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OPTIMIZATION OF EXPRESSION AND EVALUATION OF RECOMBINANT ENTEROCIN-P, AS FOOD BIO PRESERVATIVE ON SOME FOOD SPOILAGE BACTERIA

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ABSTRACT

This study optimized the expression of the enterocin P peptide (EntP) in CHO cells using medium additions. Furthermore, the minimum inhibitory concentration (**MIC**), minimum bactericidal concentration (**MBC**), and anti-biofilm effects of Ent-P were examined against several gram positive and gram-negative microorganisms. The findings indicated that increasing medium with 0.75 percent glycerol and 0.75 percent DMSO boosted Ent-P peptide synthesis by around two times. According to Ent-P antimicrobial evaluations, Shigella disentri (4 g/mL) and Escherichia coli (128 g/mL) had the lowest and highest MIC values, respectively. In comparison to *Enterococcus faecalis* and *Escherichia coli* (3+ to 2+), the Ent-P peptide shown a strong ability to suppress the formation of the biofilm generated by *Staphylococcus aureus* and *Pseudomonas aeruginosa* (4 + to +1).

Keywords: Entrocin-P peptide, CHO cells, Overexpression, Anti-biofilm, MIC, MBC

INTRODUCTION

Foodborne infections now cover a wide range of illnesses and are a major global public health issue **(Caniça, Manageiro, Abriouel, Moran-Gilad, & Franz, 2019)**. Foods undergo spoilage due to microbial, chemical, or physical actions; therefore, the use of components to inhibit microbial contamination of food has been developed. Despite the potential risks to human health, food preservatives like nitrates and sulfites are used to extend the shelf life of food goods and stop the growth of germs. Despite being effective therapies for certain illnesses, antibiotic resistance develops when they are overused **(Caniça et al., 2019; Roshanak et al., 2020)**. It can occur with various mechanisms such as biofilm formation, enzyme modification efflux and decreased permeability of bacteria **(McDermott et al., 2002)**. Consequently, the creation of novel and natural alternatives without toxic or other adverse effects is desirable.

Antimicrobial peptides (AMPs), which were first discovered in 1993, are a great substitute for preservatives and conventional antibiotics **(Dubos, 1939)**. In both prokaryotes and eukaryotes, AMPs, also referred to as host defense peptides, are a component of the innate immune response **(Javadmanesh, Mohammadi, Mousavi, Azghandi, & Tanhaiean, 2021; Rashidian et al., 2022)**. These organic compounds have a wide range of biological effects against different microorganisms, including viruses and parasites **(Roshanak et al., 2021)**. Antimicrobial peptides kill microbes in a variety of ways, and their effects on gram-positive and gram-negative bacteria may vary **(Tanhaeian, Azghandi, Mousavi, & Javadmanesh, 2020; Tanhaeian, Nazifi, Shahriari Ahmadi, & Akhlaghi, 2020)**.

Bacteriocins, which are produced in large quantities by a variety of lactic acid bacteria, are one of these antimicrobial peptides. These peptides have been used as biotechnological tools in either food preservation or the prevention and control of bacterial infectious diseases **(Evangelin, Venkateswarulu, Babu, & Kasturi, 2015)**. However, for some reasons, such as hemolytic toxicity, the utilization of antimicrobial peptides should be done cautiously **(Hou et al., 2013)**. It should be mentioned that some research has indicated that bacteriocins may be used as safe, natural food preservatives **(Drider & Rebuffat, 2011)**. A large number of bacteriocins that are called Enterocins produced by *Enterococcus* sp. Enterocin-P (Ent-P), a cationic amphiphilic peptide secreted by *Enterococcus faecium* P13, is classified under Class IIa sec-Dependent Bacteriocin **(De Kwaadsteniet, Fraser, Van Reenen, & Dicks, 2006)**. This peptide has antimicrobial activity against bacteria, particularly foodborne pathogens such as *Listeria monocytogenes*, *Clostridium perfringens*, *Clostridium botulinum*, and *Staphylococcus aureus*, according to various investigations. Furthermore, this peptide is stable at pH and lyophilization **(Franz, Van Belkum, Holzapfel, Abriouel, & Galvez, 2007; Xia, Dong, & Xiao-lan, 2011; Mousavi et al., 2022)**. **Tanhaeian et al. (2019)** found that recombinant Ent-P suppressed the growth of various bacteria, including multidrug-resistant bacteria, in high salt medium and human plasma while maintaining adequate thermal stability **(Tanhaeian, Damavandi, Mansury, & Ghaznini, 2019)**.

