VILNIUS UNIVERSITY

AUDRIUS GEGECKAS

CHARACTERIZATION AND APPLICATION OF KERATINOLYTIC PEPTIDASES FROM *GEOBACILLUS* SP. AND *BACILLUS* SPP.

Summary of doctoral dissertation
Biomedical sciences, biology (01B)

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VILNIAUS UNIVERSITETAS

AUDRIUS GEGECKAS

GEOBACILLUS SP. IR *BACILLUS* SPP. SEKRETUOJAMŲ KERATINOLIZINIU AKTYVUMU PASIŽYMINČIŲ PEPTIDAZIŲ CHARAKTERIZAVIMAS IR PRITAIKYMAS

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Table of contents

INTRODUCTION

Solid keratin-rich waste management is one of essential research area in nowadays. Conventional chemical and high thermal keratin waste decomposition methods are fully explored and not enough effective for future biotechnology perspectives [1]. However, traditional keratin-rich waste decomposition methods could be replaced by environmentally-friendly and economical microbial keratin waste biodegradation methods without energy wastage and essential amino acids and nutrition elements loss [2]. Therefore, microbial bio-decomposition are attractive approach to keratin or keratin-like waste manage without any nutrition loss in eco-friendly process environment.

Keratinolytic peptidases or keratinases (EC 3.4.21/24/99.11) are a particular class of proteolytic enzymes that displays the capability of degrading insoluble substrates such as fibrin, keratin, elastin, collagen and soluble substrates such as sodium caseinate, albumin and gelatin or other keratin-like substrates with different efficiency [3]. Keratinolytic peptidases are produced by various microorganisms including bacteria, actinomycetes and fungi and belongs to metallo and serine or metallo-serine proteases based on their catalytic type with precedence for aromatic and hydrophobic residues at the P1 position [1, 4, 5].

Keratin is the insoluble fibrous hard-to-degrade protein of feathers, wool, hair including other epidermal appendages which can be degraded by keratinolytic peptidases [6, 7]. The recalcitrance of keratins derives from compact packing of α-helices (α-keratins, 40-60 kDa) or β-sheets (β-keratin, 10 kDa) into a supercoiled polypeptide chains. Compact structure is stabilized by high degree of cross-linking disulfide bonds, hydrogen bonds and hydrophobic interactions. These proteins show high stability and resistance to proteolytic enzymes such as trypsin, pepsin or papain [6, 8].

Increased attention has been diverted to these keratinolytic peptidases because of their important potential uses in biomedicine, pharmaceutics, cosmetics and keratin waste bioconversion industries associated to the hydrolysis of keratin [1]. Keratinolytic proteinases are next generation molecular tools for keratin hydrolysis and production of small value-added peptides. It is important to find suitable biocatalysts with proper

physical and chemical properties for effective keratin-rich waste biodegradation and replace current not enough efficient biocatalysts.

Unfortunately, most naturally produced enzymes are not effective or suitable for biotechnology processes. For decades, protein engineering has been utilized to modify natural enzymes to meet the needs of different industrial biotechnology. It is therefore a powerful tool in synthetic biology through the altering of enzyme properties to suit the requirements of any hydrolysis process. Enzyme modification or construction of chimeric proteins open up new possibilities for industrial application [9, 10].

The aim of the dissertation work is to evaluate application possibilities of a newly identified keratinolytic peptidases in biodegradation processes of proteinaceous materials.

The following **tasks** have been formulated to achieve this aim:

- To evaluate physico-chemical properties and enzymatic activity of keratinolytic peptidases from thermophilic and mesophilic microorganisms;
- To evaluate the hydrolysis efficiency of proteinaceous waste by keratinolytic peptidases from thermophilic and mesophilic microorganisms;
- To design chimeric keratinolytic biocatalysts by protein engineering methods;
- To create laboratory-scale raw keratin waste biodegradation system.

Scientific novelty:

For the first time, we identified, purified and characterized native keratinolytic peptidase from *Bacillus thuringiensis* AD-12 (BtKER). Similarly, we analyzed and evaluated features of three unique enzymes from *Geobacillus* sp. AD-11 (RecGEOker), *Bacillus* sp. AD-W (BPKer) and *Bacillus* sp. AD-AA3 (BAKer) for industrial application. For the first time, we created chimeric (SynKer-TT and SynKer-TM) keratinolytic peptidases with improved physicochemical properties. Eventually, we demonstrated that metabolically active secretomes from keratinolytic microorganisms can be used for eco-friendly enzymatic keratin waste management.

Practical value:

Our findings and results expand scientific knowledge about keratinolytic peptidases produced by thermophilic and mesophilic microoganisms. Our created keratin and other proteinaceous material biodegradation system is particularly relevant to industrial biotechnology associated with conversion of large scale wastes to value-added products.

MATERIAL AND METHODS

Chemicals. All chemicals were of analytical reagent grade or higher quality and were purchased from Merck, Sigma-Aldrich and AppliChem, unless otherwise stated. All enzymes were purchased from Thermo Fisher Scientific, unless otherwise stated.

Strains. AD-11, AD-12, AD-W and AD-AA3 bacterial strains were identified by author. *E. coli* DH5α, *E. coli* BL21 (DE3) and *E. coli* Rosetta (DE3) pLysS were purchased from Novagen. *E. coli* BW25113 was obtained from prof. dr. Edita Sužiedėlienė.

Plasmids. Cloning plasmid vector used in this work: pTZ57R/T (Amp^r). Expression vectors used in this work: pET-28c(+) (Kan^r), pET-21c(+) (Amp^r) and pBAD30 (Amp^r, gift from prof. dr. Edita Sužiedėlienė).

Oligonucleotides. All oligonucleotides (Metabion) used in this work are listed in Table 1.

Table 1. Summary of oligonucleotides used in this work.

Name	Sequence (5' → 3')	Target
27F	GAG AGT TTG ATC CTG GCT CAG	16S rDNA
1495R	CTA CGG CTA CCT TGT TAC GA	16S rDNA
GeoKer-proF	GGA AAG CCG CAA AGA GGG AAA AAC GGA AA	*geoker*
GeoKer-proR	CGG GAC GCG TGA CGT CAG AAA CTT AA	*geoker*
GeoKerF	CAT ATG AAG GAA AGA TCA ATG GTG TGG GGC (*Nde*I)	*geoker*
GeoKerR	CTC GAG TTA ATA CAC TCC AAC CGC ATT GAA (*Xho*I)	*geoker*
GeoKer2F	AAA ACA TAT GGT CGC CGG CGC GTC GAC G (*Nde*I)	*geoker2*
GeoKer3F	CAT ATG ATT TTT ACG TAT GAC GGA CGA AAC CGC (*Nde*I)	*geoker3*
GeoKer4F	CAT ATG AAA AAT GTG CAC GGC CGG (*Nde*I)	*geoker4*
BtKER-proF	ATA TAG TTG AAA AGG AAT GCG ACA TT	*btker*
BtKER-proR	GTA ATA TAG TTT GTA AGA AGG AGA GCC	*btker*
BacKerNde32F	GCG CCA TAT GGA AAC ATC ATC TAC TGA TTA CG (*Nde*I)	*btker*
BacKerSTOPXho31R	GCG CCT CGA GTT AAG AGG CTT TAT TTT CTT G (*Xho*I)	*btker*
BacKerXho31R	GCG CCT CGA GAG AGG CTT TAT TTT CTT GTA A (*Xho*I)	*btker*
BacKerEcoR31F	GCG CGA ATT CGA AAC ATC ATC TAC TGA TTA C (*Eco*RI)	*btker*
BacKerSTOPXba31R	GCG CTC TAG ATT AAG AGG CTT TAT TTT CTT G (*Xba*I)	*btker*

TT2	GCG CGA GCT CAC TTC CTC CAT ACA CTC CAA GCG CAT T (*Sac*I)	*geoker*
TT3	GCG CGA GCT CAA GGA AAG ATC AAT GGT GTG G (*Sac*I)	*geoker*
TM1	GCG CGA GCT CGA AAC ATC ATC TAC TGA TTA C (*Xho*I)	*btker*

Underlined sequences indicate the restriction sites.

Bacterial growth conditions. Solid keratin medium (B) containing NH_4Cl, 0.05 %; NaCl, 0.05 %; K_2HPO_4, 0.03 %; KH_2PO_4, 0.03 %; yeast extract, 0.01 %; keratin from wool (TCI Europe), 0.1 %; $MgSO_4$, 2 mM; agar 1.8 %, pH 7.8 was used for isolation of keratinolytic peptidase producing bacterial strains. The bacterial isolates were purified and checked for keratinase production. Stock culture of the bacterial isolates were maintained in 15 % glycerol stored at -70 ºC.

Inoculum (preculture) preparation:

Geobacillus sp. AD-11 strain: cells from solid medium are inoculated to 5 mL of the A medium containing K_2HPO_4, 0.3 %; NaCl, 0.05 %; NH_4Cl, 0.1 %; glucose, 0.25 %; Tris, 0.6 %; yeast extract, 0.5 %; peptone, 1.5 %; $MgSO_4$, 2 mM; $CaCl_2$, 0.1 mM; pH 7.8. Culture was incubated for 14-16 h at 55 ºC with agitation 200 rpm.

Bacillus sp. AD-12 strain: cells from solid medium are inoculated to 50 mL of the LB medium. Culture was incubated for 12-16 h at 30 ºC with agitation 150 rpm.

Bacillus sp. AD-W strain: cells from solid medium are inoculated to 50 mL of the LB medium. Culture was incubated for 12-16 h at 30 ºC with agitation 180 rpm.

Bacillus sp. AD-AA3 strain: cells from solid medium are inoculated to 50 mL of the LB medium. Culture was incubated for 12-16 h at 30 ºC with agitation 180 rpm.

Cells cultivation:

Geobacillus sp. AD-11 strain: keratinolytic peptidase production was carried out in 2 L of previously described liquid medium A and distributed in 500 mL Erlenmeyer flasks. Each flask contained 100 mL of the A medium and inoculated with 1 % of the preculture (3×10^5 cfu mL^{-1}). Flasks were incubated for 12-14 h at 60 ºC with agitation 180 rpm. The culture supernatant was recovered by centrifugation two times at 8500 × *g* for 15 min at 4 °C.

Geobacillus sp. AD-11 strain: peptidase production was carried out in 2 L of previously described liquid medium B and distributed in 500 mL Erlenmeyer flasks. Each flask contained 100 mL of the B medium and inoculated with 1 % of the preculture (3×10^5 cfu mL^{-1}). Flasks were incubated for 80-92 h at 60 ºC with agitation 180 rpm.

The culture supernatant was recovered by centrifugation two times at 8500 × *g* for 15 min at 4 °C.

Bacillus sp. AD-12 strain: peptidase production was carried out in 2 L of previously described liquid medium B (pH 7) and distributed in 500 mL Erlenmeyer flasks. Each flask contained 100 mL of the B medium and inoculated with 1 % of the preculture. Flasks were incubated for 80-96 h at 30 ºC with agitation 180 rpm. The culture supernatant was recovered by centrifugation at 8500 × *g* for 15 min at 4 °C.

Bacillus sp. AD-W strain: peptidase production was carried out in 2 L of previously described liquid medium B (pH 8) and distributed in 500 mL Erlenmeyer flasks. Each flask contained 100 mL of the B medium and inoculated with 1 % of the preculture. Flasks were incubated for 48 h at 30 ºC with agitation 150 rpm. The culture supernatant was recovered by centrifugation at 8500 × *g* for 15 min at 4 °C.

Bacillus sp. AD-AA3 strain: peptidase production was carried out in 2 L of previously described liquid medium B (pH 7) and distributed in 500 mL Erlenmeyer flasks. Each flask contained 100 mL of the B medium and inoculated with 1 % of the preculture. Flasks were incubated for 48 h at 30 ºC with agitation 150 rpm. The culture supernatant was recovered by centrifugation at 8500 × *g* for 15 min at 4 °C.

Protein precipitation. All steps were carried out at 4 ºC. After all strains cultivation in previously described liquid medium A or B the culture medium was centrifuged at 8500 × *g* for 15 min and the supernatant was filtered through filter paper to obtain the crude enzyme filtrate. The filtrate containing the keratinolytic activity was collected. Proteins present in the crude enzyme filtrate were precipitated by using solid ammonium sulfate at desired saturation level. Precipitated proteins were collected by centrifugation at 20000 × *g* for 20 min. The pellet was dissolved in a 10 mL buffer (50 mM Tris-HCl, pH 7.8) and dialyzed in dialysis tubes (3.5K MWCO, Thermo Fisher Scientific) overnight against the same buffer for 24 h and the buffer was changed at 8 h intervals. After dialysis crude extract was concentrated with PEG 35000 to obtain ~2 mL final protein extract.

DNA manipulation. Genomic DNA of AD-11, AD-12, AD-W and AD-AA3 was extracted according to the method described by Sambrook and Rusell [11]. Plasmid DNA from *E. coli* transformants was isolated with the GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific). Gene JET™ Gel Extraction Kit (Thermo Fisher Scientific)

was used to recover DNA fragments from agarose gels. Electrocompetent *E. coli* strains preparation, ligation of DNA and all molecular methods were performed using standard molecular biology techniques [11].

