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Improvement of Enterocin P Purification Process

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ABSTRACT. Purification and heterologous expression of enterocin P (EntP), a sec-dependent bacteriocin produced by *Enterococcus faecium*, in *Escherichia coli* is described. PCR-amplified product of the enterocin P structural gene *entP* was cloned into plasmid pET-32b under the control of the inducible T7*lac* promoter. The neo-synthesized EntP was genetically modified by an addition of 3 extra amino acids, leading to recombinant EntRP. Active EntRP was recovered from the cytoplasmic soluble fraction of *E. coli* harboring appropriate recombinant plasmid, characterized by ELISA and Western-blot analysis and purified by immunoaffinity chromatography. The use of *E. coli* as heterologous host and pET-32b as expressing vector offers promising tools for heterologous production of class IIa bacteriocin.

Abbreviations

EntP enterocin P EntRP recombinant EntP

In the last decade, class IIa bacteriocins produced by lactic acid bacteria have received a particular attention because of their potential application in the food industry as natural preservatives (Cleveland *et al.* 2001; Todorov and Dicks 2004), and in medical area as antibiotic supplements or antiviral agents (Wachsman *et al.* 1999, 2003; Bujňáková *et al*. 2004). The classification of bacteriocins has designed the class IIa as small peptides with 30–60 amino acids including a consensus sequence YGN GVX CXX XXC XVX XXX A (X = any amino acid) in their N-terminal part, heat stable, non lanthionine and active against *Listeria* spp. (Nes and Holo 2000; Drider *et al*. 2006). Class IIa bacteriocins consist of an N-terminal β-sheet domain, which is structurally stabilized by the conserved disulfide bridge, and a C-terminal domain consisting of 1 or 2 α-helices often ending with a structurally extended C-terminal tail. Remarkably, the C-terminal part of a few classes IIa bacteriocins (such as sakacin G, plantaricin 423, pediocin PA-1/AcH, divercin V41 and enterocin A) contains an additional C-terminal disulfide bridge, which plays an important role in stabilizing the 3D structure. Often, but not always, the class IIa bacteriocins containing 2 disulfide bridges display higher antimicrobial potency as compared with those containing only 1 disulfide bridge. The importance of amino acid sequence in the folding and bactericidal activity of bacteriocins has been illustrated in different reports (Cotter *et al.* 2005; Drider *et al*. 2006). The cysteinyl and tryptophanyl residues play a determinant role in disulfide bridge formation and antimicrobial activity (Bhugaloo-Vial *et al*. 1999; Ennahar *et al*. 2000). EntP produced by *Enterococcus faecium* P13 (Cintas *et al.* 1997) was shown to inhibit the growth of spoilage and pathogenic microorganisms (Herranz *et al.* 2001). Studies carried out at a physiological level revealed that optimum conditions for EntP production included cultivation in MRS medium, a temperature range between 16 and 45 °C, and pH 2–11. Otherwise, studies at a biochemical level have shown that a multistep purification process composed of diammonium persulfate precipitation, exclusion chromatography, ion-exchange chromatography, hydrophobic interaction and reversed phase (RP)-HPLC, is required to obtain pure EntP. However, this process is time-consuming and relevant progress has been made to facilitate expression and purification of EntP in heterologous systems such as *Lactococcus lactis*, *Escherichia coli*, *Pichia pastoris* and *Methylobacterium extorquens* (Gutierrez *et al*. 2005*a–c*, 2006).

The present work describes another alternative method based on cloning after genetic modification of the structural EntP gene (*entP*). The active peptide was recovered from the soluble cytoplasmic fraction of *E. coli* Origami harboring the appropriate recombinant plasmid named pCR05.

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MATERIALS AND METHODS

Bacterial strains, plasmids and cloning procedures. *Enterococcus faecium* P13 (isolated from Spanish dry fermented sausage; Cintas *et al.* 1997) was used as EntP producer strain, while *Listeria innocua* F (obtained from *Pasteur Institute Collection*, Paris, France) and a set of *L. monocytogenes* strains (Table I) were used as bacteriocin-sensitive indicator strains. Producing and indicator strains were grown without shaking at 30 °C in MRS medium (de Man *et al.* 1960) (*Biokar*). The bacteriocin activity was determined by the agar diffusion test (ADT) according to Pilet *et al.* (1995). Briefly, 25 mL of molten tryptic soy yeast extract containing 0.75 % (*W*/*V*) agar was cooled at 47 °C and seeded with 1 % (*V*/*V*) of an overnight culture of *L. innocua* at 10^7 cells per mL. Seeded agar was then poured into a sterile Petri plate and allowed to solidify at room temperature. Wells (diameter 7 mm) were cut in the solidified agar and filled with 80 µL of sample. The plates were left for 2 h at 5 °C to allow diffusion of the tested aliquot, and incubated aerobically for 18 h at 30 °C. The absence or presence of inhibition zones (plus their diameter) were measured.

