Optimized expression of the antimicrobial protein Gloverin from *Galleria mellonella* using stably transformed *Drosophila melanogaster* S2 cells

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Abstract Antimicrobial proteins and peptides (AMPs) are valuable as leads in the pharmaceutical industry for the development of novel anti-infective drugs. Here we describe the efficient heterologous expression and basic characterization of a Gloverin-family AMP derived from the greater wax moth *Galleria mellonella*. Highly productive single-cell clones prepared by limiting dilution achieved a 100% increase in productivity compared to the original polyclonal *Drosophila melanogaster* S2 cell line. Comprehensive screening for suitable expression conditions using statistical experimental designs revealed that optimal induction was achieved using 600 μM CuSO_4 at the mid-exponential growth phase. Under these conditions, 25 mg/L of the AMP was expressed

at the 1-L bioreactor scale, with optimal induction and harvest times ensured by dielectric spectroscopy and the online measurement of optical density. Gloverin was purified from the supernatant by immobilized metal ion affinity chromatography followed by dialysis. In growth assays, the purified protein showed specific antimicrobial activity against two different strains of *Escherichia coli*.

Keywords Antimicrobial protein · *Galleria mellonella* Gloverin · Stably transformed *D. melanogaster* S2 cells · Recombinant protein expression · Online process monitoring · Process optimization

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Introduction

Antimicrobial proteins and peptides (AMPs) are a diverse group of polypeptide molecules with highly conserved roles in the innate immune systems of bacteria, fungi, plants, insects and vertebrates (Lehrer and Ganz 1999). AMPs often show broad activity against microbes but their mechanisms of action differ from those of conventional antibiotics (Li et al. 2012a). Due to the spread of antibiotic resistance among common pathogens, AMPs therefore offer an alternative route for the development of novel anti-infective drugs (Aoki et al. 2012; Li et al. 2012a). Current research focuses on screening for AMPs and the development of suitable production processes

(Müller et al. 2015). The greater wax moth *Galleria mellonella* is a useful model for the identification and testing of AMPs, and here we investigated the properties of *G. mellonella* Gloverin (*GmGlv*), a member of the lepidopteran-specific family of Gloverin-like proteins (Seitz et al. 2003; Brown et al. 2009; Vogel et al. 2011). In order to make such AMPs accessible for further research and commercial development, a satisfactory expression level must be achieved in one of the several host species available for recombinant protein expression, which include bacteria (*Escherichia coli*), yeasts (such as *Pichia pastoris*) and insect cells (Müller et al. 2015). These expression systems differ in terms of complexity, space–time–yield and the ability to support protein folding and posttranslational modification (Schmidt 2004). Bacterial and yeast systems are considered the simplest, but because the host cells are microbes they are often susceptible to the effects of AMPs. This is the case for the Gloverin family because the antimicrobial activity of these proteins is based on interference with bacterial lipopolysaccharides (Axén et al. 1997) and fungal membrane components (Xu et al. 2012). Although *Bombyx mori* Gloverins have been expressed in microbes (Yi et al. 2013), the antimicrobial properties of the product hamper production. As an alternative, Gloverins have been expressed in insect cells using baculovirus vectors (Lundström et al. 2002; Kawaoka et al. 2008). However, this is not suitable for large scale production either because Gloverins can also inactivate baculoviruses (Moreno-Habel et al. 2012).

Insect cells should be ideal for the expression of Gloverins because they closely resemble the natural AMP-producing tissue in terms of protein folding and pro-peptide cleavage, and are unlikely to be susceptible to AMP toxicity. In this context, stable recombinant *Drosophila melanogaster* S2 cells (rS2 cells) provide an effective system for protein expression (Moraes et al. 2012; de Jongh et al. 2013) and these cells are particularly suitable for the production of biologically active small peptides, such as spider toxins (Escoubas et al. 2003) and insect AMPs. Accordingly *Manduca sexta* and *Plutella xylostella* Gloverins have already been expressed successfully in rS2 cells (Xu et al. 2012, 2015). We therefore evaluated rS2 cells for the functional expression of *GmGlv* and also addressed bottlenecks such as the polyclonal nature of the transformed cell line, which

results in an inconsistent expression profile during long-term cultivation (Cherbas and Cherbas 2007). Following the selection of a monoclonal production cell line, we used a statistical experimental design approach to screen for optimal induction conditions. The resulting data were then used to scale the process for the production of *GmGlv* in a 1-L bioreactor, supported by additional online monitoring techniques. The secreted and functional AMP was recovered using a simple three-step procedure comprising cell removal, chromatography and dialysis, allowing the purified protein to be tested for biological activity against *E. coli*.

Materials and methods

Maintenance of *D. melanogaster* S2 cells

Unless otherwise stated, *D. melanogaster* S2 cells (Schneider 1972) were grown at 27 °C in ExCell 420 serum-free medium (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 8 mM L-glutamine (Biochrom, Berlin, Germany). The medium for rS2 cells also contained 10 µg/mL blasticidin S (Invivo-gen, Toulouse, Germany) to maintain selection pressure. All cell lines were adapted to grow in suspension and were split every 3–4 days to obtain cultures with a density of 1.5×10^6 cells/mL. For long-term preservation, 1.5×10^7 cells were suspended in 1 mL freezing medium (1:1 mixture of spent and fresh ExCell 420 medium plus 7.5% DMSO) for storage in liquid nitrogen.

