Biotechnology International 3 (1):18-25,March 2010
ISSN 0974-1453
Published by Biotechnology Society Online www.bts.org.in

Purification and activity of phage induced lysin against mastitogenic strains of Staphylococcus aureus

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Summary: A protein/proteins preparation from a phage lysate of a mastitogenic *Staphylococcus aureus* isolate was purified by physico-chemical methods. The protein/s were found to possess a greater lytic range against different staphylococcal isolates and demonstrated significantly higher lytic efficacy in presence of raw cow-milk than its/their inducing phage. The results indicate good potential of the phage lysins to be used as therapeutic agents against mastitis.

Key words: Mastitis, Staphylococcus aureus, Bacterioiphage, lysin

Introduction

Ruminant mastitis caused by *Staph. aureus* is considered to be a difficult disease to treat with conventional antibiotics and has been identified as a target animal disease against which bacteriophage therapy (BT) is expected to be more effective either alone or in combination with the other agents including antibiotics (Barrow, 2001). Various laboratories throughout the globe are engaged in research on BT of mastitis. But direct use of a phage or cocktail of phages for eliminating the organism infecting udder has certain limitations due to presence of milk. It has been perceived therefore; that a lytic system independent of phage absorption will not only increase the lytic efficacy of phage in udder but will also act synergistically with it. Endolysins have been identified as the most potent candidates for the purpose.

Endolysins are dsDNA bacteriophage-encoded enzymes that are produced in phage-infected bacterial cells during the later stages of the lytic cycle. Their major function is cleavage of peptidoglycan covalent bonds, which results in lysis of the host bacterial cell and ensures the successful release of progeny virions. These are peptidoglycan hydrolyzing enzymes have been reported to be a very interesting alternative to the classical phage therapy (Fischetti, 2005). The feasibility of using these proteins as antibacterial agents results from the fact that they are capable of cleaving peptidoglycan even

when applied exogenously (as purified or recombinant proteins) to the bacterial cell wall. Their lytic effect is very rapid and potent, especially in Gram-positive bacteria. (Fischetti, 2005).

The present paper reports concentration, partial purification and *in vitro* lytic activity of lysin protein/proteins from a phage lysate of a mastitogenic *Staph aureus*. The results obtained indicate that the lysin preparations possess excellent potential to be used as a therapeutic against mastitis.

Materials & Methods

PHAGE-HOST SYSTEM

Staphylococcus aureus (IVRI-82), initially designated as Sa1 (Chilamban *et al.*, 2004), available in the collection of Bacteriophage Laboratory was selected for the study. This isolate is the indicator organism against which a lytic phage PSa1, having a broad host-range activity was isolated.

Standardization of viable counts of IVRI-82

Viable count of IVRI-82 growing in NZCYM broth (Difco), the medium which was used for all *in vitro* lysis experiments, was standardized because all calculations with respect to determination of phage counts and quantity of lysin protein depend on the number of viable counts of the target organism. The viable counts of three 18 hrs incubated (late log phase) NZCYM broth cultures of the organism were determined by serial dilution method.

Determination of multiplicity of infection (MOI) of PSa1

To approximately 10^{10} IVRI 82 cells/ml (in log phase) suspended in sterilized NZCYM medium in 5 separate tubes, phage PSA1 (10^{10} pfu/ml) was added in amounts to make final phage : bacterial cell ratios ranging from $1:10^4$, $1:2x10^3$, $1:10^3$, $1:5x10^2$ and $1:10^2$ respectively, and the mixtures were incubated at 37^oC. The phage-bacterial cell mixtures were examined visually for clearance of NZCYM broth medium, and total bacterial counts were determined at 30 min intervals using BP agar medium (Difco) until total lysis of the suspension was visible as complete clearance of turbidity. The lowest phage-bacteria ratio which showed complete lysis of the organism with shortest period of time was considered as MOI of PSA1.

PRODUCTION AND STORAGE OF HOMOGENOUS STOCK OF psa1.

A homogenous stock of the phage was prepared by liquid culture method of Eisenstark(1967), filter sterilized and stored at 4°C after checking the sterility by usual method. The phage titer of the homogenous stock was determined by following the methodology of Chilamban *et al* (2004).

In vitro lytic EFFICACY of PSA1 against IVRI-82 in NZCYM broth and raw cow milk.

In vitro lytic efficacy of PSA1 against its indicator IVRI-82 strain in NZCYM broth was compared with its lytic efficacy in unpasteurized cow milk, as per the method described for determination of MOI.

PREPARATION OF CRUDE PHAGE LYSIN (SPAL)

Crude lysin (CL) was concentrated from IVRI-82-PSa1 lysate according to the method recommended by Doughty and Mann (1967) and Sonstein *et al.* (1971), with appropriate modifications.

