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Pigment stoichiometry of a newly isolated D1–D2–Cyt b_{559} complex from the higher plant *Beta vulgaris* L.

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Two D1–D2–Cyt b_{559} complexes with different pigment stoichiometry were isolated from the higher plant *B. vulgaris*. The procedures for isolating both complexes only differed in the washing time of the DEAE column with 50 mM Tris-HCl, pH 7.2, 0.05% Triton X-100 and 30 mM NaCl. When the column was washed until the eluate had an absorbance of 0.01 at 670 nm, the isolated D1–D2–Cyt b_{559} complex presented a pigment stoichiometry of 6 chlorophyll *a*, 2 β-carotene, and 1 cytochrome b_{559} per 2 pheophytin *a*. In contrast, when the column was exhaustively washed until the eluate reached an absorbance of 0.005 at 670 nm, the complex had a stoichiometry of 4 chlorophyll *a*, 1 β-carotene, and 1 cytochrome b_{559} per 2 pheophytin *a*. We think that the former stoichiometry corresponds to that of the native D1–D2–Cyt b_{559} complex. Moreover, both preparations showed 2 mol of pheophytin *a* per 1 mol of reaction center protein.

Beta vulgaris; Photosystem II; Pigment; Reaction center; Stoichiometry

1. INTRODUCTION

The isolation of a pigment-protein complex consisting of D1, D2 and cytochrome (Cyt) b_{559} subunits has recently been described in spinach [1], pea [2] and *Spirodella oligorrhiza* [3]. The isolated D1–D2–Cyt b_{559} complex was able to photoaccumulate reduced pheophytin (Phe) *a* in the presence of ambient reducing conditions [1–3] and $P680^+$ in the presence of silicomolibdate (SiMo) as an artificial electron acceptor [4,5]. Furthermore, the photoinduction of a spin-polarized triplet state [6] and the 2–3 ps rise time of the primary charge separation [7] were very similar to those of the bacterial reaction center. These features confirmed the prediction that the D1–D2 and L–M heterodimers are structural and functional analogues [8,9], leading to the general belief that the D1–D2–Cyt b_{559} complex constitutes the photosystem (PS) II reaction center.

The first stoichiometric analysis of the D1–D2–Cyt b_{559} complex isolated from spinach [1] and pea [2] indicated that this pigment-protein complex harbored 4–5 chlorophyll (Chl) *a*, 1 β-carotene and 1 Cyt b_{559} per 2 Phe *a*. Quinones were not detected in these preparations. Later on, other stoichiometries were reported, i.e. Dekker et al. [10] found approximately 11 Chl *a* and 2 Cyt b_{559} per 2–3 Phe *a*, and Shuvalov et al. [11] reported 4 Chl *a* and 2 Cyt b_{559} per 2 Phe *a*. More recently, Gounaris et al. [12] and Kobayashi et al. [13] have proposed a pigment stoichiometry of 6 Chl *a*, 2 Phe *a*, and 2 β-carotene for the D1–D2–Cyt b_{559} complex.

In the present work, we report the chromophore stoichiometric analysis of a newly isolated D1–D2–Cyt b_{559} complex from the higher plant *B. vulgaris*. Two different stoichiometries were obtained depending on the period of time used for washing the anion-exchange column, a step in the purification process of the D1–D2–Cyt b_{559} complex.

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2. MATERIALS AND METHODS

2.1. *Preparation of PS II membranes and the D1–D2–Cyt* b_{559} *complex*

Highly purified PS II membranes were obtained from chamber-grown *Beta vulgaris* L. (cv. Monohill) leaves. Membranes were prepared basically as described by Berthold et al. [14] but using a centrifugation at 13 350 × *g* for 10 min to obtain the thylakoid fraction.

