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Heterologous Expression of CVS Rabies Virus Glycoprotein Gene in *Pichia pastoris*

S.N. Nagesha†*, P.H. Ramanjini Gowda†*, T.M. Ningaraju†, S.N. Madhusudana, N. Deepak†, H.M. Mahadeva Swamy†, K.N. Yogendra†, T. Nagaraju‡ and Anita S. Desai‡

†*Department of Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, Karnataka, India-560 065.*
‡ *Department of Neurovirology, NIMHANS, Bangalore, Karnataka, India-560 029.*
**Corresponding Author E-mail: ramanjini@yahoo.com, nagisn@gmail.com*

Abstract

The glycoprotein of rabies virus is most antigenic and immunogenic determinant present in rabies virion and can serve as effective target for development of vaccine. Hence the CVS rabies glycoprotein gene (1575 bp) was cloned into pBKS$^+$ plasmid vector which was characterized by PCR and by sequencing, further the rabies glycoprotein gene was sub cloned into yeast transfer vector (pPICZαA) for expression. Linearised recombinant vector containing glycoprotein gene was introduced into yeast (*Pichia pastoris*, GS115) by electrophoration and the recombinant yeast clones were identified by PCR analysis. The recombinant protein expressed in yeast at a yield of 200mg per liter was confirmed by SDS-PAGE, ELISA and western blot. These results allow us to conclude that *P.pastoris* is a convenient system for the efficient production of heterologous CVS rabies glycoprotein for using as recombinant vaccine.

Keywords: CVS rabies virus; Glycoprotein gene; *Pichia pastoris*; SDS-PAGE

Introduction

Rabies is an important disease, fatal in 100% of cases if no treatment is administered. The disease spreads through domestic and wildlife animals. Despite the existence of effective pre- and post-exposure treatments, the annual number of deaths worldwide caused by rabies is estimated to be 55,000 mostly in rural areas of Africa and Asia. In India, the annual incidence of human rabies is estimated to be 20,000. Based on expert group advice, an additional 20% was added to this to include paralytic/a typical form of rabies, providing an estimate of 20,565 or about 2 per one lakh population

(Sudarshan *et al*., 2007).The first rabies vaccine (Pasteur, 1885), consisted of subcutaneous inoculation of spinal cord suspension derived from rabid rabbits. Since then, improved vaccines have been developed (Perrin *et al*., 1990; Plotkin, 1993). Despite this, the disease continues to be a problem because it spreads through wildlife, and effective vaccines are unaffordable for animals and poorer sections of human population. The nerve tissue vaccine often leads to neurological complications (Swamy *et al*., 1984). It is desirable to develop safer, cheaper and efficacious vaccine against rabies.

Rabies virus belongs family, Rhabdoviridae, order, Mononegavirales and genus Lyssavirus. The primary structure of the 3' half of rabies viral genome, present in order, the genes encoding for (+) leader RNA, the nucleoprotein 'N', the phosphoprotein 'M_1', the matrix protein 'M_2', the glycoprotein 'G' and the beginning of the larger protein 'L' which is an essential component of the rabies virus RNA dependent RNA polymerase (Kawai, 1977). The native G protein is 524 amino acids long, consisting of 19-residue long signal peptide at N-terminus ((Anilionis *et al*., 1981), a 22 amino acid transmembrane domain and a 44 amino acid cytoplasmic domain (Fishbein and Robinson, 1993; Wunner *et al*., 1988). Glycosylation of the G protein is required for immune protection by the rabies vaccines (Foley *et al*., 2000). The recombinant rabies virus glycoprotein (rRVGP) has been produced in several expressions systems: [transgenic plants (Ashraf *et al*., 2005), yeast cells (Sakamoto *et al*., 1999), mammalian cells (Kankanamage *et al*., 2003), and insect Sf9 cells infected with Baculovirus (Drings *et al*., 1999)].

Vaccines are primary tools in programmes of health intervention for both humans and animals (Varshney *et al*., 2000). Available vaccines for rabies are costly and involve risk of handling live viruses, Hence developments of safe, efficacious and inexpensive oral vaccines are desired. The ideal approach would be to produce glycoprotein of CVS rabies virus with proper folding, glycosylation and use as a vaccine. This presumption and other success report on yeast expressed recombinant Polio virus empty Capsid (Rombaut and Jore 1977) Bovine herpes virus-1 (Zhu, 1997) and Dengue virus particle (Surgue *et al*., 1997) has encouraged us to select yeast, to express the Rabies glycoprotein gene. Yeast system has the technical advantages of site specific integration, high amount of protein expression, leader sequence for the secretion of heterologous protein in the medium which can be purified easily (Hollenberg and Gellissen 1997), less sophistication for the maintenance of cells and easy to scale up using large scale fermentation system.

