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Isolation, purification and characterization of Streptolysin-*O* from *Streptococci*

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ABSTRACT

Streptolysin–O is an oxygen labile hemolysin present in broth filtrate after the growth of Hemolytic *streptococci.* In this study, *Streptococcus* was grown on Dextrose Infusion Broth from the broth filtrate; the streptolysin-O activity was confirmed by Hemolytic assay. Purification was done by ammonium sulphate precipitation, followed by column chromatography. Quantification of the purified protein was done by Lowry's method. Finally characterization of streptolysin-O was done through pH stability, temperature Stability and enzymatic assay. The optimum pH activity is 7 and the optimum temperature is 60˚C further the enzymatic assay was done for complete hemolysis.

Keywords: streptolysin, chromatography, hemolysin.

INTRODUCTION

Streptococcus is a genus of spherical Gram-positive bacteria belonging to the phylum firmicutes and the lactic acid bacteria group. Streptococci are facultative anaerobic and catalase-negative. They metabolized carbohydrates fermentative; lactic acid is the major metabolite. Streptococci are Gram-positive cocci (spherical or ovoid) often occurring in pairs and chains. Toxins called hemolysins [*haima,* blood, and Greek *lysis,* dissolution], can be secreted by pathogenic bacteria. Many hemolysins probably form pores in the plasma membrane of erythrocytes through which hemoglobin and/or ions are released (the erythrocytes lyses or, more specifically, hemolyze).

Streptolysin-O (SLO) is a hemolysin, produced by *Streptococcus pyogenes,* that is inactivated by O_2 (hence the "O" in its name). SLO causes beta hemolysis of erythrocytes on agar plates incubated anaerobically. A complete zone of clearing around the bacterial colony growing on blood agar is called beta hemolysis (Prescot 2002). On blood agar, the species exhibit various degrees of haemolysis, haemolysis produced by colonies on blood agar and Lancefield serological grouping are important factors in presumptive identification.

The strategies by which GAS adhere, and later invade, the human host are multiple and complex, involving several different adhesions and invasions. At least 17 adhesin candidates have been described,(Courtney HS et.al.,(2002) but the most extensively studied have been lipoteichoic acid (LTA), M protein, and fibronectin binding proteins.LTA adheres to fibronectin on human buccal epithelial cells.LTA and anti-LTA inhibit such adhesion, and deacylated LTA neither binds nor inhibits adhesion to oral epithelial cells (Beachey et.al., 1982). GAS surface proteins that bind fibronectin have been studied extensively and are important in adherence to both throat and

skin. These include protein F1 (PrtF1), (Hanski et al., 1992) also known as SfbI (streptococcal fibronectin binding protein I).

Once adherence has been achieved the microbe must maintain itself on the pharynx and/or skin to establish a state of prolonged colonization. M-positive strains (but not isogenic M-negative mutants) form aggregates on buccal or tonsillar epithelium, which are postulated to promote micro colony formation (Caparon et al., 1991). This event may explain reports that M-positive strains persist longer in the oropharyngeal cavity of baboons59 (Ashbaugh et al., 2000) and rats42 than do M negatives, even though the protein is apparently not required for initial adherence. In an animal model of colonization and infection 59 mice inoculated intra nasally with an encapsulated GAS strain had more persistent throat colonization and higher mortality than did those inoculated with an a capsular mutant.

Although GAS are not generally thought of as intracellular pathogens, experiments over the past few years have shown that the microorganisms can penetrate a variety of cultured human respiratory epithelial cells (Penta et al., 1999). As with bacterial adherence, multiple mechanisms seem to be involved in cellular penetration. Both M protein (Sedgewick et al., 1999) and PrtF164 have been implicated in the internalization process, which involves cytoskeleton rearrangements and interactions between the streptococcal adhesions, host integrins, and integrin ligands (Dombek et al., 1998).

