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Expression and purification of 15 kDa granulysin utilizing an insect cell secretion system

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Abstract

Granulysin is an antimicrobial and proinflammatory protein expressed in activated human T cells and natural killer cells. A single mRNA produces the 15 kDa isoform which is then cleaved at the amino and carboxy termini to produce the 9 kDa isoform. Recombinant 9 kDa granulysin has been studied in detail but little is known about the function of the 15 kDa isoform, and no protocol has been published describing expression and purification of this form. Two commercially available preparations of the recombinant 15 kDa granulysin contain tags that may affect function. Here we describe for the first time a method to produce 15 kDa granulysin as a secreted protein from insect cells. The 15 kDa granulysin is purified using a HiTrap Heparin column and a Resource S column. A typical yield of purified 15 kDa granulysin is 0.6 mg per liter of insect cell supernatant.

Keywords

Granulysin; Recombinant protein; Monocytes; Insect cell expression; T-cells; NK-cells

Introduction

Granulysin is an antimicrobial and proinflammatory protein expressed in human natural killer and activated T cells [1-3]. The 15 kDa protein is cleaved at the amino and carboxy termini to produce a 9 kDa isoform [3-5]. 9 kDa granulysin lyses gram positive and gram negative bacteria, parasites, fungi, yeast and a variety of tumor cell lines [6-8]. 9 kDa granulysin is sequestered in cytolytic granules while the 15kDa isoform is constitutively secreted from cells [9,10].

Until recently the study of 15 kDa granulysin has been hampered by the inability to express and purify it. Commercially available recombinant 15 kDa granulysin from R&D Systems and Novus Biologicals both contain tags that may affect function. The 15 kDa granulysin from R&D Systems contains a 10-Histidine tag at the C-terminus while the recombinant molecule from Novus Biologicals includes an intact GST tag at the N-terminus. The 15 kDa granulysin made by our protocol has no tag and is purified from insect cell supernatants. We show here that the intact untagged molecule behaves differently from the commercially available tagged forms in a monocyte activation bioassay, suggesting that further understanding the physiologic role of 15 kDa granulysin will be advanced by using the intact, untagged form. This work

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represents the starting point for structural studies as well as further functional characterization of this isoform of granulysin.

Materials and methods

Commercially available 15 kDa granulysin

15 kDa granulysin was purchased from R&D Systems (Cat # 3138-GN/CF) and Novus Biologicals (Cat # H00010578-P01).

Construction of 15 kDa granulysin baculovirus expression vector

A cDNA clone of 15 kDa granulysin was generated from human peripheral blood lymphocytes and cloned into pet28A *Escherichia coli* expression vector. This previously unpublished pet28A construct containing the 15 kDa granulysin was used as the template to generate an insect cell secretion expression system. The first subcloning from pet28A was to Gateway Donor vector pDonr253 which was modified from pDonr201 (Invitrogen). pDonr253 replaces the kanamycin resistance cassette with a gene encoding spectinomycin resistance and contains several sequencing primer sites to help in verification of entry clones. The 15 kDa granulysin gene was cloned by PCR from the pet28A-15 kDa construct using the primers 5'-GCTTCTGGCCGCTGCAGCCCATCTGTCAT TTGCGCGTCTGAGCCCTGAGTACTACGAC'3 and 5'GGGGACAACCTT TGTACAA GAAAGTTGATTAGAGGGGACCTGTAGAAGGTATAC'3. A baculovirus GP67 secretion leader was engineered at the 5' end of the granulysin gene by adapter PCR. Initial PCR was done using Phusion DNA polymerase (New England Biolabs) under standard conditions using a 30 second extension time. After 5 cycles the adapter primer 5' GGGGACAACCTTTGTACAAAAAAGTTGGCACCATGGTAAGCGCTATTGTTCT GTACGTGCTTCTGGCCGCTGCAGCCC3' was added, and the amplification reaction continued for 15 additional cycles. The PCR product contained the complete 15 kDa granulysin gene with a 5' GP67 leader sequence. This construct is flanked by Gateway recombination signal sequences attB1 at the 5' end and attB2 at the 3' end. The PCR product was purified using the QiaQuick PCR purification kit (Qiagen), and recombined into the pDonr253 vector using the manufacturer protocol for the Gateway BP recombination reaction (Invitrogen). This reaction mixture was transformed into *E. coli* DH5 α cells (Invitrogen) and plated on LB plates containing 50 μ g/ml spectinomycin. plasmid DNA was sequenced and verified.

