THESES OF DOCTORAL (Ph.D.) DISSERTATION

Modification of the activity of hydrolytic enzymes by the nature of the non-endogenous/non-native metal ions

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Összefoglaló **Zeyad Hasan Abdullah Nafaee "Hidrolitikus enzimek aktivitásának módosítása nem endogén/idegen fémionok révén"** c. PhD disszertációjához.

A PhD munka kétféle hidrolitikus enzim vizsgálatát tűzte ki célul, különös tekintettel a fémionok katalitikus aktivitásra gyakorolt hatására. A TEM-1 β-laktamáz, a β-laktám antibiotikumok hidrolízisét segíti elő, azokkal szemben rezisztenciát okozva. Az NColE7 a vetélytárs baktériumok DNS-ének hidrolízise révén védi meg a gazdasejtet.

A TEM-1 β-laktamáz nem metalloenzim, de számos potenciális fémkötőhely található benne. A fehérjetisztítás sikere fémion-affinitás kromatográfiával bizonyította a Ni(II) ionok kölcsönhatását a felszíni hisztidin oldalláncokkal. Ezt spektroszkópiás vizsgálatok is alátámasztották. A fehérjében található kén donoratomok szoft jellegű fémionokat köthetnek meg, mint a Cd(II), vagy Hg(II) ionok. Míg a Hg(II) ionok csökkentették az enzim katalitikus aktivitását, a Ni(II) és Cd(II) ionok elősegítették a szubsztrát hidrolízisét. A fémion természete meghatározó: a Hg(II) ionok az aktív központ közelében kötődve gátolták a reakció végbemenetelét, míg a másik két ion a szubsztrátot aktiválta. Az enzimkinetika felülvizsgálatára is sor került.

Az NColE7 metalloenzim aktív központjában egy cinkion kötődik három hisztidin oldallánchoz és a szubsztrát hasítandó csoportjához, tetratéderes elrendeződésben. A rögzített geometria miatt a vizsgált Ni(II), Cd(II) és Cu(II), idegen fémionok kötődését a fehérjéhez csak annak apo (fémmentes) formájában mutattuk ki. Kompetitív körölmények között a fehérje cink-kötött formában van. Az eddigi ismeretektől eltérően, úgy találtuk, hogy a fehérje fémion távollétében is működőképes. Ugyanakkor, a Ni(II) ion lényegesen megnöveli a katalitikus aktivitást, valószínűleg a folyamat mechanizmusának megváltoztatásával. Ez a fémion a fehérje és a szubszrát mellett egy vízmolekulát is megköthet, amelyet aktiválva hatékony nukleofil támadó ágens jön létre.

A tanulmányozott enzimek a bakteriális védekezőrendszer részét képezik, így reményeink szerint, eredményeink hozzájárulnak hatékonyabb antibiotikumok kifejlesztéséhez.

I. Introduction and objectives

It is estimated that 50% of all proteins contain metal ions, and 30% of all proteins require metal ions in for their functions associated with life. A protein that binds a metal ion in complex form is called a metalloprotein. Metal ions are coordinated by nitrogen, oxygen, or sulfur donor atom containing functional groups in the side chains of amino acid residues of the proteins. These groups are mainly the imidazoles of histidine residues, thiolates of cysteine residues, and carboxylates of aspartates and glutamates. Metalloproteins that perform a catalytic function are called metalloenzymes, promoting chemical transformations in a molecule (substrate).

Metalloproteins, as well as other proteins offering sites for potential metal ion binding, may be affected by non-endogenous/non-native metal ions found in the environment. Metal contamination therefore, may change the behavior of biological systems *e.g.*, bacteria. Bacteria, antibiotics, as well as metal ions are released into the environment through many ways leading to severe contamination. As a consequence of interaction of bacterial enzymes with metal ions, highly aggressive bacteria may develop in the environment or highly resistant bacteria evolve towards the antibiotic drugs.

In this PhD thesis, the two main topics include the study of the interaction of non-endogenous/non-native metal ions with two types of bacterial hydrolytic enzymes: (i) TEM-1 β-lactamase and (ii) NColE7 nuclease mutants that are common targets of scientific research due to their relationship to antibacterial action and resistance. Both enzymes are part of the defense systems of the bacteria. TEM-1 β-lactamase protects bacteria from β-lactam antibiotics, while NColE7 is the nuclease domain of colicin E7 that cleaves chromosomal DNA of the other bacteria under stress conditions and in cases of lack of nutrition. TEM-1 β-lactamase hydrolyzes the β-lactam ring, while NColE7 is a metalloenzyme, and cleaves the phosphodiester bond of DNA non-specifically.

