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Protection of blue color in a spirulina derived phycocyanin extract from proteolytic and thermal degradation via complexation with beet-pectin

Michael J. Selig, Nicholas M. Malchione, Sara Gamaleldin, Olga I. Padilla-Zakour, Alireza Abbaspourrad*

Department of Food Science, Cornell University, Stocking Hall, Ithaca, NY, 14853, USA

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ABSTRACT

The focus of the presented work was on improvement of spirulina based phycocyanin (PhyC) extracts as a potential natural blue colorant by enhancement of color stability via polysaccharide complexation. Beet pectin (β-pectin), guar gum and soluble soy polysaccharides (SSPS) were assessed for their ability to protect the color of a commercial phycocyanin (PhyC) extract from thermal and proteolytic degradation. Both the beet pectin and guar gum studied formed stable and homogenous solutions with the PhyC extract; SSPS did not. Color change was assessed throughout by measurement of L, a, and b color space values (Hunter, 1948) before and after thermal or proteolytic treatment. Beet pectin improved color stability, over PhyC alone, with respect to heating at 65 °C (20 min) and proteolysis by Alcalase 2.4L, papain and bromelain. For these treatments beet pectin reduced color change values (ΔE_{ab}) from 13.0 ± 0.1 to 6.2 ± 0.4 following thermal treatment and from 12.7 ± 0.2 to 5.9 ± 0.7, 19.6 ± 1.3 to 12.5 ± 0.8, and 10.8 ± 0.4 to 4.6 ± 0.6 for the Alcalase, papain and bromelain treatments, respectively. Intrinsic fluorescence (excitation at 285 nm, emission at 325 nm) of beet pectin complexed PhyC indicates an increased presence of exposed tryptophan residues compared to PhyC alone. Furthermore, measured zeta-potentials following complex heating generally diverged to slightly more negative values for PhyC-beet pectin complexes at elevated temperatures compared to PhyC alone, suggesting that maintenance of a negatively charged environment may be important to pigment color stability in PhyC complexes.

1. Introduction

Spirulina (*Arthrospira* sp.) cyanobacterial biomass has grown in popularity as a nutritional supplement due to its high protein content (~60%) and numerous reported health benefits; many associated with the anti-oxidative effects of pigmented compounds present therein (Abalde, Betancourt, Torres, Cid, & Barwell, 1998; Begum, Yusoff, Banerjee, Khatoon, & Shariff, 2016; Belay, Ota, Miyakawa, & Shimamatsu, 1993). C-Phycocyanins, blue phycobiliprotein pigment-protein complexes, are of specific interest due to the relatively limited number of natural blue pigments available to markets compared to other natural colorants such as the red-purple anthocyanins found in purple produce (grapes, berries, black carrots, cabbage, etc.). Currently, their use is limited due to the fairly low thermal stability of the pigment which rapidly loses its color with the destabilization of the protein complex it is housed in by thermally driven and other denaturation processes and/or proteolysis (Chaiklahan, Chirasuwana, & Bunnag, 2012; Eriksen, 2008; Li, Yang, & Cao, 2009).

The pigment in phycocyanins is a water-soluble open-chained tetrapyrole chromophore, phycobilin, which is covalently attached to the apo-protein via thioether linkages to cysteine residues on the two main sub-units of phycobiliproteins, denoted α (1 chromophore at Cys 84) and β (2 at Cys84 and Cys155); the αβ complex (~44,000 Da) has a tendency to aggregate into dimer, trimer, and even dodecamer quaternary structures leading to relatively large molecular weight structures (Chaiklahan, Chirasuwan, Loha, Tia, & Bunnag, 2011; Kupka & Scheer, 2008; Martelli, Folli, Visai, Daglia, & Ferrari, 2014; Padyana, Bhat, Madyastha, Rajashankar, & Ramakumar, 2001). It is currently believed that maintenance of

* Corresponding author.
E-mail address: Alireza@cornell.edu (A. Abbaspourrad).

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the covalent thioether between the pigment and protein sub-unit complex is not necessarily required to maintain spectral stability and that maintenance of the more rigid extended form of the tetrapyrole is most critical for blue color to be exhibited. Initiation of protein unfolding causes a reduction in the hydrogen bonding network between the pigment and the protein that maintains this rigid conformation, and denaturation/degradation processes that reduced this stabilization result in the tetrapyrole taking on a more helical conformation which results in color loss (Kupka & Scheer, 2008; Ma, Xie, Zhang, & Zhao, 2007).

Structural studies indicate that stabilizing the rigid conformation of the phycobilins within the phycobiliprotein complex is likely critical for maintenance of blue color in PhyC. Color degradation in extracted PhyC is readily initiated under conditions typical for food processing such as pasteurization at 73 °C or higher temperatures (Martelli et al., 2014). To date, a handful of studies have addressed this issue and only with moderate success; for instance, Chaiklahan et al. (2012) have shown the addition of high concentrations of small sugars of numerous forms can improve color stability in PhyC solutions at 65 °C and that the stability is related to sugar concentration and not any specific carbohydrate structure; others have reported benefits of low molecular weight preservatives (Kannaujiya & Sinha, 2016). Beyond this, little work has been focused on improving PhyC thermal stability in addition to resistance to proteolysis. The development of means to protect the native rigid conformation of the phycobilins from protein loosening and unfolding due to heat or the presence of denaturants, as well as proteolytic degradation by residual proteases is much needed.