In contrast to M protein and fibronectin-binding proteins, the hyaluronate capsule impedes GAS adherence to and internalization within Hep-2 cells and human keratinocytes, (Wessels et al., 1996) even though encapsulation is associated with greater lethality in a mouse model of invasive soft-tissue infection (Wessels et al., 1998) This apparent paradox was clarified when it was seen that binding of the streptococcal capsule to the hyaluronic-acid-binding protein CD44 on human epithelial cells (Schrager et al., 2000) induces cytoskeleton rearrangements, resulting in disruption of intercellular junctions and allowing the microorganisms to remain extracellular as they penetrate the epithelium (Cywes et al., 2001).

With the earliest recordings coming from the Fifth Century B.C., *streptococcus pyogenes*, and more frequently, its symptoms have been prevalent among doctors and historians for hundreds of years. The first mentioning of *streptococcus pyogenes* is to be credited to Hippocrates, in which he describes the relative symptoms of the flesh-eating bacteria in its early stages.

The group A streptococcus (GAS) (*Streptococcus pyogenes*) is among the most ubiquitous and versatile of human bacterial pathogens. The litany of infections attributable to it includes such common clinical illnesses as pharyngitis, impetigo, cellulitis, and scarlet fever. It may also, however, produce acute, life-threatening illnesses, among which are puerperal sepsis, necrotising fasciitis, myositis, and toxic shock. In a five-state laboratory and population-based surveillance study between 1995 and 1999, invasive group A streptococcal infections occurred at a rate of 3·6 per 100 000 population annually in the USA, accounting for 9600–9700 cases and 1100–1300 deaths (Barrett et al., 1995–1999). In this experiment we have been isolated and purify the streptolysin-O by employing chromatography and electrophoresis techniques.

MATERIALS AND METHODS

Isolation of bacteria: The inoculums of streptococcus was taken from the human wounded skin, as these bacteria feed on the blood cells it was easily available on wounded skin.

Characterization of Bacteria: Primary screening of bacteria were done by gram-staining test, which conforms the presence of gram positive bacteria. Streptococcus was further conformed by the hemolysis reactions on blood agar where lyses RBC's were found. Biochemical tests – catalase and coagulase test further confirmed the presence of streptococcus with no impurity of staphylococcus.

Mass culture of bacteria: For the isolation of streptolysin from streptococcus the mass culture of bacteria is needed which was done by taking the inoculums of streptococcus in dextrose meat infusion broth and incubated 37^{o}c for 12-16 hours. Freeze thaw procedure was used to grow the bacterial cells in the culture medium which further centrifuged at 5000 rpm for 30 minutes. The supernatant was collected and EDTA was added to stirred at the magnetic stirrer and freezed at -20^{o}C for overnight. The supernatant was thawed twice at 4^{o}C and collected.

Purification of Streptolysin: Now the supernatant contains the haemolysin, which was purified by adopting different processes-ammonium sulphate and gel filtration chromatography was used to precipitate and purify the streptolysin respectively followed by dialysis. SDS PAGE was performed to know the molecular weight of streptolysin, for protein concentration estimation BSA was taken as a standard by using Lowry's method.

RESULTS AND DISCUSSION

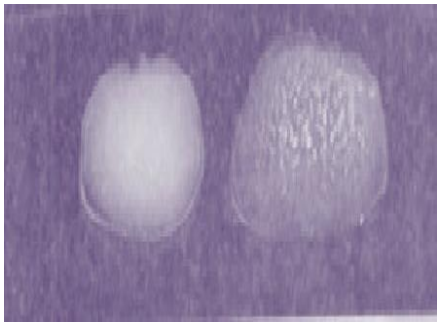
Isolation of bacteria: Bacteria present in the skin were isolated by streak plate method as mentioned earlier. Mixed culture was obtained as the normal skin flora contains many micro-organisms as shown in the figure 4.

Morphological, physiological and biochemical analysis

Characterization: Morphological, physiological and biochemical characterization of the skin swab culture was carried out to identify the Streptococci species.

Catalase test: The catalase test was negative as there was no bubble formation. Thus the culture was differentiated from staphylococcus (figure 5).

