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Purification, crystallization and preliminary X-ray analysis of phycocyanin and phycoerythrin from *Porphyra yezoensis* Ueda

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Porphyra yezoensis is one of the most important and widely cultured seaweeds in China. Phycobiliproteins exhibit excellent spectroscopic properties and play versatile roles in the biomedical, food, cosmetics and chemical synthetic dye industries. Here, the purification and crystallization of phycoerythrin and phycocyanin, two phycobiliproteins extracted from *P. yezoensis*, are described. Using a novel protocol including co-precipitation with ammonium sulfate and hydroxyapatite column chromatography, both phycobiliproteins were produced on a large scale with improved quality and yield compared with those previously reported. Native PAGE analysis indicated that phycoerythrin and phycocyanin exist as $(\alpha\beta)_3$ heterohexamers in solution. The crystals of phycoerythrin diffracted to 2.07 Å resolution and belonged to space group $R3$. The unit-cell parameters referred to hexagonal axes are $a = b = 187.7$, $c = 59.7$ Å, with nine $(\alpha\beta)_2$ heterotetramers per unit cell. The crystals of phycocyanin diffracted to 2.70 Å resolution in space group $P2_1$. Matthews coefficient analysis shows that 10–19 $(\alpha\beta)$ heterodimers of phycocyanin in the asymmetric unit would be reasonable. A self-rotation function calculation clarified this ambiguity and indicated that 12 $(\alpha\beta)$ heterodimers of phycocyanin are assembled in the asymmetric unit.

1. Introduction

Phycobiliproteins (PBPs), ubiquitously found in cyanobacteria and eukaryotic algae, are water-soluble proteins that have extraordinary fluorescence properties (Liu, Chen, Zhang & Zhou, 2005). Based on their absorption properties and chromophore groups, they are divided into four classes: phycoerythrins (PEs), phycocyanins (PCs), allophycocyanins (APCs) and phycoerythrocyanins (PECs) (Sekar & Chandramohan, 2008). PBPs not only participate in efficient energy capture and transfer (Betz, 1997; Arnold & Oppenheimer, 1950; French & Young, 1952), but also play an important role in nitrogen harvesting (Heathcote *et al.*, 1992). PBPs can be used as natural colourants in food and cosmetics to substitute for synthetic pigments (Naidu *et al.*, 1999; Bermejo *et al.*, 2003), as fluorescence probes in clinical and immunological analysis (Kronick & Grossman, 1983; Sekar & Chandramohan, 2008) and as therapeutic reagents (Sekar & Chandramohan, 2008) with antioxidant (Benedetti *et al.*, 2004), antitumour (Cai *et al.*, 1995), hepatoprotective (Nagaoka *et al.*, 2005), anti-inflammatory and antihyperalgesic (Shih *et al.*, 2009) activities. Recent studies have indicated that PBPs may have potential applications in memory enhancement and artificial neuron networks *etc.* (Burrows *et al.*, 2009; Womick & Moran, 2009).

The general process for PBP extraction consists of three steps: cell disruption, primary isolation and column chromatographic purification (Sun *et al.*, 2009). However, the product quality and yield were found to vary when using materials from different origins even if routine procedures were carried out (Padgett & Krogmann, 1987; Wang, 2002; Wang *et al.*, 2002; Sekar & Chandramohan, 2008; Ranjitha & Kaushik, 2005). For example, hydrophobic chromatography, which is effective for the purification of PBPs from *Gracilaria verrucosa*, *Palmaria palmate* and *Porphyra haitanensis*, proved to be unsuitable for those from *Porphyra yezoensis* (Niu *et al.*, 2010). Since the first structure of PC was reported in 1991 (Duerring *et al.*, 1991), structures of various PBPs from different algae have been deposited in the PDB. PBPs are primarily composed of α- and β-polypeptides

Table 1
Data collection and statistics.

Values in parentheses are for the highest resolution shell.

