# Intein Mediated Protein Synthesis and its Purification by Ni-NTA Affinity Chromatography

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**Abstract**

*An intein mediated protein purification system was established to generate His-tag free protein with high level of purity. To achieve this a synthetic intein gene (~171 bp) was constructed and fused to a histidine sequence at the C-terminus by a two-step assembly PCR. The synthetic intein gene was inserted into pEt-32a(+) vector at ECoRV and HindIII region. The target gene MBP from pMal-c5E vector fused in frame at the Hind-III and Xho I site of pEt-32a(+), containing the intein gene. The plasmid was transformed into E. coli BL21 (DE3) strain and protein expression was induced by IPTG. The MBP-Intein-His tagged fusion protein was purified from cell lysates using Ni-Nta resin affinity chromatography. The MBP was removed from intein - fused pEt vector by on-column intein-mediated cleavage with DTT.*

*Keywords: intein, histidine tag, MBP, pMal-c5E, pET32a (+), E. coli BL21 (DE3), Ni-Nta.*

## Introduction

Affinity tags are commonly used to facilitate the purification of a recombinant protein. Various methods for recombinant protein expression and purification to make the target protein to be a fusion product harboring an affinity tag, such as polyhistidine (His-tag), *Escherichia coli* maltose-binding protein (MBP), *Schistosoma* glutathione S-transferase (GST), *Staphylococcus* protein A, etc., have been developed[2]. However, these methods demonstrate a drawback that a site-specific protease is necessary to cleave the target protein from its affinity tag. The high cost and uncompleted cleavage of these proteases have limited their application[3].

Studies have elucidated that some proteins are able to self-catalyze the reactions without the need for any other protein or cofactor, known as protein splicing. In protein splicing, a segment of an inactive protein, the intein (internal protein), is excised from the rest of the protein, and the two flanking domains, the C and N-exteins, join each other, forming a biologically active protein. Protein splicing catalyzed by inteins has enabled various biotechnological applications in the field of protein engineering such as self-cleavable affinity tags to facilitate protein purification, protein ligation, differential isotope labeling of protein and cyclization of the protein backbone[4]. Recently intein-mediated expression of antimicrobial peptides like bacteriocins, β-defensins, and moricin CM4, has been successfully used[5, 6, 7]. The intein-mediated purification system is a simple, efficient option for obtaining pure, tag-free recombinant proteins in one-step purification through on-column cleavage and removal of the affinity tag under mild conditions[8].The aim of this study was to develop a system for purification and producing active recombinant protein with a good yield without the need of exogenous enzymes, whereas mediated by a self-cleaving carrier intein.

To achieve this, a mini synthetic intein was synthesized by two step assembly PCR and the intein segment was cloned into pEt-32a(+) vector at ECoRV and HindIII site. The fusion intein plasmid construct thus obtained was fused against a target protein, Maltose binding protein (MBP), obtained from pMAL-c5E vector. The MBP gene sequence with HindIII and XhoI restrictions was cloned with intein fused pET-32a(+) vector. In both the above protocols for transformation experiments *E. coli* BL21 (DE3) and *E.coli* DH5α, were used as the competent cells. The clones were checked by restriction digestion. The expression of the fusion protein was induced by isopropyl β-d-thiogalactoside (IPTG) was then separated and passed on to the Nickel (Nta) coloum for purification. The Induction of on-column cleavage, using thiol reagents, dithiothreitol (DTT), releases the target protein from the intein- histidine tag.

## Materials and Methods

### Designing the intein gene

The intein gene with ~171 bp with suitable restriction sites was constructed with 4 blocks. The sequence for the 4 blocks was obtained from gene bank database (Npu Dna-B, E acc. no: ZP_ 00110946.1 in NCBI, ZP_00108882.1). The 171 bp sequence of intein (with appropriate sequences for

restriction) is amplified in the laboratory by two step assembly PCR[9]. The 171 bp sequence was split into two single stranded fragments (1-85 and 75-171) with overlapping sequences. The following primers were used for each fragment and a two step successive PCR was carried out to assemble the intein gene.

**Primers**

Fragment 1: the forward primers were designed such that it contains sequence of fragment 1 from 1-45 (TAT TGC .....GGC) and reverse primer contains the sequence from 87-35 (-CCA AGT .....CCA) with 10 overlapping base pairs.

