Int.J.Curr.Microbiol.App.Sci (2018) *7*(7): 1643-1649

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 07 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.707.192

Prediction, Synthesis and Evaluation of an Antimicrobial Peptide from Goat Vitronectin (Vn)

Prasanta Kumar Koustasa Mishra*, Aditya Agrawal, Anil Gattani and Yapu Nijo

Division of Biochemistry, Indian Veterinary Research Institute, Bareilly, UP-243122, India

**Corresponding author*

Keywords

Antimicrobial peptide, Vitronectin, *Escherichia coli* DH5-α, MBC,

Article Info

Accepted:
10 June 2018
Available Online:
10 July 2018

A B S T R A C T

Antimicrobial resistance is an emerging challenge for health personnel and researchers. In such scenario, alternative strategies targeting novel ways to kill or inhibit growth of microbes are need of the hour. Antimicrobial peptide can be a promising candidate to deal with this crisis. Extracellular matrix protein vitronectin (Vn) is recently being established as a bactericidal agent against certain microbes. Caprine Vn has got few structural alterations as compare to human Vn. Here we have applied bioinformatics approaches to screen as well as characterise regions of goat Vn having antimicrobial peptide followed by synthesis of a predicted peptide and evaluation of the antimicrobial effect of the same against *Escherichia coli* DH5-α cells. The minimum bactericidal concentration (MBC) was determined by resazurin reduction test (RRT) and found to be 250 µg /10^7 *E. coli* cells. The peptide gave visible inhibition against the test organism.

Introduction

Antimicrobial peptides (AMPs) can be defined as a large number of small proteins (peptides) that can kill or inhibit the growth of various microbes. The peptides can be of varied length starting from 6 to 50 amino acids (Zasloff *et al*., 2002) or maximum upto 100 residues (Xiao *et al*., 2015). In case of mammals, the AMPs can be categorized under three classes as defensin, cathelicidines and histatin (Smet and Contreras, 2005). AMPs can be divided into various classes based on their structure, function and mode of action. Structure based classification categorizes AMPs as α helix forming, β sheet forming, loop former and extended conformations (Huang *et al*., 2010). Functionally, they can be again classified as anti-viral, anti-bacterial, anti-fungal and anti-parasitic. According to mechanism of action, they are grouped under two categories: (i) membrane acting and (ii) non-membrane acting. In mammals, the AMPs are distributed throughout different cell types and tissues such as neutrophils, epithelial cells, mesenchymal tissues, articular cartilage and mucosal epithelial cells (Zasloff *et al*., 2002). Recent evidences suggest extracellular matrix protein vitronectin (Vn) is capable of killing bacteria. The HBD-3 of Vn has structural similarity to HBDs of AMP like LL-37 and α-defensin (Schimdtchen *et al*., 2001). The

Cardin motif in HBD-3 of Vn was bactericidal towards *Enterococcus faecalis*. Though the mechanism of Vn mediated bacterial killing is not clear (Singh *et al*., 2011) but at low salt concentration the killing effect of HBD-3 was higher towards *E. coli*, *Candida albicans* and *Proteus mirabilis.* In the present study we have predicted the stretch(es) of goat Vn which can act as potential AMP.

Materials and Methods

Retrieval of caprine Vn sequence and prediction of AMP

The caprine Vn sequence is available in uniProt with uniProt ID Q3LRQ1 (Q3LRQ1_CAPHI). The sequence was retrieved and was analysed in an online based free server AMPA(http://tcoffee.crg.cat/apps/ampa/do). Default parameters provided by the server were used for identification of potential regions of Vn with AMP activity (Fig. 1).

Determination of biochemical properties of predicted peptides

The outputs of AMPA server were further analysed in Swiss Prot's proto-param module available online at free accession (https://web.expasy.org/cgi-bin/protparam/ protparam). The overall hydropathicity (GRAVY) values were compared and calculated (Table 1).

Synthesis of selected peptide

The chosen peptide on the basis of hydropathicity was synthesized following 9-fluorenylmethoxycarbonyl (fMoc) chemistry described by Merrifield (Merrifield, 1963). Coupling reagents 1-hydroxy benztriazole (HoBT) was purchased from Orpegen pharma (Germany), 2-(1H-benzotriazolyl-1-yl)-1,1,3,3-tetramethlyuronium hexafluoro phosphate (HBTU) was procured from GL Biochem (Shanghai, China) and Nova Biochem (Switzerland).The peptide was finally resuspended in HPLC grade water at a final concentration of 25 mg/ml.