	PE	PC
Beamline	SSRF BL17U	SSRF BL17U
Wavelength (Å)	0.9793	0.9788
Detector	MAR 225 CCD	MAR 225 CCD
No. of crystals	1	1
Distance to detector (mm)	220	220
Oscillation range per frame (°)	1.0	1.0
Total No. of images	360	360
Exposure time (s)	2	2
Temperature (K)	100	100
Resolution (Å)	50.00–2.07 (2.14–2.07)	50.00–2.70 (2.80–2.70)
Mosaicity (°)	0.18–0.33	0.42–0.77
Space group	$R3$ (hexagonal indexing)	$P2_1$
Unit-cell parameters (Å, °)	a = 187.7, b = 187.7, c = 59.7	a = 103.2, b = 123.0, c = 184.2, β = 89.3
Volume of unit cell (Å^3)	1821465	2305213
No. of ($\alpha\beta$) heterodimers per asymmetric unit	2	12
Matthews coefficient (Å^3 Da^{-1})	2.8	2.7
Data completeness (%)	99.7 (97.4)	100.0 (100.0)
$R_{\rm merge}$† (%)	14.4 (72.7)	12.7 (76.7)
$\langle I/\sigma(I)\rangle$	12.2 (1.7)	14.4 (2.7)
Multiplicity	5.5 (3.7)	6.4 (6.4)
No. of reflections collected	261871	806597
No. of unique reflections	47618 (4645)	126698 (12653)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of the intensity of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of reflection hkl.

and readily assembly into a disc shape bearing covalently attached open-chain tetrapyrroles known as phycobilins (Liu, Chen, Zhang & Zhou, 2005).

P. yezoensis is one of the major cultural seaweeds in China. In this study, we carried out a large-scale preparation of PBPs from *P. yezoensis* with high purity and implemented preliminary X-ray crystallographic studies of the PBPs that were obtained. The purification protocol developed in this paper will pave the way for the utilization of *P. yezoensis*.

2. Materials and methods

P. yezoensis seaweed was collected from an area of sea in Lvsi, Jiangsu Province, People's Republic of China. The algae were washed with fresh water and then stored at 253 K for future use. Hydroxyapatite was prepared and pre-equilibrated according to Siegelman & Kycia (1978).

All reagents used for purification were purchased from Sinopharm. Reagents for crystallization optimization were supplied by Sigma–Aldrich.

2.1. Protein purification

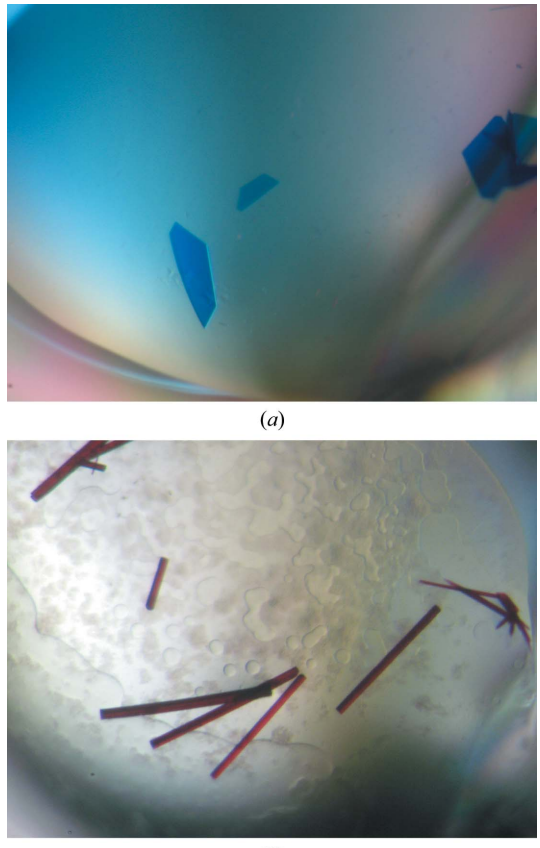
2 kg of *P. yezoensis* was crushed in 50 m*M* phosphate buffer pH 6.8 using a 4 l homogenizer (Waring Co., USA). A cell-free extract was prepared by centrifugation (Supra centrifuge, Hanil Science Corporation, Incheon, Republic of Korea) at 10 000*g* for 20 min. The supernatants were stepwise co-precipitated with ammonium sulfate at different concentrations (20, 50, 10 and 40%) to remove impurities. The final precipitant was then dissolved in 50 m*M* phosphate buffer, desalted with Sephadex G-25 (GE Healthcare Life Sciences, USA) and further purified on a home-made hydroxyapatite (HAP) column by gradient elution using a series of phosphate buffers (pH 6.8) with different concentrations. The eluates containing PE and PC were collected. The whole procedure was monitored by protein absorption using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech Inc., Piscataway, New Jersey, USA). Finally, PE was desalted again and repurified with pre-equilibrated HAP followed by native PAGE analysis using EPS601 electrophoresis equipment (Amersham Biosciences Corporation, Corston, England).

