Cytotechnology (2010) 62:547–555
DOI 10.1007/s10616-010-9305-x

ORIGINAL RESEARCH

Improvement of recombinant protein production by an anti-apoptotic protein from hemolymph of *Lonomia obliqua*

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Received: 4 June 2010 / Accepted: 3 September 2010 / Published online: 10 October 2010

Abstract Apoptosis is a major problem in animal cell culture during production of biopharmaceuticals, such as recombinant proteins or viral particles. In the present work baculovirus-insect cell expression system (BEVS/IC) is used as model to produce rotavirus like-particles, composed by three layers of three different viral proteins (VP2, VP6 and VP7). In this model baculovirus infection also induces host cell death. Herein a new strategy to enhance cell life span and to increase recombinant rotavirus protein production of BEVS/IC system was developed. This strategy relies on hemolymph from *Lonomia oblique* (total extracts or a semi-purified fraction) medium supplementation. The total extract and a purified fraction from hemolymph of *Lonomia obliqua* were able to protect Sf-9 cell culture against apoptosis triggered by oxidative stress (using the pro-oxidant agents *tert* butylhydroperoxide and hydrogen peroxide) and by baculovirus infection. Furthermore, hemolymph enhance final recombinant protein production, as it was observed by the increased amounts of VP6 and VP7, which were measured by the semi-quantitative western blot method. In conclusion, hemolymph medium supplementation can be a promising strategy to improve cell viability and productivity of recombinant protein in BEVS/IC system.

Keywords Baculovirus · Sf-9 cells · Apoptosis · Hemolymph · *Lonomia oblique* · Bioactive peptide · Recombinant protein production

Abbreviations

Hb	Crude hemolymph
Frp	Purified fraction
t-BHP	*Tert*-butylhydroperoxide
PI	Propidium iodine
$DiOC_6(3)$	3,3′dihexyloxacarbocyanine iodide
Sf-9	*Spodoptera frugiperda*
BEVS/IC	Baculovirus expression vector system/ insect cells
MOI	Multiplicity of infection
GFP	Green fluorescent protein
($\Delta\Psi$m)	Mitochondrial transmembrane potential

Introduction

Optimization of bioprocesses is essential for cost-effective production of biopharmaceuticals, such as recombinant proteins, viruses and cells. Cell death in

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bioreactors represents a major problem in animal cell culture technology because it decreases the global productivity yield (Al-Rubeai 1998; Butler 2005). Several factors have been implicated in decreasing the cell viability in bioreactors: nutrient depletion, shear stress, hypoxia, accumulation of toxic metabolites and by products, and apoptosis induced by viral infections (Arden and Betenbaugh 2004; Arden et al. 2007). There are two main approaches to improve cell viability in bioreactors: (1) optimization of the extracellular environment or (2) reprogramming gene expression to limite cell death. In the first case, culture media can be supplemented to improve cell nutrition and growth factor concentrations; toxic metabolites can be limited; or anti-apoptotic factors (chemical, peptides or proteins) can be added into the culture media (Arden and Betenbaugh 2004). The baculovirus-insect cell expression system (BEVS/IC) has emerged as a powerful tool to produce recombinant proteins and virus-like particles (VLP) due to: the easy use, ability to accommodate large gene insertions and to express high levels of recombinant protein. In addition, host insect cells are capable of processing some proteins in a manner similar to mammalian cells (Jarvis 2003). However, baculovirus infection induces oxidative stress and cell death, which limits recombinant protein production (Vieira et al. 2006). Recently some studies have demonstrated the presence of pharmacologically active substances in hemolymph of *Lonomia obliqua*, which can enhance Sf-9 insect cell growth and culture longevity (Maranga et al. 2003; Raffoul et al. 2005; Souza et al. 2005; Mendonça et al. 2008; Greco et al. 2009). In the present work, hemolymph (whole extract or a semi-purified fraction) was applied to improve BEVS/IC system by increasing rotavirus recombinant protein. Production of three different rotavirus structural proteins VP2, VP6 and VP7 (Roldão et al. 2007; Vieira et al. 2005) is the used model to assess the improvement of BEVS/IC system. Apoptosis/cell death hallmarkers, such as chromatin condensation, plasmatic membrane permeabilisation and loss of mitochondrial potential were assessed to verify the anti-apoptotic role of hemolymph. On one hand, hemolymph supplementation limited cell death induced by pro-oxidants and baculovirus infection. On the other hand, production of recombinant protein (VP6 and VP7 from rotavirus) has increased in the presence of hemolymph.

