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Thermal stability improvement of blue colorant C-Phycocyanin from *Spirulina platensis* for food industry applications

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ABSTRACT

C-Phycocyanin (C-PC) is a blue pigment in cyanobacteria, rhodophytes and cryptophytes with potential use as a value-added food colorant. Its stability was studied by examining the thermal degradation reactions in a range of temperature (25-80 °C) before and after the addition of selected edible preservatives. The natural protein crosslinker methylglyoxal does not stabilize significantly C-PC whereas addition of honey or high concentration of sugars greatly diminish the blue color degradation occurring when C-PC is exposed to high temperature.

Data show that the sugar preservative effect on the C-PC blue color is related to the final concentration of sugar added rather than the type of sugar. For this reason the best preservative was found to be fructose, which is the most soluble sugar among those tested, at saturation concentration. Exploratory sterilization studies have been carried out with six blue/green fructose syrups made by mixing C-PC with the natural yellow pigment *Carthamus tinctorius*. Both after a "low temperature" and a "high temperature" sterilization procedure the syrups remain clear and maintain their bright color with only partial blue color degradation. After the sterilization process, the syrups were monitored for two months, in such observation period the loss of blue color is minimal.

1. Introduction

In recent years the consumer concern in relation to health and safety issues about the use of synthetic colorants in foods has increased. The Food and Drug Administration (FDA) in USA, the European Food Safety Authority (EFSA) in Europe, and many other national authorities around the world have restricted the use of synthetic colorants in foods, confectionery and beverages because of their confirmed or suspected association with increased cancer development or induction of allergic reactions. For the same reasons other colorants are under study and are only provisionally allowed. The tendency in food manufacturers is therefore going progressively toward the use of natural additives. Among the different colors, the confectionary and drinks industry has a high demand in blue colorants, however, they are uncommon in nature thus leading to the use of synthetic ones. For this reason the food industry is now expressing a growing interest in the search, use, and stabilization of natural blue colorants.

The cyanobacterium *Arthrospira* (*Spirulina*) *platensis*, known mainly as a source of nutraceuticals, has recently gained considerable attention also as a source of blue pigment [1] thanks to its accessory photosynthetic blue-protein Phycocyanin (PC). The great commercial interest on PC is mainly due to the high protein yield and the relatively easy extraction procedures. PC is a protein of the phycobiliprotein family of approximately 20 kDa [2] that consists of two subunits (α and β). Biliproteins are found assembled in large, distinct granules, phycobilisomes, that are considered analogous to the light-harvesting complexes containing chlorophyll a and b in green plants. Phycobilisomes of the cyanobacterium *Spirulina platensis* consist of Allophycocyanin (A-PC) cores surrounded by C-Phycocyanin (C-PC) on the periphery. C-Phycocyanin is the major phycobiliprotein in *Spirulina* and may constitute up to 20% of the dry weight of this algae [3–5].

The chromophore, a linear tetrapyrrole (bilin) covalently attached to the apoprotein by thioether bonds to cysteine residues [6], has an absorption spectrum which depends on the protein to which is bound. Allophycocyanin, has an absorption peak at 650–655 nm whereas C-PC has its maximum at 610–620 nm.

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The interest in C-PC is increased by its nutritional and nutraceutical properties that are exploited for the preparation of food supplements and healthy foods. Beyond its nutritional value, C-PC resulted to be antioxidant both in *in vitro* and in *in vivo* studies (in experimental animals), it is suggested to have anti-inflammatory, anti-viral and anti-cancer properties, and is reported to possess the capacity to stimulate the immune defence system [7–10]. Moreover, C-PC is used as biochemical tracer in immunoassays due to its fluorescent properties [11,12].

The commercial value for C-PC applications is around 10–50 million US$ per year [13,14]. Although C-PC is already used as a blue colorant in some foods and beverages, the C-PC bright blue color is unstable to heat, preventing its use in those food products requiring high temperature processes, such as cooking or sterilization. Few studies have shown that the stability of commercial C-PC is limited [15], and only moderate increase in protein thermal stability have been achieved by adding preservatives or low concentration of sugars [16]. A recent investigation showed that citric acid is able to maintain the stability of C-PC at 35 °C even for 45 days in aqueous solution, with minimum loss, improving its shelf life [17].

The aim of this study is to find the conditions that increase the thermal stability of extracted C-PC in order to prevent color loss during high temperature industrial food processes. As an example we chose two types of process: (1) a low temperature (80 °C) sterilization for long time (30 min) and (2) a high temperature sterilization (100 °C) for short time (1 min). Such stabilization will be reflected in the optimization of the industrial production, packing and storage of the colored products.

2. Materials and methods

2.1. Materials

Dried *Spirulina* sp powder was from Martin Bauer (Nichelino (TO) Italy, www.martin-bauer.it). KH_2PO_4, K_2HPO_4, fructose, sucrose, glucose, maltose, lactose and methylglyoxal (MGO) were purchased from Sigma-Aldrich (Milan, Italy). Conventional honey and honey from manuka (*Leptospermum scoparium*) were obtained from an Italian herbalist's shop. Commercial *C. tinctorius* was from G. Mariani and C. Spa (Cellatica (BS), Italy, www.gmariani.it).

