University of South Bohemia in České Budějovice
Faculty of Science

Production of recombinant proteins using prokaryotic and eukaryotic expression systems

Bachelor's Thesis

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A recombinant enzyme PNGase H^+ was produced in *E. coli*, and several steps were tried to optimize the expression, extraction and purification. Furthermore, PCR primers were designed to detect genes of predicted sialyltransferases and DNA methyltransferases in *Ixodes ricinus* genomic DNA and plasmids were designed for future production of DNA methyltransferases in an insect expression system.

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Abstract

Recombinant protein production is an important technique in biochemistry and biotechnology, which describes the introduction of a foreign gene into an expression host, to produce proteins in high quantities. The easiest and widely used technique for the production of recombinant proteins involves bacteria expression system (for instance, *Escherichia coli)*. However, eukaryotic proteins usually require post-translational modifications, which are not performed in bacteria. In this case, other expression systems (for example, insect or yeasts) can be used. Peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidases (PNGases) are enzymes, which can cut the glycan groups from proteins, and they are often used in N-glycan analysis. PNGase H^+ is a recently found enzyme in bacteria, which has considerable higher activity at low pH in comparison to other PNGases. The aim of this work was to optimize conditions for the expression and purification of PNGase H^+ enzyme in a bacterial expression system, as well as to optimize conditions for the production of predicted Fucosyltransferase, α2,3-Sialyltransferase, α2,6-Sialyltransferase and DNA methyltransferases from *Ixodes ricinus* tick in bacterial and insect expression systems. In my work, I used bioinformatic search to design the vector constructs carrying the studied genes. Using molecular cloning and transformation of bacteria, I successfully produced the PNGase H^+ protein as confirmed by gel electrophoresis and Western Blot. For the optimal protein production, several conditions were tested: temperature and time of expression, IPTG concentration and pH of lysis buffer. Affinity chromatography on an IMAC column was used for the protein purification from the soluble fraction and from inclusion bodies. I also confirmed the presence of Fucosyltransferase, α2,3-Sialyltransferase, α2,6-Sialyltransferase and DNA methyltransferases genes in the genomic DNA from *I. ricinus*, and designed plasmids containing sequences of the predicted I. ricinus DNA methyltransferases.

Content

1 Introduction

1.1 Recombinant protein production

The production of recombinant proteins in microbial systems is an important technique in biochemistry. Before this method was developed, desired proteins had to be purified from large quantities of plant or animal tissues. Recombinant proteins on the other hand can be produced and purified in high quantities, thus they can be biochemically characterized or used in industrial processes.

The following steps are needed for the production of recombinant proteins: first, the DNA sequence of the gene of interest is amplified usually from a cDNA, then the gene is cloned into an expression vector. This DNA molecule is multiplied, usually in a prokaryotic maintaining host, and then transformed into the expression host. The expression of the gene is induced and then the protein can be purified and characterized (Figure 1).[1,2]

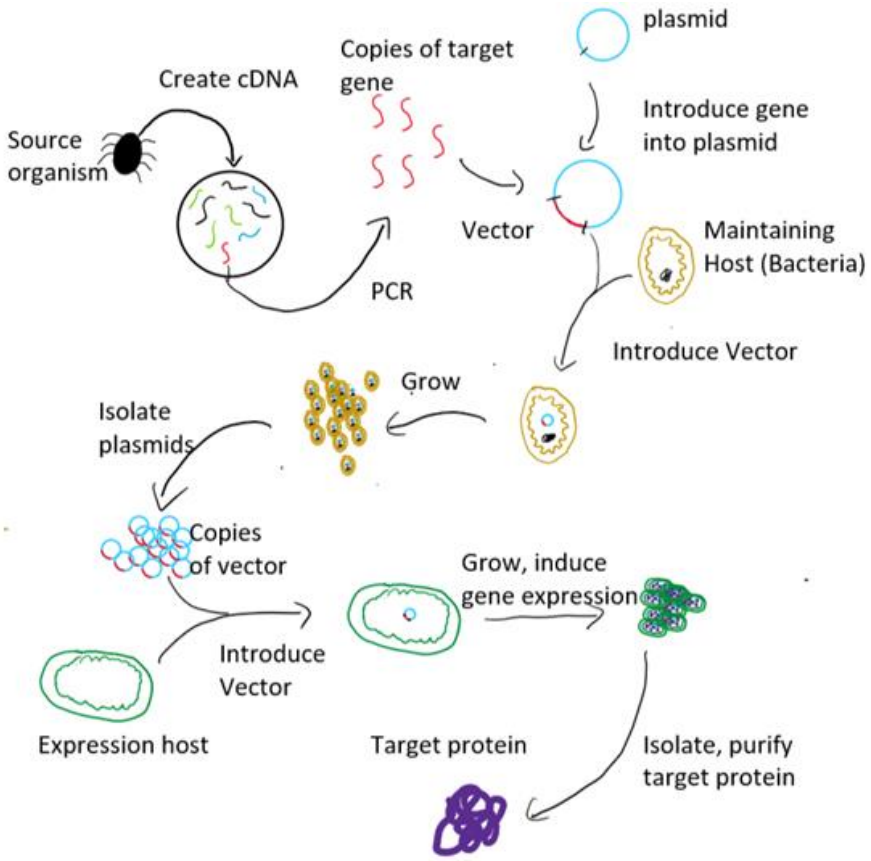

Figure 1. The scheme of recombinant protein production.

However, sometimes, the problems with the protein production occur, including low expression, inclusion body formation, protein inactivity, and others.[1,2]

The most common bacterial expression host is *Escherichia coli*, due to fast growth, cheap growth media and simple transformation methods. An expression vector for prokaryotic expression contains several sequences needed for their function (Figure 2): an origin of replication, a promoter for expression, a selection marker (mostly antibiotic resistance) and affinity tags for downstream applications.[1]

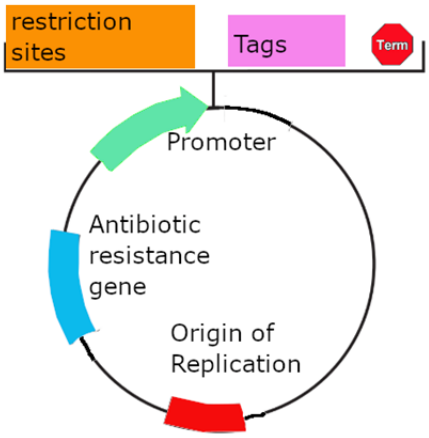

Figure 2. Scheme of expression vector for recombinant protein production (Edited)[3].

Most prokaryotes, including *E. coli*, lack the machinery for post-translational modifications, thus sometimes yielding inactive forms of eukaryotic proteins.[4] Therefore, various proteins have to be produced in eukaryotic cells. Plasmids for the protein expression in eukaryotic expression systems (for example *Drosophila* expression system or DES[3]), contain the Kozak consensus sequence, which is needed for protein translation initiation in Eukaryotes.[5] In eukaryotic expression systems, it is possible to perform transient transfection for the production of proteins after induction, or to introduce the gene into the genome to have a stable line that can produce large quantities of target protein.[3]

1.2 PNGase H^+

Many proteins produced by eukaryotic cells have small sugar oligomers attached to the N-atom of an Asparagine side chain. These attached molecules are termed "N-linked glycans" or "N-glycans".[5] Peptide-N4-(N-acetyl-β-glucosaminyl)asparagine amidases (PNGases, EC 3.5.1.52) are a group of enzymes that release N-glycans from glycoproteins and are used also in N-glycan analysis. The recombinant PNGase F is the most commonly used PNGase, as it shows high activity, but it cannot release (α1,3)core-fucosylated N-glycans that are frequently found in plants and also different eukaryotic organisms.[6,7] The glycoprotein PNGase A can release these fucosylated glycans, but has lower activity for proteins with long glycan chains.[8] PNGase A is usually extracted from almonds,[7] but a recombinant form is already commercially available, for example from New England Biolabs (Ipswich, MA, USA).

