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# Synergistic antioxidative activities of hydroxycinnamoyl-peptides

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**Antioxidants have become an important subject of study as an active ingredient for cosmetics and preservatives for food. We synthesized antioxidative peptide conjugates of hydroxycinnamic acids (HCAs) such as ferulic acid (FA), caffeic acid (CA), and sinapic acid (SA) by SPPS method. We measured their potential antioxidant properties by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging test and lipid autoxidation inhibition test. When the antioxidative peptides, such as glutathione analogue (GS(Bzl)H) and carnosine (CAR), were conjugated to HCAs, their antioxidative activities were enhanced significantly. CA-peptides exhibited the highest free radical scavenging activity by the DPPH test, and showed good antioxidative activity in the lipid autoxidation test. FA- and SA-peptides showed excellent antioxidative activity in the lipid autoxidation test. Furthermore, we demonstrated a synergistic antioxidative activity of HCA-peptide conjugates by comparing their antioxidative activity with that of a simple mixture of HCAs and the antioxidant peptides.** **Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.**

**Keywords:** antioxidant; hydroxycinnamoyl peptide conjugates; carnosine; glutathione analogue; synergistic antioxidative activity

## Introduction

Almost of all living organisms require oxygen to maintain their lives, however, unconsumed oxygen molecules can be changed to free radicals such as superoxide ($O_2^-$, OOH$^\bullet$), hydroxyl (OH$^\bullet$) and peroxyl (ROOH$^\bullet$) radicals. Severe oxidative stress caused by the active oxygen molecules bring about damage of DNA and RNA, oxidation of fatty acids in lipid constructing cell membrane, deactivation of enzyme by oxidation of cofactor, and deconstruction of protein by oxidation of amino acids [1,2]. Thus, reactive oxygen species and free radicals sequentially destroy normal cellular metabolism, and thereby accelerate the aging process and mediate serious diseases, such as chronic lung disease, diabetes, and neurodegenerative disorders (i.e. Alzheimer's and Parkinson's diseases) [3].

In the healthy human bodies, the amount of reactive oxygen species is controlled by antioxidants-mediated self-defense systems. Antioxidants prevent cellular damage by reducing free radical intermediates and by terminating chain reactions mediated by free radicals. Antioxidants have been investigated for the purpose of disease treatment and prevention. Moreover, antioxidants are utilized in industry as food additives to reduce food deterioration and in cosmetics as an ingredient to delay the skin aging process.

The use of synthetic antioxidants in food and cosmetic industries are limited because of their safety problem, although many different kinds of powerful synthetic antioxidants such as 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), 3-*tert*-butyl-4-hydroxyanisole (BHA), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ) have been developed [4,5]. Among numerous antioxidative peptides reported from the hydrolysates of natural proteins [6–11] and peptide libraries [12], the most popular industrial antioxidative peptides are carnosine and glutathione. Carnosine (CAR; $\beta$-alanyl-L-histidine) exists naturally at high concentrations (2–20 mM) in mammalian muscle tissues and in the nervous system [13]. CAR has various biological functions as a physiological buffer against lactic acid in muscles [14], a neurotransmitter [15], a regulator of glycogenolysis [16], a chelating agent for heavy metal, and a free radical scavenger. In addition, CAR is considered to be a rejuvenating agent because it can extend fibroblast life-span by reversing the signs of aging and by inhibiting lipid peroxidation within the cell membrane because of lipid peroxyradical trapping ability of imidazole. Glutathione (GSH; $\gamma$-L-glutamyl-L-cysteinyl-glycine) is a ubiquitous antioxidant in mammalian cells and can play roles as a co-substrate or as an inhibitor of several enzymes that participate primarily in cellular redox homeostasis as a result of its reduction properties [17]. Although GSH has various biological activities because of the electron donating ability of sulfur, the application of GSH as an antioxidant is restricted because of its bad pharmacological profile, low stability, and low selectivity. As a result, a variety of GSH analogues have been developed to improve its properties by chemical modification. In particular, the sulfhydryl group (SH) of cysteine exerts a prooxidative effect that with autoxidation can generate very reactive singlet oxygen ($^1O_2$) [18]. Therefore, an antioxidant peptide containing a blocked sulfhydryl group, such as S-alkylcysteine, has shown better performance in previous research [19].

Hydroxycinnamic acids (HCAs), such as ferulic acid (FA, 3-methoxy-4-hydroxy-cinnamic acid), caffeic acid (CA, 3,4-dihydroxy-cinnamic acid) and sinapic acid (SA, 3,5-dimethoxy-4-hydroxy-cinnamic) are distributed universally in plants. These are well-known antioxidants because they can readily form resonance-stabilized phenoxy radicals because of their phenolic nucleus and extended side chain conjugation [20]. They can exist in forms of

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hydroxycinnamoyl-amino acid conjugate [21] or HCA amide [22] as secondary metabolites.