Dot blot analysis of the peptide

The peptide was confirmed by immobilising 5µg of peptide on a nitrocellulose membrane followed by incubation with rabbit anti-goat Vn at 1:250 dilutions in PBST and using horse reddish peroxidase(HRPO) conjugated goat anti-rabbit antibody (GeNei, Bangalore, India) at 1:500 dilutions. The blot was developed by HRPO catalysed oxidation of diamino benzidine (DAB)(Sigma-Aldrich) as substrate. Vn, N- and C-domain of Vn were also blotted to the membrane and served as positive control whereas HPLC grade water served negative control (Fig. 2).

Circular dichroism(CD) spectroscopy

200 µl of peptide (250 µg/ml) was subjected to spectropolarimetric analysis in Jasco-180 CD spectrophotometer available at central instrumentation facility (CIF), IVRI. The data recorded were average of four scans performed from 190-310 nm. The response is presented as ellipticity in millidegree (Fig. 3). Structural details about the secondary structure of peptide were outputs of Jasco-720 software (Table 2).

Resazurin reduction test (RRT)

Reduction of resazurin to resorufin by live bacteria was proposed by Moyer and Campbell in 1963. Here we have used RRT to assess the minimum bactericidal concentration (MBC) of the peptide. *E coli* DH5-α cells were used as test organisms for the assay.

Briefly, to a 96 well polystyrene microtitre plate (Nunc MaxiSorp™ flat-bottom, Thermo

Fischer scientific)100 µl of freshly grown *E.coli* cells (OD_{600}-0.1)were transferred in triplicates as three different groups .

The peptide was diluted to three different concentrations in normal saline and 100 µl of each dilutions were added to the wells so that the groups received 250, 125 and 62.5 µg of peptide respectively. The plate was incubated at 37° C /1 hour following a brief period of exposure to 4° C in order to avoid endocytosis of the treated peptide. 30 µl of 0.1% resazurin was added to all wells and incubated further for 2 hours at 37° C. Change in colour of the resazurin solution was visually evaluated (Fig. 4).

Evaluation of antimicrobial effect

Overnight grown *E. coli* DH5- cells was sub-cultured at 1:100 and was grown till it attended OD_{600} of 0.1. 100 µl of culture was removed and was separated from the media by centrifugation at 5000 rpm/5 minutes. The pellet was resuspended with the test peptide so that the pellet received 250 µg of total peptide. The bacterial suspension was incubated for an hour at 37° C as described in the RRT test. The treated peptide solution was isolated from the bacteria by centrifuging the suspension at 5000 rpm/5 mins.

The pellet was washed twice with normal saline and finally resuspended in 1 ml of NS. Tenfold serial dilutions of the treated bacteria were prepared in NS and 10 µl from dilutions 10^{-3},$10^{-4,}10^{-5}$,10^{-6} were spotted onto a Luria Bertani (LB) agar (Sisco research laboratory, Mumbai) plate in quadruplicates. Ampicillin (25 µg) and NS served as positive and negative control respectively. Controls were prepared similarly as per the peptide and were spotted to two different LB plates. All the three plates were incubated for 16 hours at 37° C and the colonies were counted (Fig. 5).

Results and Discussion

In caprine species, C-domain of Vn is responsible for binding to *Staphylococcus aureus* (Mahwar and Joshi, 2008) whereas N domain of it showed growth inhibition in *E. coli* (Rao *et al*., 2017).

The 444 amino acid sequence of goat Vn was retrieved from uniProt server and was analysed in AMPA module. Upon analysis the server identified 5 potential stretches in Vn which might show anti-microbial effect (Fig. 1). Though the propensity of ERVYFFKGNHYW peptide was more to be an antimicrobial agent but as per grand average of hydropathicity (GRAVY) value, peptide MTKSARRHRKRYRSLRSRGRGR GRARSQ and PYRRFRSTWLSW were selected initially. But stability of *in vitro* synthesized peptides of more than 20 amino acids is a matter of concern (Gentilucci *et al.*, 2010). So the peptide with sequence PYRRFRSTWLSW was opted for synthesis. Dot blot analysis confirmed the synthesized peptide as a representative stretch of goat Vn. The predicted 3D structure of the peptide (http://bioserv.rpbs.univ-paris-diderot.fr/ services/PEP-FOLD/) is of helical contour (Fig. 6). But in contrast to that CD spectroscopy suggested presence of β strand as high as 75.7% and 24.3% of random coils which might be due to the cross linked polymeric form of the synthesized peptide. Further, the helical contour may help the peptide in better membrane permeability. Some AMPs like protegrin, maginin, cyclic and coiled indolicidin do belong to α helix forming group whereas defensins are grouped under β sheet forming proteins (Huang *et al*., 2010). The potential of RRT has been already established in determining MIC of antimicrobial agents.

