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Partial Purification and Characterization of Antimicrobial Protein from *Pseudomonas aeroginosa.*

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Abstract

Antimicrobial peptides (AMPs) are ubiquitous and natural antibiotics generated by a diverse range of microorganisms, plants, insect and mammalian cells. Recent attention has been drawn to AMPs as new antimicrobials to combat harmful microbes especially those resistant to conventional antibiotics. In the search for new antibacterial proteins, these may be used as templates for the design of novel drugs. The antibacterial protein was partially purified by ammonium sulfate precipitation. The purified antibacterial protein remained active over a wide temperature range and was resistant to all proteases tested. This antibacterial protein was characterized by direct activity on sodium dodecyl sulfate polyacrylamide gel electrophoresis. A major band on SDS-PAGE suggested that the antibacterial protein has a molecular mass of about 47kDa. It was analyzed by HPLC that the basic amino acids Lysine, Arginine and Histidine in were present in the therapeutic protein.

Keywords: *Pseudomonas aeroginosa*, AMPs, ammonium sulfate precipitation, SDS-PAGE, HPLC.

Introduction

Antibiotics are components or substance that kills or inhibits the growth of microbes produced naturally by microbes or chemically synthesized. In the last few years many cationic peptides have been isolated from a wide range of animal, plant, and bacterial species (Aley *et al.*, 1994)[1]. Antimicrobial peptides are so widespread that they are likely to play an important protective role. Marine bacterium has been recognized as important antimicrobial peptide producers which have an exceedingly bright future in the discovery of life saving drugs[2]. These peptides kill target cells through diverse mechanisms once they reach the target microbial membrane. They possess a wide array of biological mechanisms from direct killing of invading pathogens to modulation of immunity and other biological responses of the host [3]. These antimicrobial peptides are the first line of host defense in various species, have a mass of ≤10 kDa and are readily synthesized and efficiently diffuse at the point of pathogen entry or infection, hence form an invaluable component of the innate immune system [4, 5].The potential role in the production of active metabolites from soil bacteria against pathogens is a promising new topic for research; the present study reveals the partial purification and characterization of anti MRSA protein producing bacteria from soil.

Materials and Methods

Sample Collection

Soil samples were collected from Valparai, Coimbatore District, Tamil Nadu. The seventy five samples were collected and used for the isolation of bacteria which have antibacterial activity.

Isolation of bacteria

Serial dilution agar plating method (or viable plate counting method) was followed to isolate 42 bacteria from the collected samples.

Collection of MRSA

The clinical isolate of Methicillin Resistant *Staphylococcus aureus* was collected from clinical laboratory of K.M.C.H, Coimbatore, Tamil Nadu and MTTC IMTECH, Chandigarh. MRSA was preserved in agar slants for further use and stored at 4° C. Then Kirby Bauer method (agar diffusion method) was followed to determine the susceptibility of MRSA to antibiotics.

Screening of soil isolates for its antibacterial activity

Point Inoculation Method:

Muller Hinton Agar plates were prepared .The overnight culture of MRSA in nutrient broth was uniformly swabbed on the surface of Muller Hinton Agar plates using sterile cotton swab, making a lawn culture. These 42 soil isolates from the agar slants were spotted on to Muller Hinton Agar plates seeded with actively growing cells of the clinically isolated MRSA. The plates were incubated at 37 C for 24 hours. After the incubation period, the plates were observed for the zone of inhibition and the results were recorded.

Agar Well Diffusion Method

After screening by point inoculation method, the antimicrobial substance production of the soil isolates was tested by agar- well diffusion method. Muller Hinton agar plate was prepared. The overnight culture of MRSA in nutrient broth was uniformly swabbed on the surface of Muller Hinton agar plates using sterile cotton swab. Two wells of 3mm size were made with sterile cork borer on the seeded plate. Around 42 of overnight broth culture of soil isolate was added to the well aseptically. The plates were incubated without inverting for 24hrs at 30°C and the zone of inhibition was observed and recorded.

Screening of soil isolates supernatant for its antibacterial activity

The overnight grown culture of soil isolate in nutrient broth was centrifuged at 6000 rpm for 10 minutes. After centrifugation the supernatant was collected and stored at - 20 °C for further analysis. The overnight culture of MRSA in nutrient broth was uniformly swabbed on the surface of sterile Muller Hinton agar plate using cotton swab. Two wells of 3mm size were made with sterile cork borer on the seeded plate. Around 42 of overnight broth culture of soil isolate was added to the well aseptically. The plates were incubated without inverting for 24hrs at 30°C and the zone of inhibition was observed and recorded.

