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STUDY AND ANALYSIS ON CLONING OF BIRA GENE IN PET28A VECTOR AND HIS-TVE PROTEIN PRODUCTION

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ABSTRACT

In many cases, the analysis of a specific protein is hindered by the inability to purify large quantities of it from a native source. It's possible that proteins of interest are only found in trace amounts, or that attempts to purify them have been hampered by technical difficulties. DNA recombinant techniques have allowed researchers to overcome some of these limitations by producing large quantities of purified proteins from nonnative systems. Biotin labelling proteins with biotin is a useful tool for a wide range of applications because of the strong affinity between biotin and avidin or streptavidin. The biotin ligase from Escherichia coli, BirA, biotinylates a lysine side chain in a 15-amino acid acceptor peptide (also known as Avi-tag). We developed a method for producing recombinant BirA ligase for in vitro biotinylation of Avi-tag-bearing proteins. The target protein was expressed in both thioredoxin and MBP fusions, and the corresponding fusion was released by TEV protease. The HisTrap HP column was used to separate the free ligase from its carrier. In the case of thioredoxin and MBP fusion constructs, we obtained 24.7 and 27.6 mg BirA ligase per litre of culture, respectively. The recombinant enzyme was found to be extremely effective in vitro when it came to biotinylation. The procedure outlined here is an efficient way to make BirA ligase, which can be used to biotinylate a variety of Avi-tag-bearing substrates.

1. INTRODUCTION

Biotin is a small organic molecule that has an extremely high affinity for binding avidin and streptavidin [1]. Biotinylation, the chemical or enzymatic process of adding biotin to proteins, can be accomplished in two ways: chemically or biologically. In order to detect, immobilise, and purify proteins, biotinylation is a commonly used protein labelling method [2]. A biotin ligase enzyme called BirA, which is found in E. coli, can covalently attach biotin to the lysine side chain of a peptide known as the Avi-tag [3–6]. Using the Avi-tag, a variety of proteins have been labelled with biotin for a variety of applications.

There is an inducible birA gene on pACYC184 in E. coli B strain AVB101. Biotinylation in vivo requires the co-expression of BirA and an Avi-tagged protein of interest. Immobilized ligands for interaction studies can be made using this system, which we've previously employed to make biotinylated proteins. pET-32a was used to insert the gene for the protein of interest, which has an Avi-tag attached, into the pET-32a vector. When we discovered that the AVB101 strain lacks the T7 RNA polymerase expression system, we isolated the BirA encoding vector pACYC184, and co-transfected it with the pET-32a vector harbouring the target gene into an E. coli strain that had the T7 promoter (DE3). The Avi-tagged target protein was co-expressed with BirA. There were some biotinylated portions of the target protein, but the biotinylation efficiency varied from batch to batch

when done this way. When BirA was co-expressed with the Avi-tagged target protein, we saw a significant decrease in BirA expression. The labelled protein's ability to bind to a streptavidin-coated sensor chip decreases over time, suggesting that it loses biotin during storage.

Tobacco etch virus has a 27 kDa protease called tobacco etch virus protease in the Nuclear Inclusion a (NIa) gene (TEVp). TEVp has a cysteine residue (C151) in its catalytic site, unlike serineproteases. Protease activity has been completely wiped out by just one single point mutation. [7-11] The seven-amino acid long sequence EXXYXQ-S/G (TEVp cleavage Site, TS) encodes the minimal TEVp substrate specificity, where X can be any amino acid (Dougherty et al., 1988). As a result, even though this peptide appears to have low specificity, the best-known and most cleaved TSS is identical to the protease's natural substrate (ENLYFQ-G/S). It is between Q and the short aliphatic G or S residue that the proteolytic cleavage occurs [12]. The protease's C-terminal portion contains a TS with the sequence GHKVFM/S, which is recognised intramolecularly and cleaved at M218. The auto-cleaved protein fragment's proteolytic activity has been the subject of varying studies. Several TEVp mutants of amino acid 219 have been characterised [13-15] to overcome the issue of auto-cleavage.

TEVp accumulates primarily in the nucleus of plant cells during viral infection before being released from the full-length Nla precursor protein, whereas when expressed alone it primarily localises in the cytoplasm. For biotechnological applications, we engineered a mutated version of TEVp that showed strong cleavage activity on substrates that reside within the ER lumen.

2. TARGET SELECTION AND COMPUTATIONAL TARGET OPTIMIZATION

Using a 'ortholog' approach, researchers can study a given gene using a plethora of publicly available completed genome sequences and corresponding genomic DNA. Over 10 million protein sequences are included in the latest Pfam release, a collection of protein family alignments constructed automatically using hidden Markov models (HMMs). The median number of proteins in each family is 150. (Figure 1). Soluble proteins that are compatible with large-scale purification and high-throughput crystallisation are more likely to be obtained when multiple orthologs of the same target are selected for expression. Using a list of genomes for which the DNA is available, researchers can select specific targets. With these pipelines, which focus on non-membrane bacterial proteins, the targets are chosen by eliminating trans-membrane segments, signals, and large, disordered regions in the target sequence using a standard bioinformatics pipeline. Protein disorder prediction is used to design several truncations. On average, two to three constructs are created for each ortholog.

