



## Exploring and strengthening the potential of R-phycocyanin from Nori flakes as a food colourant

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# Exploring and strengthening the potential of R-phycoerythrin from Nori flakes as a food colourant

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## 26    **Abstract**

27    This study aimed to purify, characterise and stabilise the natural food colourant, R-phycocyanin (R-  
28    PC), from the red algae *Porphyra* spp. (Nori). We purified R-PC from dried Nori flakes with a high  
29    purity ratio ( $A_{618}/A_{280} \geq 3.4$ ) in native form ( $\alpha$ -helix content 53%). SAXS measurements revealed  
30    that R-PC is trimeric ( $(\alpha\beta)_3$ ) in solution. The thermal denaturation of  $\alpha$ -helix revealed one transition  
31    ( $T_m$  at 52 °C), while the pH stability study showed R-PC is stable in the pH range 4 to 8. The thermal  
32    treatment of R-PC at 60 °C has detrimental and irreversible effects on R-PC colour and antioxidant  
33    capacity (22 % of residual capacity). However, immobilisation of R-PC within calcium alginate beads  
34    completely preserves R-PC colour and mainly retains its antioxidant ability (78 % of residual  
35    capacity). Results give new insights into the stability of R-PC and preservation of its purple colour  
36    and bioactivity by encapsulation in calcium alginate beads.

37    **Keywords:** R-phycocyanin, Purification, Stability, SAXS, Immobilization, Calcium alginate.

## 38 Introduction

39       Alternative proteins have recently gained increasing attention due to substantial  
40 environmental and health impacts compared to meat consumption (Onwezen, Bouwman, Reinders,  
41 & Dagevos, 2021). Algae are a promising source of alternative proteins as they can meet health,  
42 nutritional, environmental and production needs (Diaz et al., 2022). Red macroalgae *Porphyra* spp.,  
43 commonly known as Nori, is a rich source of proteins, vitamins, minerals and antioxidants and has  
44 been used as food for centuries in Asia, and its popularity has now spread to other parts of the world  
45 (Bito, Teng, & Watanabe, 2017; Simovic, Combet, Cirkovic Velickovic, Nikolic, & Minic, 2022).  
46 Consumed as dried Nori flakes, it is frequently used to prepare sushi, soup and cakes (Bito et al.,  
47 2017). The most abundant proteins in *Porphyra* spp. are the phycobiliproteins (PBPs) R-  
48 phycoerythrin (R-PE) and R-phycocyanin (R-PC), contributing to approximately 3 % of the dry mass  
49 of *Porphyra* spp. (Cao, Wang, Wang, & Xu, 2016). Considering that nearly one million tons of  
50 *Porphyra* are produced annually (Baweja, Kumar, Sahoo, & Levine, 2016), R-PC and R-PE have  
51 great potential to be used as alternatives to animal proteins. Besides R-PE and R-PC, *Porphyra* spp.  
52 also contains the third phycobiliprotein allophycocyanin (APC), the minor protein of the PBP family  
53 (Liu et al., 1998). Compared to R-PE, which has been thoroughly studied, much less is known about  
54 the properties of R-PC of *Porphyra* spp.

55       R-phycocyanin is a strongly fluorescent and water-soluble molecule whose primary function  
56 is to transfer excitation energy to reaction centres during photosynthesis (Glazer & Hixson, 1975;  
57 Jiang, Zhang, Chang, & Liang, 2001). The intensive purple colour of R-PC arises from its covalently  
58 attached (*via* thioether bond) linear tetrapyrrole chromophores, phycoerythrobilin (PEB) and  
59 phycocyanobilin (PCB). It is composed of two subunit types forming a trimer ( $\alpha\beta$ )<sub>3</sub> or hexamer ( $\alpha\beta$ )<sub>6</sub>,  
60 with  $\alpha$  subunits of 18–20 kDa and  $\beta$  subunits of 19–21 kDa (Jian et al., 2001). One PCB chromophore  
61 is bound to both  $\alpha$  and  $\beta$  subunits, while one PEB chromophore is only attached to  $\beta$  subunits.

62       R-phycocyanin has potential bioactive properties due to the potent antioxidant,  
63 immunomodulatory and anticancer activities of covalently bound tetrapyrrole chromophores (Guo et  
64 al., 2022; Rashed, Hammad, Eldakak, Khalil, & Osman, 2023). The health benefits of R-PC, its  
65 brilliant fluorescence, vivid colour, and the need to replace synthetic dyes give R-PC great potential  
66 for food fortification and colouring. Nori seaweed represents an essential source of R-PC as natural  
67 food colourant because it can be cultivated in vast amounts, as described above. Hence, tons of R-PC  
68 could be produced annually to colour different foods and beverages. However, in this perspective, R-  
69 PC's limited stability is the major obstacle. Indeed, generally, PBPs have moderate pH stability  
70 (Munier et al., 2014; Yan et al., 2010), and their thermal stability is low: an increase in temperature  
71 above 40 °C induces a substantial decrease in protein stability and colour intensity (Faieta, Neri,  
72 Sacchetti, Di Michele, & Pittia, 2020; Munier et al., 2014; Simovic et al., 2022).

Therefore, developing R-PC as a natural food colourant and alternative protein requires a deeper understanding of its structural and functional properties and improved stability. One promising approach for stabilising R-PC and other PBPs could be encapsulation in solid food matrices, which may decrease the mobility of protein molecules, making them more resistant towards temperature and oxidation processes (Maghraby, El-Shabasy, Ibrahim, & Azzazy, 2023). In this context, alginate immobilisation is a valuable strategy since it is widely used in biotechnology and bioprocessing for the entrapment of living cells, proteins, and other biological molecules (Andersen, Auk-Emblem, & Dornish, 2015). Alginate is a natural polysaccharide derived from brown seaweed and has unique physical and chemical properties that make it ideal for immobilisation. The process involves the formation of calcium-alginate beads, which can be easily manipulated and controlled in terms of size, shape and mechanical stability (Segale, Giovannelli, Mannina, & Pattarino, 2016).

In the present study, we aim to purify, characterise and stabilise the purple protein R-PC from dried Nori flakes for the first time. We used precipitation and chromatography techniques to obtain pure protein. Optical spectroscopy and SAXS measurements conclude that purified protein is a typical R-PC, with  $\alpha$ -helix as the dominant secondary structure and trimeric oligomerisation state in solution. We determined the sequence of R-PC from Nori flakes by mass spectrometry and compared it with phycocyanins from other sources. Further, we employed absorption and far-UV CD spectroscopy to study the effects of temperature and pH on protein stability. Ultimately, we tested if R-PC immobilisation within calcium alginate beads could improve protein thermal stability and preserve its antioxidant capacity.

## 2. Materials and Methods

### 2.1 Materials

Dried Nori flakes (*Porphyra* spp.) were purchased from Fujian Friday Trading Co. LTD (China). Hydroxyapatite (HA) and diethyl aminoethyl (DEAE) Sepharose resins were bought from BioRad (CA, USA) and Sigma (MO, USA), respectively. Sodium alginate used for immobilisation was of food grade, purchased from Health Leads (France). All other chemicals were of analytical reagent grade.

### 2.2 R-PC isolation and purification

R-Phycocyanin was purified according to the previously published procedures with modifications (Boussiba & Richmond, 1979; Simovic et al., 2022; Wang et al., 2014). Nori seaweed leaves were frozen with liquid nitrogen and then pulverised using a mortar and pestle. The powdered sample was then suspended in 20 mM phosphate buffer pH 7.0 (1 g of powdered sample *per* 20 mL of buffer). Extraction of phycobiliproteins was performed overnight at 4 °C with constant stirring.

106 The resulting slurry was centrifuged at 6000 x g for 30 min at 6 °C. Proteins in the supernatant were  
107 precipitated with ammonium sulfate (65 % final concentration). The pellet was dissolved in 2.5 mM  
108 phosphate buffer pH 7.0 and was dialysed against the same buffer.