We hypothesized that the TEM-1 β-lactamase interacts with toxic borderline metal ions such as Ni(II) and soft metal ions such as Hg(II) and Cd(II), leading to a change in its function. The Zn(II)-binding site of NColE7 or its mutants is suggested to be able to accommodate other metal ions such as Ni(II), Cu(II), and

Cd(II) ions. This can lead to changes in the structural properties and function of these enzymes. The results presented here are expected contribute to the development of new strategies for protein purification, to better understanding of the kinetic behavior of β-lactamase, metal ion-protein interaction, and antibiotic development. The main objectives of my PhD work are described in the following.

1. Synthesis and purification of the proteins

TEM-1β-lactamases were extensively studied in the literature. They were purified by different techniques as recombinant proteins, but the purification method most commonly used, applied denaturing conditions and/or affinity purification tags. While the selected TEM-1 β-lactamase is not a metalloprotein, its amino acid sequence offers many metal binding sites, such as the side-chains of the His residues at its surface. So TEM-1 β-lactamase can be purified in its native fold with Ni(II)-loaded immobilized metal ion affinity chromatography (IMAC) resin. The questions addressed at this point included: (**i**) Is it possible to overexpress the protein, although its DNA gene is located outside of the multi-cloning site? (**ii**) What are the purification conditions of the protein based on its metal binding sites on immobilized metal ion affinity chromatography? (**iii**) How much pure protein can we obtain in comparison with other published methods?

Previously, KGNK protein was purified by GST affinity chromatography supplied with HPLC, while KGNK-His was purified by a batch-type immobilized metal ion affinity method. We planned to purify KGNK-His by IMAC supplied with HPLC and to express and purify new batches of the KGNK protein by GST-HPLC.

2. Characterization of the proteins by mass spectrometry, UV-absorption and CD spectroscopy, and gel electrophoresis to identify and check the quality and quantity of the purified proteins

We planned to investigate the purified proteins running along the following points: (**i**) purity, (**ii**) concentration, (**iii**) identification/molecular mass, and (**iv**) folding/secondary structure composition. The purity of the obtained proteins is primarily monitored by SDS-PAGE. While this method provides information on the concentration of the protein solution, we also planned to apply UV-absorption spectroscopy for this purpose. For determination of solution structural properties, CD

spectroscopic measurements were foreseen, followed by the evaluation of the spectra to calculate the secondary structure compositions and comparison of the results with the published crystal structures of these proteins. The advantage of the planned mass spectrometric measurements (MS) is that they can identify the expressed protein in fragmentation experiments, and they provide information on the molecular mass and in some cases on the folding of the proteins.

3. Determination of the kinetic parameters of TEM-1 β-lactamase promoted ampicillin hydrolysis process

We observed fluctuations of the molar absorbance (ε) values of the hydrolysis product of ampicillin in the catalytic experiments published in the literature. As a consequence, various values of k_{cat}, and K_M were determined for TEM-1 β-lactamase promoted ampicillin hydrolysis, while the comparison is further complicated by mutations in the enzyme amino acid sequence. Therefore, we aimed to find reasons for the fluctuation of the values of kinetic parameters by studying the kinetic activity of TEM-1 β-lactamase in the hydrolysis of ampicillin substrate under commonly used conditions. We planned to reevaluate the kinetic parameters using multi-wavelengths monitoring of the kinetic progress curves and their evaluation without preconceptions applied in classical Michaelis-Menten formalism. Tracking the hydrolysis product by mass spectrometry was expected to support the evaluation.

4. Unravelling the non-endogenous/non-native metal ion binding and their structural effects

The mature TEM-1 β-lactamase possesses 6 His residues to bind borderline transition metal ions, 2 Cys and 9 Met residues with sulfur donor groups for soft metal ion binding, and further oxygen donor groups for interaction with hard metal ions. While KGNK and KGNK-His proteins were previously shown to purify together with Zn(II) ions supposed to be their native cofactor, they can interact with other divalent metal ions, as well. Furthermore, KGNK-His contains a C-terminal 6×His affinity tag, which may affect the metal ion binding properties and thereby, the structure and function of the protein. Based on this, we aimed to study the effect of the non-endogeneous metal ions, such as Ni(II), Cd(II), and Hg(II) on non-metalloenzyme TEM-1 β-lactamase, and the non-native Ni(II), Cu(II), and Cd(II) on the NColE7

