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Isolation and characterization of the chlorophyll *a/b* protein complex CP29 from spinach

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We describe a preparative isolation method for a minor chlorophyll *a/b* protein complex from spinach Photosystem II, which we suggest is identical to the CP29 complex first reported by Machold, O. and Meister, A. (Biochim. Biophys. Acta 546 (1979) 472–480) and by Camm, E.L. and Green, B.R. (Plant Physiol. 66 (1980) 428–432). The CP29 complex was isolated in mg quantities by ion-exchange high-performance liquid chromatography and extensively characterized. The absorption maximum in the red region was at 678 nm. The purified complex retained 10–12 chlorophyll molecules per protein and had a chlorophyll *a/b* ratio of 3.0–3.2. The complex contained only one apoprotein with an apparent molecular weight between 29 and 31.5 kDa. It showed a fluorescence emission peak at 679 nm when measured at 77 K while the fluorescence excitation spectrum revealed three main peaks in the blue region at 431 nm (chlorophyll *a*), 468 nm (chlorophyll *b*) and 493 nm (carotenoids). N-terminal sequencing of fragments from the trypsin-treated CP29 gave a consensus sequence of 29 amino acids.

Introduction

The light-harvesting apparatus of Photosystem II in higher plant thylakoids consists of several integral chlorophyll-protein complexes which interact in the absorption and transfer of excitation energy to the reaction center [1]. Each chlorophyll-protein complex can be distinguished as a unit containing specific polypeptides and bound pigments [2].

Close to the reaction center are the chlorophyll *a* binding complexes, CP47 and CP43 [3,4]. These are surrounded by a secondary chlorophyll *a/b*-containing light-harvesting antenna, which consists of at least four different chlorophyll *a/b* protein complexes. Apart from the major light-harvesting chlorophyll *a/b* binding complex, designated LHC II [5], there are also the minor chlorophyll *a/b* binding complexes CP29, CP27 and CP24 [6].

4–7% of the chlorophyll in the thylakoid membrane is bound to the CP29 complex [7,8] and 80%–90% of the total amount of this complex is located in the appressed grana region of the thylakoid membrane [9], which reflects its connection to Photosystem II [cf. 10]. CP29 is reported to consist of one or possibly two polypeptides with apparent molecular weights of 22.5–31 kDa [11–15]. Furthermore it is reported to have a chlorophyll *a/b* ratio of 1.8–4 [4,7,14,16] or even to be a pure chlorophyll-*a* containing complex [6,17]. The primary amino acid sequence of the CP29 polypeptide has not been reported. However, immunological analysis indicates that CP29 have structural similarities with other chlorophyll *a/b*-binding polypeptides [13,15,18].

The structural and functional roles of CP29 within the light-harvesting antenna have not yet been fully established. However, one interesting finding is that oligomeric forms of the CP29 complex have been observed after mild SDS polyacrylamide gel electrophoresis of thylakoid membranes [7,10,19]. It has also been shown that the CP29 complex is in close connection to the reaction centre of Photosystem II [17]. In contrast to the polypeptides of LHC II, the CP29 polypeptides does not become phosphorylated [12] and does not migrate away from the Photosystem II core [9] upon the kinase mediated protein phosphorylation of the thylakoid membrane. Furthermore, based on sub-frac-

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Abbreviations: Mes; 4-morpholineethanesulphonic acid, SDS; sodium dodecyl sulphate, Tricine; *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]-glycine.

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tionation studies of phosphorylated thylakoid membranes, CP29 have been suggested to be located between the core of Photosystem II and an inner subpopulation of LHC II [20]. It has also been proposed that CP29, together with the other minor chlorophyll *a/b* complexes, forms a distinct antenna complex with a suggested function in the dissipation of excess excitation energy [14].

The chlorophyll protein complex CP29 can be resolved on the basis of the migration behaviour in different electrophoretic systems (for a review, see Ref. 1). However, no method has been available for the isolation of large amounts of the CP29 complex. This has hampered the elucidation of the molecular and structural organisation of this complex.

