Determination of Cu(II)-binding affinity of peptides containing ATCUN motif by LC ICP MS

Master's Thesis

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ATCUN motiivi sisaldavate peptiidide Cu(II)-sidumise afiinsuse määramine LC-ICP MS abil

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1 Contents

2 List of abbreviations

ATCUN - Amino-Terminal Cu(II)- and Ni(II)-binding

HSA – Human Serum Albumin

AD - Alzheimer's disease

Aβ – Amyloid Beta

Hepes - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFIP - 1,1,1,3,3,3-hexafluoro-2-propanol

LC-ICP MS - Liquid Chromatography-Inductively Coupled Plasma Mass Spectrometry

3 Introduction

It has long been known that copper has many roles in the body. Although it is an important contribution to many vital processes, its deficiency and misregulation sometimes play also a controversial role in the failure of these processes. In some cases, such as Alzheimer's disease (AD), it is difficult to say what role copper plays in its progression, but it is known that it is undoubtedly present in the equation of this disease.

In recent years, interest in ATCUN (amino-terminal Cu(II)- and Ni(II)-binding) motif models has increased due to their wide range of applications. ATCUN motif, which contains His residue in third position, has the ability to bind copper ions and is present in many body proteins. Metal-ATCUN derivative applications may be divided into two categories: redox- and non-redox-based applications. Cu-ATCUN and Ni-ATCUN derivatives are usually used to catalyse redox-dependent cleavage or modification of biomolecules. For this reason, models of the ATCUN motif may allow the creation of simple analogues of biologically active biomolecules for different purposes. They are also great models for researching peptide metal-binding binding affinities, which is of fundamental importance in the understanding competition of ATCUN-containing peptides and proteins.

Currently the determined affinities for ATCUN-containing peptides and proteins vary in a wide range. This might be caused from using of many different experimental protocols and different methods for calculating metal-binding affinities for ATCUN-containing compounds. This situation complicates the possibility for subsequent work in the study of copper binding and competition of different proteins for metal ions. Considering the wide variety of ATCUN motif involvement in biomolecules, it is essential to unify the metal-binding affinity research methods and protocols, which allows comparative analysis of binding effects for ATCUN motif-containing biomolecules.

4 Literature Review

4.1 Copper metabolism

Copper, though it can be toxic, is an important element for proper body functioning. Basically, any biochemical reaction, that depends on copper enzymes, can be considered as a role of copper. In the cell, copper can cycle between two oxidation states: Cu^{+1} and Cu^{+2}. This one-electron reaction occurs naturally in a variety of catalytic processes with varying redox potentials. Copper is involved in both the aerobic respiration and the synthesis of molecular oxygen in the photosynthetic pathway in the oxygen cycle. Copper is also a cofactor in the terminal anaerobic oxidase N2O reductase, which is involved in the nitrogen cycle (Llases, Morgada et al. 2015).

Copper is needed for lysyl oxidase, participating in crosslinking of collagen. Collagen is a main connective tissue protein in skin, cartilage, tendon, and bones. Copper contains in tyrosinase enzyme, which is required for the formation of melanin, and skin pigment. Copper takes part in cellular respiration or electron transport chain being a component of cytochrome c oxidase enzyme. Considering that ATP production depends on copper containing enzymes, it can be said that copper is essential for energy production. Copper also has a role in iron metabolism and hemoglobin synthesis.

Copper is important in superoxide dismutase function also as an antioxidant by dismutating superoxide anions to molecular oxygen and hydrogen peroxide and protects this way cellular structures.

Copper is crucial for body systems for survival, and the only way to get copper is through nutrition. Copper is absorbed in the stomach and duodenum, and enters the jejunum with the aid of metal binding protein, metallothionein. There it binds to albumin, and it is transported to liver, where it will be incorporated into ceruloplasmin. Most copper is stored in liver cells, hepatocytes (Myint, Oo et al. 2018). Any mistakes in copper absorption or its distribution can lead to serious diseases. Some of them like for example Wilson's disease is connected to copper excess, when copper accumulates in vital organs (brain, liver, etc.). Copper deficiency exists in the case of Menkes disease. However, there are illnesses where copper is considered to take part, but its role is not fully understood. In the case of Alzheimer's disease copper is known to interact with amyloid beta (Aβ), which can affect fibril formation (Bagheri, Squitti et al. 2018).

Copper interaction with different proteins depends on their metal-binding affinity, which is important to understand copper metabolism and treat the diseases linked to copper dyshomeostasis.

4.2 Human serum albumin

Human serum albumin (HSA) is a non-glycosylated, extracellular blood protein, which constitutes about 60 % of all proteins in plasma and cerebrospinal fluid. HSA plays important role in transport of hormones, nutrients and ions (Friedrichs 1997; Quinlan, Martin et al. 2005).

HSA is comprised of three homologous domains, which all include ten helices. Helices are packed in two subdomains A and B. Even though domains are structurally similar, each of them interacts

with others differently, thus HSA has an asymmetric structure containing different potential interaction sites. HSA comprises a number of different binding sites, which is the reason behind its biological multifunctionality. The most important binding site for this the study is amino terminal ATCUN motif located in N-terminus, composed of Asp-Ala-His residues (Fanali, di Masi et al. 2012; Sendzik, Pushie et al. 2017).

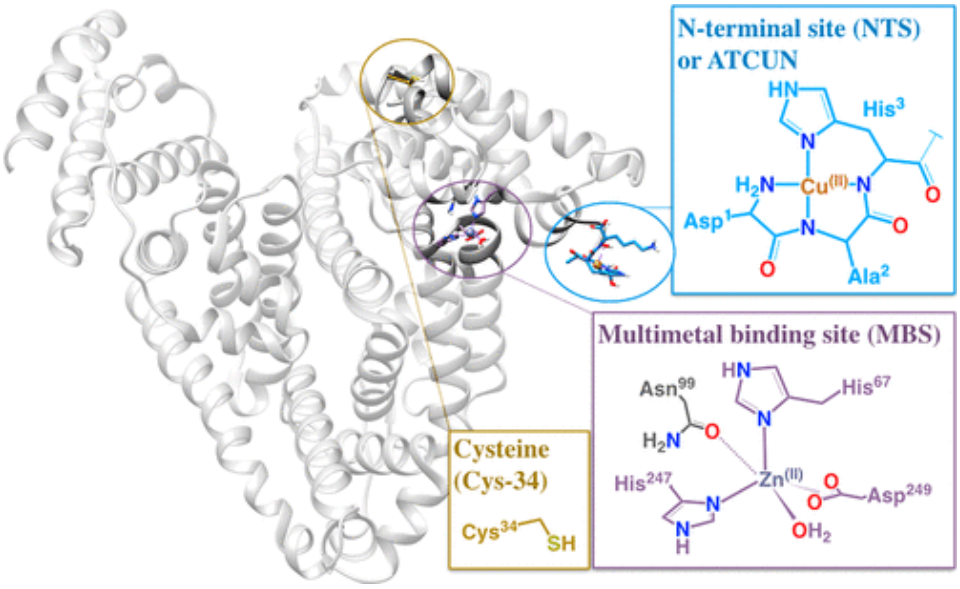
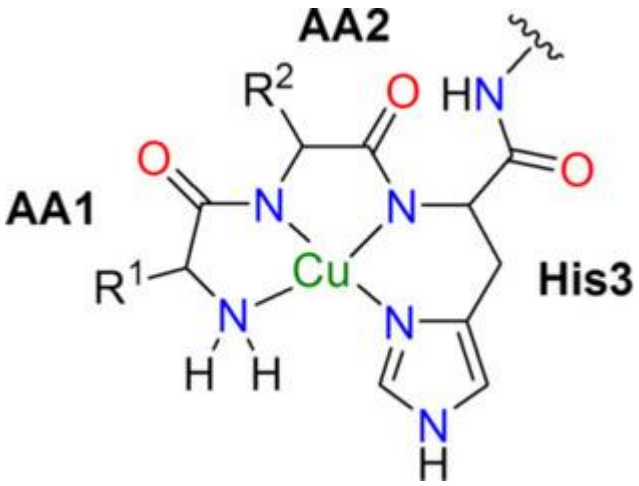


Figure 1. Metal binding sites of HSA (Sendzik, Pushie et al. 2017).

HSA multifunctionality and known metal-binding affinities make it a useful standard competitor compound in metal-binding affinity studies.

4.2.1 ATCUN motif

ATCUN motif is a metal-binding site found initially in HSA (Laussac and Sarker 1984) and later also in many other different proteins. ATCUN motif consists of a free N-terminal tripeptide, where the residue in the third position is His. A coordination donor-set constituted of a free NH2 terminal, two deprotonated amide nitrogen atoms of the two amino acids at positions 1 and 2, and an imidazole from a His residue at position 3 characterizes the ATCUN metal-binding site. (Mena, Mirats et al. 2018). These four N atoms can form stable square-planar complexes by interacting specifically and firmly with Cu(II) and Ni(II) ions. (Scheme 1) (Mena, Mirats et al. 2018)



Scheme 1. Schematic representation of the ATCUN binding site (Mena, Mirats et al. 2018).

ATCUN motif acts as a centre for strong binding of metal ions such as Cu(II). For this reason, such peptides and proteins are of great interest in studying the effect and strength of copper binding.

