# Localization of Streptolysin S (SLS) by Blood Agar Overlay Technique (Zymogram)

BADARUDDIN A. MEMON
*Department of Microbiology, Shah Abdul Latif University, Khairpur, Sindh, Pakistan.*
doctor_badar@yahoo.com

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**Summary**: Streptolysin S (SLS) is the non-antigenic, oxygen-insensitive cytolysin which is produced by Group A streptococci. It is largely responsible for a zone of beta-haemolysis surrounding colonies on blood agar media. In this paper it is described, for the first time, reliable detection, visualization and quantification of SLS using blood agar overlay (zymogram) method. This technique is sensitive and protocol describes a method with broad potential to elucidate previously undetectable biological activity of streptomycin S (SLS) on gel electrophoresis.

## Introduction

*Streptococcus pyogenes* Group A is a major etiological agent causing a variety of human diseases ranging from pharyngitis to severe and life threatening invasive diseases, such as toxic shock-like syndrome (TSLS) and necrotizing fasciitis [1]. Two major cytolytic toxins are produced by Group A Streptococci (GAS); these are the oxygen-labile streptolysin O (SLO) and the oxygen-stable streptolysin S (SLS) [2]. Streptolysin S is a potent cytolytic toxin responsible for the zone of β-haemolysis surrounding GAS colonies grown on blood agar and SLS production is now linked with severe infections including toxic shock syndrome and necrotizing fasciitis (NF), in which GAS can invade skin or soft tissues and even destroy limbs.

SLS is serum extractable, oxygen-stable membrane damaging agent of peptide nature and is non-immunogenic [3-5]. SLS is only active when complexed with certain carrier substances or stabilizers, such as RNA-core, serum components, nonionic detergents or bisazobenzidine dyes [6, 7]. These apparently unrelated molecules to which the haemolytic moiety complexes serve to remove the active peptide from the bacterial surface and bind it in an active conformation [8]. This exotoxin is a very potent membrane-damaging agent and lyses a wide variety of living cells and organelles. SLS has a membrane damaging effect not only on erythrocytes, but also on lymphocytes [9], neutrophils, platelets [10], tumor cells [11] and subcellular organelles [12, 13]. Elias *et al.,* [14] found that the treatment of erythrocytes and their ghosts with phospholipase C, followed by exposure to SLS, resulted in diminished binding of SLS suggesting a role for membrane phospholipids in SLS action.

Lai *et al.*, [15] reported that the active peptide of SLS consisted of 32 amino acid residues comprising tyrosine and phenylalanine, but was deficient in histidine, valine, leucine, cysteine, methionine and arginine residues. According to Alouf and Loridan [16], carrier free SLS is basic (pI 9.2) and the molecular weight of the denatured peptide is about 1,800. More recently, Betschel *et al*.,[17] and Nizet *et al*., [18] identified the genes encoding SLS production and showed that a gene *saga* encoded a 53 amino acid prepropolypeptide that was thought to be proteolytically cleaved to produce SLS as a 30 amino acid peptide.

The present study was undertaken to detect and localize the biological activity (haemolytic activity) of SLS by blood agar over lay (zymogram) technique using native polyacrylamide gel ectrophoresis. (PAGE).

## Results and Discussion

A summary of purification data is presented in Table-1. The elution profile shown in Fig. 1 revealed the haemolytic activity of the crude material was recovered as a single sharp peak in fractions 19-22 (each fraction of 3 ml) from hydroxylpatite column. Fraction 19 gave the highest haemolytic activity (800 HU/ml) and contained approximately 0.11 mg of purified SLS with a specific activity of approximately $3.5X10^5$ HU/mg protein.