Soluble proteins that can be used for downstream processing are one of the major bottlenecks for structural genomics. It is also difficult to obtain crystals of the purified proteins, which is another major bottleneck. Two of these two steps account for more than 80% of the pipeline attrition rate (90 percent overall attrition rate). Since the current pipeline has processed tens of thousands of protein samples using the same bioinformatics target selection procedure and standard experimental operating protocols, the accumulated data in the LIMS can be used in data mining to derive protein features that influence "crystallisation feasibility" or crystallizability. A number of methods have been used in the past to predict the protein's ability to crystallise based on its amino acid sequence.

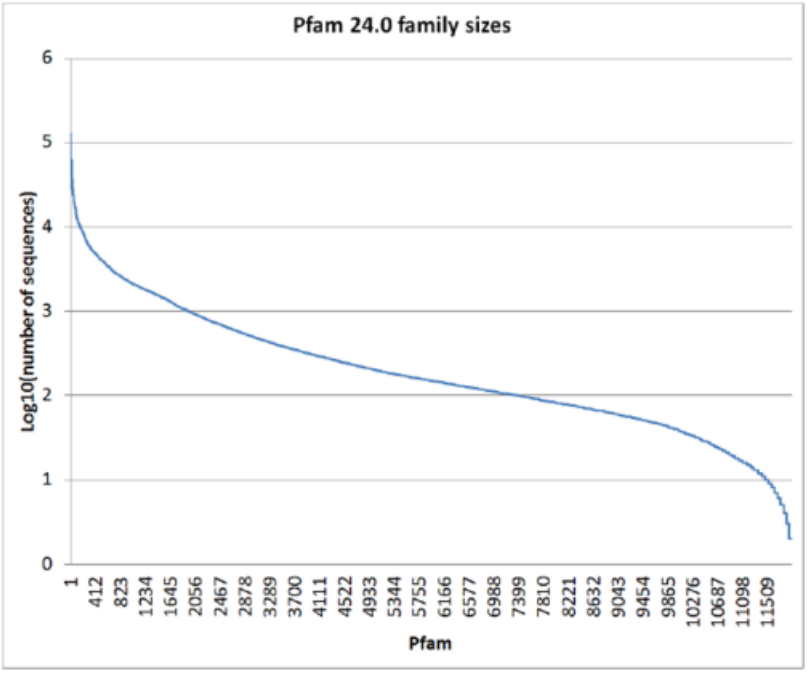


Figure 1. Family size distribution of the 12,000 protein families in the Pfam database.

In terms of data sets, the Protein Data Bank has a few hundred sequences as well as a complete protein set (PDB). As a general rule, prediction methods range from simple calculations based on isoelectric point (pI), grand average hydropathy (GRAVY), or diand tri-peptide profiles, to more complex methods that include secondary structure predictions and residue conservation predictions. Predictions using the OB-score are based on the clustering of PDB entry and UniProt sequence pI and GRAVY values for a smaller set of PDB entries. Based on our experience with JCSG-based scoring methods, we've developed our own computational method for predicting crystallisation. More than 1,700 proteins that expressed well but remained insoluble were used in the development of our crystallizability scoring method from the MCSG pipeline: a set of more than 700 proteins that had been submitted to the PDB.

2.1 Fusion Proteins

This may necessitate the addition to the coding sequence of recombinant proteins of ammo acids that are not found in the normal sequence of the peptlde under study. Recombinant proteins can sometimes incorporate a small peptide containing an antigemic site to allow immunological detection of the recombinant protein. When a similar protein is present in the host system, these "epltope flags" come in handy for monitoring recombinant proteins. Some proteins may be fused to other proteins that already have a simple purification process in place, such as peptldes or protein sequences. Because these "carriers" are proteins and/or peptlde sequences that can be easily separated by chromatography, they are often called "affinity tags."

2.2 Removal of Tag

Consideration of the effect of the carrier portion of the fusion on the target protein can influence the selection of an affinity tag. How will the protein's folding, activity, crystallographic analysts' or antigemc recognition be affected if additional amino acids are present? The final use of a fusion protein may not be affected by the affinity tag in some cases. In other cases, the recombinant protein

can't be used until the affinity tag is removed. Even though chemical cleavage is possible, the specificity requirement for chemical cleavage is low, and the protem of interest may be permanently altered by a chemical cleavage. Chemical cleavage conditions, such as pH and temperature, can alter protein properties.

