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Antibodies Purification Using ELP-zz Domain Fusions

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by

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DEDICATION

For my family who offered me unconditional love and support throughout the course of this thesis.

ABSTRACT OF THE THESIS

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by

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Dr. Wilfred Chen, Co-Chairperson
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Antibodies are immune system-related proteins called immunoglobulins (IgGs) which have applications for medical diagnostics and research. However, their purification from different sources has always been a challenge because of low antibody concentration and higher purity requirements for usage. The unique capability of Elastin like Proteins (ELPs) to reversibly precipitate at a relatively modest temperature has been utilized for purification of antibodies. This feature of ELPs to purify antibodies has already been explored using larger fusion domains such as Protein L and Protein G. However the usage of larger Protein G/L fusions with ELP resulted in 10-fold lower expression when compare to ELP or ELP fusions with shorter peptides.

In the current work, ELP fusions with a small IgG-binding peptide i.e. zz domain (a synthetic IgG binding domain derived from the Staphylococcus aureus protein A) were

generated. The production of ELP[VPGVG]-78-zz fusion in E. coli (~ 500 mg/L) was found to be five-fold higher than ELP[VPGVG]-78-ProL (~100 mg/L). In addition, the ELP[VPGVG]-78-zz fusion showed excellent binding affinity toward human, mouse, and rabbit IgGs, enabling simple purification of the different antibodies by reversible thermal precipitation. In order to recover antibody from the ELP-zz-IgG complex, different elution conditions were investigated. Close to 90% recovery was achieved using 0.5 M arginine pH 3.8 buffer.

To further increase the production of ELP-zz fusion protein, three different ELP domains (VPGXG)-40 (where X= K:V:F=1:8:1); (VPGXG)-60 (where X= K:V:F=1:8:1), (VPGXG)-80 (where X= K:V:F=1:8:1), were generated. Production of ELP(KV8F)-zz fusions were increased by two fold, while maintaining similar binding affinity for IgGs. Due to its high level production and affinity for different IgGs, we believe that these ELP-zz fusions will be useful as an economical, highly efficient, and universal platform for the purification of antibodies.

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CHAPTER 1

Introduction

1.1 Introduction

Antibodies (Ab) or immunoglobulins (Ig) are gamma globulin proteins which can be produced naturally by the immune system or can be genetically engineered and produced in artificial production systems like mammalian cells, yeast, bacteria and plant cells. Antibodies and antibody fragments find diverse applications in general research for immunochemical techniques and therapeutic and diagnostic usages. The specificity of antibody-antigen interaction and our ability to manipulate the characteristics of the interaction makes them potential tools for environmental monitoring and medical diagnostics. The purity requirements for the antibody preparation are exigent, particularly for their in vivo applications. Polyclonal antibodies are recovered directly from serum. Monoclonal antibodies are produced by fusing antibody-secreting spleen cells from immunized mice with immortal myeloma cell to create monoclonal hybridoma cell lines that express the specific antibody in cell culture supernatant. Antibody purification involves isolation of antibody from serum (polyclonal antibody), ascites fluid or culture supernatant of a hybridoma cell line (monoclonal antibody). Purification methods range from very crude to highly specific.

1.2 Antibody structure

Antibodies (also known as immunoglobulins abbreviated Ig) are gamma globulin proteins (150 kDa) that are found in blood or other bodily fluids of vertebrates, and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses. The Ig monomer is a "Y"-shaped molecule that consists of four polypeptide

chains; two identical heavy chains (H) (~50kDa) and two identical light chains (L) (~25kDa) connected by disulfide bonds. Each chain consists of two different regions: constant (C_L and C_H) and variable (V_L and V_H) domains.

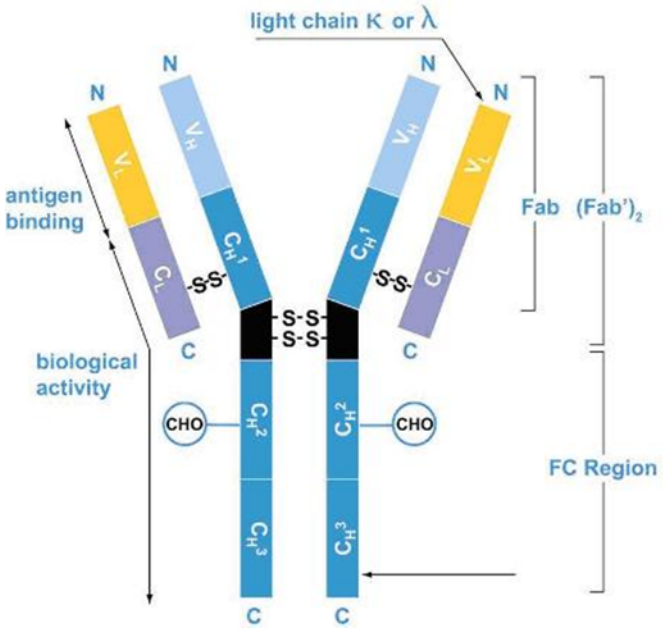


Fig.1. Structure of Antibody

The arm region of the Y is called Fab (fragment, antigen binding) region which contains the sites that can bind two antigens (in general identical) and therefore recognizes specific foreign objects. It is composed of one constant and one variable domain from each heavy and light chain of the antibody. The variable domain is also referred to as the F_V region and is the most important region for binding to antigens. The base region of the antibody called the F_C (Fragment, crystallizable) region is composed of two heavy chains that contribute two or three constant domains depending on the class of the antibody and plays a role in modulating immune cell activity.

For the purification of antibodies, various efficient strategies have been employed to target the highest purity product at the highest yield and cost effectiveness. So far, the affinity based separations i.e. affinity chromatography and non-chromatographic affinity precipitations are the most effective techniques for antibody purification. Affinity based separations are based on specific recognition between the antibody and the complementary ligand and hence minimizes the non-specific interactions which eliminates undesirable contaminants. We will discuss the existing affinity based chromatographic and non-chromatographic purification strategies in the next chapter.

In the current work, genetically engineered thermally responsive biopolymers Elastin like Polypeptides (ELPs) capable of reversible phase separation were created. ELPs are biopolymers consisting of repeat VPGVG or GVGVP units derived from mammalian protein-elastin. They are soluble in aqueous solutions below their transition temperature (T_t) but undergo a reversible phase transition into hydrophobic aggregates above the transition temperature (T_t). For these biopolymers, the transition temperature is tunable by changing polypeptide chain length and sequence. The feature to reversibly aggregate ELP above the transition temperature has been demonstrated for protein purification (Shimazu et al, 2003; Kostal et al, 2001; Meyer and Chilkoti, 1999; Meyer et al, 2001; Kostal et al., 2004; Kim et al. 2005). In our work we have created ELP fusion proteins by fusing ELPs with an IgG binding domain for antibody purification from different sources.

CHAPTER 2

Antibody Purification: Overview

2.1 Background

Antibodies are produced naturally by the immune system, however, the need for antibodies with unique specificity at a large scale has encouraged the generation of new technologies for the production of biopharmaceutical-grade antibody preparations. The production of monoclonal antibodies by the mouse hybridoma technology was the first step in the development of antibody engineering systems (Roque et al, 2004). In 1980, two monoclonal antibodies were subjected to clinical trials, but since then this number has increased considerably over the past 20 years (Roque et al, 2004). Currently, approximately 200 antibodies and their derivatives are in clinical trials for the treatment of appropriate diseases (van Dijk et al, 2001; Brekke et al, 2003). The total market for therapeutic monoclonal antibodies is a multibillion-dollar opportunity, with cancer and arthritis the major therapeutic applications with estimated total markets of $15 billion and $25 billion in 2010, respectively (Monzavi-Karbassi et al. 2001). To date, 13 monoclonal antibody based therapeutics have reached the market, although only three of them are murine/ mouse antibodies (Muromomab (OKT3) approved in 1986, Edrecolomab (Panorex) in 1995, and Zevalin approved in 2002) (van Dijk et al, 2001; Brekke et al, 2003). As a rule, a significant percentage (50-80%) of the total manufacturing cost of a therapeutic antibody is incurred during downstream processing (Roque et al, 2004). The above mentioned facts concludes that the principal task in research and industry is engineering the downstream processing of antibodies, directed toward reducing the costs and this can be addressed by exploring different types of interactions and separation techniques. Currently, many purification processes are available but they have to be

designed to target the highest yield with minimum loss of product. The purification processes are highly dependent on the type of antibody or its isotype, sources, final application and required purity criteria.

