Efficient purification and characterization of high-purity phycoerythrin 545 from Rhodomonas sp.

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Abstract

Cryptomonad phycoerythrin 545 is an important type of phycobiliprotein in basic research and technological innovations. Herein, we report a minimalistic hydrophobic chromatography method for its purification. High purity was achieved, with a purity ratio ($A_{545}/A_{280}$) of 13.66 and a recovery ratio of 78.63%. Following SDS-PAGE, Coomassie Brilliant Blue staining and Zn$^{2+}$-enhanced UV fluorescence autoradiography revealed three bands at 9 kDa, 10 kDa, and 20 kDa, corresponding to $\alpha_1$, $\alpha_2$ and $\beta$ subunits. Multiple spectral characteristics were analysed to ensure that optical activity was consistent with that of the natural protein. Absorption and fluorescence spectroscopies of purified phycoerythrin 545 displayed a strong absorption peak at 545 nm and a shoulder peak at 564 nm, and a fluorescence emission peak of at 587 nm, which confirmed unchanged energy transfer properties, and structural and functional integrity was verified by circular dichroism spectroscopy. Compared with published purification methods, this new purification protocol replaces two-step ammonium sulphate fractionation, dialysis, and size exclusion chromatography with a single chromatography step, thereby reducing the cost of large-scale kilogram-level commercial production.

Introduction

Cryptophytic phycoerythrin 545 (PE545) is a unique phycoerythrin with a maximum absorption wavelength of 545 nm without a phycobilisome structure (Gantt et al. 1971). It is present in the thylakoid lumen rather than the thylakoid membrane, and it is the only type of phycobiliprotein in unicellular cryptophyta algae of the Rhodomonas genus. These soluble proteins act as major light-harvesting antennae. PE545 is significantly different from phycobiliproteins of red algae and cyanobacteria in terms of overall structure and composition of chromophores (Doust et al. 2006; Scholes et al. 2011). High-resolution crystal structures showed that PE545 has a closed, stable quaternary structure consisting of a $\alpha_1\alpha_2\beta\beta$ heterodimer (60 kDa) containing two monomers and eight chromophores (Doust et al. 2004). The PE545 monomer is comprised of $\alpha$ and $\beta$ subunits, in which one molecule of 15,16-dihydrobiliverdin (DBV) is covalently linked to $\alpha_1$ (10.4 kDa) or $\alpha_2$ (9.2 kDa), and each $\beta$ subunit (20 kDa) is carried on three phycoerythrobilins (PEBs) at three conserved positions ($\beta_{82}$, $\beta_{158}$ and $\beta_{50}/\beta_{61}$) (Broughton et al. 2006; Overkamp et al. 2014). The two double-binding linked $\beta_{50}/\beta_{61}$ chromophores are in physical contact at the central section of the $\alpha_1\alpha_2\beta\beta$ heterodimer, and they exhibit strong electronic coupling (Wong et al. 2012; Stephen et al. 2014).

As an evolutionarily distinct phycoerythrin, PE545 is of great value and significance for research on algae photosynthesis, the evolution of algal light harvesting antennae, and the unicellular eukaryotic algae lineage (Jong et al. 2017; Rathbone et al. 2021; Jun et al. 2017; Ma et al. 2020; Sui et al. 2020). Specifically, the $\alpha$ subunit encoded by the nuclear genome originates from the linker protein of phycobilisomes, and the $\beta$ subunit encoded by the chloroplast genome originates from the $\beta$ subunit of eukaryotic red algae (Zauner et al. 2019; Rathbone et al. 2021). This knowledge not only greatly expands research on the evolution of phycobiliproteins, but also provides further evidence for the secondary
endosymbiont theory. Moreover, studies on PE545 provide important clues and an experimental basis for in-depth exploration of the optimisation of light harvesting performance by photosynthetic organisms, and the nano-level regulation principle of the excitation energy transfer process (Wong et al. 2012; Collini et al. 2010; Tong et al. 2019; Tong et al. 2020).

