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Production of enterocin P by a bioengineered Pichia pastoris to control pathogen bacteria

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ABSTRACT

Enterocin P has been characterized in class IIa bacteriocins and showed a broad spectrum of antimicrobial activity. In this study, the nucleotide sequence of enterocin P transformed and expressed in *Pichia pastoris* genomically, and the antimicrobial activity of the extracted protein was evaluated on pathogenic bacteria. The synthetic sequence of Enterocin P was ligated into pPICZαA, an integrated vector, in the *EcoRI* - *XbaI* site. The recombinant plasmid was amplified in *Escherichia coli* strain DH5α and proved for ligation and probably mutation by sequencing. The linearized plasmid was electroporated into *Pichia pastoris*. The presence of recombinant protein in yeast cell extraction was verified by SDS-PAGE and the antimicrobial activity of the purified protein. Recombinant Enterocin P tested for the inhibition of 8 avian pathogen bacteria via MIC test. Electrophoresis of purified protein and cell culture supernatant showed a protein band of about 5.4 kDa. The mass spectrometry of the sample exhibits an Amino acid sequence that corresponds to enterocin P in *Enterococcus faecium* with an accession number of OTN91526.1. Recombinant bacteriocin MIC test shows the most inhibition on *Salmonella typhimurium* and *E. coli O157*. Recombinant *Pichia pastoris* exhibited a potential for producing enterocin P in high concentrations; therefore, this antimicrobial peptide can be studied for pharmacological use or industrial applications.

Introduction

Bacteriocins are antimicrobial agents that were recognized in *Escherichia coli* media culture for the first time in 1925. These protein agents were synthesized by ribosomes and exhibited a broad spectrum of antimicrobial activity. The use of these antimicrobial molecules in food industries meets users' requirements that follow safe food with low chemical ingredients. Nisin is the first used

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bacteriocin in the industry, and many studies have been accomplished on it. Despite broad usage of this bacteriocin, some cases of bacterial resistance were reported and it is unknown a specific mechanism for this effect. Inattention to finding a method for effective control of pathogen bacteria, the study of bacteriocins is essential as a replacement for antibiotics.

Lactic acid bacteria (LAB) are used to preserve and develop flavor in food. The produced lactic acid and other antagonistic compounds, such as bacteriocins by LAB, protect the food from spoilage (Schillinger & Lucke, 1989). The LAB bacteriocins, which have been defined and characterized to date, exhibited several common properties that divide their classification into at least three well-defined classes (Ness et al., 1996): class I, the lantibiotics; class II, the small heat-stable non-antibiotics, which are divided into the subgroups IIa (pediocin like bacteriocins with strong anti listerial effect), IIb (bacteriocins whose activity depends on the complementary activity of two peptides), and IIc sec-dependent secreted bacteriocins; and class III, large heat-labile bacteriocins. The fourth class of bacteriocins consisting of an undefined mixture of proteins, lipids, and carbohydrates has been suggested.

Enterococcus faecium is one of the largest bacteriocin producers among the fecal lactic acid bacteria in flora (Ness et al., 2014). Enterocin P is produced by *E. faecium* P13 isolated from a Spanish dry fermented sausage and was characterized by pediocin-like bacteriocin. This peptide contains 44 amino acids with 44% hydrophobicity that are encoded by the 755 bp gene (Cintas et al 1997). It shows an inhibitory effect on not only *Listeria monocytogenes* but also a wide range of spoilage and food-borne gram-positive pathogenic bacteria. Moreover, enterocin P is to our knowledge the first sec-dependent pediocin-like bacteriocin characterized at the biochemical and molecular levels.

