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Expression, purification and characterization of two truncated peste des petits ruminants virus matrix proteins in *Escherichia coli*, and production of polyclonal antibodies against this protein

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ABSTRACT

Peste des petits ruminants virus (PPRV), the etiological agent of peste des petits ruminants, is classified into the genus *Morbillivirus* in the family *Paramyxoviridae*. The PPRV matrix (M) gene is composed of 1483 base pairs, encoding a 335 amino acids M protein with a molecular weight of approximately 38 kD. We have demonstrated previously that the full-length M protein was expressed at an extremely low level or not even expressed in *Escherichia coli* BL21 (DE3). In this study, the M protein was split into two truncated forms to be successfully expressed in *E. coli* at a high level using the pET30a (+) vector, respectively, by analysis of SDS-PAGE, western blot and MALDI-TOF-MS. The optimization of culture conditions led us to perform the recombinant protein induction with 0.2 mM IPTG at 28 °C for 12 h, whereby both proteins nevertheless were expressed in the insoluble form. Therefore, both His-tagged proteins were purified under the denaturing condition using a commercially available kit. Balb/c mice were immunized with the complex of purified proteins and then effectively produced polyclonal antibodies, which reached to a relatively high titer by the analysis of ELISA. The specificity of the prepared polyclonal antibodies was checked by western blot and immunofluorescence, revealing them with the desirable specificity against both non-denatured and denatured M proteins.

Introduction

Peste des petits ruminants (PPR)[2] is an acute or subacute, highly contagious and economically important viral disease of small ruminants, characterized by high fever, oculonasal discharges, pneumonia, stomatitis, and inflammation of gastrointestinal tract leading to severe diarrhea of infected animals [1–3]. Peste des petits ruminants virus (PPRV), the etiological agent of PPR, is classified into the genus *Morbillivirus* in the family *Paramyxoviridae* [4], as its biological and physicochemical features are closely related to the other morbilliviruses such as measles virus, canine distemper virus and especially rinderpest virus [5]. Structurally, PPRVs are morphologically pleomorphic particles (400–500 nm) enveloped by lipid membrane with viral glycoproteins seen as peplomers protruding from the envelope. The matrix (M) protein lies beneath the virion envelope, and interacts with the internal nucleocapsid and the cytoplasmic tails of the surface glycoproteins to form new virus particles [6–10]. In other words, the M protein serves as a bridge between the external surface viral proteins and the internal nucleocapsid, and it is believed to play a key role in the budding of virions from the infected cell surface [11].

The full genomes of several PPRV isolates [12] and vaccine strains [13] have been sequenced, showing that the full-length of their genomes was up to 15,948 nucleotides and consisted of single strand of negative sense RNA. The full-length M gene is composed of 1483 base pairs (bps), encoding a 335 amino acid (aa) M protein with a molecular weight of approximately 38 kD. We have demonstrated previously that the full-length M protein was expressed at an extremely low level or not even expressed in *Escherichia coli* BL21 (DE3) (date not shown), despite optimization of expression conditions. The extremely low yield of the full-length M protein would be attributed to many factors, such as the codon bias, mRNA secondary structure and over-length fragment. Alternatively, we herein attempted to express two truncated M proteins rather than the full-length one. Fortunately, polyclonal antibodies produced by immunization of Balb/c mice with the truncated proteins have proven to be specific against the M protein.

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[2] *Abbreviations used*: PPR, peste des petits ruminants; PPRV, peste des petits ruminants virus; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline-Tween 20; DAB, diaminobenzidine; PMFs, peptide mass fingerprintings; ELISA, enzyme-linked immunosorbent assay; RSM, response surface methodology.

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Materials and methods

Construction of two expression plasmids

The original M gene of PPRV China/tibet/geg/07-30 strain [14] was obtained from NCBI (GenBank Access No. FJ905304.1). The M protein would be split into two truncated forms to be expressed in *E. coli*, respectively. The split site was located in a weakly antigenic region (Fig. 1A) of M protein by analysis with the Lasergene Protean software. The first truncated protein (M1) contained 160 aa, ranging from the 1st to the 160th aa of M protein; the second one (M2) contained 175 aa, ranging from the 161st to the 335th aa of M protein.

Two DNA fragments corresponding to the M1 and M2 were amplified respectively from a previously constructed plasmid containing the M gene using the polymerase chain reaction (PCR) with *Pyrobest*™ DNA polymerase (Takara), according to the recommended procedure: 94 °C for 3 min, 30 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 75 s) and a final extension at 72 °C for 10 min. The primer 1/primer 2 (Table 1) and primer 3/primer 4 (Table 1) were used for the amplification of both fragments, respectively. Two PCR-amplified products were sequentially subjected to analysis with electrophoresis on a 1.5% agarose gel (Biowest), to double digestion with KpnI/XhoI restriction enzymes (NEB) and finally to subclone into the pET30a (+) expression vector (Novagen) respectively using standard molecular biology techniques [15]. The final constructs were named as pET30a-M1 and pET30a-M2, which were transformed into *E. coli* BL21 (DE3) competent cells (Life technologies), plated on Luria Broth (LB) agar containing 50 μg/mL kanamycin and incubated at 37 °C overnight. The following day, single colonies were used to inoculate 5 mL of LB liquid media containing 50 μg/mL kanamycin in culture tubes and grown overnight at 37 °C with shaking at 200 rpm, followed by sequencing using the primer 5/primer 6 (Table 1).

