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BACTERIOCIN-LIKE INHIBITORY SUBSTANCES (BLIS) FROM INDIGENOUS CLINICAL STREPTOCOCCI: SCREENING, ACTIVITY SPECTRUM AND BIOCHEMICAL CHARACTERIZATION

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Abstract

Two hundred streptococcal isolates from varied human indigenous clinical sources were screened for bacteriocinogenic activity by both direct and deferred methods (70% were showing bacteriocin-like activity). All the isolates were found highly bioactive against most Gram positive bacteria but less active against the Gram positive and totally inactive against themselves. Proteolytic enzymes rapidly inactivated the antagonistic activity whereas, glycolytic and lipolytic enzymes had no effect. Bioactivity remained stable in the presence of several organic solvents and detergents. Bacteriocin preparations could be stored at 4°C for 2 months without loss of activity and remained stable at 60°C for 60min and 80°C for 40min and at 100°C for 30min. Bioactivity was manifested within a wide range of 2-8 pH with the exception of enterocin ESF63 that was stable at low pH range of 5-8.5. All preparations were resistant to chloroform vapours. Different bacteriocin titre in terms of activity unit (AU/mL) was found against different sensitive/indicator strains.

Introduction

Bacteriocins and bacteriocin-like inhibitory substances (BLIS) are natural antibiotics produced by Gram positive bacteria. BLIS have potential applications against a wide range of human and animal diseases (Cleveland *et al.*, 2001). They are ribosomally synthesized antimicrobial peptides produced by microorganisms belonging to different eubacterial taxonomic branches (Riley & Wertz, 2002a, b). Bacteriocins produced by Gram positive bacteria fall within two broad classes: the Lantibiotics and the non-lantibiotic bacteriocins (Navarro *et al.*, 2000). Due to their resistance to temperature and low pH, the bacteriocins are digested by human and animal peptidases, thus avoiding resistance and problems associated to the presence of residues in feed and food (Russell & Mantovani, 2002).

The aim of the present study was to examine the bacteriocin-like inhibitory substances (BLIS) among indigenous clinical streptococci representing α, β and γ hemolytic groups and to understand the activity spectrum, physico-chemical characterization and activity unit of these antagonistic agents.

Materials and Methods

Bacterial strains and media: Two hundred streptococcal isolates were collected from various pathological laboratories and hospitals of Karachi (Table 1) and identified on the bases of morpho-cultural and biochemical characteristics. Bacteriocin activity was monitored on brain heart infusion agar. The indicator cultures used in this study included Gram positive, Gram negative and the yeast cells.

Table 1. Clinical nature of the indigenous streptococcal isolates.

Nature of samples	No of samples
Eye swab	27
High vaginal swab	21
Pus	06
Sputum	30
Throat swab	88
Urine	28
Total	**200**

Table 2. Bacteriocin (Streptocin) producing streptococcal strains selected for initial characterization.

Source	Identified as	Isolate Number	Lancefield group	Inhibitory Substance
Throat swab	*S. equi*	SEQ62	C	Streptocin SEQ62
Urine	*E. faecium*	ESF63	D	Enterocin ESF63
Sputum	*S. pneumoniae*	SPN83	None	Streptocin SPN83
Throat swab	*S. pyogenes*	SPY92	A	Streptocin SPY92
High vaginal swab	*S. agalactiae*	SAG152	B	Streptocin SAG152

Detection of antimicrobial activity: Four methods according to Hardy (1987) were used for the detection of bacteriocin activity: (1) Patch test (2) Stab and overlay method (3) Cross streak and (4) Agar well diffusion methods.

Physico-chemical characterization of crude preparations was done which includes effect of different temperatures i.e., 4, 60, 80, 100 and at 121°C (autoclaving); different pH values 2-10; different enzymes (catalase, trypsin, lipase and lysozyme); effect of chloroform vapours (30min); effect of surfactants including triton X-100, tween 20 and sodium dodecyl sulfate; effect of organic solvents including methanol, ethanol and propanol. Activity unit (AU/ml) was determined by bacteriocin titration. The ability of bacteriocins to pass through dialysis membrane (pore size 12,000Da), was assessed to estimate their molecular mass (Rasool *et al.*, 1996, Iqbal *et al.*, 1999, 2001).