Materials and methods

Cell lines and cell culture

Spodoptera frugiperda Sf-9 cell line was obtained from the American Type Culture Collection (ATCC, US). Cells were cultured in serum free medium SF900II (Gibco, Glasgow, UK) at 27 °C in 250 mL (working volume) spinner flasks at 170 rpm. For the fluorescence microscopy assays, cells were grown in 24 wells plates (Nunc) at 27 °C without agitation.

Baculoviruses and infections

The recombinant baculovirus vector coding for rotavirus *vp2* gene with *gfp* (green fluorescent protein) gene was kindly provided by Dr. Annie Charpilienne (CNRS-INRA, France). Multigene *Autographa californica* nucleopolyhedrovirus (*Ac*MNPV) recombinant baculovirus, coding for bovine rotavirus *vp2, vp6* and *vp7* genes was constructed and kindly provided by Prof. Polly Roy from the London School of Hygiene & Tropical Medicine, England. Infections were performed at a MOI of 5 pfu/mL and a CCI of 1×10^6 cells/mL.

Hemolymph total extract isolation

Hemolymph of *Lonomia obliqua* was collected from sixth-instar larvae after setae cut off. The collected hemolymph was centrifuged by 1,000× *g* for 10 min, the supernatant was filtered with 0.2 µm membrane filter, inactivated by heat (60 °C) during 30 min and stored at 4 °C. Hemolymph was used for medium supplementation at 1% (v:v).

Hemolymph semi-purified fraction

1 mL of total extract of hemolymph was loaded on a Superdex 75 Hr10/30 (Amersham Pharmacia Biotech) column at a rate of 0.5 mL/min and eluted with Tris-Nacl (20 mM). The eluates were harvested and monitored at 280 nm. Active fractions from Superdex 75 column were loaded on an ion change column (Resource Q). The chromatography was performed with an AKTA purifier chromatrograph (Amersham Pharmacia Biotech). The purified fractions were applied to SDS–PAGE electrophoresis for analysis.

Apoptosis induction

Apoptosis was trigged by oxidative stress induced by addition of *tert*-butyl hydroperoxide (Sigma) at 50–100 μM or by addition of H_2O_2 at 600–1,000 μM. Baculovirus infection with a MOI of 5 pfu/mL and a CCI of 1×10^6 cells/mL was also applied to induce apoptosis.

Flow cytometry

Sf-9 cells were pre-treated during 1 h with 1% (v:v) of total hemolymph extract (Hb) or with purified fraction (Frp), followed by cell death induction with t-BHP at 50–100 μM or with 600–1,000 μm of H_2O_2, during 4 h. Then 1 mL of cell culture was analysed by flow cytometry. On the other hand, samples of 0.5 mL were collected from the cell culture at different times from day 0 to day 6 post-infection. Cell death-associated changes were assessed by cytofluorometry on a BD FACSCalibur™ four colors (Becton–Dickinson), while gating the forward and the side scatters on cells (R1 region), using several fluorochromes: 3,3′ dihexyloxacarbocyanine iodide ($DioC_6(3)$, 20 nM) for mitochondrial transmembrane potential ($\Delta\Psi m$) quantification, propidium iodide (PI, 1 μg/mL) for the determination of cell viability. The acquisition and analysis of the results was performed with CellQuest (Becton–Dickinson) software.

Fluorescent microscopy

Sf-9 cells were grown in 13 mm-diameter coverslips and 24 h later they were pre-treated with total hemolymph extract (Hb) or with purified fraction (Frp) during 1 h, followed by addition of *tert*-butyl-hydroperoxide or hydrogen peroxide. After 4 h cells were stained with Hoechst 33342 (2 μM, Sigma), followed by fluorescence microscopic assessment of apoptotic nuclei. Cells were observed with a Leica DMRB microscope using a filter cube presenting UV excitation range with a bandpass of 340–380 nm of wave length.

Protein quantification

Protein quantification was done using Western blot technique. The amount of each protein was normalized with a VLP standard, with known protein concentrations which are related with the corresponding band density, for the comparison between different protein amounts. Total cellular bulk samples were reduced and resolved by denaturating electrophoresis on a 1 mm NuPAGE® Novex BIS–Tris Gel (Invitrogen) and electrically transferred to a nitrocellulose membrane (Hybond™-C extra, Amersham Biosciences). Immunochemical staining for recombinant protein detection was carried out with a polyclonal anti-Rotavirus serum, kindly provided by Dr. Annie Charpilienne (CNRS-INRA, France). Blots were developed after incubation with an alkaline phosphatase conjugated anti-Rabbit IgG antibody (Sigma) using 1-step™ NBT/BCIP blotting detection reagents (Pierce). The protein applied to Western blot was measure by Total protein assay; BCA kit (Pierce), and the area and intensity of bands in western blot gels were also quantified by densitometry analysed (GraphPad Prism 4).

