Engineering and expression of a polyprotein A construct

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ABSTRACT

Protein A is a surface protein found in the cell wall of bacteria Staphylococcus aureus and is encoded by spa gene. It is extensively used in biomedical research because of its ability to bind to immunoglobulins with high affinity. The folding of five homologous Ig- binding protein domains leads to the composition of this three-helix bundle protein and each domain is capable of binding to many mammalian proteins, essentially IgG. IgG is a type of immunoglobulins (antibodies) circulating in the blood that aid in the phagocytic destruction of antigens. The objective of this project was to create octamers of the two, out of the five, subunits of this protein: subunit B4 and B5. Starting from the monomeric unit, we applied molecular biology protocols and engineered a poly protein A made from eight repeats. The entire project can broadly be classified into three major steps: monomer to dimer, dimer to tetramer, and tetramer to octamer. Each step further had sub steps that included digesting the fragment and vector, ligating them together, screening the ligation product, finally followed by sequencing to double check the obtained product. We have finally expressed this protein and tested its antibody activity using SDS-PAGE gels and other binding assays.

1. Background Knowledge

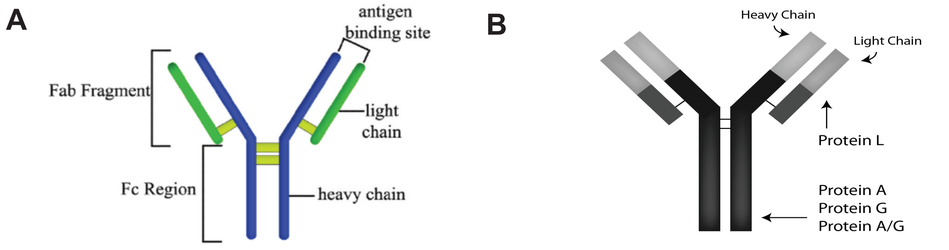

Figure 1. Immunoglobulin G (IgG) binding mechanism: A. Basic structure of Immunoglobulin G: IgG has four plypeptide chains, two identical heavy chains and two identical light chains linked by disulfide and noncovalent bonds making it a complex of 150 kDa. The placement of these chains is such that the molecule forms a Y shape. Both the ends have an identical antigen binding site. The fragment crystallizable (Fc) region of the antibody binds to Fc receptors and some proteins therebyfacilitating the activation of the immune system. The antigen binding (Fab) region binds toantigens and is composed of one constant and one variable domain of the heavy and light chain. **B.** Protein A binding to the IgG antibody: The Fc region of the IgG class antibodies from multiple species binds to Protein A with varying affinity.This binding property of protein A is widely used to isolate mammalian immunoglobulins from different species for commercial purposes.

2. Ligation scheme to make polyconstruct:

Monomer----Dimer----Tetramer----Octamer

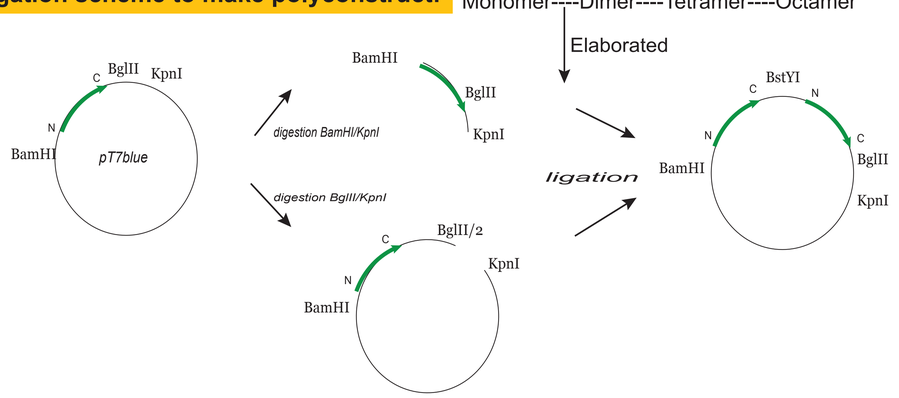

Figure 2. Construction of dimer from monomeric unit: Two restriction enzyme digestions, Bam-Kpn and Bgl-Kpn, were performed to digest the fragment and cut open the vector with the monomer respectively. The fragment was then ligated into the cut open vector resulting in a dimeric unit. The same procedure was followed further with the obtained dimeric unit to obtain the tetramer, and finally with tetramer to get to the octamer of subunit B4 and B5.

3. B4 and B5 DNA fragments on agarose gel:

B4 monomer: 180 base pairs
B4 dimer: 360 base pairs
B4 tetramer: 720 base pairs
B4 octamer: 1440 base pairs
B5 monomer: 192 base pairs
B5 dimer: 384 base pairs
B5 tetramer: 768 base pairs
B5 octamer: 1536 base pairs
pT7 blue: 2900 base pairs

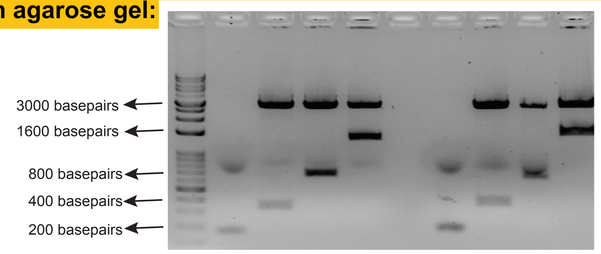

Figure 3. B4 and B5 DNA fragments on agarose gel. The first well has the DNA ladder loaded with the base pairs marked on the left. The next four wells have B4 monomer,dimer,tetramer,and octamer (dimer,tetramer,and octamer being in pT7 blue). Starting from the seventh well(sixth well skipped),the next four have B5 monomer, dimer, tetramer, and octamer (dimer, tetramer, and octamer in vector pT7 blue). As can be clearly referred from the image and the sizes of the different fragments given of the upper left side, all the fragments ran at their correct base pair number.