Table.1 Biochemical properties of the predicted peptides

Peptide sequence	Number of amino acids	Molecular weight in Da	Theoretical iso electric pH	Grand average of hydropathicity (GRAVY)
FNCQGKTYLF KGSQYW	16	1970.23	9.11	-0.762
ERVYFFKGNHYW	12	1645.84	8.6	-1.058
MTKSARRHRKRYRSLRS RGRGRGRARSQ	28	3427.96	12.65	-2.182
PYRRFRSTWLSW	12	1654.89	11.71	-1.158
RVNLRTRRVDSV	12	1470.70	12.00	-0.842

Table.2 Secondary structure content of the peptide

Structure type	Percentage
Helix	0
Beta	75.7
Turn	0
Random	24.3
Total	**100**

Fig.1 The output of AMPA server against query made for caprine Vn

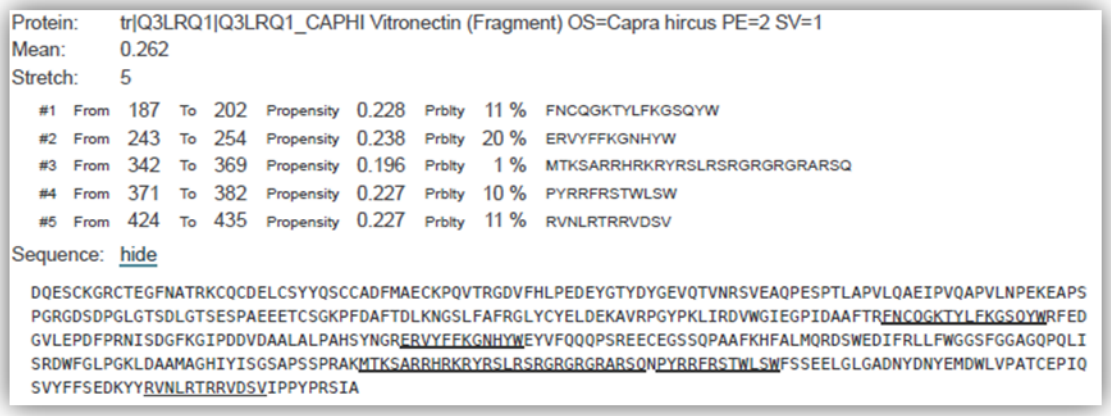