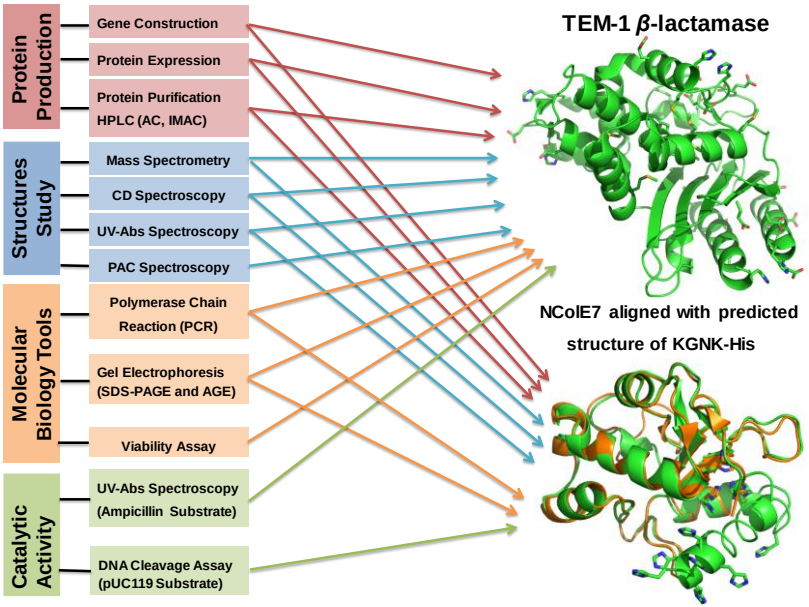
mutants. CD spectroscopic, mass spectrometric, and ^{199m}Hg(II) PAC spectroscopic experiments were planned for investigation of the structural effects of metal ions on the proteins in solution. These experiments were expected to clarify the following points: (**i**) changes in the secondary structure elements; (**ii**) the identity and number of metal ions bound to a protein; (**iii**) the relative affinity of the proteins toward the metal ions.

5. Determining the influence of metal ions on the catalytic activity of the enzymes

Interaction of TEM-1 β-lactamase with metal ions can lead to modification of the catalytic activity of the protein in ampicillin hydrolysis. We planned to study the effect of Ni(II), Cd(II), and Hg(II) ions on the kinetics of ampicillin hydrolysis catalyzed by TEM-1 β-lactamase in solution, as well as in cells in bacterial cultures. The study includes the investigation of the following effects on the hydrolysis rate: (**i**) the quality and quantity of the metal ion; (**ii**) the interaction with ampicillin; and (**iii**) the effect of the applied conditions.

KGNK has only one strong metal binding site in the active center, while KGNK-His protein has two main metal binding sites: the active center and the His-tag. Furthermore, the His-tag has previously been shown to bind to the free coordination site of the Zn(II) ion in the active center, thereby inhibiting the catalytic activity. In this work, we aimed at studying the catalytic activity of KGNK and KGNK-His proteins with pUC119 DNA as substrate in the presence of Ni(II), Cd(II), and Cu(II) ions. Ni(II) ions can occur as a contamination originated from the purification procedure by immobilized metal ion chromatography. Cu(II) ion is a strong Lewis acid and has high affinity to the His residues. Cd(II) ion was selected as the d^{10} analogue of the Zn(II) ion. We planned to investigate the following effects on the hydrolysis rate: (**i**) the role of a non-native metal ion; (**ii**) the effect of an excess amount of non-native metal ion; (**iii**) the regulation of the catalytic behavior with EDTA as a strong chelator; (**iv**) the effect of the competition between non-native and native metal ions.

The workflow of the planned experiments is depicted in Scheme 1.



Scheme 1: The workflow of this research project including the two research lines: TEM-1 β-lactamase and mutants of NColE7.

II. Experimental methods

The targeted proteins were synthesized by recombinant DNA technology, which included constructing the DNA genes of the proteins by polymerase chain reaction (PCR); ligation of the new genes into bacterial DNA carriers called DNA plasmids (vectors) such as pGEX-6P-1 and pET21a(+), which carry specific genes to express purification tags. The DNA plasmid carrying the genes of the target protein was applied for protein expression using suitable *E. coli* bacterial cells, such as BL21(DE3), and then the targeted proteins were purified by HPLC using various columns, such as glutathione and immobilized Ni(II)-affinity columns. The purification steps of the proteins were monitored using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The characterization of proteins

and their interaction with metal ions were studied by circular dichroism (CD), UV-absorption, and ^{199m}Hg(II) perturbed angular correlation (PAC) spectroscopies, and mass spectrometry (MS). The catalytic activity of TEM-1 β-lactamase in ampicillin hydrolysis in the absence and presence of metal ions was investigated by UV-Vis absorbance spectroscopy. While the catalytic activity of NColE7 mutants (KGNK and KGNK-His) in DNA hydrolysis in the absence and presence of metal ions was investigated using agarose gel electrophoresis (AGE).

III. New scientific results

T1. *We could purify TEM-1 β-lactamase directly from the bacterial pellets in its native fold without any affinity purification tags based on the surface histidine pairs as the metal binding sites by two steps of immobilized metal ion affinity chromatography (IMAC) and single step of anion exchange* [P1].

We have successfully expressed TEM-1 β-lactamase from the modified pET-21a(+) vector carrying the coding sequence of the DNA binding domain of the His-tagged Nuclear Factor I, in spite of the fact that the gene of TEM-1 β-lactamase is located outside of the multi-cloning site. Our purification strategy involved two steps of IMAC and single step of anion exchange, providing a yield of 1.9 mg/g of wet bacterial pellet weight, which is comparable to previous purification methods carried out under denaturing conditions form the periplasmic extracts. We identified the protein by mass spectrometric investigation of the trypsin digested fragments, as well as by intact protein molecular mass measurement. The results proved that purified TEM-1 β-lactamase was a mature enzyme (24-286 a.a.), in which the 23 a.a. signal sequence – required for the export of the protein to periplasm – was already cleaved. In addition, we could identify a mutation of the amino acid in position 84, which proved to be an isoleucine instead of the expected valine in the *E. coli* TEM-1 β-lactamase. The MS results also proved that the two Cys residues (Cys77 and Cys123) were oxidized in a form of a disulfide bridge.

T2. *By the secondary structures analysis of CD spectra, we proved that TEM-1 β-lactamase is folded in solution into its functional structure, similar to the published*

crystal structures. The secondary structure of enzyme is only slightly changed upon interaction with the Ni(II), Cd(II) or Hg(II) ions. While we proved by ESI-MS that Ni(II) could bind to three sites of the enzyme, one of these being preferable, while others are weakly binding sites. Both our ESI MS and ^{199m}Hg-PAC spectroscopic results proved that Hg(II) ion could bind to TEM-1 β-lactamase weakly [P1;P2].

Analyzing the CD spectra of TEM-1 β-lactamase in solution we could show that the obtained secondary structure composition *i.e.*, the percentage of α-helices, β-sheets, turns, and other conformations is close to that calculated from the published crystal structure. Thus, TEM-1 β-lactamase was folded in solution into its functional structure. We could demonstrate by CD spectroscopy, that the secondary structure of TEM-1 β-lactamase changed only slightly upon interaction with the selected Ni(II), Cd(II) and Hg(II) ions. Since the interaction with Ni(II) was already proven by the success of the IMAC purification, this supports the binding of Ni(II) and also Cd(II) ion to the surface histidine pairs that are at suitable distance for chelation. The most significant (~20%) decrease of the α-helix content was observed upon increase of the Hg(II):enzyme concentration ratio up to 10:1. This suggested that Hg(II) is most probably bound close to the methionine thioether groups inside the protein structure.

The slight but continuous change of the CD spectra upon increasing the metal ion content did not allow for determination of the number of the bound metal ions. It rather suggested that the enzyme could most likely bind more than one metal ion with not too high affinity. Therefore, using mass spectrometric experiments we proved that the major peak could be assigned to the monometallated TEM-1 β-lactamase at all the studied Ni(II):enzyme molar ratios (2:1-10:1). The relative intensity of the peaks corresponding to the two or three Ni(II)-chelating protein molecules, only slightly increased upon increasing the metal ion excess. Thus, the target protein could rather strongly coordinate the first Ni(II) ion, the second binding site is weaker, while the third site barely binds the metal ion. This strongly suggested that the sidechain donor atoms of His153 and His158 being at ~3.5 Å distance, are ideal for metal ion binding. Furthermore, Asn154 and Asp157 amino acid residues in their close vicinity may strengthen this interaction. His26 and His289 residues are at the opposite termini of the protein sequence, but become close to each other in the

folded structure at a distance of ~4.0 Å. These are suggested to form the second weak metal binding site. The third pair is formed by the His96 and His112 residues with a distance of ~10 Å between them, which might be inappropriate for significant metal ion binding.

