ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2013, *58*(6), 501-509

COPPER BINDING TO SG AND EE PEPTIDES AS A FUNCTION OF pH. IMPLICATION FOR UNDERSTANDING AMYLOIDOGENESIS

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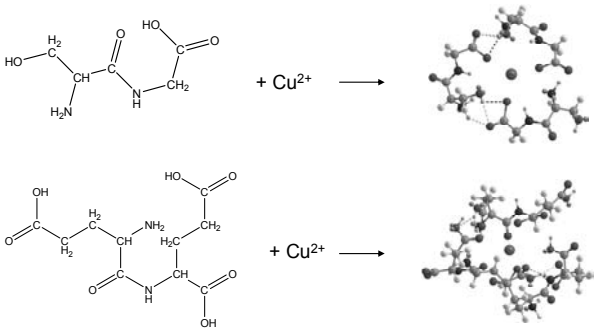
Received December 17, 2012

Abnormal metal binding to peptides is generally associated with (neuro)degenerative diseases. In these pathologies, copper ions are bound to specific sites of amyloid-β peptides (Aβ), especially to histidine residues, and induce conformational changes. Since the mechanism of metal binding to Aβ peptides under various pH conditions is still not well understood, the role of some non-histidine peptide fragments should also be investigated. Therefore, we investigated here serylglycine (SG) and glutamylglutamate (EE). Copper binding to peptides was followed by UV-Vis spectroscopy, and infrared spectroscopy. The spectral changes of supernatants containing copper-peptide complexes in the range from slightly (pH 6.0) to alkaline (pH 8.5) pH have been interpreted in terms of the equilibrium among free and complexed forms of dipeptides. The infrared spectra also confirmed pH-dependent copper binding to various chemical groups of peptide molecules. Our findings revealed that not only the histidine residues in Aβ peptides are involved in metal binding, some non-histidine peptide fragments may play an important role in copper binding via their peptide bonds.



INTRODUCTION

Copper plays an important role in the biological processes including signaling to the transcription and protein trafficking machinery, oxidative phosphorylation, iron mobilization, neuropeptide maturation, and normal development.[1,2] In fact, copper is both an essential micronutrient and a major toxicant to living cells due to its potential inhibitory effects against many physiological and biochemical processes.[3,4] Elucidating copper ion interaction with amyloid-β peptides (Aβ) is also paramount for understanding the role of Cu(II) in the pathology of AD (Alzheimer's Disease).[5] Copper, iron and aluminum are all implicated in the etiology of neurodegenerative diseases, including AD.[6,7] Several experiments with Aβ1-16 and Aβ1-28 showed that other sites than the Cu(II)-binding histidine residues may also be important for Cu(II)-induced Aβ aggregation.[8] It was shown that Cu^{2+} can change the local conformation of peptides and proteins, and the

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abnormal conformations are associated with the so-called conformational diseases, such as AD and Parkinson's disease.[9] Nevertheless, little information is available on how eukaryotic cells mobilize intracellular copper stores.[1] Brain copper, zinc, and particularly iron overload is widely accepted features of AD.[10] However, little is known about deposits formation and the role played by various amino acids in Aβ sequence.[11] The different coordination chemistry of Cu(II) compared with Zn(II) may explain the metal-specific effect on aggregation and the difference between Aβ fragment peptides.[12] The interaction between metal ions and peptides has been studied for decades in solution using spectroscopic or electrochemical methods such as circular dichroism, NMR spectroscopy, ESR or cyclic voltammetry, and more recently using mass spectrometry to understand the role of metal captions in the biochemistry of proteins.[13-16] The advantages offered by the MS methods in combination with other analytical approaches to detect and characterize Aβ were also highlighted.[17] The aim of this study was to investigate copper ion mobilization by small peptide fragments without histidine residues. The SG and EE peptide fragments might provide new insights in the extracellular aggregation mechanism of amyloid-β peptides related to neurodegenerative pathologies. Consequently, pH-dependent copper binding and mobilization by these peptides have been followed by UV-*Vis* and infrared absorption spectroscopy. Although infrared and visible spectroscopic techniques are rather common, the measurements at various pH values and peptide concentrations brought a multitude of novel information on copper binding to peptides.

