DOI 10.5012/bkcs.2010.31.5.1377

Determination of a Binding Site of Cu, Ni, Mg, and Ca Metal Ions with Angiotensin II Peptide by Electrospray Tandem Mass Spectrometry

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Received February 3, 2010, Accepted February 26, 2010

Key Words: Angiotensin II, Metal^{2+} ion, Metal^{II}-Angiotensin II complex, Mass spectrometry (MS), MS/MS

Angiotensin II (Ang II) is the main active hormone in the renin-angiotensin blood pressure regulation system.[1] Ang II is an octapeptide hormone (Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8, DRVYIHPF) that has been studied for several decades in the structure-dependent activity of the biological pathway of the Ang II receptor.[2-13] Free Ang II molecular structures in solutions have been investigated with a variety of techniques. The results have been reported as β-turn, random coil, hair-pin and other structures.[2-8] The structures of Ang II complexed with a receptor have been reported as a hair-pin or an extended structure in NMR and crystallography studies.[9-13] It is evident that several reports are not consistent with each other and that no clear agreement exists regarding the biologically active structure of Ang II during the biological process.

For the study of blood pressure control and metal-peptide interaction, the influences of metal cations on the biological activity of Ang II have been also investigated.[14-23] The biological activities of Ang II are enhanced by Li^+, Na^+, Mg^{2+}, and Ca^{2+}. It has been suggested that the cations induce conformational changes in Ang II which lead to a more active conformation.[14] As a ligand, Ang II has two strong nitrogen donor centers (the N-terminus NH_2 group and the imidazole nitrogens (N_{im}) of the histidine side chain) that are able to strengthen the interaction between Cu^{2+} (or Ni^{2+}) and the Ang II molecule. The complexes of [Cu^{II} ---(Ang II-2H^+)] and [Ni^{II} ---(Ang II-2H^+)] were both regarded to form 4 N coordination structures at pH 8 ~ 11.[22-24] Transition metal ions are also known to interact weakly with peptide carbonyl oxygens at pH values where the peptide bonds are not deprotonated.[21,25]

The present paper focuses on the binding sites between the metal ions (Cu^{2+}, Ni^{2+}, Mg^{2+}, and Ca^{2+}) and the Ang II molecule to explain the biological effect in terms of a gas-phase structural difference. We present the results of a tandem mass spectrometry and multiple mass spectrometry study regarding the interaction of metal ions with the Ang II molecule, the C-terminus amidated Ang II molecule (DRVYIHPF-NH_2), and two of its segments: the N-terminus tetrapeptide Asp1- Arg2-Val3-Tyr4-NH_2 (DRVY-NH_2) and the N-terminus hexapeptide Asp1-Arg2-Val3-Tyr4-Ile5-His6-NH_2 (DRVYIH-NH_2). Two N-terminus segments are used to confirm the [metal^{II} + Ang II]$^{2+}$ complex formation efficiency and the metal binding site.

Experimental Section

The gas-phase [metal^{II} + Ang II]$^{2+}$ cation complexes were produced by an electrospray ionization source. The experimental MS/MS and MS/MS/MS spectra for fragmentation pattern analysis were obtained using a Thermo Finnigan LTQ mass spectrometer (Thermo Electron Corp., San Jose, CA, USA). This mass spectrometer is a linear ion trap mass spectrometer equipped with an atmospheric pressure-ionization source.

LTQ conditions. The samples were introduced into the electrospray interface by a direct infusion method using a microsyringe pump (SGE, Australia) at a flow rate of 5 μL min^{-1}. The MS/MS spectra were acquired with experimental conditions of an isolation width of 0.5 - 1 mass unit, an activation time of 30 msec and q_z = 0.25. In MS/MS or MS/MS/MS mode, the parent ion molecules were manually selected one by one, and each was subjected to collision-induced dissociation (CID).