2.2 Affinity based techniques

As mentioned before, the affinity based purification strategies are the most common and capable of large scale antibody separation processes. Affinity based techniques can be classified as chromatographic and non-chromatographic techniques. In affinity chromatography, antibody interacts with ligands or proteins which are immobilized onto solid support. These affinity ligands are packed onto chromatographic columns and then the crude extracts including target antibody are run on the column. The unbound species are removed by washing the column with the elution buffers and the adsorbed target antibody is recovered. Affi-gel blue column chromatography has been employed for monoclonal antibody purification directly from ascitic on a DEAE (diethylaminoethyl). Optimal conditions were determined for the recovery of immunoglobulins free of contaminating protease and nuclease activities (Bruck et al, 1982). However, the limitations of conventional affinity chromatography are column operation, pressure drop and clogging of packed bed of adsorbents. Also, the chemical coupling of ligands onto solid support leads to reduction in binding affinity of the ligands. Protein A for example, when immobilized onto rigid ceramic composite lost 75% of its original IgG binding capacity (Guerrrier et al, 1998). Contrary to that, non-

chromatographic methods can overcome these limitations and affinity precipitation among them is a valuable technique in downstream processing for high value proteins which has depicted large scale potential in diagnostic field and immune-separation.

The application of magnetism has been actively used for antibody purification from other unclarified crude extracts. Magnetic supports with covalently immobilized affinity ligands have proved to be effective for affinity separations. Thermo-sensitive magnetic immunomicrospheres made of poly-(styrene/N-isopropylacrylamide/methacrylic acid) were synthesized for immunoaffinity purification of anti-BSA antibodies from antiserum (Kondo et al, 1994). Novel and effective superparamagnetic immunomicrospheres were developed for affinity bioseparation processes (Liu et al, 2004). However, the large scale implementation with the cost investment is limited for these processes. The potential for ion-exchange membrane adsorbers to replace traditional ion-exchange columns was also evaluated. However, anion-exchange membranes in a flow-through mode as compared to commercially available cation-exchange membranes provided a reasonable alternative to columns for the removal of low levels of impurities such as DNA, host cell protein, and virus (Knudsen et al, 2001).

Affinity based precipitation is a non-chromatographic technique which has a large scale potential in downstream processing. It utilizes a heterobifunctional ligand comprised of a polymer which possesses affinity for the target protein(s) and precipitates i.e. can be made reversibly soluble and insoluble by altering a specific parameter such as

pH or temperature. Different polymers of natural and synthetic origin have been used for this purpose. The soluble form of the ligand enables affinity binding step and then precipitation separates affinity complex from the rest undesirable components. Some of the polymers which have been used as ligand carriers are Eudragit (Taipa et al, 1998), poly-N-isopropylacrylamide (Anastase-Ravion et al., 2001). These polymers are conjugated to the IgG-binding domains and then brought to an insoluble state and precipitation of the entire affinity complex occurs in a single step. After centrifugation, the affinity complex is separated from impurities and contaminants that remain in the supernatant. Finally, the target molecule is achieved from the complex by elution and the polymer is kept at insoluble conditions in this step. After centrifugation, the target antibody can be recovered from the supernatant and the polymer precipitates which can be solubilized for reuse. This method looks operationally simple but the challenge is the complicated organic synthesis and the chemical coupling which reduces the binding affinity. This problem can be resolved by utilizing genetically engineered polymers coupled with antibody binding domains. The use of recombinant technology opens up the potential to create an infinite number of combinations between immunoglobulins, immunoglobulin fragments, tags and selected proteins, further manipulating these molecules to our advantage. Considerable efforts have been spent on finding and generating affinity ligands to obtain specific and selective purification of the required antibodies. These binding ligands vary from natural proteins A and protein G to bioengineered or fully synthetic ligands depending on the selectivity for antibody molecules.

2.3 Biospecific Ligands

Naturally available affinity ligands, such as Protein A or G for IgG purification or lectins for IgA and IgM purification, which are obtained from microorganisms or genetically modified bacteria have been mainly used as antibody binding domains. Protein L (36kDa) is isolated from bacteria *Peptostreptococcus magnus* and binds to light chain interactions. Protein L binds with high affinity and recognizes 50% of human and more than 75% of murine immunoglobulins. Roque et al., 2005 have described the interaction between artificial protein L ligand 8/7 and the target proteins IgG and Fab and demonstrated the application of this ligand as an affinity adsorbent for one step purification of immunoglobulins. The effect of pH and ionic strength of binding buffer has been studied and different eluants has been evaluated for protein recovery. This ligand additionally binds to IgG1 with κ and λ isotypes (92% and 100% of loaded protein) and polyclonal IgG from sheep, cow, goat and chicken (Roque et al, 2005).

The work also demonstrates that Protein G (~17kDa) is isolated from *Steptococcus* and binds to the F_C region of antibody. The antibody purification has been successfully demonstrated by creating ELP fusion proteins with protein G, protein L and protein LG by Kim et al, 2005. The IgG was then recovered back by .1 M sodium citrate as elution buffer. These fusion proteins were also used as a tool to purify antibody from different sources such as mouse, rabbit and hybridoma cell culture (Kim et al, 2005).

A family of linear hexapeptides has been identified for recognizing human immunoglobulin G (HIgG) through its F_C region. They are the first reported shorter

peptides binding HIgG and depicting selectivity to F_C similar to Protein A. One of the shorter domains HWRGWV was able to purify HIgG from the complex mixture of mammalian cell culture medium containing 10% fetal calf serum comparable to commercially available resins. HWRGWV demonstrated binding to all HIgG subclasses and IgGs from bovine, mouse, goat and rabbit. HWRGWV was able to bind 59.7% of HIgG in competitive mode (Yang et al, 2006; Yang et al, 2009).

Protein A (42 kDa) is a cell wall protein from Staphylococcus aureaus that binds selectively to the F_C region of IgG, but it does not bind to human IgG_3. In addition, SpA binds to Fab region of a subset of antibody with heavy chains belonging to the V_H3 family. The antibody binding characteristics of SpA was demonstrated by making ELP-SpA fusion protein (Kim et al 2005). This fusion protein showed high affinity towards IgGs and IgMs and could also preserve the ability to reversibly precipitate as antibody complex from the rest of the mixture because of ELP fused to it. A novel adsorbent coated with recombinant protein A has been used to isolate monoclonal antibodies from cell containing hybridoma fermentation broth (Thommes et al, 1996). This demonstrates high affinity of Protein A towards antibody even for low concentrations of it. Protein A-Sepharose beads have been used for isolating pure mouse IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins in nearly 100% yield (Ey et al, 1978).

A synthetic IgG binding domain zz was designed based on Staphylococcal protein A (Nilsson et al, 1987) and it has been used to make zz polyster beads for purifying mouse IgG_1, IgG_{2a} and IgG_{2b} from mouse hybridoma supernatants. The recovery of

purified IgG is 70% or greater which concludes that zz polyester beads offer a rapid and novel method to purify IgG from mouse hybridoma culture supernatants (Lewis et al, 2009). Nizard et al, 1998 have constructed a fusion protein T-zz and demonstrated the capability of zz domain binding to mouse monoclonal or rabbit polyclonal IgG. The interaction of IgG with zz was stable at pH 5 and distrupted only below pH 3.5. Fassina et al., 2001 have demonstrated the application of combinatorial technologies and molecular modeling for the discovery of synthetic ligands which has new avenues for the development of more efficient, less expensive and more importantly safer procedures for antibody purification at the industrial level. They have showed the high capacity of sorbents derivatized with PAM and ApA for certain classes of immunoglobulins such as IgM, IgA, IgE and IgY and thus expanded and facilitated the purification of immunoglobins other than IgG.

The overall objective in my current work is to synthesize and utilize zz domain which binds to the F_C region from most mammalian species. We have created the zz fusion proteins with ELP and used these fusion proteins for the purification of antibody from different sources efficiently.

CHAPTER 3

Objective/Experimental Approach

3.1 Materials and Methods

The IgGs from human, mouse and rabbit serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). The supernatant of hybridoma cell culture (C1B7) was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA, USA).

3.2 Molecular Biology, Bacterial Strains and Plasmids

Restriction endonucleases, Calf intestinal alkaline phosphatase (CIP), Phusion polymerase, Phire polymerase and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Plasmid DNA was purified using spin miniprep kits from Zymo Research (Irwine, CA). The pET-24a expression vector and the *E. coli* BLR(DE3) strain were purchased from Novagen Inc. (Milwaukee, WI). All cultures were grown and maintained in LB Broth (Tryptone 10 g/L, Sodium Chloride 5 g/L, Yeast extract 5g/L). The protein production was achieved in Terrific broth(Yeast extract 24g/L, Tryptone 12 g/L, Dipotassium phosphate 12.5 g/L, Monopotassium phosphate 2.3 g/L).Custom oligonucleotides were synthesizedby Integrated DNA Technologies, Inc. (Coralville, IA).

