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Original Article

Physical, biochemical and genetic characterization of enterocin CE5-1 produced by *Enterococcus faecium* CE5-1 isolated from Thai indigenous chicken intestinal tract

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Abstract

Enterocin CE5-1 produced by *Enterococcus faecium* CE5-1 isolated from the chicken gastrointestinal tract was active in the wide range of pH 2-10 and temperature 30-100°C and sensitive to proteolytic enzymes and α-amylase. It remained active after storage at -20°C for 2 months. Moreover, enterocin CE5-1 showed antibacterial activity against lactobacilli, bacilli, listeria, staphylococci and enterococci, especially antibiotic-resistant enterococci. *In vitro* study of enterocin CE5-1 decreased the population of *Ent. faecalis* VanB from 6.03 to 4.03 log CFU/ml. The lethal mode of action of enterocin CE5-1 appeared to be pore and filament formation in the cell wall. PCR sequencing analysis revealed the presence of two open reading frames (ORFs), containing enterocin CE5-1 (*ent*CE5-1) and enterocin immunity (*ent*I) gene. Therefore, enterocin CE5-1 from *Ent. faecium* CE5-1 could possibly be used as an antimicrobial agent to control foodborne pathogen, spoilage bacteria and antibiotic-resistant enterococci in foods, feeds and the environments.

Keywords: enterocin, *Enterococcus faecium*, chicken, antibiotic-resistant enterococci

1. Introduction

Vancomycin-resistant enterococci (VRE) are enterococci that are resistant to vancomycin. Enterococci are widespread in nature such as soil, water, food and gastrointestinal tract (GIT) in human and animal, and they have been considered as opportunistic pathogens in hospitals. They cause serious diseases such as nervous system, blood stream, neonatal, nosocomial and other infections (Sood *et al*., 2008). In addition, some of them can transmit the antibiotic-resistant gene to other bacteria and may be involved in the spread of these genes into the environment and food chain (Abriouel *et al*., 2005; Klare *et al*., 2003).

In our previous work, we found that *Ent. faecium* CE5-1 isolated from GIT of Thai indigenous chicken was suitable potential probiotic strains (Saelim *et al*., 2012). This strain able to inhibit all lactic acid bacteria (LAB) strains and some Gram-positive bacteria such as *Enterococus, Pediococcus*, *Lactobacillus* and *Listeria monocytogenes*, especially VRE. In addition, *Ent. faecium* CE5-1 was able to reduce the growth of *Ent. faecalis* VanB *in vitro* and the inhibitory effects of *Ent. faecium* CE5-1 were due to the combination of organic acid, hydrogen peroxide and bacteriocin production. Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptide or peptide complexes

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which have bactericidal or bacteriostatic effects on closely related species (Garneau *et al*., 2002). Many studies have explored the use of bacteriocins for control of pathogenic and spoilage bacteria in food. However, there is also interest in their application for controlling the dissemination of VRE.

In this study, we describe the characterization of enterocin CE5-1 produced by the probiotic *Ent. faecium* CE5-1. In addition, the efficacy *in vitro* of enterocin CE5-1 against VRE was studied.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Ent. faecium CE5-1 was isolated from gastrointestinal tract (GIT) of Thai indigenous chicken. The indicator strains used for inhibition study are listed in Table 1. All the LAB strains were grown in deMan, Rogosa and Shape broth (MRS broth, Hi Media Laboratory Pvt. Ltd., Mumbai, India, pH 6.5±0.2) at 37°C. Other strains were grown in Brain Heart Infusion broth (BHI broth, Hi Media Laboratory Pvt. Ltd., Mumbai, India) at 37°C.

2.2 Determination of bacteriocin activity

Bacteriocin activity was checked by agar well diffusion (AWD) assay (Schillinger and Lucke, 1989) using *Lactobacillus sakei* subsp. *sakei* JCM1157 as an indicator strain and expressed in arbitrary units (AU/ml). An arbitrary unit was defined as the reciprocal of highest two-fold dilution showing clear zones of growth inhibition and is expressed in arbitrary units per ml.

