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CHARACTERIZATION OF STAPHYLOCOCCIN BY PEPTIDE MASS INGERPRINTING

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ABSTRACT

Protein components such as Staphylococcin, to characterize an emerging subgroup of bacteriocins, identified by MALDI –TOF were found to have activities in the single nanomolar range. Peptide mass finger printing is a protein identification technique in which mass spectrometer is used to measure the masses of proteolytic peptide fragments. The protein is then identified by matching the measured peptide masses to corresponding peptide masses from protein or nucleotide sequence database. Staphylococcins are bacteriocins produced by Staphylococci, which are gram positive bacteria with medical and veterinary importance. Most bacteriocins produced by Staphylococci are either lantibiotics (e.g. Pep 5, epidermin, epilancin k7, epicidin 280, Staphylococcin c55/Bac R1 and nukacin ISK-1) or class II bacteriocins (e.g. Aureocins A70 and 53). Only one Staphylococcin belonging to class III, lysostaphin, has been described so far. Production of Staphylococcins is a self protection mechanism that helps Staphylococci to survive in their natural habitats. However, since these substances generally have a broad spectrum of activity, inhibiting several human and animal pathogens, it can be used as an antimicrobial agent . The aim of this work was to study the effectiveness of staphylococcin isolated from MRSA against the MRSA strain. So that the staphylococcin can be used as an novel drug molecule.Methicillin resistant Staphylococcus aureus strain, which is a bacteriocin producer was isolated, purified by ammonium sulphate precipitation test and dialysis. Molecular weight was determined by using Tricine SDS-PAGE and its activity against MRSA was confirmed by agar well assay which paved way to analyse the peptide sequence by MALDI TOF which can be further used in combination with bioinformatic tools in a novel drug design

KEY WORDS

Staphylococcin, bacteriocin, bioinformatic tools, novel drug design

INTRODUCTION

Staphylococcus aureus is a gram positive bacterium responsible for severe morbidity and mortality worldwide. The organism flourishes in the hospital setting producing blood stream and surgical wound infections. Within the genus Staphylococcus, bacteriocins have been reported from several sp., but only a few Staphylococcins have been purified and studied in detail (Lachowicz and Walczak, 1968). In recent years, there have been several reports dealing with Staphylococcal bacteriocins-Pep5 (Sahl and Brandis, 1981). Epidermin (Allgaier et al., 1986) and gallidermin (Kellner et al., 1988) containing thioether amino acids such as lanthionine and Methyllanthionine and α, β-unsaturated amino acids such as didehydroalanine and didehydroaminobutyric acid. . Epidermin and Gallidermin, produced by Staphylococcus epidermis and Staphylococcus gallinarum, respectively consists of 21 amino acids and are only different at amino acid residue position 6 (ile for epidermin and Lue for gallidermin) (Allgaier et al., 1986; Kellner et al., 1988). MALDI – TOF MS can provide relative quantification of intact proteins, particularly if the protein mixture is not complex and there are large differences in relative abundance. Mass spectrophotometer is a kind of machine which uses an analytical technique to measure the mass to – charge ratio of ions. MALDI TOF matrix assisted laser desorption/ionization (MALDI) is a soft ionization technique used in MS.Time of flight (TOF) is the method of measuring particle mass to charge ratio.

In this research work, we describe the isolation, purification, molecular weight determination, invivo activity assay and malditof assay of staphylococcin which will pave way for developing a new peptide that can be used as a drug of choice against the methicillin resistant strains.

MATERIALS AND METHODS

Bacterial cultures and media

The bacteriocin producer Staphylococcus aureus was obtained from MTCC, Chandigarh. The indicator organism, used in the bacteriocin assay was Methicillin resistant Staphylococcus aureus. Stock cultures were maintained on Brain heart infusion broth and Brain heart infusion media and stored at 4°C.