RESULTS AND DISCUSSION

MS spectrometry. MS spectra confirmed the structure of SG and EE peptides used in our experiments. SG showed characteristic signals at m/z 163 ($[M+H]^+$), m/z 145 ($[M+H-H_2O]^+$), and m/z 60 ($[HOCH_2–HC^+–NH_2]$), respectively (Fig. 1). All the small signals in the spectrum were interpreted in term of SG structure. For example, the signal at m/z 88 was assigned to $[HOCH_2–HC(NH_2)CO^+]$, whereas the signal at m/z 99 was assigned to a cyclic fragment (Fig. 2a), whereas that at m/z 114 to another one (Fig. 2b). EE also showed characteristic signals. In this case the molecule is loosing water to afford, for example m/z 259 ($[M+H-H_2O]^+$), m/z 241 ($[M+H-2H_2O]^+$), and m/z 223 ($[M+H-3H_2O]^+$), respectively.

The signal at m/z 130 was assigned to $[HOOC-(CH_2)_2–HC(NH_2)CO^+]$ fragment ion, and m/z 112 and m/z 84 were attributed to fragment ions showed in Fig. 2c and Fig. 2d, respectively.

UV-Vis spectroscopy. Even at low pH (6.0), SG peptide bound copper ions from insoluble copper phosphate to form a complex with a maximum absorbance, A = 0.208, at λ_{655}, where A is the optical absorbance and λ_{655}, the wavelength at 655 nm (Fig. 3). With more free carboxyl groups than SG, the copper-EE complex displayed a lower absorbance (A = 0.108), whereas the maximum was blue shifted (λ_{640}). It is worthwhile to note that under our experimental conditions (pH and concentration) the investigated peptides displayed no measurable absorbance in the visible range of UV-*Vis* spectrum. These data indicate the role of COOH groups in copper binding to peptides, since the amino group may have the same binding ability in case of both peptides. Under rather neutral conditions (pH 7.4), the concentrations of copper-peptide complexes increased much, as shown in Fig. 4. Once again, the absorbance of Cu(II)-SG complex formed by mobilizing copper from insoluble phosphate was more intense at λ_{657} (A = 0.723) than that of Cu(II)-EE complex (A = 0.417), which had a maximum at λ_{646}. Thus, both maxima are slightly shifted to blue wavelengths. These data suggest how important the role of pH in copper binding to various peptides is. In moderate alkaline environment (pH 8.5), both 10 μM peptides mobilized the copper ions to form complexes with the same intensity of absorbance (Fig. 5). The peaks (A = 1.01) displayed different maxima (λ_{642} nm for Cu(II)-EE, and λ_{653} nm for Cu(II)-SG).

Infrared spectroscopy. The infrared spectra also confirmed copper binding to various chemical groups in the peptide molecules as a pH function (Figs. 6 and 7). FT-IR spectra of SG peptide showed characteristic peaks (Fig. 6), at 1618 cm^{-1}, where the presence of peptide aggregates may appear (Amide I), and at 1543-1595 cm^{-1} (Amide II). Large changes in FT-IR spectra on increasing pH from acidic (pH 6.0) to alkaline (pH 8.5) were interpreted in terms of increasing concentrations of dipeptide complexes.

