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Antioxidative activities of histidine containing caffeic acid-dipeptides

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ABSTRACT

Antioxidants have been utilized in both the food and cosmetics industries to neutralize the activities of reactive oxygen species (ROS) and free radicals. Histidine-containing peptides are powerful antioxidants that exist in nature. Additionally, hydroxycinnamic acid (HCA)-peptide conjugates exhibit a synergistically enhanced antioxidative activity. Thus, caffeic acid (CA), a natural antioxidant, was conjugated to histidine-containing dipeptides (His dipeptides) in order to develop better antioxidants. The antioxidative activities were measured using 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test and lipid peroxidation test with ferric thiocyanate method. Some of the CA-His dipeptides exhibited better radical scavenging activities than CA, and all of the CA-His dipeptides showed enhanced lipid peroxidation inhibitory activities. His dipeptide enhanced the antioxidative activity of CA, and the position of histidine also affected the antioxidative activity of the compounds. CA-proline-histidine amide (CA-Pro-His-NH_2) exhibited the highest activity in both the free radical scavenging test and the lipid peroxidation inhibition test.

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Oxygen is one of the most abundant elements on the Earth and almost every living organism consumes oxygen to live. However, unconsumed oxygen molecules can be converted into free radicals such as superoxide, hydroxyl and peroxyl radicals, which are also known as reactive oxygen species (ROS). ROS initiate radical chain reactions in the human body and are responsible for aging, cancer and several chronic diseases through the oxidation of DNA, RNA, lipids, and proteins.[1–3] In the healthy human body, endogenous antioxidants can remove the continuously generated ROS by breaking the radical chain reaction either through transferring electrons or trapping hydrogen radicals in order to maintain a balance in the body. If ROS are overproduced, the balance breaks down, which leads to the severe oxidation of the living system. Many researchers, in various fields, have developed new synthetic antioxidants in order to reduce the ROS.

Phenolic compounds are widely studied as antioxidants because these compounds have a stable structure after the free radicals are quenched. A previous report showed that the antioxidant activity of the phenolic compounds increases as the number of hydroxyl (OH) and methoxy (OCH_3) groups increases.[4] Caffeic acid (CA; 3,4-dihydroxycinnamic acid) is a member of the hydroxycinnamic acid (HCA) family, and is abundant in nature. The high antioxidant activity of CA is caused by the delocalization of the unpaired electron in the extended conjugated side chain.[5] Additionally, the *ortho*-dihydroxyl group of CA forms an additional hydrogen bond, which creates a stable configuration after the O–H bond is broken.[6]

Peptide based antioxidants have also been actively studied. Antioxidative peptides have been prepared through enzymatic hydrolysis from various natural proteins[7–17] or synthetic peptide libraries.[18] Additionally, previous studies have revealed that peptides can enhance the stability of antioxidant compounds through the suppression of lipid oxidation.[19]

In our previous study, the antioxidant activity of HCAs was enhanced through conjugation with antioxidative peptides.[20] We concluded that CA-β-Ala-His-NH_2 was an ideal antioxidant in both hydrophilic and hydrophobic environments because the compound exhibited excellent free radical scavenging activity and synergistically inhibited lipid oxidation. Carnosine (CAR), which contains β-alanine and histidine, naturally exists at high concentrations (2–20 mM) in mammalian muscle tissues and nervous systems.[21–23] CAR extends the fibroblast life-span by reversing the signs of aging and inhibiting the lipid peroxidation within the cell membrane because of the lipid peroxyradical trapping ability of imidazole in histidine.[24]

In this study, we focused on histidine residue and constructed CA-histidine dipeptide libraries in order to find a better antioxidant than CA-β-Ala-His-NH_2. Several amino acids were selected based on the functional group properties. For example, cysteine was selected for its sulfhydryl side chain, phenylalanine was selected for its aromatic side group, proline was selected for its cyclic structure, and alanine was selected because it had the same molecular weight as β-alanine and only differed by one alkyl chain. The other amino acids that were selected were chosen from frequently occurring sequences in natural antioxidants.[7–19] The C-termini of all of the His-dipeptides were fixed to amides because C-terminal

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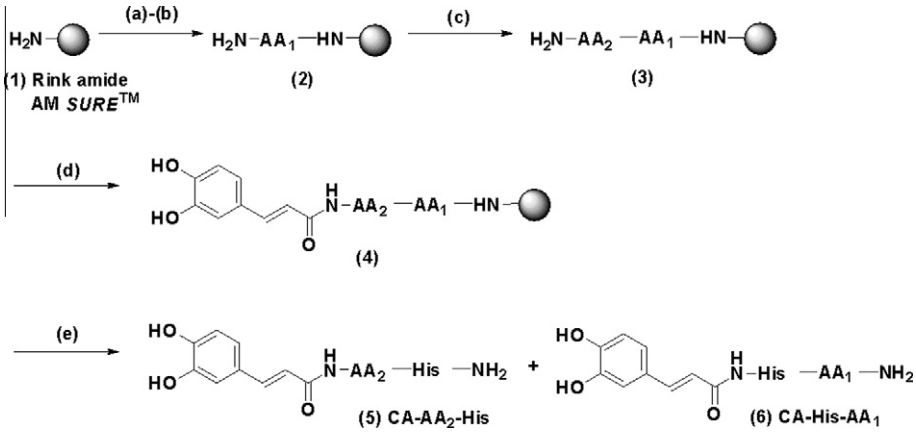

Scheme 1. Solid-phase CA-His dipeptide synthesis. Reagents and conditions: (a) Fmoc-L-amino acids (2 equiv), BOP (2 equiv), HOBt (2 equiv) and DIPEA (4 equiv) in NMP for 2 h; (b) 20% piperidine/NMP (v/v) for 3 min and 15 min; (c) repeat (a) and (b); (d) CA (2 equiv), BOP (2 equiv), HOBt (2 equiv) and DIPEA (4 equiv) in NMP for 5 h; (e) cleavage cocktail: 30% TFA/1% water in dry DCM for 1 h, and diethyl ether precipitation.

peptide amides were more stable than C-terminal free peptides under various conditions.[25–28] The anitoxidative activities of CA-His dipeptides were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test[29] and the linoleic acid auto-oxidation system with ferrous thiocyanate assay.[30,31] BHA (butylhydroxyanisole; a commercially available synthetic antioxidant), CA, CA-Phe-NH_2, and CA-His-NH_2 served as the reference compounds.[32]

First, CA-His dipeptides were synthesized through solid-phase peptide synthesis with Rink amide AM *SURE*™ resin (Scheme 1).[33]

The compounds were synthesized with high yields and purities (Table 1).

