Gene Reports 24 (2021) 101088

Contents lists available at ScienceDirect

Gene Reports

journal homepage: www.elsevier.com/locate/genrep

Cloning and the expression of the protein fusion enterocin-nisin-epidermicin $_T$ as a candidate for the treatment of gastric cancer

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ARTICLE INFO

Keywords:
Gene expression
Cloning
Gastric cancer
Bacteriocin
Enterocin
Nisin
Epidermicin

ABSTRACT

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death in the world. Because conventional therapies, including chemotherapy, surgery, radiation therapy, etc., are associated with damage to healthy cells, gastric cancer treatment remains a major challenge today. In recent years, the use of bacteriocins has become one of the emerging options in the treatment of gastric cancer. This project aims to clone and express the fusion gene, including three bacteriocins of enterocin, nisin, and epidermicin, and to produce recombinant protein as a candidate for gastric cancer treatment.

The sequence of bacteriocins was extracted from NCBI and cloning and induction of expression was performed with isopropyl thiogalactosidase (IPTG) and evaluated by SDS-PAGE and confirmed by Western blot.

The enterocin-nisin-epidermicin fusion gene was cloned in the plasmid pet 22b and its expression was confirmed using Western blot. The results showed protein fusion induced the highest level of apoptosis in AGS cells. AGS cells which treated with concentrations of nisin-enterocin-epidermicin protein fusion exhibited increasing levels of apoptosis. Today, antimicrobial peptides, including bacteriocins, have attracted the attention of scientists as an alternative treatment. Because the fusion protein has been produced and purified in this study, it can be further studied in the future as one of the suitable candidates for study of treatment of gastric cancer.

1. Introduction

Cancer is an uncontrolled growth and development of cells due to environmental factors and genetic disorders and is one of the leading causes of death in the world. More than 12 million people worldwide are infected with this deadly disease each year (Dalerba et al., 2020; Siegel et al., 2020).

Gastric cancer is one of the most common cancers caused by the abnormal accumulation of a group of cells and the formation of a clump in the stomach. According to the latest statistics from the National Cancer Research Center, gastric cancer is the most common cancer in men and the third most common cancer in women after breast and colon cancer. Most people with this cancer become aware of their illness when their disease has progressed or metastasized (Khaledi et al., 2016a, 2016b, 2016c; Lyons et al., 2019; Russo and Strong, 2019; Siegel et al., 2020).

There are a variety of treatments, including surgery, chemotherapy, and radiation therapy for stomach cancer, depending on factors such as the severity of the illness and the patient's general health (Sasaki, 2019). Routine chemotherapy drugs target dividing cells, and because they do not specifically target cancer cells, they can also damage healthy cells (Hosseini et al., 2016; Khaledi et al., 2016a, 2016b, 2016c; Saghi et al., 2016; Pourhajibagher et al., 2017; Esmaeili et al., 2019). Surgery has always been associated with the risk of metastasis, and the problem with radiation therapy is damage to natural cells and mutations in the genetic material (Sasaki, Y., et al. 2019). Therefore, for this purpose, new and specific treatment methods are needed for cancer cells that target only cancer cells (Khaledi et al., 2016a, 2016b, 2016c; Sitarz et al., 2018).

Today antimicrobial peptides including bacteriocins have attracted the attention of scientists as an alternative to treatment, and there have

DOIs of original article: https://doi.org/10.1016/j.genrep.2021.101087, https://doi.org/10.1016/j.genrep.2020.100751.
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https://doi.org/10.1016/j.genrep.2021.101088

Available online 20 March 2021

been many reports of the positive effects of antimicrobial peptides in treating infections (Abadi et al., 2016; Khaledi et al., 2016a, 2016b, 2016c; Alizadeh et al., 2019; Azadi et al., 2019; Hekmati et al., 2020; Hosseinpor et al., 2020). Clinical studies have shown the role of bacteriocins in the prevention, control, and treatment of cancers (Abadi et al., 2016; Khaledi et al., 2016a, 2016b, 2016c; Deslouches and Di, 2017; Araste et al., 2018; Leite et al., 2018). For example, in some studies, antitumor effects of LL-37 peptide against ovarian tumors, lung cancer cells, breast cancer, colon cancer cells and else survived (von Haussen et al., 2008; Chuang et al., 2009; Weber et al., 2009; Wu et al., 2010; Niemirowicz et al., 2015). Findings of other studies suggest that defensin has an anti-apoptotic effect against squamous cell carcinoma of the head and neck (SCCHN tumors), lung cancer cells, cervical cancer and prostate carcinoma (Bullard et al., 2008; Mburu et al., 2011; Hanaoka et al., 2016; Xu et al., 2016).