In this study, GSH analogue and CAR were conjugated to HCAs in order to enhance HCAs' antioxidative activities. The *C*-terminus of the two kinds of antioxidative peptides was converted to amide form because we found that *C*-terminal peptide amides were more stable than *C*-terminal peptide acids in our previous study [23]. In addition, the benzylic group was introduced to the sulfhydryl of cysteine in order to reduce the formation of disulfide bond and to increase antioxidative activity [19]. We evaluated the antioxidative activities of HCA-CAR and HCA-GS(Bzl)H by use of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test [24] and by the linoleic acid autoxidation system with ferrous thiocyanate assay [25,26]. BHA and HCAs such as FA, CA, and SA served as reference compounds.

## Materials and Methods

### Chemicals

Aminomethyl surface-layered polystyrene (AM SURE) (100–200 mesh, 0.76 mmol/g) resin, Libra tubes, Fmoc-Rink amide linker, Fmoc-His(Trt)-OH, Fmoc-Gly-OH, Fmoc-Cys(Bzl)-OH, Fmoc-Phe-OH and Fmoc-$\beta$-Ala-OH were purchased from BeadTech. (Seoul, Korea). Fmoc-Glu-OtBu was purchased from Novabiochem. FA, CA, SA, DIPEA, ninhydrin, linoleic acid (~99%) and DPPH were purchased from Sigma (St. Louis, MO, USA). BHA, ammonium thiocyanate, ferrous chloride ($FeCl_2$) and polyoxyethylenesorbitan monolaureate (Tween 20) were purchased from Aldrich (St. Louis, MO, USA). BOP, (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and HOBt were purchased from GL Bio-Chem (Shanghai, China). *N*-Methyl-2-pyrrolidone (NMP) was purchased from Junsei Chemicals (Tokyo, Japan). Piperidine, dichloromethane (DCM), diethyl ether, ethanol and methanol were purchased from Dae-Jung Chemicals (Korea). TFA was purchased from Acros Organics (NJ, USA).

### Apparatus

HCA-peptides were analyzed by high performance liquid chromatography (HPLC, Young Lin Autochro 2000), using Waters $\mu$Bondapak C18 reverse phase column (125 Å, 10 µm, 3.9 × 150 nm). They were further characterized by QUATTRO Triple Quadrupole Tandem mass spectrometer (Micromass & Waters, Milford, MA, USA). The color reaction such as DPPH and linoleic acid peroxidation test was detected by UV/visible spectrophotometer (Mecasys Co. Ltd., Optizen 2120 UV).

### Synthetic Procedure for HCA-peptides

The peptides were manually prepared by the SPPS with the Fmoc-strategy on Rink amide AM SURE resin by using LibraTube (BeadTech, Seoul, Korea). Each reaction step was monitored by Kaiser's ninhydrin test [27]. HCAs (2 eq.), BOP (2 eq.), HOBt (2 eq.) and DIPEA (4 eq.) in NMP were then added to the peptide anchored resin. The coupling reaction was performed for 5 h at 25 °C. The uncoupled free peptides, CAR and GS(Bzl)H, were acetylated at *N*-terminus with acetic anhydride and DIPEA. The resin was treated with 30% TFA/1% $H_2O$ in freshly distilled DCM for 1 h at 25 °C for detaching the product from the resin. The resin was filtered and washed with DCM and MeOH. The filtrate was concentrated and triturated with diethyl ether to obtain HCA-peptides as powder. The purity and identity of the resulting peptides or HCA-peptides were confirmed by RP-HPLC with a linear gradient elution of 0.1% TFA and acetonitrile at a flow rate of 1.0 ml/min and monitored at 230 nm, and by Quardrupole Tandem mass spectrometer. HCA-phenylalanine amides (HCA-Phe-$NH_2$) were obtained and identified by the same procedure of HCA-peptides.

### Determination of Physico-chemical Properties of HCA-peptides

We calculated LogP by using software, ChemDraw Ultra (CambridgeSoft), to determine partitioning coefficient. Also, we calculated aqueous solubility (LogS) by using online-LogP/LogS calculation software, ALOGPS 2.1 (www.vcclab.org/lab/alogps/) [28–30].

### Determination of Radical Scavenging Activity

The percent radical scavenging activity (%RSA) was estimated by measuring the decrease in the absorbance at 516 nm of the DPPH solution after adding the antioxidant. Methanolic DPPH solution (0.1 mM, 1480 µl) was mixed with 20 µl of 1.85 mM or 3.7 mM methanolic HCA-peptides in an Eppendorf tube (1.5 ml-Volume), making the final concentration to be 25 µM or 50 µM, respectively. The absorbance was monitored 10 min later. The results were expressed as $\%RSA = [\{Abs_{516\,nm}(t = 0) - Abs_{516\,nm}(t = t')\}/Abs_{516\,nm}(t = 0)] \times 100$. Control was a mixture of 0.1 mM methanolic DPPH solution (1480 µl) and 20 µl of methanol instead of the samples. Each experiment was performed in triplicate and repeated five times.

