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Real-time SPR characterization of the interactions between multi-epitope proteins and antibodies against classical swine fever virus

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ABSTRACT

The envelope glycoprotein E2 is the major immunodominant protein of the classical swine fever virus and can induce neutralizing antibodies and protective host-immune responses in infected swine. We designed, expressed, and purified multi-epitope protein (GST-BT22) that contains a tandem repeat of the E2 antigenic-determinant residues 693–704, 770–780, and 826–843, each of which is separated by a GGSSGG sequence. In the same manner, we also designed, expressed, and purified a second protein (GST-BT23) that contains a C-terminal sequence consisting of residues 1446–1460 from the classical swine fever virus nonstructural protein NS2-3 separated from the GST-BT22 sequence by a GGSSGG sequence. Western blotting of GST-BT22 and GST-BT23 with serum from a swine that had been experimentally infected with the virus showed that the proteins reacted with anti-serum, whereas GST did not. Surface plasmon resonance was used to quantify the affinities of GST-BT22 and GST-BT23 for serum antibodies ($K_a = 4.31 \times 10^8$ and 5.01×10^8, respectively). GST, used as a control, was reacted an order of magnitude less strongly than did GST-BT22 and GST-BT23. Surface plasmon resonance, therefore, appears to be a sensitive and precise method for epitope evaluation and can be used to characterize the immunogenicity of a recombinant protein.

1. Introduction

Classical swine fever (CSF) is a serious and highly contagious disease of swine, with outcomes that include acute hemorrhagic fever and acute infection [1,2]. The CSF virus (CSFV), the causative agent of CSF, is a member of the *Pestivirus* genus of the Flaviviridae family [3,4]. CSFV particles contain four structural proteins: the core protein C, and the envelope glycoproteins Erns, E1, and E2 [5–7]. E2 has four antigenic domain, A–D. Peptides representing neutralizing epitopes within A, B, and C have been used as anti-CSFV vaccines [8]. Certain multi-epitope formulations induce CSFV-specific neutralizing antibodies that protect against CSFV infection [9,10].

The utility and popularity of surface plasmon resonance (SPR)-based optical biosensors has increased greatly over the last two decades and should continue to increase as the technology becomes more accessible and its applications more varied [11]. The use of SPR-based sensors to monitor biomolecular interactions was first described in 1983 [12] and currently is the favored technology for monitoring molecular interactions, in part because binding events are monitored in real time. Other reasons for using SPR include its accuracy, rapid measurement times, low cost, and label-free reactants [13,14]. Accordingly, SPR biosensors are now also used for highly sensitive competition immunoassays [15].

For the study reported herein, we used SPR to characterize the interactions between two recombinants multi-epitope proteins and anti-CSFV antibodies in serum from a swine. Our study shows that SPR can characterize the antigenic characteristics of swine serum more accurately and objectively than other available methods and provides a substantial step forward toward improving vaccine formulas and other means of controlling CSF.

2. Materials and methods

2.1. Escherichia coli strains, plasmids, reagents, and serum

E. coli BL21 (DE3) cells and all restriction enzymes were purchased from TaKaRa (Dalian, China). T4 DNA ligase and pGEX-6p-1 were obtained from Promega (Madison, WI, USA). Glutathione Sepharose 4B resin and columns were purchased from GE Healthcare. Horseradish peroxidase-conjugated rat anti-pig immunoglobulin G (IgG) was purchased from Sigma (St. Louis, MO, USA). Immobilon-P transfer membranes were purchased from Millipore (USA). Swine sera positive and negative for anti-CSFV immunoglobulin were provided by the Lanzhou Veterinary Research

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Institute of the Chinese Academy of Agricultural Sciences. A Biacore 3000 biosensor, BIAevaluation software version 4.1, Biacore 3000 control software (ver. 4.1), amine-coupling kit reagents, and a CM5 sensor chip were from GE Healthcare.

2.2. Design and chemical synthesis of the CSFV multi-epitope-containing proteins

The multi-epitope protein BT22 contains the E2 epitopes, residues 693–704, 770–780, and 826–843 (arranged in that order), with two copies of the tri-epitope sequence arranged in tandem and with each epitope separated by the linker sequence GGSSGG (Fig. 1A), The multi-epitope protein BT23 contains the BT22 sequence attached via a GGSSGG linker at its C-terminal to residues 1446–1460 from the CSFV non-structural protein NS2-3 (Fig. 1B). The genes encoding BT22 and BT23 were commercially synthesized and individually cloned into pMD18-T plasmids at their *Bam*HI-*Eco*RI sites to form pMD-BT22 and pMD-BT23 (TaKaRa). *E. coli* JM109 cells were chemically transformed to express the plasmid constructs and preserved by puncture.