4.2.2 Biological role of ATCUN motif

Because of the presence of the ATCUN motif, HSA can be considered as copper transport protein. ATCUN motif can also be found in histamine that is acting as neurotransmitter in the brain and is present also in other organs like gut (Nieto-Alamilla, Márquez-Gómez et al. 2016). These are also some examples of the existence of the ATCUN motif in different regulatory proteins. ATCUN motifs cobe foundfind in antimicrobial peptides and they participate in production of reactive oxygen species (ROS) (Bouraguba, Glattard et al. 2020). Considering the importance of ATCUN motifs in metal binding and copper homeostasis it is not surprising that designed ATCUN motifs are of great interest in research into the regulation of copper dyshomeostasis, occurring in case of serious illnesses like for example Wilson's disease and Alzheimer's disease.

In AD Aβ peptides are known to bind copper ions and aggregate in their presence leading to Aβ toxicity. Even though it is hard to establish causation (is copper binding promoting aggregation or aggregation promotes copper binding) (Bagheri, Squitti et al. 2018), it can be clearly stated, that many truncated Aβ peptide s contain ATCUN motifs. Truncated peptides like Aβ4-x and Aβ11-x are present in amyloid plaques and present interest in researching the role of copper ions in Aβ peptide aggregation and toxicity (Cabrera, Mathews et al. 2018).

Figure 2. Example of ATCUN motif position in amino acid sequence of human hepcidin-25 (Abbas, Vranic et al. 2018).

4.2.3 ATCUN motif containing model peptides

Because the ATCUN motif is composed of the tripeptide, certain tripeptides of different proteins can serve as a great model for metal-binding studies, avoiding any interferences from more complicated structures of proteins. Design for tripeptides follows the simple rule: the first two amino acids can be any residues, and the third position must be histidine (X-Y-His).

In this study we used tripeptides GGH, MDH, DAH-COOH, and DAH-NH2. GGH, is the simplest model of naturally occurring ATCUN motif in proteins and peptides. MDH is a model of the N-terminal site of human copper transporter hCtr1. Both DAH tripeptides are human serum albumin models which differ only in the amidation of carboxyl group.

It has been shown that additional residues in the ATCUN motif models affect their metal-binding affinity. For example N-terminal DTH site containing model DTHFPI-NH2 hexapeptide and heptapeptide H-MEHFPGP-NH2 are the strongest Cu(II) binding peptides discovered so far (Płonka and Bal 2017). These peptide variants have also been studied in the current project.

Moreover, in the current project we studied also truncated Aβ 1-42 variant – Aβ 11-15. Aβ 11-15 focuses mainly on the second His part (FHH), which contains FHH ATCUN motif fragment in the sequence.

4.2.3 Binding affinity of ATCUN motifs

Binding affinity is the strength of the binding interaction between a single biomolecule to its ligand/binding partner.

One of the biggest complications when it comes to Cu(II) binding affinity is the existence of different constants for the same peptides. Some differences can be considered insignificant, others are substantial. This difference might originate from different methods or conditions of an experiment. To avoid differences, it is important to determine all constants using the same method and proceed with comparative analysis. Dissociation constants for ATCUN motif-containing peptides and proteins from the literature are presented in Table 1.

Table 1. Dissociation constants for ATCUN motif-containing peptides and proteins

	K_D, M	Method	Citation
GGH	4.0 x 10^{-13}	Potentiometric titration	(Hay, Hassan et al. 1993)
	6.3 x 10^{-13}	Combination of electronic absorption, circular dichroism, room-temperature electron paramagnetic resonance spectroscopies, potentiometric titration	(Bossak-Ahmad, Frączyk et al. 2020)
	6.3 x 10^{-13}	Potentiometric titration	(Kulprachakarn, Chen et al. 2016)

	K_D, M	Method	Citation
MDH	7.7×10^{-14}	Combination of electronic absorption, circular dichroism, room-temperature electron paramagnetic resonance spectroscopies, potentiometric titration	(Bossak-Ahmad, Frączyk et al. 2020)
	1.0×10^{-11}	Potentiometric titration	(Bossak, Drew et al. 2018)
	7.7×10^{-14}	Potentiometric titration	(Bossak, Drew et al. 2018)
DAH	1.0×10^{-12}	Competitive UV-vis spectroscopy titration	(Rózga, Sokołowska et al. 2007; Gonzalez, Bossak et al. 2018)
	1.6×10^{-14}	Competitive UV-vis spectroscopy titration	(Rózga, Sokołowska et al. 2007)
	1.6×10^{-14}	Combination of electronic absorption, circular dichroism, room-temperature electron paramagnetic resonance spectroscopies, potentiometric titration	(Bossak-Ahmad, Frączyk et al. 2020)
	2.0×10^{-14}	Combination of electronic absorption, circular dichroism, room-temperature electron paramagnetic resonance spectroscopies, potentiometric titration	(Bossak-Ahmad, Frączyk et al. 2020)
DTH	1.0×10^{-12}	Competitive UV-vis spectroscopy titration	(Rózga, Sokołowska et al. 2007)
	1.3×10^{-13}	Potentiometric titration	(Kulprachakarn, Chen et al. 2016)
VIH	1.0×10^{-13}	Potentiometric titration	(Sokolowska, Krezel et al. 2002)
YYH	4×10^{-15}	pH titration	(Miyamoto, Fukino et al. 2016)
MNH	3.1×10^{-15}	Potentiometric titration	(Bossak, Drew et al. 2018)
HSH	3.1×10^{-15}	Potentiometric titration	(Kolozsi, Jancsó et al. 2009)

	K_D, M	Method	Citation
RTH	3.1 x 10^{-15}	Potentiometric titration	(Bal, Jezowska-Bojczuk et al. 1997)
RFH	3.1 x 10^{-14}	Potentiometric and spectroscopic titrations	(Mital, Wezynfeld et al. 2015)
GGHG	1.6 x 10^{-13}	Potentiometric titration	(Kulprachakarn, Chen et al. 2016)
DSHEKR	2.2 x 10^{-15}	spectroscopic pH-metric titrations	(Frączyk 2021)
DpSHEKR	3.8 x 10^{-14} 2.6 x 10^{-13}	spectroscopic pH-metric titrations	(Frączyk 2021)
DAHKSE	2.7 x 10^{-14}	spectroscopic pH-metric titrations	(Frączyk 2021)
DAHKpSE	6 x 10^{-14}	spectroscopic pH-metric titrations	(Frączyk 2021)
MEHFPGP	1.3 x 10^{-15}	Electrospray ionization, UV–visible and circular dichroism spectroscopies	(Tabbì, Magrì et al. 2015)
DTHFPI-NH_2	2.2 x 10^{-15}	Potentiometric titration, UV–visible and circular dichroism spectroscopies	(Płonka and Bal 2017)
	2.2 x 10^{-15}	tandem mass spectrometry, high-resolution mass spectrometry, nuclear magnetic resonance spectroscopy	(Abbas, Vranic et al. 2018)
HSA	9.5 x 10^{-14}	Combination of electronic absorption, circular dichroism, room-temperature electron paramagnetic resonance spectroscopies, potentiometric titration	(Bossak-Ahmad, Frączyk et al. 2020)

The table shows that the Cu(II) binding constants of ATCUN motifs determined by different methods and by different authors vary by several orders of magnitude. There are also major differences between tripeptides and hexa/heptapeptides. Such differences may be due to the use of different methods or different experimental protocols. In order to understand the actual or comparable binding constants of ATCUN motifs, experiments with different peptides should be performed using the same methodology and experimental protocol.

5 Aims of the Study

The aim of the current study was to determine the relative binding affinities for ATCUN –containing peptides through performing direct competition experiments with HSA using Liquid Chromatography-Inductively Coupled Plasma Mass Spectrometry (LC-ICP MS) method, elaborated for the determination of Cu(II)-binding affinities of blood copper proteins.

The study has been focused on following peptides:

1. tripeptides (GGH, MDH, DAH-COOH, DAH-NH_2)
2. hexa- and heptapeptides (DTHFPI-NH_2, MEHFPGP-NH_2)
3. truncated Aβ peptide (Aβ11-15)

6 Experimental part

6.1 Materials

In Cu(II) ion binding experiments we used the following peptides: DAH-COOH, DAH-NH_2, MDH, GGH, DTHFPI-NH_2, MEHFPGP-NH_2 (all synthesized at the Institute of Technology of the University of Tartu), Aβ 11-15 (Peptide 2.0, USA). Human serum albumin (HSA) from Sigma/Merck (Darmstadt, Germany). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP); 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (Hepes), purchased from Sigma-Aldrich (St. Louis, MO, USA). Cu(II)acetate, purchased from Sigma (Sigma/Merck KGaA, Darmstadt, Germany). ICP-MS multielement calibration standard 2A in 2% HNO3 and ICP-MS internal standard mix 1 ug/mL in 2% HNO3, purchased from Agilent Technologies (USA). Ethylenediaminetetraacetic acid (EDTA, 99.995% trace metal basis), purchased from Fluka (Merck KGaA, Darmstadt, Germany). Ultrapure Milli-Q water with a resistivity of 18.2 MΩ/cm, produced by a Merck Millipore Direct-Q & Direct–Q UV water purification system (Merck KGaA, Darmstadt, Germany).

6.2 Methods

In this study we used LC-ICP MS method which can detect metallic elements at very low concentrations in liquids. System itself consists of many blocks: a sample insertion system, an inductively coupled plasma interface, ion optics, a mass analyser and a detector.

