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Designing, Cloning, and Expression of Protein G Coding Gene and Evaluating Its Function in Laboratory Conditions

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ABSTRACT:
Background and Purpose: We can obtain accurate information about a specific antigen using immunoglobulins. The absorption chromatography method is used for specific isolation of a class of immunoglobulin. The present study aims to prepare the recombinant Protein G of Streptococcus bacteria, used in absorption chromatography to separate serum IgG. **Methods**: Genomic DNA of Streptococcus bacteria was extracted and a PCR reaction was performed using primers designed based on the Protein G gene, and PCR product was cloned in PET28a plasmid. To isolate the mentioned gene, the recombinant plasmid was digested with xho I and Nco I enzymes. The Protein G gene was purified and subcloned in the expression plasmid. The Protein G gene was amplified by PCR. To examine the size of the recombinant protein, a 12% SDS page was loaded, and protein performance was examined in the ELISA test. **Results**: The PCR product, which has 641 nucleotide pairs, was successfully cloned and confirmed in the expression plasmid. The recombinant protein G is ready to be used in an absorption chromatography column for the isolation of serum IgG. Based on ELISA tests, protein G could identify mouse IgG and rabbit IgG and did not react with chicken IgY. **Conclusion**: This recombinant protein is biologically active and can be used at least for some animal species in immunology laboratories.

Keywords: Protein G, Streptococcus, Cloning, Immunoglobulin

INTRODUCTION:

Monoclonal and polyclonal immunoglobulins or antibodies are reagents used extensively in immunological techniques. Isolation of these antibodies from raw samples of serum or body fluids and culture medium makes it possible to obtain more accurate information about a specific antigen using them. Different methods are used for the specific isolation of a class of immunoglobulin. The absorption chromatography method is more common than the others. In this method, proteins G or A of microbes are used. The non-covalent binding between bacterial proteins A or G and the Fc part of immunoglobulin IgG has caused many researchers to use this feature for isolating IgG. Separation using the absorption chromatography technique is a quick and one-step purification method that separates immunoglobulins of the IgG class from the desired solution and the rest of the impurities, including other classes of immunoglobulins, do not enter the reaction and are removed.

A study by Bhide (2004) examined the possibility of using ELISA designed based on protein G to detect antibodies in deer, mountain rams, and hunting dogs. The results showed that this type of ELISA is a vital and sensitive diagnostic tool in the serological diagnosis of Lyme disease in animals and can be effectively used in serological investigations in various wild mammals (Bhide, 2004). In a study entitled "Development of an ELISA-dependent immunoassay through the use of microplates based on cells containing polyprotein G", Chen et al. (2018) concluded that polymer protein G (8pG) has a better efficiency in trapping antibodies compared to monomer protein G (1pG). The level of antibody coverage from microplates based on pG 8 cells was 23-1.5 times and 6.8-1.2 times higher than microplates based on 1pG cells, respectively. CellpG 8-based microparticles showed a significant improvement in the level of antibody coverage and maintained the homogeneous orientation of captured antibodies, making them a potential alternative to traditional microparticles in various ELISA formats (Chen 2018). Liu et al. (2018) combined citrate-coated gold nanoparticles with protein A (rProtA) and protein G (ProtG). The results revealed that these nanoparticles can be a promising alternative for magnetic-based immune trapping and form a substrate for reducing

hIgG from human plasma and the affinity of the antibody taken from the supernatant in the process control (Liu, 2018). Grishin et al. (2019) conducted a study to increase the half-life of serum lysostaphin. For this purpose, they bound serum lysostaphin to streptococcal protein G, which can be bound to albumin. The results revealed that the modified lysostaphin was only 1.5 times more active than the original enzyme and maintained 25% of its activity when bound to streptococcal protein G (Grishin, 2019).

