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Design and Construction of Recombinant ELP-Intein Cassette for Use in Simple and new Purification Methods of Recombinant Proteins

Nogol Omoumi[1], Mohammad-Ali Shokrgozar[2], Zahra Noormohammadi[1]

1. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. National Cell Bank, Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT

Background and Objective: Use of elastin-like proteins (ELPs) provides high-performance protein purification without need for chromatography. In line with cost reduction and facilitation of recombinant proteins purification, which represent a high percentage of production costs, in this project, we eliminated the need for proteases in the process of separation of recombinant proteins from ELP by designing a cassette using ELPs properties as well as insertion of autocatalytic intein protein between the recombinant protein and ELP.

***Methods*:** In this study, at first *Mxe GyrA* intein gene was amplified from pTXB1 vector by PCR method and cloned into pUC57-hEGF vector. Then, 8xELP repetitive sequences were first cloned in pUC57 vector and then into pUC57-intein-hEGF vector in the upstream of Intein-hEGF.

***Result*:** The design and construction stages of pUC57-8xELP-Intein-hEGF cassette was successful and the accuracy of 8xELP-Intein-hEGF was confirmed by sequencing.

***Conclusion*:** The use of ELP-intein cassette provides recombinant protein purification only with steps consisting of temperature, salt, and centrifugation, without need for proteolytic enzymes, and access to this technology provides the possibility of production and purification of recombinant proteins with minimum cost and facilities.

Key words: Purification, ELP, Intein

Corresponding Information: Nogol Omoumi, Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
Email: n_omoumi@yahoo.com

Introduction

Use of elastin-like proteins (ELPs) provides high-performance protein purification without need for chromatography columns. Elastomeric polypeptide consisting of vessel containing predominantly repeating units of elastin pentapeptide (VPGVG), was first studied in 1992 (11). The unique transition phase behavior of these biopolymers has made them an ideal candidate for research on purification of recombinant proteins.

ELPs consisting of repetitive VPGXG motifs (X can be any amino acid), are soluble at room temperature, but insoluble at 30-40°C. Exact transition temperature of ELPs is a variable dependent on a number of factors, including chain length, concentration, and type of the amino acid 'X'. Increase in ELP chain length or salt decreases the transition temperature. In addition, previous researchers believed that ELP tags can be used in purification of active form of integrative proteins, and consequently showed that tag deposition do not disable target protein (7, 10).

This system also allows simple purification of active proteins from cell lysate and eliminates the need for high-cost chromatography. This method is performed under a wide range of conditions, including salt concentration to reach different transition temperatures and a broad spectrum of pH, and the temperature to activate the splicing reaction (1). Therefore, by fusion of target protein to ELP, non-chromatographic protein purification can be performed only with steps consisting of temperature change, addition of salt, and centrifugation (1, 10).

ELP sequence designed by Chilkoti et al. for production of some recombinant proteins, was used in design and construction of intein-ELP gene expression cassette (2). This sequence consists of Val-Pro-Gly-Xaa-Gly pentapeptide and Xaa can be any amino acid. This pentapeptide repeat produces a polypeptide, which its solubility depends on temperature. In primary cassette valine, alanine, and glycine were placed in the position X (2).

In the most common cassette, in any 10 repeats, valine repeats 5 times, alanine 2 times, and glycine 3 times. Tandem arrangement of this sequence, produces ELPs of different lengths, which their solubility temperature is affected by factors, such as composition and length of the ELP, and there is an inverse relation between transition temperature (dissolution or transition from soluble phase to precipitate phase) and its length (2). In the present study, this sequence was used, but lysine and phenylalanine amino acids were used in the X position to reduce the transition temperature, in which ELP precipitates.

Lysine that can be ionized and is sensitive to the buffer salt, help the solubility of ELP, and phenylalanine moderates this additional solubility by its hydrophobicity. Although, this method is cost-saving and eliminates the need for expensive chromatography column, it has a limitation. ELP tag must be separated from the recombinant protein using proteolytic enzymes.

Use of these enzymes causes some problems as follows: 1) Cleavage by proteases is not always specific and may result in a cleavage at another site within the target protein; 2) Higher temperatures that are required for cleavage reaction of many proteases, may adversely affect the stability or activity of the target protein; and 3) Cleavage is sometimes inefficient due to inaccessibility of the cleavage site on the fusion protein to proteases (3). Intein mediated purification with an affinity chitin-binding tag (IMPACT) is used to resolve these problems. In contrast to other purification systems, the IMPACT systems do not need to use exogenous proteases to remove fusion tags.