Statistical analysis

The values analised represent the average of three experiments. Data are expressed as the mean ± SD. Statistical analysis was performed using ANOVA test and the level of significance was set at $p \leq 0.05$.

Results

Hemolymph purification

Previously we have described and characterized an antiapoptotic protein presented in the hemolymph of *Lonomia obliqua* (Maranga et al. 2003; Raffoul et al. 2005). The supplementation of hemolymph into the culture medium induced high levels of cell growth and cell viability, which were maintained for long periods (Souza et al. 2005). In the present study, the ability of this antiapoptotic protein in increasing recombinant protein production was investigated. Hemolymph was fractionated by gel filtration chromatography in a Superdex-G75 column. The fractions collected were applied to SDS–PAGE gels in order to analyze the resolution between the different proteins obtained by the separation process. Fractions collected were tested in Sf-9 cell culture to identify the fraction presenting antiapoptotic effect. The fraction showing the antiapoptotic actitivity was then

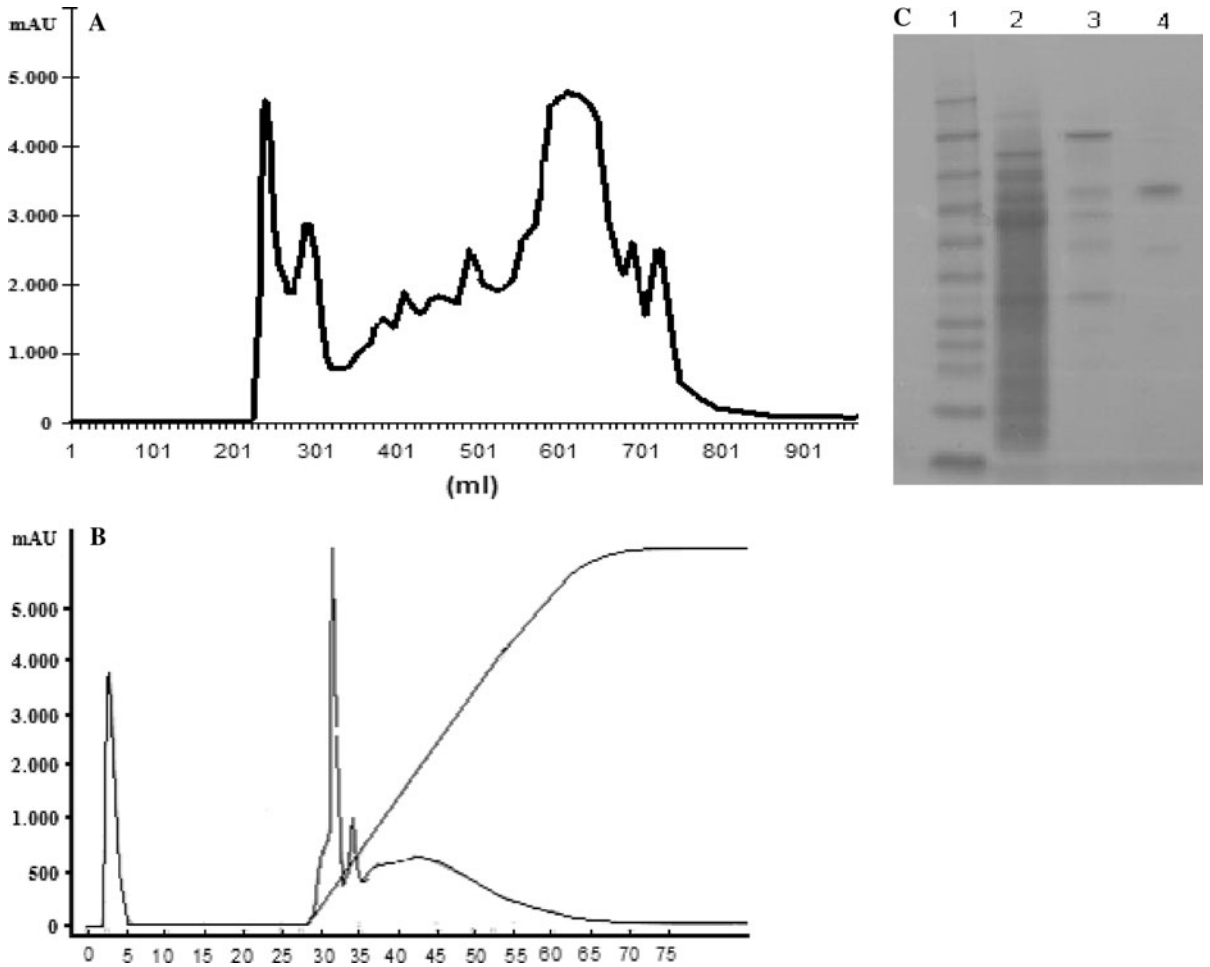


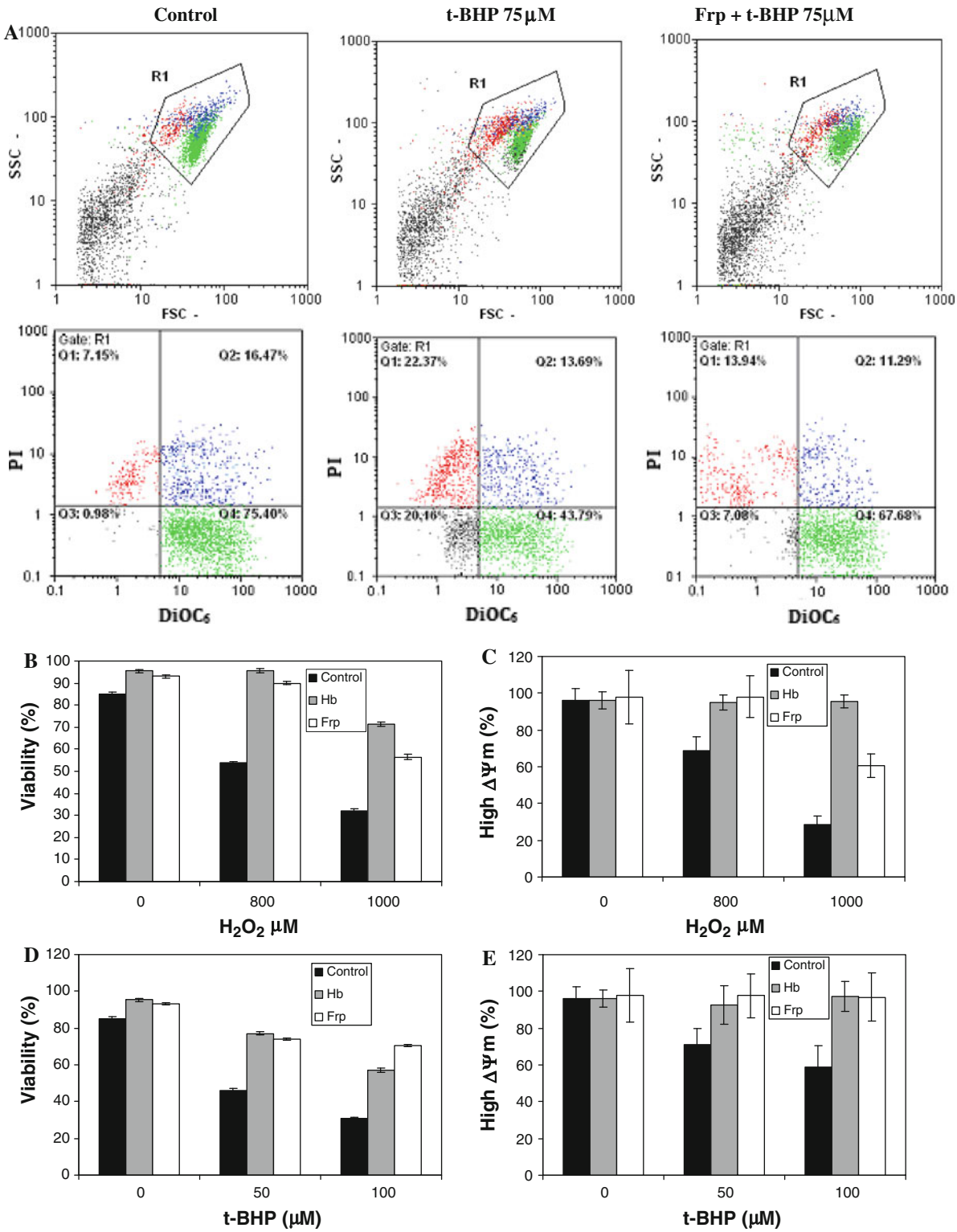
Fig. 1 *Chromatogram* shows the fractionation of L. obliqua hemolymph. Total hemolymph was loaded firstly on a gel chromatography column and was eluated at 0.5 mL/min with a sodium phosphate buffer (**a**). The fractions with antiapoptotic activity were pooled and further fractionated with a Resourse-Q ion exchange column at a rate of 1 mL/min and elution was performed with a linear gradient (0–100%) of TrisHCl 20 mM/TrisHCl 20 mM-NaCl 1 M, pH 8.0. The eluates were monitored at 280 nm (**b**). The protein fractions obtained were analysed by SDS-PAGE eletrophoresis (**c**). Lane 1, (MW) Molecular weight; lane 2, Total hemolymph; lane 3, semipurified hemolymph; lane 4, purified fraction

