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Characterization and Enhanced Production of Enterocin HJ35 by *Enterococcus faecium* HJ35 Isolated from Human Skin

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Abstract A strain named as HJ35 was isolated from the skin of sixty-five men and fourteen women for acne therapy, in order to find an effective antimicrobial agent against *Propionibacterium acnes*. Isolate HJ35 was identified as *Enterococcus faecium* based on 16 rDNA sequence and produced enterocin HJ35 having antimicrobial activities against most lactic acid bacteria, *Enterococcus* spp., *Staphylococcus aureus, S. epidermidis, Clostridium perfringens*, some bacilli, *Micrococcus flavus, Listeria monocytogenes, L. ivanovii, Escherichia coli, Pseudomonas fluorescens* and *Propionibacterium acnes*, in the modified well diffusion method. Especially, enterocin HJ35 showed a bactericidal activity against *Propionibacterium acnes* P1. The antimicrobial activity of enterocin HJ35 was disappeared completely with the use of protease XIV. But enterocin HJ35 activity is very stable at high temperature (up to 100°C for 30 min), in wide range of pH (3.0~9.0), and by treatment with organic solvents. The apparent molecular mass of enterocin HJ35 was estimated to be approximately 4~4.5 kDa on detection of its bactericidal activity after SDS-PAGE. In batch fermentation of *E. faecium* HJ35, enterocin HJ35 was produced at the mid-log growth phase, and its maximum production was obtained up to 2,300 AU/mL at the late stationary phase. By employing fed-batch fermentation, the enhanced production of enterocin HJ35 was achieved up to 12,800 AU/mL by feeding with 10 g/L glucose or 6 g/L lactate.

Keywords: *Enterococcus faecium*, bacteriocin, enterocin HJ35, *Propionibacterium acnes*, characterization, fed-batch cultivation

INTRODUCTION

Bacteriocins are defined as bactericidal proteins, which generally have a narrow spectrum of activity targeted toward a species related to the producer culture [1,2]. Thus bacteriocins are potentially useful for industrial application as biopreservatives and bioregulators of the microflora present in fermented-foods based on their antibacterial characteristics [3-8]. Following recent great interest on bacteriocin in the context of food preservation, the possibility of gene manipulation for bacteriocins is considered as one of the major reasons for undertaking bacteriocin research [9,10]. Nisin, a generally recognized as safe (GRAS) bacteriocin, produced by certain strains of *Lactococcus lactis* subsp. *lactis*, is the only bacteriocin approved for food use in many countries [11]. In addition to its food application, bacteriocins could be used as an effective antimicrobial agent for acne therapy in cosmetic industry.

Enterococci are an important group of the GRAS lactic acid bacteria (LAB) [12]. Even though *Enterococcus faecium* and *E. faecalis* are the most frequently occurring enterococcal species in the human gastrointestinal tract, they persist in the extraenteral environment, and they are ubiquitous in food processing establishments. Further enterococci display such desirable metabolic activities as lipolytic and esterolytic activities, citrate utilization and bacteriocin production [7,13-17]. Therefore, enterococci have been applied in meat and dairy products (cheese, milk, and whey starter culture), probiotic preparation for health, and other food industry. Most bacteriocins from enterococci are classified as class II bacteriocins; and are small and heat-stable non-lantibiotics [18,19]. These are further divided into three subgroups: (IIa) listeria-active peptides including enterocin A, enterocin P and enterocin CRL 35. (IIb) poration complexes consisting of two proteinous peptides for activity, and (IIc) thiol-activated peptides requiring reduced cysteine residues for activity such as enterocin B, enterocins L50A and L50B. Recently, a number of bacteriocins produced by enterococci have been reported and many informations about their inhibitory spectra, and their stabilities against heat, pH and protease are available.

In this study, a bacteriocin producer for acne therapy was isolated from skin, and its partial characterization as well as enhanced production was attempted.

