Cytokinin-Binding Proteins from Tobacco Callus Share Homology with Osmotin-Like Protein and an Endochitinase

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To study the signal transduction of cytokinins, we characterized cytokinin-binding proteins (CBPs) isolated from tobacco callus Nicotiana tabacum. Two high-affinity CBPs, CBP1 and CBP2, were isolated from the soluble fraction of tobacco callus BY-2 cells by anion exchange chromatography on a DEAE-cellulose column and affinity chromatography on a benzyladenine (BA)-linked Sepharose 4B column. Cytokinin-binding activity was determined by the equilibrium dialysis method. The degree of purification of CBP1 and CBP2 was 270 and 600-fold, respectively. These proteins had molecular masses of 34 kDa and 26 kDa, and to bind benzyladenine (BA) with dissociation constants (Kd) of $8.9 \times 10^{-6}$ M and $1.1 \times 10^{-6}$ M, respectively. Binding of BA to CBP2 was inhibited by zeatin and kinetin but not by adenine, adenosine, ATP or IAA. The optimum pH for binding of BA to CBP1 and CBP2 was approximately pH 6.5 and 7.5, respectively. CBP1 showed significant homology (90%) with endochitinase and CBP2 with osmotin-like protein (OLP). These findings and the results of immunoblotting analysis and cytokinin-binding assay of recombinant OLP indicated that CBP2 is OLP, a stress protein.

**Key words:** Benzyladenine — CBP2 — Cytokinin-binding protein — Nicotiana tabacum — Recombinant OLP — Stress protein.

Cytokinins were definitively discovered in 1955 by Miller and Skoog (review, Moore TC 1979). Their physiological roles are to promote cell proliferation and differentiation of plant cells (Jacqmard et al. 1994). However, the mechanism of their specific biological action at the molecular level has not been elucidated. In the most well characterized systems, signal molecules have been shown to bind to protein receptors and affect ion flux, phosphorylation of regulatory proteins, transcription, translation, and secretion (Libbenga and Mennes 1987, Chrispeels 1991, Blatt and Thiel 1993, Gallie 1993). However, the physiological significance of storage proteins as cytokinin-binding proteins is unclear, they may serve as a cytokinin storage compartment allowing rapid release of cytokinins upon germination. This hypothesis has not been rigorously examined.

Research on cytokinin receptors in various vegetative tissues has also yielded some purified cytokinin-binding proteins. Momotani and Tsuji (1992) purified a cytokinin-binding protein from the soluble fraction of mature tobacco leaves by successive affinity chromatography on a BA- and zeatin-linked Sepharose column. The molecular mass of this protein was 31 kDa. Using equilibrium dialysis, the Kd for BA was $1 \times 10^{-7}$ M and binding of BA to this protein was inhibited by most cytokinins. Adenine and ATP showed little competitive activity. However, further studies on the function of the 31-kDa protein have not been reported.

By analogy, it is generally thought that there are receptor proteins that bind to plant hormones and that these receptors might be important elements in models aiming to explain plant hormone action. Gardner et al. (1978) detected cytokinin-binding activity in the 80,000 × g fraction of protonemata Funaria hygrometrica, and Kobayashi et al. (1991) in carrot suspension cells. This suggested the existence of cytokinin receptor in the plasmamembrane or endoplasmic reticulum (ER). However, cytokinin-binding proteins (CBPs) have not been isolated from the plasmamembrane or ER.