CA-His dipeptides had quite different physico-chemical properties to CA (Tables 2 and 3).[34–37] The partition coefficients (Log *P*) of CA decreased through conjugation with His dipeptides. After conjugation, *C*Log *P* (calculated Log *P*) of CA decreased because of the added hydrophilic moiety in the imidazole ring. Thus, the hydrophilic nature of His dipeptides increased the aqueous solubility (Log *S*) of CA, regardless of the position of histidine. The calculated values of the partition coefficients (*C*Log *P*) and the aqueous solubilities (*C*Log *S*) of CA-His dipeptides are given in Tables 2 and 3.

Table 2
Partition coefficient (*C*Log *P*) of CA-His dipeptides

Compounds		*C*Log *P*[a]	Compounds		*C*Log *P*[a]
CA	CA	0.9750	**CA-HD**	CA-His-Asp-NH_2	−2.1002
CA-F	CA-Phe-NH_2	1.0862	**CA-FH**	CA-Phe-His-NH_2	−0.1544
CA-H	CA-His-NH_2	−1.3148	**CA-HF**	CA-His-Phe-NH_2	−0.1544
CA-AH	CA-Ala-His-NH_2	−1.5724	**CA-HH**	CA-His-His-NH_2	−2.5554
CA-HA	CA-His-Ala-NH_2	−1.5724	**CA-KH**	CA-Lys-His-NH_2	−1.8324
CA-BH	CA-β-Ala-His-NH_2	−1.6122	**CA-HK**	CA-His-Lys-NH_2	−1.8324
CA-HB	CA-His-β-Ala-NH_2	−1.6344	**CA-PH**	CA-Pro-His-NH_2	−1.1762
CA-CH	CA-Cys-His-NH_2	−1.5267	**CA-HP**	CA-His-Pro-NH_2	−0.9118
CA-HC	CA-His-Cys-NH_2	−1.4786	**CA-SH**	CA-Ser-His-NH_2	−2.5701
CA-DH	CA-Asp-His-NH_2	−2.1483	**CA-HS**	CA-His-Ser-NH_2	−2.5220

[a] Calculated by ChemDraw Ultra (CambridgeSoft).

The free radical scavenging activities (RSA) of CA, CA-Phe-NH_2, CA-His-NH_2, CA-His dipeptides were evaluated through reactions with the DPPH radicals.[38] Figure 1 shows the observed level of the %RSA. The %RSA of CA was enhanced through conjugation with His-dipeptides, and CA-Pro-His-NH_2 exhibited the highest %RSA among the CA-His dipeptide derivatives. Some of the CA-His dipeptides exhibited a higher %RSA than CA in the following order:

Table 1
Yield and purity of CA-His dipeptides

Compounds		Crude yield (%)	Purity[a] (%)	ESI-MS	
				Calculated	Found
CA-F	CA-Phe-NH_2	31	70	327.4 ($[M+H]^+$)	327.2
CA-H	CA-His-NH_2	34	77	316.3 ($[M+H]^+$)	316.2
CA-AH	CA-Ala-His-NH_2	41	72	388.4 ($[M+H]^+$)	388.3
CA-HA	CA-His-Ala-NH_2	25	78	388.4 ($[M+H]^+$)	388.4
CA-BH	CA-β-Ala-His-NH_2	25	74	388.4 ($[M+H]^+$)	388.2
CA-HB	CA-His-β-Ala-NH_2	33	71	388.4 ($[M+H]^+$)	388.6
CA-CH	CA-Cys-His–NH_2	52	66	477.1 ($[M+H]^+$)	476.2
CA-HC	CA-His-Cys-NH_2	32	78	477.3 ($[M+H]^+$)	476.2
CA-DH	CA-Asp-His-NH_2	39	81	432.4 ($[M+H]^+$)	433.3
CA-HD	CA-His-Asp-NH_2	34	80	454.9 ($[M+Na]^+$)	455.3
CA-FH	CA-Phe-His-NH_2	60	82	464.0 ($[M+H]^+$)	465.4
CA-HF	CA-His-Phe-NH_2	44	76	464.5 ($[M+H]^+$)	465.4
CA-HH	CA-His-His-NH_2	20	66	454.3 ($[M+H]^+$)	454.5
CA-KH	CA-Lys-His-NH_2	42	69	441.3 ($[M+H]^+$)	445.2
CA-HK	CA-His-Lys-NH_2	34	68	445.5 ($[M+H]^+$)	445.2
CA-PH	CA-Pro-His-NH_2	40	87	414.4 ($[M+H]^+$)	414.7
CA-HP	CA-His-Pro-NH_2	48	79	414.4 ($[M+H]^+$)	414.3
CA-SH	CA-Ser-His-NH_2	36	69	404.2 ($[M+Na]^+$)	404.4
CA-HS	CA-His-Ser-NH_2	39	67	404.5 ($[M+Na]^+$)	404.4

[a] Determined by HPLC.

