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Probing the stability of the food colourant R-phycoerythrin from dried Nori flakes

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DECLARATION OF INTEREST

Declarations of interest: none.

ABSTRACT

This study aims to characterise the stability of R-phycoerythrin (R-PE) spectroscopically, a vivid natural colourant with emerging potential for application in the food industry. High-quality ($A_{560}/A_{280} \geq 5$), native (α-helix content 75%) R-PE was purified from commercial dried Nori (*Porphyra* sp.) flakes. The thermal unfolding revealed two transitions (at 56 and 72°C), ascribed to different protein subunits. Contrary to temperature, high-pressure (HP) treatment showed a significant advantage under applied conditions: the R-PE unfolding is partly reversible, and the colour bleaching is minimised. The binding of Cu^{2+} (6.3×10^5 M^{-1}) and Zn^{2+} (1.7×10^3 M^{-1}) influenced conformational changes in protein tetrapyrrole chromophore without affecting R-PE structure and stability (colour). Results give new insights into the stability of R-PE with a good use-value in replacement of toxic synthetic dyes, preservation of red colour of this phycobiliprotein in fortified food and beverages by HP processing, and as a biosensor for Cu^{2+} in aquatic systems.

Keywords: R-phycoerythrin, High-pressure, Temperature, Binding, Stability, Metal ions.

1. INTRODUCTION

Cultivation of marine macroalgae is of significant economic and cultural importance for providing food, medicine, cosmetics, and biofuel (**Gao, Gao, Bao, Xu, & Li, 2019**). Red microalgae *Porphyra* sp., commonly known as Nori, has been used as food for centuries in many Asian countries, but its consumption has extended to Western consumers (**Cermeno, et al., 2019**; **Gao et al., 2019**). It is usually used to prepare sushi, soup, cakes, consumed as dried Nori flakes (**Bito, Teng, & Watanabe, 2017**). Dried Nori is an excellent choice of many nutrients, such as dietary fibres, polyunsaturated fatty acids, minerals, vitamins, and a large amount of proteins (approx. 40% on a dry basis).

Numerous studies have shown antioxidant, immunomodulatory, anticancer and antihypertensive effects of *Porphyra* sp. extracts and components **(Venkatraman & Mehta, 2019**). The health benefits are mainly attributed to sulfated polysaccharides, bioactive peptides, microsporine-like amino acids and R-phycoerythrin (R-PE) (**Bito, et al., 2017**). R-phycoerythrin is one of the most abundant proteins in *Porphyra* sp., representing between 1.3–1.5% of the dry biomass (**Cao, Wang, Wang, & Xu, 2016**). It is a strongly fluorescent (quantum yield ~98%) and water-soluble molecule belonging to the phycobiliprotein (PBP) family, with the primary function to transfer excitation energy to reaction centres during photosynthesis (**Apt, Hoffman, & Grossman, 1993**; **Gaigalas, et al., 2006**). The intensive reddish-pink colour of R-PE arises from its covalently attached (*via* thioether bond) linear tetrapyrrole chromophores phycoerythrobilin (PEB) and phycourobilin (PUB). It is composed of three subunit types forming a hexamer complex [(αβ)6γ]: α (18–20 kDa), β (19–21 kDa), and γ (30–33 kDa) (**Apt, et al., 1993**; **Wang, et al., 2020**). Two PEB chromophores are bound to both α and β subunits, while one PUB chromophore is found attached to the β subunit (**Fig. 1A**). Gamma subunit binds one PEB and three PUB chromophores (**Gaigalas, et al., 2006**). Besides R-PE, *Porphyra* sp. contains other PBPs: blue R-phycocyanin (R-PC) and light-blue allophycocyanin (APC). The distinction in the colour of phycobiliproteins, e.g. in its VIS absorption spectra, is the consequence of differences in the type and number of bound chromophores in protein oligomers (**Apt, et al., 1993**).

Studies have shown that R-PE exhibits significant antioxidant (**Yabuta, Fujimura, Kwak, & Watanabe, 2010**), anticancer (**Cai, et al., 2014**), and immunomodulatory effects (**Wang, et al., 2020**), mainly originating from the covalently attached chromophores. The health benefits of R-PE, its brilliant and intensive fluorescence and vivid colour, and a huge need to replace the synthetic colourants, give R-PE a great potential to be used for food

fortification and colouring. Indeed, it has been already used as a food colourant in Asia (**Fleurence, et al., 2012**). A bright yellow fluorescence of R-PE providing opportunities to prepare visually attractive fluorescent sweets and soft drinks (**Sekar & Chandramohan, 2008**).

One of the major factors for selecting a suitable food colourant is its stability. R-phycoerythrin has good functional stability through a wide pH range (from 3 to 10) (**Liu, et al., 2009**), but the thermal treatment of protein above 40°C significantly reduce its colour intensity (**Munier, et al., 2014**). Roasting dry Nori flakes induces a change of colour from red to green due to the thermal sensitivity of R-PE (**Bito, et al., 2017**). High-pressure (HP) food processing could preserve nutrients, colours, and flavours upon processing, giving it a good potential as an alternative approach for food treatment (**Wgiorgis, 2019**). Another critical factor affecting PBPs colour stability is microelements in the food due to the high propensity of tetrapyrrole chromophores to bind metal ions. Previous studies have shown the ability of various bioactive metal ions (Mn^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, etc.) to influence the colour and fluorescence properties of PBPs (e.g., **Minic, et al., 2016**; **Suresh, Mishra, Mishra, & Das, 2009**). Furthermore, the binding of microelements to R-PE could affect their bioavailability in the gastrointestinal tract and (patho)physiological processes involving these metal ions.

