DESIGN OF SECRETION VECTOR CONSTRUCTS FOR GRANZYME H EXPRESSION

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Granzima H aparţine familiei serin-proteinazelor, având un rol important ca parte a sistemului imunitar natural. Această enzimă induce apoptoză în celule ţintă, şi este produsă de către limfocitele T şi celule NK (natural killer). Rolul precis a enzimei nu este clarificată, nu există încă o structură cristalizată. Granzima H a fost clonată cu RT-PCR din RNA total izolat din limfocite umane. Diferite vectori de secreţie au fost proiectate pentru expresia optimală a Granzimei H: pET20bGraH, pETM50GraH, pETM52GraH. Enzima produsă prin intermediul vectorilor de secreţie va fi mult mai potrivită pentru măsurări de activitate în viitor.

Granzyme H belongs to the family of serine proteinases, which plays an important role as part of the natural immune system. This enzyme is produced by natural killer cells and the T lymphocytes, inducing apoptosis in target cells. The precise role of the enzyme is not yet clarified, its crystal structure is unknown. Granzyme H was cloned by RT-PCR from total RNA isolated from human lymphocytes. We designed several vector constructs for the optimal expression of Granzyme H: pET20bGraH, pETM50GraH, pETM52GraH. The enzyme produced using these vectors will be more suitable for activity measurements in the future.

Keywords: Granzyme H, apoptosis, pET secretion vectors

1. Introduction

Granzymes are released by cytotoxic T lymphocytes and by resting natural killer cells. The enzymes secreted by these two cell-types are granule associated enzymes, so called “granzymes” [1]. These cytotoxic granules contain a protein named perforin, which forms pores on the surface of cell membranes, and also contain serin proteases responsible for the breakdown of proteins [2].

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The five members of the human granzyme family (Granzyme A, B, K, M, H) are present in placental mammals, being crucial components of the immune response by elimination of host cells infected by intracellular pathogens. Granzyme B and H are evolutionary strongly related, showing a 71% structural homology in their amino acid composition, also being located on the same chromosome.

Granzyme H is a specific human enzyme, a chymotrypsin-like serine protease of which coding sequence is located on chromosome 14 [3].

Because of the odd number of cysteins, the folding of Granzyme H is not perfect, while expression in *Escherichia coli,* results a high level of inclusion body formation. The aim of this study is to design three vector constructs to produce soluble Granzyme H with the help of chaperons, and thus directing the production of the protein into the periplasmic space.

2. Materials and methods

2.1. Plasmids and bacterial strains

For optimal expression of Granzyme H we used three variants from the well-known pET system from Novagen. The gene of interest can be cloned under the control of a strong promoter of T7 bacteriophage. To avoid expression during DNA manipulation, target genes are cloned by using a host that does not contain the T7 RNA polymerase gene, such as *E. coli* DH5α: genotype: F^-φ, 80d*lac*ZΔM15, Δ(*lac*ZYA-argF) U169, *rec*A1, *end*A1, *hsd*R17 (rK^-,mk^+), *sup*E44, λ^-, *tfi*-1, *gyr*A, *rel*A1, Invitrogen. For expression of target proteins typically DE3 lysogens of *E. coli* are used. The genome of these strains contain T7 polymerase gene under the control of *lac* operator.

Plasmid vector pET20b carries an N-terminal *pelB* signal sequence for periplasmic localization of the protein, and also a C-terminal His-tag.

The *Nco I* recognition sequence from pETM50 and pETM52 vectors allows a functional expression of the target protein with an in-frame ATG codon right before the TEV cleavage site and the His-tag. DsbA and DsbC are two enzymes which facilitate disulfide bond formation while cloning into pETM50 and pETM52 vectors. Using the pETM50 vector we have the possibility of targeting the protein fused with a chaperone into the oxidizing medium of the periplasm [4].

For blue-white screening pBluescriptKS vector was used from Fermentas.

