Anti-cancer Effect of Recombinant PI-Laterosporulin10 as a Novel Bacteriocin with Selective Cytotoxicity on Triple Negative Breast Cancer Cells

Bahare Rafizadeh \cdot Somayeh Reiisi \cdot Behnaz Saffar \cdot Forough Taheri \cdot Sadegh Farhadian

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Abstract

Anti-microbial peptides (AMPs) are important factors of the innate immune system which perform a comprehensive spectrum of biological activities. The present study aims to examine the expression, purification, and cytotoxic effects of recombinant PI–laterosporulin10 (LS10) a defensing-like peptide. PI is a transmembrane peptide that helps to increase penetration and specificity of peptide targeting. PI–LS10 sequence was optimized and fused to a Xa protease site to achieve higher levels of expression. Then Xa-PI–LS10 was cloned into the pET-32a (+) vector and expressed in Escherichia coli BL21 (DE3). The fusion protein with a molecular weight of about 25.3 kDa was analyzed using the SDS-PAGE. The maximum expression was observed 8 h post-induction. After enzymatic cleavage, the purity of PI–LS10 was > 300 µg/ml as the final yield. The recombinant PI–LS10 showed anti-cancer characteristics against breast cancer cell lines. MCF7 (non-triple negative) and MDA-MB-231 (triple negative) cells were treated with different concentrations of the PI–LS10 in order to evaluate their cytotoxic activity and efficiency in targeting triple negative cells. Our results suggest that PI–LS10 had a significant cytotoxic effect against breast cancer cells in comparison to the normal cell line. Triple negative breast cancer cell (MDA-MB-231) proliferation inhibited higher in 72 h after treatment compare MCF7 cells. In conclusion, the PI–LS10 peptide was produced successfully and its favorable anti-cancer activity against a breast cancer cell line was confirmed.

Keywords Laterosporulin10 \cdot Anti-microbial peptides \cdot Breast cancer \cdot Expression and purification

Introduction

Breast cancer is on the second rank amongst the most common causes of women’s death (Dumitrescu and Cotarla 2005). Triple-negative breast cancer (TNBC), which is negative for estrogen receptor, progesterone receptor, and HER2/neu receptors, has been observed with the characteristics of the highest recurrence rate and fast growth. Prognosis rates are low for this type of pathology and its treatment is currently mainly relying on chemotherapy (Hurley et al. 2013; Marmé and Schneeweiss 2015). Considerable advancements in diagnosis, treatment and prevention of various types of cancers including the breast cancer have been reported in recent years (Miller et al. 2019). However, the side effects associated with many treatment procedures and resistance to anti-cancer drugs have caused significant challenges for a decisive treatment (Pucci et al. 2019). The main objective of the new method of cancer treatment is to discover suitable anti-cancer agents with stronger activity. Using the therapeutic peptides are seen as a promising approach in developing anti-cancer agents (Singh et al. 2022; Cicero et al. 2017). Therefore, the recent usage of small peptides such as anti-microbial peptides (AMPs) as therapeutics in cancer treatment has attracted a lot of scientific attention. AMPs are short-chain’s amino acids (usually < 100 amino acids) produced by a variety of organisms to combat diverse infectious agents including bacteria, viruses, fungi, and parasites (Mojsoska and Jenssen 2015). AMPs have various properties such as being anti-microbial, anti-viral, and anti-fungal (Vaezi et al. 2020; Felício et al. 2017). A number of AMPs
show dual activities, including anti-microbial function and selective anti-cancer peptides (ACPs) having lytic activities against the membrane; however some of them are able to penetrate the cell membrane and act on intracellular targets (Sani and Separovic 2016; Erdem Büyükkızar and Kesmen 2022; Hicks 2016). One of the limitations for the therapeutic peptides is that some AMPs have poor performance in penetrating the cell membrane. Various Cell Penetrating Peptides (CPPs) protein transduction domains have been developed in order to overcome that problem (Dinca et al. 2016). The precise mechanism of CPPs functions is still unclear, but three main mechanisms exist for transporting CPPs into the cell: Direct penetration, endocytosis, and delivery through transient structure and reverse micelle formation through CPP interaction with the cell membrane (Pizzolato-Cezar et al. 2019). In the case of PI, 11 amino acid peptide as a CCP came from the pC89 phage display library. PI is a non-polar hydrophobic amino acid that targets breast cancer cells especially TNBC and could be a potential valuable discovery in breast cancer cell therapy (Dong et al. 2008).

