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EXTRACTION AND PURIFICATION AND MOLECULAR EVALUATION OF *STAPHYLOCOCCUS AUREUS* PROTEIN A

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ABSTRACT

Staphylococcus aureus is categorized at the same range of Streptococci and Listeria. Gram-positive cocci, arranged in grape-like clusters in pairs and chains with non-motile tetrads, Aerobic, Catalase positive and oxidase-negative (OX-) so that there exist more than 47 species and subspecies of the genus Staphylococcus. This paper aims to extract and purify Staphylococcus aureus protein A. Staphylococcus aureus subsp. aureus (strain NCTC 8325) was used to extract protein A. The stages mentioned to extract protein A include three stages of break down cell walls of bacteria, its release from the bacterial wall and the last purification. The results from findings have shown that all the methods used to date found suitable to break and slip cell walls of bacteria where the amount of protein A released was substantial, yet it has to draw a particular attention that the most amount of protein gains from Lysozyme method.

Keywords: Protein A; *Staphylococcus aureus*; Molecular Evaluation

INTRODUCTION

Using immunoglobulin can obtain detailed information about a particular antigen. To isolate class-specific secondary antibodies, a variety of methods can be used, but, high binding affinity for protein A to IgG antibody can be used as a simpler, faster and common method rather than the methods like gel filtration and

sucrose density gradient centrifugation so as to isolate immunoglobulin, thus, protein A is a commercial product that is economically important and expensive with so many advantages. The other applications from protein A, can be referred to monoclonal antibody purification where the antibodies found with high importance in treatment of malignancies, treatment of inflammatory mediators and treatment of allergic diseases. According to the studies provided to date, in Iran just little studies have been conducted on Staphylococcus aureus protein A, where expression and purification of protein A from cell walls of bacteria as a commercial product with the value for IgG purification and the economic usage found of importance, reporting no study on this topic has been provided yet. Preparation and purification of Staphylococcus aureus subsp. aureus (strain NCTC 8325) protein A and getting used it in Purification of immunoglobulin G, extraction of protein A, with use of proper methods and then sticking it into a proper bed for the purification of IgG reported as the major goals of this study.

Protein A

Several virulence factors in Staphylococcus aureus have been determined where Beta-Lactamase Inhibitor Proteins (BLIP) and Protein A have been proposed as the most important virulence factors. An investigation into the correlation between Beta-Lactamase Inhibitor Proteins (BLIP) and Protein A, revealed that roughly 93.7% increase in strains contains protein A, while the strains lacking Beta-Lactamase Inhibitor Proteins (BLIP) found less about 50%; furthermore, it is revealed that Strains susceptible to oxacillin antibiotic-resistant strains called oxacillin-resistant Staphylococcus aureu lack protein A. Notably, Protein A is a protein in the cell wall of Staphylococcus aureus made of a 42 kDa antigenic polypeptide, and developed 1.7% of the Cell weight with the total 450 amino acid. 4 tyrosine units which fully exposed on the cell surface of bacteria are responsible for biological activity. This protein can be isolated using permeation chromatography and Lysostaphin digestion on IgG sepharose. In some strains of Staphylococcus aureus, such proteins are found to form Secretory and extracellular aggregates. Protein A covers the surface of most strains of Staphylococcus aureus which further protects Bacteria against antibody mainly found against with Phagocytosis property so that it acts as protective role for Bacteria. This protein has a structure resistant to PH changes ranged from 0.99 to 8.11. Further, it also shows resistant to the

detergent agents and while found with Denaturation, it can be reconstructed in 4 M urea and 6 M guanidine. Hence, useful method for serological coagglutination relies on Protein A. while Specific antibody added to Staphylococcus aureus protein A, afterwards in next stage Homologous antigen joint to it where Coagglutination developed. Protein A is encoded by spa gene. This gene contains X repeated sequences found suitable for typing Staphylococcus aureus gene. Determine typing is a priority for infection control reported essential. Protein A is in Covalent binding with peptide and the cell wall of glycans, released in medium during the process of cell development so that this amount of Protein A constitutes one third of the total Protein A produced by means of organism.

Purification of human immunoglobulin

Human antibodies that are prepared with high spending have many applications in the diagnosis, treatment and prevention of diseases. Methods for the purification of immunoglobulin G (IgG) include selective deposition of ammonium sulfate or sodium sulfate, ion chromatography, gel-filtration chromatography,

Thiophilic affinity chromatography (TAC),

affinity chromatography over a column of antigen, where methods for purification of immunoglobulin M include polyethylene glycol (PEG) precipitation and gel-filtration chromatography, and methods for the purification of IgA include Ammonium sulfate precipitation method together with Ion-exchange chromatography and chromatography on constant Jacqueline. Cell affinity chromatography has been proposed to purify IgG and IgE. To approve whether immunoglobulin purified or not, a variety of methods including Single Radial immunochemical Immunodiffusion methods and bilateral radial immunodiffusion, Two-dimensional

gel electrophoresis and immunoblotting has been utilized. Preparation for Human Serum Immunoglobulins can be used in different types of immunochemical Immunodiffusion methods to test quality and quantitiy. Immunoglobulin preparations used for the first time in 1950 for the treatment, mentioning that designed just for Intramuscular injection, but, found with side effects such as pain in the inject area and anaphylactoid reactions. Intravenous immunoglobulin was permitted to be used and widespread in the 1980s in America. More recently, immunoglobulin found with therapeutic applications prescribed for diseases such as Kawasaki disease, chronic lymphocytic leukemia, and thrombocytopenic

purpura (ITP) complicating chronic lymphocytic leukemia (CLL) and etc.

