**INTRODUCTION**

Phycobiliproteins are colored and highly fluorescent proteins that form the phycobilisome of algae, cyanobacteria, and cryptophyta. These proteins have been used as fluorescent markers (Patel, Mishra, Pawar, & Ghosh, 2005) and also for photodynamic therapy of cancer for their antioxidant, anti-inflammatory, and antitumor properties (Li et al., 2019; Pagels, Guedes, Amaro, Kijjoa, & Vasconcelos, 2019).

Allophycocyanin (APC) is the main component of the phycobilisome core. APC is a heterodimer of αβ subunits. Each subunit contains a phycocyanobilin molecule synthesized by heme oxygenase gene: nAPC, native allophycocyanin of *Agarophyton chilensis*; PB, phycobilin; pcyA, phycocyanobilin oxidoreductase gene; rAPC, recombinant allophycocyanin of *Agarophyton chilensis*.

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**Abstract**

Phycobiliproteins (PBPs) are colored fluorescent proteins present in cyanobacteria, red alga, and cryptophyta. These proteins have many potential uses in biotechnology going from food colorants to medical applications. Allophycocyanin, the simplest PBP, is a heterodimer of αβ subunits that oligomerizes as a trimer (αβ)3. Each subunit contains a phycocyanobilin, bound to a cysteine residue, which is responsible for its spectroscopic properties. In this article, we are reporting the expression of recombinant allophycocyanin (rAPC) from the eukaryotic red algae *Agarophyton chilensis* in *Escherichia coli*, using prokaryotic accessory enzymes to obtain a fully functional rAPC. Three duet vectors were used to include coding sequences of α and β subunits from *A. chilensis* and accessorial enzymes (heterodimeric lyase cpcS/U, heme oxygenase 1, phycocyanobilin oxidoreductase) from cyanobacteria *Arthrospira maxima*. rAPC was purified using several chromatographic steps. The characterization of the pure rAPC indicates very similar spectroscopic properties, λ\text{max}^\text{Abs}, λ\text{max}^\text{Em}, fluorescence lifetime, and chromophorylation degree, with native allophycocyanin (nAPC) from *A. chilensis*. This method, to produce high-quality recombinant allophycocyanin, can be used to express and characterize other macroalga phycobiliproteins, to be used for biotechnological or biomedical purposes.

