Original Article

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Design, Cloning, and Expression of PLC-Darpin Fusion Protein as Putative Immunotoxin in a Prokaryotic Host

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ABSTRACT

Background & Objective: Cancers are common genetic disease that can cause death. Bacteria, such as *Clostridium novyi*, potentially could be used in cancer treatment by producing an enzyme called phospholipase C (PLC), which causes cell lysis. The aims of this study are to cloning and expression of PLC- Darpin in *prokaryotic* host.

Materials & Methods: Briefly, the PLC- Darpin gene sequence was amplified using PCR. The amplified fragment was conducted into the *pET28a* vector, transformed into *E. coli* BL21 (DE3), and screened by the double digestion method. Protein expression was analyzed using SDS-PAGE and checked with specific antibodies using the western blotting method. The cloned fragment was confirmed using the PCR colony method.

Results: Designed PLC- Darpin protein sequence (1600 bp) was successfully amplified by specific primers taking advantage of PCR method. Then, cloned PLC-Darpin candidate sequence was reconfirmed by digestion procedure, and colony PCR. Finally, 60 KDa expressed protein in prokaryotic host reconfirmed by SDS- page and western blotting.

Conclusion: Fused PLC enzyme to Darpin protein could be considered as a main putative immunotoxin due to its enzymatic role and cytotoxicity which may be utilized as a therapeutic choice in the future with further investigations.

Keywords: Immunotoxin, Gene cloning, Darpin, Fusion protein, PLC-Darpin

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Introduction

Cancer is one of the genetic diseases that starts with mutations created in oncogenic drivers, followed by the stimulation of tumor cell growth, and by genetic or non-genetic activation or inactivation of genes that promote tumor proliferation or suppress, it is created (1). Notwithstanding the significant progress made over the past several decades in terms of reducing mortality rates through enhanced early detection techniques and remarkable advancements in therapeutic interventions, breast cancer (BC) continues to occupy the unfortunate position of being the second most prevalent cause of cancer-related fatalities among women in general, while simultaneously holding the distressing distinction of being the primary cause of cancer mortality specifically among Black and Hispanic women populations (2). On an annual basis, the global incidence of newly diagnosed breast cancer cases amounts to approximately 2.3 million, which translates to a staggering statistic wherein 1 out of every 8 cancer diagnoses across the globe is attributable to this particular malignancy, thereby underscoring its substantial impact on public health (3).BC represents the secondary most prevalent neoplasm and stands as the primary determinant of cancer-related mortality among females, encompassing 685,000 fatalities across the globe in the year 2020. Encyclopedically, BC causes about 25% of all cancer cases and 17% of all cancer deaths in women (4). BC varies because the cancer cells have differences in their genetic, epigenetic, gene expression, and protein characteristics. These things can change how tumors grow, die, spread, and respond to treatment (5).

Concerning biomarker heterogeneity, the utilization of the immunohistochemical technique within the confines of the laboratory, ascertained by the esteemed American Society of Clinical Oncology, has unequivocally affirmed that the presence of human epidermal growth factor receptor 2 (HER2), progesterone (PR), and estrogen (ER) is ubiquitously observed among individuals afflicted with the formidable and pervasive variants of BC that exhibit both aggressive and metastatic characteristics (6, 7). HER2 protein is found in high levels in stomach, breast, and ovarian cancers. When there is too much of the HER2 protein, it makes tumors grow faster and increases the chances of the tumor coming back and the person dying (8, 9). The HER2 receptor is found in large amounts on solid tumor cells and is easy to reach. This makes HER2 a good option for a type of treatment called targeted immunotherapy, as well as for using imaging techniques to study it in more detail (10). Darpins are a type of small proteins designed to be very stable and can recognize specific targets just as well or even better than proteins based on immunoglobulins (11, 12). These proteins can detect and bind to a specific part of the HER2 protein found outside cells (13-16). *Clostridium* are obligate anaerobic gram-positive bacteria that include several human pathogens. The pathogenicity of these bacteria depends on the toxin (17). According to the cytolytic effect of the toxin of *Clostridium novyi (C. novyi)* bacteria, this toxin can be considered a compound for cancer treatment. The phospholipase toxin of *C. novyi* plays a role in the hydrolysis of eukaryotic cell membranes (18). Phospholipase C (PLC) is a type of phospholipase that is distinguished from other phospholipases, such as PLA (Phospholipase A) and PLD (Phospholipase D) (which are present in bacteria), by the site that cleaves phospholipids. This enzyme is bound to the membrane (19). Because of its cytotoxic characteristics and the significance of this specific enzyme in the development of the ailment, it has become the center of extensive scrutiny within the pharmaceutical industry. Consequently, it can potentially constitute a fundamental constituent of immunotoxins or vaccines (19, 20).

The present investigation aims to amplify the designed PLC-Darpin gene within the *pET28a* vector and manifest the protein in *E. coli* BL21 as a bacterial expression host. The results of the current study will subsequently be used to design an immunotoxin based on PLC, which can be used as a therapeutic candidate in the future with further investigations used for BC.