Figure. 4 Culture from skin swab



Positive control skin swab

Figure. 5 Catalase Test

Coagulase test: Coagulase test was negative as there was no agglutination with rabbit serum. Thus the culture was identified as streptococci (figure 6)**.**

Positive control skin swab culture

Figure. 6 Coagulase Test

Beta Haemolysis of Streptococci Species: Clear zone formation was found surrounding the culture indicating the complete lysis of RBCs. Thus the streptococcus was identified as beta haemolytic.

Pure Culture of β- Hemolytic Streptococci: The Streptococci which was confirmed as group A beta haemolytic Streptococci was further sub cultured in nutrient agar plate to obtain pure culture (figure 7).

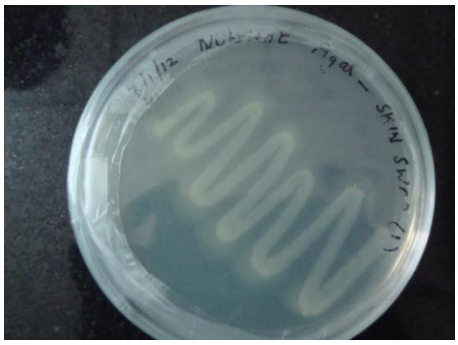


Figure. 7 Pure culture in nutrient agar plate

Streptolysin Production: Group A beta haemolytic streptococci was inoculated in Dextrose Meat Infusion broth for the production of streptolysin O (SLO). Growth of streptococci was observed after 16 hours of incubation (figure 8).

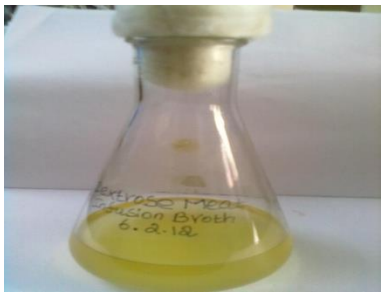


Figure. 8 Production of SLO in Dextrose meat infusion broth

Haemolytic Assay: Lysis of RBCs was observed till 5th well which confirms the presence of SLO. In the control well button formation of RBCs took place (figure 9).

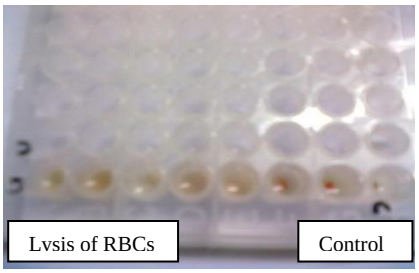


Figure. 9 Haemolytic assay for SLO

Ammonium Sulphate Precipitation: Figure 7 shows the protein pellet after entrifugation followed by ammonium sulphate precipitation.

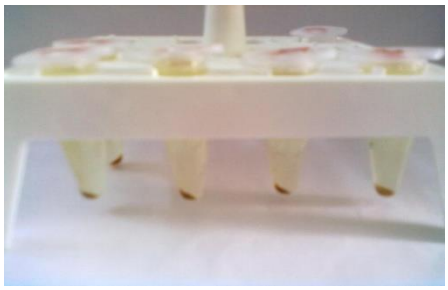


Figure.10 Pellet formed after ammonium sulphate precipitation

Gel Filtration Chromatography: The sample passed through the column was eluted and collected as different fractions. The OD was observed at 280nm and the protein concentration was found to be 10mg/ml.

Freeze thaw method: The supernatant collected from the broth was freeze thawed as shown in the figure. The 1/5th of the supernatant, which thawed first, was collected (figure 11).

Figure.11 Freeze thaw method

Protein Estimation: Estimation of protein for the dialyzed sample was carried out by Lowry method. OD was observed at 600nm and the protein concentration was found to be 12.4mg/ml. (Table.1)

SDS-PAGE: Marker was loaded in lane 1. Lane 3 and 4 shows the band for SLO which determines the molecular weight as 69kda (figure 12).