Fig.2 Dot blot showing positive reaction of the peptide with anti-goat Vn

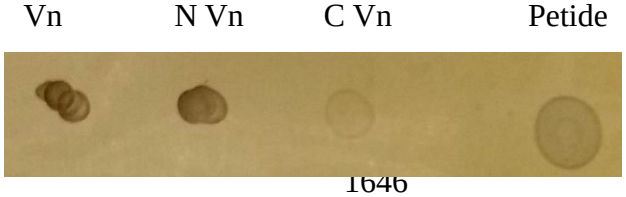


Fig.3 Circular dichroism spectroscopy analysis of the peptide

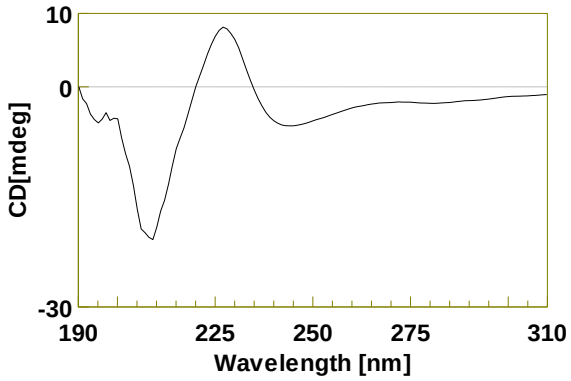


Fig.4 RRT to determine minimum bactericidal concentration of the peptide

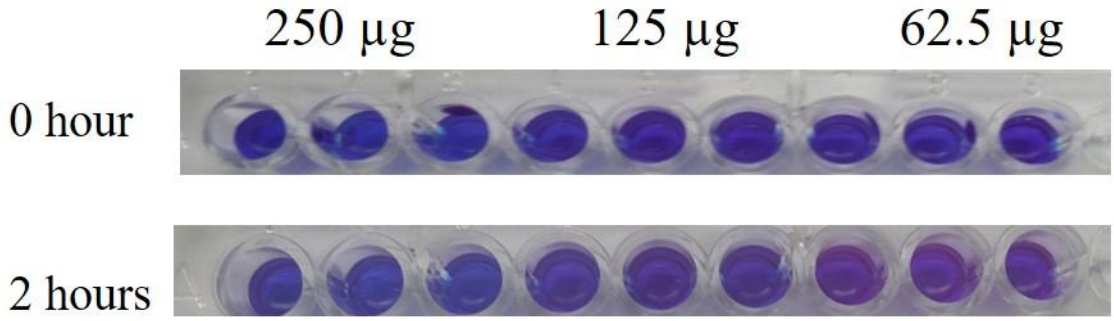


Fig.5 Drop plate method to evaluate efficacy of the peptide

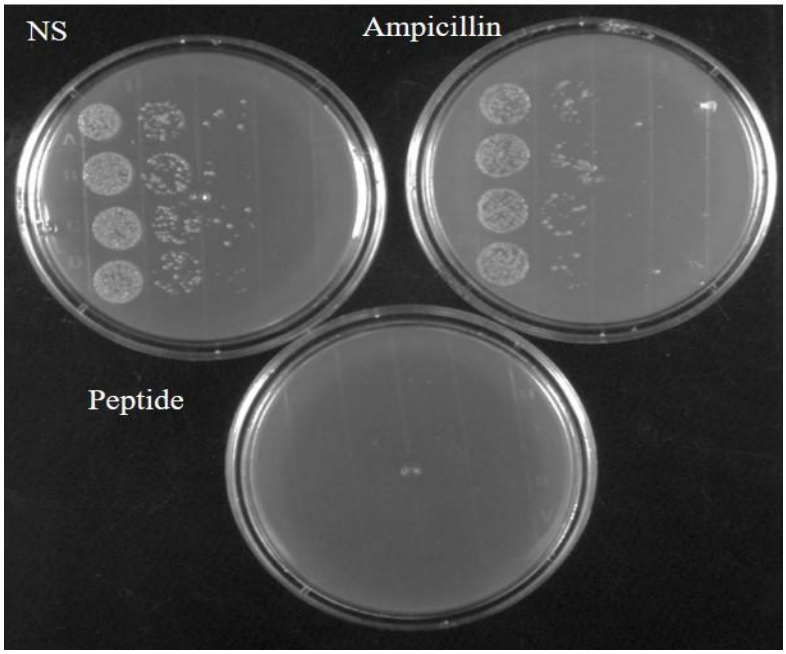


Fig.6 Predicted 3D structure of the peptide
(a) Ball and stick model (b) Cartoon structure

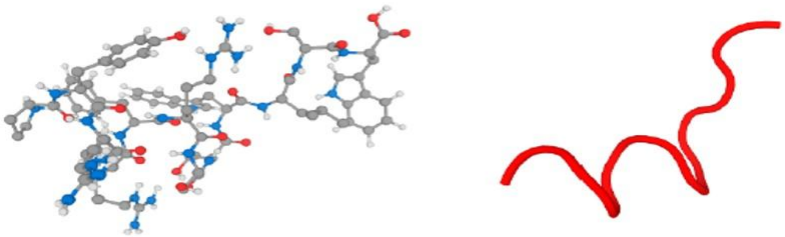
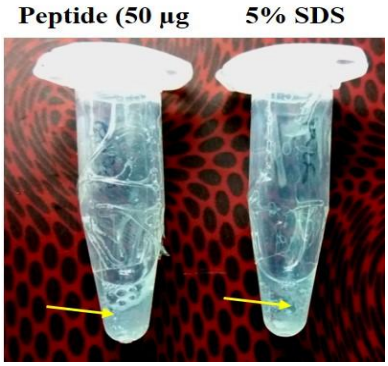


Fig.7 Bacterial lysis visible by caseous clot formation (Indicated by arrow mark)