109 The dialysed solution was applied to the CHT ceramic hydroxyapatite (HA) column,  
110 previously equilibrated with the same buffer. Non-bound proteins (mainly R-PE) were washed away  
111 with the equilibration buffer, while fractions enriched with R-PC were obtained by stepwise elution  
112 protocol employing increasing concentrations of sodium phosphate salt (pH 7.0; **Fig. 1A**). R-PC  
113 containing fraction was applied onto the DEAE Sepharose CL-6B column, previously equilibrated  
114 with 20 mM phosphate buffer pH 7.0. Unbound proteins were flushed with an equilibration buffer.  
115 Bound proteins were eluted stepwise, increasing the ionic strength of NaCl from 0.05 to 0.2 M (**Fig.**  
116 **1B**). The R-PC-rich fractions were pulled, desalted and concentrated. Purified R-PC was stored in 40  
117 % (v/v) glycerol solution at -20 °C. We analysed the obtained fractions by UV-Visible absorption  
118 spectroscopy (**Section 2.3**) and SDS-PAGE under reducing conditions. Fluorescent PBP bands were  
119 first visualised under UV light, followed by staining with Coomassie Brilliant Blue R-250.

## 120 **2.3 Determination of R-PC amino acid sequence by mass spectrometry**

121 Purified R-PC was digested by trypsin, and obtained peptides were analysed by nano Liquid  
122 Chromatography-electron spray ionisation coupled to tandem mass spectrometry (nLC-ESI-MS/MS).  
123 The protein identification was performed using PEAKS Suite X (Bioinformatics Solutions Inc.,  
124 Canada) software. Experimental details and mass spectrometry data analysis are given in  
125 Supplementary Material 1.

## 126 **2.4 UV-Visible absorbance measurements**

127 UV-Visible absorption spectra were recorded on a NanoDrop 2000c spectrophotometer  
128 (Thermo Scientific, USA) in a 1 cm pathlength quartz cell. The spectra were collected in the 250–  
129 750 nm range at room temperature. R-PC concentration was determined using the following equation  
130 (Nikolic, Minic, Macvanin, Stanic-Vucinic, & Velickovic, 2020):

$$131 \quad R - PC \left( \frac{mg}{mL} \right) = 0.154(A_{618} - A_{730}) \quad (1)$$

132 The effects of temperature and pH on visible absorption spectra of R-PC were studied using  
133 a UV-1800 Shimadzu spectrophotometer (Japan). Temperature measurements were performed using  
134 a Peltier element previously described (Simovic et al., 2022), and experimental details are given in  
135 Supplementary Material 1.

136 The pH dependence of R-PC absorption spectra was studied using the same measurement  
137 parameters described above for the thermal stability study. R-PC was incubated in the different

138 buffers (50 mM phosphate, citrate, Tris and glycine) in the pH range of 2–12 for 30 minutes, followed  
139 by the acquisition of absorption spectra at room temperature.

## 140 **2.5 Fluorescence measurements**

141 Fluorescence spectra of 1.8 µg/mL of R-PC at pH 7.0 were recorded with a FluoroMax®-4  
142 spectrofluorometer (HORIBA Scientific, Japan) under thermostable conditions (25 °C), using 5 nm  
143 excitation and 5 nm emission slit widths. The PEB chromophore was excited at 488 and 590 nm, and  
144 the emission spectra were recorded between 520 and 610 nm. Excitation of PCB chromophore was  
145 performed at 590 nm, while the emission was measured in the range 610–680 nm.

146 The spectrofluorimetric oxygen radical absorbance capacity (ORAC) assay was performed  
147 according to Ou et al. (Ou, Hampsch-Woodill, & Prior, 2001). Stock solutions of fluorescein (5 µM)  
148 as the substrate and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 300 mM) as the free  
149 radical generator were made in 75 mM sodium phosphate buffer, pH 7.4. The assay was performed:  
150 50 µL of 100 µg/mL R-PC solution (obtained as described in **section 2.7**) was mixed with 1680 µL  
151 of buffer (75 mM phosphate, pH 7.4) and 20 µL of fluorescein solution. The reaction was initiated  
152 by the addition of 250 µL of AAPH solution. Fluorescence was measured at the excitation wavelength  
153 of 485 nm and an emission wavelength of 511 nm at 37 °C. Bandwidths of slits were set to 2 nm. The  
154 fluorescence was recorded every 20 seconds for 30 min. Measurements of blank samples (**section**  
155 **2.7**) were performed in the same conditions. The blank sample's area under the curve (AUC) was  
156 subtracted from the corresponding AUC of R-PC-containing samples, and results were expressed as  
157 AUC per mg of R-PC. Measurements were performed at least in duplicate, and results were presented  
158 as mean ± standard deviation. For the evaluation of antioxidant capacity, an analysis of variance  
159 (ANOVA) of data was performed and means comparisons were made using the Tukey test.

## 160 **2.6 CD spectroscopy measurements**

161 CD measurements were carried out on a Jasco J-815 spectropolarimeter (Jasco, Japan) under  
162 constant nitrogen flow. All spectra were recorded at 25 °C. For the near-UV and visible region (250–  
163 750 nm), the concentration and pH of R-PC were set to 0.3 g/L and 7.0, respectively. Scan speed and  
164 the number of accumulations were set to 200 nm/min and two, respectively. A cell with an optical  
165 pathlength of 1 cm was used.

166 Far-UV CD spectra of 100 µg/mL R-PC have been recorded in the range 190–260 nm at a  
167 scan speed of 50 nm/min, using a cell with an optical pathlength of 1 mm and with an accumulation  
168 of three scans. Spectra were measured in different buffers in the pH range of 2–12 (**section 2.3**). The  
169 CD data analysis is given in Supplementary Material 1.

170 Thermal unfolding R-PC was performed as previously described (Simovic et al., 2022), and  
171 experimental details and data analysis are given in Supplementary Material 1.

## 172 2.7 SAXS experiments and analysis

173 SAXS spectra were obtained on the SWING beamline at the French synchrotron facility  
174 SOLEIL (St-Aubin, France) (Thureau, Roblin, & Pérez, 2021) using a wavelength of  $\lambda = 0.764 \text{ \AA}$   
175 and a sample-to-detector distance of 3 m. The achievable  $q$ -range was 0.0033 to  $0.531 \text{ \AA}^{-1}$ , where  
176  $q = \frac{4\pi}{\lambda} \sin\theta$  is the modulus of the momentum transfer, and  $2\theta$  is the scattering angle. 40  $\mu\text{L}$  of 5  
177 mg/mL of R-PC at pH 5.7 (20 mM MES containing 150 mM NaCl) and buffer were injected into a  
178 1.5 mm outside diameter quartz capillary thermalised at 15 °C.

179 The SAXS data analysis is given in Supplementary Material 1.

## 180 2.8 Immobilisation of R-PC in sodium alginate

181 The R-PC immobilisation in alginate hydrogel beads according to a previously published  
182 procedure (Zhang, Zhang, Zou, & McClements, 2016) with modifications. A solution of R-PC (4  
183 mg/mL) was prepared in 25 mM acetate buffer at pH 5. Subsequently, this solution was mixed with  
184 a 2 % aqueous solution of sodium alginate in a volume ratio of 1:1. The resulting mixture was then  
185 droplet-wise deposited into a 2 % calcium chloride solution of 50 mM acetate buffer at pH 5. The  
186 formed beads were stored at 4 °C overnight. The beads containing immobilised proteins were  
187 thoroughly washed several times with 50 mM acetate buffer pH 5. The beads were then transferred  
188 into a microtube and subjected to thermal treatment at 60 °C for 10 minutes. Beads were dissolved  
189 using 100 mM Tris-citrate buffer pH 7.3. Obtained solutions were subjected to an ORAC test (**section**  
190 **2.5**) to assess the effects of immobilisation and thermal treatment on the antioxidative capacity of R-  
191 PC. The dissolved beads without R-PC served as a blank in the ORAC assay. The control experiment  
192 comprises heating 2 mg/mL R-PC in solution (non-immobilised) at the same conditions as those used  
193 for the thermal treatment of the R-PC containing alginate beads.

