Eur Food Res Technol (2011) 233:123–129
DOI 10.1007/s00217-011-1494-9

ORIGINAL PAPER

Heterologous extracellular production of enterocin P in *Lactococcus lactis* by a food-grade expression system

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Received: 1 March 2011 / Revised: 10 May 2011 / Accepted: 14 May 2011 / Published online: 3 June 2011

Abstract In order to develop an entirely food-grade enterocin P expression system for the food industry, the enterocin P structural gene (*entP*) with or without the enterocin P immunity gene (*entiP*) was cloned in plasmid pLEB590 under control of the lactococcal constitutive promoter P_{45}. Introduction of the recombinant vectors in *L. lactis* MG1614 resulted in production of biologically active enterocin P in the supernatants of recombinant *L. lactis* MG1614. Moreover, coexpression of the *entP* and *entiP* genes could increase the production of enterocin P in all *L. lactis* MG1614 hosts. Recombinant enterocin P from *L. lactis* MG1614 (pLEB590-entP2) was purified by a three-step procedure involving ammonium sulfate precipitation, SP-Sepharose Fast Flow cation exchange, and hydrophobic adsorption chromatography. The purified bacteriocin protein concentration from recombinant *L. lactis* MG1614 (pLEB590-entP2) was 3.9-fold greater than that of *E. faecium* LM-2, and the final recovery of enterocin P activity from the supernatant of *L. lactis* MG1614 (40.2%) was dramatically improved compared with that of the native host strain (19.9%). Bacteriocin activity and Tricine-SDS–PAGE analysis revealed that purified recombinant enterocin P is biologically active and has a molecular mass corresponding to the native enterocin P from *E. faecium* LM-2, suggesting that the synthesis, process, and secretion of enterocin P progresses efficiently in recombinant *L. lactis* MG1614 hosts. The enterocin P was expressed successfully in this food-grade system.

Keywords Bacteriocin · Enterocin P · Heterologous production · *Lactococcus lactis* · Food-grade system

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Introduction

Bacteriocins are ribosomally synthesized peptides or proteins with antimicrobial activity, and considerable research interest has focused on the bacteriocins produced by lactic acid bacteria (LAB) because of their potential applications in food, pharmaceuticals, nutraceuticals, and veterinary and human medicine [1–3]. Their presence in foods would be, in general, safe for consumers because they are inactivated by pancreatic or gastric enzymes. Among bacteriocins, nisin is the most studied and is commercially available and its use as a food preservative is accepted in more than 50 countries [4]. Among the LAB, the enterococci produce a diverse and heterogenous group of bacteriocins, termed enterocins, which differ with respect to their antimicrobial activities, structures, processing, and secretion mechanisms [5–7]. The enterocins are generally active against *Listeria monocytogenes* and some bacteriocin-producing enterococci have been evaluated for use as biopreservatives in fermented dairy and meat products [6, 8]. However, enterococci may transfer antibiotic resistance genes and possess virulence factors leading to illness [9], thus, interest in the heterologous production and functional expression of

enterocins in other microbial hosts is growing rapidly. Moreover, the production of enterocins in other hosts could lead to safer production of bacteriocins, increased bacteriocin production, and provide antimicrobial capabilities to LAB that are useful as starters and protective or probiotic cultures in food [10].

As described by Franz et al. [6], the enterocins are divided into class I (lantibiotic), class II (non-lantibiotic), class III (cyclic), and class IV (large proteins). Class II enterocins can be further sub-divided into class IIa (pediocin-like), class IIb (leaderless), and class IIc (non-pediocin-like). The absence or presence (and type) of an N-terminal extension determines the secretion mechanism of class II enterocins. Class IIa enterocins are synthesized as biologically inactive precursors or prepeptides containing an N-terminal extension. Two different N-terminal extensions have been identified within class IIa bacteriocin precursors [11]. The first one is the so-called double-glycine-type leader peptides that are cleaved off concomitantly with export across the cytoplasmic membrane by dedicated adenosine triphosphate-binding cassette transporters (ABC-transporters) and their accessory proteins [12, 13]. The second is the so-called *sec*-type signal peptides, which are proteolytically cleaved concomitantly with bacteriocin externalization by the general secretory pathway or *sec*-dependent pathway [14–16].