Here we present a non-denaturing procedure by which a minor chlorophyll *a/b* binding complex, which we suggest is identical to CP29, is isolated in a single high-performance liquid-chromatography (HPLC) step. The isolation method is rapid and gives a high yield of CP29 from spinach. The purified CP29 complex has been characterized with respect to absorption and fluorescence spectra and pigment, polypeptide and amino acid composition. Furthermore, we report a partial amino acid sequence of the CP29 polypeptide.

Materials and Methods

Photosystem II-enriched particles were prepared from isolated spinach thylakoids according to the detergent method of Berthold et al. [21] with modifications according to Ford and Evans [22], and washed twice in 400 mM sucrose, 5 mM $MgCl_2$, 15 mM NaCl and 50 mM Mes (pH 6.5) to remove excess Triton X-l00. Extrinsic and also some hydrophobic polypeptides were removed from the Photosystem II particles [23.24] by successive treatments in (a) 1 M $CaCl_2$, 10 mM Mes (pH 6.5); (b) 1 M NaCl, 0.06% Triton X-100, 10 mM Mes (pH 6.5) and (c) 3 M NaSCN, 50 mM $CaCl_2$, 0.01% Triton X-100, 10 mM Mes (pH 6.5). The treatments were performed on ice at a concentration of 0.5 mg chlorophyll/ml for (a) and (b) and 0.1 mg chlorophyll/ml for (c) and lasted for 30 min. Between each successive treatment, the Photosystem II particles were collected by centrifugation at $40000 \times g$ for 30 min. The treated Photosystem II particles were washed twice in 0.4 M sucrose and 10 mM Mes (pH 6.0).

For HPLC fractionation, the treated Photosystem II particles, at a concentration of 2 mg chlorophyll/ml, were solubilized in a mixture of 2% sulfobetain SB14 (Serva), 2% digitonin (BDH) and 10 mM Mes (pH 6.0) for 30 min at 4°C during continuous stirring. Unsolubilized material was removed by centrifugation at $40000 \times g$ for 30 min. The resulting dark green supernatant was loaded on an HPLC cation-exchange column (TSK CM-3SW, 7.5 × 150 mm, LKB-products), which was preequilibrated with 0.1% sulfobetain SB14 and 10 mM Mes (pH 6.0) and kept on ice during the run. The flow rate was 0.5 ml/min. After sample application the column was washed with 70–110 ml preequilibration buffer. Material bound to the column was eluted with a NaCl gradient from 0 to 0.5 M in 0.1% sulfobetain SB14 and 10 mM Mes (pH 6.0). Relevant fractions were pooled (5–10 ml) and dialysed 3 × 1 h in 100 ml 25 mM *n*-octyl β-D-glucopyranoside, 10 mM NaCl and 10 mM Tricine (pH 7.5). The sample was concentrated using Amicon centricon concentrators with a 10 kDa cut-off filter and either used directly or frozen in liquid nitrogen with 5% dimethyl sulfoxide. All steps were performed at 4°C unless otherwise stated.

LHC II was purified according to Burke et al. [25], modified according to Mullet and Arntzen [26]. A native Photosystem I preparation was isolated following the procedure described by Mullet et al. [27].

Chlorophyll content was determined in 80% acetone according to Arnon [28]. For thin-layer chromatography, acetone-extracted pigments were separated on Kiselgel 60F 254 (Merck) using the eluant *n*-hexane/ethylacetate/triethylamine in the proportions of 58 : 30 : 12 [29]. Each band was extracted with 80% acetone and quantified spectrophotometrically.

Mild SDS-polyacrylamide gel electrophoresis was performed according to Dunahay et al. [12] using *n*-octyl β-D-glucopyranoside and small amounts of SDS to solubilize the samples in the presence of 40% glycerol. The amount of chlorophyll in the resolved green bands was estimated using a LKB Laser Densitometer.

Denaturing SDS-polyacrylamide gel electrophoresis was performed in the buffer system of Laemmli [30] at 0°C or 25°C with a 12–22.5% polyacrylamide gradient. When indicated, gels with 6 M urea and a polyacrylamide gradient from 12–21% or gels with 4 M urea and a gradient from 10–20.5% were used. Samples were solubilized at 70°C for 3 min. Gels were stained with Coomassie brilliant blue R-250. For estimation of apparent molecular weights the low molecular weight (LMW) and the polypeptide molecular weight (PMW) calibration kits from Pharmacia were used.