2.3 Preparation of crude enterocin CE5-1

Ent. faecium CE5-1 was grown in 1 L of MRS broth at 37°C for 15 h. Cells were removed by centrifugation at 12,000×*g* for 10 min at 4°C and supernatant was collected.

Table 1. Antagonistic spectrum of a crude enterocin CE5-1 of *Ent. faecium* CE5-1.

Indicator stains	Source[a]	Radius of inhibition zone (mm)	
		supernatant[c]	1 mg/ml[d]
Bacillus cereus DMST5040	DMST	0	5.7
Enterococcus durans 3L20	Our strain collection	1.5	4.6
Enterococcus faecalis 2L24	Our strain collection	1.0	2.2
Enterococcus faecalis VanB[b]	CU	2.0	4.9
Enterococcus faecium 139[b]	CU	0	0
Enterococcus faecium 174[b]	CU	1.5	3.8
Enterococcus faecium 348[b]	CU	1.5	3.7
Enterococcus faecium 4S13	Our strain collection	1.5	3.8
Enterococcus faecium 8S16	Our strain collection	2.0	4.7
Enterococcus faecium CE5-1	Our strain collection	0	0
Enterococcus faecium L7-45	Our strain collection	2.2	4.6
Enterococcus gallinarum VanC[b]	CU	2.0	4.0
Escherichia coli DMST4212	DMST	0	0
Lactobacillus plantarum D6SM3	Our strain collection	0	6.0
Lactobacillus sakei subsp. *sakei* JCM1157	JCM	6.0	6.0
Listeria monocytogenes DMST17303	DMST	5.0	5.0
Pediococcus pentosaceus 3CE27	Our strain collection	3.0	2.7
Pediococcus pentosaceus DMST18752	ATCC	0	1.0
Salmonella Typhimurium DMST16809	DMST	0	0
Salmonella Enteritidis DMST15676	DMST	0	0
Staphylococcus aureus DMST8840	DMST	0	5.6
Vibrio parahaemolyticus DMST5665	DMST	0	0

[a] Abbreviations: ATCC: American Type Culture Collection; DMST: Department of Medical Sciences; CU: The WHO Global Salm-Surv Regional Centre of Excellence: South-East Asia and Western Pacific, Faculty of Veterinary Science, Chulalongkorn University, Thailand; JCM: Japan Collection of Microorganisms.

[b] Antibiotic-resistant enterococci.

[c] Reference from Saelim *et al*. (2012).

[d] The crude enterocin CE5-1 after 60% ammonium sulfate precipitation.

Ammonium sulfate was added to the supernatant to reach 60% saturation. Then, the mixture was stirred overnight at 4°C. The precipitant was collected by centrifugation at 12,000×*g* for 20 min at 4°C. The pellet was dissolved in 50 mM sodium phosphate buffer (PB) pH 5.8 and dialyzed using dialysis tubes with molecular cut-off 1 kDa (*Spectra*/*Por*®, *Spectrum* Medical Industries, Inc., Texas, USA) against the same buffer at 4°C overnight and Iyophilized.

2.4 Characterization of enterocin CE5-1 from *Ent. faecium* CE5-1

2.4.1 Spectrum of bacteriocin activity

The crude enterocin CE5-1 powder (1 mg/ml) was used for determination of activity spectrum of bacteriocin using the method of AWD assay with various indicator strains as shown in Table 1.

2.4.2 Effect of enzyme on bacteriocin activity

The crude enterocin CE5-1 powder (1 mg/ml) was dissolved with 50 mM PB, pH 7.5 in the presence of 1 mg/ml of each enzyme including proteinase K, pronase E, trypsin, α-chymotrypsin, α-amylases and lipase. The samples were incubated in a water bath at 37°C for 3 h. After incubation, the enzyme was inactivated by heating at 100°C for 5 min.

2.4.3 Effect of pH on bacteriocin stability

The crude enterocin CE5-1 powder (1 mg/ml) was dissolved in buffer at different pH as follows: 50 mM glycine-HCl buffer (pH 2.2 to 3.6); 50 mM citric acid-sodium citrate buffer (pH 3.0 to 6.2); 50 mM M sodium phosphate buffer (pH 5.7 to 8.0); 50 mM tris-HCl buffer (pH 7.0 to 9.0); and 50 mM glycine-NaOH buffer (pH 8.6 to 10.6) and incubated at 37°C for 24 h.

