Supporting Information

β-Lactoglobulin Peptide Fragments Conjugated with Caffeic Acid Displaying Dual Activities for Tyrosinase Inhibition and Antioxidant Effect

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Materials. Fluorenylmethyloxycarbonyl (Fmoc)-Rink amide linker-coupled aminomethyl polystyrene (Rink amide AM) resin (0.47 mmol/g), filtered polypropylene tube reactors (5 or 15 mL Libra tube RT-20M), Fmoc-protected amino acids, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), and hydroxylbenzotriazole (HOBt) were obtained from BeadTech (Seoul, Korea). The compound *N,N*-diisopropylethylamine (DIPEA) was purchased from Alfa Aesar (Ward Hill, MA, USA). CA, ninhydrin, linoleic acid (~99 %), 2,2-diphenyl-1-picrylhydrazyl (DPPH), mushroom tyrosinase, 3,4-dihydroxyphenylalanine (L-DOPA), ammonium thiocyanate (NH_4SCN), ferrous chloride ($FeCl_2$), polyoxyethylenesorbitan monolaurate (Tween 20), butylated hydroxyanisole (BHA), KA, p-toluenesulfonic acid (TsOH), lithium hydroxide monohydrate (LiOH), dimethylaminopyridine (DMAP), 2,2-dimethoxypropane (DMP), 1,2-ethanedithiol (EDT), anisole, triisopropylsilane (TIPS), 3,6-dioxa-1,8-octanedithiol (DODT), thiazolyl blue tetrazolium bromide (MTT), and α-melanocyte stimulating hormone (α-MSH) were supplied by Aldrich (St. Louis, MO, USA). *N*-Methyl-2-pyrrolidone (NMP), piperidine, dichloromethane (DCM), tetrahydrofuran (THF), thionyl chloride ($SOCl_2$), ethyl acetate, hexane, benzene, diethyl ether, ethanol, methanol, calcium chloride ($CaCl_2$) were procured from Dae-Jung Chemicals (Siheung city, Korea). Trifluoroacetic acid (TFA) was obtained from Acros Organics (Morris Plains, NJ, USA). All other solvents were used without further purification.

Instrument. Synthesized products were identified using high performance liquid chromatography (HPLC, Thermo Scientific Spectra System AS3000; Thermo-Fisher, Waltham, MA, USA) equipped with an AAPPTec Spirit Peptide C18 reverse-phase column (120 Å, 5 μm, 4.6 × 250 mm; AAPPTec, Louisville, KY, USA). Mass spectrometry (electrospray ionization mass spectrometry [ESI-MS], LCQ; Thermo Finnigan, Waltham,

USA) was used to determine the mass of each peptide derivative. Linoleic acid peroxidation and enzyme inhibition tests were measured by UV/visible spectroscopy (Optizen 2120 UV, Mecasys Co. Ltd., Daejeon, Korea). A cytotoxicity assay was carried performed using an enzyme-linked immunosorbent assay (ELISA) reader (Infinite M200, Tecan, Austria). ^{1}H and ^{13}C Nuclear magnetic resonance (NMR) spectra were acquired using Bruker AVANCE-400 spectrometer (Bruker, Rheinstetten, Germany) operating at 400 MHz.

Solid phase peptide synthesis. Peptides fragments derived from β-Lactoglobulin were synthesized on Rink AM resin (0.47 mmol/g) via a solid-phase peptide synthesis method. Fmoc-amino acid (3 equivalent), BOP (3 equivalent), HOBt (3 equivalent), and DIPEA (6 equivalent) in NMP were added to the resin at room temperature (RT) and the resin mixture was shaken for 3.5 h. To remove Fmoc-protecting group on the resin, 20% piperidine/NMP solution was added and the resulting solution was shaken for 50 min at RT. The completion of each coupling reaction was determined by Kaiser's ninhydrin color test. After coupling two or three additional amino acids using the aforementioned procedure, CA (3 equivalent) was coupled to a tripeptide or two tetrapeptide using BOP (3 equivalent), HOBt (3 equivalent), and DIPEA (6 equivalent) in NMP for 3.5 h at RT. The synthesized product was cleaved from the resin using a cleavage cocktail (TFA/Anisole/TIPS/DODT = 9.5/0.3/0.1/0.1) for 2.5 h at RT. After evaporating the solvent, the desired product was precipitated with cold diethyl ether, yielding a white powder. The product was washed five times with cold diethyl ether and dried under a nitrogen atmosphere. The product was identified by ESI-MS. Peptides and peptide derivatives were analyzed by reverse-phase HPLC (RP-HPLC) using following conditions: AAPPTec Spirit Peptide C18 reverse-phase column; gradient elution with A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) from 10% to 90% B over 30 min, at a flow rate of 1.0 mL/min; detection: UV detection at 230 and 326 nm. Compounds were purified by RP-