3.3 Construction of expression vectors

3.3.1 Nomenclature of ELP

The ELP polymer is composed of Val-Pro-Gly-Xaa-Gly repeats, where Xaa is a guest residue. The various ELP constructs can be differentiated using the following notation ELP[$X_iY_jZ_k$-n], where the bracketed single letter are single letter amino acid codes specifying the guest residue, and the subscript designate the number of Val-Pro-

Gly-Xaa-Gly for each guest residue in the ELP monomer. The total number of ELP gene in number of pentapeptides is specified by n. For example, ELP[$V_5A_3G_2$-110] is an ELP of 110 pentapeptides in length that has a repeat unit consisting of 10 peptides with Val, Gly and Ala in 5:3:2 ratio respectively.

3.3.2 Synthesis of ELP

3.3.2.1 Monomer ELP synthesis

For the ELP[KV8F] library, a synthetic gene (182 bp, Figure 2) was constructed from PAGE purified synthetic oligonucleotides i.e. KV8F-FP1, KV8F-FP2, KV8F-RP1 and KV7F-RP2 (Table 1). The oligonucleotides were annealed by heating the mixture of four oligonucleotides (2 μM) to 95^{o}C and gradually cooling to room temperature with double stranded DNA with *Hind* III and *Eco* R1 compatible ends. The annealed oligonucleotides were then phosphorylated using 20 Units of T4 polynucleotide kinase in a total volume of 20 μL. pBluescript II SK(+) was codigested with *Eco*R I and *Hind* III and enzymatically dephosphorylated using CIP. The linearized pBluescript II SK(+) vector was then purified using a microcentrifuge spin column purification kit and eluted in sterile, deionized water. The digested vector (~0.1 pmole) was ligated to ~1 pmole of phosphorylated annealed oligonucleotides in a 10ul reaction with 100 Weiss Units of T4 DNA ligase and incubated at 16^{o}C overnight. The ligation mixture was transformed into chemically competent *E.coli* DH5α competent cells by heat shock. The transformants were spread on LB-Ampicillin-Xgal plates and incubated at 37^{o}C. Colonies were initially screened by blue white screening. The plasmids were isolated from white colonies and

confirmed by sequencing at Institute for Integrative Genomic Biology Instrumentation Facility, UCR.

3.3.2.2 Oligomerization of ELP

Oligomerization of ELP is achieved by recursive directional ligation. In the first round of oligomerization, ELP[KV8F-20] was constructed by ligating 10-mer insert into a vector containing 10-mer gene as follows. The pBluescript vector containing ELP[KV8F-10] was linearized with *Pfl*MI, dephosphorylated with CIP and then purified using a microcentrifuge spin column purification kit. Insert ELP[KV8F-10] was excised by digesting a separate sample of ELP[KV8F-10] with *Pfl*MI and *Bgl*I and purified using a microcentrifuge spin purification kit. The purified linearized vector and insert were ligated and transformed in *E. coli* DH5α. The transformants were initially screened by colony PCR, restriction digestion and later confirmed by sequencing. The additional rounds of ELP oligomerization proceed in a similar way, except the products of previous rounds serve as starting material for subsequent oligomerization.

3.3.2.3 Expression Vector Construction for ELP

The expression vector pET24a was modified for the compatibility for ELP insertion and insertion of restriction sites for generation of N-terminal and C-terminal ELP fusion proteins. A unique *Sfi* I site is inserted which after digestion with *Sfi* I create N-terminal *Pfl* MI compatible end and C-terminal *Bgl*I compatible end. For the construction of expression vectors having *Sfi* I site at N-terminal and MCS at C-terminal, *Sfi* I site is inserted between *Nde*I and *Bam* HI in pET24a by oligonucleotide cassette

mutagenesis (Figure 1). The pET24a is digested with *Nde*I and *Bam* HI, dephosphorylated with CIP and purified using a kit. The oligonucleotides NB-FL and NB-RL **(**Table 1) were annealed and phosphorylated using 20 U of polynucleotide kinase. The digested vector (~0.1 pmole) was ligated to ~1 pmole of phosphorylated annealed oligonucleotides in a 10 µl reaction with 100 Weiss Units of T4 DNA ligase and incubated at 16^{o}C overnight. The ligation mixture was transformed into chemically competent *E. coli* DH5α competent cells by heat shock. The transformants were spread on LB-Ampicillin-Xgal plates and incubated at 37^{o}C. Colonies were initially screened by colony PCR and later confirmed by sequencing. For the construction of expression vectors having *Sfi* I site at C-terminal and MCS at N-terminal, *Sfi* I site is inserted between *Nde*I and *Xho*I in pET24a by oligonucleotide cassette mutagenesis. The pET24a is digested with *Nde*I and *Xho* I, dephosphorylated with CIP and purified using a kit. The oligonucleotides NXFL1, NXFL2, NXRL1 and NXRL2 were annealed and phosphorylated using 20U of polynucleotide kinase. The digested vector was ligated to annealed oligonucleotides in the similar way as described previously and transformed in *E. coli* DH5α. The positive transformants were screened by colony PCR and later confirmed by sequencing.

3.3.2.4 Construction of ELP-zz fusions

The zz-domain (208 bp) was constructed by overlapping oligonucleotides ZZ-FL1, ZZ-FL2, ZZ-FL3, ZZ-FL4, ZZ-FL5, ZZ-RL1, ZZ-RL2, ZZ-RL3, ZZ-RL4 and ZZ-RL5. The nucleotides were annealed by heating the mixture of ten oligonucleotides (2

μM) to 95^{o}C and gradually cooling to room temperature with double stranded DNA with *Xma* I and *Bam* H1 compatible ends. The annealed oligonucleotides were then phosphorylated using 20 Units of T4 polynucleotide kinase in a total volume of 20 μL. pET-ELP [V-78] was codigested with *Xma* I and *Bam*HI and enzymatically dephosphorylatedusing CIP. The digested vector (~0.1 pmole) was ligated to ~1 pmole of phosphorylated annealed oligonucleotides in a 10 μl reaction with 100 Weiss Units of T4 DNA ligase and incubated at 16 ^{o}C overnight. The ligation mixture was transformed into chemically competent *E.coli* DH5α competent cells by heat shock. The transformants were spread on LB-Ampicillin plates and incubated at 37^{o}C. Colonies were initially screened by colony PCR and later confirmed by sequencing at Institute for Integrative Genomic Biology Instrumentation Facility, UCR.

3.3.2.5 Construction of N-terminal and C-terminal ELP [KV_8F-40, 60, 80]-zz and ELP[$V_5A_3G_2$-110] fusion proteins.

For the sub-cloning of the zz domain in pET-ELP[KV8F-40, 60, 80] libraries at N-terminal, the zz domain was amplified using *NdeI*-zz-FP and zz-*SacI*-RP (Table 1). The amplified zz domain was digested with *Nde* I and *Sac* I and sub-cloned in the pET-ELP[KV8F-40, 60, 80] libraries. For the sub-cloning of the zz domain at C-terminal, the zz domain was amplified using *Bam*H1-zz-FP and *Xho*I-zz-RP (Table 1) and was subcloned in the pET-ELP[KV8F-40, 60, 80] libraries using *Bam* HI and *Xho* I restriction sites. For the construction of ELP[$V_5A_3G_2$-110]-zz fusion protein, the zz-domain was

amplified with FP-zz-amp and RP-zz-amp and was cloned in between *Sac* I and *Xho* I in ELP[$V_5A_3G_2$-110]-Intein-GFP.

