Acta Biologica Szegediensis

Volume 62(1):61-66, 2018
Acta Biologica Szegediensis
http://abs.bibl.u-szeged.hu/index.php/abs

DOI:10.14232/abs.2018.1.61-66

ARTICLE

Expression and production optimization of the cationic antimicrobial peptide - indolicidin by the recombinant *E. coli* C41 (DE3) clones

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ABSTRACT The cytoplasmic granules of bovine neutrophils naturally possess indolicidin - a promising cationic antimicrobial peptide as it displays inherent inhibitory activities against a broad type of microbial pathogens. In this study, a shake flask level production and expression optimizations of the indolicidin by the recombinant *Escherichia coli* C41 (DE3) clones (transformed with pET21a(+) plasmid carrying indolicidin gene) were carried out under standard conditions, as to determine the conditions required for maximal production. It was determined that a concentration of 1 mM of IPTG was effective, the 2×YT with salts and LB media at pH 7.5 with 3-6 h of incubation were required for maximal indolicidin expression.

Acta Biol Szeged 62(1):61-66 (2018)

KEY WORDS
cationic antimicrobial peptide
drug resistance
indolicidin
optimization

ARTICLE INFORMATION
Submitted
24 February 2018.
Accepted
28 April 2018.
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Introduction

The increasing prevalence of drug resistant and multi-drug resistant bacteria has prompted to investigate and produce new compounds against bacterial pathogens as an intervention to ensure efficient infection control measures (Ahmad et al. 1995). Cationic antimicrobial peptides (CAPs) represent an exciting class of drug candidates, particularly because their mechanism of action is unlikely to induce drug resistance. As a kind of humoral immune response, such host defense peptides are synthesized by a wide range of multicellular organisms including bacteria and protect against a wide range of infectious bacteria, viruses, fungi and certain parasites (Zhang and Gallo 2016). Precisely, they are endogenous peptide antibiotics forming important components of the innate immune system (Hancock et al. 2006) of vertebrates and invertebrates. Thus, CAPs can be developed and potentially employed in medicine as new safe antibacterial compounds.

The cationic antimicrobial peptide - indolicidin is a 13-residue peptide (measures less than 2.8 kDa in size) and carries five tryptophan residues that distinguish it from α-helical and β-structured cationic peptides. It is isolated from the cytoplasmic granules of bovine neutrophils possessing a unique amino acid composition (ILPWKWPWWPWRR-NH2-H-Ile-Leu-Pro-Trp-Lys- Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH_2) with 39% tryptophan, 23% proline, and an amidated carboxyl terminus. The molecular weight of the indolicidin peptide was reported to be 2861 Da with a total of 20 amino acids (MHHHHHHILPWKWPWWPWRR) including `His'-Tag and the start codon. Indolicidin has been noted to be a promising agent since it could target a variety of bacterial and fungal pathogens. It exhibits broad-spectrum antimicrobial activity *in vitro* against Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) bacteria, fungi (*Aspergillus fumigatus, Candida albicans* and *Cryptococcus neoformans*), protozoa (*Leishmania donovani*), and human immunodeficiency virus (HIV-1) (Ahmad et al. 1995; Selsted et al. 1992; Benincasa et al. 2006; Lee et al. 2002; Bertrand-Krajewski et al. 2005). It also possesses

haemolytic and cytotoxic activities towards erythrocytes and human T lymphocytes (Schluesener et al. 1993). Despite the small size and unique composition of indolicidin, it is capable of killing bacteria, by causing disruption of the cytoplasmic membrane by channel formation.

The advent of recombinant DNA technology has conquered the traditional problems of protein research including purification of minute homogenous quantities of desired peptides from tissues (Alberts et al. 1994). It enables isolation of specific genes and to perpetuate them in host organisms besides ensuring higher expression and purity of the compound, safety, decreased cost of production (WHO 2014). Accordingly, many different host/vector systems are being developed to produce antimicrobial peptides through recombinant DNA technology. *E. coli* C41 (DE3) and C43 (DE3) strains have been employed most often due to the low cost of fermentation/expression compared to mammalian cells, and its ability to produce inclusion bodies, which aid in the purification process (Haught et al. 1998; Lee et al. 1998). Industrial biotechnology uses recombinant microbes for producing a commercial product like indolicidin in stages of upstream processing (preparation of raw material/media for fermentation), fermentation (growth of the target microorganism in a large bioreactor with the consequent production of a desired compound) and downstream processing (purification of the desired compound (Glick and Pasternak 1998). The present evaluation has majorly focused on the maximal expression and optimization of indolicidin production by the recombinant *E. coli* C41 (DE3) clone that was earlier cloned in the study to express indolicidin.

