SENRA Academic Publishers, Burnaby, British Columbia
Vol. 4, No. 1, pp. 1027-1031, 2010
ISSN: 1715-9997

Canadian Journal of pure&applied sciences

HETEROLOGOUS PRODUCTION OF SYNTHETIC CATIONIC ANTIMICROBIAL PEPTIDE IN NOVEL OSMOTICALLY INDUCIBLE *E.COLI GJ1158*

*K Haritha[1], P Udayasri, J Madhavi, KK Pulicherla[2] and KRS Sambasiva Rao
[1]Center for Biotechnology, Acharya Nagarjuna University, Guntur-522510
[2]Department of Biotechnology, R.V.R. and J.C College of Engineering, Guntur, India

ABSTRACT

Cationic antimicrobial peptides are the upcoming therapeutic molecules as alternative drugs to the antibiotics. These peptides have a good scope in current antibiotic research. In the present study *E. coli* strain GJ1158 host was chosen for the expression of gene for Insilco designed synthetic peptide, Using Modified M9 medium. Various trails were carried out to optimize the recombinant peptide production in modified M9 medium by following the Placket Burman model. The optimal media was chosen for further expression studies and the expressed antimicrobial peptide was purified using Immobilized Metal Affinity Chromatography (IMAC) system. The product was visualized on 16% Tricine SDS-PAGE. It was identified that 30% of the bacterial proteins as the recombinant protein. The expressed antimicrobial peptide was purified using Immobilized Metal Affinity Chromatography (IMAC) system. The antimicrobial activity of purified peptide using Top agar assay showed that the recombinant antimicrobial peptide has high antibacterial activity against both Gram-positive and -negative bacteria.

Keywords: Cationc synthetic peptide, immobilized metal affinity chromatography system, placket burman model, antibacterial activity.

INTRODUCTION

Many diseases are becoming difficult to treat because of the emergence of drug-resistant organisms, including bacteria, fungi, viruses and parasites (Anthony and Fauci, 1999). The pharmaceutical industry has continuously met this need by modifying existing antibiotics and developing newer antibiotics in a timely fashion. However, the rapid emergence of resistance is even a greater problem for life-threatening viral infections. Cationic antimicrobial peptides are the upcoming therapeutic molecules as alternative drugs to the antibiotics, they are widespread in nature, occur in animals, plants and bacterial species, and represent a major defence mechanism for bacteria, plants and lower animals. Recent reports have suggested that their antibiosis against bacteria is due to their positive charge and ability to adopt amphipathic conformations. Natural CPs show considerable sequence diversity, but share certain common structural features, including a high of basic amino acid content and the dispersion of hydrophobic and hydrophilic residues, which gives the peptides their amphipathic character under hydrophobic conditions (Merrifield *et al*., 1999). Even though the natural CP's so far used as a potent alternative to antibiotics they are undesirable due to fast proteolysis after application; a poor absorption due to their hydrophilicity. For these reasons there is a strong interest to develop synthetic substances that mimic the properties of AMPs, but are not as damageable as AMPs. On the other hand commercial production of natural or synthetic peptides at large scale is very expensive because it includes various extractions and purification strategies. Hence Recombinant DNA technology has been used to clone natural or synthetic genes in bacteria, fungi, plants, or yeast cells for increased production of many eukaryotic and prokaryotic proteins. Many different host/vector systems have been used to produce antimicrobial peptides through recombinant DNA technology. *E.coli* has been utilized most often due to the low cost of fermentation compared to mammalian cells, and its ability to produce inclusion bodies, which aid in the purification process (Haught *et al*., 1998). In this paper, we report the production and optimization of M9 media and purification of a synthetic antimicrobial peptide in prokaryotic strain GJ 1158 of *E. coli.*

*Corresponding author email: harithabio@gmail.com

MATERIALS AND METHODS

Strains and Plasmids

Pseudomonas aeruginosa (MTCC 424), Klebsiella pneumonia (MTCC 2405) and Streptococcus species (MTCC 389), E. coli (MTCC 1687), were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. *E. coli* GJ1158 was procured from Genei Bangalore, India. Plasmid Cloning Vector pRSET-A was procured from Invitrogen.