3.4 Expression and Purification of Fusion Proteins

For all fusion proteins, the plasmid was isolated from E. coli strain DH5α and transformed to E. coli strain BLR. A single colony was inoculated from the obtained transformants in 3 mL Luria broth cultures supplemented with 100 µg/mL ampicillin to express the fusion protein ELP [V-78]-zz. Similarly, the protein was expressed for ELP[KV8F] series using 100 µg/mL kanamycin as the antibiotic resistance. The cultures were grown overnight at 37^{o}C and 250 rpm in an orbital shaker. From this required amount of inoculum was used to inoculate 100 mL of terrific broth (12 g tryptone, 24 g yeast extract, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4/L) supplemented with 100 µg/mL ampicillin to transfer an O.D. of 0.1 for ELP [V-78]-zz. The culture was further grown for another 44-48 h at 37^{o}C and 250 rpm in an orbital shaker. The cells were then harvested by centrifuging at 5000 RPM at 4^{o}C for 15 min and resuspended back in 10 mM Tris pH 8.0 (Tb8) buffer (with 1 mg/ml lysozyme and 1X protease inhibitor) to make $O.D._{600}$ = 20. In case of ELP[KV8F] series fusion proteins, the cell pellet was resuspended back in phosphate –buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7). Cells were then lysed by sonication (5 sec pulse on, 5 sec pulse off, total 10 min run time) and cell debris were removed by centrifugation for 20 min at 4^{o}C and 15000g. The supernatant i.e. the cell lysate containing ELP fusions

was recovered and purified by repeated inverse temperature transition by modifying the procedures of Kim et al, 2005. The transition temperature was lowered by the addition of 5 M NaCl salt solution to the cell lysate for a final salt concentration of 1M NaCl (Fong et al, 2009). The samples were incubated at 37^{o}C for 20 min and then centrifuged at 15000g at 37^{o}C for 20 min to precipitate ELP fusion protein in form of pellet. Next, the pellet containing ELP and other insoluble proteins was dissolved in ice-cold PBS and centrifuged at 15000g at 4^{o}C for 15 min to remove any insoluble proteins. The ELP remains soluble at 4^{o}C and therefore the supernatant contains the purified ELP fusion protein. This ELP fusion protein is carried out through another cycle of temperature transition and removal of insoluble proteins, and finally purified ELP fusion protein is dissolved back in ice-cold PBS. The purity of final purified proteins was checked on 10% SDS-PAGE. The concentration of the fusion protein was determined by the spectrophotometric measurement at 215 nm based on the coefficient and calibration as shown by Stiborova et al, 2003.

3.5 Purification of IgG by binding with zz domain of fusion protein

For IgG purification experiments, 500 µl of IgG (1 mg/ml) was mixed with 500 µl of fusion protein and incubated for 2 hours with continuous shaking at room temperature. To recover the ELP-zz-IgG complex, 200 µl of 5M NaCl was added to the sample to make final NaCl concentration as 1M and incubated for 20 min at 37^{o} C. After that it was centrifuged at 15000g at 37^{o} C and the supernatant was removed. The complex in the

form of pellet was recovered and resolubilized in ice-cold PBS buffer. The IgG from this resolubilized complex was eluted by incubating the dissolved complex with arginine pH 3.8 buffer in the volume ratio of 1:8 for complex to elution buffer. The final concentration of elution buffer in the solution was .5M. The resolubilized complex was incubated with this buffer for 2 hours at 4^{o} C. The eluted IgG was recovered by performing the temperature transition cycle at 37^{o} C and finally all the samples at different steps were analysed on 10% SDS-PAGE. Similar binding and elution experiments were also carried for different fusion proteins with antibodies from different sources.

CHAPTER 4

Results

4.1 Production of ELP-zz fusion proteins

In the prior work conducted by our group, the antibody binding domains ProG/L were fused to ELPs, which proved to be effective for antibody purification. The production of ELP[V-78]-ProL and ELP[V-78]-ProG fusion proteins was found to be ~100 mg/L and ~400 mg/L respectively. However, the protein expression was low in case of ELP[V-78]-ProL because of the larger binding domain ProL. Moreover, ELP[V-78]-ProG/L showed limited specificity for antibodies (Kim *et al*, 2005). In order to overcome these limitations, a shorter binding domain-zz (~8 kDa) derived from Staphylococcal protein A (SpA) was fused to ELP protein and has been used for antibody purification.

The expression of ELP[V-78]-zz fusion protein was observed to be ~500 mg/L which was fivefold higher than the expression of ELP[V-78]-ProL (~100 mg/L) (Figure 3).

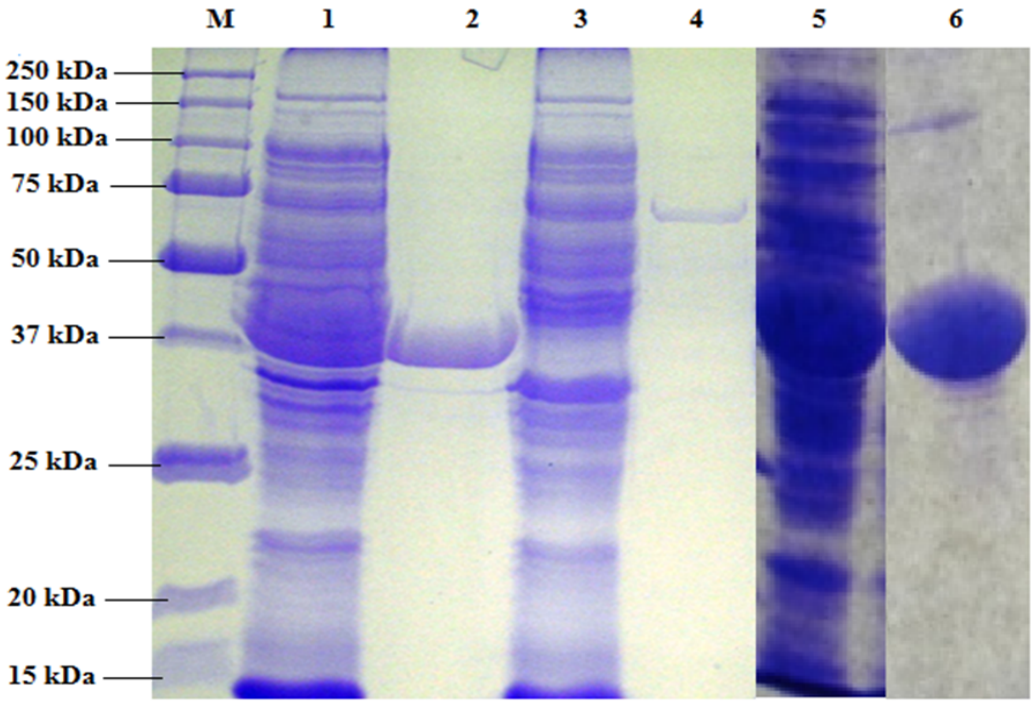


Figure 3. Production and purification of ELP-ZZ fusion proteins. The purity of protein was analyzed on 10% SDS-PAGE followed by Coomassie Blue staining. **Lane M**: Marker; **Lane 1**: cell lysate for ELP[V-78]-ZZ; **Lane 2**: Purified ELP[V-78]-ZZ; **Lane 3**: Cell lysate for ELP[V-78]-Protein L; **Lane 4**: Purified ELP[V-78]-Protein L; **Lane 5**: Cell lysate for ELP[KV8F]-79; **Lane 6**: Purified ELP[KV8F-79].

The enhanced expression of ELP[V-78]-zz fusion protein is attributed to the fact that the shorter peptides fused to ELPs result in higher recombinant protein yield. This increased expression also gave an insight to explore more on the ELP domain of fusion protein for even higher yields. The amino acid sequence of ELPs has been optimized for a protein to maximize its expression yield and purification efficiency. The incorporation of ionizable Lysine residues in ELPs enhanced salt sensitivity compared to aliphatic ELPs, thereby reducing the amount of salt needed to induce inverse phase transition in the soluble lysate. Also, it has been observed that the addition of hydrophobic

Phenylalanine residues in ELPs lowers the T_t of the ELP (Lim *et al*, 2007). Lim *et al*, 2007 had constructed libraries of ELP[KV_2F] and ELP[KV_7F] and observed that the T_t of ELP[KV_2F] series was very similar to ELP[$V_5A_2G_3$] of comparable molecular weights. However, the T_t of ELP[KV_7F] was found to be approximately 20^oC lower than ELP[$V_5A_2G_3$] having comparable molecular weights. Hence, we generated libraries of ELP[KV_8F] having repeats of 40, 60 and 80. The yield of purified protein from ELP[KV_8F] libraries (~1000 mg/L) was double the amount obtained in case of ELP[V-78](~500 mg/L). Further these ELP[KV_8F] repeats were fused to zz-domain either at N-terminal or C-terminal. The protein production from these fusion proteins ELP[KV_8F]-zz was similar to ELP[KV_8F] of comparable molecular weights (Figure 3).

