DEVELOPMENT OF ECONOMICAL PRODUCTION AND PURIFICATION PROCESSES FOR THE MINI-ANTIBODY AGAINST PROTEIN C

By

Lino K. Korah
B.S., University of Maryland Baltimore County, 2000

A Thesis
Submitted to the Faculty of the
Graduate School of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science

Department of Chemical Engineering
University of Louisville
Louisville, Kentucky

December, 2002

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A Thesis Approved on

September17, 2002

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DEDICATION

This thesis is dedicated to my parents

Mr. Korah K. Kuruvilla and Mrs. Sosamma Korah

and to my sweet brother Libin Korah

and to my wonderful brother-in-law Mr. Rejy Thomas and sister Dr. Lijo K. Thomas

and to the guiding star of my life - Dr. Kyung A. Kang

ACKNOWLEDGEMENTS

I would like to thank Dr. Kyung A. Kang for her continued guidance, inspiration and patience through-out the course of research and writing the thesis. I am very grateful to Dr. Duane F. Bruley, for his advice and encouragement over the past three years. I am grateful to my committee members, Dr. Muriel Maurer, Dr. Greg E. Rutkowski, and Dr. P. A. S. Ralston for their insight and also for reviewing my thesis. I would also like to express my gratitude to the University of Louisville technical support group, Ms. Rodica McCoy and Mr. Steve Williams, for their immeasurable help during the course of this work.

I would also like to acknowledge American Red Cross for the supply of Protein C and Dr. Michael Sierks for the 13 recombinant *E. coli* colonies. I would also like to thank Sigma Chemical for their Ni-NTA sample and Southern Biotechnology Associates, Inc. for their anti-kappa and anti-lambda antibodies.

I would like to acknowledge my family and friends who have always encouraged me and made me stick with my research. A special thanks to my best friend Naznin Vahora for her encouragements for the past seven years.

ABSTRACT

Development of Economical Production and Purification Processes

for the Mini-Antibody Against Protein C

Lino K. Korah

December 19, 2002

Protein C (PC) is a potent anticoagulant, antithrombotic, and anti-inflammatory agent found in blood plasma. PC has been proven to be efficient in the treatment of PC deficient patients and sepsis patients, with fewer side effects than the currently available anticoagulants, coumarin or heparin.

The ultimate goal of this project is to purify PC from human blood plasma in an economical way. Since blood plasma contains several homologous proteins, some of them being coagulants, traditional purification methods other than immuno-affinity chromatography cannot be employed. In an attempt to lower the PC purification cost, single chain variable fragments (mini-Ab) is being produced by recombinant *E. coli*. This mini-Ab is to be used as a ligand for the chromatographic purification of PC instead of the regular monoclonal antibody (MAb).

Thirteen recombinant *E. coli* colonies that can produce the mini-Ab by phage display method were obtained from Dr. Michael Sierks (Arizona State University). Out of the 13 colonies, the colony producing the largest amount of PC specific mini-Ab was selected using an Enzyme Linked Immunosorbant Assay (ELISA). Using this colony,

optimization of the mini-Ab production processes and culture components were performed and the production level was increased from 30 to 300 μg/ml.

From the preliminary economic analysis, the mini-Ab production based on the production time and cost of media is approximately 470 times cheaper compared to the regular MAb production.

The purification efficiency of the mini-Ab was studied using Protein A and protein L. Protein A binds predominantly to the constant region of an antibody with 10% affinity to the mini-Ab region, while protein L binds predominantly to the kappa region of mini-Ab. Using protein A, mini-Ab purification yield of 18% was achieved with the optimized elution pH of 3. However, protein L was not able to purify this particular mini-Ab, probably due to the lack of kappa region in the mini-Ab. The use of metal affinity chromatography was also tested for higher yield. However, the metals were leached out during the adsorption at the pH 7.4, possibly due to the strong binding of histidine residue of the mini-Ab to the metals. Changing the pH from 7.4 to 6.4, with the purpose of reducing the binding strength between metals and the mini-Ab, increased the yield upto 27%. Using Ni-NTA column at pH 6.4, the yield was increased upto 40%.

The purification of PC was also studied using the mini-Ab immobilized to CNBr activated sepharose and agarose gels. Using pure PC sample, the CNBr activated sepharose showed 6% PC purification yield while Agarose showed 10%. Even with this low yield, PC can still be purified using the mini-Ab at a cost of at least 22 times cheaper than that of MAb. With further optimization in the mini-Ab purification process and a newer immobilization method for the mini-Ab, this process may prove to be an efficient and economical process for PC purification.

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CHAPTER I

INTRODUCTION

Blood in an animal body supplies oxygen and nutrients to cells in various organs and removes waste products from these cells. Additionally, it plays a key role in maintaining pH balance, and distributing heat, hormones, metabolites, and components of the immune system within the body. Blood also keeps a balance between coagulation and anticoagulation (hemostasis). The closed vascular system is important for proper blood flow. Under normal conditions, an injury to the vascular system results in the formation of a blood clot at the injured site to prevent the blood from leaking out. Blood has an inherent clotting potential and, if let uncontrolled, the clots can lead to life threatening vascular occlusions. Fortunately, an anticoagulant system can regulate and oppose the coagulant system. Within intact vessels, hemostasis favors anticoagulation and, in the event of vascular injury, coagulation is favored (Colman, et al., 1994). Deficiency in any component of the coagulant system may lead to hemophilia and deficiency in any component of anticoagulant system may lead to thrombophilia.

Several blood proteins are involved in maintaining hemostasis. One of these blood proteins is Protein C (PC), which acts as an anticoagulant, antithrombotic, and anti-inflammatory agent. PC belongs to the Vitamin K dependent (VKD) family, and for this family, Vitamin K (VK) is needed during its synthesis in the hepatocyte. Most VK

dependent proteins are coagulants (Factors VII, IX, X, and prothrombin) and PC and protein S (PS) are a few anticoagulating factors (Colman, et al., 1994).

PC deficiency can lead to blood clot formation in the vascular system. Sometimes, these blood clots detach and travel through the circulatory system, initiating more blood clots leading to pulmanory embolism, deep vein thrombosis, heart attack, stroke, etc.

Currently used anticoagulants for the treatment of PC deficiency are warfarin (coumarin) and heparin. Coumarin, a VK analog, works by blocking the production pathway for VKD blood coagulating factors (Hirsh, et al., 1998a). However, coumarin not only blocks the formation of coagulants but also VK dependent anticoagulant factors including PC. Another widely used anticoagulation therapy is heparin. It prevents the activation of Factor IX and inhibits the action of thrombin (Hirsh, et al., 1998b). Heparin can prevent clots from increasing in size by reducing platelet adhesiveness. However, it has not been proven if heparin has any effect on pre-existing clots (Sang, 1995).

Even though these anticoagulants are effective in reducing the mortality rate associated with thrombo-embolism, they have some disadvantages. The major problem with these anticoagulants is excessive bleeding (Levien, et al., 1998). An individual under cumarin therapy must avoid consumption of vegetables rich in VK, since VK can interfere with the cumarin action (Watson, et al., 1984; Hirsh, et al., 1998a). Coumarin is known to react with other prescription drugs (Hylek, et al., 1998). In addition, this anticoagulant can sometimes cause skin necrosis. If taken during pregnancy, it can cross the placenta causing embryopathy, and fetal bleeding (Hirsh, et al., 1998a). Coumarin therapy should be avoided for nursing moms, since it can transfer to the newborn through

breast milk (Ginsberg and Hirsh, 1998). Heparin has also significant side effects. Long-term heparin therapy can cause osteoporosis and skin necrosis (Hirsh, et al., 1998a; Ginsberg and Hirsh, 1998). In some cases, heparin can cause heparin induced thrombocytopenia (HIT), which is an immune mediated disorder characterized by the formation of antibodies against the heparin-platelet-Factor IV complex (Hirsh, et al., 1998b).

Hence, a better anticoagulant is needed and purified PC is potentially a highly valuable therapeutic choice (Minford, et al., 1996). In the blood plasma, PC is usually present in its inactive form (zymogen), and is activated only when needed. Therefore, it does not cause internal bleeding complications as heparin or coumarin does. Purified PC concentrates have been used for the prevention and treatment of thrombosis in individuals with inherited and acquired PC deficiency (Sang, 1995). Furthermore, activated PC (APC) has antithrombotic and anti-inflammatory properties (Sang, 1995). Recently, APC has also been proven highly effective for sepsis treatment (Krishnagopalan and Dellinger, 2001). Each year in the U.S, 700,000 new cases of sepsis are diagnosed and the mortality rate is about 30-50%. In the US, sepsis is the 11th leading cause of death. No other medicine has been found to be as effective as APC.

Currently, two PC based products are available in the market. In July 2001, Baxter International Inc. obtained approval from European Medicines Evaluation Agency (EMEA) to market Ceprotin™, protein C concentrate, for people with PC deficiency (Baxter, 2001). In November 2001, Eli Lilly Co. obtained a US Food and Drug Administration (FDA) approval to use Xigris™, a recombinant form of APC, to treat sepsis patients (Eli Lilly, 2001).

Scope of the work

PC is an ideal therapeutic agent for PC deficient, sepsis patients and patients who are going through major traumas (surgeries, accidents, etc.). Assuming the heterozygous PC deficient patients have only 30% of the normal level of PC, approximately 0.0073 kg of PC is needed for a patient per year (Wu, 2000). Then, approximately 120 kg of PC is needed per year for the heterozygous patients only. The American Red Cross estimated the required PC by the patients undergoing major surgeries as 18 kg per year. For homozygous patients, approximately 10 kg of PC is needed. For all three, about 150 kg of PC per year is needed. In summary, having an inexpensive supply of PC is important for PC deficient patients, sepsis patients, and patients with traumas. Current production rate of PC from plasma using MAb is only about 12 kg (Wu, 2000) and is an expensive process due to the high cost of MAb. Therefore, the global scope of this research is to develop economical processes for the purification of PC using a cheaper form of MAb alternative, the PC-mini-Ab.

This thesis consists of the following contents. *Background* includes PC function, PC deficiency and its related diseases, the advantages and disadvantages of different PC purification methods, and the recombinant single chain fragment development and its applications. In *Materials, Methods and Instruments*, materials and experimental procedures used for the mini-Ab production and purification processes are described. Also, the instruments needed for this research are described. In the *Results and Discussion*, the research results for the PC purification are described and discussed. In the *Conclusions*, a summary of the research and concluding remarks are given. In the *Future Work*, suggested study items for the future researchers are listed.

CHAPTER II

BACKGROUND

A. Protein C and PC Related Diseases

Human PC is a glycoprotein with a molecular weight (MW) of 62,000 Dalton (Da). It is composed of a heavy chain (MW = 41,000 Da) and a light chain (MW = 21,000 Da) held together by a disulphide bond (Colman, et al., 1994).

PC circulates in the blood plasma in an inactive form. It is converted to activated Protein C (APC) by the thrombin-thrombomodulin complex on the endothelial cell surface, in the presence of calcium and Protein S. Thrombomodulin is a protein present on the endothelial cell surface, and thus localizing PC activation to the vascular endothelial surface (Sang, 1995). APC acts as an anticoagulant by inactivating two activated coagulants Factor V (FV) and Factor VIII (FVIII) (Colman, et al., 1994). Inactivation of these two factors will prevent the conversion of prothrombin to thrombin and the activation of Factor X (Stenflo, 1988). APC initiates antithrombotic activity by binding to tissue plasminogen activation inhibitor (PAI). Under normal physiological conditions PAI is bound to tissue plasminogen activator (tPA) inhibiting its fibrinolytic activity. By binding to PAI, APC leads to free tPA. The free tPA then converts plasminogen to plasmin. Plasmin then degrades the formed blood clot (De Fouw, et al., 1988). Additionally, APC has shown to have an anti-inflammatory function (Esmon, 2000; Joyce and Grinnell, 2002). Inflammation can promote coagulation, inhibit

anticoagulant responses, and diminish fibrinolysis (breaking down of fibrin). Studies have indicated that APC protects animals from death and organ failure when challenged with a lethal dose of *E. coli* (Taylor, et al., 1987). Some of the important properties of PC is given in Table 1 (Haemtech, 2002).

Table 1

Properties of Protein C

Location	Blood plasma
Concentration in plasma	4-5 μg/ml
Mode of action	Zymogen; precursor to the serine protease activated PC (APC)
Molecular weight	62,000
Extinction coefficient	$E^{1\%}_{1cm,280nm}$ =14.5
Isoelectric point	4.4-4.8

Normal concentration of PC in human blood is 4~5 μg/ml. Less than 40-60 % of the normal PC level can lead to several thrombo-embolic complications (Colman, et al., 1994). PC deficiency can lead to blood clot formation in blood vessels. These blood clots, sometimes, detach from its original site and move to various parts of the body, including vitals organs like lung, heart, brain, etc. Blood clots lodged in these organs prevent oxygen and nutrition from being transported to the organs and can eventually lead to fatal conditions, such as pulmonary embolism, heart attack, or stroke. PC deficiency has been also traced as the leading cause of recurrent deep vein thrombosis

(Reader, 1998). Protein C deficiency in the general population is estimated to be between 0.2 and 0.4% (Folsom et al., 2002).

PC deficiency can be inherited or acquired. Hereditary PC deficiency is divided into the homozygous and the heterozygous. Homozygous PC deficiency is a rare genetic disease with fatal thrombolytic complications from birth. Newborns with homozygous PC deficiency suffer from *purpura fulminas* (Kavehmanesh, et al., 2000). *Purpura fulminas* is characterized by spreading skin lesions with underlying thrombi in the blood circulation. These clots can mostly disseminate causing intravascular coagulation, which can be often fatal without immediate treatment. Usually, a homozygous PC deficient patient has less than 25% of the normal level of PC in their blood plasma. Homozygous deficiency occurs in 1 of 400,000 newborn babies (Sang, 1995). Heterozygous PC deficiency can be categorized by less than 60% of the normal PC level and may be associated with recurrent thrombo-embolic episodes (Markis, et al., 1997). Occurrence of heterozygous PC deficiency is approximately 1 in 300 people (Reader, 1998). Acquired PC deficiency can occur due to the liver diseases, since PC is produced in liver (Walker, 1990). Acquired PC deficiency can also occur due to VK deficiency, acute thrombosis, pregnancy, administration of coumarin, or estrogen-oral contraceptives (Center for Transgene Research, 2002). It is also frequently found in meningococcemia and in bone marrow transplant patients and may be the probable cause of thrombosis seen in these patients (Sang, 1995). Purified PC concentrates have been used for the prevention and treatment of thrombosis in the individuals with inherited and acquired PC deficiencies (Minford, et al., 1996). Harper, et al., (1988) reported that in some cases,

heparin did not prevent portal thrombosis in liver transplanted patients and PC replacement therapy would be the best treatment for these patients.