2.2. Spectroscopic assay

It is known that the absorption at 280 nm corresponds to the total protein in the solution and that the absorbances at 565 and 615 nm correspond to the concentrations of PE and PC, respectively (Liu, Chen, Zhang, Zhang *et al.*, 2005). Therefore, the purity of PE and PC can be monitored by the A_{565}/A_{280} and A_{615}/A_{280} ratios, respectively.

2.3. Crystallization

Initial crystallization trials were performed using the sitting-drop vapour-diffusion method at 293 and 277 K with commercially available kits from Hampton Research, Emerald BioSystems, Qiagen and XtalQuest. PE and PC crystallized in drops produced by mixing 1 µl 2 mg ml^{-1} protein in PBS buffer (1 m*M* phosphate buffer pH 6.8) and 1 µl reservoir solution. Initial crystals of PE and PC were detected in condition No. 29 of the PEGs II Suite (Qiagen) and condition C5 of Wizard III (Emerald BioSystems), respectively. After fine-tuning the crystallization conditions, PC crystals suitable for X-ray diffraction were grown from a reservoir solution consisting of 0.2 *M* ammonium chloride and 20%(*w*/*v*) PEG 3350 at 293 K (Fig. 1*a*). The PE crystals used for data collection were grown under the conditions 0.1 *M* HEPES pH 7.5, 0.1 *M* sodium acetate, 15% PEG 4000 at 277 K



(*a*)

(*b*)

Figure 1
Crystals of PC (*a*) and PE (*b*). The maximum dimensions of the PC crystals are about 0.12 × 0.04 × 0.01 mm and those of the PE crystals are about 0.08 × 0.02 × 0.02 mm.