### Determination of Antioxidative Activity

Linoleic acid emulsion (50 mM) was prepared by mixing 0.284 g of linoleic acid, 0.284 g of Tween 20 and 50 ml of sodium phosphate buffer (0.1 M, pH 7.0) by sonication for a few minutes. For oxidation test, 0.5 ml of distilled water, 2.5 ml of the aforementioned emulsion, 2.0 ml of sodium phosphate buffer (0.1 M, pH 7.0) and 0.5 ml of methanolic test samples were mixed in a glass vial (10 ml-volume). The total volume was fixed uniformly to 5.5 ml, and the samples' final concentration was 90 µM. The reaction mixture-containing vials were capped with rubber septum and closed tightly with silicon tape, then kept at 50 °C under dark condition. As a negative control, 0.5 ml of MeOH was added instead of the antioxidants. A part of the reaction mixture was withdrawn at some intervals with a microsyringe (1 ml-volume) to evaluate the antioxidative activity by slightly modified ferric thiocyanate method [27]. The ferric thiocyanate analysis was performed as follows: the reaction mixture (25 µl) was mixed with 75% ethanol (1.175 ml), 30% ammonium thiocyanate (25 µl), and 20 mM $FeCl_2$ in 3.5% HCl (25 µl) in an Eppendorf tube (1.5 ml-volume). After exact 3 min, color development with $FeCl_2$ and thiocyanate reached maximum value, and the absorbance of the mixture was measured at 500 nm. We measured the degree of oxidation at 3 h intervals at the initial phase of lipid oxidation because lipid was oxidized very fast. In the middle of the oxidation, we measured the absorbance at 8 h intervals and then, we evaluated the oxidative values at 12 h intervals at the late stage of oxidation because the rate of oxidation became slower than the initial phase. Five independent measurements were performed, and each experiment was run in triplicate. The control experiment (without antioxidant) was repeated more than seven times to obtain defined oxidation curve in the lipid emulsified autoxidation system.



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In addition, we calculated relative antioxidative activity on the basis of the induction time (h), to know exactly how many times the antioxidative activity of HCA was enhanced by peptide conjugation. Time required to attain the absorbance of 0.3 was defined as the induction time according to Chen *et al*. [26,31] The relative antioxidative activity was calculated by dividing the induction time of test samples by that of the control.

## Results and Discussion

### Synthesis of HCA-peptides

HCA-peptides were manually prepared by the SPPS with the Fmoc-strategy on Rink amide AM SURE resin (Scheme 1). After the resin was treated with 30% TFA/1% $H_2O$ in freshly distilled DCM for 1 h at 25 °C, diethyl ether precipitation of the cleavage product gave the final HCA-peptides as white powder in high yield and purity (Table 1). HCA-Phe-$NH_2$ was obtained in the same way in 30–65% yield and 75–89% purity.

### Physico-chemical Properties of HCA-peptides

HCA-peptide conjugates have different physico-chemical properties from HCA. CLogP of CAR and GS(Bzl)H increased by conjugation of HCA (Table 2). Conversely, when peptide was conjugated, CLogP of HCA decreased because of the added hydrophilic moiety such as imidazole and carboxylic acid of the antioxidant peptides. The hydrophilic nature of CAR enhanced aqueous solubility of HCA; on the other hand, GS(Bzl)H reduced its solubility (Table 3). HCA-CAR conjugates were more soluble in water than HCA, while and HCA-GS(Bzl)H conjugates were less soluble in water.

### Free Radical Scavenging Activity of HCA-peptides

The free RSA of CAR, GS(Bzl)H and HCA-peptide conjugates were evaluated by reacting with DPPH radical. The order of %RSA was observed as follows: CA ($91 \pm 2.8$) $\approx$ **5** ($89 \pm 7.8$) > **6** ($81 \pm 8.5$) > SA ($69 \pm 2.8$) > FA ($51 \pm 1.0$) > **8** ($46 \pm 5.6$) > **7** ($42 \pm 2.2$) > **4** ($30 \pm 2.3$) $\approx$ **3** ($29 \pm 3.8$) $\gg$ **2** ($3 \pm 0.7$) $\approx$ **1** ($3 \pm 0.8$) (Figure 1 (a)). The RSA was dependent upon the kinds of HCAs. Compounds **1** and **2** showed insufficient RSA by themselves. CA and CA-peptides (**5** and **6**) showed the highest %RSA. Since the *ortho*-dihydroxyl group can make additional hydrogen bonding, it can have stable conformation after breaking the O–H bond [32]. As shown in Figure 1, most of HCA-peptides maintained RSA of HCA, which means that HCA's property for releasing hydrogen radical is not much hindered by a newly formed amide bond. Although cysteine and histidine can donate hydrogen radical easily in protic solvent [2], they could not accelerate RSA of HCA under the assay condition ([Antioxidant]/[DPPH] = 0.5). Since the maximal %RSA of CA and CA-peptides were too high to be compared with each other, we performed the DPPH radical scavenging assay again at lower concentration ([Antioxidant]/[DPPH] = 0.25) (Figure 1(b)). Under this condition, we found that CA-CAR (**5)** afforded slightly higher %RSA than CA itself: **5** ($66 \pm 7.1$) > CA ($61 \pm 6.4$) > **6** ($56 \pm 7.6$).