Materials and methods

Isolation of CVS rabies glycoprotein gene

CVS Rabies virus (Challenge Virus Standard) was propagated in infected mouse brain was obtained from NIMHANS (National Institute for Mental Health and Neurosciences, Bangalore, India) and used in the present study, full length 1574 bp Rabies glycoprotein gene was isolated from the virus using the following gene specific primers employing reverse transcription polymerase chain reaction (RT-PCR).

Forward primer (*Kpn* I site underlined): 5' TGA AGG TAC CCC TCA AAA GAC TCA AGG A 3' (Base pairs: -31 to -4, NCBI accession number: NC_001542.1)

Reverse primer (*Not* I site, underlined):5' CTC GGC GGC CGC GTG ATG GTG ATG GTG ATG CAG TCC GGT CTC ACC 3' (Base pairs: +1557 to +1601)

Total RNA from infected mouse brain was extracted using Trizol reagent (Sigma, USA) method and was further subjected to cDNA synthesis at 42°C for 60 min using Murine Moloney LeukemiaVirus (M-MLV) reverse transcriptase enzyme with gene specific primers (MWG, India). The cDNA was amplified through PCR employing the same primers. PCR was carried out under the following conditions: 94°C for 30 s, 45°C for 45 s, 72°C for 60 s, for 30 cycles, and finally 72°C for 10 min.

Construction of recombinant pPICZalphaA with CVS rabies glycoprotein secretion expression vector

Bacteria, Yeast, and growth media

The *E.coli* DH5 alpha strain (Invitrogen,USA) used as host for DNA manipulation and were cultured in Luria Bertani medium (Himedia, India) that was supplemented with 50 µg/ml ampicillin for the selection of transformants. The *P. pastoris* GS115 strain (Invitrogen,USA) was cultured in YPD medium (1% yeast extract,2% peptone, 2% dextrose plus 2% agar in plates). Since GS115 has a mutant allele of the HIS4 (*P. pastoris* histidinol dehydrogenase gene) and AOX I gene, it is a histidine auxotroph with a methanol utilization slow (Muts) phenotype. The CVS rabies glycoprotein gene was cloned into $pBKS^+$ plasmid vector (Novagen,USA) at Kpn I and Not I sites and the recombinant vector was identified by PCR and sequencing (MWG,India). The gene was further sub cloned into pPICZalphaA yeast transfer vector at Kpn I and Not I sites under the control of AOX I promoter with *Saccharomyces cerevisiae* alpha factor secretion signal. Use of Kpn I and Not I recognition sequences in primers facilitated the insertion of CVS rabies glycoprotein gene into pPICZalphaA yeast transfer vector. The alpha secretory signal present in the vector upstream to the rabies glycoprotein gene was used to make the target protein to secrete into the medium. The recombinant vector was mobilized into *E.coli* DH5 alpha cells using $CaCl_2$ competent cells. The positive bacterial transformants were selected through restriction digestion of plasmid DNA using Kpn I and Not I enzymes and PCR analysis.

Transformation of yeast with pPICZalphaA containing CVS rabies glycoprotein gene and screening of Pichia pastoris expression strains through PCR.

Approximately 10 µg of recombinant expression plasmid pPICZalphaA containing CVS rabies glycoprotein gene was linearized by digesting with Sac I enzyme to get transformants in *P. pastoris* GS115 cells (Invitrogen) and used to transform competent *P. pastoris* cells by electroporation using Eppendorf electroporator at 1,500 V. After transformation, cells were plated on YPDSA plates (1% Yeast extract, 2% Peptone, 2% Dextrose, 1M Sorbitol and 2% Agar) with Zeocin (100µg/ml) and

incubated at 30°C for 3 days until colonies appeared. The parent pPICZalphaA without insert, linearized with Sac I was also transformed as negative control. The colonies obtained were restreaked on fresh YPDSA plates. Transformants bearing the chromosomally integrated copies of the pPICZalphaA containing CVS rabies glycoprotein gene were then detected by a genomic PCR assay using the gene specific primers. Genomic DNA extracted from putative positive colonies was subjected to PCR along with appropriate control samples as described earlier.

