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

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

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Abstract

The regulation of tyrosinase activity and reactive oxygen species is of great importance for the prevention of dermatological disorders in the fields of medicine and cosmetics. Herein, we report a strategy based on solid-phase peptide chemistry for the synthesis of β-lactoglobulin peptide fragment/caffeic acid (CA) conjugates (CA-Peps) with dual activities of tyrosinase inhibition and antioxidation. The purity of the prepared conjugates, CA-MHIR, CA-HIRL, and CA-HIR significantly increased to 99%, as acetonide-protected CA was employed in solid-phase coupling reactions on Rink amide resins. The tyrosinase inhibitory activities of all CA-Pep derivatives were higher than the activity of kojic acid, and CA-MHIR exhibited the highest tyrosinase inhibition activity (IC_{50} = 47.9 μM). Moreover, CA-Pep derivatives displayed significantly enhanced antioxidant activities in the peroxidation of linoleic acid as compared to the pristine peptide fragments. All CA-Pep derivatives showed no cytotoxicity against B16-F1 melanoma cells.

Tyrosinase, a copper-containing enzyme, catalyzes the hydroxylation of monophenols and oxidation of *o*-diphenols to produce reactive precursors that are essential for the formation of melanin pigments in melanocytes.[1, 2] Melanin pigments are known to protect skin cells from damage caused by ultraviolet radiation.[3] However, the excessive accumulation of melanin pigments may cause dermatological disorders such as melasma, lentigo, and freckle.[4] Moreover, tyrosinase is known to be involved in neuromelanin formation and neurodegeneration related to Parkinson's disease.[5, 6] Hence, the regulation of tyrosinase activity for the prevention of hyperpigmentation is of great importance in the field of cosmetics and medicine.

Several efforts have been directed for the development of tyrosinase inhibitors via natural resources and synthetic routes.[7] Kojic acid (KA), one of the well-known tyrosinase inhibitors, chelates the copper atom at the active site of tyrosinase.[8] Fungal metabolites, plant polyphenols, and hydroquinone derivatives that inhibit tyrosinase activity have been developed.[9] However, these inhibitors are thought to be mutagenic and cytotoxic to mammalian cells and melanocytes.[10-12] In addition, the cell penetration of KA and hydroquinone inhibitors was reported to be poor, thereby limiting their widespread use in cosmetic and pharmaceutical industries. Therefore, there is an unmet need for the development of novel tyrosinase inhibitors with high activity and reduced adverse effects.

Short peptides and their derivatives are promising alternatives for biocompatible tyrosinase inhibitors.[13-17] A milk protein β-lactoglobulin was reported to suppress melanogenesis in melanocytes [18, 19] and its peptide fragments such as YFYPEL, WYSLAMAA, YVEEL, and MHIRL were found to be the primary sites involved in tyrosinase inhibition.[20, 21] These peptide fragments may serve as good precursors for derivatization to improve the tyrosinase inhibition activity. In particular, the development of

short peptide derivatives with multi-functional activities, including tyrosinase inhibition, may be of great interest in the fields of medicine, biology, and cosmetics.

Caffeic acid (CA) is an organic compound bearing catechol and acrylic functional groups, which is known to display an antioxidant activity.[22, 23] Thereby, CA has been used to scavenge reactive oxygen species (ROS) in various biological systems.[24] As ROS are involved in both physiological and pathophysiological processes,[25, 26] it is required to keep a balance between their generation and exhaustion in biological systems using antioxidants like CA. However, CA is very susceptible to autoxidation via the conversion of *o*-diphenol into *o*-quinone and chemical oxidation, limiting its derivatization to produce a variety of CA derivatives. Hence, it is desired to develop an effective synthetic method for the facile preparation of CA derivatives with multiple bioactivities, including tyrosinase inhibition activity and antioxidant effect.

Herein, we report a synthetic approach based on solid-phase peptide chemistry for the conjugation of tetra- and tri-peptide fragments of β-lactoglobulin with CA to obtain CA-Pep conjugates with tyrosinase inhibition and antioxidant activities. The use of diol-protected CA significantly improved the purity of CA/peptide conjugates (CA-Peps). In addition, the antioxidant activity as well as the tyrosinase inhibition effect of CA-Peps were explored in this work. Furthermore, the cytotoxicity of CA-Peps against B16-F1 melanoma cells was examined.

