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การแสดงออกของเปปไทด์ LcI สายผสมจาก *Bacillus amyloliquefaciens* KKU14 และการทดสอบความสามารถในการยับยั้ง *Burkholderia pseudomallei*

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Expression of Recombinant Peptide LcI from *Bacillus amyloliquefaciens* KKU14 and Test for Its Inhibition on *Burkholderia pseudomallei*

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หลักการและวัตถุประสงค์: *Burkholderia pseudomallei* เป็นเชื้อก่อโรคเมลิออยด์ รักษาได้ด้วยยาปฏิชีวนะ อย่างไรก็ตามพบการดื้อยาในเชื้อ *B. pseudomallei* หลายสายพันธุ์ ก่อนหน้านี้พบว่า *Bacillus amyloliquefacien*s KKU14 สามารถยับยั้งการเจริญของเชื้อ *B. pseudomallei* ได้ ผู้วิจัยจึงได้โคลนยีนสร้างสารต้านจุลชีพ LcI จาก *B. amyloliquefaciens* KKU14 และทดสอบการออกฤทธิ์ต้านเชื้อ *B. pseudomallei* สายพันธุ์ P37

วิธีการศึกษา: ค้นหาข้อมูลเปปไทด์ต้านจุลชีพของแบคทีเรีย *B. amyloliquefaciens* จากฐานข้อมูล GenPept (NCBI) ออกแบบไพรเมอร์และเพิ่มจำนวนยีนจาก *B. amyloliquefaciens* KKU14 ด้วยวิธี PCR โคลนยีนเข้า เวกเตอร์พาหะชนิดต่าง ๆ แสดงออกโปรตีน และทดสอบการต้านเชื้อ *B. pseudomallei* P37 ด้วยวิธี agar well diffusion และ in-gel overlay

ผลการศึกษา: สามารถโคลนยีน *lcI* ได้สำเร็จแต่พบการแสดงออกของเปปไทด์สายผสมเฉพาะในเวคเตอร์ pQE31 เท่านั้น เปปไทด์ที่แสดงออกมีขนาดประมาณ 10 กิโลดาลตัน อย่างไรก็ดีเปปไทด์สายผสมนี้ไม่สามารถยับยั้ง *B. pseudomallei* สายพันธุ์ P37

สรุป: ในเบื้องต้นเปปไทด์ LcI สายผสมจาก *B. amyloliquefaciens* KKU14 ไม่สามารถยับยั้ง *B. pseudomallei* สายพันธุ์

Background and objective: *Burkholderia pseudomallei* is a pathogenic bacterium, causing melioidosis, a serious infectious disease. Currently, the best treatment is using antibiotic drugs. However, many clinical strains are now intrinsically resistant to almost all available antibiotic drugs. Previous study indicated that *Bacillus amyloliquefaciens* KKU14 strongly inhibits *B. pseudomallei*. In this study, antimicrobial peptide LcI of *B. amyloliquefaciens* KKU14 was cloned and expressed to test activity against *B. pseudomallei.*

Materials and Methods: Antimicrobial peptide LcI was searched from GenPept database (NCBI) and amplified from genome of *B. amyloliquefaciens* KKU14 by PCR and cloned into different cloning vectors. The recombinant clones were induced and their activity against *B. pseudomallei* strain P37 were tested by agar well diffusion and in-gel overlay.

Results: The recombinant peptide was successfully cloned. However, the expression could be observed only in pQE31 vector. The expressed peptide has the size of approximately 10 kDa. Nevertheless, the recombinant clones exposed no antimicrobial activities

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P37 ได้ อาจเกิดจากการพับตัวที่ไม่เหมาะสมของเอนไซม์สายผสม หรือเปปไทด์ที่มีฤทธิ์ต้านเชื้อ *B. pseudomallei* อาจเป็นเปปไทด์ชนิดอื่น หรืออาจต้องใช้การทำงานร่วมกันของเปปไทด์หลายชนิด ซึ่งจำเป็นต้องมีการศึกษาต่อไป

คำสำคัญ: Antimicrobial peptide, Recombinant LcI peptide, *Bacillus amyloliquefaciens*, *Burkholderia pseudomallei*

against *B. pseudomallei strain* P37.

