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An effective and simplified DO-stat control strategy for production of rabies glycoprotein in *Pichia pastoris*

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ABSTRACT

The glycoprotein (G-protein) of rabies virus is responsible for viral attachment to the host cell surface and induces virus neutralization antibodies. In the present study, the G-protein gene of rabies virus CVS strain was cloned, sequenced and expressed in the yeast, Pichia pastoris, as a secreted protein, using a simplified DO-stat control feeding strategy. This strategy involves the addition of methanol when the dissolved oxygen (DO) level rises above the setpoint avoiding methanol accumulation and oxygen limitation. The G-protein expression was evaluated by SDS-PAGE, ELISA, and western blot assays. Like native G-protein, the recombinant G-protein was found reactive when it was challenged against specific antibodies. The data indicate that the recombinant G-protein can be easily expressed and isolated, and may be useful as a safe source in the production of diagnostic kits and subunit vaccines to prevent rabies.
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1. Introduction

Rabies is a serious disease which causes a fatal form of encephalomyelitis in humans and animals when treatment is not administered. The annual number of human deaths worldwide is estimated to be 59,000 [1]. Most of these occur in developing countries, where canine rabies is enzootic, and rabid dog bite is the most common mode of transmission [2]. The existence of large reservoirs of rabies virus in stray dogs and many wild animal species represents a serious problem for humans and domestic animals [3].

Although there is evidence that controlling dog rabies through vaccination programs and eliminating stray dogs can reduce the incidence of human rabies [4], exposure to rabid dogs causes more than 99% of human deaths by rabies [5].

The rabies virus belongs in the *Lyssavirus* genus within the *Rhabdoviridae* family. This virus has a single stranded ribonucleic acid (RNA) genome in the negative sense orientation which encodes five proteins in the following order: nucleoprotein, phosphoprotein, matrix protein, G-protein, and RNA dependent RNA polymerase [6,7]. The G-protein, the only protein exposed on the surface of the viral particle, is involved in the binding to the cellular receptors, as well as the entry of the viral particle into the host cell. Structure of the G-protein (a type I transmembrane protein) consists of a cytoplasmic domain, a transmembrane domain, and an ectodomain exposed as trimers at the virus surface [8,9].

The G-protein ectodomain stimulates neutralizing antibody production to protect vaccinated animals from rabies virus infection by inducing T lymphocytes and T helper cells [10–14]. Importantly, to stimulate immune protection by vaccination, glycosylation of the G-protein is needed [15–17]. Therefore, it is necessary to produce the G-protein in a eukaryotic host. In this way, we decided to express the rabies G-protein because it is the most antigenic and immunogenic protein in the rabies.

Vaccines are a useful tool in programs of health intervention. Production of rabies vaccines in cell cultures and in suckling mouse brains (also known as Fuenzalida-Palacios) [18] involves handling live virus and is expensive. Furthermore, Fuenzalida-Palacios vaccine often leads to neurological complications [19].

Yeasts have been successfully used to produce viral proteins [20,21]. The *Pichia pastoris (P. pastoris)* expression system was selected because it has several technical advantages, such as site-

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specific integration, high levels of protein expression, eukaryotic post-translational modifications [22], a good leader sequence for the secretion of recombinant protein in the medium, and it is relatively easy to scale-up.

The closed-loop feeding strategies rely on a measurement that is an indicator of the metabolic state of the culture. The dissolved oxygen (DO) concentration and pH, in microbial fermentation are also key indicators of cellular physiology. The DO-stat control of nutrient feeding is based on the concept of DO rises (due to a reduction or cessation of oxygen consumption or respiration) upon nutrient limitation or depletion. The DO-stat control maintains the culture at a stationary DO level (the DO setpoint) by increasing the nutrient feed rate when DO rises above the setpoint and reducing the nutrient feed rate when DO drops below the setpoint. The DO-stat strategy typically works well in defined media where nutrient depletion results in rapid DO rise.

In the present study, we describe the expression of the rabies G-protein in a Mut^+ strain of *P. Pastoris,* GS115, during growth on methanol under control of the strongly induced AOX1 promoter. We also report the optimization of culture conditions for G-protein expression by means of a DO-stat feeding strategy. The DO-stat control feeding strategy used in this work involves the addition of methanol when the dissolved oxygen (DO) level rises above the setpoint. Therefore, this method avoids the methanol accumulation and oxygen limitation.

2. Materials and methods

2.1. Strains, plasmids, and culture media

Challenge virus standard (CVS-11) strain of rabies virus. *Escherichia coli* TOP10′ strain, *P. pastoris* GS115 and pPIC9 expression vector from Invitrogen Co. (USA).

Yeast extract peptone media (YPD), buffered glycerol media (BMG), buffered methanol media (BMM), Luria Bertani plates containing 100 μg/ml ampicillin (LB-Amp), and minimal dextrose plates (MD) as described in Invitrogen *Pichia expression* kit (USA).