2.3. Expression and purification of GST-BT22 and GST-BT23

The genes encoding BT22 and BT23 were excised from pMD-BT22 and pMD-BT23, respectively, using *Bam*HI and *Eco*RI and individually cloned into separate *Bam*HI-*Eco*RI–digested pGEX-6p-1 plasmids to generate pGEX-BT22 and pGEX-BT23, respectively. These constructs contained an upstream sequence for GST. The integrity of each construct was verified by restriction analysis and DNA sequencing (TaKaRa). pGEX-BT22 and pGEX-BT23 were chemically transformed into competent *E. coli* BL21 (DE3) cells. Multiple samples of transformed cells were cultured at 37 °C in LB medium contain 100 μg/mL ampicillin to seek the best expression conditions. When each culture reached an OD_{600} of 0.6–1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at one of four different concentrations (final concentration, 0.2, 0.4, 0.6, or 0.8 mM) to compare the expression levels at 37 °C after an additional 6 h of culture. Cultured cells transformed with an empty pGEX-6P-1 and cell cultures that were not induced served as controls. Cells were lysed as described below, and the proteins in the cell lysates were separated by SDS–PAGE (12% w/v acrylamide) to compare protein expression induced by the different concentrations of IPTG.

For purification of GST-BT22 and GST-BT23, cells induced with 0.2 mM IPTG and cultured as described above were centrifuged at 8000*g* for 10 min and then suspended in 50 mL of 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.3, 137 mM NaCl, 2.7 mM KCl, 1 mg/mL lysozyme (final concentration), 0.5 mM phenylmethylsulfonyl fluoride [16,17]. The cell suspensions were each lysed using a Scientz JY92-2D sonicator (400 W, suzhou, China) for 150 5-s pulse cycles followed by a 5-s resting period on ice. Cell debris was removed by centrifugation at 10,000*g* for 15 min at 4 °C. The locations of the proteins, i.e., in the supernatant and/or precipitate, were accessed by SDS–PAGE.

The supernatants, in which most of GST-BT22 and GST-BT23 were found, were individually loaded onto a glutathione Sepharose 4B column (GE Healthcare) equilibrated with phosphate-buffered saline to purify the proteins according to the manufacturer's suggested protocol. Protein concentrations for the collected fractions were measured using the Bradford method [18].

2.4. Western blotting of GST-BT22 and GST-BT23

The immunoreactivities of the proteins were assessed by western blotting [19]. Purified GST-BT22 and GST-BT23 were individually separated by SDS–PAGE and electrophoretically transferred onto a polyvinylidene fluoride membrane in a tank-blotting device (Bio-Rad, USA). After blotting, the membrane was immersed in 5% (w/v) skim milk in phosphate-buffered saline, incubated overnight at 4 °C, and then immunoblotted with a 1:40 dilution of sera from swine that had been infected with CSFV for 1.5 h at 37 °C. The membrane was washed three times (5 min each) with 0.3% (w/v) Tris, pH 7.4, 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.1% (v/v) Tween 20. The membrane was then incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-pig IgG diluted 1:10,000 in PBS. The protein bands were visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma–Aldrich).

2.5. Preparation of the biosensor surface

The binding of GST-BT22 and GST-BT23 to CSFV-specific antibodies in the sera from swine exposed to CSFV was assessed using a Biacore 3000 instrument. GST-BT22 and GST-BT23 were covalently coupled in separate flow cells to a CM5 sensor chip that contained a carboxymethylated dextran surface using reagents from a standard amine-immobilization kit as specified by the manufacturer (Biacore). All assays were performed at 25 °C, in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 Mm EDTA, 0.005% (v/v) P20 surfactant), which served as the running buffer (10 μL/min).

2.6. Optimal immobilization-pH assessment

The electrostatic attraction of a protein to a CM5 chip surface is optimal at a pH that is less than its pI; at pH values <3.5, however,