Conclusion: The recombinant peptide LcI cloned from *B. amyloliquefaciens* KKU14 does not inhibit *B. pseudomallei* strain P37 in this study. This might due to improper folding of the recombinant peptide, or the peptide that was responsible for inhibition of *B. pseudomallei* might be other peptides, or the inhibition effect might need synergistically work from several peptides together, which has to be further investigated.

Key words: Antimicrobial peptide, Recombinant LcI peptide, *Bacillus amyloliquefaciens*, *Burkholderia pseudomallei*

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Introduction

Burkholderia pseudomallei is a Gram-negative, soil saprophyte and pathogenic bacterium. It is the cause of melioidosis which is a serious community-acquired infectious disease in the endemic areas, mostly reported in Northern Australia and Southeast Asia[1]. Infection of this bacterium is acquired through direct inoculation, inhalation, and ingestion. The common features of melioidosis include skin or soft tissue infections, hepatosplenic abscess, septic arthritis, bacteraemic pneumonia and septicaemia, which is a main cause of morbidity and mortality, Almost all clinical studies have come from Thailand, northern Australia, Malaysia, and Singapore[1]. More recent bacteremia and overall mortality rates have been, respectively, 60% and 44% in Thailand, 46% and 19% in Australia, and 43% and 39% in Singapore[2]. Melioidosis is the third most common cause of death from infectious disease in northeast Thailand, exceeded only by HIV infection and tuberculosis[3]. Melioidosis treatment is difficult and frequently disappointingly slow despite administration of high dose parenteral antibiotics[1]. *B. pseudomallei* is intrinsically resistant to antibiotic drugs including penicillin, ampicillin, first-generation and second-generation cephalosporins, gentamicin, tobramycin, streptomycin, and polymyxin[4]. The antibiotic of choice is ceftazidime[5]. However, some clinical strains are highly resistant to ceftazidime such as the strain 316a, 316C, EPMN159 and 9796[6].

In 2018, Potisap and coworkers isolated bacteria from soil sample that was negative for the presence of *B. pseudomallei*. Several isolated bacteria showed antimicrobial activities against *B. pseudomallei* when tested by agar well diffusion method. They were identified as species of *Bacillus amyloliquefaciens*. *B. amyloliquefaciens* strain KKU14 showed broad-spectrum inhibitory activities against a wide range of *B. pseudomallei* strains, including the drug resistant strains 316a and 316c. The unknown antimicrobial compounds produced by *B. amyloliquefaciens* KKU14 consisted of both non-ribosomal synthesized peptides and ribosomal synthesized peptides6. However, attempts to identify the peptides from *B. amyloliquefaciens* KKU14 by Liquid chromatography–mass spectrometry (LC-MS) has not yet succeeded (data not shown).

Antimicrobial peptides of LcI family is the most reported antimicrobial peptide from *B. amyloliquefaciens* available in current protein databases. The protein LcI is named according to the order of its elution peaks from a CM-52 chromatographic column[7,8]. The first reported LcI was from *B. subtilis strain* A014. It contains 47 residues with molecular mass of 5,460 Da. Its isoelectric point is 10.25, indicating that LcI is a cationic antimicrobial peptide. It is heat stable and resistant to trypsin, pepsin and lysozyme, but susceptible to pronase E and proteinase K[8]. Its structure was different from any β-structure AMPs, namely, it contains no disulfide bridge or circular structure[9]. The size of the LcI peptide and its properties indicate that it should be a Class II bacteriocin[10]. LcI showed very strong antagonistic activity against the Gram-negative pathogen *Xanthomonas campestris* pv. *oryzea* which causes rice leaf-blight disease. Moreover, LcI also has

antagonistic activity against Gram-negative bacterium *Pseudomonas solanacearum* strain PE1, and *Ralstonia solanacearum*[14] but it cannot inhibit *Erwinia carotovora* subsp. *carotovora* and *Escherichia coli*[8].

