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Research Article

PURIFICATION OF β DEFENSIN 3 FROM CHIKEN'S BLOOD

DEVJANI BANERJEE*, TEJAS GOHIL, PRATHA BRAHMBHATT & HONEY DAVAWALA

Ashok & Rita Patel Institute of Integrated Study & Research in Biotechnology and Allied Sciences (ARIBAS), New V V Nagar, Gujarat. India. Email: devjani.chakraborty@yahoo.com

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ABSTRACT

Objective: Antimicrobial peptides are the major components of the innate immunity present in most of the organisms. Since last few decades, a considerable number of peptides, with activity against many different types of microorganisms have been found in almost all groups of animals. The present study was undertaken to isolate β defensin 3 from Chicken's Blood and to check it's efficacy against pathogenic bacterial strain mainly *Enterobacter hormaechei, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Enterococcus fecalis.*

Method: Protein was isolated from the freshly collected blood of Chicken. The crude protein was extracted using 10% TCA, followed by acid base fractionation. Further, antimicrobial activity of crude and isolated protein fractions were checked against *Enterobacter hormaechei, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Enterococcus fecalis.* The protein fractions having antimicrobial activity was subjected to PAGE. The separated fractions were further processed for mass spectroscopy and their mass was deduced by the spectral data analysis.

Results: The antimicrobial protein which was isolated from chicken blood shows partial sequence homology with respect to Gallinacin 3, a type of β defensin AMP, found in chicken. The molecular weight of this peptide was found to be 14,683 Da as per the results of LC-MS/MS. The partial sequence containing 13 amino acids is deduced by the mass spectra obtained.

Conclusions: From the present study it was concluded that the isolated antimicrobial peptide is Gallinacin 3 (β defensin) having molecular weight 14,683 Da and was able to resist the growth of few antibiotic sensitive and resistant pathogenic bacterial strain.

Keywords: Antimicrobial proteins (AMPs), Blood, Chicken, Mass spectroscopy.

INTRODUCTION

Antimicrobial peptides (AMPs) have been demonstrated to kill Gram negative and Gram positive bacteria (including strains that are resistant to conventional antibiotics), mycobacteria, enveloped viruses, fungi and even transformed or cancerous cells. Unlike the majority of conventional antibiotics it appears as though antimicrobial peptides may also have the ability to enhance immunity by functioning as immunomodulators [1]. In most of the mammals, antimicrobial peptides belong to 2 super families, Defensin and Cathelicidin. These both antimicrobial peptides are constitutively or inducibly expressed in blood cells and epithelial cells lining of the mucosal surfaces of the respiratory, digestive, and urogenital tracts [2]. Defensins peptides belongs to the family of evolutionary related vertebrate antimicrobial peptides with a characteristic β sheet rich fold and having framework of 6 disulphide-linked cysteine residues. Due to the formation of β-sheet secondary structure it may increases the amphipathicity of the peptides and enables them to act specifically with their targets. They were named 'Defensin' based upon their association with host defense settings. The two main defensin subfamilies are α and β defensins, which have different length of peptide segments between the 6 cysteines and the paring of the cysteines that are connected by disulphide bonds. Both α and β defensins consist of a triple stranded β-sheet with a distinctive 'defensin' fold [3]. The whole genome of chicken encodes total of 13 different β-defensins. These chicken β-defensin genes, named as galliancin 1-13, are clustered densely within a 86 kb distance on chromosome 3q3.5-q3.7.The deduced peptides vary from 63 to 104 amino acid residues in length sharing the characteristic defensin motif [4].

The present study was undertaken to isolate β defensin 3 from Chicken's Blood and to check it's efficacy against antibiotic pathogenic bacterial strain mainly *Enterobacter hormaechei, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Enterococcus fecalis.*

MATERIALS AND METHODS

Collection of Sample

Fresh blood sample was collected in a sterile tube containing anti coagulant (EDTA), from a registered slaughter house of Anand district, Gujarat, India. The sample was immediately carried to the laboratory in an ice box in aseptic condition for the further processing.

Extraction of Crude Protein

Blood sample was centrifuged at 3000 rpm for 10 min and buffy coat was obtained containing white blood cells. The buffy coat was carefully transferred to the fresh appendorf for protein extraction. Protein extraction was carried using TCA (10%), followed by acid - base fractionation method [5], lyophilized and stored at -20 ^{0}C. Concentration of protein was estimated at 280nm using nanodrop (Thermo Scientific). The total protein extracted from Blood was also subjected to PAGE. The gel was prepared following the standard protocol [6].

