"Characterization of the HtrA homologs from the human bacterial pathogens, *Helicobacter pylori* and *Stenotrophomonas maltophilia*"

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The HtrA (High Temperature Requirement A) proteins are conserved in evolution serine proteases, classified into family S1, subfamily S1C, sub-clan PA(S), according to the MEROPS database. Several HtrA homologs exhibit an additional, chaperone-like activity. HtrA homologs are present both in prokaryotes and eukaryotes, including humans. The model bacterial organism, *Escherichia coli*, has three homologs of HtrA: $HtrA_{Ec}$ (DegP), $DegQ_{Ec}$ (HhoA) and $DegS_{Ec}$ (HhoB). However, numerous bacterial species encode only one HtrA protein, for example *Helicobacter pylori*. In the bacterial cell, HtrAs are associated with the envelope and there they are involved in the protein quality control, mainly by removal of improperly folded proteins. This function is especially important under stressful conditions, such as heat shock, oxidative or osmotic stresses, that affect protein folding and lead to their denaturation. HtrAs are also implicated in maturation and folding of several extracytoplasmic proteins. Known substrates include outer surface proteins (A and B), RseA, neutrophil activating protein (NapA), flagellar basal body- associated protein (FliL) and other [1].

Interestingly, it has been recently demonstrated that several bacterial species secrete a fraction of HtrA out of the cell. Secreted HtrA was shown to play important roles in the pathogenesis of bacterial infections by facilitating dissemination of bacteria. A crucial step of this process is the disruption of the intercellular junctions and/or damage of the extracellular matrix (ECM). Some HtrAs are able to degrade components of adherent junctions (E-cadherin) and tight junctions (claudin-8 and occludin), as well as ECM proteins (fibronectin and aggrecan)[1,3].

HtrAs are generally homooligomeric proteins, whose functional building unit is a trimer. Trimers can assemble into higher oligomeric forms and in the case of several HtrA homologs oligomers of various sizes can be found in solution. Transitions between oligomeric forms are frequently associated with change of the enzyme activity, are substrate dependent, and are regarded as a form of the activity regulation. The typical HtrA monomer is composed of a chymotrypsin type protease domain and at least one PDZ domain (Post synaptic density protein 95, *Drosophila* disc large tumor suppressor and Zonula occludens- 1 protein domain) at the C-terminus. The N-terminal part is the least conserved part of the protein and it may play various functions [1,3].

It has been shown, that several HtrA homologs exhibit dual independent proteolytic and chaperone-like activities. As a chaperone, HtrA can be involved in holding the improperly folded polypeptides and consequently preventing their aggregation. In some cases, the chaperone activity is essential for proper folding and export of various virulence factors. The proteolytic activity is necessary for virulence of *Bordetella pertussis*, *Borrelia burgdorferi*, *Chlamydia sp.*, *Salmonella enterica* serovar Typhimurium, and pathogenic strains of *E. coli;* in many cases the lack of HtrA function causes loss of bacteria's ability to infect.

Biochemical characterization of several HtrA homologs revealed that generally these proteases require activation to cleave substrates. In the case of the model $HtrA_{Ec}$, in the resting state the enzyme is characterized by such architecture of the active site that is improper for catalysis. As a consequence, several rearrangements in the molecule are necessary for activation. The process accompanied with the conversion of the resting state hexamer to active dodecamer or 24-mer. There are two ways of activation, thermal and allosteric; the latter one requires binding of an appropriate substrate or peptide. The proteins return to an inactive state when the substrate cleavage is completed and consequently large oligomers disassemble [1,3].

This PhD thesis presents the results of research carried on the HtrA homologs derived from two pathogenic bacterial species, *Helicobacter pylori* and *Stenotrophomonas maltophilia* ($HtrA_{Sm}$ and $HtrA_{Hp}$, respectively). The biochemical properties of these proteins were compared to a model bacterial HtrA from the bacterium *Escherichia coli* ($HtrA_{Ec}$). Bacteria *H. pylori, S. maltophilia* and *E. coli* share several similarities, as they are all Gram negative bacteria capable of colonizing humans. However, the target locations in the human body and ecology of each species are different. *H. pylori* is a specialized human pathogen that resides in the stomach and duodenum; living bacterial cells were not detected in environmental conditions so far. *S. maltophilia* most commonly infects the respiratory tract, however it is generally an environmental species found in water or soil[1]. *E. coli* locates to the intestinal tract (or urinary tract in the case of UPEC) and this species includes both commensal and pathogenic strains. These bacteria can also survive for a prolonged time in water (sewage, rivers, lakes) and food.[2]

