VILNIUS UNIVERSITY

ANDRIUS JASILIONIS

The collagenolytic activity analysis of *Geobacillus thermoleovorans* DSM 15325 peptidases: characterization of M3 proteolytic enzymes family M3B subfamily oligopeptidase and U32 proteolytic enzymes family peptidase

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VILNIAUS UNIVERSITETAS

ANDRIUS JASILIONIS

Peptidazių iš *Geobacillus thermoleovorans* DSM 15325 kolagenolizinio aktyvumo analizė: M3 proteolizinių fermentų šeimos M3B pošeimio oligopeptidazės ir U32 proteolizinių fermentų šeimos peptidazės charakterizavimas

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Introduction

The importance of the proteolytic enzymes – peptide bonding hydrolases for the organisms is multifactorial. Peptidases, acting as catalysts, ensure house-keeping functions, protein degradation and uptake. The genes encoding peptidases constitute 2-5 % of all genes in the organisms' genomes (1).

Organisms' evolution determined the specialization of proteolytic enzymes and the formation of distinctive combinations of characteristics optimal for peptidases functionality, therefore the essential characteristics of peptidases structures, catalytic and regulation mechanisms is heterogeneous (2). Proteolytic enzymes according to catalytic mechanism are classified as aspartic acid, cysteine, glutamic acid, metallo, serine, threonine and mixed mechanism peptidases as well as the asparagine lyases, whereas the peptidases of uncharacterized catalytic mechanism are formally attributed to unassigned type of peptidases (3, 4). Serine and metallo-peptidases predominate in the genomes of organisms (5). The genes of peptidases with uncharacterized catalytic mechanism constitute less than one percent of all the genes of putative peptidases (3).

Proteolytic enzymes are in focus of active research studies, nonetheless the majority of the peptidases' diversity remains undescribed (6, 3). The characterization of conservative and wide-spread putative peptidases that are also homologous to the peptidases with uncharacterized catalytic mechanism remains fragmentary (6). Functions of the majority of peptidases, which are comprehensively characterized *in vitro*, in most cases remains uncharacterized *in vivo* (2).

The diversity of collagenolytic peptidases that are encoded by non-pathogenic bacteria is only fragmentary characterized, whereas the characterization of the diversity and the functional significance of collagenolytic peptidases of pathogenic bacteria are far more comprehensive (7, 8). The analysis of thermophilic proteolytic bacteria collagenolytic potential, which is typically identified when evaluating the bacterial ability to grow by uptaking proteins for nutrition, has never been performed purposefully (9). While the main biological significance of collagen degradation for prokaryotic pathogens is closely related with the virulence, the thermophilic bacteria typically use collagenolysis when uptaking collagen for nutrition. To date, the view that the collagen catabolism by thermophilic bacteria is a result of a cascade of synergistically active

inducible peptidases is predominating. Usually this model of collagen degradation disregards the collagenolytic peptidases constitutively produced by thermophilic bacteria. The overall fundamental understanding of collagenolysis process and collagenolysis biological functions for prokaryotes is reduced due to the facts that the constitutive collagenolytic activity of thermophilic bacteria was never purposefully analyzed, and the understanding of constitutive peptidases importance for collagen hydrolysis *in vivo* is obscure.

Aim of dissertation

To assess the importance of collagenolytic activity of *G. thermoleovorans* DSM 15325 constitutively produced peptidases for the collagen uptake for nutrition and characterize M3 proteolytic enzymes family M3B subfamily oligopeptidase and U32 proteolytic enzymes family peptidase.

Goals of dissertation

1. To assess the ability of *G. thermoleovorans* DSM 15325 to produce the collagenolytic peptidases constitutively and to identify the detected enzymes.
2. To characterize *G. thermoleovorans* DSM 15325 secreted M3 proteolytic enzymes family M3B subfamily oligopeptidase.
3. To assess the expression of long chain U32 proteolytic enzymes family peptidase in *G. thermoleovorans* DSM 15325 and characterize the enzyme.
4. To determine and characterize three-dimensional structure of *G. thermoleovorans* DSM 15325 long chain U32 proteolytic enzymes family peptidase.

Defensive thesis

1. *G. thermoleovorans* DSM 15325 ability to produce the collagenolytic peptidases into the extracellular environment reflects the adaptive plasticity of proteolytic geobacilli.
2. GT-SM3B is a thermostable zinc oligoendopeptidase with broad substrate specificity.
3. The determined characteristics of GT-IU32 demonstrate the structural and functional heterogeneity of U32 proteolytic enzymes family.
4. GT-IU32 *in vitro* specifically interacts with dsRNA.

Scientific novelty

Predicted ability of thermophilic bacteria to produce collagenolytic peptidases constitutively was confirmed for the first time after the detection of *G. thermoleovorans* DSM 15325 constitutively produced collagenolytic peptidases. Characterization of activity synergism of *G. thermoleovorans* DSM 15325 constitutively produced collagenolytic peptidases is the first time when the action of constitutive collagenolytic peptidase catabolic cascade was demonstrated for thermophilic bacteria. The determined cascade formed as a result of cooperative action manner of detected peptidases activity ensures the permanent collagenolytic potential of *G. thermoleovorans* DSM 15325. The ability to utilize collagen for nutrition without delay is beneficial for bacteria expanding *G. thermoleovorans* DSM 15325 adaptation plasticity and, as a consequence, increasing the efficiency of the bacteria survival strategy.

GT-SM3B from *G. thermoleovorans* DSM 15325 is the first secreted metallo-oligopeptidase from thermophilic bacteria characterized and the first metallo-oligopeptidase heterologously produced applying secretory production. The determined set of GT-SM3B characteristics is exceptional for a metallo-oligopeptidase as the combination of GT-SM3B characteristics unites the qualities of metallo-peptidases earlier separately attributed to the thermoactive metallo-oligopeptidases or to the peptidases of this catalytic type from mesophiles. GT-SM3B can be a powerful biocatalyst of collagen-derived oligopeptides with the biotechnological potential.

GT-IU32 from *G. thermoleovorans* DSM 15325 is the first U32 proteolytic enzyme family peptidase from thermophilic bacteria characterized and the first bacterial peptidase that has its specific interaction with dsRNA determined. The predicted modular domain organization of U32 proteolytic enzymes family peptidases is confirmed for the first time after the characterization of GT-IU32 domain organization. Isolated GT-IU32-CGD, being the first domain of U32 proteolytic enzymes family peptidases with solved structure, remains the only domain of U32 proteolytic enzymes family peptidases with characterized structure that does not have its functions directly associated with catalytic activity.

Scientific significance

The characterization of the constitutive collagenolytic peptidases significance for ensuring the bacterial collagenolytic potential enables to comprehend the overall collagenolytic potential‘s functional significance and the course of collagen degradation by bacteria. The characterization of GT-SM3B led to the determination of oligopeptidases biotechnological application potential. The results of GT-SM3B and GT-IU32 characterization are significant for the thorough comprehension of the characteristics and functional importance of M3 and U32 proteolytic enzymes family peptidases. The results of *G. thermoleovorans* DSM 15325 collagenolytic potential and collagenolytic peptidases characterization are relevant for the perspective development of the researches purposefully focused on the characterization of proteolytic enzymes from thermophilic bacteria.

Methods

Bioinformatic sequence analysis was performed using SignalP 4, BLAST+ 2, MEGA 6, LASERGENE 6, ClustalX 2, I-TASSER 4, NCBI CDD 3, PROSITE and Pfam 28. Softberry BPROM was used for promoter identification, while terminators were predicted using Softberry FindTerm and ARNold. Sequences genomic and operonic organization was predicted using Softberry FGENESB, MicrobesOnline and Prokaryotic Operon Database.

G. thermoleovorans DSM 15325 was cultivated in mM9 media using Erlenmeyer flask aerating at 180 rpm. The culture OD was measured at 600 nm. Precipitation with $(NH_4)_2SO_4$ was used for *G. thermoleovorans* DSM 15325 extracellular protein extraction, while geobacilli intracellular protein were extracted applying sonication for cell disruption.

Catalytic activity of peptidases was detected using zymography, azocoll or collagenous oligopeptides hydrolysis assays. Mass spectrometry was performed at the Institute of Biochemistry and Biophysics Mass Spectrometry Laboratory, Warsaw, Poland and Vilnius University Institute of Biochemistry Proteomic Centre, Vilnius, Lithuania.

Gene sequences were amplified using PCR with constructed primer pairs. Protein expression vectors pET-28c(+) and pCri7 were used. Recombinant enzymes were heterologously overexpressed in *E. coli* BL21 (DE3) applying T7 promoter induction by IPTG. GT-SM3B was purified from expression strains culture fluid whereas GT-IU32 was purified from strains' cytoplasm. Enzymes were purified applying fractionation with $(NH_4)_2SO_4$ precipitation, nickel affinity or ion exchange chromatography techniques. GT-IU32 oligomerization was characterized in detail applying analytic gel filtration. GT-SM3B kinetic constants were determined using Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH as a substrate. GT-IU32 transcription was analysed using RT-PCR.

GT-IU32-CGD was crystallized using sitting-drop vapour-diffusion method at IBMB/IRB crystallization service, Molecular Biology Institute of Barcelona, Barcelona, Spain. Complete diffraction data sets were collected from crystals using a PILATUS 6M pixel detector on beamline ID29 of the European Synchrotron Radiation Facility Grenoble, France or using an ADSC Q315R CCD detector on beamline PROXIMA 1 of synchrotron SOLEIL, Paris, France.

GT-IU32 ability to specifically *in vitro* interact with nucleic acids were evaluated using ATP-$^{\gamma 32}$P labelled selected nucleic acids applying dot blot or electrophoretic mobility shift assays.

Results and discussion

Collagenolytic peptidases constitutively produced by *G. thermoleovorans* DSM 15325

G. thermoleovorans DSM 15325 demonstrates ability to grow by utilizing proteins from media. Actively secreted proteases and peptidases ensures *G. thermoleovorans* DSM 15325 proteolytic potential, however there were no direct results confirming that proteolytic geobacilli are capable to produce peptidases constitutively. *G. thermoleovorans* DSM 15325 ability to produce proteolytic enzymes were evaluated using selective medias supplemented with native proteins like casein or collagen. Application of media supplemented with proteins usually induces expression and secretion of inductive peptidases therefore constitutive proteolytic activity of *G. thermoleovorans* DSM 15325 remained unconfirmed.