Partially Purification of antibacterial proteins by Ammonium Sulphate precipitation Method

To the cell free supernatant, ammonium sulphate was added to achieve 40% saturation. It was stirred well and incubated at 4°C for overnight. After incubation period, the precipitate was collected by centrifugation at 6000rpm for 15 minutes at 4°C.The precipitates were dissolved in sterile phosphate buffer.

Desailtig by Dialysis Method

The ammonium sulphate precipitates of various percentage of saturation (10-80%) were desalted by dialysis. Pre-treatment of dialysis bag play an essential role in dialysis. Dialysis membrane was taken and treated with distilled water at 65ºC for 10 minutes to remove glycol and then soaked in 10mM disodium Ethylene Diamine Tetra Acetic Acid (EDTA). It was then treated with 10mM Sodium bicarbonate to remove sulphur molecule and finally washed with distilled water. The membrane was clipped at one end and one third of the bag was filled with ammonium sulphate precipitates and the other end was also clipped using dialysis tubing clip. The samples were then dialyzed against phosphate buffer saline (pH 7.4) by changing buffer in every four hours for overnight. Then the dialysis bag was taken and the samples were carefully drawn and collected for further analysis. The inhibitory activity and protein content of 10-80%

saturated dialyzed protein were examined and the percentage of saturation which exhibited maximum inhibitory activity was taken for the further study.

Characterization of Antibacterial Proteins

Analysis of SDS PAGE

12% separating gel was prepared and poured inside the chamber between the glass plates without any air bubbles and allowed to polymerize for 30-60 minutes. After polymerization, 3% stacking gel was prepared and poured over the resolving gel. Comb was inserted between the glass plate and the gel was allowed for polymerization. After polymerization of stacking gel, comb was removed gently without destroying the shape of the wells. The spacer at the bottom of the glass plate was removed and the plate was fixed in the electrophoretic apparatus. Running buffer was poured into the electrophoretic tank without any air bubbles. The sample to be loaded was prepared by mixing sample and loading dye in 1:2 concentrations. Marker was also prepared in the same way. It was then denatured for 3 minutes and loaded in the wells. Marker was added in the first well and power was supplied. Once the bromophenol blue dye reaches 1cm above the bottom of the gel, current was put off. The gel was then carefully removed for staining.

MALDI-TOF analysis

The samples was mixed with MALDI matrix (cyano-4 hydroxy cinnamicacid) at 1:1 concentration and the mixture was applied to a target slide placed inside the instrument and was irradiated with the laser. The matrix observed the laser light and the sample was vapourized by ionization. Peptides was then accelerated through the flight tube under vaccum in a reflector mode (600 - 4000 Daltons) and received at the detector. Measurement was performed with the Bruker microflex LT MALDI-TOF using flex control software with compass flex series version1.3 software and a 60% Hz nitrogen laser (337nm). The spectra were collected in the linear positive mode in a mass range converting m/z 1,960 - 20,132. Spectra range ratio m/z 1,000 - 4,500 was analysed using Bruker Biotyper automation control and Bruker Biotyper 3.1 software. Based on their mass to charge ratio (m/z), the actual masses of the peptides were assigned.

Peptide Mass Fingerprinting (PMF) Analysis

The obtained MALDI spectrum was then searched and compared against existing databases like Swiss Prot and NCBI (National Centre for Biotechnology Information) using Mascot software (Matrix Science) and the protein is identified by comparing the molecular weight against a database of known peptides. Mascot computes the score based on the probability that the peptides from a sample match those in the selected protein database. Protein score greater than 68 was considered significant with a value of $p<0.05$. The more peptides the Mascot identifies from a particular protein, the higher the mascot score for that protein.

HPTLC Analysis

The antimicrobial proteins were analyzed by HPTLC technique using three amino acids lysine, asparagine and histidine as the standards. The separation was performed on HPTLC silica gel readymade plates. Sample application was done using fully automated sample applicator. A total sample volume of 7 µl was applied as 6mm bands at a dosage speed of 50 nL S^{-1}. This gave the total of 14 µg protein per band. The samples were applied 10mm from the bottom of the plate and the distance between bands was 15 mm. The development of chromatogram of the HPTLC plate was carried out in the chamber using 2-butanol/acetic acid/water(39:34:26,v/v/v).Then the plates were dried and sprayed with 0.2% ninhydrin in 2-propanol using TLC sprayer and heated to 120ºC for 2 minutes. After that plate was analyzed by HPTLC detector in 200-280 nm detection mode. Rf values were calculated and recorded.