**KEYWORDS**

*Agarophyton chilensis*, functional characterization, recombinant allophycocyanin, structural characterization
bound to cysteine 82 by a heterodimeric lyase S/U (Schluchter, 2010). This pathway has been reported only for cyanobacteria. The subunits oligomerize as a trimers (αβ)3. The trimeric form of APC is the biologically functional state. This oligomeric state is necessary to provide the specific conformation and relative location of the chromophores to present the typical absorption and emission spectra of APC with λmax(Em) at 651 nm and λmax(Abs) at 660 nm (MacColl, 2004; Samsonoff & MacColl, 2001). For its spectroscopic characteristic, purified APC is an important candidate for the biotechnological, pharmaceutical, cosmeceutical (Li et al., 2019; Pagels et al., 2019), and food industry (Dumay, Moroançais, Munier, Le Guillard, & Flurence, 2014). The recovery of allophycocyanin and other phycobiliproteins from natural sources requires large-scale cultures of cyanobacteria or considerable amount of eukaryotic algae to be processed. Nevertheless, the production of recombinant phycobiliproteins in E. coli would reduce the costs and time required to obtain them with the necessary quality for biotechnological purposes. In the last decade, different protocols to obtain recombinant phycobiliproteins have been published in order to obtain molecular species with properties similar to the proteins purified from native organisms (Biswas, 2010; Liu et al., 2010). The production of a phycocyanin subunits with λmax(Em) at 625 nm and λmax(Abs) at 641 nm (Tooley & Glazer, 2002), subunits of αAPC (Hu, Lee, Lin, Chiuhe, & Lyu, 2006; Liu et al., 2009), βAPC subunits of Synechocystis sp PCC6803 with λmax(Abs) at 611 nm and λmax(Em) at 642 nm (Chen, Lin, Li, Jiang, & Qin, 2013) are examples of these attempts. It has been reported also the obtaining of trimeric rAPC of Synechocystis sp PCC6803 in E.coli, by using multiple duet vectors (Liu et al., 2010) as well as from Synechococcus in E. coli (Biswas, 2010). These vectors contained the sequences for the subunits α and β of the phycobiliproteins, the enzymes to produce phycocyanobilin and the subunits of a heterodimeric lyase for the covalent binding of the chromophore to the corresponding cysteine. It has been reported that the isolated heterodimer (αβ) of a rAPC of Synechocystis sp PCC 6803 obtained in E.coli has an absorption maximum at 615 nm, but when rAPC recovers its trimeric state (αβ)3, also recovers its absorption maximum at 650 nm (Liu et al., 2010). There is not enough information on the metabolic pathway for the synthesis and binding of the phycobilin (PB) to the holo-APC in eukaryotic red algae. The whole genome of P. cruentum (Bhattcharava et al., 2013) and the plastid genome and transcriptome of A. chilensis (Hagopian, Silva Reis, & Kitayima, 2004; Vorpal et al., 2017) have been reported, but it was not possible to find the sequences of the enzymes of the pathway. In this article, we modify the methodology by using heterologous enzymes, to improve the chromophorylation of the trimers (αβ)3 we used three duet expression vectors and a His-tag only in the β subunits to avoid steric hindrance. Our group previously had studied APC of Gracilaria chilensis (Dagnino-Leone, 2017), from now on called Agarophyton chilensis (Le, Fredericq, Norris, Gurgel, & Schmidt, 2018). According to this information, native APC (nAPC) present in A. chilensis is trimeric. In eukaryotic red algae, APC is extracted in lower amount than the other phycobiliproteins because, as part of the core, it is associated to membranes through the linker core-membrane (Li et al., 2016; Tang et al., 2015). For biotechnological purposes, it is necessary that the recombinant APC (rAPC) be in a single oligomerization state as a trimer, highly chromophorilated, with the correct spectroscopic properties, and with a high yield after the purification process. In this article, we present an approach to obtain a trimeric allophycocyanin from a eukaryotic macroalgae in a prokaryotic system. To do that, three duet expression vectors were used, which contains coding sequences of A. chilensis allophycocyanin α and β subunits, and enzymes to obtain holo-APC (heterodimeric lyase S/U, hemoxigenase 1, phycocyanobilin oxido reductase) from the cyanobacteria Arthospira maxima. The expression was accomplished, and the protein rAPC was purified and compared with native allophycocyanin from A. chilensis (nAPC) by absorption and emission spectroscopy, circular dichroism, and molecular sieve chromatography. This method leads to the production of rAPC with very similar properties to nAPC, which can be used for biotechnological purposes.

2 | MATERIALS AND METHODS

2.1 | DNA extraction and PCR conditions

The coding sequences of the six genes needed were amplified by PCR using KAPA HiFi polymerase. apcA and apcB were obtained using A. chilensis DNA as template. The purification of DNA from A. chilensis was performed according to the literature (Ramakrishnan, Fathima, & Ramya, 2017). PCR were performed using the following primers: apcA: 5ʹ-CCATGGGTATTATTACTAAATCAATCGTTA-3ʹ;
apcB: 5ʹ-GGATCCTTTCATGTTCATTCC-3ʹ. In bold are shown the restriction sites for subcloning.

Expression vectors were constructed as follows: apcB and apcU in their cloning vectors were digested with BamHI and Sacl for apcB

2.2 | Construction of the expression vectors

Expression vectors were constructed as follows: apcB and apcU in their cloning vectors were digested with BamHI and Sacl for apcB
and Ndel and XhoI for cpcU and then ligated at the cloning site 1 and 2 in pETDuet-1 vector ( novagen), respectively. apcA and cpcS were digested with Ncol and BamHI, and cpc5 with Ndel and XhoI to be then ligated in the cloning site 1 and 2 of pCDFDuet-1 vector (Novagen), respectively. Finally, pcyA and ho1 were digested with Ncol and BamHI and ho1 with Ndel and xhol, they were cloned in pRSFDuet-1 cloning sites 1 and 2 of, respectively. The expression vectors were sequenced at the Department of Ecology from Pontificia Universidad Católica de Chile and analyzed with Bioedit software to confirm the absence of mutations.