Sugar concentrations throughout the paper are expressed in % (w/v).

2.2. C-PC extraction and purification

Extraction, separation and purification of C-PC were carried out using the procedures reported by Doke [18], with minor modifications. Extraction was done by mixing the dried algae in 100 mM phosphate buffer (pH 7.0) at a ratio of 2:100 (w/v). The mixture was continuously stirred overnight at room temperature. The sample was then centrifuged at 4800 × *g* for 15 min to remove the cell residue. The crude extract was first filtered through a standard 5 μm filter paper membrane and then microfiltrated using a 0.2 μm syringe filter. The purity ratio of C-PC (A_{620}/A_{280}) was increased from 0.45 to 1.40 by an ultrafiltration step using a membrane cutoff of 10 kDa (Amicon cell equipped with a YM10 cellulose membrane). This value is consistent with the quality of food and cosmetic grade C-PC [19,20]. Aliquots of the resulting solution containing concentrated C-PC were stored at −20 °C and thawed right before each experiments.

2.3. Thermal stability

To evaluate the effect of temperature on C-PC stability, 100–200 μL aliquots of purified protein were incubated in a gradient thermocycler (Mastercycler gradient, Eppendorf AG, Hamburg, Germany) at different temperatures (50, 60, 70, 80 ± 1 °C) for 30 min. Samples were also monitored at 25 ± 5 °C by leaving the protein at room temperature for 30 min. The MGO effect was tested by mixing extracted C-PC with 100 mM MGO for 24 h at 37 °C. The thermal stability was then evaluated as described above.

In a different experiment 100–200 μL aliquots of purified C-PC were incubated in the same thermocycler at a fixed temperature (80 ± 1 °C) and samples were taken for examination after 1, 3, 10, 30 and 60 min of incubation.

The pH of the solution was adjusted to the desired value by using 100 mM solutions of acid or basic phosphate buffer.

2.4. Analysis

The purity and the concentration of C-PC were measured using a Varian Cary 50 Scan UV/Vis Spectrophotometer (Agilent Technology, Santa Clara (CA), United States). The concentration of C-PC was calculated based on a extinction coefficient at 620 nm equal to 7.3 mL mg^{-1} cm^{-1} [21].

The thermal degradation of C-PC was monitored by following the decay of the absorbance peak at 620 nm. The irreversible process shown to be non-cooperative and the melting temperature (Tm) of denaturation was calculated by fitting the data with a sigmoidal four-parameters equation, characteristic for a simple two-state model [22–24], using the software Sigma Plot (Systat Software Inc., San Josè (CA) United States). To assess statistical significance, results from independent experiments conducted in triplicate were analyzed using the analysis of variance (ANOVA) test with a confidence level of 95% ($P < 0.05$).

2.5. Sterilization

Colored syrups were obtained by mixing 3.25 g of fructose with different amount of C-PC (stock solution 8 mg/mL) and a fixed amount of the commercial colorant *C. tinctorius*. Water was added to a final volume of 5 mL. The amount of colorants added was as follow: blue, 1000 μL of C-PC; green1, 300 μL of C-PC and 15 μL of *C. tinctorius;* green2, 200 μL of C-PC and 15 μL of *C. tinctorius;* green3, 120 μL of C-PC and 15 μL of *C. tinctorius;* green4, 50 μL of C-PC and 15 μL of *C. tinctorius*; yellow, 15 μL of *C. tinctorius*.

Low temperature sterilization: glass vials containing the colored syrup solutions were placed in a water bath at 80 ± 3 °C. The temperature of the samples was monitored by immersing an electronic thermometer into an additional sample, identical to the six syrups undergoing sterilization, that was prepared only for temperature measurement purpose and was discarded after the sterilization process. High temperature sterilization: the colored syrup solutions were placed in 0.2 mL tubes and then into a thermocycler (Mastercycler gradient, Eppendorf AG, Hamburg, Germany) that was setup for keeping the tubes at 100 ± 1 °C for 60 s. After the 60 s the thermocycler brings the temperature of the tubes immediately to room temperature (25 °C).

3. Results and discussion

3.1. Temperature and pH dependence

As a reference point for C-PC thermal stabilization, we tested the thermal stability of purified C-PC at different pH values. Assuming a two-state protein folding, the denaturation midpoint can be defined as that temperature (Tm) at which the blue absorbance at 620 nm is at the midpoint of the sigmoid denaturation curve. The calculated Tm for the purified C-PC at pH 7 was 57.5 °C (Fig. 1). C-PC is slightly more stable at pH 5 (Tm 61.8 °C) whereas pH 9 strongly diminishes the stability of C-PC (Tm 49.9 °C), in accordance with