Bacteriocins are protein metabolites, usually with a molecular weight of less than 10 kDa, that are produced by almost all bacterial groups and show their antimicrobial activity against near-productive strains. Fortunately, in recent years, the use of bacteriocins has become another emerging option in the treatment of gastric cancer. Therefore, due to the low side effects of using bacteriocins, they can be used as an independent treatment method or as an adjunct to other treatment methods (Cornut et al., 2008; Baindara et al., 2018).

It should be noted that the use of a bacteriocin alone cannot kill all pathogens. Therefore, the combination of several bacteriocins using biotechnology in a single bacteriocin and the production of a multifunctional bacteriocin is important to obtain the appropriate drug to produce the new drug combination.

The present study aimed to clone and express the fusion protein derived from the three bacteriocin nisin, enterocin, and epidermicin for later use in the treatment of gastric AGS. Enterocin is small (65 kDa) heat-stable, non-Lan containing, and membrane-active peptide of Class II bacteriocins. The enterocins killed susceptible cells rapidly, but cell lysis does not appear to be involved directly. Enterocins reduced biosynthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and inhibited protein synthesis (McAuliffe et al., 2001). Nisin of Class I bacteriocins is small (65 kDa) peptide which has unusual amino acids lanthionine (Lan), *L*-methyllanthionine (MeLan) and several dehydrated amino acids. The action of nisin is by disrupting the membrane integrity of target organisms. Nisin is active against most of the bacteria including Lactococcus, Streptococcus, Staphylococcus, Listeria and, Mycobacterium (Gross and Morell, 1971; Hurst, 1981). Epidermicin (type-A lantibiotics) is elongated, cationic peptide up to 34 residues in length, epidermicin act by disrupting the membrane integrity of target organisms (Kumar et al., 2017; Agarwal et al., 2016).

2. Materials and methods

2.1. Microorganisms and plasmids used in the study

The *E. coli* BL21 bacterium was used to propagate and maintain plasmids and recombinant vectors. The pET-22b plasmid was also used for cloning and gene expression.

2.2. Design of nisin-enterocin-epidermicin fusion gene

The sequence of all three genes including epidermicin protein EpiB (*Staphylococcus aureus subsp. aureus* NCTC8325) Nisin is gene (Lactococcus lactis subsp. lactis strain RP359). Enterocin B (*Enterococcus faecium* strain 64/3) was extracted from the NCBI site at https://www.ncbi.nlm.nih.gov and saved in FASTA format. To design the fusion gene, the sequences of all three bacteriocins were linked together by linkers, and the anticancer sequence was designed using I-TASER software and added to the bacteriocin sequence. *Xho*I and *Nde*I restriction enzymes were also used at the beginning and end of the gene cassette.

2.3. Amplification of fusion genes using PCR

In this study, fusion gene amplification was performed using PCR-designed primers. Primer sequence: SEQ-R, GGCTTGGAGCCTCTGTG.

CGTC and, left primer sequence (SEQeF), GGGAGTCCGGGGCTGTTC GAC. Amplification of the desired gene fragment was added with PCR in a volume of 20 μl, 12.5 μl master mix, 1 μl of right and left primers, 1 μl of template DNA and 4.5 μl of distilled water. The thermal gene amplification program was performed using the thermocycler device as follows. The initial denaturation stage at 95 °C for 3 min, the secondary denaturation 1-min at 94 °C, the 1-minute connection at 67 °C, the 1-minute extension stage at 72 °C for 35 cycles, and the final extension stage for 5 min at 72 °C was done. After amplifying the desired gene fragment, a sequence was taken on the agarose gel and examined and confirmed.