## 194 3. Results

### 195 3.1 Purification of R-PC from Nori seaweed

196 We combined precipitation and chromatographic techniques to purify R-PC from Nori flakes.  
197 Phycobiliproteins from Nori seaweed exhibit the characteristic absorption peaks: R-PE (peaks at 498  
198 and 560 nm, and shoulder at 545 nm), R-PC (peaks at 550–560 and 618 nm) and APC (peak at 652  
199 nm with a shoulder at 620 nm) (Glazer et al., 1975; Simovic et al., 2022). Obtained raw extract had  
200 a strong absorption in the region from 250 to 450 nm due to the presence of various pigments (**Fig.**  
201 **1C**). Precipitation with ammonium sulphate (65 %) increased the purity ratio ( $A_{618}/A_{280}$ ) from 0.31



202 to 0.61 (**Fig. 1C**) by separating most of the non-protein related pigments absorbed in the region 250–  
203 450 nm. Different PBPs were not distinguished in this step since ammonium sulphate induces non-  
204 selective precipitation of all PBPs presented in the extract (**Fig. 1C**). Separation of R-phycocyanin  
205 from R-PE was achieved by applying HA chromatography (**Fig. 1A**). Most R-PE is eluted by  
206 equilibration buffer. At the same time, R-PC exhibits much stronger binding to the HA column and  
207 100 mM phosphate is required for its elution. This chromatography step substantially increases  
208  $A_{618}/A_{560}$  ratio (1.10) compared to the sample before chromatography (0.67). Although this step  
209 removed a significant amount of R-PE, the ratio of  $A_{618}/A_{280}$  was still low (1.32), suggesting the  
210 presence of other proteins in the R-PC enriched fraction. The same findings are observed by SDS-  
211 PAGE after the fluorescence visualisation of PBPs and CBB staining (**Fig. 1D**). Hence, anion-  
212 exchange chromatography on DEAE-Sepharose was employed to purify R-PC further. The  
213 chromatogram displayed two peaks, with the R-PC fraction eluting ionic strength of 150 mM NaCl.  
214 The fraction that was eluted with 200 mM NaCl corresponded to allophycocyanin and R-PE (high  
215 binding fraction).

216 Combining HA and ion-exchange chromatography resulted in the preparation of R-PC with a  
217 purity index of  $A_{618}/A_{280}$ , around 3.4. The absence of a shoulder at 498 nm in absorption spectra  
218 (Simovic et al., 2022), which indicates the complete removal of R-PE (**Fig. 1C**) and the presence of  
219 solely two characteristic bands in the electrophoretic profile (**Fig. 1D**) served as confirmation that R-  
220 PC of the high purity is obtained. The two observed bands at ~18 and 20 kDa arise from  $\alpha$  and  $\beta$   
221 subunits of R-PC. Interestingly, visualisation of SDS-PAGE gel by UV lamp revealed that  $\beta$  subunits  
222 exhibit much stronger fluorescence than  $\alpha$  subunits. This result could be ascribed to the higher  
223 quantum yield of PEB chromophore (Grabowski & Gantt, 1978; Saraswat et al., 2011), exclusively  
224 bound to the R-PC  $\beta$  subunits, compared to the PCB chromophore, which is attached to both  $\alpha$  and  $\beta$   
225 subunits.

### 226 3.2 Spectroscopic and structural characterisation of purified R-phycocyanin

227 The purified R-PC was digested by trypsin, and we analysed obtained peptides by tandem  
228 mass spectrometry. Based on this analysis (**Supplementary Material 2**), the R-PC sequence of both  
229 subunits ( $\alpha$  and  $\beta$ ) was determined (**Fig. 2A**). Furthermore, we identified the binding sites for  
230 tetrapyrrole chromophores:  $\alpha$  subunit binds one chromophore *via* Cys84, while two chromophores  
231 are attached to  $\beta$  subunit by Cys82 and Cys153 (**Fig. 2A**). Considering the same molecular mass of  
232 PCB and PEB, we could not make a distinction between them in MS spectra, but only the position of  
233 Cys residue involved in chromophore binding. However, based on the previously published crystal  
234 structure of R-PC from *Polysiphonia urceolata* (Jiang et al., 2001), we were able to assign PCB  
235 binding to Cys84 and Cys82 of  $\alpha$  and  $\beta$  subunits, respectively, while PEB chromophore is bound to

236 the Cys153 of  $\beta$  subunits. BLAST analysis revealed the high sequence identity (around 90% or  
237 higher) between obtained R-PC from *Porphyra* spp. and other phycocyanins belonging to the  
238 different classes of *Rhodophyta* division (**Tables S1 and S2**). We also detected about 70% of  
239 sequence homology with the C-phycocyanin from cyanobacteria *Spirulina* with the identical position  
240 of Cys residues involved in the chromophore binding sites (Minic et al., 2016).

241 As previously described, the VIS absorption spectrum of purified R-PC exhibited two  
242 characteristic peaks at 552 and 618 nm, originating from PEB and PCB chromophores, respectively  
243 (**Fig. 1C**). The presence of two PCB chromophores per  $\alpha\beta$  heterodimer of R-PC gives higher  
244 absorption of the peak at 618 nm compared to the peak intensity at 552 nm, because only one PEB  
245 chromophore is bound to  $\beta$  subunit. Excitation of PEB and PCB chromophores in R-PC at 488 and  
246 590 nm gives two intensive emission peaks at 580 and 640 nm, respectively (**Fig. 2B**). In accordance  
247 with the absorption spectra, the higher content of PCB compared to PEB chromophores in R-PC (2  
248 vs 1, respectively) gives stronger emission at 640 nm compared to 580 nm. PEB and PCB  
249 chromophores are in the asymmetric environment within the R-PC structure, which induces strong  
250 signals in the near-UV/visible CD spectrum of this protein. In the visible region, two peaks appear  
251 that originate from PEB (540 nm) and PCB (634 nm) chromophores (**Fig. 2C**). We also observed two  
252 peaks at 306 and 341 nm, arising from a higher excitation state of the PEB and PCB chromophores,  
253 respectively (**Fig. 2C**) (Glazer et al., 1975). Interestingly, we observed the differences in the shape  
254 and the position of peaks in the CD spectra of PEB and PCB chromophores (10–20 nm) compared to  
255 absorption spectra, especially in terms that the relative intensity of PEB band is stronger in CD  
256 compared to the absorption spectra. This finding indicates the strong interactions of tetrapyrrole  
257 chromophores between different subunits within R-PC oligomers (Glazer et al., 1975).

258 Far-UV CD spectra were recorded to characterise the secondary structure of purified R-PC.  
259 As shown in **Fig. 2D**, the far-UV CD spectra of R-PC have negative ellipticity between 202 and 240  
260 nm, with minima at 209 and 222 nm and also show a positive ellipticity at 192 nm, which corresponds  
261 to the signals of  $\alpha$ -helical proteins. Indeed,  $\alpha$ -helix is the main secondary structure in R-PC (53 %).

262 SAXS measurements were performed to determine the R-PC shape and oligomerisation state  
263 in solution at pH 5.7. A well-defined plateau characterises the SAXS profile of R-PC at  $Q < 0.01 \text{ \AA}^{-1}$   
264 (**Fig. 2E**), confirming the absence of aggregates in the purified protein. We also observed the large  
265 shoulder at  $0.1 \text{ \AA}^{-1}$ , arising from the central hole in the R-PC structure (**Fig. 2E**). Indeed, we  
266 demonstrated by CRY SOL software that our experimental SAXS curve fits well with the theoretical  
267 curve of the crystal structure of trimeric R-PC (**Figs. 2E and 2F**) from *Polysiphonia urceolata* (Chang  
268 et al., 1996), which resembles the shape of the hollow cylinder. Indeed, SAXS intensities are well  
269 fitted to a hollow cylinder analytical model (**Fig. S1**), enabling us to determine the R-PC dimensions  
270 (**Fig. 2F**). We also performed the *ab initio* modelling by GASBOR software (**Figs. 2G and 2H**)

without making any assumption of R-PC structure and obtained a model that closely resembles the R-PC crystal structure and hollow cylinder analytical model (**Fig. 2F**). Radii of gyration value ( $R_g = 39.3 \text{ \AA}$ ), obtained from Guinier plots, is in very good agreement with the previously determined  $R_g$  values for trimeric phycocyanin (**Saxena, 1988**).

### 3.3 R-phycocyanin thermal stability

The thermal stability of the R-PC has been determined using CD and UV/VIS absorption spectroscopy. CD spectroscopy has allowed us to follow the temperature variation of the ellipticity at 222 nm, indicating the loss of the  $\alpha$ -helical structure. The resulting melting curve, shown in **Fig. 3A**, exhibits one steep transition with a melting temperature of 52 °C (325 K) and an apparent unfolding enthalpy of 385.5 kJ/mol.

In an alternative approach, we evaluate R-PC's thermal stability by studying the absorption spectra changes. The results, shown in **Fig. 3B**, indicate that up to 40 °C, no significant differences in the spectral characteristics of the protein are observed. However, as the temperature is increased further (above 45 °C), a substantial decrease in absorption intensity is observed in the region between 500 and 700 nm, followed at 55 °C and above, by the disappearance of the characteristic bands associated with PCB and PEB. Additionally, at temperatures above 50 °C, a substantial increase of absorbances at lower wavelengths (below 500 nm) indicates the aggregation of unfolded proteins. These findings suggest that R-phycocyanin undergoes significant structural changes at higher temperatures, leading to the loss of its characteristic spectral features. Reducing the temperature to 20 °C did not lead to the recovery of distinct absorption bands, indicating an irreversible protein denaturation (**Fig. 4A**).

### 3.4 R-phycocyanin pH stability

The pH stability of R-PC was assessed using UV/VIS absorption spectrometry. Spectral analysis of R-PC in the pH range 4–8 revealed well-defined absorption maxima. A noteworthy absorbance increase at 552 nm was observed for pH between 4 and 5. In contrast, the peak at 618 nm displayed similar absorbance intensity throughout the pH range 4–8 (**Fig. 3C**), indicating that moderate acidic conditions do not induce conformational changes in the vicinity of the PCB chromophore. Further increase or decrease of pH (9 and 3, respectively) triggered a significant reduction in the absorption intensities of both PEB and PCB chromophores, but without pronounced changes in the peak shapes. In extreme pH conditions (pH 2 and pH 10–12), both spectral bands of R-PC were substantially disturbed, indicating protein unfolding (**Fig. 3C**). At pH 2, a significant red shift in the spectrum occurs, suggesting the protonation of pyrrole rings within tetrapyrrole chromophores (Dietzek et al., 2004), while, at alkaline conditions, a blue shift in the R-PC spectrum indicates chromophores oxidation (Minic et al., 2018).

We examined the effects of pH on R-PC's secondary structures by far-UV CD spectroscopy (**Fig. 3D**). No significant change in the far-UV CD spectra shape was observed for pH values ranging from 3 to 9. Although at pH 3 and 9, the  $\alpha$ -helical bands at 209 and 222 nm are still pronounced, a significant decrease in ellipticity (absolute values) is observed at these pH values (**Fig. 3D**), confirming the reduction in R-PC  $\alpha$ -helical content. Furthermore, at both low acidic conditions (pH 2) and high alkaline conditions (pH above 9), we note the absence of the  $\alpha$ -helix characteristic bands (**Fig. 3D**), suggesting a complete denaturation of the protein.

### 3.5 Thermal stability of R-phycocyanin immobilised in calcium alginate beads

We immobilised R-PC in calcium alginate beads, and obtained product was subjected to thermal treatment at 60 °C for 10 minutes. Our results demonstrate that the colour of R-PC immobilised in alginate beads does not change upon heating (**Fig. 4B**). On the other hand, as described above, heating of R-PC induces the detrimental loss of the protein colour (**Fig. 4A**). Therefore, immobilised R-PC exhibit greater thermal stability (**Fig. 4B**) compared to a protein that was not immobilised (**Fig 4A**). Furthermore, the absorption spectrum of the R-PC extracted from the calcium alginate matrix after thermal treatment showed no significant change, except a small increase in absorbance at wavelengths below 500 nm, which indicates a slight protein aggregation within alginate beads upon thermal treatment. Therefore, our findings provide strong evidence of the substantial thermal stabilisation of protein in the calcium alginate matrix.

We also employed an ORAC assay to test the effects of immobilisation and heating on the antioxidative capacity of R-PC. We did not observe any significant changes in the ORAC values between R-PC and immobilised R-PC protein before thermal treatment (**Fig. 5**). Heating R-PC has detrimental effects on the protein's antioxidant activity (**Fig. 5**). On the other hand, heating the immobilised R-PC preserved the antioxidant action in comparison to the heating R-PC in solution. However, the heat treatment decreased for ~20 % the antioxidant activity of immobilised R-PC compared to unheated immobilisate (**Fig. 5**), indicating that the immobilisation approach could not preserve the antioxidant capacity of R-PC upon heating completely.

## 4. Discussion

In this study, we purified and characterised the R-phycocyanin (R-PC) from dried Nori flakes. Further, we tested the stability of purified, food-derived R-PC using the combination of several experimental approaches. We observed the low-temperature stability of R-PC since a moderate thermal treatment induces irreversible loss of protein colour and antioxidant capacity. On the other hand, protein immobilisation inside alginate beads significantly improves R-PC thermal stability.

337 In the cells of algae, R-phycocyanin and homologous PBPs, like R-PE and APC, form large  
338 photosynthetic complexes called phycobilisomes (Saluri, Kaldmäe, & Tuvikene, 2019). Therefore,  
339 an important obstacle in R-PC purification is removing the other PBPs, especially R-PE, the most  
340 abundant protein in *Porphyra*. We tackled this challenge with a combination of HA and IEC  
341 chromatography. HA chromatography removed the majority of R-PE, while IEC separated R-PC  
342 from other proteins, including APC. The achieved R-PC purity ratio of 3.4 ( $A_{618}/A_{280}$ ) was  
343 significantly higher than the purity of R-PC ( $A_{618}/A_{280} < 3.0$ ) isolated from red macroalgae  
344 *Polysiphonia urceolata* (Wang et al., 2014). However, the commonly accepted criterion for the  
345 analytical grade purity of C-phycocyanin (C-PC) is 4 (Nikolic et al., 2020). C-PC and R-PC have  
346 three and two PCB chromophores, respectively. Hence, obtained R-PC purity between 3 and 4 could  
347 not be ascribed to impurities but to a lower number of PCB chromophores bounds compared to C-  
348 PC, which induces a lower  $A_{618}/A_{280}$  ratio. Additionally, SDS-PAGE justified the R-PC purity and  
349 its heteromeric structure with two bands arising from  $\alpha$  and  $\beta$  subunits. The heteromeric structure of  
350 purified R-PC has also been confirmed by mass spectrometry (MS). Moreover, we utilised MS to  
351 determine the sequence of R-PC from Nori flakes, and high sequence homology with phycocyanins  
352 from different classes of red algae and cyanobacteria has been detected.