LITERATURE REVIEW

Protein A isolated for the first time in 1972 from cell walls of Staphylococcus aureus. Ankarest and colleagues in 1976 recognized more than 95% IgG serum join to Staphylococcus aureus protein A. Seven lofthel et al. (1982) cloned protein A gene into Escherichia coli PbR322. Mota and Guss(1982)sought to extract Protein A using bacterial mutant strains in culture which used to have extracellular protein production and secretion. Sting et al.(1990) sought to extract Protein A from cell walls of bacteria. Warnes et al.(1993) during the process of cloning this protein, observed that expressing this protein in total in Escherichia coli bacteria leads this protein aggregates in bacteria membranes whereby it would be avoided from Septa development where if the gene in membrane removes, there would be no matter. Frenay and his associates(1994) proposed a useful and fast method to determine typing of Staphylococcus aureus resistant to methicillin using the sequence of Staphylococcus aureus protein A which was determined previously. Hariss et al.(2002) conducted several studies on the cell wall of Staphylococcus aureus and how to extract the proteins. Hober et al.(2006) addressed purification of antibodies, IgG class, using column chromatography and protein A. Kobayesh and colleagues(2009) showed the gene existing in this protein in different geographic areas contain Polymorphism where based on the Polymorphism in X gene of this bacteria, 42 clinical strains isolated and this point emphasized the impact of Protein A in outbreak of this bacteria. Bahram Kazemi and his colleagues (2009) provided Staphylococcus aureus protein A cloning in Expression plasmid with the use of Affinity chromatography. Garofalo et al.(2011) investigated the role of Spa gene and protein A in induction of inflammation and osteomyelitis-consequently recognized a direct correlation between this protein and pathogenicity. Watanab and his colleagues (2013) recognized that optimization of protein A purification to cope with bottleneck in the process of high-cost production to treat antibody is of importance.

METHODOLOGY

Preparation of Bacterial strains and its culture

Staphylococcus aureus strains lyophilized and provided from Pasteur Institute of Iran, Department of bacterial vaccines and antigen preparation with number NCTC8325 and then cultured. According to the World Health Organization protocol, roughly 0.5 ml of BHI

broth culture medium added with Syringe into the vial of lyophilized bacteria and inoculated to Vials containing 50 cc of BHI broth medium, where incubated at 37 ° C for 3 hours at the next stage whereby 0.5ml of this culture accomplished in 50 cc of BHI broth culture medium. Once again, 0.5 ml of the second culture inoculated to the 50 cc of BHI broth culture medium and incubated at 37 ° C for 1 hour so that In the final stage, 0/1 ml of the culture of the third tube on BHI culture was incubated on the plate, held at 37 ° C for 24 hours in incubator to develop single colons. At the three stages of culturing with time interval as 1, 2 and 3 hours, the strain activated and then incubated for 24 hours where isolated culture prepared from bacterial suspensions at brain-heart infusion broth at 37 ° C for 24 hours. Single Gram Stain colonies to be assured of cocci and culturing on the Mannitol salt agar and Coagulase test to get assured of Staphylococcus, and control tube to produce spore have been all used **(Figure 1)**.

Figure 1: Opening the lyophilized strain of *Staphylococcus aureus*, NCTC8325

Figure 2: Inoculated the lyophilized strain of *Staphylococcus aureus*, NCTC8325 to the 50 cc of BHI broth culture medium

Brain-heart infusion broth culture medium

Commercial BHI broth culture medium made by Himedia has been used in this study. To provide this environment, 51 g of powder in one liter of heated distilled water is heated to be steady. The mixture of this environment for 1 liter volume is as follows:

1-200 g Bovine Brain Extract (BBE)

2-10 g protease peptone

3- 5 grams of dextrose chloride

4-2/5 grams disodium phosphate

5-250 grams beef heart extract

Methods for Identification of *Staphylococcus aureus*

Coagulase test:

1. Put a drop of rabbit plasma on a clean and dry slide provided using EDTA or nitrate
2. With the use of Sterile applicator, provide some of the colony in each drop as emulsion to provide a steady suspension
3. On the basis of bacteria aggregation where plasma coagulase placed, it can observe the bacteria aggregation indicates the Agglutination of the organism.

