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ORIGINAL ARTICLE

Construction, heterologous expression, partial purification, and in vitro cytotoxicity of the recombinant plantaricin E produced by *Lactococcus lactis* against *Enteropathogenic Escherichia coli* K.1.1 and human cervical carcinoma (HeLa) cells

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Abstract

Lactobacillus plantarum produces bacteriocin called plantaricin that can kill or inhibit other bacteria. Plantaricin E (Pln E), a recombinant bacteriocin, has been successfully constructed and produced by a GRAS host, *Lactococcus lactis*. A polymerase chain reaction (PCR) overlapping technique has been used to construct a ligation of signal peptide gene, Pln A and bacteriocin encoding gene, Pln E. Furthermore, the fusion fragment were cloned into pNZ8148 vector and transformed into *L. lactis* NZ3900. Molecular expression study shows that recombinant *L. lactis* NZ3900 is able to express the mature pln E at transcription level with size of 168 bp. Plantaricin E is purified by ammonium sulphate precipitation followed by gel filtration chromatography. Purified fractions were proven to be active against *Enteropathogenic Escherichia coli* K.1.1. The other fractions of Pln E also have antibacterial activity against several Gram positive and Gram negative bacteria. Purified recombinant plantaricin E is 3.7 kDa in size. The cytotoxicity assay shows purified Pln E inhibits 46.949 ± 3.338% of HeLa cell lines on 10 ppm dose whilst the metabolite inhibits 53.487 ± 2.957% of HeLa cell line on 100 ppm dose. The IC_{50} calculation of Pln E metabolite is 107.453 ppm, while the purified protein is 11.613 ppm.

Keywords Plantaricin E · Gene fusion · Purification · Cytotoxicity assay · *Enteropathogenic Escherichia coli* K.1.1 · *Lactococcus lactis*

Introduction

Diarrhea is a serious health problem in the world especially in developing countries. In Indonesia, as one of the developing country, diarrhea has gained serious attention from the government because of its high morbidity and mortality rates. Besides, diarrhea ranked as the main cause of death on infants (31.4%) and toddlers (25.2%). Diarrhea can be transmitted through contaminated water and food. Poor sanitation also plays important role on transmitting this disease [1].

Escherichia coli is Gram-negative bacteria belongs to *Enterobacteriaceae* group, rod shaped, motile, aerobic facultative, and grows well on 37 °C. *E. coli* is harmless commensal of the gastrointestinal tract in human body, but there are several strains of *E. coli* that acquired several virulence attributes. The *Enteropathogenic E. coli* (EPEC) is a pathogenic *E. coli* strain and also the main cause of diarrhea among infants and toddlers. EPEC infections are known as attaching and effacing (A/E). The bacteria will attack the intestinal epithelial cell and cause drastic changes on cytoskeletal including the accumulation of polymerized actin directly beneath the adherent bacteria [2–5]. EPEC K.1.1 is an *E. coli* strain isolated from 55% feces sample of

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infants and toddlers severe diarrhea in Purwodadi, Depok, Ciamis, Ciawi, and Sambas Indonesia. These bacteria produced extracellular protease, have ability to degrade mucin substrate and show resistancy against tetracycline and amphicilin [6–8].

Bacteriocin is a ribosomal synthesized peptide produced by bacteria. It kills or inhibits other bacteria as a defense mechanism and a part of innate immunity to maintain their ecological niche. Bacteriocin mostly produced by Lactic Acid Bacteria (LAB). It has profitable traits, namely; natural, safe, nontoxic, and easily degraded by protease in human gastrointestinal tract; attracting many considerable interests for food preservatives, probiotic, and clinical application [9, 10]. Several bacteriocin have been found on previous researches, such as bacteriocin AMA-K produced by *L. plantarum* AMA-K [11], plantaricin ASM-1 produced by *L. plantarum* A-1 [12], and plantaricin IIA-1A5 produced by *L. plantarum* IIA-1A5 [13]. Several studies have shown the cytotoxic traits of bacteriocin against human cancer cell, such as a partially purified bacteriocin from *Pediococcus acidilacti* K2a2–3 against human colon adenocarcinoma (HT29) and human cervical carcinoma (HeLa) cell, bacteriocin GM3 produced by *Lactobacillus sakei* 3 against human colon adenocarcinoma cancer (HT2 plantaricin A produced by *Lactobacillus plantarum* C11 against human T cell leukimia cell line, and also nisin produced by *Lactococcus lactis* subspecies *lactis* against human breast adenocarcinoma cell line (MCF-7) and HepG2 [14–17].

From the previous study, Mustopa et al. successfully isolated *L. plantarum* S34 and *L. plantarum* S31 from bekasam, Indonesian fermented meat. *L. plantarum* S31 acts as the source of pln A signal peptide DNA sequence and has been characterized for its antimicrobial activity and protease activity. Whilst, *L. plantarum* S34 serves as the source of plantaricin E (Pln E) sequence and also has been characterized for its antimicrobial activity, protein stability, and its mode of action [18–20]. Moreover, from other previous study, Pln E has been cloned into *E. coli* host to improve the production yield [21].

