Determination of the Molar Extinction Coefficients of the B800 and B850 Absorption Bands in Light-harvesting Complexes 2 Derived from Three Purple Photosynthetic Bacteria *Rhodoblastus acidophilus*, *Rhodobacter sphaeroides*, and *Phaeospirillum molischianum* by Extraction of Bacteriochlorophyll *a*

Yoshitaka SAGA*,**† and Keiya HIROTA*

*Department of Chemistry, Faculty of Science and Engineering, Kinki University, Higashi-Osaka, Osaka 577-8502, Japan
**PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

The molar extinction coefficients of light-harvesting complex 2 (LH2) have been ambiguous in spite of its fame and wide utilization. Herein we determine the molar extinction coefficients of the LH2 proteins derived from the three purple photosynthetic bacteria *Rhodoblastus acidophilus*, *Rhodobacter sphaeroides* and *Phaeospirillum molischianum* at 298 K by direct extraction of bacteriochlorophyll (*BChl*) *a* from the lyophilized proteins, followed by estimation of *BChl a* amounts from their electronic absorption spectra.

**Keywords** Bacteriochlorophyll, B800, B850, molar extinction coefficient, photosynthetic protein

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**Introduction**

The light-harvesting complex 2 (LH2) in purple photosynthetic bacteria is one of the famous photosynthetic proteins,1,4 and has been studied in diverse research fields such as basic photosynthetic research, biochemistry/biophysics of membranous proteins, and artificial photosynthesis.3–11 The LH2 protein has a circular form, which basically consists of nine or eight pigment-peptide units; the numbers of the units in LH2 depend on the bacterial species. Bacteriochlorophyll (*BChl*) *a* and carotenoids are embedded in the protein scaffolds. BChl *a* in the LH2 protein is classified into two groups: one is called B800 possessing the *Qy* peak around 800 nm and the other is called B850, whose *Qy* band is positioned around 850 nm. B800 BChl *a* is monomeric, whereas B850 BChl *a* molecules interact excitonically in the circular form.

Purple photosynthetic bacteria comprise many species. Among them, *Rhodoblastus* (Rbl.) *acidophilus* (formerly known as *Rhodopseudomonas acidophila*) and *Phaeospirillum* (Phs.) *molischianum* (formerly known as *Rhodospirillum molischianum*) have commonly been used in the study of the LH2 proteins because of elucidation of their three-dimensional structures.1,2 *Rhodobacter* (Rba.) *sphaeroides* is also a common species and has been extensively investigated in photosynthetic research.3–5 In the study on the LH2 proteins from these species, their molar extinction coefficients (*ε*) are basic and crucial information for estimation of their amounts.

In spite of such importance, the molar extinction coefficients of the LH2 proteins have been ambiguous, and various values were noted in previous reports on the LH2 proteins.11–20 Such confusion would originate from poor experimental evidence and/or chain citations; some reports just referred to the previous publications for the *ε* values of LH2. Therefore, it is necessary to reconsider the *ε* values of the B800 and B850 bands of the LH2 proteins based on precise experimental measurements. To the best of our knowledge, four research groups actually estimated the molar extinction coefficients of LH2 from *Rba. sphaeroides*,12–14 *Rbl. acidophilus*,13,15 and *Allochromatium* (Alc.) *vinosum* (formerly known as *Chromatium vinosum*)13 as summarized in Table S1 (Supporting Information). However, the lack of structural information on the LH2 proteins, including the number of pigments per protein, would require some assumptions and/or a little circuitous measurements for the estimation of the *ε* values. Such situations might produce inconsistency for the *ε* values of the LH2 proteins. To overcome these problems, we directly determined the molar extinction coefficients of the B800 and B850 absorption bands in the LH2 proteins containing 27 BChl *a* pigments, which were isolated from two purple photosynthetic bacteria *Rbl. acidophilus* and *Rba. sphaeroides*,12,14 by extraction of BChl *a* from lyophilized proteins. In addition, we newly determined the *ε* values of the two *Qy* bands in the LH2 protein containing 24 BChl *a* from a photosynthetic bacterium *Phs. molischianum*.5,22