In this research, we study the thermal and HP stability of food-derived R-PE isolated from dried Nori flakes for the first time. We used a combination of precipitation and chromatography techniques to obtain the high-purity protein. Absorption, fluorescence, and CD spectra revealed that purified phycobiliprotein was a typical R-PE with high α-helical content. High-pressure and high-temperature studies, established by absorption measurements, indicated that pressure treatment has substantially fewer adverse effects on R-PE stability than thermal processing. A fluorescence quenching approach characterises the binding of selected microelements (Zn^{2+} and Cu^{2+}) to R-PE. At the same time, the effects of metal ions on the stability of R-PE were evaluated by absorption and CD spectroscopy.

2. MATERIALS & METHODS

2.1. Materials

Dried Nori flakes (*Porphyra purpurea* collected from off the coast of the Galicia region in Spain) were purchased from Porto-Muíños company (Spain). Hydroxyapatite (HA) resin and DEAE-Sepharose were bought from BioRad (CA, USA) and Sigma (MO, USA), respectively. Spectroscopic characterisation of R-PE, high-pressure, and metal-binding experiments were performed in 20 mM HEPES (pH 7.0), while 20 mM phosphate (pH 7.0)

was used for the R-PE thermal unfolding. All other chemicals were of analytical reagent grade, and Milli-Q water (Millipore, Molsheim, France) was used throughout the experiments.

2.2. R-phycoerythrin isolation and purification

Nori flakes were homogenised with liquid nitrogen, and obtained powder was resuspended in 20 mM phosphate buffer, pH 7.0. The suspension was mixed for 4 hours on the magnetic stirrer, followed by sonication with the ultrasonic probe at 30W (Branson Sonifier 150, Emerson, MO, USA) for 5 minutes (intervals of 20 seconds of sonification and 10 seconds of pause). We centrifuged obtained suspension and precipitated the proteins from the supernatant with ammonium sulfate (65% of final concentration). The obtained pellet is resuspended and dialysed against 2.5 mM phosphate buffer, pH 7.0 overnight. We applied dialysed sample on HA resin (previously equilibrated with dialysis buffer) to separate R-PE from other PBPs (R-PC and APC). Non-bound fraction contains R-PE, while R-PC and APC are bound to HA resin. The phosphate buffer concentration in the R-PE fraction is adjusted to 40 mM to obtain the same ionic strength as the equilibration buffer for IEC. R-phycoerythrin was further purified on DEAE-Sepharose at pH 7.0. Non-bound proteins are washed with equilibration buffer, while R-PE is eluted with 40 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. We analysed obtained fractions using UV/VIS absorption spectroscopy (**Section 2.3**) and SDS-PAGE under standard reducing conditions. Gels were first incubated in 1 M $ZnSO_4$ for 15 min (**Minic, et al., 2018**), then visualised fluorescent PBP bands on Typhoon FLA 7000 imager (GE Healthcare, Sweden). The same gels are then stained using Coomassie Brilliant Blue R-250.

2.3. UV/VIS absorbance measurements

UV/VIS absorption spectra were recorded on a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) in a quartz cuvette with a 1.0 cm pathlength. The spectra were collected in the range 190–750 nm at room temperature. R-PE concentration was determined using equation (**Nikolic, Minic, Macvanin, Stanic-Vucinic, & Velickovic, 2020**):

$$R-PE\ \left(\frac{mg}{mL}\right) = 0{,}1247 \times \left((A_{564} - A_{730}) - 0{,}4583 \times (A_{618} - A_{730})\right) \quad (\mathbf{1})$$

The absorption spectra of R-PE (0.25 µM) were measured in the absence or the presence of Zn^{2+} or Cu^{2+} metal ions (both 10 µM). Absorption of Cu^{2+}/Zn^{2+} solutions without R-PE was subtracted to correct background.

The effects of temperature on VIS absorption spectra of R-PE were studied on UV-1800 Shimadzu spectrophotometer (Japan), using Peltier element for temperature control. The

absorbance of R-PE solution (0.37 μM) was recorded in a quartz cuvette with a 1.0 cm pathlength, between 450 and 650 nm, with a bandwidth of 1 nm and a data interval of 0.2 nm, at a scanning speed of 60 nm/min. Spectra were recorded at various temperatures (between 25 to 85°C, with steps of 10°C). The temperature increased at a 5°C/min speed, while equilibration time was set to 3 minutes for each temperature point. Measurements of buffer solution were performed at the same conditions as above, and recorded spectra were subtracted from the corresponding protein spectra.