Bacteriocin produced in the end of log phase or in early stationer phase and usually affected by the quorum sensing mechanism or the presence of stress. Plantaricin was encoded by pln gene and there were five operons in plantaricin loci named plnABCD, plnEFI, plnJKLR, plnMNOP, and plnGHSTUVW with different roles on plantaricin production. PlnABCD acts as regulatory operon. Pln EFI, plnJKLR, and plnGHSTUVW related to plantaricin biosynthesis and their cognate immunity. PlnGHSTUVW encodes the ATP binding cassette (ABC) transport system [10, 22].

Nowadays, several studies have found antibiotic resistance bacteria. To overcome this hindrance, we need a new substance that has no resistance effect to bacteria, such as bacteriocin. Bacteriocin has many properties which is suitable for alternative antibiotics. However, the lack of sufficient investment has been a significant problem to the medical application of bacteriocin, such as plantaricin. This condition leads us to one of the biggest challenge in bacteriocin research, which is the development of cost-effective approach for commercial production. Plantaricin produced by its native host has some limitations. Self-toxicity has prevented the production beyond its producer strain limit. Furthermore, the production yield will be low and the protein purification is an arduous task. To overcome these obstacles, some strategies such as genetically engineered producer strain and heterologous host need to be applied to improve the production yield. However, there are only several reports about recombinant bacteriocin, and most of them are using *E. coli* as a heterologous host. In this study, we are reporting for the first time that recombinant plantaricin E produced by a GRAS host, *L. lactis*. The *L. lactis*, well known as a safe host for its generally recognize as safe (GRAS) status also has been utilize for several biomedicines such as vaccines and antigens and other therapy agents [21, 23–29].

Thus, the aim of this study is to contrive a genetically engineered plantaricin producer strain by constructing a fusion of plnA signal peptide with Pln E, to express it using Nisin Controlled Expression (NICE) system, as well as to investigate suitable purification process. Besides, this study observes the antimicrobial activity of recombinant Pln E against EPEC K.1.1 and cytotoxicity trait against HeLa cells.

Materials and methods

Bacterial strains and growth conditions

Several bacteria strains and plasmids w required for this research. *E. coli* TOP 10 (Invitrogen) was used for standard cloning procedures. *E. coli* MC1061 (MoBiTec), a host strain with recA+ site, was used to stabilize recombinant plasmid. *E. coli* pGEM-T EF, source of Pln E gene, and *L. plantarum* S31, source of Pln A gene (kindly provided by Dr. Apon Zaenal Mustopa from LIPI). *L. lactis* NZ3900 (MoB c), a plasmid free host with LacF-pepN-nisRK sites, was used as heterologous host for recombinant gene, *Enteropathogenic E. coli* (EPEC) K.1.1 was used as indicator strain (kindly provided by Dr. dr. Sri Budiarti from IPB).

Plasmids required on this research: pGEM-T A for carrying Pln A gene; pNZ8148 with Cm^r site, an inducible expression vector with nisA promoter; pNZ8148 Sp_{pln} A–E with Cm^r site, a recombinant plasmid carrying SP Pln A and Pln E gene fusion.

Escheri ia coli TOP 10 grown in Luria–Bertani (LB) medium (10 g/L tryptone [Oxoid, England], 10 g/L sodium chlori Merck, Denmark], 5 g/L yeast extract [Oxoid, England]) supplemented with ampicillin 100 µg/mL (Bio Basic,

Canada) at 37 °C with agitation. *L. plantarum* S31 g[13]vn in deMann-Rogosa-Sharpe medium (Oxoid, England) at 37 °C without agitation. *L. lactis* grown in M17 medium (Himedia, India) supplemented with 0.5% glucose (Merck, Germany) at 30 °C without agitation. For recombinant *L. lactis*, A 10 µg/L chloramphenicol was added to media as a selection marker. Pathogenic EPEC K.[6] grown in Mueller–Hinton (MH) broth (Himedia, India) at 37 °C with agitation. Agar plates were made by addition of 1.5% (w/v) bacteriological agar (Oxoid, England) to the liquid medium.

[5]

Basic genetic techniques and enzymes

The isolation of genomic DNA from *L. plantarum* S31 was carried out using CTAB method with mo[73]cation [30]. Plasmid isolation of *E. coli* PGEM-Pln EF was carried out usin[53]esto Mini Plasmid Kit (Geneaid). Recombinant plasmid isolation from *L. lactis* pNZ8148-Pln E was carried out using a rapid method [31]. Competent cell preparation from *E. coli* MC1061 was adopting $CaCl_2$ method and plasmid introduction was carried out using heat shock method [32]. *L. lactis* electrocompetent cells preparation and plasmid introduction (el[5]troporation method) based on MoBiTec protocol, using Gene Pulser™ and Pulse Controller apparatus (Bio-Rad Laboratories, Hercules, CA). DNA restriction enzymes (*Nco*1 and *Hin*dIII) supplied by New England Biolabs, Inc (NEB) and Thermo Fischer Scientific, respectively. The T4 DNA ligase for ligation supplied by Kapa Biosystem.

