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ABSTRACT

ENDOTOXIN REMOVAL FROM PROTEIN SOLUTIONS BY IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY

by
Yun Kang

In the biotechnology/pharmaceutical industry, The removal of endotoxins from final protein product has always been a challenge. A novel method for endotoxin removal from protein solutions, immobilized metal (ion) affinity chromatography (IMAC) has been developed in this study. Fe^{3+} was charged to a chelating gel that contains iminodiacetic acid (IDA). The IDA-Fe^{3+} IMAC column was exploited to remove endotoxin from protein solutions. This method involves the adsorption of the endotoxin on IMAC column. More than 99% endotoxin is removed and protein yield is higher than 90%. IDA-Fe^{3+} IMAC has proved to be an economical and efficient method to remove endotoxin from protein solutions.

The study consists four segments: endotoxin assay method development, the evaluation of current endotoxin removal methods, adsorption isotherm and capacity study regarding IMAC column, and process development including a leaching study.

First, to properly detect endotoxin content in experimental solutions, the effect of protein on Limulus Amebocyte Lysate (LAL) test was discussed. Subtraction of protein absorbance from total LAL reaction mixture is a very efficient way. The inhibition of phosphate ion on LAL test was observed. This was circumvented by a proper sample dilution before the test.

Second, the currently available technology, Sterogene Acticlean Etox was used in a comparison study. The column capacity for endotoxin is very low (less than 100 EU/ml gel). The efficiency to remove endotoxin from protein solution with IMAC column has proved to be better.

Third, adsorption isotherms for both endotoxin and bovine serum albumin on IDA-Fe^{3+} IMAC were investigated. This IMAC column has a high capacity for endotoxins: more than 10,000 EU/ml gel. It was found that endotoxin adsorption mechanism was due to the interaction between Fe^{3+} and phosphate group in lipid A, one of the important parts of endotoxins. The IMAC resin also has high capacity for proteins. The column adsorbs endotoxins more strongly than proteins at certain condition. The buffer conditions for selectively removing endotoxins from protein solutions were determined.

Fourth, the endotoxin or protein elution process was developed. Different elution schemes were investigated including buffer pH, ionic strength, and elution competing agent. The change of solution ionic strength and introduction of NH_4Cl in elution buffer are not efficient to elute protein from the IMAC column. N-octyl-β-D-glucopyranoside (OBDG) at a low concentration could not elution protein while at a high concentration ferric ion leaching was observed. It was found that phosphate buffer was a very efficient elution agent. To prevent the leaching of metal ion from the column, elution buffer pH should be lower than 5.5.

ENDOTOXIN REMOVAL FROM PROTEIN SOLUTIONS BY IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY

by
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ENDOTOXIN REMOVAL FROM PROTEIN SOLUTIONS BY IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY

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To my beloved family

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CHAPTER 1

BACKGROUND AND RATIONALE
ENDOTOXINS AND ENDOTOXIN REMOVAL

1.1 Endotoxins

1.1.1 Structure and Origin of Endotoxins

Bacterial endotoxins (ET) are lipopolysaccharides (LPS), which is a main component of outer cell membranes of Gram-negative bacteria (Shiba, 1983), such as *Escherichia coli, Salmonella typhosa, Salmonella typhimurium* and *Bordetella pertussis*. LPS is a large amphipathic molecule consisting of three distinct regions: lipid A, the core oligosaccharides, and the O-antigenic polysaccharide side chain as shown in Figure 1.1. The hydrophobic lipid A section is made of six or seven saturated fatty acid chains linked to two phosphorylated glucosamine residues. This region has been shown to be responsible for the toxic properties of LPS (Galanos et al., 1977). The hydrophilic polysaccharide portion is made of a core oligosaccharide and an antigenic polysaccharide O side chain. The O side chain consists of repeating units of four or five sugar sequences and is unique to each bacterial serotype. The core oligosaccharide is structurally less diverse than the O chain, yet both contain a number of sugars that are rarely seen elsewhere in nature (Shiba, 1983).

LPSs derived from wild-type bacteria are classified into three categories based on their molecular structures. Type I LPS is composed of an O-antigen-specific polysaccharide, outer and inner core polysaccharides, and lipid A, and is found in *Enterobacteriaceae and Pseudomonas* species. Type II LPS, which lacks an O-antigen-specific polysaccharide, is distributed in *Hemophilus influenzae, Acinetobacters* and

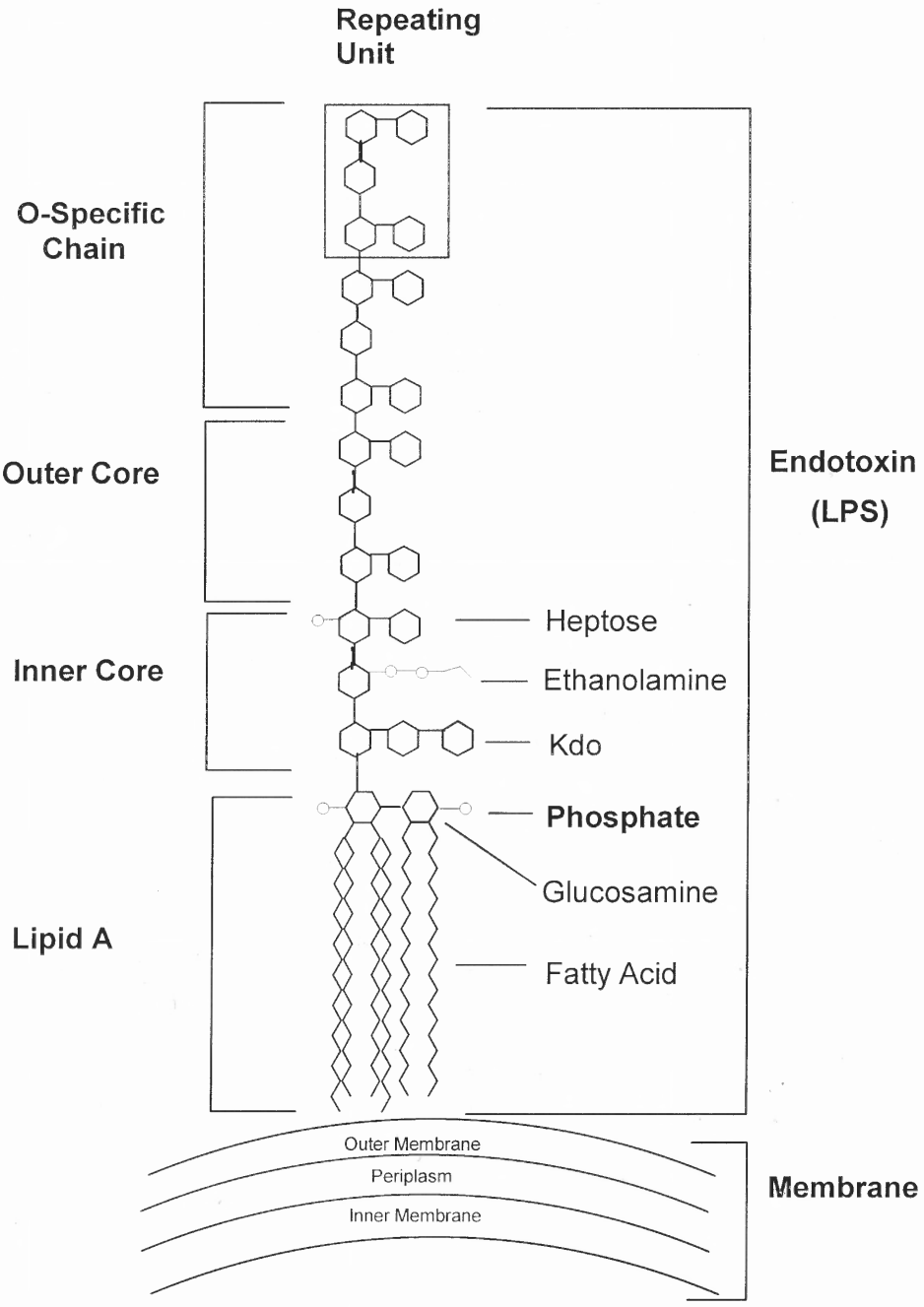


Figure 1.1 Lipopolysaccharide core structure and location in the bacterial membrane

Bordetella pertussis. The type III LPS as seen in *Chlamydia* consists only of inner core oligosaccharide and lipid A. Type I LPS is tightly polymerized and is not easily depolymerized by heat exposure, while aggregates of type II LPS are heat labile. Furthermore the molecular size of monomeric form of type II LPS is much smaller than that of type I LPS and seems more difficult to reject via sieving (Rietschel et al., 1993).

Many kinds of endotoxins with different origins are involved in previous researches such as *Haemophilus influenzae* type b (Hib) (Rienstra et al., 1990), *Enterobacteriaceae coli* (Issekutz, 1983; Hou and Zanieski, 1990; Hirayama et al., 1994; Evans-Strickfaden et al., 1996; Petsch et al., 1997), *Salmonella typhimurium* (Aida et al., 1990), *Bordetella pertussis* (Hirayama et al., 1992) and *Pseudomonas aerugiosa* (Felice et al., 1993).

1.1.2 Endotoxin and Toxicity

Endotoxins can be an indirect cause of fever because they stimulate host cells to synthesize and release endogenous proteins. These proteins then interfere with the mammalian thermoregulatory brain center to produce fever. Endotoxins are very toxic. After intravenous application at concentrations less than 1 ng/ml (10^{-9} g/ml) endotoxins produce fever in human beings and animals that in extreme cases resulted in death. As little as 150 μg endotoxin can be lethal to a horse (Li, 1999). The physiopathological effects shown by infected humans or animals include pyrogenicity, endotoxemia, leucopenia, leucocytosis, lower blood pressure, allergic reactions, sepsis, septic shock and multiorgan dysfunction syndrome. Every year more than 500,000 hospitalized patients in United States develop the sepsis. An estimated 200,000 sepsis patients develop septic

shock and nearly one-half die from multiple organ dysfunction syndrome. It was estimated that 400,000-500,00 episodes of sepsis resulted in 100,000-175,00 human deaths in the U.S. alone in 1991 (Mitzner et al., 1993; Lonergan et al., 1994). Annual costs associated with septicemia and septic shock range as high as 10 billion dollars per year (caused by the extra length of intensive care unit (ICU) stay and the intensity of clinical management, which includes antibiotics, cardiovascular support (intravenous agents), ventilatory support, kidney dialysis, and in some cases, surgery to remove the source of infection).

Besides from Gram negative bacteria, endotoxins are also possibly derived from the Gram positive bacteria and fungi. The toxicity or pyrogen function of ET is related to its origin and structure. The endotoxin from the fungi is the most lethal to human beings and animals (Kodama et al., 1997) as shown in Table 1.1. The structure and toxic effects of endotoxins from the Gram positive bacteria and Fungi should be investigated.

Table 1.1 Phase 3a clinical trial: treatment of critically ill patients contaminated with endotoxins with different origins (adapted from Kodama et al., 1997)

Clinical Trial	Survival Rate
28 patients with Gram negative infections	61%
9 cases of Gram positive infections	56%
2 cases of fungal infections	0%
13 cases of concomitant Gram positive and Gram negative infection	38%
4 cases of concomitant Gram positive and fungal infection	0%
3 cases of concomitant Gram negative and fungal infection	0%
3 cases of combined Gram positive, Gram negative and fungal infection	0%

1.2 Endotoxin Contamination

The solutions contaminated with endotoxins can be divided into five categories: aqueous solutions, parenteral solutions, bacteria filtrates, products / biomolecular solutions and plasma, according to molecular weight and the complexity of solution composition. These solutions can be found in following processes: biotech/pharmaceutical processes, pharmacy processes and therapeutic processes.

1.2.1 Biotechnological/Pharmaceutical Processes

(1) Process water

Different methods have been used to remove endotoxin from process water. Less than 0.02 EU/ml ET in water was achieved with Milli-Q system from Millipore Incorporation by the author's test. The endotoxin removal from Water for Irrigation was achieved by using the 6000 MW cutoff Polyacrylonitrile (PAN) and Polysulfone (PS) ultrafilters (Evans-Strickfaden, et al., 1996). The high quality endotoxin free water was provided by various manufacturers such as BioWhittaker Incorporation, Cape Cod Associates, etc.

(2) Buffers and raw materials

Different buffers or raw materials are used in biotech/pharmaceutical processes. Various methods should be taken to prevent the contamination of endotoxins.

(3) End products

Biotechnology provides variety of ways to produce bioproducts such as proteins. The most striking method is to express various proteins with recombinant DNA techniques in

different hosts, 90% of which is *E. Coli*. Endotoxin removal is key issue in downstream processing. The endotoxin removal process was studied by different researchers for various products, namely, conjugated vaccine Pedvax HIB (Rienstra et. al, 1990), filamentous hemagglutinin and pertussis toxin (Hirayama et al., 1992), albumin (Hou and Zaniewski, 1990; Aida, and Pabst, 1990; Hirayama et al., 1994; Anspach and Hilbeck, 1995; Morimoto, 1995; Wakitu, 1996; Sakata, 1996; Legallais et. al 1997; Petsch et al., 1997), γ-globulin (Hou and Zaniewski, 1990; Hirayama et al., 1994; Morimoto et al. 1995), IgG (Petsch et al., 1997; Legallais et. al, 1997), cytochrome C (Aida and Pabst, 1990; Hirayama et al., 1994; Morimoto et al., 1995), catalase (Aida and Pabst, 1990), myoglobin (Hirayama et al., 1994; Morimoto et al., 1995), lysozyme (Petsch et al., 1997), β-lactoglobulin (Sakata, 1996), Insulin (Sakata, 1996), superoxide dismutase (SOD), and amylopectin (Held, 1997).

1.2.2 Pharmacy: Parenteral Solutions and Diagnostics/dialysis Buffer

Parenteral solutions and diagnostics/dialysis buffers can be depyrogenated mainly by membranes. Endotoxins from the peritoneal dialysis solutions were removed using polysulfone ultrafilter (Rafiee-Tehrani et. al, 1996) and hemodialysis processes (Yamamoto and Kim, 1996) with semipermeable membrane. The endotoxin removal from the bicarbonate dialysis buffer was investigated by Cappelli et al. (1993). Brown studied depyrogenation of water for injection, Iohexol 350 mgI/ml active and placebo with submicron filters (1993).

1.2.3 Therapeutic Processes

Extracorporal endotoxin removal from the plasma or blood was used to treat the sepsis, septic and multiorgan failure (Lonergan et al., 1994; Shoji et al., 1998; Jaber et al., 1998). It is the only practical method to treat the diseases caused by endotoxin contamination.

In summary, endotoxin removals from water, buffer solutions and dialysates have been carried out successfully. However, endotoxin removal from biological solutions such as proteins and plasma proved to be very difficult due to several reasons. First, proteins form complexes with endotoxins, which are difficult to break down; Second, the interaction mechanism between the endotoxin and protein is not clear; Finally the endotoxin exists in different aggregation states, which are function of solution conditions.

1.3 Endotoxin Removal Methods

1.3.1 Traditional Methods

Traditional methods for endotoxin removal include chemical decomposition of pyrogen with acids, alkali, or oxidizing agents, which is commonly practiced in sterilization (Hou and Zaniewski, 1990), distillation, reverse osmosis (Evans-Strikfaden et. al, 1996) and adsorption by asbestos or other media.

Distillation and reverse osmosis are currently used to produce endotoxin free water (low endotoxin contents) for injection. However, distillation processes are highly capital intensive and expensive to operate. Reverse osmosis offers a less expensive method of pyrogen removal but presents substantial problems of cleaning, depyrogenating, and maintaining a non-pyrogenic permeate over extended operational time periods. Distillation and reverse osmosis have the further drawback that neither can

be used to depyrogenate parenteral solutions or targeted bioproducts (Evans-Strikfaden et al., 1996).

1.3.2 Filtration and Membrane Separations

1.3.2.1 Ultrafiltration membrane (1 to 200 nm)

In aqueous solutions a majority of endotoxin molecules aggregate into vesicles ranging from 300,000 to 1,000,000. In their lowest aggregation states, endotoxins exist in monomeric form (10,000 to 20,000). Theoretically endotoxins may be removed by ultrafiltration membranes.

Ultrafiltration membranes with low molecular weight cut-off may be more effective than submicron filters because of their ability to remove pyrogens based on size exclusion in addition to adsorption to the membrane. Ultrafiltration has been approved for the manufacture of water for injection (WFI) in Japan, and for WFI quality rinse water in Europe (Evans-Strikfaden et al. 1996). Several companies in U.S. have commercial products to produce water with very low endotoxin contents, such as Millipore's Milli-Q system with an ultrafiltration (MW cutoff 5,000) to reduce the pyrogen from high endotoxin level in tap water to an endotoxin content of lower than 0.02 EU/ml (determined by the author). However, the ultrafiltration processing has some disadvantages: low filtration rates, especially for viscous products (e.g. iodinated radiopaque products) and thus time consuming, as well as high cost of ultrafiltration equipment. Ultrafiltration membranes have no specificity to bind endotoxin. Other contaminating particles are also removed from the solution, which results in fouling.

Tetta et al. (1993) evaluated the long-term (360 h) efficacy of ultrafiltration in the reducing endotoxin using a polysulfone ultrafilter, and the impact of daily routine sterilization on the ultrafilter efficacy. The removal of endotoxin from bicarbonate dialysis fluids by ultrafiltration is an efficient, reliable, and only practical on-line procedure to circumvent microbial contamination during a conventional dialysis, on-line hemofiltration and hemodialfiltration. Bacterial products reactive to LAL test were always below the level of 0.125 EU/ml in all post-ultrafilter samples. However, the issue of endotoxin (or its subunits) permeability through polysulfone membranes has been a matter of controversy.

Polyacrylonitrile (PAN) and polysulfone (PS) hollow fiber ultrafilters with 6,000 MW cut-off are useful (Evans-Strikfaden et al. 1996) in endotoxin removal from the water for irrigation based on size exclusion. The high-flux polysulfone ultrafilter (dialyzer) represents an efficient system to remove Lipid A and *Pseudomonas aeroginosa* endotoxin from the dialysis fluids (the contaminated peritoneal solutions) to produce endotoxin free dialysate (Refiee-Tehrani et al., 1996). The effect of ion activities in hemodialysis solutions on the efficiency and behavior of polysulfone ultrafilters was not significant.

1.3.2.2 Submicron filters

The majority of ET molecules in aqueous solutions aggregate into vesicles ranging from 300,000 to 1,000,000. Endotoxins may be removed by ultrafilters with MW cut-off > 300 K (Li and Luo, 1999) or by submicron membrane filters (Brown and Fuller, 1993). The use of submicron filtration in place of ultrafiltration would provide significant cost

benefits in terms of less filtration time and equipment costs. The removal of pyrogen by the positively charged membranes with nominal pore sizes much greater than the pyrogenic material in solution has been documented. However, the filters were not shown to be efficient for removing ET when electrolytes or proteins were present in the solution (Pearson, 1985). The pyrogen removal by adsorption to submicron filters was examined. Endotoxin level after removal varies depending on the process conditions (Evans-Strikfaden et al. 1996). Brown et al. (1993) evaluated the practicality of increasing the MW cut-off of ultrafilters and efficiency of endotoxin removal using submicron filters, including a Pall Posidyne charge modified filter. The submicron filter may be capable of efficiently removing the endotoxins from the pharmaceutical products. However, the endotoxin aggregate size increases as the ionic strength increases, which is different with the results of most other researchers.

1.3.2.3 Membrane Adsorber

Ultrafiltration methods using four different hollow fiber membranes: polyester polymer alloy (PEPA), polymethylmethacrylate (PMMA), polyacrylonitrile (PAN) and polysulfone were investigated to examine the LPS rejection capability (Yamamoto and Kim, 1996). Complete rejection of LPS can be achieved by hydrophobic membrane of high adsorption capacity with an appropriate sieving property due to hydrophobic properties of lipid A. Membrane absorbers have the advantage of high throughput compared to traditional adsorption beds.

Anspach et al. (1997) prepared membrane adsorber, isotropic Nylon microfiltration membranes coated with Dextran, hydroxyethyl-cellulose (HEC), and

polyvinyl alcohol (PVA) as the matrix, and immobilized histidine (His), histamine (Him), polymyxin B (PMB), α-amylase, poly-L-lysine (PLL), polyetheleneimine (PEI), deoxycholate (DOC) as the ligands. The Nylon has advantages of high mechanical properties and low non-specific adsorption. The prepared membrane adsorber was used to remove ET from the buffers and protein solutions. To remove endotoxin from protein free solutions (20 mM PBS, pH7), all ligands comprising a net positive charge show very good endotoxin removal except the Bis:Him. In contrast, for the negative charge ligand, almost no endotoxin is removed. Mechanism for endotoxin removal is electrostatic attraction due to the negative charge properties of LPS in experimental pH range. Varying the solution ionic strength and pH results in the changes of the charge distribution of both ligands and endotoxin molecules and thus affects of endotoxin removal efficiency.

For the endotoxin removal from protein solutions, the interaction of protein with the positively charged adsorber should be considered. The poor endotoxin recovery in the presence of basic proteins was found. This was explained by masking of lysozyme on the endotoxin activity (Anspach et al., 1997). In other words, protein-bound endotoxin is not LAL-active. This is questionable. The low recovery could be an inhibition of LAL test by phosphate ions, which will be discussed in the later chapter.

The depyrogenation of different IgG solutions using the histidine-linked hollow fiber membrane was presented (Legallais, et al., 1997). Endotoxin removal from the 1mg/ml non histidine-binding mouse monoclonal IgG1 (MabCD4) solution was achieved in the presence of acetate buffer (pH 5.0) without any protein loss. The mechanism of endotoxin removal was considered as selective adsorption and adsorption replacement.

1.3.3 Adsorption & Chromatography

1.3.3.1 Adsorption

Ultrafiltration requires a large size difference between product and contaminant. It is not effective when the contaminant binds with proteins strongly (Anspach and Hilbeck, 1995). To remove endotoxin from protein solution, adsorption has proven to be the most effective method (Morimoto et al., 1995).

Commercial available adsorbents, such as immobilized histidine and polymyxin-Sepharose, have high endotoxin adsorption capacity at low ionic strength. As the ionic strength increases, endotoxin adsorption capacity decreases. Endotoxin adsorbents capable of retaining a high endotoxin capacity over a wide range of ionic strength conditions are not available. The adsorption of acidic proteins such as BSA follows the same adsorption behavior as that of endotoxins. Commercially available beads can not selectively remove endotoxin from these protein solutions.

Rienstra et al. (1990) used a hydrophobic resin HP 20 (highly porous polymer of styrene and divinylbenzene copolymer in the form of 20-50 mesh beads) to develop a method for endotoxin removal from the conjugated vaccine PedvaxHIB®, in which Hib polyribosylribitol phosphate capsular polysaccharide (PRP) is covalently bound to outer membrane protein complex of B11 strain of *Neisseria meningitidis* serogroup B. Treating LPS with chelating agents (sodium citrate) can disaggregate LPS into micelles which can be further reduced with detergents (sodium deoxycholate) to its primary molecular size of 10,000 to 20,000. It was presumed that under these conditions, LPS would be small enough to enter the pores of a macroreticular resin and bind hydrophobically to the non-

ionic surface with the ionic PRP remaining free in the solution. Optimal conditions are 6-7% sodium citrate dihydrate, 0.75 % DOC and pH above 8.0 in order to prevent DOC gelation. Low endotoxin level and high PRP yield can be achieved using HP20 adsorption process.

Hirayama et al. (1992, 1994, and 1996) developed various affinity resins for endotoxin removal from different solutions. The poly (γ-methyl L-glutamate) (PMLG) spheres with diaminoethane as a ligand have a high endotoxin-removing activity even at high ionic strength (μ=0.2-0.8). It is suitable for the endotoxin removal from antigenic proteins in the culture supernatant of *Bordetella pertussis* and tetanus and diphtheria toxoids, which are vulnerable substances and sometimes aggregate at low ionic strength (μ =0.01-0.1 M) because of their hydrophobicity (Hirayama et al., 1992). However, the adsorbent regeneration is not satisfactory.

A cross-linked N, N-dimethylaminopropyacrylamide (DAA) spherical adsorbent was prepared by suspension copolymerization of N, N – dimethyl amino propyacrylamide (DMAPAA) with N-allylacrylamide (AAA) (Hirayama et al., 1995). When $M_{lim} < 300$, and amino group content was 4.5 meq./g, DAA adsorbent showed endotoxin-removing activity at μ=0.05-0.4 and pH = 5-9. It could selectively adsorb endotoxin from BSA solution at a low ionic strength. However, the adsorption capacity for endotoxin is low.

PEI-cellulose fiber, which is suitable only for low ionic strength condition (Morimoto et al., 1995) has higher adsorbing capacity than histidine-immobilized cellulose and polymyxin-immobilized Sepharose.

All above affinity resins are suitable to remove endotoxins from specific solutions. The functional group NH_2 has interactions with the proteins, which is

responsible for the non-specific binding. The protein recovery is low. Endotoxin was considered to be adsorbed on the surface of the resin while the protein molecule was adsorbed in pores. The adsorption behaviors of resins with different pore sizes: 4,000 (Sakata et al., 1996), 5,000 (Morimoto et al., 1995), 300 (Hirayama et. al, 1994) indicates that this mechanism is problematic.

Lipid membrane has been receiving considerable attention as a model for various chemical investigations in the areas of chemical sensing and separation due to its hydrophilic and hydrophobic properties. A method using carboxylated porous supports and ligands such as cationic lipid membrane of N-octadecylchitosan consisting of 2-deoxy-2-octadecylamino-D-glucopyranose(GlcNC18),2-amino-2-deoxy-D-glucopyranose (GlcN), 2-acetamino-2-deoxy-D-glucopyranose (GlcNAc) to remove endotoxin from the protein solutions was developed (Wakitu et al., 1996). LPS were selectively adsorbed with 99.2% to 99.9% removal efficiency and 91% to 100% recovery of BSA. The residual LPS concentration varied with the pH and ionic strength.

Anspach et. al (1995) removed endotoxins by using histamine, histidine, polymyxin B and poly-L-lysine and DEAE-Sepharose affinity adsorbents. Immobilized histamine, histidine and polymyxin B were effective for endotoxin removal from the *E. coli* filtrate. However their effectiveness decreased dramatically in the presence of proteins. DEAE-Sepharose was the most suitable adsorbent for the decontamination of positively charged proteins, such as lysozyme. Clearance of negatively charged proteins is always problematic.

At very high endotoxin contamination, direct application of protein solutions to these adsorbents is not effective. In such extreme cases ultrafiltration should be used as

an initial step in order to reduce the endotoxin concentration, especially for large agglomerates of endotoxins, whereupon the endotoxin-specific adsorbents can be employed in subsequent step.

1.3.3.2 Ion-exchange

The charge character of a biomolecule can be either enhanced or inhibited by a proper adjustment of solution conditions, with pH level and salt concentration as major controlling factors (Hou and Zaniewski, 1990). The enhanced adsorption of endotoxin in the presence of 100 mM salt concentration may be due to aggregation of endotoxin molecules. As the salt concentration further increases, reduction in endotoxin removal caused by the salt counterions is observed.

In process development of endotoxin removal, original source and existing form of endotoxin as well as the nature of protein molecules contaminated with pyrogen dominate the efficiency and level of pyrogen reduction. The importance of pH, salt and flow dynamics in pyrogen removal by anion-exchange filters should be considered.

1.3.4 Phase Separation

Phase separation was widely used in the separation and purification of protein products. It provides a rapid and gentle method for removing endotoxin from protein solutions. Protein treated by this unit operation retains its normal functions. Endotoxin contamination of protein solutions was reduced by a phase separation technique using a detergent, Triton X-114 (Aida and Pabst, 1990). The disadvantage of this method is that

small amount of detergent persists in the aqueous phase after the separation and is very difficult to remove.

1.3.5 Miscellaneous Methods

In pharmaceutical or biotech industry a variety of methods were used to remove pyrogen from the raw materials, intermediates and final products. Two case studies of endotoxin removal from the SOD and amylopectin by Held et. al (1997) gave us a very good example.

1.3.6 Treatment of Sepsis, Septic Shock and Septic Multiple Organ Failure by Extracorporeal Endotoxin Removal Methods

Antibiotics can not prevent the toxic effects of lipid A in endotoxins. Current therapeutic strategies such as anti-ET, anti-cytokine have not been shown to be consistently beneficial in reducing the morbidity/mortality of patients suffering from sepsis. The complexity of inflammatory network, and lack of clinical success with the use of specific inhibitors of mediators, have kindled interest of extracorporeal therapies for treatment of sepsis (Jaber et al., 1998). Lonergan et al. (1994) induced an endotoxic shock in an animal model and attempted to treat it by direct hemoperfusion over a modified anion sorbent column. It has been shown that the reversal of septic shock correlates with the efficiency of extracorporeal endotoxin removal.

Kodama et al. (1997) summarized the methods of treating critically ill patients and their efficacy. Attempts have been made to remove the endotoxin, the main cause of sepsis, from the circulation using polymyxin B immobilized fiber, charcoal

hemoperfusion, and plasma or whole blood exchange. Attempts have also been made to remove proinflammatory cytokines, eicosanoides, and coagulative factors from the circulation in human body. Hemofiltration has been applied to treat severe sepsis and septic multiple organ failure (MOF). The low MW of the serum mediators can be removed by hemofiltration. However, no clinical study has shown its direct effect on severe sepsis. Plasma or whole blood exchange in child may be helpful because it reduces the concentration of endotoxins, amines, cytokines, and protein bound toxins. However, to reduce concentrations effectively, a large amount of whole blood or plasma must be used. Furthermore, post perfusion complications such as hepatitis or acquired immuno-deficiency syndrome may occur. Toraymyxin, a biomaterial prepared by fixing polymyxin B to polystyrene fibers, is now widely used in Japan. It has the potential for the treatment of septic patients and those with endotoxemia, which leads to MOF. However, Toraymyxin still has some unsolved problems (Kodama et al., 1997).

Polymyxin B immobilized Fiber Cartridge (PMX-F) (Shoji et al., 1998) could detoxify many kinds of endotoxins in vitro, due to its interaction with the lipid A portion of endotoxin. Utilization of fibrous adsorbents enabled Shoji et al. (1998) to design the PMX cartridge with a large surface area and low blood pressure drop in the blood flow compartment and to apply it safely to the direct hemoperfusion procedure. Multicenter clinical studies indicate that the blood endotoxin level has been significantly decreased with the application of this method. Hemodynamic abnormality such as low blood pressure and low systemic vascular resistance were significantly improved. However, polymyxin B has strong neurotoxity and nephrotoxicity.

1.4 Endotoxin and Endotoxin-Protein Complex

1.4.1 Endotoxin Aggregates and Pyrogenicity

The structure of bacterial LPS is apparently stabilized by divalent cations, because removal of Ca^{2+} and Mg^{2+} with EDTA causes the bilayer to break down into what appears to be micelles of 300,000 to 1,000,000 molecular weight (Sweadner et al., 1977). The micelles exist as small rods or disks in the electron microscope. The micelles can be further broken down in the presence of detergent or bile salts. The particles can then no longer be seen in the electron microscope, but the size and shape, as calculated from sedimentation velocity, density, and viscosity measurements are 0.8 to 1.2 nm in diameter and 20 to 70 nm long, consistent with what is known of their chemical structure. Their molecular weight is 10,000 to 20,000.

Rienstra et al. (1990) investigated resin adsorption to remove endotoxin from the conjugated vaccine PedvaxHIB. Treating LPS with chelating agents (sodium citrate) can disaggregate the LPS into micelles, which can be further reduced with detergents (sodium deoxycholate) to its primary molecular size of 10,000 to 20,000.

After sonication, the size of endotoxin aggregation became smaller, and was within the range of 10 nm to 20 nm (Sawada et al., 1986; Komuro et al., 1987).

Biological activity of LPS is greatly affected by the particle size or state of dispersion (Komuro et al., 1987). The sonication of LPS for 2 or 3 min significantly enhanced its pyrogenicity. Sonication might disperse the LPS aggregates into smaller and more uniform particles with optimum size for manifesting biological activity in aqueous medium, resulting in an enhancement of pyrogenicity (Komuro et al., 1987). However, Sawada et al. (1986) reported that LPS with a small size has less reactivity to LAL test

and low pyrogenicity in rabbits. This phenomenon (Sweadner et al. 1977) is also noteworthy because action of surfactants such as sodium deoxycholate, dissociates the aggregates of LPS mainly by chemical forces into subunits and simultaneously reduces the pyrogenicity (Oroszlan and Mora, 1963; McIntire et al., 1969; Sweadner et al. 1977). There is a possibility that active sites of LPS are masked by forming a complex with surfactants (Reynolds, et al., 1968; Hannecart-Pokorni et al. 1973; Sweadner et al. 1977), resulting in a reduction of pyrogenicity. Therefore the relationship between the biological activity and the size has to be further elucidated.

1.4.2. Endotoxin-protein Complex

It is shown that LPS associates with some proteins to form complex through electrostatic and hydrophobic interactions. There are several methods to determine if there is a complex formed between endotoxin and biomolecule such as protein.

Spectrophotometer scanning

This method is qualitative. First, the absorbance scanning of pure protein and pure endotoxin was conducted to get the base line of wavelength scanning. Second, these two pure solutions are mixed. The mixture has the same amount of endotoxin or protein as the pure endotoxin or protein control solution. If there is no complex formed, addition of absorption of two pure substances should be same as that of mixture. Otherwise, the complex formed. The scanning experiments were done in this research. The results were shown as Figure 1.2 and Figure 1.3 for bovine albumin serum (BSA)-LPS and human hemoglobin A_0 (HbAo)-LPS respectively. This result matches the research of Miyagawa (1991) and Kaca (1994).

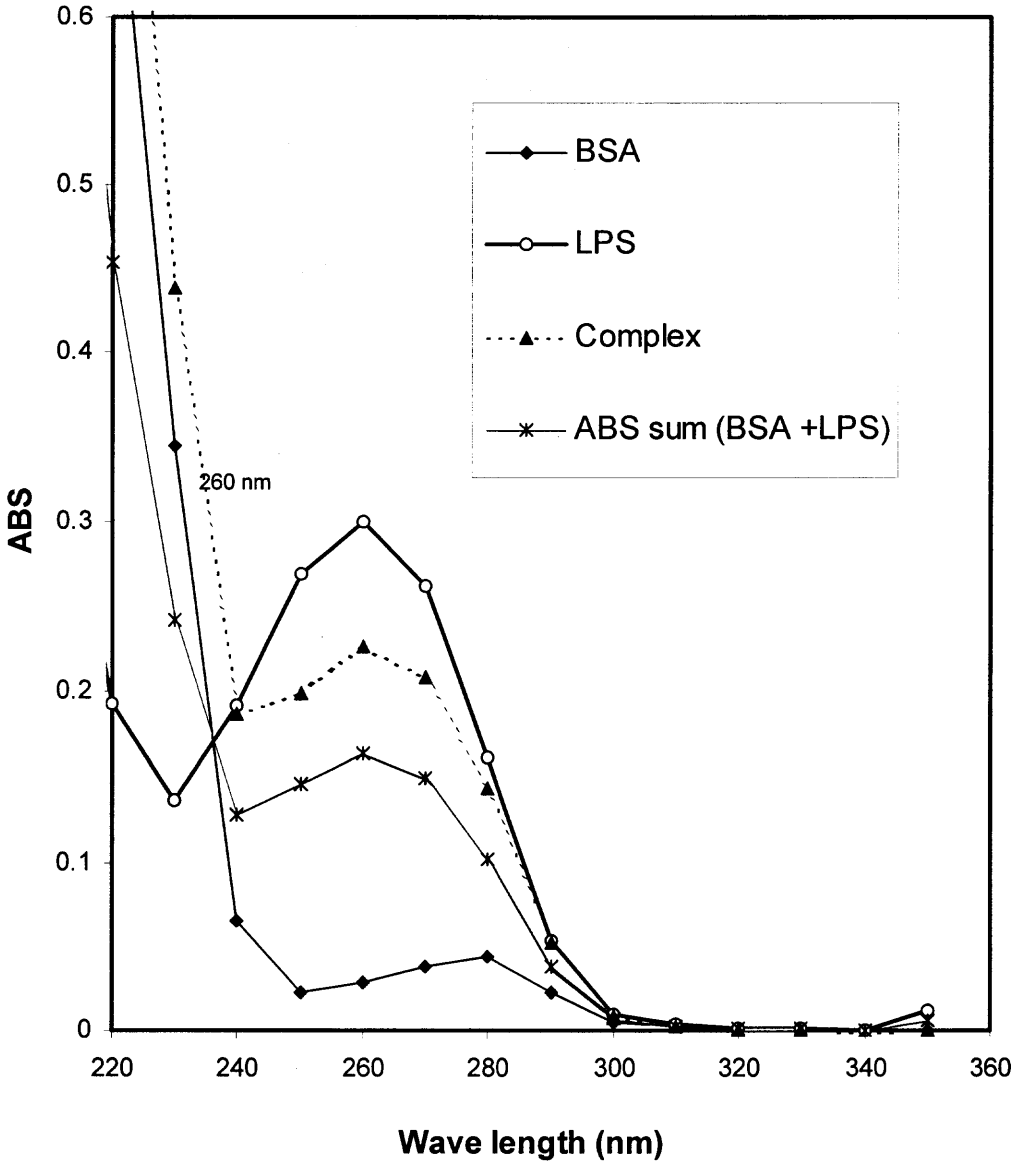


Figure 1.2 Absorbance spectrum of BSA, ET and ET-BSA complex. LPS concentration: 2 EU/ml. BSA concentration: 100 μg/ml.

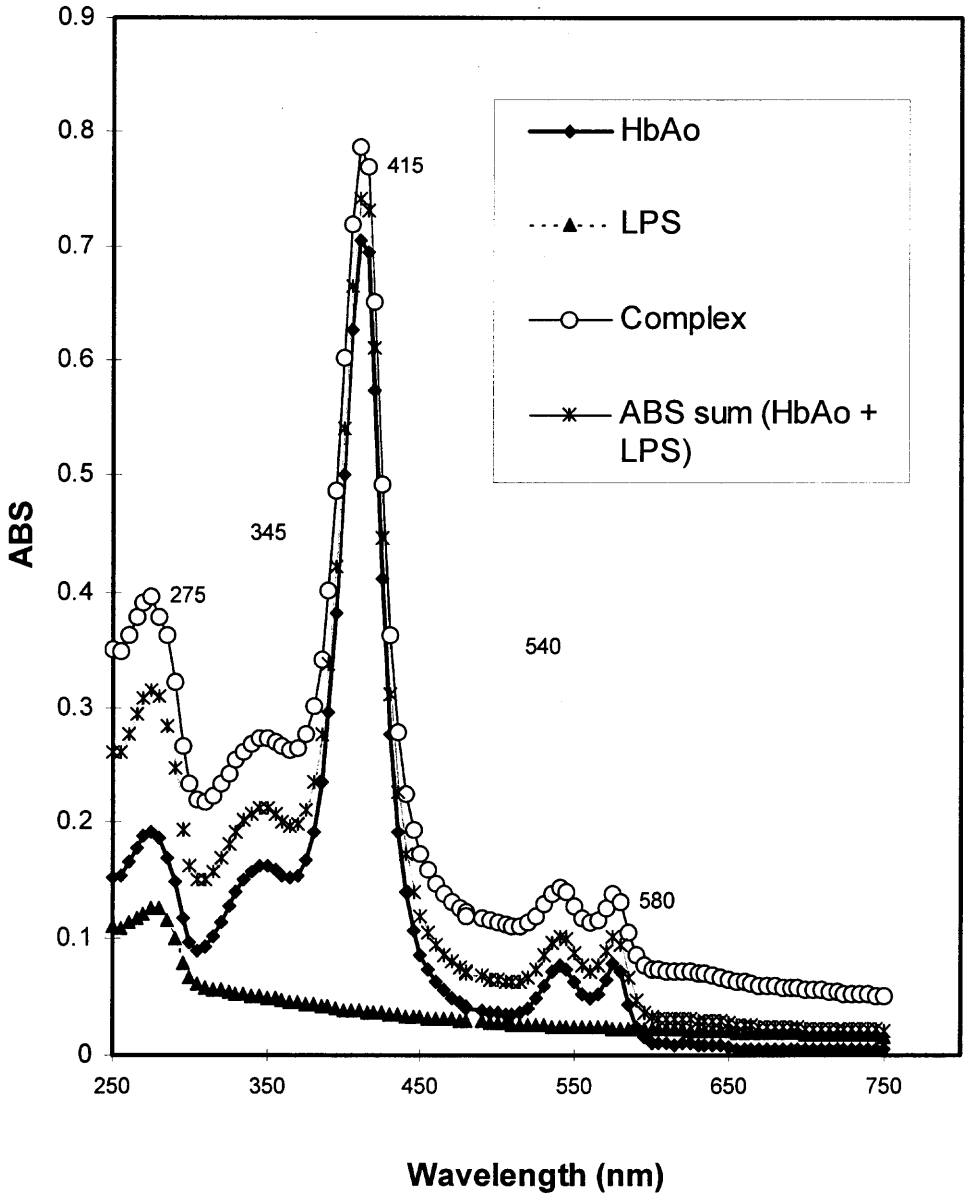


Figure 1.3 Absorbance spectrum of HbAo, ET, HbAo-ET complex. LPS concentration: 2 EU/ml. HbAo concentration: 100μg/ml.

Dynamic light scattering (Hofer et al., 1991)

Scattering intensity and diameter can be obtained by light scanning instrument. In the presence of BSA, scattering intensity and LPS diameter decrease with the increase of BSA concentration. The aggregation states of endotoxins were changed when the BSA was added into LPS solution.

Ultrafiltration membrane (Li and Luo, 1997)

If the molecular weight of the protein is known, e.g. HbAo, M.W. 68.5 K, a membrane with MW cutoff of 100K could be used. First, HbAo is applied to the membrane and the HbAo concentration in the filtrate is measured as the control. Second, mixture of the HbAo and endotoxin is applied to the membrane. If the HbAo concentration in the filtrate is quite different from the control, the complex forms. The regenerated cellulose membrane can be used due to its low protein binding.

1.4.3 Dissociate the Endotoxin-biomolecule Complex

In separation of proteins from the subcellular components, such as membrane, which is phospholipid, mild ionic or nonionic detergents such as deoxycholate, Brij, and the Triton compounds (polyoxyethylene ethers) must be used at low concentration, sometimes with gentle sonication to aid dismantling the subcellular structure (Scope, 1987, 1994).

LPS is believed to be arranged in a bilayer, analogous to phospholipid, in which the hydrophilic components are exposed to the aqueous environment while the hydrophobic fatty acid tails are sandwiched together in the center of the bilayer (Sweander et al., 1977). Similar to protein dissociation from lipid membranes, the same

method can be used to dissociate the endotoxin from the protein complex. Then endotoxin can be removed from the protein solution by phase separation with the aid of Triton-114 (Aida and Pabst, 1990). The main problem of this technique is that small amount of detergent persisted in the aqueous phase. When the detergent is removed from the solution, the small sized endotoxins will re-aggregate into a larger size of ET (Sawada et al., 1986).

Detergents such as DOC, SDS or triton X-100 are not recommendable since they disturb the LAL test with a concentration as low as 0.1% (Petsch et al., 1997). SDS is difficult to remove because of its low critical micelle concentration (CMC) (Karplus et al., 1987). Sodium deoxycholate (Issekutz, 1983) may be an effective eluent because it extensively dis-aggregates endotoxins. However, through its detergent action, it may interfere with the lipid A affinity and binding to resins. Furthermore, these detergent methods would also be unsatisfactory when one wishes to remove endotoxin from a solution, which contains other desirable high molecular weight components.

All of the above mentioned chemical methods have the following problems. Chemicals have some effects on pyrogenicity of LPS or LAL test results. The masking of LAL test by the surfactants were reported (Hannecart-Pokorni et al., 1973; Sweadner et al. 1977). The effects of chemicals on the targeted biomolecular products should be determined. Removal of chemicals has proved to be time-consuming and somewhat ineffective.

Ultrasonic irradiation was shown to be an effective way to dissociate the endotoxin aggregates and has been effects on the LAL test results (Sawada et al.,1986; Komuro et al., 1987). Haga et al. (1987) reported that effect of ultrasonic irradiation on

dissociation of antigen and antibody complex, which may be used to dissociate the endotoxin-protein complex due to the similarity of ET-protein and antigen-antibody.

Microwave radiation (2450 MH) was found to potentates the lethal effects of endotoxin in mice (Smialowicz et al., 1980; Riddle et al., 1982). McCullough et al. (1993) used microwave to dissociate antigen-antibody complex.

When antigen-antibody complex was dissociated with super critical fluid (SCF), good results were obtained (Howlett et al., 1992). The ET-protein complex may be dissociated with SCF. However, the effects of SCF on the structure and function of protein or ET, should be elucidated first.

1.5 Summary

Methods for removing endotoxins depend on the origin, structure and molecular weight of endotoxins, as well as the nature and molecular weight of biomolecules such as proteins. The structure and toxic effects of endotoxins from the Gram positive bacteria and fungi should be investigated. The relationship between the biological activity and the size of endotoxin has to be elucidated. The characterization and dissociation of endotoxin-protein should be finished before the effective separation.

The chemical decomposition of endotoxins with acids, alkali, or oxidizing agents is commonly practiced. However, such methods cannot be used for blood, blood products and bioproducts. Approaches of endotoxin removal from the aqueous solutions include physical methods such as ultrafiltration by size or MW differences, and chemical methods such as adsorption to activated carbon and ion exchange resins. Hemofiltration

has been used to treat septic shock and multiple organ failure. However, it has some problems.

Adsorption of endotoxin from target solutions by affinity resin or membrane adsorber appears to be a practical way to remove ET from the biological products. Adsorption is based on either the charge induced from phosphoryl groups or hydrophobic interaction. The resin applicable to endotoxin removal from all the biomolecules is not available. A special affinity resin which can dissociate endotoxin-protein complex and selectively remove the endotoxin from protein solution needs to be developed. The present investigation focuses on this aspect.

CHAPTER 2

BACKGROUND AND RATIONALE IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

2.1 Historical Background

The separation of biomolecules is as old as the human history. There are no records for the year when first product separation was made. The invention of chromatography makes it easy to separate and purify protein products. Martin and Synge (1941) introduced the chromatographic concept. Ion exchange chromatography, size exclusion chromatography, affinity chromatography or affinity partition and hydrophobic interaction chromatography were introduced gradually afterwards. All the separations by chromatography were based the properties of proteins such as charge, size or shape and surface characteristics. The ion exchange chromatography is based on the charge of protein. Gel filtration or size exclusion chromatography uses size or shape difference of proteins to separate and purify them. The protein surface properties such as contents of hydrophobic residues provide the basis of the hydrophobic interaction chromatography. Affinity chromatographic separation is also based on the surface properties of proteins. In 1975 Porath and his collaborators introduced an approach for protein fractionation, metal chelate affinity chromatography, which is now well known as the immobilized metal (ion) affinity chromatography (IMAC, Porath et al., 1983). IMAC is based on metal and protein interactions.

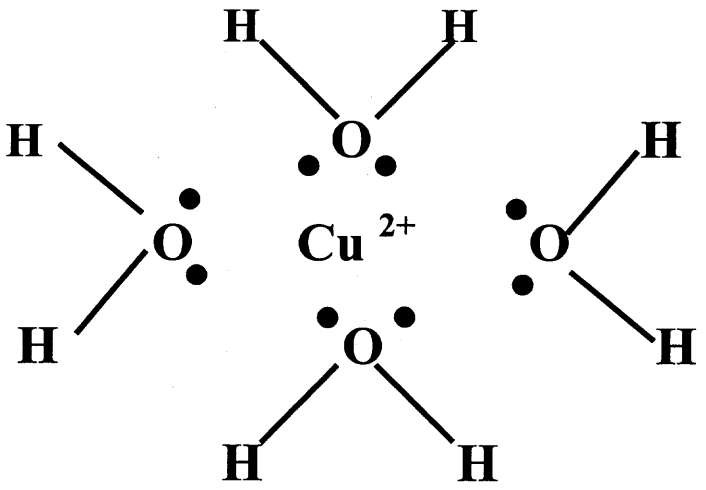
Metal-protein interaction is a very important phenomenon in biochemistry. There are two types of metal-protein interactions. First, the metal ion is an integral part of the protein structure, as in the case of metalloprotein such as hemoglobin. Second, a protein

forms a reversible complex with a metal ion, as in the case of metal chelate. The latter case was used to purify proteins from the solutions. In an efficient purification process, the protein retarded by the metal ion can be recovered by changing solution conditions. IMAC was developed using a rigid microporous stationary phase in which metal ion was immobilized. It can be used to purify bioproduct via chelating functional groups bound to their surface.

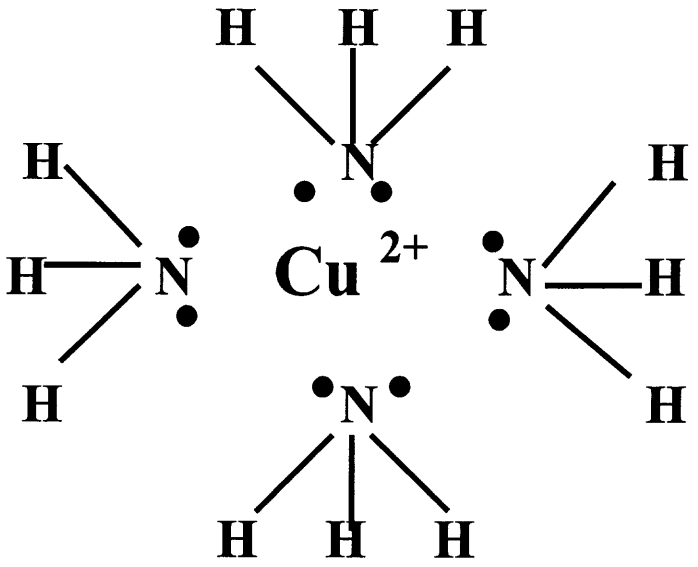
2.2 Immobilized Metal Affinity Chromatography

2.2.1 Principle

Metal ions are highly solvated in an aqueous solution as a result of coordination of water molecules (Sulkowski, 1985). In solution, metal ions can be considered as a Lewis acid (electron-pair acceptor) and the water molecule as Lewis base (electron pair donor). The water molecule can be replaced by a stronger base, resulting in a metal complex. The group combining with the metal ion is called a ligand (electron pair donor group). A donor atom (N, S, O) with a free pair of electrons constitutes a simple or monodentate ligand. When two or more donor atoms are present in one ligand molecule, such a ligand is polydentate and its coordination with the metal ion results in a metal chelate. The binding of metal ion to a ligand is much stronger in a metal chelate than in a metal complex. The principle of metal chelating is shown in Figure 2.1. The commonly used chelating ligand for metal ion immobilization in IMAC is iminodiacetic acid (IDA) (Porath and Olin, 1983) and tris (carboxymethyl) ethylene diamine (TED) as shown in Figure 2.2. Binding metal ions such as Cu^{2+}, Zn^{2+} and Ni^{2+} are often used.