Isolation of bacteriocin producer
Stab overlay method

Standard methicillin resistant Staphylococcus aureus, which is bacteriocin producer, was taken for the study. The bacteriocin producing strain was stabbed in pre-poured BHI agar plates and incubated at 37°C for 24 hrs. The plates were exposed to chloroform in order to kill the producing strain. For this, chloroform soaked filter paper (What man No.1) was placed at the lid of the plate and allowed to stand for 10-15 mins. 3 ml BHI soft agar containing 0.1 ml of standardized inoculum (2×10^8 CFU/ml) of the sensitive culture was poured over the plates and incubated at 37°C. After overnight incubation, zones of inhibition around the producer colonies were measured and documented. This method has an advantage of testing a number of producer strains against a sensitive strain.

Partial purification of Bacteriocin
Ammonium sulphate precipitation test (salt-

out process):

Brain heart infusion broth (100 ml) was prepared and was inoculated with the producer culture, Staphylococcus aureus and incubated for 24 hrs at 37°C. After incubation, the broth was centrifuged at 10,000 rpm fro 10 mins. The supernatant was taken and the pellet was discarded. To the supernatant (100 ml), 52.3 gms of Ammonium sulphate was added and dissolved completely. Then it was kept at 4°C for 24 hrs. The precipitate was recovered by centrifugation at 10,000 rpm fro 10 mins. The pellet was solubilized in 200ml 50mM sodium phosphate buffer pH 7 and it was designated as crude preparation
.

Complete purification of bacteriocin

Dialysis:

BHI broth, inoculated with the producer culture and incubated overnight was centrifuged at 10,000 rpm for 10 mins. The supernatant was taken and the pellet was discarded. To the supernatant (100 ml), 52.3 gms of Ammonium sulphate was added and dissolved completely and kept overnight at 4°C. It was again centrifuged at 10,000rpm for 10 mins. The supernatant and the pellet both were checked for its molecular weight by using dialysis membrane. Phosphate buffer saline (pH-7.2) was prepared. Dialysis membrane was cut and samples were loaded in the dialysis bag and tightened with thread. The dialysis bags were hanged and dipped inside the phosphate buffer and the whole setup was placed on the magnetic stirrer and putting magnetic beads, it was kept at 4°C for 4-5 hrs.

Estimation of protein by Lowry's method

The samples were analyzed for protein using Lowry's method. 5 tubes which serve as standard and one tube for the supernatant and one tube for the pellet were taken and 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1 ml of protein solution were added to the standard tubes marked as S1, S2, S3, S4 and S5. 0.2 ml of supernatant and 0.8 ml of pellet were also added to the respective tubes and each of the tubes were made up to 4ml by adding water. Then 5.5 ml of reagent C was added to all the tubes and kept at room temperature for 10-15 mins. Then 0.5ml of reagent D was added to all the tubes and kept in dark for 30 mins. Then OD was taken for all the tubes at 650 nm.

Lyophilization

The precipitate obtained after ammonium sulphate precipitation test was then lyophilized for further studies.

HPLC

The lyophilized sample was then sent for HPLC in Central Electrochemical Research Institute, Karaikudi for further purification.

Molecular Weight Determination:

Tricine SDS PAGE:

1. The SDS apparatus and glass plate were set up.
2. The separating gel was set in the glass plate by overlaying 0.5ml of 10% SDS solution.
3. Then the stacking gel was poured and the comb was inserted and it was allowed to set.
4. Cathode buffer was filled in the upper chamber.
5. Anode buffer was filled in the lower chamber.
6. Then the gel plate was fixed in the apparatus.
7. The sample and the protein marker were loaded.
8. The volts was set at 15mv and then to 30mv.
9. The gel was run and after running, the gel was transferred into the staining solution for 30 mins.
10. After 30mins the gel was transferred into the distaining solution, until the stains were removed. Therefore it was kept in shaker.

