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Antimicrobial activity of bacteriocin isolated and purified from rumen liquor collected from slaughtered goats

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ABSTARCT

Bacteriocin like substance with antimicrobial activity was purified from freshly collected rumen liquor using 60% ammonium sulphate precipitation followed by ion exchange(SP-Sepharose) and gel filtration (Sephadex G25) chromatographic techniques. Purity of the product was checked on SDS-PAGE, having molecular weight of 6.5 kDa. Anti-microbial activity was demonstrated using *Bacillus subtilis* by gel overlay method and agar cut well diffusion method. Proteomic analysis confirmed the substance as bacteriocin. The purified sample was resistant to the action of protease. The substance was active at pH 4, 7 and 10. It was also active at autoclave temperature. The peptide purified was found to inhibit the growth of *Staphylococcus aureus* (MTCC87), *Listeria monocytogenes* (MTCC 657) and *Pseudomonas aeurginosa* (MTCC 424).

Key words: Anti-microbial, Bacteriocin, Goat, Peptide, Rumen.

INTRODUCTION

The broad use of antimicrobials in medicine, animal husbandry and agriculture is developing several antibiotic resistant organisms. This makes the necessity to develop novel antibiotics and alternative therapeutic strategies. Ribosomal synthesized antimicrobial polypeptides, bacteriocins are usually inhibitory to strains closely related to the producing bacteria (Berlina Dhas and Vimalin Hena, 2012). These compounds have received much attention mainly because of their potential use as 'natural' food preservatives (Cleveland *et al*., 2001). The incidence of bacteriocins produced by lactic acid bacteria (food isolates) against pathogenic or opportunistic pathogenic oral bacteria was reported by Georgia *et al.* (2013). Several bacteriocins have been isolated from microorganisms from different habitats, ranging from fermented food products to soil samples.

Rumen is a rich source of anaerobic microbes. A number of bacteriocins have been identified in ruminal fluid bacteria and all these antimicrobial peptides show antimicrobial activity to related rumen microbes and other related microorganisms (Mantovani *et al.* 2002; Junqin *et al.* 2004). They are more specific and wide spectrum of activity. These antimicrobial peptides have potential to be used as a food preservative as they can inhibit various kinds of food born pathogens. They can also be used as probiotics to improve the production in ruminants (Rodriquez *et al.* 2003).

Every year large numbers of animals are being slaughtered to meet the animal protein requirement. For example, in India alone, about 130.96 million livestock species are slaughtered every year, of which 82.17 millions are goats (Basic Animal Husbandry Statistics, 2012). During slaughter significant amount of animal by-products are resulted which are either under utilized or being wasted. Rumen content is one of the animal by-products available from the slaughter house in large quantities. At present rumen content is either being completely wasted or under utilized as compost. As already proved that the rumen harbors bacteriocin producing organisms and most of the rumen micro flora interact and compete with each other for their survival and secrete anti-microbial substances, such as bacteriocins to the rumen liquor. Therefore, the rumen liquor obtained from slaughtered goats was explored as an alternate source of bacteriocins and antimicrobial activity of the bacteriocin purified on the micro-organisms of economic importance was also assessed.

MATERIALS AND METHODS

Collection of ruminal fluid and checking its Bacteriocin like activity: About 300ml of ruminal fluid was collected from healthy goat, immediately after slaughter in clean collection bottles. The collected ruminal fluid was filtered to remove coarse materials. Then, the filtrate was subjected to three rounds of centrifugation at 14500Xg for 20min at 4°C

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to get a clear supernatant free from bacteria and fine food particles. The supernatant was filtered through 0.22 micron membrane filter. This filtrate was then used to test for its bacteriocin like activity using agar cut well diffusion method with *Bacillus subtilis (*MTCC 441) as an indicator organism. The supernatant showing antimicrobial activity was subjected for further purification.

Agar cut well diffusion method: A single colony of indicator organism, *Bacillus subtilis* (MTCC 441) was inoculated into sterile Luria broth and incubated overnight at 37 º C. Then, the prepared inoculums of bacteria were evenly spread on the Mueller Hinton (MH) agar plate. With the use of a sterile well cutter, well was made in the media. The bottom of the well was sealed using the same MH Agar. Into these wells, 250ml of the testing samples were added and plates were incubated overnight at 37 º C.

Reading and interpretation of results: Clear zone around the well indicated the presence of bacteriocin like activity. The diameters of the zones of complete inhibition, including the diameter of the well, were measured to the nearest whole millimeter with ruler in non-reflecting background. The zone margin was the area where no obvious growth was visible and the readings were recorded.