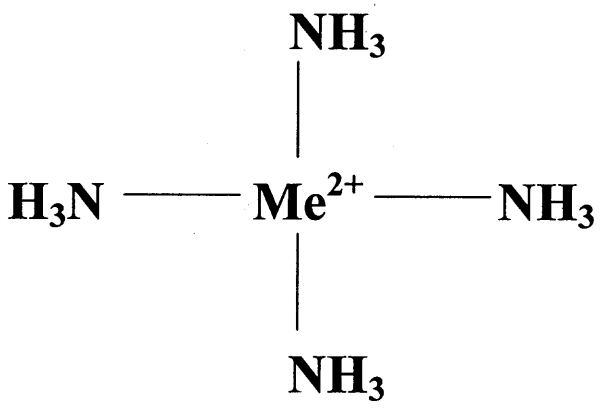


(A) Metal Ion and Water Molecules

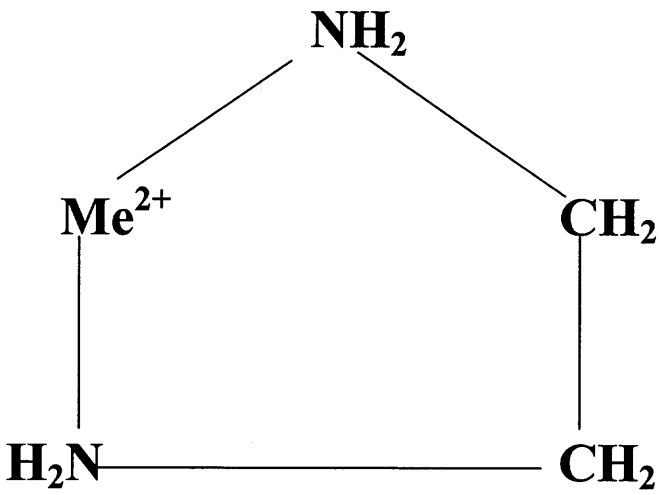


(B) Metal Ion and Basic Molecules

Figure 2.1 Metal chelating principle



C) Monodentate Metal Complex (Sulkowski, 1985)



D) Bidentate Metal Chelate

Figure 2.1 Metal chelating principle (Continue)

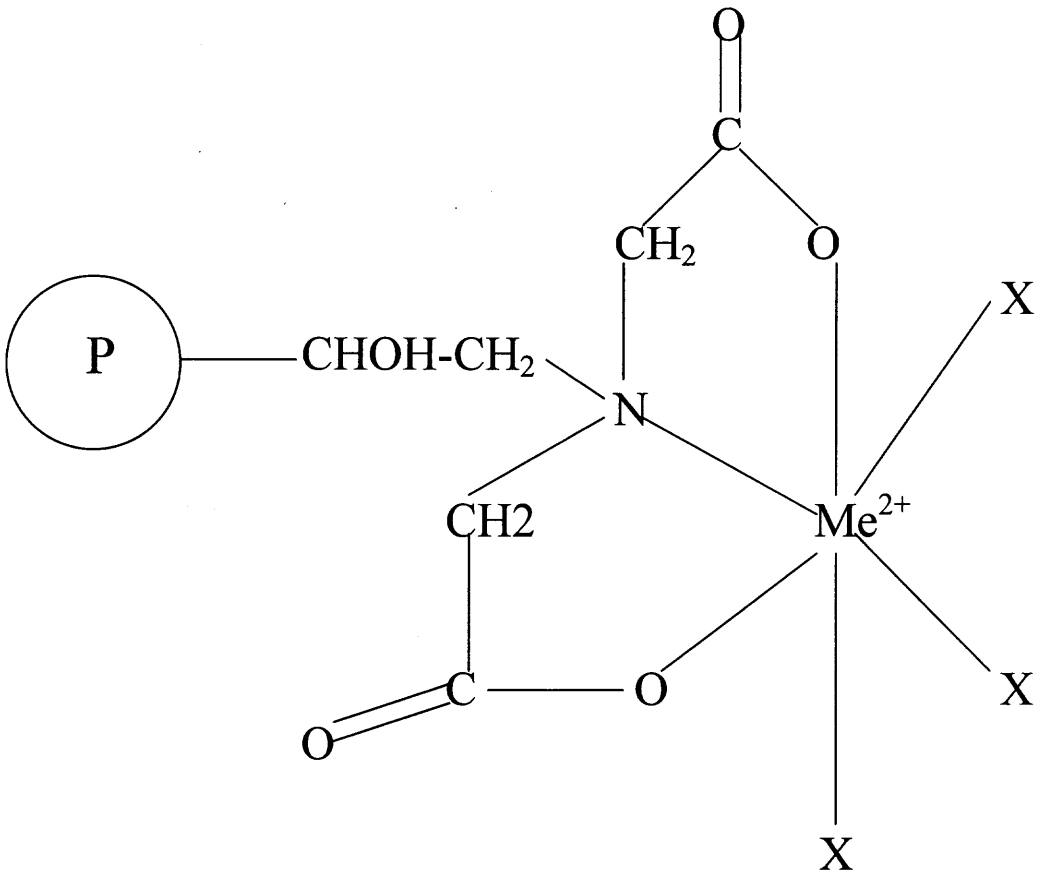


Figure 2.2 Structure of IMAC (Scope, 1994), P is the support, Me is metal ion and X is protein

The amino acids such as histidine and their residues form complexes with many transition metal ions. Therefore immobilized metal ions on the matrix will selectively retain proteins if amino acid residues such as histidine are exposed on the surface of the protein.

2.2.2 Application

IMAC is generally used to characterize protein structure or separate the histidine residue containing proteins from other constituents in a solution (Sulkowski, 1985). IMAC has been applied in the purification of variety of proteins. Examples include serum proteins (Porath et al., 1975, 1983), interferons (Edy et al. 1977; Bollin and Sulkowski, 1978; Chadha et al., 1979; Krempien et al., 1985; Coppenhaver, 1986), phosphoproteins (Muszynska et al., 1986; Shriner and Brautigan, 1987; van Heusden et al., 1991; Jiang et al., 1999), lactoferrin (Carlsson et al., 1977), glycoprotein (Lebreton et al. 1977, 1978, 1979; Corradini et al., 1988; Gmeiner et al., 1995; Wojczyk et al., 1996; Herve et al., 1996; Sedzik et al., 1999), recombinant protein (Ford et al., 1991), and fusion protein (Wizemann and von Brunn, 1999). For other proteins, reviews (Sulkowski, 1989; Enfors et al., 1990; Porath, 1990, 1992; Jones et al., 1995; Sawadogo et al., 1995; Turkova, 1999; Hage, 1999) should be consulted.

Since IMAC separation of protein is based on the interactions of histidine, cysteine and tryptophan residues with metal ions, IMAC is very useful in topological studies aimed at the determination of the number and location of amino acid (histidine) residues exposed on the surface of proteins (Hemdan et al., 1989). A variety of proteins were characterized with this method, such as, an enzymatically active immobilized

derivative of myosin (Elgart et al., 1975), cell-cycle-regulated protein calcyclin (Filipek et al., 1991), chimeric rDNA proteins (Evans et al., 1991), Zn (II)-transport protein (Yip and Hutchens, 1991; Vosters et al., 1992), recombinant human interferon gamma (Zhang et al., 1992), a monomeric HIV-1 reverse transcriptase (Sharma et al., 1994), lymphocyte chymase I (Woodard et al., 1998), and a β-peptide of Alzheimer's disease (Liu et al., 1999) etc. There is a special method to apply IMAC in protein purification: reversed IMAC (Nishino and Powers, 1979). A blank gel without metal ion was used instead of regular IMAC. It is a very effective method to purify metalloproteins. Metal Ion Transfer (MIT) based on the IMAC principle, may offer an efficient way to deplete metal ions from a metalloprotein or, conversely, to charge it with metal ions (Sulkowski, 1989).

The most widely used commercially available matrices for IMAC are Chelating Sepharose CL-6B, Chelating Sepharose Fast Flow and Chelating Superose from Pharmacia LKB, IDA or TED gels from Pierce, IDA agarose and IDA Sepharose 6B from Sigma and TSK Chelating-5PW (silica based) from Toyo Soda.

2.2.3 Protein-metal Chelate Interactions

As mentioned in Section 2.1, the binding of proteins to IMAC stationary phase is due to the substitution of weakly bonded small molecule such as water or mobile phase ions on the surface of metal chelate stationary phase, with electron-rich groups (ligands) on the surface of proteins as the electron donor. The metal ions are acting as the electron acceptor in this process.

The interaction strength between protein and immobilized metal ions is mainly determined by the number of histidine residues exposed on the surface of the protein. It

is also on that of cysteine or tryptophan (Porath et al., 1975; Sulkowski, 1985) in some extent. The chromatographic behavior of selected model proteins on IMAC with different metal ions was evaluated. The affinity of protein with immobilized metal ions mainly depends on the histidyl side chain distribution on the proteins: (i) either interior or surface; (ii) if localized on the surface, accessible or inaccessible for coordination; (iii) single or multiple; (iv) if multiple, either distant or vicinal (Hemdan et al., 1989). Proteins displaying single histidyl side chain on their surfaces may, in some instances, be resolved by IMAC (Hemdan et al., 1989). Affinity retention of proteins by immobilized metal ions increased with their number of His residue (Scully and Kakkar, 1982; Hutchens and Yip, 1990). The accessibility of the residue to IMAC interaction is very important (Berna et al., 1997). In some cases, contribution of other amino-acid side chains such as cysteine (Hutchens et al., 1989; Kipriyanov et al., 1994, 1995), trytophan (Sulkowski, 1985; Hansen et al., 1992), and occasionally, terminal amino group (tyrosine, serine, threonine and perhaps arginine) (Bianchi et al., 1994) should be considered as the source of interactions, or enhance or reduce protein-metal interactions. When ferric ion is used, phosphate groups on the surface of protein enhance the strength of IMAC affinity (Andersson and Porath, 1986). Contribution of net charge and hydrophobic properties of protein should be investigated (Belew and Porath, 1990; Turkova, 1999).

2.2.4 Stationary Phases in IMAC

2.2.4.1 Solid matrix or choice of chelating

Like the matrix of affinity chromatography, the solid matrix for IMAC must possess several properties, such as a macroporous structure, which provides a large surface area,

limited mass transfer resistance, high mechanical strength and chemical resistance. The chemical inertness is required to ensure that nonspecific adsorption does not happen during the separation. The matrix must be chemically stable and possess sidechains that can easily be modified for ligand or spacer arm attachment (Phillips, 1992). The generally used matrix for IMAC (Porath, 1990) is agarose (Porath and Olin, 1983; Andersson and Porath, 1986; Muszynska et al., 1986), dextran, and TSK gel (Porath et al., 1983; Belew et al., 1987). The regular soft agarose gel can not withstand high pressure, while highly cross-linked Sepharose beads or silica based gel can be used in high-pressure conditions.

2.2.4.2 Nature of chelating groups

Chelating groups are used to hold metal ions onto the matrix. To ensure that there is no metal ion leaching during the separation process, the binding between the chelating group and metal ions should be very strong. However, too strong binding will make the interactions between metal and protein weak. The resulting low adsorption capacity is not suitable for practical IMAC process.

Various chelating groups have been used in IMAC. The most commonly used chelating group has been iminodiacetic acid (IDA). The dissociation constant between the IDA and metal ion is very small. The dissociation constant is $10^{-10.57}$ M for IDA-Cu^{2+} and $10^{-10.72}$ M for IDA-Fe^{3+} (Martell and Smith, 1974). The strong binding of metal ions to IDA minimizes the bleeding of metal ions from the column in IMAC processes. Tris (carboxymethyl) ethylenediamine (TED) has found some applications (Chaga et al., 1996; Yip and Hutchens, 1991; Porath et al., 1983; Porath and Olin, 1983) with Ni (II) used as

the chelating ion. Nitriloacetic acid (NTA) was also tried (Ramadan and Porath, 1985; Pos et al., 1994).

2.2.4.3 Nature of chelated metal ions/choice of metal ions

Another key issue in IMAC is to choose metal ions for immobilization. The chosen metal should form a stable complex with the chelating ligand and thus maintain high affinity to protein to be separated.

Metal ions were divided into three categories: hard, soft and intermediate (Pearson, 1973). Hard metal ions such as Mg(II), Ca(II), Fe(II), Al(III), and Ga(III) and In(III) have a strong preference for oxygen-containing compounds. Soft metal ions such as Cd(II), Hg(II) and Tl(III) have a tendency to coordinate sulfur-containing compounds. The ions Cu(II), Zn(II), Ni(II) and Co(II), which belong to intermediate category, preferentially coordinate with nitrogen. The application of transition metals (Co, Ni, Cu, and Zn) was reviewed by Sulkowski (1989). Adsorption of proteins on these metal ions can be rationalized in terms of the coordination of histidine residues. Various metal ions were investigated by researchers (Andersson and Porath, 1986).

According to the classification of Pearson (1973), Fe (III) is a hard metal ion and can be considered as a Lewis acid or electron-pair acceptor. It has a very strong tendency to bind oxygen-containing compounds. IMAC-Fe(III) was used by several researchers to purify proteins, such as lysozyme, cytochrome c, avidin, bovine pancreatic RNase, myoglobin, ovalbumin and human serum albumin (Ramadan et al ., 1985; Kastner an Neubert, 1991; Zachariou and Hearn, 1995). In most cases IMAC-Fe (III) was used to separate or purify phosphoproteins, phosphoamino acids (Andersson and Porath, 1986),

and phosphopeptides (Scanff et al., 1991). Because of very low dissociation constant between Fe(III) and phosphate group, IMAC–Fe(III) has very strong binding to phosphate group containing proteins. This may be very useful in the adsorption of endotoxins.

2.2.5 Elution Process of IMAC

In ion exchange chromatography, a gradient of increasing salt concentration is used to elute target product, while in hydrophobic interaction chromatography, a decreasing salt concentration is used in most cases. For reversed phase chromatography, a gradient of increasing organic eluent concentration is widely employed (Scope, 1994). In IMAC, the adsorption mechanism is possibly a combination of interactions of metal chelate, electrostatic, hydrophobic and special groups, such as phosphate group. Of course the metal chelate affinity contributes most. The development of elution procedure is complex and time consuming compared with other chromatographic processes.

In affinity chromatography, elution of the adsorbed solutes (in author's case, protein or protein mixture) can be accomplished by differentially modifying the affinity of protein for the stationary phase (Scope, 1994). The same principle was applied to IMAC. Elution in IMAC process is done either by lowering pH to protonate the donor groups on the adsorbed protein, or by use of a stronger complexing agent such as imidazole, glycine (Scope, 1987). Since use of pH elution has some limitations (Vunnum and Cramer, 1997), the effective elution can be obtained by inclusion of a competing solute in the elution buffer. However, the choice of an appropriate competing agent is very difficult. The biggest problem is metal ion leakage caused by the competing displacer (Belew et al., 1987).

2.3 Rational Significance and Scope of Study

2.3.1 Previous Studies and Problems

As mentioned in Chapter 1, endotoxin removal depends on its origin, structure, molecular weight, as well as the nature and molecular weight of biomolecules to be purified. The endotoxin removal from water, buffer solutions and dialysate solutions has been solved. However, endotoxin removal from biological solutions such as protein solutions and plasma, has proved to be very difficult due to several reasons. The endotoxin exists in different aggregation states with molecular weight ranging from 10,000 to 1,000,000, which varies greatly as a function of solution conditions. Many proteins form complexes with endotoxins, which are extremely difficult to break down. Finally, the interaction mechanism between the endotoxin and protein is not well understood.

Ideal affinity chromatography ligands in endotoxin removal from protein solutions should a) dissociate the ET-protein complex; b) capture the ET/protein molecule with high affinity, high capacity, and high selectivity; c) either not capture or allow differential elution of protein/ET; d) allow controlled release of the ET/protein under conditions that preserve (i.e., do not degrade or denature) the protein; e) permit cleaning and reuse of the chromatography matrix. Currently available endotoxin removal affinity resins or gels such as Acticlean Etox gel from Sterogene Bioseparation Inc., have many problems, which will be described in detail in Chapter 4:

- Low capacity, even at low ionic strength the ET adsorption capacity is about 100 EU/ml gel.

- Removal mechanism is affinity and ion exchange or mainly ion exchange mechanism, which is problematic. When the ionic strength is low both the protein and ET are adsorbed. As the ionic strength increases both protein and ET are eluted.
- Affinity resin may dissociate the ET-protein complex. However the dissociation effects are not notable. It is very difficult to get endotoxin concentration less than 0.5 EU/ml in HbAo solution.
- Regeneration problems. For regeneration, the column must be treated with 1 M NaOH solution for 24 hrs, which is very time consuming and inconvenient.
- Low flow rate: 0.3 ml/min and therefore time consuming procedure.
- Very expensive.

Therefore highly efficient and cost effective ligands for removing endotoxins from protein solutions need to be developed.

Conventionally, Cu^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+} are used in IMAC. Fe^{3+} was chosen in this work, based on dissociation constant between Fe^{3+} and different groups, which is one of the most important parameters in affinity chromatography. Lipid A is a very important part in endotoxin, in which the phosphate group is vital to the structure and functionality of endotoxins. Ferric ion and the phosphate group form a complex which has a dissociation constant $K_d = 10^{-13}$ M (Andersson and Porath, 1986). For the complex between ferric ion and histidine group in the proteins, the K_d is about 10^{-6} to 10^{-8} M (Scopes, 1987), depending on how many histidine residues in the protein are exposed to the metal ion. The dissociation constant of complex between endotoxin and protein is around 10^{-8} M (Kaca, 1994). Theoretically, the Fe^{3+} ion may break down the endotoxin-protein complex and separate endotoxin from protein.

2.3.2 Main Objectives

The main objective of present study was to develop an IMAC method to remove the endotoxin from protein solutions. Optimization of the chromatography process will be the second step. The effects of different solution conditions, such as pH, ionic strength, and protein types on endotoxin removal from the protein solutions should be investigated. The following contributions will be made:

I) ET removal from protein solutions by IDA-Fe^{3+} column with model proteins: bovine serum albumin (BSA), human hemoglobin A_0 (HbAo) and immunoglobulin G (IgG).

- The effects of mobile phase conditions such as ionic strength and pH on endotoxin removal from protein solutions will be evaluated;
- The effects of feed concentration and volume on the endotoxin removal from protein solutions will be investigated to determine the column capacity;
- The effects of flow rate on the endotoxin removal from protein solutions will be investigated to determine the process throughput.

II) For comparison, ET removal from protein solutions will be conducted by a current commercially available affinity column.

III) Leaching study of the IDA-Fe^{3+} IMAC column will be conducted.

CHAPTER 3

ANALYTICAL METHOD DEVELOPMENT

3.1 Historical Background and LAL Test

Bacterial endotoxins have been recognized by industry as a major cause of pyrogenic reactions that can be encountered during the administration of biotherapeutics. It is crucial to accurately detect and monitor endotoxin contents in biomolecule-based parental therapeutics. Limulus Amebocyte Lysate (LAL) test is a widely used method to detect endotoxin level in solutions.

LAL was based on the discovery that bacterial endotoxins activate a clotting cascade in a lysate of horseshoe crab (*Limulus polyphemus*) amoebocytes reported by Bang (1956) and Levin and Bang (1964, 1968). Stanley Watson, working at Woods Hole Oceanographic Institution, developed the LAL test (Novisky, 1985, 1994). In December 1987, the United States Food and Drug Administration (FDA) published the "Guideline on validation and use of the Limulus Amebocyte Lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices". In early 1990s the switch was complete when the USP monographs were changed to specify the bacterial endotoxin (LAL) test in place of the pyrogen (rabbit) test.

There are three different methods (Sharma, 1986; Cross, 1993; Guo, 1997) of endotoxin determination utilizing LAL assay. They are gel-clot, kinetic turbimetric and chromogenic substrate assays.

3.1.1 Gel-clot LAL test

In gel-clot test (Karprus, 1987; Aida et al., 1990; Lahiri et al., 1994; Liu et al., 1997) lyophilized LAL is reconstituted by adding it to the test solution and incubating for 1 hr at 37°C. If a solid gel-clot is formed and is able to withstand a 180° inversion of the tube, the test is considered positive. This method is qualitative or semiquantitative. The detection limit of gel-clot is about 0.025 EU/ml (Guo, 1997) or 0.03 EU/ml (Cross, 1993). This test process is very tedious.

3.1.2 The Kinetic Turbidimetric LAL Assay

The kinetic turbidimetric LAL assay (Chapman et al., 1992; Brown and Fuller, 1993; Hirayama et al., 1994; Evans-Strikfaden et al., 1996; Refiee-Tehrani, 1997) is a sensitive and quantitative technique comparing with gel-clot assay. It measures the increases of turbidity during the development of a gel-clot. The rate of turbidity increase is proportional to the endotoxin concentration. Hence turbidimetric method determines endotoxin levels in solutions based on the time needed to reach a specific degree of turbidity. The detection limit for kinetic turbidimetric assay is 0.001 EU/ml.

The following kinetic turbidimetric assay equipment and kits are provided by the several manufacturers: Wako Toxinometer ET-201 (Hirayama et al., 1994; Morimoto et al., 1995), LAL 500 series 1 from Atlas Bioscan (Associates of Cape Cod) as well as LAL 500 series 2 automatic endotoxin detection system (Associates of Cape Cod). For LAL 500 series 2, test sensitivity of the assay was 0.0031 EU/ml.

3.1.3 Chromogenic Substrate Assay

In a chromogenic substrate test, a synthetic chromogenic substrate is used. This substrate is a short synthetic polypeptide with an amino acid sequence that mimics a nature cleavage site on the coagulogen. The chromogenic p-nitroaniline (pNA) moiety is attached to the end of the peptide as Ac-Ile-Glu-Gly-Arg-pNA. In the chromogenic LAL test, the proenzyme is activated by the endotoxin in a water bath at 37°C for 10 min. Chromogenic substrate is then added, and the active enzyme causes the release of pNA from the substrate, producing a yellow color. The reaction is stopped with acetic acid. The intensity of the color change produced by the substrate cleavage is measured spectophotometrically at 405 nm. The correlation between the absorbance and endotoxin concentration is linear in certain range. This method is widely used in the determination of endotoxin concentration because of its high sensitivity and easy operation (Burger et al., 1989; Hou, and Zaniewski, 1990; Vanholder et al., 1992; Mitzner et al., 1993; Roth, and Kaca, 1994; Longerman et al., 1994; Rafiee-Tehrani et al., 1996).

Main manufacturers of chromogenic kits are BioWhittaker, Seikagku (Japan), Chromogenix (Sweden).

3.1.4 The effects of LPS on protein assays

Protein concentration is determined with the method of Stanford with BSA or lysozyme as a standard (reference), or measured at 280 nm for its absorbance. The latter causes a problem. The LPS has absorbance at 280 nm, which interferes with the protein assay. The protein concentration determined by this method is higher than its actual value.

3.2 Accurate Chromogenic Measurement of Endotoxin Concentration in A Hemoglobin-Lipopolysaccharide Complex Solution

Chromogenic LAL assay is widely used in the determination of endotoxin concentration because of its high sensitivity and easy operation (Hou, Zaniewski, 1990; Cliff et al., 1995; Refiee-Tehrani et al., 1996). Problems arise when chromogenic LAL test is used to determine endotoxin contents in some protein solutions with color. It is known that hemoglobin forms a complex with endotoxin (Kaca et al., 1994; Li and Luo, 1997) and has a strong absorbance at 405 nm, which causes interference with the reading of the color intensity generated by the LAL reaction. Although previous studies reported by Cliff et al. (1995), Roth and Levin (1994) mentioned that the chromogenic LAL test required a deduction of HbAo absorbance from the total absorbance of the mixture, no detailed descriptions have been provided about the compensation. More importantly, the compensation approach has not been verified experimentally. This approach was described in details theoretically by considering both color sources introduced by the hemoglobin and the LPS activation reaction in this Section. It was verified experimentally by comparing the compensation curve to an endotoxin standard curve.

3.2.1 Materials and Methods

Reagents

Endotoxin standard from QCL-1000 LAL test kit (BioWhittaker, Inc., Walkersville, MD, USA) was used in all studies. All solutions were prepared with endotoxin free water (LAL reagent water, BioWhittaker Inc., Walkersville, MD, USA). Hemoglobin Ao (HbAo, ferrous) was purchased from Sigma.

Labware

All glassware used was autoclaved at 15 psig for 30 minutes with an electric pressure steam sterilizer (Model No. 25X, Wisconsin Aluminum Foundry Co., Inc., WI, USA) and followed with heating in an oven (Model 16, Precision Scientific Co., IL, USA) at 210°C for 3 hours. All solution transfers were performed by endotoxin free devices. Sterile, disposable plastic ware was used at all times to prevent endotoxin contamination.

Protein Assay

The absorbance scanning from 250 nm to 750 nm in our laboratory shows that the endotoxin sample has a small absorbance peak at around 280 nm (absorbance spectrum not shown). Therefore HbAo concentration was measured with a spectrophotometer (U-2000, Hitachi, Japan) at 415 nm to avoid the effect of endotoxin absorbance interference.

Endotoxin Standard Curve of Chromogenic LAL Assay

The endotoxin standard curve was generated following the procedure of BioWhittaker (1998). After LAL incubation, the absorbance (ABS) of endotoxin standard solutions at a series of endotoxin concentrations, i.e., 0.05, 0.1, 0.25, 0.5 EU /ml was measured by spectrophotometer individually. Using endotoxin free water as the blank, standard endotoxin curves were obtained by plotting each ABS versus the corresponding concentration, i. e., 0.05, 0.1, 0.25 and 0.5 EU/ml.

The two performance characteristics of standard curve were used as suggested by BioWhittaker (1998): linearity and reproducibility. The linearity of the standard curve within the concentration range used to predict endotoxin values was verified based on the least squares method. The reproducibility can be verified by comparing the different curves.

Interference of HbAo on Chromogenic LAL Assay: Shifted Curves

Two sets of endotoxin standard solutions with a series of endotoxin concentrations as mentioned above, i.e., 0.05, 0.1, 0.25, 0.5 EU/ml, were used. Each solution of the first set was spiked with HbAo to 10 μg/ml (Case 1) and each solution of the second set was spiked with HbAo to 20 μg/ml (Case 2). Therefore eight HbAo spiked endotoxin solutions were prepared. After the LAL incubation the ABS of each HbAo spiked endotoxin solution was measured by the spectrophotometer. At each endotoxin concentration, the experiment was conducted in duplicate and total absorbance, ABS^{Mix}, was obtained using endotoxin free water as the blank. The blank was also incubated with LAL following the same steps for the samples. The experimental curves (the shifted curves) were constructed by plotting each ABS^{Mix} versus the corresponding endotoxin concentration for samples spiked to 10 μg/ml and 20 μg/ml HbAo, respectively.

Compensation of HbAo Interference on Chromogenic LAL Assay: Theory

In this study three kinds of solutions were used: endotoxin standard solution (pure endotoxin solution), the HbAo solution and the mixture of endotoxin standard solution and the HbAo solution. The mixture was prepared so that the HbAo concentration in it was the same as that in the HbAo solution. In the endotoxin standard solution, the endotoxin concentration is C_{LPS}^{STAN} and the absorbance after LAL incubation is ABS_{LPS}^{STAN}. The interference of HbAo on the chromogenic LAL assay can be compensated by considering both the absorbance caused by HbAo and the absorbance caused by the LPS in the HbAo solution. The method of compensation can be mathematically described as follows.

In the HbAo solution, which contains small amount of LPS, the endotoxin concentration is C_{LPS}^{HbAo}. The total absorbance of the HbAo solution after LAL incubation, ABS^{HbAo}, has two contributions:

$$ABS^{HbAo} = ABS_{HbAo}^{HbAo} + ABS_{LPS}^{HbAo} \tag{3.1}$$

Where ABS_{HbAo}^{HbAo} is the absorbance of the HbAo solution contributed by the color of HbAo itself, which is the same before and after the LAL incubation. ABS_{LPS}^{HbAo} is the absorbance of the HbAo solution contributed by the small amount of LPS that generated the color after the LAL incubation. For the mixture of the HbAo solution and endotoxin standard solution, we have

$$C_{LPS}^{Mix} = C_{LPS,STAN}^{Mix} + C_{LPS,HbAo}^{Mix} \tag{3.2}$$

$$ABS^{Mix} = ABS_{LPS,STAN}^{Mix} + [\,ABS_{LPS,HbAo}^{Mix} + ABS_{HbAo,HbAo}^{Mix}\,] \tag{3.3}$$

Where C_{LPS}^{Mix} is the total endotoxin concentration of the mixture. $C_{LPS,STAN}^{Mix}$ is the endotoxin concentration of the endotoxin standard solution in the mixture. $C_{LPS,HbAo}^{Mix}$ is the endotoxin concentration of the HbAo solution in the mixture. ABS^{Mix} is the total absorbance of the mixture. $ABS_{LPS,STAN}^{Mix}$ is the absorbance of endotoxin standard solution in the mixture. $ABS_{LPS,HbAo}^{Mix}$ is the endotoxin absorbance in the original HbAo solution in the mixture. $ABS_{HbAo,HbAo}^{Mix}$ is the absorbance of HbAo in the mixture. The experiments were designed so that the last two terms of Eq. (3.3) should have the following relationship with the ABS^{HbAo} in Eq. (3.1):

$$[\,ABS_{LPS,HbAo}^{Mix} + ABS_{HbAo,HbAo}^{Mix}\,] = ABS^{HbAo}$$

$$= ABS_{HbAo}^{HbAo} + ABS_{LPS}^{HbAo} \tag{3.4}$$

Therefore, the absorbance of the endotoxin standard solution in the mixture after the compensation for HbAo interference is

$$ABS^{Mix}_{LPS,STAN} = ABS^{Mix} - (ABS^{HbAo}_{LPS} + ABS^{HbAo}_{HbAo}) \quad (3.5)$$

The values of $ABS^{Mix}_{LPS,STAN}$ and their corresponding endotoxin concentrations can be plotted as the curve of compensation, i.e., the corrected curve. The position of this curve is expected to be very close to that of the original endotoxin standard curve. Eq. (3.5) can also be rearranged as

$$ABS^{Mix}_{LPS,STAN} + ABS^{HbAo}_{LPS} = ABS^{Mix} - ABS^{HbAo}_{HbAo} \quad (3.6)$$

or

$$ABS^{Mix}_{LPS} = ABS^{Mix} - ABS^{HbAo}_{HbAo} \quad (3.7)$$

Where ABS^{Mix}_{LPS} is the absorbance of the total endotoxin in the mixture, which corresponds to the total endotoxin concentration of the mixture, C^{Mix}_{LPS}, in Eq. (3.2). For a mixture of HbAo and endotoxin, Eq. (3.7) can be used to calculate the total endotoxin concentration of the mixture. Since ABS^{HbAo}_{HbAo} can be measured from the mixture before the LAL incubation and ABS^{Mix} can be measured from the mixture after the LAL incubation, ABS^{Mix}_{LPS} can be calculated using Eq. (3.7). The corresponding C^{Mix}_{LPS} can then be obtained from the endotoxin standard curve.

Compensation of HbAo Interference on Chromogenic LAL Assay: Corrected Curves

Two series of endotoxin standard solutions as above were spiked to 10 μg/ml and 20 μg/ml HbAo, respectively. This time during the chromogenic LAL assay, instead of endotoxin free water, HbAo solutions were used as the blanks to compensate the effect of

HbAo in the mixtures being assayed. The HbAo solution of a concentration of 10 μg/ml was used as the blank for the assay of the series of endotoxin standard solutions spiked to 10 μg/ml, and a HbAo solution with a concentration of 20 μg/ml was used as the blank for assay of the series of endotoxin standard solutions spiked to 20 μg/ml. The blanks were also incubated with LAL following the same steps for the samples. It is expected that the experimental curves (corrected curves) for both Case 1 and Case 2 should be very close to the original endotoxin standard curve.

3.2.2 Results

Endotoxin Standard Curve of Chromogenic LAL Assay

The endotoxin standard curve was shown in Figure 3.1 which was the average of thirteen groups of experimental data considering standard deviation. R is much higher than the BioWhittaker's requirement, which is $R \geq 0.98$. This indicates that the data points for both curves are highly linear. The line above the standard curve gives the up end (+ standard deviation) of the standard curve while the line below it is the low end (-standard deviation).

Interference of HbAo on Chromogenic LAL Assay: Shifted Curves

Figure 3.2 shows the interference of HbAo on the chromogenic assay. In Case 1, where endotoxin standards were spiked to 10 μg/ml HbAo, although the linearity of the curve is high (R=0.986), the curve has shifted away from the position of the standard curve due to the interference of HbAo. In Case 2, the linearity of the curve is also high (R=0.984),

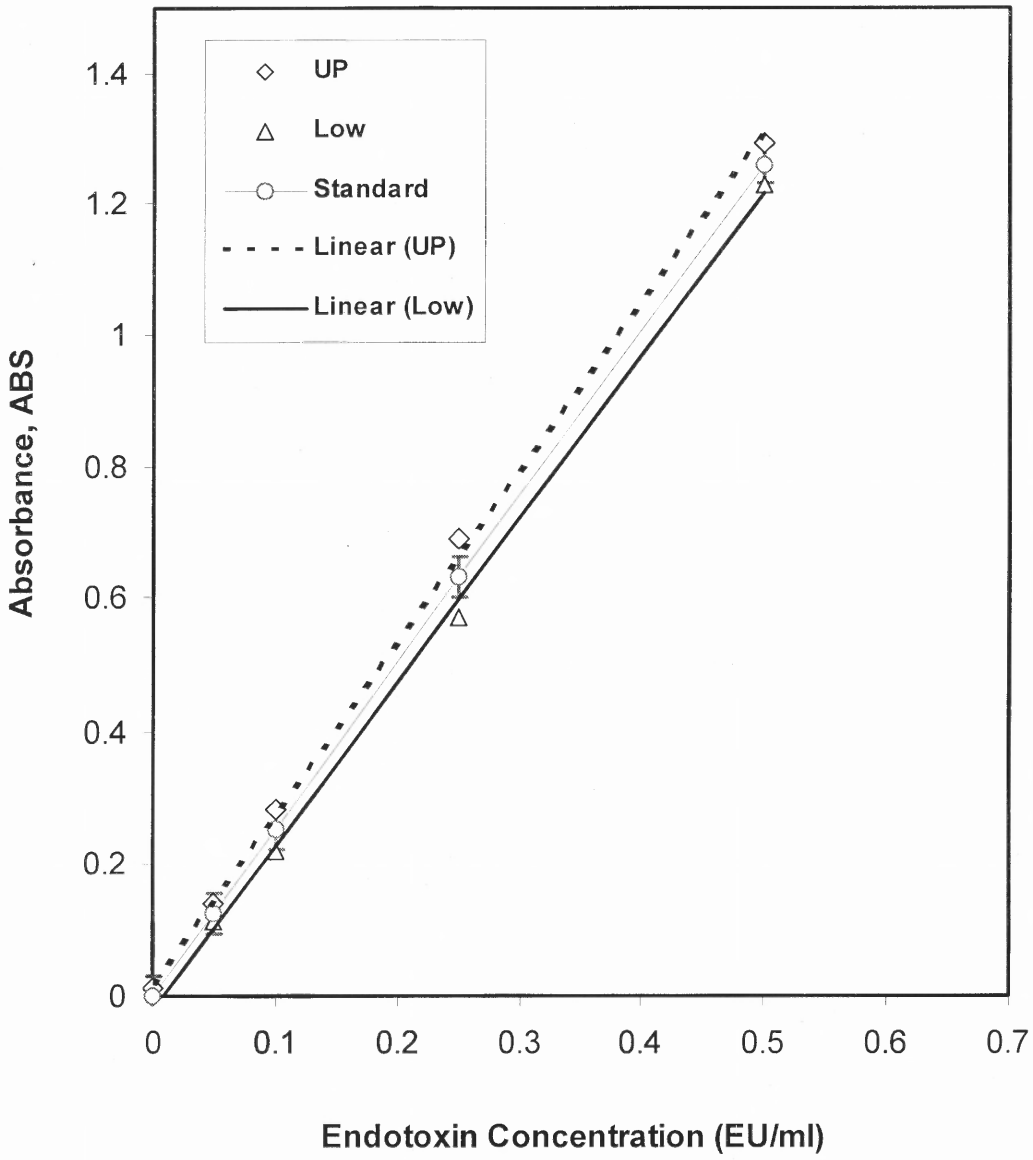


Figure 3.1 Endotoxin standard curves generated using endotoxin standard solutions. Blank: endotoxin free water.

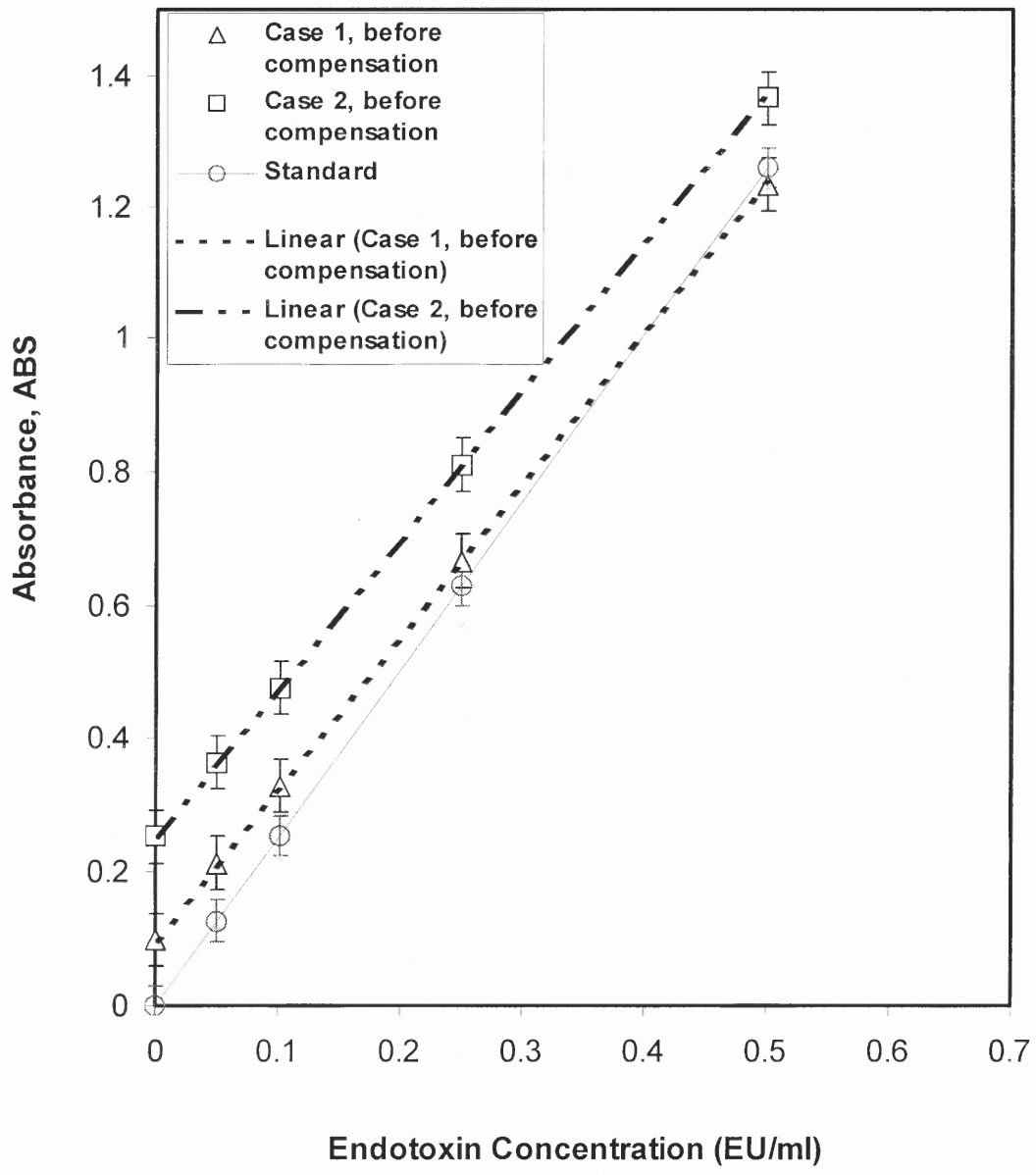


Figure 3.2 Shifted curves when the endotoxin standard solutions were spiked to 10μg/ml HbAo (Case 1) and 20 μg/ml HbAo (Case 2). Blank: endotoxin free water.

but the curve has shifted even further away compared to Case 1. This is expected due to the higher spiking HbAo concentration in Case 2.

Compensation of HbAo Interference on Chromogenic LAL Assay: Corrected Curves

As discussed before, the interference of HbAo on the chromogenic LAL assay can be compensated using HbAo solutions at corresponding concentrations as the blanks. Figure 3.3 shows the results of the compensation, i.e., the corrected curves for Case 1 (10 μg/ml HbAo spike) and Case 2 (20 μg/ml HbAo spike). It can be seen that both the corrected curves are very close to the standard curve. The linearity for both curves is high ($R \geq 0.98$). In Figure 3.4, the corrected curve and the shifted curve, both for Case 2, are plotted together for comparison. The figure shows that the position of corrected curve is much closer to the standard curve than that of the shifted curve.

3.2.3 Summary

In this study, a simple method was demonstrated to compensate the interference of hemoglobin on the chromogenic LAL assay using a series of mixtures of the endotoxin standard solutions and human hemoglobin Ao (HbAo) solutions. This approach has been described mathematically by considering both the color sources introduced by the hemoglobin and the endotoxin activated reaction. The approach has also been verified experimentally by comparing the compensated curve to an endotoxin standard curve. Further, it is expected that both the theoretical and experimental approaches can be applied to the mixtures of endotoxin and other proteins with color that interfere the chromogenic LAL assay. It is noticed that the concentrations (10 μg/ml and 20 μg/ml) of

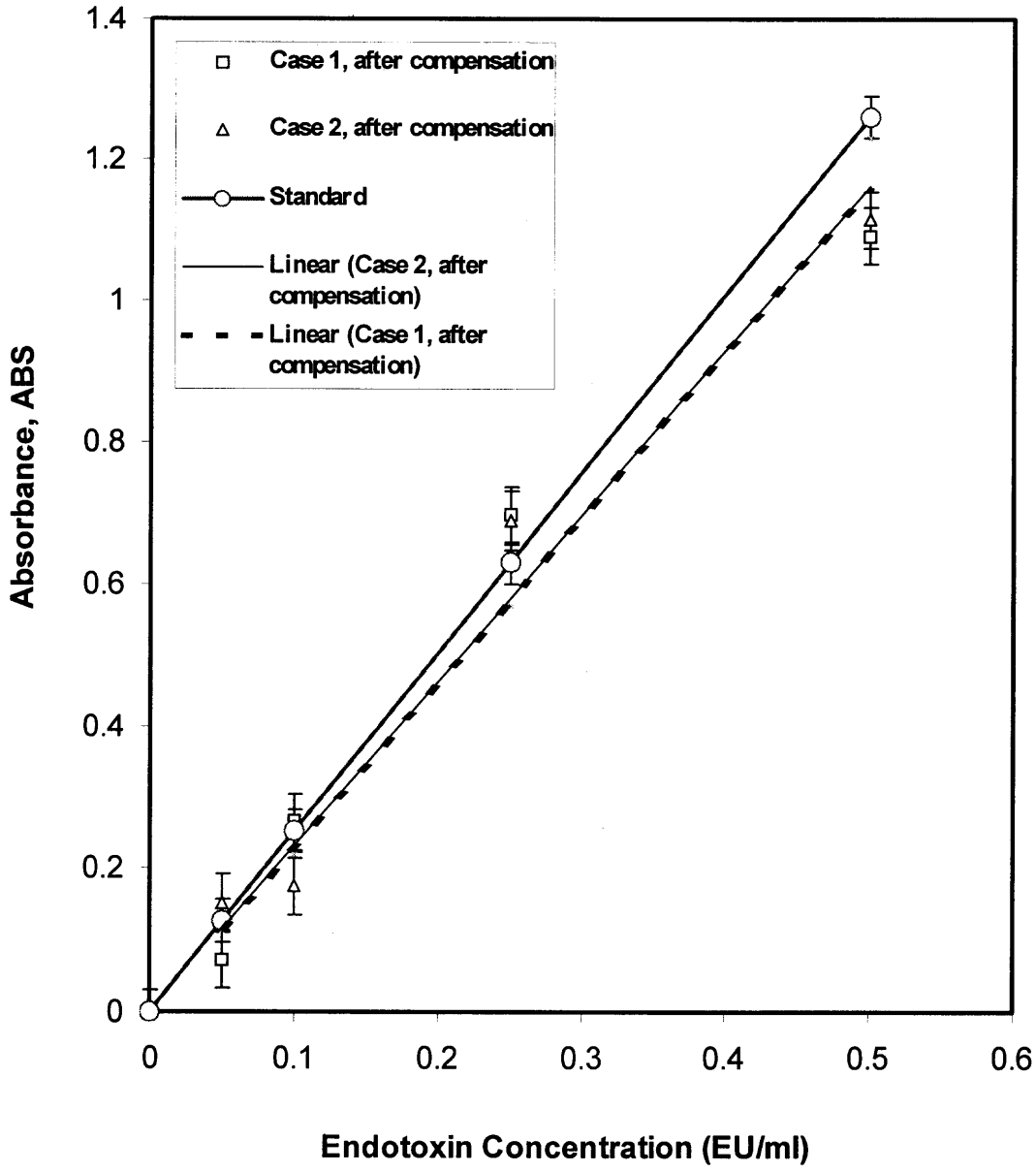


Figure 3.3 Corrected curves using the method of compensation. Case 1: endotoxin standard solutions spiked to 10 μg/ml HbAo; Blank: 10 μg/ml HbAo solution. Case 2: endotoxin standard solutions spiked to 20 μg/ml HbAo; Blank: 20 μg/ml HbAo solution

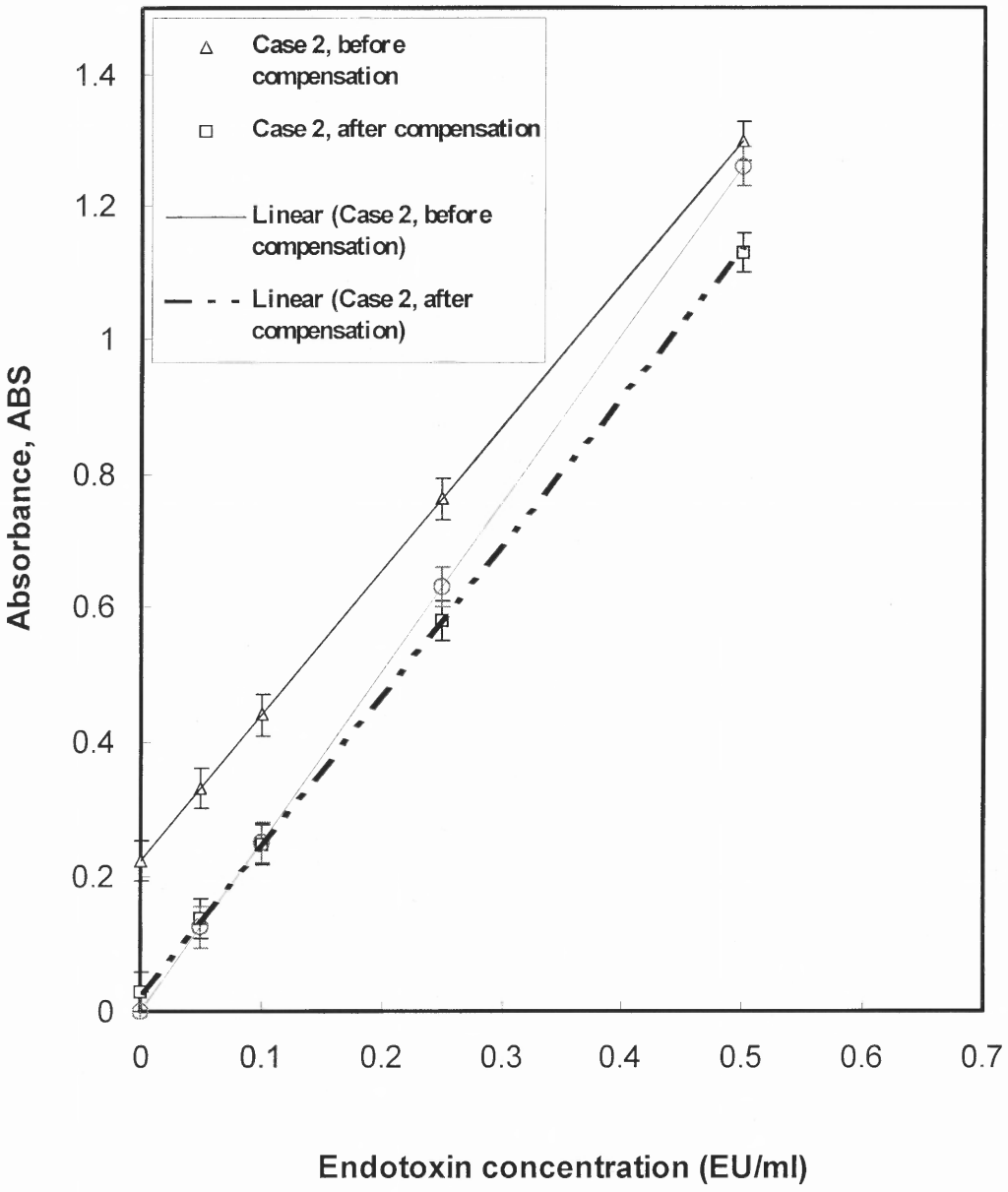


Figure 3.4 Comparison of shifted curve (before compensation) and the corrected curve (after compensation) of Case 2 (endotoxin solutions spiked to 20 μg/ml HbAo).

HbAo spikes used in this work were not very high. If hemoglobin concentration is higher, a proper sample dilution before the compensation process is required.

3.1 Inhibitions of phosphate ion on the detection of LAL test

Limulus Amebocyte Lysate (LAL) assay is widely used to determine the endotoxin content in all kinds of samples such as water, buffers, and protein solutions. Some types of samples inhibit the LAL assay. Sample inhibition occurs when substances in the sample interfere with the LAL test results. In the chromogenic assay, this inhibition results in a lower final absorbance, indicating lower level of endotoxin than what exists in the test sample. The lack of sample inhibition should be determined for each specific sample, either undiluted or at an appropriate dilution (BioWhittaker, Inc., QCL-1000 Quantitative Chromogenic LAL manual). In this research, sodium phosphate buffer was found to have some inhibition effect on LAL assay. Hence if the lipopolysaccharides (LPS) solution was prepared with phosphate buffer, proper methods are required to remove the inhibition: these are described below.

3.3.1 Materials and Methods

Reagents

Endotoxin standard from QCL-1000 LAL test kit (BioWhittaker, Inc., Walkersville, MD, USA) was used in all studies. All solutions were prepared with endotoxin free water (LAL reagent water, BioWhittaker Inc., Walkersville, MD, USA).

Labware

All glassware used was autoclaved at 15 psig for 30 minutes with an electric pressure steam sterilizer (Model No. 25X, Wisconsin Aluminum Foundry Co., Inc., WI, USA) and followed with heating in an oven (Model 16, Precision Scientific Co., IL, USA) at 210°C for 3 hours. All solution transfers were performed using endotoxin free devices Sterile, disposable plastic ware was used at all times to prevent endotoxin contamination.

Endotoxin Assay

QCL-1000 Chromogenic LAL test kit (BioWhittaker, Inc., Walkersville, MD, USA) was used to determine the LPS content in the solutions.

Experimental Methods

To determine the phosphate buffer inhibition to LPS LAL test, an equal volume of phosphate buffer with different concentrations was spiked with a known amount of standard endotoxin. The spiked solution was assayed with the control, which is endotoxin free water spiked with the same amount of standard endotoxin. Then endotoxin concentrations in both sample and control were determined. The difference between these two calculated endotoxin concentrations should be lower than ± 25% of the known LPS spike concentration. The endotoxin recovery is defined as (LPS detected in sample/ LPS in the control) x 100%. LPS concentration in the sample solution by LAL test should be in the range of 75% to 125% of control solution. Otherwise the inhibition has strong effect on the LAL test, and LAL test results are not acceptable.

3.3.2 Results and Discussions

Inhibition of phosphate buffer on the LAL assay

The experimental results of LAL assay with different concentration of sodium phosphate at fixed endotoxin spike are shown in Figure 3.5. From the figure, the strong inhibition of sodium phosphate on the LAL test is observed when the sodium phosphate concentration is higher than 10 mM. However, as the phosphate solution was diluted to lower than 8.33 mM, 90% recovery of LAL assay was obtained. Therefore, proper dilution is a good way to overcome the inhibition of sodium phosphate on LAL test. Thus, this inhibition is reversible. When the sample is properly diluted, the pyrogenicity of LPS is measurable.