Fragment 2: the forward primers were designed such that it contains sequence of fragment 2 from 75-118 (-AAT TTA TGA ...GTT) and reverse primer contains the sequence from 171-100 (-GTG TTG CGT....CGG) with 18 overlapping base pairs.

**Target gene synthesis**

Maltose binding protein was used as test or target protein to fuse with intein[11]. The sequence of maltose binding protein was derived from pMAL-c5E vector (Fig. 3). MBP sequence was amplified from pMAL-c5E vector with forward primer "*NNNNN-XXX-ATGAAAATCGAAGAAGGTAAA*" and reverse primer "*NNNNNN-XXXGACGGA TCCGAATTCCCTGCAGGTAATTAA*" for the codoing region of MBP between 1528 to 2757 bp (Fig. 4). The amplified gene with recognization sequences of Hind-III and Xho I was cloned in pET-32a (+) containing the intein gene. After confirming the colonies by colony PCR, the positive clones were further subjected to expression studies.

The respective plasmids a) pEt-32a(+) with fragment 1, b) pEt-32a(+) with fragment 2, c) the recombinant pEt-32a(+) containing the ligated intein gene (both fragment 1 and 2) and d) p pEt-32a(+) with the intein and the gene for MBP, were transformed into *E.coli* DH5α [13]for cloning purpose then the selected positive clones were transformed into *E. coli* BL21 (DE3) by the method described by Len. y[13] cells for expression studies.

**Expression**

The positive white clones from the plate were inoculated in 5 ml LB broth and incubated at 37°C until $OD_{600}$ was 0.5 (exponential phase). Induction was performed by adding 5µl of 0.4mM isopropyl β-d-thiogalactoside (IPTG) followed by incubation at 15°C for 16 hrs[14]and Centrifuged at 3000 rpm for 3 min. The pellet was resuspended in 1ml of lysis buffer and sonicated on ice for 5 min after which the sonicated sample was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred into a separate tube. Forty micro liter of supernatant and the pellet was mixed with 20 µl 3X SDS-PAGE sample buffer and boiled for 5 min and 15 µl of the samples were electrophoresed on SDS PAGE and stained with Coomassie brilliant blue[15]. The inoculum before adding IPTG was used as a control.

**Purification**

The supernatant which contains the his-tagged protein was purified (free of his-tag) through Ni-Nta column[8] purification system of Invitrogen. The target protein was eluted with 8-12 ml of native elution buffer through a nickel Nta column with column buffer containing 50 Mm of dithiothreitol (DTT) and electrophoresed with SDS PAGE.

**Results and Discussion**

The nucleotide sequence (171 bp) of the synthetic intein gene was generated using the Npu Dna-B, E acc. no: ZP_ 00110946.1 in NCBI, ZP_00108882.1, genebank database. The gene was synthesised without stop codon.

**N-** GAT ATC TAT TGC CTG AGC TAT GAA ACC GAA ATT CTG ACC GTG GAA TAT GGC AGC CTG ATT CGC GCG CGA CCA AAG ATC ATA AAT TTA TGA ACC GTT GTG TAT GAT ATT GGC GTG GAA CGC GAT CAT GTT TTT GCG CTG GGC TTT ATT GCG AGC TTT TGC GTT GAG AAG CTT**- C**

The N terminal sequence of the resulted intein was observed to restrict cleavage in its N terminal and allows cleavage only at the C -terminal. The gene contained 4 blocks A, B, F and G for splicing. A and B are located in the N-terminal splicing region, whereas F and G located in the C-terminal splicing region. Synthesis of such mini-intein gene segment has been carried out to identify and evolve proteins with novel functions[16]. Various intein fusion systems have been developed using different synthetically produced inteins, e.g., the *Sce* VMA intein[17,18], the *Mycobacterium xenopi* gyrase A intein[19], the *Synechocystis*s p. *dnaB* and *dnaE* gene inteins[20,21] and the *Mycobacterium tuberculosis* RecA intein[22,23].

It was observed from the results of the synthetic intein gene cloning with pEt-32a(+) by two step assembly PCR and further restriction digestion, that both the intein gene fragment 1 and 2 was precisely inserted into the plasmid at ECoR-V and Hind-III site (Fig. 1). The experiments also revealed that the target MB protein was cleaved from pMAL-c5E vector at Hind-III and Xho I site and was cloned successfully in pEt-32a(+) containing the

intein. The restriction of recombinant pET-32a (+) vector at HinD-III and Xho1 site and subsequent PCR amplification and electrophoresis (Fig. 2) shown that the intein gene with the gene MBP (approx. 1400 bp) was successfully cloned and cleaved from the pET-32a (+) vector (5900 bp).The study also established that each of these recombinant plasmids was effectively transformed in *E.coli* BL21 (DE3) as estimated by electrophoresis. The appearance of white color colonies on LB agar demonstrated that the recombinant pET-32a (+) vector was transformed successfully into *E.coli* BL21 (DE3) cells.

The induction study by IPTG on the positive recombinant clones containing the fused protein in pET -32a (+) vector with target gene MBP and further SDS-PAGE was represented in Fig.3. The findings (lane 3 & 4) demonstrated that induction by 0.4Mm IPTG at 15°C for 16 hrs showed significant increase in protein band compared to the uninduced cells. A gradual increase of a protein band, approximately 72 kDa, was observed under IPTG induction but was not detected in the uninduced culture (without IPTG). Subsequent purification of the fused protein crude exract through Nickel (Nta) colum individually have demonstrated that (Fig. 4) that elution through the Nickel column resulted in target protein with improved purity (Fig. 4, lane 6). The PET-intein fusion vector revealed a higher concentration and has the expected size of the MBP (~ 44 kDa) and intein- pet vector proteins (~ 28 kDa) was eluted in traces. Whereas the flow through from nickel column (Fig. 4, lane 2) contained intein-MB fused protein in lesser traces as well as additional binding impurities and no cleavage of MBP from intein fused vector was observed with reference to the final eluent from Nta column. Further the elution with DTT 50mM at $4^{\circ}C$ for 20 hours incubation cleaved maximum amount of MBP from intein – fused his tag vector Fig.4, lane4 and 5) as compared with DTT flush at $4^{\circ}c$ without incubation. This was evident when the Ni-nta column material was loaded (Fig4, lane 6) where the intein- pet vector proteins(~28kda) was still present in higher traces in the column .

This study demonstrated a simple and cost-effective streamlined intein-mediated cloning, expression and one-step purificaion protocol that combines affinity chromotography and on-column (Nta) tag clevage to generate a His tag – free protein with improved level of purity. This method thus provides a simple and reliable method for the purification of target proteins, and very well applied at the research scale[2,24]. However by manipulating the bacterial cells to effectively produce both the affinity resin and tagged target protein, the cost associated with the purification of recombinant proteins could be greatly reduced and a significant breakthrough in both large-scale production of purified proteins and enzymes can be achieved.

**Fig.1: Electrophoretic analysis of expression *E.coli* DH5α pET-32a (+) vector**

Lane 1: DL 3000
Lane 2: Recombinant pET -32a (+) vector.
Lane 3: Plasmid digested by ECoRV & HindIII
Lane 4: KB ladder

**Fig.2: Electrophoretic analysis of expression of the intein gene with MBP segment from *E.coli* BL21 (DE3)/ pET-32a (+) vector**

Lane 1: DL 3000
Lane 2: Recombinant pET -32a (+) vector.
Lane 3: Plasmid digested by HindIII & Xho1.
Lane 4: KB ladder

**Fig. 3: Expression of fused intein pet vector with target gene MBP**

Lane 1: Protein marker, broad range (NEB# P 77 02, 15µl)
Lane 2: Crude extract from uninduced cells (20 µl)
Lane 3 & 4: Crude extract from cell induced at $15^{\circ}C$ for 16 hrs

**Fig. 4: Purification through nickel Nta column**

Lane 1: Protein marker, broad range (NEB#P7702, 15µl)
Lane 2: Flow through from nickel column (6µl)
Lane 3: Wash (6µl)
Lane 4: DTT flush (6µl), (at $4^{0}c$) the stop the column flow
Lane 5: Elution of MBP after inducing cleavage at $4^{\circ}C$ for 20 hrs
Lane 6: Nickel NTA column material after elution (6µl)