Reagents. Ang II human (96%, Fluka), DRVYIHPF-NH_2 (> 90%, Peptron, Daejeon, Korea), DRVY-NH_2 (> 90%, Peptron, Daejeon, Korea), DRVYIH-NH_2 (> 90%, Peptron, Daejeon, Korea), $CuCl_2{\cdot}2H_2O$ (99%, Sigma-Aldrich Korea), $Ni(NO_5)_2{\cdot}6H_2O$ (97%, Junsei chemical Co., Tokyo, Japan), $CaCl_2{\cdot}2H_2O$ (98%, Daejung Chemical Korea), $Mg(NO_3)_2{\cdot}6H_2O$ (98%, Junsei chemical Co., Tokyo, Japan), and H_2O (HPLC grade, Merck) were used in experiments. Ang II was dissolved in water to prepare a 2×10^{-5} M solution. The three metal (Ni^{2+}, Mg^{2+}, and Ca^{2+}) solutions were prepared at a final concentration of 4×10^{-5} M in water. These two solutions were mixed together prior to obtaining the mass spectra. Cu^{2+} solution was prepared at a final concentration of 4×10^{-4} M in water.

Results and Discussion

The two-dimensional S-shape (with two turns) structural feature of Ang II was reported in a NMR experiment.[13] Each turn could be an adequate binding site for metal ions. [metal^{II} + Ang II]$^{2+}$ complexes are dominantly observed in the positive-mode ESI-MS spectrum in Figure 1. In the formation of [metal^{II} + Ang II]$^{2+}$ complex, the neutral structure of Ang II molecule is supposed to be a more favorable form compared to the deprotonated form (Ang II − H^+) of the Ang II molecule in an aqueous solution (The intensity of [metal^{II} + (Ang II − H^+)]$^{1+}$ complex is negligible in Figure 1). The metal binding sites of the [metal^{II} + Ang II] complex were analyzed as the nitrogen atom coordination of the peptide bond in potentiometric experiments.[22-24] In contrast to the potentiometric experimental results, the metal binding sites of the [metal^{II} + peptide]$^{2+}$ complex were proposed as the oxygen atom coordination of the

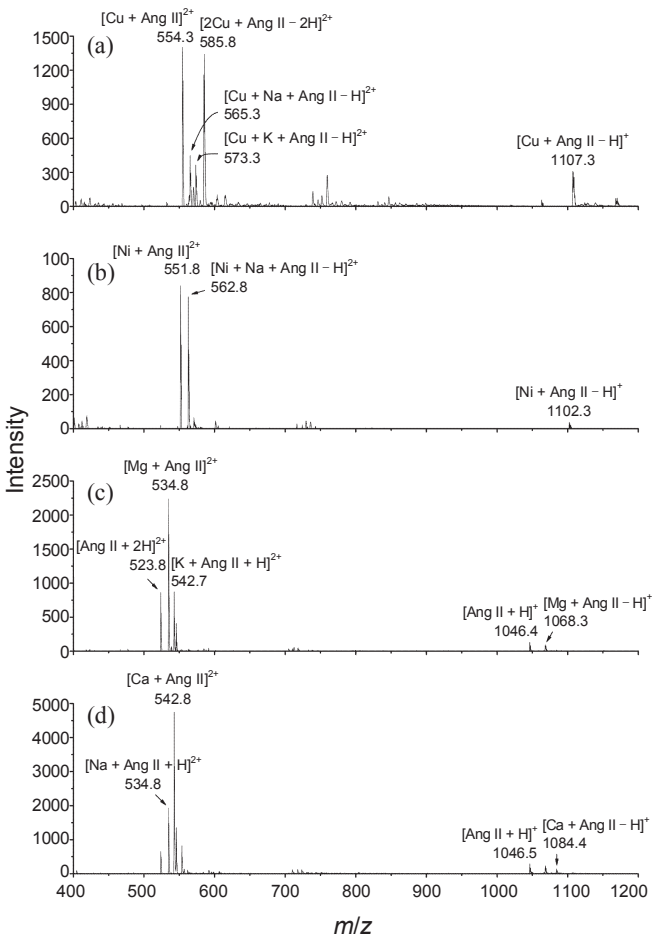

Figure 1. MS spectra in positive mode: (a) Cu^{2+} ion + angiotensin II, (b) Ni^{2+} ion + angiotensin II, (c) Mg^{2+} ion + angiotensin II, and (d) Ca^{2+} ion + angiotensin II.