2.4.4 Effect of temperature on bacteriocin stability

The crude enterocin CE5-1 powder (1 mg/ml) was dissolved in different buffers including glycine-HCl buffer pH 3.0, phosphate buffer pH 6.5 and glycine-NaOH buffer pH 8.6. The sample was heated under different temperature at 30 to 100°C for 2 h and sterilizing temperature at 110°C for 20 min and 121°C for 15 min using an autoclave (Tommy Seiko Corp., Tokyo, Japan).

For effect of storage temperature and time on bacteriocin activity, entericin CE5-1 was dissolved in different buffers including glycine-HCl buffer pH 3.0, phosphate buffer pH 6.5 and glycine-NaOH buffer pH 8.6. Then, the samples were stored at 4°C, -20°C and room temperature (29±2°C) for a period of three months.

2.5 *In vitro* antibacterial effect of enterocin CE5-1 on the survival of *Ent. faecalis* VanB

Ent. faecalis VanB was incubated in 50 ml of MRS broth at 37°C. After 6 h of incubation in the early logarithmic phase, enterocin CE5-1 was added to the culture broth of *Ent. faecalis* VanB at a final concentration of 2,560 AU/ml. The viability of *Ent. faecalis* VanB was determined by using the drop plate method (Hoben and Somasegaran, 1982) in triplicate onto MRS agar and incubated at 37°C for 48 h. *Ent. faecalis* VanB cultivated in 50 ml of MRS broth without added enterocin CE5-1 was used as control.

Morphological changes of *Ent. faecalis* VanB after treatment with enterocin CE5-1 were observed with scanning electron microscopy (SEM; *FEI Quanta* 400, Oxford. Instruments, Oxford). The cells were centrifuged at 12,000×*g* at 4°C for 10 min and resuspended in 0.2 M phosphate buffer pH 7.0. The samples were fixed in 2.5% gluteraldehyde, dehydrated with a graded ethanol series (10, 30, 50, 70, 90 and 100%), critical-point dried with CO_2 and mounted on SEM stubs. Then, the samples were observed with SEM.

2.6 PCR and DNA sequencing

The genomic DNA of *Ent. faecium* CE5-1 was extracted using the Genomic DNA Mini Kit (Geneaid, Tao-Yuan, Taiwan). The *ent*CE5-1 genes were amplified from specific primers: EntF 5′- AGGGGTGATTAGATTATGAAAC-3′ and EntR 5′- TTAAAATTGAGATTTATCTCCATAATC-3′. The PCR reactions composed of the following steps: initial denaturation at 94°C for 5 min and then performed in 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 3 min, followed by final extension at 72°C for another 10 min, then immediately cooled at 4°C. PCR products were determined by agarose gel electrophoresis and purified using a HiYield TM Gel/PCR extraction kit (Real Biotech Corporation, Taipei, Taiwan). The purified DNA fragments were sequenced and compared to nucleotide sequence database of GenBank via BLAST search (http://www.ncbi.nlm.nih.gov).

3. Results and Discussions

3.1 Inhibitory spectrum of enterocin CE5-1

The crude entercin CE5-1 showed antibacterial activity against Gram-positive bacteria (*Enterococcus*, *Lactobacillus*, and *Pediococcus* strains) and foodborne pathogenic and spoilage bacteria (*Bacillus cereus, L. monocytogenes* and *Staphylococcus aureus*), but not against Gram-negative bacteria (Table 1). Usually, bacteriocins from *Enterococcus* are active against Gram-positive foodborne pathogenic and food spoilage bacteria, whereas they are inactive against

Gram-negative bacteria. Similar results were reported for enterocin from *Ent. faecium* MTCC513 (Badarinath and Halami, 2011). The resistance of Gram-negative bacteria to bacteriocins could be due to the relative impermeability of their outer membranes (Jack *et al*., 1995). This result indicates that entercin CE5-1 from *Ent. faecium* CE5-1 can be used as biopresevatives and to extend shelf life of foods.

