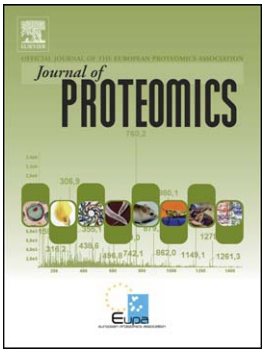

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Digestion by pepsin releases biologically active chromopeptides from C-phycocyanin, a blue-colored biliprotein of microalga Spirulina

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Abstract:

C-phycocyanin, the major protein of cyanobacteria Spirulina, possesses significant antioxidant, anti-cancer, anti-inflammatory and immunomodulatory effects, ascribed to covalently attach linear tetrapyrrole chromophore phycocyanobilin. There are no literature data about structure and biological activities of released peptides with bound chromophore in C-phycocyanin digest. This study aims to identify chromopeptides obtained after pepsin digestion of C-phycocyanin and to examine their bioactivities. C-phycocyanin is rapidly digested by pepsin in simulated gastric fluid. The structure of released chromopeptides was analyzed 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. Following separation by HPLC, chromopeptides were analyzed for potential bioactivities. It was shown that all five chromopeptide fractions have significant antioxidant and metal-chelating activities and show cytotoxic effect on human cervical adenocarcinoma and epithelial colonic cancer cell lines. In addition, chromopeptides protect human erythrocytes from free radical-induced hemolysis in antioxidative capacity-dependant manner. There was a positive correlation between antioxidative potency and other biological activities of chromopeptides. Digestion by pepsin releases biologically active chromopeptides from C-phycocyanin whose activity is mostly related to the antioxidative potency provided by chromophore.

Key words: C-phycocyanin, phycocyanobilin, digestibility, peptides, bioactivity.

Abbreviated running title: Biological activities of chromopeptides from Spirulina.

Introduction

Spirulina (genus *Arthrospira*), photosynthetic, filamentous cyanobacteria (blue-green microalga), has been used as food for centuries. It is one of the richest known source of proteins and essential amino acids, excellent source of vitamins, macro- and micro-elements, essential fatty acids, glycolipids and sulfated polysaccharides, and numerous *in vitro* and *in vivo* studies have shown various health benefits of Spirulina **[1, 2].**

The health beneficial properties of Spirulina are mainly attributed to calcium spirulan and C-phycocyanin (C-PC) **[3]**. C-PC is the most abundant protein of Spirulina, representing about 20% of the dry biomass **[4]**. It is highly fluorescent and water soluble protein with main function to transfer excitation energy to reaction centers during photosynthesis. Intensive blue color of protein arises from its covalently attached (*via* thioether bond) linear tetrapyrrole chromophore phycocyanobilin (PCB) **[5]**. One PCB molecule (**Figure 4A**) is attached to α subunit *via* Cys84, while β subunit binds two molecules of PCB *via* Cys82 and Cys153 **[5, 6]**.

A large number of studies have shown that C-PC exhibits significant anti-inflammatory, anti-cancer, nephroprotective and hepatoprotective effects **[5]**, ascribed to its antioxidant and radical scavenging properties **[4, 7]**. As PCB, *per se*, efficiently scavenges the most of reactive species, chromophore should be responsible for the most of health benefits of C-PC **[8]**. Indeed, recent studies have shown that PCB exhibits significant anti-cancer **[9]**, anti-inflammatory **[10]**, atheroprotective **[11]** and nephroprotective effects **[12]**. Interestingly, the chromosphere unit of C-PC is also responsible for binding of metal ions, such as Hg^{2+}, Pb^{2+}, Cu^{2+}, Ag^{+} **[13, 14].**

In recent years, there is a growing number of studies about protein hydrolysis as an effective method to produce bioactive peptides **[15]**. The bioactive peptides produced from enzymatic hydrolysis can exhibit excellent antioxidant capacity **[16]**, antihypertensive, anti-cancer, immunomodulatory and metal chelating activities **[17]**. Protein hydrolysis is usually performed by enzymes derived from microorganisms or plants **[18]**, but digestion by enzymes of gastro-intestinal tract (GIT), such as pepsin or trypsin, is of physiological relevance.

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 is an interesting research topic. There are scarce literature data about bioactivities of peptides obtained after C-PC digestion, as well as bioactivities of peptides

with covalently bound bioactive chromophore (chromopeptides) in general. Therefore, we examined digestibility of C-PC by pepsin in simulated gastric fluid. Chromopeptides, obtained by pepsin digestion, were purified and identified by tandem mass spectrometry. These chromopeptides demonstrated significant antioxidant, Cu^{2+}-chelating and anti-cancer activities.

Materials and methods

C-phycocyanin and phycocyanobilin purification and quantification

Analytical grade C-PC (the absorbance ratio A_{620}/A_{280} of 4.4) was purified from protein extract of commercial Spirulina powder (from Hawaiian Spirulina Pacifica, Nutrex, USA) according to the previously described protocol **[19]**. Extraction of crude phycocyanins was done using 20 mM sodium phosphate buffer, pH 6.8; the powder-buffer suspension was mixed during 3 hours at room temperature. The concentration of C-PC was determined by UV/VIS spectrophotometry **[20]**.

PCB was isolated from the same starting material by the method of Fu et al. **[21]**. The PCB concentration was determinated using a molar absorption coefficient of 37900 $M^{-1}cm^{-1}$ at 680 nm **[22]**.

Pepsin digestion of C-phycocyanin

Pepsin digestion of C-PC was performed in simulated gastric fluid (SGF) **[23].** Briefly, 80 μL of C-PC (5 mg/mL) was added to 760 μL of SGF (84 mM HCl and 35 mM NaCl, pH 1.2), containing 1 unit of pepsin (from porcine gastric mucosa, 2546 U/mg; Sigma-Aldrich, USA) per μg of C-PC. Mixture was incubated at 37^{o}C and aliquots of 60 μL were taken at 0.5 min, 5 min, 30 min, 1 h, 2 h, 5 h and 24 h after initiation of the incubation. Each aliquot was quenched by addition of 20 μL of 300 mM $NaHCO_3$, pH 11. Aliquots at zero time were prepared by quenching the pepsin containing SGF before adding C-PC. SDS polyacrylamide gel electrophoresis (SDS−PAGE) of digests under reducing conditions was performed **[24]** and gels were stained using Coomassie Brilliant Blue R-250. For identification of chromopeptides structure and their bioactivity evaluations, largeer scale digestion mixtures were prepared and digestion was done during 24 h incubation period.

Separation of chromopeptide fractions from pepsin digest by semi-preparative HPLC

Separation of pepsin digest of C-PC was conducted on HPLC Agilent 1260 system (Agilent, USA). Chromopeptides were separated using semi-preparative Zorbax Eclipse XDB

C-18 column (9.4 mm x 250 mm, 5 μm particles; Agilent, USA) connected with HPLC system. Elution of peptides and pepsin was done using gradient elution in the following order: 100% solvent A (0.1% formic acid) - one column volume, gradient from 100% solvent A to 20% solvent B (0.1% formic acid in acetonitrile) - one column volume, gradient from 80% solvent A to 40% solvent B - twelve column volume, gradient from 60% solvent A to 100% solvent B - three column volume. The flow rate was 3 mL/min with simultaneous detection at 215, 280 and 615 nm. Each chromopeptide fraction (with absorbance at 615 nm) was collected and further analyzed by mass spectrometry. For bioactivity assays, solvent was evaporated and resulting pellet was dissolved in 20% DMSO. Rechromatography of each fraction was performed under analytical conditions (experimental details were described in Supplementary Materials). Relative purity of chromopeptides in comparison to other peptides (absence of peak at 615 nm) was estimated according to absorbance at 215 nm.

Quantification of chromopeptides

All fluorescence measurements were done using FluoroMax®-4 spectrofluorometer (HORIBA Scientific, Japan). Chromopeptides quantification was done by spectrofluorimetry using whole pepsin digest of C-PC as standard, considering that the concentration of the chromophore in the digest is known. Standard curve (**Figure S0**) was obtained by preparing a series of digest dilutions (between 0.1 and 1.1 μM chromophore concentration) in 20 mM phosphate buffer pH 7.4. Emission of standard and samples (chromopeptides solutions in the same buffer) was recorded at 638 nm (with excitation wavelength at 578 nm and slits width 5 nm). At this wavelengths standard shows maximum of emission.

Identification of amino acid sequence of the chromopeptides

Chromopeptides, separated by semi-preparative HPLC, were analyzed by high resolution tandem mass spectrometry using LTQ Orbitrap XL (Thermo Fisher Scientific Inc., USA) mass spectrometer. Ionization was done in positive mode on heated electrospray ionization (HESI) probe. HESI parameters were: capillary temperature 275^{o}C, source voltage 4 kV, capillary voltage 5 V, tube lens voltage 70 V, sheath and auxiliary gas flow 12 and 3 (arbitrary units), respectively. Acquisition was 5 minute per sample. Samples were injected directly with flow 10 μL/min. MS spectra were acquired between m/z 100 and m/z 2000. Ionized peptides were fragmented with CID (collision-induced dissociation) in order to obtain MS2 and MS3 spectra. CID was performed with helium gas at a normalized collision energy of 35% and the parent ions were activated for 30 ms. The ESI-MS, ESI-MS2 and ESI-MS3 data were acquired with Xcalibur version 2.1 (Thermo Fisher Scientific Inc., USA).

Identification of chromopeptides was done by manual *de novo* sequencing using NIST Mass and Fragment Calculator Software (Version: 1.3) for calculations the fragments mass of an input peptide sequence along with m/z ions corresponding to 1+, 2+, and 3+ charge states (http://www.nist.gov/mml/bmd/bioanalytical/massfragcalc.cfm).

Antioxidant assays

The spectrofluorimetric oxygen radical absorbance capacity (ORAC) assay was performed according to Ou at al. **[25]**. Stock solutions of fluorescein (153 mM; Sigma-Aldrich) and free radical generator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 5 μM; Sigma-Aldrich) were made in 75 mM potassium phosphate buffer, pH 7.4. Excitation and emission wavelengths were of 485 and 511 nm, respectively, and slits were set to 2 nm. The relative ORAC value for chromopeptides was expressed as Trolox equivalents (TE) **[26]**.

The reducing power of chromopeptides was measured according to the method of Oyaizu **[27]**. 20 μL of sample or Trolox in concentration range of 50-400 μM was added to 50 μL of 0.2 M phosphate buffer, pH 6.6 and 50 μL of 1% potassium ferricyanide. After incubation at 50^{o}C during 20 min, 25 μL of 20% trichloroacetic acid was added to the reaction mixture. A volume of 100 μL from each incubated mixture was mixed with 100 μL of milliQ water and 12 μL of 0.1% $FeCl_3$. After a 10 min absorbance was measured at 670 nm. Reducing power, expressed as Trolox equivalents (TE), was calculated by dividing the slope of sample curve with slope of Trolox curve.