To synthesize CA-Peps, solid-phase Fmoc chemistry on Rink amide resins was employed [27]. When unprotected CA was coupled to the tetra-peptide (MHIR) synthesized on Rink amide resins using Fmoc-amino acids, several side products were observed in the chromatogram, as evident from HPLC analysis (Figure S1a). To improve the purity of desired CA-Peps, the catechol group of CA was protected using DMP and TsOH to produce

acetonide-protected CA (CA[acetonide]) after esterification of the carboxylic acid of CA (Steps (1) and (2) in Figure 1a). A white powder of CA(acetonide)-OMe was obtained at 55% yield. After the hydrolysis of CA(acetonide)-OMe to produce CA(acetonide)-OH, it was coupled to tetra-peptides (MHIR

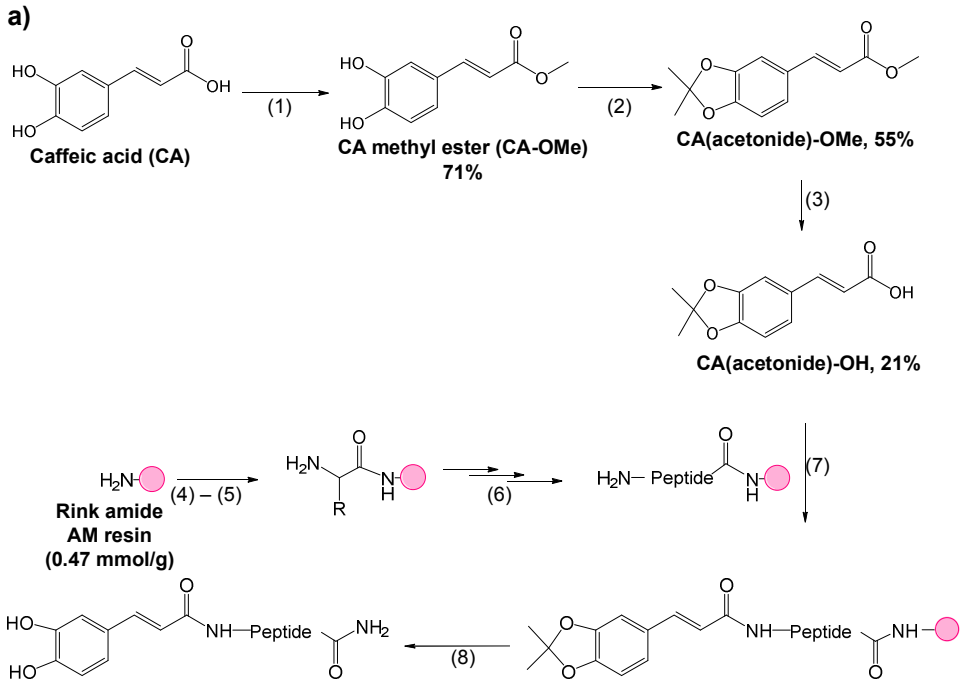

Figure 1. Synthesis of acetonide-protected CA and CA-Pep derivatives. **a)** Synthesis of acetonide-protected caffeic acid (CA[acetonide]-OH). (1) $SOCl_2$ (2 equivalent) and DMAP (0.2 equivalent) incubated in anhydrous MeOH for overnight at RT. (2) DMP (4 equivalent) and TsOH (0.05 equivalent) dissolved in benzene with reflux for 3 h. (3) LiOH (3 equivalent) treated with THF/H_2O (2:1) in ice bath for 24 h, and each step was purified by column chromatography. Solid-phase synthesis of caffeoyl-containing peptides. (4) Fmoc-L-amino acid (3 equivalent), BOP (3 equivalent), HOBt (3 equivalent), and DIPEA (6 equivalent) dissolved in NMP for 3 h. (5) 20% piperidine/NMP (v/v) for 50 min. (6) Repeat (4-5), (7) CA(acetonide)-OH (3 equivalent), BOP (3 equivalent), HOBt (3 equivalent), and DIPEA (6 equivalent) were treated in NMP for 3 h. (8) Cleavage cocktail: 95% TFA, 3% anisole, 1% TIPS, 1% DODT/DCM (v/v) for 2 h and diethyl ether precipitation. R: side chain of amino acid. **b)** Structure of synthesized CA-peptide derivatives.

and HIRL) or a tri-peptide (HIR) on Rink amide resins using solid-phase Fmoc chemistry to produce three different types of CA-Peps (Figure 1b). After the cleavage of the products from the resin using an acid solution containing water as the carbocation scavenger, the purity of CA-MHIR was determined to be over 99% (Figure S1b). CA-MHIR was completely converted to DODT adduct (Figure S1c) when an acid solution containing DODT as the scavenger was used in the cleavage step, as confirmed by ESI-MS. This side product may be obtained via 1,4-addition of a thiol compound to α,ß-unsaturated ketones.[28, 29] These results reveal that the protection of the catechol moiety of CA and use of the proper scavenger during the course of the reaction are essential for the synthesis of high-purity CA-Peps.

This optimized reaction enabled us to synthesize high-purity CA-MHIR, CA-HIRL, CA-HIR, MHIR, HIRL, and HIR (Table 1). The molecular weights of the products were confirmed by ESI-MS.