Moreover, bacteriocin-producing *Ent. faecium* CE5-1 was not inhibited by its own bacteriocin due to the production of immunity protein to protect itself. From genetic characterization of enterocin A from *Ent. faecium* CE5-1, the enterocin A immunity gene in genomic DNA of *Ent. faecium* CE5-1 was observed (Figure 1).

Currently, many recent studies have demonstrated bacteriocins for application in food, but there have been a few studies on their application in therapeutics. Unakal (2013) reported that bacteriocin produced from *Ent. faecium* was active against multiple resistant bacteria isolated from different clinical specimens i.e. *Pseudomonas aeruginosa*, *E. coli*, *S. aureus*. Interestingly, our results showed that enterocin CE5-1 exhibited inhibitory activity against antibiotic-resistant enterococci, especially *Ent. faecalis* VanB. *Ent. faecalis* VanB, known as VRE, is more resistant to streptomycin, tetracycline and vancomycin (Saelim *et al*., 2012). Most often, VRE have been isolated from patients and food animals in many countries and acknowledged as the leading cause of hospital-acquired infections. Based on this spectrum of inhibitory activity, enterocin CE5-1 from *Ent. faecium* CE5-1 may be used as a therapeutic agent to control antibiotic-resistant enterococci.

3.2 Effect of enzyme

Bacteriocin activity of the crude enterocin CE5-1 was inactivated by proteolytic enzyme and α-amylase (Table 2). After addition of proteolytic enzyme or α-amylase, the bacteriocin activity was lost. Similar results were also reported for enterocin ON-157 produced by *Ent. faecium* NIAI157 (Ohmomo *et al*., 2000). Lee and Kim (2010) also reported that inactivation of bacteriocin activity from *Ent. faecium* DB1 was observed after treatment with proteolytic enzymes and α-amylase. This suggested that carbohydrate and protein moieties were required for the bacteriocin activity of enterocin CE5-1. The sensitivity of our bacteriocin to digestive enzymes suggests that enterocin CE5-1 from *Ent. faecium* CE5-1 could be safely used.

3.3 Effect of pH

Bacteriocin activity of the crude enterocin CE5-1 at different pH levels is shown in Table 2. The bacteriocin activity of enterocin CE5-1 was active in a wide pH range (2.2-10.6). These results are supported by Franz *et al*. (1996), who showed that enterocin 900 retained activity at pH ranging from 2.0-10.0. The maximum bacteriocin activity of enterocin CE5-1 was observed at acidic (glycine-HCl buffer pH 3.0-3.6), neutral (sodium phosphate buffer pH 5.7-6.5) and alkaline (glycine-NaOH buffer pH 8.6-9.6) pH conditions after 1 h of incubation at 37°C. Moreover, the decrease in bacteriocin activity after 24 h of incubation was observed in acidic condition, while inactivation occurred in PB pH 6.5-

Figure 1. Nucleotide sequence of enterocin A structural (ORF1) and immunity (ORF2) genes. The arrows are indicated as the start codons. The asterisks are indicated at the end of protein synthesis. Leader peptides are indicated by bold type. Transmembrane helices are enclosed in boxes.

Table 2. Effect of enzyme and pH on bacteriocin activity.

Treatments		Residual bacteriocin activity (AU/ml)	
Effect of enzyme			
Control		80	
Trypsin		0	
α-chymotrypsin		0	
Proteinase K		0	
Pronase E		0	
α-amylases		0	
Lipase		80	
Effect of pH	pH	Incubation time (hours)	
		1	24
Glycine-HCl buffer	2.2	160	160
	3.0	320	160
	3.6	320	40
Citric buffer	3.0	160	160
	4.2	160	80
	6.2	160	40
Sodium phosphate buffer	5.7	320	80
	6.5	320	0
	8.0	80	0
Tris-HCl buffer	7.0	160	0
	8.0	80	20
	9.0	80	80
Glycine-NaOH buffer	8.6	320	80
	9.6	320	80
	10.6	160	0

8.0, tris-HCl buffer pH 7.0 and glycine-NaOH buffer pH 10.6. These results indicate that enterocin CE5-1 retained its bacteriocin activity in acidic condition rather than neutral and alkaline conditions and can be applied in several products.