Table 3
Aqueous solubility (*C*Log *S*) of CA-His dipeptides

Compounds		*C*Log *S*[a]	Compounds		*C*Log *S*[a]
CA	CA	−1.41	**CA-HD**	CA-His-Asp-NH_2	−1.49
CA-F	CA-Phe-NH_2	−2.76	**CA-FH**	CA-Phe-His-NH_2	−2.88
CA-H	CA-His-NH_2	−2.88	**CA-HF**	CA-His-Phe-NH_2	−2.88
CA-AH	CA-Ala-His-NH_2	−1.75	**CA-HH**	CA-His-His-NH_2	−1.72
CA-HA	CA-His-Ala-NH_2	−1.75	**CA-KH**	CA-Lys-His-NH_2	−2.13
CA-BH	CA-β-Ala-His-NH_2	−1.64	**CA-HK**	CA-His-Lys-NH_2	−2.13
CA-HB	CA-His-β-Ala-NH_2	−1.64	**CA-PH**	CA-Pro-His-NH_2	−1.77
CA-CH	CA-Cys-His–NH_2	−2.40	**CA-HP**	CA-His-Pro-NH_2	−1.77
CA-HC	CA-His-Cys-NH_2	−2.40	**CA-SH**	CA-Ser-His-NH_2	−2.59
CA-DH	CA-Asp-His-NH_2	−1.49	**CA-HS**	CA-His-Ser-NH_2	−2.59

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

CA-Pro-His-NH_2 (71 ± 3.1) > CA-β-Ala-His-NH_2 (70 ± 10.8) > CA-Lys-His-NH_2 (65 ± 4.8) > BHA (62 ± 1.0) > CA-Phe-His-NH_2 (59 ± 3.8) > CA-His-Lys-NH_2 (54 ± 5.2) > CA-Asp-His-NH_2 (52 ± 3.1) > CA-Phe-NH_2 (51 ± 7.7) > CA-His-Asp-NH_2 (49 ± 5.8) > CA (47 ± 2.1). The %RSA of the CA-His dipeptides were similar depending on the position of histidine. A higher %RSA was observed when histidine was separated from CA by one amino acid (CA-X*H*), a higher %RSA compared to when histidine was directly attached to CA (CA-*H*X).

Though the DPPH radical is mainly affected by the antioxidant, the solvent system cannot be underestimated because it also has electron donating or withdrawing groups. Some research groups have examined the kinetic solvent effects (KSE) on phenolic antioxidants.[39] The magnitude of the KSE is determined by the strength of the interaction between the hydrogen bond donor, the phenolic antioxidant, and the hydrogen accepting solvent. Hydrogen bonding plays an important role on the hydrogen atom donating activities of the phenolic antioxidants. When the phenolic antioxidants meet the free radicals, they quench the radicals through a hydrogen atom transfer from the phenolic hydroxyl to the peroxyl radicals.[40,41] Therefore, selecting the solvent system for the RSA measurements might be important for screening antioxidant compounds. The %RSA was examined in different solvents with various structural features.[42] Methanol, acetonitrile, and *tert*-butanol were chosen because they exhibit similar polarities and differ only in structure. Methanol is a broadly used protic polar solvent that can donate a proton to form a hydrogen bond with both the antioxidant and the free radical. Acetonitrile is able to form hydrogen bonds as a hydrogen bond acceptor from the antioxidant.[46,47] *tert*-Butanol is a protic polar solvent that has a bulky *tert*-butyl group, which causes steric hindrance, and therefore it cannot actively exchange protons as fast as methanol. Figure 2a shows the %RSA of the selected antioxidant compounds in different solvent systems. The %RSA exhibited similar tendencies to antioxidant strengths for the three solvent systems. However, the absolute values of the %RSA were quite different and decreased in the following order: $RSA_{methanol} > RSA_{acetonitrile} > RSA_{tert\text{-}butanol}$. As expected, *tert*-butanol exhibited the poorest %RSA because its bulkiness prevented the antioxidant from reacting with the free radical. However, it donated a proton to the radical. Methanol was a proper solvent for studying the RSA of antioxidants because the antioxidative activities were easily compared. The RSA of the selected compounds were also examined in acidic conditions in order to determine the pH effect on the antioxidant activities (Fig. 2b). An acetate buffer (pH 5.5) was used to make acidic buffered methanol, which exhibited a better RSA than methanol with some compounds in previous studies.[43,44] As expected, the RSA in buffered methanol was higher for most of the compounds.

A lipid peroxidation inhibitory assay was performed using Tween 20-emulsified linoleic acid (>99%) in order to measure the antioxidative activity (Fig. 3).[45] CA-Pro-His-NH_2 exhibited the highest percentage of lipid peroxidation inhibition (%Pi), which was even greater than BHA, a strong synthetic antioxidant. CA-Phe-NH_2, which exhibited an enhanced antioxidative activity in previous studies,[25,26] and CA-His-NH_2, which was a CA derivative containing only the His moiety, were synthesized, and their activities were compared to CA-His dipeptides. The %Pi of CA, CA-Phe-NH_2, CA-His-NH_2, and CA-His dipeptides decreased in the following order: CA-Pro-His-NH_2 (87 ± 6.1) > BHA (86 ± 2.5) > CA-Lys-His-NH_2 (84 ± 6.0) ≈ CA-Cys-His-NH_2 (84 ± 7.3) ≈ CA-Ala-His-NH_2 (84 ± 5.4) > CA-Ser-His-NH_2 (83 ± 5.2) > CA-Asp-His-NH_2 (82 ± 4.5) > CA-Phe-His-NH_2 (80 ± 10.9) ≈ CA-His-His-NH_2 (80 ± 2.6) > CA-His-Cys-NH_2 (79 ± 4.0) > CA-His-Lys-NH_2 (76 ± 10.0) > CA-Phe-NH_2 (73 ± 8.3) ≈ CA-His-Asp-NH_2 (73 ± 2.0) > CA-His-Phe-NH_2 (72 ± 9.9) > CA-His-Ser-NH_2 (71 ± 3.3) ≈ CA-His-β-Ala-NH_2 (71 ± 5.1) > CA-His-NH_2 (67 ± 4.0) > CA-His-Ala-NH_2 (66 ± 12.0) > CA-β-Ala-His-NH_2 (64 ± 9.1) > CA-His-Pro-NH_2 (61 ± 7.8) > CA (51 ± 9.5).

