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Review

The bioactive properties of *Spirulina*-derived phycobiliproteins and phycobilins

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Summary. *Arthrospira* (*Spirulina*), a photosynthetic, filamentous cyanobacteria, has been used as a food for centuries. It is one of the richest known natural sources of protein and essential amino acids, as well as an excellent source of vitamins, macro-and micro-elements, pigments, essential fatty acids, glycolipids, and polysaccharides. C-phycocyanin (C-PC), the most abundant protein in *Spirulina*, is a highly fluorescent and water-soluble heterodimeric phycobiliprotein. Its blue colour arises from a covalently attached (*via* a thioether bond) tetrapyrrole chromophore, phycocyanobilin (PCB). Numerous studies have shown that C-PC exhibits significant anti-inflammatory, immunomodulatory and anticancer effects which could be ascribed to the powerful anti-oxidative activities of PCB. In fact, investigation of C-PC digestion in simulated gastrointestinal conditions has shown that the resulting chromopeptides possess significant anticancer, anti-oxidative and metal-binding properties. Furthermore, it has been shown that various proteins (human and bovine serum albumins, bovine catalase, bovine lactoglobulin) can bind PCB, which could influence the pharmacokinetic behaviour, bioavailability and oxidative stability of PCB. On the other hand, the presence of tetrapyrrole chromophore increases the thermal, oxidative and digestion stability of these proteins. This work reviews recent findings on the bioavailability, distribution and bioactive properties of C-PC and its chromophore, with an emphasis on their fundamental significance and potential applications.

Keywords: binding, chromopeptides, C-phycocyanin, phycocyanobilin, *Spirulina*.

PHYCOBILIPROTEINS: STRUCTURE AND PHYSIOLOGICAL FUNCTION

Phycobiliproteins (PBP) are photosynthetic antenna pigments in cyanobacteria, red and cryptophyte algae that efficiently harvest light energy, which is subsequently transferred to chlorophyll during photosynthesis. Phycobiliproteins are deeply-coloured, highly fluorescent, water-soluble proteins with a high propensity to form oligomers (hexamers), which constitute the building blocks of extra-membranous antenna complex phycobilisomes. Its intensive colour arises from covalently attached linear tetrapyrrole chromophores (phycobilins) *via* thioether bonds to cysteine residues (Stanic-Vucinic et al. 2018).

Cyanobacteria *Arthrospira platensis* (*Spirulina*) contains only two PBPs: C-phycocyanin (C-PC) as the major pigment and allophycocyanin (APC), which is present in much smaller quantities (Sotiroudis andSotiroudis 2013). C-phycocyanin and APC are homologous proteins that both bind phycocyanobilin (PCB) chromophore. C-phycocyanin consists of α and β subunits, which associate with high affinity and form αβ monomers. These monomers further aggregate to form trimmers $(\alpha\beta)_3$ and hexamers $(\alpha\beta)_6$. The α subunit contains one molecule of PCB, while two PCB molecules are bound to the β subunit (Fig. 1). Both subunits of C-PC are predominantly α helical . The tertiary structure of C-PC subunits is well-defined, with a globin-like domain comprised of seven α-helices, folded similarly to myoglobin. Furthermore, PCB binding sites within C-PC correspond to the heme binding site in myoglobin. The globin domain is supplemented with two additional helices at the N-terminus, which stabilise the αβ monomer (Padyana et al. 2001).

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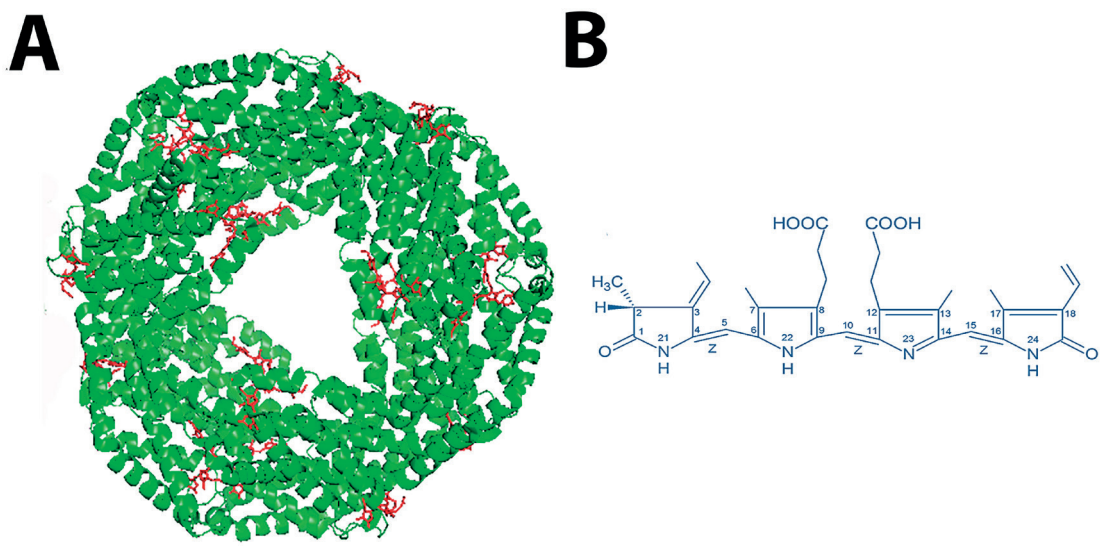

Fig. 1. A, Ribbon model of the crystal structure of C-PC hexamer (PDB: 1GH0). Phycocyanobilin is shown in red; **B**, Chemical structure of PCB.

