Protein Expression and Purification 76 (2011) 221–228

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Intein-mediated one-step purification of *Escherichia coli* secreted human antibody fragments

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ARTICLE INFO

Article history:
Received 31 August 2010
and in revised form 13 December 2010
Available online 16 December 2010

Keywords:
Intein
Chitin-binding domain
Escherichia coli secretion
Recombinant protein purification
Self-cleaving affinity tag
Disulfide bonds

ABSTRACT

In this work, we apply self-cleaving affinity tag technology to several target proteins secreted into the *Escherichia coli* periplasm, including two with disulfide bonds. The target proteins were genetically fused to a self-cleaving chitin-binding domain–intein tag for purification via a chitin–agarose affinity resin. By attaching the intein-tagged fusion genes to the PelB secretion leader sequence, the tagged target proteins were secreted to the periplasmic space and could be recovered in active form by simple osmotic shock. After chitin-affinity purification, the target proteins were released from the chitin-binding domain tag via intein self-cleaving. This was induced by a small change in pH from 8.5 to 6.5 at room temperature, allowing direct elution of the cleaved target protein from the chitin affinity resin. The target proteins include the *E. coli* maltose-binding protein and β-lactamase enzyme, as well as two human antibody fragments that contain disulfide bonds. In all cases, the target proteins were purified with good activity and yield, without the need for refolding. Overall, this work demonstrates the compatibility of the ΔI-CM intein with the PelB secretion system in *E. coli*, greatly expanding its potential to more complex proteins.

Introduction

When producing therapeutic proteins, such as monoclonal antibodies (usually IgG) or human cytokines, correct disulfide bond formation and/or glycosylation patterns are often required for activity [1]. Proteins that require these modifications must typically be produced in mammalian or other eukaryotic cells. Currently, therefore, more than 60% of all therapeutic recombinant protein products on the market are produced in mammalian cell lines, with Chinese Hamster Ovary (CHO) cells being the most popular and well-developed system [2]. Although mammalian cells have the ability to produce complex and highly modified proteins in good yield, mammalian cell culture processes are slow, costly, and require long cell line development times. Thus, when dealing with simpler protein targets, *Escherichia coli* (*E. coli*) is a more attractive alternative. Although *E. coli* cannot make many post-translational modifications, it does have the ability to secrete properly folded and disulfide-bonded proteins through a number of secretion pathways [3–5]. These secretion pathways aid proper folding and disulfide bond formation of the target protein, allowing expression of more complex targets, such as tissue plasminogen activator (tPA) [6] and single-chain antibodies (scFv) [7,8].

Downstream recovery and purification steps typically contribute the bulk of the overall production costs for most therapeutic proteins. One way to simplify these steps is through the use of affinity-tag methods, which have become very popular due to their universal applicability and good product purity [9]. In order to obtain a native product, however, the affinity tag must be cleaved from the target protein after the purification. Although many tag removal methods have been developed, they each have their own strengths and weaknesses, and therefore no truly universal method has been developed [9,10].

In the last decade, several self-cleaving protein modules have been developed and combined with conventional affinity tags to create new and simple affinity purification methods [10]. In particular, a number of engineered self-cleaving inteins have been successfully used in bioseparation processes [11–15]. In practice, the self-cleaving reaction can be induced at the intein's N-terminus by thiol addition [11,16] or its C-terminus by a mild pH shift [17]. The pTWIN vectors and the IMPACT™ system from New England Biolabs are the most published commercial intein systems to date, and are often paired with a chitin-binding domain as the affinity tag [11,16]. A majority of the NEB systems are based on thiol-induced inteins, which can be induced by compounds including 2-mercaptoethane sulfonic acid (MESNA), hydroxylamine, thiophenol, β-mercaptoethanol, 1,4-dithiothreitol (DTT) or free cysteine [11,18]. Typically, 15–30 mM DTT addition is used to trig-

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doi:10.1016/j.pep.2010.12.004

ger the cleaving reaction for N-terminally cleaving inteins [11]. This concentration of DTT will generally reduce disulfide bonds in proteins containing them, effectively inactivating those targets. Other compounds such as MESNA, hydroxylamine or free cysteine can be also used as cleaving triggers, but they tend to leave modifications at the C-terminus of the target protein [18], which could affect product activity in some cases. Therefore, for disulfide-bond-containing protein targets, the thiol-induced inteins are not ideal unless the target protein can maintain activity after thiol treatment and extra modifications at the C-terminus can be tolerated.

