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Enhanced cell permeability of kojic acid–phenylalanine amide with metal complex

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ABSTRACT

Kojic acid–phenylalanine amide (KA–F–NH_2), which showed an excellent tyrosinase inhibitory activity, did not inhibit melanogenesis in melanocyte due to its low cell permeability. To enhance its cell permeability by increasing lipophilicity, we prepared metal coordination compounds of KA–F–NH_2 and characterized them by FT-IR and ICP analysis. The metal complex of KA–F–NH_2 inhibited mushroom tyrosinase activity as much as KA–F–NH_2 and reduced melanin contents in melanocyte efficiently.

Cellular internalization of large molecule weight and hydrophilic molecules is still a formidable challenge due to the nature of plasma membrane. Although the biological barriers are necessary to inhibit invasion of xenobiotics into cells, therapeutic agents are limited to delivery at the targeted sites due to impermeability of the barrier. In order to circumvent this problem, numerous delivery methods have been intensively developed. For example, the systems based on cationic lipids[1,2] peptide bond modification[3,4] or cell-penetrating peptides[5–11] have drawn a great deal of attention. However, there have not been any studies addressing the use of metal chelation.

Kojic acid (KA), which is a natural fungal metabolite, has been extensively studied as a cosmetic material for skin whitening and as a food additive to prevent enzymatic browning because it acts as a potent metal chelator and a scavenger of free radicals.[12,13]

In our previous study, we reported that kojic acid–tripeptide amides (KA–FWY–NH_2) demonstrated 100-fold tyrosinase inhibitory activity compared to kojic acid itself.[14] However, when KA–FWY–NH_2 was applied to cell system, they exhibited no melanogenesis inhibitory activity. Since we thought that the molecular weight was too large to penetrate the cell membrane, kojic acid-amino acid amide (KA–AA–NH_2) library was also prepared to reduce molecular weight. Kojic acid–phenylalanine amide (KA–F–NH_2) did not inhibit melanin synthetic pathway at all in cell system probably due to its poor cell-penetrating ability, although it indicated the highest tyrosinase inhibitory activity as much as KA–FWY–NH_2 by mushroom tyrosinase inhibitory test.[15] Therefore, we prepared copper or zinc coordinated compounds to increase the lipophilicity[16] of KA–F–NH_2 and evaluated its melanogenesis inhibitory activity in cell system.

First, we obtained KA–F–NH_2 (**3**) in 75% yield and 95% purity by previously reported procedure[17,18] from the Rink amide AM SURE™ resin by acid cleavage condition. Cu–[KA–F–NH_2] (**4**) and Zn–[KA–F–NH_2] (**5**) were obtained by the direct chelation method[19,20] as green copper complex (**4**) and dull white zinc complex (**5**) in 40% and 20% yield, respectively (Fig. 1).[21] The molar ratio of KA–F–NH_2 (**3**) to metals was approximately 2, which is characterized by inductively coupled plasma-atomic emission spectrometer (ICP-AES) analysis.

Although kojic acid has been known to bind weakly to metalloenzymes such as tyrosinase in vivo, the chelation with free metals proceeded relatively faster and tighter.[22] Even though the complexation between metal ion and kojic acid is reversible, its equilibrium was shifted toward the complex formation due to its solidified product.[23]

