2014; 4 (1): 13-19
doi: 10.5799/ahinjs.02.2014.01.0117

RESEARCH ARTICLE

Cost effective purification of intein based syntetic cationic antimicrobial peptide expressed in cold shock expression system using salt inducible *E. coli* GJ1158

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ABSTRACT

Objective: Synthetic cationic antimicrobial peptide (SC-AMP) is an important and upcoming therapeutic molecule against conventional antibiotics. In this study, an attempt was made to purify the SC-AMP without the enzymatic cleavage of the affinity tag, by using an intein-based system.

Methods: The intein sequence was amplified from pTYB11 vector using PCR methodologies and the N-terminal of intein was ligated with SC-AMP. The designed construct, intein-SC-AMP was cloned into MCS region of cold shock expression vector, pCOLDI and the recombinant peptide was purified on a chitin affinity column by cleaving intein with 50 mM DTT without applying enzymatic cleavage. Later the peptide was quantified and its antibacterial activity of the purified peptide was studied using well diffusion method.

Results: Initially, intein-SC-AMP was expressed as a fusion protein in both IPTG inducible *E. coli* BL21(DE3) and salt inducible *E. coli* GJ1158. Single step purification using CBD (chitin binding domain) - intein tag in salt inducible *E. coli* GJ1158, yields the SC-AMP in the soluble form at a concentration of 208 mg/L. The antibacterial activity and minimal inhibitory concentration (MIC) of the purified SC-AMP was studied against both Gram positive and Gram negative microorganisms.

Conclusion: For the first time, single step purification of soluble SC-AMP was carried out using chitin-binding domain affinity tag in salt inducible *E. coli* GJ1158 without an application of enzymatic cleavage. *J Microbiol Infect Dis 2014;4(1): 13-19*

Key words: Synthetic cationic antimicrobial peptide, intein sequence, pCOLDI, chitin affinity column, *E. coli* BL21(DE3) and *E. coli* GJ1158.

Soğuk şok ekspresyon sistemi kaynaklı intein tabanlı katyonik antimikrobiyal peptitin tuz tarafından uyarılan *E. coli* GJ1158'den maliyet etkin şekilde saflaştırılması

ÖZET

Amaç: Sentetik katyonik antimikrobiyal peptid (SK-AMP) önemi gittikçe artan ve konvansiyonel antibiyotiklere alternatif olması beklenen terapötik bir moleküldür. Bu çalışmada SK-AMP'yi intein tabanlı bir sistem ile enzimatik parçalanma olmaksızın saflaştırmak amaçlanmıştır.

Yöntemler: İntein sekansı pTYB11 vektöründen PZR kullanılarak amplifiye edildi ve inteinin N-terminal ucu SK-AMP ile bağlandı. Oluşturulan bu yapı soğuk şok ekspresyon vektörü MCS bölgesine klonlandı. pCOLDI ve rekombinan peptid kitin afinite kodonundan 50 mM DDT ile ayrılarak enzimatik ayrıştırma olmaksızın toplandı. Daha sonra toplanan peptitin miktarı belirlendi ve saflaştırılan pepitd yapının antimikrobiyal aktivitesi difüzyon yöntemi ile belirlendi.

Bulgular: Başlangıçta intein bağlı SK-AMP, IPTG indüklenebilir *E. coli* BL21(DE3) ve tuz indüklenebilir *E. coli* GJ1158'de bir füzyon protein olarak eksprese edildi. Tuz ile indüklenen *E. coli* GJ1158'de kitin bağlayan kodonun kullanıldığı tek basamaklı saflaştırma ile 208 mg/L konsantrasyonunda çözünebilir SK-AMP elde edildi. Saflaştırılmış SK-AMP'nin antibakteriyel etkisi hem gram pozitif hem de gram negatif bakterilere karşı gösterildi ve minimum inhibitör konsantrasyonu belirlendi.