2.2. Obtaining the correct inserts for subcloning

Two restriction endonucleases were chosen to be used for all the three plasmid vectors. Cloning our gene of interest into an *Nco I* and *Xho I* digested pETM50 or pETM52 vector results in a plasmid DNA where the Granzyme H encoding site is located right before the TEV site, the His-tag and the Dsb chaperon encoding site of the vectors. Digesting the pET20b vector with these enzymes directs the Granzyme H gene before the pelB signal sequence.

15 μL of desired plasmid vectors for cloning were digested: 10 U of *Nco I* and *Xho I* in 2X Tango buffer in a total volume of 30 μL reaction at 37°C, overnight. To terminate the digestion, loading buffer for agarose electrophoresis was applied. The digested vectors were purified and eluted from gel using DNA Extraction Kit (Fermentas).

Preparation of the small fragment was more complicated, because it contained an *Nco I* restriction site inside the gene. To avoid the *Nco I* restriction site, we used the method of TA cloning, where the PCR amplified insert will have an adenine at the 3' end, by using Taq polymerase, and the cloning vector has a single base T overhang on each end. The insert encoding the Granzyme H sequence was amplified from GraHpET17c with GraH-R2 *Xho I* (GGCCTCGAGGAAGGTTAGTCTCATGCCTGCTG) and pETM5'*Nco I* (CGCCATGGCTTTTCCCGTGGACGATGAC) primers. pBluescriptKS vector was cleaved at the single *EcoR V* site and to its 3` ends dTTP was added by Terminal deoxynucleotidyl Transferase. The vector and insert were ligated by T4-ligase (Fermentas) in a total volume of 20 μL at 4°C, overnight. 10 μL of the reaction was transformed by heat shock into competent *E. coli* DH5α cells and incubated 16 hours at 37°C on plates containing 100 ug/mL Ampicillin. LB agar plates were prepared for blue-white screening by adding 150 μL LB, 20 μL of 20 mg/mL Xgal and 30 μL of 100 mM IPTG. Twelve white colonies were chosen for DNA isolation to verify the correct assembly of the pBluescriptKS-GraH vector. High Speed Mini Kit (Geneaid) was used for DNA isolation according to the protocol of the manufacturer. Correct sized DNA was digested with *Xho I* overnight at 37°C. The reaction was brought to halt by isopropanol precipitation. 60 μL of plasmid DNA was partially digested 1 h at 37°C with *Nco I* using a 3 fold serial dilution of the enzyme. Reaction was stopped by adding gel loading buffer . 5 μl of digested DNA was loaded into an 1% agarose gel for verification.

2.3. Ligation and digestion for verification

Restriction enzyme treated DNA fragments encoding Granzyme H were ligated with the plasmid vectors digested by the same enzymes resulting complementary ends by adding 14 μl of small fragment and 2 μl of digested

vectors to a total volume of 20 μL. A control reaction was also prepared, containing only the large fragment, each one of the digested vectors: pET20b, pETM50, pETM52. Ligation was performed using ATP-dependent T4 Ligase (Fermentas) and the provided buffer at 4°C overnight.

10 μL of each ligation mixture (including the control) was then transformed by heat shock into an appropriate host strain, competent *E. coli* DH5α cells and incubated 16 hours at 37°C on plates containing the proper antibiotics: 100 μg/mL LB Ampicillin plates for pET20b vector, and 100 μg/mL Kanamycin containing LB agar plates for pETM50 and pETM52 vectors. To analyze the presence of the insert and the correct assembly of DNA, four colonies from each plate were grown overnight at 37°C, 200 rpm for plasmid isolation with the appropriate antibiotics. High-speed Plasmid Mini Kit was used for plasmid preparation (Geneaid) according to the protocol described by the manufacturer. Plasmid DNA was eluted in 50 μL TE and verified by 1% agarose gel.