Materials and Methods

Bioinformatic studies

For our bioinformatic study, we obtained the PLC protein sequence for *C. novyi* (ABK60388.1) and Darpin from the NCBI (gene bank) database, which we saved in FASTA format (https://www.ncbi.nlm.nih.gov/genbank/fastaformat/). We then connected two pieces of Darpin and PLC using a linker consisting of 8 amino acids (GGSGSGGG) to create a fusion protein called PLC-Darpin. This immunotoxin was evaluated using various methods that we explain below. To investigate the protein's properties, we uploaded the amino acid sequences to the (https://www.imed.med.ucm.es) database and used the ProtParam and ProtScale tools in the (https://www.expasy.org) database to assess their hydrophilicity. We also used the Kyte & Doolittle method to upload possible hydrophilicity properties and obtain protein properties from the (https://web.expasy.org/protparam) server. To predict the second and third structures of the protein, we used the GOR IV server (https://npsa-prabi.ibcp.fr) the NCBI site BLAST tab, and the Swiss Model (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6030848/). After designing the sequence, we sent it for synthesis. We then used the Primer3Plus website (https://www.bioinformatics.nl/cgi bin/primer3plus/primer3plus.cgi) to create primers and increase the number of desired gene sequences using the PCR (polymerase chain reaction) method. The primers were designed for cloning tests, and we checked their characteristics using the oligo7 software.

Cloning process of the PLC-Darpin gene

To prepare Luria-Bertani (LB) agar culture medium containing kanamycin (100 µg/ml), "MERK" agar powder was used. The pH of the environment should be 7. Once the medium was prepared, kanamycin (Merck, Germany) was added in a concentration of 100 mg/ml in distilled water. The solution was then cooled, and NaOH was added to bring the pH to 7. The medium was then autoclaved to ensure sterility. To purify and evaluate the specificity of the recombinant protein, the reverse primer was added at the end after the cutting site. The sequences of the PLC, Darpin, and fusion primers can be found in Table 1. Enzymes such as *HindIII* and *XhoI* for PLC, *NdeI* and *EcoRI* for Darpin, and *NdeI* and *XhoI* for fusion were used to cut the gene fragments. By increasing the sequence number of the sequence responsible for the synthesis of PLC-Darpin, PCR was used to create the desired sequence. The product was then observed on a 1% agarose gel to confirm the presence of the desired fragment based on size.

Table 1. Sequence of PLC, Darpin, and Fusion primers (F = forward, R = reverse)

Primer sequence	Primer name
AAACATATGATGAGAGGATCGCATCACCAT	Fusion-F
AAA CTCGAG ATATAGTATTTTTCATTGTTT	Fusion-R
AAACATATGATGAGAGGATCGCATCACCAT	Darpin-F
AAAGAATTCGTCCTGAGCGTTAACGTC	Darpin-R
AAACATATGATGAGAGGATCGCATCACCAT	Fusion-F

AAA CTCGAG ATATAGTATTTTTCATTGTTT	Fusion-R

Amplification of the PLC-Darpin gene

We carried out a PCR reaction to amplify the PLC-Darpin gene fragment. We used Taq DNA polymerase enzyme and other required materials, including 9.5 µl of DDW, 1 µl of P-F, 1 µl of P-R, 1 µl of DNA, and 12.5 µl of Master Mix (Biolab, New England, UK). The reaction was conducted under the following conditions: we started with an initial cycle of denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s. We ended the reaction with a final extension at 72°C for 10 min, using a thermocycler. After that, we incubated the mixture for 10 min at 4°C (Applied Biosystem, USA). Finally, we visualized the resulting PCR product with a 1% agarose gel in 0.5X TBE (Tris-borate-EDTA) buffer at a current of 100 volts for 40 min. We used a PCR product washing kit to wash the PCR product and to evaluate the test, we electrophoresed 2 µl of the washed product on a 1% agarose gel.

Connection of the PLC-Darpin inside the expression vector

After increasing the number of desired sequence fragments, which included a nucleic acid fragment encoding PLC with a weight of 1194 bases and Darpin, a fusion protein consisting of 332 bases, their confirmation was done using gel electrophoresis to determine their size. To prepare the product, cutting sites were designed at the 5' end of the forward and reverse primers *NdeI* and *XhoI* for phospholipase C-Darpin (PLC-Darpin), and the resulting product was separated and purified on a 1% gel. Subsequently, the product was further purified and concentrated to reach a desired product concentration of approximately 95 ng/µl using a concentrator device.

Preparing the vector to perform the connection process

The BL21 strain containing *pET28a* plasmid was used to increase the amount of the desired plasmid. First, the bacteria were inoculated in an LB medium with kanamycin and then incubated at 37°C in a shaker incubator for 24 h at 70-100 rpm. Next, 20 ml of the medium containing bacteria was centrifuged at 8000 rpm for 2 min to extract the plasmid from the culture medium. Finally, following the instructions of the manufacturer of the plasmid extraction kit (AccuPrep Nano Plus), the desired plasmid was purified and concentrated. The concentration was measured using a nanodrop device, which showed a concentration of 90 ng/µl.

Enzymatic digestion and ligation reaction

The PCR product and plasmid genes were digested together in a 30 µL volume containing 15 µL PCR product, 6 µL distilled water, 1.5 µL *Xho1*, 1.5 µL *NdeI*, and 6 µL fasting digest buffer. The mixture was incubated at 37°C for overnight or 2 h. To verify the cleavage of the plasmid, the mixture was loaded onto a 1% electrophoresis gel and observed using a gel dock device. The desired product was extracted from the gel to remove any excess plasmid pieces. For the ligation stage, the Thermo Scientific Rapid Ligation Kit was used following the manufacturer's instructions. A mixture was prepared containing 2 µl of the digested PCR product, 4 µl of the prepared plasmid, 2 µl of the ligation buffer from the kit, and 2 µl of T4DNA ligase enzyme. This mixture was heated to a final volume of 12 µl with distilled water and incubated for 1 h at a temperature of 22°C. The desired fragment was then successfully integrated into the vector.

