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A simple method for recombinant protein purification using self-assembling peptide-tagged tobacco etch virus protease

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ABSTRACT

Recombinant protein purification remains to be a major challenge in biotechnology and medicine. In this paper we report a simple method for recombinant protein purification using self-assembling peptide-tagged tobacco etch virus protease (TEVp). After construction of an *N*-terminal ELK16 peptide fusion expression vector, we expressed ELK16-TEVp fusion protein in *E. coli*. SDS-PAGE analysis showed that ELK16-TEVp was expressed as active protein aggregates which could be purified to 91% purity with 92% recovery by centrifugation in the presence 0.5% Triton X-100. By using His-tagged bovine interferon-γ (His-BoIFN-γ) as the substrate, we demonstrated that EKL16-TEVp had a protease activity of 1.3×10^4 units/mg protein with almost 100% cleavage efficiency under the optimized conditions. More importantly, EKL16-TEVp could be removed from the cleavage reaction by single-step centrifugation. After removing the His-tag by nickel-conjugated agarose bead absorption, the recombinant BoIFN-γ (rBoIFN-γ) was purified to 98.3% purity with 63% recovery. The rBoIFN-γ had an antiviral activity of 1.6×10^3 units/mg protein against vesicular stomatitis virus. These data suggest that ELK16-TEVp may become a universal tool for recombinant protein purification.

1. Introduction

Recombinant proteins are often fused to various tags to facilitate their detection and purification, increase yields and/or enhance solubility. However, it is almost always desirable to obtain the native protein free of fusion partner since most fusion tags are expected to interfere with the structural studies and/or the biological activity of the target protein [1,2]. This is achieved commonly by introducing a protease recognition sequence and then cleaving at the junction between the fusion tag and the target protein. The commonly used proteases for this purpose include thrombin, factor Xa, enterokinae and tobacco etch virus protease (TEVp). Among them, TEVp is a popular protease targeting the recognition sequence ENLYFQG/S with several advantages, including stringent sequence specificity, overproduction in *E. coli* and adaptability to different buffer conditions [3–5].

As the tool for fusion tag removal, wild type TEVp suffers from a few intrinsic defects, including poor expression, low solubility in *E. coli* and self-activation to generate a truncated enzyme with diminished activity [6]. Therefore, various approaches have been investigated to overcome these limitations and several TEVp mutants have been generated, including S219V or S219N mutant with significantly reduced auto-proteolysis, L56V/S135G double mutant, T17S/N68D/I77V triple mutant and T17S/L56V/N68D/I77V/S135G quintuple mutant with improved solubility and thermal stability [6–9]. Although these TEVp mutants have been expressed in *E. coli* as 6× His, glutathione S-transferase (GST) or lectin fusion protein with high enzyme activity, their purification from bacterial extracts and removal from the cleavage reactions require expensive affinity chromatographic columns [10–12].

Bacterially expressed inclusion bodies (IBs) are generally considered to be mis-folded protein aggregates without biological activities. However, recent studies have revealed that some protein aggregates, known as non-classical or active IBs, contain correctly folded and functionally active proteins [13–15]. More recently, several self-assembling peptides have been used as the efficient inducer of active protein aggregates [16–18]. Compared with other aggregating fusion partners, self-assembling peptides are much

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smaller in size and structurally simple, and have higher "pull-down" efficiencies. For example, self-assembling peptide ELK16 $(LELELKLK)_2$ can spontaneously form β-sheet structure in aqueous solution, and function as an inducer of active protein aggregates when terminally attached to model proteins, including lipase A, amadoriase II, β-xylosidase and green fluorescent protein [17]. Therefore, self-assembling peptide-induced active aggregation is expected to have attractive application potentials in biotechnology, including *in-situ* immobilization of carrier-free enzymes, production of toxic proteins, and protein expression and purification [18]. In this study, we developed a simple method for recombinant protein purification by using self-assembling peptide ELK16-tagged TEVp (ELK16-TEVp). The proof of concept was provided by purifying recombinant bovine interferon-γ (rBoIFN-γ), which has been used to control many infectious cattle diseases [19–21]. We demonstrated that the ELK16-TEVp could be expressed as active protein aggregates and purified to a high purity with high protease activity.

2. Materials and methods

2.1. Vector construction

To construct an *N*-terminal self-assembling peptide fusion expression vector, the coding sequence for ELK16 peptide flanked by 17 proline (P) or threonine (T) rigid linkers [17] was adapted to *E. coli* codon usage using JAVA Codon Adaption Tool [22]. The synthetic sequence was cloned into pET-30a vector (Novagen, WI, USA) as a *Bgl*II/*Kpn*I segment, and the resultant vector was called pET-P16P.

To construct an ELK16-TEVp expression vector, the coding sequence for T17S/L56V/N68D/I77V/S135G quintuple mutant of TEVp [9] was adapted to *E. coli* codon usage, and the synthetic sequence was cloned into pET-P16P vector as an *Eco*RI/*Xho*I segment. The resultant vector was called pP16P-TEVp (Fig. 1a).