A novel approach to control and prevent disease is the use of bioengineered probiotics that ability to produce antimicrobial factors. For instance, to design a recombinant *E. coli* that produces bacteriocin when it detects signals from pathogen bacteria (Saeidi et al., 2011). Olejnik-Schmidt et al. (2014) engineered an *E. coli* that expressed the bacteriocin of Divercin AS7. This bacteriocin originated from *Carnobacterium divergens* AS7 and showed antimicrobial activity against pathogens like *Listeria* spp. In a study, broad antimicrobial spectrum bacteriocins were cloned in the host of *P.pasturis* and showed anti-pathogenic activity against *Pediococcus damnosus* but did not exhibit significant antimicrobial activity against *L.monocytogenes* CECT4032, *E. coli* O157:H7, *Yersinia ruckeri* LMG3279, *Campylobacter jejuni* ATCC33560, and *C. jejuni* NCTC11168 (Arbulu et al., 2015). Human lactoferrin showed antimicrobial activity and was cloned and expressed in *Lactobacillus casei* as a probiotic. This recombinant probiotic displayed antimicrobial activity in the digestive tract against enteric pathogen bacteria (Chen et al., 2010). *Rhodococcus rhodnii* exists in the intestine of *Rhodnius prolixus* insects as a symbiosis and in a study, transformation and expression of Cecropin A in *R. rhodnii* could control *Trypanosoma cruzi* in the intestine of insects. It is remembered that *R. prolixus* is a carrier of *Trypanosoma cruzi* the agent of Chagas disease (Durvasula et al., 1997).

In this study, cloning and expression of enterocin P, a bacteriocin originating from *Enterococcus faecium*, in *Pichia pastoris* was followed. The production of enterocin P on a large scale was aimed and the ability of this bacteriocin was evaluated in the control and prevention of pathogen bacteria.

Materials and Methods

Microbial Strains, vectors, and growth conditions

The bacterial strains and plasmids used in this investigation are listed in Table 1. A low salt Luria-

Bertani (LB) medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, and 10 g l^{-1} NaCl) was used for the culture of *E. coli* (DH5a). LB medium containing Ampicillin (50µg/ml) was used for the selection of transformants DH5α. The BMGY (Buffered Glycerol Complex Medium), BMMY (Buffered Methanol Complex Medium), and YPD (Yeast Extract Peptone Dextrose Medium) were used for the yeast culture (Invitrogen, Carlsbad, USA).

Gene identification, codon optimization, and recombinant expression vector construction

The coding region of enterocin P was obtained from the NCBI database with an accession number of AF005726 (www.ncbi.nlm.nih.gov). This sequence originated from *Enterococcus faecium* P13 containing 216 bp oligonucleotide fragments. The signal sequence was removed from the whole sequence and the main sequence had a mature bacteriocin sequence obtained based on the previous study (Cintas et al., 1997). The codons were optimized for expression in *P. pastoris* by the Genscript website (www.genscript.com) and the nucleotide sequence was synthesized by Bioneer Company (Daejeon, South Korea).

The pPICZαA vector was digested in *the EcoRI - XbaI* site and the digested product was purified by electrophoresis on 1% agarose gel. Enterocin P was ligated into the *EcoRI-XbaI* site in pPICZαA at 16°C for 24 h using T4 DNA ligase (Fig. 1). The ligation mixture was transformed into DH5α by the heat shock method (Sambrook et al., 2001). Positive colonies were isolated on a selective LB medium containing Ampicillin and screened with *3'AOX1* primer (GCAAATGGCATTCTGACATCC) and *5'AOX1* (GACTGGTTCCAATTGACAAGC) by colony PCR (Trower, 1996). The plasmid DNA was purified from positive colonies and subjected to DNA sequencing.

Transformation of the recombinant plasmid to P. pastoris

The electro-competent cells of *P. pastoris* strain KM71H were prepared according to the method described by Invitrogen (California, USA). The pPICZαA- enterocin P vector was linearized with *PmeI* and purified afterward. 80 µl of competent cells were mixed with 10 µg of the linearized vector, transferred into a 0.2 cm electroporation cuvette, and incubated on ice for 5 min. Cells were then pulsed by an electroporator instrument (Bio-Rad, USA) (4 ms and 2 kV) and immediately, 1.0 ml of 1 M cold sorbitol was added to the cuvette and the mixture was incubated at 30°C for 1 h without shaking. Then 1ml YPD medium was added to the yeast cells and was incubated at 30°C for 1 h. The cell suspension was spread in the volume of 200µl on YPDS agar plates containing 100µg ml $^{-1}$ Zeocin and incubated at 30°C until colonies appeared (around 2-4 days). The integration of enterocin P into the genome of recombinant *P. pastoris* was confirmed by genomic PCR using AOX1 primers.

