Research Article

Purification and Characterization of A Novel Bacteriocin Against Vancomycin Resistant Enterococci Produced by *Enterococcus Hirae* HM02-04

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Abstract
Enterococcus hirae HM02-04 isolated from breast milk exerted growth inhibition against especially vancomycin resistance enterococci (VRE). The aims of this study were to purify and characterize the antimicrobial substance produced by the strain HM02-04. One active peptide was successfully purified by 3 steps of Amberlite XAD-16 adsorption-desorption, cation exchange chromatography, and reverse-phase HPLC. It had 2605.298 Da peptide by MALDI-TOF MS analysis. The peptide sequence determined by LC-MS/MS contained only 23 amino acid residues providing the molecular mass of 2312.67 Da by *in silico* analysis. However, its amino acid sequence obtained showed no homology to other bacteriocins including enterocins previously reported and proposed as a novel one named Hiracin HM02-04. This is the first report to discover *E. hirae* isolated from breast milk that was able to produce a bacteriocin against VRE. Hiracin HM02-04 was stable at a high temperature of 121°C for 15 min and at a wide pH range of 3–9. It was sensitive to actinase E, pepsin, proteinase K and trypsin. The Hiracin HM02-04 has the narrow inhibition spectrum and no inhibition against *Listeria*. This bacteriocin was also found to have a bactericidal mode of action with concomitant cell lysis against the strain VRE 426. The present research addresses Hiracin HM02-04 as a promising alternative to conventional antibiotics in the treatment of enterococcal infections.

Keywords: Characterization, *Enterococcus hirae*, Hiracin, Purification, Vancomycin resistance enterococci

1 Introduction

Enterococci is one of the most commonly occurring antimicrobial-resistant nosocomial pathogens. The glycopeptide vancomycin resistance in the *Enterococcus* genus is rapidly increasing worldwide. Infections with vancomycin-resistant enterococci (VRE) are not only difficult to treat but also develop a strong tendency to propagate and spread from patient to patient in the hospital surrounding [1]–[3]. So far a few antibiotic are used for VRE treatment. However, it is expensive and later causes antibiotic resistance as well. Hence, VRE has become a major global health threat [4]. The problem of multiple drug resistance posed many challenges for clinicians due to the lack of effective antibiotics. Therefore, searching or developing new effective antimicrobial agents is becoming increasingly important. In recent years, bacteriocins of lactic acid bacteria (LAB) are much attractive interest since they cause non-resistance in the host system [5]. They are promising to use as the antibacterial agents to replace current antibiotics. Bacteriocins are peptide toxins

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secreted by bacteria and active against other bacterial strains closely-related or unrelated to produce bacteria. However, the number of study on the bacteriocin applications in therapeutic as the antimicrobial agent was less than in the food industry due to its natural and safe food preservatives concerns [6].

Human milk is considered as the best nutrition source for the growth and development of the neonate to provide nutritional and immunological needs. *Staphylococcus*, *Lactococcus*, *Enterococcus*, and *Lactobacillus* are species which found frequently in human milk. They are also evaluated as potential probiotic species [7]. *Enterococcus hirae* is a common pathogen in mammals and birds. It is rarely collected from the human. However, all cases related to bacteremia with severe illness [8]–[10]. *E. hirae* was also mentioned as a minority component of the human intestinal flora [11]. It was found from Brazilian artisanal cheese as well but rarely isolated from other fermented food [12]. In our laboratory, we isolated a bacteriocin producing LAB from several sources including breast milk that are able to inhibit the growth of VRE. This strain was isolated from human breast milk and identified as *E. hirae* HM02-04 (submitted). It was kept at –80°C at culture collection of Specialized Research Unit Probiotics and Prebiotics for Health, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University. Hence, further studies on purification and characterization of that bacteriocin targeted were the aims of this presentation.

