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Analysis of photooxidized pigments in water-soluble chlorophyll protein complex isolated from *Chenopodium album*

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Abstract

A water-soluble chlorophyll (Chl) protein complex isolated from *Chenopodium album* is converted to another form of the pigment protein (CP740) containing bacteriochlorin type pigments by irradiation with visible light in an aqueous aerated solution. In order to investigate the photoconverted pigments in CP740, all the chlorophyllous pigments were extracted with organic solvents from CP740 and were analyzed by high-performance liquid chromatography and mass spectrometry. Two separated products were mono-oxygen adducts to Chl *a* at the B-ring. Their visible spectral analysis combined with model calculation supported that they would be 8-oxo- (P700) and 7,8-epoxy-derivatives (P726). During extraction, covalent bonds of pigments with proteins in CP740 would be cleaved to produce the above mono-oxygenated Chls *a*. Based on the molecular structures of P700 and P726, bacteriochlorophyll type pigments in CP740 were proposed.
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1. Introduction

Water-soluble chlorophyll proteins (WSCPs) have been isolated from many kinds of plants [1]. WSCPs are categorized into two classes: (i) the visible absorption spectrum of the Class I is changeable by irradiation with visible light and (ii) that of the Class II is not changed upon illumination [1]. A WSCP (Class I) was first extracted from leaves of *Chenopodium album* by Yakushiji and his colleagues, which has a visible absorption peak at 668 nm, so-called CP668 (CP = chlorophyll protein). The molecular weights of CP668 were reported to be 16 kDa by SDS-PAGE and 78 kDa by gel-filtration chromatography [1], indicating that CP668 is a tetramer of 16 kDa subunits. It was also reported that one CP668 had about six chlorophyll (Chl) *a* and one Chl *b* molecules (see left drawing of Fig. 1) but no carotenoids in a protein [1], showing that each subunit possesses about two Chl molecules. The physiological role of CP668 is yet unknown but similar WSCPs (Class II) from other species were identified as a drought induced protein [3], a heat stress protein [4] and proteinase inhibitors [5,6]. One of the WSCPs (Class II) has been studied by X-ray crystallographic analysis and the detailed structure has been reported [1,7].

When CP668 was irradiated with visible light in an aqueous aerated solution, the absorption band at 668 nm decreased and concomitantly 740- and 564-nm peaks appeared [2]. The photoconverted protein was called CP740. Such a conversion from CP668 to CP740 required both photoexcitation and oxygen molecule [8]. The photoconversion ratio of CP668 to CP740 was dependent on the pH of the aqueous solution [9]. CP740 was reversibly changed to CP668 by addition of reducing agents [8]. Recently, Noguchi et al. reported that the photoconversion did not change the protein conformation but the Chl *a* structure to give any bacteriochlorin skeleton pigments possessing a single C7–C8 bond from vibrational analyses [10]. The molecular structures of chlorophyllous pigments in CP740 are still unknown.

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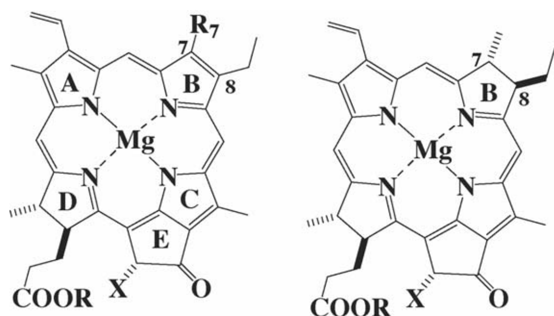

Fig. 1. Molecular structures of Chl *a* ($R^7 = CH_3$, left), Chl *b* ($R^7 = CHO$, left) and [3-vinyl]-BChl *a* (right). $X = COOCH_3$ and R = phytyl.