### Antioxidative Activity of HCA-peptides

The lipid autoxidation assay using Tween 20-emulsified linoleic acid (>99%) is a common method to evaluate the antioxidative activity indirectly. The alkylperoxyl radicals, which are spontaneously induced by air, cause lipid peroxidation during the experimental period. We delineated the antioxidative activity of the compounds in terms of the ability to restrict the early stage of lipid peroxidation (Figure 2), and thus, the percent lipid peroxidation inhibition (%Pi) is calculated when the absorbance of the control (without antioxidant) reaches approximately 1 (0.950–1.050). Unlike the result of DPPH radical scavenging test, **1** and **2** showed moderate lipid peroxidation inhibitory activities by themselves in the lipid emulsified system. However, they did not suppress lipid peroxidation sufficiently at 90 μM. FA-peptides, **3** and **4,** indicated the highest %Pi equal to BHA, a synthetic antioxidant widely used in industry due to its strong antioxidative activity. The %Pi of HCAs, the peptides, and HCA-peptides decreased in the following order: **4** ($87 \pm 2.9$) > **3** ($83 \pm 1.3$) > **8** ($80 \pm 5.1$) > **7** ($76 \pm 3.3$) > FA ($74 \pm 3.0$) > **6** ($66 \pm 3.5$) > SA ($48 \pm 3.3$) > **5** ($54 \pm 4.5$) > CA ($29 \pm 3.3$) > **1** ($18 \pm 6.7$) $\approx$ **2** ($16 \pm 1.2$). This %Pi order much differs from the % RSA order. In hydrophilic environment such as methanol in the DPPH radical scavenging assay, CA with two hydroxyl groups exerted the highest activity, whereas it exhibited the lowest antioxidative activity in the lipid autoxidation assay, probably because of the hydrophilicity. In addition, less hydrophilic HCA-GS(Bzl)H indicated better antioxidative activity than HCA-CAR in lipid emulsion system (Figures 1 and 2).

Table 2 showed that HCA-peptide conjugates have lower CLogP than HCA, which means that HCA-peptides are more hydrophilic than HCA. However, as can be seen in Figure 2, all HCA-peptides enhanced the antioxidative activity of HCA in lipid autoxidation system. Therefore, this result was hardly explained by such a simple correlation, although the hydrophobicity of the antioxidant is an important factor for increasing its accessibility toward lipophilic fatty acids. CAR (**1**) is very hydrophilic, and GS(Bzl)H (**2**) is moderately hydrophilic because of imidazole or carboxylic acid moiety, respectively. Also, HCA-peptide conjugates contained hydrophobic part such as alkyl chain of $\beta$- or $\gamma$-type peptide bond and phenyl ring. This nature could generate suitable structure for biphasic water-oil system because HCA-peptides contained both hydrophilic and hydrophobic moiety. Antioxidants can be divided into two classes depending on their solubility. Hydrophilic antioxidants act in cytoplasm and blood plasma, while hydrophobic ones function at cell membrane. Thus, if one compound contained both hydrophilic and hydrophobic parts, this property could be a great advantage to act as a potent antioxidant when applied in cell or *in vivo* system.

Furthermore, we synthesized HCA-Phe-$NH_2$, which showed enhanced antioxidative activity in previous works [33,34], and compared to our HCA-peptides under the same conditions. We found that %Pi decreased in the following order:

**4** ($87 \pm 2.9$) > **3** ($83 \pm 1.3$) > FA-Phe-$NH_2$ ($79 \pm 3.1$) > FA ($74 \pm 3.0$).

**6** ($66 \pm 3.5$) > CA-Phe-$NH_2$ ($60 \pm 1.3$) > **5** ($54 \pm 4.5$) > CA ($29 \pm 3.3$).

**8** ($80 \pm 5.1$) > **7** ($76 \pm 3.3$) > SA-Phe-$NH_2$ ($65 \pm 1.7$) > SA ($48 \pm 3.3$).

A phenylalanine moiety surely enhanced the antioxidative activity of HCA, although the activity was slightly inferior to GS(Bzl)H conjugate. This result suggested that phenyl ring moiety of the benzyl group might have a positive effect on the antioxidant activity of HCA-GS(Bzl)H.

Figure 3 shows the antioxidative activities of the test compounds as a function of time in order to compare their antioxidative