3.4 Effect of temperature

In this context, we were interested in investigating the effects of temperature on bacteriocin stability in different pH. Figure 2 showed that the crude enterocin CE5-1 was stable to heating at 30-90°C for 30 min, except at pH 8.6, in which reduced activity occurred at temperatures higher than 60°C. The thermal stability of enterocin CE5-1 was similar to another enterocin produced from *Ent. faecium* (Gupta *et al*., 2010). Heating above 80°C for 2 h caused the loss or decrease of bacteriocin activity, depending on the pH of the enterocin preparation. Moreover, the enterocin CE5-1 retained bacteriocin activity under sterilization condition at 110°C for 20 min and 121°C for 15 min, whereas at pH 6.5 and 8.6 it was partially or completely inactivated under sterilization condition. Similarly, enterocin 900 activity from *Ent. faecium* BFE 900 was heat stable, retaining activity after heating at 121°C for 15 min (Franz *et al*., 1996). Heat stability of bacteriocin may be due to the formation of small globular structures and the occurrence of strongly hydrophobic regions, stable cross-linkages and high glycine content (de Vuyst and Vadamme, 1994). The heat stability of enterocin CE5-1 indicates that it could be used as a preservative in foods and feeds because many processing procedures involve a heating step.

Stability of enterocin CE5-1 during storage was also investigated. It was found that the enterocin CE5-1 was stable at low temperature. Enterocin CE5-1 could be stored in pH 3.0 at -20°C for at least 2 months, at 4°C for at least 1 week and at room temperature for at 3 days (Figure 3). Similar results were found in another study conducted by Casaus *et al*. (1997). The antimicrobial activity of enterocin P was not lost in long-term storage at 4 and -20°C.

3.5 *In vitro* antibacterial effect of enterocin CE5-1 on the survival of *Ent. faecalis* VanB

Effect of enterocin CE5-1 on the survival of *Ent. faecalis* VanB is shown in Figure 4. *Ent. faecalis* VanB dramatically declined from 6.03 to 4.03 log CFU/ml and remained stable until 18 h of cultivation after enterocin CE5-1 was added. However, the regrowth of *Ent. faecalis* VanB was observed after 18 h of enterocin CE5-1 treatment, resulting from the bacteriocin concentration used or the development

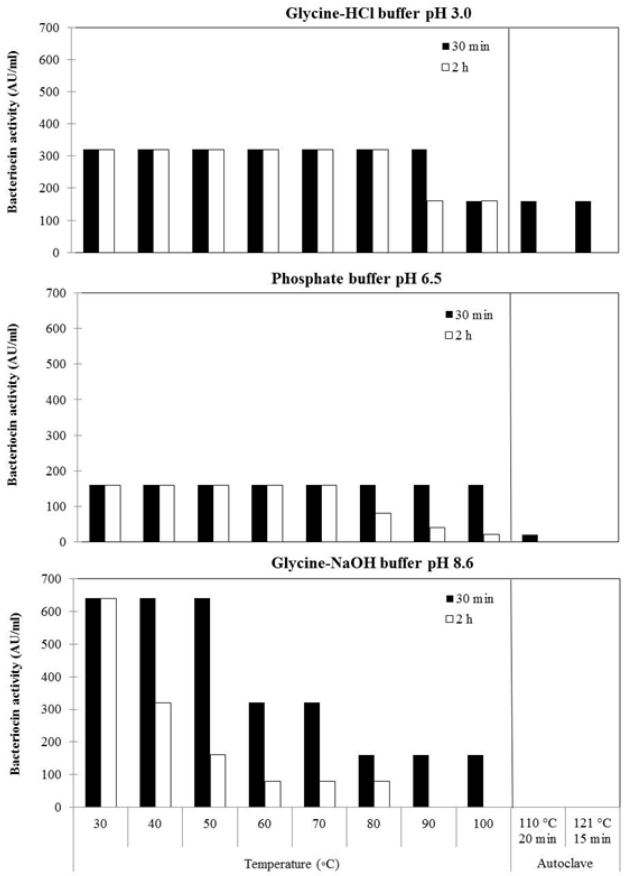

Figure 2. Effect of temperature on bacteriocin activity.