An efficient C-terminal cleaving intein is the ΔI-CM intein derived from the *Mycobacterium tuberculosis recA* intein [17]. It is 18 kDa in size, and has been paired with conventional affinity tags [19,20] as well as non-chromatographic purification tags [21–23]. Compared to the DTT-induced inteins, the cleaving activity of the ΔI-CM intein is induced by a mild pH change from pH 8.5 to pH 6.0–6.5 [17,20], suggesting its compatibility with disulfide-bonded targets. The ΔI-CM intein is also temperature dependent [20], allowing the purification conditions to be adjusted according to the needs of each specific target.

In this work, we demonstrate the use of the self-cleaving ΔI-CM intein for the expression and purification of recombinant affinity-tagged proteins secreted in *E. coli* cells. This intein provides complete tag removal triggered by a simple pH change, potentially allowing its use with targets that contain disulfide bonds. By fusing intein-tagged test proteins to a PelB leader sequence, we were able to target them to the *E. coli* periplasmic space, allowing the recovery of properly folded and disulfide-bonded targets by simple osmotic shock. These proteins were then affinity purified using a chitin-binding domain (CBD) tag, followed by pH-induced self-cleavage of the CBD tag. The targets include *E. coli* maltose-binding protein (MBP) and β-lactamase, as well as a single disulfide-bonded human domain antibody composed of the variable region of the heavy chain (V_H) portion of a single chain antibody (scFv), and a human light chain fragment with two disulfide bonds. Overall, this work presents the first examples of intein-mediated purifications of human antibody fragments expressed in *E. coli*, and demonstrates the potential use of self-cleaving inteins for the purification of disulfide-bond-containing therapeutic proteins.

Material and methods

Plasmid construction

The DNA sequence encoding the chitin-binding domain (CBD) in fusion to the ΔI-CM intein was PCR-amplified by forward primer 5′-atcgccatggatatgacgacaaatcctggtgtatc-3′ and reverse primer 5′-atc gctgcagctgcacctgcatgttgtgtacaacaaccccttc-3′ from the template plasmid pET/CI-aFGF [19]. The resulting DNA segment was digested with NcoI/PstI and ligated into NcoI/PstI cut pET27b/PNNL-EGF 5-2 V_H dAb [7], resulting in plasmid pET27b/PelB-CI-EGF V_H dAb. This plasmid encodes a single fusion protein comprising the PelB leader sequence, CBD, ΔI-CM intein, and a domain antibody (EGF V_H dAb) that is composed of the V_H fragment of the scFv against human epidermal growth factor (EGF). The protein sequence is followed by c-myc, HSV and 6× His tags to facilitate detection and activity assays for this target protein (Fig. 1, and see ELISA assay method below). The genes encoding the maltose-binding protein (MBP) and β-lactamase enzyme (β-lac) were digested with BsrGI and HindIII from the plasmids pET/EI-MBP and pET/EI-β-lac [23] and ligated into the same sites in pET27b/PelB-CI-EGF V_H dAb to replace the gene encoding EGF V_H dAb. The resulting plasmids are referred to as pET27b/PelB-CI-MBP and pET27b/PelB-CI-β-lac. The cDNA encoding human light chain κ (hLCκ), the κ subtype of the light chain fragment of the human immunoglobulin, was acquired from BAC B.V. (Naarden, The Netherlands). The hLCκ gene was PCR-amplified using the forward primer 5′-atcgtgtacacaacatgga aattgtgatgactcagtctc-3′ and the reverse primer 5′-atcgaagcttttag ctctctcccctgttgaagc-3′. The resulting PCR product was digested with restriction enzymes BsrGI and HindIII, and ligated into BsrGI/HindIII cut plasmid pET27b/PelB-CI-EGF V_H dAb to replace EGF V_H dAb, resulting in pET27b/PelB-CI-hLCκ.