On the other hand, there have been many reports on the presence of CBPs in the soluble portion of the subcellular fraction (Takegami and Yoshida 1975, Polya and Davis 1978, Moore FH 1979, Chen et al. 1980, Erion and Fox 1981, Hamaguchi et al. 1985, Kulaeva et al. 1990, Romanov et al. 1990, Momotani and Tsuji 1992, Mitsui and Sugiuara, 1993). Some of the earliest reports concerned cytokinin-binding protein from wheat germ (Polya and Davis 1978, Erion and Fox 1981), and one of these proteins (CBF-1) had an amino acid sequence similar to vicilin-type seed storage protein (Brinegar et al. 1988). This protein had high affinity (Kd = $10^{-7}$ M) for cytokinins, but low affinity for nonactive analogues of cytokinin or other plant metabolites. Photoaffinity labeling using a benzyladenine (BA) derivative resulted in the labeling of a single histidine residue, indicating a specific interaction of cytokinin with the protein. Although the physiological significance of storage proteins as cytokinin-binding proteins is unclear, they may serve as a cytokinin storage compartment allowing rapid release of cytokinins upon germination. This hypothesis has not been rigorously examined.
from *Nicotiana sylvestris*. This complex consisted of two subunits with molecular masses of 57 and 36 kDa and bound cytokinin with a Kd of approximately $2.1 \times 10^{-5}$ M. The amino acid sequence obtained from a cDNA encoding the 57-kDa subunit shared high homology with a parsley S-adenosyl-l-homocysteine (SAH) hydrolase (Mitsui et al. 1993). This enzyme is the major adenosine/cAMP-binding protein that controls the intracellular S-adenosylmethionine (SAM)/SAH ratio and thereby influences the efficiency of methylation in mammalian cells (Hohman et al. 1985). This suggested the possibility that some aspects of cytokinin activity could be mediated through the control of methylation of protein or DNA. This was the first, although not yet direct, indication of a possible function of cytokinin-binding proteins in the signal transduction of cytokinin. However, Romanov and Dietrich (1995) have reported that the major cytokinin-binding proteins from maize are not associated with SAH hydrolase activity. Using different fractionation and purification procedures and direct biochemical assays, they showed that cytokinin-binding activity and SAH hydrolase activity can easily be separated by fast protein liquid chromatography (FPLC). It therefore seems unlikely that the previously characterized 57-kDa cytokinin-binding protein possesses SAH hydrolase activity. Recently, Kakimoto identified a gene (CKII) in *Arabidopsis* which is involved in cytokinin signal transduction (Kakimoto 1996) and is similar in function to the ethylene receptor (ETR1) (Kieber et al. 1993). Also, Dymock suggest that a plant seven-transmembrane receptor is implicated in cytokinin signaling (Dymock et al. 1998). Although CKII and seven-transmembrane receptor are possible candidates of cytokinin receptor, the cytokinin-binding activity of the gene product has not been demonstrated.

As described above, a number of cytokinin-binding proteins have been identified, but none of these proteins had any function to explain the physiological action of cytokinin. In this paper, we present a method for the purification of cytokinin-binding proteins from tobacco callus, resulting in the isolation of two high affinity cytokinin-binding proteins, CBP1 and CBP2, from the soluble fraction. CBP2 was purified to homogeneity and the characteristics of the protein were investigated. Amino acid sequences of proteolytic fragments of CBP2 were found to be nearly identical to osmotin-like protein (OLP). Recombinant OLP bound to cytokinin and western blotting of recombinant OLP against anti-CBP2 antibodies showed the same behavior as that of CBP2. As OLP is classified as a stress protein, the possible physiological role of CBP2 is discussed.

**Materials and Methods**

*Plant materials*—Tobacco callus BY-2 was grown on a solid medium supplemented with 6 μM IAA, 0.5 μM BA and 0.8% of agar (Murashige and Skoog 1962). Three callus pieces of about 300 mg fresh weight were implanted on 50 ml of solid medium and maintained at 26°C in darkness for 28–30 d.

**Preparation of BA-linked Sepharose 4B—CNBr-activated Sepharose 4B gel (7.5 g) (Pharmacia Fine Chemicals, Upsalla, Sweden) was treated with $10^{-3}$ M HCl (200 ml of gel dry gel) for 15 min with gentle stirring and then washed extensively with distilled water. The gel was suspended in 300 ml of distilled water that contained $2 \times 10^{-4}$ M BA. The mixture was then shaken for 20 h at 37°C and washed successively with 0.1 M NaOH, 5% (v/v) TCA and extensively with distilled water. The gel was packed into a column (1.2 × 15.0 cm), loaded with 1.0 M ethanolamine (pH 8.0) for 2 h to block uncoupled sites on the Sepharose, and then equilibrated with TBS buffer (50 mM Tris-HCl buffer, pH 7.5, containing 10 mM NaCl).

**Purification of cytokinin-binding proteins (CBPs)—** The following procedures were performed at 4°C. Tobacco callus (200 g FW) was collected on filter paper with a Buchner funnel, and suspended in 100 ml of extraction buffer [50 mM Tris-HCl buffer, pH 7.5, containing 2 mM 2-mercaptethanol, 1 mM dithiothreitol (DTT), 1 mM Na2EDTA, 0.12 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM MgCl2], and homogenized with 200 g of sea sand and 3.3% (w/v) Polyclar AT using a mortar and pestle. The homogenate was centrifuged at 1,000 × g for 10 min and the supernatant was centrifuged at 18,000 × g for 30 min. The supernatant was precipitated by 80% saturated ammonium sulfate and stored overnight. The precipitate was collected by centrifugation at 18,000 × g for 15 min, dissolved in 50 mM Tris-HCl buffer, pH 7.5, containing 6.5 mM NaCl and 0.1 mM EDTA, and dialyzed against the same buffer. The precipitate formed during dialysis was removed by centrifugation at 18,000 × g for 15 min. The supernatant (crude extract) was processed as described below.

