The metallacrowns as templates for spontaneous self-assembly of polypeptides into a tetrahedral bundle†

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Amphiphilic peptide sequences with β-aminohydroxamic acid (βahx) at their C-termini were complexed with Cu2+ ions resulting in formation of stable metallacrowns composed of βahx and copper. The ESI-MS data for the obtained compounds show formation of supramolecular structures of Mw approx. 7000 Da with the compositions corresponding to metallacrowns. The addition of Cu2+ to a solution of βahx-containing peptides results in distinct changes in the CD spectra indicating a shift of their conformational equilibria toward helical structures. The experiments with enzymatic hydrolysis show that formation of metallacrown systems significantly increases the proteolytic stability of investigated peptides.

A successful attempt to design polypeptides which form a defined structure (triple helix bundle) in the presence of a metal ion has been previously reported. A amphiphatic peptide sequence was conjugated with a derivatized bipyridyl moiety. Conjugates synthesized in the presence of transition metal ions formed a triple helix bundle which was evidenced by CD spectra as well as by an ESI-MS technique. Additionally, the independent determination of molecular mass by gel filtration confirmed that the trimeric form is predominant in solution. The above-mentioned system was further studied by various methods and applied as a platform for the search for stable arrangements of triple helices by dynamic combinatorial chemistry.

Herein we present a new dynamic scaffold which can be used for designing self-assembling systems. This scaffold is based on a metallacrown – a stable structure formed by aminohydroxamic acids in the presence of copper(II) ions. The metallacrown assembly has been previously described. This supramolecule is formed by 4 aminohydroxamate ligands and 5 Cu2+ ions. Many papers have documented the stability and geometry of the formed metallacrown. The literature data suggest that the metallacrown scaffold may be an interesting template for self-assembly of peptide chains. This molecule is stable in water solution. The complex with the L4Cu5 stoichiometry is reported to be the only species at pH from 5 to 10. The metallacrown is a relatively rigid molecule and its geometry is similar to that of the template described by Mutter. The structure of this molecule enables the formation of the models of tetrahedral bundles – the supersecondary structures common in many proteins. Another interesting property of the metallacrown system is its possible chirality resulting from the application of chiral building blocks as the supramolecule components. In this case the relative orientation of peptide backbones is controlled by the chirality of the aminohydroxamate moiety.

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Introduction

Despite the accumulation of a large body of information on the protein folding and structures, designing stable artificial proteins is a difficult task. This problem can be simplified significantly by preorganization of relatively short peptides with predictable conformational preferences on a rigid template ensuring the orientation of peptide chains optimal for formation of defined secondary structures. This approach was initiated by Manfred Mutter who introduced the concept of TASP (template assembled synthetic proteins). The first molecules based on the TASP concept resulted from attachment of short amphiphatic peptides with high helical propensities to a cyclic peptide playing the role of a relatively rigid template. The structure of the template was modified in further investigations and currently there are many examples of applying various types of molecules including peptides, carbohydrates, porphyrins, steroids, calixarenes, and cavatins as scaffolds. The important characteristic of a TASP molecule is that the peptides are attached to the template by stable covalent bonds. On the other hand, such structures can be formed by peptides containing moieties with a large hydrogen bond or metal-complexation potential. Such a design makes the TASP molecule a reversible, dynamic system, which can be studied by the methods of combinatorial chemistry.

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Results and discussion

Design and synthesis of peptides

The literature data show a high stability of complexes formed by aminohydroxamic acids with ions of transition metals. The interactions of Cu$^{2+}$ ions with aminohydroxamic acids result in formation of supramolecular structures with the L$_4$Cu$_5$ stoichiometry. The stability of metallacrowns formed by α, β, and γ aminohydroxamic acids and Cu$^{2+}$ ions changes in the following order: β > γ > α. There are also reports on formation of stable supramolecular structures by histidine hydroxamic acid, but in this case the metallacrown structure is formed by the hydroxamic and imidazoyl moieties. In our study we decided to combine a stable metallacrown formed by the β-hydroxamic acid with the amphiphilic peptide sequences applied by Mutter as the constituents of TASP. To study the influence of the side chain charge on formation of the “dynamic TASP”, in addition to the hydroxamic acid-containing modified peptide sequence used by Mutter ([AD]-NHOH), we synthesized also [ADE]-NHOH and [ADK]-NHOH sequences composed of only negatively or only positively charged side chains, respectively. All the synthesized peptides were acetylated on their N-termini to avoid the interactions of Cu$^{2+}$ ions with N-terminal α-amino groups. All peptides were obtained on a ChemMatrix resin derivatized according to a procedure described recently. The sequences of all peptides investigated in this study are presented in Scheme 1.

