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Research Article

PARTIAL PURIFICATION OF BACTERIOCIN LIKE SUBSTANCE FROM RUMEN LIQUOR OBTAINED FROM THE SLAUGHTERED CATTLE FOR MEDICAL APPLICATIONS

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ABSTRACT

Objective: Rumen is rich source of anaerobic microbes and these microbes has ability to produce bacteriocin like substances ,The present study was aimed at Partial Purification of bacteriocin like substance from Rumen Liquor obtained from the slaughtered cattle.

Methods: Rumen liquor samples were collected from slaughtered cattle of fifteen samples collected the anti- microbial activity was seen only in those animals which were fed just before slaughter. The samples were precipitated by 60% ammonium sulphate saturation these precipitates were further purified using ion-exchange chromatography (SP-Sepharose) and eluted with 1.0 M sodium chloride as a single peak. The purity of protein was confirmed by SDS-PAGE analysis.

Results: Antimicrobial activity was obsereved in at 60% ammonium sulphate saturation and these The protein with anti-microbial activity was eluted with 1.0 M sodium chloride as a single peak using ion exchange chromatography, molecular size of these protein was around 6.5kDa, . Further the purified protein was subjected for in gel tryptic digestion and nano LC-MS analysis of the peptides released followed by the identification of protein using MASCOT analysis which had shown similarity with the fimbrial protein of *Pseudomonas aeruginosa* (*SO4440*) and oligopeptide ABC transporter, ATP binding protein (*B72300*)) [23] . Bacteriocin (BViA) falls within the superfamily of ABC transporters and ATP binding proteins demonstrate highest homology matches with the BViA.

Conclusion: Thus the observations made in this study clearly demonstrate that the peptide purified from rumen liquor was indeed bacteriocin like substance.

Keywords: Rumen liquor, anti-microbial activity, purification, protein analysis,

INTRODUCTION

Every year large numbers of animals are being slaughtered to meet the animal protein requirement. During slaughter significant amount of animal by-products are resulted which are either underutilized or being wasted [1, 2]. The sale of animal based by-products constitutes a source of economic return which would help the meat industry to remain economically viable [3, 4, 5]. Nearly 11.4% of the gross income of beef industry and 7.5% of the gross income of pork industry are based on by-product utilization (Dept. of Agriculture Economic research Service, USA, 1997/98).

Rumen is a rich source of anaerobic microbes. A number of antimicrobial peptides have been identified in ruminal fluid bacteria and all these antimicrobial peptides show antimicrobial activity to related rumen microbes and other related microorganisms [6, 7, 8]. 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 borne pathogens [9, 10]. They can also be used as probiotics to improve the production in ruminants [11, 12, 13].

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 underutilized as compost [14, 15, 16]. Therefore isolation and purification of anti-microbial peptides from rumen liquor could be economically beneficial [17,18]. Thus the present study demonstrates the isolation and partial purification of bacteriocin like substance from rumen liquor collected from the slaughtered cattle [19, 20, 21].

MATERIAL AND METHODS

Collection of Ruminal Fluid

About 500ml of ruminal fluid was collected from apparently healthy well fed slaughtered cattle in sterile collection bottles. The ruminal fluid was filtered through coarse muslin cloth in order to remove the larger feed particles. Then the filtrate was subjected to centrifugation at 14500×g for 20 minutes at 4°C. The supernatant was transferred to another tube and centrifugation was repeated thrice to remove bacteria and finer feed particles. Aliquot of ruminal supernatant was taken and tested for antimicrobial activity using cut well agar method after filtering through a 0.22 micron membrane filter. The supernatant showing antimicrobial activity is taken for further purification processes.

Fig. 1: Rumen liquor collection from the cattle

Detection of Anti-Microbial activity using Cut-Well Diffusion Method

In this method MH Agar plates cultured with *Bacillus subtilis* (MTCC 441) were taken. With the use of a sterile well cutter, wells were made in the media. The bottom of the well was sealed using the same MH Agar. Into these wells specified quantity of the testing samples (100µl of the ruminal supernatant, lyophilized 60% Ammonium Sulphate precipitated sample (20mg/ml), ion-exchange purified and lyophilized sample (10mg/ml)) were added and kept at 4°C for 4 hours for diffusion to occur. After 4 hours of incubation, the inoculated plates were incubated at 37ºC for 24-48 hours and the extent of inhibition was recorded.

Ammonium Sulphate Precipitation

Rumen liquor free of feed particles and microbes which are tested positive for antimicrobial activity was subjected for 40%, 60% and 80% ammonium sulphate precipitation overnight at 4°C sequentially. The protein precipitated at each step was separated by centrifugation at 10,000×g for 20 minutes at 4°C. The suspended precipitate was subjected for dialysis against ultra pure water. The dialyzed samples were checked for the presence of antimicrobial activity as detailed above. The sample showing antimicrobial activity was lyophilized and further purified.