Result and Discussion

In this study the detection of bacteriocin-like inhibitory activity was monitored using spot, stab-overlay, cross streak and agar-well diffusion assay techniques (Fig. 1, 2, 3, 4). Five bacteriocinogenic strains were selected for detailed study representing α, β and γ hemolytic groups of streptococci (Table 2). Bacteriocinogenic activity was demonstrated in all four types of assays against different Gram positive and Gram negative sensitive strains thus, having a broad-spectrum antagonistic activity (Table 3). Most of the Gram negative bacteria viz., *Agrobacterium tumefaciens*, *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirablis*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella dysenteriae* were not inhibited by the five selected streptocin producing strains, while *Neisseria meningitidis* and *Xanthomonas maltophila* were inhibited. In our studies 140 out of 200 i.e. 70% were found bacteriocinogenic. The individual streptocin production potential statistics is 57, 14, 65, 89, 90 and 86% by group A, B, C, D streptococci, *S. viridans* and *S. pneumoniae*, respectively.

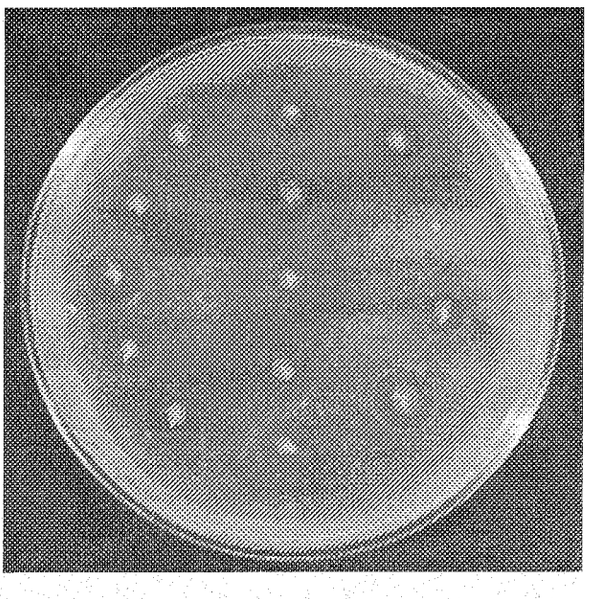

Fig. 1. Patch test demonstrating streptocin bioactivity against *Streptococcus mutans* VSMD.

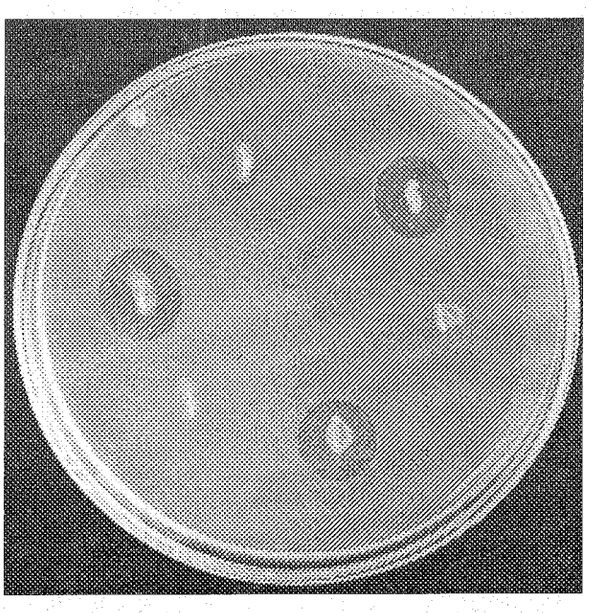

Fig. 2. Stab and Overlay method demonstrating streptocin bioactivity against *Streptococcus mutans* VSMD.

Neisseria meningitidis	1/4	14	1/4	14	NT	NT	NT	NT	NT	NT
Proteus mirabilis	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Proteus vulgaris	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Pseudomonas aeruginosa	0/17	0	0/17	0	0/17	0	0/17	0	0/17	0
Ps.aeruginosa PAO286	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Pseudomonas syringae	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Salmonella typhi	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Salmonella typhi para A	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Salmonella typhi para B	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Shigella dysenteriae	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Shigella flexneri	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Shigella sonnei	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Xanthomonas maltophila	1/1	10	1/1	12	1/1	10	1/1	10	1/1	10
Yeast										
Candida albicans	0/8	0	0/8	0	0/8	0	0/8	0	0/8	0

Inhibitory activity was determined by Stab and overlay method

A, Number of producer strains/number of tested strains; B, Average zone size (mm); 0, No zone of inhibition; NT, Not tested