Two restriction endonucleases were used to detect the presence of the Granzyme H gene: 5 μl of plasmid DNA was digested with 10 U of *Xho I* and 10 U of *Nco I* in a total volume of 10 μL restriction reaction at 37°C, overnight in 2X Tango buffer, according to the double digest tool of Fermentas. The reaction was then verified by agarose gel electrophoresis.

3. Results and discussion

3.1. TA cloning in pBluescriptKS-T vector

The amplified PCR product with an A overhang was ligated with a pKS- T vector. DNA was isolated from the positive clones of this TA-cloning. As a result of the TA cloning, plasmid DNA from positive clones was digested with *Xho I* to verify the insert direction and size. The correct vector can be seen in lane 4. from Fig. 1.

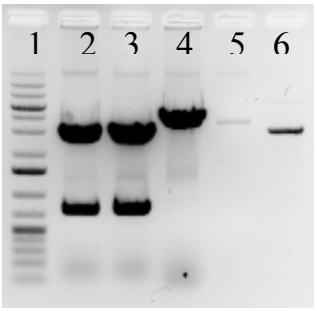

Fig. 1. Verifying the TA-ligation results. Lane 1: 1 kb molecular weight marker (Fermentas), lane 2: digestion of plasmid DNA from colony 1. with *Xho I*, lane 3: digestion of plasmid DNA from colony 5. with *Xho I*, lane 4: digestion of plasmid DNA from colony 7., lane 5: digestion of plasmid DNA from colony 11, lane 6: *Xho I* digested pKS vector

Results of the partial digestion of the correct vector can be observed in Fig. 2. Insert from plasmid DNA partially digested with *Nco I* from lane 2. and 3. was isolated from the gel.

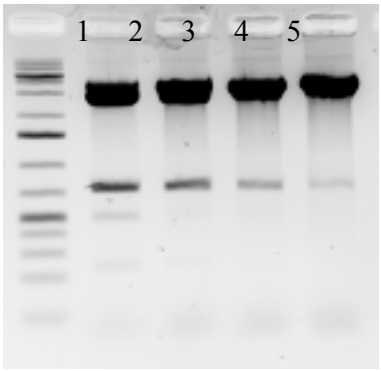

Fig. 2. Partial digestion of the correct pKS-GraH vector. Lane 1: 1 kb molecular weight marker, lane 2,3,4,5: digestion dilution at 1, 1/3, 1/9, 1/27 of the *Nco I* enzyme concentration

After ligating the correct insert with the digested vectors, plasmid DNA was isolated and verified. Fig. 3. illustrates the restriction digestion of plasmid DNA in order to verify the presence of the GranzymeH coding sequence.

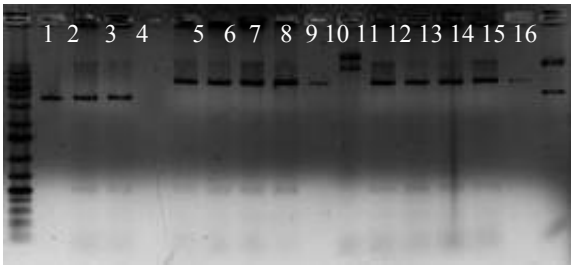

Fig.. 3. Verification of the correct DNA assembly. Lane 1: 1 kb DNA ladder, Fermentas, lane 2,3,4: digested pET20b-GraH Minipreps, lane 5-6-7-8: digested pETM50-GraH Minipreps, lane 9: digested pETM50 vector, lane 10: pETM50, lane 11-12-13-14: digested pETM52-GraH Minipreps, lane 15: digested pETM52, lane 16: pETM52 vector

Maps of the three different vectors containing Granzyme H can be seen in Fig. 4.

