

Inhibitory Activity of *Lactobacillus plantarum* U10 Isolated from Tempoyak (Fermented Durian) Made in Indonesia against *Salmonella typhi*

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Lactobacillus plantarum U10 produced bacteriocin was isolated from a traditionally fermented food “tempoyak” from Sumatera Island in Indonesia. Production of the bacteriocins started at early exponential phase and reached maximum level at early stationary phase. Furthermore, plantaricins U10 was purified by ammonium sulphate precipitation followed by gel filtration chromatography. *L. plantarum* U10 produced two bacteriocins with a molecular mass of approximately 4.5 and 9.8 kDa by SDS-PAGE. The mode of action of plantaricins U10 was identified as bactericidal agents against *Salmonella typhi* ATCC25241 as proven by CFU counting and SEM micrographs that showed differences in cell structures between treated cells and the non-treated control. SEM examination also confirmed structural destruction of membrane cells integrity and considerable morphological alteration of *S.typhi*.

Key words: bacteriocin, *L. plantarum* U10, mode of action, purification

Lactobacillus plantarum U10 yang diisolasi dari makanan tradisional fermentasi “tempoyak” berasal dari Pulau Sumatera di Indonesia menghasilkan bakteriosin. Produksi bakteriosin dimulai pada awal fase eksponensial dan mencapai titik maksimum pada awal fase stasioner. Selanjutnya, plantarisin U10 dimurnikan melalui presipitasi amonium sulfat dilanjutkan dengan kromatografi filtrasi gel. *L. plantarum* U10 menghasilkan dua bakteriosin dengan bobot molekul berkisar 4,5 dan 9,8 kDa berdasarkan SDS-PAGE. Mekanisme aksi plantarisin U10 telah berhasil diidentifikasi yaitu memiliki sifat bakterisidal terhadap *Salmonella typhi* ATCC25241 yang dibuktikan dengan jumlah CFU dan SEM mikrograf yang menunjukkan perbedaan struktur sel antara sel dengan perlakuan dan kontrol. Pemeriksaan menggunakan SEM juga menunjukkan kerusakan integritas struktur membran sel dan perubahan morfologi sel.

Kata kunci: bakteriosin, *L. plantarum* U10, mekanisme aksi, pemurnian

Tempoyak is a fermented dish that originated from Sumatera Island. It is mainly made from unpasteurised durian (*Durio zibethinus*) flesh mixed with 2.5% salt (w/v) and placed in a sealed container (Urnemi *et al.* 2010). Dominant taste of Tempoyak is acid due to fermentation process in durian flesh. Tempoyak is usually consumed as a side dish when eating time. Tempoyak is not only found in Sumatera but also in other parts of Kalimantan Island with different names. In addition, this food is also known in Malaysia as well (Amiza *et al.* 2006).

The main lactic acid bacteria (LAB) strains isolated from tempoyak is *Lactobacillus* spp (Leisner *et al.* 2001; Coeuret *et al.* 2003). Recently, many investigators have shown that various fermented food-isolated *Lactobacillus plantarum* species isolated could be considered as molecular resources for various end products such as bacteriocins, biomass,

polysaccharides, and lipid for instant *L. plantarum* KLDS 1.0391 isolated from “Jiaoke” (Gong *et al.* 2010); *L. plantarum* S34 from “Bekasam” (Mustopa *et al.* 2010); *L. plantarum* LB-B1 isolated from “Koumiss” (Xie *et al.* 2011); *L. plantarum* GJ7 isolated from “Kimchi” (Chang and Chang 2011), and *Lactobacillus* isolated from cheeses and yogurts (Yang *et al.* 2012).

The most end products from these LAB which could be benefit for medicinal purpose is bacteriocins (Gillor *et al.* 2005). This is secondary metabolites of lactic acid bacteria are ribosomally synthesized peptides that are active against similar or genetically close related bacteria (Hata *et al.* 2010).

Salmonella enterica serotype Typhi (*S. typhi*) is the bacteria that caused typhoid fever (Bhan *et al.* 2005). Typhoid fever occurs both in the tropical and subtropical countries, especially in developing countries where improperly sanitary conditions and inadequate health facilities commonly found (Kidgell *et al.* 2002). The Incidence of typhoid fever in the

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world is estimated at more than 2.16 million cases with a mortality rate of 216000 and more than 90% of them occur in Asia. In Indonesia itself the estimated incidence is 180 / 100000 per year in children aged 5-15 years (Ochiai *et al.* 2008).