HEALTH-PROMOTING ACTIVITIES OF C-PC AND WPCB

Anti-oxidative effects

Proteins bearing coloured prosthetic groups, such as the highly conjugated linear tetrapyrrole chromophore in C-PC, can be both a source and target of reactive species in biological systems. C-phycocyanin's extremely high antioxidant capacity has been clearly shown based on experiments carried out both *in vivo* and *in vitro*. In addition to scavenging peroxyl, hydroxyl, and superoxide radicals among others, it also inhibits lipid peroxidation mediated by reactive oxygen species. The bilin group seems to be the main target because *in vitro* radical-assisted bleaching of PCB colour in proteins strongly suggests its involvement in the scavenging of reactive species (Romay et al. 2003; Stanic-Vucinic et al. 2018).

Anticancer effects

In numerous studies, C-PC has been shown to display anticancer activity under both *in vivo* and *in vitro* conditions. C-phycocyanin exerts its anticancer effects through three mechanisms: 1) Induction of apoptosis and necrosis, 2) Reduction of tumour cell migration and invasion, and 3) Induction of cell cycle arrest and reduced proliferation (Braune et al. 2021).

As recently reviewed by Braune and colleagues, C-phycocyanin has been shown to induce apoptosis in numerous cancer cell lines (Braune et al. 2021). Specifically, C-PC was found to induce apoptosis through down-regulation of Bcl-2 (anti-apoptotic) and up-regulation of Bax (pro-apoptotic) proteins. Furthermore, C-PC induces releasing of cytochrome c into the cytoplasm, as well as increased expression of caspase-3 (Li et al. 2006; Roy et al. 2007). Additionally, the presence of C-PC increases PARP-1 (Poly (ADP-ribose) polymerase) cleavage, which could inhibit immediate cellular responses to DNA damage, such as DNA excision repair (Beneke et al. 2000; Roy et al. 2007).

C-phycocyanin induces down-regulation of cyclooxygenase 2 (COX-2), which converts arachidonic acid to prostaglandins and plays a crucial role in tumour progression and chemical resistance (Braune et al. 2021). Prostaglandin E2 increases the expression of P glycoprotein (MDR1), which is responsible for the development of drug resistance in cancer cells. In fact, C-PC decreases the expression of MDR1 and increases the accumulation of the anticancer drug doxorubicin in hepatocellular carcinoma cells (HepG2). Furthermore, HepG2 cells treated with C-PC have decreased levels of nuclear localized NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), a positive regulator of MDR1 (Nishanth et al. 2010).

Angiogenesis represents a critical process in tumour development and metastatic progression. Vascular Endothelial Growth Factor (VEGF), a potent angiogenic molecule, is a leading factor in the development of colorectal cancer. Treatment with dimethylhydrazine (DMH) induces colorectal cancer in rats. However, oral administration of C-PC induces apoptosis (Saini et al. 2012) and inhibits angiogenesis by decreasing VEGF-A expression. One plausible explanation for the down-regulation of VEGF-A by C-PC is inhibition of COX-2, which is involved in production of PGE_2 and promotion of angiogenesis. Interestingly, molecu-

lar modeling suggests that PCB directly interacts with the VEGF-A receptor (Saini and Sanyal 2014). It is well-known that copper stimulates angiogenesis by increasing VEGF-A expression (Xie and Kang 2009). Considering the ability of C-PC to bind Cu^{2+} ions with high affinity (Minic et al. 2016), the antiangiogenic effects of C-PC could be based on its ability to bind copper ions.

Cell cycle regulation is essential in normal cell proliferation, differentiation, and apoptosis, while dysfunction of cell cycle regulation is closely related to tumour development. While the cell cycle in normal cells is well-regulated, tumour cells can proliferate infinitely (Braune et al. 2021). One strategy to inhibit tumour growth is to induce cell cycle arrest through treatment with cell cycle targeting anticancer drugs. C-phycocyanin induces cell cycle arrest in human breast cancer cells by inhibiting cyclin-dependent kinase (CDK), an enzyme responsible for cell cycle progression. Additionally, C-PC increases the levels of p21 protein, which inhibits CDK (Ravi et al. 2015).

Irradiation of a solution of C-PC with visible light induces the formation of reactive oxygen species due to the presence of a tetrapyrrole chromophore (He et al. 1996). Because of this photosensitivity, C-PC is a good candidate for photodynamic therapy applications. In a study using breast cancer cells (MCF-7) injected subcutaneously into mice, the anticancer activity of C-PC was enhanced upon irradiation with a He-Ne laser at a wavelength of 632.8 nm. Furthermore, it has been shown that immune cells are involved in eradicating cancer cells, whereas C-PC has an immune-stimulatory effect. Considering that C-PC is a natural pigment without toxic side effects, it may be a good substitute for highly toxic conventional photo-sensitizers or chemotherapeutic anticancer drugs in the future (Li et al. 2010).

Co-administration of C-PC with other molecules could have synergistic effects in the treatment of cancers. Concomitant administration of C-PC and tretinoin has been shown to have more pronounced cytotoxic effects on the growth of A549 lung cancer cells than single administration of these molecules alone (Li et al. 2016). A similar effect was observed when C-PC was co-administrated with topotecan for treatment of LNCaP prostate cancer cells (Gantar et al. 2012).