Figure 4 shows coomassie blue stained 10% SDS-PAGE gels of purified ELP[V-78] and ELP[KV8F] at all stages of purification. These ELP fusion proteins were purified using two-step temperature transition cycles at a salt concentration of 1M NaCl and at temperature 37^oC. All the ELP was precipitated in the form of pellet during both thermal cycles without any loss of ELP in the supernatant (Figure 4). Also, it was observed that ~100% ELP present in the pellet was solubilized at 4^oC and hence there was no loss of ELP along with the insoluble proteins.

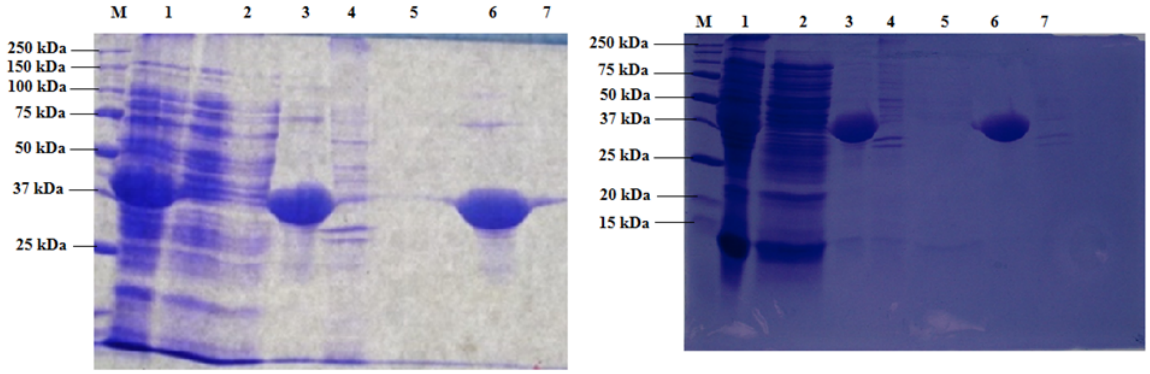


Figure 4. Purification of protein using two-step temperature transition cycles at a salt concentration of 1M NaCl and at temperature 37°C. **A.** Purification of ELP[V8F]-zz. **Lane M**: Marker; **Lane 1**: E.coli BLR ELP[V8F]-zz cell lysate; **Lane 2**: 1st cycle supernatant; **Lane 3**: 1st cycle pellet; **Lane 4**: 1st cycle insoluble pellet; **Lane 5**: 2nd cycle supernatant; **Lane 6**:2nd cycle pellet; **Lane 7**: 2nd cycle insoluble pellet. **B.** Purification of E.coli BLR ELP[V-78]. **Lane M**: Marker; **Lane 1**: E.coli BLR ELP[V-78]-zz cell lysate; **Lane 2**: 1st cycle supernatant; **Lane 3**: 1st cycle pellet; **Lane 4**: 1st cycle insoluble pellet; **Lane 5**: 2nd cycle supernatant; **Lane 6**: 2nd cycle pellet; **Lane 7**: 2nd cycle insoluble pellet.

4.2 IgG Purification

ELPs fused with antibody binding domains (ProG/L) have been utilized for the purification of antibodies using inverse transition cycling property of ELP fusion proteins (Kim *et al*, 2005). In the current work, we utilized ELPs fused with zz domain for the efficient purification of antibody. In order to compare the binding efficiency of fusion domains ProL and zz, antibody binding experiments were conducted in parallel utilizing their respective fusion proteins ELP[V-78]-ProL and ELP[V-78]-zz. For the antibody binding experiments, 500 µl of human IgG (1 mg/ml) was incubated with 500 µl of fusion protein ELP[V-78]-zz in the molar ratio of 1:7 at room temperature with continuous shaking for 2 h. After incubation, 1 M NaCl was added followed by thermal precipitation cycle at 37°C to precipitate the antibody in form of binding complex

(ELP[V-78]-zz-HIgG). This complex was then resolubilized in ice-cold PBS. For comparison, similar experiments were carried out using ELP[V-78]-ProL. All the fractions were analyzed by 10% SDS-PAGE and then quantified by Image J software. As shown in Figure 5, 85% HIgG was recovered by ELP[V-78]-ProL in the form of binding complex and 15% of unbound antibody was left in the supernatant. Also, there was no loss of fusion protein in the supernatant during the thermal cycle precipitation. This clearly depicts the fact that with the molar ratio (7:1) of fusion protein and antibody, complete recovery of HIgG is not possible. However, in case of ELP[V-78]-zz, 90% of HIgG is recovered in the form of pellet as binding complex and an equal proportion of antibody and fusion protein is retained in the supernatant. This indicates that with the molar ratio (7:1) of fusion protein and HIgG, ELP[V-78]-zz could bind all the HIgG but a fraction of binding complex (ELP[V-78]-zz-HIgG) could not precipitate in form of pellet. Also, the ELP concentration in the binding reaction is insufficient to achieve complete precipitation of ELP[V-78]-zz-HIgG.

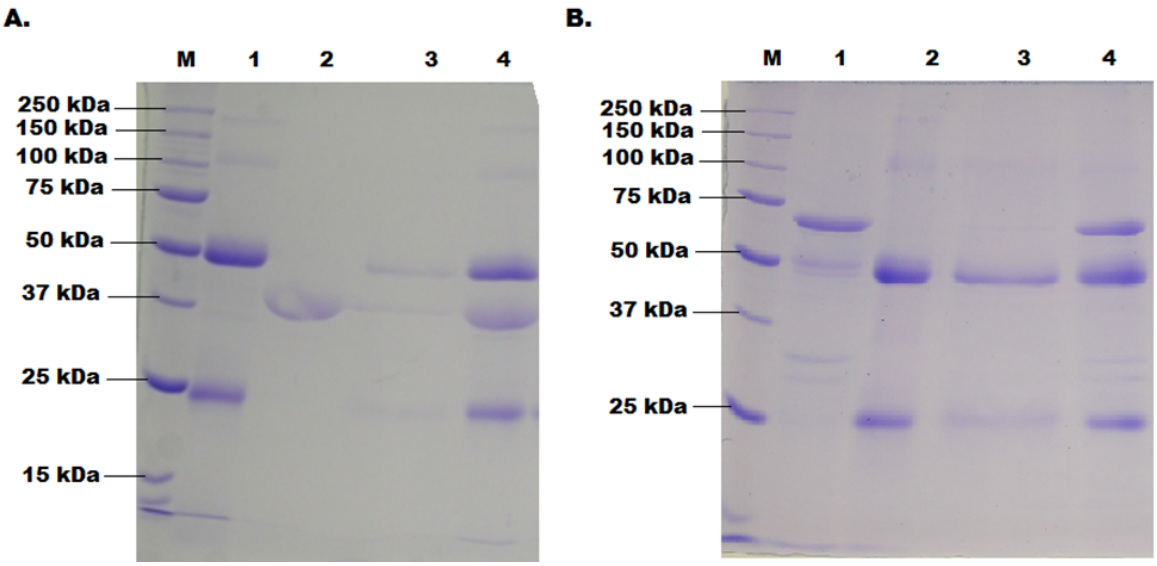


Figure 5. Comparison of Human IgG binding with ELP-fusion proteins (zz and Protein L). **A.** Binding of HIgG with ELP[V-78]-zz. **Lane M**: Marker; **Lane 1**: HIgG; **Lane 2**: ELP[V-78]-zz; **Lane 3**: Fraction of antibody complex ELP[V-78]-zz-HIgG left in supernatant; **Lane 4**: Pellet of antibody complex ELP78-zz-HIgG. **B.** Binding of HIgG with ELP[V-78]-ProL. Lane M: Marker; **Lane 1**: ELP[V-78]-ProL; **Lane 2**: HIgG; **Lane 3**: Unbound HIgG in supernatant; **Lane 4**: Pellet of antibody complex ELP[V-78]-Pro L-HIgG.

This issue was resolved by increasing the final molar ratio of ELP and HIgG in the binding reaction from 7:1 to 14:1. The molar ratio of 14:1 was achieved by using a cocktail made of ELP[V78] and ELP[V78]-zz in the same molar ratio instead of only fusion protein(ELP[V78]-zz). Figure 6 shows 100% recovery of antibody in the form of binding complex. This explains the amount of fusion protein (ELP[V78]-zz) present in the cocktail was sufficient enough to bind all the antibody and presence of ELP[V78] in the binding reaction mixture helped in complete precipitation of the binding complex.