Gene and protein sequence analysis. Chromosomal DNA of investigated organisms was used as template for amplifying the 16S rRNA gene. The 16S rRNA gene was amplified by PCR using primers 27F and 1495R. The PCR process was carried out according to Kuisiene *et al* [12]. The purified amplicons were sequenced at the Institute of Biotechnology (Vilnius, Lithuania). The DNA and protein sequences similarities were assessed by BLASTn (Basic Local Alignment Search Tool for Nucleotides) and BLASTp (Basic Local Alignment Search Tool for Protein), respectively [13, 14]. Multiple sequence alignments were performed with ESPript 3.0 [15]. A phylogenetic tree was constructed by using the neighbor-joining (NJ) algorithm in MEGA 7.0 [16-19]. Each dataset was bootstrapped 1000 times. The signal peptide sequence was analyzed by SignalP (www.cbs.dtu.dk/services/SignalP/) [20]. Possible transcription promoters were analysed with Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html) [21].

SDS-PAGE analysis. Molecular weight (MW) and protein analyses were determined by 12 % SDS-PAGE using the Laemmli method [22]. A broad range of protein standards PageRuler™ Unstained Protein Ladder (Pierce) and PageRuler™ Unstained Low Range Protein Ladder (Pierce) were used as a molecular mass markers. After electrophoresis gels were stained with PageBlue™ Protein Staining Solution (Pierce). Low and ultra low molecular weight SDS-PAGE (LMW-T-EG-PAGE and uLMW-T-EG-PAGE) were prepared and performed according to Thermo Fisher Scientific recommendations. Tricine SDS-PAGE was performed according to Schagger [23].

Zymography. Keratinolytic activity was determined on SDS-PAGE gels using keratin from wool as a substrate. After electrophoresis renaturation procedure was carried out following Fakhfakh-Zouari *et al* [24]. The renaturated gel was placed into Petri dish and covered with 50 mL B-agar medium. The gel was incubated overnight at desired temperature. The molecular weight of hypothetical keratinolytic peptidase was determined by the comparison of stained gel and the obtained zymogram.

Mass spectrometry and protein identification. Proteomic analysis was carried out at the Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics,

Polish Academy of Sciences, Warsaw. Gel bands of interest were excised, followed by in-gel trypsin digestion. Resulting peptides were separated on nano-LC, directly coupled with ESI-LTQ-Orbitrap mass spectrometer. Proteins were identified by Mascot (Matrix Science) searching mass spectrometry data against NCBInr database.

Assay of keratinase activity. Keratinase assay was done according to Cai *et al* [25] using keratin azure (Sigma-Aldrich) or keratin from wool as the substrate. The 4 mg of substrate was suspended in a total volume of 1 mL buffer (50 mM Tris-HCl at desired pH). The reaction was initiated by the addition of 10 U of enzyme. The reaction tubes were incubated at desired temperature for 1 h and the reaction was stopped by the addition of 400 μL of 10 % trichloroacetic acid (TCA). After 15 min at 4 ºC, the tubes were centrifugated for 5 min at 16000 × *g* at 25 ºC. The supernatant was spectrophotometrically measured for release of the azo dye at 595 nm or 280 nm for keratin from wool. The control was prepared by previously described method without addition of the enzyme. One unit (U) of keratinase activity was defined as the amount of enzyme causing 0.001 absorbance increase between sample and control under the standard conditions.

Biochemical characterization of keratinolytic peptidases. A range of various temperature (20-90 ºC) was used for determining optimum temperature. Similarity, optimum pH was determined by assaying the enzyme at different pH buffers (pH 5-6, 50 mM piperazine; pH 7-9, 50 mM Tris-HCl; pH 10-11, 50 mM sodium bicarbonate; 12 pH, 50 mM potassium chloride/NaOH). Thermostability and pH stability were measured by incubating the enzyme at different temperatures and pH, respectively. The effects of various metal ions, detergents, organic solvents and inhibitors on keratinolytic activity were investigated by pre-incubating the enzyme with these chemicals for 1 h at 25 ºC. Keratinolytic peptidase activity determined without additions was considered as 100 %.

Substrate specificity. The specificity of the keratinolytic peptidases toward various substrates including bovine serum albumin (BSA), Na-caseinate, gelatin (Bio-Rad), keratin from wool and collagen (Proteina) was determined on the basis of the previously described standard assay conditions. One unit (U) of keratinase activity was defined as described previously.

Gene cloning. Primers were constructed according to results obtained from mass spectrometry and database analysis for *Geobacillus* sp. AD-11 and *Bacillus* sp. AD-12,

respectively. First set of primers (GeoKer-proF/GeoKer-proR and BtKER-proF/BtKER-proR for AD-11 and AD-12, respectively) was used to amplify target gene with upstream and downstream sequence fragments for further gene analysis. Second set of primers (GeoKerF/GeoKerR and BacKerNde32F/BacKerSTOPXho31R (BacKerXho31R) for AD-11 and AD-12, respectively) was used to amplify target gene with complete ORF without signal sequence and incorporate *Nde*I and *Xho*I restriction sites for cloning into expression vectors. Obtained PCR products were purified using a GeneJET^{TM} PCR Purification Kit and ligated with pTZ57R/T cloning vector. The ligated product was transformed into *E. coli* DH5α cells using a standard protocol [11]. Recombinant clones were detected by direct blue/white screening. For target protein expression, the gene fragment was cleaved from pTZ57R/T plasmid with *Nde*I and *Xho*I restriction enzymes and ligated into the pET expression vector digested with the same restriction enzymes and dephosphorylated with alkaline phosphatase (Thermo Fisher Scientific). The resulting expression plasmid with target gene was transformed into *E. coli* DH5α cells and then retransformed into *E. coli* BL21 (DE3) or *E. coli* Rosetta (DE3) pLysS cells for protein expression. The clones were screened for the positive recombinants using colony PCR followed by double digestion with *Nde*I and *Xho*I restriction enzymes. The recombinant plasmid containing the target keratinase gene was confirmed by sequencing.

Gene expression. A transformant of *E. coli* BL21 (DE3) and *E. coli* Rosetta (DE3) pLysS harboring pET plasmid with inserted target gene was incubated at 37 ºC overnight with agitation 180 rpm in LB medium containing appropriate antibiotic. 1 % of seed culture was transferred into fresh medium and cultured at the same conditions until OD_{600} reached 0.4 (unless otherwise stated), then IPTG (to a final concentration of 0.01-5 mM) was added for recombinant protein induction. The bacterial cells were cultivated at desired temperature for another 5 hours (unless otherwise stated) before harvesting by centrifugation. For protein expression visualization cells samples obtained at different times after induction were adjusted so that their OD_{600} value were 0.4, mixed with 4× SDS-PAGE sample loading buffer and subjected to SDS-PAGE analysis. The negative control was prepared by previously described method using cells without IPTG induction.

Protein purification. *Purification of recombinant proteins:* the recombinant proteins were purified by affinity chromatography using immobilized nickel ions

(Profinity™ IMAC Resins, BIO-RAD). Cells were collected after 2× culture centrifugation at 8500 × *g* for 15 min at 4 °C in 50 mM Tris-HCl (pH 7.8). Cells were suspended in buffer A (50 mM Tris-HCl (pH 8)) at 1:4 buffer cells ratio. Supernatant and cell debris with formed inclusion bodies were collected after sonication (10 s : 20 s) of the induced cells. The column with resin was prepared and equilibrated following the manufacturer's purification protocol with slightly modifications. The bounded target protein was eluted from the column by passing buffer A (with 6 M urea) having a range of 50-500 mM imidazole. The fractions were collected and analyzed on SDS-PAGE.

Purification of native proteins: obtained keratinolytic peptidase extract was applied to DEAE-Sepharose (GE Healthcare) column (2.5 × 12 cm) equilibrated with the buffer B (25 mM Tris-HCl, pH 8). The column was washed with same buffer and eluted with step wise buffer B containing 100 mM, 250 mM, 500 mM, 750 mM and 1 M NaCl. The active fractions were pooled and dialyzed against the buffer A for 24 h. Gel filtration chromatography was performed using Bio-Gel P-60 (BIO-RAD) column (1 × 60 cm) equilibrated with buffer B1 (50 mM Tris-HCl, pH 8). The above concentrated sample was loaded on to the column followed by elution with same buffer with fraction size of 1.5 ml. The eluted active fractions were pooled and concentrated with PEG 35000 to 2 mL.

Thin-layer chromatography: 10 mg of keratin from wool was incubated with 10 U of peptidase in a total volume of 1 mL buffer (50 mM Tris-HCl) at desired temperature and pH for 12 h. The samples were collected and analyzed on silica plate (ProteoChrom® HPTLC plates, Merck) by Thin-layer chromatography (TLC) according to manufacturer's protocol and recommendations using solvent system: 2-butanol:pyridine:acetic acid:water (30:20:6:24). The documentation and evaluation of the TLC plate was done using UV visualizer under UV, using fluorescamine.

Statistical analysis. All data were analyzed using GraphPad Prism 5.0. Values are expressed as means ± standard deviation of results from three independent experiments. Data were considered as statistically significant for *P* values of 0.05 or less.

RESULTS AND DISCUSSION

1. Keratinolytic peptidases produced by thermophilic and mesophilic microorganisms

1.1. Isolation and identification of keratinolytic bacterial strains

57 thermophilic bacterial isolates were selected from collection of microorganisms (Department of Microbiology and Biotechnology, Faculty of Natural Sciences, Vilnius university, Lithuania). 19 mesophilic bacterial isolates were recovered from soil in Lithuania (Vilnius and Palanga). Their keratinolytic activities were tested by growing the isolates on keratin meal plates. The largest clearing zone on keratin was observed for AD-11 (thermophile) and three mesophilic isolates: AD-12, AD-W and AD-AA3.

16S rDNA phylogeny studies were carried out. The partial 16S rDNA sequences were determined and compared to the GenBank database in the National Center for Biotechnology Information (NCBI) using BLAST program. The AD-11 isolate was found to belonging to the genus *Geobacillus stearothermophilus* with 99 % homology (data not shown). The isolate was assigned to *Geobacillus* sp. AD-11.

Similarly, AD-12 isolate was found to belonging to the *Bacillus cereus* group with 99 % homology (data not shown). AD-12 isolate was further identified to be *Bacillus thuringiensis* based on parasporal crystal formation observed under optical and electron microscope. The isolate was assigned to *Bacillus* sp. AD-12. Additionally, AD-W and AD-AA3 isolates were found to belonging to *Bacillus altitudinis* DSM 21631^{T} / *Bacillus stratosphericus* MTCC 7305^{T} and *Bacillus amyloliquefaciens* DSM 7^{T}, respectively. Therefore, AD-W and AD-AA3 were assigned to *Bacillus* sp. AD-W and *Bacillus* sp. AD-AA3, respectively.

The amplified 16S rDNA gene sequences of all strains were sequenced and have been submitted in GenBank under Accession Nos. listed in Table 2.

Table 2. Accession numbers of 16S rDNA sequences.

Strain	Accession Nos.
AD-11	KJ789443
AD-12	KJ636471.1
AD-W	KU950739
AD-AA3	KU950740

1.2. Production and mass spectrometry analysis of peptidase from *Geobacillus* sp. AD-11

Keratinolytic peptidase are largely produced in a synthetic B medium with keratinous substrate as the sole source of carbon and nitrogen and basal A medium. The native keratinolytic peptidase (NatGEOker) was successfully produced using previously described induction medium B and non-induction medium A. The crude protein extract was characterized by SDS-PAGE and zymography methods (Fig. 1.).

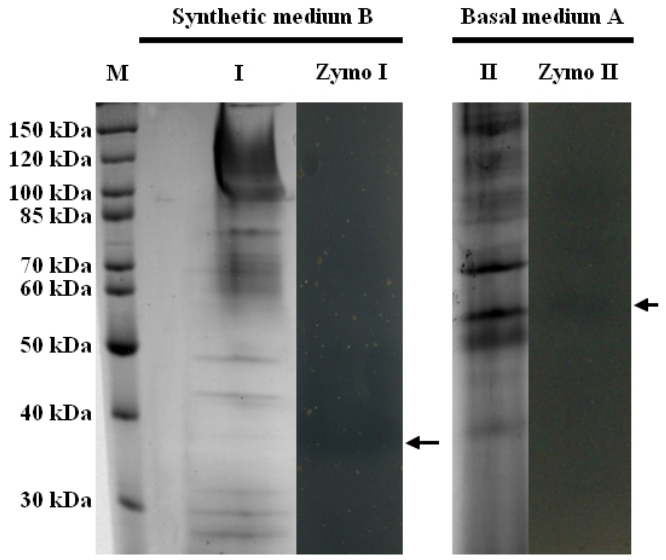

Fig. 1. SDS-PAGE and zymogram analysis of NatGEOker. M – PageRuler^{TM} Unstained Protein Ladder. I – secretome profile from B medium. II – secretome profile from A medium. Zymo I and Zymo II – peptidase analysis by zymography. Black arrows indicate zones of hydrolysis.

