Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary X-ray analysis of a water-soluble chlorophyll protein from *Brassica oleracea* L. var. acephala (kale)

Daisuke Horigome, Hiroyuki Satoh and Akira Uchida*

Department of Biomolecular Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan

Correspondence e-mail: auchida@biomol.sci.toho-u.ac.jp

Received 20 April 2003
Accepted 1 September 2003

A water-soluble chlorophyll protein (WSCP) with a chlorophyll *a*:*b* ratio of 6:1 from *Brassica oleracea* L. var. *acephala* (kale) was purified and crystallized by the hanging-drop vapour-diffusion method using PEG 8000 and zinc acetate as precipitants. The crystal belongs to the hexagonal space group $P6_422$, with unit-cell parameters $a = b = 162.2$, $c = 38.7$ Å. A native data set was collected to 2.80 Å resolution at 293 K using Cu $K\alpha$ radiation from a rotating-anode generator. Preliminary analysis *via* molecular replacement identified one kale WSCP monomer in the asymmetric unit. The crystal packing showed a tetrameric structure for kale WSCP, as suggested by previous biochemical studies of WSCPs from *Brassicaceae* plants.

1. Introduction

In green leaves there are large amounts of chlorophyll (Chl) *a*/*b* protein complexes that harvest and convert solar energy to chemical energy. These photosynthetic Chl proteins, *i.e.* light-harvesting chlorophyll *a*/*b* protein complexes, are hydrophobic membrane-bound proteins. In contrast, there are several kinds of hydrophilic Chl proteins that have been found and named water-soluble Chl proteins (WSCPs). Although their physiological function is unclear, these proteins may not participate in photosynthesis because of their high hydrophilicity and low Chl content.

Class II WSCPs (for a description of the classification, see the review by Satoh *et al.*, 2001) have been found in *Brassicaceae* plants, including cauliflower (Murata *et al.*, 1971; Nishio & Satoh, 1997; Satoh *et al.*, 1998), Virginian peppercress (*Lepidium virginicum*; Murata & Ishikawa, 1981), rapeseed (Nishio & Satoh, 1997), Brussels sprout (Kamimura *et al.*, 1997) and Japanese radish (Shinashi *et al.*, 2000, 2002). All class II WSCPs so far characterized possess a homotetrameric structure consisting of approximately 20 kDa subunits. The Chl content and its *a*:*b* ratio are variable. For example, Brussels sprout WSCP contains only one Chl in the tetramer and shows a Chl *a*:*b* ratio of >10. In contrast, there are 2–4 Chls in the tetramer of cauliflower WSCP (Chl *a*:*b* = 6.3) and in *L. virginicum* WSCP there are four Chl molecules in the tetramer (Chl *a*:*b* = 1–2). The low Chl content of WSCPs may indicate that they contribute little to the harvesting of solar energy during photosynthesis. Light-harvesting Chl *a*/*b* protein complexes in the pea plant have been found to contain 12 Chl molecules in each subunit of the trimer (Kühlbrandt *et al.*, 1994).

At present, the physiological role of WSCP is uncertain, although some interesting properties have been found. For instance, cauliflower WSCP is induced by drought stress, suggesting a possible protective role during the stress response (Nishio & Satoh, 1997). The light-induced singlet-oxygen formation of WSCP-bound Chl is lower by a factor of 4 than that of unbound Chl (Schmidt *et al.*, 2003), also suggesting a protective role for WSCP. A notable molecular function of cauliflower WSCP has also been reported, with monomeric recombinant apo WSCP directly removing Chl from the thylakoid membrane protein and forming a tetramer in aqueous solution (Satoh *et al.*, 1998). This result may suggest that WSCP regulates the Chl content of the photosynthetic apparatus in the thylakoid membrane and may play a role as a carrier of Chl during the stress response (Satoh *et al.*, 1998). In another possibility, WSCP may transport Chl molecules from thylakoid Chl proteins to a chloroplast envelope, in which the Chl-degrading enzyme chlorophyllase is located (Matile *et al.*, 1997). So far, there is no other known protein that can carry or transport Chl molecules in plant tissues. Thus, WSCP possibly participates in Chl metabolism or catabolism, including its synthesis, transport, sorting, removal, Chl *a*/*b* conversion or degradation processes.