The aim of this study is cloning and investigating the antimicrobial activity of LcI against *B. pseudomallei* because even though *B. amyloliquefaciens* KKU14 showed antimicrobial activities against *B. pseudomallei*, the antimicrobial compounds is still unknown. LcI is hypothesized to be the effective antimicrobial compound because is the most reported antimicrobial peptide from *B. amyloliquefaciens* available. This work can be the first work towards systematic identification of antimicrobial peptide against *B. pseudomallei*.

Materials and Methods

Database search for a potential antimicrobial peptide

A list of antimicrobial peptides found in *B. amyloliquefaciens* was searched from GenPept database (NCBI). Data of the mostly reported peptide was used for cloning the corresponding gene from genome of *B. amyloliquefaciens* KKU14 by polymerase chain reaction (PCR).

Primer design and gene amplification

The primers were designed from the DNA sequence encoding antimicrobial peptide LcI of *B. amyloliquefaciens* strain C13 (FJ904931.1). Oligonucleotide properties including melting temperature, hairpins, dimers and mismatches were analyzed using the program OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer). The PCR primers consisted of forward and reverse primer sequences with restriction enzyme recognition site at their 5'-end. The restriction sites chosen for forward and reverse primers include *Nco*I /*Xho*I sites for pET28a, and *Bam*HI/*Hin*dIII sites for pET28a, pET32, and pQE31, as shown in Table 1. Gene amplifications were done by using RBC Taq DNA polymerase (Real Biotech Corporation (RBC), Taiwan) with a suitable PCR reaction and cycling parameters suggested by manufacturer's manual as shown in Table 2 and 3. Genomic DNA of *B. amyloliquefaciens* KKU14 was used as a template. The correct size of PCR product was verified by agarose gel electrophoresis. The PCR product was purified by QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

The expression vectors and the purified PCR product were digested by two restriction enzymes based on the vector and cloning site. The digested products were purified by QIAquick PCR Purification Kit (Qiagen) and then ligated to the plasmid by T4 DNA ligase (New England Biolabs) using a 1:5 molar ratio of vector and insert DNA, and incubated for 4 h at room temperature. The ligation product was further transformed into the competent *E. coli* DH5α cells by heat shock method[11].

Bacterial strains and vectors

Five bacteria used in this experiment were obtained from the department of Microbiology, faculty of Medicine, Khon Kaen university, Khon Kaen, Thailand as follows: *E. coli* DH5α was used as a cloning host, BL21 (DE3) was used as the host for protein expression processes, *R. solanacearu*m and *B. pseudomallei* strain P37 were used as indicator strains. *B. amyloliquefaciens* KKU14 was previously isolated from soil sample that contained no *B. pseudomallei*[6]. The expression vectors used in this study including pET28a (+) (Novagen), pET32a (+) (Novagen) and pQE31 (Qiagen, Hilden, Germany). Antibiotics used as selective markers for selections of recombinant clones include kanamycin and ampicillin based on vector (kanamycin for pET28a (+), ampicillin for pET32a (+) and pQE31) which were added in LB medium at 30 µg/ml and 100

Table 1 Primers and vectors for cloning of the *lcI* gene

Restriction sites	Primers	Sequence (5'-3')	Vectors
NcoI/XhoI	LcI-FW1	GATATACCATGGGCAAATTCAAAAAAGTGTTAACC	pET28a
	LcI-RV1	GGGGCGCTCGAGTTTATCTACACTTTCATAAATCC	
BamHI/HindIII	LcI-FW2	GGGCGCGGATCCATGAAATTCAAAAAAGTGTTAAC	pET28a pET32a
	LcI-RV2	CCGCCGAAGCTTTTATTTATCTACACTTTCATAAATTCC	
BamHI/HindIII	LcI-FW3	GGGCGCGGATCCGATGAAATTCAAAAAAGTGTTAAC	pQE31
		CCGCCGAAGCTTTTATTTATCTACACTTTCATAAATTCC	

Table 2 PCR reaction components

Components	Volume	Final concentration
10x reaction buffer	2 µl	1x
10 mM dNTP	0.2 ul	0.1 um
Primer mix (10 µM each)	0.4 ul	0.2 um
Template DNA (100 ng/ul)	1 ul	50-100 ng
RBC Taq DNA polymerase (5U/ul)	0.1 ul	0.5 units
ddH_2O	16.3 ul	
Total volume	20	

Table 3 PCR Reaction parameters

Segment	Number of cycles	Temperature	Time
Denature of template	1	94 °C	3 min
Denaturation		94 °C	1 min
Annealing	3	46 °C	30 sec
Extension		72 °C	2 min
Maintain	1	72 °C	10 min
Store O/N	1	4 °C	O/N

µg/ml final concentrations, respectively.