Competent cell preparation and plasmid transformation

A single colony of the *E. coli* BL21 strain was cultured in a tube containing 5 mL of LB broth medium. It was then placed in a shaker incubator at a temperature of 37°C/overnight. For the next step, 1500 µL of the *E. coli* BL21 strain was cultured in a tube containing 50 mL of LB broth medium and placed in a shaker incubator at 37°C for 3 h. When the OD of the cells reached 0.4 to 0.6 at 600 nm, which is the beginning of the logarithmic phase, 50 mL was put into a Falcon 50 mL and incubated in ice for 45 min. Following this, the supernatant solution was taken out and the cell sediment was dissolved in 1 mL of "1 M" calcium chloride solution. The volume was then increased to 50 mL with calcium chloride before being placed on ice for 30 min. afterward, it was centrifuged in a refrigerated centrifuge for 15 min at 5000 rpm and a temperature of 4°C. Next, the supernatant solution was removed and 1 mL of 1 M CaCl2 solution was added. After dissolving, it was separated into 100-200 µL vials. These cells can be kept at 4°C for 4 to 48 h. In this particular case, the *E. coli* cell strain BL21 was susceptible, so the wall was partially destroyed and Cl^- and Ca^+ ions were placed between different parts of the membrane. This step prepares the bacteria for the entry of the plasmid construct. To prepare the medium for maintaining the transformed strain (105 colonies/ml), start by preparing 50 µl of the medium containing bacteria, which should also contain the host strain for the transformation process. Once prepared, add 2 µl of the conjugation mixture to the transformation strain while keeping the conjugation mixture on ice. Slowly mix the desired product and then subject it to a heat shock of 42° for 90 s, without shaking. It is important to note that *E. coli* strain BL21 (DE3) should be used as a prokaryotic host by the instructions (21). After inoculating the resulting mixture into 250 µl of prepared protection medium, leave it to incubate at 25°C overnight. The following day, incubate the inoculated medium at 37°C for 1 h in a shaker incubator with 200 rpm/min, placed horizontally.

Selection of colonies with expression vector

To check for the presence of a fragment inside a transformed vector, 10 µl of protective medium containing the transformed strains were inoculated into a plate containing LB agar kanamycin medium. The PCR colony method was used for this purpose. A small

amount of the colony was mixed with 9.5 µl of DDW, 1 µl of P-F, 1 µl of P-R, and 12.5 µl of Master Mix to prepare the PCR reaction mixture. The T7 strong promoter primers were used for colony PCR. The T7 forward and reverse promoter primer sequences can be found at (https://www.addgene.org/mol-bio-reference/sequencing-primers/). The PCR reaction was performed using a thermocycler under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 20 s, 54°C for 20 s, and 72°C for 60 s. Finally, the reaction was subjected to a final extension at 72°C for 5 min, followed by incubation at 4°C for 5 min (Bio-Rad, USA).

Confirming the presence of the PLC-Darpin gene

After growing colonies in an antibiotic-containing medium, they were transferred to an LB broth medium with 100 µg/ml kanamycin and cultivated at 37°C for 12 h. The commercially available plasmid purification kit (GenNet Bio South Korea) was used for plasmid extraction following the provided recipe. The concentration of the extracted plasmid was measured to be 75 ng/µl using a nanodrop device.

Recombinant plasmid extraction

The contents of the microtube containing BL21 with the plasmid were made uniformly by vortexing with 250 µl of PR buffer for 10 s. Next, 250 µl of PL buffer was added and the microtube was inverted 4 to 5 times. Then, 350 µl of PN buffer was added and inverted 4-6 times. After centrifugation at 8000 rpm for 10 min, the supernatant was removed and poured into the Spin Column. The pellet remained in the microtube. The Spin Column was placed inside the microtube and after closing the lid, it was centrifuged at 8000 rpm for 1 min so that all the contents of the supernatant entered the microtube. 500 µl of PO buffer was poured on the Spin Column and centrifuged again for 30 s. The purified plasmid was collected in the lower microtube. 500 µl was added to the previous volume in the lower microtube, then 700 µl of PW buffer was added to the Spin Column and centrifuged again for 30 s. The centrifugation was continued for 1 min to ensure no material remained. The purified plasmid was then used for additional tests to confirm the presence of the target fragment in the vector.

Evaluation of the presence of the PLC-Darpin

The extracted plasmid was evaluated for the presence of the fragment responsible for synthesizing the desired protein by identifying the cutting sites of specific enzymes. For PLC Darpin, the presence of *XhoI* and *NdeI* cutting sites was checked; for PLC, *HindIII,* and *XhoI*; and Darpin, *EcoRI,* and *NdeI* using a fast-cutting enzyme buffer as per the manufacturer's instructions. The enzyme digestion process was carried out in the shortest possible time by preparing the enzymes separately and in pairs using the fast buffer.