The bands between 30-40 kDa (Fig. 1. ; Zymo I) and 50-60 kDa (Fig. 1.; Zymo II) from SDS-PAGE were subjected to ESI-LTQ-Orbitrap analysis. 138 (from medium A) and 73 (from medium B) peptide mass fingerprints of the hypothetical keratinolytic peptidase were obtained from the MS analysis. NCBInr and SwissProt protein sequences databases were used in the MASCOT search tool to look for proteins that match the peptide spectrum of the NatGEOker. The peptide analysis showed similarity to two proteolytic enzymes from *Geobacillus* sp. strains: 99 % similarity to thermolysin (M04.001, GeneBank: M21663.1) and 98 % simalarity to bacillolysin (M04.014, GeneBank: CP008934.1). Thermolysin-like peptidases (TLP) are synthesized as a precursor with a propeptide. Maturation of TLP occurs through autoprocessing and this processing pathway is mediated by the propeptide [26-28].

It was determined that in both medium was produced the same proteolytic enzyme – thermolysin-like peptidase. The obtained gene product was assigned to GEOker (*Geobacillus* keratinase).

1.3. Cloning and bioinformatic analysis of GEOker gene

The first set of primers (GeoKer-proF/GeoKer-proR) was constructed according to similar sequences obtained from NCBI GenBank databases. Primers were used to amplify target gene with upstream and downstream sequence fragments for further bioinformatic analysis. The open reading frame (ORF) of GEOker is 1638 bp length which encodes a protein of 546 amino acids with 59761.72 Da molecular weight and an isoelectric point (pI) of 5.375. According to the SignalP 4.1 web server (http://www.cbs.dtu.dk/services/SignalP/; [20]), a 25 a. a. stretch with the features of the typical *Bacillus* and *Geobacillus* signal peptides at the N- terminal region of the protein was found. The putative signal peptide (prepeptide) cleavage site was predicted to be between Ala25 and Lys26. This results indicates that the proenzyme is composed of 521 a. a. with 57231.60 Da molecular weight and an isoelectric point is 5.47. Mature enzyme (after autoprocessing) is composed of 319 a. a. with 34685.03 Da and pI 5.47 (Fig. 2.).

Described GEOker has consensus zinc-binding $H^{372}ELTH^{376}$ (the positions are indicated without the cleavage of the signal peptide) motif and Glu373 and His461 amino acids are essential form enzymatic reaction. This TLP belongs to zinc metalloproteinases.

The required gene targets were amplified using second sets of primers constructed according to sequence analysis (Fig. 3.). Amplicons were purified and cloned into pTZ57R/T cloning vector. Then the target gene fragments were cleaved from pTZ57R/T vector and ligated into pET-28c(+) expression vector. The constructed plasmids containing GEOker gene fragments were used for target recombinant peptidase expression.

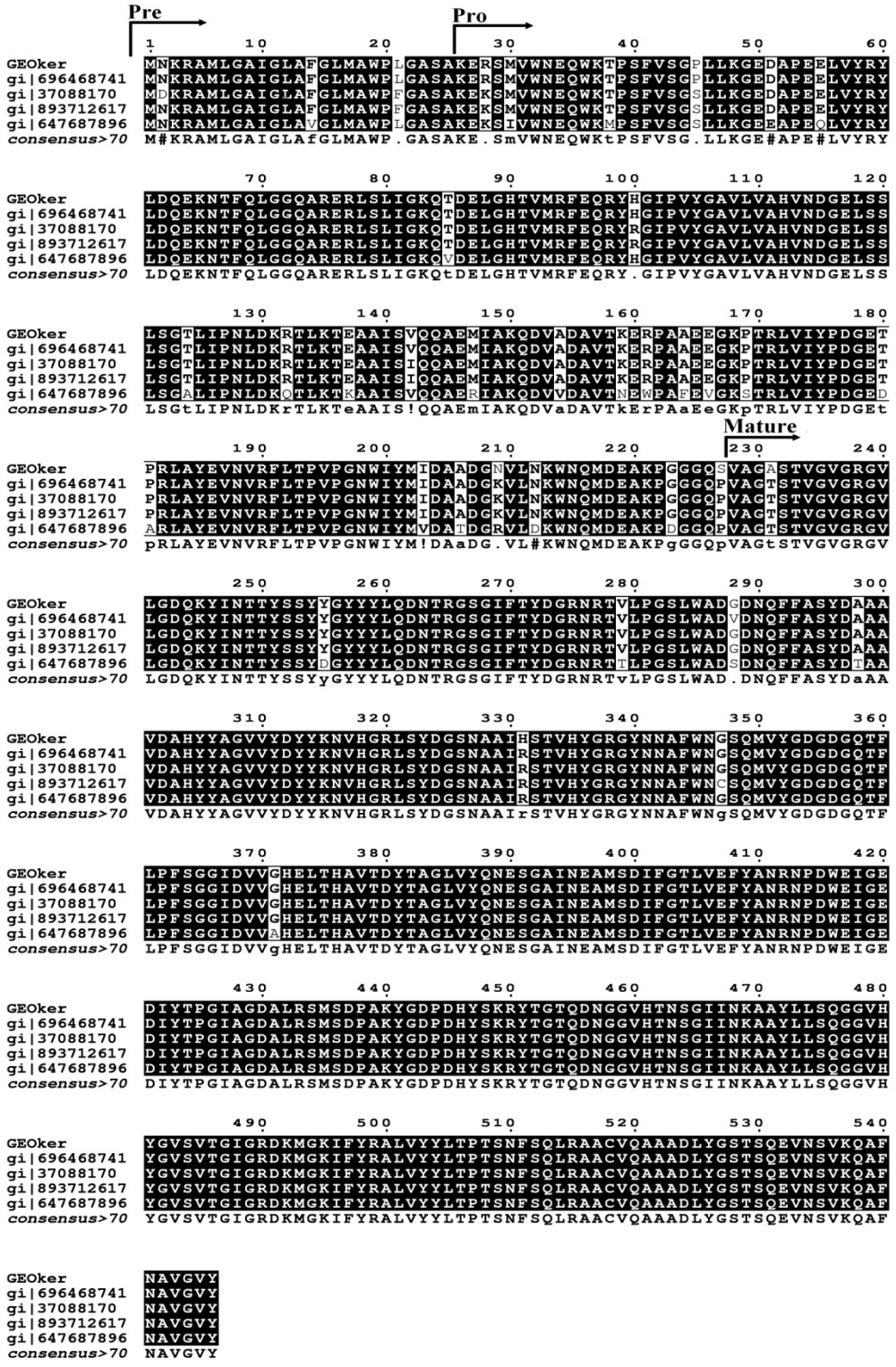

Fig. 2. Amino acids sequence alignment of GEOker from *Geobacillus* sp. AD-11 with other *Bacillus* and *Geobacillus* peptidases. The used peptidases are: gi|696468741 – bacillolysin (*G. stearothermophilus*); gi|37088170 – thermolysin, thermostable neutral protease (*B. caldolyticus*); gi|893712617 – alkaline metalloprotease (*Bacillus subtilis*); gi|647687896 – bacillolysin (*Geobacillus thermocatenulatus*). The putative starting residues of prepeptide, propeptide and mature GEOker are indicated. The figure was produced with ESPript 3.0 [15].

Fig. 3. GEOker sequence. Black arrows indicate different protein variants.

1.4. Expression and purification of the GEOker proteins

pET-28c(+) construct harboring *geoker1* gene fragment was transformed into *E. coli* BL21 (DE3) cells and protein expression was monitored and analyzed by SDS-PAGE. Analysis showed the appearance of a large amount of new protein in *E. coli* cells sample after induction with IPTG (Fig. 4A).

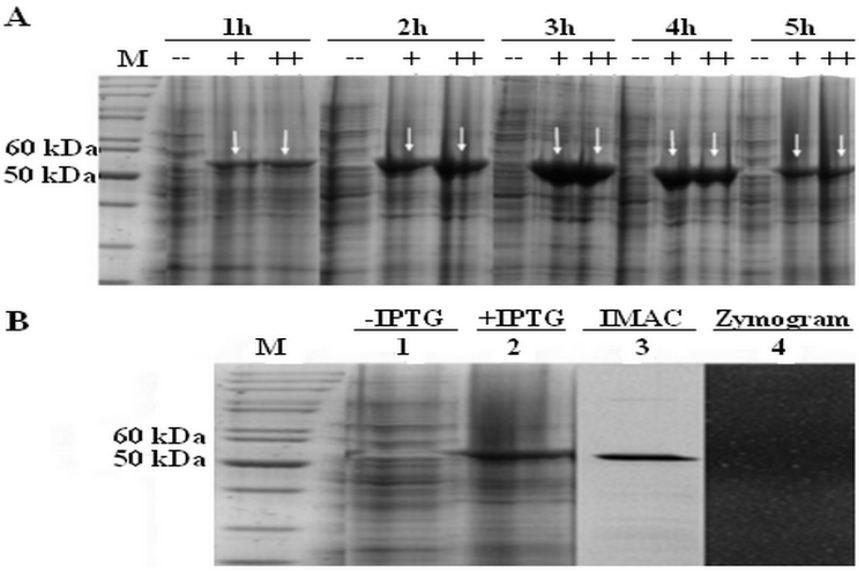

Fig. 4. SDS-PAGE analysis of expression (A) and purification (B) of GEOker1. (A) GEOker1 expression profiles at 1 h to 5 h. (--) – without IPTG addition. (+) – with 0.5 mM IPTG addition. (++) – with 1 mM IPTG addition. White arrows indicate target GEOker1 protein. M – PageRuler^{TM} Unstained Protein Ladder. (B) purification of GEOker1 and zymogram analysis. M – PageRuler^{TM} Unstained Protein Ladder. Lane 1 – proteins profile without IPTG induction. Lane 2 – proteins profile with 0.5 mM IPTG induction. Lane 3 – purified GEOker1 with IMAC. Lane 4 – zymogram of the purified GEOker1.

The size of this new expressed protein GEOker1 agreed well with predicted size of GEOker approximately 57 kDa. The optimum expression level was detected at 3 h post-induction with 0.5 mM IPTG at 30 °C. It was noted that the protein expression level decreased 4 h post-induction. For the large-scale preparation of GEOker1 as a biocatalyst for wide biotechnology application, expression must be performed for no longer than 3 h after induction. Unfortunately, expression of other GEOker protein variants (GEOker2, GEOker3 and GEOker4) was not detected.

The target recombinant N-terminus His-tagged GEOker1 protein was purified using immobilized Ni^{2+} metal affinity chromatography (IMAC). The purity of protein was analyzed by SDS-PAGE and keratinolytic activity was determined by zymography (Fig. 4B). obtained protein band approximately at 57 kDa indicated the purification of the target GEOker1. The recombinant protein was eluted from Ni^{2+} resin at 100 mM of imidazole and observed as a single band on SDS-PAGE. The zymogram analysis further confirmed the presence of active keratinolytic peptidase. The purified recombinant GEOker1 (RecGEOker) protein showed specific keratinolytic activity of 1437.6±7.5 U mg^{-1} with an overall yield of 61.2 % and a purification fold of 6.2.

1.5. Biochemical characterization of RecGEOker

The purified RecGEOker showed a typical bell-shaped curve with the keratinolytic activity in the temperature range of 20-90 °C with the maximal activity at 60 °C (Fig. 5A) It was shown that thermolysin from *G. stearothermophilus* ATCC 31197^{T} displayed maximal activity at 75 °C [29]. The activity rapidly decreased at lower temperatures. At 40-50 °C the activity of RecGEOker was reduced to 75 % and 33 %, respectively. At 70-80 °C the activity was reduced to 87 % and 71 %, respectively. The effect of temperature on the stability of purified RecGEOker was examined by measuring the remaining activity after incubation for 1-4 h at various temperatures at pH 8 (Fig. 5B). After 4 h incubation at 60 °C the enzyme showed no loss of activity. The purified RecGEOker retained higher than 50 % remaining activity after incubation for 2 h at 70-80 °C and retained lower than 20 % activity after 4 h incubation at the same temperature. These results suggested that the purified RecGEOker is stable at higher temperatures and belongs to thermostable thermolysin-like proteinases [30].

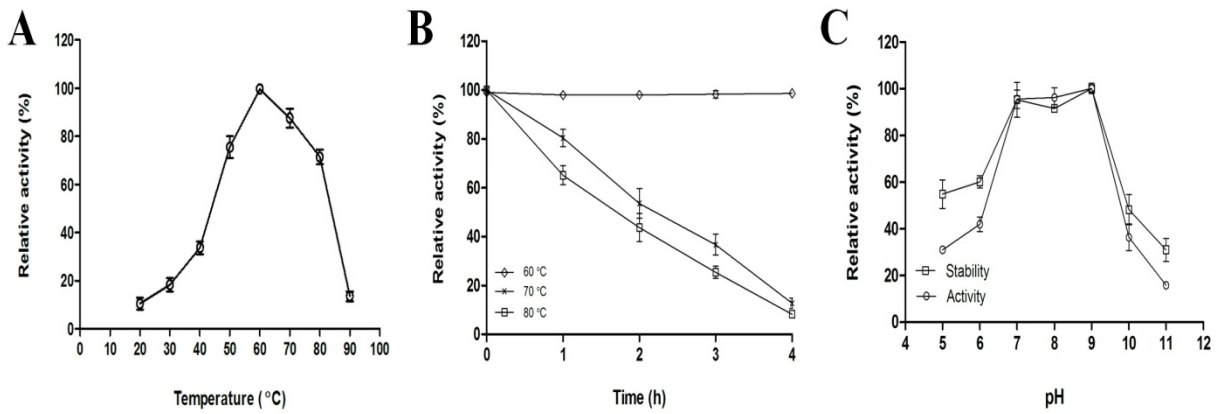

Fig. 5. Biochemical characterization of RecGEOker. (A) Effect of temperature on the activity. (B) Effect of temperature on the stability. (C) Effect of pH on activity and stability. Values represent the mean of three replicates.