L. plantarum U10 from Tempoyak (fermented durian fruit) has previously been isolated in our lab (Urnemi *et al.* 2010). Moreover, this strain also showed broad adaptive response to environmental stressors (Margareta *et al.* 2015). Furthermore, this strain exhibited good antibacterial activity against pathogenic bacteria such as *E. coli* NBRC 14237, *Staphylococcus aureus* NBRC 13276 and *Bacillus subtilis* BTCCB 612, thus this strain has potential application as natural antibacterial agent (Urnemi *et al.* 2010). However, until recently nothing is known about the antibacterial activity of *L. plantarum* U10 against typhoid infection. Therefore, the objective of this study was to purify the bacteriocin from the bacterial strain and to provide a preliminary investigation of its mode of action against *S. typhi*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Strain U10 (collection of Research Center for Biotechnology, Indonesian Institute of Science), the bacteriocin producer strain used in this study, was isolated from Tempoyak, a traditionally fermented food from Indonesia. It was cultured in deMan Rogosa Sharpe (MRS) broth (Oxoid, England), and incubated at 37 °C. Changes in pH and optical density (600 nm) were recorded every 2 h for 32 h without agitation. Bacteriocin activities were measured at the same time intervals. Pathogenic bacteria used is *S. typhi* ATCC25241 grown in Nutrient Agar (NA) containing pepton 1% (Oxoid, England), Beef Extract 0.3% (HIMEDIA, India), NaCl 0.5% (Merck, Germany), and Agar 1.5% (Oxoid, England) as slab cultured stock were stored at 20 °C. Subcultured twice in the media Nutrien Broth (NB) containing Pepton 1% (Oxoid, England), Beef Extract 0.3% (HIMEDIA, India), and NaCl 0.5% (Merck, Germany) and was incubated at 37 °C for 14 h with agitation before use.

Bacteriocin Activity. Bacteriocin activity was determined by the well-diffusion assay. Cell-free supernatants adjusted to pH 6.5 by the addition of sterile 1N NaOH using a digital pH meter (Eutech pH510), and treated with catalase (1 mg mL⁻¹) to exclude the inhibition due to hydrogen peroxide production. Three hundred supernatants were then

spotted onto paper discs (diameter 6 mm; Filtres Fioroni, France) and loaded onto soft agar plates. Soft agar media containing the Gram-positive and Gram-negative bacteria strains were then poured into the plates. These plates were incubated at 37 °C and examined for inhibition zones. In some experiments, antibacterial activity was expressed as arbitrary units (AU). To obtain the titer (AU mL⁻¹), serial dilutions of bacteriocin were prepared and dispensed in wells. The titer (AU mL⁻¹) was defined as the reciprocal of the highest dilution which gave a definite zone (Xie *et al.* 2011).

Production of Crude Bacteriocin. Cell-free supernatants for antibacterial assay was prepared by growing *L. plantarum* U10 isolates (1%, v/v) into 300 mL MRS broth medium and incubated at 37 °C for 20 h. The cells were harvested with centrifuged at 12000 g, 4 °C for 15 min (Xie *et al.* 2011). The plantaricins were purified from the cell free supernatant by ammonium sulphate precipitation and gel filtration Sephadex G-50 (GE Healthcare, Sweden). Ammonium sulphate was added until 80% saturation at 4 °C. The precipitated protein was centrifugated at 12000 g, 4 °C for 15 min and the resulting pellets were solubilized by buffer Tris-HCl 10 mM pH 7.4.

Purification of Plantaricin U10 and SDS-PAGE. For column chromatography, crude plantaricin U10 was applied to a Sephadex G-50 column (25 cm with diameter 2 cm) equilibrated with 50 mmol Tris-HCl buffer (pH 7.4) at 4 °C. The column was eluted into 5 mL fractions at a flow rate of 1 mL min⁻¹. Protein concentrations in collected fractions were determined by measuring the absorbance at 280 nm and bacteriocin activities in the collected fractions that were measured. The molecular weight of the purified plantaricin U10 was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Gels consisted of a 3.9% (v/v) stacking gel and 16% (v/v) separating gels that were 1 mm thick, 9 cm long, and 9 cm wide. Low range protein ladder (Thermo, Spectra Multicolor, EU) was used as the molecular size standard and stain with Silver Staining Kit (Fermentas, EU).