Zhang et al. (2012) conducted a study entitled "Inhibition of IgG Aggregation-induced Stimulation by Protein A and Protein G" to increase the stability of the specific binding of protein A and protein G to the FC region of IgG. They concluded that protein A provides almost complete inhibition of aggregation-induced stimulation, while protein G and two other peptides provide partial inhibition. Also, this study revealed that the binding site of protein A with IgG is the site involved in the IgG accumulation-induced stimulation. It also indicates that this site plays a major role in colloidal interactions (Zhang, 2012). To detect infectious diseases in animal and bird populations, Vaillant et al. (2013) added immunoglobulin binding protein Gs (IBP) including staphylococcal protein A (SpA), streptococcal protein G (SpG), peptostreptococcal protein L (SPL) and recombinant protein LA (SpLA) to the serum of the studied species of birds and mammals. The results revealed that in comparison with other proteins, SpLA had the highest reaction rate with animal serum and pure immunoglobulins, and SpL showed the lowest reaction. SpL reacted with raccoon serum, rabbit IgG, and chicken and pigeon IgY by direct ELISA, while SpA reacted with sera from skunk, coyote, raccoon, mule, donkey, and human by indirect ELISA. Sandwich ELISA showed a high reaction with both SpG and SpLA with mammalian serum titers ranging from 1:32 (raccoon serum) to 1024:1 (donkey and donkey serum). These results indicate that IBP can be used to detect immunoglobulin using different immunological assays (Justiz-Vaillant, 2013).

A homogeneous product can be obtained from impure and heterogeneous initial solution using the absorption chromatography technique. Protein G plays a significant role in the above purification process. All the factors affecting the production of a protein and the gene expression of that protein must be identified in advance to prevent the occurrence of inhibitory factors as much as possible. Thus, cloning the protein G gene seems necessary. Given the usefulness of proteins binding to immunoglobulins, protein A of Staphylococcus aureus and protein G of Streptococcus were produced through recombinant DNA technology using an expression plasmid in the form of fusion with MBP at the Faculty of Veterinary Medicine of Shahid Chamran University of Ahvaz, and it was labeled with peroxidase enzyme. Streptococcus Protein G is a 30 kDa protein found in the wall of this bacterium. This protein belongs to a group of bacterial proteins that can bind to IgG molecules. This protein can absorb IgG molecules of several animal species through the Fc region (4). Also, it can resist phagocytosis by escaping the immune response. The potential capability and specific and selective binding of protein G to the FC part of immunoglobulin G has caused this protein to be used in many qualitative and quantitative immunological tests by researchers. Hence, the present study was conducted to clone and express protein G (6).

MATERIALS AND METHODS:

In this study, LB liquid and solid culture medium and kanamycin and ampicillin antibiotics were used to examine contamination in the culture medium. Escherichia coli strain TOP10 was used to prepare susceptible cells and keep plasmids in them. E. coli strain BL21 (DE3) was also used for expression in the prokaryotic system. Plasmid pET-28a was prepared from the Vector Bank of Green Gene Laboratory for expression in the prokaryotic system.

Preparation of nickel column and the start of purification and cultivation in high-volume

The recombinant protein was purified by Ni-NTA affinity chromatography column (GenScript Company) based on the method provided by GeNetBio Company. Materials required for purification included Ni-NTA resin, lysing buffer, washing buffer, and extraction buffer. Purification by denature method (based on pH change):

1) 50 ml of E. coli BL21 (DE3) bacteria, which received the pET28a plasmid containing the recombinant gene, were centrifuged at 3500 x g for 10 minutes and 5 hours after induction with IPTG.

2) Bacteria precipitation was dissolved in 1 ml of lysing solution (in denatured condition) and placed on a horizontal shaker for 30 minutes.

3) After centrifugation, the supernatant solution was added to the Ni-NTA column.

4) The column was washed with 10 ml of washing buffer (pH: 5.9) and the output was collected.

5) The column was washed with 2 ml of extraction buffer (pH: 4.5) and the output was collected.

6) A sample of the column outputs was electrophoresed on a 12% SDS-PAGE gel.

7) To remove the urea in the protein solution, the purified protein was dialyzed by PBS buffer with a pH of 7.2. Then, using the Bradford method, the target protein concentration was determined.

8) Finally, the purified protein was kept at -70°C.