Ent-P's broad antibacterial spectrum suggests that it could be used as a natural antimicrobial additive in the food sector. Therefore, the present study aimed to evaluate the overexpression of Ent-P peptide in serum-free CHO cell lines with different additives and its comparison with normal expression in CHO and HEK293 cells. Besides, the antibacterial effect of this peptide on foodborne bacteria was assessed.

MATERIAL AND METHODS

Bacterial strains

For minimum inhibitory and bactericidal concentration estimation, the American Type Culture Collection (ATCC) provided Listeria monocytogenes (ATCC 19111), Escherichia coli and E. coli O157 H (ATCC 700728 and 25922), Staphylococcus aureus (ATCC 25923 and 13567), Enterococcus faecalis (ATCC 29212), Salmonella enterica (ATCC 14028) Bacillus cereus (ATCC 11778) and Shigella disentri (ATCC 13313) were used for biofilm formation tests.

The recombinant peptide synthesis

Tanhaeian et al. (2019) used Chinese hamster ovary (CHO) cells transfected with the pcDNA 3.1+ vector (GenScript, USA) to clone and express the Ent-P gene. The transfected cell lines were capable of synthesizing and secreting the peptide into the medium based on the secretion signal and the coding sequence of the recombinant EnP in the vector. After the transfected cell lines were harvested, the medium containing the recombinant peptide was collected for the antimicrobial experiment. Prior to transfection, $2.5x10^5$ CHO cells were seeded into 35 mm wells

and cultured in DMEM until 60-80% confluent. Lipofectamine 2000 (Invitrogen, USA) was used to transiently transfect CHO cells with 10 μg of pcDNA3.1(+)-EntP in serum-free medium according to the manufacturer's protocol. The transfected Chinese hamster ovary cells were grown at 37 °C with 5% CO2 and a relative humidity of 95% in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen, USA).

Overexpression in CHO cells

To optimization of expression of Ent-P peptide in CHO cells with serum-free media was conducted. The experiment was carried out in a cell culture plate which had 12 wells with three replications. Following cell counting with 0.5% trypan blue (Sigma Aldrich, USA), approximately 1×10^5 mL/cell CHO cells were implanted with culture media. Then plate placed in an incubator. When cells growth reached to 80% of capacity, supernatant was discarded **(Tanhaeian et al., 2019)**. After that, the plates were rinsed twice in phosphate-buffered saline (PBS). The culture mixture was then supplemented with serum-free medium (SFM) containing 0.75 concentrations of glycerol and dimethyl sulfoxide (DMSO, Sigma) and cultivated for 72 hours. The supernatant was discarded, and the plate was frozen at -80°C.

Purification of Ent-p protein

For protein purification, the supernatant was filtered using a 0.45 m membrane filter (Sartorius Stedim, Germany) after being pH-adjusted to 7.0 with 1 M sodium phosphate buffer. The filtered supernatant was washed with binding buffer containing 20 mM sodium phosphate, pH 7.0, and then loaded to a HiTrap column and chromatographed at a flow rate of 3 ml/min. The bound proteins were extracted using a buffer containing 100 mM sodium citrate, pH 4.6, and collected in Eppendorf tubes using a buffer containing 1 M tris-HCl, pH 9. A 6-His-tailed recombinant Ent-P peptide from QIAGEN (USA) was purified using Ni-NTA agarose. After being cleaned with distilled water, the Ni-NTA agarose column was rinsed with lysis buffer, which included 50 mM potassium phosphate, 400 mM sodium chloride, 100 mM MKCl, 10% glycerol, and 0.5 percent Triton X-100.

SDS-PAGE and Dot Blot analysis

The pure peptide protein was accessed using a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in duplicate. The gel was stained with silver staining to show protein bands. Dot blot analysis was used to assess the level of peptide expression between CHO and HEK293 cells cultured in regular culture medium and modified culture medium to determine whether there was an overexpression.