Both the ESI MS and ^{199m}Hg-PAC spectroscopic results proved that Hg(II) ion could bind to the TEM-1 β-lactamase weakly. However, no specific Hg(II) ion adduct could be detected, suggesting that Hg(II) ion can be bound to one or more similar binding sites. Inspecting the available crystal structures, indeed several similar potential binding sites, composed of methionine side chains complemented with further weakly coordinating amino acid sidechains were identified.

T3. *Using the full set of the stoichiometric and rate equations, instead of the usual Michealis-Menten formalism, for calculations describing the hydrolytic process of ampicillin catalyzed by TEM-1 β-lactamase we could prove that the primary hydrolysis product of ampicillin is converted to second product, which needs also to be considered in the evaluation process* [P2].

The hydrolytic process of ampicillin catalyzed by TEM-1 β-lactamase was described by the kinetic analysis of the set of catalytic progress curves recorded at multiple wavelengths, including various concentrations and concentration ratios of the enzyme and substrate. These experiments showed that the primary hydrolysis product of ampicillin is converted to second product, which needs also to be considered in the evaluation process. Thus, instead of the usual Michealis-Menten formalism, the full set of the stoichiometric and rate equations were used in the calculations:

$$\mathrm{S+E} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{(ES)} \quad , r_1=k_1\cdot[\mathrm{S}]\cdot[\mathrm{E}] \text{ and } r_2=k_2\cdot[(\mathrm{ES})]$$

$$\mathrm{(ES)} \overset{k_3}{\rightarrow} \mathrm{P_1} \quad , r_3=k_3\cdot[(\mathrm{ES})]$$

$$\mathrm{P_1} \overset{k_4}{\rightarrow} \mathrm{P_2} \quad , r_4=k_4\cdot[\mathrm{P_1}]$$

By this treatment we could determine the kinetic parameters, as well as the molar absorbance values with high reliability, while we showed that the classic evaluation method results in significant variations in the Michaelis-Menten parameters, based on the choice of the final absorbance (A_∞) value.

We have also identified the $\mathbf{P_1}$ and $\mathbf{P_2}$ hydrolysis products by mass spectrometry. These experiments revealed that $\mathbf{P_1}$ is, as expected, the ampicilloic acid with an m/z value of the monoprotonated species of 368.1 being 18 units higher than that of the m/z value of ampicillin. The m/z value of the peak assigned to $\mathbf{P_2}$ was obtained to be 324.1, revealing decarboxylation of ampicilloic acid (decrease of the m/z value by 44.0 units) to yield the non-enzymatic ampicillin degradation product denoted as ampilloic acid.

T4. *We showed that the catalytic activity of TEM-1 β-lactamase is slightly reduced by the gradual increase of concentration of Hg(II), while Ni(II) and Cd(II) ions slightly promoted the enzyme activity. Thus, the coordination properties of metal ions determine their effect on the catalytic process both through the interaction with the enzyme and the substrate* [P2].

The catalytic activity of TEM-1 β-lactamase was slightly reduced by the gradual increase of concentration of Hg(II) ion. These catalytic progress curves could be fitted allowing the change of the active enzyme concentration, yielding identical kinetic parameter values as those obtained in the absence of Hg(II) ion. The decreased active enzyme concentration suggested that the presence of Hg(II) ion does not affect the substrate binding, but influences the concentration of the catalytically effective enzyme possibly by blocking a fraction of the enzyme molecules. Based on these, we hypothesized that Hg(II) ion may interact with the sulfur donor atoms of Met residues close to the active center, thereby interfering with the catalytic process.

We have found that Ni(II) and Cd(II) ions slightly promoted the enzyme activity. We suggest that the most probable scenario is the binding of these metal ions to the β-lactam ring of antibiotics with a concomitant activation of the substrate for the nucleophilic attack by the enzyme, due to their strong Lewis acid property. It shall be noted that we could not observe a significant hydrolysis of ampicillin in the presence of Hg(II), Ni(II) or Cd(II) ions but absence of TEM-1 β-lactamase at identical conditions to those applied in our kinetic assays.

T5. *It was shown that the viability of the bacterial cells in metal ion containing cultures is affected by numerous factors. The concentration of the toxic metal ions, and their interaction with antibiotics exerted the major effects* [P2].