In the synthesis of [AD]-NHOH, [ADE]-NHOH, and [ADK]-NHOH, the first amino acid attached to the hydroxylamine resin was Boc-Asp-EDA-Fmoc – the derivative of aspartic acid in which the β-carboxylic group is attached to the resin, while the α-carboxylic group is derivatized by N-Fmoc-ethylenediamine (Fmoc-EDA).

We also synthesized peptides containing histidine hydroxamate at the C-termini ([AH]-NHOH and [AH1]-NHOH) as well as peptide [AD]-NH$_2$ – the analogue of peptide [AD]-NHOH, in which the C-terminal hydroxamate moiety was replaced by an amide group. The last compound was used as a negative control.

The details of synthesis of peptides has been described in a separate paper. The analytical data of all obtained compounds are presented in the Experimental section.

ESI-MS study on metallacrown formation

The obtained peptides were investigated by the ESI-MS technique for their ability to form supramolecular structures. All the compounds were dissolved in the water–methanol 1:1 system containing ammonium carbonate (10$^{-2}$ M). The concentration of the peptide was 10$^{-4}$ M and the concentration of Cu$^{2+}$ ions was 1.25 × 10$^{-4}$ M (ligand : Cu$^{2+}$ ratio = 4 : 5). The solution was incubated for 30 min and then infused directly into the ion source. An analogical measurement was also performed for the 1:1 mixture of peptides [ADK]-NHOH and [ADE]-NHOH. The spectrum obtained for the [AD]-NHOH peptide and copper(ii) ions is presented in Fig. 1. The data for other ligands are presented in the ESI. In each case, the spectra show formation of complexes with the L$_4$Cu$_5$ stoichiometry. The obtained compounds form a series of ions with a charge in the range 2 to 8.
from +5 to +10. Only the [ADE]-NHOH peptide does not show
the peaks of a complex in the positive ion mode. However, even
in this case the complex with the same stoichiometry was observed
in the negative ion mode. The molecular formula of each complex
was verified on the basis of HR-MS. The calculated and experimental
values of $m/z$ for the most abundant isotopic peaks presented in
Table 1, as well as in the ESI† (Table S1) demonstrated a good
agreement with the calculated masses. The L$_4$Cu$_5$ systems were
predominant complexes observed by the ESI-MS method.

Selected complexes were also studied by the MS/MS technique.
The obtained data demonstrate that these systems are relatively
stable in the gas phase, in contrast to the peptide clusters often
observed in the ESI spectra of peptides at high concentrations. Observed fragmentation patterns show breaking of the peptide
bonds rather than dissociation of complexes with a free ligand
formation. In the ESI-MS spectrum of the “mixed” system,
composed of [ADK]-NHOH and [ADE]-NHOH peptides, mainly
the ([ADK]-NHOH)$_2$([ADE]-NHOH)$_2$Cu$_5$ and ([ADK]-NHOH)$_3$([ADE]-
NHOH)Cu$_5$ complexes were found (Fig. 2).

An additional experiment was performed for the [AD]-NH$_2$
peptide. This compound has an identical amino acid sequence to
the [AD]-NHOH peptide, but contains an amide instead of a
hydroxamic moiety at the C-terminus. The ESI-MS spectrum does
not show the presence of supramolecular structures. The abundance
of the complex with Cu$^{2+}$ ions is low and the predominant Cu$^{2+}$
containing system is a mononuclear complex – LCu. The spectrum of
the analysed compound is presented in the ESI† (Fig. S6).