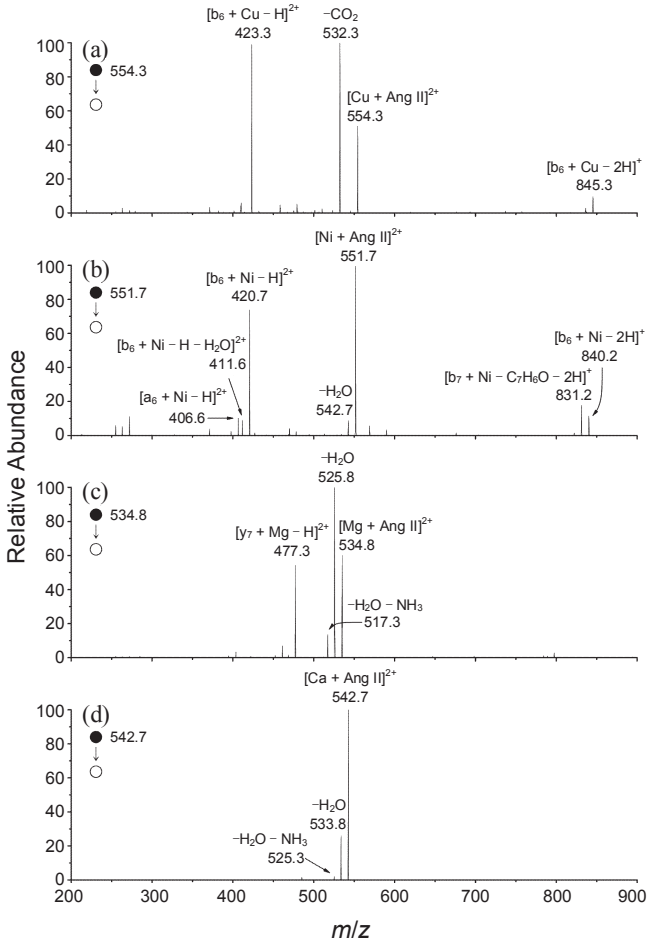

Figure 2. MS/MS spectra of (a) $[Cu^{II} + Ang\ II]^{2+}$, (b) $[Ni^{II} + Ang\ II]^{2+}$, (c) $[Mg^{II} + Ang\ II]^{2+}$, and (d) $[Ca^{II} + Ang\ II]^{2+}$ parent ions.

peptide bond of the neutral peptide molecule according to mass spectrometry and molecular dynamics simulation results.[21,25]

In Figure 2, the $[b_6 + Cu, Ni - H]^{2+}$ fragment and $[y_7 + Mg + H]^{2+}$ fragment ions were observed as the typical fragment ions in the MS/MS spectra of the parent $[Cu^{II}, Ni^{II} + Ang\ II]^{2+}$ and $[Mg^{II} + Ang\ II]^{2+}$ ions, respectively.[8] The y_7 and b_6 ions were the main fragments in the MS/MS spectra of the parent $[Ang\ II + H]^+$ ions due to the five-membered ring cleavage of the Asp1 residue[8,26-27] and the His6-containing cleavage,[28] respectively. The single observation of the $[b_6 + Cu, Ni - H]^{2+}$ (except $[y_7 + Cu, Ni + H]^{2+}$ fragment ion) in Figure 2a and 2b might indicate that the approximate metal (Cu^{2+} and Ni^{2+}) binding sites contain the Asp1 residue. Correspondingly, the dissociation process (for the observation of $[y_7 + Cu, Ni + H]^{2+}$ fragment ion) originated from the Asp1 five-membered ring cleavage is not supposed to be activated in the MS/MS spectra of the parent $[Cu^{II}, Ni^{II} + Ang\ II]^{2+}$ ions.