To perform detailed biochemical characterization of the HtrA proteins, substantial amounts of high purity preparations were necessary. For this purpose several expression

plasmids were constructed. The pET26b vector was used to obtain the *htrA* gene fusions. The resultant HtrA protein variants contained the N-terminal *E. coli* PelB signal sequence (for the periplasmic export of the resultant protein) and the sequence encoding the C-terminal His tag (to facilitate protein purification). The plasmids allowed recombinant expression of the following HtrA homologs: HtrA$_{Hp}$ derived from three *H. pylori* strains (26695, J99 and N6) and HtrA$_{Sm}$ form the *S. maltophilia* K279a strain. HtrA$_{Ec}$ was expressed from the previously constructed plasmid, deposited in the department's collection. To obtain the proteolytically inactive variants, we exchanged the codons of the active site serines (S229 for *S. maltophilia* and S221 for *H. pylori*) for alanine (S/A substitution) in the *htrA* genes using site-directed mutagenesis. The proteins were purified by the nickel-affinity chromatography under native or denaturing conditions. The latter method was used to obtain protein preparations devoid of co-purifying peptides [2,3].

HtrAs, as the quality control proteases, generally select unfolded proteins as substrates. To verify if HtrA$_{Hp}$ and HtrA$_{Sm}$ show similar preferences, proteolytic activities of these proteins were tested using β-casein and two forms of lysozyme: native and chemically denatured by reduction. β-casein is naturally unstructured and is known as a universal protease substrate, including HtrA. Lysozyme contains four S-S bridges which are necessary for the enzyme native conformation. HtrA$_{Ec}$ was used as a control protease. All tested HtrA homologs showed proteolytic activity towards β-casein and denatured lysozyme, but native lysozyme was not degraded. It means that HtrA$_{Hp}$ and HtrA$_{Sm}$ are capable of degradation of unfolded polypeptides, but not properly folded proteins [2,3].

Next, the pH preference of the HtrA homologs was determined using β- casein as a substrate. HtrA$_{Hp}$ had the highest activity in the pH range of 5.5 - 7.0 (maximum at 6.0- 6.5), HtrA$_{Sm}$ was active in a narrow pH range of 6.0- 6.5. In comparison, HtrA$_{Ec}$ works efficiently in the pH range of 5.5 - 6.5 (maximum at pH = 5.5). Then, the temperature preference of the proteases was determined. At temperature 37 °C (physiological for the human body) the apparent cleavage rates of all HtrAs were comparable, but at higher or lower temperatures significant differences were observed. First of all, maximal activities of the tested HtrAs were found at different temperatures: 35-37 ^{o}C (HtrA$_{Sm}$), 70-75 ^{o}C (HtrA$_{Hp}$) or 55 ^{o}C (HtrA$_{Ec}$). The efficiency of substrate cleavage by HtrA$_{Sm}$ dropped dramatically at temperatures above 37 ^{o}C; however, it is important to note that at low temperature (25 ^{o}C) this enzyme showed the highest activity (as compared to HtrA$_{Hp}$ and HtrA$_{Ec}$) [2,3].

The exceptionally high activity of HtrA$_{Hp}$ at elevated temperatures suggest that this enzyme is characterized by very high stability of its molecule (both pH and temperature resistant). On the other hand, a drop of activity of HtrA$_{Hp}$ at elevated temperatures indicates a low stability of this protein. To verify this hypothesis, the melting temperature (T_m) of each HtrA was calculated by means of circular dichroism. At the optimal pH value (pH 6.5) T_m of HtrA$_{Hp}$ was 87,5 °C; the remaining HtrA homologs lost their native secondary structures at lower temperatures: HtrA$_{Ec}$ (73,3 °C) and HtrA$_{Sm}$ (57,91 °C). These results explain the extremely high proteolytic activity of HtrA$_{Hp}$ at high temperatures and low proteolytic activity of HtrA$_{Sm}$ (maximum at 37 °C)[2,3].

The properties of the studied HtrA homologs seem to reflect adaptation to environmental conditions encountered in habitats typical for each bacterial species. Preference for moderate temperatures in the case of HtrA$_{Sm}$ suggests that the function of this protease is important for bacteria residing in waters and soil, as well as colonizing the human body. High resistance to denaturing conditions observed for HtrA$_{Hp}$ correlates well with the need to act both in the bacterial cell and in the harsh stomach environment, as a secreted fraction.