Construction of the polyclonal expression cell line

The *D. melanogaster* expression system (DES[®], Thermo Fisher Scientific, Darmstadt, Germany) was set up as previously described (Kollewe 2013). Briefly, wild-type S2 cells in Schneider S2 medium plus 5% FBS (Thermo Fisher Scientific) were co-transfected with the selection plasmid pCoBlast (Thermo Fisher Scientific) and an expression plasmid containing the *GmGlv* gene (pMT-flGmGlv-V5/His). This was derived from pMT/BiP/V5-His B (Thermo Fisher Scientific) and contained the full-length *GmGlv* sequence under the control of the copper-inducible *D. melanogaster* metallothionein promoter. The original BiP signal sequence was removed before inserting the

GmGlv sequence because the insert contained its own signal sequence (Fig. 1). To generate a stable polyclonal rS2 cell line, the transfected cells were cultured under selection pressure for 30 days before adapting them to grow in ExCell 420 serum-free medium.

Single-cell cloning and production screening

Monoclonal cell lines for efficient protein production were prepared by limiting dilution as previously described (Uribe et al. 2013; Wang et al. 2012; Scotter et al. 2006). The method is based on the co-cultivation of single transformants with untransfected feeder cells, followed by antibiotic selection of the clones. Ten 96-well plates (Eppendorf, Hamburg, Germany) were prepared by transferring 100 μ L antibiotic-free medium to each well and seeding with 5×10^4 untransfected feeder cells and approximately one transformed cell. The cells were allowed to proliferate for 3 days to ensure proper conditioning of the medium before 15 μ g/mL blasticidin S was added for selection. The feeder cells decayed over the next 14 days and the resistant colonies became visible. After validation by microscopy, monoclonal colonies were picked and scaled up in 48-, 24-, 12- and 6-well plates. The cells were then induced using 900 μ M CuSO_4 . After 24 h, the supernatant was collected from each well and dot blot analysis was used to identify the most productive cell lines. Cell growth during cultivation was determined by measuring the absorption at OD_{600} (Bédard et al. 1994). Production clones were tested against the parental polyclonal cell line to

confirm their enhanced productivity. The relative amount of His-tagged protein in the supernatants of six independent cultures was determined by quantification of the dot blot luminescence intensity. Western blot analysis was used to resolve the *GmGlv* distribution between the culture supernatant and cell interior after lysing the cell pellets (1 mL cell suspension) using 1 mL lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P40).

Optimization of induction conditions

The induction conditions for the production line were optimized by testing the concentration of inducer (CuSO_4) and the time of induction (cell concentration). The experiments were carried out in 24-well plates (Infors Celltron) with a 500- μ L working volume, shaking at 85 rpm. Based on preliminary experiments, the harvest time was set to 4 days after induction. The response surface method (RSM) was used based on a face-centered, central composite design. The design space covered ranges of 400–1000 μ M CuSO_4 for induction and $5\text{--}10 \times 10^6$ cells/mL. The experimental design and statistical evaluation is described in more detail in the “Appendix 2”. In addition to the experimental design, several additives that may enhance protein production in rS2 cells were also evaluated, namely DMSO (1 or 5%), glycerol (1 or 5%), sodium butyrate (5 or 10 mM), and the presence or absence of 2 μ L/mL Carl Roth His-tag inhibitor cocktail (Jeon et al. 2012; Lemos et al. 2009; Swiech et al. 2008; Chang et al. 2002; Park et al. 2002).

Fig. 1 **a** Maps of the vectors pCoBlast and pMT-flGmGlv-V5/His. **b** Full-length amino acid sequence (AA sequence) of *Galleria mellonella* Gloverin with its own signal peptide

(underlined), putative propeptide convertase recognition sequence (dark gray background) and cleavage site (gray triangle), modified from (Kollewe 2013)

Protein expression at the 1-L bioreactor scale

The laboratory-scale expression of recombinant *GmGlv* was achieved in a 2-L Labfors bioreactor (1-L working volume, Infors HT, Einsbach, Germany) equipped with one pitched-blade impeller ($3 \times 45^\circ$, $d = 65$ mm) and standard sensors for temperature, pH and oxygen saturation. A bubble-free aeration system was used, comprising a gassed silicon tube (4 m, inner diameter = 0.76 mm, outer diameter = 1.65 mm) and additional head space aeration. The aeration system was provided with a controlled gas mix of compressed air, nitrogen and pure oxygen. The process was kept at 27 °C, pH 6.4 and 40% air saturation. The stirrer speed was increased from 100 to 150 rpm during the process. The cultivation was designed as a two-step procedure: the cells were inoculated at 1.5×10^6 cells/mL and grown to the previously determined optimal density before induction with 600 μ M CuSO_4 using a bolus feed of 150 mL fresh medium. During cultivation, the offline viable cell count was determined using a Guava flow cytometer (Merck Millipore, Darmstadt, Germany). Living and dead cells were distinguished by propidium iodide staining (5 mg/L, Carl Roth, Karlsruhe, Germany). Cell growth was also monitored online by dielectric spectroscopy (Incyte system, Hamilton, Bonaduz, Switzerland) and optical density probing (ExCell 230, Exner Process Equipment, Ettlingen, Germany). The permittivity of the medium was measured at 1 and 10 MHz, and the difference between the signals was the output of the dielectric spectrometer. The increase in optical density was measured at 880 nm by light transmission through a 5-mm slit. As an additional offline parameter, the glucose concentration was determined using an enzymatic-amperometric analyzer (BiosenC, EKF Diagnostics, Barleben, Germany).