Sonuçlar: İlk kez çözünebilir SK-AMP tuz ile indüklenebilir *E. coli* GJ1158'den kitin bağlayan kodon kullanılarak tek basamakta enzimatik ayrışma olmaksızın elde edildi.

Anahtar kelimeler: Sentetik katyonik antimikrobiyal peptid, intein sekansı, pCOLDI, kitin bağlayan kodon, *E. coli* BL21 (DE3) ve *E. coli* GJ1158.

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Received: 18.09.2013, Accepted: 15.11.2013
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INTRODUCTION

Conventional antibiotics in general are produced by majority of molds and actinomycetes. Instead of regular antibiotics, new classes of antibiotics, peptide antibiotics are encoded by the genes of various organisms.[1-4] Peptide antibiotics are produced by lower vertebrates, various species of plants, insects and mammals. Approximately more than 575 peptide antibiotics have been found up to date. Majority of the peptide antibiotics are small length and cationic peptides with molecular weight less than 5 kDa.[5-7] Recently, different types of synthetic cationic peptide antibiotics have also been developed. These constructed synthetic cationic antimicrobial peptides contribute to the innate host defense against a number of harmful microbial pathogens, even some of the bacterial and fungal pathogens are resistant to conventional antibiotics. These synthetic cationic peptide antibiotics target pathogenic microbial cellular membranes of different species of plants and animals.[8] Some synthetic antimicrobial peptides have proved their activity remarkably even in animal models of infection against diverse pathogens viz., methicillin resistant *Staphylococcus aureus* (MRSA) and malarial parasites.[9-11] The important features like specific activity of SC-AMP without hemolytic activity hinder the resistance of microbes in infectious diseases and are useful in medical practice. Hence lot of research is going on the production of the peptide antibiotics as new class of therapeutics owing to their medical applications.[12-14] On the other hand, enzymatic cleavage dramatically reduces the production range.

In our previous work, soluble form of synthetic cationic peptide antibiotics had been successfully expressed in *E. coli* and purified using 6X histidine tag (data not shown here). Purification using 6X histidine tag and enzymatic cleavage reduced the production yield. Based on its potentiality in medical microbiology, the heterologous expression and cost effective purification of synthetic cationic antimicrobial peptide has never been reported. In the present work, synthetic cationic antimicrobial peptide was expressed efficiently in the soluble fraction in *E. coli* GJ1158 and intein tag was used as a fusion tag for one step purification, without enzymatic cleavage and quantity of the recombinant peptide was enhanced.

METHODS

Strains and plasmids

E. coli DH5α, *E. coli* BL21(DE3) and *E. coli* GJ1158 were used in the present study. *E. coli* DH5α was used as the maintenance host and the remaining two cultures were used as expression hosts. The synthetic cationic antimicrobial peptide was constructed in our previous study (data not shown here). Plasmid pTYB11 was procured from New England Bio Labs and oligos were obtained from Sigma-Aldrich, Bangalore. Plasmid pCOLDI was purchased from TAKARA Biosciences, Japan. *E. coli* GJ1158 was provided by Genei, Bangalore, India. *E. coli* DH5α, *E. coli* BL21(DE3), Streptococcus pyogenes, *Clostridium tetani, Listeria monocytogenes, Salmonella enteric, Shigella dysenteriae, Staphylococcus aureus and Enterobacter aerogenes* were procured from MTCC, Chandigarh, India.

Media preparation & culture conditions

Modified GYE medium (mGYEON) containing K_2HPO_4 - 6g/L, NaH_2PO_4 - 3 g/L, NH_4Cl - 1 g/L, Yeast extract - 5 g/L, Glucose - 5g/L, 1M $MgSO_4$ - 2 mL, TMM - 1 mL ($CuSO_4.H_2O$ - 2 mg/L, $Al_2(SO_4)_3.7H_2O$ - 10mg/L, H_3BO_4 - 1 mg/L, $NiCl_2.6H_2O$ - 1 mg/L, $MnCl_3.4H_2O$ - 20 mg/L, $ZnSO_4.7H_2O$ - 50 mg/L, $Na_2MoO_4.2H_2O$ - 50 mg/L, $FeSO_4$ - 50 mg/L). After media sterilization, appropriate amount of ampicillin (100 µg/µl) was added at room temperature aseptically. The initial pH of the medium was not adjusted to any value before autoclaving at 121°C for 15 to 20 min at 15 lbps. NaCl was excluded from the media composition while working with *E. coli* GJ1158.