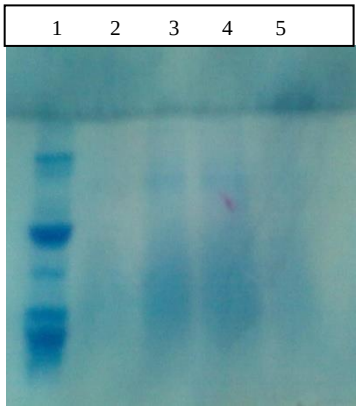


Fig. 12 SDS PAGE

P^{H} and temperature stability: pH and temperature stability was checked for SLO. The optium pH was found to be 7 and SLO was found more stable at temperature 30-40^{o}C. The activity of SLO was completely destroyed from 50^{o}C and pH 10 Figure 1 &2.

SLO inhibition assay: Enzymes trypsin and lysozyme inhibited the activity of SLO resulting in settling down of RBCS Figure 3.

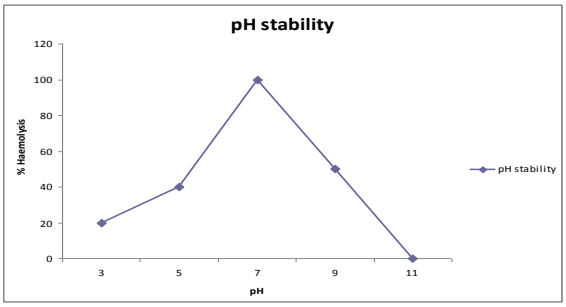


Fig. 1 pH stability of SLO

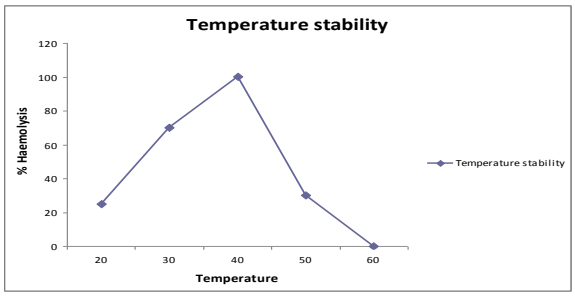


Fig. 2 Temperature stability of SLO

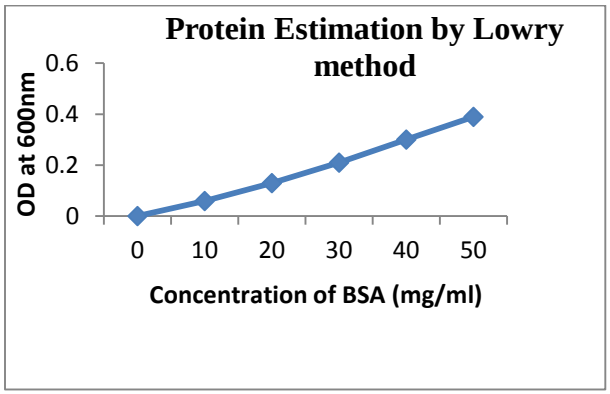


Fig. 3 Protein Estimation by Lowry method

Table 1: Protein estimation by Lowry's method

Reagent used	B	S1	S2	S3	S4	S5	SLO	SLO
BSA(µL)	0	10	20	30	40	50	-	-
Conc(µg)	-	10	20	30	40	50	-	-
Sample (µL)	-	-	-	-	-	-	25	50
D.H_2O (µL)	500	490	480	470	460	450	475	450
Lowry's reagent (mL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Folin's reagent (µL)	250	250	250	250	250	250	250	250
OD at 600nm	0	0.06	0.13	0.21	0.3	0.39	0.39	0.62

CONCLUSION

The study concludes that the streptolysin O which was isolated from the group A beta haemolytic streptococci has the ability to lyse the RBCs which was confirmed by haemolytic assay. The maximum stability of Streptolysin 0 was at pH 7. The activity of SLO was most stable between 30-40^{o}C and above 50^{o}C the activity was rapidly destroyed. It was confirmed through SDS PAGE that the molecular weight of streptolysin O is 69kDa. The enzymes trypsin and lysozyme has the ability to inhibit the

activity of SLO. Streptolysin O has many applications like cancer treatment adhesions, scars, and fibrosis.

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