In contrast to C-PC, literature data focused on the anticancer activities of PCB alone are scarce. A study dealing with PaTu-8902 human pancreatic adenocarcinoma cells reported that PCB inhibits cell proliferation and decreases oxygen free radical generation in mitochondria. At the same time, reduced glutathione levels were elevated following PCB administration (Konickova et al. 2014). A recent study reported that both C-PC and PCB induce apoptosis in MCF-7 cells (Hussein et al. 2021), suggesting that PCB is mainly responsible for the anticancer activity of C-PC

Anti-inflammatory and immunomodulatory effects

C-phycocyanin and PCB utilise several mechanisms to execute their anti-inflammatory and immunomodulatory activities. One important mechanism explaining the anti-inflammatory activity of C-PC is its ability to inhibit COX-2, which leads to decreased levels of PGE_2. However, PCB is a poor inhibitor of COX-2 (Reddy et al. 2000). C-phycocyanin inhibits acute inflammation and hyperalgesia in rats through down-regulation of inducible nitric oxide synthase and tumour necrosis factor-alpha (TNF-α), as well as inhibition of myeloperoxidase. The proposed mechanism includes inhibition of phosphorylation of p38 protein by C-PC (Shih et al. 2009). A study conducted on mast cells in rats suggests that C-PC can also inhibit histamine release: a phenomenon related to the potent antioxidant activity of C-PC (Remirez et al. 2002).

In vitro co-culture model of Caco-2 cells and differentiated macrophages in the presence of sodium butyrate (SB) and lipopolysaccharide (LPS) resulted in damage to the tight junction of co-cultured Caco-2 cells. However, C-PC works to maintain the tight junction of the Caco-2 cells and contributes to attenuating inflammation and regulating the mucosal immune responses by suppressing inflammatory cytokines, such as IL-6 and IL-8, and conversely enhancing the production of TGF-β1 as an anti-inflammatory and immunomodulatory cytokine (Yoshimoto et al. 2019).

C-phycocyanin is a potent inhibitor of platelet aggregation induced by collagen. Collagen increases the concentration of Ca^{2+} in platelets, an effect abrogated by the presence of C-PC. C-PC also inhibits the synthesis of thromboxane B2 and cGMP, which are responsible for intracellular mobilisation of Ca^{2+}. Additionally, electron paramagnetic resonance spectroscopy showed C-PC's ability to inhibit hydroxyl radical formation in collagen-activated platelets, which could decrease Ca^{2+} mobilisation, leading to inhibition of platelets aggregation (Hsiao et al. 2005).

The ability of C-PC to activate regulatory T cells could be used for the treatment of autoimmune diseases. C-phycocyanin inhibits experimental encephalomyelitis in rats. Furthermore, mononuclear cells isolated from the peripheral blood of patients with multiple sclerosis and incubated with C-PC showed a phenotype that is typical for T regulatory cells. Indeed, RT-PCR analysis showed that C-phycocyanin increases the expression of genes responsible for the development of regulatory T cells (Penton-Rol et al. 2011).

C-phycocyanin can influence the synthesis of particular classes of antibodies. It has been shown that C-PC decreases the level of antigen-specific IgE and IgG1 antibodies in the serum of mice immunised with ovalbumin. However, C-PC induces an increase in antigen-specific IgA antibodies in the intestinal mucosa. Therefore, C-PC could inhibit allergens in

two ways: 1) by increasing the level of IgA to suppress the invasion of allergens; or 2) by inhibition of IgE and IgG1 production in the systemic immune system to minimise excessive responses to allergens (Nemoto-Kawamura et al. 2004).

Some of the anti-inflammatory activities of C-PC could be due to its chromophore, PCB. It has been shown that biliverdin reductase transforms PCB to phycocyanorubin, a bilirubin analogue that is a potent inhibitor of NADPH oxidase, an enzyme involved in numerous inflammatory processes (McCarty 2007). Phycocyanobilin increases the viability of PC12 cells (derived from rat adrenal medulla) upon induction of oxidative stress with glutamate and hydrogen peroxide. The same study investigated the effects of PCB on the oxidative and inflammatory state of rats after induction of brain stroke. Phycocyanobilin decreases the expression of cytokines (IL-1β, TNF-α), chemokines (CXCL2), and adhesion molecules (ICAM-1) involved in the inflammation response induced by a brain stroke. Additionally, the level of malondialdehyde is decreased upon PCB administration, indicating that tetrapyrrole pigment decreases oxidative stress levels. On the other hand, PCB induces an increase in the level of the anti-oxidative enzyme superoxide dismutase (Marin-Prida et al. 2013). The anti-inflammatory activities of PCB are also based on its ability to increase expression of heme oxygenase 1, an enzyme involved in the prevention of atherosclerosis (Strasky et al. 2013).

BEHAVIOUR OF C-PC IN SIMULATED GASTROINTESTINAL CONDITIONS

Recently, research into the effects of oral administration of C-PC through consumption of *Spirulina* dietary supplements, its potential susceptibility to GIT proteolysis, and the structure and bioactivities of released chromopeptides, has attracted considerable interest. SDS-PAGE analyses have shown that C-phycocyanin is rapidly digested by pepsin in simulated gastric fluid (Fig. 2A). The structure of chromopeptides released by this digestion was analysed by high-resolution tandem mass spectrometry, and peptides varying in size from 2 to 13 amino acid residues were identified in both subunits of C-phycocyanin (Fig 2B) (Minic et al. 2016).

Following separation by high-performance liquid chromatography, chromopeptides were analysed for potential bioactivities. It was shown that all five chromopeptide fractions have significant antioxidant activities (Fig. 3A) and show cytotoxic effects against human cervical adenocarcinoma and epithelial colonic cancer cell lines (Fig. 3B). Furthermore, all chromopeptides exhibited the ability to bind Cu^{2+} ions with high affinity, in the range 0.5-1 x 10^6 M^{-1} (Fig. 3C). In addition, chromopeptides protect human erythrocytes from free radical-induced hemolysis in an anti-oxidative capacity dependant manner. There was a positive correlation between anti-oxidative potency and the other biological activities of chromopeptides. Thus, digestion by pepsin releases biologically active chromopeptides from C-phycocyanin, whose activity is mainly correlated with the anti-oxidative potency provided by the chromophore (Minic et al. 2016).

