+2}, endotoxin removal efficiency was increased without sacrificing the protein yield.

PROTEIN AND ENDOTOXIN INTERACTIONS AND ENDOTOXIN REMOVAL FROM PROTEIN SOLUTIONS

by
Liping Li

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This dissertation is dedicated to
my beloved parents
and my brother

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CHAPTER 1

INTRODUCTION

1.1 Endotoxin and Pyrogenic Reactions

Bacterial endotoxins are lipopolysaccharide (LPS) derived from the outer cell membranes of Gram-negative bacteria (Hou and Zaniewski, 1990). Endotoxins are known to have potent biological effects in man and in many animal species when administered systemically (Issekutz, 1983). Endotoxins are pyrogenic; that is, they produce fever in man or other mammals. Even if the bacteria are killed, endotoxin removal is essential for the safe parenteral administration of products produced from natural sources or by recombinant DNA technology. The sensitivity of mammals to LPS pyrogens is extraordinary. Contamination levels of less than 1 ng/ml elicit a strong fever response and can even result in death (Nelsen, 1978).

The property of the LPS pyrogen explains why elimination of pyrogens from aqueous solution is problematic. The molecule is unusually thermally stable, although exposure to dry heat at 250°C for periods up to an hour will destroy it. The molecule is relatively insensitive to changes in pH as well, although high concentrations of acids and bases (usually at levels that would also destroy the desired material containing pyrogen) may deactive the molecule (Nelsen, 1978).

The LPS molecule is anionic or negatively charged because of its phospholipid group. It may, therefore, be eliminated at least in theory, by binding on positively charged adsorbents (Nolazn et al., 1975).

In practice, however, this binding is relatively inefficient, presumably because of its relatively low charge density and because of its unusual molecular weight characteristics. Pyrogen range in size from subunits of 10, 000–20, 000 to aggregates of 0.1 μm in diameter (Hannecart-Pokorni et al., 1973). In aqueous solutions, the hydrophobic regions of LPS molecules tend to associate and form large aggregates; the unaggregated LPS subunit is seldom found. The degree of aggregation can be either enhanced or inhibited by the presence or absence of protein due to protein and endotoxin interaction, divalent cations, chelating agents, and detergents (Nelsen, 1978; Sweadner et al., 1977).

Divalent cations, such as Ca^{2+} or Mg^{2+}, cause LPS molecules to aggregate into large vesicles. In the absence of these ions, the vesicles dissociate into micelles of 300,000-1,000,000 molecular weight. (Sweadner et al., 1977).

The presence of chelating agents, such as EDTA, bind up the divalent ions in solution and cause the subsequent disaggregation of vesicles into micelles. (Nelsen, 1978; Rogers et al., 1969). The presence of strong detergents or bile salts also cause the disaggregation of vesicles and/or micelles into basic LPS subunits (10,000-20,000 Da) (Nelsen, 1978; Oroszian et al., 1963).

In the presence of some proteins, such as hemoglobin and serum albumin, endotoxin aggregate states also change and form complexes with proteins. This has been proved by sucrose density gradient, ultrafiltration, and chromatography method (Roth et al., 1994; David et al., 1995).

These facts make decontamination or removal of endotoxins from solutions a problem in biological research as well as in the commercial production of biopharmaceuticals by recombinant DNA technology.

1.2 Endotoxin and Biotechnology

Bacterial endotoxins are lipopolysaccharides (LPS) derived from the outer membranes of gram negative bacteria (Pearson, 1985a). Endotoxin contamination during manufacture of water for injection (WFI) and parenteral solutions is of great concern to the pharmaceutical industry. Endotoxins are responsible for most pyrogenic reactions if they occur in sufficient amounts in large volume parenterals (infusion fluids) (Baggerman et al., 1985; Evans-Strikfaden et al., 1996). Endotoxins in distilled water cause serious complications as they can be present in kidney dialysis machines and intravenously infused nutrients, pharmaceutical agents and various other products. Endotoxins were also shown to cause disease in animals (Gerba and Goyal, 1981). The physiopathological effects shown by affected humans or animals include pyrogenicity, endotoxemia, leucopenia, leucocytosis, shock, allergic reactions and lowered blood pressure (Enterline et al., 1985).

Gram-negative bacteria are widely used in biotechnology industry to produce recombinant DNA products such as peptides and proteins. Bacterial endotoxins have been recognized by the industry as a major cause of the pyrogenic reactions that can be encountered during the administration of biotherapeutics. The removal of these physiologically active agents from final bioproducts has always been a challenge.

Because proteins are biologically active and sensitive to pH or temperature changes, endotoxin removal from protein solutions is more complicated than that from water.

The applications of genetic engineering fall into two broad categories: (a) modification of the structure or amplification of the production of scarce biological products by cloning the relevant genes in bacteria. This method use bacteria as factories for the production of target compounds. (b) Alteration of selected organisms to improve their utility in a desired environment, such as the development of a bacterium which imparts novel texture or flavor when used in cheese production (Sharma, 1986).

There is growing interest in the production and isolation of enzymes and other proteins from microorganisms. This has been stimulated by the increasing use of enzymes as industrial catalyst or in clinical analysis and the potential applications of pharmacologically active polypeptides resulting the advent of recombinant DNA techniques.

Recombinant DNA technology used for the production of pharmaceutically useful polypeptides such as insulin, human growth hormone (Goeddel et al., 1979), and interferons (Nagata et al., 1980; Goeddel et al., 1980; Goeddel et al., 1981; Gray et al., 1982) has been intimately linked to the E. coli expression system. The microbe was the first prokaryote used for the commercial production of insulin via a cloned gene. The gram-negative organism has been a favorite for at least three reasons. It has relatively simple genetics, is well characterized, and has a rapid growth rate. However, E. coli as a host for an eukaryotic gene encoding a useful polypeptide or protein has defects. One defect which received early extensive notoriety is the production of an endotoxin by E.

coli. Obviously, there are some inherent problems associated with this organism as it relates to the purification of products produced by recombinant DNA technology.

Administrating human proteins derived by recombinant DNA technology has the potential of unknown risks, concerned with the production of biopharmaceuticals derived from E. coli. The questions that are relevant are: what are the clinical implications? How serious are such problems? Bacterial contaminants, even at a relatively low concentration, could give rise to undesirable side effects. As mentioned above contamination levels of less than 1 ng/ml elicit a strong fever response and can even result in death (Nelsen, 1978).

In the biotechnology industry, Gram-negative bacteria are widely used to produce recombinant DNA products, such as peptides and proteins, bacterial endotoxins have been recognized as a major cause of the pyrogenic reactions that can be encountered during the administration of biological therapeutics. The removal of these physiologically active agents from final bioproducts has always been a challenge to the industry. Then the final problem is: how to remove the contaminants such as endotoxins.

To solve the problem it is essential to understand endotoxin characteristics in biological solution. It is known that some proteins can disaggregate endotoxins and form complexes with lipopolysaccharide in biological solutions, which makes the removal of endotoxins from protein based biopharmaceutical products more complicated. Some of such proteins have been identified, such as lipoproteins (Schlichting *et* al., 1996) and monoclonal antibodies (Ziegler *et* al., 1991). It is reported that hemoglobin not only can bind but also disaggregate endotoxins, and enhance endotoxin activation of *Limulus* amebocyte lysate (LAL) in a concentration dependent manner (Kaca *et* al., 1994; Roth *et*

al., 1994). Variable effects of human serum albumin (HSA) on endotoxin biological activity have also been reported. The biological activity of endotoxin is changed in the presence of HSA, depending on HSA concentration (Kaca *et* al., 1994).

1.3 Research Objectives

1.3.1 Endotoxin Disaggregation by Protein and Protein Concentration Effect on Protein-LPS Binding and Endotoxin Removal

To understand endotoxin characteristics in a biological solution, studies were carried out using ultrafiltration membrane to investigate whether other proteins also have interactions with endotoxin and disaggregate endotoxin. Several protein preparations (human hemoglobin, bovine serum albumin, lysozyme, and cytochrom C) at different concentrations were mixed with endotoxin from *Escherichia* coli 026:B6 respectively, and then ultrafiltered using a 300,000 molecular weight cut–off polysulfone membrane. Membrane filtrates were subjected to *gelation Limulus amebocyte lysate* (LAL) test. If protein interacted with endotoxin and disaggregated endotoxin into smaller fragments, the endotoxin content in the membrane filtrate would be detected by LAL test. The results are shown in Chapter 3.

However, how the concentration of these proteins, which have interaction with endotoxin and disaggregate endotoxin, affects protein-LPS binding and endotoxin removal is unknown. The information will be very useful for design and improvement of ultrafiltration membrane processes for removing endotoxin from protein solutions. In the following study, an ultrafiltration method was used to investigate protein concentration effect on protein-LPS binding and endotoxin removal. Protein samples at various concentrations were incubated with endotoxin samples from *Escherichia coli* 026:B6 at a

fixed concentration. After incubation, the mixtures were filtered with 300,000 nominal molecular weight cut-off (NMWCO) polysulfone ultrafiltration membranes. *Limulus* amebocyte lysate (LAL) test was employed to detect endotoxin in the filtrates. This is the first time that the relationship between protein concentration and endotoxin disaggregation is determined. The results are shown in Chapter 3.

1.3.2 Endotoxin Disaggregation by Bovine Serum Albumin

Although we already know that some proteins have interaction with endotoxin and disaggregate endotoxin, the following questions are still unknown: what is endotoxin disaggregation rate; whether endotoxin is partially or completely disaggregated; how does the endotoxin form change before and after disaggregation, and what elements affect such disaggregation. To further elucidate how protein disaggregates endotoxin, size exclusion high performance liquid chromatography (SE-HPLC) was employed. This is the first time size exclusion chromatography was combined with UV detection has been used to study endotoxin disaggregation. The results are presented in Chapter 4.

Size exclusion high performance liquid chromatography (SE-HPLC) method was used to investigate LPS disaggregation in the presence of bovine serum albumin (BSA). Ultraviolet (UV) absorbance was employed as a detection method to obtain the chromatography profile. This is the most elucidative way to demonstrate LPS disaggregation. Protein and endotoxin mixture samples at various concentration ratios, which were incubated at 37 °C for 30 min or directly set at room temperature without incubation, were analyzed by size exclusion high-performance chromatography (SE-HPLC). Size exclusion chromatography separates molecules on the basis of molecular

size, that is, molecules are eluted in order of decreasing molecular size. If endotoxin is disaggregated by protein to form smaller size aggregates, such smaller size aggregates will be eluted at longer retention time compared with the not-disaggregated endotoxin aggregates. From the chromatograph profile, it can be seen see that the area of the first LPS aggregate peak, which is produced by the larger aggregates, will decrease under the effect of bovine serum albumin (BSA) and switch to longer retention time. Therefore the area of LPS peaks, which are smaller than the first peak and eluted at longer retention time, will increase. It was also found that at high BSA/LPS ratio under incubation condition, the smallest size new peak groups were formed.

1.3.3 Use of Ca^{+2} to Re-Aggregate Endotoxin in Protein Solution and Subsequent Removal of Endotoxin by Ultrafiltration

We already know that some proteins, such as hemoglobin and albumin, have interaction with endotoxin and disaggregate endotoxin. This makes endotoxin removal in such protein solution more complicated. Another problem is how to remove endotoxin in the presence of protein. Here investigation was performed to find a way to re-aggregate endotoxin in protein in order to remove it with ultrafiltration membrane. Some studies have shown that the addition of divalent cations enhanced the LPS aggregation in water (Galanos and Lüderitz, 1975). However, whether the LPS subunits bound to protein molecules in a biological solution will re-aggregate by adding divalent cations is unknown. Finding an answer to this problem will help to find an easy and non-toxic way to remove endotoxin from biological solution. The following study was focused on this problem. In that study, a method of two-stage ultrafiltration was used to investigate the effect of Ca^{+2} on LPS re-aggregation in hemoglobin solutions and the removal of

endotoxin. Gelation *Limulus amebocyte lysate* (LAL) test was used to quantified the Ca^{+2} effect. Before this study, all other studies concentrated on Ca^{+2} influence on endotoxin aggregate size in aqueous solution. This study found a new application of Ca^{+2} on improving endotoxin removal in protein solution. The qualitative endotoxin removal results are shown in Chapter 5.

1.3.4 Quantitative Determination of Ca^{+2} Effects on Endotoxin Removal and the Subsequent Removal of Endotoxin by Ultrafiltration

From the above studies, the qualitative effect of Ca^{+2} on endotoxin removal would be already known. To get quantitative Ca^{+2} effect on endotoxin removal and protein yield, another experiment was carried out. Since the chromogenic LAL assay was employed to detect endotoxin, it was possible to determine endotoxin concentration quantitatively. The endotoxin removal efficiency after re-aggregation in the presence of Ca^{+2} was studied. The yield of hemoglobin was also investigated. The results are shown in Chapter 6.

CHAPTER 2

LITERATURE REVIEW OF ENDOTOXIN, PROTEIN AND ENDOTOXIN INTERACTION, ENDOTOXIN REMOVAL

2.1 Chemistry and Structure of Endotoxin

About 100 years ago (1892-95), Richard Pfeiffer investigated host-parasite interrelationships, especially in Cholera infections. He showed that Vibrio cholerae produce two quite different types of toxic principles, heat-labile endotoxin which is released from living organisms during growth, and a heat-stable toxin which is released only when the bacterial cells undergo disintegration, then called endotoxin (Rietschel, 1984). In the available literature there is no authentic paper in which Pfeiffer actually introduces the term endotoxin. After 1904 many articles and reviews referred to Pfeiffer (Wolff, 1904), but in none of the quoted articles is endotoxin really mentioned. In any case, there is no doubt that it was Pfeiffer who first introduced the term endotoxin.

A bacterial or prokaryotic cell is surrounded by an envelope that maintains the organism's shape and integrity. In gram-negative bacteria, this envelope consists of two lipid layers. The inner layer is composed of phospholipids and proteins, and the outer layer contains proteins and lipopolysaccharide (LPS), an endotoxin (Glauert and Thornley, 1969). As is shown in Figure 2.1, lipopolysaccharide (LPS) or endotoxin is a macromolecular substance tightly bound to the surface of gram-negative bacteria and forming an integral part of the membrane structure (Galanos and Lüderitz, 1984).

This outer layer reacts to changes in the environment. It inhibits the entrance of toxic compounds, such as antibiotics (Rietschel et al., 1994) and is important in nutrient

transport. This outer layer mediates the physiological and pathophysiological interaction of the bacterium with the host organism. LPS is the main antigen of gram-negative bacteria, carrying the antigenic determinants of O specificity (Westphal et al., 1983). It is responsible for many pathophysiological activities that accompany gram-negative infections (Lüderitz et al., 1982). Furthermore, LPS is receptor for many bacteriophages (Lindberg, 1973).

Chemically, LPS consists of two parts of contrasting chemical and physical properties; a hydrophilic polysaccharide and a hydrophobic lipid, the lipid A (Figure 2.2). The polysaccharide may be further subdivided into the O-specific polysaccharide and the core oligosaccharide. LPS shows considerable heterogeneity, even when isolated from a single bacterial culture. This heterogeneity occurs because bacteria synthesize various LPS molecules that differ in the length of the O-specific chains (Rietschel et al., 1994). The hydrophobic moiety of LPS, lipid A, is responsible for a great number of biological processes observed in infections, inflammation, and some chromic diseases.

The O-specific chain is a polymer of oligosaccharides with repeating units consisting of 1 to 8 glycosyl residues. The structure of the repeating units differs among bacterial strains (Malchesky et al., 1995). Compared with the O-antigen, the core oligosaccharide has a conserved structure with an inner KDO-heptose region and an outer hexose region, In E. coli species, five different core types are known. Salmonella species share only one core structure.

The most conservative part of endotoxin is lipid A, which, apart from few exceptions (Mayer and Weckesser, 1984), shows very narrow structural relationship in different bacterial genera. It consists of a β-1,6 linked disaccharide of glucosamine,

covalently linked to 3-hydroxy-acyl substituents with 12 to 16 carbon atoms via amid and ester bonds; these may be further esterified with saturated fatty acids. This hydrophobic part of endotoxin adopts an ordered hexagonal arrangement, resulting in more rigid structure compared with the rest of the molecule.

The supramolecular conformation of lipid A allows the optimal expression of endotoxic activity. Lipid A, as an amphophilic molecule, behaves similarly to phospholipids, which adopt different physical structures under different ambient conditions, such as changes in temperature, pH, and charge state (Malchesky et al., 1995).

The early work on the supramolecular structure of LPS has been reviewed by Shands. It focused on the characterization of LPS particles with typical molecular weights of 1×10^6 to 20×10^6 Da as estimated using physicochemical data on diffusion coefficients and intrinsic viscosity. The investigation by Lipka et al (Lipka et al., 1988) on the phase behavior of aqueous dispersions of lipid X, a pyrogen precursor, indicated the critical micellar concentration to be 4×10^{-5} mol/L in a physiological saline solution; the two pK values are $pK_1 = 1.3$ and $pK_2 = 8.2$. The sugar phosphoryl moieties account for the low pH value and can be considered as the functional groups that interact with positively charged matrices in LPS removal, whereas the long-chain alkyl groups in lipid A, as the hydrophobic tail, cause micelle formation and molecular aggregation. (Malchesky et al., 1995). It was proposed that aggregation is governed by non—polar interactions between neighboring alkyl chains as well as the bridges generated among phosphate groups by bivalent cations (De Pamphilis, 1971; Wang and Hollingsworth, 1996)

Electron microphotographs showed endotoxins as "snake-, donut- or rod-like filaments and flat sheets" (Shands et al., 1967, Hannecart-Polorni et al., 1973). Modern analytical methods, such as X-ray diffraction, FT-IR spectroscopy, neutron scattering, NMR (Seydel et al., 1993) and molecular modeling (Kastowsky et al., 1992), revealed a more detailed image of the three-dimensional organization of endotoxins. Although these studies did not convey a uniform picture, it is evident that endotoxins aggregate in lamellar, cubic and hexagonal inverted arrangements, such as micelles and vesicles with diameters up to 0.1 μm (Figure 2.3).

2.2 Biological Activity of Endotoxin

Endotoxins elicit their biological response by stimulating a reaction in the host cells, not by killing them or by inhibiting cellular functions. Host cells of primary importance are mononuclear cells, such as monocytes and macrophages (Galanos et al., 1993; Galanos et al., 1992). For example, LPS-induced activation of macrophages results in the production of bioactive lipids, reactive oxygen species, and especially peptide mediators, such as tumor necrosis factor alpha (TNF), and several interleukins (Rietschel et al., 1994). These potent hormone-like proteins can induce many of the typical endotoxin effects by acting independently, in sequence, synergistically, or antagonistically. Clinical effects include high fever, hypotension, and irreversible shock (Malchesky, 1995).

Endotoxins are pyrogenic; that is, they produce fever in man or other mammals. Even if the bacteria are killed, their removal is essential for the safe parenteral administration of products produced by natural sources or by recombinant DNA technology. The sensitivity of mammals to LPS pyrogens is extraordinary. Contamination

levels of less than 1 ng/ml elicit a strong fever response and can even result in death (Nelsen, 1978).

Since endotoxins have profound toxicity, this forbade a safe use in man so far. Therefore any superfluous endotoxin exposure must be strictly avoided to prevent complications. This is especially true for medicaments applied intravenously.

In order to prevent the endotoxin-caused reactions, limitations were set as shown in Table 2.1.

It needs to be pointed out that endotoxins may also have beneficial effects. Endotoxins have been used in artificial fever therapy, to destroy tumors and to improve nonspecifically the immune defense.

Normally, man comes into contact with endotoxins by drinking and swimming in contaminated water (Sykora et al., 1980; Sykora and Keleti, 1981). In addition, man may come into contact with endotoxin aerosols released by spray irrigation of contaminated water via skin or eyes (Gerba and Goyal, 1981). Endotoxin concentration in water sources closely related to daily life is shown in Table 2.2.

From Table 2.2 it can be seen that human beings can be obviously in contact with endotoxins at any time and apparently can handle this without problems. As long as these are in contact with the skin or digestive system, they are tolerated quite well.

Table 2.1 Endotoxin limitation.

Item	Limitation
Breath	20 ng/m^3 (Hasday et al., 1996)
Intravenous application	5 EU (Endotoxin Unit)/kg body per hour (Pearson, 1985)
Water for injection (WFI)	≤ 0.25 EU/ml *
Zinc Sulfate Injection	≤ 25 EU/ml *
Factor IX	≤ 0.1 EU/ml (5 EU/dose) *
Immune serum globulin	≤ 0.91 EU/ml (≤ 5 EU/dose) *

*Pearson, F. C. (1985) FDA Guideline for LAL End-Product Testing of Biologicals, Parenteral Drugs, and Medical Devices. In *Pyrogens: endotoxins, LAL testing, and depyrogenation*. Marcel Dekker, Inc. New York. p. 185.

Table 2.2 Endotoxin concentration in daily life source.

Source	Concentration (EU/ml)
Tap and mineral water	1 –20 (Müller-Calgan, 1989)
Water in open-air swimming pool	25, 600 (Müller-Calgan, 1989)

LPS is removed from the body over many weeks. It is degraded mainly in the liver and subsequently excreted through the gut (Klein et al., 1985). Biliary excretion has been identified as one route by which degraded LPS reaches the gut. Several mechanisms of detoxification alleged to begin in the blood stream have been proposed, but conclusive evidence for degradation and detoxification of LPS has yet to be presented.

2.3 Endotoxin and Protein Interaction

Endotoxin removal from protein solution encounters additional difficulty: endotoxins have interaction with some proteins, even bind or form complexes with proteins, which may be the target compounds of interest in recombinant biopharmaceuticals. That makes endotoxin removal from protein solution more complicated.

Endotoxins show a remarkable capability to interact with proteins (Table 2.3). One category of proteins is anti-endotoxin antibodies and proteineous endotoxin receptors (Morrison et al., 1994). Molecular recognition can be assumed for such interaction.

Many basic proteins (pI > 7) were reported to interact with endotoxin. Electrostatic interactions can be assumed as the main driving force for such interaction. While some studies supported that hydrophobic is the major driving force and only limited ionic binding is involved (Ohno and Morrison, 1989).

Neutral protein, as hemoglobin and even acidic proteins, as serum albumin (pI < 7) (Roth et al., 1994; David et al, 1995) also were reported to interact with endotoxin. The mechanisms of such interactions are not quite clear now. Generally it is assumed that

hydrophobic interaction between protein and endotoxin is the main driving force. For serum albumin, fatty acid binding domains might be involved (David et. al., 1995).

2.4 Endotoxin Removal Techniques

Methods for removing endotoxins depend on the molecular weight of the endotoxin, its chemical properties, or both. The chemical decomposition of endotoxins with and acid, alkali (Niwa et al., 1969), or oxidizing agent (Cherkin, 1975) is commonly practiced; however, such methods cannot be used on blood or other biological products because of their destructive nature.

Nondestructive approaches to endotoxin removal from aqueous solutions include physical methods based on ultrafiltration by size and molecular weight differences (Sweadner et al., 1977) and by adsorption to activated carbon and ion-exchange resins (Nolazn et al., 1975; Pearson, 1985b).

Basically, four different approaches have been used to remove pyrogens from aqueous solutions. These include (a) ultrafiltration, (b) adsorption to asbestos and activated charcoal, (c) ion-exchange chromatography, and (d) affinity chromatography (Table 2.4).

(a) Ultrafiltration. Ultrafiltration is a highly efficient method for removing pyrogens from contaminated solutions. This technique is dependent on a difference between the physical size of the pyrogen molecules and of the product being purified.

The contaminated solution is pressed through a finely porous polysulfone membrane that retains high molecular–weight molecules and allows the low-molecular-weight molecules to pass through. Selection of the appropriate filter size is dependent on

the composition of the solution being purified and the state of aggregation of the endotoxin molecules.

The degree of aggregation of endotoxin molecules is affected by the presence of protein, due to endotoxin and protein interaction, divalent cations, chelating agents, and detergents in the solution.

Endotoxin can be removed effectively from parenteral products with ultrafilters with a 10,000 Da nominal molecular weight limit. In aqueous solutions, the majority of endotoxin molecules aggregate into vesicles ranging from 300,000 to 1,000,000 Da (Nelson, 1978).

Four filtration systems can be used in ultrafiltration: tangential-flow filtration, stirred-cell filtration, hollow-fiber filtration, or thin-channel filtration. Conventional filtration is not recommended because retained material builds up on the membrane surface and causes a drastic reduction in flow rate and volume processing capacity (Sharma, 1986). Ultrafiltration membranes can be cleaned and depyrogenated by pumping NaOH (0.1-1.09 M) or HCl (0.1 M) through the system. Most systems are available for both general laboratory use and large-scale industrial use.

(b) Adsorption by asbestos and activated charcoal. The adsorption of pyrogens to asbestos fiber and to activated charcoal are pyrogen elimination procedures that preceded the use of ultrafiltration system (Nolazn et al., 1975). These systems, however, are inconsistent, and highly dependent on the pyrogen concentration in the solution. They are accompanied by high losses of the substance being purified. Due to its toxicity, in 1975, FDA prohibited the use of asbestos fiber for pyrogen elimination from parenterals.

Even though activated charcoal adsorption was effective in elimination pyrogens, however, it was of limited value because it was difficult to remove the activated charcoal from the purified solution and, as with any adsorption technique, there was a high risk of adsorption of the desired product to the adsorbent.

(c) Endotoxin elimination using ion-exchange chromatography. Ion-exchange chromatography is frequently used in the purification of products of recombinant DNA technology. The advantages of this technique are its ability to process large sample volumes, its high capacity, and the relatively mild conditions employed. Since endotoxins are negatively charged due to the phosphate groups in its molecules, anion exchangers were successfully used to remove endotoxins.

The use of DEAE-Sephadex for the removal of pyrogens was first described by Grabner (Grabner, 1975). The pyrogens were irreversibly adsorbed on this resin during the passage of a pyrogenic aqueous solution of high ionic strength (0.1 to 0.2 M). Likewise, the anion-exchange resin DEAE-Sepharose CL-6B has been shown to effectively adsorb pyrogens from a solution containing urokinase, which appeared in the flow-through volume (Shibatani et al., 1983).

Cation exchangers do not appear to adsorb pyrogens.

(d) Endotoxin removal by affinity chromatography. Chemical adsorption of endotoxins from whole blood or plasma is the only practical clinical treatment for septic patients. In industry, adsorption has found wide use too. Sorption is based on either the charge induced from phosphoryl groups or on hydrophobic interaction as a result of the presence of lipid A groups (Malchesky et al., 1995).

The cyclic lipophilic peptide antibiotic, polymyxin B sulfate, is known to neutralize the biological activity of endotoxin (Rifkind, 1967; Corrigan et al., 1971; Morrison et al., 1976) presumably due to its binding with high affinity to the toxic lipid A moiety of endotoxins (Sharma, 1986). The hydrophobic components of polymyxin B can be accounted for as coming from the isobutyl group in leucine and the phenyl group in phenylalanine (Malchesky et al., 1995).

The bactericidal activity of the antibiotic polymyxin B against gram-negative bacteria is based on the ability to deorganize the bacterial wall after insertion (Newton, 1956). The surface active cyclic peptide can break down endotoxin aggregates (Lopes and Inniss, 1969). Owing to the interaction of polymyxin B with lipid A, the most conservative part of endotoxin, it should be a group selective ligand with potential to recognize endotoxins from different origins. Its use as a ligand in affinity sorbents displays endotoxin clearance factors of $> 10^5$ from heavily contaminated culture filtrates (1-10 μg/ml) of different gram-negative bacteria (Issekuzs, 1983). However, treating endotoxemia with polymyxin B is difficult because it is toxic effect on the kidneys and nervous system (Hanazawa et al., 1984).

One other affinity chromatography method was introduced by Minobe et al. (Minobe et al., 1983). Histamine was immobilized to aminohexyl-Sepharose CL-4B with glutaraldehyde treatment. This adsorbent has a high affinity for pyrogens from various gram-negative bacteria. Results from studies examining the characteristics of the immobilized histamine for pyrogen adsorption have suggested that the interaction between the adsorbent and the pyrogen is both hydrophobic and ionic. Although the

histamine sorbent was favored, it was switched to histidine owing to the biological activity of histamine (Minobe et al., 1988).

An immunoaffinity chromatography in which the ligand is an antibody against the component of interest may selectively remove the antigen while pyrogens remain in the feed solution. Because of the high costs involve in the production of large quantities of immobilized antibodies, their use is likely to remain restricted to those applications where a low-cost ligand is not appropriate, and the compound to be isolated has a high value. Hence, the technique has been used for the purification of pharmaceutical proteins such as human interferon (Sbcher and Burke, 1980), vaccines, and antibodies.

Table 2.3 Proteins that interact with endotoxin.

Protein	Category	Reference
CD14 CD16 CD18	Anti-endotoxin antibody or proteineous endotoxin receptors (Molecular recognition)	Morrison, et al., 1994
Lysozyme Lactoferrin Transferrin Alkaline phosphatase	Basic proteins (pI > 7) (Electrostatic interaction or hydrophobic interaction)	Ohno and Morrison, 1989 Elass-Rochard, et al., 1995 Berger and Berger, 1988 Poelstra, et al., 1997
Hemoglobin Serum albumin	Neutral protein (pI ~7) or Acidic protein (pI <7) (Hydrophobic interaction or other mechanism is under development)	Hou and Zaniewski (1990) David, et al., 1995

Table 2.4 Endotoxin removal methods.

Method	**Principle**	**Reference**
Ultrafiltration	Physical method: based on difference between physical size of the pyrogen molecules and the product be purified	Malchesky et al., 1995
Adsorption by asbestos and activated charcoal	Non-selective adsorption	Nolazn et al., 1975; Kazden, H. 1975;
Endotoxin elimination using anion-exchange chromatography	Ionic interaction e.g. DEAE- Sephadex	Grabner, 1975
Affinity chromatography	Hydrophobic interaction e.g. Polymyxin-B	Rifkind, D. 1967; Corrigan, et al., 1971; Morrison et al., 1976
	Hydrophobic and ionic e.g. Histamine and histidine	Minobe et al., 1983; Minobe et al., 1988
	Immunoaffinity e. g. Antibody ligand	Sbcher and Burke, 1980

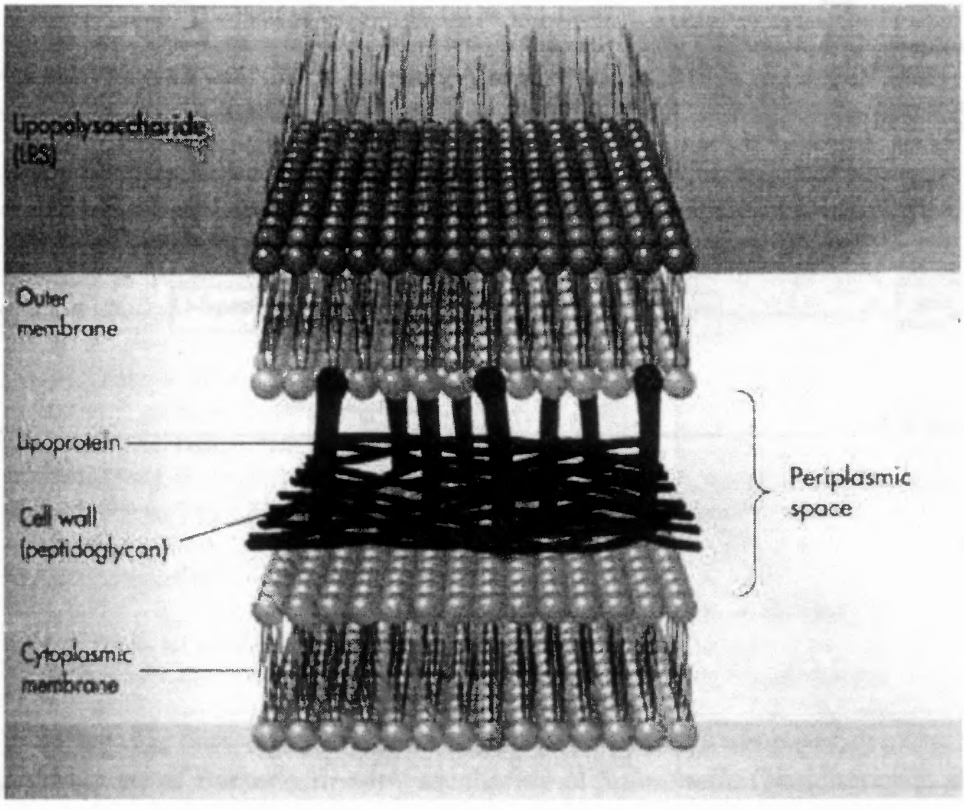


Figure 2.1 Structure of gram-negative bacteria cell wall and membranes.

