A Water-Soluble Chlorophyll Protein in Cauliflower May Be Identical to BnD22, a Drought-Induced, 22-Kilodalton Protein in Rapeseed

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A water-soluble chlorophyll protein (WSCP) in cauliflower (Brassica oleracea L.) was purified and its N-terminal sequence was determined. Forty-six of 48 residues of the sequence completely matched those of the drought-induced 22-kD protein (BnD22) in rapeseed (Brassica napus L.). All 40 sequenced residues of WSCP from rapeseed were perfectly matched to those of BnD22. Thus, WSCP may be identical to BnD22. The abundance of WSCP was increased in detached cauliflower leaves.

The existence of a WSCP, with an absorption spectrum that changed drastically with illumination, was first reported in Chenopodium album (Yakushiji et al., 1963). This type of WSCP is found in some Chenopodiaceae and Amaranthaceae species (Takamiya, 1972-1973). A different type of WSCP, with an absorption spectrum that showed no conversion by illumination, was found in cauliflower (Brassica oleracea var Botrys) (Murata et al., 1971), wild mustard (Brassica nigra; Murata and Murata, 1971), and Lepidium virginicum (Murata and Ishikawa, 1981). This latter type of WSCP is known to be widespread among Brassicaceae plants, suggesting that it is important in plant metabolism. Physicochemical and biochemical characterization of these chlorophyll proteins has been performed (Murata and Murata, 1971; Murata et al., 1971; Murata and Ishikawa, 1981; Murata, 1986), but almost nothing is known of their physiological function in plant tissues. In this study we purified the WSCP from cauliflower and determined its N-terminal amino acid sequence. Comparisons of the sequence with protein databases revealed a near-identity of WSCP to a proteinase inhibitors, because they possess a complete sequence signature motif for this family (Downing et al., 1992; Reviron et al., 1992). The physiological role of these proteins is not known, but it is predicted that they are proteinase inhibitors, because they possess a complete sequence signature motif for this family (Downing et al., 1992; Lopez et al., 1994). Since the N-terminal amino acid sequence of cauliflower WSCP was nearly identical to that of BnD22 in rapeseed, we hypothesized that BnD22 and WSCP in rapeseed are identical. The occurrence of WSCP in rapeseed has not been reported; therefore, this protein was purified, and its N-terminal amino acid sequence was compared with that of BnD22.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of cauliflower (Brassica oleracea L. var botrys) and rapeseed (Brassica napus L. var oleifera) were germinated on moistened vermiculite containing 50% of no. 1034 plant culture soil (Green Tec, Tochigi, Japan). The seedlings were grown under controlled environmental conditions at a temperature of 25°C, a RH of 50%, and a photon flux density of 200 μmol m⁻² s⁻¹, during a 16-h light photoperiod (6 AM-10 PM), and were watered every day at the same time (8 PM) to maintain soil moisture at field capacity.

Purification of WSCP from Cauliflower Plants

Purification of WSCP from cauliflower was performed during vegetative growth, 2 months after germination. One kilogram of plants, including leaves, stems, and roots, was homogenized with a blender in 100 mM phosphate buffer (pH 7.4) containing 850 mM NaCl. The homogenate was filtered through two layers of gauze. Solid (NH₄)₂SO₄ was added to the filtrate and a fraction between 40 and 70% saturation was collected by centrifugation. The precipitate was dissolved in 100 mM phosphate buffer (pH 7.4) and dialyzed against the same buffer. The dialysate was passed through a DEAE-cellulose column (DE-52, Whatman), which had been equilibrated with 100 mM NaCl. The homogenate was filtered through two layers of gauze. Solid (NH₄)₂SO₄ was added to the filtrate and a fraction between 40 and 70% saturation was collected by centrifugation. The precipitate was dissolved in 100 mM phosphate buffer (pH 7.4) and dialyzed against the same buffer. The dialysate was passed through a DEAE-cellulose column (DE-52, Whatman), which had been equilibrated with 100 mM phosphate buffer (pH 7.4). The unbound fraction was concentrated and dialyzed against 10 mM phosphate buffer (pH 7.4). The dialysate was separated by PAGE (detergent-free) according to the method of Laemmli (1970). A major green band was excised with a razor blade and the protein was recovered from the gel by the crush-and-soak method. The WSCP fraction was concentrated and subjected to gel-filtration chromatography on a Superdex 200 HR column. Fractions corresponding to the major peak of the elution profile were pooled and subjected to biochemical analyses. WSCP was purified from rapeseed by the same procedure as that for cauliflower.

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Abbreviations: BnD22, Brassica napus L. drought-induced 22-kD protein; WSCP, water-soluble chlorophyll protein.
The molecular masses of WSCPs from cauliflower and rapeseed were determined by Superdex 200 HR column chromatography using chymotrypsinogen A (25 kD), aldolase (158 kD), and ferritin (440 kD) as the standards.

