STABILITY OF FREE AND ENCAPSULATED C-PHYCOCYANIN IN DIFFERENT pH, TEMPERATURE AND STORAGE CONDITIONS

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ABSTRACT

Phycocyanin-C (C-PC) is a blue fluorescent bioactive biliprotein originated from cyanobacteria with applications as natural colorant in food, medicine, and cosmetics. However, C-PC sensitivity to stress conditions still limits its commercial applications. Hence, this work aimed to evaluate the properties and stability of C-PC encapsulated in chitosan matrices to select formulations capable of increasing its resistance to stress conditions. The colorimetric and fluorometric properties of free C-PC and encapsulated C-PC in chitosan (SPD-1) and in chitosan with tripolyphosphate (SPD-2) were evaluated under stress conditions (pH 2 to 10 and temperature 25 to 70°C), as well as their stability in the presence and absence of light for two and four weeks, respectively. The encapsulation improved the thermal stability of C-PC, preserving around 70% of its absorbance at 70 °C. Moreover, the encapsulated samples were more stable under stress in alkaline environments. Therefore, the encapsulation of C-PC in polymeric matrices can improve the maintenance of its colorimetric properties in adverse conditions and be an alternative for developing new sustainable technologies to expand the application of natural colorants in foods and cosmetics.

Keywords: Natural colorants. Natural polymers. Encapsulation Spray-drying. Protein stability.

1 INTRODUCTION

The growing interest in natural colorants derives from the consumer's rejection of products with synthetic and chemical substances, motivated by concerns surrounding health and environmental impacts. [1] Through the advances in biotechnology, researchers and industries are heading towards more sustainable alternatives, such as substituting synthetic additives for natural compounds in their products to improve the quality of their goods and the perception of consumers toward their brands. C-Phycocyanin (C-PC) is an example of a natural colorant used to substitute synthetic colorants in foods and cosmetics.

C-PC is a blue-colored protein produced by cyanobacteria that belongs to the group of phycobiliproteins. This protein class comprises light-harvesting macromolecules linked to open-chain tetrapyrroles as a prosthetic group. More recently, C-PC has reached a global annual market of 250 million dollars. Its increasing popularity is due to its many possible applications in several fields, such as food technology and cosmetics, acting as a natural dye, and pharmaceutical industries for its antioxidant, anti-inflammatory and anticancerous capabilities. [2]

Unfortunately, natural colorants such as C-PC are susceptible to stability issues, showing quick degradation in stress conditions. Encapsulation technologies using natural polymers, like chitosan, have been emerging as a successful sustainable solution to stability problems that natural colorants face, proving to be successful in enhancing resistance to high temperatures, acidic and alkaline pH, and increasing storage time. [1]

With this in mind, this work sought to evaluate the stability of free and encapsulated C-PC chromoprotein in chitosan matrices using colorimetric analysis aiming to contribute to the development of natural and bifunctional products with advantages and higher resistance, reducing health risks and environmental impacts.

2 MATERIAL & METHODS

Initially, free C-PC (Xian Pincredit Bio-Tech Co. Ltd, A_{620}/A_{280}= 1.79) was evaluated at a concentration of 0.3 mg/mL, pH 6, and 25 °C to obtain an absorption spectrum from 310 to 760 nm using a plate reader. Then a calibration curve was established (concentrations 0.1, 0.3, 1, 2, 5, and 15 mg/mL) at the absorbance peak (wavelength (λ) of 620 nm). With the calibration curve, an initial absorption of 0.3 AU was determined for the following experiments. Stability studies of free C-PC under stress conditions such as high temperatures and alkaline and acidic environments were performed. Samples were prepared at pH 6.0 ± 0.1 to study the effect of temperature on the free and encapsulated C-PC and maintained at the temperatures of 25°C, 50°C, 60°C and 70°C, then analyzed by their absorbance at $\lambda_{620\ nm}$ in intervals of 0.5 h, 1.0 h, 2.0 h, and 2.5 h. The effect of pH was evaluated by

preparing samples at pH 2, 4, 6, 8, 10, and 12 (± 0.2), kept at a temperature of 25 °C for 30 minutes before assessment of the absorbance spectra.