Furthermore, the thermal stability of PhyCs is higher (up to 70 °C) when present in its native state where they exist as membrane associated intercellular protein aggregates; particularly in thermophilic cyanobacteria (Chen & Berns, 1978; Jespersen, Stromdahl, Olsen, & Skibsted, 2005; Martelli et al., 2014). The intercellular space they exist in is also noted for containing significant amounts of polysaccharide in the form of the acidic spirulan, which is noted for its anti-viral properties (Wackett, 2013). In this context Yan and co-workers (2014) have shown alginate based microcapsules can provide some extended thermal stability to isolated PhyCs. Thus, complexation with similarly anionic or even neutral polysaccharides may also have potential to assist in PhyC color stability, potentially through maintenance of this extended tetrapyrrole structure, and the work herein investigates this notion. Numerous studies to date have shown the protein stabilizing effects of materials such as the beet pectin, guar gum and soluble soy polysaccharides evaluated here (Wong & Wong, 1992), but not to our knowledge in the context of PhyC stability. The work presented herein attempts to complex a commercial PhyC extract with these polysaccharides and assess their ability to maintain the blue color of the extract under thermal and proteolytic conditions and should be of interest to researchers and industries looking for effective and stable means to deliver blue color in natural products.

2. Materials & methods

2.1. Natural colorant, enzymes & carbohydrates

Liquid “Spirulina Blue” phycocyanin extract from DDW The Color House (Louisville, KY) was used as the source of phycocyanin natural blue colorant for this study. The phycocyanin content of this source was estimated to be 43 ± 5 mg/g liquid extract following protocol outlined by Bennett and Bogorad (1973). Complexes of such were prepared with beet pectin (β-Pectin) that was graciously provided by CP Kelco (Copenhagen, Denmark), guar gum from Colony Gums (Monroe, NC), and soluble soy polysaccharide (SSPS) provided by Fuji Oil (Japan). The commercial protease mix used, Alcalase 2.4 L, was provided as a sample by Novozymes A/S (Bagsværd, Denmark); the preparation is reported by Novozymes to have an activity of 2.4 Anson units/g. Food grade papain and bromelain were obtained from Makwood Inc (Grafton, WI) and have manufacturer reported activities of 2400 gelatin digesting units (GDU)/g and 800 tyrosine units (TU)/g, respectively.

2.2. Preparation of phycocyanin-carbohydrate complexes

Phycocyanin complexes were prepared at 20 °C by mixing the Spirulina extract into 50 mM citrate buffer at pH 6.8 to achieve a 2% (w/w) solution of the extract and then dispersing either beet pectin, guar gum or SSPS into the solution to a concentration of 2% (w/w). Solutions were then homogenized by hand until the preparation was uniform and evenly dispersed. Singular batches of each complex were prepared for use during the analyses presented within this work. Furthermore, multiple preliminary observational rounds of polysaccharide complex batches were prepared and analyzed with observations similar to those seen with the final batches prepared and used for the assessment presented herein.

2.3. Thermal stability testing

The thermal stability of the PhyC extract and carbohydrate complexed forms prepared herein was tested by heating 2% solutions (w/w) of the PhyC extract, with and without complexation, at 50 and 65 °C for 20 min, cooling on ice, diluting to 0.5% extract by weight, and then comparing color changes; testing was also conducted at 80 and 90 °C but no color data is reported. Heated PhyC extract, beet pectin, and guar gum complexes were diluted 1 in 10 and further assessed with respect to zeta-potential and intrinsic fluorescence. All treatments were run and analyzed in triplicate.

2.4. Protease treatments

The original phycocyanin extract and the extract-carbohydrate complexes were diluted to a final extract concentration of 0.5% (w/w) for all protease treatments. Initially, the native extract and both beet pectin and guar gum complexes were treated for 45 min with a 0.1% (w/w) loading of Alcalase 2.4L commercial serine endopeptidase. Treatments were run at 40 °C in 50 mM citrate buffer adjusted to pH 6.8. Further time course data on the hydrolysis of the PhyC extract and that complexed with beet pectin was additionally run; in this set samples were taken at 5, 10, 15, 30, 45, 60 and 120 min and analyzed for color changes. Additional and separate hydrolyses with papain and bromelain were also run on both the extract and the beet pectin complexed extract; these hydrolyses were run for 30 min under the same conditions noted above. All treatments were run and analyzed in triplicate.

2.5. Gel electrophoresis

The molecular weight distribution of proteins in complexes was analyzed by SDS-Page in a Mini-Protean gel electrophoresis system from Bio-Rad (Hercules, CA). TGX Fast Cast acrylamide solutions from Bio-Rad were used to hand-cast 1 mm thick tris-glycine acrylamide gels. 10X Tris-glycine running buffer (pH ~ 8.2) was prepared by mixing water, tris base, glycine, and sodium dodecyl sulfate in a ratio of 100:3:14.4:1 and then diluted 1 in 10 for running gel electrophoresis. Standard 2X Laemmli buffer was used as the sample buffer with β-mercaptoethanol as the reducing agent. 12 μL of each sample was loaded into the gel wells and electrophoresis was then run for approximately 30 min at a constant voltage of 250 V.