Cloning strategies of the *lcI* gene

Four constructs were designed to generate different features of the *lcI* gene products (Table 4).

Analysis of the inserted DNA

The inserted DNA was preliminary analyzed by the colony PCR method. Briefly, the selected clones grown on selective plate were suspended in 10 µl sterile deionized water, boiled in 100°C water for 5 min, centrifuged at 13,000 rpm for 1 min, and 1 µl supernatant was then used as DNA template. The inserted DNA was amplified using the same primer pair and PCR conditions with amplification of the expected gene. Recombinant plasmids from positive clones showing expected PCR product were isolated and analyzed by restriction analysis. The recombinant plasmid contained an expected size of inserted DNA was subsequently confirmed by DNA sequence analysis (Macrogen, Korea).

Protein expression and analysis

The confirmed recombinant plasmid with correct inserted DNA sequence was transformed into *E. coli* BL21 (DE3) by heat shock method. A single colony of the transformants was cultured in LB broth containing suitable antibiotic with shaking at 200 rpm, 37 °C for 16 h. The bacterial culture was diluted with LB medium (OD_{600} = 0.2), grown with shaking at 200 rpm until mid-exponential phase (OD_{600} = 0.6-0.8), and then 1 mM final concentration of isopropyl-β-d-thiogalactopyranoside (IPTG) (Sigma, St. Louis, Missouri, USA) was added. Cells were further cultured for 4 h at 37 °C. After that, 1 ml culture suspension was further separately harvested by centrifugation at 13,000 rpm for 5 min and 100 µl loading buffer was added into the precipitation before the mixture was boiled in 100°C water for 10 min. The treated suspension was centrifuged at 13,000 rpm for 5 min and 10 µl supernatant was subsequently analyzed by glycine-SDS-PAGE or tricine-SDS-PAGE for small peptide product as described previously by Hames (1998)[12] and Shangger (2006)[13], respectively.

Test of antimicrobial activity against *B. pseudomallei*

The induced recombinant cells cultured in 50 ml were centrifuged at 4,000 rpm for 15 min at 4°C and the pellets were resuspended in 5 ml 1x phosphate buffer saline (PBS) containing 0.5 mM EDTA, 0.5 mM PMSF and then lysed by ultrasonication on ice using 30% maximal amplitude pulse on 5 sec and off 5 sec for 6 min. Then 20 µl of the sonicated suspension was separated by centrifugation at 13,000 rpm for 10 min at 4°C. After that the expressed protein in lysed cells and supernatant fractions were checked by SDS-PAGE. The supernatant of the cell extracts was used for

Table 4 Cloning constructs and features of *lcI* gene products

Cloning constructs	Vector/ promoter	Restriction sites	Fusion protein	His-Tag position	Product size
1	pET28a/T7	*NcoI*/*Xho*I	-	C-terminal	~10 kDa
2	pET28a/T7	*Bam*HI/*Hind*III	-	N-terminal	~20 kDa
3	pET32a/T7	*Bam*HI/*Hind*III	Trioredoxin	N-terminal	~30 kDa
4	pQE31/T5	*Bam*HI/*Hind*III	-	N-terminal	~10 kDa

antimicrobial activity test by agar well diffusion method as described in previous experiment and directly detected on polyacrylamide gel as described previously[14] using *B. pseudomallei* strain P37 and *R. solanacearum* as indicator strains.

Results

Database searching for antimicrobial peptides of *B. amyloliquefaciens*

Three ribosomal synthesized antimicrobial peptides of the species *B. amyloliquefaciens* were reported in GenPept database including LcI, plantazolicin and subtilosin A. Among those predicted antimicrobial peptides, the antimicrobial peptide LcI was most frequently found, exhibited in 36 strains of *B. amyloliquefaciens* as showed in Table 5. LcI was therefore selected for cloning in this study. LcI is a small peptide containing 92-94 amino acid (~10 kDa) by which the first 25 amino acid was predicted as a signal peptide[14].