2.3 | In vivo heterologous expression of recombinant Allophycocyanin (rAPC)

30 ng of each expression vectors, pCDF-apcA-cpcS, pET-apcB-cpcU y pRSF-pcyA-ho1, were co-transformed in electrocompetent E.coli BL21 (DE3). A Bio-Rad Micropulsar electroporator was used with a 2.5 kV transformation protocol for five milliseconds using a 2-mm gap cuvette. The transformed bacteria were grown in LB-Agar plates supplemented with 15 mg/L kanamycin, 25 mg/L streptomycin, and 50 mg/L ampicillin. The plates were incubated for 16 hr at 37°C. Starter culture (SC) was prepared with one colony in 20 ml TB (Terrific broth) under ampicillin. The plates were incubated for 16 hr at 37°C. The expression vectors were sequenced at the Department of Ecology from Pontificia Universidad Católica de Chile and analyzed with Bioedit software to confirm the absence of mutations.

2.4 | Purification of rAPC

The proteins were precipitated with ammonium sulfate (60% saturation) at 4°C, during 16 hr; the sample was then centrifuged for 20 min at 15,000g. The protein pellet was dissolved in 5 ml of Buffer A (50 mM K₂HPO₄/KH₂PO₄ pH 7, 150 mM KCl, 10% glycerol, and 5 mM dithiothreitol) in presence of proteases inhibitors (Complete mini EDTA free, Roche) and then lysed by sonication in ice bath, for 5 min (10 s sonication, 30 s pause). The total lysate was centrifuged at 15,000 x g for 20 min. The supernatant was used as input for the following purification steps.

2.5 | Characterization of rAPC

Purified proteins were analyzed by native PAGE(not shown), de-naturating SDS-PAGE and Western blot, using the His-tag on the β subunit for detection. Absorption and emission spectra of rAPC and nAPC were recorded in a Jasco V-650 spectrometer and a SHIMADZU RF-5301 PC spectrofluorimeter. The samples with a ratio A651/A280 >4 and with emission at 660 nm upon excitation at 651 nm were used for the oligomeric characterization.

The oligomerization state of rAPC and nAPC was determined in a molecular sieve chromatography with a Superdex 200 HiLoad 16/60 column (Amersham). The oligomer size was estimated using a calibration curve of molecular standard. Three replica of MW standards from 1,750–670,000 Da (Gel filtration standard, Bio-Rad) were performed.

Circular dichroism spectra (190–250 nm) of nAPC and rAPC were recorded in a Jasco J-1500 spectropolarimeter with PM-539 detector and Peltier PTC-517 at the Centro de Estudios para el Desarrollo de la Química (CEPEDEQ), Universidad de Chile. For measurement of the thermal stability, the circular dichroism signal following the changes in ellipticity at 222 nm between 25 and 75°C (0.5°C/min) was recorded. The protein concentrations were 0.1 mg/ml in both experiments.

The fluorescence lifetime was measured in a Fluoro Time 200 (PicoQuant Inc) fluorimeter, with diode Lasers and LEDs as excitation source and an ultrafast MCP detector at Molecular Physics Lab, Christian Texas University. The measurements were performed with an angle of 54.7°, and the data were adjusted with software FluorFit4, for multicomponent systems (t) = ∫(A(t)·e⁻⁄τ·dt). The flavoprotein degree was determined for nAPC and rAPC based on (Biswas, 2010; Glazer, 1988) in two independent experiments.

3 | RESULTS

3.1 | Construction of the expression system for rAPC

The sequences of the genes heme oxygenase 1 (ho1), phycocyanobilin oxidoreductase (pcyA), lyase S subunit (cpcS), and lyase U subunit (cpcU) of A. chilensis have not been identified yet. The similitude among proteins involved in the phycobilisome system, for example APC of A. chilensis and A. maxima (82% and 83% identity for the a and β subunit, respectively) or heme oxygenase 1 of Gracilaria sp and
DAGNINO-LEONE E et al. suggested that the metabolic pathways for their synthesis could be also similar although \( apcA \) and \( apcB \) genes are located at the chloroplast in \( A. \) \textit{chilensis} and in the genomic DNA in \( A. \) \textit{maxima}. It was not possible to find sequences reported for eukaryotic phycocyanobilin oxide reductase or lyases in public databases. The genome of \( A. \) \textit{maxima} (Xu et al., 2016) was available, so as a thoughtful alternative, the three necessary enzymes from \( A. \) \textit{maxima} were used. All the expression vectors show no mutations in the coding sequences.