The verified clone was subcloned by Gateway LR recombination (Invitrogen) into pDest-670 for insect cell expression. The final expression clone was verified by size and restriction digest pattern. The expression clone was then transformed into *E. coli* DH10Bac (Invitrogen), and plated on LB agar plates containing kanamycin, gentamycin, tetracycline, X-gal, and IPTG as per manufacturer's protocols. White colonies were selected and bacmid DNA was generated by alkaline lysis plasmid preparation. The bacmid DNA was verified by PCR amplification across the bacmid junctions.

Expression and purification of 15 kDa granulysin

All insect cells were grown in Hyclone SFX-Insect medium (Thermo Scientific). The bacmid DNA described above was transfected into Sf9 insect cells to create the recombinant baculovirus. Large-scale direct transfections were done using Insect Gene Juice Reagent (Novagen) with Sf9 insect cells at 1.5×10^6 /100 ml SFX medium. The ratio of Gene Juice to bacmid was 3:1. The rest of the protocol was carried out according to the Insect Gene Juice protocol and virus was harvested 4 days after transfection. The resulting baculovirus was then titrated in Sf9 Easy Titer cells [11]. Large scale expression was done using Hi5 insect cells grown in 3L Erlenmeyer flasks (Corning). A one liter cell culture was set at 8.5×10^5 /ml. The cells were grown overnight at 27 °C. The next day the cells were counted and infected at a

Multiplicity of Infection (MOI) of 3. The cells were kept at 27 °C for 4 hours then shifted to 21 °C and grown for 48 hours. The cells were spun at 1500 × g for 10 min at 4 °C. The insect cells were discarded and the supernatant containing the secreted 15 kDa granulysin are filtered using a 0.45 μ M filter and stored at -20 °C.

The purification protocol is optimized for 500 ml of insect cell supernatant and all steps were carried out at 4 °C. The purification of the 15 kDa protein requires two columns and both columns use the same purification buffers. Purification Buffer A is 20 mM Hepes, pH 7.3 and Buffer B is 20 mM Hepes, 2M NaCl, pH 7.3. The first column is a 5 ml HiTrap Heparin HP (GE Health Care Cat # 17-0406-01). The heparin column is equilibrated with 25 ml of Buffer A. 500 ml of insect cell supernatant is then loaded onto the column at a flow rate of 5ml/min and the column is washed with 25 ml of Buffer A. The elution is a linear gradient of 0-100% Buffer B over 20 column volumes and fractions are collected at 2 ml intervals. The 15 kDa granulysin elutes between 35 and 50% Buffer B which corresponds to a conductivity range of 55-68 mS/cm.

The fractions are pooled and then buffer exchanged with 100% Buffer A using an Ultracel -10K Amicon Ultra Centrifugal filter according to the manufacturer's protocol. The buffer exchanged material is then loaded onto a 1 ml Resource S column (GE Health Care Cat # 17-1178-01) previously equilibrated with 5 ml of Buffer A. After the material is loaded, the column is washed with 2 ml of Buffer A, and a 20 ml linear gradient from 0-100% Buffer B applied to the column. 1 ml fractions are collected and the protein elutes between 40-50% Buffer B with a conductivity range of 48-53 mS/cm. The purified protein is then concentrated using a concentrator similar to the Ultracel -10K Amicon Ultra Centrifugal filter. The protein was frozen in liquid nitrogen and stored at -80 °C.

SDS-PAGE gels

The protein purity was evaluated by SDS-PAGE using Biorad 15% Tris-HCl gels in a BioRad miniprotein Tetra Cell. The protein was stained using SimplyBlue Safe Stain (Invitrogen). Precision Plus Protein Standards (BioRad) were used for SDS-PAGE. Protein concentration was determined by Pierce Bicinchoninic Acid method kit (Thermoscientific 23225).

LPS- Detection

After completion of protein purification all samples were tested for LPS using the Limulus Amebocyte Lysate Kinetic-QCL kit (Lonza 50-650U).