Expression of recombinant enterocin P and SDS-PAGE analysis

The positive colony of *P. pastoris* was grown in 5 ml of BMGY medium at 30°C for 24 h. Then, the isolated cells were further cultured in 100 ml of BMGY, incubated at 30°C, and stirred at 250 rpm for about 20 h until the optical density reached to 2–6 at 600 nm (OD600). Cells were harvested by centrifugation at 3000×*g* at room temperature for 5 min. The cell pellet was then re-suspended in 25 ml of BMMY medium and transferred into a 500 ml flask. The culture was shaken at 30°C for 96 h. Absolute methanol was added every 24 h to a final concentration of 5 µl ml $^{-1}$. Cultures were centrifuged at 5000×*g* at 4°C for 5 min and the final supernatant was collected. A 15 µL of the supernatant of the culture medium from each transfected cell (transfected cells by recombinant or self-ligated pPICZαA) was electrophoresed on polyacrylamide gel (16%) contained SDS and stained with Coomassie Brilliant Blue (Laemmli, 1970).

Purification of enterocin P

The supernatant of a culture medium used for protein precipitation by ammonium sulfate (70% w/v) for the transfected cells was proved by SDS-PAGE. Precipitated proteins were dissolved in phosphate buffer saline (PBS) pH 7.2 and dialyzed against PBS overnight. The protein solution was purified over a Ni-NTA agarose column (Qiagen, Hilden, Germany) corresponding to the manual, and imidazole was used (20 mM) to elute the His-tagged proteins. To determine the protein's molecular mass in the supernatant of yeast medium, the eluted purified fractions with antimicrobial activity were subjected to mass spectrometry MALDI-TOF (Tabriz University, Tabriz, Iran).

Antibacterial activity tests

The antibacterial activity of recombinant enterocin P was tested on eight avian pathogenic bacteria. These bacteria were acquired from the bacterial collection in the veterinary faculty, Ferdowsi University of Mashhad, and include *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 20271, *Escherichia coli* K99 (ATCC 31618), *Klebsiella pneumoniae* ATCC 700603**,** *Salmonella enteritidis ATCC 1624*, *Bacillus cereus*, *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* O157 H7.

The Minimum Inhibitory Concentrations (MIC) of the bacterial isolates were determined using the micro broth dilution method in a 96-well microtiter with four replications, according to Vipra *et al*. (2013). The pathogenic strains were grown overnight on Nutrient agar and individual colonies were isolated and suspended in Nutrient broth to match the 0.5 McFarland standard (~1×10^8 CFU ml^{-1}), 5 µl of bacterial suspensions were added to 95 µl of media into a 96-well microtiter plate. A volume of 100 µl from the stock solution of enterocin P (886.4 µg/ml) was diluted to make different concentrations. A volume of 100 µl from each dilution was added to the wells containing bacterial cells. Corresponding bacterial controls and media controls were provided. The microtiter plates were incubated at 28 ℃ overnight without shaking. The lowest concentration of enterocin P with no bacterial turbidity was considered as a MIC record for different tested pathogen bacteria.

Results

Construction of Recombinant vector and t The ligation reaction was confirmed by sequencing and colony PCR. Nucleotide sequencing of the recombinant product revealed that there was no mutation in the enterocin P sequence. Colony PCR of recombinant E. coli DH5α showed a nucleotide band corresponding to the size of enterocin P (180 bp) by bacteriocin-specific primers (Fig. 2). Also, insertion was demonstrated in the plasmid by PCR with plasmid-specific primers (AOX1 primers). The PCR product was ≈700 bp if there was any insertion in the plasmid. The empty plasmid shows a band of about 520 bp.