Materials and Methods

Competent *E. coli* C41 (DE3) cells (Lucigen, USA) were prepared and transformed with the pET-21a (+) recombinant plasmids (Fig. 1). The optical density (OD) checked transformed cells were subsequently assessed to optimize indolicidin expression with (flasks A & B) and without (flask C) 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) induction. After everyone hour, 1 ml of culture was withdrawn from each flask to check the OD and indolicidin expression (Froger and Hall 2007).

Confirmation of indolicidin expression by the recombinant E. coli

Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (tricine-SDS-PAGE) was employed for the separation and confirmation of indolicidin. A 20% separating gel (1 cm length) followed by 16% separating gel (5 cm length) was overlaid with 10% 'spacer gel' (to

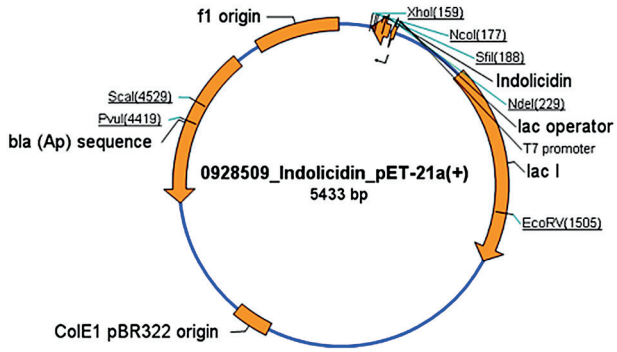

Figure 1. pET-21a (+) expression vector map showing the restriction sites, the gene insert (indolicidin), selectable marker [bla (Ap)] and the origin of replications (f1 origin & ColE1-EMD Biosciences). Gene insert: Indolicidin; Host: *E. coli* K12 XL10 gold (dam+ dcm+); Gene size: 63 bp. Vector backbone: pET-21a (+) Cloning sites: XbaI/XhoI

considerably sharpen the bands for proteins and peptides of 1-5 kDa). The electrophoresis was started after loading 10 µL of the prepared samples at 90 V. After separation, the protein was visualized by directly staining the gel by Coomassie Brilliant Blue (Schägger and von Jagow 1987; Schägger 2006). The culture from A & B flasks (induced) were centrifuged, before and after induction for 10 min, 10 000 rpm at 4 °C. The supernatants were discarded, and the samples of each cell pellet were normalized at appropriate OD, boiled and run on two sets of 16% tris-tricine gels simultaneously. The gels were suitably stained, destained and visualized. The un-induced samples (from the flask C) were also subjected to tricine-SDS-PAGE. The samples possessing the expressed indolicidin of the study were screened for their antimicrobial activity against a set of bacterial pathogens along with un-inoculated media samples as controls so as to determine the presence and antimicrobial activities of the expressed indolicidin of the study.

Effect of the inducer - IPTG on indolicidin production by the recombinant E. coli

A contaminant free, confirmed *E. coli* clone was involved in all the optimization studies. The *E. coli* culture (OD_{600} value of 1.5 - 2.5) in LB medium was induced with different concentrations (0.5 mM, 1.0 mM, 2 mM, 5 mM, and 10 mM) of the IPTG and were incubated at 37 °C in duplicates. Pre and post induction samples were collected and were analyzed quantitatively and qualitatively by electrophoresis.