Purification of Crude protein extract

Crude protein was purified using cation exchange column chromatography. Crude protein extract was dissolved in 20 mM ammonium acetate and loaded on to Macro-prep High S- Strong Cation exchanger column. Elution was performed using linear gradient of ammonium acetate (100-1500 mM) and eluted fractions were lyophilized.

Antimicrobial activity of protein fractions

Antimicrobial activity of each fraction was determined by well diffusion method using N-agar. Zone of inhibition (ZOI) were obtained by using agar well diffusion method standardize by National Committee for Clinical Laboratory Standards [7]. Activity of total and isolated fractions along with control antibiotic was checked against all of the five microorganisms. Control antibiotic used was Penicillin (30 µg/ml). Bacteria were obtained from Microbial Type Culture Collection, Chandigarh, India. Microorganisms used were *Escherichia coli* (MTCC no. 443), *Pseudomonas aeruginosa* (MTCC no.741), *Bacillus subtilis* (MTCC no. 2423), *Enterococcus fecalis* (MTCC no. 439), *Enterobacter hormaechei* (Clinical isolate). The column eluted fraction which showed the significant antimicrobial activity was further processed for Acid Urea PAGE and mass spectroscopy for molecular weight deduction using LCQ Fleet Ion Trap Mass Spectrometer. The data obtained by mass spectrometer was analyzed using Bioworks Software.

Amplification of β defensin 3 gene

DNA isolation from chicken blood: For DNA isolation from chicken blood Hi PurA™Mammalian genomic DNA purification kit (Hi Media) was used.

Table 1: Preparation of reaction mixture

Components	Volume (25 µl reaction)
2X PCR Taq Polymerase	12.5 µl
Blood DNA	4 µl
Forward Primer(gf)	3 µl
Reverse Primer(gr)	3 µl
Nuclease Free Water	2.5 µl

Table 2: Conditions for PCR

Steps	Temperature	Time	No. of cycles
Initial denaturation	94^{0}C	04:00	1
Denaturation	93^{0}C	01:00	40
Annealing	60^{0}C	00:45	40
Extension	72^{0}C	01:00	40
Final extension	72^{0}C	10:00	1
Hold	4^{0}C	04:00	_

Quantification and Quality assessment of DNA: The DNA stock samples was quantified using Nanodrop spectrophotometer at 260nm-280nm using the convention that one absorbance unit at 260nm wavelength equals 50µg DNA per ml. Quality and purity of DNA were checked by Agarose gel electrophoresis [8]. *Polymerase Chain Reaction*: PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube in Eppendorf Thermal Cycler. Composition of reaction mixture for PCR is given in Table 1. The PCR protocol designed for 40 cycles for the primers (Forward primer: ATGCGCATTGTGTATCTGCT and Reverse primer: TCAGCGCATCCACTTCATAC) used is given in Table 2. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

RESULT AND DISCUSSION

Crude Protein Fraction

The isolated crude protein fraction from blood was subjected to 1 D SDS PAGE (Fig 1). In all, four different fractions were obtained: Fraction 1: Acidic pellet fraction (Lane 1), containing insoluble basic protein. Fraction 2: Acidic supernatant fraction (Lane 2), containing soluble basic protein. Fraction 3: Basic supernatant fraction (Lane 3), containing soluble acidic protein. Fraction 4: Basic pellet fraction (Lane 4), containing insoluble acidic protein.

All the four fractions were subjected to antimicrobial activity. Around 30 µg/ ml concentration of protein fraction was used to study antimicrobial activity. Out of four maximum antimicrobial activities was observed in fraction 2. As fraction 2 is believed to contain soluble basic protein, it was further subjected to Acid Urea PAGE for the isolation of low molecular weight cationic protein (Fig 2). Further, fraction 2 was subjected to Column chromatography (Cationic). In all, 15 fractions were collected; lyophilized and further all of them were subjected to antimicrobial activity. Out of 15, 6 fractions showed maximum activity against *E.coli*. Whereas, fraction 12 showed antimicrobial activity against all the five bacteria: *Enterobacterhormaechei, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Enterococcus faecalis* (Table 3).