The method used for tube coagulase test

Principles: *Staphylococcus aureus* produces Coagulase enzyme with the ability to Plasma clotting so that this makes it differentiated from other members of the family Micrococcaceae.

Test method: put 0.5ml human or rabbit plasma in a glass tubes (13 mm 100 mm) and add an amount of colon to it. This plasma can be held for 10 days at refrigerator temperature and for several months at - 20 º C. The suspension was incubated for 4-1 hours at a temperature of 37-35 ° C, where the formation of clots was examined. If the clot not formed in 4 hours, a tube is placed in incubator for a night.

Identification of *Staphylococcus aureus* by means of Mannitol salt agar (MSA) medium culture:

In this environment due to 7.5% salt, development of many bacteria avoided, but, Staphylococcus can grow well. This environment contains glucose-mannitol and red phenol agent at which Staphylococcus aureus can cause Mannitol fermentation appears whereby acidification of the environment and changing the color to yellow would be resulted.

Preparation of mannitol salt agar:

The culture medium used exist as prepared powder from Gibco company where it needs to pour 5.5 g of each culture medium with 50 ml distilled water in Meyer flask and mix the powder produced to not have power in pellets

to have just the solution. As agar exists in culture medium, it has to be dissolved in container where it is essential to boil the mixture for one minute to have transparent color of container; the container has to be mixed in continuous not let the agar burned so that while the container found necessary to be sterilized by means of autoclave pressure of 15 pounds per square inch and 121 º C for 15 minutes. After sterilization and cooling container at 45ºc, it is better to pour it in the sterilized pilots of solution. After solidification of culture media, the container found suitable to be at 37ºc for 24 hours in order to control pollution. If contamination not seen in container, better to keep it far from 37ºc and culture the bacteria whereby the result would be observed after 24 hours so that change of color from red to yellow indicates *Staphylococcus aureus*.

The method of extraction of toluene /Triton X-100, ethylenediaminetetraacetic acid (EDTA)

50 ml Tris-Hcl 0.02 M, PH7.5 with 2.5 ml of toluene,0.025 micro liter Triton X100 and 0.05 mg of EDTA added to 5 g of Cellular weight, and put for 30 minutes in shaking incubator at 37 ° C, centrifuged at 3500 × g round for 20 min so that Protein measurement using Lowry method was performed on the supernatant. Absorption at 560 nm was equal to 0.1. To determine protein, Lowry method and Linear equation (=0.0055X+0.0008) calculated where found 18.23 mg per 0.5 ml.

Freez and thaw method

This includes two stages of cellular Lysis and Freez and thaw. 5 g of cellular weight suspended with 50 ml of cold water, 50 ml of 0.5 M Tris base and 05 0.0% Triton, put in shaking incubator at 30 ° C, where read during 5 hours every 30 minute in wavelength of 580nm. Decrease at absorption in the last time interval displayed Lysis. At second stage, Suspension put in the freezer for -12 ° C for 12 hours and then carried out at labrotorary and vortex temperature and then centrifuged at 1300 × g round so that Protein measurement using Lowry method was performed on the supernatant. Absorption at 560 nm was equal to 0.1. To determine protein, Lowry method and Linear equation (=0.0055X+0.0008) calculated where found 18.23 mg per 0.5 ml.

Absorption amount	Hours
0.298	17
0.295	17:30
0.292	18
0.223	18:30
0.065	19
0.225	19:30
0.077	20
0.058	20:30
0.032	21
0.016	21:30
0.039	22

Lysozyme extraction

Suspension prepares for 20 ml Phosphate-buffered Saline with 0/0015 mol/l PMSF, 50mg sodium azide and 50 mg lysozyme. This suspension is placed in shaking incubator at 37 ° C for 22 hours. Then bacteria centrifuged at 13000 round where on bacteria are deposited. And the solution on the surface passes through filter with the Pore around diameter of 45/0 mm, and Protein measurement using Lowry method was performed on the supernatant. Absorption at 560 nm was equal to 0.781. To determine protein, Lowry method and Linear equation (=0.0055X+0.0008) calculated where found 141.85 mg per 0.5 ml.

Extraction of Sodium metaperiodate

Bacteria with 40 ml of 0.1 ml/l Sodium metaperiodate solution with the content of mol/l PMSF0/0015 and mg 100 sodium azide suspended where centrifuged at 13000 round for 20 minutes and the solution on the surface appeared with Hemodialysis against PBS for 24 hours at 5 ° C, and Protein measurement using Lowry method was performed on the supernatant. Absorption at 560 nm was equal to 0.220. To determine protein, Lowry method and Linear equation (=0.0055Y+0.0008) calculated where found 39.85 mg per 0.5 ml.

Measurement of protein using micro-Bradford procedure

Bradford Assay Procedure: 100 ml of 85% phosphoric acid with 200 mg Coomasie Brilliant Blue Solution-G 250 received to 200 ml with distilled water and then passed through Filter paper.

