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Stabilization study of inactivated foot and mouth disease virus vaccine by size-exclusion HPLC and differential scanning calorimetry

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ABSTRACT

The inactivated foot and mouth disease virus (FMDV), which has a sedimentation coefficient of 146S, is crucial to the efficacy of vaccine preparations, but extremely unstable in vitro. It is prone to dissociate into smaller particles referred to as 12S with a concomitant decrease in immunogenicity; therefore, it is of great importance to find the best condition for stabilizing the FMDV. In the present work, the effects of solution pH and temperature on the dissociation of 146S was investigated and potential stabilizers were screened, with aid of high-performance size-exclusion chromatography (HPSEC) for rapid and quantitative determination of 146S, together with differential scanning calorimetry (DSC) technology for thermal stability analysis. The most stable pH was found between 7.5 and 8.0. Among excipients tested, sucrose and glycerol provided the best protection, such that the half-life of 146S in solution at 45 °C could be prolonged from less than 30 min to more than 3 days by adding 20% sucrose. The stabilization mechanism was confirmed using DSC analysis, which showed that the transition temperature related to 146S dissociation was increased by 5.4 °C in the presence of 20% sucrose. The physical stabilization effects afforded by these stabilizers would allow for the retaining of effective 146S antigens during transportation and storage under relative harsh condition.

1. Introduction

Foot and mouth disease (FMD) is a highly contagious disease of livestock that causes severe economic loss in susceptible cloven-hoofed animals such as cattle, swine, sheep and goats [1]. The highly infectious nature of the disease, combined with its ability to cause persistent infections and to exist as multiple types and variants, makes FMD difficult to control. Foot and mouth disease remains one of the costliest diseases of livestock worldwide, billions of doses of vaccines are administered each year [2]. Currently new vaccines, for example, vaccines prepared from recombinant proteins and peptides based on the VP1 structural protein of the virus have been reported [3,4]. However, they have not been proven as effective as the conventional inactivated FMDV products [5].

The poor stability of the inactivated FMDV is one of the facing challenges for the vaccine production. It has been recognized that the assembly structure of the intact virus, which has a sedimentation coefficient of 146S, is crucial to the efficacy of vaccine preparations and permits the induction of a protective antibody response [6]. However, the 146S is sensitive to solution conditions, the assembly is easily disassembled by mild heating or at low pH [7,8], forming smaller dissociated products referred to as 12S with severely reduced immunogenicity [9]. Such instability put forward high requirement on cold chain transportation and storage, which will increase the production cost and even make vaccination difficult in areas where FMD is endemic and the environment is relatively harsh. Moreover, poor stability is often companioned with short shelf life of the vaccine products.

Although stabilization of the inactivated FMDV vaccine antigens is demanded, there were few researches focusing on this issue. It was reported that inactivation of FMDV with formaldehyde can stabilize the physical integrity of the intact FMDV without affecting its immune activity [10]. However, formaldehyde residual may raise concerns on safety of the vaccine. Acid-resistant FMDV mutants was also reported [11], which helps to better understanding the molecular determinants of increased resistance to acid-induced disassembly. However, because of the multiple serotypes and variants of the virus, a lot of work and researches are needed for selection of the mutant with improved stability. Physical stabilization of the live virus was also reported. Several additives such as inositol, glycerol, and sodium glutamate were found to protect

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the live virus against inactivation in aerosols and during freeze-drying [12] or cryopreservation [13]. However, few researches were reported about physical stabilization of inactivated FMDV. The lack of rapid method for in vitro detection of the inactivated virus could be one reason, since the conventional quantification of 146S conducted by ultracentrifugation is unsuitable for high-throughput screening for effective excipients due to complex operation and low detection efficiency [14]. Recently, a double antibody sandwich ELISA method was developed for quantitative determination of 146S and 12S [15]. Based on this ELISA analysis, excipients including sucrose combined with bovine serum albumin (BSA) was found to protect the 146S from dissociated into 12S to certain degree during storage as oil-emulsion vaccine [16]. Although the method is useful, results based on ELISA analysis are known to be often influenced by preparation procedure, and possible nonspecific or incomplete binding. Therefore, the accuracy of quantification by ELISA is limited. Moreover, the protection mechanism of sucrose and BSA for 146S was not investigated.

In one of our previous work, a high-performance size-exclusion chromatography (HPSEC) method was developed and had been proven effective for rapid and quantitative determination of 146S during purification [17] and for monitoring the dissociation of 146S [14]. Therefore, it can also be a valuable tool for rapid determining the best stabilization conditions for 146S by minoring changes in 146S content in solutions, as well as for efficient screening of suitable excipients that can stabilize the inactivated virus.