Metal chelating activity of chromopeptides was tested by fluorescence spectroscopy. Slits were set to 5 nm, excitation wavelength was 578 nm and emission was recorded at 638 nm. Chromophore concentration in analyzed samples (chromopeptides, C-PC or PCB in 20 mM phosphate buffer, pH 7.4) was kept constant (1 μM), and concentration of $CuCl_2$, $FeSO_4$ or $FeCl_3$ varied from 0.5 to 6 μM. Binding constants (K_a) were calculated using equation **[28]**:

$$K_a[Me] = \frac{F_0 - F}{F - F_i}$$

where F_0, F and F_i represent emission intensities (at 638 nm) of chromopeptides (or C-PC) without metal cation (Me), with addition of Me and at an infinite concentration of Me, respectively.

Inhibition of AAPH-induced erythrocyte hemolysis by chromopeptides was performed as previously described **[29]**. Heparinized blood samples were obtained by venipuncture from healthy human donors, after obtaining informed consent. Erythrocytes were isolated by centrifugation at 2000g for 10 min, washed three times with phosphate saline buffer (PBS)

and finally re-suspended in the same buffer to obtain hematocrit level of 5%. Cells were preincubated with chromopepetides, PCB or Trolox at final concentration of 5 μM for 15 min at 37^{o}C. After that, mixtures were incubated with 50 mM (final concentration) AAPH during 4 hours at 37^{o}C for the purpose of induction the free radical chain oxidation. Erythrocytes incubated with PBS served as control, and 100% hemolysis was obtained by cells incubation in distilled water. At 30, 60, 120, 180 and 240 min after beginning of hemolysis, 200 μL of reaction mixture was removed and centrifuged at 3000g for 2 min. Hemoglobin content in supernatants was determined at 540 nm using Drabkin's reagent.

Cell culture and cytotoxicity assay

Human cervical adenocarcinoma (HeLa) and human epithelial colonic carcinoma (Caco-2) cells were cultured according to Krstic at al. **[30]** and Stojadinovic et al. **[31]**, respectively. Cytotoxicity of each chromopeptide fraction on these cell lines was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay **[32]**. Cells were seeded in 96-well plates at a density of 10,000 cells per well and left overnight to allow cell attachment. Next day, cells were incubated with chromopeptide solution (90 μM) for the next 24 h. 2% DMSO in medium was added to the cells in the control wells and cell culture medium was added to blank wells. After 24 h, 20 μL of 5 mg/mL MTT (Sigma-Aldrich) solution in phosphate-buffered saline was added and the resulting mixture was incubated for the next 1 h at 37°C. The medium was carefully removed and 200 μL of DMSO were added to dissolve the formed formazan complexes; absorbance was read at 540 nm. Data are expressed as percentage of viability with untreated cells taken as 100%.

Statistics

All experiments were performed at least in duplicate and results were presented as mean ± standard deviation. For evaluation of chromopeptides cytotoxicity and antioxidant capacity, an analysis of variance (ANOVA) of data was performed and means comparisons were done using Turkey test. Relationship between variables has been assesed by means of Pearson's product moment correlation coefficient. In all statistical analyses, differences were considered significant if $p<0.05$.

Results and discussions

C-phycocyanin is readily digested by pepsin

C-PC digestibility was analyzed by SDS-PAGE after pepsin digestion in simulated gastric conditions **(Figure 1A**). The results demonstrate that pepsin rapidly (after 0.5 minutes) releases small peptides, visible as smears at the bottom of separating gel that gradually decrease with increasing of digestion incubation time. This suggests that C-PC is very susceptible to pepsin digestion under applied conditions, and it is in agreement with the C-PC instability below pH 4; after unfolding at acidic pH, protein becomes fully accessible to enzyme **[33]**.

Released peptides were separated using semi-preparative HPLC. Five dominant fractions of peptides containing PCB chromophore (I-V) were isolated (**Figure 1B**). There is a good agreement between shapes of peaks at 615 nm (absorption of chromophore), 280 (absorption of chromophore and aromatic amino acid residues) and 215 nm (absorption of chromophore and peptide bonds). Rechromatography of isolated fractions has shown that relative purity of chromopeptides (according to absorbance at 215 nm) in fractions II-V was between 91 and 98 %, while chromopeptide in fraction I had 72% purity (**Figures S14-S18**). Therefore, it can be concluded that chromopeptides obtained after semi-preparative HPLC have reasonoble purity grades.

Identification of chromopeptides released by pepsin digestion

Chromopeptides separated by HPLC were sequenced using tandem mass spectrometry. Sequences of chromopeptides were determinated from MS spectra of chromopeptide fractions (**Figures 2A, S1A-S5A**), using their calculated molecular masses (**Table 1**), known sequences of α (UniProtKB-P72509) and β (UniProtKB-P72508) subunits of C-PC (**Figure 4B**) and position of PCB binding. Confirmation of sequences was done by analysis of MS2 and MS3 spectra of parent ions, and MS and MS2 spectra of pure PCB. MS spectrum of PCB shows characteristic dominant ion at m/z 587.29; in PCB MS2 spectrum the most dominant ions with m/z ratios 464.22 and 299.14 are generated by CID fragmentation of PCB (**Figures S6A and S6B; Table S6**); MS3 spectrum of ion with m/z 299.14 contains ion with m/z 271.14 (**Figure S6C**). Similarly to other bilins, ion with m/z ratio 464.22 is the result of loss of the terminal two pyrrole rings, while ion with m/z 299.14 results from cleavage of the C-C bond between the central bridge methylene carbon to either of the inner two pyrrole rings in PCB molecule. Aditional ion with m/z ratio 271.14 (z=1) corresponds to natural loss of CO from the product ion at m/z 299.14 **[34**]. CID cleaves thioether bond between PCB chromophore and peptide,

thus ions representing PCB and peptides without chromophore occur in MS2 spectra of chromopeptides (**Figures 3B, S1B-S5B; Tables S1B-S5B**). Further fragmentation of ion with m/z ratio 587.29 gives ions with m/z ratio 464.22, 299.14 and 271.14 in appropriate MS3 spectrum (**Figures 3D, S1D-S5D; Tables S1D-S5D)**. These ions were confirmation that parent ion is chromopeptide and only ions which fragmentation gives ions derived from chromophore were further analyzed. Fragmentation of peptide ions without chromophore in MS2 spectrum gives MS3 spectrum with series of y and b ions, confirming chromopeptide sequences (**Figures 3C, S1C-S5C; Tables S1C-S5C**).