Tyrosinase is the key enzyme that catalyzes the following two oxidation steps in melanogenesis: hydroxylation of tyrosine to L-DOPA and the subsequent oxidation of L-DOPA to L-dopaquinone, which is cyclized into dopachrome. The inhibition activity of three CA-Peps, two tetrapeptides, and one tripeptide against mushroom tyrosinase was assessed by measuring the characteristic absorption of dopachrome formed in the oxidation and cyclization reactions of L-DOPA (2.5 mM) at 475 nm.[30]

As shown in Figure S2, the formation of dopachrome from L-DOPA was significantly suppressed in the presence of 50 μM of CA-MHIR. Moreover, no dopachrome was formed upon the treatment of L-DOPA solution with higher concentration of CA-MHIR (100 μM). These results clearly suggest that CA-MHIR was able to effectively inhibit the catalytic activity of tyrosinase. To compare the inhibitory activity of CA-Pep derivatives and peptides, their inhibition efficacies were measured as a function of concentration (Figure 2a). All CA-Pep

Table 1. Purity and Molecular Weights of Peptides and CA-Pep Derivatives

Compounds	**Purity (%)**	**Mass ($[M+H]^+$)**	
		Calculated	**Found**
CA-MHIR	>99	717.3	717.5
MHIR	>99	555.3	555.5
CA-HIRL	95	699.7	699.7
HIRL	94	537.3	537.5
CA-HIR	94	586.6	586.6
HIR	>99	424.2	424.4

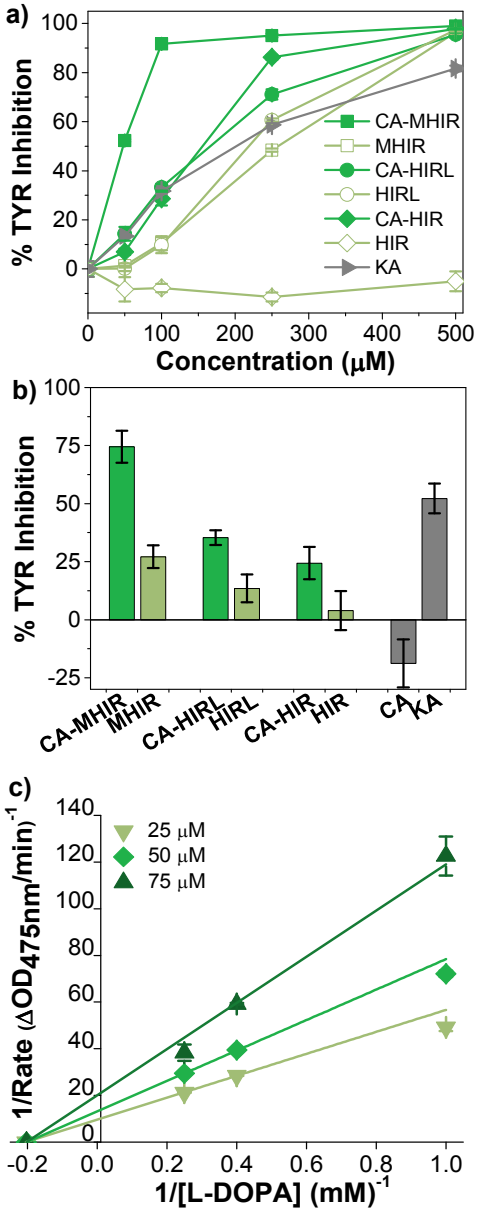

Figure 2. Tyrosinase inhibition activity of CA-Pep derivatives. a) Tyrosinase inhibitory activity of CA-Pep derivatives, pristine peptides, and KA as a function of concentration. b) Quantitative comparison of tyrosinase inhibitory activity at 100 μM of each compound. A total of 2.5 mM L-DOPA and 100 μg/mL tyrosinase were incubated at 25°C for 10 min. c) Lineweaver-Burk plot of tyrosinase inhibition reaction in the presence of CA-MHIR at

various concentrations.

derivatives (CA-MHIR, CA-HIRL, and CA-HIR) exhibited higher inhibition activities than pristine peptides (MHIR, HIRL, and HIR). Note that CA itself had no tyrosinase inhibitory activity. This result reveals that the conjugation of CA to the peptides was very effective in improving their tyrosinase inhibitory activities. Notably, CA-MHIR exhibited higher inhibitory activity (75 ± 7%) than KA (52 ± 6%) (Figure 2b).

We calculated the half maximal inhibitory concentrations (IC_{50}) of CA-Peps, pristine peptides, and KA (Table S1). As expected, all CA-Peps exhibited lower IC_{50} values than pristine peptides. CA-MHIR had the lowest IC_{50} value of 47.9 μM, which was five times lower than that of pristine MHIR. Thus, CA-MHIR had the highest potency to inhibit tyrosinase activity. We speculate that CA conjugation may improve the accessibility of the peptides to tyrosinase to induce deformation of the active site. Based on IC_{50} values, the tyrosinase inhibitory activity was reported in the following order:

CA-MHIR (47.9 μM) > CA-HIR (154.8 μM) > CA-HIRL (166.2 μM) > KA (201.7 μM) > HIRL (218.8 μM) > MHIR (257.1 μM) > HIR (none)