2.3 Proteinases Commonly Used for Tag Removal

To break down fusion proteins, four different endoproteinases are commercially available. A specific proteolytic process is required to activate the two (thrombm and FactorXa) that come from eucaryotic cell extracts. Life Technologies, Rockville, MD, makes TEV NIa proteinase, which is commercially available under the trade name TEV protease, recombinant, in the laboratory using genetic engineering techniques. Purified cell extract protein or recombinant form of enterokinase are both available.

3. MATERIALS AND METHODS

3.1 Constructs

A codon-optimized version of TEVp was cloned into pcDNA3 upstream the SV5-tag (GKPIPNPLLGLD) and used as the basis to perform all TEVp mutants, obtained by site-directed mutagenesis (QuikChange Site Directed Mutagenesis Kit, Stratagene), using primers: N23Q fw/rv (CTATCTGCCACCTGACTcAgGAAAGCGACGGACATACC/GGTATGTCCGTCGCTTTCcTgA GTCAGGTGGCAGATAG); C130S fw/rv (GTCTCCGACACCTCTTcTACATTCCCTTCTAGTG/CACTAGAAGGGAATGTAgAAGAGGTG TCGGAGAC); T173G fw/rv (CCATTCAGCCAGCAACTTCggAAATACTAACAATTACTTCACCTC/(GAGGTGAAGTAATT GTTAGTATTTccGAAGTTGCTGGCTGAATGG); C110S fw/rv (GAGGGAGGAACGCATCaGCCTGGTGACTACCAACTTCC/GGAAGTTGGTAGTCACCAGGC tGATGCGTTCCTCCCTC). TEVp was modified by inserting at the N-terminus the coding sequences of an immunoglobulin secretion signal (Li et al., 1997) and cloned into pcDNA3 vector, yielding sec-TEVp. Sec-TEVp 235 was obtained by substitution of the wild type (wt) C-terminal sequence with AACGAAGGGGGCCTGGAA.

The SV5-TS-MHC-I-roTag, and the SV5-MHC-I-TS-roTag were derived from a construct encoding the cDNA ofthe A2 allele of the human MHC-I chain previously described, containing at the N-terminus the coding sequences for a secretion signal and the SV5 tag (GKPIPNPLLGLD) and at the C-terminus the roTag tag (SISSSIFKNEG). The TEV cleavage site (ENLYFQ/G) was included either in the ER luminal position downstream of SV5 or in the cytosolic tail just upstream roTag. The construct for Tetherin (N65,92A) (Petris et al., 2014b), with a deletion of the 20C-terminal residues that serve as a signal for the GPI anchor was C-terminal tagged with SV5 followed by TS and roTag. The single ORF pcDNA3 vector, encoding the full length L and H chains from mAb 14F7 (Carr et al., 2000), was obtained by cloning the coding sequences of the two chains (Rodríguez et al., 2007), engineered by the addition of an immunoglobulin secretion signal peptide at the N-terminus and including the linker SSGSENLYFQGT with a TS (underlined) between the two chains327.

3.2 Plasmids Construction

Primer sets C19S1, C110S1 and C110S2 and C130S1 and C130S2 were designed for site-directed mutation of the TEVp5M (Table S1) by PCR using the plasmid pET28-TEVp5M as the template. Using NcoI and XhoI to remove the fragment encoding the TEVp construct, the plasmid was

subcloned into the NcoISalI site of the pET28-EmGFP plasmid to express the TEVp variant fused to the EmGFP protein. As Origami(DE3) was resistant to kanamycin, the XbaI-XhoI excised fragment was subcloned into the pET-22b with the same restriction enzyme treatment to confer ampicillin resistance on the transformants. It was found that the HRP's amino acid sequence matched up well with the mPerx (Fig. 2).

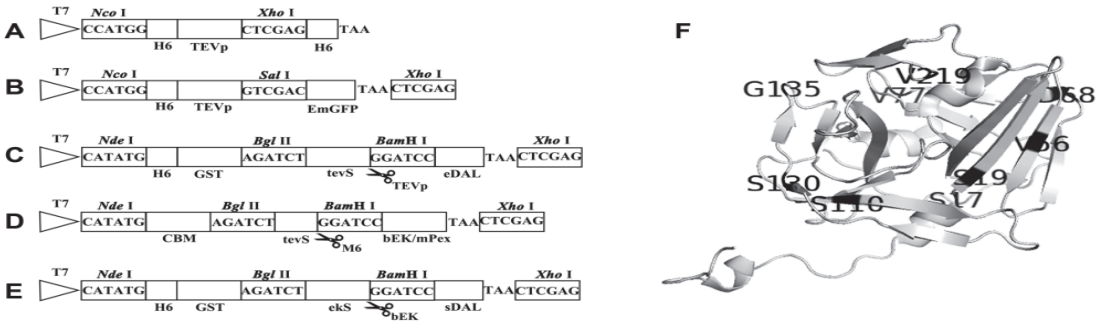


Fig. 2. Design of fusion proteins.

