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Purification and Characterization of a Novel 21 kD Calcium Binding Protein from *Dunaliella salina*

Ko, Jae Heung and Sun Hi Lee*
Department of Biology, Yonsei University, Seoul 120-749, Korea

A novel calcium binding protein (CaBP) was purified to electrophoretic homogeneity from *Dunaliella salina*. In the course of purification experiment, this CaBP was identified as a monomer and its molecular weight was about 21 kD and isoelcetric point (pI) value was about 4.1 using isoelectrofocusing. This CaBP was able to bind Ca^{2+} even in the presence of an excess $MgCl_2$ and KCl both in solution. In the SDS-PAGE, the Ca^{2+}-bound form migrates more faster than the Ca^{2+}-free form. On the contrary, the Ca^{2+}-bound form was slower than the Ca^{2+}-free form in the nondenaturing PAGE. This means that the CaBP undergoes conformational change in the Ca^{2+}-bound condition. Furthermore, UV absorption spectrum and fluorescence intensity of this CaBP was investigated. UV absorption peak was appeared at about 258 nm and decreased somewhat in Ca^{2+}-bound condition. In the measurement of fluorescence, maximum intensity was appeared at 303 nm and decreased in Ca^{2+}-bound state, similarly as UV absorption spectrum. These show distinct changes upon Ca^{2+}-binding, which indicate of structural and/or dynamic changes largely reminiscent of other members of the EF-hand Ca^{2+}-binding protein family.

Keywords : *Dunaliella salina*, Calcium, Calcium-binding protein

A variety of endogenous and exogenous stimuli causes an influx of Ca^{2+} into the cytosol of plant cells. One of the mechanisms by which this influx of Ca^{2+} is transduced to response elements within the cell is through a family of proteins, known as the EF-hand family of Ca^{2+}-binding proteins (Poovaiah and Reddy, 1987). In animals, there are over 160 members of the EF-hand protein family such as calmodulin, troponin C, and a number of S 100 proteins identified. The function of calmodulin in Ca^{2+} signal transduction has been studied extensibly, and many target enzymes have been identified (Hepler and Wayne, 1985; Poovaiah and Reddy, 1987; Roux *et al.*, 1987). Calcium binding to calmodulin induces a conformational change, thus exposing hydrophobic sites that are involved in the interaction with target proteins. The fact that other EF-hand CaBPs also expose hydrophobic regions upon calcium binding (Chiba and Mohri, 1989; Roberts and Harmon, 1992) suggests that this model may represent a general mechanism for the function of these proteins as Ca^{2+} signal mediators. However, calmodulin and a calcium dependent protein kinase are the only well-characterized member of the EF-hand protein family known in plants. Clearly, to establish the hierarchy of how the signals initiated by changes in cytosolic Ca^{2+} concentration are transduced in plant cells, it is of central importance to understand the number and nature of the protein receptors for Ca^{2+} in plant cells. Recently, several plant proteins that share amino acid sequence similarites with calmodulin were identified by cDNA cloning and inferred to be members of the EF-hand family. These reports demonstrated that plants possess potential Ca^{2+}-receptor proteins in addition to calmodulin that may transduce signals to a more limited group of target proteins or in response to a selective number of stimuli.

In this report, we present the purification and characterization of molecular weight of 21 kD calcium binding protein from *D. salina*, halotolerant, green algae, which by structural similarity and in-

*Corresponding author: Fax +82-2-312-5657
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direct biochemical assay we infer to be a member of the EF-hand family of Ca^{2+}-modulated proteins.

MATERIALS AND METHODS

Growth condition

Dunaliella salina was grown in media containing 1.7 M NaCl previously described (Pick and Chitlaru, 1991). Cultures were carried out at 26°C under continuous illumination with white fluorescent lamps giving 3,800 lux with axenic air bubbling and maintained at the logarithmic growth phase.