Results and Discussion

Isolation of Bacteria

Soil bacteria were isolated from seventy five soil samples from Valparai, Coimbatore district, Tamil Nadu, using nutrient agar by serial dilution spread plate method. A total of 42 bacteria were isolated based on their morphological and structural characteristics.

Antimicrobial Susceptibility of MRSA to antibiotics

Antimicrobial susceptibility of MRSA to bacteria, Kanamycin, Vancomycin, Metronidazole, Neomycin, Gentamicin, Erythromycin, Chloramphenicol, Streptomycin, Tetracycline and Methicillin was carried out by Kirby Bauer disc diffusion assay. The results were tabulated **(Table:1)**

Table: 1 Antimicrobial Susceptibility of MRSA to antibiotics

Sl.No:	Organisms	Zone Of Inhibition (mm)										
		A	Cf	C	G	K	M	N	Ox	P	T	V
1	*C. diphtheria*	-	35	-	-	-	-	10	-	-	9	-
2	*E. coli*	-	-	-	-	15	-	16	-	-	R	-
3	*K. pneumoniae*	-	-	-	25	22	-	22	-	-	-	-
4	MRSA	-	-	-	-	-	R	-	-	R	-	16
5	*M. smegmatis*	-	R	-	-	-	-	-	-	-	-	-
6	*P. vulgaris*	-	-	-	9	R	-	-	-	-	R	-
7	*p. aeruginosa*	-	-	-	17	8	-	11	-	-	-	-
8	*Shigella sp*	R	13	14	-	-	-	-	-	-	-	-
9	*S. aureus*	-	-	-	-	-	-	R	R	-	-	14
10	*S. epidermidis*	-	-	-	-	-	R	-	R	R	-	-

A-Ampicillin, Cf-Ciprofloxacin, C-Chloramphenicol, G-Gentamicin, K-Kanamycin, M-Methicillin, N-Neomycin, Ox-Oxacillin, P-Penicillin, T-Ticarcillin, V-Vancomycin

Screening of soil isolates for its antibacterial activity

All of the soil isolates were tested for their ability to produce antibacterial substance against MRSA. Soil isolates from agar slants were spotted onto the Muller Hinton agar. Out of the 42 isolates, only one isolates (A) showed inhibitory activity against MRSA. Bacterial isolate (A) which showed antibacterial property was identified as *Pseudomonas aeruginosa* by 16s rDNA sequencing. Zone of clearance of about 17 mm was observed after 24 hours of incubation. After screening by point inoculation method, the antibacterial substance production was confirmed by agar well diffusion method. The overnight culture of isolate A was added to the wells in Muller Hinton agar (MHA) plate. A clear zone of inhibition of 17 mm was observed against MRSA. **(Table:2)**

Table: 2 Soil isolate against MRSA by point inoculation and agar well diffusion method

S.No.	Soil isolate A	Zone of inhibition against MRSA (mm)
1	Spotted Soil isolate	17mm
2	Culture broth	17mm

Antibacterial Activity of the Protein Precipitated by Ammonium Sulphate Method

The protein of *Pseudomonas aeruginosa* was precipitated by ammonium sulphate at 40% saturation.The pellet was resuspended in phosphate buffer (pH 7). The protein of the *Pseudomonas aeruginosa* was screened for its antibacterial activity against the 3 clinical pathogens.The inhibition activity was almost similar to the inhibition activity of the supernatant, *Corynebacterium diptheriae* (19mm), *Staphylococcus aureus* (13mm), *Klebsiella pneumoniae* (10mm) *Mycobacterium smegmatis* (17mm). The comparitive analysis of the antibiotic sensitivity and antibacterial activity of the protein of *Pseudomonas aeruginosa* reveals that the protein has relatively more activity against *Staphylococcus aureus, Mycobacterium smegmatis* and *Corynebacterium diptheriae* that has become resistant to the prescribed antibiotics used. The protein has only moderate activity against *Klebsiella pneumoniae*. **(Table:3)**

Table: 3 Antibacterial Activity of *Pseudomonas aeruginosa*

S.No	Name of the organism	Zone of Inhibition(mm)	
		Supernatant	Protein
1	*MRSA*	-	-
2	*S. aureus*	19	13
3	*S. epidermidis*	-	-
4	*E. coli*	-	-
5	*P.aeruoginosa*	-	-
6	*Proteus sp.*	-	-
7	*K. pneumoniae*	10	10
8	*Shigella sp.*	-	-
9	*C. diptheriae*	19	17
10	*M. smegmatis*	17	17

SDS-PAGE

The molecular weight of the protein was determined by SDS-PAGE and was found to be 47 kDa.The antibacterial protein produced by *Pseudomonas aeroginosa* was purified by sequential precipitation and preparative chromatography process. In SDS-PAGE the protein was observed as a single band on staining the gel with comassie brilliant blue with an estimated mass of 47 kDa**.**