Amplification of the *lcI* gene

The *lcI* gene from the genome of *B. amyloliquefaciens* KKU14 was successfully amplified. Size of the expected PCR products was approximately 300 bp (Figure 1).

Analysis of inserted DNA and sequencing

Five random transformants from each construct were analyzed by colony PCR. PCR products with the size of approximately 300 bp were detected, which was the expected size of the *lcI* gene. When confirmed by restriction analysis, inserted DNA with the size of approximately 300 bp were found, indicating a successful cloning result. Two recombinant plasmids from clones of each construct including pET28a-LcI, pET32a-LcI, and pQE31-LcI were sequenced, and the correct open reading frame (ORF) of the *lcI* gene was confirmed. The *lcI* gene of *B. amyloliquefaciens* KKU14 contains 285 nucleotides encoding a 94 amino acid peptide with a molecular weight of 9.93 kDa comprising of 25 amino acid signal peptide and 69 amino acid mature peptide (7.38 kDa). It has an isoelectric point of 9.44 when calculated by ExPASy's Compute pI/Mw program. The gene showed 100% identity with the *lcI* gene of *B. amyloliquefaciens* strain Y14 (CP017953.1) and CAUB946 (HE617159.1), 99% identity with *B. amyloliquefaciens* strain FZB42 (CP000560.1) and C13 (FJ904931.1). This result confirms that the strain KKU14 was *B. amyloliquefaciens*.

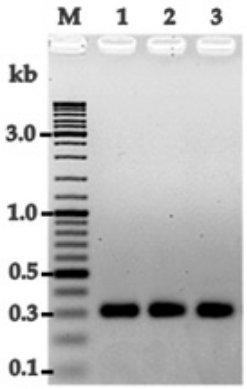

Figure 1 Agarose gel electrophoresis (1.5% agarose) represents the PCR products of the *lcI* gene. Lane M: DNA MW marker, lane 1-3: PCR products from different primer pairs; LcI-FW1 and LcI-RV1 (lane 1), LcI-FW2 and LcI-RV2 (lane 2), and LcI-FW3 and LcI-RV3 (lane 3).

Protein expression

To compare the expression of the recombinant peptide under different promoters and fusion tags, 3 different vectors were used in this work. The small peptide from the pQE31-LcI constructs was completely expressed in *E. coli* BL21 (DE3), while no expression was observed in the pET28a-LcI and pET32a-LcI constructs. The expressed peptide showed a size of approximately 10 kDa in tricine SDS-PAGE (Figure 2). Slight expression of recombinant LcI was also observed in uninduced cell.

Antimicrobial activity

Under initial experiment conditions, no antimicrobial activities against *R. solanacearum* and *B. pseudomallei* strain P37 were detected by agar well diffusion test so far. The supernatant with the expected LcI peptide exposed no antimicrobial activity against *B. pseudomallei* strain P37 (Figure 3).

Table 5 Antimicrobial peptides found in the species *B. amyloliquefaciens**

AMP name	Gene name	Number of strains	Accession no.	Predicted size (kDa)
LcI	*lcI*	*36*	AFJ60377.1	9.8
			KOS50567.1	9.9
			KTF59913.1, etc.	10.0
Plantazolicin	*pznA*	1	D3VML5.1	4.4
Subtilosin A	*sboA*	1	ABW83032.1	4.3