Two extra fragmentation channels, $[Cu^{II} + Ang\ II - CO_2]^{2+}$ and $[Ca^{II}, Mg^{II} + Ang\ II - H_2O]^{2+}$ ions, were also observed in the MS/MS spectra of the parent $[Cu^{II}, Ca^{II}, Mg^{II} + Ang\ II]^{2+}$ ion. In the MS/MS spectra of the parent $[Ca^{II} + Ang\ II]^{2+}$ ion (Figure 2d), no observation of the y_7- or b_6-related fragment ions suggest that the dissociation processes originated from the Asp1 five-membered ring cleavage and the His6-containing cleavage are not activated due to the Ang II binding sites of the Ca^{2+} ion. The Ang II binding sites of the Ca^{2+} ion were reported to be two carboxyl groups (Asp1 and Phe8) and two peptide carbonyl oxygens (Arg2 and His6) in the NMR experiment.[15]

In the MS/MS/MS spectra of the $[b_6 + Cu - H]^{2+}$ and $[b_6 + Ni - H]^{2+}$ ions (Figure 3a and 3b), the observation of the $[a_5 + Cu - H]^{2+}$ fragment and the $[b_4 + Ni - 2H]^+$ fragment ion suggests that the Asp1-Arg2-Val3-Tyr4 sequence of Ang II could be assigned as a main binding site of Cu^{2+} and Ni^{2+} ions, respectively, in the $[Cu^{II}, Ni^{II} + Ang\ II]^{2+}$ gas-phase complex. In the case of the $[y_7 + Mg + H]^{2+}$ ion (Figure 3c), the observation of the $[y_5 + Mg]^+$ fragment and the $[y_6 + Mg]^+$ fragment ions suggests that the Ile5-His6-Pro7-Phe8 sequence of Ang II is a main binding site of the Mg^{2+} ions in the $[Mg^{II} + Ang\ II]^{2+}$ gas-phase complex. In Figure 3d, NH_3 loss (−17 amu) and $[Ca^{II} + Ang\ II - H_2O - 60\ ]^{2+}$ fragment ions are observed at the MS/MS/MS spectrum of $[Ca^{II} + Ang\ II - H_2O]^{2+}$. A loss of 60 amu is regarded as a combinational (NH_3 + side alkyl group) loss like as $[NH_3 + C(NH_2)NH]$ or $[NH_3 + CH(CH_3)_2\ ]$. Two main fragment ions (−17 and −60 amu) are also observed in the MS/MS/MS spectrum (Figure 3e) of $[Ca^{II} + Ang\ II - H_2O - NH_3\ ]^{2+}$ ion. So, it is difficult to analyze the Ca^{2+} ion binding sites of