Absorption spectra and derivative spectra were recorded on a Shimadzu UV-3000 spectrophotometer at room temperature. Analysis was made on samples in 25 mM *n*-octyl β-D-glucopyranoside, 10 mM NaCl and 10 mM Tricine (pH 7.5) at a chlorophyll concentration of 5 μg chlorophyll per ml. To determine absorption spectra of chlorophyll complexes resolved by mild electrophoresis in slab gels, whole slots (1 × 5 cm) were cut out and placed in a gel cuvette. A vertical slit (0.1 × 5 mm), parallel to the chlorophyll bands, was mounted in front of the cuvette and spectra were recorded.

Fluorescence excitation- and emission-spectra were recorded at 77 K as previously described [31].

Polyclonal antibodies against HPLC-purified CP29 were obtained through standard immunological methods.

Amino acid analysis on dried and hydrolyzed samples (6 M HCl, 24 h, 110 °C) were performed on a Beckman System 6300 high performance amino acid analyzer according to the standard procedure of the instrument.

Trypsin treatment of the HPLC purified fractions was performed at room temperature with a chlorophyll concentration of 400 μg/ml, in 25 mM *n*-octyl β-D-glucopyranoside, 10 mM NaCl and 10 mM Tricine (pH 7.5). A trypsin (Sigma, type III) to chlorophyll ratio of 0.05 (w/w) was used. The reaction was terminated after 10 min by addition of hot (70 °C) Laemmli solubilization buffer, directly followed by incubation at 70 °C for 5 min. The fragments were separated by denaturing electrophoresis (25 °C, without urea) and transferred to a polyvinylidene difluoride membrane (PVDF, Millipore) [32] using a JKA-Biotech semidry electroblotter. After Coomassie staining of the PVDF membrane, protein bands were cut out and sequenced on an Applied Biosystem model 470 sequenator.

Results

Upon HPLC cation exchange chromatography of detergent solubilized Photosystem II particles (Fig. 1) most of the protein and chlorophyll passed the column without binding. When the bound proteins were eluted the most prominent chlorophyll and protein containing fraction were found at approx. 0.35 M NaCl. When this chlorophyll containing HPLC-fraction was analyzed by mild SDS-polyacrylamide gel electrophoresis, i.e., without disrupting the pigment–protein interaction, 80–95% of the chlorophyll remained bound to the protein and migrated as one distinct chlorophyll-protein complex. The rest of the chlorophyll was found as free pigment (Fig. 2). Comparison showed that the single chlorophyll-protein band comigrated with the chlorophyll *a/b* binding complex CP29 in Photosystem II particles. The absorbance spectra of the resolved green chlorophyll-protein complexes after mild electrophoresis are also shown in Fig. 2. The HPLC-isolated chlorophyll-protein complex and the CP29 complex from Photosystem-II particles showed similar absorption spectra with the main peak at 672–673 nm and a low absorption around 650 nm. CP27 (CP II) and CP 24 had their absorption peaks at 671 and 669 nm, respectively, with a clearly visible shoulder around 650 nm. Based on these results we suggest that the HPLC-purified chlorophyll-protein complex is identical to the CP29 complex of Photosystem II.

CP29 corresponding to 1 mg chlorophyll could be purified from 50 mg-treated Photosystem II particles. One prerequisite for a successful purification was that the Photosystem II particles were extensively treated to remove extrinsic and some hydrophobic proteins (Fig. 3, lanes A and B), that otherwise would have co-purified with CP29. Furthermore, it was essential to elute loosely bound proteins from the column before applying the salt gradient.