We have found that the presence of ampicillin influences the toxic effect of the metal ions through complexation, leading to adducts with lower toxicities than that of the metal ions themselves. While 10 μM Cd(II) ion did not affect the bacterial growth, neither 10 μM Ni(II) nor 10 μM Hg(II) ions allowed the bacteria to proliferate. We observed a significant decrease of metal ion toxicity when ampicillin was also present in the above solutions. The differences in the neutralization effect of ampicillin was more pronounced for Hg(II) ion than for Ni(II) ion, which was suggested to be attributed to different coordination modes of the two metal ions in their ampicillin complexes.

T6. *We have established an optimized purification method for KGNK-His enzyme using a single step immobilized metal ion affinity chromatography (IMAC) supplied with HPLC technique. CD spectroscopy and ESI-MS proved that KGNK enzyme was able to bind a single Ni(II), Cu(II) and Cd(II) non-native metal ions in its active center. By ESI-MS we proved that none of the non-native metal ions could replace Zn(II)ion in the active site of the KGNK enzyme* [P3].

We successfully purified KGNK-His protein by single step of immobilized metal ion affinity chromatography (IMAC) supplied with HPLC technique with optimized purification conditions. Mass spectrometry demonstrated that the purified KGNK-His was obtained in its apo form and high quality. The yield of the purified protein was ~3 mg/1 g of wet bacterial pellets. We could prove that KGNK enzyme was able to bind a single non-native metal ion, such as Ni(II), Cu(II) and Cd(II) ion in its active center. Addition of one equivalent of non-native metal ions caused slight but significant changes in the circular dichroism spectrum, reflected in a red shift of about 2 nm and accompanied by a small change in the intensity similarly to those of the effects caused by Zn(II) ion coordination. The main MS peak was assigned to the KGNK bound to a single metal ion. We have found that Cd(II) ion is a weaker interacting agent than Ni(II) or Cu(II). We could show that under competitive

conditions none of the foreign metal ions could replace Zn(II) in the active site of the NColE7 mutants.

T7. *Our experiments proved that the secondary structures and catalytic activity of NColE7 mutants (KGNK and KGNK-His) are affected by the presence of complex anions, such as phosphate ion or EDTA* [P3].

We could demonstrate the interaction of KGNK enzyme with anions, such as phosphate ion or EDTA through changes in CD spectra resulting in a similar red shift to that caused by the metal ions. In contrast, even large excess of chloride ions did not affect the spectrum of KGNK. The above phenomena were attributed to the interaction between the negatively charged complex anions with the positively charged amino acid sidechains of the enzyme. We have found that the catalytic activity of both the apo KGNK and the apo KGNK-His enzymes was only slightly inhibited by EDTA addition in equimolar amount – to be enough to remove any metal ion impurity from the enzyme. The enzymes were only inhibited, when ~ 10 eq of EDTA was supplied. Thus, we could demonstrate, that the mutant enzymes exhibited catalytic activity in their apo-forms under the applied conditions. We suggest that the competition between the negatively charged EDTA and the substrate towards the positively charged amino acid side-chains of the enzyme is responsible for the inhibition at large excess of the chelator.

T8. *We have shown that addition 1eq of Zn(II), and Cu(II) ions to the apo-NColE7 mutants resulted in decreased catalytic activity compared to that observed with the apo-form. The decrease of the activity due to the presence of Cd(II) ion was less pronounced, but increasing the metal ion concentration caused further decrease in the activity. As a surprising result, the addition 1eq of Ni(II) ion caused extremely high enzyme activity, and the excess of the Ni(II) ions could not significantly inhibit this highly active enzyme* [P3].

Zn(II) ion was suggested to be essential for the nuclease activity of NColE7. We have found that addition 1 eq of Zn(II), and Cu(II) ions to the catalytically active apo-enzyme surprisingly, resulted in a less active enzyme compared to the apo-form.