Assay procedure

Assay performed at 48-units micro plate. The volume of each sample in Sink found 200 micro liter where 100 micro liter contains reagent and Dilution in each raw carried out from 1 micro liter to 5 micro liter of the sample with the full volume of protein and volume of distilled water from 99 micro liter to 95 micro liter. Then, the results examined with ELISA reader. Micro-Bradford procedure carried out for the method used where an investigation using ELISA reader showed that the most amount of protein used in Methods of Cell Lysis found with Lysozyme.

Protein measurement using Lowry method:

With use of Bovine serum albumin (BSA), Sigma examined as the standard potential protein contamination in polysaccharide where this examined using Lowry method. Generalities and Principles of Lowry method:

This method includes two reactions where the first is the reaction of copper ions combined

with protein molecules whereby a Mixed-valence Copper Complex with protein develops. In second reaction, phosphotungstic acid , phosphomolibdic acid Folin Solution caused a specific color appears in the complex. In protein-copper complex, amino acids tryptophan, tyrosine, phenylalanine are restored and blue color which its concentration relies on the amount of protein, is built. Lowry method used has been very sensitive and its accuracy is between 1 to 100 micrograms of protein per ml.

Preparation of necessary standards

Solution A: 20 g of sodium carbonate (Na2CO3) and 5 g Potassium sodium tartrate in double, reached to 1 liter using 1.0M NaOH.

Solution B: 5 g of copper sulfate with 5 water molecules (CuSO4. 5H2O) reached to 1 liter using distilled water.

Solution C: 50 ml of solution A plus 1 ml Solution B.

Solution D: Folin- ciocalteu diluted with distilled water with ½ ratio and should be consumed fresh.

Methods of doing experiments:

To provide standard, Doses of 10 to 100 micrograms per ml bovine serum albumin (BSA) used to provide the dilutions of 10, 20, 40, 60, 80 and 100 ml per ml in Duplicate where Doses of 0, 2/0, 4/0, 6/0, 8/0 and 9/0 ml distilled water added to each dilution so far as the final volume reaches to 1 ml in each tube.

To prepare sample, 1 ml of sample taken and added to what prepared till now. 5 ml solution C added to each tube and incubated for 10 minutes in room temperatures. Thereafter, 1 ml solution D is added and kept in room temperatures for 30 minutes. OD of samples measured immediately at 540 nm wavelength. Blank sample OD subtracted from OD for the main dilutions so as the standard blank OD subtracted from standard OD dilutions.

Standard curve depicted using Excel software regarding the data shown above.

PHA tests:

4/2 g of sodium citrate, 24/1 NaCl and 142 g / 0 g citric acid dissolved in 270 ml distilled water and then PH modulated in 6.1. This Solution was autoclaved for 20 min at 121 ° C, and then 30 ml of 20% sterilized glucose solution with 0/22 microns filters added to the solution so that this Solution was used after filtration.

Preparation of sheep red blood cells:

100 ml defibrinated sheep blood was obtained from Darvash company with production date 2013; Best consumption date mentioned 20 days after the date of manufacture where defibrinated sheep blood kept in 100 ml ulcer solution at 4 121 ° C. Then centrifuged for 15

min at g × 1500, and Packed red blood cells were centrifuged three times with 7.4 PBS PH, and centrifuged two times with ulcer solution.

The cells in 160 ml of Formalin ulcer solution (450 mL ulcer +30 mL formalin 37%) as a suspension prepared and placed for 24 hours on Shaker–Incubator. After this time the cells were centrifuged and suspended in 160 mL of preservative solution (Solution containing 300 mL ulcer solution, 150 mL ethanol and 45 ml of formalin with 4 PH) and placed in laboratory conditions for 48 hours. The cells were then centrifuged in 160 mL of 6.4 PBS, and in the end of process centrifuged two times with 7.4PH PBS and centrifuged two times with 7.4 PH PBS and in the end Cell density of 20% prepared with 7.4 PH PBS.

A protein binding to red blood cells:

Mix 1 ml of the suspension of SRBC 20% with 2 ml of protein A and put it in water bath at 37 ° C for 2 hours so that mix it every 30 minutes. The cells rinsed twice with 20 volumes of buffer 7.4 PH and twice with 20 volumes of buffer 7.4 PH containing 0.1% Normal rabbit serum and finally the cells rinsed with 20 volume of 7.4 PH buffer containing 0.1% Normal rabbit serum prepared as suspension. Cells Divided in hemolysis tubes and stored at 4 ° C. it has to state that the cells found susceptible would not last for over 48 hours.

PHA test method:

In the first vertical row of 96-Well Polystyrene Microplates, 25 microliters of serum of rabbits immunized with protein A poured and prepared using buffer PH 7.4. 25 ml of red cells sensitized added to each dilution and then kept for two hours in laboratory temperature after while mixed. Similarly, in simultaneous, Monospecific Antiserum of protein A used as positive control serum and Normal rabbit serum and buffer PH 7.4 used as negative control.