RT-PCR was used to amplify the leaderless mPex Q45-S350 encoding fragment using maize leaves as a template and primers mPex1 and mPex2 as the primers. For the CBMtevS-mPerx expression, the BamHI and XhoI incubation was followed by insertion into the pCBM-tevS vector with the same treatment. It was created by using BamHI and XhoI to cut out the bEK sequence from the plasmid pET41a-EK, and inserting it into the pCBM-tevS plasmid, which had been treated in the same way. For each plasmid, the corrective mutations were sequenced.

3.3 Construction of fusion expression plasmids

Amplified BirA gene using pACYC-184 plasmid as a template, using primers 1 and 2 (Table 1) EcoRI and NotI sites are found in both the forward and reverse primers. The TEV protease cleavage site is also encoded in the forward primer. It was digested with EcoRI and NotI and ligated into pET-32a twice digested with the same enzymes, and the PCR amplified birA gene was inserted. One of the results of this experiment was the creation of BirA/pET-32a, which allowed the target protein to be expressed in a thioredoxin-fusion form (Fig. 3A).

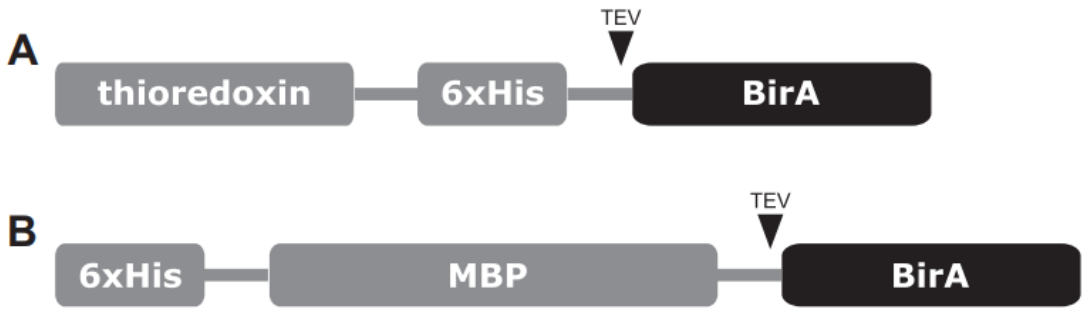


Fig. 3. Schematic representation of the two constructs in which the target protein is expressed as a fusion protein with (A) thioredoxin or (B) MBP.

Table 1 Primers used for birA gene amplification and vector modification

Primers	Sequences (5′–3′)
1	CCG*GAATTC*GAAAACCTGTATTTCCAGGGCATGAAGGATAACACCGTGCCACTG *Eco*RI TEV
2	ATAAGAAT*GCGGCCGC*TTATTTTTCTGCACTACGCAGGGATAT *Not*I
3	GGAATTC*CATATG*AAAATCGAAGAAGGTAAACTGGTA *Nde*I
4	CGC*GGATCC*TCCCCTTCCCTCGATCCCGAGGTTGTTGTT *Bam*HI

Note: The restriction sites used for cloning are shown in italics, with the name of the enzyme under the sequence. In primer 1, the coding sequence for TEV protease cleavage site is underlined.

The TEV protease cleavage site is also encoded in the forward primer. It was digested with EcoRI and NotI and ligated into pET-32a twice digested with the same enzymes, and the PCR amplified birA gene was inserted. One of the results of this experiment was the creation of BirA/pET-32a, which allowed the target protein to be expressed in a thioredoxin-fusion form (Fig. 3A).

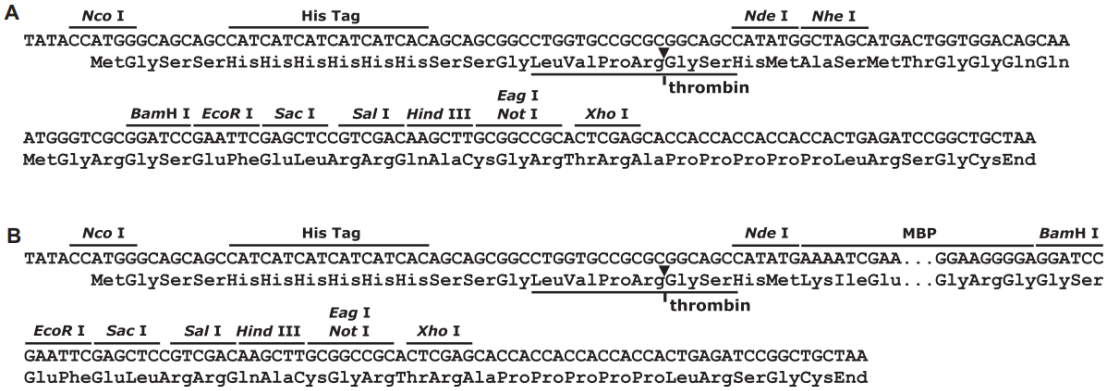


Fig. 4. Expression/cloning region of (A) original pET-28a and (B) its modified version, in which a sequence encoding MBP was inserted between the NdeI and the BamHI sites. This modification allows the vector to be used for MBP fusion expression

As shown in Figure 3B, an MBP-coding sequence was first added to the commercial vector pET28a before the fusion expression of the protein could proceed (Fig. 4). Amplification of the MBP coding sequence using primers 3 and 4 (Table 1) and inserting the NdeI and BamHI double-digested PCR product into pET-28a was accomplished using vector pRK793 as the template. Once EcoRI and NotI had been used to double-digest the PCR-amplified birA gene into the modified PET-28a plasmid, it was ligated into the plasmid. MBPBirA/pET-28a was the name given to the fusion expression construct.