Protein purification from the cell culture supernatant

The harvested cell suspension was centrifuged and the supernatant was passed through a sterilizing filter before storing at 4 °C. The protein carried a His₆ tag and was therefore purified by immobilized metal ion affinity chromatography (IMAC) using an FPLC system (NGC Discover, Biorad, Munich, Germany) equipped with an affinity column suitable for insect

cell culture medium (1-mL HisTrap excel column, flow velocity 1 mL/min, GE Healthcare Life Science, Freiburg, Germany). The column was equilibrated with 5 column volumes (CV) of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) before the clarified supernatant was applied to the column at a flow rate of 0.3 mL/min. Contaminating proteins were removed by flushing with 50 CV of binding buffer supplemented with 15 mM imidazole, and pure *GmGlv* was recovered by eluting with 8 CV of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4, 500 mM imidazole). Fractions containing *GmGlv* were identified by SDS PAGE and dialyzed against PBS to remove the imidazole (Slide-a-Lyzer, Thermo Fisher Scientific). This three-step procedure comprised sequential dialysis steps of 2, 2 and 12 h. The concentrations of pure *GmGlv* were determined by spectrophotometry at 280 nm (Synergy HTX Multi-Mode Reader, Biotek, Bad Friedrichshall, Germany) using the predicted extinction coefficient for Gloverin (<http://web.expasy.org/protparam/>) and by using a standard Bradford assay with BSA as the reference (Applichem, Darmstadt, Germany). High-purity fractions were used as standards to enable direct protein quantification by SDS-PAGE in further experiments.

SDS-PAGE, dot blot and western blot analysis

Proteins from the cell culture supernatants and cell lysates were resolved by reducing SDS-PAGE on 4–20% polyacrylamide gradient gels (Criterion XTTM, Biorad) and detected using Biorad stain-free technology (Kazmin et al. 2002; Ladner et al. 2004). After electrophoresis for 25 min at 250 V, the gels were transferred to the imaging system (ChemiDocTM, Biorad) and activated for 2.5 min with UV light for documentation. For western blotting, proteins were transferred to polyvinylidene difluoride membranes (7 min at 25 V and 2.5 A, Trans-Blot[®] TurboTM, Biorad). The membranes were blocked for 1 h with PBS containing 5% BSA, stained for 2 h with a His₅-HRP-antibody conjugate (Qiagen, Hilden, Germany) diluted 1:5000 in PBS with 0.05% Tween 20, and washed three times with PBS containing 0.1% Tween 20. Proteins were detected by enhanced chemoluminescence (Clarity Western ECL substrate, Biorad). The same staining procedure was also used for vacuum-assisted dot-blot experiments (VWR dot blot device, Darmstadt, Germany) in which culture supernatants

(a) Dot blot analysis of different cell lines

Fig. 2 a Dot blot analysis for the screening of single cell clones. The signal intensity is a direct measure of the overall expression of His₆-tagged proteins and is determined by staining with a His₅-HRP antibody followed by chemiluminescent

from different cell lines were directly blotted onto a membrane (Amersham Protan 0.2 μ m, GE Healthcare).

In vitro antimicrobial assay

The antimicrobial activity of *GmGlv* was investigated using two separate growth assays based on previous reports (Xu et al. 2012; Yi et al. 2013). The assays were carried out using shake-flask cultures (37 °C, 25 mL, 250 rpm) of *E. coli* Rosetta Gami B (B-strain derivative) and *E. coli* Rosetta Gami 2 (K-12 derivative), both grown in LB medium (Sigma-Aldrich). Cultures of *Saccharomyces cerevisiae*, grown in YPD medium (Sigma-Aldrich) were used as a non-bacterial reference. For the first assay, cells were collected from mid-exponential phase cultures and diluted to OD₆₀₀ = 0.01 in fresh medium containing either 10 μ M *GmGlv* (dissolved in PBS) or same amount of pure PBS as a reference. Three replicates of 100 μ L each were transferred to a 96-well plate and the OD₆₀₀ was monitored for 20 h during incubation in a well-humidified environment at 37 °C (Synergy HTX Multi-Mode Reader, Biotek). The dose-dependent activity of *GmGlv* (2.8, 8.5, 11.0 and 17.8 μ M) against *E. coli* Rosetta Gami 2 was determined in the same way, but with a shorter incubation time of 10 h. For the second assay, actively growing cells from all three cultures were centrifuged and resuspended in 10% medium diluted in PBS to simulate non-ideal growth conditions. Again, the mixtures contained either 10 μ M *GmGlv* or PBS as a reference. The cells were cultivated and monitored as described above.

(b) Frequency distribution of the cell specific expression of His-tagged proteins

detection. The red circle indicates a highly productive clone. **b** Frequency distribution of the cell-specific expression of His₆-tagged proteins for wild-type, polyclonal and the different monoclonal S2 cell lines

Results

Single-cell cloning for the generation of a highly productive cell line

The successful generation of stable polyclonal transformants (Kollewe 2013) was followed by a limiting dilution assay to generate stable clones suitable for scaling up to monocultures. The polyclonal cultures were seeded into 960 wells and we recovered 42 monoclonal cell lines, which were induced with 900 μ M CuSO₄. Dot blot analysis of the corresponding supernatants revealed that most of the clones were no more productive than the original polyclonal culture (Fig. 2a), as confirmed by the frequency distribution of cell-specific *GmGlv* expression (Fig. 2b). However, a small number of monoclonal lines showed much greater productivity as well as vigorous growth, and the most promising clone (indicated by a red circle in Fig. 2a) was selected for expansion and was preserved for further experiments.