The crude extract was applied to a DEAE-cellulose column (2.5 × 8.0 cm, DE 52 Whatman, Madistone, U.K.) which had been equilibrated with TBS (50 mM Tris-HCl, pH 7.5, 10 mM NaCl). The column was washed with 60 ml of TBS and eluted with a linear gradient of 0.01 to 1.0 M NaCl in TBS. Fractions containing cytokinin-binding activity were combined and the proteins were concentrated by precipitation with ammonium sulfate (80% saturation). The precipitate was dissolved in a minimum volume of TBS and centrifuged at 18,000 × g for 15 min to remove the denatured insoluble proteins. The supernatant was applied to a column of BA-linked Sepharose 4B (1.2 × 15.0 cm) that had been equilibrated with TBS. The column was washed with 120 ml of TBS and then eluted with TBS containing 1.0 M NaCl and 25% (v/v) ethylene glycol. Each 3-ml fraction was collected at a flow rate of 0.2 ml min⁻¹.

**Solubilization of recombinant OLP—** E. coli transfected with recombinant pET-3d plasmid containing OLP cDNA (Takeda et al. 1991) were grown overnight in LB medium containing 100 μg ml⁻¹ ampicillin at 37°C. The culture was diluted 1 : 9 in fresh LB medium containing ampicillin and grown at 37°C for 2 h, at which time, optical density was measured to determine logarithmic bacterial growth. At the end of this period, 1 mM isopropyl β-d-galactopyranoside (IPTG) was added for induction of transcription from the tac promoter. The culture was then grown at 37°C for 3 h, after which bacteria were harvested by centrifugation at 8,000 × g for 10 min at 4°C. The bacteria were homogenized in 1 ml PBS including 1% Triton X-100 solution (150 mM NaCl, 16 mM NaH2PO4, 4 mM Na2HPO4, pH 7.3, 1% Triton X-100) per 50 ml *E. coli* culture. *E. coli* was centrifuged at 10,000 × g for 5 min at 4°C. The bacterial pellet was resuspended in lysis buffer and suspended in 1 ml TBS.
solution [400 μg ml⁻¹ lysozyme, 10% sucrose, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0)], incubated for 30 min, and centrifuged at 12,000 × g for 15 min. The resulting pellets were resuspended in 1 mM DTT, 100 μM PMSF, 100 mM Tris-HCl (pH 8.0) and cells were broken open by freeze-thawing. To this suspension, DNase (50 K units ml⁻¹) and 10 mM MgCl₂ was added, and debris were centrifuged at 12,000 × g for 15 min. The pellets were resuspended in 1% TritonX-100, 1 mM EDTA, 100 mM NaCl, 20 mM Tris-HCl (pH 8.0) and centrifuged at 12,000 × g for 15 min. The supernatant (Triton X-100 solubilized fraction) and the pellet, which contains inclusion bodies, were separated.

Inclusion bodies were resuspended in 2 M urea (2 M urea solubilized fraction) and centrifuged at 12,000 × g for 15 min. The pellets were solubilized with 8 M urea containing 1 M DTT were centrifuged at 12,000 × g for 15 min. The pH of the supernatant was reduced 5.0 with Tris-acetate in 6 M urea containing 1 M DTT, and centrifuged at 18,000 × g for 20 min. The supernatant was applied to a SP-Sepharose column in running buffer (8 M urea, 1 mM EDTA, 25 mM Tris-acetate, pH 5.0) and soluble recombinant OLP in 8 M urea was slowly dialysed against buffer for 4-5 d at 4°C to completely remove urea, and purified soluble recombinant OLP was obtained.

Estimation of cytokinin-binding activity—Cytokinin-binding activity was determined by the method of equilibrium dialysis of Reinard and Jacobsen (1989). Test cells were made from commercially available 1.5 ml microtubes (Eppendorf), where the lid is used as a reservoir for binding buffer and the tube serves as a lid. First, the lid and tube are separated, and a hole is made on the tube with a hot pin. Dialysis membrane (e.g. Visking from Serva) were cut into 1.5 × 1.5 cm squares and soaked in the Tris buffer without ligand. Next, the membrane was placed on the lower lid, which had been filled with 260 μl of binding buffer containing 50 mM Tris-HCl buffer, pH 7.5, and [³H]benzyladenine (³H-BA, 57 mCi mmol⁻¹, Moravek Biochemicals Inc., CA, U.S.A.) as well as unlabelled BA as ligand. The dialysis membrane was fixed between the lower lid and the upper tube. The binding assay was started by introducing 260 μl of protein solution from the hole in the upper tube. The cell was incubated for 20 h at 4°C in a humid chamber. Following dialysis, 100 μl of the protein solution was collected and the radioactivity was measured in a liquid scintillation counter with Beckman LS 6500 (Beckman Ins. Inc., Fuller- ton, CA, U.S.A.). Aliquots from both compartments of the cell were measured in the same liquid scintillation counter. Aequasol-2 (Biotechnology Systems NEN Research Products, Boston, U.S.A.) was used as scintillant liquid.

