Supplementary material

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

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2. MATERIALS AND METHODS: DATA ANALYSIS

All data analyses were performed using OriginPro (MA, USA) software.

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

High-pressure data were analysed using two approaches:

The first approach was based on studying the HP-induced disappearance of the fine structure of the peak at 560 nm. To perform such analysis, it was necessary to determine the λ_{start} of the beginning of the peak at 560 nm. λ_{start} corresponded to the local minimum in the 2nd derivative spectrum. The specific area of the fine structure of the peak at 560 nm (A_s) in the basic spectra was determined in the interval between λ_{start} and λ_{end}, where λ_{end} represented the end of the fine structure of the 560 nm band. The absorbance at λ_{start} and λ_{end} was the same, and it has been used as a baseline to calculate A_s. The total area (At) was calculated similarly to As but without baseline subtraction. The relative area (Ar) is the ratio between As and At. The percentage of R-PE unfolding/dissociation was determined using the following equation:

$$\%_{R-PE\ unfolding/disscociation} = \frac{Ar_{0.1MPa} - Ar_P}{Ar_{0.1MPa}} \quad (\mathbf{1})$$

where $Ar_{0.1MPa}$ and Ar_P represent the relative area of the 560 nm band at 0.1 MPa and a given pressure, respectively. The results were expressed as the percentage of unfolded/dissociated R-PE as a function of pressure. The Gibbs free energy (ΔG at 0.1 MPa) and the apparent volume change of unfolding/dissociation (ΔV) were determined by fitting the pressure denaturation curves with the two-transition model using an equation adapted from (**Minic, et al., 2020**):

$$\%_{R-PE\ unfolding/disscociation} = 100 - \left(\frac{a}{1 + e^{-\frac{\Delta G_I + \Delta V_I x P}{RT}}} + \frac{b}{1 + e^{-\frac{\Delta G_{II} + \Delta V_{II} x P}{RT}}} \right) (\mathbf{2})$$

where a and b represent a relative contribution of the first and second transition, respectively, while a+b=100. The pressure value at which one-half of proteins is unfolded/dissociated (50%) represents the half-denaturation/dissociation pressure ($P_{1/2}$) of R-PE.

The second approach was based on monitoring the HP-induced redshift of the 498 nm band. The centre of spectral mass of the 498 nm-band was calculated using the equation:

$$\lambda = \frac{\sum_j A_j \lambda_j}{\sum_j A_j} \quad (\mathbf{3})$$

where Aj is absorbance intensity at wavelength λj in the range from 490 to 510 nm.

The percentage of protein unfolding/dissociation was determined using the following equation:

$$\%_{R-PE\ unfolding/disscociation} = \frac{\lambda_P - \lambda_{0.1MPa}}{\lambda_{330MPa} - \lambda_{0.1MPa}} \ (4)$$

where λ_P, $\lambda_{0.1MPa,}$ and λ_{330MPa} represent the centre of spectral mass at a given pressure, 0.1 MPa and 330 MPa, respectively. The absorption at 330 MPa was used because the redshift transition of the 498 nm band was completed at this pressure. The results were expressed as the percentage of unfolded BLG as a function of pressure. The obtained curve was fitted into a one-transition model as described previously (**Minic, et al., 2020**):

$$\%_{R-PE\ unfolding/disscociation} = 100 - \frac{100}{1 + e^{-\frac{\Delta G_{0.1\ MPa} + \Delta V * P}{RT}}} \ (5)$$

2.5. Fluorescence measurements

The binding of metal ions to R-PE was studied by the fluorescence quenching titration method using intrinsic fluorescence of R-PE at a constant concentration (0.59 nM) and various Zn^{2+} (0–1 mM) and Cu^{2+} (0–10 μM) concentrations. The change in fluorescence emission spectra was measured after precisely 1 min of adding each aliquot of metal ion to the protein solution. Emission of Cu^{2+}/Zn^{2+} solutions without R-PE was subtracted to correct background fluorescence.