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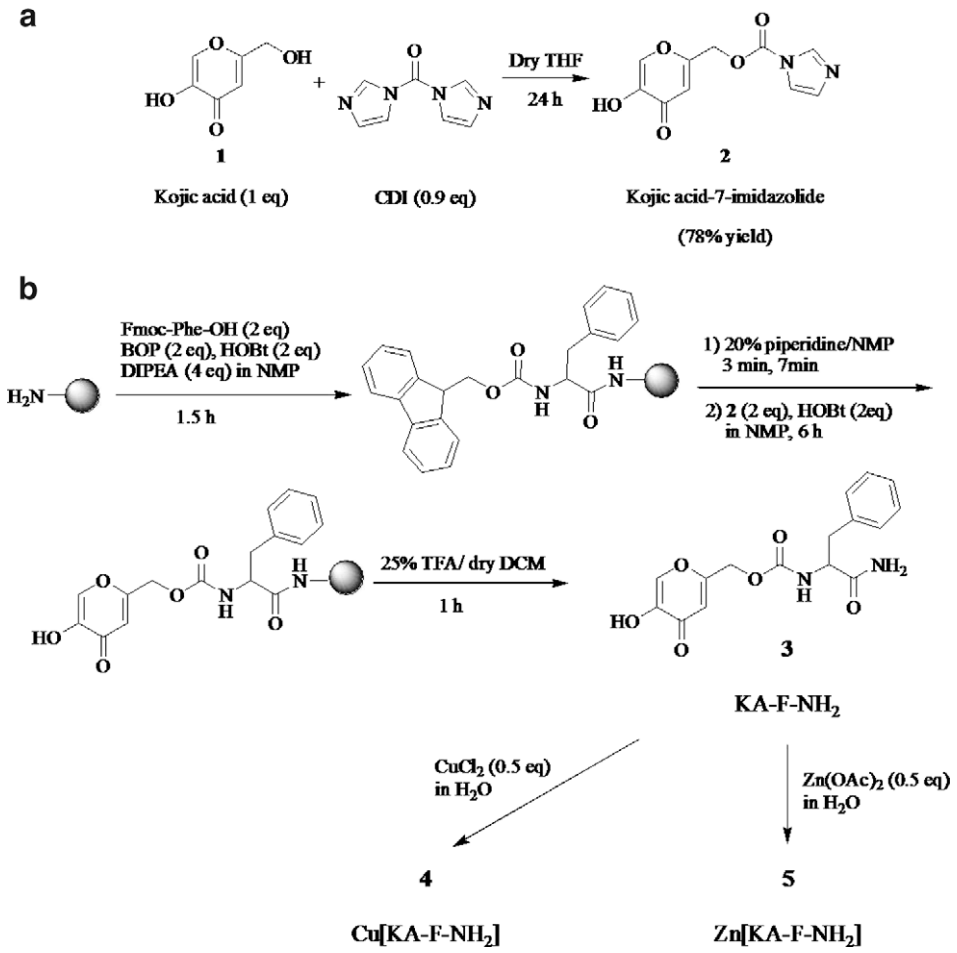

Figure 1. Synthetic scheme. (a) synthesis of kojic acid-7-imidazolide (KA-Im, **2**); (b) preparation of KA–F–NH_2 (**3**), Cu[KA–F–NH_2](**4**) and Zn[KA–F–NH_2](**5**).

The kojic acid derivatives (**2**, **3**) and metal complexes (**4**, **5**) were characterized by FT-IR analysis. When the carbonyl group participates in bonding to copper(II) or zinc(II) ion, its stretching band should be down-shifted. Thus, new bands at 1567 cm^{-1} and 1513 cm^{-1} appeared after copper coordination in the spectrum (Fig. 2(d)). The bands that appeared at 1725 cm^{-1} of carbamate bond and at 1678 cm^{-1} of amide C-terminus were unchanged, which indicated that these carbonyl groups participated very little in coordinating the metal complexation. The IR spectra of Zn–[KA–F–NH_2] (**5**) were very similar to those of Cu–[KA–F–NH_2] (**4**) (Fig. 2(e)).

To evaluate tyrosinase inhibitory activity of the metal complex (**4**, **5**), we performed a mushroom tryosinase inhibition test.[24] Like KA–F–NH_2 (**3**), this showed much higher mushroom tyrosinase inhibitory activity than KA (**1**) itself (Fig. 3). It is known that the ketone group and hydroxyl group of KA (**1**) are coordinated with the divalent copper ion at the active site of tyrosinase, and consequently the enzyme is deactivated.[25] Since Cu–O coordination are competing between dinuclear copper of tyrosinase and copper of Cu–[KA–F–NH_2] (**4**), **4** revealed slightly lower activity than KA–F–NH_2 (**3**).

Prior to the evaluation of tyrosinase inhibitory activity in cell system for metal complexes, we performed crystal violet assay[26] in order to examine the complexes' cytotoxicity. In fact, KA–F–NH_2 (**3**) and the metal complexes (**4**, **5**) were not cytotoxic by concentration of 200 μM (Table 1).