2.4. *In situ* high-pressure VIS absorbance measurements

Visible (VIS) absorption spectra under *in situ* HP were recorded on a Cary 3E spectrometer (Varian, Palo Alto, CA, USA) using an HP optical bomb with sapphire windows and HP generator as previously described (**Minic, et al., 2020**). A square quartz cell (with an optical pathlength of 5 mm) containing the sample was positioned within the HP optical bomb. A plastic membrane on the top of the cell separated the sample from the pressure-transmitting liquid (H_2O). The absorbance of R-PE solution (0.37 μM) was recorded between 450 and 650 nm, with a bandwidth of 1 nm and a data interval of 0.2 nm, at a scanning speed of 60 nm/min. Spectra were recorded at various pressures (between 0.1 to 450 MPa, with steps of 10 or 30 MPa) at 20°C. The pressure was increased at 10 MPa/min speed, while equilibration time was set to 3 min for each pressure point. Measurements of buffer solution were performed at the same conditions as above, and recorded spectra were subtracted from the corresponding protein spectra. An increase of pressure induces compression of the sample and, consequently, measured absorbances are higher. Therefore, we corrected the spectra by multiplying the experimental absorbance values by the relative solvent volume ($V(P_{measured})/V$ (0.1 M Pa)). The dependence of the relative volume of water was taken from (**Vedam & Holton, 1967**). Data analyses are provided in the Supplementary material.

2.5. Fluorescence measurements

The fluorescence spectra were recorded with a FluoroMax®-4 spectrofluorometer (HORIBA Scientific, Japan) under thermostable conditions (25°C), using 5 nm excitation and 5 nm emission slit widths. The excitation wavelength was set at 488 nm, and the emission spectra were recorded between 510–650 nm. The synchronous fluorescence spectra were collected with interval $\Delta\lambda = 10$ nm, where $\Delta\lambda = \lambda em - \lambda ex$.

The binding of metal ions to R-PE was studied by the fluorescence quenching titration method using intrinsic fluorescence of R-PE. The experimental details are provided in the Supplementary material.

2.6. CD spectroscopy measurements

CD measurements were carried out on Jasco J-815 spectropolarimeter (Jasco, Japan) under constant nitrogen flow. All spectra were recorded at 25°C. For the near-UV and visible region (250–600 nm), the concentration of R-PE was 0.25 µM, while the concentration of Cu^{2+}/Zn^{2+} was 10 µM. Buffer and metal ion spectra were subtracted from spectra of R-PE and R-PE/metal complexes, respectively. Scan speed and number of accumulations were set to 200 nm/min, and two accumulations. A cell with an optical path length of 1 cm was used.

Far-UV CD spectra of 0.67 µM R-PE in the absence or presence of 27 µM Zn^{2+} or Cu^{2+} were recorded in the range 185–260 nm, at a scan speed of 50 nm/min, using a cell with an optical path length of 0.1 mm and with an accumulation of two scans. Buffer and metal ion spectra were subtracted from spectra of R-PE and R-PE/metal complexes, respectively. Secondary structure content was calculated by CD Pro software, using the SELCON algorithm and SP37 database.

Thermal unfolding of 75 nM R-PE was performed at the temperature range of 32–95°C, with a temperature increasing rate 2°C/min, while equilibration time was set to 1 min before each measurement. Ellipticity was measured in the far-UV region at 222 nm with pathlength cells of 1 cm. Results were normalised between 0 and 100 and expressed as a dependence of the percentage of R-PE unfolding on temperature. We fitted obtained data using the model, which comprises two transitions described previously (Greenfield, 2006), with slight modifications (see Supplementary Materials for further details on data analysis).

3. RESULTS

3.1. Purification of R-phycoerythrin

We performed a combination of precipitation and chromatography techniques to obtained pure R-PE from Nori flakes. Purification of R-PE has been monitored using UV/VIS absorption spectroscopy and SDS-PAGE. The raw extract exhibited strong absorption in the 220–400 nm region due to various pigments. Ammonium sulfate precipitation (65%) induced a substantial increase in rations A_{560}/A_{280} (from 0.2 to 1.0; **Fig. 1B**) by removing most of the non-protein impurities absorbing in the region 220–400 nm. This step did not distinguish between different PBPs, as could be concluded from the presence of R-PC and APC absorption bands in the region 600–700 nm (**Fig. 1B**). The bands on SDS-PAGE at 18 kDa after visualisation of PBPs fluorescence **(Fig. 1D**) and CBB staining (**Fig. 1D**) confirmed the presence of R-PC and APC. R-phycoerythrin has been mostly separated from R-PC and APC using hydroxyapatite chromatography in the batch mode (**Fig. 1**). The non-bound fraction

with light reddish-pink colour (**Fig. 1C**) contained R-PE with a remarkable increase of A_{560}/A_{620} ratio (8.9) in comparison to the sample before chromatography (2.1). At the same time, R-PC and APC remained bound to the HA matrix (**Fig. 1C**), and the band's absence at 18 kDa was observed (**Fig. 1D**). Although R-PC and APC were removed in this step, the A_{560}/A_{280} ratio was still low (1.1), indicating other proteins not belonging to the PBP family. Therefore, we have performed anion-exchange chromatography on DEAE-Sepharose to purify R-PE further. R-phycoerythrin has been eluted with 150 mM NaCl, and a significant increase in A_{560}/A_{280} (5.1) has been observed, indicating that pure R-PE is obtained. Additionally, SDS-PAGE of IEC fraction of R-PE showed only bands arising from R-PE: a strong band at ~20 kDa, corresponding to α and β subunits, and two discrete bands between 30 and 35 kDa corresponding to γ subunits (**Fig. 1D**). Overlapped α and β subunits at 20 kDa resulted from similar molecular masses (18.9 and 20.2 kDa, respectively) that could not be separated under applied conditions.