All of the CA-His dipeptides exhibited higher %Pi than CA, unlike the %RSA because of the differences in the hydrophilicity of the compounds. The DPPH radical scavenging assay was carried out in methanol, which was a hydrophilic solvent, whereas the lipid peroxidation inhibitory assay was performed in Tween 20-emulsified

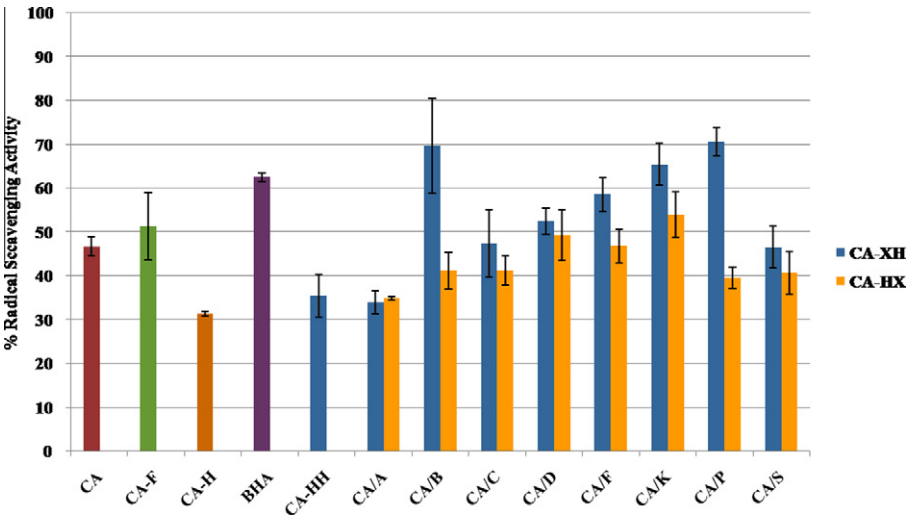

Figure 1. DPPH radical scavenging activity of CA-His dipeptides. [Antioxidant]/[DPPH](mol/mol) = 0.25. Each experiment was performed in triplicate and repeated five times. The values are given as the mean ± standard error.

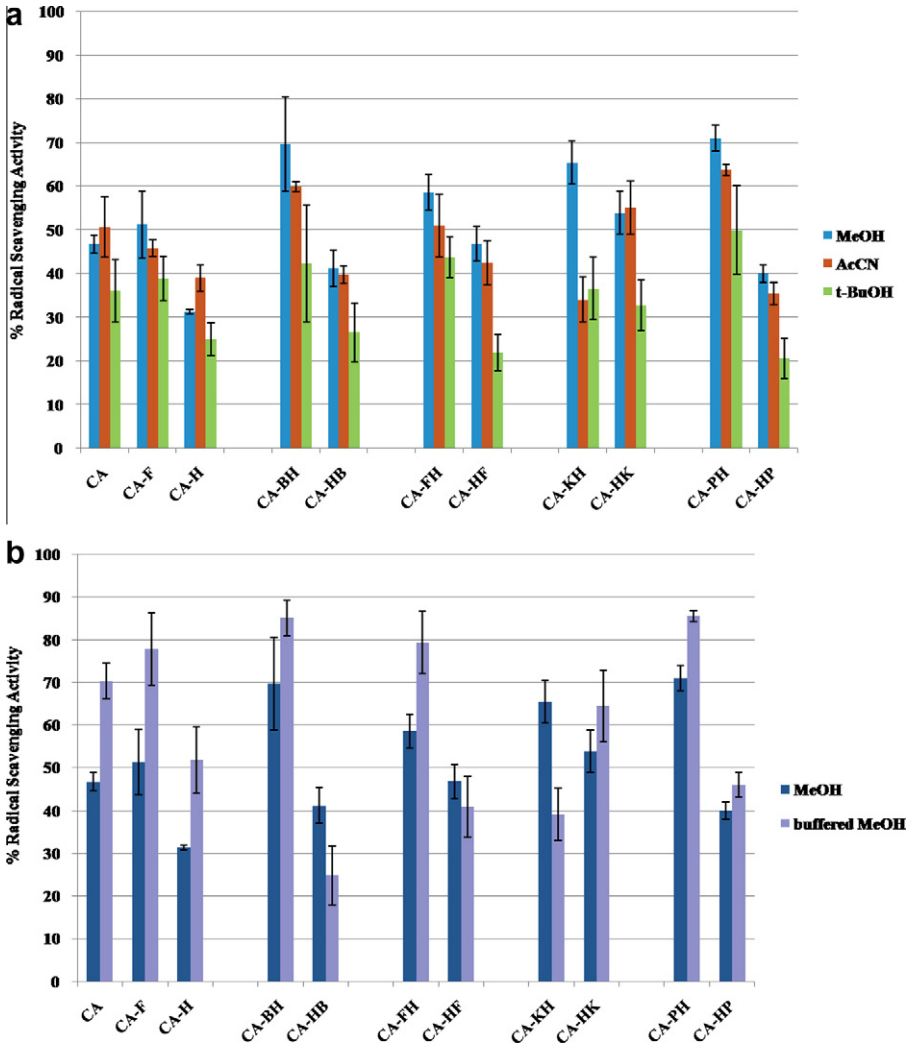

Figure 2. DPPH radical scavenging activity of selected CA-His dipeptides in different solvent systems. [Antioxidant]/[DPPH](mol/mol) = 0.25. Each experiment was performed in triplicate and repeated five times. The values are given as the mean ± standard error.

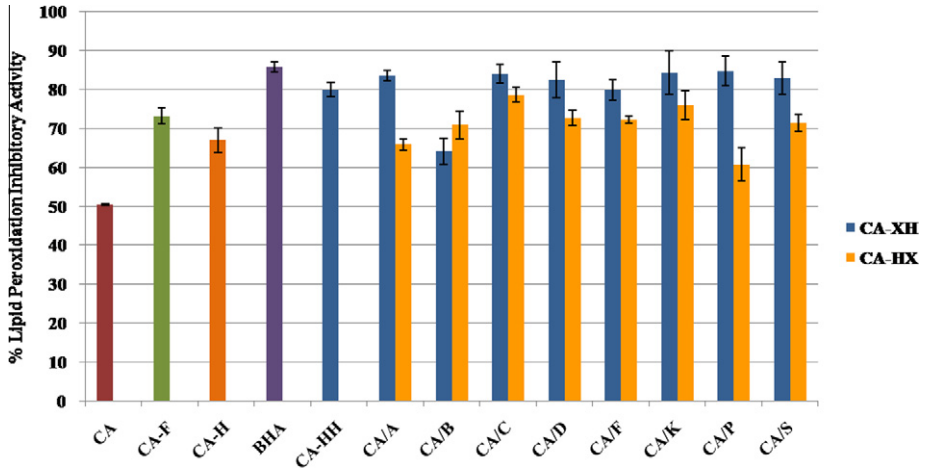

Figure 3. Antioxidative activity of CA-His dipeptides at 10 h in lipid autoxidation system measured by the ferric thiocyanate method. Conditions: the final concentration of each antioxidant was 90 μM; kept at 50 °C under dark conditions for 10 h. Each experiment was carried out in triplicate, and the values are given as the mean ± standard error.