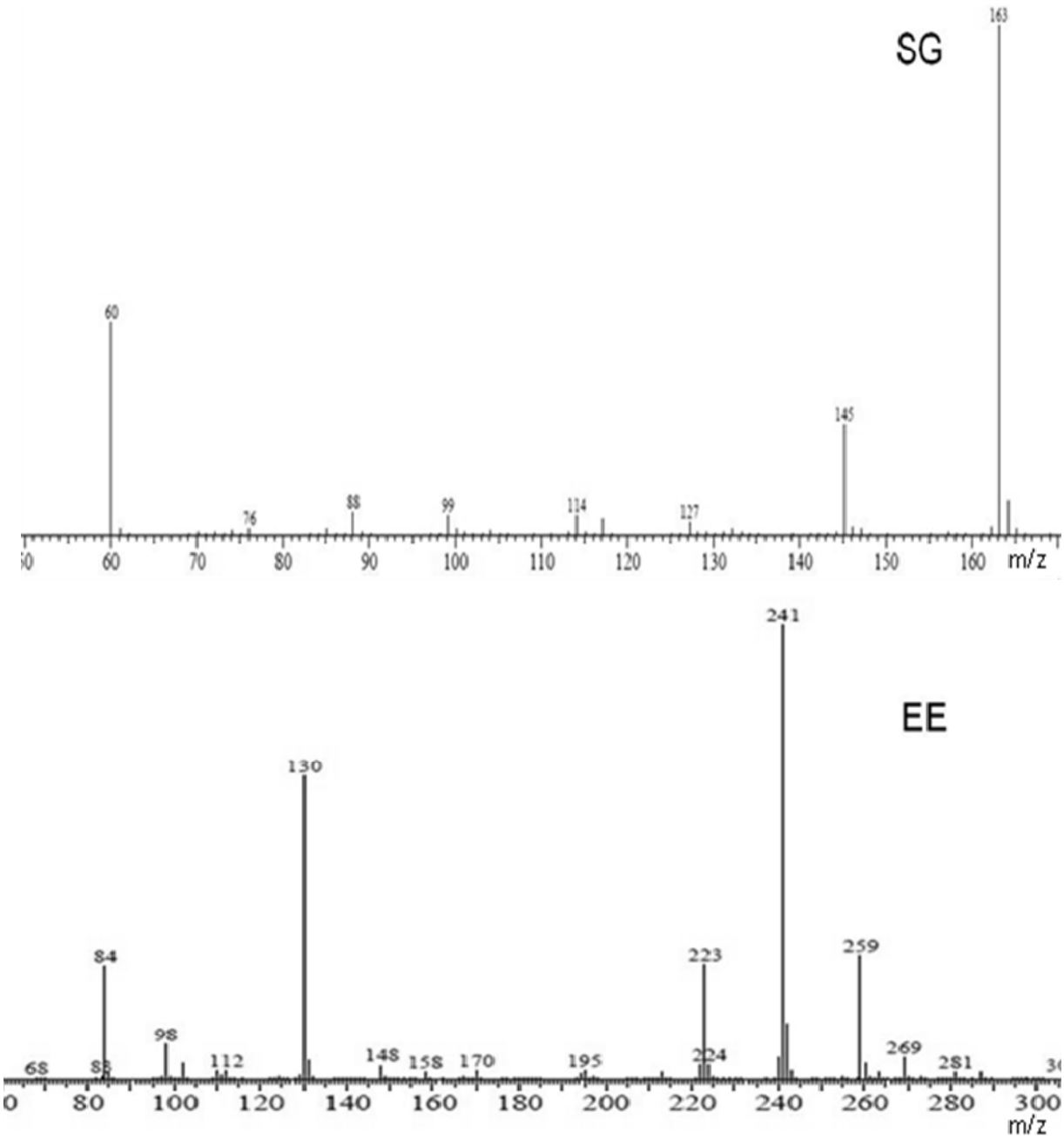
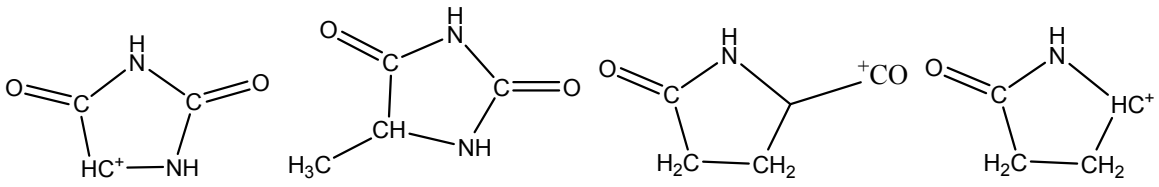


Fig. 1 – MS spectra of pure SG and EE peptides.



a) m/z 99 b) m/z 114 c) m/z 112 d) m/z 84

Fig. 2 – Proposed structure of some fragment ions resulted from serylglycine and glutamylglutamate in the MS measurement.

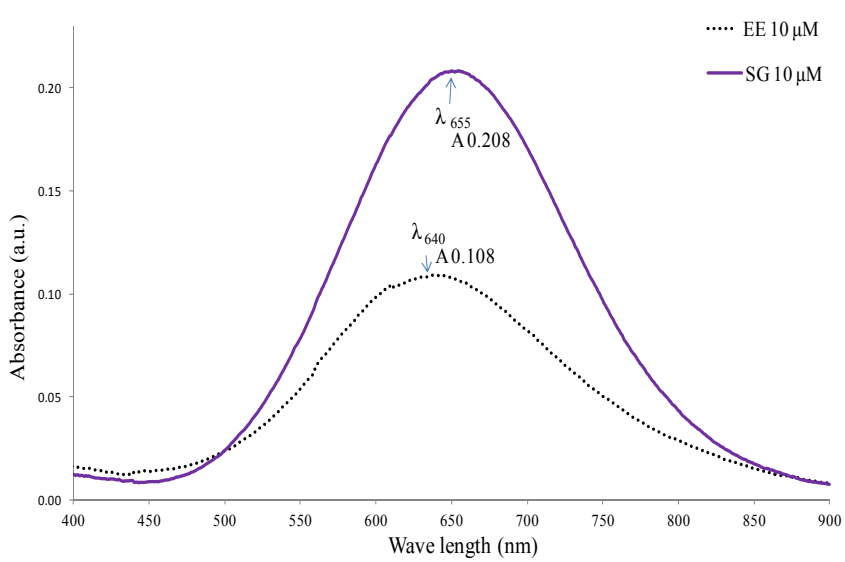


Fig. 3 – UV-*Vis* spectra of supernatants containing copper complexes of SG and EE peptides at pH 6.0.