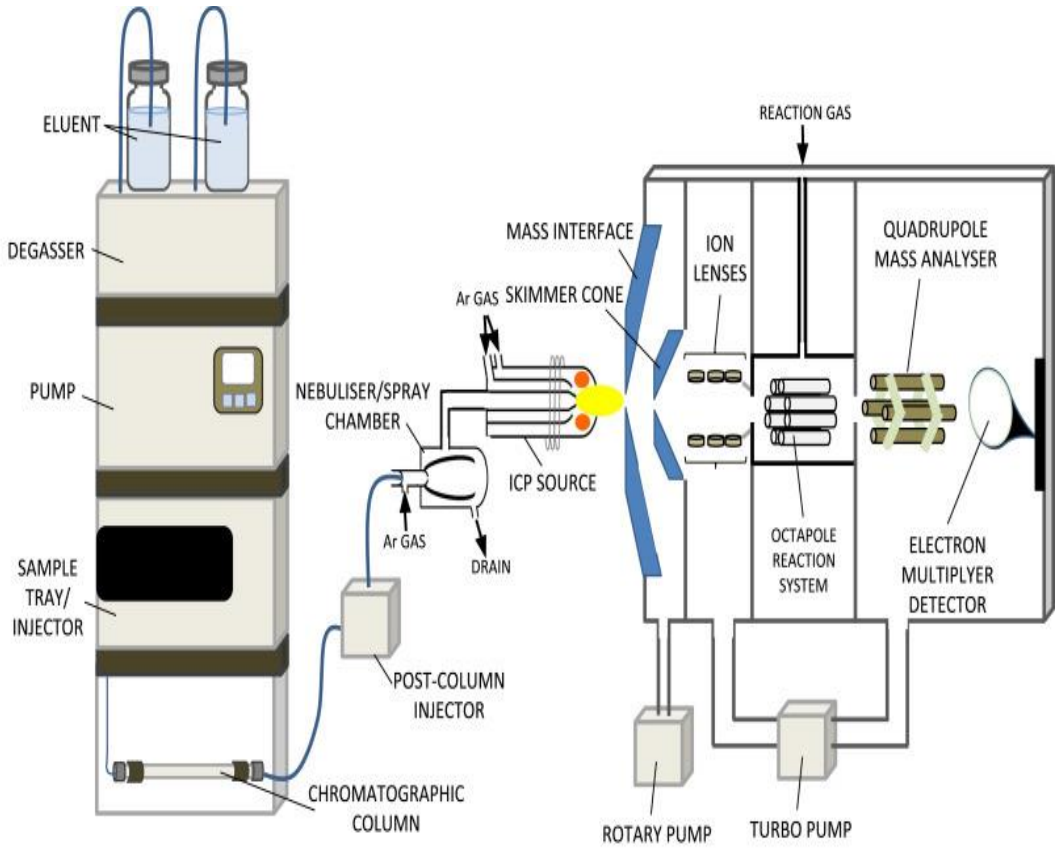


Figure 3. A Brief Overview of LC-ICP MS instrumentation (Delafiori, Ring et al. 2016).

LC-ICP MS analysis was performed using an Agilent Technologies (Santa Clara, USA) Infinity HPLC system consisting of a 1260 series µ-degasser, 1200 series capillary pump, Micro WPS autosampler, and 1200 series MWD VL detector with Agilent 7800 series ICP-MS instrument (Agilent, Santa Clara, USA). Instrument ICP-MS MassHunter 4.4 version C.01.04 (Agilent) was used to check instruments and analyse data. ICP-MS was operated under conditions: RF power 1550 W, nebulizer gas flow 1.05 l/min, auxiliary gas flow 0.90 l/min, plasma gas flow 15 l/min, nebulizer type: MicroMist, elements monitored: Cu-63.

Separation of different copper proteins was conducted using Sephadex G-25, which separates compounds by weight, by using an injection volume of 10 µl. ICP MS compatible flow rate was 0.4 ml/min. In order to get rid of contaminating metal ions in the buffer, the mobile phase was eluted through the Chelex®100 Chelating Ion Exchange resin (Sigma, Merck KGaA, Darmstadt, Germany) before liquid chromatographic separation. Mobile phase and reagent solutions were prepared daily before the experiment.

6.2.1 Sample preparation

All lyophilized peptides were dissolved in HFIP at a concentration of 100 µM to disassemble preformed aggregates. The solution was split into aliquots, HFIP was vacuum evaporated, and the tubes containing the peptide film were kept at 80 °C until needed.

For all experiments, the HSA stock solution was prepared in type II water and then diluted in reaction buffer solution (50 mM Hepes, 50 mM NaCl, pH 7.4). HFIP-treated peptide aliquots were dissolved in 10 mM NaOH, incubated on ice for 10 minutes, then diluted to final concentration using 50 mM Hepes and 50 mM NaCl, pH 7.4. Cu(II)aetate was used to metalate HSA, Aβ (11–15), and Aβ (4–16). In 50 mM Hepes, 50 mM NaCl, pH 7.4, a suitable quantity of Cu(II)acetate was added to HSA, Aβ (11–15), and Aβ (4–16).

7 Results and discussion

7.1 Determination of incubation time.

By using peptide (Aβ11-15) we determined the time necessary for reaching the equilibrium between peptides and HSA for Cu(II) binding (Figure 4).

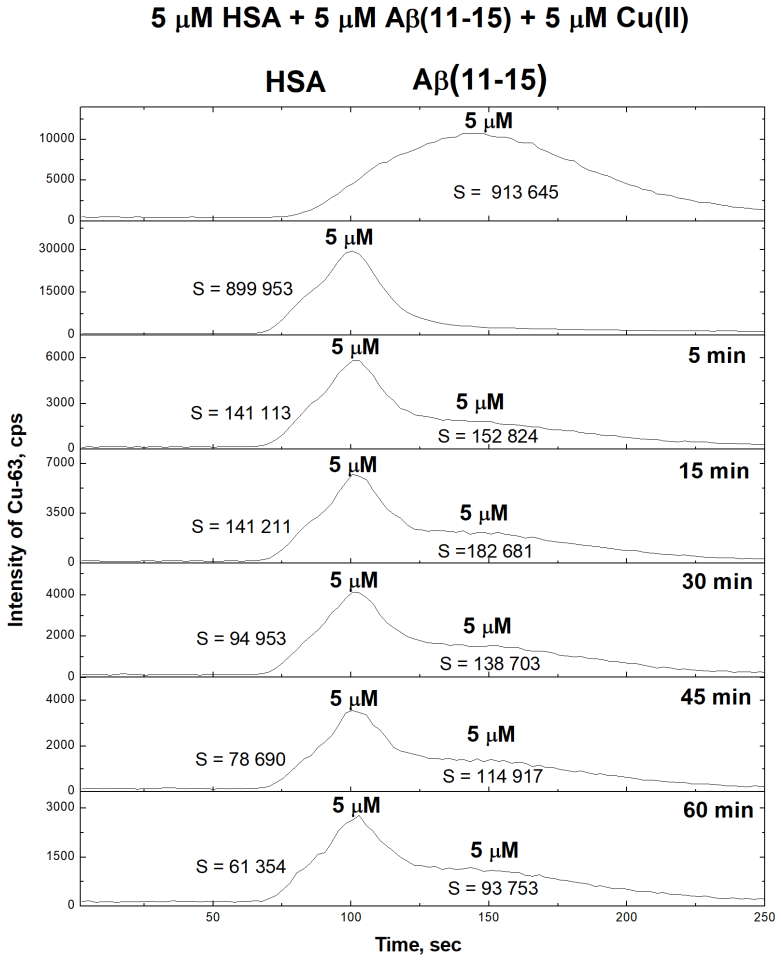


Figure 4. Competition kinetics between HSA and Aβ11-15 for Cu(II) ions determined by LC-ICP MS. Conditions: 5 μM HSA, 5 μM Aβ11-15, 5 μM Cu^{2+} in 50 mM HEPES, 50 mM NaCl pH 7.4; ICP MS: column - 1 ml Sephadex G-25 Superfine; elution buffer 200 mM NH_4NO_3, pH 7.4; flow rate 0.4 ml/min; injection volume 10 μl, Cu-63 was monitored by ICP MS.

It is seen from Figure 5 that at 30 minutes reaction reaches equilibrium between competitor compounds and in all further experiments incubation period of 30 minutes was used.

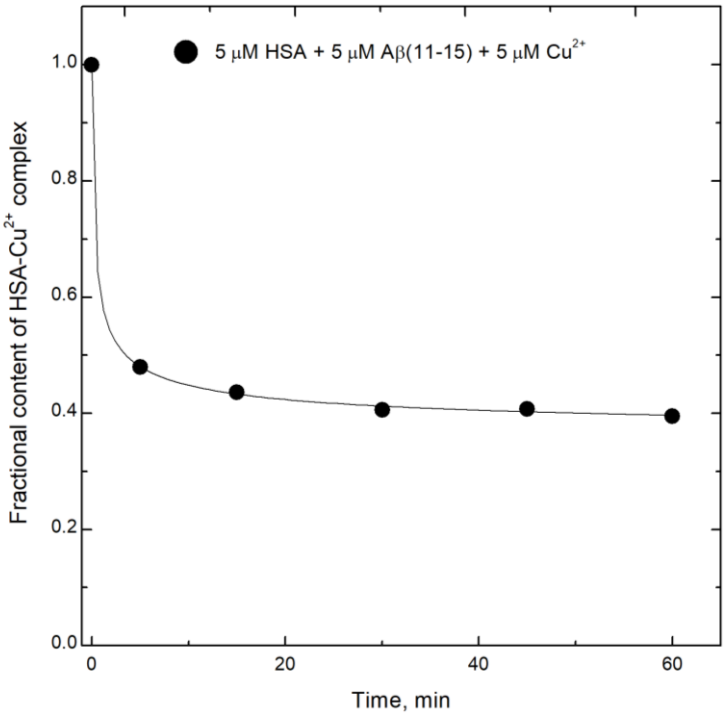


Figure 5. Competition of Aβ11-15 and HSA for binding of Cu (II) ions. Conditions: 5 µM HSA, 5 µM Aβ11-15, 5 µM Cu^{2+} in 50 mM HEPES, 50 mM NaCl pH 7.4; ICP MS: column - 1 ml Sephadex G-25 Superfine; elution buffer 200 mM NH_4NO_3, pH 7.4; flow rate 0.4 ml/min; injection volume 10 µl, Cu-63 was monitored by ICP MS.