Subsequently, we investigated inhibition kinetics of CA-MHIR to understand the mechanism underlying tyrosinase inhibitory activity. As shown in Figure 2c, inhibition kinetics was measured at three different concentrations of CA-MHIR and the Lineweaver-Burk plots were drawn to extract a Michaelis constant (K_m) and a maximum velocity (V_{max}). V_{max} value for the inhibition reaction of CA-MHIR decreased from 0.10 and 0.073 to 0.056 $nm \cdot min^{-1}$ as the concentration of CA-MHIR increased from 25 and 50 to 75 μM, whereas K_m

value remained constant (2.0 mM). These kinetic parameters reveal that CA-MHIR is a non-competitive inhibitor for tyrosinase. Therefore, CA-MHIR may bind to sites other than the active site of tyrosinase for oxidation, leading to the deformation of the three dimensional structure of the active site.

Antioxidant activities of CA-Pep derivatives were investigated using the linoleic acid oxidation assay with FTC, a common method for the evaluation of antioxidant activity in hydrophobic environment.[31, 32] In this assay, the peroxidation of linoleic acid in Tween 20 emulsion spontaneously occurs in the presence of molecular oxygen, resulting in an increase in the absorption of the solution at 500 nm. The antioxidant activities of CA-Peps and pristine peptides were examined by measuring the absorbance at 500 nm over time (Figure 3). We hardly observed any increase in the absorbance of the solution containing CA-MHIR, indicating that the peroxidation of linoleic acid was prevented by CA-MHIR (Figure 3a). However, pristine MHIR exhibited no antioxidant activity. Thus, CA conjugation to the peptide imparts antioxidant and tyrosinase inhibitory activities to the resulting peptide conjugate. Similar results were observed for CA-HIRL and CA-HIR, showing significant enhancement in the antioxidant activity after CA conjugation (Figure 3b and 3c). The extent of the inhibition of lipid peroxidation (%Pi) in the presence of CA-Pep derivatives was calculated when the absorbance of the control reached approximately 1. As shown in Figure 3d, the antioxidant activity of CA-MHIR was more than four times higher than that of MHIR. In addition, both CA-HIRL and CA-HIR exhibited two-three times higher antioxidant activity than pristine peptides. The antioxidant activity of CA-Pep derivatives was higher than CA, but lower than BHA, a well-known antioxidant for preventing lipid peroxidation. As BHA has been known as a potential carcinogenic and toxic agent,[33] CA-Pep derivatives would be more beneficial for applications in various biological systems. The order of the antioxidant activity of CA-Peps based on %Pi values is as given below:

BHA (86 ± 1) > CA-MHIR (79 ± 0.2) > CA-HIR (78 ± 0.8) > CA-HIRL (76 ± 0.3) > CA (73 ± 1) >> HIRL (32 ± 6) > HIR (26 ± 5) > MHIR (-23 ± 4)