Culture Media

Luria-Bertani (LB) medium was used in seed culture. The expression studies used Glucose yeast extract medium [GYE: yeast extract (5g/l), NH4Cl (1g/l), NaCl (0.5g/l), KH2PO4 (3g/l), K2HPO4 (6g/l), 1M MgSO4 (2ml/l), Trace element mix (1ml/l)]. 2% D-Glucose was supplemented to the media that required Glucose. Modified M9 media (MM9) was used for the production of recombinant antimicrobial peptide from *E. coli* expression host GJ1158. The medium was prepared as per the given composition. Na_2HPO_4(6 g/l), KH_2PO_4(3 g/l), NH_4Cl(2 g/l), Yeast Extract(4 g/l), Glucose(4 g/l), 1M $CaCl_2$-0.1ml,1M $MgSO_4$-1ml, Trace Metal Mix-1ml, pH-7.4-7.5

Analysis of antimicrobial peptide expression

For the expression of gene for *insilico* designed synthetic cationic antimicrobial peptide, it is required to transform the rDNA (synthetic gene-pRSET-A*)* in to the expression host system. In the present study *E. coli* strain GJ1158 host was chosen for the expression of synthetic gene. The rDNA was transformed into the competent bacterial system by heat shock method. Recombinant synthetic gene bearing GJ1158 bacterial expression host was grown in GYE and MM9 media till the OD_{600} reaches 1.0. GJ1158 culture grown in various media, was induced with NaCl (50 mM, 100 mM, 200 mM, and 500 mM) respectively. Induction was carried out at 37^{o}C for various time intervals (3, 4, and 5 hours). 2ml of bacterial culture was taken and centrifuged at 7000rpm for 10 minutes to harvest the bacterial cells. The pellet was suspended in 100µl Phosphate Buffered Saline. To this, 100µl of 2X Sample Solubilizing Buffer was added and used for the protein analysis.

Optimization studies for recombinant protein production from GJ1158

Modified M9 medium was chosen for the production of recombinant antimicrobial peptide from GJ1158.The medium was chosen with the literature support of Janardhan *et al*. (2007). In addition to the reported modified M9 medium tryptone was also added to the medium. The media was prepared with the composition described earlier. To optimize the media and to find out the key regulators in the growth and production of the desired protein molecules, 9 sets of media were designed by following the Placket Burman model. All 9 sets of media were inoculated with 5% of freshly grown culture and at regular time intervals of time, samples were collected to analyze the parameters viz., OD, dry cell weight, substrate reduction, and product formation.

Purification of the expressed peptide

The cloning vector pRSET-A used in the present study has given the opportunity to easily purify the expressed protein by containing the 6X *His* tag at the N-terminal end. The purification strategy is based on the natural affinity of Histidine towards the nickel ion. In the present study, Immobilized metal affinity chromatography (IMAC) system containing Nickel-Sepharose column provided by Bangalore Genei was used to purify the desired expressed antimicrobial peptide. With this system, by inducing the gene with NaCl can give the product of fusion protein containing desired antimicrobial peptide with 6X His tag at the N-terminal end. Bacterial lysate was prepared using either frozen or fresh bacterial pellet from 100ml of culture in 10ml of equilibration buffer by sonication. The lysate was centrifuged at 14,000rpm for 30 minutes to get rid of cell debris. Nickel CL Agarose column was equilibrated with 10ml of 1x equilibration buffer by removing the cap at the top of the column and pour off the storage solution. The clarified lysate was applied from step 2 of the column to allow the flow of the sample completely in to the gel bed. Then the column was washed with 1 ml of 1x equilibration buffer. Again wash the column with 20ml of 1x equilibration buffer. The column was eluted with 10ml of 1x elution buffer to collect the samples as fractions. Firstly 0.5ml was collected as the first fraction and the rest was collected as 1ml fraction. The eluted His tag protein was now analyzed by means of tricine SDS-PAGE.