Several enterocins have been heterologously produced in *Lactococcus lactis* using the expression vectors associated with antibiotic resistance genes [12–17]; however, antibiotic resistance has become a major clinical and public health problem [1] and antibiotic resistance genes cannot be present in food-grade systems, so food-grade selective markers should be developed for enterocins expression. Compared with the antibiotic resistance selection markers, use of a nisin resistance selection marker for developing bacteriocin-resistant food-grade starters or probiotics for the food industry is preferred.

Enterocin P is a *sec*-dependent class IIa bacteriocin produced by *E. faecium* LM-2 isolated from a traditional cheese produced in Inner Mongolia, China [18], and by other *E. faecium* strains of diverse origin [19, 20]. It is synthesized as a 71-amino acid pre-peptide consisting of a 44-amino acid mature bacteriocin and a 27-amino acid signal peptide. The mature enterocin P shows a broad antimicrobial spectrum against *L. monocytogenes* and a wide range of gram-positive spoilage and foodborne pathogenic bacteria, suggesting its potential application as a natural food antimicrobial agent.

In this work, we report heterologous production and functional expression of enterocin P in *L. lactis* using a food-grade expression vector with the nisin immunity gene *nisI* as a selection marker.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The LAB strains and plasmids used in this work are listed in Table 1. *E. faecium* LM-2, the enteriocin P producer, was previously isolated from "Byaslag", a traditional cheese of Inner Mongolia in China [18]. It was grown in MRS broth (BD Biosciences, Shanghai, China) and incubated at 37 °C. *L. monocytogenes* 54002 was used as the indicator strain in the bacteriocin activity assay. Both bacteria were stored at −80 °C in MRS and Trypticase Soy Broth supplemented with 0.6% Yeast Extract (BD Biosciences, Shanghai, China), respectively, containing 15% (v/v) glycerol. *L. lactis* MG1614 was propagated at 30 °C in M17 broth (Oxoid) supplemented with 0.5% (wt/vol) glucose (GM17). Transformants of *L. lactis* were selected with 200 IU/mL nisin (Sigma, Shanghai, China). The food-grade expression vector pLEB590 was supplied by Dr. T. M. Takala, Department of Applied Chemistry and Microbiology, University of Helsinki, Finland [21].

Construction of the recombinant plasmids pLEB590-entP1 and pLEB590-entP2

Total genomic DNA from *E. faecium* LM-2 was isolated by using the Genomic DNA Isolation Kit (Bio-Rad Laboratories, Hercules, CA, USA) and was used as a template for PCR amplification of a 311-bp *BamH*I-*Xho*I fragment (P1) carrying the structural gene *entP* with its signal sequence and putative ribosome binding site (RBS) and another 529–bp *BamH*I-*Xho*I fragment carrying the structural-plus-immunity gene (P2, *entP,* and *entPi*). Oligonucleotide primers are shown in Table 2 and were obtained from Laboratory Service Division, University of Guelph. Plasmid DNA isolation was performed with a QIAprep Spin miniprep kit (Qiagen, Mississauga, Canada) or a High Pure plasmid isolation kit (Roche Molecular Biochemicals, Mississauga, Canada), as described by the manufacturer, with the addition of lysozyme (20 mg/mL). All DNA restriction enzymes were from New England BioLabs (Mississauga, Canada) or Roche Molecular Biochemicals and were used as recommended by the supplier. The PCR products were digested by *BamH*I/*Xho*I and inserted in digested pLEB590. Ligations were performed with T4 DNA ligase (Roche) and competent *L. lactis* MG1614 cells were obtained according to the method of Holo and Nes [22]. Ligation mixtures were used to transform electrocompetent cells with a Gene Pulser and a pulse controller apparatus (Bio-Rad Laboratories, Mississauga, Canada). Firstly, to verify the size of inserts, a part of the mini-prepared recombinant plasmid was used as template DNA for PCR amplification, and another part was doubly