Isolation of intein and synthetic gene

The gene coding for the chitin binding domain - intein tag was amplified using pTYB11 with the following primers. Intein forward primer; 5' CGCGGATCCTGCTTTGCCAAGGGTAC 3' (26 mer; BamHI cleavage site is in bold) and intein reverse primer; 5' CCCGAATCCGTTCTGTACAACAACCTG 3' (27 mer; EcoRI cleavage site is in bold). The synthetic cationic antimicrobial gene (78 bp) was amplified using the parental clone pPJB-KSR (pRSETA: synthetic cationic antimicrobial peptide) by PCR with the following primers. SC-AMP forward primer; 5' CGCGGATCCATGTGCCTTAAAGTC 3' (24 mer; EcoRI cleavage site is in bold) and reverse primer; 5' GGGAAGCTTTTACATCTTGAACCAGAT 3' (27 mer; HindIII cleavage site is in bold)

The N-terminus of the intein was fused with the C-terminus of the gene (SC-AMP). On ligation, the construct was created with BamHI and HindIII at forward and reverse region for easy cloning into the multiple cloning site of highly expressible cold shock expression vector pCOLD I with T_4 DNA ligase, followed by the transformation into maintenance host DH5α.

Expression of intein-SC-AMP fusion protein

For expression studies, the recombinant plasmid was transformed from maintenance host to salt inducible expression host *E. coli* GJ1158. The recombinant culture was grown at 37°C using glucose yeast extract medium (GYEON) in incubator shaker. When the OD_{600} reaches to 0.4 - 0.5 refrigerate the culture at 15°C and allow standing for 30 minutes. Later, sterile NaCl (at the final concentration of 200 mM) was added aseptically and continue the incubation on shaking at 15°C for 24 hours. After 24 hrs of induction, both uninduced and induced samples were pellet down, suspended in PBS (pH 8.0) and boil the samples at 100°C for 10 min. The protein analysis was carried out using 12% SDS-PAGE against a standard protein marker.

Purification of synthetic cationic antimicrobial peptide in *E. coli* GJ1158

The harvested *E. coli* GJ1158 cells were suspended in 100 mM Tris-HCl, 1 mM EDTA (pH 8.0) and 1 mM PMSF and mixed well. The sample was subjected to sonication of 5 cycles on time, 5 cycles off time and 5 min of total time. After sonication, the samples were centrifuged at 13,800 rpm for 10 min at 4°C. If any inclusion bodies are observed in the pellet, they are solubilized and refolded by using standard procedure for *E. coli*.[15,16]

Inclusion bodies require solubilization using 8M urea in 100 mM Tris-HCl buffer (pH 8.0). After solubilization, the total peptide is purified using affinity chromatography on chitin matrix. Initially, the soluble form of recombinant peptide was diluted to five times in TEN buffer (100 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0] and 500 mM NaCl; [pH 8.0]) for optimal re-folding and efficient binding to the column. In the procedure, 10 ml chitin beads (New England Biolabs) were equilibrated with buffer and the soluble refolded protein solution was loaded with a binding capacity of 2 mg/ml. The unbound proteins were removed by washing with 100 mM Tris/HCl buffer containing 1 mM EDTA and 500 mM NaCl (pH 8.0), followed by 50 mM DTT which induced on-column cleavage of synthetic cationic antimicrobial peptide from the intein tag at 4°C between 38 - 42 hrs in a static condition. The synthetic cationic antimicrobial peptide was eluted from the column using wash buffer. With the use of 2 kDa molecular weight cut off (MWCO) membrane, the DTT was removed gradually by dialysis using eluted fractions (purified synthetic cationic antimicrobial peptide) against deionized water.

