SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

The use of recombinant protein substrates for the examination of HIV-1 and SARS-CoV-2 proteases and for the development of a bio-layer interferometry-based protease assay

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1. Introduction

1.1. The proteolytic enzymes

The proteolytic enzymes (also referred as proteases or proteinases) have been studied since the late 18th century. The proteases have important role in the daily life of the mankind, as they are involved in the processes of the living organism that maintain homeostasis. In terms of their biochemical properties, they are able to hydrolize peptide bond between amino acid residues. In the case of digestive enzymes the proteolytic activity results degradation of the target molecules due to the cleavage at multiple positions. In addition, proteases may be able to cleave target proteins at specific site(s) through so-called limited proteolysis, where proteins are cleaved at one or only a few specific sites, which may play an important role in the regulation of protein function, often involving activation of the target protein (e.g. the blood-clotting cascade). During evolution, proteases have adapted to a wide range of conditions in organisms with different structures (e.g. pH changes, reductive environment, ionic strength) and use different catalytic mechanisms (serine, cysteine, threonine or aspartyl, metallo- and glutamic acid proteases) to hydrolyse the target molecule (substrate). Proteases play an important role in the development of various infectious diseases. As an example, the function of proteases in promoting the replication cycles of various viruses is to promote maturation (virus maturation), and it is therefore important to understand the structure and functionality of these enzymes in detail.

1.2. HIV-1 protease

Retroviral proteases are the members of the aspartyl protease A2 family. The protease of human immunodeficiency virus type 1 (HIV-1) functions as a homodimer, the active enzyme is formed by the interaction of two monomers having identical sequence and structure, in most cases the monomers consist of 99 residues. The viral protease plays a major role in the replication cycle through irreversible post-translational modification of viral gag and gag-pol polyproteins. The cleavage of the gag and gag-pol proteins results in the formation of functional units, such as the matrix (MA), capsid (CA), nucleocapsid (NC) structural proteins, and the enzymes that also play crucial role in the viral replication, namely reverse transcriptase (RT), integrase (IN), and protease (PR). Therefore, the HIV-1 PR in maturation has great importance in the viral replication cycle. The catalytic diad consists of the aspartate amino acid residues (at 25 and 25' positions) of the monomer of HIV-1 protease, this diad is responsible for the

hydrolysis of peptide bond. Besides the catalytic motif, there are other structurally important regions that are involved in the formation of functional homodimer and substrate binding, such as the flap region, the loop being responsible for the formation of 'fireman's grip' interactions at the active site, the dimerization region composed of antiparallel β-strands, and the consensus α-helix near the C-terminus of the enzyme. Determination of the substrate specificity and amino acid preferences of HIV-1 PR remains a challenge to date, despite considerable efforts has been made to characterize cleavage sites *in vitro*. The S1 pockets of retroviral proteases are characterized primarily by a preference for hydrophobic amino acids, but the amino acid preferences of other (S2-S4) substrate-binding pockets show more significant differences. With respect to HIV-1 PR substrates, the P2 position is critical, characterized mainly by the presence of asparagine or β-branched-chain amino acid residues. In addition to the active site motifs, other distal regions (e.g. substrate groove) are also involved in substrate binding of the HIV-1 and human T-lymphotropic virus type 1 (HTLV-1) proteases. The substrate groove allows the recognition and binding of P12-P5 and P5'-P12' residues of the substrate. The substrate groove has effect on the efficacy of the enzyme by increasing its affinity for binding to the substrate.

1.3. The nucleicapsid as an HIV-1 protease substrate

Based on the previous assumptions, the HIV-1 PR plays role in post-maturation *via* the cleavage of capsid and nucleocapsid proteins. The HIV-1 nucleocapsid (NC) protein is a small basic protein containing two zinc finger motifs. The NC has several functions in viral replication, it is involved in the reverse transcription, the dimerization and packaging of the genomic RNA, in viral assembly and also has chaperone activity to promote nucleic acid folding. Proteolysis has been shown to occur between phenylalanine (F16) and asparagine (N17) residues at 16^{th} and 17^{th} positions of the proximal zinc finger motif. The hydrolysis of the NC zinc finger motif has been previously assumed to play a role in the early stage of the replication cycle, but this role is not clearly understood yet, despite the importance of this mechanism in the life-cycles of HIV viruses. In accordance with this, the NC cleavage site cleavage site sequence is highly conserved and the importance of the protease was implied ny the studies that have investigated its role in the early phase. Furthermore, the study of NC processing may provide important insights into the specificity of HIV-1 PR.

1.4. SARS-CoV-2 main protease

The main protease (Mpro) of SARS-CoV-2 (Severe acute respiratory syndrome coronavirus-2) belongs to the cysteine protease family, which contains two monomers, each consisting of three domains (domains I, II and III). The substrate binding site is located in the groove between domains I and II. The active site is located in this groove and the catalytic site is formed by the cysteine (C145) activated by the histidine (H41) residue, which is characteristic of cysteine proteases and which in this case forms the highly conserved catalytic diad. The most important function of SARS-CoV-2 Mpro is to process the viral polyproteins into functional units. Understanding the substrate specificity of the proteolytic enzymes is crucial for both *in vitro* and *in silico* identification of natural substrates and for drug design at the molecular level. The SARS-CoV-2 Mpro prefers "small amino acid"-X-(L/F/M)-Q*(G/A/S)-X cleavage motif, where X indicates any amino acid and * indicates the cleavage site. The SARS-CoV-2 Mpro shows a strong preference for glutamine (Q) at the P1 position of the cleavage site sequence, but recent experimental data suggest that not only glutamine but histidine may also occupy the P1 position. There is mainly leucine in P2 position, but methionine was also described in several cases, while glycine/alanine/serine residues are the most frequent in the P1' position. Considering the critical role of the Mpro in the replication cycle and the fact that there are no such human proteins which are its structural homologs, the Mpro is considered as a main target of antiviral therapies.