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MATERIALS AND METHODS

Bacterial Strains and Media

A strain named as HJ35 was isolated from the skin of sixty-five men and fourteen women of Korean volunteers, aged between 16 to 25 with scrub method. For the general spreading method, serial dilutions were made in 0.05% Triton X-100 solution, spread onto propionate and gentamicin sulfate agar (PAGSA) plates, incubated in an anaerobic jar at 32°C for 48 h and colonies were then streaked on fresh sodium lactate broth (NLB; Difco Laboratories, MI, USA) agar plates, and maintained as stock cultures in NLB broth with 20% glycerol at -70°C. Working cultures for growth and bacteriocin production were propagated in NLB broth at 32°C for 24 h. *Micrococcus flavus* ATCC 10240 grown in nutrient broth (Difco Laboratories, MI, USA) at 32°C was used as the indicator strain.

Microbiological Identification

Isolate HJ35 was identified on the basis of Gram staining, catalase test, the 16S rDNA sequence comparison and scanning electron microscopy (SEM).

Enterocin HJ35 Assay

The modified well diffusion method was performed for enterocin HJ35 [20]. After pouring the basal agar (2.5% agar and 0.1% Tween 80) layer in depth of 5 mm, plates were incubated for 24 h at room temperature. The wells having 7-mm diameter were cut out from plates and then plates were incubated at 37°C for 2 h or at room temperature for 2 days to dry and to facilitate sample diffusion into the agar. Cell-free supernatants (200 μL) were added to the wells, allowed to diffuse at 4°C, and the base agar was flipped into the petri dish lid before the overlay was applied. The indicator strain, *M. flavus* ATCC 10240, was mixed with 5 mL of NLB soft agar (0.7% agar), and about 10^7 cells were overlaid at each plate, then cultivated for 2 days at 32°C. Enterocin HJ35 activity was assayed by spotting serially two-fold diluted enterocin HJ35 in a well. The reciprocal of the greatest inhibitory dilution was used to calculate the activity units (AU) per mL of the original cultures. All experiments were performed in duplicate, and the results presented are the mean values of duplicate trials.

Production and Preparation of Crude Enterocin HJ35

Enterocin HJ35 production was performed in a 1-L bottle having 700 mL fermentation medium of NLB [8]. Fermentation was started with 1% (v/v) inoculum of 18-h cultured *E. faecium* HJ35, and incubated for 7 days at 32°C with pH control at 7.0 ± 0.1 by 3 N HCl and 3 N NaOH. Agitation speed was 150 rpm without aeration. Samples were aseptically removed every 12 h to determine the viable cells and bacteriocin activity. Cell growth was checked spectrophotometerically, and enterocin HJ35 activity of the culture broth was evaluated using the method described previously.

Culture broth from a jar fermenter was centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was sterilized by passing through 0.22-μm cellulose acetate filter. Crude enterocin HJ35 was isolated by ammonium sulfate precipitation [20]. Solid ammonium sulfate was slowly added to culture supernatant until reached 75% saturation level at 4°C, with constant stirring, over a period of 5 h. Slow stirring was continued for an additional 1 h at 4°C. Precipitated proteins were then recovered by centrifugation at 10,000 rpm for 20 min at 4°C, and resuspended in 100 mM phosphate buffer (pH 7.0), followed by extensive dialysis against 2 L of the same buffer for 12~24 h in dialysis tubing (molecular weight cut-off, 1,000; Spectrum Medical Industries, CA, USA). Dialyzed sample was stored at -70°C.

Antimicrobial Spectrum of Enterocin HJ35 Activity

The antimicrobial activity of the partially purified enterocin HJ35 was assessed using several Gram-positive and Gram-negative bacteria, and two molds, which are involved in food spoilage or pathogenicity by modified well diffusion method [20]. All strains were previously subcultured in the appropriate growth agar medium, and propagated in liquid medium prior to inoculation into soft-agar medium (0.75% agar) of the same composition.

Physico-chemical Properties of Enterocin HJ35

To confirm the nature of enterocin HJ35, the partially purified enterocin HJ35 fraction was treated with 1 mg/mL of various hydrolytic enzymes at 37°C for 1 h. All enzyme reactions were performed in buffers as recommended by the supplier (Sigma Chemical Co., MO, USA). To determine heat stability, aliquots (500 μL) of the partially purified enterocin HJ35 were incubated at different temperatures (40, 50, 60, 70, 80, 90, and 100°C) for 30 min, or at 121°C for 15 min [21]. To estimate pH stability, the partially purified enterocin HJ35 was stored for 4 h of storage at 4°C in the following buffers: 50 mM citrate buffer at pH 3~6; 50 mM phosphate buffer at pH 7.0; and 50 mM Tris-HCl buffer at pH 8~9 [21]. The partially purified enterocin HJ35 was also treated at 30°C for 1 h with a series of organic solvents in water (50:50, v/v) including ethanol, methanol, acetone, toluene, isopropanol, and chloroform. After evaporating residual solvents at 30°C for 2 h, the residual enterocin HJ35 activity was determined by the modified well diffusion method. Results are presented as means of duplicate tests.

