Charles University, Faculty of Science
Department of Biochemistry

Doctoral study programme: Biochemistry

Summary of Ph.D. Thesis

Structural characterization of biotechnologically and medicinally important proteins

Strukturní charakterizace biotechnologicky a medicinálně významných proteinů

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Abstract (in English)

A large number of biological processes depends on dynamics of protein structure and specific protein-protein and protein-ligand interactions occurring under specific native conditions in or outside of cells. Standard methods for protein structure analysis like x-ray crystallography, nuclear magnetic resonance or cryo-EM are able to obtain important atomic or near- atomic resolution protein structures, however these are usually a static snapshot of protein locked in a specific conformation and mostly in non-native conditions.

Structural mass spectrometry on the other hand, allows to describe protein structure dynamics, protein-protein and protein-ligand interactions and obtain inter- and intraprotein distance constraints between amino acid residues, all while working with proteins in their native conditions and needing only a fraction of sample.

In this work, hydrogen/deuterium exchange mass spectrometry (HDX-MS) and classical proteomic approaches were used together with other methods to analyse biotechnologically important proteins of fungal cellulolytic system Lytic Polysaccharide Monooxygenase (LPMO) and Cellobiose Dehydrogenase (CDH) as well as plant-derived photosensitizer protein LOV2 with potential use in biologically targeted photodynamic therapy.

These methods allowed us to follow cellulolytic reaction of reduced LPMO even in heterogeneous solution of crystalline cellulose, obtaining insights into structural changes accompanying LPMO catalysis, mainly its notorious instability which we determined to be caused by oxidative modification of the protein, as well as verifying and structurally describing previously reported stabilisation of LPMO by suitable substrate. The recently speculated role of hydrogen peroxide as true LPMO cosubstrate was also confirmed.

Analysis of LOV2 protein then explained previously reported gradually increasing production of singlet oxygen upon protein irradiation as caused by a release of flavin cofactor into the solution, with interesting implications for biologically targeted photosensitizers.

Keywords: Structural mass spectrometry, hydrogen/deuterium exchange mass spectrometry (HDX-MS), lytic polysaccharide monooxygenase (LPMO), cellobiose dehydrogenase (CDH), oxidative modification, cellulose degradation, turbidimetry, photosensitizer, light oxygen and voltage sensing domain (LOV)

Abstrakt (In Czech)

Velké množství biologických procesů je závislé na strukturní dynamice proteinů a specifických protein-proteinových, nebo protein-ligandových interakcích závislých na specifických podmínkách uvnitř, či vně buněk. Analýza struktury proteinů klasickými metodami jako je rentgenová krystalografie, NMR, nebo nově kryo-EM poskytuje důležité struktury s atomárním rozlišením, avšak většinou ukazuje pouze statický obrázek bez detailů o dynamice, nebo transientních interakcích, a navíc často v nenativních podmínkách.

Tyto detaily může doplnit strukturní hmotnostní spektrometrie, která umí poskytnout informace o proteinové dynamice, interakcích proteinů s jinými molekulami a také o specifických meziatomových vzdálenostech v samotných proteinech, nebo mez interakčními partnery, to vše při relativně nativních podmínkách.

V této práci byla použita vodík/deuteriová výměna spojená s hmotnostní spektrometrií (HDX-MS), klasická proteomická analýza a turbidimetrie ke studiu biotechnologicky užitečných proteinů houbového celulolytického systému lytické polysacharidové monooxygenasy (LPMO) a celobiosadehydrogenasy (CDH) a rovněž fotosensitivní domény LOV2 pocházející z rostlinného fototropinu využitelné při fotodynamické terapii.

Pomocí těchto metod byla sledována celulolytická reakce katalyzovaná redukovaným enzymem LPMO v heterogenním roztoku mikrokrystalické celulosy a byly získány poznatky o změnách a dynamice struktury LPMO během reakce. Podstata destabilizace enzymu byla určena jako oxidativní degradace způsobená vedlejší reakcí hydroxidových radikálů generovaných aktivním centrem enzymu a byla rovněž strukturně popsána a vysvětlena stabilizace enzymu polysacharidovým substrátem. Byla rovněž potvrzena spekulovaná role H_2O_2 jako pravého kosubstrátu LPMO.

Analýza LOV2 pomohla vysvětlit dříve publikované zvýšení produkce singletového kyslíku ($^1O_2$) při dlouhodobém ozáření, které je způsobené uvolněním FMN kofaktoru s vysokou efektivitou produkce $^1O_2$ do roztoku po oxidativní modifikaci cysteinu v blízkosti kofaktoru v molekule proteinu.

Klíčová slova: strukturní hmotnostní spektrometrie, vodík deuteriová výměna, lytická polysacharidová monooxygenasa (LPMO), cellobiosadehydrogenasa (CDH), oxidativní modifikace, rozklad cellulosy, turbidimetrie, fotosenzitivní protein, “light oxygen and voltage sensing domain” (LOV)

1. Introduction

1.1. Protein Structure

The function of proteins is tightly bound to their structure and often also to the dynamics of this structure. Proteins are often changing their structure locally upon substrate binding or during interaction with other proteins, with some proteins even undergoing vast structural rearrangements[1] or relative domain movements during their function[2]. In the last decades, the ever-improving high-resolution methods like x-ray crystallography, NMR and cryo-EM enabled to obtain thousands of precise atomic or near-atomic resolution structures of various proteins. While these structures are crucial for our understanding of protein function and form a basis upon which hypotheses of protein function are often built, they are more-or-less limited to a static picture, as structural dynamics and various more transient protein-protein and protein-ligand interactions elude detection by these methods. Moreover, the technical limitations of high-resolution methods necessitate the use of highly concentrated protein samples, often in a solution with a composition and ionic strength far from their native conditions, making direct correlation of obtained structure and function unreliable.

To obtain additional information about protein conformational dynamics, especially in conditions as native as possible, lower resolution structural methods based on mass spectrometry are utilized contributing to the emerging "integrative structural biology" field. These methods are characterized by rapidly analysing small amounts of sample in near-native conditions including heterogenous solutions and can provide information about localized solvent accessibility or hydrogen bonding in proteins, as well as obtain inter- and intraprotein distance constraints for model refinement of detecting and localizing protein-protein interactions[3].