1.5. Protease assays

The proteolytic enzymes play key role in many biological processes, they are important drug targets and have a wide range of biotechnological and industrial applications. For this reason, there is a great demand for efficient protease assays. Numerous different methods are available to study proteases, which can be differentiated according to the assay design, the assays can be classified as homogeneous and heterogeneous assays. In homogeneous systems, both the substrate and the enzyme are in the reaction solution (e.g. FRET-based techniques), whereas in heterogeneous methods the substrate is immobilized to a solid surface (e.g. surface plasmon resonance), while the enzyme is in the solution. The recombinant protein technology has opened new opportunities in the field of enzymatic studies via the application of recombinant proteins as protease substrates. Our research group has also been involved in the development and application of similar substrate systems in the recent past.

1.6. Applications of a recombinant protein substrate-based system

There are several different types of substrate systems that are available to study proteolytic enzymes, one of these are the recombinant protein substrates which have a wide range of applications and many advantages. The recombinant substrates developed by our research group consist of a maltose-binding protein (MBP) fused with an N-terminal hexahistidine tag (His_6), followed by the natural cleavage site of the tobacco etch virus (TEV) protease and the natural or modified cleavage site sequence of the protease to be studied. The substrate contains a fluorescent protein at its C-terminus. Each part of the fusion protein has an important function. The His_6 tag allows the purification with immobilized metal affinity chromatography (IMAC). The MBP tag increases the solubility of the protein and promotes its folding. The cleavage site of TEV PR can be used i) as a control cleavage site to test whether the cleavage site is accessible for the proteolysis, and ii) it can be used e.g. in the case of cleavage site identification when after cleavage of the protein (e.g. by TEV and the protease of interest) the molecular masses of the cleavage fragments are determined using mass spectrometry.

These substrates can be used in multiple types of measurements, such as in a separation-based heterogeneous system where the proteolysis can be detected by fluorimetry or electrophoresis, but can be used in homogeneous assays, as well. In the case of in-solution digestion, the substrates and the products can be analyzed by SDS-PAGE. Although, these substrates can be applied in different experimental systems, none of these applications enable real-time detection of product formation.

1.7. The bio-layer interferometry

The bio-layer interferometry (BLI) is an optical analytical method that allows real-time monitoring of molecular interactions by measuring interference. The method is mainly used for quantitative and qualitative analysis of various molecular interactions, but alternative applications also exist. To date, only few studies have been reported for the analysis of enzyme activity using the BLI method. To our knowlwedge, there is only one example for the use of proteases in BLI measurements, but this in this study the proteolytic enzyme was used only for enzymatic removal of molecules remaining on the sensor surface rather than for the measurement of protease activity by BLI. To our knowledge, the use of BLI in protease studies has not been published to date.

2. Aims

Based on the use of the recombinant fluorescent protein substrate system developed by our research group, we aim to perform the following studies.

I.) Examining the HIV-1 and SARS-CoV-2 proteases

I/A We aimed to investigate the substrate specificity of HIV-1 PR using recombinant substrates representing the cleavage site of the proximal zinc finger motif of HIV-1 nucleocapsid. We aimed to perform cleavage reactions using wild-type and modified substrates, and to study the conformational states of the zinc finger motif.

I/B Our aim was to design and produce substrates containing known or *in silico* predicted cleavage site sequences of the SARS-CoV-2 Mpro and to identify cleavage positions and determine enzyme kinetic parameters.

II.) Development of a new method for real-time detection of proteolytic cleavage based on the use of recombinant protein substrates.

We aimed to use the recombinant fluorescent protein substrates to develop a biolayer interferometry (BLI)-based measurement method, and – after the optimization of the reaction conditions - we aimed to apply this assay to study the substrate specificity and the substrate groove (substrate-binding site at the enzyme surface) of HIV-1 PR.

3. Materials and Methods

3.2. Expression and purification of HIV-1 protease

The catalitically inactive HIV-1 PR (HIV-1 $PR_{\Delta N \Delta C}$) - containing a modified dimerization region - was expressed in *Escherichia coli* BL21(DE3) cells which were cultured in ampicillin-containing Luria-Bertani (LB) medium. The protein expression was induced by the addition of isopropyl-ß-D-thiogalacto-pyranoside (IPTG), followed by incubation at 37°C for 3 h, and harvesting of cells by centrifugation. The pelleted bacterial cells were lysed by sonication in the presence of lysozyme. The protein was purified by reversed-phase chromatography (ÄKTA Purifier) using a C18 column at a flow rate of 1 ml/min using a 0-40% increasing gradient elution using eluents containing water and 0.05% trifluoroacetic acid (TFA) and acetonitrile and 0.05% TFA. The purities of the fractions were checked by 14% SDS-PAGE. Fractions with sufficient purity (>90%) were renatured in two steps and the protein concentrations were determined using a NanoDrop instrument and BCA kit.

