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

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26 **Abstract**

27 This study aimed to purify, characterise and stabilise the natural food colourant, R-phycoyanin (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 (α -helix content 53%). SAXS measurements revealed
30 that R-PC is trimeric ($(\alpha\beta)_3$) in solution. The thermal denaturation of α -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-phycoyanin, 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-phycoyanin (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 allophycoyanin (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-phycoyanin 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 ($\alpha\beta$)₃ or hexamer ($\alpha\beta$)₆,
60 with α subunits of 18–20 kDa and β subunits of 19–21 kDa (Jian et al., 2001). One PCB chromophore
61 is bound to both α and β subunits, while one PEB chromophore is only attached to β subunits.

62 R-phycoyanin 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).

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

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

93 **2. Materials and Methods**

94 **2.1 Materials**

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

100 **2.2 R-PC isolation and purification**

101 R-Phycocyanin was purified according to the previously published procedures with
102 modifications (Boussiba & Richmond, 1979; Simovic et al., 2022; Wang et al., 2014). Nori seaweed
103 leaves were frozen with liquid nitrogen and then pulverised using a mortar and pestle. The powdered
104 sample was then suspended in 20 mM phosphate buffer pH 7.0 (1 g of powdered sample *per* 20 mL
105 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 \AA^{-1} , where
176 $q = \frac{4\pi}{\lambda} \sin\theta$ is the modulus of the momentum transfer, and 2θ is the scattering angle. 40 μL 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-phycoerythrin
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 allophycoerythrin 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 α and β
221 subunits of R-PC. Interestingly, visualisation of SDS-PAGE gel by UV lamp revealed that β subunits
222 exhibit much stronger fluorescence than α 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 β subunits, compared to the PCB chromophore, which is attached to both α and β
225 subunits.

226 **3.2 Spectroscopic and structural characterisation of purified R-phycoerythrin**

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 (α and β) was determined (**Fig. 2A**). Furthermore, we identified the binding sites for
230 tetrapyrrole chromophores: α subunit binds one chromophore *via* Cys84, while two chromophores
231 are attached to β 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 α and β subunits, respectively, while PEB chromophore is bound to

236 the Cys153 of β 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 β 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 α -helical proteins. Indeed, α -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 \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**)

271 without making any assumption of R-PC structure and obtained a model that closely resembles the
272 R-PC crystal structure and hollow cylinder analytical model (**Fig. 2F**). Radii of gyration value ($R_g =$
273 39.3 Å), obtained from Guinier plots, is in very good agreement with the previously determined R_g
274 values for trimeric phycocyanin (**Saxena, 1988**).

275 **3.3 R-phycocyanin thermal stability**

276 The thermal stability of the R-PC has been determined using CD and UV/VIS absorption
277 spectroscopy. CD spectroscopy has allowed us to follow the temperature variation of the ellipticity
278 at 222 nm, indicating the loss of the α -helical structure. The resulting melting curve, shown in **Fig.**
279 **3A**, exhibits one steep transition with a melting temperature of 52 °C (325 K) and an apparent
280 unfolding enthalpy of 385.5 kJ/mol.

281 In an alternative approach, we evaluate R-PC's thermal stability by studying the absorption
282 spectra changes. The results, shown in **Fig. 3B**, indicate that up to 40 °C, no significant differences
283 in the spectral characteristics of the protein are observed. However, as the temperature is increased
284 further (above 45 °C), a substantial decrease in absorption intensity is observed in the region between
285 500 and 700 nm, followed at 55 °C and above, by the disappearance of the characteristic bands
286 associated with PCB and PEB. Additionally, at temperatures above 50 °C, a substantial increase of
287 absorbances at lower wavelengths (below 500 nm) indicates the aggregation of unfolded proteins.
288 These findings suggest that R-phycocyanin undergoes significant structural changes at higher
289 temperatures, leading to the loss of its characteristic spectral features. Reducing the temperature to
290 20 °C did not lead to the recovery of distinct absorption bands, indicating an irreversible protein
291 denaturation (**Fig. 4A**).

292 **3.4 R-phycocyanin pH stability**

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

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

312 **3.5 Thermal stability of R-phycoyanin immobilised in calcium alginate beads**

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

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

331 **4. Discussion**

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

337 In the cells of algae, R-phycoyanin 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-phycoyanin (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 α and β 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 α -
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 α -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 α -helices in comparison to R-PE (>70 %)
398 (Simovic et al., 2022), suggesting the presence of γ subunit is the main contributor to the higher
399 percentage of α -helix in R-PE.