1.2. Structural Mass Spectrometry

Mass spectrometry allows to measure exact mass of ionized analyte in the form of mass to charge ratio (m/z). Structural mass spectrometry methods then utilize various techniques to covalently or non-covalently label proteins to induce localized mass change and deduce structural information from the location of these labels on the protein upon proteolytic cleavage ("bottom-up" approach), or to observe proteins in their intact form ("top-down" approach) and obtain structural information such as protein complex subunit composition or

protein collisional cross-section. In this work, mostly hydrogen/deuterium exchange mass spectrometry method was utilized so it will be described in detail in this summary.

Hydrogen/deuterium exchange coupled with mass spectrometry (HDX-MS) is a non-covalent labelling method based on the exchange of hydrogens in a protein for deuterium from solvent D_2O, and subsequent mass spectrometric analysis which detects increase in mass associated with the exchange. The rate of exchange is influenced by solvent accessibility of exchanging hydrogens and their possible hydrogen bonding and local conformational dynamics[4]. In folded proteins, the exchange can thus wary wildly on the timescales from minutes to months[5]. The measured rate of exchange in specific parts of protein over a time period can yield information about anything from protein structure and its dynamics[6] to protein-protein[7,8]or protein-ligand interactions[9]. The analysed sample can in principle be labelled in any ESI compatible solvent and even in heterogenic solution[10], giving the HDX-MS the advantage of using more native conditions than higher-resolution methods like X-ray crystallography or NMR.

Most HDX-MS experiments are done in *continuous labelling* mode (**Figure 1, page 5**)[11], where protein sample is incubated in typically 80-90% D_2O for various time intervals. The continuous increase in deuteration is observed and changes to the exchange under different conditions or in presence of different other molecules are detected. After a set incubation time, the exchange reaction is "quenched" by adjusting pH to around 2.5 where the exchange is minimal and usually also rapidly frozen in liquid nitrogen to stop the exchange and avoid back-exchange of deuterium for hydrogen during subsequent analysis[11].

The analysis of deuterated samples is usually done using a "bottom up" approach characterized by protease digestion of the sample and subsequent HPLC-ESI-MS. The deuteration is calculated from mass increase of peptides and spatial resolution therefore depends on proteolysis. Because the digestion needs to be performed under low pH, typical protease used is pepsin, which also has the advantage or relatively low specifity, creating overlapping peptides that enable to further increase the resolution by calculating deuteration of overlapping regions[12]. A range of alternative proteases was also developed specifically to allow digestion under HDX quench conditions[13,14].

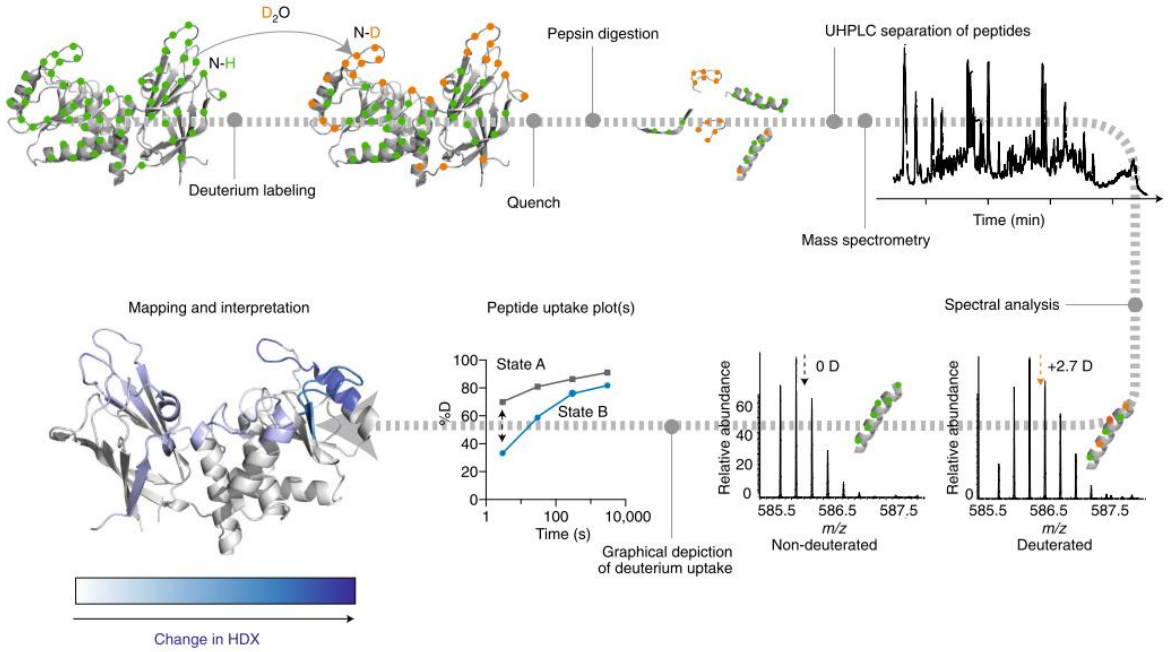

Figure 1: Schema of the common "bottom -up" HDX experiment. *Sample is incubated in D_2O and aliquots are taken at specific incubation times. Deuteration is stopped by lowering pH and temperature. Samples are subsequently analyzed by LC-MS after proteolytic cleavage, often done "online" with protease columns connected to LC-MS setup. Rate of deuteration is calculated from the average mass change of any given peptide. Adapted from (104).*

1.3. Fungal Cellulolytic System – LPMO and CDH

The example of complex enzymatic system, where protein dynamics play an important role is fungal extracellular cellulolytic system. Two of the enzymes involved are lytic polysaccharide monooxygenase (LPMO) and cellobiose dehydrogenase (CDH) (**Figure 2**).