Constitutive collagenolytic potential of *G. thermoleovorans* DSM 15325 was confirmed by detecting constitutive collagenolytic peptidases in secretome of geobacilli which were cultivated in mM9 media that cannot cause the induction of peptidases expression. The optimal values of pH along with temperature – pH 6.5 ir 60 °C (10) were used for *G. thermoleovorans* DSM 15325 large scale cultivation in mM9 media for intracellular as well as extracellular protein extraction. The culture inoculum was prepared under identical conditions using mM9 media as well. When accomplishing a purpose to identify the collagenolytic peptidases produced by *G. thermoleovorans* DSM 15325 constitutively by mass spectrometry, the protein fraction of geobacilli culture supernatant was extracted by precipitation using an 80 % saturation of $(NH_4)_2SO_4$ which was selected during optimization as an optimal saturation level for *G. thermoleovorans* DSM 15325 produced peptidases extraction. The intracellular proteins were also extracted with a goal to evaluate the constitutive expression of strains intracellular collagenolytic peptidases. The *G. thermoleovorans* DSM 15325 cultivation conditions ensured the extraction of geobacilli non-inductive collagenolytic peptidases at the late exponential phase. When performing the initial evaluation of *G. thermoleovorans* DSM 15325 collagenolytic activity of extracted peptidases 50 mM TRIS-HCl, pH 7.4/60 °C buffer supplemented with 20 mM $CaCl_2$ and 10 μM $ZnCl_2$ and 200 mM NaCl and 40 μM Brij 35 was used as an incubation buffer for gelatine zymography. *G. thermoleovorans* DSM 15325 constitutive collagenolytic peptidases demonstrating 130 (GT-KP130) and 120 (GT-KP120) kDa

molecular mass were detected (Fig. 1). The gelatine zymography parameters were optimized for *G. thermoleovorans* DSM 15325 collagenolytic peptidase detection. Optimization of zymography incubation buffer results states that 20 mM $CaCl_2$ is necessary for the ensurance of GT-KP120 and GT-KP130 activity. There was no influence of 10 μM $ZnCl_2$ for the gelatinolytic activity of latter collagenolytic peptidases, but when the zymogram gels were incubated in 50 mM TRIS-HCl pH 7.4/60 °C buffer supplemented only with 20 mM $CaCl_2$, a collagenolytic peptidase of approximately 100 kDa molecular mass (GT-KP100) was detected in the extract of geobacilli extracellular proteins (Fig. 1). The influence of 200 mM NaCl and 40 μM Brij 35 for the activity of GT-KP100, GT-KP120 and GT-KP130 constitutively produced by *G. thermoleovorans* DSM 15325 was not detected. The intracellular *G. thermoleovorans* DSM 15325 collagenolytic peptidases were not detected.

A collagenolytic peptidase GT-KP120 that was constitutively produced by *G. thermoleovorans* DSM 15325 and detected by using gelatine zymography demonstrated a gelatinolytic activity at 11-98 °C. The gelatinolytic activity of larger molecular mass GT-KP130 constitutively produced by geobacilli was determined at 37-98 °C.

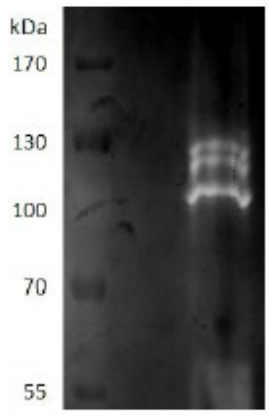

Fig. 1. Activity detection of extracted collagenolytic peptidases

Activity of GT-KP120 and GT-KP130 peptidases constitutively produced by *G. thermoleovorans* DSM 15325 was completely inactivated by EDTA and EGTA (in both cases 10 mM), whereas Pefabloc SC (5 mM), phenantroline (10 mM) and phosphoramidone (10 μM) had no influence on GT-KP120 and GT-KP130 gelatinolytic activity.

GT-KP100, which was completely inhibited by 10 μM Zn^{2+} cation with an application of 50 mM TRIS-HCl pH 7.4/60 °C buffer with 20 mM $CaCl_2$, demonstrated substrate specificity analogical to GT-KP120 and GT-KP130 at 19-80 °C by catalyzing peptidolysis of gelatin and partially denatured collagen type I from rat tail tendon, partially denatured casein and partially denatured albumin (the latter caseinolytic activity was not detected for GT-KP120 and GT-KP130), but did not catalyze peptidolysis of partially denatured elastin or keratin. GT-KP100 gelatinolytic activity was completely inactivated by EDTA and EGTA elastin or keratin. GT-KP100 gelatinolytic activity was completely inactivated by EDTA and EGTA elastin or keratin. GT-KP100 gelatinolytic

Table 1. *G. thermoleovorans* DSM 15325 peptidases identified with mass spectrometry.

Enzyme, sequence accesion	Molecular mass, M_r	Number of identified peptides	Sequences of identified peptides	Coverage, %	Score
Bacillolizine homologue GenBankAEV20496.1	59789	6	N^{66}TFQLGGQAR^{75} G^{266}SGIFTYDGR^{275} N^{413}PDWEIGEDIYTPGIAGDALR^{433} Y^{441}GDPDHYSK^{449} Y^{451}TGTQDNGGVHTNSGIINK^{469} A^{500}LVYYLTPTSNFSQLR^{515}	15	332
Pz-peptidase A homologue DDBJ BAD99433.1	66849	8	Y^{16}ERPDIAQLQASFQEALDSFR^{36} A^{38}GSAALQHEAMK^{49} Q^{119}LFALAETQLKT^{129} Y^{130}APAVVEDLQK^{141} T^{166}LAQLQPFVESPDR^{179} E^{199}LDELYDELVHVR^{211} L^{218}GFQNFVELGYAR^{230} S^{546}PFADGAVASVVGHIER^{562}	19	319
Pz-peptidase B homologue DDBJ BAD99434.1	70213	2	L^{448}YLLNHYLEGFR^{459} A^{563}GSSDYPIEVLK^{574}	3	82

activity was completely inactivated by EDTA and EGTA (in both cases for 1 mM), phenantroline (1mM) and phosphoramidone (10 μM), whereas Pefabloc SC (1 mM) had no influence on GT-KP100 ability to hydrolyze gelatine.

The collagenolytic activity of peptidases produced by *G. thermoleovorans* DSM 15325 into cultivation media were detected by using zymography of gelatin and collagen between approximately 100 and 130 kDa. Gelatin and acid solubilized collagen have influence on the electrophoretic mobility of proteins (11, 12), therefore the probable localization of collagenolytic peptidases between 100 and 130 kDa in SDS-PAGE gel was validated by measuring the intensity of azocoll hydrolysis with a purpose to confirm the molecular mass of collagenolytic peptidases identified by zymography. Collagenolytic peptidases were washed out from homogenized SDS-PAGE gel slices excised between mass standards of protein molecular mass marker. The intensity of azocoll hydrolysis assay was applied in order to measure a collagenolytic activity of prepared protein extracts from distinct molecular mass region. The localization of GT-KP100, GT-120 and GT-KP130 between 100 and 130 kDa in SDS-PAGE gel was confirmed as protein extract from 100-130 kDa slice demonstrated the maximum hydrolytic activity against azocoll.

The SDS-PAGE slice with *G. thermoleovorans* DSM 15325 constitutively produced collagenolytic peptidases was subjected for mass spectrometry in order

to identify collagenolytic peptidases. MALDI TOF/TOF-MS/MS ESI-TRAP of protein from gel sample led to the identification (p= 0.01) of 48 proteins (http://proteom.pl/lookatthis/index.php; sample 207192165kuis_h7). The *in silico* homology analysis of identified protein sequences enabled to determine that peptidases constitutively produced by *G. thermoleovorans* DSM 15325 are homologous (Table 1) to the comprehensively characterized zinc peptidases from M3 proteolytic enzymes family M3B subfamily and M4 proteolytic enzyme family (3). GT-KP120 and GT-KP130 are the homologues of Pz-peptidase A (DDBJ BAD99433.1; MEROPS M03.010; PDB 3AHM) and B (DDBJ BAD99434.1; MEROPS M03.007), respectively, and GT-KP100 is a bacillolysine (GenBank AEV20496.1; MEROPS M04.014) homologue.

The assumption that moderate thermophile *G. thermoleovorans* DSM 15325 possess permanent collagen decomposition potential by constitutively producing extracellular peptidases with collagenolytic specificity was confirmed by the successful detection and identification of collagenolytic peptidases produced by proteolytic geobacilli cultivated in the medium without any peptidase expression inductors with glucose as a single source of carbon and energy. *G. thermoleovorans* DSM 15325 constitutively produced collagenolytic peptidases are homologues of comprehensively characterized zinc peptidases, which constitutive production was previously only predicted (13). The substrate specificities of Pz-peptidases and bacillolysine suggest that these *G. thermoleovorans* DSM 15325 hydrolases may act in a cooperative activity manner. According to the proposed model, the bacillolysine collagenolytic activity against collagen polypeptide chains should produce short collagen peptides, which are suitable substrates for Pz-peptidases lacking proteolytic activity. The formed constitutive catabolic cascade enables the collagen and other proteins from cellular environment to be effectively and immediately used by *G. thermoleovorans* DSM 15325 for the nutritional purposes. The activity of catabolic cascade may also ensure the increase of protein uptake as the oligopeptides of degraded proteins after the transportation to the geobacilli cytoplasm may induce peptidases expression, resulting in significant increase of the environmental oligopeptide and/or the protein uptake efficiency. Characterized *G. thermoleovorans* DSM 15325 ability of a constitutive production of the extracellular collagenolytic metallo-peptidases that acts by forming a constitutive catabolic cascade reflect the overall adaptive plasticity of proteolytic geobacilli survivals strategy.

Characterization of *G. thermoleovorans* DSM 15325 secreted M3 proteolytic enzyme family M3B subfamily oligopeptidase (GT-SM3B)

The assumption that secreted zinc-dependent oligopeptidase is encoded in the *G. thermoleovorans* DSM 15325 genome was proven by GT-SM3B sequence amplification from chromosome of this thermophilic bacteria. The sequence of the GT-SM3B gene was 1,857 bp in length, coding a protein of 618 amino acids in length. The molecular mass was calculated to be 70.2 kDa, while the molecular mass of the enzyme without the 23-residue signal sequence was calculated to be 67.7 kDa. The nucleotide sequence of gene GT-SM3B has been deposited in GenBank under accession no. KF779146, GenBank attributed protein ID AHG94995.1 for GT-SM3B. GT-SM3B sequence was the least different from sequences of putative oligopeptidases F with predicted signal peptides of other geobacilli. A comparison of the amino acid sequences revealed 99.2% identity of GT-SM3B to oligoendopeptidase F (GenBank AEV19421.1) of *G. thermoleovorans* CCB_US3_UF5 and 98.7% identity to oligoendopeptidase F (GenBank ADI26721.1) of *Geobacillus* sp. C56-T3. When analyzing the metallo-oligopeptidases with the known biochemical characteristics, the GT-SM3B amino acid sequence was the most identical sharing 40.3% and 38.5% identity, respectively to $PepF_{Ba}$ (GenBank AAQ08885.2; MEROPS M03.007) and Pz-peptidase (DDBJ BAA13561.1; MEROPS M03.007) secreted by mesophilic bacteria *B. amyloliquefaciens* 23-7A (14) and *B. licheniformis* N22 (15). A comparison of the GT-SM3B sequence with those of the only studied metallo-oligopeptidases from thermophiles revealed 39.5% sequence identity with *G. collagenovorans* MO-1 intracellular Pz-peptidase B and a notably lower identity of 26.2% with *G. collagenovorans* MO-1 intracellular Pz-peptidase A (16). Identity of 34.2% was observed between sequences of GT-SM3B and M3 proteolytic enzymes family M3B subfamily's comprehensive characterized holotype peptidase *Lactococcus lactis* subsp. *lactis* IL1403 oligopeptidase F (GenBank AAK05825.1; MEROPS M03.007) (17). Results of GT-SM3B sequence homology analysis indicated enzyme novelty for characterization, as the enzyme amino acid sequence identity with the metallo-oligopeptidases studied so-far did not exceed 41% identity. The GT-SM3B sequence *in silico* analysis revealed zinc metallo-peptidase consensus motif His-Glu-Xaa-Xaa-His

(His^{400}-Glu^{401}-Leu-Gly-His^{404}) and Glu (Glu^{428}), positioned after 23 amino acids downstream of the consensus motif as essential sequence elements governing metal-binding and in consequence catalytic mechanism of GT-SM3B catalytic activity. Identified sequence elements are typical for gluzincin metallo-peptidases (18). All to date known metallo-oligopeptidases are gluzincins, so GT-SM3B is no exception. The GT-SM3B sequence *in silico* analysis also revealed domain organization typical for M3 proteolytic enzymes family M3B subfamily oligopeptidases F (3). The regulation of GT-SM3B gene transcription is independent as gene encoding characterized oligopeptidase *in silico* was not assigned to operon. Sequence homology and conservative sequence elements as well as domain organization assign GT-SM3B to the M3 proteolytic enzymes family M3B subfamily of the only bacterial metallo-peptidases limited to oligopeptidolytic specificity.

Recombinant GT-SM3B expressed in *E. coli* BL21 (DE3) accumulated in inclusion bodies. The attempt to enhance the solubility of GT-SM3B accumulated in the cytoplasm of *E. coli* BL21 (DE3) cells failed. However, *E. coli*, as predicted, recognized native signal peptide of GT-SM3B *in silico*, despite the introduced N-terminal hexahistidine thrombin-cleavable tag. Optimization of recombinant GT-SM3B secretion was performed in order to increase yield of recombinant enzyme in culture fluid. An appreciable amount of secreted GT-SM3B was detected when 0.5 mM IPTG induced GT-SM3B expression for 4 h at 30 °C, starting the induction after recombinant *E. coli* BL21 (DE3) reached OD_{600} 0.5. When producing GT-SM3B, LB medium was replaced by M9 medium with casamino acids 1% (w/v), since the latter medium substantially intensified oligopeptidase secretion. GT-SM3B production using secretion by an expression strain in order to achieve soluble and active enzyme is the first time when secretory production was used for metallo-oligopeptidase. M3 proteolytic enzymes family M3B subfamily peptidases that were produced by heterologous overexpression remained soluble in the *E. coli* cytoplasm (19). The only bacterial metallo-oligopeptidase with signal peptide produced in *E. coli* (PgPepO from P. gingivalis 381) was also smoothly expressed remaining intracellularly soluble, but was not secreted by *E. coli* BL21 (DE3) pLysS (20). The secretory production of GT-SM3B granted the active and not aggregation prone enzyme's final mature form to be not affected by additional amino acids and was beneficial for enzymes purification. Active recombinant peptidase was purified from

expression culture fluid to 36-fold by $(NH_4)_2SO_4$ precipitation fractionation combined with two consecutive ion-exchange chromatography purification steps. Assessment by SDS-PAGE and gelatin zymography confirmed GT-SM3B homogenous purity, revealing homodimerization (Fig. 2).

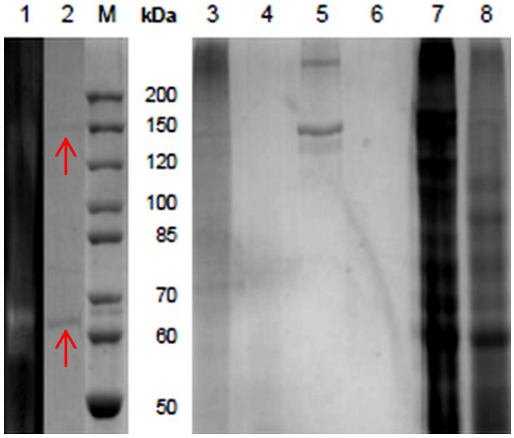

Fig. 2. Results of GT-SM3B purification and hydrolysis of collagenous peptides. 1: GT-SM3B gelatin zymography; 2: Purified GT-SM3B; M: Marker; 3: Gelatin control; 4: Gelatin-derived oligopeptide hydrolysis with GT-SM3B; 5: Type I collagen control; 6: Type I collagen-derived oligopeptide hydrolysis with GT-SM3B; 7: Raw collagen extract control; 8: Raw collagen extract-derived oligopeptide hydrolysis with GT-SM3B.

The optimum temperature for maximum GT-SM3B peptidolytic activity was 40 °C, among the ten different temperatures tested (Fig. 3A). The GT-SM3B temperature optimum is similar to $PepF_{Ba}$ optimum at 45 °C (14) and comparable with *B. licheniformis* Pz-peptidase and PgPepO activities at 37 °C (15, 20). Only *G. collagenovorans* MO-1 thermoactive intracellular Pz-peptidases B and A significantly exceed the GT-SM3B optimum, 30 °C and 25 °C, respectively (16). Oligopeptidase demonstrated a typical bell-shaped curve with the hydrolytic activity in the temperature range of 10-80 °C. The thermoactivity of the GT-SM3B at temperatures 50 °C and 60 °C was 84% and 64% relative to the optimum enzyme thermoactivity. The peptidase activity of 18% was detected at 10 °C, while GTSM3B activity decreased at the temperature over 60 °C. The characterized metallo-oligopeptidase was inactive at 95 °C (Fig. 3A), but still demonstrated 34% of its activity at 70 °C and 2% at 90 °C. The range of GT-SM3B effective activity is one of the widest ever reported for metallo-oligopeptidases. The high thermoactivity of GT-SM3B, for 20 °C above optimum, is a distinctive characteristic of GT-SM3B. The temperature of GT-SM3B activity maximum is low, as for an enzyme from the obligate thermophile *G. thermoleovorans* DSM 15325 that has growth maximum

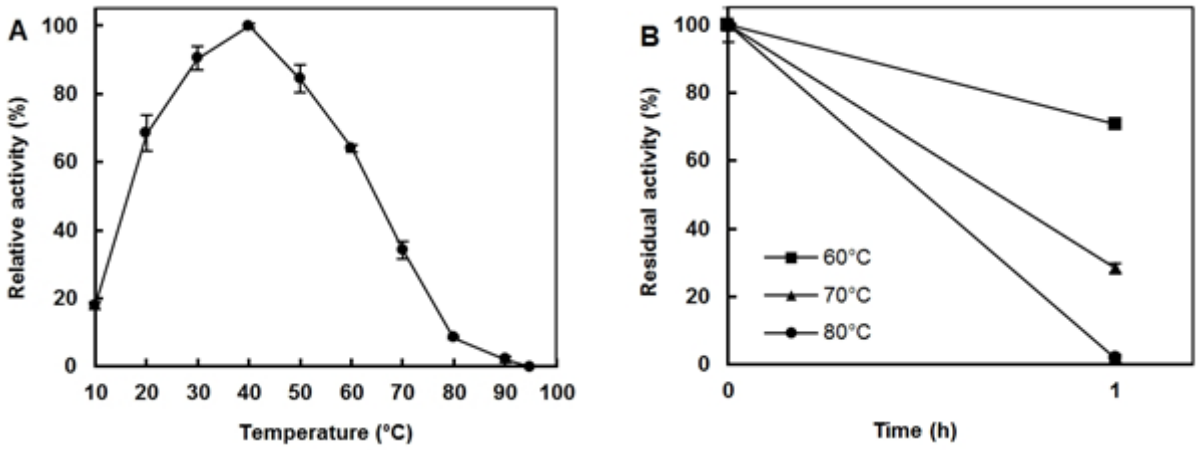

Fig. 3. Effects of temperature on GT-SM3B. A: The effect of temperature on activity of GT-SM3B. The purified oligopeptidase was assayed at temperatures ranging from 10 ºC to 95 ºC in 50 mM HEPES-NaOH, pH 7.3 with Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH peptidolysis assay to determine the effect of temperature on activity. Peptidolysis at optimal temperature (5.3 ± 0.03 U/mg) was taken as 100% of oligopeptidase activity. B: The effect of temperature on stability of GT-SM3B. The thermostability of GT-SM3B was investigated after incubation of the enzyme solutions in the absence of substrate at indicated temperatures for 1 h in 50 mM HEPES-NaOH, pH 7.3. Residual peptidolytic activity was measured after the incubation. Peptidolysis at 60 °C (3.6 ± 0.06 U/mg) was taken as 100% of oligopeptidase activity.

between 55 °C and 60 °C with growth minimum at 55 °C (10). Previously characterized bacterial metallo-oligopeptidases secreted by mesophiles, namely $PepF_{Ba}$, *B. licheniformis* Pz-peptidase, and PgPepO, were the most active at or near the temperature of microorganism growth optimum.