In 2014, Wang, et al. discovered PNGase H^+ with a pH optimum at 2.6, in the bacterium *Terriglobus roseus*.[9] The PNGase H^+ gene was successfully cloned and expressed in *E. coli*. This recombinant enzyme liberates high mannose-, hybrid- as well as complex-type *N*-glycans. The protein is more efficient in releasing N-glycans with core α1,3-fucose than the established PNGase A. PNGase H^+ is not a glycosylated protein itself, therefore it does not release N-glycans from itself that would contaminate the results.[9]

PNGase H^+ is a useful tool in N-glycan analysis, because it can be expressed using protein expression systems and is non-glycosylated. The low pH optimum makes it a good candidate for cleavage in acidic conditions.[9] The enzyme is not commercially available yet. Therefore, in my work, I aimed to produce an active recombinant PNGase H^+ for the deglycosylation of several proteins, which are in use in the Laboratory of Applied Biochemistry.

1.3 Predicted glycosyltransferases from the tick *Ixodes ricinus*.

Other proteins of our interest are sialyltransferases, and DNA methyltransferases of tick *I. ricinus*; for this reason, my work focussed also on production of these proteins. Previously, in the Laboratory of Applied Biochemistry, several sequences of these enzymes were identified in the transcriptomes from *I. ricinus*.[10, 11]

Sialyltransferases are enzymes that catalyse the biosynthesis of sialylated oligosaccharides, like glycans of glycoproteins.[12] These sialylated glycoproteins are common in several different organisms, especially higher Eukaryotes.[13] Some pathogens and parasites, including ticks, use these molecules to mask them from the host's immune system.[14] Sterba, et al. showed that the majority of sialylated glycoprotein present in ticks is produced by its host, but a small amount is produced by the arthropod itself.[15]

Cytosines and adenines of a DNA molecule can be methylated by different DNA methyltransferases. In eukaryotes, this epigenetic modification influences chromatin architecture and gene transcription.[16] Homologues to two cytosine-methylating enzymes (DNMT1 and DNMT3) as well as homologues to an adenine-methylating enzyme (DAMT) have been identified in the gDNA of *I. ricinus*.[11]

2 Aims

The aim of my work was to optimize conditions for the production of recombinant PNGase H^+ in a bacterial expression system and to establish approaches to the production of predicted Fucosyltransferase, α2,3-Sialyltransferase, and α2,6-Sialyltransferase and other proteins from tick *I. ricinus* in bacterial and insect expression systems.

3 Materials and Methods

3.1 Production of PNGase H^+

3.1.1 Bacteria expression system

A pET vector with synthetic PNGase H^+ gene was produced by GenScript (Piscataway, NJ, USA). Glycerol stocks of transformed *E. coli* TOP10 competent cells (home-made) and Rosetta-gami 2 (DE3) Competent Cells (Novagen, Merck Millipore, Darmstadt, Germany) were prepared by my co-supervisor previously and stored at -80 °C for 2 years before the start of my work.

3.1.2 Plasmid isolation

5 mL Luria Bertani broth (LB) media with antibiotics were inoculated with cells from a glycerol stock or single colony from LB agar under sterile conditions and allowed to grow overnight at 37 °C on a shaker at 190 rpm. The overnight culture was centrifuged for 15 min at 4 °C, 4 500 x g. The plasmids were extracted by following the procedure of the used High Pure Plasmid Isolation Kit (Roche Life Science, Basel, Switzerland). After the procedure, the purified plasmid DNA was diluted in 100 µL Elution Buffer. The concentration and purity were determined by UV spectroscopy with a Nanophotometer Pearl (Implen, Munich, Germany).

3.1.3 Polymerase Chain Reaction (PCR)

PCR was performed to confirm the presence of the PNGase H^+ gene. 2 µL of the sample (e.g. isolated plasmids), 10 µL of PCR Master Mix (Roche Life Science), 1 µL 10 µM Forward and 1 µL 10µM Reverse primer for the PNGase H^+ gene and 6 µL water to make a 20 µL reaction volume. The used primers are the same as for the preparation of a pRSET vector (Section 3.1.10, Table III). The template for the positive control was the pET vector produced by GenScript and for the negative control water was used. The temperature program for the PCR test for the presence of the PNGase H^+ gene was the following: 4 min at 95 °C, 30 cycles with 30 s at 95 °C, 30 s at 65 °C and 1:45 min at 72 °C, final elongation at 75 °C for 5 min.

The samples were put on ice and mixed with DNA Gel Loading Dye (6x) (Thermo Fischer Scientific, Waltham, Massachusetts, USA) with 1x SYBR Green. (Thermo Fischer Scientific) Electrophoresis was performed using 1.5 % agarose gel and 1 kb O' Gene Ruler DNA ladder (Thermo Fischer Scientific) with SYBR Green.

3.1.4 Optimal conditions for PNGase H^+ expression

An overnight bacterial culture was prepared as described above. 250 µL of this culture were used to inoculate 50 mL of LB media containing 1 % glucose (to minimize basal expression) and 30 µg/mL Kanamycin (Kan); bacteria were then grown at 37 °C at 190 rpm on a shaker. Every hour, an aliquot of 1 mL was taken from the culture and the optical density was determined at 600 nm (OD600), until the value was between 0.6 and 1. The growth was centrifuged for 15 min at 4 500 x g at 4 °C; the pellet was resuspended in LB with 30 µg/mL Kan and again centrifuged under the same conditions to wash away glucose. Pellet was resuspended in 50 ml of LB medium with Kan. The growth was continued at 37 °C for 15 min; 6 aliquots with 7 mL were taken and further manipulated according to the conditions tested. The concentration of Isopropyl-β-D-1-thiogalactopyranosidepyl (IPTG) was either 1 mM or 0.1 mM, the induction happened at 18 °C, 25 °C (RT) and 37 °C. For 18 °C and 25 °C, 1 mL aliquots were taken 3, 9, 15 and 24 h after induction, for 37 °C, 1 mL aliquots were taken 1, 2, 3 and 4 h after induction; the Zero-point is an aliquot before induction. The OD600 of the aliquots was determined before they were centrifuged for 1 min at 14 000 x g; the supernatant was discarded, and the pellet was frozen at -20 °C.

A lysis buffer with 5 % acetic acid and 1 % Triton X-100 was prepared, according to Wang et al., 2014.[9] Protease inhibitor Phenylmethylsulfonylfluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA) was added to lysis buffer to a concentration of 1 mM directly before cell lysis. 100 µL of this buffer were added to each pellet. The samples were sonicated 3x for 10 s with a probe with 30 s rests on ice in between. Then, suspensions were centrifuged for 30 min at 14 000 x g at 4 °C; both the pellet and the supernatant were collected.

3.1.5 Measurement of protein concentration

Total protein concentration of the supernatants was determined with a Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific) following the manufacturer's protocol. Measurements were performed using a Synergy H1 Hybrid Multi-mode Reader (BioTek, Winoosky, VT, USA).

3.1.6 SDS-PAGE

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed in a 12 % acrylamide gel created in the Mini-PROTEAN Tetra Handcast system (Bio-Rad, Hercules, CA, USA). The separation gel was prepared according to Table I and filled between two clean glasses with 0.75 mm interspacing. The gel was overlaid with distilled water and allowed to polymerize for about 20 minutes. The water on top was removed and the stacking gel according to Table I was filled over the separation gel. A 10-well comb was placed in the stacking gel and the gel was left to polymerize for at least 20 minutes.

Table I. Composition of separation and stacking gel for SDS-PAGE

Component	Separation gel 12%	Stacking gel 5%
Distilled water	1.7 mL	1.213 mL
Acrylamide (30%,37.5:1)	2 mL	0.266 mL
4x Separation buffer (1.5 M TRIS, 4 g/L SDS, pH 8.8)	1.25 mL	-
4x Stacking buffer (0.5 M TRIS, 4g/L SDS, pH 6.8)	-	0.5 mL
10 % Ammonium persulfate	100 µL	20 µL
Tetramethylethylendiamine	5 µL	2 µL
Total volume per one gel	~5 mL	~2 mL

Supernatant samples were mixed with 5x reducing Loading Buffer (40 g/L SDS, 0.25 M Tris(hydroxymethyl)aminomethane (TRIS), 0.6 g/L bromophenol blue, 0.0773 g/L Dithiothreitol (DTT), 378 g/L Glycerine, pH 6.8) and pellets were suspended in 2x reducing Loading Buffer. All samples were heated to 95 °C for 10 min and then placed on ice. The gel was placed in the electrophoresis chamber, which was filled with SDS running buffer (prepared from 10x SDS running Buffer: 30 g/L TRIS, 144 g/L Glycine, 10 g/L SDS). 20µL of the supernatant sample or 10 µL of the pellet sample were loaded; the used marker was Protein Marker VI (Applichem, Darmstadt, Germany). The chamber was closed, and 120 V were applied until the bromphenol blue reached the end of the gel (~45 min). The gels were stained with Coomassie Brilliant Blue (CBB) solution.