Synthetic medium (SM) containing glycerol 32 g/l; KH_2PO_4 20 g/l; $MgSO_4 \cdot 7H_2O$ 2 g/l; $CaCl_2 \cdot 2H_2O$ 0,3 g/l; $(NH_4)_2SO_4$ 15,7 g/l; 1 ml/l of 1000× vitamins solution (biotin 0.002 g/l; pantothenate calcium 0.4 g/l; acid folic 0.002 g/l; niacin 0.4 g/l; *p*-aminobenzoic acid 0.2 g/l; pyridoxine (HCl) 0.4 g/l; riboflavin 0.2 g/l; thiamine (HCl) 0.4 g/l; Inositol 2 g/l) and 1 ml/l of 1000× micronutrients solution (boric acid 0.05 g/l; copper sulphate 0.004 g/l; potassium iodide 0.01 g/l; ferric chloride 0.02 g/l; manganessium sulphate 0.04 g/l; sodium molybdate 0.02 g/l; zinc sulphate 0.04 g/l).

2.2. Amplification of rabies G-protein gene by RT-PCR

Viral RNA was isolated using TRIzol® (Invitrogen). Reverse transcription was carried out using 1 μg of RNA, random hexamer primers, RNAsin® (Promega), and Moloney Murine Leukemia virus reverse transcriptase (Promega, USA). PCR on the cDNA was performed with GRVF (5′ ATGTCGACAAAAGAATGGTTCCTCAGGCTCTCCTGTTTGTACC 3′) and GRVR (5′ ATGAATTCTCACAGTCCGGTCTCACCCCCGCTCTTG 3′) containing a *Sal*I and *Eco*RI sites, respectively. PCR was for 30 cycles (94 °C for 45″, 58 °C for 45″ and 72 °C for 1.5 min). Electrophoresis was in 1.5% agarose with ethidium bromide.

2.3. Generation of a recombinant P. pastoris

The purified RT-PCR product was digested with *Sal*I and *Eco*RI and ligated to the pPIC9 vector using *Xho*I and *Eco*RI restriction sites. *E.coli* TOP10′ competent cells were transformed by electroporation with the pPIC9 and the G-protein open reading frame (ORF) insert to create the pPIC9-G expression vector. Transformation was confirmed by colony-PCR using specific primers to the AOX1 region: 5′AOX1 (5′ GAC TGG TTC CAA TTG ACA AGC 3′) and 3′AOX1 (5′ GCA AAT GGC ATT CTG ACA TCC 3′).

For the electroporation, 10 μg of pPIC9-G plasmid DNA was digested in the AOX1 promoter with *Sac*I and used to transform 40 μl of the *P. pastoris* competent cells (1×10^9 cells/ml). Recombinant His^+ colonies were selected on MD plates (without histidine) for 48 h at 28 °C.

Colony-PCR was performed using the AOX1 primers. The PCR sequence was compared to the rabies G-protein sequence of the CVS strain (GenBank).

2.4. Small scale protein expression

PCR positive clones were cultured overnight at 28 °C, 100 rpm in 5 ml of YPD medium. Cells were collected by centrifugation (2000*g*, 15 min) and resuspended in 50 fml of BMG in a 250 ml Erlenmeyer flask for an additional 24 h. These cells were similarly resuspended in 10 ml of BMM in a 100 ml Erlenmeyer flask for growth under the same conditions for 72 h. Methanol was added every 24 h to a final concentration of 0.5% (v/v). Non-transformed *P. pastoris* was also cultured under similar conditions in BMG/BMM mediums supplemented with histidine, and used as a negative control. Supernatant samples were taken every 24 h (0, 24, 48 and 72) and then analyzed by SDS-PAGE and ELISA.

2.5. Detection of recombinant G-protein by ELISA

Culture supernatant protein from different time-points and cultures were coated in a 96-well plate by mixing 100 μl of sample with 100 μl of coating buffer (25 mM Na_2CO_3, $NaHCO_3$, pH 9.6) and incubated at 4 °C overnight. Then, the plate was blocked at 37 °C for 1 h with a blocking buffer containing 3% of skimmed milk powder in 0.05% PBS-Tween followed by three washings with 0.05% PBS-Tween.

As primary antibody, a 1:500 dilution of a monoclonal antibody to the rabies G-protein (Santa Cruz cat. sc-57995) was added and the samples were incubated at 37 °C for 1 h. The plate was washed three times. Then a 1:2000 dilution of an Anti-Mouse IgG – Peroxidase conjugated antibody produced in rabbit (Sigma cat. A9044) was added and the samples were incubated at 37 °C for 1 h. Finally, the plate was washed three times and treated with a solution containing 0.03% of ATBS and 0.02% of H_2O_2 for 1 h in the dark before measuring the absorbance at 405 nm.

2.6. High cell density fermentation

Batch and fed-batch cultures were performed in a 7.5 L BioFlo 310 Benchtop Bioreactor (New Brunswick-Scientific, Edison, NJ, USA).

A starter culture (150 ml) of a SM medium was inoculated with 5 ml of an overnight culture in YPD medium of the selected clone, and incubated at 28 °C overnight. 3.85 L of fresh SM in the fermentor was inoculated with 150 ml of a SM starter culture ($OD_{600nm} = 10$). An optical density of 1 unit corresponds to approximately 0,24 g/l of dry cell. The temperature was set at 28 °C, and the pH was adjusted to 5.

The propeller rate was set to increase to maintain the dissolved oxygen (DO) in 40% of the saturation value during the glycerol batch. Once glycerol depleted, a methanol feeding strategy was designed to maintain ~40% DO automatically with a controller regulating the methanol feed rate (BioCommand software, New Brunswick). For 48 h of methanol induction, the feed pump was set

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