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source and/or reference
Strains		
E. faecium LM-2	Enterocin P producer	Liu et al. [23]
L. monocytogenes 54002	Indicator, enterocin P^s	NICPBP
L. lactis MG1614	Plasmid-free derivative of NCDO712, $nisin^s$, enterocin P^r	Takala and Saris [21]
Plasmids		
pLEB590	3.1 kb, nis^r, *nis*I as a selection marker	Takala and Saris [21]

NICPBP National Institute for the Control of Pharmaceutical and Biological Products

s sensitive, *r* resistant

digested by *BamHI*/*Xho*I. And then the proper recombined plasmids with inserts were confirmed by nucleotide sequencing. Furthermore, the proper clones containing plasmids pLEB590-entP1 and pLEB590-entP2 were confirmed by a direct antimicrobial test.

Heterologous production of enterocin P in *L. lactis*

Recombinant *L. lactis* MG1614 (pLEB590-entP1) and *L. lactis* MG1614 (pLEB590-entP2) were inoculated (1%, v/v, respectively) into 100 mL GM17 broth and incubated at 37 °C for 48 h. Samples were taken at appropriate intervals to determine the optical density (at 600 nm) of the culture and the antimicrobial activity of the recombinant bacteriocin produced.

Purification of recombinant enterocin P

The enterocin P produced by *L. lactis* (pLEB590-entP2) cells was purified according to the method of Liu et al. [23] with slight modification. Briefly, supernatants from 24 h cultures grown in GM17 broth at 30 °C were subjected to precipitation with ammonium sulfate (80%, w/v). The sample was kept at 4 °C with stirring for 2 h. After centrifugation at 12,000 g for 30 min at 4 °C, the pellet and floating materials were harvested and dissolved in 50 mM sodium phosphate buffer pH 6.5 and dialyzed using a 1.0 kDa cut-off membrane against the same buffer at 4 °C overnight. This mixture was then injected into a SP-Sepharose Fast Flow cation exchange column (GE Healthcare, Mississauga, Canada) equilibrated with 50 mM of sodium phosphate buffer (pH 6.5) in AKTA FPLC system (GE Healthcare, Mississauga, Canada). The column was washed with the same buffer and the absorbed proteins were then eluted by the application of a linear salt gradient (0–1 M of NaCl) in the same buffer for 30 min. The flow rate was 1 mL/min and the absorbance was recorded at 220 and 280 nm. Fractions of 5 mL were collected and the antimicrobial activity was determined. The active fractions from the cation exchange column were further subjected to hydrophobic interaction chromatography using Octyl-Sepharose CL-4B (GE Healthcare, Mississauga, Canada) in AKTA FPLC system. The column was equilibrated with 1.7 M of $(NH_4)_2SO_4$ and then eluted with a linear increasing gradient using H_2O and ethanol at a flow rate of 1 mL/min. The absorbance was recorded at 280 nm and bacteriocin activity of each fraction (3 mL) was determined. Enterocin P produced by *E. faecium* LM-2 grown in MRS was purified by the same procedure and used as a control.

Table 2 Primers and PCR products used in this study

Primer or PCR product	Nucleotide sequence (5′–3′) or description[a]	Purpose
Primers		
EntP-1	GGCGGATCCAAGGAGGTATTGATTTAT	Amplification of P1 and P2
EntP-2	TAGTCGAGTGGCTAATGCTGTTTCAGTT	Amplification of P1
EntP-3	CCGTCGAGTATCATAACTCAAAGTCCCG	Amplification of P2
PCR products		
P1	311-bp *BamHI*-*Xho*I fragment carrying the structural gene *entP* with its signal sequence and putative RBS	Cloning in pLEB590
P2	529-bp *BamHI*-*Xho*I fragment carrying the structural-plus-immunity gene (*entP* and *entPi*) with its signal sequence and putative RBS	Cloning in pLEB590