Purification of 21 kD CaBP

Cells were harvested (2,000×g/4°C) and homogenized in 5 vol. of extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM EGTA, and 2 mM DTT) using sonicator (Vibra Cell, Sonics and Materials Inc.). The homogenate was centrifuged at 12,000×g for 60 min and the supernatent was centrifuged again at 100,000×g for 2h. All steps were performed at 4°C. The supernatent, adjusted to 3 mM $CaCl_2$ was loaded onto a column of phenyl-Sepharose CL-4B that had been pre-equilibrated with 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.2 mM $CaCl_2$, and 0.5 mM DTT. The column was washed and proteins were eluted with the same buffer containing 5 mM EGTA instead of $CaCl_2$. The resultant fractions containing 21 kD CaBP were determined by $^{45}Ca^{2+}$-overlay methods of Maruyama *et al.* (1984). These fractions were pooled, dialyzed, and loaded onto DEAE-Sephacel column pre-equilibrated with 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, and 0.5 mM DTT. The proteins were eluted with same buffer containing linear gradient of 0-0.5 M NaCl. The 21 kD CaBP containing fraction eluted at about 0.4 M NaCl was pooled and heated at 80°C for 2 min after addition of 5 mM $CaCl_2$. The heated sample was quickly chilled on ice and the denatured proteins were removed by centrifugation. Final purification was achieved by gel filtration on a sephacryl S-200 column. Purity was assessed by SDS-PAGE and nondenaturing PAGE.

Detection of Ca^{2+}-binding proteins using $^{45}Ca^{2+}$-overlay method

SDS-PAGE was performed using the discontinuous buffer system of Laemmli (1970) in gels containing 12.5% or 15% acrylamide. After SDS-PAGE, electrophoretic transfer of proteins to nitrocellulose membrane was carried out with an electrophoretic blotting apparatus (Hoefer TE50X) at 10 V/cm for 20 h at 4°C. The nitrocellulose membrane, with proteins transferred to it, was washed with a solution containing 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl buffer (pH 6.5) and then incubated in the same solution with 1 mCi/L of $^{45}CaCl_2$ for 10 min at 25°C by the method of Maruyama *et al.* (1984). The membrane was rinsed with 50% ethanol and dried for autoradiography.

Spectroscopic Measurement

UV and fluorescence spectra were measured using spectrophotometer (Hitachi U2000) and spectrofluoremeter (Hitachi F2000). The conditions of measurement was described in each Figure legend.

Calcium bound mobility shift experiment

Samples were resuspended in PAGE gel sample buffer containing 5 mM $CaCl_2$ or 5 mM EGTA and run a SDS-PAGE or nondenaturing PAGE.

RESULTS AND DISCUSSION

In view of the central role of Ca^{2+} in the regulation of various cellular activities, the structural and functional characteristics of Ca^{2+}-binding proteins (CaBPs) have been the subject of intense research interest.

Purification of a 21 kD CaBP.

Calcium-binding proteins are known to bind to a hydrophobic matrix in a Ca^{2+}-dependent manner, and this property was utilized in developing a scheme for purifying 21 kD CaBP from *D. salina*.

The homogenate of salt stress-treated cells after ultracentrifugation was applied to a phenyl-Sepharose column in the presence of Ca^{2+}. Since this protein shows up-expression in the salt stress (Ko and Lee, 1995), we used salt stress treated cells. The 21 kD CaBP was eluted from the column by using EGTA. This protein was further purified on a DEAE-Sephacel column followed by gel filtration chromatography. In addition to its apparent calcium-binding properties, 21 kD CaBP was found to be heat stable, a property that is also shares in common both calmodulin and other members of the calcium-mo-

dulated family of protein (Haiech and Capony, 1988). As shown in Fig. 1, when the fraction containing 21 kD CaBP from DEAE-Sephacel column was heated for 2 min to 80°C, followed by centrifugation to pellet heat-denatured proteins, 21 kD CaBP was quantitatively recovered in the heat-stable supernatant fraction. The isolated protein was homogenous and its molecular weight was about 21 kilodalton, when tested by SDS-PAGE (Fig. 1). Nondenaturing PAGE and molecular weight calibration using gel filtration chromatography suggest that this protein exists as a monomer. Also, isoelectric point (pI) value of this protein was about 4.1 in the experiment of isoelectric focusing (data not shown).

Size determination by sephacryl S-200 gel filtration

Purified protein was found to have a molecular mass of 21 kD as determined by electrophoretic migration in the presence of SDS. However, it typically eluted from a sephacryl S-200 column at 0.68 column volume as a symmetric peak, which corresponds to an apparent molecular mass of 26.7 kD based on a calibration of the column with globular proteins (Table 1). These results suggested that this 21 kD CaBP was likely to be an elongated molecule with a domain architecture similar to calmodulin in which the long axis of the molecule determined its behavior on a sizing column (Weber *et al.*, 1994).

The elution pattern of CaBP on the gel filtration was dependent on the Ca^{2+} content. In the presence of Ca^{2+}, the movement of this CaBP was more faster than the absence. This has been interpreted as the change of molecular conformation through Ca^{2+} binding.