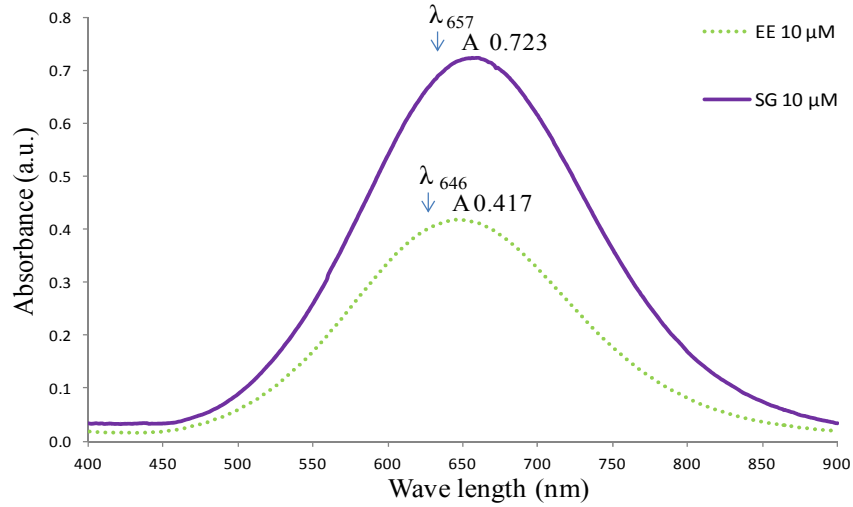


Fig. 4 – The mobilization of copper ions by SG and EE peptides from insoluble copper phosphate at pH 7.4.

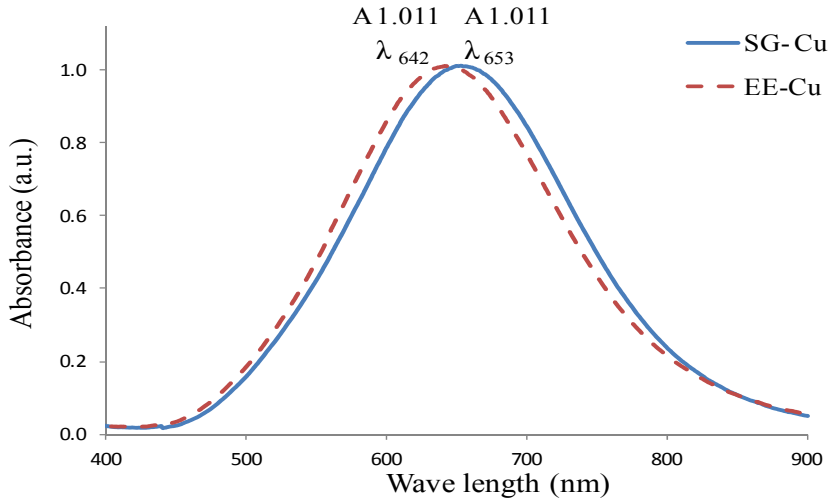


Fig. 5 – UV-*Vis* spectra of supernatants containing copper complexes of SG and EE peptides at pH 8.5.

EE peptide also gave characteristic infrared spectra, depending on the pH values (Fig. 7, left) the most significant signals were found at 1620 cm^{-1} (shoulder at pH 8.5), 1680 cm^{-1} (at pH 7.4), and 1685 cm^{-1} (at pH 6.0) characteristic to Amide I band. The amide I band is clearly distinguished at

pH 6.0 and 7.4, and less visible at higher pH. The signals attributed to Amide II band were 1593 cm^{-1} (at pH 8.5), 1583 cm^{-1} (at pH 7.4), and 1579 cm^{-1} (at pH 6.0) respectively. The FT-IR spectra of SG and ED in the presence of copper phosphate reported in Figs. 6 and 7 gives direct evidence of the formation of copper (II) complexes. In the Cu(II) complexes formed for pH 6.0 the metal-ligand bonding occurs through the amino nitrogen and peptide oxygen (Fig. 6). As pH further increased, the structure of the copper complexes changed into a form in which the metal-ligand bonding occured through the amino nitrogen, peptide nitrogen (deprotonated), and carboxyl oxygen.