4. Protein expression:

- Expressed B4 and B5 octamer DNA fragments, ligated into pQE plasmid (expression vector), were transformed into BLR cells for protein expression.Cultures were grown to population density (OD280) of 0.6-0.8 in Luria Broth media before induction with IPTG.
- The cells were collected by centrifugation before undergoing chemical lysis with Lysozyme followed by sonication, which uses high intensity sound waves to disrupt cell wall.
-The mixture of proteins was crudely purified on a Nickel column which binds the 6x his tag on the protein, followed by final purification on a size exclusion column using FPLC (Fast Paced Liquid Chromatography).

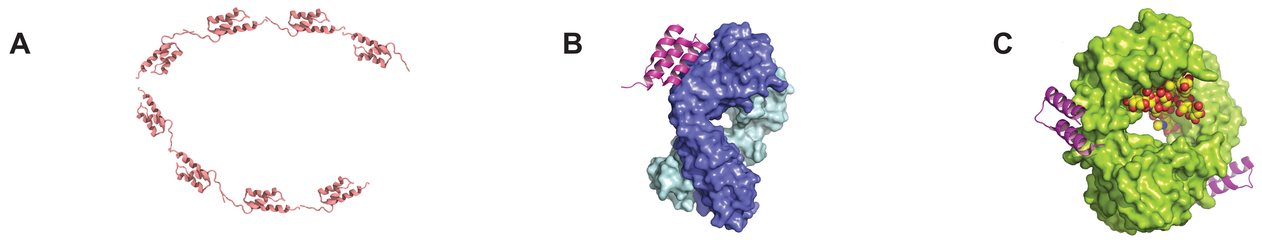

Figure 4. **A** represents a rendering of the protein A octamer molecule forming a three- helix bundle protein. **Figure B** represents the binding of protein A to the antigen binding (Fab) region of an antibody. **Figure C** represents the binding of Protein A to the crystallize fragment (Fc) region of an antibody.

5. Proteins on SDS PAGE gel and Western Blot:

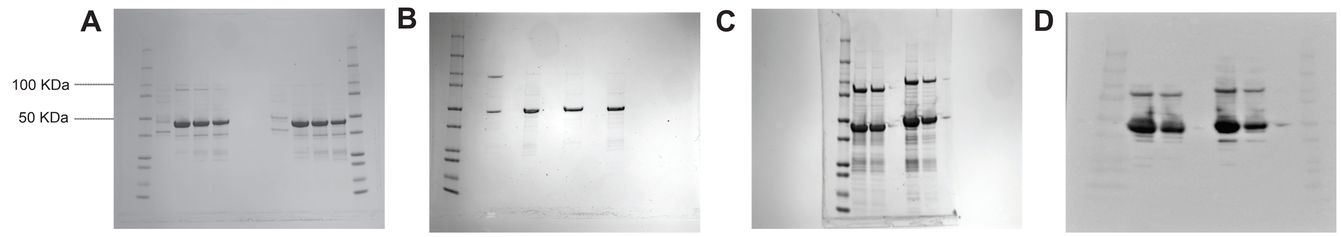

Figure 5. Protein B4 octamer and B5 octamer coomassie stained and western blotted: A. The four peak samples of protein B4 octamer were run on an SDS PAGE gel and comassie stained to check if the protein ran at the correct molecular size. It ran slightly lower than the expected size (around 47KDa).The first well has the standard loaded, then the non denatured protein in the next four wells, skipped two wells, then the denatured protein the the next four wells finally followed by the standard in the last well. **B**. The two peak samples of protein B5 octamer were run on an SDS-PAGE gel and comassie stained to check if the protein ran at the correct molecular size. It ran slightly lower than the expected size (around 50 KDa) but it ran higher than protein B4 octamer as expected due to its slightly larger molecular size. The first well has the standard loaded followed by the non denatured and the denatured protein the next four wells all loaded in an alternate pattern. **C and D.** Two equal sets of concentrations of protein B4 octamer(10 and 20μM) and B5 octamer (10 and 20μM) were run on SDS PAGE gel, half of which was coomassie stained (C) and the other half western blotted (D). The purpose of coomassie staining the gels again is to serve as a replica for the western blotting experiment to be certain that it was the protein that the secondary antibody bound to. The purpose of western blotting the proteins is to check their viability. In the western blotting technique, the proteins were first transferred from the SDS gel on to a PVDF membrane. The membrane was blocked to prevent any non specific binding of antibodies. Finally, a peroxidase conjugated anti-rabbit IgG antibody was allowed to bind to the blotted proteins, which, when reacted with ELC substrate produced a chemiluminescent signal.. As can be seen in figures C and D, both the proteins appear to be at the same level when coomassie stained and western blotted (protein B5 being slightly above protein B4 as expected)thereby showing that the protein A octomers actively bound to the anti-rabbit IgG as detected by the application of ECL reagent.There were bands observed corresponding to base pairs double the actual size showing that these proteins also form viable dimers and these dimers also bind the anti-rabbit IgG antibody as can be seen in figure D.

Conclusions and Outlook

- We successfully engineered protein A to make polyconstructs of its two subunits, B4 and B5, using pT7 blue to make octamer and then pQE to express the protein
- We successfully showed that the proteins made bind to the anti-rabbit IgG antibody.
- Both the subunits actively form dimers and these dimers also bind to the anti- rabbit IgG antibody.

References: 1. Graille M et. al (May 2000). "Crystal structure of a Staphylococcus aureus protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity". 97 (10): 5399–404.
2. Idusogie EE et. al(April 2000). "Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc".164 (8): 4178–84.