The polypeptide composition of the isolated CP29 fraction was analyzed by denaturing SDS-polyacrylamide gel electrophoresis. To find optimal resolution the analysis was performed with three different gel types at 0 °C and 25 °C. Under all six conditions only one polypeptide could be detected (Fig. 3). However,

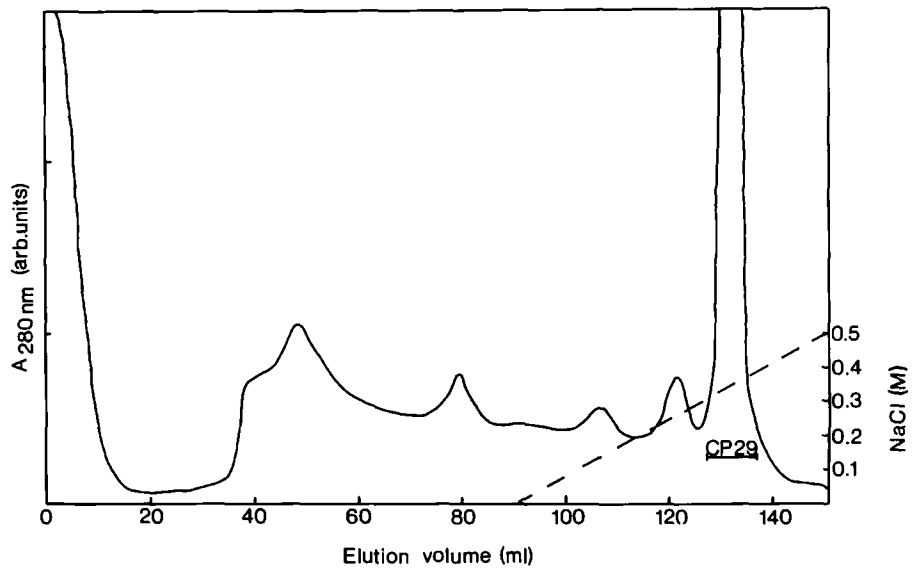

Fig. 1. Chromatogram of treated Photosystem II particles, solubilized and then fractionated on a cation exchange column (TSK CM-3SW). After sample application the column was washed with 90 ml buffer followed by a NaCl gradient from 0 to 0.5 M. The most prominent chlorophyll-protein containing fractions are marked as CP29.

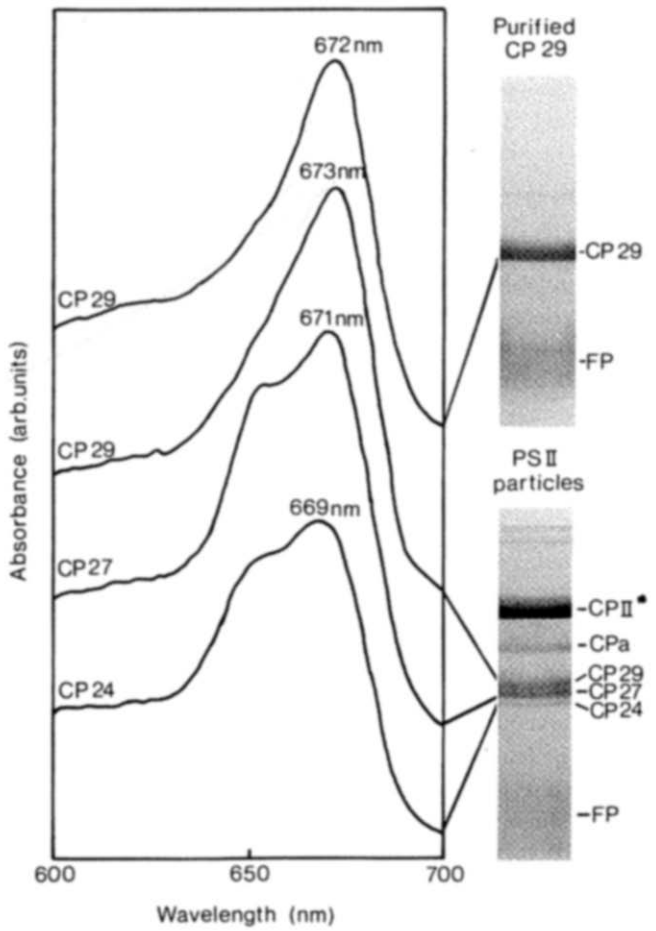

Fig. 2. Mild electrophoresis on the pooled HPLC fractions in Fig. 1, here marked as purified CP29 (10 μg chlorophyll) and treated Photosystem II (PS II) particles (25 μg chlorophyll). CPII*, LHC II trimer; and CPa, CP43 and CP47; CP27, LHC-II monomer and CP27, FP, free pigment. Absorption spectra of the marked chlorophyll-protein complexes, analyzed directly in the gels.