LPMO is cellulolytic copper-dependent redox enzyme, found mainly in bacteria[15] and fungi[16] but also in several other organisms[17,18]. When their active site copper ion, found on a flat surface of the protein, is reduced from Cu^{+2} to Cu^{+1} by either small-molecular reductants or its redox partner CDH, LPMO binds to its polysaccharide substrate[19,20] and reduced copper ion binds either oxygen[21,22] or hydrogen peroxide[23,24] to produce ROS. Produced radicals are directed at either C1 or C4 position of β-(1→4) glycosidic bonds of polysaccharides, depending on the type of LPMO, causing oxidative cleavage of the

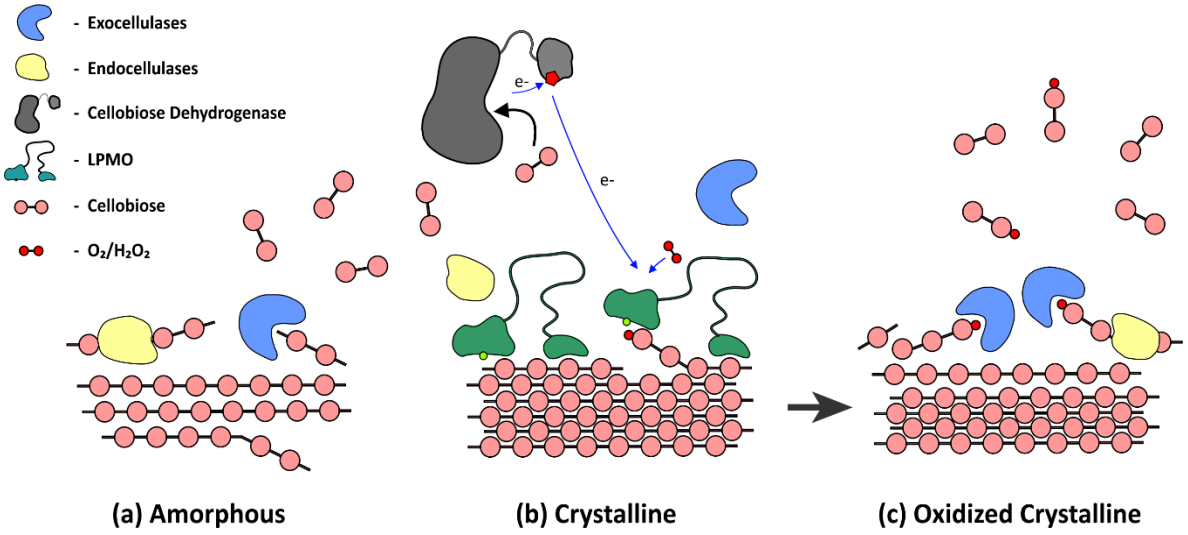

Figure 2: Schematic representation of fungal cellulolytic system employing CDH and LPMO. *Amorphous cellulose structures* ***(a)*** *are easily accessible by endo- and exocellulases and therefore are readily broken down into mono- and disaccharides. Crystalline cellulose* ***(b)*** *is however sterically hardly accessible and LPMO enzymes with flat catalytic sites are needed to oxidatively cleave these parts of cellulose to make them accessible* ***(c)****.*

bond[25,26]. While LPMO enzymes are already utilized biotechnologically, they have a serious limitation in the form or low stability during catalysis and their application is challenging to optimize. This low stability was not researched thoroughly yet with only a handful of studies concerning thermal stability and possible structural changes during catalysis[20,27], however oxidative damage due to off-pathway catalytic processes is suspected to be involved[23]. The nature of the cosubstrate is also a topic of heated discussion, with increasing number of reports stating that H_2O_2 is in fact the true cosubstrate and O_2 is only an intermediate, which would in fact make the enzyme peroxygenase and not monooxygenase[19,23,24,28,29]. This is in contrast to previous belief of O_2 being the polysaccharide substrate oxidizing cosubstrate of LPMO enzymes directly[2,22,30]. Thus, study of structural dynamics of LPMOs during catalysis and the nature of its cosubstrate are currently both an important topic to tackle.

CDH (EC 1.1.99.18) is so far the only identified extracellular flavocytochrome[31]. It is composed of two functional domains: N-terminal cytochrome domain (CYT) harbouring heme *b* and C-terminal dehydrogenase domain (DH) containing flavin adenine dinucleotide (FAD) cofactor with a flexible linker connecting the two domains[2,32]. Cellobiose or other small sugars including lactose and glucose[33] can be oxidized at the DH domain while reducing the FAD cofactor to $FADH_2$. Electrons from $FADH_2$ are then channelled in two

one-electron steps to CYT domain via direct interdomain electron transfer (IDET). Once at the CYT domain, electrons can be transferred to terminal acceptor, which is usually LPMOs active-site copper ion, reduction of which starts LPMOs oxidative depolarization of cellulose[2]. The IDET is the crucial step in CDH function and dictates the rate at which CDH supplies electrons to other molecules[34]. It was found, that this transfer is occurring as a flip-flop mechanism during which CDHs separate domains alternate between a "closed" to "open" conformation using the flexible linker as a separator[32]. The extent to which domains interact was proposed to be largely governed by distribution of electrostatic charges on the interaction interfaces of the domains giving the protein specific pH optimum when domain repulsion is lowest[35–37]. Importantly, the electrons can be transferred not only to LPMO molecule, but to any other molecule with suitable redox potential and even to the surface of electrode[33]. This has been utilized in the production of CDH based saccharide biosensors in the form of electrodes covered in immobilized CDH. Details of structural changes occurring in CDH during IDET and knowledge of parameters, which influence the transfer, are important in future engineering and biotechnological adaptation of CDH enzymes.

1.4. Biological Photosensitiser LOV2

Another part of this work relates to the study of photosensitizer domain LOV2 of *Avena sativa* phototropin. Photosensitizers (PS) are light absorbing compounds that produce specific reactive oxygen species (ROS) from oxygen upon irradiation with light of specific wavelength. They have found use in antimicrobial photodynamic inactivation (aPDI)[38], photodynamic therapy (PDT)[39] cancer treatment or in the study of cellular ROS mediated signalling[40]. Using exogenous dyes as PS for PDT is not effective, due to inability to effectively target specific cells, general toxicity and limited pharmacokinetics. To address this issue, genetically encoded PS were developed with majority of those currently used and studied being flavoproteins carrying FMN or FAD derived from microbial and plant "light, oxygen and voltage sensing" (LOV) photoreceptor domains of phototropin[41]. The LOV photoreceptors, occurring in archaea, bacteria, fungi and plants are responsible for phototropism, chloroplast movement, circadian rhythms or general stress response, all of these physiological responses being driven by light or reactive oxygen species stimuli[42]. Upon light irradiation, LOV2 produces mainly highly reactive singlet oxygen $^1O_2$ that can induce oxidative modification of various macromolecules leading to cell death when

produced in high quantity[43]. During the photocycle of LOV, the signal is transduced as a structural rearrangement of the domain following a formation of a covalent bond between conserved Cys residue in the active site of the protein and C4a atom of the present flavin isoalloxazine ring upon irradiation of FMN or FAD by blue light. Importantly, cysteine devoid variants were also reported showing higher levels of radical species detected upon irradiation. To increase the quantum yield of the ROS production, proteins like miniSOG (mini Singlet Oxygen Generator) are engineered and the exact knowledge of protein structure and mechanistic details of ROS generation in natural photosensitizing domains are crucial for effective development of new photosensitizers[44]. In this work, contribution to structural and functional study of one such domain from *Avena sativa – As*LOV2[45] was done with the aim of explaining the gradually increasing singlet oxygen production upon irradiation detected for this protein.