To determine the type of quenching, Stern-Volmer's (SV) quenching constant was calculated according to the relationship (Lakowicz, 2006):

$$\frac{\mathbf{F_0}}{\mathbf{F}} = \mathbf{1} + \boldsymbol{k_q}\boldsymbol{\tau_o}[\mathbf{Q}] = \mathbf{1} + \boldsymbol{K_{SV}}[\mathbf{Q}] \ (6)$$

where F_0 and F are protein emission fluorescence at 573 nm without and with the addition of metal ion, respectively, k_q is the quenching rate constant of the biomolecule, τ_o is the lifetime of the phycoerythrin without quencher ($7.1x10^{-9}$ s) (**Brody & Brody, 1961**), [Q] is the concentration of Cu^{2+} or Zn^{2+}, and K_{SV} is SV quenching constant. K_{SV} is equal to the slope of the SV plot.

The association (binding) constant (K_a) for the R-PE: metal ion complex was calculated using the equation (**Bia, et al., 2004**):

$$\log\frac{\mathbf{F_0} - \mathbf{F}}{\mathbf{F}} = -\boldsymbol{n}\log\frac{\mathbf{1}}{[\mathbf{M}] - [\mathbf{P}]\frac{\mathbf{F_0} - \mathbf{F}}{\mathbf{F_0}}} + \boldsymbol{n}\log\boldsymbol{K_a} \ (7)$$

where [P] and [M] are the total concentrations of protein (R-PE), and metal ion (Cu^{2+} or Zn^{2+}), respectively, and K_a is the binding constant.

All experiments were performed in triplicate. Data were analysed using the Student's *t*-test. A value of $p < 0.05$ was considered significant.

2.6. CD spectroscopy measurements: Thermal unfolding of R-PE

Thermal unfolding of R-PE was studied by measuring ellipticity at 222 nm. Experimental data were normalised between 0 and 100 % using the equation:

$$w\ (\%\ of\ unfolded\ R-PE) = \frac{\theta_{start} - \theta_T}{\theta_{start} - \theta_{end}}\ (\mathbf{8})$$

where θ_{start}, θ_{end} and θ_T, represents ellipticity in mdeg at 32 °C, 95 °C, and a given temperature, respectively.

R-phycoerythrin unfolding data, obtained by far-UV CD spectroscopy, were fitted into a two-transition model, as described previously (**Greenfield, 2006**), with slight modifications:

$$y = \frac{k_1}{k_1 + 1} * U_1 + \frac{4 * C * k_2 + 1 - \sqrt{8 * C * k_2 + 1}}{4 * C * k_2} * U_2\ (\mathbf{9})$$

$$k_1 = e^{\left(\frac{H_1}{1.987 * T}\right) * \left(\frac{T}{T_{m1}} - 1\right)}\ (\mathbf{10})$$

$$k_2 = e^{\left(\frac{H_2}{1.987 * T}\right) * \left(\frac{T}{T_{m2}} - 1\right) - \ln(C)}\ (\mathbf{11})$$

In all parameters, 1 and 2 in subscripts correspond to the first and second transition during R-PE unfolding, respectively.

C, k, T, T_m, and H represent R-PE concentration (7.5×10^{-8} M), unfolding constant, the variable temperature in Kelvin, melting temperature in Kelvin and enthalpy of unfolding in cal/mol, respectively.

U_1 and U_2 represent a relative contribution of the first and second transition, respectively, while $U_1 + U_2 = 1$.