Specific binding was calculated by subtracting the non-specific binding from the total binding. Total binding was determined in 1.0 × 10⁻⁷ M ¹⁴C-BA (57 mCi mmol⁻¹) and non-specific binding in 1.0 × 10⁻⁷ M (57 mCi mmol⁻¹) with 200-fold molar excess of non-radioactive BA. In the kinetic investigation of the binding of BA to CBPs, total binding was determined with ¹⁴C-BA (57 mCi mmol⁻¹) over a range of concentrations of 1.0 × 10⁻⁷ M to 1.0 × 10⁻³ M, and non-specific binding was determined with ¹⁴C-BA (57 mCi mmol⁻¹) over the same range of concentrations with 200-fold molar excess of non-radioactive BA. The dissociation constant and the number of binding sites on the protein molecule were calculated from Scatchard plots (Scatchard 1949, Rosenthal 1967) of the data obtained.

Measurement of chitinase activity—Ethylene glycol chitin, was used as a substrate. To 1 ml of 0.05% (w/v) ethylene glycol chitin, 0.5 ml of protein solution was added and incubated for 15 min at 37°C. Color reagent [2 ml, 0.05 g liter⁻¹ K₂Fe(CN)₆ in 0.5 M Na₂CO₃] was added and the mixture was immediately boiled for 15 min. After the mixture was cooled, the absorbance at 420 nm was read. The change in absorbance was directly proportional to the enzymatic product, N-acetylglucosamine (GlcNAc) concentration (Imoto and Yagishita 1971). Chitinase activity was calculated from a standard curve of GlcNAc (1 Unit = 1 μmol GlcNAc product min⁻¹).

Protein quantitation—The protein content of each fraction was determined by the method of Bradford (1976) using a protein assay kit (Bio-Rad Labolatories, Richmond, CA, U.S.A.) with bovine serum albumin (Sigma) as the standard.

Polyacrylamide gel electrophoresis—SDS-PAGE was performed using slab gels according to the method of Laemmli (1970). A 12% polyacrylamide gel (bisacrylamide : acrylamide—0.8 : 30.0) was prepared. After electrophoresis, protein bands were stained in Coomassie Brilliant Blue R 250 (CBB) in 50% (v/v) methanol and 7% (v/v) acetic acid overnight with gentle shaking, and destained with 20% (v/v) methanol and 7% (v/v) acetic acid.

Estimation of molecular mass—The molecular mass of purified CBPs was estimated by gel filtration on a 1.0 × 72.0 cm column of Sephadex G-75 (Pharmacia Fine Chemicals) that had been equilibrated with TBS. The column was calibrated with a molecular mass markers (MW-GF-200KIT, Sigma, St. Louis, Missouri, U.S.A.), consisting of β-amylose (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa).

Western blotting analysis—Purified CBP2 (130 μg) was diluted with 500 μl of phosphate buffer and mixed well with 750 μl of Freund’s complete adjuvant. The mixture was introduced into four positions of the abdomen of a guinea pig by subcutaneous injection. After one month, dot blot was performed to check the formation of antibody. If the antibody was formed at that time, an equal amount of CBP2 was injected again into the same guinea pig in the same manner as described above (in this step, Freund’s incomplete adjuvant was used). After one week, dot blot was performed again. Blood (about 7–8 ml (guinea pig)⁻¹) was collected from the heart with a syringe. The blood was incubated at 37°C for one hour and centrifuged at 18,000 × g for 10 min at 4°C. Antiserum were obtained as supernatant and stored at −20°C until use. This antiserum was specific for CBP2 when tested against total protein from tobacco callus in a western blotting experiment (data not shown). However, a week response was also observed for CBP1.