3.3. Production of purified recombinant protein substrates

3.3.1. Cloning

The circular pDest-His_6-MBP-mApple (mApple, monomeric red fluorescent protein) and pDest-His_6-MBP-mEYFP (mEYFP, monomeric yellow fluorescent protein) expression vectors were cleaved with PacI and NheI restriction endonucleases, while the pDest-His_6-MBP-$(GGGGS)_4$-mApple vector was cleaved with PacI and BamHI enzymes. The cleavage products were separated on a 1% agarose gel and the linearized plasmids were purified from the gel using a DNA extraction kit. The forward and reverse complementation primers encoding the cleavage sites of the proteases and the purified linearized expression plasmid were ligated using T4 DNA ligase, and the mixture was transformed into BL21(DE3) bacterial expression cells using heat shock. The transformants were spread onto LB-agar plates and incubated at 37°C for at least 16 hours, and the plasmids were isolated from separate colonies. The success of cloning was verified by sequencing.

3.3.2. Expression in bacterial cell cultures

For the HIV-1 PR assays, we designed such recombinant protein substrates that contained a wild-type or modified cleavage site prepresenting the proximal zinc finger motif of the HIV-1 NC protein, or a 9- or 24-mer wild-type or modified HIV-1 MA/CA cleavage site. The substrates designed for the assays of SARS-CoV-2 Mpro contained either the natural autoproteolytic cleavage site of Mpro or the unknown natural cleavage site predicted by *in silico* methods.

For the production of HIV-1 PR and SARS-CoV-2 Mpro substrates, *E. coli* BL21(DE3) cells transformed with the plasmid encoding the corresponding substrate. Cultures were grown in LB medium and protein expression was induced by IPTG. The protein expression was carried out by incubation for 3 hours, followed by harvesting of cells by centrifugation.

3.3.3. Cell lysis

Cells were lysed by sonication in lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing lysozyme, DNase and protease inhibitor (PMSF). After centrifugation, the supernatants (cell lysates free from cell debris) containing the recombinant substrate were used for purification or for analyses.

3.3.4. Protein purification with Ni-NTA magnetic beads

The purification of the recombinant substrates was performed using Ni-NTA magnetic agarose beads. The substrates were purified from the total cell lysates, and repeated washing steps were applied to remove the majority of aspecifically or weakly bound proteins. The purified substrates were eluted from the beads by using a solution containing EDTA, followed by buffer exchange. The protein concentrations were determined using NanoDrop instrument, based on the molecular weights and extinction coefficients of the recombinant substrates. The purities of the samples were checked by SDS-PAGE.

3.4. In-solution digestion of the recombinant HIV-1 nucleocapsid substrates

The catalytically active HIV-1 PR (containing stabilizing mutations) was in-house stock. The concentration of the active enzyme was determined by using an oligopeptide

substrate representing the 9-mer sequence of HIV-1 MA/CA cleavage site (VSQNY*PIVQ). The in-solution digestions were performed using His_6-MBP-$(GGGGS)_4$-mApple substrates representing the HIV-1 NC cleavage site sequences. The cleavage reactions were performed in 20 µl final volume in HIV-1 reaction buffer (the enzyme and substrate were used in 250 nM and 1.4 mM final concentrations, respectively, the buffer contained $ZnCl_2$ or dithiothreitol (DTT) and EDTA). The reaction mixtures were incubated for 16 h at 37°C, followed by sample preparation under native conditions. Samples were analyzed on a 14% polyacrylamide gel. For the in-gel renaturation of the proteins, the gel was rinsed in distilled water for 30 min in two cycles. The partially renatured proteins were detected under UV light or by a blue light transilluminator.

3.5. Digestion of the recombinant SARS-CoV-2 main protease substrates

In the cleavage reactions, the His_6-MBP-TSAVLQ*SGFRKM-mEYFP (representing the autoproteolytic cleavage site of SARS-CoV-2 nsp4) and the His_6-MBP-REGTRVQ*SVEQIRE-mEYFP (containing the predicted cleavage site sequence in the human C-terminal binding protein 1 (CTBP1) protein) recombinant proteins were used as substrates for SARS-CoV-2 Mpro. The final concentrations of the substrate and enzyme in the cleavage reaction were 1.5 and 2 µM, respectively. Due to the lack of any selective, tight-binding SARS-CoV-2 Mpro inhibitor, it was not possible to determine the active site concentration, therefore, the enzyme activity was considered to be 100%. A solution of 20 mM Tris, 100 mM NaCl, pH 7.8 was used as reaction buffer and the cleavage reactions were performed in 40 µl final volume. The reaction mixtures were incubated at 37°C for 1 hour, then the mixtures were aliquoted (20-20 µl). Some samples were treated using denaturing conditions, in this case the enzymatic reactions were stopped by the addtion of buffer containing SDS and 2-mercaptoethanol, and the samples were heated to 95°C. These samples were analyzed by 14% SDS-PAGE. The remaining samples were used for molecular weight determination by matrix-assisted laser desorption ionization time of fligth mass spectrometry (MALDI-TOF-MS) measurements.

3.6. Kinetic measurments of the recombinant SARS-CoV-2 substrates

The kinetic parameters were determined by magnetic bead-based assays which were performed by using His_6-MBP-TSAVLQ*SGFRKM-mEYFP and His_6-MBP-REGTRVQ *SVEQIRE-mEYFP substrates, the SARS-CoV-2 Mpro was applied in 0.074 µM and 0.74 µM

final concentrations, respectively. The product formation was followed by measuring the fluorescence at 510 nm excitation and 540 nm emission wavelengths using a Biotek Synergy H1 plate reader.

3.7. Bio-layer interferometry-based assay

3.7.1. Preparation of the Ni^{2+}-, Cu^{2+}-, Zn^{2+}-, and Co^{2+}-NTA biosensors

The equilibrated Ni-NTA biosensors were treated with solution containing EDTA in high conentration, and after the removal of nickel ions, the sensors were placed into a solution of a divalent cation (10 mM $NiSO_4$ / 10 mM $ZnCl_2$ / 10 mM $CoCl_2$ / 10 mM $CuCl_2$).