After evaluating the properties and stability of free C-PC, the effects of encapsulation with chitosan on the colorimetric properties of the blue protein under different stress conditions were determined. The encapsulation occurred using chitosan microcapsules prepared by spray-dry technique, in which C-PC was added to a solution of chitosan and 5% acetic acid and, later on, dried in a spray-dryer. Samples of C-PC free or encapsulated in chitosan (SPD-1 and SPD-2) were prepared in purified water at pH 6 ± 0.1 with initial absorbance at λ_{620nm} of 0.3 AU. The samples were kept at 25°C and 70°C for 0.5 h and evaluated by their absorbance spectra to understand the effect of encapsulation on their thermal stability.

As for the study of the effect of different pH on free and encapsulated C-PC, samples of free C-PC, SPD-1, and SPD-2 at pH 2 ± 0.2, 6 ± 0.2, and 10 ± 0.2 were prepared and kept at 25°C for 0.5 h. Again, analysis using absorbance spectra was carried out considering an initial absorbance at λ_{620nm} of 0.3 AU for analysis in plate readers.

3 RESULTS & DISCUSSION

First, it was determined the absorbance spectrum of free C-PC at pH 6 and 25 °C to define the parameters for the following studies (**Fig. 1.A**). Then, the effect of different temperatures (**Fig. 1.B**) and pH (**Fig. 1.C**) were evaluated on the absorbance of C-PC to select the best stress conditions for the test with the encapsulated samples.

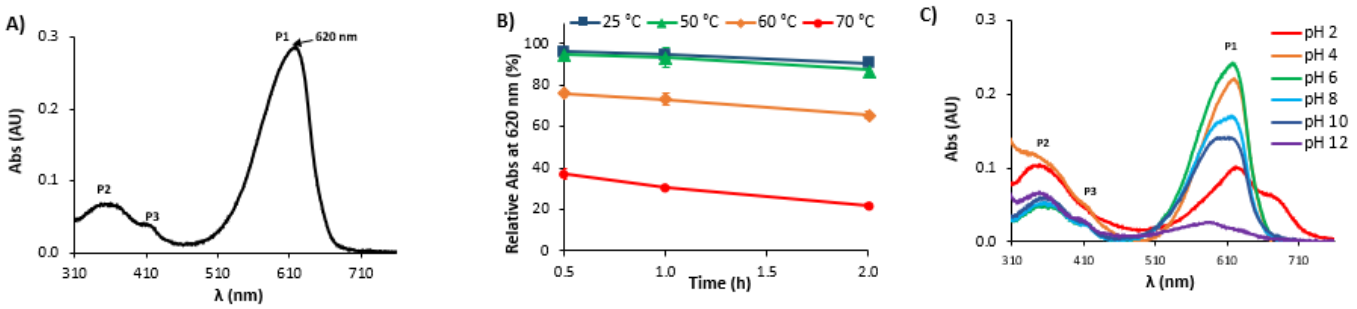

Fig. 1. Characterization and stability of C-PC. A) Absorbance spectrum of C-PC. **B)** Relative Abs (at λ 620 nm) with respective standard deviations of C-CP at different times and temperatures. **C)** Absorbance spectra of C-PC after 30 min at different pH (± 0.2). Initial Abs was 0.3 AU at λ 620 nm. Except for **B)**, the temperature was maintained at 25 ± 2 °C, and except for **C)**, the pH of solutions was 6.0 ± 0.1.

Fig. 1.A illustrates the absorbance spectrum of C-PC. This spectrum shows the presence of three absorbance peaks for the CP-C solution, with the peak at $\lambda_{620\ nm}$ (P1) presenting the highest absorbance for C-PC, as it is the absorbance of its characteristic blue chromophore.[3] The peaks near $\lambda_{300\ nm}$ are related to the presence of amino acids with intrinsic absorbance and fluorescence in the structure of this protein, such as tryptophan, phenylalanine, and tyrosine, reported in the amino acid sequence of C-PC.[4]

As observed in **Fig.1.B**, C-PC maintained above 90 % of its absorbance at $\lambda_{620\ nm}$ for the two hours of evaluation. In contrast, C-PC at 70°C only presented 37.2% of its initial value after 0.5 h, thus supporting the fact that C-PC has low stability at high temperatures. As for the pH, **Fig. 1.C** illustrates that very extreme pH values, such as 2 and 12, significantly alter the absorbance spectra of C-PC, showing lower values and shifted peaks.