Minimum inhibitory and bactericidal concentration tests

A 96-well microtiter plate with four replications was used to carry out the minimum inhibitory concentration (MIC) experiment, which was done utilizing the micro broth dilution method. The bacteria were serially diluted and then distributed into a 96-well microtiter plate after being grown overnight on mueller hinton medium and suspended to a concentration of approximately 1×10^8 CFU/mL. The MIC for each bacteria was the lowest amount of Ent-P that could destroy the bacteria. Following MIC, all dilutions of every type of bacteria were cultivated individually on Mueller Hinton agar medium, and the minimum bactericidal concentration (MBC) was established as the absence of growth **(Tanhaeian, Azghandi, et al., 2020)**.

Biofilm assay

Utilizing a colorimetric microtiter plate assay, the strains' ability to produce biofilms was evaluated **(Peeters, Nelis, & Coenye, 2008)**. For the preparation of microbial suspension, the amount of 20 µl of bacteria was added to the Trypticase Soy Broth (TSB). The culture was then inoculated for 24 hours at 37 °C. The peptide was serially diluted and inoculated to the wells before being infected for 24 hours at 37 °C. Following that, 150 mL of this bacterial culture was used to inoculate sterile 96-well polystyrene microtiter plates. Microwells were cleaned three times with 200 mL of phosphate-buffered saline (PBS) and dried after 24 hours of incubation at 37 °C without shaking. 100 mL of 99% methanol was applied to fix biofilms, and after 15 minutes, supernatants were removed and the plate was air-dried. The wells were then filled with 100 mL of 1% crystal violet (Merk, USA). After 20 minutes, the plate was rinsed under running tap water. By adding 150 mL of 33% acetic acid, the related CV was released. The optical density of each well was measured using a microplate reader (Epoch, BioTek, USA) at 590 nm. The biofilm experiment was carried out in two stages, one with and one without Ent-P. All assays were performed three times. Medium was used as a control to determine background OD. Three standard deviations above the control was designated as the cut-off OD (ODc). Based on the results of the microtiter plate test, the bacteria were divided into four groups: non-biofilm producers (OD test < ODc), weak biofilm producers (ODc < OD < 2X ODc), moderate biofilm producers (2X ODc < OD < 4X ODc), and strong biofilm producers (4X ODc < OD).

RESULT AND DISCUSSION

Overexpression of Ent-P

The expression of Ent-P peptide in serum-free CHO cells with additives including DMSO and glycerol was investigated by the SDS-PAGE test. As illustrated in figure 1, Ent-P was successfully expressed. The 5.453 kDa protein band shows the proper expression of protein in CHO cells and secretion into the culture medium.

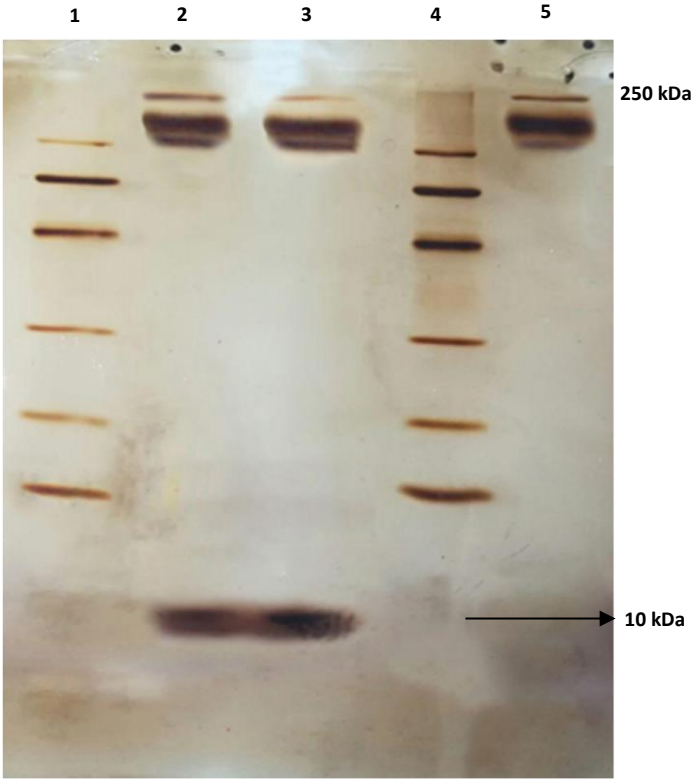

Figure 1 SDS-PAGE test of Ent-p peptide secreted from CHO cells into the medium culture. **Lanes 1 and 4)** Low molecular weight protein marker **lane 2) and 3)** Overexpressed Ent-p peptide secreted from CHO cells. **Lane 5)** Negative control

The overexpression of Ent-P peptide in CHO cells (culture medium with additives) in comparison with the normal expression in CHO and HEK293 cells (normal culture medium) was assessed by dot-blot analysis (Figure 2).