3. RESULTS

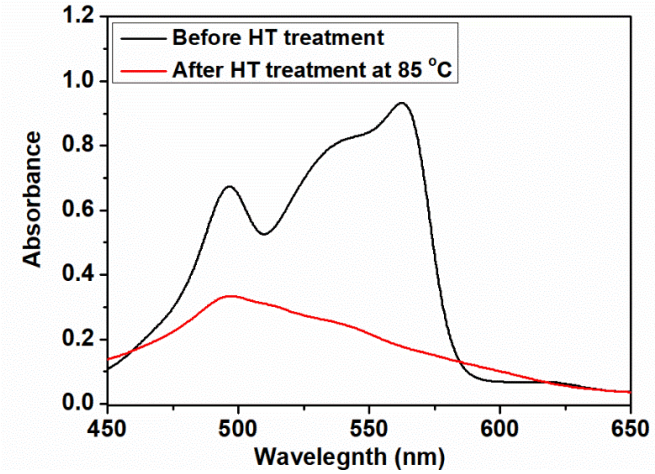

Figure S1. The absorption spectra of 0.37 µM R-PE before and after treatment at 85 °C (optical pathlength 1 cm).

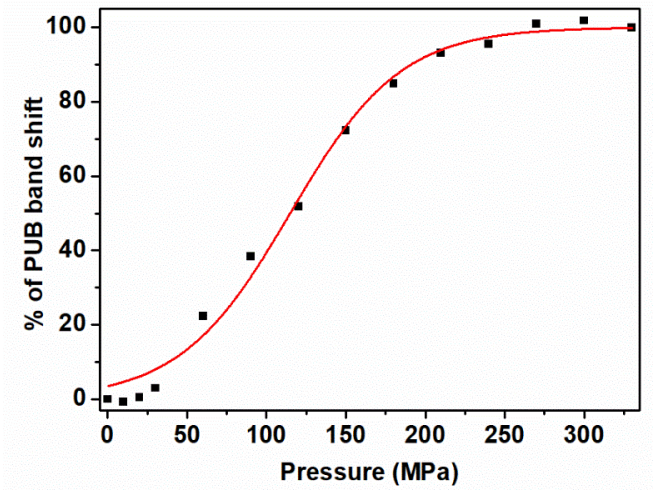

Figure S2. The R-PE dissociation/unfolding curve with the corresponding fit (full red line) was obtained by monitoring the redshift of the PUB band at 498 nm.

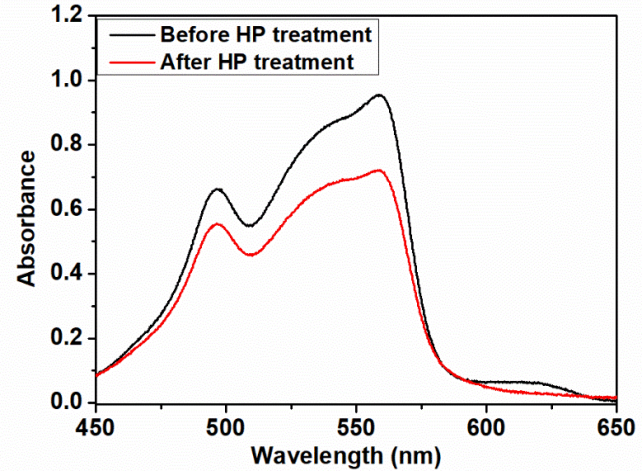

Figure S3. The absorption spectra of 0.37 µM R-PE before and after HP treatment at 450 MPa and 20 °C (optical pathlength 1 cm).

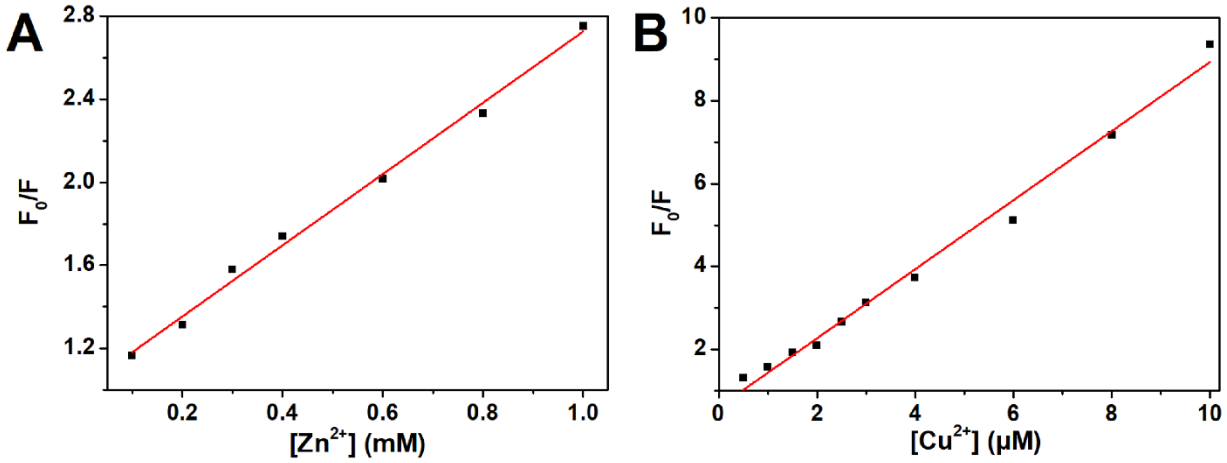

Figure S4. Stern-Volmer plots of R-PE fluorescence quenched by Zn^{2+}(**A**) and Cu^{2+}(**B**) at pH 7.0 and at 25 °C.

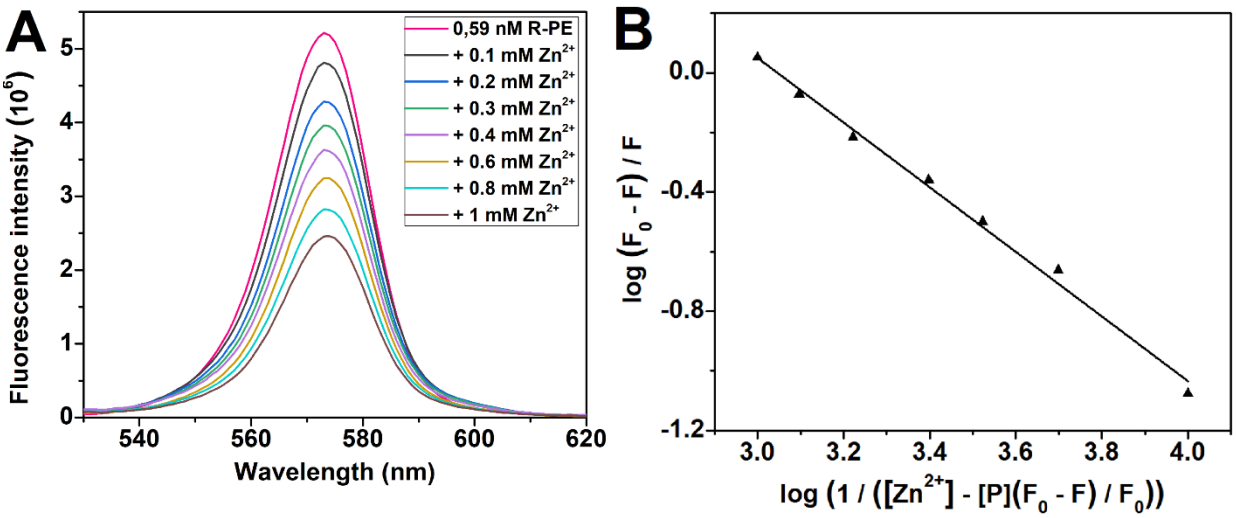

Figure S5. (**A**) Quenching of synchronous fluorescence spectra (Δλ=10 nm) of R-PE by Zn^{2+} at pH 7.0 and at 25 °C; (**B**) Synchronous fluorescence quenching based plots for determination of binding constants of Zn^{2+} to R-PE.

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