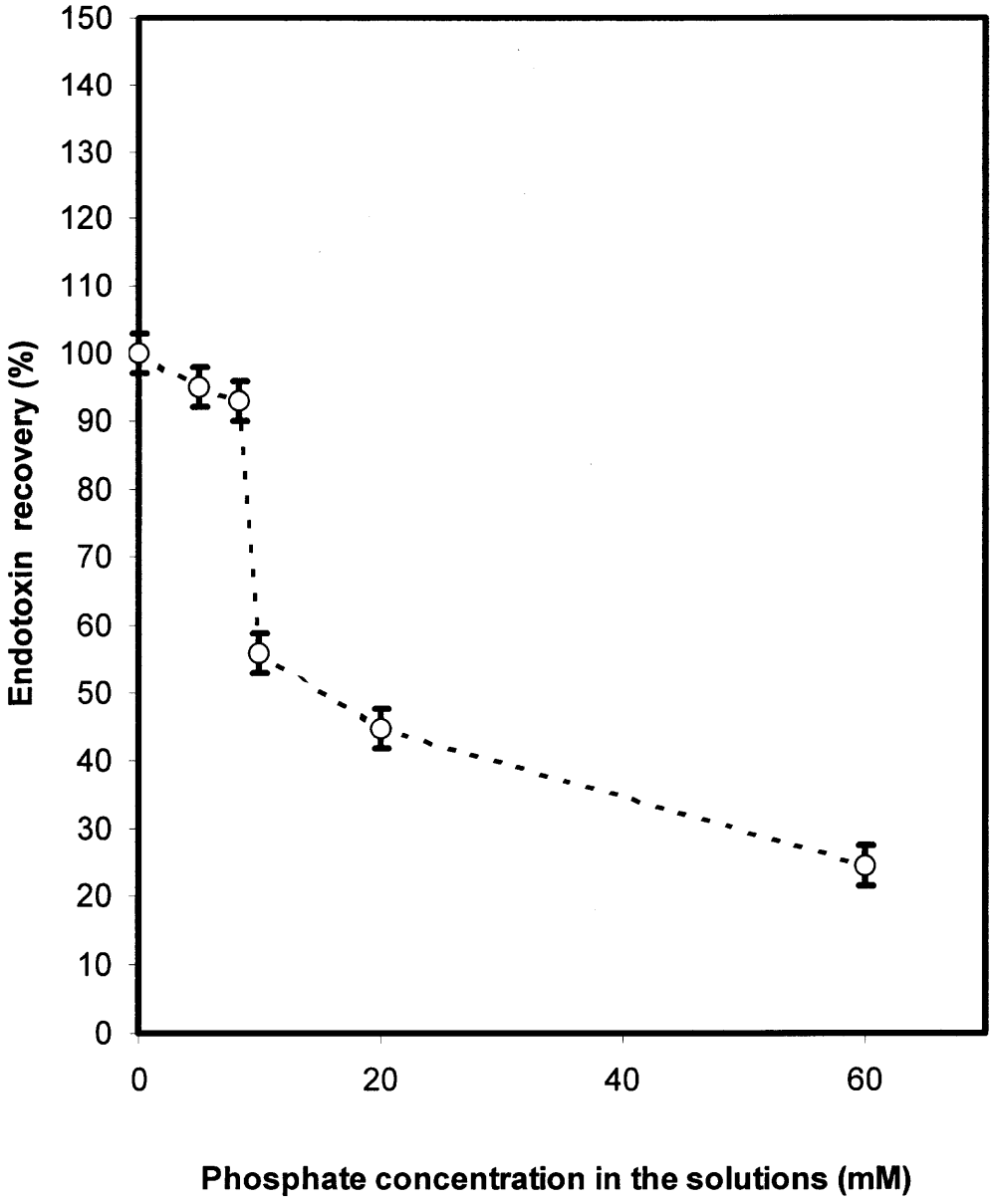


Figure 3.5 Phosphate concentration on the endotoxin concentration determination: Endotoxin concentration recovery

CHPATER 4

EFFECT OF SOLUTION CONDITIONS ON ENDOTOXIN REMOVAL AND PROTEIN RECOVERY BY ACTICLEAN ETOX COLUMN

4.1. Introduction

Lipopolysaccharide (LPS) or endotoxin is known to have potent biological effects in human. Serious endotoxin infection can cause sepsis and septic shock, leading to severe hypertension, cardiovascular collapse, multiple organ failure and death (Billiau and Vandekerckhove, 1991). In biotechnology industry, Gram-negative bacteria are widely used 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 (Hou and Zaniewski, 1990). The removal of these physiologically active agents from final bioproducts has always been a challenge, especially in the situations where endotoxins bind product proteins. Significant product loss and low product yield can result from the separation steps employed to remove endotoxins. It is 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. 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 previous studies, researchers in Luo's research group investigated the concentration effects of hemoglobin and albumin on protein-LPS binding and endotoxin removal (Li and Luo, 1997). It was found that the proteins interacted with LPS and formed protein-LPS complexes, resulting in the

disaggregation of LPS vesicles. Researchers in this group also reported the use of Ca^{+2} to re-aggregate lipopolysaccharide (LPS) in hemoglobin solutions and the subsequent removal of endotoxin in a two-stage ultrafiltration process (Li and Luo, 1998, 1999).

Although numerous methods such as ion exchange adsorption (Weber et al., 1995), ion exchange membrane (Belanich et al., 1996), ion-exchange filter (Hou and Zaniewski, 1990), ultrafiltration (Li and Luo, 1998, 1999), and extraction (Aida and Pabst, 1990) have been studied for endotoxin removal from protein solutions, affinity adsorption has proven to be the most effective technique. Polymyxin B has been investigated intensively as an endotoxin binding ligand since Issekutz (1983) reported its ability to remove endotoxin from solutions. In subsequent studies, polymyxin B has been immobilized on chromatographic supports such as Sepharose (Karprus et al., 1987) to remove endotoxin from protein solutions. Membranes (Petsch et al., 1997) or fibers (Tani et al., 1992) have been used as the support as well. Immobilized histidine has also been employed to remove endotoxin from protein solutions. Sepharose resin (Matsumae et al., 1990), filter-paper (Guo et al., 1997), and hollow fiber membranes (Legallais et al., 1997) have been used as the support. Hirayama et al. (1994) studied the use of cross-linked N, N-dimethylaminopropylacrylamide (DMAPAA) spherical particles for selective removal of endotoxin from protein solutions. They found that ionic strength had less effect on the endotoxin removal efficiency with DMAPAA than with immobilized histidine. In another study, investigators from the above group (Morimoto et al., 1995) studied the application of polyethyleneimine (PEI)-immobilized cellulose fibers for endotoxin removal from protein solutions. Matsumae et al (1990) applied immobilized histidine to remove endotoxin from protein solutions. The effects of pH and ionic

strength on the removal of endotoxin were investigated. Although the influences of ionic strength on endotoxin removal were reported in the above articles, the effects of divalent cations on endotoxin removal and protein recovery were not studied. At the same ionic strength, divalent cations have much stronger influences on endotoxin aggregation state due to the bridging effect (Li and Luo, 1998).

The use of Acticlean Etox affinity column from Sterogene Bioseparations (Carlsbad, CA) to remove endotoxin from hemoglobin preparations where the protein formed complex with LPS subunits (Kang and Luo, 1998) was reported. The effects of various solutions such as endotoxin-free water, NaCl and $CaCl_2$ on the endotoxin removal efficiency and protein recovery in the chromatographic process were investigated. However, the study was carried out with a fixed ionic strength at neutral pH. Further experiments were needed to see how the column would behave at various ionic strength and pH conditions.

In this study, endotoxin was removed from hemoglobin preparations using an Acticlean Etox affinity column. The effects of ionic strength and pH on endotoxin removal efficiency and protein recovery have been investigated. The LPS-resin and protein-resin adsorption mechanisms have been discussed. The effects of protein-LPS complexation on protein recovery in the chromatographic column had also been studied.

4.2. Experimental

4.2.1 Materials

4.2.1.1 Reagents

Endotoxin from *Escherichia coli* 026:B6 (Sigma, St. Louis, MO, USA) was used in all

studies. All solutions were prepared with endotoxin free water (LAL reagent water, BioWhittaker Inc., Walkersville, MD, USA). Hemoglobin Ao (HbAo, ferrous), NaCl (>99%) and $CaCl_2$ (>99%) were purchased from Sigma.

4.2.1.2 Column and Experimental Setup

The experiments were conducted on an Acticlean Etox column (Product No. 4AA04), which was kindly provided by Sterogene Bioseparations, Inc. (Carlsbad, CA, USA). The column dimensions were 3.5x0.7 cm. The column was packed with an Acticlean Etox affinity resin, which had a Actigel ALD support matrix (4% agarose) linked to a proprietary ligand through a secondary amine. The experimental setup was shown in Figure 4.1. Before each use the column was cleaned by perfusion with 30 ml of 1.0 M NaOH, allowed to stand at 4°C overnight and washed with endotoxin free water until neutrality. Samples were applied to the column at an approximate flow rate of 0.3 ml/ min. Elution was carried out at above flow rate with endotoxin-free water as the mobile phase.

4.2.1.3 Labware

All glassware used was autoclaved at 15 psig for 30 minutes with an electric pressure steam sterilizer (Model No. 25X, Wisconsin Aluminum Foundry Co., Inc., WI, USA) and followed with heating in an oven (Model 16, Precision Scientific Co., IL, USA) at 210°C for 3 hours. All solution transfers were performed by endotoxin free devices. Sterile, disposable plasticware was used at all times to prevent endotoxin contamination.

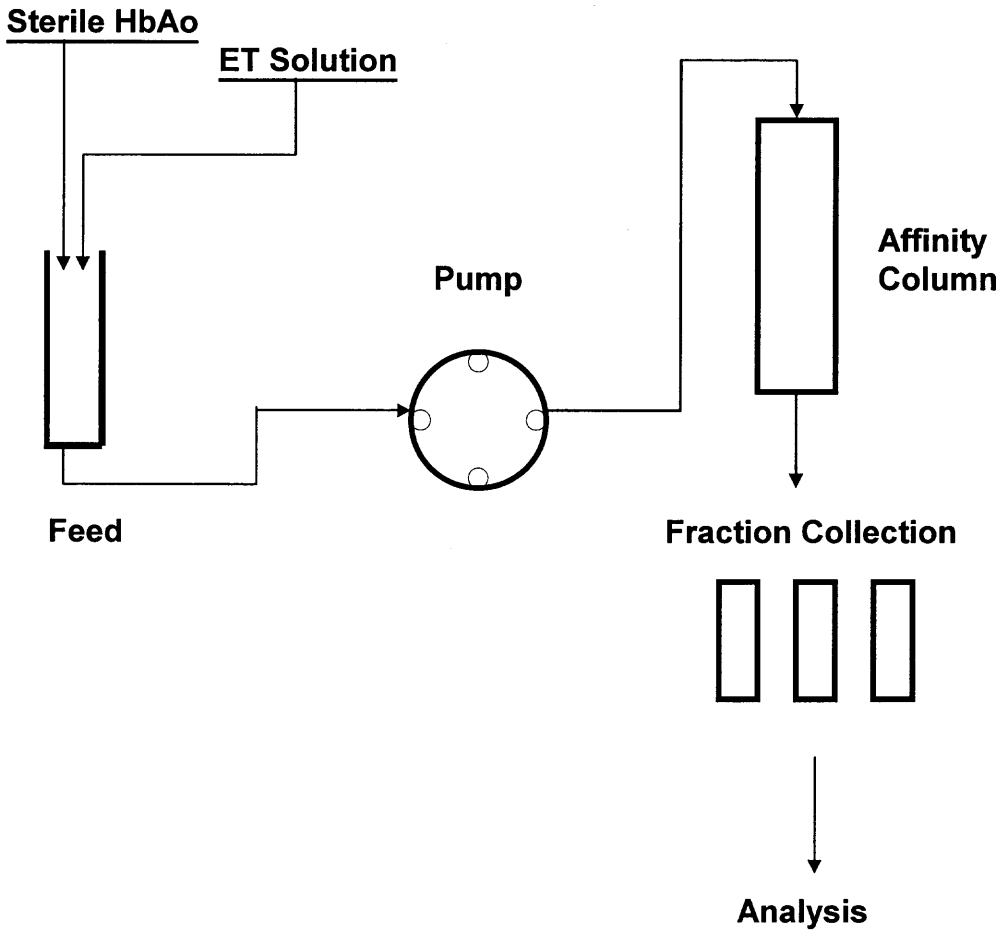


Figure 4.1 Experimental Setup

4.2.2 Protein Assay

The absorption scanning from 250 nm to 700 nm by author shows that the endotoxin sample only has the absorbance peak at around 280 nm (The absorption spectrum is not shown here). Therefore HbAo concentration was measured with a model U-2000 UV-Vis spectrophotometer (Hitachi Instruments Inc., Danbury, CT, USA) at 415 nm to avoid interference by the endotoxin absorbance.

4.2.3 Endotoxin Assay

To determine endotoxin concentration, a chromogenic *limulus* amebocyte lysate (LAL) test kit QCL-1000 from BioWhittaker (BioWhittaker Inc., Walkersville, MD) with a modified procedure was used. The sensitivity of this test is 0.01 EU/ml. The reaction mixtures were measured at 405 nm using the Hitachi U-2000 spectrophotometer and the results were compared to a standard curve to obtain endotoxin concentrations. However, HbAo also has a strong absorbance at 405 nm and subtraction of HbAo absorbance is necessary. The correction was done by subtracting the HbAo absorbance from total absorbance of the reaction mixture (Kang and Luo, 1999).

The endotoxin removal ability of the process was quantitatively evaluated using Endotoxin Removal Efficiency, ERE, which was determined by the following equation,

$$ERE = \frac{m_{LPS}^{feed} - m_{LPS}^{frac}}{m_{LPS}^{feed}} X100\%$$

Where m_{LPS}^{feed} is the amount of endotoxin (in endotoxin unit, EU) in the feed loaded on the column. m_{LPS}^{frac} is the amount of endotoxin (EU) in all the elution fractions.

4.2.4 Endotoxin Removal from HbAo-LPS Feeds Prepared with Endotoxin Free Water

HbAo solutions were mixed with endotoxin to reach about 2.00 EU/ml final endotoxin concentration and 90 to 100.00 μg /ml HbAo and incubated for 30 minutes at 37°C in water bath in order to form HbAo-LPS complex (Li and Luo, 1998). 10 ml feed of such HbAo solution was perfused through the column at 0.3 ml /min. The eluents were collected at 3 ml (Fraction I), 13 ml (Fraction II), and 23 ml (Fraction III). Fraction II is the main fraction, or product. Proper dilutions of samples taken from the fractions were needed in order to obtain the maximum endotoxin assay sensitivity.

4.2.5 Endotoxin Removal from HbAo-LPS Feeds Prepared with NaCl Solutions at Various Ionic Strength

HbAo solutions prepared with NaCl solutions at different ionic strength, 0.05, 0.10, 0.15 and 0.25 M, were mixed with endotoxin to reach about 2.00 EU/ml final endotoxin concentration and 90 to 100.00 μg /ml HbAo concentration. The mixtures were incubated for 30 minutes at 37°C. The ionic strength of the solution was calculated according to Debye-Huckel theory (Pimmetel and Spratley, 1971). 10 ml feed was applied to the column. Three fractions were collected in the same way as described in Section 4.2.4.

4.2.6 Endotoxin Removal from HbAo-LPS Feeds Prepared with $CaCl_2$ Solutions at Various Ionic Strengths

HbAo solutions prepared with $CaCl_2$ solutions of different ionic strengths, 0.05, 0.10, 0.15, 0.25 M, were mixed with endotoxin to reach about 2.00 EU/ml final endotoxin

concentration and 90.00 to 100.00 μg /ml HbAo concentration. The mixtures were incubated for 30 minutes at 37°C. 10 ml feed was applied to the column. Three fractions were collected in the same way as described in Section 4.2.4.

4.2.7 Elution of Pure HbAo Feed Prepared with Endotoxin Free Water

In the second part of the study on ionic strength effect on protein recovery in the affinity process, pure protein solutions were employed as feeds, which served as controls to those involving HbAo-LPS mixtures as feeds. HbAo solutions without an endotoxin spike were prepared with endotoxin free water to reach final HbAo concentrations from 90 to 100 μg /ml. After incubation at 37°C for 30min., 10 ml feed was applied to the column. Three fractions were collected in the same way as described in Section 4.2.4.

4.2.8 Elution of Pure HbAo Feeds Prepared with NaCl Solutions at Various Ionic Strength

HbAo solutions without endotoxin spike were prepared with NaCl solutions at 0.05, 0.10, 0.15, 0.25 M ionic strength to reach final HbAo concentration from 90 to 100 μg /ml. The solutions were incubated for 30 minutes at 37°C. 10 ml feed was applied to the column. Three fractions were collected in the same way as described in Section 4.2.4.

4.2.9 Elution of Pure HbAo Feeds Prepared with $CaCl_2$ Solutions at Various Ionic Strength

HbAo solutions without endotoxin spike were prepared with $CaCl_2$ solutions at 0.05, 0.10,0.15, 0.25 M ionic strength to reach final HbAo concentration from 90 to 100 μg /ml. The solutions were incubated for 30 minutes at 37°C. 10 ml feed was applied to the

column. Three fractions were collected in the same way as described in Section 4.2.4. All feeds applied to the Acticlean Etox column in the study of ionic strength effect were summarized in Table 4.1.

4.2.10 Endotoxin Removal from HbAo-LPS Mixtures Prepared with Buffers at Various pH

In order to study the effect of pH on the endotoxin removal efficiency and protein yield, HbAo-LPS feeds were prepared with buffers at various pH: 4.5, 6.0, 7.0, 8.0 and 9.0. The buffers used were acetate buffer (pH 4.5, Cases 4.1), phosphate buffer (pH 6.0, Case 4.2; pH 7.0, Case 4.3; and pH 8.0, Case 4.4), and This-HCl buffer (pH 9.0, Case 4.5). The concentration was 0.05 M for all buffer solutions. First, the stock solutions of HbAo and endotoxin were prepared separately with the same buffer at a specific pH. A certain volume of the HbAo stock was then mixed with a certain volume of endotoxin stock to reach final endotoxin concentration at about 2.00 EU/ml and HbAo at 90 to 100 μg /ml. The HbAo-LPS mixtures at pH 4.5, 6.0, 7.0, 8.0 and 9.0 were incubated at 37°C for 30 min., respectively, to form HbAo-LPS complex. 10 ml feed from each mixture was applied to the column at a flow rate of 0.3 ml/min in individual runs. Three fractions were collected in the same way as described in Section 4.2.4.

4.2.11 Elution of Pure HbAo Feeds Prepared with Buffers at Various pH

In the second part of the study on pH effect on the protein recovery in the affinity process, pure protein solutions at various pH were employed as feeds, which served as controls to those involving HbAo-LPS mixtures as feeds. The buffers used were acetate

buffer (pH 4.5, Case P-4.1), phosphate buffer (pH 6.0, Case P-4.2; pH 7.0, Case P-4.3; and pH 8.0, Case P-4.4) and Tris-HCl buffer (pH 9.0, Case P-4.5). The concentration was 0.05 M for all buffers. Five pure HbAo solutions were prepared with above buffers at pH 4.5, 6.0, 7.0, 8.0 and 9.0, respectively. In the control experiments, 10 ml of the pure HbAo feed from each solution were applied to the column at a flow rate of 0.3 ml/min in individual runs. Three fractions were collected in the same way as described in Section 4.2.4.

4.3. Results and Discussion

4.3.1 Endotoxin Removal Efficiency of HbAo-LPS Feeds Prepared with NaCl Solutions at Various Ionic Strength

The endotoxin concentrations in the feeds and the products of the chromatography process are shown in Table 4.1 for Cases 2.1, 2.2, 2.3 and 2.4 in which feeds were prepared with NaCl solutions at various ionic strengths. The endotoxin removal efficiency, ERE, as a function of ionic strength, μ, for all these cases is shown in Figure 4.2. It can be seen from Figure 4.2 that ERE was around 71% as the ionic strength was increased from 0 to 0.05 M. When the ionic strength was increased from 0.05 to 0.15 M, ERE dropped significantly from 72% to 43%, which indicates the mechanism of ion exchange in this range of ionic strength. When ionic strength was increased from 0.15 to 0.25 M, ERE was kept almost constant at a level of 40%. This indicates affinity interactions other than ionic exchange had taken place.

Table 4.1 Effect of solution ionic strength on LPS concentration in feed and product, HbAo recovery, and endotoxin removal efficiency, ERE

Cases	Ionic Strength I (M)		HbAo recovery R (%)		LPS Concentration (EU/ml)		ERE (%)
	NaCl	$CaCl_2$	Without LPS	With LPS	Feed	Product	
Case 1	0	0	94.0	90.0	2.19	0.64	71.8
Case 2.1	0.05	0	100.0	99.7	2.27	0.69	69.6
Case 2.2	0.10	0	100.0	100.0	2.31	0.99	56.4
Case 2.3	0.15	0	99.0	96.0	2.44	1.30	42.7
Case 2.4	0.25	0	99.0	92.9	2.12	1.37	39.6
Case 3.1	0	0.05	97.0	97.6	2.85	1.19	54.0
Case 3.2	0	0.10	100.0	99.0	2.43	1.56	39.7
Case 3.3	0	0.15	99.0	99.0	2.75	1.60	38.2
Case 3.4	0	0.25	96.0	96.9	2.67	1.61	37.8

4.3.2 Endotoxin Removal Efficiency of HbAo-LPS Feeds Prepared with $CaCl_2$ Solutions at Various Ionic Strength Conditions

The endotoxin concentrations in the feeds and the products of the chromatography process are shown in Table 4.1 for Cases 3.1, 3.2, 3.3 and 3.4 in which feeds were prepared with $CaCl_2$ solutions at various ionic strengths. The endotoxin removal efficiency, ERE, as a function of ionic strength, I, for all above cases was shown in Figure 4.2. It can be seen from Figure 4.2 that ERE dropped significantly from 71% to around 38%, as the ionic strength was increased from 0 to 0.10 M, which indicates the mechanism of ion exchange in the range of ionic strength. When ionic strength was increased from 0.10 to 0.25 M, ERE was kept almost constant at a level of 38%. This indicates affinity interactions other than ionic exchange had taken place.

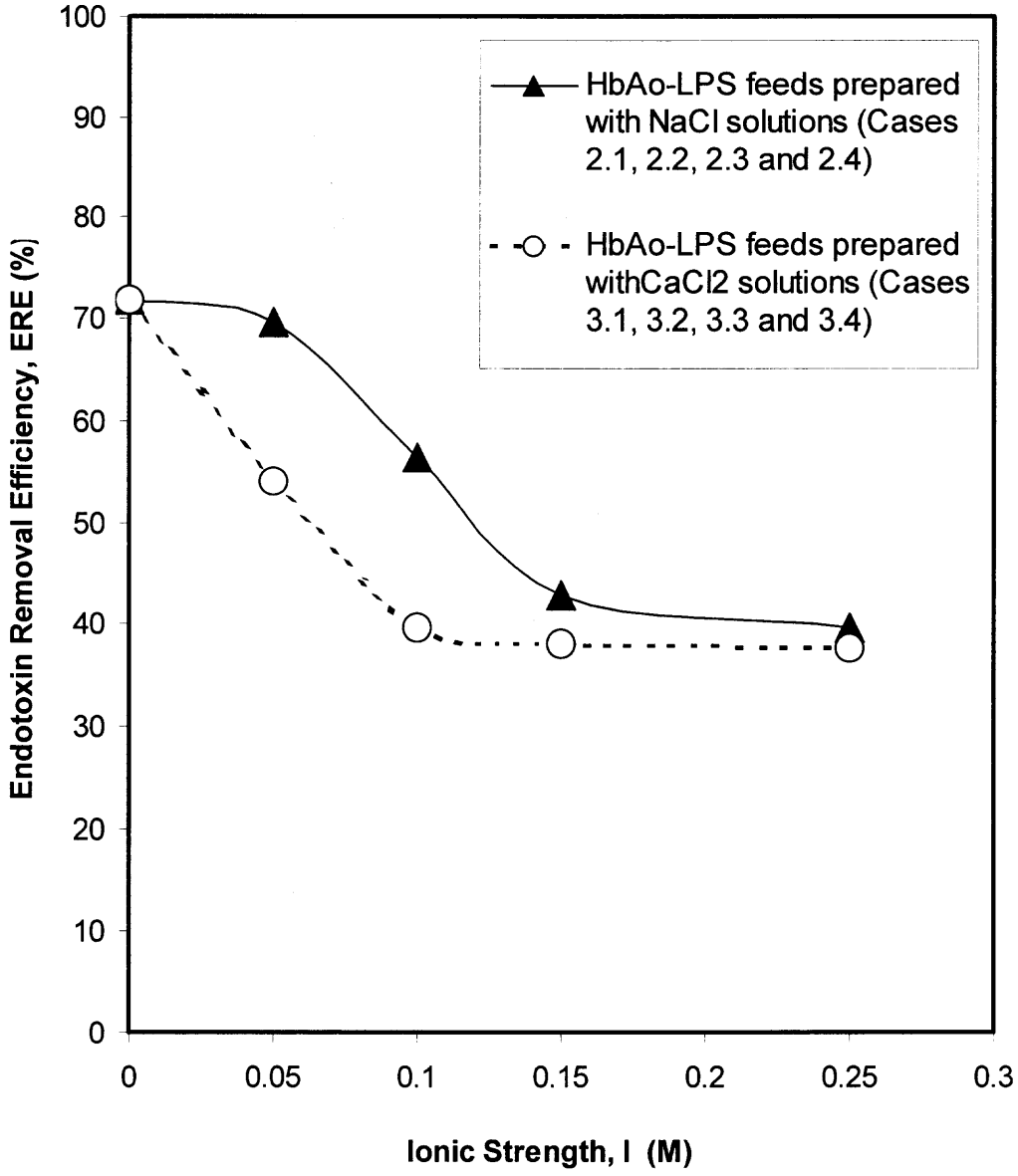


Figure 4.2 Effect of solution ionic strength on endotoxin removal efficiency, ERE, of HbAo-LPS feeds prepared with NaCl and $CaCl_2$ solutions at various ionic strength, respectively

4.3.3 Protein Recovery of HbAo-LPS Feeds Prepared with NaCl at Various Ionic Strength Conditions

The HbAo recovery, R, of the chromatography process is shown in Table 4.1 for Cases 2.1, 2.2, 2.3 and 2.4 in which HbAo-LPS feeds were prepared with endotoxin free water (Case 1) and NaCl solutions (other cases) at various ionic strength. It can be seen that HbAo recovery was around 90% when the feed was prepared with endotoxin free water. When ionic strength was increased from 0 to 0.10 M, HbAo recovery was also increased to 100%, which indicates a very weak ion exchange interaction in this range of ionic strength. When ionic strength was increased from 0.10 to 0.25 M, HbAo recovery was decreased slightly and then kept almost constant at a level of 94%. This indicates that possible hydrophobic interactions might have taken place. Within the ionic strength range studied, i.e., 0 to 0.25 M, R was always above 90% and it reached the maximum point, 100% at I = 0.1 M. The largest discrepancy of HbAo recovery at different ionic strength was less than 10%. There was a very limited HbAo adsorption to the column in the cases reported.

4.3.4 Protein Recovery of HbAo-LPS Feeds Prepared with $CaCl_2$ at Various Ionic Strength Conditions

The HbAo recovery in the chromatography process is shown in Table 4.1 for Cases 3.1, 3.2, 3.3 and 3.4 in which HbAo-LPS feeds were prepared with endotoxin free water (Case 1) and $CaCl_2$ solutions (other cases) at various ionic strength. It can be seen that HbAo recovery was increased from 90% to around 99%, as the ionic strength was increased from 0 to 0.10 M, which indicates the mechanism of ion exchange in this range of ionic strength. When ionic strength was increased from 0.10 to 0.25 M, R was

decreased slightly and retained constant at a level of 97%. Similar to the situations in Section 4.3.3, this indicates possible hydrophobic interactions between the HbAo and the resin. Within the ionic strength range from 0 to 0.25 M, R was always above 90% and it reached the maximum point, 99%, at I = 0.1 M. The largest discrepancy of HbAo recovery at different ionic strength was less than 9%, which represents a very limited HbAo-resin interaction in the whole range of ionic strength studied in the experiments.

By comparing the HbAo recovery of above two situations, i.e., feeds prepared with NaCl solutions and feeds prepared with $CaCl_2$ solutions, the changes of R versus ionic strength followed a similar pattern. When the ionic strength was increased from 0 to 0.25 M, R was first increased to a maximum value at I = 0.1 M, then decreased slightly and kept at a constant level thereafter. The largest difference of R values between the two situations was about 3% at I = 0.25 M.

4.3.5 Protein Recovery of Pure HbAo Feeds Prepared with NaCl at Various Ionic Strength Conditions

Table 4.1 shows the HbAo recovery in the chromatographic process in which pure HbAo feeds were prepared with endotoxin free water (Case P-1) and NaCl solution (Cases P-2.1 to P-2.4). Here pure HbAo feeds were used as controls for the HbAo-LPS feeds described in Section 4.3.3. From the table, HbAo recovery was 94% when the feed was prepared with endotoxin free water (I = 0). When the ionic strength was increased from 0 to 0.05 M and further to 0.15 M, HbAo recovery was close to 100% and stayed there. This indicates a weak ionic interaction between the protein and resin. When the ionic

strength was increased to 0. 25 M, HbAo recovery dropped slightly to 99%, which implies a minimum affinity interaction between HbAo and the resin.

4.3.6 Protein Recovery of Pure HbAo Feeds Prepared with $CaCl_2$ at Various Ionic Strength Conditions

Table 4.1 also shows the HbAo recovery in the chromatographic process in which pure HbAo feeds were prepared with endotoxin free water (Case P-1) and $CaCl_2$ (Cases P-3.1 to P-3.4). Here pure HbAo feeds were used as controls for the HbAo-LPS feeds described in Section 4.3.4. From table, HbAo recovery was 94% when the feed was prepared with endotoxin free water (I = 0). When the ionic strength was increased from 0 to 0.05 M and further to 0.15 M, HbAo recovery was close to 98% and stayed there. This indicates a weak ionic interaction between the protein and resin. When the ionic strength was increased to 0. 25 M, HbAo recovery dropped slightly to 96%, which implies a minimum affinity interaction between HbAo and the resin.

4.3.7 Effect of pH on Endotoxin Removal Efficiency of HbAo-LPS Feeds

In this part of the study (Sections 4.3.7 and 4.3.8), HbAo-LPS mixtures prepared with buffers at various pH were applied to the column, as described in Section 4.2.10. Figure 4.3 shows how the change of pH effected the endotoxin removal efficiency, ERE. When pH = 4.5, ERE was 92%. As pH was increased from 4.5 to 8.0, ERE decreased gradually from 92% to 83%. As pH was increased further from 8.0 to 9.0, ERE dropped drastically from 83% to 43%. The results indicate that ion exchange and other interactions had occurred between the endotoxin molecules and the resin.

4.3.8 Effect of pH on Protein Recovery of HbAo-LPS Feeds

Figure 4.3 shows how the change of pH affected the HbAo recovery, R. When pH = 4.5, R was 97%. As pH was increased from 4.5 to 7.0, R increased to 100%. As pH was increased further from 7.0 to 9.0, R decreased slightly to 97%. Therefore, there was only less than 3% of HbAo being retained in the column across the pH range in the study.

4.3.9 Effect of pH on Protein Recovery of Pure HbAo Feeds

As described in Section 4.2.11, pure HbAo solutions prepared with buffers at various pH were employed as the feeds to the column. These pure HbAo feeds served as controls to the HbAo-LPS mixtures mentioned in Section 4.3.8. When pure HbAo feeds were applied to the column, HbAo recovery, R, was 100% for all of the pH values.

4.3.10 Discussion

The results in Section 4.3.1 and 4.3.2 have revealed several aspects of endotoxin removal from HbAo-LPS mixture in this affinity column. First, endotoxin removal efficiency, ERE, was a strong function of ionic strength in both situations (feeds prepared with NaCl solutions and $CaCl_2$ solutions). ERE had a general decreasing trend when ionic strength was increased. Second, cations in the feeds have significant influences on ERE of the column. Moreover, $CaCl_2$ solutions affected endotoxin binding to the column much more than NaCl solutions at all ionic strengths (except I = 0 M). Third, the results indicate that the adsorption mechanism between endotoxin molecules and the resin includes ion exchange as well as other interactions such as hydrophobic interaction.

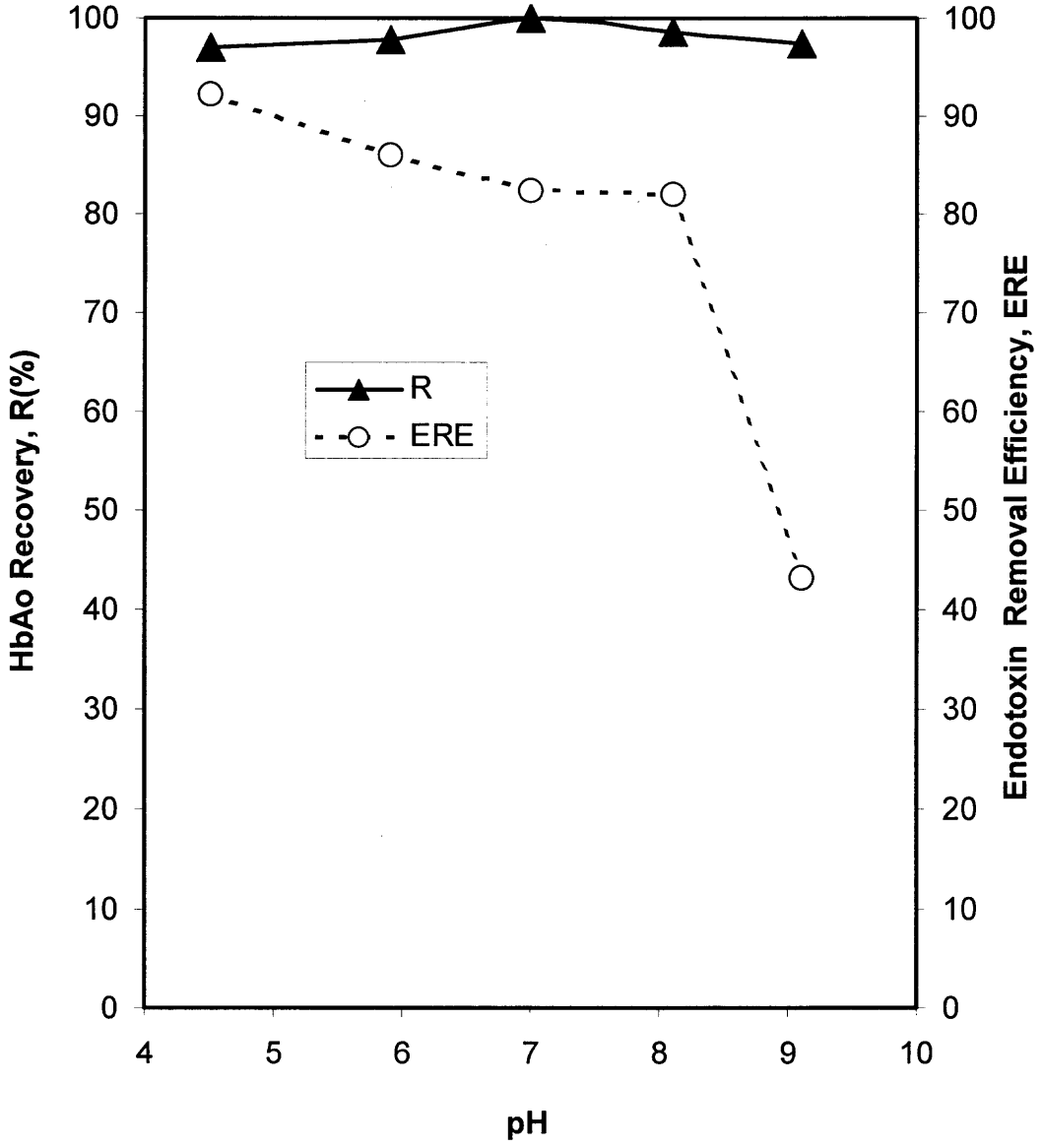


Figure 4.3 Effect of pH on endotoxin removal efficiency, ERE, and protein recovery, R, of HbAo-LPS feeds prepared with buffers at various pH, m=0.05 M

Ionic strength also affects protein recovery, R, in both situations of HbAo-LPS feeds and pure HbAo feeds as presented in Sections 4.3.3, 4.3.4, 4.3.5 and 4.3.6. However, the effects of ionic strength on protein recovery are very limited. When HbAo-LPS mixtures were used as the feeds, R was always above 94% within the ionic strength range studied. The results indicate that the column has very weak affinity to HbAo. Further, it was also shown that the protein-LPS interactions affected protein recovery. By comparing HbAo recovery of HbAo-LPS mixtures to the HbAo recovery of pure HbAo solutions, the presence of endotoxin in protein solutions decreased the protein recovery. The decrease of R is apparently due to the complexation between HbAo and LPS. Since LPS was adsorbed by the resin in the column, the HbAo molecules in the HbAo-LPS complex were also retained in the column.

The results in Section 4.3.7 show a strong influence of pH on endotoxin removal efficiency. The adsorption mechanism may be explained as follows. Since the pI of endotoxin is about 2 (Pearson, 1985), endotoxins are always negatively charged within the pH range in this study. According to the manufacturer, the resin has a pKa around 8.0. Therefore the resin was positively charged when pH was less than 8.0, neutral when pH was equal to 8.0, and negatively charged when pH was greater than 8.0. At pH = 4.5, the resin had a strong positive charge and adsorbed majority of the endotoxin in the HbAo-LPS mixture, resulting in a very high ERE = 92%. As pH was increased from 4.5 to 8.0, the resin gradually became less positively charged and eventually neutral. As a result, the resin adsorbed less endotoxin than before, and ERE slowly decreased to 83%. However, other affinity interactions were still at play, which contributed to the 83% ERE even though the resin was not charged at this point. As the pH was increased further

from 8.0 to 9.0, the resin was negatively charged, which created a repulsion between endotoxin molecules and the resin. Consequently, there was a sharp 40% drop of ERE. At pH 9.0, only 43% of the endotoxin was adsorbed by the column. The adsorption was likely due to other affinity interactions such as hydrophobic interactions between the endotoxin and the resin.

The pH has a very limited effect on protein recovery in both situations of HbAo-LPS feeds and pure HbAo feeds, as presented in Sections 4.3.8 and 4.3.9. The influence of pH on R was even smaller than that of ionic strength. Further, from the results of both ionic strength study and pH study, it can be seen that the resin has almost no affinity to HbAo. When pure HbAo feeds were applied to the column, R was 100 % at all pH values. When HbAo-LPS feeds were applied to the column, R was above 97% at all pH values. The fact that applying HbAo-LPS mixture had reduced the protein recovery once again demonstrated the effect of protein–LPS complex on protein recovery as discussed earlier in this section.

4. 4 Summary

In this study, an affinity chromatographic column Acticlean Etox was used to remove endotoxin from HbAo preparations. It was found that ionic strength and pH conditions of the feed solutions had significant effects on endotoxin removal from HbAo solutions. For HbAo-LPS feeds prepared with NaCl solutions, endotoxin removal efficiency decreased from 71% to 38% when ionic strength increased from 0 to 0.25 M. For HbAo-LPS feeds prepared with $CaCl_2$ solutions, endotoxin removal efficiency decreased from 71% to 37% when ionic strength increased from 0 to 0.25 M. When pH was varied from

4.5 to 9.0, endotoxin removal efficiency of the column was significantly reduced from 92% to 43%. The adsorption mechanism has been discussed for both situations. Protein recovery of the endotoxin removal process was also investigated. Contrary to the finding on endotoxin removal efficiency, both ionic strength and pH had only very limited effects on protein recovery in the process, for HbAo-LPS feeds as well as pure HbAo feeds. The column adsorbed less than 6% of HbAo in all cases while removing significant amounts of endotoxin. Finally, it was found that the presence of endotoxin in protein solutions could reduce the protein recovery of this affinity process, due to the complexation of HbAo and LPS.

Current available endotoxin removal affinity resins or gels such as Acticlean Etox gel from Sterogene Bioseparation Inc., have following problems.

1) Low capacity. Even at low ionic strength the ET adsorption capacity is about 100EU/ml gel, which is very low.

2) Removal mechanism is affinity and ion exchange or mainly ion exchange mechanism, which is problematic. When the ionic strength is low both the protein and ET are adsorbed. As the ionic strength increases both protein and ET are eluted out.

3) Affinity resin may dissociate the ET-protein complex. However the dissociation effects are not notable. It is very difficult to reduce endotoxin concentration below 0.5 EU/ml in HbAo solution.

4) Regeneration problems. The column must be treated with 1 M NaOH solution for 24 hrs, which is very time consuming and inconvenient.

5) Low flow rate: 0.3 ml/min and therefore time consuming procedure.

6) Very expensive.

Therefore highly efficient and cost effective ligands for removing endotoxin from protein solutions need to be developed.

CHAPTER 5

ADSORPTION ISOTHERM OF ENDOTOXIN IN IDA-Fe^{3+} IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY COLUMN

5.1 Introduction

Immobilized metal ion affinity chromatography (IMAC) is a special affinity chromatographic method based on the interactions between biomolecules (mainly proteins) and metal ions. It was first introduced in 1975 by Porath et al. In IMAC, the interactions are due to coordination between immobilized metal ions and electron donors on the surface of adsorbates such as surface-located histidine, tryptophan and cysteine residues. IMAC has been applied in the purification of many proteins, which was reviewed by Sulkowski in 1985 and Porath in 1992.

The removal of bacterial endotoxins from final bioproducts such as proteins and peptides has always been a challenge, especially in the situation where endotoxins bind product proteins.

IMAC has found a wide application in protein purification process. However, there is no report applying IMAC to remove endotoxin from protein solutions, and here a novel IMAC method is developed to removal endotoxin from protein. Fe^{3+} was charged to a chelating gel that contains iminodiacetic acid (IDA). The IDA-Fe^{3+} IMAC column was exploited to remove endotoxin from the mixtures of proteins and endotoxin. In most cases more than 90% protein yield and higher than 99% endotoxin removal efficiency were obtained. It is an efficient, cost effective, user friendly method to selectively remove endotoxin from protein solutions as compared with traditional endotoxin removal methods (Issekutz, 1983; Karprus et al., 1987; Aida and Pabst, 1990; Hou and

Zaniewski, 1990; Matsumae et al., 1990; Tani et al., 1992; Webber et al., 1995; Belanich et al., 1996; Petsch et al., 1997; Legallais et al., 1997; Guo et al., 1997; Kang and Luo, 1998; Li and Luo, 1998, 1999).

To improve the efficiency of IDA-Fe^{3+} IMAC in endotoxin removal from protein solutions, the endotoxin adsorption isotherms were investigated under different solution conditions. Isotherm models were tested and the adsorption mechanism was discussed. Good agreement between the experimental data and model predictions was achieved.

5.2 Model

In order to allow quantitative analysis of the effect of buffer conditions such as ionic strength and pH on the endotoxin adsorption, equilibrium isotherm models were considered. The frequently used equilibrium models are illustrated as follows. More sophisticated models can be found in the literature (Ruthven, 1984; Valenzuella and Myers, 1989).

5.2.1 Linear Isotherm

$$q^* = K\,C^* \tag{5.1}$$

Where q^* is the average solute concentration in the adsorbent at equilibrium and C^* is the equilibrium concentration of the solute in bulk liquid. K is the equilibrium constant. This linear isotherm is a very ideal situation. Generally, adsorption follows linear isotherm when the solution concentration is very low.

5.2.2 Langmuir Isotherm

The most widely used model is the Langmuir isotherm, shown in equation 5.2.

$$q^* = \frac{q_m C^*}{K_D + C^*} \tag{5.2}$$

Where q* is the average solute concentration in the adsorbent at equilibrium and C* is the equilibrium concentration of the solute in bulk liquid. This model contains two isotherm parameters: q_m, which is the maximum binding capacity of the adsorbent, and K_D, which is the apparent dissociation constant of the adsorbate-adsorbent complex.

Langmuir model is applicable when following conditions are satisfied: (1) the solution is ideal; (2) the solute gives monolayer coverage; (3) the adsorption layer is ideal; (4) no solute-solute interactions in the monolayer; (5) there are no solvent-solute interactions. These conditions cannot be valid in liquid-solid adsorption at high concentrations. However, the Langmuir isotherm was in excellent approximation for single-component adsorption equilibrium in liquid-solid adsorption.

One method for testing the validity of Langmuir isotherm is by Scatchard analysis, which simply involves linearization of the isotherm model to the form as shown in Equation 5.3.

$$\frac{q^*}{C^*} = \frac{q_m}{K_D} - \frac{1}{K_D} q^* \tag{5.3}$$

As indicated in this equation, a linear relation between q*/C* and q* would indicate Langmuir adsorption. If the Langmuir isotherm does not fit the experimental data adequately, the consideration of alternative approaches is required.

5.2.3 Freundlich Isotherm

As shown in equation 5.4, the Freundlich isotherm model employs two isotherm parameters.

$$q^* = k' C^{*(\frac{1}{n'})} \tag{5.4}$$

The parameter k' is very similar to the capacity parameter in Langmuir isotherm, and is numerically equal to the adsorbed solute concentration in equilibrium with a solution having a concentration of 1.0 mg/ml. It is more difficult to assign a physical meaning to the exponential parameter n'. Perhaps the best interpretation is that 1/n' indicates the affinity of the adsorbate-adsorbent interaction, similarly to the dissociation constant in the Langmuir model. In this regard, a high value of 1/n' indicates a strong interaction while a low value represents a weak interaction.

5.3 Determination of Endotoxin Adsorption Isotherm

5.3.1 Frontal Analysis

Frontal analysis was first independently developed by James and Philips (1954) and by Schay and Szekely (1954) for determination of adsorption isotherm. Successive abrupt step changes of increasing concentration are performed at the column inlet and the breakthrough curves are determined. The preparation of the series of solutions of known concentrations needed is labor intensive. As an alternate, it is possible to use a liquid

chromatograph with a gradient system that can deliver step gradients of known concentrations, which are not only precise but also accurate.

The area from the dead time to the inflection point of the breakthrough curve gives the amount adsorbed:

$$Q_{i+1} = Q_i + \frac{(C_{i+1} - C_i)(V_{F,i+1} - V_0)}{V_a} \tag{5.5}$$

Where Q_i and Q_{i+1} are the amounts of compound adsorbed by the column packing after the ith and the (i+1)th step, when in equilibrium with the concentrations C_i and C_{i+1}, respectively, $V_{F,i+1}$ is the retention volume of the inflection point of the (i+1)th breakthrough curve, V_o is the column void volume, and V_a is the volume of adsorbent in the column, which is 1ml for 1 ml IMAC column and Vo is 0.75 ml. For the case of one feed concentration, equation 5.5 can be modified to

$$Q = C\,(V_F - 0.75) \tag{5.6}$$

5.3.2 Frontal Analysis by Characteristic Point (FACP)

Measurement of a single-component isotherm can be performed from the rear boundary recorded in FA. This is frontal analysis by characteristic points (Glueckauf, 1955). One large concentration solution is pumped into the column. After a long enough plateau has been formed in the column, pure mobile phase is again pumped into the column. The diffusion profile is recorded and the isotherm is derived from this profile using Equation 5.5 to 5.6. Contrary to conventional practice, it is not necessary to wait until the steady

response of the detector indicates that the eluate concentration is the same as the concentration in the stream of mobile phase pumped into the column to return to a pure mobile phase system.

A mass balance can be established for each point on the rear part of the elution profile of this rectangular injection pulse. The amount adsorbed is calculated by integration of the area under the peak, starting from the tail end (C = 0). Following equation might be written:

$$Q_C = \frac{1}{V_a} \int_0^C (V - V_0) dC \tag{5.7}$$

Where Q_C is the amount of compound adsorbed by the column packing when in equilibrium with the concentration C, Va is the volume of adsorbent in the column, and V is the retention volume of the point of the diffusion profile at concentration C, or the characteristic point. Thus, each point of the rear profile gives one point of the isotherm. With the data acquisition system, several hundred points of an elution profile can be conveniently recorded and stored, providing as many data points for the isotherm as needed.

5.3.3 Static Method

This method consists of immersing a known weight of adsorbent in a solution of known concentration in a closed vessel and waiting until equilibrium is reached. After the equilibrium is reached, the solution concentration is determined. In many aspects the

static methods is comparable to frontal analysis. However, it has disadvantages such as being time consuming and requiring a large amount of materials.

5.4 Materials and Methods

5.4.1 Materials

Endotoxin from *Escherichia coli* 026: B6 (Sigma Chemical Co., St. Louis, MO) was used in all experiments. $FeCl_3$, NaCl, NH_4Cl and EDTA were also purchased from Sigma. All solution transfers were performed with endotoxin free devices. Sterile, disposable plasticware was used at all times to prevent endotoxin contamination. All solutions were prepared with Milli-Q water generated by a Biocel water purification system (Millipore Corporation, Bedford, MA). The endotoxin concentration in the Milli-Q water was below 0.05 EU/ml based on the results of LAL tests in this research.

5.4.2 Experimental Apparatus

A GradiFrac chromatographic system (Amersham Pharmacia Biotech, Piscataway, NJ) was used in all the experiments. The experimental setup was shown in Figure 5.1. Feed and mobile phase solutions were transferred by an adjustable peristaltic P-1 pump. The ultraviolet absorbance of the eluent from the column was measured by UV-1 monitor at 280nm. The flow cell used in the monitor was a standard cell with an optical path length of 1.00 cm. The UV absorbance was recorded by two means. The first one was a REC 102 2-channel recorder, which was used to show chromatograms directly during the experiments. The second one was a data acquisition system M1101 (Keithley Metrabyte,

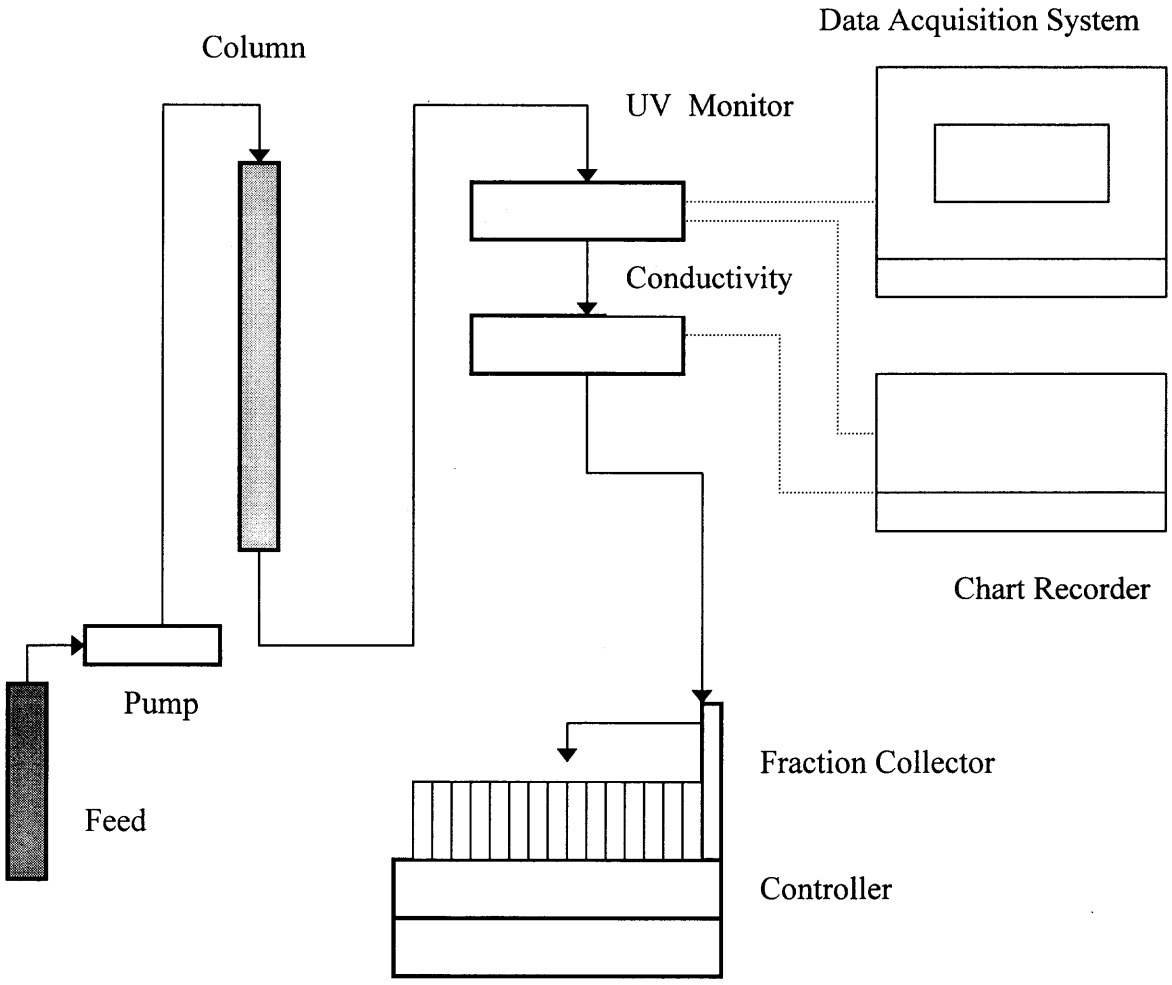


Figure 5.1 IMAC experimental setup

Cleveland, OH) connected with a computer, which was used to record the chromatograms in digital forms so that they could be transferred later for data processing and analysis.