HPLC (A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile from 10% to 90% B over 30 min at a flow rate of 4.0 mL/min).

Synthesis of CA methyl ester (CA-OMe). CA (15 mmol) was dissolved in methanol (100 mL) in a 100 mL round-bottom flask and thionyl chloride (2 equivalent) was added dropwise in an acetonitrile/dry ice bath, followed by the addition of DMAP (0.2 equivalent). The resulting mixture was stirred at RT for overnight. After evaporation of methanol, the product mixture was overnight precipitated using cold chloroform/hexane (1:3) solution. The precipitate was filtered and washed several times with chloroform/hexane (1:3), and dried in vacuum. Crude CA-OMe was purified by silica gel chromatography (71 %) using an eluent of ethyl acetate and hexane at 1:2 ratio.

^{1}H NMR (400 MHz, $(CD_3)_2CO$, 297K): δ 3.69 (3H, s), 6.27 (1H, d, J = 16), 6.758 (1H, d, J = 8), 6.99-7.0 (1H, m), 7.01-7.05 (1H, m), 7.46-7.50 (1H, m), 9.13 (1H, s), 9.6 (1H, s)

^{13}C NMR (400 MHz, $(CD_3)_2CO$, 297K): δ 51.18 ($COO\underline{C}H_3$), 113.65 (C-8), 114.76 (C-2), 115.69 (C-5), 121.38 (C-6), 125.45 (C-1), 145.14 (C-4), 145.54 (C-7), 148.39 (C-3), 166.97 (C-9)

Synthesis of acetonide-protected CA-OMe (CA[acetonide]-OMe). Purified CA-OMe (10 mmol) dissolved in anhydrous benzene (100 mL) was reacted with DMP (4 equivalent) in a two-neck 250 mL flask. One neck of the flask was connected to a Soxhlet extractor and the thimble was filled with anhydrous $CaCl_2$ to trap methanol and deionized (DI) water. The other neck was sealed with a rubber septum for sampling. Argon gas was injected into the flask for 5 min and the solution was heated at reflux for 5 min, followed by the addition of TsOH (0.05 equivalent). During the reaction, ferric chloride test was performed to check the degree of reaction. After 3 h, the reaction mixture was cooled to RT and benzene was removed by rotary evaporation. The crude product (deep yellowish liquid) was purified by

silica gel chromatography and eluted with ethylene acetate and hexane (1:9). CA(acetonide)-OMe was obtained as a white powder.

^{1}H NMR (400 MHz, $(CD_3)_2CO$, 297K): δ 1.68-1.77 (6H, s), 3.7 (3H, s), 6.478 (1H, d, J = 16), 6.86-6.88 (1H, m), 7.14-7.17 (1H, m), 7.32 (1H, d, J = 1.6), 7.563 (1H, d, J = 16)

^{13}C NMR (400 MHz, $(CD_3)_2CO$, 297K): δ 25.74 (acetonide C($\underline{C}H_3)_2$), 51.51 (COO$\underline{C}H_3$), 106.84 (C-5), 108.61 (C-2), 115.57 (C-8), 119.19 (C-6), 124.84 (C-1), 128.43 (acetonide $\underline{C}(CH_3)_2$), 144.94 (C-4), 148.10 (C-7), 149.49 (C-3), 167.40 (C-9)

Methyl ester deprotection of CA(acetonide)-OMe (CA[acetonide]-OH). Purified CA(acetonide)-OMe (2 mmol) was dissolved in THF/DI water (2:1) and cooled in an ice bath, followed by the addition of LiOH (3 equivalent). The mixture was overnight stirred in an ice bath. CA(acetonide)-OH was purified by silica gel chromatography (21 %) and eluted with ethylene acetate and hexane (1:2).