3.1.6.1 Western blot analysis

After identifying the conditions with the highest relative concentration of the expressed protein, another SDS-PAGE in a 12 % acrylamide gel was performed with the Protein Marker III 'Prestained', peqGOLD (VWR Peqlab, Radnor, PA, USA) and subsequently a Western blot to a Polyvinylidene fluoride (PVDF) membrane was performed in a Trans-blot Turbo transfer system (Bio-Rad) using the pre-programmed Standard SD blotting program (30 min, 25V, up to 1 A). After blotting, membrane was blocked in 10 ml of 5% skimmed milk in Phosphate Buffered Saline (PBS) (blocking solution) in a 50 ml centrifuge tube for 1 h at RT on the rotating shaker. After blocking, the membrane was washed in PBS for 10 min with rotation. Monoclonal mouse antibodies against the Histidine tag were diluted 1:1000 in 5 mL blocking solution and transferred onto the membrane for 1 h incubation on a rotator. Next, the membrane was washed 3 times for 10 min in PBS-T (PBS with 0.05% Tween 20). Tween 20 was added to improve the washing of membrane. Anti-mouse antibodies conjugated with Horseradish peroxidase (HRP) were diluted 1:1000 in 5 mL blocking solution and applied to the membrane. Then the membrane was incubated for 1 h at RT or overnight at 4 °C and then washed 3 times for 10 min in PBS-T. The signal was developed using WesternBright Quantum HRP substrate kit (Advansta, Menlo Park, California, USA) and the signal was detected using a G: BOX Chemi XX6 gel documentation system (Syngene, Cambridge, UK). The gel used for blotting was stained with CBB afterwards.

3.1.7 Optimal lysis conditions

50 mL of bacterial suspension produced as described above was split into four equal aliquots. Three aliquots were induced under optimal conditions (18 °C, 24 h, 0.1 mM IPTG), while the other aliquot was not induced and serves as negative control. One induced aliquot was used for inclusion body protein purification using ProteoSpin™ Inclusion Body Protein Isolation Micro Kit (Norgen Biotek, Thorold, Canada), according to the manufacturer's protocol for acidic proteins. Theoretical pI of the protein is 5.55.

Bacteria were pelleted, and the supernatant was discarded; the cell pellet was frozen at -20 °C until use. On the day of protein isolation, cells were thawed at room temperature and 200 µL of the Cell Lysis Reagent were added to the pellet and suspended by passing it 15 times through a 1 mL syringe with a needle. The suspension was centrifuged for 10 min at 14 000 x g and the supernatant was discarded. 200 µL of the Cell Lysis Reagent were again added to the pellet and it was again suspended by passing it a few times through the same needle and syringe from above. 600 µL of a 10-fold dilution of the Cell Lysis Reagent were added to the suspension and the mixture was passed through the needle a few times. The suspension was centrifuged for 10 min at 14 000 x g and the supernatant was again discarded. The pellet was suspended again in 800 µL of the Cell Lysis Reagent using the needle and syringe. The suspension was again centrifuged like before and the supernatant was discarded.

50 µL of IB Solubilization Reagent were added to the pellet and it was dissolved by pipetting and vortexing. 50 µL of sterile deionized water was added and the sample was mixed by vortexing. 50 µL of this dissolved protein sample were transferred into a microcentrifuge tube and 200 µL Milli-Q water were added. 7.5 µL of pH Binding Buffer (Acidic) were added to the sample and it was mixed by vortexing.

A spin column was assembled with a collection tube and the cap of the column was opened. 250 µL of Column Activation and Wash Buffer (Acidic) were added to the column and the cap was closed. The column was centrifuged for 1 min at maximum speed and the FT was discarded. Another 250 µL of the same buffer were added and centrifuged as described and the FT was discarded again.

The 257.5 µL of the sample were applied onto the column and centrifuged for 1 min and the FT is discarded. 250 µL of Column Activation and Wash Buffer (Acidic) were added to the column and the cap was closed. The column was centrifuged for 1 min at maximum speed and the FT was discarded. This step was repeated once again.

5 µL of Neutralizer was added to a fresh microtube and the column was transferred into this microcentrifuge tube. 25 µL of Elution Buffer were added to the column and it was centrifuged for 1 min; this step was repeated with the same microtube.

The second induced aliquot was lysed by sonication in a basic buffer (20 mM Tris HCl, 150 mM NaCl, 1mM PMSF pH 8.0).

The remaining induced aliquot and the negative control was lysed by sonication in acidic buffer as described above.

Protein concentration was determined with a Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific) as described above, SDS-PAGE in a 12 % acrylamide gel with the marker Protein Marker III (Applichem) and Western blot was performed from the lysed samples as well as the remaining pellets boiled in 2x reducing Loading Buffer.

3.1.8 Production and purification of PNGase H^+

Bacterial cell suspension (50 mL) was induced with IPTG for 18 °C, 0.1 mM for 24 h and after protein expression, bacteria were lysed un basic lysis buffer. His-tag purification was performed from the supernatant using an IMAC HyperCel column (PALL life sciences, Port Washington, New York, USA), following the protocol for Medium-Pressure Column Purification of Histidine tagged (His-tagged) Proteins for Profinity IMAC resins (Bio-Rad). Compositions of buffers for protein purification are described in Table II.

Table II. Composition of buffers for protein purification

Name	Buffer composition
Equilibration Buffer	50 mM sodium acetate, 0.3 M NaCl, pH 4.0
Binding Buffer	50 mM NaH_2PO_4, 0.3 M NaCl, 5 mM Imidazole, pH 8.0
Wash Buffer	50 mM NaH_2PO_4, 0.3 M NaCl, 20 mM Imidazole, pH 8.0
Elution Buffer	50 mM NaH_2PO_4, 0.3 M NaCl, 500 mM Imidazole, pH 8.0
Sanitation Buffer	1 % acetic acid 0.12 M phosphoric acid, pH 1.5
Metal ion solution	0.2 M $NiSO_4$
Regeneration Buffer	50 mM NaH_2PO_4, 0.3M NaCl, 0.2 M EDTA, pH 7.5

The IMAC HyperCel column (1 ml volume) was equilibrated with 5 mL Equilibration Buffer and loaded with 3 mL nickel ion solution. Excess ions were washed away with 5 mL of Equilibration Buffer. The column was washed with 10 mL of deionized water and equilibrated with 5 mL of Binding Buffer. The supernatant after basic lysis was diluted to a volume of 1 mL with Binding Buffer and loaded to the column with a flow rate of 1 mL/min and the FT was collected. The column was washed three times with 2 mL of wash buffer and the fractions were collected. Bound proteins were eluted from the column three times with 5 mL Elution buffer each and 1 mL fractions were collected. To regenerate the column, it was washed with 5 mL of Regeneration Buffer and 5 mL of deionized water. The column was stripped of metal ions with 10 mL Regeneration Buffer, washed with 5 mL Sanitation Buffer and 10 mL deionized water at 2 mL/min and stored in 20 % ethanol.

The purified proteins were analysed by SDS-PAGE in a 12 % acrylamide gel with Protein Marker III (Applichem) and Western blot analysis.

3.1.9 Activity of PNGase H^+

The activity of the recombinant PNGase H^+ protein was tested by a set of overnight reactions with a glycoprotein transferrin. The following fractions from the purification process were tested: acidic lysis, 1:10 dilution of acidic lysis buffer (pH 2.6), slightly basic lysis (pH 8), flowthrough after IMAC purification, wash step (W6), elution steps E1, E2, and E3.