### 3.2 Purification of rAPC

The purification of rAPC was performed from a pellet of 3.94 g of recombinant bacteria. Fractions of the semipurified rAPC from the ionic exchange chromatography account for 7 mg/L of bacterial culture, following its absorption at 651 nm. The following chromatography step with IMAC was also followed at 651 nm; two turquoise fractions were identified, the fraction retained in the column and the flow-through fraction. Both protein fractions were analyzed. Even though both fractions have a \( \lambda_{\text{max}} \) close to 651 nm, only the retained fraction showed an identical spectrum with nAPC. This fraction accounts for 0.124 mg/L of bacterial culture. The characterization of this fraction is presented below.

### 3.3 Characterization of rAPC

The purified rAPC showed the characteristic turquoise color. Its absorption and emission spectra are shown in Figure 1a. For both, nAPC and rAPC, their spectroscopic characteristics are very similar, with an absorption maximum at 651 nm, a shoulder at 620 nm and an emission maximum at 661 nm. Figure 1b shows the electrophoretic characterization of the purified protein (A651 nm/A280 nm >4). In SDS-PAGE (Figure 1b), two bands at the estimated size of 17–19 kDa corresponding to \( \alpha \) and \( \beta \) subunit of APC can be observed. The sample of rAPC presents a band of a slightly higher size. The Western blot shows signal only in the rAPC sample (Figure 1b).

The oligomerization state of nAPC and rAPC, determined by size exclusion chromatography, shows an estimated size of each protein, corresponding to MW of 112 kDa and 150 kDa, respectively (Figure 2).
The circular dichroism spectra for rAPC and nAPC are very similar, showing the profile of predominant α helices as secondary structures, with minima at λ 208 and 222 nm and a maximum at 190 nm (Figure 3). The temperature of melting (Tm) determined by circular dichroism shows a Tm for nAPC of 64°C and a Tm of 56°C for the rAPC (Figure 4).

The fluorescent lifetime (τ) for each protein was measured, and the values are τ_{nAPC} = 1.65 ns and τ_{rAPC} = 1.64 ns indicating a completely functional protein.

The degree of chromophorylation was close to 50%, (nAPC: 52%, rAPC: 57%). Figure A1 shows the spectra for the denatured nAPC and rAPC that were used for the calculations. rAPC and nAPC showed similar degree of chromophorylation. Table 1 shows a comparison of the characteristics of rAPC and nAPC.

4 DISCUSSION

Phycobiliproteins have an enormous biotechnological potential, their applications go from food colorant to biomedical uses because they possess antioxidant and antitumorous properties. They are also used in photodynamic therapies as fluorescent probes because their spectroscopic characteristics. Allophycocyanin is the most simple phycobiliprotein, it possesses only one phycocyanobilin molecule per subunit attached to the peptide backbone, and its native functional oligomer is a trimer (De Marsac, 2003; MacColl, 1998, 2004). Phycobiliproteins from red algae are much less studied than cyanobacterial. An exception is the red microalgae *Porphyridium cruentum* (Bermejo, Ruiz, & Acien, 2007; Bermejo, Talavera, & Alvarez-Pez, 2001; Nagy, Bishop, Klotz, Glazer, & Rapoport, 1985), but for eukaryotic macro algae the studies of phycobiliproteins are only a few (Galland-Irmouli et al., 2000; Lüder, Knoetzel, & Wiencke, 2001). Allophycocyanin is the less abundant in eukaryotic phycobilisomes (Glazer, 1988) that presents a problem for the study and biophysical characterization of this protein.