Recombinant protein expression

After transferring the expression vector into the bacterial host, the bacterial fragment was grown in an LB agar medium containing kanamycin and confirmed for qualification. The bacteria were then inoculated into 5 ml of LB broth medium containing 100 µg/ml kanamycin and incubated in an incubator at 37°C for one night to bring them to the logarithmic growth phase. After the bacteria grew in the medium, a ratio of 1 to 10 bacteria was inoculated into a 1-liter Erlenmeyer flask containing 100 ml of LB broth with a concentration of 100 µg/ml kanamycin. The medium was then heated for 1, 3, and 5 days with a final concentration of 1 mM of β-thioisopropyl β-thiodigalactoironoside. After protein synthesis was induced, the Erlenmeyer flask was quickly cooled in ice. Finally, cells were separated using a centrifuge at 8000 rpm for 10 min at 4°C. The culture medium was then evaluated for the presence of protein in the prokaryotic host. It is worth noting that inoculation was used first to evaluate expression and standardization in a low volume of the growth medium (5 ml), and then standardization of protein expression will be done in a high volume in future studies.

Evaluation of the presence of synthesized protein sites in bacteria

The evaluation of protein expression in the host bacterium was conducted by using a small volume of bacteria before and after induction. The protein expression was evaluated by measuring light absorption following centrifugation and the addition of lysing buffer. After evaluating protein expression in the host bacterial sample, the presence and accumulation of the protein expressed in the transformed strain were investigated. To begin the analysis, the bacterial suspension was prepared in 30 mM PBS (phosphate-buffered saline). Next, the sonicated bacterial suspension was analyzed for the presence of protein in the supernatant and in the bacterial body. This was done by adding sample lysis buffer and performing SDS-PAGE (Sodium dodecyl-sulfate polyacrylamide gel electrophoresis) with 10% acrylamide. The protein accumulation was then evaluated. All evaluation steps were carried out according to the protein purification protocol (GE Health Care).

Evaluation of the presence of recombinant protein (PLC-Darpin) by SDS-PAGE

To ensure the purity of the protein extract and determine its molecular weight, we utilized the electrophoresis method on a polyacrylamide gel with the presence of sodium dodecyl sulfate (SDS). We prepared the buffers required for the polyacrylamide gel method along with the SDS. Then, we took 16 µl of the desired sample and mixed it with 4 µl of the loading buffer before placing it in the wells. We separated the proteins according to molecular weight for 1 h at 125 V and 25 Amp.

Western blotting

To observe a protein band in a polyacrylamide gel, the unknown sample (30 µl) was prepared at a 10% concentration and poured into a well. An electric current was established and the gel was left for 45 min

with a voltage of 150 and amperage of 30. The gel was then cut and the paper was activated in pure methanol for 2 min. The paper and gel were placed in a cold transfer buffer for equilibration for 5 min. After soaking the sponges in the device to catch bubbles and preparing the sandwich, the transfer cold buffer was filled up to the top of the sandwich and ice was poured around the tank. An electric current of 150 Ampraz was used for 2 h, after which the protein marker band was observed. The paper was then incubated in a blocking suspension, which was prepared in a mild shaker for 1 h at room temperature and then for 18 h at 4°C. After completing the blocking step, the paper was washed 3 times with TBST solution, each for 5 min with gentle shaking. The primary antibody solution, dissolved in blocking, was then incubated with shaking for 18 h at 4°C. The paper was washed 3 times with washing solution, each for 5 min, and then heated in a secondary antibody solution for 3 h at room temperature using a shaker. To observe the protein band bound to the specific antibody on the gel, a diaminopeptidase reagent was used at a concentration of 0.5. It is important to note that this step of the work was done in the dark and the presence of the band was checked 1 min after the dye was in contact with the paper.

Results

Results of bioinformatics studies

Design and making of protein-fusion:

The design and construction of protein fusion along with identification regions of restriction enzymes to cut and separate the sequence, some tags and communication regions required for cloning, expression of recombinant protein was done.