Determination of Protein. Determination of protein concentration performed quantitatively using bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, US) with bovine serum albumin (BSA) as the standard protein using a microplate. Microplate was incubated at 37 °C for 30 min, then the reaction proceeds read at a wavelength of 540 nm using an ELISA reader (Pierce 2013).

Mode of Action Plantaricin U10. To study the effect of the antibacterial compound on *S. typhi* ATCC25241, 10 mL cell-free supernatants of *L. plantarum* U10 was adjusted to pH 6.5. A treatment with catalase (1 mg mL⁻¹) was added to the 50 mL culture of *S. typhi* ATCC25241 at early exponential phase and then incubated at 37 °C. Samples were taken every 2 h to record the optical density at 600 nm and determine the viable cells (CFU mL⁻¹) by counting CFU on nutrient agar plates after incubation at 37 °C overnight. All experiment were carried out in triplicate (Xie *et al.* 2011).

Scanning Electron Microscope (SEM) Analysis. *S. typhi* ATCC25241 bacteria was suspended in plantaricin and later incubated for 24 h at 37 °C. The solution was centrifuged, the supernatant discarded, 2% glutaraldehyde added and soaked for several hours. After the solution was centrifuged, the supernatant was discarded, added a solution of 2% tannic acid was added and soaked for 2 h. The solution was centrifuged, fixative solution was discarded, caccodylate buffer was added and soaked for 20 min. Then solution was

centrifuged, buffer was discarded, 1% osmic acid was added and soaked for 1 h and subsequently dehydrated with a graded ethanol series. The samples were lyophilized, coated with gold in an ion coater, and then examined by scanning electron microscopy JEOL JSM-5310 LV series (Zengin and Baysal 2014; Wang *et al.* 2014).

RESULTS

Antibacterial Activity Spectrum. The antibacterial activity spectrum of cell-free supernatant obtained from 20 h *L. plantarum* U10 culture was assayed using wells diffusion agar methods against a wide range of microorganisms (Table 1). The antibacterial activity of cell-free supernatant not only give clear zone for Gram-positive bacteria but also for Gram-negative bacteria. Based on this observation *L. plantarum* U10 was able to inhibit several bacteria and food-borne pathogens, such as *Bacillus subtilis*; *Escherichia coli*, and *Pseudomonas aeruginosa*. Particularity *Salmonella typhi* and *Staphylococcus*

Table 1 Antibacterial activity spectrum of plantaricin U10 produced by *Lactobacillus plantarum* U10

Species	Strain	Source ¹	Diameter (mm) of zone of inhibition ²
<i>Salmonella typhi</i>	ATCC 25241	ATCC	11.33 ± 1.15
<i>Staphylococcus aureus</i>	ATCC 6538	ATCC	11.00 ± 0.00
<i>Bacillus subtilis</i>	ATCC 19659	ATCC	9.33 ± 0.58
<i>Pseudomonas aeruginosa</i>	ATCC 15442	ATCC	9.67 ± 0.58
<i>Escherichia coli</i>	MC	Mobitech	-
<i>Escherichia coli</i>	DH5α	Mobitech	9.33 ± 0.58
<i>Escherichia coli</i>	TOP10	Mobitech	8.67 ± 0.58
<i>Staphylococcus epidermis</i>	-	Lab stock	12.67 ± 0.58
<i>Salmonella typhi</i>	-	Lab stock	10.33 ± 0.58
<i>Listeria monocytogenes</i>	BTCC B693	BTCC	10.67 ± 0.58
<i>Lactobacillus plantarum</i>	S34	Lab stock	-
<i>Lactobacillus fermentum</i>	U11	Lab stock	-
<i>Lactobacillus plantarum</i>	S12	Lab stock	-
<i>Lactobacillus acidophilus</i>	C _{9,9}	Lab stock	-
<i>Pediococcus pentosaceus</i>	Gr3	Lab stock	-
<i>Pediococcus acidilactici</i>	S23	Lab stock	-
<i>Pediococcus acidilactici</i>	R24	Lab stock	-
<i>Lactococcus lactis</i>	LAC 2	Lab stock	-
<i>Lactococcus lactis</i>	Nz3900	Mobitech	-

¹ ATCC, American Type Culture Collection; BTCC, Biotechnology Type Culture Collection.