In addition to its importance for theoretical studies, PE545 has valuable application features, such as a unique optical activity, a high fluorescence quantum yield (> 80%), and a variety of biological activities including antioxidant activity and inhibition of cancer cells (Senthilkumar et al. 2013; Sonani et al. 2014; Liu et al. 2015; Mukesh et al. 2019). Compared with other types of phycoerythrins, PE545 has a low molecular weight and high stability. So it will be useful for fluorescent dyes, fluorescent probes, and photodynamic therapy (Li et al. 2019; Abidizadegan et al. 2021). The efficient separation and purification of PE545 is therefore of primary importance, especially improving purity and recovery yield.

Thus, PE545 is valuable not only for fundamental research and applications, hence technological innovations are needed for its further development and utilisation, especially high purity (analytical grade, \( A_{545}/A_{280} > 4.0 \)), low cost, and large-scale preparation techniques (Li et al. 2019). Existing method for PE545 separation and purification is a cumbersome process, including freeze-thawing for cell disruption and crude PE545 extraction, followed by ammonium sulphate fractionation, 24 h dialysis, and ion-exchange and size-exclusion chromatography (Doust et al. 2004; Wilk et al. 1999). Although high purity (\( A_{545}/A_{280} > 10.0 \)) is achieved using this purification approach, it is complicated and time-consuming, and recovery is low, which greatly restricts PE545 applications. Thus, preparation of naturally occurring PE545 from *Rhodomonas* must be optimised to make it faster and more efficient, with high purity and a high recovery yield.

Herein, we report a new protocol for the purification of PE545 using a Butyl-S Sepharose 6 Fast Flow hydrophobic interaction column. This one-step chromatographic procedure is easily scaled up, and it benefits from simplicity, high efficiency, low cost, high protein yield, analytical-grade purity, and no impact on optical properties.

**Materials And Methods**

**Algae culture and harvest**

A unialgal culture of *Rhodomonas salina* preserved in our laboratory was grown in f/2 artificial seawater culture media. The culture medium and algae biomass were separated by centrifuging (4000 rpm for 10 min at 4 °C). Fresh algal cells were stored at -80°C and used on subsequent purification steps (Fig. 1).

**Coarse separation of PE545**

At the beginning of the purification process, 0.02 mol/L phosphate buffer solution (pH 6.9) was added to thawed algae cells at a mass: volume ratio of 1:10. After three freeze–thawing cycles, centrifugation (12000 rpm, 30 min, 4 °C) was employed to obtaining supernatant containing PE545. This was subjected to precipitation with 50% saturated ammonium to facilitate the binding and dissociation of PE545 with
the hydrophobic medium. The supernatant, generated by another centrifugation step (12000 rpm, 20 min, 4 °C), was filtered through a cellulose acetate membrane (0.22 µm) for subsequent fast protein liquid chromatography (FPLC) purification.

**Hydrophobic interaction chromatography purification**

The PE545 solution was passed through a 0.22 µm filter and further purified by FPLC using an AKTA pure 25 M2 instrument (GE Healthcare, USA) equipped with a Butyl-S Sepharose 6 Fast Flow hydrophobic interaction chromatography column (GE Healthcare). Both 280 nm and 545 nm wavelengths were used for real-time monitoring and detection. Briefly, to equilibrate the column, five column volumes of equilibration buffer consisting of 0.02 mol/L phosphate buffer containing 2.5 M (NH₄)₂SO₄ was pumped through the media at a flow rate of 2 mL/min. The PE545 solution was then applied using a cryo-loop. The column was developed with 0.02 mol/L phosphate buffer containing 2.0 M (NH₄)₂SO₄ at the same flow rate so that impurities could be fully eluted from the column. Finally, the red target proteins were eluted with 0.02 mol/L phosphate buffer, immediately frozen in liquid nitrogen, and stored at -80 °C.