2. Aims of the Thesis

The aim of this thesis was to explain structural aspects of activity of fungal enzymes CDH and LPMO involved in cellulose degradation and changes accompanying singlet oxygen generation by LOV2, using structural mass spectrometry methods.

The specific goals were:

- To characterize the interaction of cellobiose dehydrogenase and lytic polysaccharide monooxygenase
- To characterize structural stability of LPMO and structural changes accompanying LPMO inactivation during catalysis
- To obtain information about the nature of LPMO co-substrate
- To characterize changes occurring on *As*LOV2 during its light irradiation and production of reactive oxygen species

3. Results and Discussion

3.1. Study of fungal cellulolytic enzyme system

Fungal cellulolytic enzymes LPMO and CDH are heavily studied proteins, mainly due to their potential use in saccharification of lignocellulosic biomass and for biosensor production respectively. Current understanding of their activity is, however, still lacking mainly due to the complexity of the system involving ROS and insoluble substrates. The interaction of these enzymes during their electron transfer and notorious instability of LPMO are examples of two factors that have not yet been explained well[20,23,27].

We decided to implement HDX-MS and also other methods and shed more light on the interaction of CDH with LPMO, on structural changes occurring on the proteins during protein catalysis on a native substrate and explain its low stability in reducing conditions as well as to confirm the nature of its cosubstrate.

After the initial optimization of quench buffer and protease for optimal digestion sequence coverage, series of HDX-MS experiments was conducted, with the aim of detecting the interaction interfaces of *Neurospora crassa* CDH (*Nc*CDH-IIA) and *Neurospora crassa* LPMO9C (*Nc*LPMO9C) during electron transfer from CDH to LPMO. We observed several effects, however none of them was clearly pointing to a defined interaction interface between CDH and LPMO. However, weak signs of interaction were detected on LPMO, manifesting as structural relaxation around the active site when CDH and LPMO were mixed in the absence of substrate or reducing agents. Unfortunately, even repeated experiments with added substrates did not indicate any clear interaction interface under reducing conditions on either LPMO or CDH. While the protein-protein interaction was not detected, the structural effects of reduction of CDH by cellobiose alone have been detected as lower deuteration on the presumed interfaces of both CDH domains and around the FAD cofactor, indicating interdomain electron transfer is occurring and FAD is being reduced influencing the local structure (**Figure 3**). We utilized part of these results in the study of electron transfers in CDH, which is discussed below, and then concentrated on the structural dynamics of both enzymes separately. The CDH-LPMO cross-talk was further studied by our collaborators using computational approaches combined with mutagenesis and enzyme assays and was recently published in *ACS Catalysis*[46].

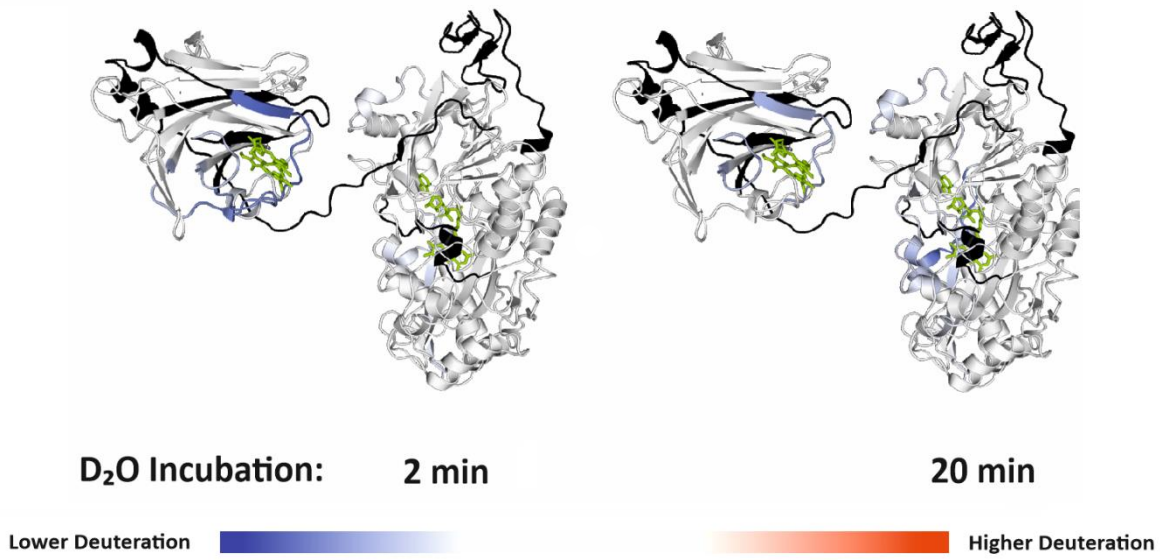

Figure 3: Effect of reduction of CDH by cellobiose measured by HDX-MS. *Protection from deuteration is apparent on the interaction interfaces of cytochrome and dehydrogenase domains. Black parts of the protein were uncovered peptides.*

We also extended the study published by Kádek et al.[37] and performed detailed HDX-MS analysis of glycosylated and deglycosylated wild-type and mutant CDH from *Crassicarpon hotsonii* confirming the hypothesis of charge repulsion-caused inhibition of interdomain electron transfer at higher pH and validating the possibility to engineer CDH proteins with shifted pH optimum. This data is now being complemented with functional enzyme kinetic analyses and will form the basis of another publication.

3.1.1. Study of LPMO structural dynamics during catalysis (Publication I)

While studies on CDH-LPMO interaction at reducing conditions provided no decisive protein-protein interaction induced effects, massive increase in deuteration was detected for LPMO molecule. We hypothesized that this might be linked to previously suggested LPMO degradation by reactive oxygen species[47], therefore we decided to investigate more.

