

Proposition of Ph.D. thesis

**COORDINATION AND REDOX
PROPERTIES OF MODEL PEPTIDES
OF METAL BINDING SITES OF
PROTEINS**

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I. INTRODUCTION AND THE AIM OF THE WORK

It is well-known that essential metal ions play important roles in the synthesis and transport of organic molecules and in the catalysis of acid-base and redox processes in biological systems. Such interactions are present in the metalloenzymes, too. On the other hand the redox active metal ions (such as Cu(II)) related to neurodegenerative disorders (such as Alzheimer's or prion disease). It is also obvious that among the organic compounds proteins are the most suitable for binding these metal ions and histidine imidazole ring is the most frequent binding site.

The Cu,Zn SOD enzyme and the human prion protein have different function, but the anchoring donor atoms of both metalloproteins are imidazole nitrogens and in both cases the copper ion binding is more preferable in the Cu(II) ion form. Cu(II)/Cu(I) redox reaction, however, plays a major role in their function as well.

The Cu,Zn-SOD enzyme is a metalloenzyme and is present in the eukaryotic cells. In the active centre of this enzyme the Cu(II) ion is bound through four imidazole nitrogens while the zinc(II) ion is coordinated through three imidazole nitrogens and the carboxylate group of an aspartic acid. These two binding sites are connected by an imidazolato bridge. Cu,Zn-SOD is one of those specific enzymes that catalyze the disproportionation of superoxide ion to hydrogen peroxide and dioxygen in the cells.

The other protein is the human prion protein (Hu-PrP^C). There is a general consensus about that PrP can bind at least six copper(II) ions; four copper-binding sites are present in the octarepeat region (residues 60-91), and the region between the octarepeat and the C-terminal structured domain (residues 90-126) provides two further copper(II) binding sites. Its function relating to copper homeostasis is not well established yet. It was assumed that the Hu-PrP^C takes part in the copper transport, homeostasis and protects the cells against harmful oxidative effect. Nowadays it is accepted that it is a redox sensor, which binds the Cu(II) ion excess and protects the neurons against oxidative damage.

It is well known that the metal catalyzed oxidation (MCO) can lead to damage of bio-molecules and this is implicated in

oxidative stress, biological aging and neurodegenerative diseases. The metal ions and the free radicals can play an important role in various oxidative reactions of proteins. Accordingly, the proteins can lose their function and role in the living system. In addition they can resist to proteases, aggregate in different tissues. They may lead to damage of tissues and development of disorders most likely.

The function and the structure of the above mentioned proteins have been studied extensively in various research groups earlier. One of the research fields of the *Bioinorganic Research Group at the University of Debrecen* is the investigation of metal binding sites of both proteins and my work joined to this field.

Our aim was

- synthesis of human prion protein 103-112 fragment and its four mutants and characterization of their Cu(II), Ni(II) complexes,
- the planning and synthesis of protected peptides containing histidyl residues in different number and positions,
- the solution equilibrium studies of their Cu(II) and Ni(II) complexes by potentiometric titration, UV-Vis and CD spectroscopy,
- the determination of the redox properties and SOD activity of the Cu(II) complexes of these ligands,
- oxidation of human prion protein 103-112 fragment and its four mutants containing oxidation-sensitive methionine and histidine,
- the statement of the role of these reactions in prion disease.

II. EXPERIMENTAL METHODS

The protected peptides (Figure 1) were synthesized by means of a microwave-assisted Liberty 1 **Solid Phase Peptide Synthesizer** (CEM, Matthews, NC) using the Fmoc/tBu technique and the TBTU/HOBt/DIPEA activation strategy. In the case of the peptides the free amino terminus was treated with DMF containing 5 V/V% Ac₂O and 6 V/V% DIPEA to obtain the acetylated amino group. The side chain protecting groups were cleaved with the mixture of TFA/TIS/H₂O/DODT (94/2.5/2.5/1 V/V%) at room temperature for 1-1,5 hours. The solution containing the peptide was separated from the resin by filtration, followed by recovering from the pertinent solution by precipitation with cold diethyl ether. The precipitate was washed with cold diethyl ether, then was centrifuged and dried, redissolved in water and finally lyophilized.