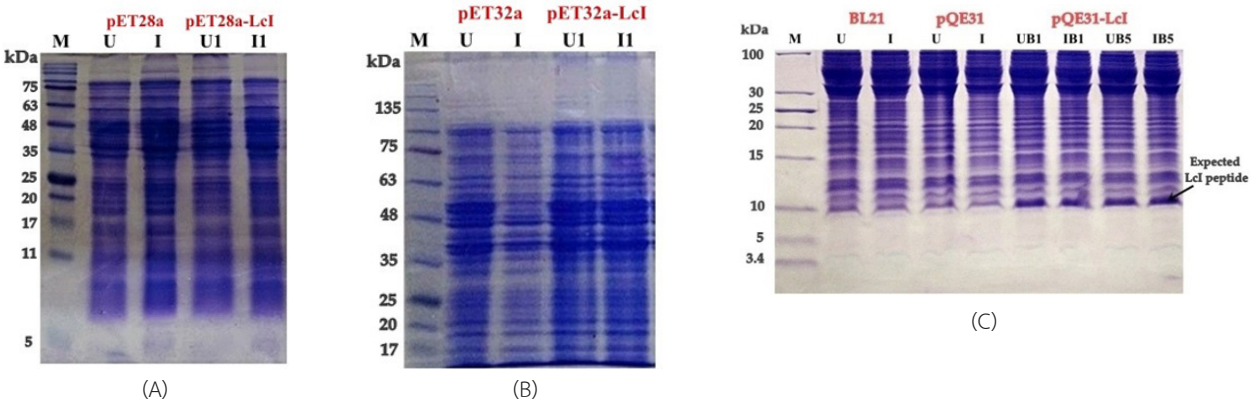

Figure 2 SDS-PAGE of the LcI protein expression using *E. coli* BL21 (DE3) as an expression host. a) pET28a-LcI, and b) pET32a-LcI (15 %, tris-glycine), c) pQE31-LcI (16 %T, 6%C, tris-tricine). U = uninduced clone. I = induced clone. B is *E. coli* BL21 (DE3) as negative controls. N and V are empty vectors as negative controls. 1 is recombinant clone 1, B1 and B5 are recombinant clone B1 and B5. Lane M: a protein molecular weight marker

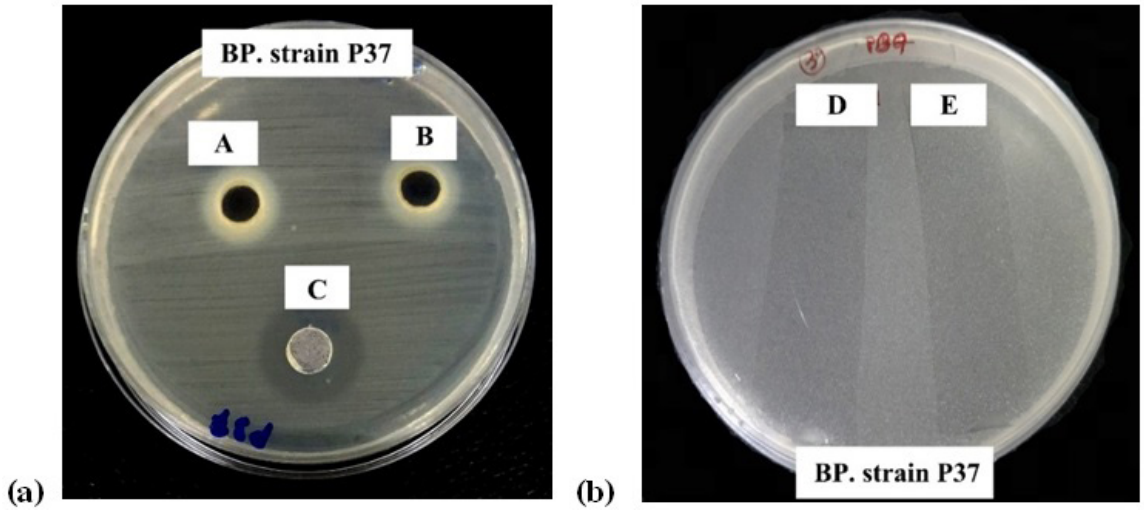

Figure 3 Antimicrobial activity test represented recombinant LcI from *B. amyloliquefaciens* KKU14 does not inhibit growth of *B. pseudomallei* strain P37. (a) Agar well diffusion: A was supernatant of sonicated pQE31-LcI clone, B was supernatant of sonicated pQE31 clone for negative control, C was ceftazidime for positive control. (b) In-gel overlay: D was supernatant of sonicated pQE31 clone for negative control, E was supernatant of sonicated pQE31-LcI clone.