Agar Gel Immunodiffusion

To approve whether protein A was specific, Agar Gel Immunodiffusion has been used. Phenobarbital buffer 6/8 PH was added to One gram agarose containing 100 ml, heated to melt well. Glass slides or plates washed well with soap and water, and then cleaned with alcohol. 20 ml of molten agarose added on Glass slides or plates by means of pipette. And then it was allowed the gel remained closed and be in the refrigerator at 4 ° C for 30 minutes, whereby a sample of pattern is depicted in order to add protein A and Positive control serum antibodies against protein A and negative control. Then slides and the plate kept in a desiccator to Maintain moisture of gel and let the bands emerge

between the positive control and protein A for 24 to 48 hours. For better observation, the bands emerged at gel colored with Coomasie brilliant blue.

Buffer prepared phenobarbital 6/8 PH:

Solution A: 12 g of sodium phenobarbital were dissolved in 800 ml of distilled water.

Solution B: 4/4 g of thiobarbituric acid in 150 mL of distilled water at a temperature of 95 ° C was solved.

Solution C: Solution A and B mixed together and PH with 5 M NaOH reached to 2/8 and then 0.15 g Merthiolate added to it and finally the final volume with distilled water reached to one liter, and filtrated with Filter paper.

Preparation of Coomasie Brilliant Blue Solution and Dye Removal from Solutions

Dye Removal from Solutions: To 400 ml ethanol, 70 ml glycine acetic acid, and 530 ml distilled water added and then kept in a dark-colored container at 4 ° C.

Preparation of Coomasie Brilliant Blue Solution: 125 g Preparation of Coomasie Brilliant Blue Solution dissolved in 500 ml solution removing color and then kept in a dark-colored container at 4 ° C, after filtered.

Preparation of 5 M NOAH

200 g of sodium hydroxide dissolved in one liter of distilled water and then Autoclaved at 121 ° C for 30 min after filtration.

monospecific gamma globulins from blood serum

to avoid from interference in serum total, 1000 g of solid ammonium sulfate was added to 1000 ml of distilled water at 50 ° C and put for 5 hours in Magnetic stirrer. It was kept one night in room in order to be saturated where it was kept in room temperature for one night aimed to prepare it in saturation and then Ph solution using 4% Ammonia reached to 7.2 after Precipitation, kept at 4° C. Then, 150 ml of serum containing Monospecific antibodies with ratio of 1 to 2 with PBS 2/7 was diluted. After 30 minutes, the solution kept at 4° C for one night so as the reaction gets finished. After Centrifuge, the suspension over ×3500g at 4° C for 60 minutes and removing the solution participated with 400 ml 45% saturated ammonium sulfate, rinsed and then Centrifuged one more time. And Deposits dissolved in 110 ml 7/2 PH PBS where Centrifuge found necessary to remove unnecessary materials existing in solution whereby the deposits from solution mixed and then Centrifuged with Ammonium sulfate with 40% saturation. 10 ml buffer added to the deposit in order to be prepared as suspension and then the serum containing monospecific antibody transmitted to dialysis bag and dialysis sustained on for 24 hours, aimed to remove ions existing in solution.

After the dialysis completed, centrifuge finished and the supernatant passed through filter of 0.45 micron in 50 ml vial, kept at 4° C so far as get used.

Deposition of rabbit IgG serum using Ammonium sulfate

Blood sampling about 5 cc from Young rabbits heart reported and the blood kept in a refrigerator at 4 ° C for 2 hours, then centrifuged at 3500× g for 30 minutes and the serum separated from it so that 1.4 g Ammonium sulfate added to 4 ml rabbit serum at 4 ° C so far as deposition develops and the Ammonium sulfate solution found indissoluble. The mixture centrifuged for 20 minutes at 3500× g and deposition dissolved in 2 ml PH 7/2 buffer. To remove Ammonium sulfate from serum, dialysis sustained on for 24 hours. To prepare IgG in deposition, 50% saturated Ammonium sulfate has been used. To calculate the saturation percent of Ammonium sulfate, the software calculating referred to http://www.encorbio.com/protocols/AM-SO4.htm has been used.

SDS-PAGE method using Coomasie Brilliant Blue Solution and Silver nitrate

SDS-PAGE method using Silver nitrate

Electrophoresis accomplished using Silver nitrate as band not observed in the hot acid and freez and thaw methods.