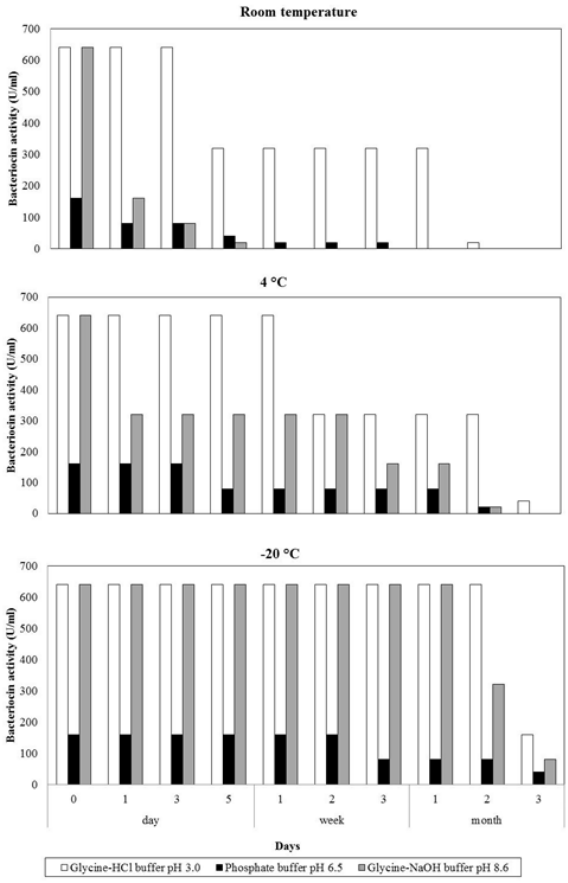

Figure 3. Effect of storage temperature and time on bacteriocin activity in different pH.

of resistant cells. Similar results were in agreement with previous study obtained by Enan (2006), who found that *L. monocytogenes* regrowth was observed only in samples treated with a single bacteriocin (nisin or plantaricin UG1) and proved to be resistant variants at a resistance frequency in the range of 10^{-5} to 10^{-6}. The resistance to nisin or curvaticin 13 may be due to change in cytoplasmic membrane fatty acid and phospholipid compositions in the cell wall (Bouttefroy and Millière, 2000).

However, the decrease of cell counts after treatment with enterocin CE5-1 was caused by cell death, confirming any morphological change of cell wall of *Ent. faecalis* VanB by SEM analysis. Apparently, the cell wall of *Ent. faecalis* VanB treated with enterocin CE5-1 was damaged due to formation of pores and filaments (Figure 5). Generally, class II bacteriocins have an amphiphilic/hydrophilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarization and death (Cotter *et al*., 2005). According to these results, enterocin CE5-1 showed bactericidal mode of action against *Ent. faecalis* VanB.

3.6 Analysis of enterocin CE5-1 encoding gene

Nucleotide sequence of PCR product (about 540 pb) showed 100% homology with enterocin A gene of *Ent. faecium* (GenBank code AF240561). According to the nucleotide sequence analysis, two ORFs were predicted to encode proteins (Figure 1). The ORF1, designated *ent*CE5-1, encoded the 65 amino acids residues (pre-peptide), including 18 amino acids of leader peptide in the putative N-terminus and 47 amino acids of the mature *ent*CE-1. The first 18 amino acids were identified as leader peptide or signal peptide containing Gly-Gly motif at position -1 and -2 and glutamic

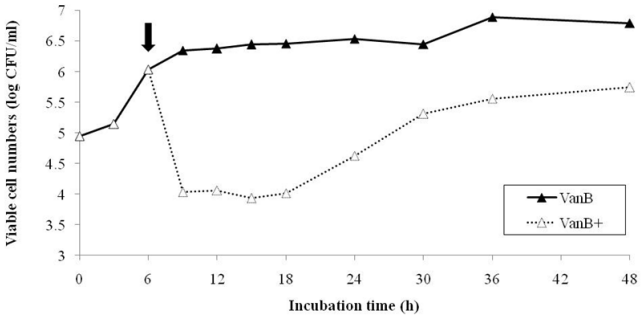

Figure 4. Growth of the *Enterococcus faecalis* VanB in MRS broth with enterocin CE5-1 (VanB+) and without enterocin CE5-1 (VanB). The arrow indicates the point at which the enterocin CE5-1 was added.