GT-SM3B retained 71% of the initial activity after 1 h exposure to 60 °C (Fig. 3B), giving extrapolated half-life ($t_{1/2}$) estimates in excess of 1.5 h. At 70 °C, the $t_{1/2}$ value of the oligopeptidase was approximately 40 min. The thermal inactivation of GT-SM3B at 80 °C was almost complete, with an observed residual activity below 2% (Fig. 3B). The comparison of GT-SM3B thermostability with the previously reported for other metallo-oligopeptidases shows that GT-SM3B oligopeptidase is as thermostable as Pz-peptidase A and slightly less stable than Pz-peptidase B of *G. collagenovorans* MO-1. GT-SM3B is the first thermostable extracellular metallo-oligopeptidase, because it is more thermostable than *B. licheniformis* Pz-peptidase and $PepF_{Ba}$ secreted by mesophiles. The thermostability of GT-SM3B is also the highest among the non-thermoactive metallo-oligopeptidases.

Demonstrating an effective activity level above 50% within a wide pH range of 5.0-8.0, GT-SM3B demonstrated pH optimum at pH 7.3 (Fig. 4). Below neutral pH, the GT-SM3B activity had tendency towards gradual decline to 44% of hydrolytic activity

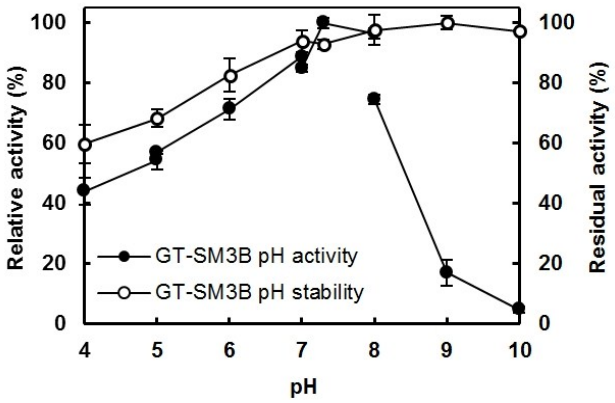

Fig. 4. The effect of pH on the activity and stability of GTSM3B. The influence of different pH values on the GT-SM3B activity was tested at 60 °C with the Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH peptidolysis assay. GT-SM3B pH stability was described by determining residual activity after enzyme pre-incubation in the absence of substrate for 1 h at 40 °C in buffer of pH 4-10 range with the specified hexapeptide peptidolysis assay at 60 °C. Peptidolysis at 60 °C (3.6 ± 0.06 U/mg) was taken as 100% of oligopeptidase activity.

at pH 4. The slight shift of the pH profile of GT-SM3 to more alkaline pH was noticed, because activity at pH 8.0 was by approximately 7% higher than measured at pH 7.0. GT-SM3B peptidolysis declined sharply above pH 8.0, and at pH 10.0 it was not more than 5% (Fig. 4). The purified oligopeptidase was stable at pH from 5.0 to 8.0 (Fig. 4). The treatment of GT-SM3B at strongly acidic or alkaline conditions led to the reduction of enzyme activity. Residual activity of GT-SM3B at pH 4.0 and 9.0 was 60% and at pH 10.0 was 40%. The GT-SM3B pH optimum – pH 7.3 is closer to optimums of PgPepO a pH 6.8-7.0 (20) and $PepF_{Ba}$ at pH 7.0 (14), as *B. licheniformis* Pz-peptidase requires pH 7.8 for maximal activity (15). If compared with the pH optimums of intracellular metallo-oligopeptidases, the GT-SM3B pH optimum is almost identical to PepB, a streptococcal oligopeptidase F with pH optimum at 7.4 (21). The profiles of GT-SM3B pH activity and stability have more resemblance to the profiles known for intracellular metallo-oligopeptidases from lactococcci and *G. collagenovorans* MO-1 when compared to extracellular metallo-oligopeptidase from mesophiles, regarding the profiles' widths and characteristic shift to alkaline pH (Fig. 4) (17, 16).

The effects of metal chlorides and other reagents on GTSM3B activity are shown in Tables 2 and 3, respectively. The presence of Li^+, K^+, Sn^{2+}, and Ba^{2+} at concentration of 1 mM as well as K^+ at 10 mM concentration had no effect on the activity of recombinant oligopeptidase. The addition of Ca^{2+} up to 1 or 10 mM had negligible stimulation on GT-SM3B catalytic activity. The Mg^{2+} and Na^+ ions at 1 mM concentration were stimulators, enhancing GT-SM3B enzymatic activity by about 20%. Interestingly, at 10 and 350 mM, Na^+ cation had the same stimulatory activity on GT-SM3B as at 1 mM concentration. The Co^{2+} ion at 1 mM was a weak inhibitor of GT-SM3B, because it had decreased oligopeptidase activity only by about 6%. Other divalent cations tested

Table 2. Effect of metal cations on the GT-SM3B activity.

Metal ion*	Relative activity, %
Control	100**
Li^{+}	105.23 ± 0.63
Na^{+}	127.67 ± 3.79
Na^{+} (10 mM)	118.81 ± 0.8
Na^{+} (350 mM)	118.55 ± 1.28
Mg^{2+}	121.33 ± 3.09
K^{+}	99.73 ± 1.34
K^{+} (10 mM)	101.47 ± 2.42
Ca^{2+}	102.73 ± 0.84
Ca^{2+} (10 mM)	105.56 ± 0.61
Mn^{2+}	49.12 ± 3.98
Fe^{3+}	23.83 ± 2.45
Co^{2+}	94.38 ± 1.41
Ni^{2+}	57.7 ± 5
Cu^{2+}	53.16 ± 1.78
Zn^{2+}	58.47 ± 0.69
Sn^{2+}	96.78 ± 1.33
Ba^{2+}	102.91 ± 2.02

*Final concentration, 1 mM or as indicated.
**Hexapeptide cleavage at 60 °C was taken as 100% corresponding to 3.4 ± 0.06 U/mg.

Table 3. Effect of various compounds on the GT-SM3B activity.

Compound	Concentration	Relative activity,%
Control	–	100*
DTT	1 mM	28.09 ± 6.59
TCEP	1 mM	110.21 ± 3.32
2-ME	1 mM	86.75 ± 3.39
Urea	1 mM	99.69 ± 0.58
	10 mM	98.47 ± 0.32
Guanidine HCl	1 mM	78.74 ± 1.92
SDS	1 mM	52.83 ± 0.9
	10 mM	ND
Tween 20	0.1%	67.08 ± 3.17
	1%	36.09 ± 4.57
Tween 40	0.1%	58.42 ± 2.76
	1%	38.47 ± 0.39
Tween 60	0.1%	61.97 ± 0.57
	1%	22.76 ± 1.65
Tween 80	0.1%	42.82 ± 1.36
	1%	14.53 ± 0.86
Ethylene glycol	0.1%	89.95 ± 3.03
	1%	76.04 ± 1.21
Glycerol	1%	115.52 ± 3.81
	5%	74.62 ± 1.4
Triton X-100	0.1%	84.14 ± 0.85
	1%	63.62 ± 2.42
Triton X-305	0.1%	40.69 ± 1.97
	1%	1.94 ± 0.8
Brij 35	1 mM	ND
Brij 58	1 mM	ND

*Hexapeptide cleavage at 60 °C was taken as 100% corresponding to 3.4 ± 0.06 U/mg.
ND not detectable

acted as GT-SM3B inhibitors at 1 mM concentration, reducing the enzymes activity by approximately 50%. Fe^{3+} was the strongest oligopeptidase metal ion inhibitor, with inhibition activity of over 75%. M3 proteolytic enzyme family M3B subfamily secreted or thermostable oligopeptidases of the were similarly susceptible to Fe^{3+}, Co^{2+}, Ni^{2+}, and Cu^{2+}, like GT-SM3B. Pz-peptidases from *G. collagenovorans* MO-1 and *B. licheniformis* N22 were more tolerant to Mn^{2+} than GT-SM3B (16, 15). The characterized metallo-oligopeptidase was more intensively stimulated by Mg^{2+} than Pz-peptidase B from *G. collagenovorans* MO-1 (16).

The amount of Na^+ up to at least 350 mM was a positive activity modulator for GT-SM3B. However, 1 M of NaCl decreases the activity of lactococcal oligopeptidase F by 25% (22). Chelation by EDTA caused complete GT-SM3B inhibition, but the same 1 mM concentration of EGTA had no influence on the hydrolase catalysis. Reducing agents modulated GT-SM3B differently at 1 mM. Enzyme activity was suppressed by DTT and 2-ME by more than 65% and 9%, respectively. TCEP stimulated GT-SM3B activity by about 10%. All tested detergents inactivated recombinant oligopeptidase at a different extent; from approximately 14% inhibition by 0.1% Triton X-100 up to the complete elimination by 10 mM of SDS and 1 mM of Brij 35 or Brij 58. Urea at 1 mM and 10 mM concentrations had no impact on GT-SM3B activity. However, the other chaotropic agent guanidine-HCl at 1 mM inhibited the characterized enzyme by nearly 20%. The presence of glycerol at 1% concentration ensured the enhancement of GT-SM3B activity by more than 11%. The glycerol solution of 5% concentration and both applied concentrations of ethylene glycol produced weak inhibitory effects on GT-SM3B activity, ranging from 7% to 24%. The analysis of the impact of compounds often used in biotechnology on GT-SM3B activity is the most detailed ever reported for metallo-oligopeptidase. Results of GT-SM3B sequence *in silico* analysis were confirmed by the effect of chelators, divalent metal ions and phosphoramidone, indicating this enzyme to be a zinc-containing metallo-peptidase. The crucial importance of the divalent ion was demonstrated by complete GT-SM3B activity inhibition by EDTA and phosphoramidone, but the characterized enzyme was not Ca^{2+} dependent. Milimollar excess of Zn^{2+} strongly inhibited GT-SM3B, and this is typical for zinc metallo-peptidases (23).