3.4 Target protein release and isolation

Protease TEV cleaved both thioredoxin-BirA and MBP-BirA fusion proteins at a mass ratio of 100:1 (i.e, for 1 mg of purified fusion protein, 10 lg of protease were added). Cleavage was allowed to

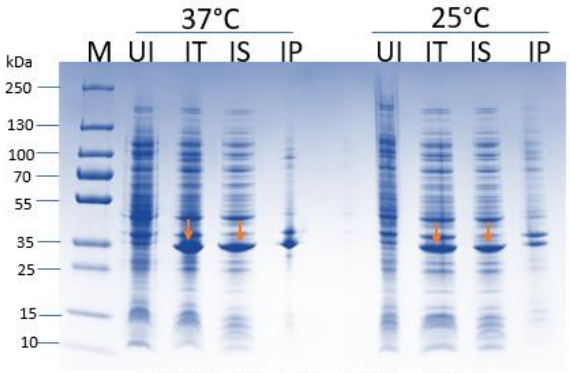
proceed for 16 hours at room temperature. A HisTrap HP column was used to analyse the reaction mixture after cleavage (5 ml). It was decided to store the unbound BirA recombinant protein in 50% glycerol at 20 C for further use.

3.5 Activity test

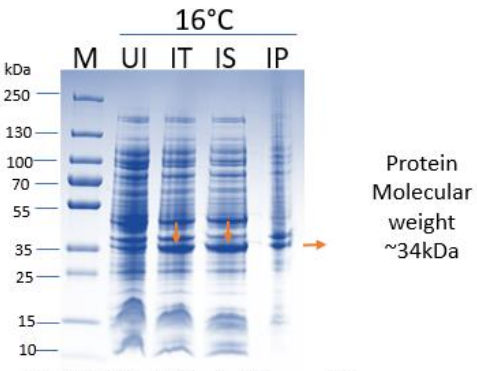
Different amounts of recombinant BirA were applied to 30 lMAvi-tagged substrate (in cell lysis buffer, pH 8.8) in the presence of biotin (0.3 mg/l) and ATP (0.5 mg/l) in order to evaluate the enzyme's biological activity. Room temperature reaction was allowed to proceed for 14 hours. When Streptavidin Sepharose was added to the reaction mixture, the biotinylated substrate was captured. A Zeba spin desalting column was used to remove any free biotin from the solution before the sample was applied to the Streptavidin Sepharose (0.5 ml).

4. RESULTS AND ANALYSIS

BirA protein expression at 37°C, 25°C and 16°C:



4-20%SDS-PAGE gel stained with Coomassie blue

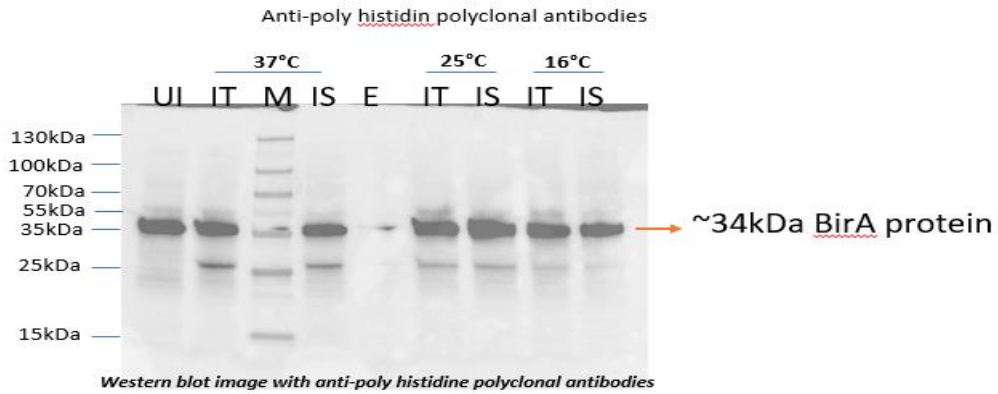


4-20%SDS-PAGE gel stained with Coomassie blue

Observation:

4-20% SDS-PAGE gels show that the expression of BirA protein in soluble form at all tested temperatures, around 34kDa, but expected molecular weight is around 40kDa.

