Protein & Peptide Letters, 2009, *16*, 339-341

Crystallization and Preliminary Crystallographic Analysis of Recombinant Human Calcyphosine

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Abstract: Human calcyphosine was cloned into the pET-28a vector and highly expressed in *Escherichia coli* BL21 (DE3) cells. The protein was purified and crystallized. The crystal diffracted to 2.8 Å and belonged to space group $P2_12_12$, with the unit cell parameters a=70.39 Å, b=132.02 Å, c=46.20 Å.

INTRODUCTION

Calcium is a very important messenger involved in many eukaryotic cellular processes such as contraction, secretion, fertilization, cell proliferation and apoptosis. In prokaryotic cells, although calcium is not essential for the growth of many microorganisms, it can help in stabilizing the bacterial cell wall and plays an important role in the heat stability of endospores [1]. It has also been reported that calcium is critical for some bacterial cellular processes such as chemotaxis in *Escherichia coli*, and heterocyst differentiation in cyanobacteria [2]. Ca^{2+} signals mediated through Ca^{2+}-binding proteins such as calmodulin and recoverin are thought to be an important mechanism involved in regulating cellular processes. In 1979, Lefort and colleagues found a novel Ca^{2+}-binding protein, named calcyphosine, from a dog thyroid cDNA library [3]. Calcyphosine has been identified in certain mammals such as human [4], bovine, rabbit [5], dog and sponge [6], but is absent from mouse and five other rodents [7]. It has been demonstrated that calcyphosine is a major phosphorylated substrate for protein kinase A in a cyclic AMP (cAMP)-dependent manner in response to stimulation of dog thyroid cells by thyrotropin. Calcyphosine might thus be implicated in cross-signaling between the cAMP and calcium-phosphatidylinositol cascades to coordinate cellular proliferation and differentiation [8]. Although calcyphosine plays an important role in cellular processes, the precise mechanism of calcyphosine activation remains unclear. No structure to date has been reported for any member of the calcyphosine family. Here we report the cloning, expression, purification, crystallization and preliminary X-ray analysis of recombinant human calcyphosine, consisting of 189 amino acid residues with a theoretical molecular weight of 22 kDa.

MATERIALS AND METHODS

Cloning, Expression and Purification

The commercial cDNA of human calcyphosine was inserted into the pET-28a plasmid and calcyphosine was expressed at high levels in *Escherichia coli* BL21 (DE3) cells as a fusion protein with a 6×His tag at the N-terminal. *Escherichia coli* BL21 cells grown in LB medium containing 50 μg/ml kanamycin were induced overnight for 20 h at an optical density of OD_{595}=0.6-0.8 by the addition of 0.2 mM isopropyl-*ß*-D-thiogalactopyranoside (IPTG). Harvested cells were suspended in lysis buffer (50 mM Tris-HCl pH8.0, 300 mM NaCl, 5% glycerol) and lysed by sonication. The soluble cell lysate was centrifuged at 15,000 rpm for 30 min at 277 K. The supernatant was loaded onto a 20 ml Ni-NTA column equilibrated in lysis buffer. After washing away the unbound protein with three column volumes of wash buffer (50mM Tris-HCl pH 8.0, containing 300 mM NaCl, 5% glycerol, 20 mM imidazole), tightly bound proteins, mainly calcyphosine, were then eluted with 20 ml elution buffer (50mM Tris-HCl pH 8.0, containing 300 mM NaCl, 5% glycerol, 50 mM imidazole). Calcyphosine was further purified using Resource Q column (Pharmacia Biotech). The purity of calcyphosine was examined by 12% SDS-PAGE at each purification step.

Crystallization

For crystallization purposes, the protein with a His tag fused at the N-terminus was concentrated using a 10K ultrafiltration membrane (Filtron) in a buffer containing 20 mM Tris-HCl pH 8.0 to 20 mg/ml by ultrafiltration (Millipore Amicon, USA) and subjected to Crystal I, II, Index Screen, and PEG/ion Screen (Hampton Research, USA) as initial screening kits. Crystallization experiments were performed at 16 °C using the hanging-drop vapour-diffusion method. 1.5 μl of protein solution were mixed with 1.5 μl of reservoir solution and equilibrated against 0.2 ml of the reservoir solution.

Data Collection

The preliminary X-ray diffraction analysis of calcyphosine was performed at room temperature using an in–house Mar345dtb image plate with a Rigaku RU2000 rotating Cu anode X-ray generator at 40 kV and 20 mA (λ=1.5418 Å). The crystals were flash-frozen by direct transfer from the drop to liquid nitrogen. The indexing and data processing were performed with *HKL2000* [9].

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RESULTS AND DISCUSSION

Expression and Purification of Recombinant Human Calcyphosine

Calcyphosine fused with an N-terminal His-tag was solubly expressed in *E.coli* with a yield of about 20 mg/L culture. After purification using a Resource Q column (Fig. **1**), the purified calcyphosine protein was calculated to be >95% pure based on SDS-PAGE analysis (Fig. **2**).

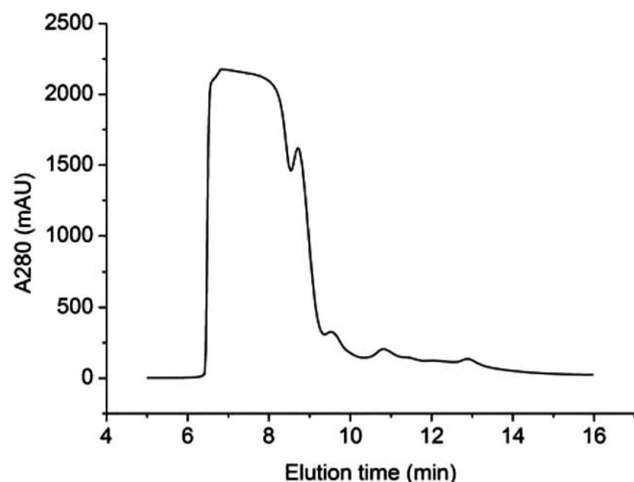

Figure 1. The purification profile of calcyphosine on Resource Q.

