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Purification and assembling a fused capsid protein as an enterovirus 71 vaccine candidate from inclusion bodies to pentamer-based nanoparticles

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ABSTRACT

An efficient preparation process for a novel Enterovirus 71 (EV71) vaccine was developed in this paper, which is a fused antigen by connecting the truncated capsid proteins of VP1, VP2 and VP3 into one molecule through flexible peptide linkers and expressed in *E. coli* as inclusion bodies. The fused protein was purified at denatured state through two-step ion exchange chromatography, with final purity above 95%, host cell proteins below 0.003% and residual DNA less than 50 ng/mL. During the following refolding and assembling process through dilution, the fused antigen precipitated completely, while the precipitation was efficiently inhibited with 2 M urea or 0.5 M arginine as an additive. Size exclusion chromatography analysis indicated the protein formed soluble aggregates with linear or rod-like appearance in transmission electron microscopy. These aggregates transformed into pentamers with a size of 15 nm at pH 8.0 after the additive removing. Moreover, most of the pentamers assembled as sphere-like particulates about 25–40 nm after being induced by calcium chloride. High antigen-specific IgG titer was elicited by immunization with the nanoparticles in mouse model. Splenocytes proliferative responses and cytokines analysis indicated this particulate antigen could induce humoral and cellular immune responses. These results lay foundations for developing the fused antigen as an alternative vaccine against hand-foot-and-mouth disease (HFMD) and for the large-scale production for *E.coli*-based vaccines.

1. Introduction

Human enterovirus 71 (EV71) is one of the main causative pathogens for hand-foot-and-mouth disease (HFMD) which often infects infants and children under 5 years old [1,2]. Since it was first found in 1969, it has caused several HFMD outbreaks in Asian countries and resulted in severe morbidity and mortality [1,3,4]. Although some compounds could be used to cure HFMD, prophylactic vaccine is believed as the most effective way to eradicate this disease [5,6].

Until now, a number of EV71 vaccine candidates have been developed at different stages. Among these candidates, traditional inactivated EV71 virus was researched most extensively and just received the license to be used in clinic in China. Inactivated virus was reported to exhibit almost 100% protective efficacy, however, low productivity and safety concerns still prompt scientists to develop other vaccine substitutes [7]. Through epitope mapping analysis, some vital antigen epitopes of EV71 were identified, some of them were synthesized to be explored as vaccine candidates, such as SP55 and SP70, but these subunit vaccines often have low immune responses [8,9]. With repeated antigens on the surface but no infective genetic materials inside, recombinant virus-like particles (VLPs) represent a safer and even more effective vaccine platform [10–13]. Several EV71-derived VLPs were prepared by infecting Sf9 insect cells with a recombinant baculovirus vector containing P1 and 3CD genes of EV71 [14,15]. However, insect

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expression system is usually cost expensive and low productive. Recently, two chimeric VLP vaccines for EV71 were prepared in the bacterial system of *E. coli* by inserting the core and highly conserved antigen peptides into hepatitis B virus core protein (HBc) as a carrier [16,17]. These chimeric VLP vaccine candidates were reported to be able to induce neutralization antibodies protection for female BALB/c mice, but limited types of antigen epitope might not guarantee an overall protection against virus infection.

With the benefit of growing fast and easy control, prokaryotic system is increasingly explored to produce VLPs. Virus capsid proteins could be expressed in prokaryotic cells as organized VLPs such as HBc, or as capsomeres such as L1 protein of human papillomavirus [18]. But more often, for bacterial system, foreign proteins tend to form insoluble aggregates as inclusion bodies that need to be firstly denatured and solubilized before reassembling *in vitro*. VLPs assembling from denatured protein might be more difficult than from capsomeres of pentamer or hexamer units, but on the other hand, there are some advantages for expressing capsid proteins as inclusion bodies. Firstly, they are easy to be separated from cell debris simply by centrifugation [19]; secondly, they could be purified at denatured state with efficient removal of the nucleic acids and some host cell proteins (HCPs) that may be enveloped inside the particles when assembled *in vivo* [20]; thirdly, that may be the most important point, they could be produced fast, in large scale and in low cost through the *E. coli* system [11]. Hence, it is of great importance to investigate the mechanism and develop general techniques of assembling capsid proteins from inclusion bodies for bacterial-based vaccine preparation.