² Wells (6 mm in diameter) were filled with 25 µl samples cell-free supernatant which pH neutralized to 6.5 and H₂O₂ eliminated; mean counts of tri-trials (mean ± SD); "-" no inhibitory zone was observed.

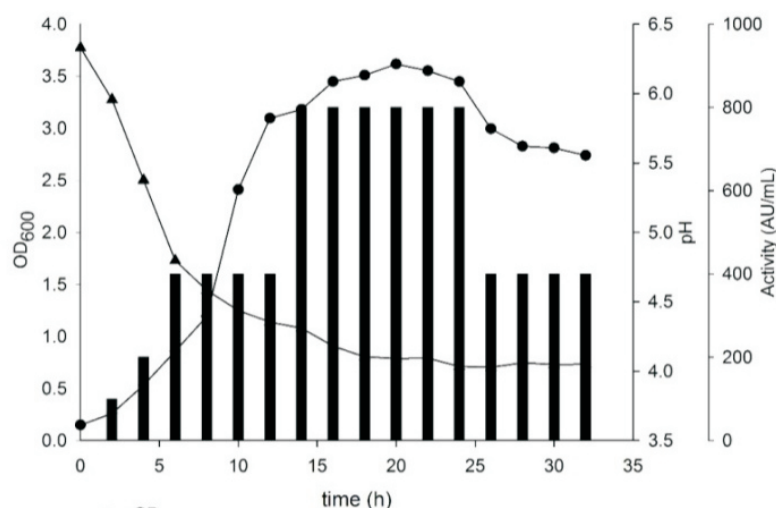


Fig 1 Bacteriocin production during the growth of *Lactobacillus plantarum* U10 in MRS broth at 37 °C. The optical density (absorbance at 600 nm: ●) and pH (▲) of culture were measured at the time intervals as indicated. The antibacterial activity (■) was also assayed and expressed as AU mL⁻¹.

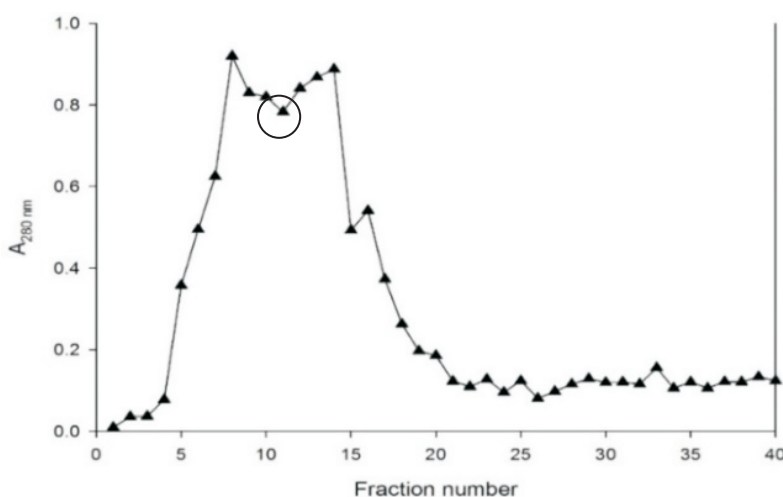


Fig 2 Elution profile of purified plantaricin U10 on Sephadex G-50 column monitoring by absorbance at 280 nm. The purified plantaricin is shown by a circle.

aureus both of them were more sensitive to *L. plantarum* U10's cell-free supernatant treatment.

Biomass and Bacteriocin Production. Biomass measurement and bacteriocin production result are shown. *L. plantarum* U10's cell density increased from 0.1 up to 3.6 (OD₆₀₀) during 20 h at 37 °C and gradually decreased until 2.7 (OD₆₀₀) when incubation was continuous until 32 h. For pH medium, it decreased rapidly from 6.5 to 4.0 at the same period of measurement. Furthermore, plantaricin U10 production was started at the early exponential phase and reached maximum (800 AU mL⁻¹) after 20 h of incubation (early stationary phase) when cells biomass was at optimum OD (Fig 1).

