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A two-step purification strategy using calmodulin as an affinity tag

Lianyun Lin[a,b,c,d,1], Chen Liu[b,1], Bidhan Chandra Nayak[b,1], Weiyi He[a,c,d], Minsheng You[a,c,d,*], Zhiguang Yuchi[a,b,*]

[a] State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, Institute of Applied Ecology, Fujian Agriculture and Forestry University, Fuzhou, 350002, China
[b] Tianjin Key Laboratory for Modern Drug Delivery & High-Efficiency, Collaborative Innovation Center of Chemical Science and Engineering, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, 300072, China
[c] Joint International Research Laboratory of Ecological Pest Control, Ministry of Education, Fuzhou, 350002, China
[d] Fujian-Taiwan Joint Centre for Ecological Control of Crop Pests, Fujian Agriculture and Forestry University, Fuzhou, 350002, China

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ABSTRACT

Calmodulin (CaM) is a Ca^{2+}-binding protein that plays an important role in cellular Ca^{2+}-signaling. CaM interacts with diverse downstream target proteins and regulates their functions in a Ca^{2+}-dependent manner. CaM changes its conformation and hydrophobicity upon [Ca^{2+}] change and consequently changes its interaction with CaM-binding domains from the targets. Based on these special properties of CaM, it was used as an affinity tag to develop a novel purification strategy by using it for two sequential orthogonal purification steps: 1) an affinity purification step, in which CaM-tag interacts with an immobilized CaM-binding domain; and 2) a hydrophobic interaction chromatography step, during which CaM binds to a phenyl sepharose column. In both steps, the CaM-tagged protein binds in the presence of Ca^{2+} and unbinds in the presence of ethylenediaminetetraacetic acid (EDTA). An optional third step can be added to remove the CaM-tag if necessary. We used green fluorescent protein (GFP) as a test protein to demonstrate the effectiveness of the method. High yield and high purity of GFP with proper function was obtained using this novel strategy. We believe that this method can be applied to a wide range of protein targets for structural and functional studies.

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1. Introduction

Affinity tags play vital roles in many research areas of life science, especially in the purification of recombinant proteins. The primary advantage of using affinity tags in protein purification is their reversible and removable nature. At present, although a handful of different affinity tags have been widely used, none is universal [1]. All affinity tags have their pros and cons. Most commonly used affinity tags include histidine (His) tag, glutathione-S-transferase (GST) tag and Maltose binding protein (MBP) tag. Despite their use in routine purification workflows, no single tag can be used in orthogonal purification steps.

Calmodulin (CaM) is a small calcium binding protein (~17 kDa) comprised of 148 amino acid residues. It is a highly conserved dumbbell-shape protein consisting of two lobes, N-lobe and C-lobe, connected by a linker region [2]. CaM is a vital calcium ion sensor for various target proteins including ion channels, kinases and phosphatases [3–7]. It plays important roles in muscle contraction, inflammation, memory and immune response [8–16]. CaM possesses the capability to bind targeting proteins with high affinity (Kd in pM to μM range). The CaM-binding domains (CBD) within the targeting proteins share some unique signature structural features, including the α-helical propensity and specific distribution of hydrophobic residues [17–21]. In many cases, the binding property changes in the presence or absence of calcium ions, which makes the binding a reversible event [22]. In addition, calcium binding induces a conformational change in CaM, transforming it from a hydrophilic structure to a more hydrophobic state, which changes its solubility and the binding affinity to hydrophobic columns [23,24].

In the present study, we utilized the reversible interaction between CaM and CBD in addition to the hydrophobicity change of CaM upon Ca^{2+}-binding to develop a novel two-step purifica-

* Corresponding authors at: State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, Institute of Applied Ecology, Fujian Agriculture and Forestry University, Fuzhou, 350002, China, and Tianjin Key Laboratory for Modern Drug Delivery & High-Efficiency, Collaborative Innovation Center of Chemical Science and Engineering, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, 300072, China.
E-mail addresses: msyou@iae.fjau.edu.cn (M. You), yuchi@tju.edu.cn (Z. Yuchi).
[1] These authors contributed equally to this work.