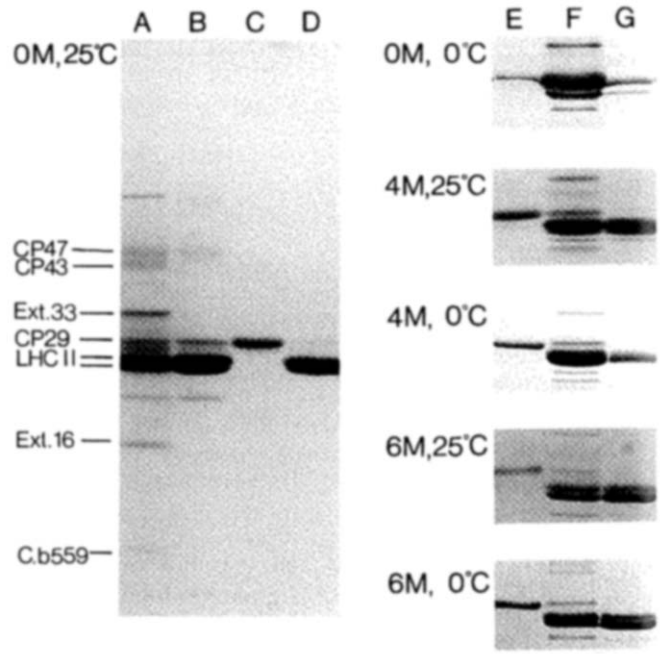

Fig. 3. Denaturing electrophoresis performed under six different conditions, without urea (0 M), 4 M urea and 6 M urea at 0 °C and 25 °C each. (A) and (F), Photosystem II particles; (B), treated Photosystem II particles; (C) and (E), purified CP29, (D) and (G), purified LHC II. (E), (F) and (G) show only a part of each gel.Sample corresponding to approx. 20 μg chlorophyll was loaded on each slot. Ext. 33, extrinsic 33 kDa protein; Ext.16, extrinsic 16 kDa protein, C.*b*559: large subunit of cytochrome *b*-559.

the relative mobility of the CP29 polypeptide compared to the LHC II polypeptides was influenced by the conditions used. Thus the polypeptides of CP29 and LHC II showed good separation when the electrophoresis was run at 25 °C, without urea, while comigration was observed at 0 °C. Such comigration was also sometimes found for the 4 M urea gels when performed at 0 °C, although not in the gel shown in Fig. 3. Sufficient resolution was obtained when 4 M urea gels at 25 °C and 6 M urea gels at 0 °C and 25 °C were used. Depending on the gel system used, the polypeptide of CP29 had an apparent molecular weight between 29 and 31.5 kDa. The identity of CP29 was further strengthened by comigration in denaturing electrophoresis of the HPLC-purified CP29 and of CP29 excised from mild electrophoresis of Photosystem II particles (not shown).

The absorption spectrum of the isolated CP29 in the range of 400–720 nm shows one dominating peak at 678 nm and two maxima in the blue region at 437 nm and 465 nm (Fig. 4). Furthermore, a prominent shoulder is seen at 490 nm, indicating the presence of carotenoids. The spectrum also reveals a peak at 640 nm, which has been suggested to be characteristic for the CP29 complex [6,33]. The lack of a second peak or pronounced shoulder in the red region of the spectrum might be