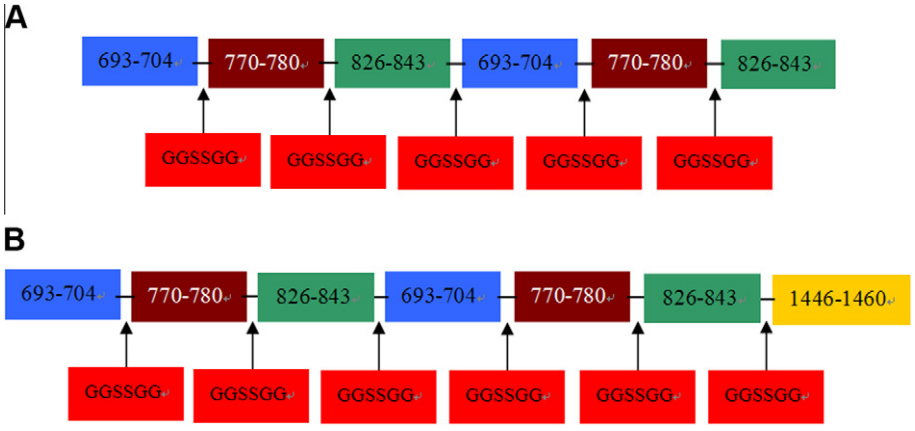

Fig. 1. Design of CSFV multi-epitope proteins. Schematics of the (A) BT22 and (B) BT23 constructs. Residues 693–704, 770–780, and 826–843 are from the CSFV E2 glycoprotein. Residues 826–843 are from the CSFV non-structural protein NS2-3. Each epitope is separated from the next by the linker, GGSSGG.

the carboxymethylated dextran matrix loses its net negative charge, and then proteins cannot bind to the flow cell surface [20]. Therefore, the optimal pH for immobilization of GST-BT22 and GST-BT23 was first identified. To do so required three steps: GST-BT22 and GST-BT23 were each diluted to 0.28 mg/mL in 10 mM sodium acetate at a pH of 4.0, 4.5, 5.0, or 5.5. Each of these solutions (60 μL) was individually injected into the sensor at a flow rate of 10 μL/min. After each sample application, 25 μL of 50 mM NaOH (10 μL/min) was used to clean the sample loop.

2.7. Immobilization of proteins on the CM5 sensor chip

In order to eliminate the GST reaction, We immobilized GST, GST-BT22 and GST-BT23 on a CM5 chip different channel after activating its carboxymethyl dextran surface on one side of each flow cell compartment with a 115-μL mixture (1:1, v/v) of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 0.1 M *N*-hydroxysuccinimide. We then individually injected 210 μL samples of GST, GST-BT22 and GST-BT23 (0.28 mg/mL protein in 10 mM sodium acetate, pH 4.5) into flow cells 1, 2 and 3, respectively, to obtain 8000 resonance units (RU) for each immobilized protein (1 RU equals 1 pg of bound protein/mm^2 of sensor surface). All remaining activated groups were blocked by injection of 75 μL of 1 M ethanolamine into each of the cells. Finally, we removed noncovalently bound GST, GST-BT22 and GST-BT23 by injecting 50 μL of 50 mM NaOH at 10 μL/min. Flow cell 1 was left unmodified to provide a reference, GST-BT22 in flow cell 2, GST-BT23 in flow cell 3 and GST in flow cell 4. Preparation of the cells for the immunoassay was monitored using the Biacore 3000 control software.

2.8. GST-BT22 and GST-BT23 immunoassays

Serum samples were first diluted in HBS-EP buffer (pH 7.4) at ratios of 1:80, 1:160, 1:320, 1:640, and 1:1280. Starting with the largest dilution (lowest concentration) and ending with the smallest dilution (highest concentration), we injected the diluted serum over the GST, GST-BT22, GST-BT23, and reference surfaces, for 180 s at 10 μL/min, respectively. We then observed the rate of dissociation for 150 s, during which only HBS-EP buffer was passed through the cells. Experiments were performed in duplicate. After each binding cycle, the GST-, GST-BT22- and GST-BT23-coated chips were regenerated with 10 mM glycine–HCl, pH 3.0, at 20 μL/min for 30 s. Compared with 50% (v/v) ethylene glycol regeneration, glycine–HCl regeneration was superior. All SPR runs were performed at 25 °C.

The sensorgrams for the interactions between GST-BT22, GST-BT23, or GST and positive serum were recorded in real time and analyzed using BIAevaluation Software version 4.1. Association and dissociation rate constants (k_a and k_d, respectively) and the association equilibrium constant (K_a) were obtained by fitting the association and dissociation rates from the experiments performed with the various dilutions of the CSFV-positive sera to a single-site binding model (1:1 Langmuir binding).

3. Results

3.1. Expression and purification of GST-BT22 and GST-BT23

The recombinant plasmids, pGEX-BT22 and pGEX-BT23, were individually transformed into *E. coli* BL21(DE3), which, after addition of IPTG, expressed recombinant proteins with the calculated value of ~31 kDa (GST-BT22) and ~33 kDa (GST-BT23). Cells that contained an empty pGEX-6P-1 and cells that had not been induced with IPTG did not express a recombinant protein. The observed molecular masses are consistent with those expected for the two proteins. SDS–PAGE results shows that the optimal amounts of the expressed proteins were obtained by induction with 0.6 mM IPTG and subsequent culture for 6 h (data was not shown). The proteins were mostly present in the extract supernatants rather than in the pellets, which allowed the direct purification of the proteins by glutathione Sepharose 4B chromatography. The tagged proteins were eluted with 50 mM Tris–HCl, pH 8.0, 10 mM reduced glutathione. