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High-yield production of the VP1 structural protein epitope from serotype O foot-and-mouth disease virus in *Escherichia coli*

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Abstract For effective control of foot-and-mouth disease (FMD), the development of rapid diagnostic systems and vaccines are required against its etiological agent, FMD virus (FMDV). To accomplish this, efficient large-scale expression of the FMDV VP1 protein, with high solubility, needs to be optimized. We attempted to produce high levels of a serotype O FMDV VP1 epitope in *Escherichia coli*. We identified the subtype-independent serotype O FMDV VP1 epitope sequence and used it to construct a glutathione *S*-transferase (GST) fusion protein. For efficient production of the FMDV VP1 epitope fused to GST (VP1e–GST), four *E. coli* strains and three temperatures were examined. The conditions yielding the greatest level of VP1e–GST with highest solubility were achieved with *E. coli* BL21(DE3) at 25 °C. For high-level production, fed-batch cultures were conducted in 5-l bioreactors. When cells were induced at a high density and complex feeding solutions were supplied, approximately 11 g of VP1e–GST was obtained from a 2.9-l culture. Following purification, the VP1 epitope was used to immunize rabbits, and we confirmed that it induced an immune response.

Keywords *Escherichia coli* · Fed-batch cultivation · FMDV · VP1 epitope

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious and major pathogen of cloven-hoofed animals such as cattle, swine, sheep, and goats [12]. FMD can be spread by infected animals through aerosol transmission or by close contact with contaminated farming equipment, vehicles, and other animals; it has the potential to cause severe epidemics. Containment of FMD involves considerable efforts with respect to strict monitoring of animal movement, trade restrictions, and quarantines, and occasionally the elimination of millions of animals, thereby resulting in significant economic losses for affected countries [22]. To control this disease, rapid and early diagnosis and vaccination strategies are necessary. Currently, serological tests such as enzyme-linked immunosorbent assays (ELISAs) are used to monitor viral infection and the immune status of animals. For effective vaccination and to develop more rapid and reliable diagnostic tests, appropriate viral antigens need to be identified and produced in vitro.

The FMD virus (FMDV), the causative agent of FMD, is an RNA virus (8,500 nt) and is classified as a *picornavirus* within the genus *Aphthovirus* [2]. This non-enveloped virus has an icosahedral capsid consisting of 60 copies of structural proteins (VP1, VP2, VP3, and VP4). FMDV is classified by antigenic diversity, with seven distinct serotypes [O, A, C, Asia 1, South African Type (SAT) 1, SAT 2, and SAT 3]. These serotypes can be further subdivided

by antigenicity and molecular topotype [8]. Serotype O is the most prevalent serotype among the seven FMDV serotypes and occurs in many parts of the world [25]. The VP1 structural protein is highly immunogenic, and has been proposed to play a major role in serotype specificity, virus attachment, and in eliciting an immune response in affected individuals [2, 12, 27, 29]. In particular, a portion of the VP1 G-H loop (amino acids 140–160 at the C terminus) has been identified as a major antigenic site [1, 9, 10]. This finding suggests that certain VP1 epitopes are responsible for the induction of protective neutralizing antibodies and could be useful for the development of vaccines against FMDV, and for better, more reliable diagnostic systems [13].

Over the last decade, there have been concerted efforts to efficiently produce FMDV VP1 proteins in various hosts, including plants, yeast, and bacteria [3, 7, 20, 21, 26]. Shi et al. (2006) reported the secretory production of FMDV VP1 in *Pichia pastoris* with a high yield (approximately 1.0 g/l of culture medium) [26]. However, the actual culture volume was small (100–200 ml), and relatively long cultivation times compared with *E. coli* were necessary. More recently, Gao et. al. (2010) reported the production of three VP1 epitopes (amino acids 140–160, 200–213, and a combination of both these epitopes), using flask cultivation of an *E. coli* host, as glutathione *S*-transferase (GST) fusion constructs. They achieved a high production yield with relatively moderate solubility [11]. However, in many reports, the expression level of target protein was not satisfactory, and in most cases the proteins were produced as insoluble inclusion bodies requiring labor-intensive and time-consuming refolding processes before they could be of any use [3, 21]. It is also worth noting that all previous cultivations were conducted in flasks with no reports about large-scale production of VP1 proteins as yet.

In our study, we aimed to produce a VP1 epitope (VP1e) of serotype O FMDV as a GST fusion protein in *E. coli*, and using fed-batch cultivation attempted to achieve high-level production of the protein. We also sought to determine the antigenicity of the identified VP1e.