Gene expression and SDS-PAGE analysis

The integration of enterocin P into the genome of recombinant *P. pastoris* was confirmed by genomic PCR using AOX1 primers (Fig. 3A). The PCR product was sequenced and the gene of enterocin P showed no mutation in the nucleotide sequence. The expression of the recombinant enterocin P peptide in *P. pastoris* was evaluated by SDS-PAGE. As expected, a 5.4 kDa band corresponding to the size of enterocin P peptide was observed in the acrylamide gel (Fig. 3B). The result of mass spectrometry exhibits an Amino acid sequence corresponds to enterocin P in *Enterococcus faecium* with an accession number of OTN91526.1. The result was blasted by BlastN, an online software (www.ncbi.nlm.nih.gov/blast). The query sequence (43 Amino acids) covered from 28 to 70 amino acids in the origin sequence with 100% similarity.

Antibacterial assay

The extracted enterocin P from recombinant yeast showed antibacterial activity against some of the major pathogenic bacteria as presented in Table 2. Recombinant enterocin P showed the most

inhibitory effect on *Salmonella typhimurium*, *Salmonella enteritidis*, and *Escherichia coli* (O157 H7).

DISCUSSION:

Bacteriocins have different applications in the food, safety, and pharmaceutical industries. In natural conditions, they were produced in the trace by specific bacteria, and purification of them involves numerous steps like precipitation and chromatography processes that some processes are expensive and time-consuming. Production of bacteriocin by gene transformation is a way to solve this issue. In this study, entrerocin P was transformed into *P. pastoris* by an integration vector. The sequence of the recombinant protein showed %100 homology with enterocin P produced by *enterococcus facium* P13. It reflected correct codon optimization for the nucleic acid sequence for expression in *Pichia pastoris*. Recombinant bacteriocin exhibited %92 homologies with bacteriocin M (*Enterococcus faecium*) and %80 homology with sakacin-A (*Lactobacillus sakei*).

A conserved motif of YGNGV was determined from 5 to 9 amino acids. It is typical of bacteriocin class II a. The ten amino acids of the N-terminal were ATRSYGNGVY and the 10 amino acids of the C-terminal were WASGLAGMGH. The amino acid chain of recombinant bacteriocin is followed by polyhistidine and it is expected to some changes in the properties of protein like net charge and hydrophobicity (Table 3). Both net charge and hydrophilicity increased in recombinant bacteriocin and these reflected an increase in the antimicrobial property of this molecule. Recombinant enterocin P includes 20% basic amino acids, 4% acidic amino acids, and 32% hydrophobic amino acids showing the character of an amphipathic molecule. Some studies showed that amphipathicity is more essential than hydrophobicity for connecting to the membrane of bacteria. (Vipra et al., 2013; Fernandez-Vidal et al. 2007).

Enterocin P is a bacteriocin with well recognized biochemically and genetically character. This bacteriocin showed a broad spectrum of pathogenic inhibitory (Cintas et al., 1997). This bacteriocin was tested on eight bacteria based on the MIC procedure. All bacteria showed resistance to the achievable serum drug level except *Salmonella typhimurium* with a sensitive level. Sensitive implies that the organism is inhibited by the serum concentration of the drug. *Escherichia coli* (O157 H7) with intermediate level showed that entrerocin P is usually attainable at blood and tissue levels for which response rates may be lower than for susceptible isolates. It was shown that all pathogenic food bacteria were inhibited by pure enterocin P from 4 to 559 ng/ml at the MIC test. In a recent study, 20-fold concentrated supernatant of *E. faecium* p13 and crud supernatant had no inhibitory effect on gram-negative bacteria like *Salmonella typhimurium, Escherichia coli, and Pseudomonas fluorescens*. Also, isolated bacteriocin of *E. feacalis* TG2 had no inhibitory effect on *Salmonella* and *E. coli* (Xi et al., 2018). In contrast, the character of recent bacteriocin was similar to the sec-dependent bacteriocin class IIa. Attention to Table 3 clears some changes in recombinant bacteriocin structure due to adding polyhistidine to the amino acid chain. There is an increase in the net charge of recombinant molecules about the native molecule. It was revealed that the net charge of antimicrobial proteins correlates with their attachment to the negatively charged bacterial membrane [Giangaspero et al., 2001; Tossi et al. 1994). More net charge in recombinant bacteriocin probably increased attachment and its antimicrobial activity. Also, hydrophobicity is essential to the penetrance of antimicrobial protein into the bacterial membrane (Glukhov et al., 2008). This property increases interaction with the fatty acyl chain in bacterial membranes and exhibits higher toxicity. However, it is declared that high hydrophobicity may show toxicity in mammalian cells or host cells and loss of antimicrobial activity (Chen et al., 2007). Hydrophobicity of recombinant enterocin P