3.2. Spectroscopic characterisation of purified R-phycoerythrin

The absorption spectra of purified R-PE showed the typical peak at 560 and shoulder at 545 originating from the PEB chromophore. In comparison, the PUB chromophore gave a characteristic peak at 496 nm (**Fig. 1B**). Excitation of R-PE at 488 nm revealed the broad, distinct peak at 573 nm with the shoulder at 620 nm (**Fig. 2A**). The absence of symmetry in emission spectra of R-PE has been already described as the consequence of multiple interacting PEB and PUB chromophores in the molecule (**Gaigalas, et al., 2006**). A synchronous fluorescent spectrum could overwhelm challenges in interpreting classical fluorescence spectra of molecules with different fluorophores. Indeed, the synchronous spectrum of R-PE gave a narrow, symmetric peak at 573 nm without shoulder at 620 nm (**Fig. 2B**). The asymmetric environment of tetrapyrrole chromophores within R-PE induced strong signals in its near-UV/VIS CD spectra. Consistent with absorption spectroscopy, three peaks were observed in the VIS region originating from both PEB (540 and 570 nm) and PUB (498 nm) chromophores (**Fig. 2C**). Additionally, a strong peak at 306 nm is observed from a higher excitation state of the PEB chromophore (**Fig. 2C**) (**Glazer & Hixson, 1975**).

Secondary structures of R-PE was characterised by CD spectroscopy. Far-UV CD spectrum of R-PE at pH 7.0 showed negative ellipticity between 200 and 240 nm, with two minima at 208 and 221 nm and a positive peak at 193 nm (**Fig. 2D**), a typical characteristic of α-helical proteins. The calculated α-helical content (75%) in R-PE is in agreement with previously published data, where the percentage of α-helix varies in the range 60–79%

(**D'Agnolo, Rizzo, Paoletti, & Murano, 1994**; **MacColl, et al., 1999**; **Ritter, Hiller, Wrench, Welte, & Diederichs, 1999**).

3.3. R-phycoerythrin thermal stability

The decrease in R-PE ellipticity at 222 nm following heating, as a consequence of α-helical loss, helps to study protein stability. The representative melting curve of R-PE is shown in **Fig. 3A**. Interestingly, the unfolding curve of R-PE showed two transitions with melting points at 56.6 and 72.6°C, and corresponding unfolding enthalpies of 263.6 and 575.4 kJ/mol. This phenomenon could be ascribed to the heteromeric structure of R-PE.

In the second approach, we studied the effects of temperature on VIS absorption spectra of R-PE. An increase of temperature up to 45°C induced a slight decrease in absorbance in the whole spectrum, without changes in the shape of spectra (**Fig. 3B**). Further heating of the R-PE sample caused a significant reduction of absorption intensity, followed by the complete disappearance of the PEB band (545-560 nm) at 85°C (**Fig. 3B**). In comparison, the PUB band (496 nm) at 85°C still existed, but its spectral shape was significantly changed as the peak became broader (**Fig. 3B**). Absorbances at 560 and 496 nm were decreased by 86 and 52%, respectively, at 85°C. Therefore, PUB chromophore exhibits better thermal stability in comparison to PEB chromophore. Lowering the temperature from 85 to 25°C, the PEB band could not recover, while the PUB band was still broad (**Fig. S1**), indicating irreversible denaturation of R-PE.

3.4. R-phycoerythrin high-pressure stability

We performed *in situ* HP VIS absorption spectroscopy to probe R-PE stability at HP conditions. Changes in a well-defined VIS absorption spectrum of R-PE could give valuable information on the effect of the treatment on protein dissociation and unfold. A moderate increase of pressure, up to 90 MPa, did not induce significant changes in the absorption spectra of R-PE, except that shoulder at 545 nm became more visible, and the slight redshift (from 496 to 498 nm) of the PUB band has been observed (**Fig. 3C**). Further increase of pressure, up to 150 MPa, induced a slight decrease of absorption intensity at 560 and 498 nm, while an additional increase in absorbance at 545 nm has been observed. Above 150 MPa, there was a significant decrease in absorbance in the whole spectrum. The most noticeable change in the protein spectrum was the disappearance of the fine peak structure at 560 nm: at 360 MPa, it is wholly merged with the rest of the PEB band. An increase of pressure to 450 MPa induced an additional decrease in absorption intensities: at 560 and 498 nm, absorbances were lower for 37 and 26%, respectively, than 0.1 MPa (**Fig. 3C**). This result indicated that

the PUB band is better preserved at HP conditions, in line with high-temperature measurements.