Protein splicing is a post-translational process, which involves excision of the intein sequence and ligation of the flanking sequences (N- and C-exteins). Since the discovery of this phenomenon in 1995, about 200 inteins have been identified. Their length varies from 100 to 800 amino acids and contain conserved sequences, due to engineerings performed on some types of inteins, they lost the ability of ligation of exteins, and one type of intein has been made, which separetes an extein from one of its terminals. This type of intein is used in the IMPACT protein purification kit (New England Biolabs Ltd.) (13).

The inteins used in this system are induced by temperature, pH, or thiol compounds, such as dithiothreitol (DTT), which make a cleavage between intein and target protein. Given that human epidermal growth factor (hEGF) is the most important growth factor in construction of connective tissue, epithelium, and living artificial skin, in this research, this protein was used as target protein. Insertion of intein between hEGF and ELP purification tag, can non-enzymatically separate the hEGF (4). The present study was conducted with the purpose of designing and construction of recombinant ELP-Intein cassette for use in new purification methods of recombinant proteins.

Materials & Methods

In this study, *Escherichia coli* Top10F' (Invitrogen) was used as cloning host. *Mxe GyrA* intein sequence with an intein having N-terminal restriction site, was amplified from pTXB1 vector (New England Biolabs Ltd.). The hEGF coding sequence was obtained from GenBank database, and was ordered to Gene Fanavaran Company with C-terminal *Nsi*I, *Dra*III, and *Bgl*II restriction sites, in pUC57 vector between NdeI and BamHI restriction sites.

In the design of ELP fragment, 9 VPGXG repeats were designed, so that valine (7 times), 1 phenylalanine (1 time), and Lysine (1 time), were places in the Xaa position. Then, this set was repeated 8 consecutive times during several cloning cycles [(VPG(KV7F)G)9]8. The transition temperature of the ELP in the presence of 0.4 mM ammonium sulfate is about 20°C. 1xELP fragment (containing an ELP coding 140-bp repeat) was ordered between *Dra*III and *Bg*lI restriction sites in pUC57 vector. Given that the restriction site sequence is GCCNNNNNGGC for *Bg*lI, and CACNNNGTG for *Dra*III, N-terminal restriction sites were designed, so that they could match and pair to each other, in the form of 5'CACGGC|GTG 3′ and 5'CACGGC|GTG 3′, respectively. GGC is repeated at the beginning and end of each ELP sequence, and provides the consecutive insertion of the inter-sequence, without additional base.

Mxe GyrA intein gene cloning into pUC57-hEGF vector

Primers of intein gene were designed using Gene Runner program and then synthesized. Oligonucleotide sequence of the forward primer was 5′ACACGGCGTGATCTACTTCCTGCAGTCGC 3′(the underlined region is *NsiI* restriction site), and the oligonucleotide sequence of the reverse primer was 5′ACACGGCGTGATCTACTTCCTGCAGTCGC 3′ (the underlined region is *Dra*III restriction site). PCR (Eppendorf) reaction was performed with an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 35 s and annealing at 54°C for 40 s, and a final extension step at 72°C for 84 s (9). The PCR product was purified using Fermentas Gel extraction kit. Then, the ligation between linear pUC57-hEGF vector and intein was performed using T4 ligase at room temperature, and then 5 µl of ligation solution was used to transform the bacteria Top10F' (Invitrogen) using heat-shock method. Finally, 10 colonies grown on medium containing ampicillin were selected, and after plasmid purification by miniprep (Fermentas) method, the cloning results were confirmed by enzymatic digestion.

During several cycles of cloning (by enzymatic digestion of pUC57 vector with *Dra*III and *Bgl*I enzymes, ligation reaction between linear vector and fusion fragment, transformation into *E. coli*, and plasmid purification, respectively), the 140-bp 1xELP was repeated 8 times and resulted in a 1120-bp 8xELP fragment. At first, pUC57-1xELP were digested with *Dra*III and *Bgl*I enzymes, in other words, both ligated fragment and vector were prepared by enzymatic digestion, and pUC57-2xELP was produced after ligation reaction, transformation into bacterium, and plasmid purification.