The mass cultivation of these bacteria was done in the presence of the antibiotic kanamycin (50 μg/ml). After the culture reached an OD of 0.6, IPTG (final concentration of 1 mM per cc) was added and the bacteria were allowed to shake for 5 hours at 37°C (2500 g).

The procedure for purification by native method (based on change of imidazole concentration):

1) 50 ml of E.coli BL21 (DE3) bacteria, which received the pET28a plasmid containing the

recombinant gene, was centrifuged overnight at 3500 x g for 10 minutes after induction with IPTG.
2) Bacteria precipitation was dissolved in 1 ml of lysing solution (in native conditions) and lysozyme was added to it with a concentration of 1 mg/ml. Then, it was placed on the shaker for 30 minutes.
3) After centrifugation, the clear supernatant solution was added to the Ni-NTA column.
4) The column was washed with 10 ml of washing buffer containing 30 mM imidazole and the output was collected.
5) The column was washed with 2 ml in four parts of 500 µl extraction buffer containing 300 mM imidazole and the output was collected.
6) A sample of the column output was electrophoresed on a 12% SDS-PAGE gel.
Finally, the purified protein was stored at -20°C.
Determination of protein solubility
The basis for determining protein G solubility is that 2 ml of native buffer and 50 µl of lysozyme enzyme were added to the protein, and it was placed at room temperature for 1 hour. At the same time, to break the cell wall well, it was put in the freezer for 10 minutes to freeze and take out to melt. This was repeated twice. After 1 hour, the glass bead was added and vortexed 3 times for 3 minutes, and the sample was taken from it. After sampling, 2-3 ml of Denature buffer was added to it and the glass bead was added again and vortexed. The supernatant solution was carefully collected in 1.5 ml microtubes and centrifuged at 13000 rpm for 5 minutes. The supernatant contains protein in the native phase, and the protein precipitation is in the denature phase. Then, the denature was transferred to another Falcon and centrifuged 2 or 3 times. The supernatant solution is denatured and insoluble. The pellet was discarded. Sample buffer was added to native and denature samples and transferred to the column. Using the SDS-PAGE method, it was found that the expression was done either in denature or native phase. Expression in the native phase indicates that the protein is soluble, and expressed in the denature phase indicates that the protein is insoluble. If it is expressed in both phases, it is both soluble and insoluble. After adding the denature, it is sometimes taken on the column and purified to determine what state it is in.
Antibody purification using protein G G column
Human blood serum is needed to perform the Protein G efficiency test in ELISA. Accordingly, 3 ml of human blood was taken and placed at 37°C for 1 hour. The serum was separated by centrifugation at 10000 rpm for 5 minutes and stored at -70°C. To obtain more accurate results in the efficiency test, the resulting serum was loaded on the G purification column for the isolation of IgG antibodies based on the following guidelines.

RESULTS:

1. Enzymatic digestion of plasmid pUC57 with gene G and plasmid pet28a lacking fragment
Plasmid pUC 57 containing protein G G gene fragment and carrying plasmid pET28a were digested by XhoI and NcoI cleavage enzymes. Enzymatic digestion products were observed on 1% agarose gel. Plasmid pET28a lacking the fragment formed a 5300 bp band (Figure 1a) and plasmid pUC57 with the gene fragment formed two bands after digestion, in which the 2710 bp band was related to the linearized PU57 vector and the 641 bp band was related to the protein G gene fragment (Figure 1b).

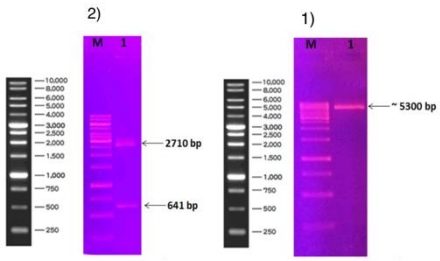

Figure 1- Enzymatic digestion reaction of well 1 a) linearized pET28a vector and b) pUC57 vector containing G gene (641 bp) using XhoI and NcoI enzymes. Well 1: B) pUC57-G vector, Well M: 1kb marker