Materials and methods

Bacterial strains and plasmids

All bacterial strains and plasmids used in this work are summarized in Table 1. *E. coli* XL1-Blue was used as a main host for cloning, with other *E. coli* strains used for protein production. Codon optimization for the selected VP1e gene was performed using a program in the Web site of EnCor Biotechnology Inc. (www.encorbio.com). The codon-optimized sequence (105 bp) was synthesized by Bioneer (Daejeon, Korea). To amplify the codon-optimized VP1e gene, polymerase chain reaction (PCR) was conducted with a C1000 thermal cycler (Bio-Rad, Hercules, CA, USA) using Primestar DNA polymerase (Takara Bio, Otsu, Japan). Primers used for amplification were 5′-GCGTGGATCCTGCAAATATGGCGAAAGC-3′ (forward; *Bam*HI site underlined) and 5′-GCCGCTCGAG TTATCAAATCGCGCCATAGTTAAAGCT-3′ (reverse; *Xho*I site underlined). The resulting PCR product was digested with *Bam*H1 and *Xho*I and then cloned into pGEX-4T-1 (Stratagene, La Jolla, CA, USA) to yield pGST-VP1e. This resulting plasmid was used to express the VP1 epitope fused to GST (termed here VP1e–GST). Restriction digestion, ligation, and agarose gel electrophoresis were carried out using standard procedures [24].

Flask cultivation

Escherichia coli harboring pGST-VP1e were pre-cultured in 3 ml of Luria–Bertani (LB; 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) broth containing 2 % (w/v) glucose at 37 °C with shaking at 200 rpm. After overnight cultivation, 500 µl of pre-culture was inoculated into 50 ml of fresh LB broth in a 250-ml flask and incubated at 37 °C with shaking at 200 rpm. When the optical density, at 600 nm (OD_{600}), of the culture reached 0.5, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma Chemical Co., St. Louis, MO, USA) was added to induce expression. At 4 h post-induction, cells were harvested by centrifugation (6,000 rpm for 10 min at 4 °C). Ampicillin (50 µg/l) was included as a supplement in all flask cultures.

Fed-batch cultivation

A seed culture was prepared in a 1-l flask containing 200 ml of defined R/2 medium [17] and 2 % (w/v) glucose. The R/2 medium contained 2 g/l $(NH_4)_2HPO_4$, 6.75 g/l KH_2PO_4, 0.85 g/l citric acid, 0.7 g/l $MgSO_4{\cdot}7H_2O$, and 5 ml of a trace metal solution (10 g/l $FeSO_4{\cdot}7H_2O$, 2.25 g/l $ZnSO_4{\cdot}7H_2O$, 1 g/l $CuSO_4{\cdot}5H_2O$, 0.5 g/l $MnSO_4{\cdot}5H_2O$, 0.23 g/l $Na_2B_4O_7{\cdot}10H_2O$, 2 g/l $CaCl_2{\cdot}2H_2O$, and 0.1 g/l $(NH_4)_6MO_7O_{24}$). Fed-batch cultivations were conducted in 5-l bioreactors (BioCNS, Daejeon, Korea) containing 1.8 l of R/2 medium with 2 % (w/v) glucose and 10 % (v/v) of inoculum (200 ml). Cells were cultivated at 37 °C during the pre-induction period with the temperature reduced to 25 °C during the post-induction period. Except for periods when the pH increased because of glucose depletion, the pH was maintained at 6.8 by adding 50 % (v/v) of ammonia water. The dissolved oxygen (DO) concentration was maintained at 40 % of air saturation by automatically increasing the agitation speed to 1,000 rpm, and by changing the percentage of pure oxygen. Two different

Table 1 Bacterial strains used in this study

Strain or plasmid	Genotype	Reference or source
E. coli strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacI^{q}ZΔM15 Tn10 (Tet^r)]*	Stratagene
BL21(DE3)	*F$^-$ompT hsdS$_B$($r_B^-m_B^-$)gal dcm (DE3)*	Novagen
W3110	*F$^-$ lambda$^-$ IN(rrnD-rrnE)1 rph-1*	Lab stock
MG1655	*F$^-$ lambda$^-$ilvG- rfb-50 rph-1*	Lab stock
Plasmids		
pGEX-4T-1	Ap^r, tac promoter, lacI^q	GE Healthcare
pGST-VP1e	FMDV VP1 gene epitope in pGEX-4T-1	This study

feeding solutions were supplied by a pH–stat feeding strategy: the defined feeding solution containing 700 g/l glucose and 20 g/l $MgSO_4 \cdot 7H_2O$; and the complex feeding solution containing 500 g/l glucose, 75 g/l yeast extract, and 20 g/l $MgSO_4 \cdot 7H_2O$. When the pH increased to a value greater than its set point (pH 6.8) by 0.08 because of glucose depletion, the appropriate volume of feeding solution was automatically added to the culture. Foam was suppressed as necessary by manual addition of sterilized antifoam (Sigma). Cell growth was monitored by measuring the OD_{600} using a UV/Vis spectrophotometer (Optizen POP, Mecasys, Daejeon, Korea). Expression of VP1e–GST was induced by adding IPTG to a final concentration of 1 mM when the cell density reached 110 at OD_{600}. Culture samples were periodically collected by centrifugation, and the cell pellets and supernatants were stored at −20 °C for further analysis.