Subsequent statistical verification experiments showed that the monoclonal cell line expressed and secreted significantly more His₆-tagged protein than the parental polyclonal cells (Fig. 3a, “Appendix 1”). The difference between the mean expression values of the monoclonal and polyclonal cell lines was ~100% with a 99% confidence interval of 82–142%. Western blotting was carried out using an antibody that recognizes the His₆-tag, revealing a band of the anticipated molecular weight (~17.4 kDa) in both

Fig. 3 Expression screening of the parental polyclonal cells and the derived monoclonal cell line. **a** Comparison of *dot blot* intensity representing cell culture supernatants from six independent experiments indicates statistically significant differences using Student's two-sample *t* test by assuming

normality and homoscedasticity (mean $\pm$ SD, $p < 0.001$). **b** Western blot of cell lysates (L) and cell culture supernatants (S) from wild-type S2 cells, polyclonal rS2 cells and monoclonal rS2 cells, stained with a His₅-HRP antibody

Fig. 4 Contour plots of the response surfaces. **a** *GmGlv* concentration after 4 days of expression as a function of CuSO_4 concentration and induction cell density. **b** Cell viability after 4 days of expression as a function of CuSO_4 concentration and induction cell density

recombinant cell lines but no band in the wild-type S2 control. The presence of the correct band in the supernatant indicated the successful synthesis of full-length *GmGlv* and correct processing, i.e. propeptide cleavage and secretion.

Optimization of expression conditions and harvest time in a small-scale culture

The monoclonal S2 cells were induced with different concentrations of CuSO_4 at different cell densities

according to a central composite design. The resulting data were used to generate a response surface showing *GmGlv* production as function of both input variables (Fig. 4a, “Appendix 2”). The resulting model predicted that the maximum protein yield would be achieved by inducing the cells with 600 μM CuSO_4 at a density of $8\text{--}9.5 \times 10^6$ cells/mL. Additional investigations (Fig. 4b) showed that high concentrations of CuSO_4 are cytotoxic, and the effect is more severe at higher cell densities. This may reflect the partial exhaustion of the medium when induction is carried

Fig. 5 The influence of different medium additives on *GmGlv* expression based on the analysis of supernatants by SDS-PAGE following induction with 600 μM CuSO_4

out at high cell densities, which restricts cell growth and regeneration capacity.

The presence of additives (DMSO, glycerol or sodium butyrate) did not boost the production of *GmGlv* and even had a negative effect on cell growth and viability at elevated concentrations (Fig. 5). Based on these data, the three additives were excluded

from subsequent experiments and the concentration of CuSO_4 was kept below cytotoxic levels.

Scale up to a well-monitored 1-L bioreactor culture

The data from the small-scale experiments allowed us to scale up the process to 1-L bioreactors. Standard parameters such as dissolved oxygen, pH and temperature were maintained at constant levels throughout the cultivation to ensure optimal cell growth (“Appendix 3”). For the first 3 days, the cells proliferated with a specific growth rate of 0.027 h^{-1} , which is in the range of previous reports (Moraes et al. 2012). Based on the RSM data, the cells were induced with 600 μM CuSO_4 before the density reached 1×10^7 cells/mL. The post-induction growth rate dropped to 0.016 h^{-1} , but viability remained above 95% until harvest. Signals from both online cell monitoring methods correlated with the offline values and could be fitted using second-order polynomials (Fig. 6a, c).

Fig. 6 Analysis of cell growth and *GmGlv* production.

a Representative time course of cell growth in a *GmGlv*-expressing monoclonal rS2 cell line at the 1-L bioreactor scale. The two-step procedure comprised a 3-day growth phase followed by induction with 600 μM CuSO_4 and a subsequent 3-day protein expression phase. **b** *GmGlv* production as function of time reveals an optimal harvest window 3–4 days after induction. **c** Online optical density and permittivity signals plotted against the corresponding offline cell densities and corresponding second-order polynomial fits. **d** *GmGlv* production shows a linear dependency on cell growth

Fig. 7 SDS-PAGE analysis of IMAC fractions confirming the successful capture and concentration of *GmGlv* from the culture supernatant. Lanes culture supernatant (S), flow through (FT), wash fractions 1–2, and elution fractions 1–6

The *GmGlv* concentration in the supernatant was monitored to determine the optimal window for harvesting (Fig. 6b). The highest concentration of *GmGlv* (25 mg/L) accumulated on days 3–4 after induction. The growth curve then flattened as the cell population entered the stationary phase. During the production phase, the amount of *GmGlv* showed a linear relationship with cell growth, indicating a strong relationship between cell proliferation and product generation (Fig. 6d). Prolonged cultivation during the stationary phase led to product degradation and should be avoided in an optimized process.

Dielectric spectroscopy and the online measurement of optical density were both suitable for the accurate timing of key operational transitions (induction and harvest). However, the measurement of residual glucose concentration was not suitable for this purpose. Cell growth declined before the main carbon source was exhausted and no drop in glucose consumption was detected at the optimal harvest time (“Appendix 3”). The early decline in cell growth may reflect either the prolonged exposure to the cytotoxic CuSO_4 or the depletion of another growth-limiting substrate.

Capture of recombinant GmGloverin from the supernatant

Recombinant *GmGlv* was captured from culture supernatant by IMAC. The protein fraction was

then dialyzed against PBS. Analysis of the fractions by SDS-PAGE indicated the successful binding of *GmGlv* to the column and its concentrated elution with 500 mM imidazole. However, several host cell proteins that are naturally rich in histidine residues were secreted during cultivation, and these were also captured on the column and co-eluted with *GmGlv*, as shown by the minor contaminating bands (Fig. 7).