Table-1: Purification and Recovery of Streptolysin S.

| Purification Step | Volume (ml) | Total activity (Hu) | Total Protein (mg) | Specific activity | Purification Factor | Recovery (%) |
|---|---|---|---|---|---|---|
| Starting material | 220 | 220000 | 2.5 | $9x10^4$ | 1 | - |
| Hydroxylaptite column | 9<br>18, 19, 20 Three fractions | 49152 | 0.13 | $3.5x10^5$ | 4.7 | 11 |
| Fraction 19 | 3 | 36864 | 0.11 | $3.5x10^5$ | 2.5 | 11 |

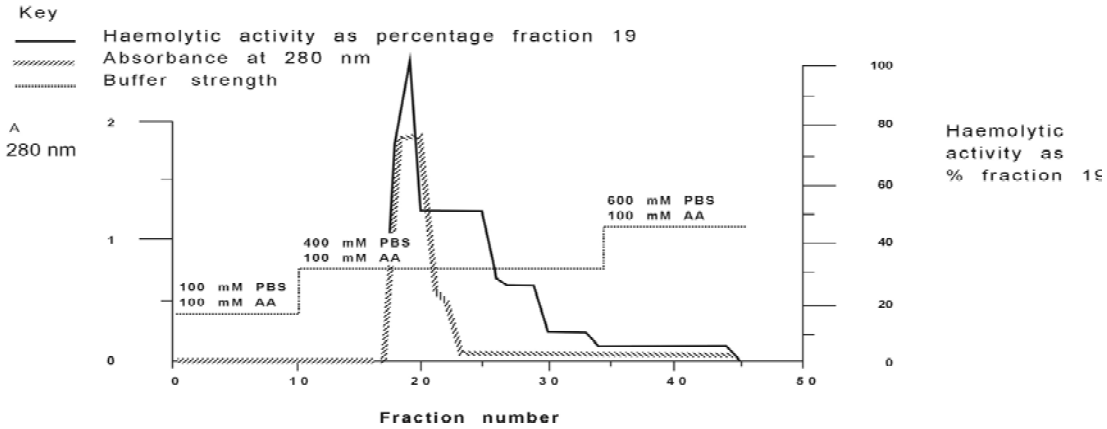


Fig. 1: Elution profile from hydroxylapatit column.

The degree of purity of peak fractions from hydroxylapatite chromatography was assessed by 15% SDS-polyacrylamide gel electrophoresis. The peak haemolytic fractions contained a low molecular weight peptide which proved difficult to visualize by coomassie blue staining of SDS-PAGE gels but was detected by the silver staining technique. The low molecular weight peptide in crude and purified preparation (Fig. 2 and 3) always ran at the dye front of the gel as visualized through silver staining whereas appearance of other extra bands were all components of yeast RNA-core, the carrier molecule. The molecular weight of SLS was reported 4000 Daltons.

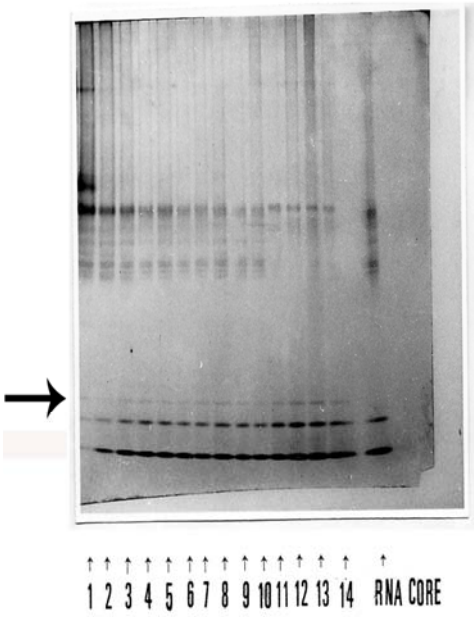


Fig. 2: Analysis of crude preparation of SLS by Native Polyacrylamide gel. Samples from fourteen (14) inductions were analyzed on 15% native polyacrylamide gel. RNA-core was also analyzed as control. The gel was silver stained. Arrow indicates the suspected band of SLS.