Calculation of antibacterial activity: Antibacterial activity was expressed as arbitary units (AU/ml), calculated as a^bx1000, where "a" represents the dilution factor and "b" represents the last dilution that produces an inhibition zone. Activity was expressed per ml by multiplication with 1000. One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (Todorov and Dicks 2007).

Precipitation of protein using ammonium sulphate: Clear supernatant of rumen liquor (100ml) was taken in a beaker and subjected to 60% ammonium sulphate precipitation, overnight at 4°C with continuous stirring using magnetic stirrer. After 24 hr the precipitated rumen liquor was subjected for centrifugation at 10,000Xg for 20 min at 4°C. After centrifugation the sediment was dissolved in sterile ultra pure water and dialyzed against ultra pure water, overnight. The dialyzed sample was filtered through a 0.22 micron membrane filter and the filtrate was tested for its bacteriocin like activity using agar cut well diffusion method. The sample showing bacteriocin like activity was subjected for lyophilization.

Ion exchange chromatography-SP Sepharose: Re-suspended ammonium sulphate (60%) precipitated sample (10 ml) was mixed with 5 ml of equilibrated SP Sepharose with 0.1 M Citrate buffer, incubated at room temperature for 1 hr and centrifuged at 12000Xg for 15 min at 25°C, then the supernatant was collected in a sterile tube. To the precipitate, 20 ml of 0.1 M Citrate buffer was added, incubated at room temperature for 1 hr and centrifuged at 12000Xg for 15 min at 25°C and then the supernatant was collected in a sterile tube. The process was repeated with 0.5 M, 1.0 M and 1.5 M NaCl. The collected supernatants were dialyzed and tested for bacteriocin like activity using agar cut well diffusion method. The supernatant showing bacteriocin like activity was subjected to gel filtration chromatography.

Gel filtration chromatography-Sephadex G50: The swollen Sephadex G50 (10g/50 ml) was packed into a column (30cm x 1 cm) and was allowed for 2-4 hrs for proper packing. 60ml of 50mM Sodium Phosphate buffer (pH 7.0) with 0.15 M NaCl was used to equilibrate the column. Then, the column was calibrated with BSA (66.409 KDa) and lysozyme (14.7 KDa) as molecular marker. Ion exchange purified sample was subjected for further purification using same column. The proteins were eluted using 50 mM Sodium Phosphate buffer (pH 7.0) with 0.15 M NaCl. 1 ml fractions were collected in an automatic fraction collector. Absorbance of each fraction was measured at 280 nm. The readings were plotted in a graph and the fractions with protein were identified. The fractions showing anti-microbial activity were pooled, dialyzed and lyophilized.

SDS-PAGE analysis of purified sample: The lyophilized bacteriocin sample (4 to 5 µg) was subjected for 12% SDS-PAGE (Laemmli, 1970) at 100 volts for 1.5 hr and the protein bands were visualized using silver staining technique (Schevchenko *et al.* 1996).

Gel overlay method: The gel containing the purified protein fraction was washed twice with sterile ultrapure water, 20 minutes each time, and placed in a sterile Petri plate. The MH media was autoclaved in conical flask and was immediately placed in a water bath. When the temperature reached 45-50°C, 5 ml of Luria broth containing uniform suspension of indicator organism was added into it and poured into petri plate contained the gel and allowed to cool. Then the plate was kept in the incubator overnight and checked for the zone of clearance.

Proteomic analysis: This was carried out as a custom service (The Centre for Genomic Application, New Delhi, India). The only one protein band seen in the SDS-PAGE corresponding to the 6.5 kDa protein marker was subjected for in-gel tryptic digestion and the peptides released were analyzed using nano LC-MS. The peptide mass fingerprints were searched against the non-redundant protein database (MSDB) to identify the protein-using MASCOT.

Effect of proteolytic enzymes on the bacteriocin like activity of purified peptide: Proteolytic enzymes -α-Chymotrypsin, Trypsin, Protease, and Proteinase K were prepared at the concentration of 10 mg/ml of sterile water. Phosphate buffer (0.01 M, pH 7.0), (230 µl) was taken in four different eppendorf tubes into which 10 µl of purified sample(5 µg) and 10 µl of the prepared enzymes were added separately and also 240 µl buffer with 10 µl lyophilized sample and 250 µl buffer alone were taken in two other eppendorf tubes. Then, the tubes were incubated at 37ºC for 2 hrs and then the enzymes were inactivated at 100°C. After incubation the bacteriocin like activity was assessed using agar cut well diffusion method.