**Experimental**

**Apparatus**

Electronic absorption spectra were measured with a Shimadzu UV-2450 spectrophotometer under the temperature control with a Shimadzu thermoelectric temperature-controlled cell holder TCC-240A. High-performance liquid chromatography (HPLC)
was performed with a Shimadzu LC-20AT pump, an SPD-M20A detector, and a CTO-20A column oven. Size exclusion chromatography (SEC) was carried out with a GE Healthcare AKTAprime plus system. Cell disruption was carried out with a Stansted pressure cell homogenizer FPG12800. Centrifugation and ultracentrifugation were performed with a Kubota centrifuge Model 7000 and a Hitachi CP80MX ultracentrifuge, respectively. Lyophilization was done with a Tokyo Rikakikai FDU-1200 freeze dryer equipped with paper filters.

**Materials**

The three purple photosynthetic bacteria *Rbl. acidophilus* DSM137, *Rba. sphaeroides* 2.4.1, and *Phs. molischianum* DSM120 were provided by Dr. Jiro Harada, Kurume University, and were photosynthetically grown by irradiation with high light in our laboratory. An anion exchange resin Whatman DE52 was purchased from GE Healthcare Life Sciences. Organic solvents were obtained from commercial suppliers and used in experiments without further purification.

**Isolation of LH2**

The LH2 proteins were isolated from the three purple photosynthetic bacteria as follows.21 Harvested bacterial cells were washed with 20 mM Tris-HCl buffer (pH 8.0) and were disrupted with a pressure cell homogenizer at 125 MPa. Unbroken cells and large debris were removed by centrifugation at 4350 × g for 30 min at 277 K, followed by ultracentrifugation of the supernatant at 200000 × g for 60 min at 277 K to collect the membranes. The pellets were then resuspended with 20 mM Tris-HCl buffer (pH 8.0), and solubilized by addition of a detergent *N,N*-dimethyldodecylamine-N-oxide (LDAO). The final concentrations of LDAO in the suspensions of the membranes from *Rbl. acidophilus, Rba. sphaeroides,* and *Phs. molischianum* were 0.35, 0.35, and 1.0%, respectively. After incubation for 1 h at room temperature in the dark, unsolubilized components were removed by ultracentrifugation of the supernatant at 200000 × g for 90 min at 277 K. LH2 proteins were isolated from the supernatant by sucrose gradient centrifugation using 0.2 – 0.8 M continuous gradient in 20 mM Tris-HCl buffer containing 0.1% LDAO and 150 mM NaCl (pH 8.0) at 175000 × g for 18 h at 277 K. The LH2 band was then collected and sucrose was removed by ultrafiltration, followed by purification with anion-exchange chromatography using DE52 resins. The LH2 proteins were analyzed by SEC on a HiPrep 16/60 Sephacryl S-300 HR column with 20 mM Tris–HCl buffer containing 0.1% LDAO and 150 mM NaCl (pH 8.0) at a flow rate of 0.4 mL min⁻¹.21

**Spectroscopic measurements of LH2 and extracted BChl a**

LH2 was dispersed in 20 mM Tris-HCl buffer containing 0.1% LDAO (pH 8.0) and the electronic absorption spectra were measured at 298 K. Then, the 9.0-mL LH2 solution was lyophilized as follows: the LH2 solution was rapidly frozen by using liquid nitrogen, and dried under the reduced pressure for 5 h in the dark. Pigments were rapidly extracted with a 4.0-mL mixture of acetone and methanol (1:1), followed by filtration with a Millipore Millex-LS filter (pore size: 5 μm). The solution was mixed with acetone to adjust the ratio of acetone and methanol to 7:2 (v/v), and its electronic absorption spectra were measured. The concentration of BChl a in acetone/methanol (7:2) was determined by using its molecular extinction coefficient 7.60 × 10⁴ M⁻¹ cm⁻¹ at the Qₜ peak.12,13,15,24

**HPLC measurements**

Extracted BChl a was analyzed on a reverse-phase column 5C18-AR-II (6 mm i.d. × 250 mm), which was kept at 303 K, with methanol at a flow rate of 1 mL min⁻¹.