Table I. Antagonistic activity exerted by EntP and EntRP against various target strains of *L. monocytogenes*

Strain[a]	Inhibition zone, mm[b]	Strain[a]	Inhibition zone, mm[b]
DSMZ 12464	3.1	ENITIAA 526[d]	2.0
CIP 7835	2.0	ENITIAA 342[d]	1.0
ATCC 19115	–	ENITIAA 523[d]	2.7
LO 28	1.5	ENITIAA 532[d]	1.5
Scott A	–	ENITIAA 348[d]	2.7
ENITIAA 535[c]	–	ENITIAA 332[d]	2.0
ENITIAA 525[d]	1	ENITIAA 530[d]	2.5

[a]ATCC *American Type Culture Collection*
CIP *Collection Institut Pasteur*
DSMZ *German Collection of Microorganisms and Cells Cultures*
ENITIAA *Ecole Nationale des Industries Agricoles et Alimentaires*
LO 28 Clinical strain.
Scott A Reference strain for dairy product studies (CIP 103 575).
[b]Values after treatment with EntP and with EntRP are identical (average of 2 independent experiments).
[c]Isolated from smoked salmon and displaying resistance against divergicin M35, a new class IIa bacteriocin produced by *Carnobacterium divergens* M35 (strain was obtained from *Ismail Fliss Laboratory, Université Laval*, Québec, Canada).
[d]Derivatives of ENITIAA strain 535.

Oligonucleotide design and DNA manipulations. Total DNA isolated from *E. faecium* P13 was amplified by PCR using sense primer MO104 (5′-GCC ATG GGA GCT ACG CGT TCA TAT GG-3′) and antisense primer MO105 (5′-GCT CGA GTT AAT GTC CCA TAC CTG CCA AAC C-3′). Primer MO104 contains *Nco*I restriction site and an additional GCA triplet, and the antisense primer MO105 contains *Xho*I restriction site (Fig. 1A). The amplified DNA fragment was digested with *Xho*I and *Nco*I restriction enzymes (*Biolabs*) and cloned in pDrive vector (*Qiagen*) giving plasmid pCR04. Plasmid pCR04 was digested with *Xho*I and *Nco*I and restriction enzymes. The DNA fragment containing the putative structural *entRP* gene was purified from agarose gel and cloned in pET-32b expressing vector giving plasmid pCR05. Then, this plasmid was transferred to *E. coli* Origami (DE3) pLysS (*Novagen*) for further analysis.

The design and cloning strategy set up in this work led to a slight modification of EntP by the addition of three amino acids (Fig. 1B). Transformation and storage of *E. coli* competent cells were done according to Chung *et al.* (1989). Transformants containing pDrive vector or its derivative were selected in Luria–Bertani (LB) agar medium with ampicillin (100 mg/L); those containing pET-32b or its derivative were selected in LB agar medium with ampicillin (100 mg/L) and chloramphenicol (30 mg/L). *E. coli* strain JM109 (*Stratagen*) was used for standard cloning procedures, and *E. coli* Origami (DE3) pLysS (*Novagen*) for gene expression. *E. coli* strains were aerobically grown in LB medium at 37 °C (Sambrook *et al.* 1989).

The nucleotide sequence of the cloned *entRP* gene was determined by the dideoxynucleotide chain termination method (Sanger *et al.* 1977) with an ABI 370 automated sequencer using the Taq Dye-Deoxy™ terminator cycle sequencing kit (*Perkin-Elmer*).

Heterologous expression of EntRP in E. coli. Overnight cultures of *E. coli* Origami/pCR05 were diluted to 3 % (*V*/*V*) in TB medium containing 100 mg/L ampicillin and 30 mg/L chloramphenicol (Sambrook *et al.* 1989), and aerobically grown until the absorbance (A_{600}) reached the value of 0.8. Then, the cul-

tures were induced by an addition of isopropyl β-thiogalactoside (IPTG) at a final concentration of 1 mmol/L. Samples of 20 mL were withdrawn after 0, 1, 2, and 3 h of induction. Cells were recovered from samples by centrifugation (8000 ***g***, 6 min, 4 °C), resuspended in 2 mL of 10 mmol/L imidazole binding buffer (pH 7.9) (*Amersham-Biosciences*), and disrupted by sonication in ice-cold water until the required viscosity was obtained. The cytoplasmic soluble fraction (CSF) was separated from the insoluble fraction and cell debris by centrifugation (14 000 ***g***, 15 min, 4 °C). The CSF was filtrated (0.45-μm pore size; *Sartorius*) and loaded onto a 1-mL Nickel His-Trap chelating column (*Amersham-Biosciences*). The column was washed with 10, 20 and 60 mmol/L binding buffer (pH 7.9). The TRX-$(His)_6$-EntRP was eluted with 2 mL of binding buffer at 500 mmol/L imidazole (pH 7.9).

