Microbiological Research 207 (2018) 211–225

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Revealing the inhibitory potential of *Yersinia enterocolitica* on cysteine proteases of the papain family

Mateusz Kędzior[a,*], Aleksandra Pawlak[b], Rafał Seredyński[a,c], Jacek Bania[d], Aleksandra Platt-Samoraj[e], Magdalena Czemplik[a], Elżbieta Klausa[f], Gabriela Bugla-Płoskońska[b], Jan Gutowicz[a]

[a] Department of Physical Chemistry of Microorganisms, Institute of Genetics and Microbiology, Faculty of Biological Sciences, University of Wrocław, Przybyszewskiego 63/77, 51-148 Wrocław, Poland
[b] Department of Microbiology, Institute of Genetics and Microbiology, Faculty of Biological Sciences, University of Wrocław, Przybyszewskiego 63/77, 51-148 Wrocław, Poland
[c] Department of Physiology, Wrocław Medical University, Chałubińskiego 10, 50-368 Wrocław, Poland
[d] Department of Food Hygiene and Consumer Health Protection, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Norwida 31, 50-375 Wrocław, Poland
[e] Department of Epizootiology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego 13, 10-718 Olsztyn, Poland
[f] Regional Centre of Transfusion Medicine and Blood Bank, Czerwonego Krzyża 5, 50-345 Wrocław, Poland

ARTICLE INFO

Keywords:
Cysteine protease
Papain
Cathepsin
Inhibitor
Yersinia enterocolitica
Chaperone

ABSTRACT

Cysteine proteases of the papain family, including mammalian cathepsins, play important physiological roles, however, their excessive activity may contribute to the development of various pathologies. Therefore, cysteine cathepsin inhibitors are being considered as promising drugs to treat cathepsin-driven diseases. Diverse saprophytic and parasitic microbes produce such inhibitors, which target the host's proteases playing pivotal roles in immune responses, thus leading to the survival of microbes within their host. *Yersinia enterocolitica* is a Gram-negative zoopathogenic coccobacillus, which has developed several mechanisms to evade the host's immune system. Nevertheless, the bacterium has not yet been shown to produce any cysteine protease inhibitors. Here we demonstrate that *Y. enterocolitica* strains of different bioserotypes and genotypes synthesize papain and human cathepsin L inhibitors, but not bovine cathepsin B inhibitors. By employing fluorimetry and zymography, the cell-surface inhibitors were shown to associate peripherally with the outer membrane, while the inhibitors present in cell-free extracts proved to: interact reversibly with their target enzymes, exhibit thermolability and stability in a range of pH values (5–9), and have high molecular weights. Batch affinity chromatography on papain–agarose resin was then undertaken to isolate putative inhibitors of cysteine proteases from the bacterial extract. The isolated 18 kDa protein was identified by LC–MS/MS as the periplasmic chaperone Skp. The Skp-containing eluate inhibited the activity of cysteine cathepsins produced by human dermal fibroblasts. The homologous Skp protein was also isolated from the extract of *Escherichia coli*. Our results point to a possible new biological role of the bacterial chaperone Skp.

1. Introduction

Cysteine proteases are one of the major types of proteolytic enzymes. Their hallmark is the nucleophilic thiol group of the catalytic cysteine residue, which enables the hydrolysis of peptide bonds in various proteins (Brömme, 2001). The most abundant cysteine proteases share a structural fold with papain, a plant endopeptidase being accumulated in the latex of *Carica papaya* fruits. Therefore, they are grouped into the clan CA and named papain-like proteases, which comprise 38 families, with the most numerous C1 papain family

Abbreviations: α-MEM, (alpha-modified Eagle's minimum essential medium); Ail, (attachment-invasion locus); BPW, (buffered peptone water); E-64, (*L-trans*-epoxysuccinyl-*L*-leucylamido(4-guanidino)butane); LC–MS/MS, (liquid chromatography coupled to tandem mass spectrometry); LDH, (lactate dehydrogenase); M9, (minimal medium); MWCO, (the molecular weight cut-off); NHDF, (normal human dermal fibroblasts); NHS, (normal human serum); PFGE, (pulsed-field gel electrophoresis); RFU, (relative fluorescence units); RT, (room temperature); SEM, (standard error of the mean); T3SS, (type III secretion system); Yop, (*Yersinia* outer protein); Yst, (*Yersinia* stable toxin); Z-Phe-Arg-AMC, (benzyloxycarbonyl-*L*-phenylalanyl-*L*-arginine-7-amido-4-methylcoumarin hydrochloride)

