Supplementary material for the article:

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Covalent binding of food-derived phycocyanobilin to bovine β-lactoglobulin under physiological conditions

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Supplementary materials

1. Materials and methods

1.1. CD measurements

For the measurements in the near-UV and VIS region (250–750 nm), concentration of dialyzed BLG and BLG-PCB complex (Section 2.6.) was 50 μM. Buffer and PCB spectra were subtracted from spectra of BLG and BLG-PCB complex, respectively. Scan speed and number of

accumulations were set to 200 nm/min and 3, respectively, while a cell with an optical path length of 1 cm were used.

Far-UV CD spectra of 40 μM BLG and 40 μM BLG-PCB complex (dialyzed sample; Section 2.6.) were recorded in the range 185–260 nm, at a scan speed of 50 nm/min, using a cell with an optical path length of 0.1 mm and with an accumulation of three scans. Buffer and PCB spectra were subtracted from spectra of BLG and BLG-PCB complex, respectively. Secondary structure content was calculated by CDPro software, using CONTIN algorithm and SD43 database.

1.2. Determination of yield of BLG-PCB reaction

Two approaches were used in order to determine the yield of BLG-PCB adduct formation (Section 2.7.).

In first, 60 μL of BLG-PCB adduct or free BLG (dialyzed samples, obtained according to section 2.6.) was mixed with 30 μL of 1 M Tris buffer (pH 8.0), 180 μL of water and 30 μL of 2 mM DTNB (dissolved in 50 mM sodium acetate). After 1 hour incubation at room temperature absorbance was measured at 405 nm. BLG-PCB adduct *per se* shows absorption at this wavelength; therefore, we performed two controls. First control (AI) did not contain DTNB reagent, while the second control (AII) did not contain protein. The resultant absorbance (Ar) was obtained by equation:

$$Ar = As - AI - AII \quad \textbf{(1)}$$

where As is absorbance of the sample (protein + water + buffer + DTNB).

Free cysteine was used as standard in the concentration range between 100 and 800 μM. Based on standard curve (**Fig. S1**) and resultant absorbance, concentrations of free SH group in

BLG and BLG-PCB adduct were determined. Percentage of SH groups in adduct, modified by PCB (the yield of reaction), was calculated using equation:

$$\text{Yield (\%)} = 100\% - [\text{BLG-PCB}] / [\text{BLG}] \times 100\% \quad \textbf{(2)}$$

where [BLG-PCB] and [BLG] represent concentrations of free SH groups in BLG-PCB adduct and free BLG, respectively.

In second approach, C-PC and dialyzed BLG-PCB adduct (Section 2.6.) were digested by pepsin in simulated gastric fluid (SGF). Briefly, 125 μL of C-PC (8 mg/mL) or BLG-PCB adduct (2 mg/mL) was added to 375 μL of SGF (84 mM HCl and 35 mM NaCl, pH 1.3), containing 10 units of pepsin (from porcine gastric mucosa, 2800 U/mg; Sigma-Aldrich, USA) per μg of protein. Mixtures were incubated at 37°C during 4 hours. Reaction was stopped by addition of 60 μL of 1 M $NaHCO_3$. Standard curve (**Fig. S2**) was constructed by preparing a series of digest dilutions (between 0.2 and 1.2 μM chromophore concentration) in 20 mM phosphate buffer pH 7.2. Emission of standard and sample (50 fold dilution of BLG-PCB digest in the same buffer) was recorded at 640 nm, with excitation wavelength at 580 nm and slits width 5 nm. Yield was estimated using equation:

$$\text{Yield (\%)} = [\text{chromophore}] / [\text{protein}] \times 100\% \quad \textbf{(3)}$$

where [chromophore] represent concentration of PCB bound to BLG determined by standard curve, while [protein] is total BLG concentration in dialyzed adduct.