Quantification of the purified peptide

The purified peptide was quantified by the standard Bradford's method to identify right fraction of the eluate containing the peptide and also to determine the expression level of the cloned antimicrobial peptide. The experiment was carried out as per the given protocol.

Antimicrobial assays

Antimicrobial activity was tested by an by top agar assay described by Kim *et al.* (2005) and also by radial diffusion method which was developed by Asoodeh *et al.* (2004) on both Gram-positive (*Streptococcus)* and Gram-negative *(Pseudomonas aeruginosa,and Klebsiella pneumonia)* bacteria. Bacteria were first grown in LB broth to an OD_{600} nm of 0.8. A 10µl aliquot of the bacteria was then taken and added to 8ml of fresh LB broth with 0.7% agar and poured over a 90mm Petri dish containing 25ml of 1.5% agar in LB broth. After the top agar hardened, a 20µL aliquot of the test sample filtered on a 0.22µm Millipore filter was dropped onto the surface of the top agar and completely dried before being incubated overnight at 37°C. If the designed peptide contained antimicrobial activity, a clear zone formed on the surface of the top agar representing inhibition of bacterial growth and ampicillin was used as a control.

RESULTS AND DISCUSSIONS

In the present work the prokaryotic strain GJ 1158 of *E. coli* host was used for the recombinant AMP expression. GJ1158 is a salt inducible expression system developed by Gowrishankar, and proven to be a good expression

Table 1. Media design by Placket Burman method for the optimal production of cationic peptide.

Flask No	Glucose (%)	Yeast Extract (%)	Tryptone (%)	Na_2HPO_4 (%)	KH_2PO_4 (%)	NH_4Cl (%)	1M $MgSO_4$ (ml/100ml)	1M $CaCl_2$ (ml/100ml)	Trace Metal Mix (ml/100ml)
1	H 1	H 0.8	H 0.8	L 0.5	H 0.3	L 0.1	L 0.1	0.01	0.1
2	L 0.4	H 0.8	H 0.8	H 0.7	L 0.3	H 0.4	L 0.1	0.01	0.1
3	L 0.4	L 0.2	H 0.8	H 0.7	H 0.3	L 0.1	H 0.1	0.01	0.1
4	H 1	L 0.2	L 0.2	H 0.7	H 0.3	H 0.4	L 0.1	0.01	0.1
5	L 0.4	H 0.8	L 0.2	L 0.5	H 0.3	H 0.4	H 0.1	0.01	0.1
6	H 1	L 0.2	H 0.8	L 0.5	L 0.3	H 0.4	H 0.1	0.01	0.1
7	H 1	H 0.8	L 0.2	H 0.7	L 0.3	L 0.1	H 0.1	0.01	0.1
8	L 0.4	L 0.2	L 0.2	L 0.5	L 0.3	L 0.1	L 0.1	0.01	0.1
9	0.4	0.4	0.4	0.6	0.3	0.2	0.1	0.01	0.1

host for heterologous protein production compared to BL21 (DE3) (Jawahar *et al.,* 2008). The rDNA was extracted from the DH5α bacterial cells and was successfully transformed into GJ1158 bacterial cells. The expression host containing the synthetic gene was grown up to 1 OD in different sets of media which were designed as per the Placket Burman model (Table 1). Various growth parameters like OD, Dry cell weight, Substrate consumption and product formation rate were analyzed (data not shown). Based on the results, it was identified that the flask no. 2 media composition was the optimal combination of nutrients suitable for the expression of AMP. The optimal media was chosen for further expression studies. The bacterial cultures were grown in the selected media and induced with various concentrations of NaCl as described in materials and methods for different intervals of time at 37°C in order to optimize the inducer concentration and time required for the production of recombinant protein. The protein expressions were resolved in 15% SDS-PAGE. As we failed to clearly visualize the desired band size of 11 KDa on 15 % SDS-PAGE, technique of Tricine SDS-PAGE was adapted for our present investigation. The Synthetic gene product was visualized on 16% Tricine SDS-PAGE (Fig. 1), and in turn the inducer concentration of 200mM NaCl for GJ1158 was identified as the optimal inducer concentration. And the time of induction was also optimized and the induction time of 4 hours was identified as the optimal time required for the inducer for the maximal production of recombinant synthetic gene (Fig. 1).