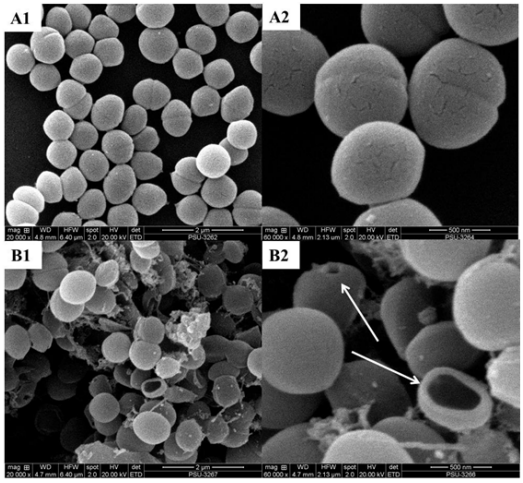

Figure 5. Scanning electron microscopy (SEM) of *Enterococcus faecalis* VanB cells. (A) untreated and (B) treated with enterocin CE5-1. An arrow indicates pore formation.

acid motif (Glu) at position -8. Most of bacteriocins are synthesized in inactive form called pre-bacteriocin or pre-peptides, which carry a leader peptide at the N-terminus. There are many types of bacteriocin leader, such as double-glycine-type (G-G) leader peptide, leader peptide and signal peptide (Franz *et al*., 2007). However, some bacteriocins are synthesized without a leader peptide, such as enterocin L50A, enterocin Q (Cintas *et al*., 2000), and aureocin A53 (Netz *et al*., 2002). The leader peptide is cleaved during secretion of enterocin on the cytoplasmic membrane. In addition, the putative mature *ent*CE5-1 was composed of 47 amino acid residues. The prediction of the three-dimensional structure of the putative mature *ent*CE5-1 showed 98.8% confidences with the pediocin-like antimicrobial peptide sakacin P (PDB code 1OG7) using Phyre2 software. Enterocin CE5-1 was grouped as a Class IIa bacteriocins because it contains YGNGVXC motif near the N-terminus.

The relationships between structure and function were investigated by diversity of Class IIa enterocins, activity spectrum, amino acid sequences and predicted secondary structure. After alignment of amino acid sequences of Class IIa bacteriocins, the results showed that Class IIa bacteriocins contained many conserved regions (Figure 6). The first region at amino acids 8 to 14 is a region of YGNGVXC motif at the N-terminus, which is normally found in Class IIa bacteriocins. However, Class IIa bacteriocins have a strong effect against *L. monocytogenes*. The YGNGVXC motif does not play a role in inhibition of *L. monocytogenes*. For example, enterocin EJ97 did not show the YGNGVXC motif, but it can restrain the growth of *L. monocytogenes* (Gálvez *et al*., 1998). Though, the replacements made in the YGNGVXC motif showed the decrease of antibacterial activity against target organisms (Franz *et al*., 2007). The antimicrobial activity was reduced after replacement of tyrosine (Y) residue in the YGNGV motif of carnobacteriocin B2 to phenylalanine (F) residue (Quadri *et al*., 1997).

The second region at Cys-14 and Cys-19 residues forms the disulfide bridge, which plays an important role in spectrum of activity. Two disulfide bonds have broader spectra of activity than those with a single bond (Ennahar *et al*., 2000; Simon *et al*., 2002). Moreover, addition of reducing agents, such as α-mercaptoethanol or dithiothreitol reduced the inhibitory activity of enterocin KP (Isleroglu *et al*., 2012) and enterocin A (Badarinath and Halami, 2011).

The third regions at Val-21, Trp-23, Ala-26 and Ile-30 are the hydrophobic amino acid residues. However, still little is known about the function of these regions. The three-dimensional structure shows that Val-21 and Trp-23 is a hinge region of Class IIa enterocins. Moreover, Ala-26 and Ile-30 locate in the transmembrane helices that binds to the target membrane.

