Journal of Experimental Sciences 2011, 2(2): xx-xx
ISSN: 2218-1768
www.scholarjournals.org

journal of experimental sciences
www.jexpsciences.com

JES-Physical Sciences

X-Ray Diffraction Studies of Staphylococcin for Novel Drug

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Article Info

Article History

Received : 13-03-2011
Revised : 16-03-2011
Accepted : 17-03-2011

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Abstract

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 . X ray crystallography can determine the size of atoms , the lengths and types of chemical bonds , and the atomic scale difference among various materials. The aim of this work was to study the effectiveness of staphylococcin isolated from MRSA against the MRSA strain.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 XRD analysis of staphylococcin was done.

Key Words: Bacteriocin, Lantibiotic, Staphylococcin, MRSA, Antimicrobial activity

Introduction

Staphylococcus aureus is a gram positive bacterium responsible for severe morbidity and mortality worldwide. It is one of the leading cause of human infection in skin, soft tissues, bones and joints, abscess and normal heart valves. 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. These lanthionine containing antimicrobial peptides belongs to the group termed lantibiotics, to distinguish them from other bacteriocins which do not contain these modified residues (Schnell *et al.*, 1988). The biosynthesis of lantibiotics includes the post-transitional modification of a ribosomally synthesized precursor (Jung, 1991); this feature clearly distinguishes lantibiotics from both peptide antibiotics produced by multi enzyme complexes and other antibiotics which are more typically the products of a secondary metabolism (jack *et al.*, 1995). 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). Studies of these lantibiotics have suggested that they may be biologically active against pathogenic bacteria such as *Propionibacterium acne*, *Staphylococci* and *Streptococci* (Jung, 1991).

X- ray crystallography is a method of determining the arrangement of atoms within a crystals, in which a beam of X – rays strikes a crystals and diffracts into many specific directions.From the angles and intensities of these diffracted beams, a crystallographer can produce a three dimensional picture of the density of electrons within the crystals.From this electron density, the mean positions of the atoms in the crystal can be determined , as well as their chemical bonds, their disorder and various other information.Since many materials can form crystals such as salts,metals,minerals,semi conductors, as well as various inorganic, organic and biological molecules.X- ray crystallography has been fundamental in the development of many scientific fields.The method also revealed the structure and functioning of many biological molecules, including vitamins, drugs,proteins and nucleic acids such as DNA.

In this research work, we describe the isolation, purification, molecular weight determination and XRD analysis of staphylococcin.

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 dissolve 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 is 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 tight with thread. The dialysis bag were hanged and dipped inside the phosphate buffer and the whole setup was placed on the magnetic stirrer and putting a magnetic bead it was kept at 4°C for 4-5 hrs.

Estimation of protein by Lowry's method

The samples were then taken for protein estimation by 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 marks as S1, S2, S3, S4 and S5. 0.2 ml of supernatant and 0.8 ml of pellet were also added to the respectively tube 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 forHPLC in central electrochemical research institute, Karaikudi for further purification.

Molecular Weight Determination:

Tricine SDS PAGE:

1. The SDS apparatus and glass plate was 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 in to the staining solution for 30 mins.
10. After 30mins the gel was transferred in to the distaining solution, until the stains removes, therefore it was kept in shaker.
11. Then it was viewed under white illuminator.
12. The gel was stored in phosphate buffer

Results and Discussion

Stab overlay method

Zone of inhibition around the producer colonies were observed and were measured. This confirms that the MRSA strain producers bacteriocin and can inhibit the strain by the bacteriocin isolated from it.

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 fro 10 mins. The pellet was solubilized in 200 ml of 50mM sodium phosphate buffer (pH7) and it is designated as crude preparation.

Complete purification of bacteriocin:

Dialysis:

After the incubation of the proteins in dialysis bags kept 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. The concentration of protein was given in table no.1.

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.

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 peak 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 u v 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 are 2 peaks and the concentration of compound A was found to be 0.005 which is very negligible and the concentration of sample b was found to be 20.01µg/ml

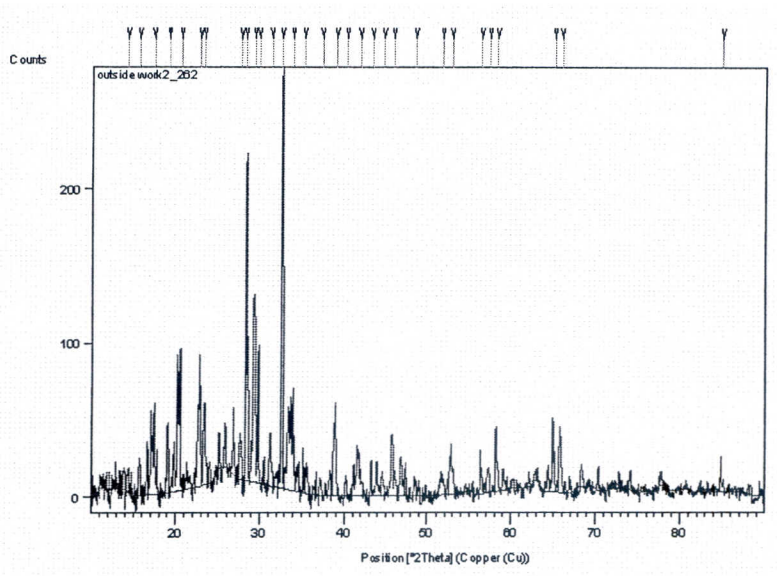
Sample A:

SF = AREA/CONC
= 1.10/20 =.005
CONC = AREA/SF
= 1.10/0.005 =220µg/ml

Sample B:

SF = 13.11/20
= 0.655
CONC = 13.11/0.655 = 20.01µg/ml

XRD chromatogram:



XRD yields the atomic structure of material based on the elastic scattering of x ray from the electron clouds of the indeructual atom in the system . PD is commonly used to identify unknown substances by comparing diffraction data against A and B maintained by IC for DD .It is also used to characterize heterogeneous solid materials . The diffraction pattern of the AMP staphylococcin that are tabulated shows that the lattice parameter value a,b,c are 16.956950 ,16.956950 ,12.461836 respectively is that A=B≠C so the structure of protein is tetragonal crystal system.

Conclusion

It may be 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 concluded that it has strong anti –Staphylococcal activity against MRSA strain. And from XRD we co0nform that the proyein has got a tetragonal crystal system.

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