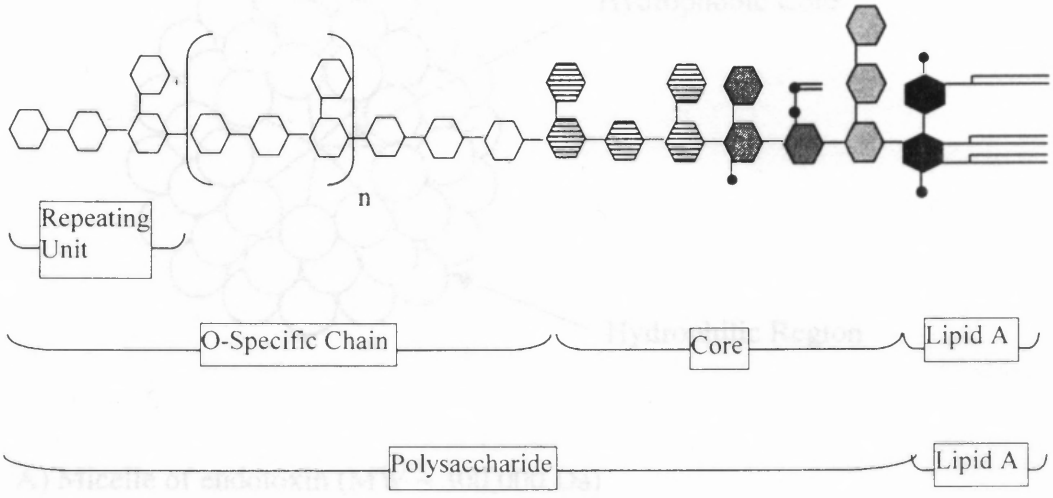


Figure 2.2 Structure of Bacteria lipopolysaccharide of *Salmonella* (Malchesky et al., 1995).

Sugar residue

L-Glycero-D-Manno-heptose

2-Keto-3-deoxy-D-manno-octonate

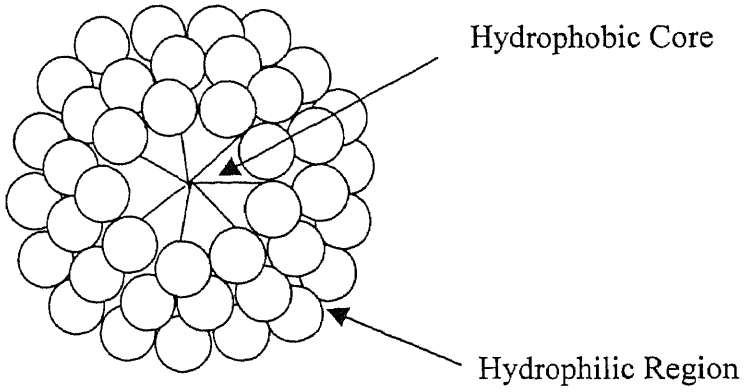
D-Glucosamine

Phosphate

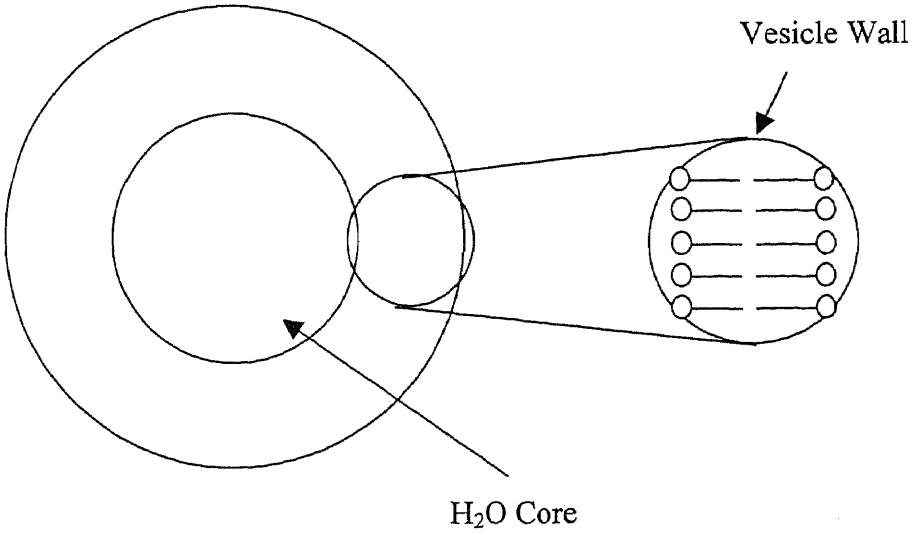
Ethanolamine

3-Hydroxyacyl group

3-Acyloxyacyl group



A) Micelle of endotoxin (MW ~ 300,000 Da)



B) Vesicle of endotoxin (MW > 10^6)

Figure 2.3 Structure of endotoxin aggregate (Millipore Co.).

CHAPTER 3

ENDOTOXIN DISAGGREGATION BY PROTEIN AND PROTEIN CONCENTRATION EFFECT ON PROTEIN-LIPOPOLYSACCHARIDE BINDING AND ENDOTOXIN REMOVAL

3.1 Objective

Bacterial endotoxins are lipopolysaccharide (LPS) derived from the outer cell membranes of Gram-negative bacteria (Hou and Zaniewski, 1990). Endotoxins are known to have potent biological effects in man and in many animal species when administered systemically (Issekutz, 1983). In biotechnology industry, Gram-negative bacteria are widely used to produce recombinant DNA products, such as peptides and proteins. Bacterial endotoxins have been recognized as a major cause of the pyrogenic reactions that can be encountered during the administration of biological therapeutics. The removal of these physiologically active agents from final bioproducts has always been a challenge to the industry.

It is known that some proteins can disaggregate endotoxins and form complexes with lipopolysaccharide (LPS). Some of such proteins have been identified, such as lipoproteins (Schlichting *et* al., 1996) and monoclonal antibodies (Ziegler *et* al., 1991). It is reported that hemoglobin not only can bind but also disaggregate endotoxins, and enhance endotoxin activation of *Limulus* amebocyte lysate (LAL) in a concentration dependent manner (Kaca *et* al., 1994; Roth *et* al., 1994). Variable effects of human serum albumin (HSA) on endotoxin biological activity have also been reported. The biological activity of endotoxin is changed in the presence of HSA, depending on HSA concentration (Kaca *et* al., 1994).

Nevertheless, whether other proteins also interact with endotoxin and disaggregrate endotoxin, and how protein concentration affects protein-LPS binding and endotoxin removal are unknown. In this study, an ultrafiltration method was used to investigate interaction of protein and endotoxin and protein concentration effect on protein-LPS binding and endotoxin removal. Protein samples (human hemoglobin, bovine serum albumin, lysozyme, and cytochrome C, Table 3.1) at various concentrations were incubated with endotoxin samples from *Escherichia coli* 026:B6 at a fixed concentration. After incubation, the mixtures were filtered by 300,000 nominal molecular weight cut-off (NMWCO) polysulfone ultrafiltration membrane. *Limulus* amebocyte lysate (LAL) test was employed to detect endotoxin in the filtrates.

3.2 Materials and Methods

3.2.1 Materials

Proteins and endotoxin. Endotoxin from *Escherichia coli* 026:B6, human hemoglobin A_o (HbA_o, ferrous) and bovine serum albumin (BSA, fraction V), cytochrome C from bovine heart, lysozyme from chicken egg white were purchased from Sigma.

Other materials. 0.1 M endotoxin-free HCl, 0.1 M endotoxin-free NaOH, NaCl (>99.0%), endotoxin-free water and endotoxin detection kit E-TOXATE were purchased from Sigma. Ultrafree-CL polysulfone 300,000 NMWCO ultrafiltration membranes were purchased from Millipore. All glassware used was autoclaved (series 300, Harvard/LTE) for 1 hour and followed with heating in an oven (Thelco Model 16, Precision Scientific Co.) at 175 °C for 3 hours. All solution transfers were performed with endotoxin-free devices.

Endotoxin-free 0.9% NaCl solution was prepared with NaCl (>99.0%) and endotoxin-free water. Protein solutions at various concentrations were prepared with endotoxin-free 0.9% NaCl solution. 50 μg LPS /ml endotoxin solution was prepared with endotoxin-free 0.9% NaCl solution.

Ultrafiltration membrane. Ultrafiltration membranes are rated on the basis of molecular weight exclusion limits. Endotoxins that exceed the molecular weight exclusion limit of a given membrane are retained on the surface of the membrane. Normally, LPS molecules aggregate into vesicles with a molecular weight around 1 million Da (Pearson, 1985b). Therefore, pure endotoxin in aqueous solutions can not pass through the ultrafiltration membranes rated at 300,000 molecular weight. If endotoxin aggregates are broken-down by protein molecules and form much smaller protein-LPS complexes as expected, the complexes will pass through the 300,000 NMWCO membrane and endotoxin will be found in the filtrate. In this study, 300,000 NMWCO polysulfone membranes were used to filter all samples (Figure 3.1). The filtrates were subject to LAL test.

3.2.2 Endotoxin Detection

Endotoxin was detected using *Limulus* amebocyte lysate (LAL) test (Sigma, 1994). Gelation was taken as the endpoint. Endotoxin from *Escherichia coli* 055:B5 was used as the standard. For standard endotoxin detection, when endotoxin concentration was greater than 0.06 Endotoxin Unit/ml, it formed solid gel and gave positive result; when endotoxin concentration was less than 0.06 Endotoxin Unit/ml, it did not form solid gel and gave negative result. Before the formation of the gel, the solution might be cloudy in

the presence of endotoxin. The intensity of the cloudiness increases with the increase of endotoxin concentration in the solution (Figure 3.3).

3.2.3 Pure Protein Study

0.9 ml protein preparations at various concentrations (Table 3.2, 3.3, 3.4, 3.5) were added to 1.5 ml sterile Eppendorf microcentrifuge tubes respectively, then 0.1 ml 0.9% endotoxin free NaCl was added to each tube. The mixtures were incubated in a water bath at 37 °C for 30 minutes. After that the solutions were placed on 300,000 NMWCO polysulfone ultrafiltration membranes and centrifuged at 1800g for 5 minutes. 0.1 ml filtrate was taken from each solution and LAL test was employed to detect the endotoxin in it (Figure 3.2).

3.2.4 Pure Endotoxin Study

0.1 ml 50 μg LPS/ml endotoxin solution was put into 1.5 ml sterile Eppendorf microcentrifuge tube, then 0.9 ml endotoxin free 0.9% NaCl solution was added to the tube. The solution was incubated in a water bath at 37 °C for 30 minutes. The following steps were the same as those in the pure protein study (Figure 3.2).

3.2.5 Protein-LPS Binding Study

0.9 ml protein preparations at various concentrations (Table 3.6, 3.7, 3.8, 3.9) were added to 1.5 ml sterile Eppendorf microcentrifuge tubes respectively, then 0.1 ml 50 μg LPS/ml endotoxin solution was added to each tube. The mixtures were incubated in a water bath

at 37 °C for 30 minutes. The following steps were the same as those in the pure protein study (Figure 3.2).

3.3 Results and Discussion

3.3.1 Pure Hemoglobin Study

Pure human hemoglobin A_o (HbA_o) solutions were filtered by 300,000 NMWCO membranes and the filtrates were tested by LAL method. The results are shown in Table 3.2. When HbA_o concentrations were 19.2 μg/ml, 38.3 μg/ml and 75.6 μg/mg, the LAL test gave negative results. It suggested that the filtrates of pure HbA_o were endotoxin free.

3.3.2 Pure Albumin Study

Pure bovine serum albumin (BSA) solutions were filtered by 300,000 NMWCO membranes and the filtrates were tested by LAL method. The results are shown in Table 3.3. When BSA concentrations were 6.8 μg/ml, 10.2 μg/ml and 20.4 μg/ml, the LAL test gave negative results. It suggested that the filtrates of pure BSA solutions were endotoxin free.

3.3.3 Pure Lysozyme Study

Pure chicken egg white lysozyme solutions were filtered by 300,000 NMWCO membranes and the filtrates were tested by LAL method. The results are shown in Table 3.4. When lysozyme concentrations were 28.1 μg/ml, 56.3 μg/ml and 112.5 μg/ml, the LAL test gave negative results. It suggested that the filtrates of pure BSA solutions were endotoxin free.

3.3.4 Pure Cytochrome C Study

Pure bovine heart cytochrome C solutions were filtered by 300,000 NMWCO membranes and the filtrates were tested by LAL method. The results are shown in Table 3.5. When cytochrome C concentrations were 20.1 μg/ml, 50.7 μg/ml and 101.3 μg/ml, the LAL test gave negative results. It suggested that the filtrates of pure BSA solutions were endotoxin free.

3.3.5 Pure Endotoxin Study

Pure endotoxin solution was filtered by a 300,000 NMWCO membrane and the filtrate was tested by LAL method. The filtrate was clear in the presence of LAL lysate and LAL test gave negative result. This indicated that the endotoxin was in an aggregated state and could not pass through the membrane, so no endotoxin was found in the filtrate.

3.3.6 HbA_o-LPS Binding Study

The HbA_o-LPS mixtures were filtered with 300,000 NMWCO membranes after incubation. The filtrates were tested by LAL method and the results are shown in Table 3.6. In this study, the HbA_o concentration in the mixture was increased while the endotoxin concentration was kept the same at 5 μg LPS/ml in every mixtures. It was observed that when HbA_o concentration was increased, the cloudy intensity of the filtrate-LAL solution increased too. When HbA_o concentration was 19.2 μg/ml and 38.3 μg/ml in the HbA_o-LPS mixture, the filtrates of the mixtures formed slightly cloudy solutions in the presence of LAL lysate, and the test gave negative results. When HbA_o

was at 76.5 μg/ml in the HbA_o-LPS mixture, the filtrate of the mixture formed a solid gel in the presence of LAL lysate, and the test gave a positive result.

The physical meanings of above results may be explained as follows. Pure endotoxin is usually in an aggregated state with molecular weight around 1 million Da and is retained by a 300,000 NMWCO membrane. If a small amount of hemoglobin is added to the pure endotoxin solution, the protein molecules break the endotoxin aggregates and form protein-LPS complexes with endotoxin subunits. These complexes have much smaller size than the original pure endotoxin aggregates and can easily pass the 300,000 NMWCO membrane. Therefore, the endotoxin content in the protein-LPS complexes in the filtrate result in a cloudy solution in the presence of LAL lysate. If the hemoglobin concentration is increased in the protein-LPS mixture, more protein-LPS complexes will form and pass the membrane. The intensity of the cloudiness of the filtrate-LAL solution increases as well. If the hemoglobin concentration is increased further, it will reach a point that the filtrate forms a solid gel with LAL lysate, i.e., a positive LAL test result.

3.3.7 BSA-LPS Binding Study

The BSA-LPS mixtures were filtered with 300,000 NMWCO membranes after incubation. The filtrates were tested by LAL method and the results are shown in Table 3.7. When BSA concentration was 6.8 μg/ml and 10.2 μg/ml in the mixtures, both of them formed clear solutions with LAL lysate and the test gave negative results. This means no endotoxin was detected in the filtrates. When BSA was at 20.4 μg/ml in the mixture, it formed solid gel with LAL lysate and the test gave a positive result. These

results may also be explained by the above discussions about HbA_0. It is noticed from Table 3.6 and Table 3.7 that BSA is more effective in disaggregating endotoxin at a lower concentration than HbA_0. One explanation is that a BSA molecule binds more endotoxin subunits than a HbA_0 molecule does in the solution. The exact mechanism is not well understood yet.

3.3.8 Lysozyme-LPS Binding Study

The lysozyme-LPS mixtures were filtered with 300,000 NMWCO membranes after incubation. The filtrates were tested by LAL method and the results are shown in Table 3.8. When lysozyme concentrations were 28.1 μg/ml, 56.3 μg/ml, 112.5 μg/ml in the mixtures, all of them formed cloudy solutions with LAL lysate and the test gave negative results. If endotoxin was disaggregated by lysozyme to form small aggregates, the small aggregates would pass through ultrafiltration membrane and would be detected. The negative LAL test results mean almost no endotoxin was found in the filtrate. That indicated endotoxin was not disaggregated by lysozyme.

3.3.9 Lysozyme-LPS Binding Study

The cytochrome C-LPS mixtures were filtered with 300,000 NMWCO membranes after incubation. The filtrates were tested by LAL method and the results are shown in Table 3.9. When cytochrome C concentrations were 20.1 μg/ml, 50.1 μg/ml, 101.3 μg/ml in the mixtures, all of them formed clear solutions with LAL lysate and the test gave negative results. If endotoxin was disaggregated by cytochrome C to form small aggregates, the small aggregates will pass through ultrafiltration membrane and were detected. The

negative LAL test results means almost no endotoxin was found in the filtrates. That indicated endotoxin was not disaggregated by cytochrome C.

3.4 Conclusions

These results demonstrate that some proteins (hemoglobin and albumin in this study) can break endotoxin aggregates. Also concentration of the protein has a significant effect on the amount of endotoxin disaggregated and the amount of protein-LPS complex formed. Ultimately, protein concentration will affect the rate of endotoxin removal by ultrafiltration membrane processes. Knowing this mechanism is very important to ultrafiltration membrane process design for removing endotoxin from biological solutions.

The above results also show that chicken egg white lysozyme and bovine heart cytochrome C did not disaggregate endotoxin, that is, these two proteins did not break endotoxin into small fragments.

Table 3.1 Properties of proteins and endotoxin.

Name	**Molecular weight (Da)**	**Isoelectric point**
Hemoglobin from human	64,500	7.0
Albumin	67.000	4.9
Lysozyme	13,370	11.1
Cytochrome C	12,327	10.7
LPS from E. coli 026:B6	$\sim 10^6$	at pH above 2, LPS is negatively charged (Pearson,1985c)

Table 3.2 LAL test results of pure HbA_o solutions.

HbA_o concentration (μg/ml)	Endotoxin concentration (μg LPS/ml)	LAL test result of the membrane filtrate
19.2	0	_
38.3	0	_
76.5	0	_

Table 3.3 LAL test results of pure BSA solutions.

BSA concentration (μg/ml)	Endotoxin concentration (μg LPS/ml)	LAL test result of the membrane filtrate
6.8	0	–
10.2	0	–
20.4	0	–

Table 3.4 LAL test results of pure lysozyme solutions.

Lysozyme concentration (μg/ml)	Endotoxin concentration (μg LPS/ml)	LAL test result of the membrane filtrate
28.1	0	–
56.3	0	–
112.5	0	–

Table 3.5 LAL test results of pure cytochrome C solutions.

Cytochrome concentration (μg/ml)	Endotoxin concentration (μg LPS/ml)	LAL test result of the membrane filtrate
20.1	0	–
50.7	0	–
101.3	0	–

Table 3.6 LAL test results of HbA_o-LPS binding study.

HbA_o concentration in HbA_o-LPS mixture (μg/ml)	Endotoxin concentration in HbA_o-LPS mixture (μg LPS/ml)	LAL test result of the membrane filtrate
19.2	5	–
38.3	5	–
76.5	5	+

Table 3.7 LAL test results of BSA-LPS binding study.

BSA concentration in BSA-LPS mixture (μg/ml)	Endotoxin concentration in BSA-LPS mixture (μg LPS/ml)	LAL test result of the membrane filtrate
6.8	5	–
10.2	5	–
20.4	5	+

Table 3.8 LAL test results of lysozyme-LPS binding study.

Lysozyme concentration in lysozyme-LPS mixture (μg/ml)	Endotoxin concentration in lysozyme-LPS mixture (μg LPS/ml)	LAL test result of the membrane filtrate
28.1	5	–
56.3	5	–
112.5	5	–

Table 3.9 LAL test results of cytochrome C-LPS binding study.

Cytochrome C concentration in cytochrome C-LPS mixture (μg/ml)	Endotoxin concentration in cytochrome C-LPS mixture (μg LPS/ml)	LAL test result of the membrane filtrate
20.1	5	–
50.7	5	–
101.3	5	–

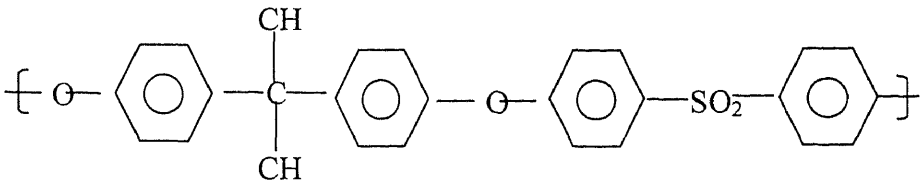


Figure 3.1 Molecular structure of hydrophobic polysulphone membrane (Howell et al., 1993).

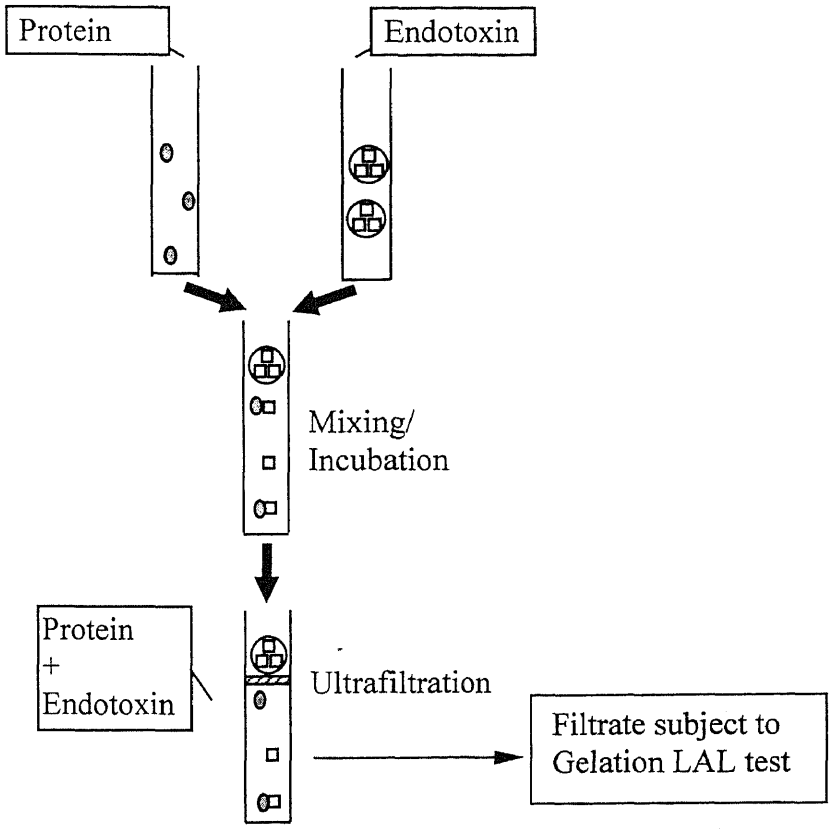


Figure 3.2 Schematic diagram of experimental procedure. The experimental procedure: protein/LPS mixtures at various concentration ratios were incubated at 37 °C for 30 minutes. After incubation, the mixtures were applied to ultrafiltration. The filtrate was applied to gelation LAL test.

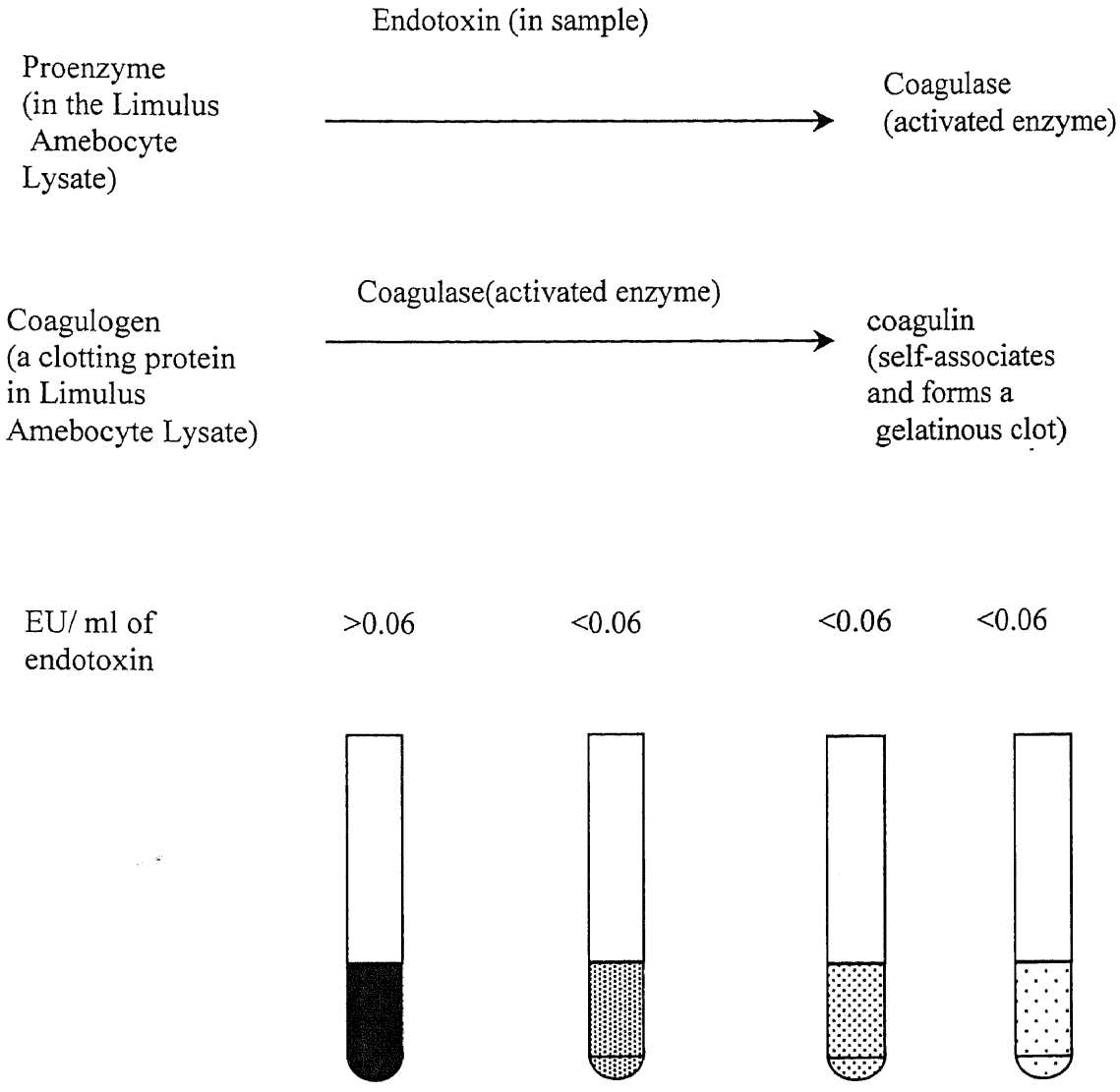


Figure 3.3 Principle of *Limulus amebocyte lysate* (LAL) test.

CHAPTER 4

SIZE EXCLUSION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (SE-HPLC) STUDIES ON ENDOTOXIN DISAGGREGATION BY BOVINE SERUM ALBUMIN (BSA)

4.1 Objective

Endotoxin subunits have molecular weights of 10,000 or less, depending on the composition of the solution in which they are present (Gerba and Goyal, 1981). Aggregated forms of endotoxin range from 300,000 to 1,000,000 daltons (Brown and Fuller, 1993; Abramson et al., 1981; Sweadner et al., 1977; Hannecart-Pokorni et al., 1973). In Chapter 5 it is shown such endotoxin aggregation is facilitated by cations in protein solution; and the aggregation is obliterated by protein, such as hemoglobin and albumin, in a protein concentration dependent manner. It is hypothesized that an equilibrium exists between aggregated endotoxin and disaggregated endotoxin in the presence of protein. Using ultrafiltration method, previous studies described in Chapter 3 have shown that protein concentration has a significant impact on the equilibrium shifting: as protein concentration increased, the equilibrium was disturbed and shifted to form more disaggregated endotoxin. However, some questions are still unsolved, that is, what is the endotoxin disaggregation rate? Is it partially or completely disaggregated. Before and after disaggregation, how do endotoxin aggregated forms change and what elements affect such disaggregation.

Studies of proteins that interact with LPS have used a variety of methods to demonstrate and quantify such interaction. For example, fluorescent-labeled LPS was used to study LPS binding to lipopolysaccharide-binding protein (LBP) (Tobias et al.,

1995; Yu and Wright, 1996). Other nondestructive methods of analysis, such as native gel electrophoresis was used with biosynthetically isotope labeled LPS (Juan et al., 1995). Membrane ultrafiltration was used to study the effect of protein concentration on protein and LPS binding as described in Chapter 3.

Size-exclusion high-performance liquid chromatography (SE-HPLC) is another nondestructive method for studying protein and LPS interaction. The advantage of the size-exclusion HPLC technique is that it neither structurally alters the LPS by adding a fluorescent adduct nor disrupts the native state of the proteins and LPS. In addition, the size-exclusion HPLC method can measure the size of LPS aggregates, determine an exclusion limit, and resolve protein from LPS.