Characterization of antimicrobial activity of *GmGlv*

Two different microbial growth assays were used to confirm the activity of the recombinant *GmGlv* under optimal growth conditions and nutrient-delimited conditions, respectively. Both of the *E. coli* strains showed significantly inhibited growth in the presence of 10 μM *GmGlv* (Fig. 8 upper panel) but the effect was more severe under nutrient-delimited conditions, where growth was nearly completely inhibited (Fig. 8 lower panel). Incubation with different concentrations of *GmGlv* showed that its antimicrobial activity against the K12 derivative *E. coli* Rosetta Gami 2 was concentration dependent, and could be observed to concentrations as low as 2.8 μM (Fig. 9).

As already described in other studies, the C-terminal tag has a negligible effect on the activity of Gloverin (Xu

Fig. 8 Growth profiles of *E. coli* Rosetta Gami 2, *E. coli* Rosetta Gami B and *S. cerevisiae* following treatment with 10 μM GmGlv (blue) or PBS as a reference (black). The test was performed either in fresh medium (upper panel, $n = 3$,

mean $\pm$ SD) or in 10% medium diluted in PBS (lower panel, single measurement). Cultivation and time-resolved OD measurements were carried out in a tightly-sealed 96-well plate with a working volume of 100 μL

Fig. 9 Concentration-dependent activity of GmGlv. **a** Growth profiles of *E. coli* Rosetta Gami 2 in fresh LB medium following treatment with 0, 2.8, 8.5, 11 or 17.8 μM GmGlv. Cultivation and time-resolved OD measurements were carried out in a

tightly sealed 96-well plate with a working volume of 100 μL. **b** Optical density after 10 h growth in GmGlv-containing LB medium as function of the GmGlv concentration

et al. 2012, 2015). In contrast to Gram-negative bacteria, *S. cerevisiae* with its mannan-based outer barrier was not susceptible to GmGlv under ideal growth conditions and only partially affected under nutrient-delimited conditions. Our results suggest that GmGlv binds specifically to Gram-negative bacteria, most likely through an interaction with bacterial lipopolysaccharide (Axén et al. 1997; Yi et al. 2013; Xu et al. 2015).

Discussion

Early process optimization achieved by single-cell cloning and screening induction conditions

Stable polyclonal cell lines can be generated easily and used as a straightforward basis for protein production, but the expression level in a polyclonal

cell population is often heterogeneous and the most productive clones become outnumbered by the less-productive but faster-growing majority (Cherbas and Cherbas 2007). The isolation of single-cell clones is therefore an important step in early process optimization, and is often achieved by limiting dilution (Wang et al. 2012; Uribe et al. 2013). However, for insect cells no data are available thus far that show a comprehensive comparison between different clones and the polyclonal parental culture. Furthermore, single-cell cloning does not always achieve enhanced protein production in rS2 cells, and the considerable effort may therefore be wasted (Schetz and Shankar 2004). We therefore isolated more than 40 monoclonal cell lines and compared their expression profiles. The resulting frequency distribution (Fig. 2b) confirms that most clones do not outperform the parental polyclonal culture. However, this is unsurprising because the frequency of highly-productive cells in the parental cell pool is usually low and the likelihood of selecting such cells reflects this initial ratio. Nevertheless, some clones showed the expected increase in productivity and were used to seed monocultures that produced significantly larger amounts of target protein. It is therefore advisable to isolate and screen an appropriate number of clones. Because limiting dilution usually takes several weeks to months, less cumbersome alternatives have been evaluated by other groups. One is the re-selection of rS2 subpopulations by repeated treatment with high concentrations of the selection antibiotic (Lemos et al. 2009). This is a straightforward approach but it does not prevent further rearrangements in the still polyclonal culture, so the most suitable application may be as an additional step before single-cell cloning to enhance the final yield of highly productive clones. Another recently introduced method is immune-magnetic selection, which achieves the rapid selection of highly productive cells but is limited to rS2 cells expressing membrane-bound proteins (Santos et al. 2016).

The second step in early process development is the optimization of induction conditions. For the Mt promoter, induction is carried out with CuSO_4 or CdCl_2 , both of which act in a dose-dependent manner but are cytotoxic at high concentrations (Bunch et al. 1988; Lim and Cha 2006). Although Cd^{2+} is a potent inducer (Li et al. 2012b; Yang et al. 2012; Wang et al. 2012; Uribe et al. 2013) it also activates endogenous

heat-shock promoters (Bunch et al. 1988) and is highly toxic to humans, a reason why this substance was not used routinely in the past. The evaluation of 37 publications dealing with the expression of proteins in rS2 cells under the control of the Mt promoter revealed that the majority of authors ($n = 24$, 65%) used CuSO_4 at concentrations of 400–600 μM , with smaller numbers using higher ($n = 6$, 16%) or lower concentrations ($n = 3$, 8%) of CuSO_4 and 11% ($n = 4$) used CdCl_2 (“Appendix 4”). Our RSM results confirmed that the optimal CuSO_4 dose is within the common range of 400–600 μM . We also showed that there is an optimal induction cell density, but this parameter has less impact on the final yield. The main reason for the decline in protein production at higher inducer concentrations is the loss of cell viability. Efficient protein production therefore requires a balance between induction strength and toxicity. The relationship between cell growth and recombinant protein production exists because both processes require efficient protein synthesis (Gnoth et al. 2007). This was clearly demonstrated in our bioreactor-scale experiment, where there was a linear correlation between cell growth and *GmGlv* production.

