

Dizajn linearnih i cikličkih katalitičkih peptida

Sepčić, Sabino

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UNIVERSITY OF RIJEKA
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Master's degree
"Medicinal chemistry"

Sabino Sepčić

Design of linear and cyclic catalytic peptides

Graduate thesis

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Graduate thesis was defended on September 19th before the committee:

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The thesis has 65 pages, 47 pictures, 10 tables and 40 references

Sažetak

Pozadina: Enzimi su proteini koji ubrzavaju kemijske reakcije stvaranjem povoljnih uvjeta za pretvorbu supstrata u produkte. Enzimi su velike makromolekule građene od aminokiselina povezanih peptidnim vezama. Peptidi, koji su također sastavljeni od aminokiselina, su manji i jednostavniji od proteina i mogu imati katalitička svojstva.

Cilj: Glavna svrha ovoga rada je istraživanje promjena svojstva katalize i samoorganizacije kod peptida koje proizlaze iz izmjena aminokiselina.

Metode: Tehnike koje su korištene su sinteza na čvrstom nosaču, tekuća kromatografija, masena spektrometrija, infracrvena spektroskopija, katalitički kinetički test i računalna simulacija molekularne dinamike.

Rezultati: Dva linearna i dva ciklička peptida su sintetizirana i karakterizirana. Strukture sintetiziranih peptida su kromatografski potvrđene što dokazuje mogućnost ciklizacije oktapeptida na čvrstom nosaču. Linearni peptidi formiraju supramolekularne strukture u obliku β -ploče sa cinkom. Daljnja analiza peptida sa katalitičkim kinetičkim testom je potrebna za dokazivanje njihovih katalitičkih svojstava.

Zaključak: Kombinacija računalnih i laboratorijskih tehnika je koristan alat u dizajnu peptida. Dizajn katalitičkih peptida relativno je novo polje proučavanja, stoga ovaj rad ima za cilj steći detaljnija saznanja o području.

Ključne riječi: Peptid, kataliza, dizajn peptida, ciklizacija

Summary

Background: Enzymes are proteins that help speed up chemical reactions by creating favourable environment for the conversion of substrates into products. Enzymes are large macromolecules composed of amino acids connected with peptide bonds. Peptides, which are also made up of amino acids, are smaller and easier to synthesize than proteins and can display catalytic properties.

Aim: The main purpose of this thesis is to explore the changing of self-assembly and catalytic properties of peptides by introducing amino acid alterations.

Methods: Techniques used are solid phase peptide synthesis, liquid chromatography, mass spectrometry, infrared spectroscopy, catalytic kinetic assay and computational molecular dynamics simulation.

Results: Two linear and two cyclic peptides were synthesized and characterized. The chromatography data validates the synthesized peptides which proves the possibility of octapeptide on-resin cyclization. Linear peptides formed supramolecular β -sheet-like structures with zinc. Further analysis of the peptides using the catalytic kinetic assay is needed to confirm their catalytic properties.

Conclusions: The combination of computational and laboratory techniques is a useful tool in peptide design. Catalytic peptide design is a newly emerging field of study and for this reason, this thesis aims to obtain a better understanding of the matter.

Key words: Peptide, catalysis, peptide design, cyclization

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Introduction

Peptides, from Greek *peptós* – digested, are short amino acid chains with lengths between two and fifty amino acids connected by peptide bonds. Amino acids are organic molecules that contain two functional groups, the amino ($-NH_2$) and the carboxyl ($-COOH$) group along with a side chain. The elements which can be found in every amino acid are carbon (C), hydrogen (H), oxygen (O), nitrogen (N). Additional atoms, such as sulphur (S) and selenium (Se) can be found in the side chains of specific amino acids, such as methionine and cysteine containing sulphur or selenocysteine, a non-universal proteinogenic amino acid, containing selenium. There are twenty amino acids that are used as building blocks for proteins, called proteinogenic amino acids, and they are encoded in the human genetic code. The amino acids produced and used in biological systems as well as being prevalent in laboratory research are L-amino acids. Each amino acid is unique, with a different mass and electrostatic interactions with neighbouring molecules. Amino acids can have electrically charged side chains, with positive and negative charges, polar uncharged side chains or hydrophobic side chains. The uniqueness of each amino acid stems from their side chains, since their main parts, the amino, carboxyl group and hydrogen, are the same in every amino acid. All amino acids have at least one ionisable positive charged group (by protonation of the α -amino group) and negatively charged group (by deprotonation of the α -carboxyl group). When the amino acids form a peptide bond between the amino and carboxyl groups, the groups are no longer able to ionize. The ionizable groups of peptides and large proteins are on the side chains and the α -amino and α -carboxyl terminal groups of the polypeptide chains. Peptides with different amino acid compositions have different physical and chemical properties. Therefore, it is possible to create peptides with a vast array of different properties and applications. The absolute quantity of different possible peptides increases by 20-fold with each additional amino acid which

increases the difficulty of analysing most of the possible amino acid alterations in larger peptides.

What are enzymes

Enzymes are biological catalysts which are prevalently large proteins. They increase the speed of chemical reactions by reducing the activation energy, the minimum energy needed for the reaction to occur. They are essential for most of the chemical processes in our bodies. For enzymes to accelerate the rate of a reaction, the substrate should bind to the active site of the enzyme and form the transient enzyme-substrate complex after which the reaction occurs and a product molecule dissociates from the enzyme leaving it intact for another reaction¹. Enzymes work only in certain conditions and have an optimal range of temperature and pH depending on their function and location in an organism. For example, human intestinal enzymes function optimally at 37°C and at pH 8, while human stomach enzymes work best at pH 1.5².

Enzymes are classified according to the reaction they catalyse. Each enzyme or a group of enzymes that catalyse the same reaction is given a four-part EC number, each part of which provides information about the catalytic reaction. Enzymes with different structures that catalyse the same type of reaction have the same EC number, as the structure of the catalytic molecule is not taken into account in the classification system. The enzymes can be classified into 7 main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases and translocases with the EC numbers from 1-7 respectively³. Our bodies need a large variety of different enzymes to function.

Many enzymes cannot function by themselves unless they have a specific non-protein molecule attached to them called cofactor⁴. There are two types of cofactors: inorganic ions (such as zinc and copper ions), and

organic molecules known as coenzymes which are often vitamins⁴. Metalloprotein is a generic term for a protein that contains a metal cofactor. The proportion of metalloproteins in the proteome is still widely unknown⁵. One of the most common types of metalloproteins are the zinc-finger proteins (ZNFs). The amino acids coordinate the zinc ions to stabilize the fold. They are an abundant group of proteins with a wide array of molecular functions. Due to the wide variety of zinc-finger domains, ZNFs are able to interact with proteins, DNA, RNA and PAR (poly-ADP-ribose). Thus, ZNFs are involved in the regulation of several cellular processes⁶.

A commonly used model for enzymatic reactions is the Michaelis-Menten (MM) equation.

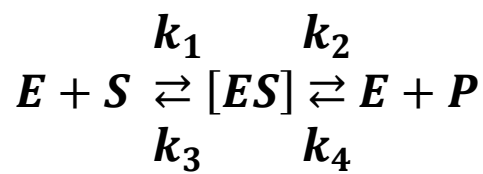


Figure 1. General enzyme reaction mechanism.

As shown in Figure 1, the enzyme-substrate complex (ES) is formed by combining the enzyme E with substrate S at a rate constant k_1 . The ES complex can either dissociate into a free enzyme E and S or form a product P at a rate constant k_3 and k_2 respectively. The enzyme and product are capable of reverse reaction of catalysis which reverses the catalytic reaction and creates ES at a rate constant k_4 ⁷. The concentration of ES complex is relatively constant through the catalytic cycles due to the complex's slow formation and rapid consumption. Therefore, at steady rate, with high concentrations of substrates in comparison with the concentration of enzyme, the rate of formation of ES can be approximated with the steady-state approximation as equal to the rate of breakdown of ES.

K_M implies that half of the active sites on the enzymes are filled with substrates. Different enzymes have different K_M values, ranging from 10^{-1}

to 10^{-7} M. The factors that affect K_M are: temperature, ionic strength, pH and the nature of the substrate⁷.

$$v = V_{\max} \frac{[S]}{K_M + [S]}$$

Figure 2. The Michaelis-Menten equation.

The Michaelis-Menten equation (Figure 2) is used to calculate the rate of an enzymatic reaction (v) using the concentration of the substrate S . The V_{\max} and the K_M are dependent of the enzyme and are the maximum speed of the chemical reaction which happens at saturating substrate concentrations (V_{\max}) and the substrate concentration at which half of the maximum velocity is achieved (K_M).

Catalytic constant (K_{cat}) value is the number of molecules of product produced per catalysis site per minute. It is defined as equal to the maximum catalytic velocity V_{\max} divided by the total concentration of the enzyme ($V_{\max}/[E]$)⁸.

The two values of K_M and K_{cat} are used to calculate the rates of the enzyme catalysed chemical reactions.

Hydrolitic enzymes

Hydrolases are a class of enzymes which utilize water as a hydroxyl group donor during the breakdown of substrates. They catalyse the hydrolysis of a chemical bond in biomolecules, thus dividing a large molecule into two smaller molecules. Hydrolases are the largest and most diverse class of enzymes with more than 200 enzymes which catalyse the hydrolysis of multiple types of compounds. They catalyse the hydrolytic cleavage of carbon-carbon (C-C), carbon-nitrogen (C-N), carbon-oxygen (C-O) bonds, etc. Examples of some common hydrolases include proteases, glycosidases,

lipases and esterases. Esterases, which are a main theme of this graduate thesis, are hydrolases which split esters into an acid and an alcohol⁹, as illustrated in Figure 3.

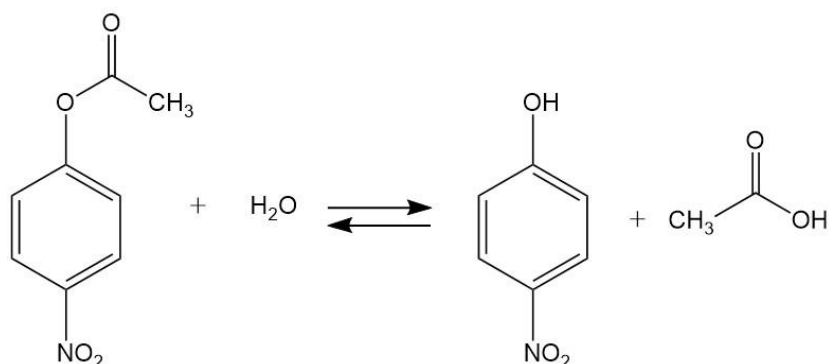


Figure 3. The graphical representation of the hydrolytic cleavage of 4-nitrophenyl acetate (pNPA) into 4-nitrophenol (pNP) and acetic acid.

The synthesis of peptides

Peptides are naturally synthesized in the process of translation in the ribosomes. A transcribed polymer chain of messenger RNA, which is copied from a segment of the DNA, is decoded in the ribosome outside the nucleus which creates a complementary polypeptide chain. In the laboratory, peptides are synthesized using chemical, instead of biological methods. In the early 1960s, Robert Bruce Merrifield, an American biochemist, proposed the use of solid polystyrene-based support for peptide synthesis. Peptide synthesis became a more practical part of modern research following the appearance of solid-phase techniques. Peptides could be assembled from the carboxyl terminus to the amino terminus using N α -protected amino acids¹⁰. For his work, Merrifield was awarded the Nobel Prize in chemistry in 1984.: „For his development of methodology for chemical synthesis on a solid matrix“¹¹.

Construction of a peptide chain on an insoluble solid phase has advantages including: the possibility of automation; the ability to wash and filter the soluble reagents and solvents from the intermediate peptides; excess

reagents and solid support for peptides can bring the reaction to completion with minimal peptide losses¹². The solid-phase synthesis approach still has its limitations. By-products from incomplete reactions, side reactions or impure reagents, will accumulate on the resin during the chain assembly and elongation phases and contaminate the final product. These impurities are similar to the desired product and are difficult to remove¹².

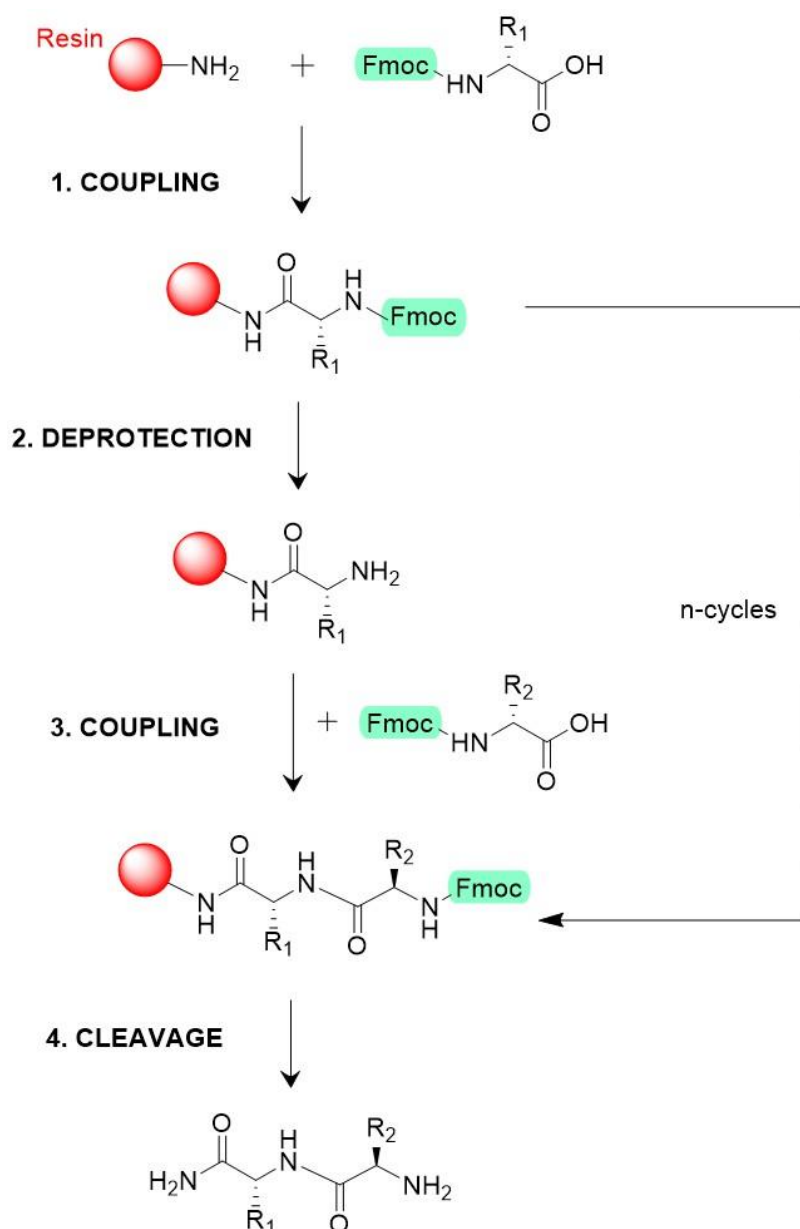


Figure 4. Schematic representation of the solid phase peptide synthesis (SPPS) principle.