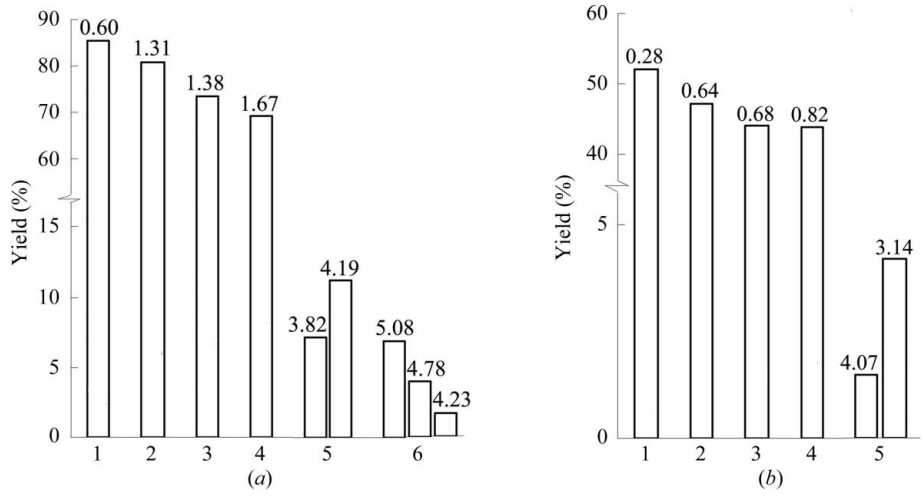


Figure 2
Yield and purity analysis of PE (*a*) and PC (*b*) during the purification steps. Lanes 1–4, co-precipitation with ammonium sulfate at concentrations of 20, 50, 10 and 40% sequentially; lane 5, eluate after the first HAP chromatography; lane 6, eluate after the second HAP chromatography. The bars represent the yield of the target protein after every purification step. The elates after HAP chromatography were carefully collected in different purity grades. The numbers on the bars indicate the corresponding purity of PE and PC as monitored by the A_{565}/A_{280} and A_{615}/A_{280} ratios, respectively.

(Fig. 1*b*). The crystals of both proteins usually appeared within 1 d and grew to full size in 4–5 weeks.

2.4. Data collection

All crystals were mounted in nylon loops and flash-frozen in liquid nitrogen prior to data collection on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF; People's Republic of China). The cryoprotectant for the PC crystals consisted of 0.14 *M* ammonium chloride, 17.5% PEG 3350 and 30% ethylene glycol, while the PE crystals were cryoprotected with Paratone-N oil (Hampton Research). The data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The data statistics for PE and PC are listed in Table 1.

3. Results and discussion

3.1. Preparation of PBPs from *P. yezoensis*

A crude fraction containing approximately 17.26 g PE and 10.81 g PC was obtained from homogenization of 2 kg *P. yezoensis*. After a four-step co-precipitation with ammonium sulfate, the purities of the two target proteins, indicated by the A_{565}/A_{280} and A_{615}/A_{280} ratios, kept increasing and finally reached 1.67 and 0.82, corresponding to 13.89 and 8.77 g PE and PC, respectively. The purity was improved remarkably using HAP chromatography, with yields of 0.184% and 0.057% (Fig. 2). Moreover, higher purity was easily achieved when HAP chromatography was implemented again. For example, the purity of PE was above 4.5 with a yield of 0.109% when the purification procedure using HAP chromatography was repeated. According to native PAGE analysis, the purified PE and PC existed homogeneously in solution with an approximate molecular weight of ~120 kDa, coresponding to an $(\alpha\beta)_3$ heterohexamer (Fig. 3).

Although the extraction and purification of PBPs has been extensively studied (Padgett & Krogmann, 1987; Wang, 2002; Wang *et al.*, 2002; Niu *et al.*, 2007), the procedures used for other species of algae did not work well when applied to proteins from *P. yezoensis*. The most recent protocol involved the application of hydrophobic chromatography. For example, 28 g *P. yezoensis* was homogenized and applied onto a phenyl Sepharose column to obtain 27 mg PE with a purity of 2.0–2.5 (yield of 0.096%), followed by purification on a DEAE-Sepharose column to reach a purity of 4.5 with a yield of 0.082% (Niu *et al.*, 2010). The improved approach described in this paper yielded PBPs in higher quality and quantity, which greatly decreased the purification time and cost.

3.2. Self-rotation function analysis

The crystals of PE belonged to the primary rhombohedral space group *R*3, with unit-cell parameters (in the equivalent hexagonal setting) $a = b = 187.7$, $c = 59.7$ Å. Matthews coefficient analysis (Matthews *et al.*, 1968; Kantardjieff & Rupp, 2003) suggested that there are two $(\alpha\beta)$ heterodimers in the asymmetric unit ($V_M = 2.81$ Å^3 Da^{-1}), corresponding to a solvent content of 56%. A self-rotation function was calculated using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP*4 suite (Winn *et al.*, 2011), which clearly showed noncrystallographic symmetry (NCS). The peaks in the $\chi = 180^\circ$ section (Fig. 4*a*) indicate the NCS twofold axes, which