* Corresponding author.
E-mail address: mateusz.kedzior@uwr.edu.pl (M. Kędzior).

https://doi.org/10.1016/j.micres.2017.12.005
Received 10 July 2017; Received in revised form 29 November 2017; Accepted 4 December 2017
Available online 08 December 2017
0944-5013/

(Brömme, 2001; Rawlings et al., 2012). Members of this family are widespread in nature, being found in almost every group of living organisms, including bacteria, fungi, protists, plants and animals (Rawlings et al., 2012). Animal cysteine proteases of the papain family, named cathepsins, localize predominantly to the lysosome, where they are involved in a number of physiological processes, such as intracellular protein turnover, proenzyme activation, prohormone maturation, bone remodeling, apoptosis and immune responses (phagocytosis, antigen presentation) (Berdowska, 2004; Müller et al., 2014). However, when the activity of cysteine cathepsins is upregulated, they may contribute to the development of different pathologies, including rheumatoid arthritis, osteoarthritis, osteoporosis, atherosclerosis, muscular dystrophy and cancer (Berdowska, 2004; Brömme, 2001). Therefore, these enzymes are considered as new diagnostic markers and promising drug targets to treat the aforementioned diseases (Berdowska, 2004).

A number of selective cysteine cathepsin inhibitors, isolated from living organisms or synthesized in chemical laboratories, have been successfully used for the experimental treatment of many pathologies. Several of them have reached the stage of clinical trials, e.g., odanacatib (developed by Merck & Co., Inc.) as a potent and nontoxic inhibitor of cathepsin K to treat osteoporosis and bone metastasis (Gauthier et al., 2008). Different small-molecule inhibitors of cysteine cathepsins, isolated from cellular extracts or conditioned culture media of various microorganisms, have been applied in science, medical research and biotechnology. For instance, leupeptin and E-64, secreted by soil actinomycetes and *Aspergillus japonicus*, respectively, are common ingredients of commercially available protease inhibitor cocktails. These compounds and their derivatives have also been employed in the investigation of cysteine cathepsin biological functions, as well as in several therapeutic approaches. Additionally, saprophytic and pathogenic microbes may synthesize proteinaceous inhibitors of cysteine proteases in order to facilitate their adaptation to the host environment. Indeed, such inhibitors may regulate the activity of microbial endogenous proteases involved in host invasion, restrain the activity of host proteases produced as defense factors against infections or suppress both innate and adaptive immune responses of the host by inactivating lysosomal cathepsins (Kędzior et al., 2016).

Given the importance of cysteine cathepsin inhibitors as putative therapeutic molecules and microbial virulence factors, we chose to investigate whether *Yersinia enterocolitica* may be a potent producer of such inhibitors. *Y. enterocolitica* is the most heterogeneous species within the genus *Yersinia*. It comprises Gram-negative coccobacilli, which cause yersiniosis – the third most common zoonotic bacterial disease of gastrointestinal tract in the European Union and other countries in temperate climate zones (Rahman et al., 2011). Swine are the primary reservoir of both presumably nonpathogenic (biotype 1A) and pathogenic (biotypes 1B, 2–5) *Y. enterocolitica* strains, which can be transmitted to humans via contaminated food, such as raw or undercooked pork, raw or pasteurized milk, liquid egg products and vegetables (Bottone, 1997; Rahman et al., 2011). The bacterium may then colonize the distal small intestine and proximal colon, where it penetrates the epithelium and, subsequently, is internalized by phagocytes and transported within them to the mesenteric lymph nodes, liver and spleen (Bottone, 1997; Fàbrega and Vila, 2012). The virulence factors of *Y. enterocolitica*, which are encoded by the genes located on the bacterial chromosome and the 70 kb virulence plasmid pYV, include: Ail (attachment-invasion locus) enabling adhesion to and invasion of mammalian cells, as well as conferring resistance to the bactericidal activity of serum; Yst (*Yersinia* stable toxin) damaging the intestinal mucus layer; Yops (*Yersinia* outer proteins) limiting immune and inflammatory responses (Fàbrega and Vila, 2012; Skorek et al., 2013).