B. PC sources and PC Purification

1. PC Source

PC is currently purified from human blood plasma (Baxter, 2002). It can also be obtained from recombinant mammalian cell culture systems (Lubon and Paleyanda, 1997).

Since the plasma fractionation process was developed by Cohn in 1940s, it has become a vital industry. Approximately nine million liters of blood plasma are collected each year in United States and processed for various plasma proteins (Bruley and Drohan, 1990). One of the plasma-fractionating products, Cohn fraction IV-1 paste, is an offline discard stream that contains more than 90 percent of total PC in plasma (Bruley and Drohan, 1990). Hence, it may be an ideal, more cost effective source for PC than blood plasma.

A variety of mammalian cells have been studied for the production of recombinant PC. However, due to the complexity involved in the accurate post translational modifications involved in PC molecules and the complicated production process, its potential use is limited (Bruley and Drohan, 1990; Freshney, 2000). Fully functional PC has been produced from a kidney cell line (293 cells) by Eli Lilly Co. of Indianapolis, Indiana.

Research has also been performed to produce PC in the milk of transgenic animals. Two different genomic pig lines have expressed 0.3 and 1-2 g/l of active PC in its milk (Van cott, et al., 1996).

2. PC Purification

For this research, PC from human blood plasma is chosen as the primary source of PC due to its easy availability. Since it is a natural resource, it has less immunogenic reactions and the best acceptance by clinicians and users. Nonetheless, the purification technique that will be described developed by this author may also be applied to other sources of PC.

Blood plasma contains several proteins homologous to PC and most of these are coagulants. The concentration of PC in human blood is only 4 μg/ml, and as a pharmaceutical product, the final product should have a very high purity. Therefore, a highly specific chromatography, immuno-affinity chromatography, would be the ideal choice over the other traditional protein purification methods. The purification of PC from plasma or Cohn fraction IV, economically, is an on-going research project for many researchers. Purification of PC from plasma using an affinity chromatography using Concanavalin A is currently under research (Perez-Compos, et al., 1996). However, the yield of PC purification was not reported. Another purification method is using an immobilized metal ion affinity chromatography (IMAC) column from Cohn Fraction IV (Wu, et al, 1998b; 1999; Wu, 2000). This is based on the fact that PC contains 12 surface accessible histidines that can play a role in metal binding. However, this process requires ion-exchange chromatography and prothrombin was not completely removed using

IMAC (Bruley, 2002). Prothombin is a homologous protein to PC and has opposite biological functions. Neither IMAC nor concavalian A is not yet used in the industrial scale to make a licensed product.

Current commercial PC purification methods use monoclonal antibodies (MAb) produced by animal cells. This process is expensive due to the slow doubling time of animal cells, high cost of the culture media, and maintenance of a highly sterile environment for the animal cell culture (Biopharmaceutical Production, 2001; Freshney, 2000). Hence, a new approach is to use the single chain variable region of an antibody molecule (mini-antibodies), which is the smallest fragment with a complete antigen binding function (Worn and Pluckthun, 2001).

C. Antibody and Antibody Purification

1. Antibody

In response to a foreign high molecular weight molecule (antigen), the body produces antibodies highly specific to that particular antigen. The basic unit of antibodies or immunoglobulins (Ig) consists of four-protein chains in the shape of the capital letter "Y" (Figure 1) and linked by disulphide bonds. There are two pairs of chains: heavy and light chains. The light chain consists of either a kappa or lambda region. Heavy chains have five different types, which divide Ig's into five different classes: IgG, IgM, IgA, IgD, and IgE. Each class of heavy chain can combine with either of light chain. The only exceptions are for camels and llamas and their antibodies sometimes, lack light chains (Saldanha, 2000). The smaller (light) polypeptide chain has

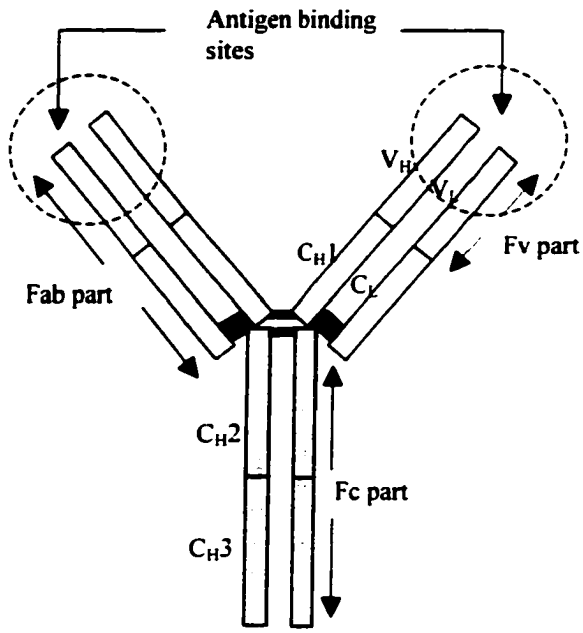

Figure 1. The basic structure of an antibody. There are three heavy chain constant domains (C_H1, C_H2 C_H3) and one variable constant domain (V_H). The light chain domain consists of a variable domain and a constant domain. The variable region (heavy and light chain) forms the specific antigen-binding site (modified from Roitt, et al., 1985).

a molecular weight of 25 KDa while the larger (heavy) polypeptide chain has a molecular weight varying from 50 to 77 KDa. These polypeptide chains are connected together by covalent and non-covalent forces to give four-chain structure based pairs of identical heavy and light chains (Roitt, et al., 1985).

The most common immunoglobulins class is IgG. The light chain has two domains (a variable domain and a constant domain) and the heavy chain has four domains (one variable domain and three constant domains). The tip of the arms of "Y" shape on both the heavy and light chain is called the variable domains (VL and VH). The other domains are called constant domains (CL, CH1, CH2, CH3) (Saldanha, 2000).

With the extent of knowledge and technology available, antibody engineering can join separate segments of heavy and light chains of the variable domains. These domains are linked by a peptide to form a single chain variable fragment (ScFv) with the ability to bind highly specifically to a particular antigen.

Using phage display method (Griffiths, et al., 1998; Cwirla, et al. 1990), a single chain variable fragment (ScFv; mini-Ab) with high specificity to PC was obtained (Figure 2; Wu, et al., 1998a). They can be produced by recombinant *E. coli* as a soluble protein. First, *E. coli* cells are grown in the growth media, where they multiply. Then, the cells are transferred to the production media to stop the growth of the cells. In the presence of inducer, the phageparticles in the *E. coli* produce the mini-Ab as a soluble protein. This mini-Ab consists of a heavy chain (V_H) and a light chain (V_L) of the variable region of the antibody, connected by a linker (Figure 2).

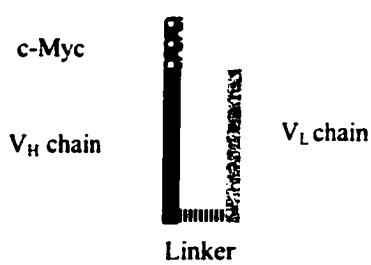

Figure 2. Schematic diagram of a single chain variable fragment (ScFv; mini-Ab).

The molecular weight of the mini-Ab was found to be around 29 kDa (Wu, et al., 1998a). As a part of their structure, the mini-Ab also contains a c-myc tag. The c-myc tag can be used for identification of the protein in ELISA and/or for the purification of protein, using an anti-c-myc antibody. Advantages of mini-Abs using *E. coli* over regular

monoclonal antibodies include less contamination problems during the production stage, less shear sensitivity of the host microorganism, less expensive culture medium, much higher production rate, and easier scaling of the process (Freshney, 2000).

This mini-Ab can also be used for the purification of PC from the transgenic milk (Degener, et al., 1998; Pollock et al., 1999) and also in PC immuno-biosensor (Piervincenzi, et al., 1998; Spiker and Kang, 1999).

2. Antibody Production and Purification

Antibodies have been used for the passive immunization against anticipated viral and bacterial infections (e.g., anthrax, diptheria, small pox etc.). Recently, antibodies have also been used as therapeutic agents, such as anti-tumor antibodies to deliver diagnostics (Huennekens, 1994) or even toxic isotopes *in vivo* (Sanchez, et al., 1999). Furthermore, antibodies are important tools for purifying antigens used in research and in disease treatment. Antibodies can be obtained from animal cell culture or ascites fluid and they can be purified in numerous ways. Conventional techniques, such as precipitation (with ammonium sulfate and/or caprylic acid), ion exchange chromatography (for example, DEAE), and gel filtration chromatography, have been widely used, in the past. However, today, the most commonly used purification method is affinity chromatography (Wilkinson, 2000). Affinity chromatography uses the specific interactions of an agent (ligand) highly specific only for the biological protein of interest (usually >95% of the pure product). The most commonly used ligands in IgG purification are protein A or G. Other ligands include protein L, peptidomimetic ligands,

anti-immunoglobulins, mannan binding proteins, and the relevant antigen (Wilkinson, 2000).

Protein A and G: Protein A and Protein G are bacterial cell wall proteins and both predominantly bind to the Fc region of IgG. Protein A (MW = 43 kDa) can be obtained from *Staphylococcus aures* and protein G, from *Streptococcus spp.* (Huse, 2002). Protein A contains five similar domains, designated as E, D, A, B and C. Each region is capable of binding to the Fc region of the immunoglobulins from different mammals. However, steric hindrance may prevent the binding of more than one or two IgG molecules to the immobilized protein A (Solomon, et al., 1992). Protein A also has approximately 10 % affinity to the V_H domain of ScFv (Akerstrom, et al., 1994; Wilkinson, 2000).

Protein A affinity chromatography is probably the most common method for antibody purification (Huse, et al., 2002). The reasons are: it is well characterized; can be obtained in large quantities from recombinant bacteria; is stable over the pH range of 2-11; it refolds after the treatment with denaturing solutions with urea or guanidinium salts; one can clean the purification system using highly basic (0.5M NaOH) solution with minor decrease in functional capacity; the leakage of protein A from the column is negligible and, therefore, it allows several cycles of binding and eluting antibodies increasing operational efficiency. Wu, et al., (1998a) has used protein A for the purification of the mini-Ab against PC. Therefore, in this study protein A was tested as a ligand for the PC mini-Ab purification.

Protein G has binding sites not only for antibodies but also for albumin and 2-macroglobulin, which can contaminate the antibody product. Currently, however,

genetically modified protein G is available with these additional binding sites removed. In general, protein G does not bind to ScFv (Akerstrom, et al., 1994)

Protein L: Protein L (MW= 92kDa) is a cell wall protein expressed by some strains of the anaerobic bacterial species of *Peptostreptococcus magnus*. Protein L binds to antibody light chains. More specifically, it binds to the framework kappa (κ) region of V_L domain, having a broader IgG binding activity than protein A (Nilson, et al., 1993; Wikstrom, et al., 1996).

Anti-c-myc MAb 9E10: Laroche-Traineau, et al., (2000) reported the use of 9E10 as a ligand for the purification ScFv. The yield using 9E10 affinity column was only 12%. Since 9E10 itself is an expensive ligand and the yield is low, its use as a ligand for the mini-Ab purification was not recommended. However, this can be used for Enzyme Linked Immunosorbent Assay (ELISA) of mini-Ab because not many antibodies are available for mini-Ab.

Immobilized metal ion affinity chromatography (IMAC): IMAC was introduced in 1975 by Porath and coworkers (Porath, et al., 1975) under the name of Metal Chelate Affinity Chromatography. They describe the use of nickel and copper metal ions for the fractionation of proteins from human serum. Since then, IMAC has been studied as a tool for the purification of target proteins and peptides in both analytical and large scales (Chaga, 2001; Luo, et al., 2001). Currently, IMAC is one of the most popular methods for the purification of recombinant proteins (Chaga, 2001).

IMAC utilizes the differential affinity of proteins for immobilized metal ions (Figure 3). The affinity of proteins to metal ions is due to the functional amino acids located on the surface of the proteins such as imidazole group of histidine, thiol group of

cysteine, and indolyl group of tryptophan. Since the affinity between the amino acid side chains of proteins and metal ions is reversible, it can be used for the protein adsorption and then for the elution under mild conditions. IMAC has several advantages over other affinity chromatography: different metals can be immobilized on the same chelating media after the metal ions are stripped from the gel matrix using EDTA; can reuse IMAC hundreds of times without any loss of metal chelating properties; various chelators [eg., iminodiacetic acid (IDA), tris(carobxymethyl) ethylenediamine (TED), nitrolotriacetic acid (NTA), etc.] can be used to immobilize metal ions.

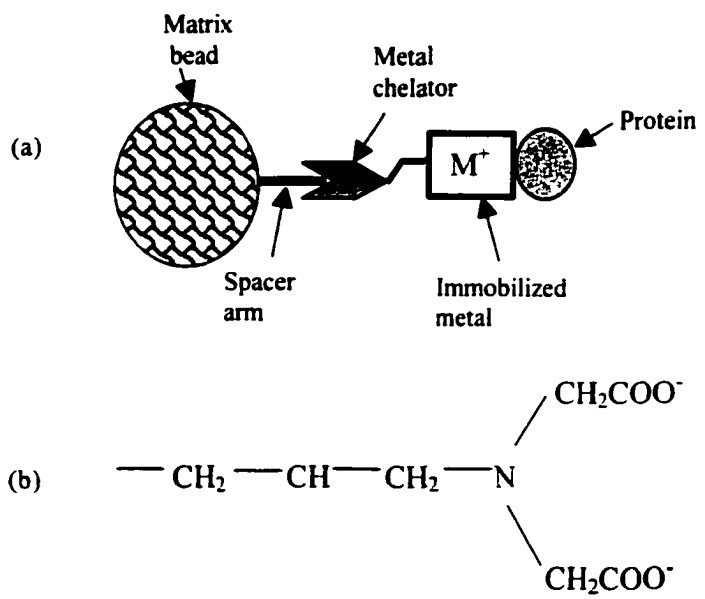

Figure 3. (a) A schematic diagram of the basic principle of IMAC. A certain type of protein can be retained on the column via the formation of coordinate bonds with the metal ion. (b) The structure of the most commonly used metal chelator, iminodiacetic acid (IDA).