**Results and Discussion**

Figure 1 shows electronic absorption spectra of the LH2 proteins isolated from *Rbl. acidophilus* (A), *Rba. sphaeroides* (B), and *Phs. molischianum* (C) in 20 mM Tris-HCl buffer containing 0.1% LDAO (pH 8.0) at 298 K.

![Figure 1](image1.png)

**Fig. 1** Electronic absorption spectra of the LH2 proteins isolated from *Rbl. acidophilus* (A), *Rba. sphaeroides* (B), and *Phs. molischianum* (C) in 20 mM Tris-HCl buffer containing 0.1% LDAO (pH 8.0) at 298 K.

Figure 2 shows SEC elution patterns of the LH2 proteins of *Rbl. acidophilus* (A), *Rba. sphaeroides* (B), and *Phs. molischianum* (C). The proteins were eluted on a HiPrep 16/60 Sephacryl S-300 HR column with 20 mM Tris-HCl buffer containing 0.1% LDAO and 150 mM NaCl (pH 8.0) at a flow rate of 0.4 mL min⁻¹.

![Figure 2](image2.png)

**Fig. 2** SEC elution patterns of the LH2 proteins of *Rbl. acidophilus* (A), *Rba. sphaeroides* (B), and *Phs. molischianum* (C). The proteins were eluted on a HiPrep 16/60 Sephacryl S-300 HR column with 20 mM Tris-HCl buffer containing 0.1% LDAO and 150 mM NaCl (pH 8.0) at a flow rate of 0.4 mL min⁻¹. Additionally, the ratios of absorbance of B850 BChl a to that at 280 nm were more than 3.0, 2.9, and 2.0,
injection of the extract solutions. Pigments including BChl absorption spectra of the pigment extracts from LH2 are measured at a rate of 1 mL min\(^{-1}\). The signals denoted by (B), and (C) in acetone/methanol (7/2, v/v).

Fig. 3 Electronic absorption spectra of the pigments extracted from the lyophilized LH2 proteins of *Rbl. acidophilus* (A), *Rba. sphaeroides* (B), and *Phs. molischianum* (C) in acetone/methanol (7/2, v/v).

Fig. 4 HPLC elution patterns of BChl \(a\) extracted from the lyophilized LH2 proteins of *Rbl. acidophilus* (A), *Rba. sphaeroides* (B), and *Phs. molischianum* (C). BChl \(a\) was eluted on a reverse-phase column 5C18-AR-II (6 mm i.d. × 250 mm) with methanol at the flow rate of 1 mL min\(^{-1}\). The signals denoted by \(x\) were due to the direct injection of the extract solutions.

For LH2 of *Rbl. acidophilus*, *Rba. sphaeroides*, and *Phs. molischianum*, respectively. Figure 2 shows the SEC elution patterns of the LH2 proteins in this study. LH2 from *Rbl. acidophilus*, *Rba. sphaeroides*, and *Phs. molischianum* was eluted at 60, 51, and 61 mL, respectively, under the present SEC conditions, and other fractions were hardly detected. These results indicate the high purity of the LH2 proteins used in this study.

Lyophilization of the LH2 proteins allowed us to extract the pigments including BChl \(a\) with high efficiency. Electronic absorption spectra of the pigment extracts from LH2 are depicted in Fig. 3. The Soret and \(Q_y\) peaks of BChl \(a\) in this solvent were observed at 363 and 770 nm. Note that absorption bands around 400 – 550 nm are derived from carotenoids that are co-present in the LH2 proteins. Typical elution patterns of BChl \(a\) extracted from the lyophilized LH2 proteins are shown in Fig. 4. BChl \(a\) was eluted at 13 min accompanying very slight amounts of allomerized BChl \(a\) around 11 min.\(^{25}\) No other fraction, which originated denaturation of BChl \(a\), was detected in these HPLC chromatograms. The electronic absorption spectroscopy and HPLC analysis revealed that little denaturation of BChl \(a\) occurred through the extraction process.