This is the first effort at using size exclusion high performance liquid chromatography (SE-HPLC) combined with UV absorbance detection to study endotoxin disaggregation. SE-HPLC method was used to investigate LPS disaggregation in the presence of bovine serum albumin (BSA). Ultraviolet (UV) absorbance was employed as the detection method to obtain the chromatography profile. This is the most elucidative way to demonstrate LPS disaggregation. Protein and endotoxin mixture samples at various concentration ratios, which were incubated at 37 °C for 30 min or directly set at room temperature without incubation, are analyzed with SE-HPLC. Size exclusion chromatography separates molecules on the basis of molecular size, that is, molecules are eluted in order of decreasing molecular size. If endotoxin is disaggregated by protein to form smaller size aggregates, such smaller aggregates will be eluted at longer retention times, in comparison with the original endotoxin aggregates. From the chromatographic profile, we can see that the area of the first LPS aggregate peak, which is produced by the

larger aggregates, will decrease under the effect of BSA and move to longer retention time. The area of LPS peaks, which are smaller than the first peak and eluted at longer retention times, will increase. Under certain conditions, new peak groups of smaller size species formed.

4.2 Materials and Methods

4.2.1 Materials

Glassware. All glassware used was sterilized in an autoclave (Series 300, Harvard/LTE) for 1 hour and followed with heating in an oven (Thelco Model 16, Precision Scientific Co., Chicago, IL) at 175 °C for 3 hours. All solution transfers were performed by endotoxin-free device. Sterile, disposable plasticware was used to prevent endotoxin contamination.

Reagents. Endotoxin-free water from BioWhittaker (BioWhittaker Inc., Walkersville, MD) was used for endotoxin dilution and all sample solution preparations. Endotoxin-free water produced by Milli-Q water system (Millipore Corporation, Bedford, MA) was used for high performance liquid chromatography (HPLC) mobile phase preparation. Endotoxin from *Escherichia coli* 026:B6, bovine serum albumin (BSA, fraction V, cell culture reagent), NaCl (molecular biology reagent) were from Sigma (Sigma Chemical Co., St. Louis, MO). Bovine serum albumin and endotoxin solutions were prepared with endotoxin-free 0.15 M NaCl solution.

4.2.2 Pure LPS Study

Pure LPS solution was prepared by placing 0.25 ml LPS stock solution of 1 mg/ml in a 15 ml endotoxin-free sterile polystyrene tube; then adding 0.75 ml endotoxin-free 0.15 M NaCl.

Pure LPS study without incubation: After pure LPS solution was prepared, 1 ml sample were taken from the tube containing pure LPS solution, and then transferred to the HPLC sampler vials for HPLC analysis.

Pure LPS study with incubation: After pure LPS solution was prepared, the tube containing LPS solution was incubated at 37°C in a water bath for 30 min. 1 ml sample was taken from the tube containing pure LPS solution, and then transferred to HPLC sampler vials for HPLC analysis (Figure 4.1).

4.2.3 Pure Protein Study

Bovine serum albumin (BSA) stock solution at various concentrations was prepared by serial dilution with endotoxin-free 0.15 M NaCl (Table 4.1). Stepwise dilution was used to make dilution accurately. 0.5 ml BSA solution at various concentrations was taken and put into 15 ml endotoxin-free sterile polystyrene tube individually. Then 0.5 ml endotoxin-free 0.15 M NaCl was added to each tube to prepare pure BSA solution. The concentrations of these BSA solutions were 0.25, 0.5, 1, 2 and 4 mg/ml (Table 4.2).

Pure BSA study without incubation: After pure BSA solutions were prepared, 1 ml samples were taken from all solutions, and then transferred to HPLC sampler vials for HPLC analysis.

Pure BSA study with incubation. After pure BSA solutions were prepared, the tubes containing BSA solutions were incubated at 37°C in a water bath for 30 min. 1 ml samples were taken from all solutions, and then transferred to HPLC sampler vials for HPLC analysis (Figure 4.1).

4.2.4 BSA-LPS Interaction without Pre-Incubation

BSA solution at various concentrations was prepared by series dilution with endotoxin-free 0.15 M NaCl (Table 4.2). Stepwise dilution was used to make dilution accurately. 0.5 ml BSA solution at various concentrations was taken and put into a 15 ml endotoxin-free sterile polystyrene tube individually. 0.25 ml LPS solution at 1mg/ml was added to each tube, then 0.25 ml endotoxin-free 0.15 M NaCl was added to each tube to prepare BSA and LPS mixtures at various concentration ratios (Table 4.3). In such BSA and LPS mixtures, the BSA concentration was 0.25, 0.5, 1, 2 and 4 mg/ml, while endotoxin concentration is the same for each mixture solution, that is 0.25 mg/ml (Table 4.4).

After BSA and LPS mixtures were prepared, 1 ml samples were taken from all mixtures and transferred to HPLC sampler vials for HPLC analysis (Figure 4.2).

4.2.5 BSA-LPS Interaction with Pre-Incubation

BSA solution at various concentrations was prepared by series dilution with endotoxin-free 0.15 M NaCl (Table 4.2); such stepwise dilution was used to make dilution accurately. 0.5 ml BSA solution at various concentrations was taken and put into a 15 ml endotoxin-free sterile polystyrene tube individually. 0.25 ml LPS solution at 1mg/ml was added to each tube, then 0.25 ml endotoxin-free 0.15 M NaCl was added to each tube to

prepare BSA and LPS mixtures at various concentration ratios (Table 4.3). In such BSA and LPS mixtures, the BSA concentration was 0.25, 0.5, 1, 2 and 4 mg/ml, while endotoxin concentration is the same for each mixture solution, that is 0.25 mg/ml (Table 4.4).

After BSA and LPS mixtures were prepared, the tubes containing the mixture solutions were incubated at 37°C in a water bath for 30 min. 1 ml samples were taken from all mixtures and transferred to HPLC sampler vials for HPLC analysis (Figure 4.1).

4.2.6 Size Exclusion High Performance Liquid Chromatography (SE-HPLC)

The basis for the separation mechanism in size exclusion chromatography is 'sieving'. Chromatographic separations based on size are achieved by controlling the size of the pores in the stationary phase. If the molecules are too large for the pores, they never enter the gel and move outside the gel bed with the eluting solvent. Thus, the very large molecules in a mixture move the fastest through the gel bed. The smaller molecules, which can enter the gel pores, are retarded and move more slowly through the gel bed. Molecules of intermediate size will be partially excluded from the pores and can be separated from each other based on the fractional extent of their exclusion (Figure 4.3). In size exclusion chromatography, molecules are, therefore, eluted in order of decreasing molecular size (Figure 4.4).

Hewlett-Packard series II 1090 HPLC system (Hewlett-Packard Co., Wilmington, DE) was used to analyze LPS disaggregation in protein solutions. The size exclusion column, TSK-GEL G2000SWXL, was from TosoHaas (TosoHaas, Montgomeryville,

PA). Diode array detector was used for determination the UV absorbance of column eluents.

BSA and LPS mixture solutions at various concentration ratios (Table 4.4), which were pre-treated without incubation or with incubation, were analyzed by HPLC on a TosoHaas TSK-GEL G2000SWXL column in an isocratic 0.1 M phosphate buffer at pH 6.7. The buffer consisted of 0.05 M KH_2PO_4, 0.05 M Na_2HPO_4, 0.1 M Na_2SO_4 and 0.05 % NaN_3. HPLC assay was performed at flow rate of 1 ml/ml. The injection volume was 25 μl. Diode array detector was set at 260 nm for detection UV absorbance.

4.3 Results and Discussion

4.3.1 Pure LPS Study

To investigate how LPS was disaggregated by BSA, it is necessary to examine how the LPS molecule changes after being mixed with BSA. Therefore a study of pure LPS with SE-HPLC was performed. Since in size exclusion chromatography molecules are eluted in order of decreasing molecular size. The size distribution of pure LPS aggregates can be determined. If LPS was composed of uniform size aggregates, it would elute as one peak. If LPS was composed of various size aggregates, it would elute as different size peak complexes, and the eluting peaks would be detected at different retention times. The larger size aggregates would move fastest through the gel bed and elute first. The smaller molecules, which can enter the gel pores, would be retarded and move more slowly through the gel bed, and be eluted later.

From the chromatography we can see that pure LPS was composed of different size aggregates. The pure LPS eluted as several peaks, detected at different retention

times. When LPS alone was chromatographed on TEK-GEL G2000SWXL, at the concentrations employed, detected by absorbance at 260 nm, two peaks were detected. The peak eluted at 6.9 min was the first peak group, another peak eluted at 9.3 min was the second peak group (Figure 4.5). Further regardless of pre-incubation or not, pure LPS peaks were the same.

4.3.2 Pure Protein Study

Using the ultrafiltration membrane method, BSA and LPS interaction was studied in the previous chapter. The study showed that endotoxin was disaggregated by BSA. According to that study, when pure BSA solutions were filtered by 300,000 nominal molecular weight cut-off (NMWCO) membrane, and the filtrates tested with LAL method, the test gave negative results. First, this indicated that pure BSA was endotoxin free; second, it showed that BSA molecules were small enough to pass through the membrane. When pure LPS solution was filtered through a 300,000 NMWCO membrane and tested with LAL method, the LAL test gave negative result. This indicated that LPS was too large to pass through the membrane, so no LPS was detected in the membrane filtrate. But when BSA and LPS mixtures were filtrated with 300,000 NMWCO membranes and tested with LAL method, the LAL test gave positive results at certain BSA/LPS ratio. This indicated LPS was disaggregated by BSA and passed through the membrane. To further investigate how LPS size changed after disaggregation by BSA, size exclusion liquid chromatography (SE-HPLC) method was developed on the basis of previous qualitative studies.

To investigate how LPS is disaggregated by BSA, it is essential to know BSA status before it is mixed with LPS. As described above, BSA preparations at various concentrations were injected for HPLC assay (Table 4.2).

From the chromatogram at Figure 4.6 we can see, first, at the concentrations of 4, 2, 1 and 0.5 mg/ml, pure BSA eluted at 8.1 min and 9.7 min. At concentration of 0.25 mg/ml, pure BSA peak only was detected at 9.7 min at absorbance 260 nm, as shown in Figure 4.5. Second, regardless of pre-incubation or not, pure BSA peaks were the same.

4.3.3 Endotoxin Disaggregation by BSA

From the previous chapter, it is known that BSA and endotoxin have interaction. Using ultrafiltration membrane method, it can be seen that endotoxin was disaggregated by BSA. Such disaggregated endotoxin could pass through 300,000 molecular weight cut off membrane and was detected by gelation L*imulus Ambocyte Lysate* (LAL) test. But such quantitative study can not demonstrate 1) endotoxin existing form changed after such disaggregation 2) endotoxin disaggregation rate 3) endotoxin disaggregation completeness.

Using size exclusion high performance chromatography (SE-HPLC) to demonstrate BSA interaction with LPS, advantage was taken of the significant differences in molecular weight of these two molecules, Whereas LPS exists as an aggregated macromolecular structure with molecular weight of the order of 10^6 or greater, BSA is a medium molecular mass protein with a molecular mass of about 67,000 Da. These molecules are readily separable on columns of TSK-GEL G2000SWXL (Tosohaas Co., Montgomeryville, PA). When LPS alone was chromatographed using TEK-GEL

G2000SWXL, at the concentrations employed was detected by absorbance at 260 nm, two peaks were eluted. The peak eluted at 6.9 min was the first peak group and another eluted at 9.3 min was the second peak group, as shown in Figure 4.6. When BSA was run individually, it eluted at 8.1 min and 9.7 min, at the concentration 4, 2, 1 and 0.5 mg/ml. While at concentration 0.25 mg/ml, the eluted peak at 8.1 min was very small.

As described above, albumin disaggregated endotoxin study was performed at a BSA to endotoxin concentration ratio of 16 (Table 4.4), that is, BSA concentration in the mixture solution was 4 mg/ml, endotoxin concentration in the mixture solution was 0.25 mg/ml. From the chromatogram of pure LPS, pure BSA, BSA and LPS mixture (Figure 4.7), it can be seen, comparing with the pure endotoxin profile, the peak group 1 of the mixture partially shifted from retention time 6.9 min to a longer retention time; the peak group 1 area of the mixture decreased distinctly (Figure 4.12, Figure 4.13, Figure 4.14); the peak group 2 area of the mixture increased (Figure 4.15); the peak group 3 area of the mixture increased (Figure 4.16); and a new peak group, which was named peak group 4, formed only in the mixture solution (Figure 4.17)

First, from the result it is concluded that endotoxin was disaggregated by BSA. The disaggregation led to the formation of smaller size endotoxin fragments. Because size exclusion chromatography separates molecules in decreasing order of molecular size, Those fragments eluted at longer retention time. So from the chromatography profile and analyzing figures, one can see peak area of peak group 1 decreased, while peak area of peak group 2 and 3 increased. Furthermore, due to such disaggregation, a smaller size peak group, which was named peak group 4, formed.

Second, from these results, it is concluded that such BSA and endotoxin interaction caused BSA and endotoxin binding. Pure BSA has a peak at 9.7 min. Pure endotoxin has a peak at 9.3 min. The two peaks can not be separated by the column employed. So we totaled the peak area of peak group 3 of pure BSA and pure endotoxin, and compared it with peak group 3 area of BSA and endotoxin mixture. Comparing peak area sum of pure BSA and pure endotoxin with BSA and endotoxin mixture (Figure 4.16), we can see the peak area of BSA and endotoxin mixture is larger than area sum of pure BSA and pure endotoxin. Therefore we concluded that the BSA and endotoxin interaction led to BSA and endotoxin binding. Another experiment, which was carried out at without incubation condition for the same BSA and endotoxin concentration, also assured such conclusion (Figure 4.23).

Third, from the result we can see peak 1 of pure endotoxin was the major peak that was disaggregated (Figure 4.7, Figure 4.18). After the disaggregation, the peak area of the BSA and endotoxin mixture was only 17.2% of the original pure endotoxin peak area (Figure 4.29). Also we can see peak 1 was not completely disaggregated, some remaining part still can be found under the experiment conditions (Figure 4.7, Figure 4.14, Figure 4.29).

4.3.4 Effect of BSA Concentration on Endotoxin Disaggregation

To examine the question further, BSA concentration was varied in BSA and endotoxin mixture solutions to investigate the effect of BSA concentration on endotoxin disaggregation.

From the previous chapter, it is known that BSA concentration has effects on BSA and endotoxin interaction. Using ultrafiltration membrane method, one can see that as BSA concentration increased in the BSA and endotoxin mixture solutions, more endotoxin was disaggregated by BSA. Therefore more disaggregated endotoxin passed through 300,000 nominal molecular weight cut off (NMWCO) membrane. As the amount of endotoxin passing through the membrane above the detection limit of gelation *Limulus Amebocyte Lysate* (LAL) test, the endotoxin can be detected. This study can not demonstrate 1) at different BSA concentrations in the BSA-endotoxin mixture, what is the difference between endotoxin aggregate sizes? 2) at what BSA concentration in the BSA and endotoxin mixture, or at what BSA and endotoxin concentration ratio, endotoxin is disaggregated significantly.

First, from the chromatography profile (Figure 4.8, Figure 4.9) one can see that at different BSA concentrations in the BSA-endotoxin mixture solutions, or at different BSA/LPS concentration ratios in the mixture solutions, the peak area changes for each peak group were different. The more BSA concentration in BSA and endotoxin mixture, or the higher the BSA/LPS concentration ratio, the smaller the peak group 1 area (Figure 4.12, Figure 4.13, Figure 4.14), the larger the peak group 2 area (Figure 4.15), the larger the peak group 3 area (Figure 4.16), and the larger the peak group 4 area (Figure 4.17). ΔS is defined as peak area of BSA and LPS mixture minus corresponding peak area sum of pure BSA and pure LPS. $\Delta S = S_{mixture} - \Sigma S_{pure.}$ As shown in Figure 4.18, when BSA concentration increased, ΔS of PG1 decreased, ΔS of PG2, PG3 and PG4 increased. The peak area percentage was defined as percentage of mixture peak area divided by sum peak area of pure BSA and pure endotoxin. For instance, at BSA concentration 0.25, 0.5,

1, 2, 4 mg/ml in the mixture, the peak area percentage of peak group 1 is 72.3, 42.1, 18.4, 15.2 and 17.2% respectively (Figure 4.29). Those results showed as BSA concentration increased, more LPS was disaggregated, more smaller size endotoxin fragments formed, therefore peak area of peak group that eluted at shorter retention time decreased, for example, peak group 1. Peak area of peak group that eluted at longer retention time increased, for example, peak group2, 3 and 4.

Also, from the results we can see when BSA concentrations were 2 mg/ml and 4 mg/ml in the mixture solution, or at BSA/LPS concentration ratio of 8 and 16, the LPS disaggregation was significant. From the above study it is known that more LPS was disaggregated by BSA, the greater mixture peak group 1 area decreased, the higher was the mixture peak group 4.

As defined above, the peak area percentage is mixture peak area divided by peak area sum of pure BSA and pure endotoxin, then multiplied by 100. For peak group 1, when BSA at 0.25 and 0.5 mg/ml, the peak area percentage is 72.3% and 42.1%, as BSA concentration increased to 2 and 4 mg/ml, the peak area percentage decreased to 15.2% and 17.2% (Figure 4.29).

For peak group 4, at BSA concentration at 0.25 mg/ml, if assumed peak area as 1, when BSA concentration increased to 2 and 4 mg/ml, the peak area was 53.0 and 63.2. This means that, as BSA concentration increased, the peak group 4 area increased by factor of 53 and 63 times respectively.

4.3.5 Effect of Sample Pre-Incubation on Endotoxin Disaggregation

The experiment was designed to investigate pre-incubation influence on BSA and endotoxin interaction. As described above, the study was performed at various BSA and endotoxin concentration ratio as shown in Table 4.4. In contrast, a set of control experiments without incubation was carried out. All the experiment procedures were the same except no sample incubation was done before HPLC assay.

As described in 'Endotoxin Disaggregation by BSA', after the endotoxin disaggregation by BSA, the peak group 1 of the mixture partially shifted from retention time 6.9 min to longer retention time. The peak group 1 area of the mixture decreased distinctly (Figure 4.8, Figure 4.9, Figure 4.14). The peak group 2 area of the mixture increased (Figure 4.15). The peak group 3 area of the mixture increased (Figure 4.16). New peak group, which was peak group 4, formed only in mixture solutions (Figure 4.17, Figure 4.12, Figure 4.13).

Also from Section 4.3.4, it is known that more endotoxin was disaggregated in BSA and endotoxin mixture, the smaller the peak group 1 area (Figure 4.29, Figure 4.14), the larger the peak group 2 area (Figure 4.15), the larger the peak group 3 area (Figure 4.16), the larger the peak group 4 area (Figure 4.17, Figure 4.12, Figure 4.13).

Comparing the chromatography profiles with and without incubation at each BSA/LPS concentration ratio (Figure 4.8, Figure 4.9, Figure 4.10, Figure 4.11), it is clear that incubation will increase the LPS disaggregation. Under incubation condition, peak group 1 area is smaller than that without incubation (Figure 4.25), and the peak percentage is smaller than that without incubation (Figure 4.29). For example, at BSA/LPS concentration ratio equaled to 1, without incubation, the peak percentage of

peak group 1 was 98.1%; while with incubation, the peak percentage of peak group 1 was 72.3% (Figure 4.29). That means that more LPS was disaggregated with incubation. Peak group 2 and 3 area was larger than that without incubation, that means that more LPS was disaggregated and formed binding with BSA (Figure 4.26, Figure 4.27). Also only with incubation, a new peak group, peak group 4, formed (Figure 4.28, Figure 4.12, Figure 4.13). In contrast, without incubation, no peak group 4 existed.

From ΔS, which is defined as peak area of BSA and LPS mixture minus corresponding peak area sum of pure BSA and pure LPS, $\Delta S = S_{mixture} - \Sigma S_{pure.}$ As shown in Figure 4.18 and Figure 4.24, when BSA concentration increased, at with pre-incubation condition, ΔS of PG1 is less, and ΔS of PG2, PG3 and PG4 are larger than that without incubation.

From the results of LPS disaggregation without incubation, it is also noticed that LPS disaggregation started right after endotoxin was mixed with BSA, no matter whether the BSA and LPS mixture was incubated or not. Without incubation, such disaggregation is not obvious at low BSA concentration, i. e. 0.25, 0.5, 1 mg/ml, but as protein concentration increased in the mixture solutions, LPS disaggregation is easily detectable. Without incubation, LPS disaggregation is mainly due to the disaggregation of peak group 1. With incubation, disaggregation is detectable even at low BSA concentrations. This fact, from another view, gave evidence that incubation improved LPS disaggregation.

4.3.6 Discussion

The mechanism of BSA and endotoxin interaction is not clear yet. BSA is a basic protein (pI < 7). Some studies suggested that interaction between basic protein and endotoxin involves hydrophobic interaction. Fatty acid binding domains might be involved also (David et al., 1995).

Due to the phosphate group in endotoxin molecule, at pH above 2, the endotoxin is always negatively charged. The investigation by Lipka et al (Lipka, et al 1988) on the phase behavior of aqueous dispersions of lipid X, a pyrogen precursor, indicated the critical micellar concentration to be 4×10^{-5} mol/L in a physiological saline solution. The two pK values are $pK_1 = 1.3$ and $pK_2 = 8.2$. The sugar phosphoryl moieties account for the low pH value. The long-chain alkyl groups in lipid A, as the hydrophobic tail, cause micelle formation and molecular aggregation. (Malchesky et al., 1995). Normally endotoxin exists in vesicles. Under the hydrophobic interaction, the vesicles are broken into micelles.

4.4 Conclusions

The results of this study have shown that bovine serum albumin can disaggregate endotoxin. Such disaggregation was affected by BSA concentration. As BSA concentration increased, more endotoxin was disaggregated, and more smaller size endotoxin fragments were formed. Also pre-incubation of BSA and endotoxin mixture facilitated such endotoxin disaggregation. At the same BSA concentration in the mixture, pre-incubation caused more endotoxin to be disaggregated.

The results are significant for endotoxin removal process design. For endotoxin removal design, not only should solution composition be considered, but also concentration of each component be taken into account. Especially for membrane process or chromatography process taking advantage of endotoxin molecular size, from this study, it is clear that at different concentration composition, the endotoxin aggregate size will vary. Such endotoxin size changes will affect endotoxin removal significantly.

Table 4.1 BSA stock solution preparation.

No	BSA concentration (mg/ml)	Original BSA solution	Endotoxin-free 0.15 M NaCl solution volume (ml)	*Dilution time*
1	8	1.6 ml 10mg/ml BSA solution	0.4	1.25
2	4	1 ml of (1)	1	2
3	2	1 ml of (2)	1	2
4	1	1 ml of (3)	1	2
5	0.5	1 ml of (4)	1	2

Table 4.2 Pure BSA solution preparation.

No	BSA concentration (mg/ml)	BSA stock solution volume (ml)	Endotoxin-free 0.15 M NaCl solution volume (ml)
1	0.25	0.5 ml BSA at 0.5 mg/ml	0.5
2	0.5	0.5 ml BSA at 1 mg/ml	0.5
3	1	0.5 ml BSA at 2 mg/ml	0.5
4	2	0.5 ml BSA at 4 mg/ml	0.5
5	4	0.5 ml BSA at 8 mg/ml	0.5

Table 4.3 BSA and endotoxin mixture solution preparation.

No	BSA stock solution volume (ml)	Endotoxin stock solution volume (ml)	Endotoxin-free 0.15 M NaCl solution volume (ml)
1	0.5 ml BSA at 0.5 mg/ml	0.25 ml LPS at 1mg/ml	0.25
2	0.5 ml BSA at 1 mg/ml	0.25 ml LPS at 1mg/ml	0.25
3	0.5 ml BSA at 2 mg/ml	0.25 ml LPS at 1mg/ml	0.25
4	0.5 ml BSA at 4 mg/ml	0.25 ml LPS at 1mg/ml	0.25
5	0.5 ml BSA at 8 mg/ml	0.25 ml LPS at 1mg/ml	0.25

Table 4.4 Composition of BSA and endotoxin mixture solutions.

No	BSA concentration in the mixture solution (mg/ml)	Endotoxin concentration in the mixture solution (mg/ml)	BSA/LPS concentration ratio
1	0.25	0.25	1
2	0.5	0.25	2
3	1	0.25	4
4	2	0.25	8
5	4	0.25	16

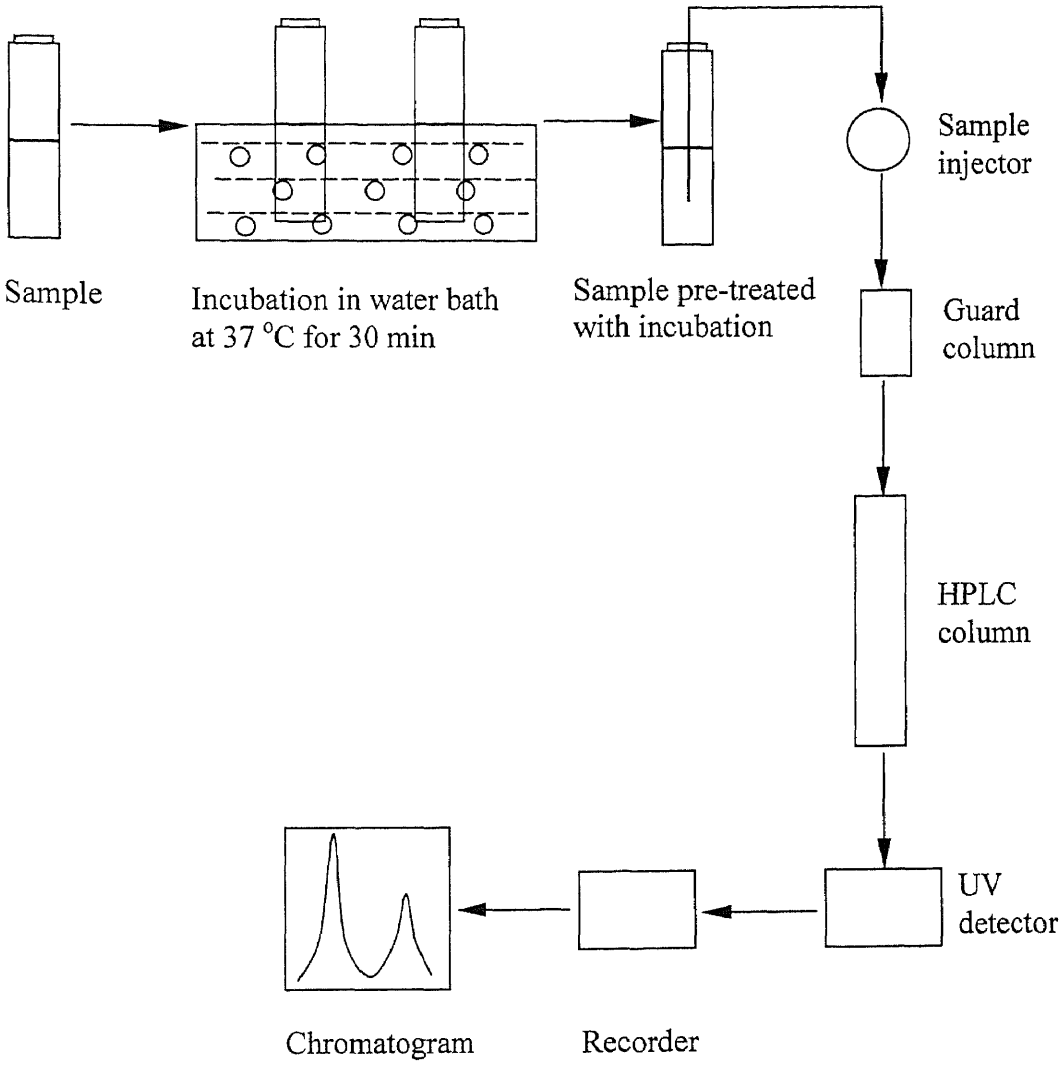


Figure 4.1 Illustration of pre-incubated sample study.

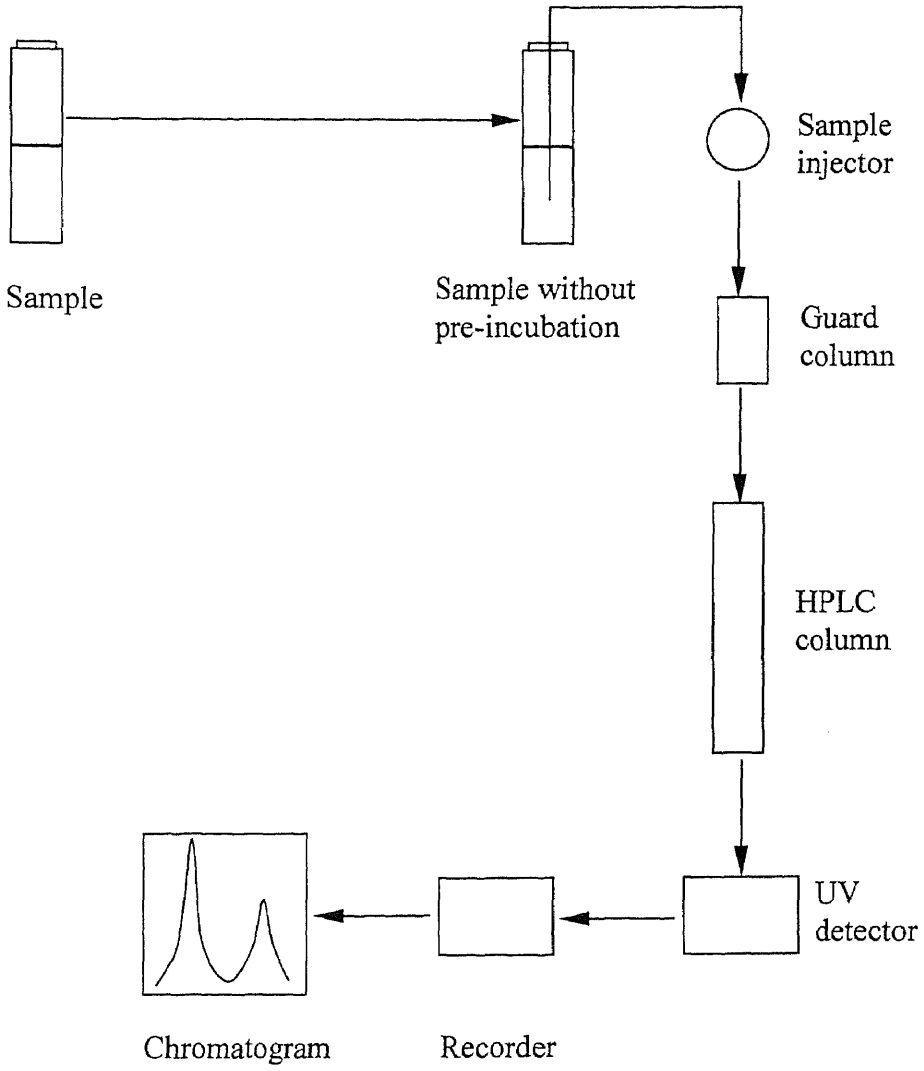
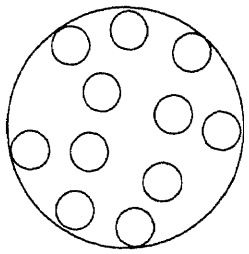


Figure 4.2 Illustration of without pre-incubated sample study.