7.2 Determination of relative dissociation constants.

Experimental results for competition for Cu(II) ions between HSA and ATCUN-containing peptides were used for calculation of the relative dissociation constant for ATCUN-containing peptides. In calculation following equation was used (Bossak-Ahmad, Frączyk et al. 2020):

$$\frac{K^{Cu}_{Cu(HSA)}}{K^{Cu}_{Cu(Peptide)}} = \frac{[Cu(HSA)]([Peptide]_{theoretical} - [Cu(Peptide)])}{[Cu(Peptide)]([HSA]_{theoretical} - [Cu(HSA)])} \quad (1)$$

which was then modified to:

$$Y = \frac{Kd(Peptide)}{Kd(HSA)} \cdot X \quad \text{, where} \quad (2)$$

$$X = [Cu(HSA)]([Peptide]_{theoretical} - [Cu(Peptide)]),$$

$$Y = [Cu(Peptide)]([HSA]_{theoretical} - [Cu(HSA)]).$$

Competition between HSA and GGH. GGH is a model peptide, which competition with HSA is presented in Figure 6.

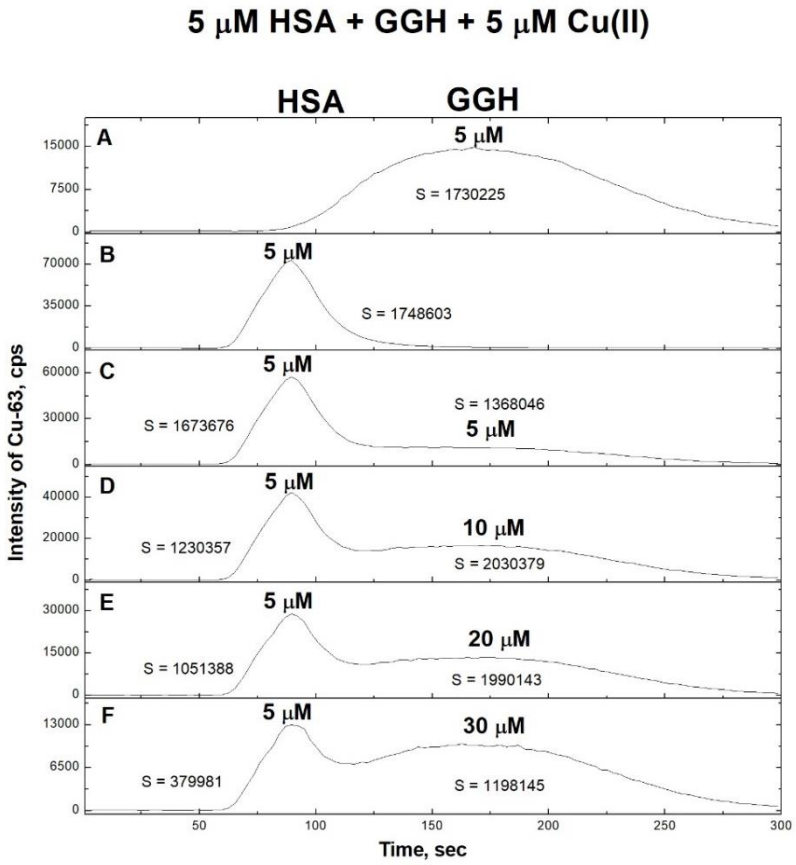


Figure 6. Competition between HSA and GGH for Cu(II) ions determined by LC-ICP MS. Conditions: 5 μM HSA, 5 – 30 μM concentrations of GGH, 5 μM Cu^{2+} in 50 mM HEPES, 50 mM NaCl pH 7.4; ICP MS: column - 1 ml Sephadex G-25 Superfine; elution buffer 200 mM NH_4NO_3, pH 7.4; flow rate 0.4 ml/min; injection volume 10 μl, Cu-63 was monitored by ICP MS.

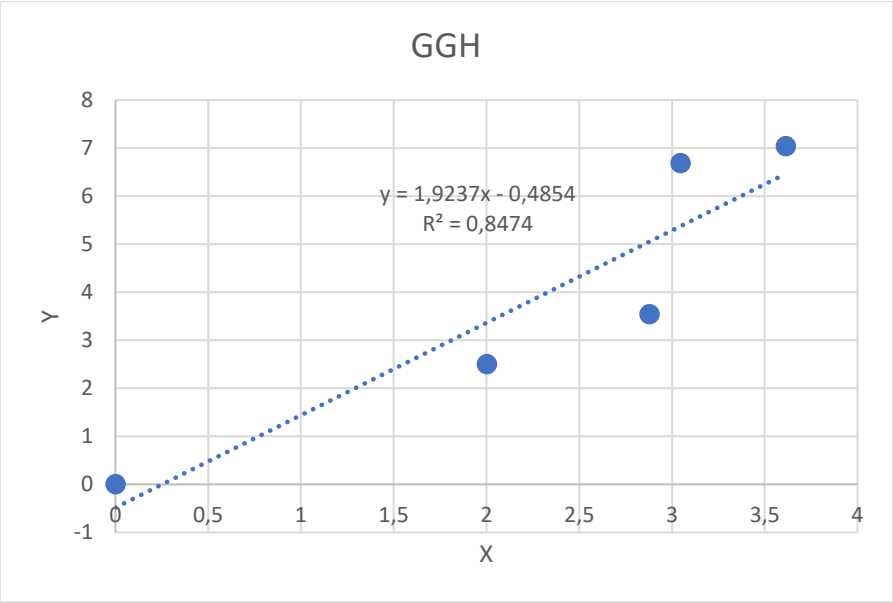


Figure 7. Determination of relative dissociation constant for GGH-Cu(II) complex according to Equation (2). Conditions: Buffer - 50 mM HEPES, 50 mM NaCl pH 7.4.

By using the Equation (2) the linear correlation between X and Y was calculated, which showed that K_D(Peptide) / K_D(HSA) is equal to 1.92 (Figure 7). This means that Cu(II)-peptide dissociation constant K_D = 1.82 x 10-13 M.

Competition between HSA and MDH. MDH is a N-terminal tripeptide from copper transporter Ctr1, which competition with HSA is presented in Figure 8.

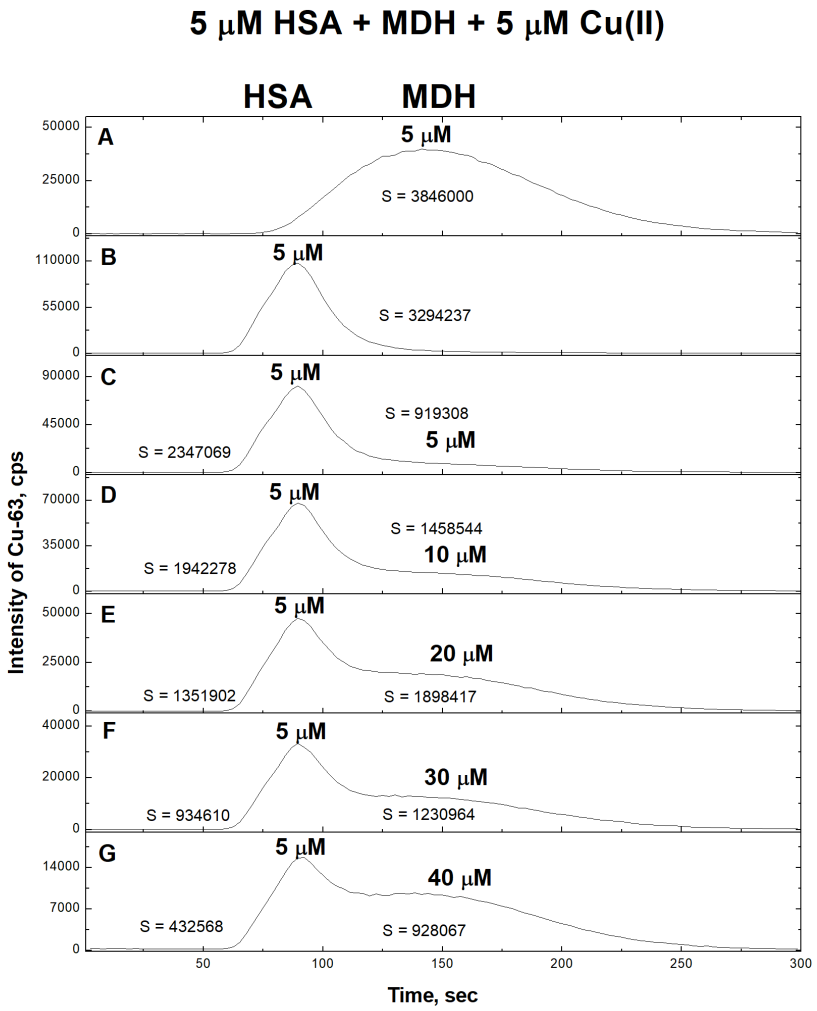


Figure 8. Competition between HSA and MDH for Cu(II) ions determined by LC-ICP MS. Conditions: 5 μM HSA, 5 – 40 μM concentrations of MDH, 5 μM Cu^{2+} in 50 mM HEPES, 50 mM NaCl pH 7.4; ICP MS: column - 1 ml Sephadex G-25 Superfine; elution buffer 200 mM NH_4NO_3, pH 7.4; flow rate 0.4 ml/min; injection volume 10 μl, Cu-63 was monitored by ICP MS.