Several immobilized metal ions can be used for the purification of biomolecules. Based on the preferential reactivity towards nucleophiles, metal ions can be divided into three categories - hard, intermediates and soft. The group of hard metal contains Fe^{3+}, Ca^{2+}, and Al^{3+} and they show binding preference to oxygen. Soft metal ion group includes Cu^{2+}, Hg^{2+}, and Ag^{2+} and they prefer sulfur. Intermediate metal ions are Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} and they coordinate nitrogen, oxygen, and sulfur. Histidine residues are the major targets for intermediate metal ions. Studies were also done on the type of gel matrices (silica or agarose) for the immobilization of the chelating group and were found that gel matrix only played a little role on the selectivity or capacity of adsorbents (Chaga, 2001).

The mini-Ab may contain histidine residue on its surface and hence the feasibility of using metal affinity column for the purification of mini-Ab was tested. Intermediate metal ions were tested, since it has been reported to successfully purify ScFv's. The metals used for the purification of ScFv include Sepharose-IDA-Cu (Sanchez, et al., 1999; Laroche-traineau, et al., 2000), Ni sepharose column (Kipriyanov, et al., 1997), Ni-NTA-agarose (Tan, et al., 1998; Goel, et al., 2000; Vancan, et al., 2002), Zn-IDA-sepharose (Kramer, et al., 2002), Co charged sepharose (Chaga, et al., 1999).

Immuno Affinity Chromatography for PC: For immuno affinity chromatography a ligand (e.g., MAb, mini-Ab) is immobilized on the gel matrix and acts as a stationary phase. The immobilized ligand reacts with the protein of interest via the antigen-antibody reaction. The protein of interest (e.g., PC) in the source material will bind to the ligand specifically and all other unbound proteins are washed off during the washing

stage. The elution step is done by changing condition of the gel matrix and the protein of interest can be eluted from the column, giving highly pure protein.

D. Project Flow Chart

An over view of the research project is shown in Figure 4. The *E. coli* cells are first cultured and once an optical density of 0.9 is reached, the cells are transferred to the induction media where mini-Ab is secreted to the culture media. The cells are then removed from the culture media and the supernatant is used for the purification of the mini-Ab. The purified mini-Ab was immobilized to gel matrices and then used for the PC purification.

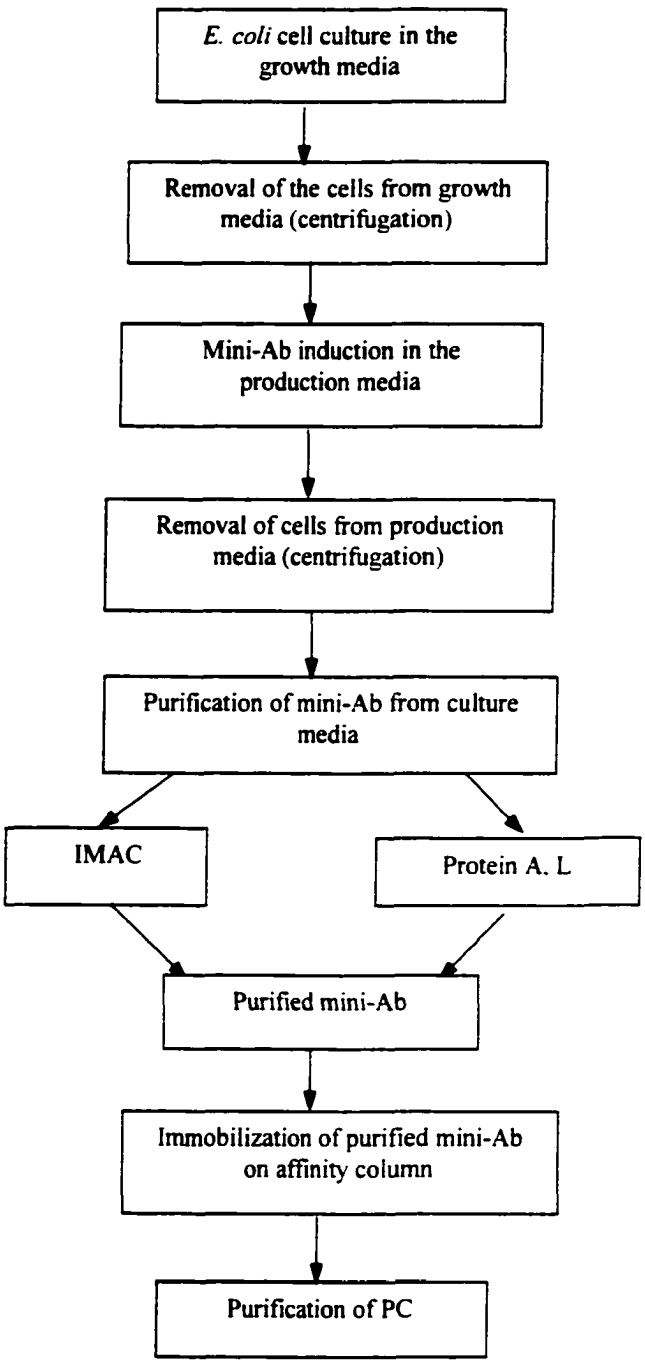

Figure 4. An overview of the project.

E. Research Objectives

The objectives for this research study are:

1. To select the best mini-Ab producing *E. coli* colony out of the three highest producing colonies and to optimize the production process.
2. To purify the mini-Ab from the culture broth using affinity chromatography.
3. To perform a feasibility study of PC purification using the mini-Ab.
4. To perform a preliminary economic analysis of PC purification using the mini-Ab.

CHAPTER III

MATERIALS, INSTRUMENTS, AND METHODS

A. Materials

1. Materials for *E. coli* Culture

Thirteen recombinant *E. coli* colonies producing mini-Ab by phage display method were obtained from Dr. M. Sierks at the Arizona State University. The *E. coli* colonies were grown in 2XTY media. This media (cell *growth media*) contains 16 g tryptone (Sigma; St. Louis, MO), 10 g yeast extract (Bacto Laboratories; Liverpool, Australia), and 5 g NaCl (Sigma) in one liter of distilled water. To that, 1% glucose (Sigma) and the antibiotic 0.01% ampicillin (Sigma) were added.

The mini-Ab is produced in the *production media*. This media contains the same composition of the media shown above. However, it also contains 0.1% glucose instead of 1%, 0.01% ampicillin, and also 1 mM (this concentration was later modified to 0.1mM) isopropyl β-D-thiogalactopyranoside (IPTG; ICN Biomedical, Inc.; Irvine, CA) as an inducer, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor.

The cells are separated from the media using a centrifuge in a 50 ml plastic tube (Greiner Bio-one; Longwood, FL). The media was sometimes concentrated using a centrifuge centricon filter™ (Millipore; Schwalbach,Germany) of molecular cut off range of 10,000.

2. Biochemicals for ELISA

For the ELISA, 96 well, microtiter plate were purchased from Nalge Nunc International (Roskilde. Denmark). PC was provided by American Red Cross (Rockville. MD). The following chemicals/biochemicals were purchased from Sigma: horseradish peroxidase-conjugated goat anti mouse IgG. OPD (o-phenylenediamine dichloride) solution containing urea hydrogen peroxide (yielding 0.4 mg/ml of OPD, 0.4 mg/ml of urea hydrogen peroxide, and 0.05 M phosphate-citrate buffer), bovine serum albumin (BSA), anti-human PC rabbit antibodies and anti-human PC mouse monoclonal antibodies. Mouse anti-human lamda antibodies and mouse anti-human kappa antibodies was generous gifts of the Southern Biotechnology Associates. Inc. (Birmingham. AL). Anti-c-myc antibody, 9E10, were purchased from the Santa Cruz Biotechnology (Santa Cruz, CA).

Buffers used are:

1. PBS (phosphate buffered saline; Sigma: pH 7.4)
2. PBST (phosphate buffered saline; Sigma containing 0.05% Tween 20: Sigma: pH 7.4)

3. Biochemicals for SDS-PAGE

All chemicals, including the molecular marker needed for this study was purchased from Sigma. The buffers used for the study are as follows:

a. Tris-Cl/SDS pH 6.8 - Stacking Gel buffer

b. Tris-Cl/SDS pH 8.8 - Separating Gel buffer

c. SDS electrophoresis buffer (Tris, SDS, and glycine)

d. SDS sample buffer (Tris, SDS, bromophenol, and glycerol)

e. Coomassie blue staining solution

4. Biochemicals for Protein Assay

The reagents for the Bicinchoninic Acid (BCA) protein assay and BSA for standard was purchased from Pierce (Rockford, IL).

5. Materials used for Chromatographic Purification

For mini-Ab purification: CNBr Activated Sepharose™ gel matrix was purchased from Amersham Biosciences (Piscataway, NJ). Protein A and Protein L were purchased from Sigma. HiTrap chelating sepharose column was purchased from Amersham Biosciences. Unless otherwise specified, all other chemicals for various buffers were purchased from Sigma.

For PC purification: CNBr activated sepharose™ gel matrix was purchased from Amersham Biosciences and an agarose gel matrix (Actigel ALD™) was purchased from Sterogene (Carlsbad, CA). Unless otherwise specified, all other chemicals for various the buffers were purchased from Sigma.

B. Instruments

The culture media for *E. coli* cells was autoclaved using sterilmatic (Market Forge; Everett, MA). The cells were cultured in a shaking incubator (C24 incubator shaker; New Brunswick Scientific; Edison, NJ). The cells were separated from the culture media using Marathon 3200 (R) general purpose centrifuge (Fischer Scientific; Hanover Park, IL). For the studies of ultrasonication of cells, Sonic Dismemberator™ (Fischer Scientific) was used. The pH of the buffers and samples were measured using a Accumet™ pH meter (Fischer Scientific).

The optical densities of the samples were measured using a DU SERIES 500 Spectrophotometer purchased from Beckman Instruments, Inc. (Fullerton, CA). For the incubation of ELISA plates, an Isotemp Incubator was used (Fischer Scientific). ELISA plates were read by an ELISA plate reader (Bio-Rad; Hercules, CA).

Chromatography was performed by a fraction collector (Pharmacia FRAC-100) Pharmacia Peristaltic Pump P-1, a Control Unit UV-1, an Optical Unit UV-1, and the Recorder REC 101.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using a Mini-Protean 3 Cell (BioRad; Hercules, CA).

C. Methods

1. Methods for *E. coli* Culture

Culture Medium Preparations. The 2XTY media is autoclaved for 20 minutes and to that filtered glucose (1%) and ampicillin was added. Glucose was not autoclaved but was filtered and was added separately to the autoclaved media. When sugars are heated in the presence of other medium ingredients, especially phosphates, they partially decompose to substances that are very toxic to some microorganisms (Bailey and Ollis, 1986). To this prepared cell growth media, the *E. coli* colonies were added and were grown at 37°C in a shaking incubator at 300 rpm. When the optical density (O.D.) of the media reached 0.9, the cells were separated using a centrifuge at 4000 rpm for 20 minutes. The separated cells were then transferred to the production media and cultured in the shaking incubator at 300 rpm for 24 hrs at 30°C. The cells were then separated using a 50 ml centrifuge tube at 4000 rpm for 20 minutes. The supernatant was then collected and concentrated to a desired concentration using Centricon Filter™ at 4000 rpm for 20 minutes.

Ultrasonication of *E. coli* cells: Ultrasonication was used to loosen the cell membrane in order to release the mini-Ab present inside the cell. Two different sets of experiments were performed using the ultrasonicator. For the first set, the cells were suspended in 5 ml of PBS buffer after being separated from the medium (optical density 2.5). A vibration with amplitude ranging from 48 to 98 microns was applied to the sample for 5 minutes. The sample was immersed in an ice-bath to avoid heating during ultrasonication. Once ultrasonicated, the cells were separated and the supernatant was

concentrated using ultrafiltration and the mini-Ab concentration was checked using ELISA.

2. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA for the mini-Ab quantification. This ELISA protocol was originally developed by Wu (1998a) and optimized by Ahn (Korah. et al., 2001)

(1) Coat each well of a mictrotiter plate with 100 μl/well of PC at a concentration of 1 μg/ml-PBS.

(2) Incubate the plate overnight at 4°C.

(3) Wash each well three times with the washing buffer (PBS; 250 μl/well).

(4) Block each wells with blocking buffer, [1% Bovine Serum Albumin (BSA) in PBS: 250 μl/well] and incubate the plate for 90 minutes at room temperature.

(5) Wash each well three times with the washing buffer.

(6) Apply 100 μl of PBS to all wells except to the wells on the first row. 200 μl of the sample of the desired concentration was applied to the first row and diluted across the plate using the half serial dilution scheme.

(7) Incubate the samples at the room temperature for 2 hrs.

(8) Wash each well three times with the washing buffer.

(9) Apply 100 μl/well of 9E10 antibody (from mouse) at the concentration of 1 μg/ml-PBS.

(10) Incubate the plate for 2 hours at room temperature.

(11) Wash the plate three times with the washing buffer.

(12) Add 100 µl/well of horseradish peroxidase-conjugated goat anti mouse IgG at 1:1000 dilution in PBS.

(13) Incubate the plate for 30 minutes at room temperature.

(14) Wash each well six times with the washing buffer.

(15) Add 100 µl/well of OPD solution.