Electrophoresis revealed oligomerization of the purified enzyme (Fig. 2). The molecular mass of the GT-SM3B oligomer was close to 150 kDa. The estimation of GT-SM3B homodimerization was expected. The homologous secreted hydrolases from bacilli $PepF_{Ba}$ (14) and *B. licheniformis* Pz-peptidase (15) were purified as homodimers. It is likely that the *in vivo* homodimer is a native state of GT-SM3B, but the *in vitro* monomer is also catalytically active at the same extent as the oligomer (Fig. 2). Secreted M13 family oligopeptidase PgPepO was found to be a monomeric (20). Intracellular bacterial metallo-oligopeptidases are mainly known as monomers. The substrate specificity of the oligopeptidase towards the hexapeptide Carbobenzoxy-Gly-Pro-Gly-

Gly-Pro-Ala-OH and mixtures of short-chain collagen-derived oligopeptides was determined at 60 °C (Table 4). The specific peptidolytic activity at 60 °C was by almost 36% lower comparing with the GT-SM3B peptidolysis maximum of 5.3 ± 0.03 U/mg at optimal temperature and pH. The dissolvent of gelatin, type I collagen, and raw collagen extract in acetic acid as well as exposure to 60 °C during inspection of GT-SM3B hydrolytic activity were the manipulations that produced short oligopeptides accessible for the GT-SM3B active center. M3 proteolytic enzyme family M3B subfamily peptidases cleave peptides ranging from 5 to 23 amino acids in length (15, 17). Having broad oligopeptidolytic specificity to collagen derived oligopeptides (Fig. 2), GT-SM3B did not hydrolyze azocoll, an intact dye-impregnated type I collagen. Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH, the optional substrate for oligopeptidase activity evaluation (15), was actively hydrolyzed by GT-SM3B between Gly-Gly and/or Pro-Gly. The V_{max} of this reaction was 2.65 ± 0.03 $\times 10^{-3}$ μM/min, with K_M and k_{cat} values of 2.17 ± 0.04 × 10^{-6} M and 5.99 ± 0.07 s^{-1}, respectively. M3 proteolytic enzyme family M3B metallo-oligopeptidases lack aminopeptidolytic activity and require a minimum of three amino acids from the peptide carboxyl terminus (17); this enables to predict the hexapeptide cleavage sites with high degree of confidence.

Taken together, the GT-SM3B substrate specificity and accordance of enzyme's characteristics with *G. thermoleovorans* DSM 15325 optimal growth conditions suggest a role of the characterized oligopeptidase in bacteria nutrition. The GT-SM3B encoding thermophile is a proteolytic bacterium (10). The described secreted oligopeptidase functioning extracellularly would increase the efficiency of *G. thermoleovorans* DSM 15325 collagen catabolism cascade. According to this model, GT-SM3B would take intermediate place as a cascade member, acting in partnership with extracellular *G. thermoleovorans* DSM 15325 collagenolytic proteases and intracellular metallo-oligopeptidases. Possible partial

Table 3. Activity of GT-SM3B towards oligopeptide substrates.

Substrate	Specific activity, U/mg	Relative activity, %
Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH	3.4 ± 0.06	100*
Gelatine derived oligopeptide mixture	2.27 ± 0.03	66.8 ± 1.32
I type collagen derived oligopeptide mixture	2.19 ± 0.07	64.4 ± 3.2
Raw collagen extract oligopeptide mixture	1.42 ± 0.2	41.8 ± 14.08

*Hexapeptide cleavage at 60 °C was taken as 100%.

overlapping substrate specificity of GT-SM3B and intracellular *G. thermoleovorans* DSM 15325 metallo-oligopeptidases, as usual for lactococci and lactobacilli metallo-oligopeptidases, would be fully reasonable. Housekeeping functions, not necessary protein turnover, are attributed to a part of characterized bacterial M3 proteolytic enzymes family M3B peptidases that are constitutively expressed. GT-SM3B is not a constitutively secreted enzyme. The hydrolase was not identified by mass spectrometry analysis in the *G. thermoleovorans* DSM 15325 secretome during characterization of geobacilli constitutive collagenolytic potential characterization. Secreted metallo-oligopeptidases from bacilli $PepF_{Ba}$ (14) and *B. licheniformis* N22 Pz-peptidase (15) are supposed to participate in the degradation of peptides for cell nutrition, whereas *P. gingivalis* 381 secreted PgPepO ensures invasion of this pathogen (23).

Characterized GT-SM3B is the first secreted thermostable metallo-oligopeptidase with possible function in *G. thermoleovorans* DSM 15325 to respond to environmental changes. The determined combination of GT-SM3B characteristics unites the distinctive qualities of metallo-peptidases earlier separately attributed to the thermoactive metallo-oligopeptidases or metallo-oligopeptidases from mesophiles. GT-SM3B is a powerful thermostable biocatalyst with board substrate specificity. The outlined GT-SM3B characteristics suggest that hydrolase may be implicated in biotechnological processes.

Characterization of *G. thermoleovorans* DSM 15325 long chain U32 proteolytic enzymes family peptidase (GT-IU32)

The assumption that long chain U32 proteolytic enzyme family peptidase is encoded in the *G. thermoleovorans* DSM 15325 genome was proven by GT-IU32 sequence amplification from chromosome of these thermophilic bacteria. The sequence of the GT-IU32 gene was 1,269 bp in length, coding a protein of 422 amino acids in length. The molecular mass was calculated to be 47.9 kDa. The nucleotide sequence of gene GT-IU32 has been deposited in GenBank under accession no. JN628020, GenBank attributed protein ID GenBank AEP39604.1 for GT-IU32. GT-IU32 was the least different from sequences of putative U32 proteolytic enzymes family long chain peptidases of other geobacilli. A comparison of the amino acid sequences revealed 99.5% identity of GT-IU32 to putative U32 proteolytic enzymes family long chain peptidases

(DDBJ BAD76834.1; GenBank ACX77635.1; GenBank ADI25985.1; GenBank ADU94963.1; GenBank AEV20175.1; GenBank AGE23134.1; GenBank ESU70462.1; DDBJ GAD12620.1; DDBJ GAJ58075.1; GenBank KDE47364.1) and 99.3% identity to U32 proteolytic enzymes family long chain peptidase (GenBank EQB96026.1) of *Geobacillus* sp. A8. When compared to typical U32 proteolytic enzymes family peptidases GT-IU32 was the most identical, 34.6 % identity, to yrrN (*Bacillus subtilis*) protein group typical yrrN peptidase (GenBank AIY94059.1; EMBL CAB14677.1; MEROPS U32.A02) encoded in *B. subtilis* subsp. *subtilis* 168 genome. Identity of 32.7% was observed between sequences of GT-SM3B and U32 proteolytic enzymes family holotype *P. gingivalis* ATCC 53977 characterized peptidase PrtC (GenBank AAA25650.1; MEROPS U32.001) (24). Out of all U32 proteolytic enzymes family peptidases with the known biochemical characteristics, the GT-SM3B amino acid sequence was the most identical sharing 48.8 % identity to U32 proteolytic enzymes family long chain peptidases which sequences are identical smcol1 (GenBank AAT66437.1; MEROPS U32.002) from *S. mutans* GS-5 ser. c (25) and SMU.761 (GenBank AAN58483.1; MEROPS U32.002) from *S. mutans* UA159 (26). GT-IU32 displayed a relatively low 26.7% identity with U32 proteolytic enzymes family putative cytoplasmic peptidase (GenBank AAM02119.1; MEROPS U32.UPW; PDB 5D88) of *M. kandleri* AV19 that crystal structure was solved and characterized (27). Results of GT-IU32 sequence homology analysis indicated enzyme novelty for characterization, as the enzyme amino acid sequence identity did not exceed 49% when compared with the so-far studied U32 proteolytic enzymes family peptidases. The GT-IU32 sequence *in silico* analysis revealed U32 proteolytic enzymes family peptidases conservative motif Glu-Xaa-Phe-Xaa-Xaa-Gly-[Ser/Ala]-[Leu/Ile/Val/Met]-Cys-Xaa-Xaa-Xaa-Xaa-Gly-Xaa-Cys-[Leu/Ile/Val/Met]-Ser (Glu^{179}-Ala-Phe^{181}-Ile-His-Gly^{184}-Ala^{185}-Met^{186}-Cys^{187}-Ser-Ala-Tyr-Ser-Gly^{192}-Arg-Cys^{194}-Val-Leu^{196}-Ser^{197}) and sequence domain organization typical for putative U32 proteolytic enzymes family long chain peptidases from geobacilli (3). The genome of *G. thermoleovorans* DSM 15325 is not sequenced, however the genome of *G. thermoleovorans* CCB_US3_UF5 being phylogenetically close to *G. thermoleovorans* DSM 15325 is annotated. Therefore GT-IU32 gene genomic and operonic organization was characterized by *in silico* analysis of GT-IU32 homologue gene from *G. thermoleovorans* CCB_US3_UF5 genomic and operonic organization. *G. thermoleovorans* CCB_US3_UF5 as all geobacilli whose genomes was annotated as

well as *G. thermoleovorans* DSM 15325 encodes two U32 proteolytic enzymes family peptidases. Long chain U32 proteolytic enzymes family peptidases are encoded after the gene encoding short chain, 309 amino acids length, U32 proteolytic enzymes family peptidases. GT-IU32 homologue gene from *G. thermoleovorans* CCB_US3_UF5 *in silico* along with short chain U32 proteolytic enzymes family peptidase gene and genes of *O*-methyltransferase and Udk kinase were assigned to operon. Genes of characterized U32 proteolytic enzymes family peptidases are usually identified in operons (28, 29). GT-IU32 homologue gene from *G. thermoleovorans* CCB_US3_UF5 assignment to operon was expected as all U32 proteolytic enzymes family peptidases encoded in genomes of geobacilli are identified in operons with identical organization as *in silico* identified for GT-IU32 homologue gene in *G. thermoleovorans* CCB_US3_UF5 chromosome. The transcription regulation *in silico* analysis revealed that GT-IU32 homologue gene transcription is coordinated with Udk kinase gene as terminator was predicted only after Udk kinase gene. Meanwhile promoters were predicted for both genes. Long chain U32 proteolytic enzymes family peptidases from *G. thermoleovorans* DSM 15325 sequence homology and conservative sequence elements as well as domain organization assigns GT-IU32 to the U32 proteolytic enzymes family of peptidases of unknown catalytic mechanism demonstrating collagenolytic activity typically.