Because most of the Chl-binding proteins are hydrophobic, few crystal structures of Chl proteins have been determined; our knowledge of the ways in which Chls bind to proteins is therefore very limited. As WSCP exhibits high hydrophilicity and low Chl content, it may be a good material for elucidating the interactions between Chl and proteins at the atomic level. We report here the purification, crystallization and preliminary X-ray study of kale WSCP. The structure determination of WSCP will

provide an insight into the way in which the hydrophilic protein binds Chl molecules. Furthermore, the crystal structure should reveal the essential role of Chl during WSCP tetramerization. Although recombinant apo WSCP binds chlorophyllide, it fails to form a tetramer, indicating that the phytol tail is essential to WSCP tetramerization (Schmidt *et al.*, 2003).

2. Experimental

2.1. Purification

The WSCP used in the present study was purified from the leaves of kale (*Brassica oleracea* L. var. *acephala*) cultivated in Chiba, Japan. After three months of germination, 3 kg of leaves were harvested, washed and homogenized in 100 m*M* phosphate buffer pH 7.0. The homogenate was filtered through two layers of gauze. Solid ammonium sulfate was added to the filtrate and a fraction between 20 and 90% saturation was collected by centrifugation. The precipitate was dissolved in 5 m*M* phosphate buffer pH 7.0 and dialyzed against the same buffer. After the removal of insoluble precipitates by centrifugation, the supernatant was loaded onto a DEAE-cellulose column (DE-52, Whatman) that had been equilibrated with the same buffer. The bound protein was eluted with 350 m*M* phosphate buffer pH 7.0 and was further separated by PAGE (detergent-free) according to the method of Laemmli (1970). The major green band was excised and homogenized in 100 m*M* phosphate buffer pH 7.0 in order to extract the protein from the gel. The extracted WSCP fraction was then subjected to gel-filtration chromatography on a Sephacryl S-200 HR column pre-equilibrated with 100 m*M* acetate buffer pH 4.2. Fractions with $A_{673}/A_{280} \geq 1.3$ were pooled and their purity was confirmed by SDS–PAGE.

2.2. Crystallization and X-ray diffraction

Transparent green crystals of kale WSCP were grown by the hanging-drop vapour-diffusion method at 293 K. The initial crystallization conditions [160 m*M* zinc acetate, 80 m*M* sodium cacodylate buffer pH 6.5, 14.4%(*w*/*v*) PEG 8000, 20%(*v*/*v*) glycerol] were obtained using Crystal Screen Cryo from Hampton Research. Larger crystals were obtained by refinement of the concentrations of PEG 8000 and zinc acetate. Prior to collecting the diffraction data, crystals were soaked for 3 d in harvesting solution [80 m*M* zinc acetate, 100 m*M* sodium cacodylate pH 6.1, 30.0%(*w*/*v*) PEG 4000 and 30.0%(*v*/*v*) glycerol]. After soaking, the crystal was sealed in a glass capillary. The diffraction data were collected at 293 K on a Rigaku R-AXIS IIC image-plate detector using graphite-monochromated Cu $K\alpha$ radiation from a Rigaku RU-200 rotating-anode X-ray generator operating at 50 kV and 80 mA (λ = 1.5418 Å) with a 0.3 × 0.3 mm focal spot. The crystal-to-detector distance was set at 155 mm. The exposure time was 100 min for each image covering 1° of oscillation. The data were processed with *d*TREK* v.7.3 (Pflugrath, 1999).