The purified peptidase displayed keratinolytic activity within a broad pH range of 5-11 with an optimum at pH 9 (Fig. 5C). The activity rapidly decreased at pH 6 and pH 10 to 40 % and 37 %, respectively. The pH stability profile indicated that the purified peptidase was highly stable in the pH range between 7 to 9. Generally, the commercial microbial proteinases have pH optima in the alkaline range between 8 and 12 and the RecGEOker fell in this range [31].

In response to various modulators, results indicate the stimulated activity by 1 mM Mg^{2+} and 10 mM Mn^{2+} ions up to 112.6±2.8 % and 116.6±1.9 %, respectively (data not shown). The enzyme activity was decreased up to 50 % by 1 mM K^{+}, 10 mM Mg^{2+}, 1 mM and 10 mM Zn^{2+}, 1 mM and 10 mM Ni^{2+}. Among the ions tested Mn^{2+}, Mg^{2+} and Ca^{2+} ions positively regulated enzyme activity and stability. This phenomenon might be attributed to these ions involvement in stabilization of the keratinolytic peptidase molecular structure as reported Kojima [27]. It is well known that tertiary structure of the enzyme ant the substrate-enzyme complex may be stabilized and maintained by salt or ions bridge formed by metal ions.

The stability of RecGEOker toward detergents and organic solvents was determined by incubating the peptidase with detergents (1 % and 5 %) and organic solvents (10 % and 25 %) for 1 h at 25 °C (data not shown). The RecGEOker produced by *Geobacillus* sp. AD-11 was stable toward detergents like Tween 40 (5 %), Tween 60 (5 %), Tween 80 (5 %), Triton X-100 (1 %), Triton X-305 (1 %) and Brij 35 (5 %) and the relative keratinolytic enzyme activities were 180.1±3.9 %, 133.5±3.6 %, 122.2±1.5

%, 115.3±1.9 %, 153.4±1.7 % and 105.1±2.6 %, respectively. Studies revealed that the purified RecGEOker was slightly unstable toward tested organic solvents and relative activities were decreased. High remaining activity of recombinant peptidase treatment with detergents and/or organic solvents suggested that this peptidase can be a powerful biocatalyst in various white biotechnology areas associated with small peptide application or directly affect the surface of skin.

Peptidase can be classified based on their sensitivity to various inhibitors [32]. Accordingly, further assays were performed to evaluate the effects that various inhibitors might have on RecGEOker activity (data not shown). The studies indicated that RecGEOker was strongly inhibited by 1,10-phenanthroline, which are well-known inhibitor of metalloproteinases. This suggested that metal ions were involved in the catalytic activity. Other inhibitors, such as leupeptin and Pefabloc® SC, displayed slightly inhibitory effects. This inhibition profiles further confirmed that the keratinolytic peptidase belongs to the metallopeptidase family. Moreover, the thiol regents, such a 2-mercaptoethanol and DTT showed no effect of increased activity, respectively. Several reports have shown that keratinolytic peptidases are generally unable to hydrolyse keratin in the absence of reducing agents which help in sulfitolysis by breaking disulfide bonds [24]. However, such effect is usually attributed to the reduction of cysteine bridges in the keratinous substrate rather than direct effect on the enzyme. The RecGEOker noted to retain 39.1±1.8 % and 37.8±2.4 % of its activity in the presence of EDTA and EGTA as metallopeptidase inhibitors, respectively. This results suggests that metal cofactors were required for peptidase function and/or stability.

The activity of the purified RecGEOker toward various substrates including soluble substrates such as bovine serum albumine (BSA), sodium caseinate, gelatin and insoluble substrates such as collagen and keratin from wool was determined (Table 3.).

Table 3. Relative activity of RecGEOker toward different protein substrates.

Protein substrate	Relative activity (%)
Keratin from wool	100
Collagen	98±1.1
Sodium caseinate	95±1.4
Gelatin	92±2.5
BSA	37±1.9

The purified keratinolytic peptidase showed the highest preferentially toward keratin from wool > collagen > sodium caseinate > gelatin > and BSA in descending order. Keratinolytic peptidases can be divided into two types based on their hydrolysis of soluble and insoluble keratin-rich and keratin-like substrates. Purified RecGEOker efficiently hydrolyzed both soluble and insoluble substrates.

Qualitative analysis of the hydrolysis of various soluble and insoluble protein substrates was performed by SDS-PAGE (Fig. 6.).

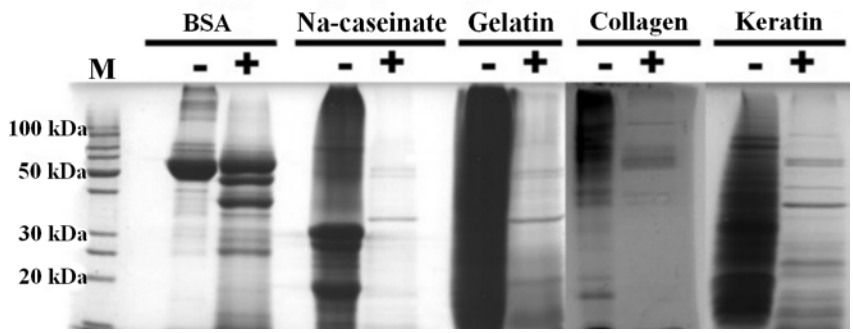

Fig. 6. SDS-PAGE analysis of RecGEOker substrate specificity. (-) – samples without treatment. (+) – samples with treatment. M – PageRuler^{TM} Unstained Protein Ladder.

The purified RecGEOker showed high hydrolysis efficiency toward analyzed substrates and completely cleaved keratin from wool, collagen, gelatin and sodium caseinate in descending order. Partially hydrolysis was achieved with BSA as a substrate. Obtained result indicate high RecGEOker appliance in various biotechnology areas associated with hydrolysis of both globular and fibrous native proteins.

The goal of RecGEOker study was to use keratinolytic peptidase from *Geobacillus* sp. AD-11 in enzymatic process to obtain high value bio-active keratin hydrolysates. Currently, the commercial keratin hydrolysates are obtained by chemical treatment with essential amino acids and nutrition element loss. Moreover, the obtained peptide length play most important role in various use. Protein hydrolysates with a molecular weight lower than 5 kDa are characterized by reduced allergenicity and it is desirable to obtain peptide fractions with molecular masses bellow 3 kDa [33].

The first step in bio-active peptide production involved transforming keratin from wool into small peptides by RecGEOker (Fig. 7.).

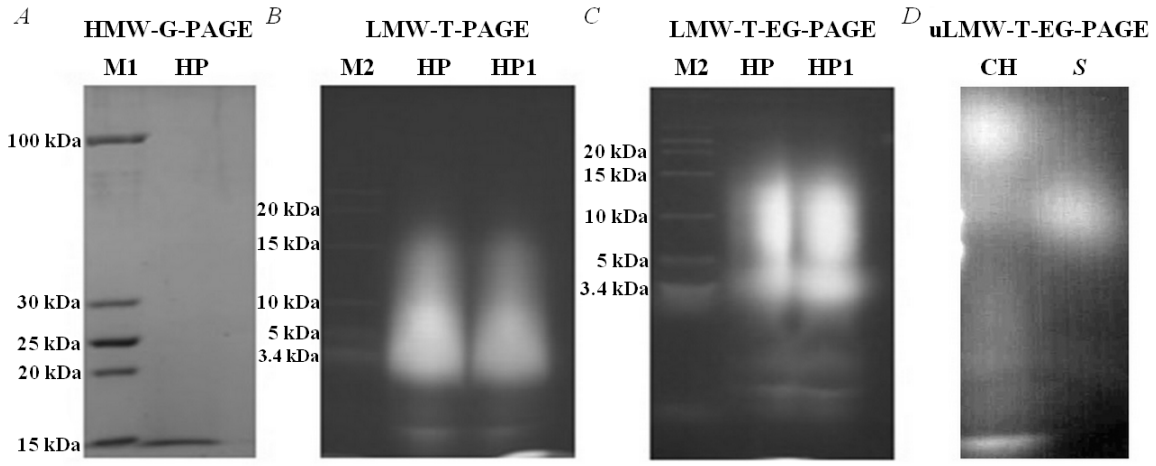

Fig. 7. Analysis of hydrolysis products. M1 – PageRuler^{TM} Unstained Protein Ladder. M2 – PageRuler^{TM} Unstained Low Range Protein Ladder. (A) HMW-G-PAGE – high molecular weight glycine PAGE. (B) LMW-T-PAGE – low molecular weight tricine PAGE. (C) LMW-T-EG-PAGE – low molecular weight tricine PAGE with incorporated ethylene glycol. (D) uLMW-T-EG-PAGE – ultra low molecular weight tricine PAGE with incorporated ethylene glycol. HP/HP1 – hydrolysis products obtained by enzymatic degradation. CH – commercial hydrolysate. S – small peptides.

Firstly, obtained keratin hydrolysis products (HP/HP1) were analyzed by high molecular weight glycine PAGE (HMW-G-PAGE) (Fig. 7A). Results showed that all hydrolysis products are less than 15 kDa. Further, peptides were analyzed by low molecular weight tricine PAGE (Fig. 7B) and low molecular weight PAGE with incorporated ethylene glycol (Fig. 7C) with fractionation capability between 1 kDa and 20 kDa. LMW-T-PAGE results confirmed that all peptides molecular masses are below 15 kDa. Keratin hydrolysis products can be divided into two groups: large (between 5 kDa and 15 kDa; *L*) and small (about 3.4 kDa; *S*) according to results obtained from LMW-T-EG-PAGE. Finally, obtained keratin hydrolysis products were analyzed in ultra-low molecular weight tricine PAGE with incorporated ethylene glycol (Fig. 7D) and compared with commercial peptides (CH). In comparison to commercial hydrolysate obtained by chemical degradation of keratin *S* products form one group of peptides and lack higher or lower molecular weight peptides which can be allergic or have no positive effect, respectively. Therefore, RecGEOker is a powerful biocatalyst for eco-friendly enzymatic biodegradation of keratin-rich substrates and produce a wide range of defined length small peptides for biotechnological applications in broad industry areas.

1.6. Production and purification of keratinolytic peptidases from mesophilic microorganisms

All mesophilic strains were growth in previously described synthetic medium B. Proteins in secretome were partially purified by ammonium sulfate precipitation (Table 4.). Common names were given to these enzymes: BtKER (*Bacillus* sp. AD-12 keratinase), BPKer (*Bacillus* sp. AD-W keratinase) and BAKer (*Bacillus* sp. AD-AA3 keratinase).

Table 4. Protein precipitation by ammonium sulfate.

Saturation (%)	Specific activity (U mg^{-1})		
	AD-12	AD-W	AD-AA3
0-20	19.67±5.03	31±3.46	15.67±3.21
20-40	43.33±6.11	160±26.06	290.33±14.05
40-60	48±5.29	255.67±11.59	209±16.82
60-80	30.33±4.16	205±13.23	160±13.75
0-75	120.4±4.1	ND	ND
20-80	ND	526±15.5	520±12.01

ND – no data.

The keratinolytic peptidase BtKER was purified by ammonium sulfate fractionation followed by DEAE-Sepharose and Bio-Gel P-60 chromatography. The purity of protein was analyzed by SDS-PAGE and keratinolytic activity was determined by zymography (Fig. 8.).

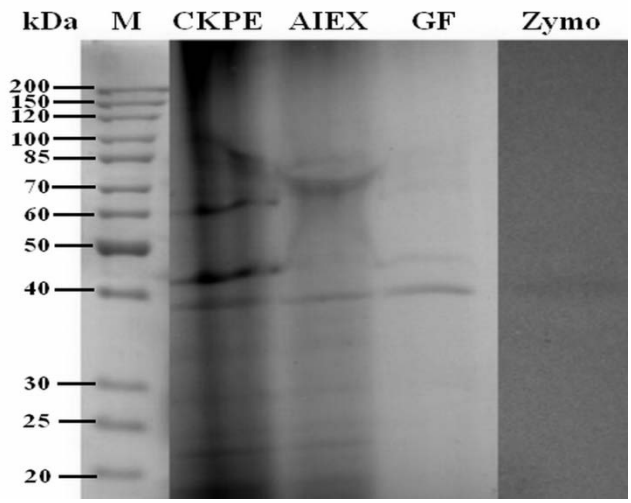

Fig. 8. SDS-PAGE and gel zymography of BtKER purification. M – PageRuler™ Unstained Protein Ladder. CKPE – crude keratinolytic peptidase extract. AIEX – partially purified BtKER by anion exchange chromatography. GF – purified BtKER by gel filtration. Zymo – zymogram analysis of purified BtKER.