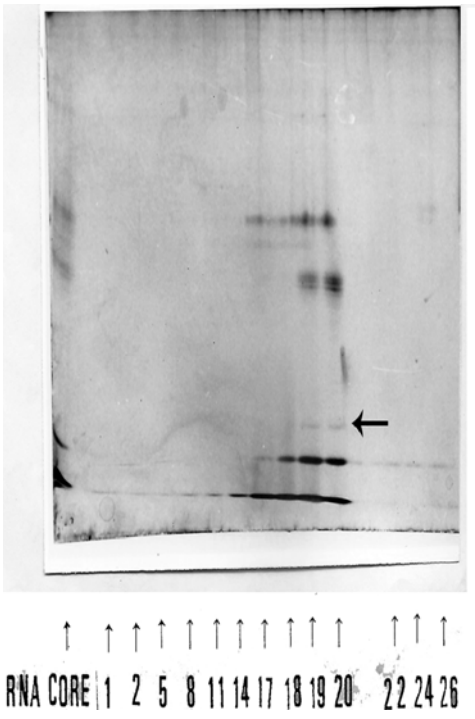


Fig. 3: Analysis of purified preparation of SLS by Native polyacrylamide gel. Different fractions of purified SLS were analyzed on 15 % native PAGE. RNA-core was also run. The gel was silver stained. Arrow indicates the suspected band of SLS.

In order to detect the biological activity of SLS, native PAGE was first run and developed by the silver staining technique. It was found that the crude material from the induction buffer (Fig. 3) and the purified preparation (Fig. 4) both contained a band which was not present in the sample of RNA-core which was included as a control.

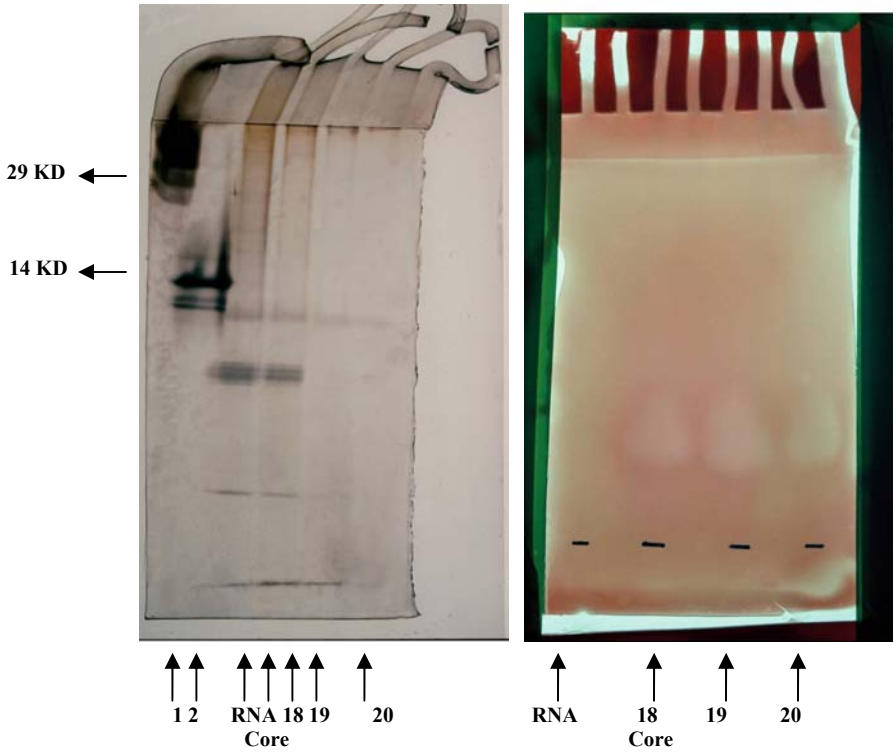


Fig 4: Native polyacrylamide gel: Key: 4a: The fractions (18, 19, & 20) loaded as duplicate of zymogram gel with mol: wt standard markers (carbonic anhydrase and α-lactalbumin and were run in 15% native polyacrlamide gel. RNA-core was run as control. The gel was silver stained. Lane 1 and 2 represent α-lactalbumin and Carbonic anhydrase respectively. 4b. Three haemolytic fractions of purified SLS (18, 19 and 20) were run on 15% native polyacrylamide gel. 1% purified agar containing 2% v/v washed sheep erythrocytes was overlaid on the gel to detect haemolytic activity after electrophoretic separation had occurred.