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tion strategy using CaM as an affinity fusion tag. The CBD we chose is from an intracellular calcium-release channel, ryanodine receptor (RyR) [25]. RyR opening is regulated by cytosolic and luminal calcium concentration, and CaM has a major contribution to the fine-tuning of the channel activity [26–35]. There are three CBDs in RyR1 (skeletal isoform). We chose CBD1 as the bait because of the good reversibility of the interaction between CBD1 and CaM upon [Ca^{2+}] change [36]. We demonstrate the effectiveness of our strategy by purifying the green fluorescent protein (GFP) as a test protein so that the folding and activity of the target can be monitored by the fluorescence signals.

Earlier reported studies have shown the use of the CaM-CBD interaction as an affinity binding tag, but the usage was confined to phage display sorting [37]. Our strategy aims to use CaM as an affinity tag in a two-step tandem purification workflow, which would be more efficient and convenient than the existing protocols. This concept would have potential application in yielding highly pure protein required for structural and functional studies.

2. Materials and methods

2.1. Materials

DNA fragments of CBD, CaM, and GFP, codon optimized for *E. coli* expression and DNA primers for PCR amplification were synthesized from GENEWIZ (Suzhou, China). Q5 DNA polymerase, restriction enzymes, and DNA ladder were purchased from New England Biolabs (Ipswich, MA, USA); E.Z.N.A gel extraction kit and plasmid extraction kit were purchased from Omega Bio-Tek (Norcross, GA, USA); T4 DNA polymerase was purchased from Novagen (Darmstadt, Germany); Luria Bertani (LB) – broth and agar were purchased from Oxoid (Basingstoke, Hampshire, UK); Opti-Protein XL protein marker were purchased from ABM (Richmond, BC, Canada). HisTrap (NiNTA) column, amylose resin and HiPrep 16/60 Superdex 75 gel filtration column were purchased from GE Healthcare (Little Chalfont, UK); Talon resin was purchased from Clonetech laboratories Inc. (Mountain View, CA, USA). All chemicals used in the experiments were of analytical grade.

2.2. Cloning, vector construction, and transformation

To clone CBD expression vector, CBD-MBP DNA fragment was assembled by fusion PCR and inserted into HT vector, a modified pET-28a vector containing an N-terminal hexahistidine tag and a TEV protease cleavage site [38], using ligation-independent cloning (LIC) technique. The final construct contains an N-terminal His-tag and a C-terminal MBP-tag (Fig. 1A top). To clone CaM-GFP expression vector, first, a new HCT vector containing a N-terminal His-tag followed sequentially by CaM-tag, TEV cleavage site, and LIC cloning site was created. Subsequently, GFP gene was amplified by PCR and inserted into HCT vector by LIC method to create His-CaM-GFP construct (Fig. 1A bottom). The CaM expression vector was created by inserting CaM gene into HMT vector, a modified pET-28a vector containing an N-terminal hexahistidine tag, a MBP-tag and a TEV protease cleavage site using LIC method [38]. The recombinant vectors were transformed into the New England Biolabs Turbo competent cells (Ipswich, MA, USA) and their sequences were confirmed by sequencing reactions.

2.3. Expression of His-CBD-MBP, His-CaM-GFP, and CaM

E.coli BL21 (DE3) cells transformed with respective expression vectors were grown at 37 °C in 2YT medium supplemented with 50 μg/ml kanamycin until OD_{600} reached ~0.6. Followed by induction with 0.4 mM IPTG the cells transformed with His-CBD-MBP and CaM were grown for another 20 h at 18 °C prior to harvesting. The expression of His-CaM-GFP protein was performed at 30 °C for 6 h after induction. Cells were harvested by centrifugation at 8000*g* for 10 min.