Addition of 1 eq of Cd(II) ions did not decrease the catalytic to the same extent due to the lower affinity towards the catalytic site than those of Zn(II) or Cu(II) ions. Addition of further equivalents of Zn(II), Cu(II), or Cd(II) ions caused further slight inhibition of the enzyme. We have demonstrated that the enzyme has extremely high activity in the presence of 1 eq of Ni(II) ion, and the enzyme was not inhibited by the addition of an excess of the Ni(II) ions. We proved that Ni(II) ion cannot initiate DNA cleavage in the absence of the enzyme under the applied conditions. Based on these results, we suggested that new hydrolytic mechanisms have evolved. The KGNK mutants lack Arg447, which is suggested to have an important role in proton transfer process – promoting the generation of the nucleophilic OH^- ion by His545. The ability of the excess of the applied metal ions (Zn(II), Cu(II), and Cd(II) ions) to inhibit the enzyme is due to prevention of the His545-mediated OH^- generation via weak coordination to this His residue. Therefore, we suggested that in apo-KGNK and KGNK-His, the free His residues participate in protonation and deprotonation processes, while in the Ni(II)-bound KGNK or KGNK-His, Ni(II) ion may promote the deprotonation of a coordinated water molecule, which can serve as an efficient attacking agent for DNA hydrolysis.

In agreement with the MS results of KGNK, we could show that the catalytic activity was characteristic for the Zn(II)-bound enzymes both for KGNK, and KGNK-His under competitive conditions supplementing Zn(II) and a non-native metal ion together. Thus, Zn(II) ion could displace all the applied non-native metal ions from the active center of the enzyme. The above phenomena were independent of the order of addition of metal ions, reflecting that the thermodynamic relations govern the metal ion binding in the active center of these mutants.

IV. Scientific publications

The Hungarian Scientific Bibliography (MTMT) identifier: **10069594**

Published full papers related to the dissertation:

[P1] **Z.H. Nafaee**, É. Hunyadi-Gulyás, B. Gyurcsik: Temoneira-1 β-lactamase is not a metalloenzyme, but its native metal ion binding sites allow for purification by immobilized metal ion affinity chromatography, *Protein Expression and Purification*, DOI: 10.1016/j.pep.2022.106169; 201 (2023) 106169. **IF= 1.6 - Q3**

[P2] **Z.H. Nafaee**, V. Egyed, A. Jancsó, A. Tóth, A.M. Gerami, T.T. Dang, J. Heiniger-Schell, L. Hemmingsen, É. Hunyadi-Gulyás, G. Peintler, B. Gyurcsik: Revisiting the hydrolysis of ampicillin catalyzed byTemoneira-1 β-lactamase, and the effect of Ni(II), Cd(II) and Hg(II). *Protein Science*, DOI: 10.1002/pro.4809; 32 (2023) e4809. **IF= 8.1 - Q1, D1**

[P3] **Z.H. Nafaee**, B.Hajdu, É. Hunyadi-Gulyás, B. Gyurcsik: Hydrolytic mechanism of a metalloenzyme is modified by the nature of the coordinated metal ion, *Molecules*, DOI: 10.3390/molecules28145511; 28 (2023) 5511. **IF= 4.6 - Q1**

ΣIF= 14.3

Two further manuscripts are in progress for publication: (i) the first article is related to His-tagged NColE7-R447G (KGNK-His), and (ii) the second is a literature review related to nuclease domains of colicin E7 and E9.

Full paper not related to the dissertation:

1. A. Tóth, K. Sajdik, B. Gyurcsik, **Z.H. Nafaee**, E. Wéber, Z. Kele, N.J. Christensen, J. Heiniger-Schell, J.G. Correia, L. Hemmingsen, A. Jancsó: From disorder to order – Turning on the signaling pathway in the AsIII sensor protein AfArsR. Under revision in *Journal of the American Chemistry Society*, (2024)

Oral presentations and posters related to the dissertation:

1. **Z.H. Nafaee**, É. Hunyadi-Gulyás, B. Gyurcsik, (Onsite Lecture) TEM-1 β-lactamase is not a metalloenzyme but metal ion binding to the histidine-pairs exposed at the protein surface may count, 8th International Symposium on Metallomics (8-ISM), 2022, July 11-14, The Kanazawa Chamber of Commerce and Industry, Kanazawa, Japan.
2. **Z.H. Nafaee**, É. Hunyadi-Gulyás, B. Gyurcsik, (Onsite Lecture), Effect of metal ions Ni(II), Cd(II), and Hg(II) on TEM-1 β-lactamase, XLV Chemistry Lectures of Hungarian Chemical Society, 2022, October 25-27, Szeged, Hungary.
3. **Z.H. Nafaee**, V. Egyed, É. Hunyadi-Gulyás, B. Gyurcsik, (Online Lecture), Properties of TEM-1 β-lactamase under heavy metal pollution conditions, 28th International Symposium on Analytical and Environmental Problems (28th ISAEP), 2022, November 14-15, Szeged, Hungary.
4. **Z.H. Nafaee**, V. Egyed, A. Jancsó, A. Tóth, A.M. Gerami, T.T. Dang, J. Heiniger-Schell, L. Hemmingsen, É. Hunyadi-Gulyás, G. Peintler, B. Gyurcsik (Onsite Lecture), Effects of metal ion binding on Temoneira-1 β-lactamase, 56th Complex Chemistry Symposium, 2023, May 30 - June 01, Szeged, Hungary.
5. **Z.H. Nafaee**, B. Hajdu, É. Hunyadi-Gulyás, B. Gyurcsik, (Online Lecture), Nuclease domain of colicin E7 under heavy metal, 29th International Symposium on Analytical and Environmental Problems (29th ISAEP), 2023, November 13-14, Szeged, Hungary.