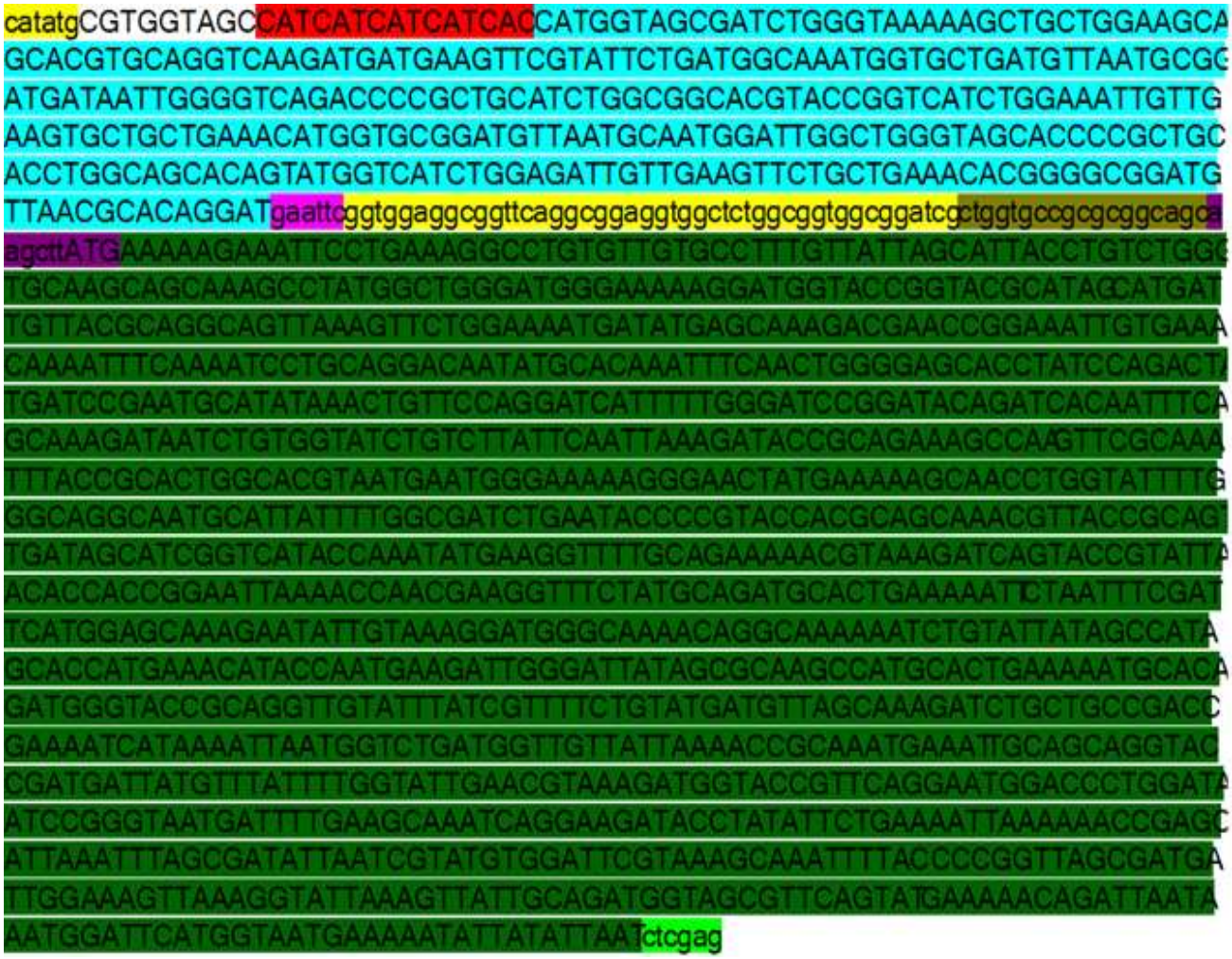


Figure 1. Design and making of protein-fusion.

Nde1 **Hexa-Histidin** **Darpin** ***EcoR1*** **Linker** **Thrombin** **aagctt: *HindIII*, ATG: codon methionine) If expressed separately)** **PLC** ***Xho1***

The size of the PLC protein is 50 kDa, Darpin is 10 kDa, and the fusion protein is 60 kDa.

Evaluation of sequences and their structural analysis

Examination of antigenicity:

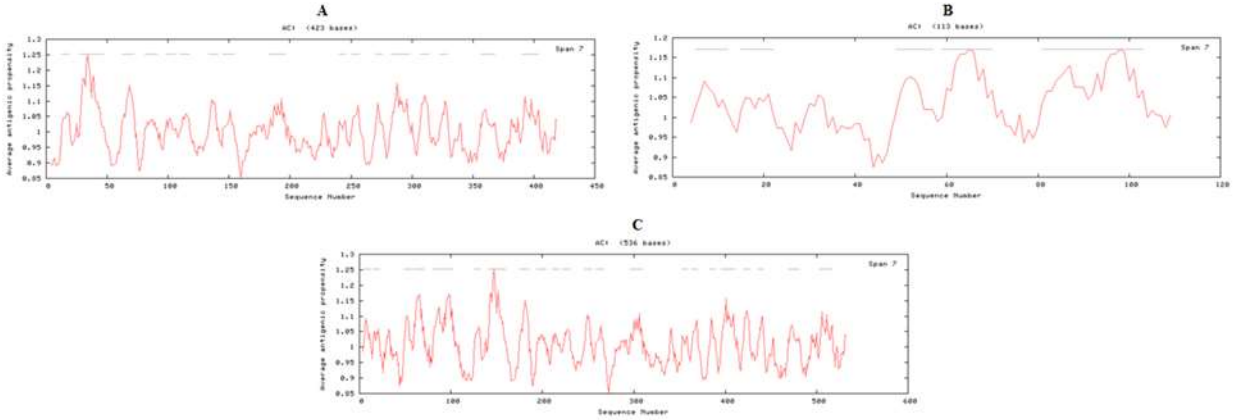


Figure 2. Examination of proteins antigenicity.The upper values of the graph are the areas with antigenic properties. A) Investigation of the antigenicity of PLC that score=1.0008 on the vertical axis has the property of antigenicity. B) Examination of the antigenicity of Darpin that score=1.0294 on the vertical axis has the property of antigenicity. C) Investigation of the antigenicity of PlC-Darpin that Score=1.0068 on the vertical axis has the property of antigenicity. According to the algorithm defined for the software, in the case of PLC, 17 regions, and in the case of Darpin, 5 regions, and the case of PLC-Darpin, 22 regions are of high antigenicity.

Investigation of hydropathicity:

The grand average of hydropathicity (GRAVY, which shows the degree of hydrophobicity of a peptide, was used in the investigation of protein hydrophobicity. Positive values of GRAVY indicate hydrophobicity and negative values mean hydrophilicity. Amino acids with a score<0 are acceptable because these regions have hydrophilic amino acids that are accessible to antibodies *(in vivo)*.