Per each reaction, 10 µL Glycine/HCl buffer (0.4 M, pH 2.6), and 5 µL transferrin solution (10µg/µL) were denatured at 100 °C for 10 minutes and 5 µL of each tested sample were added. PNGase F reaction prepared according to the manufacturer's manual with 5 µL transferrin solution (10 µg/µL) was used as positive control. The negative control consists of the same buffer as the samples and transferrin filled up to the volume with water. All reactions were incubated at 37 °C overnight. 1.2 µL (0.6 µL for positive control) of each sample were diluted to 15µL with distilled water, 5x reducing leading buffer was added and SDS-PAGE in a 12 % acrylamide gel with Protein Marker III (Applichem) was performed.

3.1.10 Circular polymerase extension cloning (CPEC)

To create a pRSET vector with the gene of PNGase H^+ for better yield, CPEC was performed. First, PCR was performed to amplify the PNGase H^+ gene from the pET vector and the pRSET counterpart of a pRSET vector. The primers used for the reaction prolonged the sequence at the ends to create an overlap of 15 – 17 bp with a melting temperature between 60 - 70 °C in accordance with CPEC protocol,[17] according to Table III.

Table III. Primers for PCR to perform CPEC to create a pRSET vector with PNGase H^+ gene

Name	Forward primer	Reverse primer	Product length
PNGase H^+ gene	CACCATGCCCCGCAT CTTGTGCCGC	TTCTCGAGGCGTTT CACCGGGCAGCC	1716 bp
pRSET vector	GTGAAACGCCTCGA GAAGCTTGATCCGGC	ATGCGGGGCATGGT GGCGAATTCGGAT	2900 bp

After PCR, electrophoresis was run in a 1% agarose gel and the bands of the right size were cut out. The DNA was purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Life Sciences, Chalfont St. Giles, United Kingdom) in accordance with the protocol. 10 µL (for PNGase H^+ Gene) or 25 µL (for pRSET) Elution buffer type 4 was used for elution. The samples were purified by precipitation in 96% ethanol. 10 % of the volume of 3 M Sodium acetate and 3x the volume of ice-cold ethanol was added and kept overnight. This solution was centrifuged at 4 °C at 14 000 x g and the supernatant was discarded. The pellet was washed with 70% ethanol, which was discarded afterwards. The pellet of the pRSET plasmid was diluted in 25 µL and the one of PNGase H^+ was diluted in 10 µL water. The purity and concentration of the DNA samples was controlled via UV-spectroscopy with a Nanophotometer Pearl (Implen). 50 - 100 ng of linearized plasmid and 10-fold molar amount of the insert were mixed with 5µL Q5 reaction buffer, 2.5 µL dNTPs, 0.25 µL Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs) and water reach a reaction volume of 25 µL. The molecules were connected and amplified with CPEC. The temperature conditions for the CPEC reaction were 98 °C for 3 min, 25 cycles of 98°C for 10 s, 72°C to 50 °C with speed of 0.1K/s, 50 °C for 10s and 72 °C for 2min 15 s and a final extension step at 72 °C for 5 min. Electrophoresis was performed in a 1.5 % agarose gel as described above.

3.2 Production of *Ixodes ricinus* proteins

3.2.1 Confirmation of target enzyme genes in genome of I. ricinus

To confirm the presence of the target genes in *Ixodes ricinus* gDNA, PCR was performed with primers designed for amplification of conserved regions of the genes. The temperature protocol used was the following: 4 min at 95 °C, 30 cycles with 30 s at 95 °C, 30 s at the annealing temperature (Table IV) and 30s at 72 °C, final elongation at 75 °C for 5 min. Table IV lists the tested genes and length of PCR products and the predicted annealing temperature. Primers to actin gene of *Ixodes* spp. were provided by RNDr. Pavlina Vechtova Ph.D. Electrophoresis was performed in a 1.5 % agarose gel as described above.

Table IV. PCR primers for conserved regions of DNA methyltransferases and Sialyltransferases genes of *I. ricinus*.

Gene	Forward primer	Reverse primer	Prod. length	Ta [°C]
DNMT1 (MH795945)	GACAACACCCT GATCCCCTG	CCTGTTTTCCCA TCGGCTCT	136 bp	55
DNMT3 (MH926034)	ACATCGTCTGG GGCAAGATC	CATGTACGACG AGGTGGACG	143 bp	55
DAMT (MH926033)	CCTGGTTGCTG TGTGGTGTA	TGCTTCGTCAC CTTAACCCA	120 bp	55
α-2.3-Sialyltransferase	TCGTCGGTTTCT CTGGACAC	AGCGTCGATCA GCCTGAAG	140 bp	55
α-2.6-Sialyltransferase	GCACGAGGACC TGGGATGC	CCTCCGCCAAG AGGAAGACG	132 bp	58
Actin	CGCCATCCTCC GTCTGGACTT	CGTCGGGAAGC TCGTAGGACTT	160 bp	60

3.2.2 Creation of plasmids containing target enzyme genes

pMT/V5-His A vectors including DNMT and DAMT sequences were created in Geneious 10.0.5, in accordance with the protocol for *Drosophila* expression system (Thermo Fischer Scientific). Primers for Gibson assembly were designed to the vector sequences (similarly to primers for CPEC as mentioned above) and ordered from Generi Biotech (Třebeš, Czech Republic).

Insert sequences for DNMT1 and DNMT3 were amplified from *I. ricinus* cDNA and by PCR reaction using Q5 High-Fidelity DNA polymerase (New England Biolabs). For a 20 µL reaction volume, the following components were mixed: 4 µL Q5 Reaction buffer, 0.4 µL10 mM dNTPs, 1 µL 10 µM Forward primer, 1 µL 10 µM Reverse primer, 1 µL DNA template, 0.2 µL Q5 High-Fidelity DNA Polymerase, 12.4 µL nuclease free water. The temperature protocol was in accordance with the melting temperature of the designed primers and the length of the sequence: 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 72 °C for 150 s (DNMT1) or 84 s (DNMT3), after the circles 72 °C for 2 min and then hold at 4 °C. Electrophoresis in a 1.5 % agarose gel was performed as described above.

4 Results

4.1 Production of PNGase H^+

4.1.1 Confirmation of the presence of PNGase H^+ gene

The concentration of the isolated plasmid was 31.4 ng/µL, the absorbance ratio 260/280 was 1.882. The PCR product of isolated plasmids from transformed *E. coli* cells and stock plasmids (with primers for PNGase H^+) each had a similar size of approximately 1.7 kbp. (Figure 3). Thus, we confirmed that Rosetta-gami competent cells contain pET plasmid with the PNGase H^+ gene, and we used them for further work.