Effect of pH on the bacteriocin like activity of purified peptide: Purified sample (5µg) was treated at pH 2.0, 4.0, 7.0, 10.0 and 12.0 for 2 hrs at RT after which the pH was adjusted to 7.0 and the bacteriocin like activity was assessed using agar cut well diffusion method.

Effect of temperature on the bacteriocin like activity of purified peptide: Equal amount of lyophilized sample (5 µg) was taken in two different tubes and out of which one tube was incubated at room temperature for 1hour and the other tube was subjected to autoclave (121°C for 15 minutes). Then bacteriocin like activity of the sample was assessed using agar cut well diffusion method.

Bacteriocin like activity of purified sample on other pathogenic microbes: The antimicrobial activity of purified sample was tested against the growth of some common food borne pathogens like *Listeria monocytogens* (MTCC 657), Staphylococcus *aureus* (MTCC 87), *E.coli* (MTCC 40) and *Salmonella typhimurium* (MTCC 98) and also against *Pseudomonas aeuroginosa* (MTCC 424) using agar cut well diffusion method as stated above.

RESULTS AND DISCUSSION

Freshly collected rumen liquor from the slaughtered goats was subjected to three rounds of centrifugation and the supernatant collected was tested against the growth of indicator organism, *Bacillus subtilis* (MTCC 441) and produced zone of inhibition of 24 mm as compared to blank control.

The substance responsible for bacteriocin activity was purified to homogeneity using 60% ammonium sulphate precipitation, ion-exchange chromatography (SP-Sepharose) followed by gel filtration chromatography (Sephadex G50). The 60% ammonium sulphate precipitated protein produced zone of inhibition around 22mm, against the growth of indicator organism.

The lyophilized sample after 60% ammonium sulphate precipitation was subjected to Ion exchange chromatography (SP-Sepharose) and eluted with different concentrations of NaCl. The purified sample (eluted with 0.5M NaCl), produced the zone of clearance of 23 mm in size, against the growth of indicator organism.

Ion exchange purified sample was further subjected for gel filtration chromatography using SephadexG50. SephadexG50 column was calibrated using known concentration of BSA (66.409 kDa) and lysozyme (14.7 kDa). The separation profile resulted in two peaks (Fig.1) of which peak II had shown the zone of clearance (22mm)(Fig.2) against the growth of indicator organism and the peak appeared after elution of lysozyme, thereby indicating the low molecular weight of the bacteriocin like substance.

Table 1 shows the purification profile of the bacteriocin like substance purified from rumen liquor. The profile shows that bacteriocin like substance was purified to the extent of 37 fold and the recovery of the substance was only about 68%.

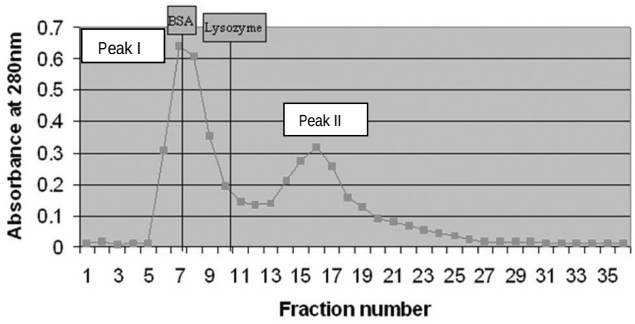

FIG 1: Gel filtration profile of ion exchange purified sample.

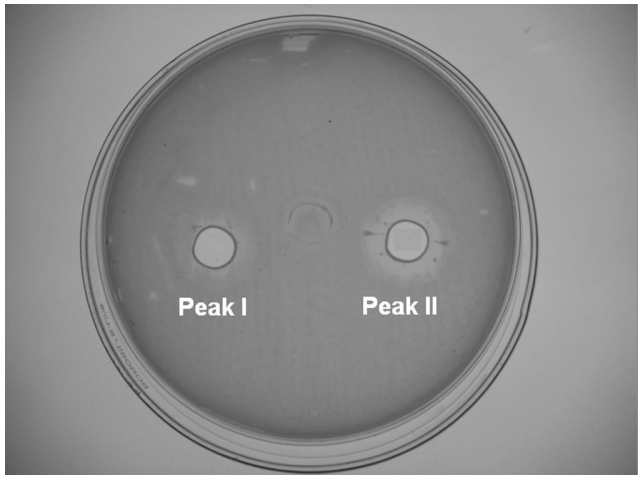

FIG 2: Gel filtration purified sample showing zone of clearance against the growth of indicator organism. Protein Peak II Showing very significant bactericoin activity.