By repeating these steps, pUC57-3xELP, pUC57-5xELP, and finally pUC57-8xELP, were obtained. Considering that the ELP sequence is rich in cytosine and guanine (GC rich) and the risk of error in the synthesis of repetitive sequences, colony PCR method cannot be used to verify the cloning of repetitive sequences of 8xELP in the pUC57 vector. Therefore, at first colonies containing larger plasmid were determined by a simple method (boiling the colonies in water and loading them on agarose gel) using Quick check method, and then to determine the size of cloned ELP after digestion with *Dra*III and *Bgl*I enzymes, microtube was incubated at 37°C for 3.5 hours. After this period of time, to be ensured of enzyme function, 5 µl of the reaction product was loaded on agarose gel, and size of the fragments were determined, and band of

the larger fragments appeared higher on the gel.

Cloning of repetitive ELP sequence into pUC57-1xELP vector

After verification of 8xELP cloning in pUC57 vector, the fusion fragment was extracted from the gel using gel extraction kit. After digestion of pUC57-intein-hEGF vector by *Dra*III and *Bgl*I enzymes, it were loaded on agarose gel and extracted. The ligation reaction between linear vector and fusion fragment was performed by T4 ligase at room temperature, and was transformed into Top10F' (Invitrogen) bacterium. After plasmid purification, cloning of the final cassette was confirmed by enzymatic digestion and sequencing (9).

Results

The PCR product of intein gene amplification was loaded on agarose gel 1% along with marker. The cloning of pUC57-intein-hEGF vector was performed by double enzymatic digestion with *Nsi*I and *Dra*III enzymes in order to separate the 620-bp intein from the 2710-bp length pUC57 vector. To confirm that whether the ELP sequence was repeated 8 times or not, each enzymatic digestion with *Dra*III and *Bgl*I enzymes was repeated, and finally 1120-bp length 8xELP was observed on the gel. The cloning of 8xELP fragment into pUC57-intein-hEGF vector was confirmed by double enzymatic digestion with *BamH*I and *Nde*I enzymes, followed by sequencing (Figs. 1 and 2).

A, Column M, 1 kb marker; Column 1, *Mxe GyrA* intein gene PCR product produced by specific primers and pTXB1 reference vector (620

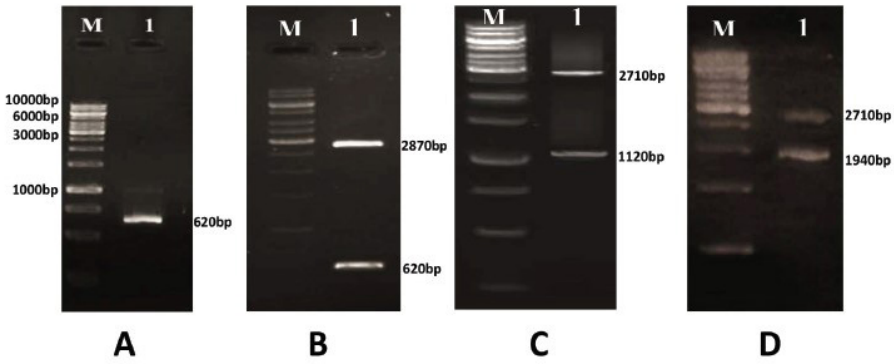

Fig. 1
PCR rpoducts and digestion. A. PCR amplification of Mxe GyrA intein gene by specific primers, Lane M: 1kbp DNA marker, Lane 1: Mxe GyrA intein gene. B. Confirmation of Mxe GyrA intein gene presence in pUC57-hEGF vector by dobul digestion of NsiI and DraII. Lane M: 1kbp DNA marker, Lane 1: Digested vector. C. Confirmation of Mxe GyrA intein gene presence in pUC57 vector by dobul digestion of BgiI and DraII. Lane M: 1kbp DNA marker, Lane 1: Digested vector. D. Confirmation of pUC57-8xELP-intein-hEGF vector by dobul digestion of NdeI and BamH1. Lane M: 1kbp DNA marker, Lane 1: Digested vector