SDS-PAGE method using Coomasie Brilliant Blue Solution

SDS-PAGE (Sodium dodecyl sulfate) gets used for protein samples. As protein A's molecular weight varies from 42 kDa to 52 kDa and according to what mentioned above, protein A's molecular weight would be equal to 52 kDa in NCTC8325 strain. Hence, 12% gels made but it has to take into consideration where the protein's molecular weight is less, Percent of polyacrylamide and gel rises to prepare gel in compressed form in package. This method includes preparing gel in two stages entitled stacking and resolving gels. Before the solutions prepared, in initial, spacer and the comb found essential to be rinsed with water and soap and then cleaned with distilled water and ethanol, drained with gas in the end. Hence, Spacers placed in straight among glasses and closed on the stands and then clips tightly closed. At first, Resolving gel prepared for 20 ml where the way to prepare it has been brought as following:

Resolving Gel:

Distilled water	6.6**ml**
Acryl amide	**8 ml**
Solution B	**5 ml**
Ammonium persulfate	**200**

	microliters
Temed	**8 microliters**
SDS	**200 microliters**

The amount of Ammonium persulfate and SDS are always equal with each other. Temed acts as glue while preparing gel for solution, thus, it adds in the end to the solution, but, 1.5 ml of resolving is removed before temed added, poured in a microtube and added for about 4-6 microliter. Observation needed at this stage to specify at what time interval would be closed while the gel poured between two glass flats whereby at the bottom of both flats blocked with Agarose or Temed. Hence, the resolving get filled with ethanol of 96%, to have Polymerization reaction away from the air while gel closed, ethanol has to be removed and stacking solution has to be prepared.

Method of Preparation of Stacking
Stacking Gel

Distilled water	**2.7 ml**
Acryl amide	**670 micro liters**
Solution C	**500 micro liters**
Ammonium persulfate	**40 micro liters**
Temed	**4 micro liters**
SDS	**40 micro liters**

Preparation of samples

40 ml sample with 10 ml of sample buffer in a microtube vortexed and boiled for 5 minutes and then samples placed in Wells using Hamilton syringe(Sample buffer size should be one fifth of the sample).

cast was connected to a power source with 90 volts and 0.08 amps and after 2 hours while get loaded up to the bottom of flat, the gel removed and colored at laboratory temperature for 5 minutes on shaker on the Basin containing Coomasie Brilliant Blue Solution or staining solution and then the gel rinsed several times with distilled water and placed for 5 minutes in the basin containing the color removal solution or Destaining solution so that waiting for the bands to be emerged.

RESULTS

Figure 3: Bacteria aggregation in plasma

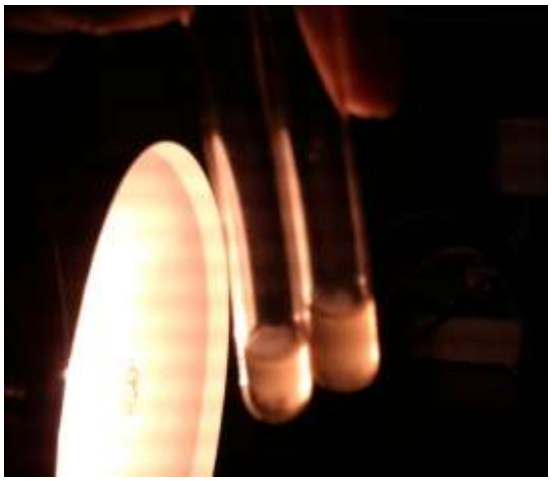

Figure 4: Clot formation after 4 hours

Figure 5: Change of color from red to yellow after *Staphylococcus aureus* cultured

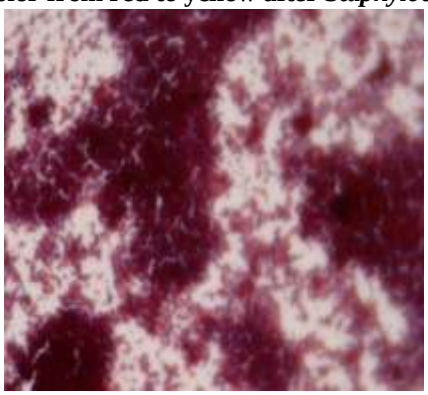

Figure 6: Microscopic slides of Triton lyses method for the cell wall of toluene, Triton and EDTA