linoleic acid, which was biphasic. Tables 2 and 3 show that CA-His dipeptides had lower *C*Log *P* and lower *C*Log *S* than CA because CA-His dipeptides were more hydrophilic and more soluble in water than CA. Therefore, CA-His dipeptides might have a good accessibility for the lipid emulsion because they contained both hydrophobic and hydrophilic parts in one molecule.[36–38]

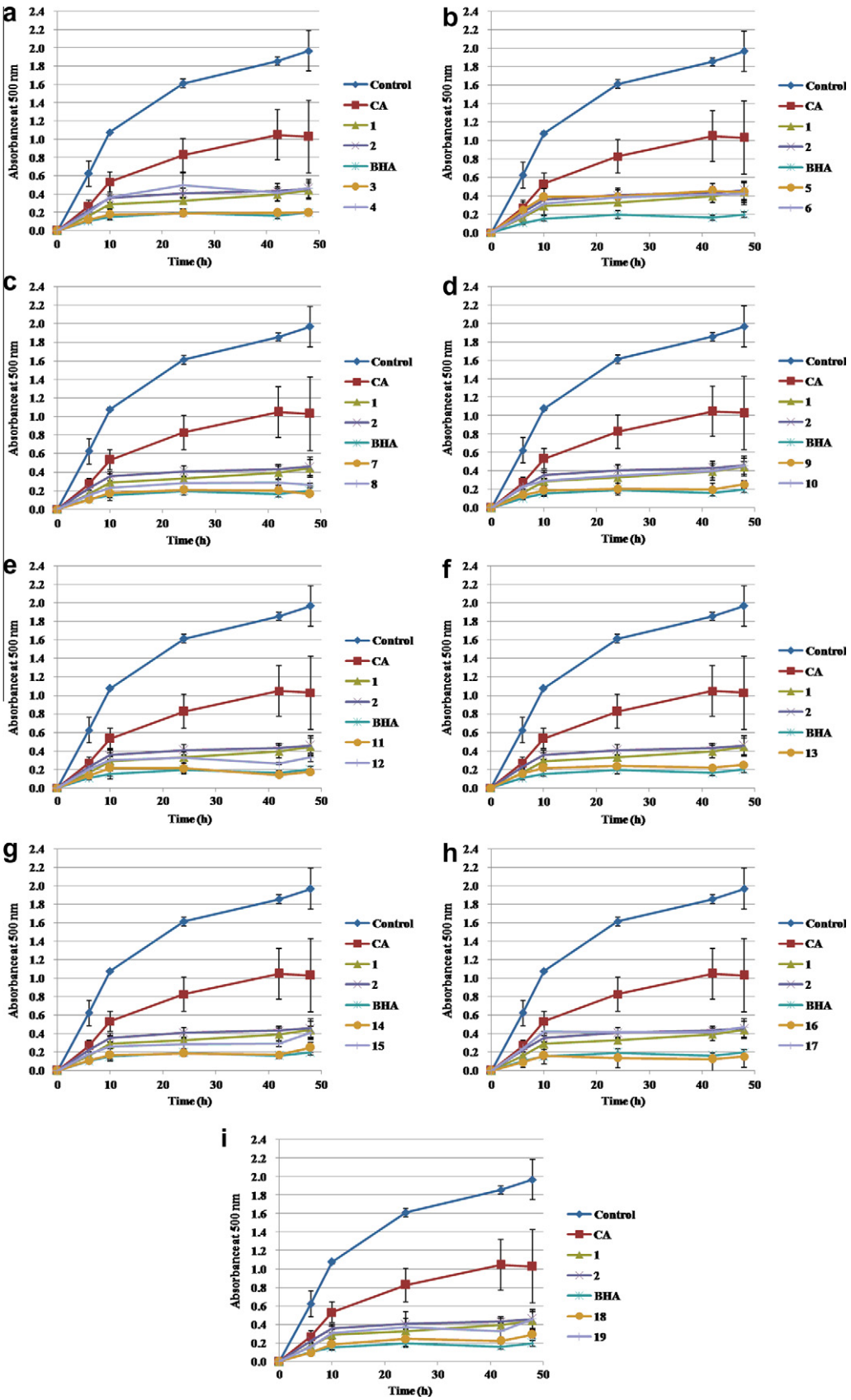

Figure 4. Antioxidative activity of CA-His dipeptides as a function of reaction time in lipid autoxidation system measured by ferric thiocyanate method. (a) CA-Ala-His-NH_2 (**3**), CA-His-Ala-NH_2 (**4**); (b) CA-β-Ala-His-NH_2 (**5**), CA-His-β-Ala-NH_2 (**6**); (c) CA-Cys-His-NH_2 (**7**), CA-His-Cys-NH_2 (**8**); (d) CA-Asp-His-NH_2 (**9**), CA-His-Asp-NH_2 (**10**), (e) CA-Phe-His-NH_2 (**11**), CA-His-Phe-NH_2 (**12**); (f) CA-His-His-NH_2 (**13**); (g) CA-Lys-His-NH_2 (**14**), CA-His-Lys-NH_2 (**15**); (h) CA-Pro-His-NH_2 (**16**), CA-His-Pro-NH_2 (**17**); (i) CA-Ser-His-NH_2 (**18**), CA-His-Ser-NH_2 (**19**). 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.