Laterosporulin10 (LS10), as defensin like class IIα bacteriocin, is an antimicrobial and anticancer peptide that firstly isolated from the Brevibacillus SKDU10 strain (Baindara et al. 2016). It has been revealed that LS10 peptide has anti-cancer activity against some cancer cells, including human breast cancer cell line (MCF-7), human embryonic kidney cancer cell line (HEK293T), human fibrosarcoma cell line (HT1080), human cervical cancer cell line (HeLa) and human lung cancer (H1299). The LS10 acts on the cancer cells through a mechanism of membrane disintegration and induces apoptosis (Baindara et al. 2017a). In the present study, we describe the procedure of developing a functional recombinant LS10 anti-microbial peptide and targeting of TNBC cells by adding PI sequence to LS10 upon purification.

### Materials and Methods

#### Bacterial Strains, Plasmids, and Reagents

The bacterial strains used in this study were *Escherichia coli*-DH5α and -BL21 (DE3). Two plasmids, pUC57 (Metabion, Germany) and pET32a (+) (National Institute of Pasteur, Iran) were used. Bacterial strains were grown on Luria–Bertani (LB) broth or in LB agar. Growth efficiency of the bacteria was determined by spectrophotometry and/or plating method followed by a serial dilution procedure. Restriction enzymes HindIII, NcoI and T4 DNA ligase for cloning were purchased from Invitrogen (Invitrogen, USA). Factor Xa protease was purchased from Thermo Scientific (Thermo Scientific, USA). Oligonucleotides were synthesized by Metabion Company. For plasmid DNA extraction and purification of gel fragments and PCR products, Favorgen Kit (Favorgen, Taiwan) was used.

### Construction of the Fusion Expression Plasmid pET32a(+)–Xa–PI–LS10

Sequences of LS10 gene (Baindara et al. 2017b), PI Oligonucleotide (Gao et al. 2015), and factor Xa Protease site, were obtained by the literature and database nucleotide sequence. The rare codons in *E. coli* were compared with LS10, PI and factor Xa Protease site sequence were designed by web based software such as Gene script (http://www.genescript.com), *E. coli* codon usage analyzer 2.1 (http://www.faculty.ucr.edu), and OPTIMIZER (Puigbo et al. 2007) (a web server utility that optimizes a DNA or Protein sequence) as well as, the iACP (Chen et al. 2016) server was used to study the anti-cancer capabilities of the PI–LS10 peptide.

According to codon usage in *E. coli* the site of the factor Xa Protease protein fused to a PI–LS10 gene (Xa–PI–LS10) was synthesized by the Generay Biotech Company (Shanghai, China) and was inserted into a pUC57 vector. The synthesized fusion gene fragment in the vector was transformed into *E. coli* DH5α competent cells (Russell and Sambrook 2001). After PUC57 (Xa–PI–LS10) extraction, the synthesized fusion gene fragment was extracted from the PUC57 using double digestions by NcoI and HindIII. In addition, the restriction enzymes NcoI and HindIII were used to digest the pET32a (+) plasmid, then the digested fragment and vector was determined on the agarose gel: it was recovered from the gel using a Favorgen Agarose Gel DNA Extraction Kit. Afterward, the purified fragment was ligated to the digested pET32a (+) at 16 °C overnight and then the ligation product was transformed into *E. coli* BL21 (DE3) competent cells. Colony-PCR confirmed the gene cloning in pET32a (+) (T7-pro and T7-ter primers) and DNA sequencing verified the result of the positive clones (Fig. 1).

### Expression of Fusion Protein and Dot Blot Analysis

A single colony of *E. coli* BL21 transformed with pET32a (+)–Xa–PI–LS10 was inoculated into 20 ml LB media containing 100 μg/ml ampicillin, and was cultured at 37 °C overnight inside an incubator shaker with rotation speed of 130 RPM. Next, the overnight culture was inoculated with a ratio of 1 to 10 into fresh 200 ml LB medium with 100 μg/ml ampicillin inside the incubator shaker. When the optical density of the culture at 600 nm (OD600) reached 0.5 to 0.6, to induce the protein expression, a final concentration of 1 mM isopropylthio-β-d-galactoside (IPTG) was added to the medium. Following the optimization of expression condition, bacterial cells were cultured at 22 °C under vigorous shaking and IPTG induction for 10 h as incubation time. Cells were harvested by centrifugation at 4000 rpm for
20 min at 4 °C and were suspended in 5 ml buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cell suspension was lysed using the sonication (Ultrasonic Cell Crusher) then recombinant protein was analyzed by 12% SDS-PAGE. The quantities of fusion product were estimated by protein content in the liquid supernatant (Bradford protein assay) in 595 nm wavelength and through using standard protein BSA (bovine serum albumin). For Dot blotting with anti-His antibody, the protein sample was spotted to a polyvinyl difluoride (PVDF) membrane (BioRad, USA) and left to dry at room temperature. The membrane was incubated with a dilution of mouse anti-(6 × His) peroxidase antibody (1:2000, Sigma, USA). Tetramethyl benzidine (TMB) (CytoMatin Gene, Iran) substrate was added and the protein was recognized by the appearance of the blue band on PVDF membrane.