The **pH-potentiometric titrations** were performed in order to determine the protonation constants of the ligands and the stability constants of the Cu(II), Ni(II) complexes. The pH-potentiometric titrations were performed in 4.00 mL samples at $3 \cdot 10^{-3}$ M ligand concentration with the metal ion to ligand ratio 1:2, 1:1, 2:1. All pH-potentiometric measurements were carried out at a constant ionic strength of 0.2 M KCl and at a constant temperature (298 K). Protonation constants of the ligands and overall stability constants of the metal complexes were calculated by means of the general computational programs SUPERQUAD and PSEQUAD. According to the determined values complex distribution curves of the systems were plotted using the MEDUSA program.

UV-vis spectra of the complexes were recorded from 200 to 900 nm on Hewlett Packard HP 8453 diode array and Perkin Elmer Lambda 25 double beam spectrophotometers and quartz cuvettes with 1.000 cm path length. The same concentration range was used as for pH-potentiometry.

Circular dichroism spectra of the complexes were registered on a JASCO J-810 spectropolarimeter using 1.00 mm and 1.000 cm cells in the 200–800 nm wavelength range at the same concentrations as used in pH-potentiometry.

The redox potential values of the copper(II) complexes were determined by **cyclic voltammetry**. The cyclic voltammograms of the copper(II) complexes were obtained by means of a Metrohm VA 746 Trace Analyzer. The measurements were carried out at 25°C with a three electrode assembly, different scan rates, 800–(–600) mV potential range and 140 μ A limiting current. For the evaluation of the voltammograms we used the CACYVO program that was provided by the distributor of the instrument.

The **superoxide dismutase (SOD) activity** of the complexes was determined by the indirect method of NBT reduction. The superoxide anion was generated *in situ* by the xanthine/xanthine oxidase reaction and detected spectrophotometrically by monitoring the reduction of NBT. The tests were carried out in phosphate buffer ($5 \cdot 10^{-2}$ mol/dm³) at pH 6.8 and 7.4. For comparison the activity of Cu,Zn-SOD enzyme and the Cu(HPO₄) was also measured in control experiments. The changes in the absorbance were registered by a Perkin Elmer Lambda 25 double beam spectrophotometer at 560 nm. The results were plotted in Excel and the data were fitted by the Scientist program.

The **oxidation** reaction mixture containing 1.1 mM of peptides and metal-to-ligand molar ratio 1:1.1 was incubated at 25 °C for 1 h in the presence of hydrogen peroxide at metal to H₂O₂ molar ratio 1:4 for all peptides. The pH was adjusted to 7.4. After incubation the reaction was stopped by addition of Na₂EDTA at ligand to Na₂EDTA ratio 1:5.

The purity of the ligands and the products of oxidized samples were checked by using **High Performance Liquid Chromatography (HPLC)**. The purity of multihistidine ligands were carried out on a Grace Vydac C18 (250 mm $\times$ 4.6 mm, 300 Å, 5 μ m) column. The oxidized products were conducted on a Teknokroma Europa Protein C18 (250 $\times$ 4.6 mm, 300 Å, 5 μ m)

column. The samples were analyzed by analytical RP-HPLC using a Jasco instrument, equipped with a Jasco MD-2010 plus multiwavelength detector. The separation of products of oxidation samples were analyzed by semipreparative RP-HPLC using a Jasco instrument, equipped with a Jasco UV-2077 plus UV-vis detector and Grace Vydac C18 (250 mm × 10 mm, 300 Å, 5 µm) column. Mobile phases were water (A) and acetonitrile (B) containing 0.1% TFA. The flow rate was 1 and 2 ml/min.

A MicroTOF-Q type Qq-TOF MS instrument (Bruker Daltonik, Bremen, Germany) was used for the **MS and MS/MS** measurements in positive ion mode. The spectra were evaluated with the DataAnalysis 3.4 software from Bruker.