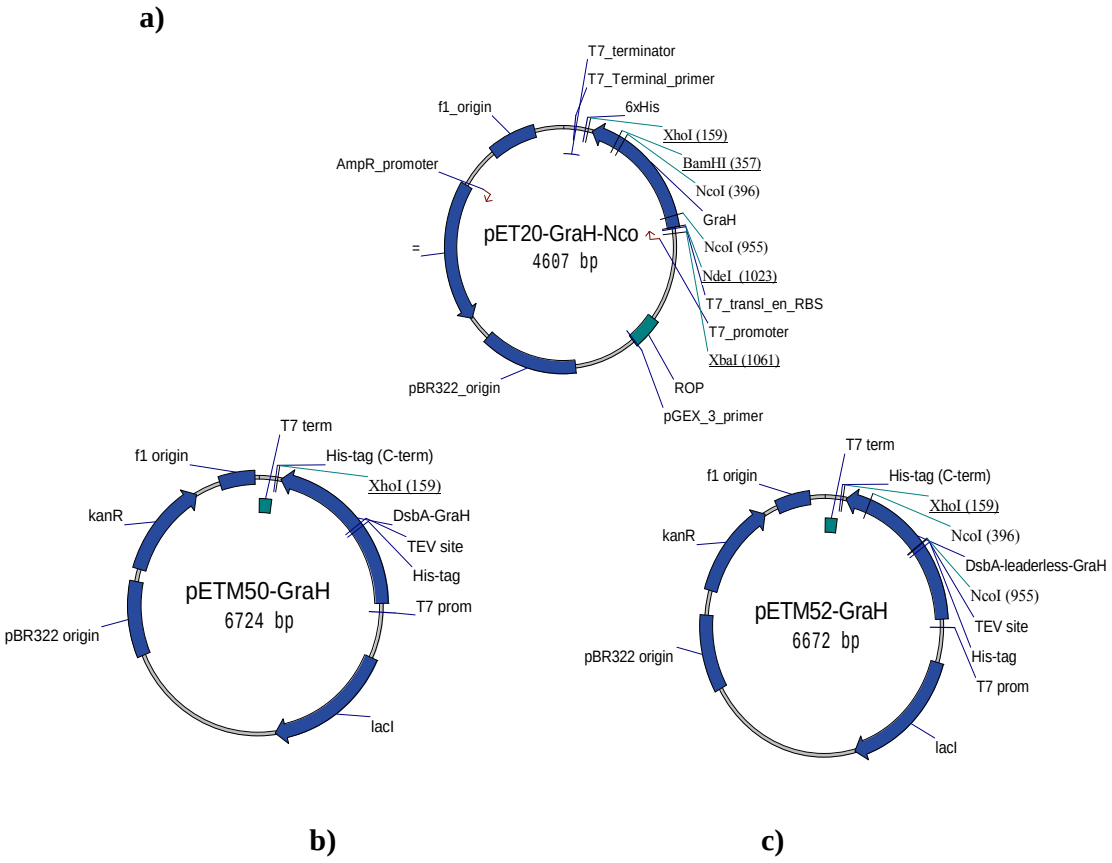

Fig. 4. Vector maps. a) vector map of the pET20bGraH vector, b) vector map of the pETM50GraH vector, c) vector map of the pETM52GraH vector

4. Conclusions

We designed three expression vectors: with the help of the pET20bGraH vector, we will be able to direct the protein production into the periplasmic space with the *pelB* signal sequence of the vector. Also, the vector contains a 6XHis affinity tag, which will reduce the time of purification. A pETM50GraH and pETM52GraH construct was also obtained, each of them containing a fusion chaperon protein (DsbA), which will help the GranzymeH in correct folding

In the future we will optimize the expression of Granzyme H with the help of these secretion vectors: pET20bGraH, pETM50GraH, pETM52GraH. Efficient

expression will allow activity measurements and crystallization to determine the 3D structure of the enzyme.

Acknowledgments

The work has been funded by the Sectorial Operational Programme Human Resources Development 2007-2013 of the Romanian Ministry of Labour, Family and Social Protection through the Financial Agreement POSDRU/88/1.5/S/60203, by the University of Sapientia, Department of Technical Sciences, and the Hungarian Academy of Sciences, Domus Programme.

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