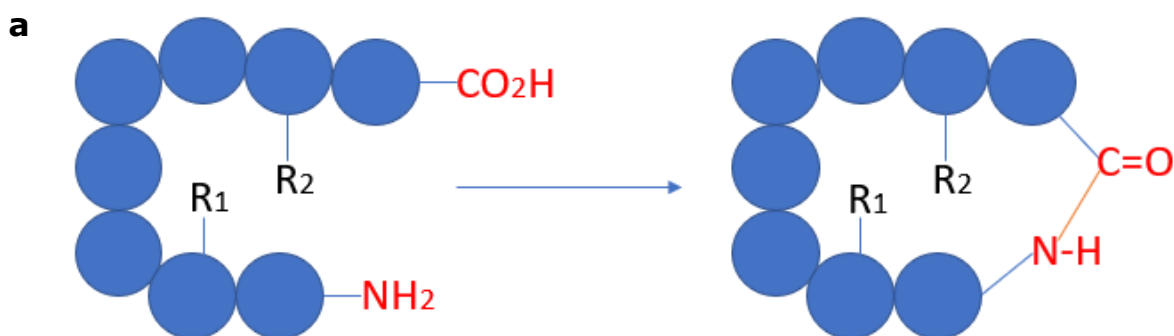
The SPPS method consists of several steps which are repeated in order to construct the desired peptide (Figure 4). The resin, a solid phase support for the construction of the polypeptide chain, is added and contained in a reaction vessel. The peptide and soluble reagents are added portion wise through the top of the reaction vessel and removed by vacuum or under positive nitrogen pressure. The C-terminal amino acid residue of the peptide is attached to the insoluble resin via its carboxyl group. The side chains of the amino acids which are added to the reactions have to be masked with semi-permanent protecting groups which are not affected by the reactions employed during the chain elongation processes¹². The temporary protecting group masking the α -amino group, such as *tert*-butoxycarbonyl (Boc) or fluorenylmethyloxycarbonyl (Fmoc), is removed during the process of deprotection. An excess of the amino acid is introduced, with the now free carboxy group activated for amide bond formation through the generation of an activated ester or by reaction with a coupling reagent. After the activated amino acid is coupled to the resin, excess reagents are removed by washing and the process of deprotection, activation and coupling is repeated until the desired peptide sequence is synthesized. In the final step, the side chain protecting groups are deprotected and the peptide is released from the support. Typically, the resin linkage and side chain protecting groups are chosen so that the peptide is deprotected and released under the same conditions¹².

Peptides can be modified at the N-terminus and the C-terminus groups. The C-terminus group can be amidated with the right choice of resin linker and the N-terminus group can be acetylated with acetic anhydride following the synthesis of the linear peptide chain. The overall charge of the peptide is reduced, so the modified peptides more closely mimic the native protein. Another advantage is the increase of metabolic stability as well as their ability to resist enzymatic degradation by synthetases, exopeptidases and aminopeptidases¹³.

This study used the method of Fmoc/tBu SPPS, which is based on an orthogonal protecting group strategy. The N-Fmoc group is used for the protection of the α -amino group and is base-labile and the side chain protecting groups (such as *tert*-butyl and trityl) and resin linkage agents are acid-labile. The deprotection of the α -amino group is performed in basic conditions using the solution of 20% piperidine in *N,N*-dimethylformamide (DMF). The side chain protecting groups and the resin linkage agent are removed with trifluoroacetic acid (TFA), an excellent solvent for peptides which can be used in standard glass laboratory glassware, as well as being volatile and removable by evaporation. These attributes make the Fmoc/tBu approach to SPPS popular¹².

Cyclization

Cyclic peptides are polypeptide chains with intra-cyclic bonds which are formed between two or more amino acid residues. Cyclic peptides can be formed in nature, with the example of: vancomycin, romidepsin, cyclosporin A and griselymicin¹⁴. Cyclization is used in synthetic peptides to create a desired three-dimensional structure and stability. Cyclic peptides are formed in four different ways: head-to-tail, side chain-to-side chain, tail-to-side chain and head-to-side chain cyclizations¹⁵.



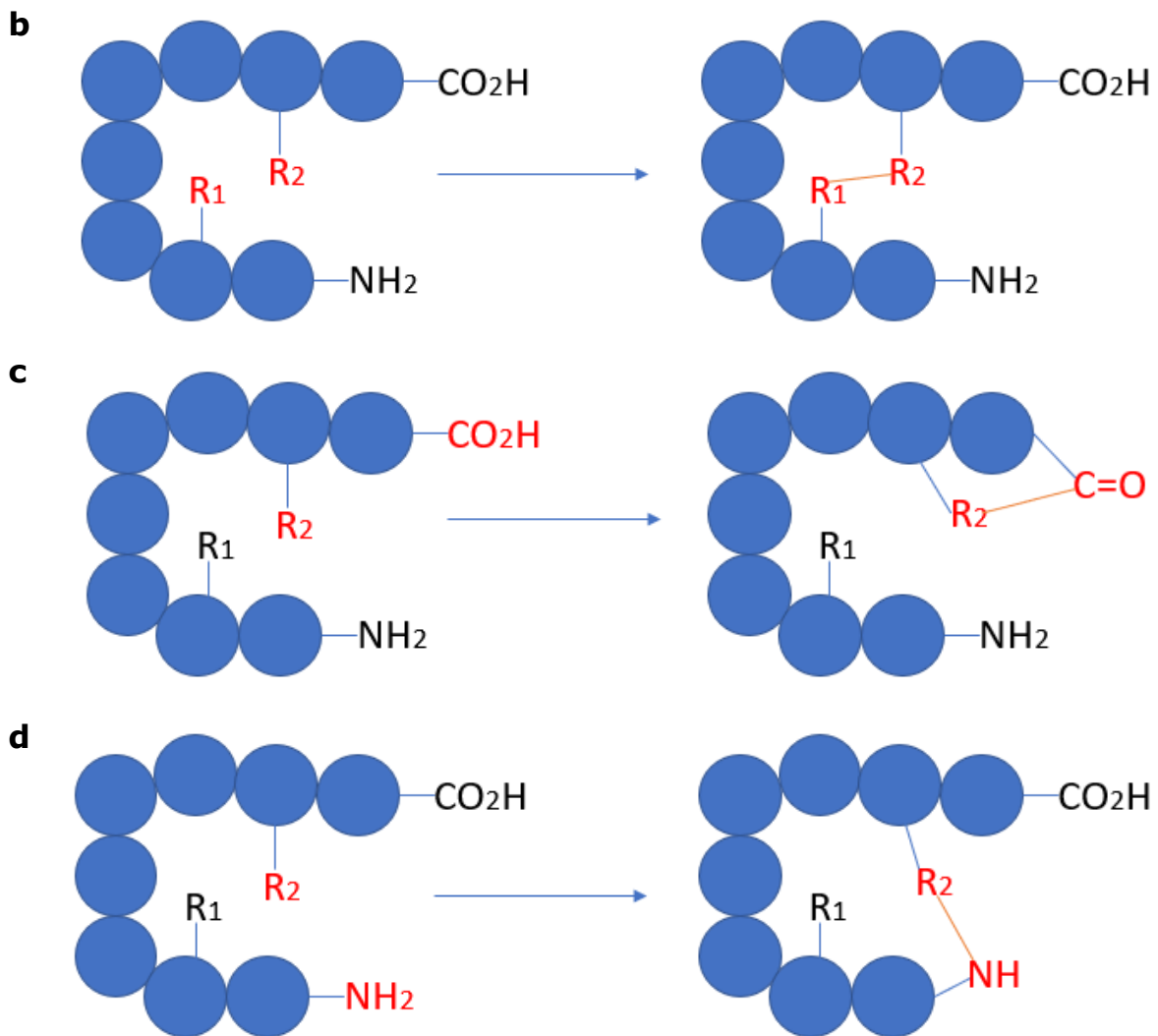


Figure 5. Schematic illustration of different types of cyclization: **a)** Head-to-tail; **b)** Side chain-to-side chain; **c)** Tail-to-side chain; **d)** Head-to-side chain

Head-to-tail cyclization, or terminal-to-terminal cyclization, involves the formation of a peptide bond between the carboxyl and amino terminal groups (Figure 5a). Terminal-to-side chain (Figure 5c, d) and side chain-to-side chain (Figure 5b) involves the formation of a bond between the side chain of an amino acid residue and either the carboxyl/amino terminal group or another side chain inside the polypeptide chain. Cyclization is typically performed following the synthesis of the linear peptide and can be performed while the peptide is attached to the solid resin support or in solution¹⁵. Disulfide bond formation between two thiol groups of the

cysteine residues is the most common chemical method of cyclisation. About 30% of eukaryotic proteins have at least one disulfide bond which regulates the protein function and stabilizes the structure.

According to the International Union of Pure and Applied Chemistry (IUPAC), no summary on the approaches to cyclic peptide nomenclature has been agreed upon. The cyclic peptides are divided into two types: homodetic, whose ring consists solely of amino acid residues in eupeptide linkage, such as the one in head-to-tail cyclization, and heterodetic, whose ring-forming linkages are not solely eupeptide bonds; but one or more is an isopeptide, disulfide ester, or other bond^{16,17}. Cyclic peptides such as the isopeptide bond cyclized PC3 (cyclo-(IHIHINIE)) and PC4 (cyclo-(IHINIHIE)) peptides and peptides with disulfide bonds are in the category of heterodetic cyclic peptides. The representation shown in Figure 6 is possible and more informative, but the simpler cyclo-(IHIHINIE) and cyclo-(IHINIHIE) were chosen to note the cyclic peptides PC3 and PC4.

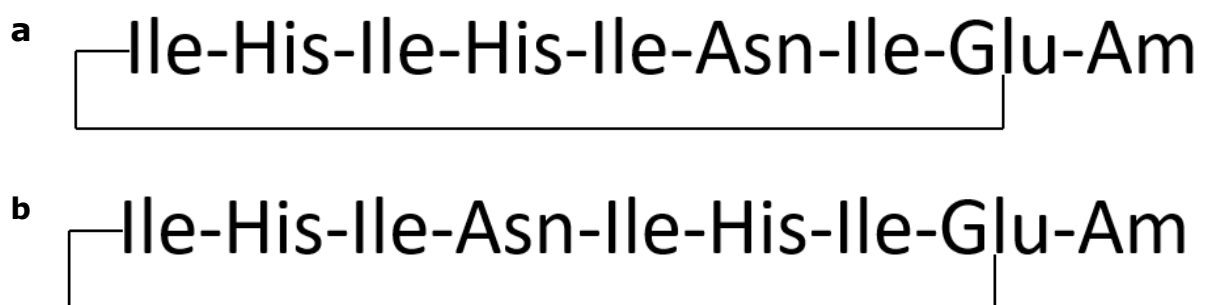


Figure 6. Representation of the possible heterodetic cyclic peptides nomenclature

Catalytic peptides

It is possible that the earliest proteins might have been formed from short peptides with repeating amino acid sequences that relied on self-assembly to achieve a folded structure¹⁸. Self-assembly is the process by which an organized structure spontaneously forms from individual components, as a result of local and specific interactions among the components¹⁹. Self-

assembling peptides comprise monomers of short amino acid sequences that assemble to form nanostructures. The simplest building blocks of self-assembled peptide structures are dipeptides, such as diphenylalanine (FF), which forms nanotubes and nanovesicles²⁰. Simple heptapeptides with alternating polar and nonpolar residues self-assemble into extended beta sheets which are stabilized through intermolecular association of the polar residues in the sequence²¹. In addition, structural characterization of amyloid-forming peptides has revealed a multitude of conformational variations of the basic cross- β conformation²². It is hence possible that the first protein enzymes were short peptides which built the amyloid structures that provided the frameworks to sustain their catalytic activities²³.

Molecular self-assembly is known to result in new emerging properties and the swift rise of nanotechnology is a proof of its fundamental and practical significance²⁴. Peptides provide numerous routes for self-assembly due to the interplay between hydrogen bonding, van der Waals forces, electrostatic and stacking interactions²⁵ to allow for formation of diverse supramolecular structures determined by subtle changes in charge, hydrophobicity and size of the peptides²⁶.

Despite the obvious appeal, it is not easy to create efficient catalysts through self-assembly of peptides. The same factors that provide advantages to self-assembled peptide catalysts can be unfavourable to their development. The astronomical sequence space of even the small sequences makes the establishment of meaningful structure-activity relationships difficult²⁷.

Metals might have figured largely in the advent of protein catalysis^{28,29}. As well as catalysing reactions, transition metal ions influence the protein folding through the interaction with ligating sidechains. Therefore, influenced by Rufo *et al.*, 2014., we have explored the metal interactions, design and catalytic properties of Zn²⁺- binding amyloidogenic peptides. Zinc was chosen as a cofactor due to its high abundance and frequent occurrence in modern metal-dependent hydrolases such as

metalloproteases and carbonic anhydrase. The Zn^{2+} ion lowers the pK_a of bound water thus stabilizing and positioning hydroxide for a nucleophilic attack of the substrate. The Zn^{2+} binding site in enzymes frequently contains two His ligands projecting from positions i and $i+2$ of a β -sheet and a third neighbouring His completes the primary ligand environment. Such 3-His metal coordination sphere is observed in proteins such as erythrocyte carbonic anhydrase²³.

Rufo *et al.*, 2014., tested the effect of changing the amino acid residues in a heptapeptide on catalytic activity. The two His ligands are polar residues in an alternating polar and nonpolar residue peptide. Different nonpolar residues were tested, Val, Ala and Ile. Of those, Ile produced peptides with the highest activity. The third polar residue was permuted, and out of Asp, Glu, Gln, Tyr, His, Lys and Arg, the Gln residue variant had the highest activity. The terminal acetyl and carboxamide groups also appeared to be important for activity, as the peptide lacking the groups was catalytically inactive. The peptide with the highest catalytic activity out of the tested peptides is Ac-IHIHIQI-Am²³, with the alternating nonpolar Ile, the i and $i+2$ His and Gln polar residues. The zinc coordination sphere of the peptide Ac-IHIHIQI-Am consists of two His residues from one peptide and one His residue from the second peptide which coordinate a single Zn^{2+} ion as was tested in a computational simulation and proven in a zinc-dependent kinetic assay with peptide and different concentrations of zinc. The activity of the peptide was saturated at values higher than 0.5 molar equivalents of $ZnCl_2$ to the peptide²³.