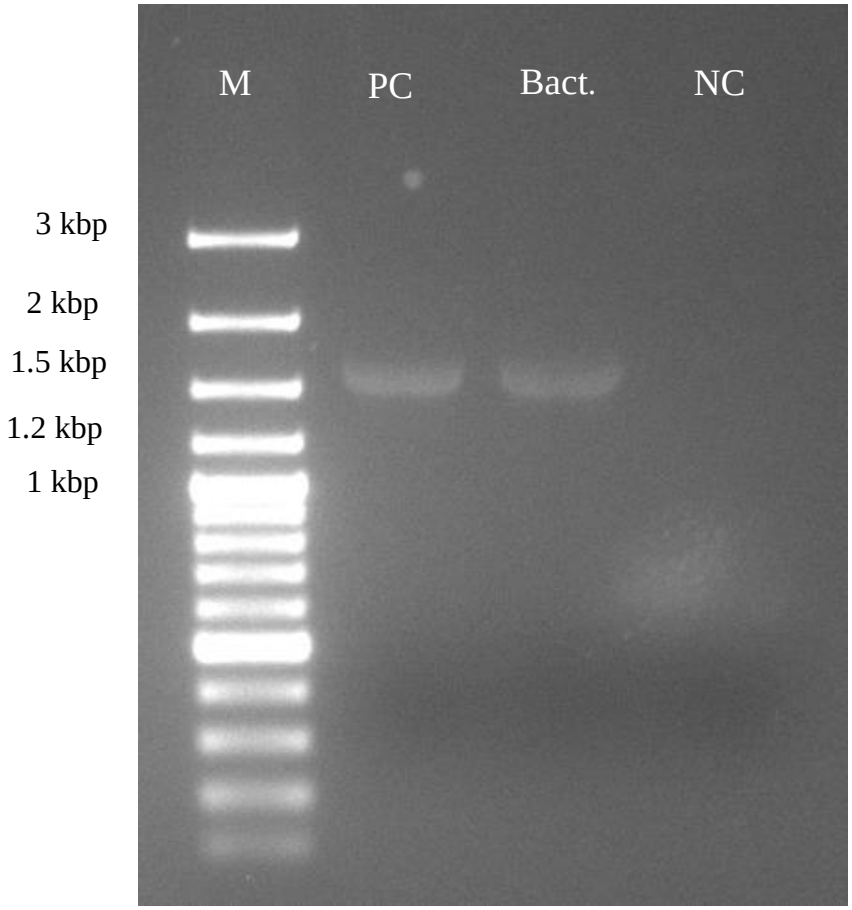

Figure 3. Agarose gel (1.5 %) electrophoresis of PCR products with primers for PNGase H^+ gene: M – GeneRuler DNA Ladder; PC – positive control, plasmid stock containing gene; Bact. – plasmid extracted from bacterial growth; NC – water instead of DNA template.

4.1.2 Optimal conditions for PNGase H^+ expression

Three temperatures, different time of expression and two concentrations of IPTG (Table V) were tested for the overexpression of PNGase H^+. According to Table V, the amount of proteins in the supernatant samples after lysis in acidic lysis buffer (5 % acetic acid, 1 % Triton X-100, 1 mM PMSF) grown for shorter times is generally lower. Expression at 18 °C with cells grown for 24h and induced with 0.1 mM IPTG yielded the highest concentration of protein.

Table V. Total protein concentration in supernatants of acidic lysates from bacteria after induction of PNGase H^+ expression in Rosetta-gami cells using different temperature and concentration of IPTG. Samples marked in bold were used for the further analysis.

Number	t (h)	T (°C)	IPTG (mM)	c (µg/mL)
1	0			206.2
2	1	37	0.1	1208.6
3	1	37	1	327.0
4	2	37	0.1	327.0
5	2	37	1	526.1
6	3	37	0.1	163.5
7	3	37	1	604.3
8	**4**	**37**	**0.1**	**391.0**
9	**4**	**37**	**1**	**526.1**
10	3	25	0.1	334.1
11	3	25	1	263.0
12	**16.5**	**25**	**0.1**	**661.2**
13	16.5	25	1	646.9
14	**24**	**25**	**0.1**	**583.0**
15	24	25	1	753.6
16	3	18	0.1	327.0
17	3	18	1	369.7
18	16.5	18	0.1	455.0
19	**16.5**	**18**	**1**	**440.8**
20	**24**	**18**	**0.1**	**831.8**
21	24	18	1	718.0

Cells grown at all tested conditions were sonicated in the acidic lysis buffer (5 % acetic acid, 1 % Triton X-100, 1mM PMSF) and both supernatants and pellets were analysed using a 12 % acrylamide gel by SDS-PAGE. The six most promising conditions were chosen for further analysis (marked in bold in Table V). The conditions of these samples were 4 h at 37 °C with 1 and 0.1 mM IPTG (samples 8 and 9), 16.5 h and 24 h at 25 °C with 0.1 mM IPTG each (samples 12 and 14), 16.5 h at 18 °C with 1 mM IPTG (sample 19), and 24 h at 18 °C with 0.1 mM IPTG (sample 20).

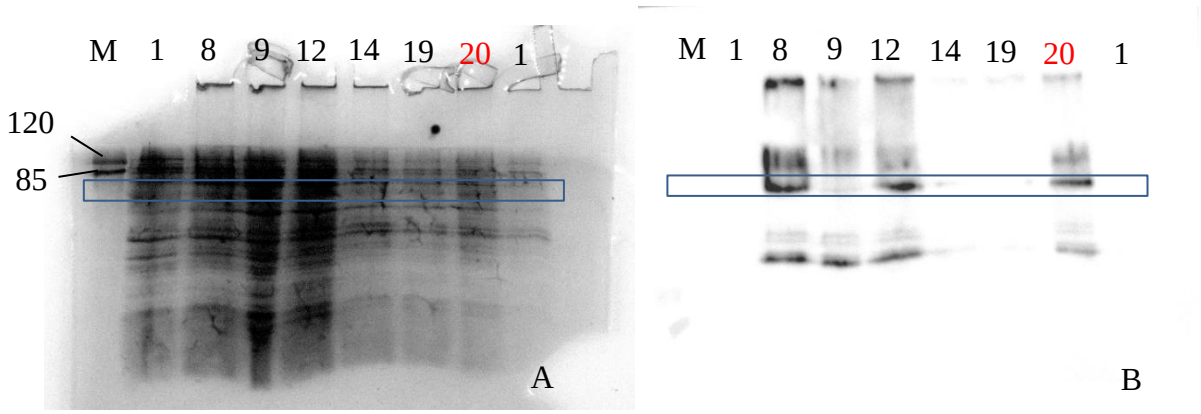

Figure 4. SDS-PAGE in 12 % acrylamide gel (A) and western blot (B) of insoluble protein fractions after acidic lysis from Rosetta-gami cells containing PNGase H^+ gene induced at different conditions. M – unstained protein marker, 1 – uninduced sample; 8 – 4 h, 37 °C, 0.1 mM IPTG; 9 – 4 h, 37 °C, 1 mM IPTG; 12 – 16.5 h, 25 °C, 0.1 mM IPTG; 14 – 24 h, 25 °C, 0.1 mM IPTG, 19 – 16.5 h, 18 °C, 0.1 mM IPTG; 20 – 24 h, 18 °C, 0.1 mM IPTG; Blue Box indicates expected size for PNGase H^+ (~60 kDa[9]); red number indicates the determined optimal condition.

The SDS-PAGE of the supernatant samples after lysis in the acidic lysis buffer (not shown) only shows very weak bands unsuitable for Western blot. This result also infers that proteins were mainly present in insoluble fraction (inclusion bodies). The western blot of the pellets shows signals above and below the theoretical size of PNGase H^+ (60.4 kDa[9]) (Figure 4).

Strong signals of the correct size are present in the conditions 37 °C, 4h, 0.1 mM IPTG (8); 25 °C, 16.5h, 0.1 mM IPTG (12) and 18 °C, 24 h, 0.1 mM IPTG (20). The bands in the SDS-PAGE of sample 20 are less intense than sample 8 and 12; this sample contains more target protein in relation to total protein in the pellet after acidic lysis.

4.1.3 Optimal lysis conditions

Basic Lysis buffer can extract more than two times as much protein as acidic lysis buffer. The ProteoSpin™ Inclusion Body Protein Isolation Micro Kit (Norgen Biotek) solubilizes little protein compared to the other isolation methods (Table VI).

Table VI. Total protein concentration after basic lysis, acidic lysis and inclusion bodies extraction from induced Rosetta-gami growth

Protein isolation conditions	c (µg/mL)
Non-induced Acidic Lysis	1053.4
Acidic Lysis	901.3
Basic Lysis	2343.2
Inclusion bodies extraction	171.3

An SDS-PAGE in a 12 % acrylamide gel shows intense bands in the basic lysate (Figure 5, A). Samples of acidic Lysis (1,2) present weak bands compared to the concentration of protein measured(Figure 5, Table VI), possibly, the proteins are not stable in the buffer and are degraded. In a Western blot, a signal appears at about 35 kDa in the basic lysis and all applied pellets, lower than the expected size of PNGase H^+ (60.4 kDa[9]). A weak signal at about the right size can be seen in the pellet of the basic lysate and a very weak signal at the right size may be present in the basic lysate. The signal present in the Western blot of the pellet of the non-induced sample can be attributed to the low specificity of used antibodies or high basal expression. There is no signal in the acidic lysate or dissolved inclusion body proteins, therefore, lysis will be performed in basic lysis conditions.