DEAE-Sepharose chromatography resulted in a 2-fold purification with a 61.1 % recovery rate. After Bio-Gel P-60 chromatography purification was 5.2-fold with a final recovery to 28 % and 623.7±12.9 U mg $^{-1}$ specific activity. The molecular weight of this purified BtKER was determined to be 39 kDa by SDS-PAGE. An activity gel analysis confirmed that the purified BtKER is a single monomeric protein.

Many biotechnological processes requires considerable investment and resource. It is important to create environmentally friendly and economically attractive biodegradation process. Keratin material is now processed by combination of mechanical and chemical treatment methods. This approach is inefficient and uncontrolled. Therefore, we offer to use secretome for profitable keratin-rich material biodegradation. In that case, *Bacillus* sp. AD-W and *Bacillus* sp. AD-AA3 secretomes were used for further experiments.

1.7. Biochemical characterization of BtKER, BPKer and BAKer

The purified BtKER showed a typical bell-shaped curve with the keratinolytic activity in the temperatures range of 10-50 °C, with the maximal activity at 30 °C (Fig. 9A.). Optimal temperature for BPKer and BAKer – 50 °C (Fig. 9C, E). The thermal stability of keratinolytic peptidases were evaluated by incubating the enzymes at different temperatures (between 30 and 70 °C). The BtKER was stable at 40 °C and also retained more than 80 % of the initial activity after 4 h of incubation at that temperature. The half-life time of the BtKER was 2 h at 50 °C. Additionally, BtKER retained 22 % and 13 % residual activity after 1 h incubation at 60 °C ad 70 °C, respectively. Our observation showed that partially purified BPKer and BAKer exhibited more than 80 % of relative activity between 47-60 °C and 43-61 °C, respectively.

The purified BtKER displayed keratinolytic activity within a broad pH range of 5-11 with an optimal at pH 7 (Fig. 10.). BPKer showed pH optimal at 10 pH and it had more than 80 % of relative activity at pH range 9-11. BMKer showed optimal at pH 8 and it had more than 80 % of relative activity at pH range 7-11.

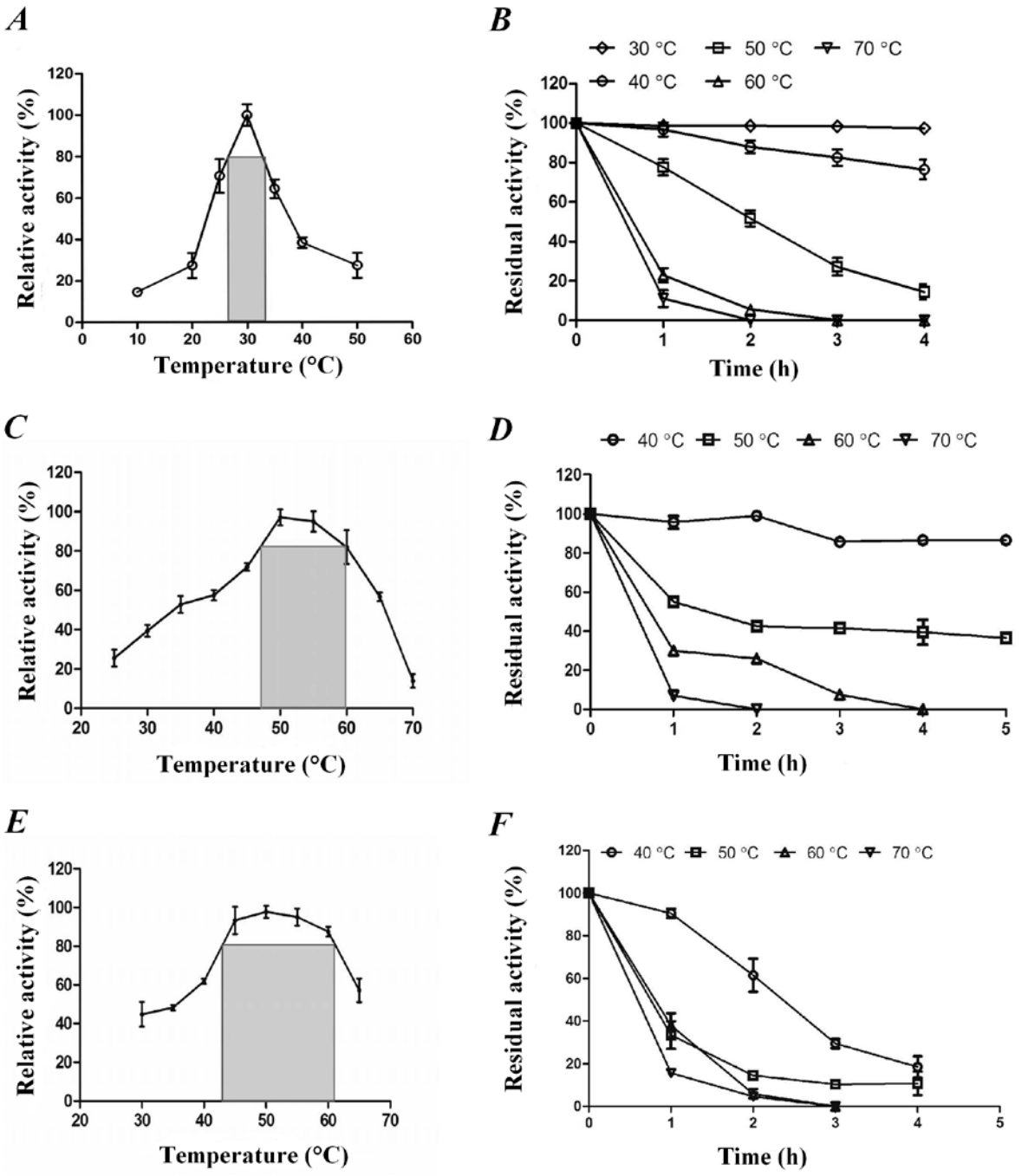

Fig. 9. Effect of temperature on activity (A, C, E) and stability (B, D, F). A and B – BtKER. C and D – BPKer. E and F – BAKer. Grey square means more than 80 % relative activity.

Therefore, the keratinolytic peptidase BtKER from *Bacillus* sp. AD-12 is a new kind of keratinase. The purified enzyme showed some novel characteristics comparing with other authors reported results. Previous results suggested that BtKER had a molecular weight 39 kDa and the highest enzyme activity was at pH 7 and 30 °C. BtKER showed lowest optimal working temperature comparing with all known reported

keratinolytic peptidases from *Bacillus*. For this reason, BtKER can widely be used in biotechnological and industrial processes where 30 °C or room temperature is required.

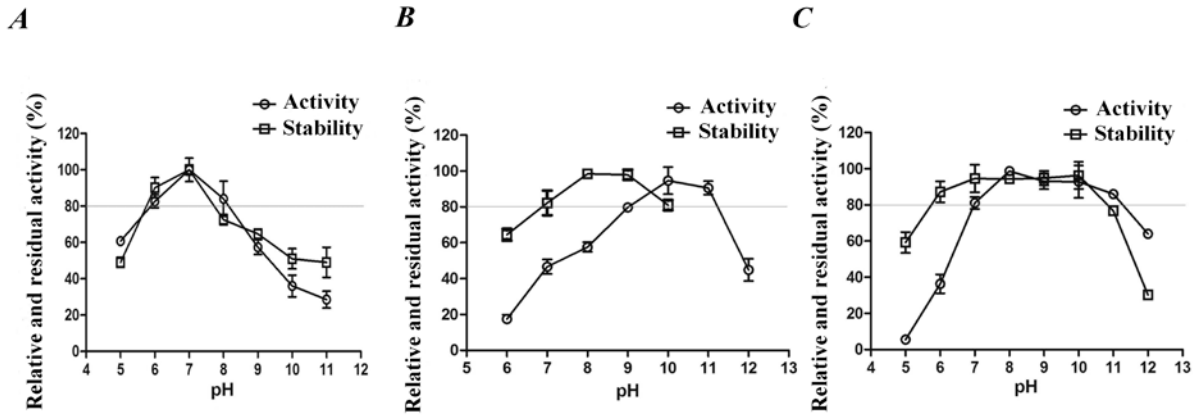

Fig. 10. Effect of pH on activity and stability. A – BtKER. B – BPKer. C – BAKer. Grey horizontal line means more than 80 % relative or residual activity.

Brandelli [1] declare that the presence of divalent metal ions such as Ca^{2+}, Mg^{2+} or Mn^{2+} often stimulate the keratinolytic peptidases. BtKER was positively stimulated only by 10 mM Mn^{2+} (data not shown). Mn^{2+} also stimulated BPKer (120.4±16.4 %). Obtained results suggested that Fe^{2+} negatively regulated enzyme activity and/or stability of all investigated peptidases.

Keratinolytic peptidase can be used in various biotechnology areas, including detergent and leather industries. Therefore, stability and relative activity of all peptidases toward different detergents were determined (data not shown). Positive effect on BtKER was obtained with detergents such as Triton X-100 (1 %), Triton X-305 (1 %) and Brij 58 (5 %); BPKer – Tween 40 (1 %); BAKer – no positive effect. Obtained results suggested that all peptidases is detergent-stable and can be used as detergent additive or in leather processing.

Various organic solvents were examined for their effects on the activity of the peptidases. In the reaction mixture containing organic solvents, enzyme deactivation can be caused by the disruption of the enzyme molecule hydrophobic core due to the change of medium hydrophobicity. Additionally, polar solvents such as DMF, DMSO, methanol, ethanol and propanol can penetrate into the protein and induce structural changes for the interaction between the active site and substrate [6]. Obtained results

suggested that all keratinolytic peptidases were unstable in the presence of polar protic solvents (methanol, ethanol and propanol) and only BtKER was positively effected in the presence of polar solvent DMF 25 % (up to 134.8±2.9 %).

Keratinolytic activity of studied peptidases were significantly inhibited by Pefabloc® SC and leupeptin (data not shown). Consequently, these peptidases were classified as serine peptidases. Meanwhile, EDTA and EGTA also showed similar effects suggesting that peptidases are metal ion related enzymes. In fact, serine peptidases are known to contain Ca^{2+} binding site and removal of this metal from the strong binding site is associated with a significant decrease in enzyme stability [6]. Obtained results indicated that all peptidases belongs to serine keratinolytic peptidases and require metal cofactors for hydrolysis process. Most of all known characterized *Bacillus* species had also been reported as a serine peptidases [1].

The keratinolytic peptidase BtKER showed the highest preferentially toward keratin from wool > sodium caseinate > collagen > BSA > and gelatin in descending order (Table 5.). Similar results were obtained with BPKer and BAKer. Qualitative analysis of the hydrolysis of various soluble and insoluble protein substrates was performed by SDS-PAGE (Fig. 11.).

Table 5. Relative activity of peptidases toward different protein substrates.

Protein substrate	Relative activity (%)		
	BtKER	BPKer	BAKer
Keratin from wool	100	100	61.5±4.8
Sodium caseinate	84±2.4	96.7±1.8	100
Collagen	17±1.1	91.9±5.9	52.1±0.7
BSA	12±1.2	18±5.7	34±1.7
Gelatin	7±0.8	10.9±3.5	73.7±2.9

According to Kornillowicz-Kowalska [34] all investigated peptidases can be classified as keratinolytic peptidases with ability to hydrolyse insoluble protein substrates in preference to keratin. Hydrolysis specificity results suggested that BtKER, BPKer and BAKer may have potential applications in the efficient biodegradation of keratinous waste and eco-friendly dehairing in the various white biotechnology industries.