III. STUDIED LIGANDS

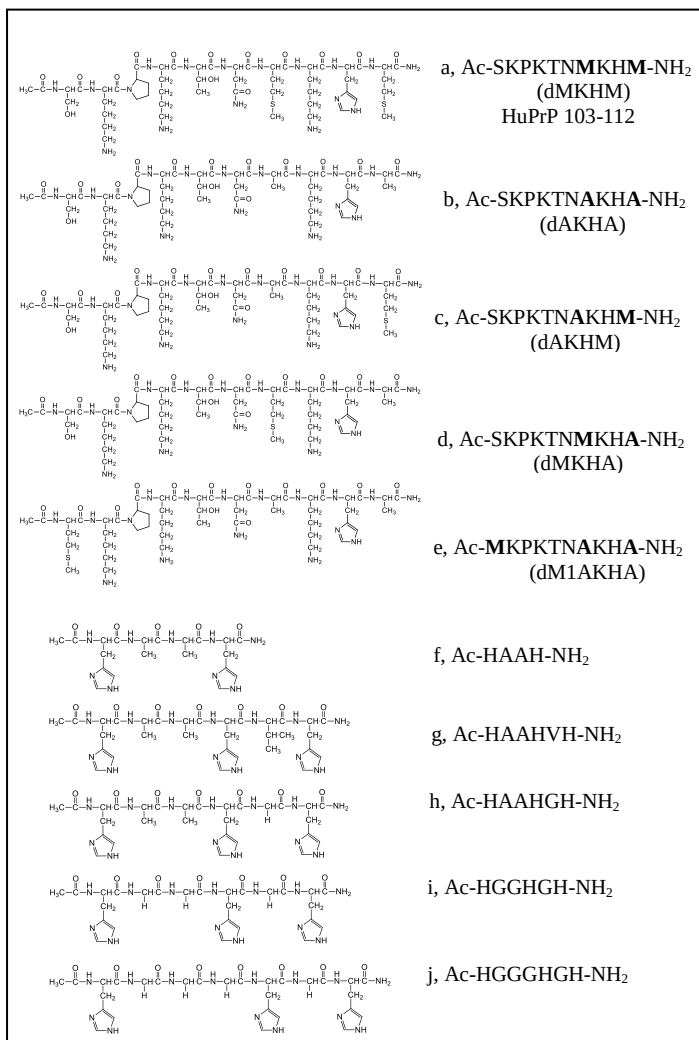


Figure 1: Structure of the studied ligands

IV. NEW SCIENTIFIC ACHIEVEMENTS

1. **The complex formation properties of the human prion protein fragment and its mutants**

We determined the structures and stability constants of Cu(II) and Ni(II) complexes of HuPrP(103-112) fragment and its four mutants and we compared the results with those of previously studied ligands.

- In the case of the studied peptides it was stated that the His111 is the major anchoring group for both metal ions. Based on the experimental results we can say that Met109 and Met112 do not affect the stability and composition of metal ion complexes.
- The spectroscopic measurements made it possible to detect a weak interaction of Met109 in the [N⁻, N⁻, N(Im)] coordinated Cu(II) species. It was stated that this interaction is weaker than in the case of the previously studied HuPrP 109-112 (Ac-MetLysHisMet-NH₂), but higher than that of the larger HuPrP(84-114)H96A prion fragment.
- The studied Cu(II) complexes have higher stability than the Ni(II) complexes; this result is in agreement with the Irving-Williams series.
- The studied peptides are able to bind the Ni(II) ions contrary to the coordination sphere modeling Ac-MetLysHisMet-NH₂ tetrapeptide.

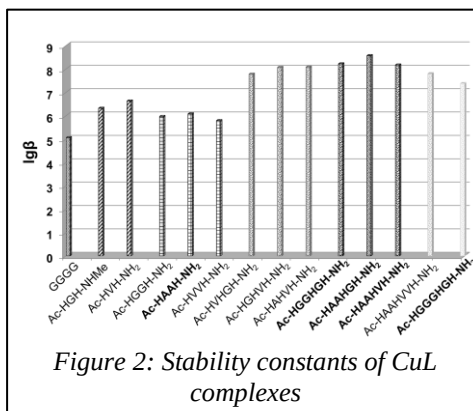
2. **Complex formation properties of the model peptides of Cu,Zn SOD enzyme**

Our aim was the synthesis of the active centre modeling multihistidine peptides of the Cu,Zn SOD enzyme. We determined the acid-base properties of these ligands and the stability, the composition of their Cu(II); Ni(II) complexes. We characterized the structure of metal ion complexes and estimated the ratio of isomeric structures of 1:1 complexes.

- The imidazole nitrogen donor atoms of the histidine side chain in the studied ligands are the primary binding site for Cu(II) and Ni(II) ions.