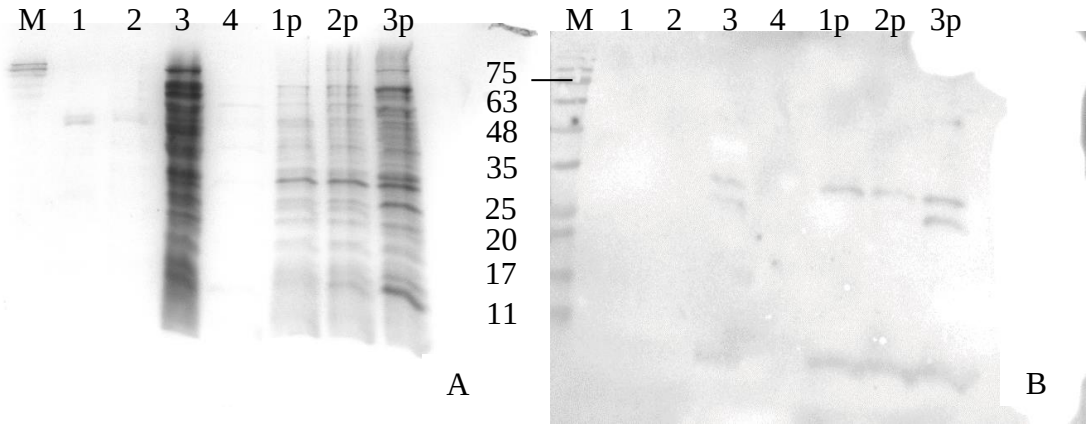

Figure 5. SDS-PAGE in 12 % acrylamide gel (A) and Western blot (B) of lysates from different methods. M – unstained protein marker 1 – non-induced cells, acidic lysis buffer, supernatant fraction; 2 – IPTG-induced cells, acidic lysis buffer, supernatant fraction; 3 – IPTG-induced cells, basic lysis buffer, supernatant fraction; 4 – extraction with inclusion body protein isolation kit; 1p – pellet of non-induced cells after lysis in acidic buffer; 2p – pellet of IPTG-induced cells after lysis in acidic buffer; 3p – pellet of IPTG-induced cells after lysis in acidic buffer.

4.1.4 Production and purification of PNGase H$^+$

After purification with an IMAC HyperCell column (PALL life sciences) of the basic lysate of Rosetta-gami cells with pET vector containing the PNGase H$^+$ gene induced at optimal conditions (18 °C, 24 h, 0.1 mM IPTG), four proteins remain in higher concentration, two of which are in the vicinity of the theoretical mass of PNGase H$^+$.

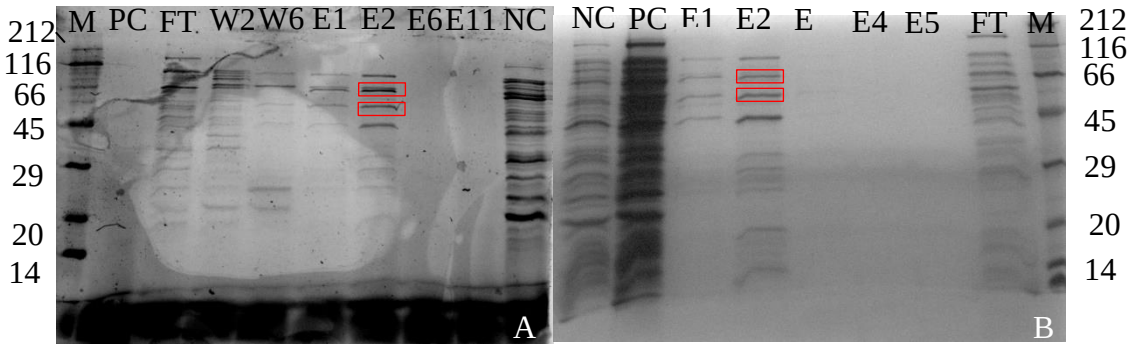

Figure 6. SDS-PAGE in 12 % acrylamide gel (A, B) of different steps of purification of basic lysate of induced bacteria containing the PNGase H$^+$ gene in an IMAC Hypercell column. M – prestained protein marker; PC – basic lysis of induced bacteria; FT – flowthrough; W+number – fractions from the washing step; E+number – fractions from the elution step; NC – lysis of non-induced bacteria; red boxes mark bands at about the right size.

The flowthrough (FT) and the second fraction from the washing step (W2) contained several proteins of different sizes. The bands in the sixth fraction from the washing step (W6) are similar to the bands present in the elution steps (E1-5), therefore weakly bound proteins are effectively washed away by the washing Buffer. Four bands are visible in the first 3 mL (E1 - E3) of the elution step, these signals are most intense in E2. In this sample there are several other bands of lower size present, probably due to degradation or mechanical destruction of the target protein. The bands in the E3 fraction are weak and and no proteins were detected in the tested fractions afterwards (E4, E5, E6, E11). The bands in the red boxes appear close to the theoretical size of PNGase H$^+$ (60.4 kDa[9]) (Figure 6).

4.1.5 Activity of PNGase H$^+$

To determine the potential deglycosylation activity of PNGaseH+ recombinant protein, different protein samples (bacterial lysates and eluted fractions after column purification) were mixed with glycoprotein transferrin. PNGaseF activity was used as a positive control for deglycosylation. A band at about the size of glycosylated transferrin (80 kDa[18]) appears in the negative control (NC), an overnight reaction of 50 µg transferrin with water at acidic conditions (pH 2.6). The band in the positive control (PC) appears lower, around the size of deglycosylated transferrin (78 kDa[18]). The bands in basic lysate (Lb), flowthrough (FT), fraction of unbound protein from the washing step (W), and the first eluted fraction (E1), are the same size as glycosylated transferrin. The protein in the reactions with the elution fractions E2 and E3 appears slightly smaller than the glycosylated protein, but still above the size of the deglycosylated transferrin. The reaction with acidic lysate (La) exhibits a very weak band at the size of glycosylated transferrin and a band at the size of the deglycosylated transferrin. In the diluted acidic lysate (1:10), several bands are visible at the sizes of the glycosylated and deglycosylated transferrin (Figure 7). Although all reactions were performed with the same amount of transferrin, different intensities are observed; the band in the positive control is the most intense.

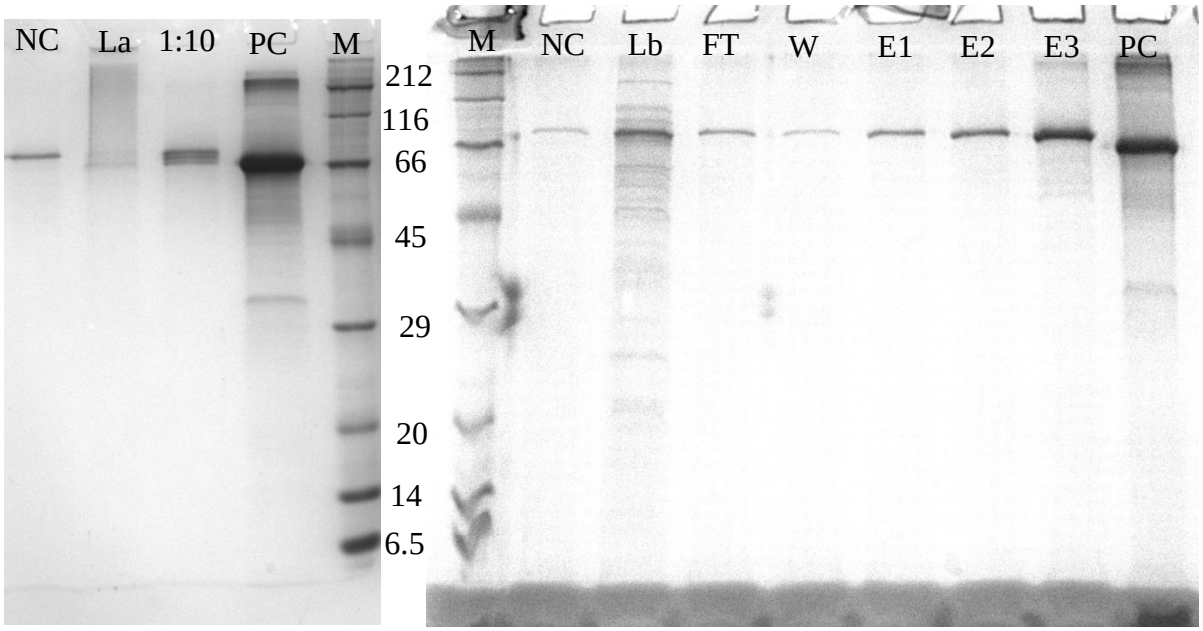

Figure 7. SDS-PAGE in 12 % acrylamide gel of overnight reactions of 50 µg transferrin with acidic lysis samples and steps of purification under basic conditions. M – prestained protein marker; NC – negative control; La – acidic lysis supernatant; 1:10 – ten times diluted sample of acidic lysis; PC – positive control (PNGase F); Lb – basic lysis supernatant; FT – flowthrough after purification with IMAC column; W – washed fraction; E1, E2 and E3 – eluted fractions.