2.4. Purification of His-CBD-MBP and CaM

The harvested cells were resuspended in lysis buffer (10 mM HEPES pH 7.4, 250 mM KCl, 25 mg/ml DNaseI, 25 mg/ml lysozyme, 1 mM PMSF and 0.1% Triton-x100) and lysed by sonication (50% amplitude, two rounds of 4 min cycle, 1 s on and 1 s off). The cell debris was removed by centrifugation for 30 min at 40,000*g*. For the purification of His-CBD-MBP, the soluble fraction was loaded onto HisTrap HP column (GE Healthcare) equilibrated with binding buffer (10 mM HEPES, pH 7.4, 250 mM KCl) and eluted using elution buffer (10 mM HEPES, pH 7.4, 250 mM KCl, 500 mM imidazole). The elution peak corresponding to the target protein was collected and loaded onto amylose column (New England Biolabs) equilibrated with binding buffer (10 mM HEPES, pH 7.4, 250 mM KCl) and then eluted with elution buffer (10 mM HEPES, pH 7.4, 250mMKCl, 10 mM maltose). CaM protein was first purified by HisTrap HP and amylose columns using a similar protocol, and the His-MBP tag was cleaved by TEV protease. The cleaved sample was loaded on a Talon column (Clonetech) to remove the His-MBP tag, and pure CaM protein was collected from the flow through.

2.5. Isothermal titration calorimetry

The interaction between CaM and His-CBD-MBP was characterized by isothermal titration calorimetry using Malvern PEAQ-ITC instrument (Malvern, UK). The purified CaM and His-CBD-MBP proteins were dialyzed either against a buffer containing 150 mM KCl and 10 mM HEPES (pH 7.4) with 5 mM $CaCl_2$ or 5 mM EDTA at 4 °C. Titrations consisted of 20 injections of 2 μL of 150 μM CaM into the cell containing either 15 μM His-CBD-MBP or control buffer.

2.6. Purification of His-CaM-GFP

Cells containing His-CaM-GFP were lysed as described in Section 2.3, except for the removal of Triton-x100 in the lysis buffer. After centrifugation, $CaCl_2$ was added in the soluble fraction to a final concentration of 5 mM, followed by loading onto amylose column, equilibrated with 5 CV (column volume) binding buffer (10 mM HEPES pH 7.4, 250 mM KCl, 5 mM $CaCl_2$) and pre-bound with CBD-MBP protein. The column was then washed with 5 CV washing buffer (10 mM HEPES pH 7.4, 250 mM KCl) and eluted using elution buffer (10 mM HEPES pH 7.4, 250 mM KCl, 5 mM EDTA). The CaM-GFP protein was collected and dialyzed for one hour in dialysis buffer (10 mM HEPES pH 7.4, 150 mM KCl) to remove EDTA. After dialysis, $CaCl_2$ was added into the CaM-GFP protein to a final concentration of 10 mM followed by loading onto phenyl sepharose column pre-equilibrated with 5 CV binding buffer (10 mM HEPES pH 7.4, 150 mM KCl, 10 mM $CaCl_2$). The column was then washed with 5 CV washing buffer (10 mM HEPES pH 7.4, 150 mM KCl) and then the CaM-GFP protein was eluted by elution buffer (10 mM HEPES pH 7.4, 75 mM KCl, 10 mM EDTA). The collected protein was cleaved with recombinant TEV protease and then loaded onto Talon column pre-equilibrated with 5 CV binding buffer (10 mM HEPES pH 7.4, 150 mM KCl) and eluted by elution buffer (10 mM HEPES, pH 7.4, 250 mM KCl, 500 mM imidazole). Finally, the size of the protein was examined by a gel filtration column (Superdex 200 16/600). The purity of proteins collected in each step was examined on a 15% (w/v) reducing SDS-PAGE.

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