353 Overall, we successfully optimised a protocol for producing high-purity vibrant purple-  
354 coloured R-PC. Although, to the best of our knowledge, there is no analysis on the costs of R-PC  
355 extraction and purification, the purity grade of phycocyanins, in general, strongly influences its  
356 commercial value. For example, the prices of C-PC from *Spirulina* range from \$0.35 to \$135 per  
357 gram in the food and cosmetics industries and up to \$4600 per gram for therapeutic and diagnostic  
358 applications (Zittelli, Lauceri, Faraloni, Benavides, & Torzillo, 2023). However, the  
359 commercialisation of R-PC and other phycocyanins faces challenges due to their chemical instability  
360 and the high costs and time-consuming nature of extraction and purification methods (Fernandes et  
361 al., 2023, Zittelli, Lauceri, Faraloni, Benavides, & Torzillo, 2023). Therefore, there is a pressing need  
362 to scale R-PC production for industrial purposes to enable its sustainable production and meet market  
363 demands, representing essential aspects for future studies.

364 Optical spectroscopy and SAXS curves show typical features of R-PC: well-defined  
365 absorption, CD bands in the near-UV-VIS region and intensive fluorescence detectable at low nM  
366 concentrations (Wang et al., 2014). The specific feature of R-PC is the presence of additional bands  
367 in absorption, fluorescence and CD spectra, arising from the PEB chromophore, not presented in the  
368 cyanobacterial phycocyanins (Yan et al., 2010). The different peak positions between near-UV-VIS  
369 CD and absorption spectra of R-PC indicate strong exciton-type interactions between tetrapyrrole  
370 chromophores, confirming the oligomeric structure of the protein (Glazer et al., 1975; Li et al., 2020).  
371 Additionally, the stronger intensity of the PEB band compared to the PCB band in CD spectra, which

372 is opposed to the intensity ratio of these two bands in absorption spectra, could be ascribed to the  
373 deviation of the PEB chromophore D ring from BC rings conjugate plane, as observed in the crystal  
374 structure of R-PC from *Polysiphonia urceolata* (Jiang et al., 2001; Pescitelli et al., 2003). In this  
375 regard, it was previously shown that dihedral angles within and between chromophores significantly  
376 contribute to the intensities in CD spectra (Berova, Di Bari, & Pescitelli, 2007). Such an effect could  
377 be especially relevant within oligomeric PBPs where chromophores of different subunits could  
378 interact with each other. Indeed, our SAXS experimental curve of R-PC in solution (pH 5.7), purified  
379 from Nori (*Porphyra*), fits well with the theoretical curve of R-PC crystal structure from *Polysiphonia*  
380 *urceolata* in trimeric oligomer state, indicating conservation of R-PC 3D structure between different  
381 classes of organisms. Indeed, this finding is in accordance with the above-stated high sequence  
382 homology between *Porphyra* R-PC and phycocyanin from other sources. We also elucidated the  
383 structure of R-PC by the *ab initio* approach and found very good agreement with the model obtained  
384 by crystallography (Jiang et al., 2001). However, we observed that R-PC from red algae and C-PC  
385 from cyanobacteria do not have the same propensity towards oligomerisation. According to Saxena  
386 (Saxena, 1988), C-PC from the cyanobacterium *Synechocystis* sp. has a hexameric structure at pH  
387 5.7, whereas R-PC is in a trimeric state here. Moreover, our SAXS results (data not shown)  
388 demonstrated that C-PC from *Spirulina*, the most cultivated microalgae for food purposes, also has a  
389 hexameric form at pH 5.7. This difference indicates that the unique presence of PEB chromophore in  
390 R-PC, but not in C-PC, could provoke the subtle conformational changes that influence the  
391 oligomerisation trend.

392 The native structure of R-PC was preserved in dried Nori flakes, as confirmed by high  $\alpha$ -  
393 helical content (>50 %). Although (to the best of our knowledge) no previous studies are focusing on  
394 the far-UV CD spectra of R-PC, our results are in good agreement with the obtained  $\alpha$ -helical content  
395 for C-PC from *Spirulina* and other cyanobacteria (Chen, Liu, MacColl, & Berns, 1983; Chi et al.,  
396 2020; Edwards, Hauer, Stack, Eisele, & MacColl, 1997; Li, Gillilan, & Abbaspourrad, 2021).  
397 However, R-PC has a substantially lower amount of  $\alpha$ -helices in comparison to R-PE (>70 %)  
398 (Simovic et al., 2022), suggesting the presence of  $\gamma$  subunit is the main contributor to the higher  
399 percentage of  $\alpha$ -helix in R-PE.

400 Temperature increase induced a steep reduction in  $\alpha$ -helix ellipticity, showing a one-transition  
401 unfolding of R-PC at  $T_m \sim 52$  °C. Although (to the best of our knowledge) there is no data about the  
402 thermal stability of R-PC, the studies conducted on phycocyanins from *Spirulina* and other  
403 cyanobacteria (C-PC) obtained similar thermodynamic data with the melting temperature of C-PC in  
404 the range of 50-60 °C (Chen et al., 1983; Faieta et al., 2020). On the other hand, our previous CD  
405 study demonstrated higher thermal stability of the R-PE from *Porphyra* spp. due to a more stable  $\gamma$   
406 subunit, a unique feature of the R-PE oligomer (Simovic et al., 2022). Absorption measurements

407 revealed that the R-PC colour is unstable above 40 °C. At the same time, the thermal treatment at 55  
408 °C or above induces the complete and irreversible loss of R-PC-specific bands in the absorption  
409 spectra. Although previous studies also confirmed that R-PE colour from Nori flakes starts to  
410 diminish above 40 °C (Simovic et al., 2022), the R-PE specific absorption peaks, especially the band  
411 arising from  $\gamma$  subunit, could be observed up to 75 °C. Therefore, the presence of  $\gamma$  subunit is R-PE's  
412 key structural feature, making its structure substantially more stable than R-PC. In this context, the  
413 previous studies demonstrated that phycocyanin from *Spirulina*, with the same subunit composition  
414 as R-PC, also has unstable colour at higher temperatures with the onset of colour loss above 40 °C  
415 (Chaiklahan, Chirasuwan, & Bunnag, 2012; Faieta et al., 2020).

416 The colour of R-PC exhibits good stability in the pH range of 4–8, previously shown for  
417 phycocyanin from *Spirulina* (Li et al., 2021; Yan et al., 2010). The secondary structures ( $\alpha$ -helices)  
418 of R-PC are also well preserved in the same pH range, confirming that tetrapyrrole chromophores are  
419 good indicators of the conformational state of PBPs. R-PE from red macroalgae has a slightly wider  
420 pH stability range (Munier et al., 2014), which is in accordance with the higher thermal stability of  
421 R-PE. Shifting the pH to more acidic (pH 3) or alkaline conditions (pH 9) will induce loss in  $\alpha$ -helical  
422 structure and colour intensity of R-PC. However, the main spectral feature of R-PC is still preserved,  
423 indicating the absence of complete protein denaturation. A similar observation was made on C-PC  
424 from *Spirulina*, which was not entirely unfolded at pH 3 and 9 (Li et al., 2021; Zhang, Cho,  
425 Dadmohammadi, Li, & Abbaspourrad, 2021). Moreover, in contrast to R-PC, the  $\alpha$ -helical structure  
426 in C-PC is mostly preserved at pH 9 (Li et al., 2021). Still, C-PC becomes more flexible, and  
427 dissociation from hexamers/trimers to monomers was observed at pH 9 (Li et al., 2021). Increasing  
428 the pH above 10 or acidification to pH 2 entirely unfolds R-PC, while tetrapyrrole chromophores  
429 oxidation at high pH was also observed. Considering that moderate acidic conditions (pH 3–4) do not  
430 have detrimental effects on R-PC colour and most fruit juices display pH in this range (Reddy, Norris,  
431 Momeni, Waldo, & Ruby, 2016), R-PC have a promising potential for applications as a colouring  
432 agent for soft beverages, even if its properties can be improved.