To homogenize, enzymatic binding between the gene fragment and pET28a was done by the T4 ligase enzyme. Then, the recombinant carrier was transferred to the susceptible E. coli TOP10 host. Two methods of PCR and enzymatic digestion were used to examine the correctness of the homology of the gene fragment in the pET28a expression carrier.
2. Confirmation of homogenization by PCR method
The PCR reaction was performed using the T7 primer of the desired fragment. The reaction product was examined on 1% agarose gel and the results of the PCR colony showed that only colonies 4, 6, 7, and 8 received the desired structure and it is positive. Based on Figure 2, the bp950 band can be seen on 1% agarose gel. In PCR with T7 primers on the recombinant vector pET28a-G, 309 bp are added to the desired gene.

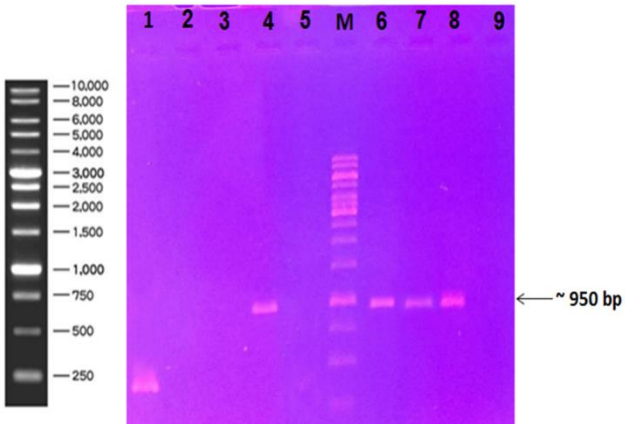

Figure 2: Colony PCR to confirm the presence of G gene using T7 primers on 1% agarose gel. Well 1: positive control (pET28a vector without gene), Well 2: negative control, Wells 3 to 9: recombinant colonies. Only colonies 4, 6, 7, and 8 have received the structure. M well: DNA=1kb marker

3. Confirmation of homogenization by enzymatic digestion method

To reconfirm the homogenization, the plasmid was extracted from one of the positive colonies (colony No. 2). To ensure the correct homogenization, the recombinant PET28a vector containing the G gene was cut with NcoI and XhoI enzymes. The 5300 bp band of the linearized PET28a plasmid and the presence of 641 bp bands for the G gene can be seen after digestion on agarose gel (Figure 3).

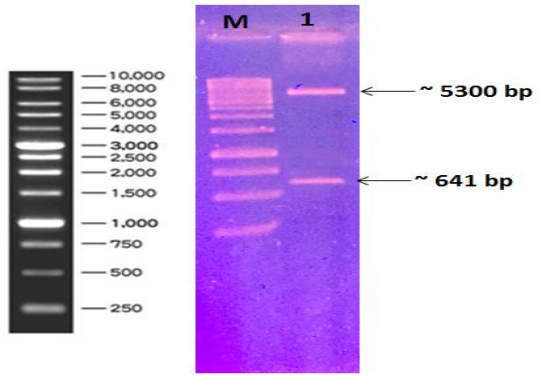

Figure 3. Confirmatory digestion on PET28a recombinant vector with NcoI and XhoI enzymes, Well M: DNA=1kb marker and Well 1: linearized plasmid and Protein G gene fragment

4. Transformation of pET28a-G vector into E. Coli BL21 (DE3) bacteria

The recombinant plasmid containing the G gene was transformed into susceptible BL21 (DE3) cells. The PCR reaction was performed with T7 primers to confirm the transfer (Figure 4). Colony PCR results showed that only colonies 3 to 7 received the desired structure and were positive. Based on Figure 4-4, a 950 bp band can be seen on 1% agarose gel. About 309 bp are added to the desired gene PCR with T7 primers on the pET28a-G recombinant vector.