^{1}H NMR (400 MHz, $(CD_3)_2CO$, 297K): δ 1.68 (6H, s), 6.36 (1H, d, J = 15.6), 6.81 (1H, d, J = 7.6), 7.1-7.12 (1H, m), 7.13-7.18 (1H, s), 7.58 (1H, d, J = 16)

^{13}C NMR (400 MHz, $(CD_3)_2CO$, 297K): δ 25.52 (acetonide C($\underline{C}H_3)_2$), 106.44 (C-5), 108.27 (C-2), 116.62 (C-8), 118.79 (C-6), 124.25 (C-1), 128.19 (acetonide $\underline{C}(CH_3)_2$), 144.01 (C-4), 147.56 (C-7), 148.75 (C-3), 167.80 (C-9)

Measurement of tyrosinase inhibitory activity. A total of 25 μL CA-Pep was mixed with 250 μL of 0.1 M phosphate-buffered saline (PBS, pH 6.8), 250 μL L-DOPA (2.5 mM), and 200 μL DI water. The solution was treated with 25 μL aqueous mushroom tyrosinase solution (400 U/mL) and the resulting mixture was incubated for 10 min at RT. The absorbance of the mixture was measured at 475 nm. The tyrosinase inhibition activity of each substance was calculated based on the following equation:

%Inhibition = (1 – [$Abs_{475\ nm}$ of test sample/ $Abs_{475\ nm}$ of the control]) × 100

A blank solution containing the same components without the enzyme and inhibitor was used. As a negative control, 25 μL of methanol was added instead of samples.

Measurement of antioxidant activity. The lipid peroxidation inhibition activity was measured for the antioxidant activity of CA-Pep derivatives. First, linoleic acid emulsion (50 mM) was prepared by dissolving 0.284 g of linoleic acid and 0.284 g of Tween 20 in 0.1 M PBS (50 mL, pH 7.0). The emulsion (2.5 mL) was mixed with 0.5 mL DI water, 2.0 mL PBS, and either 0.5 mL CA-Pep, peptide, or methanol. The final concentration of CA-Pep was 100 μM. Each emulsion sample in a glass vial (10 mL) with a rubber septum was placed in the dark at 50°C for 50 h. For measurement of the antioxidant activity using the modified ferric thiocyanate (FTC) method, the emulsion sample (25 μL) at each time interval was mixed with 1.175 mL ethanol (75%), 25 μL a NH_4SCN aqueous solution (30%), and 25 μL $FeCl_2$ (20 mM)/10% HCl aqueous solution. After the resulting mixture was incubated for 3 min at RT, the absorbance of the solution was measured at 500 nm and the lipid peroxidation inhibition activity (%Pi) was determined by comparing the absorbance value when the absorbance of negative control (containing methanol) reached approximately 1. The value of %Pi was calculated based on the following equation:

%Pi = (1 – [Abs_{500nm} of test sample / Abs_{500nm} of control]) × 100

Cytotoxicity test. Mouse melanoma cells (B16-F1) were cultured in a serum-free medium for 24 h. B16-F1 cells were seeded into six-well plates. The culture medium was replaced with α-MSH-containing medium and the cells were incubated with CA-Peps or peptides (100 μM) for 72 h. Following incubation, cells were washed several times with PBS and treated with MTT solution. After incubation for 4 h, isopropyl alcohol was added to all wells and the

cell solution was thoroughly mixed to dissolve the dark blue crystals. Water-soluble formazan was measured by recording the absorbance at 570 nm using ELISA reader.