Determination of Antimicrobial Inhibition Mode

Cells of *P. acnes* P1 in log-phase were suspended in sterile 100 mM phosphate buffer (pH 7.0), and 500 or 1,000 AU/mL of partially purified enterocin HJ35 was added at 30°C. After specified times, the viable cells (CFU/mL) were determined on NLB agar plates by the

plate counting method.

Scanning Electron Microscopic (SEM) Observation

Morphological change of enterocin HJ35-treated cells was investigated by SEM (S-4200 FEG-SEM, Hitachi, Japan). After *P. acnes* P1 cells in exponential-phase were treated with 800 AU/mL of enterocin HJ35 for 10 h, samples were fixed in 25% (v/v) glutaraldehyde at 4°C for 12 h, washed three times in a sterile 10 mM phosphate buffer (pH 7.0), and dehydrated by passage through a graded ethanol series (60, 70, 80, 90, 95, and 100% (v/v)). Specimens were then coated with Pt-Pb in an ion spatter (Hitachi, Japan) [22].

Molecular Weight Determination by SDS-PAGE

To estimate the molecular weight of partially purified enterocin HJ35, 16% SDS-PAGE was performed in duplicate with loading 20 μL of sample and molecular weight standard. Protein standards and their molecular weights were as follows: ovalbumin, 43,000; carbonic anhydrase, 29,000; lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor, 6,200; and insulin, 3,000. The gel was run in a vertical slab gel apparatus (Protein Cell II; Bio-Rad, CA, USA) at constant voltage (100 V) for 2 h. Half of the gel was stained with a Coomassie brilliant blue R-250, while the other was assayed for bacteriocin activity using the direct method as described previously [11].

Batch and Fed-Batch Fermentations

Small-scale batch and fed-batch fermentations were performed in a fermenter (1.5 L working volume). The fermentation medium was NLB with 1.2% sodium lactate as substrate. The fermentation was started with 1% (v/v) inoculum of 24-h culture in NLB, and the temperature of incubation was kept at 32°C and the pH was maintained at 7.0 ± 0.1 by the addition of 3 N NaOH. The agitation rate was 150 rpm in the fermenter and no aeration was provided [23,24]. Fed-batch fermentation was operated as batch fermentation at start and incubated until 250 h with feeding glucose or lactate firstly at about 48 h and next at every 12 h.

RESULTS AND DISCUSSION

Identification of the Enterocin HJ35 Producer Isolated from Human Skin

Enterococci were reported to be isolated from various environments; fish, vegetable, human wound exudate, rice bran, dairy product, meat, and so on. [9,19]. Bacteriocin-producing strains against *P. acnes*, which plays a central important role in the etiology of acne, were screened on PAGSA media from various human skins of Korean volunteers. One isolate named as HJ35 was identified to produce bacteriocin against *P. acnes*. In biochemical and morphological observation, this isolate was confirmed to be Gram-positive, nonmotile and catalase-negative coccus (data not shown). In phylogenetic analysis based on the 16S rDNA sequence, 400 bp sequence of strain HJ35 was placed within the region occupied by the genus *Enterococcus faecium* (Fig. 1). Therefore, isolate HJ35 was identified as a species of *E. faecium* HJ35 having 97% similarity at 16S rDNA level and the isolate was tentatively assigned as *E. faecium* HJ35. The bacteriocin produced by this strain was also named as enterocin HJ35 [5,18].