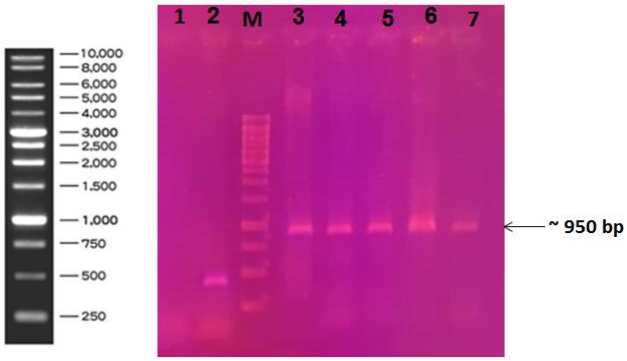

Figure 4: Colony PCR to confirm the presence of G gene with T7 primers in BL21 strain on 1% agarose gel. Well 1: negative control, Well 2: positive control (pET32a vector without fragment), Wells 3 to 7: recombinant colonies, and Well M: DNA=1Kb marker

5. The analysis of recombinant Protein G expression in 5 ml culture medium

After confirming the gene homogenization in the pET28a plasmid carrier, the recombinant carrier was transferred to the E. coli BL21 (DE3) expression host. Among the obtained colonies, after culturing bacterial cells and induction with IPTG with a final concentration of 1 mM, gene expression was analyzed on a 12% SDS-PAGE gel in the second, fourth, and sixteenth hours after induction. The size of protein G is 25 kDa. Due to the protein's acidity (pI=4.8), it is mostly placed higher than usual on the SDS-PAGE gel. Figure (5) shows a 30 kDa band related to the protein G.

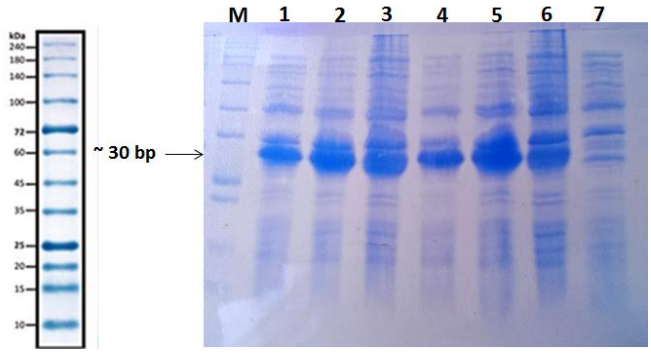

Figure 5: Protein G expression analysis on 12% SDS-PAGE gel. Wells 1, 2, and 3: four-hour sample, Wells 4, 5, and 6: overnight sample, Well 7: sample before induction, and Well M: protein marker

6. Recombinant protein purification using affinity chromatography (Ni-NTA column) by the denature method (based on pH change):

The purification of the recombinant protein marked with histidine sequence was performed by Ni-NTA affinity chromatography. Due to the formation of body inclusion during protein G expression, protein purification was done under denatured conditions and with the help of changing the medium pH. To purify recombinant protein G, bacteria with plasmid pET-28a-G were cultured on a large scale (50ml) in a shaker incubator for one day and night at 37°C. The precipitation of Arlene solution, obtained from native and denature phases, was examined in the previous step. In this step, the desired protein was transferred in the native phase and the denature phase on the nickel column and purified using changes in the concentration of the desired protein. Finally, the purified samples were run on a 12% SDS-PAGE gel until the purity of the protein was determined. As shown in Figure 6 and Figure 7, the 30kDa band was observed in the purified samples both in the native and denature phases.

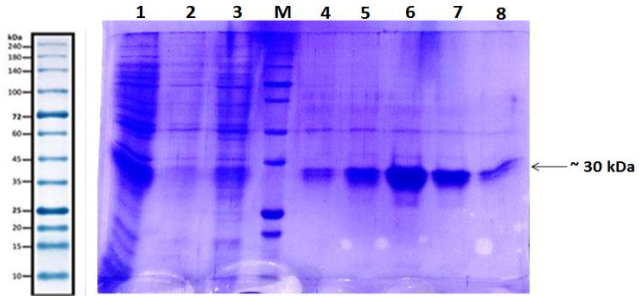

Figure 6: Purification analysis of protein G in denature conditions on 12% SDS-PAGE gel. Well 1: lysate solution, Well 2: column output after protein loading, Well 3: washing by buffer containing 8 M urea, Wells 4 to 7: elution 1-4 outputs, respectively, Well 8: MES output, and Well M: protein marker