The reduction of resazurin by various dehydrogenases in live bacteria results in formation of pinkish coloured resorufin. But dead bacteria are unable to bring such changes in the reaction medium. The wells with 62.5 µg of peptide showed pink colour after incubation with resazurin solution whereas the 125 µg wells were slightly purplish. But the colour was relatively unchanged in 250 µg peptide treated wells. The MBC of the peptide was taken as 250 µg/10^7 DH5-α cells and used in subsequent study. The drop plate method indicated a strong antimicrobial effect of the peptide as there was no growth even at 10^{-3} dilutions as compare to ampicillin and NS which had two and eight colonies respectively at 10^{-5} dilutions. Decreasing the concentration of peptide to 50 µg as a total dose to the same bacterial mass resulted cloudy clots which was comparable to 5 % SDS treated bacterial sample (Fig. 7). Further exploration with higher numbers of replicates and different microbes can help in establishing the anti-microbial potential of this peptide.

The conclusions and future directions are as follows:

Finding newer antimicrobial agents can help in combating the antibiotics resistance strains of microbes. AMP is definitely a potent weapon against such organisms. The peptide identified in the study was found to have potent bactericidal efficacy. Mass spectrometric analysis can help in detailed structural composition of the peptide and testing the peptide against structurally different microbes can explore its range as well as mechanism of action.

Acknowledgments

The authors are highly thankful to Director, IVRI and Dr Paritosh Joshi, Principal scientist, Division of Biochemistry for providing necessary funds and facilities. Mishra PKK is supported by ICMR-SRF fellowship, Nijo Y and K.C.Amith by ICAR-JRF.

References

Gentilucci, L., De Marco, R., and Cerisoli, L. (2010). Chemical modifications designed to improve peptide stability: incorporation of non-natural amino acids, pseudo-peptide bonds, and cyclization. *Current pharmaceutical design*, *16*(28), 3185-3203.

Huang, Y., Huang, J. and Chen, Y. 2010. Alpha-helical cationic antimicrobial peptides: relationships of structure and function. Protein and cell. 1: 143-152.

Mahawar, M., and Joshi, P. (2008). Goat vitronectin: characterization and binding to Staphylococcus aureus. *Comparative Biochem. Physio. Part B: Biochem. Mol.Biol.*, *149*(3), 410-418.

Marc Torrent, Paolo Di Tommaso, David Pulido, M. Victòria Nogués, Cedric Notredame, Ester Boix, David Andreu; AMPA: an automated web server for prediction of protein antimicrobial regions, *Bioinformatics*, Volume 28, Issue 1, 1 January 2012, Pages 130–131,

Memfield RB. 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. JAm Chem Soc 852149-2154.

Moyer, R. H., and Campbell, J. J. R. (1963). Mechanism of resazurin reduction in milk of low bacterial content. *Journal of Dairy Science*, *46*(9), 897-906.

Prasada, R. T., Lakshmi, P. T., Parvathy, R., Murugavel, S., Karuna, D., and Paritosh, J. (2017). Identification of second arginine- glycine- aspartic acid motif of ovine vitronectin as the complement C9 binding site and its implication in bacterial infection. *Microbiol immunol.*, *61*(2), 75-84.

Schmidtchen, A., Frick, I. M. and BjoÈrck, L. 2001. Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial α- defensin. Mol. Microbial. 39: 708-713.

Singh, B., Su, Y. C. and Riesbeck, K. 2011. Vitronectin in host pathogen interactions and antimicrobial therapeutic applications. Centr. Eur. J. Biol. 6: 973-980.

Smet, K. and Contreras, R. 2005. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol. Lett. 27: 1337-1347.

Xiao, H., Shao, F., Wu, M., Ren, W., Xiong, X., Tan, B. and Yin, Y. 2015. The application of antimicrobial peptides as growth and health promoters for swine. J. Ani Sci. Biotech. 6: 19-26.

Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. Nature. *415*: 389-395.

How to cite this article:

Prasanta Kumar Koustasa Mishra, Aditya Agrawal, Anil Gattani, Yapu Nijo. 2018. Prediction, Synthesis and Evaluation of an Antimicrobial Peptide from Goat Vitronectin (Vn). *Int.J.Curr.Microbiol.App.Sci.* 7(07): 1643-1649. doi: https://doi.org/10.20546/ijcmas.2018.707.192