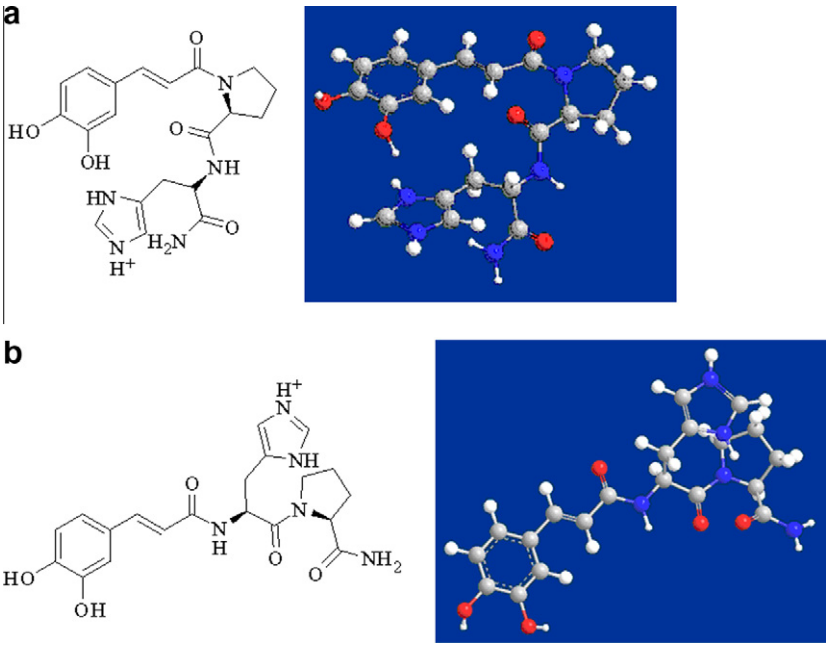

Figure 5. Proposed molecular structures of (a) CA-Pro-His-NH_2 (CA-PH); (b) CA-His-Pro-NH_2 (CA-HP). The figures depicted were prepared with ChemDraw Ultra (CambridgeSoft).

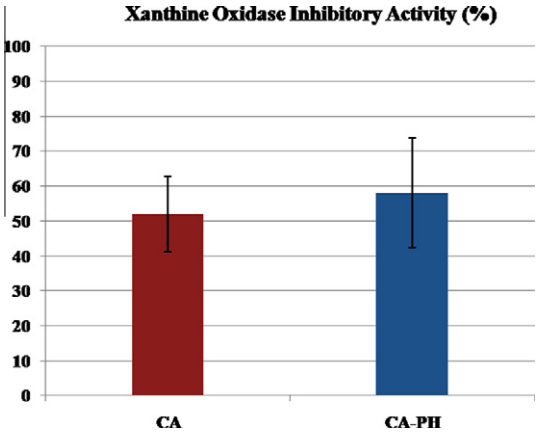

Figure 6. Xanthine oxidase inhibitory activities of CA and CA-PH (0.1 mM). Each experiment was carried out in triplicate, and the values are given as the mean ± standard error.

The DPPH assay results showed that the position of histidine in the dipeptide also affected the antioxidative activity of CA. With the exception of CA-β-Ala-His-NH_2, the %Pi of the CA-His dipeptides were similar depending on the position of histidine (Fig. 4). The %Pi increased when an amino acid was placed between histidine and CA.

Figure 3 shows the percentage of lipid peroxidation inhibition (%Pi) of the compounds at 10 h in order to compare the antioxidative activities during the early stages of the lipid peroxidation. While the absorbance of the negative control dramatically increased, the absorbance of the reaction mixture with CA moderately increased during the early stages and then acutely increased. An enhanced antioxidative activity was also observed when one amino acid, such as phenylalanine and histidine, was conjugated to CA. CA-His dipeptides exhibited better activities than CA-His-NH_2 because only one histidine could not enhance the antioxidative activity of CA. CA-His dipeptides exhibited excellent antioxidative activities, and several compounds had promising activities as potent antioxidants.

In the CA-His dipeptide libraries, compound CA-Pro-His-NH_2 had an excellent antioxidative activity in both the free radical scavenging activity test and the lipid peroxidation inhibition test. Compound CA-Pro-His-NH_2 consisted of proline and histidine and exhibited a higher %Pi than the commercial antioxidant, BHA. Proline commonly appears in many peptide-based natural antioxidants.[17,18] Interestingly, CA-Pro-His-NH_2 exhibited a notable antioxidative activity enhancement, whereas CA-His-Pro-NH_2 exhibited a poor antioxidative activity, even though their amino acids compositions were the same. We here suggest a structure (Fig. 5) to explain the significant difference in the antioxidant activities between CA-Pro-His-NH_2 and CA-His-Pro-NH_2 which was caused by the order of the amino acids. In the proposed structures, proline in CA-Pro-His-NH_2 provided a tilted structure that provided additional stability to the hydroxyl radical of CA by the imidazole ring after the free radicals were quenched, whereas CA-His-Pro-NH_2 did not have the same beneficial structure.

When we performed DPPH free radical scavenging test and lipid peroxidation inhibition test, CA-Pro-His-NH_2 exhibited promising activities. For further studies in biological system, we examined the antioxidative activity of CA-Pro-His-NH_2 by a well-known antioxidant evaluating system with enzyme, xanthine oxidase.[48–51] Xanthine oxidase (XO, EC 1.2.3.2) oxidizes hypoxanthine to xanthine, then to uric acid, and once uric acid is accumulated, it can cause hyperuricacidemia. When we performed XO inhibitory assay by using modified Noro's method,[48] CA-Pro-His-NH_2 exhibited a tendency to enhance the antioxidative activities of CA from 52% to 58% (Fig. 6).

In conclusion, conjugating histidine-containing dipeptides to CA enhanced the antioxidative activity of CA. Several compounds exhibited predominant RSA compared to CA because of the radical trapping ability of imidazole in histidine. All of the compounds exhibited better lipid peroxidation inhibitory activities than CA. CA-Pro-His-NH_2 exhibited excellent results in both the RSA and the lipid peroxidation inhibitory activity. Therefore, CA-Pro-His-NH_2 was an ideal antioxidant in both hydrophilic and lipophilic systems probably because the structure of proline enabled histidine to stabilize the hydroxyls of CA, leading to the enhanced antioxidative activities.