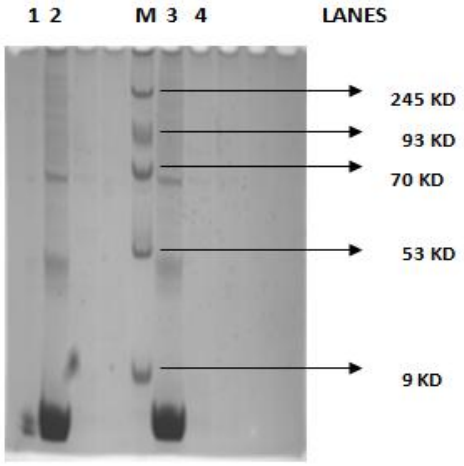

Fig 1: 1- D SDS PAGE of Protein Fraction

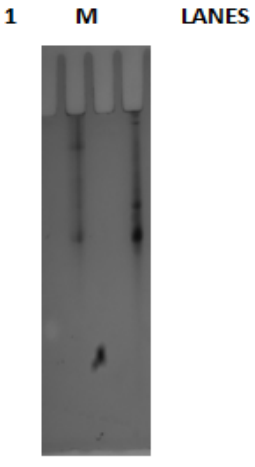

Fig 2: Urea page of fraction 2acidic: supernatant fraction

Later on fraction 12, which gave efficient antimicrobial activity, was further subjected to SDS page, the obtained band revealed that the isolated protein fraction was having peptide of 14.6 KD (Fig 3).

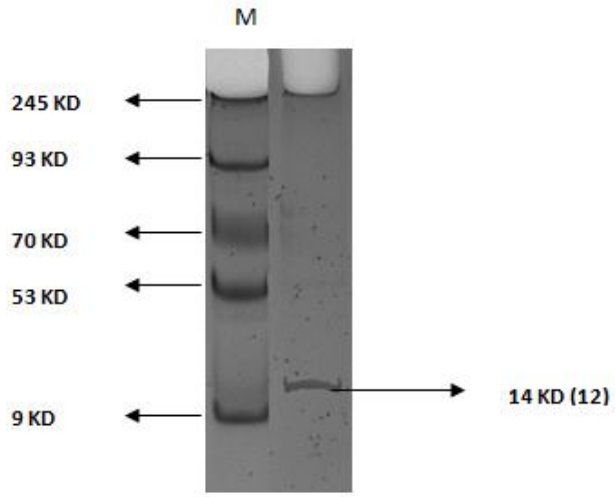

Fig 3: 1- D SDS PAGE of isolated fraction 12

Table 3: Zone of Inhibition of crude and Column eluted fractions

Microorganism	Zone of Inhibition of crude fraction	Zone of inhibition of column eluted fraction
Escherichia coli	++	++
Pseudomonas aeruginosa	++	+
Bacillus subtilis	++	+
Enterococcus fecalis	-	-
Enterobacter hormaechei	++	+

No ZOI (-); Less ZOI (+); Clear ZOI (++)

The peptide fraction was also subjected to LC MS – MS. The accurate molecular weight of isolated peptide was deduced by Applied Biowork system, which was found to be 14,683 Da. The graph of the same has been shown below along with the m/z ratio. Later on partial sequence of amino acid was deduced (Fig 4 and 5). The homology was matched using NCBI protein BLAST.

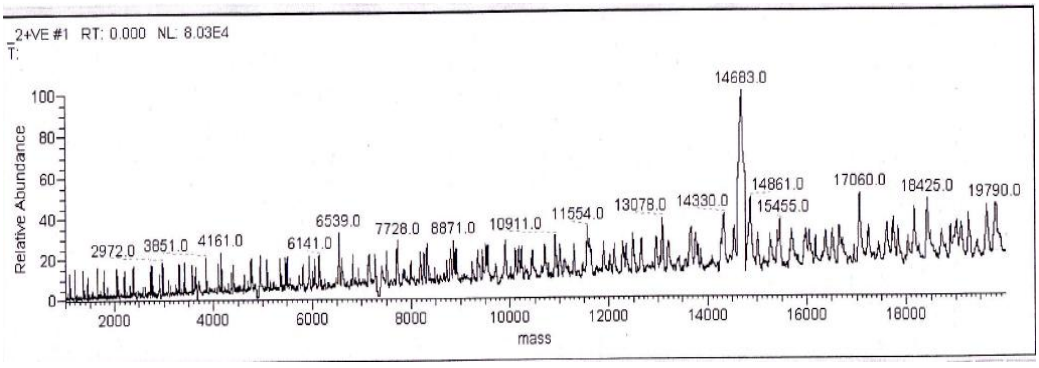

Fig. 4: Molecular weight of the isolated peptide (Applied Biowork system)