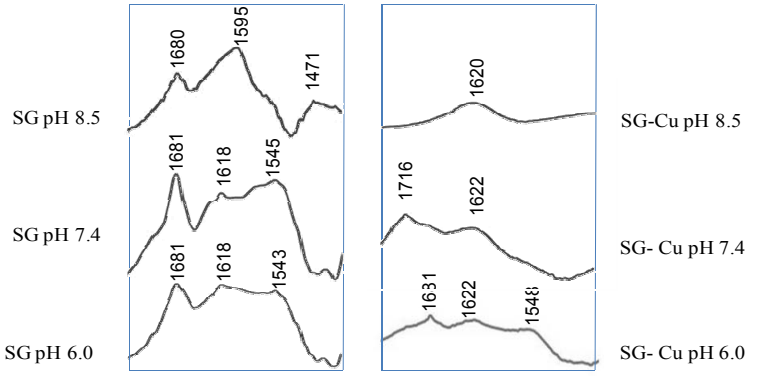


Fig. 6 – FT-IR spectra of SG peptide(left) and SG-Cu complexes (right) at various pH values: 6.0; 7.4 and 8.5.

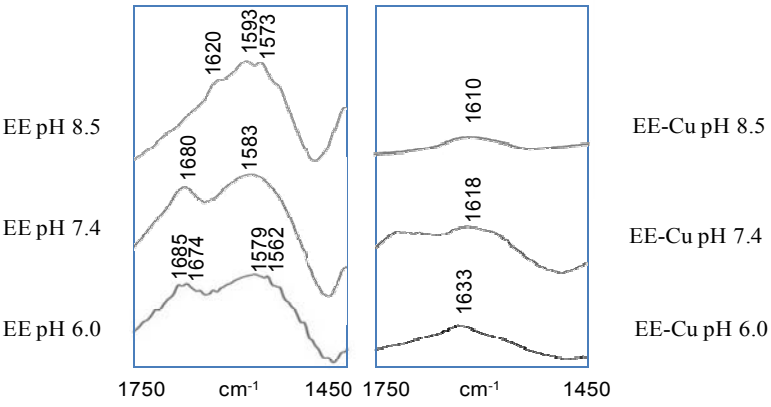


Fig. 7 – FT-IR spectra of EE peptide at various pH values: free EE peptide (left), as well as EE at pH 6.0; 7.4 and 8.5 (right).

The characteristic band for amide II decreased or totally disappeared in the Cu(II)-SG complex spectra due to copper binding to –NH- group. At pH 6.0 the high signal from 1543 cm^{-1} decreased and shifted to 1548 cm^{-1}, which shows a small proportion of complex at acidic pH. At the same time, the signals from 1545 cm^{-1} (pH 7.4) and 1595 cm^{-1} (pH 8.5), characteristic of peptide bond, disappeared when copper phosphate was added. The signal from 1618 cm^{-1} shifted to 1622 cm^{-1} (pH 6.0 and 7.4) and 1620 cm^{-1} (pH 8.5), without much change in intensity, indicating that small changes occur at C=O level in the presence of copper ions. EE peptide in the presence of copper ions also showed characteristic changes in the IR spectra. The band at 1685 cm^{-1} shifted to 1633 cm^{-1} indicating complexation by carboxylic group of glutamate moiety copper ion (Fig. 7). The signal at 1579 cm^{-1}, characteristic to amide II vibration in EE spectra (pH 6.0) decreased considerably in the presence of copper ions.

In the neutral range of pH (pH 7.4), new bands are observed at 1718 and 1618, whereas, the band at 1680 cm^{-1} corresponding to amide I vibration, and 1583 cm^{-1} corresponding to amide II vibration decreased. The new bands are unambiguously from complex of copper with EE peptide. In the alkaline medium, a band at 1610 cm^{-1} was observed and assigned to the C=O stretching vibration, while the signals from 1573 cm^{-1} and 1593 cm^{-1} assigned to amide II vibration disappeared. The lack of characteristic bands suggested the copper-peptide complex formation. The most common structural motif of these complexes is built up by the deprotonation and metal ion coordination of subsequent amide groups in the form of fused five-member chelate rings. The metal ion selectivity of this process and the role of various anchoring

groups should be discussed. The highest metal binding affinity of SG peptide is connected to the presence of two anchoring groups in proximity (the amide bond and COOH or OH groups). The FT-IR spectra showed unambiguously that copper ions induce deprotonation and metal ion coordination of the –CONH–peptide bond.