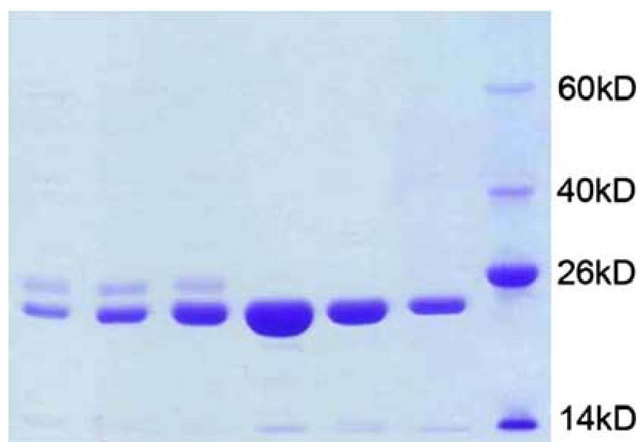

Figure 2. SDS-PAGE of the purified calcyphosine.

Crystallization and Data Collection

Initial crystals were obtained using PEG/ion Screen solution No. 7, containing 0.2 M Calcium Chloride dehydrate and 20% w/v Polyethylene Glycol 3350 at 293 K. Microneedle-like crystals grew with a few days. Optimization of the condition to 0.1M PIPES buffer, pH 6.3, 0.2 M Calcium Chloride dehydrate and 20% PEG8000 greatly improved the quality and the size of the crystal, but we still failed to obtain single crystals. Finally, crystals suitable for high-resolution X-ray crystallographic analysis were obtained by seeding and microcrystal selection techniques. Some high quality crystals were produced in two weeks (Fig. **3**). A data set was collected from a single calcyphosine crystal (Fig. **4**). The crystal diffracted to a resolution of 2.8 Å and belonged to space group $P2_12_12$, with unit cell parameters of a=70.39 Å, b=132.02 Å, c=46.20 Å. Assuming the presence of two molecules per asymmetric unit, as in the case of other Ca^{2+}-binding proteins with four EF-hand motifs, the Matthews coefficient is 2.4 $Å^3$ Da^{-1} and the solvent content is 49.8%. Data statistics are listed in Table **1**.

Figure 3. Typical crystals of calcyphosine. Scale bar represents 100 microns.

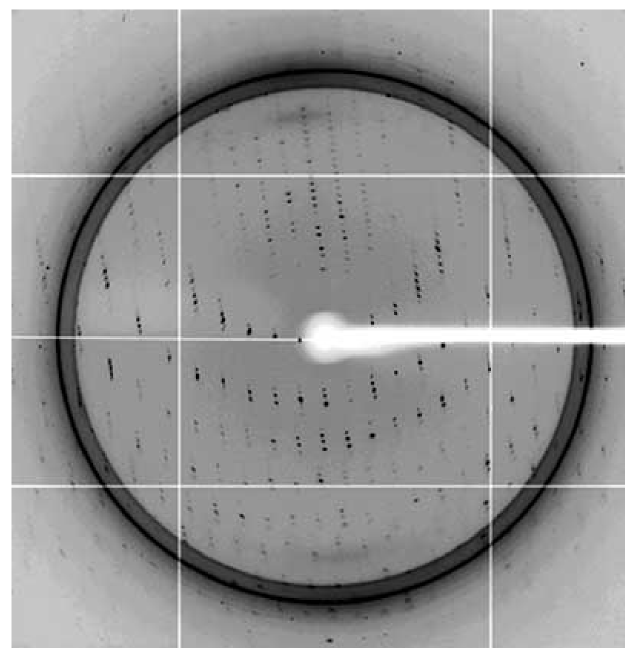

Figure 4. Typical X-ray diffraction pattern from a calcyphosine crystal.

Calcyphosine shares 29% sequence identity with calmodulin [10]. Molecular replacement using calmodulin and other related Ca^{2+}-binding proteins as search models has been attempted but without success so far. Crystallization of seleno-methionine (Se-Met)-substituted calcyphosine is in progress.

Table 1. Data-Collection Statistics for Calcyphosine

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Resolution (Å)	50-2.8
Space group	$P2_12_12$
Unit-cell parameters (Å)	a=70.39, b=132.02, c=46.20
R_{merge} (%)	7.0 (25.5)
Average $I/\sigma(I)$	23.7 (5.0)
Completeness (%)	94.5 (94.0)
redundancy	3.8 (3.8)
No. of observed reflections	37953
No. of unique reflections	10453
Molecules per ASU	2
V_M (Å^3 Da^{-1})	2.4
Sovent content (%)	49.8

$R_{merge} = \Sigma_i |I_i - \langle I \rangle| / \Sigma \langle I \rangle$, where I_i is an individual intensity measurement and $\langle I \rangle$ is the average intensity for all the reflection i.

ACKNOWLEDGMENTS

This work was supported by funds from the National Natural Science Foundation of China and the Ministry of Science and Technology 973 Project.

Received: July 01, 2008 **Revised: August 01, 2008** **Accepted: August 04, 2008**

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