2 Materials and Methods

2.1 *Bacterial strains and their growth conditions*

E. hirae HM02-04 isolated from breast milk were used as a source of bacteriocin while VRE 426 was as an indicator strain to determine the inhibition activity for purification and characterization study. Both were cultivated in the MRS medium (Difco, USA) at 37°C for 18 h without agitation. The growth conditions of other bacterial strains were shown in Table 1. Their

Table 1: List of indicator strains, their growth condition and inhibition activity of the bacteriocin produced by *E. hirae* HM02-04

Indicator Strain	Growth Condition	Bacteriocin Activity (AU/mL)
VRE 426, VRE 328, VRE4841	MRS, 37°C	800
Enterococcus. fecalis JCM 5803^T	MRS, 37°C	3200
Enterococcus faecium JCM 5804	MRS, 37°C	200
Enterococcus fecalis TISTR 927	MRS, 37°C	200
Listeria innocua ATCC 33090	TSYE, 37°C	0
*Staphylococcus aureus*118	NB, 37°C	0
Bacillus coagulans JCM 2257	NB, 37°C	400
Bacillus cereus JCM 2152	NB, 37°C	0
Bacillus subtilis TISTR 025	NB, 37°C	0
Streptococcus equinus JCM 5802	NB, 37°C	0
Leuconostoc mesenteroidessubsp.mesenteroides JCM6124	MRS, 30°C	0
Lactococcus lactic 5805	MRS, 37°C	800
Lactobacillus corynifomis JCM 1164	MRS, 37°C	100
Lactobacillus saliva D4	MRS, 37°C	0
Lactobacillus saliva I49	MRS, 37°C	0
Lactobacillus plantarum KL1	MRS, 37°C	0
Lactobacillus plantarum ATCC 14917	MRS, 37°C	400
Lactobacillus fermentum D18	MRS, 37°C	0
Lactobacillus johnisonii NUN 19-2	MRS, 37°C	0
Salmonella Enteritidis S003	NB, 37°C	0
Salmonella entiritica DMST 17368	NB, 37°C	0
Pseudomonas auruginosa 15442	NB, 37°C	0
Pseudomonas acidophila	NB, 37°C	0
Escherichia coli ATCC 8739	NB, 37°C	0
Escherichia coli E010	NB, 37°C	0
Acinetobacter calcoaceticus TISTR 360	NB, 37°C	0

ATCC, American Type Culture Collection, Rockville, Md; JCM, Japan Collection of Microorganisms, Wako, Japan; TISTR, Thailand Institute of Scientific and Technological Research, Thailand; DMST, Department of Medical Sciences Thailand, Thailand. MRS, de Man Rogosa Sharpe; TSYE, Trypticase soy yeast extract; NB, Nutrient Broth.

stock culture was maintained in 40% glycerol and stored at –80 and –20°C for a long and daily use, respectively. All vancomycin-resistant strains studied were obtained as gifts from the Faculty of Medicine, Chiang Mai University.

2.2 *Determination of bacteriocin activity*

Bacteriocin activity was determined by using a spot on lawn method as described by Ennahar *et al*. [13]. Ten microliters of indicator strains grown overnight were seeded into 6 mL of 0.7% soft agar medium and overlaid on 1.5% agar plate containing the same medium. Ten microliters of bacteriocin solution were spotted on the overlaid plate and incubated for 18 h at 37°C. The activity of bacteriocin was expressed as an arbitrary unit (AU/ mL).

2.3 *Purification of bacteriocin*

Hiracin HM02-04 was purified from an 18 h culture of *E. hirae* HM02-04 grown in 1 L of MRS broth containing 0.5% (w/v) of $CaCO_3$ (pH 6.5) at 37°C. The cell-free supernatant (CFS) was collected by centrifugation (11,000 × g,15 min), heated at 70°C for 30 min to destroy the living cell and further purified by the modified method of Rumjuankiat *et al*. [14]. In brief, 20 g of Amberlite XAD-16 (Sigma-Aldrich, Germany) activated by 70% isopropanol was poured in 1 L of CFS and gently stirring at 4°C for 12 h to allow bacteriocin binding. Subsequently, the unbound compounds were removed using deionized water and 40% ethanol once. The bacteriocin was eluted with 100 mL of 70% isopropanol in 0.1% trifluoroacetic acid (TFA) and then removed isopropanol by a rotary evaporator (R-300, Buchi Ltd., Switzerland) to obtain the concentrated sample which was loaded into a cation-SP-Sepharose Fast Flow cation-exchange column (XK16 column, Amersham Bioscience, USA). The latter column was pre-equilibrated with phosphate buffer at pH 5.5 (buffer A) at the flow rate of 1 mL/min. The unbound was removed with 5 column volumes of buffer A. The bacteriocin fractions were eluted by a gradient of 1 M NaCl in the same buffer (buffer B). The active bacteriocin fractions obtained were applied to reverse phase HPLC (Walter, USA) using a reverse-phase column (Resource RPC 3 mL, Amersham Bioscience, Sweden) and eluted with a gradient of MilliQ water and acetonitrile in 0.1% TFA at a flow rate of 1 mL/min as follows: 0–15 min, 0–65% (v/v); 15–25 min, 65–75% (v/v); and 25–30 min, 75–100 % (v/v) and then kept for further 1 min by 100% (v/v) acetonitrile. The active fractions were injected to RP-HPLC again to obtain pure bacteriocin. To avoid the effect of acetonitrile, its residual was further removed by a Speed-Vac concentrator (Uivapo100H, Uniequip, Germany).