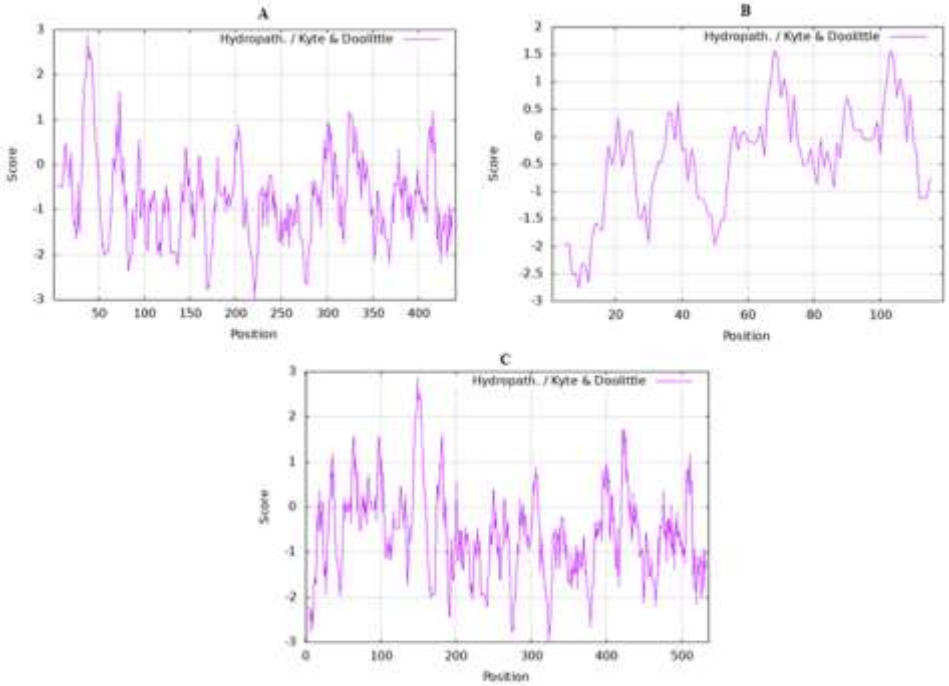


Figure 3. Investigating protein hydropathicity. A) PLC protein hydrophobicity check chart with GRAVY: -0.764. B) Hydrophobicity chart of Darpin protein with GRAVY: -0.472. C) PLC-Darpin hydrostaticity check chart with GRAVY: -0.639.

Investigation of pH:

The isoelectric point of a protein refers to the pH level at which the protein's net charge is zero. Below the isoelectric point, proteins have a positive charge, and above it, they have a negative charge. The protein's isoelectric point can range from very acidic values, around 4.0, to very alkaline values, around 12.0. pI for PLC, Darpin, and PLC-Darpin are 5.90, 5.37, and 6.35 respectively.

Prediction of two-dimensional structure:

This structure refers to the regular and repetitive spatial arrangement of adjacent amino acids in a polypeptide chain. This protein structure is created by hydrogen bonds between the hydrogens of the amide group and the oxygens of the carbonyl group, which form the body of the protein. Two large groups of secondary structures include α-helices and β-structures.

3D structure prediction:

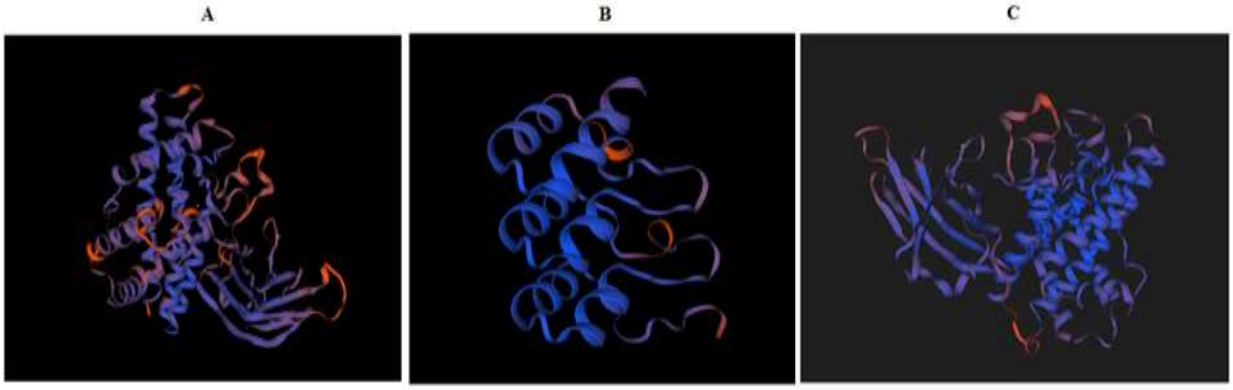


Figure 4. 3D structure of target proteins. A) 3D structure of PLC. B) 3D structure of Darpin. C) 3D structure of PLC-Darpin.

The results of laboratory research

After the synthesis of the PLC-Darpin sequence by the company, the PCR method using specific primers was used to increase the number of desired sequences for cloning, and the desired fragment was confirmed using size and sequencing methods**.**

Cloning results and confirmation of the desired fragment presence in the selected colony and protein expression