5.4.3 IMAC Column and Adsorbent Resin

A HiTrap Chelating column with dimensions of 0.7 x 2.5 cm was purchased from Amersham Pharmacia Biotech. The column was packed with Pharmacia Chelating Sepharose High Performance resin consisting of highly cross-linked agarose beads to which iminodiacetic acid (IDA) was coupled by stable ether bounds via a seven atom spacer arm. From the manufacturer, the mean diameter of the adsorbent particle is approximately 34 μm. The average pore size is around 100 nm.

5.4.4 Buffers

Three kinds of buffer solutions were used in the experiments. All LPS feeds were prepared with buffer A - 0.5 M NaCl in 0.02 M acetate buffer at pH 5.5. The starting buffer was buffer A. The elution buffer, buffer B, was 0.5 M NH_4Cl in 0.02 M acetate buffer at pH 5.5. The stripping buffer, buffer D, was 0.05 M EDTA (ethylenediaminetetraacetic acid) in buffer A. All buffers were filtered through a 0.22 μm filter and degassed under vacuum for at least 25 min prior to being applied to the column.

5.4.5 Column Preparation and Operation

The HiTrap Chelating column was stored in 20% ethanol when purchased. The column was first washed with 5 ml Milli-Q water at a flow rate of 1 ml/min to remove the

ethanol. Then the column was loaded with 0.5 ml of 0.1 M $FeCl_3$ solution at 1 ml/min to charge Fe^{3+} to the resin. Finally, the column was washed with 5 ml Milli-Q water at 1 ml/min to remove the unbound Fe^{3+} from the column. The column was now charged with metal ion and ready for use. Before applying the feed, a blank run was performed to elute nonspecifically bound metal ions that might otherwise be eluted during desorption. The column was washed with 2.5 ml of buffer A at 2 ml /min followed by 5ml of buffer B at 1 ml/min. The column was then equilibrated with 2.5 ml of buffer A at 1 ml/min. The feed (LPS solution) was applied to the column at 1 ml /min until inflection point was reached in elution profile. The column was then washed with the starting buffer (buffer A) at 1 ml/min for 10 min. The column was regenerated by washing with buffer D to strip all the metal ions, followed by washing with 5 ml of Milli-Q water, both at a flow rate of 1 ml/min.

5.4.6 Endotoxin Assay

To determine endotoxin concentration, a chromogenic Limulus Amebocyte Lysate (LAL) test kit QCL-1000 from BioWhittaker (BioWhittaker Inc., Walkersville, MD) with a modified procedure was used. The sensitivity of this test is 0.01 EU/ml. The reaction mixtures were measured at 405 nm by a Hitachi U-2000 spectrophotometer (Hitachi Instruments Inc., Danbury, CT) and the results were compared to a standard curve to obtain endotoxin concentrations.

A series of solutions of known endotoxin concentrations were prepared as the endotoxin has strong absorption at 280 nm on UV detector. Endotoxin standard curve could be also obtained by drawing the endotoxin concentration (EU/ml or μg/ml) vs.

absorbance (ABS) at 280 nm. Endotoxin ABS in the whole process can be obtained from a data acquisition system M1101 (Keithley Metrabyte, Cleveland, OH) incorporated with a computer, which was used to record the chromatograms in digital forms so that they could be transferred later for data processing and analysis. By comparing with the standard curve, the endotoxin concentration was easily obtained. Both methods were used in this research.

5.5 Results

The experimental results of endotoxin adsorption at different solution conditions by frontal analysis are depicted in Figure 5.2. In order to allow quantitative analysis of the effects of buffer conditions such as ionic strength and pH on the endotoxin adsorption, different equilibrium isotherm models were investigated.

5.5.1 Langmuir Isotherm

The most widely used model, Langmuir isotherm, was investigated in this study. The derivation of Langmuir isotherm includes the assumption of monovalent and homogeneous adsorption on the adsorbent. The assumption of monovalent adsorption is valid only when each adsorbate molecule is bound by interaction with single immobilized ligand. Homogeneous adsorption implies that each adsorbate molecule is bound by an identical interaction. Consequently, the binding energy and dissociation constant must be the same for each adsorbed molecule. Furthermore, Langmuir isotherm requires that the adsorbed molecules do not interact with other unbound molecules. Thus it is obvious that Langmuir isotherm is truly valid only when the adsorption is simple and well behaved.

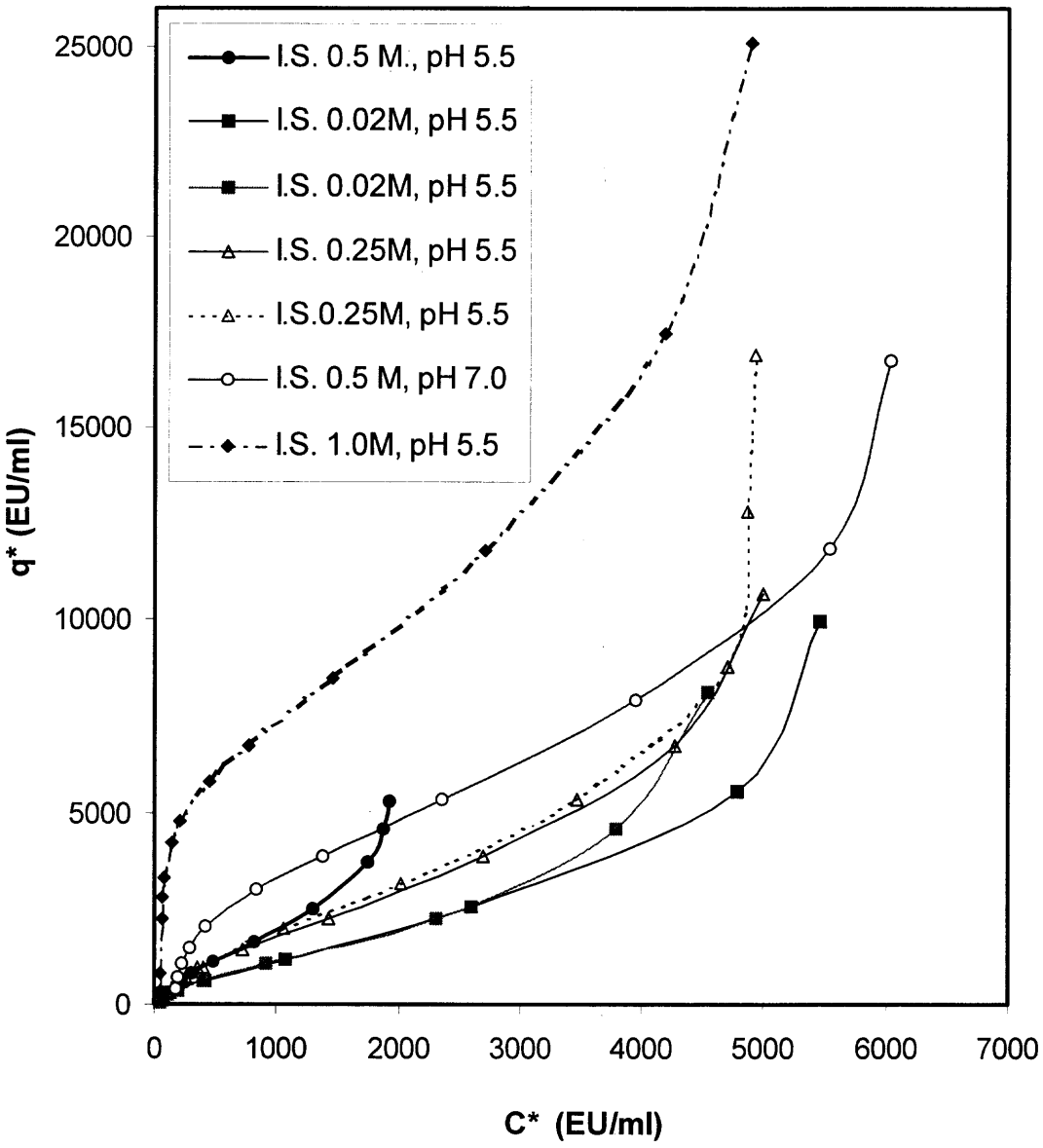


Figure 5.2 Isotherms of endotoxin on IDA-Fe^{3+} IMAC column, acetate buffer at different ionic strength and pH conditions at room temperature, flow rate 0.5 ml/min

Regardless of these limitations, Langmuir isotherm has been widely used for protein adsorption processes and quite often has been found to fit equilibrium data adequately. Other factors, which contribute to the popularity of Langmuir isotherm, are simplicity, the physical significance of the parameters, and the ability to use this model in conjunction with complex rate models. One possibility of the Langmuir isotherm used in the characterization of the endotoxin adsorption process is that the immobilized ligand, Fe^{3+}, is homogenous in the IMAC matrix.

A typical Langmuir isotherm model which best fits the experimental equilibrium data was determined by non-linear regression analysis. The result was shown in Figure 5.3. Solid points represent the experimental data. The dash line is the result predicted by Langmuir model. The q_m is 1.43 x 10^4 EU/ml while K_d is 6.66x 10^{-3} EU/ml, about $6.66x10^{-6}$ M. The Langmuir isotherm did not fit the experimental data very well. Disagreement between experimental data and the modeling prediction was found especially at high endotoxin concentration. In most cases, predictions for maximum endotoxin binding capacity were well below measured values of adsorbed endotoxin concentration. The Scatchard analysis was used to test the adequacy of Langmuir isotherm as mentioned before. The Scatchard analysis corresponding to the experimental data in Figure 5.3 was repeated in Figure 5.4, which indicates that Langmuir isotherm yielded an inadequate fit to the experimental data. All the Scatchard analyses of experimental data at different buffer ionic strength and pH conditions are shown in Figure 5.5. All the curves are not linear and have same trends. At same q*, as the ionic strength increases, q*/c* increases. This means that higher q* can be achieved with higher ionic strength at same C*. The Langmuir isotherm is truly valid only when the adsorption is

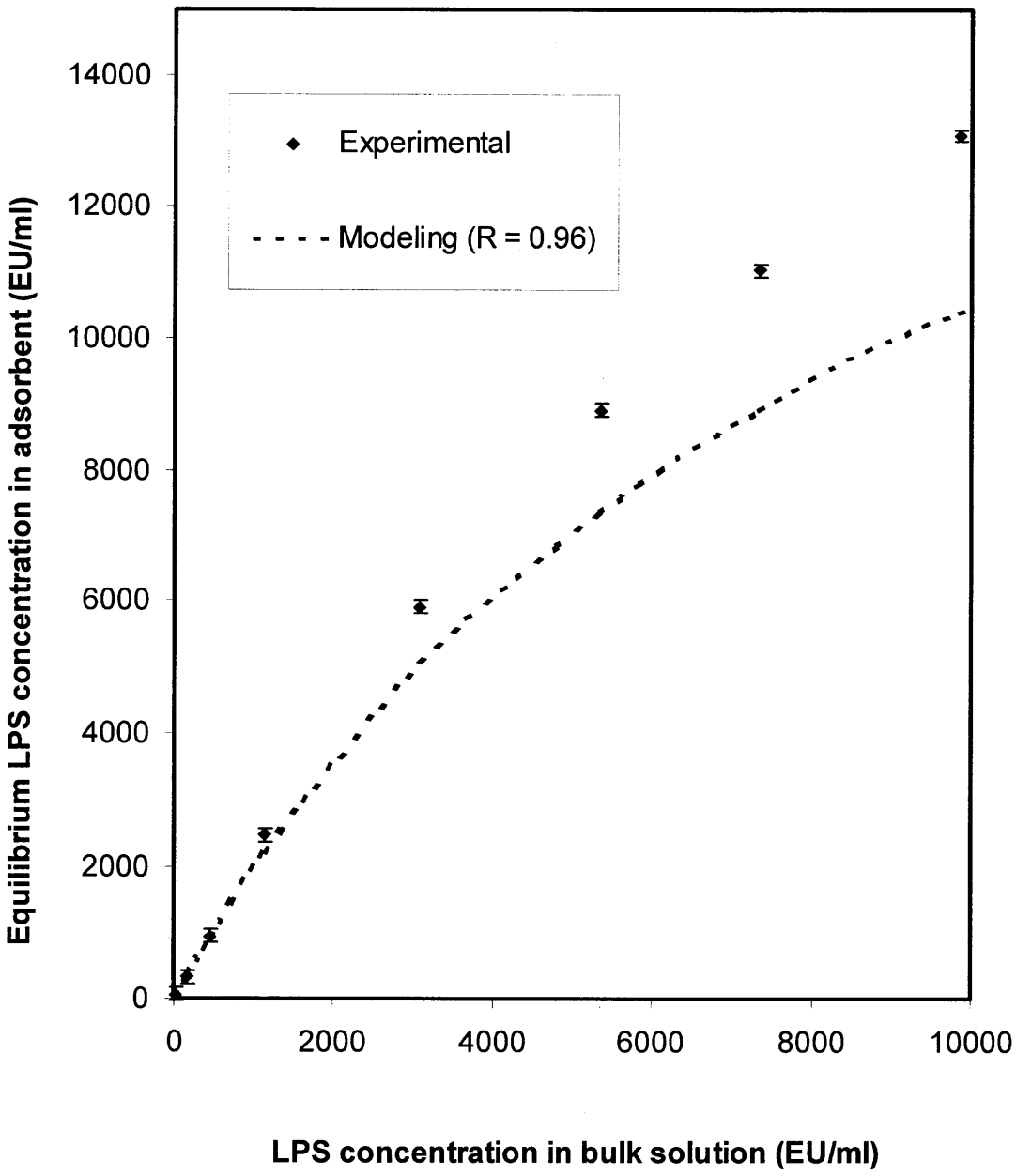


Figure 5.3 Langmuir isotherm yielded an inadequate fit to the experimental data

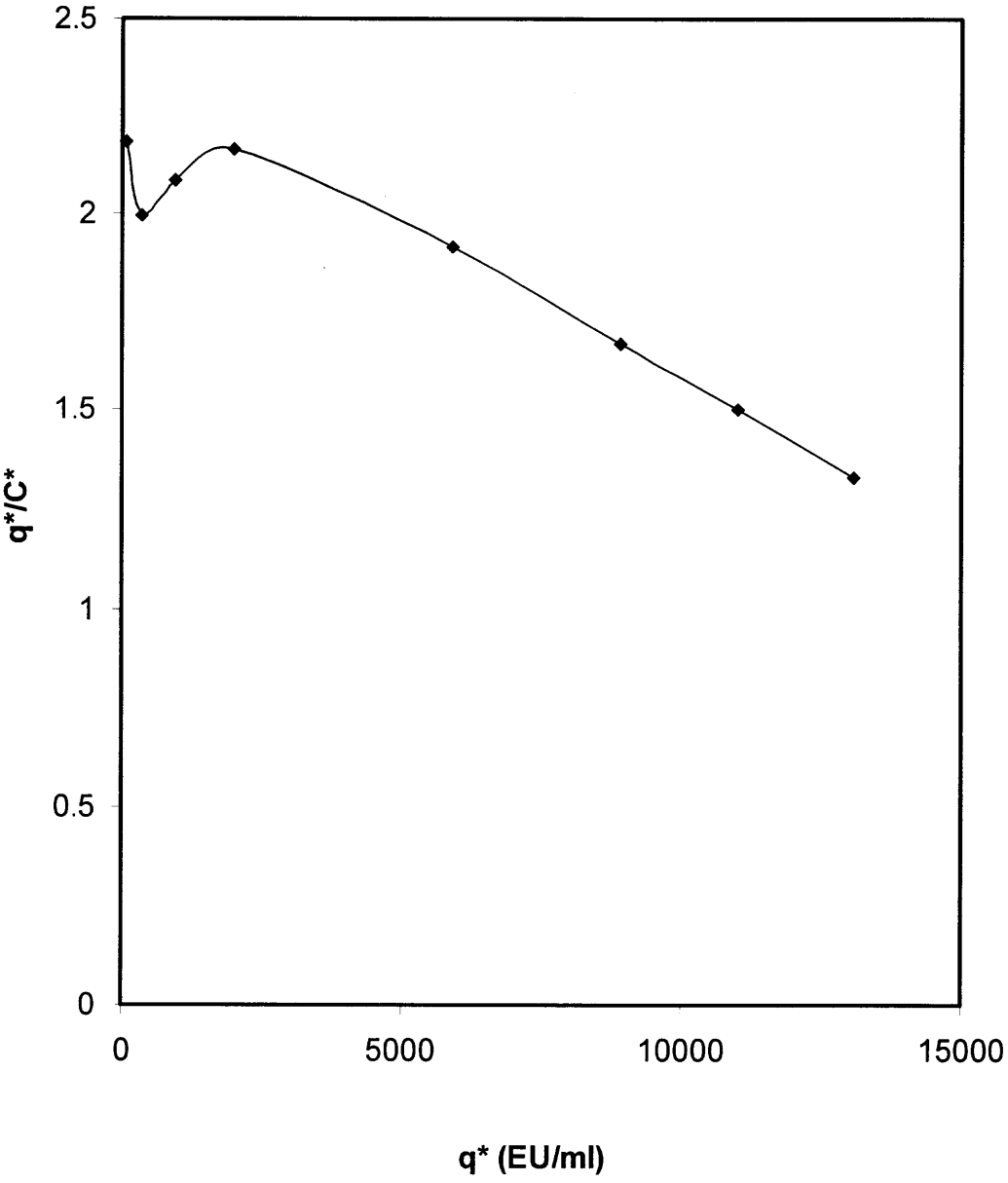


Figure 5.4 Scatchard analysis of LPS adsorption on IDA-Ferric IMAC column

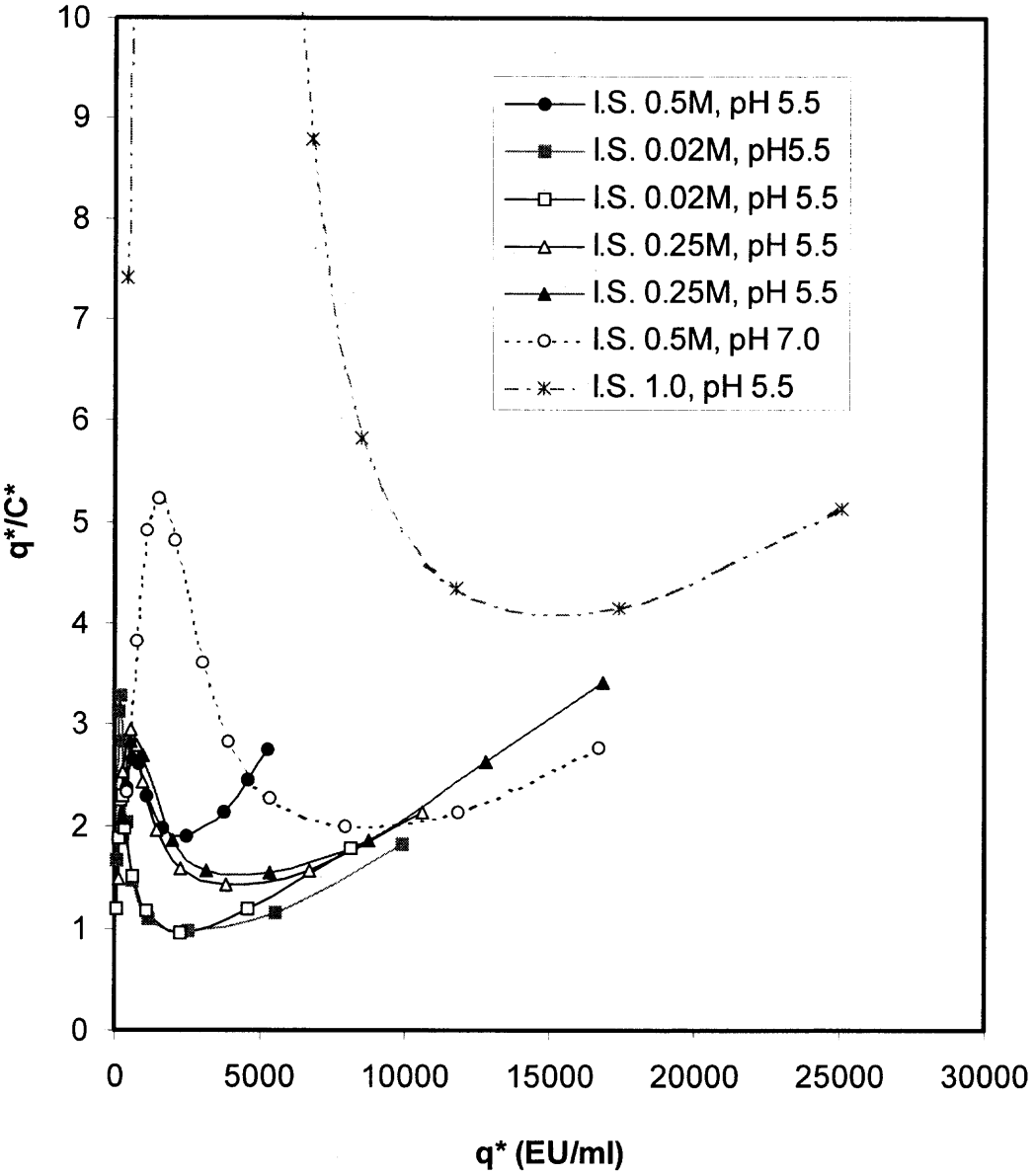


Figure 5.5 Scatchard analysis, adsorption of endotoxin on IDA-Fe^{3+} IMAC column from acetate buffer with different ionic strength and pH conditions at room temperature, flow rate: 0.5 ml/min

simple and well behaved. It is obvious that this is not the case with the adsorption of endotoxins, due to their large and labile size, a wide variety of existing forms and a wide variety of surface sites on endotoxin for interactions with the ligands.

From the analysis of Langmuir isotherm, several conclusions were obtained. First, endotoxin adsorption capacity on the IMAC-Fe^{3+} is more than 14,000 EU/ml gel, which is very high. Second, the non-linear Scatchard plots can be interpreted as an indication of heterogeneous adsorption (Sada et al., 1986). Heterogeneous adsorption of endotoxin by IDA-Fe^{3+} IMAC is not surprising when a variety of LPS existing states and correspondingly various LPS-ligand interactions contributing to adsorption. Third, the interactions among the endotoxin molecules could be possible in the experimental conditions.

5.5.2 Freundlich Isotherm

Freundlich isotherm model is an empirical model and can be derived from the case of heterogeneous adsorption. It has been used extensively in liquid adsorption systems where the assumptions of non-interactive solutes and homogeneous binding energies are often not valid (Maron and Lando, 1974). The Freundlich isotherm parameters which best fit experimental data for LPS adsorption on IMAC-Fe^{3+} were determined by non-linear regression analysis similar to that used in the Langmuir analysis. These parameters are presented in Table 5.1. At pH 3.3, the endotoxin adsorption capacity of IMAC-Fe^{3+} is very high. In the experiments it is impractical to get endotoxin breakthrough the column because the process takes such a long time.

Table 5.1 Variation of Freundlich parameters for LPS adsorption with buffer and ionic strength

pH	I (M)	k'	n'	R^2
5.5	0.02	4.924	1.203369	0.9646
	0.02	4.030	1.183432	0.9725
	0.25	3.457	1.090513	0.9701
	0.25	2.668	1.027855	0.9629
	0.5	2.256	1.078749	0.9817
	1.0	11.233	1.194743	0.964
5.5	0.5	2.256	1.078749	0.9817
7.0		10.381	1.212415	0.9495

In Table 5.1, duplicate experiments were conducted for ionic strength 0.02 M, pH 5.5 and 0.25 M, pH 5.5, respectively. The Freundlich analyses for each duplicate of data gave similar k' and n', which indicated that frontal analysis of endotoxin was reproducible. The dependence of k' and n' on buffer pH and ionic strength provides the basis for endotoxin adsorption and removal. As shown in Table 5.1, the capacity parameter k' and exponential parameter n' both show weak dependence on ionic strength and pH.

The isotherm determined by these analyses was found to give fair agreement with the experimental data, as typically shown in Figure 5.6, where the dash line represents Freundlich model prediction. The Freundlich modeling gave a fair match to the

experimental data at different ionic strengths and pH, especially in the low endotoxin concentration range. The isotherm predictions of endotoxin adsorption behavior at a high endotoxin concentration are very poor. Other isotherm models should be considered. Fortunately, in practical applications, the endotoxin concentration is in the range where Freundlich isotherm is acceptable.

5.5.3 The Interaction between Endotoxin and IDA–Fe^{3+}

The effects of buffer conditions on the endotoxin adsorption, as discussed previously, may indicate the type of the interaction, which is responsible for the endotoxin adsorption. All the model predicted results under different ionic strength conditions are shown in Figure 5.7, compared with one group of experimental data. The circles are the experimental data at ionic strength of 0.02M, pH 5.5. The main trend of the effect of the ionic strength is that as the ionic strength increases q* increases except when ionic strength is changed from 0.25 to 0.5 M at the same C*. The result indicates that electrostatic interactions might be involved in endotoxin adsorption. The effect of pH on endotoxin adsorption to IDA-Fe^{3+} IMAC column was shown in Table 5.1, which is more complex than that of ionic strength on endotoxin adsorption.

The interactions described above are quite surprising as physical properties of endotoxins are considered. Endotoxin has an isoelectric point of 1.3 and consequently carries a net negative charge in the studied pH range (Petsch et al., 1997). Ligand, Fe^{3+}, is positive charged. Ion exchanger exploits different net charges on biomolecules at a given pH, and interacts with the biomolecules principally by electrostatic attraction.

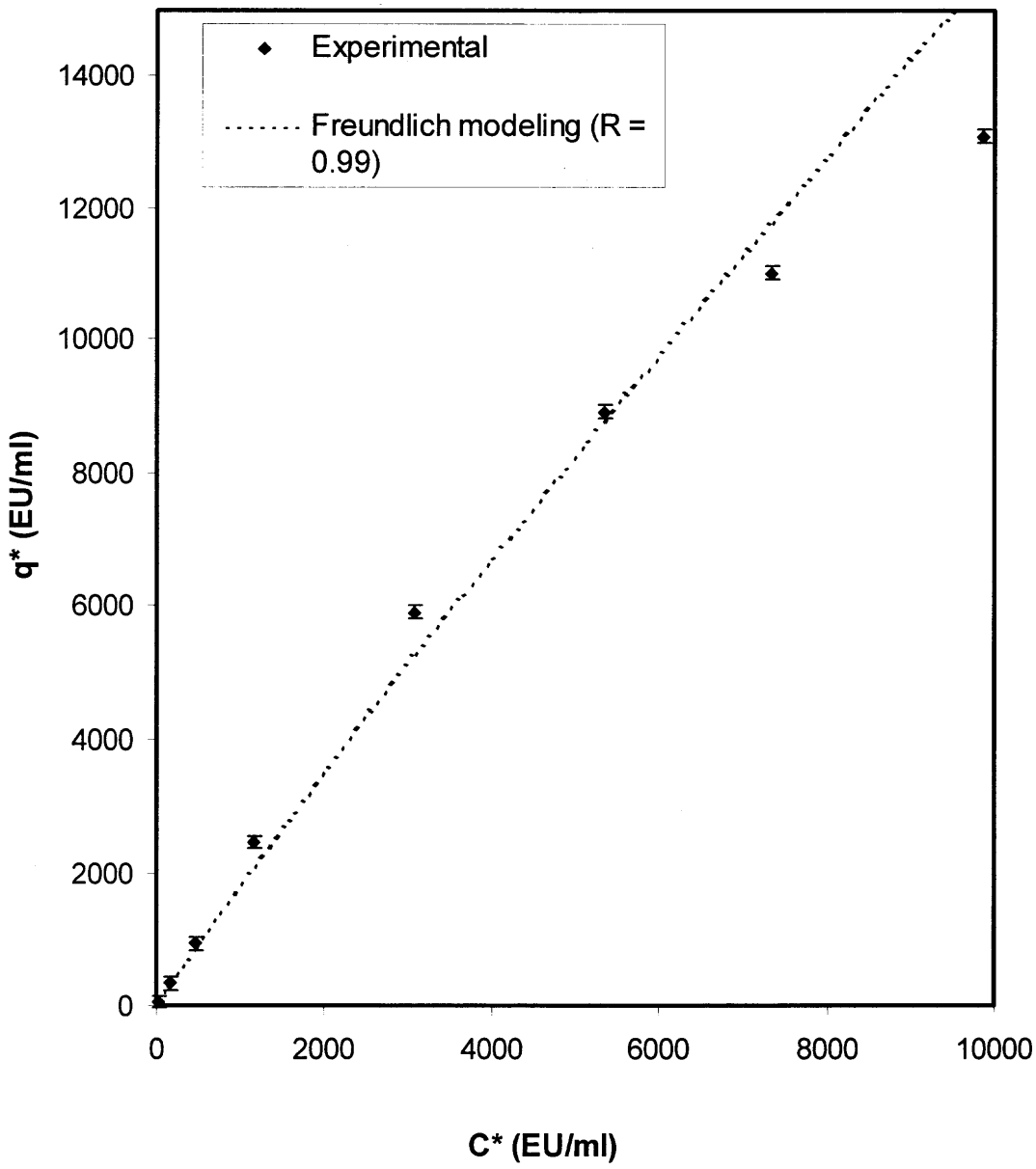


Figure 5.6 The comparison of experimental data and Freundlich modeling, pH 5.5, IS = 0.5 M, buffer flow rate, 0.5 ml/min.

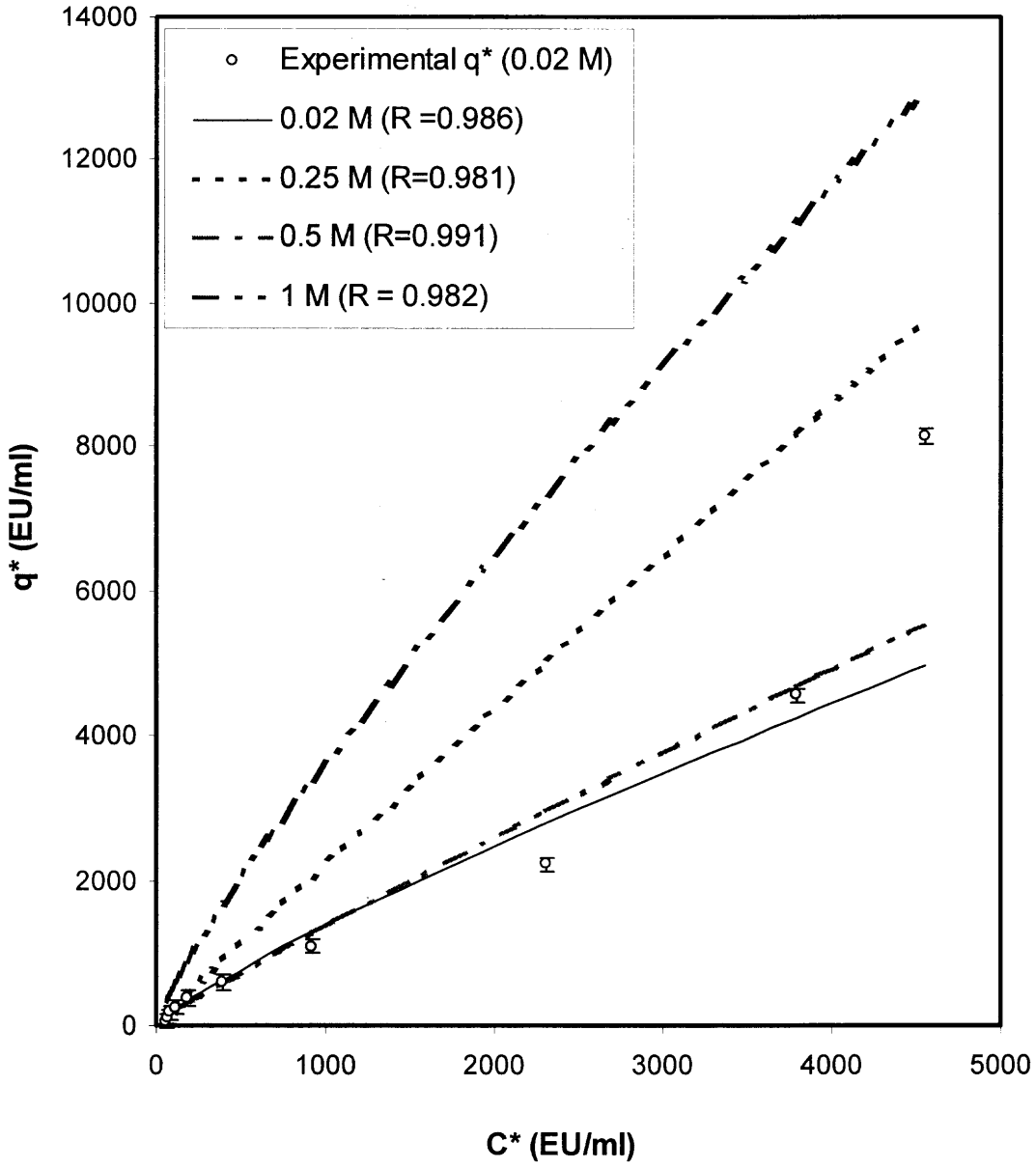


Figure 5.7 The effects of ionic strength on the model of endotoxin adsorption

There are two main classes of ion exchangers: anion exchanger with positively charged adsorbents and cation exchanger with negatively charged adsorbents. Therefore, anion exchange might be one part of the mechanism for endotoxin adsorption to IDA-Fe^{3+}. However, as shown above, as buffer ionic strength is increased from 0 to 0.5 M, the adsorption capacity increases. Therefore the ion exchange mechanism above can not provide satisfactory explanation to endotoxin adsorption.

The endotoxin is usually described as both hydrophobic and hydrophilic molecule. In aqueous solvents, hydrophobic patches on adsorbates seek out other hydrophobic surfaces (on adsorbents) preferentially. Hydrophobic interaction chromatography (HIC) exploits this hydrophobic interaction between the adsorbate and adsorbent. Obviously HIC mechanism can be excluded in the adsorption process of endotoxin to IMAC-Fe^{3+} column, except for nonspecific binding.

In protein purification with IMAC, the underlying principles of the binding of proteins to the metal chelate stationary phase is believed to be the result of the ability of electron-rich ligands on the surfaces of proteins to substitute weakly bonded ligands such as water or mobile phase ions in the metal complexes on the surface. In this process, the chelating metal plays the role of accepting unshared pair of electrons from the side chains of amino acids at the surface of the protein molecule. Phosphate groups have been shown to strengthen protein interactions with immobilized metal (Sulkowski, 1988). The contribution of phosphate groups to the overall binding strength was also reported (Andersson, 1986). For the endotoxin adsorption on IMAC-Fe^{3+}, it is believed in this research that the mechanism is similar to the latter.

The adsorption mechanism can be analyzed as follows. At the beginning, the endotoxin was adsorbed on the IMAC gel as a monolayer. In this step the adsorption follows Langmuir isotherm. The endotoxin concentration is low and the interaction between adsorbed endotoxin molecules is very weak. Also as explained above, the ligand, Fe^{3+} is very homogeneous. Therefore Langmuir isotherm can predict the adsorption behaviors of endotoxin, as long as endotoxin concentration is low. As the adsorption continues, the monolayer condition is not satisfied any more. Then the adsorption breaks the conditions indicated by Langmuir isotherm. Since the interaction among endotoxin molecules are very strong at this time, the endotoxin adsorption continues. Therefore adsorption of endotoxin to the column includes two periods. First, endotoxin is adsorbed to Fe^{3+}, which is due to the interaction between the Fe^{3+} and the phosphate group in the endotoxin. Second, additional endotoxin in solution is adsorbed on the already adsorbed endotoxin because of the strong interactions among the endotoxin molecules. In the first step it is reasonable to assume Langmuir adsorption mechanism. The saturation adsorption capacity is 1.43×10^4 EU/ml gel. In second step the adsorption continues, and more endotoxin will be adsorbed. Therefore endotoxin adsorption capacity of IDA-Ferric is very high.

5.5.4 Discussion –Implications in Adsorption Process Design

The work in this chapter was to determine the isotherm of endotoxin adsorption on the IDA-Fe^{3+} IMAC column. The results provided more reliable and efficient theoretical basis for buffer selection and adsorption process design, optimization, simulation and process scale-up. Isotherm model provides deep insights into the character of the

endotoxin and IDA-Fe^{3+} interactions. If the isotherm is known, the process development and optimization have a theoretical basis and can be completed in a more rational way. In this study, the endotoxin exhibited heterogeneous interactions with immobilized IDA-Fe^{3+} IMAC column. Variation of ionic strength and pH in the study was to identify the adsorption mechanism, types of the forces involved in the adsorption. As described above, adsorption of endotoxin on the IDA-Fe^{3+} IMAC column was mainly due to the phosphate group in the endotoxin and the interaction mechanism was essentially metal chelating. Hydrophobic interactions might also be involved as nonspecific binding.

5.6 Summary

Frontal analysis studies have been shown to be an effective way of characterizing the effects of buffer conditions on the endotoxin adsorption process as well as the Fe^{3+}-endotoxin interactions. By comparison of experimental data with different isotherm models, endotoxin adsorption mechanism was put forward. Then isotherm parameters were calculated. The developed isotherm models are in good agreement with experimental results.

The adsorption isotherm of endotoxin provides a theoretical basis for endotoxin removal. In this chapter, the adsorption of endotoxin, LPS, by IDA-Fe^{3+} IMAC column has been investigated. It was found that endotoxin was bound mainly by interactions between the phosphate group in endotoxin and ligand Fe^{3+} on the beads. The interaction mechanism is believed to be the metal chelating. The adsorption of endotoxin to the IDA-Fe^{3+} IMAC column may be also due to the hydrophobic property of the lipid A, a key part of endotoxin, and maybe net negative charge of the endotoxin. The properties

of the endotoxin are important in discussing the mechanism of its adsorption on IDA-Fe^{3+}. Interactions among endotoxin molecules are very important in discussing the adsorption of the endotoxin to IMAC-column. The interactions result in multi-layer adsorption of endotoxin on IDA-Fe^{3+} IMAC column.

CHAPTER 6

ADSORPTION CAPACITY OF ENDOTOXIN IN IDA-Fe^{3+} IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY COLUMN

6.1 Introduction

The removal of bacterial endotoxins from final bioproducts has always been a challenge, especially in the situations where endotoxins bind product. IMAC was widely used for protein purification (Porath, 1985, 1992; Sulkowski, 1985). However, there is no reported application of IMAC to remove endotoxin from protein solutions except that a novel IMAC method was developed to removal endotoxin from protein by the author. Fe^{3+} was charged to a chelating gel that contains iminodiacetic acid (IDA). It is an efficient, cost effective method to selectively remove endotoxin from protein solutions compared to traditional endotoxin removal methods (Issekutz, 1983; Karprus et al., 1987; Aida and Pabst, 1990; Hou and Zaniewski, 1990; Matsumae et al., 1990; Tani et al., 1992; Webber et al., 1995; Belanich et al., 1996; Petsch et al., 1997; Guo et al., 1997; Legallais et al., 1997; Li and Luo, 1998, 1999).

To the author's knowledge, there is no report concerning the quantitative aspect of interactions between endotoxin and IMAC adsorbent. In this chapter the investigation of quantitative interactions was reported. The frontal analysis was used as a reliable method to obtain the column capacity under different solution conditions such as the ionic strength and pH.

6.2 Materials and Methods

6.2.1 Reagents

Endotoxin from *Escherichia coli* 026:B6 (Sigma, St. Louis, MO, USA) was used in all studies. All solutions were prepared with water from Milli-Q system (BioWhittaker Inc., Walkersville, MD, USA). All other chemicals were purchased from Sigma.

6.2.2 Experimental Apparatus

The experimental apparatus was used as Chapter 5.

6.2.3 IMAC Column and Adsorbent Resin

IMAC column and adsorbent resin was described in Chapter 5.

6.2.4 Buffers

Three kinds of buffer solutions were used in the experiments as Chapter 5.

6.2.5 Column Preparation and Operation

Column preparation and operation follow the same procedure outlined in Chapter 5.

6.2.6 Frontal Analysis

Frontal analysis in this research was performed according to the standard procedure. Each equilibrated column, loaded with Fe^{3+}, was fed continuously with a endotoxin solution at a constant concentration and the absorbance of the effluent at 280 nm was continuously monitored and recorded on a chart paper and in computer data acquisition

system. The sample was fed until the absorbance of the effluent reached a constant plateau level equaling to the absorbance of the feed solution. The elution volume of the each test endotoxin feed was then determined from inflection point of the front of the elution profile, taking into account each column's void volume as below.

Figure 6.1 shows the principle of frontal analysis. The column capacity is calculated according to following equation:

$$\text{Amount of endotoxin} = (V_e - V_0)\, C \qquad (6.1)$$

Where Ve is frontal retention volume of the endotoxin solution, Vo is the void volume of the column, i.e., retention volume of unretained solutes, and C is the concentration of endotoxin in the feed solution. Ve was determined from the breakthrough curve at the median bisector of the frontal curve (inflection point) as shown in Figure 6.1. As will be demonstrated below, this method yielded accurate data. If the chromatogram was described as the endotoxin response vs. elution time. The equation 5.5 to 5.7 should be used.

6.2.7 Endotoxin Assay

To determine endotoxin concentration, a chromogenic Limulus Amebocyte Lysate (LAL) test kit QCL-1000 from BioWhittaker (BioWhittaker Inc., Walkersville, MD) with a modified procedure was used. The procedure was described in Chapter 5.

6.2.8 Determination of the Amount of Immobilized Ligand (Fe^{3+})

The amount of immobilized Fe^{3+} was calculated as the difference between the total amount of Fe^{3+} applied to the column and the amount washed out as the excess during

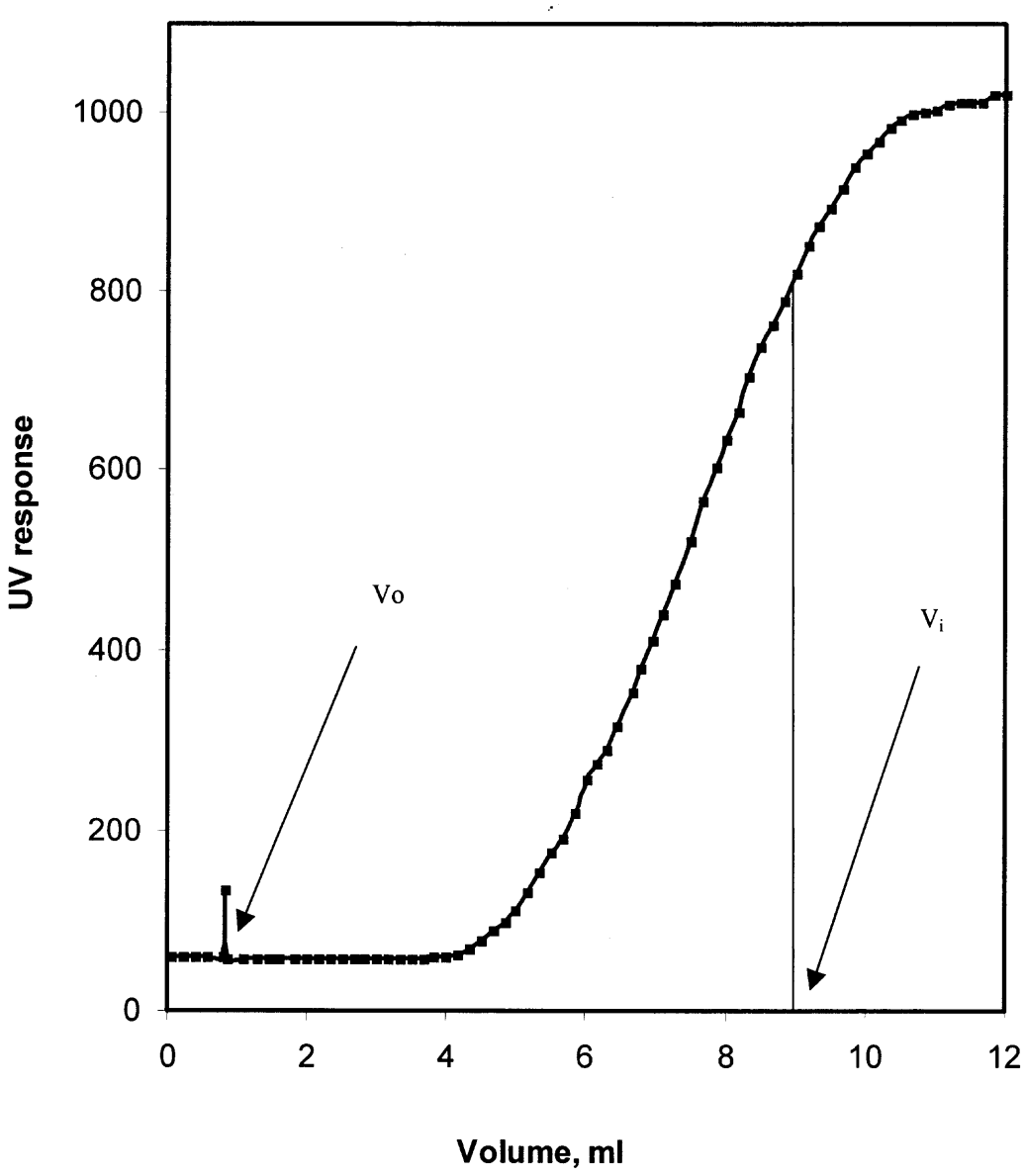


Figure 6.1 The principle of frontal analysis

washing process. The Fe^{3+} concentration is determined by Sigma Ferrozine total iron ion kit. The leaching of the column can be investigated by determination of the ferric ion concentration in all elution fractions.

6.2.9 Data Analysis

The results obtained from chromatographic experiments were used as a basis to calculate the dissociation constants for endotoxin to IMAC-Fe^{3+}. Kasai et al. equation (1986) was used:

$$K_d = \frac{B_t}{V - V_0} - [A_0] \tag{6.2}$$

Where K_d = dissociation constant of endotoxin-ferric, $[A_0]$ = initial concentration of endotoxin applied to the column, B_t = total amount of immobilized ligand, ferric, V = variable volume, V_0 = elution volume of endotoxin under the conditions where its interaction with the immobilized ligand ferric is abolished. K_d can be obtained by plotting 1/(V-Vo) vs. $[A_0]$.

The experimental data were also treated according to a modified form of Nichol equation:

$$\frac{1}{V - V_0} = \frac{1}{(V_0 - V_m)[X]K_{ax}} + \frac{[Ao]}{(V_0 - V_m)[X]} \tag{6.3}$$

Where V = variable volume, V_0 = elution volume of endotoxin under the condition where its interaction with the immobilized ligand X is abolished, Vm = elution volume of neutral, completely excluded molecule, [Ao] = concentration of endotoxin in the applied solution, [X] =concentration of adsorption sites, K_{ax} = associate constant for the adsorption of endotoxin with the immobilized ligand ferric.

6.3 Results and Discussions

6.3.1 Concentration of Immobilized Ligand

Using the procedure outlined above, the total amount of immobilized Fe^{3+} on the IDA IMAC column was determined to be 34 μmol/ml of adsorbent. As provided by the manufacturer, the total Cu^{2+} amount immobilized is about 23 μmol/ml of adsorbent for IDA-Cu^{2+} IMAC column. The results obtained in this research are within the range stated by the manufacturer.

6.3.2 Frontal Analysis

Figure 6.2 shows the frontal analysis profile for endotoxin after the chromatographic process on the IDA-Fe^{3+} column when mobile phase flow rate is 0.3 ml/min with the feed concentration of 6054 EU/ml. This profile is a representative of endotoxin elution for all different feeds under different solution conditions. From the experimental result, the reproducibility is very good if the Fe^{3+} was stripped and recharged every time after use. The shape of the elution front is sigmoidal. This is generally caused as a consequence of imperfection in the column packing as well as a lack of local distribution equilibrium mainly due to large variations in the size of gel beads. The technical and operational advantages of adsorbents packed with small and uniform sized beads are thus obvious. The HiTrap chelating beads are adsorbents with big pores. However, large pores are easily accessed by the endotoxins, which is a big advantage over small pore adsorbents.

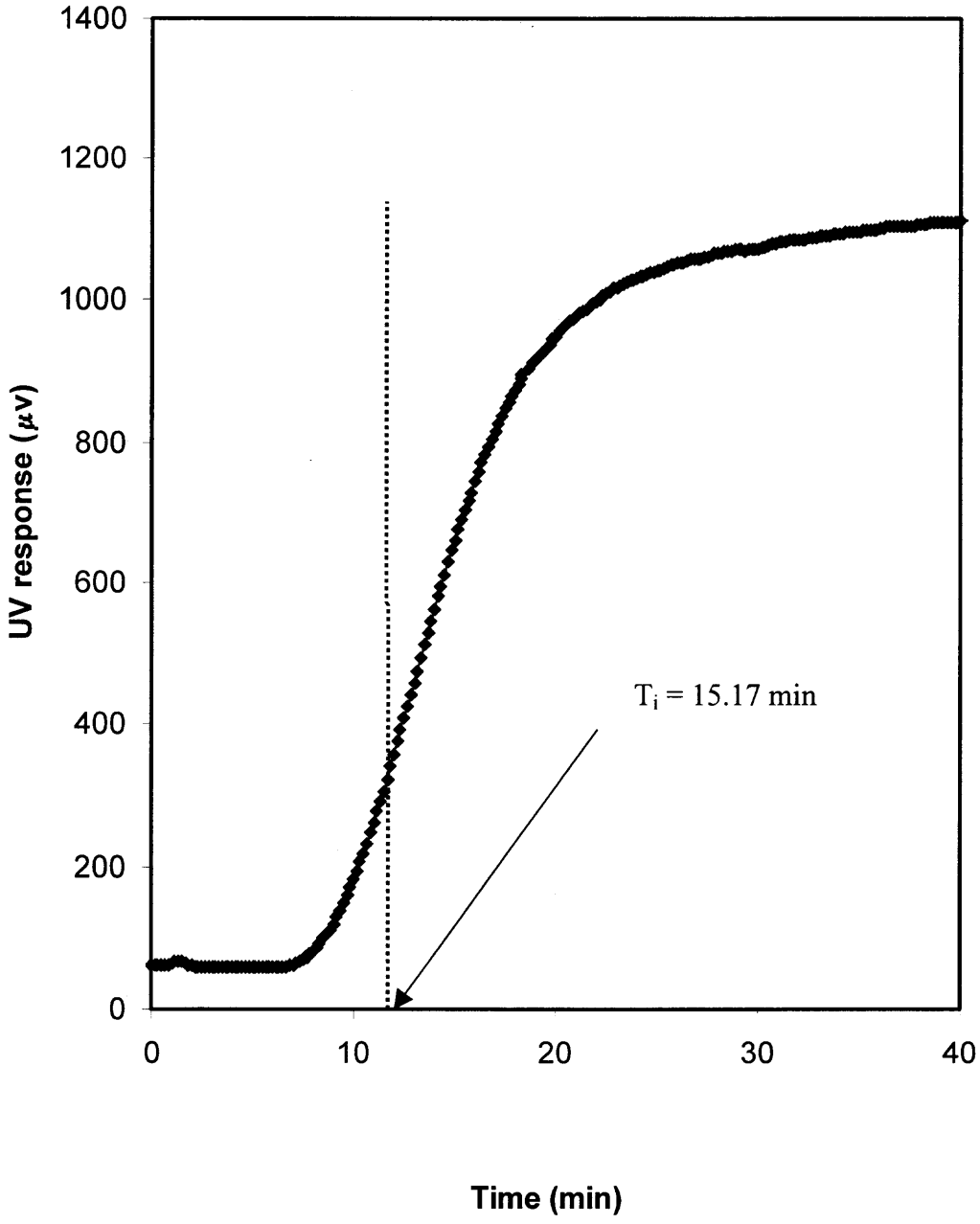


Figure 6.2 Breakthrough curve of ET with IDA-FERRIC IMAC column, mobile phase: 0.5M NaCl, 0.02 M acetate buffer, pH 5.5, flow rate: 0.3 ml/min

6.3.3 The Effects of Mobile Phase Flow Rate on the Column Capacity of Endotoxin Adsorption to IDA- Fe^{3+} IMAC Column

The adsorption capacity of the IDA-Fe^{3+} IMAC column at different feed flow rates are shown in Figure 6.3 and Table 6.1. Four flow rates, 0.2, 0.3, 0.5 and 1.0 ml/min were investigated to discuss the effect of flow rate on the column adsorption capacity to endotoxin. At a flow rate of 0.3 ml /min, the highest capacity was obtained: 6054 EU/ml. Considering the productivity of the IMAC column, 0.5 ml/min flow rate was used in following experiments.