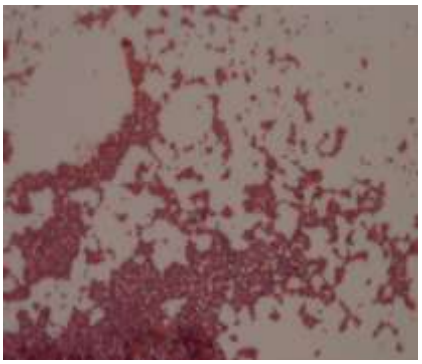

Figure 7: Microscopic slides of Cellular lysis of Freez and Thaw method

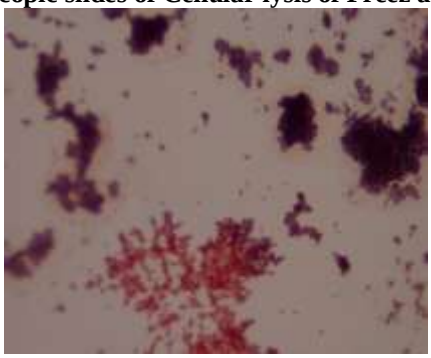

Figure 8: Lyses method of cell walls using Lysozyme method

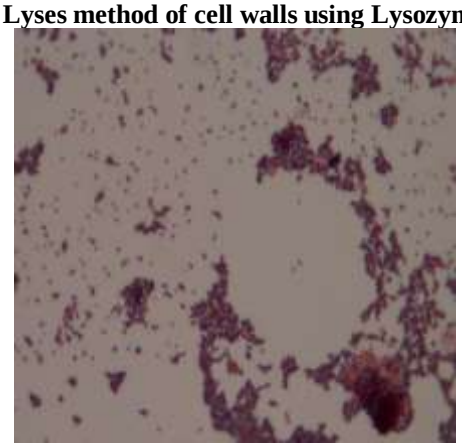

Figure 9: Microscopic slides of Cellular lyses method with Sodium metaperiodate

Figure 10: Blood sampling from rabbit in order to prepare serum

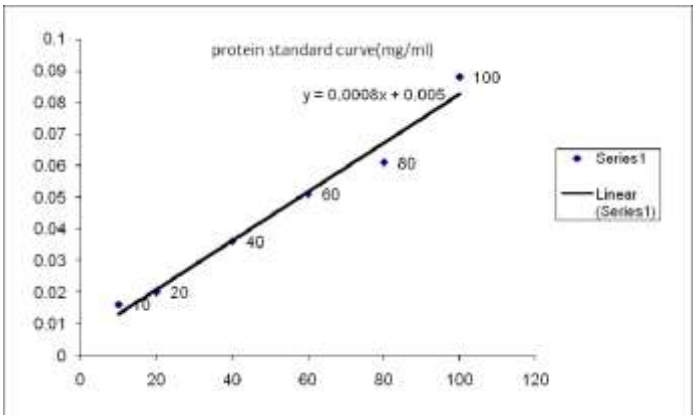

Figure 11: Standard protein curve

To determine the amount of protein, Lowry method and Y=0.0055X+0.0008 have been used.