INTERACTIONS OF PCB WITH SELECTED PROTEINS

The pharmacokinetics of bioactive molecules, such as PCB, is strongly influenced by their interactions with proteins present in circulation. Additionally, the bioavailability of food-derived PCB could be affected by interactions with food proteins. This chapter reviews the recent findings of PCB interactions with proteins that could influence the stability, bioavailability and pharmacokinetics of PCB.

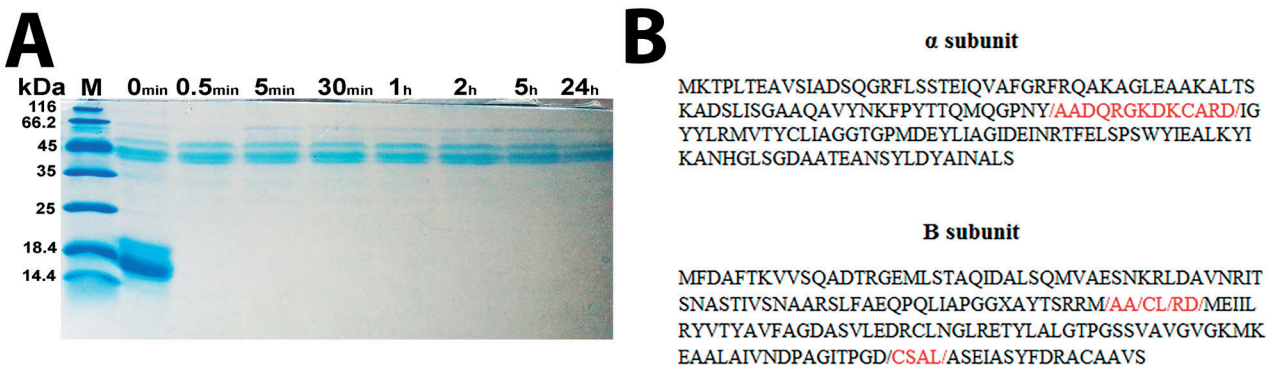

Fig. 2. A, SDS-PAGE analysis (16% PAA gel) of time-dependent pepsin digestion of C-PC (17-19 kDa). The band at about 40 kDa represents pepsin. M and 0–24 h denote molecular weight markers and digestion time, respectively; **B,** Amino acid sequences of C-PC α and β subunits with chromopeptide sequences (red), obtained after pepsin digestion.

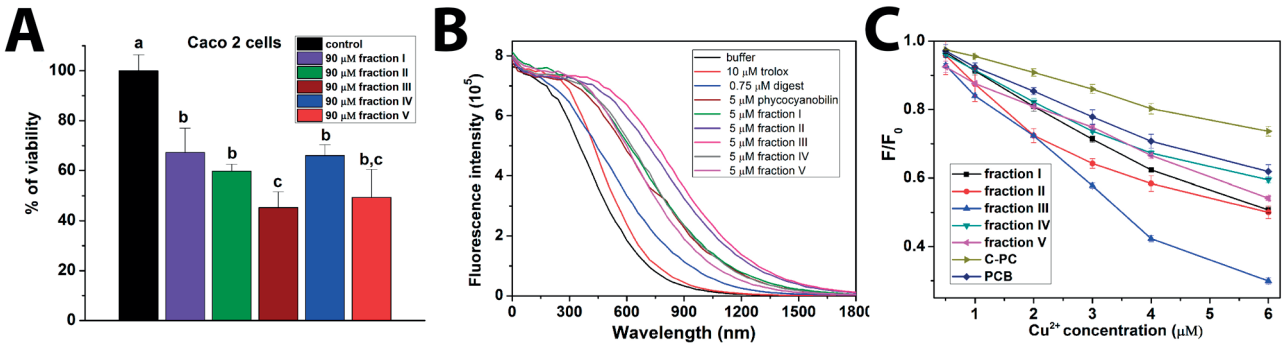

Fig. 3. A, Cytotoxic effects of chromopeptides on Caco-2 cell line during 24 h incubation; **B,** Results of ORAC anti-oxidative test of chromopeptides, PCB and C-PC digest in comparison to Trolox as positive control; **C,** Quenching of chromophore fluorescence (emission and excitation wavelengths were 638 and 578 nm, respectively) in chromopeptides, C-PC and PCB by Cu^{2+} ions. F_0 and F represent fluorescence of chromophore in the absence and presence of different concentrations of Cu^{2+}. The concentration of chromophore was 1 μM.

Characterisation and effects of binding of PCB to human and bovine serum albumins

Human and bovine serum albumins are single polypeptide, α-helical proteins, composed of three homologous alpha-helical domains (I, II and III), each containing two subdomains (A and B). The extraordinary binding capacity of serum albumins for various ligands makes these proteins important regulators of the pharmacokinetic properties of drugs, and model proteins for the study of interactions with natural bioactive compounds (Varshney et al. 2010; Maciazek-Jurczyk et al. 2018).

Based on both a computational approach and spectroscopic titration, we demonstrated the presence of two putative high-affinity binding pockets on human serum albumin (HSA) for anionic forms of PCB (Fig. 4). Computational prediction of phycocyanobilin pKa values suggested that the monoanionic form is the most stable form under physiological conditions. The computationally predicted binding sites for PCB are identical to the two identified binding sites for bilirubin (subdomains IB and IIA) (Minic et al. 2015).

Results obtained by protein and pigment fluorescence measurements, circular dichroism, and competition experiments confirmed high affinity (binding constant of 2.2 x 10^6 M^{-1}; Fig. 5A), stereoselective binding of the PCB *M*-conformer (Fig. 5B) to has, as well as competition with bilirubin, warfarin and hemin. Experimental data confirm that phycocyanobilin binds to HSA with an affinity similar to bilirubin. Under conditions characterised by increased bilirubin plasma concentrations, or intake of drugs binding to the IB or IIA binding site, the pharmacokinetics of PCB may also be changed (Minic et al. 2015).

Furthermore, PCB binding to HSA induces structural changes in both the protein and ligand. UV-visible absorbance measurements indicate that bound PCB has a more extended conformation in comparison to the free form. Experimental data show an increase in thermal (circular dichroism (CD) melting curves, Fig. 6A) and proteolytic stability (trypsin digestion study data; Fig. 6C), as well as α-helical content (CD and FT-IR spectra data; Fig. 6B) of the protein upon ligand binding. Therefore, the binding of PCB to HSA may provide higher stability and longevity to the protein in plasma (Radibratovic et al. 2016).

Protein fluorescence quenching and microscale thermophoresis (Fig. 7A) also demonstrate that PCB binds to bovine serum albumin (BSA; K_a = 2 x 10^6 M^{-1}) with high-affinity.

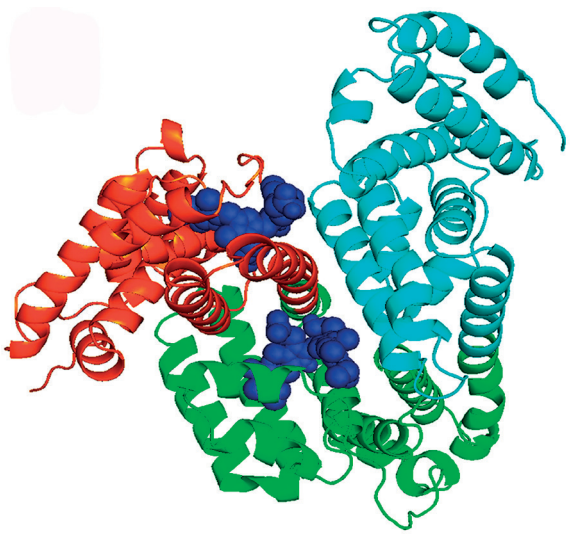

Fig. 4. Molecular modelling of PCB bound HSA. The domains in the ribbon model of protein HSA (PDB: 1BM0) are colour coded as follows: I, orange; II, green; III, turquoise blue. Structural models depicting two potential binding sites for a monoanionic form of PCB to HSA: bound PCB are shown in blue colour space-filling representation.

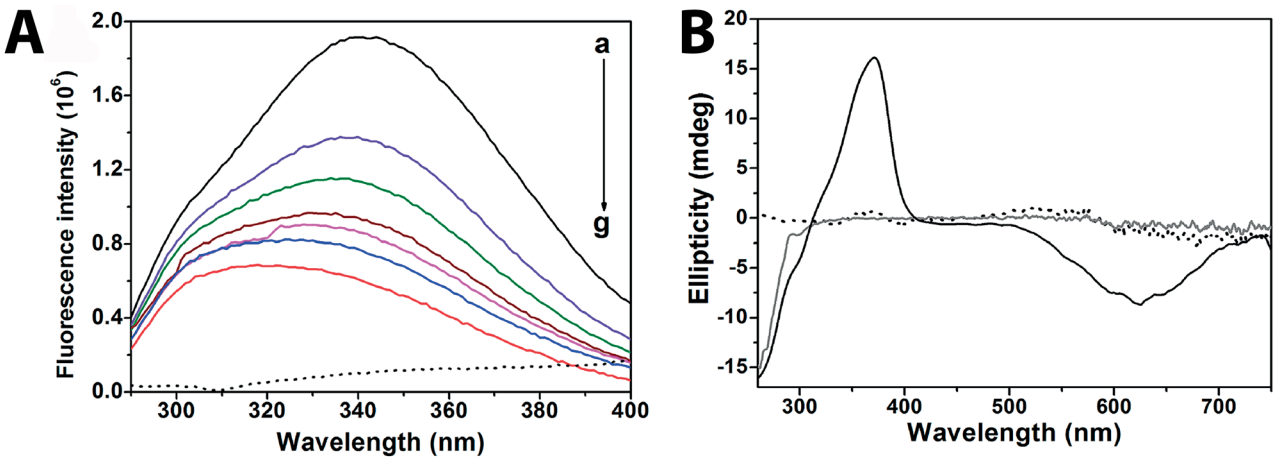

Fig. 5. A, Emission spectra (excitation at 280 nm) of HSA (0.375 μM) in the presence of different concentrations of PCB (0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.5 μM, for curves a to g, respectively). Dot line curve shows the emission spectrum of 1.5 μM PCB; **B,** Near UV-visible CD spectra of PCB (18 μM) in the presence (solid black line) and absence of 18 μM HSA (dot line). The grey line represents the CD spectrum of 18 μM HSA.