The core parameters of PE545 purification, namely concentration, purity ratio, and yield, were calculated using Eqs. (1) and (2), respectively. The extinction coefficient of PE545 was 12.6. The PE545 recovery expressed as a percentage of the amount of PE545 obtained after purification to the total amount of PE545 in the crude extract (Tang et al. 2016; Zang et al. 2020).

\[
C_{PE545} (\text{mg/mL}) = \frac{OD_{545}}{12.6} \quad (1)
\]
\[
P_{PE545} = \frac{A_{545}}{A_{280}} \quad (2)
\]

**SDS-PAGE and spectrometric analyses**

Denatured samples were separated by 4–20% gradient SDS-PAGE and protein bands were detected by UV-fluorography after soaking the gel in 20 mM zinc sulphate for 10 min. After UV-fluorography analysis, the same gel was stained with Coomassie Blue solution.

The characteristics of light-harvesting, energy transfer and chromophores were monitored by absorption spectroscopy, fluorescence spectroscopy and circular dichroism (CD) spectroscopy, respectively. In detail, absorption spectra were acquired using a Cary 60 UV-Vis spectrophotometer (Agilent, USA) at 260–700 nm, and absorption values of PE545 were measured at 280 and 545 nm. Fluorescence spectra were acquired using a Cary Eclipse fluorescence spectrophotometer (Agilent, USA) at an excitation wavelength of 545 nm. CD spectra were acquired using a Chirascan CD spectrometer (Applied Photophysics Corporation, UK) with an optical path length of 1 mm.

**Results And Discussion**

**Extraction of PE545**
Cells of *R. salina* grew well with obvious accumulation of PE545 (Fig. 1). The purity ratio of crude extract following repeated freeze-thawing was 1.46 (Fig. 2a). In order to facilitate the binding and dissociation of PE545 with the medium under appropriate conditions in the next step of hydrophobic chromatography, the sample needs to be treated with high salt. On the basis of a large number of experiments in the early stage, ammonium sulfate with 50% saturation was selected for sample treatment. After adding an appropriate amount of ammonium sulfate to the crude extract and centrifuging, the purity of PE545 in the supernatant increased slightly to 1.69 (Fig. 2a), and the recovery of PE545 reached 81.35%.

**Hydrophobic interaction chromatography**

In the next essential step, an FPLC instrument equipped with a Butyl-S Sepharose 6 Fast Flow column was employed for further purification, following preliminary purification via 50% saturated ammonium sulphate fractionation. A change in ionic strength of the mobile phase alters the polarity of proteins, and impurities and PE545 were eluted at different times depending on their differential interactions with the n-butyl groups of the resin (Fig. 2c). The elution volumes of the three different mobile phases were 100 mL, 100 mL and 50 mL.

Absorbance at 545 nm was used for real-time surveillance of PE545 elution. All PE545 was bound to the column in starting buffer containing 2.5 M ammonium sulphate, while a large amount of impurities accounting for ~47% of total protein was eluted ($A_{280}$, peak 1 in Fig. 2c). Following the passage of 20 mM phosphate buffer containing 2.0 M (NH$_4$)$_2$SO$_4$ through the column, more impurities (~9% of total protein) were eluted ($A_{280}$, peak 2 in Fig. 2c). During this process, the absorption value at 545 nm remained zero, and no red component was observed, which indicated that the target protein was still bound to the column material (Fig. 2d). When the mobile phase was changed to 20 mM phosphate buffer, the increase in absorption value at 545 nm and the appearance of a red component in the eluate indicated that PE545 was eluted (Fig. 2d). The results of 545 nm absorption curve integration showed that more than 99% of PE545 was eluted. After PE545 collection in multiple fractions, the fraction with the highest purity ($A_{545}/A_{280} = 13.66$) was collected separately, and this accounted for around half of all collected target protein. The final recovery of PE545 was 78.63% (Table 1). Moreover, alongside the improvement in protein purity, there were no significant changes in the characteristics of visible light absorption spectra and fluorescence emission spectra for all three samples (Fig. 2a, b), which indicates that this purification process did not significantly affect the biological properties of PE545. Experimental results showed that the purification effects were superior to others (Doust et al. 2004).
Table 1
Purification efficiency of PE545 from *Rhodomonas salina*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Amounts of PE545 (mg)</th>
<th>PE545 yield (%)</th>
<th>Purity ratio ($\frac{A_{545}}{A_{280}}$)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extracts</td>
<td>63.08 ± 3.09</td>
<td>100</td>
<td>1.46 ± 0.15</td>
<td>1</td>
</tr>
<tr>
<td>After n-butyl hydrophobic chromatography</td>
<td>49.60 ± 1.98</td>
<td>78.63</td>
<td>13.66 ± 0.52</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD (n = 3).