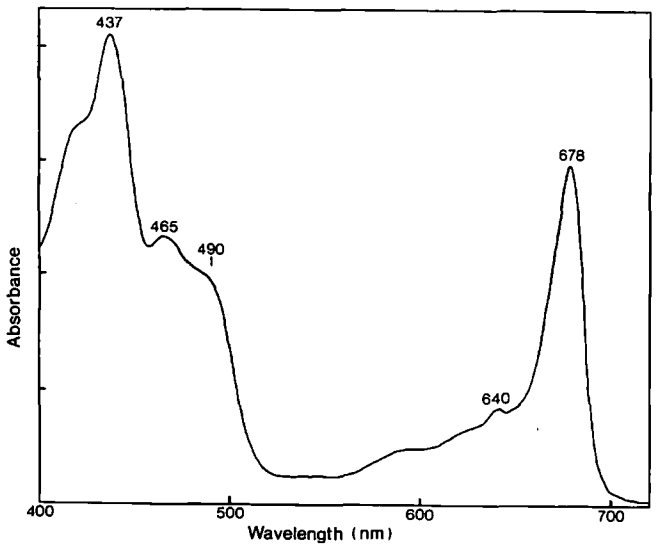

Fig. 4. Absorption spectrum of isolated CP29 in a medium composed of 25 mM *n*-octyl β-D-glucopyranoside, 10 mM NaCl and 10 mM Tricine (pH 7.5).

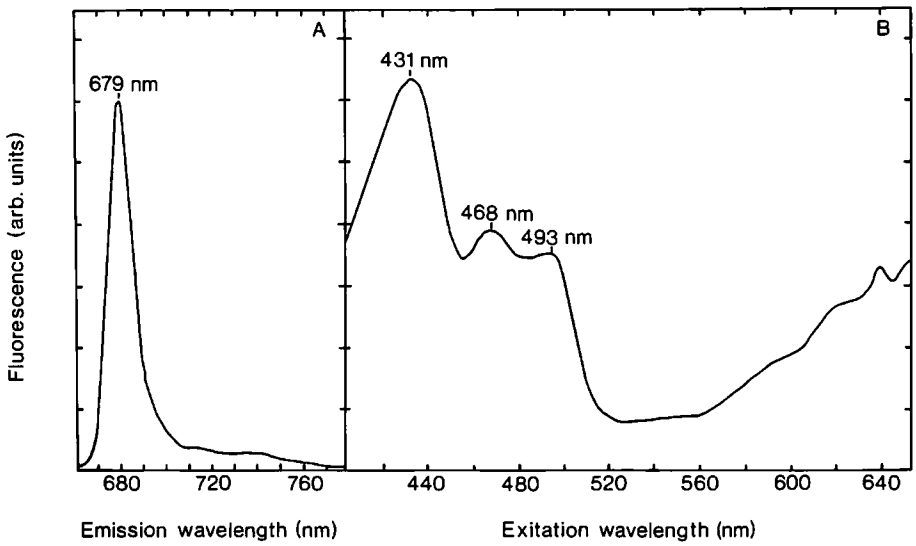

Fig. 5. Fluorescence spectra of the isolated CP29, recorded at 77 K. (A) Emission spectrum obtained with a broad band excitation, 390–560 nm; (B) excitation spectrum with emission measured at 679 nm.

taken as an evidence for the absence of chlorophyll *b*. However, a peak at 650 nm in the 4th derivative spectrum reveals the presence of chlorophyll *b* (not shown). Furthermore, a chlorophyll *a*/*b* ratio of 3.0–3.2 was obtained by extraction and determination in 80% acetone.

The thin layer chromatography of acetone extracted pigments revealed substantial amounts of chlorophyll *a*, chlorophyll *b* and xanthophylls (not shown). The xanthophylls showed mobilities corresponding to lutein, violaxanthin and neoxanthin. The different xanthophylls were found in approximately equal amounts and corresponded each to 0.5–1 xanthophyll per CP29 complex. Thus, CP29 has a similar carotenoid composition as the LHC II complex (cf. Ref. 6), but different from the chlorophyll *a* complex CP47, which contains β-carotene (cf. Ref. 34).

The low-temperature fluorescence emission spectrum (Fig. 5) of the isolated CP29 complex shows a main peak at 679 nm, in accordance with an earlier report [14], and two minor components at 713 nm and 740 nm. The fluorescence excitation spectrum (Fig. 5) shows strong peaks at positions corresponding to chlorophyll *a* (431 nm), chlorophyll *b* (468 nm) and carotenoids (493 nm). The pronounced carotenoid peak shows that these pigments transfer energy to the fluorescing chlorophyll *a*. We therefore suggest that the association of xanthophylls to the CP29 complex is functional (cf. Ref. 35).