Purification of VP1e–GST

Cells were harvested by centrifugation (6,000 rpm for 20 min at 4 °C) and the cell pellet resuspended in an appropriate volume of ice-cold phosphate-buffered saline (PBS) at pH 7.4. Cells were then disrupted by sonication (VC750, Sonics & Materials Inc., Newtown, CT, USA) for 30 min at a 20 % amplitude (3-s pulses at 7-s intervals). After centrifugation (10,000 rpm for 20 min at 4 °C), soluble protein fractions were collected and VP1e–GST was purified by affinity column chromatography using Glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. Eluted fractions were pooled and dialyzed (MWCO 7,000; Snakeskin, Pierce Biotechnology, Rockford, IL, USA) against PBS (pH 7.4) for 24 h. All purification procedures were performed at 4 °C.

Western blot and ELISA

Solubility and functional activity were confirmed by Western blot and ELISA. Briefly, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were transferred to polyvinyl difluoride (PVDF) membranes (Roche, Penzberg, Germany) for 90 min at 60 mA using a Bio-Rad transblot apparatus (Bio-Rad, Hercules, CA, USA). The membrane was incubated with a blocking solution (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 % skim milk, and 0.05 % Tween-20) for 1 h at room temperature (RT). Membranes were incubated with a serotype O FMDV VP1-specific monoclonal antibody (Jeno Biotech Inc., Chuncheon, Korea), and washed four times with 1× TBS-T (150 mM NaCl, 50 mM Tris–HCl pH 7.5, and 0.05 % Tween-20) for 30 min. Detection or further blotting with a HRP-conjugated anti-mouse antibody (Sigma) was then carried out.

ELISAs were conducted in 96-well microtiter plates (Nunc Invitrogen Ltd., Carlsbad, CA, USA) coated with 0.1 μg/well of purified VP1e–GST in 0.1 M $NaHCO_3$ (pH 9.0) at 4 °C. Plates were briefly washed, followed by blocking with 4 % (w/v) skim milk in PBS-T (135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 with 0.05 % Tween-20) for 1 h at RT. The serotype O FMDV VP1-specific antibody (Jeno Biotech Inc.) was then added and incubated for 1 h at RT. Plates were washed and incubated with a HRP-conjugated anti-mouse monoclonal antibody (Sigma) for 1 h at RT. Finally, the plates were washed four times with 1× PBS-T. TMB peroxidase substrate (100 μl; BD Biosciences, San Jose, CA, USA) was added to initiate the peroxidase reaction and 50 μl of 2 M H_2SO_4 used to stop the reaction. The absorbance at 450 nm in wells was determined using an ELISA reader (Infinite M200 PRO, Tecan, Grodig, Austria).

Analytical methods

In fed-batch cultures, the dry cell weight (DCW) was determined. Culture broth was collected in three 1.5-ml microcentrifuge tubes, centrifuged, and pellets were washed with distilled water, then dried in a vacuum oven at 80 °C for 24 h. For analysis of protein production, cells were disrupted by sonication in 300 μl of 1× PBS, with a

small aliquot of the total fraction removed for further analysis. The remaining suspension was centrifuged (10,000 rpm for 10 min at 4 °C) and the soluble fractions were prepared from the supernatant. The amount of protein was determined with a Bio-Rad Protein Assay Kit (Bio-Rad) using bovine serum albumin (BSA) as a standard. Protein samples were analyzed by SDS-PAGE. The proportion of VP1e–GST to all other proteins was calculated based on the densities of VP1e–GST bands, which were quantified by GS-800 imaging densitometry (Bio-Rad).

Animal immunization and ELISA evaluation of VP1e

Rabbits were injected with 1.0 mg of purified VP1e–GST emulsified in Freund's Complete Adjuvant (FCA) via an intradermal injection (250 μg/rabbit). Three booster shots were given at 2-week intervals, consisting of the same amount of VP1e–GST but emulsified in Freund's Incomplete Adjuvant (FIA). Rabbits were bled 2 weeks after the last immunization. The titer of polyclonal antiserum raised against VP1e–GST was analyzed by a sandwich ELISA. Serotype O FMDV VP1-specific antibody (Jeno Biotech Inc.) was used to coat the wells of 96-well microtiter plated overnight at 4 °C. VP1e–GST (2 μg/ml) was added to each well and plates were incubated at RT for 1 h then washed with 1× PBS-T. Serum samples from immunized rabbits were added, with the first sample from each rabbit diluted 1/100 and then serially diluted fivefold across the plate. Plates were washed and incubated with a HRP-conjugated anti-rabbit monoclonal antibody (Sigma) for 1 h at RT. Plates were washed four times with 1× PBS-T and TMB peroxidase substrate (100 μl) was added to initiate the peroxidase reaction. To stop the reaction, 50 μl of 2 M H_2SO_4 was added to each well. The absorbance at 450 nm in wells was determined using an ELISA reader (Infinite M200 PRO).