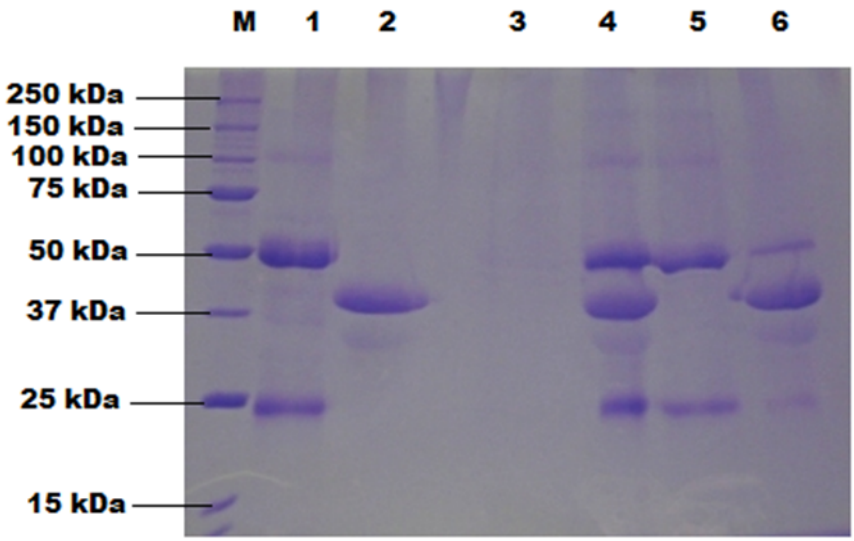


Figure 6: Enhanced recovery of Human IgG using helper ELP (ELP[V-78]) along with ELP[V78]-zz. **Lane M**: Marker; **Lane 1**:HIgG; **Lane 2**: ELP(ELP[V-78]) &ELP[V78]-zz; **Lane 3**: Supernatant after precipitation of antibody complex ELP[V-78]-zz-HIgG with helper ELP (ELP[V-78]); **Lane 4**: Pellet of antibody complex ELP78-zz-HIgG with helper ELP (ELP[V-78]); **Lane 5**: Recovered HIgG after elution with 0.5 M Arginine pH 3.8; **Lane 6**: Recovered ELP[V78]-zz and helper ELP (ELP[V-78]) after elution with 0.5 M Arginine pH 3.8.

To recover the bound HIgG from the binding complex (ELP[V-78]-zz-HIgG), different elution conditions were investigated (Figure 6). Approximately, 91% recovery of HIgG was achieved using 0.5 M arginine pH 3.8 buffer (Figure 6). However, the buffers 1M acetic acid (pH 2.8) and 0.1 M Glycine (pH 2.0) could not recover any antibody (Figure 7). Arakawa *et al*, 2004 have optimized elution conditions of humanized monoclonal IgG4 from Protein A affinity column and reported that 92% of IgG4 was recovered efficiently by using 0.5 M arginine pH 3.8. The use of arginine suppresses aggregation of unfolded or partially folded structures but does not denature proteins

(Shiraki*et al*, 2002). Arginine also enables dissociation of protein from protein-protein complexes (Arakawa *et al*, 2004).

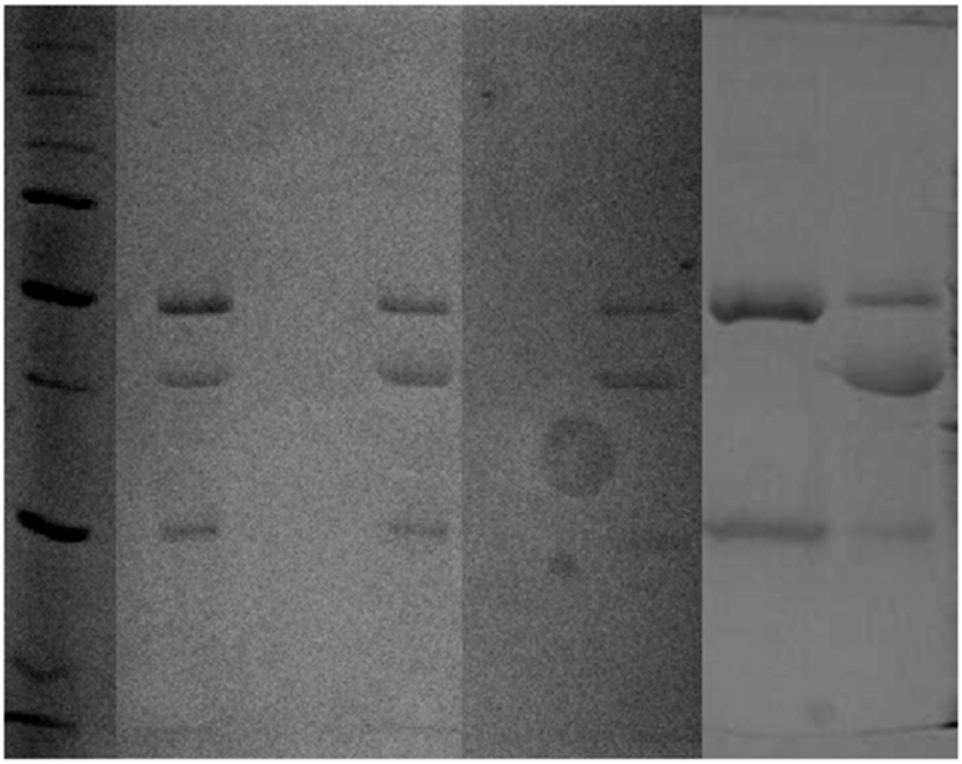


Figure 7: Recovery of Human IgG from binding complex (HIgG-ELP-zz) after elution. **Lane M**: Marker; **Lane 1**: Binding complex (HIgG-ELP-zz); **Lane 2**: Recovered HIgG after elution with 1M acetic acid (pH 2.8); **Lane 3**: Recovered ELP-zz after elution with 1M acetic acid (pH 2.8); **Lane 4**: Recovered HIgG after elution with 0.1 M Glycine pH 2.0; **Lane 5**: Recovered ELP-zz after elution with 0.1 M Glycine pH 2.0; **Lane 6**: Recovered HIgG after elution with 0.5 M Arginine pH 3.8; **Lane 7**: Recovered ELP-zz after elution with 0.5 M Arginine pH 3.8.

In order to utilize the benefit of two-fold higher protein yield of ELP[KV_8F]-zz series and lower T_t, binding and elution studies were also carried out using ELP[KV_8F]-zz repeats. As shown in Figure 8, 90% of the HIgG was precipitated in the form of binding complex (ELP[KV_8F-80]-zz-HIgG) using molar ratio of 7:1 for fusion protein (ELP[KV_8F-80]-zz) and antibody. Also, 90% HIgG was recovered by using 0.5 M

arginine pH 3.8 buffer. Similar HIgG binding results were also obtained with ELP[KV_8F-60]-zz (Figure 8) and ELP[KV_8F-40]-zz (data not shown). Therefore, the quantity of fusion protein produced from the same amount of culture in case of ELP[KV_8F]-zz series enables recovery of more antibody as compared to ELP[V-78]-zz .