EV71 is a kind of non-enveloped, single-stranded RNA virus. Matured virus is an icosahedral sphere which consists of 60 copies of VP1, VP2, VP3 and VP4. VP4 is buried inside the icosahedron with the function of binding nucleic acids, having little impacts on the structure of icosahedral particles [21–23]. VP1, VP2 and VP3 contain most of the epitopes and constitute the virus outer shell through a pentameric assembly unit. Recently, antigen epitopes mapping for EV71 was investigated in detail, and we found that combining parts of the three capsids together could provide effective protections on neonatal mice against virus infection [24]. However, being expressed as inclusion bodies in *E.coli* and meanwhile missing an efficient production procedure impede further developing this fused capsid protein as a new potential EV71 vaccine candidate. Here, based on the previous study, the fused capsid protein containing the truncated VP1, VP2 and VP3 was constructed and expressed. After extraction, the inclusion bodies were solubilized and purified at denatured state. Then a refolding and assembling procedure was developed through an intermediate step with 2 M urea or 0.5 M arginine to suppress precipitate. Transmission electron microscopy (TEM) demonstrated that this fused protein could assemble into pentamer-derived particulate with a size of 25–40 nm. High antigen-specific IgG titer could be induced by immunization with the particulate antigen in mouse model. For the first time, to our knowledge, the fused truncated EV71 capsid protein was demonstrated to assemble itself into sphere-like nanoparticles which hold great promise to be developed as a new kind of vaccine candidate against HFMD. Furthermore, the robust purification and assembling approaches described here lay a foundation for the large-scale production of *E. coli*-based vaccines.

2. Materials and methods

2.1. DNA manipulation

EV71 FY0805, isolated from Anhui province of China at 2008 (Genbank accession number: HQ882182.1), was used as the template for protein designation. Briefly, the total RNA was extracted with TRIzol from virus stock and reverse-transcripted into cDNA with random primers. Then, the encoding region of 70–249 (a peptide consists of 180 amino acids located at N-terminal of VP2), 324–443 (a peptide consists of 120 amino acids located at N-terminal of VP3), and 746–876 (a peptide consists of 131 amino acids located at C-terminal of VP1) of the viral polypeptides were cloned respectively with fusion primers shown in Table 1. Subsequently, the DNA sequence encoding the truncated fused protein was amplified from the PCR products of former step by fusion PCR. The target fragment from the PCR product was extracted and digested with NdeI and EcoRI and ligated into the pET30a(+) (Novagen, USA). Then the resulting recombinant plasmid pET30a-vacA was transformed into *E. coli* BL21 (DE3).

2.2. Expression and induction of EV71 fused capsid protein

The recombinant *E.coli* BL21 (DE3) was first grown at 37 °C in five shake-flasks each containing 100 mL LB medium supplemented with 100 μg/mL kanamycin and then inoculated in a 20 L bioreactor (NBS, USA) when cell density reached OD_{600} of 5.0–8.0. Cells were continuously grown in 15 L fermentation medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L) supplemented with 100 μg/mL kanamycin. EV71 fused capsid protein expression was induced when cell growth reached mid-exponential phase with 1 mM isopropyl-D-thiogalactopyranoside (IPTG). After 4 h induction, cells were harvested by centrifugation at 4,000*g* for 20 min at 4 °C.

2.3. Inclusion bodies extraction and solubilization

The cell pellets were resuspended by Lysis Buffer (20 mM Tris-HCl, 1 mM EDTA, pH 8.5) at 1: 10 (w/v). The suspension was disrupted by high pressure homogenization (APV2000, Germany) at 3 runs and the pellets of inclusion bodies were collected by centrifugation at 10,000*g* for 30 min. The inclusion bodies of the fused capsid protein were washed three times by Lysis Buffer containing 1% Triton X-100, 1 M NaCl and 2 M urea, respectively.

For each purification experiment, the inclusion bodies were solubilized and denatured in the denaturant buffer (6 M guanidine chloride, 50 mM dithiotretiol (DTT), 20 mM Tris-HCl, 1 mM EDTA, pH 8.5) at room temperature by continuous shaking using rotary drum for 8 h. After centrifugation at 10,000*g* for 20 min, the suspension was collected and stored at −70 °C. After each centrifugation, the supernatant and pellets were collected and detected by SDS-PAGE.

2.4. Purification of solubilized denatured EV71 fused capsid protein

Two-column chromatography of ions exchange was used to purify the denatured fused capsid protein. Before ion exchange chromatography, the denatured protein was firstly exchanged into buffer A (8 M urea, 5 mM DTT, 10 mM PB, 1 mM EDTA, pH 7.0) by a desalting column (XK 200 × 16 mm ID, GE Healthcare) containing 50 mL Sephadex G25 (GE Healthcare, USA) and connecting to a ÄKTA Purifier 100 (GE Healthcare, USA). Then the fused protein in buffer A was loaded onto a column containing 20 mL Poros HQ (ABI, USA) equilibrated by buffer A. The pass-through peak was adjusted to pH 6.5 and applied to another column containing 20 mL Poros HS (ABI, USA) equilibrated by buffer B (8 M urea, 5 mM DTT, 10 mM PB, 1 mM EDTA, pH 6.5). The adsorbed protein was eluted by buffer B containing 0.3 M NaCl through linear gradient elution of 5 CV. Eluted peaks were collected and subjected to SDS-PAGE analysis.