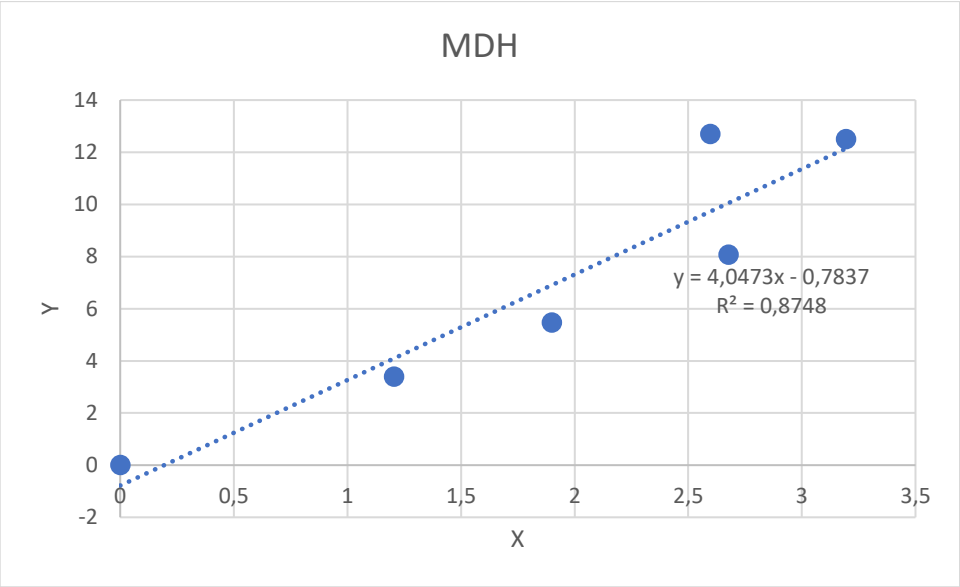


Figure 9. Determination of relative dissociation constant for MDH-Cu(II) complex according to Equation (2). Conditions: Buffer - 50 mM HEPES, 50 mM NaCl pH 7.4.

By using the Equation (2) the linear correlation between X and Y was calculated, which showed that K_D(Peptide) / K_D(HSA) is equal to 4.05 (Figure 9). This means that Cu(II)-peptide dissociation constant $K_D = 3.84 \times 10^{-13}$ M.

Competition between HSA and DAH-COOH. DAH-COOH is a N-terminal tripeptide from HSA, which competition with HSA is presented in Figure 10.

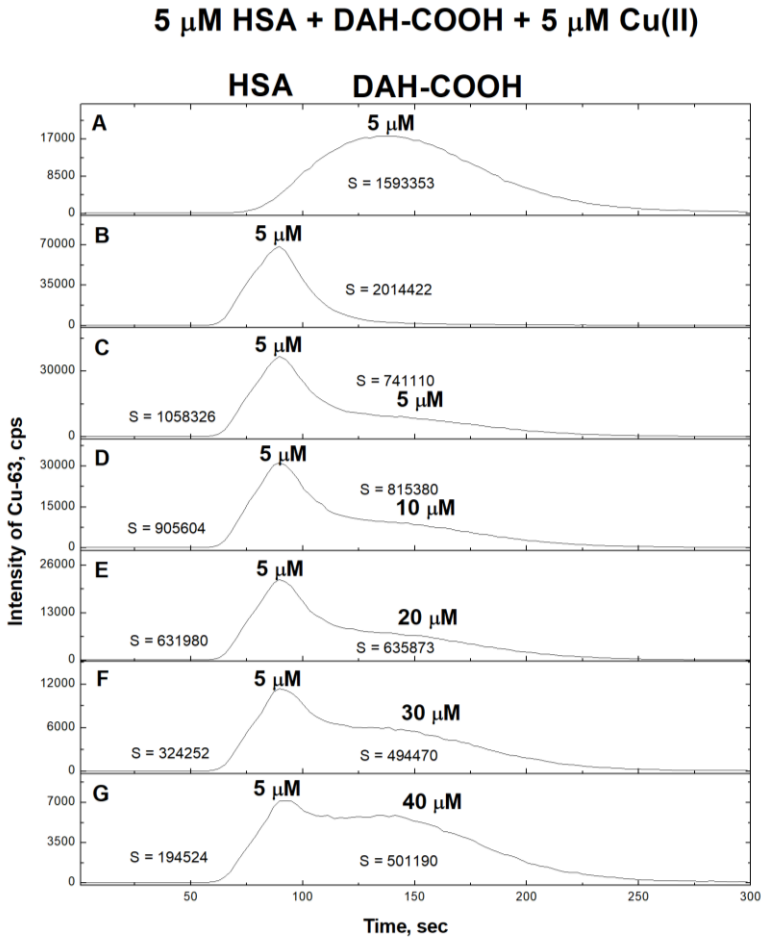


Figure 10. Competition between HSA and DAH-COOH for Cu(II) ions determined by LC-ICP MS. Conditions: 5 μM HSA, 5 – 40 μM concentrations of DAH-COOH, 5 μM Cu^{2+} in 50 mM HEPES, 50 mM NaCl pH 7.4; ICP MS: column - 1 ml Sephadex G-25 Superfine; elution buffer 200 mM NH_4NO_3, pH 7.4; flow rate 0.4 ml/min; injection volume 10 μl, Cu-63 was monitored by ICP MS.

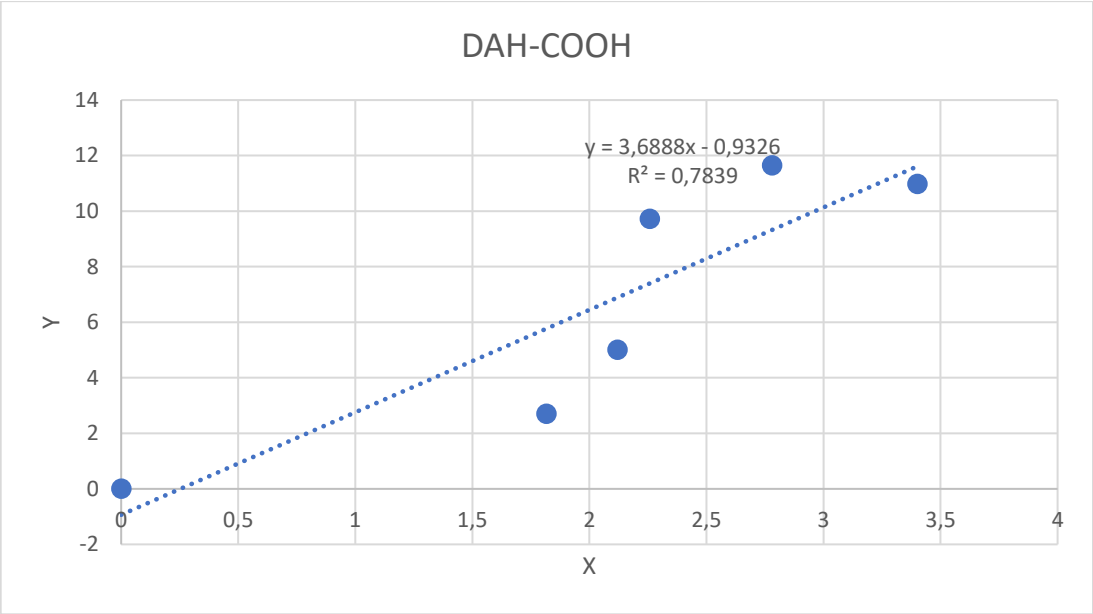


Figure 11. Determination of relative dissociation constant for DAH-COOH-Cu(II) complex according to Equation (2). Conditions: Buffer - 50 mM HEPES, 50 mM NaCl pH 7.4.

By using the Equation (2) the linear correlation between X and Y was calculated, which showed that K_D(Peptide) / K_D(HSA) is equal to 3.69 (Figure 11). This means that Cu(II)-peptide dissociation constant $K_D = 3.51 \times 10^{-13}$ M.

Competition between HSA and DAH-NH₂. DAH-NH_2 is an amidated N-terminal tripeptide from HSA, which competition with HSA is presented in Figure 12.