To expand characteristics of bacterial HtrAs, their cleavage site specificity was determined. According to literature, HtrA$_{Hp}$ preferentially cleaves its natural substrate, E-cadherin, at the consensus motif [VITA]↓[VITA]-x-x-D-[DN][3]. Substrate specificity of HtrA$_{Sm}$ was not determined previously. To compare specificities of all HtrAs studied in this work we used β-casein and reduced lysozyme as model substrates. The resulting cleavage products were identified using mass spectrometry technique (LC- MS). This method enabled us to determine the preferences of the enzymes towards amino acids flanking the cleaved peptide bonds. HtrA$_{Hp}$ and HtrA$_{Ec}$ showed very similar specificity toward amino acids located directly before and after the cleaved peptide bond. Both proteases cut preferentially after nonpolar amino acids at the P1 position, such as valine, or leucine and isoleucine. Interestingly, despite the aforementioned similarities, the enzymes produced different peptides and location of their cleavage sites within substrates was different. It indicates the possibility that HtrA$_{Hp}$ and HtrA$_{Ec}$ have different specificities at the non-prime sites in the substrate proteins. In the case of HtrA$_{Sm}$ the ratio of polar and non-polar amino acids at the P1 position was similar; the enzyme preferred valine, serine and leucine at P1, but not isoleucine. At the P1`position (after cleaved peptide bond), HtrA$_{Sm}$ showed an increased preference for charged amino acids, compared to HtrA$_{Hp}$ and HtrA$_{Ec}$. This different substrate specificity is correlated with a single amino acid substitution within the substrate S1 specificity pocket, which is responsible for

selective binding of a side chain of an amino acid at P1. For HtrA$_{Hp}$ and HtrA$_{Ec}$ this region was conserved and the S1 specificity pockets for HtrA$_{Ec}$/HtrA$_{Hp}$ contain the following amino acids: I205/I216, A227/238 and I228/I239 [2,3].

In the case of HtrA$_{Sm}$, the chaperone-like activity of this protein was also characterized and compared to this of HtrA$_{Ec}$. HtrA$_{Ec}$ is known to prevent aggregation of the chemically denatured lysozyme molecules. HtrA$_{Sm}$ showed similar properties and efficiently decreased amount of aggregates formed, as judged by the light scattering assay and measurement of amounts of precipitated lysozyme particles [2].

In the next step, the quaternary structure of HtrAs was examined. For this purpose, two methods were used: size exclusion chromatography (SEC) and analytical ultracentrifugation. Using the first method, the elution profiles of HtrA from *H. pylori* (26695 strain), *S. malthophilia* and *E. coli* were compared. Well studied HtrA$_{Ec}$ is eluted in one peak at the position corresponding to the hexamer. HtrA$_{Hp}$ and HtrA$_{Sm}$ behaved in this experiment differently. The proteolytically inactive variant of HtrA$_{Sm}$, HtrA$_{Sm}$S229A, was eluted in three fractions (possibly trimers, hexamers and higher order oligomers of undetermined by SEC size). However, when the active variant of HtrA$_{Sm}$ was used, only two clearly defined peaks were observed (probably trimers and hexamers with a dominance of the smaller forms). HtrA$_{Hp,}$ was eluted as a mixture of trimers and hexamers with a strong dominance of trimers.

The SEC experiments were verified by more accurate technique, sedimentation velocity centrifugation. This experiment was performed by Dr Anna Modrak-Wójcik at the University of Warsaw (Division of Biophysics). In this case we analyzed two inactive HtrA$_{Hp}$ variants (derived from the 26695 and N6 strains) as well as active and inactive variants of HtrA$_{Sm}$. The experiments confirmed that both HtrA homologs, HtrA$_{Hp}$ and HtrA$_{Sm}$, were present as mixtures of oligomers of varying size. Proteolytically active HtrA$_{Sm}$ migrated in two dominant peaks, corresponding to trimers and hexamers. The proteolytically inactive variant of HtrA$_{Sm}$ formed trimers and hexamers, but larger particles were detected as well. These discrepancies result most probably from the presence of peptide ligands which are very difficult to remove from a preparation of the proteolytically inactive HtrA variants [2,3].