Biomass and wet cell weight was monitored at OD_{600}. Peptide estimation was carried out using Lowry's method[17]. Peptide analyses of the purified and unpurified samples were done using 18% Tricine-SDS-PAGE.

Antimicrobial activity analysis

Antimicrobial activity of the desired peptide was checked by top agar assay and also by the well diffusion method on pathogenic microorganisms such as Staphylococcus aureus and *Enterobacter aerogenes*[18]. Initially bacteria was grown in appropriate broth (Luria-Bertani Broth) to an OD_{600} of 0.8 - 1.0. At OD_{600}, 20 µL of both bacterial cultures was added to 8 mL of LB broth with 0.7 % agar and poured in petridish containing 25 mL of 1.5 % LB agar separately. After the hardening of top agar, 10 µL of unpurified intein SC-AMP was loaded on the wells containing *Enterobacter aerogenes* and 6 µL of purified synthetic cationic antimicrobial peptide was loaded on the wells containing Staphylococcus aureus and completely dried before incubating overnight at 37°C. If sample has antimicrobial activity, a zone of inhibition was observed. PBS (Phosphate Buffer Saline) acts as negative control.

Determination of MIC

The purified peptide was further analysed for its minimal inhibitory concentration on various bacterial strains19. Different concentrations ranging from 5 to 30 µg/ml of peptide (5 µg, 10 µg, 15 µg, 20 µg, 25 µg and 30 µg/ml) was added to the optimal diluted pathogenic bacterial cultures (*Streptococcus pyogenes*, *Clostridium tetani*, *Listeria monocytogenes*, *Salmonella enterica* and *Shigella dysenteriae*) incubated at 37°C for 3 hrs. After incubation, the five bacterial cultures were transferred and spread on agar plates and incubated at 37°C for overnight. The MIC of peptide concentration was recorded.

Hemolytic assay

The hemolytic activity of synthetic cationic antimicrobial peptide was determined with sheep erythrocytes based on radial diffusion assay.[20] The samples were loaded on well in the solidified blood agar medium and the 0.2% triton X-100 was used as positive control (hemolytically active). The plates were incubated at 37°C for overnight to determine the hemolytic activity of the peptide.

RESULTS

Construction of intein-SC-AMP expression plasmid

The Chitin Binding Domain-intein tag was amplified from T7 promoter based pTYB11 expression system (Figure 1) and synthetic cationic antimicrobial

gene was also amplified (Figure 2). Later N and C terminus of intein and synthetic cationic antimicrobial gene was ligated and the desired construct is cloned in MCS region of BamHI and HindIII of highly expressible cold shock expression vector pCOLDI. The recombinant plasmid used for expression of synthetic cationic antimicrobial peptide as a fusion protein. Transformation of recombinant plasmid was carried out in the *E. coli* and the transformants were confirmed using colony lysate PCR and also by sequencing using gene specific primers. The sequence was 5'- CGCGGATCCATGTGCCTTA-AAGTCCGTAT TTGGTTTAAAATGGAGGCGGAG-GACATGTGCCTGAAGGTGCGCATCTGGTTCAA-GATGAAGCTTCCC - 3'.