Our findings revealed that beside the histidine residues in Aβ peptides, some other residues different from the histidine ones may be involved in metal binding. Copper binding to various sites in peptide sequence, such as serine residue may affect significantly the conformation and, consequently, the biological properties of Aβ peptides, as well as other proteins of physiological relevance. Thus, it is well known that copper ions interact with peptides and protein, including albumin and a macroglobulin in blood to produce conformational changes, namely an increase in the proportion of β conformers.[18] Such data may explain Cu(II)-induced aggregation of $A\beta_{1-40}$, which depends on the Cu(II):Aβ ratio.[8] Indeed, not only histidine residues, but also serine, tyrosine, and glutamic acid residues may be involved in the aggregation process of peptides. Dynamic morphological changes of the aggregates appear to be a common morphological end point independent on the Cu(II) concentration, which shifted from fibrillar to non-fibrillar at increasing Cu(II):Aβ ratios.[8] High concentrations of copper are found in amyloidal plaques linked to amyloid-β peptides. Although copper binding to peptides depends on pH, few studies relate to property of peptides to bind more copper ions at higher pH. It has been suggested that proteins are able to mobilize copper ions from insoluble precipitates at increasing pH. Copper ions are found in blood and other body fluids at low concentration, because copper ions form precipitates in the presence of phosphates. Therefore, a complex relationship between the concentration of copper ions in various cells and pH may exist in the presence of proteins and inorganic salts (phosphate, carbonate, iron, bicarbonate, etc.). Since the pH of body fluids may differ from one organ or cellular organelle to another (pH 2 in stomach, pH 8.5 in mitochondria) a deep investigation of metal binding to peptides and proteins in a range of physiological pH could be paramount for understanding the pathological changes of metal concentrations in the body.

MATERIALS AND METHODS

All chemicals were of analytical reagent grade. The dipeptides serylglycine (SG) and glutamylglutamate (EE) were purchased from Biochemica GmbH (Germany). The copper phosphate was prepared by mixing stoichiometrically copper sulfate and trisodium phosphate. Sodium and potassium phosphates, copper sulfate, and sodium carbonate were from Carl ROTH, Sigma Aldrich or Bucharest Reagent S.A. Cu(II) complexes of SG and EE have been obtained by mixing the corresponding dipeptides with copper phosphate at pH 6.0, 7.4, and 8.5, respectively. Working solutions, when necessary, were carried out with deionized water (18.2 MΩ cm) from a Milli-Q system (Millipore, Bedford, MA). The structure and purity of peptides have been proved by mass spectrometry (Fig. 1).

UV-Visible measurements. The absorption spectra in the UV-*Vis* region were performed with a Libra SP35 spectrophotometer (Biochrom Ltd, UK), using quartz cuvettes of 10 mm (volume of 1 mL) in the range from 200 to 900 nm; however only the visible domain (400-700 nm) was relevant to our study.

Infrared absorption measurements were done in solid KBr using a Shimadzu Model 8400S FT-IR spectrophotometer. All spectra were recorded in the frequency region 500–4000 cm^{-1}, under a resolution of 2 cm^{-1} and with a scanning speed of 2 mm sec^{-1} and 20 scans per sample. The obtained spectra were compared with standard spectra of functional groups.

Mass spectrometric measurements were carried out using a GC-MS Schimadzu QP2010 mass spectrometer with direct inlet and chemical ionization. Methan was the reagent gas. The pH values were determined using a Hanna Instruments pH 211 Microprocessor pH-meter with a combination electrode and a set of pH test paper, and no correction for the obtained values has been made. Copper phosphate powder and the corresponding peptide solutions were mixed using a ultrasonic bath (from Selecta model Ultrasons). To obtain clear solution of copper-peptide complexes the mixtures were centrifugated using a Hettich Zentrifugen Model Mikro 22R (Germany), and the supernatants were used in the UV-*Vis* measurements or evaporated to dry for FT-IR analysis. Buffer solutions in all cases were prepared from $K_2H_2PO_4$/ $Na_2H_2PO_4$ at different ratio.

Procedure. The SG and EE peptides were solved in various buffer solutions to obtain 10 μM peptide solutions at pH = 6.0, 7.4, and 8.5, respectively. To 1 mL of each solution, 20 mg of copper phosphate was added. The resulted mixtures were placed in the ultrasonic bath and sonicated for 10 minutes, followed by a 10-min

centrifugation at 8000 rpm. The supernatant was used for UV-*Vis* measurements. The infrared measurements were done on the residues obtained after evaporation of each supernatant. The residues were mixed with solid KBr, and the infrared spectra were recorded in the wavenumber range from 500 to 4000 cm^{-1}. However, only the 1500 – 1700 cm^{-1} domain was important to our study.