In this work, we have obtained an eukaryotic recombinant allophycocyanin, rAPC, from *A. chilensis* using prokaryotic accessory enzymes (heterodimeric lyase S/U, hemeoxigenase 1 and phycocyanobilin oxido reductase) from *A. maxima* in *E.coli* with their spectroscopic and biochemical properties comparable to the purified native allophycocyanin. The expression system designed is based on the literature but with changes in order to obtain a fully functional protein. We designed an expression system to produce equivalent number of copies for α and β subunits. This was confirmed by the SDS-PAGE (Figure 1) in which the intensity of the bands stained with Coomassie blue was also similar. The expression system was also selected to obtain a higher number of copies for the enzymes responsible for the synthesis and binding of the chromophores by using the vector pRSF which has a replication time 5 times faster than the vector that contained the α and β subunits of rAPC. This is important because the objective was to obtain a high degree of chromophorylation. Another important difference was the addition of a His-tag only to the N-terminal of β subunits, instead of the N-terminal of α subunits (Biswas, 2010) or to the N-terminal of both subunits as described in Liu et al., (2010). The structural information we had on APC (Dagnino-Leone, 2017) was used in order to have less effect in the oligomerization state as a trimer. Figure 5 shows the molecular model of *A. chilensis* rAPC trimer, the position of the His-tag is indicated and it was designed to eliminate the steric hindrance that could be produced in the organization of the subunits, and it would account for the trimeric oligomerization state obtained with this protocol. In previous reports (Biswas, 2010), a different combination of three duet vectors were used; in (Liu et al., 2010) the authors included the six necessary genes in two expression vectors, inserting...
cpcS and cpcU in tandem in the cloning site 2 of pCDF vector and also ho1 and pcyA in the cloning site 2 of vector pRSF. In both cases, lower chromophorylation efficiency (27%) was reported, compared with the expected for a native protein. In our case, we reached similar chromophorylation degree for the recombinant protein in comparison with the native one using the same methodology.

rAPC showed the characteristic spectrum of native APC, an absorption maximum at $\lambda = 651$ nm with a shoulder at $\lambda = 620$ nm, an emission maximum at $\lambda = 660$ nm, and very similar to those reported in literature (MacColl, 1983, 2004; MacColl, Csatorday, & Berns, 1981) for cyanobacterial APC.

Experimental information about the oligomeric state of nAPC in other red alga was not available even though in (Murakami, Mimuro, Ohki, & Fujita, 1981) the authors reported a trimeric state for the functional APC purified from Anabaena cylindrica. Our results from molecular sieve chromatography point to a trimeric state for nAPC from A. chilensis and for rAPC. This result agrees with the models obtained by X-ray crystallography which report $(\alpha\beta)_3$ as the biological unit (Dagnino-Leone, 2017), (Brejc, Ficner, Huber, & Steinbacher, 1995; McGregor, Klartag, David, & Adir, 2008; Murray, Maghlouai, & Barber, 2007; Schmidt, Krasselt, & Reuter, 2006). The circular dichroism spectrum for nAPC as well as for rAPC agrees with the secondary structure reported for the crystallographic structures of APC for Agarophyton chilensis (PDB ID: STIJ; Bhattacharva et al., 2013), and also agrees with other APC structures from cyanobacteria reported at the PDB (Brejc et al., 1995; McGregor et al., 2008; Murakami et al., 1981; Murray et al., 2007), with predominance of helical structures revealed by the two minima at 222 and 208 nm. The deconvolution of the Far-UV CD spectrum of rAPC reveals a 71% of helical content, in complete agreement with the 76% reported for the crystallographic structure of the nAPC considering that the His-tag contributes to the spectra lowering the percentage of helical structures.

The main difference between nAPC and rAPC of A. chilensis is the melting temperature. The value of Tm for nAPC was 64°C and Tm for rAPC was 56°C. This difference could be assigned to the presence of the His-tag used to facilitate the purification of the recombinant protein.

The His-tag is associated at the N-terminal of the A.chilensis allophycocyanin $\beta$ subunit (Sequence added: MGSSHHHHHHHSDP), and there are three in each trimer. Considering that the analysis of the trimer showed a distance between tags of 40 Å; so it is possible that they could clash among them and its mobility would increase along with temperature, this fact would account for a decrease of 8°C in Tm for the rAPC as compared with nAPC. This behavior has been reported before (Khan, Legler, Mease, & Duncan, 2012) for native and recombinant proteins involving His-tag for recognition. The presence of the tags also could explain the differences in the circular dichroism spectrum and the difference in the elution time during

| Characteristic                  | nAPC       | rAPC       |
|--------------------------------|------------|------------|
| $\lambda_{\text{max}}$ Abs     | 651 nm     | 651 nm     |
| $\lambda_{\text{max}}$ Em      | 662 nm     | 662 nm     |
| $\tau$                         | 1.65 ns    | 1.64 ns    |
| Oligomeric state               | $(\alpha\beta)_3$ | $(\alpha\beta)_3$ |
| Tm                             | 64°C       | 56°C       |
| Chromophorylation degree       | 52%        | 57%        |

### TABLE 1
Characteristics of nAPC and rAPC

**FIGURE 5** Molecular model of the structure of rAPC from Agarophyton chilensis. Yellow: $\alpha$ subunits, light blue: $\beta$ subunits, red: the phycocyanobilins. Schematically, the position of the His-tag is shown in green. On the left, frontal view, on the right a lateral view.
the molecular sieve chromatography in which the His-tag should be responsible of the change in the hydrodynamic volume. Molecular models of the rAPC suggest this possibility.