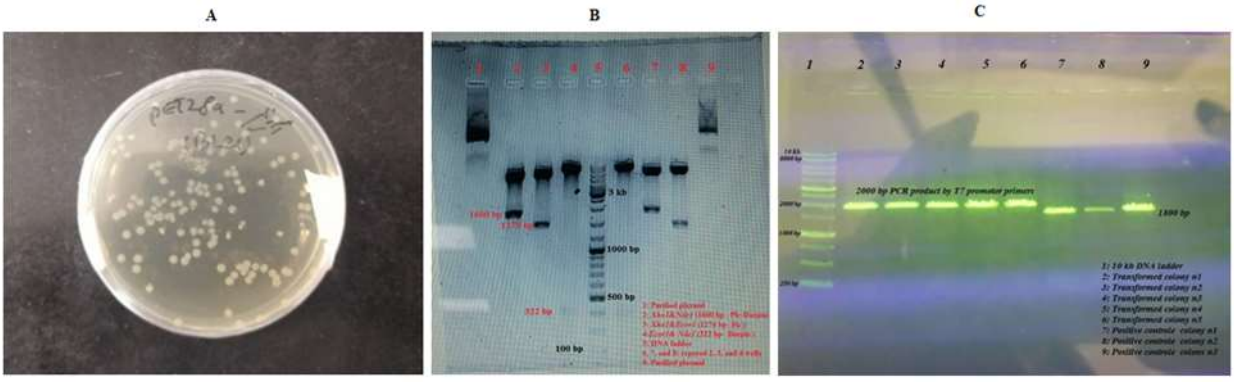


Figure 5. Cloning results and confirmation of the desired fragment presence in the selected colony. A) BL21 culture that contains an expression vector, which is being grown in a medium that contains kanamycin. B) The process of enzymatic digestion used to separate the sequence of PLC and Darpin. C) Result of a colony PCR. After using the PCR colony method, the sequence of 1194 bases and 1600 base pairs in the colony sample grown in the differential medium was confirmed and the sequence of the above sequence indicated the accuracy of cloning.

Protein extraction, purification, and measurement

After evaluating the presence of the protein in the host, it was determined that the desired protein was secreted in the cytoplasm of the host bacteria in the form of a solution, and to prevent the destruction of the second and third structures, purification with imidazole solution was performed after sonication and from the upper liquid sample. After reaching the desired optical density, the protein expressed in the prokaryotic host (in the medium containing kanamycin) was induced by IPTG with a concentration of 1 mM for 3 h, followed by sonication and purified by affinity chromatography. Further, after removing the impurities (salt, etc.), the dialysis method measured the protein concentration and stored and preserved it after the dry freezing method.

Confirmation of purified protein

To finally confirm the purified protein, western blot, and SDS-PAGE tests were performed. Its molecular weight was estimated using SDS-PAGE, and then the sample was evaluated using specific antibodies for the C-terminal hexahistidine present in the corresponding protein amino acid sequence, and the presence of hexahistidine was confirmed by comparing it with an internal standard. The results showed that the protein expression was successful.

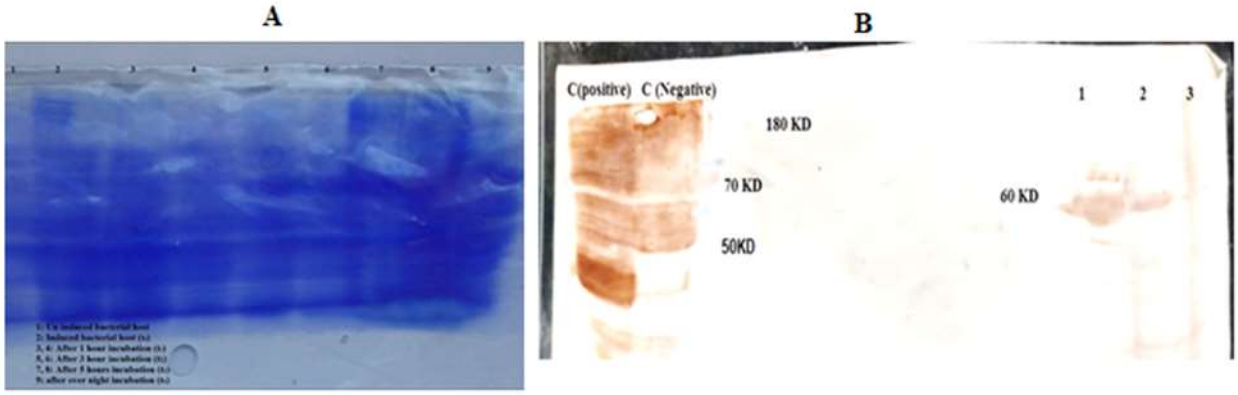


Figure 6. Confirmation of purified protein. A) SDS-PAGE results. Based on the image and wells 5 and 6, IPTG induced target protein expression after 3 h) the western blotting method was used to detect the presence of a histidine sequence in the protein. The protein in well 2 has a size of approximately 60 kDa.

Discussion

Due to drug resistance in chemotherapy and the destruction of natural body tissue in radiation therapy (22, 23), we can create a toxic agent that is specifically delivered to these areas and destroys tumor cells (24). Treatments targeting and destroying cancer cells must recognize the differences between normal body tissues and cancer cells (22). Using *C. novyi* bacteria is an effective approach to treat cancer cells. Acting on cancer cells, this bacterium can create a local and adaptive inflammatory response and cytotoxic effects against tumor cells. Considering the undeniable role of cloning and gene expression in the production of recombinant products (drugs, vaccines, and gene therapy) and the role of phospholipase toxin of *C. novyi* in the hydrolysis of eukaryotic cell membranes, with further studies, the expression of active site targeted toxin can be a suitable candidate for evaluating the effects be cytolytic in tumors (18).

Due to the important role and special features of HER2, it is possible to target it for targeted cancer treatment (25). So, a therapy targeting HER2 is effective and specifically treats certain cancers, like HER2-positive BC (26).

Darpin is a low molecular weight protein and is a new class of non-IgG protein, highly soluble in water, has high chemical and thermal stability, and potentially low production cost, which specifically binds to various tumor-associated antigens such as the HER2 receptor and its affinity is higher than antibody, so it is a good substitute for immunoglobulin proteins (12, 27, 28).