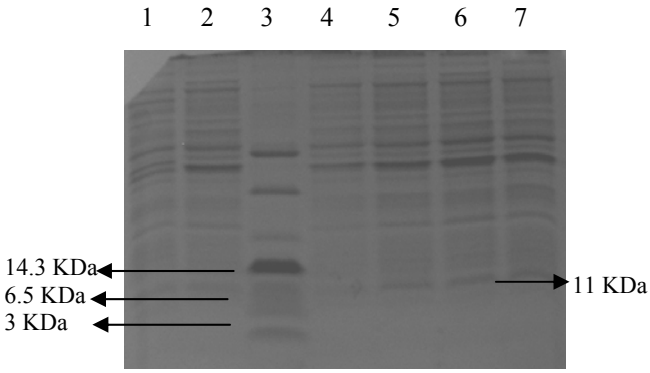

Fig. 1 Tricine SDS – PAGE analysis of recombinant peptide expression from GJ1158 host at different time intervals.

1= GJ1158 uninduced.
2= GJ1158 induced.
3= Low molecular weight protein marker.
4=Syn g pRSET-A GJ1158 uninduced.
5= Syn g pRSET-A GJ1158 induced-2 hours.
6= Syn g pRSET-A GJ1158 induced-4 hours.
7= Syn g pRSET-A GJ1158 induced-6 hours.

Purification of the peptide

Polyhistidine tags on the other hand are extremely small and therefore do not in most cases affect the folding of the attached protein. It also has very strong reversible binding attributes allowing for a very rapid and single-step purification. The tag usually consists of six consecutive histidine residues, but can vary in length from 2 to 10. Indeed, there has been great debate with regard to the length of the polyhistidine repeat and their

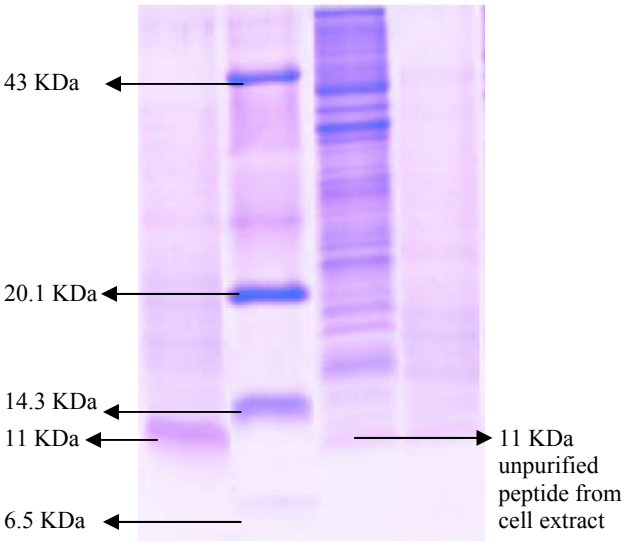

Fig. 2. Tricine SDS-PAGE analysis of IMAC purified Cationic antimicrobial peptide.
1= Purified peptide
M=Low molecular weight protein marker
2= Cell extract of syn g pRSET-A GJ1158 induced

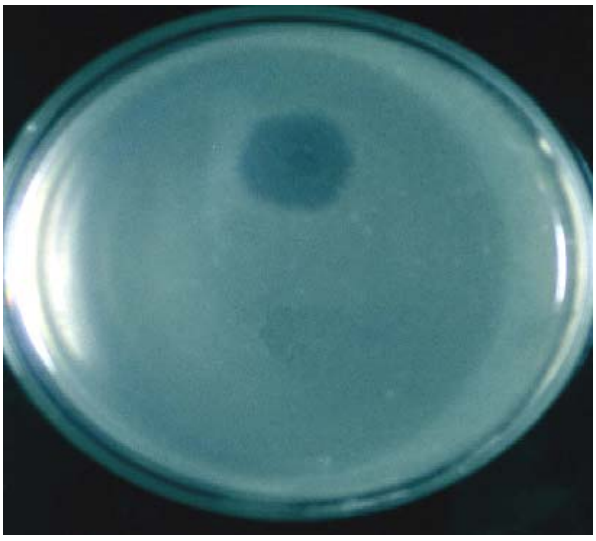

Fig. 3. Activity analysis of antimicrobial peptide by Top agar assay on *Pseudomonas aureginosa*.