Construction of expression vector for Pln E

The recombinant of Pln E was constructed by isolation of Pln A and Pln E genes as described previously, followed by an ov[25]apping PCR technique under the following conditions. Pre denaturation at 94 °C, 3 min; denaturation at 94 °C, 1 min; annealing at 54 °C, for Pln A, 58 °C for signal [24]tide, 57 °C for Pln E and SPpln A–E fusion, all of them for 1 min; extension at 72 °C, 30 s; and final extension at 72 °C for 6 min. Primer pairs SP-Pln A_F (CAACCCATGG CCATGAAAATTCAAATTA[18]GGT) and SP-Pln A.F_R (CGATTAAATCCACCTACT) were used to amplify a 66 bp SP A from plasmid pGEM-T Pln A (product 1). Primer pairs M-Pln E_F (AGTAGGTGGATTTAATCG) an[18]-Pln E_R (GATC*AAGCTT*TTAACGAATACTTTTCA) were used to amplify a 102 bp mature Pln E from plasmid pGEM-T Pln EF (product 2). Primer pairs SP-plnA F and M-Pln E_R were used to amplify a mixture of product 1 and 2 (a 168 bp, SP A-Pln E fragments).

Then, the fusion gene fragments were digested by *Nco*1-*Hin*dIII restriction enzymes and cloned into the pNZ8148 vector. Before inserting the gene into the vector, it was already adjusted beforehand by adding two base pair CC to allow translation fus[64]at *Nco*1 (CCATGG) site [26]. Thus, the ligation mixture were transformed into *E. coli* MC1061 to get a stable recombinant plasmid [33]. The precise clones containing pNZ8148-Pln E fusion was confirmed using PCR reaction and sequencing. Positive clones of pNZ8148 SP A-Pln E was isolated using Presto Mini Plasmid Kit (Geneaid) before transformed into *L. lactis* NZ3900 using electroporation method [31]. Selection of transformant was using LB agar with the addition of chloramphenicol. Positive clones of *L. lactis* transformant were screened by colony PCR using primer pairs Promoter8148 and Terminator8148.

DNA sequencing analysis was held by FirstBASE Laboratories[38]st BASE Company, Malaysia). Similarity sequence was performed by BLAST analysis on NCBI (http://www.ncbi.nlm.nih.gov/BLAST). DNA sequence's assembly and translation performed by DNAMAN version 4.0 (LynnonBiosoft).

Heterologous expression of recombinant Pln E

To express the protein at transcription level, RNA from *L. lactis* pNZ8148 SP Pln A–E were isolated using *Ambion® Totally RNA™ Total RNA Isolation Kit*. Purified RNA was qualitatively tested using 2% agarose gel electrophoresis. RNA concentration's value was determined by spectrophotometry at 260 nm. Obtained RNA were used for reverse transcription PCR (RT PCR) reaction at 45 °C, 30 min. The cDNA was amplified at following [10]nditions: pre denaturation at 94 °C, 3 min; denaturation at 94 °C, 1 min; annealing at 57 °C, 1 min; extension at 72 °C, 30 s; and final extension at 72 °C, 5 min. The result was visualized on 2% agarose gel [20].

Production of recombinant Pln E

The recombinant bacteria grown in a flask overnight at 30 °C in 10 mL GM17 medium contained chloramphenicol (100 µg/mL). The whole culture inoculated i[47]100 mL fresh GM17 + Cmp medium and grown at 30 °C until the optical dens[63](OD_{600}) of the culture reached 0.5. Protein expression induced by adding 10 ng/mL Nisin inducer (MoBiTec, Germany) and incubated [52]0 °C without agitation. After incubation period (5 h), the cells harvested by centrifugation at 16,000×*g* and the cell free supernatant (then [62]be called as metabolite) pH was adjusted into 6.5 using 1 M NaOH and stored at 4 °C before use [9].

Purification of Pln E

The purification of Pln E was conducted in ammonium sulphate (Merck, Germany) precipitation and gel filtration chromatography using Sephadex G-50 (GE Healthcare). Metabolites from 100 mL of *L. lactis* pNZ 8148 SP Pln A–E

obtained from earlier production brought to 60% saturation by adding ammonium sulphate and overnight incubation at 4 °C. Precipitated protein harvested by centrifugation at 16,000×*g*, 30 min at 4 °C and resulted in pellet which was solubilized into 1.5 mL by 25 mM Tris HCL buffer pH 7.4. For gel filtration chromatography, precipitated protein of Pln E was applied to Sephadex G-50 column equilibrated with 25 mM Tris HCl buffer (pH 7.4) at 4 °C. The column was eluted into 1 mL sample at flow rate of 40 mL/min. Protein concentration in collected fractions were determined by measuring the absorbance at 280 nm and the activity of collected fractions were measured using single disk diffusion assay against EPEC K.1.1 as indicator strain [34–36].

Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine SDS-PAGE) analysis

SDS-PAGE analysis performed by Tricine SDS 16% (v/v) polyacrylamide gel [37]. The fusion proteins suspended in SDS loading buffer and heated at 70 °C for 10 min. As much as 5 µL of SDS loading buffer loaded per 10 µl sample. After vertical electrophoresis, the gel stained with Silver Staining Kit (Thermo Scientific).

Protein concentration determination

Determination of protein concentration performed quantitatively using bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, USA) with bovine serum albumin (BSA) as the standard protein. Working reaction was made by mixing Reagent A and Reagent B with 1:50 ratio. Then, sample and working reaction were mixed in 96-well plates with 1:20 ratio. The plate was incubated at 37 °C for 30 min and the result was read at 540 nm using an ELISA reader.

Bacteriocin activity assay

Bacteriocin activity determination by the single disk diffusion assay using EPEC K.1.1 and other Gram positive and Gram negative bacteria as the strain indicator [35]. A 20–50 microlitres of fractions collected at each step of purification process spotted in a 6 mm diameter sterile paper disk (Filtres Fioroni, France). After the sample dried, paper disk transferred into soft media agars containing 1×10^8 CFU/mL strains indicator. After an hour incubation at 4 °C, the plates transferred into 37 °C incubation for 24 h. The activity of bacteriocin expressed as arbitrary units per mL (AU/mL) and calculated by:

$$\text{Bacteriocin activity (AU) mm}^2\text{/mL} = \frac{Lz - Ls}{V}$$

where LZ: clear zone area (mm^2); LS: well area (mm^2) and V: volume of sample (mL) [38].

Cytotoxicity Assay

The citotoxicity assay of Pln E was conducted in vitro using MTT assay method on HeLa cell line. Two samples of Pln E, a freeze-dried purified Pln E and encapsulated Pln E metabolites were prepared for this assay. HeLa cells were cultured on RPIM media, suplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin–streptomycin at a CO_2 incubator. The confluent cells were washed and harvested before they were cultured on a 96-well plates on 1×10^4 cells/well density. The confluent HeLa cells then washed and harvested with tripsin-0.01% EDTA before cultured on 96-well plate on 10^4 cell/100 µL density for 10–12 h. The cells then treated with various concentration of sample. After 24 h of incubation, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was added to each well. Plate was incubated for 4 h and 10% SDS was added as stopper and incubated for overnight. Then the absorbance was measured on 570 nm using an microplate reader (Thermo Scientific) [39].

Statistical data analysis

Data were expressed as mean ± standard deviation (SD) for three trials. Group of comparisons were performed using one-way analysis of variance (ANOVA) followed by Fisher's LSD test.

Results and discussions

Construction of expression vector for Pln E

Pln A is a pheromone peptide that can induce bacteriocin production in several bacteria strains [40]. Based on characterization of Pln A, we assume that this peptide is secreted outside the cells. The extracellular expression of Pln A proves that there is a leader sequence or signal peptide (SP) on Pln A gene. In this study, we isolate SP Pln A gene from *L. plantarum* S31 and generate 66 bp gene fragment after PCR amplification. Then, we also isolate Pln E from *E. coli* pGEM-T EF and generate 102 bp gene fragment (Fig. 1a).

The PCR overlapping technique was used for this construction vector and it generated 168 bp of fusion gene fragment of SP Pln A–E (Fig. 1a). SP Pln A gene is placed in front of Pln E gene because its function as signal peptide and can induce production of Pln E protein. To get a stable recombinant plasmid, we introduced the recombinant plasmid into *E. coli* MC1061. This step was intended to obtain higher transformation efficiency when we introduced the recombinant plasmid into *L. lactis* [41].

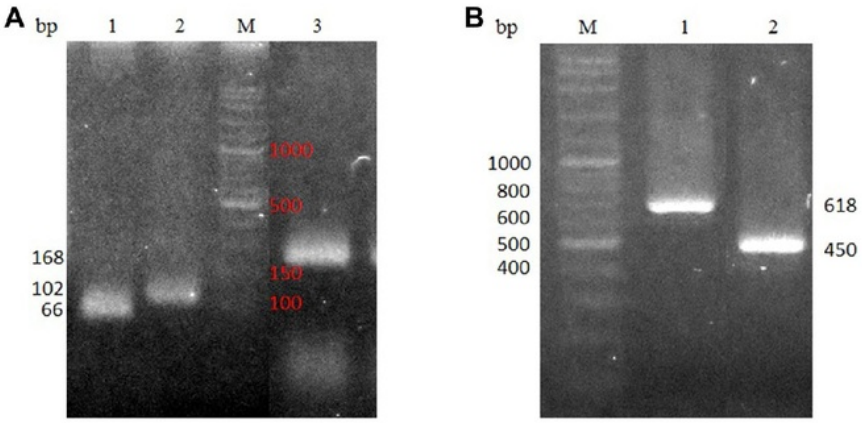

Fig. 1 Results of PCR amplification gene fusion **a** PCR product of 1: signal peptide pln A (66 bp) from pGEM-T pln A, 2: plantaricin E (102 bp) from pGEM-T pln EF, 3: fusion fragment containing the SP Pln A and mature plantaricin E (168 bp). **b** Identification of plasmid *L. lactis* NZ3900 (pNZ8148-SP_{plnA}-plnF) by PCR, 1: PCR product of gene fusion (618 bp), 2: negative control (*L. lactis* pNZ8148) (450 bp)