### Circular dichroism

The obtained peptides were investigated by the CD method.
The measurements were performed in ammonium bicarbonate
(10$^{-2}$ M) and the concentration of peptides and copper(II) ions
was the same as in the MS experiments. The results obtained
for [AD]-NHOH and [AD]-NH$_2$ peptides in the presence and
absence of Cu$^{2+}$ ions are presented in Fig. 3. The data concerning
other ligands are given in the ESI.†

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**Table 1** The comparison of $m/z$ values (experimental and calculated) for the most abundant isotopic peaks of metallacrowns formed by the [AD]-NHOH peptide

| Peptide     | z  | Experimental $m/z$ value | Calculated $m/z$ value | Elemental formula of complex |
|-------------|----|-------------------------|------------------------|-----------------------------|
| [AD]-NHOH   | 5+ | 1454.5836               | 1454.5764              | C$_{312}$H$_{563}$N$_{88}$O$_{88}$Cu$_5$ |
|             | 6+ | 1212.3155               | 1212.3149              | C$_{312}$H$_{563}$N$_{88}$O$_{88}$Cu$_5$ |
|             | 7+ | 1039.2741               | 1039.2710              | C$_{312}$H$_{563}$N$_{88}$O$_{88}$Cu$_5$ |
|             | 8+ | 909.4885                | 909.4880               | C$_{312}$H$_{563}$N$_{88}$O$_{88}$Cu$_5$ |
|             | 9+ | 808.5459                | 808.5457               | C$_{312}$H$_{563}$N$_{88}$O$_{88}$Cu$_5$ |

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**Fig. 2** The isotopic pattern of the peak of $m/z = 908.5404$ for a “mixed” system (composed of [ADK]-NHOH, [ADE]-NHOH, and copper$^{2+}$) ions. On the top the experimental data are shown and compared with the calculated ones (lower panels). The stoichiometries for all complexes are given on the right.

**Fig. 3** The CD spectra of [AD]-NHOH and [AD]-NH$_2$ peptides and their complexes with copper$^{2+}$ ions. The black line corresponds to the ligand, the red line corresponds to the stoichiometric addition of copper$^{2+}$ ions, and the blue one corresponds to the excess of copper$^{2+}$ ions.
The spectra of investigated compounds (here presented for peptides [AD]-NHOH and [AD]-NH\(_2\)) show a strong negative band, which is accompanied by a negative shoulder at 210–220 nm. The CD spectra of [ADK]-NHOH and [ADE]-NHOH peptides demonstrate that the conformational equilibrium of these compounds is dominated by open conformations while peptides [AD]-NHOH, [AH]-NHOH, and [AH1]-NHOH are characterized by some fraction of the helical or \(\beta\)-turn conformation. The addition of Cu\(^{2+}\) ions (ratio corresponding to the metallacrown stoichiometry) drastically changes the CD spectrum of [AD]-NHOH (Fig. 3). There are two negative bands at 206 and 220 nm accompanied by a strong positive band at 190 nm. The spectrum is very similar to that characteristic of the \(\alpha\)-helical conformation. The spectra of peptides [AH]-NHOH and [AH1]-NHOH in solutions containing Cu\(^{2+}\) as well as the spectrum of [ADK]-NHOH (ESI†) are essentially the same. However, the spectral changes of the [ADE]-NHOH peptide induced by addition of Cu\(^{2+}\) ions are much less pronounced. Interestingly, a mixture of the [ADE]-NHOH and [ADK]-NHOH peptides in the presence of Cu\(^{2+}\) shows the spectrum suggesting the domination of the helical conformation (Fig. 4). Unfortunately, a partial precipitation of the reaction product influences the measured ellipticity and makes measurements less reproducible. A control experiment was also performed on the [AD]-NH\(_2\) peptide. In this case, the changes in the CD spectrum upon Cu\(^{2+}\) addition were negligible (Fig. 3).

**Enzymatic hydrolysis**

Peptides [AD]-NH\(_2\) and [AD]-NHOH were incubated with trypsin in the presence and absence of Cu\(^{2+}\) ions. The results after 20 min of incubation are presented in Fig. 5. In panels B–D (peptide [AD]-NHOH without Cu\(^{2+}\) ions and peptide [AD]-NH\(_2\) with and without Cu\(^{2+}\)), the predominant peaks correspond to proteolytic fragments. Only in panel A, representing a spectrum of peptide [AD]-NHOH in the presence of Cu\(^{2+}\) ions, the predominant peak corresponds to an unhydrolyzed ligand, which suggests that the complexation resulting in formation of an ordered helical structure protects the peptide chains from the enzymatic hydrolysis. In the spectrum (Fig. 5A) metallacrown is not observed due to acidic pH. The addition of 10% TFA was needed to quench the enzymatic hydrolysis reaction. At acidic pH the complex bonds in metallacrown systems were decomposed. Moreover MS measurements were performed in the mixtures containing 0.1% HCOOH, which prevented the reconstruction of metallacrowns.