2.4 *Determination of protein concentration*

The concentration of protein was quantified by the Bradford method [15]. Bovine Serum Albumin was used to perform a standard curve.

2.5 *Molecular weight determination by SDS-PAGE and MALDI TOF mass spectrometry*

To evaluate the molecular weight of the active bacteriocin, the active fraction was analyzed with 15% Tris-Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Mi-Protean® Tetra system, Bio-rad, USA), using Fermentas Spectra™ Multicolor Low Range Protein Ladder (Thermo Scientific, USA) as a size reference ranging from 1.7–40 kDa according to the method of Therdtatha *et al*. [16]. After electrophoresis, the gel was cut into two parts. One part was to check the bacteriocin purity by silver staining. The other one was assayed for inhibition activity by activity gel. The gel was washed three times with sterile deionized water for 1 min and overlaid with 7 mL of soft MRS (0.7% agar) seeded with 10^5 CFU/mL of VRE 426. After incubation at 37°C for 18 h, the gel was examined for the presence of an inhibition zone [16]. Its molecular weight was also analyzed by matrix- assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Germany)

2.6 *Amino acid sequence determination*

The bacteriocin HM02-04 was analyzed by LC-MS/MS at the National Center for Genetic Engineering and Biotechnology (BIOTEC) (Pathum Thani, Thailand). The 100 ng of the peptide was analyzed using a System of Ultimate3000 Nano/Capillary LC (Thermo

Scientific, UK) coupled to a Hybrid quadrupole Q-Tof impact II™ (Bruker Daltonics GmbH, Germany) equipped with a Nano-captive spray ion source. One microliter of the peptide was subjected to the trapping column (Thermo Scientific, PepMap100, C18, 300 µm i.d. × 5 mm), using full loop injection. The sample was resolved on an analytical column (PepSwift Monolithic Nano Column, 100 µm × 5 cm i.d.) at a column temperature of 60°C. A linear gradient for elution method was used to elute peptides into the mass spectrometer, mobile phases A (0.1% formic acid) and B (0.1 % formic acid in 80% acetonitrile) at a constant flow rate of 1 µL/min. The gradient condition was performed as follows; the mobile phase gradients were conditioned as follows: separation period (0–8.5 min: 0–45% B), washing period (8.6–10.6 min: 90% B), and re-equilibration period (10.7–20 min: 1% B). Electrospray ionization was carried out at 1.6 kV using the CaptiveSpray. Mass spectra (MS) and MS/MS spectra were fully acquired in the positive-ion mode over the range m/z 150–1200 (Compass 1.9 for OTOF series software, Bruker Daltonics). Mass accuracy in positive detection mode after tune/internal calibration with Sodium trifluoroacetate (NaTFA) was within 1.6 ppm. Briefly, LC-MS/MS spectra were acquired using a data-dependent auto-MS/MS method, used a dynamic method, with a fixed cycle time of 3 s. The mass spectra were acquired and analyzed using Bruker compass data analysis 4.4 software and Bruker Bio tool 3.2 software (Bruker Daltonics GmbH, Germany).

2.7 *Determination of pH and thermal stability and enzymatic toleration*

To determine bacteriocin temperature stability, it was heated to 80°C for 30 min, 100°C for 15, 30 min and 121°C for 15 min. The residual bacteriocin activity at each temperature was determined and compared with the unheated bacteriocin used as the control referring to 100% relative activity.

For pH stability, bacteriocin solution was adjusted to pH 3–9 using citrate phosphate (pH 3, 4, 5 and 6), sodium phosphate (pH 7), Tris-HCl (pH 8 and 9) and maintained at the corresponding pH for 2 h at 37°C. The residual bacteriocin activity at each temperature was determined and compared with the ones at 0 h considered as 100% relative activity.