Heterologous expression of recombinant Pln E

The utilization of a compatible expression host vector on heterologous expression process is very important. On previous study, several expression vectors such as pMG36c, pMSP, and pNZ8048 on *L. lactis* host and pNZ8048 is the most effective one [9]. Hence, we chose to use pNZ8148 as the vector, because it's a derivative of pNZ8048. The pNZ8148 is a shuttle vector that can replicate in *E. coli* and LAB [9].

The SP Pln A–E on pNZ8148 is successfully transformed in *L. lactis*. *L. lactis* is a microorganism with large biotechnological potential, so it's a suitable host for heterologous production. *L. lactis* produces no endotoxins or other toxic substances, no inclusion bodies, no spores, no extracellular proteinaces and has a GRAS status (100% food grade). It also means that cellular component or enzymes can be used with only partial purification or directly in the crude cell extract without further purification steps [29]. *L. lactis* is also a good candidate for heterologous protein delivery in foodstuff or in the digestive tract [42].

We confirmed the transformation result by PCR reaction using F/R primer on recombinant *L. lactis*. *L. lactis* pNZ8148 SP Pln A–E is in a higher band (618 bp) compared to *L. lactis* pNZ8148 as control (450 bp) (Fig. 1b). The result confirms that the gene successfully inserted into vector. Another confirmation method, the DNA sequence analysis shows the start position (pNisA promotor) and end position (terminator) of transcription and translation, as well as the fusion gene that has been inserted according to *Nco*1-*Hin*dIII restriction sites, as shown on Fig. 2. Furthermore, RT-PCR reaction was used to investigate the heterologous expression of Pln E. The templates for this reaction are total RNA from *L. lactis* pNZ8148 SP Pln A–E under nisin induction and control strains which has been successfully isolated. The RT-PCR result shows a 168 bp sized band of Pln E gene fusion indicating the gene

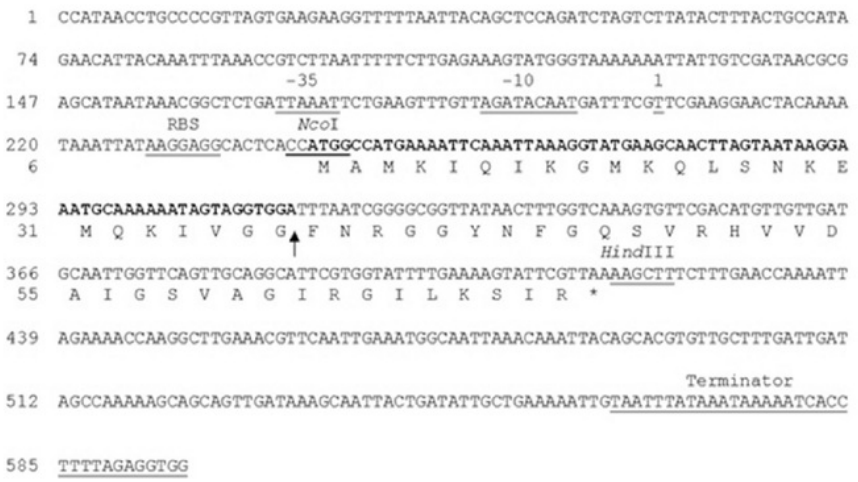

Fig. 2 DNA construction analysis of pNZ8148 SP Pln A-Pln E. Sequence analysis of plnE, RBS: Ribosom Binding Site; *: stop codon; +1: transcription start position. GxxxG motive on amino acid nos. 5–9 and 20–24

fusion of Pln E encoded by derivatives pNZ8148 has successfully induced by nisin and expressed on transcription process (Fig. 3).

Production and purification of Pln E

The first step to purify the protein starts with production. The genetically engineered protein Pln E is expressed on pNZ8148 vector, a NICE expression system vector, through an autoregulation mechanism. Induction with nisin stimulates the transcription process on nisA promoter through nisK and nisR regulon which are integrated to *L. lactis* DNA chromosome [43]. This process allows us to control the expression level by controlling nisin concentration. NisK gene encoded histidin kinase, and acts as a nisin receptor and proceed the response to nisR. The result of this response will create a transcription factor which activated nisA promotor, so the transcription process by RNA polymerase will be proceed [43]. If the concentration of nisin is too low, nisin cannot activate nisK gene in the host and if the concentration is too high, it has negative effect on *L. lactis* host by inhibit the growth of the bacteria [42].