Reduction of LPMO followed by HDX-MS revealed two distinct effects. First one was manifesting as decrease in deuteration on peptides around the copper active site occurring at the very short time points presumably connected to the previously observed structural changes caused by reduction of copper ion[20]. The second effect was an extensive increase in deuteration, starting at peptides around the active site and progressing to the rest of the

molecule with longer intubation time, with signal intensities of involved peptides diminishing at the same time. We believe this effect to be the result of structural unfolding and degradation caused by oxidative damage induced by ROS produced at the active site, as we identified a number of oxidative modifications predominantly on peptides around the active site using LC-MS/MS analysis, including oxidative peptide cleavages (**Figure 4**).

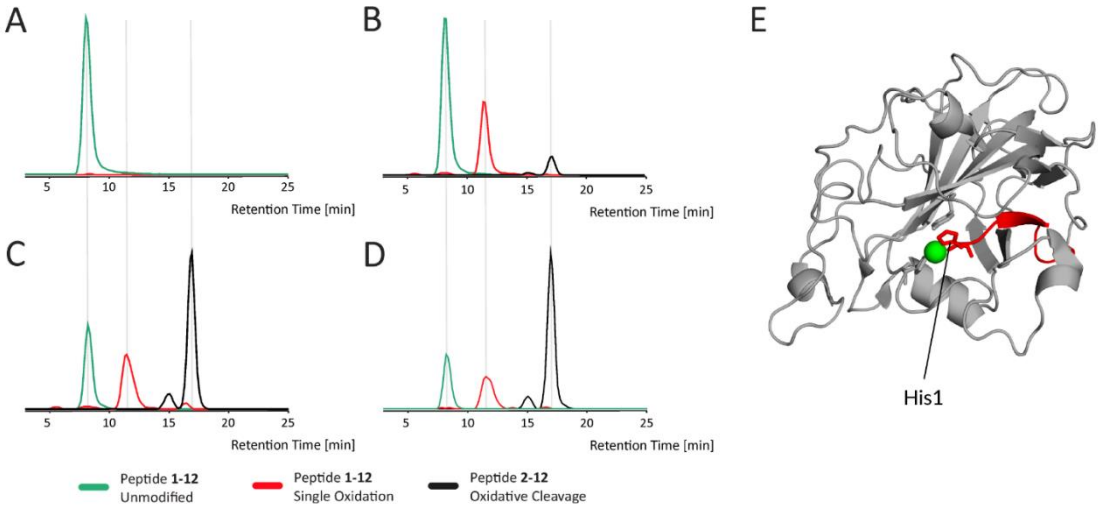

Figure 4. Monitoring kinetics of LPMO oxidative modifications by mass spectrometry*. Extracted ion chromatograms for the N-terminal LPMO peptide (1–12) that contains His1 residue involved in copper ion binding. LPMO were incubated (A) alone for 30 min, or with 5 mM ascorbic acid for (B) 10 min, (C) 20 min, (D) 30 min. Subsequently, it was digested with Asp-N and analyzed by LC-MS/MS. Chromatographic traces show a signal for unmodified (green), oxidized (+O1; red) and oxidatively cleaved (−His, +C1O1-H2; black) peptides. Localization of the peptide and the His residue is shown on a structure in (E). Copper ion is shown as green ball.*

It was previously reported, that cellulosic substrate increases thermal stability of LPMO[20]. To probe the effect of substrate on the stability of LPMO, another HDX-MS experiment was performed (**Figure 5**). To be able to include insoluble polysaccharide substrates in the reaction mixture, we added a fast spin filtration step in-between quenching and freezing to allow us to separate the insoluble particles from the proteins before LC-MS analysis, with as little hydrogen back exchange as possible. This required careful selection and testing of compatible spin filter chemistry was necessary as we have for example found that nylon based centrifuge tube filter membranes leech a significant amount of contaminants to the sample and seriously affect the LC-MS analysis[48].