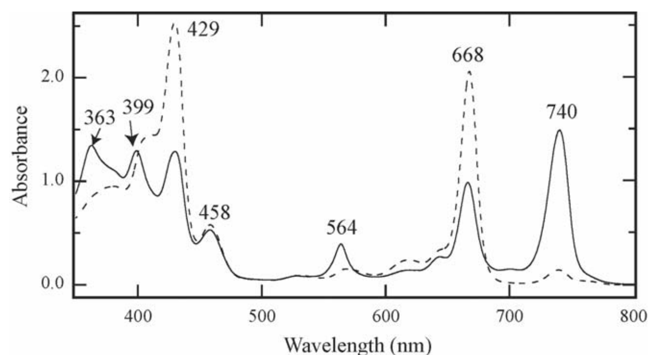

Fig. 2. Electronic absorption spectra of WSCP before (broken line) and after light illumination (solid line) in aerated 10 mM phosphate buffer (pH 7.0).

In the present work, the structures of chlorophyllous pigments extracted from CP740 could be determined from observed high-performance liquid chromatography (HPLC)–visible and mass (MS) spectra and their calculated peak positions. The local structure around the chlorophyllous pigment in CP740 was also proposed.

2. Materials and methods

2.1. Purification of WSCP (Class I) and its photoconversion

The leaves of *C. album* were collected along the banks of the Katsura (Kyoto) and Kino rivers (Wakayama). The WSCP was purified according to the reported methods [10]. The WSCP was dissolved in air-saturated 10 mM phosphate buffer (pH 7.0) and was irradiated with a 250 W metal halide lamp (Sumita Kogaku Glass Ltd., Saitama) for 5 min.

2.2. MS spectrometry of WSCP

The molecular weight of WSCPs were analyzed with matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF)–MS spectrometry (AXIMA-CFR PLUS, Shimadzu Co. Ltd., Kyoto) in positive ion mode. The matrix used was α-cyano-4-hydroxycinnamic acid which was dissolved in acetonitrile and 0.1% aqueous trifluoroacetic acid (1:1, v/v) to a final concentration of 10 μg/μl. The WSCP solution was mixed with the same volume of the matrix solution.

2.3. Analysis of chlorophyllous pigments

All the chlorophyllous pigments of illuminated WSCP were extracted with methanol and acetone (1:1, v/v) and the extract was centrifuged to remove almost all proteins under ambient conditions (in the dark and at room temperature). HPLC was done with a Shimadzu LC-10AS pump and a Shimadzu SPD-10AV photodiode array detector. Liquid chromatography (LC)–MS was done with a Shimadzu LCMS-2010X. A normal phase packed column 5SL-II (4.6 mm × 150 mm, Nacalai Tesque, Kyoto, Japan) was used and the mobile phase was hexane and acetone (92:8, v/v) at 1 ml/min. The molecular models were calculated according to the reported method [11].

3. Results and discussion

3.1. Preparation of photooxidized WSCP and its analysis

Fig. 2 shows absorption spectra of the WSCP isolated from *C. album*. In the WSCP before illumination (broken line of Fig. 2), CP668 was major with 429- and 668-nm peaks ascribable to Chl *a* and a 458-nm peak of Chl *b*, and a small amount of CP740 was observed which was accidentally produced by slight illumination during the purification. When the aerated solution was irradiated with visible light, absorption bands at 429 and 668 nm of CP668 decreased and new bands at 363, 399, 564 and 740 nm appeared (see solid line of Fig. 2). The peak height at 668 nm decreased to about half of the original after 5-min illumination and no more change was observed upon prolonged irradiation. As the photoconversion ratio of CP668 to CP740 depended on the experimental conditions [9], the present samples could not be photoconverted to more than this stage. It is noteworthy that the absorption band at 458 nm ascribable to Chl *b* was little changed and no photoreaction of Chl *b* occurred in CP668.