The reactivity of polyclonal antiserum raised against VP1e–GST was analyzed against inactivated serotype O FMDV by both indirect and competitive ELISAs. For both analyses, serotype O FMDV Manisa strain-coated plate (Prionics AG, Schlieren-Zurich, Switzerland) was used. In indirect ELISA, the serially diluted serum samples from immunized rabbits were added on each well of the plate. After incubation at RT for 1 h, plates were washed four times with 1× PBS-T and incubated with a HRP-conjugated anti-rabbit monoclonal antibody (Thermo Fisher Scientific, Rockford, IL, USA) for 1 h at RT. Plates were washed four times with 1× PBS-T and TMB peroxidase substrate (100 μl) was added to initiate the peroxidase reaction. To stop the reaction, 50 μl of 2 M H_2SO_4 was added to each well. The absorbance at 450 nm in wells was determined using an ELISA reader (Infinite M200 PRO). In competitive ELISA, the serially diluted serum samples from immunized rabbits were added on each well of the plate. After incubation at RT for 1 h, plates were washed four times with 1× PBS-T. Then serotype O FMDV-specific monoclonal antibody (Prionics AG) were added and plates were incubated for 1 h at RT. All the other steps (peroxidase reaction and detection) were done in the same way as described earlier. The percentages of inhibition (PI) of the control and test sera were calculated according to the following formula:

$$\mathrm{PI}(\%) = 100 - \left(\frac{\mathrm{OD}_{450}\text{test sample}}{\mathrm{OD}_{450}\text{max}}\right) \times 100$$

If PI is higher than 50 %, it means that the specific antibodies against serotype O FMDV are present in the test sera. In both ELISAs, non-immunized rabbit serum was used as a negative control.

Reactivity of the GST fusion tag was also confirmed using a direct ELISA method. Recombinant GST (2 μg/ml) or VP1e–GST (2 μg/ml) were coated on plates and the residual binding sites blocked with 5 % (w/v) skim milk. After 1 h, the plates were washed with 1× PBS-T. Serum samples from immunized rabbits were added, with the first sample from each rabbit diluted 1/100 and then serially diluted fivefold across the plate. Plates were washed and incubated with a HRP-conjugated anti-mouse monoclonal antibody (Sigma) for 1 h at RT. Plates were washed four times with 1× PBS-T and TMB peroxidase substrate (100 μl) was added to initiate the peroxidase reaction. To stop the reaction, 50 μl of 2 M H_2SO_4 was added to each well. The absorbance at 450 nm in wells was determined using an Infinite M200 PRO ELISA reader.

Results and discussion

Serotype O FMDV VP1 sequences

In FMDV, the VP1 capsid protein is known to be a major immunodominant region that can activate immune systems [12]. The GH loop containing the RGD motif is responsible for cell–virus interactions and for recognizing FMDV-specific antibodies [14]. Because of its important functions, researchers have tried to control FMDV infections by targeting VP1. However, the diversity of the VP1 immunodominant regions across subtypes makes it difficult to determine epitope sequences. Moreover, the variation in length of the GH loop needs to be optimized before VP1 can be fully exploited in serological assays [23, 28, 30, 32]. To analyze the conserved region of serotype O-specific VP1, 1,687 FMDV VP1 sequences were obtained from the National Center for Biotechnology Information (NCBI) database and aligned with Jalview version 2 [31]. The alignment revealed 224 sequences lacking the GH loop;

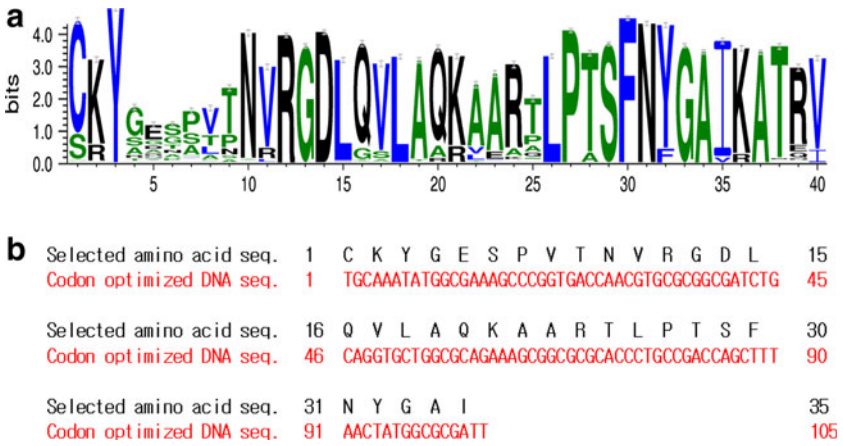

Fig. 1 **a** Sequences from over 1,000 strains of serotype O FMDV were aligned and used to generate a logo using Weblogo. *Black letters* indicate the hydrophilic amino acids (R, K, D, E, N, Q), *green letters* indicate the neutral amino acids (S, G, H, T, A, P), and finally *blue letters* indicate the hydrophobic amino acids (Y, V, M, C, L, F, I, W). The *size of each letter* represents the frequency of that amino acid at corresponding position. **b** Selected VP1e amino acid and codon-optimized gene sequences (color figure online)

therefore, they were removed from further analyses, with 1,463 sequences remaining. To examine conservation and variation in the GH loop, we analyzed the profile of the GH domain and its neighboring sequences with WebLogo version 3.2 [6]. The profile of the most conserved region is presented in Fig. 1a. This region spanned amino acids 134–173 of FMDV VP1 and was identified as a B cell epitope for FMDV (Fig. 1b).