The zymogram technique was used to locate haemolytic activity of SLS on native gels. A native SDS- PAGE gel was run with a duplicate set of bands at side of gel. After electrophoresis was completed, the gel was cut into two halves, one was stained by the silver technique, (Fig. b) and the duplicate half was used for the zymogram assay (Fig.1a). A suspension of washed sheep RBCs and agar was mixed and overlaid on the gel. After incubation, an area of lysed RBCs appeared in a position on the gel equivalent to that occupied by the stained band, identified as SLS. Three fractions were assayed by this method and fraction 19 produced a larger haemolytic zone than fractions 18 and 20, which also corresponded to the result of the assay for total haemolytic activity.

Estimated molecular weights of less than 4,000 have been reported for SLS [19-21]. But clear and direct supporting evidence has been not published. Alouf and Loridan [16] first time presented the data concerning the analysis of SLS on SDS and native- PAGE. According to their findings, a band which they identified as SLS migrated with the dye front in SDS and native PAGE, although the gel profile of RNA-core carrier molecule was not reported. From these results the molecular weight of SLS was estimated to be below 4000.

In current study, when crude and purified SLS was analyzed by native-PAGE with RNA-core as control, it was observed that RNA-core profile lacked one band as compared to the SLS gel profile and this band was suspected of being SLS. Comparison of the pattern of haemolysis with that of the stained bands after silver staining showed that the haemolytic activity resided in a component not at the dye front at a position expected of a small peptide. Three fractions were assayed by this method and fraction 19 produced a relatively larger haemolytic zone than fractions 18 and 20 as expected from the haemolytic assay of these fractions.

Nizet *et al.* [18] identified the genetic basis for SLS production by GAS as requiring a group of nine linked genes in SLS production. A mutation in any one of the genes rendered GAS unable to make the toxin. Also Kazuhiro *et al.,* [22] reported a new

gene cluster for the lantibiotic streptin that is possibly involved in SLS formation. The toxicity of SLS to a variety of cells and smooth muscle has qualified this extracellular product as accessory virulence factor. It is now well-accepted that this potent bacterial toxin plays an important role in producing necrotizing fasciitis (NF), the rapid infection of soft tissue referred to as "flesh-eating" disease.

In this paper it is described, for the first time, reliable detection, visualization and quantification of SLS using blood agar overlay (zymogram method). This technique is sensitive and protocol describes a method with broad potential to elucidate previously undetectable biological activity of SLS on gel**.**

## Experimental

*Strain*

Strain C203S of *Streptococcus pyogenes* group A was obtained from Professor Joseph E. Alouf, Institut Pasteur, Paris. It was selected because it produces a high level of SLS [12]. The strain was stored lyophilized or as broth culture supplemented with 20% glycerol at-20°C.

*Media*

The culture medium (BHI-BM) used for toxin production and the details of the preparation were as follows: 50 ml of Brain heart infusion broth (Difco) was supplemented with 1% (w/v) maltose (BDH) and 2% (w/v) sodium bicarbonate (May and Baker) and was abbreviated as BHI-BM. Stock solutions of sodium bicarbonate (10% w/v) were sterilized by membrane filtration (0.45 µm pore size) and stored at $4^0$ until used. Volumes of BHI_BM sterilized by autoclaving (15 1b/15 ml) were supplemented with sterile maltose (1% final concentration) and sterile sodium bicarbonate (2% final conc) before inoculation. Inocula were grown on agar plates consisting 200 ml of BHI broth (Difco) solidified with o.7 % w/v technical agar (Oxoid) and sterilized by autoclaving at $121^0$. After pouring, solidifying and drying the plates were stored at $4^0$C until used.

*Cultural Conditions*

BHI agar plates were inoculated from a loopful of thawed glycerinated broth culture and incubated at $37^0$C for 10 h. Simultaneously, plates of Sheep blood agar (Oxoid Blood Agar base No.2, Oxoid Ltd, London, England) plus BHI Blood agar were also inoculated to confirm the presence of β-haemolytic phenotype of the colonies. After incubation, 50 ml of BHI. BM (Difco) was inoculated as a starter culture from the growth harvested from one BHI agar plate. After incubation for 10 h at $37^0$ without shaking, the starter culture was added to 2 l of BHI-BM in a 2 l Erlenmayer flask which was then incubated at $37^0$ for 6-7 h without shaking.