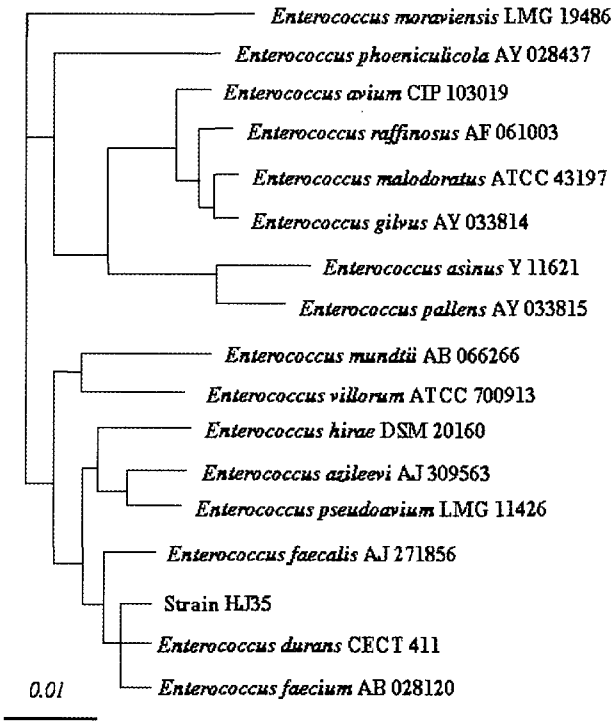

Fig. 1. Phylogenetic tree based on 16S rDNA sequences of strain HJ35, *Enterococcus* species, and representatives of some related taxa, with a scale of 0.01 means substitutions per nucleotide position.

Production and Partial Purification of Enterocin HJ35

E. faecium HJ35 was cultured in NLB broth at 32°C for 7 days and the culture broth was separated by centrifugation. Enterocin HJ35 was partially purified from culture broth by ammonium sulfate precipitation. The activity of the partially purified enterocin HJ35 was 32-fold increased to 51,200 AU/mL.

Antimicrobial Spectrum of Enterocin HJ35 Activity

To determine the antimicrobial spectrum, the partially purified enterocin HJ35 was tested against various non-pathogenic and pathogenic bacteria, and two molds using the modified well diffusion method (Table 1) [25]. Enterocin HJ35 showed a broad antibacterial activity against

Table 1. Antimicrobial spectrum of enterocin HJ35 by the modified well diffusion method

Indicator organism	Culture medium[a]	Incubation temp. (°C)	Inhibition by partially purified enterocin HJ35
Gram positive bacteria			
Lactobacillus delbrueckii KCCM 15808	MRS	37	+
Pediococcus acidilactici KCTC 1626	MRS	37	-
Leuconostoc mesenteroides KCCM 11324	MRS	25	+
Lactococcus lactis KCCM 40104	MRS	30	-
Propionibacterium acidipropionici P200910	NLB	30	-
Bacillus subtilis IFO 12113	NB	37	+
Bacillus subtilis BR 40	NB	37	+
Bacillus pumilis	NB	37	-
Bacillus cereus	NB	30	-
Staphyloccous aureus KCCM 32359	NB	30	-
Streptococcus mutans	BHI	37	+
Lactococcus lactis KCCM 32406	MRS	30	+
Listeria monocytogenes	TSB-YE	30	+
Micrococcus flavus KCCM 10240	NB	30	+
Propionibacterium acnes P1	NLB	30	+
Propionibacterium acnes P2	NLB	32	+
Propionibacterium acnes P3	NLB	32	+/-[b]
Propionibacterium acnes P5	NLB	32	-
Propionibacterium acnes KCTC 3320	NLB	32	-
Propionibacterium acnes KCTC 5212	NLB	32	+
Propionibacterium acnes KCTC 3314	NLB	32	-
Gram negative bacteria			
Escherichia coli KCCM 32396	NB	30	+
Escherichia coli JM 109	LB	37	+
Pseudomonas cepacia SBB 9611	NB	30	+-
Pseudomonas cepacia SBA 9613	NB	30	+
Pseudomonas fluorescens SBB 9631	NB	30	+
Chryseomonas luteola SBA 9634	NB	30	+
Xanthomonas maltophila SBC 9611	NB	30	-
Molds			
Aspergillus niger KCCM 11239	PDB	25	-
Aspergillus oryzae KCCM 11371	PDB	25	-

[a] Abbreviations: MRS is commercially available medium; NLB, sodim lactate broth; NB, nutrent broth; TSB-YE, trypticase soy broth with 0.6% yeast extract; LB, luria broth; PDB, potato dextrose broth.
[b] Not clear.