Despite the fact that KA–F–NH_2 (**3**) has more than 90% tyrosinase inhibitory activity at 100 μM, there was no reduction in melanin formation activity in cell system because its low lipophilicity did not allow penetrating the cell membrane. According to Lipinski's rule,[27] KA–FWY–NH_2 has low cell permeability because its molecular weight is 682 Dalton and there are several hydrogen bonding donors (OHs, NHs) and acceptors (Os, Ns). Although the molecular weight of KA–F–NH_2 (**3**) is only 332 Dalton, its *C* log *P* value is −0.3254, which means that KA–F–NH_2 (**3**) is too hydrophilic to permeate the cell membrane. Due to the hydrophilic nature of **3**, there was no melanin reducing activity in cell system.

In contrast, the metal complex (**4**, **5**) reduced melanin levels in Mel-Ab cell (Fig. 4).[28] This result demonstrates that metal complex of KA–F–NH_2 (**3**), whose *C* log *P* values of **4** and **5** are 1.2536 and 1.4536, respectively, is able to penetrate cell membrane by decreasing its hydrophilicity by metal chelation. This process blocks hydrogen bonding donors and acceptors of KA–F–NH_2 (**3**). When the metal complexes penetrated the cell membrane and reached the inside of melanocyte, coordinated metal ions would be released and the resulting free KA–F–NH_2 (**3**) would bind competitively to the dinuclear copper in tyrosinase.

Cu–[KA–F–NH_2] (**4**) exhibited its superior activity in cell system as well as during the in vitro tyrosinase inhibition test. However, Zn–[KA–F–NH_2] (**5**) showed relatively weak depigmenting activity in cell system compared to that of enzyme assay. This is probably due to weaker Zn–O coordination ability than Cu–O coordination,

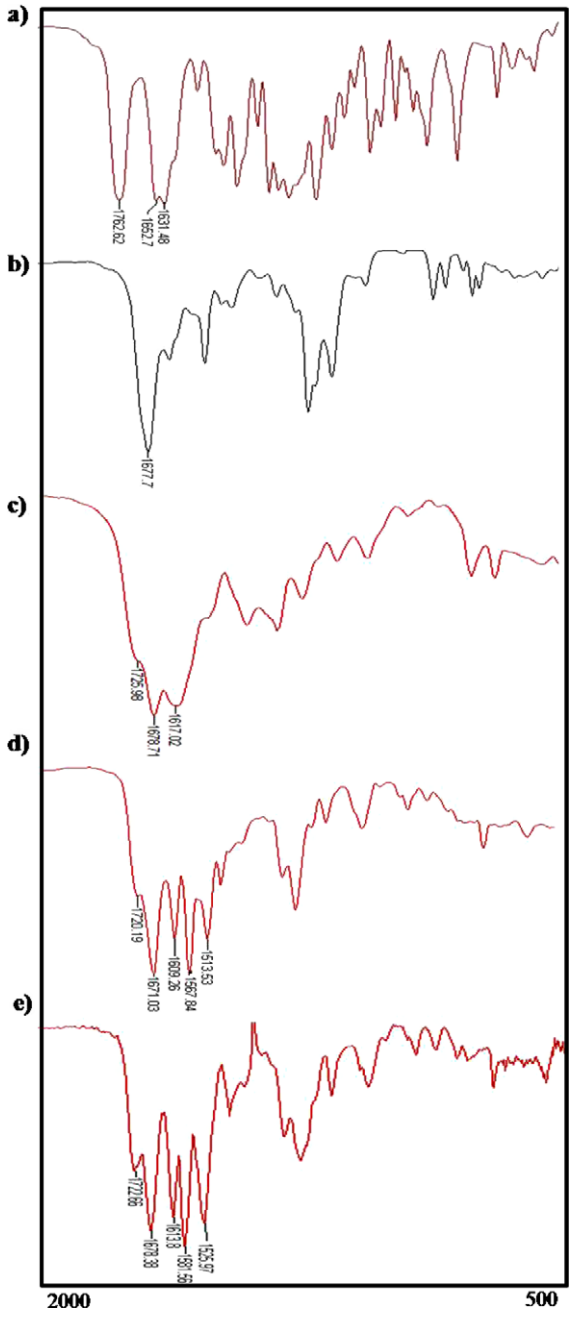

Figure 2. FT-IR spectra. (a) KA-Im (**2**); (b) phenylalanine amide; (c) KA–F–NH_2 (**3**); (d) Cu–[KA–F–NH_2] (**4**); (e) Zn–[KA–F–NH_2] (**5**).