1.3. Detection of BLG-PCB adduct by mass spectrometry

Heated electrospray ionization (HESI) was done in positive mode. HESI parameters were: capillary temperature 275°C, source voltage 4.21 kV, capillary voltage 41.92 V, tube lens voltage 110 V, sheath and auxiliary gas flow 15.01 and 3.99 (arbitrary units), respectively. Acquisition was 1 min per sample. Samples were injected directly with flow 20 μL/min. MS

spectra were acquired between m/z 200 and m/z 2000. MS2 and MS3 spectra were obtained by collision-induced dissociation (CID) fragmentation of ionized proteins. CID was performed with helium gas at normalized collision energy of 35%, while the parent ions were activated for 30 ms. The ESI-MS and ESI-MS2 data were analyzed in Xcalibur version 2.1 (Thermo Fisher Scientific Inc., USA).

1.4. Computational details

To predict the binding site(s) of PCB on BLG, molecular docking simulations were performed using Autodock Vina program (**Trott & Olson, 2010**). Protein and pigment molecules were prepared for docking experiment as described in our previous paper (**Minic, Milcic, Stanic-Vucinic, Radibratovic, Sotiroudis, Nikolic, et al., 2015**). In order to cover total protein volume, a grid box (28×28×28 Å) was moved over the rectangular matrix containing protein with points 8 Å apart. From each docking run 9 binding modes with highest scoring function were kept for further analysis.

In order to account for disulfides interchange in BLG a steered molecular dynamics simulation was performed. Using the collective variable module (colvar in NAMD 2.9) (**Phillips, Braun, Wang, Gumbart, Tajkhorshid, Villa, et al., 2005**) restraints on three cysteine residues (Cys106, Cys119 and Cys121) were applied. For the first 150 ns of the simulation, restraints were maintained in order to simulate for simultaneous disulfide interchanges between Cys119 and Cys121. After that, new Cys106-Cys121 disulfide bond was created and the system was simulated for further 150 ns without any restrains. At the end, interactions between PCB covalently bound to the Cys119 and rest of the protein were investigated by covalent docking method, more specifically flexible side chain method (**Bianco, Forli, Goodsell & Olson, 2016**),

using docking engine from AutoDock 4.2 program (**Morris, Huey, Lindstrom, Sanner, Belew, Goodsell, et al., 2009**).

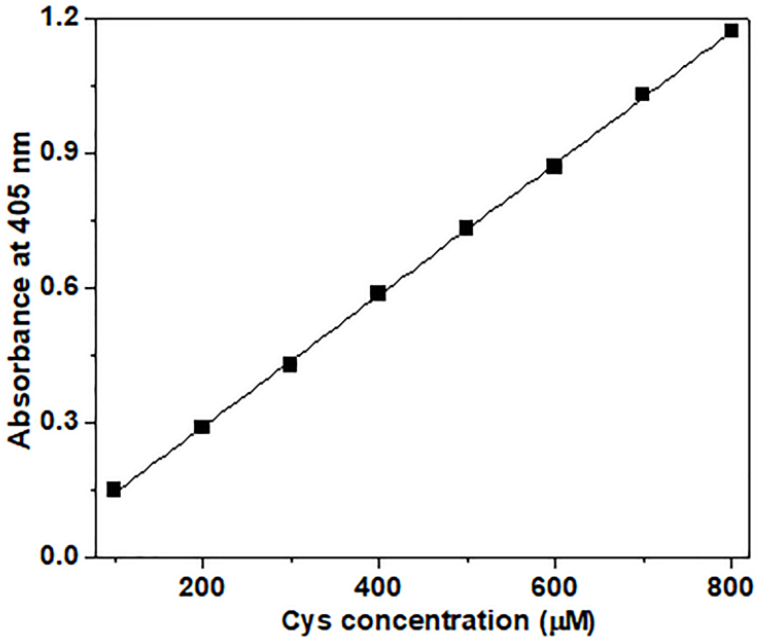

Figure S1. Standard curve for determination of free SH groups in BLG.