Production of VP1e–GST in flasks

The coding gene (105 bp) of the selected epitope was synthesized de novo and contained codons that had been modified such that they were favorable for *E. coli* (Fig. 1b). The gene was cloned into pGEX-4T-1 to yield pGST-VP1e. This construct contained the VP1e gene linked to the 3′ end of the GST gene. Protein expression was controlled by the IPTG inducible *tac* promoter (P*tac*). It is well known that the production of recombinant proteins in *E. coli* can be affected by various culture conditions including culture temperature, concentration of the inducer, and host strains [5, 18]. To choose the best host and optimal temperature for VP1e–GST expression, four *E. coli* strains [XL1-Blue, BL21(DE3), W3110, and MG1655] widely used for recombinant protein production were examined at three temperatures (25, 30, and 37 °C). After IPTG induction, each strain was cultivated at the three temperatures for 4 h, and cell growth, production, and solubility of proteins were analyzed. For most of the strains, cell growth rates were higher at 25 °C than at the higher temperatures (Fig. 2a). In particular, when *E. coli* BL21(DE3) was cultivated at 25 °C, the cell density was approximately 1.6-fold greater than that of the other strains. The yield of VP1e–GST was highly correlated with the density of the cultures (Fig. 2b).

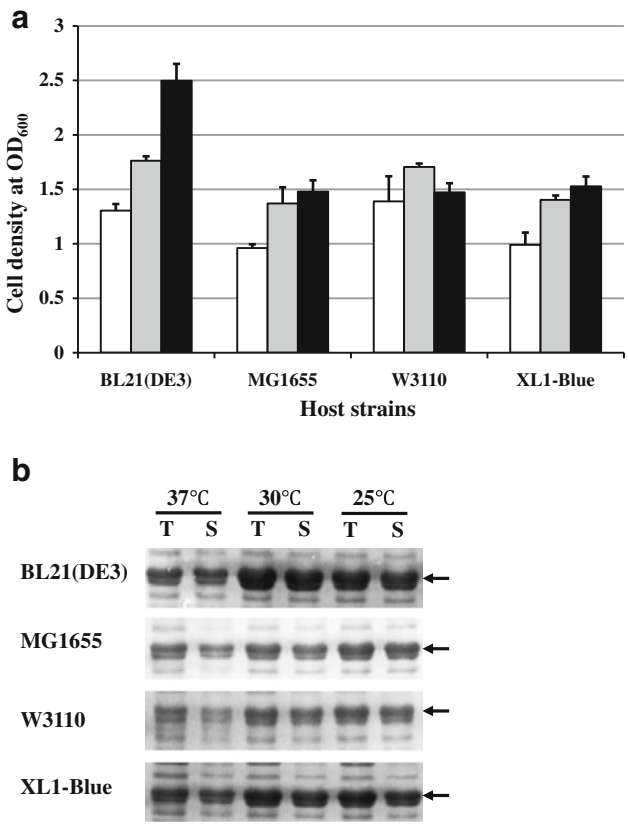

Fig. 2 **a** The final cell density (OD_{600}) of each culture producing VP1e–GST at various temperatures (*white*, 37 °C; *gray*, 30 °C; *black*, 25 °C). **b** SDS-PAGE analysis of protein production and solubility. *Lane T* total fraction; *S* soluble fraction. *Numbers on top* indicate the culture temperatures. *Arrows* indicate the band corresponding to VP1e–GST

At all temperatures and for all strains, VP1e–GST was highly soluble and the highest yields were obtained from cultures grown at 25 °C (Fig. 2b). Among the four *E. coli*

strains, BL21(DE3) produced the highest yield of recombinant fusion protein. Based on our results BL21(DE3), which has been widely used for fed-batch cultivation, was identified as the optimal host for VP1e–GST production.

VP1e–GST reactivity

The recombinant VP1e–GST produced in flask cultures was purified by affinity column chromatography (Fig. 3a), and specific reactivity was confirmed by Western blots and ELISA. In the Western blots, VP1e–GST was successfully detected by a serotype O VP1-specific monoclonal antibody. However, the GST protein alone (negative control) was not detected by the same antibody (Fig. 3b). Our ELISA results corresponded with the Western-blot analysis, with VP1e–GST successfully recognized by the serotype O FMDV VP1-specific antibody, and GST detected at basal levels (Fig. 3c). We concluded that VP1e–GST produced by *E. coli* was highly reactive and specific.