Table 6.1 Column endotoxin adsorption capacity at different feed conditions

Flow rate (ml/min)	**Feed Concentration**		**Column Capacity** (EU/ml)
	(μg/ml)	(EU/ml)	
0.2	100	2797	5801
0.3		2883	6054
0.5		3080	4876
1.0		2877	4074
0.5	1	27	51
	10	172	343
	25	456	950
	50	1146	2482
	200	5344	8909
	300	7339	11009
	400	9853	13082

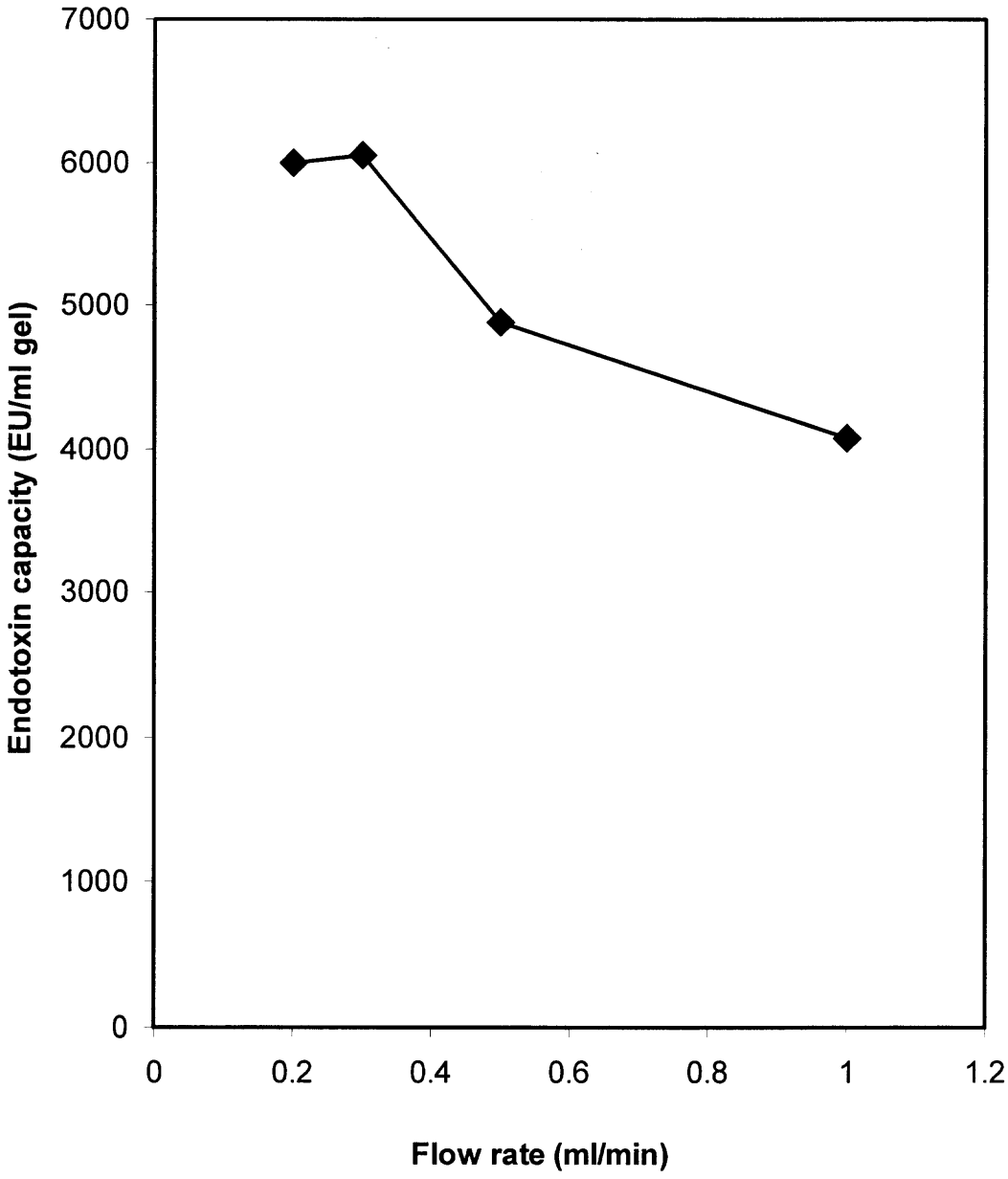


Figure 6.3 Endotoxin adsorption capacity on IDA-Fe^{3+} IMAC column vs. mobile phase flow rate, feed concentration : 100 μg/ml, buffer: 0.5 M NaCl, 0.02 M acetate buffer, pH 5.5

6.3.4 The Effects of Ionic Strength on the Column Capacity of Endotoxin Adsorption to IDA-Fe^{3+} IMAC Column

The effect of ionic strength on the column capacity was shown in Figure 6.4 at the same buffer pH and same level of endotoxin feed concentration. Four different ionic strength conditions were tried. Some of them were duplicated, and experimental reproducibility was very good. At lowest ionic strength, 0.02 M, the column capacity was lowest. As the ionic strength increased the column capacity increased as well. At an ionic strength of 1.0 M, the column capacity is 1.1×10^4 EU/ml, which is the 10 times of that at ionic strength of 0.02 M.

6.3.5 The Effects of pH on the Column Capacity of Endotoxin Adsorption to IDA-Fe^{3+} Column

The effect of pH on the column adsorption capacity of endotoxin was shown in Figure 6.5 with the same ionic strength and same level of endotoxin concentration in feed solution. At pH 3.3, the column capacity was highest. As solution pH increased the column capacity decreased sharply. At pH 7.0, the column capacity is 6656.7 EU/ml, which is the 1/5 of that at pH 3.3. This result was very useful in applying IMAC in protein-LPS separation. The elution of the protein should be implemented in low pH (< 4.0) to prevent the LPS from being eluted from the column. However, during the adsorption period, a higher pH can be applied to keep the adsorption amount of LPS as small as possible. Therefore, the selective removal of LPS from protein solutions can be conducted by choosing appropriate ionic strength and pH conditions.

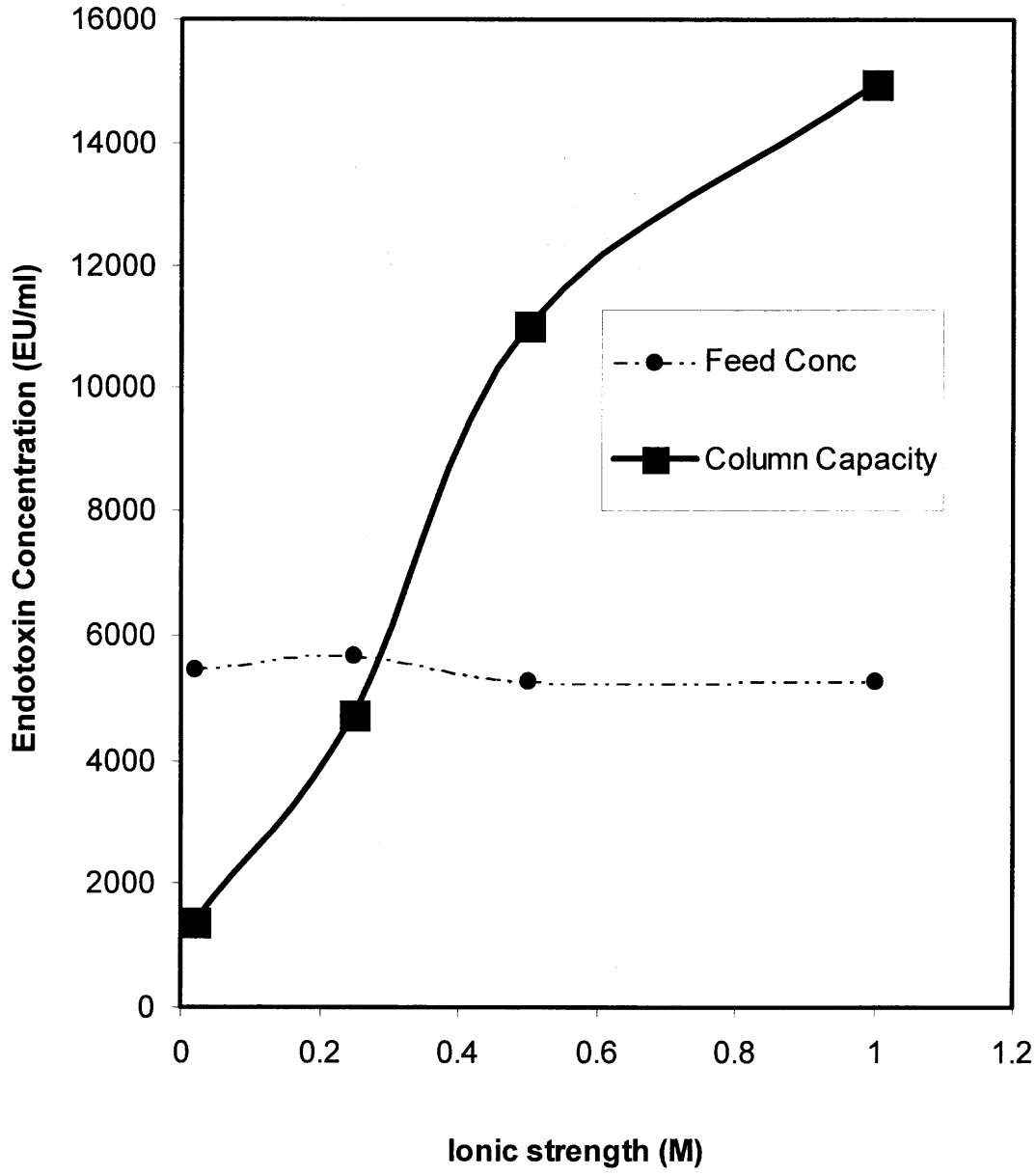


Figure 6.4 Endotoxin adsorption capacity on IDA -Fe^{3+} IMAC column vs. mobile phase ionic strength, pH 5.4

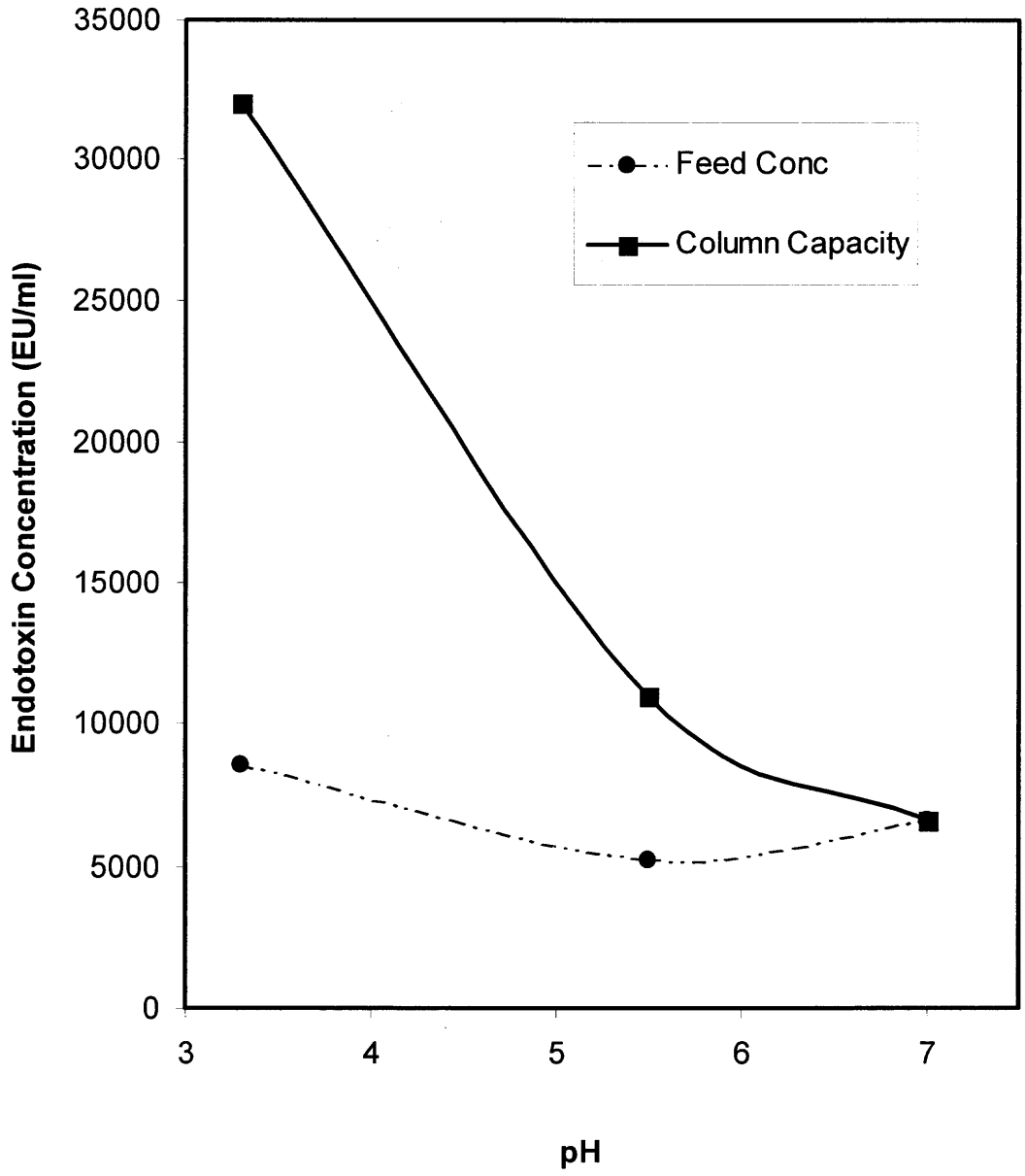


Figure 6.5 Endotoxin adsorption capacity on IDA-Fe^{3+} IMAC column vs. mobile phase pH, ionic strength 0.5 M

6.3.6 Quantitative Affinity Data

The plot of the experimental data according to Kasai equation is shown in Figure 6.6. From the plot, the total concentration of ligand that has interacted with endotoxin, B_t, is obtained directly from the intercept on the ordinate. K_d can be calculated from the slope and B_t. If the molecular weight of endotoxin is assumed to be 1,000,000, $K_d = 4.47 \times 10^{-7}$ M and $B_t = 9.7 \times 10^{-4}$ μmol. If the molecular weight of endotoxin is 100,000, $K_d = 4.47 \times 10^{-8}$ M. Further if the molecular weight of endotoxin is 10,000, $K_d = 4.47 \times 10^{-9}$ M.

If the equation of Nichol et al. is used, the results are shown in Figure 6.7. In the figure, $1/(V-V_0)$ is plotted against endotoxin concentration in the feed solution. K_{ax}, which is $1/K_d$, is calculated by slope/intercept. $(V_0-V_m)[X]$, which is similar to B_t in Kasai equation is reciprocal of the slope. Finally parameters were obtained: $K_d = 5.6 \times 10^{-7}$ M and $(V_0-V_m)[X] = 1.286 \times 10^{-3}$ μmol as MW of endotoxin is 1,000,000. Therefore the calculations from Kasai and Nichol equations gave similar results.

K_d shows stability of the complex of endotoxin (adsorbate) and Fe^{3+} (ligand). K_d is very low in the case, which means the complex is very stable. B_t is obviously too low compared to the total Fe^{3+} amount which is immobilized in the column as the adsorption ligands. This means that most of the active sites were not used.

As well known, the interaction of proteins with IMAC adsorbents is governed by the number or density of exposed histidine, cysteine or tryptophan residues. Unlike proteins, endotoxin has no amino residues. However, K_d of endotoxin to IDA-Fe^{3+} is higher than that to ordinary proteins. As discussed in previous chapters, the endotoxin adsorption to the IMAC column is due to interactions of phosphate group in lipid A potion of endotoxin with the ligand, Fe^{3+}. This can explain its adsorption behavior.

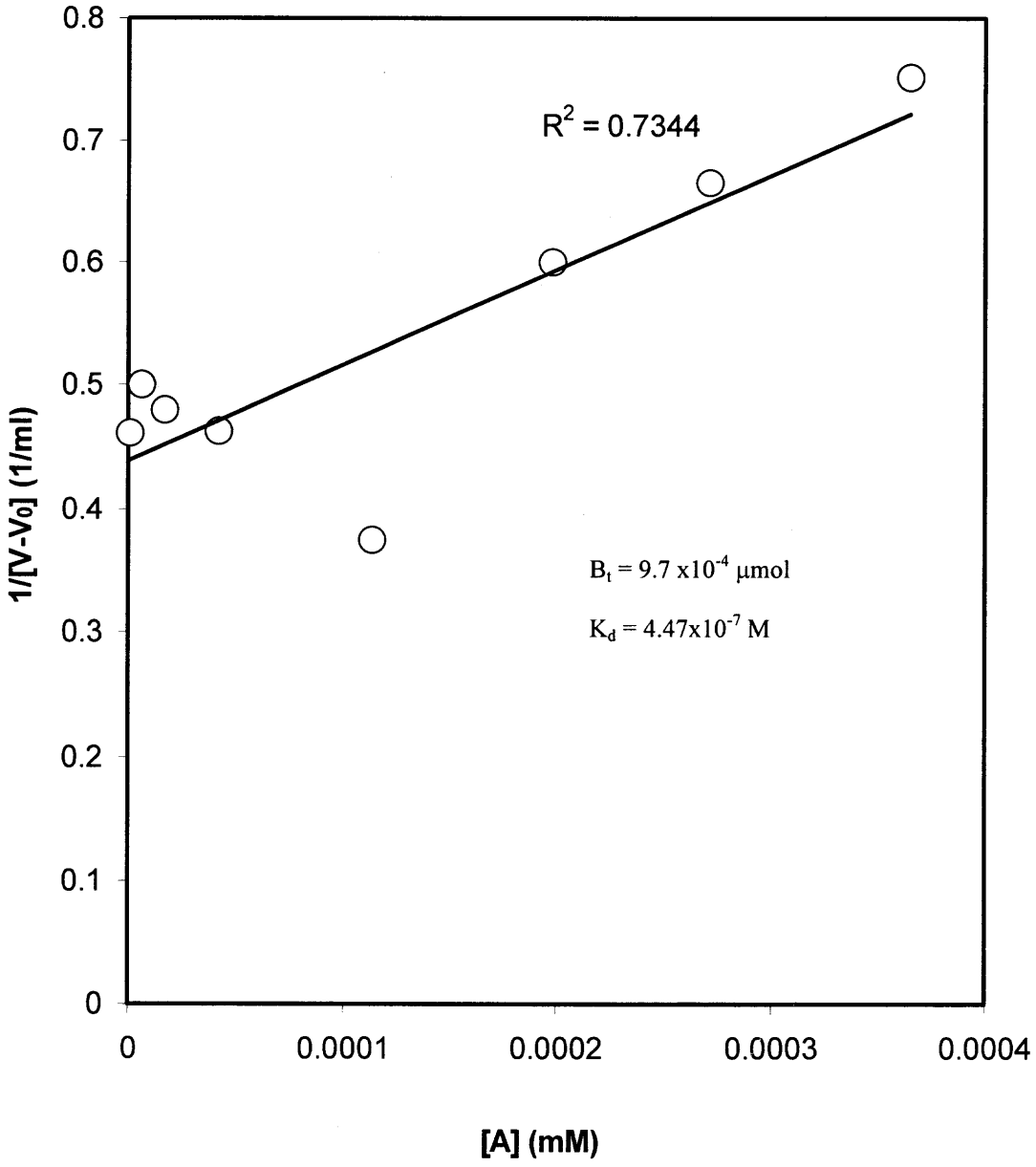


Figure 6.6 Determination of quantitative affinity parameters for the interaction between the endotoxin and IDA-Fe^{3+}, MW of ET: 1000K (Kassai eq.)

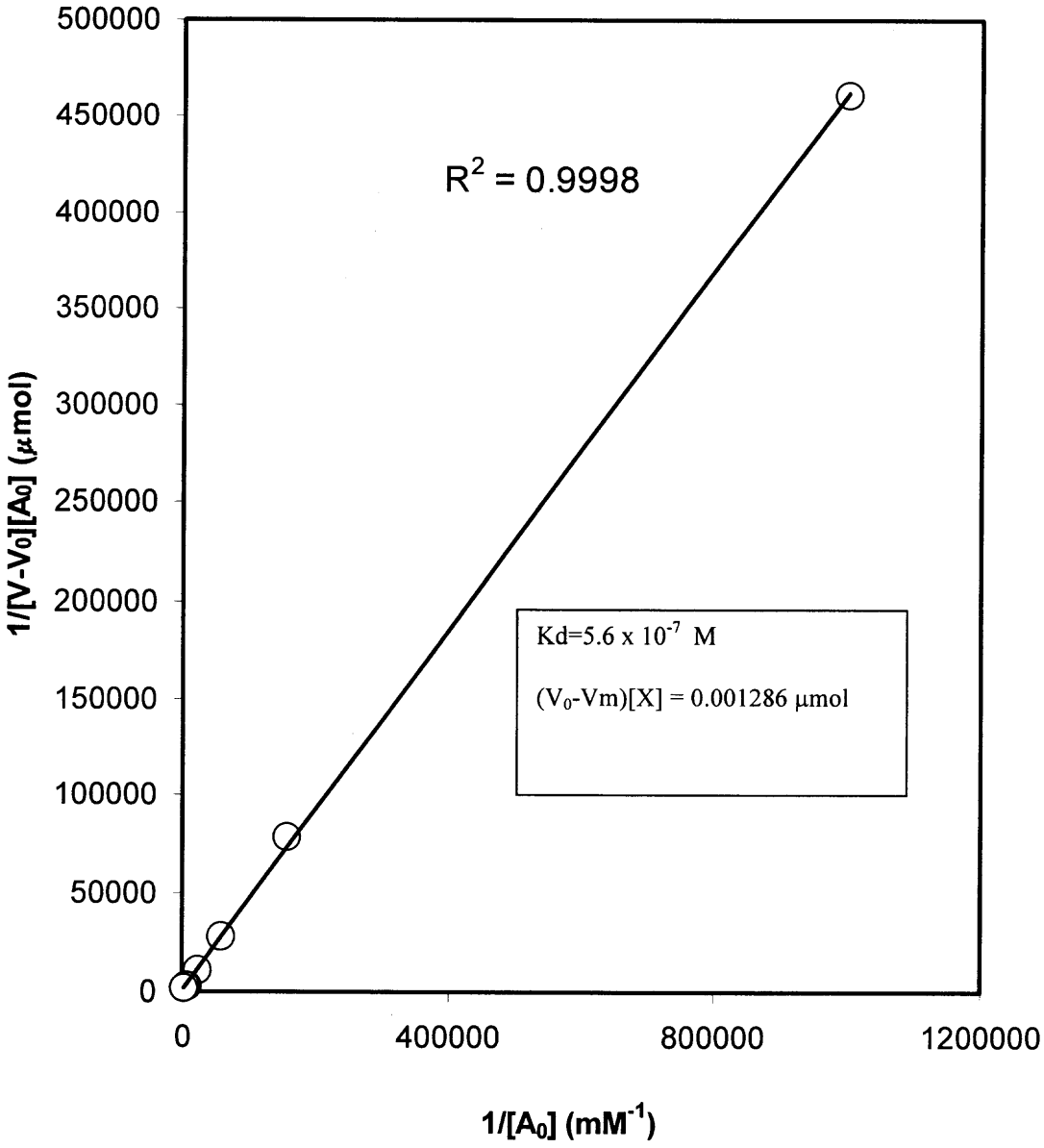


Figure 6.7 Determination of quantitative affinity parameters for the interaction between the endotoxin and IDA-Fe^{3+}, MW of ET: 1000K (Nichol eq.)

At low ionic strength, the endotoxin exists as vesicle or micelle. Its molecular weight is very high, i.e., around 1,000,000. Many lipid A groups were encased in the vesicles or micelles and have no chance to interact with Fe^{3+}. The adsorption capacity to IMAC column is very low. However, as ionic strength is increased, e.g. 1M, the situation is quite different. The endotoxin exists in small subunits with molecular weight around 10,000 to 20,000, which bring many lipid A groups to active sites on adsorbents. Thus, the adsorption capacity of the column for endotoxin at higher ionic strength condition is much larger that at low ionic strength. It might be argued that the low capacity of IMAC IDA-Fe^{3+} adsorbents for endotoxin at low ionic strength is a consequence of steric effects. This may be caused by an efficient shielding of the adsorption sites by the bulk of endotoxin molecules since endotoxin molecules are too big to get into the pores of the adsorbents. It seems that the author's results also show some support for this argument. As reported in the literature (Sweadner, 1977), the size of endotoxin aggregates is around 10 to 70 nm while the endotoxin subunit (existing in high ionic strength solution) is much smaller. Pores of the adsorbents should be large enough that free diffusion is not hindered significantly. This normally can be met with pores that are 10 to 20 times larger than the size of the adsorbate molecule (Satterfield et al., 1973). For the case of endotoxin, pore should be larger than 1.4 μm. From manufacturer, the pore size of IDA adsorbent is around 100 nm. Therefore, the steric effect is one of the reasons for low endotoxin adsorption capacity at low ionic strength.

Figure 6.8 shows the column capacity and capacity/Fe^{3+} used at different feed concentration. At low feed concentration, ET/Fe^{3+} = 0.02. This suggests the presence of many extra binding sites in IMAC- Fe^{3+} gel. At high feed concentration, five endotoxin

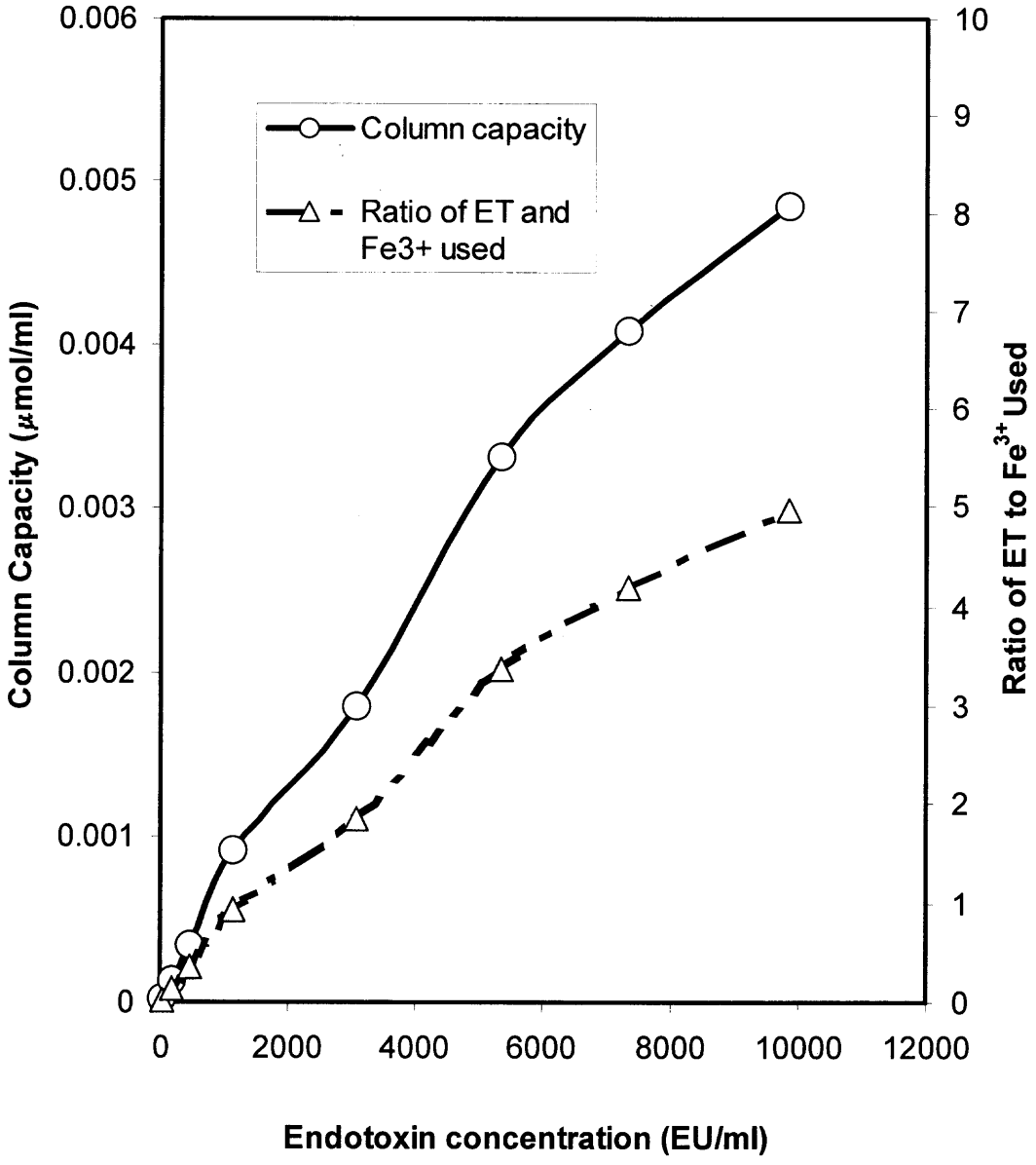


Figure 6.8 Column capacity at different initial endotoxin concentrations and ratio of ET to Fe^{3+} in the complex

molecules interact with one molecule of ferric ligand. This means that ferric ion can interact with multi-molecules. There are two possibilities. First, one endotoxin binding site interactions with an endotoxin complex, which is composed of several endotoxin molecules. Another possibility is one site can bind several endotoxin molecules at the same time. The first mechanism is more convincing.

6.4 Summary

The work in this chapter was to determine the capacity of endotoxin adsorption on the IDA-Fe^{3+} IMAC column under different solution conditions. The results provided more reliable and efficient theoretical basis for buffer selection and adsorption process design, optimization, simulation and process scale-up. It also provided deep insight into the character of the endotoxin and IMAC-Fe^{3+} interactions. In this study, the endotoxin exhibited heterogeneous interactions with immobilized IMAC-Fe^{3+}. Variation of ionic strength and pH in the study was to identify the adsorption mechanism, types of the forces involved in the adsorption. As described above, adsorption of endotoxin on the IMAC-Fe^{3+} is mainly due to the phosphate group in the endotoxin and the interaction mechanism is essentially metal chelation. Hydrophobic interactions might also be involved as nonspecific binding. The very small dissociation constant indicates that affinity between ET and Fe^{3+} is very strong. Steric effect should be considered in process development of endotoxin removal, as ET aggregate is a big molecule. The interactions among the ET molecules are very important in ET adsorption process.

As adsorption capacity under different conditions is known, the process development and optimization have a theoretical basis and can be completed in a more

rational way. In the loading period of endotoxin removal process, in order to get low endotoxin adsorption and higher protein adsorption, a higher pH buffer condition should be chosen. During elution period lower pH will be used to prevent the endotoxin from eluting together with protein targets.

CHAPTER 7

ADSORPTION ISOTHERM OF BSA IN IDA-Fe^{3+} IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY COLUMN

7.1 Introduction

To more efficiently apply of IMAC-Ferric in removal of endotoxin from protein solutions, protein adsorption isotherm was investigated at different solution conditions in this chapter. Different isotherm models were tried and adsorption isotherm was obtained. Good matches between the experimental data and model predictions were reached.

7.2 Models

Different models were summarized in Chapter 5.

7.3 Determination of the Isotherm

Different methods to determine the endotoxin adsorption isotherm were described in Chapter 5.

7.4 Materials and Methods

7.4.1 Materials

Bovine Serum Albumin (BSA) (Sigma Chemical Co., St. Louis, MO) with very low endotoxin content was used in all experiments. $FeCl_3$, NaCl, NH_4Cl and EDTA were also purchased from Sigma. All solution transfers were performed with endotoxin free devices. Sterile, disposable plasticware was used at all times to prevent endotoxin

contamination. All solutions were prepared with Milli-Q water generated by a Biocel water purification system (Millipore Corporation, Bedford, MA). The endotoxin concentration in the Milli-Q water was below 0.05 EU/ml based on the author's test results.

7.4.2 Experimental Apparatus

The same experimental apparatus as described were used.

7.4.3 IMAC Column and Adsorbent Resin

IMAC column and adsorbent resin was described in Chapter 5.

7.4.4 Buffers

Three kinds of buffer solutions were used in the experiments as chapter 5.

7.4.5. Column Preparation and Operation

Column preparation and operation follow same procedure outlined in Chapter 5 except one difference. BSA was used as the feed in stead of LPS in Chapter 5.

7.4.6 Protein Assay

To determine protein concentration, an UV monitor was used. A series of solutions of known BSA concentrations were prepared. BSA standard curve was plotted by drawing absorbance (ABS) vs. BSA concentration (μg/ml) at 280 nm. ABS of BSA in the whole process was also obtained from a data acquisition system M1101 (Keithley Metrabyte,

Cleveland, OH) incorporated with a computer, which was used to record the chromatograms in digital form so that they could be transferred later for data processing and analysis. By comparing with standard curve, protein concentration was easily obtained.

7.5 Results

Adsorption isotherms of BSA under different solution conditions were obtained experimentally with frontal analysis and depicted in Figure 7.1. For all the solution conditions except pH 3.4 and pH 7.0, the adsorption isotherms are very similar. This indicates that BSA adsorption under different solution conditions on IDA-Ferric IMAC column follows the same isotherm model. In order to allow quantitative analysis of the effect of buffer conditions on the BSA adsorption, different equilibrium isotherm models were investigated as follows.

7.5.1 Langmuir Isotherm

The most widely used isotherm model, Langmuir isotherm, was used for adsorption of BSA on the IMAC-Fe^{3+} column. It is obvious that the Langmuir isotherm is truly valid only when the adsorption is simple and well behaved. One concern is that Langmuir isotherm may be too simple for the adsorption of the protein, due to interactions among the protein molecules. Regardless of these limitations, the Langmuir isotherm has been widely used for the protein adsorption processes and quite often has been found to fit the equilibrium data adequately. As mentioned before one reason that the Langmuir isotherm

might be used in the characterization of the protein adsorption process is that the immobilized ligand, Fe^{3+}, is homogeneously distributed in the matrix.

The adsorption isotherm under different solution conditions was determined by experiments as shown in Figure 7.1. The Scatchard analysis was used to test the adequacy of the Langmuir isotherm as mentioned before. The typical Scatchard analysis corresponding to the experimental data at pH 5.5, 0.5 M solution condition was conducted as seen in Figure 7.2, which indicated that Langmuir isotherm yielded an inadequate fit to experimental data. All Scatchard analyses of experimental data at different buffer ionic strength (0 to 1.0 M) and pH (3.4 to 7.0) are not linear and have the same trends (not shown here) as Figure 7.2.

The non-linear Scatchard plots can be interpreted as an indication of heterogeneous adsorption (Sada et al., 1986). As heterogeneous adsorption by IMAC-Ferric ligand is identified, Freundlich isotherm might be a model to describe the adsorption of protein on IMAC column.

7.5.2 Freundlich Isotherm Model

Freundlich isotherm model is an empirical model and can be derived from the case of heterogeneous adsorption. The Freundlich isotherm parameters which best fit the experimental data for protein adsorption in IDA-Fe^{3+} IMAC column were determined by non-linear regression analysis similar to that used in Langmuir analysis. These parameters were presented in Table 7.1. The isotherms determined by these analyses were found to give fair agreement with the experimental data, as typically seen in Figure

7.3. In Figure 7.3 the isotherm model with Freundlich at different ionic strength was shown.

Table 7.1 Variation of Freundlich parameters for BSA adsorption with buffer pH and ionic strength conditions

pH	I.S. (M)	k'	n'	R^2
3.4	0.5	2.7475	0.796876	0.9875
4.4		3.5353	0.788706	0.9826
5.5		3.6943	0.799808	0.9749
7.0		3.0858	0.755173	0.9854
5.5	0.02	5.9044	0.869338	0.9813
	0.25	7.5511	0.913159	0.9873
	0.5	3.6943	0.799808	0.9749
	1.0	7.7106	0.902039	0.9886

As shown in Table 7.1 and Figure 7.3, the capacity parameter k' and exponential parameter n' both showed very weak dependence on ionic strength. As the ionic strength increased from 0.25 M to 1.0 M, fluctuation of the k' and n' was observed. However, all the models derived from the experimental data at different ionic strength conditions gave very similar predictions. If the same BSA concentration at different ionic strength is used in the feed, almost the same BSA concentration on the IMAC beads could be obtained.

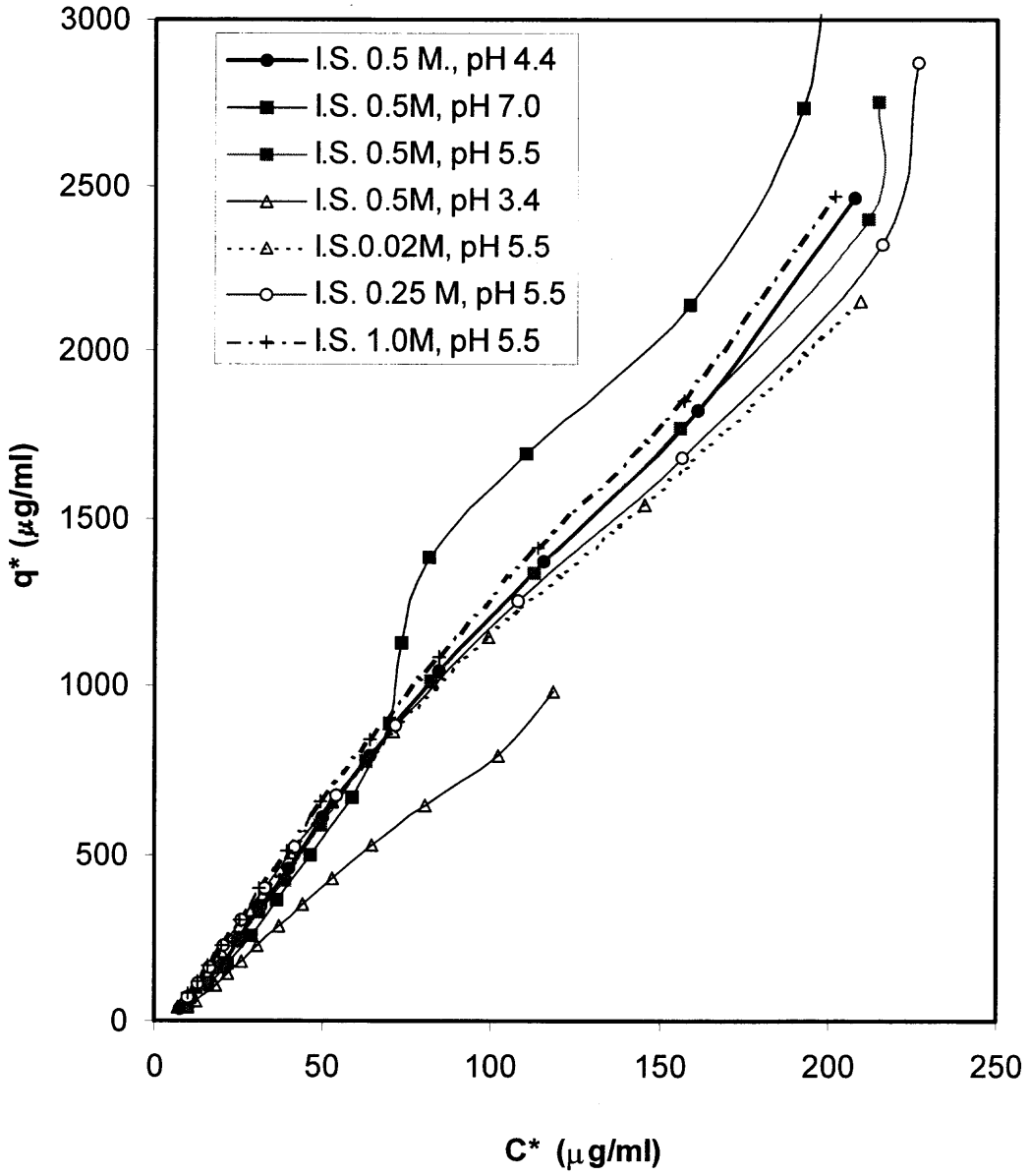


Figure 7.1 Isotherms of BSA on IDA-Fe^{3+} IMAC column, acetate buffer under different ionic strength and pH conditions at room temperature, flow rate 0.5 ml/min

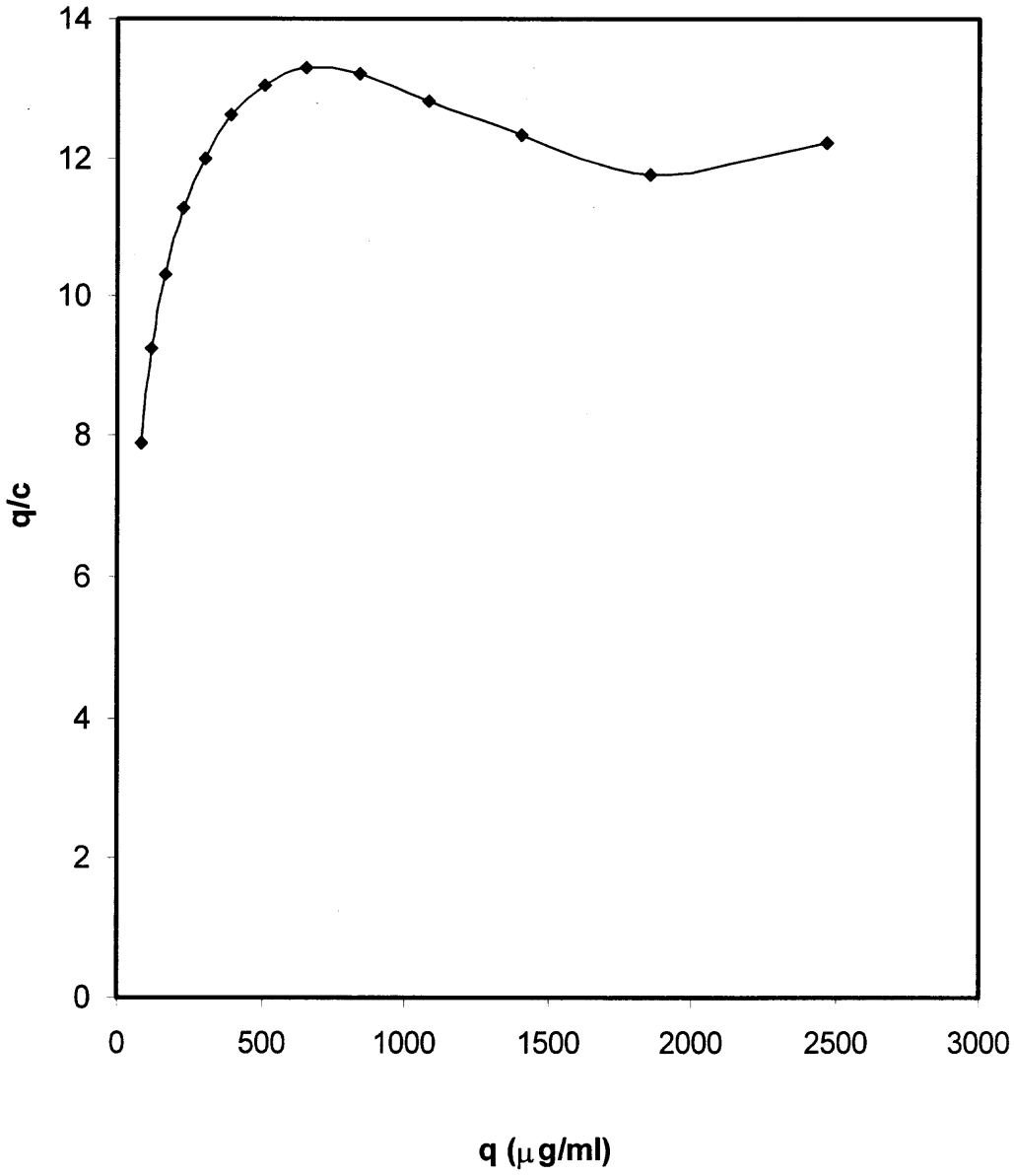


Figure 7.2 Scatchard analysis for experimental data at buffer condition of pH 5.5, I.S. 0.5 M

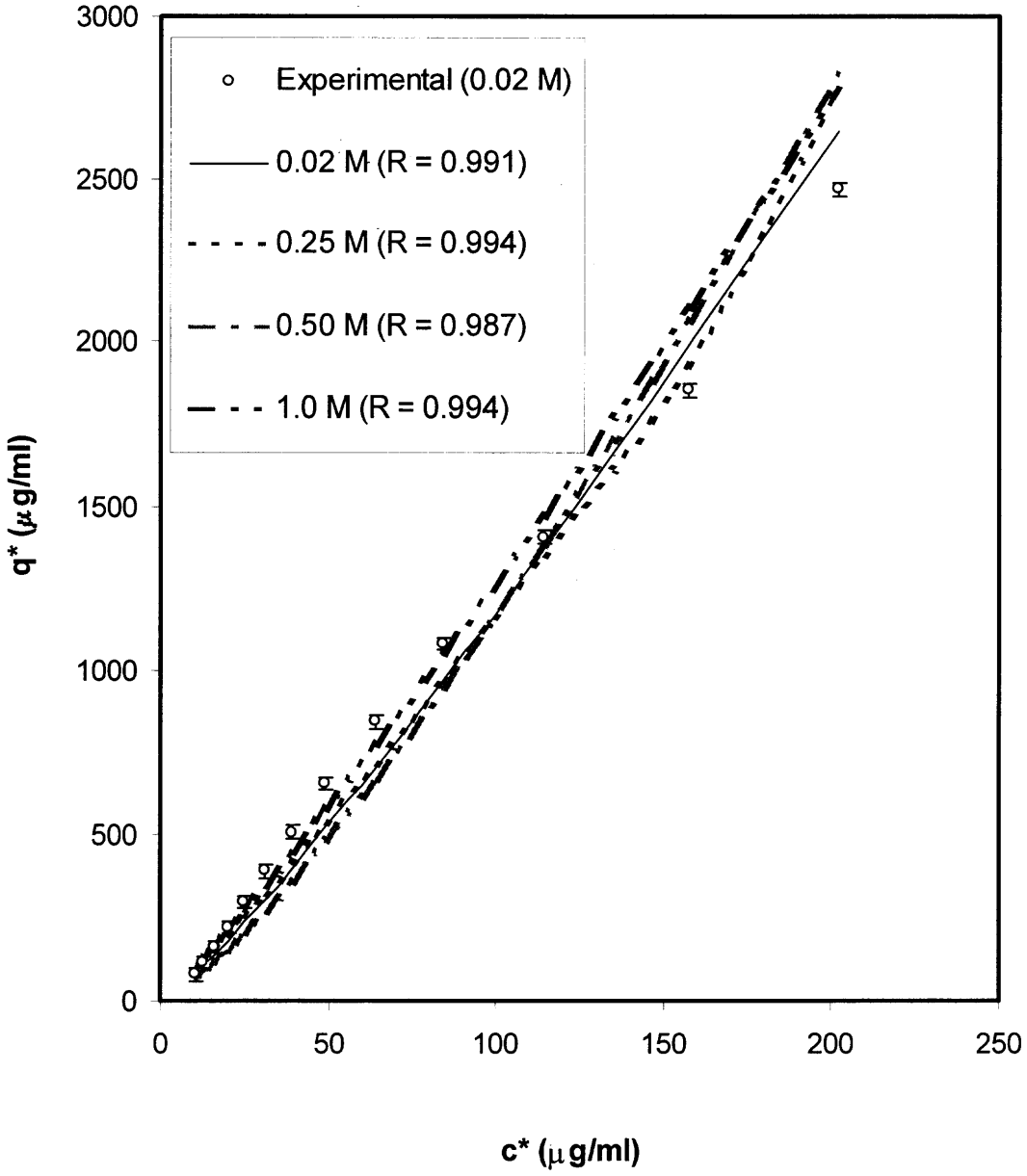


Figure 7.3 The effect of buffer ionic strength on the isotherm model of BSA adsorption to IDA-Fe^{3+} IMAC column

Therefore the prediction of BSA adsorption on IMAC by Freundlich model is not ionic strength sensitive.

More dependence of k' and n' on pH was observed as compared with that of ionic strength. The dependence of k' and n' on buffer pH and ionic strength provided the basis for BSA adsorption process.

7.5.3 The Interaction between BSA and IMAC–Fe^{3+}

The effect of buffer conditions on the BSA adsorption, as discussed previously, may indicate the type of interactions, which are responsible for the BSA adsorption. The behavior of BSA adsorption presents an interesting picture. The effect of ionic strength on the BSA adsorption is shown in Figure 7.3. As the solution ionic strength increases from 0.02 M to 1.0 M, the adsorption isotherm of BSA changes very little. Thus, the results indicate that electrostatic interactions might be involved in BSA adsorption on the IMAC-Fe^{3+}. However, it does not play a major role. The effect of pH on BSA adsorption is shown in Table 7.1, which is more complex than that of ionic strength on BSA adsorption. In whole pH range, the IMAC-Fe^{3+} has a very high BSA concentration on the beads. This indicates that there are strong interactions between the IMAC-Fe^{3+} and BSA.

The interactions described above are quite surprising when the physical properties of the BSA are considered. BSA has an isoelectric point of 4.6 and consequently carries a net negative charge at pH > 4.6 and a positive charge at pH < 4.6. Ligand, Fe^{3+}, is positive charged. Ion exchanger exploits the different net charges on the proteins at a given pH, and interaction with the proteins principally by electrostatic attraction. At pH <

4.6, both Fe^{3+} and BSA have net positive charge. The corresponding adsorption capacity of BSA on IMAC-Fe^{3+} should be low. On the other hand, as buffer pH > 4.6, BSA is negatively charged. Due the electrostatic attraction of Fe^{3+} and BSA, the BSA adsorption capacity on the column could be higher than that at pH < 4.6. Furthermore as the buffer pH increases, the BSA adsorption capacity should increase. Therefore ion exchange might explain BSA adsorption to IDA-Fe^{3+} beads. However, as shown above, as buffer ionic strength was increased from 0.02 M to 1.0 M, the adsorption behavior has little change. Therefore the ion exchange mechanism can not provide satisfactory explanation for the BSA adsorption behavior. Obviously HIC mechanism can be excluded in the adsorption process of BSA to IMAC-Fe^{3+} column except the situation of nonspecific binding.

In IMAC, the underlying principles of the binding of proteins to the metal chelate stationary phase is believed to be the result of the ability of electron-rich ligands on the surfaces of proteins to substitute weakly bonded ligands such as water or mobile phase ions in the metal complexes on the surface. In this process, the chelating metal plays the roles of accepting unshared pair of electrons from the side chains of the amino acids at the surface of the protein molecule.