3. Results and discussion

3.1. Characterization

5 mg of WSCP was purified from 3 kg of kale leaves. The 22 N-terminal amino-acid residues of kale WSCP were sequenced and 19 residues were identified as R(K)E-QV*x*DSNGNPV*xx*GAKYFIQP, with the exceptions being residues 5, 13 and 14. The first residues, Arg and Lys, show heterogeneity (Arg was the major residue), which implies the existence of isoproteins or allelic variation. Similar heterogeneity has also been observed in cauliflower WSCP at residue 15 (Gly major and Ala minor) and at residue 25 (Ser major and Lys minor) (Nishio & Satoh, 1997). Two cDNAs for kale WSCP have been cloned (Zanma & Satoh, unpublished results), Q8H0F0 and Q8H0E9 (SWISS-PROT/TrEMBL). The amino-acid sequences deduced from the two cDNAs were found to be highly homologous, sharing 95.3% identity. The cDNA analysis revealed that kale WSCP possesses a 13-residue N-terminal extension sequence. Detailed analysis of the kale WSCP cDNA will be published elsewhere. Residues 14–35 of the deduced sequence from the cDNA (Q8H0F0) matched one of the partial N-terminal amino-acid sequences (REQV) perfectly. The other protein sequence (KEQV) was unmatched only at the third residue deduced from the other cDNA (Q8H0E9) (Gln in protein and His from cDNA). These results indicate that the purified kale WSCP did not contain a protein from the mRNA entered as Q8H0E9 cDNA. We therefore assumed that the cDNA (Q8H0F0) encoded the major kale WSCP purified in this study.

The exact molecular weight of the monomeric kale WSCP was measured by MALDI–TOF mass spectrometry to be 18 897.03 Da. This value was approximately consistent with the calculated molecular weight of residues 14–189 (176 residues) of Q8H0F0, 18 882.5 Da. These results may indicate that the 23 C-terminal amino-acid residues have been processed and removed in the mature form of WSCP. Similar processing has been reported in rapeseed WSCP (Ilami *et al.*, 1997) and cauliflower WSCP (Satoh *et al.*, 1998).

The purified kale WSCP co-migrated with a WSCP isolated from Brussels sprout (78 kDa) on non-denaturing PAGE (data not shown), indicating that kale WSCP may form the same quaternary structure, *e.g.* tetrameric conformation, as class II WSCPs from the other *Brassicaceae* plants. The absorption spectrum of kale WSCP is shown in Fig. 1. The peak wavelengths of kale WSCP were nearly identical to those of cauliflower WSCP. Chl of kale WSCP was extracted with 2-butanone as described by Murata *et al.* (1968) and a Chl *a*:*b* ratio of 6:1 was determined spectrophotometrically (Porra *et al.*, 1989).

3.2. Crystallization

The optimal crystallization conditions for growing crystals suitable for X-ray analysis were as follows: 3 µl protein solution (5.0 mg ml^{-1}) containing 5 m*M* sodium acetate pH 4.2 was mixed with an equal volume of reservoir solution containing 5.0%(*w*/*v*) PEG 8000, 80 m*M* zinc acetate

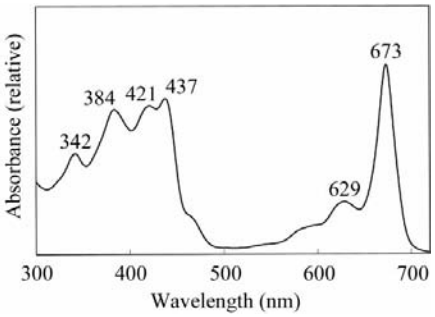

Figure 1
Absorption spectrum of kale WSCP.

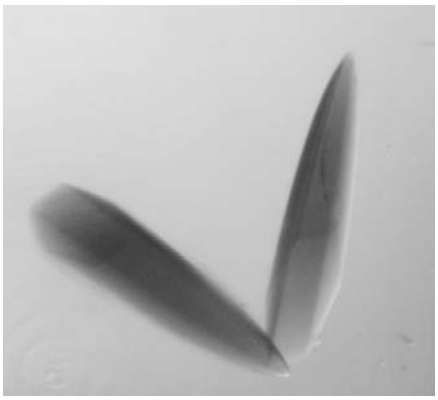

Figure 2
Hexagonal crystals of kale WSCP.

Table 1
Summary of X-ray data for kale WSCP.

Values in parentheses are for the last resolution shell.

Resolution range (Å)	70.2–2.80 (2.90–2.80)
Unique reflections	7692
Completeness (%)	98.2 (99.9)
Redundancy	5.7
$R_{\rm merge}$	0.093 (0.411)
$\langle I\rangle/\langle\sigma(I)\rangle$	14.9 (3.5)

and 100 m*M* sodium cacodylate pH 6.1. The droplet was then equilibrated against 0.5 ml reservoir solution. Single crystals appeared within 10 d and reached maximum dimensions after one month. The average crystal dimensions were 0.8 × 0.2 × 0.2 mm for hexagonal cone-shaped crystals (Fig. 2).