The sensitivity of bacteriocin to different enzymes, including proteolytic enzymes of actinase E, pepsin, protease K and trypsin, as well as α-amylase and lipase (Sigma-Aldrich,USA) was tested by incubating bacteriocin in the presence of above enzymes at final concentration of 1 mg/mL, at their optimal temperature and pH for 2 h. The mixture was subsequently heated for 5 min at 100°C to stop reactions. The residual bacteriocin activity was determined and compared to the reaction without enzymatic treatment used as the control.

2.8 *Mode of action of bacteriocin*

The indicator strain VRE 426 was grown in 10 mL MRS at 37°C. The 20 AU/mL bacteriocin corresponded to 2.17 µg bacteriocin was added to VRE culture after 8 h incubation. The cell density and viable cells were determined every 2 h for 24 h by spectrophotometer at OD_{600nm} and the standard plate count method, respectively [14]. The culture without bacteriocin addition was used as the control. The percentage of killing efficiency was calculated as follow:

$$\%\text{Killing} = \frac{\text{Initial viable cells} - \text{Final viable cells}}{\text{Initial viable cells}} \times 100\%$$

Where: Initial viable cells were determined at the beginning of bacteriocin addition

3 Results

3.1 *Purification of bacteriocin*

Proteinaceous structure of antimicrobial substance produced by *E. hirae* HM02-04 was preliminary confirmed by proteinase K treatment resulting in no remaining inhibition activity against VRE [17]. This target antimicrobial compound was further purified by a three-step purification protocol summarized in Table 2. Partial purification by amberlite adsorption and cation exchange chromatography using SP-Sepharose resulted in low yields 2–5 times (47.41–17.81%) of CFS while increasing purification to 1.48–5.3 folds. All active fractions were subsequently purified for the final stage of RP-HPLC twice. The active fraction was eluted by 65–70% acetronitrile to obtain a single peak (Figure 1) with the purification of 26.12 folds and a yield of 1.05%.

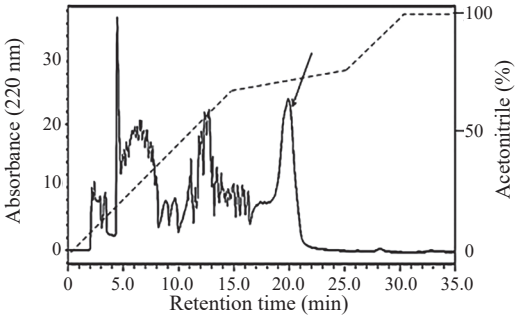

Figure 1: Reverse-phase chromatogram of the Hiracin HM02-04. The dash line is gradient condition of acetonitrile. The arrow indicated active fraction.

3.2 *Molecular weight and amino acid sequence determination*

To confirm the purity and estimate the molecular weight of the purified bacteriocin, the Tris-Tricine SDS-PAGE and zymogram analysis were performed (Figure 2). Only a single band was obtained and indicated the homogeneity of bacteriocin with the molecular mass of around 3 kDa. In addition, it displayed an inhibition zone around 3 kDa, where the gel was overlaid on the MRS plate containing VRE 426. However, its molecular mass analyzed by the MALDI-TOF mass spectrophotometer was 2605.298 Da (Figure 3) namely Hiracin HM02-04.

The amino acid sequence of Hiracin HM02-04 performed by LC MS/MS resulted in a complete sequence of 23 amino acids, PSATGMARWNPGGRAMMNAPPAA with its MW of 2312.67 Da by *in silico* analysis which was smaller than the one analysed by MALDI-TOF mass (2605.298 Da). Possibly, some modification reaction of a few missing amino acids could occur at N-terminal. The sequence obtained in this study showed 71.43% similarity to ATP synthase beta subunit of virus Dioscorea trifida with only 30% coverage by protein BLAST against the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). However, it did

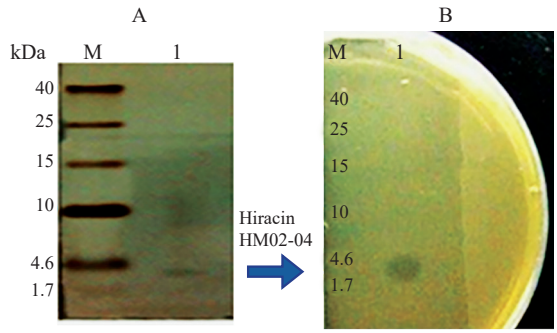

Figure 2: SDS-PAGE of purified protein. A, Silver stained Tris-Tricine SDS-PAGE containing 15% polyacrylamide gel, M: Spectra Multicolor Low Range Protein Ladder (Thermo Scientific Pierce, USA), Lane 1: purified protein. B, Protein with antibacterial activity visualized by overlaying soft MRS agar containing the cells of VRE 426 used as an indicator strain.