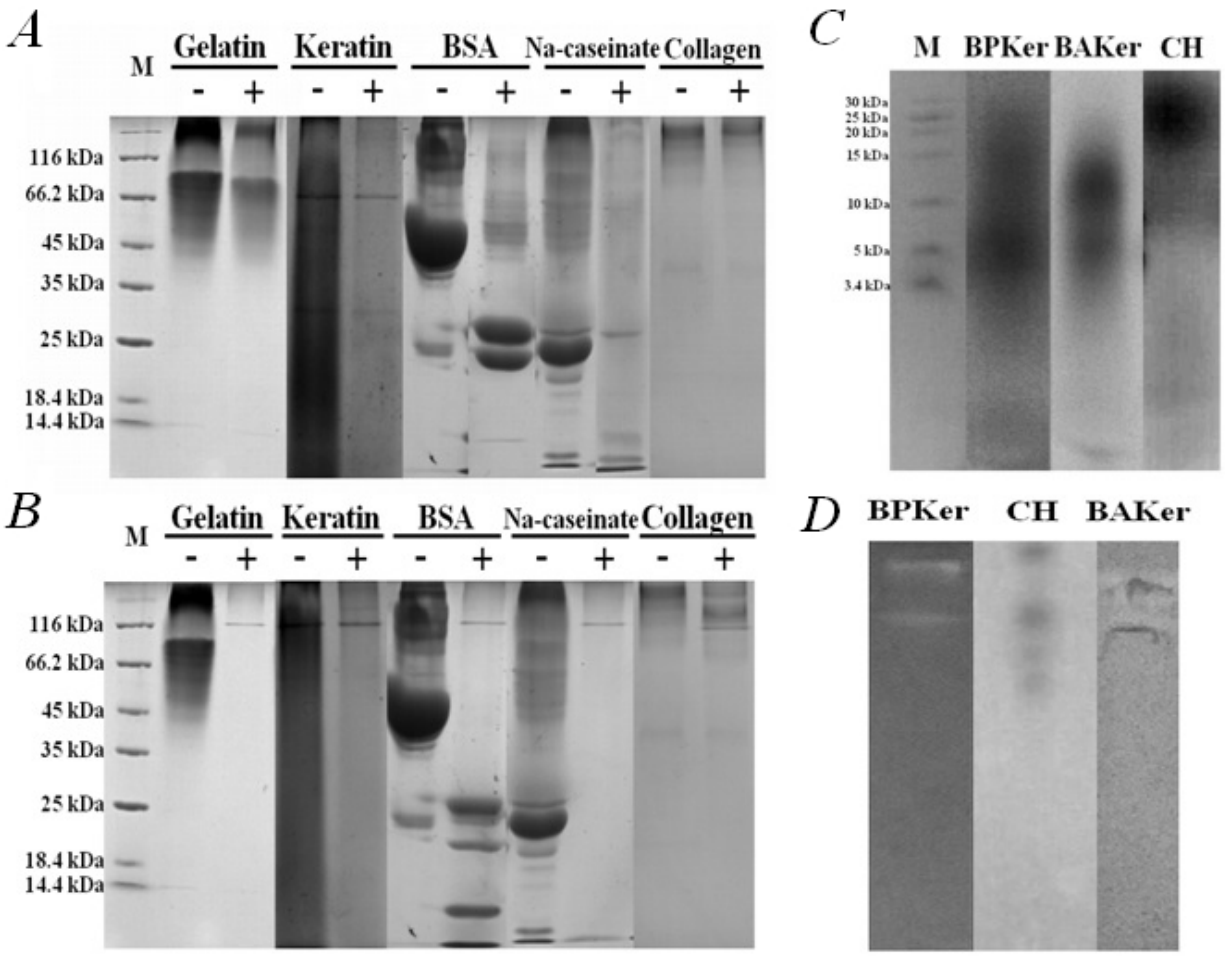

Fig. 11. SDS-PAGE analysis of BPKer (A) and BAKer (B) substrate specificity. SDS-PAGE (C) and TLC (D) analysis of hydrolysis products. M – Pierce^{TM} Unstained Protein MW Marker. (-) – samples without treatment. (+) – samples with treatment. CH – commercial hydrolysate. BPKer – hydrolysis products obtained by enzymatic degradation with BPKer. BAKer – hydrolysis products obtained by enzymatic degradation with BAKer.

The goal of our study was to use keratinolytic peptidases BtKER, BPKer and BAKer in enzymatic process to obtain high value bio-active keratin hydrolysates. Preliminary analysis of the keratin hydrolysates and commercial peptides was done using thin layer chromatography (Fig. 11D). Results suggested that commercial hydrolysate products can be separate into four different peptide groups, whereas peptides produced by BPKer or BAKer form two groups of peptides.

Obtained keratin hydrolysis products were analyzed by high molecular weight glycine PAGE (data not shown). Results showed that all hydrolysis products are less than 25 kDa. Further, peptides were analyzed by LMW-T-EG-PAGE (Fig. 11C). Results confirmed that all peptides molecular weight are below 20 kDa. In comparison to commercial hydrolysate obtained by chemical degradation of keratin, BPKer and BAKer

hydrolysis products formed two heterogeneous groups, whereas CH formed one peptide group in size range 20-25 kDa.

Physical and chemical characterization of native BtKER, BPKer and BAKer suggested that new keratinolytic peptidases are powerful biocatalysts for efficient keratin waste biodegradation and can replace conventional insufficient non-biological hydrolysis processes without energy, important amino acids and nutritional element loss. High value bio-active hydrolysis products – peptides obtained from keratin waste biodegradation are suitable for industrial applications in white and green biotechnology.

2. Chimeric keratinolytic peptidases

2.1. Synthetic homodimer SynKer-TT

Bioinformatic methods were used for analyze keratinolytic peptidase RecGEOker gene. SynKer-TT (novel synthetic keratinase) was constructed by protein engineering joining two protein domains (GEOker1) by a protein linker ELGGS (Fig. 12.).

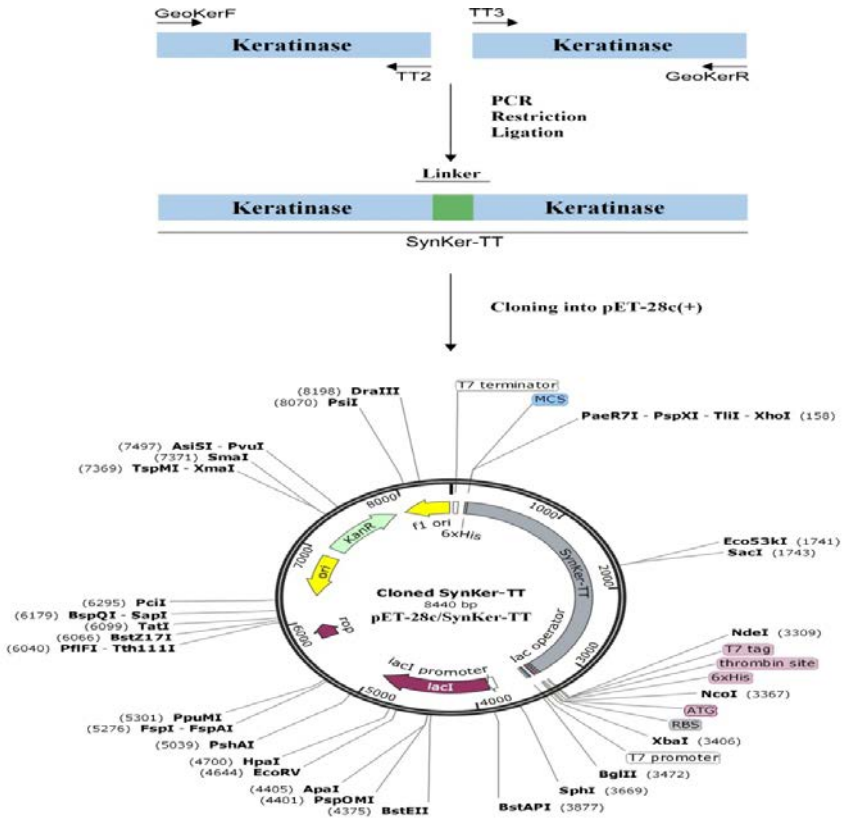

Fig. 12. Strategy for construction of synthetic keratinase homodimer SynKer-TT. Prepared with SnapGene® (http://www.snapgene.com/).

The target recombinant N-terminus His-tagged SynKer-TT protein was purified using immobilized Ni^{2+} metal affinity chromatography (IMAC). The purity of protein was analyzed by SDS-PAGE (Fig. 13.). Obtained protein band approximately at 116 kDa indicated the purification of the target SynKer-TT. The recombinant protein was eluted from Ni^{2+} resin at 250 mM of imidazole and observed as single band on SDS-PAGE. The purified recombinant SynKer-TT protein showed specific keratinolytic activity of 223.8±27. U mg^{-1} with an overall yield of 39.5 % and a purification fold of 2.58.

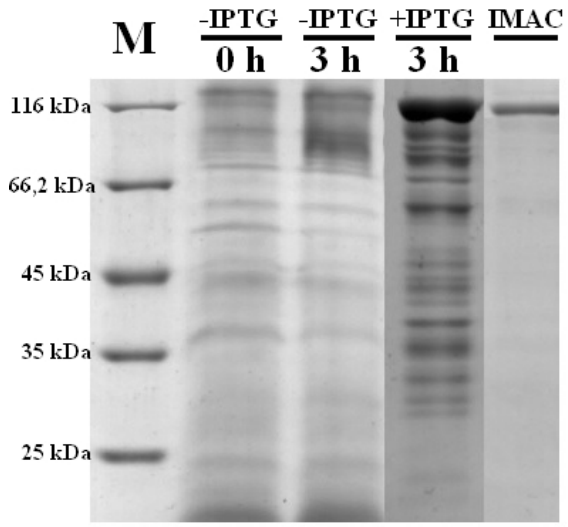

Fig. 13. Purification of SynKer-TT. M – Pierce™ Unstained Protein MW Marker. -IPTG – proteins profile without IPTG induction. +IPTG – proteins profile with IPTG induction. IMAC – purified SynKer-TT with immobilized metal ion affinity.

Specific keratinolytic activity of recombinant SynKer-TT was reduced about 6.4 times (comparing to RecGEOker). This phenomenon could be attributed to the complex structure of SynKer-TT. As a results, the interaction between enzyme and substrate, as well as the subsequent hydrolysis is dramatically influenced by non-physiological structure of GEOker1. It is well-known that selection of a suitable linker to join the protein domains together can be complicated. Therefore, the selection or rational design of a linker to join fusion protein domains is an important part for construction of chimeric proteins.

Protein thermal stability is an important factor considered in pharmaceutical and industrial applications. Synthetic linker which was used in our study hold fusion protein

domains in short distance. Thermal stability of SynKer-TT could be greatly improved due to this feature (Fig. 14.).

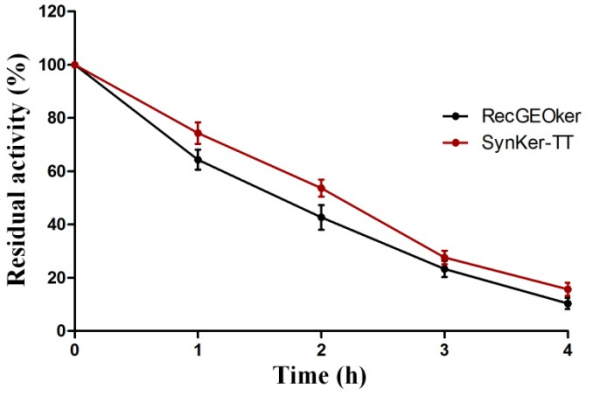

Fig. 14. Effect of temperature (80 °C) on the stability of RecGEOker and SynKer-TT.

RecGEOker and SynKer-TT were incubated at 80 °C for 4 hours. Our results demonstrate that thermal stability of SynKer-TT was increased up to 11 %. These results suggested that linker provided the conformation, flexibility and stability needed for a protein biological function in higher temperatures. Moreover, results suggested that hydrolysis products obtained by SynKer-TT are similar to peptides obtained by RecGEOker and are suitable for biotechnological application (Fig. 15.).

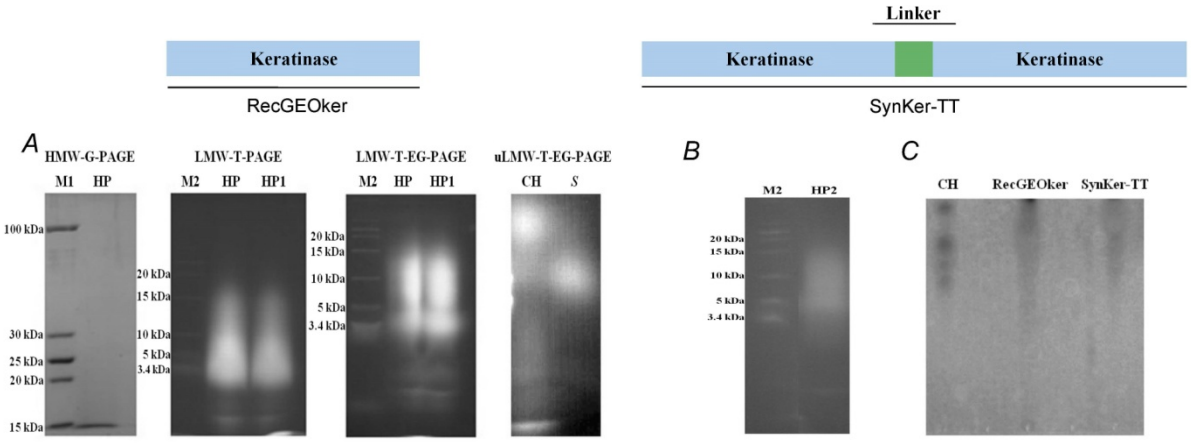

Fig. 15. Analysis of hydrolysis products obtained by RecGEOker (A) and SynKer-TT (B and C). A and B – SDS-PAGE analysis. C – TLC analysis. M1 – PageRuler^{TM} Unstained Protein Ladder. M2 – PageRuler^{TM} Unstained Low Range Protein Ladder. HP/HP1 – hydrolysis products obtained by enzymatic degradation with RecGEOker. HP2 – hydrolysis products obtained by enzymatic degradation with SynKer-TT. CH – commercial hydrolysate. *S* – small peptides.