In this work, the change of self-assembly and catalytic properties of linear and cyclic peptides were investigated as follows:

- a) The effect of changing the glutamine (Q) to asparagine (N)
- b) The effect of changing the position of histidine (H) within the sequence
- c) The effect of cyclization on the overall activity of the peptide

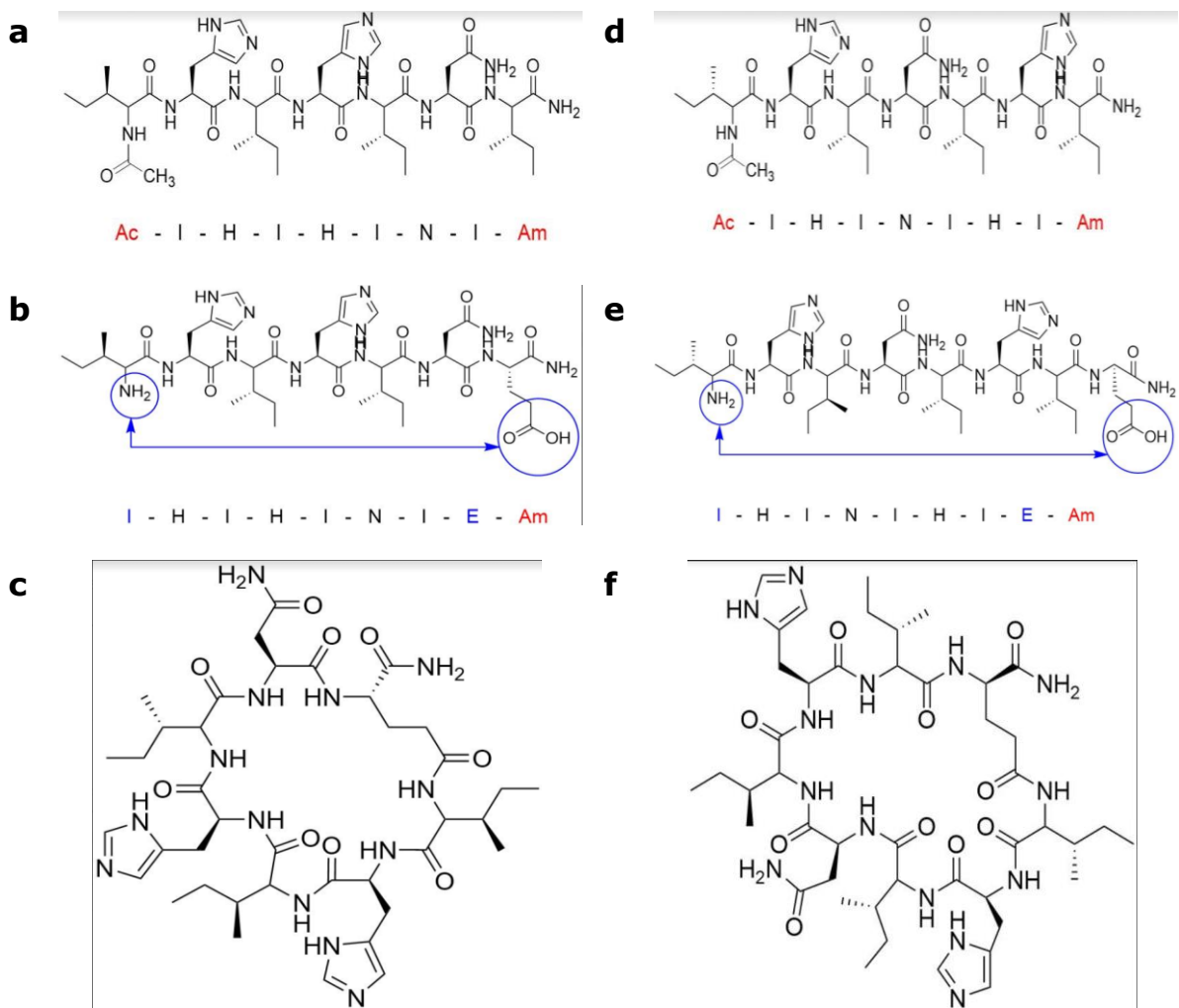


Figure 7. The structure of peptides: **a)** Ac-IHIHINI-Am; **b)** Linear IHIHINIE-Am; **c)** Cyclo-(IHIHINIE-Am); **d)** Ac-IHINIHI-Am; **e)** Linear IHINIHI-E-Am; **f)** Cyclo-(IHINIHI-E-Am). The modified chemical groups acetyl (Ac) and amide (Am) are highlighted as red. The blue line connecting the N-terminal group and the glutamate carboxyl group of the peptides b) and e) shows the chemical groups which are coupled by cyclization.

Purpose of the thesis

The main objective is the design, synthesis and characterization of novel linear and cyclic catalytic peptides. The catalytic activity *i.e.*, the ability to catalyse the ester hydrolysis of the proposed peptides was assessed through the model reaction of pNPA hydrolysis. This substrate was chosen for the measurement of the peptide catalytic activity to allow for comparison with natural and designed enzymes²³. For this purpose, we have chosen the peptides Ac-IHIHINI-Am and Ac-IHINIHI-Am, inspired by the most catalytically active peptide from a work by Rufo *et al.*, 2014.²³, Ac-IHIHIQI-Am. The cyclic versions of the linear peptides, cyclo-(IHIHINIE-Am) and cyclo-(IHINIHIIE-Am) were obtained through an isopeptide bond formation between the carboxyl group of the glutamic acid and the amino group of the N-terminal. By using cyclization to form intra-connected peptide structures, we expect that the increased rigidity introduced by cyclization will improve the catalytic efficiency of short peptides. These sequences will be tested for their ability to self-assemble into catalytic amyloids that could act as Zn²⁺ dependent esterases. The formation of supramolecular peptide structures is tested using infrared spectroscopy. The amide I vibrational region (1600-1700 cm⁻¹) is widely used because of the intense protein signal. Lastly, to directly compare the zinc coordination data from a computational simulation performed by Rufo *et al.*, 2014.²³, we ran a computational simulation with peptides and Zn²⁺.

Materials and methods

General information

Peptide synthesis was performed using the following materials: *N,N*-diisopropylethylamine (DIPEA; VWR, USA), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP; Apollo Scientific, UK), piperidine (Acros Organics, Belgium), 2-(1*h*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; Thermo Fisher Scientific, USA), 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-aminomethyl resin (Rink Amide AM resin; Sigma-Aldrich, USA), diethyl ether (DEE; Lachner, Czech Republic) and TFA and triisopropylsilane (TIS; Sigma-Aldrich, USA).

Infrared spectroscopy was performed using the Cary 630 ATR-FTIR spectrometer (attenuated total reflectance Fourier-transform infrared spectroscopy; Agilent Technologies, USA).

Peptide purification was performed using the 1260 Infinity HPLC (Agilent Technologies, USA). The column used is ZORBAX SB-C18 (Agilent Technologies, USA) with the specifications 5 μ m, 4.6 x 150 mm.

Peptide characterisation was performed using two instruments: Bruker MALDI-TOF/TOF ultrafleXtreme mass spectrometer (Bruker, USA) and coupled LC-MS 1260 Infinity (liquid chromatography-mass spectrometry; Agilent Technologies, USA) with 6460 Triple Quad Mass Spectrometer (QqQ; Agilent Technologies, USA). The column used is XSelect CSH C18 (Waters, USA) with the specifications 3.5 μ m, 4.6 x 150 mm.

The material used for MALDI-TOF/TOF (matrix assisted laser desorption/ionization – time-of-flight/time-of-flight) analysis is α -cyano-4-hydroxycinnamic acid (CHCA; Sigma-Aldrich, USA) and for infrared (IR) analysis zinc chloride (Gram-Mol, Croatia) and deuterium oxide (D₂O; Sigma-Aldrich, USA).

Catalytic activity analysis was performed on the HIDEX sense (HIDEX, Finland) microplate reader. Chemicals used for catalytic activity analysis are: pNPA (Sigma-Aldrich, USA) and pNP (Acros Organics, Belgium)

Solvents used for synthesis and characterisation: Milli-Q water, dichloromethane (DCM), DMF and acetonitrile (ACN).

Peptide synthesis

The peptides were manually synthesized using the method of Fmoc solid phase peptide synthesis on a Rink amide AM (100-200 mesh) resin. The Rink resin loading capacity is 0.78 mmol/g. Amino acid coupling was performed using a three-fold excess of Fmoc protected amino acids over the resin in DMF with the HBTU and DIPEA as activating and coupling reagents in a 2.9 and a 6 ratio over the resin respectively. The deprotection of the Fmoc was performed using the solution of 20% piperidine in DMF by submerging the peptide in the solution and mixing for ten minutes. Between each of the protocol processes, the peptide synthesis column was washed three times with DMF and DCM. Double coupling was performed for the isoleucines of the linear peptides and the asparagine, isoleucines and histidines of the cyclic peptides by repeating the process of the addition of the amino acid, HBTU and DIPEA in DMF to the resin without the deprotection with piperidine. The N-terminal amino acid of the linear peptides was acetylated using the 10% solution of acetic anhydride in DMF. The cleavage of the peptide was carried out in a chemical cleavage cocktail: 95% TFA, 2.5% TIS and 2.5% water. The duration of the cleavage is three hours, during which the peptides are submerged in the cleavage cocktail solution. The cleaved peptide was precipitated and washed in a cold DEE after which it was centrifuged at 4700 RPM at a temperature of 4°C for 10 minutes. DEE and the cleavage cocktail supernatant were decanted from the precipitate. The precipitation and washing in cold DEE and the

subsequent centrifugation were performed two more times. The crude peptide was dissolved in a solution of 20% ACN in water.

Peptide cyclization

The cyclization of peptides was performed on resin before the cleavage of the peptide from the solid resin support. The method of peptide cyclization consists of two parts: I) the deprotection of the glutamic acid side chain and II) the subsequent cyclization of deprotected glutamic acid and the free amino terminal group. The glutamic acid (E), with its protecting group *O*-2-PhiPr (*O*-2-phenylisopropyl), was selectively deprotected in a solution of 1% TFA in DCM three times with a duration of ten minutes and subsequently neutralised with a solution of 5% DIPEA in DCM three times with a duration of five minutes. The cyclization of the unprotected on-resin peptide was performed using 5 molar equivalents of PyBOP and 10 molar equivalents of DIPEA over the resin for two hours. The resin was subsequently washed three times with DMF and DCM.

ATR-FTIR spectroscopy

The peptides for the IR experiments were dissolved to a starting concentration of 2 mM in two batches. Crude peptides were used in the IR measurements. One batch contained peptides and Zn²⁺ at a concentration of 5 mM by dissolving zinc chloride in D₂O and the other batch contained peptides dissolved in pure D₂O. The peptides were serially diluted to the final concentration of 0.25 mM and left for 1 hour at room temperature. Peptide samples were recorded on the IR instrument with the following parameters: measurement range (1300-2000 cm⁻¹), resolution 4 cm⁻¹, 2 background scans between each measurement and 100 number of sample scans in transmission measurement mode.

LC-MS QqQ

The crude peptides were dissolved in a solution of 20% ACN/H₂O to a concentration of 1 mg/mL and subsequently filtered through a 0.2 μm filter. The filtered solution was further diluted with water to the final concentration of 0.5 mg/mL. The mobile phases used are: I) water with 0.1% formic acid and II) ACN with 0.1% formic acid. The elution used for the analysis of the linear peptides is a gradient 5-100% change of concentration of ACN in water with a duration of 17 minutes and a flow of 1 mL/min. The elution used for the analysis of the cyclic peptides is a gradient 20-70% change of concentration of ACN in water with a duration of 20 minutes and a flow of 1 mL/min. The injection volume of the sample is 20 μL. Following the gradient elution of the peptide analysis, the mobile phase is changed into a 5 minute 95% ACN in water. The diode array detector (DAD) detector is set to measure at 214 nm. The measuring range of the coupled mass spectrometer is set to 3000 Da.

MALDI-TOF/TOF

The crude peptide sample is dissolved in 50% ACN/H₂O with 0.1% TFA to a concentration of 100 ppm. The CHCA solution is prepared by dissolving a small amount of pure CHCA powder in a solution of 50% ACN/H₂O with 0.1% TFA with progressively more volume until the final solution is saturated with CHCA. An intermediate solution consisting of 50% CHCA and 50% peptide is prepared, and a drop of the solution is added to a MALDI-TOF MS target metal plate. Each of the linear peptide spectra consists of 500 laser shots. The laser intensity is 30%.

Preparative HPLC

The crude linear peptides were dissolved in a solution of 20% ACN/H₂O to a concentration of 4 mg/mL and subsequently filtered through a 0.45 µm filter. The filtered solution was used as a sample for preparative HPLC. The mobile phases used are: I) Water with 0.1% TFA and II) ACN with 0.1% TFA. The elution used for the purification is a gradient 10 to 70% change of concentration of ACN in water with a duration of 30 minutes and a flow of 3 mL/minute. The injection volume of the sample is 500 µL. Following the gradient elution, the mobile phase is changed into a 5 minute 95% ACN in water cleaning run followed by a 5-minute post run which returns the conditions of the mobile phase to the starting 10% ACN in water. The peptides are manually collected into test tubes by collecting the peaks which can be detected on a DAD detector set to measure at a wavelength of 214 nm. Multiple runs were performed per peptide in order to collect more pure peptides from the crude peptide.

Catalytic activity

Lyophilized pure peptide was dissolved in 10 mM HCl to a concentration of 1.1 mM. Isopropanol was added to the solution in a ratio of 1:9. 200 µL of the solution was further diluted 10x with the addition of 1800 µL of pH 8 Tris Buffer (25 mM). Following the dissolution, 8 different samples were prepared in triplicates by combining 50 µL of the peptide solution with 100 µL of 25 mM Tris and 5 µL of zinc solution to a final concentration of 0, 3.125, 6.25, 12.5, 20, 25, 30, 40 µM of ZnCl₂. Finally, 50 µL of pNPA solution in 25 mM Tris buffer was added to the samples to the final concentration of 200 µM of pNPA and immediately measured. A control was created by exchanging the 50 µL of peptide solution with further 50 µL of 25 mM Tris solution. Samples were prepared in a 96 well plate and measured at a wavelength of 405 nm for 30 cycles up to a total of 30

minutes. The calibration curve was prepared by dissolving pNP in a buffer solution and serially diluting the solution from a concentration of 200 μM to 6.25 μM and measuring the absorbance at 405 nm.

Computational modeling

Molecular dynamics simulations of the linear peptides were performed in Gromacs 2022.2³⁰ using the force field CHARMM36m and the temperature 303.15K. The thermodynamic ensemble used in the simulations is the canonical ensemble (NVT ensemble). The simulations were carried by the supercomputer Bura based in The University of Rijeka. The peptides were minimized in the molecular editor program Avogadro³¹. The web-service program CHARMM-GUI³² was used to create files which can be read by GROMACS. Simulations were carried with two peptides and a Zn^{2+} cation per simulation. Peptides were solvated in a rectangular water box type with edge distances from the peptide of 10.0 Å and with added KCl ions at a concentration of 0.15 M placed with the Monte-Carlo method of ion placement. The integration step in the simulation was 2 fs and the coordinates were saved every 100 ps. Production runs were carried for a total simulation time of 50 ns with 500 frames. To minimize the error which arises from minimization of structure towards the stable conformer the first 100 frames (10 ns) were deleted. The programs used for molecular visualization are Pymol³³ and Visual Molecular Dynamics (VMD)³⁴. Pymol was used for model-generating and the visualisation of peptide and zinc, and VMD was used to measure the distance between the Zn^{2+} ion and the peptide and to generate the root mean square deviation (RMSD) and root mean square fluctuation (RMSF) data.

Results

We have designed, synthesized and characterized two linear and two cyclic peptides (Table 8).

Table 8. The synthesized peptides and their abbreviations

Abbreviation / name	Sequence
PL1	Ac-IHIHINI-Am
PL2	Ac-IHINIHI-Am
PC3	cyclo-(IHIHINIE-Am)
PC4	cyclo-(IHINIHIE-Am)

Cyclic versions of the linear peptides, PC3 and PC4, were prepared through the N-terminal to glutamic acid side chain cyclization. The glutamic acid with the protecting group *O*-2-PhiPr was selectively deprotected in a mildly acidic solution of 1% TFA in DCM. The deprotected carboxyl group was subsequently coupled with the free N-terminus.

The validity of the synthesized peptides was analysed with liquid chromatography and mass spectrometry techniques.

Characterization of the peptides

Reverse phase liquid chromatography analysis was performed with the peptides PL1, PL2, PC3 and PC4. The peptides were eluted in the acidic polar mobile phases of water with 0.1% formic acid and ACN with 0.1% formic acid through the nonpolar column CSH C18. The mass analysis was performed using two techniques, the QqQ coupled mass spectrometer with electrospray ionization (ESI) and the MALDI-TOF/TOF mass spectrometer. The MALDI-TOF-MS/MS analysis was performed to confirm that the correct peptides were synthesized, which was used to validate the sequence of the linear peptides. Both linear peptides have the same mass but show a

different disposition of the amino acids within the sequence. To clearly distinguish them, the MS/MS analysis was needed to confirm the primary structure of the peptides.