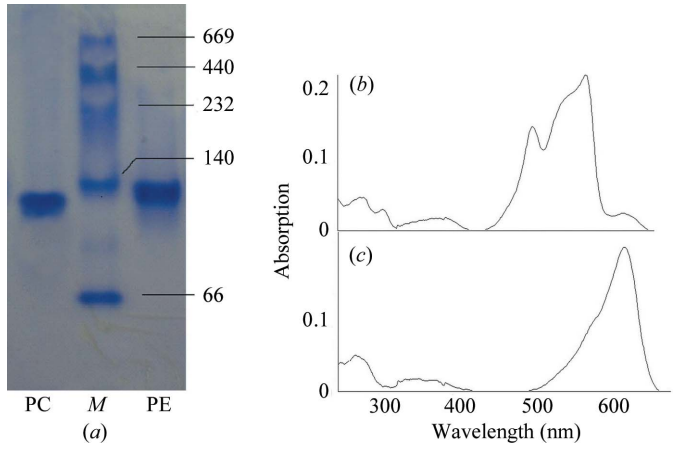
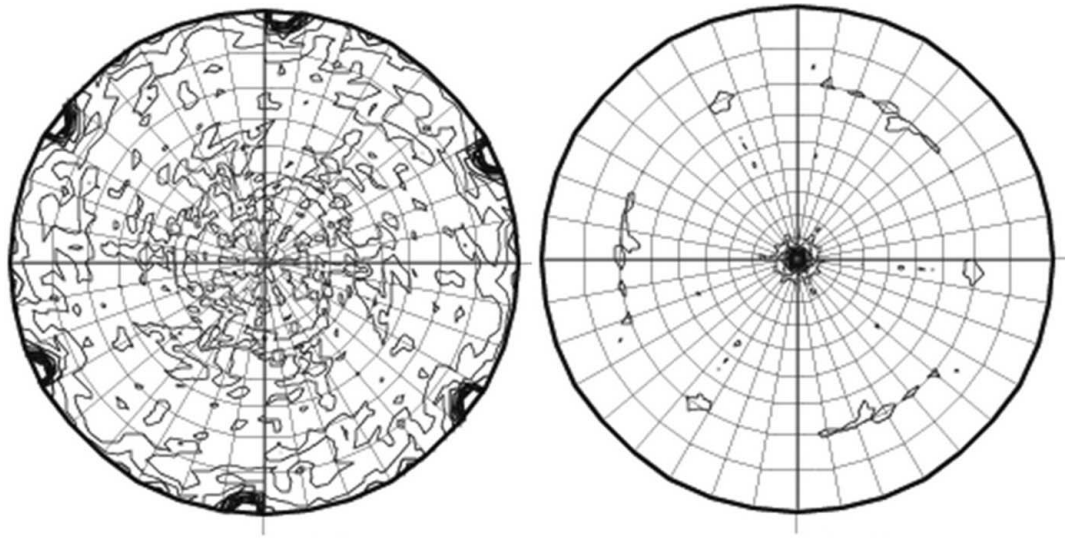


Figure 3
Native PAGE and spectral analysis of PE and PC. (*a*) Native PAGE analysis on a 5% separating and stacking gel. The results shown indicate that PC and PE exist as heterohexamers in solution. Lane *M* contains molecular-weight markers (labelled in kDa). (*b*) and (*c*) show the characteristic absorption of PE and PC from *P. yezoensis* after purification by HAP chromatography.