Purification of the target rabies glycoprotein

In order to achieve the purification and concentration of the secreted proteins for further use, Single colony of yeast transformant showing high level of secreted expression was cultured under optimal expression conditions and the culture supernatant was collected after methanol induction. The polyhistidine containing recombinant protein was purified from the secreted media by Affinity column containing the ProBond™ resin (Invitrogen, USA) using polyhistidine tag from the fusion protein. The final concentration of the protein in the purified solution was estimated by The Lowry's method utilizing the Folin-Ciocalteau reagent.

Analysis of the expressed products through SDS-PAGE and confirmation of the expressed pPICZalphaA containing CVS rabies glycoprotein through Western blot and ELISA

The expressed purified protein samples were separated by electrophoresis on a 12% denaturing sodium dodecyl sulfate polyacrylamide gel and also SDS-PAGE gel was stained with silver stain as per Sambrook *et al*., 2001. The secretary expression of rabies glycoprotein was confirmed with Rabies-positive serum through Western blot and Enzyme Linked Immuno Sorbent Assay (ELISA). Briefly, proteins were transferred from the gel onto nitrocellulose membrane. After transfer, the membrane were probed with 1:400 dilution of the rabies glycoprotein specific monoclonal antibody raised in rabbit (obtained from NIMHANS, Bengaluru) and 1:1,000 dilution of horse radish peroxidase labeled goat anti-mouse immunoglobulin G (IgG). Protein samples were visualized with ODD/H2O2 (O-Dianisidine dihydrochloride-hydrogen peroxide) chromogen substrate solution. Further the proteins present in the culture supernatants of cloned yeast cells with or without insert was detected by the ELISA using rabies positive serum against rabies glycoprotein and with goat anti-mose IgG HRPO.

Results

Isolation of CVS rabies glycoprotein gene and construction of pPICZalphaA containing CVS rabies glycoprotein recombinant vector

The 1575 bp full length gene of rabies glycoprotein gene was isolated through RT-PCR. The gene was first cloned and confirmed in $pBKS^{+}$ plasmid vector and then the

full length gene nucleotide sequence was submitted to NCBI genebank (Accession No. GQ233040). The gene was further sub cloned into pPICZalphaA yeast transfer vector at Kpn I and Not I sites and the recombinant construct; pPICZalphaA containing CVS rabies glycoprotein gene was mobilized into *E.coli* DH5 alpha for its mass production before transforming in Pichia. Transformation and selection of recombinant Pichia clones carrying foreign gene was confirmed through PCR and the recombination efficiency of Pichia through electroporation was 2x 10^2/ µg of linearized plasmid DNA. Seven out of twelve genomic DNA samples isolated from randomly selected yeast transformants amplified the expected 1575 bp DNA along with known positive control whereas negative sample did not show any bands (Fig. 1).

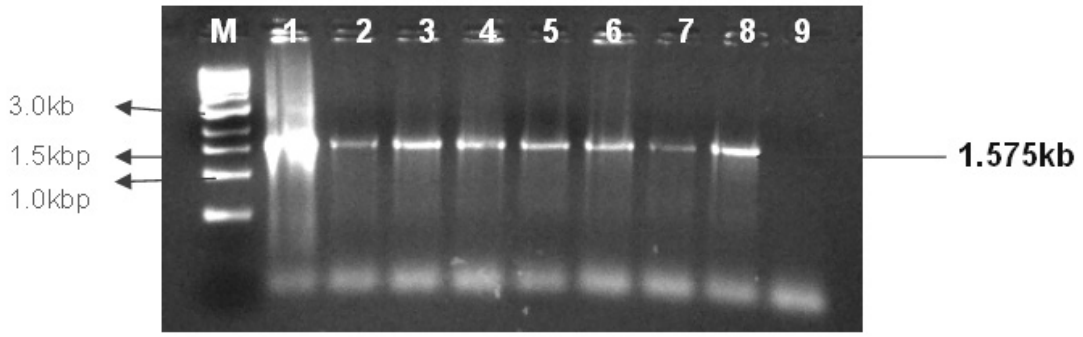

Figure 1: PCR amplified products from Pichia colonies positive for Rabies Glycoprotein gene insert.