**Fig.4**

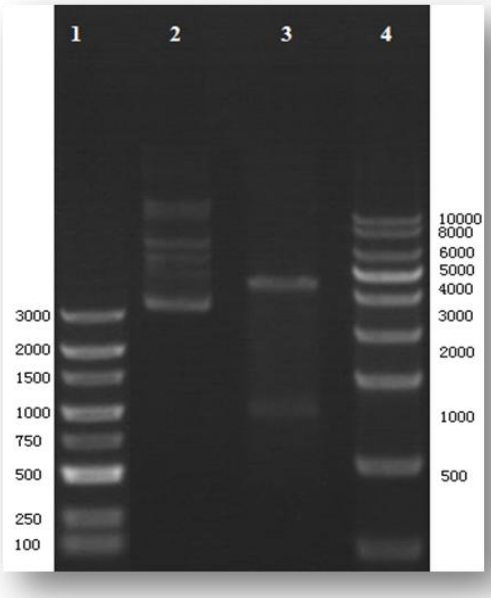



**Fig.5**

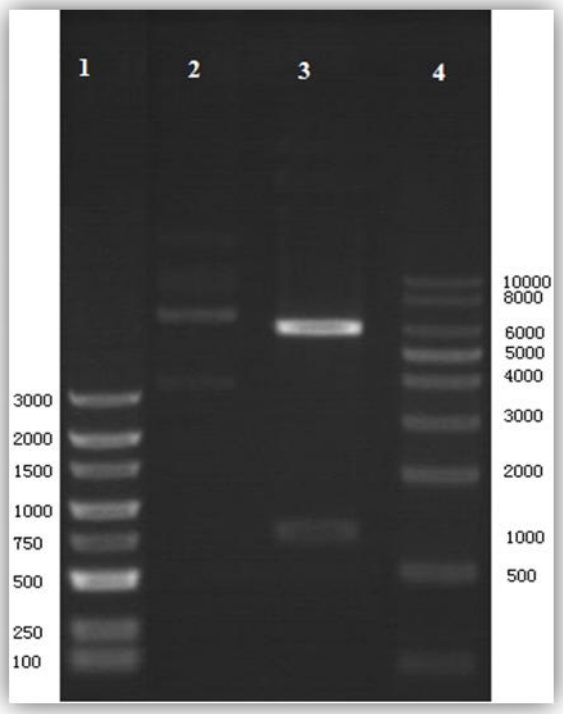



**Fig. 6**

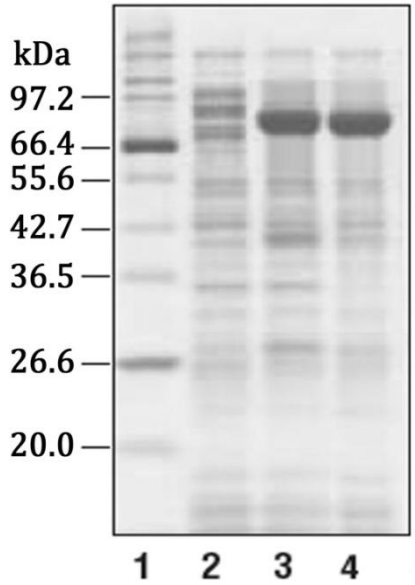
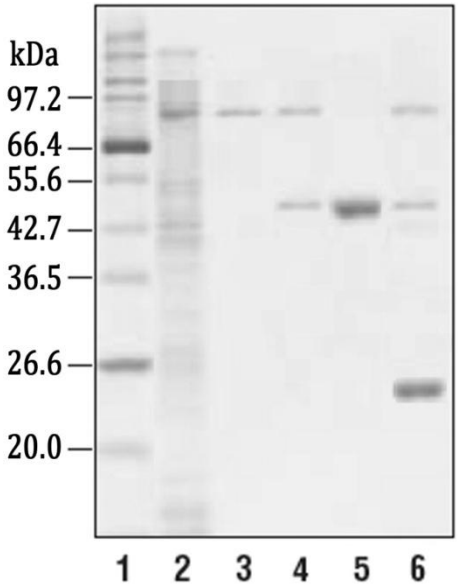



**Fig. 7**

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