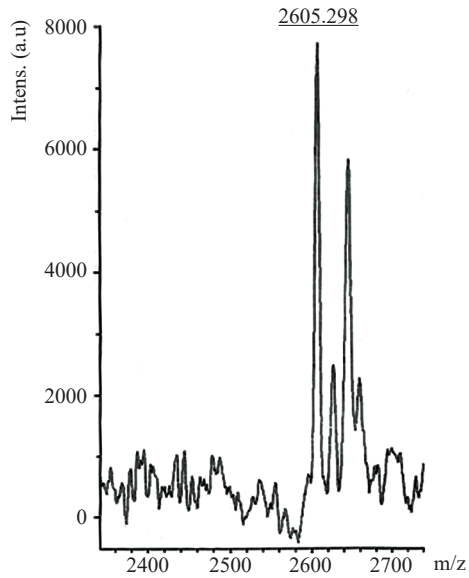

Figure 3: MALDI TOF mass spectra of Hiracin HM02-04.

Table 2: Purification of bacteriocin produced by *E. hirae* HM02-04

Purification Stage	Total Protein Concentration (mg)	Total Activity (AU)	Specific Activity (AU/mg)	Yield (%)	Purification (fold)
Cell free supernatant	764.1	270000	353.36	100	1
Amberlite XAD-16 resin	244	128000	524.59	47.41	1.48
Cation exchange	12.08	22800	1886.79	17.81	5.34
RP-HPLC	0.03	240	9230.77	1.05	26.12

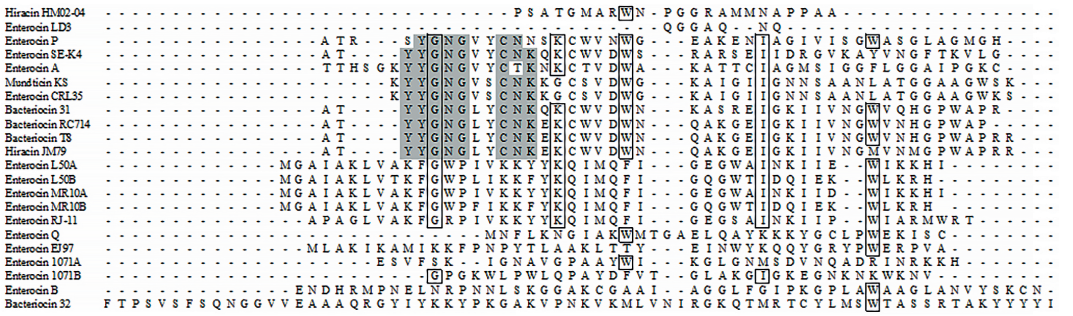

Figure 4: Protein sequence alignments of the mature peptides of Hiracin HM02-04 and Enterocins produced by *Enterococcus* where the conserved sequence is indicated by highlight. While the boxes were indicated as sequence identity.

not show homology with other known antimicrobial peptides and especially the enterocin group shown in Figure 4. Some bacteriocins such as, Enterocin CRL35 [18], bacteriocin 31 [19], Enterocin A [20], bacteriocin RC714 [21], bacteriocin SE-K4 [22], Mundticin KS [23], bacteriocin T8 [24] and Hiracin JM79 [25] had a common sequence of "YYGNG-CNK" which was not found from Hiracin HM02-04. Thus, Hiracin HM02-04 could be a novel LAB bacteriocin which provided a unique amino acid sequence with VRE specificity.

3.3 *Effect of hydrolytic enzymes, pH and temperature on Hiracin HM02-04 stability*

Hiracin HM02-04 was sensitive to all 4 proteolytic enzymes but resistant to amylase and lipase. This suggested that it had a proteinaceous nature [17]. Moreover, the presence of α-amylase or lipase did not affect bacteriocin activity which revealed that the inhibitory compound did not contain lipid or carbohydrate moieties.

Table 3: Characters of bacteriocins from *Enterococcus*.