The LC and MS chromatogram data of the linear PL1 (Ac-IHIHINI-Am), PL2 (Ac-IHINIHI-Am) and cyclic peptides PC3 (cyclo-(IHIHINIE-Am) and PC4 are presented in Figures and Tables 9-21.

The retention time of the peptide PL1 is 8:30 minutes (blue framework, Figure 9a) with the used elution method of 5-100% change of concentration of ACN in water with 0.1% formic acid with a duration of 17 minutes.

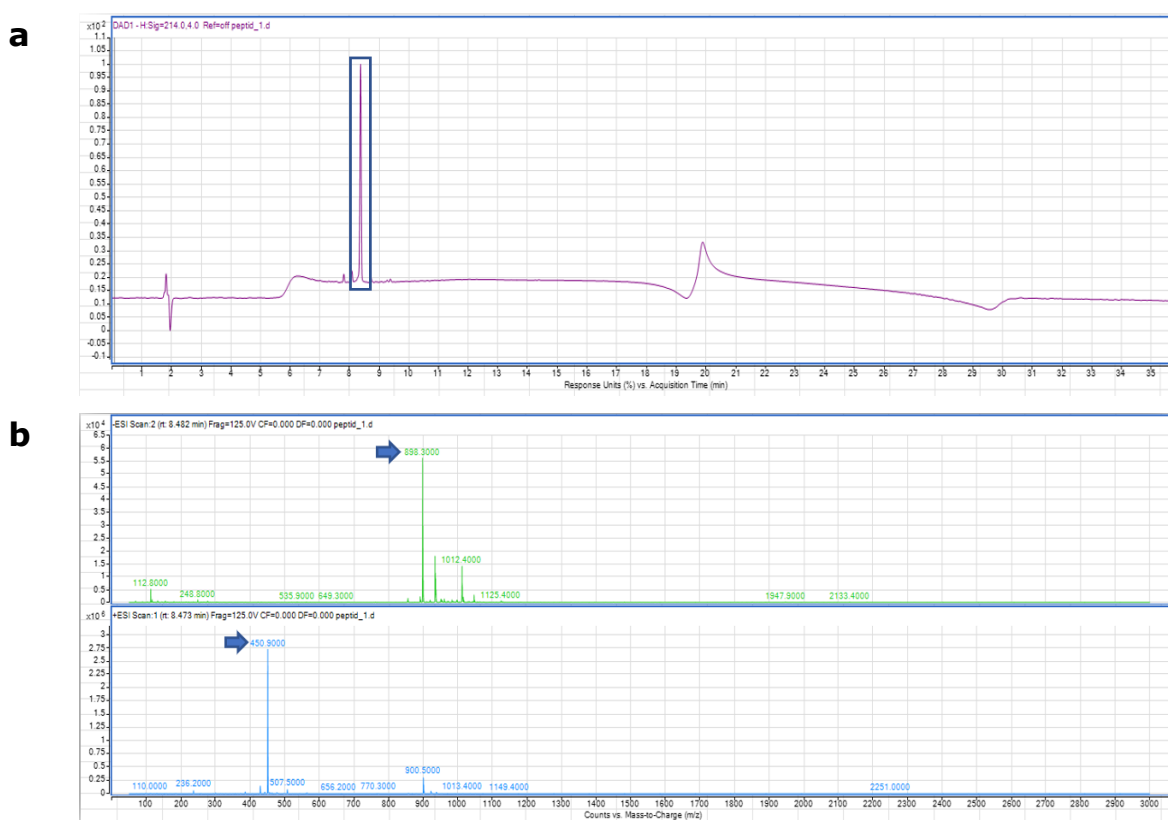


Figure 9. LC and MS characterization of the peptide PL1 **a)** HPLC-DAD chromatogram ($\lambda = 214$ nm). Blue framework is the retention time which was analysed in b). **b)** MS ESI (electrospray ionization) negative and positive ion mass-to-charge (m/z) ratios (range 50-3000 Da). The main peaks are marked with blue arrows.

The molecular mass of the peptide is 900.074 Da for the average mass and 899.523 Da for the monoisotopic mass. The main peaks from Figure 9, which are marked with blue arrows, are presented in Table 10 and correspond to the expected m/z ratio for the peptide PL1.

Table 10. Expected and measured m/z of peptide PL1. Abbreviations: Avg.= Average, Mono. = Monoisotopic, Meas. = Measured

Charge	Mass (Avg.)	Mass (Mono.)	m/z (Meas.)
-1	899.0833	898.5258	898.3000
+2	451.0453	450.7741	450.9000

The masses of nine peptide fragments can be marked out on the mass chromatogram data given in Figure 11. The peptide fragments are marked with blue arrows and their m/z ratios are presented in Table 13.

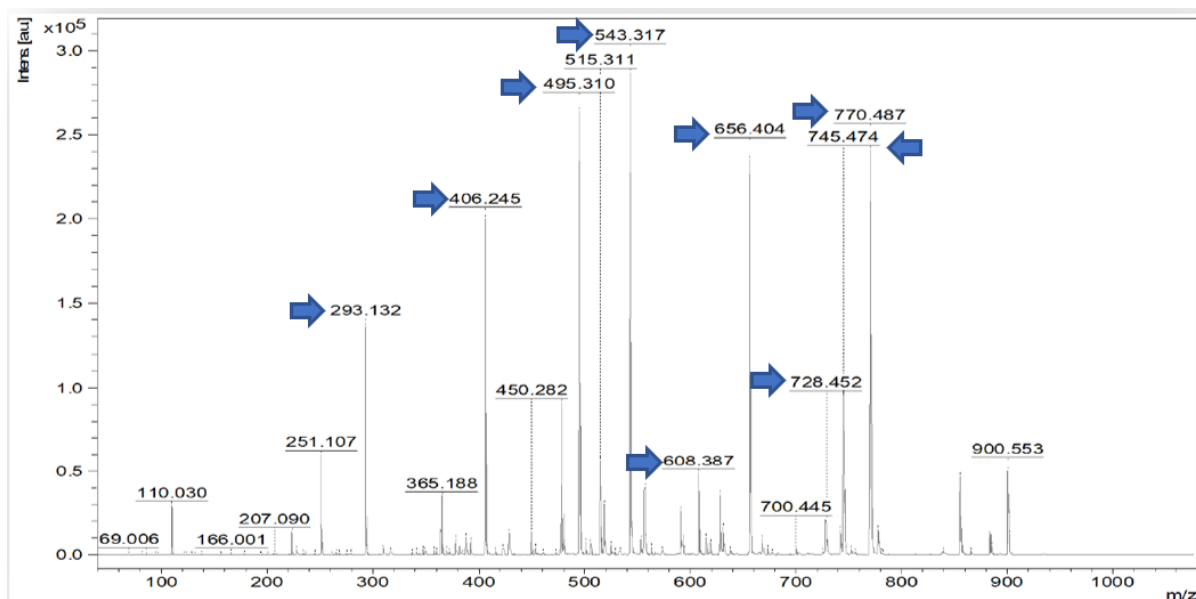


Figure 11. MALDI-TOF-MS/MS mass chromatogram of peptide PL1 (range 50-1000 Da). The identified fragments are marked with blue arrows.

Peptides consist of amino acid residues linked together by amide bonds. Collision-induced dissociation technique carried out by the MALDI-TOF/TOF mass spectrometer fragments peptides mostly along the peptide backbone, often with a transfer of one or two hydrogens to create stable ion structures. The system for peptide fragments is illustrated in Figure 12. N-terminal fragment ions (those containing the N-terminus of the peptide, or in this case the modified acetyl group) are labelled with the first three letters of the alphabet (a, b, c), while the C-terminal fragment ions (which contain the C-terminus of the peptide, or in this case the modified amide group) are labelled with the last three letters of the alphabet (x, y, z). Numerical subscripts identify the position of the amino acid at which fragmentation has occurred, which for N-terminal fragment ions coincides with the standard numbering of peptide residues, whereas the C-terminal fragment ions are numbered sequentially from the C-terminal amino acid³⁵.

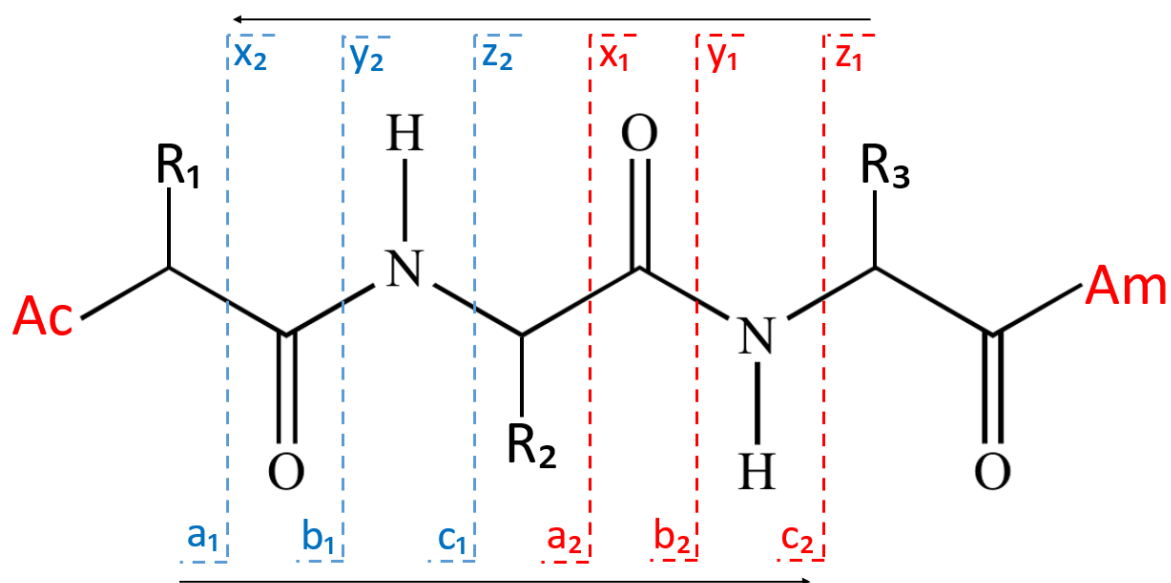


Figure 12. Schematic illustration of different types of fragment ions.

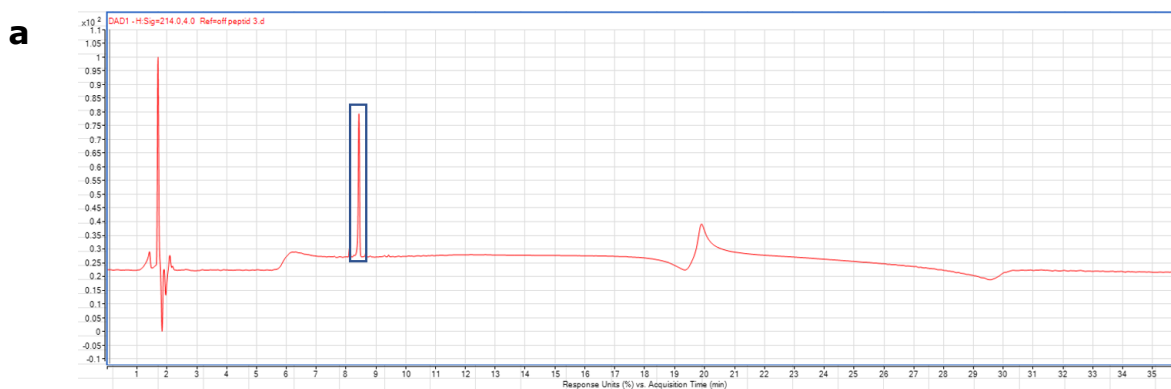
The list of the detected b, y and z fragments for the peptide PL1 can be found in Table 13 confirming that the analysed sequence has the correct amino acid sequence. Five b, three y and one z fragments can be found in Figure 11 mass chromatogram. The peptide fragments b_4 [Ac-IHIH(C=O)]⁺ and b_5 [Ac-IHIHI(C=O)]⁺ which are underlined in the Table 13 differ from

the possible PL2 peptide fragment ions and prove the validity of the PL1 peptide sequence.

Table 13. Expected and measured m/z of peptide PL1 fragments. The fragment ionic charge is +1.

Fragment	Sequence	Mass (Avg.)	Mass (Mono.)	m/z (Meas.)
<i>b</i> ₂	Ac-IH(C=O)	293.345	293.160	293.132
<i>b</i> ₃	Ac-IHI(C=O)	406.504	406.244	406.245
<i>y</i> ₄	(NH)HINI-Am	495.586	495.304	495.310
<u><i>b</i>₄</u>	Ac-IHIH(C=O)	543.645	543.303	543.317
<i>y</i> ₅	(NH)IHINI-Am	608.746	608.388	608.387
<u><i>b</i>₅</u>	Ac-IHIHI(C=O)	656.805	656.387	656.404
<i>z</i> ₆	(R)HIHINI-Am	728.421	728.873	728.452
<i>y</i> ₆	(NH)HIHINI-Am	745.887	745.447	745.474
<i>b</i> ₆	Ac-IHIHIN(C=O)	770.909	770.430	770.487

The retention time of the peptide PL2 is 8:30 minutes (blue framework, Figure 14a) with the used elution method of 5-100% change of concentration of ACN in water with 0.1% formic acid with a duration of 17 minutes.



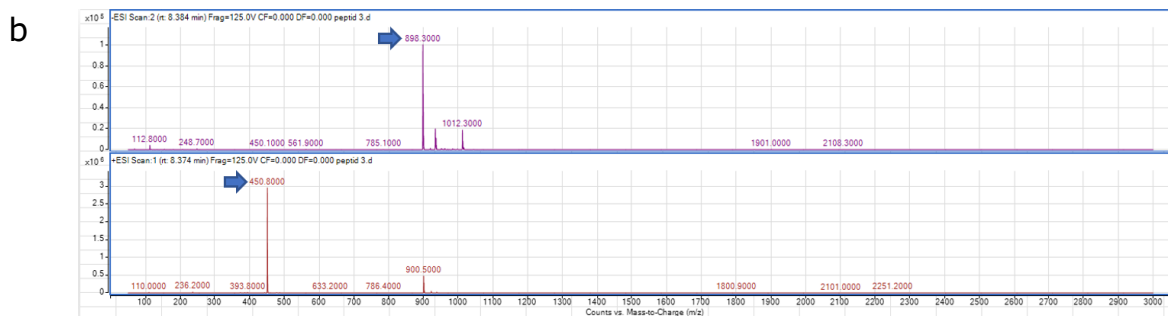


Figure 14. LC and MS characterization of the peptide PL2 **a)** HPLC-DAD chromatogram ($\lambda = 214$ nm). Blue framework is the retention time which was analysed in b). **b)** MS ESI negative and positive ion m/z ratios (range 50-3000 Da). The main peaks are marked with blue arrows.

The molecular mass of the peptide is 900.074 Da for the average mass and 899.523 Da for the monoisotopic mass. The main peaks from Figure 14, which are marked with blue arrows, are presented in Table 15 and correspond to the expected m/z ratio for the peptide PL2.

Table 15. Expected and measured m/z of peptide PL2

Charge	Mass (Avg.)	Mass (Mono.)	m/z (Meas.)
-1	899.0833	898.5258	898.3000
+2	451.0453	450.7741	450.8000

The masses of ten peptide fragments can be marked out on the mass chromatogram data given in Figure 16. The peptide fragments are marked with blue arrows and their m/z ratios are presented in Table 17.