The D1–D2–Cyt b_{559} complex was isolated from PS II membranes following essentially the procedure described by Nanba and Satoh for spinach [1]. Membranes at a total Chl concentration of 1 mg/ml in 50 mM Tris-HCl, pH 7.2, were solubilized in 4% Triton X-100 for 1 h in the dark. The resultant supernatant was loaded onto a 1.6 × 15 cm Fractogel TSK-DEAE 650S anion-exchange column (Merck). The column was washed with buffer containing 50 mM Tris-HCl, pH 7.2, 0.05% Triton X-100 and 30 mM NaCl, until the absorbance of the eluate was 0.01 at 670 nm in a 1-cm pathlength cuvette. The D1–D2–Cyt b_{559} complex was eluted with a 30–200 mM NaCl linear gradient in the same buffer. The green band which eluted at about 110 mM NaCl contained the D1–D2–Cyt b_{559} complex. We will refer hereafter to RC1 as the complex obtained in this way. When we washed the DEAE column thoroughly until the eluate absorbance was 0.005 at 670 nm we obtained a D1–D2–Cyt b_{559} complex that we will call RC2 hereafter. In both cases, the fractions with more intense green colour were pooled out and subjected to the polyethylene glycol (PEG) precipitation [15]. The precipitate was resuspended in 50 mM

Tris-HCl, pH 7.2, and stored at 77 K. All purification steps were done at 4°C in the dark.

2.2. *Spectroscopic quantitation of Chl a, Phe a and Cyt* b_{559}

Pigments and Cyt b_{559} of the D1-D2-Cyt b_{559} complex were quantitated from their absorption spectra, which were recorded with a Beckman DU-64 spectrophotometer. Total pigments were extracted with 100% acetone at 4°C in the dark, sonicated for 2-3 min to favor the release of pigments and centrifuged for 1 min with a microfuge. Then, 1.5 mM HCl final concentration was added to the supernatant to induce total pheophytinization of the Chl *a*. Concentration of total Phe *a* was determined from the pheophytinized extract at 666 nm using an extinction coefficient of 49.3 $mM^{-1} \cdot cm^{-1}$ [16]. Content of Phe *a* in the non-pheophytinized acetonic extract was determined using an extinction coefficient of 6.504 $mM^{-1} \cdot cm^{-1}$, calculated from the absorption spectra of the pheophytinized extract based on the comparison between the absorbances at 666 nm and 535 nm. The concentration of Chl *a* corresponded to the difference in Phe *a* contents between the pheophytinized and the non-pheophytinized extract.

The amount of Cyt b_{559} was calculated from the dithionite-reduced minus ferricyanide-oxidized difference absorption spectra using an extinction coefficient of 21.0 $mM^{-1} \cdot cm^{-1}$ at 559 nm (the α-band) [17]. Concentration of Cyt b_{559} was also determined by the alkaline-pyridine haemochrome method, using an extinction coefficient of 23.4 $mM^{-1} \cdot cm^{-1}$ at 556 nm [18].

2.3. *Quantitation of Chl a and β-carotene by HPLC*

HPLC analysis and quantitation of the pigments were done according to de las Rivas et al. [19]. Pigments were extracted with 100% acetone, sonicated for 2-3 min and the extract filtered through a 0.22 μm Millex filter (Millipore). All steps were carried out at 4°C in the dark. The chromatography was developed on a 100 × 8 mm Waters C-18 Novapack column (4-μm particle size) with a radial compression module attached to it. Peaks were detected at 450 nm.

2.4. *Protein determination*

Protein concentration of D1-D2-Cyt b_{559} complex preparations was determined by the method of Markwell et al. [20], using bovine seroalbumin as the standard.

3. RESULTS

Two types of D1-D2-Cyt b_{559} complex preparations were obtained from purified PS II membranes of the higher plant *B. vulgaris*, depending on for how long we washed the anion-exchange column with buffer containing 50 mM Tris-HCl, pH 7.2, 0.05% Triton X-100 and 30 mM NaCl. Absorption spectra of these preparations were obtained immediately after elution from the column, just before the PEG treatment. Absorption spectra of the RC1 and RC2 preparations were similar, exhibiting the maximum red peak at 675.5 and 673.5 nm, respectively. The spectra were comparable to that of the D1-D2-Cyt b_{559} complex isolated from other higher plants [1-3].