The bacteriocins purified so far was reported to be precipitated at 60% ammonium sulphate precipitation which was further purified using ion-exchange chromatography followed by gel filtration type of chromatography. For example bacteriocin like inhibitory substance produced by ruminal anaerobe, *Butrivibrio fibrosolvens* JL5 (Rychlik and Russel 2002) and *Ruminococcus albus* 8 (Odenyo *et al*. 1994) reported to be precipitated with 60% ammonium sulphate and the bacteriocin produced from *Lactobacillus plantarum* SR18 was found to be precipitated by 75% ammonium sulphate (El-Shouny *et al*., 2013). Bovicin HC5 produced by *Streptococcus bovis* HC5 was purified using SP Sepharose and the antimicrobial substance was eluted by 0.4 M NaCl (Mantovani *et al.* 2002). The crude extract of Bovicin HJ50 was first applied to SP Sepharose column and followed by purification with Sephadex G50 column (Xiao *et al*. 2004). Staphylococcin 188 was purified using Sephadex G75 column and after equilibration and elution with 50mM sodium phosphate buffer (pH 7.0), the separation profile resulted in two peaks in which peak II had more antimicrobial activity when it was checked by agar cut well diffusion method (Saeed *et al*. 2007).

The substance purified with anti-microbial activity was found to be 6.5 kDa in size in SDS-PAGE analysis, as shown in fig.3a, which is very well corroborate with the gel filtration profile as the fraction with anti-microbial activity was appeared after the elution of lysozyme (14.7 kDa) protein. Most of the bacteriocins reported so far were also found to have molecular size at the range of 6 to 6.5 kDa. For example, molecular mass of Bovicin 255 produced by rumen *Streptococcus species* was estimated to be 6 kDa in size (Whitford et al. 2001) and the bacteriocin produced by *Bacillus subtilis* was also found to have the molecular weight of 6.4Kda (Berlina Dhas and Vimalin Hena, 2012). The antimicrobial activity of 6.5 kDa protein was further confirmed by gel overlay method wherein, the protein band present in the SDS-PAGE gel corresponding to 6.5 kDa molecular size found to produce zone of clearance against *Bacillus subtilis* as shown in Fig.3b.

The identity of the bacteriocin purified was further confirmed by proteomic approach. In gel tryptic digestion of the protein band seen and nano LC-MS analysis of the peptides released, followed by the identification of protein using MASCOT analysis had shown high similarity with the fimbrial protein of *Pseudomonas aeruginosa* (*SO4440*) and oligopeptide ABC transporter, ATP binding protein (*B72300*) (Table 2). Fath and Kolter (1993) had demonstrated that bacteriocin (BViA) belongs to the super family of ABC transporters and the ATP binding proteins demonstrate highest homology matches with the BViA. This clearly supports that the peptide purified is indeed a bacteriocin.

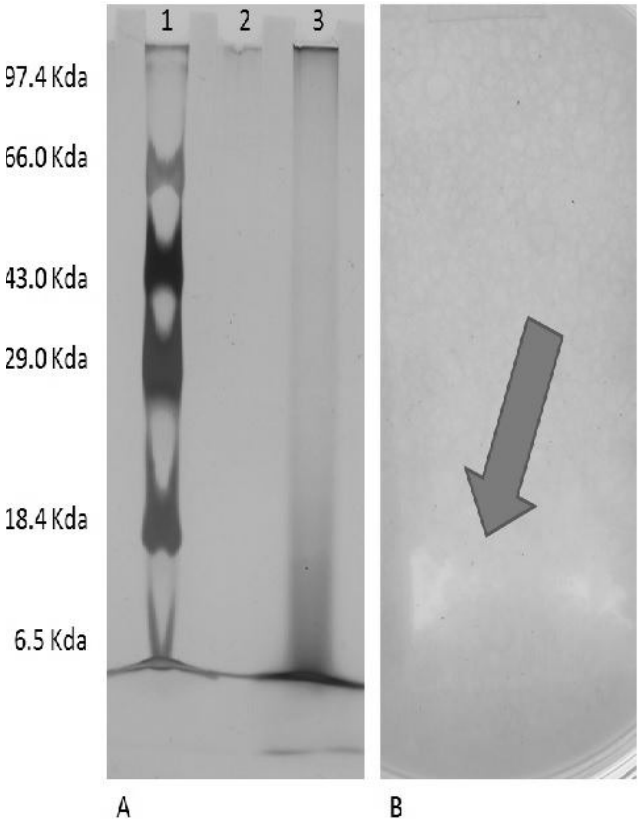

FIG 3a: SDS-PAGE analysis of purified bacteriocin like substance. (Lane 1: Protein molecular weight marker, Lane 3: Purified bacteriocin like substance [~6.5 kDa]).