Oral presentations and posters not related to the dissertation:

1. **Z.H.A. Nafaee**, B. Gyurcsik, K. Nagata, (Virtual Poster) Purification and characterization of NFI-BD, 35th Anniversary Symposium of the Protein Society (PS35), 2021, July 7-14, United States of America.
2. **Z.H.A. Nafaee**, K. Nagata, B. Gyurcsik, (Virtual Lecture) Synthesis and characterization of nuclear factor I, a potential metal ion binding protein, XLIV Chemistry Lectures of Hungarian Chemical Society, 2021, October 26-27, Szeged, Hungary.

Coauthored oral presentations and posters related to the dissertation:

1. B. Hajdu, **Z.H. Nafaee**, É. Hunyadi-Gulyás, B. Gyurcsik, (Onsite lecture) Metal binding sites in proteins, 55th Complex Chemistry Symposium, 2023, May 25 - 27, Debrecen - Hungary.
2. B. Gyurcsik, **Z. Nafaee**, B. Hajdu, É. Hunyadi-Gulyás, (Onsite poster) Metal ion selection by enzymatic systems, Biomolecular Technology of Proteins (BioToP) Conference, 2023, June 19 - 20, Vienna, Austria.

Coauthored oral presentations and posters not related to the dissertation:

1. Tóth A., B. Gyurcsik, **Z.H. Nafaee**, É. Hunyady-Gulyás, A. Jancsó, (Onsite lecture) Investigation of the functional selectivity and depression mechanism of the half-metal regulator AfArs protein, 56th Complex Chemistry Symposium, 2023, May 30 - June 01, Szeged, Hungary.
2. A. Tóth, B. Gyurcsik, **Z.H. Nafaee**, É. Hunyady-Gulyás, A. Jancsó, (Onsite poster) Investigation of the functional selectivity and depression mechanism of the metalloid regulator AfArsR protein, Biomolecular Technology of Proteins (BioToP) Conference, 2023, June 19 - 20, Vienna, Austria.

Declaration of the supervisor

Undersigned Dr. Béla Gyurcsik, as the supervisor of the PhD candidate **Zeyad Hasan Abdullah Nafaee** in connection with the PhD dissertation entitled **Modification of the activity of hydrolytic enzymes by the nature of the non-endogenous/non-native metal ions** I declare that the following publications connected to the PhD dissertation:

Z.H. Nafaee, B.Hajdu, É. Hunyadi-Gulyás, B. Gyurcsik: Hydrolytic mechanism of a metalloenzyme is modified by the nature of the coordinated metal ion, *Molecules*, DOI: 10.3390/molecules28145511; 28 (2023) 5511.

Z.H. Nafaee, V. Egyed, A. Jancsó, A. Tóth, A.M. Gerami, T.T. Dang, J. Heiniger-Schell, L. Hemmingsen, E. Hunyadi-Gulyás, G. Peintler, B. Gyurcsik: Revisiting the hydrolysis of ampicillin catalyzed by Temoneira-1 β-lactamase, and the effect of Ni(II), Cd(II) and Hg(II). *Protein Science*, DOI: 10.1002/pro.4809, 32 (2023) e4809.

and all the thesis points T1–T8 *i.e.*, the results used in the PhD dissertation demonstrate the contribution of the candidate.

In the list of the publications related to the PhD dissertation only such publications are considered, which were not previously used, and will not be used in the future in a doctoral procedure in the Chemistry Doctoral School of the University of Szeged.

Szeged, March 08th, 2024

Dr. Béla Gyurcsik
supervisor