The peak position at 740 nm characteristic of CP740 indicates that Chl *a* molecules in CP668 would be photoconverted to some chlorophyllous pigments possessing a bacteriochlorin π-system whose Q_y peaks were located at around 720–770 nm in a monomeric state [12]. Recent resonance Raman spectral analysis also supported that the photoproducts in CP740 would be bacteriochlorin skeleton pigments [10]. All four peak positions of the photoproducts in CP740 at 740, 564, 399 and 363 nm were similar to Q_y, Q_x and Soret peaks of 3-deacetyl-3-vinyl-bacteriochlorophyll *a* ([3-vinyl]-BChl *a*, see right drawing of Fig. 1) in diethyl ether at 745, 560, 389 and 351 nm [13] (see Table 1). Considering that [3-vinyl]-BChl *a* is 7,8-trans-dihydrogenated Chl *a*, the double bond between C7 and C8 positions of Chl *a* in CP668 was changed to a single bond upon illumination to give CP740 possessing bacteriochlorin chromophores like [3-vinyl]-BChl *a*.

Fig. 3 shows a MALDI–TOF–MS spectrum of the isolated WSCP before the light illumination with two peaks at 15,862 Da (major) and 15,880 Da (minor): no apparent peaks were observed at the region over 20,000 Da. One subunit of the WSCP was reported to have a molecular weight of 16 kDa [1]. These observed peaks were ascribable to the subunit. The

Table 1
Electronic absorption peaks (nm) of the isolated WSCPs[a] and chlorophyllous pigments[b]

	Q_y	Q_x	Soret
CP668	668		458, 429
CP740	740	564	458, 399, 363
Chl *a*	661		429
Chl *b*	642		452
[3-Vinyl]-BChl *a*[c]	745	560	389, 351

[a] In 10 mM phosphate buffer (pH 7.0).
[b] In diethyl ether.
[c] Ref. [13].

WSCP prior to illumination was a mixture of CP668 (major) and of CP740 (minor), as described above (see also broken line of Fig. 2). Therefore, the molecular weight of a CP668 subunit was 15,862 Da and the peak at 15,880 Da could be ascribed to its photoconverted subunit. The photoreaction of CP668 increased by about 18 mass numbers in the molecular weight of its subunit. Since the photoreaction of CP668 to CP740 occurred in the presence of air, one oxygen atom would be added to one CP668 subunit by illumination. One Chl *a* molecule in a CP668 subunit was photooxidized to give its mono-oxygenated species. Combining with the above absorption spectral analysis, an oxygen species would attack the C7–C8 double bond of the B-ring in the Chl *a*.

3.2. Structural analysis of extracted pigments from CP740

To investigate the chemical structure of the photoproducts in CP740, the pigments were extracted with organic solvents from an aqueous illuminated WSCP solution. Fig. 4 shows the absorption spectrum of the extract in methanol and acetone (1:1, v/v). The extracted pigments gave absorption bands at 431 and 664 nm ascribable to Chl *a* and also a broad band at 727 nm, but no peak at 740 nm could be detected. The remaining aqueous phase was colorless and showed no visible absorption including a 740-nm peak. All of the chlorophyllous molecules were thus extracted from the illuminated WSCP, indicating that the chlorophyllous species with a 740-nm peak in CP740 would be changed to other pigments possessing an absorption band at 727 nm during extraction. It could not be ruled out that the 727-nm absorbing species would interact with the protein to give red-shifted

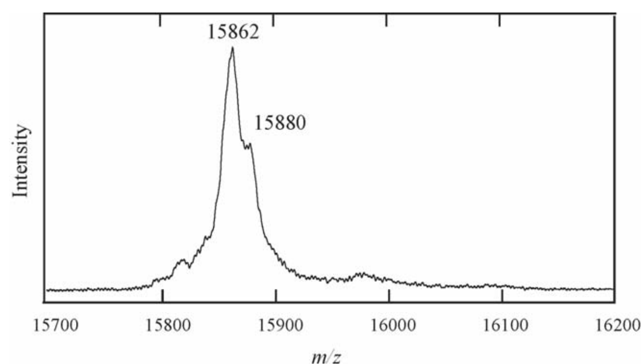

Fig. 3. MALDI–TOF–MS spectra of isolated WSCP (before illumination).