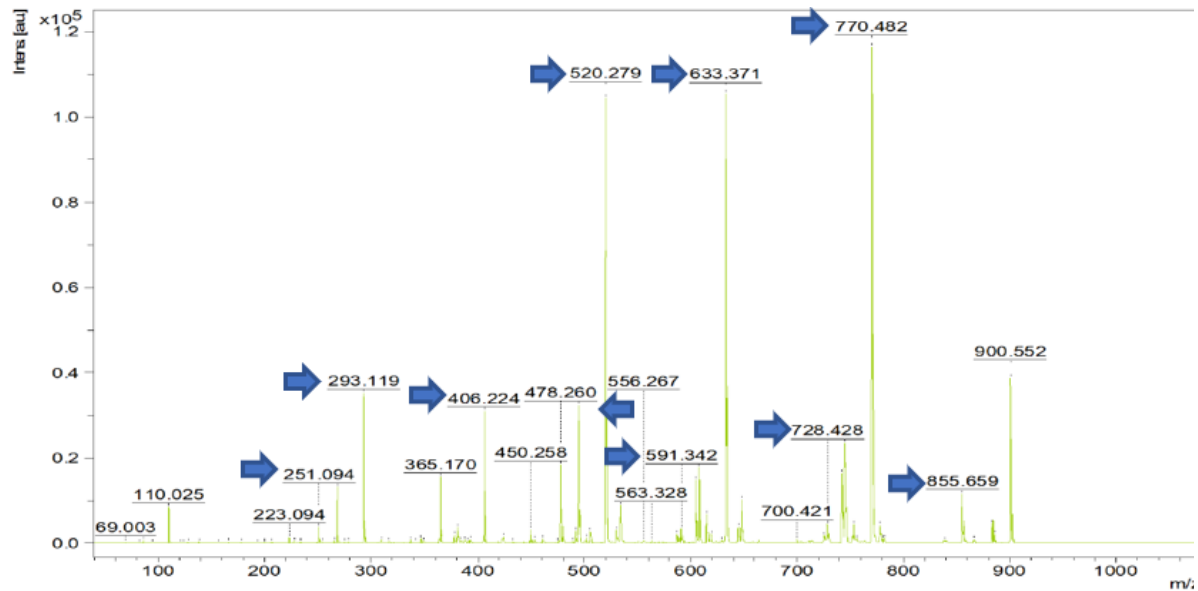


Figure 16. MALDI-TOF-MS/MS analysis of peptide PL2 (range 50-1000 Da). The identified fragments are marked with blue arrows.

The list of the detected a, b, z fragments for the peptide PL2 can be found in Table 17 confirming that the analysed sequence has the correct amino acid sequence. Five b, four z and one a fragments can be found on the Figure 16 mass chromatogram. The peptide fragments z_2 [(R)HI-Am]⁺, b_4 [Ac-IHIN(C=O)]⁺ and b_5 [Ac-IHINI(C=O)]⁺ which are underlined in Table 17 differ from the possible PL1 peptide fragment ions and prove the validity of the PL2 peptide sequence.

Table 17. Expected and measured m/z of peptide PL2 fragments. The fragment ionic charge is +1.

<i>Fragment</i>	Sequence	Mass (Avg.)	Mass (Mono.)	m/z (Meas.)
<u>z</u>₂	(R)HI-Am	251.309	252.151	251.094
<i>b</i> ₂	Ac-IH(C=O)	292.345	293.160	293.119
<i>b</i> ₃	Ac-IHI(C=O)	405.504	405.244	406.224
<i>z</i> ₄	(R)NIHI-Am	478.572	478.278	478.260
<u>b</u>₄	Ac-IHIN(C=O)	519.608	519.287	520.279
<i>z</i> ₅	(R)INIHI-Am	591.732	591.362	591.342
<u>b</u>₅	Ac-IHINI(C=O)	632.766	632.371	633.371
<i>z</i> ₆	(R)HINIHI-Am	728.873	728.421	728.428
<i>b</i> ₆	Ac-IHINIH(C=O)	769.909	769.430	770.482
<i>a</i> ₇	Ac-IHIHINI(R)	855.073	854.535	855.659

The retention time of the peptide PC3 is 7:20 minutes (blue framework, Figure 18a) with the used elution method of 20-70% change of concentration of ACN in water with 0.1% formic acid with a duration of 20 minutes.

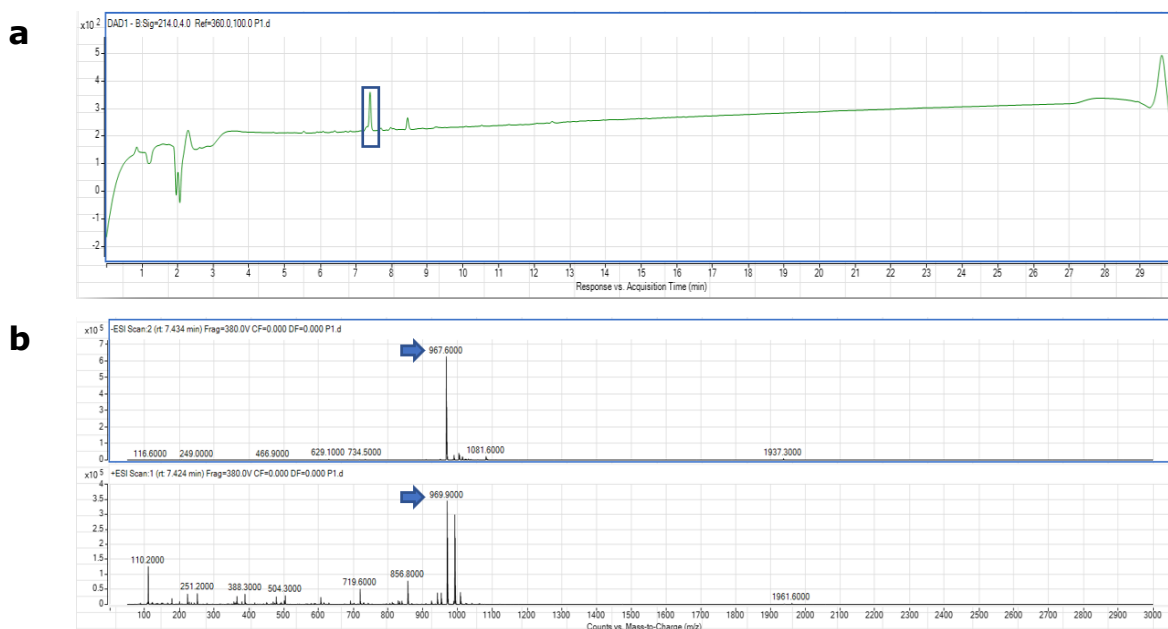


Figure 18. LC and MS characterization of the peptide PC3 **a)** HPLC-DAD chromatogram ($\lambda = 214$ nm). Blue framework is the retention time which was analysed in b). **b)** MS ESI negative and positive ion m/z ratios (range 50-3000 Da). The main peaks are marked with blue arrows.

The molecular mass of the peptide is 969.1543 Da for the average mass and 968.5536 Da for the monoisotopic mass. The main peaks from Figure 18, which are marked with blue arrows, are presented in Table 19 and correspond to the expected m/z ratio for the peptide PC3.

Table 19. Expected and measured m/z of peptide PC3

Charge	Mass (Avg.)	Mass (Mono.)	m/z (Meas.)
-1	968.1465	967.5458	967.6000
+1	970.1615	969.5735	969.9000

The retention time of the peptide PC4 is 7:10 minutes (blue framework, Figure 20a) with the used elution method of 20-70% change of concentration of ACN in water with 0.1% formic acid with a duration of 20 minutes.

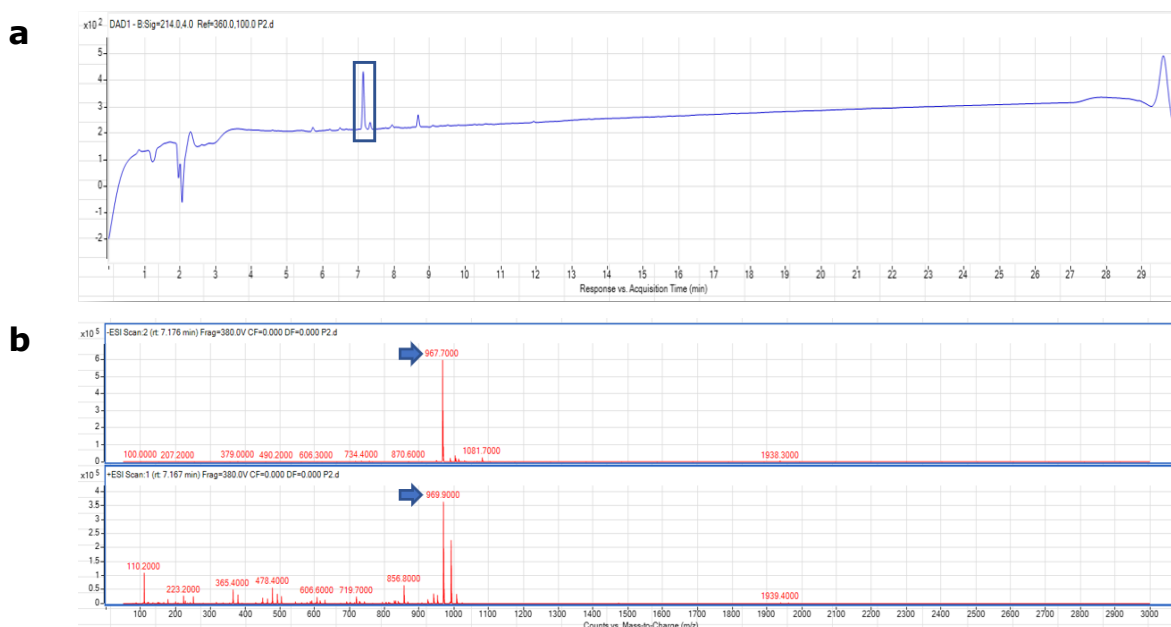


Figure 20. LC and MS characterization of the peptide PC4 **a)** HPLC-DAD chromatogram ($\lambda = 214$ nm). Blue framework is the retention time which was analysed in b). **b)** MS ESI negative and positive ion m/z ratios (range 50-3000 Da). The main peaks are marked with blue arrows.

The molecular mass of the peptide is 969.1543 Da for the average mass and 968.5536 Da for the monoisotopic mass. The main peaks from Figure 20, which are marked with blue arrows, are presented in Table 21 and correspond to the expected m/z ratio of the peptide PC4.

Table 21. Expected and measured m/z of peptide PC4

Charge	Mass (Avg.)	Mass (Mono.)	m/z (Meas.)
-1	968.1465	967.5458	967.7000
+1	970.1615	969.5735	969.9000

The purity of the analysed crude peptide samples was assessed with the manual integration of chromatogram peaks (Table 22). The main peak (blue framework, Figure 9, 14, 18, 20a) was used as the major peak for purity calculations. Linear peptides PL1 and PL2 are 63.00 and 72.29% pure respectively and the cyclic peptides PC3 and PC4 are 56.88% and 67.74% pure respectively. The peptide with the highest purity is PL2 (72.29%) and the lowest purity PC3 (56.88%).

Table 22. The purity of the peptides calculated by chromatogram peak integration

Peptide	Purity
PL1	63.00%
PL2	72.29%
PC3	56.88%
PC4	67.74%

Following the liquid chromatography and mass spectrometry analysis, the linear peptides PL1 and PL2 were purified. The peptides were purified by manually collecting the main peaks (blue framework, Figure 9, 14a) by collecting at a time lapse around the peak retention time of 8:30 minutes. The final purification mass after lyophilisation is 1 mg, which after 10 purifications with in total 20 mg of crude peptides, gives the purification yield of 5%.

Infrared (IR) spectroscopy

Infrared analysis was performed with crude peptides PL1, PL2, PC3 and PC4 to analyse the formation of supramolecular peptide structures. Having established the purity of the peptides, we used IR to evaluate the fibril forming in the presence of Zn^{2+} ²³. The absorbance spectra with the range of 1300-2000 cm^{-1} was measured, as it contains the intense amide I vibrational region (1600-1700 cm^{-1}).

Different conditions were tested. The peptides were, at varying concentrations, dissolved in two solutions: pure D_2O and D_2O with 5 mM $ZnCl_2$. The table of prepared solutions for IR analysis of the peptides is given in Table 23.

Table 23. Table of prepared solutions for IR analysis of the peptides PL1, PL2, PC3 and PC4

1) Pure D_2O	
2) D_2O with 5 mM $ZnCl_2$	
Concentrations of peptide in the first measurement batch / peptide dissolved in D_2O	Concentrations of peptide in the second measurement batch / peptide dissolved in D_2O with 5 mM $ZnCl_2$
3a) 2 mM	3b) 2 mM
4a) 1 mM	4b) 1 mM
5a) 0.5 mM	5b) 0.5 mM
6a) 0.25 mM	6b) 0.25 mM

The infrared measurement data of the linear PL1 (Ac-IHIHINI-Am), PL2 (Ac-IHINIHI-Am) and cyclic peptides PC3 (cyclo-(IHIHINIE-Am)) and PC4 (cyclo-(IHINIHIIE-Am)) is given In Figures 24-25.

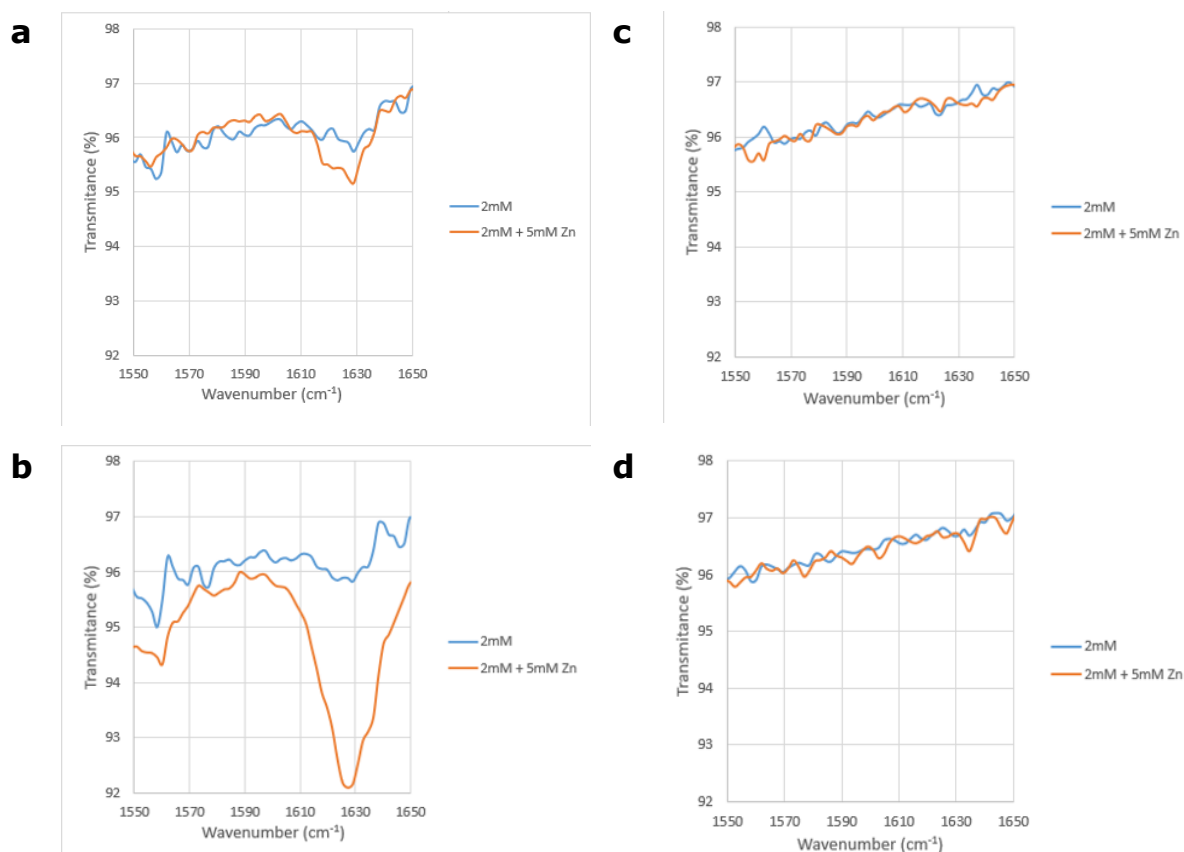


Figure 24. IR spectra for peptides dissolved in D₂O and D₂O with 5 mM ZnCl₂ to a concentration of 2 mM: **a)** PL1, **b)** PL2, **c)** PC3, **d)** PC4. Measurement range is 1550–1650 cm⁻¹.