7- Recombinant protein purification using affinity chromatography (Ni-NTA column) by native method (based on the change in imidazole concentration):

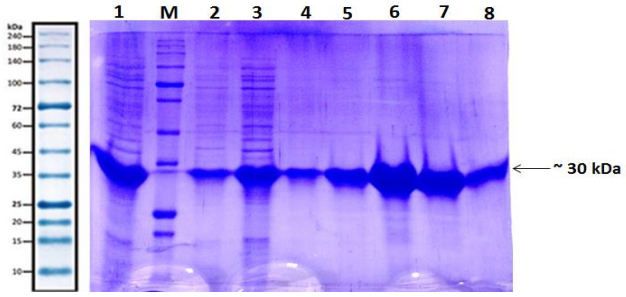

Figure 7: Purification analysis of protein G in native solution condition on 12% SDS-PAGE gel. Well 1: lysate solution, well 2: column output after protein loading, well 3: washing by buffer containing 30 mM imidazole, well 4 to 7: Elution outputs 1-4, respectively, Well 8: MES output, and Well M: protein marker

8. Bradford method for measuring protein G concentration

The protein concentration of the unknown sample in µg/ml is determined according to the standard curve. The amount of protein in 1 ml of the extracted protein solution is measured by multiplying it by the dilution factor and the initial volume of the unknown sample. The denature and native conditions in the output components collected from the column are presented in the table. The total content of the purified protein in the denature phase (Table 2) and native phase (Table 1) is 551 and 412 µg, respectively.

Table 1: Bradford calculation in the native phase

Native phase		
sample	concentration (ug/ml)	content (ug)
E1	167	67
E2	269	108
E3	299	120
E4	169	67
MES	126	50
Total		**412**

Table 2: Bradford calculation in the denature phase

	Denature phase	
sample	concentration (ug/ml)	content (ug)
E1	153	61
E2	256	102
E3	422	169
E4	316	126
MES	231	92
Total		**551**

9. Western blotting to confirm the expressed protein

To confirm the expression of recombinant protein G, the presence of protein G was examined based on the western blotting method using HRP 127 antibody (Anti-His tag HRP-conjugate). Figure 8 shows the confirmation of the protein. With presence of recombinant Protein G was confirmed by the appearance of a 30 kDa brown band on the PVDF paper.

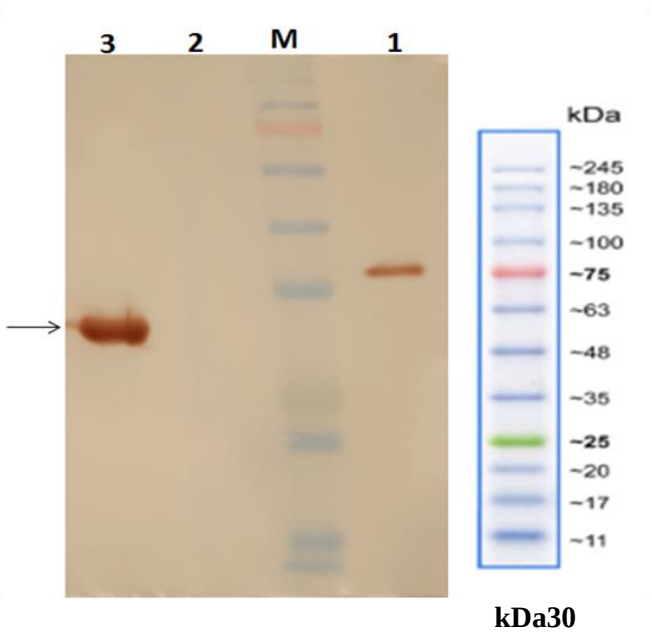

Figure 8: Analysis and confirmation of purified G recombinant proteins using western blotting with anti-histidine antibody. Well M: protein marker, well 1: positive control, well 2: negative control, and well 3: sample of protein G

10. Antibody purification using Protein G column

Human blood serum is needed to perform the Protein G efficiency test in ELISA. To obtain more accurate results in the efficiency test, the resulting serum was loaded on the G purification column to separate IgG antibodies. To examine the quality of the purified antibody, the different fractions collected from the column were loaded on a 12% SDS-PAGE gel. The purified fractions, antibody heavy chain with a size of about 50 kDa, and light chain with a size of about 25 kDa can be observed in Figure 9.