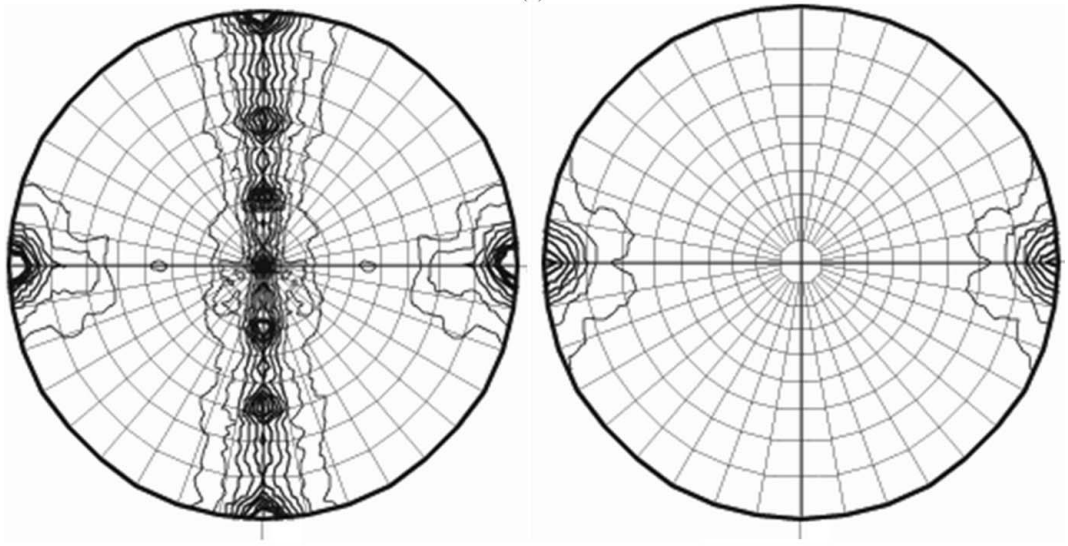
are regularly repeated owing to the crystallographic threefold symmetry. A unique NCS twofold axis exists in the *xy* plane at 55° from the *x* axis, which is perpendicular to the crystallographic threefold symmetry axis shown in the $\chi = 120°$ section. A preliminary attempt to solve the structure was carried out using molecular replacement with the *Phaser* program (McCoy *et al.*, 2007) and the search model was composed of chains *A* and *B* from the published structure of PE from *Gracilaria chilensis* (PDB entry 1eyx; Contreras-Martel *et al.*, 2001). An unambiguous solution was obtained and rigid-body refinement was subsequently performed using *REFMAC* (Murshudov *et al.*, 2011) with a final *R* factor of 0.30 and $R_{\rm free}$ of 0.28.

The crystals of PC belonged to space group $P2_1$ and the Matthews coefficient (Matthews *et al.*, 1968; Kantardjieff & Rupp, 2003) suggested that 10–19 ($\alpha\beta$) heterodimers in an asymmetric unit would give a reasonable $V_{\rm M}$ (1.73–3.28 Å^3 Da^{-1}). Therefore, a self-rotation function was calculated as described above. The results are shown in Fig. 4(*b*). The two peaks at $\varphi = \pm 90°$ and $\psi = 90°$ in the $\chi = 180°$ section represent a crystallographic twofold symmetry axis. The presence of strong peaks in the $\chi = 120°$ section indicate that there is an NCS threefold axis which is superimposed with the crystallographic twofold axis. The peak with $\varphi = 0$, $\psi = 0$, $\chi = 180°$ represents a unique NCS twofold axis perpendicular to the crystallographic twofold axis and the NCS threefold axis and their combination generates a set of six peaks with $\varphi = 0°$ and $\chi = 180°$. The crystallographic twofold axis combines with the twofold and threefold NCS axes to give 12 ($\alpha\beta$) heterodimers in the asymmetric unit.

Initial attempts to solve the PC structure by molecular replacement implemented in *Phaser* (McCoy *et al.*, 2007), *MOLREP* (Vagin & Teplyakov, 2010) and *AMoRe* (Navaza, 1994) within the *CCP*4 suite (Winn *et al.*, 2011) using any of the known $\alpha\beta$ heterodimers (PDB entry 2bv8 chain *A* and chain *B*; Contreras-Martel *et al.*, 2007), $\alpha_2\beta_2$ heterotetramers (PDB entry 1eyx; Contreras-Martel *et al.*, 2001) or $\alpha_6\beta_6$ heterododecamers (PDB entry 2bv8; Contreras-Martel *et al.*, 2007) as the search model did not succeed. A successful solution was obtained using the automatic molecular-replacement program *BALBES* (Long *et al.*, 2008; Fig. 5*a*). The resulting model was composed of two toroids with different numbers of subunits: one was an $\alpha_6\beta_6$ heterododecamer and the other was an $\alpha_4\beta_4$ heterooctamer.