Spatial structure of the complexes SG-Cu and EE-Cu

Using software HyperChem and Chem3D Ultra 10.0, we calculated arrangement in space, energy and chemical bonds distances to find possible structures of the complexes (Fig. 8).

SG

EE

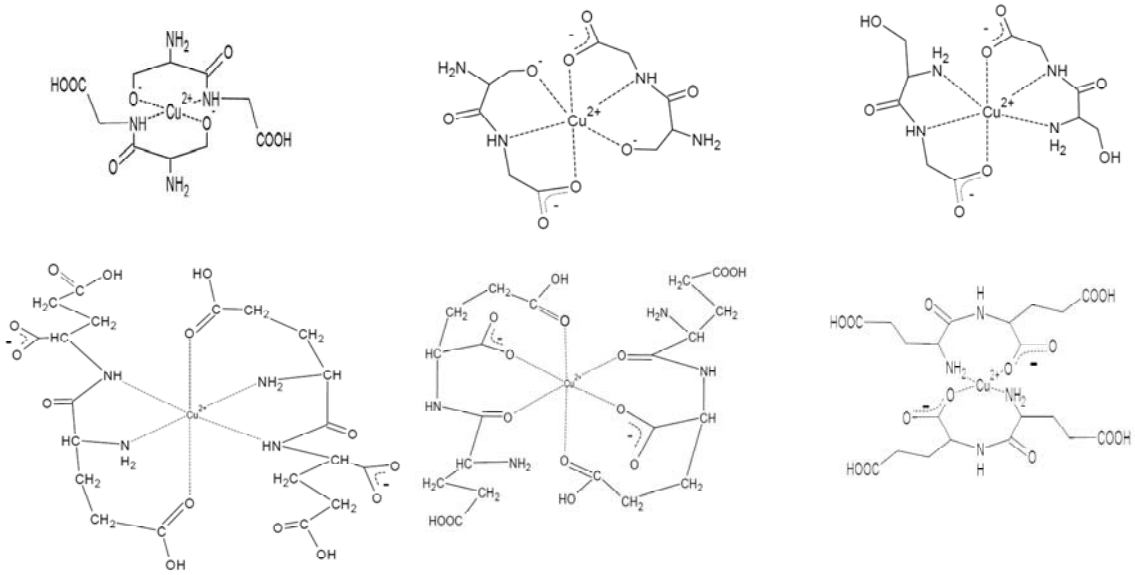


Fig. 8 – Possible structures of peptides complexes with copper ions.

SG-Cu(II)

EE-Cu(II)

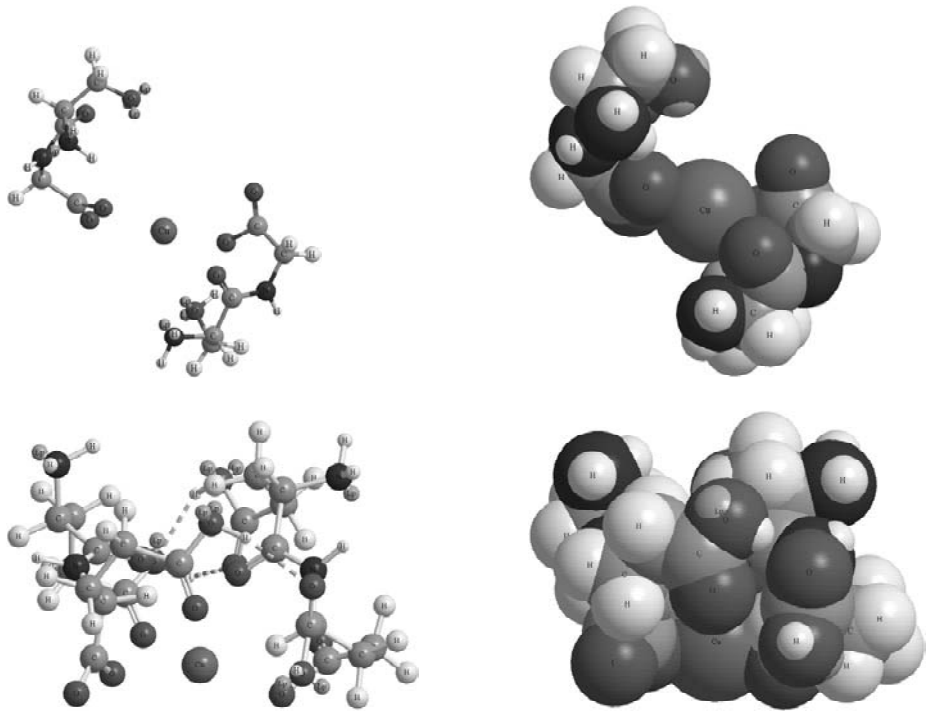


Fig. 9 – Computer simulation using the Chem3D Ultra 10.0 program;
probable tertiary structure of peptides complexes with copper ions, when two peptide molecules bind a copper ion.

