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Ph.D. Thesis title: **„Analysis of the inhibitory potential of the coccobacilli *Yersinia enterocolitica* on cysteine proteases of the papain family"**

## ABSTRACT

Cysteine proteases of the papain family are widespread in nature and play important physiological roles in many organisms. Animal cysteine proteases, named cathepsins, localize mostly to the lysosomes and are involved in cellular processes, such as: protein turnover, proenzyme activation, prohormone maturation, bone remodeling, apoptosis and immune responses (phagocytosis and antigen presentation). However, upregulation of their activity may contribute to the development of different pathologies, including: osteoarthritis, osteoporosis, atherosclerosis, muscular dystrophy and many types of cancer. Therefore, cysteine cathepsin inhibitors are considered as promising drugs to be used for the effective treatment of aforementioned diseases. Such inhibitors are usually synthesized in laboratories or isolated from various organisms. Saprophytic and parasitic microbes may produce the inhibitors of the host's cysteine cathepsins playing pivotal roles in both innate and adaptive immune responses, thus leading to the limitation of such responses and survival of microbes within their host.

A Gram-negative coccobacillus of the species *Yersinia enterocolitica* is an intracellular pathogen of animals and humans. It has developed several mechanisms to evade phagocytosis by the host's innate immune cells. So far, however, the bacterium has not been shown to produce any cysteine protease inhibitors. Due to the prospective importance of such inhibitors in bacterial pathophysiology, as well as their possible applications in medicine, the major objective of this work was to analyze the inhibitory potential of *Y. enterocolitica* on cysteine proteases of the papain family.

As shown by a fluorimetric assay, the environmental *Y. enterocolitica* strains, which had previously been isolated from aborting sows and aborted fetuses, and belonged to different bioserotypes and genotypes, produced papain and human cathepsin L inhibitors, but not bovine cathepsin B inhibitors. Their inhibitory properties were discovered in the bacterial crude extracts and on the cellular surface. The strains also secreted papain inhibitors into the culture medium. They synthesized peptidase inhibitors during both exponential and stationary growth phases, and at both 28 °C (the optimum growth temperature) and 37 °C (the human

body temperature). Culturing the strains under nutrient limitation (in a minimal medium) resulted in the increased secretion of papain inhibitors.

Biochemical and molecular characterization of the inhibitory potential of *Y. enterocolitica* was then undertaken by employing fluorimetry and zymography. The analysis of the cell surface inhibitors of papain and cathepsin L revealed that they were peripherally bound to the outer membrane via hydrophobic and/or electrostatic interactions. The peptidase inhibitors present in the bacterial extracts were shown to be thermolabile (sensitive to temperatures higher than 40 °C) and stabile in a range of pH values from 5 to 9 (over 90% stability). They interacted reversibly with their target enzymes and proved to be high molecular weight factors; four proteinaceous papain inhibitors with molecular masses in a range from less than 50 kDa to over 120 kDa were identified by reverse zymography.

Subsequently, a comparison of the inhibitory effects of *Y. enterocolitica* and other Gram-negative bacteria on cysteine proteases was performed. The crude extracts of the clinical *Escherichia coli* H64 and reference *Pseudomonas aeruginosa* PAO1 strains inhibited papain, cathepsin L and cathepsin B, the latter feature not being observed for the extracts of *Y. enterocolitica* strains. The bacterial cell surfaces restrained papain and cathepsin L activity similarly to *Y. enterocolitica* cells. Both strains also secreted papain inhibitors into the culture medium (*P. aeruginosa* additionally secreted cathepsin L inhibitors).

Finally, a protein with molecular mass of about 18 kDa was isolated from the extracts of *Y. enterocolitica* and *E. coli* by batch affinity chromatography on papain-agarose resin. The protein interacted strongly and specifically with immobilized papain. It was identified by LC--MS/MS as the periplasmic chaperone Skp, which assists in proper folding of many virulent outer membrane proteins in bacteria of the family *Enterobacteriaceae*. The chromatographic eluates containing Skp, but not the ones without this protein, inhibited papain and cathepsin L. Additionally, the eluate obtained from the extract of *Y. enterocolitica*, containing Skp and stripped of low molecular weight compounds by dialysis, inhibited cysteine cathepsins produced by normal human dermal fibroblasts (NHDF). Therefore, it was suggested that the chaperone Skp is a putative inhibitor of cysteine proteases, hence pointing to its possible new biological role. However, further research is needed in order to unequivocally confirm this assumption.

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