Certain medium additives are often used to enhance protein expression in rS2 cells. The histone deacetylase inhibitor sodium butyrate inhibits chromatin condensation and thus promotes transcription in less active regions of the genome (Dorner et al. 1989; Chen et al. 2002; Li and Li 2006; Zhao et al. 2006). Protein expression in rS2 cells is thus enhanced if the transgene is integrated into silent regions of the genome (Chang et al. 2002; Lemos et al. 2009). However, our results show that this effect is negligible if the insert is located in an active genomic region, as it is the case for highly productive single-cell clones. Other common additives include DMSO (Chang et al. 2002; Jeon et al. 2012) and glycerol (Swiech et al. 2008). Both substances have been shown to stabilize proteins and act as chemical chaperones during folding (Yoshida et al. 2002). DMSO is also an effective permeabilizing agent that promotes the release of intracellular products into the medium. However, neither of these compounds affected *GmGlv* production at concentrations of 1%, indicating that *GmGlv* requires no further stabilization or assisted release. The protease inhibitor cocktail also showed no effect, supporting reports that Gloverins are generally intrinsically stable (Axén et al. 1997). Accordingly we

found that *GmGlv* was secreted efficiently from rS2 cells as demonstrated by western blot analysis. Higher concentrations of the additives (5% glycerol or DMSO, and 10 mM sodium butyrate) reduced cell viability and thus the protein yield. Our data emphasize the importance of maintaining physiological conditions for optimal cell growth in order to maximize protein expression even in the presence of cytotoxic inducers. Special additives are not required in the case of stable AMPs such as *GmGlv* but may be necessary for membrane-bound proteins that are otherwise expressed at low levels (Smith 2011; Santos et al. 2016).

Optimal timing guided by process analytical technology is necessary during scale up

Appropriate process analytical technology is required during scale-up to monitor the physiological state of the cells, which is a critical process parameter for protein production (Junker et al. 1994). Dielectric spectroscopy (Druzinec et al. 2013, 2014; Justice et al. 2011) and online OD measurement (Akhnoukh et al. 1996; Bédard et al. 1994) are valuable tools for the real-time monitoring of processes based on insect cells, such as the baculovirus mediated protein expression in *Spodoptera frugiperda* cells. Exemplarily, in a corresponding process for recombinant adeno-associated virus vectors, the permittivity signal was used to determine the optimal time of infection and harvest (Negrete et al. 2007). The same techniques were successfully adapted herein to report the physiological status of a stable rS2 cell line. Both techniques were able to determine optimal windows for induction and harvest and their outputs could be used interchangeably. However, using both techniques simultaneously provides added value, because the signal ratio is an indicator of cell viability.

The optimal induction point in our bioreactor system was during the mid-exponential growth phase 70 h after inoculation. This is comparable to simulations of human transferrin production using rS2 cells in a spinner flask system, where the optimal induction time point was similarly in the exponential phase 70–80 h after inoculation (Lim and Cha 2006). The optimal time for harvest was determined to be between 75 and 100 h post-induction in our system. Other studies have shown that yields reach a peak at 100–160 h post-induction (Park et al. 1999, 2008;

Lim and Cha 2006; Lee et al. 2007). However, these cultivations were carried out in non-controlled cultivation systems, with prolonged growth phases. Furthermore, when *GmGlv* was expressed in shake-flask cultures the optimal harvest time shifted to 100–120 h post-induction (data not shown). The production peak may therefore correspond to the end of the exponential growth phase as previously reported (Lee et al. 2007), which is consistent with our *GmGlv* expression profile. Other studies suggest that the production peak corresponds to the beginning stationary phase (Lim and Cha 2006; Park et al. 2008). Nevertheless, most of the protein is synthesized during exponential cell proliferation in all systems and further process intensification could therefore be achieved by extending the growth phase after induction, e.g. using an extended fed batch process (Søndergaard 1996) or a perfusion culture (Wang et al. 2012).

Assessment of the achievable AMP yield

In order to compete with other platforms, a sufficient protein yield is necessary. Common yields for the recombinant production of different mature AMPs (including those of non-insect origin) using the hosts *E. coli* and *P. pastoris* varied between 10 µg/L and 700 mg/L (Parachin et al. 2012). If only AMPs of insect origin were considered, the same hosts showed expression levels between 0.5 and 68 mg/L (Parachin et al. 2012; Müller et al. 2015). Despite potential high yields, the susceptibility of microorganisms to AMPs can hamper their suitability as expression hosts and higher eukaryotic expression systems are necessary, as proposed in this study. Therefore, mammalian cell lines, such as CHO-K1 (Horwitz et al. 2000; Chiou et al. 2009), and insect cell lines, such as S2 or Sf9 (Lundström et al. 2002; Kawaoka et al. 2008; Carballar-Lejarazú et al. 2008; Xu et al. 2012, 2015), were frequently used for AMP expression. However, there are less comprehensive data on achieved yields because the main focus of these studies was basic functional analysis of the proteins. Generally, the expression level for stably transformed insect cell lines and the baculovirus expression vector system varies between 0.2 and 20 mg/L (Wickham et al. 1995; Kollwe 2013), optimized processes can also exceed 100 mg/L.

As a concrete example, a spider peptide toxin (4.7 kDa) was expressed in recombinant S2-cells at

the 12-L scale and yielded 0.48 mg/L after purification (Escoubas et al. 2003). The recombinant expression of a bacteria binding C-type lectin (~16 kDa) in rS2-cells resulted in 1.5 mg/L (Uribe et al. 2013), while another C-type lectin-like protein (14–17 kDa) was expressed at around 95 mg/L (Scotter et al. 2006). The latter is an example of rS2 cells surpassing all previous attempts to produce this protein in *E. coli*, baculovirus-infected fall armyworm cells or *P. pastoris* (Scotter et al. 2006). Another approach is the expression in CHO cells, but despite their widespread and very successful use in antibody production they are not always superior to rS2 cells. Exemplarily a recent comparison showed that rS2 cells were more suitable for the production of human coagulation factor IX (Vatandoost and Bos 2016). With respect to the production of an antimicrobial protein, named rBPI, CHO-K1 cells yielded 6 mg/L (Horwitz et al. 2000).