Polyclonal antibodies, raised against the HPLC-purified CP29, were found to crossreact with several polypeptides resolved by denaturing electrophoresis (not shown). Apart from crossreaction with the 27 kDa and 25 kDa polypeptides of LHC-II, the antibodies also reacted with two Photosystem I polypeptides in the 24–20 kDa range probably belonging to the light-harvesting complex of Photosystem I (LHC I).

The overall amino acid composition of the isolated CP29 (Table I) revealed the presence of three histidines and a high content of asparagine/aspartic acid and glutamine/glutamic acid. Histidine, asparagine and glutamine have been suggested to be important for chlorophyll binding [36]. Interestingly, a high proportion (43%) of polar amino acids, defined as in Ref. 37, was found. This is a high value considering that the protein requires detergents to be solubilized and kept in solution.

TABLE I

Amino acid composition of the isolated CP29

Residues per protein is calculated for a molecular weight of approx. 29.5 kDa. Cysteine, tryptophane and tyrosine were not determined.

Amino acid	Mol%	Residues per protein (mol/mol)
Asx	9.1	25.4 (25)
Thr	5.8	16.2 (16)
Ser	4.8	13.5 (13)
Glx	11.3	31.8 (32)
Pro	7.1	19.9 (20)
Gly	12.0	33.6 (34)
Ala	10.3	28.8 (29)
Val	4.7	13.2 (13)
Met	0.3	0.7 (1)
Ile	4.1	11.6 (12)
Leu	13.2	36.8 (37)
Phe	5.7	16.1 (16)
His	1.1	3.1 (3)
Lys	6.5	18.2 (18)
Arg	4.0	11.1 (11)
	100.0	280.0 (280)

TABLE II

N-Terminal amino acid sequences from trypsin fragments of CP29

The three overlapping N-terminal sequences were combined to give a consensus sequence of 29 amino acids. Note that residue 10 and 17 were not firmly established but strengthened by the fact that trypsin preferably cleaves after arginine or lysine.

	Fragment 1	Fragment 2	Fragment 3	Consensus sequence	
1	Asn			Asn	N
2	Leu			Leu	L
3	Ala			Ala	A
4	Gly			Gly	G
5	Asp			Asp	D
6	Ile			Ile	I
7	Ile			Ile	I
8	Gly			Gly	G
9	Thr			Thr	T
10	Arg?			Arg (?)	R
11	Thr	Thr		Thr	T
12	Glu	Glu		Glu	E
13	Ser	Ser		Ser	S
14	Ala	Ala		Ala	A
15	Asp	Asp?		Asp	D
16		Val		Val	V
17		Lys?		Lys (?)	K
18		Ser?	Ser	Ser	S
19		–	Thr	Thr	T
20		–	Ser	Ser	S
21		Leu?	Leu	Leu	L
22		Gln	Gln	Gln	Q
23		Pro	Pro	Pro	P
24		Tyr	Tyr	Tyr	Y
25			Ser	Ser	S
26			Glu	Glu	E
27			Val	Val	V
28			Phe	Phe	F
29			Gly	Gly	G

The isolated CP29 polypeptide was found to be N-terminally blocked and thus not possible to sequence directly. To circumvent this problem, the CP29 was treated with trypsin. The trypsin treatment produced, among others, three distinct fragments with apparent molecular weights in the range 17–19 kDa, of which the two heavier fragments could be further digested to the 17 kDa fragment upon prolonged digestion (not shown). The fragments were N-terminally sequenced and the result is shown in Table II. The N-terminal sequences of the three fragments turned out to be overlapping and could therefore be combined to a consensus sequence with a length of 29 amino acids, which is about 10% of the mature protein. The arginine in position 10 and the lysine at position 17 was not firmly established by the sequence analysis alone but was supported by the fact that the site for trypsin degradation is at arginine or lysine.