Fig. 1 shows the electronic absorption spectra of the pigment acetonic extract of RC1 and RC2 preparations in the 440-630 nm spectral region. It is clear that both preparations had different absorbance in the carotenoid region around 490 nm. The preparation washed for a longer time (RC2) displayed lower levels of carotenoid compared to RC1. Spectra were normalized at 535 nm,

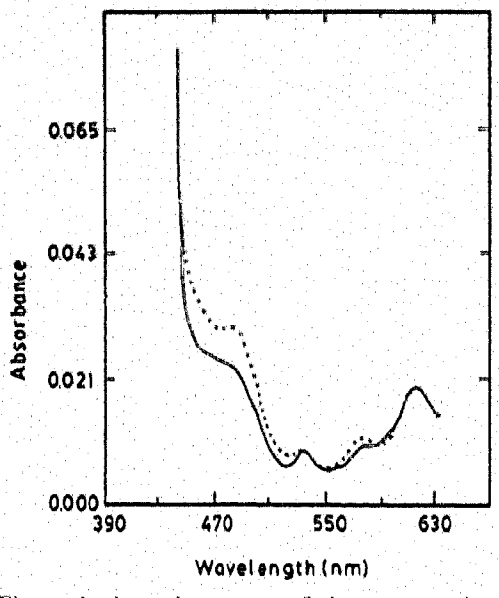

Fig. 1. Electronic absorption spectra of pigment acetonic extract of RC1 (- - - -) and RC2 (——) in the 440-630 nm spectral region. Spectra were normalized at 535 nm (Q_x band of the Phe *a*).

which corresponded with the maximum peak of the Q_x band of the Phe *a*.

The typical spectra of an acetonic extract before and after its pheophytinization with 1.5 mM HCl are shown in Fig. 2. Chlorophyll *a* was completely converted to Phe *a* in acidic conditions. From these spectra (see Materials and Methods) we calculated the content of Chl *a* and Phe *a* in the RC preparations. The preparations were found to consist of 6 Chl *a* per 2 Phe *a* for RC1, and 4 Chl *a* per 2 Phe *a* for RC2. The content of Chl *a* and carotenoid were also calculated from the HPLC chromatograms of the acetonic extracts (see Materials and Methods). A typical chromatogram detected at 450 nm of a pigment acetonic extract is shown in Fig. 3. The two major peaks at 8.2 min and 9.9 min retention time were due to Chl *a* and β-carotene, respectively. The minor peak with a retention time of 9.2 min was due to Phe *a*. From these chromatograms we calculated a ratio of 2 β-carotene per 6 Chl *a* and 1 β-carotene per 4 Chl *a* for RC1 and RC2, respectively.

The cytochrome content of the RC1 and RC2 preparations was calculated from both the reduced-oxidized difference spectra and the alkaline-pyridine haemochrome method. The dithionite-reduced minus ferricyanide-oxidized spectra showed a maximum around 560 nm and the alkaline-pyridine haemochrome spectra at 556 nm (Fig. 4). Both methods of measurements gave similar results. The concentration of Cyt b_{559} was comparable for both RC1 and RC2 preparations, i.e. 1 Cyt b_{559} per 2 Phe *a*, and was independent of the elution fraction obtained from the 30-200 mM NaCl gradient. Thus, the Cyt b_{559} content remained quite constant regardless of the type of RC preparation and the elution fraction.