Appropriate quantities of CBP2 and recombinant OLP were fractionated by 12% SDS-PAGE, and transferred from the gels onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Co. Bedford, MA, U.S.A.) in a blotting apparatus (Bio-Craft, Model BE-310). Transfer was carried out at 200 mA for 45 min in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 15% (v/v) methanol. A portion of the membrane was cut for Coomassie Brilliant Blue (CBB) staining and the remainder was used for immunoblotting analysis. Membranes were blocked for 1 h in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20] and 4% defatted milk powder (blocking solution) at 37°C with gentle shaking, then washed 3 times with the same solution. The first antibody reaction was carried out for 1 h in blocking solution at 37°C. We generally used 20 ml of this solution with antiserum at a dilution of 1 : 1,000. The membrane was washed with blocking solution without antibody 3 times for 10 min each. The second antibody reaction was performed for 30 min at 37°C in 10 ml of blocking solution containing 5 μl of
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affinity purified rabbit anti-guinea pig IgG coupled to alkaline phosphatase (Zymed Laboratories, Inc. San Francisco, CA, U.S.A.). The membrane was rinsed 3 times for 10 min each with TBST, and once for 5 min with AP buffer [100 mM Tris-HCl (pH 9.5), 5 mM MgCl₂, 100 mM NaCl]. Staining of membrane was performed in 5–10 ml of AP buffer containing [0.4 mM NBT (nitroblue tetrazolum), 0.4 mM BCIP (5-bromo-4-chloro-3-indolyl phosphate)] at 37°C for 5 min with gentle shaking. When color appeared on the membrane, the reaction was stopped by washing with water.

Determination of the NH₂-terminal amino acid residues of CBPs—

The N-terminal sequence of the purified CBPs was determined after a 12% SDS-PAGE gel and electroblotting onto PVDF membrane (Immobilon-P, Millipore). The CBP band visualized by CBB-staining was destained with 20% (v/v) methanol and 7% (v/v) acetic acid, excised from the PVDF membrane with a sharp razor, and amino acid sequences were determined using an automated protein sequencer (ABI 477A protein sequencer with an online 120A PTH analyzer).

Determination of amino acid sequences of partial peptides from CBP2—CBP2 purified by electrocution through Centolutor (Amicon, Grace Company, Beverly, MA, U.S.A.) was separated on a SDS-PAGE gel. Peptide fragments of the purified CBP2 were obtained by the digestion of the protein at 37°C with 2.5 μg prolyl endopeptidase (Wako Pure Chemical Industries, Osaka, Japan) and their separation was performed according to the methods of Herman (Herman 1987). A 16.5% acrylamide gel for separation and 4% gel for concentration were used. After electrophoresis, electroblotting onto PVDF membrane (Immobilon-P, Millipore) was performed and peptide fragments were stained for 1 h with 0.025% Seva Blue G in 10% acetic acid and destained for 2 h with 10% acetic acid. RPN 755 (Amersham) was used as low molecular mass markers. Then, amino acid sequences were determined using an automated protein sequencer 492-HOC (Applied Biosystem, Branchburg, NJ, U.S.A.).

Results

Isolation of cytokinin-binding proteins from the soluble fraction of tobacco callus—Two main peaks were observed upon DEAE-cellulose chromatography of the soluble fraction of tobacco callus. The first peak eluted at 0.01 M NaCl, and the second peak eluted at approximately 0.4 M NaCl. Cytokinin-binding activity fractions were obtained. These active fractions from the DEAE-cellulose column were collected and applied to a BA-linked Sepharose 4B column. The column was washed with TBS buffer prior to the elution with 25% ethylene glycol in the presence of 1 M NaCl until no UV-absorbing substance was detected in the eluate. As shown in Figure 1a, three main peaks (in absorbance at 280 nm) were obtained. Two of them (one eluted by washing and the other by elution buffer) showed cytokinin-binding activity. The second peak (fractions 18–30) had a relatively high cytokinin-binding activity. The third peak was eluted in fractions 45–54 with TBS containing 1 M NaCl and 25% ethylene glycol.

Protein fractions at each step were analyzed by SDS-PAGE (Fig. 1b). Lane 1 and lane 2 show the electrophoresis pattern of the supernatant after 1,000 x g and 18,000 x g centrifugation in the purification steps, respectively. Fractions from the second peak contained a single band of protein with a molecular mass of 34 kDa (lane 5), which we named CBP1. Fractions from the third peak contained a major band of protein with a molecular mass of 26 kDa (lane 6), which we named CBP2.

The molecular masses of the purified CBP1 and CBP2

Fig. 1a Affinity chromatography on BA-Sepharose 4B column of the active fractions obtained from the column of DEAE-cellulose. The column was washed with 50 mM TBS and eluted with 50 mM TBS containing 1.0 M NaCl and 25% ethylene glycol. The arrow indicates the start of addition of elution buffer. The solid circle (○) indicates the absorbance at 280 nm, and the open circle (●) indicates chitinase activity. BA-binding activity (shaded area) was determined by equilibrium dialysis.