M: DNA Molecular marker (NEB).1: Positive Control 2 to 8: Positive Pichia colonies containing Rabies Glycoprotein gene insert. 9: Negative colony without insert.

Analysis of the expressed product through SDS-PAGE

Four colonies that were found positive with PCR were selected for inducing the expression of the target gene. In order to study the expression of rabies glycoprotein gene fraction, the optimal method and growth conditions necessary for expression were standardized. All the four clones showed expression of the expected 70 kDa (Rai *et al.,* 2006) protein after 108 hr post induction (Fig. 2A), whereas there was no specific protein band detected in pPICZalphaA vector transformed yeast at that range. The protein bands were clearly visible only in case of the sample that was incubated for 108 hrs and no clear bands were observed before or after 108 hrs of incubation. Studies conducted to scale up the expression level with increased induction period and methanol concentration revealed that 108 hrs of post induction incubation period with 0.5% methanol concentration is ideal for large amount of rabies glycoprotein expression (200 mg/L of the culture supernatant). However, the level of expression could not be further boosted above this scale with either increased methanol concentration or increased duration of incubation.

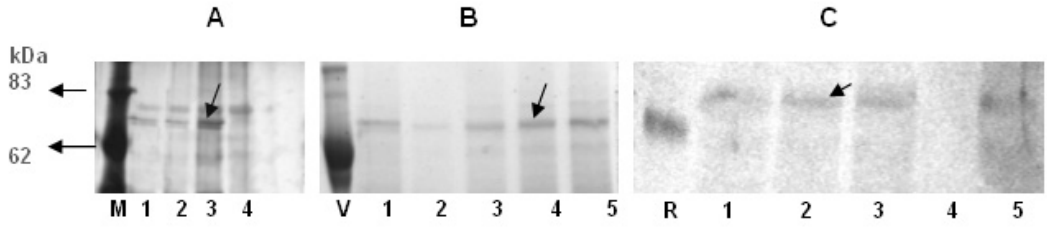

Figure 2: Electrophoretical analysis of recombinant CVS rabies glycoprotein produced in *P.Pastoris*.

A. Silver staining of recombinant rabies glycoprotein.

M: Standard protein molecular weight marker (NEB). 1, 2,3: Culture supernatant of yeast cells carrying vector with Rabies Glycoprotein gene insert. 4: Culture supernatant of yeast cells carrying vector without insert.

B. SDS-PAGE of purified recombinant rabies glycoprotein

V: Crude animal vaccine (IAH&VB, Bangalore) 1, 2, 3, 4, 5: Purified proteins from culture supernatant of yeast cells carrying vector with Rabies Glycoprotein gene insert

C. Western blot analysis of purified recombinant rabies glycoprotein R: Positive control (Rabipur human vaccine).1, 2, 3, 5: Purified proteins from culture supernatant of yeast cells carrying vector with Rabies Glycoprotein gene insert. 4: Culture supernatant of yeast cells carrying vector without insert.

Western blot assay for the expressed protein

Purified protein from the positive transformant that showed expected band of 70 KDa (Rai *et al.*, 2006) through SDS-PAGE (Fig.2B) was analyzed through Western blotting to confirm the specificity of the expressed protein. Appropriate positive signal was obtained in case of the positive transformant, whereas the protein sample transferred from Pichia transformed with pPICZalphaA negative control did not develop any signal on the membrane (Fig. 2C). The culture supernatants of positive Pichia colonies confirmed the presence of recombinant protein through ELISA (Fig.3).

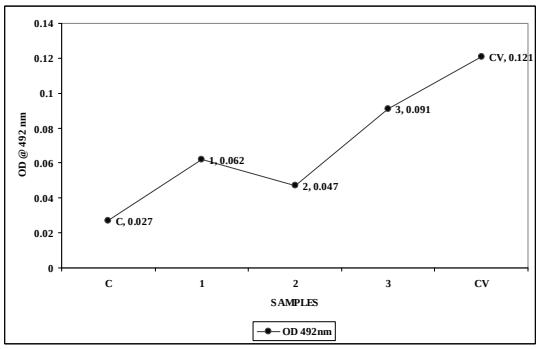

Figure 3: ELISA for recombinant CVS rabies virus glycoprotein produced in yeast supernatant.