Based on the disappearance of the fine structure of the PEB peak at 560 nm, we constructed a dissociation/unfolding curve (**Fig. 3D**), and the obtained result is well fitted with the two-transition model (equation 2 in Supplementary Materials). This model enables us to extract the volume changes (ΔV), Gibbs free energies of dissociation/unfolding (ΔG) and the values of half-denaturation/dissociation pressure ($P_{1/2}$) (**Table 1**). There were significant differences in ΔG and $P_{1/2}$ values, while the difference in the volume change was not so pronounced between the two transitions (**Table 1**). Interestingly, plotting the dependence of the redshift of the PUB band on pressure gave the one-transition curve (**Fig. S2**) with the fitting parameters very similar to the values obtained by fitting the first transition of the PEB band (**Table 1**; Equation 5 in Supplementary Materials). R-phycoerythrin unfolding is followed by a significant decrease in its PUB and PEB bands absorbance (**Liu, et al., 2009**). At ~110 MPa, where the first transition of the PEB band and the redshift of the PUB band occur, there was a negligible decrease in absorbance. Therefore, it is unlikely that the first transition is due to protein unfolding. A more pleasurable explanation is that a change in interactions between chromophores and subunits occurs at lower pressures (~110 MPa) during the first transition. Indeed, the previous studies proved that the fine structure of peak at 560 nm arises from dimers of PEB chromophores at the subunit interfaces within R-PE hexamer (**Gaigalas, et al., 2006**; **Womick, Liu, & Moran, 2011**). Therefore, partial dissociation of R-PE hexamer induced the loss of fine structure of PEB band at 560 nm through dissociation of PEB dimers at the subunit interfaces. The second transition at ~250 MPa was followed by the disappearance of the fine structure of the PEB peak at 560 nm and with a significant decline in absorption intensity, suggesting that both proteins unfolding and dissociation occur.

Decreasing the pressure from 450 to 0.1 MPa induced recovering the fine structure of the peak at 560 nm. However, the absorption intensity was only partially recovered: R-PE's absorbances after HP treatment at 560 and 496 nm were lower for 24 and 16%, respectively, compared to its spectrum before treatment (**Fig. S3**). Therefore, similar to *in situ* study, *ex-situ* measurement showed better preservation of the PUB band than the PEB band upon HP treatment.

3.5. The binding of Cu^{2+} and Zn^{2+} ions to R-phycoerythrin

R-Phycoerythrin exhibited a strong fluorescence at 573 nm, but adding Zn^{2+} and Cu^{2+} ions strongly quenched its fluorescence intensity (**Figs. 4A** and **4B**). In general, there are two

types of quenching: static (contact) and dynamic (collisional). Stern-Volmer (SV) plots (**Fig. S4**) for both metal ions were linear (r^2 is 0.993 and 0.989 for Zn^{2+} and Cu^{2+}, respectively), proving that only one type of quenching was present under applied conditions. The slope of these curves represented an SV quenching constant of $1.7x10^3$ M^{-1} (Zn^{2+}) and $0.83x10^6$ M^{-1} (Cu^{2+}), with a bimolecular quenching rate constant of $2.4x10^{11}$ (Zn^{2+}) and $1.2x10^{14}$ $M^{-1}s^{-1}$ (Cu^{2+}), one and four orders of magnitude higher than the diffusion rates of biomolecules (10^{10} $M^{-1}s^{-1}$). These data suggest that static (contact) quenching of R-PE fluorescence by Zn^{2+} and Cu^{2+} ions occurs through the stable complex formation between R-PE fluorophores and metal ions. Changes in absorption and near-UV/VIS CD spectra of R-PE (**Figs. 5A** and **5B**) in the presence of these metal ions also confirmed a static mechanism of quenching of protein fluorescence. Cu^{2+} shows a more substantial quenching effect on R-PE fluorescence than Zn^{2+} (**Figs. 4A** and **4B**). Consequently, the binding constant of Cu^{2+} to R-PE ($6.3x10^5$ M^{-1}; **Fig. 4D**) is much higher than the affinity of Zn^{2+} ($1.74x10^3$ M^{-1}; **Fig. 4C**).

The effects of metal ions binding on the conformation of tetrapyrrole chromophores of R-PE were evaluated by absorption and CD spectroscopy. The presence of Cu^{2+} induces a moderate decrease of absorbances at 496 nm (13%), 560 nm (10%), and 545 nm (14%) in comparison to free R-PE (**Fig. 5A**). On the contrary, there was a discrete effect of Zn^{2+} on the absorption spectrum of protein: the slight decrease of band intensity at 496 nm was observed (5%). The changes of absorbances at 560 and 545 nm were negligible, except for the slight change in the shape of the shoulder at 545 nm. The binding of Cu^{2+} significantly influenced near-UV/VIS CD spectra of R-PE (**Fig. 5B**). The most considerable reduction in band intensities was at 306 nm (33%) and 545 nm (25%), suggesting the preferential Cu^{2+} binding to PEB chromophore. In opposite to absorption spectra, near-UV/VIS CD spectra of R-PE were sensitive to the presence of Zn^{2+} (**Fig. 5B**). The intensity of the PUB band (498 nm) was reduced by 23%, while the shape of the PEB band at 545 nm was changed entirely, becoming more narrow and redshifted by 5 nm, followed by a 6% decrease of intensity. The changes in the shape and intensities of PEB bands at 306 and 570 nm upon Zn^{2+} addition are almost negligible. Therefore, zinc ions are preferably bound to the PUB chromophore, although Zn^{2+} binding to PEB chromophore could also be observed, with an absorption/ellipticity peak at 545 nm. The binding constant of R-PE for Zn^2, based on synchronous fluorescence spectra data (**Fig. S5**) when PEB chromophore is selectivity excited, was lower by 36% ($1.1x10^3$ M^{-1}) compared to the value using classical fluorescence spectra (**Figs. 4A** and **C**; $1.7x10^3$ M^{-1}). Zinc ions have a higher preference to bind PUB chromophores, and selective excitation of