Peptide Mass Fingerprinting (PMF) Analysis

In that molecular mass of each spots were determined and 14 masses were observed in PMF. These mass values (peptides) were subjected for homology search in protein databases. The search results showed the 5matches among 14 query sequences. The molecular masses of matched peptides are 1168.4882, 1037.6243, 2273.0010, 2288.9946 and 2192.8508. Sequences of matched Peptides are,

M S R T V M C R K (Met- Ser-Arg-Thr-Val-Met-Cys-Arg-Lys)

L N M M N A……E D R K F L Q Q E M…… D K F L S G E D Y A……K (Leu-Asn-Met-Met-Asn-Ala……Glu-Asp-Lys-Phe-Leu-Gln-Gln-Glu-Met…..Asp-Arg-Lys-Phe-Leu-Ser-Gly-Glu-Asp-Tyr-Ala……Lys). **(Fig:1)**

Fig :1 Homology search for Peptide mass fingerprinting

Mascot Search Results

Protein View

Match to: **FETP_PSEA7** Score: **47** Expect: **3.2**
Probable Fe(2+)-trafficking protein OS=Pseudomonas aeruginosa (strain PA7) GN=PSPA7_5885 PE=3 SV=1
Nominal mass (Mr): **10675**; Calculated pI value: **6.10**
NCBI BLAST search of FETP_PSEA7 against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Pseudomonas aeruginosa PA7
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **14**
Number of mass values matched: **5**
Sequence Coverage: **40%**
Matched peptides shown in **Bold Red**
1 **MSRTVMCRK**Y HEELPGLDRP PYPGAKGEDI YNNVSRKAWD EWQKHQTMLI
51 NERR**LNMMNA EDRKFLQQEM DKFLSGEDYA K**ADGYVPPSA

Residue Number Increasing Mass Decreasing Mass
Start - End Observed Mr(expt) Mr(calc) ppm Miss Sequence
1 - 9 1168.4882 1167.4809 1167.5675 -74 2 -.MSRTVMCRK.Y
2 - 9 1037.6243 1036.6170 1036.5270 87 2 M.SRTVMCRK.Y
55 - 72 2273.0010 2271.9937 2272.0286 -15 2 R.LNMMNAEDRKFLQQEMDK.F 2 Oxidation (M)
55 - 72 2288.9946 2287.9873 2288.0235 -16 2 R.LNMMNAEDRKFLQQEMDK.F 3 Oxidation (M)
64 - 81 2192.8508 2191.8435 2192.0459 -92 2 R.KFLQQEMDKFLSGEDYAK.A Oxidation (M)
No match to: 906.4180, 995.4596, 1020.4265, 1097.9470, 1138.6760, 1153.4830, 1229.6719, 1490.6354, 2162.9126

HPTLC

The antimicrobial protein from *Pseudomonas aeruginosa* has been analyzed High Performance Thin Layer Chromatography (HPTLC) using three amino acids Lysine, Arginine and Histidine as the standards. Rf values of antifungal protein were obtained as - 0.07,0.59,0.68 and 0.80. By comparing the Rf values of Lysine, Arginine and Histidine to that of antifungal protein it is assumed that the basic amino acids Lysine, Arginine and Histidine in that protein. Further studies should be carried out to find the aminoacid sequence present in the antifungal protein. **(Fig:3) & (Table: 4)**