Enterocin/Host Cell	MW (Da)	Inhibitory Spectrum	Physical Characteristic	Reference
Hiracin HM02-04/*E. hirae* from human milk	2605.29	VRE, *Lc. lactic* 5805, *Lb. corynifomis* 1164 and *Lb. plantarum* 14917, *Bacillus coagulans*	80–100°C for 30 min 121°C for 15 m, pH 3–9	This study
Bacteriocin T8/*E. faecium* T8	5100	Enterococci, *Listeria*, *Lb sakei*, *Propioni bacterium* sp.	60 min at 100°C, pH 4.0–10.0	[24]
Enterocin LD3/E.hirae/from dosa batter	4114.6	*S.aureus*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *L.monocytogenes*, *E. coli* O157:H7 and *Vibrio* sp	121°C, pH 2–6	[26]
Hiracin JM79/*E. hirae* DCH5 from ducks	5093.7	*Enterococcus*, *Clostridium botulinum*, *L. monocytogenes*, and S. aureus	ND	[25]
Hiraecin S/*E. hirae* C311 from bovine intestinal	ND	Enterococci, *Listeria*	ND	[27]
Enterocin F-420 (Enterocin P)/ *E. hirae* F420 from goat milk	4493	*Listeria*, *Bacillus*, *Brochothrix* and enterococci	100°C for 30 min, pH4–8	[28]
Enterocin EJ97/*E. faecalis* EJ97	5340	enterococci, several species of *Bacillus*, *Listeria*, and *S.aureus*	pH 2.0–9.5, 80°C for 1h	[29]
Enterocin 1071A/*E. faecalis* BFE	4285	*Enterococcus*, *Lb. salivarius* subsp. *Salivarius*, *L. innocua*, *Micrococcus* sp., *Peptostreptococcus aerogenes*, *Streptococcus agalactiae*, *Propionibacterium freudenreichii* subsp. shermanii	50% of activity remained after 15 min at 121°C, pH 3–12	[30]
Enterocin B/*E. faecium* T136	5465.2	*Listeria*, staphylococci and most lactic acid bacteria, *Clostridium sporogenes*, *C. lostridium tyrobutyricum*, *Propionibacterium* spp.	ND	[20]
Bacteriocin 32/*E. faecium* VRE200	7998	*E. faecium*, *E. hirae*, and *E. durans*	ND	[31]

Thermal stability of Hiracin HM02-04 was performed at various high temperatures of 80, 100 and 121°C. The results showed that the stabilities at 80 and 100°C for 30 min, as well as 121°C for 15 min, exhibited 100% relative activity.

The Hiracin HM02-04 was assayed in the pH range 3–9 at 37°C for 2 h. They showed 100% relative activity at pH 3–9. This indicates that bacteriocin would active and further applied in acidic as well as nonacidic processing conditions.

Its physicochemical characters were compared to other bacteriocins produced by *Enterococcus* listed in Table 3. All including Hiracin HM02-04 were tolerant at high temperature and displayed at a wide pH range of acid to alkaline.

3.4 *Inhibitory spectrum of Hiracin HM02-04*

The antagonistic activity of Hiracin HM02-04 against several strains of LAB as well as pathogens is shown in Table 1. It exhibited various bacteriocin activities of 200–3200 AU/mL against enterococci including VRE, *E. faecium* and *E. faecalis* while *E. fecalis* JCM 5803T was the most sensitive. In addition, it also inhibited the growth of other LAB including *Lc. lactis* 5805, *Lb. corynifomis* 1164 and *Lb. plantarum* 14917 as well as other gram-positive bacteria *Bacillus coagulans*. However, it had no effect on *Listeria* and gram-negative bacteria. This indicated that Hiracin HM02-04 displayed a narrow inhibition spectrum against only gram-positive bacteria. To compare its inhibition spectrum to other bacteriocins produced by *Enterococcus* previously reported (Table 3), most displayed inhibition activity against only gram-positive bacteria except Enterocin LD3 from *E.hirae* did against gram-negative bacteria such as *Salmonella typhi, Shigella flexneri*, *Escherichia coli* O157:H7 and *Vibrio* sp as well. So far, only pumilicin 4 produced by *Bacillus pumilus* strain WAPB4 in samples collection from soil and water exhibited growth inhibition to both VRE and MRSA [32]. While enterocin CE5-1 produced by *E. faecium* CE5-1 isolated from the chicken intestinal tract [33], Lactacin 3147 [34], bacteriocin-like inhibitory substance DSH20 from *E. faecium* strain DHS20 [35] exhibited growth inhibition to VRE. This is the first report to propose bacteriocin from *E. hirae* which displayed inhibition activity against VRE.