- The increasing number of histidines in the amino acid sequence increases the stability of imidazole coordinated species. This observation is in agreement with the previously published data. The position and the quantity of histidyl residues in the peptide chain are able to affect the complex stability.
- High stability CuL complexes are formed. From a structural point of view these complexes are considered to be adequate models of the copper(II) binding site of the Cu,Zn SOD enzyme.

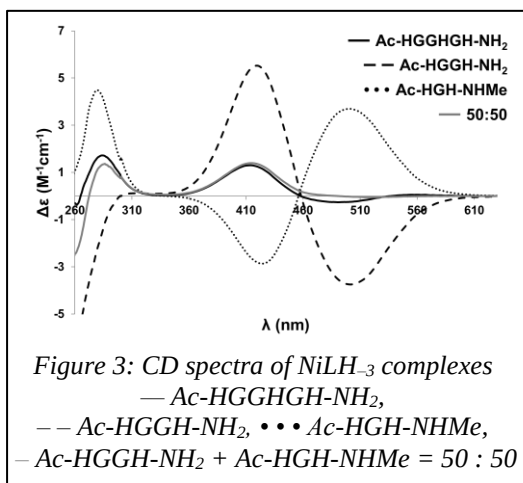
- The stability series of the imidazole coordinated species are (Figure 2): Ac-HXXH-NH₂ < Ac-HXH-NH₂ < Ac-HXXHZZH-NH₂ ~ Ac-HGGGHGH-NH₂ < Ac-HXHZH-NH₂ < Ac-HXXHZH-NH₂ (X,Z = Gly, Ala, Val).



- The Cu(II) ions induce the deprotonation of amide nitrogen in slightly basic pH range. Simultaneously, in the case of three histidine containing peptides coordination isomers are formed. The ratio of coordination isomers was estimated by CD spectroscopy. The metal ions bind to the C-terminal histidine residue.
- The consequence is that the metal binding affinity of C-terminal HZH sequence of CuLH₂ and CuLH₃ complex follows the Ac-HGGGHGH-NH₂ > Ac-HGGHGH-NH₂ > Ac-HAAHGH-NH₂ > Ac-HAAHVH-NH₂ order.
- In the case of three histidine containing ligands dinuclear species are formed in the presence of excess of metal ions.
- In the presence of Ni(II) ions the deprotonation of the two amide nitrogens takes place in a cooperative process. In the case of NiLH₃ with [N⁻, N⁻, N⁻, N(Im)] coordinated mode

complexes with isomeric structures are formed. The ratio of isomers can be calculated by means of CD spectroscopic technique. According to the results the Ni(II) ion prefers the binding to the internal histidine residue better than Cu(II) ion.

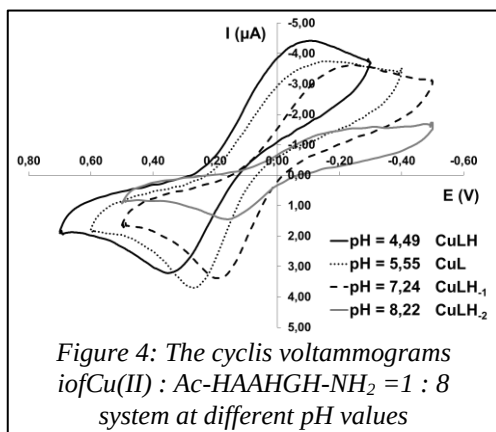
- The ratio of isomers of exclusively glycine containing peptides is ~50:50 % (Figure 3). In the case of Ac-HAAHVH-NH₂ the Ni(II) ion binds to the internal histidine residue and in the case of Ac-HAAHGH-NH₂ the metal ion binds to the C-termini.
- The ratio of isomers depends on the size of the amino acid side chain. This trend shows a significant preference for metal binding at C-terminal histidine in the next series: Ac-HAAHVH-NH₂ < Ac-HGGHGH-NH₂ ~ Ac-HGGGHGH-NH₂ < Ac-HAAHGH-NH₂.
- These ligands are not able to bind two equivalents of Ni(II) ion contrary to Cu(II) ion.



3. The redox properties of Cu(II) complexes of the model peptides of Cu,Zn SOD enzyme

After the equilibrium studies we investigated the redox parameters of 1:1 Cu(II) complexes. The obtained redox potential values can give information about the SOD activity of the Cu(II) complexes. Those complexes can be good functional models of the enzyme that can decompose superoxide similarly to the native enzyme, which means that the redox potential of the complex falls between -0.16 V and +0.89 V.