The next studies assessed the effects of encapsulation on the thermal and pH stability of C-PC, as presented in **Fig. 2**.

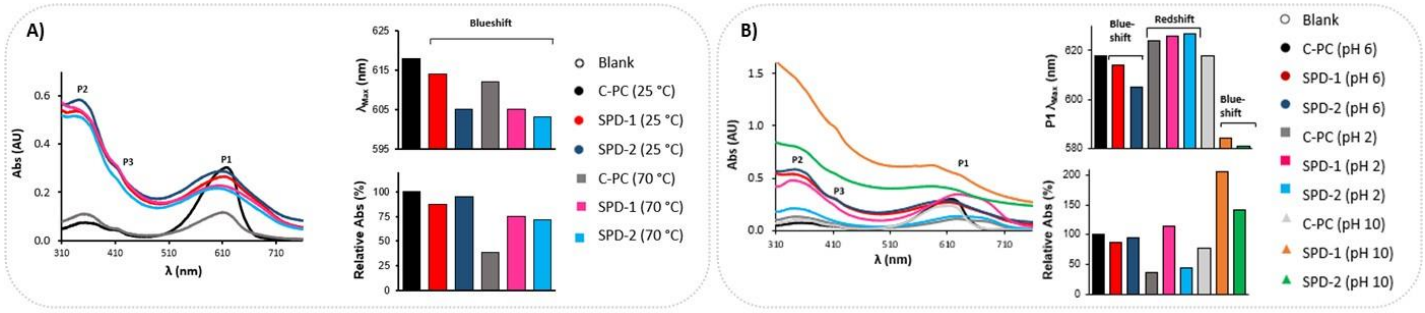

Fig. 2. Stability of encapsulated C-PC at different temperature pH. UV-Vis absorbance spectra with P1 λ_{Max} and its relative Abs of free (C-PC) and encapsulated (SPD-1 and SPD-2) C-PC at **A)** 30 min at 25 °C or 70 °C and **B)** 30 min at pH 6, pH 2, and 10.

The impact of high temperatures (70°C) on encapsulated C-PC can be seen in **Fig. 2.A**, showing the absorbance spectrum, its relative absorbance, and maximum λ of P1 (λ_{Max}). In contrast to free C-PC, encapsulation preserved the absorbance of the colorant after 30 minutes, keeping the relative absorbance above 70 % in SPD-1 and SPD-2 samples. However, the encapsulation technique also caused a "blueshift", *i.e.* a decrease in the maximum wavelength of P1. Encapsulation can cause an increase in the thermal stability of different compounds by acting as a protective physical barrier, thus reducing the exposure of colorants to

light, temperature, humidity, and other substances. In this way, encapsulation proved to be effective in protecting C-PC molecules from the negative effects of exposure to high temperatures.

Meanwhile, **Fig.2.B** shows that free C-PC and SPD-2 had a redshift (*i.e.*, decrease in maximum wavelength) and loss of half of their absorbance for P1 at acidic pH. Although the SPD-1 sample also presented a redshift for P1, it was able to conserve its absorbance. Therefore, encapsulation with C-PC was able to preserve the absorbance of C-PC in acidic environments.

As for the alkaline environments, 30 min at pH 10 reduced around 20 % of the relative absorbances of P1 for the free C-PC. For the encapsulated samples, there was an intense blueshift and an increase in the absorbance of P1, especially for SPD-1. Extreme pH can cause structural and conformational alterations of biomolecules such as proteins and polymers, which could explain the increase of absorbance and blueshift observed at pH 10 for the encapsulated C-PC.

4 CONCLUSION

Encapsulating C-PC in chitosan and chitosan with tripolyphosphate matrices improved the protein stability in stress conditions such as acidic and alkaline pH and high temperatures. Therefore, encapsulating natural colorants in polymeric matrices can be an effective strategy to enhance their stability and potentially expand their applications in foods and cosmetics.

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ACKNOWLEDGEMENTS

FAPESP (2021/06686-8, 2023/01368-3, 2023/10479-3, 2023/13069-0), CNPq (312463/2021-9, 309955/2022-0 and INCT MIDAS/CNPq 465594/2014-0), CAPES (001 and CAPES/PDPG 88881.708195/2022-01), and FAPESB (INCITE PIE0006/2022).