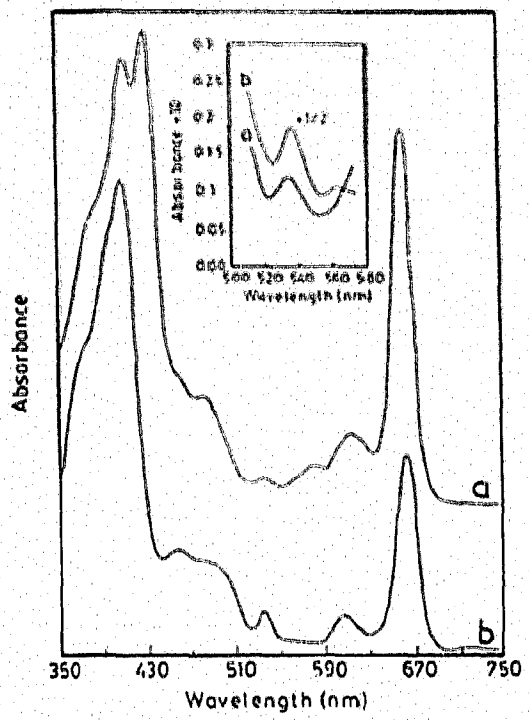

Fig. 2. Electronic absorption spectra of a pigment acetonic extract of RC1 preparation. (a) Pigment acetonic extrac with no addition. (b) The same as in (a) after addition of 1.5 mM HCl final concentration. The insert represents a magnification of the Q_x band region of the Phe *a*. Spectra a of the insert were used to quantitate the content of Phe *a* of the non-pheophytinized extracts.

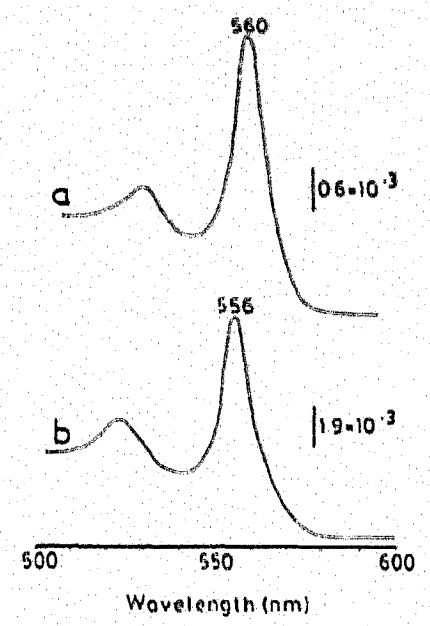

Fig. 4. (a) Dithionite-reduced minus ferricyanide-oxidized difference spectra of the D1-D2-Cyt b_{559} complex from *B. vulgaris* in the 500–600 nm spectral range. The concentration of RC corresponded to 3 μg Chl *a*/ml. (b) Dithionite-reduced minus ferricyanide-oxidized difference spectra of the pyridine haemochrome derivative of the D1-D2-Cyt b_{559} complex. The sample (0.76 μg Chl *a*/ml) was treated with a reaction mixture containing 0.1 M NaOH, 33% pyridine and 0.27 mM $K_3Fe(CN)_6$ in 50 mM Tris-HCl, pH 7.2, and the spectrum was recorded. Then, a few crystals of dithionite were added to the sample and the spectrum was run again. The difference between the second and the first spectra is shown.

Table I summarizes all the calculations done through this work. Table I shows the chromophore and protein mass stoichiometry per 2 Phe *a* for both RC1 and RC2 preparations. Notable differences in the amounts of Chl *a* and β-carotene were observed between the preparations RC1 and RC2. As much as one third of Chl *a* and half of β-carotene were lost by prolonging the washing time. In contrast, the Cyt b_{559} content was independent of the type of RC preparation. Moreover, protein determination indicated the presence of 2 Phe *a* per about 80 kDa, which matches very well with the addition of the apparent molecular masses, as determined by electrophoresis, of D1, D2 and Cyt b_{559} subunits of the RC1 or RC2 preparations. This finding shows that 2 mol of Phe *a* are present per 1 mol of D1-D2-Cyt b_{559} complex.