In the case of HtrA$_{Hp}$, trimers were a dominant, but other oligomeric forms were present as well. Interestingly, the content of individual oligomeric forms in the two analyzed variants of HtrA$_{Hp}$ differed significantly. For HtrA$_{Hp}$ form the 26695 strain we observed three major peaks corresponding to trimers, hexamers, and nonamers, with a dominance of trimers. For

HtrA$_{Hp}$ from the N6 strain we obtained different results. Two major peaks were observed, corresponding to trimers and 18-mers. Strain-dependent differences in HtrA$_{Hp}$ oligomer abundance were also observed in casein zymograms. A high content of oligomeric forms was detected in HtrA$_{Hp}$ from strains N6 and J99, whereas HtrA$_{Hp}$ from strain 26695 migrated predominantly as monomers in the gel. This observation implies that the HtrA$_{Hp}$ oligomers are remarkably stable as they are able to withstand the denaturing conditions of gel electrophoresis. Comparison of amino acid sequences of the HtrA$_{Hp}$ variants from the 26695, J99 and N6 strains revealed that the proteins are 99% identical and differ only by five amino acids. Possibly, these substitutions are responsible for the observed differences in the stability of the HtrA$_{Hp}$ oligomers [2,3].

In the case of several well characterized HtrA homologs, the presence of substrates leads to rearrangements of oligomers and formation of cage-like large assemblies. This phenomenon can be easily observed using SEC. For example, the elution profile of HtrA$_{Ec}$ co-incubated with β-casein shows additional fractions which correspond to 24-mers. In our experiments, the presence of substrates also resulted in the formation of higher order oligomers in the case of all studied proteolytically inactive HtrA S/A variants. In preparations of active HtrA$_{Sm}$ and HtrA$_{Hp}$ large assemblies (with or without substrates) were not observed. Most probably, higher order oligomers of HtrA$_{Sm}$ disassemble after degradation of substrate molecules, similarly to HtrA$_{Ec}$. Comparison of the theoretical model of HtrA$_{Sm}$ with the known structure of HtrA$_{Ec}$ supported assumption that interactions between HtrA$_{Sm}$ trimers are labile. In the presence of a substrate, HtrA$_{Hp}$ (S/A) forms stable oligomers similar to HtrA$_{Ec}$, and this analysis suggested, that substrate- dependent structural rearrangements are similar for these two enzymes. This result was independent of the tested temperature and pH [2,3].

Determination of the physiological function of the HtrA$_{Hp}$ protein in the bacterial cell has so far been significantly hampered due to the lack of strains lacking the functional *htrA*$_{Hp}$ gene. Previous attempts to inactivate the *htrA*$_{Hp}$ gene were unsuccessful, despite the usage of more than 100 *H. pylori* strains of various origin. In collaboration with Dr hab. Anna Zawilak- Pawlik from the Polish Science Academy in Wroclaw one more attempt to knock out the *htrA*$_{Hp}$ gene was made. In the case of a single *H. pylori* strain, N6, our efforts have been successful and the N6Δ*htrA*$_{Hp}$ strain were constructed. For control, the complementing strain with the re-introduced functional *htrA*$_{Hp}$ gene was obtained (N6Δ*htrA*/*htrA*$_{N6}$) [unpublished data]. Thanks to the internship in the laboratory of Prof. Dr. Steffen Backert from University of Erlangen- Nuremberg, a detailed characterization of the *htrA* mutation

phenotypes was possible. When bacteria were grown under standard conditions for *H. pylori*, no visible growth defects of the *htrA* mutant strains were observed. Then, effects of various stress conditions were tested, including heat shock, oxidative, osmotic and pH stresses and treatment with puromycin. The knock-out strain was generally more sensitive to the majority of the tested stressful conditions when compared to the wild type or complementing N6 strains. The only exception was oxidative stress induced by H_2O_2 or cumene hydroperoxide; in this case all tested strains were affected by the oxidants equally. All stressors used in our experiments are known to affect the structure of proteins, leading to their denaturation and eventually aggregation. Therefore, it can be assumed that $HtrA_{Hp}$, similarly to the other bacterial HtrA homologs, is an important player in the protein quality control system of *H. pylori* [3].

In summary, the greatest achievements of my work include:

(1) Detailed characterization and comparison of the proteolytic activities of two HtrA homologs from pathogenic bacteria *H. pylori and S. maltophilia*.

(2) Characterization of the chaperone activity of $HtrA_{Sm}$.

(3) Determination of quaternary structures of $HtrA_{Hp}$ and $HtrA_{Sm}$.

(4) Determination of the effects of the $htrA_{Hp}$ gene deletion on the growth and survival of *H. pylori* under stressful conditions *in vitro.*

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