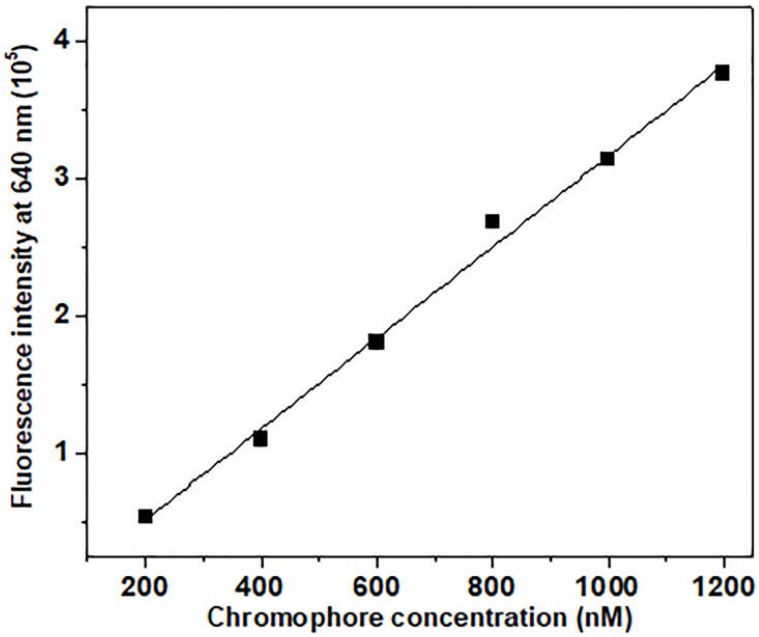

Figure S2. Standard curve for determination of neo-chromopeptides concentration in BLG-PCB pepsin digest (excitation at 580 nm).