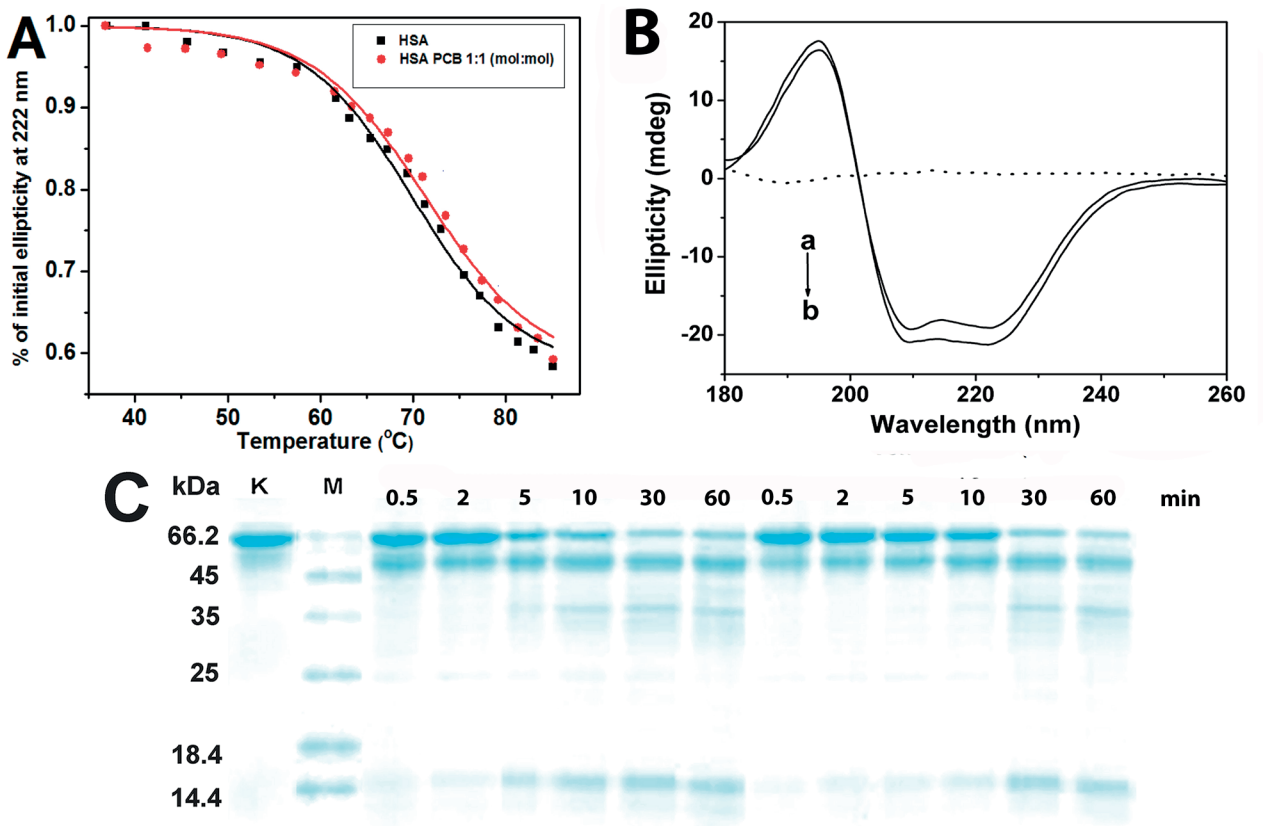

Fig. 6. A, Temperature dependence of 0.5 μM HSA ellipticity at 222 nm in the presence and absence of 0.5 μM PCB; **B,** Far-UV circular dichroism spectra of HSA in the absence (a) and presence (b) of PCB; **C,** SDS-PAGE profile after trypsin digestion of 3.8 μM HSA in the presence and absence of 3.8 μM PCB. Lane K: HSA without trypsin; lane M: MWmarkers; lines 0.5-60 min: digestion time.

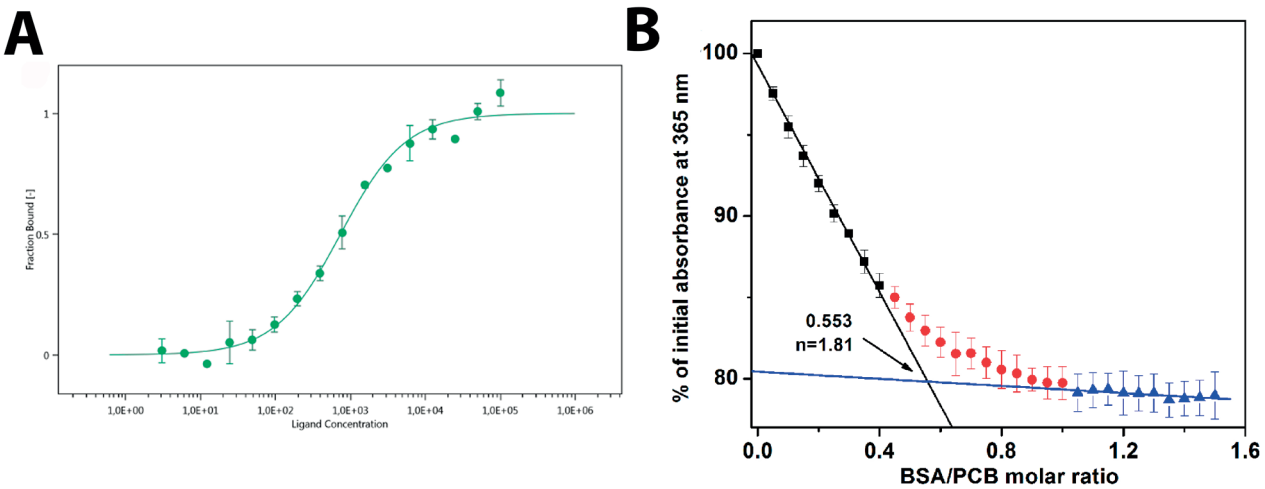

Fig. 7. A, Microscale thermophoresis binding curve for PCB interaction with BSA; **B,** Determination of binding stoichiometry for BSA-PCB complex by monitoring decreasing PCB absorbance at 365 nm. Points coloured in black and blue were used for extrapolation to determine the intersection point.

Spectroscopic titration (Fig. 7B) with computational analysis revealed two binding sites on BSA (Minic et al. 2018a).

However, compared to HSA, binding sites for PCB on BSA are located at the inter-domain cleft (between domains I and III) and the subdomain IB (Fig. 8), while CD spectroscopy indicate stereoselective binding of the *P* conformer of pigment to BSA. Therefore, these results highlight subtle differences between PCB binding for bovine vs human serum albumin. CD spectroscopy revealed that BSA displays increased thermal stability in the presence of PCB. Additionally, the proteolytic stability of BSA is slightly increased upon PCB binding (based on pepsin digestion study data) (Minic et al. 2018a) .