applied to an anion exchange column (Resource Q). The chromatograms and the SDS–PAGE gel are showed at Fig. 1.

Hemolymph prevents Sf-9 cell death induced by oxidative stress

Baculovirus infection of Sf-9 insect cells induces oxidative stress, as demonstrated by increased levels of lipid peroxidation and protein oxidation (Wang

Fig. 2 Flow cytometry analysis of Sf-9 cells, in growth▶ phase, was treated or not with 1% (v/v) of purified fraction of hemolymph (Frp): Apoptotic death of the cells was induced with 75 μM of t-BHP for 4 h (**a**) or with 800–1,000 μm of H_2O_2 (**b**). After this period, the cultures were marked with 1 μg/mL of PI and 20 μM of $DiOC_6(3)$. After 20 min of contact the samples were analysed by flow cytometry, with different cell populations: apoptotic cells (Q1), necrotic cells (Q2), cell debris (Q3), or viable cells (Q4) (**a**). The percentage of viability and cells presenting high $\Delta\Psi m$ were determined and are showed at (**b–e**) (average of three experiments. The difference observed was significant for $p \leq 0.05$)


A
Control
t-BHP 75μM
Frp + t-BHP 75μM
R1
SSC -
FSC -
PI
DiOC6
Gate: R1
Q1: 7.15%
Q2: 16.47%
Q3: 0.98%
Q4: 75.40%
Q1: 22.37%
Q2: 13.69%
Q3: 20.16%
Q4: 43.79%
Q1: 13.94%
Q2: 11.29%
Q3: 7.08%
Q4: 67.68%
B
Viability (%)
Control
Hb
Frp
H2O2 μM
C
High ΔΨm (%)
D
t-BHP (μM)
E

et al. 2001, 2004). Moreover, an increase on the cellular oxygen uptake rate, due to the baculovirus infection, has also been observed, which can be related to the oxidative stress induced in the virally infected cells (Saarinen and Murhammer 2002). Therefore, in order to evaluate the cytoprotective effect of hemolymph against oxidative stress and cell death, Sf-9 cells were pre-treated with crude hemolymph (Hb) or purified fraction of hemolymph (Frp) for 1 h followed by addition of *tert*-butylhydroperoxide or hydrogen peroxide to trigger oxidative stress. Apoptotic hallmarkers were assessed by flow cytometry as described in Fig. 2. Representantive flow cytometer diagrams obtained with or without Frp pre-treatment, followed by cell death induction with t-BHP are shown in Fig. 2a. Quantification of hemolymph-mediated cell death prevention is described in Fig. 2b–e. Hydrogen peroxide at 800 or 1,000 μM and t-BHP at 50 or 100 μM induced loss of viability (Fig. 1b, d) and $\Delta\Psi m$ dissipation (Fig. 2c, e) in Sf-9 cells for which these effects were partially prevented by pre treatment with whole extract of hemolymph (Hb) or purified fraction (Frp). The difference observed in all experiments was significant to $p \leq 0.05$.

Nuclear chromatin condensation is another event of apoptotic cell death process. This process can be detected by fluorescent microscopy using Hoechst 33324, which binds to DNA and is an useful tool for apoptotic nuclei determination. Similarly, addition of Hb or Frp into the culture medium partially prevents chromatin condensation in the nuclei when cell death