PEB chromophores disabling the energy transfer from PUB to PEB, which seems to produce a less pronounced quenching effect Zn^{2+} on PEB chromophore in synchronous spectra.

Finally, far-UV CD spectra were recorded to estimate the effects of metal ions binding on secondary structures of R-PE. The binding of metal ions did not crucially change the spectral region between 208–222 nm (**Fig. 5C**). The only change was observed in band intensity at 193 nm upon adding Cu^{2+} (**Fig. 5C**). As described before, the principal secondary structure in free R-PE is α-helix (75%), while the presence of Cu^{2+} (74%) and Z^{n2+} (77%) has a discrete effect on the α-helical content in protein.

4. DISCUSSION

In the present study, we combined different experimental approaches to probe the stability of food-derived nutraceutical R-PE. We report that high-pressure treatment had significantly less detrimental effects on R-PE stability and colour than thermal treatment. The binding of selected bioactive metal ions (Cu^{2+} and Zn^{2+}) to R-PE induced substantial conformational changes in covalently bound tetrapyrrole chromophores without affecting protein colour and only minor changes in protein secondary structure content.

R-phycoerythrin is purified from commercial dried Nori flakes, obtained from red algae *Porphyra purpurea* collected at the Atlantic coast of Spain. In the algal cells, R-phycoerythrin with other homologous PBPs (R-PC and R-PE) forms phycobilisomes, large photosynthetic complexes (**Apt, et al., 1993**). Therefore, the significant obstacle in R-PE purification is to remove R-PC and APC from the extract. We tackle this challenge by simple sample application of HA resin in batch mode, under the condition where R-PE is the only PBP not bound to the matrix. IEC removed the other proteins. The obtained R-PE's purity ratio of 5.1 (A_{560}/A_{280}) was considerably higher than the commonly accepted criterion for R-PE purity of 3.2 (**Nikolic, et al., 2020**). SDS-PAGE justified the heteromeric structure of R-PE with two bands arising from γ subunit (γ33 and γ31), belonging to the isoforms of R-PE containing two different γ subunits (**Apt, et al., 1993**).

We further utilised absorption, fluorescence and CD spectroscopy to characterise purified phycobiliprotein. It exhibited a typical characteristic of R-PE: well-defined absorption and near-UV/VIS CD bands, and strong fluorescence detectable at pM concentration (**Gaigalas, et al., 2006**; **Liu, et al., 2009**; **Munier, et al., 2014**). The high α-helical content confirmed that the native R-PE structure is preserved in dried Nori flakes. An increase in a temperature-induced gradual decrease of α-helix ellipticity, giving the unfolding R-PE curve with two transitions: at 56°C and 72°C. A previous CD study on the thermal

unfolding of B-PE from Porphyridium cruentum also demonstrates two transitions (**Gonzalez-Ramirez, et al., 2014**). The thermal unfolding of co-isolated R-PC exhibited only transition at ~55°C (data not shown). R-phycocyanin only contains α- and β-subunits (homologous to R-PE subunits) but not γ subunit. Therefore, the second transition (72°C) in the R-PE unfolding curve seems to result from a more stable γ subunit in the protein oligomer structure. The BLAST analysis (**https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins**) found high homology only between α and β subunits, implying the different stability of the γ subunit. Absorption measurements confirm findings from previous studies (e.g., **Munier, et al., 2014**) that the R-PE colour is not stable above 40°C and that the PEB band in the spectrum was much more sensitive to thermal treatment than the PUB band. With three PUB chromophores, γ-subunit mainly contributes to the overall absorption of the PUB band (**Gaigalas, et al., 2006**). Therefore, combining results from absorption and far-UV CD thermal unfolding measurements, it is evident that the γ subunit of R-PE is more stable than the α and β subunits.