To examine the expression of CBD–intein-tagged fusion proteins under the control of the P_{tac} promoter, the fusion protein genes with the PelB leader were moved to the pMAL-c2x vector (New England Biolabs) by NdeI and HindIII restriction sites. This resulted in deletion of the encoded MBD in the native pMAL, and its replacement with the PelB–CBD–intein fusion protein cassettes. The resulting plasmids are referred to as pMAL/PelB-CI-EGF V_H dAb, pMAL/PelB-CI-MBP, pMAL/PelB-CI-β-lac, and pMAL/PelB-CI-hLCκ.

Plasmid transformation, protein expression and periplasmic protein recovery

The expression vectors were transformed into *E. coli* BLR (DE3) (Novagen) or Rosetta (DE3) (Novagen). For expression, single colonies were used to inoculate 5 mL LB medium supplemented with 100 μg/mL of ampicillin (for pMAL-based expression vectors) or 20 μg/mL kanamycin (for pET27b-based expression vectors) and grown at 37 °C overnight as seed cultures. The next day, 1 mL of each seed culture was used to inoculate 100 mL of 2 × LB medium (20 g tryptone 10 g yeast extract, 10 g NaCl per L, pH 7.0), supplemented with 100 μg/mL ampicillin or 20 μg/mL kanamycin. Cultures were grown at 37 °C for 2.5 h, and then equilibrated to 20 °C for 30 min before induction by 0.1–1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG). Precursor proteins were expressed at 20 °C for 16–24 h, followed by cell harvest by centrifugation at 3000*g* and 4 °C for 10 min.

The cell pellets were resuspended in 0.2 times the initial culture volume of ice-cold sucrose–EDTA buffer (30 mM Tris–HCl pH 8.5, 20% w/v sucrose, 2 mM EDTA), followed by incubation on ice for 20 min. Afterward, the cells were pelleted by centrifugation for 45 min at 3000*g* and 4 °C. The supernatant was decanted, and the pellets were resuspended in 0.2 times the initial culture volume of 0.2 mM $MgCl_2$, incubated on ice for 20 min, and centrifuged for 30 min at 3000*g* and 4 °C. Target proteins were purified from the recovered supernatant.

For β-lactamase experiments where clarified lysate samples were taken, half of the cell pellet was used for periplasmic recovery as above, using 0.1 times initial culture volume for resuspension. The other half of the cell pellet was resuspended in 0.1 times initial culture volume of ice cold lysis buffer (10 mM Tris–HCl, pH 8.5, 2 mM EDTA) and frozen at −20 °C overnight. Cells were subsequently thawed and sonicated on ice (typically 3–4 pulses of 15 s each at a power setting of 0.4–0.5 W RMS). Cell lysates were clarified by centrifugation at 14,000*g* for 20 min at 4 °C.

Chitin column purification for maltose-binding protein, β-lactamase and EGF V_H dAb

To avoid premature intein cleavage and non-specific protein–protein association, all of the following purification steps were performed at 4 °C with ice-cold buffers. For high salt purification conditions, the periplasmic extract was adjusted to 20 mM Tris–HCl pH 8.5, 500 mM NaCl, 1 mM EDTA by addition of 0.2 mL 1 M Tris–HCl, 1 mL 5 M NaCl and 20 μL 500 mM EDTA per 10 mL of periplasmic fraction. For low-salt purification conditions, the periplasmic extract was adjusted to 20 mM Tris–HCl pH 8.5, 50 mM NaCl, 2 mM EDTA by addition of 0.2 mL 1 M Tris–HCl, 0.1 mL 5 M NaCl and 40 μL 500 mM EDTA per 10 mL of periplasmic fraction. The extracts were then loaded onto a column packed with 2 mL chitin–agarose resin (New England Biolabs). After loading,