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Expression and purification of the calcium binding photoprotein mitrocomin using ZZ-domain as a soluble partner in *E. coli* cells

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ABSTRACT

We constructed a vector for soluble protein expression in the cytoplasm of *Escherichia coli* cells using the cold induced system. The vector, named pCold-ZZ-P-X, consists of a histidine tag sequence, IgG binding domain of protein A (ZZ domain), the cleavage site of human rhinovirus 3C protease followed by the multiple cloning sites under the controlled of the cold shock protein A (*csp*A) promoter and the *lac* operator. Using this expression vector, the calcium binding photoprotein mitrocomin from luminous jellyfish was successfully expressed as a soluble ZZ fusion protein and purified. After removing the ZZ domain by protease digestion, recombinant apomitrocomin was obtained and then regenerated to mitrocomin by incubation with coelenterazine. The luminescence properties of recombinant mitrocomin were characterized and compared to other photoproteins including aequorin, clytin-I and clytin-II.

Introduction

The calcium binding photoprotein emits visible light by an intramolecular reaction in the presence of Ca^{2+}. The cDNA clones of many calcium binding photoproteins from luminous coelenterates including aequorin [1], clytin-I [2], clytin-II [3], mitrocomin [4] and obelin [5] have been isolated. All these photoproteins are a complex of apoprotein with 2-peroxide of coelenterazine [6]. Among these photoproteins, aequorin is the most studied [6–8] and has been applied to immunoassays development [9] and the drug screening systems [10]. At present time, the apoproteins of photoproteins have been expressed in *Escherichia coli* cells and the highly purified proteins were obtained, except for mitrocomin [4]. Previously, we showed that apoprotein of aequorin was highly expressed as a soluble form into the periplasmic space using the signal peptide sequences of the outer membrane protein A (OmpA) for secretion [11,12]. Similarly, previous our reports have shown that *Renilla* luciferin binding protein [13], apoclytin-I [14] as well as apoclytin-II [3] were expressed efficiently as a soluble form with six histidine-tagged sequence in the periplasmic space of *E. coli* cells. On the other hand, apoobelin was expressed in the cytoplasm as inclusion bodies [15].

Mitrocomin (also known as Halistaurin) was isolated from the luminous jellyfish *Mitrocoma cellularia* (formerly *Halistaura mitrocoma*) [16,17] and the primary structure of mitrocomin consists of 190 amino acid residues containing six cysteine residues, with three EF-hand domains for Ca^{2+} binding and a tyrosine residue at the carboxyl terminus [4]. Previously, we examined the apomitrocomin expression in the cytoplasm or periplasmic space of *E. coli* cells using several bacterial expression systems and the luminescence activity of mitrocomin was detected in the crude extracts of *E. coli* cells by adding of Ca^{2+}. However, the expression level of apomitrocomin was below 0.01 μg per ml of cultured cells. To characterize the luminescent properties of recombinant mitrocomin comparing with other photoproteins, a few milligrams of mitrocomin is required. Recently, we demonstrated that the fusion proteins with the synthetic IgG-binding domain (ZZ domain: 116 amino acid residues) of Protein A derived from *Staphylococcus aureus* are expressed as a soluble protein in the cytoplasm of *E. coli* cells using the cold induced expression system [18]. The ZZ domain in the fused protein plays an important role as a solubilizing partner and a protein stabilizer in the cytoplasm of *E. coli* cells at low temperature. The ZZ domain fusion to cysteine-rich proteins shows more effective solubilization in *E. coli* cells [18].

To purify the expressed protein without the ZZ domain, usually it is applied twice to nickel chelate columns (Fig. 1). Firstly, the soluble fraction of ZZ-fused protein expressed in *E. coli* cells at 15 °C is directly applied to the first nickel chelate column. Secondly, the histidine-tagged ZZ fusion protein eluted from the column is digested with a specific proteinase. Then, the digested fraction of ZZ fusion protein is applied on the second nickel chelate column and the flow-through fraction containing the target protein is recovered. The undigested histidine-tagged ZZ protein and the cleaved histidine-tagged ZZ domain are adsorbed to the nickel chelate column. To make this process much easier, we constructed a new expression vector, pCold-ZZ-P-X, which contains a protease cleavage site for removal of the ZZ domain (Fig. 1). The pCold-ZZ-P-X vector is a derivative of pCold-ZZ-X [18] and consists of a histidine tag se-