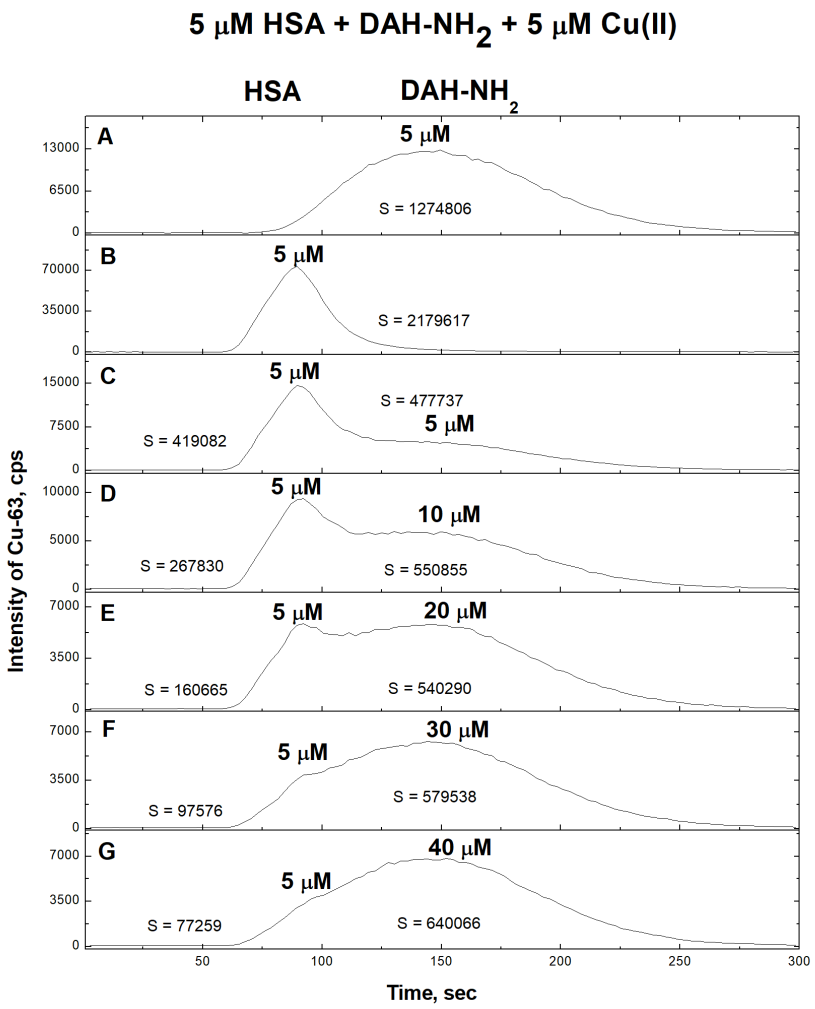


Figure 12. Competition between HSA and DAH-NH_2 for Cu(II) ions determined by LC-ICP MS. Conditions: 5 μM HSA, 5 – 40 μM concentrations of DAH-$CONH_2$, 5 μM Cu^{2+} in 50 mM HEPES, 50 mM NaCl pH 7.4; ICP MS: column - 1 ml Sephadex G-25 Superfine; elution buffer 200 mM NH_4NO_3, pH 7.4; flow rate 0.4 ml/min; injection volume 10 μl, Cu-63 was monitored by ICP MS.

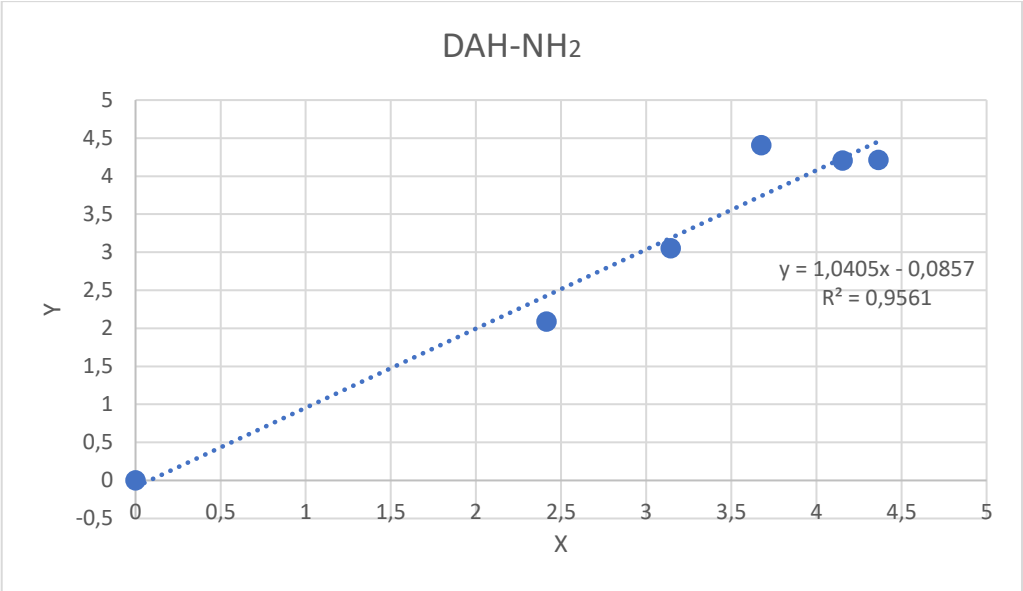


Figure 13. Determination of relative dissociation constant for DAH-NH_2-Cu(II) complex according to Equation (2). Conditions: Buffer - 50 mM HEPES, 50 mM NaCl pH 7.4.

By using the Equation (2) the linear correlation between X and Y was calculated, which showed that K_D(Peptide) / K_D(HSA) is equal to 1.04 (Figure 13). This means that Cu(II)-peptide dissociation constant K_D = 9.88 x 10^{-14} M.

Competition between HSA and DTHFPI. DTHFPI is an amidated hexapeptide from human hepcidin-25, with exposes according to literature (Płonka and Bal 2017) the second highest affinity for Cu(II) ions and its competition with HSA for Cu(II) ions is presented in Figure 14.

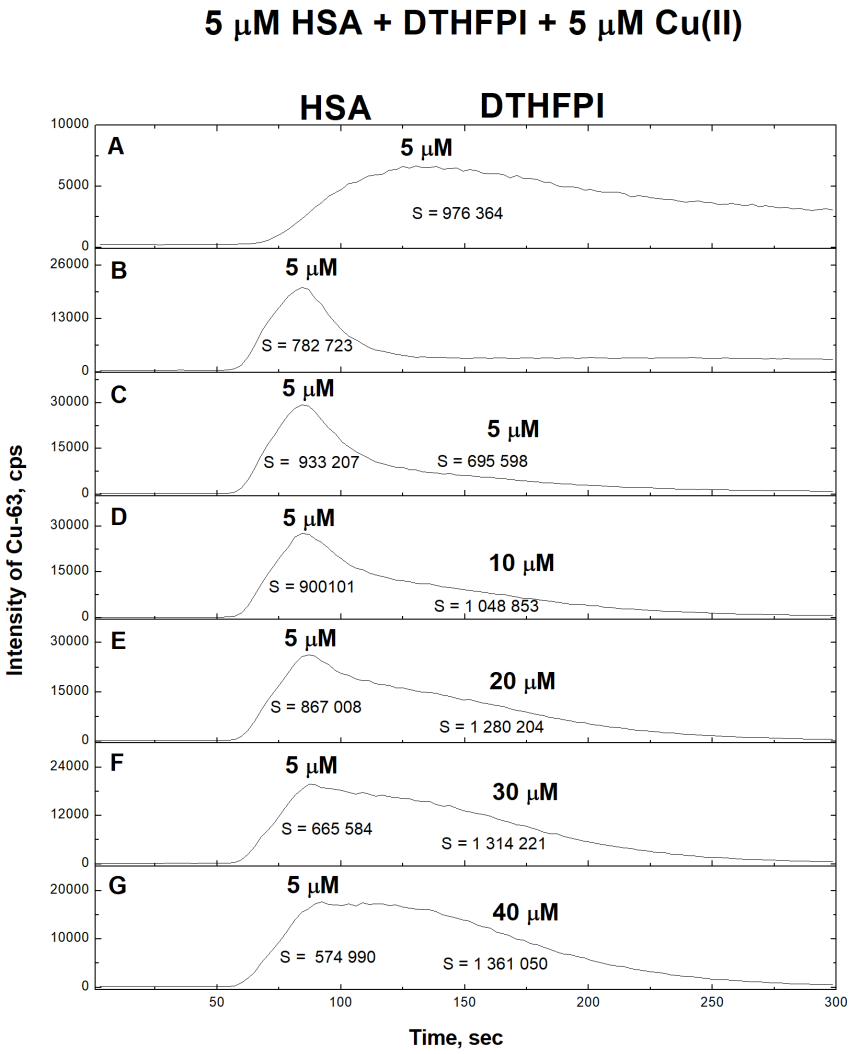


Figure 14. Competition between HSA and DTHFPI for Cu(II) ions determined by LC-ICP MS. Conditions: 5 μM HSA, 5 – 40 μM concentrations of DTHFPI, 5 μM Cu^{2+} in 50 mM HEPES, 50 mM NaCl pH 7.4; ICP MS: column - 1 ml Sephadex G-25 Superfine; elution buffer 200 mM NH_4NO_3, pH 7.4; flow rate 0.4 ml/min; injection volume 10 μl, Cu-63 was monitored by ICP MS.

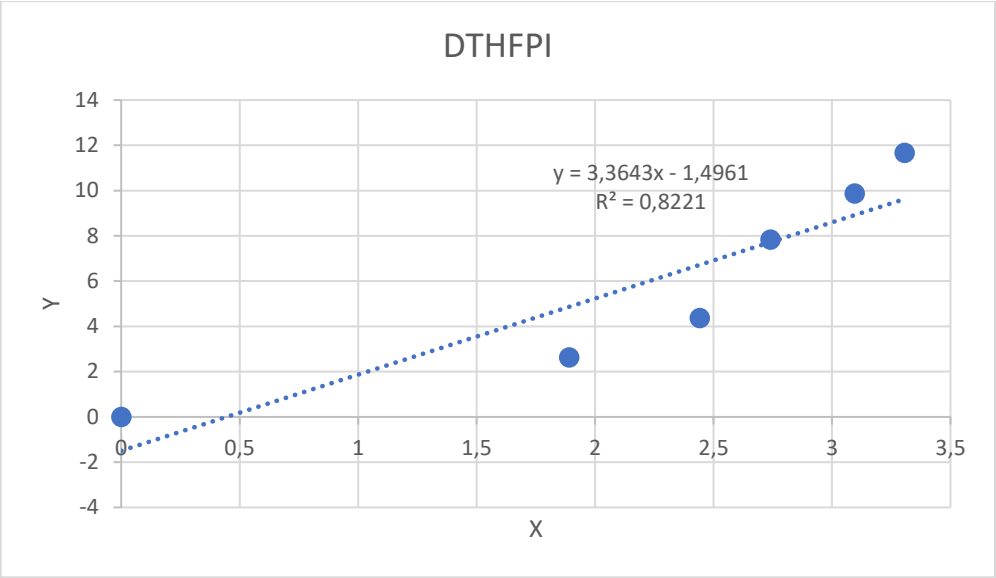


Figure 15. Determination of relative dissociation constant for DTHFPI-Cu(II) complex according to Equation (2). Conditions: Buffer - 50 mM HEPES, 50 mM NaCl pH 7.4.