The amounts of PE545 were determined with equation system by measures of absorbances at 545 nm.

Previous studies demonstrated that Butyl-S Sepharose 6 Fast Flow resin is a relatively mild hydrophobic medium that selectively adsorbs PE545 without altering its structural conformation (Hjerten et al. 1974). This type of hydrophobic attraction between PE545 and the n-butyl matrix is due to a net increase in entropy of the environment, which enhances the thermodynamic stability by releasing ordered water molecules surrounding non-polar protein groups into the surrounding solutions, and reducing the non-polar surface area exposed to water. Although hydrophobic forces are affected by many factors, the primary factor here is the amino acid sequence composition of PE545, especially the hydrophobic amino acid distribution on the surface of PE545 (Novoderezhkin et al. 2010).

**Gel electrophoresis**

Following SDS-PAGE, Coomassie Brilliant Blue staining and Zn$^{2+}$-enhanced UV-fluorescence autoradiography revealed three bands at the expected positions ($\alpha_1$ and $\alpha_2$ subunits around 10 kDa, and $\beta$ subunit around 20 kDa) (Fig. 3). Because each $\alpha$ subunit contains only one DBV, its autofluorescence is weaker than that of the $\beta$ subunit (Scholes et al. 2011). Coomassie Brilliant Blue staining revealed additional impurity protein bands following freeze-thawing extraction of PE545 (lane 4 in Fig. 3), and were absent in the final PE545 (lane 3 in Fig. 3), consistent with the UV-Vis absorption spectra.

**Spectral analyses of PE545**

Although PE545 with high purity and high yield was obtained using the above-mentioned optimised purification process, we wished to ensure that the purified protein maintained its spectral properties before follow-up studies on energy transfer.

The characteristics of light-harvesting and energy transfer were monitored by absorption spectroscopy and fluorescence spectroscopy. The purified PE545 in the study has a visible light absorption range from 450 nm to 650 nm, with a strong absorption peak at 545 nm and a shoulder peak at 564 nm (Fig. 4a). A fluorescence emission peak of purified PE545 was observed at 587 nm following excitation at 545 nm (Fig. 4b). Comparing the absorption and fluorescence emission spectra showed that the $\alpha_1\alpha_2\beta\beta$
heterodimer of PE545 has a 42 nm red shift from 545 nm to 587 nm. These spectra revealed that the purified protein retained its complete energy transfer properties, consistent with previous reports for purified PE545 (Novoderezhkin et al. 2010; Tong et al. 2020). As the basis of the energy transfer function of PE545, the main absorption band of PE545 is due to the PEB chromophore, and the main fluorescence emission peak at 587 nm is derived from the DBV chromophore, consistent with the results of previous studies (Wilk et al. 1999; Doust et al. 2004). Meanwhile, the fluorescence spectrum can also identify the quantum state of specific chromophore molecules, in addition to reflecting the internal energy transfer ability. The fluorescence properties were similar to those reported previously, indicating that the energy transfer function of PE545 purified by the new method was unchanged (Marrone 1999). Again, because the function of PE545 is closely related to its structure, these results also proved that the protein microenvironment and quantum states of the chromophore in PE545 were not altered, which further confirms the reliability of the new purification method.