In the present work, we aimed to find the best condition for stabilizing the FMDV. With aid of HPSEC for rapid and quantitative determination of 146S, the effects of solution pH and temperature on the dissociation of 146S was investigated and potential stabilizers were screened. The stabilization mechanism of the solution pH and additives were discussed based on differential scanning calorimetry (DSC) analysis, which is a powerful technique for optimization of formulation of proteins including vaccine antigens [18,19].

2. Materials and methods

2.1. Materials

FMDV strain O China 1999 supernatant was kindly provided by Lanzhou Veterinary Research Institute (Chinese Academy of Agricultural Sciences, China) after cultivation, inactivation and pretreatment [17].

D-sucrose, dextrose, sorbitol, mannitol, D (+)-trehalose dehydrate, bovine serum albumin (BSA), amino acids including arginine (Arg), lysine (Lys) and glycine (Gly) hydrochloride were purchased from Sigma-Aldrich (MO, USA). Tween 20, Tween 80, and glycerol were obtained from Shantou Xilong Chemical Co., Ltd (Guangdong, China), and polyethylene glycol with molecular weight of 400–200,000 were from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). All other chemicals were analytical grade reagents, and all solutions were prepared using Mili-Q grade water (Millipore, USA).

2.2. Preparation of FMDV standard and its quantification by HPSEC

FMDV standard was collected by the sucrose gradient ultracentrifugation according to previous reports [5,20]. The sucrose gradients were set as 15–45% (w/w), and the detailed preparation procedure was described in previous work [14]. The concentration of the purified FMDV was calculated from UV absorbance, using extinction coefficient $E^{1\%}_{259,1cm} = 76$ [21].

The purified FMDV by sucrose gradient ultracentrifugation was conducted to serial dilution and was then analyzed by HPSEC as described previously [14,17]. The analysis was performed on a TSK G4000 SW_{XL} (300 × 7.8 mm I.D.) analytical column (Tosohaas, Stuttgart, Germany) using an Agilent 1100 HPLC series system (Agilent, USA) with an inline degasser, a sample cooler, and variable-wavelength detector operating at 259 nm. FMDV samples were kept at 4 °C prior to analysis and the column temperature was kept below 20 °C to avoid dissociation of FMDV during detection. To acquire the molecular weight (MW) of the samples, the column was also connected to a multi-angle laser light scattering (MALLS) detector (DAWN EOS, λ = 690 nm, Wyatt Technology Corp., USA) and a refractive index (RI) detector (OPTILAB DSP, Wyatt Technology Corp., USA). The MW was obtained using the ASTRA® software (Wyatt Technology, USA). Samples of 100 μL were injected and eluted at 0.6 ml/min with 50 mM phosphate buffer pH 7.2 containing 100 mM Na_2SO_4. The peak area of FMDV at 259 nm was linearly proportional to FMDV concentration, with R^2 = 0.998 over the tested range between 0 and 60.2 μg/mL. Therefore, the FMDV concentration in testing sample could be calculated from the peak area at 259 nm according to the calibration curve.

2.3. Screening of excipients to stabilize inactivated FMDV

The stabilizers of FMDV were screened at pH 8.0 and 45 °C. FMDV with addition of various excipients were prepared at a 146S concentration of 50 μg/mL in 20 mM pH 8.0 sodium phosphate buffer solution. After being stored at 45 °C for 20 min, the residual 146S content was detected by HPSEC and compared with the control samples in absence of any excipients. Those excipients with more residual 146S than the control were considered to be stabilizers and were studied further. All experiments were performed in duplicate, and the results were expressed as average value with standard deviation.

2.4. Differential scanning calorimetry analysis

Calorimetric experiments were performed with a MicrolCal™ VP-DSC system (GE Healthcare, USA) to study the thermal stability of FMDV in different solution conditions. The FMDV solution with or without excipients at an initial 146S concentration of 0.5 mg/mL in 20 mM PB of different pH was heated from 20 to 90 °C at a scan rate of 60 °C/h. The thermogram for each of the corresponding buffer was subtracted from that for virus-containing solution. Data analysis was performed using the MicrolCal Origin 7.0 (OriginLab Corp., MA) software.

2.5. Transmission electron microscopy analysis

The structure of FMDV was also studied by Philips FEI Tecnai 20 transmission electron microscopy (TEM, Royal Philips Electronics, Amsterdam). The samples before and after heating were applied to a 400-mesh copper grid, dried, and stained with 1% uranyl acetate before TEM characterization.

3. Results and discussions

3.1. Thermal stability of FMDV studied by HPSEC

The thermal stability of FMDV at different temperature was firstly studied by monitoring the changes in 146S content with HPSEC method. Fig. 1A shows the typical changes in chromatograms of inactivated FMDV during storage at 45 °C. At beginning, the 146S purified from sucrose density gradient ultracentrifugation showed only one peak at about 13.4 min with average Mw determined by MALLS about 5500 kDa as reported previously [14]. During storage, this peak decreased gradually. In

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