In context with the information discussed above, the achieved Gloverin yield of 25 mg/L is not superior but still competitive to other expression hosts, especially when considering the easy recovery of the active product. As discussed before, the yield reported in this study is also not a dead end, but will increase with further process intensification and the use of advanced process modes (Wang et al. 2012).

Specific antimicrobial activity of GmGlv

GmGlv is a member of the Gloverin family. These are heat-stable proteins of approximately 14 kDa. They are rich in glycine but lack cysteine residues, hence there are no disulfide bonds. The Gloverins usually have a basic isoelectric point (pI). GmGlv showed the typical characteristics of this family: 17.4 kDa (14.7 kDa without tags), a predicted pI of 9.74 (9.79 without tags) and ~50% sequence identity with other Gloverins in the UniProt database (<http://www.uniprot.org/blast/>). Gloverins have been identified in several lepidopteran species, including *Hyalophora gloveri* (Axén et al. 1997), *Helicoverpa armigera* (Mackintosh et al. 1998), *Trichoplusia ni* (Lundström et al. 2002), *M. sexta* (Xu et al. 2012), *B. mori* (Yi et al. 2013), *Diatraea saccharalis* (Silva et al. 2010), *P. xylostella* (Xia et al. 2015) and *Spodoptera exigua* (Hwang and Kim 2011). The general mechanism of their

antimicrobial activity is not fully understood, but it is based on interactions with bacterial LPS that increase membrane permeability or inhibit the formation of the bacterial outer membrane. The activity of Gloverins differs according to the origin of each protein. Gloverins are often identified in the hemolymph of larvae challenged with *E. coli* or LPS, suggesting that most of them are more or less active against Gram-negative bacteria (e.g. HgGlv, HaGlv, TnGlv, BmGlv, DsGlv and PxGlv). However, some Gloverins are essentially inactive against *E. coli*, but are active against Gram-positive bacteria or fungi (e.g. MsGlv and SeGlv). In addition to its antibacterial activity, TnGlv was also shown to inactivate a common insect virus: *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Moreno-Habel et al. 2012). Little is known about the activity of GmGlv, although the hemolymph of LPS-challenged *G. mellonella* larvae contains GmGlv and significantly inhibits the growth of *E. coli* on agar plates (Altincicek et al. 2007). This effect may reflect the presence of a mixture of GmGlv and other AMPs (Altincicek et al. 2007). We found that pure GmGlv also specifically inhibits the growth of two *E. coli* strains that are frequently used for protein expression, suggesting that *E. coli* would be an unsuitable host for the expression of this AMP. Antimicrobial activity was observed down to a concentration of 2.4 µM GmGlv, which is consistent with the minimum inhibitory concentration of 0.5–3 µM reported for other Gloverins (Axén et al. 1997; Mackintosh et al. 1998; Lundström et al. 2002). According to the *antimicrobial peptide database* (<http://aps.unmc.edu/AP/main.php>), AMPs are classified as active, if they have a minimum inhibitory concentration below 100 µM (Wang et al. 2016). In terms of this definition GmGlv is regarded as active and suitable for further research. Our data strongly support the likelihood that GmGlv acts specifically against Gram-negative bacteria such as *E. coli* because it had a negligible effect on the growth of *S. cerevisiae*, as previously shown for BmGlv (Yi et al. 2013). This specificity reflects the binding of Gloverin to LPS (“Appendix 5”) which probably induces conformational changes in the AMP (Axén et al. 1997; Yi et al. 2013). The complete mechanism of action is not yet understood, and crystallization studies will be required to determine the specific nature of these interactions.

Conclusion

The establishment of a robust and efficient production process for functional AMPs is a major challenge, but this is required to produce enough material for crystallization studies and for the development of AMPs as anti-infective drugs. We found that rS2 cells were ideal for the production of *GmGlv* and that the production system benefited significantly from single-cell cloning. Optimized induction conditions and process monitoring facilitated the reliable production of pure *GmGlv* that can be used for further research or for the development of complete downstream processes. The incorporation of online monitoring techniques at this early stage not only helps us to understand the process characteristics, but can also be used for the validation and qualification that becomes necessary when the product enters clinical development.

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Author contributions JZ conceived, designed and conducted the experiments, and wrote the manuscript; TW helped to draft

and to revise the manuscript; PC helped to draft and to revise the manuscript, and supervised the research; All authors have given their approval for this final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Appendix 1: Statistical assessment of normality and homoscedasticity for the comparison of the expression of the parental polyclonal cells and the monoclonal cell line D7

Statistical assessment of expression data was conducted using R v3.2.3 (R Core Team 2014). Because the normal q - q plots in Fig. 10 do not show any points outside their confidence intervals, there is no reason to doubt the validity of the assumption of normality for the data in both groups. The high p value of 0.9619 in a subsequent F-test indicates that there is insufficient evidence to reject the assumption of homoscedasticity. Consequently, Student's two-sample t test was used to compare the means of both datasets.

Fig. 10 Normal q - q plots for the expression datasets used to compare the monoclonal cell line and polyclonal culture

Appendix 2: Response surface method for the determination of optimal induction conditions

See Tables 1 and 2.