Finally, the GXXXG motif at the C terminus is found in the transmembrane helices. This motif can interact with the membranes of the bacterial cells. The GXXXG-containing sequence variants cause the antimicrobial activity of bacteriocin. The replacement of glycine that are part of the $G_{13}XXXG_{17}$-motif in plantaricin J (plnJ) and plantaricin K (plnK) with large residue was detrimental to the antimicrobial activity. However, similar replacements of the other residues (Gly-20 in plnJ and Gly-7, Gly-25 and Gly-26 in plnK) had little

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                    10        20        30        40        50
                    ....|....|....|....|....|....|....|....|....|....|...
ent CE5-1           TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSI------GGFLGGAIPGKC-
ent A               TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSI------GGFLGGAIPGKC-
ent P               ---ATRSYGNGVYCNNQKCWVNWNEAKQQIAG-----IVIGGWASGLASMGR-
ent SE-K4           ----ATYYGNGVYCNKQKCWVDWSRARSEIIDRGV-KAYVNGFTKVLGGIGGR
ent CRL35           -----KYYGNGVSCNKKGCSVDWGKAIGIIGNNSA-ANLATGGAAGWKS----
mut F-59.1          -----KYYGNGVTCGKHSXSVDWXKXT--------------------------
mes Y105            -----KYYGNGVHCTKSGCSVNWGEAASAGIHR-----LANGGN-GF------
lac MMFII           -----TSYGNGVHCNKSKCWIDVSELETYKAG-----TVSN--PKDILW----
cur A               ----ARSYGNGVYCNNKKCWVNRGEATQSIIG-----GMISGWASGLAGM---
leu C               -----KNYGNGVHCTKKGCSVDWGYAWTNIANNSV-MNGLTGGNAGWHN----
wei A               -----KNYGNGVYCNKHKCSVDWATFSANIANNSVAMAGLTGGNAGNK-----
sak P               -----KYYGNGVHCGKHSCTVDWGTAIGCIGNNAA-ANWATGGNAGWNKC---
ped PA-1            -----KYYGNGVTCGKHSCSVDWGKATTCIINNGA-MAXATGGHQGNHKX---
Consensus                 YGNGVXC    C V W  A   I           GXXXG
```

Figure 6. Alignment of the putative amino acid of Class IIa bacteriocin. (ent: enterocin; mut: mutacin; mes: mesenteriocin; lac: lactococcin; cur: curracin; leu: leucocin; wei: weissellin; sak: sakacin; ped: pediocin).

effect on the antimicrobial activity of the bacteriocin (Rogne *et al*., 2009).

The ORF2, termed *ent*I, was located downstream of *ent*CE5-1. The *ent*I is 312 bp in length that encodes 103 amino acid sequences and the reported sequence makes it very similar to the enterocin A immunity protein. Genetic analyses showed that prediction of the three-dimensional structure of the putative enterocin CE5-1 exhibited 100% confidence with the enterocin A immunity protein (PDB code 2BL8) using Phyre2 software. Immunity protein of Class IIa bacteriocins are located intracellularly, with a small proportion being associated with cell membrane (Soliman *et al*., 2007). It was produced to protect bacteriocin-producing cells against the antimicrobial activity of their own bacteriocin (Johnsen *et al*., 2005). However, unknown mechanisms of the immunity protein interact with the bacteriocin. It is hypothesized that the immunity proteins recognize a bacteriocin receptor and directly or indirectly inhibit its action by altering the receptor conformation or by hiding its bacteriocin-binding site (Drider *et al*., 2006).

4. Conclusion

Enterocin CE5-1 produced from *Ent. faecium* CE5-1 displays a narrow inhibitory spectrum, tolerance to a wide range of pH and temperature and sensitivity to proteolytic enzymes and á-amylase. In addition, it can inhibit the growth of VRE *in vitro*. Therefore, enterocin CE5-1 may be potentially suitable for use as an antibacterial agent to control foodborne pathogen, spoilage bacteria in food and feed processes and to decrease the dissemination of VRE in the environments. However, purification of enterocin CE5-1 should be studied to prove its structure, safety profile and mode of action. In addition, the ability of the enterocin CE5-1 or enterocin CE5-1-producing cells to control foodborne pathogen, spoilage bacteria or VRE *in situ* has to be investigated.

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