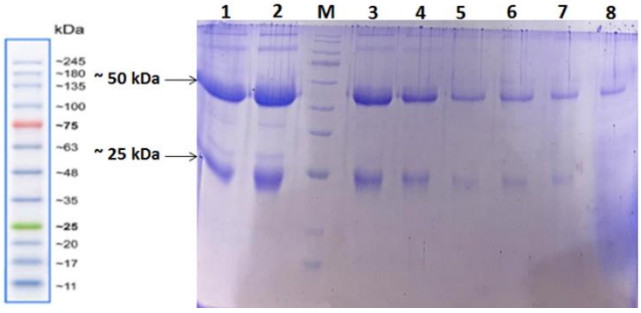

Figure 9: Purification of IgG antibody from human serum using purification column G. Wells 1 to 8: purified fractions; Antibody heavy chain with a size of about 50 kDa and light chain with a size of about 25 kDa can be observed. Well M: protein marker

11. Measurement of purified antibody concentration
Table 3 presents the concentration of heavy and light chains of IgG after estimation by THE Bradford method. The total content of purified protein is 2645 µg.

Table 3: Bradford calculation of IgG antibody

sample	concentration (ug/ml)	content (ug)
E1	606	606
E2	1049	1049
E3	469	469
E4	265	265
E5	131	131
E6	126	126
Total		**2645**

Determining the function of protein G by ELISA method
Indirect ELISA and sandwich ELISA methods (Table 1) were used in this experiment to measure Protein G function. Given the studies conducted in this regard, the function of recombinant protein G can be measured using purified human IgG antiserum and evaluating the interaction between recombinant protein G with it in the ELISA method. Finally, the reaction was stopped with 2.5 M sulfuric acid and the yellow color turned to orange after changing the color of the solution to yellow. Then, the absorption was read at 492 nm. It was used to determine specific antibody or antibody titration in serum samples.
Figure 10 shows the reaction of recombinant Protein G with mouse and rabbit IgG and chicken IgY. Chicken IgY is used as a control and has the lowest detection rate by Protein G, and Protein G can identify the FC part of the mouse antibody compared to the FC of the rabbit antibody.

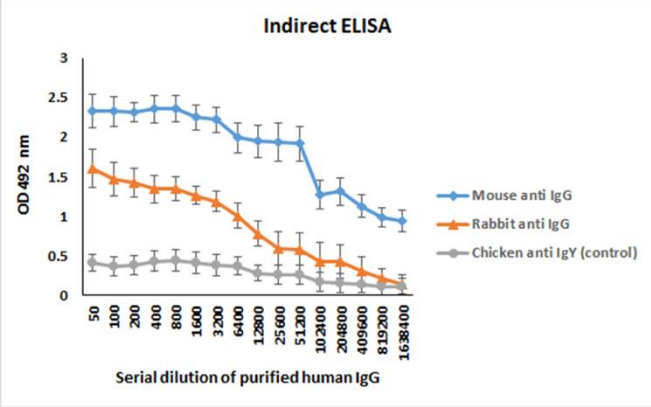

Figure 10: Investigating the function of recombinant Protein G produced in immunized mice by indirect ELISA method.

The anti-human globulin is coated in the well and is trapped by adding the antibody sample. Then, an immune complex is formed by adding the specific antigen to the medium. Then, the specific labeled antibody against the antigen is used as a recognition system. This method was used in this experiment based on Figure 11. Accordingly, anti-human IgG was coated in the well, and a labeled IgG antibody was added to the well, Protein G was added as an antigen, and an immune complex was formed. Figure 11 illustrates the reaction of mouse and rabbit antiserum with purified Protein G. Based on this figure, rabbit antiserum has higher absorption than mouse. Chicken IgY was used as a control, which showed very little absorption. Protein G showed the highest absorption rate at a concentration of 5000 ng. The absorption rate of both samples decreased with decreasing concentration, indicating the optimal efficiency of the recombinant protein.