VP1e–GST production in fed-batch cultures

We cultured *E. coli* BL21(DE3) harboring pGST-VP1e using fed-batch techniques within a 5-l bioreactor to produce a sufficient amount of protein for downstream applications. In fed-batch cultivation, the protein yield at high cell densities is strongly affected by nutrient composition of the feeding solution [15, 16, 19]. To investigate the effect of nutrient composition on VP1e–GST production under these conditions, two feeding solutions (defined and complex) were examined. In both cases, the same defined feeding solution was supplied during the pre-induction period. Following induction with 1 mM IPTG once the OD_{600} of the culture reached 110, the two feeding solutions were supplied separately to individual reactors. For each fermentation, the time profiles of cell density (OD_{600}), dry cell weight (g DCW/l), and the proportion (%) of VP1e–GST among total proteins were determined (Fig. 4; Table 2). When the defined feeding solution was supplied during the post-induction period, cells grew up to 62 g DCW/l at 8-h post-induction with a specific growth rate (μ) of 0.044 h^{-1}

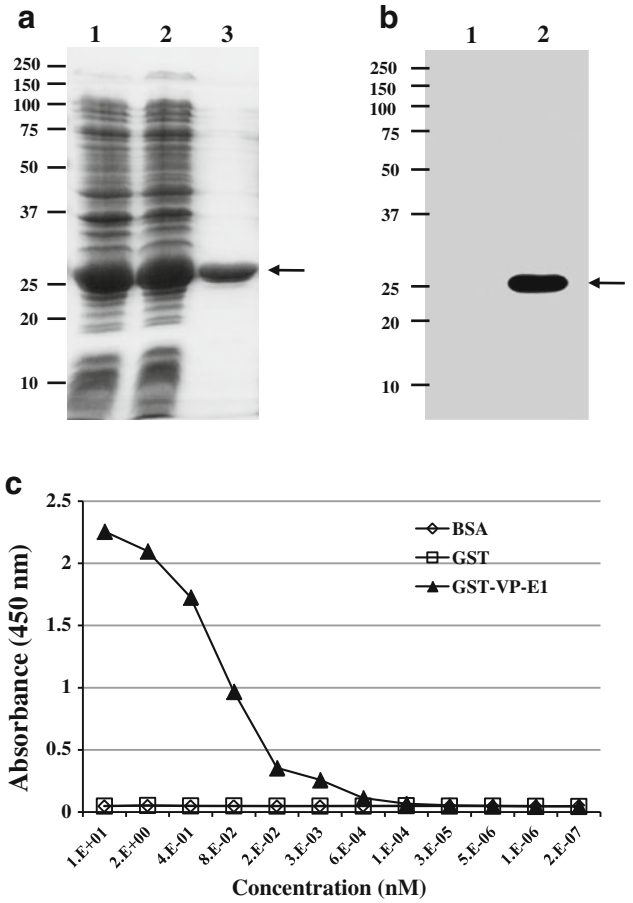

Fig. 3 **a** SDS-PAGE analysis of VP1e–GST production and purification. *Lane 1* total fraction; *lane 2* soluble fraction; *lane 3* purified sample. **b** Western-blot analysis of purified VP1e–GST. *Lane 1* purified GST; *lane 2* purified VP1e–GST. **c** ELISA results using the purified VP1e–GST as the coating antigen. *Filled triangle* VP1e–GST; *unfilled square* GST; *unfilled diamond* bovine serum albumin. *Arrows* indicate the band corresponding to VP1e–GST

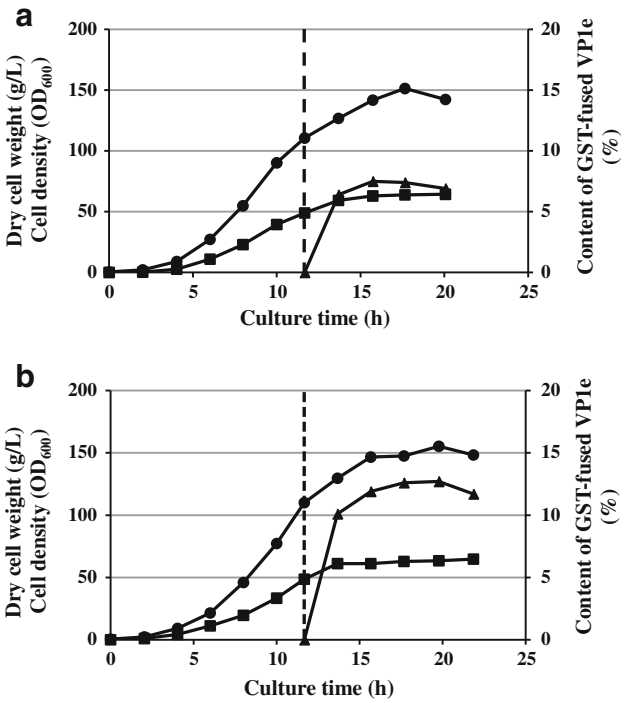

Fig. 4 Profiles of cell growth, dry cell weight, and protein production during fed-batch cultivations of *E. coli* BL21(DE3) harboring pGST-VP1e. Cultures were supplemented with **a** defined feeding solution or **b** complex feeding solution. Cells were induced at an OD_{600} of 110 (*dashed line*). *Filled circle* cell density according to OD_{600}; *Filled square* dry cell weight (g/l); *Filled triangle* VP1e–GST as a percent of total protein content

Table 2 Summary of the fed-batch cultures using the defined and complex feeding solutions

Feeding solution	Induction (OD_{600})	Max. cell density (OD_{600})	Specific growth rate in post-induction (h^{-1})	Max. production yield (g/l)
Defined[a]	110	151	0.044	2.3
Complex[b]	110	156	0.057	3.8

[a] Defined feeding solution (700 g/l glucose and 20 g/l $MgSO_4 \cdot 7H_2O$)

[b] Complex feeding solution (500 g/l glucose, 20 g/l $MgSO_4 \cdot 7H_2O$, and 75 g/l yeast extract)

(Fig. 4a). Immediately after induction, production of VP1e–GST was observed and reached a maximal level of 7.5 % of total proteins, corresponding to approximately 2.3 g of VP1e–GST/l at 4-h post-induction. Protein yields were maintained at relatively high levels after this time point. In this cultivation, the highest productivity of VP1e–GST was 0.16 g/l/h. To supply more nutrients, particularly nitrogen source, in fed-batch cultures, a complex feeding solution (500 g/l glucose, 20 g/l $MgSO_4 \cdot 7H_2O$ and 75 g/l yeast extract) was supplied during the post-induction period. At 4-h post-induction, cells grew without a significant reduction in μ (0.057 h^{-1}), which was higher than that observed for the cells fed the defined solution (Fig. 4b). The maximum cell density was as high as 63 g DCW/l at 8-h post-induction. The fraction of VP1e–GST among all proteins increased immediately after induction, and was found to be up to 12.7 % of the total protein content (approximately 3.8 g of VP1e–GST/l) at 6-h post-induction (Fig. 4b). The productivity of VP1e–GST was 0.21 g/l/h, which was much higher than that obtained by supplementation with the defined feeding solution. Under both conditions, we were unable to observe any significant changes in cell growth following IPTG induction. Similar cell densities (62–63 g DCW/l) were obtained at 8-h post-induction; however, there was a large difference in VP1e production between the two conditions with the yield more than 1.65-fold higher for the complex feeding solution. In general, protein production requires many resources, which can be exacerbated by high cell densities such as those that occur in bacterial cell cultures. This can be one limiting factor in the production of recombinant proteins. Our results indicate that supplementation with more nitrogen sources from a complex feeding solution can be used for the biosynthesis of extra amino acids, and consequently resulted in enhanced production of VP1e–GST. Using a complex feeding solution, approximately 11 g of VP1e–GST was obtained from 2.9 l of culture.

Validation of immunogenicity

It is important to verify whether recombinant proteins produced in bacterial hosts have appropriate immunogenicity similar to that elicited by the whole virus [16]. To determine whether purified VP1e–GST was capable of inducing an immune response, serum was taken from rabbits immunized with purified VP1e–GST and applied to sandwich and direct ELISAs. In the sandwich ELISA, sera from the immunized rabbits were highly reactive and specific for VP1e–GST (Fig. 5a). Although GST is known to be a weakly immunogenic protein [4], rabbits immunized with VP1e–GST likely induce a non-specific immunogenic response against the GST portion of the fusion protein along with the VP1 epitope itself, and it might give high signal in sandwich ELISA independent of VP1 epitope. The possible contamination of GST-specific antibody in sera was

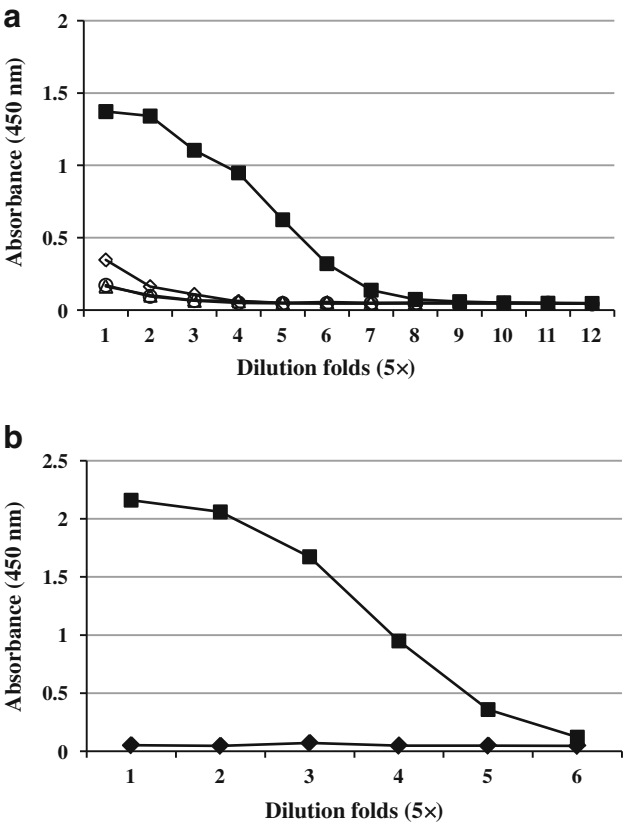

Fig. 5 Induction of an immune response in rabbits immunized with VP1e–GST. **a** Sandwich ELISA. *Filled square* serum from immunized rabbits, with VP1e–GST used to coat microtiter plates; *unfilled diamond* serum from immunized rabbits but VP1e was not used to coat the microtiter plates; *unfilled circle* non-immunized rabbit serum on VP1e–GST-coated plates; *unfilled triangle* non-immunized rabbit serum on plates lacking VP1e. **b** Direct ELISA with serum from immunized rabbits. Sera were added to wells that had been coated with GST (*filled diamond*) or VP1e–GST (*filled square*)