[a] Restriction sites for *BamHI* and *Xho*I are underlined

Protein electrophoresis

Expression of the target genes was confirmed by tricine-sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Tricine-SDS–PAGE) according to the method of Zhang et al. [24] with minor modification. Protein electrophoresis was performed on 10–20% precast gradient Tris–Tricine gel (Bio-Rad) in a Mini-PROTEAN electrophoresis unit (Bio-Rad). The running buffer (100 mM Tris, 100 mM Tricine, 0.1% SDS, pH 8.3) was poured into the buffer tank and 21 μL samples were loaded in wells. A low molecular mass protein marker with size ranging from 1.4 to 26.6 KDa (Bio-Rad) was used as standard. The unit was run at 100 V for 2 h and the gel was stained with the Silver Stain Plus reagent (Bio-Rad). To confirm the bacteriocin band, a bioassay was performed at the same time. The identical gel, which was run in the same unit, was fixed in 20% isopropanol–10% acetic acid in water for 30 min at room temperature and washed six times (15 min each time) with deionized water to remove SDS. And then the gel was used for direct detection of antimicrobial activity by overlaying with soft agar (0.75%) seeded with indicator strain (*L. monocytogenes* 54002) and incubated at 37 °C overnight.

Antimicrobial activity and protein concentration assays

Antimicrobial activity of individual colonies was examined by the stab-on-agar test, as described by Gutierrez et al. [15]. The activity of the supernatant or purified bacteriocin was assayed by agar well diffusion, as described by Mayr-Harting et al. [25]. The bacteriocin activity, expressed as AU (arbitrary unit) per milliliter, was defined as the reciprocal of the highest serial twofold dilution showing a clear zone of growth inhibition of the indicator strain. Protein concentrations were measured by using the RC DC™ protein assay kit (Bio-Rad).

Results

Construction and identification of the recombinant plasmids pLEB590-entP1 and pLEB590-entP2

Cloning of PCR fragments containing the enterocin P structural gene (*entP*) with or without the immunity gene (*entiP*) into the vector pLEB590, resulted in the plasmids pLEB590-entP1 and pLEB590-entP2. The proper recombinant plasmids were constructed, as evidenced by plasmid isolation, restriction enzyme analysis, and nucleotide sequencing. Results from an antimicrobial test showed recombinant *L. lactis* MG1614 cultures containing either pLEB590-entP1 or pLEB590-entP2 displayed antimicrobial activity against *L. monocytogenes* 54002, while the supernatant of *L. lactis* MG1614 (pLEB590) control strain did not display any antagonistic effect (data not shown).

Heterologous production of enterocin P in *L. lactis*

As shown in Fig. 1, the highest production and antagonistic activity of enterocin P in the supernatants of the recombinant *L. lactis* cultures were observed during the stationary phase (18–32 h), after which the concentration of enterocin P and the activity of the supernatants decreased. Furthermore, the antimicrobial activity of the supernatant from *L. lactis* MG1614 (pLEB590-entP2) was twofold higher than that found in the supernatant of *L. lactis* MG1614 (pLEB590-entP1). Thus, *L. lactis* MG1614 with plasmid pLEB590-entP2 was selected as the recombinant host strain in subsequent studies.

Purification of recombinant enterocin P

The results of the purification of enterocin P from *E. faecium* LM-2 and *L. lactis* MG1614 supernatants are shown in Table 3. Compared with the native strain (*E. faecium* LM-2), coexpression of *entP* and *entiP* from pLEB590 did not result in a dramatically increase of specific activity of enterocin P in *L. lactis* MG1614. However, the purified bacteriocin protein concentration from recombinant *L. lactis* MG1614 with pLEB590-entP2 (5.66 mg) was 3.9-fold greater than that of *E. faecium* LM-2 (1.45 mg), and the total activity (4.10×10^5) of purified recombinant bacteriocins was fourfold higher than that of the host strain (1.02×10^5). The final recovery of enterocin P activity was 40.2% of the initial activity in *L. lactis* MG1614, whereas the corresponding value for *E. faecium* LM-2 was 19.9%.