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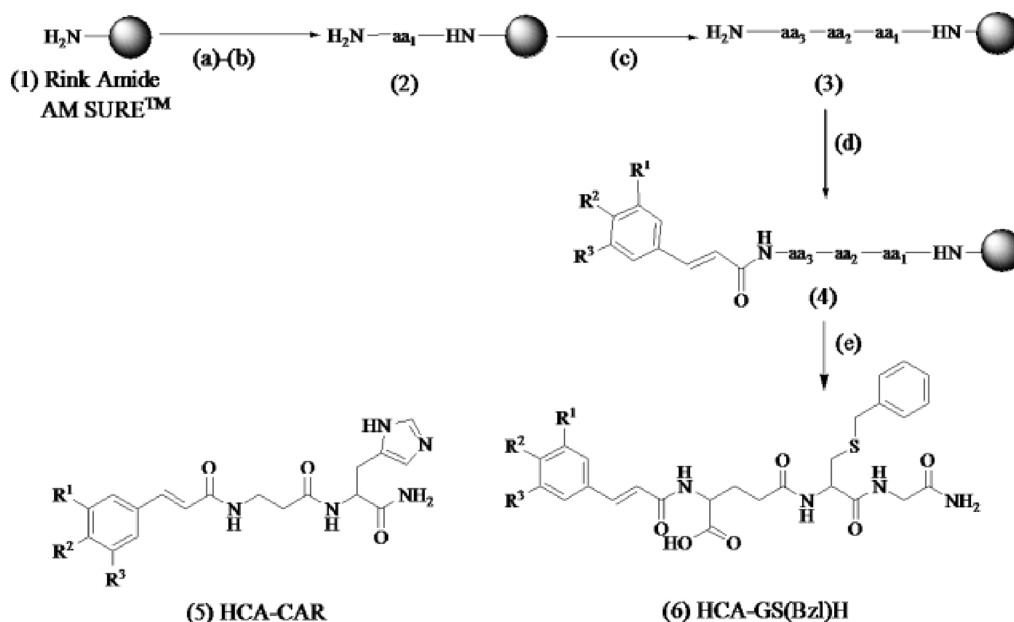


**Scheme 1.** Solid-phase HCA-CAR and HCA-GS(Bzl)H synthesis. Reagents and conditions: (a) Fmoc-L-amino acids (2 eq.), BOP (2 eq.), HOBt (2 eq.) and DIPEA (4 eq.) in NMP for 1.5 h, (b) 20% piperidine/NMP (v/v) for 3 min and 15 min, (c) repeat (a) and (b) once or twice, (d) HCA (2 eq.), BOP (2 eq.), HOBt (2 eq.) and DIPEA (4 eq.) in NMP for 5 h, (e) cleavage cocktail : 30% TFA/1% water in dry DCM for 1 h, and diethyl ether precipitation. FA: $R^1 = H$, $R^2 = OH$, $R^3 = OCH_3$; CA: $R^1 = H$, $R^2 = R^3 = OH$; SA: $R^1 = R^3 = OCH_3$, $R^2 = OH$.

**Table 1.** Yield and purity of HCA-peptides

| Compounds | | Crude yield (%) | Purity (%)[a] | ESI-MS | |
|---|---|---|---|---|---|
| | | | | Calculated | Found |
| **1** | CAR | 31 | 95 | 268.3 ($[M + H]^+$) | 269.3 |
| **2** | GS(Bzl)H | 67 | 95 | 461.5 ($[M + Na]^+$) | 461.1 |
| **3** | FA-CAR | 48 | 85 | 402.4 ($[M + H]^+$) | 403.7 |
| **4** | FA-GS(Bzl)H | 59 | 76 | 595.6 ($[M + Na]^+$) | 594.9 |
| **5** | CA-CAR | 43 | 73 | 388.4 ($[M + H]^+$) | 389.7 |
| **6** | CA-GS(Bzl)H | 66 | 69 | 581.6 ($[M + Na]^+$) | 581.7 |
| **7** | SA-CAR | 41 | 81 | 432.4 ($[M + H]^+$) | 433.9 |
| **8** | SA-GS(Bzl)H | 62 | 89 | 625.7 ($[M + Na]^+$) | 625.9 |

[a] Determined by HPLC.

**Table 2.** Partition coefficient (CLogP) of HCA-peptides

| Compounds | CLogP[a] | Compounds | CLogP | Compounds | CLogP |
|---|---|---|---|---|---|
| | | **1** | −2.9478 | **2** | −1.0384 |
| FA | 1.4212 | **3** | −1.1660 | **4** | 0.5690 |
| CA | 0.9750 | **5** | −2.5972 | **6** | 0.1228 |
| SA | 1.2043 | **7** | −1.3829 | **8** | 0.3521 |

[a] Calculated by ChemDraw Ultra (CambridgeSoft).

performances from the early stage of lipid autoxidation to the late stage. The absorbance was measured over time, until the negative control reached its maximum value, which reached approximately 2 (1.950–2.150) at the 48 h; then the absorbance decreased over time. Compounds **1** and **2,** which showed insufficient antioxidative activities, however, maintained moderate and steady activities for the given period (Figure 3(a)). The reason why **2** could display constant activity is that the benzyl group of cysteine may prevent GSH from forming the oxidized form GSSG, which has no antioxidative activity [18].

HCAs and HCA-peptides did not show a large difference at the very early stage of lipid peroxidation. However, the lipid oxidation inhibitory activity of HCAs was reduced rapidly after 10 h, while HCA-peptides inhibited lipid oxidation steadily without significant fluctuation until 48 h (Figure 3(b–d)). This result demonstrated that peptide conjugation might contribute to enhanced stability of HCAs under the assay condition, as well as their accessibility to emulsified linoleic acid. However, there were some ambiguities to explain this result, as to whether the increased antioxidative activity of HCA-peptides was just due to additional functional groups or due to a synergistic effect from peptide conjugation. Therefore, we measured the antioxidative activity of a simple mixture of HCA and CAR or GS(Bzl)H to address this issue. Although the simple mixtures of CA or SA with the antioxidant peptides displayed better performances than CA or SA alone, they were much inferior to CA- or SA-peptide conjugates. In the case of FA