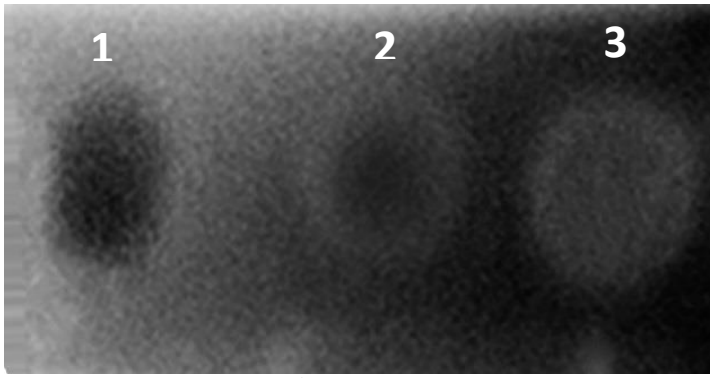

Figure 2 The dot-blot analysis of Ent-p peptide production in medium culture. **Lane 1**) Overexpression of Ent-p peptide in CHO cells (culture medium with additives). **lane 2)** Ent-p peptide secreted from CHO cells (normal culture medium). **lane 3)** Ent-p peptide secreted from HEK293 cells (optimized media).

Different peptide expression was observed among three experimental treatments. Accordingly, peptide expression in the optimized condition of CHO cells with additives outperformed peptide expression in normal culture medium. Furthermore, estimation of peptide concentration with BCA suggested that the amount of peptide expression in CHO and HEK293 cells without additives in the medium was 921 µg/mL, 447 µg/mL, respectively. Moreover, a positive effect of glycerol and DMSO on Ent-P peptide production by CHO cells was observed. Besides, Ent-P peptide production was escalated two times using glycerol 0.75%

and DMSO 0.75% (approximately 1720 µg/mL) in comparison with no additives in the media.

Although the use of food preservatives and antibiotics is unavoidable due to their beneficial influence on food half-life extension, their drawbacks are evident. In addition to its antibacterial, antifungal, and antiviral capabilities, antimicrobial peptides can display antibacterial efficacy against antibiotic-resistant bacteria (Javadmanesh et al., 2021). Ent-P is a peptide with a wide range of antimicrobial activities. This peptide is lowly secreted by lactic acid bacteria. The use of molecular techniques now allows the recombinant peptide's high molecular weight to be expressed within recombinant prokaryotic and eukaryotic systems. When a protein is expressed in mammalian cells, it will be more compatible and safer for human consumption than the same product from other hosts such as bacteria, plants, and yeast **(Walsh, 2010)**.

However, eukaryotic systems may be slightly less efficient than prokaryotic systems **(Liu, Chu, & Hwang, 2001)**. To solve this issue, numerous strategies for optimizing the generation of recombinant peptides in mammalian cells have been developed. Increasing the level of peptide expression can significantly lower the cost and time required to produce recombinant peptides. Because they grow quickly, are easily transfected, and may undergo complicated post-translational modifications essential for biological activity, CHO cells are the favored system for manufacturing therapeutic recombinant peptides on a large scale **(Walsh, 2010)**.

In this study, we tried to produce a recombinant peptide in a serum-free medium. Because serum is required not only for maintenance but also for the proliferation and differentiation of human and animal cells in culture. However, the use of serum might be challenging for several reasons, including cost, intervention in the purification of the recombinant products, and the possibility of viral contamination **(Froud, 1999)**.

Liu et al. (2007) found that by including 1% glycerol in the medium, the level of recombinant macrophage-colony stimulating factor (M-CSF) synthesis in CHO cells will increase by 38% one day after cell growth **(Liu & Chen, 2007)**. In addition, glycerol can protect proteins from heat and chemicals (Timasheff, 2002). Additionally, glycerol can induce proper protein fusion and increase protein synthesis **(Tieman, Johnston, & Fisher, 2001)**.

Dimethyl sulfoxide (DMSO) was found to have a positive impact on the expression of extracellular genes in recombinant CHO cells when its effect on this expression was studied **(Liu et al., 2001)**. **Strätling (1976)** reported that DMSO can affect RNA synthesis by reducing a polar interaction between histone and chromatin and enhancing transcription and gene expression **(Strätling, 1976)**.