**Purification and Proteolytic Cleavage of Fusion Protein**

The purification procedure was performed using Ni2+ affinity chromatography method (Nickel NTA Agarose Resin ABT, Spain). The fusion protein sample was delivered through a column and comprehensively washed with binding buffer (100 mM Tris base, 500 mM NaCl, and 20 mM Imidazole, pH 8), and then, the fusion protein eluted with elution buffer (500 mM NaCl, 20 mM Tris, and 250 mM imidazole, pH 8.0). The eluted fractions were collected to further analysis by SDS-PAGE. The purified fusion proteins were dialyzed overnight at 4 °C using a 14 kDa cutoff dialysis membrane. Afterward, dialyzed peptides in PBS buffer were concentrated by Amicon Ultra-15 10K centrifugal filter (Merck Millipore, Ireland). The protein concentration was determined through the Bradford test. The Xa protease enzyme was added to the produced recombinant protein proportion to the protein concentration. Concentrated peptides were dissolved in equal proportions of 2×TBS buffer followed by addition of factor Xa 1:100 ratio and then incubated at 25 °C for 5 h according to the manufacturer’s instructions (Thermo Scientific, USA). PI–LS10 peptide was again purified with a Amicon Ultra-15 10K centrifugal filter at 4000 RPM centrifugation, for 20 min at 4 °C. The PI–LS10 peptide was detected on SDS-PAGE 15%.

**Cell Culture and Cytotoxicity Assay**

The human dermal fibroblasts (HDF) as normal cell line and human breast cancer cell lines, MCF-7 and MDA-MB 231 were obtained from the Cell Bank of Institute Pasteur of Iran. The cells were grown in RPMI 1640 (Gibco, USA) media supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin–streptomycin (Sigma, USA) and incubated at 37 °C with 5% CO2 and 95% humidity. Cell cytotoxicity was evaluated using MTT assay. Briefly, MCF-7 and MDA-MB-231 cells (1 × 10⁴ cells/well) were seeded in 96 well plates. After 24 h of incubation, the cells were treated by increasing concentrations of PI–LS10 (1–10 μg). While the medium without peptide was used as a negative control. The plates were incubated for 24, 48, and 72 h and each well was added with 20 μl of MTT solution (5 mg/ml in PBS) then incubated for 4 h at 37 °C. Afterward, the medium was removed, and 100 μl of Dimethyl sulfoxide (DMSO; Merck, Germany) was added to each well to dissolve the formazan crystals. An ELISA plate reader measured the absorbance at 490 nm wavelength and cell viability was estimated.

**Statistical Analysis**

Descriptive data were expressed as mean ± SEM. The significance of difference was evaluated with ANOVA and post hoc Bonferroni. Data were analyzed by Graph Pad Prism software (version 7.01). Significant difference was set at P < 0.05.

**Results**

**Bioinformatics Analysis**

Analysis of solubility prediction of the PI–LS10 fusion protein and cleaved LS10 and PI–LS10 showed solubility
rates of 45% and 71%, 66% respectively as compared to the average solubility of *E. coli* soluble proteins (40%). The average solubility prediction of soluble proteins (*E. coli*) was obtained from experimental data (Niwa et al. 2009). Protein-sol patches online software was used to estimate the hydrophobic parts on the protein surface based on the PDB model (Fig. 2). The results suggested the ratio of polar and non-polar regions on the LS10 and the fusion protein. The hydrophobic areas are identified in green as depicted in Fig. 2.

**Sub-cloning of Recombinant Vector**

Recombinant plasmid of pET32a (+)–Xa–PI–LS10 was constructed through cloning of the synthetic fragment. The results of colony PCR showed that there was successful insertion of the target fragments into the vector (Fig. 3). Additionally, the DNA sequencing confirmed that the recombinant plasmid was successfully constructed. In addition, DNA sequencing confirmed that no changes had occurred in the sequence of the targeted gene (data not shown).