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**Table 3.** Aqueous solubility (CLogS) of HCA-peptides

| Compounds | CLogS[a] | g/l | Compounds | CLogS | g/l | Compounds | CLogS | g/l |
|---|---|---|---|---|---|---|---|---|
| | | | **1** | −1.17 (±1.05) | 50.89 | **2** | −2.96 (±0.80) | 0.96 |
| FA | −2.02 (±0.31) | 2.15 | **3** | −2.65 (±0.93) | 2.19 | **4** | −4.06 (±0.93) | 0.13 |
| CA | −1.76 (±0.32) | 3.80 | **5** | −2.39 (±0.89) | 3.90 | **6** | −3.73 (±0.70) | 0.19 |
| SA | −2.15 (±0.41) | 2.06 | **7** | −2.71 (±0.93) | 2.14 | **8** | 0.27 (±0.91) | 0.07 |

[a] Calculated by the ALOGPS 2.1 Program. www.vcclab.org/lab/alogps/.

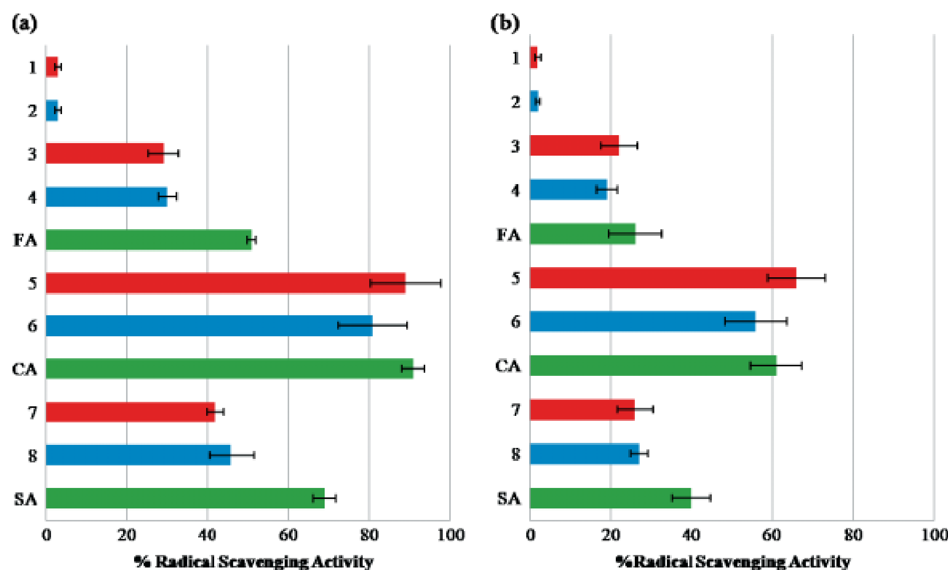


**Figure 1.** DPPH radical scavenging activity of HCA-peptides. (a), [Antioxidant]/[DPPH](mol/mol) = 0.5; (b), [Antioxidant]/[DPPH](mol/mol) = 0.25. Each experiment was performed in triplicate and repeated five times. The values are given as the mean ± standard error.

and the peptide mixtures, it was worse than FA itself. This result clearly proves that antioxidant peptide conjugation synergistically contributed to the antioxidative activity of HCA. The synergistic effect was superior in SA- and CA-peptide conjugates, even though FA-peptides indicated the highest %Pi.

As can be seen in Figure 4, HCA-peptide conjugates show higher relative antioxidative activity than HCA because they have much longer induction time. The induction time of the control to reach an absorbance of 0.3 was 2.2 (±0.1) h. The relative antioxidative activity of HCAs, the peptides, and HCA-peptides decreased in the following order: **4**(21.8) > **3**(18.6) > **8**(7.0) > FA(5.9) ≈ **7**(5.7) > **6**(4.5) > **5**(2.5) ≈ SA(2.3) > CA(1.6) ≈ **1**(1.6) ≈ **2**(1.1). This order is very similar to that of %Pi value discussed above.

Compound **1** exerted its antioxidative activity in a concentration-dependent manner. At the highest concentration of 1 mM and at the lowest concentration of 0.1 mM, %Pi of **1** was 51 (±7.5) and 25 (±2.3), respectively (Figure 5(a)). Also, FA and FA-peptide conjugates, **3** and **4**, showed better antioxidative activity at higher concentration (Figure 5(b)). In particular, **3**

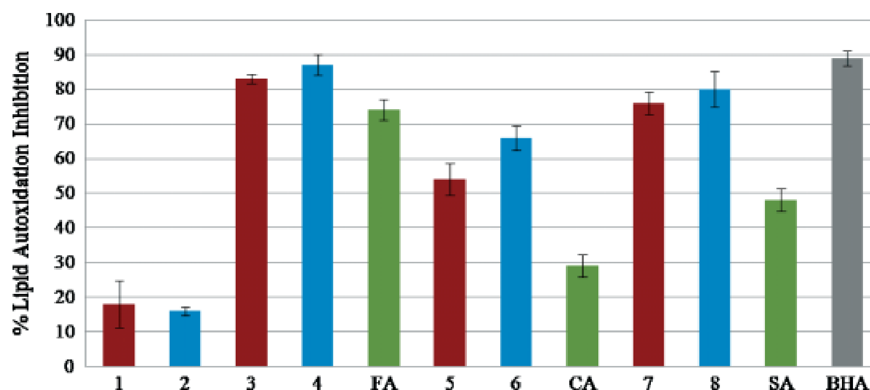


**Figure 2.** Antioxidative activity of HCA-peptides at early stage of lipid autoxidation system measured by the ferric thiocyanate method. Conditions: the final concentration of each antioxidant was 90 μM; kept at 50 °C for 10 h under dark conditions. Each experiment was carried out in triplicate, and the values are given as the mean ± standard error.