The association phase was performed in assay buffer (containing buffer A (50 mM sodium acetate, pH 5) and buffer B (0.275 M Na_2HPO_4, 0.2 M NaH_2PO_4, 4 M NaCl, pH 5.6) in a 1:1 ratio) for 120 seconds, in 4 µl volume of His_6-MBP-VSQNY*PIVQ-mEYFP substrate (0.7 µM). The dissociation step was performed in assay buffer for 120 seconds in 250 µl volume. For the regeneration of the biosensors, phosphoric acid solution (pH 2.5) was used according to the manufacturer's instructions. The data were evaluated using BLItz Pro 1.2 software and mathematical fitting evaluation of the data was performed using a 1:1 binding model (Langmuir) with local fitting.

3.7.2. Measurments with purified substrates

The measurements were started by recording the baseline in assay buffer for 30 seconds in 250 µl volume, followed by immobilization of substrates to the biosensor (using 14 µM purified substrate solution) for 60 seconds in 4 µl volume (drop mode). The immobilization was followed by washing steps, the biosensors were washed with assay buffer to remove excess substrates and non-specifically or weakly bound molecules. The first washing step lasted 60 seconds (250 µl volume, tube mode), while the second wash step lasted 30 seconds (4 µl volume, drop mode). A second washing step was introduced to minimize the aspecific signal from baseline shift. The HIV-1 PR used for the measurements was diluted 1:1 with the B component of the assay buffer before proteolysis and further dilutions were performed with assay buffer. Each measurement was performed in 4 µl volume (drop mode).

The atazanavir inhibitor was dissolved in dimethyl sulfoxide (DMSO) therefore, the assay buffer was supplemented with 5% DMSO. The final concentration of HIV-1 PR used in

our assay was 9 nM, the atazanavir applied in 2 nM and 2 μM final concentrations. The HIV-1 PR was diluted in assay buffer containing 5% DMSO. The control samples contained only 5% DMSO-containing assay buffer. For measurments with trypsin, we used human cathionic trypsin, which was provided by András Szabó. All measurements were performed on a BLItz instrument (Pall Forté Bio, Fremont, CA, USA) at room temperature (25°C). The primary experimental data were exported from BLItzPro 1.2 and further analyzed using Microsoft Excel.

3.7.3. Measurments using cell lysates

The BL21(DE3) cells expressing the His_6-MBP-VSQNY*PIVQ-mEYFP recombinant protein as well as the non-transformed cells were lysed as described above, and the supernatants obtained from the centrifugation of the cell lysates were used for the measurements and SDS-PAGE analyses.

The baseline was set for BLI measurements in lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5) for 20 seconds. The substrate immobilization from cell lysate was then performed for 30 seconds. The biosensor was washed in three consecutive washing steps, i) in lysis buffer for 30 seconds, ii) in assay buffer for 30 seconds (250 μl volume, tube mode), and iii) in assay buffer again for 30 seconds (4 μl volume, drop mode). The same conditions were applied as in the case of measurements with the purified substrate.

4. Results

4.1. Examination of the specificity of the HIV-1 protease using substrates representing the nucleocapsid cleavage site

4.1.1. Determination of relative cleavage efficiency

We investigated the processing of a sequence representing the HIV-1 NC proximal zinc-finger motif. We designed recombinant protein substrates to determine whether our protein substrate-based system can be used to study the conformational properties of structural motifs (in this case zinc-finger) and whether the cleavage preferences can be determined using these recombinant proteins. We designed His_6-MBP-$(GGGGS)_4$-mApple substrates containing only the proximal zinc finger sequence of HIV-1 NC. The recognition sequences of HIV-1 PR represented the full-length zinc finger motif (^{11}KIVKCCFNCGKEGHTARNCRAPR^{32}), which enabled to study the conformational states of the structural motif and its proteolysis. In addition to the wild-type sequence, N17T, N17L, N17F, I14_ins-N17G, and K14I-N17G mutants were also investigated.

To investigate the effects of the zinc ion on the processing of the recombinant fluorescent proteins, the cleavage reactions were performed in the presence of DTT and EDTA, as well as in $ZnCl_2$-containing buffer. The EDTA was used as a chelator to ensure the formation of an „opened" conformation in the metal ion-free state of the zinc fingers, while DTT was added to reduce the cysteine side chains and prevent the formation of intra- and inter-monomeric disulfide bonds. In contrast to this, the zinc-containing buffer was used to ensure the formation of zinc finger motifs, a „closed" conformation was expected to inhibit the substrate cleavage.

The proteolysis of substrates by HIV-1 PR was observed only in the buffer containing DTT and EDTA. This implied that the cleavage site is accessible in the zinc finger having „opened" conformation, and chelation of zinc ions with EDTA and reduction of cysteine side chains with DTT ensured the „opened" conformation of the zinc finger. In contrast, the substrates were resistant to proteolysis in the presence of zinc, suggesting that the presence of zinc triggered the formation of the „closed" conformation for the structural motif, making the proximal NC cleavage site inaccessible for the protease and preventing proteolysis. In the presence of $ZnCl_2$, HIV-1 PR did not process any of the substrates, but such bands appeared on the gel images in both cleavage reactions and substrate control samples that were not observed in samples containing DTT and EDTA. It was hypothesized that these bands correspond to

oligomers formed via intermolecular interactions under native (non-denaturing) conditions, whereas in the buffer containing DTT and EDTA, the formation of such intermolecular disulfide bridges may be inhibited.