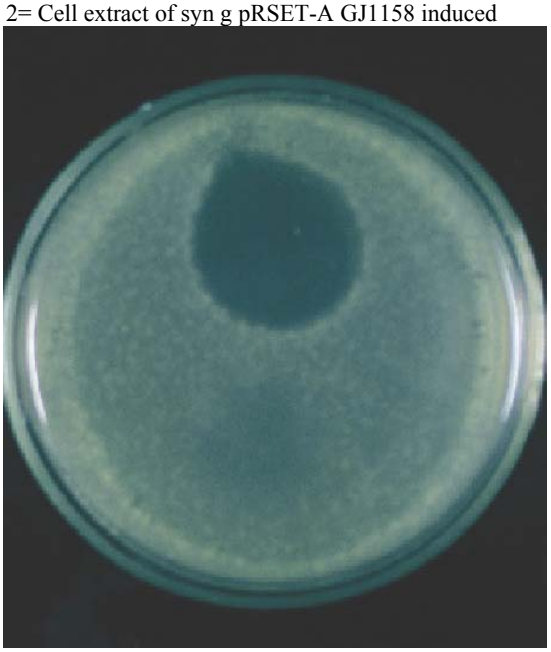

Fig. 4. Activity analysis of antimicrobial peptide by Top agar assay on *Klebsiella pneumonia.*

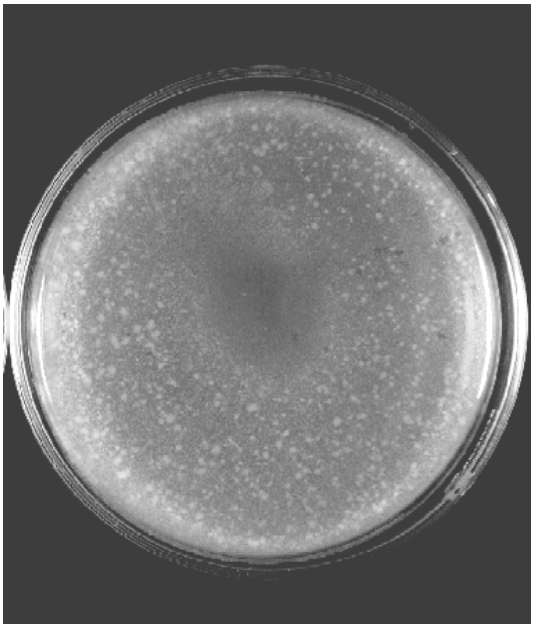

Fig. 5. Activity analysis of antimicrobial peptide by Top agar assay on *Streptococcus species.*

respective capacities to bind metal affinity resins. Recent data however appear to show there is no difference between the length of the repeat and the purification level (Mohanty *et al*., 2004). Polyhistidine tags can be placed on either the N or C-termini of recombinant proteins, although the optimal location does vary depending on the folding and biochemical characteristics of the adjacent recombinant protein. The hexahistidine tag enables the uses of immobilized metal affinity chromatography (Cao *et al.,* 2005; Marzena pazier and Jack Lubkowski, 2006; Moon *et al.,* 2007; Zhou *et al.,* 2007) for the purification of the recombinant peptides. This affinity chromatography is based upon interaction of immobilized metal ions with amino acids residues such as tryptophan, histidine, and cysteine, exposed on the protein surface. The use of immobilized metal affinity chromatography with expanded bed adsorption technology was recently described for recombinant His-tag proteins (Clemmitt *et al*., 2000; Sahin *et al*., 2005) further, the expressed peptide was purified by means of immobilized metal ion

affinity chromatography and purified sample was confirmed on Tricine SDS-PAGE (Fig. 2). It was identified that 30% of the bacterial proteins as the recombinant protein. The N- terminal histidine tag of the peptide facilitated the purification by binding to the immobilized nickel ion in the column. The same procedure was followed for the purification of Sterptolysin O (Camprubi *et al.,* 2006) the purified peptide concentration was estimated as 23mg/l by using Bradford's method by comparison with a standard curve of known amounts of bovine serum albumin (Mattos Areas *et al*., 2002). The antimicrobial assay of CP revealed that it was active against both Gram-positive and -negative bacteria (Figs. 3-5).