Western Blot

15% SDS-PAGE gels were used for all Western blots. Each lane was loaded with 88 μ g of protein. Protein was transferred to Immobilon-P PVDF membrane (Millipore) for 1 hour at 10 volts using a BioRad Trans-Blot SD Semi Dry Transfer Cell. The membrane was washed in PBST (phosphate buffered saline, pH 7.4 + 1 % Tween 20; GIBCO and Sigma Aldrich, respectively) and blocked for 1 hour in PBST + 5 % milk (BioRad Blotting grade blocker non-fat dry milk). Polyclonal anti-granulysin rabbit antiserum [3,5] was added at a 1/10,000 diluted in PBST+ 5% milk and incubated for 1 hour. The membrane was washed 3 times with PBST and the ECL-Anti-Rabbit IgG horseradish peroxidase-linked whole antibody from donkey (GE healthcare) was added at 1/5000 dilution for 1 hour. Proteins were detected with ECL-western blotting detection reagents (GE healthcare) and Amersham Hyperfilm ECL.

Monocyte Activation with 15 kDa granulysin

Elutriated monocytes were obtained from the Cell Processing Section of the Clinical Center of the NIH. Monocytes were cultured at 2×10^6 cells/ml in 24 well plates in RPMI-1640

supplemented with 10% heat-inactivated FBS (Hyclone), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin (complete medium). Cells were incubated with medium or with the indicated concentration of 15 kDa granulysin at 37 °C for 4 hours. Cells were then washed one time with PBS and the cell pellet frozen at -80 °C until use.

rtPCR

RNA was purified from monocytes using the RNeasy MiniKit (Qiagen). Qiashredder columns (Qiagen) were used to lyse cells and the on column DNase I digest was always used to purify the RNA. cDNA was generated using the iScript cDNA Synthesis kit (BioRaD) using 1 µg of RNA in a reaction volume of 20 µl. The rest of the steps followed the manufacturer's suggestions.

After cDNA was generated, rtPCR reactions were set up in 384 well plates (Applied Biosystems) in a final reaction volume of 10 µl. Each gene was tested in triplicate and GUS was the gene chosen as the house keeping gene. The reaction contained the Power SyBR Green PCR Master Mix (Applied Biosystems) with either set of these primers. Gus1 5' CCGAGTGAAGATCCCCTTTTTA'3 Gus2 5'CTCATTTGGAATTTGCCGATT'3 or IL61 5'AACCTGAACCTTCCAAAGATGG'3 IL62 5'TCTGGCTTGTTCTCACTACT'3. CCL201 5'TCCTGGCTGCTTTGATGTCA3' CCL202 5'CAAAGTTGCTTGCTTGCTTCTGA3'. rtPCR reactions were done in a 7900 HT Fast Real-Time PCR System (Applied Biosystems). The cycle method used was 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 sec, 60 °C for 1 min, a dissociation curve was added (to insure specificity of primers used for each selected gene), 95 °C for 15 sec, 60 °C for 15 sec, 95 °C for 15 sec. This was repeated 40 times. Data were analyzed using SDS 2.3 software package (Applied Biosystems).

Results

A cDNA clone of the 15 kDa granulysin was isolated from human peripheral blood lymphocytes and cloned into the pet28A *E. coli* expression vector. Several expression studies were carried out using *E. coli* but poor yields due to protein degradation indicated the pet28A expression system was not feasible (data not shown). The 15 kDa gene was subcloned into an *E. coli* expression vector containing a GST- tag but instability of the protein and degradation of the GST tag resulted in poor yields of protein (data not shown). Attempts to express 15 kDa granulysin using the yeast *Kluyveromyces lactis* also were unsuccessful.

The failure of *E. coli* and *K. lactis* as viable systems to express 15 kDa granulysin prompted us to explore an insect cell secretion system. The original pet28 clone was used as a template to amplify the 15 kDa granulysin into a modified pDonr201 vector called pDonr253 (see Materials and Methods). The 15 kDa gene was amplified by PCR and a baculovirus GP67 secretion leader sequence was engineered at the 5' end of granulysin by adapter PCR (see Materials and Methods). The PCR product was purified and recombined into pDonr253 vector according to the manufacturer's protocol. The vector was transformed into *E. coli* DH5α and plated on LB plates that contained spectinomycin. The plasmid was purified from *E. coli* and the 15 kDa granulysin sequence was verified.