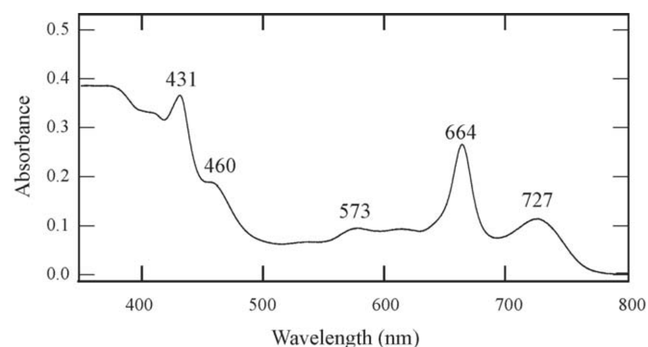

Fig. 4. Electronic absorption spectra of pigments extracted from illuminated WSCP in methanol and acetone (1:1, v/v).

peaks in CP740, while the 240 cm^{-1} shift (727–740 nm) is much larger than that of Chl *a* in CP668 (90 cm^{-1}). Such a red-shift might be ascribable to electronic interaction among the 727-nm species but this situation is less probable because of interaction of Chl molecules in CP668 suggested in [1]. As a result, it was proposed that 740-nm chlorophyllous species evidently strongly interacts with a protein in CP740. It is noteworthy that Chls *a* and *b* in CP668 interact less with apoprotein because they were easily extracted and the resulting apoprotein was reconstituted with additional Chls.

The resulting extract was analyzed with HPLC and LC–MS. The normal phase HPLC gave four main peaks as shown in Fig. 5. From their electronic absorption and mass spectra as well as retention times of the authentic samples, peaks 1 and 2 were identified as Chls *a* and *b*, respectively. These two peaks were also detected from the extract from the unilluminated WSCP. The other two peaks (3 and 4) appeared during irradiation to

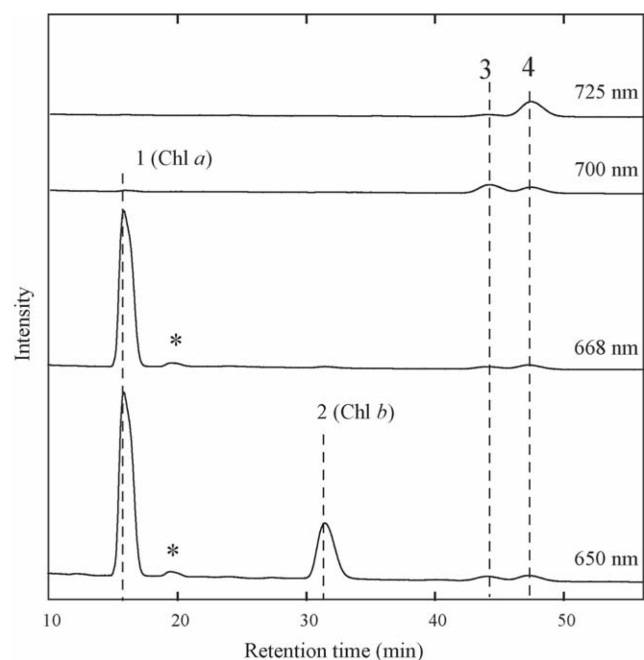

Fig. 5. Normal phase HPLC elution profiles of extracts from illuminated WSCP detected at 650, 668, 700 and 725 nm (lower to upper). See Section 2.3 for HPLC conditions. Peak (*) was ascribable to Chl *a*' (13^2-(*S*)-epimer of Chl *a*) produced during extraction.