Fig. 1b SDS-PAGE of cytokinin-binding proteins. The proteins were stained with Coomassie Brilliant Blue. (1) 5 μg of supernatant after centrifugation at 1,000 x g. (2) 5 μg of supernatant after centrifugation at 18,000 x g. (3) 5 μg of crude extract. (4) 3 μg of pass-through fraction from BA-Sepharose 4B. (5) 2 μg of CBP1 fraction. (6) 2 μg of CBP2 fraction. (7) Molecular mass markers: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and a-lactalbumin (14.4 kDa).
Fig. 2 Determination of molecular masses of cytokinin-binding proteins (CBP1, CBP2) on Sephadex G-75 column. The gel filtration column was eluted with TBS (described in Materials and Methods). The column was calibrated with molecular mass markers, which were blue dextran (Vo), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa).

in their native forms were also estimated by gel-filtration chromatography on a Sephadex G-75 column (Fig. 2). The column was calibrated with molecular mass markers. From this standard curve, we obtained the values of 34 ± 1 kDa and 26 ± 1 kDa for CBP1 and CBP2, respectively.

From a total of 200 g of tobacco callus, 1.06 mg of CBP1 and 0.45 mg of CBP2 were obtained (Table 1). The value of total cytokinin-binding activity of crude extract (3.63 x 10³ dpm) is consistent with the sum of activity of CBP1 and CBP2 (1.86 x 10³ dpm + 1.79 x 10³ dpm each). These chromatographic processes efficiently removed other proteins, but a large part of cytokinin-binding activity in the original fraction was recovered during this procedure, possibly because most proteins present in the original fraction were not adsorbed by the BA-linked Sepharose 4B column. This procedure resulted in a 270-fold purification of CBP1 and 600-fold purification of CBP2, calculated from the value of specific cytokinin-binding activity (dpm (mg protein)⁻¹).

Characterization of cytokinin-binding properties of CBPs—The effect of pH on the binding of BA is shown in Figure 3. The specific cytokinin-binding activities of CBP1 and CBP2 were measured from pH 4.0 to pH 10.0, using several buffers (50 mM sodium-acetate, 50 mM phosphate, 50 mM Tris-HCl and 50 mM Tris-glycine). The optimum pH values for binding of BA to CBP1 and CBP2 were approximately pH 6.5 and pH 7.5, respectively.

In order to characterize the affinity of CBPs for BA, Scatchard analysis was carried out. The dissociation constant (Kd) and the number of binding sites on the protein molecule were determined over a range of concentrations from 1.0 x 10⁻⁷ M to 1.0 x 10⁻⁵ M BA (Fig. 4). This analysis yielded biphasic curves, indicating that both CBP1 and CBP2 have at least two binding sites for BA. The dissociation constant (Kd) and frequency (number of binding sites) of CBP1 for high affinity binding were 8.87 x 10⁻⁶ M.

Table 1 Purification of CBPs from tobacco callus

<table>
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<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (dpm)</th>
<th>Specific activity (dpm mg⁻¹)</th>
<th>Purification (-fold)</th>
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<td>BA-Sepharose 4B</td>
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<tr>
<td>CBP1</td>
<td>1.06</td>
<td>1.86 x 10³</td>
<td>1.76 x 10³</td>
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<tr>
<td>CBP2</td>
<td>0.45</td>
<td>1.79 x 10³</td>
<td>3.98 x 10³</td>
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Purification of CBPs from tobacco callus. At each step in extraction and purification, protein contents and cytokinin-binding activities were determined as described in Material and Methods. Purification of CBPs was calculated from the value of specific binding activity (dpm (mg protein)⁻¹).
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CBPs bind to cytokinins with high and low affinity. The high-affinity binding site for CBP1 has a dissociation constant (Kd) of $1.08 \times 10^{-6}$ M, and for CBP2 it is $1.46 \times 10^{-6}$ M. The low-affinity binding site for CBP1 has a Kd of $1.35 \times 10^{-7}$ M, and for CBP2 it is $1.15 \times 10^{-7}$ M.

Fig. 4 Scatchard plot for BA binding to CBP1 and CBP2. The binding of $1.0 \times 10^{-7}$ to $1.0 \times 10^{-5}$ M BA to CBP1 and CBP2 was determined using 10 µg of CBPs for each assay, as described in Materials and Methods. The graphs show moles of BA bound/concentration of free BA versus moles of BA bound. The apparent dissociation constant (Kd) of CBP1 and CBP2 for BA were calculated according to the method of Scatchard (1949). The values for CBP1 were $1.08 \times 10^{-6}$ M and $2.13 \times 10^{-7}$ M, respectively, for high and low affinity. For CBP2, the values were $1.35 \times 10^{-7}$ M and $2.13 \times 10^{-8}$ M, respectively.

The binding affinity (Kd) of CBP2 for BA was comparable to previously reported values for cytokinin-binding proteins. Furthermore, binding to the cytokinin molecule was reversible and saturable (data not shown).