No difference in absorbance is observable between pure water and water-zinc solution. Dissolved zinc salt has no influence on the absorption band of the peptides so the difference between absorbances come from peptides themselves. The absorbance peak at 1630 cm⁻¹ is characteristic for the amide I parallel β -sheet²² and can be seen absorbed by peptides PL1 and PL2 while dissolved in water-zinc solution (Figure 24a, b; Figure 25). The same absorption band is not observable with the linear peptides dissolved in pure heavy water solution. On the other hand, no observable absorption is visible with the cyclic peptides (Figure 24c, d). The β -sheet like spectrum is seen only in linear peptides PL1 and PL2 dissolved in deuterium oxide with dissolved zinc, which suggests that zinc promotes the formation of the structure.

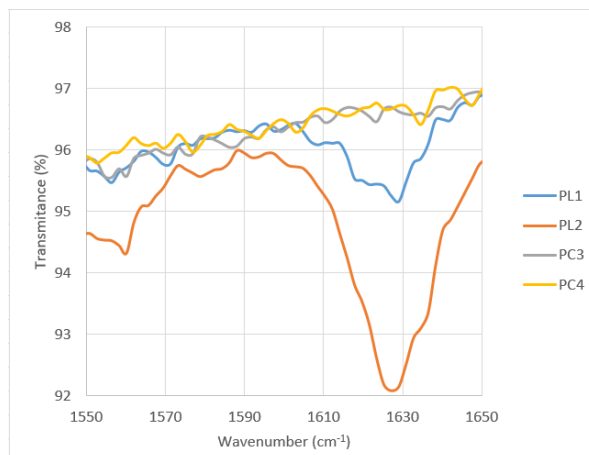


Figure 25. IR spectra for peptides PL1, PL2, PC3, PC4. Peptides were dissolved to a concentration of 2 mM in D₂O with 5 mM ZnCl₂. Measurement range is 1550-1650 cm⁻¹.

The peptide with the highest absorbance in the 1630 cm⁻¹ band is the PL2 (Ac-IHINIHI-Am) (Figure 25), which could suggest that the changing of the position of asparagine within the sequence between two histidine residues could increase the β -sheet forming capabilities of the peptide. The peptide PL2, with the asparagine in the middle of the sequence is more symmetrical than the peptide PL1 (Ac-IHIHINI-Am), which could suggest that it helps in forming the β -sheet structure.

Computational simulation

Computational simulation was performed with two peptides, PL1 and PL2, with two peptides per single simulation. The duration of the simulation is 50 ns, with the first 10 ns of the simulation being discarded for reasons of energy minimization in the zinc-peptide distance measurements. Rufo *et al.*, 2014., showed that their most active peptide Ac-IHIHIQI-Am coordinates two peptides between a single Zn²⁺ ion. Two His residues from one peptide and one His from the second peptide in vicinity constitute the primary coordination sphere and the building block of the intermolecular peptide structure²³.

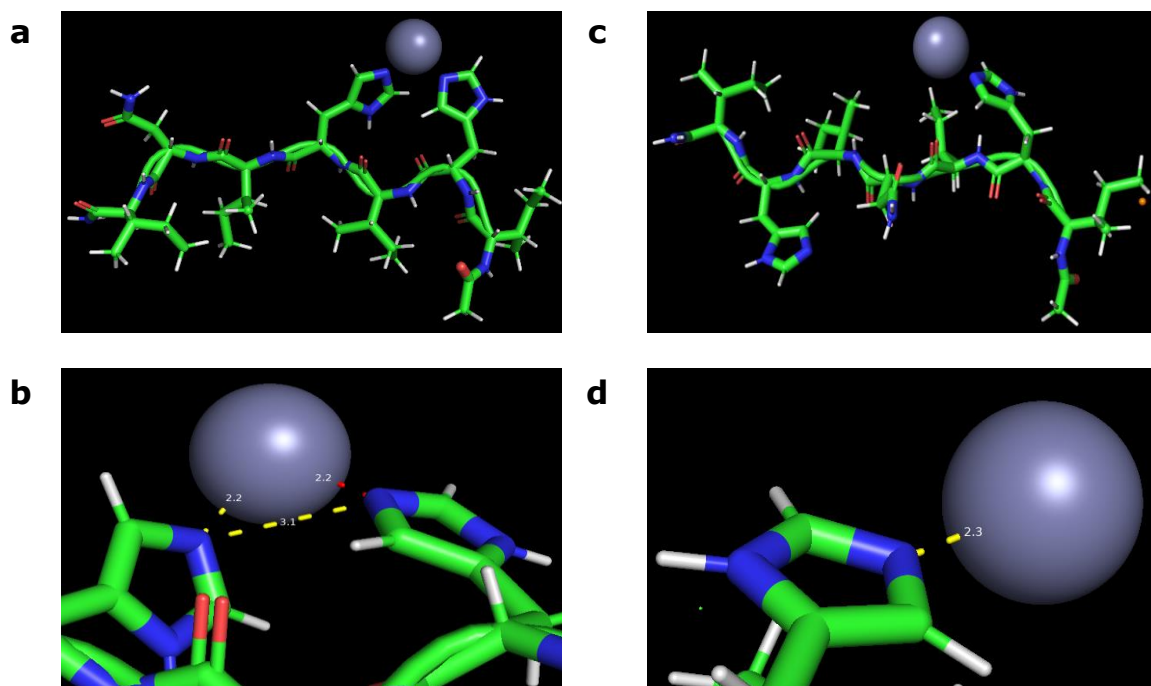


Figure 26. Computationally derived model of zinc metal coordination. Zn^{2+} ion is shown as a grey sphere. Dotted line between the histidine nitrogen and the Zn^{2+} ion is the straight line between the centre of the atoms.

Peptide PL1: **a)** A model of the designed peptide with the Zn^{2+} ion. **b)** The distance between the metal sphere centre and the histidine side chain's nitrogen [\AA].

Peptide PL2: **c)** A model of the designed peptide with the Zn^{2+} ion. **d)** The distance between the metal sphere centre and the histidine side chain's nitrogen [\AA].

In this work, the simulation was repeated twice and the zinc was coordinated with a single peptide (PL1 and PL2) (Figure 26a, c). Two His residues of the peptide PL1 with the i and $i+2$ His residues coordinate a single Zn^{2+} ion (Figure 26b), while a single His residue of the peptide PL2 with the more far-off i and $i+4$ His residues coordinate the Zn^{2+} ion (Figure 26d).

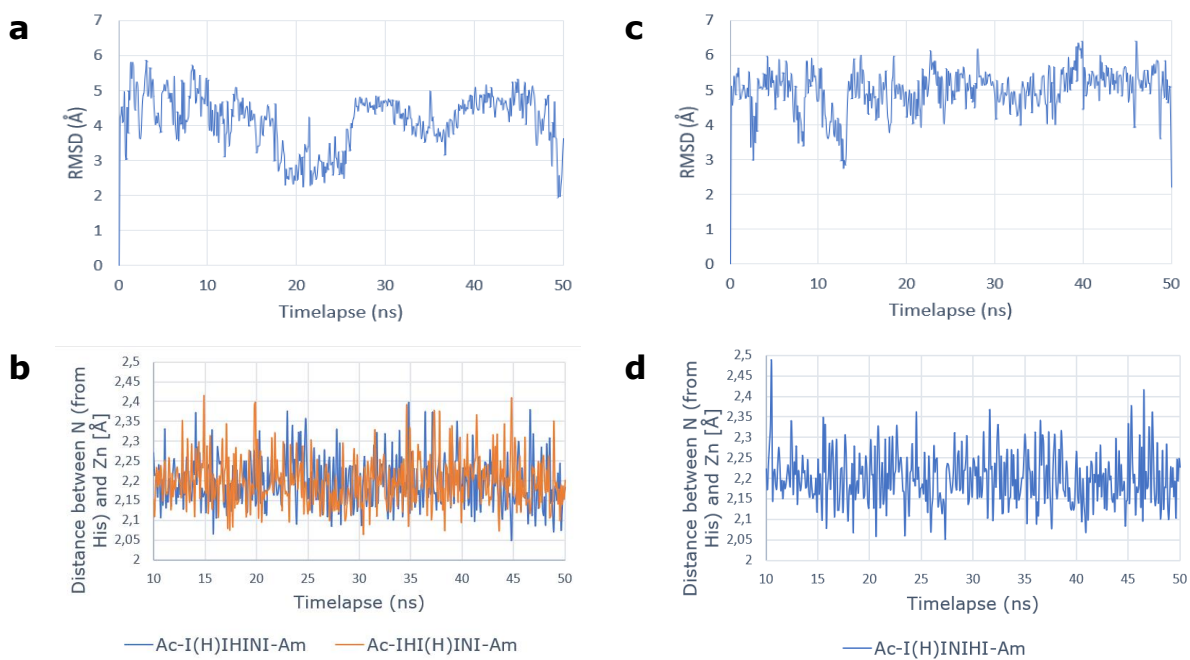


Figure 27. RMSD diagram and the distance between Zn^{2+} ion and the histidine in peptides PL1 and PL2. The RMSD diagram represents a simulation for 50 ns while the zinc-histidine distance measurements represents the simulation for 40 ns.

Peptide PL1: **a)** RMSD analysis of molecular dynamics simulation trajectory. **b)** The distance change between the centre of the metal sphere and the closest histidine side chain's nitrogen. The measured histidine is labelled with (H).

Peptide PL2: **c)** RMSD analysis of molecular dynamics simulation trajectory. **d)** The distance change between the centre of the metal sphere and the closest histidine side chain's nitrogen. The measured histidine is labelled with (H).

RMSD is the average displacement of the atoms at an instant of the simulation relative to a reference structure³⁶, in this case to the first frame of the simulation. The RMSD data plotted in Figure 27a, c diagrams shows that, after the highly mobile start of the simulation, a relatively small oscillation (3 Å) in the RMSD value is observable which means that the molecular dynamics simulation has undergone relaxation and the system has reached a stationary state with the peptides being equilibrated. As

shown in Figure 27b, d, the average distance between the nitrogens of the histidines and zinc is 2.2 Å, which is consistent with the study by Rufo *et al.*, 2014., which showed the distance of 2.2 Å.

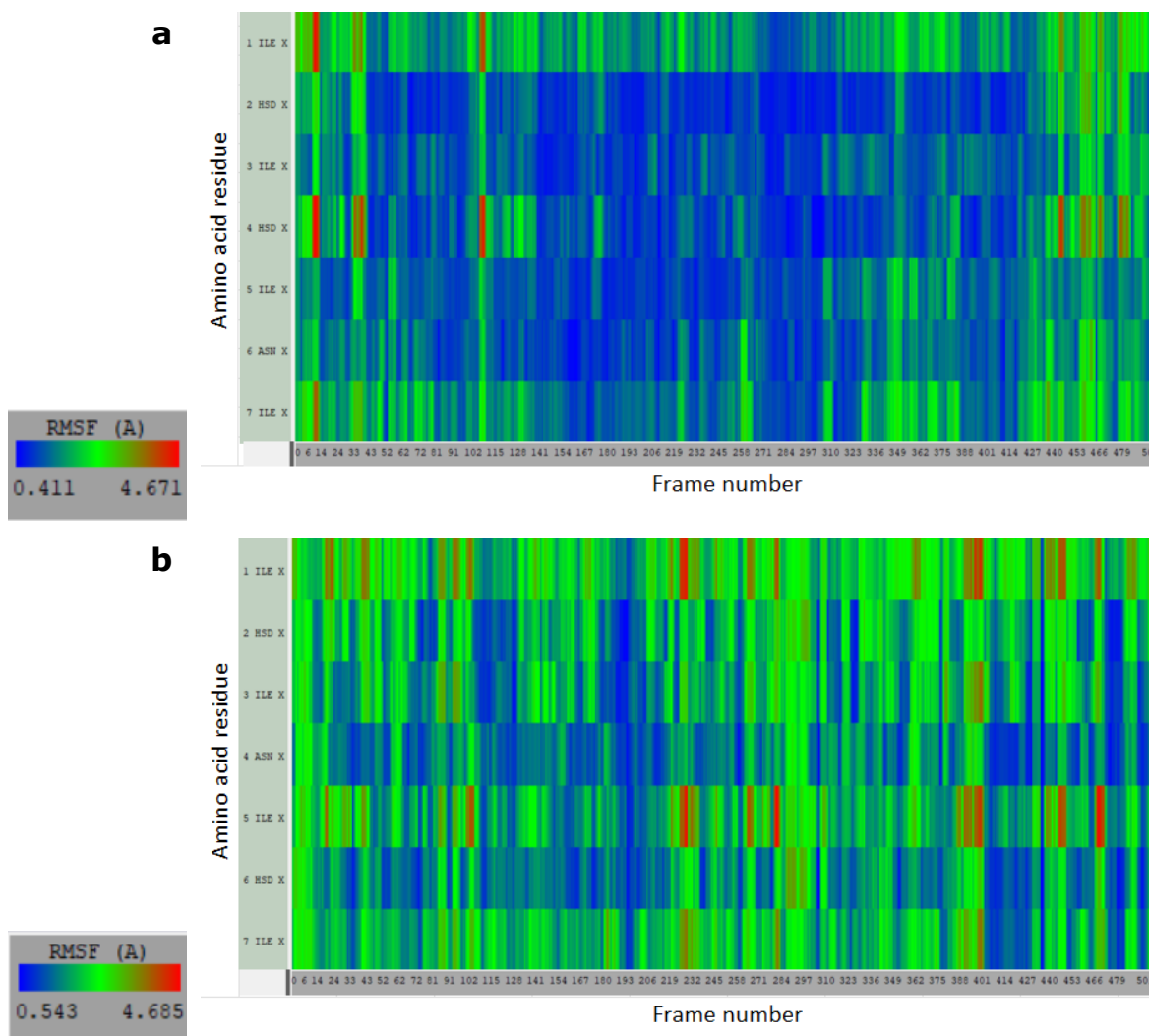


Figure 28. RMSF diagram representing molecular dynamics simulation for 50 ns representing: **a)** Peptide PL1; **b)** Peptide PL2. The RMSF numerical legend is shown at the bottom left corner of the diagrams.