7.5.4 Discussion: Implications in Adsorption Process Design

The work in this chapter was to determine the isotherm of BSA adsorption on the IMAC-Fe^{3+} column. The results provided more reliable and efficient theoretical basis for buffer selection and adsorption process design, optimization, simulation and process scale-up. Isotherm model provides deep insights into the character of the BSA and IDA-

Fe^{3+} interactions. In this study, the BSA exhibited heterogeneous interactions with immobilized IDA-Fe^{3+}. Variation of ionic strength and pH in the study was to identify the adsorption mechanism, types of the forces involved in the adsorption. As described above, adsorption of BSA on the IDA-Fe^{3+} IMAC beads was mainly due to metal chelating affinity between the metal ion Ferric and BSA. Since at higher pH, adsorption capacity is higher, adsorption process can be executed at higher pH and elution process at lower pH. To prevent possible ion exchange interactions, higher ionic strength should be maintained.

As isotherm is known, the process development and optimization has the theoretical basis and can be completed in a more rational way. It also provides the basic data for process simulation, which is a very important step in process scale-up. The isotherm studies comprise the first step in the process model evaluation.

7.6 Summary

Frontal adsorption studies have been shown to be an effective way of characterizing the effects of buffer conditions on an adsorption process as well as the Fe^{3+}-BSA interactions. The adsorption isotherm of BSA also provides theoretical basis for the purification of the BSA. In this chapter, the adsorption of BSA by IMAC-Fe^{3+} was investigated.

The Langmuir isotherm cannot predict adsorption behaviors of BSA, even at low BSA concentration as the adsorption does not follow the conditions assumed by Langmuir isotherm. The Freundlich isotherm model provides very good predictions of the BSA adsorption behavior in IDA-Fe^{3+} IMAC column.

It was found that BSA was bound mainly by metal chelating interactions between BSA and ligand Fe^{3+}. The effects of buffer ionic strength and pH conditions on the BSA adsorption behaviors indicate that ion exchange mechanism might be involved in process. However, it doesn't play a major role. To prevent possible ion exchange interactions, the higher ionic strength should be maintained.

CHAPTER 8

ADSORPTION CAPACITY OF BSA IN IDA-Fe^{3+} IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY COLUMN

8.1 Introduction

To the author's knowledge, there is no report concerning the quantitative aspects of interactions between protein and IMAC-Ferric adsorbents. In this chapter, BSA was used as a model protein. The frontal analysis was used as a reliable method to obtain the endotoxin adsorption capacity on IMAC column under different solution conditions. The effects of solution ionic strength and pH on BSA adsorption capacity of IMAC-Ferric were obtained. The adsorption mechanism was investigated quantitatively by adapting previously reported equation to IMAC-Ferric system. Then interaction mechanism between proteins and IMAC-Ferric was discussed.

8.2 Materials and Methods

8.2.1 Reagents

Bovine Serum Albumin (BSA) (Sigma, St. Louis, MO, USA) with low endotoxin content was used in all studies. All solutions were prepared with Mill-Q water (BioWhittaker Inc., Walkersville, MD, USA). All the other chemicals were purchased from Sigma.

8.2.2 Experimental Apparatus

Experimental apparatus was described in Chapter 5.

8.2.3 IMAC Column and Adsorbent Resin

A HiTrap Chelating column with dimensions of 0.7 x 2.5 cm was purchased from Amersham Pharmacia Biotech. The resin was described in Chapter 5 in details.

8.2.4 Buffers

The same buffers were used as Chapter 5.

8.2.5 Column Preparation and Operation

The column preparation and operation procedure was described in Chapter 7.

8.2.6 Frontal Analysis

The frontal analysis method was described in Chapter 5.

8.2.7 BSA Assay

The optical density of BSA was monitored by UV-1 in Pharmacia GradiFrac System, which was collected by a data acquisition system M1101 (Keithley Metrabyte, Cleveland, OH) incorporated with a computer. This system was used to record the chromatograms in digital forms so that they could be transferred later for data processing and analysis. By comparing with standard curve, BSA concentration was easily obtained.

8.2.8 Determination of the Amount of Immobilized Ligand (Fe^{3+})

The Fe^{3+} concentration is determined by Sigma total iron kit. First, the concentration of feed $FeCl_3$ solution was determined and total amount of Fe^{3+} applied to column was

calculated. Second, the Fe^{3+} amount eluted in loading and washing process was determined. The amount of immobilized Fe^{3+} was calculated as the difference between the total amount of Fe^{3+} applied to the column and the amount washed out during washing process. The leaching of the column can be investigated by determination of the ferric ion concentration in all elution fractions

8.2.9 Data analysis

The same equations described in Chapter 5 were used.

8.3 Results and Discussions

8.3.1 Concentration of Immobilized Ligand, Fe^{3+}

Using the procedure outlined above, the total concentration of immobilized Fe^{3+} on the IDA IMAC column was determined to be 34 μmol/ml of adsorbent. As provided by the manufacturer, the total Cu^{2+} concentration is about 23 μmol/ml of adsorbent for IDA-Cu^{2+} IMAC column. Author's measured results are within the range stated by the manufacturer.

8.3.2 Frontal Analysis

The shape of the elution front is also sigmoidal as the elution front of endotoxin. This is generally caused as a consequence of imperfection in the column packing as well as a lack of local distribution equilibrium mainly due to large variations in the size of the gel beads. The technical and operational advantages of adsorbents packed with small and uniform sized beads are obvious. The HiTrap chelating beads are adsorbents with big

pores, which is easily accessed by BSA molecules, which is a big advantage over the small pore adsorbents. The separation of BSA and endotoxin might be achieved using their size difference.

8.3.3 The Effects of Ionic Strength on the Column Capacity of BSA Adsorption to IDA-Fe^{3+} Column

The effect of ionic strength on the column capacity was shown in Figure 8.1 and Table 8.1 at the same buffer pH and BSA feed concentration (ca. 550 μg/ml). Four different buffer ionic strength conditions, 0.02, 0.25, 0.5 and 1.0 M, were used in the experiments. At the lowest ionic strength condition, 0.02 M, the column capacity was highest. As the ionic strength was increased from 0.02 to 0.25 M, the column capacity decreased from 71.4 mg/ml gel to 53.4 mg/ml. This indicates that electrostatic interaction might partly contribute to BSA adsorption to the IMAC-Fe^{3+} beads at low ionic strength buffer condition. This seems reasonable considering that BSA is negatively charged at pH 5.5. However, as ionic strength increased further from 0.25 to 1.0 M, the column capacity remains constant, 52 to 53 mg/ml gel. This result indicated that the IMAC-Fe^{3+} was quite different from regular ion exchange chromatography. Electrostatic interactions can not satisfactorily explain the adsorption behavior of BSA on IDA-Fe^{3+} IMAC column.

8.3.4 The Effects of pH on the Column Capacity of BSA Adsorption to IDA-Fe^{3+} Column

The effect of buffer pH on the column capacity was shown in Figure 8.2 and Table 8.1. Four different pH conditions were tried with the same ionic strength (0.5 M) and same level of BSA concentration in feed solution (ca. 550 μg/ml). At lowest pH 3.4, the

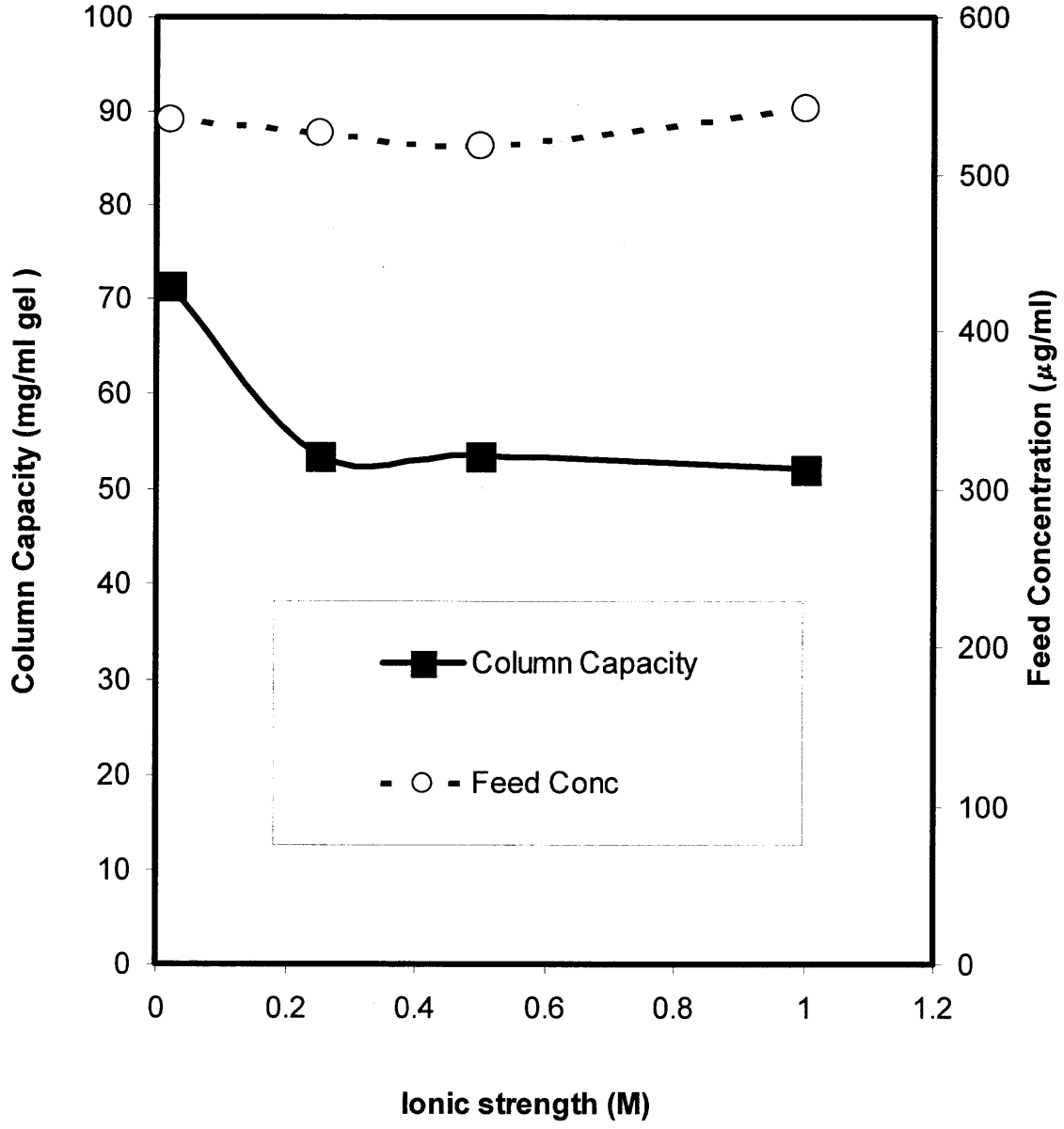


Figure 8.1 The effect of mobile phase ionic strength on BSA adsorption capacity on IDA-Fe^{3+} IMAC beads, pH 5.5

column capacity was highest. As the pH was increased from 3.4 to 4.3, the column capacity decreased slightly from 69.8 to 67.5 mg/ml gel. As the pH increased further to 5.5, the column capacity decreased to 53.5 mg/ ml gel. Finally as buffer pH was increased to 7.0, the column capacity is lowest, 18.2 mg/ml. As the properties of BSA were considered, the experimental results are very interesting. pI of BSA is about 4.6. At pH< 4.6, BSA is positively charged while negatively charged at pH > 4.6. Since Fe^{3+} is positively charged, ion exchange mechanism cannot explain the adsorption behavior of BSA on IMAC-Fe $^{3+}$. This research was very useful in applying IMAC in protein-LPS separation. To keep the protein adsorption capacity at very high level the buffer pH for adsorption process should be less than 7.0.

Table 8.1 Column capacity for BSA adsorption with buffer pH and ionic strength conditions

pH	I.S. (M)	Capacity(mg/ml)
3.4	0.5	69.8
4.4		67.5
5.5		53.5
7.0		18.2
5.5	0.02	71.4
	0.25	53.4
	0.5	53.5
	1.0	52.0

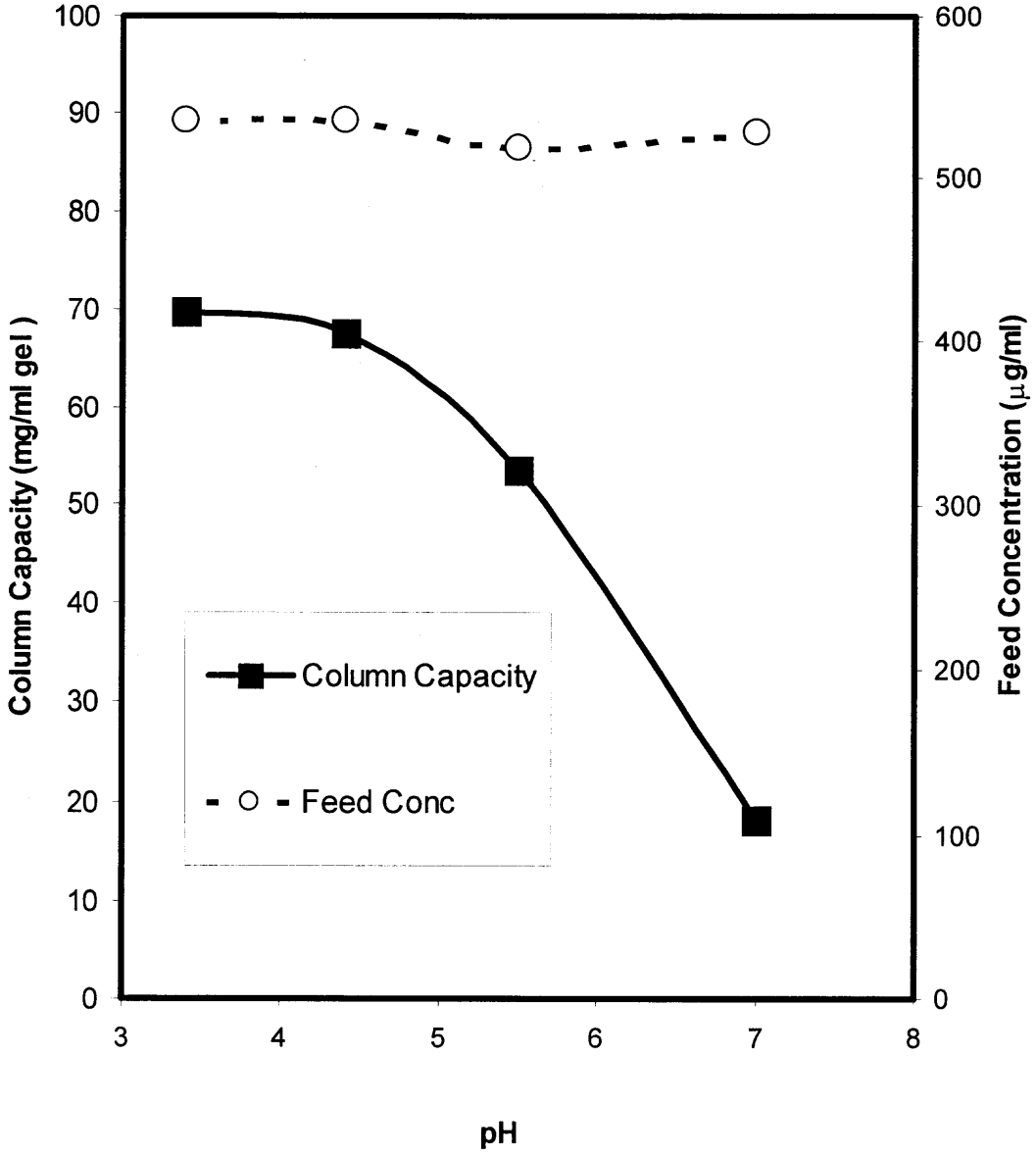


Figure 8.2 The effect of mobile phase pH on the adsorption to IDA-Fe^{3+} IMAC column, Ionic Strength 0.5 M

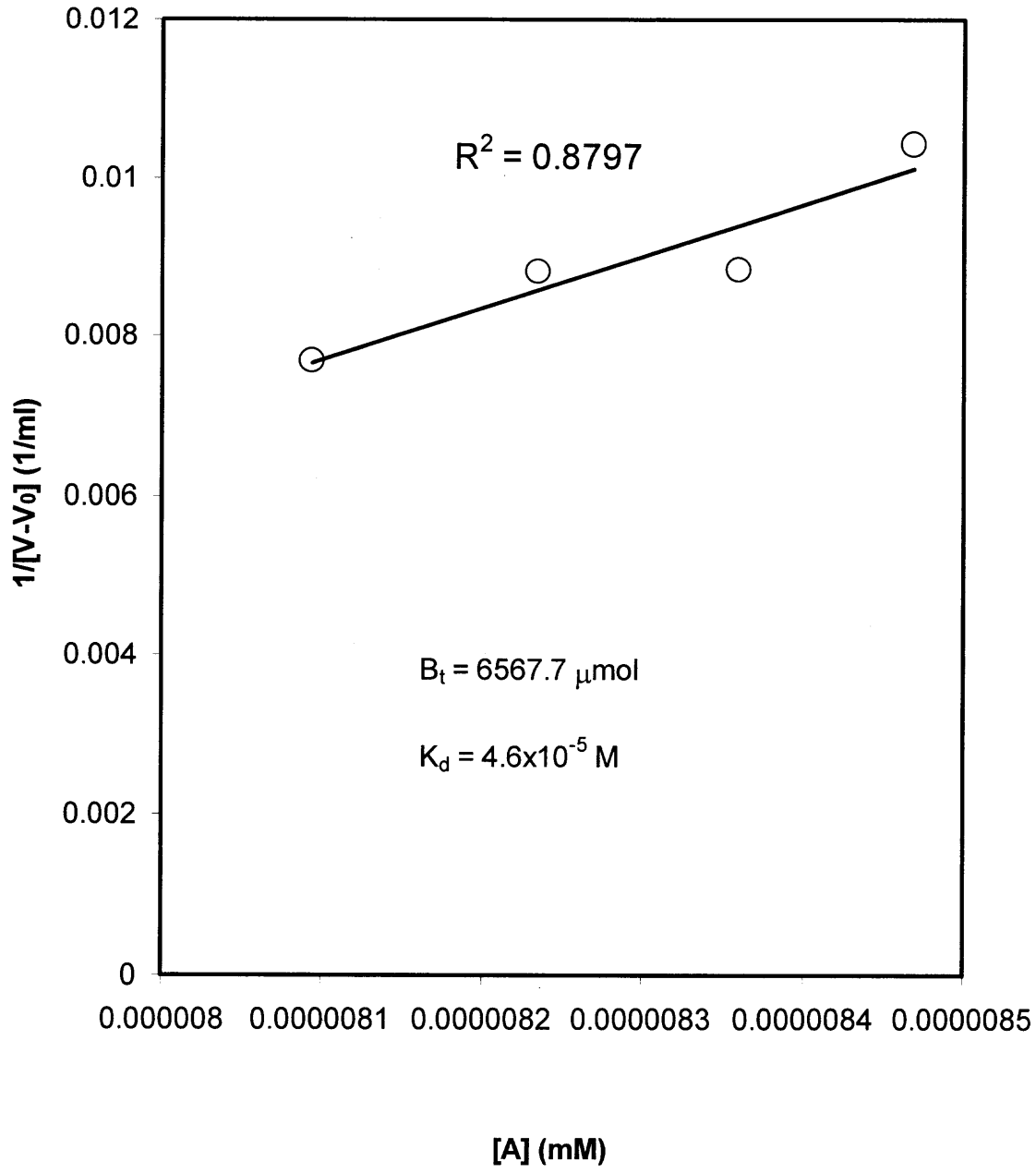


Figure 8.3 Determination of quantitative affinity parameter for the interaction between BSA and IDA-Fe^{3+}, MW of BSA: 64.5K, Kassai eq.

8.3.5 Quantitative Affinity Data

The plot of experimental data fitting to the equation of Kasai et al. is shown in Figure 8.3. From the plot, the total concentration of ligand that has interacted with BSA, B_t, is obtained directly from the intercept on the ordinate. K_d can be calculated from the slope of the plot and the B_t. As the molecular weight of BSA is 64,500, $K_d = 4.6 \times 10^{-5}$ M and B_t = 6567.7 μmol.

K_d shows stability of the complex of BSA, the adsorbate and Fe^{3+}, the ligand. If K_d is very low, the complex is very stable. B_t is obviously very large compared to the total Fe^{3+} amount, which was immobilized in the column as the adsorption ligands. This means that many BSA molecules were bound to one single adsorption site

As well known, the interaction of proteins with IMAC adsorbents is governed by the number or density of exposed histidine, cysteine or tryptophan residues. This can explain protein's adsorption behaviors. As reported in the literature, the size of BSA molecule is around 5 nm (Creighton, 1984). Pores of the adsorbents should be large enough so that free diffusion is not hindered significantly. This normally can be met with pores that are 10 to 20 times larger than the sizes of the adsorbate molecule (Satterfield et al., 1973). For the case of BSA, pore should be larger than 50nm. The pore size of IDA adsorbent is around 100 nm. Therefore, the steric effect is not a problem for BSA adsorption.

8.4 Summary

The work in this article was to determine the capacity of BSA adsorption on the IMAC-Fe^{3+} column under different solution conditions. The results provided more reliable and

efficient theoretical basis for buffer selection and adsorption process design, optimization, simulation and process scale-up. It also provided deep insight into the character of the BSA and IMAC- Fe^{3+} interactions. In this study, the BSA exhibited heterogeneous interactions with immobilized IMAC- Fe^{3+}. Variation of ionic strength and pH in the study was used to identify the adsorption mechanism, types of the forces involved in the adsorption. As described above, adsorption of BSA on the IMAC- Fe^{3+} was mainly due to histidine group in the BSA and the interaction mechanism was essentially metal chelating. Electrostatic forces might also be involved at the low solution ionic strength.

As adsorption capacity under different conditions is known, the process development and optimization has the theoretical basis and can be completed in a more rational way. It also provides the basic data for process simulation, which is a very important step in process scale-up. In the loading period of BSA removal process, in order to get high BSA adsorption capacity, buffer with a very high pH should be avoided.

BSA is a typical model protein, its adsorption on IMAC- Fe^{3+} is due to metal chelating interaction between BSA and Fe^{3+}. As the bead pore size is very big, steric effect on BSA adsorption can be eliminated. Other proteins, such as HbAo (neutral protein) and IgG (alkaline protein) were known for strong endotoxin binding. It is very difficult to remove endotoxin from these protein solutions. Like BSA, these proteins have many common properties. First, the protein size is smaller than 10 nm. In other words, there is no steric effect on mass transfer in the pores of IMAC-Fe^{3+} beads. Second, all these proteins have histidine group(s) on the surface, which could contribute

the metal chelating interactions. This indicates that IMAC-Fe^{3+} can be used to purify proteins and at same time remove endotoxins.

CHAPTER 9

ELUTION PROCESS DEVELOPMENT AND OPTIMIZATION IN IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY FOR ENDOTOXIN REMOVAL FROM PROTEIN SOLUTIONS

9.1 Introduction

Bacterial endotoxins are lipopolysaccharides (LPS) derived from the outer cell membranes of Gram-negative bacteria (Vaara and Nikaido, 1984). In biotechnology industry, Gram-negative bacteria are widely used 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 (Hou and Zaniewski, 1990). The removal of these physiologically active agents from final protein products has always been a challenge, especially in the situations where endotoxins bind proteins.

Although numerous methods such as ion exchange adsorption (Webber et al., 1995), ion exchange membrane (Belanich et al., 1996), ion-exchange filter (Hou and Zaniewski, 1990), ultrafiltration (Li and Luo, 1998, 1999), extraction (Aida and Pabst, 1990) have been studied for endotoxin removal from protein solutions, affinity adsorption has proven to be the most effective technique. As to the endotoxin binding affinity ligands, immobilized Polymyxin B (Issekutz, 1983; Karprus et al., 1987; Tani et al., 1992; Petsch et al., 1997) and Immobilized histidine (Matsumae et al., 1990; Guo et al., 1997; Legallais et al., 1997) have been investigated intensively with variety of supports. Both adsorbents have high capacities for adsorbing endotoxin in solutions with low ionic strength. However, it is difficult to selectively remove endotoxin from the protein solutions. The nephrotoxicity and neurotoxic reaction of polymyxin B

(Physicians' Desk Reference, 1993; Kodama et al., 1997) further prevent its application in endotoxin removal from bioproducts. The author (Kang and Luo, 1998) has reported the use of Acticlean Etox affinity column from Sterogene Bioseparations (Carlsbad, CA) to remove endotoxin from hemoglobin preparations where the protein formed a complex with LPS subunits. The effects of various solutions such as endotoxin-free water, NaCl and $CaCl_2$ on the endotoxin removal efficiency and protein recovery in the chromatographic process were investigated. All the above study indicates that an effective and economic adsorbent, which can give both high endotoxin removal efficiency and high protein recovery, is yet to be developed.

Since its introduction by Porath et al. in 1975, immobilized metal ion affinity chromatography (IMAC, also know "metal chelate chromatography") has gained wide acceptance in purification of proteins and peptides (Sulkowski, 1985; Yip and Hutchens, 1994; Scopes, 1994). Much of the earlier work concentrated on using Cu^{2+} or Zn^{2+} as the chelating metal ion (Sulkowski, 1985; Scopes, 1987). Later Fe^{3+} was found to be a very effective chelating ion for isolation of phosphorylated amino acids, peptides and proteins based on the preferential affinity between the immobilized iron (III) ion and the phosphate groups in the biomolecules (Andersson, 1986; Muszynsha, 1992). It was proposed by author and coworkers that LPS also had phosphate groups in its structure, which might interact strongly with immobilized Fe^{3+}. However, to the best of author's knowledge, there are no reported studies on the specific use of IMAC for endotoxin removal. In this chapter a novel IMAC method or process for endotoxin removal from protein solutions (acidic protein, BSA, neutral protein, HbAo and basic protein, IgG) was developed. Fe^{3+} was charged to a chelating gel that contains iminodiacetic acid (IDA).

The IDA-Fe^{3+} IMAC column was exploited to remove endotoxin from the mixtures of bovine serum albumin and endotoxin (BSA-LPS), and from the mixtures of human hemoglobin Ao and endotoxin (HbAo-LPS), as well as from the mixture of IgG and endotoxin (IgG-LPS). Endotoxin removal efficiency and protein yield were investigated (Kang and Luo, 1999).

The selectivity and retention of biomolecules in IMAC could be altered in several ways (Anspach, 1994; Vunnum and Cramer, 1997). First, the different type of stationary phase and chelating function between the ligand and the spacer arm can be used when the methods are first developed. Second, changing pH is regularly used to selectively elute the adsorbed molecules from IMAC column. Third, changing the nature and concentration of the salts is frequently used in the research. Fourth, with different nature and concentration of competing agent in elution buffer, the selectivity and retention of the adsorbed molecules can be modulated or adjusted. In this chapter the elution process development and optimization were reported to obtain high endotoxin removal efficiency and high protein yield. Different elution strategies were tried. Phosphate ion is a very efficient modulator in the elution of protein from the IDA-Fe^{3+} column. The experimental results were used to confirm the theory which was put forward in Chapter 5 to 8. Adsorption mechanism was further discussed.

9.2 Materials and Methods

9.2.1 Materials

Endotoxin from *Escherichia coli* 026: B6 (Sigma Chemical Co., St. Louis, MO) was used in all experiments. Human Hemoglobin Ao (HbAo, ferrous), bovine serum albumin

(BSA), Goat immunoglobulin (IgG), $FeCl_3$, NaCl, NaH_2PO_4, Na_2HPO_4, Na_3PO_4, NH_4Cl and EDTA (ethylenediaminetetraacetic acid, sodium) were also purchased from Sigma. N-octyl-β-D-glucopyranoside (OBDG) was purchased from CALBIOCHEM (Calbiochem-Novabiochem International, San Diego, CA). All solution transfers were performed with endotoxin free devices. Sterile, disposable plasticware was used at all time to prevent endotoxin contamination. All solutions were prepared with Milli-Q water generated by a Biocel water purification system (Millipore Corporation, Bedford, MA). The endotoxin concentration in the Milli-Q water was below 0.05 EU/ml based on author's test.

9.2.2 Experimental Apparatus

A GradiFrac chromatographic system (Amersham Pharmacia Biotech, Piscataway, NJ) was used in all experiments. Feed and mobile phase solutions were transferred by an adjustable peristaltic P-1 pump. The ultraviolet absorbance of the eluent from the column was measured by an UV-1 monitor at 280 nm. The flow cell used in the monitor was a standard cell with an optical path length of 1.00 cm. The UV absorbance was recorded by two means. The first one was a REC 102 2-channel recorder, which was used to show chromatograms directly during the experiments. The second one was a data acquisition system M1101 (Keithley Metrabyte, Cleveland, OH) incorporated with a computer, which was used to record the chromatograms in digital forms so that they could be transferred later for data processing and analysis. A GradiFrac fraction collector was used to collect fractions for protein and endotoxin assay.

9.2.3 IMAC Column and Adsorbent Resin

A HiTrap chelating column with dimensions of 0.7 x 2.5 cm was purchased from Amersham Pharmacia Biotech. The column was packed with Pharmacia chelating Sepharose High Performance resin consisting of highly cross-linked agarose beads to which iminodiacetic acid (IDA) was coupled by a stable spacer arm. The mean diameter of the adsorbent is approximately 34 μm. The average pore size is around 100 nm.

9.2.4 Buffers

Buffers A, B, C and D were used in the experiments, which were listed in Table 9.1. All feed solutions were prepared with buffer A – 0.5 or 1.0 M NaCl in 0.02 M acetate buffer at pH 5.5. The starting buffer was buffer A. To develop an effective chromatographic process, which keeps endotoxin in the column, four elution buffers were used individually or in combination for protein elution study. The first elution buffer, buffer B, was 0.5 M NH_4Cl in 0.02 M acetate buffer at pH 5.5. The second elution buffer, buffer C_0, was OBDG in buffer B. The third elution buffer, buffer C_1, was sodium phosphate in buffer A. The fourth elution buffer, buffer C_2 was sodium phosphate buffer. The stripping buffer, buffer D, was 0.05 M EDTA in buffer A. All buffers were filtered through a 0.22 μm filter (Nalgene, MA) and degassed under vacuum for at least 25 min prior to being applied to the column.

9.2.5 Column Preparation and Operation

The HiTrap chelating column was stored in 20% ethanol when purchased. The column was first washed with 5 ml Milli-Q water at a flow rate of 1 ml/min to remove the ethanol. Then the column was loaded with 0.5 ml of 0.1 M $FeCl_3$ solution at 1 ml/min to

Table 9.1 Summary of buffers used in the IMAC process

Buffer	Composition	pH
A (starting buffer)	0.5 M or 1.0M NaCl in 0.02 M acetate buffer	5.5
B (elution buffer)	0.5 M NH_4Cl in 0.02 M acetate buffer	3.3~5.5
C_0 (elution buffer)	OBDG in buffer B	5.5
C_{01}	0.17 mM OBDG	
C_{02}	0.34 mM OBDG	
C_{03}	0.69 mM OBDG	
C_{04}	1.03 mM OBDG	
C_{05}	8.60 mM OBDG	
C_1 (elution buffer)	0.06 M Sodium phosphate in buffer A	3.5 5.0 5.4, 5.5 8.0
C_2 (elution buffer)	0.06 M Sodium phosphate buffer	5.5
D (stripping buffer)	0.05 M EDTA in buffer A	

charge Fe^{3+} to the resin. Finally, the column was washed with 5 ml Milli-Q water at 1 ml/min to replace the unbound Fe^{3+} from solution. The column was now charged with metal ion and ready for use. Before applying the feed, a blank run was performed to elute nonspecifically bound metal ions that might otherwise be eluted during desorption. In the blank run, the column was washed with 2.5 ml of buffer A at 2 ml/min followed by 5ml of buffer B at 1 ml/min. The column was then equilibrated with 2.5 ml of buffer A

at 1 ml/min. The feed (10 ml of pure protein, pure LPS or protein-LPS mixture) was applied to the column at 1 ml /min. The column was then washed with the starting buffer (buffer A) at 2 ml/min for 10min. Finally, the column was eluted by elution buffers (buffer B, C or combination of B and C) at 1 ml/min using step gradients. The column was regenerated by washing with buffer D to strip all the metal ions, followed by washing with 5 ml of Milli-Q water, both at a flow rate of 1 ml/min.

9.2.6 Protein Assay and Protein Yield of Protein-LPS Mixtures by the Column

Protein concentrations in all eluents from the IMAC column were measured by two means. First, the ultraviolet absorbance of the eluent was monitored continuously at 280 nm for protein peaks. Second, fractions were collected and protein concentrations in all fractions or fraction pools were assayed using Bradford method (Bradford, 1976). Protein yield, *Y*, of the IMAC process was determined by the following equation:

$$Y = \frac{m_{pro}^{peak}}{m_{pro}^{feed}} \times 100\% \qquad (9.1)$$

Where m_{pro}^{feed} is the amount of protein (μg) in the feed loaded to the column. m_{pro}^{peak} is the amount of protein (μg) in the protein peak collected.

9.2.7 Endotoxin Assay and Endotoxin Removal Efficiency of Protein-LPS Mixtures by IMAC Column

To determine endotoxin concentration, a chromogenic *limulus* amebocyte lysate (LAL) test kit QCL-1000 from BioWhittaker (BioWhittaker Inc., Walkersville, MD) with a modified procedure was used. The reaction mixtures were measured at 405 nm with a

results were compared to a standard curve to obtain endotoxin concentrations. However, HbAo has a strong absorbance at 405 nm and subtraction of its absorbance is necessary. The correction was done by deducting the HbAo absorbance from total absorbance of the reaction mixture (Kang and Luo, 1999).

The endotoxin removal performance of this IMAC process was quantitatively evaluated using two parameters: Local Endotoxin Removal Efficiency, *LERE,* and Global Endotoxin Removal Efficiency, *GERE.* *LERE* was determined by the following equation (Li and Luo, 1999):

$$LERE = \frac{m_{LPS}^{feed} - m_{LPS}^{peak}}{m_{LPS}^{feed}} \times 100\% \qquad (9.2)$$

Where m_{LPS}^{feed} is the amount of endotoxin (in endotoxin unit, EU) in the feed loaded to the column. m_{LPS}^{peak} is the amount of endotoxin (EU) in the protein peak collected.

GERE was determined by the following equation:

$$GERE = \frac{m_{LPS}^{feed} + m_{LPS}^{buffer} - m_{LPS}^{frac}}{m_{LPS}^{feed} + m_{LPS}^{buffer}} \times 100\% \qquad (9.3)$$

Where m_{LPS}^{buffer} is the amount of endotoxin (EU) in buffer solutions used to wash and elute the column. m_{LPS}^{frac} is the amount of endotoxin (EU) in all elution fractions collected. *LERE* and *GERE* were calculated based on endotoxin concentrations in the feeds, buffers and elution fractions of chromatographic runs for BSA-LPS, HbAo-LPS or IgG-LPS.

9.2.8 Assay of Ferric Ions in the Eluents

To check if there is any leaching of ferric ions during the elution process, a total iron test

kit based on a ferrozine colorimetric principle (Sigma Chemical Company, St. Louis, MO) with a modified procedure was used. The detection limit is 0.05μg/ml. Milli-Q water, iron standard and test sample were placed in three cuvets individually and then mixed with iron buffer reagent and color reagent respectively. After 10 minutes the mixtures were measured at 560 nm with a Hitachi U-2000 spectrophotometer. The total iron concentration in the sample was obtained by comparing the absorbance of sample to that of the iron standard.

9.2.9 Elution with Buffer B (NH_4Cl in 0.02 M Acetate Buffer)

In this section, the elution of protein and endotoxin from IMAC with buffer B was investigated. Five different feeds were used: pure BSA, pure HbAo, pure LPS, BSA-LPS mixture and HbAo-LPS mixture. Protein concentrations were determined by Bradford assay while endotoxin contents were obtained by LAL test. 10 ml feed solution was loaded to the column at 1 ml/min and fraction collection started at the beginning of the loading. The subsequent washing and elution steps were described in Section 9.2.5. The fraction collection program was set in the following fashion. Both features of "fraction collection" and "peak collection" of the Gradifrac system were used. The fractions were collected at every 5 min before the last "peak collection" was activated. The fractions were collected at every one-minute after the last "peak collection" was finished. All fractions or fraction pools were assayed for protein and endotoxin contents. To check the effect of NH_4Cl concentration on the elution of protein or endotoxin, gradient elution runs were also conducted with 0 to 1.0 M NH_4Cl in buffer A.

9.2.10 Elution with Buffer C_0 (OBDG in Buffer B)

It was reported that OBDG could dissociate protein-LPS complex (Karplus, 1987), a feature that might be used to improve the endotoxin removal efficiency and protein recovery in a process of endotoxin removal from protein solutions. In this work, OBDG was added into buffer B to form buffer C_0. Buffer C_0 solutions with various OBDG concentrations were applied to IMAC column. BSA-LPS mixtures and HbAo-LPS mixtures were used as feeds. The chromatographic process was carried out in the same way as mentioned in Section 9.2.9.

9.2.11 Elution with Buffer C_1 (Sodium Phosphate in Buffer A)

As mentioned before, phosphate might be an efficient modulator for protein elution from IDA-Fe^{3+} IMAC. Sodium phosphate in buffer A under different solution conditions was tried. Buffer C_1 with different pH, i.e., pH 3.5, 5.0, 5.5 and 8.0 were prepared to investigate the effect of buffer pH on the elution of protein. In another set of experiments, Buffer B or C_0 was used followed by buffer C_1 in the elution process of IMAC for a comparison.

9.2.12 Elution with Buffer C_2 (Phosphate Buffer)

BSA-LPS feed was prepared as the same procedure in Section 9.2.9. 10 ml feed solution was loaded to the column at 1 ml/min and the fraction collection started at the beginning of the loading. The subsequent washing and elution steps were described in Section 9.2.5. 0.06 M phosphate buffer was used instead of sodium phosphate in the buffer A to

selectively elute protein and endotoxin from the column. The protein and endotoxin contents were determined in all fractions or fraction pools. This experiment is used to show which component in Buffer C_1, modulator or acetate buffer, is more important to elute the protein from the IMAC column.

9.2.13 Phosphate Buffer (Buffer C_2) as both the Starting Buffer and the Elution Buffer

The feed, the mixture of BSA and LPS, was prepared as same procedure in Section 9.2.9 except that phosphate buffer was used in stead of acetate buffer. 10 ml feed solution was loaded to the column at 1 ml/min and the fraction collection started at the beginning of the loading. The subsequent washing and elution steps were described in Section 9.2.5 except this time the phosphate buffer was used for washing and elution. All fractions or fraction pools were assayed for protein and endotoxin contents.

9.3 Results and Discussions

9.3.1 Effect of NH_4Cl on the Elution Process

NH_4Cl is routinely used as an eluting agent in IMAC for protein purification. However, buffer B (containing NH_4Cl) was not able to elute proteins in the experimental runs for both pure feeds (pure BSA, HbAo and LPS) and mixture feeds (BSA-LPS and HbAo-LPS). Neither stepwise elution (0.5 M or 1.0 M NH_4Cl) nor linear gradient elution from 0 to 1.0 M could elute proteins or endotoxin. Hence, NH_4Cl is not an effective agent to elute proteins out of the IMAC-Fe^{3+} column. The reason might be that affinity between IMAC-Fe^{3+} and protein/LPS is very high compared with affinity between IMAC-Fe^{3+} and NH_4^+.

9.3.2 Effect of OBDG in Elution Buffer on the Elution Process

Buffer C_0 solutions containing OBDG at different concentrations were prepared as shown in Table 9.1. They were tested to see if any of them could selectively elute the protein from the column after binding of protein-LPS mixture on the IDA-Fe^{3+} resin. The experiments were summarized in Table 9.2. The OBDG concentration in elution buffer B ranges from 0.17 mM to 8.6 mM. An example of elution profiles was shown in Figure 9.1 in which OBDG concentration is 0.17 mM. The major peak, i.e., first peak, is elution buffer front. The corresponding elution time equals time for buffer C_0 to displace the column. Other small changes in the chromatogram were caused by buffer changes or changes of operating flow rate. Bradford assay and LAL test confirmed that there was no protein and endotoxin eluted in all fractions. As OBDG concentration in elution buffer B is 0.34, 0.69, and 1.03 mM, and similar results were obtained. No protein or endotoxin was eluted. Further from Table 9.2, when acetate buffer pH ranges from 3.5 (Case 1.2, OBDG 0.34 mM) to 5.0 (Case 1.3, OBDG 0.34 mM), the similar results were obtained as Figure 9.1.

As OBDG concentration in buffer B was increased to 8.5 mM, a different elution profile was obtained (not shown here). Two major peaks were found in the profile. The first was the peak corresponding to the dead volume. The second peak indicated that there was ferric ion leaching from the column. This was confirmed by a blank run (with the starting buffer as the feed instead of the protein or endotoxin feed) with same elution buffer. Nearly the same elution profile was obtained (not shown here).

Tables 9.2 Removal of endotoxin from protein solutions using buffer C_0 (OBDG in buffer B) as the elution buffer

	Feed	Elution buffer	pH
Case 1.1	BSA-LPS	C_{01}	3.5
Case 1.2	BSA-LPS	C_{02}	3.5
Case 1.3	BSA-LPS	C_{02}	5.0
Case 1.4	BSA-LPS	C_{03}	5.5
Case 1.5	BSA-LPS	C_{04}	5.5
Case 1.6	BSA-LPS	C_{05}	5.5

9.3.3 Effect of Phosphate on the Elution Process

The use of phosphate as an eluting agent to selectively elute proteins from the column was studied. Buffer C at various pH conditions was used as shown in Table 9.3. In all experiments, phosphate was dissolved in buffer A.

Three strategies were used in elution of protein or endotoxin from IMAC column. First, only one elution buffer, C_1, was used. Second, buffer C_1 was used after buffer B. Third, buffer C_1 was used after buffer C_0. The experimental results were summarized in Table 9.3. HbAo-LPS feed was applied in Case 2.1. BSA-LPS feed was used in Case

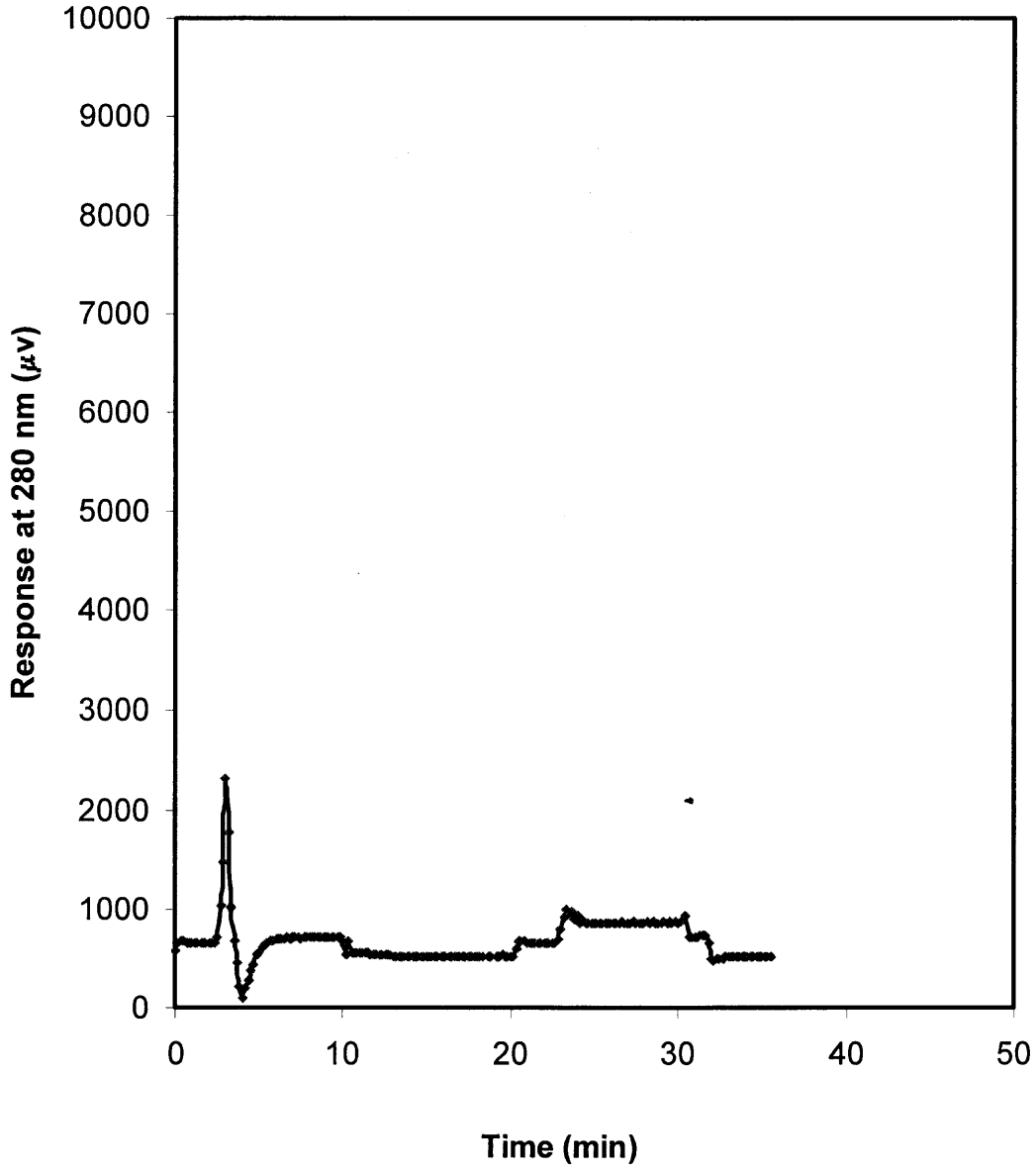


Figure 9.1 The chromatogram of BSA-LPS mixture eluted with buffer C_{01} (Case 1.1). Loading period: feed, 10 ml mixture containing 16.14 μg/ml BSA and 18.26 EU/ml LPS at 1 ml/min, 10 min. Washing period: 20 ml buffer A at 2 ml/min, 10 min. Elution period, 15 ml buffer C_{01} at 1 ml/min, 15 min.

2.2. IgG-LPS was used in Case 2.3. Two elution buffers, buffer B or buffer C_0 and buffer C_1 in series or only one buffer C_1 were used in the experiments, respectively. Table 9.3 shows that over 97% of endotoxin was removed from the protein solutions and protein yield was more than 90% in all the cases.

Table 9.3 Removal of endotoxin from protein solutions using phosphate as elution buffer

Case	Feed	Elution Buffer	LERE (%)	GERE (%)	Y (%)
Case 2.1					
2.1.1	HbAo-LPS	C_1 (pH5.0)	99.9	97.7	95.9
2.1.2	HbAo-LPS	B (pH5.3)+C_1 (pH5.0)	99.3	86.4	96.5
Case 2.2	BSA-LPS				
2.2.1	BSA-LPS	B (pH5.3)+C_1 (pH5.0)	99.7	97.5	90.0
2.2.2	BSA-LPS				
2.2.2.1	BSA-LPS	C_1 (pH5.0)	99.5	86.6	94.5
2.2.2.2	BSA-LPS	C_1 (pH5.4)	99.9	84.8	98.0
2.2.2.3	BSA-LPS	C_1 (pH5.5)	99.8	88.5	100.0
2.2.2.4	BSA-LPS	C_1 (pH8.0)	97.3	82.1	100.0
2.2.3	BSA-LPS				
2.2.3.1	BSA-LPS	C_{02}+C_1 (pH3.5)	99.7	97.5	93.6
2.2.3.2	BSA-LPS	C_{02}+C_1 (pH5.0)	99.8	94.7	90.0
2.2.4	BSA-LPS	C_2 (pH5.5)	99.8	90.2	100.0
Case 2.3	IgG-LPS	C_2 (pH5.5)	99.9	92.3	92.1

9.3.3.1 Endotoxin Removal from HbAo Solution

Figure 9.2A shows elution profile of endotoxin of HbAo-LPS mixture separated by the IMAC column (Case 2.1.1). It can be seen that endotoxin concentrations in all eluted fractions were very low. During the loading period (0 to 10 min) and the washing period (10 to 20 min), the output endotoxin concentration was between 0.6 and 0.7 EU/ml. This indicated that in loading and washing periods some endotoxin was not adsorbed to the column and washed out. During the first elution period (20-30 min) with buffer B, some endotoxin was also found in the eluants. However, in the second elution period (after 35 min), in which buffer C_1 was used, the eluted endotoxin was less than 0.1 EU/ml. This is low enough to satisfy all the endotoxin limit requirements (Held et al., 1997; Williams, 1996). Figure 9.2B shows that the elution profile of HbAo of the HbAo-LPS mixture separated by the IMAC column (Case 2.1.1). The chromatogram in Figure 9.2B (up) was recorded directly by the data acquisition system and the elution profile in Figure 9.2B (down) was obtained by Bradford assay of fractions and fraction pools. It can be seen that the HbAo peak came out at around 36 min. The peak was sharp with protein concentration of 95.69 μg/ml (35-40 min), 48.33 μg/ml (40-44 min) 5.62 μg/ml (44-49). During the loading (0 to 10 min), washing (10 to 20 min) and early elution (20 to 36 min) periods, the baseline of the chromatogram was very stable. This indicated that there was no protein content in this period. Total protein yield is 96.5%.

Figure 9.3A also shows elution profile of endotoxin of HbAo-LPS mixture separated by the IMAC column (Case 2.1.2). Similar to the situation in Figure 9.2A, endotoxin concentrations were low during the loading period (0-10 min), the washing period (10-20 min) and the elution period (20-42 min).

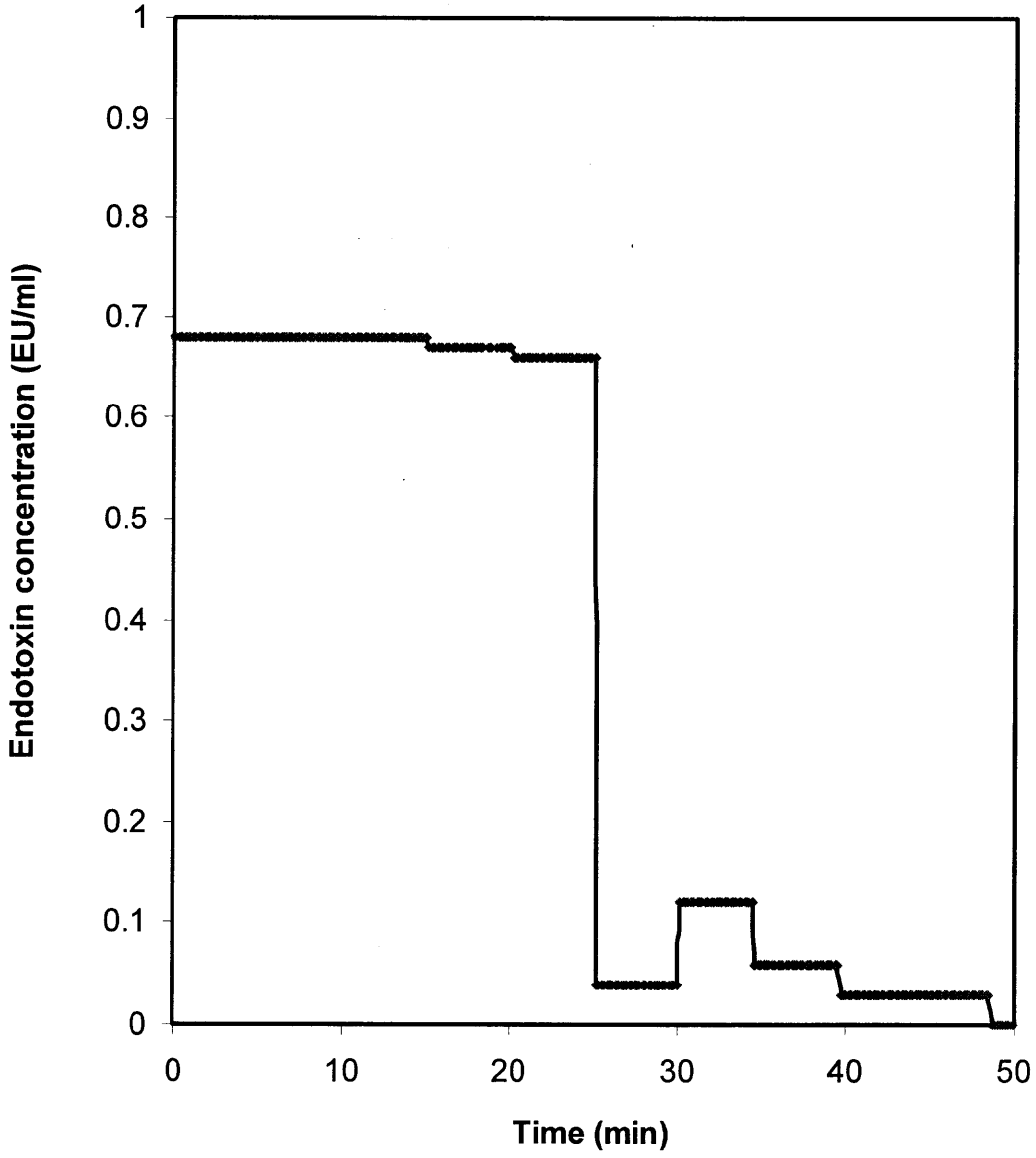


Figure 9.2A Endotoxin elution profile of HbAo-LPS mixture (Case 2.1.1). Loading period: feed, 10 ml mixture containing 76.72 μg/ml HbAo and 7.4 EU/ml LPS at 1 ml/min, 10 min. Washing period: 20 ml buffer A at 2 ml/min, 10 min. Elution period, 10 ml buffer B at 1 ml/min, 10min. Elution period: 20 ml buffer C_1 at 1 ml/min, 20min.