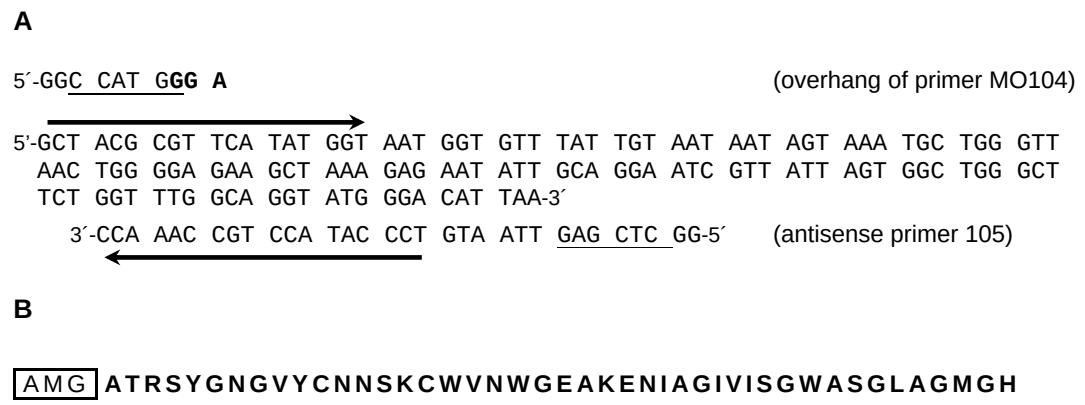

Fig. 1. **A**: The positions (indicated by appropriate *arrows*) of the sense primer MO104 + its overhang containing (*Nco*I restriction site + additional triplet **GGA**), and antisense primer 105 + its overhang which contains *Xho*I restriction site; in each primer, the restriction site is underlined; additional triplet is shown in **bold**; **B**: amino-acid sequence of pre-EntP (fine characters) and mature EntP (**bold characters**); the extra amino acids introduced in enterocin P are boxed.

After this first immobilized metal affinity chromatography (IMAC) purification, the fraction containing the fusion protein was desalted against distilled water using PD-10 column (*Amersham-Biosciences*). The TRX-$(His)_6$-EntRP was cleaved with enterokinase EKMax™ according to the manufacturer's instructions (*Invitrogen*). Imidazole and NaCl were added to the cleaved fusion protein to a final concentration of 10 and 500 mmol/L, respectively, and the pH was adjusted to 7.4 with 1 mol/L HCl. Separation of EntRP from fusion protein "TRX-$(His)_6$-EntRP" was achieved by the 2nd IMAC step. EntRP was found in the flow-through fraction and the non-cleaved TRX-$(His)_6$ in fraction eluted by 500 mmol/L binding buffer.

Proteins and immuno-blotting assays. Proteins were separated under reducing conditions by Tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16.5 % polyacrylamide) (Schagger and von Jagow 1987). Proteins were stained by $AgNO_3$ (Blum *et al*. 1987). Transfer of proteins to nitrocellulose membrane and immuno-blotting analysis was done according to Richard *et al*. (2004*a*).

RESULTS AND DISCUSSION

The last decade has witnessed, through the impressive literature dedicated to class IIa bacteriocins, a strong rise in the interest of these antimicrobial peptides and their potential applications as food additives. All class IIa bacteriocins can be purified by standard methods consisting in at least three steps, which is time-consuming and usually yielding very low amount of pure peptide (about 100 μg/L of culture supernatant) (Métivier *et al*. 2000). All disadvantages and drawbacks related to purification methods of class IIa bacteriocins are highlighted and discussed in Carolissen-Mack Kay *et al*. (1997). It is evident that heterologous expression systems for class IIa bacteriocins stand as interesting and promising alternative methods. The heterologous systems are expected to offer several advantages over native systems, such as the control of bacteriocin gene expression, enhancement of production levels and rapid purification methods.