Western Blot:

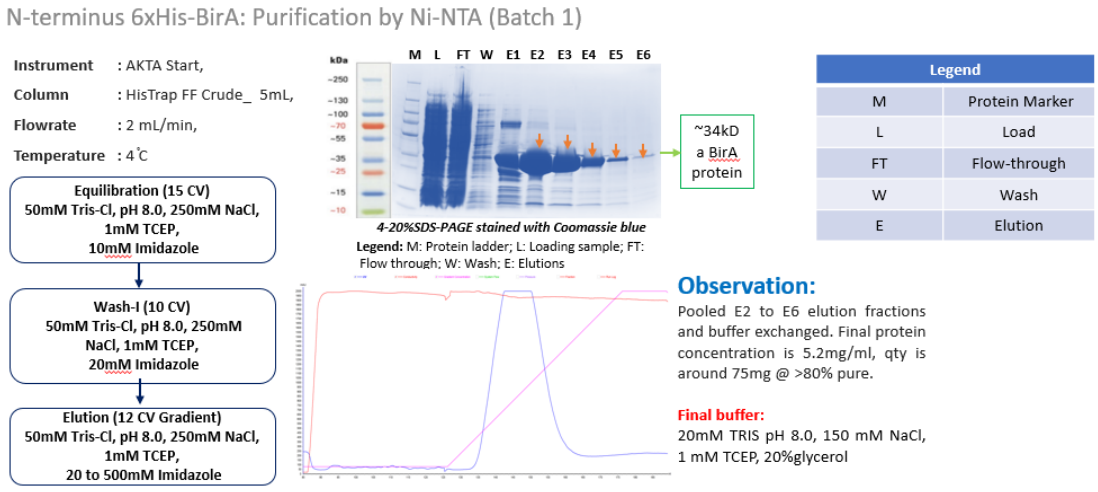


Western blot image with anti-poly histidine polyclonal antibodies

Observation:

4-20% SDS-PAGE gels show that the expression of BirA protein in soluble form at all tested temperatures, around 34kDa, but expected molecular weight is around 40kDa.

4.2 Purification of N terminal 6xHis- BirA: Batch1



4.1 Purification of His-TEV protein production

Purification of His-MBP by NiNTAcolumn : Entire process was executed at 4°C cold room.

Lysis Buffer: 50mM Sodium Phosphate buffer, pH8.0, 1mM EDTA, 300mM NaCl, 0.3mM TCEP, 1µM E64.

Equilibration: 20mM Sodium Phosphate buffer, pH7.5, 500mM NaCl, 0.3mM TCEP.

Elution buffer: 20mM Sodium Phosphate buffer, pH7.5, 350mM NaCl, 0.3mM TCEP. , 500mM imidazole (Low UV absorbance).

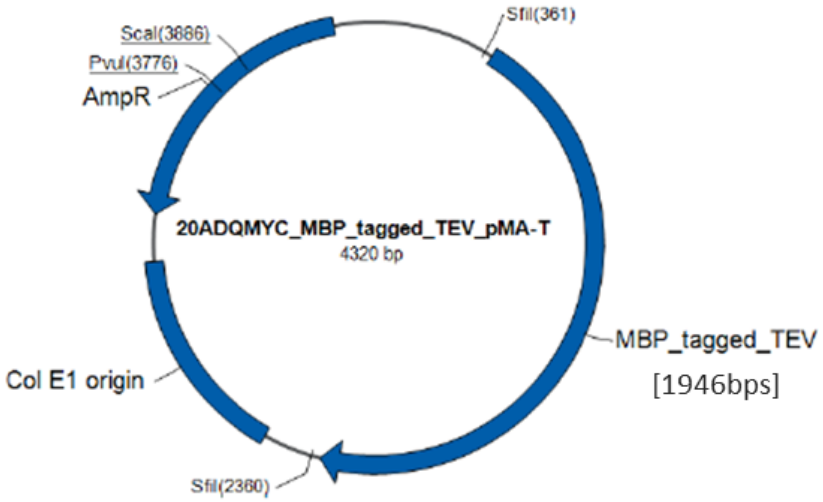
Cell pellet was lysed using high pressure homogenizer at 1000 bars, and clarified by centrifugation at 90,000xg for 25min at 4°C.

Loaded the clarified lysate onto pre-equilibrated NiNTA resin @2ml/min flow rate.

Washed the column with 4% Elution buffer until the OD at 280nM >0.1. Eluted the protein in a linear gradient of 0mM-500mM Imidazole collecting 7.4ml sized fractions.

Resolved all the peak fractions by SDS PAGE, pooled the fractions having His-TEV and concentrated to resolve further by SEC.