Discussion

Up to now only small quantities of the CP29 complex have been isolated. This has been achieved by different electrophoretic techniques (see Ref. 1). With the isolation method described here, the CP29 complex has been obtained in a homogenous form in mg quantities. It shows a red absorption maximum as high as 678 nm (Fig. 4) which is at a 4–6 nm longer wavelength than that seen for CP29 after mild electrophoresis (Refs. 11 and 14 and Fig. 2). This suggests that the HPLC-purified CP29 is in a more native state.

The isolated CP29 complex is distinguished from LHC II [5] with respect to (a) chlorophyll *a* and *b* content, (b) absorption spectrum, (c) mobility in denaturing electrophoresis, (d) trypsin digestion pattern and (e) partial amino acid sequence. It is distinguished from CP24 (Ref. 38; Spangfort, M., unpublished results) with respect to (a) chlorophyll *a*/*b* ratio, (b) absorption spectrum (c) mobility in denaturing electrophoresis and (d) partial amino acid sequence. Finally it is distinguished from the less characterized CP27 [14] with respect to (a) absorption spectrum and (b) mobility in denaturing electrophoresis.

An interesting question is the number of pigment molecules that are present in the protein. Based on quantitative amino acid analysis and pigment analysis 8–9 chlorophyll *a*, 2–3 chlorophyll *b* and 2–3 xanthophylls were found per CP29 protein. We suggest that CP29 has 9 chlorophyll *a* and 3 chlorophyll *b*, since these values are in agreement with the measured chlorophyll *a*/*b* ratio of 3.0–3.2. This should be compared with the suggestion that LHC II contains 7 chlorophyll *a* and 6 chlorophyll *b* [6]. Furthermore, quantification of the Coomassie stained polypeptides after denaturing electrophoresis of purified complexes suggest that CP29 and LHC II contains approximately the same amount of chlorophyll per protein. However, the value of chlorophyll per protein for CP29 should be taken as a lower limit as it cannot be excluded that some pigments has been lost during isolation.

A discrepancy regarding the chlorophyll *b* content of CP29 exists. Thus some reports suggest that CP29 does not contain chlorophyll *b* [6,17] while other argue for its presence [5,11]. The presence of chlorophyll *b* in the CP29 isolated here was established by (a) the method of Arnon [28], (b) fourth-derivative spectrum of the native complex and (c) thin-layer chromatography. It should be noted that the contribution from chlorophyll *b* is not directly evident in the red region of the native absorption spectrum.

CP29 has been reported to contain one [6,11] or two [12,13,15] apoproteins. Our results favours the former view, since the CP29 isolated here consists of one single apoprotein, despite that six different electrophoretic

conditions have been employed. However, differences between species can not be excluded.

Structural similarities between the CP29 complex and other chlorophyll binding proteins were indicated by immunological crossreactivity. Thus, polyclonal antibodies raised against CP29 also reacted with polypeptides from LHC II and polypeptides probably belonging to the light-harvesting antenna of Photosystem I. This crossreactivity is in accordance with earlier observations [13,15,18]. However, when the consensus amino acid sequence of HPLC-purified CP29 was compared with the sequences of LHC II [5], CP24 (Ref. 38; Spangfort, M., unpublished results), LHC I [39], LHC I-15 [40], CP43 [41] and CP47 [41], no significant homology was found. As the different chlorophyll *a/b* binding proteins have both regions of high and low homology, we suggest that the consensus sequence found here is in a region of the CP29 that shows low homology with other chlorophyll *a/b* binding proteins.

The organization of CP29 in Photosystem II is not yet fully established. However, the value of 10–12 chlorophyll per CP29 found here can be used to estimate the number of CP29 molecules present in Photosystem II. The assumption made is that Photosystem II contains some 250 chlorophyll [42] of which 6–10% is located in CP29 (recalculated from thylakoid values from Refs. 5 and 7 and also obtained here after mild electrophoresis). Using these values 1.2–2.5 CP29 molecules per Photosystem II is obtained. Interestingly, a dimer formation of CP29 has been observed under mild electrophoresis conditions [7,10,19]. However, if such a dimer formation also occurs in the native Photosystem II remains to be established.

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