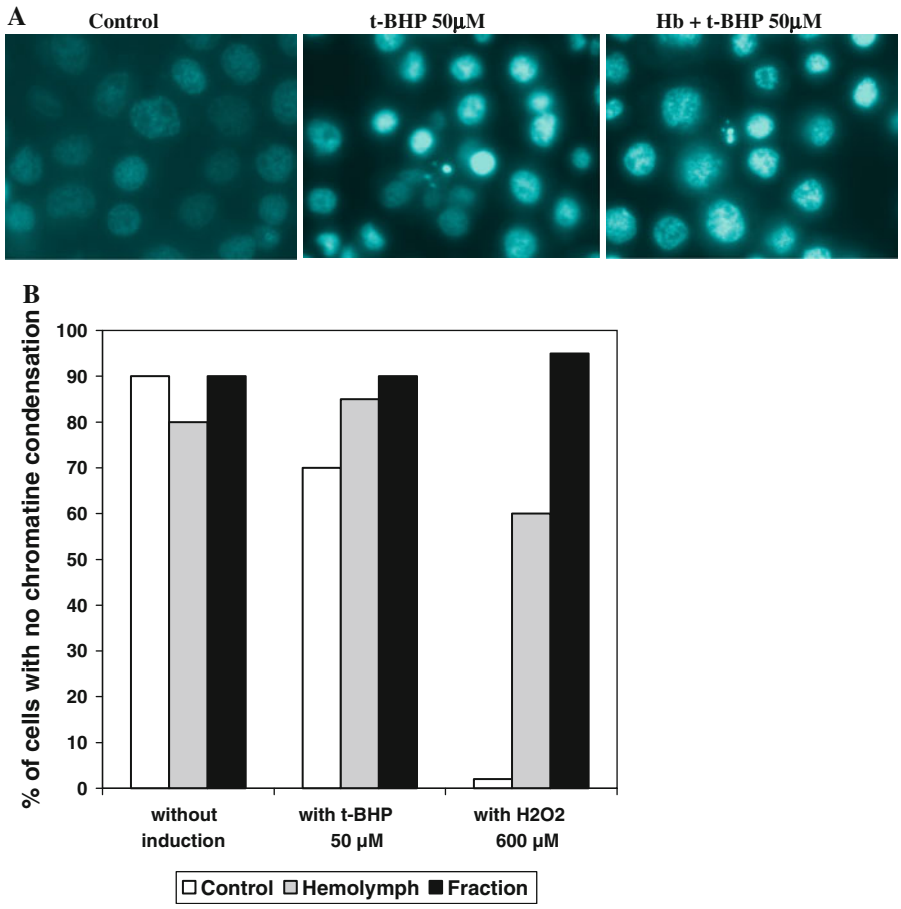


Fig. 3 Effect of the hemolymph in the protection against cell death induced by oxidative stress. The Sf-9 cells were previously treated or not with 1% (v/v) of crude (Hb) or purified fraction of hemolymph (Frp). After this period the cell death was induced with 50 μM tert-butylhydroperoxide (t-BHP) or 600 μM of H_2O_2 for 4 h. After this period the cultures were marked with 20 μM of Hoechst for assessment of chromatine condensation by fluorescent microscopy. Representative pictures of cells are shown in Fig. 3a. Quantification of condensed nuclei is presented in Fig. 3b