(16) Incubate the plate in a dark room for 30 minutes at the room temperature.

(17) Read the absorption of the wells at 450 nm using the ELISA plate reader. The concentration of the mini-Ab in the sample was calculated using a standard curve of the known mini-Ab concentration.

ELISA for PC quantification. The washing buffer used for this assay is Tris buffered saline (TBS. 12.5 mM Tris buffer, 0.05 M NaCl, pH 7.2; Sigma) with 0.05% Tween 20 (TBST) and the blocking /dilution buffer was TBS with 0.1% BSA. Coating buffer was 0.1 M $NaHCO_3/Na_2CO_3$ (Sigma) at pH 9.2.

(1) Coat the wells of a mictrotiter plate with 100 µl/well of 1:2300 (5 µl of the antibody into 11.5 ml of coating buffer) of the anti-human PC rabbit antibody in the coating buffer.

(2) Incubate the plate overnight at 4°C.

(3) Wash each well three times with 250 µl of the TBST washing buffer.

(4) Block each wells with the blocking buffer for 60 minutes at room temperature.

(5) Wash each well three times with the washing buffer.

(6) Apply 100 µl of dilution buffer (TBS; 0.1% BSA) to all wells except the first row. 200 µl of the PC standard as well as the sample were applied to the respective rows and were diluted across the plate using the half serial dilution scheme.

(7) Incubate the samples at 37°C incubator for 90 minutes.

(8) Wash each well three times with the washing buffer.

(9) Apply 100 µl/well of monoclonal anti-human PC mouse antibody (HP-4) at a concentration of 1 µg/ml-dilution buffer.

(10) Incubate the plate for 90 minutes at 37°C incubator.

(11) Wash it three times with the washing buffer.

(12) Add 100 µl/well of goat anti-mouse IgG peroxidase conjugate at 1:1000 dilution in the dilution buffer.

(13) Incubate the plate in 37°C incubator for 20 minutes.

(14) Wash each well at least six times with the washing buffer.

(15) Add 100 µl/well of the OPD solution.

(16) Incubate the plate in a dark room at room temperature for 30 minutes.

(17) Read the absorption of wells at 450 nm using a ELISA plate reader. The PC concentration in the sample was calculated using the standard.

Mini-Ab characterization using ELISA. To characterize the light chain of mini-Ab, either having a kappa chain or not, the following protocol was developed. It is similar to the one for the mini-Ab quantification, except for the step 9, which is given below.

(9) Apply 100 µl/well of either anti-kappa human antibodies or anti-lambda antibodies (both from mouse) at a concentration of 1 µg/ml- PBS in dilution buffer.

Affinity comparison of MAb and mini-Ab using ELISA. A protocol was developed to compare the affinity of MAb and the mini-Ab to PC. The protocol is similar to the mini-Ab quantification ELISA assay, except for the following steps. A schematic diagram is shown in Figure 5.

(6) Apply 100 μl of the PBS dilution buffer to all wells except to the first row. 200 μl of the mini-Ab sample and the MAb sample at a desired concentration were applied to two of the designated rows, respectively, and serially diluted across the plate.

(7) Incubate the plate at room temperature for 2 hrs.

(8) Wash each well three times with the washing buffer.

(9) Apply 100 μl/well of 9E10 antibody at a concentration of 1 μg/ ml in the dilution buffer to the wells contained mini-Ab. Apply PBS to the wells containing MAb.

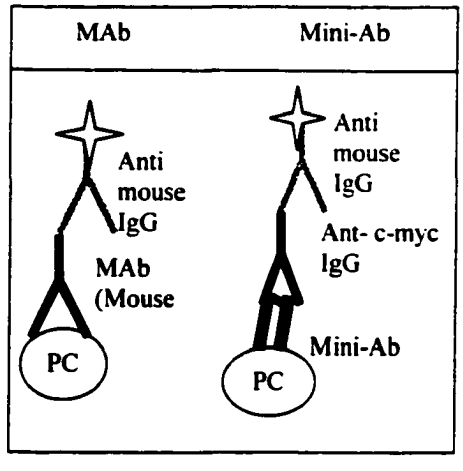

Figure 5. Schematic for the ELISA scheme for the affinity comparison of a MAb and the mini-Ab.

3. Quantification of mini-Ab in the samples

Spectrophotometric reading. The mini-Ab from chromatographic purification was measured at 280 nm using the spectrophotometer. The extinction coefficient of variable light chain from the literature was $E^{0.1\%}_{280nm} = 1.16$ (Fasman, 1989). Since the exact extinction co-efficient was not available, the closest one to mini-Ab was that of the variable light chain and hence was chosen as the extinction co-efficient for this study.

Protein Assay. The purified mini-Ab were also measured using Bicinchoninic Acid (BCA) protein assay. 100 µl of sample was applied to 2 ml of working reagent. This solution was mixed well and was incubated at 37°C for 30 minutes. It was then cooled to room temperature for one hour prior to the absorbance measurement at 562 nm. From the optical density of the sample, the concentration of protein was calculated from the standard curve using bovine serum albumin.

4. SDS-PAGE

The most common method for obtaining the molecular weight of a protein, as well as the sample purity, is by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The protein is denatured by placing it in negatively charged Sodium Dodecyl Sulfate (SDS). The resulting denatured protein has a negative charge and will migrate through the polyacrylamide gel from the cathode towards the anode. The factors affecting the protein migration rate are the pore size of the gel, and the size, shape and charge density of the molecule. The purity of the sample is

determined by the number of bands. The molecular weight can be obtained by comparing the position of the molecule markers of known molecular weight.

Electrophoresis under non-reducing conditions was done on the fraction obtained from the protein A column. The separation gel was made with 30% acrylamide solution, 20% ammonium per sulfate, water, TEMED, and Tris Cl buffer at pH 8.8. Once the gel is ready, the required amount of sample was applied to each well. The amount in each well should be at least 1-3 μg/ml to get a clear band. If the sample was too dilute, it was concentrated before the experiment. After the electrophoresis, the gel was stained with Coomassie Blue to visualize the bands.

5. Method for the mini-Ab purification

Immobilization of ligand to gel matrix. The gel preparation and ligand coupling protocol was obtained and followed according to the manufactures instructions. In short, the required amount of gel was weighed and suspended in 1 mM of HCl for 15 minutes and washed with 1 mM of HCl on a sintered glass filter. It was then washed with the coupling buffer. The required ligand was mixed with coupling buffer (0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl). The coupling solution was mixed with gel and was allowed to react in a shaking incubator at room temperature for 3-4 hours. The excess ligand was washed with 5 CV of coupling buffer. Any remaining active group of gel was blocked with 0.1 M Tris Cl buffer at pH 8.0 for 2 hours at room temperature. The gel was then loaded to a column and was washed with 5 CV of three cycles of alternating pH. Each cycle contained a wash with 0.1M acetate buffer (pH 4.0 containing 0.5 M NaCl) followed by 0.1 M Tris HCl buffer (pH containing 0.5 M NaCl).

Purification of the mini-Ab using Protein A. The protocol for mini-Ab purification was obtained from Sambrook et al. (1989).

1. Apply the prepared gel to a column and equilibrate it with 100 mM Tris Cl (pH 8.0).
2. Add 1% volume of 1 mM Tris Cl (pH 8.0) to the mini-Ab source material.
3. Load the source material in the column and wait for 10 minutes, and then wash the column with 10 column volume (CV) of 100 mM Tris Cl (pH 8.0) followed by 10 CV of 10 mM Tris Cl (pH 8.0).
4. Add one CV of 100 mM glycine (pH 3.0) and collect 500 µl fractions of the column eluate in microfuge tubes containing 50 µl of 1M Tris Cl.
5. Just as the column runs dry, add another 1 CV of 100 mM glycine (pH 3.0) and continue collecting samples. Continue this process until 5 CV have eluted from the column or until the absorption profile at 280 nm is flat.
6. Remove residual proteins and other biomolecules from the column by washing it sequentially with 10 volumes of 3 M urea, 1 M LiCl, and 100 mM glycine (pH 2.5). Finally, readjust the pH of the column to 8.0 by washing it with 10 CV of 100 mM Tris Cl. After the completion of purification process, store the column at 4 °C in 100 mM Tris Cl containing 0.02% sodium azide.

Purification of mini-Ab using Protein L. The mini-Ab purification protocol using protein L was obtained from the manufacturers.

1. Once Protein L is immobilized on the CNBr Sepharose gel according to the manufacturers protocol, a column was prepared by loading gel matrix into it.

2. Once the gel settles, wash it with 5 CV of 10 mM PBS (pH 7.2, 0.12 M NaCl).
3. Load the mini-Ab source material at a pH of 8.0 for adsorption.
4. Wash away unbound biomolecules with 10 CV of PBS.
5. Elute the bound mini-Ab with 20 ml elution buffer (0.1 M glycine at pH 3.0) and immediately neutralize the eluate with Tris-base until pH 7.5.
6. The column may be regenerated as previously explained in the "mini-Ab purification using protein A" section.

Purification of mini-Ab using IMAC. The HiTrap Chelating IMAC column contained 1 ml of gel with pre-immobilized IDA chelator.

1. The HiTrap chelating columns was washed with 5 column volumes (CV; 5 ml) of distilled water and then the column was saturated with 0.5 ml of the desired metal salt solution in distilled water. Metal ions were applied to the column as follows:

   IDA-Ni^{2+}: The HiTrap column was washed with distilled water and then 0.5 ml of 0.1 M $NiSO_4$.

   IDA-Cu^{2+}: The column was saturated with 0.1 M $CuSO_4$.

   IDA-Co^{2+}: The gel was saturated with 0.1 M $CoCl_2$.

   IDA-Fe^{2+}: For this column, 0.01 M $Fe_2(SO_4)_2$ was used to saturate the column.
2. After washing it with 5 ml of distilled water to remove the loosely bound metals, the column was equilibrated with 5-10 CV binding buffer (0.02 M PBS, 0.5 M NaCl, pH 7.4).
3. The mini-Ab sample was then loaded. After adsorption step the column was washed with 5 to 10 CV of binding/ washing buffer.

4. 5 CV of the elution buffer was applied to elute the bound proteins. The sample was then applied and was then washed with 5 to 10 CV of binding buffer or until the baseline was flat.
5. 5 CV of the elution buffer (0.5 M imidazole in the binding buffer) was applied to elute the bound proteins.
6. The column was then washed for the next use. If the metals needed to be stripped it was washed with 0.05 M EDTA in the binding buffer.

Mini-Ab purification using Ni-NTA column. This column contained pre-immobilized Ni on NTA chelator. The purfication steps are similar to that of as explained in "Purification of mini-Ab using IMAC". Follow the steps of 2-6 for purification of mini-Ab.

6. Method for PC Purification

The mini-Ab was coupled to CNBr-Sepharose as explained in p.g. 31, section immobilzation of ligand to gel matrix.

The mini-Ab was coupled to Actigel according to the manufacture's instructions. In short, the gel was washed with 5 CV of coupling buffer (0.1 M PBS pH 7.2). Mix the ligand to equal volume of the coupling buffer. Add ALD-coupling solution (1 M $NaCNBH_3$) to a final concentration of 0.1 M. Mix this to the gel and let it react in a shaking incubator for 3-4 hours. Filter the resin and wash with 5 CV of 0.5 M NaCl. Load the gel to a column.

Purification of PC Using Mini-Ab. PC was purified according to the protocol of Kang (1992).

1. Once the mini-Ab immobilized gel is prepared, load it into a column and equilibrate it with washing/adsorption buffer (0.02 M sodium citrate; 0.08 M NaCl, pH 6.0). The adsorption pH of 6 is chosen because the proteases in blood plasma are less active at this pH.
2. Wash the column using washing buffer until the UV spectrometer shows a constant absorbance.
3. Apply the sample and give 30 minutes for adsorption.
4. Wash unbound biomolecules using the washing buffer until the UV spectrometer shows a constant absorbance.
5. Elute protein C using elution buffer (0.1 M sodium carbonate buffer; 0.15 M NaCl, pH 10.0) and neutralize the eluate immediately by 3 M HCl.
6. Residual protein can be eluted with a high salt (atleast 0.5 M NaCl) content in the washing buffer.

CHAPTER IV

RESULTS AND DISCUSSION

A. Optimization of mini-Ab production

1. Selection of the Highest Mini-Ab Producing Colony

As previously stated, thirteen colonies producing the mini-Ab against PC were obtained from Dr. Michael Sierks at the Arizona State University (Wu, et al., 1998a). Researchers in Dr. Kang's laboratory have tested all thirteen colonies to confirm whether they produced PC specific mini-Ab at a reasonable rate. Out of the 13 colonies, three colonies that show high production of the mini-Abs were E4FX, H3FIX and H3FX (Korah, et al., 2001).

To select the colony producing the most mini-Ab, three colonies were cultured and the mini-Ab concentration in the culture broth was measured by ELISA. The supernatant of the E4FX culture broth contained approximately 35 μg/ml of mini-Ab, H3FIX, 15 μg/ml, and H3FX, 11 μg/ml (Figure 6). The colony E4FX was selected for further optimization studies.