The effect of DMSO, glycerol, sodium butyrate and temperature on the rate of beta-interferon production was assessed in CHO cells and it was found that a temperature of 30 °C, with a maximal effect, increased the production by 4-folds, DMSO increased the production rate by 2 times, sodium butyrate increased the production by 3-folds and glycerol increased the production by 0.85-fold **(Rodriguez, Spearman, Huzel, & Butler, 2005)**. In this study, as expected, by adding DMSO 0.75% to the medium, the expression of Ent-P peptide increased twofold in comparison with control.

Antimicrobial effect

Table 1 summarizes the outcomes of the MIC and MBC testing against several gram-positive and gram-negative bacteria. As a result, *Shigella disentri* and *Escherichia coli* had the lowest and highest MIC values, respectively. It has been discovered that this peptide has advantageous antibacterial activity against some foodborne bacteria as a result (Table 1).

Table 1 Minimum inhibitory concentration and minimum bactericidal concentration results against foodborne pathogens (µg/mL)

Bacteria		MIC	MBC
Listeria monocytogenes	ATCC 19111	16	16
Escherichia coli O157:H	ATCC 700728	64	-
Escherichia coli	ATCC 25922	128	-
Staphylococcus aureus	ATCC 13567	8	8
Staphylococcus aureus	ATCC 25923	8	8
Enterococcus faecalis	ATCC 29212	16	32
Salmonella enterica	ATCC 14028	8	32
Bacillus cereus	ATCC11778	8	8
Shigella disentri	ATCC 13313	4	8

The purified Ent-P peptide was tested for antibacterial activity. In a previous study by **Tanhaiean et al. (2019)**, the antibacterial activity of this peptide was examined and it was found that this peptide was effective against antibiotic-resistant bacteria and some gram-positive and food-borne bacteria (Tanhaeian et al., 2019). In this study, the Ent-P peptide showed antibacterial activity against all tested species.

Antibiofilm activity

Table 2 displays the outcomes of the biofilm assay. *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed the greatest biofilm reduction by bacteriocin Ent-P (4+ to 1+ and 4+ to 1+, respectively), whereas *E. coli* and *Enterococcus faecalis* showed the least biofilm reduction (3+ to 2+ and 3+ to 2+, respectively).

Table 2 The effects of the bacteriocin Ent-P peptide on the inhibition of biofilm formation by some bacteria

Bacteria	**PA**	**PA+EN**	**SA**	**SA+EN**	**E**	**E+EN**	**EC**	**EC+EN**
Biofilm	101(4+)	9(1+)	97(4+)	5(1+)	34(3+)	11(2+)	24(3+)	3(1+)

EN: Ent-P, **PA**: pseudomonas aeruginosa, **SA**: staphylococcus aureus, **E**: Enterococcus, **EC**: E coli

Studies have revealed that AMPs have potent anti-biofilm capabilities against bacteria as well as antibiotic isolate resistance. In order to stop bacterial adherence during the initial phases of biofilm development, the AMPs can function as an inhibitor **(Batoni, Maisetta, & Esin, 2016)**. By removing or eliminating the bacteria, they can also eliminate mature biofilms **(Segev-Zarko, Saar-Dover, Brumfeld, Mangoni, & Shai, 2015)**. According to studies, bacteriocins such as nisin A, lacticin Q, and nukacin ISK-1 can damage the cell walls of adherent cells in biofilms (for example, methicillin-resistant Staphylococcus aureus) and cause these cells to release ATP, which is more effective than antibiotic therapies **(Okuda et al., 2013)**.

Tanhaeian et al. (2019) reported that the Ent-P peptide is resistant to heat and high salt concentration. Besides, the presence of His-taq, for purification purposes raises its positive charge and, as a consequence, increases the antibacterial property of the peptide **(Tanhaeian et al., 2019)**. These characteristics, together with antibacterial and anti-biofilm qualities, and expression in CHO cells as a friendly host for human consumption, indicate that this peptide could be employed as an effective preservative in the food industry.

CONCLUSION

Overall, the result of this study showed that glycerol and DMSO increased the production of Enterocin-P peptide by around twofold. Furthermore, Results demonstrated the potential of the Enterocin-P peptide to act as antibacterial and anti-biofilm agents and for other biomedical applications.

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