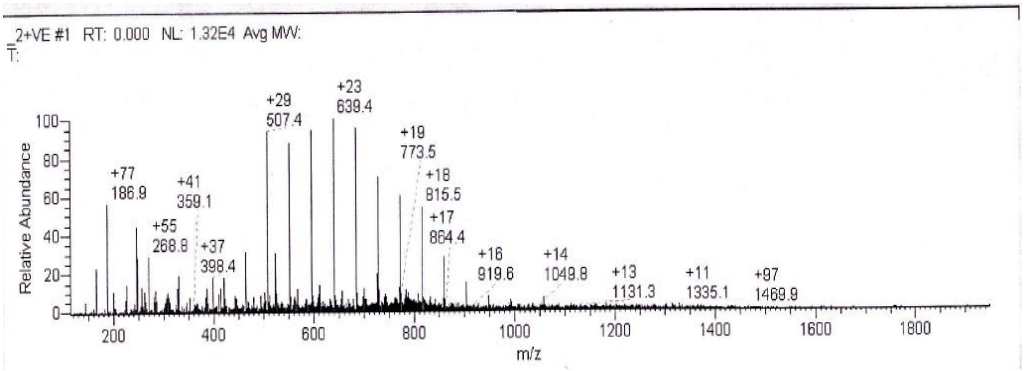

Fig. 5: The m/z ratio of the obtained peptide fraction 12 (Applied Biowork system) Analysis by Mass Spectra

Fig. 6: DNA Bands

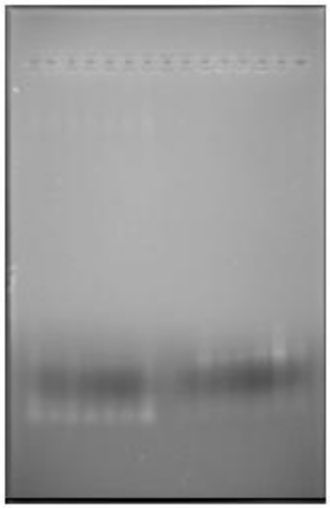

Fig. 7: PCR Bands

The above mentioned graph gives us an idea regarding the partial amino acid sequence which is LFCRVGSCKFCPH. The obtained partial sequence showed 83% homology with the β Defensin -3 of *Gallus gallus,* also known as Gallinacin.

FCRVGSCKFCPH – Deduced partial sequence of β defensin

FCRVGSC_F_ PH – Sequence in NCBI databank

The gene of same was also amplified (Fig 6 – 7) and run on 2% Agarose gel with 1kb marker.

Gallinacins are β-sheet cationic peptides having conserved cysteine rich defensin motif, which have antimicrobial activity against wide range of microorganisms. In the present study the deduced amino acid sequence shows partial similarity with gallinacin i.e. defensin present in chicken blood. It is reported that gallinacin have potent antimicrobial activity against gram negative as well as gram positive bacteria. In total 13 different gallinacin genes have been identified i.e. from Gal 1 to Gal 13 [9]. They are present as part of innate immune system ti fight against microbial infection. Expression of gallinacin is different in various organs i.e. Gal 1 to Gal 7 are mostly expressed in bone marrow, tongue, trachea and bursa of fabricus. Gal 8-13 are expressed in liver, kidney, male and female reproductive tissue [10].

Some variations in the sequence of amino acid determine their specificity to various microbial targets in different microorganisms. The partial sequence resulted had some difference with respect to the sequence of NCBI which may be due to intra species variation which may affect the structure motif including disulfide bond within the structure.

CONCLUSION

The antimicrobial protein which was isolated from chicken blood shows partial sequence homology with respect to gallinacin 3 which is one of the gallinacin genes from Gal 1 to Gal 13 gallinacins in chicken which all have different expression rate. The gallinacin is rich in cystein and arginine residues and can be purified using cationic exchange column chromatography. The molecular weight of this peptide is almost 14,683 Da as per the results of LC-MS/MS. The partial sequence containing 13 amino acids is deduced by the mass spectra obtained. The capability of this column eluted fraction named as gallinacin as an antimicrobial agent was demonstrated by well diffusion method, by evaluating the effect of the peptide on bacterial growth. The action of the potent gallinacin was found to be bactericidal.

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