**NMR experiments**

**Free peptide.** The NMR investigations carried out in the free form of the [AD]-NHOH peptide dissolved in D\(_2\)O allowed us to assign all amino acid spin systems. The H\(_2\)–C\(_2\) chemical shifts of the free peptide are in agreement with the CD data indicating predominantly the open conformation. The addition of paramagnetic Cu\(^{2+}\) ions caused significant changes in 1D \(^1\)H and 2D \(^1\)H–\(^1\)C HSQC. Several \(^1\)H–\(^1\)C correlations were broadened beyond the detection limit due to the dipole–dipole interaction between the nuclei and the unpaired electron of Cu\(^{2+}\) and the long electron relaxation time of Cu\(^{2+}\) (Fig. 6A–C) Disappearance of the \(\beta\)-amino and adjacent Leu signals from the 2D \(^1\)H–\(^1\)C HSQC spectrum allowed us to unambiguously identify the binding sites of the metal that is in line with metallacrown formation. While the majority of H\(_2\)–C\(_2\) correlations are moved downfield, the side-chain signals after Cu\(^{2+}\) addition occupy the same positions as in the free peptide indicating a fast exchange between the bound and free peptides. Only the C\(\beta\) chemical shifts of alamines are moved slightly upfield by \(~0.3\) ppm. The downfield and upfield changes of the C\(\alpha\) (\(~0.9–1.4\) ppm) and C\(\beta\) chemical shifts of selected residues relative to the free peptide...
This conclusion. The only resonance that splits into two after Cu\textsuperscript{2+} addition (4 : 5 ratio) is the N-terminal acetyl moiety what indicates a possible exchange between different conformations of the less ordered N-terminal part of the folded peptide.\textsuperscript{14} After further addition of Cu\textsuperscript{2+} ions (4 : 10 ratio), the relative intensities of these two peaks are not changed. The lack of changes in positions of the side-chains of leucines 3, 7, and 11, as well as of lysines and glutamic acids, suggests that the resulting tetrahedral bundle despite its compact nature is more a molten globule than a well packed structured hydrophobic core (Fig. 6C). Therefore, also the possible salt-bridges between the side chains of Glu and Lys stabilizing the four-helices tertiary structure can be excluded.\textsuperscript{15}

**Discussion.** The mass spectrometric measurements demonstrate unambiguously that peptides containing a β-amino-hydroxamic or an imidazolo hydroxamic moiety at C-termini formed the supramolecular structures with the compositions corresponding to those of metallacrowns. The molecular masses of formed supramolecules (approx. 7200 Da) are comparable with those of ubiquitin or cytochrome c. The relatively high abundance of the L\textsubscript{4}Cu\textsubscript{5} complex suggests that this system is predominant in solution. In addition, the metallacrown obtained is the only form of Cu\textsuperscript{2+}-hydroxamate complexes detected by the ESI-MS method. The collision-induced-dissociation experiment revealed a high stability of the investigated supramolecule. This result is similar to that obtained previously for trimers of peptides containing a bipyridyl moiety.\textsuperscript{16} The high stability distinguishes the systems formed here from unspecific clusters of peptides often observed in ESI experiments. The literature reports on the metallacrowns formed by the hydroxamic acids indicate a good correlation between the composition of complexes in solution and that observed in the gas phase by the ESI method.\textsuperscript{17} A control experiment performed on the [AD]-NH\textsubscript{2} peptide proves that the metallacrown formation requires the hydroxamate moiety and cannot be considered as a property of the peptide sequence itself.

Mass spectrometry is a well established method for studying supramolecular assemblies, including cation-bound systems. Recently, the binding of an ammonium ion by DNA G-quadruplexes has been analysed.\textsuperscript{18} In this system, the stoichiometry of the complex formed in the gas phase depends significantly on the instrument type and ion source conditions, e.g. declustering potential. In our case, the spectra measured using two different instruments (microTOF-Q and Apex-ultra) were almost the same and the influence of experimental settings on spectra was negligible which may indicate that mass spectra reflect the stoichiometry of supramolecules in solution. Based on those results, we consider mass spectrometry as a method suitable for studying the interactions of peptides bearing the aminohydroxamate group with Cu\textsuperscript{2+} ions.