Briefly, 200 ml of NZCYM broth grown IVRI 82 culture was inoculated with PSA1 in such an amount that final phage: bacterial ratio was about 1:100. The phage-bacterial suspension was incubated at 37°C with intermittent shaking till complete lysis, as indicated by clearance of turbidity was obtained. The proteins of this lysate were concentrated by repeated ammonium sulphate precipitation, high-speed centrifugation and dialysis against 0.07 M sodium phosphate buffer saline for 3 days. The protein concentration of the final preparation (CL) was determined by Lowry's method. The lytic activity of CL was tested by spotting it against IVRI 82. Finally, the semi purified lysin proteins were subjected to Sephadex G-200 gel filtration for further purification.

Purification of Crude lysin: Sephadex G-200 Gel filtration

The CL preparation was further purified by Sephadex G-200 Gel filtration. The eluted fractions were collected at every 5 minutes interval in sterilized tubes. Protein

concentrations in each of the fraction elute was monitored by recording optical density (OD) at 280 nm wave length (G.B.C Spectrophotometer). Elution profile was plotted in relation to fraction number and their respective ODs. Elutes constituting a peak were pooled. A total protein content of each pool was determined by Lowry's method which were then stored at –20^{o}C.

In vitro lytic activity of purified fractions against IVRI-82

Lytic activity of fraction pools against indicator IVRI-82 strain of *Staphylococcus aureus* in NZCYM broth was assessed by the method described for determination of MOI. Briefly, 10 µl the pool was added to each tube with 5 ml of standardized culture suspension with 10^6 cfu/ml, except the control, and incubated at 37^{o}C for 180 minutes. Total counts were determined at various time intervals using freshly prepared BP agar. The fraction pool having lytic activity was designated as SPAL (Staphylococcal phage-associated lysin)

L*ytic range of SPAL against heterologous isolates of *Staphylococci

All isolates of staphylococci available with collection were tested for activity of SPAL to determine its lytic range . The across strain lytic efficacy was also ascertained visually for *S aureus* ATCC 6538, *S. intermedius* IVRI 17 and 42, *B. subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9341 and *E. coli (wild isolate)* by observing the clearance of culture medium after different time intervals of inoculation.

Results & Discussion

Staphylococcus aureus IVRI-82, initially isolated from a mastitis cow not responding to antibiotic treatment was selected for the study because it was the indicator strain for the phage which showed lytic activity against majority of the staphylococci strains available in the lab (Rawat and Verma, 2007). The mean viable count of 18 h NZCYM broth culture of IVRI-82 was determined as 1.088×10^{10} cfu/ml. This value was used for all future experiments determining lytic efficacies of phage and lysin preparations.

The first mandatory step for developing a universal phage-based therapeutic system that precludes the necessity of *in vitro* sensitivity determination of pathogens associated with individual cases, is collection of broad host range lytic phages against the strains of pathogen isolated from a different geo-climatic regions of a country or an area (Barrow, 2001).Phage tentatively named as PSa1, which was isolated from farm yard slurry of IVRI Dairy against IVRI 82 was found to be lytic against most strains of the collection from a different geographical regions (Rawat and Verma, 2007), and was therefore, singled out for present trials**.**

From the previous experiences and experimental work with BT, it has been concluded that BT can be very successful in the conditions in which the infection process mimics the conditions under which phages multiply optimally *in vitro*. Therefore, the infection of udder by either true pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae,* or opportunistic pathogens such as *E. coli* and *S. uberis* become the preferred targets for BT (Barrow, 2001). Phage SA1: IVRI 82 ratio, optimum for maximum lytic efficacy was found to be $1:10^2$. For all future *in vitro* lysis assays, the phage-bacteria ratio was maintained to about 1:100.

Since, in clinical settings, phage is expected to be lytic against the target organism in milk medium in its various phases of growth, it was found essential to compare the lytic efficacy of PSa1 against log and stationary phases IVRI 82 growth in unpasteurized milk and normal growth medium i.e., NZCYM broth.

The repeated tests for *in vitro* lytic efficacy of the phage revealed that the efficacy was significantly reduced in milk as compared to NZCYM medium. Results are presented in Table 1. Similar results have recently been reported by O'Flaherty *et al*. (2005a). The major causes of this reduction of lytic efficacy of candidate phages in milk were attributed to inhibition of adsorption of phage to bacterial cells in presence of some thermo-labile milk proteins, and presence of pre-existing anti-*Staphylococcus aureus* antibody titer in milk, that block the receptor sites for phages.

Staphylococcal phage associated lysin was prepared using IVRI-82 and PSA1 host phage system. The lytic enzymes induced during the terminal stages of phage replication in host IVRI-82 were purified by a 2-step procedure. Initially, the crude enzymatic protein having lytic activity, were salted out by precipitation and high speed centrifugation to eliminate SA1 population from the lysate. This preparation was designated as phage induced crude lysin (CL) which was further purified by Sephadex G200 filtration and designated as Staphylococcal phage induced lysin (SPAL). The protein concentration CL prepared from 200 ml of IVRI 82 and PSA1 was found to be 28 µg/10 µl. When subjected to Sephadex G-200 filtration, a single sharp peak immediately after void volume was obtained. The protein content of the pooled SPAL was found to be 25 µg/10 µl.