4.1.6 Circular Polymerase Extension Cloning (CPEC)

The linear sequences of PNGaseH+ gene and pRSET vector were successfully amplified by PCR using primers, listed in Table III. The concentration and purity of the PCR products needed for the assembly of a pRSET vector with PNGase H^+ gene are shown in Table VII. Despite the high purity and concentration of the produced PCR products, no band with the expected size appears after electrophoresis in a 1.5 % agarose gel after CPEC.

Table VII. Concentration and absorption of PCR products of linearized plasmid and PNGase H^+ gene using designed primers

Sample	Concentration(ng/µL)	Adsorption (260/280)
pRSET vector	90.3	1.757
PNGase H^+ gene	66.9	1.942

4.2 Production of *Ixodes ricinus* proteins

4.2.1 Confirmation of the presence of target genes in the genome of I. ricinus

The PCR reactions of gDNA with primers for conserved regions of DNA methyltransferases (DNMT1, DNMT3, DAMT) and α-2,6-Sialyltransferase yield products in the expected size (Figure 8, Table IV). The product for α-2,3-Sialyltransferase (S2.3) appears around a size of 700 bp, but the expected length of the conserved region is about 140 bp (Table IV), This difference is due to introns in the conserved region. All tested target genes are present in the genome of *I. ricinus*.

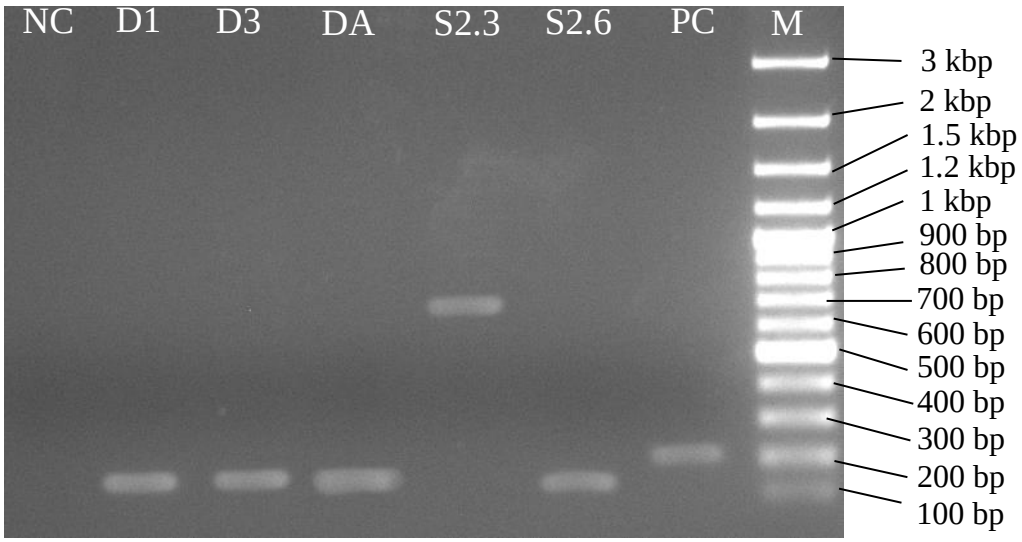

Figure 8. Agarose electrophoresis of PCR products from *I. ricinus* gDNA. NC – Negative control, primers for actin without DNA template; D1 – primers for DNMT1; D3 – primers for DNMT3; DA – primers for DAMT; S2.3 – primers for α-2,3-Sialyltransferase; S2.6 – primers for α-2,6-Sialyltransferase primers with gDNA; PC – positive control with primers for actin.

4.2.2 Creation of plasmids containing target enzyme genes

We suggested that the efficient production of recombinant *I. ricinus* enzymes can be done using insect expression systems. For instance, the *Drosophila* expression system (ThermoFischer Scientific), which we planned to use in the further work, uses pMT vectors for successful transfection of insect cells. Thus, we designed primers to assemble pMT/V5-His A plasmids containing the genes of interest with Gibson Assembly in the program Geneious 10.0.5 (Figure 9).

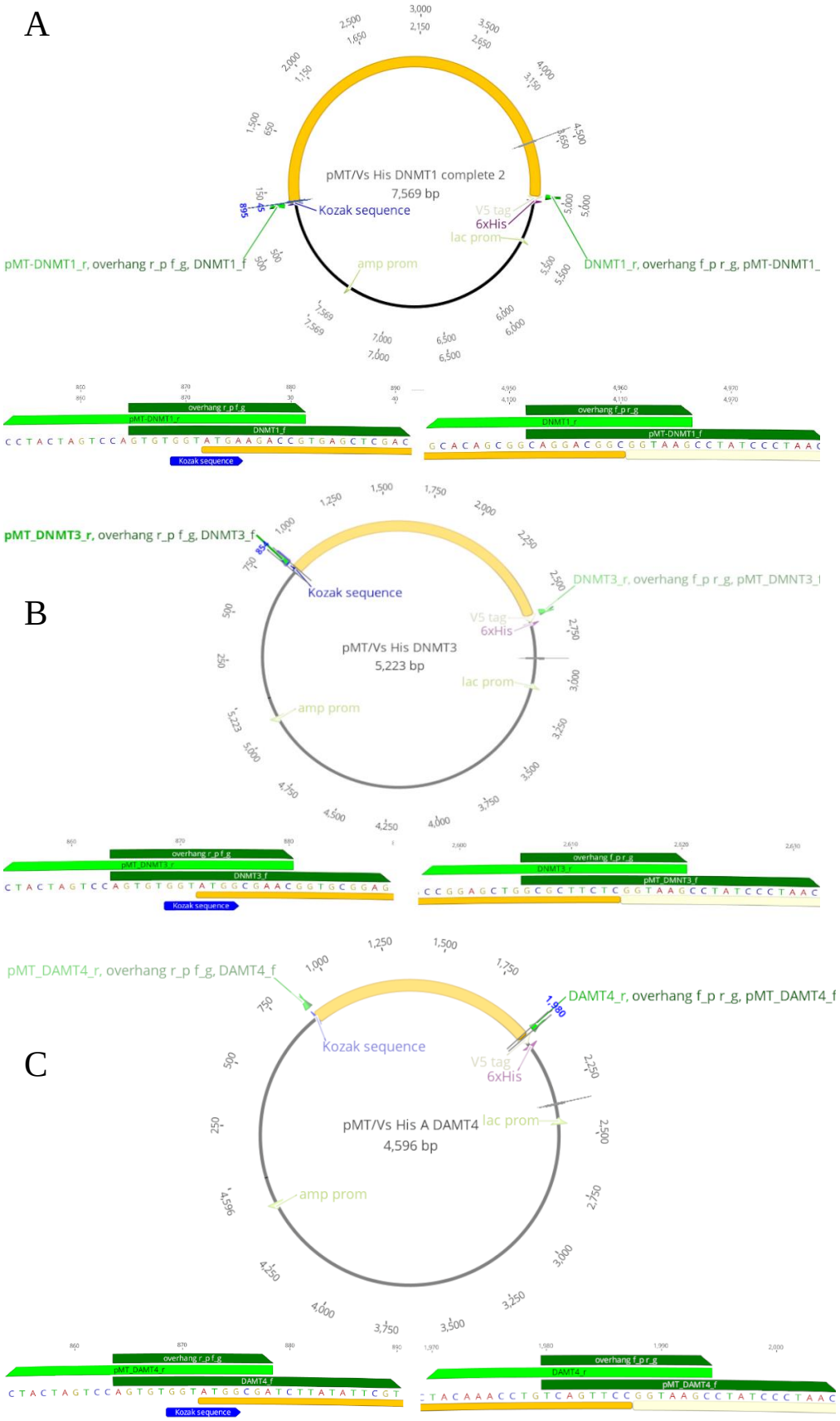

Figure 9. Designed pMT plasmids and primers for DNMT1 (A), DNMT3 (B), and DAMT4 (C)

Primers were designed to overlap the gene and plasmid sequence and the reverse primer present the reverse complement sequence (Table VIII). After PCR of cDNA with designed primers for DNMT1 and DNMT3, no band appears in a 1.5 % agarose gel.