Although complex formation partly masked the antioxidant properties of PCB and BSA (Fig. 9A), a mutual protecting effect against free radical-induced oxidation was found (Figs 9B and 9C). Hence, BSA could serve as a suitable delivery system for PCB as a food colourant and bioactive component (Minic et al. 2018a).

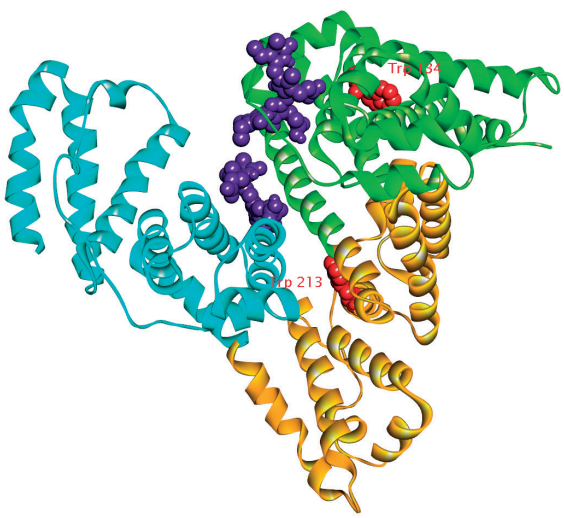

Fig. 8. Molecular modelling of the BSA-PCB complex: the ribbon model of the BSA protein (PDB: 3V03). Domains are coloured as follows: I, green; II, orange; III, turquoise blue. Bound PCB and Trp residues are shown in purple and red in the space-filling representation, respectively.

Characterisation and effects of binding of PCB to catalase

Typical catalases are homotetramers with molecular masses ranging from 200 to 350 kDa. Catalases are essential antioxidant enzymes involved in defence mechanisms against oxidative damage, and convert hydrogen peroxide (H_2O_2) to oxygen and water (Glorieux and Calderon 2017). Phycocyanobilin is quite sensitive to oxidation, and its binding to catalase could reduce free-radical induced pigment oxidation.

Fluorescence quenching experiments demonstrate that PCB binds to catalase with moderate affinity (Ka of 3.9×10^4 M^{-1}). The presence of the tetrapyrrole pigment does not influence the secondary structure content or thermal stability of the protein. CD spectroscopy revealed alterations in catalase tertiary structure, but without adversely affecting enzyme activity (Fig. 10). Computational analyses indicate that the ligand most likely binds to amino acid residues near the cavity between enzyme homotetramer subunits far from the active site. Finally, complex formation protects the pigment from free-radical induced oxidation (bleaching) suggesting a possible role in prolonging its half-life and bioactivity *in vivo* in the complex with catalase (Gligorijevic et al. 2021).

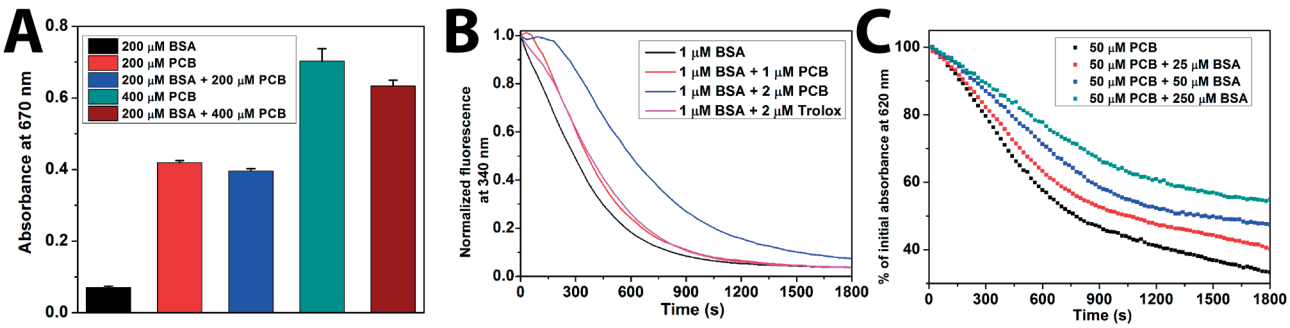

Fig. 9. A, Masking of antioxidant activity of PCB in the BSA-PCB complex: Reducing power (absorbance) of free BSA, free PCB, and BSA-PCB complexes; **B,** Effects of BSA-PCB binding on protein oxidative stability: BSA (1 μM) fluorescence decay after addition of the free radical generator AAPH in the absence and presence of PCB (1 or 2 μM) and Trolox (2 μM); **C,** Monitoring of PCB oxidative degradation by AAPH, with and without BSA, as a percentage of the initial absorbance at 620 nm after AAPH addition.

Covalent binding of PCB to bovine lactoglobulin

Bovine lactoglobulin (BLG) is a major whey protein that can bind various lipophilic bioactive compounds, including vitamins, fatty acids, cholesterol and polyphenols, etc. (Kontopidis et al. 2004). One of the main structural features of BLG is the presence of a free cysteine, Cys-121, that is responsible for the polymerisation properties of BLG due to its capability to form intermolecular disulfide bonds and promote disulfide exchange (Hoffmann and vanMil 1997). It is well-known that PCB spontaneously reacts with compounds bearing free SH groups, such as cysteine (Bishop et al. 1991), indicating that PCB is a good candidate for covalent binding to BLG.