*Measurement of Bacterial Growth*

Growth was estimated by measuring the $A_{600nm}$ of samples of culture withdrawn at 1 h intervals from 5-9 h. When the $A_{600nm}$ exceeded 0.3 the sample was diluted with BHI-BM to bring the absorbance value to within measuring range. Absorbance values were measured on a Simadazu recording spectrophotometer (Graphicord-UV 240) using cells with a 1 cm light path.

*Induction of Streptolysin S*

The method used was essentially that of Alouf and Loridan [16].except that centrifugation time was reduced to 10 min. A 50 ml volume of overnight culture grown statically was inoculated into 2. 5 l (20 ml in 1 l or 10 ml in 500 ml) and grown unshaken for 6-7 h at $37^0$C. The culture was centrifuged at 12000g (Sorval RC-5B) FOR 20 min at $4^0$C and the cell pellet was washed in 100 mM potassium phosphate buffer, pH 7.0 before resuspension to a final volume of 40 ml (or 16 ml in case of 1 l, 8 ml in case of 500 ml) of induction buffer (IB) ($KH_2PO_4$ 100mM, $MgSO_4$ 2mM, the final volume of 40 ml was adjusted to pH 7.0 with NaOH) supplemented with 30 mM-maltose. The cell suspension was incubated for 5 min at $37^0$C and then induced by adding 0.5 mg/ml RNA-core (Sigma). After 5 min the cell suspension was centrifuged (Sorval RC-5B) at 15000g for 10 min at $4^0$C. The supernate (crude SLS) was collected and made 100 mM with ammonium acetate (sigma) to stabilize [20]. The pellet was then resupended in 40 ml (16 or 8 ml) of induction buffer supplemented with maltose (15 mM final concentration) and induced as described above. Ten to 14 inductions could be made on the same pellet. The supernates from the inductions were kept at 4°C.

*Purification of Streptolysin S*

All steps were done at $4^0$C. Crude SLS preparation i.e., pooled supernates from 14 induction cycles was applied to a column (2.5x14 cm) of hydroxylapatite (biogel HTP, from Bio-Rad) equilibrated with 100 mM potassium acetate / 100

mM ammonium acetate (pH. 7.0). After application of the crude toxin the column was washed with 1 column volume (100 ml) of this buffer and then the toxin was eluted with 400 mM-potassium phosphate/100 mM ammonium acetate buffer (pH. 7.0). The flow rate was 30 $ml^{-1}$ and 3.0 ml fractions were collected and analysed by $A_{280}$ and spot haemolysis. Haemolytic fractions were then further characterized by haemolytic titre, molecular weight estimation on SDS-PAGE and haemolytic activity on native gels. The peak fraction gave the highest haemolytic activity (8000 HU/ml) and contained approximately 3.5 x $10^5$ HU/mg protein. This material was used to determine haemolytic activity on native gels.

*Polyacrylamide Gel Electrophoresis*

Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [23]. After trying different thickness, 15% resolving gels were used after obtaining best result. Stacking gel contained 4.5% (w/v) acrylamide. Samples were solubilized in a water bath at $100^0$C for 90 seconds. The same procedure was applied to the molecular weight standards (SDS-7 Kit) obtained from Sigma. Loading volume of sample s was adjusted according to the capacity of respective gel i.e., 50 µl for 1.5 thick gel and 25-30 µl for 0.8 mm thick gel. Samples were electrophoresed into the stacking gel at 20 mA and at 25 mA in the separating gel for 0.8 mm thick gels. For 1.5 mm thick gel, the current for the stacking gel was increased to 30 mA and for the separating gel 45 mA. Gels were run until the tracking dye reached the bottom of the gel, after which gels were fixed and stained either by Coomassie blue or by the silver staining method. This silver staining method was adapted and modified from the procedures of Oakley *et al*., [24].

*Native Polyacrylamide Gel*

The procedure described was modified for native polyacrylamide gel as follows. The two denaturants, 2-mercaptoethanol and SDS were omitted from the sample, resolving, stacking and running buffers. Samples were not heated and gels were run at $4^0$C. The gels were used for zymogram assay.