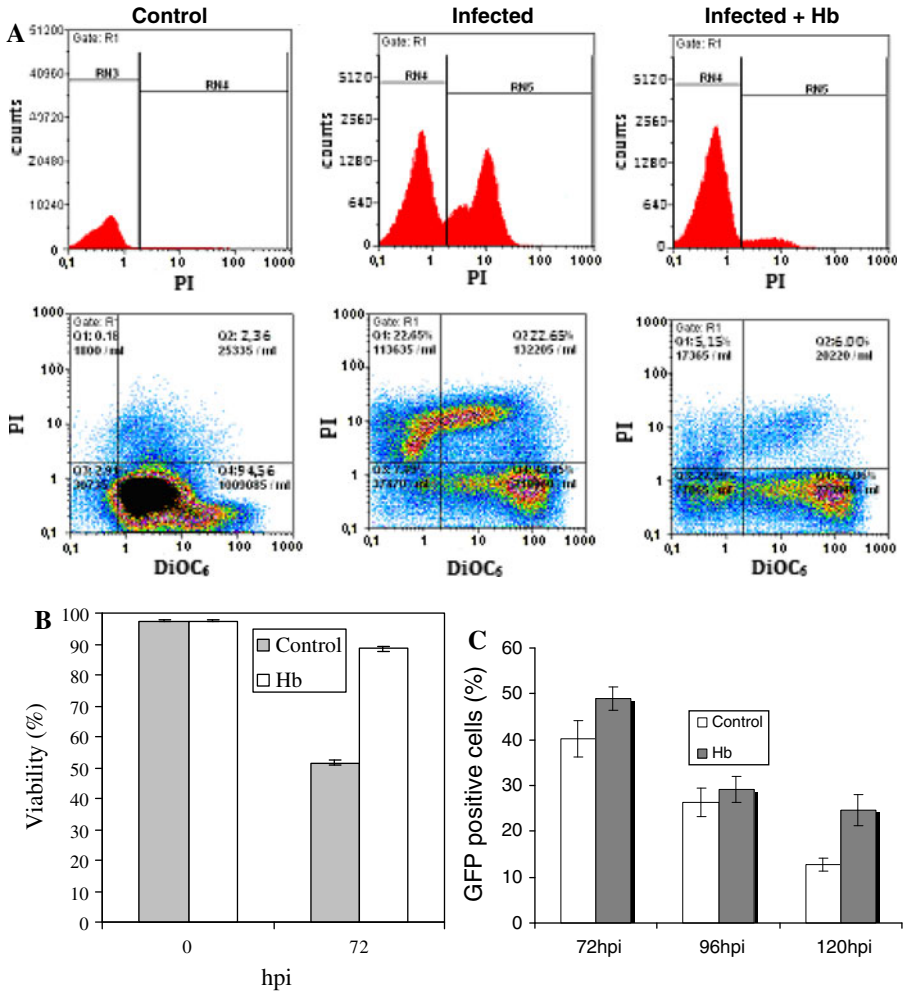


Fig. 4 Sf-9 cells pre-treated or not with hemolymph were infected with baculovirus expressing GFP-Vp2 recombinant protein (5 MOI) and analysed by flow cytometry. At 72, 96 and 120 hpi, cell death was assessed by propidium iodide and $DIOC_6$ (**a** and **b**) and the levels of insect cells producing virus were followed by green fluorescent due to GFP expression (**c**). **a**) Flow cytometry diagrams of cell viability analysis (PI) at 72 hpi. **b**) Viability of infected cells at 72 hpi, in treated or not treated culture with hemolymph. **c**) GPF positive cells in cultures treated or not treated with hemolymph at 72, 96 and 120 hpi. (Average of three experiments. The difference observed was significant for $p \leq 0.05$)

was induced by the pro-oxidants t-BHP or H_2O_2 (Fig. 3).

Hemolymph inhibits cell death induced by baculovirus infection and increases baculovirus infected cell population

The use of baculovirus-insect cell expression system (BEVS/IC) can be limited by cell death induced by baculovirus infection. In Fig. 4, the ability of hemolymph in protecting insect cells against death induced by baculovirus infection is demonstration, using a baculovirus vector expressing GFP-VP2 from rotavirus. Cell viability was assessed by flow cytometry using propidium iodide as dye to verify plasma membrane integrity and 3,3′ dihexyloxacarbocyanine iodide ($DioC_6(3)$), for mitochondrial transmembrane potential ($\Delta\Psi m$) quantification. Representantive flow cytometer diagrams obtained with or without Frp pre-treatment, followed by baculovirus infection at a MOI of 5/cell are shown in Fig. 4a. Figure 4b presents there is the quantitative data concerning cell death prevention by Hb at 0 and 72 h post infection (hpi). At 72 hpi there are around 50% of viable cells