Table VIII. Sequences and melting temperatures of designed primers for Gibson Assembly of a pMT vector with DNMT1, DNMT3 or DAMT genes

Primer	Sequence 5’ – 3’	Tm [°C]
Forward DMNT1_f	GTGTGGTATGAAGACCGTGAGCTCGAC	66.9
Reverse DNMT1_r	CTTACCGCCGTCCTGCCGCTGTGC	72.1
Forward pMT_DNMT1_f	CAGGACGGCGGTAAGCCTATCCCTAAC	68.0
Reverse pMT_DNMT1_r	CGGTCTTCATACCACACTGGACTAGTAGG	65.6
Forward DMNT3_f	AGTGTGGTATGGCGAACGGTGCGGAG	70.7
Reverse DNMT3_r	CTTACCGAGAAGCGCCAGCTCCGG	69.3
Forward pMT_DNMT3_f	GCGCTTCTCGGTAAGCCTATCCCTAAC	66.7
Reverse pMT_DNMT3_r	GTTCGCCATACCACACTGGACTAGTAG	64.5
Forward DAMT_f	AGTGTGGTATGGCGATCTTATATTCGT	62.1
Reverse DAMT_r	GCTTACCGGAACTGACAGGTTTGTA	63.0
Forward pMT_DAMT_f	TCAGTTCCGGTAAGCCTATCCCTAAC	63.6
Reverse pMT_DAMT_r	TCGCCATACCACACTGGACTAGTAG	63.4

5 Discussion

5.1 Production of PNGase H^+

5.1.1 Confirmation of presence of PNGase H^+ gene

The size of the PNGase H^+ gene is 1707 bp[9]; the product after PCR of extracted plasmids from the provided Rosetta-gami cells with the primers against PNGase H^+ is at about 1.7 kbp, at the same size as the product of the positive control with template plasmids. Therefore, the vector containing the gene for PNGase H^+ is present in the used Rosetta-gami expression host and expression can be started with this strain.

5.1.2 Optimal conditions for PNGase H^+ expression

The varying intensities of the bands in the SDS-PAGE of the pellet from different conditions can be explained by loading errors of the viscous samples. As the intensity of the signal in the western blot is high in comparison with the signal in the SDS-PAGE, the optimal conditions for expression of PNGase H^+ with the used pET expression system in Rosetta-gami cells are 18 °C and 0.1 mM IPTG for 24 h (Figure 4). Wang et al. determined the same conditions for time and temperature, but 1 mM IPTG.[9] A possible explanation for this difference is the use of different expression hosts. The signals above the size of PNGase H^+ in the Western blot of the SDS-PAGE of pellets from different expression conditions can be explained by posttranslational modifications of the PNGase H^+ protein, or by unspecificity of anti-His antibodies, which recognized bacterial proteins with several histidine residues. The signals smaller than the size of PNGase H^+ on the Western blots can be explained by degradation of the target protein or, probably by unspecific binding of antibodies (Figure 4)

Small amounts of PNGase H^+ can be produced in Rosetta-gami cells with a pET expression system; an overexpression of the target gene was not observed.

5.1.3 Optimal lysis conditions for extracting PNGase H$^+$

The usage of an acidic lysis buffer as described by Wang et al.[9] (5 % acetic acid, 1 % Triton X-100, 1 mM PMSF) produces some issues; the supernatant after lysis shows low amounts of protein and performing SDS-PAGE at a low pH does not yield clean results. The usage of a kit for inclusion bodies extraction can only extract low amounts of protein from the produced pellet. The most protein is extracted by a basic lysis buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM PMSF, pH 8). A weak signal appears at a lower size in the western blot for these lysis conditions, either due to low specificity or degradation of the target protein. Although these results don't confirm the presence of the protein without doubt, we still assume that the product is extracted by a basic lysis buffer, as proteins at about the size of PNGase H$^+$ remain after affinity chromatography against the attached His-tag.

5.1.4 Purification of PNGase H$^+$

Four proteins show a high affinity to the IMAC HyperCell column (PALL Life Sciences) and are can be washed out within the first 3 mL at a higher imidazole concentration (500 mM). Two of these four proteins appear around the theoretical size of PNGase H$^+$ (60.4 kDa[9]). It is assumed that one of these bands represents the target protein.

5.1.5 Activity of PNGase H$^+$

The lower intensity of the bands in the activity assay samples and negative control can be explained by insoluble protein after denaturation in acidic conditions without suitable denaturation buffer. Lysate in acidic condition shows activity in deglycosylation of transferrin. As the basic samples showed extremely low activity, the low pH optimum of PNGase H$^+$ [9] is a probable explanation. The 1:10 diluted sample of the acidic lysis also shows activity, but the deglycosylation is incomplete after reaction overnight. This shows that the concentration of the target protein in acidic lysis is not high, as already mentioned before.

5.1.6 Circular Polymerase extension cloning (CPEC)

CPEC did not result in the assembly of a pRSET vector containing the PNGase H$^+$ gene; Gibson Assembly with the same vectors might yield better results. The pRSET expression system may have resulted in a higher production of the target protein.

5.2 Production of *Ixodes ricinus* proteins

5.2.1 Confirmation of the presence of target enzyme genes in the genome of I. ricinus

The presence of the genes of α-2,3-Sialyltransferase, α-2,6-Sialyltransferase, DNMT1, DNMT3, and DAMT in the genome of *Ixodes ricinus* was confirmed by using primers to the conserved gene regions. The size difference between the PCR product of α-2,3-Sialyltransferase primers and gDNA and the size of the conserved region can be explained by the presence of introns.

5.2.2 Creation of plasmids containing target enzyme genes

A Drosophila expression system was considered promising for the production of these recombinant proteins from *Ixodes* ticks. Therefore, I designed the pMT plasmid vectors, containing genes of *I. ricinus* DNA methyltransferases, for the expression in the *Drosophila* cells.

6 Conclusion

Several steps of optimization were performed to produce an active PNGase H^+ enzyme in *E. coli* cells. Different temperatures of expression, concentrations of IPTG, and lysis buffers were tested. However, these conditions did not result in a valuable amount of the expressed protein. Thus, I found that PNGase H^+ belongs to proteins, which are hard to produce using the standard bacterial expression system, therefore, testing of other expressing cells, plasmids and different lysis buffers should be considered.

I also confirmed the presence of predicted sequences of sialyltransferases and DNA methyltransferases in *Ixodes ricinus* genome using PCR. Drosophila expression system was considered promising for the production of these recombinant proteins. Therefore, I designed the vectors, containing studied *I. ricinus* genes, for the expression in the insect cells.

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8 List of abbreviations

Table IX. Used abbreviations

Abbreviation	Meaning
Amp	Ampicillin
CBB	Coomassie Brilliant Blue
CPEC	Circular Polymerase Extension cloning
DES	Drosophila expression System
DNMT, DAMT	DNA Methyltransferases
DTT	Dithiothreitol
FT	Flowthrough
Fuc	Fucose
HRP	Horse Radish Peroxidase
IPTG	Isopropyl-β-D-1-thiogalactopyranosidepyl
Kan	Kanamycin
LB	Lysogeny broth
NC	Negative Control
OD600	Optical Density at 600 nm
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PC	Positive Control
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonylfluoride
PNGase	Peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase
PVDF	Polyvinylidenfluoride
RT	Room temperature
SDS	Sodium dodecyl sulphate
TRIS	Tris(hydroxymethyl)aminomethane