4.3 MBP-His-TEV gene information:



Gene Sequence:

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GCAACCGGTGATGGTCCGGATATTATCTTTTGGGCACATGATCGTTTTGGTGGTTATGCACAGAGCGGTC
TGCTGGCAGAAATTACACCGGATAAAGCATTTCAGGACAAACTGTATCCGTTTACCTGGGATGCAGTTCG
CTATAACGGTAAACTGATTGCATATCCGATTGCAGTTGAAGCACTGAGCCTGATCTATAACAAAGATCTGC
TGCCGAATCCGCCTAAAACCTGGGAAGAAATTCCGGCACTGGATAAAGAACTGAAAGCAAAAGGTAAAA
GCGCACTGATGTTTAATCTGCAAGAACCGTATTTTACCTGGCCTCTGATTGCAGCAGATGGTGGCTATGCA
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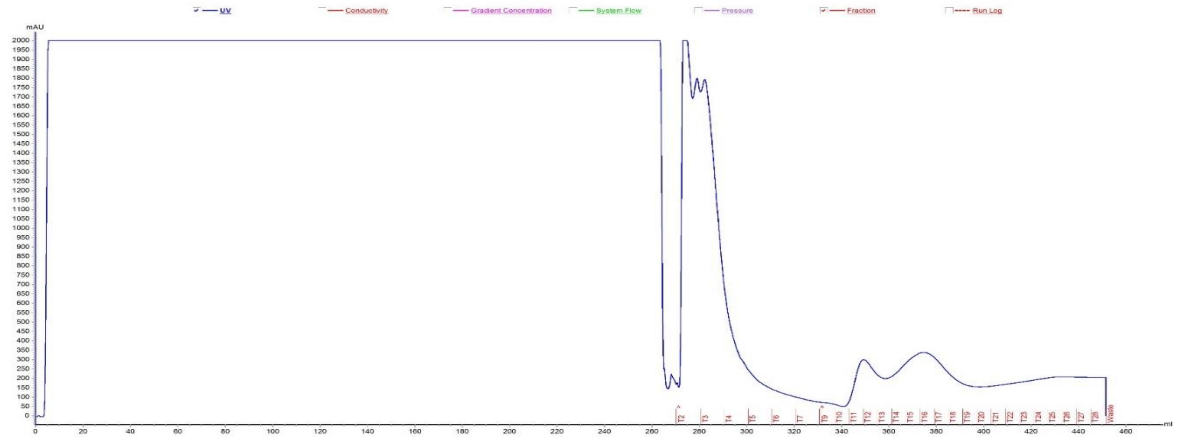
ATGAATCGTCGTCGTCGCCGTTAATAACTCGAGGAGCTCGGAGCACAAGACTGGCCTCATGGGCCTTCCG
CTCACTGCC

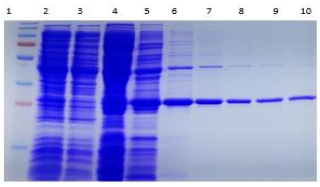
4.4 His-TEV_Ni-NTA purification: Chromatogram



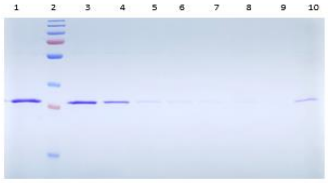
Observation:

The Ni-NTA purification of His-TEV protein was carried out AKTA START purification platform to load, washing of unbound protein and elution of POI.

4.5 TEV protease purification with 25% Imidazole wash

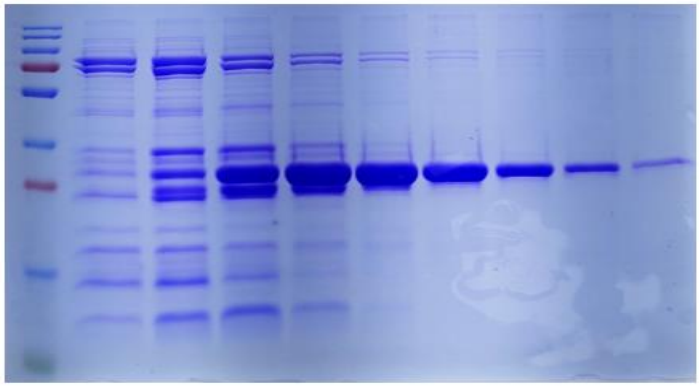


GEL 1
Lane 1: marker
Lane 2: : Loading sample
Lane 3 :Flow through
Lane 4-5: wash fracs
Lane 5-10: Peak fractions