The character of the CD spectra of peptides [AD]-NHOH, [AD]-NHOH, [AH]-NHOH, and [AH1]-NHOH in the presence of Cu\textsuperscript{2+} indicates a high content of helical structures in the conforma-tional equilibrium. The helix content for peptide [AD]-NHOH was estimated according to the procedure described in the literature.\textsuperscript{19} The α-helix content is about 61% for the stoichiometric amount of Cu\textsuperscript{2+} ions and 65% when the twofold excess of Cu\textsuperscript{2+} ions with respect to the stoichiometric ratio was applied. The residual
molar ellipticity measured in our system in the presence of Cu$^{2+}$ ($[\Theta] = -21 600$ deg cm$^2$ dmol$^{-1}$) is very close to that reported by Mutter$^{20}$ ($[\Theta] = -22 000$ deg cm$^2$ dmol$^{-1}$) for TASP molecules based on a sequence similar to that used in our study (EALEKALKEALKG). These data may indicate that in the metallacrown system investigated herein amphiphilic peptide chains interact in the same way as was reported for TASP. In a CD experiment the [AD]-NH$_2$ peptide does not show distinct changes upon addition of Cu$^{2+}$ ions. This result is similar to that obtained by the MS method and indicates the crucial role of the hydroxamate moiety in the investigated interactions.

The analysis of ESI-MS, NMR, and CD data indicates that the peptides containing the hydroxamic moiety in the C-terminal part form tetrameric structures in the presence of Cu$^{2+}$ ions. Tetramerization observed in this system is connected with a shift in the conformational equilibrium toward helical structures (Fig. 7). This behaviour is also reflected by a much higher enzymatic stability of the tetrameric complex in comparison to other systems which are not able to form metallacrowns.

Conclusions

In summary, the peptide hydroxamic acids based on the amphiphilic structures form a relatively simple and convenient system for studying the self-assembly of oligopeptides into tetrahelical bundles. The simplicity of ligand synthesis and preparation of complexes, possibility of mixed complexes formation, as well as geometrical properties similar to that of TASP make this system attractive for dynamic combinatorial chemistry.

Experimental section

General peptide synthesis

SPPS of peptides was performed manually in polypropylene syringe reactors (Intavis AG) equipped with polyethylene filters, on a modified ChemMatrix resin$^{11}$ designed for synthesis of hydroxamic acid peptides. The synthesis was carried out according to the standard FMoc procedure. The FMoc-protecting groups were removed using 25% piperidine solution in DMF. The coupling reactions were performed in DMF using TBTU (3 eq.) in the presence of DIEA (3 eq.). The peptides were cleaved from the resin using a solution of TFA-H$_2$O-Tis (95:2.5:2.5) at room temperature for 2 h. The peptides were isolated after evaporation of TFA in a nitrogen stream.

The analytical data of obtained peptides are as follows:

- Peptide [AH]-NHOH: HRMS (ESI) = m/z calcd for $C_{74}H_{130}N_{22}O_{21} + 2H^+ = 860.5276$ [M + 2H]$^{2+}$; found: 860.5226; $R_{11}$ 28.2 min, $R_{12}$ 26.0 min.
- Peptide [AH]-NH$_2$: HRMS (ESI) = m/z calcd for $C_{81}H_{142}N_{22}O_{22} + 2H^+ = 896.0462$ [M + 2H]$^{2+}$; found 896.0466; $R_{11}$ 30.4 min, $R_{12}$ 25.6 min.
- Peptide [AD]-NHOH: HRMS (ESI) = m/z calcd for $C_{78}H_{140}N_{22}O_{22} + 2H^+ = 870.5407$ [M + 2H]$^{2+}$; found 870.5404; $R_{11}$ 30.4 min, $R_{12}$ 25.6 min.
- Peptide [ADK]-NHOH: HRMS (ESI): m/z calcd for $C_{81}H_{137}N_{22}O_{22} + 2H^+ = 872.4360$ [M + 2H]$^{2+}$; found: 872.4363; $R_{11}$ 36.6 min, $R_{12}$ 30.2 min.
- Peptide [AD]-NH$_2$: HRMS (ESI): m/z calcd for $C_{78}H_{142}N_{22}O_{22} + 2H^+ = 862.5433$ [M + 2H]$^{2+}$; found: 862.5433; $R_{11}$ 31.0 min, $R_{12}$ 27.4 min.