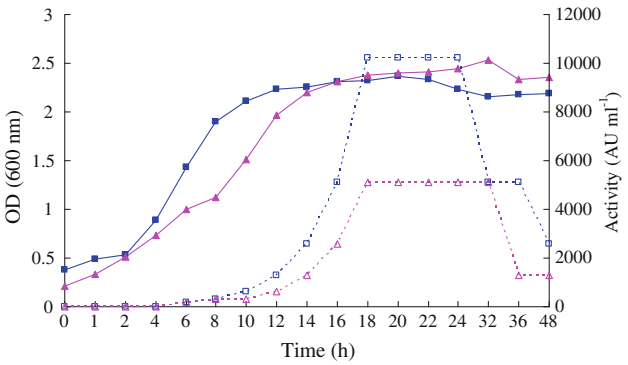

Fig. 1 Growth kinetics of recombinant *L. lactis* strains with plasmid pLEB590-entP1 (*filled triangle*) or pLEB590-entP2 (*filled square*). *Solid lines* indicated the optical density at 600 nm, *dashed lines* indicated the activity of enterocin P (AU/mL)

Table 3 Purification of enterocin P from *E. faecium* LM-2 or *L. lactis* supernatant

Purification stage	Volume (mL)	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Purification fold	Recovery (%)
E. faecium LM-2						
Culture supernatant	100	578	5.12×10^5	8.86×10^2	1.00	100
Ammonium sulfate precipitation	10	269	4.10×10^5	1.52×10^3	1.72	80.1
Cation-exchange chromatography	15	3.36	1.54×10^5	4.58×10^4	51.7	30.1
Hydrophobic interaction chromatography	5	1.45	1.02×10^5	7.06×10^4	79.7	19.9
L. lactis MG1614						
Culture supernatant	100	832	1.02×10^6	1.23×10^3	1.00	100
Ammonium sulfate precipitation	10	377	8.19×10^5	2.17×10^3	1.76	80.3
Cation-exchange chromatography	15	12.5	6.14×10^5	4.91×10^4	39.9	60.2
Hydrophobic interaction chromatography	5	5.66	4.10×10^5	7.24×10^4	58.9	40.2

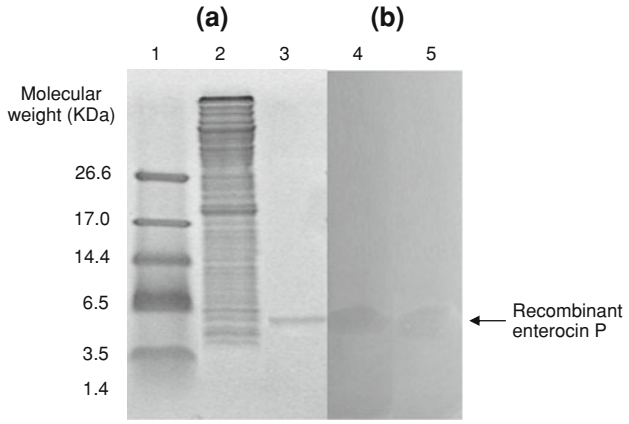

Fig. 2 Tricine-SDS–PAGE of the purified recombinant enterocin P and detection of antimicrobial activity on the gel. **a** Silver-stained gel (*1* low molecular mass protein marker with size ranging from 1.4 to 26.6 KDa; *2* crude recombinant enterocin P; *3* purified recombinant enterocin P); **b** The gel was overlaid with *L. monocytogenes* 54002 for determining antimicrobial bacteriocins (*4* crude recombinant enterocin P; *5* purified recombinant enterocin P)

Tricine-SDS–PAGE

Further characterization of enterocin P from *E. faecium* LM-2 and *L. lactis* MG1614 was performed by electrophoresis and an overlay assay. Results from Tricine-SDS–PAGE showed the size of the expressed recombinant protein corresponded to the expected size of mature enterocin P (between 3.5 and 6.4 kDa). Both the crude and purified recombinant enterocin P was shown to be biologically active, which was confirmed by the inhibitory zone observed when the gel was half overlaid with an indicator strain (Fig. 2).