GEL 2
Lane 2: Marker
Lane 1,3-10 Peak fractions

4.6 TEV protease purification with 25% Imidazole wash



GEL 1
Lane 1: marker
Lane 2: : Peak fractions

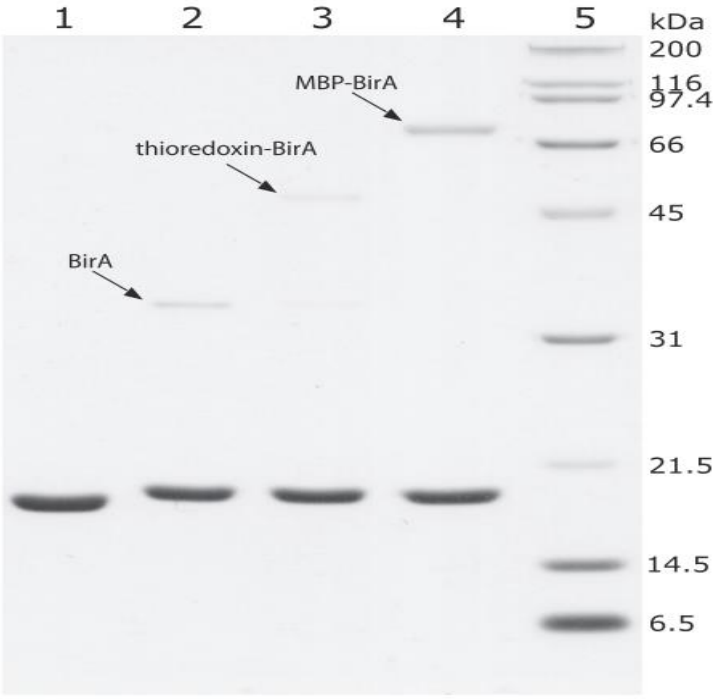


Fig.4. Activity assay for thioredoxin-BirA and MBP-BirA fusion proteins. Lane 1, negative control of the biotinylation reaction, which contains all the reagents except for the biotin ligase; Lane 2–4, biotinylation by released BirA (positive control), thioredoxin-BirA and MBP-BirA, respectively (enzyme to substrate molar ratio was 1:50 in all three cases and the reactions were proceeded at room temperature for 4 h); Lane 5, protein standards.

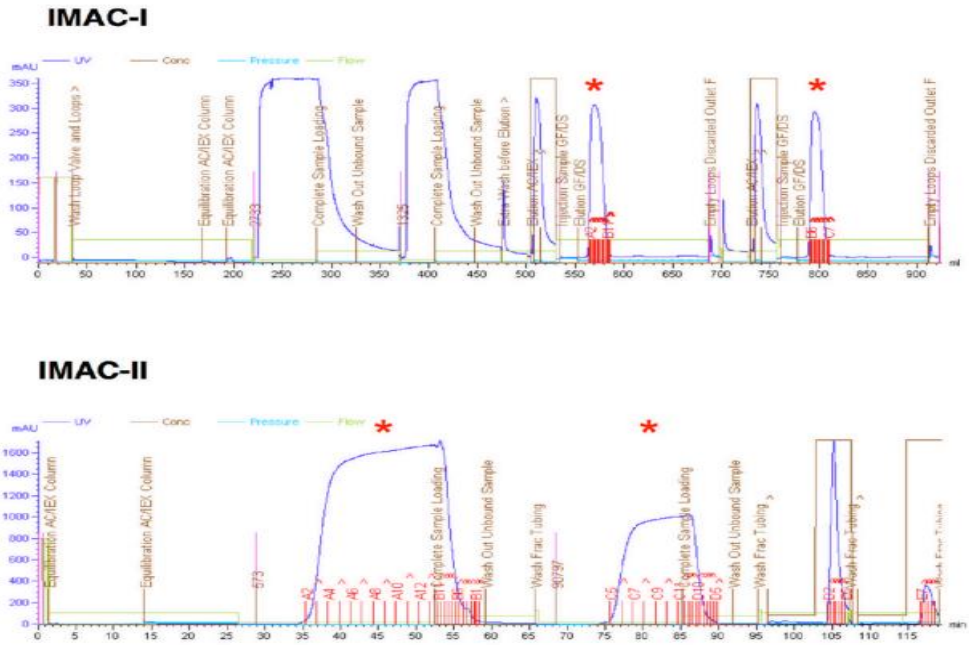


Figure 5. Purification of two His6-labeled proteins on IMAC-I (top) and IMAC-II after cleavage with TEV protease (bottom). Red asterisks show collected protein peaks.

CONCLUSION

In order to produce large quantities of highly purified BirA ligase, we devised a procedure. In vitro biotinylation of different Avi-tagged substrates can be performed using this recombinant ligase, which is enzymatically active. There are numerous uses for a TEV protease that functions along the secretory pathway. Our results show that the complete IgG mAb 14F7, originally derived from a single ORF, can be correctly assembled and secreted when the sec-TEVp QSG is coexpressed. In the secretory pathway, this unique sec-TEVp variant could be used to conditionally activate pro-enzymes or inactivate protein molecules or complexes, as it has been done in the cytosol.

- Successfully cloned BirA gene fragment of 975bps in pET28a vector at BamHI/ XhoI restriction enzymes site.
- Two of the tested clones of pET28a-BirA were released expected gene fragment confirming the gene integration.
- Small scale over expression was carried out in BL21(DE3) star cells at 37°C, 25°C and 16°C.
- BirA was expressed at all temperatures tested (37°C, 25°C and 16°C) in soluble form, also confirmed with anti-poly histidine polyclonal antibodies in Western blot.

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