- Based on the redox parameters of the imidazole coordinated Cu(II) complexes we confirmed that the increasing number of histidine residues in the coordination sphere decreases the redox potential values. This statement is in good agreement with earlier results. The greater the stability of the complex is the more difficult to reduce the metal centre.
- It was stated that the redox potential values of the formation of [N(Im), N⁻, N(Im)] and [N(Im), N⁻, N⁻, N(Im)] coordinated Cu(II) complexes are positive or small negative values, because of their slightly distorted geometry. The cyclic voltammograms of imidazole coordinated complexes, CuLH₋₁ and CuLH₋₂ are presented on Figure 4.

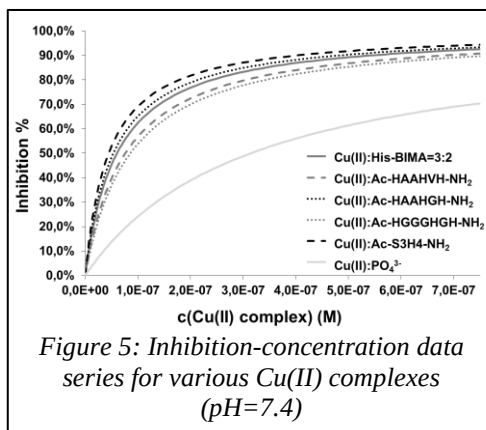


- All the determined redox potential values fall into the positive redox potential range so these complexes can be good SOD models.
- The positive reduction potential of CuLH_{-1} or CuLH_{-2} species of present studied peptides established the assumption that copper(II) binding is preferred on the HZH sequence of the molecule.

4. The SOD activity of Cu(II) complexes of the model peptides of Cu,Zn SOD enzyme

As a continuation of our electrochemical experiments, we measured the SOD activity of the Cu(II) complexes of hexa- and heptapeptides at pH 6.8 and 7.4.

- It was established that the relative activity of Cu(II) complexes is around 2-4 % at pH 6.8.
- Increasing pH increases the SOD activity.
- The activity of Cu(II) complexes of studied hexa- and heptapeptides is similar to those values, which were determined previously for CuL complexes of Ac-HisSarHisSarHisSarHis-NH₂ and the imidazolato bridged complex existing in the Cu(II)-His-BIMA 3:2 system at pH 7.4. This similarity is presented on the Figure 5. For better overview the signs of measuring points are omitted.

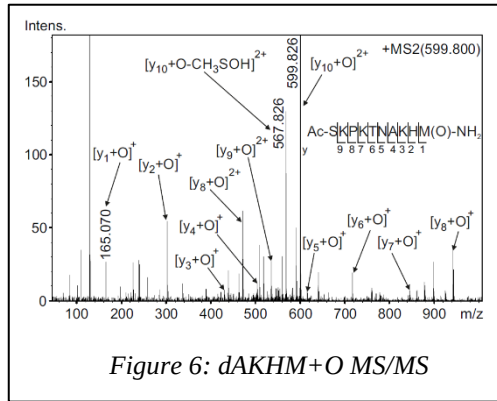


- All exclusively imidazole coordinated and [N(Im), N⁻, N(Im)], [N(Im), N⁻, N⁻, N(Im)] coordinated Cu(II) complexes could be responsible for the SOD activity. The amide nitrogen coordinated complexes with slightly distorted geometry results in the reduction reaction of $\text{Cu(II)} + \text{e}^- \rightarrow \text{Cu(I)}$ and in the $\text{O}_2^{\cdot-}$ binding to metal ion centre.

5. Oxidation of the human prion protein fragment and its mutants

As a continuation of studies of Cu(II) complexes of one histidine containing prion fragment and its mutants, we investigated the oxidation of the ligands. The histidine and the methionine amino acids are sensitive to oxidation in the chosen ligands in the presence of Cu(II)/H₂O₂. The oxidized products were isolated by LC-MS and LC-MS/MS.