Fig. 2
Sequecing of recombinant cassete by CLC software

bp); B, Column M, 1 kb marker; Column 1, verification of the presence of intein gene in the pUC57-hEG vector by double enzymatic digestion with *Nsi*I and *Dra*III enzymes (2870- and 620-bp fragments); C, Column M, 1 kb marker; Column 1, verification of cloning of 8xELP repetitive sequences in pUC57 vector by double enzymatic digestion with *DraIII* and *BglI* (2710- and 1120-bp fragments); D, Column M, 1 kb marker; Column 1, verification of the final recombinant pUC57-8xELP-intein-hEGF cassette by double enzymatic digestion with *BamH*I و *Nde*I (2710- and 1940-bp fragments).Verification of final recombinant cassette by sequencing method

Discussion

In the 1990s, purification of recombinant proteins using affinity tags was a common and widely used method (5). In this method, the target protein was being expressed as fusion protein-affinity tags, which could be purified by affinity column chromatography. Different affinity tags have been used for this purpose, such as glutathione S-transferase, maltose binding domain, *Staphylococcus* protein A, polyhistidine, and calmodulin-binding peptides (3). Following purification of proteins, affinity tags are usually separated from target protein by proteases using specific sites in their sequence (5). Additional chromatography steps are required to separate the target protein from affinity tags and proteases. To avoid restrictions on the use of proteolytic enzymes for separation, use of inducible self-cleavage activity of protein splicing element (intein), was investigated in purification (3).

Comparison with other methods

In our research, use of chromatographic methods is a common method for purification of recombinant proteins.

Momeu and Zakhratsev used consecutive chromatography to separate and obtain glucose oxidase from broth fermentation, and explained the specific purification method (14).

Loughran et al. produced histidine-tag containing BX-1 recombinant protein, which can be simply purified by immobilized metal affinity chromatography (6).

Although, this type of chromatography was being widely used for purification of proteins, it was always laborious and required multiple steps and expensive equipments. To resolve the problems caused by chromatography, non-chromatographic methods were suggested for purification of proteins. Designation of intein-based constructs was first performed by New England Biolabs Company, on the basis of which, Wood and banki and other researchers have carried out various successful researches in this area. This technology is a new and cost-saving method for protein purification (1, 12)

Problems of cassette designing

PCR technique is used to produce fusion fragment and its cloning into a vector (9), especially in cases where the size of fusion fragment is small and enzymatic digestion method does not work, while a significant amount of the fragment can be achieved by PCR method. In this case, the reason of not using PCR method was the probability of mutation during amplification. Despite the use of *pfu* exonuclease enzyme for amplification, mutation was likely to occur due to repetitive sequences and the high number of cytosine and guanine. Therefore, although the enzymatic digestion method has low efficiency, it was repeated several times and high amounts of fusion fragments were used in ligation reactions.

When the vector was digested with *Dra*III enzyme to be prepared for ligation to fusion fragment, due to low concentration of the fusion fragment and self-ligation of the vector, all the colonies grown on the medium after transformation contained only vector without fusion fragment. In other words, after screening of a significant number of colonies, it was found that no cloning was done. Most strains of *E. coli*, including Top10F', which are used as cloning host, naturally has cytosine and adenine methylation

activity in some sequences, and given that some enzymes, such as *Dra*III, are not able to identify the enzyme digestion site due to sensitivity to methylation, it is better that engineered strains of *E. coli*, such as GM2163 (dam- and dcm-), be used as cloning host, thus the enzymatic digestion by *Dra*III is completely done apart from the inhibition caused by methyl group against cytosine in the CpG sequence, and accuracy of the site in the fusion fragment is confirmed .Hence, ELP containing pUC57 plasmid was transformed to GM2163 strain, but self-ligation problem remained after all the steps. After rejecting the CpG methylation hypothesis, calf Intestinal alkaline phosphatase (CIAP) was used to remove the 5' phosphate from the pUC57 vector and resolve the self-ligation problem.

Conclusion

In protein purification process, replacement of common digestion sites, such as factor Xa and enterokinase by intein reduces the use of proteolytic enzymes during separation of target protein from affinity tag. Also, there is no need to separate the used enzyme. Both mentioned cases reduce finished production price and are cost-saving. In addition, use of ELP tag with self-association property eliminates the need for expensive chromatography column in recombinant protein purification process.

Use of this vector provides purification of recombinant proteins, only by steps including temperature, salt, and centrifugation, without the need for proteolytic enzymes. Therefore in near future it is expected that non-chromatographic protein purification will be an alternative method for the production of recombinant proteins for academic and industrial use in biotechnology.

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