FIG 3b: Gel overlay method: Purified bacterocin like substance showing zone of clearance.

The purified sample was treated with proteolytic enzymes and tested for the retention of bacteriocin like activity. Trypsin, chymotrypsin and proteinase K were partially inhibiting the bacteriocin like activity of the sample but the enzyme protease did not affect the activity of the sample. The purified sample lost its bacteriocin like activity (no zone of inhibition) at pH 2 and 12 but the treatment at pH 4, 7 and 10 did not affect the activity of the sample. The bacteriocin from *Lactobacillus plantarum* and *Pediococcus pentosaceus* was also active at the pH range of 2 to 6 (Fatima and Mebrouk, 2013). The purified bacteriocin retained its activity after treating to autoclave temperature but the activity was comparatively lesser than the activity of the bacteriocin maintained at room temperature. Ravi Sankar and his colleagues (2012) also observed that the bacteriocin produced from *Lactobacillus plantarum*, isolated from cow milk was showing the activity even after autoclaving. Bacteriocin purified was found to be resistance to the activity of proteolytic enzymes and was found to be stable at pH 4 to 10 and at autoclave temperature of 121°C for 15 minutes which suggest possible use of this substance as an anti-microbial agent outside the rumen as a probiotic and/or food

TABLE 1: Purification profile of Bacteriocin like Substance from rumen liquor.

Sample/step	Total volume (ml)	Total protein (mg)	Total activity units[1]	[2]Specific activity (AU/mg)	[3]Purification fold	[4]%yield (Bacteriocin activity recovered)
Ruminal supernatant	470	1521	15040	9.88	1	100
Ammonium sulphate precipitation (60%)	100	338	12800	37.87	4	85
SP-Sepharose Ion exchange chromatography	90	136.44	11520	84.43	8.5	76.5
Sephadex G50 Gel filtration chromatography	80	28	10240	365.71	37	68

[1]Activity units (AU/ml): Reciprocal of the highest dilutionx1000/volume of bacteriocin added.
[2]Specific activity (AU/mg): Total activity of subsequent step/total protein of the same step.
[3]Purification fold: Specific activity of subsequent step/ specific activity of crude preparation.
[4]Yield: (Total activity of subsequent step/ total activity of crude preparation) x100.

TABLE 2: Results of Database Searching for Protein Identification (First five hits).

Protein ID/Protein Name	Score*
S04440/ Fimbrial Protein – P.aeruginosa(strain1244)	43
B72300/ Oligopeptide ABC tranporter, ATP binding protein – T.maritima(Strain MSB8)	36
O1J1I5_DEIGD/ Hypothetical Protein – D.geothermalis(Strain DSM11300)	35
O49VJ6_STAS1/ Putative dihydroxyacetone kinase – S.saprophyticus subsp. Saprophyticus(strain ATCC1)	33
O7V580_PROMM / Possible Hpt domain – P.marinus(Strain MIT 9313)	32

preservative. Therefore the ability of the substance to inhibit the growth of the other micro-organisms was tested.

The purified sample also showed the bacteriocin like activity against the growth of some common food borne pathogens like *Listeria monocytogens* (MTCC 657)(16 mm of zone of inhibition) and *Staphylococcus aureus* (MTCC 87) (20 mm of zone of inhibition) and also against *Pseudomonas aeuroginosa* (MTCC 424) (29 mm of zone of inhibition) but it did not inhibit the growth of *E.coli* and *Salmonella typhimurium* .The ability of the substance inhibiting the growth of *Pseudomonas aeroginosa* and *Staphylococcus aureus* is important as there are reports of emergence of multi-drug resistance of the above bacterial species. The bacteriocin produced from *Lactobacillus paracasei* subsp. *Paracasei* BGUB9 also found to inhibit the growth of various Gram-positive and Gram-negative pathogenic bacteria like *Streptococcus, Staphylococcus, Shigella, Listeria, Pseudomonas* (Tolinacki *et al*. 2010)

Thus the study clearly demonstrates the utilization of rumen liquor obtained from slaughtered goats as a source of bacteriocin, although the specific organism(s) responsible for bacteriocin purified in this study is not known and demonstrates further the efficacy of this substance in inhibiting the growth of the pyogenic organisms in the aerobic atmosphere which supports the possible use of this substance as an anti-microbial agent in an aerobic atmosphere.

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