The investigation of PLC-Darpin fusion proteins as potential therapeutic modalities in oncological treatment uncovers a complex interaction between cellular viability and targeted protein expression. Eijkenboom *et al.* (2021) illustrated that DARPins can proficiently eliminate neoplastic cells from ovarian tissues without jeopardizing cellular integrity, successfully achieving the total eradication of EpCAM-positive BC cells while acknowledging a reduction in oocyte viability during in vitro proliferation assays attributable to the minimal expression of EpCAM on oocytes (29). This indicates that although targeted cytotoxicity can be attained

against malignant cells, there exists a concomitant risk of inadvertent consequences on adjacent non-malignant cells, particularly within sensitive tissue environments. There are 13 types of PLC in mammals. Types of PLCs are divided into six isotypes according to their structure. These isotypes include: β, γ, δ, ε, ζ, η. Each PLC possesses distinctive and intersecting regulatory authority regarding the manifestation and spatial allocation within the cell. The precise regulation and responsiveness of the PLC reaction to various extracellular and intracellular inputs with appropriate kinetics are necessary due to its extensive range of functions. This need has driven the evolution of six PLC isotypes in animals, each with a distinct mode of regulation. PLC pre-mRNA can undergo alternative splicing, resulting in the potential existence of up to 30 PLC enzymes in a single mammalian organism. Most bacterial PLC variants are distinguished by one of four protein groups that share structural similarities (30). Toxic PLC demonstrates the capability to engage with the eukaryotic cellular membrane and initiate the hydrolysis of phosphatidylcholine and sphingomyelin, resulting in cell lysis's ultimate occurrence (19). The first bacteria that were introduced as anticancer agents were streptococci and clostridia. The anti-cancer potential of bacteria is very diverse, in this regard, genetically modified bacteria can be used in anti-cancer treatments. It is possible to cause apoptosis or necrosis inside the tumor by using the toxins of anti-cancer bacteria (31-33). *C. novyi* NT bacteria can be used in cancer treatment with special enzymes or special toxins it produces (34).

The clinical utilization of targeted therapeutic agents associated with phospholipase C (PLC) signaling pathways is further underscored in investigations concentrating on antibody-drug conjugates (ADCs), exemplified by mirvetuximab soravtansine. This particular ADC has demonstrated significant efficacy in managing platinum-resistant ovarian carcinoma, attaining an overall response rate of 44% (35). The research underscores the clinical ramifications of focusing on specific neoplastic markers, a methodological approach that modulators of PLC activity could potentially enhance.

In this study, a PLC-Darpin structure was made, which was analyzed with online software regarding antigenicity, hydrophobicity, second and third structure, and some chemical properties. Based on the results, it has 22 regions with high antigenic properties, and GRAVY is negative, which shows that this structure has acceptable hydrophilic areas that can be accessible to antibodies (in vivo environment). The PLC-Darpin gene sequence was amplified by PCR and using specific primers designed with Primer3Plus software. Plasmid *pET-28a* was cut with restriction enzyme *XhoI*/*NdeI,* and the size of the fragments created on the agarose gel confirmed the correctness of the enzyme reaction and double digestion. Recombinant plasmids were transformed into BL21 (DE3) expression strain bacteria, and protein expression was done under specific conditions and by adding 1 mM IPTG and using the SDS-PAGE method. Protein expression was evident from 2 h PBS after induction according to the protein size. Then, finally, the correctness of protein expression was confirmed by the western blot method, which is with hexa-histidine-specific antibody.

Considering the role of PLC in cell lysis and Darpin, which has a high affinity for binding to various tumor-related antigens such as the HER2 receptor, the results of the current study can subsequently be used to design an immunotoxin based on PLC that can be used in the future and with more studies, it can be used as a candidate for BC treatment.

Conclusion

In this study, a PLC-Darpin structure was developed and analyzed for its antigenicity, hydrophobicity, and stability using online software. It was found to have 22 highly antigenic regions and a negative GRAVY score, indicating favorable hydrophilicity for antibody accessibility *in vivo*. The protein exhibited relative stability, with a two-dimensional structure comprising approximately 30% alpha helices. The PLC-Darpin gene was amplified via PCR, confirmed through agarose gel electrophoresis, and ligated into the PET-28a plasmid before transforming into *E. coli* (DE3). Protein expression was induced with 1 mM IPTG, and SDS-PAGE analysis confirmed successful expression, which was further validated by Western blotting using a hexahistidine antibody.

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Conflict of interest

The authors declare no conflict of interest.

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Ethics approval and consent to participate

This study complies with ethical principles and the standards for conducting clinical research and is approved by The Ethics Committee of Zanjan University of Medical Sciences (IR.ZUMS.REC.1400.030). In the current study; all ethical guidelines including Ethics and Consent to participate have been collected. Informed consent was obtained from the patients/participants whose clinical samples have been used in the study. All results of this study have been classified and maintained by a dissertation in the Zanjan University of medical Sciences. We have indeed provided all raw data on which our study is based. In addition, the datasets analyzed during the current study available from the

corresponding author on reasonable request. The data that support the findings of this study are available from an Educational Hospital but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Zanjan University of Medical Sciences.

Authors' Contribution

All authors read and approved the manuscript. Contributions of the authors in this study were as follows: B M: supervision, visualization, data curation, formal analysis, validation, software, methodology, conceptualization; S Sh: investigation, formal analysis; N M: writing—original draft preparation; M Sh: writing—review and editing; D A: supervision, formal analysis, software, methodology.

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