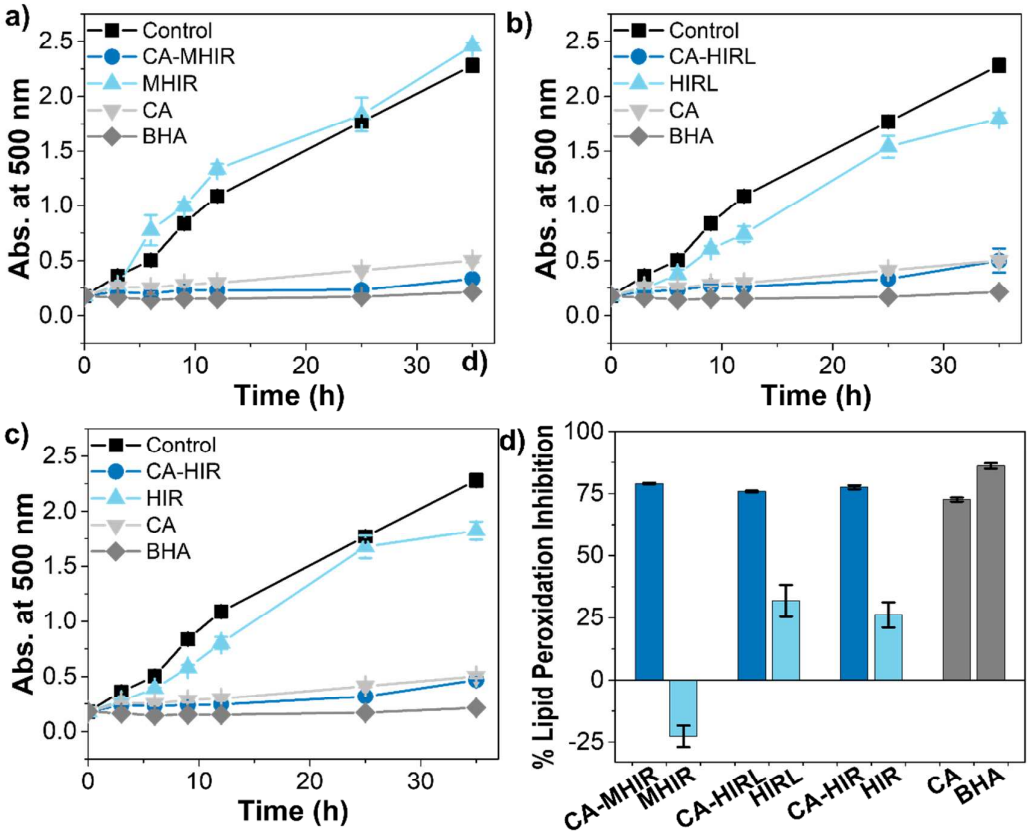

Figure 3. Antioxidant activities of CA-Pep derivatives in terms of linoleic acid peroxidation. The absorbance of peroxidized linoleic acid at 500 nm over time was recorded in the presence of a) CA-MHIR and MHIR, b) CA-HIRL and HIRL, and c) CA-HIR and HIR. d) Efficacy of CA-Pep derivatives at 100 μM concentration for the inhibition of lipid peroxidation. As a negative control, 25 μL of methanol was added instead of CA-Pep derivatives.

The biological activities of peptides, proteins, and their derivatives have been widely

explored, however, most of them exhibited a single activity.[34-37] Although some molecules were reported to have a dual activity on tyrosinase inhibition and antioxidant effect, their dual activity was inferior to that of CA-Peps.[38, 39] In addition to the superior dual activity of CA-Peps, the synthetic method for CA-Peps was more effective and versatile to produce diverse bioactive conjugates than the extraction-based methods for the aforementioned products.

The cytotoxicity of CA-Pep derivatives and pristine peptides was examined in B16-F1 melanoma cells using MTT assay. CA-Pep conjugates displayed no noticeable cytotoxicity at 100 μM concentration (Figure 4). In addition, pristine peptides as well as CA exhibited no cytotoxicity against melanoma cells. This result clearly suggests that CA-Pep derivatives may be applied for the regulation of tyrosinase catalytic activity and scavenging of reactive oxygen species in various biological systems.

In conclusion, high-purity CA-Pep derivatives were successfully synthesized by coupling CA(acetonide)-OH to β-lactoglobulin peptide fragments on resin supports for tyrosinase inhibition and antioxidant effects. All CA-Pep derivatives showed much higher tyrosinase inhibition than pristine peptides and KA. In particular, CA-MHIR exhibited the highest inhibitory activity. Moreover, CA-Pep derivatives showed excellent antioxidant activities in the lipid peroxidation assay and dispalyed no cytotoxicity against B16 melanoma cells. The synthetic approach for designing CA-Pep derivatives may be applicable for the synthesis of various CA-functionalized molecules. In addition, the utility of CA-Pep derivatives with dual activities may be extended to cosmetics and medicines to inhibit tyrosinase and scavenge reactive oxygen species.

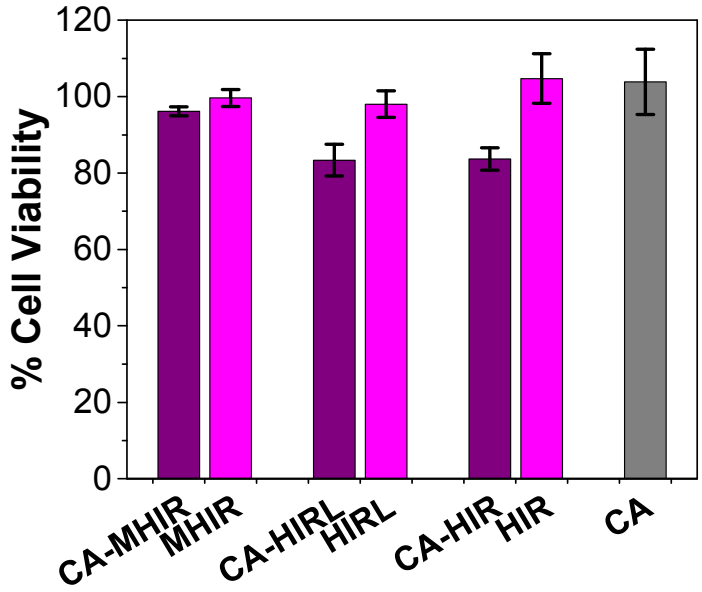

Figure 4. Cytotoxicity of CA-Pep derivatives and peptides against B16 melanoma cells. A total of 100 μM of each compound was incubated with cells for 72 h at 37°C.

Supporting Information

Synthetic procedures and characterizations for all the CA-Pep derivatives, in vitro assay for the evaluation of tyrosinase inhibition and antioxidant activity of CA-Pep derivatives, and a cytotoxicity test are available free of charge on the ACS Publications website at.

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Conflict of interest

The authors have no conflict of interest regarding the publication of this paper.