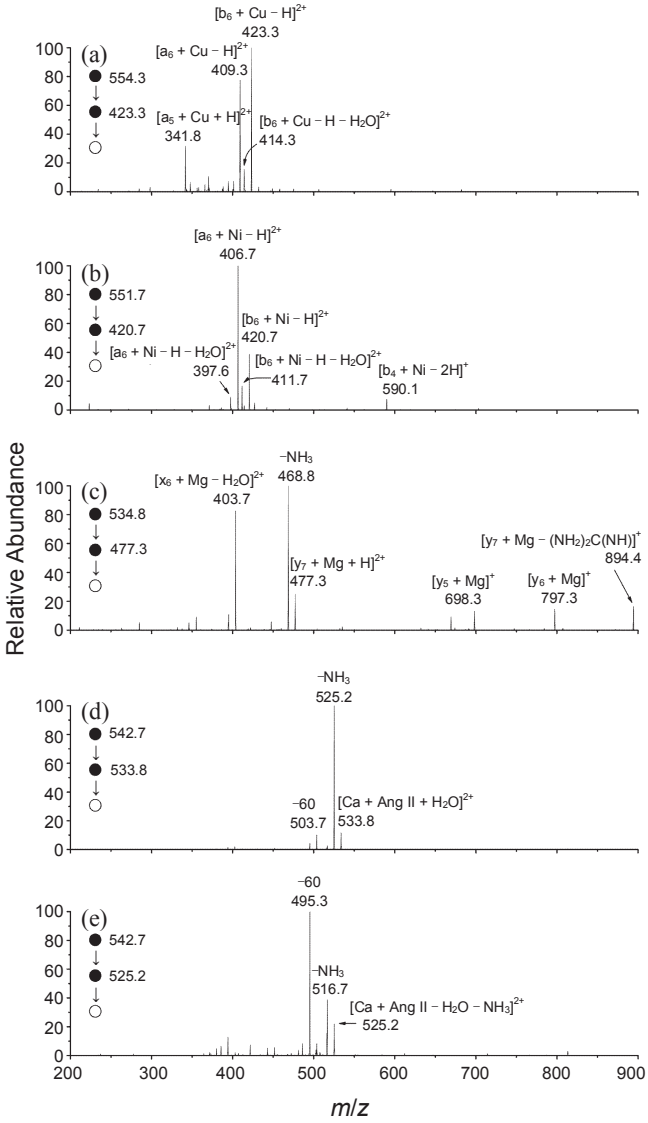

Figure 3. MS/MS/MS spectra of (a) $[b_6 + Cu - H]^{2+}$, (b) $[b_6 + Ni - H]^{2+}$, (c) $[y_7 + Mg + H]^{2+}$, (d) $[Ca^{II} + Ang\ II - H_2O]^{2+}$, and (e) $[Ca^{II} + Ang\ II - H_2O - NH_3]^{2+}$ ions.

Ang II due to the dissociation channels related to H_2O and NH_3 loss instead of the peptide backbone dissociation channel in the MS/MS and MS/MS/MS spectra.

The MS/MS spectrum of $[Cu^{II} + DRVYIHPF\text{-}NH_2]^{2+}$ is observed to investigate the CO_2 dissociation process. Figure 4a spectrum is identical to that in Figure 2a except for the −44 amu (CO_2 loss) fragment ion. C-terminus amidation blocking prominently decreases the −44 amu fragmentation channel. Therefore, it is concluded that the fragmentation channel of CO_2 loss arises from the C-terminus carboxyl group instead of from the Asp1 side chain carboxyl group, which involves intramolecular hydrogen bonding with the Arg2 backbone peptide bond.[8,26-27] The analogous MS/MS fragmentation patterns of $[Ni^{II}, Mg^{II}, Ca^{II} + DRVYIHPF\text{-}NH_2]^{2+}$ (Figure 4b, 4c, and 4d) by comparing MS/MS spectra of $[Ni^{II}, Mg^{II}, Ca^{II} + Ang\ II]^{2+}$ ions (Figure 2b, 2c, and 2d) suggest that the C-terminus ami-