2. IPTG Effect on the Mini-Ab Production

For the production the mini-Ab, the culture is performed in two different stages and two different media are used respectively: the *growth media* (for *E. coli* growth)

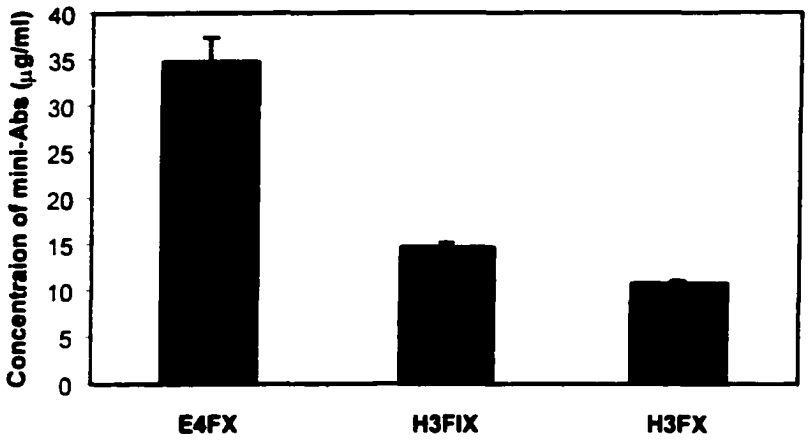

Figure 6. Concentrations of mini-Ab in the culture broths of three *E. coli* colonies.

and *production media* (for the production of the mini- Ab). For the production of mini-Ab, initially 1 mM concentration of inducer isopropyl β-D-thiogalactopyranoside (IPTG) was used. To study the effect of IPTG concentration on the production rate of the mini-Ab, IPTG concentration in the production medium was varied from 0 to 2 mM. After the completion of the culture, the concentration of the mini-Ab in the supernatant of the culture broth was measured by ELISA. The results are shown in Figure 7. At the IPTG concentration of 0 mM, the production was almost zero, as expected. The production rates of the mini-Ab was similar and highest at 0.1 and 1 mM of IPTG. At IPTG concentrations 0.05 mM and 2 mM, approximately 40 % of reduction in the mini-Ab production was observed. Higher IPTG concentration than the optimum can be toxic since it can overwhelm the secretion process of the produced mini-Ab or it may affect in the aggregation of poorly folded mini-Abs (Wang, 2002). By using 0.1 mM of IPTG as opposed to 1 mM, the production cost of mini-Ab was lowered by about 30%.

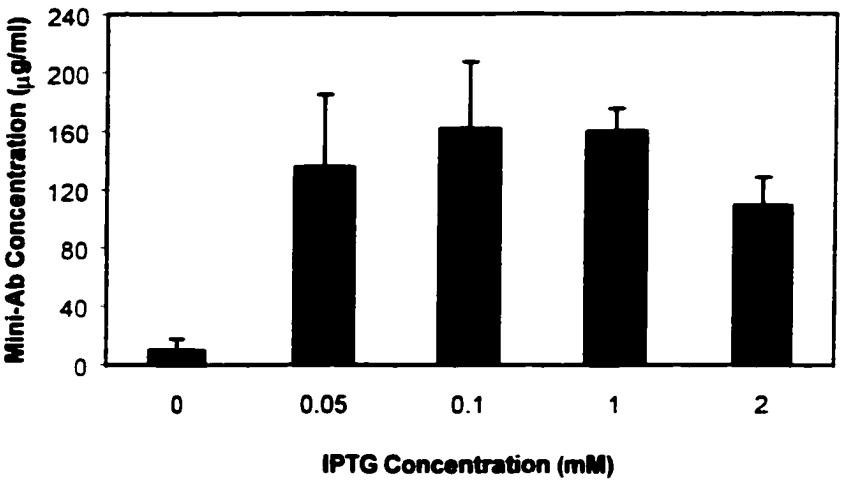

Figure 7. The effect of IPTG (inducer) on the mini-Ab production

3. Ultrasonication Study

The location of the secreted antibody can be in either the bacterial periplasm or the culture media (Kripriyanov, et al., 1997). The produced PC mini-Ab from *E. coli* was initially designed to be secreted from the *E. coli* cells to the culture medium (Wu, et al., 1998a). To verify whether there is any extensive amount of mini-Abs in the *E. coli* cells, ultrasonication was applied to the cell to loosen the cell membrane. Ultrasonication was used, rather than breaking the cells, to avoid the release of other biomolecules including nucleic acids in the cells (Oxender and Fox, 1987). These bio-molecules in the source materials can further complicate the purification process of mini-Ab.

After separating the cells from the culture broth, the cells were washed in PBS buffer. Cells were then subjected to ultrasonication in the amplitude range of 48-96 microns. After the ultrasonication, the cells were separated from the media and the mini-Ab concentration in the media was checked using ELISA. The optical density values of

ELISA for all samples were very low (below 0.2 of O.D.). The cells were observed under microsope to check for cell damage. No cell damage was observed.

An experiment was designed to verify whether the cause of the low ELISA signal was due to the little mini-Ab released from the cell or due to the denaturation of the mini-Ab by ultrasonication. The supernatant of the cell broth containing mini-Ab alone was subject to the same conditions as in the previous experiment and the result is shown in Figure 8. As the ultrasound amplitude increased, the affinity of mini-Ab decreased. This shows that the mini-Ab may have been denatured due to the shear stress generated by the ultrasound, implying that the mini-Ab is pressure (or shear) sensitive.

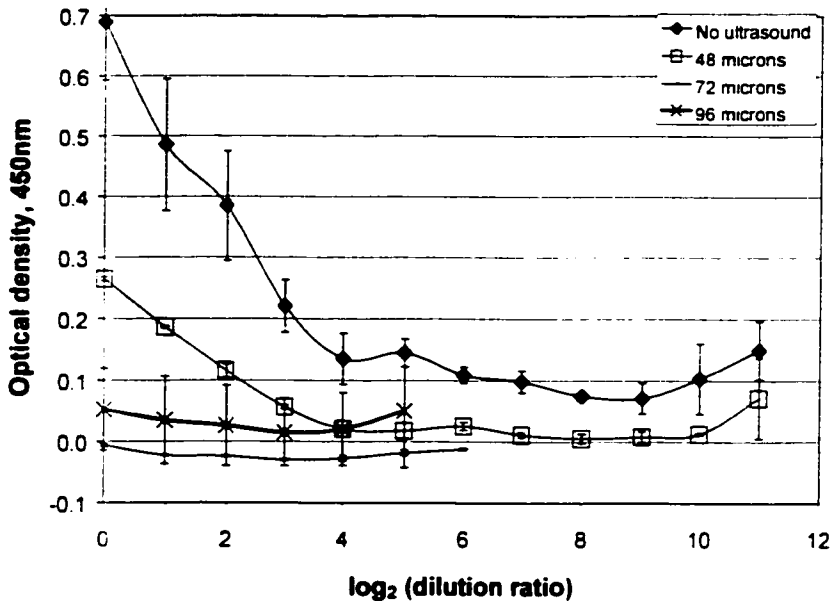

Figure 8. The optical density of ELISA when the sample was subject to ultrasound at various power amplitudes.

4. Other Parameters Affecting the Mini-Ab Production

The optimization of the mini-Ab production was a joint effort with another researcher, Mr. D. G. Ahn. Parameters, such as glucose concentration, temperature, and pH were studied by him (Korah, et al., 2001; Ahn, 2002). For the glucose concentration in the production media, the mini-Ab production was the highest at 0.1% glucose concentration. The temperature effect on the mini-Ab production was also studied. At 30°C and 25°C, the production was the highest. The initial pH of the mini-Ab producing media was studied and was found that at pH 7 the production was highest. After the optimization of these parameters, the current production rate of the mini-Ab is in the range of 300 μg/ml.

B. Mini-Ab Purification

1. Purification of the mini-Ab by Protein A

After completing the mini-Ab production, *E. coli* cells were separated from the reactor broth by centrifugation. The produced mini-Ab in the media was purified using protein A immobilized CNBr-Sepharose chromatography. Approximately 0.45 g of protein A was immobilized on 1 ml of the gel matrix and the gel was loaded in a 0.5 cm diameter chromatography column.

For the initial study, the supernatant was 32 times concentrated by ultrafiltration and was then applied to the protein A column, to reduce the sample volume. After the adsorption and the washing steps, the glycine buffers at the pH range of 2 to 4 were used for elution. The purified mini-Ab was quantified by ELISA. The purification yields [(mini-Ab mass in the eluate)/(mini-Ab mass in the source material) x 100] of the three-elution pH's are shown in Table 2. The elution pH of 3 gave the highest mini-Ab purification yield (12%). To eliminate the concentration step by ultrafiltration, the supernatant was applied directly to the protein A column and the mini-Ab was eluted at

Table 2

Mini-Ab purification using protein A column at the pH range of 2 to 4.

Sample	32 x concentrated supernatant by ultrafiltration			Supernatant
pH	2	3	4	3
Yield of mini-Ab purification (%) *	10±2	12±1	8±1	18 ±2

* The values are an average of two experiments.

pH 3.0. The purification yield was then increased to 18%. The less purification yield while using concentrated sample may be due to the loss of the mini-Ab activity by the shear stress during the ultrafiltration process, as shown in ultrasonication studies and also due to the mini-Ab adhesion to the filtrate membrane surface. When regular antibodies are purified using protein A affinity column, the range of purification yield are from about 50% (Poiesi, et al., 1989) to 90% (Lindmark, et al., 1983). Considering the fact that protein A has only 10% affinity to the ScFv part, 18% purification yield of the mini-Ab appears to be reasonable.

Purified mini-Ab's were also characterized by SDS-PAGE. The mini-Ab purified without ultrafiltration showed a single band around the molecular weight of 29 kDa (Figure 9). The purified sample was then concentrated by ultrafiltration, and that sample showed a band around 60 kDa besides the 29 kDa, indicating a possible dimer formation. Two small bands around 16 kDa was also found and they may be the small fragments produced by the degradation of the mini-Ab into light and heavy chains by ultrafiltration.

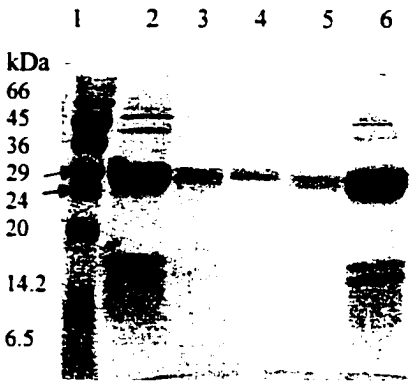

Figure 9. An SDS-PAGE analysis of mini-Ab samples. Lanes: 1, molecular mass markers (values in kDa are shown on the left); 2 and 6, ten times concentrated purified mini-Ab samples; 3 and 5, purified mini-Ab from supernatant using protein A column; 4, standard mini-Ab sample.

2. Purification of the mini-Ab by Protein L

When the antibody library was initially used for the PC mini-Ab construction of light chain, both kappa and lambda region genes were supposed to be coded (Griffiths, et al., 1994). However, it was never verified whether this mini-Ab produced by E4FX included kappa or lambda chain. Protein L has much higher affinity for the Fv part of the antibody containing kappa chain than protein A. Mini-Ab contains only Fv part, and hence protein L was also tested for the purification of mini-Ab. However, protein L chromatography column adsorbed little of the PC mini-Ab. To check whether mini-Ab has affinity to the free form of protein L, an ELISA was performed using protein A and protein L. The control was (PC)-(mini-Ab)-(9E10)-(anti mouse IgG horseradishperoxidase). The ELISA signal intensity is shown in Table 3.

Table 3

The affinity of the mini-Ab to protein A and protein L

	9E10	Protein A	Protein L
ELISA Signal Intensity	1.666±0.01	0.645±0.01	0.211±0.01

Based on the result, the mini-Ab has little affinity to protein L. To confirm whether mini-Ab contained a kappa or lambda light chain, a specially designed ELISA was also performed. For this study the mini-Ab was immobilized on the plate surface and then anti-kappa or anti-lambda antibody was applied instead of 9E10. Since the mini-Ab is humanized, a mouse anti-human antibody was used and then anti-mouse IgG

horseradishperoxidase conjugate was added for coloring reaction. However, little signal was obtained for both antibodies. Possible reasons are: (1) antibodies used were anti-*human* kappa or lambda antibody and may not be suitable for this humanized mini-Ab; (2) the epitopes on light chain may not be available for the anti-kappa or lambda antibody. Possible mini-Ab's binding configuration in the ELISA is such that the light chain is immobilized on the surface (Figure 10), it may not be available for binding during ELISA (Actigen, 2002).

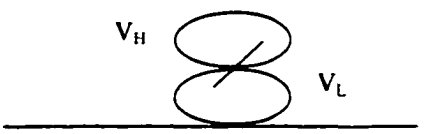

Figure 10. A schematic diagram for a possible mini-Ab binding configuration on the ELISA plate: adsorption of the light chain on the ELISA plate.

To avoid the immobilization of the light chain on the ELISA plate surface, PC was coated on plate followed by the mini-Ab, then anti-kappa or lambda, followed by anti-mouse IgG horseradishperoxidase conjugate. However, still no signal could be obtained for either kappa or lambda antibodies. The mini-Abs are produced from the large synthetic repertoires built from the majority of human V genes segments (Griffiths, et al., 1994). However, an analysis of the synthetic antibodies shows that some segments of light chains, ($V_{L\kappa}$ segments DPK-1 and DPK-2; and $V_{L\lambda}$ segments DPL-5 and DPL-23) that are common in the natural antibodies were not seen in the synthetic antibodies (Griffiths, et al., 1994). Hence the anti-kappa and anti-lambda antibodies that are developed against human natural antibodies may not work for the synthetic mini-Ab. In

summary, it appears that protein L is not appropriate for the purification of PC specific mini-Ab.

3. Mini-Ab Purification Using IMAC Columns

(a) Initial IMAC study

Four metals, Cu^{2+}, Ni^{2+}, Fe^{2+}, and Co^{2+}, which are frequently used in the IMAC are tested for the mini-Ab purification. The usual purification capacity of one ml of HiTrap Chelating IMAC gel matrix is about 10 mg of histidine tagged proteins. Initially, for the adsorption of the protein to IMAC column pH 7.4 was chosen, since the adsorption of histidine to the metal is reported to be the highest at this pH (Luo, et al., 2001).

IDA-Ni: After washing and equilibrating the Ni-IMAC column, the mini-Ab sample was loaded. Initially, 10 ml of the reactor broth was used. However, after adsorption and washing, most of the Ni leached out of the column. Ni^{2+} has a light greenish color and leaching of Ni^{2+} is visible. During the elution with 0.5 M imidazole, no peak was obtained.

After stripping any remaining Ni^{2+} with EDTA, the column was reloaded with the metal ions ($NiSO_4$). This time, pure mini-Ab sample (350-400 μg) in the binding buffer was applied to the column. Again, Ni^{2+} was leached out during the adsorption stage.

IDA-Co^{2+}: Co-IMAC column had a pinkish color and during the adsorption of purified mini-Ab sample (350-400 μg), Co^{2+} was also leached out.