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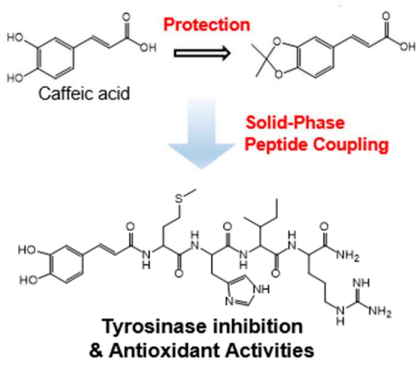

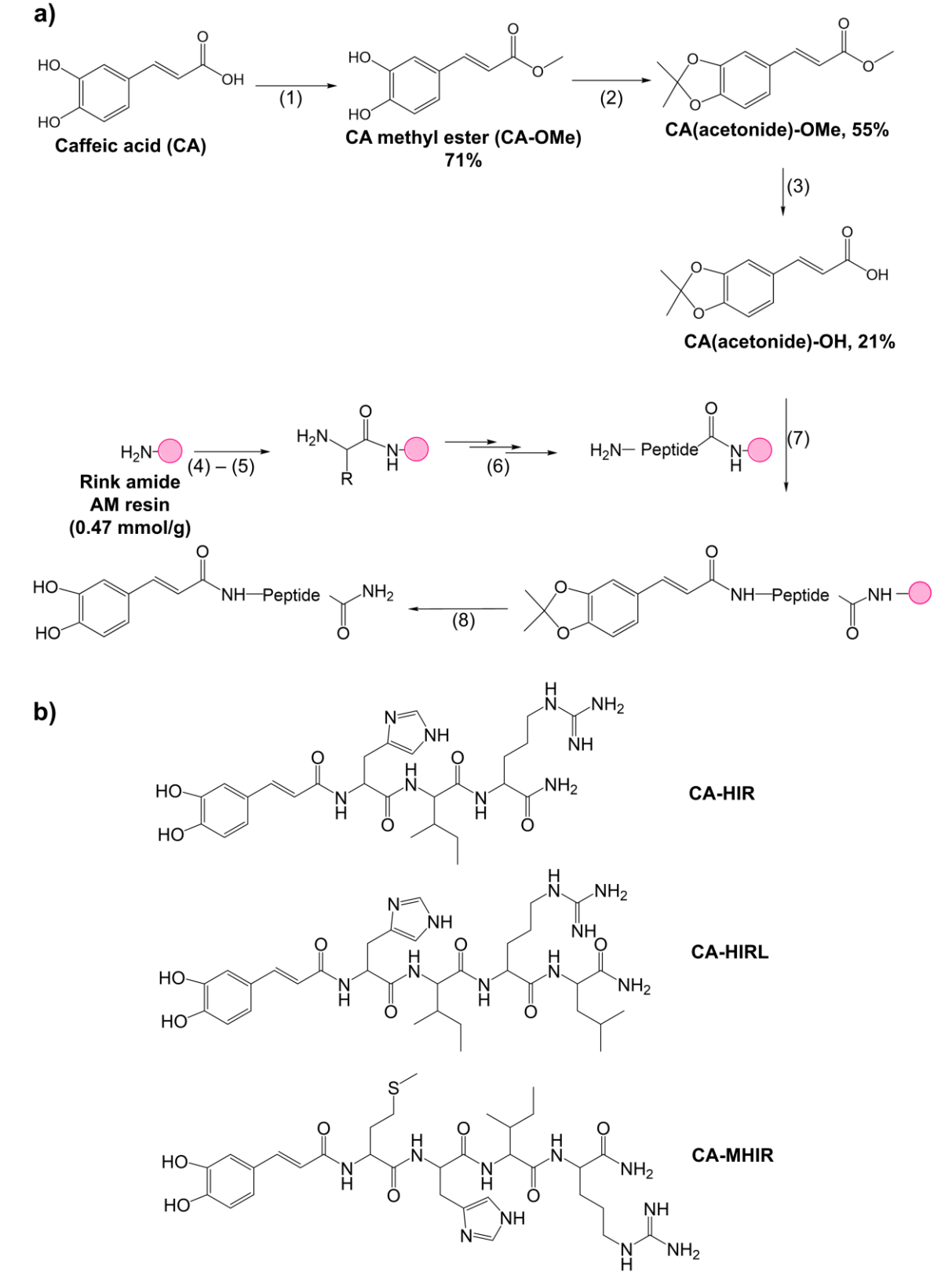
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Caffeic acid (CA)
(1)
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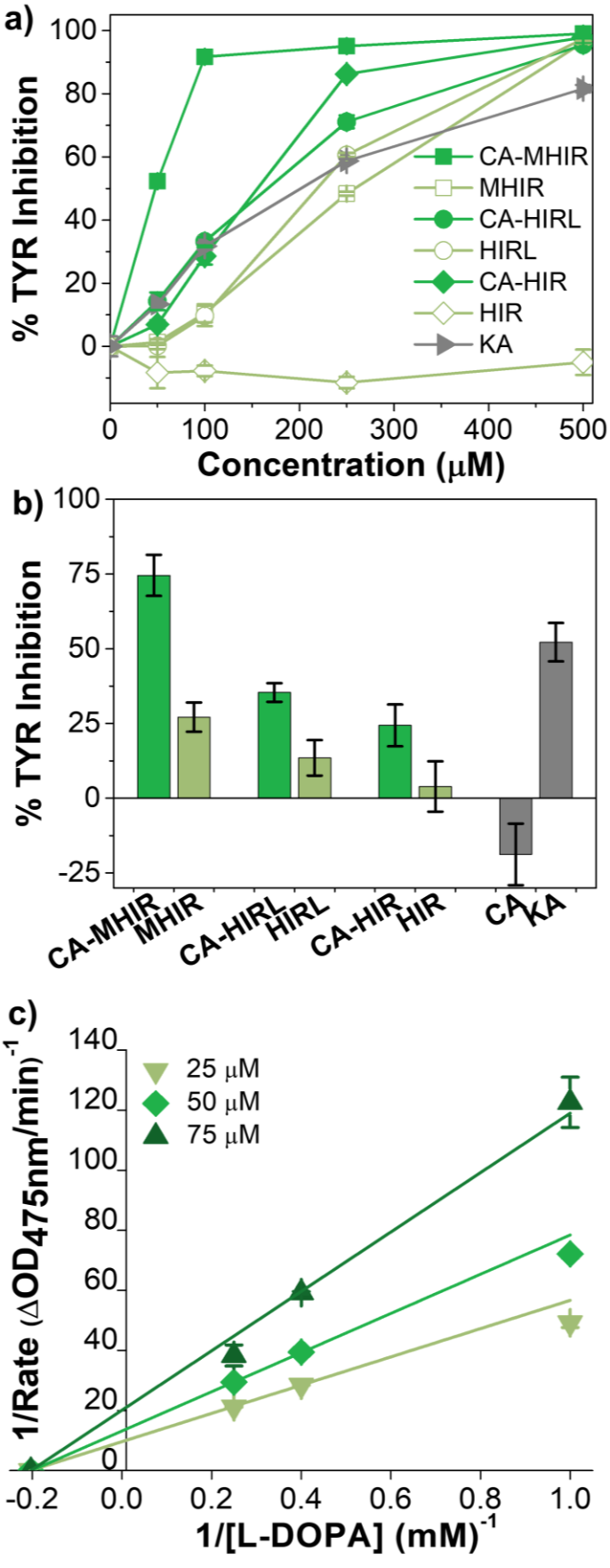
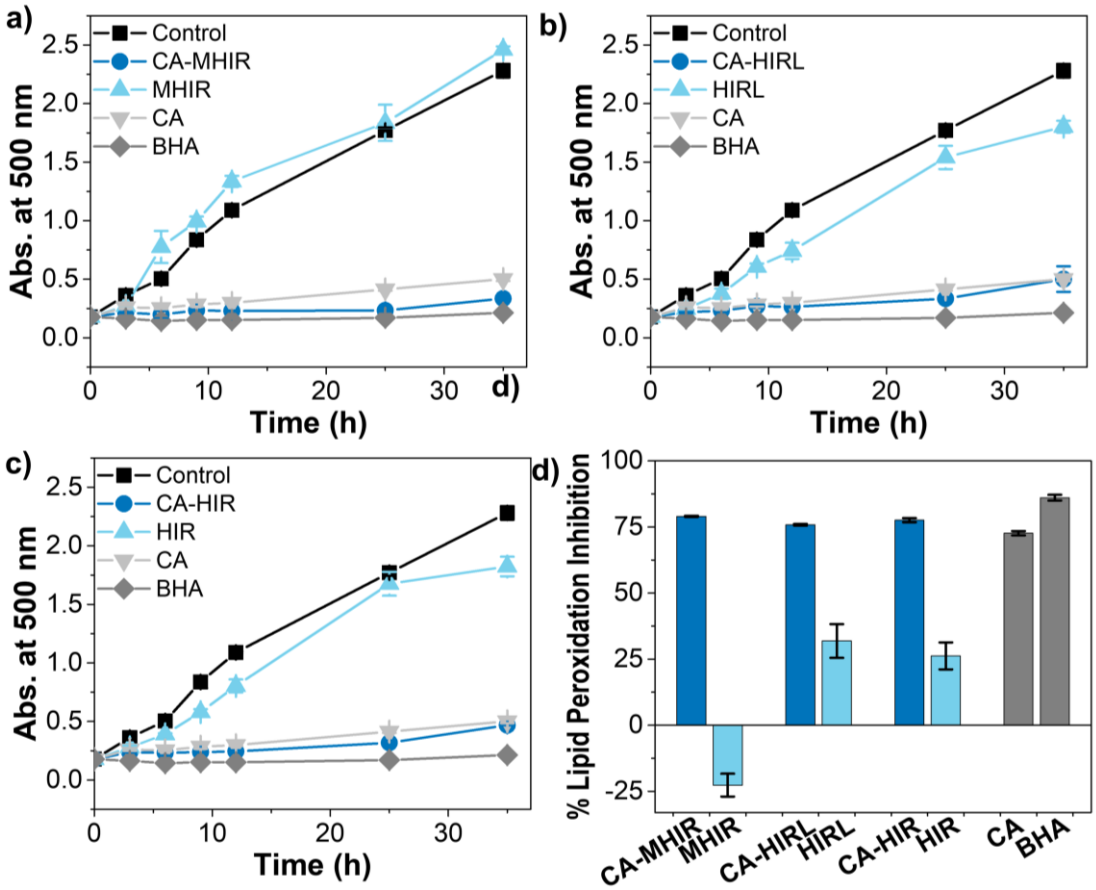
