Recombinant Expression, Isotope Labeling, and Purification of Cold Shock Protein from *Colwellia psychrerythraea* for NMR Study

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Cold shock proteins (Csps) are a subgroup of the cold-induced proteins on reduction of the growth temperature below the physiological temperature. They preferentially bind to single-stranded nucleic acids to translational regulation via RNA chaperoning. Csp plays important role in cold adaptations for the psychrophilic microorganism. Recently, Cold shock protein from psychrophilic bacteria, *Colwellia psychrerythraea* (CpCsp) has been identified. Three dimensional structures of a number of Csps from various microorganisms have been solved by NMR spectroscopy or X-ray crystallography, but structures of psychrophilic Csps were not studied yet. Therefore, cloning and purification protocols for further structural study of psychrophilic Csp have been optimized in this study. CpCsp was expressed in *E. coli* with pET-11a vector system and purified by ion exchange, size exclusion, and reverse phase chromatography. Expression and purification of CpCsp in M9 minimal media was carried out and ^{15}N-labeled proteins with high purity over 90% was obtained. Further study will be carried out to investigate the tertiary structure and dynamics of CpCsp.

Key Words: Cold shock protein, *Colwellia psychrerythraea*, Psychrophile, Overexpression, Purification

Introduction

All living organisms have ability for the survival from environment change, such as cold shock, heat shock and the other stresses.[1,2] When organism was attacked by dramatical cold stress, normal protein products are decreased, but some special protein products are dramatically increased until adaptation. This is called cold shock protein (Csp). Csps are divided into two types, Class I and II. Class I Csps are expressed low level at 37 °C and are dramatically expressed after a shift to lower temperature. In contrast, class II Csps are present at a certain level at 37 °C and after temperature falls, expression is increased a little from their steady-state levels.[3] These proteins found in a gram-positive, negative bacteria and they have a highly homologous identity with each other.[4] Molecular mass of Csps is approximately 7.4 kDa and preferentially bind to single-stranded nucleic acids to translational regulation *via* RNA chaperoning.[5-8] They are consisting of a β-barrel structure that includes five antiparallel β-strands.[9,10] Cold shock proteins have been found in many bacteria, including hyperthermophilic, thermophilic, mesophilic and psychrophilic species.[11] The three-dimensional structures of a number of bacterial Csps have been solved by NMR and X-ray.[12-15] In case of *Bacillus subtillis* Csp, three dimensional structure has been determined in complex with hexathymidine.[9,11]

Colwellia psychrerythraea 34H is considered as an obligate psychrophile, Gram-negative bacteria. This flagella-containing organism can be found in continually cold marine environments from arctic, antarctic deep oceans, sea water, sea ice, and sediment.[16] *Colwellia psychrerythraea* cold shock protein (CpCsp) consists of 68 amino acids and molecular mass is 7.2 kDa. It shows sequence identities of 60% with *E. coli* Csp. When temperature is lowered from 37 °C to 10 °C, it is expressed much more than at higher temperature. It starts expression at cold shock and slowly decreased after 2 hours later. None of the three dimensional structures of psychrophilic Csp has been studied yet. In this study, we performed cloning, expression, isotope labeling, and purification of CpCsp for further NMR study.

Methods

Construction of the CsCsp expression plasmid. The plasmid DNA encoding CpCsp protein was kindly provided by Dr. Hak Jun Kim at Korea Polar Research Institute. To increase the production of CpCsp protein in *E. coli*, we cloned the CpCsp gene into pET-11a(+) expression vector containing an IPTG inducible promoter and resistance to ampicillin using *Nde*I and *Bam*HI restriction enzyme sites (Figure 1). PCR was per-

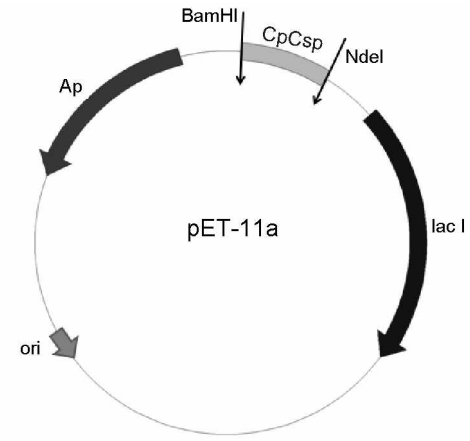

Figure 1. Plasmid map of CpCsp.