An 100 mL of metabolite is obtained from *L. lactis* pNZ8148 SP Pln A–E culture. The metabolite was further concentrated by ammonium sulphate precipitation (60%, w/v) and after centrifugation process, 0.6 g of protein pellets obtained from this purification step. The pellet was loaded into a Sephadex G-50 matrix for gel filtration chromatography. Thirteen fractions (F1–F13), each fraction is 1 mL, was eluted from the column. There are two distinct peaks named

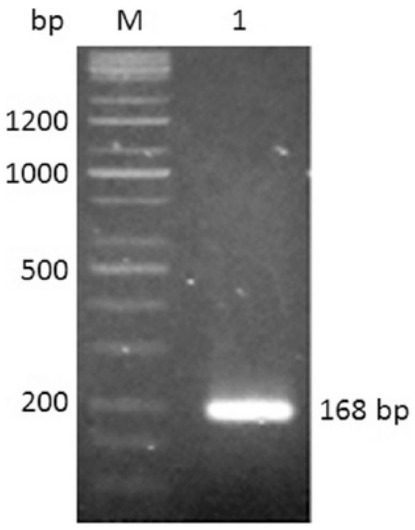

Fig. 3 Result of reverse transcription of mRNA *L. lactis* pNZ8148 fusion to examine transcription level. M: 100 bp DNA Marker; 1: Fusion fragment of SPplnA and mature plantaricin E

fraction 3 and fraction 9 (Fig. 4). Fraction 4–5 and fraction 7–8 have the same relative absorbance value, then the two fractions can be combined (Fig. 5).

Bicinchoninic acid assay (BCA assay) shows the protein concentration value decreased in every step of purification. The summary of purification process is presented on purification table, as shown on Table 1. The highest specific activity is in gel filtration chromatography step which is 787.83 AU/mg with purification fold 8.59, but the yield percentage shows a low recovery value, which is only 2.79%. This low value was obtained because only small amount of Pln E (1 mL) were eluted from the purification process compared to other purification steps. Because of the low yield, we cannot prove that gel filtration chromatography is effective in purifying and recovering most of bacteriocin.

The pln E yield is decreased a lot from supernatant to gel filtration chromatography (100–2.79%). The yield is quite low from other documentation 20ult. Borrero et al. [9] did several purification steps of enterocin P from *L. lactis* NZ9000 (with pMUPE plasmid) and hiracin JM79 from *L. lactis* NZ9000 (with pMUHE plasmid). The yield of enterocin P is increased into 130% from supernatant to ammonium sulphate precipitation and decreased into 36% in gel filtration chromatography. While the yield of hiracin JM79 is decreased into 30% in ammonium sulphate precipitation and 26% in gel filtration chromatography [44]. The yield differences might due to the bacteriocin type and plasmid that used in the study.

Another confirmation method to detect Pln E is using SDS-PAGE. In metabolite sample analysis, we compare metabolite from three different sources. The metabolites were obtained from *L. lactis* NZ3900 as host cell, *L. lactis* pNZ8148 SP Pln A–E without nisin induction, and *L. lactis* pNZ8148 SP Pln A–E with nisin induction (Fig. 4b). Metabolite from host cell do not show any protein band. It means *L. lactis* NZ3900 do not express any protein into metabolite. On the other hand, recombinant *L. lactis* which contained Pln E gene, secretes a protein in 3.7 kDa band size, as shown on Fig. 4b. Nisin induced sample shows thicker protein band than non-induced sample. It can be concluded that nisin can induce Pln E production. Purified Pln E from filtration gel chromatography fraction also shows a 3.7 kDa protein band on SDS-PAGE analysis (Fig. 4c). It means Pln E successfully purified even though within a small amount. Several studies also reported that the molecular weight of bacteriocins of lactic acid bacteria belonging to class II have molecular weight less than 10 kDa [45].

Bacteriocin activity and cytotoxicity assay

The inhibitory effect of each fraction in purification process was assayed using single disk diffusion assay against EPEC K.1.1 [26]. All fraction show inhibitory activity by