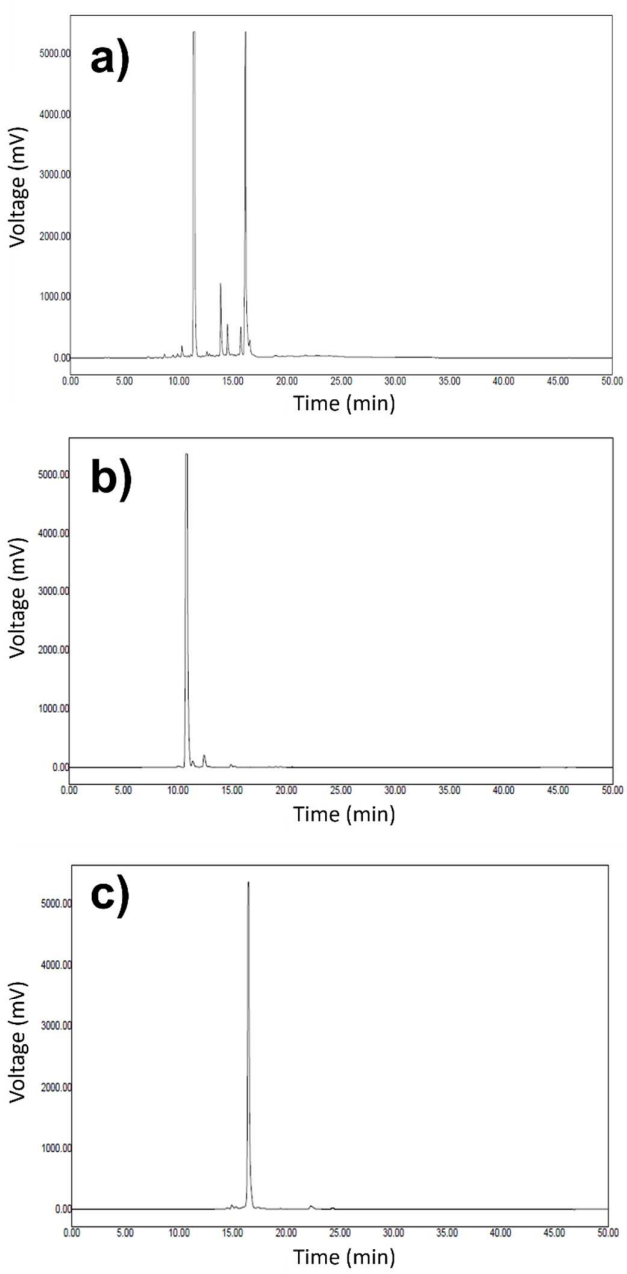

Figure S1. RP-HPLC chromatograms of CA-MHIR synthesized by using a) unprotecte d CA, b) CA(acetonide)-OH with cleavage using a water-containing acid solution, and c) CA(acetonide)-OH with cleavage using a DODT containing acid solution.

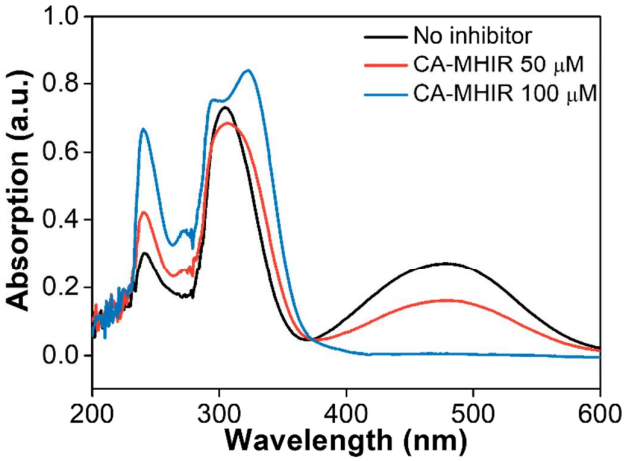

Figure S2. UV absorption spectra of the solution containing mushroom tyrosinase and L-tyrosine with no inhibitor (black line), 50 μM CA-MHIR (red line), or 100 μM C A-MHIR (blue line).

Table S1. IC_{50} Values of CA-Peps and Pristine Peptides

Compound	**IC_{50} (μM)**
CA-MHIR	47.9
MHIR	257.1
CA-HIRL	166.2
HIRL	218.8
CA-HIR	154.8
HIR	-
KA	201.7