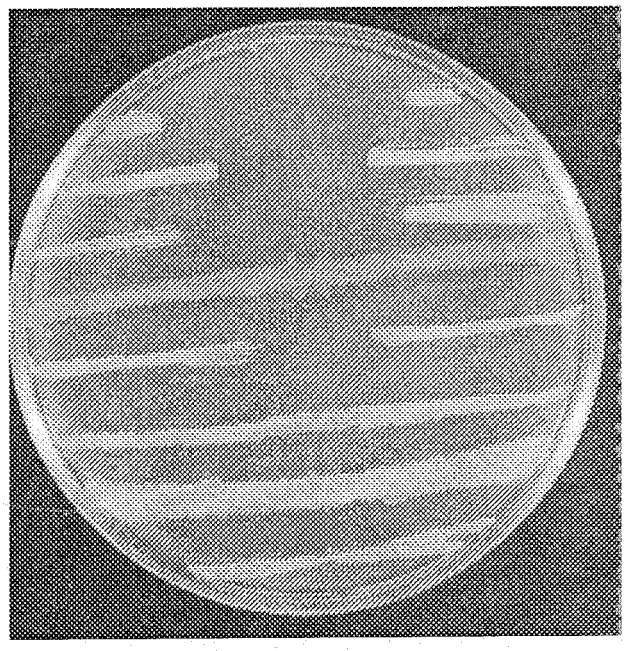

Fig. 3. Cross streak method demonstrating streptocin bioactivity against different indicator bacteria.

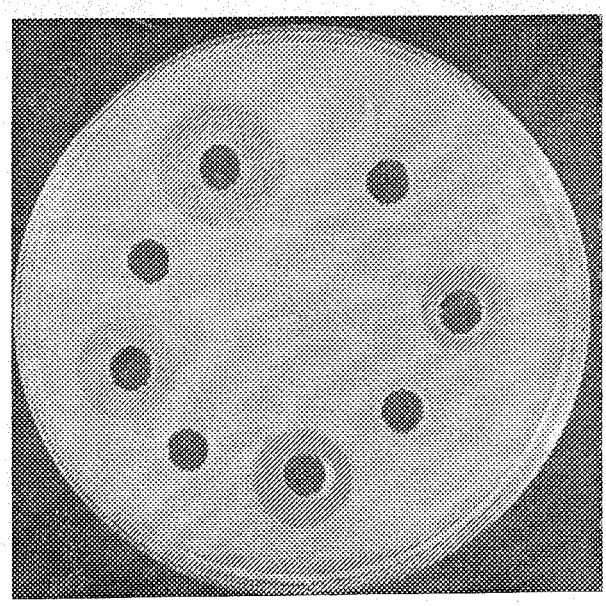

Fig. 4. Agar well diffusion method demonstrating streptocin bioactivity against *Saphylococcus aureus* AB211.

Table 3. Inhibition spectrum shown by bacteriocinogenic strains of streptococci against Gram positive and Gram negative bacteria and yeast cells.

	Bacteriocinogenic strains									
	SEQ62		ESF63		SPN83		SPY92		SAG152	
Sensitive organism	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Gram positive bacteria										
Bacillus subtilis	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Bacillus cereus	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Clostridium perfringens	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Corynebacterium diphtheriae	1/1	14	1/1	13	NT	NT	NT	NT	0/1	0
Lactobacillus acidophilus	2/6	10	2/6	10	0/2	0	0/2	0	0/6	0
Micrococcus lysodiecticus	6/10	18	6/10	18	7/10	18	5/10	15	3/10	8
Listeria monocytogenes	0/1	0	0/1	0	0/1	0	0/1	0	NT	NT
Staphylococcus aureus	35/40	19	35/40	18	35/50	18	35/40	16	32/40	16
Staphylococcus epidermidis	2/12	10	5/12	8	4/12	8	5/12	10	3/12	6
Staphylococcus saprophyticus	0/1	0	0/1	0	NT	NT	NT	NT	NT	NT
Streptococcus agalactiae	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Streptococcus equi	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
Enterococcus faecalis	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
Enterococcus faecium	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
Streptococcus mutans	0/30	0	0/30	0	0/30	0	0/30	0	0/30	0
Streptococcus pneumoniae	0/26	0	0/26	0	0/26	0	0/26	0	0/26	0
Streptococcus pyogenes	0/30	0	0/30	0	0/30	0	0/30	0	0/30	0
Streptococcus sanguis	0/4	0	0/4	0	0/4	0	0/4	0	0/4	0
Gram negative bacteria										
Agrobacterium tumefaciens	0/1	0	0/1	0	0/1	0	0/1	0	NT	NT
Enterobacter aerogenes	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Escherichia coli AB712	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Escherichia coli BU40	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Escherichia coli 5014	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Escherichia coli WT	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
Klebsiella pneumoniae	0/12	0	0/12	0	0/12	0	0/12	0	0/12	0
Neisseria meningitidis	1/4	14	1/4	14	NT	NT	NT	NT	NT	NT
Proteus mirabilis	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Proteus vulgaris	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Pseudomonas aeruginosa	0/17	0	0/17	0	0/17	0	0/17	0	0/17	0
Ps.aeruginosa PAO286	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Pseudomonas syringae	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Salmonella typhi	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Salmonella typhi para A	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Salmonella typhi para B	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Shigella dysenteriae	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Shigella flexneri	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Shigella sonnei	0/1	0	0/1	0	0/1	0	0/1	0	0/1	0
Xanthomonas maltophila	1/1	10	1/1	12	1/1	10	1/1	10	1/1	10
Yeast										
Candida albicans	0/8	0	0/8	0	0/8	0	0/8	0	0/8	0