IDA-Fe^{2+}: After the column was prepared, 400-450 μg of purified mini-Ab sample was applied to the column and washed with binding buffer. Fe-IMAC column

has a light yellowish color. The mini-Ab did strip the Fe^{2+} ions but not as much as Ni^{2+} or Co^{2+}. Since Fe^{2+} ions have more affinity to oxygen group than histidine, the ion binds more strongly to the chelator than to the protein. This may have been the reason why Fe^{2+} ions were not stripped as much as the Ni^{2+} or Co^{2+}. However, when the eluted mini-Ab was quantified by ELISA the purification yield was only 0.3%.

IDA-Cu^{2+}: 350-400 μg of purified mini-Ab was used as sample source. This column has a light bluish color. Even with Cu column, some leaching of Cu ions was observed at the adsorption pH of 7.4. However, approximately 2% of the mini-Ab was retained in the column and was eluted with elution buffer.

(b) pH effect on mini-Ab adsorption to IMAC column

When the binding of metal ion to the protein is stronger than the chelator, the metal ions transfer the electrons from the chelator to the protein. This may cause the ions to leach out of the column (Oxender and Fox, 1987). It appears that Cu^{2+} was retained in the column better than the other metals since the binding force of Cu^{2+} to IDA (chelator) is stronger than those of Ni^{2+} or Co^{2+}, i.e., $Cu^{2+} > Ni^{2+} > Co^{2+}$ (Vancan, et al., 2002). Therefore, the strong binding of the mini-Ab to Cu^{2+}, Ni^{2+} and Co^{2+} was suspected to be the cause of the metal leaching during the adsorption stage. As a next step, the binding of protein to metal ions needs to be weakened in order for the metal to remain in the column. In IMAC, this binding force is strongly dependent on pH (Luo, et al., 2001) and, hence, the effect of the pH on the mini-Ab adsorption to the metal was studied. For this study, IDA-Cu column was chosen as a model column because of the copper's higher affinity to IDA. At the pH lower than 7, the protein binding is weakened due to the

protonation of amino groups of the protein. At a higher pH (>8), the affinity may be reduced due to the evolution of negative charges at the binding site of protein to metals (Vancan, et al., 2002). Therefore, additional adsorption pH's of 5.4, 6.4 and 8.6 of mini-Ab in PBS solution were tested. Note here, the pH of the sample solution was changed rather using a buffer of that pH range since it has been reported (Bioprocessing, 2002) that the buffer composition can also affect the adsorption of histidine residue to metal ion. The pH of the sample solution was verified the sample was added. The purification yields were 27% at pH 6.4, 7% at pH 8.6 and 6% at pH 5.4 (Figure 11) and, therefore, 6.4 was the optimal pH for mini-Ab adsorption. Even at these pH's some metal stripping was shown by a peak in the chromatogram at 280 nm (the metal as well as proteins has an absorbance at 280 nm). To verify, whether buffer composition had an effect, sodium acetate buffer (pH 6.4) was tested and the yield (30%) was within the error range of pH 6.4 (sodium phosphate), indicating the pH is more important that the buffer composition tested.

(c) Purification using Ni-NTA column

Since the binding of metal to the chelator NTA is stronger than to IDA, Ni-NTA column was also used to test the purification efficiency. The pH tested were 7.4 and 6.4. pH of 7.4 was used since it was the recommended pH by the manufactures. At the adsorption pH of 6.4 the yield was 40% yield while at pH 7.4 it was only 3% (Figure 12). Because the binding of metal ions to NTA is stronger than IDA a better performance, as expected, was obtained. From the two columns tested (Cu-IDA and Ni-NTA), the pH of 6.4 was the optimal pH for the mini-Ab adsorption.

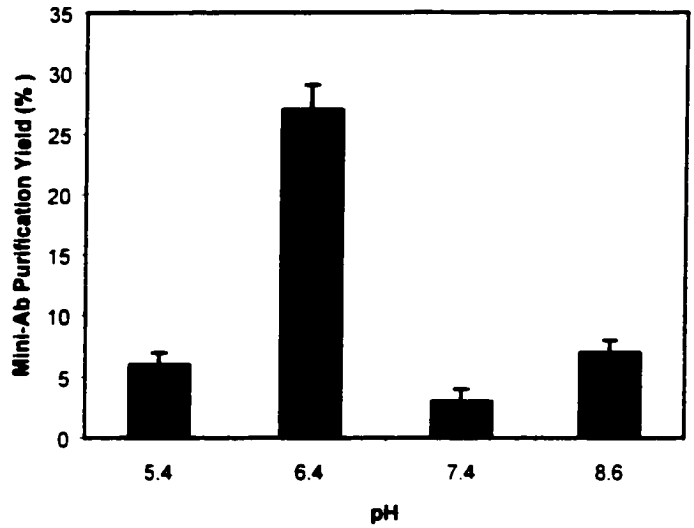

Figure 11. pH effect on the purification of mini-Ab using Cu-IDA IMAC column. The adsorption buffer used for this study is sodium phosphate.

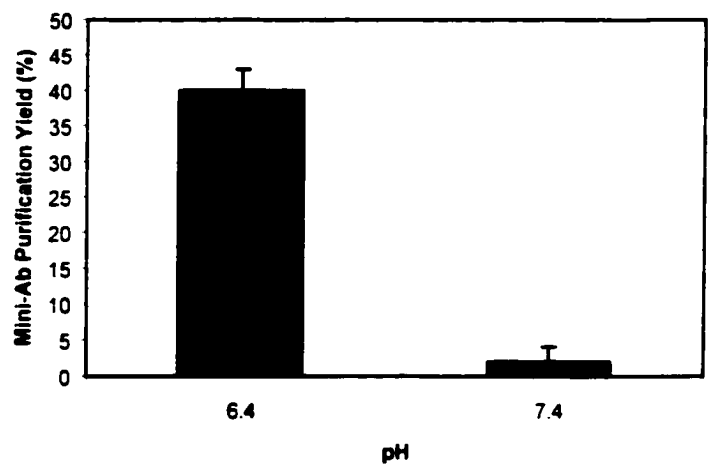

Figure 12. pH effect on the purification of mini-Ab using Ni-NTA IMAC column. The adsorption buffer used is sodium phosphate.

C. Affinity of the Mini-Ab to PC

To compare the affinities of mini-Ab and MAb to PC molecule, an ELISA was designed at the same molar concentration (Figure 5). Since the molecular weight of MAb (150 kDa) is five times greater than that of mini-Ab (29 kDa), the concentration of 10 μg/ml for MAb and 2 μg/ml for mini-Ab were used. It should be noted that because there are not many antibodies available for mini-Abs the ELISA for the mini-Ab is slightly different from that of MAb. Since the ELISA for the mini-Ab usually needs 9E10 antibody and therefore, the ELISA assay for the mini-Ab has one more step than that of MAb. This may cause less secondary antibody binding (less signal intensity) at the same affinity. Therefore, this was only a simple and rough comparison of these two antibodies. Figure 13 shows the ELISA results normalized by the mini-Ab affinity to PC. The affinity of MAb is about three times higher than that of mini-Ab.

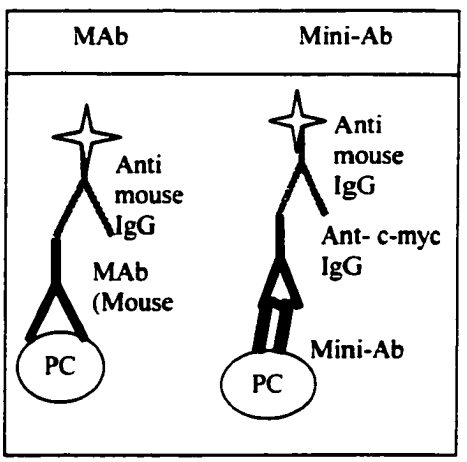

Figure 5. Schematic for the ELISA scheme for the affinity comparison of a MAb and the mini-Ab

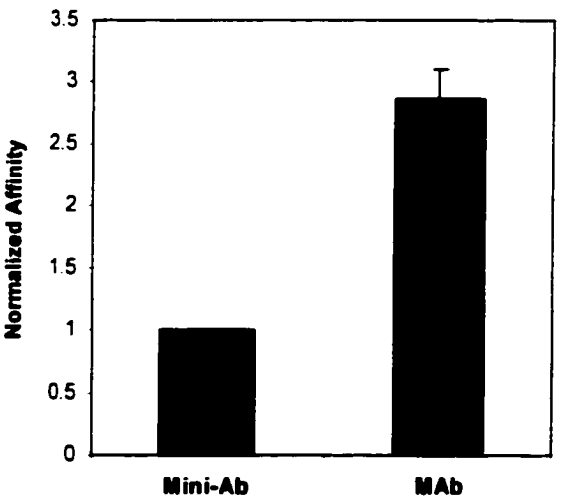

Figure 13. Affinity comparison of a MAb and the mini-Ab (same molar concentration) to Protein C.

D. Feasibility Study for Protein C Purification Using Immobilized Mini-Ab

Two different chromatography gel matrices were used for mini-Ab immobilization: CNBr activated sepharose and monoaldehyde-agarose (Actigel). The binding mechanism for CNBr Sepharose gel is the reaction of CNBr on the gel with the primary amines of proteins. Actigel uses the monoaldehyde to bind the primary amines of proteins to the gel and this binding process is much milder than CNBr binding (Sterogene, 2002). Wu, et al., (1998a) has used 1.25 mg of mini-Ab in 5 ml of PBS was immobilized on 0.4 g of CNBr Sepharose gel. A higher immobilized mini-Ab concentration (4.8 mg/ml of gel) have shown poor purification performance compared to a lower mini-Ab concentration (2.2 mg/ml of gel) (Canaan-Haden, et al., 1995).

As a preliminary study, approximately 1.25 mg of the purified mini-Ab (2.5 ml of 0.5 mg/ml of mini-Ab) was used for 1 ml of the gel matrix. The immobilization efficiency of the mini-Ab on CNBr Sepharose gel was 75-85% and for Actigel, it was 45-50% (Table 4).

Table 4

Mini-Ab immobilization efficiencies of CNBr Sepharose and Actigel.

	CNBr Sepharose		**Actigel**	
	Trial 1	Trial 2	Trial 1	Trial 2
Immobilization Efficiency	75%	85%	45%	50%

Initially PC/human serum albumin (HSA) mixture was used as a PC source. However, the PC purification yield using PC/HSA was only 3% for CNBr Sepharose and 5% using Actigel (Table 5). Since, HSA can interfere with the binding efficiency of PC to antibodies (Kwon, 2002), the PC source used for the future study will be Cohn fraction IV, which has little HSA (Wu, 2000). To test whether the poor purification efficiency of PC by mini-Ab was due to the interference of HSA, 20 μg of pure PC was then used as the sample. The PC purification efficiencies of CNBr Sepharose column was 6% and Actigel, 10% (Table 5). This result indicates that HSA does interfere with the binding of PC to the mini-Ab and Cohn fraction IV may be a better choice than the blood plasma for PC sample and being an offline product it can be obtained at a much cheaper cost.

Table 5

Purification efficiency of PC by CNBr Sepharose and Actigel columns. The values are an average of two experiments.

	CNBr Sepharose	Actigel
Yield of PC/HSA (%)*	3±1	5±1
Yield of pure PC (%)*	6±1	10±2

For both cases (PC/HSA and pure PC) Actigel gives the better performance than CNBr Sepharose. In the process of the mini-Ab immobilization, any primary amino group of a single mini-Ab can covalently bind to the CNBr Sepharose gel surface (Amersham Biosciences, 2002). Therefore, during the immobilization process random

multi-point binding of a mini-Ab molecule on the gel matrix may occur and the mini-Ab can change its three-dimensional structure. On the other hand, Actigel has a six-atom spacer arm and uses the monoaldehyde coupling chemistry to bind the proteins (Sterogene, 2002; Figure 14). Hence the mini-Ab is not directly bound to the gel surface and hence there is no multiple binding. Therefore, the binding of protein to Actigel preserves the activity of immobilized proteins better (Kang, et al., 1992; Sterogene, 2002) than CNBr, thereby keeping the affinity between the mini-Ab and PC molecule better.

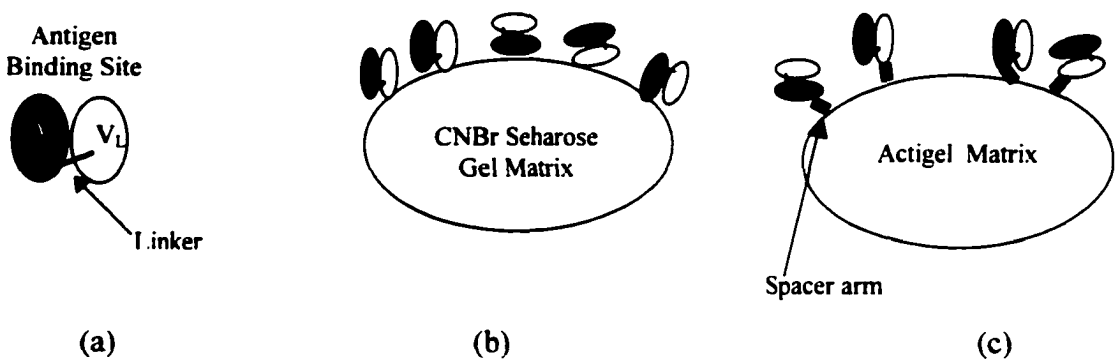

Figure 14. Schematic diagram for the mechanism of mini-Ab immobilzation to gel matrix. (a) A schmatic diagram of the mini-Ab. (b). Mini-Ab is directly bind to the gel marix via primary amine group. (c) Binding of mini-Ab is via a spacer arm on the actigel surface.

From the affinity comparison of MAb and the mini-Ab to PC molecules by ELISA showed that MAb had an affinity for PC only about three times higher than the mini-Ab, at the same molar concentration (Figure 16). However, for this initial PC purification study, the yield using the mini-Ab immobilized column was 10%. Even

though Actigel showed a better purification yield, probably due to the small size of the mini-Ab and the random nature of the mini-Ab binding to the gel surface, the affinity of the mini-Ab may have been reduced during the immobilization. Hence, a better immobilization technique as well as gel matrix should be explored for future studies.