11. Then it was viewed under white illuminator.
12. The gel was stored in phosphate buffer

MALDI-TOF/TOF

The tryptic digests were prepared by mixing equal amounts (2:2) of peptide mixture with the matrix solution (α-cyano-4-hydroxycinnamic acid) saturated with 0.1% TFA and acetonitrile (1:1). Then the samples were analyzed in reflectron mode with delay time of 90 ns and 25 Kv accelerating voltage in the positive ion mode. To improve the signal to noise ratio, summation of 300 laser shots were taken for each spectrum. External calibration was done using peptide I calibration standard with masses ranging from 1046-3147 Da. Mass spectras were acquired using ULTRAFLEX-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), equipped with a 337 nm pulsed nitrogen laser. MS-MS spectra were acquired by selecting the precursor mass with 8 Da windows.

2.8. Data Base Analysis

Spectra were processed using Flex Analysis software. Monoisotopic peptide masses were assigned and used in the database search. The protein identification was accomplished utilizing the MASCOT data base search engine (Matrix Science, London, UK) (http//www.matrixscience.com, search engine). Probability-based MW search scores were estimated by a comparison of search results against an estimated random match population and were reported as 10 log10 (P), where P is the absolute probability. Scores >63 were shown to be significant (P<0.05) in the mascot search. Proteins identified with scores less than the significant level were reported as unidentified.

Stab overlay method

Zone of inhibition around the staphylococcin producer colonies were observed on the plates which were swabbed with MRSA strain and were measured which suggest that MRSA is sensitive to staphylococcin.

Partial purification of bacteriocin

The partial purification of bacteriocin was done by ammonium sulphate precipitation test i.e. salt-out method. The precipitate was recovered by centrifugation at 10,000 rpm for 10 mins. The pellet was solubilized in 200 ml of 50mM sodium phosphate buffer (pH-7) and it is designated as crude preparation.

Complete purification of bacteriocin:
Dialysis:

After the incubation of the proteins in dialysis bags, maintain in phosphate buffer at 4°C for 5 hrs, the proteins were estimated by Lowry's method. Then the OD was taken at 650 nm.

Molecular weight determination

SDS PAGE

The protein was then run in Tricine SDS-PAGE and protein bands were obtained and viewed under white illuminator which showed the molecule to have 3000 da molecular weight when compared with the low molecular weight marker.

HPLC

The HPLC chromatogram for the AMP bacteriocine (staphylococcin) shows 2 peak one with an retention time of 2.743 and the other with 2.967 . The height of peaks are 0.168 and 2.413 .The area of peaks are 1.101 and 13.115 respectively. The stationary phase used in the column is silica gel and the mobile phase is acetonitrite. The detector used was uv spectrophotometric detector (254nm). The sample was forced under a pressure of (125kgb/cm^2) and the amount of sample injected was With all these data it was found that 2 different compounds were present in the sample as there were 2 peaks and the

concentration of compound A was found to be 0.005 which was very negligible and the concentration of sample b was found to be 20.01µg/ml. Other protein is present in negligible amount when compared to staphylococcin.

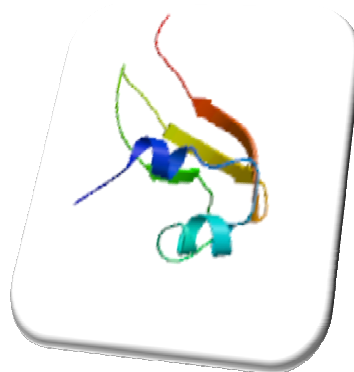
MALDI TOF

The 20kda protein when characterized by MS/MS fragmentation to obtain more structural information. The amino acid sequences were obtained and subjected to BLAST search in order to find out the nature of the protein in which it was found that the mass range of 41777 was related to hypothetical protein SAVO 344 of Staphylococcus aureus sub species Mu50 which is a strain less sensitive to vancomycin.

The sequence details are: it has got 158 amino acids which are as follows..

MNIFSRMTAALAAAAISTLALLGCDEQKIKE
LEEGLSTEADVRAKFGEPERIWNEQDGSRTL
EYNRQPAGAKNYMITIGADGKMSALRQVLAP
HVFARIVPGMEENEVRRMLGKPAKMMTYQLK
QETDWDWNYIDPPTREMQFTVTFGSDGRVL
RTLNRERLPDESRG

The structure of the bacteriocin got by sequence analysis using Swiss prot.