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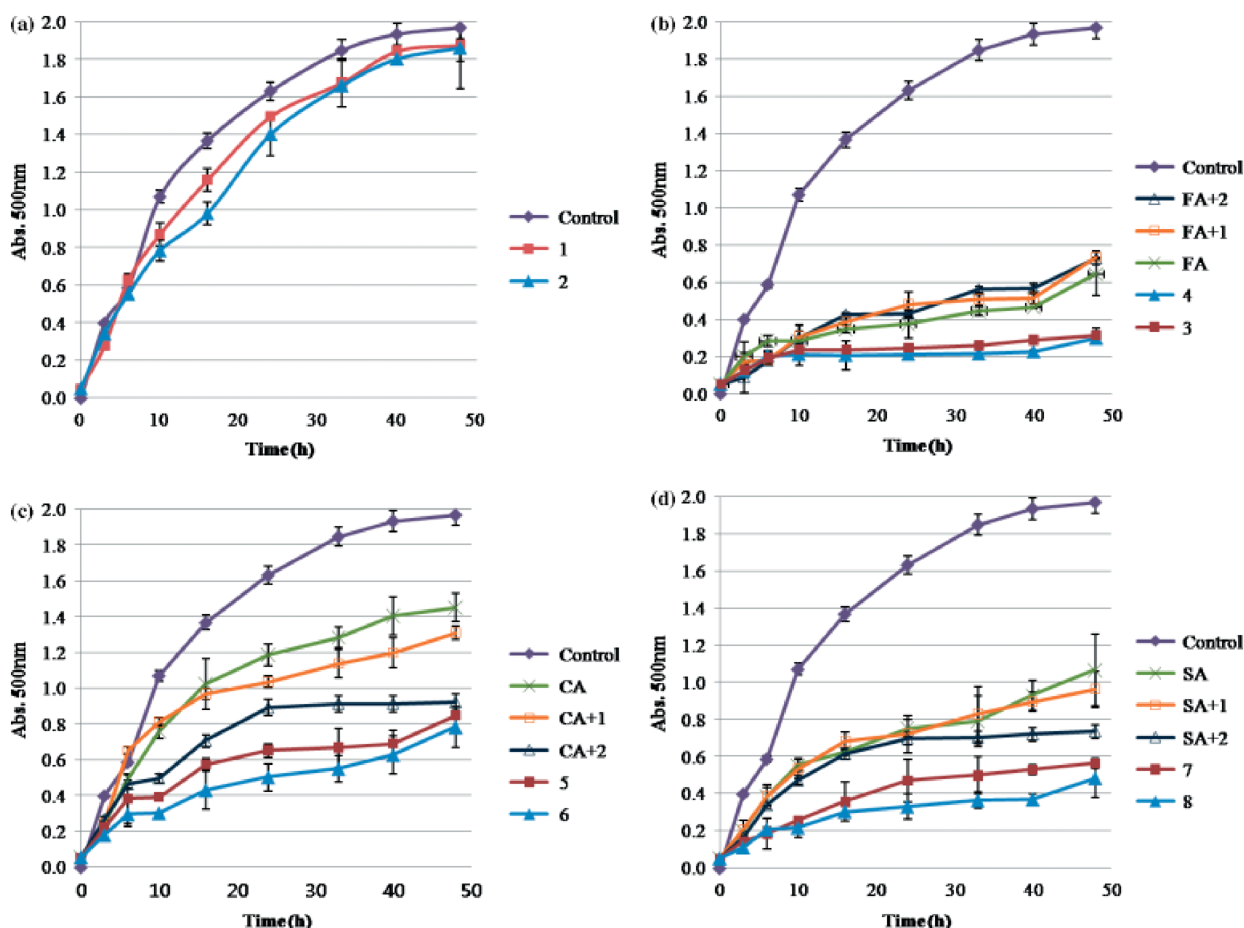


**Figure 3.** Antioxidative activity of HCA-peptides as a function of reaction time in lipid autoxidation system measured by ferric thiocyanate method. (a), GS(Bzl)H and CAR; (b), FA, FA-peptides and mixture of FA and the peptides; (c), CA, CA-peptides and mixture of CA and the peptides; (d), SA, SA-peptides and mixture of SA and the peptides. Conditions: the final concentration of each antioxidant was 90 µM; kept at 50 °C under dark conditions. Each experiment was carried out in triplicate, and the values are given as the mean ± standard error.