The verified clone was then subcloned into pDest-670 insect cell expression vector. This expression clone was verified by size and restriction digest pattern. The new expression construct was transformed into *E. coli* DH10Bac cells and plated on LB plates containing kanamycin, gentamycin, tetracycline, X-gal, and IPTG as per manufacturer's protocols. Only white colonies were selected from the plates and bacmid DNA sequences across the bacmid junctions.

Generation of baculovirus containing 15 kDa granulysin

The purified bacmid DNA described above was transfected into Sf9 insect cells to generate the recombinant baculovirus. Sf9 insect cells were grown to 1.5×10^6 cells/100 ml in SFX medium. Insect Gene Juice was mixed in a ratio of 3:1 to bacmid DNA for the transfection into the Sf9 cells. After 4 days the virus was harvested and the baculovirus was then titrated in Sf9 easy titer cells.

Expression of 15 kDa granulysin in Hi5 insect cells

Both Sf9 and Hi5 insect cells were tested for expression of 15 kDa granulysin. In addition to cell type, the effects of time of infection and temperature were also tested (Fig. 1). Based on these studies, we selected Hi5 cells and, after infection, shifted the temperature from 27 °C to 21 °C and allowed the cells to grow for 48 hours.

Hi5 insect cells were grown in a disposable 3 liter flask containing 1 liter SFX medium. The cell culture was set to 8.5×10^5 /ml and allowed to grow overnight at 27 °C. The next day the cells were counted and then a MOI of 3 was used to infect the cells. The cells were kept at 27 °C for 4 hours to allow the maximum infection, and then the culture was shifted to 21 °C and grown for 48 hours. The cells were then spun down and only the cell supernatant was used for purification.

Purification of 15 kDa granulysin

A purification protocol was optimized using 500 ml of insect cell supernatant (Table 1). The material was thawed at 4 °C and filtered through a 0.45 μ m filter. To initially concentrate the 15 kDa granulysin, we elected to take advantage of the high isoelectric point of 15 kDa granulysin (pI = 9.39). The filtered insect supernatant was loaded onto a 5 ml HiTrap Heparin HP column at a loading speed of 5 ml per minute. The protein was eluted using NaCl and 15 kDa granulysin eluted at a conductivity range between 55-68 mS/cm.

This material was then pooled and buffer exchanged back into Hepes buffered solution containing less than 50 mM NaCl. Again taking advantage of the positive charge of 15 kDa granulysin, the cation exchange column Resource S was used as the final step in the purification of 15 kDa granulysin. The material was then injected onto an equilibrated 1 ml Resource S column. Protein was eluted in the same buffer used in the heparin purification and 15 kDa granulysin eluted over a conductivity range of 48-53mS/cm. At this stage the protein was concentrated and a BCA assay was used to determine the concentration of the protein. The protein was then run on a 15% SDS-PAGE gel to verify purity (figure 2). A Western blot and mass spectrometry were also performed on the purified protein to verify that it as the 15 kDa form of granulysin (data not shown).

Table 1 shows a typical yield from 500 ml of insect cell supernatant. Normally two 500 ml purifications are combined, resulting in 0.6 mg of purified 15 kDa granulysin from 1 liter of material. Aliquots of the purified protein were prepared, snap frozen in liquid nitrogen and stored at -80 °C. The purified protein was tested for LPS using the Limulus Amebocyte Lysate Kinetic -QCL kit by Lonza(see Materials and Methods); all preparations contained <1 EU LPS/ml.

We attempted to scale up the purification protocol by either concentrating the insect supernatant prior to loading onto a column or using larger columns for the purification. A variety of concentration methods failed because the protein either precipitated out of solution or stuck to membranes used to concentrate the protein. In other cases it was quicker to directly load the material onto a column instead of trying to concentrate it under a stream of nitrogen.

The use of larger columns also proved to be problematic. The best yield was obtained using a 5 ml HiTrap Heparin HP and loading 500 ml of insect supernatant at a time. A larger 20 ml heparin column is available but it only comes in a fast flow bead form instead of the high performance type. Simply connecting two or three 5 ml HiTrap columns also resulted in decreased purity of 15 kDa granulysin for reasons we do not understand.