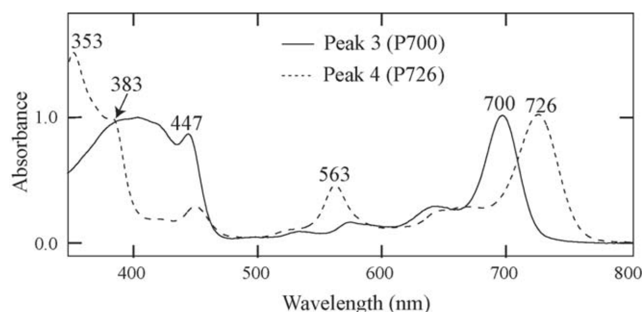

Fig. 6. Electronic absorption spectra of peaks 3 (solid line) and 4 (dashed line) in hexane/acetone (92:8). Normalized at Q_y peak intensities.

WSCP, and were ascribable to extracted pigments from the photoconverted WSCP. These peak intensities were much smaller than those of peaks 1 and 2. These resulting pigments were unstable under the extracting conditions and decomposed quickly during the operation. Fig. 6 shows electronic absorption spectra of peaks 3 and 4 in hexane:acetone (92:8). They have Q_y maxima at 700 and 726 nm, called P700 and P726 (P = pigment), respectively. From LC–MS analyses, both the mass numbers observed were the same to be 909, which was 16 mass larger than that of Chl *a*. Assuming from the above result that a Chls *a* molecule in CP668 was photooxidized to give its mono-oxygen adduct at the B-ring in CP740, the molecular structures of P700 and P726 were also mono-oxygenated Chls *a* at the B-ring. As their candidates, 7-oxo-, 8-oxo- and 7,8-epoxy-Chls *a* are proposed (see Fig. 7).

Recently, Kunieda and Tamiaki reported the electronic absorption spectra of zinc 8-ethyl-8-methyl-7-oxo- and 7-ethyl-7-methyl-8-oxo-bacteriochlorins [11]. The absorption spectral feature of P700 is very similar to that of zinc 7-ethyl-7-methyl-8-oxo-bacteriochlorin (see Table 2). The similarity indicated that P700 would be 8-oxo-Chl *a* (middle drawing in Fig. 7). The feature of P726 is different from those of zinc 7- and 8-oxo-bacteriochlorins but its Q_x and Soret bands are similar to those of [3-vinyl]-BChl *a* possessing sp^3-carbons on the 7- and 8-positions (see Table 2). Therefore, P726 would be 7,8-epoxy-Chl *a* (right drawing in Fig. 7) having sp^3-C7 and C8.

The above structural expectation was confirmed by molecular modeling. Q_y absorption maxima of the three mono-oxygen adducts were estimated from MM+/PM3 and ZINDO/S calculation [11]; λ_{max} = 745 nm (7-oxo-Chl *a*), 694 nm (8-oxo-Chl *a*) and 729 nm (7,8-epoxy-Chl *a*). The observed Q_y peak positions of P700 or P726 were almost the same as the estimated 694 or 729 nm for 8-oxo- or 7,8-epoxy-Chls *a*, respectively. Unfortunately, neither P700 nor P726 could be isolated as pure samples from their HPLC elutions and their NMR and IR spectra are not available.

3.3. Estimation of photooxidized products in CP740

From Sections 3.1 and 3.2, mono-oxygenated Chls *a* on the B-ring possessing a bacteriochlorin π-system tightly interacted with a protein in CP740 and the photooxidized products were transformed by extraction with organic solvents to give 8-oxo-

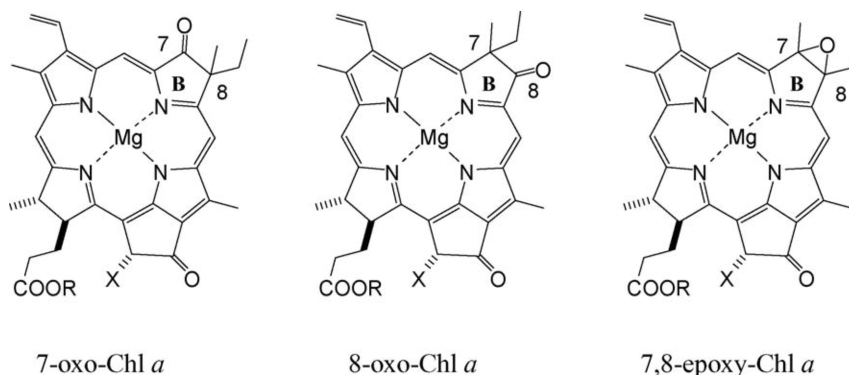

Fig. 7. Molecular structures of mono-oxygenated Chl *a* at the B-ring. X = $COOCH_3$ and R = phytyl.