all non-pathogenic and pathogenic bacteria tested, but did not show antifungal activity against the molds. The bacterial strains inhibited by enterocin HJ35 included most lactic acid bacteria, *Enterococcus*, *Staphylococcus aureus* ATCC 25923, *S. aureus* KCCM 32359, *S. epidermidis* ATCC 12228, *Clostridium perfringens* ATCC 3624, some bacilli, *M. flavus* ATCC 10240, *Listeria monocytogenes* ATCC 15313, *L. ivanovii* ATCC 19119, *Escherichia coli* KCCM 32396, *E. coli* JM 109, *Pseudomonas fluorescens*, and *P. acnes*. In general, most enterocins have the capability of growth inhibition against Gram-positive bacteria, such as *L. monocytogenes*, *S. aureus*, *Clsotridium* spp. including *C. botulinum* and *C. perfirngens* [6,7]. Also, enterocin AS-48 has been reported to have antimicrobial activity against some Gram-negative bacteria such as *Salmonella choleraesuis* in combination with acidic or alkaline pH condition, sublethal heat, or outer membrane-permeabilizing treatment [26]. Therefore,

Table 2. Stability of enterocin HJ35 against various enzymes, heat, pH, and organic solvents

Treatment (Enzyme)	Residual activity (AU/mL)	Treatment (Heat)	Residual activity (AU/mL)	Treatment (pH)	Residual activity (AU/mL)	Treatment (Organic solvent)	Residual activity (AU/mL)
Control	51,200	Control	51,200	Control	51,200	Control	51,200
Protease I	51,200	40°C[a]	51,200	3	51,200	Ethanol	51,200
Protease IV	51,200	50°C[a]	51,200	4	51,200	Methanol	51,200
Protease XIII	51,200	60°C[a]	51,200	5	51,200	Acetone	51,200
Protease XIV	0	70°C[a]	51,200	6	51,200	Chloroform	51,200
Pepsin A	51,200	80°C[a]	51,200	7	51,200	Toluene	51,200
α-Chymotrypsin	51,200	90°C[a]	51,200	8	51,200		
β-Chymotrypsin	51,200	100°C[a]	51,200	9	51,200		
α-Amylase	51,200	121°C[b]	0				
Ribonuclease A	51,200						

[a]Heat treatment for 30 min.
[b]Autoclaved for 15 min.

enterocins themselves or their producers have great potentials as biopreservatives or bioprotectives.

Physico-chemical Properties of Enterocin HJ35

The treatment of partially purified enterocin HJ35 (51,200 AU/mL) with protease XIV resulted in a complete loss of bacteriocin activity (Table 2). However, any change of activity was not observed when enterocin HJ35 was treated with the other proteolytic enzymes including protease I, protease IV, protease XIII, pepsin A, α-chymotrypsin, and β-chymotrypsin. Enterocin HJ35 did not affected by treatment with amylase and ribonuclease A. These results confirm that enterocin HJ35 is an antimicrobial substance having the proteinaceous nature, and that no carbohydrate moieties are essential for its activity [25].

Enterocin HJ35 proved to be relatively heat stable (Table 2). It was completely stable even when treated at 100°C for 30 min. This stability of bacteriocins is known to be attributed to its structures having strongly hydrophobic regions, stable cross-linkage, and a high glycine content [23]. The heat stability of enterocin HJ35 also rules out the possibility that the inhibitory activity may be due to a bacteriophage.

Finally, enterocin HJ35 was stable between pH 3.0 and 9.0, and not affected by treatment with any of organic solvents, which can provide its usefulness for the industrial application. From these findings, enterocin HJ35 seems to be classified as class II bacteriocin.

Inhibition Mode of Enterocin HJ35

To determine whether enterocin HJ35 had a bactericidal or a bacteriostatic effect, partially purified enterocin HJ35 was added to *P. acnes* P1 cells suspended in phosphate buffer (pH 7.0). A decrease in CFU/mL was observed by expose to enterocin HJ35 (Fig. 2), which suggest a bactericidal action of enterocin HJ35. In SEM observation, *P. acnes* P1 cells in exponential phase showed distinct morphological change by treatment with 800 AU/mL of enterocin HJ35 (Fig. 3). However, the intrinsic nature of this change has not been identified and further investigation will be required [24].