Ion Exchange Chromatography

The 60% Ammonium Sulphate precipitated protein which found positive for antimicrobial activity was further purified using ion-exchange chromatography (SP-Sepharose). The column (1×10cm) is equilibrated with 0.1 M Citric acid buffer, pH 4.2 and eluted with 0.5 M, 1.0 M and 1.5 M NaCl solution (Step wise gradient method). 2.0 ml fractions were collected and screened for protein at 280 nm. The fractions containing the protein were screened for anti-microbial activity.

SDS-PAGE Analysis

The purity of ion exchange purified proteins was checked on SDS-PAGE. The purified protein was resolved on 10% SDS-PAGE [18] and silver stained [19].

Proteomic Analysis

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 formed were analyzed using nano LC-MS (Custom Service). The peptide mass fingerprints were searched against the non redundant protein database (MSDB) to identify the protein using MASCOT [20].

RESULTS AND DISCUSSION

Anti-microbial activity of rumen liquor collected from the slaughtered cattle was tested using cut well diffusion method with the *Bacillus subtilis (MTCC* 441) as an indicator organism (Fig.2A) because this organism is an aerobic bacteria and can be cultured easily. It is one of the major food spoilage organisms and also found to be sensitive to various anti-microbial peptides [22].

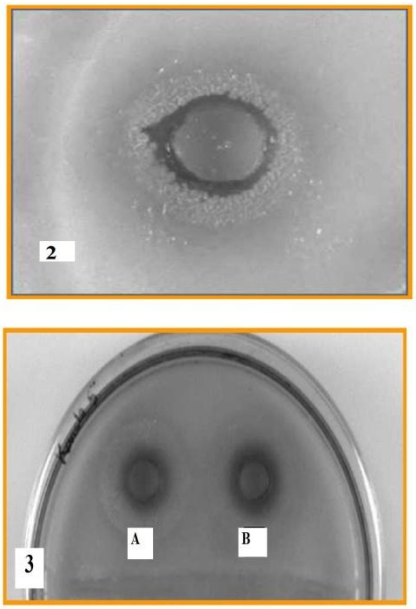

Fig. 2: Anti-Microbial Activity of Rumen Liquor (Supernatant of Rumen Liquor Centrifuged thrice at 14500×g for 20 minutes at 4°C). Fig. 3: Anti-Microbial Activity of 60% Ammonium Sulphate Precipitated Protein (A& B – Two different preparations of Protein).

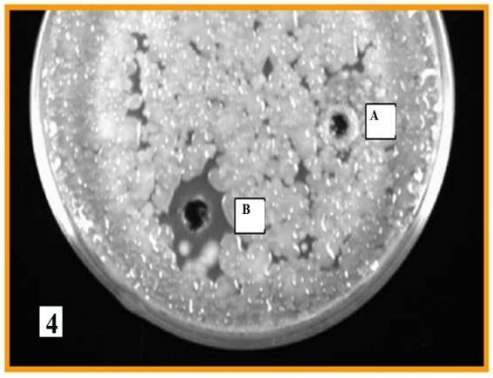

Fig. 4: Anti-Microbial Activity of Purified Protein (B) and Negative Control (A)

Rumen liquor sample which showed anti-microbial activity was subjected for ammonium sulphate precipitation and the precipitated protein at 60 % ammonium sulphate saturation was found to exhibit anti-microbial activity against *Bacillus subtilis (*MTCC 441) (Fig.2B). The result was consistent with repeated testing of different rumen liquor samples and also corresponds with other findings. Mantovani *et al.* (2002) had demonstrated the isolation of bovicin HC5 from the culture of *S.bovis* at 40-60 % ammonium sulphate precipitation. The bacteriocin, Pediocin was also found to be precipitated at 60 % ammonium sulphate saturation.

The samples having anti-microbial activity were further purified using Ion-exchange chromatography. The protein with anti-microbial activity was eluted with 1.0 M sodium chloride as a single peak (Fig.3A & 3B) which supports the cationic nature of this protein as the case with other types of bactericoins identified.

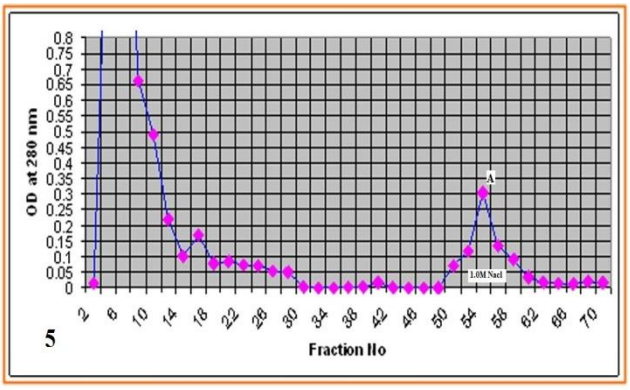

Fig. 5: Ion-Exchange Chromatographic Purification of Anti-Microbial Protein from Rumen Liquor. A. The graph indicating the elution of anti-microbial protein using 1.0 M NaCl.