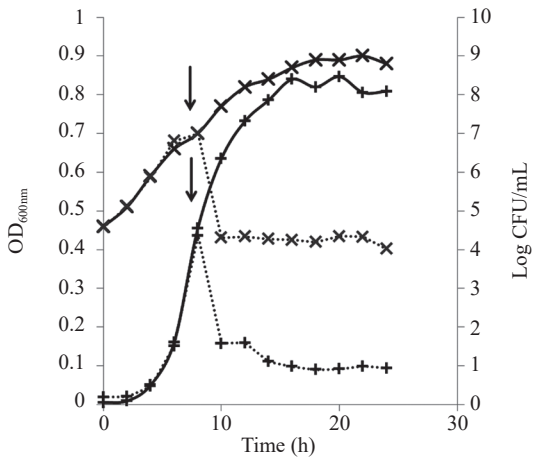

Figure 5: Effect of Hiracin HM02-04 on the growth of vancomycin resistance *enterococcus* VRE 426. The growth were determined by the optical density at 600 nm (+) and viable cell concentration (X) under the condition of without bacteriocin (____) and with bacteriocin (……). The arrow indicated the addition of Hiracin HM02-04 at 8 h cultivation.

3.5 *Mode of action of Hiracin HM02-04*

The addition of 20 AU/mL bacteriocin to the mid-logarithmic phase (8 h) of VRE culture solution resulted in decreases in both the number of viable cells for about 2log CFU of the initial cell population and in optical density when compared with the control shown in Figure 5. These reductions corresponded to 99.8% of the viable cells after treatment (Table 4). In contrast, both viable cell concentration and optical density of the control increased during 24 h. These results indicated that bacteriocin has bactericidal activity with cell lysis.

4 Discussion

The genus *Enterococcus* have become a major concern of nosocomial infections. They show multiple drug resistance, especially vancomycin resistance named vancomycin resistance enterococci (VRE) which cause serious infections. They are more difficult to treat by regular infections due to only a few antibiotics applied for. Campo *et al*. isolated 218 isolates from different origins. They all belonged to *E. faecalis* and *E. faecium* for 93 and 125 isolates, respectively. Fifty-six isolates showing vancomycin resistance were from human clinical, human fecal, sewage and chicken

samples for 12.6, 19.2, 17.8 and 100%, respectively indicating high potential detection of vancomycin resistance isolates from each area which represents an additional risk for human health [21].

Table 4: Killing activity of Hiracin HM02-04 against VRE426

Time (h)	Cell Viability (CFU/mL)	Killing Efficiency (%)
8	9.9×10^6	
10	2.0×10^4	99.8 ± 0.034
12	2.2×10^4	99.78 ± 0.022
14	1.9×10^4	99.81 ± 0.087
16	1.8×10^4	99.82 ± 0.053
18	1.6×10^4	99.84 ± 0.098
20	2.2×10^4	99.77 ± 0.107
22	2.1×10^4	99.79 ± 0.0367
24	1.4×10^4	99.89 ± 0.000

Therefore, it was very important to find out the active compound like bacteriocin which would not cause antibiotic resistance later on. In this study, Hiracin HM02-04 produced by *E. hirae* exhibited bacteriocin activity against VRE with the action mode of the bactericidal activity causing cell lysis which had similar action mode of Lacticin 3147 [34] and Pumilicin 4 [32]. However, Pumilicin 4 and Lacticin 3147 exhibited only bactericidal activity against VRE at high concentration of 80 AU/mL and 20,000 AU/mL, respectively. Under these concentration, they had bacteriostatic activity while Hiracin HM02-04 exhibited bactericidal activity against VRE at only 20 AU/mL. Up to date, only a few bacteriocins produced by *E. hirae* were reported for example hiraecin S from bovine intestinal [27], Hiracin JM79 produced by *E. hirae* DCH5 isolated from Mallard ducks [25], *E. hirae* F420 from Moroccan raw goat milk [28] and bacteriocin from *E. hirae* LD3 from dosa batter [26]. However, none displayed inhibition activity against VRE. In addition, *E. hirae* is actually known to cause infections in animals but is rarely isolated from human clinical samples [9]. Therefore, this is the first report to discover *E. hirae* isolated from breast milk that was able to produce a bacteriocin against VRE.