2.2. Synthetic heterodimer SynKer-TM

SynKer-TM was constructed by protein engineering joining two protein domains (GEOker1 and BtKER) by a protein linker ELGGS (Fig. 16.). BtKER protein is hypothetical keratinolytic peptidase from *Bacillus* sp. AD-12. *btker* gene analysis was performed by NCBI database (data not shown). *btker* gene was cloned and expressed in *E. coli* BL21 (DE3) cells. However, no significant results were observed due to complete hydrolysis of all recombinant cell proteins. We declare, that BtKER protein might be toxic for *E. coli* cells. Therefore, construction of SynKer-TM could improve solubility of RecGEOker and reduce toxicity of BtKER.

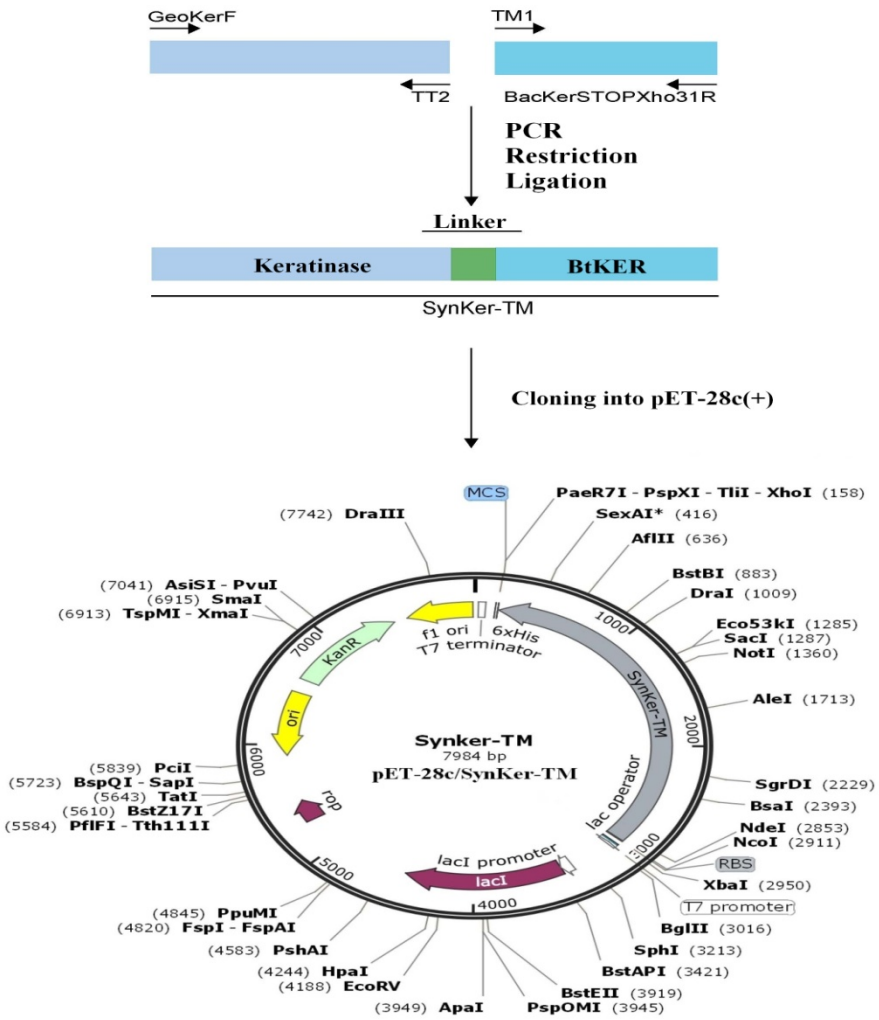

Fig. 16. Strategy for construction of synthetic keratinase homodimer SynKer-TM. Prepared with SnapGene® (http://www.snapgene.com/).

Synthetic heterodimer SynKer-TM was expressed in *E. coli* BL21 (DE3) cells. The induction of recombinant protein synthesis was performed at OD_{600} of 0.4 with 0.5 mM IPTG. The recombinant protein synthesis was continued for 2 h at 30 °C (Fig. 17.).

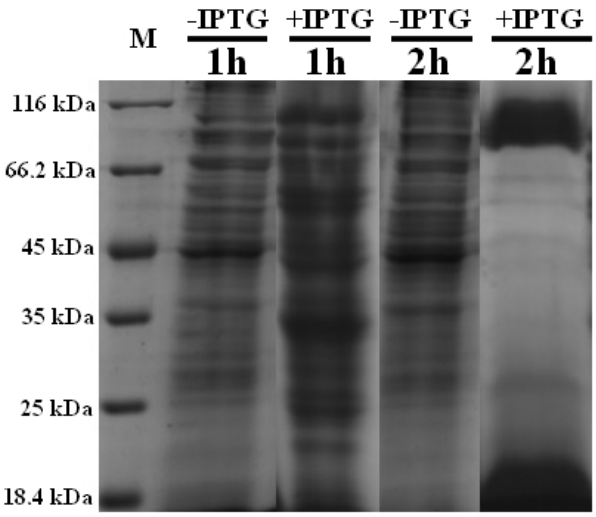

Fig. 17. Expression of synthetic heterodimer SynKer-TM. M – Pierce™ Unstained Protein MW Marker. -IPTG – proteins profile without IPTG induction. +IPTG – proteins profile with IPTG induction.

After 2 h of induction obtained protein band approximately at 100 kDa indicated the target recombinant SynKer-TM protein. However, recombinant SynKer-TM enhanced *E. coli* protein degradation that occurs after induction. His-tagged SynKer-TM was expressed as inclusion bodies. The washed inclusion bodies were solubilized by the slow addition of 6 M urea (Fig. 18.).

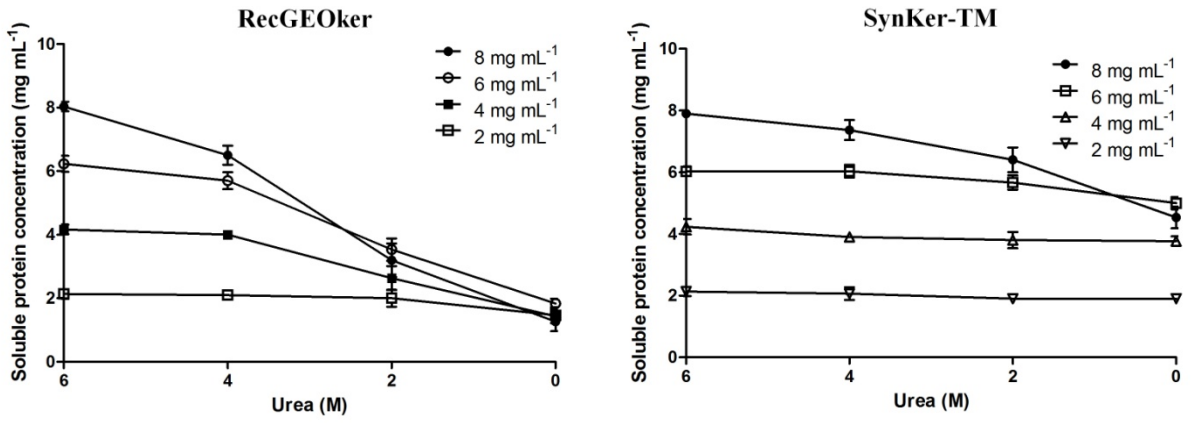

Fig. 18. Solubility of RecGEOker and SynKer-TM.

Synthetic heterodimer SynKer-TM was successfully cloned and expressed in heterologous system. Obtained results showed that expression of SynKer-TM result in the formation of insoluble aggregates known as inclusion bodies. It is reliable that protein aggregates prevented *E. coli* cells from toxic BtKER effect. Additionally, fusion protein technology increased solubility of high hydrophobic RecGEOker protein up to 2.78 times (about 5 mg mL^{-1}). Although the expression of SynKer-TM was positive, BtKER protein functional activity was not detected.

The successful construction of a recombinant fusion proteins requires two indispensable parts – the component protein and the linker. The selection of a suitable linker to join the proteins together can be complicated. The reduced activity of both SynKer-TT and SynKer-TM was only one unfavourable subsequence. However, our results showed that either solubility or thermal stability can be improved by joining two identical or structurally different protein domains.

3. Lab-scale keratin biodegradation process

Investigation and application of keratinolytic peptidases in biotechnology related processes are the main tasks of this dissertation work. In our recent work we argued and proposed to use microbial secreted proteins – secretome – as biologically active component for efficient keratin or related waste biodegradation. Either genetically modified organisms or recombinant proteins are suitable for many biotechnologically important processes. However, these biological units are preparation-related expensive and undesirable for many wide use products. It is important to create biodegradation system in eco-friendly manner. Therefore, for this purpose we explored our keratinolytic peptidases BtKER, BPKer and BAKer and designed lab-scale biodegradation system (Fig. 19.). Three main factors were investigated:

- Synergistic effect of different microbial secretomes;
- Effect of process temperature;
- Effect of process pH.

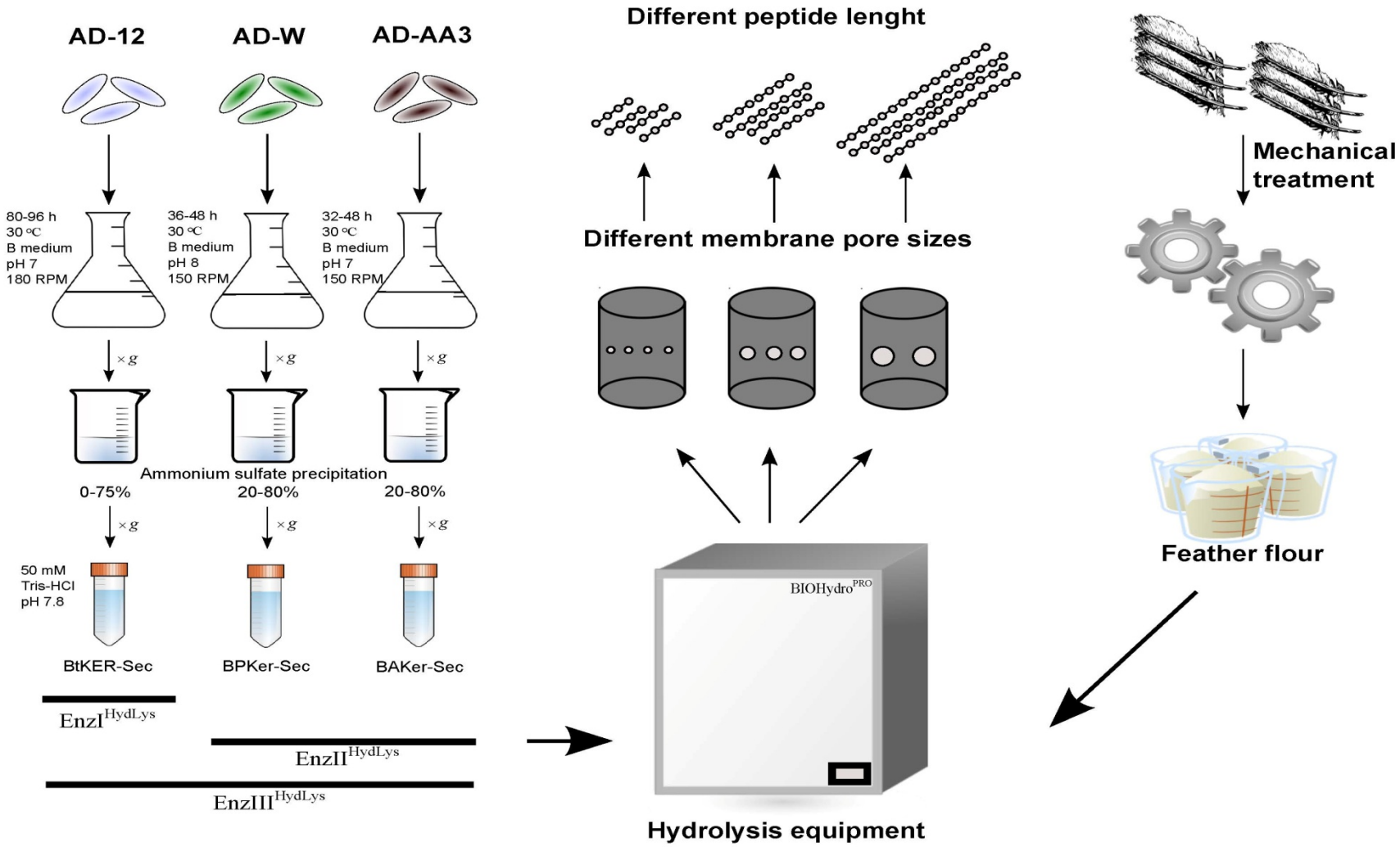

Fig. 19. Lab-scale keratin biodegradation system.

Recommendations: optimal conditions were chosen according to experimental results:

- $EnzII^{HydLys}$ enzyme complex;
- 30-35 °C hydrolysis temperature;
- pH 8;
- 3 h;
- 5-10 mg mL^{-1} proteinaceous substrate.