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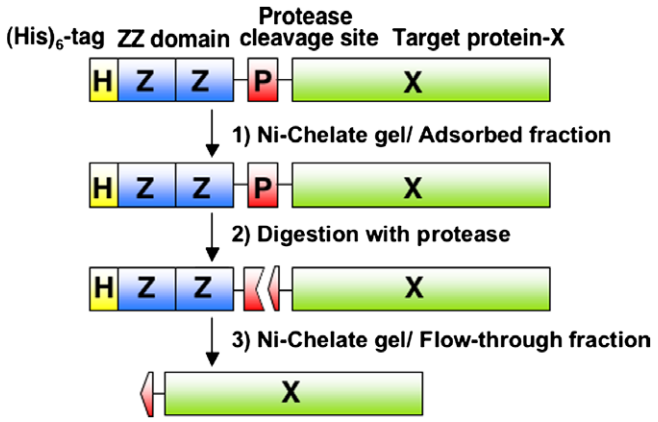

Fig. 1. Purification of a target protein from ZZ domain fusion protein expressed in *E. coli* cells.

quence for the nickel chelate affinity purification, an IgG binding domain of protein A (ZZ domain), and the cleavage site of human rhinovirus 3C protease followed by the multiple cloning sites (Fig. 2).

In this paper, we constructed the apomitrocomin expression vector, pCold-ZZ-P-MI, and expressed ZZ-apomitrocomin in *E. coli* cells at 15 °C. After the cleaving of the ZZ domain, the apomitrocomin without ZZ domain was purified and recombinant mitrocomin was prepared and characterized.

Materials and methods

Materials

The recombinant aequorin [12], clytin-I [14] and clytin-II [3] were prepared as previously described. The sources of chemicals were as follows: ethylenediaminetetraacetic acid disodium salt (EDTA · 2Na),[1] $CaCl_2 \cdot 2H_2O$, 100 mM $CaCO_3$ standard solution, tris(hydroxymethyl)aminomethane, (±)-dithiothreitol (DTT), 2-mercaptoethanol, imidazole, $NiSO_4 \cdot 6H_2O$, $(NH_4)_2SO_4$ (Wako Pure Chemicals, Osaka, Japan), *n*-coelenterazine (*n*-CTZ); chelate Sepharose Fast Flow, PreScission protease (GE-Healthcare Bio-Science, Piscataway, NJ); *cp*-coelenterazine (*cp*-CTZ), *hcp*-coelenterazine (*hcp*-CTZ), *f*-coelenterazine (*f*-CTZ) and *fcp*-coelenterazine (*fcp*-CTZ) (Promega, Madison, WI); coelenterazine (CTZ), *h*-coelenterazine (*h*-CTZ) and *Bis*-coelenterazine (*Bis*-CTZ) (Chisso, Yokohama, Japan); *e*-Coelenterazine (*e*-CTZ) and methoxycoelenterazine (*MeO*-CTZ) was kindly provided by Dr. K. Teranishi (Mie Univ., Japan).

Construction of pCold-ZZ-P-X vector

The cold induced expression vector, pCold-ZZ-P-X, consists of a histidine tag sequence for a nickel chelate affinity chromatography, the cleavage sequence of human rhinovirus 3C protease (PreScission protease) between ZZ domain and a target protein, followed by multiple cloning sites (EcoRI/XhoI/HindIII/SalI/PstI/XbaI). The vector was constructed from pCold-ZZ-X [18] as follows. The recognition sequence of Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro for PreScission protease was inserted into the BamHI–EcoRI site of pCold-ZZ-X using the synthetic linkers of PreScission B/E–F (5′ GA TCT CTG GAA GTT CTG TTC CAG GGG CCC G 3′) and PreScission B/E–R (5′ AA TTC GGG CCC CTG GAA CAG AAC TTC CAG A 3′) to give pCold-ZZ-P-X (Fig. 2B). The vector contains the cold shock protein A promoter (cspA) controlled by the *lac* operator [19].