We identified in total six chromopeptides from C-PC pepsin digest (fraction II contained two chromopeptides), varying in size from 2 to 13 amino acid residues (**Table 1**). In fraction IV two additional chromopeptides were found from traces of allophycocyanin, protein with sequence very similar to C-PC and with the same chromophore **[35]**. Chromopeptide from the most abundant fraction I arises from α subunit of C-PC, while others fractions originate from digestion of β subunit of C-phycocyanin or α subunit of allophycocyanin (**Table 1**). These results are in agreement with those of SDS-PAGE digest, confirming that pepsin efficiently cleaves C-PC into very small (chromo)peptides. Small size of peptides is obtained due to broad specificity of pepsin and therefore numerous cleavage sites. In β subunit of C-PC pepsin cleaves after Met79, Ala81 and Leu83, resulting in short chromopeptides (AACLRD, CLRD, AACL and CL). In contrast, in α subunit at homolog positions pepsin does not act, as it rarely cleaves when Lys is in P1 position (Lys81 and Lys83) and in P3 position (with Ala85 at P1) **[36]**, resulting in single long chromopeptide AADQRGKDKCARD. In general, bioactive peptides frequently contain 3-20 amino acid residues **[17**], and thus small size of obtained chromopeptides indicate their potential bioactivity.

Antioxidant activities of chromopeptides

ORAC assay is based on ability of antioxidants to inhibit degradation of fluorescence molecule induced by free radicals and ORAC value, as a measure of substance antioxidant capacity, is usually expressed as Trolox equivalents. As shown in **Figure S7A** and **Table 2**, chromopeptides effectively quenched peroxyl radicals with scavenging activities from 8.2 to 13.2 greater than Trolox, a hydrosoluble analogue of vitamin E. Due to presence of other peptides and pepsin, whole digest showed higher ORAC value (25.4 TE) in comparison to chromopeptides from C-PC and PCB itself (8.4 TE). On average, chromopeptides contributed to the total mass of digest (all peptides from C-PC and pepsin) by 3.5%, but to the ORAC

value of the digest with 40%. Therefore, chromophore part of peptides (from chromopeptides and PCB) is mainly responsible for radical scavenging activity of digested C-PC. Tyr, Trp, Cys, Met and His are the most potent free radical scavenging residues in ORAC assay, due to their electron/hydrogen donating abilities **[37]**. Identified chromopeptides do not contain mentioned amino acid residues (except Cys, whose sulfhydryl group is involved in thioether bond with PCB), and their antioxidative activities originate almost completely from chromophore. In whole digest, beside chromophore, sixteen Tyr, one Trp, three Cys (with free sulfhydryl group), ten Met and one His residues (**Figure 4B)** contribute to total antioxidative activity in ORAC assay, resulting in higher TE value in comparison with chromopeptide fractions.

In reducing power assay, antioxidants reduce Fe^{3+}-ferricyanide complex to Fe^{2+} ions, and increased absorbance of reaction mixture is directly proportional to the reducing power capacity. Chromopeptides showed significant and dose depending reducing power in this test, from 2.7 to 4.7 higher than standard antioxidant Trolox, whereby the fractions II and III were the most active (**Figure S7B; Table 2)**. PCB also showed significant reducing power (4.5 TE), while activity of whole C-PC digest (3.2 TE) only corresponded to the averaged activity of all chromopeptide fractions (3.2 TE), suggesting that reducing capacity is also almost entirely derived from PCB chromophore. Of all protein amino acid residues, only Cys shows substantial reducing power capacity **[37]**. In contrast to ORAC assay, in whole digest only three Cys residues and chromophore contribute to reducing power, having as consequence similar TE value as chromopeptide fractions.

It is well known that tetrapyrroles, including phycocyanobilin, show potent antioxidant activity *in vitro* **[38, 39]**. However, there is no literature data about antioxidant activities of peptides with covalently bound tetrapyrrole chromophore. In comparison to other low molecular weight (LMW) peptides obtained after pepsin digestion of food proteins, isolated C-PC derived chromopeptides show moderate to high ORAC values **[40-45]**, and high reducing power capacity **[46-48]**.

Fluorescence quenching is a useful approach to study interactions between proteins/peptides and various ligands. In contrast to Fe^{2+}/Fe^{3+} ions (results not shown), Cu^{2+} efficiently quenched chromopeptides fluorescence (**Figure S7C**), indicating its specific binding to all chromopeptides. Indeed, calculated binding constants were $0.5 – 1.0 \times 10^{6}$ M^{-1} (**Table 2**). Obtained binding constants are moderately higher in comparison to N-terminal peptides of prion proteins that specifically bind Cu^{2+} ions **[28]**. Cupric ions quenched C-PC

fluorescence with lower average value for the binding constant compared to chromopeptides and PCB (**Table 2**), indicating that the chromophore is main contributor for the binding of Cu^{2+} ions. Higher binding of Cu^{2+} ions for PCB than C-PC can be explaned by the fact that PCB in native protein has extended conformation, while upon denaturation (in this case digestion) chromophore forms cyclic-helical conformation which is more prone to bind Cu^{2+} ions **[13]**. Inability of Fe^{2+} and Fe^{3+} ions to quench neither chromopeptides nor PCB fluorescence indicates that chromopeptides selectively chelate Cu^{2+} ions. Accordingly, C-PC derived chromopeptides may be useful in preventing pro-oxidative effect of copper, and in increasing its bioavailability in the GIT.