Ca^{2+}-dependent electrophoretic mobility shift

It has been previously documented that the CaBP shows a calcium-dependent alteration in the electrophoretic mobility, a characteristic feature of calmodulin and other member of the EF-hand calcium-binding protein family (Burgess, 1982). In the presence of SDS, purified CaBP was found to migrate with an apparent molecular mass of 19 kD in the presence of added $CaCl_2$ and was slightly more retarded in its migration in the presence of added EGTA (Fig. 2A). Under non-SDS, native electrophoretic conditions, this CaBP exhibited a more distinct mobility shift and in this case, calcium containing CaBP migrates more slower (Fig. 2B). This important Ca^{2+}-dependent shift in mobility is consistent with the prediction that metal-depleted form is more acidic than the Ca^{2+}-saturated protein (Cox and Stein, 1981).

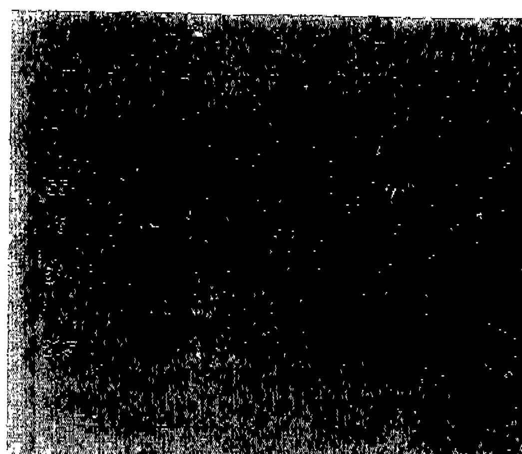

Fig. 1. Purification of 21 kD CaBP from *D. salina*. Fractions in the purification step were analyzed by SDS-PAGE (12.5%). Lane 1: Crude extract, Lane 2: Precipitate of 70% ammonium sulfate, Lane 3: Active fraction of phenyl-sepharose chromatography, Lane 4: Active fraction of DEAE-Sephacel chromatography, Lane 5: Heat treated pellet of lane 4, Lane 6: Heat treated supernatent of lane 4, Lane 7: Purified 21 kD CaBP from Sephacryl S-200 chromatography. Molecular size markers are indicated in left side.

Table 1. Size determination by Sephacryl S-200 gel filtration. Measurements were performed on a 1×100 cm sepharcryl S-200 column at a flow rate of 8 mL/h. in 25 mM Tris-HCl (pH 7.5), 50 mM NaCl in the presence of either 5 mM EGTA or 5 mM $CaCl_2$, as indicated. 1 mg protein was loaded and run

Protein	EGTA/$CaCl_2$	^{a}Kav	$^bM_{app}$
23kD CaBP	EGTA	0.46	26.7
23kD CaBP	$CaCl_2$	0.39	28.8

^{a}Kav, distribution coefficient.
$^bM_{app}$, apparent molecular weight, based on calibration of the column with a set of globular proteins.

UV absorption spectrum of 21 kD CaBP and effect of calcium

In the UV absorption spectrum (Fig. 3), it was guessed that this protein has no tryptophan since tryptophan-containing proteins generally will have a shoulder around 290 nm. In addition, the fine structure that one usually associates with calcium-binding proteins, like calmodulin, is not noticeable in the

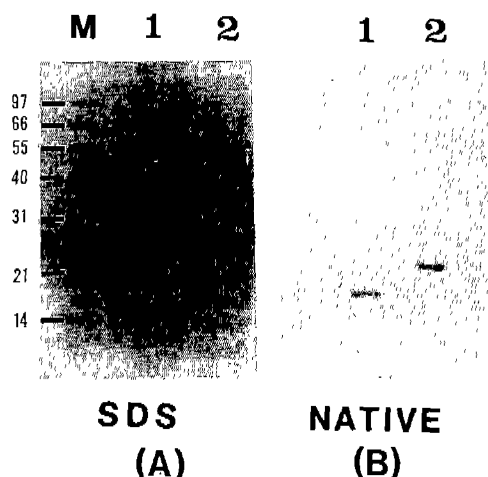

Fig. 2. Calcium-dependent electrophoretic mobility shift. (A), PAGE in the presence of SDS. (B), native PAGE in the absence of SDS. Lanes 1 and 2; 2 μg of CaBP run in the presence of 5 mM $CaCl_2$ (lane 1) or 5 mM EGTA (lane 2) in sample loading buffer. ※(Lane M; mokular marker)