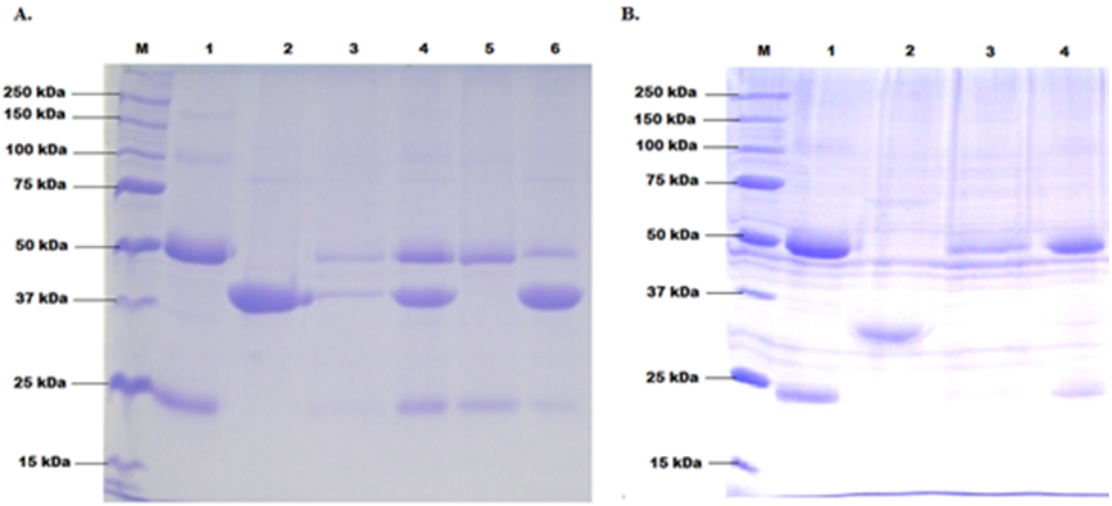


Figure 8: Binding of Human IgG with ELP[KV8F-60,80]-zz and elution of HIgG from ELP[KV8F-60, 80]-zz-HIgG. **A.** Binding of Human IgG with ELP[KV8F-80]-zz and elution of HIgG from ELP[KV8F-80]-zz-HIgG. **Lane M**: Marker; **Lane 1**: HIgG; Lane 2: ELP[KV8F-80]-zz; **Lane 3**: Fraction of antibody complex ELP[KV8F-80]-zz-HIgG left in supernatant; **Lane 4**: Pellet of antibody complex ELP[KV8F-80]-zz-HIgG; **Lane 5**: Recovered HIgG after elution with 0.5 M Arginine pH 3.8; **Lane 6**: Recovered ELP-zz after elution with 0.5 M Arginine pH 3.8. **B.** Binding of Human IgG with ELP[KV8F-60]-zz and elution of HIgG from ELP[KV8F-60]-zz-HIgG. **Lane M**: Marker; **Lane 1**: HIgG; **Lane 2**: ELP[KV8F-60]-zz; **Lane 3**: Fraction of antibody complex ELP[KV8F-60]-zz-HIgG left in supernatant; **Lane 4**: Pellet of antibody complex ELP[KV8F-60]-zz-HIgG.

Further, similar binding studies were also carried out using antibody from different sources. As shown in Figure 9, 92%, 95% and 100% of rabbit IgG was recovered using fusion proteins ELP[V-78]-zz, ELP[KV_8F-60]-zz and ELP[KV_8F-80]-zz respectively. Also, both fusion proteins ELP[V-78]-zz and ELP[KV_8F-80]-zz enabled

85% recovery of mouse IgG in one step process (Figure 10). The antibody recovery efficiencies from different sources using fusion proteins ELP[V-78]-zz, ELP[KV_8F-60]-zz and ELP[KV_8F-80]-zz were higher than reported earlier using fusion domains ProG/L and other chromatographic separations (Lewis and Rehm, 2009; Kim *et al*, 2005; Thomas *et al*, 2002; Dancette *et al*, 1999).

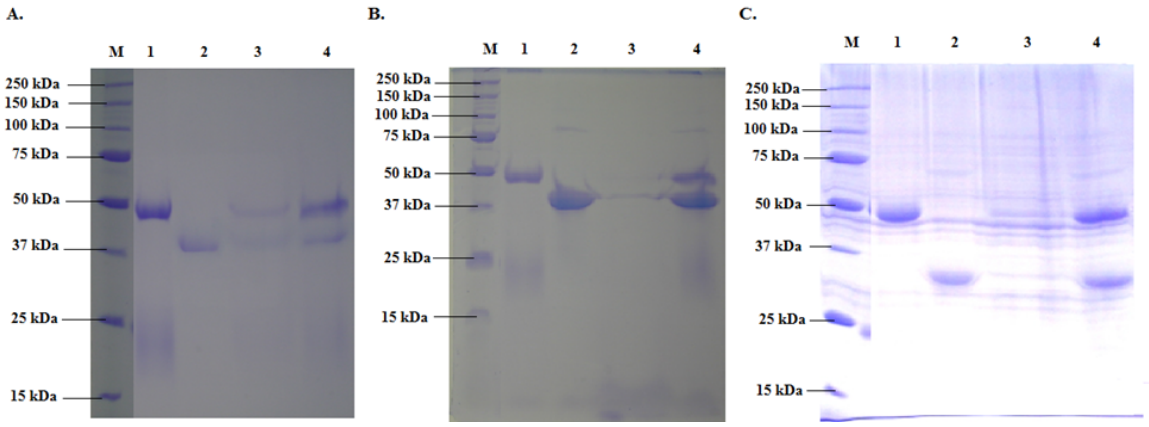


Figure 9: Recovery of Rabbit IgG using ELP[V-78]-zz, ELP[KV8F-80]-zz and ELP[KV8F-60]-zz. **A.** Binding of RIgG with ELP[V-78]-zz. **Lane M**: Marker; **Lane 1**: RIgG; **Lane 2**: ELP[V-78]-zz; **Lane 3**: Fraction of antibody complex ELP[V-78]-zz-RIgG left in supernatant; **Lane 4**: Pellet of antibody complex ELP[V-78]-zz-RIgG. **B.** Binding of RIgG with ELP[KV8F-80]-zz. **Lane M**: Marker; **Lane 1**: RIgG; **Lane 2**: ELP[KV8F-80]-zz; **Lane 3**: Fraction of antibody complex ELP[KV8F-80]-zz-RIgG left in supernatant; **Lane 4**: Pellet of antibody complex ELP[KV8F-80]-zz-RIgG. **C.** Binding of RIgG with ELP[KV8F-60]-zz.Lane M: Marker; **Lane 1**: RIgG; **Lane 2**: ELP[KV8F-60]-zz; **Lane 3**: Fraction of antibody complex ELP[KV8F-60]-zz-RIgG left in supernatant; **Lane 4**: Pellet of antibody complex ELP[KV8F-60]-zz-RIgG.

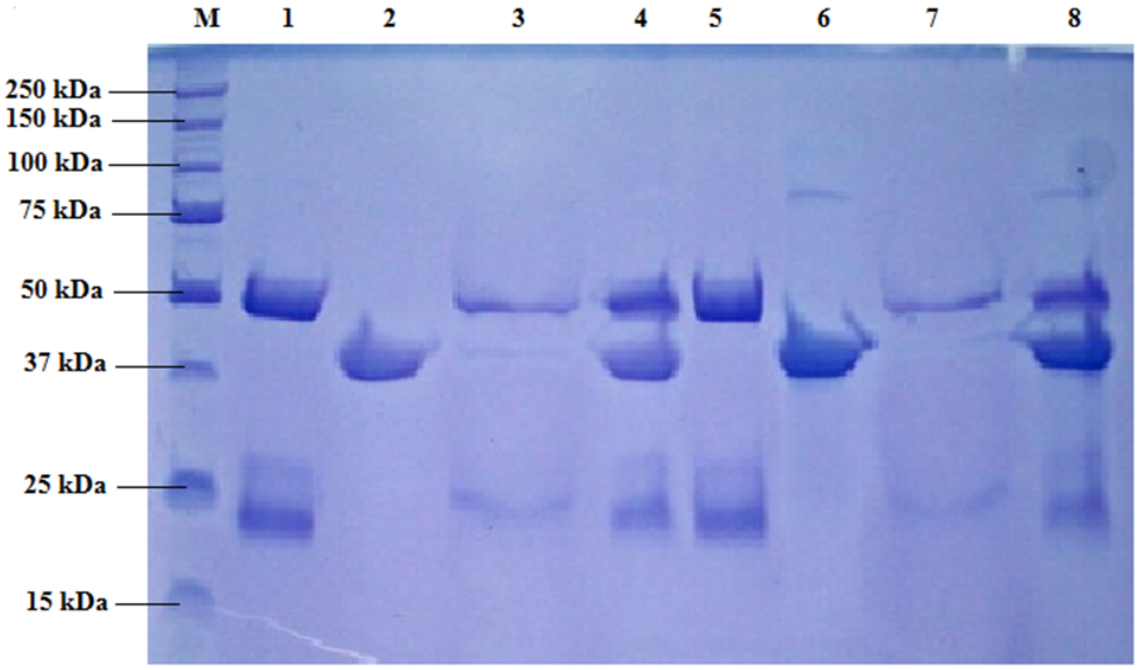


Figure 10. Recovery of Mouse IgG using ELP[V-78]-zz, ELP[KV8F-80]-zz. **Lane M:** Marker; **Lane 1**: RIgG; **Lane 2**: ELP[V-78]-zz; **Lane 3**: Fraction of antibody complex ELP[V-78]-zz-RIgG left in supernatant; **Lane 4**: Pellet of antibody complex ELP[V-78]-zz-RIgG; **Lane 5**: RIgG; **Lane 6**: ELP[KV8F-80]-zz; **Lane 7**: Fraction of antibody complex ELP[KV8F-80]-zz-RIgG left in supernatant; **Lane 8**: Pellet of antibody complex ELP[KV8F-80]-zz-RIgG.