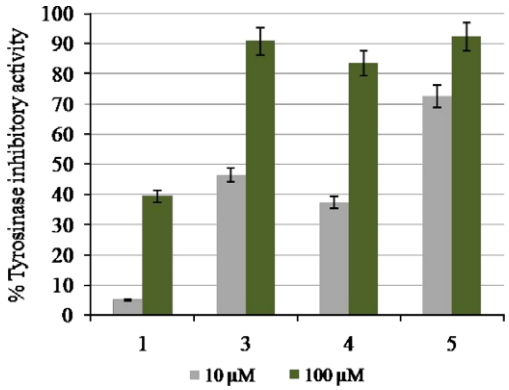

Figure 3. Mushroom tyrosinase inhibitory activity; % Inhibitory activity was measured after 10 μM (grey solid bars) or 100 μM (green solid bars) of each sample, respectively. Each experiment was performed in triplicate and averaged.

Table 1
Cell viability in Mel-Ab cell[a]

Concentration (μM)	KA–F–NH_2 (**3**)	Cu–[KA–F–NH_2] (**4**)	Zn–[KA–F–NH_2] (**5**)
1	96.6 (±3.4)	97.7 (±7.6)	106.0 (±0.9)
10	90.1 (±9.3)	103.6 (±11.1)	108.3 (±1.6)
50	105.3 (±1.4)	112.6 (±8.3)	108.3 (±1.8)
100	103.4 (±4.4)	104.8 (±7.9)	114.0 (±1.5)
200	102.4 (±7.3)	117.7 (±6.3)	113.3 (±5.5)

[a] Percent (%) of control (±S.D.).

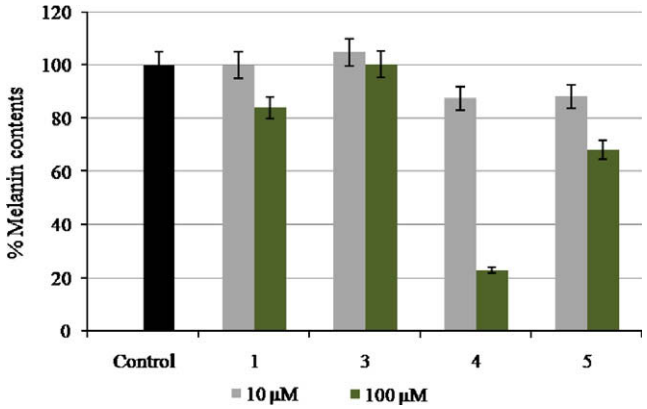

Figure 4. Melanogenesis inhibitory activity in Mel-Ab cell; % Melanin contents were measured after 10 μM (grey solid bars) or 100 μM (green solid bars) of each sample, respectively. Black solid bar means the result of control sample without any inhibitor. Each experiment was performed in triplicate and averaged.

and Zn^{2+} ion is easily released before penetrating cell membrane when Zn–[KA–F–NH_2] (**5**) is treated on the cell. The general order of metal complexes' stability with oxygen is known as following; Hg > Pd > Cu^{2+}/Fe^{3+} > Ni>Co > *Zn* > Cd > Fe^{2+} > Mn > Mg > Ca.[23] This proves that Cu–[KA–F–NH_2] (**4**) has better inhibitory activity in cell system than Zn–[KA–F–NH_2] (**5**).

In conclusion, the metal complexes of KA–F–NH_2 exhibited potent tyrosinase inhibitory activity both in vitro enzyme test and in cell-based assay system. These results demonstrated that metal complex formation could be applied as a delivery system for hydrophilic molecules which have low cell permeability into cells. In addition, these new materials can be used as an effective whitening agent in the cosmetic industry or applied on irregular hyperpigmentation. Further investigations on various metal complexes of kojic acid–amino acid derivatives are underway, and the results will be reported elsewhere.