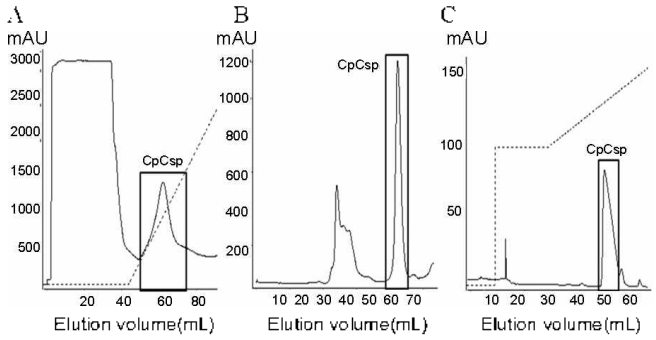

Figure 2. The elution profile of CpCsp from ion exchange (A), size exclusion (B), and hydrophobic interaction chromatography (C). The peak corresponding to CpCsp is marked with a box. The dotted line denotes the concentration of gradient elution.

formed under the following conditions: 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 1 min at 72 °C. The pET-11a/CpCsp plasmid was transformed into the expression host, *E. coli* BL21 (DE3) for expression of CpCsp.[17]

Expression of CpCsp from an LB medium. Transformed cells were grown on Luria-Bertani (LB) agar plates containing 50 μg/mL ampicillin. SDS-PAGE analysis was used to select a colony for overexpression of CpCsp. One colony was used to inoculate 50 mL of LB medium with 50 μg/mL ampicillin, and grown overnight in 37 °C shaking incubator. 10 mL of the fully grown culture was mixed with 1 L of fresh LB medium with 50 μg/mL, and grown at 37 °C until optical density reached 0.7 at 600 nm. The culture was induced with 1 mM IPTG and was grown 36 more hours at 10 °C. The cells were harvested and the cell pellet was stored at −80 °C.

Expression of [^{15}N]-labeled CpCsp from a minimal medium. One colony was used as an inoculums to 4 mL of LB medium supplemented with 100 μg/mL and grown overnight in a 37 °C shaking incubator. The fully grown 4 mL culture was used as an inoculum to 100 mL of minimal medium supplemented with the same antibiotics, and the culture was grown overnight. The minimal medium contained $^{15}NH_4Cl$ as a sole nitrogen source. The next morning, this 100 mL culture was mixed with 900 mL of minimal medium. The culture was grown at 37 °C, and IPTG was added to 1 mM final concentration when OD_{600} was 1.0. The temperature was switched to 10 °C, and the culture was grown for another 36 hours. The cells were harvested and the cell pellet was stored at −80 °C. The cells were resuspended in 6 mM Tris-HCl, 2 mM DTT, and 2 mM EDTA buffer (pH 6.8).

Isolation and purification of CpCsp. All lysis and purification processes were carried out at 4 °C. A frozen pellet was resuspended and lysed by ultrasonication in buffer A (6 mM Tris-HCl, 2 mM DTT, 2 mM EDTA, pH 6.8). Supernatant after centrifugation at 16,000 rpm and 4 °C was retained for 50 min. The supernatant was loaded onto a Hitrap QFF column (anion exchange column, GE) that had been preequilibrated with buffer A. The column was washed with buffer B and then the bound protein eluted with linear gradient from 0 to 300 mM NaCl. The CpCsp-containing fraction were pooled and concentrated by AmiconUltra (Millipore). Concentrated CpCsp was purified

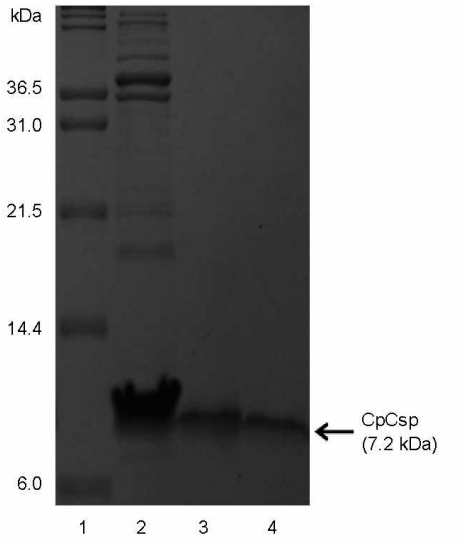

Figure 3. Purification of CpCsp produced from 2 L of LB medium as monitored by Tricine SDS-PAGE. lane 1, size marker; lane 2, result of ion exchange chromatography; lane 3, result of size exclusion chromatography; lane 4, result of hydrophobic interaction chromatography.

using size exclusion chromatography on HiLoad 16/60 Superdex75 column (Pharmasian). Finally, CpCsp-containing fractions were concentrated and purified by hydrophobic interaction chromatography on Resource RPC (GE). SDS-PAGE analysis was applied to identify CpCsp-containing fraction.