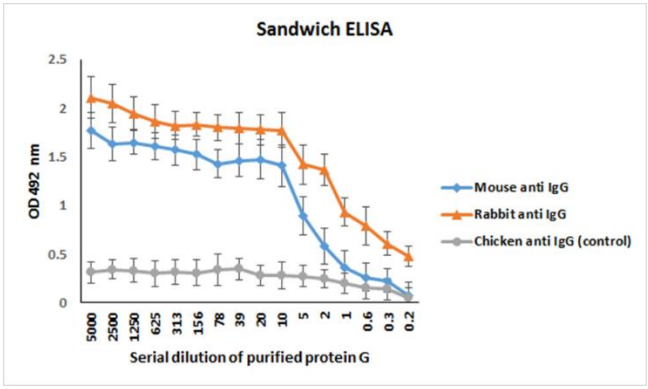

Figure 11: Investigating the function of recombinant Protein G produced in immunized mice by sandwich ELISA method

CONCLUSION:

The present study was performed to evaluate the activity of these proteins for binding to different types of animal IgG. This protein reaction in a fixed dilution of 1.5000 was investigated with different amounts of mouse, rabbit, and chicken IgG. As expected, this protein did not react with chicken IgG, but it could identify mouse and rabbit IgG. Among the immunoglobulins of the animal species tested, this protein had the highest affinity for rabbit IgG in sandwich ELISA and mouse IgG in indirect ELISA. Yamamoto et al. (1985) had already shown the favorable reaction of protein A with cat IgG. In the study by Shapouri et al., given the dispersion of E.coli in contact and the dispersion of different animal species with this bacterium, after covering the wells of the ELISA plate with soluble antigens of E.coli from cat and dog sera as the primary antibody and protein A as the labeled tracer in this part of the study, serum sample was prepared from each animal species of dog, cat, and chicken and examined in indirect ELISA test using soluble antigen of E.coli, assuming that different

animal species have more likely anti-antigens of soluble E.coli.

The results obtained from animal species revealed that cats and dogs had antibodies against E.coli soluble antigen. However, the reaction of chicken serum with this antigen could not be detected (Shapouri, 2015). The production of recombinant protein G in prokaryotic and eukaryotic systems is very limited. This study is the first report of cloning, expression, and purification of streptococcus Protein G in E.coli. pET28a plasmid vector was used in this study. The pET system is one of the most powerful systems for gene expression and recombinant protein production in E.coli bacteria. In this system, the target gene is under the control of a strong T7 promoter. In the host genome of the E.coli (BL21-De3) strain, transcription of the gene was performed under the control of this promoter by RNA of T7 phage polymerase cloned in the bacterial cell. Thus, the production of protein in this system is not affected by the mRNA of cellular factors involved in protein synthesis due to the independence of the gene transcription system from the host cell. Finally, it can be concluded that the product evaluated in this study (recombinant Protein G) has sufficient potential to be used in some immunological tests and can potentially replace some commercial items. In addition to the FC part, Protein G also reacts with a part of the Fab, which will reduce the sensitivity of diagnostic tests. Another advantage is that Protein G has a sequence that binds to albumin. If we want to use Protein G for IgG purification, this part of the protein should be removed. However, more efforts should be made to increase the efficiency of this product as much as possible and especially to expand the capability to identify animal IgG species.

CONCLUSION:

In examining the expression test at different hours after induction with IPTG, very good expression was observed using the SDS-PAGE test. The molecular weight of the recombinant protein was about 30 kDa. The results of the expression and purification of this protein in BL21 (DE3) E.coli host showed that this recombinant protein can be purified in denature and native conditions according to predictions and has a very good expression level in E.coli BL21 (DE3) host. Finally, it can be concluded that the Protein G product evaluated in this study can be used in some immunological tests. Based on the results of examining the efficiency of the recombinant Protein G and the reaction of the immune system of the laboratory animal to it, it is recommended to investigate its protective power.

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