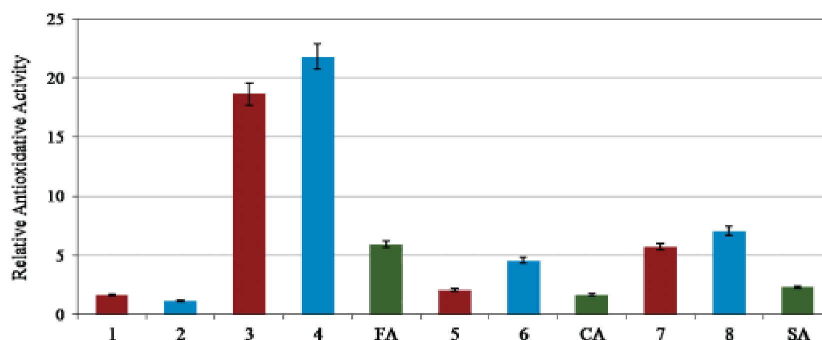


**Figure 4.** Relative antioxidative activity of HCAs, the peptides, and HCA-peptides based on the induction time (h). Conditions: the final concentration of each antioxidant was 90 µM; kept at 50 °C under dark conditions. The values are given as the mean ± standard error.

and **4** showed excellent antioxidative activity at very low concentration, 12.5 µM. Compound **2** showed 28 (±1.4) of %Pi at the lowest concentration, 0.1 mM (Figure 5(a)). However, compound **2**'s antioxidative activity continuously decreased at higher concentration, and it accelerated the rate of lipid autoxidation instead of inhibiting it at concentrations of 0.5 mM and higher. This might be due to the result of a prooxidative effect, which is stimulated as the amount of cysteine increased [11,18]. Even though sulfhydryls were blocked with benzyl group to prevent formation of oxidized glutathione (GSSG), this was not enough to eliminate the prooxidative effect of cysteine completely. However, conjugation with FA completely neutralized the negative effect of **2** (Figure 5(b)).

## Conclusion

Our results demonstrated that HCA-CAR or HCA-GS(Bzl)H synergistically enhanced the antioxidative activity and the free RSA of HCA. SA- and CA-peptide conjugates exhibited better synergistic effect than FA-peptides, even though FA-peptides indicated the



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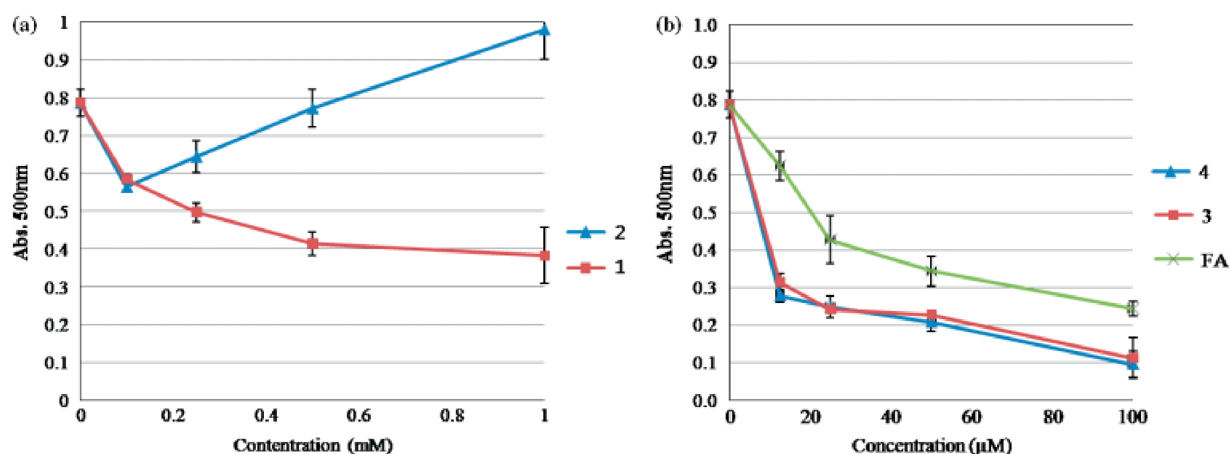


**Figure 5.** Antioxidative activity of the peptides and FA-peptides at different concentrations. (a), lipid peroxidation inhibitory activity of GS(Bzl)H and CAR at more than 100 μM; (b), lipid peroxidation inhibitory activity of FA and FA-peptides at less than 100 μM. Conditions: the concentration of each antioxidant was final concentration; kept at 50 °C for 9 h in the dark conditions. Each experiment was carried out in triplicate, and the values are given as the mean ± standard error.

highest %Pi. HCA-CAR exhibited good antioxidative activity in both methanol and lipid emulsion system because of hydrophilic nature and lipid peroxyradical trapping ability of imidazole group, while HCA-GS(Bzl)H showed potent antioxidative activity in lipid emulsion system because of sulfur and phenyl ring moiety. In particular, CA-CAR showed the highest %RSA and synergistically enhanced antioxidative activity, and hence CA-CAR could be an ideal antioxidant in both hydrophilic and hydrophobic environment. Further cell-based studies on the antioxidative ability of HCA-peptides are underway.

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