Proceedings of

International Conference on Beneficial Microbes
ICOBM 2014

Microbes for the Benefits of Mankind

USM UNIVERSITI SAINS MALAYSIA

ICOBM 2014

27th - 29th May 2014
PARKROYAL Penang Resort
Penang, Malaysia

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Effectivity of Plantaricin from Indonesian *Lactobacillus plantarum* As Antimicrobial Substances against *Escherichia coli*

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ABSTRACT

Indonesian indigenous strains of *Lactobacillus plantarum* IIA-1A5 was isolated from Indonesian local beef, and identified by 16S rRNA sequencing. *L.plantarum* IIA-1A5 are able to produce plantaricin, termed plantaricin IIA-1A5. The aim of this research was to evaluate effectivity of plantaricin IIA-1A5 as antimicobial substances against *Escherichia coli*. Parameters analyzed were the effect of the presence of lipoteichoic acid (LTA) on plantaricin IIA-1A5 adsorption, analysis of *N-acetyl-glucosamine*, bacterial cell leakage, and changes in bacterial cell morphology by scanning electron microscope. The result showed that LTA significantly improve the absorption of plantaricin to cell wall of *E. coli* 3-fold higher than in the absence of LTA. It is acceptable that the addition of LTA might facilitate the binding of plantaricin to the bacterium. Plantaricin IIA-1A5 could disrupt the *N-acetyl-gucosamine* (NAG) on peptidoglican of cell wall of *E.coli*. Microscopic morphology by Scanning Electron Microscope of *E.coli* treated with plantaricin IIA-1A5 was remarkably different compared to that of the untreated cells. This is hypothesized may be due to ability of plantaricin IIA-1A5 to disrupt cell membrane and promote cell leakage of *E.coli*.

Keywords: *Lactobacillus plantarum,* plantaricin, *Escherichia coli*

1. INTRODUCTION

Bacteriocins of lactic acid bacteria are potent food biopreservative agents and can be effective in controlling the incidences of food poisoning outbreaks. Bacteriocins are considered to be safe natural biopreservatives, because it is assumed that they are degraded by the proteases in the gastrointestinal tract and may be useful asa primary hurdle for controlling food-borne pathogens (Cleveland *et al*., 2001). *Lactobacillus plantarum* is lactic acid bacteria which produces bacteriocin, called plantaricin. Plantaricin A, Plantaricin EF and Plantaricin JK are included into Class II bacteriocin (Diep *et al*., 2009). Whereas Plantaricin W from *Lactobacillus plantarum* LMG 2379 belongs to a new family of two-peptide bacteriocin (Holo *et al*., 2001).

Our previous research showed that Indonesian indigenous strains of *Lactobacillus plantarum* was isolated from Indonesian beef e g. *L.plantarum* IIA-1A5, *L.plantarum* IIA-1B1, *L.plantarum* IIA-2B2, and *L.plantarum* IIA-1C4. They were identified species and strains by molecular technique using PCR and 16S rRNA sequencing, and have high similar identify as *L.plantarum* JDM 1 (97%) by phylogenetic tree analysis using Kimura model.The exploration about their functions have been done, such as antibacterial activities against pathogenic bacteria (*Escherichia coli, Staphylococcus aureus* and *Salmonella Typhimurium*). It was reported that all strains produces antibacterial substances that could inhibit growth of pathogenic bacteria. *L. plantarum* IIA-1A5 have better antimicrobial activities than others (Arief, 2011). It is being important to explore it completely. Purification the plantaricin as bacteriocin from indigenous *L. plantarum* IIA-1A5 is very important to describe their function as antimicrobial substances. *Escherichia coli* is Gram negative bacteria that commonly contaminate foods. Purification and biochemichal characterization of plantaricin is essential to evaluate its potential for antimicrobial agents against *E. coli*.

2. MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus plantarum IIA-1A5 was grown in MRS broth and agar media. *Escherichia coli* ATCC 25922 was grown in Nutrient Broth and Nutrient agar as slab cultured stock. Stock cultures are stored in media at -20^{0}C, subculture twice for 24 hrs at 37^{0}C in the same media and incubate at suitable temperature before use.

Purification of plantaricin

This experiment was carried out according to Tiwari and Srivastava (2008); Hata *et al*. (2010) modified by Arief *et al* (2013). *L. plantarum* IIA-1A5 was grown in deMan Rogosa Sharpe (MRS) broth, supplemented with 3% yeast extract and incubated at 37^{0}C without agitation for 20 hours for the production of bacteriocin. The cell are removed by centrifugation (10.000 rpm for 20 min, 4^{0}C), followed by filter-sterilized (0,2 µm membrane). The plantaricin was purified from the cell free supernatant by ammonium sulfate precipitation and cation-exchange chromatography. In order to determine the molecular mass, purified plantaricin was subjected to SDS-page electrophoresis.