3.3. Data collection and X-ray diffraction analysis

A native data set was collected to 2.80 Å resolution at 293 K. The space group of the crystal was determined to be hexagonal $P6_422$, with unit-cell parameters $a = b = 162.2$, $c = 38.7$ Å. The statistics of data collection are summarized in Table 1. $V_{\rm M}$ values were calculated with a molecular weight of 19.8 kDa (residues 14–189 plus one Chl molecule). There could be one or two kale WSCP monomers in the crystallographic asymmetric unit, with corresponding $V_{\rm M}$ values of 3.7 or 1.9 Å^3 Da^{-1}, respectively (Matthews, 1968).

Preliminary analysis *via* molecular replacement was performed using the program *AMoRe* (Navaza, 1994; Collaborative Computational Project, Number 4, 1994). The search model used was obtained from the model of the *L. virginicum* WSCP monomer, which is now in the final stages of refinement (Uchida *et al.*, 2002). The amino-acid sequence of kale WSCP (Q8H0F0) and *L. virginicum* WSCP share 41.0% identity. The model contained a single Chl *a* molecule in the monomer of folded *L. virginicum* WSCP, the amino acids of which were completely replaced by those of kale WSCP (Q8H0F0). One WSCP monomer was found in the asymmetric unit and simulated-annealing refinement using the *CNS* program (Brünger *et al.*, 1998) resulted in an *R* factor of 38.4% (70–2.8 Å data used). The reasonable crystal packing showed tetrameric kale WSCP, as in the case of *L. virginicum* WSCP (Uchida *et al.*, 2002). Further refinement of the crystal structure is now under way.

References

Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Ilami, G., Nespoulous, C., Huet, J.-C., Vartanian, N. & Pernollet, J.-C. (1997). *Phytochemistry*, **45**, 1–8.

Kamimura, Y., Mori, T., Yamasaki, T. & Katoh, S. (1997). *Plant Cell Physiol.* **38**, 133–138.

Kühlbrandt, W., Wang, D. N. & Fujiyoshi, Y. (1994). *Nature (London)*, **367**, 614–621.

Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.

Matile, P., Schellenberg, M. & Vicentini, F. (1997). *Planta*, **201**, 96–99.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Murata, T. & Ishikawa, C. (1981). *Biochim. Biophys. Acta*, **635**, 341–347.

Murata, T., Odaka, Y., Uchino, K. & Yakushiji, E. (1968). *Comparative Biochemistry and Biophysics of Photosynthesis*, edited by K. Shibata, A. Takamiya, A. T. Jagendorf & R. C. Fuller, pp. 222–228. Tokyo University Press.

Murata, T., Toda, F., Uchino, K. & Yakushiji, E. (1971). *Biochim. Biophys. Acta*, **245**, 208–215.

Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.

Nishio, N. & Satoh, H. (1997). *Plant Physiol.* **115**, 841–846.

Pflugrath, J. W. (1999). *Acta Cryst.* D**55**, 1718–1725.

Porra, R. J., Thompson, W. A. & Kriedemann, P. E. (1989). *Biochim. Biophys. Acta*, **975**, 384–394.

Satoh, H., Nakayama, K. & Okada, M. (1998). *J. Biol. Chem.* **273**, 30568–30575.

Satoh, H., Uchida, A., Nakayama, K. & Okada, M. (2001). *Plant Cell Physiol.* **42**, 906–911.

Schmidt, K., Fufezan, C., Krieger-Liszkay, A., Satoh, H. & Paulsen, H. (2003). *Biochemistry*, **42**, 7427–7433.

Shinashi, K., Kiyono, S., Ito, O., Uchida, D., Igari, R., Kuroki, J., Satoh, H., Oonishi, I. & Uchida, A. (2002). *Acta Cryst.* A**58**, C119.

Shinashi, K., Satoh, H., Uchida, A., Nakayama, K., Okada, M. & Oonishi, I. (2000). *J. Plant Physiol.* **157**, 255–262.

Uchida, A., Horigome, D., Sasaki, T., Meguro, E., Kaneko, N., Ito, N., Satoh, H. & Oonishi, I. (2002). *Acta Cryst.* A**58**, C120.