- A method was developed for the separation of oxidized products of human prion protein fragment and its four mutants by HPLC experiment technique.
- The fragmentation of only one histidine containing mutants (dAKHA) occurred, the formation of 2-oxo-histidine derivative was not detected in contrast with literature studies in the MS spectra, of the peptide chain. The fragmentation of the ligand by the α -amidation pathway could be observed.
- In the case of methionine containing peptides (dMKHA, dAKHM, dMKHM, dM1AKHA) the oxidation of methionine to methionine sulfoxide occurred. The oxidation of the methionine residue is proved by MS/MS (*Figure 6*).
- The presence of methionine residues protects the peptides from fragmentation. The oxidation process occurs randomly, it does not depend on the position of the methionine; methionine residues undergo oxidation either involved in the coordination of the copper(II) ions, or near or far the binding sites. The histidine oxidation was not occurred in all cases.
- It was concluded that Cu(II) ions have catalyzing role in the above mentioned process.



- On the other hand, the presence of methionine residues protects the prion protein and other peptides around these proteins from fragmentation, oxidation and degradation. In these cases copper(II) ions catalyze the oxidation of methionine to methionine sulfoxide.

V. POSSIBLE APPLICATIONS OF THE RESULTS

The investigation of peptides for different enzyme models has been going for decades in the *Bioinorganic Research Group*. These results give molecular level analysis about real metal binding sites of protein/metalloprotein. The studies can clarify the interaction between studied metal ions and peptide side chains (there are either in the coordination sphere or far away). Thus the effect of the side chains can be specified on the complex formation processes.

During our research work we synthesized histidine containing ligands and studied their Cu(II) and Ni(II) complexes and redox properties. Summarizing our results we can say that these multihistidine ligands are good starting points for the planning of such molecules and their Cu(II) complexes of which can be good structural and functional models of the SOD enzyme.

On the other hand the investigation of oxidation of human prion fragment (Hu PrP^C 103-112) and its mutant can give good information of prion disease. These can provide valuable information for biological investigations aimed at determining the cause of neurodegenerative diseases.

TUDOMÁNYOS PUBLIKÁCIÓK (PUBLICATIONS)

Az értekezés alapját képező közlemények (Articles connected to the thesis) (3):

Tudományos folyóiratban megjelent közlemények (Published articles) (2):

1. Gizella Csire, József Demjén, Sarolta Timári, Katalin Várnagy
Electrochemical and SOD activity studies of copper(II) complexes of bis(imidazol-2-yl) derivatives, Polyhedron, **61**, 202-212 (2013)
IF:2,047
2. Gizella Csire, Lajos Nagy, Katalin Várnagy, Csilla Kállay
Copper(II) Interaction with the Humán Prion 103-112 Fragment – Coordination and Oxidation, Journal of Inorganic Biochemistry, **170**, 195-201 (2017)
IF: 3,205

Tudományos folyóiratban még meg nem jelent közlemények (Not published articles) (1):

1. Gizella Csire, Sarolta Timári, József Asztalos, Judit Király, Mariann Kiss, Katalin Várnagy
Coordination, redox properties and SOD activity of Cu(II) complexes of multihistidine peptides
(közlésre beküldve)

Az értekezés alapját képező előadások (Lectures connected to the thesis) (10):

1. Gizella Csire, Sarolta Timári, Katalin Várnagy
Coordination, redox properties and SOD activity of Cu(II) complexes of multihistidine peptides
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2. Csire Gizella, Kállay Csilla, Nagy Lajos, Várnagy Katalin, Sóvágó Imre
A humán prion fehérje 103-112 fragmense és a Cu(II)-ion közötti kölcsönhatás vizsgálata – koordináció és oxidáció
50. Komplexkémiái Kollokvium, Balatonvilágos, Magyarország, 2016.05.30-06.01., E41

Coordination and redox properties of model peptides of metal binding sites of proteins