400 Temperature increase induced a steep reduction in α -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 γ
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 γ subunit, could be observed up to 75 °C. Therefore, the presence of γ 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 (α -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 α -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 α -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

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

465 **5. Conclusions**

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

473 Our results suggest that R-PC has a promising potential for colouring soft beverages at
474 slightly-acidic conditions. However, further studies are needed to further improve its pH stability.
475 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.

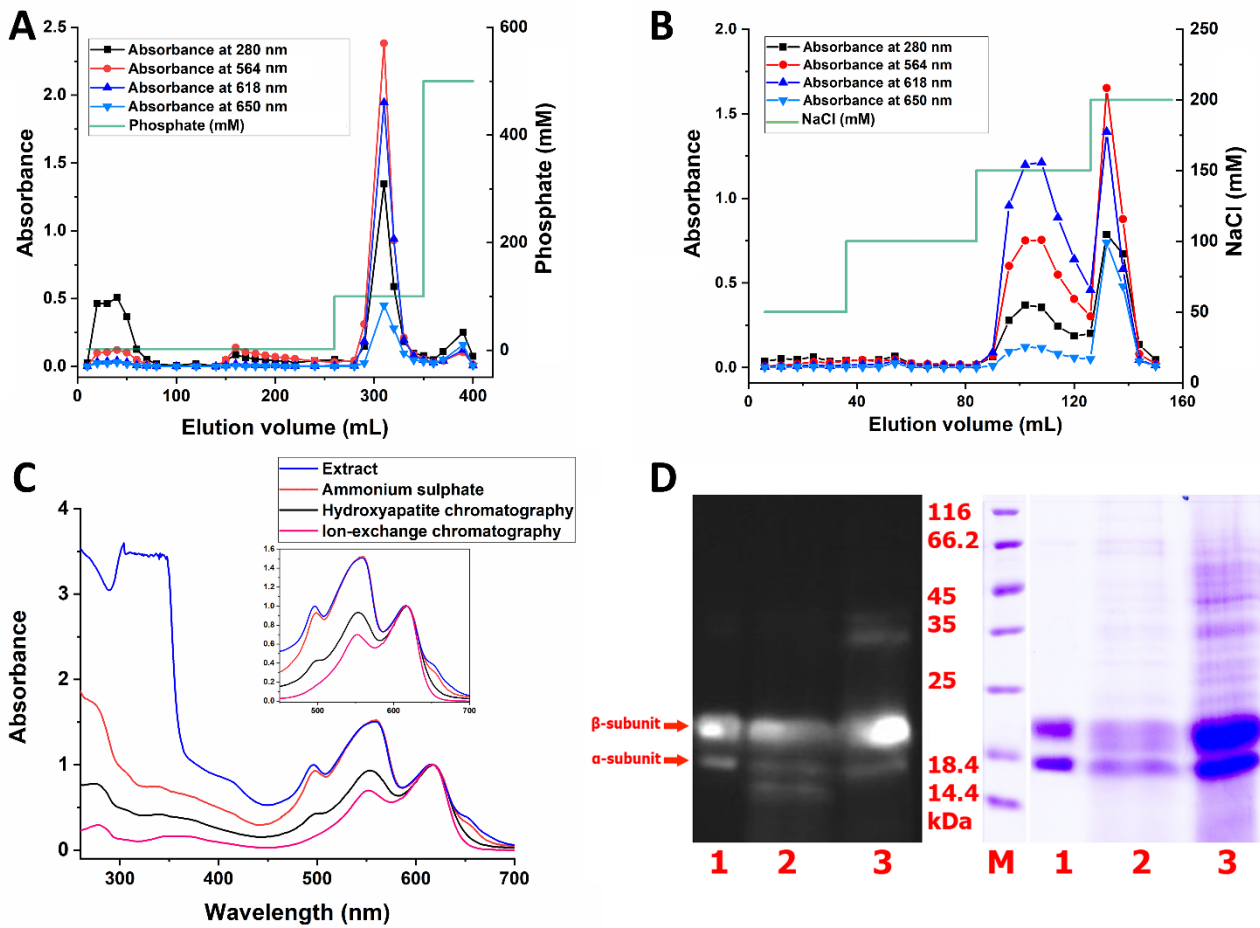
490 **Declaration of Competing Interest**

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.

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498 47/2023-01/200168 and 3) a short-term FEBS fellowship to Simeon Minić.

499 **Figure legends**



500

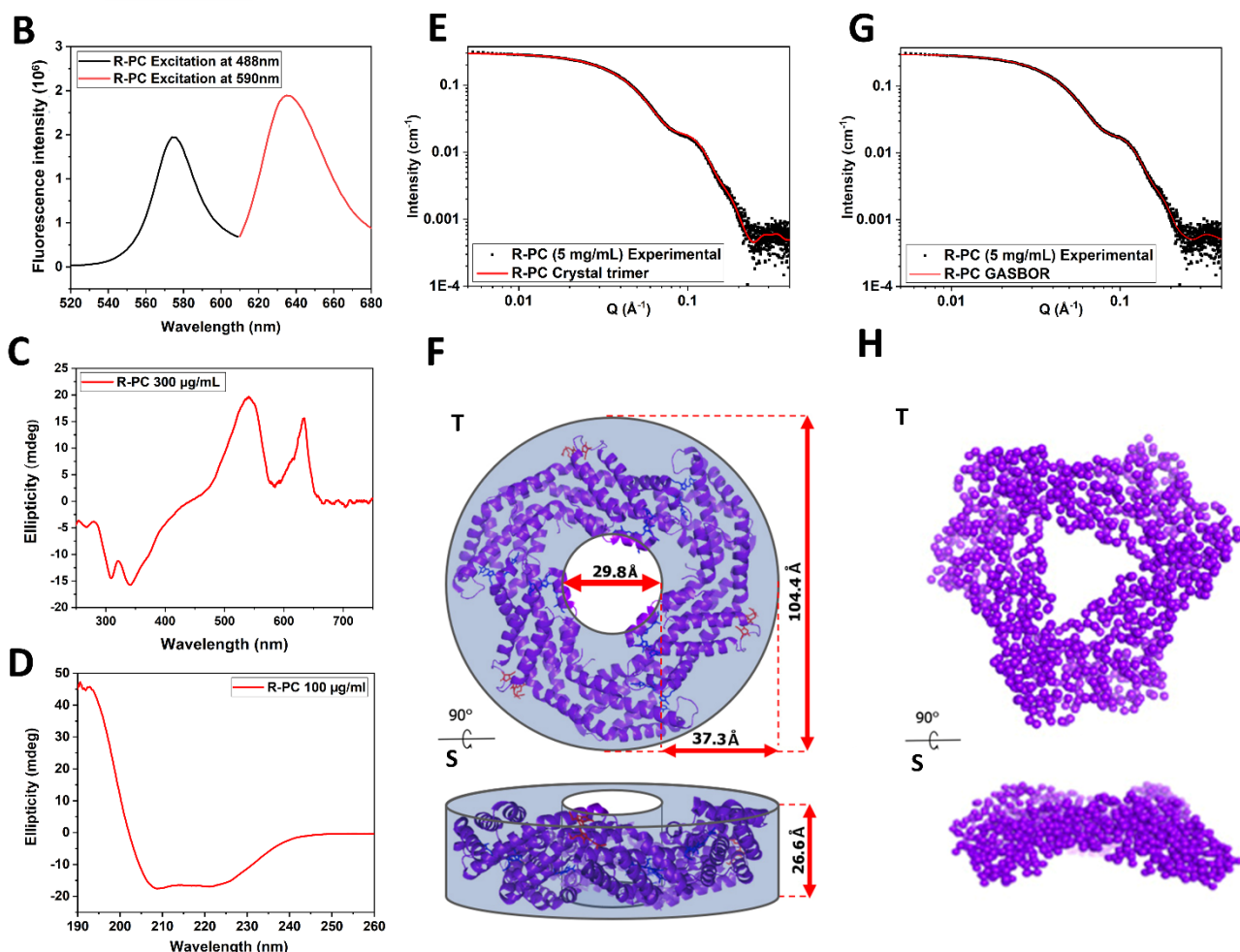
501 **Figure 1.** The chromatograms obtained after (A) hydroxyapatite and (B) ion-exchange
 502 chromatography of R-PC; (C) Absorption spectra of R-PC after each purification step; (D) SDS-
 503 PAGE electrophoresis under UV light (left) and after CBB staining (right) - lane 1: Purified RPC,
 504 lane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

A Alpha chain:

1 MKTPITEAIA SADSQGRFLS NGELQAINGR YQRAAASLGA ARSLTNNNAQR LITGAAQSVY TKFPYVTQMP GPTYASSAIG
 81 KAKCARDIGY YLRMVTYICLV VGATGPMDEY LVAGLEEINR SFELSPSWYV EALQYIKGSH GLSGQIGNEA NVYLDYAINI
 161 LS

Beta chain:

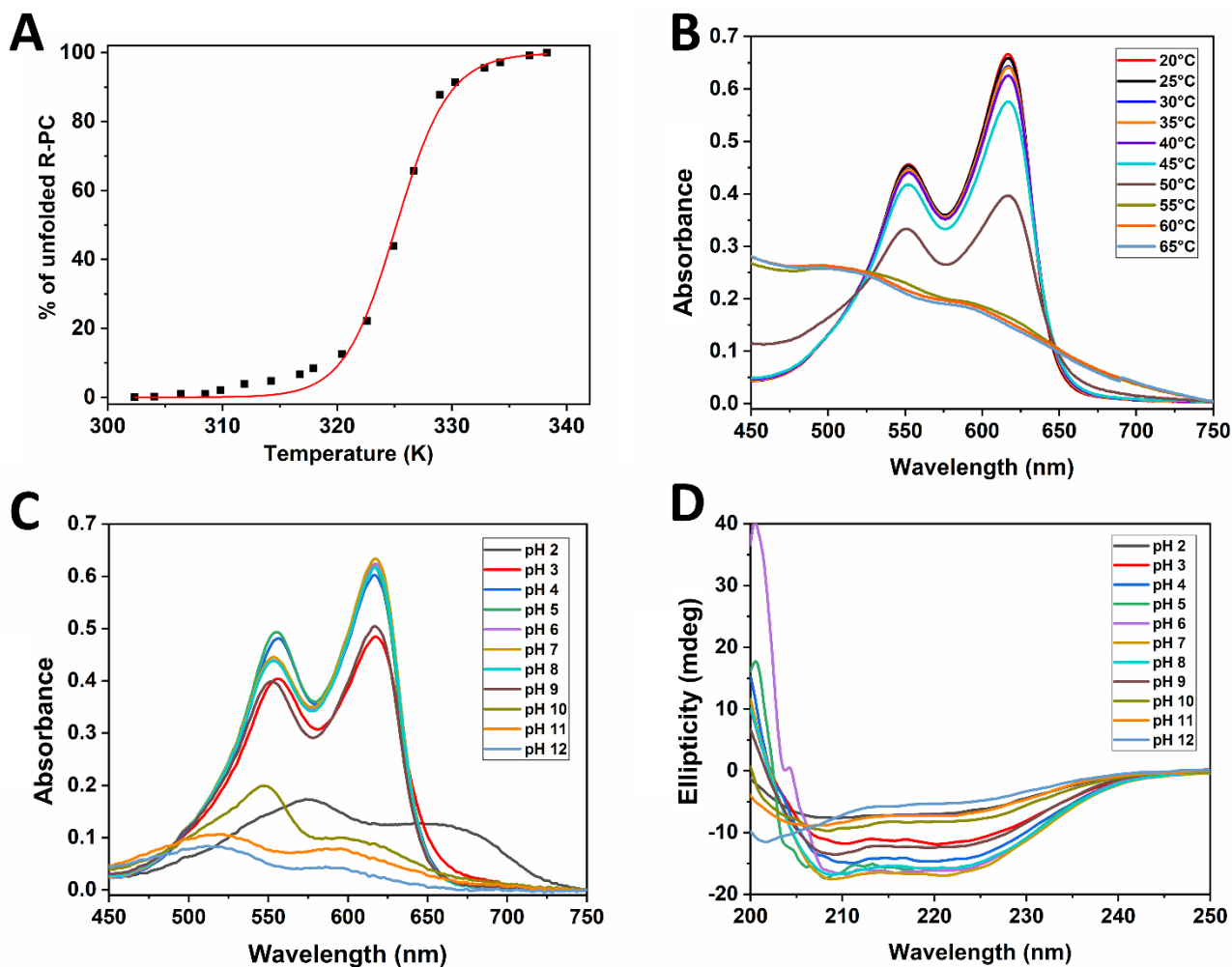
1 MLDAFAKVVA QADARGEFLS NTQLDALSSM VAEGNKRLDV VNKINSNNSA IVTNSARALF AEQPQLIQPG GNAYTSRRMA
 81 ACLRDMEIVL RYVSYAMIAG DSSVLDDRCL NGLRETYQAL GTPGSSVSVA VQKMKEASVA LANDLTGTPQ GDICALVAEL
 161 GSYFDRAAVS VV