Analysis of mode of action plantaricin IIA-1A5 against *E.coli*

Effects of detergents, organics solvents and plantaricin IIA-1A5 on the cell leakage of E. coli. To test the effect of organic solvents on subsequent adsorption of plantaricin IIA-1A5, cells were suspended in methanol, ethanol, hexan and choloroform. Suspensions obtained after detergent or organic solvent treatments were mixed with plantaricin IIA-1A5 and incubated at 30°C for 30 min. After centrifugation, residual plantaricin IIA-1A5 was assayed in supernatant (Atrih et al., 2001).

Effect of the presence of lipoteichoic acid on plantaricin IIA-1A5 adsorption. Purified lipoteichoic acid (2 mg) prepared from *Staphylococcus aureus* (Sigma) was added to 2.6 ml of *E. coli* cell suspension (10^8 cfu mL^{-1}). Plantaricin is added at a final concentration of 160 AU mL^{-1} and the mixture was incubated for 40 min at 30°C. Following centrifugation (15,000X *g*, 10 min), the supernatant were measured for residuals non-adsorbed plantaricin. Controls were cells mixed with plantaricin or lipoteichoic acid mixed with plantaricin (Atrih et al., 2001).

Analysis of N-acetyl-glucosamine. The experiment was conducted to determine the effect of plantaricin IIA-1A5 of the bacterial cell wall by measuring the levels of *N-acetyl-glucosamine* as a precursor mukopeptida cell wall constituent.

Analysis of bacterial cell leakage. Observations leaks was carried out to study how plantaricin IIA-1A5 interfere with cell membrane permeability. The mechanism of destruction of the cell membrane is one sign of abnormal cells after treatment of plantaricin. Analysis of cell leakage spectrographs were performed using an UV-VIS Spectrophotometer at a wavelength of 280 nm and 260 nm (Atrih et al., 2001).

Analysis of changes in bacterial cell morphology by Scanning Electron Microscope. Analysis of cell morphology due to the treatment of plantaricin IIA-1A5 include damage to cell morphology, structure of bacteria, as well as damage to the cell wall.

Data Analyzes

Data was collected and analyzed by Analysis`of variance (ANOVA). If there were significantly different, the Duncan test was used for further analyzis (Steel and Torrie, 1995)

3. RESULTS AND DISCUSSION

Effects of detergents, organics solvents and plantaricin IIA-1A5 on the cell leakage of E. coli

Cell leakage was investigated to study how plantaricin IIA-1A5 interferes with cell membrane permeability. The mechanism of destruction of the cell membrane is a commmon feature of the plantaricin-treated cells. Analysis of cell leakage spectrographs were performed using an UV-VIS Spectrophotometer at a wavelength of 260 nm and 280 nm to detect the presence of cellullar proteins and genetic material (DNA or RNA), respectively. Higher absorbance value at these wavelengths indicates that more celullar content (protein or genetic materials) released from the cell due to more severe membrane cell disruption. The effectivity of plantaricin IIA-1A5 was similar with SDS and better than ethanol, hexane and methanol (Table 1).

Table 1. *E.coli* cell leakage

	Treatments	*Absorbance*	
		280 nm	260 nm
Organic solvent	80% Ethanol	$1.41 + 0.32^{c}$	2.15 ± 0.16^{c}
	80% Chloroform	$7.20 + 1.16^{b}$	11.19 ± 3.42^{a}
	80% Hexane	$2.18 + 0.80^{d}$	2.99 ± 1.18^{c}
	80% Methanol	$0.52 + 0.39^{e}$	0.94 ± 0.14^{d}
Detergen	1% SDS	$4.81 + 1.15^{c}$	7.58 ± 2.19^{b}
	2% Triton X-100	$12.27 + 3.51^{a}$	9.55 ± 3.42^{a}
Plantaricin IIA-1A5	20 AU/ml	$5.33 + 1.23^{c}$	6.26 ± 2.45^{b}

Value represents mean ± sd (n=6). Means in the same column with different superscript indicates significance differences ($p < 0.05$).

Effect of the presence of lipoteichoic acid on plantaricin IIA-1A5 adsorption

Plantaricin to Gram negative absorption was smaller (25%) compared with Gram positive (40%) (Table 2). Plantaricin has low absorption due to Gram negative bacteria because Gram negative bacteria do not have the LTA on the cell wall peptidoglycan layer. The role of LTA is apparently to mediate interaction (attachment) bacteriocin to the cell wall. LTA located in peptidoglycan in large quantities on Gram positive bacteria. These results were in line with the inhibition of plantaricin to *E. coli* was smaller than *S.aureus*, and *N-acetyl-glucosamine* (NAG) that regardless of the *E.coli* was much less than the *S.aureus.* (Figure 1).