CONCLUSIONS

1) Evaluated impact of environmental conditions and various modulators on functional activity of explored keratinolytic peptidases (RecGEOker, BtKER, BPKer and BAKer);
2) All investigated keratinolytic peptidases are suitable for hydrolysis of various soluble and insoluble proteinaceous materials into value-added peptides;
3) Features of thermal stability and solubility of our created chimeric (fused) keratinolytic biocatalysts SynKer-TT and SynKer-TM were improved;
4) Designed lab-scale biological hydrolysis systems (EnzII^{HydLys} and EnzIII^{HydLys}) are applicable for bioconversion of renewable keratin-rich waste.

LIST OF PUBLICATIONS

The thesis is based on the following original publications:

1) **Gegeckas A**., Gudiukaitė R., Debski J., Citavicius D. Keratinous waste decomposition and peptide production by keratinase from *Geobacillus stearothermophilus* AD-11. *Int. J. Biol. Macromol*. 2015:75, 158-165;
2) **Gegeckas A**., Gudiukaitė R., Citavicius D. Keratinolytic proteinase from *Bacillus thuringiensis* AD-12. *Int. J. Biol. Macromol*. 2014:69, 46-51.

Other publications:

1. Gudiukaitė R., **Gegeckas A**., Sadauskas M., Citavicius D. Detection of Asp371, Phe375, and Tyr376 influence on GD-95-10 lipase using alanine scanning mutagenesis. *Appl. Biochem. Biotechnol*. 2016:178(4), 654-69;
2. Gudiukaitė R., **Gegeckas A**., Kazlauskas D., Citavicius D. Influence of N- and/or C-terminal regions on activity, expression, characteristics and structure of lipase from *Geobacillus* sp. 95. *Extremophiles*, 2014:18(1), 131-45.

CONFERENCE PRESENTATIONS

Dissertation theme was presented in 7 conferences (6 of them – international):

1. **Gegeckas A**., Simkute A., Gudiukaite R., Citavicius DJ. Keratin waste biodegradation and peptide production by keratinolytic proteinase from *Bacillus methylotrophicus* AD-AA3. VAAM 2015 Annual Conference of the Association for General and Applied Microbiology, 13-16 March 2016. Jena, Germany;
2. Simkute A., **Gegeckas A**., Gudiukaite R., Citavicius DJ. Keratinolytic proteinase from *Bacillus pumilus* AD-W with promising keratin waste biodegradation activity. Vita Scientia 2016, 04 January 2016. Vilnius, Lithuania;
3. **Gegeckas A**., Simkute A., Gudiukaite R., Citavicius DJ. Keratinolytic proteinase from *Bacillus pumilus* AD-W with promising peptide-production activity.

BioMicroWorld 2015 6th International Conference on Environmental, Industrial and Applied Microbiology, 28-30 October 2015. Barcelona, Spain;

4. **Gegeckas A**., Gudiukaitė R., Šimkutė A., ČitaviČius DJ. Keratiniloziniu aktyvumu pasižymintys fermentai: įvairovė ir biotechnologinis potencialas. 2015 m. Jaunųjų mokslininkų konferencija BIOATEITIS: gamtos ir gyvybės mokslų perspektyvos, Gruodžio 10 d., 2015. Vilnius, Lietuva;
5. **Gegeckas A**., Gudiukaite R., Citavicius DJ. Synthetic homodimer of GEOker keratinase for efficient biodegradation of keratin by-products. BioMicroWorld 2015 6th International Conference on Environmental, Industrial and Applied Microbiology, 28-30 October 2015. Barcelona, Spain;
6. **Gegeckas A.**, Gudiukaite R., Čitavičius D. Keratinolytic proteinase as a powerful biocatalyst for bio-active peptide production. CBM2014 2th Congress of Baltic Microbiologists, 16-19 October 2014. Tartu, Estonia;
7. **Gegeckas A.**, Gudiukaitė R., Čitavičius D. Production, purification and partial characterization of keratinolytic serine peptidase from newly isolated *Bacillus* sp. K1-2 strain. FEMS2013 5th Congress of European Microbiologists, 20-25 July 2013. Leipzig, Germany.

Other conferences (as co-author):

1. Malunavicius V., Gudiukaite R., **Gegeckas A**, Citavicius DJ. Construction of new chimeric lipase using protein engineering methods. Vita Scientia 2016, 04 January 2016. Vilnius, Lithuania;
2. Gudiukaite R., **Gegeckas A**., Malunavicius V., Citavicius D. Functionality analysis of structural domains from GD-95 lipase by site specific and random mutagenesis. FEMS2015 6th Congress of European Microbiologists, 07-11 June 2015. Maastricht, The Netherlands;
3. Gudiukaite R., **Gegeckas A**., Čitavičius D. GD-95 lipase - new biocatalyst in wide industry areas. CBM2014 2th Congress of Baltic Microbiologists, 16-19 October 2014. Tartu, Estonia;
4. Gudiukaitė R., **Gegeckas A**., Čitavičius D. *Geobacillus* spp. kamienų rekombinantinių lipazių fizikinių bei cheminių savybių įvairovės įvertinimas.

Mokslas Gamtos Mokslų fakultete, 8-ta mokslinė konferencija, Spalio 3 d., 2014. Vilnius, Lietuva;

5. Gudiukaitė R., **Gegeckas A**., Čitavičius D. Cloning, purification and esterification capability determination of lipase produced by *Geobacillus* sp. 76. FEMS2013 5th Congress of European Microbiologists, 20-25 July 2013. Leipzig, Germany.

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REZIUMĖ

Šiame darbe buvo siekiama identifikuoti, charakterizuoti ir pritaikyti skirtingų mikroorganizmų – termofilinių ir mezofilinių – sekretuojamas keratinolizines peptidazes. Mokslinių straipsnių duomenų bazėse nuolat atsiranda naujos informacijos apie iki šiol neaprašytas keratinolizines peptidazes ir jų pritaikymo galimybes.

Nustatyta, kad pirmasis mūsų identifikuotas mikroorganizmas yra priskiriamas termofilinių mikroorganizmų *Geobacillus* genčiai. Išsamesnė analizė leido sąlyginai AD-11 izoliatą priskirti *G. stearothermophilus* rūšiai. Keratinolizinė peptidazė, sekretuojama *Geobacillus* sp. AD-11 kamieno buvo pavadinta GEOker. Sėkminga buvo ir šios keratinolizinės peptidazės produkcinės terpės paieška. Sukurta ir pritaikyta minimali sintetinė terpė, kurioje vienintelis anglies, azoto, sieros ir energijos šaltinis yra vilnos keratinas. Mikroorganizmui esant tokioje aplinkoje yra maksimaliai sintetinamas ir sekretuojamas į aplinką fermentas, galintis vykdyti efektyvią šio vienintelio substrato hidrolizę iki mažos molekulinės masės junginių, kurie jau gali būti panaudojami ląstelės metabolizmui.

Gavus masių spektrometrijos rezultatus buvo galutinai įsitikinta, kad *Geobacillus* sp. AD-11 kamienas sekretuoja GEOker keratinolizinę peptidazę. Detali gautų peptidų sekų analizė duomenų bazėse leido identifikuoti hipotetinį GEOker baltymą. Nustatyta, kad šis baltymas yra sintetinamas kaip Pre-Pro-fermentas, kuris galutinai įgyja savo funkciškai aktyvią konformaciją tik po sėkmingos sekrecijos į užląstelinę aplinką ir po autokatalizės, kurią vykdo paties fermento N-galinis domenas (Pro-peptidas). GEOker baltymas savo aminorūgščių seka yra panašiausias į baciloliziną (*G. stearothermophilus* ir *G. thermocatenulatus*), termoliziną (*thermolysin-like*), termostabilią neutralią proteinazę (*B. caldolyticus*) ir šarminę metaloprotеazę *(B. subtilis*).

Įvykdžius *geoker* geno klonavimo ir raiškos eksperimentus nustatyta, kad aktyvią šio baltymo formą įmanoma gauti tik klonavus GEOker baltymą kartu su jo Pro-seka (GEOker1). Tikėtina, kad Pro-seka nulemia baltymo hidrofobiškumą ir netirpių baltyminių kūnelių susiformavimą rekombinantinių *E. coli* BL21 (DE3) ląstelių citoplazmoje. Dažnu atveju netirpūs baltyminiai kūneliai yra didžiulė problema vykdant heterologinę ar homologinę genų raišką. Mūsų tirtu atveju, šių kūnelių susidarymas

nulėmė funkciškai aktyvaus rekombinantinio baltymo RecGEOker gavimą. Taip pat pademonstruota, kad neįvykus autokatalizei – baltymas yra aktyvus.

Fizikinių ir cheminių savybių įvertinimas ir gauti rezultatai papildo literatūros duomenis apie termofilinių mikroorganizmų sekretuojamas keratinolizines peptidazes. Nustatytos optimalios savybės (temperatūra, pH), įvairių cheminių junginių poveikis (metalų jonai, detergentai, organiniai tirpikliai, slopikliai) ir gebėjimas hidrolizuoti įvairius baltyminius substratus. Pademonstruota, kad RecGEOker yra efektyvus ir tinkamas biokatalizatorius mažos molekulinės masės produktų gamybai.

Identifikuotos ir detaliai išanalizuotos 3 mezofilinių mikroorganizmų sekretuojamos keratinolizinės peptidazės. Nustatyta, kad visi 3 izoliatai – AD-12, AD-W ir AD-AA3 – priklauso *Bacillus* genties bakterijoms. Detalesnė analizė leido AD-12 izoliatą priskirti *B. thuringiensis,* AD-W – *B. altitudinis*/*B. stratosphericus,* o AD-AA3 – *B. amyloliquefaciens* rūšims. Pasinaudojus *Geobacillus* sp. AD-11 kamieno augimo terpės sudėtimi, buvo modifikuotos ir pritaikytos sintetinės terpės mezofilinių mikroorganizmų sekretuojamų keratinolizinių peptidazių sekrecijai. Pritaikius tą pačią produkavimo strategiją, buvo sėkmingai gauti fermentai, kurie pavadinti BtKER (*Bacillus* sp. AD-12), BPKer (*Bacillus* sp. AD-W) ir BAKer (*Bacillus* sp. AD-AA3).

Atlikus BtKER baltymo gryninimą, gautas apie 39 kDa dydžio baltymas, kurio funkcinis aktyvumas patvirtintas zimografijos metodu. Siekiant sukurti optimalų biologinės degradacijos procesą, nuspręsta BPKer ir BAKer negryninti, o eksperimentams ir pritaikymui naudoti šias keratinolizines peptidazes sintetinančių mikrooganizmų sekretominius baltymus – sekretomą. Detali fizikinių ir cheminių savybių analizė leido išskirti potencialias fermentų pritaikymo galimybes. Iki šiol literatūroje nebuvo skelbiama informacijos apie *B. thuringiensis* sekretuojamas keratinolizines peptidazes, todėl mūsų gauti rezultatai apie BtKER fermentą yra svarbus indėlis, papildantis informaciją apie šių fermentų įvairovę.

Šiama darbe buvo sukurti du chimeriniai baltymai: homodimerinis SynKer-TT (sintetinė keratinazė, sudaryta iš dviejų GEOker baltymų) ir heterodimerinis SynKer-TM (sintetinė keratinazė, sudaryta iš GEOker ir BtKER baltymų). Homodimerinis SynKer-TT baltymas pasižymėjo iki 11 % didesniu temperatūriniu stabilumu 80 °C temperatūroje, lyginant su monomeriniu RecGEOker baltymu. Suliejus RecGEOker baltymą su BtKER (SynKer-TM) pavyko padidinti RecGEOker baltymo tirpumą 2,78

karto (iki 5 mg mL^{-1}). Taip pat pavyko sumažinti BtKER baltymo tirpumą, tačiau nustatyti šio baltymo funkcionalumo nepavyko. Gauti rezultatai leidžia teigti, kad baltymų inžinerija yra galingas molekulinis įrankis, leidžiantis sukurti patobulintus biokatalizatorius. Literatūroje yra mažai duomenų apie keratinoliziniu aktyvumu pasižyminčius chimerinius baltymus, todėl mūsų gauti rezultatai yra svarbūs moksliniu aspektu.

Keratinolizinių peptidazių pritaikymas ir hidrolizės potencialo panaudojimas yra vienas svarbiausių šios disertacijos uždavinių. Yra tikslinga neefektyvius mechaninius ir cheminius keratino atliekų (žaliavų) perdirbimo procesus pakeisti į efektyvius ir aplinkai saugius biologinius perdirbimo procesus. Buvo siekiama sukurti hidrolizės procesą, kuriame sinergistinis identifikuotų keratinolizinių peptidazių poveikis būtų maksimaliai išnaudojamas mažos molekulinės masės produktų gamybai. Eksperimentiškai nustatytos baltyminės kilmės substrato (vilnos keratino) hidrolizės sąlygos ($EnzII^{HydLys}$ fermentų kompleksas, 30-35 °C temperatūra, pH 8 buferinė sistema, 3 inkubavimo valandos, 5-10 mg mL^{-1} vilnos keratino) leido sukurti laboratorinio lygio keratino biodegradacijos sistemą. Mūsų sukurta aplinkai draugiška biokonversijos sistema yra tinkama peptidų gamybai, kurie gali būti panaudojami įvairiose biotechnologijos srityse.

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