Magnified beaded gel with pores

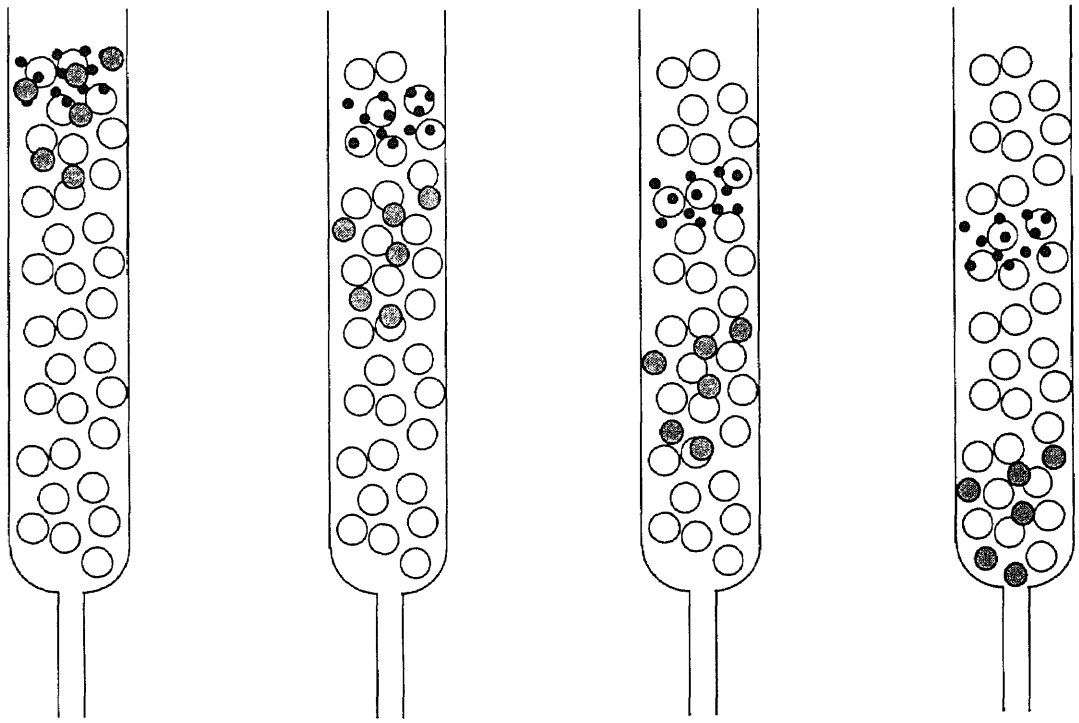


Figure 4.3 Illustration of size exclusion liquid chromatography procedure.
Size exclusion chromatograph. Open circles represent porous gel molecules; large solid circles represent molecules too large to enter the gel through the pores, and smaller solid circles represent molecules capable of entering the gel pores.

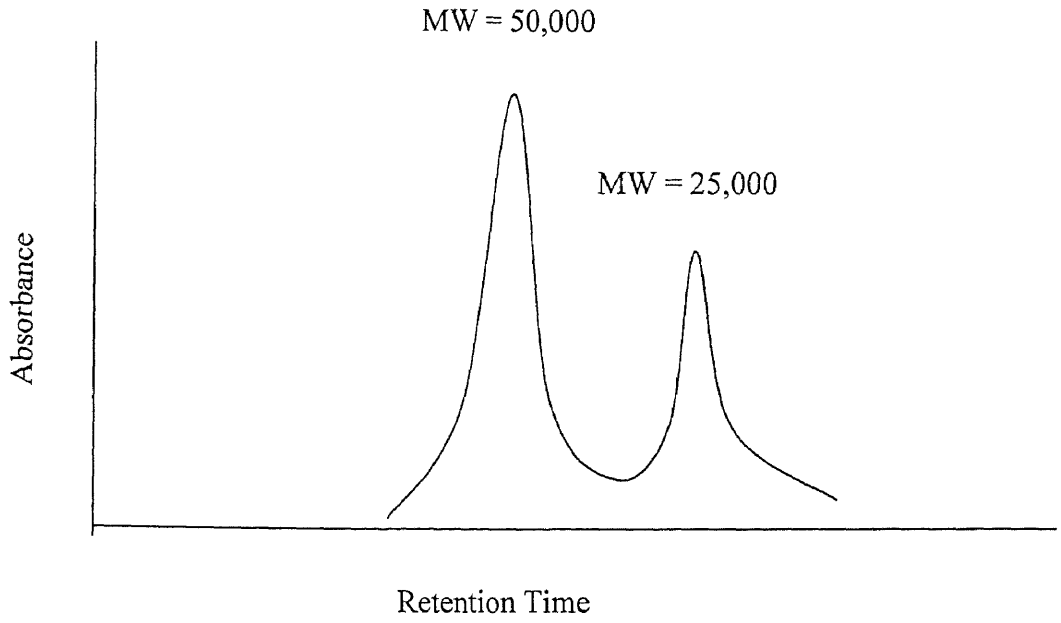


Figure 4.4 Illustration of size exclusion chromatogram.

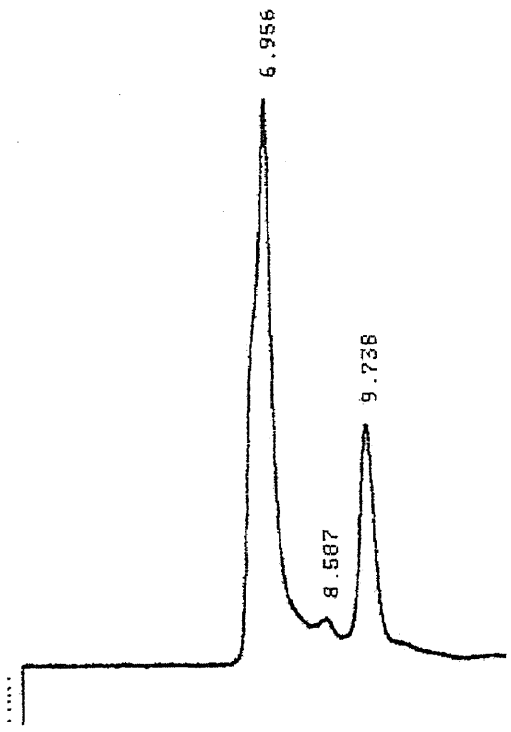


Figure 4.5 Chromatograms of pure LPS sample at a concentration of 0.25 mg/ml.
PG1: Peak group1, retention time, Tr = 6.0 –7.7 minutes.
PG2: Peak group2, retention time, Tr = 8.0 –8.8 minutes.
PG3: Peak group3, retention time, Tr = 9.3 –9.7 minutes.

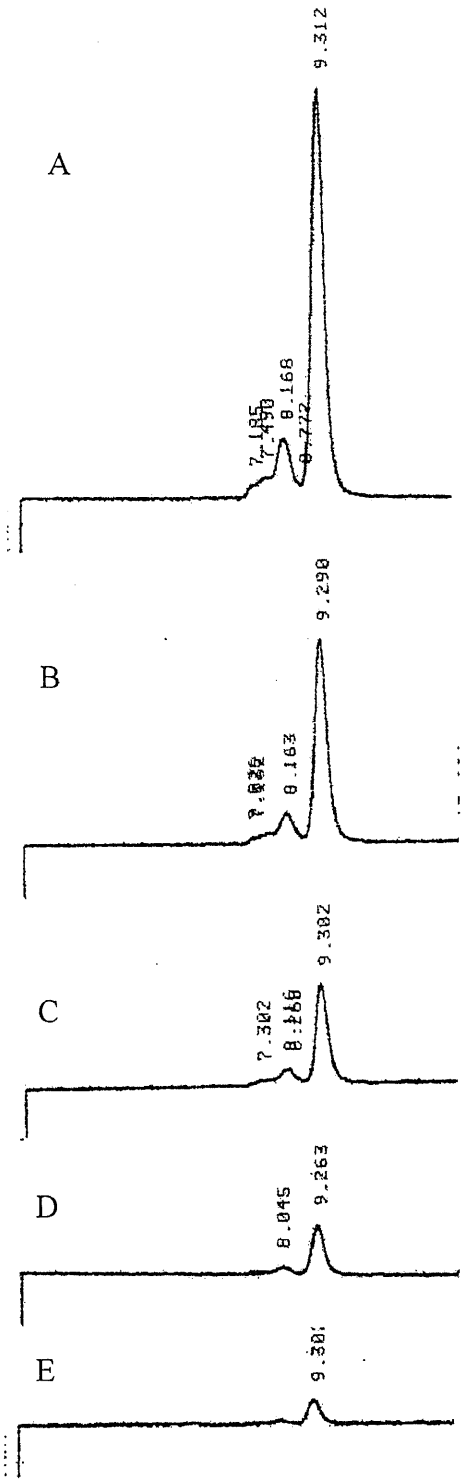


Figure 4.6 Chromatograms of pure BSA samples at various injecting concentrations. A: BSA concentration, C_{BSA}= 4 mg/ml; B: BSA concentration, C_{BSA} = 2 mg/ml; C: BSA concentration, C_{BSA} =1 mg/ml; D: BSA concentration, C_{BSA} = 0.5 mg/ml; E: BSA concentration, C_{BSA} = 0.25 mg/ml.

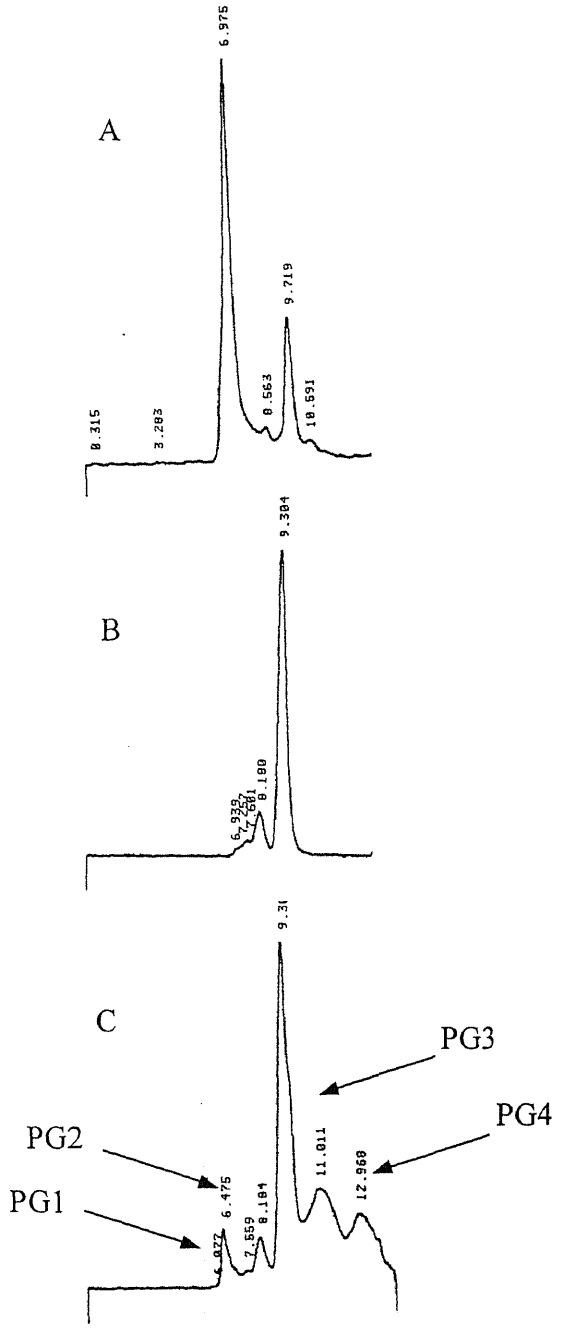


Figure 4.7 Chromatograms of LPS disaggregation by BSA (see 4.3.3).
A: Pure LPS concentration, C_{LPS} = 0.25 mg/ml;
B: Pure BSA concentration, C_{BSA} = 4 mg/ml;
C: BSA and LPS concentration ratio, C_{BSA}/C_{LPS} = 16.
PG1: Peak group1, retention time, Tr = 6.0 –7.7 minutes.
PG2: Peak group2, retention time, Tr = 8.0 –8.8 minutes.
PG3: Peak group3, retention time, Tr = 9.3 –9.7 minutes.
PG4: Peak group4, retention time, Tr = 10.5 – 12.9 minutes.

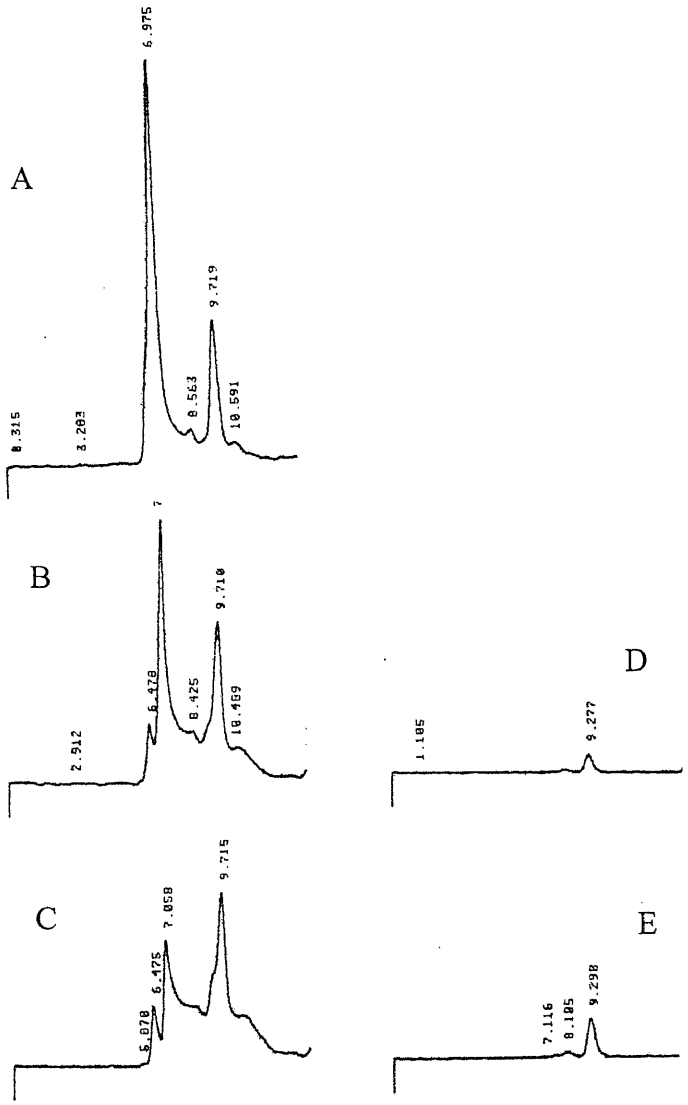


Figure 4.8 Chromatograms of BSA and LPS mixture samples, pure LPS sample and pure BSA samples at incubation condition.
A: Pure LPS concentration, C_{LPS} = 0.25 mg/ml;
B: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =1;
C: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =2;
D: Pure BSA concentration, C_{BSA} = 0.25 mg/ml;
E: Pure BSA concentration, C_{BSA}= 0.5 mg/ml.

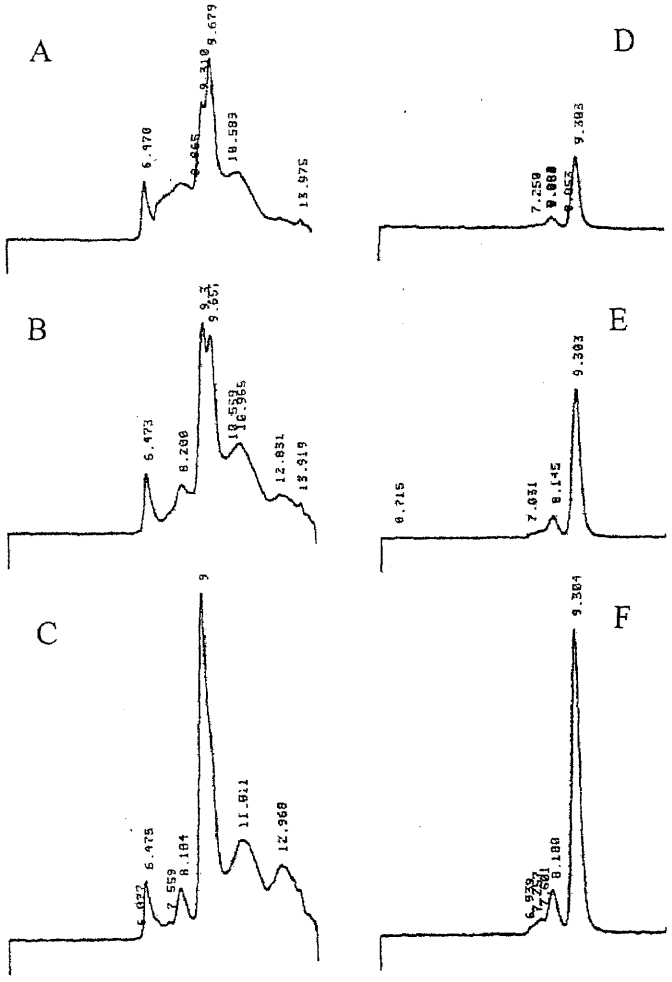


Figure 4.9 Chromatograms of BSA and LPS mixture samples, pure BSA samples at incubation condition.
A: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =4;
B: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =8;
C: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =16;
D: Pure BSA concentration, C_{BSA} = 1 mg/ml;
E: Pure BSA concentration, C_{BSA} = 2 mg/ml;
F: Pure BSA concentration, C_{BSA} = 4 mg/ml.

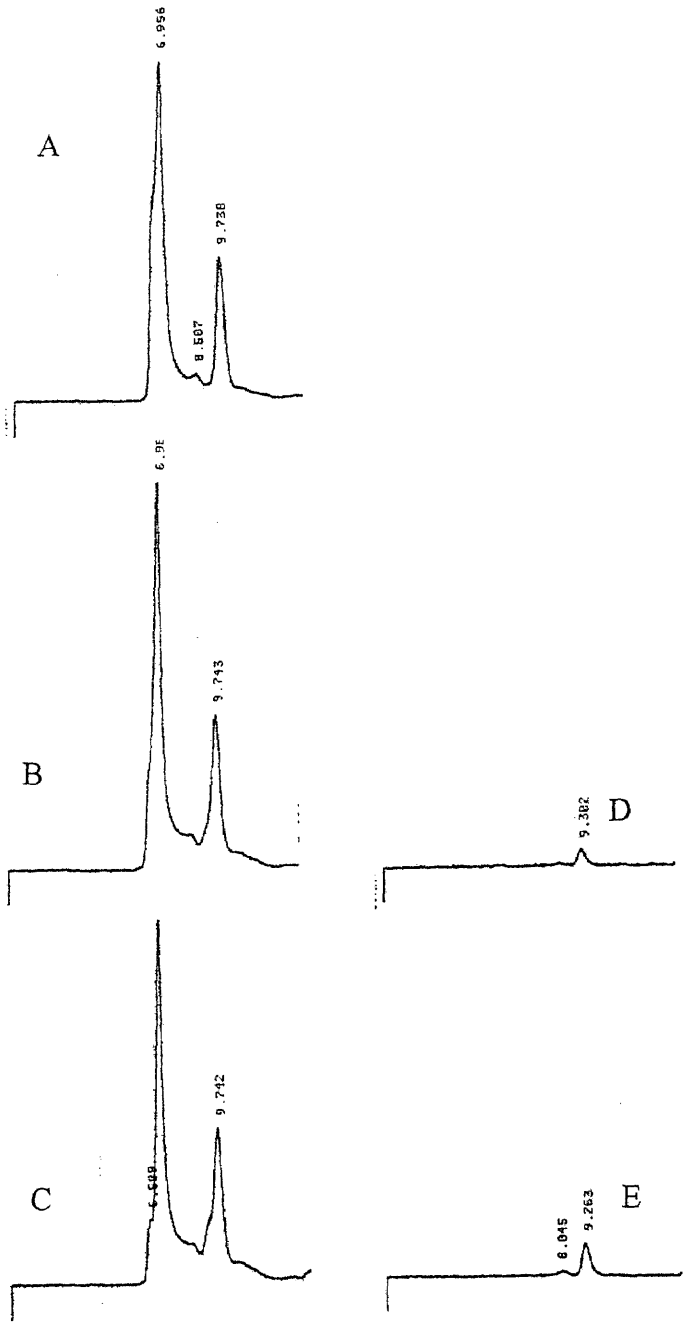


Figure 4.10 Chromatograms of BSA and LPS mixture samples, pure LPS sample and pure BSA samples at without incubation condition.
A: Pure LPS concentration, C_{LPS} = 0.25 mg/ml;
B: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =1;
C: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =2;
D: Pure BSA concentration, C_{BSA} = 0.25 mg/ml;
E: Pure BSA concentration, C_{BSA}= 0.5 mg/ml.

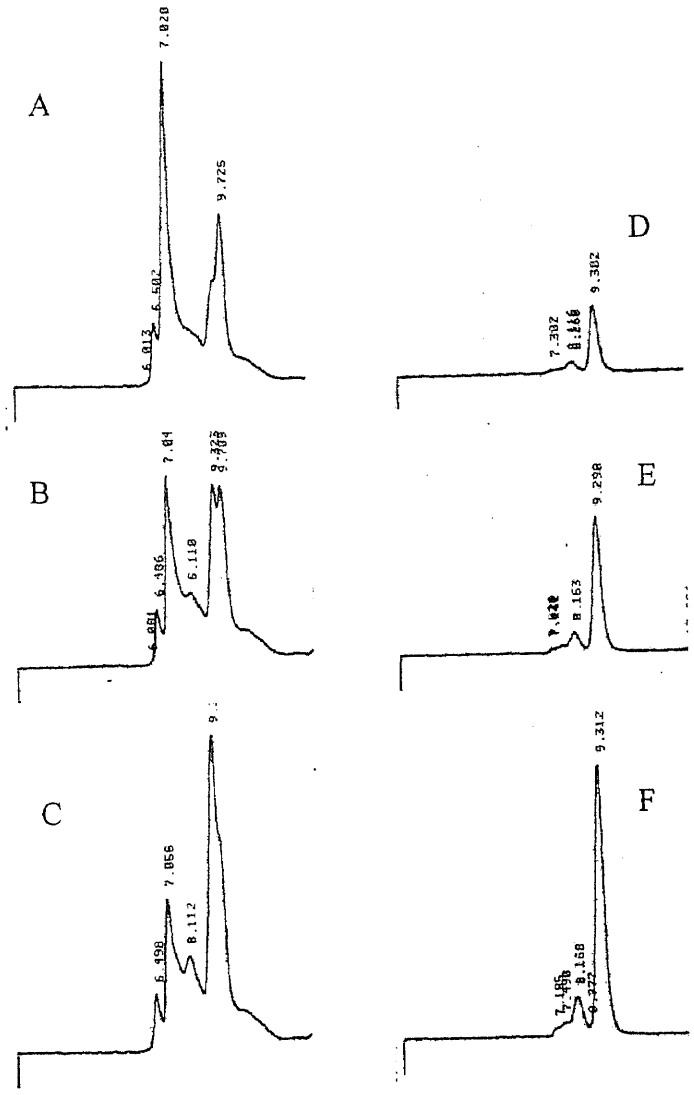


Figure 4.11 Chromatograms of BSA and LPS mixture samples, pure BSA samples at without incubation condition.
A: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =4;
B: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =8;
C: BSA and LPS mixture at concentration ratio, C_{BSA}/C_{LPS} =16;
D: Pure BSA concentration, C_{BSA} = 1 mg/ml;
E: Pure BSA concentration, C_{BSA} = 2 mg/ml;
F: Pure BSA concentration, C_{BSA} = 4 mg/ml.

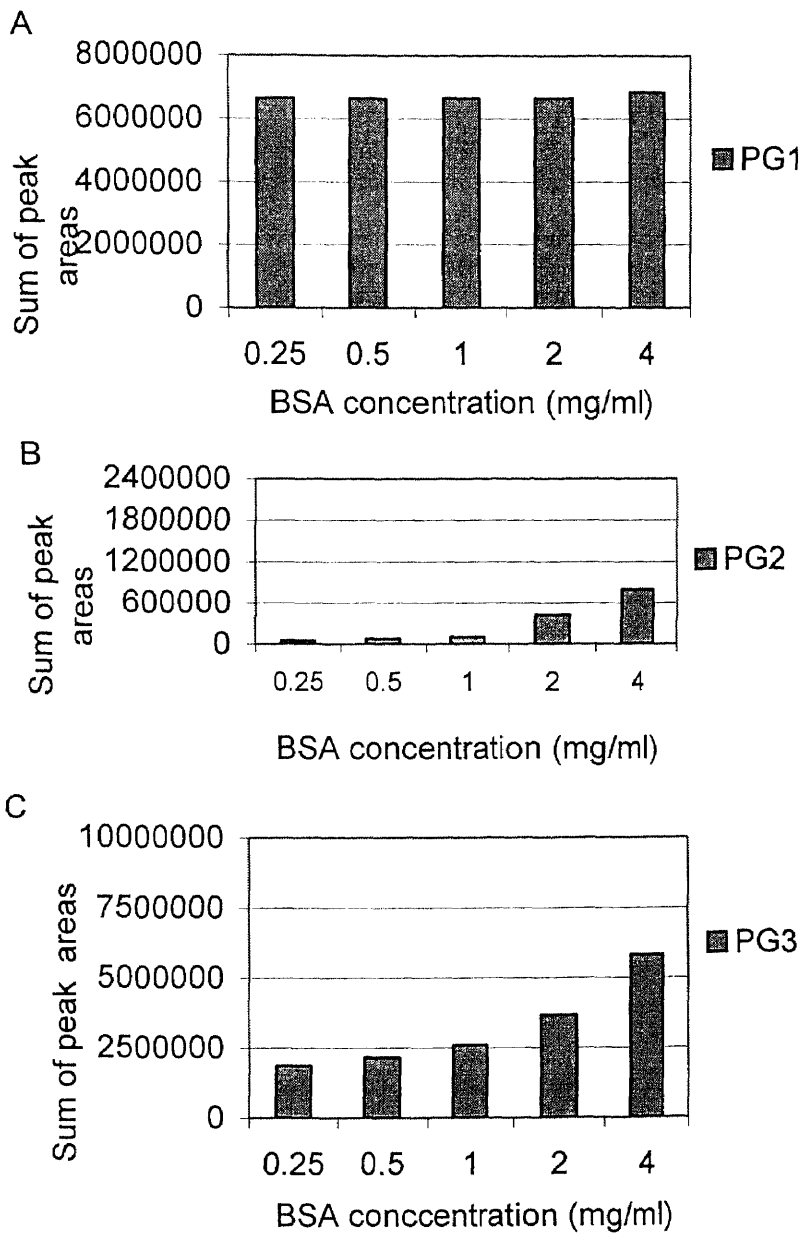


Figure 4.12 Sum of peak areas at specific elution periods for samples prepared with incubation. A peak area sum was calculated by summing up the total peak areas of corresponding peak groups in both pure LPS and pure BSA chromatograms. A: peak group 1, PG1, corresponding to elution period, $T_r = 6.0 - 7.7$ min. B: peak group 2, PG2, corresponding to elution period, $T_r = 8.3 - 8.8$ min. C: peak group 3, PG3, corresponding to elution period $T_r = 9.3 - 9.7$ min.

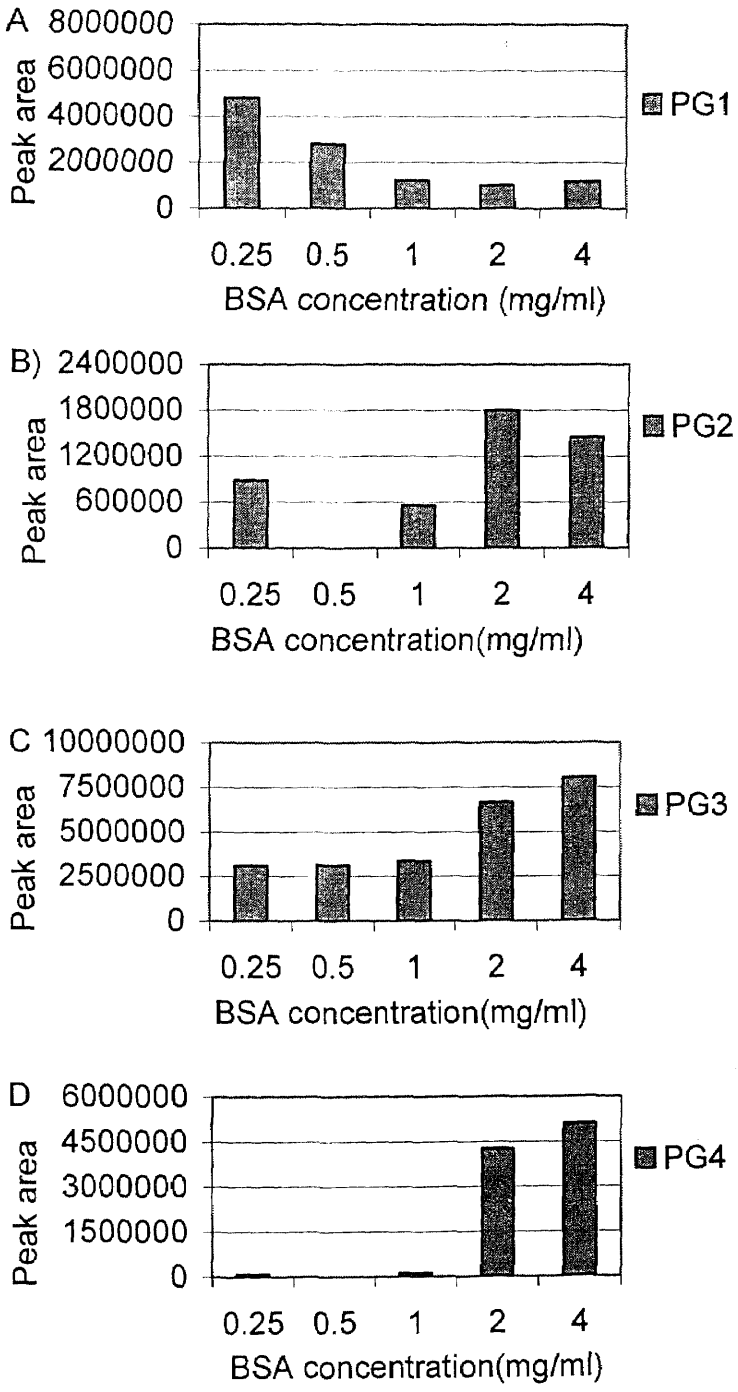


Figure 4.13 Peak areas at specific elution periods for BSA-LPS mixtures prepared with incubation. A: peak group 1, PG1, corresponding to elution period, T_r = 6.0-7.7 min. B: peak group 2, PG2, corresponding to elution period, T_r = 8.3-8.8 min. C: peak group 3, PG3, corresponding to elution period, T_r = 9.3-9.7 min. D: peak group 4, PG4, corresponding to elution period, T_r =10.5 – 12.9 min.