433 *Porphyra* spp. proteins have a high essential amino acid index, representing a sustainable  
434 alternative to animal proteins (Rawiwan, Peng, Paramayuda, & Quek, 2022). Further, the added value  
435 of *Porphyra* spp., as the source of alternative proteins, is the high abundance of PBPs characterised  
436 by the vivid colours and strong bioactive properties (Venkatraman & Mehta, 2019). These specific  
437 properties of PBPs substantially strengthen the consumer acceptance of *Porphyra* spp. as an  
438 alternative source of proteins. However, preserving the PBPs' colour and bioactivities during the food  
439 treatment is a significant challenge for enhancing their application in the food industry. Alginate  
440 hydrogel beads represent a promising system for encapsulating, protecting, and delivering food  
441 proteins (Zhang et al., 2016). Our study unambiguously confirmed that encapsulation of R-PC into

alginate beads completely preserves the protein colour upon thermal treatment at 60 °C. Moreover, the antioxidative capacity of encapsulated R-PC is maintained largely upon thermal treatment but not wholly, probably due to slight protein aggregation within alginate beads, which could mask R-PC antioxidant activity. On the other hand, heating of R-PC that was not immobilised induces detrimental loss of protein colour and antioxidative activity, followed by aggregation of unfolded R-PC subunits. Indeed, previous studies demonstrated the ability of alginate-encapsulated C-PC from *Spirulina* to preserve its antioxidant capacity upon thermal treatment (Qiao et al., 2022). Therefore, encapsulating R-PC or other temperature-sensitive, food-derived PBPs into alginate beads could be an excellent approach to preserve and modulate its techno-functional properties, such as colour, aggregation and antioxidant capacity. Furthermore, this approach could be a good strategy for controlling the release of bioactive PBPs in the gastrointestinal tract (GIT) (Qiao et al., 2022). Alginate immobilisation in the presence of calcium ions represents the non-covalent entrapment of the protein of choice by making a gel-like network that surrounds the protein, thus entrapping it within (Zhang et al., 2016). Factors like pH and mechanical treatment could significantly affect the system's stability in this context. By having R-PC non-covalently immobilised, easier protein leakage is expected. This could be a double-edged sword. While the risk of leakage limits conditions at which alginate: R-PC beads can be stored and prevents rigorous mechanical treatments, this could be beneficial during their potential consumption since during the mechanical eating process of chewing and different pH values of GIT could easily release bioactive R-PC. On the other hand, the utilisation of composite gels which combine alginate with other biopolymers, such as chitosan, gelatin, and various proteins, improves the physical and mechanical properties of the gel, enhancing encapsulation efficiency, resulting in better protection and controlled release of bioactive compounds (McClements, 2018; Ramdhan, Ching, Prakash, & Bhandari, 2020).

## 5. Conclusions

We successfully purified and characterised the R-PC from commercially dried Nori flakes. The purified protein exhibited optical and structural characteristics of typical R-PC in the oligomeric (trimeric)  $\alpha$ -helical structure. R-PC has moderate pH stability while being more stable in acidic than in alkaline conditions. It also has relatively low-temperature stability, and an irreversible colour change and antioxidant capacity loss were observed upon thermal treatment at 60 °C. However, immobilisation of the R-PC inside calcium alginate beads significantly improved protein thermal stability and preserved its antioxidant capacity and colour.

Our results suggest that R-PC has a promising potential for colouring soft beverages at slightly-acidic conditions. However, further studies are needed to further improve its pH stability. Encapsulating R-PC into solid matrices could be an excellent approach to preserve protein colour and



476 bioactivities upon thermal processing, strengthening its potential for food colouring and positioning  
477 it as an ideal alternative protein.

#### 478 **Credit authorship contribution statement**

479 **Luka Velickovic:** Investigation, Conceptualisation, Formal analysis, Writing – original draft. **Ana**  
480 **Simovic:** Investigation, Conceptualisation, Formal analysis, Writing – review and editing. **Nikola**  
481 **Gligorijevic:** Investigation, Conceptualisation, Formal analysis, Writing – review and editing.  
482 **Aurélien Thureau:** Investigation, Formal analysis. **Milica Obradovic:** Investigation. **Tamara**  
483 **Vasovic** – Investigation, Formal analysis. **Georgios Sotiroudis:** Investigation, Writing – review and  
484 editing. **Maria Zoumpanioti:** Writing – review and editing. **Annie Brûlet:** Investigation, Formal  
485 analysis, Writing – review and editing. **Tanja Cirkovic Velickovic:** Writing – review and editing.  
486 **Sophie Combet:** Investigation, Formal analysis, Writing – review & editing. **Milan Nikolic:**  
487 Conceptualisation, Funding acquisition, Supervision, Writing – review and editing. **Simeon Minic:**  
488 Investigation, Conceptualisation, Formal analysis, Funding acquisition, Supervision, Writing –  
489 review and editing.

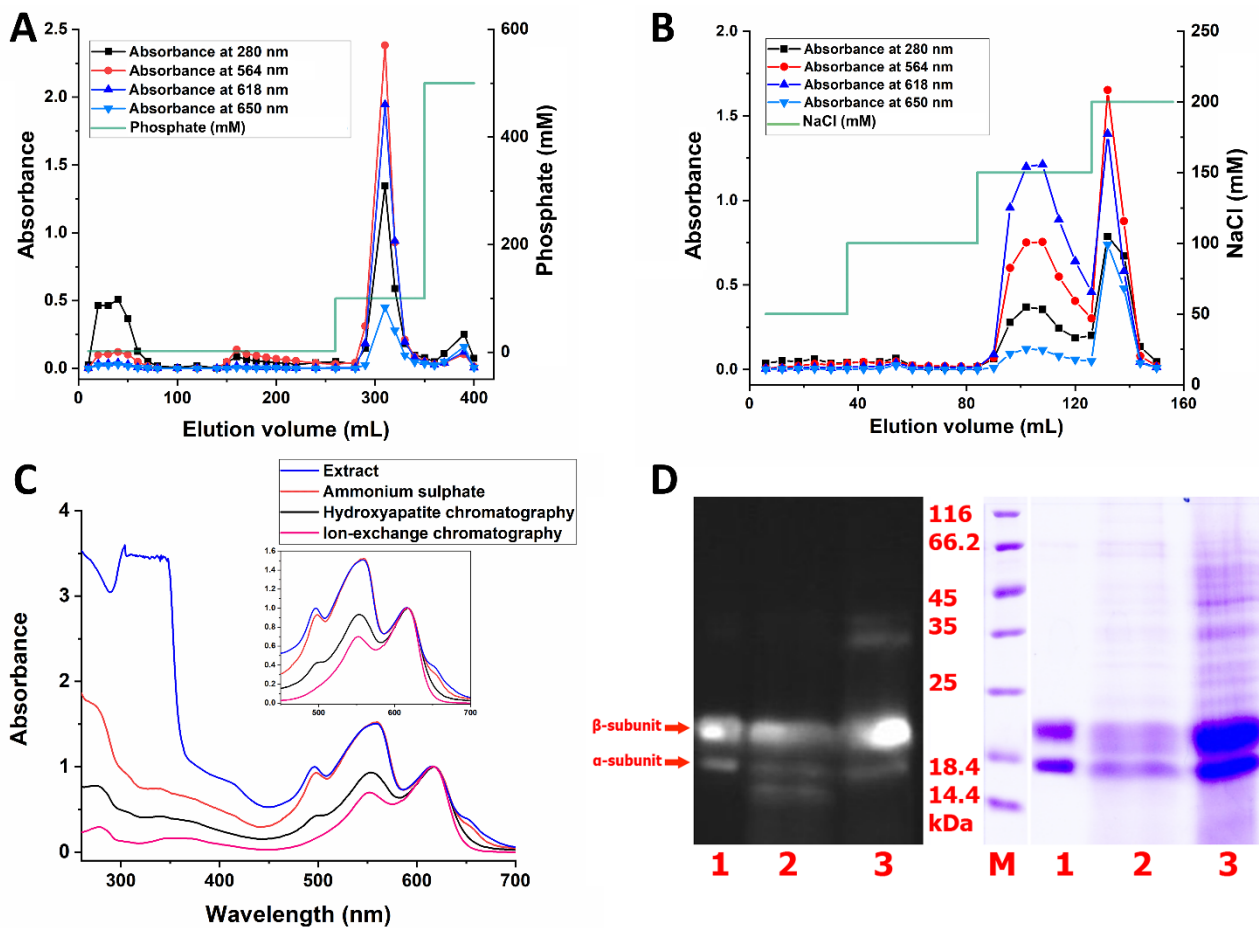
#### 490 **Declaration of Competing Interest**

491 The authors declare that they have no known competing financial interests or personal relationships  
492 that could have appeared to influence the work reported in this paper.