GT-IU32 transcription in *G. thermoleovorans* DSM 15325 exponential growth phase, applying optimal cultivation temperature 60 °C for strains growth, was confirmed. Proteolytic geobacilli transcribed GT-IU32 constitutively. The degradation of GT-IU32 transcript in *G. thermoleovorans* DSM 15325 death phase was also revealed while performing characterization of GT-IU32 transcription in *G. thermoleovorans* DSM 15325. GT-IU32 gene transcript was also successfully identified applying RT-PCR with primer pairs GEOCOL and LITCOL (Fig. 5) when total RNA used for RT-PCR was isolated from geobacilli cells in exponential growth phase cultivated at minimum growth temperature 55 °C and maximum growth temperature 70 °C (10). The GT-IU32 gene transcription at 55 and 70 °C was constitutive as *G. thermoleovorans* DSM 15325 for total RNA isolation was cultivated in mM9 media with glucose as a single carbon and energy source. Proteomics analysis was also carried out to confirm GT-IU32 expression in *G. thermoleovorans* DSM 15325. Native GT-IU32 was identified in lysates of

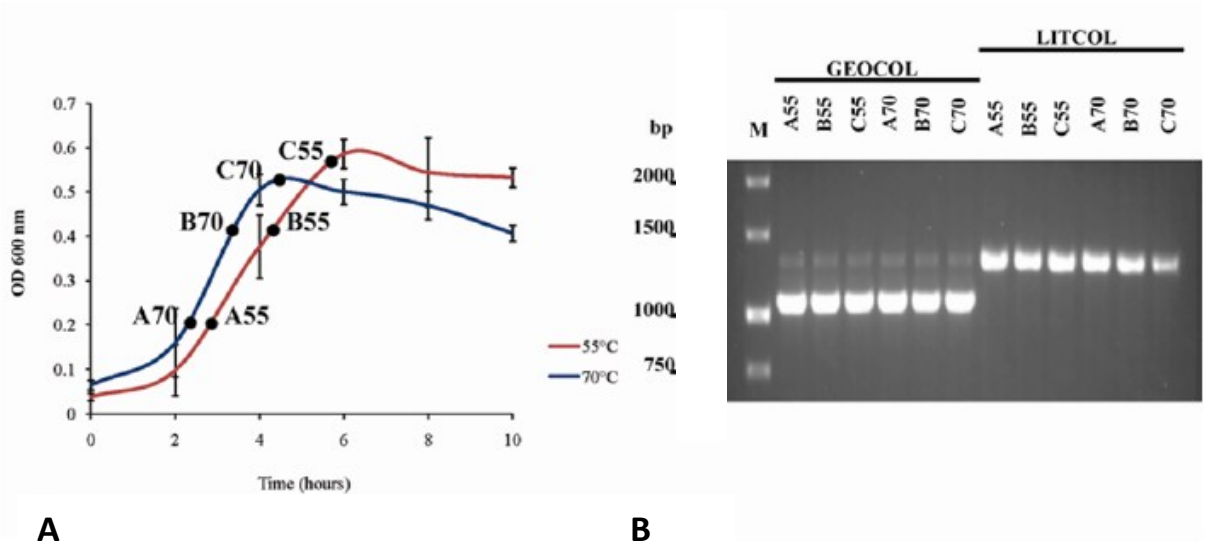

Fig. 5. Transcriptional analysis of GT-IU32 at the minimum, 55 °C, and maximum, 70 °C, growth temperature. A: Growth curves of *G. thermoleovorans* DSM 15325. The cultures were grown in mM9 medium. The circles on the curves indicate the sampling points for the isolation of total RNA. B: RT-PCR products, obtained using GEOCOL and LITCOL.

geobacilli. Expression of GT-IU32 in *G. thermoleovorans* DSM 15325 lysates was assayed by a linear ion trap survey mass spectrometry approach and a more sensitive multiple reaction monitoring method using triple quadruple option of a QTRAP 4000 mass spectrometer. The initial approach, using survey mass spectrometry, did not lead to identification of any GT-IU32 tryptic peptides in geobacilli cell lysates, although a number of other lysate proteins were identified (data not shown). Therefore, a more sensitive and direct MRM method (30), designed to find selected GT-IU32 tryptic peptides in complex cell lysate mixtures, was applied. This led to the identification of three tryptic peptides: Lys-Pro-Glu-Leu-Leu-Ala-Pro-Ala-Gly-Asn-Leu-Glu-Lys (Mascot įvertis 32, *p*= 0,01), Ala-Asn-Ala-Asp-Asn-Phe-Thr-Ile-Glu-Glu-Ile-Arg (Mascot įvertis 31, *p*= 0,01) and Ala-Ile-Pro-Val-Met-Ile-Glu-Leu-Gly-Val-Asp-Ser-Leu-Lys (Mascot įvertis 35, *p*= 0,01), out of eighteen peptides analyzed, that correspond to GT-IU32. Thus, the data show that GT-IU32 is translated in *G. thermoleovorans* DSM 15325, though the expression level is apparently low. Native GT-IU32 was not identified in geobacilli extracellular protein concentrated fraction even when sensitive multiple reaction monitoring method was applied for GT-IU32 identification. Secretion of GT-IU32 was not predicted *in silico* as well.

The evaluation of recombinant GT-IU32 expression in *E. coli* BL21 (DE3) revealed oligomerization of expressed enzyme (Fig. 6). Fragmentary *in vitro*

characterized U32 proteolytic enzymes family peptidases are mostly monomeric hydrolases (31, 32), however homodimerization of PrtC and smcol1 is also characterized (24, 25). GT-IU32 homooligomerization observed in SDS-PAGE gels was confirmed by

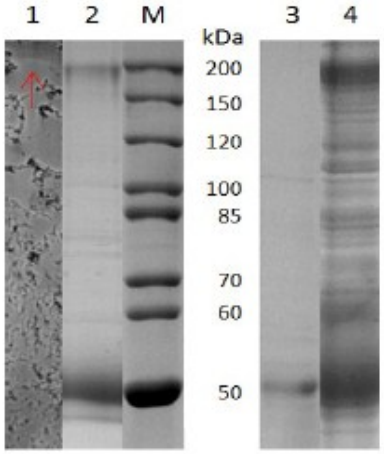

Fig. 6. Evaluation of recombinant GT-IU32 purity, homooligomerization and collagenolytic activity of GT-IU32 oligomers. 1: GT-IU32 activity detection applying partial denatured collagen type I zymography, without reduction; 2: Purified GT-IU32 electrophoretic profile, without reduction; M: marker; 3: Purified GT-IU32 electrophoretic profile, with reduction 2-ME; 4: Evaluation of GT-IU32 expression in *E. coli* BL21 (DE3) without reduction.

analyzing purified enzyme sample with analytic gel filtration (Fig. 7). Reduction suppressed homodimerization or recombinant GT-IU32 (Fig. 6). The ability of GT-IU32 monomers to form homodimer by forming extramolecular disulfide bridges was also confirmed by analytic gel filtration when purified enzyme sample supplemented with reductors was analyzed (Fig. 8ABC). It should be noted that the GT-IU32 has eight cysteine residues, and some of them could be involved in disulfide bond formation. High ionic strength had no impact on GT-IU32 homooligomerization.

Catalytic mechanism of U32 proteolytic enzymes family peptidases remains unknown, however EDTA acted as inhibitor of U32 proteolytic enzymes family peptidases activity (24, 32). PrtC peptidases activity was slightly stimulated by effect of Ca^{2+} while Zn^{2+} inhibited PrtC activity (24). Characterization of calcium and zinc ions on GT-IU32 monomers thermostability enable to outline, that Zn^{2+} is important for GT-IU32 molecule thermostability (Fig. 9). Recombinant GT-IU32 was unstable above 55 °C. Enzymes $t_{1/2}$ at 55 °C was determined to be 1.2 h. Calcium ions had no enhancing effect on the thermostability of GT-IU32 – $t_{1/2}$ of recombinant peptidase at 55 °C was 1.1 h. The presence of zinc ions increased GT-IU32 stability substantially – $t_{1/2}$ at 55 °C raised to 1.7 h. The possible synergistic effect of calcium and zinc ions to GT-IU32 monomer thermostability was not observed (Fig. 9).

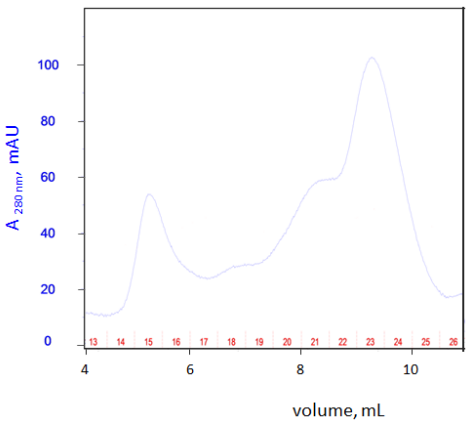

Fig. 7. Molecular masses of GT-IU32 oligomers determined applying analytical gel filtration, without reduction. Elution volumes according to column calibration: till 6 mL –above 440 kDa, 7-8.5 mL – 75-158 kDa, 8.5- 9.5 mL – 44-75 kDa.

GT-IU32 catalytic activity was confirmed applying partially denatured collagen type I from rat tail tendon zymography (Fig. 6). Only GT-IU32 dimer but not monomeric recombinant GT-IU32 showed collagenolytic activity at 50 °C. GT-IU32 is highly substrate specific hydrolase capable of hydrolyzing partially denatured collagen type I from rat tail tendon but inert towards partially denatured collagen type I from bovine skin. Recombinant GT-IU32 homodimer was unable to degrade albumin, casein, elastin, gelatine or keratin either. GT-IU32 demonstrated weak collagenolytic activity as it was necessary to incubate zymography gels for at least 12 h in order to observe clear bands confirming collagenolytic activity of GT-IU32 dimer (Fig. 6). Proteomic analysis was also applied to confirm the presence of recombinant GT-IU32 in the zymogram and to correlate it with enzymatic activity. The clear band from the zymogram gel was excised and subjected to proteomic analysis. The proteins in the gel slice were digested with trypsin and analyzed with mass spectrometer. Proteomic analysis confirmed collagenolytic activity of GT-IU32 dimer. U32 proteolytic family peptidases that *in vitro* demonstrated catalytic activity are weak collagenolytic endopeptidases so GT-IU32 activity, as well as substrate specificity, characterizes GT-IU32 as typical U32 proteolytic family peptidase (24, 33).