is reduced in comparison to native bacteria of course. This property in native bacteriocin is low based on the Wimley-White scale. Probably hydrophobicity has no major role in the antimicrobial activity of enterocin P. The target receptor for class IIa bacteriocins is proteins of the sugar transporter mannose phosphotransferase system (Man-PTS), this transporter was found in the membrane of *Listeria spp* (Kjos et al., 2011). thus, enterocin P has been shown the most inhibitory potential on this pathogen bacterium.

In contrast to another study, *S. typhimurium* was sensitive to recombinant enterocin P. Of course, it is shown that *E. faecium* GM-1 produces a bacteriocin-like enterocin P with a broad antimicrobial spectrum effect including *S. typhimurium* (Kang & Lee, 2005). The recent bacteriocin exhibited 85.7% similarity of amino acid sequence to the mature enterocin P protein. Also, class II bacteriocins produced by lactic acid bacteria can inhibit a limited number of gram-positive bacteria. These bacteriocins are usually placed in the Pediocin family and inhibit *Aeromonas hydrophila* (Elegado et al., 1997).

Conclusion

In this study, cloning and expression of enterocin P originated from *E. faecium* p13 *was* accomplished in *p. pastoris* as a host. Enterocin P was detected in the supernatant of recombinant *p. pastoris* medium. Recombinant enterocin P showed inhibitory effects on gram-negative bacteria in MIC tests ranging from 2.65 to 465.75.

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Table 1. Bacteria and plasmid used in this study

Bacterium or plasmid	Relevant feature	Source and reference
Bacteria and yeast		
Escherichia coli strain DH5α	*fhuA2 lacΔU169 phoA glnV44 Φ80' lacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	
P. pastoris strain KM71H	Muts strain, i.e., slow-growing on methanol	Invitrogen-USA
Plasmids		
pPICZαA	Amr; E. coli-yeast shuttle vector; X kb	Invitrogen-USA
pPICZEntrosin	Amr; pPICZαA contain Enterocin P ; X kb	This study

Table 2. The minimum inhibitory concentration (MIC) test for enterocin P and some antibiotics (CLSI-Vet08) on eight pathogenic bacteria

	MIC (µg/ml)							MBC (µg / ml)
Pathogenic bacteria	Enterocin P	Ampicillin	Gentamicin	Penicillin	Vancomycin	Oxacillin	Enrofloxacin	Enterocin P
S. aureus ATCC29213	58.23	≤0.25	0.12-1	≤0.5	≤2	≤2	0.03-0.12	58.23
P. aeruginosa ATCC20271	465.75	ND	≤4	ND	ND	ND	1-4	931.5
E.coli ATCC	68.9	2-8	0.25-1	ND	ND	ND	0.008-0.03	137.86
K. pneumoniae ATCC700603	116.89	ND	ND	ND	ND	ND	ND	
S. enteritidis ATCC1624	18.23	≤2	≥16	≤2	≤2	ND	ND	36.46
Bacillus cereus	18.36	ND	ND	ND	ND	ND	ND	73.44
S. typhimurium (ATCC 14028)	2.656	≤2	ND	≤2	ND	ND	ND	42.66
E. coli (o157 H7)	10.648	ND	ND	ND	ND	ND	ND	42.59