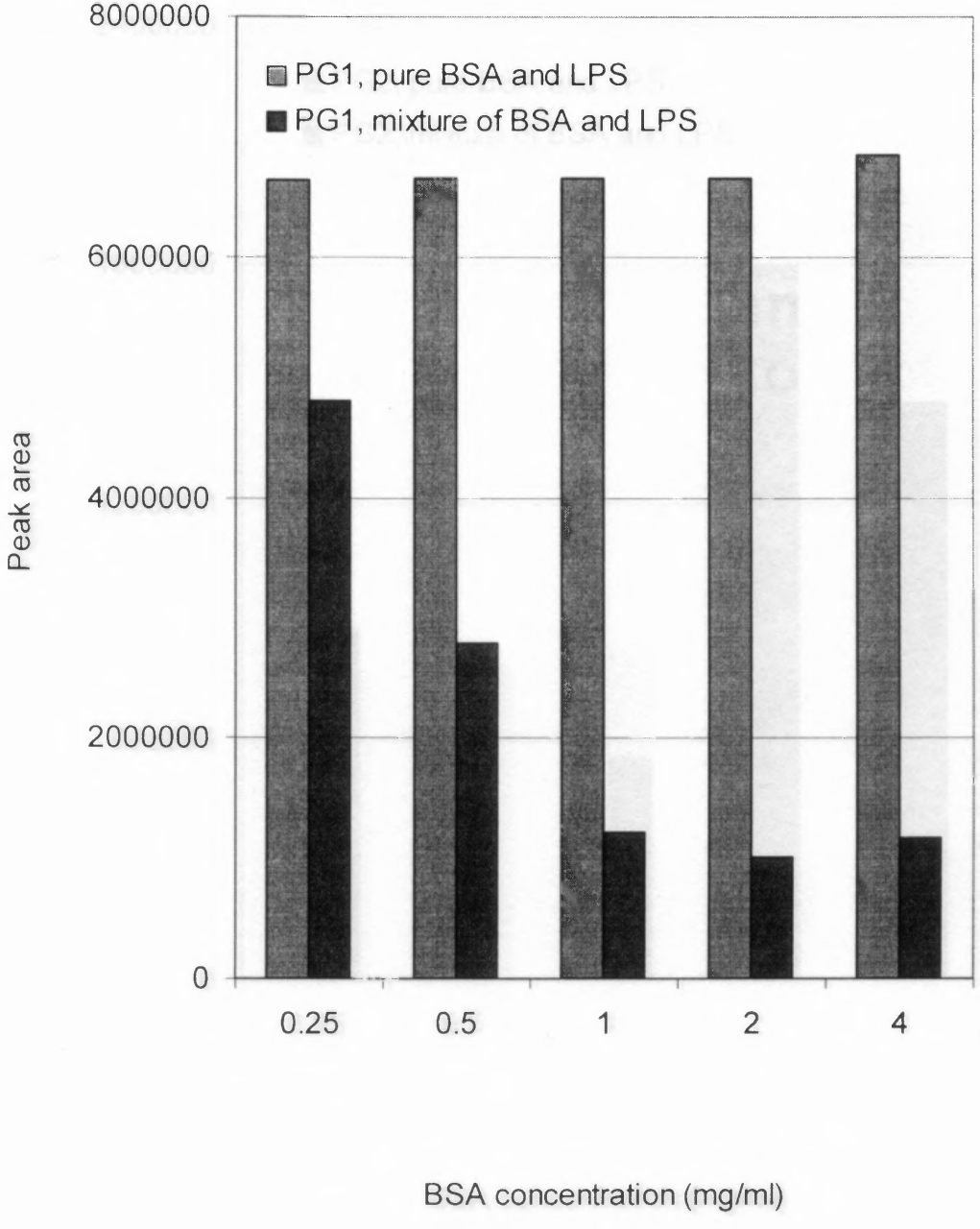


Figure 4.14 Peak area comparison of PG1 with incubation.

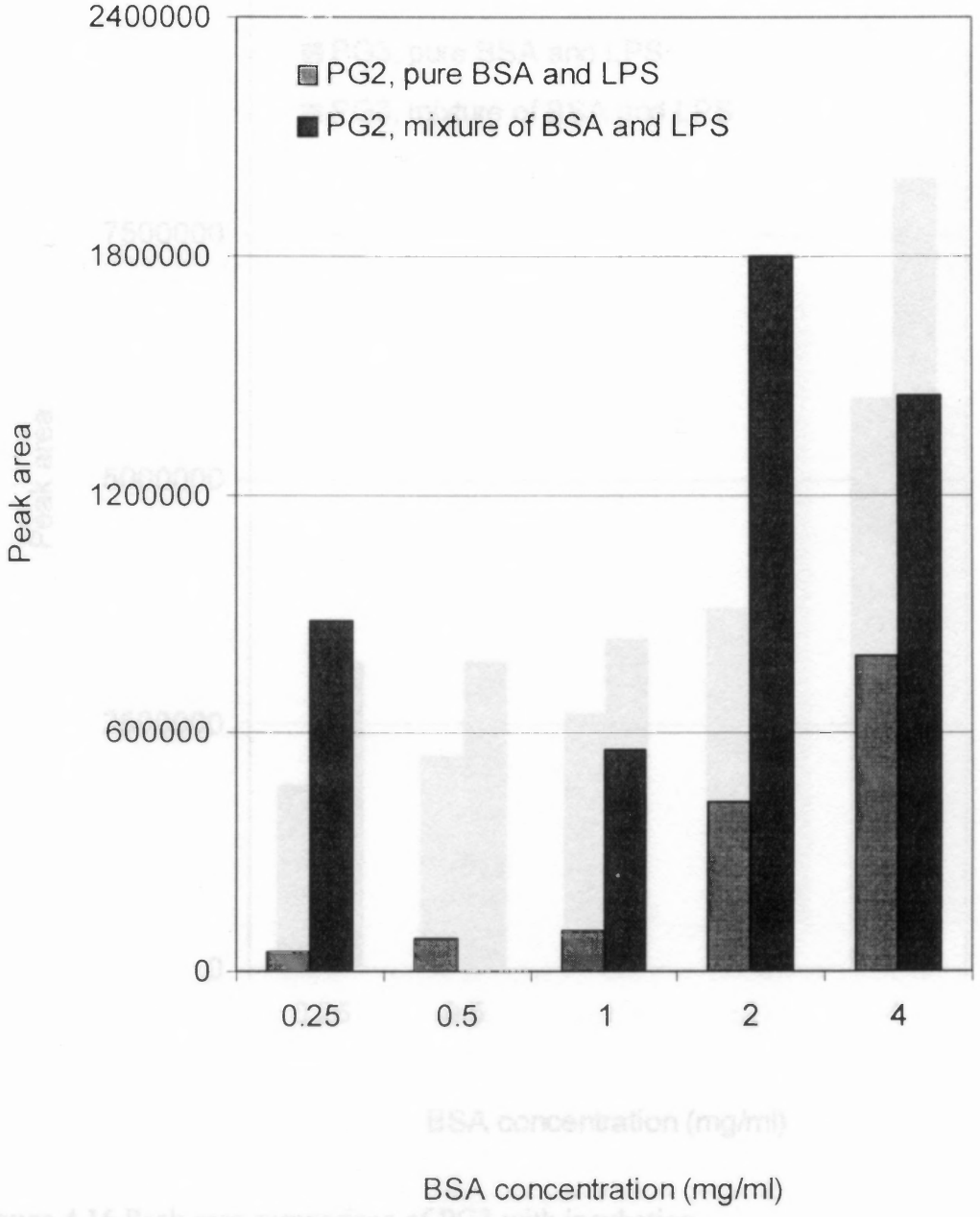


Figure 4.15 Peak area comparison of PG2 with incubation.

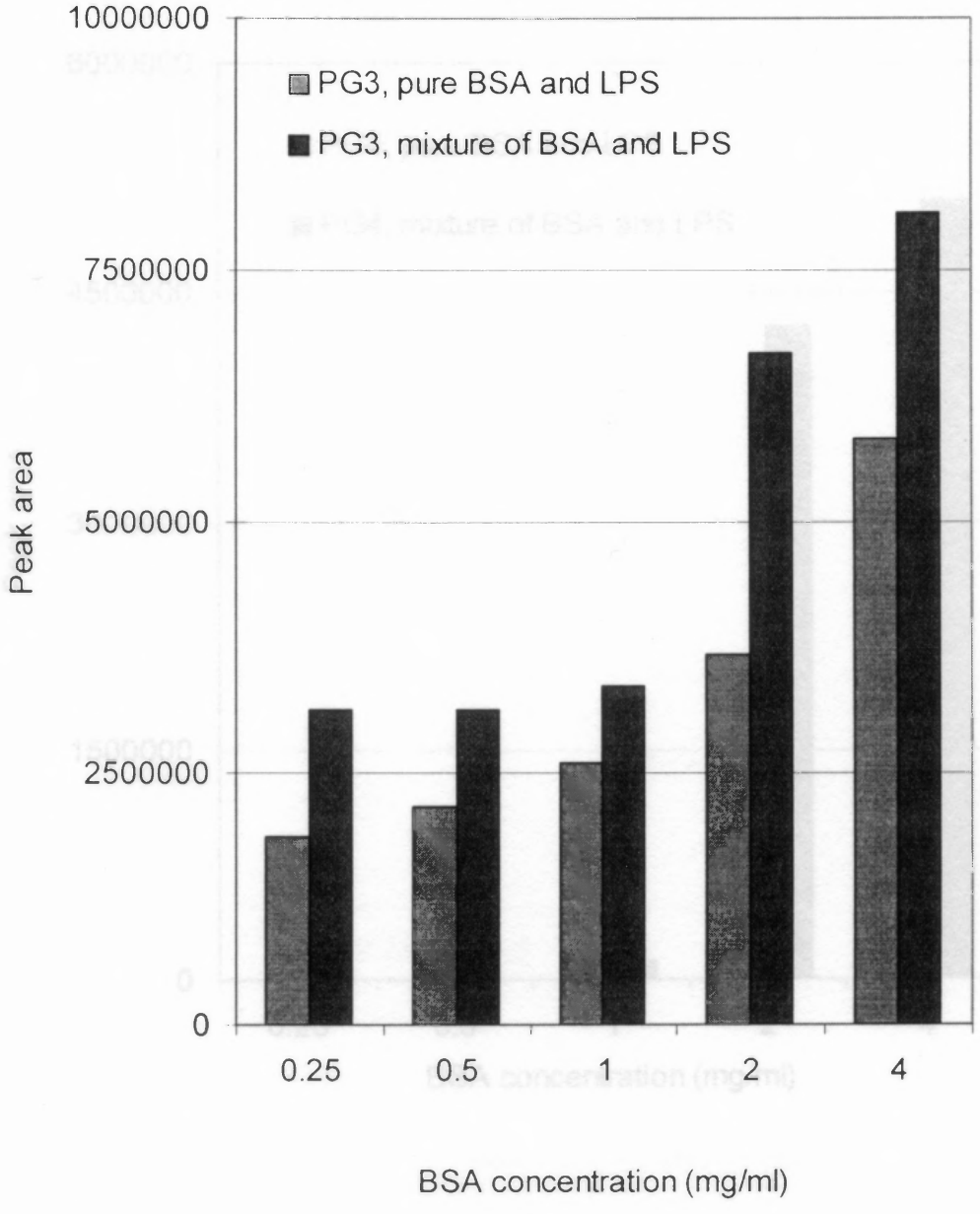


Figure 4.16 Peak area comparison of PG3 with incubation.

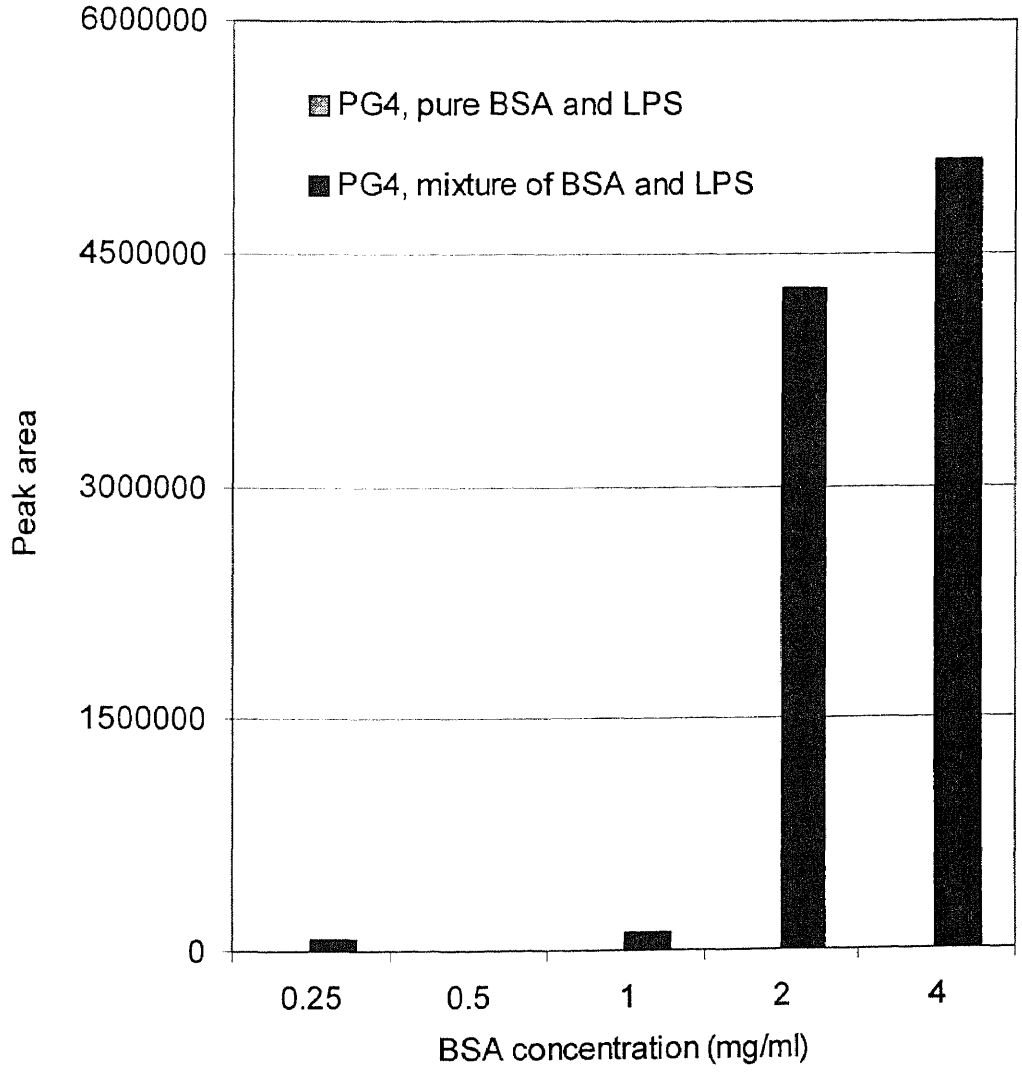


Figure 4.17 Peak area comparison of PG4 with incubation.

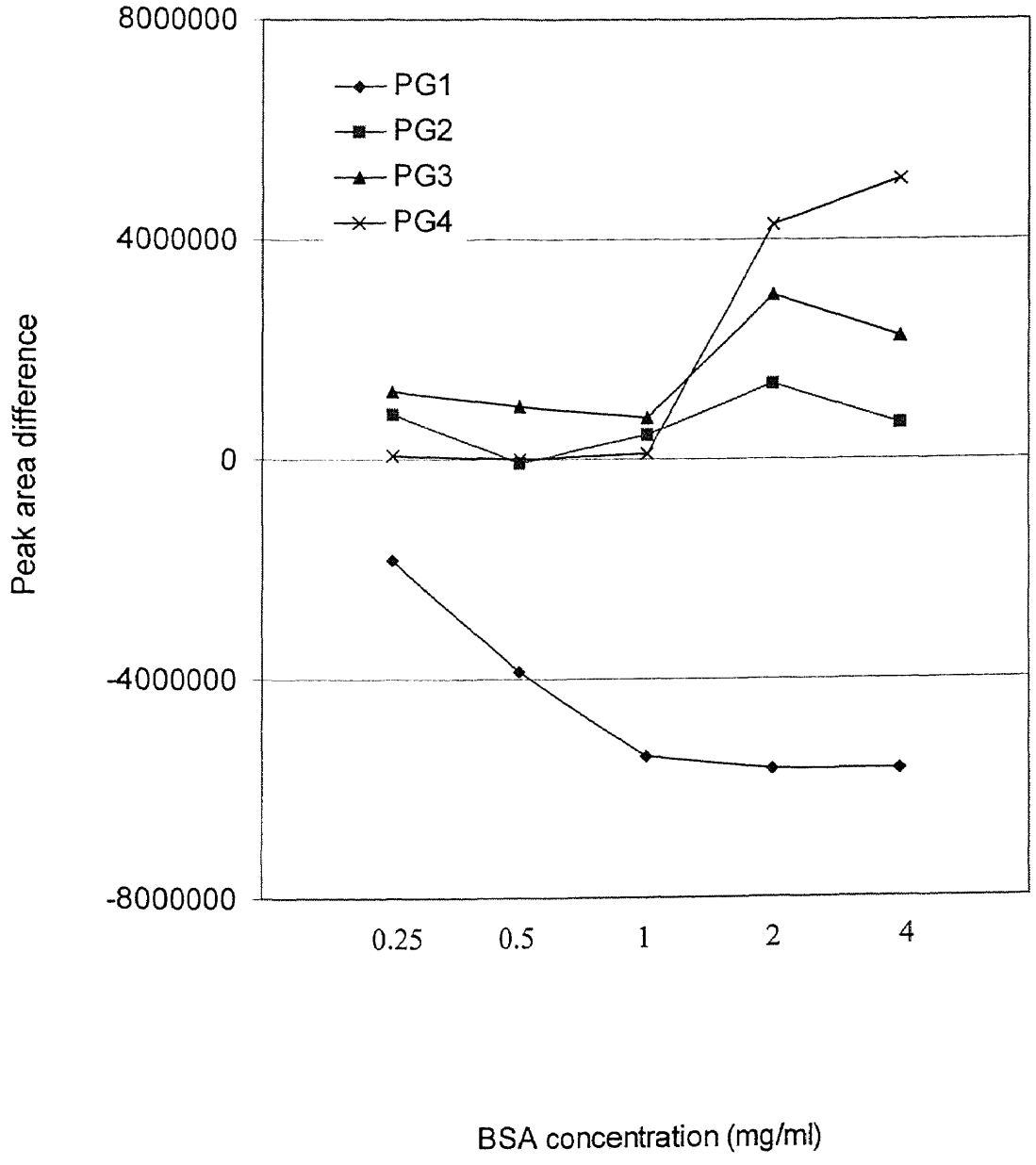


Figure 4.18 Δ S of PG1, PG2, PG3 and PG4 with incubation.

Δ S= Peak area of BSA and LPS mixture – Corresponding sum peak area of pure BSA and pure LPS

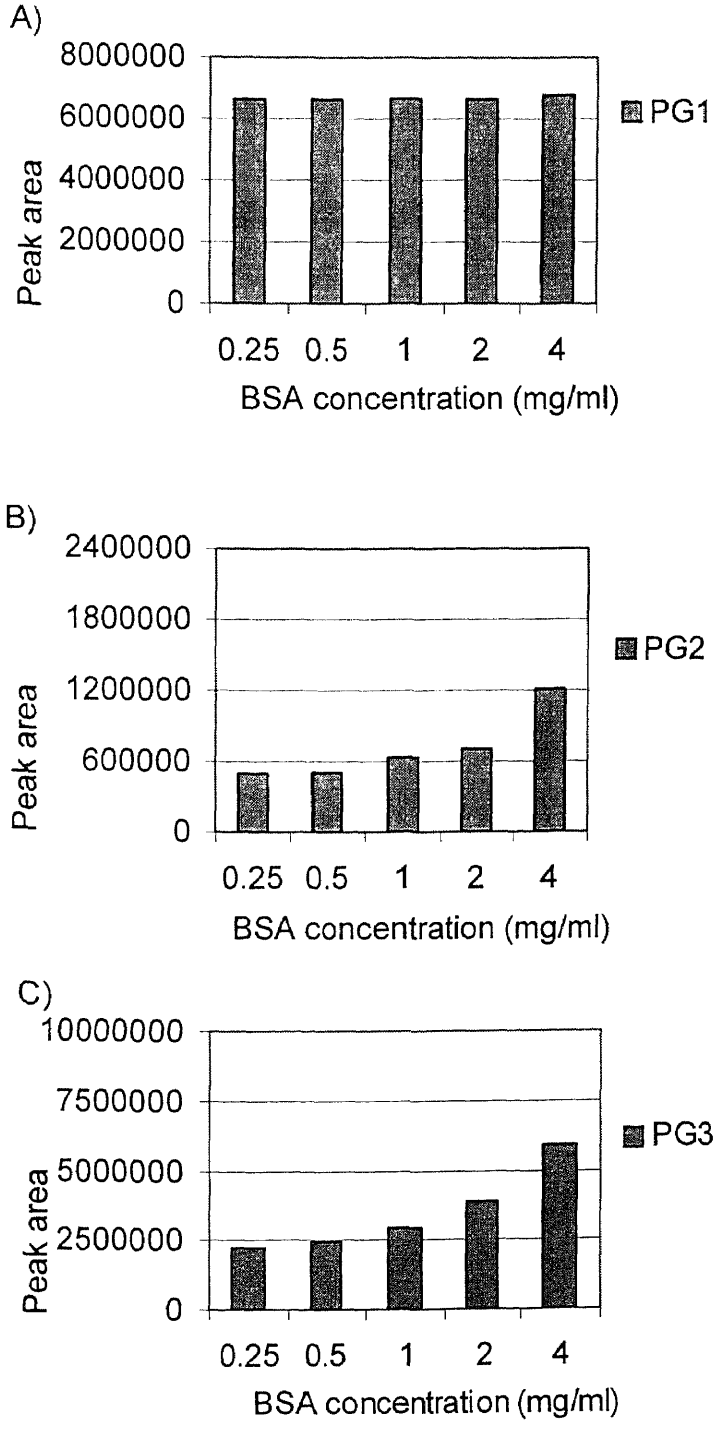


Figure 4.19 Sum of peak areas at specific elution periods for samples prepared without incubation. A peak area sum was calculated by summing up the total peak areas of corresponding peak groups in both pure LPS and pure BSA chromatograms. A: peak group 1, PG1, corresponding to elution period, $T_r = 6.0 - 7.7$ min. B: peak group 2, PG2, corresponding to elution period, $T_r = 8.3 - 8.8$ min. C: peak group 3, PG3, corresponding to elution period $T_r = 9.3 - 9.7$ min.

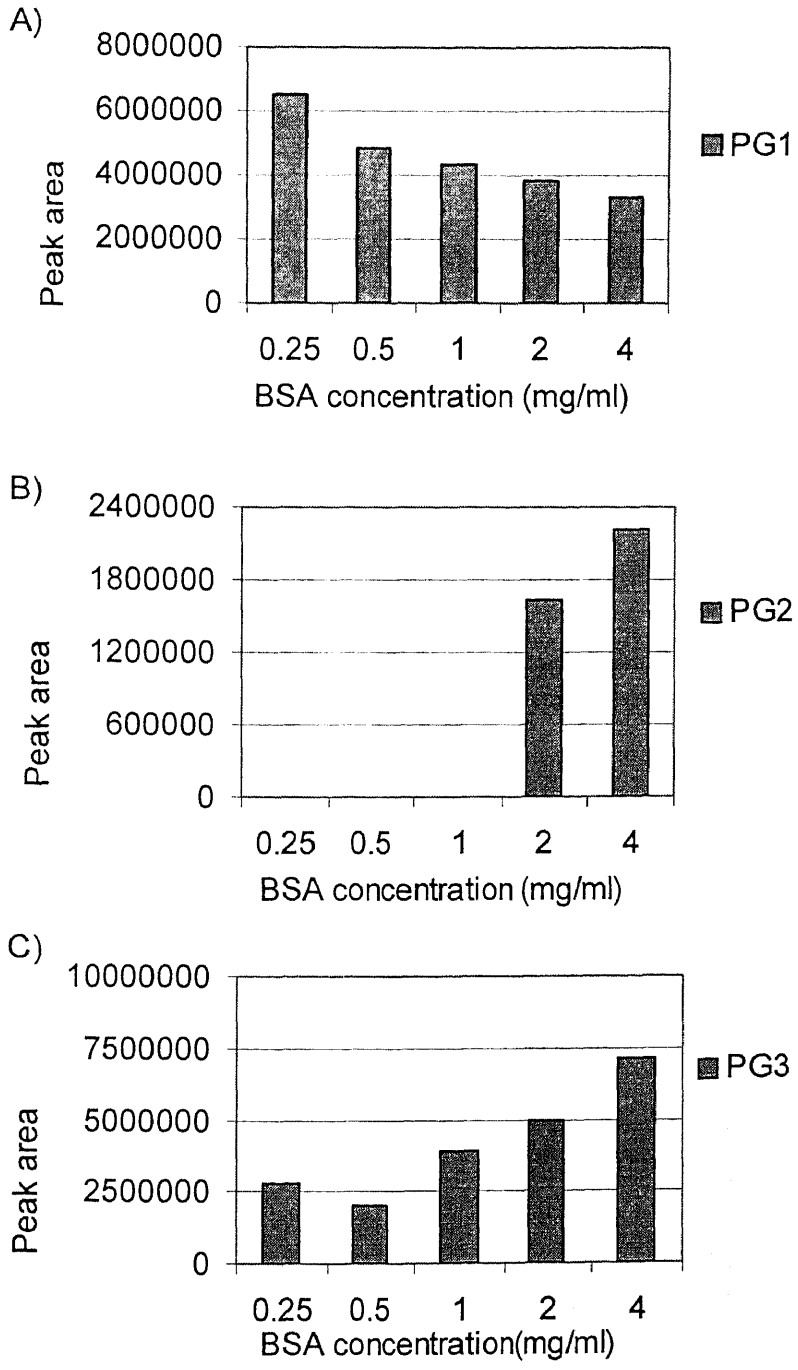


Figure 4.20 Peak areas at specific elution periods for BSA-LPS mixtures prepared without incubation. A: peak group 1, PG1, corresponding to elution period, T_r = 6.0-7.7 min. B: peak group 2, PG2, corresponding to elution period, T_r = 8.3-8.8 min. C: peak group 3, PG3, corresponding to elution period, T_r = 9.3-9.7 min.

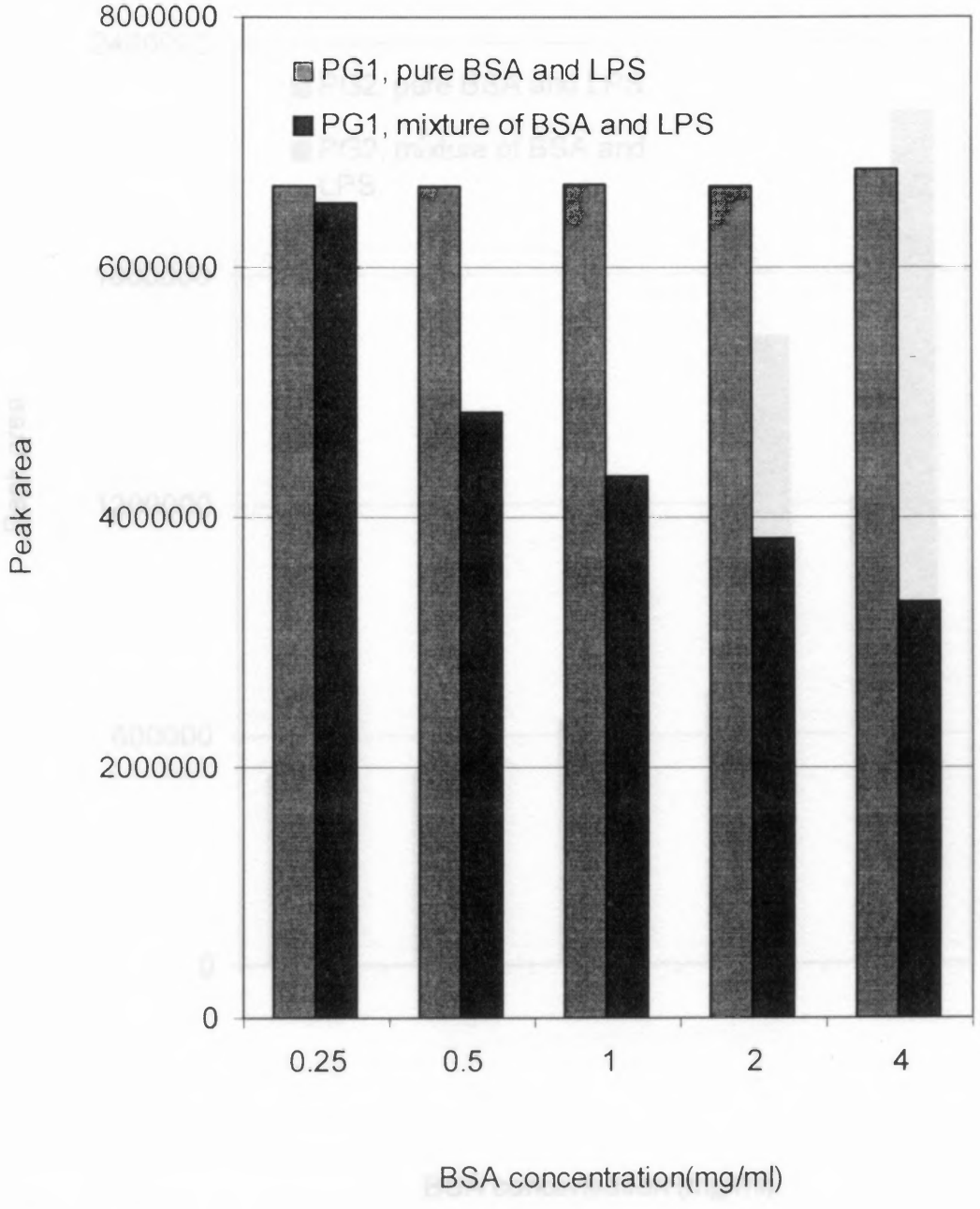


Figure 4.21 Peak area comparison of PG1 without incubation.