CD spectroscopy is associated with the absorbance of a target substance, and it is also affected by molecular asymmetry or conformational asymmetry. In the visible region (450–750 nm), the main contribution to the CD spectrum is from the chromophore, hence it is very sensitive for reflecting the structure of the chromophore. Thus, CD spectra are suitable for in-depth confirmatory testing of the integrity of chromophore conformation, chromophore structure, and chromophore function. As can be seen from Fig. 4c, the CD spectra of purified PE545 had a pronounced positive peak at 536 nm and a negative peak at 569 nm, with the positive and negative peaks intersecting at 554 nm, consistent with literature reports (Maccoll et al. 1994; Doust et al. 2006). Furthermore, the intersecting point at 554 nm suggests a strongly coupled chromophore pair with exciton splitting and excitation delocalisation. These results agree well with the absorbance of two double-bound PEB groups (β50/β61) at the central interface of the PE545 dimer, which further demonstrates the retained spatial conformation of the chromophores (Maccoll et al. 1994). This verifies that the purified PE545 had good structural and functional integrity, and the complete energy transfer function was maintained.

To verify these results in more depth, the second-order derivative Gauss formula was used to deconvolute the absorption spectrum, the fluorescence emission spectrum, and the CD spectrum of the final high purity PE545 (Fig. 4). In the absorption spectrum, two spectral components at 545 nm and 569 nm were obtained by Gaussian deconvolution with $R^2 = 0.99256$ (Fig. 4a). In the fluorescence emission spectrum following excitation at 545 nm, two peaks (582 nm and 609 nm) were also obtained with $R^2 = 0.99898$ (Fig. 4b). Deconvolution of the CD spectrum yielded three peaks at 516 nm, 536 nm and 569 nm, with an $R^2$ value of 0.96767 (Fig. 4c). All deconvolution data were also in good agreement with reported spectral components (Maccoll et al. 1994), and the results further confirmed that the optical activity was not altered by the purification method.

**Conclusion**

In this study, we developed a highly valuable purification protocol for PE545. Based on crude PE545 extraction following three freeze-thaw cycles, analytical grade PE545 ($A_{545}/A_{280} = 13.66$) was obtained
using one-step hydrophobic interaction chromatography, with a high recovery yield of 78.63 ± 0.2%. Purity was verified by SDS-PAGE and spectral analyses. Furthermore, absorption, fluorescence and CD spectra confirmed that the final PE545 retained intact optical activity consistent with that of the native protein. The purified material was therefore suitable for subsequent detailed studies on energy transfer and various practical applications.

Compared with published purification methods, this new method is much simpler than two-step ammonium sulphate fractionation, dialysis, and size-exclusion chromatography, which is useful for reducing complexity, increasing recovery yield, improving the level of automation, and maintaining natural activity. In addition, this new purification protocol for PE545 could lower the cost of large-scale production, and may even facilitate commercial kilogram-scale production. Additionally, this method provides a paradigm for purification procedures for phycobiliproteins with similar properties.

Declarations

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Declaration of competing interest The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material Data available on request from the authors


References


**Figures**
Figure 1

Schematic process for PE545 purification
Figure 2

Absorption spectrum of PE545 at checkpoints of purification (a); fluorescence spectrum of PE545 at checkpoints of purification with 545 nm excitation (b); chromatograms of purification process by HPLC on Butyl-S Sepharose 6 column (monitored with 280 nm) (c); chromatograms of purification process by HPLC on Butyl-S Sepharose 6 column (monitored with 545 nm)
Figure 3

Zn$^{2+}$-enhanced UV-fluorescence image of SDS-PAGE (a); Coomassie Brilliant Blue stained SDS-PAGE image (b)
Figure 4

Deconvolution of absorption spectrum of purified PE545 (a); deconvolution of fluorescence spectrum of purified PE545 (b); deconvolution of CD spectrum of purified PE545 (c)