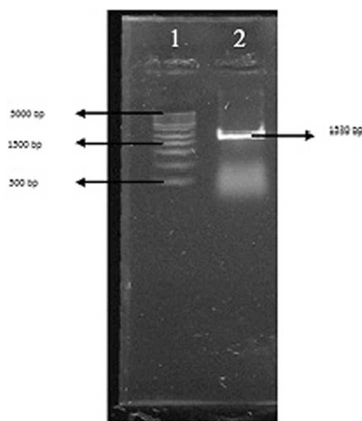

Figure 1. Amplification of intein

Lane 1: DNA Ladder; Lane 2: Intein amplification

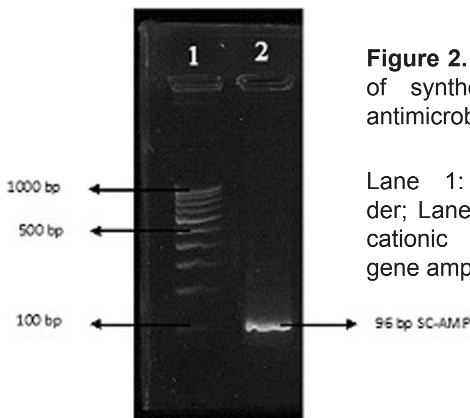

Figure 2. Amplification of synthetic cationic antimicrobial gene

Lane 1: DNA Ladder; Lane 2: Synthetic cationic antimicrobial gene amplification

Expression of recombinant synthetic cationic antimicrobial peptide in *E. coli*

Recombinant *E. coli* expressing the intein - synthetic cationic antimicrobial peptide was cultivated in mGYEON medium for cost effective production. The recombinant intein - synthetic cationic antimicrobial peptide was expressed in soluble form instead of insoluble aggregates in *E. coli* GJ1158. Smith and Johnson (1988) have reported that lower temperatures remarkably improve the soluble recombinant expression21. On the other hand, *E. coli* BL21(DE3) express the recombinant synthetic cationic antimicrobial peptide in inclusion body form at 37°C. The expression of 59.2 kDa fusion protein was confirmed using 12% SDS-PAGE (Figure 3).

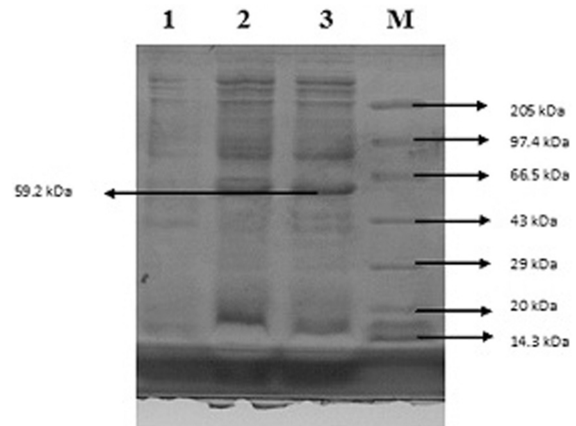

Figure 3. 12% SDS-PAGE Analysis of recombinant protein (Intein SCAMP)

1: Uninduced culture of synthetic cationic antimicrobial peptide pCOLDI *E. coli* GJ1158
2, 3: Induced culture of synthetic cationic antimicrobial peptide pCOLDI *E. coli* GJ1158
M: Protein marker

Purification of recombinant peptide

Generally, recombinant peptides are expressed in soluble form. But negligible amount of peptide is expressed as insoluble aggregates in *E. coli* GJ1158. After incubation, protein quantification yields the 208 mg/L of recombinant peptide. The purified 2.9 kDa peptide was confirmed using 18% Tricine-SDS-PAGE is shown in Figure 4. The final yield of the purified recombinant peptide was less than around 25% of the sample taken for purification.[15]

The recombinant peptide over expressed with salt inducible *E. coli* GJ1158 is comparatively less expensive than IPTG inducible BL21(DE3). The purification was easy due to the expression of peptide in soluble form using *E. coli* GJ1158, which has not been reported till to date using intein based single step purification.

Antimicrobial Activity

The antimicrobial activity was performed to determine the activity of unpurified intein synthetic cationic antimicrobial peptide and purified synthetic cationic antimicrobial peptide. Fig. 5 shows the zone of clearance around the well containing recombinant intein-SC-AMP, where control well shows no zone of inhibition. Fig. 6 shows the zone of clearance around the well containing purified SC-AMP, where control well shows no zone of inhibition.