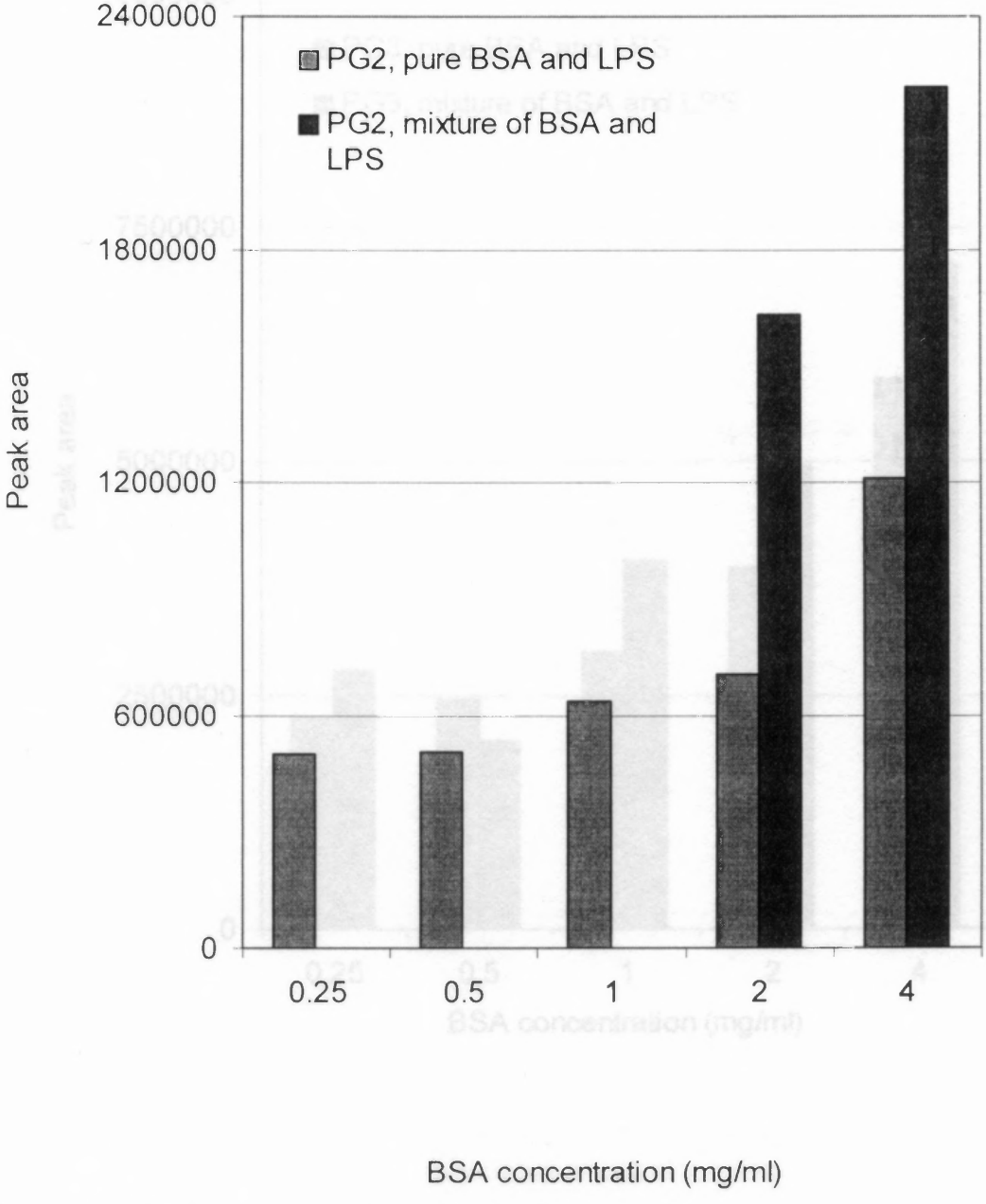


Figure 4.22 Peak area comparison of PG2without incubation.

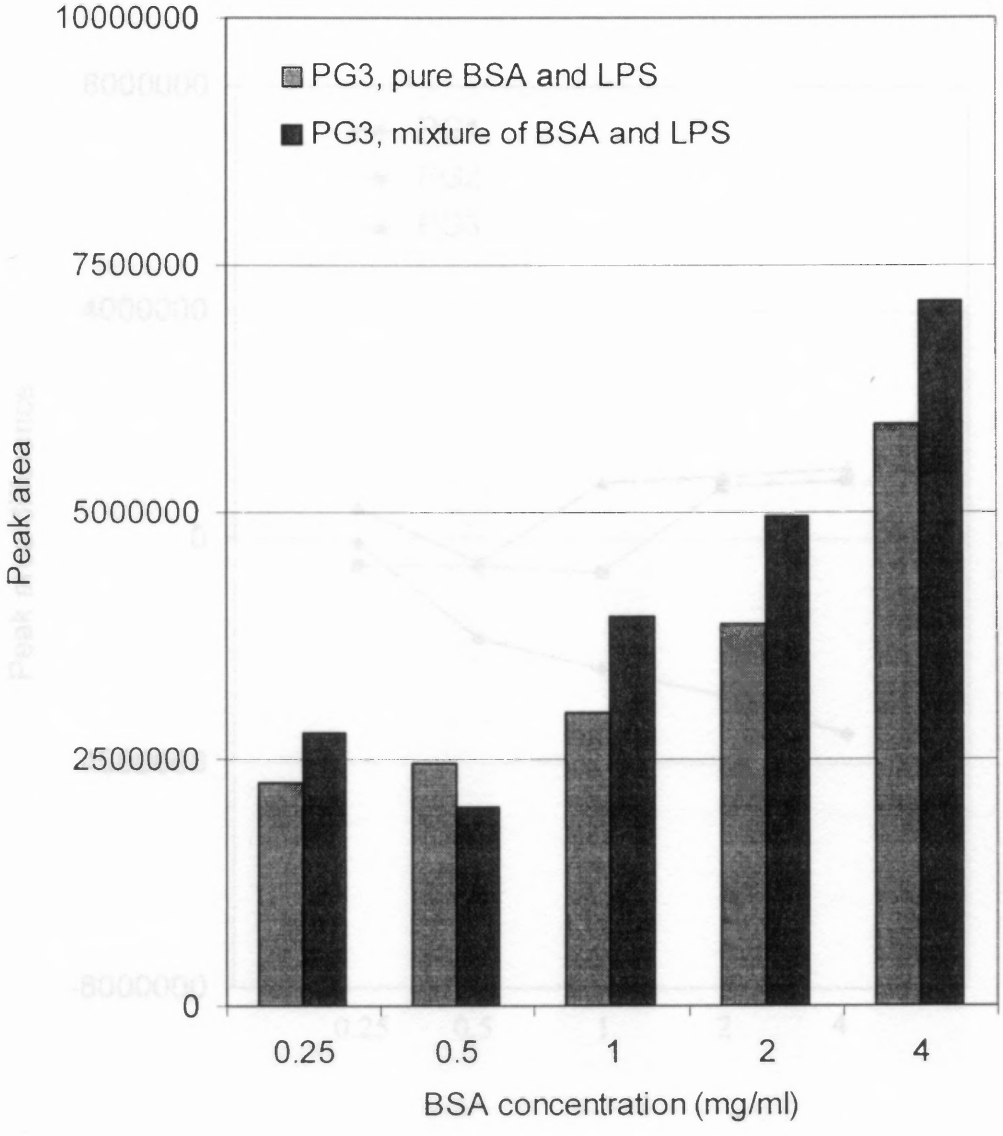


Figure 4.23 Peak area comparison of PG3 without incubation.

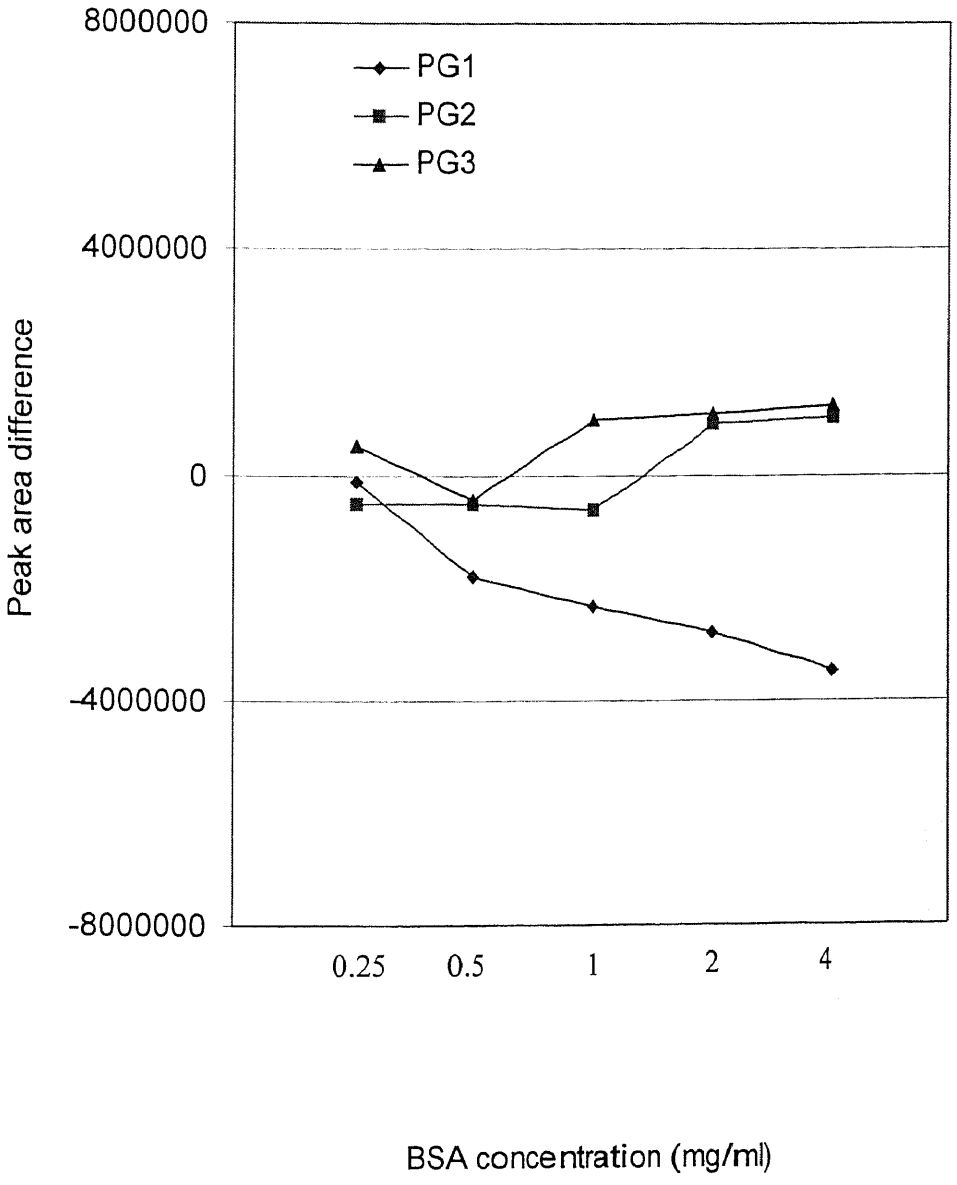


Figure 4.24 Δ S of PG1, PG2 and PG3 without incubation.
Δ S= Peak area of BSA and LPS mixture – Corresponding sum peak area of pure BSA and pure LPS

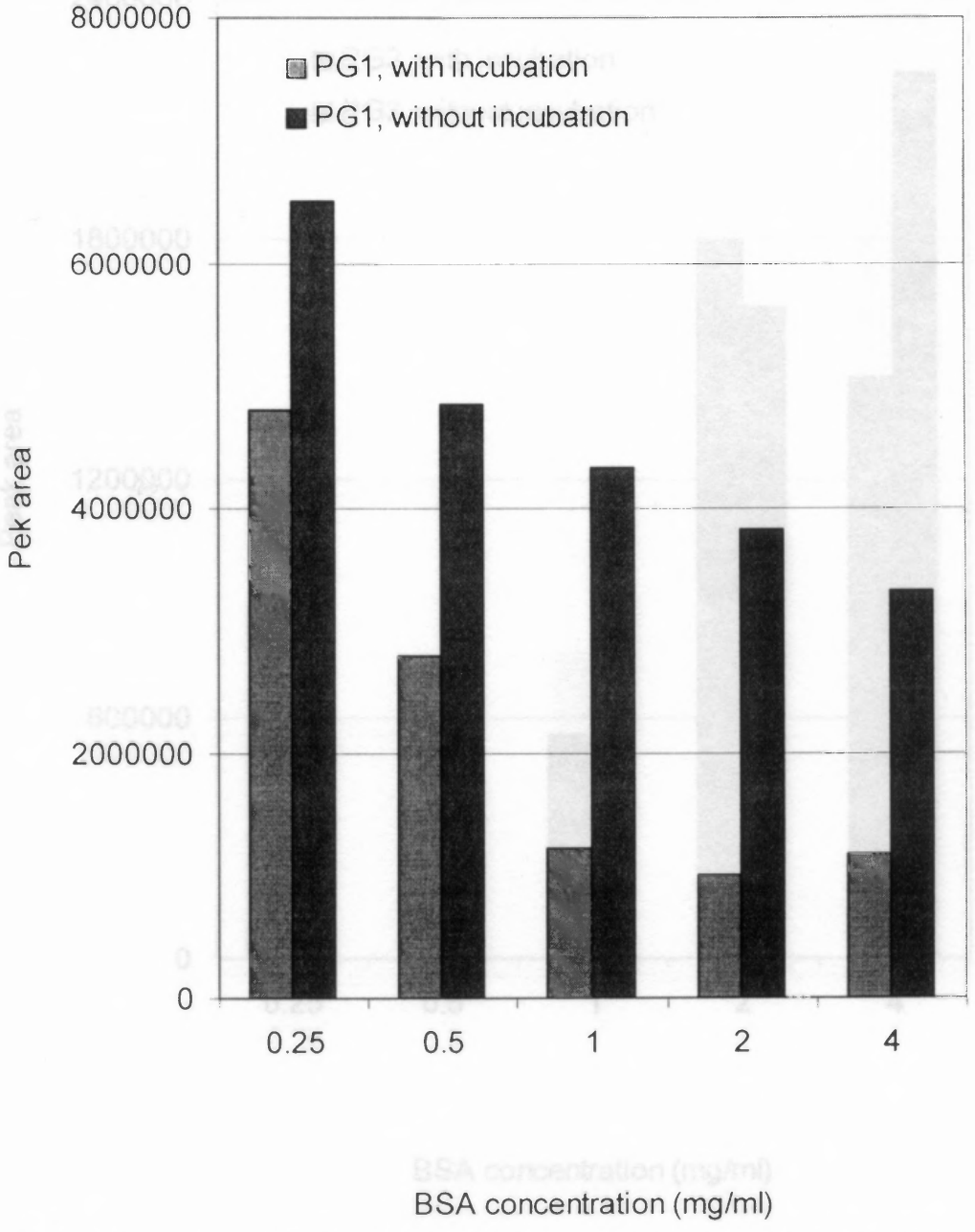


Figure 4.25 Peak area comparison of PG1 with incubation and without incubation.

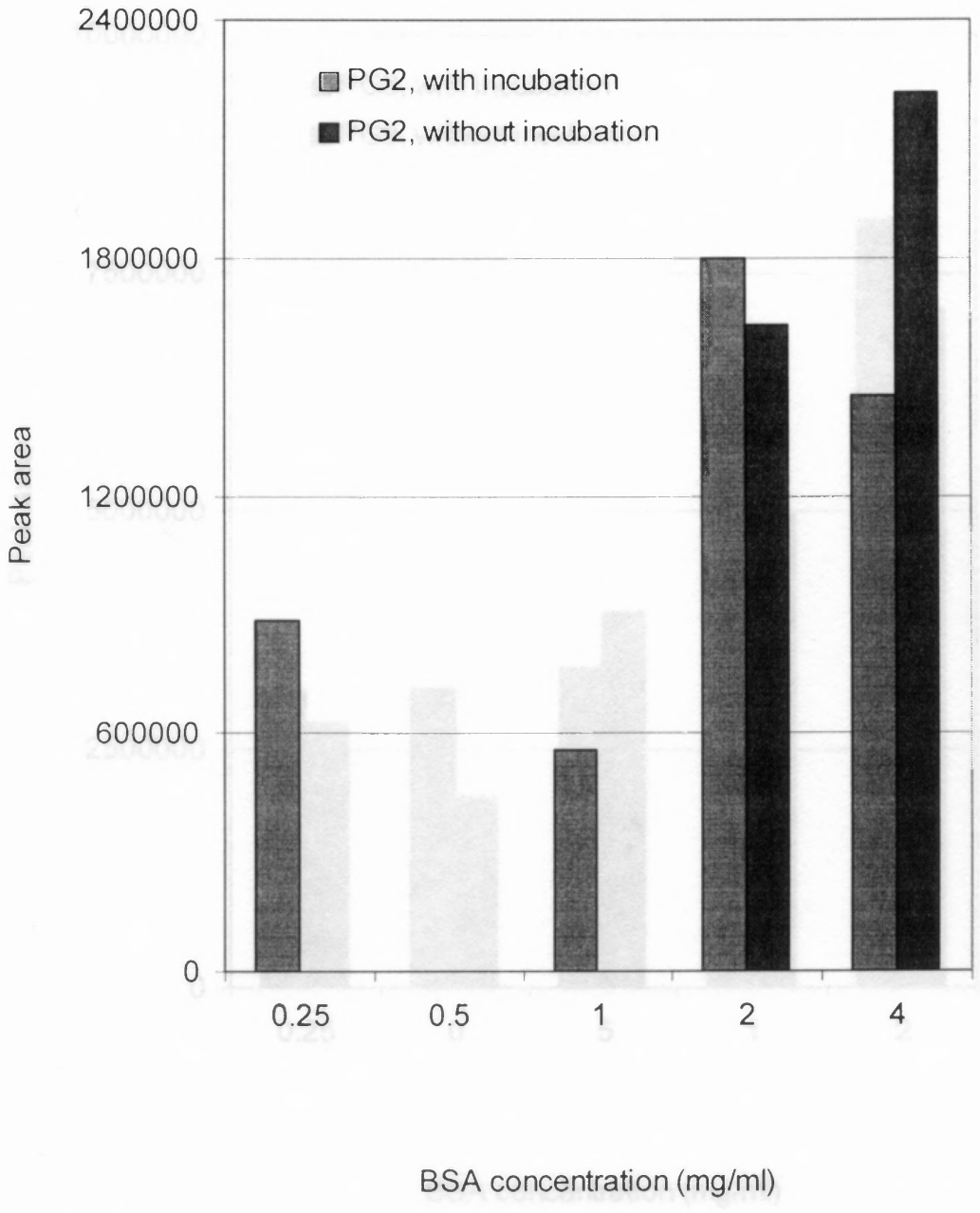


Figure 4.26 Peak area comparison of PG2 with incubation and without incubation.

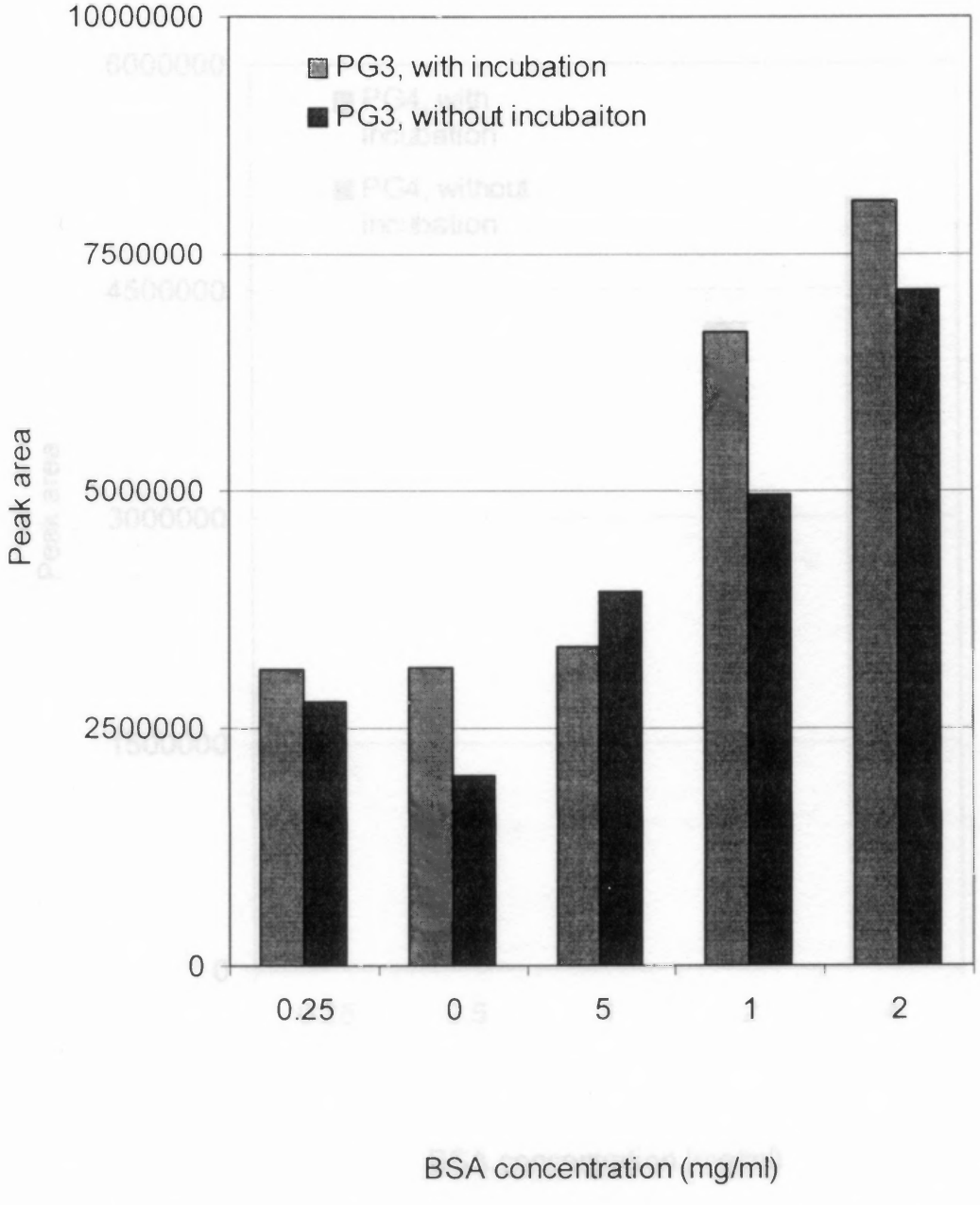


Figure 4.27 Peak area comparison of PG3 with incubation and without incubation.

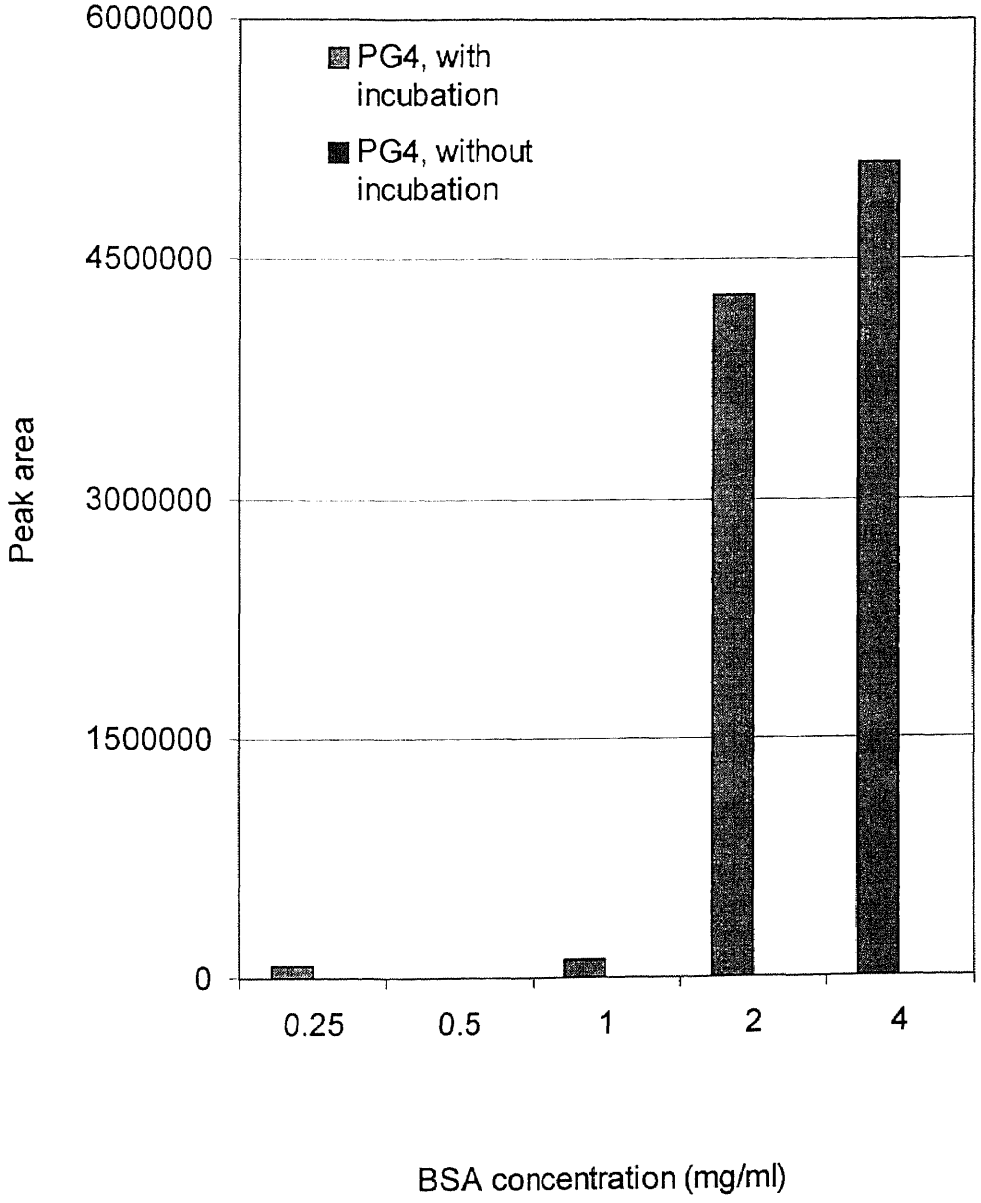


Figure 4.28 Peak area comparison of PG4 with incubation and without incubation.

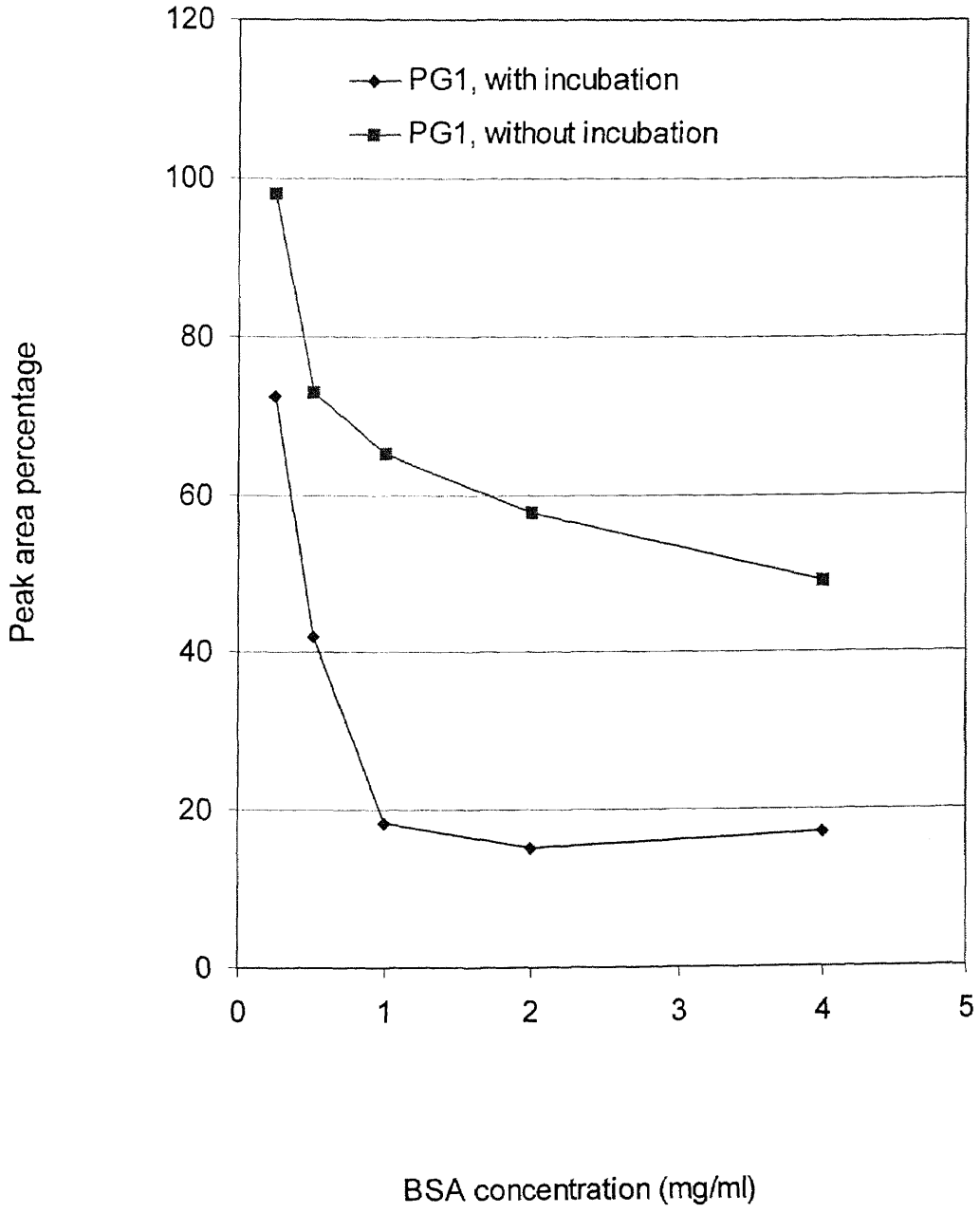


Figure 4.29 Peak area percentage comparison of PG1 with incubation and without incubation.

CHAPTER 5

USE OF CA^{+2} TO RE-AGGREGATE LIPOPOLYSACCHARIDE (LPS) IN HEMOGLOBIN SOLUTIONS AND THE SUBSEQUENT REMOVAL OF ENDOTOXIN BY ULTRAFILTRATION

5.1 Objective

It is known that LPS subunits (monomers) have a molecular weight of approximately 10,000 to 20,000 daltons (Evans-Strickfaden and Oshima, 1996). Normally, these subunits aggregate into vesicles with a molecular weight around 1,000,000 daltons (Pearson, 1985b). The aggregation is believed to be facilitated by cations. Because LPS has negatively charged phosphate groups, cations, especially divalent cations such as Ca^{+2} and Mg^{+2} can act as "bridges" between LPS subunits (Schindler and Osborn, 1979), resulting in LPS bilayer sheets or vesicles with a diameter of the order of 0.1μm in water (Nelsen, 1978; Pearson, 1985b). It is also known that some proteins can disaggregate endotoxins and form complexes with LPS in biological solutions, which makes the removal of endotoxin from protein-based biopharmaceutical products more complicated. In Chapter 3, the concentration effect of hemoglobin on protein-LPS binding and endotoxin removal was studied.

Some studies have shown that the addition of divalent cations enhanced the LPS aggregation in water (Galanos and Lüderitz, 1975). However, whether the LPS subunits bound to protein molecules in a biological solution will re-aggregate upon adding divalent cations is unknown. In this study, a method of two-stage ultrafiltration was used to investigate the effect of Ca^{+2} on LPS re-aggregation in hemoglobin solutions and the

removal of endotoxin. Pure endotoxin in aqueous solutions can not pass through the ultrafiltration membranes rated at 300,000 nominal molecular weight cut-off (NMWCO). If endotoxin aggregates are broken down by protein molecules and form smaller protein-LPS complexes, the complexes will pass the membrane and endotoxin will be found in the filtrate. If LPS subunits in complexes are re-aggregated and form large vesicles again in the solution, then the large vesicles will not pass the membrane and endotoxin will not be found in the filtrate.