The purification of bacteriocin from a culture of *S. bovis* using SP-Sepharose chromatography and found that the peptide with anti-microbial activity was eluted with high concentration of sodium chloride. Other bacteriocins – Pediocin and Bovicin HJ50 [20] were also purified using SP-Sepharose. These peptides were also found to be eluted with 1.0 M sodium chloride [21].

The purified sample was shown as a single band (~ 6.5 kDa) on SDS-PAGE analysis (Fig.4). Therefore further purification of sample was not attempted. Instead, it was directly subjected for in gel tryptic digestion and nano LC-MS analysis of the peptides released followed by the identification of protein using MASCOT analysis which had shown similarity with the fimbrial protein of *Pseudomonas aeruginosa* (*SO4440*) and oligopeptide ABC transporter, ATP binding protein (*B72300*) (Table 1). Bacteriocin (BViA) falls within the superfamily of ABC transporters and ATP binding proteins demonstrate highest homology matches with the BViA . Thus the

observations made in this study clearly demonstrate that the peptide purified from rumen liquor was indeed bacteriocin like substance.

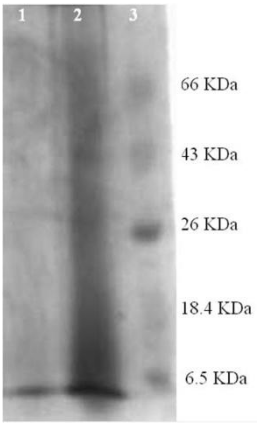

Fig. 6: 12% SDS-PAGE analysis of Ion-Exchange Purified anti-microbial peptide from Rumen Liquor (Lane 1: Ion-Exchange Purified sample; Lane 2: 60% Ammonium Sulphate Precipitated Protein; Lane 3: Mol.Wt Protein Marker)

Table 1: Results of Database Searching for Protein Identification (First five hits).

S.No.	Protein ID/Protein Name	Mass	Score*
1	S04440/ Fimbrial Protein – *P.aeruginosa*(strain1244)	16382	43
2	B72300/ Oligopeptide ABC tranporter, ATP binding protein – *T.maritima*(Strain MSB8)	---	36
3	O1J1I5_DEIGD/ Hypothetical Protein – *D.geothermalis*(Strain DSM11300)	47496	35
4	O49VJ6_STAS1/ Putative dihydroxyacetone kinase – *S.saprophyticus subsp. Saprophyticus*(strain ATCC1)	21068	33
5	O7V580_PROMM / Possible Hpt domain – *P.marinus*(Strain MIT 9313)	---	32

* Score = MASCOT probability based scoring system

This study further demonstrates the efficiency of anti-microbial peptide isolated from ruminal supernatant in the atmospheric conditions as indicated by the exhibition of anti-microbial activity in the aerobic condition. The efficacy of the bacteriocin isolated as a food preservative and/or as a probiotic need to be studied nevertheless this study supports that the rumen liquor directly obtained from the slaughtered cattle would definitely form the alternative source of anti-microbial agent and effective utilization will improve the economy of the meat industry.

CONCLUSION

In the present study, the rumen liquor was collected from fifteen different slaughtered cattle and tested for their anti-microbial activity. The anti-microbial activity was seen only in those animals which were fed just before slaughter. Anti-microbial activity was tested against *Bacillus subtilis* in cut well diffusion method. The samples which were found positive for anti-microbial activity. The proteins precipitated at 60 % ammonium sulphate saturation were found to be positive for anti-microbial activity against *Bacillus subtilis.* The samples were dialyzed and lyophilized. The lyophilized samples were also positive for anti-microbial activity.

The lyophilized proteins obtained at 60 % ammonium sulphate saturation were subjected to SDS-PAGE and silver stained. The region of PAGE containing the peptide corresponding to 6.5 KDa was found to possess anti-microbial activity.

The anti-microbial peptide was subjected for further purification using SP-Sepharose cation exchange chromatography. The anti-microbial peptides were eluted at 1.0 M sodium chloride. These fractions were lyophilized and tested for anti-microbial activity, Thus the anti-microbial peptide isolated and purified from rumen liquor is a bacteriocin like substance and is proteinaceous in nature and cationic. The molecular size was found to be ≤ 6.5 KDa. Importantly, these peptides were found to be resistant to the gut enzymes. Therefore the purified peptides can be used as a feed additive.

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