505

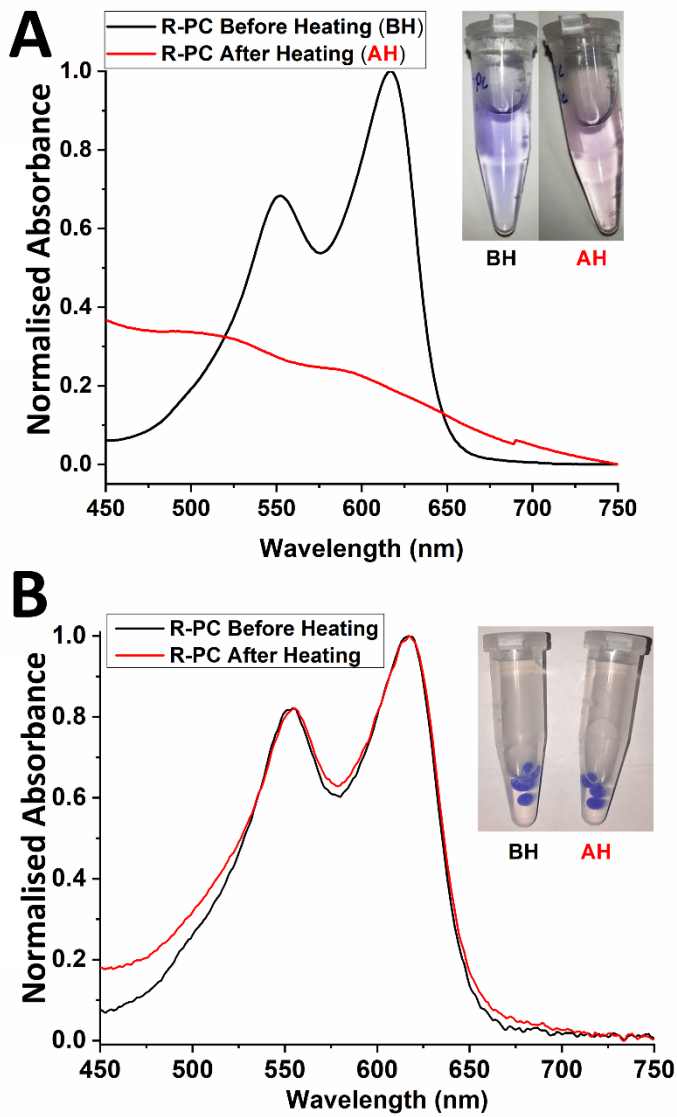
506 **Figure 2.** (A) Amino acid sequences of R-PC α and β subunits determined by mass spectrometry. P
 507 denotes the attachment site for PCB (Cys84 and Cys82 in α and β subunits, respectively) and PEB
 508 chromophores (Cys153 in β subunit); (B) Fluorescence emission spectra of purified R-phycocyanin
 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 χ^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 χ^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.



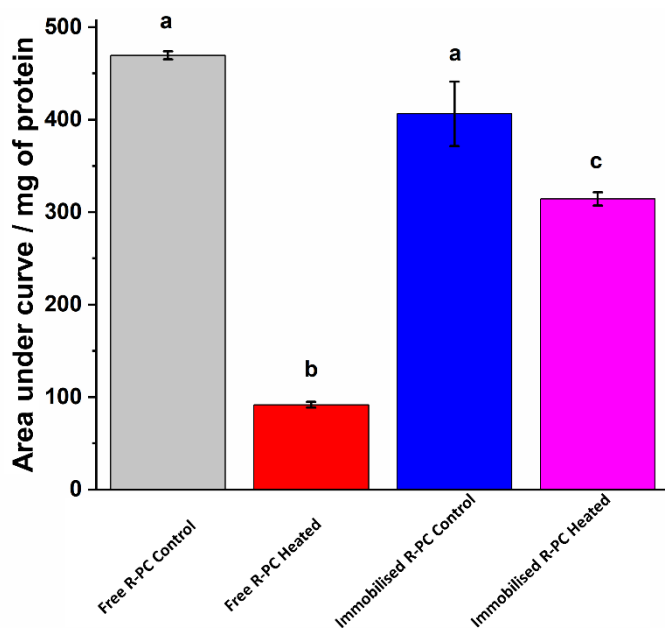
518

519 **Figure 3.** (A) 20 µg/mL (182 nM) R-PC thermal unfolding curve with the corresponding fit (full red
520 line), obtained by measuring ellipticity at 222 nm and pH 7.0; (B) The effects of temperature on
521 visible absorption spectra of 100 µg/mL (0.9 µM) R-PC at pH 7.0 (optical pathlength 1 cm); (C) The
522 effects of pH on visible absorption spectra of 100 µg/mL R-PC at 25 °C; (D) The effects of pH on
523 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.



528

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

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