Similar to ORAC assay, AAPH through peroxyl radical generation induce oxidative damage of RBC, inducing hemolysis in time-dependent manner. Hemolysis was significantly lower ($p<0.05$) after 2 h of incubation in presence of chromopeptides and in particular PCB. Moreover, C-PC derived chromopeptides and PCB showed significantly higher RBCs protective activities than Trolox (**Figure S7D, Table 2**). The addition of the PCB and chromopeptides to the RBC suspension in absence of AAPH did not cause hemolysis even after 6 h of incubation (data not shown). The highest protective activity of PCB chromophore is most likely due to its hydrophobic nature, as antioxidants with greater lipophilicity have better ability to inhibit RBC hemolysis **[49]**. High protective activity of PCB in this assay further confirms chromophore responsibility for antioxidant properties of chromopeptides. As expected, there was a strong correlation between inhibition of hemolysis and ORAC value ($R=0.99$; **Fig. S8**), reducing power ($R=0.99$; **Fig. S9**) and Cu^{2+}-chelating activity ($R=0.97$; **Fig. S10**). This suggests that chromopeptides protect erythrocyte membrane structure and function by scavenging AAPH-generated free radicals and this activity implicated their potential for *in vivo* inhibition of lipid peroxidation.

Cytotoxicity of chromopeptides on cancer cell lines

In this study, Caco-2 and HeLa cell lines were used to evaluate cytotoxic activities of C-PC derived chromopeptides. In general, chromopeptides showed more cytotoxic effect on Caco-2 than HeLa cells (**Figure 3**). All five chromopeptide fractions (90 μM) significantly ($p<0.05$) reduced viability in Caco-2, while fractions II, III and V were significantly ($p<0.05$) cytotoxic on HeLa cells. The most potent cytotoxicity to Caco-2 cells had fraction III (reduced viability to 45±6%; IC_{50} value about 70 μM), and fraction V was the most cytotoxic for HeLa cells (reduced viability to 52±2%). These data are comparable with cytotoxity of PCB on pancreatic cancer cell lines **[9]**. There are large literature differences between doses of C-PC

required to induce cell death, depending on cancer cell lines used in evaluation of cytotoxicity **[50, 51]**. C-phycocyanin has shown significant effects on growth inhibition of HeLa cells at much lower concentrations **[51]** in comparison to concentrations of chromopeptides required to induce cell death in our study, indicating that apoprotein part of C-PC is an important contributor to the anti-cancer activity of protein. On the other hand, LMW peptides obtained from different food sources exhibited significant cytotoxic activities on several cancer cell lines at various concentrations, from few μM **[52]**, hundreds of μM **[53-55]** or even few mM **[56]**, suggesting that isolated chromopeptides show moderate cytotoxic activities in comparison to other food LMW peptides.

Interestingly, there is a good correlation between chromopeptides cytotoxic activity on Caco-2 cells and ORAC value (R=0.93; **Fig. S11**), reducing power (R=0.88; **Fig. S12**) and Cu^{2+}-chelating activity (R=0.93; **Fig. S13**), suggesting that chromopeptides with higher antioxidative activities have stronger cytotoxic effect(s). This implies that C-PC-derived chromopeptides reduced viability of cells by disturbing free radical balance specific for cancer cells. In contrast to normal cells, many types of cancer cells produce high levels of reactive species: anti-cancer activity of PCB on pancreatic cancer cell lines was due to inhibition of mitochondrial production of ROS and improvement of glutathione redox status **[9]**.

Conclusion

The present study has shown that C-PC is easily digestible by pepsin. In chromopeptide fractions, obtained after separation of pepsin digest of C-PC, identified chromopeptides contained 2 to 13 amino acid residues. Obtained chromopeptides show potent antioxidant activities, with chromophore portion being most responsible for these effects. Moreover, chromopeptides efficiently protected erythrocytes from free radical-induced hemolysis, as well as had cytotoxic activity on cancer cells, in parallel to their antioxidative capacity. These results indicate that chromopeptides released after pepsin digestion of C-PC could substantially contribute to the promotion of human health by increasing the organism's antioxidant load.