E. Preliminary Economic Evaluation

1. Production Cost Analysis for Mini-Ab and MAb

Currently, PC purification from blood plasma is performed using PC specific MAb's (Ceprotein™). To determine the feasibility of the usage of the PC-mini-Ab for PC purification, its production cost is estimated and compared with MAb's. The costs for screening the cell lines and for the antibody purification are assumed to be approximately the same for both. In this estimation, the cost difference in bioreactors, the labor, and utility are not included, although they will be much higher for the MAb production. Table 6 shows a simple production cost comparison between the mini-Ab and MAb. For the mini-Ab production time after optimization, currently, one batch culture take a day to complete. For MAb production it takes, at least, 4-6 days (Freshney, 2000; Jackson, et al., 2001). At the end of the production the mini-Ab concentration is at least 300 mg/L. For MAbs, 100 mg/L was reported as the average production rate (Monoclonal antibodies, 2001; Jackson, et al., 2001; Karu, et al., 1995). The molecular weight of mini-Ab (MW=30 kDa) is one fifth of MAb (MW=150 kDa). The major consideration for this estimation was the media cost and the production rate. Here, the number of available binding sites for a MAb molecule is assumed to be only one since the other site may not be available due to the steric hinderence once one site is occupied. A rough estimation of the production rate of the mini-Ab is 1.3×10^{-3} mole/day·$. For MAb, the rate is 2.8×10^{-6} mole/day·$. Therefore, the production rate of mini-Ab is 470 times higher than MAb's.

Table 6

The cost comparison for mini-Ab from *E. coli* and MAb from animal cell culture.

Mini-Ab		MAb	
Media component	Cost / liter ($)	Media component	Cost / liter ($)
2XTY	3.45	DMEM[a]	27.50
IPTG	1.82	Calf (bovine) serum	30.00
Ampicillin	0.59	Antibiotic	2.68
Glucose	0.23		
PMSF	1.58		
Total cost of medium/L	7.66	**Total cost of medium/L**	60.18
Doubling time	20 minutes	**Doubling time**	18-20 hours
Production rate (mg/L)	300	**Production rate (mg/L)**	100
Specific production rate (mole/day·dollar)	**$1.3x10^{-3}$**	**Specific production rate (mole/day·dollar)**	**$2.8x10^{-6}$**

[a] Dulbecco's modification of Eagle's Media

2. Cost Comparison of the PC Purification using Mini-Ab and MAb.

To study the feasibility of the mini-Ab as an alternate ligand for PC purification, a cost analysis for the PC purification using mini-Ab and MAb was performed. Table 7 shows cost comparison of the PC purification for mini-Ab and MAb.

The cost estimation for the antibody purification is based on protein A affinity column. although the Ni-NTA provides better yield. It is because the protocol for IMAC still needs further optimization. Using Protein A column, Poiesi and his coworkers (1989) reported about 60% of MAb purification yield. For the mini-Ab, the yield was 18%.

Van Cott, et al., (1996) has used MAb for PC purification and has reported about 70% of yield. Using the mini-Ab the PC purification yield was 10%. Without any further optimization of the PC purification process the PC purification using the mini-Ab is approximately 22 times (470/21) cheaper than using regular MAb. Taking into consideration that the IMAC purification of mini-Ab yields higher and is less expensive than protein A column, the cost of PC purification may be further reduced in the future.

Table 7

The cost comparison for the PC purification using regular MAb and mini-Ab.

	Mini-Ab	**MAb**
Production cost ratio	1	470
Antibody purification ratio	3	1
PC purification ratio	7	1
Total cost ratio	**21**	**470**

CHAPTER V

CONCLUSIONS

Protein C is a potent anticoagulant, antithrombotic, and anti-inflammatory with several important medicinal applications. It has been proven to be effective in the treatment of patients with PC deficiency and sepsis. It may also have the potential to treat patients with many other thrombotic complications. Therefore, an inexpensive supply of PC can be important for the treatment of these patients. A significant part of the PC purification cost is from the expensive monoclonal antibody used for the purification. To reduce the cost, an alternate ligand, a single chain variable fragment (mini-Ab), was developed by Dr. Michael Sierks. This mini-Ab is designed to be secreted to the culture media during the induction stage of *E. coli* cells.

Optimization of Mini-Ab Production. One of the three colonies that showed the highest production, E4FX, was selected for the optimization studies. The cost of the mini-Ab production was reduced by 30% using 0.1 mM of IPTG compared to 1 mM of IPTG, maintaining the same production rate.

Mini-Ab Purification. Protein A and protein L affinity chromatography purification methods were tested for mini-Ab purification. Using protein A, the mini-Ab was purified from the supernatant with 18% purification yield. Protein L was found to be unsuitable for this mini-Ab purification.

Metal affinity chromatography was also tested for the mini-Ab purification, since the mini-Ab may contain histidine residues. During the adsorption step at pH 7.4, the metals were leached out significantly due to the strong binding of histidine residue to the metals. By decreasing the pH to 6.4 with the purpose of reducing the binding force of histidine to metals, 27% of mini-Ab purification yield was obtained with Cu-IDA column. Using Ni-NTA column under the same conditions, a purification yield of 40% was achieved.

Feasibility study for Protein C Purification using Immobilized Mini-Ab. The mini-Ab was immobilized on CNBr Sepharose and Actigel gel. The immobilization efficiency of CNBr Sepharose was better than Actigel by about 25-30%. Pure PC was then purified using these columns using the source material of PC/HSA mixture and pure PC. For PC/HSA sample, the purification yield using CNBr Sepharose was 3% and Actigel was 5%. For pure PC sample, mini-Ab immobilized on CNBr Sepharose showed 6% purification yield as compared to Actigel, 10%. For both PC sources, Actigel showed a better purification efficiencies than CNBr Sepharose. From these results, HSA appear to interfere with the binding of PC to mini-Ab. Hence, Cohn fraction IV may be a better source for PC rather than blood plasma since Cohn fraction IV has little HSA. The affinity of regular antibodies was only three times higher than mini-Ab at same molar concentration. However, the PC purification yield was almost six times higher, indicating a significant affinity reduction of the mini-Ab to PC during immobilization to the gel surface.

Preliminary Economic Evaluation. A preliminary cost estimation based on the production media cost and cell doubling time shows that mini-Ab production is 470 times

cheaper compared to regular MAb. Incorporating the purification cost for the mini-Ab and PC, without even any optimization of the current purification process, PC purification using the mini-Ab was found to be 22 times cheaper than using regular MAb. With further process optimization in mini-Ab purification and PC purification using the mini-Ab, this new ligand has the high potential to reduce the PC production cost.

CHAPTER VI

FUTURE WORK

For further improvements in PC production using the mini-Ab, the following studies are suggested.

Optimization of mini-Ab production. Mini-Ab contains a *lac* operon and hence the possibility of using lactose as an inducer should be studied. Chemical permiabilization of *E. coli* cells with detergents (triton X-100 and tetraethylene-glycol-monodecyl-ether) and membrane active peptides [e.g., polymyxin-β-nonapeptide (PMBN), melittin, phospholipase A_2] should be tested as alternatives to ultrasonication to test whether there is any significant amount of the mini-Ab remaining in the *E. coli* cells, as Morbe and Riesenberg (1997) suggested.

Purification of mini-Ab. To achieve better purification yield of the mini-Ab, protein A purification of the mini-Ab needs to be optimized. The parameters that to be studied include the adsorption pH and time, and salt concentration in the buffer, etc. IMAC column for the mini-Ab purification may be the best method because of its low cost. Parameters such as the adsorption and the elution pH, buffer, adsorption time, chelators (e.g., NTA, TED) should be optimized. Also, Cu-NTA column should be studied to improve the purification yield.

Immobilization of the mini-Ab. Parameters, such as, pH and time of coupling, concentration of ligand and buffer composition should be studied to optimize the

immobilization efficiency of mini-Ab to gel matrix. A better immobilization method should be to be explored in the near future to reduce the lose of mini-Ab activity during immobilization.

REFERENCES

Actigen. (2002). Affinity proteins applications booklet. 16-17.

Ahn. D.G. (2002). Personal communication.

Akesson M. Karlsson EN. Hagander P. Axelsson JP. Tocaj A. (1999). On Line detection of acetate formation in *Escherichia coli* cultures using dissolved oxygen responses to feed transients. *Biotechnol. Bioeng.* 64. 590-598.

Akerstrom. B.. Nilson. B.H.K.. Hoogenboom. H.R.. Bjork. L. (1994). On the interaction between single chain Fv antibodies and bacterial immunoglobulin-binding proteins. *J. Immunol. Methods.* 177. 151-163.

Arndt. K. M.. Müller. K. M.. and Plückthun. A. (1998). Factors influencing the dimer to monomer transition of an antibody single-Chain Fv fragment. *Biochemistry.* 37. 12918-12926.

Biopharmaceutical Production. (2001). www.ul.ie/~biopharm/bioprod.html

Bioprocessing. (2002). Fractogel EMD process media: Introduction to metal chelate affinity chromatography. http://www.merck.de

Bruley. D.F.. and Drohan W. N. (1990). Protein C and related anticoagulants. *Advances in Applied Biotechnology Series.* Eleventh Volume. Gulf Publishing Company.

Canan-Haaden. L.. M.. Ayala. M. E.. Fernandez-de-Cossio. I.. Pedroso. and L. Rodes. (1995). Purification and application of a single-chain Fv antibody fragment specific to Hepatitis B virus surface antigen. *Biotechniques.* 19. 606-614.

Chaga. G.. Hopp. J. and Nelson. P. (1999). Immobilized metal ion affinity chromatography on Co^{2+}-carboxymethylaspartate-agarose superflow. as demonstrated by one step purifcation of lactate dehydrogenase from chicken breast muscle. *Biotechnol. appl. Biochem.* 29. 19-24.

CTR Research. (2002). http://www.nd.edu/~transgen/research/

Cwirla. S.E.. Peters. E.A.. Barrett. R.W.. Dower. W.J. (1990). Peptides on phage: a vast library of peptides for identifying ligands. *Proc Natl Acad Sci USA.* 14. 496-499.

Degener, A. et al., (1998). Zn^{2+}-selective purification of recombinant proteins from milk of transgenic animals. *J. Chromatogr. A.* 799, 125-137.

Eli Lilly. (2001). www.lilly.com.

Esmon, C. (2000). Protein C pathway. *Crit. Care. Med.* 28(9), S44-S48.

Fasman, G.D. (1989). *Practical Handbook of Biochemistry and Molecular Biology.* CRC Press, Boca Raton, Florida, 265.

Freshney, I. R. (2000). *Culture of Animal Cells: A manual of basic Technique.* Fourth Edition. Wiley-Liss, New York, 387-390.

Ginsberg, J.S., and Hirsh, J. (1998). Use of antithrombotic agents during pregnancy. *Chest.* 114, 524S-530S.

Goel, A., Colcher, D., Koo, J., Booth, B. J. M., Pavlinkova, G., and Batra, S. K. (2000). Realtive position of the hexahistidine tag effects binding properties of a tumar-associated sing-chain Fv construct. *Biochim. Biophy.s Acta.* 1519, 13-20

Griffiths,A.d., Williams, S.C., Hartlet,O., Tomlinson, I.M., Waterhouse, P., Crosby, W. L., Kontermann, R.E., Jones, P.T., Low, N.L., Allison, J., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, J.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E., and Winter, G. (1994). Isolation of high affinity human antibodies directly from large synthetic repertories, *EMBO journal.* 13(14), 3245-3260.

Harper, P.L., Edgar, P.F., Luddington, R.J., Seaman, M.J., Carrell, R.W., Salt, A.T., Barnes, N., Rolles, K., and Calne, R. Y. (1988). Protein C deficiency and portal thrombosis in liver transplantation in children. *Lancet.* 2(8617), 924-927.

Hirsh, J., Warkentin, T.E., Raschke, R., Granger, C., Ohman, E.M., and Dalen, J.E. (1998a) Heparin and low-molecular weight heparin: Mechanisms of action, pharmakokinetics, dosing considerations, monitoring, efficacy and safety. *Chest.* 114, 489S-510S.

Hirsh, J., Dalen, J.E., Anderson, D.R., Poller, L., Bussey, H., Ansell, J. Deyking, D., and Brandt, J.T. (1998b). Oral anticoagulants: Mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest.* 114, 445S-469S.

Hoogenboom H.R., Chanes. (2000). Natural and designer binding sites made by phage display. *Curr Opin Biotechnol.* 9, 102-108.

Hudson, P. J., and Kortt, A. A. (1999). High avidity scFv multimers; diabodies and triabodies. *J. Immunol. Methods.* 231,177-189.

Jackson, L.R., Trudel, L.J., and Lipman, N.S. (2001). Small Scale Monoclonal Antibody Production In Vitro: Methods and Resources. www.labanimal.com

Joyce, D.E., and Grinnell, B.W. (2002). Recombinant human activated protein C attenuates the inflammatory response in endothelium and monocytes by modulating nuclear factor-κB. *Crit Care Med.* 30(5), S288-S294.

Kang, K.A., Ryu, D., Drohan, W.M., and Orthner, C.L. (1992). Effect of matrices on affinity purification of protein C. *Biotechnol Bioeng.* 39, 1086-1096

Korah, L., Ahn, D. G., and Kang, K.A. (2001). Optimization of the Culture Conditions and Purification of Anti-Protein C-miniantibodies Produced from *E. coli.* Proceeding of the Annual 2001 ISOTT Meeting, Kluwer Academic/Plenum Publishers. (In Press).

Karu, A. E., Bell, C.W. and Chin, T. E. (1995). Recombinant Antibody Technology. *ILAR J.* 37, 127-132.

Kavehmanesh, Z., Abolghasemi, A. and Khalili Matinzadeh, Z. (2000). Neonatal purpura fulminans in a neonate with protein C deficiency. *Irn. J. Med. Sci.* 25(3&4), 169-171.