Following electrophoresis, the intensities of the bands were determined by densitometry for the reactions that were performed in the presence of DTT and EDTA. Our results indicate that none of the studied mutations prevented the formation of the zinc finger structural motif and proteolytic cleavage.

The highest substrate conversion was obtained for the N17F mutant, which was found to be a better substrate of HIV-1 PR as compared to the wild-type sequence, which was in agreement with the results of oligopeptide substrate-based measurements. With the exception of the N17F mutant, the substrate conversions obtained for the other mutants were similar, probably due to the relatively lower sensitivity of the gel-based assay. The determination of kinetic parameters by the fluorescent protein-based assay was not possible because the optimal conditions for the cleavage reaction were not compatible with the Ni-NTA-based assay, as DTT and EDTA have a negative effect on substrate immobilization, resulting in substrate dissociation. In additon, the use of these agents facilitates the formation of a „closed" conformation of the motif, which prevents proteolysis.

Based on our results, the substrate-based system was found to be suitable to study the conformational states as well as cleavage preferences, but in the latter case we could investigate the differences between the modified substrates only to a limited extent.

4.2. Examination of the SARS-CoV-2 main protease

4.2.1. Preparation of recombinant substrates

We studied the cleavage of a natural (nsp4) and a putative proteolytic cleavage site of SARS-CoV-2 Mpro, for these cleavage reactions His_6-MBP-mEYFP recombinant fusion proteins were designed. The His_6-MBP-TSAVLQ*SGFRKM-mEYFP substrate represented the natural nsp4 autoproteolytic cleavage site of the SARS-CoV-2 polyprotein, while the His_6-MBP-REGTRVQ*SVEQIRE-mEYFP protein contained the putative cleavage site sequence of the human C-terminal binding protein 1 (CTBP1) protein which was identified by *in silico* prediction and is identical in human CTBP1 and CTBP2 isoforms (^{153}GTRVQ*SVEQI^{162}).

Based on the results of electrophoretic assays, the His_6-MBP-TSAVLQ*SGFRKM-mEYFP substrate was a better substrate of the SARS-CoV-2 Mpro. Only a low cleavage

efficiency was observed for the His_6-MBP-REGTRVQ*SVEQIRE-mEYFP protein, indicating a lower preference for the CTBP1 cleavage site.

4.2.2. Determination of SARS-CoV-2 Mpro's natural cleavage sites

We performed in-solution digestion of the full-length recombinant CTBP1 protein and the His_6-MBP-TSAVLQ*SGFRKM-mEYFP and His_6-MBP-REGTRVQ*SVEQIRE-mEYFP protein substrates with SARS-CoV-2 Mpro. The cleavage positions were identified based on the molecular weights determined *in vitro* and calculated from the amino acid sequences of the proteins. The analytical measurements for molecular weight determination were carried out by Dr. Tibor Nagy (University of Debrecen, Faculty of Sciences, Deptartment of Applied Chemistry).

As it was expected, the recombinant His_6-MBP-mEYFP substrate representing the nsp4 cleavage site sequence (TSAVLQ*SGFRKM) was cleaved at the desired position within the incorporated sequence. Following the cleavage of the recombinant CTBP1 protein, the analysis of cleavage fragments indicated that the full-length protein was cleaved at the *in silico* predicted position (^{153}GTRVQ*SVEQI^{162}). The SARS-CoV_2 Mpro cleaved the recombinant His_6-MBP-mEYFP substrate containing, as well, at the same position. Our results confirmed the presence of the cleavage site that was predicted by computational methods, and in our studies we identified first a previously unknown natural host cell substrate of SARS-CoV-2 Mpro, the CTBP1 protein, and the cleavage site sequence. The fluorescent substrates were further used to determine enzyme kinetic parameters to test substrate preference.

4.2.3. Kinetic measurments

After demonstrating that SARS-CoV-2 Mpro cleaves recombinant substrates at the desired positions, Ni-NTA magnetic bead-based enzyme kinetic measurements were performed with His_6-MBP-TSAVLQ*SGFRK-mEYFP and His_6-MBP-REGTRVQ*SVEQIRE-mEYFP substrates to compare cleavage site preferences. The kinetic analysis showed that the His_6-MBP-TSAVLQ*SGFRKM-mEYFP substrate containing the natural cleavage site of the polyprotein is a better substrate for SARS-CoV-2 Mpro than the substrate containing the CTBP1 cleavage site. This is in agreement with the results of the gel-based assay, which showed a higher cleavage efficiency of the His_6-MBP-TSAVLQ*SGFRKM-mEYFP substrate as compared to the His_6-MBP-REGTRVQ*SVEQIRE-mEYFP substrate. Our results

demonstrated that the designed substrates can be used for proteolytic studies and showed that the substrate is processed at the incorporated CTBP1 cleavage site with lower efficiency as compared to the autoproteolytic cleavage site.

4.3. Development of a bio-layer interferometry-based protease assay

4.3.1. Purification of the mutant HIV-1 protease

The HIV-1 protease was used as a model enzyme for the development of the bio-layer interferometry-based assays. Besides the catalytically active enzyme, we used a modified HIV-1 PR as control. This mutant enzyme containes deletion of 1-4 and 96-99 residues (HIV-1 $PR_{\Delta N \Delta C}$), due this modification the mutatnt enzyme is unable to form a functional dimer, and lacks catalytic activity. The active enzyme was in-house stock, the purification of HIV-1 $PR_{\Delta N \Delta C}$ was performed by reverse phase chromatography.