Production of active EntRP in E. coli *Origami/pCR05*. *E. coli* Origami/pCR05 cultures were induced by IPTG. The induction of the T7 RNA polymerase promoter yielded *de novo* synthesis and expression of 2 neo-synthesized polypeptides, which displayed apparent molar mass of 22 and 20 kDa. These neo-synthesized polypeptides should correspond to the fusion protein TRX-$(His)_6$-EntRP and thioredoxin (TRX) since

their respective sizes are in good agreement with the theoretical sizes. ELISA analyses using anti-divercin V41 polyclonal antibodies allowed detection of EntRP within the CSF of *E. coli* Origami/pCR05.

Treatment of the fusion protein TRX-$(His)_6$-EntRP with enterokinase EKMax^{TM} resulted in an accumulation of thioredoxin, EntRP and a small amount of fusion protein TRX-$(His)_6$-EntRP that was not hydrolyzed (Fig. 2A, lane *2*). The EntRP was purified from the mixture by immuno-affinity chromatography (Richard *et al*. 2004*b*); the purity of EntRP was checked on SDS-PAGE (*data not shown*) and confirmed by Western-blot analysis (Fig. 2B, lane *2*).

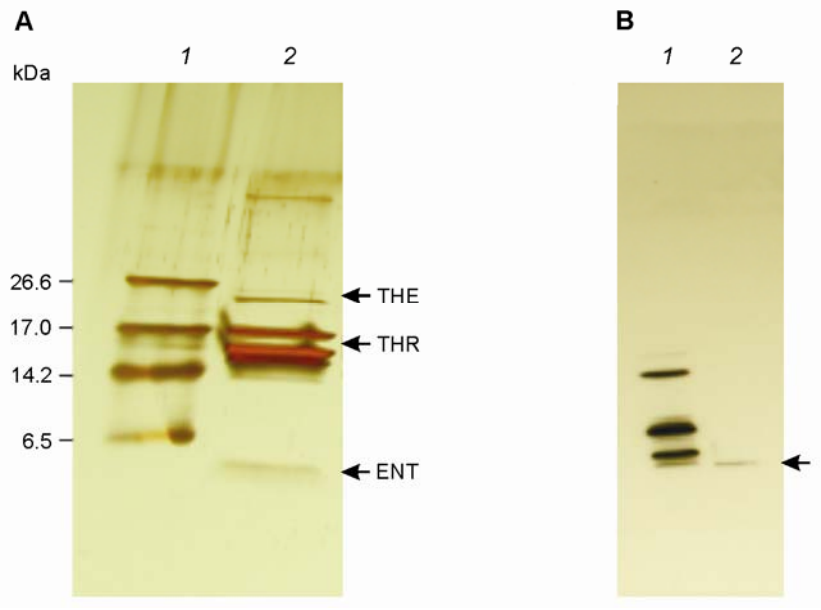

Fig. 2. Treatment of the fusion protein TRX-$(His)_6$-EntRP with enterokinase EKMax^{TM}. **A**: 15 % Tricin–SDS-PAGE; *1* – ultra-low-range molar-mass-markers (*Sigma*), *2* – sample: THE – TRX-$(His)_6$-EntRP, THR – thioredoxin, ENT – EntRP released upon enzymic treatment; **B**: immunoblotting analysis of the EntRP purification by immobilized-metal-affinity chromatography (IMAC); *1* – TRX-$(His)_6$-EntRP, *2* – EntRP (*arrow*) obtained after the second IMAC.

The potential anti-listeria activity of pure EntRP and fusion protein TRX-$(His)_6$-EntRP was assessed against the indicator organism *L. innocua* F. EntRP was active but the fusion protein TRX-$(His)_6$-EntRP did not generate any antimicrobial activity. On the one hand, we observed that the slight genetic modification performed on native EntP did not interfere with its antimicrobial potency. On the other hand, the fact that the fusion protein TRX-$(His)_6$-EntRP is devoid of antimicrobial activity is questionable because divercin V41 under similar conditions TRX-$(His)_6$-DvnRP exhibited high antimicrobial activity. It is tempting to hypothesize that differences could exist between EntRP and TRX-$(His)_6$-EntRP in terms of folding, and access to the target cells. Interestingly, we observed that EntP and EntRP listericidal spectra are identical (Table I).

The results of this work report on the cloning and expression of (EntRP) in *E. coli* Origami (DE3) (pLysS) using a strategy that deserves consideration for reasons including (*i*) the use of *E. coli* Origami as heterologous host and pET-32b as expressing vector, (*ii*) the inherent instability observed in the case of divercin V41 DNA structural gene *dvnV41* (Richard *et al.* 2004*b*) is not a general trait for class IIa bacteriocins, and (*iii*) the purification process by immuno-chromatography is rapid and convenient.

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