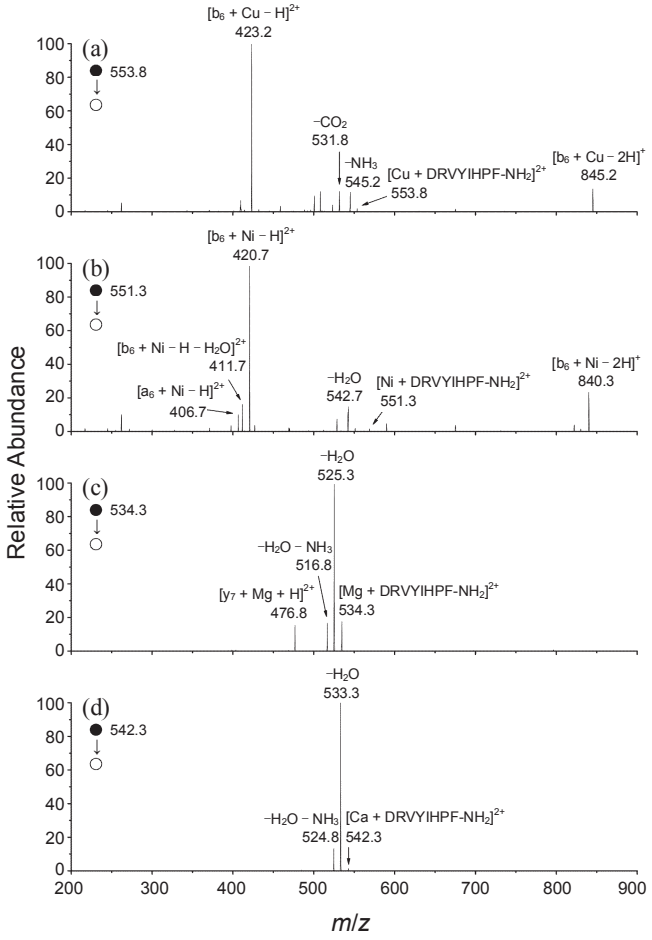

Figure 4. MS/MS spectra of (a) $[Cu^{II} + DRVYIHPF\text{-}NH_2]^{2+}$, (b) $[Ni^{II} + DRVYIHPF\text{-}NH_2]^{2+}$, (c) $[Mg^{II} + DRVYIHPF\text{-}NH_2]^{2+}$, and (d) $[Ca^{II} + DRVYIHPF\text{-}NH_2]^{2+}$ parent ions.

dation blocking does not make any influence in the binding site propensities of Ni^{2+}, Mg^{2+}, and Ca^{2+} ions regarding the formation of $[metal^{II} + Ang\ II]^{2+}$ complexes.

Positive-mode ESI-MS spectra of DRVY-NH_2 and DRVYIH-NH_2 complexed by Cu^{2+} or Ni^{2+} ions are shown in Figure 5a and 5b. The metal (Cu^{2+} or Ni^{2+}) complex formation efficiency (which is observed as an $[Cu^{II}, Ni^{II} + DRVY\text{-}NH_2]^{2+}$ ion intensity) of peptide segment DRVY-NH_2 is observed to be similar to that of peptide segment DRVYIH-NH_2. The MS/MS spectra (Figure 5c and 5d) of the parent $[Cu^{II}, Ni^{II} + DRVYIH\text{-}NH_2]^{2+}$ ions show that the Cu^{2+} and Ni^{2+} binding sites of Ang II is identical to that of $[Cu^{II}, Ni^{II} + DRVYIHPF\text{-}NH_2]^{2+}$ (Figure 4a and 4b). Therefore, it is supposed that the N-terminus vicinity of Ang II is the better binding site in the competition between the N-terminus vicinity and the His6 vicinity of Ang II to anchor Cu^{2+} or Ni^{2+} metal ion because the metal ion (Cu^{2+} or Ni^{2+}) are regarded to interact with peptide carbonyl oxygens instead of peptide nitrogens at neutral pH (where the peptide bonds are not deprotonated).