Discussion

Since identification of the protein band observed in SDA-PAGE by mass spectrometry has not been successful yet, other attempt has been done to directly test some known antimicrobial peptides from *B. amyloliquefaciens*. According to GenPept database search result, the most frequently reported antimicrobial peptide of *B. amyloliquefaciens* was the LcI protein. Therefore, LcI was chosen to be cloned from *B. amyloliquefaciens* KKU14 into *E. coli* in this study. LcI peptides of *B. amyloliquefaciens* differ from those of *B. subtilis* A014 both in size and their encoding DNA sequences. LcI peptide of *B. subtilis* A014 consists of 47 amino acids, which refer to a peptide of a mass about 5.464 kDa. Whereas LcI peptides among *B. amyloliquefaciens* species contain 92-94 amino acids, resulting in a peptide with a mass of about 10 kDa, which was resistant to heat[14].

We successfully cloned the *lcI* gene of *B. amyloliquefaciens* KKU14 into various cloning vectors. However, despite using 3 different expression vectors including pET28a, pET32a and pQE31 with 4 constructs, the recombinant LcI could be expressed from only one construct. When LcI was cloned into pQE31 vector with N- terminal 6xhistidine tag, under the control of T5 promoter, an overexpressed protein with the size of 10 kDa was observed. However, slight expression of recombinant LcI was also observed in uninduced cell, indicating that the T5 promoter of pQE31 might not be tight enough to control the expression. Furthermore, N-terminal poly-His tag itself

might interfere with the expression of the downstream protein, which are in agreement with a report that a poly-His-tag placed at N-terminal region can induce overexpression of recombinant protein in *E. coli* under no induction conditions[15].

In this study, LcI fusion protein with N-terminal or C-terminal 6x histidine tag was not expressed in pET28a and pET32a constructs under the control of T7 promoter, although it was reported earlier to be expressed in this vector system[14]. One could argue that the T7 promoter might not suit the expression of LcI under our laboratory condition, since the protein was well expressed under T5 promoter of the pQE31 vector. Furthermore, host strains play a major role in the process of efficiently expressing large amounts of recombinant antibacterial peptides. Currently, although various constructs have been used for production of antibacterial peptides, difficulties have been experienced in antimicrobial peptide expression since they might be toxic to its expression hosts[16,17]. The fusion protein method was used for reducing the peptide's toxicity to improve the expression level[18]. However, a position of the fusion partner attaching to the amino or the carboxyl end of bacteriocins affected the antimicrobial activity[19]. The bacteriocin possessed antimicrobial activity when its amino end was attached to fusion protein[14,19]. As activity of some peptides has been linked to the penetrating function of the carboxyl end, this may provide an explanation as to why the bacteriocin attached to the carboxyl end fusions produce no activity[19]. Activities of recombinant proteins with the fusion tag maybe depend largely on the nature of proteins.

In our preliminary agar well diffusion test, no inhibition zones were observed when tested with *R. solanacearum* as positive control and *B. pseudomallei* strain P37. However, Hu and coworkers reported activity of recombinant LcI against *R. solanacearum*, when the peptide is expressed in pET32a vector, which contains histidine and additional thioredoxin tag[14]. The thioredoxin tag has been reported to facilitate protein folding[20]. This might suggest an improper folding of the recombinant peptide in our study since it was expressed in pQE31, which provides only a histidine tag to the recombinant protein. Further optimizations to gain properly folded and functional recombinant LcI peptide are needed to be done. Thereafter, the recombinant LcI protein can be purified, concentrated, then test for antimicrobial activity against *R. solanacearum* on polyacrylamide gels to confirm the activity of the expressed LcI protein. The antimicrobial test with *B. pseudomallei* will be done after a successful confirmation with *R. solanacearum*.

Conclusion

The recombinant peptide LcI cloned from *B. amyloliquefaciens* KKU14 does not inhibit the growth of *B. pseudomallei* strain P37 so far. This might due to improper folding of the recombinant peptide, or the antimicrobial peptide that was responsible for growth inhibition of *B. pseudomallei* might be other peptides or the effect could be involved by several peptides that work together synergistically, which has to be further investigated.

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