The conditions ensuring crystallization of GT-IU32 peptidase was not selected. However, the evolvement of GT-IU32 three-dimensional structure determination strategy enabled to use partial proteolysis for GT-IU32 monomer domain isolation for subsequent crystallization trials successfully. Spontaneous proteolytic fragmentation of the recombinant full length protein led to isolation of N-terminal (GT-IU32-NGD) and C-terminal (GT-IU32-CGD) GT-IU32 domains with 37.5 and 10.4 kDa molecular mass respectively. GT-IU32-NGD was unstable *in vitro*, fortunately purified GT-IU32-CGD remained stable. Selection of conditions for GT-IU32-CGD crystallization was successful. Native GT-U32-CTD crystallized in space group $P2_12_12_1$ using 100 mM sodium acetate, 200 mM ammonium acetate, 30% (w/v) polyethylene glycol 4000 pH 4.6 as the reservoir

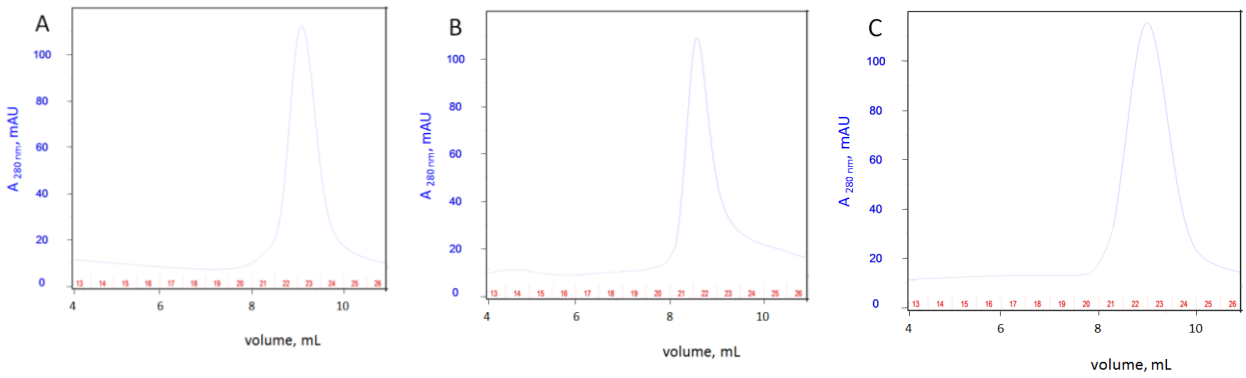

Fig. 8. Influence of reduction with A: 5 mM DTT; B: 10 mM 2-ME; C: 1 mM TCEP on GT-IU32 oligomerization. Elution volumes according to column calibration: till 6 mL – above 440 kDa, 7-8.5 mL – 75-158 kDa, 8.5- 9.5 mL – 44-75 kDa.

solution and the crystals diffracted to 1.1 Å resolution. GT-IU32-CGD structure was determined by single-wavelength anomalous diffraction of a selenomethione-derivatized variant of GT-IU32-CGD, which crystallized in space group $P3_22_1$ using 100 mM sodium citrate, 2 M ammonium acetate pH 5.5 as the reservoir solution; the crystals diffracted to 1.15 Å resolution. PDB references for GT-IU32-CGD native 4HE6; selenomethionine variant 4HE5.

GT-IU32-CGD three-dimensional structure shows a compact distorted open β-barrel made up of eight β-strands (β1-β8), into which the first eight residues (Ser^{334}-Phe^{341}) and the last single residue (Asn^{422}) are inserted (Fig. 10A). The first six residues are present with a refined occupancy of 60%, which is in accordance with mass spectrometric analysis of carefully washed crystals. The barrel is actually arranged as a strongly twisted, curled and arched antiparallel β-sheet (β6-β5-β7-β2-β1-β8-β3-β4; connectivity -1, +3, +1, -6, -1, +2, +3); the flanking strands, β4 and β6, do not interact with each other through their main chains. In addition, β4 and β7 are close to giving rise to a perfect barrel with β1 β3 and β8 (Fig. 10A), but they do not contact each other through their main chains either. Instead, the gap is closed by the side chains Phe^{379}, Gln^{381}, Lys^{409} and Arg^{410} (Fig. 10B). The sheet wraps around a central hydrophobic core formed by the side chains of residues Ala^{342}, Val^{345}, Tyr^{348}, Ala^{355}, Val^{357}, Ala^{359}, Phe^{363}, Val^{369}, Phe^{371}, Ile^{376}, Phe^{379}, Ile^{383}, Gln^{381}, Ala^{397}, Val^{404}, Phe^{406}, Val^{408}, Leu^{412}, Asn^{416} and Met^{418}. In contrast, the surface is mainly hydrophilic and shows seven lysines, five arginines, five aspartates and ten glutamates, which contribute to four salt bridges ($Asp^{367}\cdots Arg^{364}$, $Glu^{370}\cdots Arg^{419}$, $Glu^{377}\cdots Arg^{410}$ and $Asp^{395}\cdots Arg^{398}$; Fig. 10C). Furthermore,

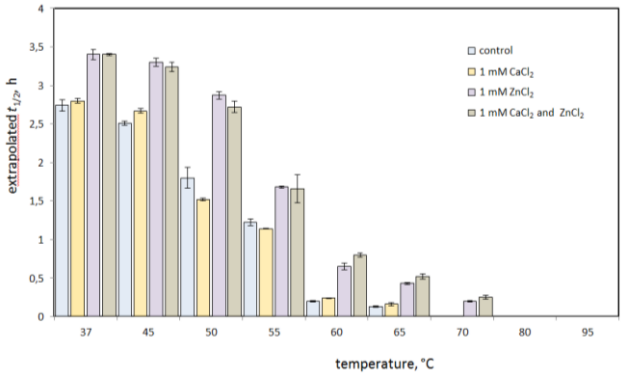

Fig. 9. Influence of calcium and zinc ions on GT-IU32 thermostability.

a potential sodium cation was tentatively assigned on the surface. Finally, an acetate ion was likewise found bound to the protein surface.

Structure-similarity searches with GT-U32-CGD identified the LepA/EF4 protein (PDB 3CB4), eRF3 (PDB 3E20), EF-1α (PDB 1SKQ) and EF-Tu (PDB 2C77) as the most similar structures, with Z-scores of 10.9-11.0, r.m.s.d. values of 1.7-2.0 Å and aligned stretches of 78-80 residues. Superposition of these structures onto GT-U32-CGD revealed equivalent architectures, topologies and connectivities despite negligible sequence identity (8-19%). These structural relatives are all found in proteins that further comprise an additional N-terminal guanine-nucleotide binding domain and they participate in ribosomal protein translation elongation or termination (34). For the elongation factors, it has been shown that the GT-U32-CGD like domains interact with aminoacyl-loaded tRNAs and with antibiotics that target the protein-synthesis machinery (34, 35). Accordingly, a similar function to a protein engaged in binding is conceivable for GT-U32-CGD of GT-IU32 from *G. thermoleovorans* DSM 15325.

GT-IU32-CGD structural similarity to domains of translation factors interacting with RNA enabled to formulate the assumption that GT-IU32 are able to interact with nucleic acids specifically. The initial evaluation of GT-IU32 interaction with RNA was performed by filter binding assay using *E.* coli bulk tRNA as a potential interaction partner. GT-IU32 was able to interact with tRNA in a concentration dependent manner, K_d 2.86 ± 0.43 nM. The binding was specific as it was determined with competitor-binding assay using unlabeled tRNA as a competitor. The positive initial evaluation of GT-IU32 binding activity served as a basis for further peptidase interaction specificity analysis. For the in-depth specificity of GT-IU32 interaction with RNA characterization, the peptidases interaction as well as interaction specificity with 44 nt sarcin-ricin loop fragment, 26 bp dsRNA, polyuridylic acid, and a 27 nt ssRNA unable to establish secondary structure were tested. The ability of recombinant GT-IU32 to form complexes with dsDNA fragment of 136 bp in length was as well considered for the description. GT-IU32 *in vitro* was able to bind with all the tested nucleic acids including

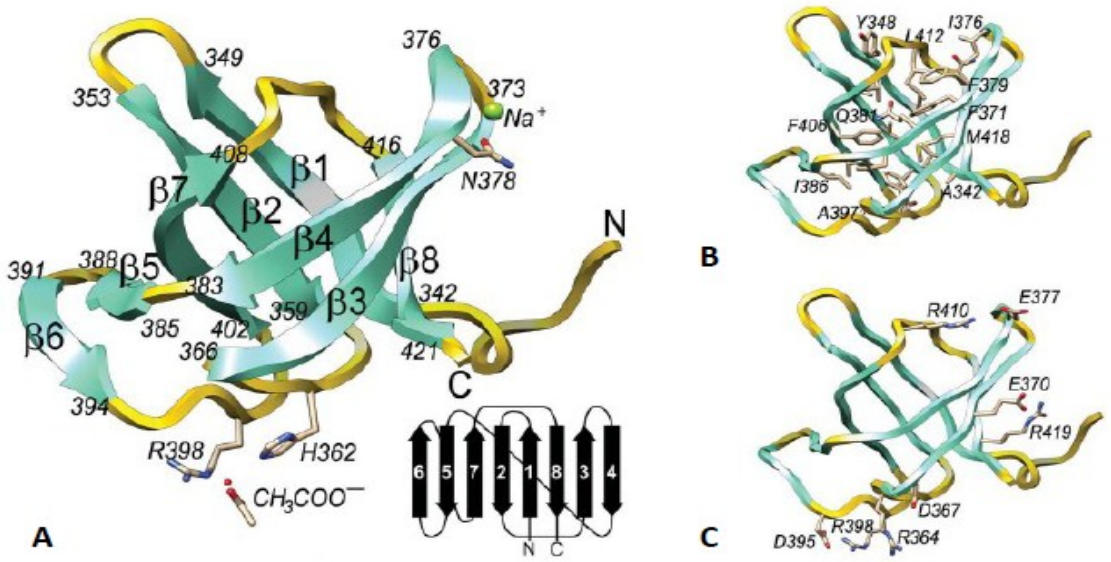

Fig. 10. Overall three-dimensional structure of GT-IU32-CGD. A: Ribbon-type plot of GT-U32-CGD with the eight *β*-strands labelled and marked with their flanking residues. Residues participating in ion binding are also shown and labelled. Inset (lower right): topology scheme of the protein. B: Cartoon showing the side chains participating in the central hydrophobic core. C As B but showing the charged residues of the protein engaged in salt bridges.

dsDNA fragment. However, GT-IU32 specifically interacted only with dsRNA. K_d 3.25 ± 0.19 nM of interaction with sarcin-ricin loop fragment was observed while GT-IU32 interaction affinity with 26 bp dsRNA fragment was slightly lower – K_d 3.97 ± 0.21 nM. The gel shift mobility assays completely confirmed the results of the GT-IU32 U32 peptidase binding activity specificity (Fig. 11). The detailed analysis of the shift patterns enabled to conclude that both GT-IU32 monomer and homodimer were able to demonstrate binding activity. GT-IU32 specificity for dsRNA sequence was not detected as characterized peptidase interacted with dsRNA of different sequence.

Taken together, the GT-IU32 substrate specificity, constitutive expression, localization as well as an ability to specifically interact with dsRNA suggests a role of the peptidase in bacteria housekeeping functions ensurance. It is reasonably possible that GT-IU32 native substrates are *G. thermoleovorans* DSM 15325 intracellular proteins with domains that are similar to collagen according structural organization. Perhaps functions of GT-IU32 native substrates are related to RNA functionality.