**Expression of the Recombinant Protein**

The web based software (http://web.expasy.org/compute.pi) predicted molecular weight of Trx–His–Xa–PI–LS10 as approximately 25.3 kDa. The expected molecular mass of recombinant protein (25.3 kDa) is shown in Fig. 4A, and indicates successful expression of the fusion protein. ImageJ software was used for estimating the density of the bands on the gel. According to ImageJ and SDS-PAGE the high expression of Trx–His–Xa–PI–LS10 protein occurred at 6 and 8 h post-induction. The fusion protein consisted of approximately 30% total cellular proteins. The Dot-blot test with anti His antibody also confirmed the existence of recombinant protein in comparison with the positive and negative controls (Fig. 4B).

**Purification and Proteolytic Cleavage of the Fusion Protein**

Purification of recombinant PI–LS10 was performed through ABT Ni2+–NTA affinity chromatography using the His-tag in the recombinant protein. The fusion protein Xa–PI–LS10 was eluted efficiently from the column and verified at 12% SDS-PAGE (Fig. 5A). The final yield of Xa–PI–LS10 was 373.62 μg of recombinant protein, indicating that the fusion protein was expressed at a high level. The measured purity of the protein was higher than 95%. The Xa–PI–LS10 protein...
was cleaved efficiently using the Xa protease at 25 °C for 5 h, as confirmed by a 15% SDS–PAGE. After the cleavage, the digested peptide, PI–LS10 was again purified with a Amicon Ultra-15 10K centrifugal filter. A band at 7.4 kDa was detected using SDS-PAGE 15% which corresponds to the recombinant PI–LS10 that was predicted by the web based software (http://web.expasy.org/compute.pi) (Fig. 5B).

**Cell Viability Assay**

The cytotoxic activity of recombinant LS10 and PI–LS10 was investigated against HDF as normal a cell line and two human breast cancer cell lines of MCF7 and MDA-MB-231 as non-triple negative and triple negative cancer cells. Cytotoxicity was estimated through treating the cells with serial dilutions of LS10 and PI–LS10 followed by a MTT assay after 24, 48, and 72 h. Dose-dependent cytotoxicity effect curves for the breast cancer cell lines are shown in Fig. 6A–D. For each experiment, cell viability was evaluated in at least three independent experiments for every cell culture. We measured the half maximal inhibitory concentration (IC50) as the peptide concentration at which cell viability is reduced by 50% in comparison to untreated cells (Table 1). The most significant cytotoxicity was observed for the MDA-MB-231 in comparison to MCF7 cell line at the time of 72 h (IC50=3.1 μg) (Fig. 6E). Also, cell cytotoxicity of LS10 compare with PI–LS10 estimated in MDA-MB-231 cell line (Fig. 6F). The normal cell (HDF) was much less sensitive to PI–LS10, and showed 80–90% viability in all concentration (Fig. 6G).

**Discussion**

LS10 is a class-IIId bacteriocin pseudo-defensin peptide with anti-microbial and cancer repression properties. Defensins are a pivotal group of host-defense peptides expressed mainly in neutrophils and epithelial cells (Xu and Lu 2020). Numerous bacterial peptides with anti-microbial properties have been observed that act similar to defensins. Defensin-like peptides (i.e., AMPs) have shown a combination of anti-microbial and anticancer properties. However, there has been little success in development of the AMPs for therapeutic applications. On the other hand, development of a new group of anti-cancer drugs with low cytotoxicity to normal cells would be a major challenge as well. Therefore, AMPs are favorable potential candidates for cancer treatment (Bruggeman et al. 2019). Nevertheless, isolation of AMPs and their chemical synthesis could be complicated and expensive; therefore, it seems that production by recombination technology can be a promising pathway for high
Fig. 6 The concentration-dependent cytotoxicity curves of LS10 and PI–LS10 toward cancer cell lines. A, B MCF7, and C, D MDA-MB-231 assessed by the MTT test. E Comparison cell cytotoxicity of PI–LS-10 between MCF7 and MDA-MB-231 cells. F Comparison cell cytotoxicity LS10 vs PI–LS10. G Cytotoxicity curves of PI–LS10 on normal cell HDF.
yield production, with low cytotoxicity to healthy cells (Kuzmin et al. 2018).