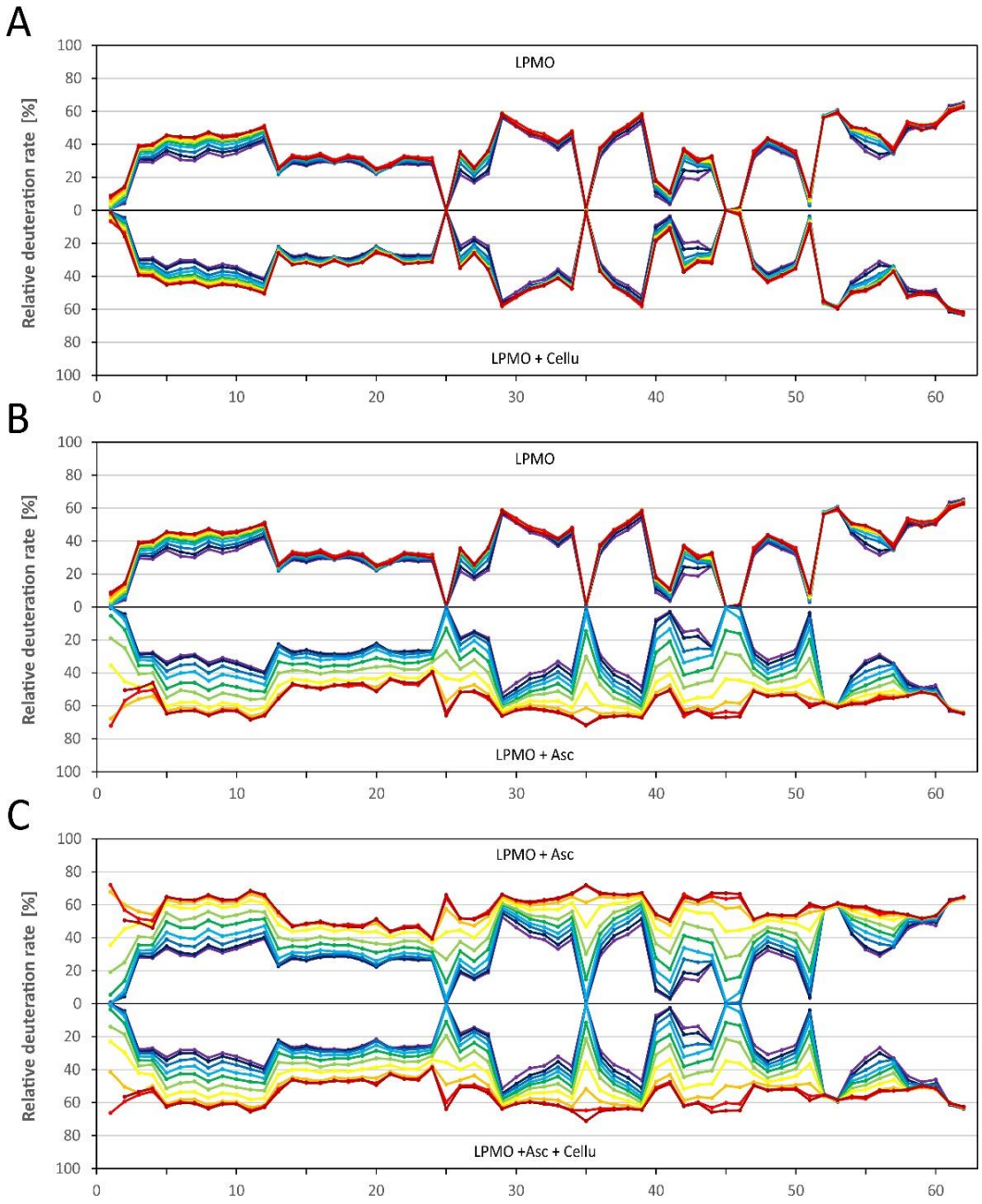

Figure 5: HDX-MS experiment featuring LPMO, ascorbic acid and crystalline cellulose. *Timepoints are 3, 5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes and correspond to the colours starting at purple and ending with red according to sequence of colours in the visible light spectrum. X-axis shows all analysed peptides in their sequential order. Y-axis shows the rate of deuteration.*

The increase in deuteration upon LPMO reduction manifested on the same peptides as before, however the presence of crystalline cellulose slowed down the increase globally. We believe, that the presence of suitable amount of cellulose substrate limits the oxidative damage incurred by the enzyme by absorbing bulk of the radicals created. Similar results

obtained with a different methodology were reported during our manuscript preparation[19].

We additionally analyzed apo form of LPMO to address the potential structural change induced by its loss, that could contribute the observed protein unfolding. We found that the removal of copper ion causes characteristic change to the structure of LPMOs active site while lowering the overall thermal stability of the protein. However recent publication showed that the copper remains bound to the protein for tens of minutes even at elevated temperature[20]. We can therefore conclude, that while copper ion clearly plays an important role in active site conformation, its loss is not the primary cause of protein degradation

3.1.2. Study of LPMO H_2O_2-dependent cellulolytic activity (Publication II)

Next experiments were aimed to confirm or disprove, whether H_2O_2 reported recently[23,28] is indeed a cosubstrate to LPMO. Previously reported turbidimetric workflow was modified to a continuous measurement[49] and analyse LPMO cellulolytic activity under various conditions in real time. Ascorbic acid, widely used as a reductant in LPMO activity assays, also slowly produces H_2O_2. We examined the activity of LPMO with an increasing concentration of ascorbic acid, all of which are presumed to be saturating, and with glucose oxidase (GOX) to observe the effect of increasing H_2O_2 production. The increase in activity proportional to the amount of ascorbic acid present was observed. Importantly, when catalase was also present, the cellulolytic activity was severely limited (**Figure 6**).

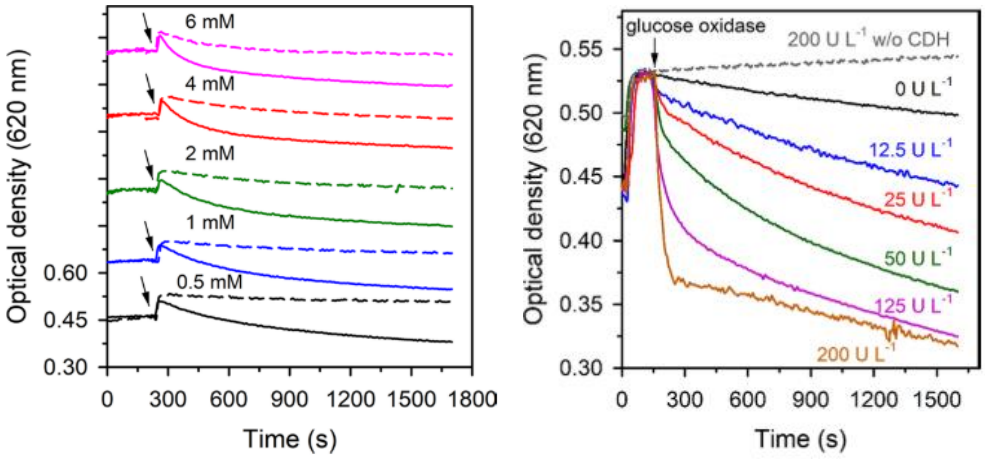

Figure 6: Turbidimetry measurement of LPMO activity with various concentrations of ascorbic acid or GOX. *A) Ascorbic acid addition. Time of addition of ascorbic acid is indicated by black arrow. Initial increase in absorbance is caused by binding of LPMO to the substrate. Gradual decrease of absorbance from that point corresponds to the activity of LPMO. Dashed lines represent identical reactions with the addition of 2000 U/ml catalase. B) GOX addition. Time of addition of GOX is indicated by black arrow. Reduction is facilitated by small amount (0.5 μM) CDH.*

Interestingly, catalase also reduced LPMO activity when its natural redox partner CDH was employed as a reductant showing a role of H_2O_2 in native conditions. Increased production of oxidized oligosaccharides in presence of H_2O_2 was confirmed by MALDI-MS measurement and consumption of H_2O_2 during reaction was observed electrochemically.

3.1.3. Study of electron transfers in CDH (Publication III)

The focus of this work currently in review in *ASC Catalysis* is the investigation of the two electron transfer steps in *Neurospora crassa* CDH, interdomain electron transfer (IDET) between CYT domain and DH domain and interprotein electron transfer (IPET) from CYT domain to the final electron acceptor. Chimeric proteins consisting of various swapped domains from *Nc*CDHIIA and *Nc*CDHIIB were produced and their properties were analysed electrochemically and by molecular docking.

Kinetic measurements showed, that there are differences in presteady-state reduction rates of FAD by cellobiose between the different DH domains, but no additional effect was observed by CYT domain swapping. Chimeric CDH proteins showed shifted pH optimum and reduced IDET rates and modelling of domain contact showed that domain edge-to-edge distance is the key factor in effective IDET, and low-surface complementarity and longer linker causes the protein to prefer open conformation with domains further apart.

Data from our previous HDX-MS measurements of the interaction of non-chimeric *Nc*CDHIIA with *Nc*LPMO9C was utilized in this study (**Figure 7**) showing that the interaction is only affecting peptides in the vicinity of the active site, disproving previous theory of interaction occurring on the opposite side of the protein[50].