4.3.2. Preparation and cleavage of recombinant substrates

For the HIV-1 PR assay, we designed His_6-MBP-mEYFP and His_6-MBP-mApple recombinant protein substrates representing the 9- (P5-P4') or 24-mer (P12-P12') cleavage site sequence of the HIV-1 MA/CA cleavage site. Both wild-type (wild-9 and wild-24) and mutant (mut-9 and mut-24) substrates were generated. Of the sequences containing the shorter HIV-1 MA/CA cleavage site, the mutant sequence contained leucine at P2 position (VSQLY*PIVQ). The 24-mer mutant MA/CA cleavage site sequence represented the residues of HIV-1 PR CA/p2 cleavage site at P12-P6 and P5'-P12' positions, respectively. Using the latter substrates, we were able to investigate the substrate-binding to the substrage groove binding site.

To test whether purified proteins can be used as substrates for HIV-1 PR, in-solution digestion reactions were performed. After electrophoresis of the samples, the appearance of a proteolytic fragments indicated that the recombinant proteins were cleaved by the HIV-1 PR at the incorporated cleavage site and therefore can be used in enzymatic reactions. No cleavage was observed in the case of HIV-1 $PR_{\Delta N \Delta C}$. In the downstream experiments we used these proteins as substrates in cleavage reactions to develop a new assay for continuous measurement of proteolytic activity.

4.3.3. Otpimization of the conditions of the BLI-based protease assay

4.3.3.1. Digestion with catalitically active and inactive HIV-1 protease

The development of the bio-layer interferometry-based protease assay was based on the assumption that substrates immobilized on the biosensor surface containing the HIV-1 PR cleavage site are sensitive to proteolysis, and that the release of proteolytic fragments reduces the size of the immobilized substrates on the surface, thereby reducing the thickness of the biomolecular layer, and that the change in biolayer thickness can be detected as a measurable signal. The His_6-MBP-VSQNY*PIVQ-mEYFP substrate was used to test and develop the method, using HIV-1 PR as the model enzyme. Catalytically active and inactive enzymes were used to validate the signal, which can be used to distinguish the changes that occur as a result of proteolysis. Ni-NTA-based biosensors were used for the measurements.

The BLI-based protease assay consists of 3 main steps. The first step is the immobilization of the substrate to the Ni-NTA-based sensor *via* the His_6 affinity tag of the substrate. In the second step, the proteins weakly or aspecifically bound to the biosensor are eliminated. In a third step, enzymatic hydrolysis of the substrate is performed, starting with immersion of the sensor in HIV-1 PR solution. The substrates were processed at the built-in cleavage site, causing decrease of the thickness of the biolayer on the sensor, which can be detected as a function of time. Once the immobilized His_6-MBP-VSQNY*PIVQ-mEYFP substrate (72.24 kDa) was cleaved by HIV-1 PR, the N-terminal fragment remained on the sensor (His_6-MBP-VSQNY, 44.45 kDa), while the C-terminal product containing the fluorescent protein (PIVQ-mEYFP, 27.64 kDa) was released into the solution which contained the HIV-1 PR enzyme, as well, resulting in a signal change due to a reduction in the thickness of the biolayer.

In order to exclude possible false positive results - that the decrease of the signal was caused by the spontaneous dissociation of His_6-tagged substrates rather than by proteolysis - the catalytically inactive HIV-1 PR was used in the control experiment. In measurements with the HIV-1 $PR_{\Delta N \Delta C}$ enzyme, we observed only a negligible decrease of the signal, despite the fact that the active and inactive enzymes were applied in the same concentration. This implied that the changes of the signal were observed due to the processing at the MA/CA cleavage site of the recombinant substrate, rather than spontaneous substrate dissociation. Our measurements proved that the method could be used to detect proteolytic activity, and therefore, the further studies were performed to optimise the measurement conditions.

4.3.3.2. Cleavage reaction using trypsin

In our experiment, we investigated whether the assay could be used to study other proteolytic enzyme(s) and whether the sensitivity of the system could be increased by using an enzyme that generates a larger change of signal intensity. Purified human cationic trypsin was used in our study. The HIV-1 PR cleaves the recombinant substrates only at a single position within the incorporated cleavage site, resulting in a ~26 kDa decrease of the molecular mass of the immobilized substrate. The His_6-MBP-VSQNY*PIVQ-mEYFP substrate is cleaved by the trypsin at multiple sites, which causes higher change of the substrate's molecualr weight. Accordingly, we observed a larger change in signal for trypsin compared to HIV-1 PR, which may have been due to the fact that the conditions (e.g. temperature) used during the measurements were more optimal for trypsin.

Our results suggest that the activity of proteolytic enzymes other than HIV-1 PR can also be tested using our method and that the decrease of the signal intensity can be improved by using such an enzyme or substrate which causes higher decrease of the substrate's molecular weight upon cleavage. It is important to note that the conditions of the measurements need to be optimised for each enzyme, e.g. type of sensor, type of substrate (e.g. specific or general protease substrate), or reaction conditions (e.g. buffer components, pH, temperature).

4.3.4. The effect of different metal ions on substrate immobilization

Various affinity chromatographic techniques can be used to immobilize the His-tagged proteins with divalent cations, but the interactions between the individual cations and the His-tag are not well established in the literature. The spontaneous dissociation of the substrates from biosensors can be reduced by using the appropriate cation, a relatively lower dissociation constant may improve substrate immobilization. In addition to this, it is essential to work with a system that is compatible with regeneration, i.e. to obtain broadly similar sensorgrams after several enzyme reactions and regeneration steps (reproducibility). This is also very important from the viewpoint of cost-effectiveness and simplicity.