In the present study, we speculated that PI–LS10, as a member of the defensin-like family, may exert its anti-cancer activity by targeting highly metastatic cells. Characteristics of LS10 have been previously examined by Baindara et al. (2016, 2017a). Nonetheless, production of LS-10 as a recombinant product was not accomplished until now. Therefore recombinant expression of this peptide could pave the way toward industrialized production. To produce and evaluate the anti-cancer activity of PI–LS10, its sequence was cloned into a prokaryotic expression vector pET 32a (+) and was expressed as His-tagged fusion protein within E.coli BL21 in optimized conditions. It has been revealed that adding a fusion protein to the N-terminal segment of group IIa of bacteriocins induces a sufficient degree of flexibility (Kazazic et al. 2002). Therefore, we designed the Trx–Xa–PI in construct at the N-terminal site of the LS10 encoding gene. Various temperatures and inducer proportions were examined in order to optimize the parameters of PI–LS10 production. We recovered the pure fusion protein Trx–Xa–PI–LS10 using the standard affinity purification method. After the recovery of purified Trx–Xa–PI–LS10, we completed the digestion with the Xa factor and harvested the PI–LS10. Then, anti-cancer activity of the purified PI–LS10 evaluated on two human breast cancer cell lines and one non-cancerous cell line. The IC50 value of PI–LS10 showed its activity only in cancer cell lines rather than normal cell lines, highlighting the selective toxic effect of PI–LS10. The corresponding IC50 values associated with recombinant PI–LS10 were 5.3 and 3.1 µg for MCF7 and MDA-MB-231 cell lines, respectively. A previous study has reported the IC50 value of approximately 5 µM (up 10 µg) for MCF7 compared to the wild type sample. According to Baneyx and Mujacic, the decrease in potency of the laterosporulin might be attributed to the oligomerization in the high concentrations (Baneyx and Mujacic 2004).

The cancer cells surface is different from the healthy cells by several aspects (Rasmussen and Ditzel 2009). One of the main differences between normal and cancerous cells is the charge of the cell surface; the surface has a negative charge in cancer cells compared to the normal cells which is caused by the high expression levels of negatively charged lipid molecules (Utsugi et al. 1991; Walter et al. 2013). Therefore, most of the AMPs with cationic property display extensive of anti-cancer activities due to the highly positive charge (Mader and Hoskin 2006; Riedl et al. 2011). In addition, AMPs possess a range of mechanisms such as disintegration of mitochondrial membranes that could be used against cancer cells (Smolarczyk et al. 2010). Wild sample LS10 and our recombinant LS10 in the present study also have been shown to induce cell cytotoxicity and apoptosis in cancer cells.

In summary, we have designed and synthesized a fusion system using the Xa factor site, which lead to a considerable advantage for the production of recombinant LS10; so a large mass and potent bacteriocin is created. Furthermore, the anti-cancer activity of PI–LS10 is significantly increased in recombinant peptide. The presence of PI sequence lead to the triple-negative breast cancer cells to be targeted. However, due to the anti-cancer properties of LS10, an effect on the MCF7 cells was also observed. But, an anti-cancer activity was significantly increased on the MDA-MB-231 cells within 72 h. The recombinant PI–LS10 produced in the present study might be considered as a potential anti-cancer agent. These results suggest that the protease specifically cleaved the target peptide from the fusion protein while preserving the native N-terminus; and describe a comparative in vitro study on a potential anti-cancer peptide.

**Table 1**  IC50 values for breast cancer cell lines

| Cell line | IC50 (µg/ml) | IC50 (µg/ml) | IC50 (µg/ml) | IC50 (µg/ml) | IC50 (µg/ml) | IC50 (µg/ml) |
|-----------|-------------|-------------|-------------|-------------|-------------|-------------|
|           | 24 h        | 48 h        | 72 h        | 24 h        | 48 h        | 72 h        |
| LS10      |             |             |             |             |             |             |
| MCF7      | 8.69 ± 0.59 | 6.9 ± 0.95  | 5.8 ± 0.83  | 6.55 ± 1.45 | 6.008 ± 1.35 | 5.34 ± 0.87 |
| MDA-MB-231| 7.28 ± 0.15 | 5.99 ± 0.98 | 5.01 ± 0.4  | 6.25 ± 0.98 | 5.26 ± 1.012 | 3.12 ± 0.54 |

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**Author Contributions** BR: performed in vitro experimental prepared initial draft and data collection. SR: coordinated the study, supervised the data collection and proof read manuscript, perform data analysis and article editing. BS and SF: prepared information on complementary literature and performed data analysis. FT: performs article editing and share in prepared initial draft.

**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.
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