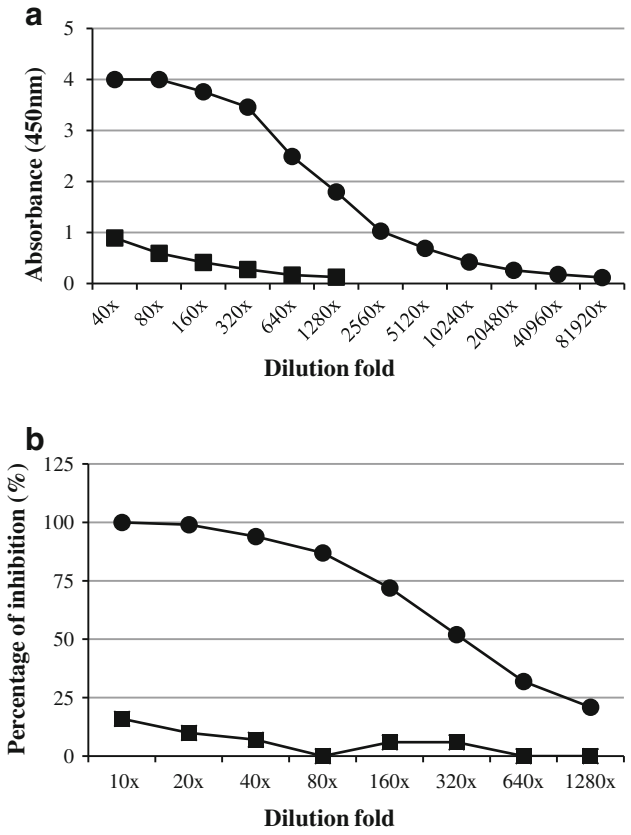

Fig. 6 Evaluation of immunized rabbit serum with VP1e–GST. **a** Indirect ELISA with serum from immunized rabbits. *Filled circle* serum from immunized rabbits with VP1e–GST; *filled square* non-immunized rabbit serum. **b** Competitive ELISA with serum from immunized rabbits. *Filled circle* serum from immunized rabbits with VP1e–GST; *Filled square* non-immunized rabbit serum

analyzed by direct ELISA. As shown in Fig. 5b, sera from the immunized rabbit showed much higher reactivity against VP1e–GST than GST alone and, this result of direct ELISA indicates that the serum samples contained a VP1e-specific antibody as opposed to a GST-specific antibody.

The immunogenic activity of VP1e–GST was also evaluated against inactivated serotype O FMDV by indirect and competitive ELISAs. In indirect ELISA, sera from the immunized rabbit showed much higher reactivity against inactivated serotype O FMDV than that by non-immunized rabbit sera (negative control) (Fig. 6a). Also, in competitive ELISA, sera from the immunized rabbit showed significantly high inhibition signal in competition with serotype O FMDV-specific monoclonal antibody (MAb) than that by non-immunized rabbit sera (Fig. 6b). These results mean that the immunization of rabbit with the purified VP1e–GST successfully raised the serotype O FMDV-specific antibody. From all ELISA results, it is concluded that the purified VP1e–GST antigen was appropriate for use in vaccines and in the development of serological assays, and that large-scale expression in *E. coli* was possible.

In conclusion, we synthesized a new recombinant VP1 epitope for serotype O FMDV and developed an efficient *E. coli* production system. In present construct (VP1e–GST), VP1e epitope (35 amino acids) itself could be obtained from VP1e–GST by thrombin digestion which cleavage site was present between VP1e and GST, and the preparation of soluble VP1e epitope was also confirmed (Supplementary material). However, we think it is not necessary to remove the GST by protease digestion, which requires labor-intensive purification in the downstream process. As proved here, the VP1e–GST was highly soluble and immunogenic and it can be a promising molecule for FMDV prevention and detection. Using fed-batch cultivation and supplementation with a complex feeding solution, a high yield (3.8 g/l) of antigen (VP1e–GST) was obtained. To the best of our knowledge, this is the first report of large-scale production and purification of soluble, functional FMDV VP1 epitopes in GST-fused format. For other serotypes of FMDV, distinct antigenic sequences, including the VP1 region, have been reported [2, 27, 33]. We believe our strategy for large-scale production can be applied to the production of these other epitopes, and will contribute towards global efforts at FMD prevention, treatment, and eradication.

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