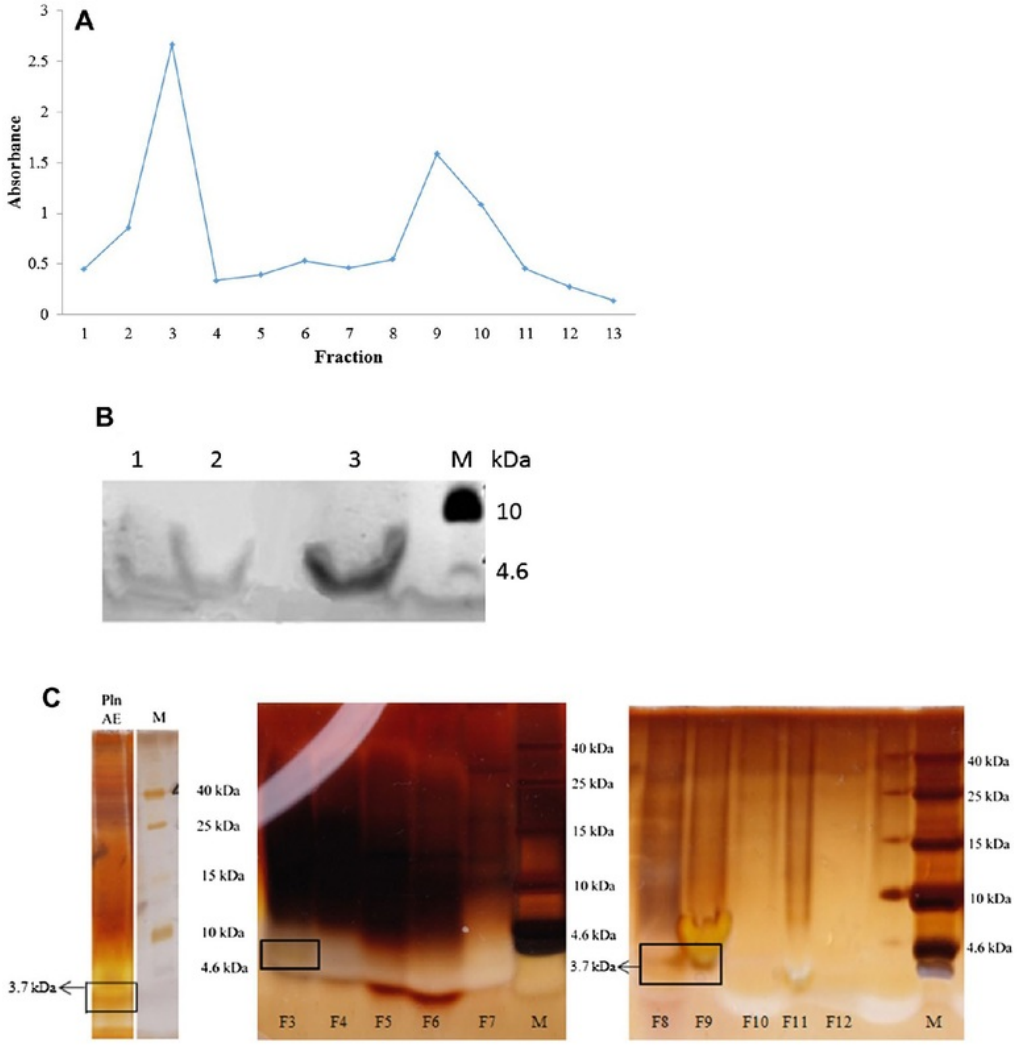

Fig. 4 Chromatogram profile of gel filtration chromatography and Tricine SDS PAGE analysis. **a** Gel filtration chromatography of pln E, measured on 280 nm; **b** Tricine SDS-PAGE of (1) Host cell (*L. lactis* NZ3900); (2) Non induction; (3) *L. lactis* recombinant Pln E with nisin induction; **c** 1. Tricine SDS-PAGE of culture supernatant and purified recombinant Pln E (fraction 3–12) from *L. lactis* pNZ8148

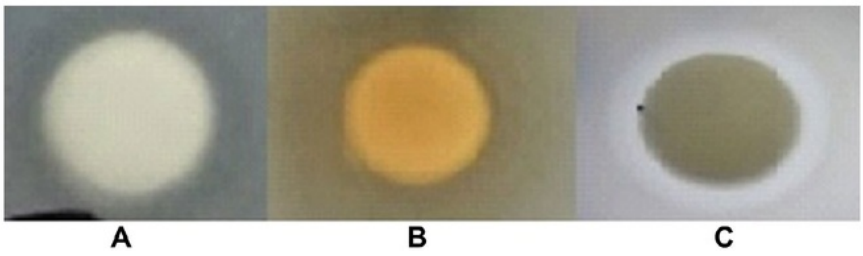

Fig. 5 Antimicrobial activity of crude extract Pln E as determined by the agar well diffusion test against Enteropathogenic *Escherichia coli* (EPEC) K.1.1. **a** Metabolite sample; **b** Pellet from ammonium sulphate precipitation; **c** Purified Pln E from fraction 7–8 of gel filtration chromatography

Table 1 Purification summary of recombinant plantaricin E

Purification steps	Volume (mL)	Total protein (mg)	Total activity (AU)	Specific activity (AU mg^{-1})	Purification fold	Yield (%)
Culture supernatant	100.00	556.62	51025.00	91.67	1.0	100.00
Ammonium sulphate precipitation	1.50	17.49	5004.38	286.13	3.12	9.81
Gel filtration	1.00	1.81	1422.81	787.83	8.59	2.79

forming a clear zone formation (data not shown). Fraction 7–8 from gel filtration chromatography forms a bigger clear zone means more inhibitory activity compared to another fractions. The antimicrobial activity of plantaricin against Gram-negative bacteria has been reported in several previous researches such as Plantaricin K25 against *Bacillus cereus* [46]; Plantaricin IIA-1A5 against EPEC K.1.1, *Salmonella* 3[illegible]nd *Shigella* A33 [13]; and Plantaricin KL-1Y against *Salmonella enterica* serovar *enteriditis* DMST 17368, *Pseudomonas aeruginosa* ATCC 15442 and 9027, *E. coli* O157:H7, and *E. coli* ATCC 8739 [47].