LTA was reported to improve the absorption of plantaricin to the cell wall of Gram positive and Gram negative bacteria. In this result, LTA was able to improve the effectiveness of absorption plantaricin to *E.coli* as 208%, while the addition of LTA to *S.aureus* only increase absorption of plantaricin by 25% (Table 2). Increased absorption of plantaricin in *S. aureus* was less than the *E. coli* because *S. aureus* has a lot LTA on the cell wall. LTA increase attachment of the plantaricin to Gram negative bacteria. For improvement purpose, plantaricin engineering might be required to create better plantaricin enable to kill Gram negative bacteria stronger, which can be done by the addition of those ways and hydrophobic groups on the sugar molecule plantaricin (LTA hydrophobic nature and had a sugar moiety).

Table 2. Analysis effect of LTA on plantaricin adsorption

Treatments	*S. aureus*	*E.coli*
Plantaricin adsorbed with LTA (%)	50	77
Plantaricin adsorbed without LTA (%)	40	25
Effectivity of LTA (%)	25	208

Analysis of N-acetyl-glucosamine

The inhibition of plantaricin to *E.coli* was smaller than *S. aureus*, and *N-acetyl-glucosamine* (NAG) regardless of the *E. coli* was much less than the *S.aureus* (Figure 1). Peptidoglycan on Gram positive bacteria is considerebely thicker than Gram negative bacteria. Figure 1 showed that plantaricin can disrupt the *N-acetyl-gucosamine* on peptidoglican of cell wall bacteria. These results were in line with diametere zone of inhibition from plantaricin.

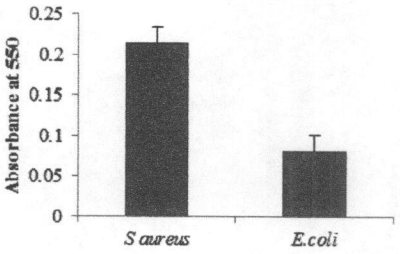

Figure 1. *N-acetyl-glucosamine* of cell wall bacteria treated by plantaricin IIA-1A5

Analysis of changes in bacterial cell morphology by Scanning Electron Microscopy (SEM)

Microscopic morphology of *S. aureus* treated with plantaricin IIA-1A5 was remarkably different compared to that of the untreated cells (Figure 2). This was hypothesized due to ability of plantaricin IIA-1A5 to disrupt cell membrane and promote cell leakage of *E.coli*. Untreated *E.coli* cell looks normal, with bacil-shaped cells clearly visible. However, after treatment with plantaricin IIA-1A5, cell rupture was visible which were smaller in size and show pores-like structures in the surface with cell material leaking from the cells. The result was similar with pediocin PD-1 acts on the cytoplasmic membrane of *Oenococcus oeni*, and its activity may be due to the generation of pores in the cell membrane (Bauer *et al.*, 2005).

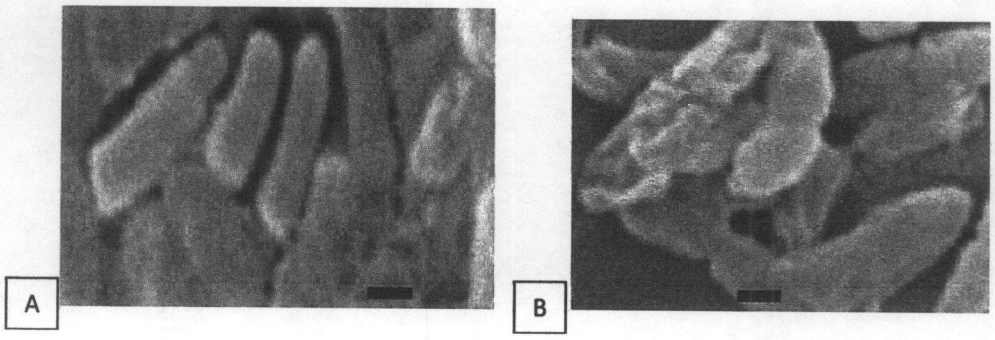

Figure 2. Microscopic analysis of morphology *E.coli* treated by plantaricin IIA-1A5 (B) in comparison to control, in the absence of plantaricin IIA-1A5 (A). The bars in each figure correspond to 1 µm in size, as reference.

5. CONCLUSION

Plantaricin IIA-1A5 shows ability to promotes leakage of *E.coli*. Cell leakage is caused by cell membrane disruption by plantaricin IIA-1A5 to the release of proteineaceus and genetic material, and *N-acetyl-glucosamine*. Lipoteichoic acid can increase the plantaricin adsorption. SEM analysis showed that plantaricin IIA-1A5 formed pores in the surface of *E.coli* cells. Mode of action plantaricin IIA-1A5 against *E. coli* seemed to be bacteriocidal rather than bacteriostatic.

6. ACKNOWLEDGEMENT

The authors are grateful to the Directorate of Higher Education (DIKTI), Ministry of Education Republic of Indonesia, through National Strategic Research Grant No046/SP2H/PL/Dit.Litabmas/III/2012 and International Toray Science Foundation through Science and Technology Research Grant 2013 for the funds.

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