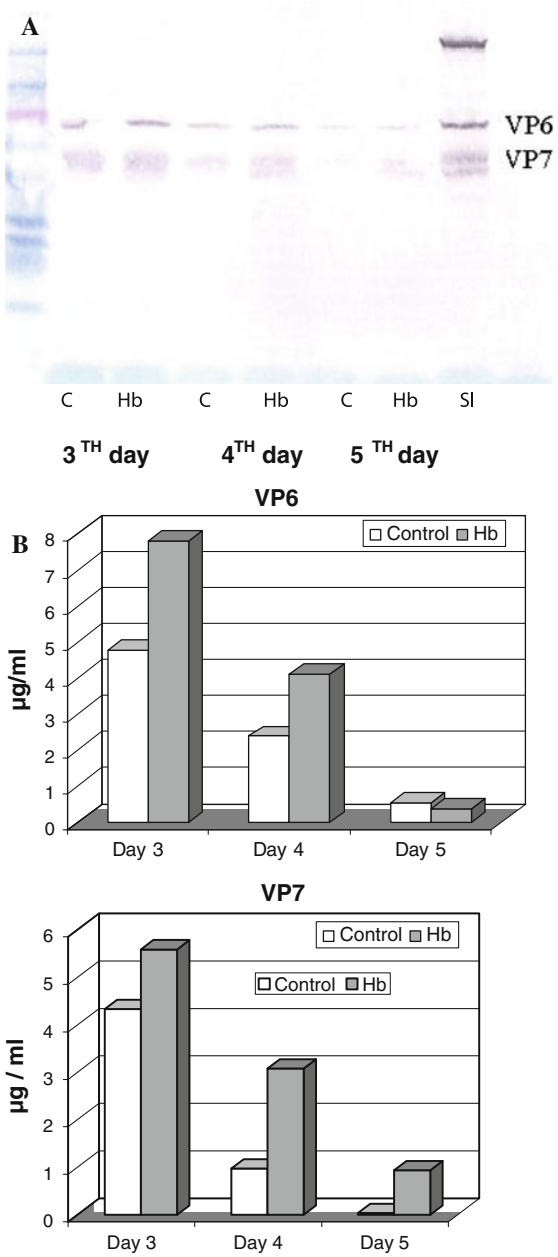


Fig. 5 Determination of proteins Vp6-Vp7 production by Western blot. For that, Sf-9 cells were infected with baculovirus expressing Vp2–Vp6–Vp7 genes (5 MOI), with or without previous treatment (for 1 h) with hemolymph (1% v/v). On the 3rd, 4th and 5th day after infection, a sample of each one of the culture was obtained, charged into a gel of SDS–PAGE, transferred to a nitrocellulose membrane and marked with specific antibodies for each protein (**a**). (MW) Molecular weight (Benchmark Prestained Protein ladder—Invitrogen); (C) Controls without hemolymph; (Hb) with hemolymph; (SI) Internal standard (purified sample of VLP containing VP2, VP6 and VP7. **b** The protein concentration was semi-quantified by densitometry

in the control culture while in Hb treated culture, the cell viability is of 89%, showing cell death prevention after Hb treatment. Figure 4c shows the percentage of cells expressing recombinant green fluorescent protein (GFP) in cultures treated or not treated with hemolymph after 72, 96 and 120 hour post infection. As can be observed for all time points, the percentage of GPF fluorescent cells is higher in cultures pretreated with hemolymph than in control cultures. The differences observed were significant at $p \leq 0.05$ by ANOVA test.

Determination of the hemolymph effect recombinant protein production in Sf-9 cells infected with baculovírus

For the determination of the hemolymph effect on multcistronic vectors, a tricistronic baculovírus expressing the bovine rotavirus glycoprotein Vp2, Vp6 (under control of the polyhedrin) and Vp7 (under control of the p10 promoter) was used in this experiment. The production of recombinant proteins was accomplished by infection of Sf-9 cells (10^6 cells/mL) with baculovírus expressing multigenes with a multiplicity of infection of 5. One hour before the infection the culture were treated or not with hemolymph. The semi-quantitative western blot method was performed using samples collected on 3, 4 and 5 days. The results are in the Fig. 5a, b. As it can be observed in Fig. 5b, an increase in the production of VP6 and VP7 was obtained by hemolymph treatment. The amount of protein was semi-quantified by measuring the area and intensity of bands after densitometry analysis.

Discussion

Previously, it has been shown that hemolymph is capable of inhibiting cell death in different models (Maranga et al. 2003; Souza et al. 2005). We have identified a protein from *Lonomia obliqua* hemolymph able to increase the production of the rabies virus glycoprotein, expressed in *Drosophila melanogaster* S2 cells, by about 59% (Mendonça et al. 2008). The highest values of RVGP production were observed in the exponential phase (Mendonça et al. 2009). However, in the present work baculovirus-insect cell

expression system was used to verify the beneficial effects of hemolymph in this recombinant protein production system. We could demonstrated in the present work that medium supplementation with total extract or a semi-purified fraction from hemolymph of *Lonomia obliqua* is a powerful strategy to improve recombinant protein production in the BEVS/IC system. Medium supplementation with total extract or a purified fraction from hemolymph prevented Sf-9 cell death induced by oxidative stress (Figs. 2 and 3) and delayed the cell death by baculovirus infection (Fig. 4a, b). Moreover, hemolymph was also able to increase the concentration of cells producing recombinant proteins (Fig. 4c). The final productivity of recombinant proteins VP6 and VP7 from rotavirus was also higher in the case of Sf-9 cells pre-treated with hemolymph. Of note, the used method is a semi-quantitative technique to measure protein expression, further work is necessary to precisely quantify the increase of recombinant protein production. Therefore, Hb appears to improve recombinant protein production by two distinct reasons: (1) by augmenting cellular life spam and preventing cell death and (2) by increasing cellular especific productivity of recombinant protein, although this last event needs to be further analysed and explored. In conclusion, culture medium supplementation with hemolymph (total extract or semi-purified fraction) appears as a novel and promising tool to improve recombinant protein production in the BEVS/IC system for biotechnological applications.

Acknowledgments The authors acknowledge Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant no. 05/5443-9, São Paulo, Brazil).

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