Acknowledgments

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References and notes

1. Jeroudi, M. O.; Hartley, C. J.; Bolli, R. *Am. J. Cardiol.* **1994**, *73*, 2.
2. Kehrer, J. P. *Crit. Rev. Toxicol.* **1993**, *23*, 21.
3. Spector, A. *J. Ocul. Pharmacol. Ther.* **2000**, *16*, 193.
4. Wright, J. S.; Johnson, E. R.; DiLabio, G. A. *J. Am. Chem. Soc.* **2001**, *123*, 1173.
5. Graf, E. *Free radic. Biol. Med.* **1992**, *13*, 435.
6. Son, S.; Lewis, B. A. *J. Agric. Food Chem.* **2002**, *50*, 468.
7. Pratt, D. E. *J. Food Sci.* **1972**, *37*, 322.
8. Yukami, S. *Agric. Biol. Chem.* **1972**, *36*, 871.
9. Rhee, K. S.; Ziprin, Y. A.; Rhee, K. C. *J. Food Sci.* **1979**, *44*, 1132.
10. Yamaguchi, N.; Yokoo, Y.; Fujimaki, M. *Nippon Shokuhin Kogyo Gakkaishi.* **1975**, *22*, 431.
11. Park, P. Y.; Jung, W. K.; Choi, Y. R.; Kim, S. K. *Korean J. Life Sci.* **2000**, *10*, 34.
12. Sakanaka, S.; Tachibana, Y.; Ishihara, N.; Juneja, L. R. *Food Chem.* **2004**, *86*, 99.
13. Kim, S. K.; Kim, Y. T.; Byun, H. G.; Nam, K. S.; Joo, D. S.; Shahidi *J. Agric. Food Chem.* **2001**, *49*, 1984.
14. Mendis, E.; Rajapakse, N.; Kim, S. K. *J. Agric. Food Chem.* **2005**, *53*, 581.
15. Hsu, K. C.; Lu, G. H.; Jao, C. L. *Food Res. Int.* **2009**, *42*, 647.
16. Suetsuna, K. *Mar. Biotechnol.* **2000**, *2*, 5.
17. Minelli, A.; Bellezza, I.; Grottelli, S.; Galli, F. *Amino Acids* **2008**, *35*, 283.
18. Saito, K.; Jin, D. H.; Ogawa, T.; Muramoto, K.; Hatakeyama, E.; Yasuhara, T.; Nokihara, K. *J. Agric. Food Chem.* **2003**, *51*, 3668.
19. Park, E. Y.; Murakami, H.; Matsumura, Y. *J. Agric. Food Chem.* **2005**, *53*, 8334.
20. Kwak, S. Y.; Seo, H. S.; Lee, Y. S. *J. Pept. Sci.* **2009**, *15*, 634.
21. Cacciatore, I.; Cocco, A.; Fontana, M.; Lucente, G.; Pecci, L.; Pinnen, F. *Amino Acids* **2005**, *28*, 77.
22. Abe, H.; Dobson, G. P.; Hoeger, U.; Parkhouse, W. S. *Am. J. Physiol.* **1985**, *249*, 449.
23. Ohtsuka, C.; Takahashi, H. *Brain Dev.* **1983**, *5*, 509.
24. Johnson, P.; Fedyna, J. S.; Schindzielorz, A.; Smith, C. M.; Kasvinsky, P. J. *Biochem. Biophys. Res. Commun.* **1982**, *109*, 769.
25. Chen, J. H.; Ho, C. T. *J. Agric. Food Chem.* **1997**, *45*, 2374.
26. Spasova, M.; Kortenska-Kancheva, V.; Totseva, I.; Ivanova, G.; Gerogriev, L.; Milkova, T. *J. Pept. Sci.* **2006**, *12*, 369.
27. Spasova, M.; Ivanova, G.; Weber, H.; Ranz, A.; Lankmayr, E.; Milkova, T. *Oxid. Commun.* **2007**, *30*, 803.
28. Noh, J. M.; Kwak, S. Y.; Lee, Y. S. *Biopolymers* **2007**, *88*, 300.
29. Pekkarinen, S. A.; Stockmann, H.; Schwarz, K.; Heinonen, I. M.; Hopia, A. I. *J. Agric. Food Chem.* **1999**, *47*, 3036.
30. Mitsuda, H.; Rasumoto, K.; Iwami, K. *Eiyoto Shokuryo* **1966**, *19*, 210.
31. Chen, H. M.; Muramoto, K.; Yamaguchi, F. *J. Agric. Food Chem.* **1995**, *43*, 574.
32. Wattenberg, L. W. *Food Chem. Toxicol.* **1986**, *24*, 1099.
33. *Synthetic procedure for CA-His dipeptides*: The peptides were prepared using conventional Fmoc solid-phase peptide synthesis on Rink amide AM *SURE*™ resin (0.69 mmol/g) using LibraTube® (BeadTech, Seoul, Korea). Each reaction step was monitored by Kaiser's ninhydrin test.[33] After His-dipeptides were anchored onto the resins, CA (2 equiv), BOP (2 equiv), HOBt (2 equiv) and DIPEA (4 equiv) in NMP were added and the coupling reaction was performed for 5 h at 25 °C. Then, cleavage cocktail (30% TFA/1% H_2O in DCM) was treated for 1 h at 25 °C to detach the product from the resin. The filtrate was concentrated and triturated with cold diethyl ether to obtain CA-His dipeptides as powder. The purity of CA-His dipeptides was verified through high performance liquid chromatography (HPLC, Young Lin Autochro 2000), using Waters μBondapak C18 reverse phase column (125 Å, 10 μm, 3.9 × 150 nm) with a linear gradient elution of 0.1% TFA and an acetonitrile (20–80%, v/v), flow rate of 1.0 mL/min, and UV detector (Mecasys Co. Ltd, Optizen 2120 UV) at 260 nm. The mass of the compounds were confirmed by using a QUATTRO Triple Quardrupole Tandem mass spectrometer (Micromass & Waters, Milford, MA, USA). CA-phenylalanine amide (CA-Phe-NH_2) and CA-histidine amide (CA-His-NH_2) were obtained and identified using the same procedure as CA-His dipeptides.
34. *Calculation of Log P and Log S of CA-His dipeptides*: The partitioning coefficient, Log *P*, was calculated using ChemDraw Ultra (CambridgeSoft). Additionally, the aqueous solubility (Log *S*) was calculated using online-Log *P*/Log *S* calculation software, ALOGPS 2.1 (www.vcclab.org/lab/alogps/).
35. Sun, H. *J. Chem. Inf. Comput. Sci.* **2004**, *44*, 748.
36. Broto, P.; Moreau, G.; Vandyke, C. *Eur. J. Med. Chem. -Chim. Theor.* **1984**, *19*, 71.
37. Tetko, I. V.; Bruneau, P. *J. Pharm. Sci.* **2004**, *93*, 3102.