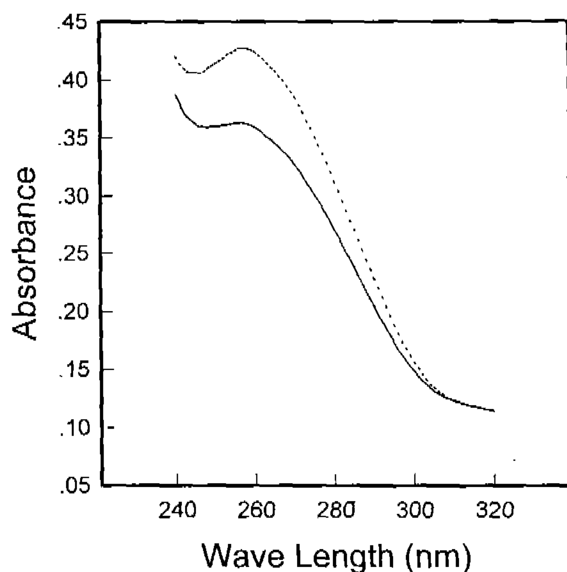

Fig. 3. UV absorption spectrum of 21 kD CaBP and effect of Calcium. The spectra were measured in 0.1 mg purified protein, 100 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.5 containing 2 mM EGTA (dashed line) or 1 mM $CaCl_2$ (solid line) and recorded between 240 and 320 nm.

250-270 nm region. Conformational change induced by Ca^{2+}-binding to 21 kD CaBP were investigated by means of UV absorption difference. In the presence of Ca^{2+}, a decrease in absorbance between 250 and 300 nm was observed (Fig. 3). Such a negative difference is similar to the Ca^{2+}-induced absorption changes in calmodulin (Klee, 1977) that are thought to indicate an increased exposure of the aromatic amino acid tyrosin to the aqueous solvent in the presence of ligand (Klee, 1977).

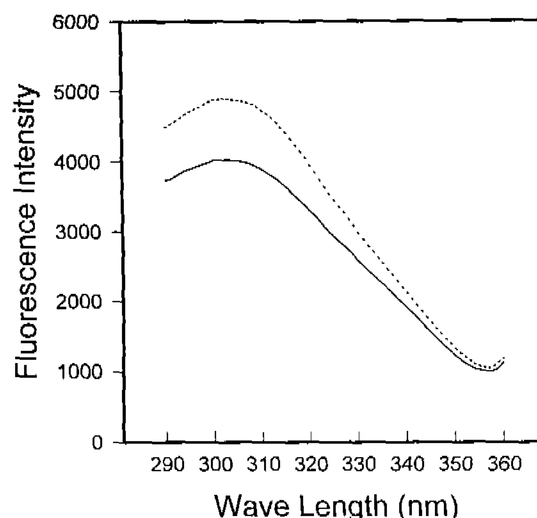

Fig. 4. Fluorescence emission spectrum of 21 kD CaBP and effect of Calcium. The spectra were measured in 0.1 mg purified protein, 100 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.5 containing 2 mM EGTA (solid line) or 1 mM $CaCl_2$ (dashed line) was excited at 290 nm. The emission spectra were recorded between 290 and 360 nm.

Effect of calcium on the fluorescence emission spectra

The effect of Ca^{2+} on the conformation of 21 kD CaBP was also studied by measurement of fluorescence spectroscopy. Using an excitation wavelength of 290 nm, we recorded the fluorescence emission in the absence or presence of 1 mM $CaCl_2$. As shown in Fig. 4, the spectra reveal emission quench after the addition of $CaCl_2$. Known fluorescence emission properties of isolated tyrosin and tryptophan residues (Cantor and Schimmel, 1980) suggest that, as above, changes in the environment of both aromatic amino acids occur upon Ca^{2+} binding. These conformational changes induced in 21 kD CaBP by Ca^{2+} are considerable importance since the Ca^{2+}-dependent exposure of a hydrophobic plate on the surface of the protein is critical for the interaction with the target proteins and subsequent activation.

Through the study of purification and characterization, we have found that this CaBP was quite similar to calmodulin in some biochemical pro-

perties. But the distinct difference was the molecular weight since most of the calmodulins in various sources, including plants and animals, were about 16 kD. So, future studies should involve the role of this CaBP in the salt stress because this protein shows up-expression in the salt stress (Ko and Lee, 1995), and cloning of its cDNA for the information of molecular level.

ACKNOWLEDGEMENTS

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