5.2 Materials and Methods

5.2.1 Materials

Endotoxin from *Escherichia coli* 026:B6 and human hemoglobin A_o (HbA_o, ferrous) were from Sigma. Hemoglobin and endotoxin solutions were prepared with endotoxin-free 0.15 M NaCl solution. 0.1 M endotoxin-free HCl, 0.1 M endotoxin-free NaOH, NaCl (molecular biology reagent), $CaCl_2 \cdot 2H_2O$ (molecular biology reagent), endotoxin-free water and endotoxin detection kit E-TOXATE were from Sigma. Ultrafree-CL polysulfone 300,000 NMWCO ultrafiltration membrane filters were from Millipore. All glassware used was sterilized in an autoclave (Series 300, Harvard/LTE) for 1 hr and followed by heating in an oven (Thelco Model 16, Precision Scientific Co.) at 175 °C for 3 hrs. All solution transfers were performed by endotoxin-free devices.

5.2.2 Endotoxin Detection

Endotoxin was detected using *Limulus* amebocyte lysate (LAL) test. Gelation was taken as the endpoint. Endotoxin from *Escherichia coli* 055:B5 was used as the standard. For

standard endotoxin detection, when endotoxin concentration was greater than 0.06 Endotoxin Unit (EU) /ml, solid gel formed and the LAL test gave positive result.

5.2.3 Protein-LPS Binding Study

Seven 0.9 ml hemoglobin A_0 solutions at various concentrations (Table 5.1) were put into 1.5 ml sterile Eppendorf microcentrifuge tubes respectively, then 0.1 ml 50.0 μg LPS /ml endotoxin solution was added to each tube. The mixtures were incubated in a water bath at 37 °C for 30 minutes. After incubation, the solutions were applied to 300,000 NMWCO polysulfone ultrafiltration membranes and ultrafiltration was performed by centrifuging at 1800 g for 5 minutes. This is the first ultrafiltration, as shown in Figure 5.1. 0.1 ml filtrate of each solution was set aside for LAL test, and the remaining filtrate was divided into two parts, one part was used for LPS re-aggregation study with $CaCl_2$. Another was used for a control experiment with NaCl.

5.2.4 Endotoxin Re-Aggregation Study

Seven 0.2 ml filtrates at various hemoglobin concentrations from the first ultrafiltration were put into 1.5 ml sterile Eppendorf microcentrifuge tubes. Then 0.2 ml of 0.09 M endotoxin-free $CaCl_2$ solution was added to each tube. The ionic strength of the $CaCl_2$ solution was 0.27 M. The mixtures were incubated in a water bath at 37 °C for 30 minutes. After incubation, the solutions were applied to 300,000 NMWCO polysulfone ultrafiltration membranes and the ultrafiltration was performed by centrifuging at 1800 g for 5 minutes. This is the second ultrafiltration as shown in Figure 5.1. 0.1 ml filtrate of each solution was subjected to LAL test. In order to conduct a control experiment that

was used as a comparison to the endotoxin re-aggregation study in $CaCl_2$ solution, 0.27 M NaCl solution was used to study the aggregation state of LPS in the mixtures. The ionic strength of NaCl solution was 0.27 M, as same as that of $CaCl_2$ solution. The experiment procedure was similar to that of the endotoxin re-aggregation study in $CaCl_2$ solution.

5.3 Results and Discussion

5.3.1 Protein-LPS Binding Study

The LAL test results of protein-LPS binding study are shown in Table 5.1. In this study, the hemoglobin A_o concentration in the mixture was changed from 144.0 μg/ml to 76.5 μg/ml, while the endotoxin concentration was kept the same at 5.0 μg LPS /ml in every mixture. Meanwhile the HbA_o and LPS concentration ratio was changed from 28.8 to 15.3. After the first ultrafiltration, the filtrates of all mixtures formed solid gels in the presence of LAL lysate, and the LAL test gave positive results. This study shows that hemoglobin can break endotoxin aggregates and form HbA_o-LPS complexes, which is consistent with the phenomenon reported in Chapter 3.

5.3.2 Endotoxin Re-Aggregation Study

Table 5.2 shows the LAL test results of the filtrates from the second ultrafiltration. When the $[HbA_o]$/ [LPS] was equal to or greater than 17.3, and the corresponding $[Ca^{+2}]/[HbA_o]/[LPS]$ was equal to or less than 8.3, the mixtures of the LAL lysate and the filtrate formed solid gels and the LAL tests of the filtrates with Ca^{+2} gave positive results, which indicated that endotoxin was found in the filtrates. When the $[HbA_o]/[LPS]$ was

equal to or less than 17.0, and the corresponding $[Ca^{+2}]/[HbA_o]/[LPS]$ was equal to or greater than 8.5, the mixtures did not form solid gel and the LAL tests of the filtrates with Ca^{+2} gave negative results. This indicated no endotoxin was found in those filtrates. In comparison, for the control experiments with NaCl at the same $[HbA_o]/[LPS]$ and $[Ca^{+2}]/[HbA_o]/[LPS]$ ratios, the mixtures of the LAL lysate and the filtrate all formed solid gels and the LAL tests of the filtrates all gave positive results. This indicated that endotoxin was found in the filtrates. By comparing the LAL test results of the filtrates with Ca^{+2} to the LAL test results of the filtrates with Na^{+}, we can see clearly the effect of Ca^{+2} on the re-aggregation of LPS subunits.

The physical meanings of above results may be explained as follows. Pure endotoxin is usually in aggregate state with a molecular weight around 1,000,000 daltons and is retained by a 300,000 NMWCO membrane. In this study, HbA_o and endotoxin mixtures at seven different concentration ratios were incubated before the first ultrafiltration. Hemoglobin molecules broke the endotoxin aggregates and formed protein-LPS complexes with LPS subunits. As we reported in our previous study (Li and Luo, 1997), protein concentration has a significant effect on protein-LPS binding and the amount of endotoxin disaggregated. In other words, the following equilibrium moved towards the RIGHT-HAND direction as the protein concentration was increased.

$$\text{LPS aggregates} + \text{Protein} \rightleftharpoons \text{Protein-LPS}$$

However, when Ca^{+2} was added to all samples after the first ultrafiltration, the cation began to move the above equilibrium towards the LEFT-HAND direction due to the "bridging effect", which results in the re-aggregation of LPS subunits. When the $[HbA_o]/[LPS]$ was decreased from 17.3 to 17.0 or the $[Ca^{+2}]/[HbA_o]/[LPS]$ was increased

from 8.3 to 8.5 (Table 5.2), most LPS subunits re-aggregated into large endotoxin vesicles and were retained by a 300,000 NMWCO membrane, thus no detectable amount of endotoxin was found in the filtrate of the second ultrafiltration.

5.4 Conclusions

The results of this study have shown that Ca^{+2} can affect the state of LPS aggregation not only in water, but also in protein solutions where the protein-LPS complexes exist. The addition of Ca^{+2} to protein-LPS mixture can result in the re-aggregation of LPS subunits, which makes it easier to remove endotoxin from protein solutions via ultrafiltration. This knowledge is also very useful to design and improvement of chromatographic endotoxin removal processes.

Table 5.1 LAL test results of HbA_o-LPS binding study.

HbA_o initial concentration in HbA_o-LPS mixture (μg/ml)	Endotoxin initial concentration in HbA_o-LPS mixture (μg LPS/ml)	[HbAo]/[LPS][a]	LAL test result of the filtrate from the first ultrafiltration
144.0	5	28.8	+[b]
108.0	5	21.6	+
90.0	5	18.0	+
86.6	5	17.3	+
84.9	5	17.0	+
83.2	5	16.7	+
76.5	5	15.3	+

LAL: *Limulus* Amebocyte Lysate.

HbA_o: hemoglobin A_o.

LPS: lipopolysaccharide.

a: [HbAo]/[LPS], the concentration ratio of HbA_o and LPS.

b: "+", formation of a gel which does not collapse after one 180° inversion.

Table 5.2 LAL test results of endotoxin re-aggregate.

[HbAo]/[LPS][a]	[Ca^{+2}]/HbAo]/[LPS][b]	LAL test result with $CaCl_2$ [c]	LAL test result with NaCl [d]
28.8	5.0	+[e]	+
21.6	6.7	+	+
18.0	8.0	+	+
17.3	8.3	+	+
17.0	8.5	–[f]	+
16.7	8.7	–	+
15.3	9.4	–	+

a: [HbAo]/[LPS], the concentration ratio of HbA_o and LPS. [LPS] = 5.0 ug/ml for all experiments.

b: [Ca^{+2}]/HbAo]/[LPS], the concentration ratio of Ca^{+2}, hemoglobin A_o and lipopolysaccharide.

c: LAL test result of the filtrate with $CaCl_2$ from the second ultrafiltration.

d: LAL test result of the filtrate with NaCl from the second ultrafiltration.

e: "+", formation of a gel which does not collapse after one 180° inversion.

f: "-", no gel formation.

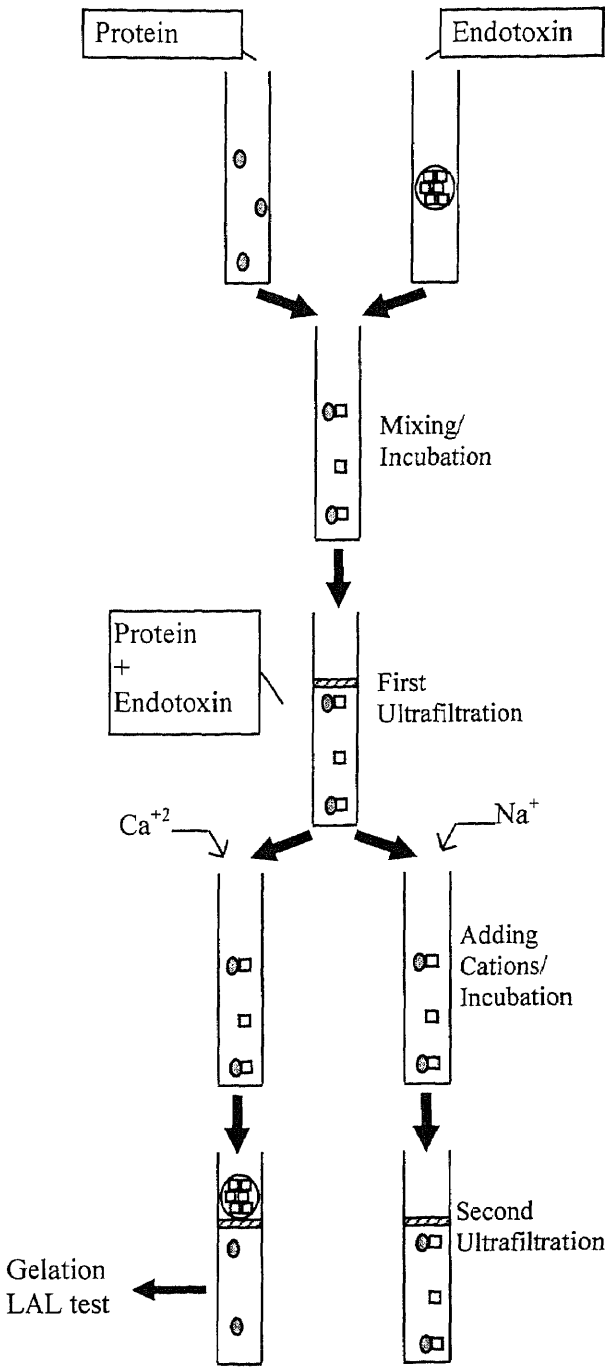


Figure 5.1. Schematic diagram of experimental procedure: protein/ LPS mixtures at various concentration ratios were incubated at 37 °C for 30 minutes. After incubation, the mixtures were applied to the first ultrafiltration. To each filtrate at various protein/LPS ratios from the first ultrafiltration Ca^{+2} or Na^{+} solution was added respectively. The resulting mixtures were incubated and the second ultrafiltration was conducted. Gelation LAL test was used.

CHAPTER 6

QUANTITATIVE DETERMINATION OF CA^{+2} EFFECTS ON ENDOTOXIN REMOVAL AND PROTEIN YIELD IN A TWO-STAGE ULTRAFILTRATION PROCESS

6.1 Objective

It is known that some proteins can bind endotoxins and form protein-LPS complexes in biological solutions (Zielger et al., 1982; Ziegler et al., 1991; Schumann et al., 1990; Wakitu et al., 1996; Schlichting et al., 1996). It was reported that hemoglobin not only can bind but also disaggregate LPS, and enhance LPS activation of *Limulus* amebocyte lysate (LAL) in a concentration dependent manner (Kaca et al., 1994; Roth et al., 1994). In Chapter 3, the concentration effects of hemoglobin and albumin on protein-LPS binding and endotoxin removal was studied. It was found that the proteins interacted with LPS and formed protein-LPS complexes, resulting in the disaggregation of LPS vesicles.

Endotoxin subunits have molecular weights of 10,000 or less, depending on the composition of the solution in which they are present (Gerba and Goyal, 1981). Aggregated forms of endotoxin range from 300,000 to 1,000,000 daltons (Brown and Fuller, 1993; Abramson et al., 1981; Sweadner et al, 1977; Hannecart-Pokorni et al., 1973). The aggregation is believed to be facilitated by cations. Because LPS has negatively charged phosphate groups, cations, especially divalent cations such as Ca^{+2} and Mg^{+2} can act as "bridges" between LPS subunits (Schindler and Osborn, 1979), resulting in LPS bilayer sheets or vesicles with a diameter of the order of 0.1μm in water (Nelsen, 1978; Pearson, 1985). In a previous study, researchers in our research group investigated

the cation effects on endotoxin removal efficiency and protein recovery in an affinity chromatographic process (Kang and Luo, 1998). In Chapter 5, studies were performed on the effects of Ca^{+2} on LPS re-aggregation in hemoglobin solutions and the removal of endotoxin. When LPS subunits in complexes were re-aggregated and formed large vesicles again in the solution, they did not pass through the membrane and endotoxin was not found in the filtrate.

In the work presented in this article, the method of two stage ultrafiltration was also used to study the effect of Ca^{+2} on LPS re-aggregation. Since the chromogenic LAL assay was employed to detect endotoxin, it was possible to determine endotoxin concentration quantitatively. The endotoxin removal efficiency after re-aggregation in the presence of Ca^{+2} was studied. The yield of hemoglobin was also investigated.

6.2 Experiment

6.2.1 Materials and Methods

Glassware. All glassware used was autoclaved in an autoclave (Series 300, Harvard/LTE) for 1 hour and followed with heating in an oven (Thelco Model 16, Precision Scientific Co.) at 175 °C for 3 hours. All solution transfers were performed by endotoxin-free devices. Sterile, disposable plasticware was used to prevent endotoxin contamination.

Reagents. Endotoxin-free water from BioWhittaker (BioWhittaker Inc., Walkersville, MD) was used for endotoxin dilution and all solution preparations. Endotoxin from *Escherichia coli* 026:B6, human hemoglobin A_o (HbA_o, ferrous), 0.1 M endotoxin-free HCl, 0.1 M endotoxin-free NaOH, NaCl (molecular biology reagent), $CaCl_2 \cdot 2H_2O$ (molecular biology reagent) were from Sigma (Sigma Chemical Co., St.

Louis, MO). Hemoglobin and endotoxin solutions were prepared with endotoxin-free 0.15 M NaCl solution. Ultrafree-CL polysulfone 300,000 NMWCO (Nominal Molecular Weight Cut-off) ultrafiltration membrane filters were from Millipore (Millipore Corporation, Bedford, MA).

6.2.2 Endotoxin Assay

To determine endotoxin concentration, a chromogenic *Limulus* Amebocyte Lysate (LAL) test kit QCL-1000 from BioWhittaker (BioWhittaker Inc., Walkersville, MD) was used. The chromogenic substrate is a short synthetic polypeptide with an amino acid sequence that mimics a natural cleavage site in a clotting protein present in the lysate. The chromogenic *p*-nitro aniline (*p*NA) moiety is attached to the end of the peptide as Ac-Ile-Glu-Ala-Arg-*p*NA. In the chromogenic LAL test the proenzyme which exists in the *Limulus* amebocyte lysate (LAL) was activated by the endotoxin in a water bath at 37 °C for 10 minutes. Chromogenic substrate was then added, and the active enzyme caused the release of *p*NA from the substrate, producing a yellow color. After 20 minutes, the reaction was stopped with 25% acetic acid. The intensity of the color change produced by the substrate cleavage was measured with the UV/VIS spectrophotometer (Hitachi U-2000, Hitachi Instruments Inc., Danbury, CT) at 405 nm (Figure 6.3). The results were compared to a calibration curve to obtain endotoxin concentration. However HbA_o also has a strong absorbance at 405 nm, and it should not be counted in the endotoxin absorbance. The correction was made by deducting the HbA_o absorbance from the total absorbance of the reaction mixture (Figure 6.2a, Figure 6.2b).

Endotoxin removal efficiency, *ERE*, of the ultrafiltration was determined by the following equation:

$$ERE = \frac{C_{LPS}^{load} - C_{LPS}^{filt.}}{C_{LPS}^{load}} \quad (1)$$

Where C_{LPS}^{load} is the endotoxin concentration in the solution loaded on the ultrafiltration membrane filter (EU/ml). $C_{LPS}^{filt.}$ is the endotoxin concentration in the filtrate of the ultrafiltration (EU/ml).

6.2.3 Protein Assay

The concentration of HbA_o in the solution was measured at 410 nm by a UV/VIS spectrophotometer (Hitachi U-2000, Hitachi Instruments Inc., Danbury, CT). Protein yield , *Y*, of the ultrafiltration was determined by the following equation:

$$Y = \frac{C_{HbAo}^{filt.}}{C_{HbAo}^{load}} \quad (2)$$

Where $C_{HbAo}^{filt.}$ is the HbA_o concentration in the filtrate (μg/ml). C_{HbAo}^{load} is the HbA_o concentration in the solution loaded on the ultrafiltration membrane filter (μg/ml).

6.2.4 The First Stage Ultrafiltration

3.6 ml HbA_o solutions at various concentrations (Table 6.1) was incubated with 0.4 ml 50.00 EU/ml endotoxin solution at 37 °C water bath for 30 minutes, the concentration ratio of HbA_o to endotoxin were 0.90, 1.80, 2.70, 3.60 μg/EU respectively. Sterile, endotoxin-free culture tubes were used for the incubation. After incubation, the mixture solutions were placed on 300,000 NMWCO polysulfone ultrafiltration membrane filters and ultrafiltration was performed by centrifuging the mixture samples at 1800 g for 5 minutes. This is the first stage ultrafiltration, as shown in Figure 6.1. 0.1 ml filtrate of each solution was set aside for LAL test, and the remaining filtrate was divided into two parts, one part was used for the second stage ultrafiltration to investigate Ca^{+2} effect on endotoxin removal and protein purification with $CaCl_2$. Another was used for a control experiment with NaCl.

6.2.5 The Second Stage Ultrafiltration

Four 1 ml filtrates at various hemoglobin concentrations from the first stage ultrafiltration were put into sterile endotoxin-free culture tubes. Then 1 ml 0.05 M endotoxin-free $CaCl_2$ solution was added to each tube. The ionic strength of the $CaCl_2$ solution was 0.15 M. The mixtures were incubated in a water bath at 37 °C for 30 minutes. After incubation, the solutions were applied to 300,000 NMWCO polysulfone ultrafiltration membranes and the ultrafiltration was performed by centrifuging at 1800 g for 5 minutes. This is the second stage ultrafiltration as shown in Figure 6.1. 0.1 ml filtrate of each solution was subjected to LAL test. In order to conduct a control experiment that was used as a comparison to the endotoxin re-aggregation study in $CaCl_2$ solution, 0.15 M NaCl

solution was used to study the aggregation state of LPS in the mixtures. The ionic strength of NaCl solution was 0.15 M, as same as that of $CaCl_2$ solution. The experiment procedure was similar to that of the study with $CaCl_2$ solution.

6.3 Results and Discussion

6.3.1 Endotoxin Removal of the First Stage Ultrafiltration

The endotoxin removal efficiency of the first stage ultrafiltration is shown in Table 6.2. As the HbA_o/LPS concentration ratio increased from 0.90 to 3.60 μg/EU, the endotoxin removal efficiency, *ERE*, decreased from 98.84% to 88.36%. This phenomena can be explained as follows: pure endotoxin in water can not pass through the ultrafiltration membranes rated at 300,000 nominal molecular weight cut-off (NMWCO). When endotoxin aggregates were broken down by protein molecules and smaller protein-LPS complexes were formed, the complexes passed the membrane and endotoxin was found in the filtrate. As observed in Chapter 3, the protein capacity of breaking down endotoxin is protein concentration dependent. Higher the protein concentration, more the endotoxin being dissociated. Therefore, at high HbA_o/LPS concentration ratios, more endotoxin was broken down to form smaller protein-LPS complexes, which passed through the membrane. This resulted in less endotoxin retained by the membrane and lower *ERE*.

6.3.2 Protein Yield of the First Stage Ultrafiltration

The protein yield of the first stage ultrafiltration is shown in Table 6.2. As the HbA_o/LPS concentration ratio increased from 0.90 to 3.60 μg/EU, the protein yield, *Y*, increased

from 79.91% to 95.19%. The binding between the HbA_o and the membrane may be the cause of protein loss during the filtration.

6.3.3 Endotoxin Removal of the Second Stage Ultrafiltration - The Effect of Ca^{+2} on Endotoxin Removal

Figure 6.4 shows endotoxin concentrations in feeds loaded on the second stage ultrafilters and endotoxin concentrations in membrane filtrates of the second stage ultrafiltration in the situations when Ca^{+2} or Na^+ was added. From Figure 6.4 we can see that at various HbA_o/LPS concentration ratios, the endotoxin concentrations in the filtrates with Ca^{+2} were much lower than those in the control samples (with Na^+). Figure 6.5 shows the amount of endotoxin removed from the solution during the second stage ultrafiltration. The amount of endotoxin removed from the solution with Ca^{+2} were much higher than those in the solution with Na^+. The effect of Ca^{+2} on endotoxin removed is clearly shown in Table 6.3, The *ERE* with Ca^{+2} was two to three times of the *ERE* with Na^+.

6.3.4 Protein Yield of the Second Stage Ultrafiltration - The Effect of Ca^{+2} on Protein Yield

Figure 6.6 shows HbA_o concentrations in feeds loaded on the second stage ultrafilters and HbA_o concentrations in filtrates of the second stage ultrafiltration. From Figure 6.6 we can see that at various HbA_o/LPS concentration ratios, the curves representing HbA_o concentrations with Ca^{+2} and with Na^+ are almost overlapped, which indicates that the HbA_o concentrations in the filtrates with Ca^{+2} were very close to those in the control experiments (with Na^+).

Figure 6.7 illustrates the protein loss during the second stage ultrafiltration. The amount of HbA_o lost from the solution with Ca^{+2} and Na^+ are almost the same, which indicates that the introduction of Ca^{+2} has almost no effect on protein yield. Table 4 shows that the HbA_o yields with Ca^{+2} are very close to the HbA_o yields with Na^+ at various HbA_o/LPS concentration ratios.

The physical meanings of above results may be explained as follows. Pure endotoxin is usually in aggregate state with a molecular weight around 1,000,000 daltons and is retained by a 300,000 NMWCO membrane. In this study HbA_o and endotoxin mixtures at four different concentration ratios were incubated before the first ultrafiltration, hemoglobin molecules broke the endotoxin aggregates and formed protein-LPS complexes with LPS subunits. As we reported in our previous study (Li and Luo, 1997), protein concentration has a significant effect on protein-LPS binding and the amount of endotoxin disaggregated. In other words, the following equilibrium moved towards the RIGHT-HAND direction as the protein concentration was increased.

$$\text{LPS aggregate} + \text{Protein} \rightleftharpoons \text{LPS subunits} + \text{Protein-LPS} \qquad (3)$$

As a result, *ERE* decreased from 98.84% to 88.36%. However, when Ca^{+2} was added to all samples after the first ultrafiltration, the cation began to move the above equilibrium towards the LEFT-HAND direction due to the "bridging effect", which resulted in the re-aggregation of LPS subunits. Therefore less endotoxin was detected in the filtrates of the second stage ultrafiltration with Ca^{+2} (Figure 6.4), and more endotoxin was removed (Figure 6.5) comparing with the control experiment (with Na^+).

6.4 Conclusions

The results of this study have shown that Ca^{+2} can affect endotoxin aggregate states not only in water as reported before, but also in protein solutions where protein-endotoxin complexes exist. The addition of Ca^{+2} to protein-endotoxin mixture can result in the re-aggregation of endotoxin subunits, which makes it easier to remove endotoxin from protein solutions through ultrafiltration. The effects of Ca^{+2} on endotoxin removal efficiency in the second stage ultrafiltration can be clearly seen by comparing the *ERE* with Ca^{+2} to the *ERE* with Na^{+} in the control experiment.

The results also show that the protein yield during ultrafiltration with Ca^{+2} or with Na^{+} are almost the same. This indicates that the protein yield was not sacrificed in the process of LPS re-aggregation in the presence of Ca^{+2}. This phenomenon has potential utility in ultrafiltration processes to increase endotoxin removal efficiency and in the mean time maintain protein yield.

Table 6.1 Protein and endotoxin concentrations in feeds loaded to the first stage ultrafiltration.

HbA_o Concentration in Feed (μg/ml)	Endotoxin Concentration in Feed (EU/ml)	$[HbA_o]$ /[LPS]* (μg/EU)
45.00	50.00	0.90
90.00	50.00	1.80
135.00	50.00	2.70
180.00	50.00	3.60

*The concentration ratio of HbA_o and LPS.

Table 6.2 Endotoxin removal efficiency, *ERE*, and HbA_o yield, *Y*, of the first stage ultrafiltration.

$[HbA_o]$/[LPS] (μg/EU)	*ERE* (%)	*Y* (%)
0.90	98.84	79.91
1.80	94.96	90.67
2.70	92.64	95.39
3.60	88.36	95.19

Table 6.3 Effect of Ca^{+2} on endotoxin removal efficiency, *ERE*, of the second stage ultrafiltration.

$[HbA_o]$/LPS (μg/EU)	*ERE* with Ca^{+2} (%)	*ERE* with Na^{+} (%)
0.90	59.09	19.60
1.80	72.73	34.17
2.70	73.10	28.15
3.60	54.78	24.98

Table 6.4 Effect of Ca^{+2} on protein yield, *Y*, of the second stage ultrafiltration.

$[HbA_o]$/[LPS] (μg/EU)	*Y* with Ca^{+2} (%)	*Y* with Na^{+} (%)
0.90	71.57	69.05
1.80	82.54	80.32
2.70	86.79	87.49
3.60	89.54	90.60

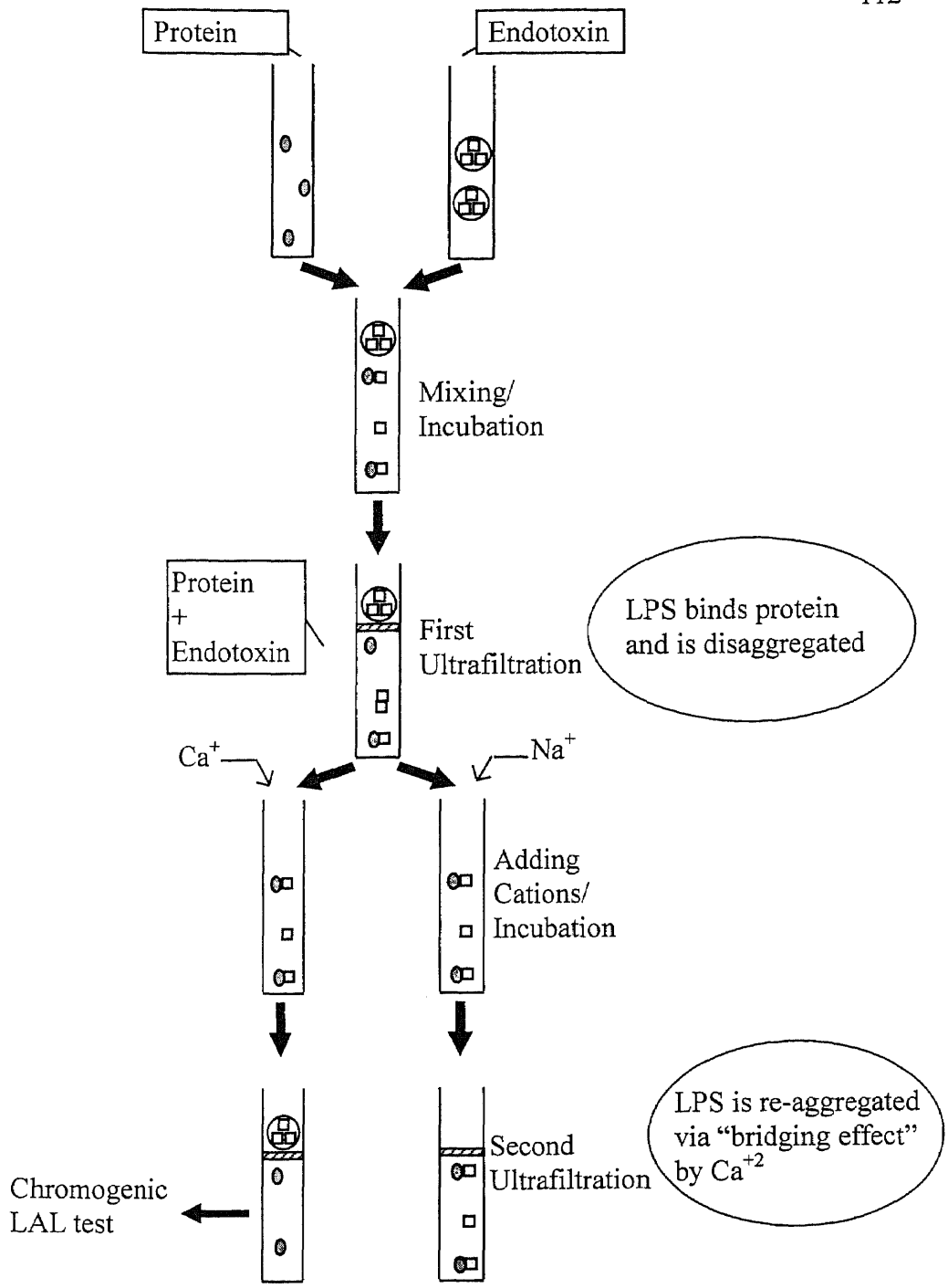


Figure 6.1 Schematic diagram of experimental procedure. The experimental procedure: protein/ LPS mixtures at various concentration ratios were incubated at 37 °C for 30 minutes. After incubation, the mixtures were applied to the first ultrafiltration. To each filtrate at various protein/LPS ratios from the first ultrafiltration Ca^{+2} or Na^{+} solution was added respectively. The resulting mixtures were incubated and the second ultrafiltration was conducted. Chromogenic LAL test was used.