Primary screening of production media - shake flask studies

A total of eight different media (nutrient broth, min A, Luria Bertani medium, 2×YT medium, 2×YT with salts,

terrific broth (TB) medium, SB medium & SOB medium (Himedia, India) containing varying concentrations of tryptone, yeast extract with different carbon sources like glycerol, glucose and with several salts/buffer combinations were involved in the preliminary screening of a best medium for expression (Sambrook and Russel 2001). Similarly, 1 ml of 100 mg/mL sterile filtered ampicillin was added just before inoculation, 0.5 mL stock culture was inoculated, and all the studies were executed at shake flask level (with 50 ml of the test medium in a 250 ml flask).

To prepare the inoculum for shake flask studies, 0.5 mL of stock culture was inoculated into 50 mL of LB-Amp medium after thawing and incubated at 37 °C, 200 rpm for overnight. From the overnight culture, 5 mL of culture was inoculated into the shake flasks of different media (100 mL) with ampicillin and incubated at 37 °C, 200 rpm. The induction was carried out using 1 mM IPTG at 1.0 ± 0.2 OD after obtaining the desired cell OD. One mL of IPTG induced (also normalized to 10 OD_{600}) culture sample was taken from each flask for 3 h (while a sampling was also done just before induction). The indolicidin peptide expression was checked for both the induced as well as the uninduced cell samples after lysis by tricine SDS-PAGE.

Effect of different pH on indolicidin production by the recombinant E. coli

In order, to study the effect of media pH on indolicidin production, initial media pH was adjusted ranging from 6 to 9 with an increment of 0.5 unit (6, 6.5, 7, 7.5, 8, 8.5 and 9). The experiment was carried out using LB media (with optimized amount of ingredients) in duplicates and indolicidin was extracted after 12 h of incubation and estimated for indolicidin activity.

Effect of incubation time on indolicidin production by the recombinant E. coli

The indolicidin production media was prepared in duplicates with all optimized parameters and inoculated with the *E. coli*. The indolicidin assay was carried out from the 8^{th} h incubation up to 32 h after every 4 h interval (*i.e.* 8, 12, 16, 20, 24, 28, and 32 h) of incubation.

Effect of temperature on indolicidin production by the recombinant E. coli

The incubation - temperature at various ranges (25, 30, 37, 40 and 45 °C) was evaluated and optimized to support a better expression of the indolicidin peptide by the recombinant *E. coli* of the study. Developmental batches were executed at 250 mL flasks with 50 mL of LB medium in duplicates inoculated with the test *E. coli* and were incubated at room temperature as well as at 30, 37, 40 and 45 °C appropriately (Shokri 2003; Schmidt 2005). Precisely, all the indolicidin fermentation processes included simultaneous gearing up of the growing cells to produce the indolicidin protein by inducing them with 1 mM IPTG so that the cellular machinery was mostly engaged in expressing the target protein(s) in abundance from of the start of the inoculation. Finally, the fermentation medium was separated by harvesting the cells, concentrated and then evaluated for the target peptide.

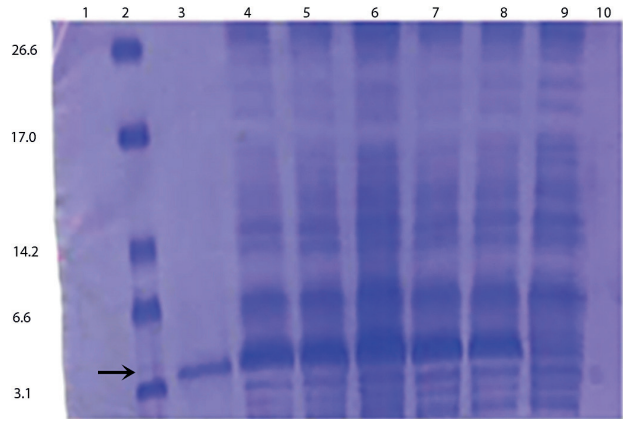

Figure 2. Tricine - SDS-PAGE profile of indolicidin expression check of 10 OD normalized *E. coli* transformants. Band representing the target peptide - indolicidin measuring 2.8 kDa.