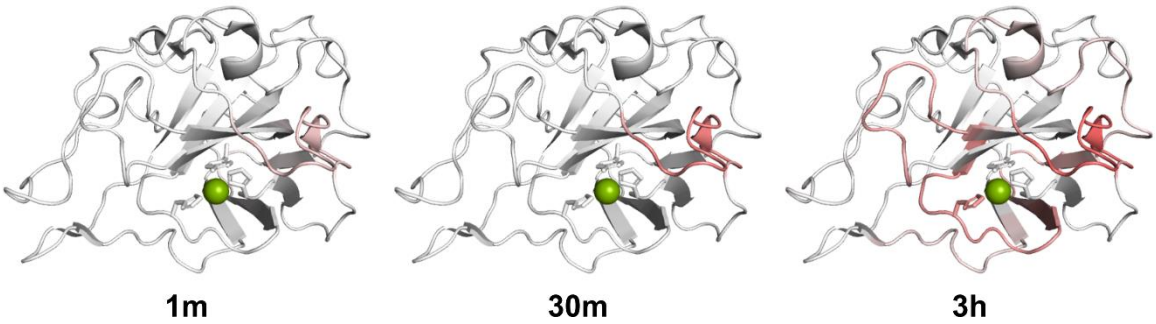

Figure 7: HDX-MS of interaction of NcCDHIIA and NcLPMO9C. *The interaction was observed in oxidized state due to protein degradation occurring in reducing conditions. Structural perturbance was detected on the LPMO in the vicinity of the active site copper ion, indicating direct contact of active sites of CDH and LPMO. No other effect was observed anywhere on the protein.*

Measurement of the IPET rate between WT and chimeric CDH and *Nc*LPMO9C was also performed and consistently with measurements of IDET, IPET rates were higher for chimeric proteins preferring open-state conformation.

3.1.4. Summary of LPMO and CDH study

From all the results obtained in **Publication I**, **II and III** it can be concluded, that H_2O_2 is indeed needed for the cellulose breakdown by LPMO to occur and O_2, previously thought as being prime cosubstrate of LPMO, is probably just an intermediate, that needs to be first reduced to H_2O_2 to be utilized by LPMO. Probable mechanism of such LPMO reaction was published very recently[19,29,51] and correlates well with data of H_2O_2 induced activity in **Publication II** and enzyme degradation by oxidative damage described in **Publication I.** Together with observed stabilization of LPMO by polysaccharide substrate it can be concluded that for effective industrial utilization of LPMO, it is crucial to maintain saturation of LPMO with substrate to eliminate radical side reaction causing protein degradation, while keeping suitable amount of H_2O_2 present to increase the enzyme activity.

Results from **Publication III** then show, that main factors influencing both the IDET and IPET in CDH are relative domain flexibility and domain surface complementarity. The recent data on the mutant version of CDH (not published yet) then show a way to engineer the enzyme so it can exert its electron transfer activity even at physiological pH and that it functions more efficiently if the aberrant hyperglycosylation introduced by *Pichia pastoris* is removed. These findings are important for engineering of CDH proteins with desired characteristics for biosensor production or industrial cellulose saccharification.

3.2. Study of oxidative processes on *As*LOV2

LOV2 domain from *Avena sativa* phototropin 1 protein (*As*LOV2) is a photosensitizer containing FMN and producing reactive singlet oxygen ($^1O_2$) upon light irradiation. It has been found for similar photosensitizer miniSOG (80% primary structure identity), that continuous irradiation gradually increases the quantum yield of $^1O_2$ production, while causing oxidative modification of amino acid residues surrounding the FMN. The increase in quantum yield is however not fully explained. Study of this gradual quantum yield increase was thus conducted with *As*LOV2 as model protein in collaboration with the group of assoc. prof. Erik Sedlák from the Center for Interdisciplinary Biosciences in Košice.

3.2.1. Study of photoinduced damage of AsLOV2

To study the production of $^1O_2$ by *As*LOV2, wild-type and variant with conserved active-site cysteine C450 replaced by alanine, resulting in limited photoswitching ability and presumably higher radical generation, was used.

The measurement of $^1O_2$ phosphorescence showed gradual increase in $^1O_2$ production upon irradiation of both *As*LOV2 WT and *As*LOV2 C450A. Complementary measurement of FMN fluorescence indicated that a release of FMN to the solution, more prominent in WT protein, is responsible for the increase in $^1O_2$, since free FMN has higher quantum yield.

To help explain the release of FMN from the protein, both bottom-up and top-down mass spectrometry was employed to analyse structural changes to *As*LOV2 WT and *As*LOV2 C450A variant upon irradiation. A range of oxidative modifications was found on the protein with similar, but more extensive modification globally detected in *As*LOV2 WT protein, concentrated in the area close to the FMN, seemingly contrary to the higher ROS production in WT protein. However, the FMN retained on protein in C450A generates radicals located directly next to the detected modified residues which could increase their extent of oxidation. Importantly, C450 of *As*LOV2 WT was also found to be extensively oxidized upon irradiation. Simulation of the modification of C450 shows, that transformation of -SH functional group to -SO2, creates sterical clash, that forces the FMN out of the protein (**Figure 8**) explaining higher release of FMN to solution in WT protein.

The details of *As*LOV2 $^1O_2$ production and release of FMN to the solution may be utilized to design more efficient genetically encoded photosensitizers for photodynamic therapy based on LOV domains, able biologically target specific tissues or cells thanks to their protein part and release FMN upon irradiation for high local oxygen radical production.