We have tested the effects of HP on R-PE stability up to 450 MPa, the value usually used for food processing (**Huang, Hsu, & Wang, 2020**). In situ HP VIS absorption study has shown that the PEB spectral band undergoes two transition processes. The first transition (~110 MPa) is ascribed to the R-PE oligomer dissociation, while the second (~250 MPa) is due to R-PE unfolding and additional dissociation. Subunit dissociation indeed preceded the protein unfolding (**Minic, et al., 2020**). Interaction between subunits in multimeric proteins (such as R-PE) causes cavities at subunit interfaces, which are eliminated at moderate pressures. Thermodynamic parameters of the first transition of the PEB band correspond with those obtained by studying the redshift of the PUB band. The HP-induced redshift of the PUB band indicates the changes in the level of the γ subunit, probably dissociating from the R-PE complex. It has been shown that the γ subunit lies in the central cavity of the R-PE disc, stabilising the αβ hexamer (**Chang, et al., 1996**). It looks that HP induces dissociation of γ subunit from R-PE and destabilises αβ hexamer, dissociating to αβ trimers, αβ monomers or individual subunits. Further increase of pressure causes elimination of cavities within subunits and, as a final consequence, protein unfolding occurs. In accordance with temperature measurements, we found that the PUB spectral band is more resistant than the PEB band to HP treatment, confirming the higher stability of γ subunits compared to α and β subunits.

High-pressure has a prominent advantage in various food treatments, preserving nutrients, colours and flavours. We have shown that after HP treatment at 450 MPa, the shape of the absorption spectrum of R-PE is completely recovered without detrimental loss of

absorption intensity. On the contrary, thermal treatment of R-PE induces irreversible, complete loss of the PEB band (colour bleaching). Therefore, HP treatment of R-PE containing food and beverages could be an excellent alternative to thermal processing to avoid losing vivid R-PE colour. It was already shown the absence of irreversible changes in B-PE structure after HP treatment of Porphyridium cruentum extract at 400 MPa (**Tran, et al., 2019**). Further, the storage stability of the blue C-PC from Spirulina in beverages was obtained by HP processing at 450 MPa (**Zhang, Cho, Dadmohammadi, Li, & Abbaspourrad, 2021**).

The binding of metal ions could substantially influence the colour and fluorescence of PBPs (**Suresh, et al., 2009**). Significant quenching of the R-PE fluorescence at micromolar concentrations of Cu^{2+} in our study (**Fig. 4B**) indicates a strong interaction between protein and this essential microelement. The measured binding constant ($6.3x10^5$ M^{-1}) was in the same order of magnitude as Cu^{2+} affinity for C-PC and phycocyanobilin from Spirulina (**Minic, et al., 2016**). The binding affinity of Zn^{2+} was significantly lower ($1.7x10^3$ M^{-1}). According to near-UV/VIS CD spectra of R-PE, binding of Cu^{2+} and Zn^{2+} induced significant changes in the chiral environment of tetrapyrrole chromophores. In the presence of these ions at 10 µM (~0.6 ppm), only minor (10% for Cu^{2+}) and negligible (<5% for Zn^{2+}) decrease in PEB band absorbance was observed. These microelements are usually present in beverages at similar concentrations (**Fernandez-Lopez, Gomez-Nieto, Gismera, Sevilla, & Procopio, 2018**). Consequently, they should not affect the colour of drinks fortified with R-PE. Tetrapyrrole chromophores in extended conformations exhibit a large fluorescence quantum yield, while its cyclisation reduces the fluorescence of PBPs (**Bocker, et al., 2020**). It seems that the binding of Cu^{2+} and Zn^{2+} caused conformational changes of chromophores from extended to cyclic ones, which are more prone to bind metal ions (**Suresh, et al., 2009**).

Near-UV/VIS CD and absorption measurements revealed that these metal ions have nonidentical preferences to different chromophores within R-PE: Zn^{2+} for PUB chromophore, while Cu^{2+} on the PEB at position 545. Both metal ions had the lowest binding to the PEB 560. This chromophore in the R-PE structure makes a dimer at the interface between α and β subunits, introducing the steric hindrance and limiting metal ions binding potency (**Gaigalas, et al., 2006**; **Womick, et al., 2011**). Although the conformation of tetrapyrrole chromophores was changed, far-UV CD measurements did not show significant changes in the secondary structure content of R-PE upon metal ions binding, confirming that R-PE is still well folded in the presence of Cu^{2+} and Zn^{2+}.

Herein presented results open up attractive possibilities for further investigation and application of R-PE. Many factors limit the bioavailability of dietary Zn and Cu in some food systems (**Wang, Zhou, Tong, & Mao, 2011**). R-phycoerythrin complexation of Zn^{2+} and Cu^{2+} could increase their bioavailability in the gastrointestinal tract. Indeed, it was shown that some proteins and peptides could form complexes with Zn^{2+} and Cu^{2+} ions, which improve their absorption and bioavailability in intestinal conditions (**Guo, et al., 2014**; **Wang, et al., 2011**). The free Cu^{2+} ions generate reactive oxygen species in vivo (**Shin, et al., 2012**). The ability of R-PE to tightly bind cupric ions could prevent their pro-oxidative action. Fluorescent biosensors for detecting heavy metal ions in aqueous media (e.g., water and beverages) have recently gained popularity. They provide high specificity and low detection limits, fast response time, and technical simplicity (**De Acha, Elosua, Corres, & Arregui, 2019**). The ability of Cu^{2+} ions to significantly decrease fluorescence of R-PE even at 0.5 μM concentration (32 ppb) in our study indicates that R-PE could be exploited as a potential biosensor for Cu^{2+} in aquatic life systems. Interestingly, the ability of C-phycocyanin, PBP from Spirulina, to serve as possible biosensors for Hg^{2+} ions was demonstrated (**Bhayani, Mitra, Ghosh, & Mishra, 2016**).