There are several reasons for selecting *Y. enterocolitica* to analyze its inhibitory potential on cysteine proteases. Firstly, the bacterium secretes virulent cysteine proteases – the effector proteins YopP and YopT (the latter being homologous to papain) (Fàbrega and Vila, 2012; Rawlings et al., 2012), but it has not been investigated whether the activity of these enzymes is controlled intracellularly by any endogenous inhibitors as is the case for cysteine proteases synthesized by other pathogenic bacteria (Kędzior et al., 2016). Secondly, diverse bacterial species secrete the homologues of α_2-macroglobulin, a homotetrameric glycoprotein capable of inhibiting proteases of any catalytic type. These homologues prevent the degradation of bacterial cells by the host's proteolytic defense system (Wong and Dessen, 2014). The homologue of α_2-macroglobulin is also encoded in the genome of *Y. enterocolitica* (Rawlings et al., 2012), but it remains unclear whether this protein is produced by the bacterium as a functional protease inhibitor. Finally, *Y. enterocolitica* may replicate effectively within phagocytes (Mills et al., 1997) and induce the apoptosis of antigen-presenting cells (Erfurth et al., 2004) by involving the secretory machinery named the type III secretion system (T3SS) (Fàbrega and Vila, 2012), thereby restraining innate and adaptive immune responses, respectively. Several pathogenic microbes impair these responses through the action of their cysteine cathepsin inhibitors (Kędzior et al., 2016), which have not yet been shown to be synthesized by *Y. enterocolitica*.

The inhibitory potential of *Y. enterocolitica* strains from pigs with reproductive disorders (Platt-Samoraj et al., 2006, 2009) was tested against papain family cysteine proteases in a few steps. Initially, we investigated the effect of *Y. enterocolitica* cell-free extracts, conditioned culture media and cellular surface on the activity of papain and cathepsins B and L. Then, the impact of different culture conditions on the activity of cysteine protease bacterial inhibitors was assessed. The inhibitory potential of the coccobacillus was also compared with the antiproteolytic activity of two other widespread and often pathogenic Gram-negative species, *Escherichia coli* and *Pseudomonas aeruginosa*, which were included in our study as the reference bacteria since their genomes have already been shown to encode the proteins affecting the activity of cysteine proteases (Garcia-Ferrer et al., 2015; Sanderson et al., 2003). Finally, the biochemical and molecular characterization of the inhibitors synthesized by *Y. enterocolitica* was performed and concluded with the identification of a proteinaceous putative inhibitor of papain and cathepsin L in both *Y. enterocolitica* and *E. coli*.

2. Material and methods

2.1. Enzymes and chemicals

Bovine spleen cathepsin B (12.5 U/mg protein), human liver cathepsin L ($\geq$ 0.5 U/mg protein), papaya latex papain (21 U/mg protein), porcine trypsin-EDTA solution, porcine skin gelatin (type A), benzyloxycarbonyl-*L*-phenylalanyl-*L*-arginine-7-amido-4-methylcoumarin hydrochloride (Z-Phe-Arg-AMC), *L-trans*-epoxysuccinyl-*L*-leucylamido (4-guanidino)butane (E-64), alpha-modified Eagle's minimum essential medium (α-MEM), fetal bovine serum and penicillin-streptomycin solution were purchased from Sigma-Aldrich, Inc. Immobilized papain (agarose resin), prestained protein molecular weight marker (20–120 kDa), Pierce™ BCA Protein Assay Kit and GelCode™ Blue Safe Protein Stain were acquired from Thermo Scientific, Inc. Buffered peptone water (BPW) was obtained from BD Diagnostic Systems, Inc. M9 minimal medium ingredients: disodium phosphate, monopotassium phosphate, sodium chloride, ammonium chloride, calcium chloride, magnesium sulfate and glucose were purchased from POCh S.A. (Gliwice, Poland). These and all other reagents were of analytical grade.

2.2. Bacterial and human cell cultures

Y. enterocolitica strains, analyzed in the present study, had been previously isolated from aborting sows and aborted fetuses in the industrial pig farms located in north-eastern Poland, and subjected to biochemical typing, serotyping and genetic marker identification (Platt-Samoraj et al., 2006, 2009). We performed further genomic characterization of the strains by pulsed-field gel electrophoresis (PFGE), in