1. Results

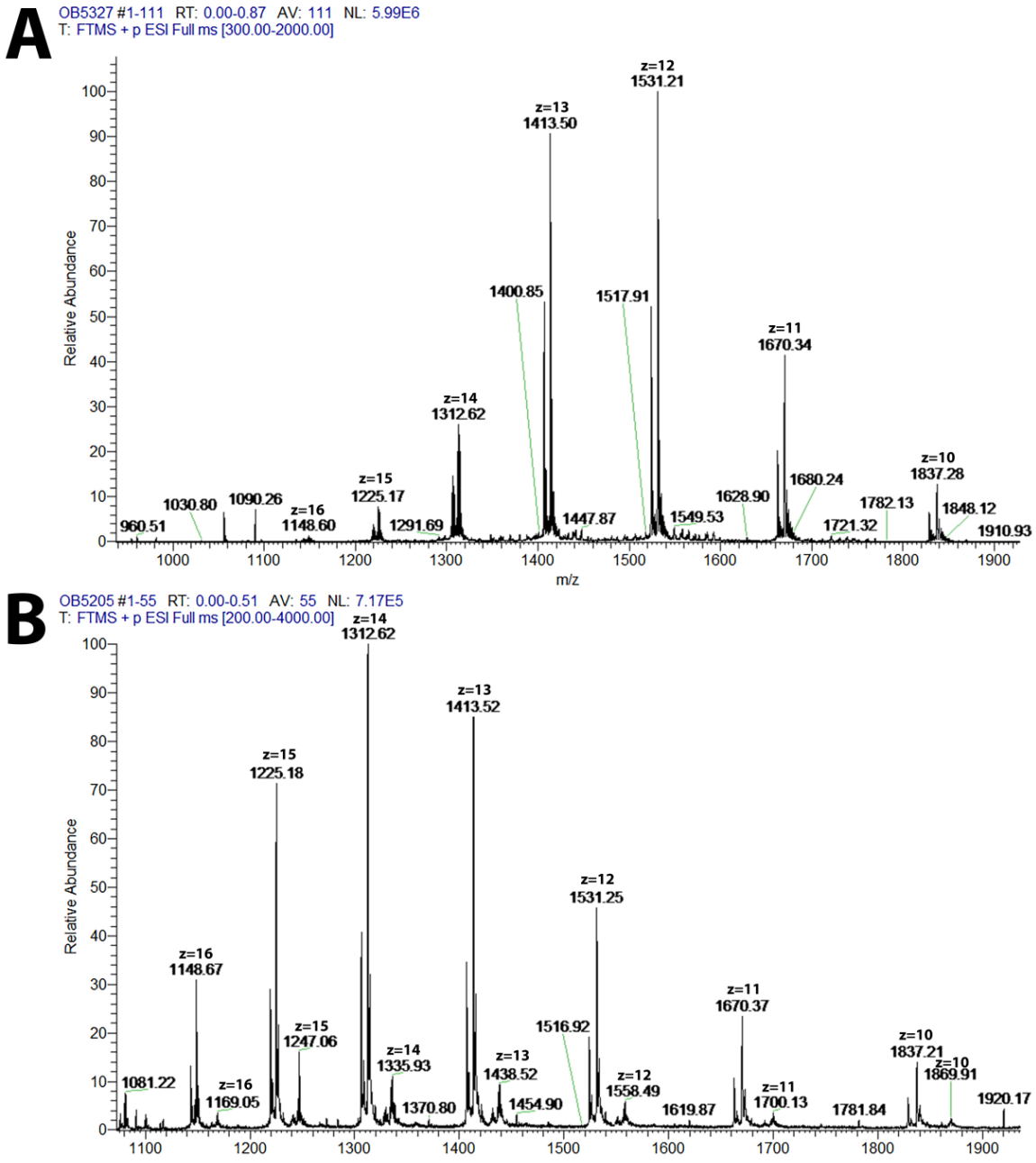

Figure S3. MS spectrum of 20 µM BLG incubated without **(A)** and in the presence of 20 µM PCB **(B)** for 2 hours at 37ºC in 20 mM phosphate buffer pH 7.2.