Circular dichroism. Circular Dichroism (CD) experiments were performed by using a J-810 (JASCO, Tokyo, Japan) spectropolarimeter using with 1 mm path length cell. The CD spectra of the CpCsp (15 mM) were recorded at 25 °C and 0.1 nm intervals from 190 to 250 nm (far-UV region). For each spectrum, the data from six scans were averaged and smoothed CD data were expressed as mean residue ellipticity [θ] given in deg·cm^2 dmol^{-1}.[18]

NMR experiments. The NMR sample contained 1 mM [^{15}N]-CpCsp in 50 mM potassium phosphate buffer, pH 6.0, containing 5 mM DTT and 0.1 mM EDTA. The ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectrum was collected at 25 °C on a Bruker DMX-400 spectrometer. The raw data acquired with 2,048 data points for t_2 and 256 for t_1 increments. NMR spectrum was processed with the NMRPipe/nmrDraw software package and analyzed using the Sparky.[19,20]

Results and Discussion

Expression of CpCsp from an LB medium. As shown in Figure 3, the purified CpCsp showed a band corresponding to a molecular mass of 7.2 kDa. To determine optimal conditions, we tried several temperatures for cell growth and various OD_{600} values for induction. The optimal temperature and OD_{600} were determined to be 37 °C and 0.7, respectively. We further optimized the post-induction growth condition by lowering the temperature to 10 °C.

Expression of [^{15}N]-labeled CpCsp from a minimal medium. Since the cell growth in minimal media is different from the

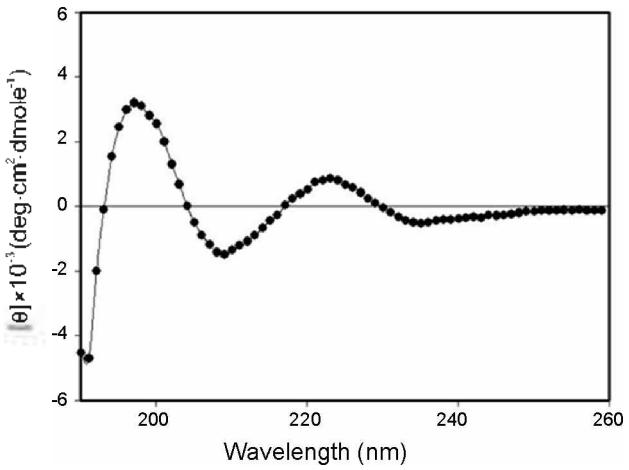

Figure 4. Circular Dichroism spectra of CpCsp in potassium phosphate buffer.

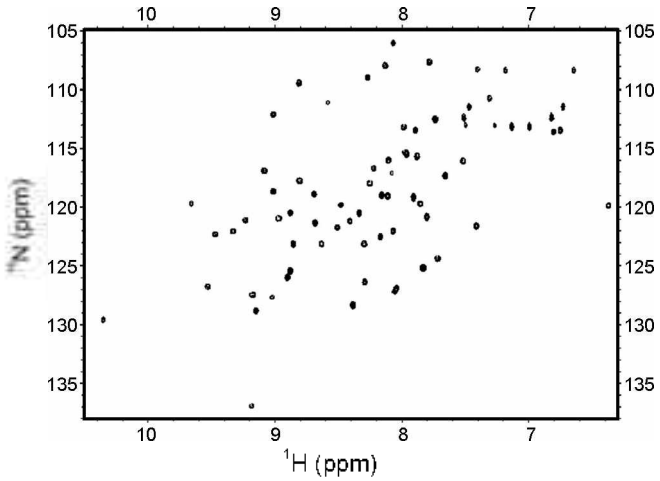

Figure 5. [^{1}H-^{15}N] HSQC spectrum of CpCsp in 50 mM potassium phosphate buffer at 25 °C.

case of LB medium, we tried to find an optimal condition again. In the case of labeled samples, the cells grew only to 70 - 80% of what could be expected from the LB medium. We found an optimized temperature of 37 °C and 1.0 OD_{600} value as the best conditions.