(*a*)



(*b*)

Figure 4
Self-rotation functions of PE (*a*) and PC (*b*). The maps were calculated using the program *MOLREP*. The maps of PE and PC are shown with polar angles of $\chi = 120°$ (right) and 180° (left).

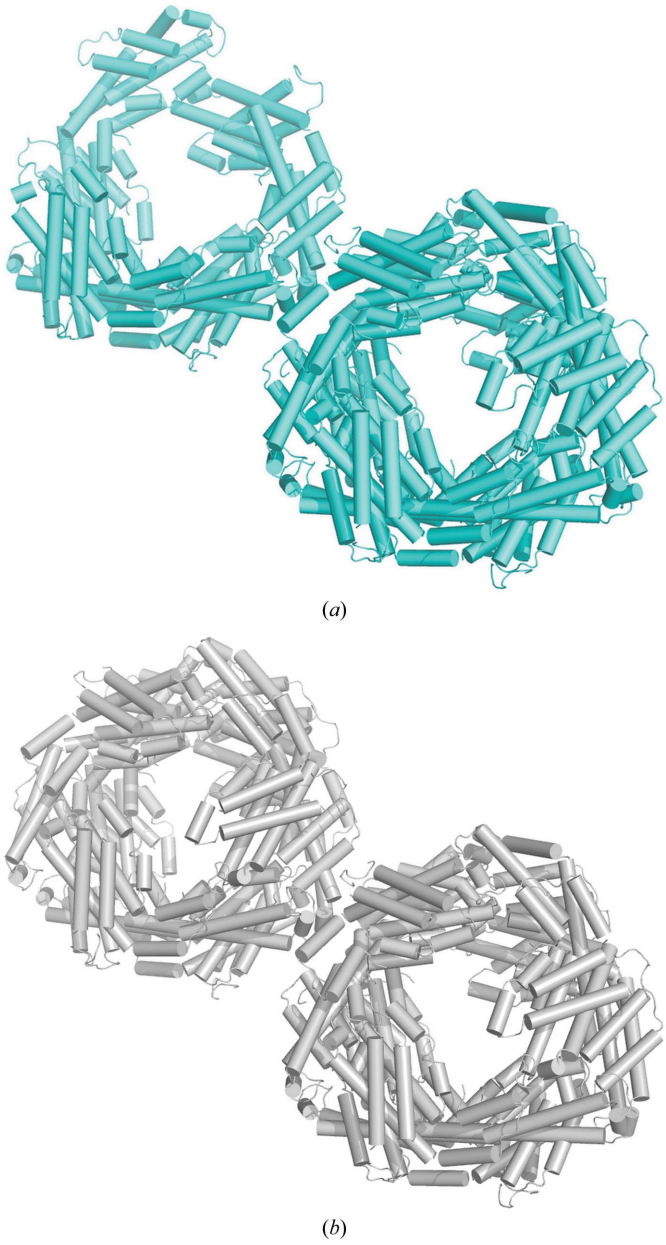


Figure 5
Presentation of the partial model of PC (*a*) automatically built using *BALBES* (Long *et al.*, 2008) and the complete model of PC (*b*). The model of PC was composed of two toroids containing one heterododecamer per toroid.

Furthermore, we found sufficient positive density near the $\alpha_4\beta_4$ heterooctamer to accommodate the missing $\alpha_2\beta_2$ part. Therefore, the structure of the heterododecamer was transformed onto the heterooctamer according to the NCS twofold axis and the missing subunits of the latter were easily patched. Finally, the whole model contained two $\alpha_6\beta_6$ heterododecamer toroids associated by an NCS twofold axis (Fig. 5*b*), which agrees with the solution from the self-rotation function. The assembly of PC described above corresponds to a V_M of 2.73 Å^3 Da^{-1} with a solvent content of 55% based on Matthews coefficient calculations (Matthews *et al.*, 1968; Kantardjieff & Rupp, 2003).

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