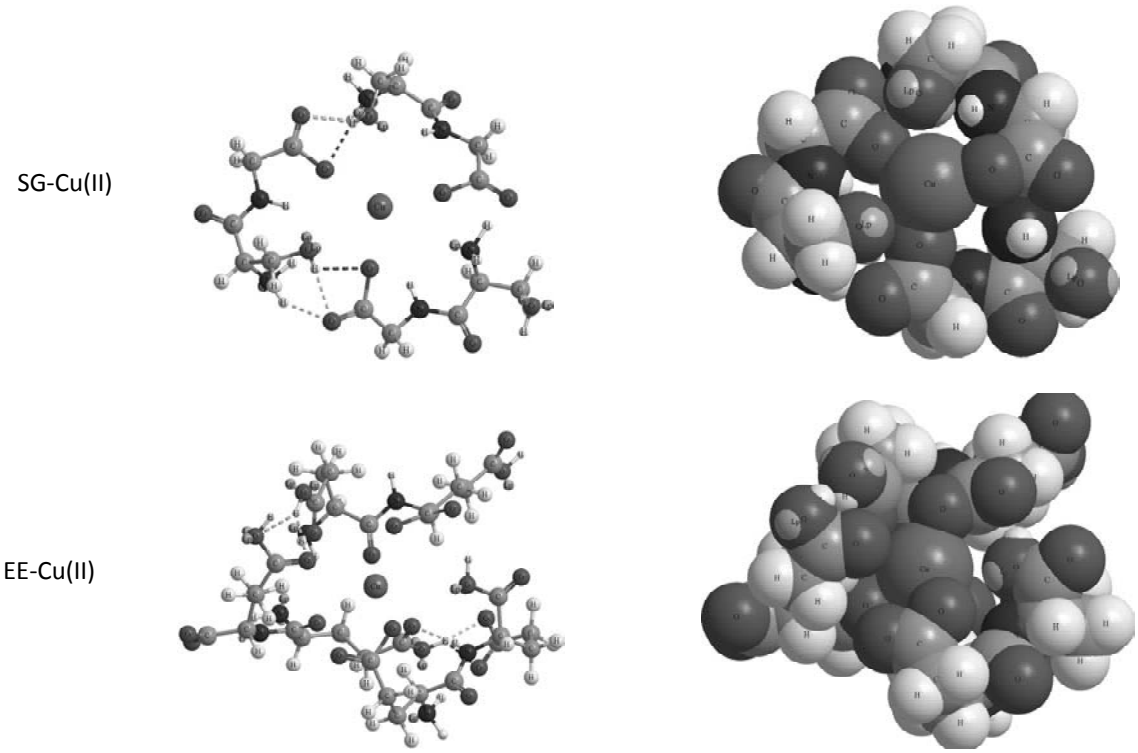


Fig. 10 – Possible spatial structure of the peptides complexes with copper ions, when three peptide molecules bind a copper ion.

From spatial arrangement we have obtained and presented some possible stable conformations of the peptide complexes with copper ion. In the first case, two peptide molecules bind one copper ion (Fig. 9) and in the second case, three peptide molecules bind a copper ion (Fig. 10). Thus, our computer simulation studies clearly show that the hydroxyl group from serine is involved in the complex formation.

CONCLUSIONS

Two non histidine peptides, SG and EE, studied in respect to their UV-vis and FT-IR spectra, showed a high binding capacity toward copper ions from the insoluble salts. However, both the stability and coordination geometry of peptide complexes are much influenced by the amino acid sequence of the ligands and pH. The large spectral changes of supernatants containing copper-peptide complexes in the range from acidic (pH 6.0) to slightly alkaline pH (pH 8.5) have been interpreted in terms of the equilibrium among free and complexed forms of dipeptides. The infrared spectra also confirmed pH-dependent copper binding to various chemical groups in the peptide molecules. The UV-vis spectroscopy studies clearly support all above mentioned observations.

Acknowledgements: The financial support of this work by Roumanian Government within the Partnership Program coordinated by UEFISCDI Bucharest (CNCS-IDEI, Contract 313/2011) is gratefully acknowledged.

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