Results

In this study, the LB-Amp plates incubated with transformation mixes of host *E. coli* C41 (DE3) cells and pET-21a (+) (EMD Biosciences, USA) with indolicidin gene construct showed a good growth of individual, isolated colonies after overnight incubation. The transformation efficiency was found to be 2.2×10^5 CFU/ng of DNA. The OD values of *E. coli* transformants grown in flasks A & B (with IPTG induction), and flask C (no IPTG induction) at different intervals both before and after induction with the inducer indicated a difference in expression between the IPTG induced and non-induced clones. The SDS-PAGE analysis done for the culture sampled from the flask cultures A & B of the induced clones confirmed the expression of 2.8 kDa indolicidin peptide as a discrete band at the bottom of the gel (Fig. 2) and the expressed peptide was also quantified by densitometry along with a standard. The expression of the peptide by the transformed cells was also confirmed after growing the preliminarily confirmed transformants in shake-flask cultures and was also found that the induction of recombinant protein (indolicidin) in *E. coli* cultures could be best obtained when induced

Table 1. Primary screening - pre and post induction OD patterns of indolicidin expressing *E. coli* clones in different production media.

Test production media	OD600 (time of sampling)			
	JBI	AI (2 h)	AI (3 h)	AI (4 h)
LB medium	0.82	1.73	2.16	2.74
SB medium	0.92	1.72	2.24	2.52
SOB medium	0.74	1.68	2.20	2.46
TB medium	0.86	1.92	2.65	3.00
2×YT medium	0.72	1.82	2.40	2.92
2×YT with salts	1.02	2.06	2.90	3.38

AI - after induction; JBI - just before induction

at OD_{600}value of 0.6 to 1.0.

The standardization of parameters governing the growth of recombinant *E. coli* C41 (DE 3) and production of indolicidin in high yields were studied at shake flask level. The effect of various media, different concentrations of IPTG inducer, suitable temperature, pH of the media, incubation duration brought useful outcomes. Of the eight different media [Nutrient broth, Min A, LB medium, 2×YT medium, 2×YT with salts, Terrific Broth (TB) medium, SB medium and SOB medium] containing varying concentrations of tryptone, yeast extract with different carbon sources like glycerol, glucose and with several salts/buffer combinations involved in the preliminary screening, it was apparent that the growth of *E. coli* was higher 2×YT with salts, TB medium and in LB medium with elevated levels of OD compared to other media. SOB medium was noted to be the least in supporting *E. coli* growth. Similarly, a difference in indolicidin expression by the *E. coli* clone was noted when grown in different media as the OD obviously varied when determined both before and after IPTG induction (Table 1). Although, each medium supported high yield of indolicidin, the 2×YT with salts, TB medium and LB media were found to support higher expressions especially after induction with IPTG. In addition, the difference in indolicidin expression was also evaluated using SDS-PAGE. It was observed that in the presence of 2×YT with salts and LB media as much as 101 mg/L and 90 mg/L of indolicidin was estimated, respectively after four hours from the time of induction.

Discussion

The alarming development of microbial drug resistance has resulted in intensive research and development of alternate antimicrobial compounds in order, to maintain a pool of effective therapeutic agents in real times so as to combat microbial infections. More importantly, these new antimicrobials should preferably possess both unique modes of action as well as different cellular targets compared with the existing antibiotics. Accordingly, good efforts have been made to develop cationic antimicrobial peptides as drugs since they possess broad spectrum ability in killing microbes and act non-specifically by targeting the sites for which development of resistance is difficult. In this line, the peptides like indolicidin is an attractive subject for drug research and hence there has been a lot of commercial interest and effort in developing indolicidin as a potential antimicrobial therapeutic. Though, a large number of antimicrobial peptides have been found in nature and been designed, relatively a few have been investigated for their clinical trials based on their promising *in vitro* and animal studies.