Table 1 ANOVA for the quadratic model that describes *GmGlv* production after 4 days as a function of cell concentration (A) and CuSO_4 concentration (B)

Source	Sum of squares	df	Mean square	F value	p value Prob > F	
Model	2387.31975	6	397.886626	125.119645	<0.0001	Significant
A	16.6083246	1	16.6083246	5.22266277	0.0301	
B	1434.54256	1	1434.54256	451.107035	<0.0001	
AB	49.5245589	1	49.5245589	15.5735197	0.0005	
A ²	107.989939	1	107.989939	33.958575	<0.0001	
B ²	724.809437	1	724.809437	227.92397	<0.0001	
AB ²	56.4182078	1	56.4182078	17.7413003	0.0002	
Residual	89.0413776	28	3.1800492			
Lack of fit	28.45301	7	4.06471571	1.40883528	0.2537	Not significant
Pure error	60.5883676	21	2.88516036			
Cor total	2558.3453	35				

Table 2 ANOVA for the cubic model that describes cell viability after 4 days as a function of cell concentration (A) and CuSO_4 concentration (B)

Source	Sum of squares	df	Mean square	F value	p value Prob > F	
Model	20,039.7564	8	2504.96955	282.350427	<0.0001	Significant
A	49.0105715	1	49.0105715	5.52428104	0.0251	
B	116.81813	1	116.81813	13.1672853	0.0010	
AB	1851.79606	1	1851.79606	208.727251	<0.0001	
A ²	23.3543994	1	23.3543994	2.63241709	0.1145	
B ²	4474.0554	1	4474.0554	504.298127	<0.0001	
A ² B	35.7292093	1	35.7292093	4.02725753	0.0533	
AB ²	828.976863	1	828.976863	93.4390485	<0.0001	
B ³	580.280857	1	580.280857	65.4070018	<0.0001	
Residual	283.899077	32	8.87184616			
LACK OF FIT	151.95109	11	13.8137355	2.19850604	0.0581	Not significant
Pure error	131.947987	21	6.28323746			
Cor total	20,469.4054	41				

Appendix 3: Time course of additional process variables during the cultivation of rS2 cells for the production of *GmGlv*

See Fig. 11.

Fig. 11 Time course of standard bioreactor parameters: **a** stirrer speed (n , black), dissolved oxygen (pO_2 , red), gas mix composition (blue) and pH (gray). **b** Glucose consumption during cultivation

Appendix 4: Comparison of the induction conditions in 37 studies

See Table 3.

Table 3 Comparison of the induction conditions in 37 studies using the Mt promoter in rS2 cells

Inducer and concentration	Number	Proportion (%)	References
$CuSO_4 < 400 \mu M$	3	8.1	Deml et al. (1999), Valle et al. (2001), Lieberman et al. (2007)
$CuSO_4$ 400–600 μM	24	64.8	Bernard et al. (1994), Nilsen and Castellino (1999), Park et al. (1999, 2008), Shin and Cha (2002), Friry et al. (2002), Chang et al. (2002), Shin et al. (2003), Schetz et al. (2003), Shin and Cha (2003), Cho et al. (2004), Lim and Cha (2006), Johansson et al. (2007), Lee et al. (2007, 2009, 2011), Kim et al. (2008), Seok et al. (2010), Gutsche et al. (2011), Chung et al. (2011), Jeon et al. (2012), Uribe et al. (2013), Xu et al. (2015), Vatandoost and Bos (2016)
$CuSO_4$ 601–800 μM	4	10.8	Dubreuil et al. (1996), Dubreuil and Grushko (1999), Perret et al. (2003), Santos et al. (2007)
$CuSO_4 > 800 \mu M$	2	5.4	Bunch et al. (1988), Graziano et al. (1998)
$CdCl_2$ 1–100 μM	4	10.8	Uribe et al. (2013), Li et al. (2012b), Yang et al. (2012), Wang et al. (2012)

Appendix 5: Site-specific binding of *GmGlv* to LPS

There is no broad agreement on the exact position at which Gloverin binds to LPS. The latter comprises three major parts: lipid A, a carbohydrate core region,

and the O-specific antigen. If all parts are present, the LPS is regarded as smooth. If the outer parts are absent, LPS is regarded as rough or deep rough depending on the degree of truncation (Ra, Rc, Rd, and Re LPS). *BmGv* is inactive against *E. coli* DH5alpha

but active against the rough mutants *E. coli* D21 (Ra-LPS), *E. coli* D21e7 (Rc-LPS), *E. coli* D21f1 (Rd-LPS) and *E. coli* D21f2 (Re-LPS) (Yi et al. 2013). Furthermore, *Tn*Glv (Lundström et al. 2002) and *Hg*Glv (Axén et al. 1997) also show activity primarily against *E. coli* D21f2. These results indicate that most (positively charged) Gloverins probably bind to the (negatively charged) inner core region or lipid A. The two *E. coli* strains used in this study have not been comprehensively characterized in terms of their LPS phenotype, but their parental strains carry short LPS (Chart et al. 2000). Furthermore *E. coli* Rosetta Gami 2 carries the *galE*–*galK* mutation, which blocks the synthesis of UDP-galactose and its incorporation in the LPS, thus producing a truncated LPS (van Die et al. 1984). These data suggest that *Gm*Glv also binds to the inner part of the LPS. However, *Ms*Glv binds to the outer core region and the O-specific antigen of LPS and also to other fungal and bacterial membrane compounds, but it does not interact with Rc, Rd or Re LPS or with lipid A (Xu et al. 2012).

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