Construction of apomitrocomin expression vector, pCold-ZZ-P-MI

To express apomitrocomin in *E. coli* cells, pCold-ZZ-P-MI was constructed as follows. The coding region of mitrocomin was obtained from pMI-17 [4] as a template by PCR amplification (25 cycles; 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) using Ex-Taq polymerase with primer sets of MI-5N/EcoRI(5′ ggc GAA TTC GTC AAG CTT ACG ACT GAC TTT 3′;EcoRI site underlined)and pCold-R primer(5′ GGC AGG GAT CTT AGA TTC TG 3′). The resultant fragment digested with EcoRI and XhoI was inserted into the same sites of pCold-ZZ-P-X to give pCold-ZZ-P-MI (Fig. 2A). The nucleotide sequence was determined with an Applied Biosystems model 310 DNA sequencer using a BigDye terminator v1.1 cycle sequencing kit.

Expression and purification of apomitrocomin from bacterial cells

(i) Expression of His-ZZ-P-apomitrocomin (His-ZZ-P-apoMI) as a soluble form in E. coli cells

The host *E. coli* strain used was BL21 (Novagen, Madison, WI). The seed culture of the bacterial strain possessing pCold-ZZ-P-MI vector was grown in 10 ml of Luria-Bertani broth containing ampicillin (50 μg/ml) at 37 °C for 18 h. This seed culture was transferred into 400 ml of LB broth in a 3L flask, incubated for 3 h and then cooled on an ice-water bath for 1 h. After adding of IPTG at the final concentration of 0.2 mM to the culture medium, the bacterial cells were incubated at 15 °C for 17 h. The cells were harvested by centrifugation at 3000g for 5 min from 800 ml culture medium and the pellet was suspended in 80 ml of 50 mM Tris–HCl (pH 7.6), and disrupted by sonication using a Branson model 250 sonifier (Danbury, CT) 3 times for 3 min on ice. After centrifugation at 12,000g for 20 min, the supernatant containing His-ZZ-P-apoMI was applied on a nickel chelate column (column size; 2.5 × 6 cm), equilibrated with 20 mM Tris–HCl (pH 7.6). After washing with 250 ml of 50 mM Tris–HCl (pH7.6), His-ZZ-P-apoMI was eluted with 0.1 M imidazole. The yield of His-ZZ-P-apoMI was 51.7 mg from 800 ml of cultured cells with over 95% purity on SDS–PAGE analysis.

(ii) Digestion of His-ZZ-P-apoMI with PreScission protease

To determine the optimal digestion conditions His-ZZ-P-apoMI (2.2 mg) eluted from a nickel chelate column was digested with various amounts of PreScission protease (1, 3, 10 and 20 μg) in 1 ml of 50 mM Tris–HCl (pH 7.0) containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT at 4 °C for 18 h. The digestion of ZZ domain was analyzed by SDS–PAGE. For apomitrocomin purification, 6 μg of PreScission protease were used for 9.3 mg of His-ZZ-P-apoMI in 3 ml of the reaction mixture.

(iii) Purification of apomitrocomin

To separate apomitrocomin from the cleaved ZZ domain and undigested His-ZZ-P-apoMI by PreScission protease, the digests (9.3 mg of His-ZZ-P-apoMI in 3 ml) were directly applied on a nickel chelate column (column size; 0.5 × 6 cm), equilibrated with 20 mM Tris–HCl (pH7.6). The flow-through fractions (9 ml) containing apomitrocomin were collected and the purity was determined by SDS–PAGE analysis.

Determination of luminescence activity of mitrocomin and semi-synthetic mitrocomins

The regeneration to mitrocomin and semi-synthetic mitrocomin from apomitrocomin (1 μg) were performed by incubation with 1 μg coelenterazine or coelenterazine analogue (1 μg/μl in

[1] *Abbreviations used:* apomitrocomin, apoprotein of mitrocomin; EDTA, ethylenediaminetetraacetic acid; histidine-tagged ZZ domain fused apomitrocomin, His-ZZ-P-apoMI; FWHM, a full width at half maximum; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; rlu, relative light units; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.