Pln E, in various forms; metabolites, pellets, and fractions from gel filtration chromatography process show an ability to inhibit the growth of EPEC K.1.1, a Gram negative bacteria without th[illegible]ddition of some substances. In previous study, Pln E was able to inhibit the growth of Gram negative bacteria if the outer membrane is weakened with some help from EDTA [48]. On the other hand, in this study, Pln E able to inhibit the growth of EPEC [illegible].1 without the addition of EDTA. Besides, several Gram negative and Gram Positive bacteria strain has been used as strain indicator. Inhibitory activity is detected in all indicator strain, even though it shows different affectivity. We calculate the bacteriocin activity value using Abbasiliasi formula [38]. The result shows that Pln E can inhibit several pathogenic bacteria with the highest activity against EPEC col 5, as shown on Table 2.

In this research, we used MTT assay method to discover the cytotoxicity traits of Pln E. MTT assay method is suitable for measuring of drug sensitivity in established cell lines as well as primary cells. HeLa cell line is a co[illegible]nuous cell line derived from human cervix cancer cell. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into a formazan crystal form, which can be solubilized for homogenous measurement. This conversion can only be done by a living cell. Mitochondria play[illegible] very important part here because the conversion will determine mitochondrial activity. For most of cell population, mitochondrial activity related to the numbe[illegible] viable cell. Therefore, the number of viable cells were used to measure in vitro [illegible]otoxicity effect of drugs. Microplate reader on 570 nm was used to determine the number of viable cells and later the percentage of cell mortality [49].

The HeLa cell lines with 10×10^4 cell/well densities were treated with different dose of freeze dried purified Pln E and encapsulated metabolites of Pln E. The IC_{50} calculation, as shown on Table 3, shows that purified Pln E saves to be consumed under the dose of 11.613 ppm and inhibits $46.949 \pm 3.338\%$ of HeLa cell lines on 10 ppm dose. It means purified Pln E can inhibit the growth of HeLa cell

Table 2 Antimicrobial activity of recombinant plantaricin E against pathogenic bacteria

Indicator organism	Strain[a]	Medium-incubator temperature (°C)	Sensitivity[b]
EPEC	K.1.1	MHB, 3[illegible]	++
Bacillus cereus	ATCC 10876	BHI-G, 37	++
Staphylococcus aureus	ATCC 6538	NB, 37	++
Escherichia coli	ATCC 8739	NB, 37	++
Listeria spp.	col 12, our strain collection	NB, 37	+
Coliforms	col 2, our strain collection	NB, 37	++
EPEC	col 5, our strain collection	NB, 37	+++
EPEC	col 10, our strain collection	NB, 37	++
Staphylococcus spp.	col 3, our strain collection	NB, 37	++
Listeria monocytogenes	BTCC B693	NB, 37	+

[a] *ATCC* American type culture collection, *BTCC* biotechnology type culture collection

[b] No inhibitory zone was observed; +: ≤9 mm inhibition; ++: 9–12 mm inhibition; +++: ≥13 mm

Table 3 Cytotoxic effect of Pln E on HeLa cells

Purification steps	Concentration (mg/mL)	O.D. of sample	% Mortality	IC_{50}
Culture supernatant	1	0.681 ± 0.014	− 18.801 ± 2.400	107.453
	10	0.746 ± 0.057	− 30.161 ± 9.889	
	50	0.643 ± 0.013	− 12.158 ± 2.311	
	100	0.266 ± 0.017	53.487 ± 2.957	
Gel filtration	0.5	0.586 ± 0.012	− 2.245 ± 2.115	11.613
	1	0.668 ± 0.027	− 16.485 ± 4.805	
	5	0.577 ± 0.028	− 0.628 ± 4.834	
	10	0.304 ± 0.019	46.949 ± 3.338	

line within only small dose. On the other side, Pln E metabolite saves to be consumed under the dose of 107.453 ppm and inhibits 53.487 ± 2.957% of HeLa cell line on 100 ppm dose. Pln E obtained from metabolite needs a higher dose to inhibit 50% of HeLa cell's growth. It may due to the activity of other substances in the metabolite. Other substances could act as an antagonist toward Pln E. Therefore, the cytotoxicity of metabolite is not as good as purified Pln E. Nowadays, not many research has been conducted to determine the cytotoxicity value of bacteriocin. In general, the observation of the cytotoxicity traits of Pln E against HeLa cell lines added functionality of this recombinant bacteriocin.

Conclusion

It can be concluded that Pln E successfully fuses with plnA signal peptide, inserts on pNZ8148 plasmid, and transforms in *L. lactis* NZ3900. Pln E also can inhibit Gram negative bacteria without the addition of other substances that could weaken the outer membrane of the bacteria. Besides that, Pln E may provide an alternative treatment for diarrhea and cervical cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human or animal subjects performed by any of the authors.

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