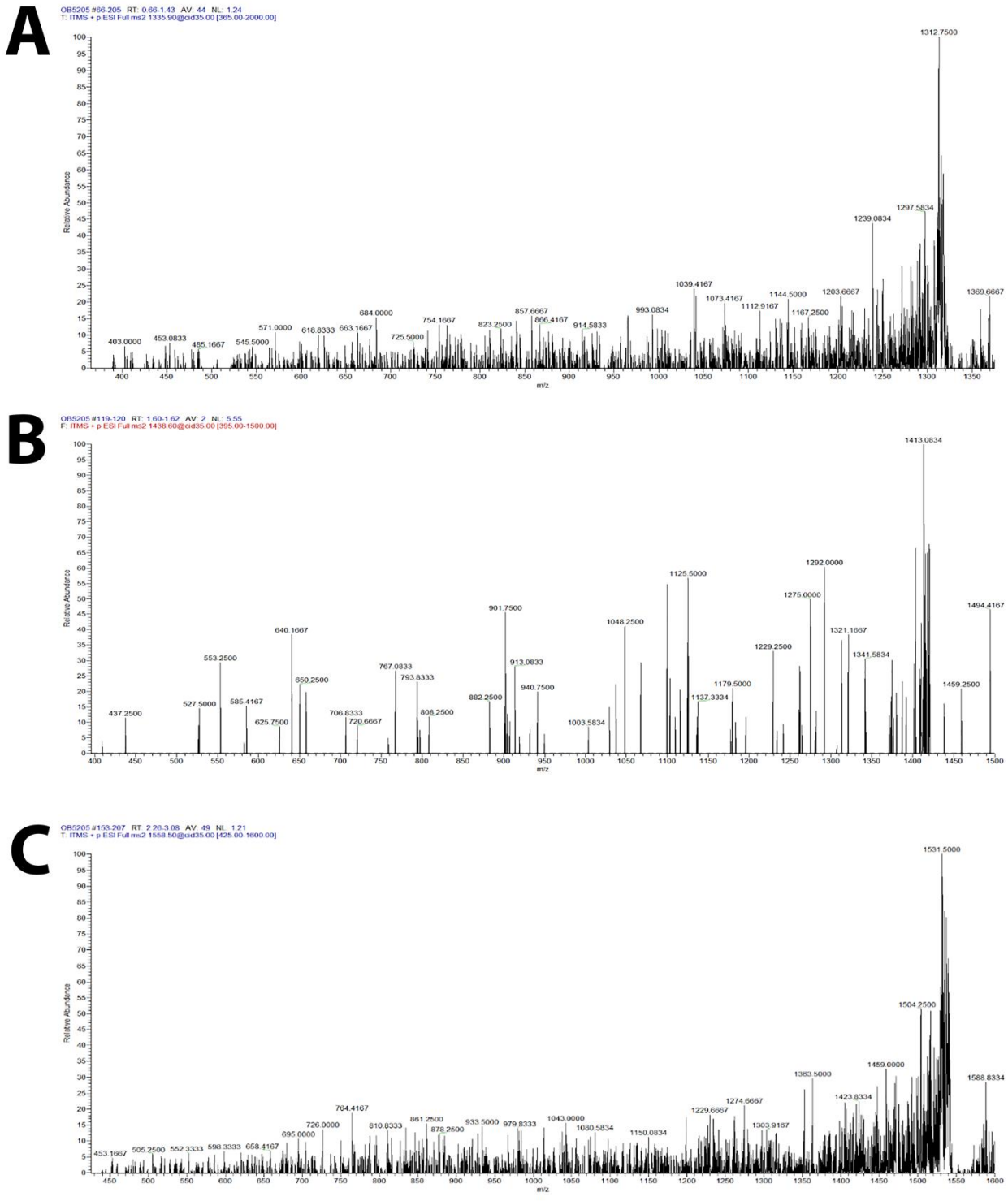

Figure S4. MS2 spectrum of molecular ion with m/z ratio **(A)** 1335.90 (z=14), **(B)** 1438.60 (z=13) and **(C)** 1558.50 (z=12) from MS spectrum of 20 µM BLG incubated in the presence of 20 µM PCB for 2 hours at 37ºC in 20 mM phosphate buffer pH 7.2.

Table S1. The list of masses from MS spectrum of 20 µM BLG incubated in the presence of 20 µM PCB for 2 hours at 37ºC in 20 mM phosphate buffer pH 7.2. BLG A – β-lactoglobulin isoform A; BLG B – β-lactoglobulin isoform B; BLG A:PCB – covalent adduct of β-lactoglobulin isoform A with PCB oxidation derivative; BLG B:PCB – covalent adduct of β-lactoglobulin isoform B with PCB oxidation derivative.

Sample	m/z	z	mass	Delta mass (BLG:PCB – BLG)
BLG A	2031.800	9	18277.20	325.403
BLG A:PCB	2067.956	9	18602.60	
BLG B	2041.276	9	18362.48	324.482
BLG B:PCB	2077.330	9	18686.97	
BLG A	1828.658	10	18276.58	323.483
BLG A:PCB	1861.006	10	18600.06	
BLG B	1837.217	10	18362.17	326.263
BLG B:PCB	1869.843	10	18688.43	
BLG A	1662.516	11	18276.67	325.4559
BLG A:PCB	1692.102	11	18602.13	
BLG B	1670.394	11	18363.34	324.1271
BLG B:PCB	1699.860	11	18687.46	
BLG A	1524.033	12	18276.40	326.2368
BLG A:PCB	1551.220	12	18602.63	
BLG B	1531.252	12	18363.02	326.7768
BLG B:PCB	1558.483	12	18689.80	
BLG A	1406.843	13	18275.95	326.56
BLG A:PCB	1431.963	13	18602.51	
BLG B	1413.519	13	18362.74	325.1313
BLG B:PCB	1438.529	13	18687.88	
BLG A	1306.476	14	18276.66	326.0096
BLG A:PCB	1329.762	14	18602.67	
BLG B	1312.621	14	18362.69	326.1776
BLG B:PCB	1335.919	14	18688.87	
BLG A	1219.511	15	18277.66	326.136
BLG A:PCB	1241.253	15	18603.80	
BLG B	1225.179	15	18362.69	328.1595
BLG B:PCB	1247.057	15	18690.85	
BLG A	1143.292	16	18276.68	326.192
BLG A:PCB	1163.679	16	18602.87	
BLG B	1148.670	16	18362.72	326.1216
BLG B:PCB	1169.053	16	18688.84	

