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Expression of an antiviral protein from *Lonomia obliqua* hemolymph in baculovirus/insect cell system

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ABSTRACT

The control of viral infections, mainly those caused by influenza viruses, is of great interest in Public Health. Several studies have shown the presence of active properties in the hemolymph of arthropods, some of which are of interest for the development of new pharmacological drugs. Recently, we have demonstrated the existence of a potent antiviral property in the hemolymph of *Lonomia obliqua* caterpillars. The aim of this study was to produce an antiviral protein in a baculovirus/Sf9 cell system. The resulting bacmid contains the sequence coding for the antiviral protein previously described by our group. Total RNA from *L. obliqua* caterpillars was extracted with Trizol and used in the reverse transcription assay with oligo(d)T primer followed by polymerase chain reactions (RT-PCR) with specific primers for the cDNA coding for the antiviral protein, based on the sequence deposited in the GenBank database. Restriction sites were inserted in the cDNA for ligation in the donor plasmid pFastBac1™. The recombinant plasmid was selected in *Escherichia coli* DH5α and subsequently used in the transformation of *E. coli* DH10Bac for the construction of the recombinant bacmid. This bacmid was used for the expression of the antiviral protein in the baculovirus/Sf9 cell system. After identifying the protein by western blot, activity tests were performed, showing that the purified recombinant protein was able to significantly reduce viral replication (about 4 logs). Studies on the optimization of the expression system for the production of this antiviral protein in insect cells are in progress.

1. Introduction

The first licensed human therapeutic protein using the recombinant DNA technology was insulin, produced in 1982 on a large scale in *Escherichia coli*. However, due to the impossibility to express complex proteins with post-translational modifications in bacteria, animal cells have become a more attractive alternative for industrial purposes (Butler, 2005). Animal cell cultures were developed in the last decade of the 19th century with the first attempts to hold pieces of fabric in plasma or biological fluids for several days or weeks. Since then, Technology of Animal Cell Cultures has achieved great progress, and is currently one of the most successful tools in biotechnology (Kretzmer, 2002).

Animal cell cultures require a complex medium, often supplemented with expensive bovine serum which provides essential proteins, such as growth factors, that have to be removed during downstream processing (Reyes-Ruiz and Barrera-Saldana, 2006). An attractive alternative is the use of the expression in the baculovirus/insect cell system described by Smith et al. (1983). This system is widely used as a tool for the production of recombinant proteins that require complex post-translational modifications (Carpentier et al., 2001). Glycosylation, which is the addition of carbohydrates (glycans) to proteins synthesized by animal cells, is one of the examples of post-translational modification. The parameters of cell culture – such as nutrients, oxygen, toxic metabolites, concentration, pH and temperature – may have significant effects on the glycan structure distribution in recombinant proteins, and therefore require efficient control (Butler, 2005).

Several proteins are also targets of the biotechnology industry due to their large commercial interest. In this context, the caterpillar *Lonomia obliqua* gained great prominence in biotechnology in Brazil, owing to the active properties identified in its venom and in its hemolymph (Veiga et al., 2005), which can interfere in blood

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coagulation and fibrinolysis (Veiga et al., 2003), enhance cell growth (Maranga et al., 2003), act as anti-apoptotic agent (Souza et al., 2005) improve recombinant protein production (Mendonca et al., 2009, 2008; Vieira et al., 2010) and demonstrate antiviral effect (Greco et al., 2009).

The present study describes a system for the protein expression in Sf9/baculovirus cells using the recombinant DNA to obtain a protein from the *L. obliqua* caterpillar that displays a potent antiviral action (Greco et al., 2009). This protein is found in the hemolymph of *L. obliqua* caterpillars, and its encoding cDNA sequence is the basic element for the construction of the expression system. The large protein expression allows the analysis of its function and biochemical characterization. This is the preliminary description of the baculovirus/Sf9 cell system used for the expression of this antiviral protein from the hemolymph of *L. obliqua* caterpillar.

2. Materials and methods

2.1. Determination of protein and cDNA sequences and construction of the recombinant protein

2.1.1. Sequence determination and primer design

The design of primers specific for the amplification of the cDNA coding for the putative antiviral protein was based on the protein and cDNA sequences. For identification of the protein sequence, *L. obliqua* hemolymph was purified and the fraction containing the antiviral property was analyzed by SDS–PAGE; the N-terminal sequence of the antiviral protein was determined by Maldi-Q-Tof mass spectrometry (Wattenberg et al., 2002). In order to identify the cDNA coding for the protein of interest, the N-terminal sequence was analyzed against cDNA libraries of *L. obliqua* tegument and spicules (Veiga et al., 2005), using all possible translation frames of each cDNA. The sequence of the respective cDNA was used for primer design and further cDNA amplification by PCR. Restriction sites were also included in the primer sequence for further ligation in the plasmid pFastBac1™ (Invitrogen), as well as a His-tag sequence.