Purification and Molecular Weight of Plantaricin U10. Plantaricin from cell-free supernatant was collected by centrifugation, then

proteins were concentrated by 80% ammonium sulphate precipitation, followed by Sephadex G-50 gel filtration column. All procedures were done in cold room. Forty fractions (F1-F40) with 2 mL each were succeeded to be eluted from a Sephadex G-50 column and showed two distinct peaks at fraction number 8 and 14 respectively (Fig 2) and were further sub-fractioned into six sample named SI (F8 and F9), SII (F10 and F11), SIII (F12-F14), SIV (F15), SV (F16 and F17), and SVI (F18-F20). Antagonistic activity of each sample was assayed using *S. typhi* ATCC25241 as bacterial indicator, yet only in sample II (F10-11) showed inhibitory activity as by clear zone formation (data not shown). Purification indicators after two steps of purification was 61.07 fold and 33.25% for yield, as summarized in Table 2. The partially purified plantaricins was analysed by the SDS-PAGE for

Table 2 Purification of plantaricin U10

Step	Volume (mL)	Total Activity (AU)	Total Protein (mL)	Specific Activity (AU mg ⁻¹)	Purification (fold)	Yield (%)
Cell free supernatant	300	800	5286	0.15	1	100
Ammonium sulphate precipitation	6	400	177.60	2.25	14.88	5.0
Gel filtration	3	133	14.39	9.24	61.07	33.25

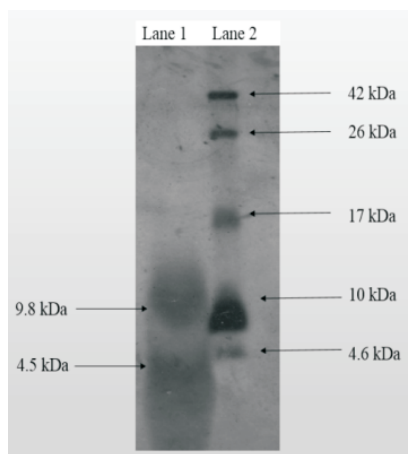


Fig 3 SDS-PAGE analysis of bacteriocin produced by *Lactobacillus plantarum* U10. Fermentas silver stained gel, Lane 1: partially purified crude extract plantaricin, and Lane 2: rainbow molecular mass markers.

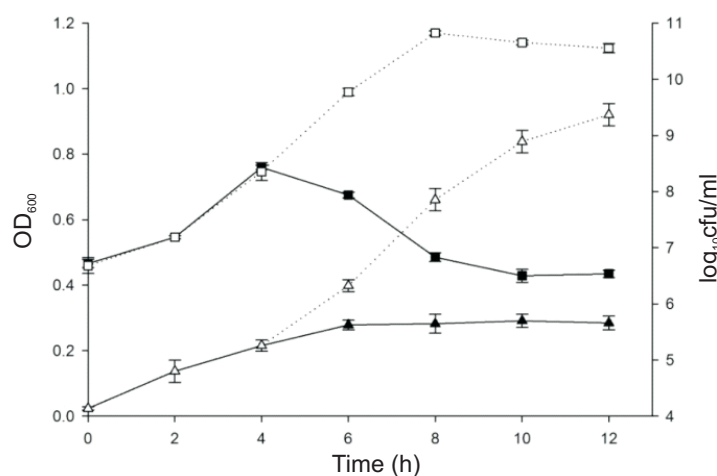


Fig 4 Mode of plantaricin U10 against *Salmonella typhi* ATCC 25241. ▲ and △, optical density measured at 600 nm presence plantaricin and absence plantaricin, respectively; ■ and □, Log CFU mL⁻¹ counts of the indicator strain treated presence plantaricin and absence plantaricin, respectively. The arrow indicates the time of addition of the plantaricin (4 h). the experiment was conducted in triplicate, the error bars were standard deviation (SD).

determination of the molecular weight (Fig 3). It clearly exhibited two protein bands with a molecular mass of approximately 4.5 and 9.8 kDa.

Mode of Plantaricin Action. After the addition of plantaricin U10 to an exponentially growing of *S. typhi* ATCC25241 culture, OD values of the treated culture demonstrated no significant increment or relatively constant. On the other hand, the addition of plantaricins U10 into the same condition caused dramatically

decrease in viable cells number (from 2.2×10^8 CFU mL⁻¹ to 3.1×10^6 CFU mL⁻¹) over the following 9 h observation (Fig 4). These results suggested that the mode of plantaricins U10 action was bactericidal effect.