CONCLUSION

It is concluded from this study that the bacteriocin –Staphylococcin isolated from MRSA can inhibit MRSA after it is purified,and it is not affected on treatment with various temperature, pH, enzymes, salts and chloroform. Its anti-Staphylococcal activity was checked by various methods and can be said that it has strong anti –Staphylococcal activity against MRSA strain and the protein s sequence can be used in for drug designing.

REFERENCES

[1] Allgaier, H., Jung, G., Werner, R.G., Schneider, U. and Zahner, H. (1986). Epidermin: sequencing of a heterodet tetracyclic 21-peptide amide antibiotic. European Journal of Biochemistry, 160: 9-22.

[2] Bastos, M.C.F., Ceotto, H., Coelho, M.L.V. and Nascimento, J.S. (2009).

Staphylococcal Antimicrobial peptides; Relevant Properties and Potential Biotechnological Applications. Current Pharmaceutical Biotechnology, 10: 38-61.

[3] Dajani, A.S. and Wannamaker, L.W. (1969). Demonstration of a bactericidal substance against β-hemolytic streptococci in supernatant fluids of Staphylococcal cultures . Journal of Bacteriology, 97: 985-991.

[4] Furmanek, B., Kaczorowski, T., Bugalski, R., Bielawski, K., Bogdanowicz, J. and Podhajska, A.J. (1999). Identification, characterization and purification of the lantibiotic staphylococcin T, a natural gallidermin variant. Journal of Applied Microbiology, 87: 856-866.

[5] Gagliano, V.J. and Hinsdill, R.D. (1970). Characterization of a Staphylococcal aureus bacteriocin. Journal of Bacteriology, 104: 117-127.

[6] Hale,E.M. and Hinsdill, R.D. (1973). Characterization of a bacteriocin from Staphylococcus aureus strain 462. Antimicrobial agents and chemotherapy, 4: 634-640.

[7] Jack, R.W., Tagg, J.R. and Ray, B. (1995). Bacteriocins of gram positive bacteria. Microbiological Reviews, 59:171-200.

[8] Jetten, A.M., Vogels, G.D. and De Windt, F. (1972). Production and purification of a staphylococcus epidermidis bacteriocin. Journal of Bacteriology, 112: 235-242.

[9] Jung, G. (1991). Lantibiotics, a survey. In Nisin and Novel Lantibiotics, ed. Jung, G. and Sahl, H.G., pp. 1-35. Leiden, Netherlands: Escom.

[10] Kellner, R., Jung, G., Horner, T., Zahner, H., Schnell, N., Entian, K.D. and Gotz, F. (1988). Gallidermin: a new lanthionine-containing polypeptide antibiotic. European Journal of Biochemistry, 177: 53-59.

[11] Lachowicz, T. and Walczak, Z. (1968). Purification and properties of Staphylococcin A. Archivum Immunologiae et Therapiae Experimentalis, 16: 855-863.

[12] Nakamura, T., Yamazaki, N., Taniguchi, H. and Fugimura, S. (1983). Production, purification and properties of a bacteriocin from Staphylococcus aureus isolated from saliva. Infection and immunity, 39: 609-614.

[13] Rogolsky,M. qnd Wiley, B.B. (1977). Production and properties of a Staphylococcin genetically controlled by the staphylococcal plasmid for exfoliative toxin synthesis. Infection and immunity, 15: 726-732.

[14] Sahl, H.G. and Brandis, H. (1981). Production, purification and chemical properties of an anti-staphylococcal agent; produced by Staphylococcal epidermidis. Journal of General microbiology, 127: 377-384.

[15] Schnell, N., Entian,K.D., Zahner, H., Kellner, R. and Jung, G. (1988). Prepeptide sequence of epidermin, ribosomally synthesized antibiotics with four sulphide rings. Nature, 333: 276-278.