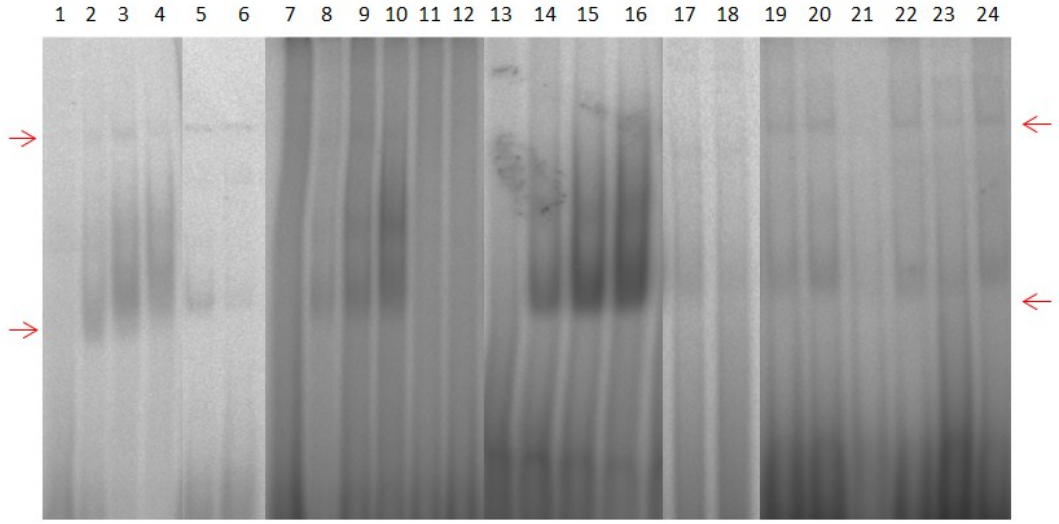

Fig. 11. Evaluation of GT-IU32 binding activity and binding activity specificity to tRNA, 44 nt sarcin-ricin loop fragment and 26 bp dsRNA fragment *in vitro* by electrophoretic mobility shift assay. 1: labeled tRNA (4 pM) control; 2-4: GT-IU32 (2-4-8 pM) binding to labeled tRNA (4 pM); 5-6: competition-binding effect of unlabeled tRNA (4-8 pM) into an GT-IU32-labeled tRNA complex (molar ratio 1:1, 4 pM); 7-12: as 1-6: except instead of tRNA 44 nt sarcin-ricin loop fragment was used for binding and binding specificity evaluation; 13-18: as 1-6: except instead of tRNA 26 bp dsRNA fragment was used for binding and binding specificity evaluation; 19: evaluation of competition-binding effect of unlabeled 44 nt sarcin-ricin loop fragment (4 pM) to GT-IU32-labeled tRNA complex (molar ratio 1:1, 4 pM); 20: evaluation of competition-binding effect of unlabeled 26 bp dsRNA fragment (4 pM) to GT-IU32-labeled tRNA complex (molar ratio 1:1, 4 pM); 21: evaluation of competition-binding effect of unlabeled tRNA (4 pM) to GT-IU32-labeled 44 nt sarcin-ricin loop fragment complex (molar ratio 1:1, 4 pM); 22: evaluation of competition-binding effect of unlabeled 26 bp dsRNA fragment (4 pM) to GT-IU32-labeled 44 nt sarcin-ricin loop fragment complex (molar ratio 1:1, 4 pM); 23: evaluation of competition-binding effect of unlabeled tRNA (4 pM) to GT-IU32-labeled 26 bp dsRNA fragment complex (molar ratio 1:1, 4 pM); 24: evaluation of competition-binding effect of unlabeled 44 nt sarcin-ricin loop fragment (4 pM) to GT-IU32-labeled 26 bp dsRNA fragment complex (molar ratio 1:1, 4 pM). Red arrows indicates GT-IU32 monomer and homodimer.

Conclusions

1. *G. thermoleovorans* DSM 15325 constitutively produced Pz-peptidases A and B homologues as well as bacillolysine homologue into the extracellular environment during the exponential growth phase under optimal growth conditions.
2. GT-SM3B is a thermostable zinc oligoendopeptidase able to form homodimers.
3. GT-SM3B monomer and homodimer has broad oligopeptidolytic activity specificity for collagen oligopeptides.
4. GT-IU32 was identified in the fraction of *G. thermoleovorans* DSM 15325 intracellular proteins by mass spectrometry. The constitutive expression of GT-IU32 during the exponential growth phase is not dependent on cultivation temperature of *G. thermoleovorans* DSM 15325.
5. Thermoactive GT-IU32 homodimer catalyze peptidolysis of denatured I type collagen. Homodimerization of GT-IU32 is ensured by disulfide bridges. GT-IU32 specifically *in vitro* interacts with dsRNA. Zinc ions increased thermostability of GT-IU32 monomer.
6. GT-IU32-CGD three-dimensional structure, when solved with high resolution, shows a compact distorted open β-barrel made up of eight antiparallel β-strands β-sheet. GT-IU32-CGD structure is similar to the structures of the domains of translation factors.

List of publications

1. Jasilionis A, Kuisiene N. 2015. Characterization of a novel thermostable oligopeptidase from *Geobacillus thermoleovorans* DSM 15325. J Microbiol Biotechnol 25:1070-1083.
2. Trillo-Muyo S, Jasilionis A, Domagalski MJ, Chrusz M, Minor W, Kuisiene N, Arolas JL, Sola M, Gomis-Rüth FX. 2013. Ultratight crystal packing of a 10 kDa protein. Acta Crystallogr D Biol Crystallogr D69:464-470.
3. Jasilionis A, Kaupinis A, Ger M, Valius M, Chitavichius D, Kuisiene N. 2012. Gene expression and activity analysis of the first thermophilic U32 peptidase. Centr Eur J Biol 7:587-595.

Jasilionis A, Kuisiene N. 2012. Collagenolytic peptidases from *Geobacillus thermoleovorans* DSM 15325. Science in Faculty of Natural Sciences: Articles of the 7th scientific conference, 5 October 2012. Vilnius: Vilniaus universiteto leidykla, 7:48-59.

1. Jasilionis A, Kuisiene N. 2015. Identification of peptidases generating constitutive collagenolytic activity of *Geobacillus thermoleovorans* DSM 15325, abstr FEMS-0763. Abstr 6th Congress of European Microbiologists. FEMS, Maastricht, The Netherlands.
2. Jasilionis A, Polacek N, Kuisiene N. 2015. Determination of the RNA binding activity of the U32 peptidase from *Geobacillus thermoleovorans* DSM 15325. J Biotechnol 208(Suppl):S45.
3. Jasilionis A, Kuisiene N. 2013. Purification and characterization of the novel thermostable oligopeptidase F homolog of *Geobacillus thermoleovorans* DSM 15325, abstr P-134. Abstr 5th Congress of European Microbiologists. FEMS, Leipzig, Germany.

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2014	merit-based scholarship Lithuanian Science Council
2013	merit-based scholarship Lithuanian Science Council
2011	ERASMUS practice scholarship Vilnius University
2010	merit-based scholarship Lithuanian Science Council
2010	merit-based scholarship Lithuanian Science Council

Articles

Jasilionis A, Kuisiene N. 2015. Characterization of a novel thermostable oligopeptidase from *Geobacillus thermoleovorans* DSM 15325. J Microbiol Biotechnol 25:1070-1083.

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Other publications

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spring semester /2015 – 2016	Enzymology, bachelor studies Vilnius University Faculty of Natural Sciences
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Reziumė

Baltymai – gyvybės struktūrinio bei funkcinio integralumo pagrindas, todėl proteoliziniai fermentai vieni svarbiausių gyvų organizmų fermentų. Siekiant nuodugniai suvokti gyvybės dėsningumus, būtina charakterizuoti proteolizinių fermentų įvairovės peptidazių struktūrą ir aktyvumo valdymą bei funkcinę svarbą. Kolagenas – vienas esminių struktūrinių organizmų baltymų, todėl kolagenolizės tyrimai išlieka aktualia tyrimų sritimi. Kolagenolizinių peptidazių gebėjimas hidrolizuoti kolageną užtikrina daugelį svarbių fiziologinių funkcijų. Kolagenolizinių peptidazių gebėjimas užtikrinti kolagenolizę *in vitro* svarbus biotechnologijai.

Eukariotų ir patogeninių prokariotų kolagenazės išlieka intensyviausiai tiriamomis kolagenolizinėmis peptidazėmis, tuo tarpu nepatogeninių prokariotų kolagenolizinių peptidazių įvairovės dėsningumai išlieka fragmentiškai charakterizuoti. Rengiant disertaciją atlikti tyrimai, siekiant charakterizuoti termofilų kolagenolizinį potencialą bei charakterizuoti termofilų kolagenolizinės peptidazes, kurios anksčiau netirtos.

Charakterizuotas *G. thermoleovorans* DSM 15325 gebėjimas konstitutyviai produkuoti kolagenolizines peptidazes, sudarančias katabolinę kaskadą, patvirtino termofilų gebėjimą būti nuolat pasiruošus įsisavinti kolageną. *G. thermoleovorans* DSM 15325 kolagenolizinių peptidazių pradinių kolageno įsisavinimo etapų įvairovės charakterizavimas taip pat leidžia papildyti kolageno skaidymo eigos modelį bei nuosekliai suvokti termofilinių bakterijų kolagenolizinio potencialo svarbą adaptaciniam plastiškumui.

M3 proteolizinių fermentų šeimos M3B pošeimio oligopeptidazės, sekretuojamos *G. thermoleovorans* DSM 15325, charakterizuotas esminių katalizinių, kinetinių bei substratinio specifiškumo savybių derinys atspindi oligopeptidazės svarbą termofilinės bakterijos mitybai prisitaikant prie aplinkos pokyčių. Charakterizuotos oligopeptidazės savybių derinys taip pat pažymi termostabilios hidrolazės biotechnologinio pritaikomumo potencialą.

U32 proteolizinių fermentų šeimos ilgosios grandinės peptidazės iš *G. thermoleovorans* DSM 15325 raiškos, katalizinės, termostabilumo bei struktūrinės charakteristikos leidžia objektyviau suvokti U32 proteolizinių fermentų šeimos peptidazių savybes ir funkcinę svarbą. U32 proteolizinių fermentų šeimos ilgosios grandinės peptidazės iš *G. thermoleovorans* DSM 15325 gebėjimas specifiškai *in vitro* sąveikauti su dgRNR, anksčiau charakterizuojant kolagenolizines peptidazes, nebuvo nustatytas.

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