Additional analytical data (NMR of [AD]-NHOH and ESI-MS/MS for all peptides) have been presented in a previous paper.$^{11}$

Purification

All crude products were analyzed by analytical HPLC using a Thermo separation HPLC system with UV detection (210 nm) on a Varian Protein Prep C18 column (250 × 4.6 mm, 5 μm) (Grace, Deerfield, IL, USA), with a gradient elution of 0–80% B in A ($A = 0.1\%$ TFA in water; $B = 0.1\%$ TFA in acetonitrile–H$_2$O 4:1) for 45 min (flow rate 1 ml min$^{-1}$, rt). For all compounds additional HPLC analysis was performed on a Varian Microsorb-MV 100-5 CN column (4.6 mm × 250 mm; Varian, Palo Alto, CA, USA) in the same gradient system. Retention times are given as $R_{t1}$ and $R_{t2}$, respectively. Preparative reversed-phase HPLC was performed on a Tosoh TSKgel ODS-120T column (21.5 mm × 300 mm) (Tosoh, Tokyo, Japan) using the same solvent system, gradient 0.5% min$^{-1}$, and a flow rate of 7 ml min$^{-1}$.
Mass spectrometry

The compounds investigated were dissolved in methanol: NH₄HCO₃ solution (1:1, 10⁻² M). The MS experiments were performed on a FT-ICR (ion cyclotron resonance) MS Apex-Qe Ultra 7T instrument (Bruker Daltonics, Germany) equipped with an ESI source. Argon was used as a collision gas. The instrument was operated in a positive and a negative ion mode and calibrated with Tunemix™ (Bruker Daltonics, Germany).

The instrumental parameters were as follows: temperature of the drying gas was 200 °C, resolution for m/z 300–1600, dry gas – nitrogen (3 L min⁻¹), temperature 200 °C, and accumulation time 1.0 s. The MS spectra were also recorded on a Bruker micrOTOF-Q mass spectrometer equipped with an Apollo II electrospray ionization source.

The instrumental parameters were as follows: scan range m/z 300–1600, dry gas – nitrogen (3 L min⁻¹), temperature 200 °C.

Circular dichroism

CD spectra were recorded on a Jasco J-600 spectropolarimeter. Peptides were dissolved in ammonium carbonate buffer (10⁻² M) at the concentration of 10⁻⁴ M. The complexes were obtained in stoichiometric mixtures of ligand:Cu²⁺ (4:5, 10⁻² M: 1.25 x 10⁻² M) and at the excess of copper(II) ions (ligand:Cu²⁺ 4:10). The pH range was 7.5–8.0. The pH content determined by elemental analysis.

Cylindrical magnetic resonance (NMR)

All NMR measurements for the [AD]-NHOH peptide and its Cu²⁺ complex were done on Bruker Avance and Varian Unity + 500 MHz spectrometers, at 25 °C in 99% D₂O ammonium carbonate buffer (10⁻² M) at pH 7.8 (2 mg peptide in 650 μL). The spectra used for resonance assignments of the [AD]-NHOH peptide spin systems before and after addition of Cu²⁺ (ligand: Cu²⁺ ratios 4:5 and 4:10), i.e. the 2D ¹H–¹H TOCSY (20 and 80 ms mixing times), ¹H–¹H ROESY (300 ms), ¹H–¹H NOESY (100 and 500 ms), and ¹H–¹³C HSQC spectra were processed by NMRpipe and analyzed in Sparky (T. D. Goddard and D. G. Kneller, http://www.cgl.ucsf.edu/home/sparky).

Enzymatic hydrolysis

Briefly, the peptides (0.1 mg) were dissolved in NH₄HCO₃ buffer solution (0.575 ml, 10⁻² M, pH 7.8). Then a 10 μl aliquot of the trypsin stock solution (0.1 mg in 1 ml of water) was added to obtain the 1:100 enzyme:substrate (w/w) ratio. The reaction mixtures were incubated at 37 °C for 20 min, 40 min, 1 h, and 2 h. The reaction was quenched by addition of 10% aqueous trifluoroacetic acid (150 μL). The resulting digest was lyophilized and used for MS experiments. The experiments with copper(II) ions were performed similarly to those mentioned above. Briefly, the peptides (0.1 mg) were dissolved in NH₄HCO₃ buffer (0.575 ml) and to this solution CuSO₄ (18 μl) was added (stock solution 1 mg per 1 ml). Then 10 μl of the trypsin stock solution (0.1 mg in 1 ml water) was added. Further conditions have been previously described.

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