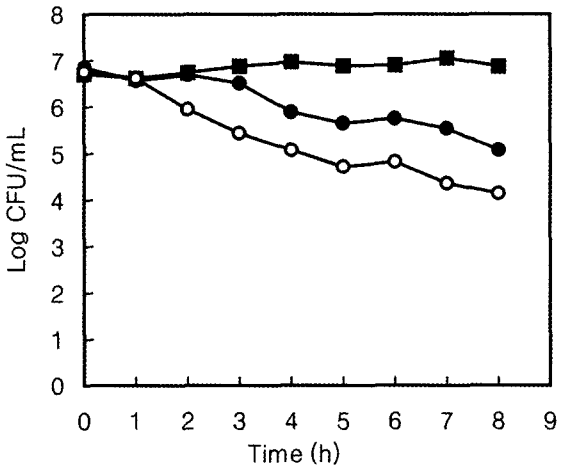

Fig. 2. Bactericidal inhibition of enterocin HJ35 against *Propionibacterium acnes* P1 in phosphate buffer. ■; Control, ●; 500 AU/mL, ○, 1,000 AU/mL.

Molecular Weight of Enterocin HJ35

Because several contaminant proteins were detected in the partially purified enterocin HJ35 (Fig. 4), A 16% SDS-PAGE gel containing enterocin HJ35 was cut into two vertical parts. One part was stained with Coomassie brilliant blue R-250 for protein detection, while the remaining part was fixed and used for direct detection of enterocin HJ35 activity using the method of Daba *et al.* [11]. The bactericidal activity of enterocin HJ35 was found in a protein band having an apparent molecular

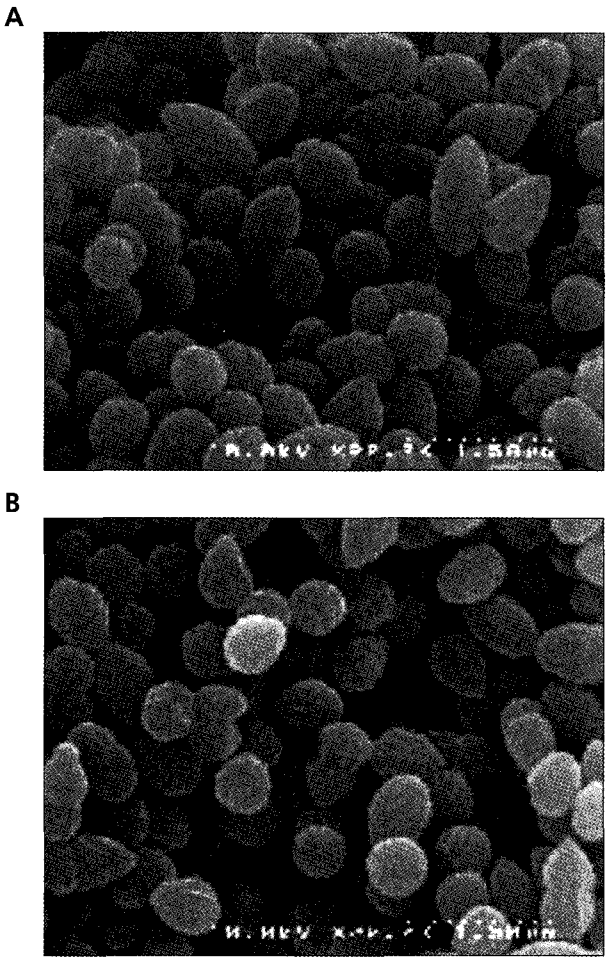

Fig. 3. Scanning electron microscopic (SEM) observations of *Propionibacterium acnes* P1 cells in exponential-phase. A; untreated cells (control), B; cells treated with enterocin HJ35.

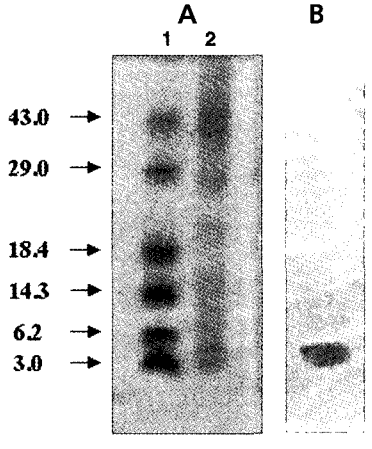

Fig. 4. SDS-PAGE of partially purified enterocin HJ35 for molecular weight determination. A; The gel was stained with Coomasie brillant blue R-250, B; The gel overlaid with indicator strain. Lane 1: protein marker; lane 2: partially purified enterocin HJ35.