2.4. Cloning of the fusion gene in the pET-22b vector

The fusion gene must be cloned into the pET-22b expression vector. For this purpose, the endonuclease enzymes XhoI and NdeI were used. After digesting the enzyme and creating a sticky end on the vector and PCR product from the previous step, the ligation between the fusion gene and the vector was performed using the T4 DNA ligase enzyme. The reaction product was then used to transform the *E. coli* BL21-competence bacteria by thermal shock. For this purpose, we first put the competence bacteria on ice for 30 min, then put them under heat shock for 90 s at 42 °C. In the next step, we added 900 μl of Luria Bretani culture medium to the vial without antibiotics and centrifuged for 3 min at 9000 RPM. We then discarded 700 micrograms of surface liquid and dissolved the precipitate in the remaining 300 μl, then cultured the recombinant bacterium on a solid laryngeal culture medium containing ampicillin and incubated for 24 h at 37 °C. PCR was used to confirm this stage and the gene was cloned, and the product was electrophoresis on the agarose gel. In this way, the colonies containing the desired plasmid were selected.

2.5. Expression of fusion protein enterocin-nisin-epidermicin

Culture media used in this study were liquid and solid Luria Brittany (LB) medium, which after preparation and autoclaving the medium, its temperature reached 50–40 °C from antibiotic stock (Ampicillin and sigma Kanamycin) with a concentration of 100, is added 1 μ per 1 μ per liter of LB content. In the next step, was cultured a colony containing recombinant plasmid in 5 ml LB broth environment containing 100 g of kanamycin (ml) and incubated it for one day in the incubator 37 °C to obtain the maximum growth rate. Then, 5 ml of the overnight culture was added to 100 ml of LB medium and placed in an incubator at 37 °C for 2–3 h. IPTG was used to induce gene expression after the number of bacteria reached the appropriate level (OD 0.4–0.6). Before induction, 1 mL was first removed from the culture medium as a control sample and after centrifugation, its sediment was collected and placed at −20 °C. To the rest of the medium, was added 5 μl per ml of culture medium from 100 mM IPTG stock and placed in a shaking incubator at 37 °C for 3 h. Samples were prepared at intervals of 1, 1.5, 2, and 3 h after induction, as well as 24 h later, and its sediments were placed at −20 °C after centrifugation.

2.6. Investigation of RNA gene expression at the RNA level by real-time PCR method

For this purpose, first RNA extraction was performed before and after induction, then cDNA was synthesized from it and then the presence of our purified mRNA was checked by the PCR test.

Gene name: Hybrid bacteriocin
(723 bp)
Codon Optimization: No
Vector: pET-22b (+)
Vector type: Custom vector
Cloning Sites: 5' BamHI / EcoRI 3'

Sequence from 5' to 3'

GGATCCGTGCGGGGAGTCCGGGCTGTTCGACATGAATTTAAAAAAAAATAATTTAGAGTATAATTTATGCA
TTTTTTTAGCAGTAATTATAAATTTAGGATTATTTATTTTTTCCGAAACTATATTAAGTAAGATTTTACTA
TTGATTGCTATTGTTTTATTAGTTATTCCTAATTTCATGCAAAAAAAGAAGAGAAAAAACTCGTGCTGTAT
GGCAGGATTTATGAAGTTAATTCAGTTCTTAGCAACTAAAGGTCAAAAGTATGTTTCACTTGCATGGAAAC
ATAAAGGTACTATTTTAAAATGGATTAACGCCGGTCAAAGTTTTGAATGGATTTATAAACAAATCAAAAAA
TTATGGTCATGCTGTATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGG
TGCATCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTGATGGGTTGTA
ACATGAAAACAGCAACTTGTCATTGTAGTATTCACGTAAGCAAAGTGGACAATAAGTTTAATAAAGAACTG
AGCGTGGCTGGGCGGGAGATAGTGACCCTGCCAAACCTCAATGACCCACAGAAAAAGGCGTTCATTTTCTC
CCTCTGGGATGACCCATCGCAGTCAGCCAACTTGCTGGCGGAAGCGAAAAAGCTTAACGACGCACAGGCTC
CCAAGCCGAATTC

Fig. 1. The fusion gene, constructed with three bacteriocin enterocin, nisin, and epidermicin.