Table 3. Molecular properties of native enterocin P and recombinant bacteriocin

	Length	Mass (Da)	Isoelectric point (pI)	Net charge	Hydrophobicity* Kcal mol^{-1}	Extinction coefficient** ($M\,cm^{-1}$)	Graphical structure
Rec-enterocin P	50	5449.5043	8.24	+2	+42.53	19605	
enterocin P	44	4627.1515	8.22	+1	+28.55	19605	

***** Wimley-White scale

** A factor used to describe how much light a molecule absorbs

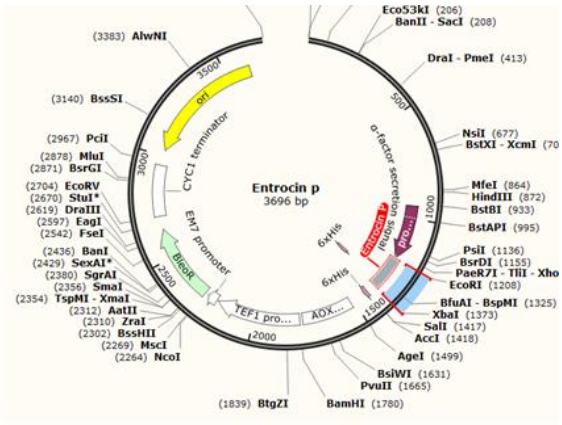


Fig. 1. Schematic figure from the virtual clone of enterocin P encoding sequences in pPICZαA vector

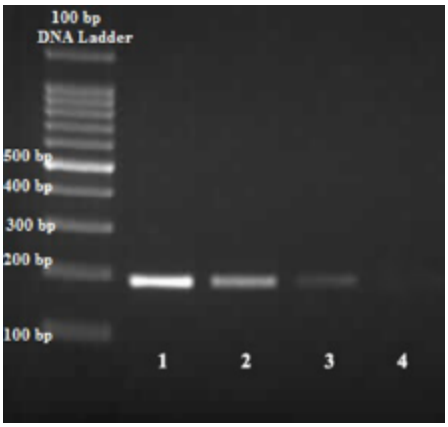


Fig. 2. Colony PCR of recombinant *E.coli* DH5α by enterocin P specific primers. Amplification of insert in recombinant plasmid showed a band about 180 bp from lanes 1 to 3. Lane 4 is the negative control

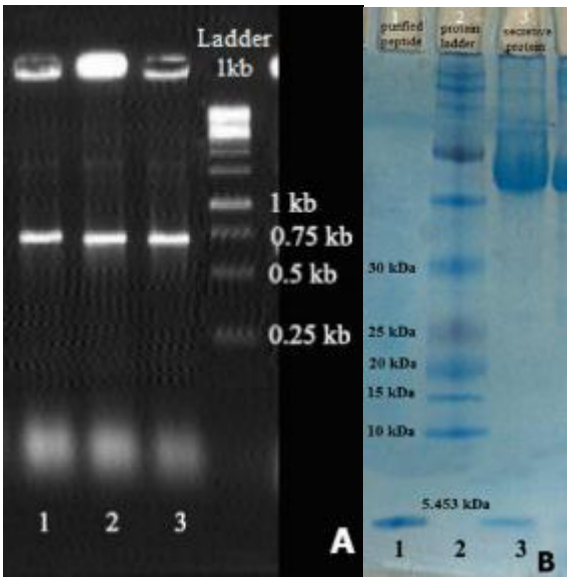


Fig. 3. (**A**) amplification of plasmid DNA extracted from recombinant *P. pastoris* by AOX primers. The plasmid contained with the insert showed an amplification band of about 700 bp from lan1 to 3. (**B**) electrophoresis of purified enterocin P (lane 1) protein ladder (lane 2) and culture media supernatant (lane 3) on the polyacrylamide gel (16%).