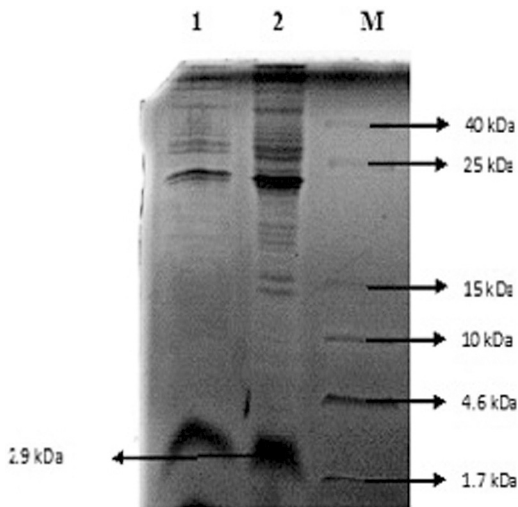

Figure 4. 18% SDS-PAGE Analysis of synthetic cationic antimicrobial peptide

1: Purified synthetic cationic antimicrobial peptide (After second level)
2: Purified synthetic cationic antimicrobial peptide (First level)
M: Protein marker

MIC and hemolytic assay

The MICs of purified recombinant synthetic cationic antimicrobial peptide against Gram-positive and Gram-negative bacteria are shown in Table 1 and the MIC values in the range of 15-25 μg/ml. Some antimicrobial peptides exhibit hemolytic activities, but no hemolytic activity was observed even after overnight incubation using the concentration more than 30 μg/ml, indicating that SC-AMP was not toxic to red blood cells.

Table 1. Minimal inhibitory concentrations of synthetic cationic antimicrobial peptide on Gram positive and Gram negative microorganisms.

Pathogenic microorganisms	MIC[a] (μg/ml) of purified peptide
Gram positive	
Streptococcus pyogenes	25
Clostridium tetani	15
Listeria monocytogenes	20
Staphylococcus aureus	25
Gram negative	
Salmonella enterica	25
Shigella dysenteriae	20
Enterobacter aerogenes	10

[a] Experiments were carried out in triplicates

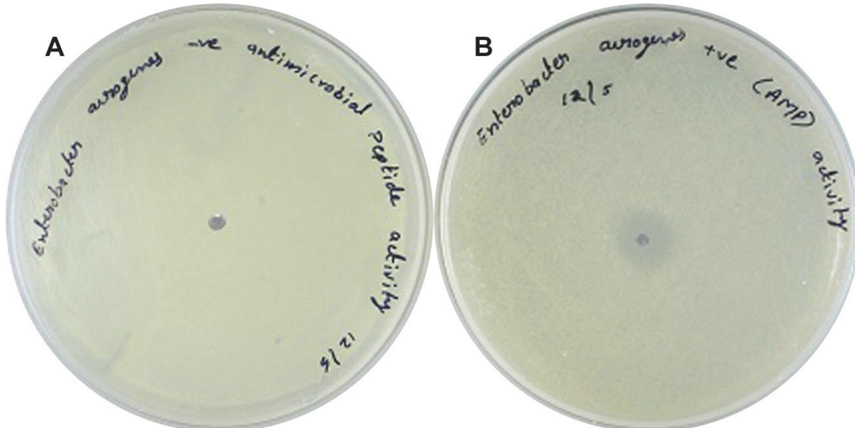

Figure 5. Activity analysis of intein synthetic cationic antimicrobial peptide on *Enterobacter aerogenes*

A: Negative control (PBS)
B: 10 μl of intein synthetic cationic antimicrobial peptide