CHAPTER 5

Conclusions

Among the non-chromatographic affinity separations, ELP biopolymers fused with antibody binding domains can be easily expressed in microorganisms and purified easily by taking advantage of the temperature transition properties. Also, the characteristics of these fusion proteins can be controlled by varying the length and amino acid composition of ELP. The antibody binding affinity has already been demonstrated using ProL/G as fusion domains with ELP. The ELP domains have been fused to Protein G or Protein L and have been used for the separation of IgG. However, ELP fused to larger antibody binding domains i.e Pro G/L, resulted in 10-fold lower protein yield. So, in the current study the ELP was fused to shorter amino acid domain (zz-domain) which gave enhanced expression. Moreover, with the change in ELP domain i.e. ELP[KV8F] series led to a further increase in production of purified fusion protein.

The increased production of fusion proteins from the same amount of culture as in ELP[V-78] led to higher recovery of antibody. It was observed that with the molar ratio (7:1) of fusion protein and HIgG, ELP[V-78]-zz could bind all the HIgG which was not possible in the case of ELP[V-78]-ProL. Also, in order to recover complete antibody in form of pellet complex ELP[V-78]-zz-HIgG, the cocktail made of ELP[V-78] and ELP[V-78]-zz in equimolar ratio was used which enabled 100% recovery of antibody. For the elution, 0.5 M arginine (pH 3.8) buffer was used which enabled 91% recovery of HIgG. The antibody was also recovered efficiently utilizing the new series of ELP[KV8F]. These fusion proteins were also employed for successful recovery of antibody from different sources like mouse and rabbit. 92%, 95% and 100% of rabbit IgG

was recovered using fusion proteins ELP[V-78]-zz, ELP[KV8F-60]-zz and ELP[KV8F-80]-zz respectively. Also, both fusion proteins ELP[V-78]-zz and ELP[KV8F-80]-zz enabled 85% recovery of mouse IgG in one step process.

In future experiments, these fusion proteins can be tested to recover antibody from complex solutions like hybridoma cell culture supernatant. The activity of the purified antibody can be checked for its further performance. The ELP fusion proteins available after purifying the antibody can be reused to evaluate their efficiency for further purification. Hence, in the present study efficient ELP fusion proteins were generated for successful recovery of antibody from different sources with maximum efficiency. Due to its high level production and affinity for different IgGs, we believe that these ELP-zz fusions will be useful as an economical, highly efficient, and universal platform for the purification of antibodies.

Table 1: Oligonucleotides used in the study

Oligonucleotide	Sequence (5'-3')
KV8F-FP1	AGCTTATATGGGCCACGGCGTGGGTGTTCCGGGCAAAGGTGTCCC AGGTGTGGGCGTACCGGGCGTTGGTGTTCCGGGCGTTGGTGTCCC AG
KV8F-FP2	GTGTGGGCGTTCCGGGCTTTGGTGTCCCAGGTGTGGGCGTACCGG GCGTTGGTGTTCCGGGCGTTGGTGTGCCGGGCGGGCTGAAATGAG
KV8F-RP1	AATTCTCATTTCAGCCCGCCCGGCACACCAACGCCCGGAACACCA ACGCCCGGTACGCCCACACCTGGGA
KV8F-RP2	CACCAAAGCCCGGAACGCCCACACCTGGGACACCAACGCCCGGA ACACCAACGCCCGGTACGCCCACACCTGGGACACCTTTGCCCGGA ACACCCACGCCGTGGCCCATATA
NB-FL	**TATGAGCAAAGGGCCGGGCTGGCCGG**
NB-RL	**GATCC**CGGCCAGCCCGGCCCTTTGCTCA
NXFL1	TATGCGCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCG
NXFL2	CAGCAAAGGGCCGGGCTGGCCGTGATAAC
NXRL1	TCGAGTTATCACGGCCAGCCCGGCCCTTTGCTGCGGCCGCAAGC
NXRL2	TTGTCGACGGAGCTCGAATTCGGATCCGCGCA
zz-FL1	CCGGGTGTAGGTGGCAGCGGCAGCG
ZZ-FL2	GCAGCGTAGACAACAAATTCAACA AAGAACAACAAAACGCGT TCTATGAG
ZZ-FL3	ATCTTACATTTACCTAACTTAAACGAAGAACAACGAAACGCCTTC ATCCA
ZZ-FL4	AAGTTTAAAAGATGACCCAAGCCAAAGCGCTAACCTTTTAGCAGA AGCTA
ZZ-FL5	AAAAGCTAAATGATGCTCAGGCGCCGAAATAAG
ZZ-RL1	GATCCTTATTTCGGCGCCTGAGCATCATTTAGC
ZZ-RL2	TTTTTAGCTTCTGCTAAAAGGTTAGCGCT
ZZ-RL3	TTGGCTTGGGTCATCTTTTAAACTTTGGATGAAGGCGTTTCGTTGT TCTT
ZZ-RL4	CGTTTAAGTTAGGTAAATGTAAGATCTCATAGAACGCGTTTTGTT

	GTTCT
ZZ-RL5	TTGTTGAATTTGTTGTCTACGCTGCCGCTGCCGCTGCCACCTACAC
CP-KV8F-FP	AGCTTATATGGGCCACGGCG
CP-KV8F-RP	AATTCTCATTTCAGCCCGCC
NdeI-zz-FP	GGAATTCCATATGGTAGACAACAAATTCAACAAAG
ZZ-SacI-RP	CCGGAGCTCGCTGCCGCTGCCGCTGCCTTTCGGCGCCTGAGCATC ATTTAG
BamHI-zz-FP	CGCGGATCCGGCAGCGGCAGCGGCAGCGTAG
XhoI-zz-RP	CACCTCGAGTTATTTCGGCGCCTGAGCATCATTTAG
FP-zz-Amp	CGCGAGCTCGGGCAGCGGCAGCGGCAGCGTAGACAAC
RP-ZZ-Amp	CCGCTCGAGTTATTTCGGCGCCTGAGCATC

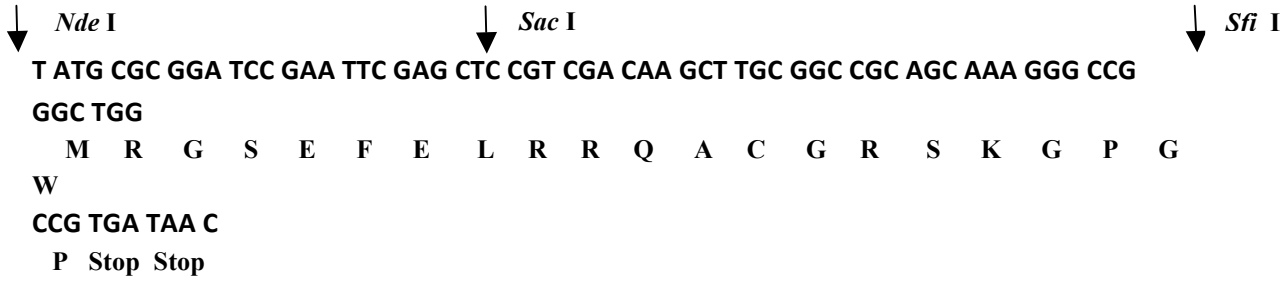
A. ELP [KV_8F-10] gene sequence

B. Modified pET vector sequences

Modified pET24a for ELP expression

T ATG AGC AAA GGG CCG GGC TGG CCG TAA GGA TCC
M S K G P G W P Stop G S

Modified pET24a for N-terminal zz-ELP fusion protein expression



Modified pET24a for C-terminal zz-ELP fusion protein expression

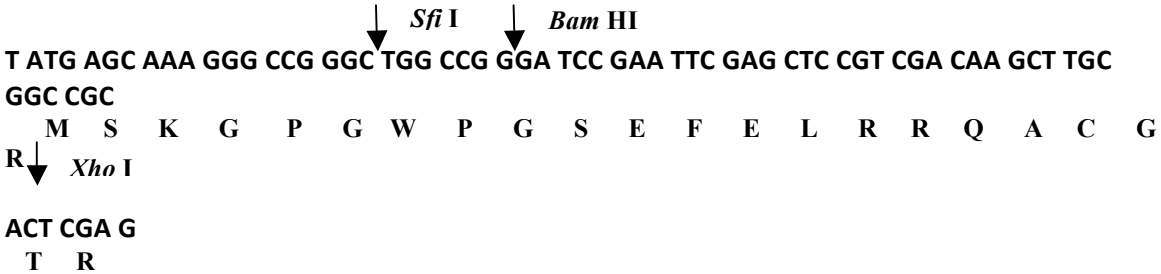


Figure 2. Cloning Strategy

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