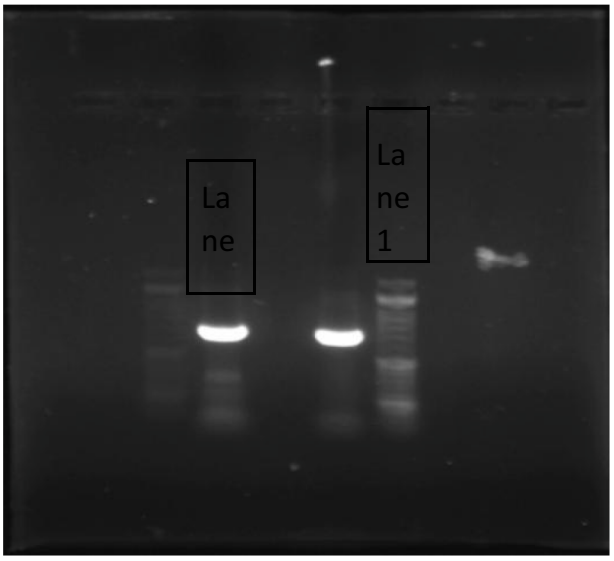

Fig. 2. Agarose gel the fusion gene of the enterocin-nisin-epidermicin gene from the PCR reaction. Lane 1 marker 50 bp, lane 2 amplified DNA 460 bp.

2.7. Investigation of gene expression at the protein level by SDS-PAGE method

The recombinant protein was purified on a nickel column and then SDS-PAGE was used to confirm the protein. For this purpose, first, the desired buffers and solutions including acrylic acrylamide solution (30.8%), high and low gel buffer, buffer electrode (tank), 10% ammonium persulfate solution, and protein sample buffer were prepared and the induction samples after induction. The products were poured into the prepared wells of the gel, and after connecting to the electric current and performing electrophoresis, the staining was done using the Coomassie blue.

2.8. Confirmation of the expression of recombinant proteins by Western blotting method

To confirm the recombinant protein and to assess its antigenicity, the Western blot test was performed. In this method, the presence of recombinant protein using the anti his tag antibody was evaluated. For this purpose, buffers and solutions were first prepared, including Tris Buffered Saline + Tween 20 (TBST), transfer buffer, and diaminobenzidine solution (DAB). First, the post-expression and non-induced samples were selected, and electrophoresed 15% on the gel. In the next step, the gel bands were transferred to nitrocellulose paper and blocked in the buffer for one day to prevent non-specific reactions. Then washed three times with PBS buffer. The His-tag antibody was then prepared and the nitrocellulose paper was immersed in it and placed on the shaker at room temperature for 1 h. In the next step, a secondary antibody was

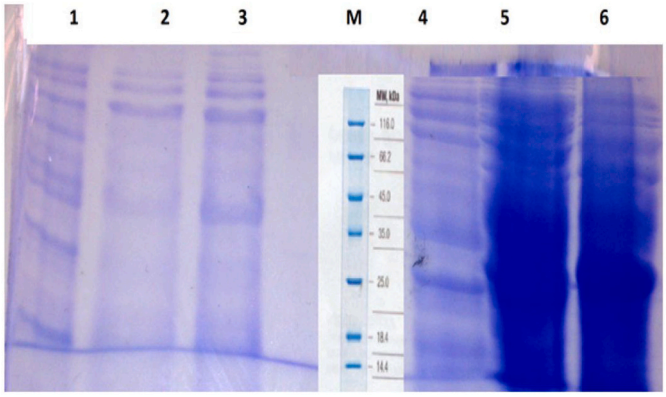

Fig. 3. Examination of gene expression. Rows 1 to 4 without induction samples, row M of protein marker, row 5 induction sample after 2 h, and row 6 induction sample after 24 h with a weight of 28 kDa.

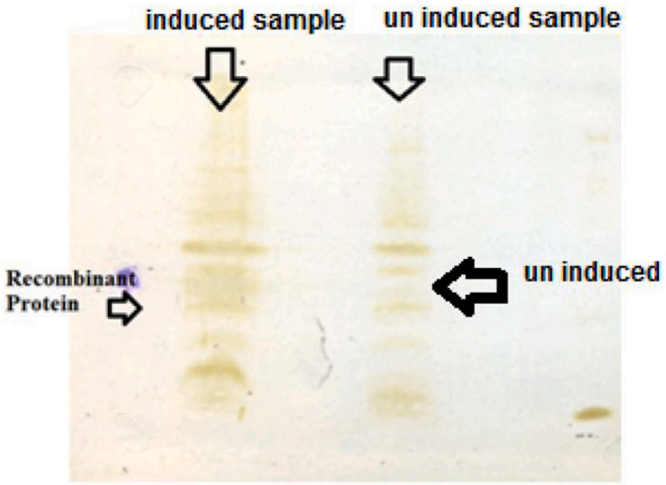

Fig. 4. Western blot results.

prepared and the paper was immersed in it. Finally, after washing the samples with PBS buffer, the paper nitrocellulose was placed in a diaminobenzidine solution to determine the bands.