As a concluding remark, the structures of $[Cu^{II}, Ni^{II} + Ang\ II]^{2+}$ complex were investigated by comparing those of $[Ca^{II}, Mg^{II} + Ang\ II]^{2+}$ complex in the gas-phase CID mass spectra. The $[b_6 + Cu, Ni - H]^{2+}$, $[a_5 + Cu + H]^{+}$ and $[b_4 + Ni - 2H]^{+}$

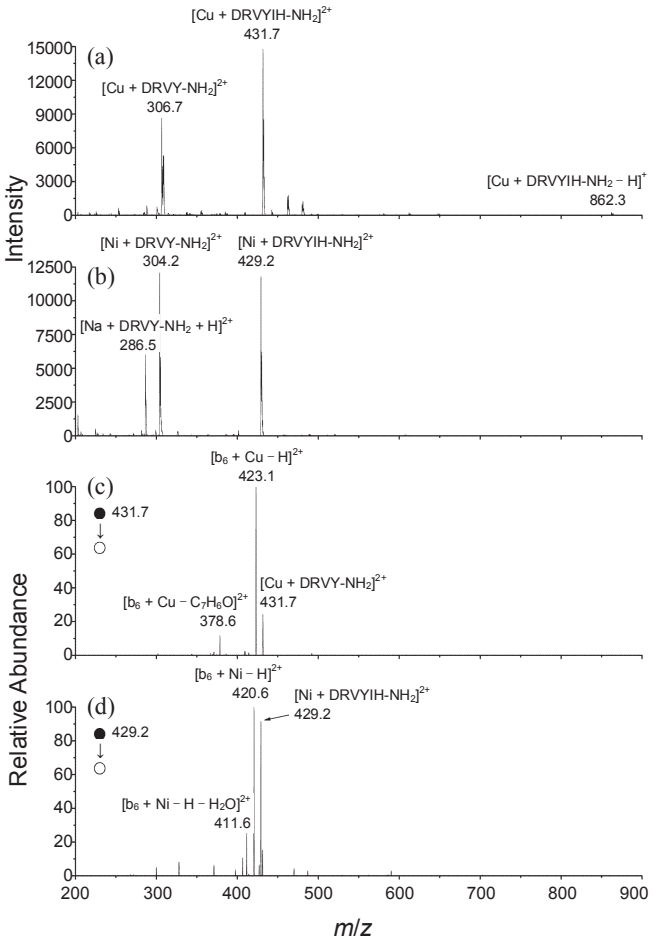

Figure 5. (a) MS spectrum of (Cu^{2+} ion + DRVY-NH_2 + DRVYIH-NH_2) in positive mode, (b) MS spectrum of (Ni^{2+} ion + DRVY-NH_2 + DRVYIH-NH_2) in positive mode, (c) MS/MS spectrum of [Cu^{II} + DRVYIH-NH_2]$^{2+}$ parent ion, and (d) MS/MS spectrum of [Ni^{II} + DRVYIH-NH_2]$^{2+}$ parent ion.

Table 1. The proposed metal binding site in the [metal^{II} + Ang II]$^{2+}$ complexes

Metal ion	Proposed binding site of Ang II (O: peptide carbonyl oxygen atom)
Cu^{2+}, Ni^{2+}	$O_{Asp1}O_{Arg2}O_{val3}O_{Tyr4}$
Mg^{2+}	$O_{Ile5}O_{His6}O_{Pro7}O_{Phe8}$
Ca^{2+}	N/A

fragment ions were respectively observed as significant fragment ions in [Cu^{II}, Ni^{II} + Ang II]$^{2+}$ MS/MS and MS/MS/MS spectra. Correspondingly, the Asp1-Arg2-Val3-Tyr4 carbonyl oxygens are suggested to be a main binding site of Cu^{2+} and Ni^{2+} ions in the [Cu^{II}, Ni^{II} + Ang II]$^{2+}$ gas-phase complex. The suggested binding sites for Cu^{2+} or Ni^{2+} ions in [Cu^{II}, Ni^{II} + Ang II]$^{2+}$ are completely different from the binding sites for Ca^{2+} or Mg^{2+} ions in the [Ca^{II}, Mg^{II} + Ang II]$^{2+}$ complex ions (Table 1). Therefore, it is concluded that the Ang II biological activity would not be enhanced by the addition of Cu^{2+} or Ni^{2+} ions.

Acknowledgments. This paper was supported by Research Fund, Kumoh National Institute of Technology.

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