The binding specificities of CBPs were examined by adding various cytokinin analogues and IAA, and analyzing their ability to displace $^{14}$C-BA. As shown in Table 2, among all active cytokinins tested, zeatin (a naturally occurring cytokinin) and kinetin (a synthetic cytokinin) were highly effective in displacing $^{14}$C-BA, especially in CBP2. Binding of $^{14}$C-BA to CBP2 was inhibited by 98.6% and 92.3% by $10^{-4}$ M zeatin and kinetin, respectively. This suggested that CBP2 interacts with cytokinins in vivo. Adenine, adenosine and ATP inhibited the binding of BA to both CBP1 and CBP2, but the extent of the inhibition was less than that by zeatin and kinetin. A number of CBPs have already been isolated from various plants, but binding of cytokinin to these CBPs is generally not inhibited by adenine, adenosine and ATP. cAMP showed little competitive activity in the present experiment. Cytokinins have been found to enhance cell division in the presence of IAA. However, $10^{-4}$ M IAA had no effect on the binding of BA to either CBP1 or CBP2.

Table 2 Effects of various cytokinin analogues and IAA on the BA-binding activity of CBP1 and CBP2

<table>
<thead>
<tr>
<th>Compound</th>
<th>(10^{-4} M)</th>
<th>% Displacement</th>
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<tr>
<td>BA</td>
<td>100.0</td>
<td>100.0</td>
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<td>Zeatin</td>
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Effects of various cytokinin analogues and IAA on the BA-binding activity of CBP1 and CBP2. Binding of BA was determined by equilibrium dialysis against 50 mM Tris-HCl buffer (pH 7.5) that contained $1.0 \times 10^{-7}$ M $^{14}$C-BA and the competitors described. Dialysis was performed for 20 h at 4°C. $^{14}$C-BA displaced by each competitor is shown as the percentage of the $^{14}$C-BA bound in the absence of competitor.

Fig. 5 Comparison of NH_2-terminal amino acid sequences of CBP1 and CBP2. Homologous amino acids are indicated by the asterisk. X indicates an unidentified amino acid position.
Homology of the NH$_2$-terminal amino acid sequences in CBPs to OLP and endochitinase—The NH$_2$-terminal amino acid sequences of CBP1 and CBP2 were compared with protein sequences in the National Biomedical Research Foundation Protein Sequence Data Bank of Japan. There was high homology between the NH$_2$-terminal sequence of CBP1 and the corresponding region of endochitinase (90.0%) and between the NH$_2$-terminal sequence of CBP2 and the corresponding region of osmotin-like protein (92.9%) (Fig. 5).

**Purification of CBP1**—In order to examine whether CBP1 has chitinase activity, we measured the chitinase activity of each fraction eluted from BA-linked Sepharose 4B was measured (Fig. 1a). A sharp peak of chitinase activity was detected only in the CBP1 fraction but not in the CBP2 fraction. This result is consistent with the results of SDS-PAGE (Fig. 1b) and homology analysis of NH$_2$-terminal amino acid of CBPs (Fig. 5). However, there is an obvious difference between the position of chitinase activity and cytokinin-binding activity in the CBP1 fraction. This suggests the existence of at least two or more proteins in this fraction although only a single band with a molecular mass of 34 kDa (Fig. 1b, 2) was detected. An attempt to separate these proteins by isoelectric focusing was unsuccessful. Isoelectric focusing appears to be unsuitable for separating basic proteins whose pI is over pH 7-8, showing notable tailing. NEPHGE (nonequilibrium pH gradient electrophoresis) (O’Farrell et al. 1977) resulted in the detection of three major bands in the CBP1 fraction (unpublished data). The results suggest that cytokinin-binding activity and chitinase activity arise from separate proteins. Further characterization of CBP1 is currently under way.

**Amino acid sequences of internal fragments of CBP2**—Purified CBP2 was used for the determination of amino acid sequences. CBP2 was digested with protease V8, which cleaves after glutamine, and two new internal fragments were obtained along with an N-terminal fragment (Fig. 6a, lane 2). The sequence of the 17 kDa fragment (WKGWGKPPNTLAELYNQFS) was the same as that of OLP except for the first amino acid (W), (Fig. 6b). The
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Discussion

CBP1 and CBP2 were isolated from the water soluble fraction of tobacco callus by a combination of DEAE-cellulose column chromatography and affinity chromatography using BA as a ligand (Table 1). To isolate cytokinin-binding proteins, many groups have used affinity chromatography with cytokinins as ligands (Takegami and Yoshida 1975, Polya and Davis 1978, Chen et al. 1980, Erion and Fox 1981, Momotani and Tsuji 1992). We found that an elution buffer containing 1 M NaCl and 25% ethylene glycol, which is used for affinity chromatography of antigens and antibodies (Anderson et al. 1979), was effective for the elution of CBP2, which binds strongly to cytokinin (BA) through hydrophobic interactions.