The fluorescence lifetimes (τ) are also identical showing that rAPC and nAPC have similar functional properties. It has been reported that Synechocystis sp. PCC6803 also has also a similar value for τ (Maksimov et al., 2014).

The spectrum of nAPC and rAPC at denaturing conditions (8 M urea, pH 2) and the relationship between the concentrations of phycocyanobilin chromophore allows calculating the degree of chromophorylation of nAPC and rAPC. These values were the same with an estimated value of 52% and 57%. Biswas (2010) report that for monomers, the chromophorylation rate is 40% and for the trimer totally chromophorylated is 6.4%.

To this point, the A. chilensis rAPC showed very similar properties with nAPC, as it is shown on Table 1.

We were able to produce 7 mg/L of recombinant rAPC, but only 0.124 mg/L corresponds to a functional trimeric conformation. Biswas et al. (2010) reported the production of 5 to 12.4 mg of rAPC from Synechococcus sp. strain PCC 7002, but they did not report the amount of functional protein for comparison (Biswas, 2010). More experiments are needed to fine tuning the protein expression of A. chilensis rAPC. Changing temperature and induction time would allow optimize the production of functional A. chilensis rAPC in E. coli.

In summary, we have obtained a recombinant eukaryotic allophycocyanin in its trimeric and functional conformation, by using a cyanobacterial enzymatic accessory system. rAPC has very similar properties with nAPC, and it is completely functional for biotechnological and/or biomedical purposes. In addition, this system would allow the study of the biophysical characteristics of the other subunits with different spectroscopic properties, present in the core of the phycobilisome of Agarophyton chilensis, such as αII and β18. The system also will allow the production of other recombinant phycobiliproteins from other red macroalga for biotechnological purposes.

ACKNOWLEDGMENTS
Doctoral scholarship CONICYT No 21120260 to J.D-L, FONDECYT No 113.0256 and Programa ENLACE 216.037.021-1.0 VRID_UDEC. The authors are grateful to Dr. Victor Castro (FONDEQUIP No EQM140151 CEPEDEQ. Universidad de Chile) for the access to the instrument for circular dichroism studies; to Dr. Zygmund Gryzinski (North Christian University Texas) for the fluorescence lifetime studies; to Dra. Wendy Schulchter, for fruitful advice to JD-L; to Dra. Mariela González (Universidad de Concepción) for providing the Arthrospira maxima strain used in this work; and to Mr Pablo Macaya for his technical assistance.

CONFLICT OF INTEREST
None declared.

AUTHOR CONTRIBUTION
Jorge Dagnino-Leone: Conceptualization; Investigation; Methodology; Writing-original draft. Maximiliano Figueroa: Conceptualization; Investigation; Methodology; Writing-review & editing. Elena Uribe: Methodology; Resources. María Victoria Hinrichs: Investigation; Methodology. Diego Ortiz-López: Formal analysis; Investigation; Methodology. José Martínez-Oyanedel: Conceptualization; Data curation; Formal analysis; Methodology; Supervision; Validation; Writing-original draft; Writing-review & editing. Marta Bunster: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Writing-original draft; Writing-review & editing.

ETHICS STATEMENT
None required.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this published article.

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**FIGURE A1** Spectra of denatured rAPC and nAPC for the determination of the chromophorylation efficiency. *The samples were denatured in PBS 50 mM, 8 M urea pH2 and the spectra were recorded. The method described by Biswas 2010 and Glazer 1988 used the molar extinction coefficient $\varepsilon = 35.4$ mM/cm or cyanobilins in absence of the protein environment (folded), and in this article, the molar extinction coefficient for folded rAPC and nAPC of 14.9 mM/cm was used for the calculations.*