Discussion

Class IIa bacteriocins are considered promising antimicrobial agents for use in medicine and food preservation [11]. Heterologous expression systems for the production and secretion of class IIa bacteriocins are being developed. However, an entirely food-grade class IIa bacteriocin expression system for the food industry has not been exploited. In this study, we studied the cloning, production, and secretion of enterocin P, using a food-grade expression vector with the nisin immunity gene *nisI* as a selection marker and food-grade microorganism *L. lactis* as the production host. This is the first report on heterologous extracellular production of enterocin P in *L. lactis* by an entirely food-grade expression system.

In our study, all recombinant *L. lactis* strains carrying lactococcal vectors with the enterocin P structural gene (*entP* with the signal peptide) in the presence or absence of the enterocin P immunity gene (*entiP*) displayed extracellular inhibitory activity, as evidenced by halos of inhibition against *L. monocytogenes* 54002 by agar well diffusion. This suggested that presence of *entP* is the minimum requirement for the production of biologically active enterocin P. *L. lactis* strains carrying vectors containing *entP* gene plus the *entiP* gene showed greater inhibitory activity, which indicated that the coexpression of the *entP* and *entiP* genes increased the production of enterocin P in all *L. lactis* MG1614 hosts. This is consistent with the findings of Gutierrez et al. [15]. Similarly, Sanchez et al. [17] reported that coexpression of the *hirJM79* and *hiriJM79* genes increased the production of *sec*-dependent bacteriocin HirJM79 by all lactococcal hosts. Increased bacteriocin production may be explained by assuming that *L. lactis* is relatively resistant to enterocin P, but it may endure more of the bacteriocin when it expresses the *entiP* product. Generally, bacteriocin producers are protected from their own bacteriocin by the concomitant expression of a cognate immunity protein. These proteins act either by affecting bacteriocin aggregation and pore formation or by disturbing the interaction between the bacteriocin and the membrane-located

receptor of the mannose-phosphotransferase system for class IIa bacteriocins [15, 17, 26].

The production of bacteriocins by heterologous hosts may be based on the expression of native biosynthetic genes, the exchange of leader peptides, and/or the dedicated ABC secretion and processing systems, or adding signal peptides recognized by general secretary pathways [10, 15, 16]. The higher production of enterocin P by recombinant *L. lactis* MG1614 strains as compared to that of native strain may be ascribed to the heterologous expression systems facilitating the transcriptional/translational control of bacteriocin gene expression [10]. These results indicate that extracellular production of enterocin P by the *sec*-dependent pathway is an efficient process in *L. lactis* and the signal peptide of enterocin P could drive the processing and secretion of bacteriocin in *L. lactis*. Similar observations had been reported by Gutierrez et al. [15] and Sanchez et al. [17].

As reported in this work, the production and functional expression of enterocin P in *L. lactis*, using the food-grade expression vector pLEB590, have been achieved. The *L. lactis* MG1614 (pLEB590-entP2) cells would merit consideration as an alternative experimental model for heterologous production and functional expression of enterocin P. This expression system provides a more stable enterocin P production platform, without the need for selective antibiotic pressure, and thus, may be considered safer for the production of enterocin P as a natural preservative or ingredient for the food industry. Additionally, this expression system can be used to establish new bacteriocinogenic starter cultures or probiotics for application in food processing to control both foodborne pathogens and spoilage organisms.

Acknowledgments This research was funded by Natural Science Foundation of China (30671482) and Special Fund for Agro-scientific Research in the Public Interest of China (200903012).

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