In the optimization experiments, the surfaces of individual biosensors were coated with Zn^{2+}, Ni^{2+}, Co^{2+} or Cu^{2+} divalent cations, to which His_6-MBP-VSQNY*PIVQ-mEYFP substrate was subsequently immobilized during the association step, then the dissociation was investigated. After an acidic regeneration step, the sensors were re-used for a parallel measurement.

The most stable interaction was obtained for the His_6-tagged substrate in the case of the sensors coated with nickel ions, we observed no remarkable changes in the binding capacity, dissociation and association constants as compared to the other ions.

4.3.5. The effect of enzyme concentration on the processing of the immobilized substrate

For the validation of the method, it is very important to keep in mind the general roles of enzyme kinetics. In our experiments we investigated how the reaction velocity changes upon increasing enzyme concentration, whether differences can be detected and the signal intensity changes proportionally with the amount of enzyme. The measurements were performed by loading the Ni-NTA biosensors with equivalent amounts of His_6-MBP-VSQNY*PIVQ-mEYFP substrate and then we investigated substrate processing, using different concentrations of HIV-1 PR. From the primary experimental results (sensogram), we processed the data to determine the decrease of biolayer's thickness as a function of time ($\Delta nm/\Delta t$) for each proteolysis step and performed a correlation analysis of the slope values of these results as a function of enzyme concentration. Our results showed that the system was suitable for detecting differences of enzyme activity if it was applied in different concentrations. The differences of signal changes that were observed at different enzyme concentrations confirmed that the detected changes were due to the proteolytic activity of the enzyme rather than spontaneous substrate dissociation or other aspecific effects. In our further measurements, we performed an experiments by inhibiting the enzyme activity and performed substrate specificity assays.

4.3.6. The effect of an inhibitor on the reaction rate

To further confirm that the change of the optical signal is caused by proteolysis and not by spontaneous dissociation of the substrate from the biosensor surface or by non-specific interactions, we performed cleavage reactions in the presence of atazanavir inhibitor which is capable for inhibiting HIV-1 PR.

The cleavage reactions were performed using the substrate His_6-MBP-VSQNY*PIVQ-mEYFP and the HIV-1 PR. The most intense signal changes were observed in the control reaction which contained no inhibitor. As compared to the control, the signal slope was lower when atazanavir was applied in 2 nM final concentration, which implied only partial inhibition. In contrast to this, the change of the measured signal was negligible in the presence of 2 μM

inhibitor, indicating almost complete inhibition of HIV-1 PR at the applied inhibitor concentration.

Our results confirmed that the change of the detected signal was caused by proteolysis of the substrate by HIV-1 PR, which can be inhibited by a specific inhibitor.

4.3.7. Measurments using cell lysates

The recombinant proteins containing histidine tags (e.g. His_6 or His_8) can be immobilized directly from complex biological samples (e.g. bacterial cell lysates). Measurement with unpurified proteins in this way can further reduce the cost and time of the BLI protease assays, which may be critical in the case of high-throughput methods.

We started our experiments by immobilization of His_6-MBP-VSQNY*PIVQ-mEYFP recombinant fluorescent protein substrates from cell lysate. In our control experiment, we used the lysate of non-transformed BL21(DE3) cells that did not express the substrate in order to determine the extent of background binding. The cell lysates were analyzed by SDS-PAGE, the fluorescent substrate was detectable in the Coomassie stained gel, but several bacterial proteins were also detectable in the total cell lysate, therefore the proteins visualized under UV light. After renaturation of the proteins in the gel, only the fluorescent protein was detectable in the lysate of cells expressing the substrate, whereas no band was observed in the control sample at the same molecular size. This confirmed the presence or absence of the substrate in the samples.

The experimental conditions that were applied in the case of total cell lysates were identical to those used for the purified substrates, but an extra washing step was added to eliminate most of the aspecifically bound contaminants. Different signal intensities were obtained after immersing the Ni-NTA biosensors into the different cell lysates, we observed much lower binding signal for the lysate containing no substrate as compared to the cells expressing the protein substrate. In the proteolysis step of the assay, the catalytically active HIV-1 PR caused reduction of the binding signal in the substrate-containing sample, whereas it remained unchanged in the sample lacking the substrate. Our SDS-PAGE- and BLI-based measurements implied that the designed method may be suitable for the immobilization of His_6-tagged proteins from total cell lysates, therefore, may be potentially applied to investigate the proteolytic activity of HIV-1 PR, but we did not perform detailed studies with non-purified substrates. It is important to note that primary purification of the substrates may be beneficial,

as elimination of contaminants may increase the efficiency of substrate immobilization and help to avoid interference with non-specifically bound proteins.

4.4. The use of a bio-layer interferometry based assay

4.4.1. Examination of the S2 binding pocket of the HIV-1 protease

After optimizing the conditions for substrate immobilization and the BLI-based measurements, the developed method was applied to investigate the substrate specificity of HIV-1 PR. The experiments were performed using His_6-MBP-VSQNY*PIVQ-mEYFP substrate. A modified version of this substrate was also prepared, by changing the asparagine to leucine at the P2 position of the cleavage site (VSQLY*PIVQ). A comparative analysis of the cleavage efficiency was performed by determining the initial $\Delta nm/\Delta t$ of wild-type and mutant mEYFP substrates. The decrease of the signal intensity was higher for the wild-type as compared to the mutant cleavage site-containing substrate. The significant difference between the two different cleavage sites is in good agreement with the amino acid preferences that were previously determined for HIV-1 PR on oligopeptide substrates. Our results clearly demonstrated that the real-time BLI-based protease assay can be used to study the enzyme specificity, and we have therefore designed additional substrates to study the binding sites located at the surface of HIV-1 PR.