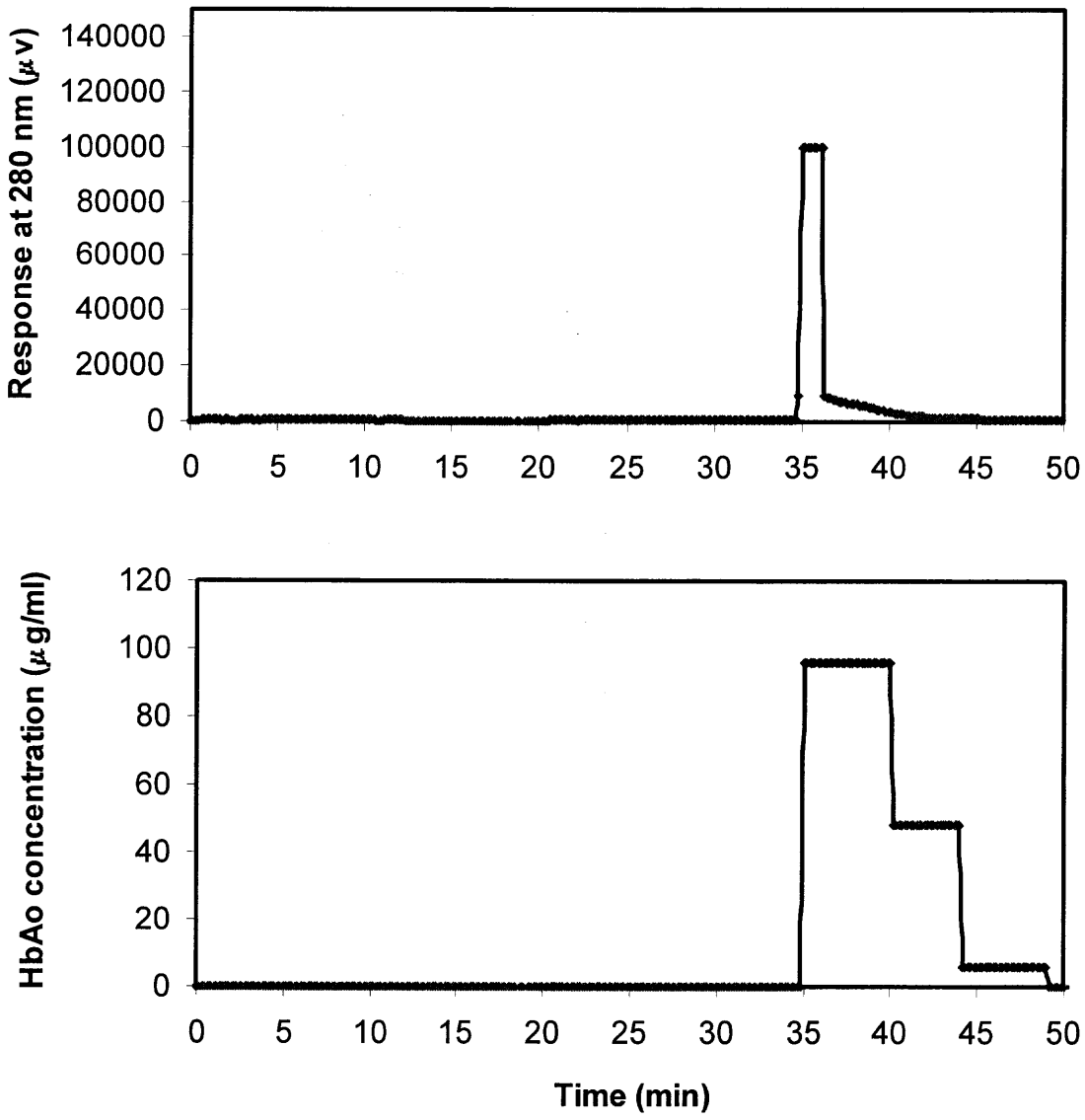


Figure 9.2B Protein elution profile of HbAo-LPS mixture (Case 2.1.1). Up: chromatogram of UV response at 280 nm, recorded by the data acquisition system. Down: chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 76.72 μg/ml HbAo and 7.4 EU/ml LPS at 1 ml/min, 10 min. Washing period: 20 ml buffer A at 2 ml/min, 10 min. Elution period: 10 ml buffer B at 1 ml/min, 10min. Elution period: 20 ml buffer C_1 at 1 min/ml, 20 min.

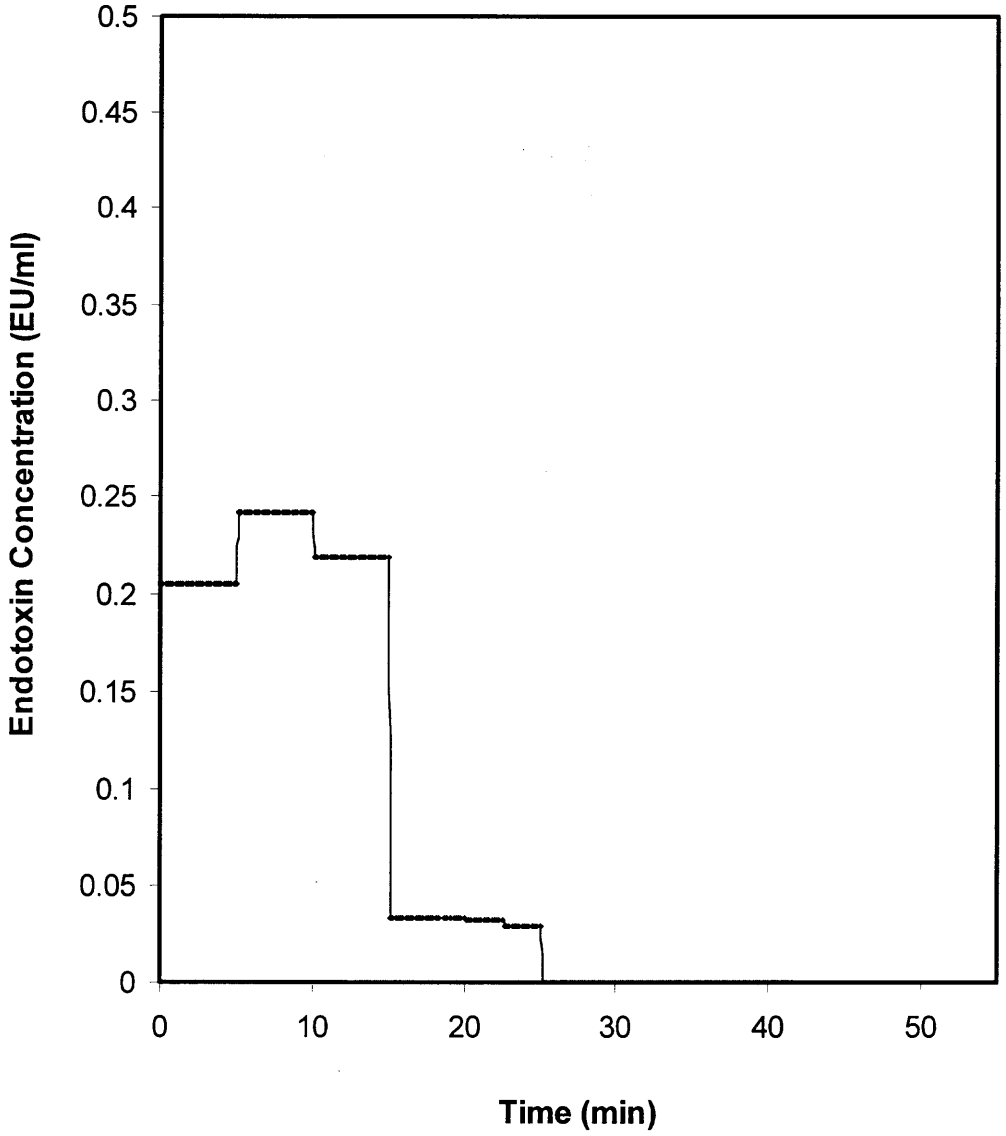


Figure 9.3A Endotoxin elution profile of HbAo-LPS mixture from the IMAC column (Case 2.1.2). Loading period: feed, 10 ml mixture containing 19.06 μg/ml HbAo and 21.26 EU/ml LPS; flow rate, 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 22 ml buffer C_1 at 1 ml/min.

Figure 9.3B shows that the elution profile of HbAo in the HbAo-LPS mixture separated by the IMAC. A sharp HbAo peak came out at around 24 min with a concentration of 36.56 μg/ml. The baseline of the HbAo chromatogram was also very stable during the loading (0 to 10 min), washing (10 to 20 min) and the early elution (20 to 24 min) periods.

9.3.3.2 Endotoxin Removal from BSA Solution

Figure 9.4A shows the elution profile of endotoxin in BSA-LPS mixture separated by the IMAC column (Case 2.2.1). It can be seen that endotoxin concentrations in all fractions were very low. During the loading period (0 to 10 min) and the washing period (10 to 20 min), the output endotoxin concentration was 0.15 EU/ml. During the elution period (20 to 52 min) while the protein was eluted out, the output endotoxin concentration was 0.05 EU/ml before 36 min and 0.03 EU/ml from 36 to 38 min. After 38 min, the endotoxin contents were below the detection limit of the LAL test. Figure 9.4B shows the elution profile of BSA in the BSA-LPS mixture separated by the IMAC column. The chromatogram in Figure 9.4B (up) was recorded directly by the data acquisition system, and the elution profile in Figure 9.4B (down) was obtained by Bradford assay of fractions and fraction pools. It can be seen that the BSA peak came out at around 36 min. The peak was sharp with a BSA concentration of 75.31 μg/ml. During the loading (0 to 10 min), washing (10 to 20 min) and early elution (20 to 36 min) periods, the baseline of the chromatogram was very stable except a small peak at 24 min. The Bradford assay result of the corresponding fraction of this peak showed no protein content.

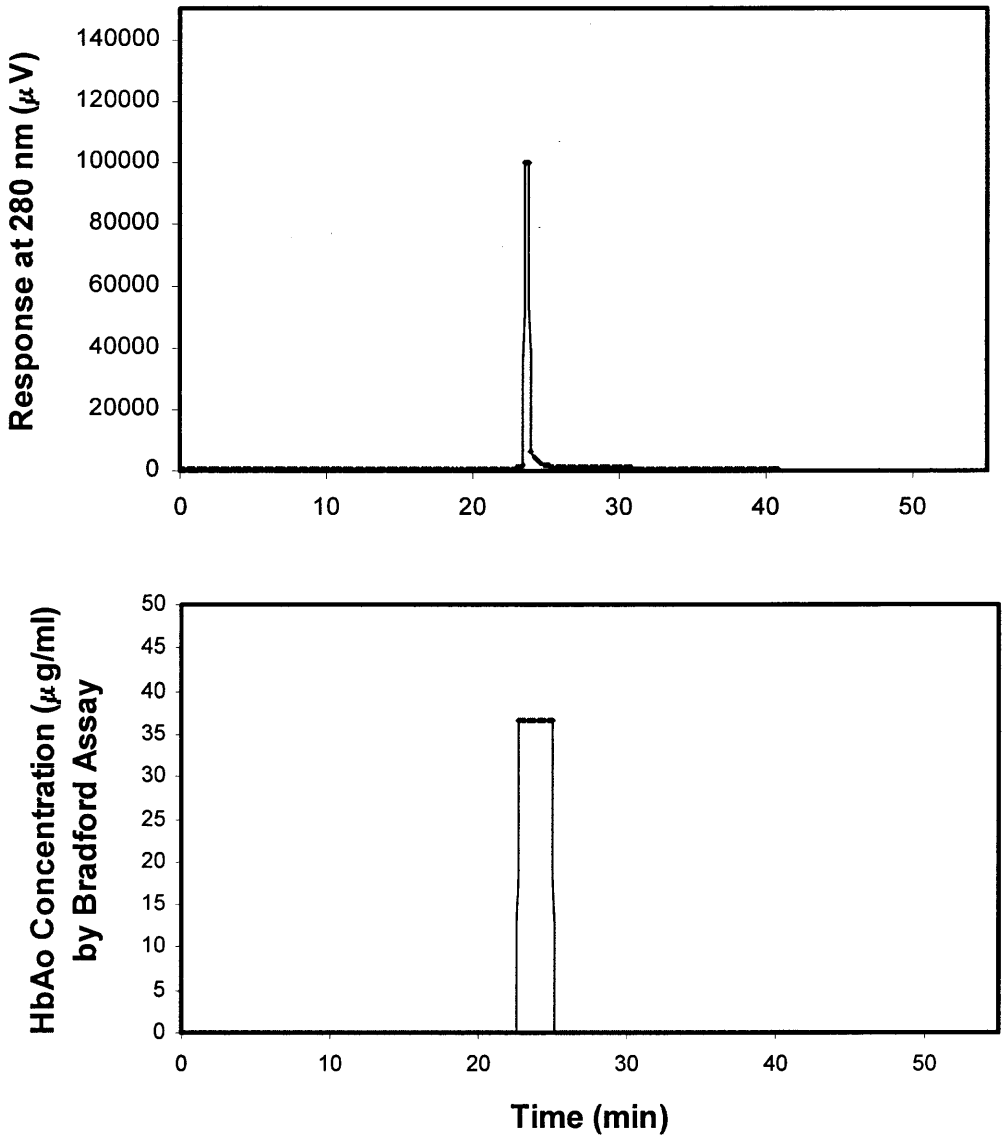


Figure 9.3B Protein elution profile of HbAo-LPS mixture from the IMAC column. Up: chromatogram of UV response at 280 nm, recorded by the data acquisition system. Down: chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 19.06 μg/ml HbAo and 21.26 EU/ml LPS; flow rate, 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 22 ml buffer C_1 at 1 ml/min.

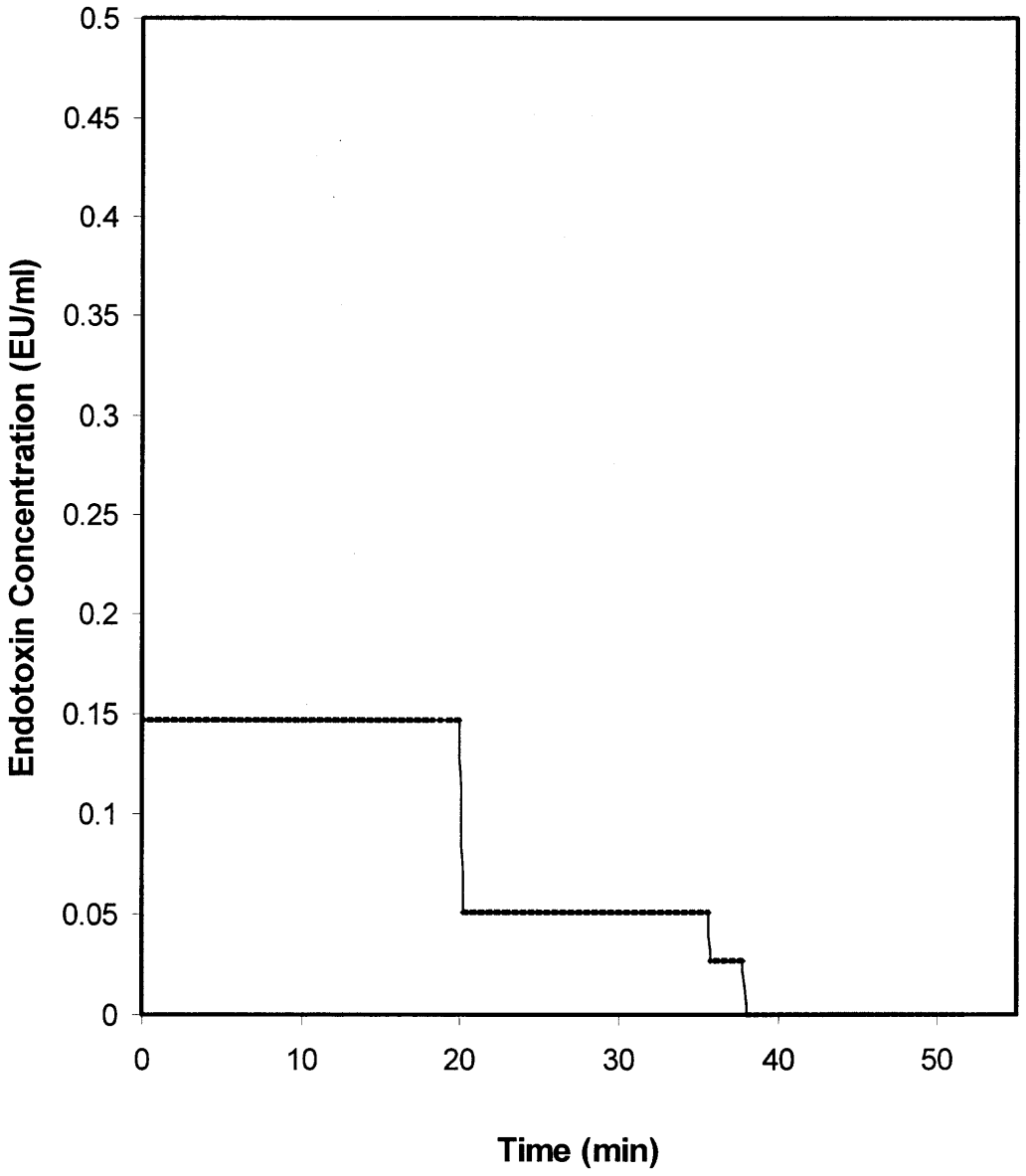


Figure 9.4A Endotoxin elution profile of BSA-LPS mixture from the IMAC column (Case 2.2.1). Loading period: feed, 10 ml mixture containing 16.14 μg/ml BSA and 18.26 EU/ml LPS; flow rate, 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 10 ml buffer B at 1 ml/min. Elution period: 22 ml buffer C_1 at 1 ml/min.

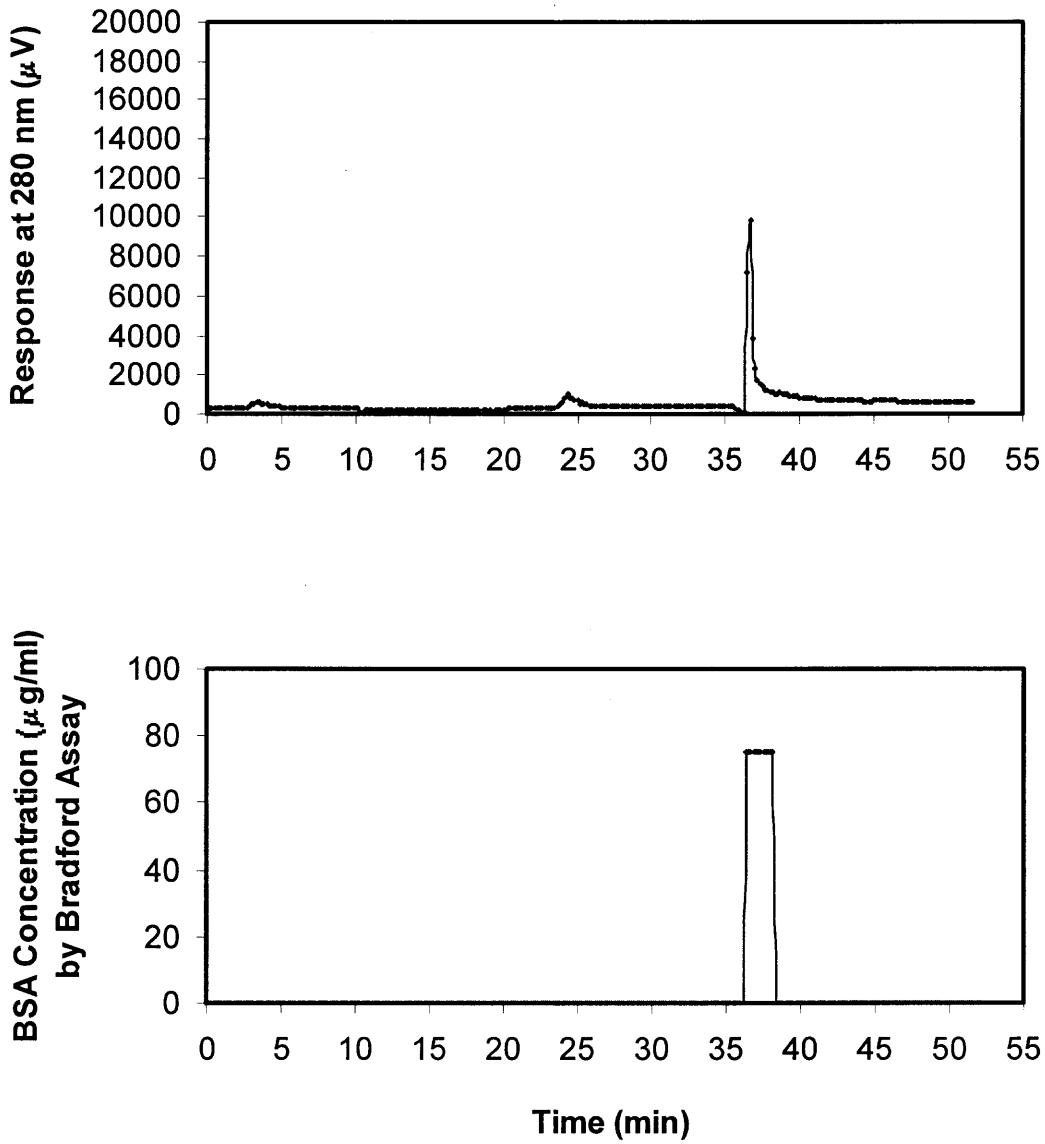


Figure 9.4B Protein elution profile of BSA-LPS mixture from the IMAC column. Up: chromatogram of UV response at 280 nm, recorded by the data acquisition system. Down: chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 16.14 μg/ml BSA and 18.26 EU/ml LPS; flow rate, 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 10 ml buffer B at 1 ml/min. Elution period: 22 ml buffer C_1 at 1 min/ml.

Figure 9.5A shows the elution profile of endotoxin of BSA-LPS mixture separated by the IMAC column (Case 2.2.2.1). Similar to the situation in Figure 9.2A, there was some endotoxin washed out in the loading period (0-10 min), the washing period (10-20 min) with starting buffer A, and first several minutes in elution period (20-24 min) with buffer B. After 24 minutes the endotoxin concentration in eluant is very low. Figure 9.5B shows the elution profile of BSA in the BSA-LPS mixture separated by the IMAC. A sharp BSA peak came out at 24 min with a concentration of 141.5 μg/ml. Similar to the situation of HbAo-LPS experiment, the baseline of the BSA chromatogram was also very stable during the loading (0 to 10 min), washing (10 to 20 min) and the early elution (20 to 24 min) periods. The difference is that elution peak is narrower than that in the case of HbAo. The protein yield was 94.5%. It was also noticed that in Case 2.2.2.1 BSA concentration in the product fraction pool was 141.5 μg/ml, which was 2.0 times of the BSA concentration in the feed (71.89 μg/ml). Therefore protein concentration was increased after IMAC processes.

As described previously, three strategies were used in elution of protein or endotoxin from IMAC column. As shown in Table 9.3, for HbAo-LPS feed, nearly the same LERE and protein yield were obtained in first two strategies. Using combination of two buffers, GERE was 86.4%, which is less than 97.7% with one buffer C_1. This means that in the process with two elution buffers, some endotoxin was washed out in loading or washing periods. Two strategies have similar good separate results. For BSA-LPS feed, high protein yield was obtained for three elution strategies. In all the cases (Cases 2.2.1, 2.2.2, 2.2.3 and 2.2.4), more than 99% LERE and above 90% protein yield was obtained.

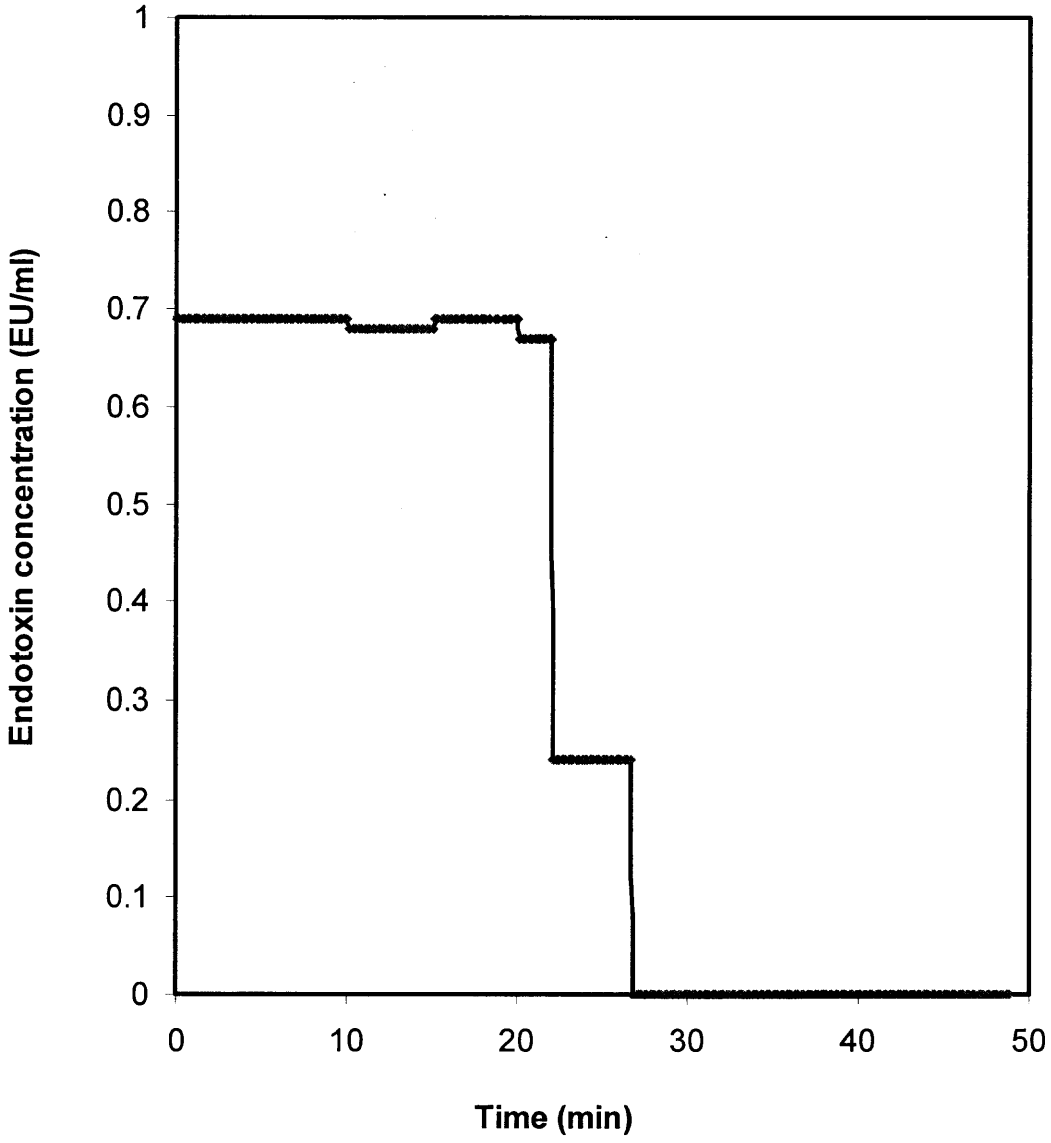


Figure 9.5A Endotoxin elution profile of BSA-LPS mixture (Case 2.2.2.1). Loading period: feed, 10 ml mixture containing 71.89 μg/ml BSA and 19.06 EU/ml LPS at 1 ml/min, 10min. Washing period: 20 ml buffer A at 2 ml/min, 10 min. Elution period: 28 ml buffer C_1 at 1 ml/min, 28min.

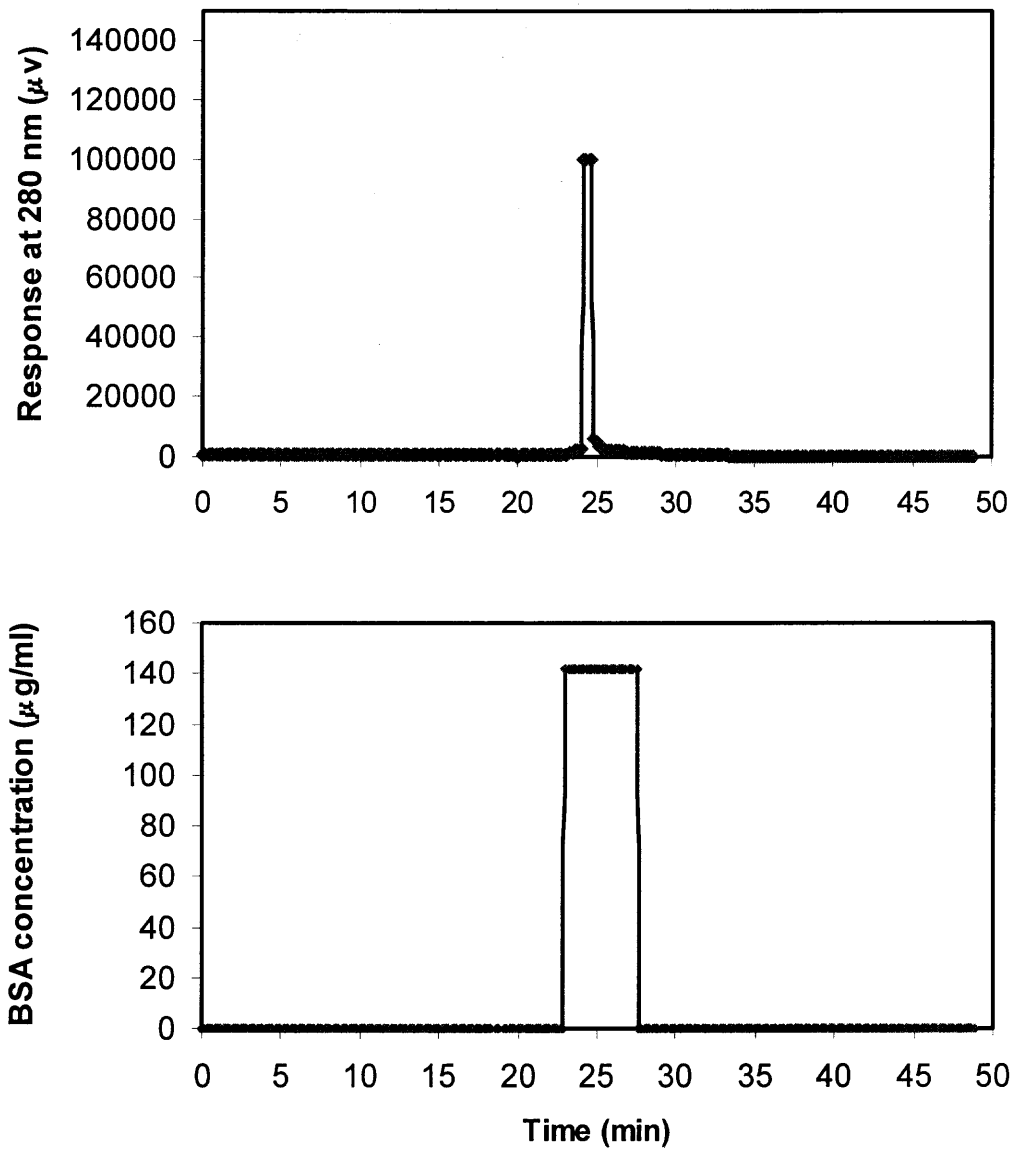


Figure 9.5B Protein elution profile of BSA-LPS mixture. Up: chromatogram of UV response at 280 nm, recorded by the data acquisition system. Down: chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 71.89μg/ml BSA and 19.06 EU/ml LPS at 1 ml/min, 10min. Washing period: 20 ml buffer A at 2 ml/min, 10 min. Elution period: 28 ml buffer C_1 at 1 ml/min, 28 min.

The endotoxin elution profiles for Cases 2.2.2.3, 2.2.2.2 and 2.2.4 were shown in Figure 9.6A, 9.7A and 9.8A, respectively. For all the cases, in the loading (0-10 min) and washing with buffer A (10 to 20 min), endotoxin concentration in eluant was less than 1 EU/ml, which indicates that some endotoxin was washed out in the process of loading and washing. The endotoxin concentration in the product fractions (after 20 min) was getting lower. These were eluted by elution buffer C_1, C_1 and C_2, respectively. The protein elution profiles were provided in Figure 9.6B, 9.7B and 9.8B, separately. The figures indicated that most of the protein was eluted in a very short time. This means the IMAC process not only removes the endotoxin from the protein solution but also concentrates the protein solutions as well.

9.3.3.3 Endotoxin Removal from IgG Solution

Figure 9.9A shows elution profile of endotoxin of IgG-LPS mixture separated by the IMAC column (Case 2.3). Similar to the cases of BSA and HbAo solutions, some of endotoxins were eluted during the loading period (0-10 min), the washing period (10-20 min). Figure 9.9B the elution profile of IgG in the IgG-LPS mixture separated by the IMAC. A sharp IgG peak came out at around 25 min with a concentration of 115.3 μg/ml. Similar to the situations in the BSA-LPS and IgG-LPS experiments, the baseline of the IgG chromatogram was also very stable during the loading (0 to 10 min), washing (10 to 20 min) and the early elution (20 to 24 min) periods. 92.1% protein yield and 99.9 % LERE were obtained in this research.

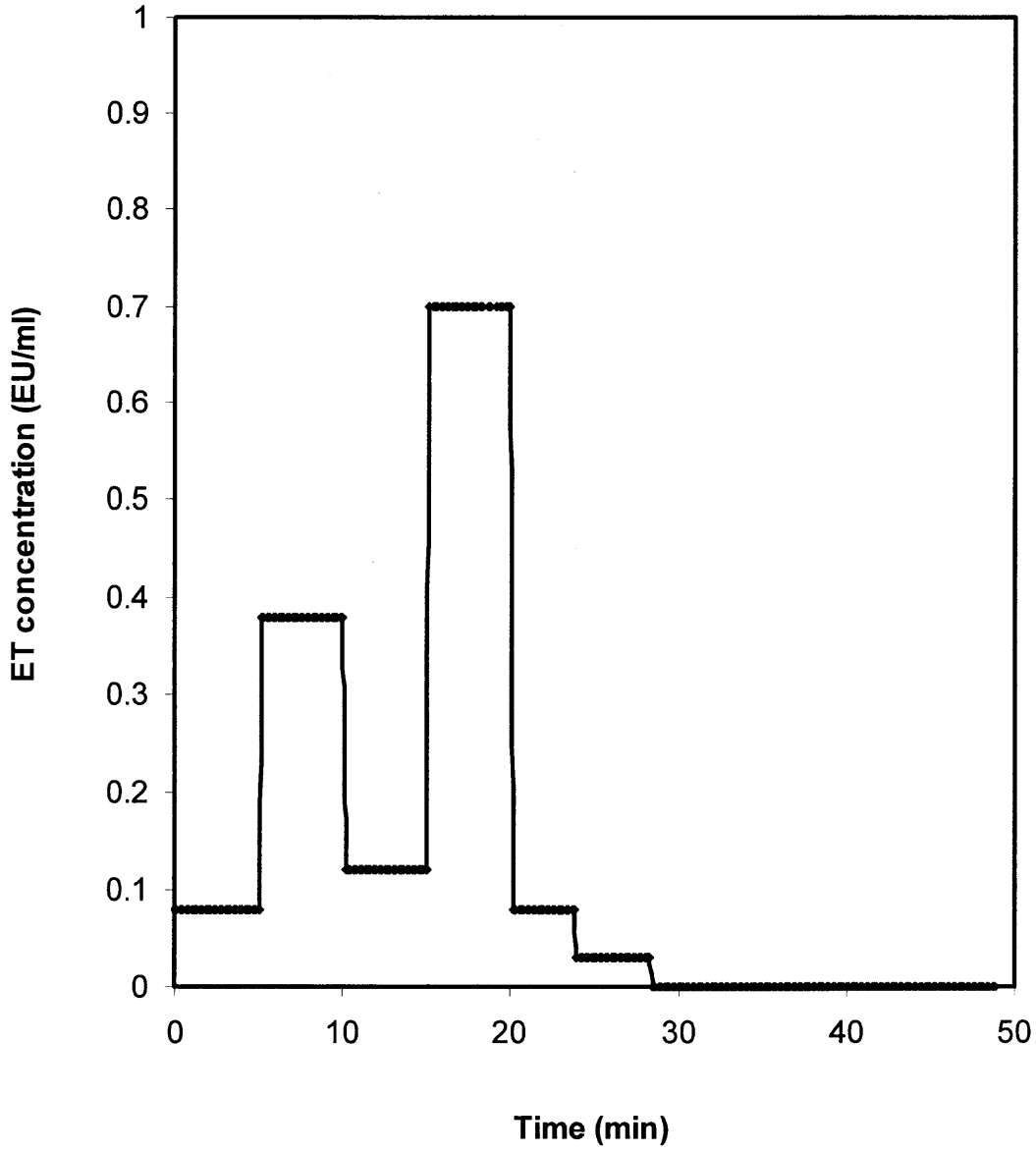


Figure 9.6A Endotoxin elution profile of BSA-LPS mixture (Case 2.2.2.3). Loading period: feed, 10 ml mixture containing 78.87 μg/ml BSA and 9.92 EU/ml LPS at 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period, 28 ml buffer C_1 at 1 ml/min.

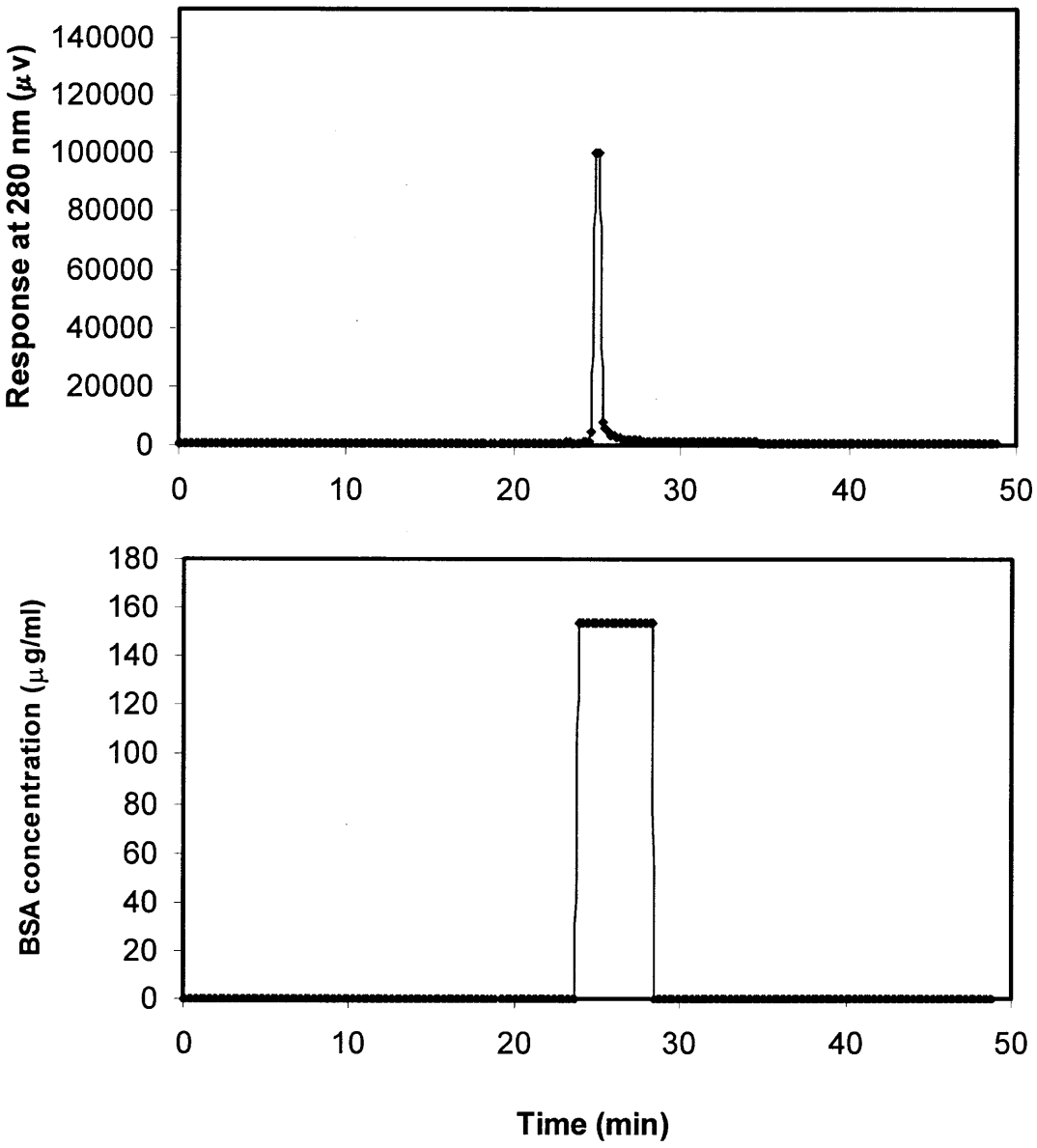


Figure 9.6B Protein elution profile of BSA-LPS mixture (Case 2.2.2.3). Up: chromatogram of UV response at 280 nm, recorded by the data acquisition system. Down: chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 78.87μg/ml BSA and 9.92 EU/ml LPS at 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 28ml buffer C_1 at 1 ml/min.

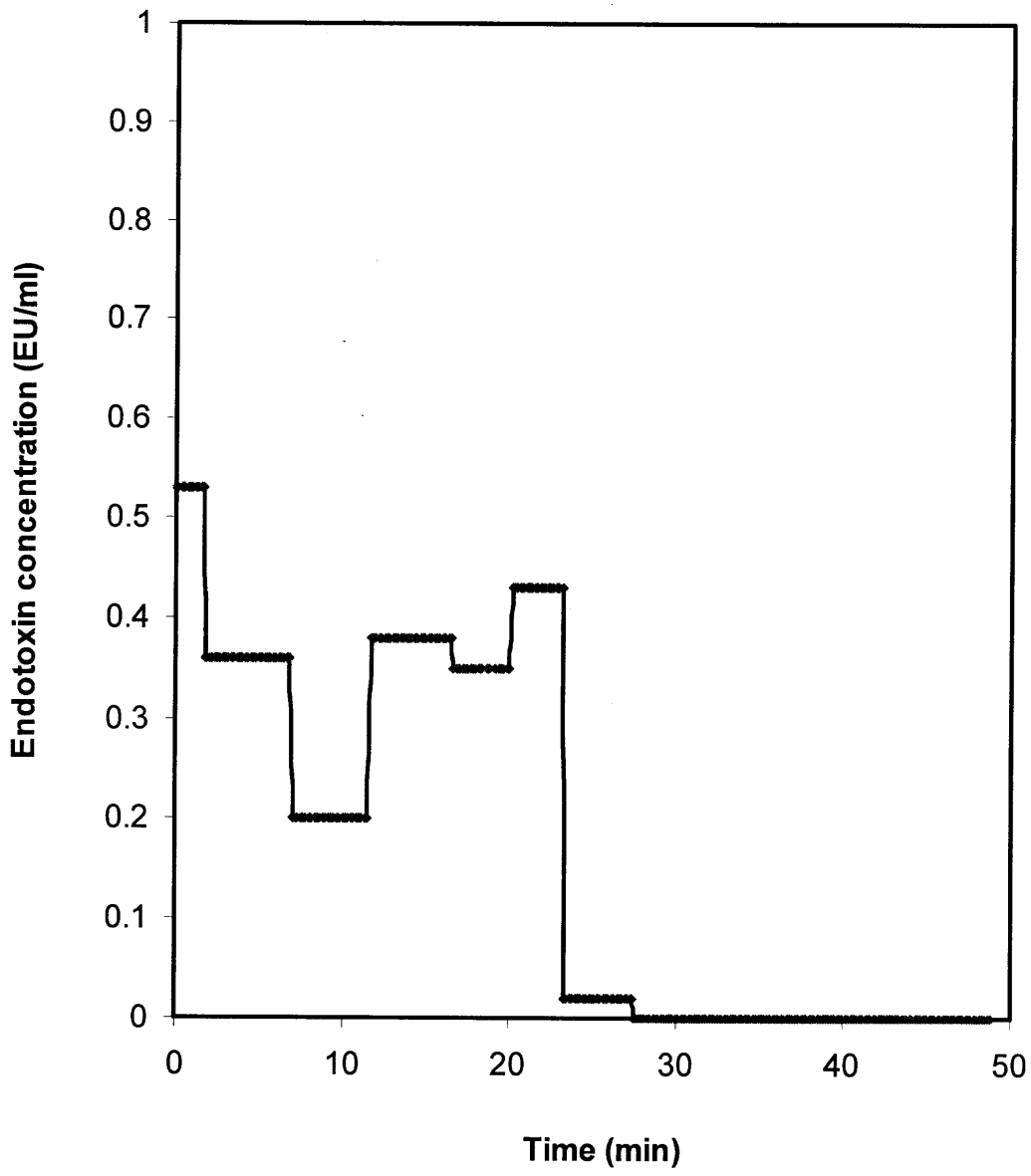


Figure 9.7A Endotoxin elution profile of BSA-LPS mixture (Case 2.2.2.2). Loading period: feed, 10 ml mixture containing 78.87 μg/ml BSA and 7.08 EU/ml LPS; flow rate at 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 28 ml buffer C_1 at 1 ml/min.

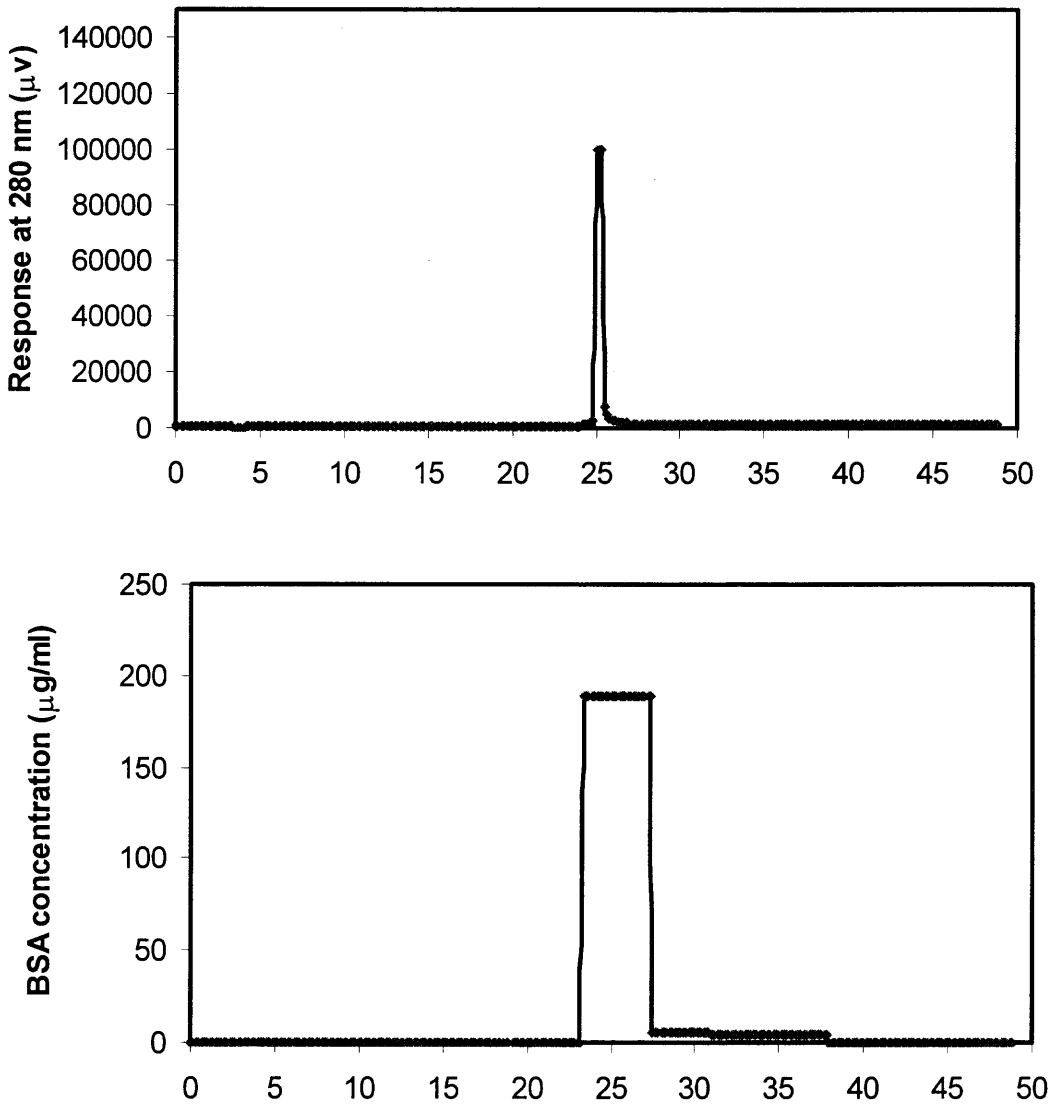


Figure 9.7B Protein elution profile of BSA-LPS mixture (Case 2.2.2.2). Up:chromatogram of UV response at 280 nm, recorded by the data acquisition system. Down:chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 78.87 μg/ml BSA and 7.08 EU/ml LPS at 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 28 ml buffer C_1 at 1 ml/min.