The *in vitro* lytic activity of purified preparation (SPAL) was tested against IVRI 82 and other cultures like standard *Staphylococcus. aureus* (ATCC-6538), *Micrococcus luteus* (ATCC 9341), *Bacillus subtilis* (ATCC 6633) *Staph. intermedius* (IVRI 17 and 42) and *E .coli* (wild isolate) to determine the lytic range. It can be concluded from the study that purified lysin (SPAL) showed a broader host range activity against staphylococci than the phage PSa1 which induced it. (Table 2).SPAL preparation alone, when incorporated to 5 ml NZCYM broth culture suspension with approximately 10^{10} cfu/ml of IVRI-82 @ 10 µl containing 25 µg of protein, was found to eliminate IVRI-82 population by 99% within 180 minutes. Similar results were obtained when raw milk in place of growth medium was used indicating that, in contrast to PSA1, SPAL preparations were equally efficacious against IVRI 82 in both growth medium and milk. CL was found to reduce the population by more than 99% in both NZCYM broth and milk within 120 min.

The data on *in vitro* lytic efficacy of CL and SPAL in growth medium and raw milk, and lytic range against different staphylococcal isolates indicate that these peptidoglycan hydrolyzing proteins can become a very effective means of mastitis therapy in future. Further studies on *in vivo* use are therefore warranted.

Table: 1.*In vitro* lytic activity of PSa1 against IVRI 82 in NZCYM broth and raw cow-milk

Phage:Bacteria ratio	Total viable counts (cfu/ml)							
	Milk Medium				NZCYM broth			
	30 min	60 min	90 min	180 min	30 min	60 min	90 min	180 min
$1:10^4$	$2.5x10^8$	$4x10^8$	$8x10^8$	$5x10^8$	$2.5x10^9$	$5.5x10^7$	$8.2x10^6$	$4.4x10^6$
$1:2x10^3$	$1.3x10^8$	$2x10^8$	$5x10^8$	$4x10^8$	$1.5x10^9$	$6x10^7$	$7x10^6$	$3.2x10^6$
$1:10^3$	$1.25x10^8$	$2.5x10^8$	$7x10^8$	$3x10^8$	$2.1x10^9$	$2x10^7$	$6x10^6$	$2.5x10^6$
$1:5x10^2$	$3x10^8$	$2x10^8$	$3x10^8$	$5x10^8$	$1.2x10^9$	$4x10^7$	$7x10^6$	$5.5x10^6$
$1:10^2$	$1.5x10^8$	$2x10^8$	10^8	$2.5x10^8$	$1.8x10^9$	$4x10^7$	$5x10^6$	$6.3x10^5$
None (Control)	$6.8x10^8$	$1.5x10^8$	$2x10^8$	$6x10^8$	4.5x109	$5.4x10^9$	$3.2x10^{10}$	$3.5x10^{10}$

Table: 2 *In vitro* lytic range of SPAL, CL and PSa1 against different isolates of *Staphylococcus*

Isolate	Lytic activity of lysin preparations		Lytic activity of phage PSa1
	SPAL	CL	
S. aureus ATCC 6538	Lytic	Lytic	Lytic
S. aureus IVRI 82	Lytic	Lytic	Lytic
S. aureus IVRI 20	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 11	Lytic	Lytic	Lytic
S. intermedius IVRI 42	Lytic	Lytic	Non-lytic
S. intermedius IVRI 17	Lytic	Lytic	Non-lytic
M. luteus ATCC 9341	Non-lytic	Non-lytic	Non-lytic
B. subtilis ATCC 6633	Non-lytic	Non-lytic	Non-lytic
E. coli (Wild)	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 36	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 38	Lytic	Lytic	Non-lytic
S. aureus IVRI 5	Lytic	Lytic	Non-lytic
S. aureus IVRI 13	Lytic	Lytic	Non-lytic
S. aureus IVRI 16	Non-lytic	Non-lytic	Non-lytic
S. aureus Chennai 26	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 19	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 47	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 31	Lytic	Lytic	Non-lytic
S. aureus IVRI 178	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 1	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 45	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 2	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 48	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 21	Lytic	Lytic	Non-lytic
S. aureus IVRI 29	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 27	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 37	Lytic	Lytic	Non-lytic
S. aureus IVRI 35	Lytic	Lytic	Non-lytic
S. aureus IVRI 83	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 84	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 129	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 112	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 55	Non-lytic	Non-lytic	Non-lytic
S. aureus IVRI 28	Non-lytic	Non-lytic	Non-lytic

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