The protein concentration was determined by the bicinchoninic acid method using a kit from Sigma. BSA was used as a standard.

**Preparation of Polyclonal Antibody**

All basic immunological techniques referred to are standard protocols (Harlow and Lane, 1988). For immunization of rabbits, the WSCP polypeptide from cauliflower was further purified by SDS-PAGE. A band containing 1 mg of 22-kD polypeptide was excised, and the gel was fragmented by passing it several times through syringe needles. An equal volume of Freund’s complete adjuvant was added to the fragments and mixed vigorously by vortexing. One intradermal and two subcutaneous injections were performed at 3-week intervals. Immunoglobulins were purified from the antiserum by (NH₄)₂SO₄ fractionation followed by protein A-Sepharose CL-4B column chromatography, according to standard methods. The titer of the antibody was assayed by western-blot analysis.

**Treatments of Cauliflower Plants under Stress Conditions**

Stress was imposed on 3-month-old well-watered cauliflower plants using two regimes. For each set of experiments, three to five leaves were collected and stored at -80°C until they were assayed.

In a first set of experiments cauliflower leaves with petioles were detached from the stem (at 8 pm) and placed on a dry paper towel under the conditions described above. The leaves were frozen (-80°C) 0, 24, and 48 h after detachment.

In another set of experiments leaves with petioles were detached (at 8 pm) and placed on a dry paper towel for 16 h under the conditions described above. They were then rewatered by soaking the cut end of the petiole in water for 8 h and again placed on a dry paper towel for 16 h.

The percentage of water content of the detached leaves was calculated as (WF – WD)/WF × 100, where WF is the weight of fresh material measured when harvested and WD is the dry weight measured after the leaf was dried at 70°C for 6 h.

**Western Blotting and ELISA**

To monitor the change of WSCP abundance after detachment, leaf proteins were analyzed by ELISA and western blotting using the specific polyclonal antibody raised in the present work. The frozen leaves (described above) were ground in a mortar in an equal volume of 0.1 M sodium phosphate buffer (pH 7.4). The homogenates were centrifuged and the supernatants were stored. An ELISA was used to quantify the expression levels of WSCP with the antibodies, using purified WSCP as a standard. WSCP content was determined relative to protein, chlorophyll, and leaf area.

In a second set of experiments aliquots of the leaf extracts containing 250 μg of protein were fractionated by SDS-PAGE. The molecular mass of the WSCP subunit was determined using BSA (66 kD), ovalbumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.2 kD) as the standards. The proteins were transferred onto a PVDF membrane, and WSCP was detected using anti-WSCP and peroxidase-conjugated anti-rabbit IgG antibodies.

**Determination of N-Terminal Amino Acid Sequence**

Two-hundred picomoles of the WSCP polypeptide was separated on SDS-PAGE. The polypeptide was transferred electrophoretically onto a PVDF membrane and stained with Coomassie brilliant blue in methanol. The band was cut from the membrane and subjected to sequencing using a gas-phase sequencer (Procise 491, Applied Biosystems).

**Assay of Trypsin Inhibitory Activity of WSCP**

The inhibitory action of purified WSCP on trypsin was measured according to the method of Kim et al. (1985). An aliquot of the WSCP solution was mixed with porcine pancreatic trypsin (Wako Pure Chemical Industries, Osaka, Japan) at molar ratios of 1:1, 5:1, and 10:1, and the mixture was adjusted to 2.5 mL with 50 mM Tris-HCl buffer (pH 7.5). The mixture was incubated at 25°C for 5 min. Two milliliters of the mixture was placed in a cuvette and 0.1 mL of 10 mM BAEE (N-α-benzoyl-L-Arg ethyl ester hydrochloride) solution in the same buffer was added. The change at A253 was recorded against a reference cuvette containing 2 mL of the buffer and 0.1 mL of the BAEE solution. Soybean (Glycine max) trypsin inhibitor (Wako Pure Chemical Industries,) was used as a positive control for the inhibitory activity.

**RESULTS AND DISCUSSION**

We purified a WSCP from cauliflower plants including leaves, stems, and roots. The absorption spectrum of purified WSCP is shown in Figure 1. The WSCP shows absorption peaks at 673.0, 629.5, 437.0, 420.5, 383.0, 341.5, and 274.0 nm. The protein contains both chlorophylls a and b, due to chlorophyll a and the relatively small peak at 465 nm for chlorophyll b. Murata et al. (1971) reported that the chlorophyll a/b ratio of cauliflower WSCP was 6:1. The molecular mass of WSCP was 22 kD by SDS-PAGE (Fig. 4B, discussed below) and 76 kD by gel-filtration chromatography on Superdex 200 HR column (data not shown), indicating that native WSCP forms an oligomeric structure.