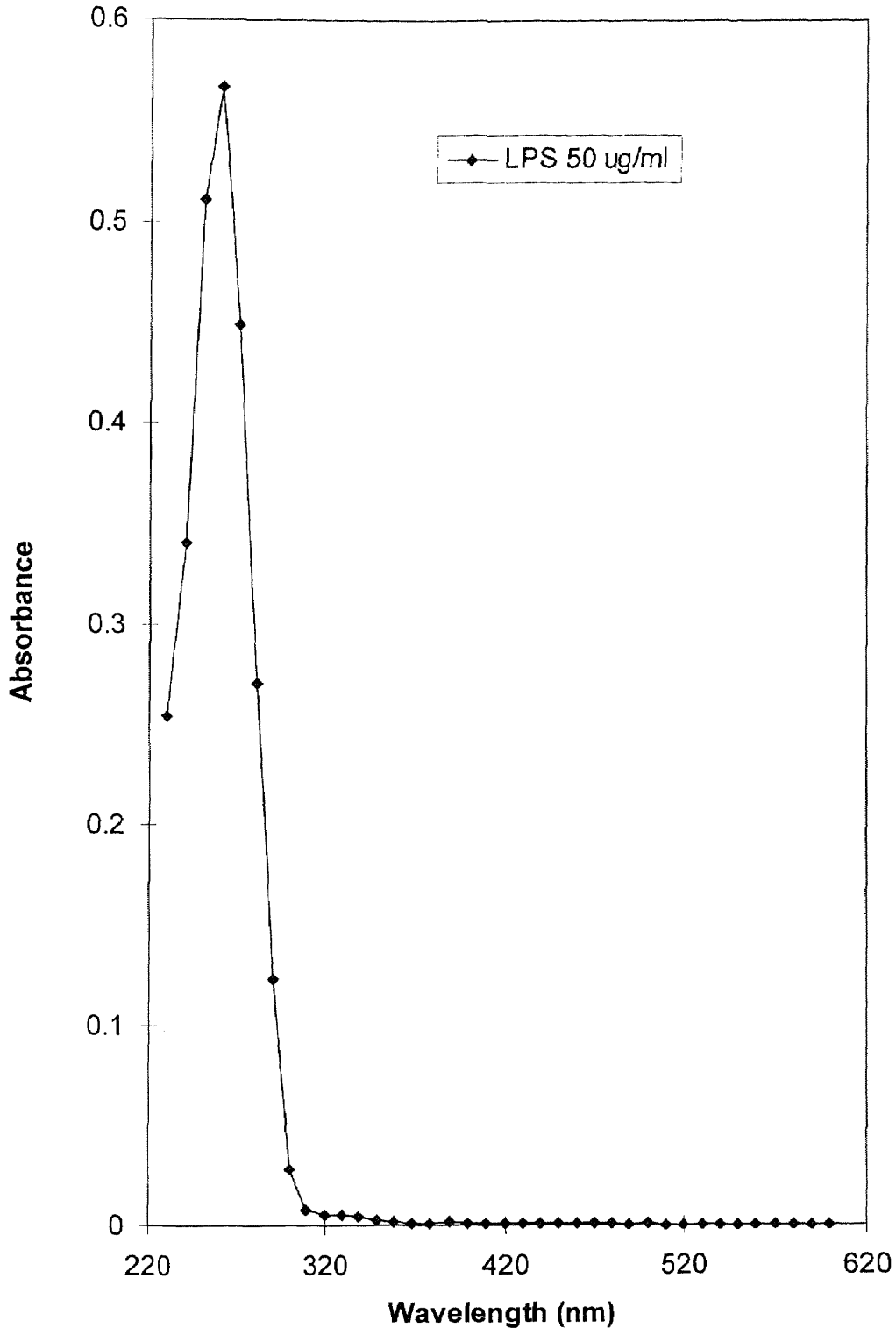


Figure 6.2a Absorption spectrum of 50 μg/ml LPS in 1 cm cuvette.

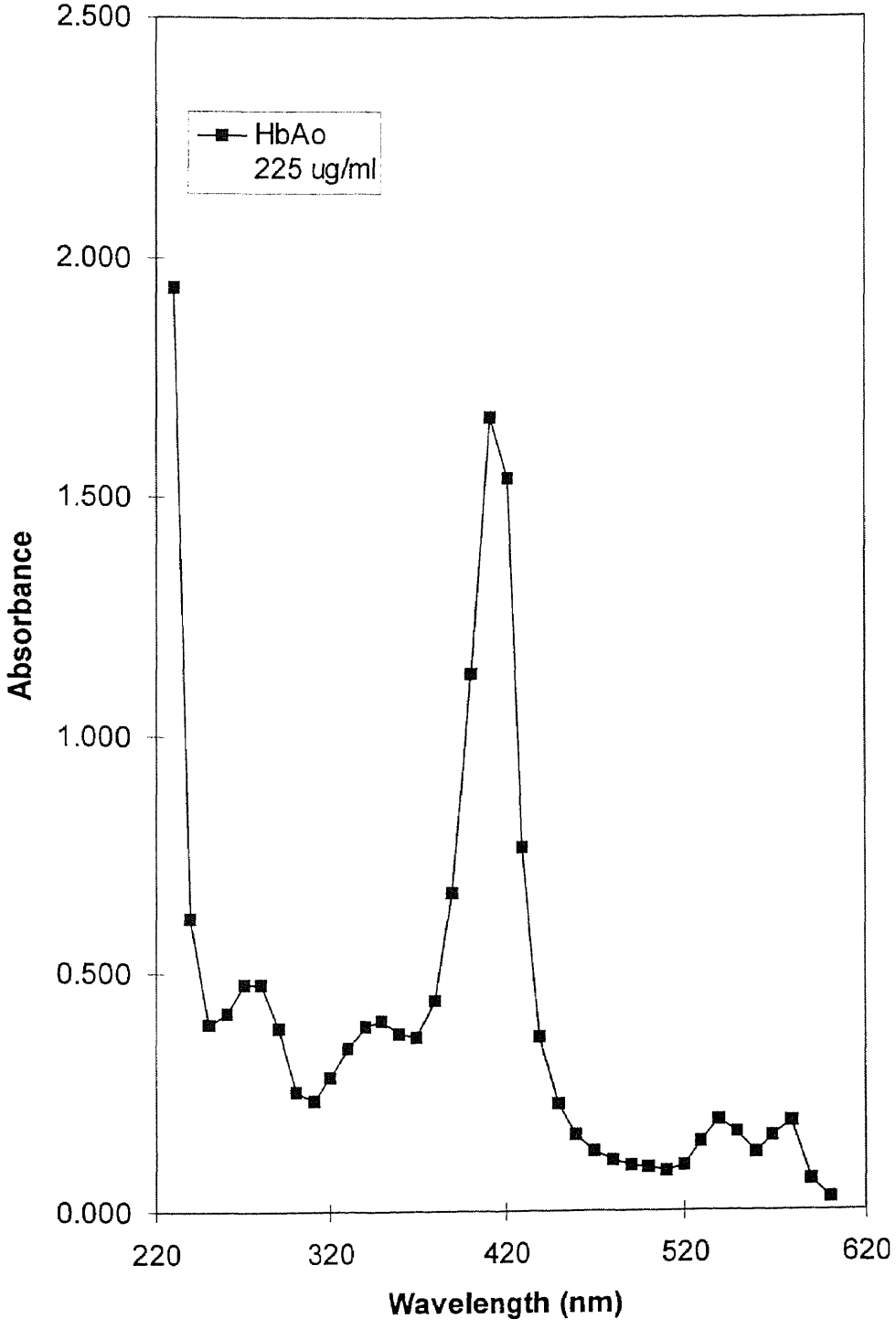


Figure 6.2b Absorption spectrum of 225 μg/ml HbA_o in 1 cm cuvette.

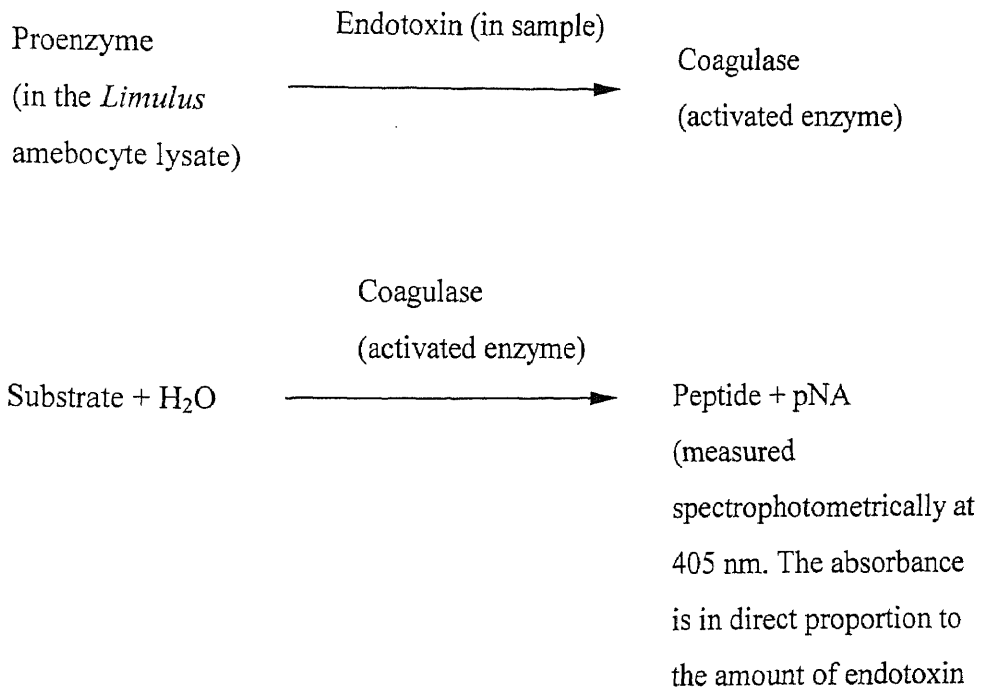


Figure 6.3 Principle of chromogenic *Limulus* amebocyte lysate (LAL) test.

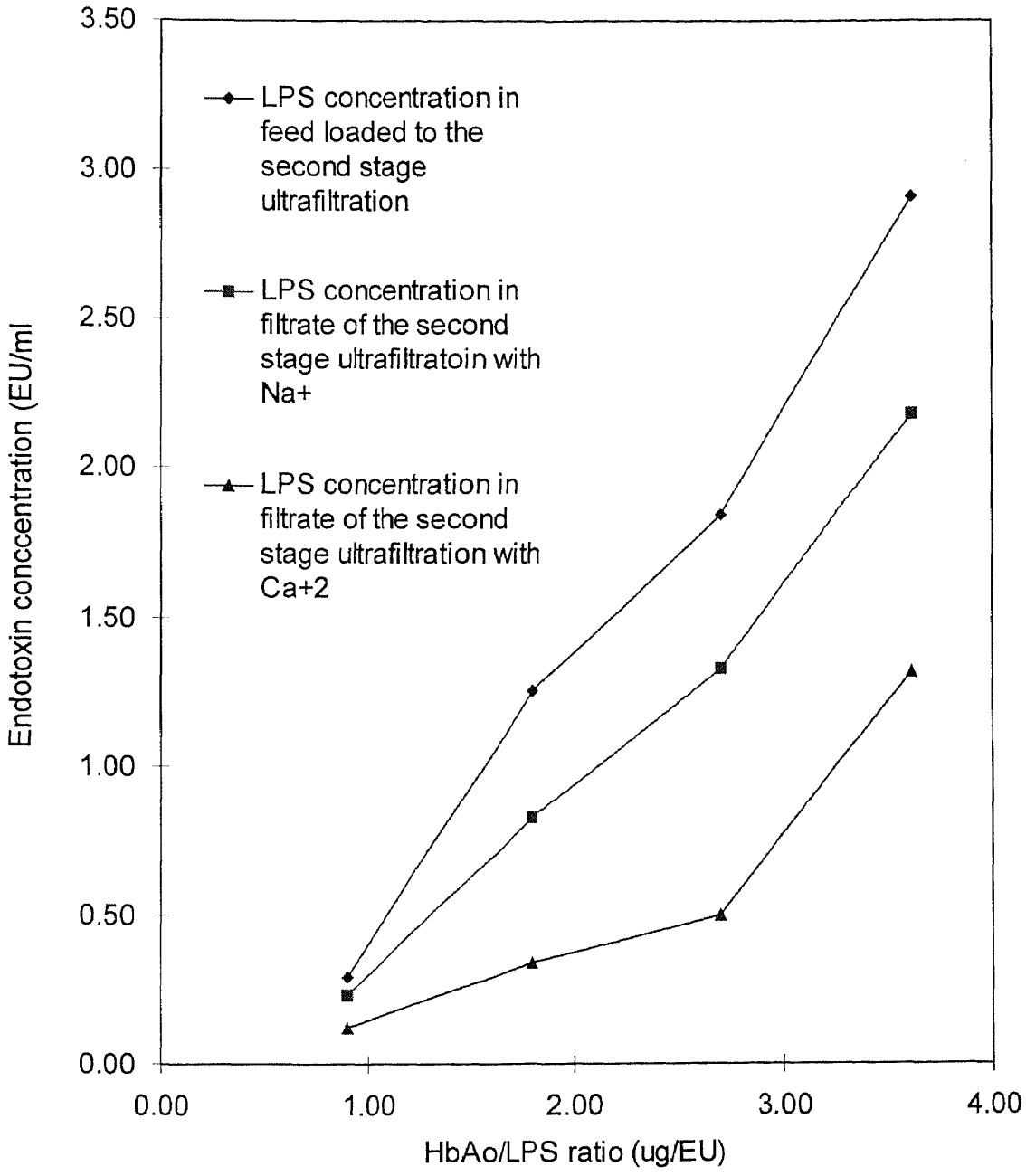


Figure 6.4 Endotoxin concentration in the second stage ultrafiltration.

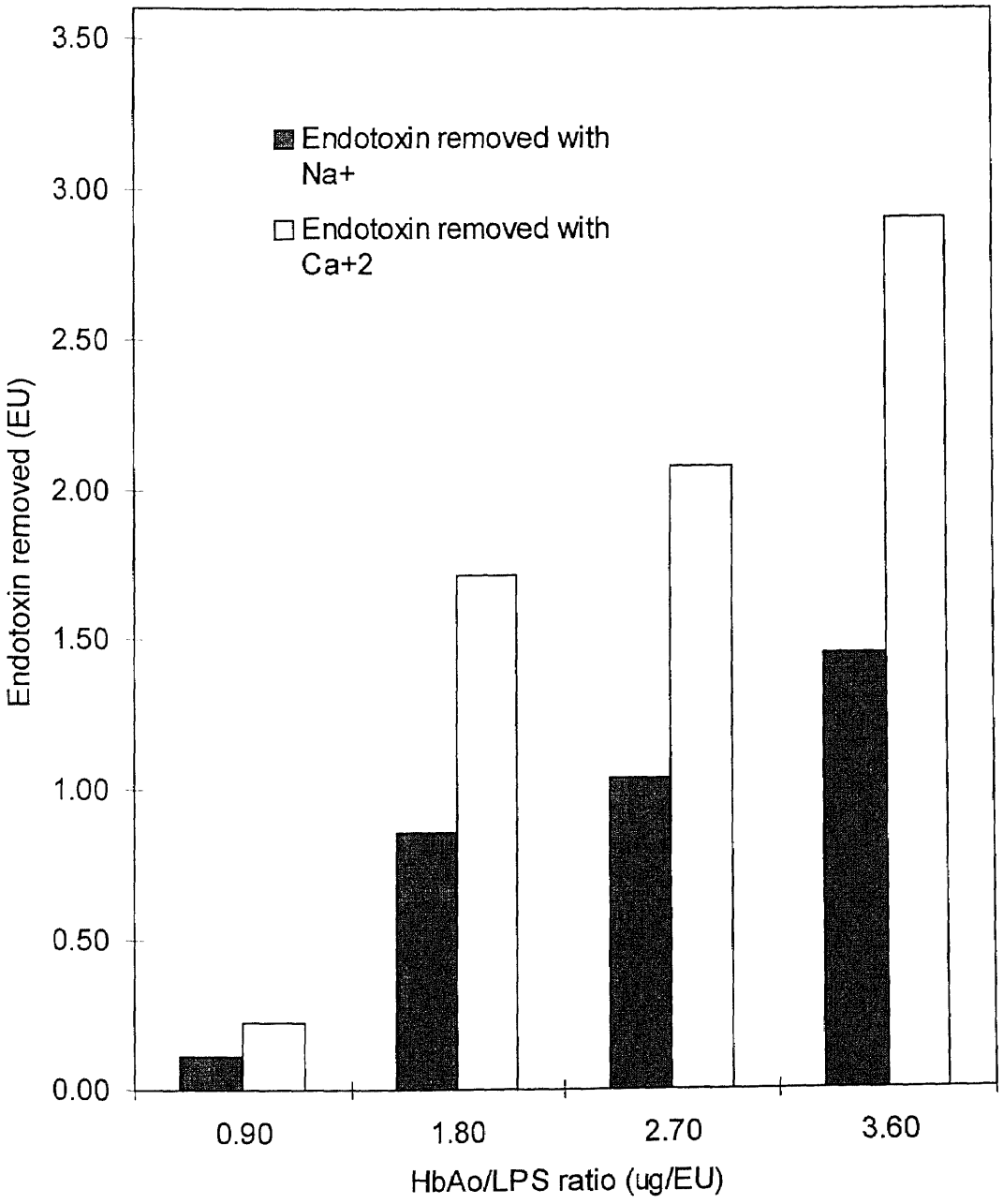


Figure 6.5 Endotoxin removed during the second stage ultrafiltration.

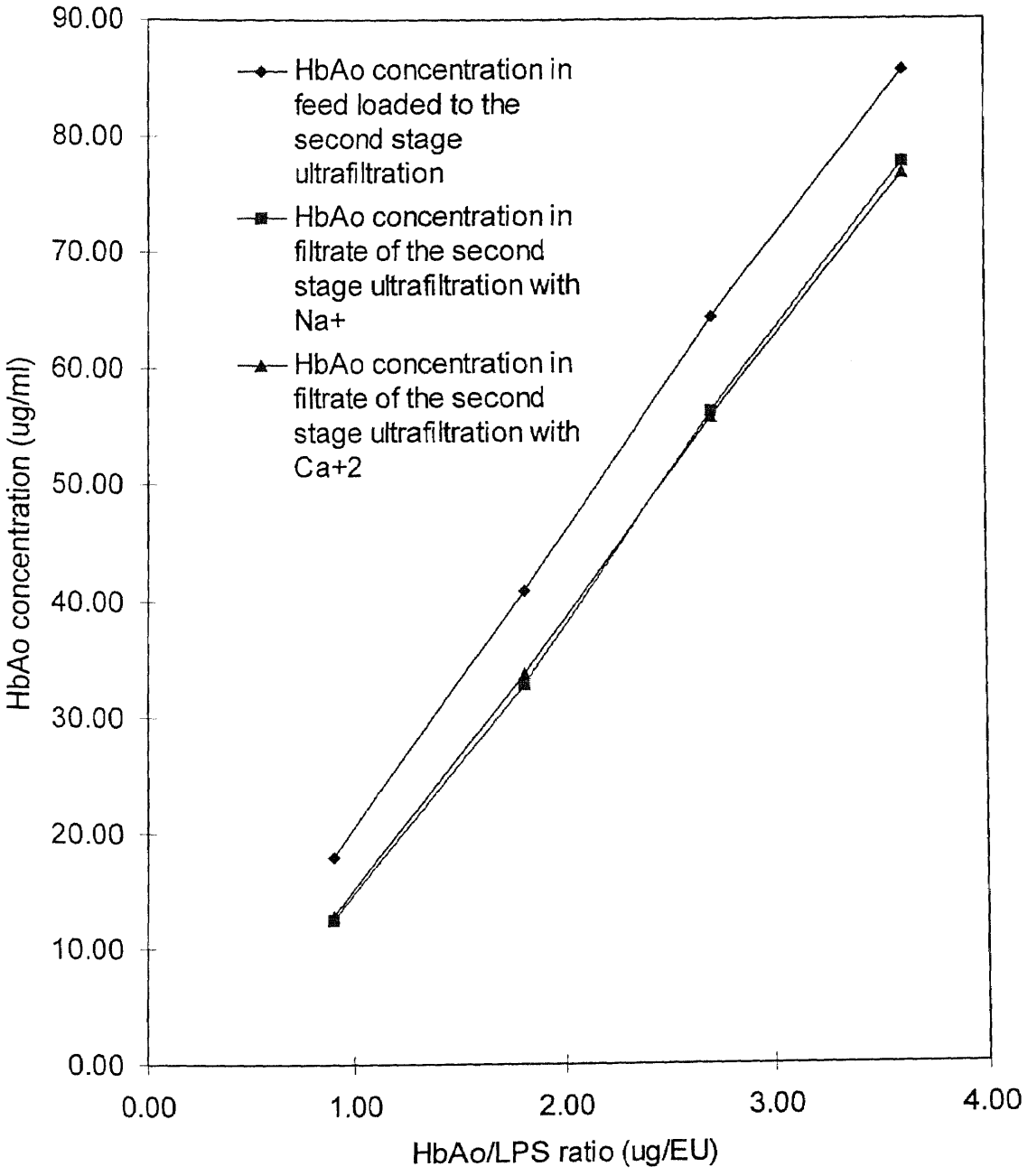


Figure 6. 6 Protein concentration in the second stage ultrafiltration.

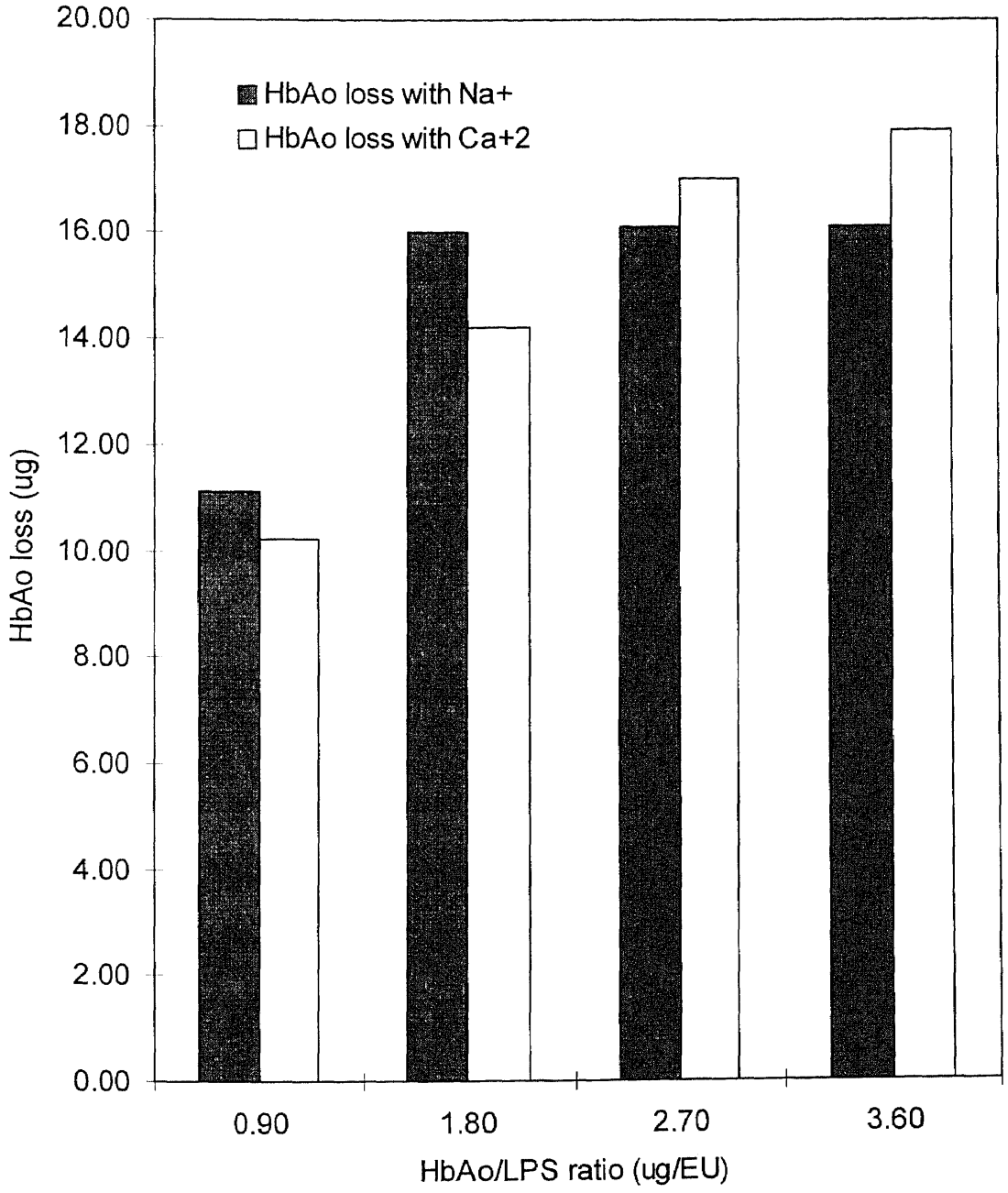


Figure 6.7 Protein loss during the second stage ultrafiltration.

CHAPTER 7

7.1 Conclusions

From this study the following conclusions can be made 1) some proteins have interaction with endotoxin while others do not. 2) Protein concentration has effect on protein-LPS binding and endotoxin removal. 3) When LPS is dissociated by BSA, large aggregates are disaggregated to form smaller size fragments. 4) Not only can Ca^{+2} act as "bridges" between LPS subunits in aqueous solution, it can also re-aggregate LPS in protein solution where LPS is disaggregated by protein. Such disaggregation improves endotoxin removal and does not lose protein recovery.

From the study of *Endotoxin Disaggregation by Protein and Protein Concentration Effect on Protein-LPS Binding and Endotoxin Removal*, we can see that chicken egg white lysozyme and bovine heart cytochrome C did not disaggregate endotoxin, that is, these two proteins did not break endotoxin into small fragments. Hemoglobin and albumin could break endotoxin aggregates. Also concentration of these two proteins had a significant effect on the amount of endotoxin disaggregated and the amount of protein-LPS complex formed. Ultimately, protein concentration affected the rate of endotoxin removal by ultrafiltration membrane processes.

In the study of *Size Exclusion High Performance Liquid Chromatography (SE-HPLC) Studies on Endotoxin Dissociation by Bovin Serum Albumin (BSA)*, BSA size exclusion high performance liquid chromatography (SE-HPLC) was used to investigate LPS disaggregation by BSA. Under the study experiment condition, which pre-treated BSA and LPS mixtures with incubation at 37°C for 30 minutes, BSA to LPS

concentration ratio (C_{BSA}/C_{LPS}) was 16. First, it was found that LPS was disaggregated by BSA. After disaggregation, the major LPS peak, peak group 1, retention time T_r = 6.0 – 7.7, was disaggregated and peak area reduced to 17.2% comparing with non-disaggregated LPS peak area.

Second, BSA concentration in the BSA and LPS mixtures had effect on LPS disaggregation. As BSA concentration increased in the BSA and LPS mixtures, more major peak of LPS, peak group 1, which retention time T_r= 6.0 – 7.7, was disaggregated, and more smaller size fragments were formed. Under the study experiment condition, which pre-treated BSA and LPS mixtures with incubation at 37°C for 30 minutes. When BSA to LPS concentration ratios (C_{BSA}/C_{LPS}) were at 1, 2, 4, 8, 16 in BSA and LPS mixture solutions, the major peak area of LPS, peak group 1, which retention time T_r = 6.0 – 7.7, peak area reduced to 72.3%, 42.1%, 18.4%, 15.2% and 17.2 % respectively comparing with the non-disaggregated LPS peak area.

Third, pre-incubation of BSA and LPS mixture solutions had effect on LPS disaggregation. Under pre-incubation condition, much more LPS was disaggregated by BSA comparing with at non pre-incubation condition. When BSA to LPS concentration ratio (C_{BSA}/C_{LPS}) were at 1, 2, 4, 8 and 16, at pre-incubation condition, peak group 1, which retention time T_r = 6.0 – 7.7, peak area reduced to 72.3%, 42.1%, 18.4%, 15.2% and 17.2% comparing with non-disaggregated LPS peak. With same BSA to LPS concentration ratio, at non pre-incubation condition, peak group 1 area only reduced to 98.1%, 72.9%, 65.2%, 57.7% and 49.0%

In *Qualitative Study on Ca^{+2} Re-Aggregate Endotoxin in HbA_o Solutions and Subsequent Removal of Endotoxin by Ultrafiltration*, a method of two-stage ultrafiltration

was used to investigate the effect of Ca^{+2} on the state of LPS aggregation in HbA_o solutions. Qualitative gelation *Limulus* amebocyte lysate (LAL) test was used to detect endotoxin. Some studies have shown that in aqueous solution, divalent cation can act as "bridges" between LPS subunits, so that LPS form large vesicles. The reason is LPS has negatively charged phosphate groups, cations, especially divalent cations such as Ca^{+2} and Mg^{+2} can act as "bridges" between LPS subunits, resulting in LPS bilayer sheets or vesicles with a diameter of the order of 0.1μm in water. Such property has potential utility in endotoxin removal. Through this study, it was found that Ca^{+2} re-aggregated LPS subunits into large vesicles in protein solution, hence the re-aggregated LPS was retained by a 300,000 nominal molecular weight cut-off ultrafiltration membrane, therefore endotoxin was removed subsequently. Such re-aggregation occurred at concentration ratio of HbA_o / endotoxin (C_{HbAo}/C_{LPS}) equal to or less than 17.0, or at concentration ratio of Ca^{+2} / HbA_o / lipopolysaccharide ($C_{Ca+2}/C_{HbAo}/C_{LPS}$) equal to or greater than 8.5

In the study of *Quantitative Determination of Ca^{+2} Effects on Endotoxin Removal and Protein Yield in A Two-Stage Ultrafiltration*, the method of two-stage ultrafiltration was also used to study the effect of Ca^{+2} on LPS re-aggregation. By using of chromogenic LAL assay, it is possible to get LPS removal efficiency. It was found that, at various protein to endotoxin concentration ratios, after the LPS re-aggregation by Ca^{+2}, endotoxin removal efficiency was in the range of 54.78 to 73.10%, meanwhile protein yield was in the range of 71.57 to 89.54%.

Those results are significant for endotoxin removal process design. For endotoxin removal design, not only should solution composition be considered, but also

concentration of each component should be taken into account. Especially for membrane process or chromatography process taking advantage of endotoxin molecular size, from this study it is clear that at different concentration composition, endotoxin aggregate size will vary, such endotoxin size change will affect endotoxin removal remarkably.

7.2 Recommendations

Further studies are recommended: First, to investigate size and molecular weight of LPS aggregates and protein-LPS mixture. Since no proper LPS molecular size standards were available, the size of LPS aggregate was not studied. The experiments can be carried out by using Minidawn Laser Scattering Device (Wyatt Technology Corporation, Santa Barbara, CA). Second, to investigate incubation temperature effects on LPS disaggregation by protein. Third, to investigate incubation time effects on LPS disaggregation by protein.

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