The molar extinction coefficients of the B800 and B850 absorption bands of the three kinds of LH2 proteins were estimated from the concentrations of BChl \(a\) extracted from the lyophilized proteins and information on the number of BChl \(a\) in the LH2 proteins. Table 1 summarizes the \(\epsilon\) values of the \(Q_y\) absorption bands of LH2 from the three purple bacteria. The \(\epsilon\) values of the LH2 B800 bands derived from *Rbl. acidophilus*, *Rba. sphaeroides*, and *Phs. molischianum* were 2.57 \(\times 10^6\), 2.43 \(\times 10^6\), and 1.68 \(\times 10^6\) M\(^{-1}\) cm\(^{-1}\), respectively; and the \(\epsilon\) values of the LH2 B850 bands were 3.19 \(\times 10^6\), 3.23 \(\times 10^6\), and 2.22 \(\times 10^6\) M\(^{-1}\) cm\(^{-1}\), respectively (the average of six measurements). The present \(\epsilon\) values of the B850 bands in LH2 from *Rbl. acidophilus* and *Rba. sphaeroides* were close to that of LH2 in the previous reports.\(^{12,13,15}\) The \(\epsilon\) values of both the B800 and B850 bands in LH2 of *Phs. molischianum* were smaller than those from the other two bacteria. The main reason for the decrease of the \(\epsilon\) values in LH2 of *Phs. molischianum* is the difference in the number of BChl \(a\) molecules. The differences in the microenvironmental structures of the BChl \(a\) binding sites would also change the \(\epsilon\) values of the B800 and B850 bands in the LH2 proteins.

The recovery yields in the BChl \(a\) extraction processes were checked by measurements of electronic absorption spectra of rinse solutions of the filters through the extraction process by methanol, which was able to solubilize chlorophyllous pigments with high efficiency. As a result, the averaged amounts of residual BChl \(a\) were 1.3, 1.4, and 1.1% of BChl \(a\) amounts in the pigment extracts, determined by using the molar extinction coefficient 6.00 \(\times 10^6\) M\(^{-1}\) cm\(^{-1}\) at the \(Q_y\) peak.\(^{26}\) These values were much lower than that in the loss (7%) reported previously,\(^{12}\) indicating the advantage in the present study.

To summarize, we successfully determined the molar extinction coefficients of the B800 and B850 absorption bands of the LH2 proteins derived from the three purple photosynthetic bacteria. These data will be helpful for quantitative studies using the LH2 proteins.

**Table 1** The molar extinction coefficients of the B800 and B850 absorption bands of the LH2 proteins isolated from the three purple photosynthetic bacteria in 20 mM Tris–HCl buffer containing 0.1% LDAO (pH 8.0) at 298 K.

| Bacteria                        | \(\epsilon\) (B800 band)/ \(10^6\) M\(^{-1}\) cm\(^{-1}\) | \(\epsilon\) (B850 band)/ \(10^6\) M\(^{-1}\) cm\(^{-1}\) |
|---------------------------------|----------------------------------------------------------|----------------------------------------------------------|
| *Rbl. acidophilus* DSM137       | 2.57 \pm 0.03                                           | 3.19 \pm 0.17                                           |
| *Rba. sphaeroides* 2.4.1        | 2.43 \pm 0.02                                           | 3.23 \pm 0.03                                           |
| *Phs. molischianum* DSM120      | 1.68 \pm 0.03                                           | 2.22 \pm 0.04                                           |

a. The average of six measurements.

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Supporting Information

The molar extinction coefficients of the B800 and B850 absorption bands of the LH2 proteins, which were estimated on the experimental basis previously, are provided in supporting information. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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