The bacteriocin was commonly produced and secreted into the medium with a very small quantity [36]. Based on bacteriocin purification process commonly used, it was indicated that it had a strong cationic hydrophobic nature [37]. The 23 amino acid sequence of Hiracin HM02-04 contained 3 glycine residues and 12 hydrophobic residues consisting of 5 alanine, 4 proline, and 3 methionine residues which strongly confirmed for its hydrophobic properties. Hydrophobic regions play a role in the inactivation of microoganism by hydrophobic intereaction between the sensitive bacterial cells and bacteriocin molecules [38].

Only 23 amino acids obtained fulfilled its mature peptide of 2312.67 Da which was smaller than the one of 2600.47 Da analyzed by MALDI TOF Mass. Some occurrence of post translational modification may cause an error by mass spectrometry-based analysis [39]. However, based on amino acid sequence obtained, Hiracin HM02-04 lack of the YGNGVXC motif at N-terminal which is a leader peptide for bacteriocin secretion [40]. This character was similar to enterocin B [20], enterocin EJ97 [29], enterocin 1071A [30], enterocin 1071B [30], bacteriocin 32 [31] and enterocin LD3 [41]. Its small size of less than 10 kDa and thermal-stable could belong to either class I or II of bacteriocin [5]. However, it was protease sensitive to protease K, trypsin, pepsin and actinase E that were different from cyclic bacteriocin to which more proteolytic resistance [42]. It also had no effect on growth inhibition of *Listeria* and displayed its inhibition activity by the one-component system. These characters were different from those bacteriocin belonging to class IIa, b, or c. Considering to class I, lantibiotics can perform by post translational modification reaction and its biosynthesis starts with the enzymatic reaction by 2–8 H_2O molecule lost to obtain mature peptide. However, in this study, the calculated mass of the sequence obtained was 2312.67 Da, approximately 293 Da less than those observed by LC-MS/MS suggest that a few amino acid residues should be included to complete its structure. Based on *in silico* analysis, additional at least two amino acids at N-terminal should be possible to obtain the complete amino acid sequence with or without hydration reaction like lantibiotic class I. Further study by PCR cloning by specific primers of known sequence and sequencing as well as cyanogen bromide treatment for possible initial formulated methionine [43] are suggested.

Hiracin HM02-04 displayed a narrow inhibitory spectrum mainly towards *Enterococcus* and some species of LAB. Several studies previously reported that bacteriocins produced by *Enterococcus* are able

to inhibit *Listeria* spp. [27] for example bacteriocin from *E. faecium* IM1 and *E. hirae* IM1 strains isolated from home made Egyptian dairy products [44], enterocin L11 [45] and enterocin LD3 [41]. However, Hiracin HM02-04 performed inhibitory activity which was similar to bacteriocin 32 produced by *E. faecium* VRE200 isolated from a patient [31]. These findings suggested that the dominant type of bacteriocin produced by clinical isolates might differ from the one by food-grade isolates. These difference type of bacteriocin might become dominance within any environments that are influenced by the surrounding ecology [31].

Unlike antibiotics are forwarded to target specific enzymes, most bacteriocins inhibit target cells by pore formation and subsequently allow permeation of the cytoplasmic membrane or inhibition of cell wall biosynthesis, or a combination reaction leading to cell death. The leakage of molecules from target bacterial cells may or may not cause cell lysis [5]. The mode of action of Hiracin HM02-04 against VRE was bactericidal along with cell lysis. This complex mode of action caused some difficulty for pathogens to develop resistance against bacteriocins. These activities are similar to the ones of enterocin EFS2 [46], bifidocin A [47]. Considering to the stability, Hiracin HM02-04 showed heat stable up to 121°C for 15 min. The high-temperature stability of bacteriocins is evaluated to be a positive feature, allowing them to be applied in products that will be exposed to sterilization or other heat treatment processes. In addition, Hiracin HM02-04 showed pH stability at pH 3–9. This indicated that the Hiracin HM02-04 may be useful if it will be applied as an antimicrobial agent working in acidic as well as nonacidic conditions in the future.

5 Conclusions

This study described the purification and characterization of Hiracin HM02-04, a bacteriocin produced by *E. hirae* strain HM02-04. Hiracin HM02-04 was purified by a three-step purification and it had a molecular mass approximately 2.6 kDa. Hiracin HM02-04 has pronounced antimicrobial activity against enterococci, including VRE and some gram-positive bacteria. Moreover, Hiracin HM02-04 was sensitive to proteolytic enzymes and displayed good thermal and pH stability. It had bactericidal action mode with cell lysis to indicator strain VRE 426. Further studies about the structure and sequence of the bacteriocin gene would be needed to clarify before application.

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Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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