By using the Equation (2) the linear correlation between X and Y was calculated, which showed that K_D(Peptide) / K_D(HSA) is equal to 3.36 (Figure 15). This means that Cu(II)-peptide dissociation constant K_D = 3.19 x 10^{-13} M.

Competition between HSA and MEHFPGP. MEHFPGP is an amidated heptapeptide Semax, with exposes according to literature (Tabbì, Magrì et al. 2015) the highest affinity for Cu(II) ions and its competition with HSA for Cu(II) ions is presented in Figure 16.

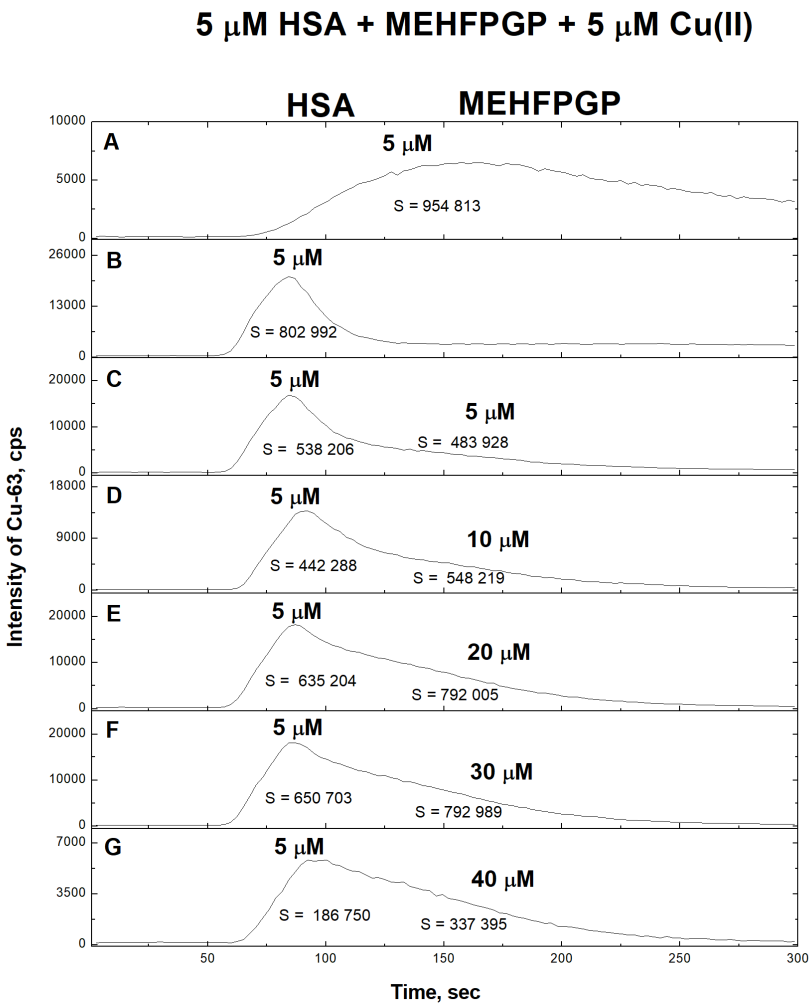


Figure 16. Competition between HSA and MEHFPGP for Cu(II) ions determined by LC-ICP MS. Conditions: 5 μM HSA, 5 – 30 μM concentrations of MEHFPGP, 5 μM Cu^{2+} in 50 mM HEPES, 50 mM NaCl pH 7.4; ICP MS: column - 1 ml Sephadex G-25 Superfine; elution buffer 200 mM NH_4NO_3, pH 7.4; flow rate 0.4 ml/min; injection volume 10 μl, Cu-63 was monitored by ICP MS.

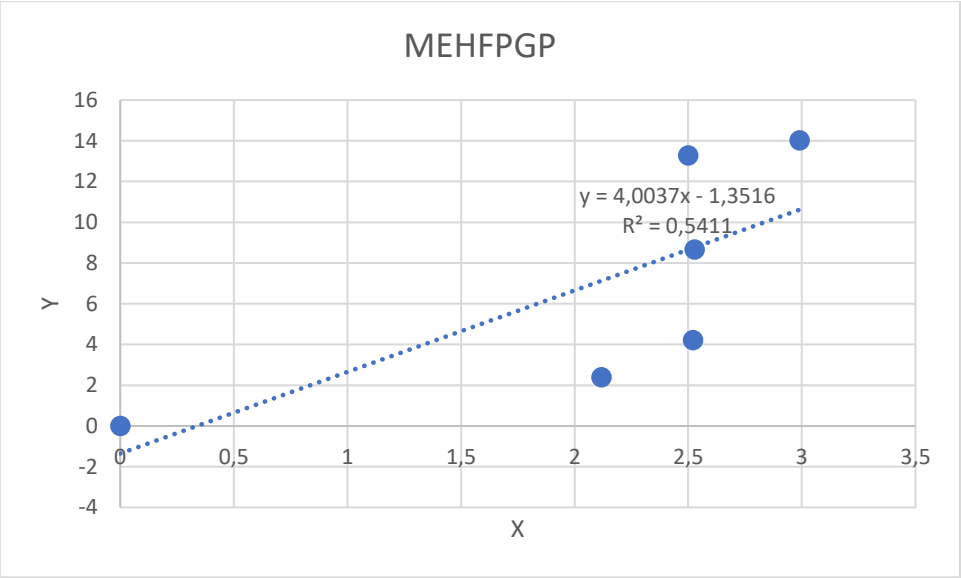


Figure 17. Determination of relative dissociation constant for MEHFPGP-Cu(II) complex according to Equation (2). Conditions: Buffer - 50 mM HEPES, 50 mM NaCl pH 7.4.

By using the Equation (2) the linear correlation between X and Y was calculated, which showed that K_D(Peptide) / K_D(HSA) is equal to 4.00 (Figure 17). This means that Cu(II)-peptide dissociation constant $K_D = 3.80 \times 10^{-13}$ M.

Competition between HSA and Aβ11-15. Aβ11-15 is an truncated model peptide from Aβ peptides, which competition with HSA for Cu(II) ions is presented in Figure 18.

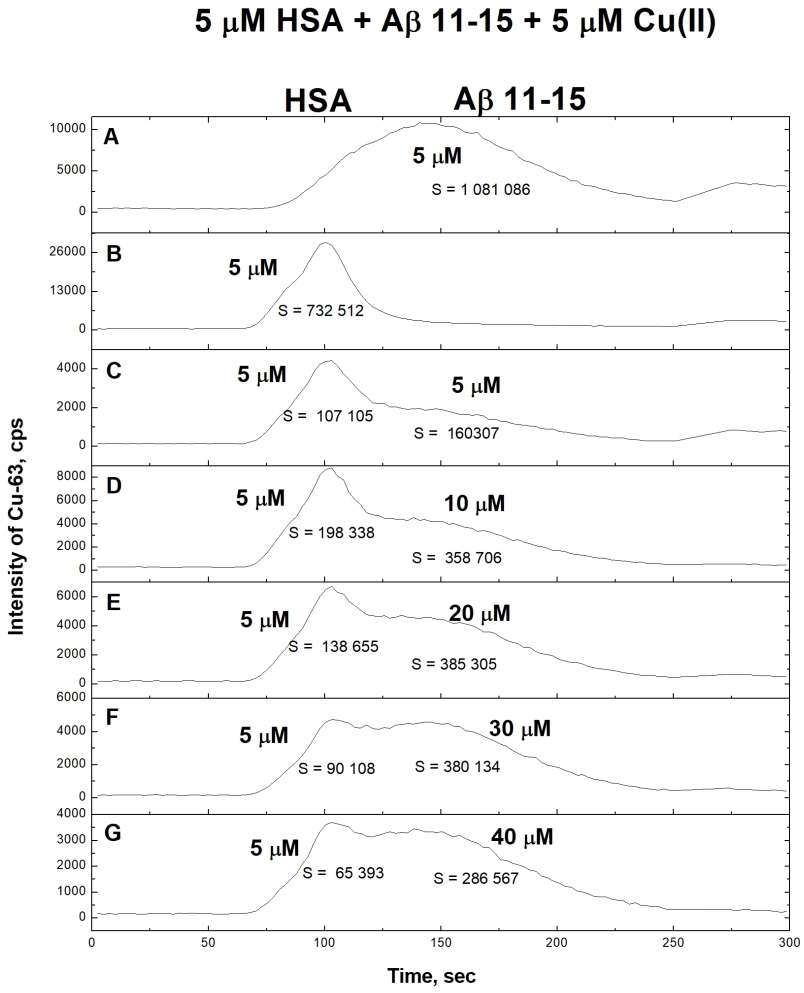


Figure 18. Competition between HSA and Aβ 11-15 for Cu(II) ions determined by LC-ICP MS. Conditions: 5 μM HSA, 5 – 40 μM concentrations of Aβ 11-15, 5 μM Cu^{2+} in 50 mM HEPES, 50 mM NaCl pH 7.4; ICP MS: column - 1 ml Sephadex G-25 Superfine; elution buffer 200 mM NH_4NO_3, pH 7.4; flow rate 0.4 ml/min; injection volume 10 μl, Cu-63 was monitored by ICP MS.