CBP1 and CBP2 were estimated to have a molecular masses of 34 kDa and 26 kDa, respectively, from their mobility during SDS-PAGE (Fig. 1b) and by gel filtration (Fig. 2). CBP1 and CBP2 are different from the CBPs reported to date. Although the molecular mass of CBP1 is similar to that of cytokinin-binding protein (32 kDa) isolated from the soluble fraction of a strain of tobacco callus cells derived from *Nicotiana tabacum* cv. Wisconsin No. 38 (Hamaguchi et al. 1985), the binding properties in terms of dissociation constant and the number of binding sites of CBP1 are different from those of this protein. A Scatchard plot showed that both CBP1 and CBP2 have two binding sites for BA per molecule. Kd values for the high-affinity sites of CBP1 and CBP2 were 8.87 x 10^-6 M and 1.08 x 10^-6 M, respectively. A high concentration of ammonium sulfate has been reported to somehow provoke the artificial binding of plant hormone to protein (Venis 1984). To avoid overestimation of the binding activity due to non-specific binding in the presence of ammonium sulfate, we used the equilibrium dialysis method for binding assays. This method allows us to distinguish specific binding from non-specific binding. Compared with other CBPs, the affinity of CBP1 for BA seems to be low. The affinity of CBP2 for BA is high, and positively correlated with the cytokinin binding activity of related cytokinin analogues. The reason for the differences in optimal pH (Fig. 3) and affinities to adenine, adenosine and ATP (Table 2) in CBP1 and CBP2 is unknown.

The N-terminal amino acid residues (Fig. 5) and amino acid sequences of internal fragments (Fig. 6a, b), indicated that CBP2 is the same as OLP. The results of immunoblotting analysis (Fig. 7) and cytokinin-binding assay (Fig. 8) using recombinant OLP indicate that it has the same function as CBP2 in structure and recognition of binding site to cytokinin. This is the first report that a stress protein, OLP, is related to a cytokinin-binding protein, CBP2.

Expression of genes encoding tobacco osmotin and osmotin-like protein is induced by ABA, NaCl, ethylene, and also by wounding and viral infection (Larosa et al. 1992, Zhu et al. 1995). In addition, the promoter of osmotin gene exhibits specific temporal and spatial expression patterns both during normal plant development and after adaptation to NaCl (Nelson et al. 1992). Sequence comparison revealed that osmotin and osmotin-like protein are highly homologous to an anti-virus protein (gp22) iso-
lated from cultured tobacco cells (Kononowicz et al. 1992). Gene expression of stress protein controls tissue specificity during the late stage of embryogenesis in *Pisum sativum* L. (Barratt and Clark 1991) and specific stages in flower formation (Fluhr et al. 1991). Furthermore, a plant homedoain protein involved in transcriptional regulation of pathogen-related gene has been cloned (Korfhage et al. 1994). Thus there seems to be a close relationship between stress proteins and morphogenesis, and CBP2 may be a stress protein which plays an important role in hormone signal transduction of cytokinin related to morphogenesis. Cytokinins are synthesized mainly in roots, and the expression of osmotin and osmotin-like protein is developmentally regulated in intact plants with a high level of expression in roots (Zhu et al. 1995). Moreover, the present study demonstrates the nearly complete homology between CBP2 and OLP (a stress protein). OLP is located mainly in the roots (Koiwa et al. 1994). Fujimoto reported that cytokinin-specific binding protein belongs to a pollen allergen/pathogenesis-related protein family (Fujimoto et al. 1998). However, its physiological role has not been described. Further physiological analysis at the gene level is now under way to clarify the role of CBP2, which has high affinity for cytokinins and highly homologous to OLP, a member of stress protein family.

We thank Dr. Humihiko Sato (Department of Applied Life Sciences, Kyoto University) for gift of recombinant OLP, Dr. Noriaki Kondo (Department of Biological Sciences, University of Tokyo) for the determination of N-terminal amino acid residue of CBPs, Dr. Hirofumi Uchimiya and Dr. Sigeo Tomioka (Institute of Molecular and Cellular Biosciences, University of Tokyo) for the determination of amino acid sequences of peptide fragments of CBP2 and Dr. Kazuo Harada (Laboratory of Life Sciences, Tokyo Gakugei University) for critical reading of this manuscript.

**References**


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(Received August 23, 1999; Accepted November 14, 1999)