3. Katalin Várnagy, Gizella Csire, Sarolta Timári, Ágnes Dávid, Csilla Kállay
The role of side chains in the fine tuning of metal binding ability of peptides
12th European Biological Inorganic Chemistry Conference, Zürich, Svájc, 2014. 08. 24-28., S751. oldal, OP 6
4. Katalin Várnagy, Gizella Csire, Sarolta Timári
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5. Kállay Csilla, Csire Gizella, Nagy Lajos, Várnagy Katalin, Sóvágó Imre
Hisztidint és metionint tartalmazó peptidek kölcsönhatása réz(II)-ionokkal
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7. Katalin Várnagy, Gizella Csire, Sarolta Timári
Copper(II) complexes of peptide derivatives containing imidazole side chains, as models of Cu,Zn-SOD enzyme
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8. Csire Gizella
Cu(II)–peptidkomplexek koordinációs és redoxi sajátossága, , illetve SOD aktivitása
47. Komplexkémiái Kollokvium, Mátraháza, Magyarország, 2013. 05. 29-31., E21
9. Csire Gizella
Cu(II)-bisz(imidazolil) származékok redoxi sajátosságainak és SOD aktivitásának vizsgálata
XXXI. OTDK, Kémiai és Vegyipari Szekció, Koordinációs kémiai tagozat, Eger, Magyarország, 2013. április 4–6., 173. oldal

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Cu(II)-bisz(imidazolil) származékok redoxi sajátságainak és SOD aktivitásának vizsgálata

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Az értekezés alapját képező poszterek (Posters connected to the thesis) (5):

1. Gizella Csire, Sarolta Timári, Mariann Kiss, Katalin Várnagy
Coordination, redox properties and SOD activity of Cu(II) complexes of multihistidine peptides

13th European Biological Inorganic Chemistry Conference, Budapest, Magyarország, 2016. 08. 28.- 09. 01., 257. oldal, P104

2. Gizella Csire, Sarolta Timári, Katalin Várnagy
Coordination, redox properties and SOD activity of Cu(II) complexes of multihistidine peptides

International Symposium on Metal Complexes 2016, Barcelona, Spanyolország, 2016. 06. 07-10., 65. oldal

3. Gizella Csire, Csilla Kállay, Lajos Nagy, Katalin Várnagy, Imre Sóvágó

Copper(II) Interaction with the Humán Prion 103-112 Fragment – Coordination and Oxidation

13th International Symposium on Applied Bioinorganic Chemistry, 2015. június 12–15., Galway, Írország, 111. oldal, P13

4. Gizella Csire, Csilla Kállay, Lajos Nagy, Katalin Várnagy, Imre Sóvágó

Metal ion catalyzed oxidation of a humán prion fragment

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5. Csire Gizella, Várnagy Katalin

Cu(II)–peptidkomplexek koordinációs és redoxi sajátsága, illetve SOD aktivitása

XIX. Bolyai Konferencia, Budapest, Magyarország, 2014. 03. 22-23. 26. oldal, IX.

Az értekezésben nem tárgyalt előadások (Lectures not connected to the thesis) (2):

*Coordination and redox properties of model peptides of metal
binding sites of proteins*

1. Csire Gizella
Imidazolgyűrűt tartalmazó ligandumok Cu(II)-komplexeinek elektrokémiai és SOD aktivitás vizsgálata
DETEP konferencia, Debrecen, Magyarország 2012. 05. 10.
2. Csire Gizella
Imidazolgyűrűt tartalmazó aminosav- és peptidszármazékok átmenetifém komplexeinek elektrokémiai vizsgálata
DETEP konferencia, Debrecen, Magyarország, 2011. 05. 05.

Az értekezésben nem tárgyalt posztterek (Posters not connected to the thesis) (1):

1. Katalin Várnagy, Sarolta Timári, Gizella Csire, Norbert Lihi
Coordination and redox properties of copper(II) and iron(II/III) complexes of bis(imidazol-2-yl) ligands
11th International Symposium on Applied Bioinorganic Chemistry,
Barcelona, Spanyolország, 2011. 12. 02-05, P147



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List of publications related to the dissertation

Foreign language scientific articles in international journals (2)

1. **Csire, G.**, Nagy, L., Várnagy, K., Kállay, C.: Copper(II) interaction with the Human Prion 103-112 fragment - Coordination and oxidation.
J. Inorg. Biochem. 170, 195-201, 2017. ISSN: 0162-0134.
DOI: <http://dx.doi.org/10.1016/j.jinorgbio.2017.02.018>
IF: 3.205 (2015)
2. **Csire, G.**, Demjén, J., Timári, S., Várnagy, K.: Electrochemical and SOD activity studies of copper(II) complexes of bis(imidazol-2-yl) derivatives.
Polyhedron. 61, 202-212, 2013. ISSN: 0277-5387.
DOI: <http://dx.doi.org/10.1016/j.poly.2013.05.053>
IF: 2.047

Total IF of journals (all publications): 5,252

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