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Figures

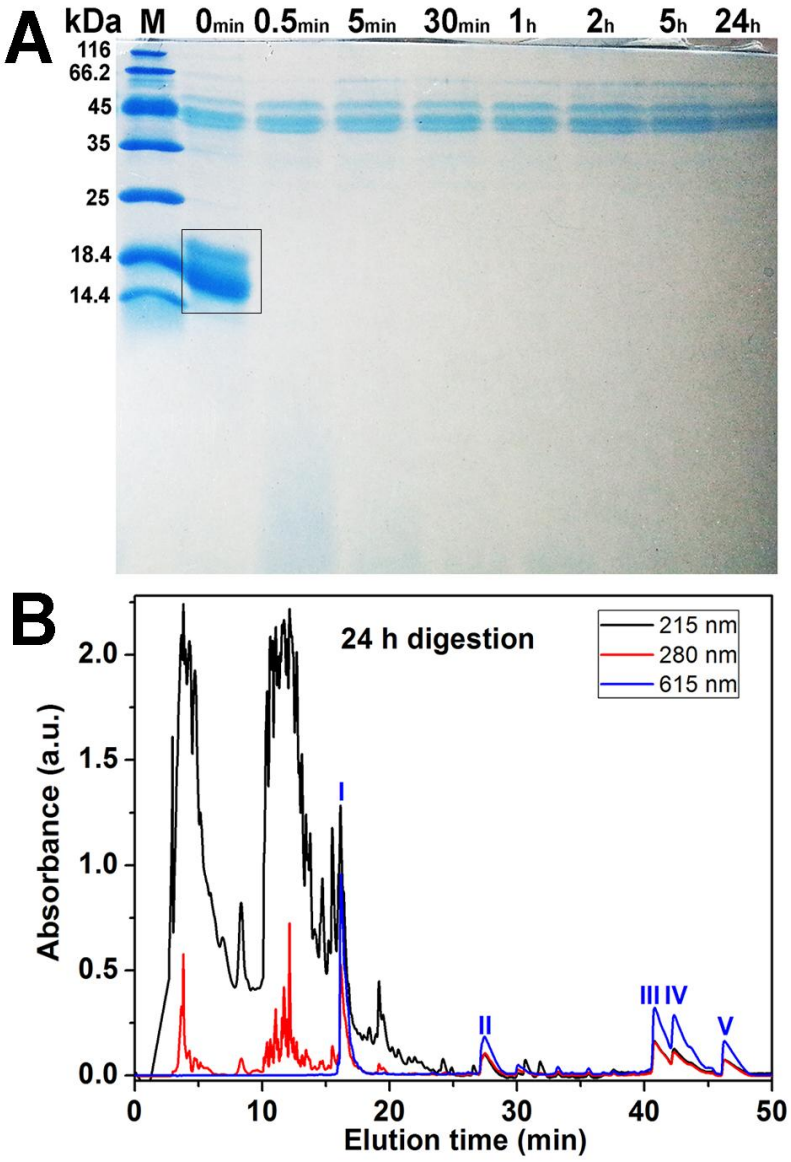


Figure 1. (A) SDS-PAGE analysis (reducing conditions, 16% PAA gel) of time dependent pepsin digestion of C-PC (1 U/μg protein). Rectangle is marking α (17 kDa) and β (19 kDa) subunits of C-PC. Band at about 40 kDa represents pepsin. M and 0-24 h denote molecular weight markers and time of digestion, respectively; **(B)** RP-HPLC (C-18 column) chromatogram of chromopeptides (fractions I-V) obtained after 24 h C-PC digestion by pepsin.