To construct a His-BoIFN-γ expression vector, the coding sequence for the mature peptide of BoIFN-γ with a TEVp recognition sequence at the 5′ end was amplified from pGEX-BoIFN-γ vector [23] by PCR using rTaq DNA polymerase (TaKaRa, Dalian, China), and the forward primer FP (5′-GAGGATCCGAAAACCTG-TACTTCCAGGGTCAGGGCCAATTTTTTAGAGAA-3′) and reverse primer RP (5′-TACTCGAGTTACGTTGATGCTCTCCGGCC-3′). The PCR product was cloned into pET-30a vector as a *Bam*HI/*Xho*I segment, and the resultant vector was called pET-BoIFN-γ (Fig. 2a).

2.2. Protein expression

pP16P-TEVp or pET-BoIFN-γ vector was transformed into *E. coli* strain BL21 (DE3), and the Luria broth culture supplemented with 50 μg/mL kanamycin was grown overnight at 37 °C in an orbital shaker. The cell culture was diluted (1:100) in 2 × YT medium (10 g yeast extract, 16 g tryptone, 5 g NaCl/L) supplemented with the same antibiotic, and grown to OD_{600} 0.6 at 37 °C. The expression of fusion protein was induced with different concentrations of IPTG for different times at different temperatures.

2.3. Cell lysis

After IPTG induction, cells were harvested by centrifugation for 10 min at 6,000*g* at 4 °C, washed two times in lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 5% glycerol, pH7.2), and disrupted in the lysis buffer by sonication treatment (40 w, 10 s, 20 s intervals, 5 min). After centrifugation for 10 min at 6,000*g* (ELK16-TEVp) or 12,000*g* (His-BoIFN-γ), the crude cell extract was collected for protein purification.

2.4. Purification of ELK16-TEVp

ELK16-TEVp was purified using the method for active protein aggregate purification [17] with slight modification. For the pilot experiment, the expression of ELK16-TEVp was induced with 0.2 mM IPTG for 6 h at 30 °C. Cells were harvested from 50 mL culture, and lyzed in 5 mL lysis buffer by sonication treatment. ELK16-TEVp was precipitated by centrifugation for 10 min at different centrifugation forces at 4 °C. For the larger scale purification, the expression of ELK16-TEVp was induced for 16 h at optimized conditions. A total of 4 g wet weight cells was harvested from 500 mL culture and lyzed in 50 mL lysis buffer by sonication treatment. ELK16-TEVp was precipitated by centrifugation for 10 min at optimized centrifugation force at 4 °C. After washing two times in 50 mL lysis buffer containing 0.5% Triton X-100, the purified ELK16-TEVp was washed one time with 50 mL TEVp cleavage buffer (50 mM Tris-HCl, pH8.0, 0.5 mM EDTA, 1 mM DTT), and dissolved in 5 mL TEVp cleavage buffer.

2.5. Purification of His-BoIFN-γ

His-BoIFN-γ was purified using Ni-NTA Agarose Column (Qiagen, USA) under native conditions by following the product instruction. One milliliter of Ni-NTA slurry (50%) was loaded into a column (1 mL), and equilibrated with 8 mL lysis buffer. The column was loaded with 5 mL cleared bacterial lysate from 50 mL culture (0.95 g wet weight), and washed two times with 8 mL washing buffer containing 40 mM imidazole. His-BoIFN-γ was eluted with 5 mL elution buffer containing 250 mM imidazole. The purified protein was dialyzed in 25 mL TEVp cleavage buffer for 9 h at 4 °C with 3 buffer changes. After centrifugation for 10 min at 16,000*g* at 4 °C, His-BoIFN-γ was diluted to 500 μg/mL in TEVp cleavage buffer.

2.6. Protease cleavage and target protein recovery

The protease cleavage reaction (100 μL) consisted of different amounts of ELK16-TEVp or GST-TEVp (BBI Life Sciences, USA) and His-BoIFN-γ in the presence or absence of different additives. The cleavage reaction was carried out for 1 or 2 h at different pH at different temperatures. After cleavage, ELK16-TEVp was removed by centrifugation for 10 min at 16,000*g* at 4 °C. Following the absorption with an equal volume of 50% Ni-NTA agarose slurry (Qiagen, USA) for 30 min at 4 °C, His-tag was removed by an additional round of centrifugation. The cleavage reaction was followed by 12% or 15% SDS-PAGE analysis. After Commassie blue staining, the protein bands of interest were scanned using the Image Lab™ Software associated with GelDoc XR System (Bio-Rad, USA). The cleavage efficiency (%) was calculated according to the percent ratio of BoIFN-γ/His-BoIFN-γ band densities. One unit of ELK16-TEVp was defined as ≥ 85% cleavage of 3 μg substrate within 1 h at optimized conditions. The purified BoIFN-γ was submitted to 1 cycle of Triton X-114 isothermal extraction to remove residual endotoxin [24]. The purified protein was mixed with 1% Triton X-114 (final concentration), incubated for 10 min on ice and then 5 min at 37 °C. After centrifugation for 1 min at 12,000*g* at 30 °C, the upper aqueous phase was collected for antiviral assay.

2.7. Antiviral assay

The antiviral activity of purified rBoIFN-γ against vesicular stomatitis virus (VSV) was determined on MDBK cells (ATCC, USA) as described previously [25]. Briefly, the cells were seeded on 96-well plates, grown to 90% confluence and serial dilutions of rBoIFN-γ were added in triplicates. After incubation for 24 h at 37 °C, an optimal concentration (100 $TCID_{50}$) of VSV was added. After