C: Control Yeast supernatant without glycoprotein gene 1 to 3: Yeast supernatant with rabies virus glycoprotein gene product CV: Commercial vaccine (Rabipur)

Discussion

Since most therapeutically relevant glycoproteins require glycosylation, mammalian cell lines are preferred. However, yeast demonstrates better protein titres; shorter fermentation times and can grow in chemically defined media. However, recombinant glycoproteins expressed from Pichia Pastoris have high mannose Noligosaccharides which have reduced half life (Gemmill *et al*., 1999).The rabies virus glycoprotein alone is sufficient to induce protective immunity (Wiktor *et al*., 1973). Purified glycosylated rabiesGprotein derived from eukaryotic cells is effective as a vaccine in animal models (Kieny *et al*., 1984). The nonglycosylated G protein prepared from bacterial cells does not give protective immunity (Yelverton *et al*., 1983; Lathe *et al*., 1984; Malek *et al*., 1984). The G protein expressed in yeast is glycosylated. Yet, it gives protection only against intramuscular and not intra cerebral virus challenge (Klepfer et al., 1993), apparently due to differences in yeast-based glycosylations. Eukaryotic expression systems, which have the potential to express the protein in the native confirmation has been tried. The yeast based expression system is unique in that it combines the advantages of both prokaryotic (high expression levels, easy scale up, inexpensive growth media) and eukaryotic (capacity to carry out most of the posttranslational modifications) expression systems. In recent years, the methylotrophic yeast, *Pichia pastoris* has emerged as a powerful and inexpensive heterologous expression system for the production of high levels of functionally active recombinant proteins. From an expression point of view, the existence of well established fermentation methods, that can generate very high cell densities using purely defined media (Wegner 1983) and the strong, tightly regulated methanol inducible alcohol oxidase (AOX1) promoter (Tschopp *et al*., 1987), make *Pichia pastoris* a very valuable host for heterologous protein expression. This presumption and other success reports on yeast expressed recombinant Polio (Rombaut and Jore 1997), Bovine herpes virus-1(Zhu *et al*., 1997) and Dengue virus (Surgue *et al*., 1997) vaccines have prompted us to evaluate *Pichia pastoris* as a host to express the Glycoprotein of CVS rabies virus for using as vaccine. Therefore, the present study was undertaken to clone and express the glycoprotein gene coding for transmembrane glycoprotein of CVS rabies virus in *Pichia pastoris* as secretary products and use the expressed protein as immunogen in experimental animals.

Four positive colonies were selected for induction, all the colonies showed expression of the target protein. SDS-PAGE and Western blotting analysis showed that a specific protein, whose molecular weight is approximately 70 kDa (Rai *et al*., 2006), is expressed in *P. pastoris* and the expressed protein can react with positive rabies glycoprotein serum. Gene expression was seen even after six passages in the medium, indicating good genetic stability of the introduced gene fragment within the recombinant yeast system. In order to increase the expression level, the expression conditions were optimized. Under the optimal condition, the expressed protein amounted to about 200 mg/L of the culture. The protein bands were clearly visible

only in case of the samples that were incubated for 108 hrs and no clear bands were observed before or after 108 hrs of incubation. This could be due to the critical incubation period, i.e., 108 hrs required for biomass accumulation and no bands were visible after 120 hrs of incubation indicating that host-specific proteases may be acting on the protein following prolonged incubation. The observed low level of expression may be partly attributed to the low copy number of the genes integrated within the yeast genome and partly to the nature of the protein (Romanos *et al*., 1991) The most important parameter for efficient expression in Pichia was found to be adequate aeration during methanol induction and hence the culture volume within the flask was kept as low as 20% of the total flask volume. It was also necessary to maintain the incubation temperature at 30°C with rotation of 250–300 rpm. With the use of fomenter and codon optimization of rabies glycoprotein gene, the expression level can be further improved. However, the recombinant protein expressed may have potential applications in developing serotype specific diagnostics and as a recombinant vaccine which needs further studies using adjuvant along with the antigen for better immunogenesity. The CVS rabies transmembrane glycoprotein has been expressed in an alien expression system namely the yeast, *Pichia pastoris*. Our studies indicate that Pichia pastoris can be an excellent alternative for large scale production of this recombinant protein. The pPICZalphaA with glycoprotein recombinant vector constructed in the present study is suitable for secretory expression of the target protein and can be employed for commercial production of recombinant glycoprotein protein for further clinical studies like vaccination with adjuvant and also for developing diagnostic kit.

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