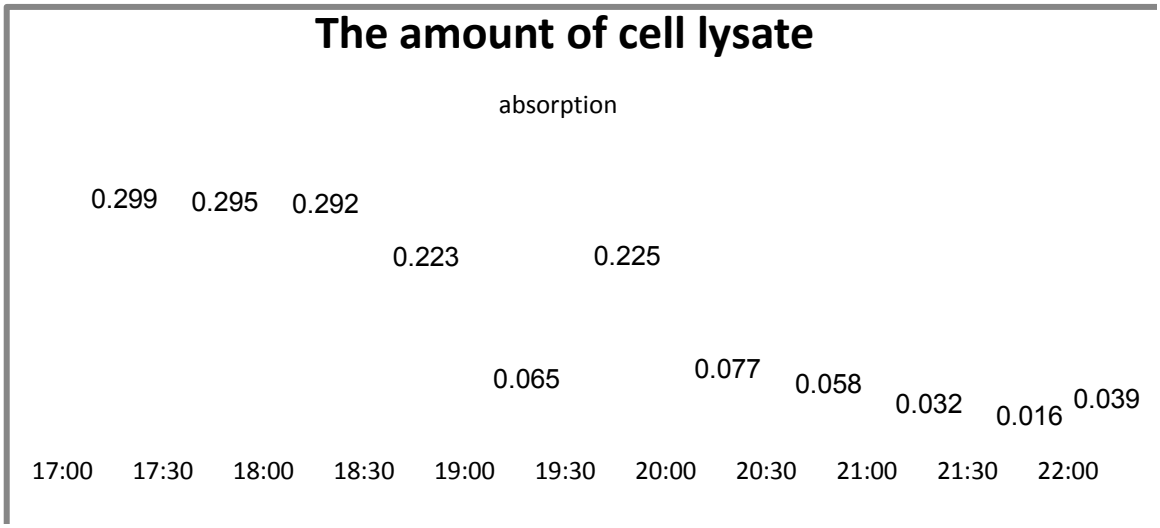

Figure 12

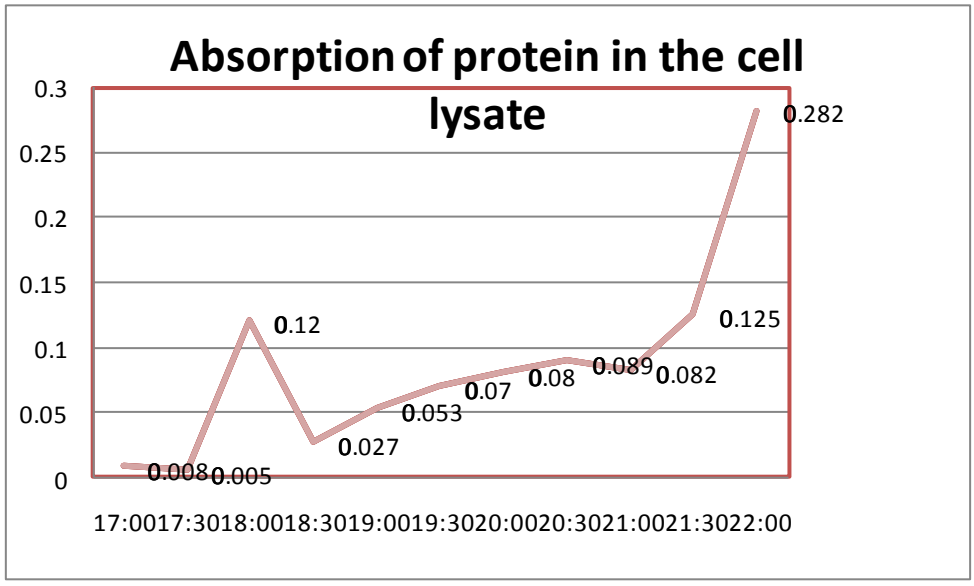

Figure 13

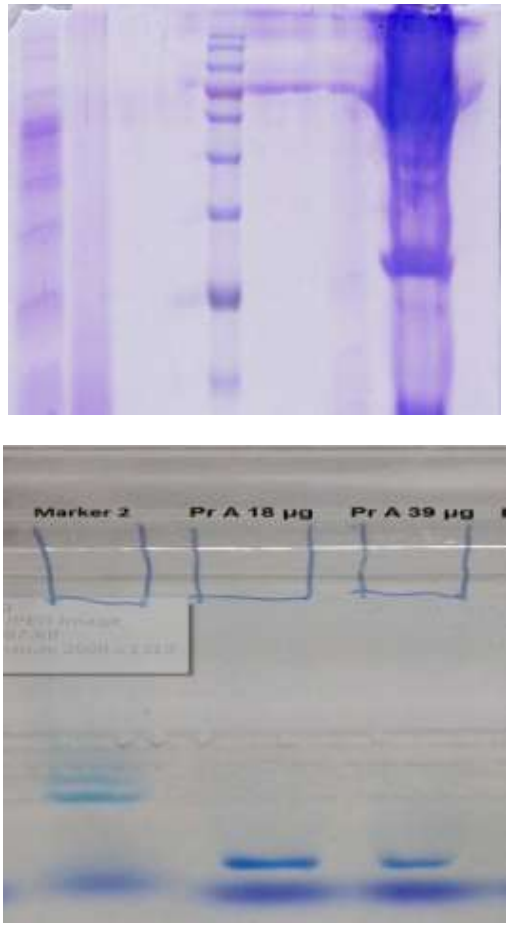

Figure 14: Protein electrophoresis using Sodium metaperiodate, Triton and EDTA

CONCLUSION

Results of SDS-PAGE

Six samples placed in wells where five wells associated to 5 methods of lysis of bacteria to extract purify *Staphylococcus aureus* protein A. *Staphylococcus aureus* subsp. aureus (strain NCTC 8325) and one well associated to the rabbit serum in order to detect IgG band where methods of Sodium metaperiodate, lysozyme and Freez and Thaw had band but methods of hot acid and Triton lacked protein. Further, the band associated to IgG observed in the sample for rabbit serum. The concentration for the sample of rabbit serum was so high so that loaded later than other bands, whereby Serum samples were distributed to the remaining samples.

The way to prepare sample buffer

sample buffer	6X	5X	1X
Tris	4gr	3/38gr	0/67gr
Glycerol	60ml	50ml	10ml
Bromophenol Blue	0/03gr	0/03gr	0/03gr
SDS	6gr	5gr	1gr

Tris initially dissolved in 30 ml of distilled water and the PH modulated to 8/6 and then SDS was added, thereby stirrer modulated with slow round whereby the color added in several stages reached finally to 100 ml.

Preparation of staining soluion

Coomasie Brilliant Blue Solution added to the ethanol and then added to acetic acid reached to 1 liter.

Polyacrylamide gel electrophoresis with silver nitrate staining:

The gel is made from a acrylamide polymers where the base acrylamide polymer in form of transverse bands let this polymer used regularly so that pores with specific and equal diameter appear in gel whereby Gel polymerization by adding ammonium persulfate (or riboflavin) started, proliferated by adding N,N,N',N'–tetra methyl ethylene diamine or 3-(Dimethylamino) propionitrile.

N,N,N',N'–tetramethyl ethylene diamine causes the free radicals of Ammonium persulfate develops whereby such radicals lead to Polymerization, because the free radicals existing in 3-dimethylamino-propionitrile is essential for this process, thus, Polymerization occurs while low Ph exists or stops.

Increasing trend of N,N,N',N'–tetramethyl ethylene diamine let the speed of Polymerization increases.

Preparation of staining soluion

Methanol	**450 ml**
Coomasie Brilliant Blue Solution	**1 g**
acetic acid	**100ml**
Distilled water	**450 ml**

Preparation of destaining solution

Methanol	**100ml**
acetic acid	**100ml**
Distilled water	**800ml**

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