Small scale expression

The *E. coli* BL21 (DE3) transformants were cultured in 4 mL LB liquid medium containing kanamycin (50 μg/mL) overnight at 37 °C, shaking at 200 rpm. Fresh LB liquid medium (5 mL) containing kanamycin (50 μg/mL) was incubated with 50 μL overnight pre-cultures until optical density 600 nm (OD_{600nm}) reached 0.5. The expression of both His-tagged M1 (His-M1) (Fig. 1B) and M2 (His-M2) (Fig. 1C) fusion proteins was induced by 0.4 mM isopropyl-b-D-thiogalactopyranoside (IPTG) (Takara) for 10 h at 28 °C, shaking at 250 rpm. Subsequently, *E. coli* cells were harvested by centrifugation at 8000*g* for 5 min at 4 °C. The pellets were resuspended in phosphate-buffered saline (PBS). The cell suspensions were mixed with 2×sample loading buffer (Takara) heated at 100 °C for 10 min. The denatured samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 12% polyacrylamide gels with a protein ladder (Thermo) followed by Coomassie brilliant blue staining and analysis of western blot. The uninduced control cultures were analyzed in parallel.

For western blot, proteins were transferred onto a 0.45 μm polyvinylidene difluoride membrane (Roche) using a semi-dry transfer apparatus (Bio-Rad). The membrane subsequently was blocked in 5% (w/v) skim milk powder shaking at room temperature for 2 h, followed by shaking incubation for 2 h at room temperature with mouse monoclonal anti-polyHistidine antibody (Sigma) (v/v: 1/10,000). The primary antibody-bound membrane was washed 3 times, 10 min each time, with Tris-buffered saline-Tween 20 (TBST) and then incubated at room temperature for 1 h with peroxidase-conjugated rabbit anti-mouse IgG (working dilution of 1:20,000, Sigma). The secondary antibody-bound membrane was washed 3 times with TBST for 30 min followed by incubation with the diaminobenzidine (DAB) solution (Thermo) for 5 min at room temperature. The reaction was stopped with ultra pure water and the resulting membrane was dried prior to photography.

Optimization of expression conditions

The temperature, IPTG concentration and induction time for the expression of foreign proteins in *E. coli* BL21 (DE3) were optimized [16]. In short, when OD_{600nm} of the liquid culture reached 0.5 after vigorous shaking at 37 °C, the culture was divided into different tubes induced with IPTG under different temperature conditions (18, 21, 24 and 28 °C) for additional 12 h for temperature optimization. Under such a condition of optimized temperature, IPTG was added to each fresh subculture (OD_{600nm} = 0.5) with different final concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mM) to be incubated for an additional 10 h for optimization of IPTG concentration. For optimization of induction time, subcultures (OD_{600nm} = 0.5) were incubated for an additional times (0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 h) under optimal conditions of IPTG concentration and induction temperature. All of liquid subcultures were collected and then mixed with 2×sample loading buffer (v/v: 1/1) heated at 100 °C for 10 min for analysis of SDS–PAGE to choose optimal culture parameters.

Solubility testing

High-level expression of foreign proteins in *E. coli* is often responsible for accumulating them as insoluble inclusion bodies [17–19]. Sonication of proteins can also lead to formation of

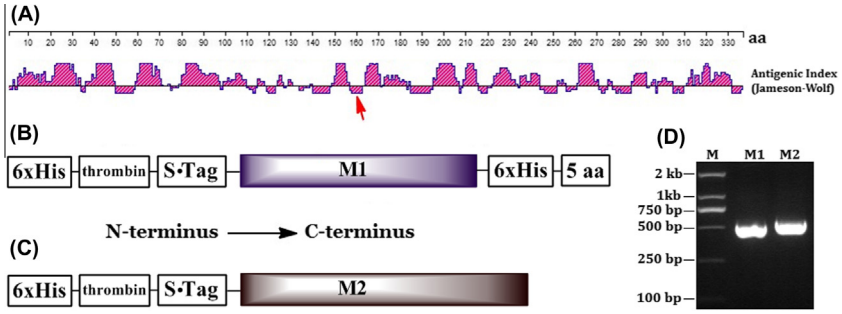

Fig. 1. Construction of two recombinant expression vectors. The antigenic index of PPRV M protein according to the Jameson-Wolf method using the Lasergene Protean software (A). A weakly antigenic region indicated by a red arrow may be split to express two truncated M protein nearly without affection on its antigenicity. Schematic diagrams of two His-tagged fusion truncated M proteins, namely His-M1 (B) and His-M2 (C). The proportions of both (B) and (C) do not actually match them. aa, amino acid. Two DNA fragments corresponding to the M1 and M2 amplified by the PCR, respectively (D). Lane M, 2,000 DNA marker (Takara); lane M1, M1 fragment; lane M2, M2 fragment.