Morphological Changes of Plantaricin U10 Exposed - *S. typhi*. *S. typhi* ATCC25241 were treated for 24 h with plantaricin (133 AU mL⁻¹) then observed by SEM to examine morphological changes

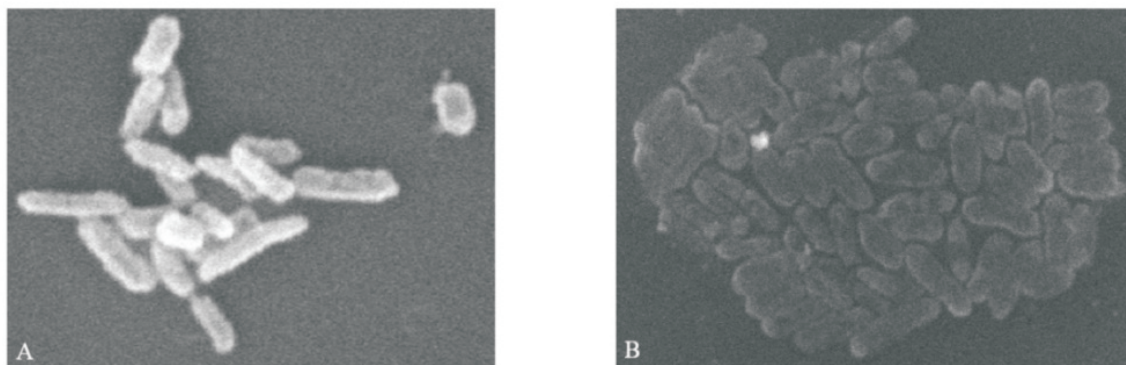


Fig 5 Morphological changes under SEM micrographs caused by plantaricin U10. A: Before treated plantaricin, B: after treated 24 h with plantaricin U10.

of the appearance of the treated cells. SEM micrographs showed difference in cell structures between treated plantaricins U10 group and the non-treated control. Non-treated cells were intact (coccus shaped or regular rod) and showed of smooth surfaces. However bacterial cell treated with plantaricins U10 underwent considerable structural changes cell that can be differentiated (Fig 5). SEM examination confirmed the structural destruction integrity of the cells and considerable morphological alteration bacterial cell. This finding showed cells death by the plantaricins U10 treatment was probably through pores formation on the outer membrane of *S.typhi*.

DISCUSSION

In this study, we described the plantaricins U10 production from early growth of the cells, purification and its mode of action. The activity of plantaricins U10 againsted Gram-negative bacteria such as *S.typhi* is a rare phenomenon. According to Todorov (2009), ineffective bacteriocin against Gram negative bacteria due to physical barrier of phatogenic bacteria's outer membrane that masked the site for bacteriocin action. Most bacteriocins produced by *L. plantarum* inhibited Gram-positive bacteria, e.g. plantaricin W (Holo *et al.* 2001) and plantaricin LR14 effectively killed *Micrococcus luteus* (Tiwari and Srivastava 2008). More importantly, plantaricin C19 (Atrih *et al.* 2001), plantaricin AMA-K (Todorov *et al.* 2008), plantaricin NA (Mills *et al.*, 2011), plantaricin LB-B1 (Xie *et al.* 2011), and plantaricin Y (Chen *et al.* 2014) have been experimentally proven to be potential as natural biopreservative to control/prevent food contamination from *Listeria monocytogenes*. In accordance with our result, others investigators showed bacteriocins activity against broad range of Gram-negative bacteria.

Such as reported by, bacteriocin AMA-K produced by *L. plantarum* AMA-K can inhibit *E. coli* (Todorov *et al.* 2007). Plantaricin LP31 produced by *L. plantarum* LP31 have broad inhibitory spectrum such as *Pseudomonas* sp, *S. aureus*, *L. monocytogenes*, *B. cereus*, *B. megaterium*, and *B. subtilis* (Muller *et al.* 2008). Plantaricin MG produced by *L. plantarum* KLDS1.0391 can inhibit *L. monocytogenes*, *S. aureus*, and *S. typhimurium* (Gong *et al.* 2010). Muhialdin *et al.* (2012) reported Lactic acid bacteria which isolated from tempoyak in Malaysian showed antibacterial activity against tested strains of Gram positive and Gram negative bacteria such as (*B. subtilis*, *E.coli*, *S. aureus*, *S. epidermidis*, *Klebsiella pneumoniae*, and *S. typhimurium*).