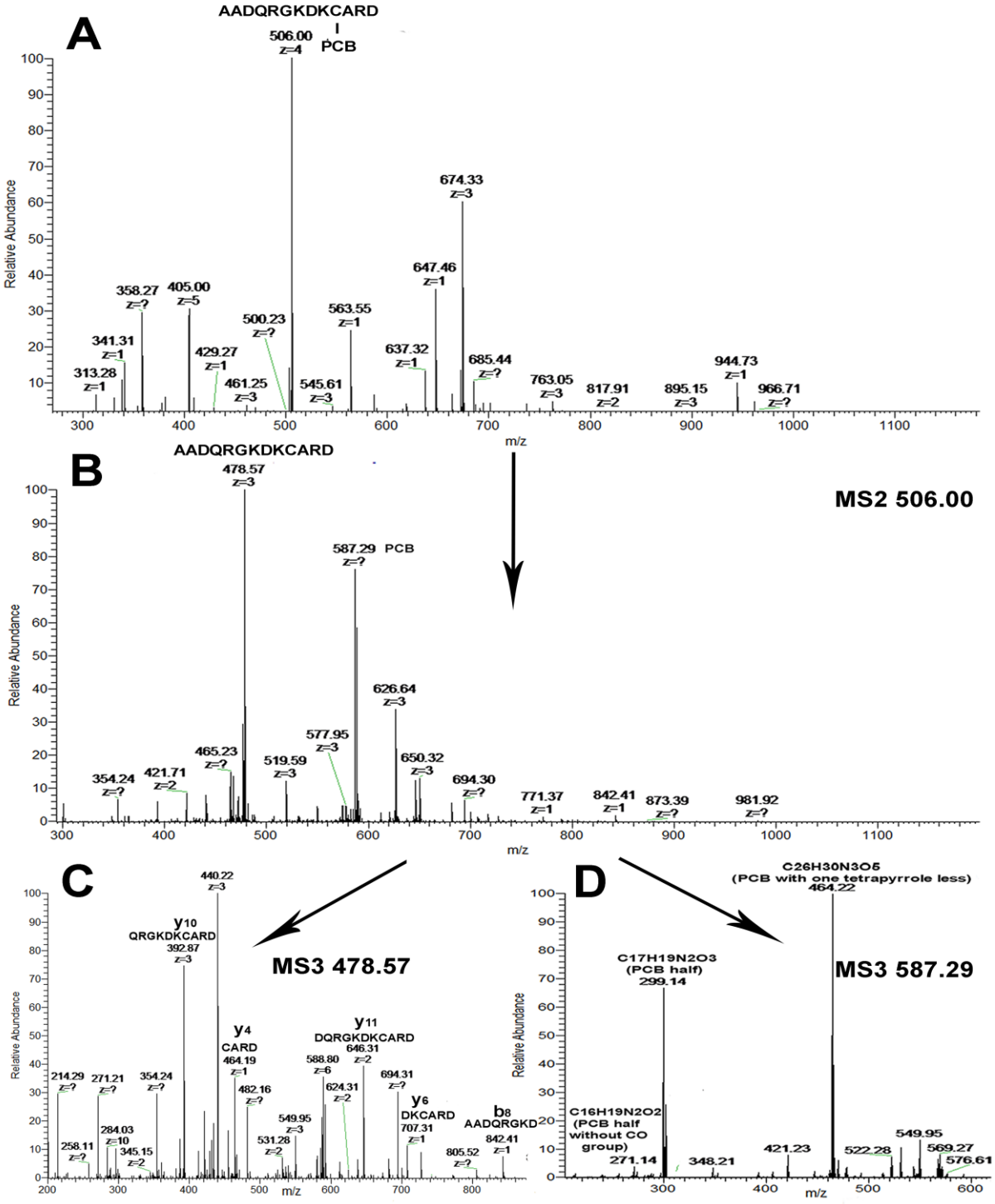


Figure 2. (**A)** MS spectrum of fraction I, after 24 h C-PC digestion by pepsin. Peaks with m/z ratios 405.00, 506.00 and 474.33 arise from AADQRGKDKCARD chromopeptide; (**B)** MS2 spectrum of molecular ion with m/z ratio 506.00 (z=4); (**C**) Fragmentation of ion with m/z ratio 478.57 (z=3), representing AADQRGKDKCARD sequence without PCB. Data were obtained after recording MS3 spectrum of this ion (MS2 506.00, MS3 478.57); (**D**) Fragmentation of ion with m/z ratio 587.29 (z=1), representing PCB. Data were obtained after recording MS3 spectrum of this ion (MS2 506.00, MS3 587.29).

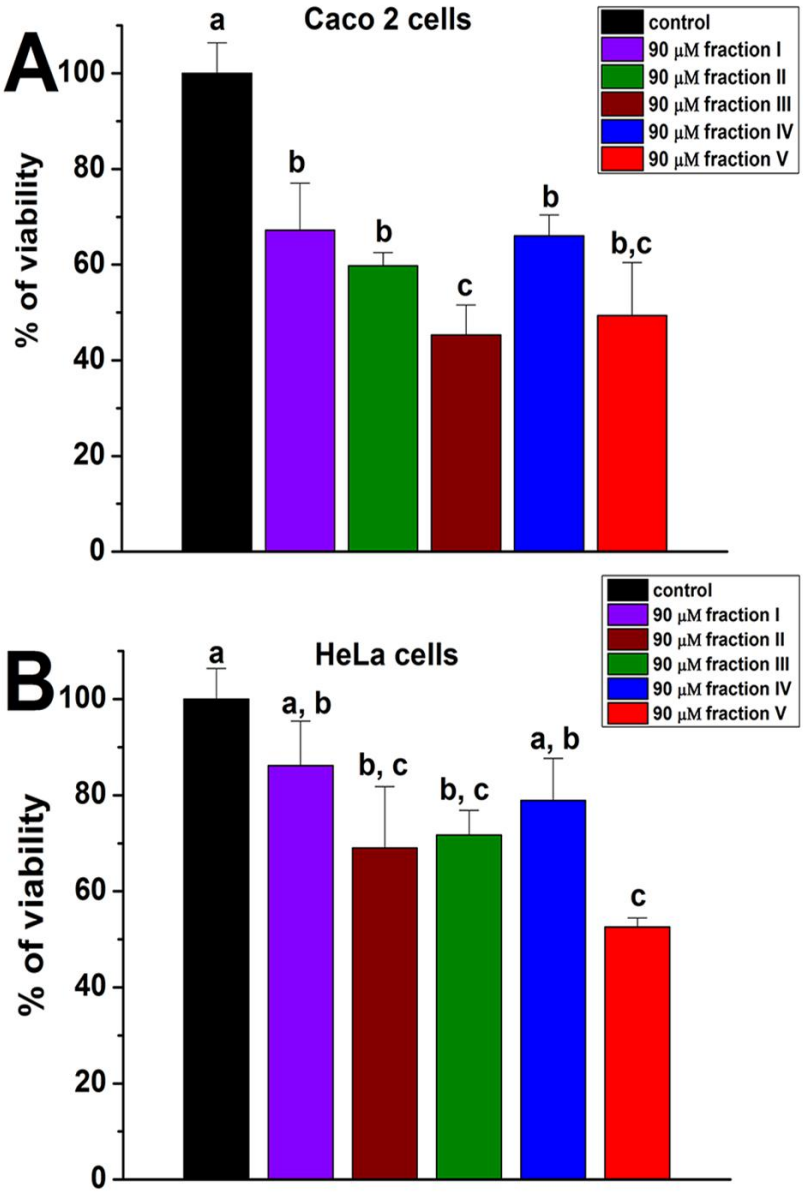


Figure 3. Cytotoxic effects of chromopeptides (90 μM) on Caco-2 (**A**) and HeLa (**B**) cell lines during 24 h incubation. The data marked by different letters are significantly different ($p<0.05$). Values are shown as means ± standard deviations.