*Blood Agar Overlay (Zymogram)*

The zymogram technique was used to locate haemolytic activity of SLS on native gels. After electrophoresis, the gel was washed briefly with 150 mM sodium phosphate buffer (pH 6.8). Defibrinated sheep blood (Becton Dickinson) agar consisting of 2% thrice washed erythrocytes and purified agar (1%) in 150 mM sodium phosphate buffer (pH 6.8) was prepared, cooled to $40^0$C and poured onto the gel to a thickness of 1 mm. The electrophoresis gel plus the blood agar overlay was supported by a glass plate and was left undisturbed for five minutes in order to solidify. A second glass plate was placed onto the blood agar overlay with maximum care to avoid trapping air bubbles between the sandwich. The sandwich was kept at $37^0$C in a humid atmosphere and inspected for haemolysis each hour for 8 h.

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**References**

1. O. Goldmann, M. Rohde, G. S. Chhatwal and E. Medina. *Journal of Infection and Immunity*, 2004, **72**, 2956
2. I. Ginsburg. Streptolysin S in Microbial Toxins (Montie, T.C., Kandis, S. and Ajil, S.J., eds) Vol.III, pp.100-167, Academic Press, New York. 1970.
3. M. A. Waqar, S. Aijaz and S. Shaukat, *Journal of the Chemical Society of Pakistan,* **32**, 681 (2010).
4. K. U. Abbasi, M. Y. Khuhawar and M. I. Bhanger, *Journal of the Chemical Society of Pakistan,* **32**, 301 (2010).
5. M. Yontem, S. Kaleli, M. Akdogan, R. Kayapinar and F. Gultekin, *Journal of the Chemical Society of Pakistan,* **32**, 353 (2010).
6. Y.Taketo and A. Taketo, *Zeitschrift fur Naturforschung*, **41**c, 258 (1986).
7. Y. Taketo and A. Taketo, *Zeitschrift fur Naturforschung*, **42**c, 599 (1987).
8. J. L. Duncan and L. Mason, *Infection and Immunity*, **14**, 77 (1976).
9. W. Hryniewicz and J. Pryjma. *Journal of Infection and Immunity,* **16**, 703 (1977).
10. I. Ginsburg, *Journal of Infectious Diseases*, **126**, 294 (1972).
11. Y. Taketo and A. Taketo. *Journal of Biochemistry*, **6**, 35 (1966).
12. A. W. Bernheimer and L. L Schwartz, *Journal of Bacteriology*, **7**, 1100 (1964).

13. H. Keiser, G. Weissmann and W. A. Bernheimer, *Journal of Cell Biology*, **22**, 101 (1964),
14. N. Elias, M. Heller and I. Ginsburg, *Israel Journal of Medical Science*, **2**, 302 (1966).
15. Y. C. Lai, M. T.Wang, J. B. Defarria and T. Akao, *Archives in Biochemistry Biophysics*, **191**, 804 (1978).
16. J. E Alouf and C. Loridan, *Journal of General Microbiology*, **132**, 307 (1986).
17. S. D. Betschel, S. M. Borgia, N. L. Barg, D. E Low and J. C. de Azevedo, *Journal of Infection and Immunity*, **66**, 1671 (1998).
18. V. Nizet, B. Beall, D. J. Bast, V. Datta, V. Kilburn, D. E. Low and J. C. de Azevedo, J. C. *I Journal of Infection and Immunity*, **68**, 4245 (2000).
19. W. Hryniewicz and J. Pryjam, N pathogenic streptococci, Parker, M.T. (ed) Reed bokks Ltd. Chertsey, Surrey, United Kingdom, Pp. 59-60 (1978).
20. J. Koyma and F. Egami, *Journal of Biochemistry*, **53**,147 (1963).
21. C. Y. Lai, M. T, Wang, J. B. Defaria and T. Akao, *Archives in Biochemistry Biophysics*, **191**, 804 (1978).
22. K. Kazuhiro, S. Tatsuhito and T. Akira, *Journal of Biochemistry*, **129**, 769 (2001).
23. I. Laemmli, *Nature*, **227**, 680 (1970).
24. B. R. Oakley, D. R. Kirsch and R. Morris, *Analytical Biochemistry*, **105**, 361 (1980).