#### 493 **Acknowledgement**

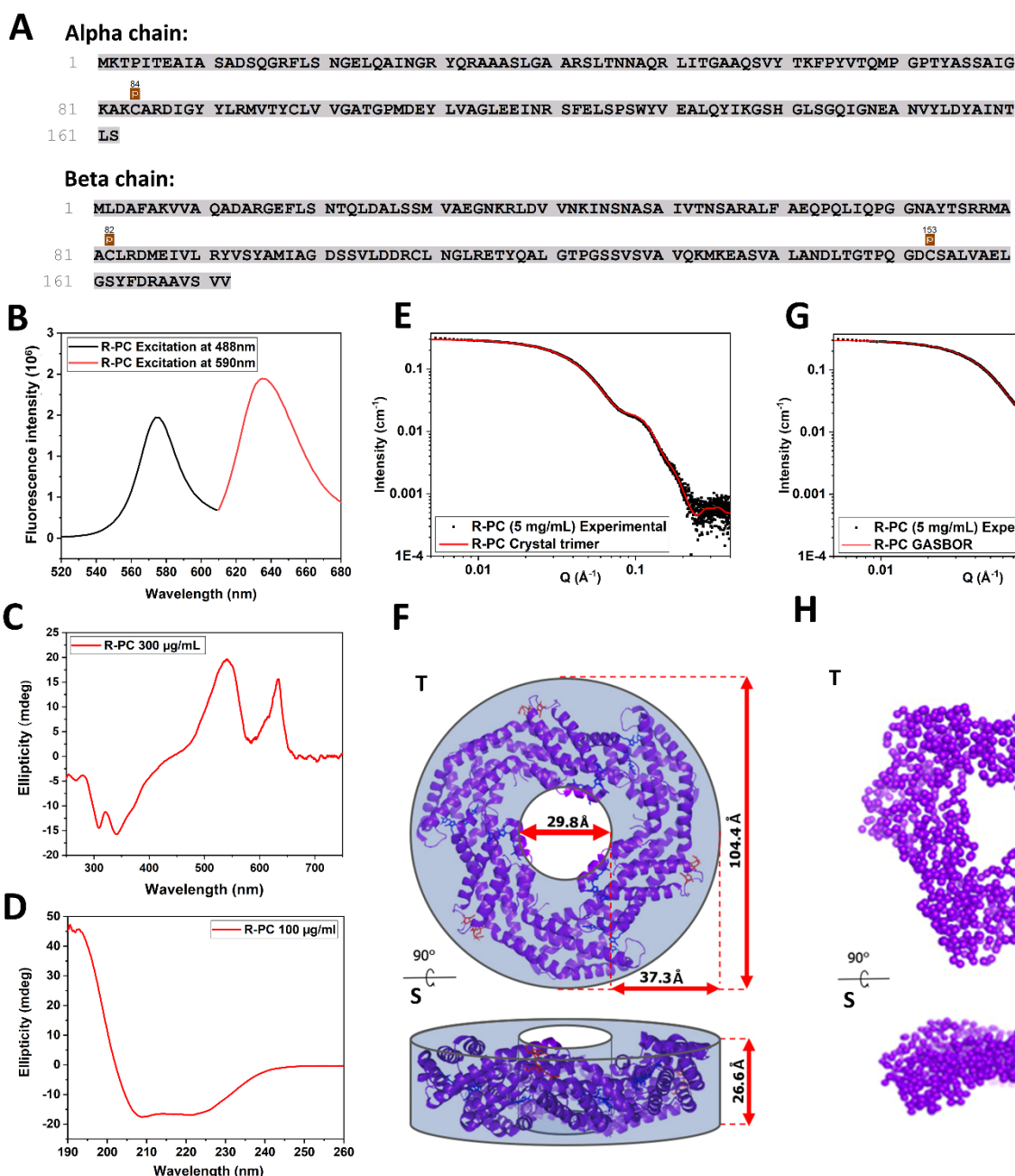
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#### 499 **Figure legends**



500

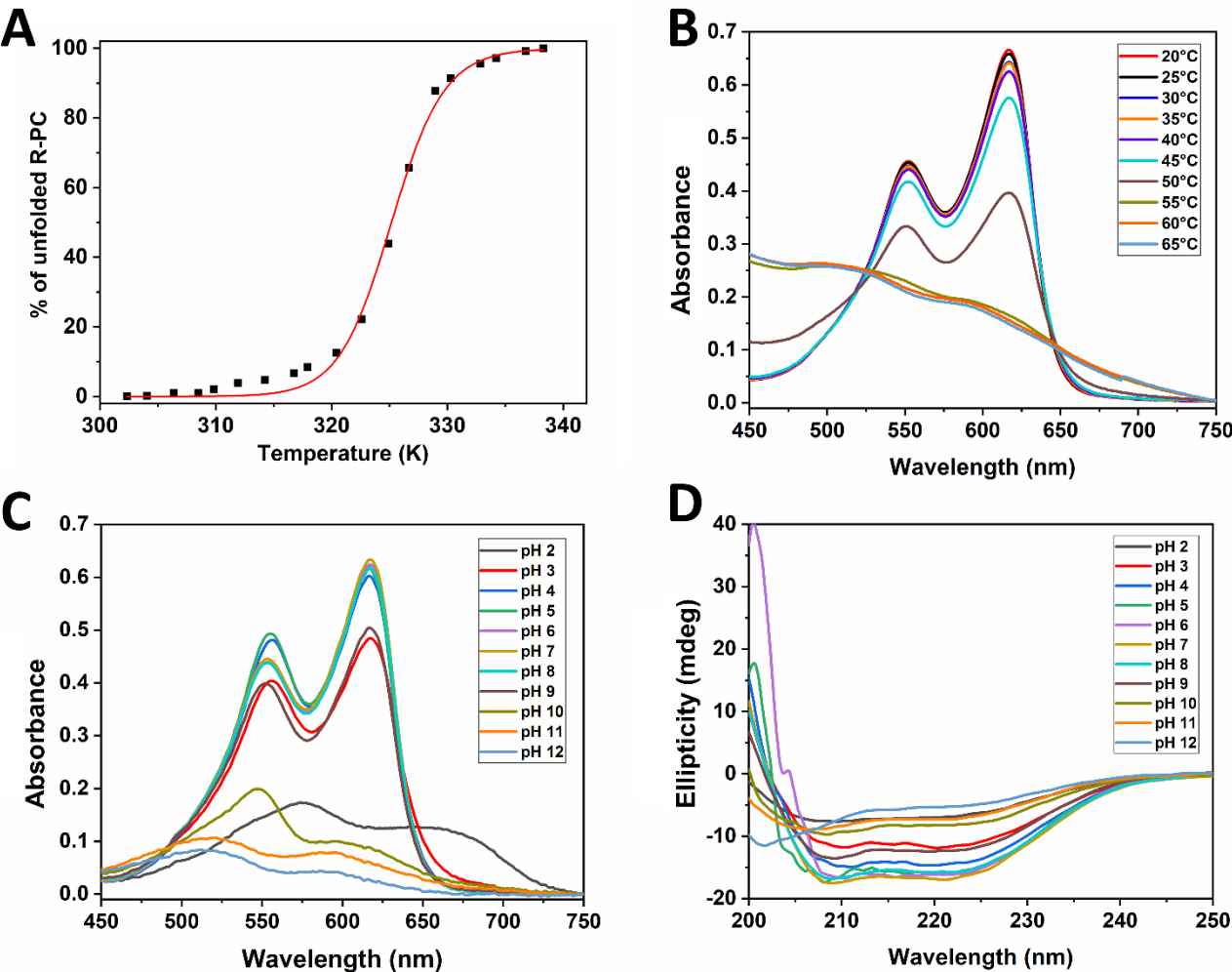
501 **Figure 1.** The chromatograms obtained after (A) hydroxyapatite and (B) ion-exchange  
 502 chromatography of R-PC; (C) Absorption spectra of R-PC after each purification step; (D) SDS-  
 503 PAGE electrophoresis under UV light (left) and after CBB staining (right) - lane 1: Purified RPC,  
 504 lane 2: hydroxyapatite eluate, lane 3: protein extract, M: molecular size markers.