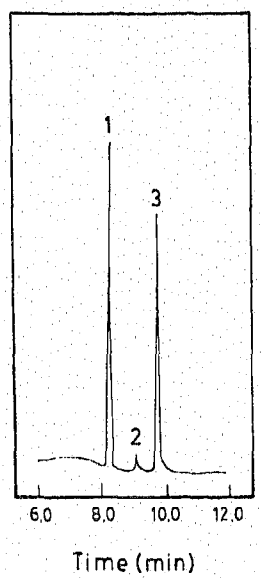

Fig. 3. HPLC chromatogram of a pigment extract (see Materials and Methods). Peaks were detected at 450 nm and identified as Chl *a* (1), Phe *a* (2) and β-carotene (3).

Table I

Pigment content per 2 Phe *a* of RC1 and RC2 preparations from *B. vulgaris*

	RC1	RC2
Chl *a*	6.01 ± 0.16	4.11 ± 0.08
Phe *a*	2.00	2.00
β-carotene	1.63 ± 0.09	0.84 ± 0.01
Cyt b_{559}	1.14 ± 0.03	1.23 ± 0.12
Phe *a*/80 kDa protein	2.03 ± 0.005	2.00 ± 0.005

Values represent means ± SE ($n = 6$).

4. DISCUSSION

The chromophore stoichiometry of the D1-D2-Cyt b_{559} complex is still a subject of debate. Our results showed that it was possible to obtain two different pigment stoichiometries in preparations of the same biological material. The two stochiometries depended only on the time used for washing the column during the purification of the D1-D2-Cyt b_{559} complexes. The stoichiometry of the RC1 preparation is in good agreement with the results recently reported by Gounaris et al. [12] and Kobayashi et al. [13] for pea and spinach, respectively. On the other hand, the pigment stoichiometry of the RC2 preparation is equivalent to that obtained for the reaction center from purple bacteria and to the earlier stoichiometries reported for spinach [1] and pea [2], and more recently for *S. oligorrhiza* [3]. However, our results are in contrast with the stoichiometry reported by Dekker et al. [10] for spinach.

A noteworthy observation of the present paper is the close similarity between the first preparations of D1-D2-Cyt b_{559} complex from spinach [1] and pea [2] and our RC2 preparation. All three preparations showed a similar pigment stoichiometry with the maximum red peak centered around 673 nm. It appears then that there is a close relationship between the absorbance of the washing eluate just before starting the 30-200 mM NaCl gradient, the pigment stoichiometry and the maximum absorption peak in the red.

The Cyt b_{559} content of the D1-D2-Cyt b_{559} complex is still controversial. We assayed this very carefully for every elution fraction from the 30-200 mM NaCl gradient of the RC1 and RC2 preparations. The content of the Cyt b_{559} remained relatively constant for all the elution fractions, i.e. approximately 1 Cyt b_{559} per 2 Pheo *a*. This is in good agreement with other reports [1-3,21], but disagrees with that from Dekker et al. [10] and Shuvalov et al. [11]. However, our results do not necessarily imply that less purified preparations from *B. vulgaris*, such as PS II membranes and the oxygen-evolving core complex, have also 1 Cyt b_{559} per D1-D2-Cyt b_{559} complex. This research is underway.

Our results show that the washing process of the anion-exchange column is an important step in the purification of the D1-D2-Cyt b_{559} complex. A rigorous control of the column washing conditions must be carried out to get a good homogeneity of the D1-D2-Cyt b_{559} complex preparation so that a general consensus on its chromophore stoichiometry can be reached. We currently believe that the pigment stoichiometry found in our RC1 preparation rather corresponds to that of a native D1-D2-Cyt b_{559} complex. The reduced number of pigments in the RC2 may, however, be advantageous to characterize the remaining chromophores, which include presumably the essential ones for the functioning of the D1-D2-Cyt b_{559} complex.

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