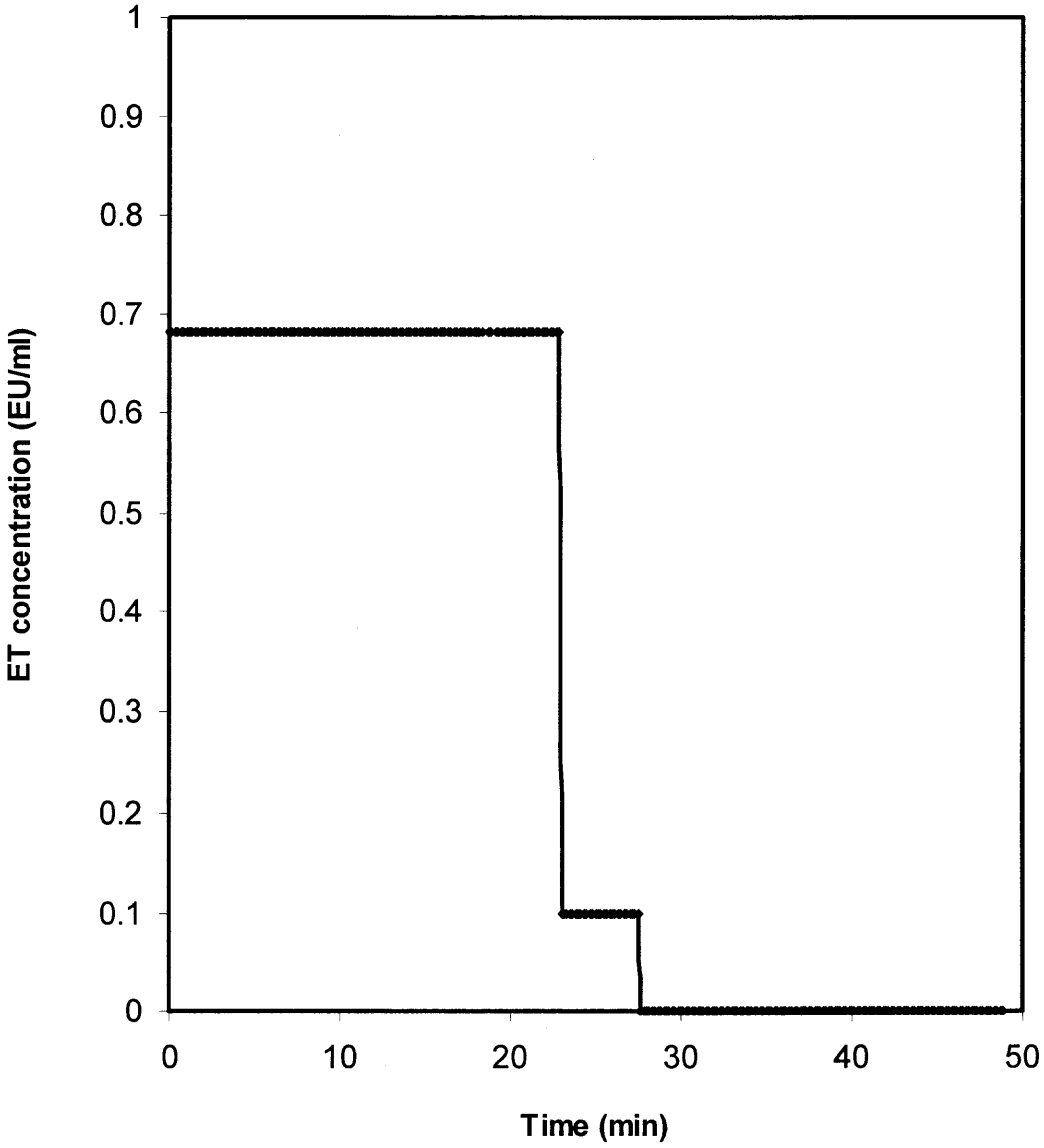


Figure 9.8A Endotoxin elution profile of BSA-LPS mixture (Case 2.2.4). Loading period: feed, 10 ml mixture containing 74.44 μg/ml BSA and 20.90 EU/ml LPS at 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 28ml buffer C_2 at 1 ml/min.

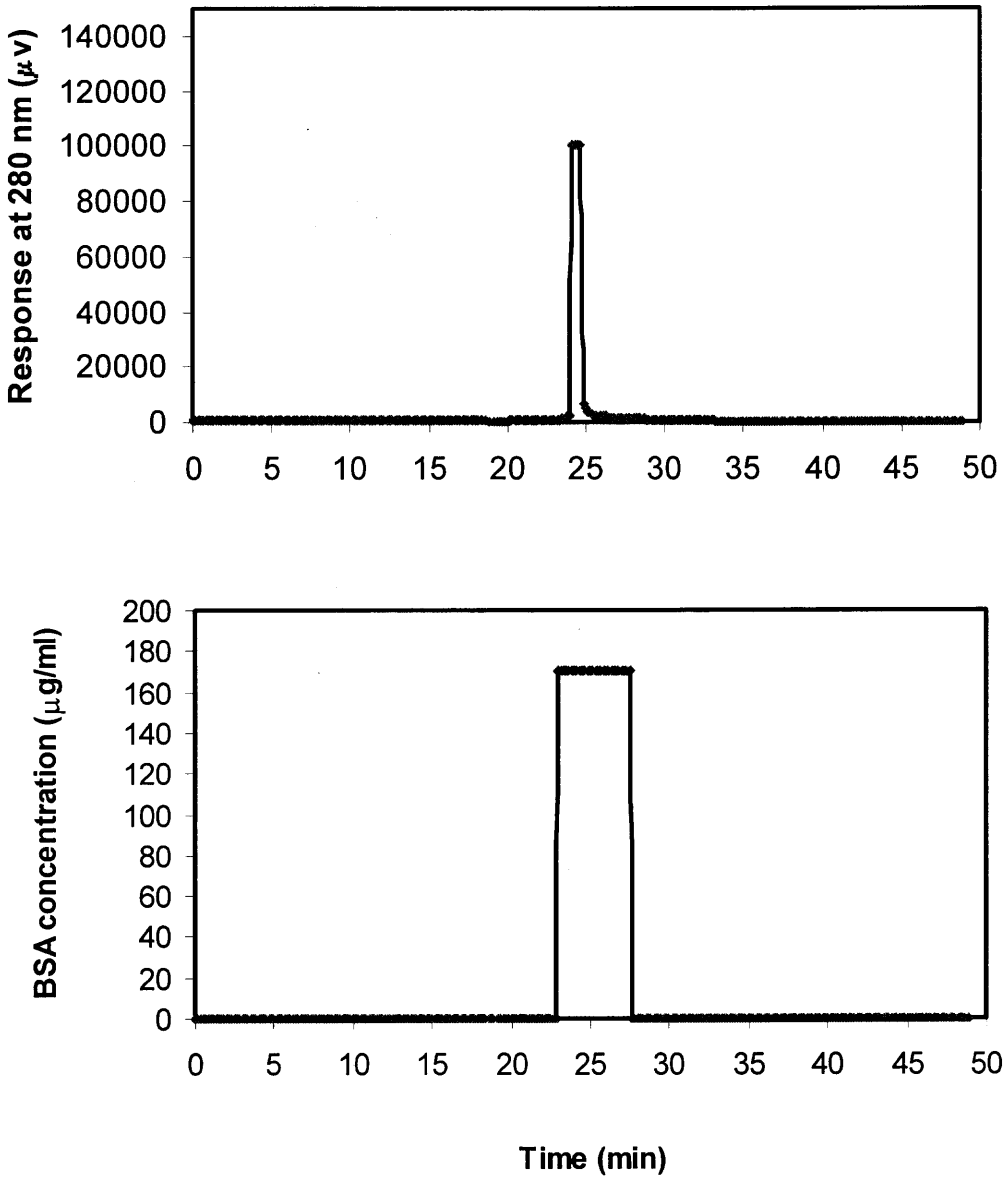


Figure 9.8B Protein elution profile of BSA-LPS mixture (Case 2.2.4). Up:chromatogram of UV response at 280 nm, recorded by the data acquisition system. Down: chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 74.44 μg/ml BSA and 20.90 EU/ml LPS at 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 28 ml buffer C_2 at 1 ml/min.

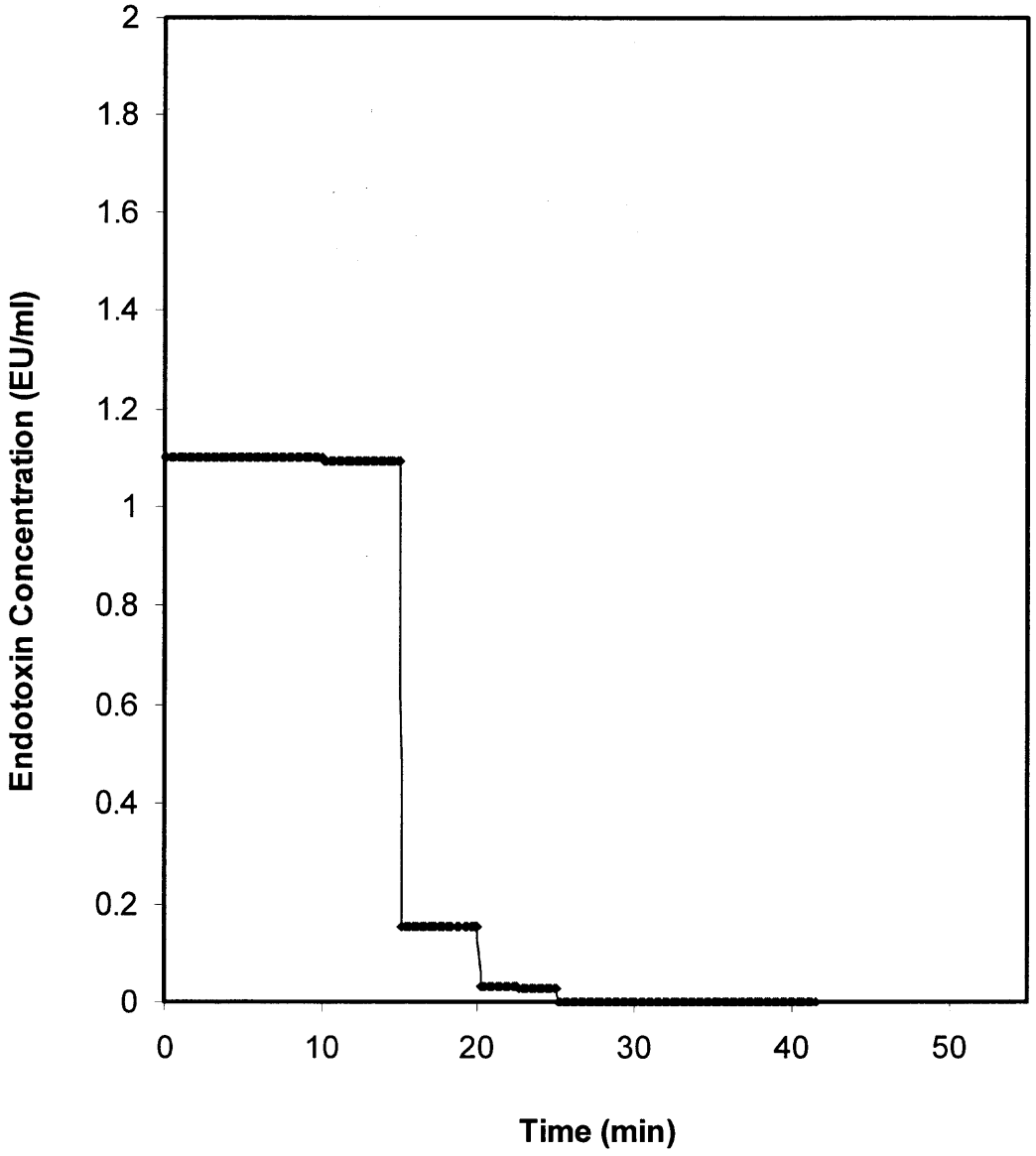


Figure 9.9A Endotoxin elution profile of IgG-LPS mixture from the IMAC column (Case 2.3). Loading period: feed, 10 ml mixture containing 93 μg/ml IgG and 31.42 EU/ml LPS; flow rate, 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 22 ml buffer C_2 at 1 ml/min.

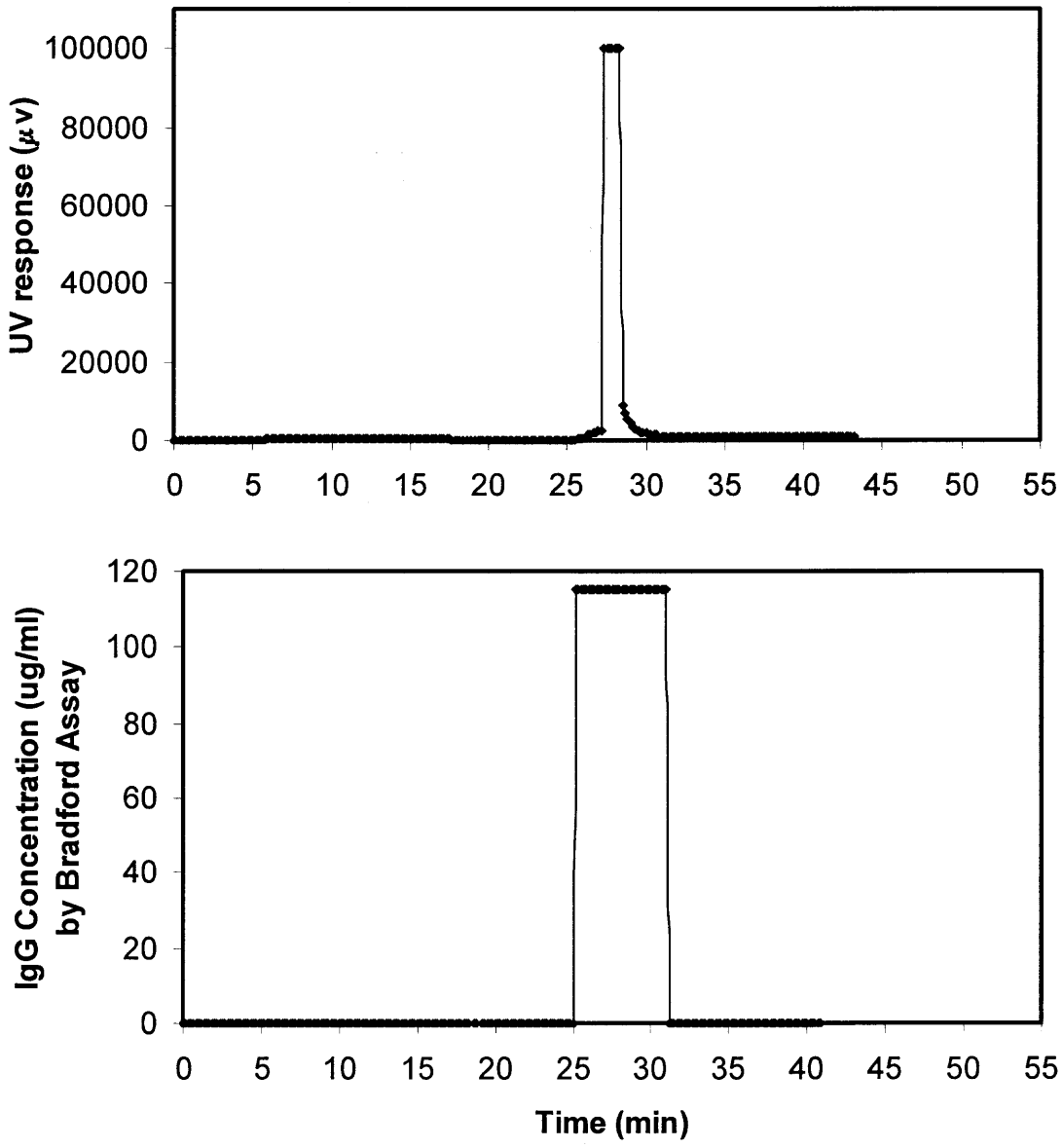


Figure 9.9B Protein elution profile of IgG-LPS mixture from the IMAC column. Up: chromatogram of UV response at 280 nm, recorded by the data acquisition system. Down: chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 75.1 μg/ml IgG and 31.42 EU/ml LPS; flow rate, 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 22 ml buffer C_2 at 1 min/ml.

9.3.4 Effect of Elution Buffer pH and Ionic Strength on the Protein Yield and Endotoxin Removal Efficiency

In this part of study, BSA-LPS mixtures prepared with buffer A were applied to the column as the feeds as shown in Table 9.3. Elution buffers with different pH (3.5, 5.0, 5.5 and 8.0) were investigated. The effect of elution buffer pH on the endotoxin removal efficiency and protein recovery was shown in Figure 9.10. As pH was increased from 3.5 to 5.5, LERE was nearly constant. As pH was further increased from pH 5.5 to 8.0, LERE decreased slightly from 99.8% to 97.3%. In the whole pH range, BSA recovery is more than 93%. Protein elution profiles were shown in Figure 9.11 (pH3.5, Case 2.2.3.1), Figure 9.12 (pH 5.0, Case 2.2.3.2), Figure 9.6B (pH5.5, Case 2.2.2.3) and Figure 9.13 (pH8.0, Case 2.2.2.4). At low pH range (pH3.5), high protein yield and endotoxin removal efficiency were obtained. Protein was eluted in a very narrow period (5 min) and its concentration is higher than corresponding concentration in the feed. At pH 8.0, ferric ions were detected in the eluents after main protein peak. This indicated that high pH could cause the leaching of ferric ions. In all experiments, the ionic strength in the starting buffer and elution buffer was 0.5 M or 1.0 M, which is very high. In all cases, endotoxin concentrations in the product fractions were very low and protein concentrations were high. The endotoxin removal efficiency from the protein solution is more than 99%, and protein yield is above 90%. The results indicate that the interactions between the adsorbed biomolecules, protein or LPS and the resin are typical for an IMAC column, which matches conclusions from Chapter 5 to Chapter 8.

9.3.5 Phosphate Buffer as both the Starting Buffer and the Elution Buffer

In order to optimize the process, experiments were carried out using buffer C_2 as both the

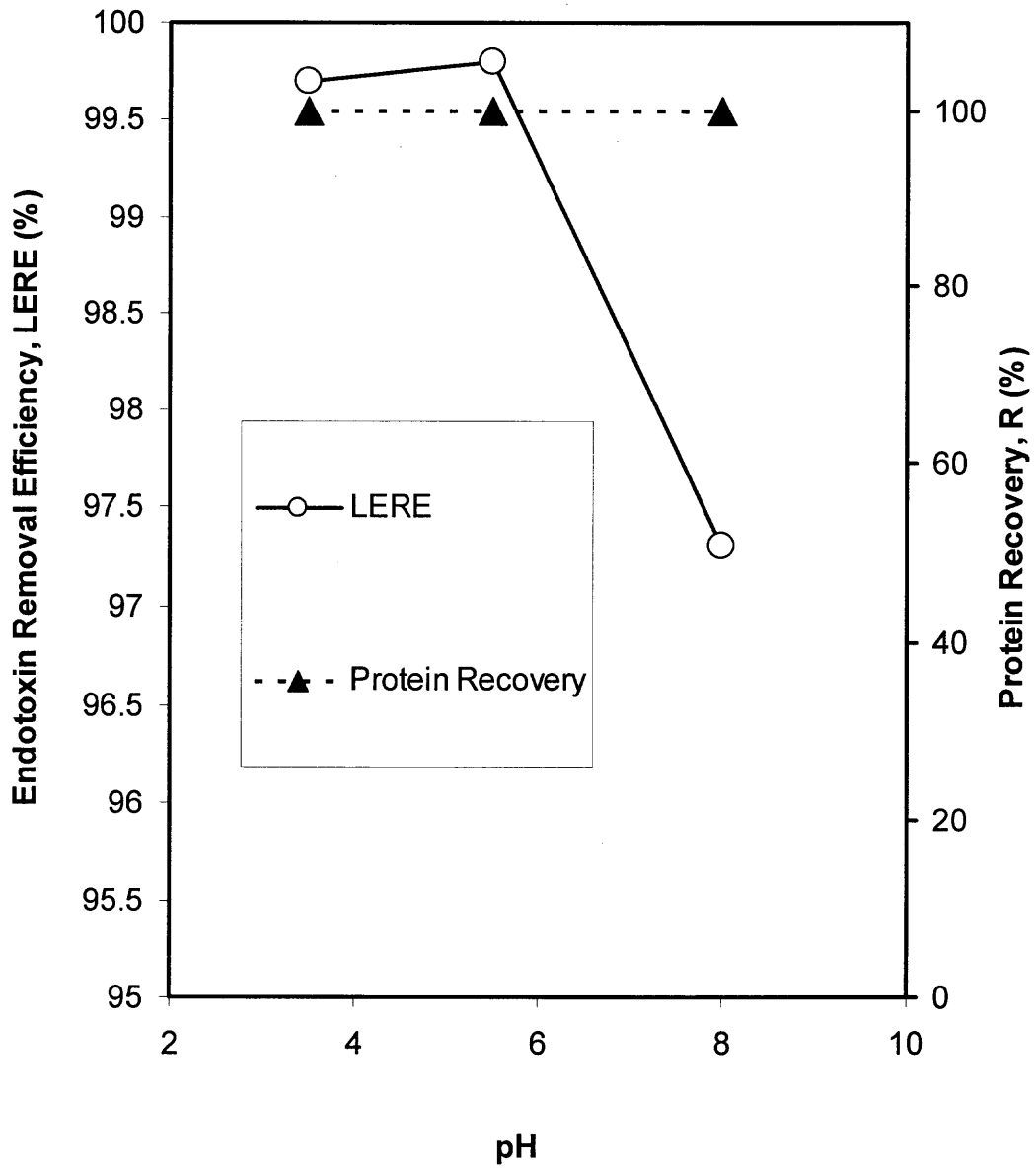


Figure 9.10 The effect of pH on the behavior of IMAC column.

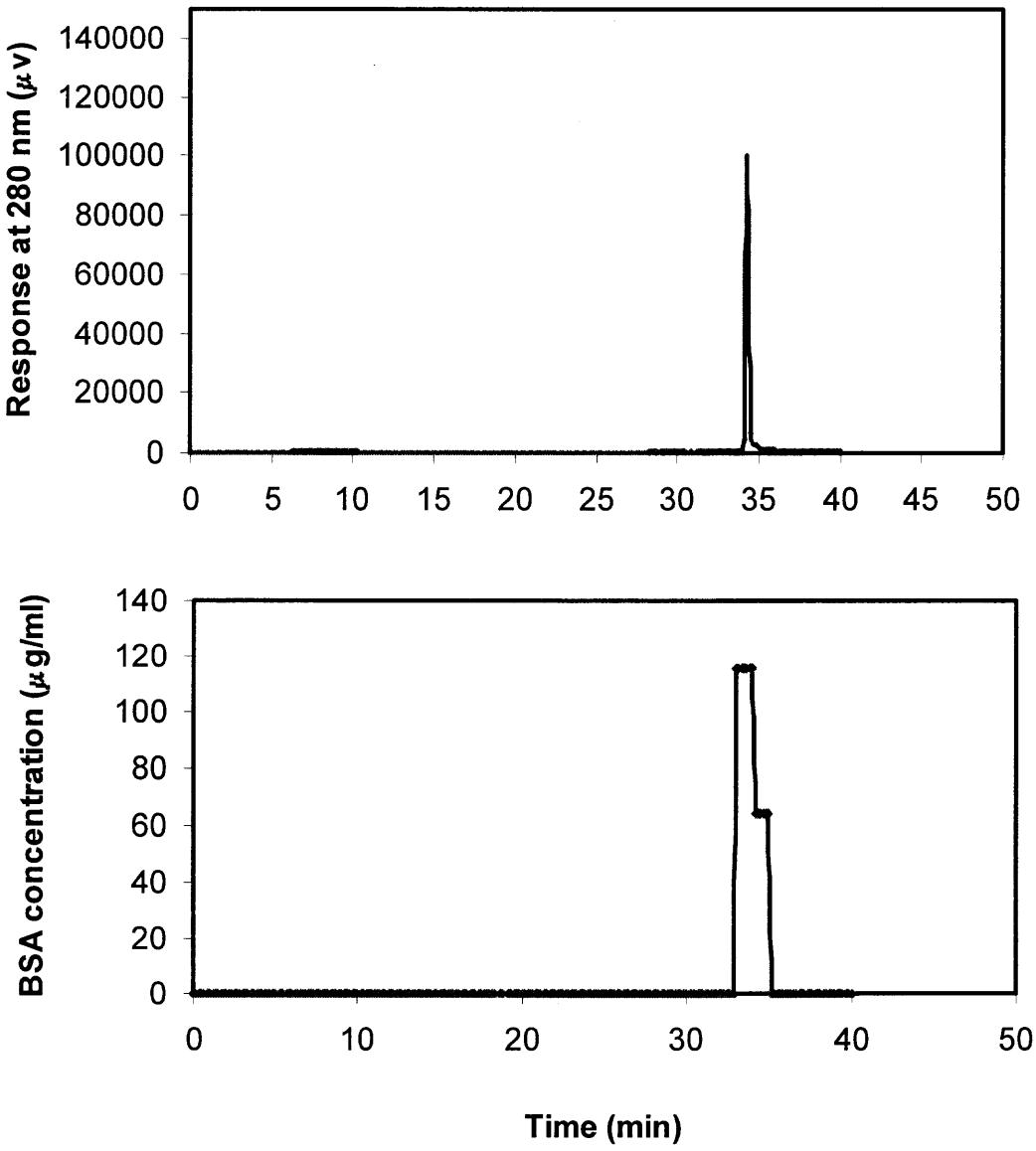


Figure 9.11 Protein elution profile of BSA-LPS mixture (Case 2.2.3.1). Up: chromatogram of UV response at 280 nm, recorded by the data acquisition system. Down: chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 19.23 μg/ml BSA and 41.23 EU/ml LPS at 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 10 ml buffer C_{02} at 1 ml/min. Elution period: 10 ml buffer C_1 at 1 min/ml.

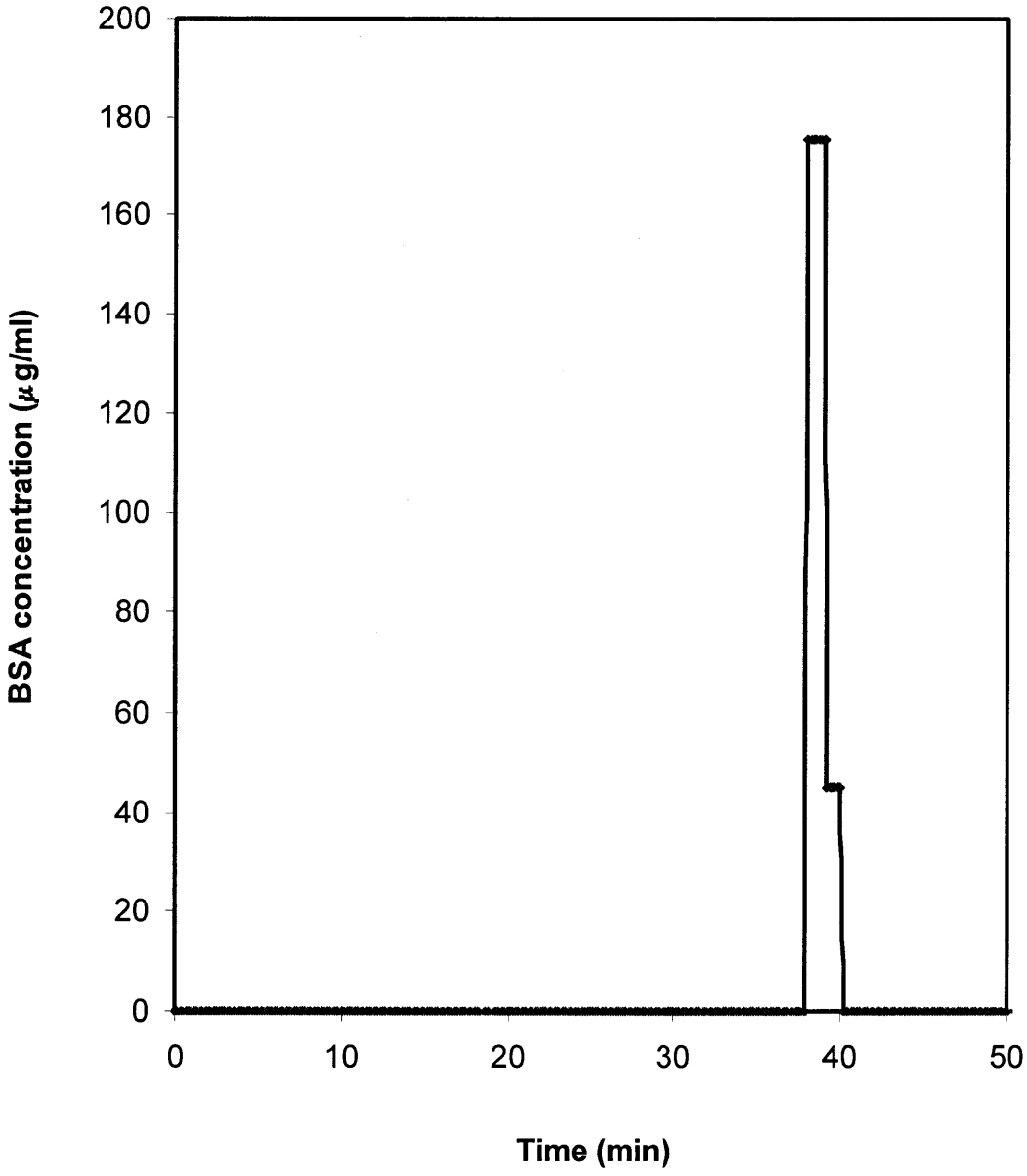


Figure 9.12 Protein elution profile of BSA-LPS mixture (Case 2.1.3.2). Chromatogram of Bradford assay results of fractions and fraction pools. Loading period: feed, 10 ml mixture containing 19.69 μg/ml BSA and 19.40 EU/ml LPS at 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 10 ml buffer C_{02} at 1 ml/min. Elution period: 20 ml buffer C_1 at 1 min/ml.

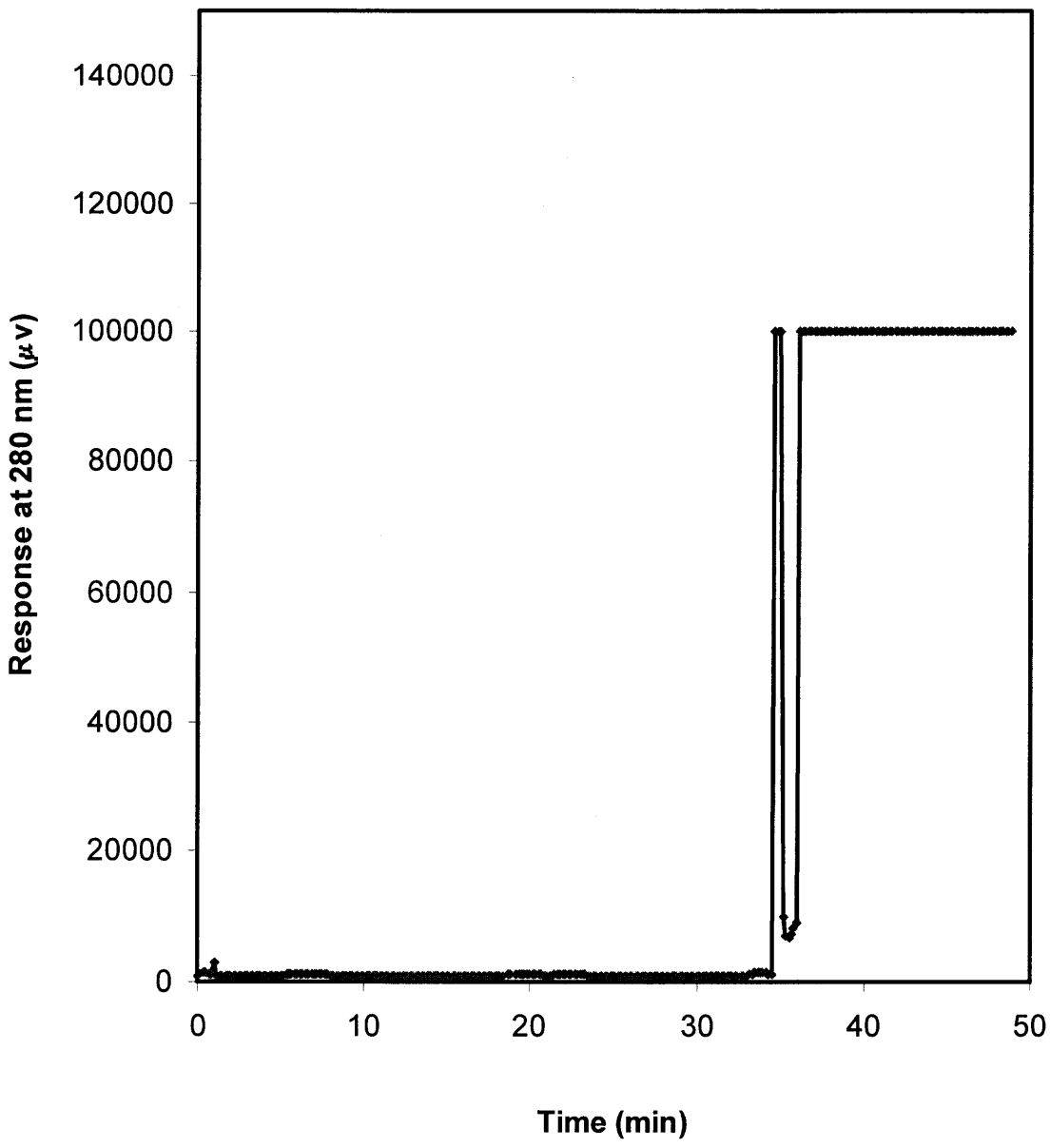


Figure 9.13 Chromatogram of BSA-LPS mixture eluted at pH 8.0 (Case 2.2.2.4). Loading period: feed, 10 ml mixture containing 83.33 μg/ml BSA and 19.16 EU/ml LPS at 1 ml/min. Washing period: 20 ml buffer A at 2 ml/min. Elution period: 10 ml buffer B at 1 ml/min. Elution period: 18 ml buffer C_1 at 1 min/ml.

starting buffer and the eluting buffer. However, this method was not successful. It is reasonable. As mentioned before, the phosphate ion in this process was used as a modulator. Phosphate ion competed with protein or endotoxin for adsorption sites on IMAC beads. Phosphate ion has higher affinity with ferric ion than protein and endotoxin. Therefore protein or endotoxin was not adsorbed to the column and washed out during loading and washing periods as detected by UV absorbance, Bradford assay and LAL test (not shown here).

9.4. Discussions

Using a novel method / process with an IMAC column, the removal of at least 99% endotoxin from HbAo, BSA and IgG solutions was achieved. During the elution process, the proteins were selectively eluted from the column by phosphate ions. The interaction mechanism between IDA-Fe^{3+} and LPS or protein was explained by authors (Kang and Luo). In this chapter different elution strategies were optimized the elution process.

NaCl was used to prevent possible ionic interactions between adsorbed molecules and resin. NH_4Cl was frequently used in IMAC as an elution reagent because NH_4^+ might form complex with metal ions and therefore elute adsorbed molecules from the column when it appears in the elution buffer. NH_4Cl is not suitable to elute the protein from IDA-Fe^{3+} column.

With the introduction of OBDG, the endotoxin-protein complex might be broken down. However, the linkage of Fe^{3+} and IDA is also broken, which causes the leaching of Fe^{3+}. This result is reasonable. As a surfactant, OBDG can reduce the affinity between ligand Fe^{3+}, and the support group IDA, which causes the leaching of Fe^{3+}. As a result,

the addition of OBDG to buffer B has no practical effect on the selective removal of endotoxin from the protein solutions in IMAC processes. New elution buffers should be investigated in order to elute the adsorbed proteins from the IDA-Fe^{3+} column.

The phosphate ions are very efficient to elute the adsorbed molecules from the IMAC column. This may be because the affinity of ferric to phosphate ion is much higher than to the protein or endotoxin. Therefore this elution can be described as displacement of adsorbed molecules with phosphate ions. Ionic strength and pH conditions were also investigated to get the optimal elution conditions. The protein yield and endotoxin removal efficiency were almost independent on the ionic strength and pH. This confirmed the adsorption mechanism put forward in previous chapters.

To obtain more efficient application of IDA-Ferric in removal of endotoxin from protein solutions, the following topics also need to be consulted from previous chapters. First, the ligand capacity and ferric amount immobilized in the column, which was determined in Chapter 6. Second, endotoxin and protein adsorption capacities to the IMAC column under different pH and ionic strength conditions were determined by frontal analysis in Chapters 6 and 8. Third, adsorption isotherms of proteins and endotoxins were determined in previous chapters.

9.5 Summary

Chromatography employing traditional affinity resins leads to significant protein loss and low protein yield in the process of endotoxin removal from protein solutions. At low solution ionic strength, the adsorption capacity of both endotoxin and protein are very high. As the ionic strength increases, the adsorption of the protein and endotoxin

decreases simultaneously. Adsorption mechanism of protein and endotoxin to traditional resins are ion exchange and affinity, mainly ion exchange.

IDA-Fe^{3+} ligand can selectively remove the endotoxin from the protein solution. Different washing and elution strategies for removing endotoxin from protein solutions in an IDA-Fe^{3+} IMAC column are reported in this chapter. Although NH_4Cl is frequently used in IMAC as an elution agent, it is not effective to elute protein from the IDA-Fe^{3+} column. OBDG can dissociate endotoxin-protein complex. However, it might cause the leaching of the ligand, Fe^{3+}, because it reduces the interactions between the ligand and support. Phosphate ion has been proved to be an efficient elution reagent for proteins. The IDA-Fe^{3+} IMAC column was exploited to efficiently remove endotoxin from the mixture of bovine serum albumin and endotoxin (BSA-LPS), and from the mixture of human hemoglobin Ao and endotoxin (HbAo-LPS) as well as from the mixture of Goat Immunoglobulin (IgG) and endotoxin (IgG-LPS). More than 95% protein recovery and above 99% endotoxin removal efficiency are found in author's newly developed process in the range of ionic strength = 0 ~ 1.0 M and pH=3.5 ~ 5.5.

The high ionic strength (0.5 M to 1.0 M) in mobile phase indicates that interaction mechanism between the adsorbed protein or LPS and IDA-Fe^{3+} is not electrostatic interaction. This IMAC behavior was explained by its adsorption mechanism mentioned in previous chapters.

CHAPTER 10

LEACHING STUDY OF IDA-Fe^{3+} IMAC COLUMN

10.1 Introduction

Leaching is an important issue for affinity chromatographic columns and should be investigated in the validation of chromatographic process. Low ligand leaching is required for an effective chromatographic column. For IDA-Fe^{3+} IMAC column, the ligand is ferric ion. Under some conditions ferric ion could be eluted and cause ligand leaching problem. The objective of this chapter is to investigate the leaching of ferric ion from IMAC column under different elution solution conditions and to get optimal operational conditions to prevent leaching in elution process. Blank runs were conducted. Operational conditions were obtained.

10.2 Experimental Methods

10.2.1 Materials

NaCl, NH_4Cl, $FeCl_3$, H_3PO_4, NaH_2PO_4, NaH_2PO_4, NaAc and EDTA were purchased from Sigma. All solution transfers were performed by endotoxin free devices. Sterile, disposable plastic ware was used at all times to prevent endotoxin contamination. All solutions were prepared with Milli-Q water generated by a water purification system (Millipore Corp., Bedford, MA). The endotoxin concentration in Milli-Q water was below 0.05 EU/ml based on author's test.

IMAC Column and Adsorbent Resin A 0.7 x 2.5 cm HiTrap chelating column was purchased from Amersham Pharmacia Biotech (NJ, USA). The column was packed with

Pharmacia chelating Sepharose High Performance resin consisting of highly cross-linked agarose beads to which iminodiacetic acid (IDA) was coupled by a stable spacer arm. **Buffer System.** Buffer A, start buffer is 0.5 M NaCl in 0.02 M acetate buffer at pH 5.5. The first elution buffer, buffer B, was 0.5 M NH_4Cl in 0.02 M acetate buffer at pH 5.5, which was used to remove the loosely bound metal ion in the column. The second elution buffer, buffer C, was 0.06 M sodium phosphate buffer (pH 3.5, 5.5, and 7.5), which was used to elution the protein products and in this experiment was used to test the metal ion leaching of the column. The stripping buffer, buffer D, was 0.05 M EDTA (ethylenediaminetetraacetic acid) in buffer A. All buffers were filtered through a 0.22 μm filter and degassed under vacuum for at least 40 min prior to being applied to the column.

10.2.2 Column Preparation and Operation

The HiTrap chelating column was stored in 20% ethanol when purchased. The column was first washed with 5 ml Milli-Q water at a flow rate of 1 ml/min to remove the ethanol. Then the column was loaded with 0.5 ml of 0.1 M $FeCl_3$ solution at 1 ml/min to charge Fe^{3+} to the resin. Finally, the column was washed with 5 ml Milli-Q water at 1 ml/min to remove the unbound Fe^{3+} from solution. The washed out Fe^{3+} solution was collected to determine the concentration of unbound ferric ion. The column was now charged with metal ion and ready for use. Before applying the feed, a blank run was performed to elute nonspecifically bound metal ions that might otherwise be eluted during desorption, the eluent was also collected. The column was washed with 2.5 ml of buffer A at 2 ml /min followed by 5ml of buffer B at 1 ml/min. The column was then

equilibrated with 2.5 ml of buffer A at 1 ml/min. The feed (start buffer solution) was applied to the column at 1 ml /min. This procedure was considered to be a blank run. The column was then washed with the starting buffers (buffer A) at 2 ml/min for 10min. Finally, the column was eluted by buffer C at 1 ml/min using step gradients, the eluant was collected for the determination of leached ferric ion. The column was then regenerated by washing with buffer D to strip all the metal ions, followed by washing with 5 ml of Milli-Q water, both at a flow rate of 1 ml/min.

10.2.3 Fe^{3+} Ion Determinations

The amount of immobilized Fe^{3+} was calculated as the difference between the total amount of Fe^{3+} applied to the column and the Fe^{3+} amount, which was washed out as the excess. The leaching of the column can be investigated by determination of the ferric ion concentration in all elution fractions. Total iron ion concentration was determined by the Sigma ferric kits (Sigma Chemical Company, St. Louis, MO, USA).

The procedure for total iron using Sigma kits was modified by author. The modified protocol is as follows.

1) Add 1.5 ml iron buffer reagent, catalog No. 565-1, to cuvets labeled Blank, Standard and Test.

2) Add 0.3 ml iron free water to Blank, 0.3 ml Iron, catalog No. 565-5, standard to Standard and , 0.3 ml sample to Test. Mix each cuvet thoroughly.

3) Read and record absorbance (A) of Test and Standard vs. Blank as reference at 560 nm. This is initial A.

4) To each cuvet add 0.03 ml (30 μl) iron color reagent, catalog No. 565-3. Mix thoroughly and place them at room temperature for 10 minutes.

5) Read and record absorbance (A) of Test and Standard vs. Blank as reference at 560nm. This is final A.

6) Calculations:

ΔA_{test}= Final A_{test} -Initial A_{test}

$\Delta A_{standard}$ = Final $A_{standard}$ -Initial $A_{standard}$

Total iron (μg/dL) = (ΔA_{test} / $\Delta A_{standard}$) x 500

Total iron (μmol/dL)= Total iron (μg/dL) x 0.175

10.3 Results and Discussion

The experimental results were tabulated in Table 10.1. The amount of ferric ion applied to the column for immobilization is 41.4 μmol. The extra unbound ferric ion was washed out with MilliQ water, the amount of which was listed in the first row. Then the ferric amount immobilized in the column was shown in the second row. The products were collected in certain volumes and corresponding ferric concentrations were determined. The ferric amount in the product was shown in the fifth row. The leaching of column is defined in the last row as the ratio of the amount of ferric in the product to that immobilized, which is expressed in percentage.

As shown in Table 10.1, the pH of elution solution has a great effect on the leaching of ferric ion from the column. At pH 3.5, no leaching was found in product collection. As pH of elution buffer increased to 5.5, 0.06% ferric ion was leached into the product. With the further increase of the pH of elution to 7.5, 0.7% of the ferric ion

was eluted out from the column. Considering the leaching effects, it is necessary to keep pH of the elution solution under 4.0 in the protein elution process from the IMAC column.

Table 10.1 The effect of elution buffer pH on the ferric ion leaching, ferric ion amount in the feed: 41.4 μmol.

pH	pH 3.5	pH 5.5	pH 7.5
Fe^{3+} washed out (μmol)	7.913	4.207	6.745
Fe^{3+} in the column (μmol)	33.490	37.193	34.650
Fe^{3+} conc. in the product (μmol /ml)	0	9.144×10^{-3}	48.634×10^{-3}
Product volume (ml)	5.0	2.8	5.0
Fe^{3+} in the product (μmol)	0	0.026	0. 243
Leaching (%)	0	0.06	0.7

10.4 Summary

The leaching experimental results were summarized in this chapter. As pH increased from 3.5 to 7.5, the ferric leaching percentage was increased from 0 to 0.7%. To avoid the leaching of ferric into the product, it is necessary to control the elution at pH < 4.0.

CHAPTER 11

FINAL CONCLUSIONS AND RECOMMENDATIONS

11.1 Conclusions

In the biotechnology industry, Gram negative bacteria are widely used to produce recombinant DNA products such as peptides and proteins. The removal of endotoxins from protein solutions has always been a challenge. The novel IMAC-Fe^{3+} method was developed to remove endotoxins from different protein solutions with a high capacity and high selectivity. More than 99% of endotoxin was removed. The protein yield is higher than 90%. Compared with traditional methods, it has the advantage of high efficiency, low cost and easy regeneration.

The endotoxin content detection method was developed considering the effects of different factors such as protein and buffer components on LAL test. Proper dilution is good way to remove the inhibition to endotoxin assay.

The adsorption mechanism of endotoxin on IDA-Fe^{3+} IMAC resin was determined by frontal analysis. The adsorption of endotoxin to the column includes two periods. First, endotoxin is adsorbed to Fe^{3+}, which is due to the interaction between Fe^{3+} and the phosphate group of lipid A in the endotoxin. Second, additional endotoxin is adsorbed on the already adsorbed endotoxin because of the strong interaction among endotoxin molecules. In the first step it is reasonable to assume Langmuir adsorption mechanism. The adsorption capacity is 1.43×10^4 EU/ml gel. In the second step the adsorption continues, and more endotoxin will be adsorbed. Therefore the adsorption capacity of endotoxin on IDA-Fe^{3+} IMAC column is very high.

It was found that endotoxin was bound mainly by interactions between the phosphate group in endotoxin and ligand Fe^{3+} on the beads. The interaction mechanism is believed to be the metal chelating. The adsorption of endotoxin to the IMAC-Fe^{3+} column may be also due to the hydrophobic property of the lipid A, and the net negative charge of the endotoxin. The properties of the endotoxin are important in discussing the mechanism of its adsorption on the IDA-Fe^{3+} IMAC beads. Interactions among the endotoxin molecules are very important in discussing the adsorption of the endotoxin to IMAC column. The interactions result in multi-layer adsorption of endotoxin on the beads. Steric effect should be considered in process development of endotoxin removal, since endotoxin aggregate is a big molecule.

In the loading period of endotoxin removal process, in order to get low endotoxin adsorption and higher protein adsorption, a higher pH buffer condition should be chosen. During elution period a buffer with a lower pH will be used to prevent the endotoxin from eluting from the column together with protein targets.

Frontal adsorption studies have been shown to be an effective means of characterization of the effects of buffer conditions on an BSA adsorption process as well as Fe^{3+}-BSA interactions. The Langmuir isotherm cannot satisfactorily predict the adsorption behaviors of BSA. The Freundlich isotherm model provides very good predictions of the BSA adsorption behavior in IMAC-Fe^{3+}. It was found that BSA was bound mainly by metal chelating interactions between BSA and ligand Fe^{3+}. The effects of buffer ionic strength and pH conditions on the BSA adsorption behaviors indicate that ion exchange mechanism might be involved in process.

To prevent possible ion exchange interactions, the higher ionic strength should be maintained. The adsorption of BSA on the IDA-Fe^{3+} IMAC beads was mainly due to histidine group in the BSA and the interaction mechanism was essentially metal chelating. Electrostatic forces might also be involved at the low solution ionic strength. In the loading period of endotoxin removal process, in order to get high BSA adsorption capacity, buffer with a very high pH should be avoided.

BSA is a typical model protein. Its adsorption on IMAC-Fe^{3+} is due to metal chelating interaction between BSA and Fe^{3+}. Since the IMAC bead pore size is very big, steric effect on BSA adsorption can be eliminated. Other proteins, such as HbAo (neutral protein) and IgG (alkaline protein) were known for strong endotoxin binding. It is very difficult to remove endotoxin from these protein solutions. All these proteins also have histidine group(s) on the surface, which could contribute the metal chelating interactions. This indicates that IDA-Fe^{3+} IMAC column can be used to purify HbAo and IgG and at same time remove endotoxins. IDA-Fe^{3+} IMAC method is quite different.

As shown in the comparison study, the traditional affinity chromatographic methods cannot remove the endotoxin from protein solutions. Adsorption mechanism of protein and endotoxin to traditional resins are ion exchange and affinity, mainly ion exchange.

The phosphate ions are very efficient to elute the adsorbed molecules from the column. The possible reason is that the affinity of ferric to phosphate ion is much higher than to the protein. Therefore this elution can be described as displacement of adsorbed molecules with phosphate ions. Ionic strength and pH conditions were also investigated

to get the optimal elution condition. The protein yield and endotoxin removal efficiency were almost independent on the ionic strength and pH.

For IMAC-Fe^{3+} column, the ligand is ferric ion. Under some conditions ferric ion could be eluted and cause ligand leaching problem. The investigation of the leaching of ferric ion from IMAC column at different elution conditions was conducted. As the pH increased from 3.5 to 7.5, the ferric leaching percentage was increased from 0 to 0.7%. To avoid the leaching of ferric into the product, it is necessary to control the elution pH < 4.0.

11.2 Recommendations

Endotoxin removal from protein solutions with IDA-Fe^{3+} IMAC column is a new and promising method. A few recommendations in this area are offered here for the future work.

Methods for endotoxin removal from protein solutions depend on the origin, structure and molecular weight of endotoxins, as well as the nature and molecular weight of proteins. The structure and toxic effects of endotoxins from the Gram positive bacteria and fungi should be investigated. The relationship between the biological activity and the size of endotoxin has to be elucidated first. The interaction mechanism between proteins and endotoxins need further investigation.

During this study, it was found that as ET concentration in the feed was higher than 500 μg/ml, the IMAC column was blocked. The possible reason is the precipitation of endotoxins on the IMAC bead surface. The solubility of endotoxin in the adsorption

on IMAC process should be further investigated. It might be helpful to use activity coefficient to discuss the endotoxin adsorption process with IMAC.

Other elution methods could be further investigated to prevent possible ferric leaching. According to the principles of IMAC, the histidine and imidazole are very good candidates as elution reagents.

The modeling of IMAC process is very complex. However, it is worth investigations. First, it is very important to identify the rate-limiting step. Second, the model can be established considering mass balance, diffusion in the pore, rate of mass transfer through the fluid film and equilibrium. Diffusion coefficients, axial dispersion coefficients can be obtained by studying the column response to pulse inputs.

The endotoxins from different origins could be tried. It is not a bad idea to include cell lysate from fermentation process as the feed to this developed IMAC column.

To demonstrate the advantages of Fe^{3+} as a chelating metal ion over other metal ions, such as IDA-Cu^{2+}, could be investigated.

Since the small scale of IDA-Fe^{3+} IMAC process for endotoxin removal from protein solutions is very successful, the next step is process development. Scale-up of IDA-Fe^{3+} IMAC process should be investigated to get it finally commercialized.

APPENDIX

ABBREVIATIONS

ABBREVIATIONS

ABS:	Absorbance
BSA:	Bovine serum albumin
DAH:	Diaminohexane
DMAPAA:	N, N-dimethylaminopropylarylamide
DOC:	Deoxycholate
ERE:	Endotoxin removal efficiency
ET:	Endotoxin
EU:	Endotoxin units
FDA:	Food and Drug Administration
HbAo:	Human hemoglobin A_0
Him:	Histamine
His:	Histidine
ICU:	Intensive care unit
IDA:	Iminodiacetic acid
IgG:	Immunoglobulin G
IMAC:	Immobilized metal affinity chromatography
LAL:	Limulus Amebocyte Lysate
LPS:	lipopolysaccharides
MOF:	Multi organ failure
NTA:	Nitriloacetic acid
OBDG:	N-octyl-β-D-glucopyranoside

ABBREVIATIONS
(Continued)

PAN:	Polyacrylonitrile
PEI:	Poly(ethyleneimine)
PEPA:	Polyester polymer alloy
PLL:	Poly-L-lysine
PMB:	Polymyxin B
PS:	Polysulfone
PVA:	Polyvinyl alcohol
SOD:	Superoxide dismutase
USP:	The United States Pharmacopoeia
TED:	Tris(carboxymethyl) ethylene diamine

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