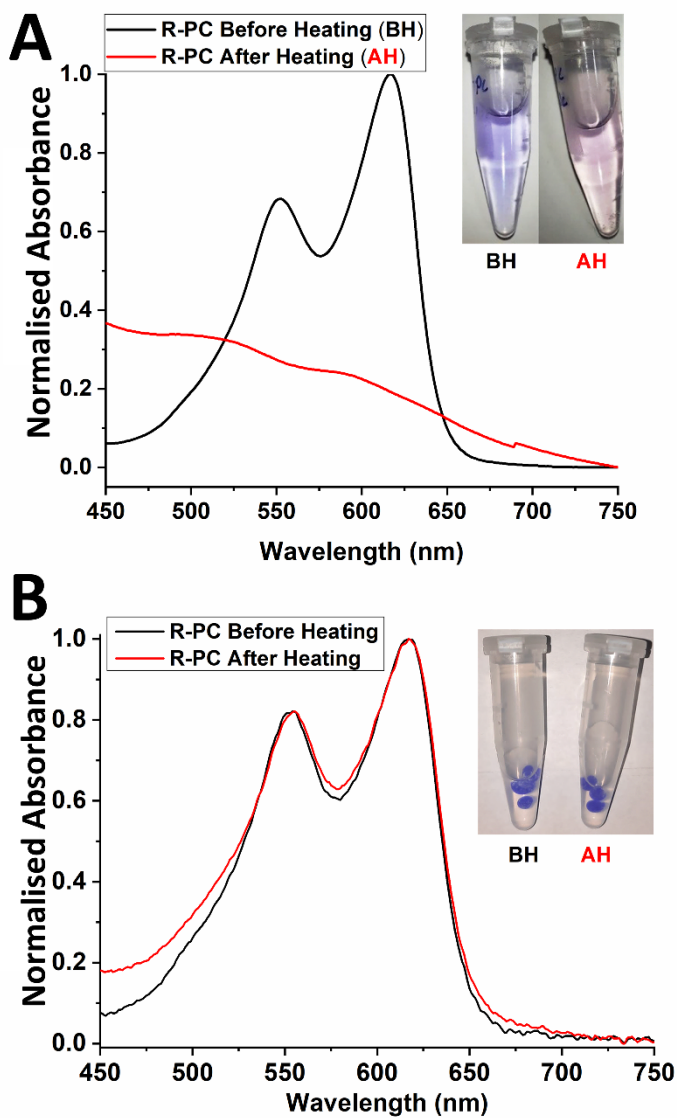
505

506 **Figure 2.** (A) Amino acid sequences of R-PC  $\alpha$  and  $\beta$  subunits determined by mass spectrometry. P  
507 denotes the attachment site for PCB (Cys84 and Cys82 in  $\alpha$  and  $\beta$  subunits, respectively) and PEB  
508 chromophores (Cys153 in  $\beta$  subunit); (B) Fluorescence emission spectra of purified R-phycocyanin  
509 (16 nM) after excitation at 488 nm (black line) and 590 nm (red line) at pH 7.0; (C) Near-UV/Visible  
510 and (D) far-UV CD spectra of R-PC (pH 7.0); (E) Comparison of the experimental SAXS curve of  
511 R-PC at pH 5.7 (dotted black line) with the theoretical curve (full red line) calculated from R-PC  
512 crystal structure (PDB:1F99) by CRY SOL software (ATSAS); (F) Ribbon model of the crystal  
513 structure of R-PC trimer superimposed with the hollow cylinder analytical model; Dimensions are  
514 obtained by the best fit to this model (Table S3). Obtained  $\chi^2$  value was 2.8; (G) Comparison of the  
515 same experimental SAXS curve with the curve obtained from *ab initio* modelling (full red line) using

516 GASBOR software (ATSAS). The obtained  $\chi^2$  value was 1.5; **(H)** Ab initio envelope of the R-PC  
 517 oligomer corresponding to the GASBOR model. T and S denote top and side views, respectively.

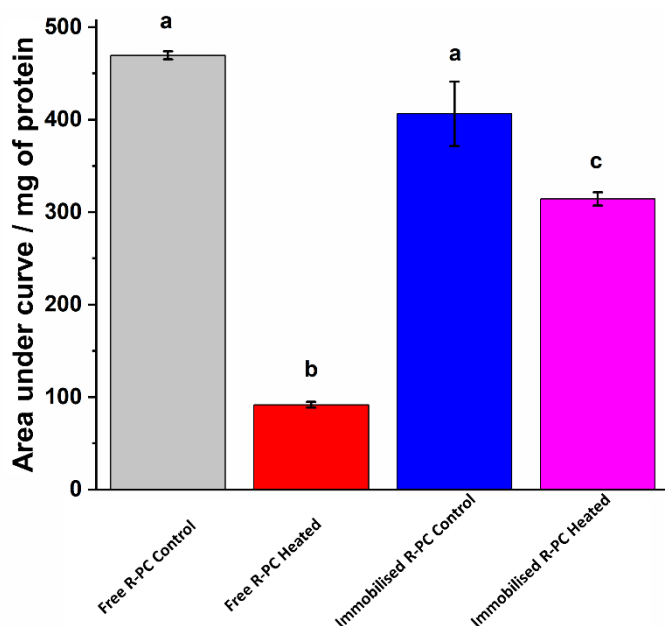


518 **Figure 3.** (A) 20 µg/mL (182 nM) R-PC thermal unfolding curve with the corresponding fit (full red  
 519 line), obtained by measuring ellipticity at 222 nm and pH 7.0; (B) The effects of temperature on  
 520 visible absorption spectra of 100 µg/mL (0.9 µM) R-PC at pH 7.0 (optical pathlength 1 cm); (C) The  
 521 effects of pH on visible absorption spectra of 100 µg/mL R-PC at 25 °C; (D) The effects of pH on  
 522 far-UV CD spectra of 100 µg/mL R-PC at 25 °C.



524

525 **Figure 4.** Absorption spectra of (A) R-PC without immobilisation and (B) R-PC extracted from  
 526 calcium alginate beads before and after heating at 60 °C for 10 min; Insets show pictures of (A) R-  
 527 PC without immobilisation and (B) immobilised R-PC before (BH) and after (AH) heating.



**Figure 5.** Histogram comparing antioxidative activity (ORAC assay) of R-PC without immobilisation (free R-PC) and R-PC immobilised in calcium alginate beads before and after heating at 60 °C. The data marked by different letters are significantly different ( $p < 0.05$ ).

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