Inhibitory activity was determined by Stab and overlay method

A, Number of producer strains/number of tested strains; B, Average zone size (mm); 0, No zone of inhibition; NT, Not tested

Crude streptocin preparations were subjected to different treatments including heat, pH, enzymes, organic solvents and surfactants (Table 4). The heat sensitivity threshold of all streptocins was similar. It may be mentioned that streptocin produced by ruminal enteococcal isolates has been reported to be heat stable (Laukova & Marekove 2001). In the present study cell free neutralized supernatants (CFNS) of producer streptococci were tested and found to be stable at pH levels between 2-8, except enterocin ESF63 which was stable between 5-8.5. Rasool *et al.*, (1996) reported that streptococcin Sam 51 and Sam 53 were not affected by pH range of 2-8. Cell free neutralized supernatants were tested for their sensitivity to various enzymes. Accordingly five streptocins were found to be completely sensitive to trypsin (1mg/mL). Loyola-Rodriguez *et al.*, (1992) reported that mutacin from *S. sorbinus* was partially inhibited by α-chymotrypsin and completely inactivated by papain or ficin digestion. Lipolytic and glycolytic enzymes had no effect on these bacteriocin preparations. Our results are in agreement with those of Laukova *et al.*, (1993) who reported that bacteriocins produced by enterococci were resistant to chloroform vapours. Further, there was no increase or decrease in the activity after the streptocins were treated with various organic solvents and surfactants. The titre (Table 5) of crude preparations of *S. equi* SEQ62, *S. pneumoniae* SPN83 and *S. agalactiae* SAG152 was 640AU/mL and for *E. faecalis* ESF63 and *S. pyogenes* SPY92 was 1280AU/mL. According to Laukova *et al.*, (2003) the titre of enterocin V24 was 51,200 AU/mL. None of the five inhibitory substances (streptocin preparations) passed through the dialysis membrane (pore size 12,000Da), suggesting their molecular mass to be >12,000Da.

Table 4. Effect of different treatments on streptocin activity.

Treatments	SEQ62	ESF63	SPN83	SPY92	SAG152
Temperature					
60°C (60min)	R	R	R	R	R
80°C (40 min)	R	R	R	R	R
100°C (30 min)	S	S	S	S	S
121°C (15 min)	S	S	S	S	S
4°C (2 months)	R	R	R	R	R
pH (2-10)	2-8	5-8.5	2-8	2-8	2-8
Enzymes					
Catalase	R	R	R	R	R
Trypsin	S	S	S	S	S
Lipase	R	R	R	R	R
Lysozyme	R	R	R	R	R
Chloroform vapours (30min)	R	R	R	R	R
Surfactants (Triton X-100, Tween 20, Sodium dodecyl sulfate)	R	R	R	R	R
Organic solvents (Methanol, Ethanol, Propanol)	R	R	R	R	R

R, Resistant; S, sensitive

Table 5. Diffusion zone method for the titration of streptocins in term of activity units (AU/mL) using *S. aureus* AB211 as indicator culture.

	Two-fold serial dilutions of bacteriocin				
Dilutions	**SEQ62**	**ESF63**	**SPN83**	**SPY92**	**SAG152**
Undiluted	+	+	+	+	+
1:2	+	+	+	+	+
1:4	+	+	+	+	+
1:8	+	+	+	+	+
1:16	+	+	+	+	+
1:32	+	+	+	+	+
1:64	+	+	+	+	+
1:128	-	+	-	+	-
1:256	-	-	-	-	-
1:512	-	-	-	-	-
1:1024	-	-	-	-	-
AU/mL*	640	1280	640	1280	640

+, zone of inhibition; -, no zone of inhibition

$$\text{*Activity unit (AU/mL)} = \frac{\text{Reciprocal of the highest dilution} \times 1000}{\text{Volume of bacteriocin added}}$$

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