Kramer, K., Fiedler, M., Skerra, A., and Hock, B. (2002). A generic strategy for subcloning antibody variable regions from the ScFv phage display vector pCANTAB 5E into pASK85 permits the economical production of Fab fragments and leads to improved recombinant immunoglobulins stability. *Biosens Bioelectron.* 17, 305-313.

Krishnagopalan, S., and Dellinger, R. P. (2001). Innovative therapies for sepsis. *BioDrugs.* 15(10), 645-654.

Kwon, H.J. (2002). Theoretical and experimental investigation on sensing performance of protein C immuno-optical sensor for physiological samples. *Ph.D. Dissertation.* Chemical Engineering, Univeristy of Louisville, Kentucky.

Laroche-Traineau, J., Clofent-Sanchez, G., and Santarelli, X. (2000). Three-step purification of bacterially expressed human single-chain Fv antibodies for clinical applications. *J. Chromatogr. B.* 73, 107-117.

Levine, M.N., Raskob, G., Landefeld, S., Kearon, C. (1998). Hemorrhagic complications of anticoagulant treatment. *Chest.* 114, 511S-523S.

Lindmark, R., Thoren-Tolling, K., and Sjoquist, J. (1983). Binding of immunoglobulins to protein A and immunoglobulins levels in mammalian sera. *J. Immonol. Methods.* 62, 1-13.

Monoclonal Antibodies. (2001). www.ul.ie/~biopharm/monoclon.html

Morbe, J.L. and Riesenberg, D. (1997). Release of miniantibodies from *E. coli* cells into the supernatant at low and high cell densities. *Microbiol. Res.* 152, 385-394

Minford, A.M.B., Parapia, A.A., Stainforth, C. and Lee, D. (1996). Treatment of homozygous protein C deficiency with subcutaneous protein C concentrate. *Brit. J. Haemat.* 93, 215-216.

Piervincenzi, R.T., Reichert,W.M., Hellinga, H.W. (1998). Genetic engineering of a single-chain antibody fragment for surface immobilization in an optical biosensor. *Biosens. Bioelectron.* 13, 305-312.

Poiesi, C., Tamanini, A., Ghielmi, S., and Albertini, A. (1989). Protein A, hydroxyapatitie and diethylaminoethyl: Evaluation of three procedures for the preparative purification of monoclonal antibodies by high performance liquid chromatography. *J. Chromatogr.* 465, 101-111.

Pollock, D. P., et al. (1999). Transgenic milk as a method for the production of recombinant antibodies. *J. Immunol. Methods.* 231, 147- 157.

Porath, J. (1992). Immobilized metal ion affinity chromatography. *Protein Expr Purif.* 3(4), 263-281.

Reaader. (1998). Hypercoagulable states and thrombotic risk assessment. *REAADS Medical Products, Inc.* 8, (2).

Roitt, Ivan M., et al. (1985). *Immunology.* Gower Medical Publishing, New York, 151-163.

Sanchez, L., Ayala, A., Freyre, F., Pedroso, I., Bell, H., Falcon, V. and Gavilondo, J.V. (1999). High cytoplasmic expression in *E. coli*, purification, and *in vitro* refolding of a single chain Fv antibody fragment against the hepatitis B surface antigen. *J. Biotechnol.* 72, 13-20.

Solomon, B., Raviv,O., Leibman, E., and Fleminger, G. (1992). Affinity purification of antibodies using immobilized FB domain of protein A. *J. Chromatogr.* 597, 257-262.

Stenflo, J. (1988). The biochemistry of protein C. In R.M. Bertina (ed.). *Protein C and Related Proteins.* Churchill Livingstone, Edinburgh, 21-54.

Tan, P.H., Chu, V., Stray, J. E., Hamlin, D. K., Pettit, D., Wilbur, D.S., Vessella, R. L., Stayton, P. S. (1998). Engineering the isoelectric point of a renal cell carcinoma targeting antibody greatly enhances scFv solubility. *Immunotechnology.* 4, 107-114.

Tallet, B., Astier-Gin, T., Castroviejo, M. and Santarelli, X. (2001). One -step chromatographic purification of a His-tag recombinant carboxyl half part of the HTVL-I surface envelop glycoprotein overexpressed in Escherichia coli as a secreted form. *J. Chromatogr. B.* 753, 17-22

Taylor, F.B., Jr., Chasing, A., Esmon, C.T., D'Angelo, A., Vigano-D'Angelo, S., and Blick, K.E. (1987). Protein prevents the coagulopathic and lethal effects of E. infusion in the baboon. *J. Clin. Invest.* 79, 918-925.

Vancan, S., Miranda, E.A., and Bueno, S. M.A. (2002). IMAC of human IgG: studies with IDA-immobilized copper, nickel, zinc, and cobalt ions and different buffer systems. *Process Biochem.* 37, 573-579.

Van Cott, K. E., Williamns, B., Velander, W.H., Gwazdauskas, F., Lee, T., Lobon, H., Drohan, W.N. (1996). Affinity purification of biologically active and inactive forms of recombinant human protein C produced from porcine mammary gland. *J. M. Recognit.* 9 (5-6), 407-414.

Walker, F.J. (1990). Protein C deficiency in liver disease. *Ann. Clin. Lab. Sci.* 20(2), 106-112.

Wang, P. (2002). http://www.mrc-cpe.cam.ac.uk/~plw/phageqa.html

Wilkinson, D. (2000). Immunochemical techniques inspire development of new antibody purification methods. *Scientist.* 14(8), 25.

Worn, A., and Pluckthun, A. (2001). Stability engineering of antibody single-chain Fv fragments. *J. Mol. Biol.* 305, 989-1010.

Wu, H., Gound, G.N., and Sierks, M.R. (1998a). Artificial antibodies for affinity chromatography of homologous proteins: application to blood proteins. *Biotechnol. Progr.* 14, 496-499.

Wu, H., Bruley, D.F. (1999). Protein C separation from human blood plasma cohn fraction IV-1 using immobilized metal affinity chromatography. *Biotechnol. Progr.* 15, 928-931.

Wu, H. (2000). Protein seperation from homologous human blood proteins, cohn fraction IV-1 using immobilized metal affinity chromatography. *Ph.D. Dissertation.* Chemical and Biochemical Engineering, University of Maryland Baltimore County, Maryland.

NOMENCLATURE

APC - Activated Protein C

BCA - Bicinchoninic Acid

BSA - Bovine Serum Albumin

CNBr - Cyanogen bromide

CV - Column volume

Da - Dalton

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme Linked Immunosorbent Assay

EMEA - European Medicines Evaluation Agency

FDA - Food and Drug Administration

HIT - Heparin Induced Thrombocytopenia

IDA - Iminodiacetic acid

Ig - Immunoglobulins

IPTG - Isopropyl β-D-thiogalactopyranoside

IMAC - Immobilized metal ion affinity chromatography

kDa - kilodalton

MAb - Monoclonal Antibody

Mini-Ab - Mini-Antibody

MW - Molecular Weight

NTA - Nitrolotriacetic acid

O.D. - Optical density

PAI - Plasminogen activation inhibitor

PBS - Phosphate buffered saline

PC - Protein C

PMSF - Phenylmethylsulfonyl fluoride

ScFv - Single Chain Fragment

SDS PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TBS - Tris buffered saline

TED - Tris(carobxymethyl) ethylenediamine

TEMED - Tetramethylethylenediamine

VK - Vitamin K

VKD - Vitamin K Dependent

V_H - Variable heavy chain

V_L - Variable light chain

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PUBLICATIONS:

Spiker, J.O., **Korah, L.**, Williams, G.M., and Kang, K.A., "Feasibility Study of Diagnosis of Leg Venous Disease using Near Infrared Continuous Spectroscopy," *Cardio Vascular Medicine and Science*, **3** (2), p. 89-95, 2000.

Korah, L., Scott, Jr., F.D. Williams, G.M., and Kang, K.A., "Preliminary Studies of the Application of Near-Infrared Spectroscopy in the Diagnose of Deep Vein Thrombosis," Proceeding of the 1999 Annual ISOTT Meeting, (In Press).

Korah, L., Ahn, D. G., and Kang, K.A., "Optimization of the Culture Conditions and Purification of Anti-Protein C-miniantibodies Produced from *E. coli*," Proceeding of the Annual 2001 ISOTT Meeting, Kluwer Academic/Plenum Publishers, (In Press).

Korah, L. and Kang, K.A., "Preliminary Study for the Protein C Purifcation Using-Miniantibodies Produced from Recombinant *E. coli*," Submitted to the Proceedings to the Annual ISOTT 2002 Meeting.

PRESENTATIONS:

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Kwon, H.J., **Korah, L.**, Scott, Jr., and Kang, K.A., "Venous Diseases Detection Using Near Infrared Spectroscopy," 12th Annual Mid-Atlantic Bioengineering Consortium's (MABEC) Meeting, April 9, 1999, University of Virginia, VA [with abstract]

Korah, L., Scott, Jr., F. D. Williams, G.M., and Kang, K.A., "Application of Near Infrared Continuous Wave Spectroscopy to Diagnose Venous Diseases in Legs," 1999 Annual International Society of Oxygen Transport to Tissue (ISOTT), Aug. 28-Sep. 2, 1999, Hanover, NH, USA [with abstract]

Kang, K.A., Scott, Jr., F. D. Williams, G.M., and **Korah, L.**, "The Monitoring of Hemoglobin Changes to Detect Venous Diseases in The Leg," AIChE Annual Meeting, Oct. 31-Nov. 5, 1999, Dallas, TX, USA [with abstract]

Korah, L., Scott, Jr., F.D. Williams, G.M., and Kang, K.A., "An Effectual Determining Method for the Detection of Deep Vein Thrombosis in Legs Using Light," The 19th Southern Biomedical Engineering Conference, Apr. 14-16, 2000, Blacksburg, VA, USA [with short paper]

Kang, K.A., **Korah, L.**, and Ahn, D. G., "Optimization of the Culture Conditions and Purification of anti-Protein C-miniantibodies produced from *E. coli*," 2001 Annual

International Society on Oxygen Transport to Tissue (ISOTT). Aug. 11-15. 2001. Philadelphia. PA. USA [with abstract]

Korah, L.. Ahn. D. G.. and Kang. K.A.. "Purification of anti-Protein C-miniantibodies produced from *E. coli* using Protein L and Protein A Column." AIChE Annual Meeting. Nov. 4-9. 2001. Reno Hilton. Reno. NV. USA [with abstract]

Korah, L. and Kang. K.A.. "Preliminary Study for the Protein C Purifcation Using-Miniantibodies Produced from Recombinant *E. coli*." 2002 Annual International Society on Oxygen Transport to Tissue (ISOTT). Aug. 24-28. 2001. Manchester. UK [with abstract]

Local/ Institutional

Korah, L.. Scott. Jr.. F.D. Williams. G.M.. and Kang. K.A.. "Detection of Venous Diseases in Legs Using Near Infrared Spectroscopy." Undergraduate Research Day. Apr. 28. 1999. UMBC. Baltimore. MD [with abstract]

Korah, L.. Scott. Jr.. F.D. Williams. G.M.. and Kang. K.A.. "A Diagnostic and Prognosis Determining Method for Deep Vein Thrombosis in Leg Using Light." The 3rd Annual Becton Dickinson Biosciences-UMBC Scientific Symposium. Nov. 10. 1999. UMBC. Baltimore. MD [with abstract]

Korah, L.. Scott. Jr.. F.D. Williams. G.M.. and Kang. K.A.. "Near Infrared Spectroscopy as a means for the Detection of Venous Diseases in Legs." Undergraduate Research Day. Apr. 28. 2000. UMBC. Baltimore. MD [with abstract]

Korah, L.. Scott. Jr.. F.D. Williams. G.M.. and Kang. K.A.. "Leg Venous System Functionality Assessment by Near Infra Red Spectroscopy." Research! Louisville Student Research Day. Oct. 31. 2000. University of Louisville. KY [with abstract]

Korah, L.. and Kang. K.A.. "Skin Cancer Classification Using Light." Engineer's Days 2001. Feb. 23-24. 2001. University of Louisville. KY [with abstract]

Ahn. D. G.. **Korah, L.**. and Kang. K.A.. "Purification of Protein C (PC) using mini-antibodies produced by recombinant *E. coli*." Engineer's Days 2001. Feb. 23-24. 2001. University of Louisville. KY [with abstract]

Vaccaro. D. J.. **Korah, L.**. and Kang. K.A.. "Detection of Leg Vein Blood Clots Using Light." Engineer's Days 2001. Feb. 23-24. 2001. University of Louisville. KY [with abstract]

Vaccaro. D. J.. **Korah, L.**. and Kang. K.A.. "Diagnosis of Venous Pathology in the leg using Near Infrared spectroscopy." Symposium 2001: Drug Design for a New Millennium. Mar. 12. 2001. Rudd Heart and Lung center. Jewish Hospital. Louisville. KY [with abstract]

Ahn. D. G., **Korah, L.**, and Kang. K.A., "Cost Effective Purification of Protein C using Mini-Monoclonal Antibodies Produced by Recombinant *Escherichia coli*." Symposium 2001: Drug Design for a New Millennium. Mar. 12. 2001. Rudd Heart and Lung center. Jewish Hospital. Louisville. KY [with abstract]

Vaccaro. D. J., **Korah, L.**, and Kang. K.A., "Detection of Deep Vein Thrombosis." Engineer's Days 2002. Feb. 23-24. 2002. University of Louisville. KY [with abstract]

Korah, L., and Kang. K.A., "Optical Detection of Skin Cancer." Engineer's Days 2002. Feb. 23-24. 2002. University of Louisville. KY [with abstract]

Ahn. D. G., **Korah, L.**, and Kang. K.A., "Optimization of Protein C Specific Mini-Antibody Production." Symposium 2002: Drug Design for a New Millennium. Mar. 12. 2002. Rudd Heart and Lung center. Jewish Hospital. Louisville. KY [with abstract]

Vaccaro. D. J., **Korah, L.**, and Kang. K.A., "Development of Protocol for DVT Detection in Trauma Patients." Summer Research Opportunity Program. July 31. 2002. University of Louisville. KY