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ORIGINAL ARTICLE

Cloning and Expression of Recombinant Tick-Borne Encephalitis Virus-like Particles in *Pichia pastoris*

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Abstract
Objectives: The purpose of this study was to verify the feasibility of using the glyceraldehyde-3-phosphate dehydrogenase (GAP) promotor based *Pichia pastoris* expression system to produce tick-borne encephalitis virus (TBEV) virus-like particles (VLPs).
Methods: The complementary DNA encoding the TBEV prM signal peptide, prM, and E proteins of TBEV Korean strain (KrM 93) was cloned into the plasmid vector pGAPZαA, then integrated into the genome of *P. pastoris*, under the control of the GAP promoter. Expression of TBEV VLPs was determined by Western blotting using monoclonal antibody against TBEV envelope (E) protein.
Results: Recombinant TBEV VLPs consisting of prM and E protein were successfully expressed using the GAP promoter-based *P. pastoris* expression system. The results of Western blotting showed that the recombinant proteins were secreted into the culture supernatant from the *P. pastoris* and glycosylated.
Conclusion: This study suggests that recombinant TBEV VLPs from *P. pastoris* offer a promising approach to the production of VLPs for use as vaccines and diagnostic antigens.

1. Introduction

Tick-borne encephalitis virus (TBEV) belongs to the *Flavivirus* genus of the *Flaviviridae* family and can cause fatal encephalitis in humans in Europe, Russia, and Far East Asia [1,2]. In South Korea, TBEV was first isolated from wild rodents in 2006 [3]. The flavivirus genome contains a single, long, open reading frame that encodes a polyprotein, which is cleaved into three structural proteins, i.e., the capsid (C), premembrane (prM) and envelope (E) proteins, and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [4]. In flaviviruses, the prM and E proteins play crucial roles in the assembly and secretion of the virions [5–8]. Several studies have demonstrated that flavivirus virus-like particles (VLPs), consisting of

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the prM and E proteins, have been shown to be similar to the native virions in the structural and functional features for infection [5,9−12]. Thus, flavivirus VLPs can be substituted for native virions in investigations into the biological features of flavivirus, such as vaccine study for the prevention of flavivirus-induced diseases.

Until recently, various expression systems, including mammalian cells and insect cells, have been used to produce TBEV VLPs as antigens [10,13,14].

Pichia pastoris is one of the most widely used systems for producing recombinant protein by heterologous expression [15]. This system offers several advantages in comparison with other eukaryotic expression systems, such as the production of large-scale target proteins in their native conformation and cost-efficiency, and the proven safety of yeast-expressed VLPs vaccines such as hepatitis B virus VLPs [16] and human papillomavirus VLPs [17]. For this reason, the *P. pastoris* expression system has been used for the production of flavivirus proteins including VLPs [18−21]. However, there are no reports on the production of TBEV VLPs from *P. pastoris*. In the current study, we investigated the expression of TBEV VLPs using the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter-based *P. pastoris* expression system.

To our knowledge, this is the first report on the successful expression of TBEV VLPs in *P. pastoris*.

2. Materials and methods

2.1. Viruses and cells

The TBEV Korean isolate, KrM 93 strain (GenBank accession No. HM535611), was propagated in the brains of suckling mice and BHK-21 cell as described previously [22]. The infected cell culture medium was used for RNA extraction.

2.2. Yeast strain and plasmid vector

The *P. pastoris* host strain X33 (Invitrogen, Carlsbad, CA, USA) was used as the expression host in this study. The expression vector pGAPZαA (Invitrogen) contains the selectable marker Zeocin (Invitrogen), which is bifunctional in both *P. pastoris* and *Escherichia coli*, the GAP promoter, and the alcohol oxidase I (*AOX1*) transcription termination regions. *E. coli* transformants were selected on low salt Luria-Bertani agar plates containing 25 μg/mL Zeocin. *P. pastoris* transformants were selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol, 2% agar, and 100 μg/mL Zeocin) and *P. pastoris* liquid cell cultures were grown in YPD broth (1% yeast extract, 2% peptone, and 2% dextrose) with Zeocin.

2.3. Construction of recombinant expression vector

The coding sequence for the signal peptide of prM, prM, and E proteins was amplified from KrM 93 strain viral RNA by reverse transcription-polymerase chain reaction (RT-PCR) using SuperScript III First-strand synthesis system for RT-PCR (Invitrogen) and *Ex Taq* DNA polymerase (Takara, Shiga, Japan) according to the manufacturer's instructions, and the following primers: KrM93SS-F 5′-GAC **TTC GAA** *ATG* GTT GGC TTG CAA AAA-3′ (*Bst* BI site in bold and start codon in italics) and KrM93E-R 5′-GAA **TCT AGA** GCT GCC CCC ACT CCA AGG-3′ (*Xba* I site in bold). The PCR product (named as 93prM-E) was first cloned into pCR 2.1-TOPO plasmid (Invitrogen) and then subcloned into pGAPZαA following enzymatic digestion using *Bst* BI and *Xba* I (New England BioLabs Inc., Beverley, MA, USA) to construct the full-length 93prM-E clone downstream of GAP promoter, which was designated as pGAPZαA/93prM-E (Figure 1A). The plasmid inserts were confirmed by DNA sequencing.

2.4. Yeast transformation

The plasmid pGAPZαA/93prM-E was linearized with *Bgl* II (New England BioLabs) and transformed into *P. pastoris* X33 using *Pichia* EasyComp Kit (Invitrogen) according to the manufacturer's instructions. The transformed yeast cells were incubated in YPDS agar containing 100 μg/mL Zeocin at 30°e for 3−4 days. Zeocin-resistant yeast colonies were selected and identification of the insert in these Zeocin-resistant transformants was checked by colony PCR.

2.5. Expression of recombinant TBEV E protein in *P. pastoris*

Positive transformants, selected as described previously, were inoculated in 5 mL YPD broth with 100 μg/mL Zeocin with shaking (250 rpm) at 30°C overnight. These cultures were transferred to 500 mL of YPD broth with Zeocin with shaking (250 rpm) at 30°C for 48 hours. The culture supernatant and cell pellet were collected by centrifugation at 10,000 × *g* for 10 minutes. The cell pellet was disrupted using Yeast PE LB (G-Biosciences, St. Louis, MO, USA) according to the manufacturer's instructions. The lysate was clarified by centrifugation at 20,000 × *g* for 30 minutes at 4°C. The culture supernatant was precipitated by using Amicon Ultra-15 Centrifugal Filter Units with 30 kDa membrane (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis

The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a reduced 4−12% Bis-Tris Gel (Invitrogen). Separated protein bands were transferred onto a 0.45-μm polyvinylidene difluoride membrane using the iBlot system (Invitrogen) according to the manufacturer's instructions. The membrane was blocked with 5% skim milk in Tris buffered saline with 0.05%