3. Results

The peptide hybrid was designed to three bacteriocins, nisin, enterocin, and epidermicin, extracted from the NCBI site. *Xho*I and *Nde*I restricted enzymes were also added to the gene cassette. The sequence of this fusion gene is shown in Fig. 1.

In the next step, the PCR reaction of the enterocin-nisin-epidermicin fusion gene was amplified using specially designed primers. The PCR gel

was then electrophoresed to confirm the amplification of the PCR gene. The presence of the band indicated the amplification of the target gene (Fig. 2).

In the next step, the recombinant plasmid containing the fusion transformed into competence *E. coli* BL-21 DE3 plysS. The PCR was used to confirm recombinant plasmid-containing colonies.

After construction, the enterocin-nisin-epidermicin in the fusion gene into the pET-22b expression plasmid, induction with IPTG was performed. Real-time PCR was then performed to ensure gene expression at the RNA level, and later the SDS-PAGE method was used to confirm the expression at the protein level. The result can be seen in the 28 kDa band in Fig. 3.

In the last step, the Western blotting method was used to ensure the confirmation of the recombinant protein produced (Fig. 4).

The results showed induced sample confirmed with Anti histag but uninduced sample don't react with Anti histag.

4. Discussion

Common treatments for cancer include surgery, chemotherapy, and radiation therapy, depending on the patient's general health and severity. Each of these treatments has its drawbacks (Delaunoit, 2011; Joharatnam-Hogan et al., 2020). Therefore, due to the spread of cancer, scientists are still looking for new treatments that are more effective in the treatment process and have fewer side effects. One of the newest treatments is the use of antimicrobial peptides, including bacteriocins (Hoskin and Ramamoorthy, 2008).

The antitumor properties of nisin in head and neck squamous cell carcinoma (HNSCC) in several studies were explored (Joo et al., 2012; Kamarajan et al., 2015). Also, the apoptotic effect of nisin on colon cancer cells (SW480) in another study evaluated (Ahmadi et al., 2017). Goudarzi et al. in another study concluded that nisin had a cytotoxic effect on gastrointestinal (AGS and KYSE-30), hepatic (HepG2), and blood (K562) cancer cell lines (Abadi et al., 2016). Also, nisin induces preferential apoptosis and decreased cell proliferation of oral squamous cell carcinoma (OSCC) (Joo et al., 2011).

Based on the results of another study nisin also could be a potential therapeutic for the breast cancer cells (Avand et al., 2018). The apoptotic effect of enterocin against HeLa, HT-29, and AGS human cancer cells was explored in a study (Ankaiah et al., 2018). Also anticancer activity of enterocin against human colon, gastric (HT-29, Caco-2, and AGS), cervical (HeLa) cancer cells survived (Ankaiah et al., 2017).

In the present study, by producing a recombinant fusion protein, we tried to examine it as an alternative treatment candidate for gastric cancer.

After the design and synthesis of nisin-enterocin-epidermicin fusion protein, cloning was performed. In this study, the expression vectors of the pET system were used for this purpose. The system used in this study was pET-22b, which is widely used for the production of recombinant drugs. *E. coli* BL21DE3 plays an expression system that was also used to express the product due to its high production and reasonable price and pelb signal peptide sequence. Finally, the recombinant protein produced and purified, and according to the results, the highest protein content was 24 h after induction.

The present study aimed to clone and express the genes of enterocin-nisin-epidermicin in the fusion gene in laboratory conditions and to confirm its accuracy with SDS-PAGE and Western blot. This recombinant protein can be used as one of the alternative treatments for stomach cancer in the future.

Declaration of competing interest

None declared.

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Abbreviations

Fusion proteins: or chimeric proteins are proteins created through the joining of two or more genes that originally coded for separate proteins
Enterocin: is a novel bacteriocin produced by *Enterococcus faecium*.
Epidermicin: is a novel antimicrobial peptide that has potent activity against Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus*, and it may have potential for use in therapy for infections caused by these bacteria.
Nisin: is a polycyclic antibacterial peptide produced by the bacterium Lactococcus lactis.
Cloning: is the process of producing genetically identical individuals of an organism either naturally or artificially.
Gene expression: is the process by which information from a gene is used in the synthesis of a functional gene product.