CONCLUSION

Towards the end of the present investigation a novel synthetic cationic peptide having broad spectrum of antimicrobial activity was produced to satisfactory levels. Further Clinical trails could prove this synthetic cationic antimicrobial peptide to be an effective antimicrobial agent and it can be effectively used on a commercial scale for control of several human pathogenic microorganisms.

REFERENCES

Anthony, S. and Fauci, MD. 1999. Antimicrobial resistance: the NIH response to a growing problem <http://www3.niaid.nih.gov/about/directors/congress/1999/0225.htm>.

Asoodeh, A., Naderi, MH., Mirshahi, M. and Ranjbar, B. 2004. Purification and characterization of antimicrobial and antifungal and non haemolytic peptide from Rana Ridibunda. Journal of sciences,Islamic republic of Iron.15(4):303-9.

Camprubi, S., Bruguera, M. and Canalias, F. 2006. Purification of recombinant histidine-tag streptolysin O using immobilized metal affinity expanded bed adsorption (IMA-EBA). International journal of biological macromolecules. 38(2):134-9.

Cao W., Zhou, Y., Ma, Y., Luo, Q. and Wei, D. 2005. Expression and purification of antimicrobial peptide adenoregulin with C-amidated terminus in *Escherichia coli***.** Protein Expression Purif. 40:404-410.

Clemmitt, RH. and Chase, HA. 2000. Immobilised metal affinity chromatography of beta-galactosidase from unclarified Escherichia coli homogenates using expanded bed adsorption. J. Chromatography A. 874:27-43.

Haught, C., Davis, G., Subramanian, R., Jackson, K. and Harrison, R. 1998. Recombinant Production and Purification of Novel Antisense Antimicrobial Peptide in *Escherichia coli*. Biotechnology and Bioengineering. 57:55-61.

Jawahar, BP., Pulicherla, KK., Rekha, VPB., Nelson, R. and Sambasiva, Rao KRS. 2008. Studies on Designing, Construction, Cloning and Expression of a novel synthetic antimicrobial peptide Current Trends in Biotechnology and Pharmacy. 2(2):334 -340.

Kim, B. 2006. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol. 3:238-250.

Marzeena, P. and Jacek, L. 2006. Expression and purification of recombinant human a-defencins in *Escherichia coli,* Protein Expression and Purification.49(1):1-8.

Mattos Arêas Ana Paula de., Maria Leonor Sarno de Oliveira., Celso Raul Romero Ramos, Maria Elisabete Sbrogio-Almeida, Isaías, Raw. and Paulo Lee Ho. 2002. Synthesis of cholera toxin B subunit gene: cloning and expression of a functional 6XHis-tagged protein in *Escherichia coli,* Protein Expression and Purification. 25 (3):481-487.

Mohanthy, AK. and Weiner, MC. 2004. Membrane protein expression and production:effect of poly His taglenth and position. Protein Expression and Purification. 33:311-325.

Moon, WJ., Hwang, Dk., Park, EJ., Kim, YM. and Chae, YK. 2007. Recombinant expression, isotope labeling, refolding, and purification of an antimicrobial peptide. Protein Expr Purif. 51(2):141.

Sahin, A., Tetaud, E., Merlin, G. and Santarelli, X. 2005. Chromatogr. J. 818:19-22.

Zhou, QF., Luo, XG., Ye, L. and Xi, T. 2007. High level production of a novel antimicrobial peptide perinerin in *Escherichia coli* by fusion expression. 54(5):366-70.

Received: Sept 18, 2009; Accepted: Dec 17, 2009