38. *Determination of radical scavenging activity*: The percentage of radical scavenging activity (%RSA) was measured through the decrease in the absorbance at 516 nm of the DPPH solution after adding the antioxidant. The methanolic DPPH solution (0.1 mM) was prepared, and 20 μL of 1.85 mM antioxidants were added to 1480 μL of the DPPH solution in an Eppendorf tube (1.5 mL-volume), making the final antioxidant concentration 25 μM. The absorbance was monitored after 20 min. The results were expressed as $\%RSA = [\{Abs_{516\ nm}\ (t = 0) - Abs_{516\ nm}\ (t = t')\}/Abs_{516\ nm}\ (t = 0)] \times 100$. The negative control was a mixture of 0.1 mM methanolic DPPH solution (1480 μL) and 20 μL of methanol instead of the samples. All of the experiments were performed in triplicate and averaged.
39. Barclay, L. R. C.; Edwards, C. E.; Vinqvist, M. R. *J. Am. Chem. Soc.* **1999**, *121*, 6226.
40. Foti, M. C.; Daquino, C. M.; Geraci, C. *J. Org. Chem.* **2004**, *69*, 2309.
41. Litwinienko, G.; Ingold, K. U. *J. Org. Chem.* **2003**, *68*, 3433.
42. *Determination of radical scavenging activity in various solvent systems*: The %RSA of the selected antioxidants was also evaluated under different solvent systems such as *tert*-butanol, acetonitrile or acetate buffered methanol (pH 5.5). The DPPH solution with acetonitrile and *tert*-butanol was prepared in the same way as the aforementioned methanolic DPPH solution. The buffered methanolic DPPH solution was prepared in the mixture of pH 5.5 acetate buffer, making its final concentration 0.1 mM.
43. Amorati, R.; Pedulli, G. F.; Cabrini, L.; Zambonin, L.; Landi, L. *J. Agric. Food Chem.* **2006**, *54*, 2932.
44. Sharma, O. P.; Bhat, T. K. *Food Chem.* **2009**, *113*, 1202.
45. *Determination of antioxidative activity*: A lipid peroxidation inhibitory assay was performed using Tween 20-emulsified linoleic acid (>99%) in order to indirectly measure the antioxidative activity. A 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 a sodium phosphate buffer (0.1 M, pH 7.0). After the emulsion was prepared, 0.5 mL of distilled water, 2.5 mL of the linoleic acid emulsion, 2.0 mL of a sodium phosphate buffer (0.1 M, pH 7.0) and 0.5 mL of the test samples dissolved in methanol were mixed in a glass vial (10 mL-volume). The vials containing the reaction mixture were sealed and stored at 50 °C under dark conditions. As a negative control, 0.5 mL of MeOH was added to the reaction mixture instead of the antioxidants. The reaction mixture was withdrawn at intervals with a microsyringe (1 mL-volume) in order to evaluate the antioxidative effect using ferric thiocyanate method. The ferric thiocyanate analysis was performed using the following procedure. The reaction mixture (25 μL) was mixed with 75% ethanol (1.175 mL), 30% ammonium thiocyanate (25 μL), and 20 mM ferrous chloride in 3.5% HCl (25 μL) in an Eppendorf tube (1.5 mL-volume). The color development of ferrous chloride and thiocyanate reached their maximum values after 3 min, and the absorbance of the mixture was measured at 500 nm. The degree of oxidation was initially measured at 6 h intervals and then at 12 h intervals in the later stages of oxidation as the rate of oxidation decreased.[20] Five independent measurements were performed, and each experiment was run in triplicate. The control experiment without antioxidant was repeated 10 times in order to define the oxidation curve in the lipid emulsified autoxidation system. The absorbance of the negative control reached approximately 1 (1.059–1.092) at 10 h, and continued to increase up to a value of approximately 2 (1.824–2.112) at 48 h before decreasing again. The antioxidative activity of the compounds was delineated in terms of the ability to restrict the early stage of the lipid peroxidation (Fig. 3) because alkylperoxyl radicals were spontaneously induced in air. The percentage of lipid peroxidation inhibition (%Pi) was calculated when the absorbance at 500 nm of the control (without antioxidant) reached approximately 1.
46. Kryachko, E. S.; Nguyen, M. T. *J. Phys. Chem. A.* **2002**, *106*, 4267.
47. Wakisaka, A.; Abdoul-Carime, H.; Yamamoto, Y.; Kiyozumi, Y. *J. Chem. Soc., Faraday Trans.* **1998**, *94*, 369.
48. Noro, T.; Oda, Y.; Miyase, T.; Ueno, A.; Fukushima, S. *Chem. Pharm. Bull.* **1983**, *31*, 3984.
49. Sweeney, A. P.; Wyllie, S. G.; Shalliker, R. A.; Markham, J. L. *J. Ethnopharmacol.* **2001**, *75*, 273.
50. Ihara, N.; Kurisawa, M.; Chung, J. E.; Uyama, H.; Kobayashi, S. *Appl. Microbiol. Biotechnol.* **2005**, *66*, 430.
51. *Determination of enzyme activity*. Xanthine oxidase inhibitory assay was performed using xanthine as the substrate by using modified Noro's method as following.[48] Sodium phosphate buffer (0.1 M, pH 7.5, 580 μL), 20 μL of xanthine oxidase from cow's milk obtained from Aldrich, and 200 μL of the 0.7 mM test samples were mixed in an Eppendorf tube at 25 °C for 15 min. Then, 400 μL of 0.15 mM xanthine solution was treated for 30 min at 25 °C, and was quenched by 100 μL of 1 N HCl. The absorbance of the reaction mixture was measured at 290 nm. Three independent measurements were performed, and each experiment was run in triplicate. The percentage of xanthine oxidase inhibitory activity was calculated when the absorbance at 290 nm of the control (without antioxidant) reached approximately 2.5 (2.547–2.558).