4.4.2. Examination of the substrate groove of the HIV-1 protease

The BLI-based protease assay was applied to study the substrate groove of HIV-1 PR, as the role of this enzyme surface binding site in substrate binding has not yet been confirmed by independent studies. We designed a His_6-MBP-mApple substrate containing the 24-mer natural sequence of the HIV-1 MA/CA cleavage site (wild-24, DTGHSNQVSQNY* PIVQNIQGQMVH). We also prepared a substrate containing the modified recognition sequence in which the MA/CA cleavage site was modified at P12-P6 and P5'-P12' positions to represent the HIV-1 CA/p2 cleavage sequence (mut-24 GVGGPGHVSQNY* PIVQSQVTNSAT). It was assumed that the modification of the cleavage sequence may alter the interactions between the substrate and the enzyme surface binding sites, as most of the polar residues in the P12-P6 positions were changed to apolar ones. The modification of the cleavage site sequence was expected to have effect on cleavage efficiency. The differences between the wild-type and the mutant substrates were determined using BLI.

The proteolysis of both wild-type and mut-24 substrates was detectable, and the substrate containing the mutant cleavage site sequence was processed much showed as compared to the wild-type. The reason for the difference was presumably that the interactions have changed between the enzyme and the substrate at the binding sites of the substrate groove due to the modification of the substrate, and the altered interactions resulted in altered efficiency of the processing. The same substrates were used to determine enzyme kinetic parameters, as well, the results of the magnetic bead-based measurements confirmed that the HIV-1 PR cleaves the wild-type cleavage site-containing substrate more efficiently. Our results imply that the BLI-based method can be used efficiently in protease assays and demonstrate that the substrate groove plays an important role in substrate binding of HIV-1 PR.

In conclusion, our BLI-based protease assay can be used to investigate proteases real time by continuous optical measurement of the activity. The number of real-time homogeneous (e.g. FRET-based) and heterogeneous (e.g. surface plasmon resonance) protease assays are limited, thus, this BLI-based system may be a good alternative for continuous measurement of product formation. This assay is a novel approach for the application of the BLI methods and the recombinant protein substrate system developed in our laboratory, as well. Due to the versatility of the recombinant substrates, the BLI-based assay may be adapted to a wide variety of substrates and proteases.

5. Summary

My PhD thesis is based on the use of a previously developed recombinant fluorescent protein substrate system. This system was used to investigate the substrate specificity of HIV-1 and SARS-CoV-2 proteases, furthermore, we used the recombinant substrates to develop a new method for real-time measurement of protease activity.

To study HIV-1 protease, **we prepared recombinant substrates representing the wild-type (N17) and mutant (N17T, N17L, N17F, I14_ins-N17G, and K14I-N17G) proteolytic cleavage sites of HIV-1 nucleocapsid's proximal zinc-finger motif. The conformational states of the substrates were investigated.** The zinc-finger motif was not cleavable by HIV-1 protease in the presence of zinc ion, whereas processing was observed in the presence of EDTA and DDT which prevented the formation of the supersecondary structure. **A comparative analysis of cleavage efficiencies was performed**, we observed the highest substrate conversion for the N17F mutant, our results are consistent with the amino acid preferences determined previously on oligopeptide substrates.

We performed the *in vitro* investigation of such substrates which represented a known or an *in silico*-predicted cleavage site sequence of SARS-CoV-2 Mpro. We confirmed that the SARS-CoV-2 Mpro was able for the processing of the substratres containing either the known or the predicted cleavage site sequences, as well. After the identification of the cleavage positions we performed enzyme kinetic measurements to investigate substrate specificity. As a result of our work, **we have identified a previously unknown substrate of SARS-CoV-2 Mpro, the human CTBP1 protein, and a novel cleavage site was also indentified in this protein**, which was processed with lower efficiency than the autoproteolytic cleavage site sequence of SARS-CoV-2 polyprotein.

The recombinant fluorescent protein substrates were used to develop a novel method for the measurement of proteolytic activity by bio-layer interferometry, using HIV-1 protease as a model enzyme. After optimization of the assay conditions, the method was successfully applied to study the specificity of HIV-1 PR by investigating its P2 amino acid preference and to demonstrate that the substrate-groove interaction site at the enzyme surface is involved in the formation of enzyme-substrate interactions. The designed method may help to expand the use of BLI for new applications by allowing real-time measurement of protease activity in small volumes and in a high throughput-compatible manner.

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7.Appendix

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List of publications related to the dissertation

1. **Miczi, M.**, Diós, Á., Bozóki, B., Tőzsér, J., Mótyán, J. A.: Development of a Bio-Layer Interferometry-Based Protease Assay Using HIV-1 Protease as a Model.
   Viruses-Basel. 13 (6), 1-20, 2021.
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3. **Miczi, M.**, Golda, M., Kunkli, B., Nagy, T., Tőzsér, J., Mótyán, J. A.: Identification of Host Cellular Protein Substrates of SARS-COV-2 Main Protease.
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4. Mahdi, M., Mótyán, J. A., Szojka, Z., Golda, M., **Miczi, M.**, Tőzsér, J.: Analysis of the efficacy of HIV protease inhibitors against SARS-CoV-2·s main protease.
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   Data in Brief. 18, 203-208, 2018.
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