5. CONCLUSIONS

In the present study, we examined the stability of R-PE obtained from commercial dried Nori flakes using spectroscopic methods. Thermal unfolding of R-PE exhibits a two-transition process due to the different stability of R-PE subunits. Protein heating at 85°C irreversibly denatures R-PE with detrimental effects on its colour. Moderate pressure (~110 MPa) induces dissociation of R-PE hexamer, while the further increase of pressure (~250 MPa) causes protein unfolding. Opposite to temperature, HP treatment at 450 MPa has far less destructive effects on R-PE colour intensity. At the same time, absorption bands are mostly recovered after a return to atmospheric pressure. R-phycoerythrin exhibited moderate-high- and low-affinity binding for Cu^{2+} and Zn^{2+} ions, respectively. Although both ions significantly influence the conformation of chromophores within R-PE, there was no substantial effect on protein colour or its secondary structure content.

Our study results suggest that HP processing of R-PE containing food and beverages could preserve protein red colour, a significant advantage compared to the thermal treatment. Further studies are needed for an additional improvement of the HP stability of R-PE. The presence of Cu^{2+} and Zn^{2+} ions does not have a high probability of impairing R-PE function as a food colourant. On the contrary, the binding to R-PE could improve their bioavailability in

the gastrointestinal tract. Therefore, food fortification with copper and zinc ions in the presence of R-PE could be a promising approach for the more efficient delivery of these microelements.

AUTHOR CONTRIBUTIONS

Ana Simovic: Conceptualisation, Formal analysis, Investigation. **Sophie Combet**: Investigation, Writing - review & editing. **Tanja Cirkovic Velickovic**: Funding acquisition, Writing - review & editing. **Milan Nikolic**: Conceptualisation, Formal analysis, Funding acquisition, Investigation, Supervision, Writing - review & editing. **Simeon Minic**: Conceptualisation, Formal analysis, Funding acquisition, Investigation, Supervision, Writing - original draft.

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FIGURE LEGENDS

Figure 1. (**A**) Ribbon model of the crystal structure of R-PE hexamer (PDB: 5B13). γ subunit occupies the central cavity of the R-PE hexamer. However, it is not visible in the electron density of R-PE crystal structure (**Miyabe, et al., 2017**); (**B**) UV/VIS absorption spectra of R-PE after each purification step, as indicated; (**C**) Separation of R-PE (light reddish pink solution) from R-PC and APC (light purple, bound fraction) using HA chromatography in batch mode; (**D**) SDS-PAGE profile of R-PE purification (14% gel) after staining with CBB (left) and Zn2+-induced fluorescence visualisation (right, λex: 473 nm, λem: 580 nm): 1: raw extract; 2: ammonium sulfate precipitation (65%); 3: HA chromatography (supernatant); 4: IEC on DEAE Sepharose (150 mM NaCl); M-molecular size markers.

Figure 2. (**A**) Classical (λex= 488 nm) and (**B**) synchronous fluorescence spectra (Δλ= 10 nm) of R-PE (pH 7.0); Near-UV/VIS (**C**) and far-UV CD (**D**) spectra of R-PE (pH 7.0).

Figure 3. (**A**) The R-PE (75 nM) thermal unfolding curve with the corresponding fit (full red line), obtained by measuring ellipticity at 222 nm and pH 7.0; (**B**) The effects of temperature on VIS absorption spectra of 0.37 μM R-PE at pH 7.0 (optical pathlength 1 cm); (**C**) The effects of HP on VIS absorption spectra of 0.37 μM R-PE at pH 7.0 and 20°C (optical pathlength 0.5 cm): Inset represents the redshift of the band at 496 nm; (**D**) The R-PE

dissociation/unfolding curve with the corresponding fit (full red line) obtained by monitoring the disappearance of peak at 560 nm.

Figure 4. R-PE fluorescence quenching (excitation at 488 nm) in the presence of Zn^{2+} (**A**) and Cu^{2+} (**B**) ions at 25°C and pH 7.0; Fluorescence quenching based plots for determination of binding constants of Zn^{2+} (**C**) and Cu^{2+} (**D**) to R-PE.

Figure 5. The effects of Zn^{2+} and Cu^{2+} binding on VIS absorption (**A**), near-UV/VIS CD (**B**) and far-UV CD (**C**) spectra of R-PE at pH 7.0 and 25ºC.

TABLES

Transition	ΔV (mL/mol)	ΔG (kJ/mol)	$P_{1/2}$ (MPa)
1^{st} transition in PEB band	-79 ± 9	8.2 ± 0.5	107 ± 7
2^{nd} transition in PEB band	-58 ± 10	14.9 ± 3.3	247 ± 16
The redshift of the PUB band	-70 ± 6	8.1 ± 0.6	115 ± 3

Table 1. Thermodynamic parameters of pressure unfolding/dissociation for R-PE sample, obtained from HP-VIS absorption spectroscopy at 20°C and pH 7.0.

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