RMSF is a measure of the displacement of a particular atom, or a group of atoms, relative to the reference structure, averaged over the number of atoms³⁶. The displacement of amino acid residues was measured relative to the starting structure of the peptides in the Figure 28. The red and green colours indicate that the residues are highly mobile and flexible at

measured time intervals, while the blue colour indicates the rigidity of the residue. As shown with the green and red colours in both peptides in Figure 28., the terminal isoleucines are more mobile than the internal residues. Apart from the terminal residues, the flexible residues include the central histidine (residue number 4) of the PL1 peptide (Figure 28a) and the 3rd isoleucine (residue number 5) of the peptide PL2 (Figure 28b). The high flexibility of the histidine and isoleucine could emerge from zinc-coordination and consequent molecular flexing.

Kinetic assay

Kinetic assay was performed using the peptide Ac-IHIHIQI-Am. The lyophilised pure peptide was dissolved in a solution of 25 mM (pH 8) Tris buffer, 200 μ M pNPA and ZnCl₂ to a concentration of 25 μ M. Measurements were performed using different concentrations of dissolved ZnCl₂, from 40 μ M to 0 μ M, as shown in Table 29. The control measurement was performed without the peptide.

Table 29. Table of prepared solutions for the kinetic assay analysis of the peptide Ac-IHIHIQI-Am

Measurement number	Zinc concentration
1.	40 μ M
2.	30 μ M
3.	25 μ M
4.	20 μ M
5.	12.5 μ M
6.	6.25 μ M
7.	3.125 μ M
8.	0 μ M

Calibration curve was constructed using six different serially diluted pNP concentrations dissolved in PBS buffer measured at a wavelength of 405 nm, from 200 μM to 6.25 μM (Figure 30).

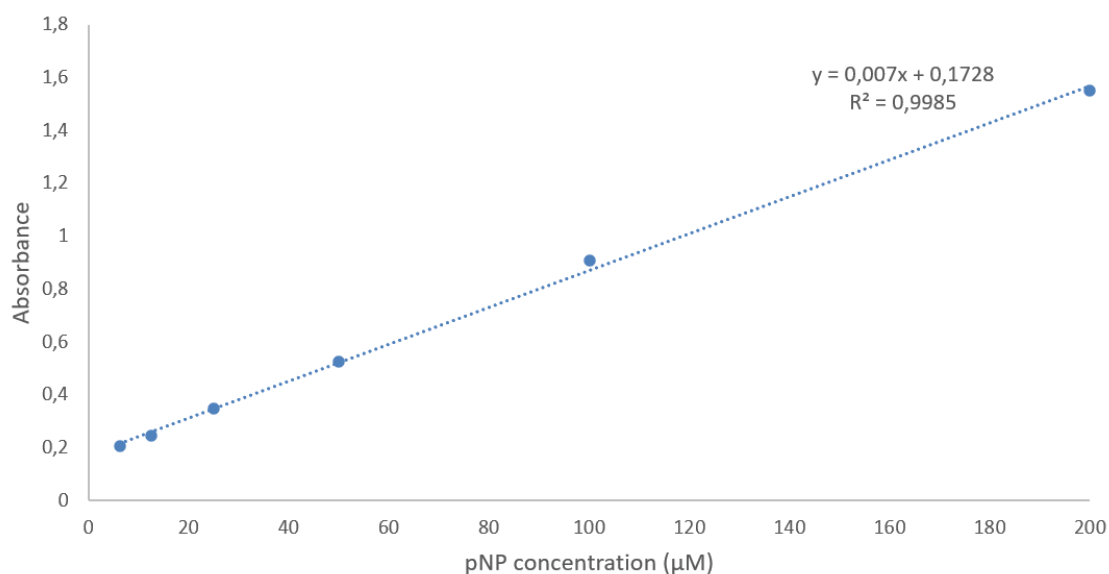


Figure 30. pNP concentration calibration curve

The linear equation for the dependence of absorbance on the concentration of pNP is $y = 0.007x + 0.1728$, where the x is the concentration of pNP in μM and the y is the absorbance at a wavelength of 405 nm. The coefficient of determination (R^2) of 0.9985 indicates that 99.85% of variance of the dependent value (absorbance) is explained by the variance of the pNP concentration. The descriptive power of the R^2 value is high and the linear regression matches well with the measured data.

The kinetic assay plots of the peptide Ac-IHIHIQI-Am is given in Figure 31. The peptide was dissolved in a Tris buffer solution as shown in Table 29. The measurements were performed at a wavelength of 405 nm for 30 cycles up to a total of 30 minutes.

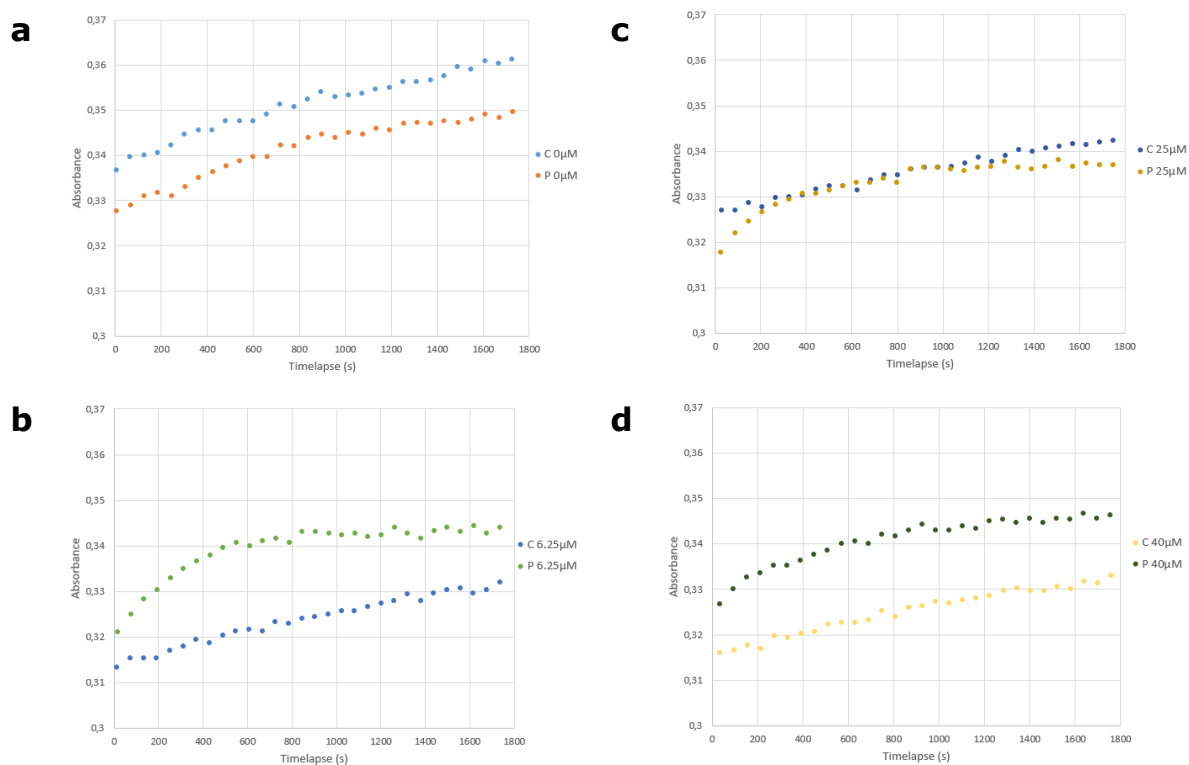


Figure 31. Kinetic assay plots of the peptide Ac-IHIHIQI-Am. The abbreviation C stands for the control measurement without the dissolved peptide while P stands for the experimental measurement with the peptide. **a)** Concentration of ZnCl_2 of $0 \mu\text{M}$; **b)** Concentration of ZnCl_2 of $6.25 \mu\text{M}$; **c)** Concentration of ZnCl_2 of $25 \mu\text{M}$; **d)** Concentration of ZnCl_2 of $40 \mu\text{M}$.

The concentration of zinc ranged from $40 \mu\text{M}$ to $0 \mu\text{M}$, which compared to the concentration of peptides of $25 \mu\text{M}$ means that the molar equivalent of zinc over the peptide is from 1.6 to 0. Rufo *et al.* discovered that two Ac-IHIHIQI-Am peptides coordinate a single zinc ion, which was shown in a computational simulation and a zinc concentration kinetic assay²³. We have expected that molar equivalents of 0.5 over the concentration of the peptide would saturate the absorbance spectrum and that the kinetic assay plots would look similar over that value as no further zinc ions could be chelated by peptides.

Even though we have changed the concentrations of zinc, no difference in absorbance spectrums of the measurements can be seen in Figure 31. The

differences between the absorbances of the control and experimental measurements are negligible.

These measurements were performed with one changing parameter, the concentration of the dissolved ZnCl_2 . The other parameters of peptide concentration and pNPA concentration remained the same. The kinetic assay measurement with the peptide Ac-IHIHIQI-Am with the concentration of 25 μM and pNPA concentration of 200 μM with these concentrations of zinc did not give measurable data.

Discussion

Enzymes catalyse a vast number of chemical reactions with high efficiency and selectivity in a diverse set of conditions. Much effort has been dedicated into developing new enzymes and changing the selectivity and scope of existing enzymes³⁷. We have designed peptides with alternating polar and nonpolar residues and modified terminal groups. The catalytic activity of the peptide utilizes metal ions as cofactors and it is zinc based. The two His residues in the peptide should coordinate the zinc metal ion. We designed four peptides to test the change of self-assembly and catalytic properties in three different areas:

- a) The effect of changing the amino acid glutamine (Q) for asparagine (N), for which a peptide PL1 was synthesized. Glutamine and asparagine are both amino acids with polar uncharged side chains, with the only difference being a single methylene group which is missing in asparagine.
- b) The effect of changing the position of asparagine within the sequence, for which a peptide PL2 was synthesized. The two His residues of the peptide Ac-IHIHIQI-Am are located at i and $i+2$ positions and are both coordinating a single Zn^{2+} ion. Changing the position of asparagine in between the two His residues puts them at positions i and $i+4$ and increases the distance between the two chelating histidines and the zinc ion.
- c) The effect of cyclization on the overall activity of the peptide, for which two peptides were synthesized: PC3 and PC4. By using cyclization to form intra-connected peptide structures, we increased the rigidity of the three-dimensional structure.

Peptide synthesis

The four peptides PL1, PL2, PC3 and PC4 were synthesized using the SPPS technique. In total, the crude peptide synthesis yield was 200 mg for the linear peptides and 150 mg for the cyclic peptides. Characterisation was performed following the full cleavage of the peptides from the resin. After the full cleavage of the cyclic peptides, an unexpectedly low yield of 50 mg was obtained. The mass chromatogram of the peptide PC4 showed the ESI-charged mass of 1209.7000 Da and the ESI+ charged mass of 1211.9000 Da. The expected average mass of the peptide PC4 is 969.1543 Da. The side chain protecting group of the histidine used in peptide synthesis (Fmoc-His(Trt)-OH) is triphenylmethyl (Trytyl, Trt), with the average mass of 242.320 Da. Adding the triphenylmethyl protecting group mass with the average expected peptide mass gives the mass of 1211.4743 Da. The cleavage was repeated once more with both cyclic peptides and a yield of 150 mg was achieved while also showing the validity of the sequence in the mass chromatogram. It is possible that an error occurred during the preparation of the cleavage cocktail, which would mean that the cocktail was not acidic enough with the high enough concentration of TFA to fully deprotect and cleave the peptide.

Characterization of the peptides

All analysed peptides show one dominant peak in the reversed phase HPLC chromatogram. The mass chromatogram data obtained from the coupled mass spectrometer indicates that the peptides of the desired mass are the ones which constitute the dominant peak. Both the positive and the negative ESI MS ion modes were used to analyse the peptides. The major advantage of ESI+ mode, which charges through protonation, lies in the fact that more compounds are expected to ionize in this mode if ESI/MS is coupled with liquid chromatography. On the other hand, a major advantage of ESI- mode, which charges through deprotonation, is the lower

background noise, improved sensitivity and potential for lower detection limits³⁸.

The tandem mass spectrometry data of the linear peptide PL1 and PL2 was used to determine the correct fragment ions, but the cyclic peptides PC3 and PC4 were also analysed using the MALDI tandem mass spectrometry. The problem arises with the searching of fragments. As the peptides do not have a free terminal group but are intra-connected with an isopeptide bond, no a, b, c and z, y and x fragments can be assessed. The only m/z ratios of major intensity which were observed in the MALDI measurements of the cyclic peptides were the 969.579 Da for the peptide PC3 which corresponds to the expected average mass of 969.1443 and the m/z ratios of 969.569 Da and 1938.127 Da for the peptide PC4. The ratio of 969.569 corresponds to the expected average mass of 969.1443 for the peptide PC4 while the 1938.127 is possibly a dimer since it has twice the mass than the expected mass for the peptide.

Peptide purification was performed with the linear peptides PL1 and PL2 by a manual collection of the main peaks. The final purification mass after lyophilisation is 1 mg, which means that the purification yield is 5% and that 95% of the before-crude sample was dissipated. One possible issue could arise from the manual collecting of peaks, as a certain amount of the dissolved peptide passes through the analytical tubing before being redirected into the peak-collecting test-tubes.

Preparative HPLC was not performed with the cyclic peptides and will be a part of future work.

Infrared (IR) spectroscopy

Infrared analysis was performed with the four peptides PL1, PL2, PC3 and PC4 to analyse the formation of fibril structures with zinc. The linear peptides are showing absorbance at the peak of 1630 cm^{-1} which suggests that the rigid nature of the cyclic peptides withholds the peptides from creating a supramolecular β -sheet-like structure. The measurements were performed with D_2O , and not with normal water because water IR band (1640 cm^{-1}) interferes with the protein amide I band. The OH vibrations caused by liquid water and water vapor would have needed to be subtracted out of the protein solution spectrum³⁹.

The peptides were left for one hour at room temperature to incubate with zinc, but the possible problem is the inability to know the appropriate time for the complete nucleation of the supramolecular structure. Rufo *et al.* showed that the introduction of Gln into the sequence and, especially the substitution of leucines with isoleucines drastically diminishes the fibrillation time, the time needed to form fibril aggregates, and the maximum activity is achieved with no need for longer incubation, as opposed to the Leu-core peptides with the lag-time of 24 hours²³. The amyloid fibril formation lag-time represents a time that is required for the nuclei that are formed early on in the reaction to grow and proliferate in order to reach detectable aggregate concentration levels⁴⁰. It is unknown if the peptides used in this study for the IR analysis have fully formed supramolecular structures, as the single methylene group missing on the Asn residue compared with the Gln, the Glu residue in the cyclic peptides and the cyclization of the peptides PC3 and PC4 could impact the amyloid fibril formation lag-time.

Computational simulation

Two simulations were performed with both peptides PL1 and PL2 to try to see if two peptides can chelate a single Zn^{2+} , as was the case in the simulation by Rufo *et al.*²³. A computational simulation with cyclic peptides and zinc has been attempted, but the web-service program CHARMM-GUI³² was not able to read the head-to-side chain cyclization files from the molecular editor program Avogadro³¹. Since CHARMM-GUI was needed to create the files for a GROMACS³⁰ simulation, the simulation could not be performed. Head-to-side chain cyclizations are less common in the literature and it is possible that the programs are not optimized to perform simulations with such types of peptides.