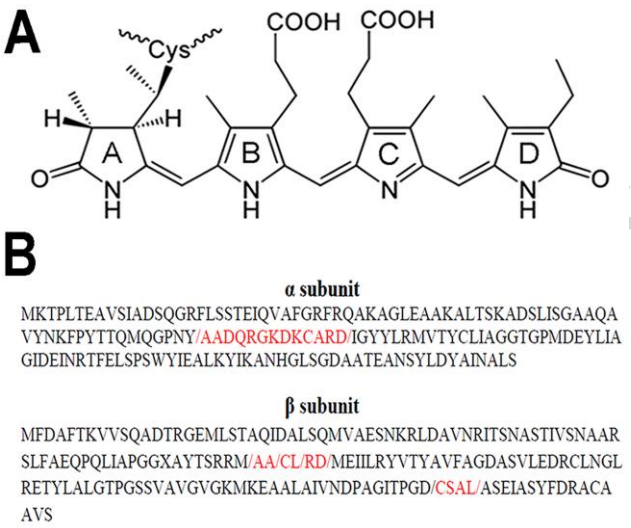


Figure 4. (A) Chemical structure of tetrapyrrol (A-D) PCB chromophore bound to C-PC *via* thioether bond; **(B)** Amino acid sequences of C-PC α and β subunits with chromopeptide sequences (red), obtained after pepsin digestion.

Tables

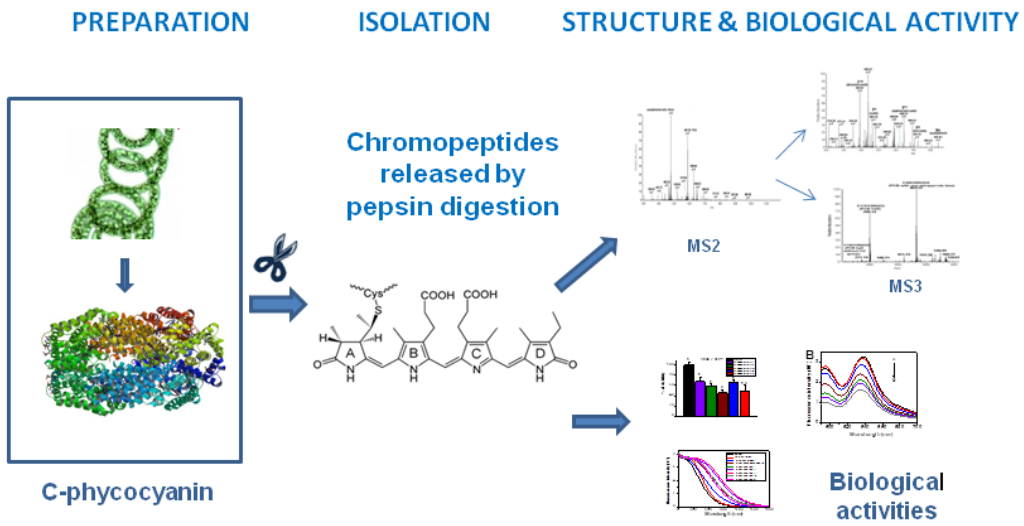
Table 1. Amino acid sequences of chromopeptides (isolated after 24h pepsin digestion of C-PC), obtained by tandem mass spectrometry. [a]Chromopeptide sequences arising from traces of allophycocyanin; [b]Glutamine residue deamidation (0.98476).

Fraction	Chromopeptide sequence	Experimental mass	Theoretical mass	Δ mass	Relative abundance
I	AADQRGKDKCARD	2019.9491	2018.9632	0.9859[b]	100
II	AACLRD	1233.5841	1233.5852	-0.0011	100
II	CLRD	1091.5099	1091.5110	-0.0011	61
III	CSAL	978.4531	978.4521	0.0009	100
IV	AACL	962.4609	962.4571	0.0038	100
IV	ATCL[a]	992.4699	992.4677	0.0022	50
IV	TATCL[a]	1093.5181	1093.5154	0.0027	32
V	CL	820.3820	820.3829	-0.0010	100

Table 2. Results of ORAC, reducing power, Cu^{2+} binding and hemolysis assays of chromopeptides, C-PC, PCB and whole C-PC digest. Except for Cu^{2+} binding, Trolox was positive control in all antioxidant tests. ORAC and reducing power values are expressed as Trolox equivalents (TE). The data marked by different letters are significantly different ($p<0.05$). Values are shown as means ± standard deviations.

Sample	ORAC assay TE (μM/μM of chromophore)	Reducing power TE (μM/μM of chromophore)	Binding constants of Cu^{2+} ions ($K_a \cdot 10^6$ (M^{-1}))	Hemolysis assay (% of hemolysis after 2h incubation)
Fraction I	9.4±0.9[a]	3.0±0.2[a]	0.60±0.03[c]	22.3±2.1[c]
Fraction II	12.0±1.3[a]	4.7±0.3[b]	0.62±0.04[c]	9.1±0.8[e, f]
Fraction III	13.2±1.1[a]	4.7±0.3[b]	1.04±0.05[d]	6.2±.0.6[f]
Fraction IV	9.1±1.0[a]	2.7±0.1[a]	0.46±0.02[b, c]	21.2±1.3[c, d]
Fraction V	8.2±0.9[a]	3.2±0.2[a]	0.49±0.02[b, c]	15.2±1.1[c, d, e]
Whole C-PC digest	25.4±2.8[b]	3.2±0.3[a]	/	/
PCB	8.4±0.7[a]	4.5±0.4[b]	0.42±0.03[b]	3.0±0.3[f]
C-PC	/	/	0.24±0.02[a]	/
Trolox	1.0±0.1	1.0±0.1	/	34.5±2.1[b]
Control (RBC with AAPH)	/	/	/	45.4±3.7[a]

Graphical abstract



Highlights

- C-Phycocyanin, blue biliprotein of Spirulina, is rapidly digested by pepsin.
- Sequences of peptic chromopeptides were determinated by tandem mass spectrometry.
- Antioxidant, metal-chelating and cytotoxic effect on cancer cell lines were shown.
- Chromopeptides protect human erythrocytes from free radical-induced hemolysis.
- Chromophore is mainly responsible for observed biological effects of chromopeptides