Table 2
Electronic absorption data of chlorophyllous pigments

Compounds	Absorption maxima[a] (nm)								
Zinc 8-ethyl-8-methyl-7-oxo-bacteriochlorin[b]	724 (100)	684 (8)	666 (8)	580 (8)	441 (32)			398 (38)	
Zinc 7-ethyl-7-methyl-8-oxo-bacteriochlorin[b]	683 (100)	630 (26)	565 (13)	525 (11)	444 (111)	420 sh (90)	409 (93)	389 sh (81)	
[3-Vinyl]-BChl *a*[c]	745 (100)		560 (35)					389 (67)	351 (113)
P700[d]	700 (100)	646 (29)	577 (16)	536 (9)	447 (86)	423 sh (93)	406 (99)	386 sh (94)	
P726[d]	726 (100)		563 (30)					383 (66)	353 (100)

[a] Parentheses indicate relative peak intensities normalized at the longest absorbing maximum (Q_y band).
[b] In tetrahydrofuran, Ref. [11].
[c] In diethyl ether, Ref. [13].
[d] In hexane and acetone (92:8, v/v).

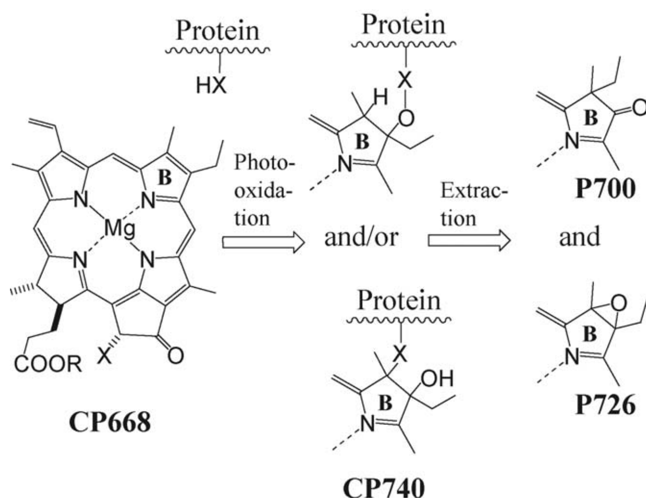

Fig. 8. Schematic drawing in photooxidation of CP668 to CP740 and extraction of P700/P726 from CP740. X = $COOCH_3$ and R = phytyl.

and 7,8-epoxy-Chls *a*. We speculate that interaction of photoproducts with a protein would be due to the cleavable covalent bonds, e.g., O–O or C–S. As 7-oxo-Chl *a* was not detected in the extracts, a reactive oxygen species should attack the 8-position of Chl *a* preferentially.

Based on the above assumption, photoconversion of CP668 to CP740 would occur as follows. Photoexcited Chl *a* in CP668 reacts with oxygen molecules to produce active oxygen, such as singlet oxygen or superoxide anion. The resulting species oxidizes at the 8-position of Chl *a* and the oxygenated Chl *a* reacted with neighboring peptidyl residue (–XH) to give supercomplexes (CP740) as shown in Fig. 8. Alternatively, photoexcited Chl *a* in CP668 was directly oxygenated by oxygen and resulting oxygenated Chl *a* was trapped by –XH. The proposed photoproducts in CP740 have sp^3 covalent bonds at the 7- and 8-positions as well as the single C7–C8 and C8–O bonds and are bonded with a protein through a relatively weak covalent bond. The photoreaction pathway is consistent with the fact that Chl *b* possessing an electron-withdrawing formyl group at the 7-position could not react with an active oxygen species at its less reactive B-ring.

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