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17. *Synthesis of KA-Im* (**2**); Kojic acid (5 g, 35 mmol) was dissolved in dry THF (100 ml), and then the solution of CDI (5.1 g, 31 mmol) in dry THF (50 ml) was added by dropping. After 24 h at rt, white precipitant KA-Im (**2**) was obtained by filtration in 78% yield. Its structure was identified by NMR; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.34 (1H, s, –OH), 8.33 (1H, s, N–CH═N), 8.13 (1H, s, –CH–O), 7.67 (1H, s, imidazole), 7.10 (1H, s, imidazole), 6.66 (1H, s, CH–C═O), 5.30 (2H, s, CH_2–O).
18. *Solid-phase synthesis of KA–F–NH$_2$* (**3**); Firstly, the Fmoc-Rink amide linker was coupled to the core-shell type of AM SURE™ resin (BeadTech Inc. Korea) with HBTU (2.0 equiv), HOBt (2.0 equiv) and DIPEA (4.0 equiv) in NMP at rt for 3 h. The Rink amide AM SURE™ resin was filtered and washed with NMP and DCM, and then dried in vacuo. The substitution level of the linker was 0.67 mmol/g determined by Fmoc titration. After removing the Fmoc group of Rink amide linker with 20% piperidine in NMP (v/v), *N*-Fmoc-phenylalanine (2.0 equiv) was introduced to the resin with BOP (2.0 equiv), HOBt (2.0 equiv) and DIPEA (4.0 equiv) in NMP. Fmoc group was deprotected, and then KA-Im (**2**, 2.0 equiv) was treated in NMP with HOBt (2.0 equiv) as a coupling additive at rt for 6 h. Each coupling and deprotection step was monitored by the ninhydrin color test. Finally, the resin was treated with 25% TFA in dry DCM (v/v) at rt for 1 h. The crude KA–F–NH_2 (**3**) in the filtrate was concentrated under low pressure, and precipitated with cold diethyl either. The white powder was further washed with diethyl ether and dried in vacuo.
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21. *Preparation of metal complexes* (**4**, **5**); KA–F–NH_2 (**3**, 0.2 mmol) was dissolved in distilled water with stirring and gentle heating. $CuCl_2$ or $Zn(OAc)_2$ (0.1 mmol) dissolved in distilled water was added into the above solution of KA–F–NH_2 (**3**) by dropwise. Then, its pH was adjusted to 6.0–7.0 with aq NaOH solution (0.25 N). The metal complexes (**4**, **5**) were completely formed at rt within 3 h. The final products were collected by filtration and washed intensively with distilled water to remove salts.
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24. *Mushroom tyrosinase inhibition assay*: Seventy microliters of phosphate buffer (pH 6.8, 0.1 M) containing inhibitor dissolved in DMSO (10 μl) were mixed with 10 μl of 100 μg/ml mushroom tyrosinase, and then 10 μl of 10 mM L-DOPA were added. Following incubation at 37 °C, absorbance was measured every 10 min for at least 1 h at 475 nm. The % inhibitory activity was calculated by the following formula; $(A - B)/A \times 100$ (A: absorbance of reference solution; B: absorbance of test sample solution). Each experiment was performed in triplicate and averaged.
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26. *Crystal violet assay*: cell viability was determined using a crystal violet assay (Dooley et al. 1994), After incubation with the samples for 24 h, culture media were removed and replaced with 0.1% crystal violet in 10% ethanol. Mel-Ab cells were then stained for 5 min at room temperature and rinsed four times. The crystal violet retained by adherent cells was then extracted with 95% ethanol, and absorbance was determined at 590 nm using an ELISA reader (TECAN, Salzburg, Austria). Each experiment was performed in triplicate and averaged.
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28. *Melanogenesis inhibition assay in Mel-Ab cell*: Mel-Ab cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) treated with 10% fetal bovine serum (FBS), 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA), 1 nM cholera toxin (CT), 50 μg/ml streptomycin, and 50 U/ml penicillin at 37 °C in 5% CO_2. Effects of the inhibitors on melanin formation in Mel-Ab cell line were estimated. When the samples were treated at the concentrations of 10 μM and 100 μM for four days, the inhibitors-treated cells produced much less amount of melanin than the cells without inhibitors. The melanin contents of Mel-Ab cells were measured spectrophotometrically in the experimental period. Each experiment was performed in triplicate and averaged.