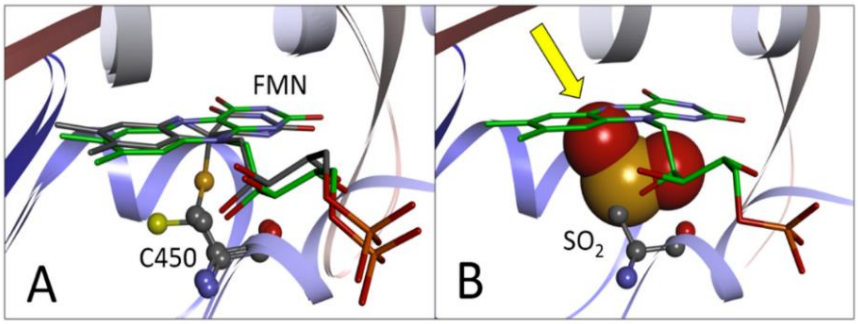

Figure 8: A) *Superposition of PDB ID: 2w0u (FMN with green carbons) and PDB ID: 2v0w (FMN bound to Cys450 with grey-coloured carbons).* ***B)*** *-SH to -SO_2^- substitution in C450 with marked atomic clash of the generated structure.*

4. Summary

The goal of this thesis was to contribute to the research of medicinally and biotechnologically important enzymes using methods of structural mass spectrometry. Following results, included in three publications, were obtained during the study of fungal cellulolytic enzymes Lytic Polysaccharide Monooxygenase and Cellobiose Dehydrogenase and natural photosensitizer *As*LOV2:

- Structural changes to *Nc*LPMO9c during its catalysis, namely reduction of its active site copper ion and subsequent degradation, were observed using HDX-MS
- Primary cause of the loss of activity and degradation of *Nc*LPMO9c during catalysis was identified as oxidative modification and peptide bond cleavage using standard proteomics and peptide intensity observation during HDX-MS
- Protective effect of substrate on NcLPMO9c was detected using HDX-MS in heterogenous mixture of *Nc*LPMO9c and crystalline cellulose, shown to slow down the onset of degradation
- Hydrogen peroxide was confirmed as a cosubstrate to NcLPMO9c using turbidimetric and electrochemical measurements of reaction mixtures containing NcLPMO9c, phosphoric acid swollen cellulose and various hydrogen peroxide generation systems, as all reaction were inhibited by catalase and activity was proportional to the amount of hydrogen peroxide added
- The rate of IDET and IPET in CDH was found to be strongly dependant on relative movement of CYT and DH domains of CDH and their structural complementarity. CDHs with less compatible domain interaction interfaces and longer linker resulting in higher preference for open conformation show higher IPET and lower IDET rates and vice versa.
- Oxidative modification of C450 in *As*LOV2 was found to be responsible for release of FMN to the solution upon extended protein irradiation, causing increase in the production of singlet oxygen

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List of Publications

Publications directly supporting this thesis:

1. **Filandr, F.**, Kavan, D., Kracher, D., Laurent, C. V. F. P., Ludwig, R., Man, P. & Halada, P. Structural Dynamics of Lytic Polysaccharide Monooxygenase during Catalysis. *Biomolecules* 10, 242 (2020). Doi:10.3390/biom10020242

2. **Filandr, F.**, Man, P., Halada, P., Chang, H., Ludwig, R. & Kracher, D. The H2O2-dependent activity of a fungal lytic polysaccharide monooxygenase investigated with a turbidimetric assay. *Biotechnol. Biofuels* 13, 1–13 (2020). doi:10.1186/s13068-020-01673-4

3. Petrenčáková, M., **Filandr, F.**, Hovan, A., Yassaghi, G., Man, P., Kožár, T., Schwer, M. S., Jancura, D., Plückthun, A., Novák, P., Miškovský, P., Bánó, G. & Sedlák, E. Photoinduced damage of AsLOV2 domain is accompanied by increased singlet oxygen production due to flavin dissociation. *Sci. Rep.* 10, 1–15 (2020). doi: 10.1038/s41598-020-60861-2

4. Felice A.K.G., Schuster C., Kadek, A., **Filandr F.**, Laurent, C.V.F.P., Scheiblbrandner, S., Schwaiger, L., Schachinger, F., Kracher, D., Sygmund, C., Man, P., Halada, P., Oostenbrink, C. & Ludwig, R. Chimeric cellobiose dehydrogenases reveal the function of cytochrome domain mobility for the electron transfer to lytic polysaccharide monooxygenase. ***ACS Catal****., under review.*

Other publications by author:

5. Kalabova, D., **Filandr, F**., Alblova, M., Petrvalska, O., Horvath, M., Man, P., Obsil, T. & Obsilova, V. 14-3-3 protein binding blocks the dimerization interface of caspase-2. *FEBS J.* febs.15215 (2020). doi:10.1111/febs.15215

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PERSONAL

Birthday	:	26th August 1991
Nationality	:	Czech
Languages	:	Czech - native speaker English - fluent (C2)
Relationship	:	Married
Hobbies	:	Photography, Cycling, Gaming, Snowboarding

SKILLS AND EXPERIENCE

Mass Spectrometry:

ESI/MALDI-FT-ICR, MALDI-TOF
LC-MS, LC-MS/MS (HPLC, UPLC)
Hydrogen/Deuterium Exchange
Chemical Crosslinking
FPOP

Other scientific techniques:

PCR, Q-PCR, Western Blotting,
Stopped-Flow, UV/VIS Spectrometry

Software and other skills:

Bruker DataAnalysis, ProteinScape
PEAKS Studio
PyMOL
CorelDRAW
MS Office
Python programming (basic)

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PROFILE

I am currently a PhD student in Prague working in mass spectrometry focused laboratory of Petr Novák (Laboratory of Structural Biology and Cell Signalling - http://peterslab.org/). I have been stuyding biotechnologically useful cellulose degrading enzymes Lytic Polysacchatide Monooxygenase (LPMO) and its redox partner Cellobiose Dehydrogenase (CDH) by determining their structural changes during catalysis. I was mainly using hydrogen/deuterium exchange mass spectrometry and standard proteomic approaches for PTM analysis, but I also worked on several projects where I performed Q-PCR, Western Blotting, FPOP-MS and stopped-flow spectrometry. I enjoy learning new technologies and solving interesting problems and I want to continue my career in science, be it in academia or company R&D.

EDUCATION

2003 - 2011: High School
Gymnázium T.G.Masaryka, Litvínov, Czechia
2011-2014: Bachelor's Degree in Biochemistry
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2014-2016: Masters's Degree in Biochemistry
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2016-2020: PhD in Biochemistry
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NON-SCIENTIFIC WORK EXPERIENCE AND CERTIFICATES

2011 - obtained Cambridge English **FCE, Grade A** (CEFR Level C1)
2011 - 2020 - **Private Tutor** of english, chemistry and mathematics
2014 - 2016 - **Teacher** of weekly english courses in language school „Point 007"

ACADEMIC ACHIEVEMENTS AND EXPERIENCE

Publications: 4 published, 1 submitted, 1 other being finalized

Internships: a total of one month in Vienna in laboratory of Ludwig Roland during two internships where I worked with Daniel Kracher. Data obtained resulted in a joint publication.

Conference talks: 2

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