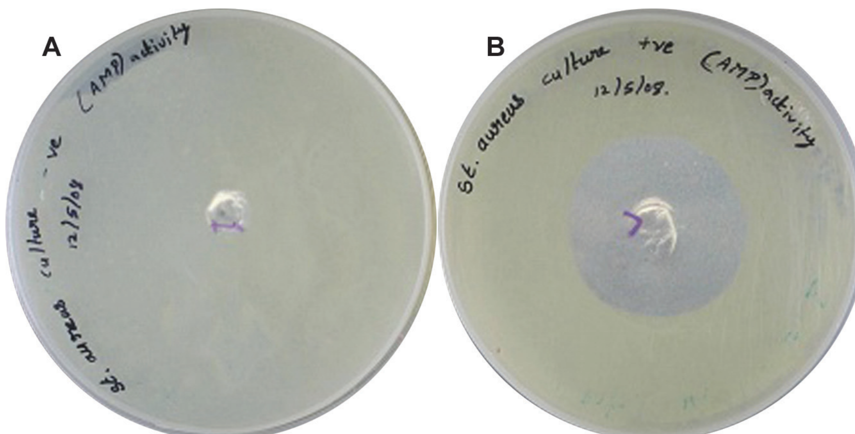

Figure 6. Activity analysis of the purified synthetic cationic antimicrobial peptide on *Staphylococcus aureus*

A: Negative control (PBS)
B: 6 μl purified synthetic cationic antimicrobial peptide

DISCUSSION

To look at the therapeutic potential of synthetic cationic antimicrobial peptide, soluble and cost effective production and purification is required. Major drawback of recombinant peptides associated with difficulty in scale-up and observed low yields after purification.[22-24] Multi-step purification reduces the yields to several folds and is not cost-effective. Using fusion his-tag thioredoxin, many AMPs have been expressed successfully in *E. coli*, but production ranges are not satisfactory.[25-27] In some studies, insoluble fractions are solubulized by using chaotropic detergent urea and guanidium hydrochloride. After refolding of the peptide using detergents, peptides show less antimicrobial activity on pathogens, because of proteolytic degradation. Different enzymatic and chemical cleavage methods are employed to cleave a peptide from its fusion partner, where chemical methods are inefficient. The N-terminal of the peptide is constructed with different proteases, such as thrombin, Factor Xa or enterokinase. In pCOLDI factor Xa is present at the upstream of the peptide. The presence of ten amino acids between factor Xa and peptide may have negative effect in terms of activity. The enzyme used to cleave the factor Xa is also expensive. Chen et al., in 2009 used the SUMO fusion system for cost effective antimicrobial peptide expression, but removal of endotoxin was difficult.[28]

In some studies, peptides of varying lengths ranging from 37 amino acids for LL-37 to 9 amino acids for HHC-10 were produced [29]. Li et al., in 2009 achieved the expression and purification of peptides in the range of 30 amino acids and more.[30] But after enzymatic cleavage and purification, the final yield was less than 10% of the sample taken for purification. In this study, we produced 26 amino acid peptide and the yield was less than ~25% of the sample taken for purification. Encouraging results of antimicrobial activity using SC-AMP was studied with Gram positive and Gram negative microorganisms. The MIC of purified SC-AMP was also studied using Gram positive and Gram negative microorganisms. Among different Gram positive microorganism, 15 µg/ml of purified SC-AMP was enough to inhibit the growth of *Clostridium tetani* and 25 µg/ml of purified SC-AMP was enough to inhibit the growth of *Streptococcus pyogenes*. But 10 µg/ml of purified SC-AMP was enough to inhibit the growth of *Enterobacter aerogenes*. Hence this cost effective purification of synthetic cationic antimicrobial peptide lacking enzymatic cleavage will have a huge demand in future if approved on clinical trials.

In conclusion, this study revealed the importance of single step purification of soluble recombinant synthetic cationic antimicrobial peptide from salt inducible *E. coli* GJ1158 using intein with a chitin binding domain affinity tag. The main advantage is expressing the soluble recombinant peptide without enzymatic cleavage and increasing peptide quantity.

Acknowledgements

We heartfully express our indebtedness to the management of Bapatla Education Society, Bapatla, Andhra Pradesh, India for their financial assistance. We also express our sincere thanks to Prof. J. Srinivasa Rao, Head of the Department of Chemical Engineering, for his technical help and support throughout the work.

Conflict of interest: Authors have no conflict of interest.

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