The N-terminal amino acid residues of WSCP, spanning 1 through 50, were sequenced and 48 amino acid residues were identified (except for two residues at 41 and 42, Fig. 2). A single amino acid peak was observed in each sequencing cycle. Amino acid recovery of the Edman degradation of WSCP was more than 30%, indicating that we sequenced a major protein in the preparation because the recovery is...
the known protein sequences in a nonredundant protein set. To identify isozymes or allelic variants of the WSCP genes, Figure 2 shows an alignment of the amino acid sequences of WSCP and related proteins that was obtained from the databases. Surprisingly, the WSCP sequence matches that of the mature form of BnD22 in rapeseed (Murata et al., 1992) and 4.6 for the cauliflower WSCP (Murata et al., 1971).

Comparisons between cauliflower WSCP and the 22-kD salt-stress-induced protein (P22) in radish (Raphanus sativus L.) showed a significant similarity. Forty of 48 residues of the cauliflower WSCP matched the sequences of P22, although residues 25, 26, 29 in P22 are missing in the WSCP sequence. Detailed comparisons of rapeseed BnD22 and radish P22 have been performed. Lopez et al. (1994) reported that both proteins are closely related.

BnD22 possesses a 19-residue signal sequence that may be responsible for targeting the precursor to ER (Downing et al., 1992), but WSCP in Lepidium virginicum has been detected in the stroma of Percoll-purified chloroplasts (Murata, 1986). The cauliflower WSCP may also exist in chloroplasts, since the protein contains chlorophyll molecules in its subunits, but it is also possible that the apo-WSCPs may be transported into chloroplasts via the ER and the Golgi apparatus, as is the case for a light-harvesting chlorophyll protein of Euglena (Kishore et al., 1993; Sulli and Schwartzbach, 1995).

BnD22 has been characterized as a 22-kD protein that is induced by progressive or rapid water stress and salinity (Downing et al., 1992; Reviron et al., 1992). To investigate

![Figure 1](image1.png)

**Figure 1.** Absorption spectrum of purified WSCP from cauliflower. The WSCP contains chlorophyll a and a relatively small amount of chlorophyll b.

usually 30 to 70%, depending on the protein species and the reaction conditions.

Two amino acid heterogeneities were detected at residues 15 (Gly is major and Ala is minor) and 25 (Ser is major and Lys is minor). These heterogeneities imply the existence of isozymes or allelic variants of the WSCP genes.

The amino acid sequence of WSCP was compared with the known protein sequences in a nonredundant protein sequence database constructed from SwissProt, PIR, pf, genpept, and genpept-upd using the BLASTp program (Altschul et al., 1990), implemented in a supercomputer at the Institute for Chemical Research (Kyoto University, Japan). Figure 2 shows an alignment of the amino acid sequences of WSCP and related proteins that was obtained from the databases. Surprisingly, the WSCP sequence matches that of the mature form of BnD22 in rapeseed (Downing et al., 1992), with the exception of residues 27 (Gly in WSCP and Ala in BnD22) and 37 (Ile in WSCP and Leu in BnD22). This result strongly suggests that the two proteins, WSCP in cauliflower and BnD22 in rapeseed, are identical. Because B. oleracea is the parent of the amphidiploid B. napus, cauliflower and rapeseed should contain many identical proteins. But it is also conceivable that their sequences are not perfectly matched, because the evolution of these two species has occurred independently.

Although WSCP and BnD22 may be identical, we cannot exclude the possibility that the C-terminal portion of WSCP, which was not sequenced in this study, differs from that of BnD22. The reported pI values of BnD22 and the cauliflower WSCP are similar, i.e. 5.1 for BnD22 (Reviron et al., 1992) and 4.6 for the cauliflower WSCP (Murata et al., 1971).

Comparisons between cauliflower WSCP and the 22-kD salt-stress-induced protein (P22) in radish (Raphanus sativus L.) showed a significant similarity. Forty of 48 residues of the cauliflower WSCP matched the sequences of P22, although residues 25, 26, and 29 in P22 are missing in the WSCP sequence. Detailed comparisons of rapeseed BnD22 and radish P22 have been performed. Lopez et al. (1994) reported that both proteins are closely related.

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![Figure 2](image2.png)

**Figure 2.** Sequence alignment of the 50 amino acid residues of cauliflower WSCP with related proteins obtained from the database search. WSCP, WSCP in cauliflower (B. oleracea). BnD22, Rapeseed (B. napus); and P22, radish (R. sativus) drought-induced 22-kD proteins. S36621 (accession no. of PIR database). Probable drought-induced protein in B. rapa (C. Shin, S. Song, and Y. Choi, unpublished data). S57810 (accession no. of PIR database). Hypothetical protein precursor (clone TPP11) in tomato (Lycopersicon esculentum) (Milligan and Gasser, 1995). KPI, Kunitz-type proteinase inhibitor in soybean (G. max). Vertical arrows indicate unmatched residues between cauliflower WSCP and BnD22. The region that contains a signature motif of the Kunitz-type proteinase inhibitor is underlined. Gaps, indicated by dashes, were introduced to obtain a maximum matching of the sequences. Amino acid residues that are identical to those of cauliflower WSCP are boxed.