After selection of the expression vector [pET-21a (+)] of the study, several different host strains were identified [*E. coli* C41DE3 derivative of BL21 (Lee et al. 1998; Ponti et al. 1999), HS174DE3 (Lee et al. 2000), BL21DE3 and AD494DE3 (Morin et al. 2006) and JM109 (Taguchi et al. 1994)] from literature and was noted that two mutant strains of *E. coli* BL21 (DE3), C41 (DE3) and C43 (DE3) were frequently used to overcome the toxicity associated with over expressing recombinant proteins using the bacteriophage T7 RNA polymerase expression system. The strains were also reported with properties of proteolytic deficiencies, solubilization of recombinant proteins, or derivation from different cell strains (Miroux and Walker 1996; Dumon-Seignovert et al. 2004). Miroux and Walker (1996) further reported that by using *E. coli* C41 (DE3), cell death during induction of the host could be avoided. Further, *E. coli* has been used successfully for the higher expression of several recombinant antimicrobial peptides like lactoferricin (Feng et al. 2006; Kim et al. 1997), calmodulin (Zheng et al. 1997), CM4 (Zhou et al. 2009) and human beta-defensin 2 (Xu et al. 2006). As has been observed in the present study, Wagner et al. (2008) reported that *E. coli* C41 (DE3) strain is acceptable for convenient over expression of any given novel protein such as indolicidin.

Haught et al. (1998) and Skosyrev et al. (2003) reported a growth maximum of 1.5 OD. Other researchers had reported expression of 0.1 to 310 mg/L of antimicrobial peptide (Haught et al. 1998; Lee et al. 1998; Skosyrev et al. 2003; Hara and Yamakawa 1996; Lee et al. 2002; Hwang et al 2001). The major goal for optimizing production of recombinant proteins is to produce the highest amount of functional product per unit volume per unit time.

In this context, most of the work was only aimed at increasing recombinant protein production in bacterial strains by increasing the biomass production and studies on the expression of recombinant proteins based on the effects of media composition are minimal. Though Swartz (2001) and Balbás (2001) reported that the fermentation and the level of intracellular accumulation of a recom-

binant protein in *E.coli* is dependent on the final cell OD and the specific activity of the protein, it is also known that the production of secondary metabolites in microbial strains can depend on the composition of the medium in which the organism is grown and the expression of all proteins will be not maximal in one medium and it has to be optimized for specific proteins.

Despite this, little attention has been paid to the effects of medium formulation on the accumulation of recombinant proteins (Broedel et al. 2001). As the industrial expression of the eukaryotic peptide - indolicidin is more at the developing stage, the present study included appropriate evaluations so as to formulate an effective medium for the maximal expression of indolicidin. While the available literature played a pivotal role, the prior knowledge on the aspect was of much help in formulating various test media of the study for indolicidin expression in *E. coli*. Further, the expression host of the study - *E. coli* C41 (DE3) that grew rapidly and at high cell densities on inexpensive substrates was an added advantage. Further, the total protein concentration in LB medium was estimated to be higher with 1 mM concentration of IPTG, while the same was noted to be insignificant with further increase in concentration of IPTG. Similarly, from the results obtained, it was evident that the growth rate or doubling time of the indolicidin clone (*E. coli*) was faster in 2×YT with salts, TB medium and LB medium with pH 7.5 at 37 °C than at lesser temperatures, with an incubation ranging from 3-6 h towards a higher production of indolicidin when compared with other tested parameters (Table 2).

Table 2. Post induction OD patterns of *E. coli* clones expressing indolicidin in different production conditions.

Ranges of media pH, incubation temperature/duration and optical density (OD)					
pH	OD	Incubation time (h)	OD (600 nm)	Temperature °C)	OD (600 nm)
6.0	0.608	3	0.485	30	0.598
6.5	0.577	6	0.548	37	0.625
7.0	0.572	12	0.452	40	0.624
7.5	0.670	16	0.512	45	0.588
8.0	0.598	20	0.513	-	-
8.5	0.515	24	0.493	-	-
9.0	0.500	28	0.495	-	-

Conclusion

In this study, lab scale optimization of indolicidin expression determined the ideal parameters for maximal expression of the peptide by pET21a(+) recombinant plasmids in *E. coli* C41 (DE3). Against the fact that industrial production optimization of any novel peptide usually preceded by lab scale production optimization, the outcomes of the present evaluation could be suitably employed during scale up events so as to maximize the indolicidin expression. Further, alternative drugs are viewed to be the remedies to redress the emerging microbial pathogens, and that other potent antimicrobial peptides could also be tried as to develop them in to promising drugs.

Acknowledgement

The authors sincerely thank the University Grants Commission (UGC), New Delhi for having funded this research work [Major Research Project - F. No. 41-1161/2012 (SR)].

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