Kinetic assay

Kinetic assay was performed with the pure lyophilised peptide Ac-IHIHIQI-Am, whose measurement was shown in this thesis, and with the synthesized peptide PL1. A preliminary measurement with the peptide PL1 with different concentrations of pNPA (from 1 to 0 mM) and constant concentration of peptide (24 μ M) and zinc (1 mM) showed no differences in absorbances between different pNPA concentrations. The peptide did not fully dissolve in a stock solution of 7.4 pH phosphate-buffered saline (PBS) buffer which means that the concentrations used in the measurements are unknown. Tris buffer was found to be a better solvent for the peptides. K_M and K_{cat} values were incalculable from the different-pNPA concentration measurements. pNPA is a sensitive chemical and more care is necessary with its management. At non-optimal pH and temperature values it is capable of decomposing into pNP and is not well soluble in water-based solutions at higher concentrations. Considering that the kinetic assay measurement with zinc has three notable parameters: the concentration of zinc, peptide and pNPA; we have chosen to optimize the method and test

the dependence of absorbance on the concentration of zinc. The used concentrations of zinc, pNPA and peptide did not give the measurable data to calculate the zinc-dependence of the peptide Ac-IHIHIQI-Am. It is possible that the error comes from poor pipetting due to the high precision necessary to pipette several different solutions into a 96-well and the speed at which it needs to be put to measurement as the catalytic reaction of hydrolysis commences as soon as the substrate and enzyme mix. Different concentrations of the peptide and repeated measurements with different concentrations of pNPA and zinc will be necessary to optimise the kinetic assay method and will be a part of future work.

Conclusion

In this thesis four peptides were synthesized, two linear and two cyclic, inspired by the most catalytically active peptide from a work by Rufo *et al.*, 2014.²³, Ac-IHIHIQI-Am. These peptides were tested for their ability to form supramolecular structures and act as Zn²⁺ dependent esterases. The chromatography and mass spectroscopy analysis of the peptides data shows the validity of the sequences of synthesized peptides. The validity of the cyclic peptide structures proved the possibility of octapeptide on-resin cyclization. Infrared analysis shows the supramolecular structure-forming abilities of the linear peptides, which are missing in the absorbance spectrums of cyclic peptides. Computational simulation showed that the linear peptides PL1 and PL2 can coordinate a zinc ion with their histidine residues. The combination of computational and „wet-lab“ techniques is a useful tool for gaining insights in the field of catalytic chemistry. Further research and experiment repeats are necessary to validate the potential catalytic activity of the peptides and will be a part of a future work.

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Životopis

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O MENI

Student diplomskog sveučilišnog studija Medicinska kemija na Odjelu za biotehnologiju Sveučilišta u Rijeci. Trenutno istražujem kemiju peptida i njihovu mogućnost uporabe kao potencijalnih katalizatora. Želim proširiti svoja znanja iz biokemijskih znanosti te u budućnosti voditi svoje vlastite znanstvene projekte. Osim kemije, zanimaju me informatika i ekonomija.

OBRAZOVANJE I OSPOSOBLJAVANJE

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Područja obrazovanja: Prirodne znanosti, matematika i statistika

Konačna ocjena : 4,421

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Konačna ocjena : 4.393

Diplomski rad: Application of Machine Learning in Peptide Design

Sveučilišni prvostupnik biotehnologije i istraživanje lijekova - Cum LaudeBacc. Biotech et. Pharm

Opća gimnazija

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RADNO ISKUSTVO

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Fidelta d.o.o. [07/2021]

Mjesto: Zagreb

Zemlja: Hrvatska

Studentska praksa u zagrebačkoj tvrtci Fidelta d.o.o. Na praksi su razvijena brojna znanja iz analitike; timski rad sa tekućinskom kromatografijom - analitičkom i preparativnom, masenim spektrometrom i optičkim emisijskim spektrometrom sa induktivno spregnutom plazmom (ICP-OES).

Studentska praksa - Odjel za biotehnologiju, laboratorij doc.dr.sc. Duška Čakare

Odjel za biotehnologiju, Rijeka [06/2020]

Mjesto: Rijeka

Zemlja: Hrvatska

Studentska praksa u laboratoriju doc.dr.sc. Duška Čakare na odjelu za Biotehnologiji Sveučilišta u Rijeci. Razvijena znanja iz spektroskopije i kemije koloidnih sustava.

Ljetni učenički i studentski poslovi - trgovac, booker apartmana, skladišno-administrativni poslovi, vođenje instrukcija iz kemije i matematike

[06/2013 - Trenutačno]

Mjesto: Cres, Rijeka, Istra

Zemlja: Hrvatska

Radim od osmog razreda osnovne škole svako ljeto u rodnome gradu Cresu. Plaćam si troškove studiranja u Rijeci studentskim poslovima tijekom godine.

PUBLIKACIJE

Application of Machine Learning in Peptide Design - Završni rad

[2020]

<https://repository.biotech.uniri.hr/islandora/object/biotechri>

:514 urn:nbn:hr:193:310606

Istraživanje uporabe računalnih tehnika u dizajnu peptida. Dizajn peptida je skup i dugotrajan proces jer istraživači provode visoko-protočni probir zajedno sa eksperimentalnom validacijom koji zahtijevaju veliku količinu vremena i sredstava. Strojno učenje i genetski algoritmi mogu biti korišteni za brži i jeftiniji dizajn programiranjem računala da radi proces dizajniranja za nas.

Design of linear and cyclic peptides for ester hydrolysis – Diplomski rad

[2022]

Glavni cilj ovog diplomskoga rada je dizajn i karakterizacija novih linearnih i cikličkih katalitičkih peptida. Peptidi su inspirirani katalitičkim peptidima iz znanstvenih radova. Tijekom rada, dobio sam iskustva u sintezi peptida na čvrstom nosaču (Solid phase peptide synthesis), u ciklizaciji peptida te u instrumentalnim metodama purifikacije i analize. Osim rada u laboratoriju, koristio sam svoja znanja iz računalne kemije te virtualno radio simulacije peptida u računalnim programima na superračunalu Bura Sveučilišta u Rijeci. Polje cikličkih peptida, pogotovo cikličkih katalitičkih peptida, je još uvijek većim dijelom neistraženo područje znanstvenih istraživanja. Koristeći ciklizaciju za formiranje povezanih cikličkih struktura, očekujemo da će povećana rigidnost dobivena ciklizacijom poboljšati katalitičku efikasnost kratkih peptida.

PROJEKTI

HRZZ projekt (uip-2019-04-7999) - Dizajn katalitički aktivnih peptida i peptidnih nanostruktura(DeShPet) - suradnik

[10/2020 - Trenutačno]

Dizajn katalitički aktivnih peptida i peptidnih nanostruktura. Projekt hrvatske zaklade za znanost pod vodstvom doc.dr.sc. Daniele Kalafatović. Sinteza peptida u laboratoriju te njihova daljnja analiza. Nakon mog završnog rada na temu Primjene strojnog učenja u dizajnu peptida nastavio sam raditi u laboratoriju na projektu DeShPet. Moj doprinos projektu je rad u laboratoriju koji je povezan sa računalnim simulacijama koje provodim na superračunalu Bura Sveučilišta u Rijeci.

Iskustvo: rad na UV-Vis spektrofotometru te fluorescentnom spektrometru, masena spektrometrija, mikroskopija, sinteza peptida na črستم nosaču (SPPS), nabava laboratorijskih kemikalija/posuda, molekularna simulacija na računalu itd.

POZNAVANJE METODA U KEMIJI

Kromatografija

Poznavanje rada na kromatografskim uređajima, HPLC, UPLC i preparativna kromatografija. Iskustvo sa kombiniranim metodama tekućinske kromatografije i masene spektrometrije. Iskustvo dobiveno radom u tvrtci Fidelta d.o.o. i na Odjelu za biotehnologiju tijekom rada u projektu DeShPeT i istraživanja za diplomski rad.

Masena spektrometrija

Poznavanje rada na uređajima za masenu spektrometriju. Triple quadrupole i maldi time-of-flight spektrometar. Iskustvo dobiveno radom u tvrtci Fidelta d.o.o. i na Odjelu za biotehnologiju tijekom rada u projektu DeShPeT i istraživanja za diplomski rad.

Spektrometrija

Iskustvo rada sa spektrometrima, UV-vidljivim (UV-Vis), fluorescentim, infracrvenim (IR) i optičko-emisijskim sa induktivno spregnutom plazmom (ICP-OES).

Iskustvo rada sa optičko-emisijskim spektrometrom dobiveno u tvrtci Fidelta d.o.o., iskustvo rada sa UV-Vis, IR i fluorescentnim spektrometrima dobiveno na Odjelu za biotehnologiju tijekom rada u projektu DeShPeT i na studentskoj praksi u laboratoriju doc.dr.sc. Duška Čakare.

Računalna kemija

Dobro poznavanje programa za računalnu kemiju: Chimera, Cresset Spark-Flare, Gromacs, Marvin, Pymol, VMD, wxMacMolPlt, Gamess...

Iskustvo dobiveno tijekom studija i istraživanja na Odjelu za biotehnologiju. Provođenje simulacija peptida i cinka na najsnažnijem superračunalu u Hrvatskoj, Buri, Sveučilišta u Rijeci

https://drive.google.com/file/d/1X_56CDhSIIMKvPIXcq-Y5JYHVHemfHLK/view?usp=sharing

Sinteza peptida na čvrstem nosaču

Iskustvo dobiveno tijekom istraživanja na Odjelu za biotehnologiju u projektu DeShPeT

KONFERENCIJE I SEMINARI

27. Hrvatski skup kemičara i kemijskih inženjera (HSKIKI) - Poster prezentacija

[Veli Lošinj, Primorsko goranska županija, 05/10/2021 - 09/10/2021]

Hrvatski skup kemičara i kemijskih inženjera je organiziran od strane Hrvatskog kemijskog društva u Velom Lošinj u otoku Lošinj u listopadu 2021. godine. Skup okuplja znanstvenike iz različitih grana kemijskih znanosti na seminarima, radionicama i predavanjima. Za ovaj skup sam pripremio plakat na temu "*Design of linear and cyclic peptides for ester hydrolysis*" (*Sabino Sepčić, Patrizia Janković, Daniela Kalafatović*) kojeg sam prezentirao na poster prezentaciji tijekom skupa. Plakat se može otvoriti u poveznici opisa.

<https://27hskiki.hkd.hr/>

<https://drive.google.com/file/d/1iWLqXWbHg6XyqOKjkjRbuJQk15UCgviE/view?usp=sharing>

Pint of Science Croatia - Popularizacija znanosti

[Rijeka, 17/05/2021 - 19/05/2021]

Pint of Science je međunarodni festival popularizacije znanosti koji se održava u svibnju i dovodi istraživače do vašeg lokalnog bara da Vam pričaju o najnovijim zbivanjima u znanosti. Ove godine je festival održavan online, pa sam pripremio video u kojemu objašnjavam cilj mojeg rada u laboratoriju u Rijeci. Video uradci se mogu pogledati na Facebook i Youtube stranicama Pint of Science Croatia.

https://pintofsciencehr.wixsite.com/2020?fbclid=IwAR0h-IF5Vsja_B7OX0u7TMzIGt2oCgROgstxzPMFdFgBeYcuWXY7l6czmRE

Solid Phase Peptide Synthesis: Design, Side-Reactions and Manufacture

[Rijeka - Online, 21/04/2021 - 22/04/2021]

Online predavanja tvrtke Tides o sintezi peptida na čvrstoj fazi. Fokus ovih predavanja su analiza sekvenci peptida, sintetička metodologija i nusprodukti koji nastaju sintezom peptida

https://informaconnect.com/tides-oligonucleotide-and-peptide-therapeutics-series/agenda/3/#webinars_solid-phase-peptide-synthesis-design-side-reactions-and-manufacture_08-30

MREŽE I ČLANSTVA

Član: Studentski zbor biotehnologije u Rijeci

[Rijeka, 09/2018 - Trenutačno]

Rad u studentskom zboru odjela za Biotehnologiju. Čuvanje interesa studenata i promoviranje odjela za Biotehnologiju

Studentski predstavnik odjela za Biotehnologiju

[Rijeka, 10/2017 - Trenutačno]

Stegovno povjerenstvo Odjela za biotehnologiju

[10/2018 - 09/2020]

Provedba stegovnih postupaka prema pravilniku o stegovnoj odgovornosti nastavnika i suradnika Sveučilišta u Rijeci

POČASTI I NAGRADE

Studentski znanstveni fond SIZIF

Zaklada Sveučilišta u Rijeci i Ured za znanost Studentskog zbora Sveučilišta u Rijeci [2021]

Otvoreni znanstveni fond Sveučilišta u Rijeci. Znanstveni fond mi je financirao aktivno sudjelovanje na skupu kemičara i kemijskih inženjera u Velom Lošinju (HSKIKI) koji se održao u listopadu 2021. godine.

<https://www.zaklada.uniri.hr/zaklada/financijske-potpore/fond-sizif/>

Državna stipendija za studente u STEM područjima znanosti - 2019-2020.

[2019]

Stipendija Ministarstva znanosti i obrazovanja za studente preddiplomskih STEM studija.

<https://stemstipendije.mzo.hr/>

Stipendija grada Cresa - 2017-2019., 2020-2022.

Grad Cres [2017]

Festival matematike 2017. - Ekipno natjecanje

Srednja škola Ambroza Haračića, PO Cres [05/05/2017]

Ekipno matematičko natjecanje u Puli. Od 63 prijavljenih ekipa srednjih škola plasirali smo se na 1. mjesto iz kategorije općih, jezičnih i klasičnih gimnazija.

<http://www.ss-aharacica-malilosinj.com.hr/?p=9589>

http://mdi.hr/festival_matematike_2017

Međunarodno natjecanje Klokane bez granica 2017.

Srednja škola Ambroza Haračića, PO Cres [23/03/2017]

Međunarodno matematičko natjecanje - Klokane bez granica 2017., plasiranje u top 5% najboljih u Republici Hrvatskoj.

http://www.ss-aharacica-malilosinj.com.hr/?page_id=14439

VOLONTIRANJE

Organiziranje sakupljanja donacija građana grada Cresa za pomoć gradu Petrinji

[Cres, 29/12/2020 - 31/12/2020]

Zajedno sa svojim ocem i dogradonačelnikom grada Cresa, Marinom Gregorovićem, organiziralo se sakupljanje potrepština za građane grada Petrinje i okolice. Sakupili smo preko četiri tone potrepština, te smo sa dva dostavna vozila na datum Nove godine odvezli potrepštine u Petrinju i okolna sela.

https://www.facebook.com/permalink.php?story_fbid=2667806683505197&id=1379554622330416

Otvoreni dan Odjela za biotehnologiju

[Rijeka, 05/2022]

Volontiranje na radionici "Fantastični peptidi i kako ih pronaći" na Odjelu za biotehnologiju. Cilj radionice je bio širenje znanosti o proteinima i peptidima učenicima osnovnoškolske i srednjoškolske dobi, gdje su učenici mogli kvalitativno testirati razne tvari iz kućanstva na sadržaj

proteina.

Volonter udruge studenata biotehnologije Sveučilišta u Rijeci (USBRI)

[Rijeka, 10/2017 - Trenutačno]

Volonter na brojnim projektima udruge studenata biotehnologije.

<http://www.usbri.uniri.hr/>

DIGITALNE VJEŠTINE

Moje digitalne vještine

MS Office (Excel, Word, PowerPoint) / poznavanje programa PyMOL, Avogadro, ChemAxon Marvin, VMD, GAMESS, MacMOLPit / Osnove programskog jezika Python / izvrsno poznavanje financijskog upravljanja računovodstva i financijskog planiranja / MedCalc statistical software / UCSF ChimeraX / Shell Script (Bash) / Timski rad

JEZIČNE VJEŠTINE

Materinski jezik/jezici: **hrvatski**

Drugi jezici:

engleski

SLUŠANJE C1

ČITANJE C1

PISANJE C1

GOVORNA

PRODUKCIJA C1

GOVORNA

INTERAKCIJA C1