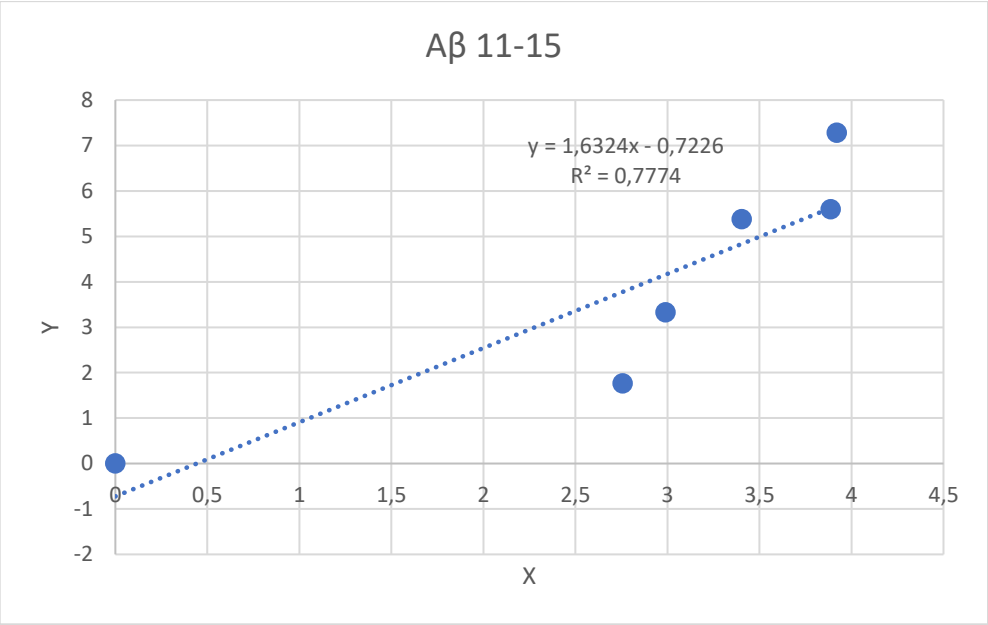


Figure 19. Determination of relative dissociation constant for Aβ(11-15)-Cu(II) complex according to Equation (2). Conditions: Buffer - 50 mM HEPES, 50 mM NaCl pH 7.4.

By using the Equation (2) the linear correlation between X and Y was calculated, which showed that K_D(Peptide) / K_D(HSA) is equal to 1.63 (Figure 19). This means that Cu(II)-peptide dissociation constant K_D = 1.55 x 10^{-13} M.

Obtained results are presented in Table 2.

Table 2. Dissociation constants for ATCUN motif-containing peptides and HSA

Peptide	K_D, M
GGH	1.82 x 10^{-13}
MDH	3.84 x 10^{-13}
DAH-COOH	3.51 x 10^{-13}
DAH-NH_2	9.88 x 10^{-14}
DTHFPI	3.19 x 10^{-13}
MEHFPGP	3.80 x 10^{-13}
Aβ 11-15	1.55 x 10^{-13}
HSA	9.50 x 10^{-14}

It follows from the Table 2 that dissociation constants for ATCUN-containing peptides and HSA vary in rather narrow range from 9.88 x 10^{-14} M to 3.84 x 10^{-13} M HSA has the highest affinity for Cu(II) ions - K_D = 9.50 x 10^{-14} M. Its N-terminal amidated tripeptide DAH-NH_2 has similar binding affinity K_D = 9.88 x 10^{-14} M, whereas tripeptide with free carboxylate exposed reduced affinity K_D = 3.51 x 10^{-13} M. Hexa- and heptapeptides, which had according to the literature (Tabbì, Magrì et al. 2015; Płonka and Bal 2017) highest affinities for Cu(II) ions did not expose high affinities and were 3.3 - 4 times weaker as HSA. Truncated Aβ11-15 peptide exposed relatively high Cu(II)-binding affinity K_D = 1.55 x 10^{-13} M, which is comparable with HSA.

Taken together our results demonstrate that all ATCUN motifs expose quite similar Cu(II)-binding affinities and there exists difference in Cu(II)-binding affinities between amidated and nonamidated tripeptides.

8 Abstract

In the body, HSA is one of the main binders and transporters of copper ions. The center responsible for the ability of HSA to bind copper ions is ATCUN (amino-terminal Cu(II)- and Ni(II)-binding) motif, located in N-terminal of the protein. However, ATCUN motif is not unique to HSA. This motif, with different sequences, is found in many peptides and proteins.

Since the interaction between peptides/proteins and copper ions is of interest in studying the nature of these biomolecules and their effects on the copper metabolism, ATCUN motif has gained much of attention. ATCUN motifs are characterized by the most fundamental parameters, which are dissociation constants (K_D) for corresponding Cu(II)-peptide complexes.

In case of well-studied proteins, like HSA, the K_D values are well known and agree in different papers, however, in case of ATCUN-containing peptides the determined K_D values differ in large range. Possible explanations for this situation could be connected with the use of different research methods and different research protocols by different authors.

This work was aimed at developing a specific protocol and using one method to obtain comparable dissociation constants for different peptides like tripeptides (GGH, MDH, DAH-COOH, DAH-NH_2), hexa and heptapeptides (H-DTHFPI-NH2, H-MEHFPGP-NH2) and truncated Aβ peptide Aβ11-15.

Obtained results show that dissociation constants for ATCUN-containing peptides and HSA vary in rather narrow range. HSA has the highest affinity for Cu(II) ions - $K_D = 9.50 \times 10^{-14}$ M. Its N-terminal amidated tripeptide DAH-NH_2 has similar binding affinity $K_D = 9.88 \times 10^{-14}$ M, whereas tripeptide with free carboxylate exposed reduced affinity $K_D = 3.51 \times 10^{-13}$ M. Hexa- and heptapeptides, which had according to the literature highest affinities for Cu(II) ions did not expose high affinities and were 3.3 – 4 times weaker as HSA. Truncated Aβ11-15 peptide exposed relatively high Cu(II)-binding affinity *K*, which is comparable with HSA.

9 Kokkuvõtte

HSA on ekstratsellulaarne verevalk, mis on üks peamisi vaseoonide sidujaid ja transportijaid. HSA vaseioone sidumisvõime eest vastutab ATCUN (aminoterminaalne Cu(II)- ja Ni(II)-siduv) motiiv, mis asub valgu N-terminaalses otsas. ATCUN motiiv koosneb vabast N-terminaalsest tripeptiidist, kus kolmandal positsioonil olev aminohappejääk on His. ATCUN motiiv pole HSA-le ainuomane, vaid seda motiivi leidub paljudes peptiidides ja valkudes.

Kuna peptiidide/valkude ja vase ioonide vaheline interaktsioon pakub huvi nende biomolekulide olemuse ja nende mõju uurimiseks vase metabolismile, on ATCUNi motiiv pälvinud palju tähelepanu. ATCUN motiive iseloomustavaks kõige fundamentaalsemaks parameetriks on vastavate Cu(II)-peptiidi komplekside dissotsiatsioonikonstandid (K_D).

Hästi uuritud valkude (näiteks HSA) puhul on K_D väärtused hästi teada ja nad on sarnased erinevates töödes, kuid ATCUN-i sisaldavate peptiidide puhul erinevad määratud K_D väärtused suures vahemikus. Selline olukord võib olla tekkinud erinevate uurimismeetodite ja erinevate uurimisprotokollide kasutamisest erinevate autorite poolt.

Käesoleva töö eesmärk oli välja töötada spetsiifiline LC-ICP MS protokoll ja kasutada seda ühte meetodit erinevate Cu(II)-peptiidide komplekside dissotsiatsioonikonstantide määramiseks. Uurimise all olid tripeptiidid (GGH, MDH, DAH-COOH, DAH-NH_2), heksa- ja heptapeptiidid (DTHFPI, MEHFPGP) ja trunkeeritud Aβ peptiid Aβ11-15 ning määrati peptiidide suhteline K_D HSA suhtes otsese HSA-ga konkurentsikatse tulemustest.

Saadud tulemused näitavad, et ATCUN-i sisaldavate peptiidide ja HSA dissotsiatsioonikonstandid varieeruvad üsna kitsas vahemikus. HSA-l on kõrgeim afiinsus Cu(II) ioonide suhtes – $K_D = 9{,}50 \times 10^{-14}$ M. Selle N-terminaalsel amideeritud tripeptiidil DAH-NH_2 on sarnane seondumisafiinsus $K_D = 9{,}88 \times 10^{-14}$ M, samas kui vaba karboksülaadiga tripeptiidi afiinsus on 3.6 korda madalam. Heksa- ja heptapeptiidid, millel oli kirjanduse andmetel kõrgeim afiinsus Cu(II) ioonide suhtes, ei omanud kõrget afiinsust ja olid 3,3 - 4 korda nõrgemad kui HSA. Trunkeeritud Aβ11-15 peptiid omas suhteliselt kõrge Cu(II) sidumisafiinsuset, mis on võrreldav HSA-ga.

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