Antiviral response of the baculovirus has been reported in the literature (Gronowski et al., 1999) and the histidine tag can stimulate the immune system response (Masek et al., 2011). Therefore, we also amplified and cloned sequences of two other proteins (LOH-19-AY829833 and 8-LOH) that have molecular weights similar to the protein with the histidine sequence, to confirm that the protective effects observed in the results would be due to the action of the antiviral protein from *L. obliqua* (20-LOH-JN807330) and not a response of the immune system to the His-tag sequence (Masek et al., 2011; Veiga et al., 2005).

2.1.2. RNA extraction and RT-PCR

A *L. obliqua* caterpillar specimen was cross-sectioned in the middle, the extremities were cut off and RNA was extracted from the remaining portion with Trizol (Invitrogen) according to the Manufacturer's instructions. The RNA was stored at −80 °C until use.

The first-strand cDNA was synthesized using Oligo$(dT)_{18}$ Primer (Fermentas) and Superscript III reverse transcriptase (Invitrogen).

For amplification of the sequence of interest, PCRs consisting of 12.5 μl PCR Master Mix (Promega), 200 ng of cDNA and 10 μM of each specific primer were carried out in a thermocycler under the following reaction conditions: initial cycle at 94 °C for 3 min; 35 cycles at 94 °C for 1 min and 30 s, a temperature gradient ranging from 45 °C to 55 °C for 1 min and 30 s, and 72 °C for 1 min and 30 s; final extension at 72 °C for 10 min.

Amplification products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide (1 μg/ml).

2.1.3. Cloning in pFastBac1

The pFastBac1™ donor vector (Invitrogen™) was used in a first cloning step. For cloning reactions, both the vector and the amplified cDNAs were digested with BamHI and HindIII restriction enzymes.

After overnight incubation at 16 °C, the ligation reaction was employed in the transformation of *E. coli* DH5α (Invitrogen™). Bacteria were grown on plates containing LB medium and ampicillin (100 μg/ml).

Twenty colonies were selected for growth in liquid Luria–Bertani (LB) containing ampicillin (100 μg/ml). For selection of colonies containing the recombinant donor plasmid, cultures were analyzed by PCR using the primers specific for the cDNA of the antiviral protein and other proteins. Agarose gel electrophoresis (1%) was performed to verify the amplified products.

To confirm that the insert was appropriately ligated into the cloning vector, clones screened by PCR and restriction enzyme digestion were also subjected to sequence analyses with primers Seq *Forward* pFastBac1TM (5′-AAATGATAACCATCTCGC-3′) and Seq *Reverse* pFastBac1TM (5′-CAAGCAGTGATCAGATCCAGACAT-3′). The cycle sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (code 4337456, Applied Biosystems). Capillary electrophoresis and sequence analyses were performed in an ABI 3730 DNA Analyzer (Applied Biosystems), using 36 cm capillaries loaded with the POP7polymer. Sequences were analyzed in the Sequencing Analysis 5.3.1 software.

2.1.4. Recombinant bacmid

After the generation of the pFastBac1™ construct (with the cDNA of the antiviral protein and of the other proteins), the purified plasmid DNAs were transformed into DH10Bac™ *E. coli* for transposition into the bacmid. Identification of the colonies containing the recombinant bacmid was based on blue/white colony selection.

2.1.5. Isolation and analysis of the recombinant bacmid

Extraction of bacmids was performed according to the Manufacturer's protocol (Bac-to-Bac® Baculovirus Expression System, Invitrogen). To verify the presence of the gene of interest after transposition, PCRs with M13 primers were used. The obtained amplicons were further sequenced using the pFastBac1™ primers for confirmation of the presence of the gene of interest in the bacmid after transposition.

2.1.6. Transfection of insect cells

Transfection of insect cells with the recombinant bacmid was performed according to the Bac-to-Bac® Baculovirus Expression System manual (Invitrogen™). Sf9 cells in the log phase (1.5–2.5×10^6 cells/ml, greater than 95% viability) were used in the experiment, using 500 ng of the recombinant baculovirus for transfection. Cell morphology was observed daily post infection for signs of viral infection. After 144 h, the supernatant was collected and considered as the first passage of the recombinant baculovirus.

To confirm the nucleotide sequence of the recombinant protein, a sample from a culture infected with a second pass was collected after 72 h. After extraction of DNA and RNA, PCR and RT-PCR were carried out respectively, as previously indicated.

DNA samples resulting from the PCR were subjected to nucleotide sequencing with the forward and reverse primers used for the amplification of the cDNAs.

The supernatant of all crops was collected daily for the determination of cell number, nutrient, titration of baculovirus and recombinant protein identification (data not shown). Western blot with anti-His antibody (GE Healthcare) and studies of cell morphology with photomicrographs were performed after each step.