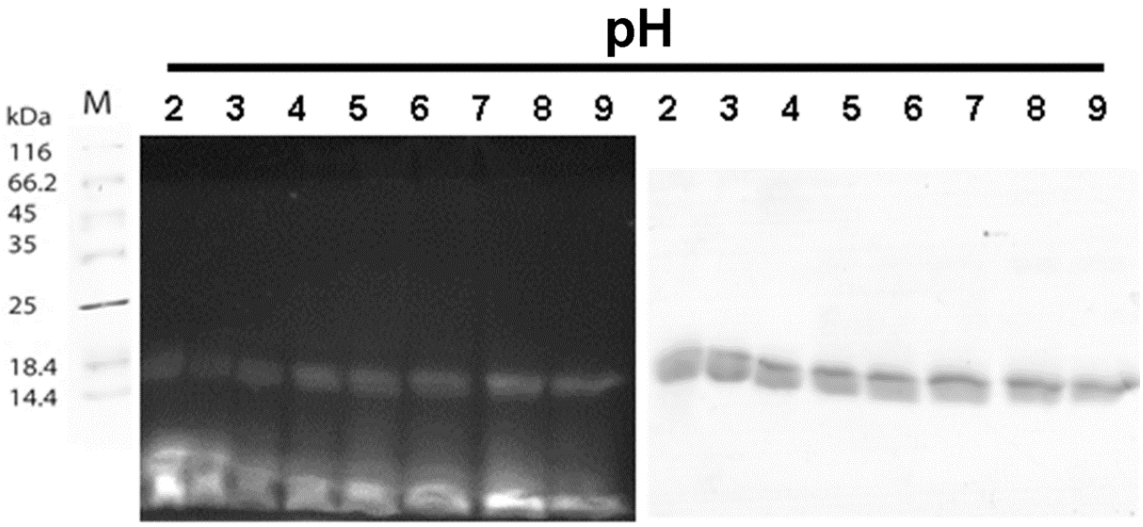

Figure S5. Effects of pH on BLG-PCB adduct formation: SDS-PAGE (16% PAA gel, non-reducing conditions). Left: Zn^{2+} staining; right: CBB staining.

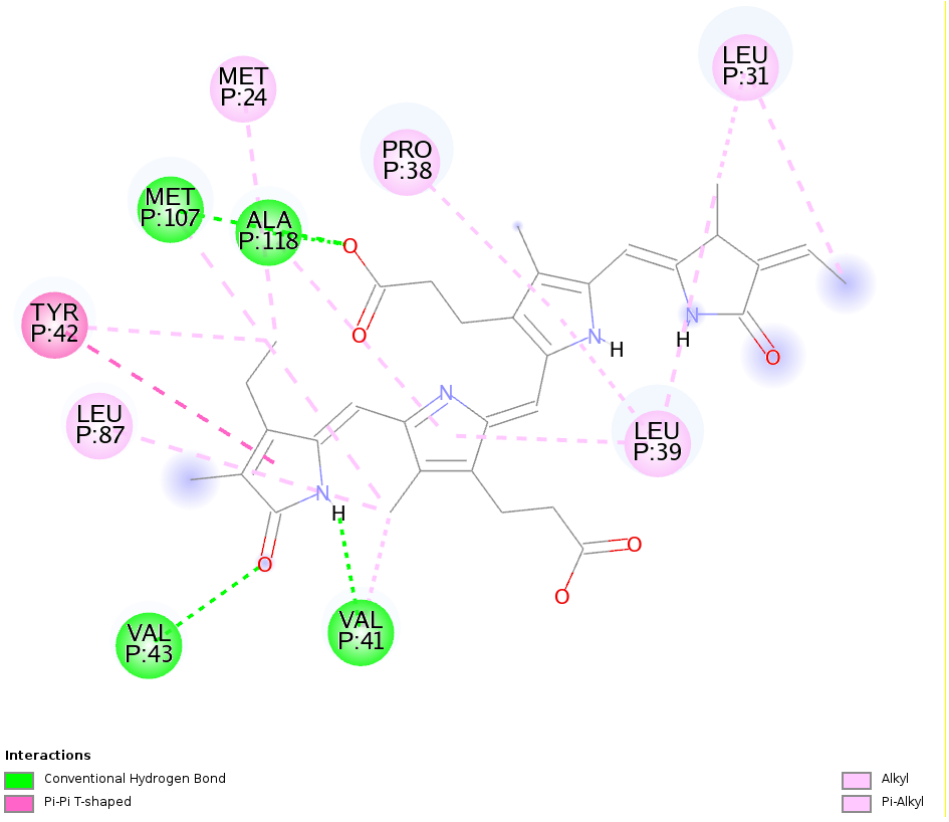

Figure S6. 2D diagram with labeled interactions of covalently docked PCB to BLG after SMD simulation of disulfide exchange (PDB ID: 3NQ3).

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