Purification of CpCsp. Purification of CpCsp consists of three steps, which are ion exchange, size exclusion, and hydrophobic interaction chromatography. We followed the general methods reported previously[21] but protocols were modified and optimized for CpCsp. Ion exchange chromatography was used by Hitrap QFF column (anion exchange column). CpCsp was eluted with gradient from 5 to 30 percent of B buffer concentration by NaCl. These fractions include many different kinds of impurities. Therefore, each fraction was concentrated by AmiconUltra (Millipore) and concentrated CpCsp was purified using size exclusion chromatography on HiLoad 16/60 Superdex 75 column (Parmasian). Most impurities are detached at this step, but the purity was not enough for the NMR study. CpCsp-containing fractions were concentrated and finally purified by hydrophobic interaction chromatography on Resource RPC column. The CpCsp fraction was collected and lyophilized. The yield of the CpCsp was about 1.5 mg/L in LB or 1.0 mg/L in minimal medium, respectively. Purified CpCsp showed a band corresponding to a molecular mass of 7.2 kDa. Presence of Ser after methionine at the N-terminus allows the removal of methionine by methionine aminopeptidase.[22]

Circular dichroism. The CD spectrum of CpCsp is given in Figure 4. This spectrum is somewhat unusual, however similar spectra have been observed for CspBs from *Bacillus subtilis*,[23] *Thermotoga maritime*,[24] and for major Csp from *E. coli*, CspA.[25-27] Generally, maximum positive band at 200 nm as well as minimum negative band at 225 nm are the characteristics of antiparallel β-sheet structure. Csp exhibits an unusual far UV-CD spectrum for a predominantly β-sheet protein. It is believed that the far-UV CD spectrum of Csp is due to the β-structure rich in aromatic amino acid residues, preventing a detailed analysis of the secondary structure. CpCsp have seven aromatic residues (four Phe, one Trp and two Tyr). There is a tendency for the aromatic side chains such as Tyr and Trp in protein to give positive CD features in the between 215 and 230 nm.[28] Therefore, weak positive ellipticity between 215 and 230 nm has been attributed to the β-sheet structure as well as these side chains of aromatic residues positioned on the surface of β-sheets[29-33] and the CD maximum between 195 and 200 nm originates from the antiparallel β-sheet structure.[3,34] Therefore, CD spectra of CpCsp adopted an antiparallel β-sheet conformation similar to other Csps.

NMR experiment. As shown in Figure 5, the HSQC spectrum confirmed the high purity of ^{15}N-labeled CpCsp by showing 60 nicely resolved peaks, which correspond to the number of the residues in CpCsp, except proline. 6 peaks were not shown because of the spectral overlapping. There were six sets of peaks from the side chain of Gln and Asn. One peak from the side chain of Trp appeared to 10.5 ppm. Although the ^{1}H NMR spectrum of CpCsp in 50 mM potassium phosphate buffer was difficult to analyze because of the severe overlapping, the amide resonances were dispersed in the ^{1}H-^{15}N HSQC spectra.

Conclusion

Cold shock proteins have been found in many bacteria, including hyperthermophilic, thermophilic, mesophilic, and psychrophilic species. The three-dimensional structures of a number of bacterial Csps have been solved by NMR and X-ray. Csp from psychrophilic bacteria has not been studied much yet. In order to understand the mechanism of cold adaptation of psychrophilic bacteria, we decided to study structure of Csp from psychrophilic bacteria, *Colwellia psychrerythraea*. We optimized the overexpression and purification conditions such as temperature, agitation velocity (rpm), and purification methods. Each condition for CpCsp was optimized and CpCsp was successfully expressed and purified. According to the CD spectra, CpCsp adopted an antiparallel β-sheet conformation similar to other Csps. However, CpCsp have unusual CD band at far-UV region because of seven aromatic residues. Isotope labeling (^{15}N) was also successfully performed for the studies of the structure and dynamics of the CpCsp in potassium phosphate buffer. Result of CD spectrum showed that CpCsp have an

antiparallel β-sheet structure and NMR spectrum showed that CpCsp have excellent dispersion of the resonances. Therefore, CpCsp was fold properly in potassium phosphate buffer. In conclusion, the expression and purification protocols for the CpCsp described in this paper could be applied to high-level expression and purification of various kinds of psychrophilic bacterial Csp. Further structural studies on CpCsp will be performed.

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