Indeed, zinc-induced fluorescence visualisation of BLG-PCB adducts after SDS-PAGE (Fig. 11A) demonstrate the ability of BLG to bind the tetrapyrrole pigment covalently *via* cysteine residues. Based on PCB fluorescence enhancement (Fig. 11B) upon BLG addition at physiological pH (7.2), it has been found that covalent pigment binding is slow (k_a=0.065 min^{-1}; Fig. 11C) and of moderate affinity (K_a=4×10^4 M^{-1}). Binding also occurs over a broad pH range and under simulated gastrointestinal conditions. Adduct formation rises with pH and in concentrated urea (k_a=0.101 min^{-1}; Fig. 11C), suggesting that protein unfolding exposes cysteine residues, which become more available for covalent binding of PCB. The BLG-PCB adduct has slightly altered secondary and tertiary protein structure, and bound PCB has higher fluorescence and a more extended conformation than the free chromophore (Minic et al. 2018b).

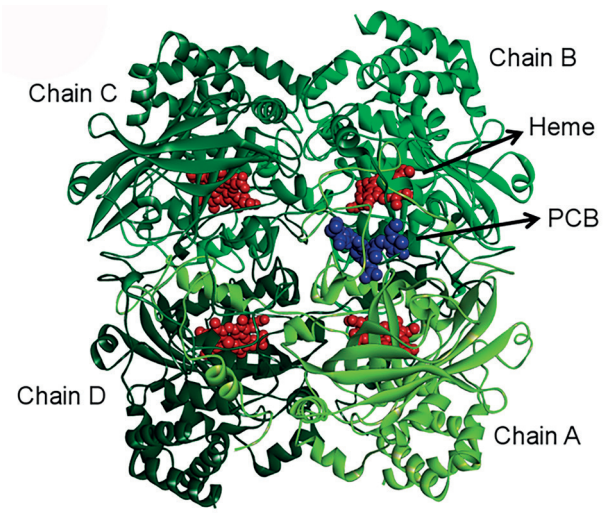

Fig. 10. Molecular modelling of the CAT:PCB complex: the binding site of PCB to CAT (PDB: 1TGU) of lowest energy.

The BLG–PCB adduct possesses enhanced antioxidant properties and bound PCB protects BLG against free radical-induced oxidation. Despite the similar thermal stabilities of BLG and BLG–PCB, BLG–PCB is less susceptible to covalent and non-covalent aggregation under moderate heat treatment (63 °C, 30 min). The blocked thiol group and reduced hydrophobicity due to the masking of hydrophobic residues by bound PCB and the heat-induced β-sheet to α-helix transition contribute to the low susceptibility of BLG–PCB to aggregation. Covalently modified protein is more resistant to pepsin and pancreatin. Hence, BLG covalent modification by PCB improves BLG's techno-functional properties (Radomirovic et al. 2018).

Therefore, the propensity toward covalent binding of PCB makes BLG an efficient vehicle for supplementation of food with PCB. In addition, covalent modification of PCB with BLG is an innovative approach for preparing a multifunctional food protein, making it a valuable ingredient for the food industry.

CONCLUSIONS

C-phycocyanin, a blue phycobiliprotein from *Spirulina*, has potential health benefits that have been demonstrated in

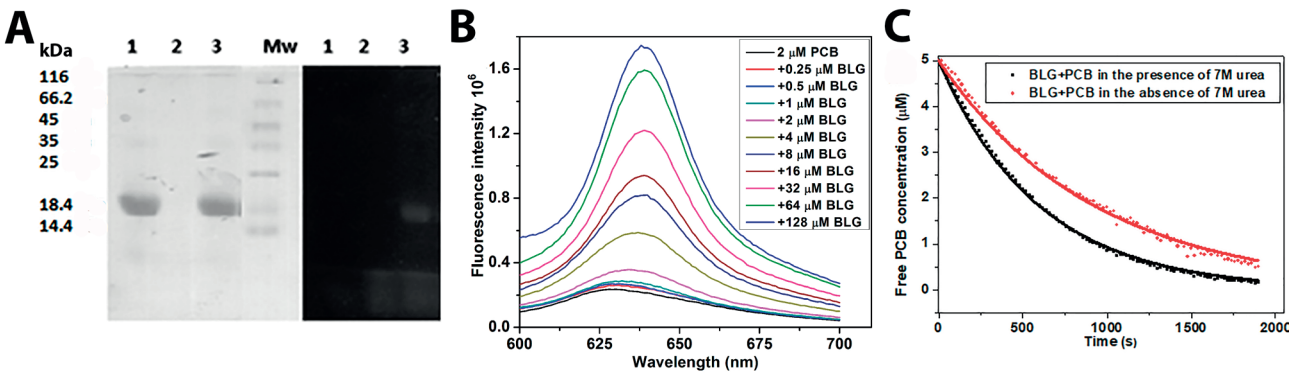

Fig. 11. A, SDS-PAGE (16% PAA gel) of free BLG (1), free PCB (2), and BLG-PCB complex (3). Left: CBB staining; Right: specific visualisation of PCB by Zn^{2+}; **B,** Emission spectra of PCB in the absence and the presence of different concentrations of BLG (excitation at 580 nm); **C**, Fitting of the free PCB concentration change during time into first-order kinetics.

numerous *in vitro* and *in vivo* studies. Most of the biological activities of C-PC arise from a covalently attached chromophore known as PCB. Indeed, simulated gastric digestion of C-PC released small chromopeptides with anticancer, antioxidant and metal-chelating activities. However, further studies are needed for complete evaluation of the bioavailability and bioactivity of PCB and chromopeptides.

High-affinity binding of PCB to HSA could significantly influence its transport and distribution, while binding to catalase protects the pigment from oxidative injury. The ability of PCB to bind BSA and BLG could be utilised for food functionalisation and would provide both colour and bioactivity. Additionally, sensitive redox-active health-promoting PCB can be better preserved in complex food matrices containing BSA and BLG.

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