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Changes in the amount of WSCP in cauliflower leaves after detachment as assayed by ELISA analyses using a polyclonal antibody. The stressed leaves were collected at 24-h intervals. At each sampling point, the proteins from five leaves were extracted and analyzed. Specific amounts of WSCP relative to total protein (open columns, per milligram of protein), leaf area (hatched columns, per square centimeter), and chlorophyll (filled columns, per microgram of chlorophyll) are displayed.

Figure 3. Changes in the amount of WSCP in cauliflower leaves after detachment as assayed by ELISA analyses using a polyclonal antibody. The stressed leaves were collected at 24-h intervals. At each sampling point, the proteins from five leaves were extracted and analyzed. Specific amounts of WSCP relative to total protein (open columns, per milligram of protein), leaf area (hatched columns, per square centimeter), and chlorophyll (filled columns, per microgram of chlorophyll) are displayed.

Figure 4. Change in the amount of WSCP in cauliflower leaves after detachment as assayed by SDS-PAGE followed by Coomassie brilliant blue-staining (A), western-blot analysis (B), and ELISA (C). Cauliflower leaves were detached (lanes and column a) and placed on a dry paper towel for 16 h (lanes and column b), rewatered for 8 h, and then placed on a dry paper towel again for 16 h (lanes and column c). Values in Figures 3 and 4 cannot be directly compared because the conditions were not identical. The arrow in B indicates a 22-kD band for WSCP.
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Figure 5. Assay of trypsin inhibitory activity of cauliflower WSCP. The trypsin activity measured as a change at A\text{253} with BAEE as a substrate without adding any protease inhibitors is displayed in a. A 5-fold molar excess of soybean trypsin inhibitor inhibits the activity completely (b). The WSCP and trypsin were mixed at molar ratios of 10:1 (c), 5:1 (d), and 1:1 (e), and the remaining trypsin activity was assayed. No trypsin-inhibitory activity of WSCP was detected.

Three sequence motif signatures for WSCPs from cauliflower, rapeseed, and soybean are displayed in Fig. 2. This motif is well conserved in BnD22 in rapeseed, P22 in radish, and in a Kunitz-type protease inhibitor in soybean (Gotor et al., 1995; Fig. 2); therefore, it is predicted that BnD22 and P22 are protease inhibitors (Downing et al., 1992; Lopez et al., 1994).

To examine whether cauliflower WSCP inhibits a vertebrate digestive Ser-protease, as do Kunitz-type protease inhibitors from leguminous plants, the inhibitory action of WSCP on trypsin was examined (Fig. 5). Kunitz trypsin inhibitors in soybean inhibit bovine trypsin activity in a 1:1 molar ratio (Kim et al., 1985). As shown in Figure 5, a 10-fold molar excess of cauliflower WSCP did not inhibit the activity of porcine pancreatic trypsin (Fig. 5c), whereas a 5-fold molar excess of soybean trypsin inhibitor inhibited it completely (Fig. 5b). This result showed that cauliflower WSCP does not possess activity as a trypsin inhibitor, even though the WSCP sequence contains a perfect signature motif of the inhibitor family (Val4 to Ile 20, underlined in Fig. 2). BnD22 should not function as a trypsin inhibitor because it lacks the conserved Arg residue, i.e. Arg 63 in soybean trypsin inhibitor, which is located in the reactive (inhibitory) region of Kunitz-type trypsin inhibitors. Cauliflower WSCP also may not possess the Arg residue in its putative inhibitory region.

Predicted physiological functions of the Kunitz protease inhibitor in plant tissues are to inhibit endogenous proteases to regulate proteolytic reactions in plant metabolism, to inhibit digestive proteases in invasive insects and microorganisms, and to act as storage proteins (for reviews, see Ryan 1973, 1981). At this time, possible functions of WSCP as a protease inhibitor and/or as a drought-stress-induced protein are unknown. However, it is likely that WSCP plays an important role in chlorophyll metabolism during drought stress because WSCP binds chlorophyll molecules with such high affinity that they cannot be removed even by an 80% acetone treatment. Why does a drought-induced protein that contains a motif of the Kunitz-type protease inhibitor need to bind to chlorophyll? To investigate the properties and functions of WSCPs during response to drought stress and adaptation, molecular cloning, expression of the recombinant WSCP, and transgenic studies of WSCP cDNA and its genes are now in progress.

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