Fig: 2 HPTLC Spectrum Of Antimicrobial Protein of *Pseudomonas aeruginosa*

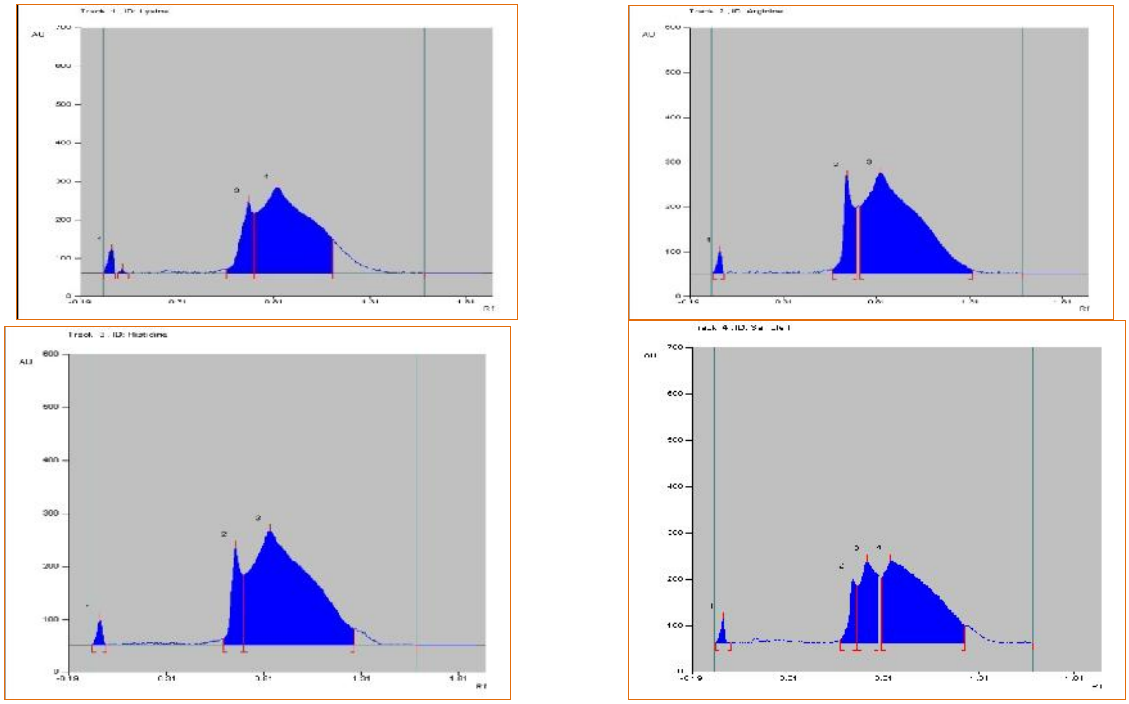

Table: 4 Comparison of Rf Values of Amino acids and Anti Bacterial Protein By HPTLC

S.No	Rf Value			
	Amino acid			Protein Sample
	Lysine	Histidine	Arginine	
1	0.00	0.61	0.58	0.59
2	0.057	0.71	0.73	0.68
3	0.71	-	-	-
4	-	-	-	0.80

Conclusion

Antimicrobial, ribosomally synthesized, cationic peptides have been recognized only recently as an important part of innate immunity. Antibacterial protein kill target cells through diverse mechanisms once they reach the target microbial membrane. Thus in current study, conclusion some inducible antibacterial proteins are present in *Pseudomonas aeruginosa*. These peptides can be used as blueprints for the design of new antimicrobial agents. Antibacterial protein based therapies will form attractive candidates as alternative antibiotic treatments, since they offer several potential advantages over currently used classes of drugs. However, further studies on purification and characterization of the intended antibiotic would be inevitable.

References

1. Aley, S.B., M. Zimmirman, E. Hetsko, Selsted and Gillin, F.D. 1994. Killing of Giardia lamblia by cryptdins and cationic neutrophil peptides. Infect Immu. 62: 5397-5403.
2. Biljana M and Havard J: Peptides and Peptidomimetics for Antimicrobial Drug Design. Pharmaceuticals 2015; 8: 366-415. Doi:10.3390/ph8030366.
3. Bevins, C., 1994. Antimicrobial peptides as agents of mucosal immunity. Ciba Found Symp. 186:250 269.
4. David R and Pascale C: How bacterial pathogens colonise their hosts and invade deeper tissues: Microbes and Infection 2015; 17(3): 173-183. http:// doi.org /10.101.6 / jmicinf. 2015.01.004.
5. Diep, D.B., and I Nes,I.F. 2002. Ribosomally synthesized antibacterial peptides in Grampositive bacteria. Curr Drugs Target. 3:107122.
6. Roberta Fontoura., Jordana Corralo, Spada, Terra Silveira, Siu Mui Tsai., Adriano Brandelli, 2008. Purification and characterization of an antimicrobial peptide produced by *Pseudomonas* sp. strain 4B. World J .Microbiol Biotechnol 25:205 213.
7. Rethnapriya E and Ravichandran S: Bioactive peptides from the grapsid crab grapsusstrigosus Sakai. Asia journal of pharmaceutical and clinical research 2014; 7(5): 305-308.
8. Guangshun W: Human Antimicrobial Peptides and Proteins. Pharmaceuticals 2014; 7(5): 545-594. Doi.10-3390/ph7050545.
9. Hanlock RE and Scott M. The role of Antimicrobial peptides in animal defenses. Proc. NatAcad Sa USA. 2000: 97:8856-8861.
10. Yeaman, M.R., and Yount, N.Y. 2003, Mechanisms of Antimicrobial peptides Action and Resistance. Pharmacol Rev. 55:27-55.

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