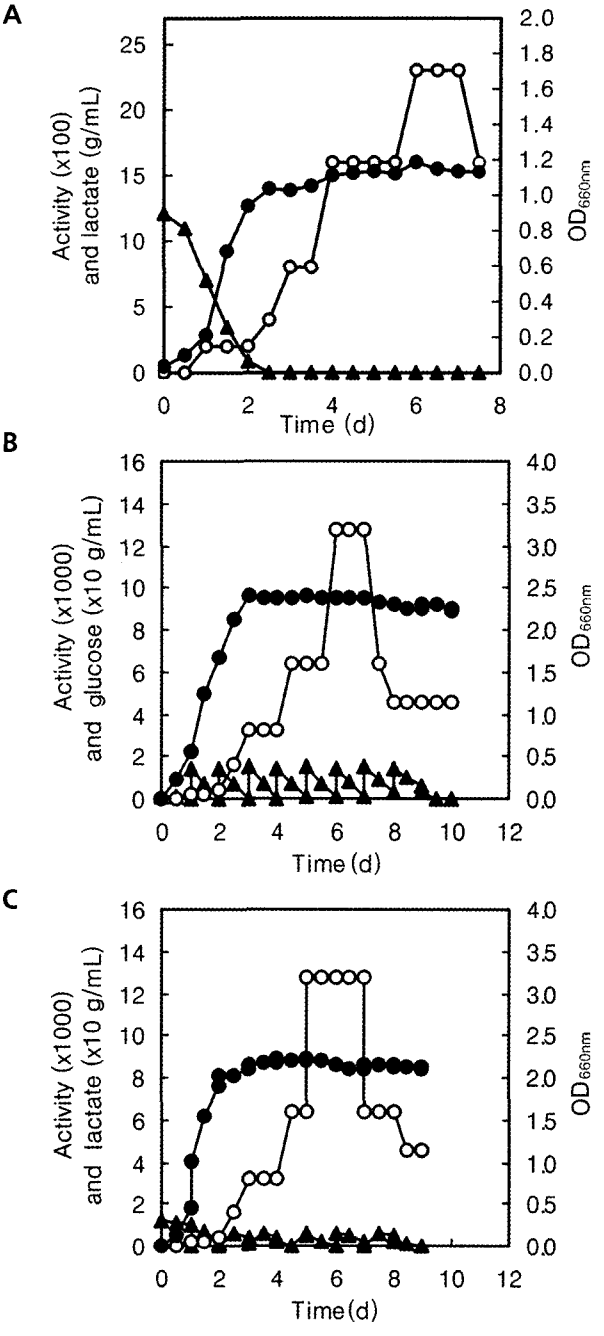

Fig. 5. Batch and fed-batch fermentation for enterocin HJ35 production. A; Batch fermentation, B; fed-batch fermentation with feeding glucose, C; fed-batch fermentation with feeding lactose. ●; Cell growth, ▲; bacteriocin activity, ○; glucose or lactose concentration.

mass of approximately 4~4.5 kDa.

Batch and Fed-Batch Fermentation

The production of enterocin HJ35 by *E. faecium* HJ35 was performed for 7 days at 32°C, pH 7.0 and 150 rpm

in NLB (Fig. 5). Enterocin HJ35 was extracellularly produced during logarithmic growth and the production profile showed a typical growth-associated pattern. The maximum activity was observed as about 2,300 AU/mL in 7 day batch culture (Fig. 5A). The cell growth was increased in fed-batch culture by feeding with 10 g/L glucose or 6 g/L lactate and the maximum activity was achieved up to 12,800 AU/mL (Figs. 5B and C). Generally, fed-batch culture prevents substrate inhibition by carbon sources or nitrogen sources [26-30]. The working volume in a fermenter is not significantly increased because the feeding volume is nearly balanced with the sampling volume taken, and glucose fed is rapidly consumed. Finally we gained an optimal dynamic feeding profile by varying the feeding-rate with pH control at 7.0.

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