Comparison of 15 kDa granulysin with commercial available forms of the protein

The purified 15 kDa granulysin was then compared to the two commercially available forms of 15 kDa granulysin. The Novus Biologicals form contains an intact GST-Tag while the R&D Systems product has a C-terminal 10-histidine tag. We have previously shown that 15 kDa granulysin activates monocytes, causing changes in gene expression (Clayberger *et. al.* submitted). We compared the ability of the three forms of granulysin to promote expression of IL-6 and CCL20, as measured by real time PCR. Over a wide range of protein (1-200 nM) our untagged 15 kDa granulysin was superior to either of the commercial preparations (Figure 3).

Discussion

Our group first identified granulysin as a gene expressed by human T lymphocytes “late” (3-5 days) after activation [12-14]. We expressed the 9 kDa isoform in *E. coli* and have published more than 45 papers describing its function in cytotoxicity and inflammation [4,5]. However, we were unable to express the 15 kDa isoform to study its function.

Recently, publications have suggested function(s) for 15 kDa granulysin, using commercially available forms of the protein which retain their affinity tags [15,16]. Our experience was that tags can impact *in vitro* effects of such proteins. We, therefore, set out to express and purify untagged, intact 15 kDa granulysin. As described elsewhere, this form of granulysin does not kill; rather it is highly proinflammatory, inducing monocytes to differentiate to dendritic cells (Clayberger *et. al.* submitted).

Dendritic cells are the “professional” antigen presenting cells of the immune system, and, as such, have tremendous potential for use in induction of active or tolerizing immune responses via vaccination. More than 100 Investigational New Drug applications currently exist for use of *in vitro* differentiated dendritic cells in vaccination, largely for tumors or HIV. In the great majority of these protocols, GM-CSF is used to differentiate dendritic cells in combination with various cocktails containing IL-1, gamma interferon, LPS, or other molecules [17-19]. Despite the large number of such protocols, results to date have been suboptimal, with less than a 10% overall clinical response rate [17].

We show elsewhere that 15 kDa granulysin induces dendritic cells that differ in a number of ways from those differentiated with GM-CSF. Preliminary studies indicate that chemokines, cytokines, adhesion and costimulatory molecules induced by these two treatments give rise to differences in antigen presentation that may result in different clinical outcomes (Clayberger *et. al.* submitted). Expression and purification of intact, untagged 15 kDa granulysin is a first step towards development of this protein as a potential immunotherapeutic and makes possible further functional and structural studies.

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Figure 1.

Western blot of 15 kDa granulysin secreted from insect cells. Different cell types, expression times, and growth temperatures were tested. Each lane contains 88 μ g of protein. Lanes 1: Sf9 cells 27 °C for 48 hours, Lane 2: Hi5 cells 21 °C 48 hours, Lane 3: Hi5 cells grown 27 °C 48 hours, Lane 4: Sf9 cells grown 27 °C for 72 hours, Lane 5: Hi5 cells 21 °C for 72 hours, Lane 6: Hi5 grown at 27 °C for 72 hours, Lane 7: Natural killer cell line YT (positive control). Lane 2 was the condition chosen for expression.

Figure 2.

15% SDS-PAGE stained with coomassie of purification of 15 kDa granulysin secreted from Hi5 insect cells. Lane 1: Standard (250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, 15 kDa, 10 kDa), Lane 2: 7 µg insect supernatant, Lane 3: 3.5 µg insect cell supernatant, Lane 4: Standard, Lane 5: 3 µg protein eluted from heparin column, Lane 6: Standard, Lane 7: 3 µg purified 15 kDa granulysin.

Figure 3.

Comparison of three different sources of 15 kDa granulysin. Monocytes were cultured for 4 hours with the indicated concentrations of granulysin. Expression of IL-6 and CCL20 was measured by rtPCR and is depicted as fold increase over expression in monocytes cultured with medium alone. Source of 15 kDa granulysin: this paper (▲), Novus Biologicals (○), or R&D Systems (■)

Table 1

Sample	mg/ml	Volume (ml)	Total protein (mg)
Insect supernatant	0.55	500	275
Combined heparin fractions	0.24	16	3.84
Resource S column fraction 11	0.15	1	0.15
Resource S column fraction 12	0.15	1	0.15