Plantaricins U10 production during growth of *L. plantarum* U10 in MRS broth at 37 °C have maximum activity at stationary phase of growth. The highest activity of a bacteriocins produced during stationary phase of growth was also similar as found at plantaricin LB-B1 (Xie *et al.* 2011), plantaricin MG (Gong *et al.* 2010), bacteriocin La-14 (Todorov *et al.* 2011), plantaricin ST194BZ (Todorov and Dicks 2005), and plantaricin ST71KS (Martinez *et al.* 2013). The decrease in the antibacterial activity at the later stationary phase (i.e. after 24 h) could be ascribed to plantaricins U10 degradation by proteolytic enzymes released during cell lyses, or binding of the peptide to proteins or producer cells (Gong *et al.* 2010). During the 32 h of growth, the pH decreased from pH 6.3 to 4.0. The optical density cell (OD 600nm) increased from 0.15 to 2.74 (Fig. 1). A similar results were reported for bacteriocin LB-B1 produced by *L. plantarum* LB-B1 (Xie *et al.* 2011), bacteriocin ST4SA produced by *Enterococcus mundtii* (Todorov and Dicks 2009), bacteriocin ST13BR produced by *L. plantarum* ST13BR (Todorov *et al.* 2004).

Plantaricins U10 in our study were obtained from partially purification steps (ammonium sulphate and size-exclusion chromatography) so the protein bands which were assumed as bacteriocins appeared as 2 bands with different molecule size (approximately 4.5 and 9.8 kDa) and those predicted palntaricins were bacteriocin class II group. This finding was within the range of most bacteriocins reported (3.0 kDa bacteriocin *L. plantarum* ST341LD and 14.0 kDa bacteriocins *L. plantarum* ST23LD) (Todorov and Dicks 2005). Further purification steps is need to be done to obtain single protein band that refer to targeted plantaricin. According to Hata *et al.* (2010) and Gong *et al.* (2010) additional steps after size-exclusion chromatography such as cation-exchange or reverse-phase HPLC are simplest way to obtain the more pure and single band protein obtained.

Addition of cell-free supernatant U10 into a 4-h-old culture (exponential phase) of *S.typhi* ATCC25241 resulted in growth inhibition for 12 h (Fig. 4), suggesting that the mode of activity of plantaricins U10 is bactericidal on sensitive strain as proven by cell count decrease in the number of viable cell (from 2.2×10^8 CFU mL⁻¹ to 3.1×10^6 CFU mL⁻¹) of *S. typhi* ATCC25241. A similar mode of actions also reported from other studies, e.g. plantaricin LB-B1 (Xie *et al.* 2011), bacteriocin ST4Sa (Todorov and Dicks, 2009) and bacteriocin La-14 (Todorov *et al.* 2011).

In general, bacteriocins function produced by Gram-positive bacteria is primarily directed to eliminate or reduce other competitors Gram-positive bacteria (Ennahar *et al.* 2000). Under normal circumstances, bacteriocins produced by Gram-positive bacteria do not have a bactericidal effect on Gram-negative species. However, in some cases, their bacterial activity against some sensitive Gram-negatives can be directly observed under SEM as membrane rupture (Wang *et al.* 2014). In agreement, our result also proved that plantaricins U10 have ability to disturb integrity of membrane of *S.typhi* which was believed to be sensitive to our plantaricins U10. According to McAuliffe *et al.* (2001), class II of bacteriocins exert their action mostly on cells targets by pore formation on membrane of target cells that induce proton motive force (PMF).

To conclude, in this study, plantaricins U10 against *S.typhi* produced by *L. plantarum* U10 isolated from the traditional fermented food “tempoyak” have been proven. Plantaricins U10 have molecular mass of approximately 4.5 and 9.8 kDa by SDS-PAGE. Plantaricins U10 production reached maximum at 20 h

incubation in media MRS broth 37 °C (800 AU mL⁻¹) at early stationary phase of the growth organism and its mode action was bactericidal, as challenged against *S. typhi* ATCC25241. According to the SEM micrographs, it showed bacteriocins form pores in the membranes of target cells. These results provide theoretical foundation for the application of plantaricins U10 as antibacterial peptide in the pharmaceutical industry.

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