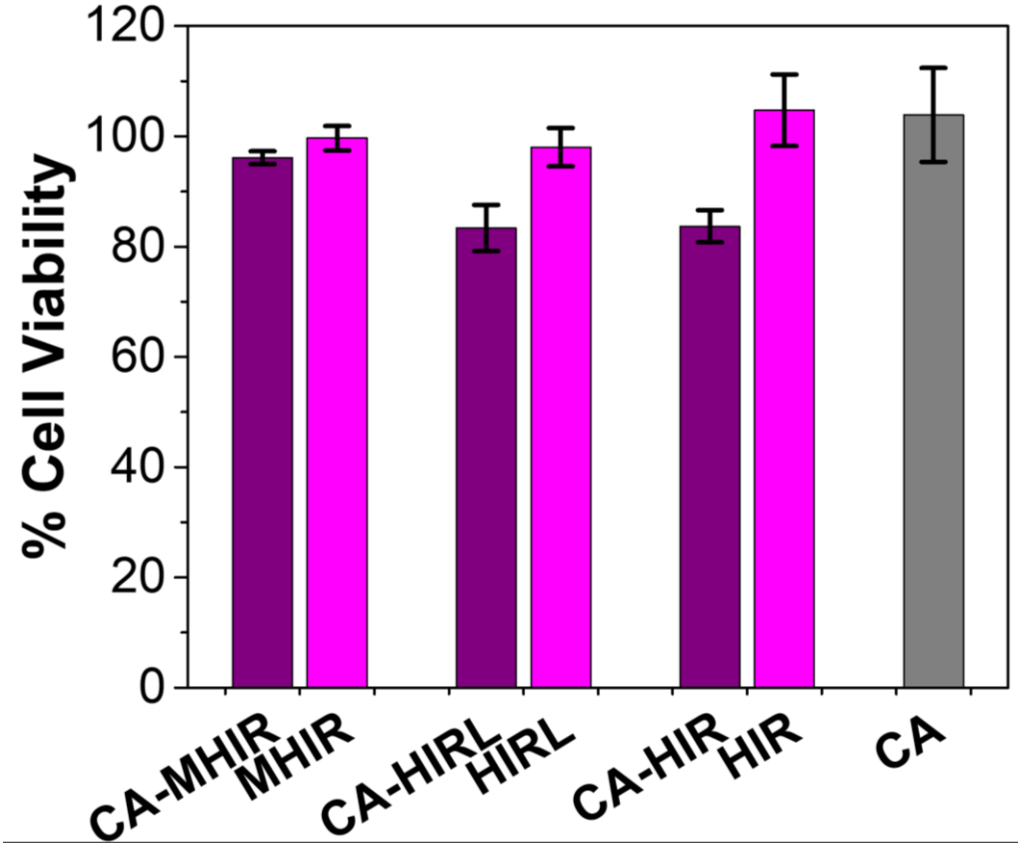
71%
(2)
CA(acetonide)-OMe, 55%
(3)
CA(acetonide)-OH, 21%
Rink amide
AM resin
(0.47 mmol/g)
(4) – (5)
(6)
(7)
(8)
b)
CA-HIR
CA-HIRL
CA-MHIR


a)
% TYR Inhibition
Concentration (μM)
CA-MHIR
MHIR
CA-HIRL
HIRL
CA-HIR
HIR
KA
b)
% TYR Inhibition
CA-MHIR
MHIR
CA-HIRL
HIRL
CA-HIR
HIR
CA
KA
c)
1/Rate (ΔOD475nm/min)-1
1/[L-DOPA] (mM)-1
25 μM
50 μM
75 μM


a)
Control
CA-MHIR
MHIR
CA
BHA
Abs. at 500 nm
Time (h)
b)
Control
CA-HIRL
HIRL
CA
BHA
Abs. at 500 nm
Time (h)
c)
Control
CA-HIR
HIR
CA
BHA
Abs. at 500 nm
Time (h)
d)
% Lipid Peroxidation Inhibition
CA-MHIR
MHIR
CA-HIRL
HIRL
CA-HIR
HIR
CA
BHA


% Cell Viability
120
100
80
60
40
20
0
CA-MHIR
MHIR
CA-HIRL
HIRL
CA-HIR
HIR
CA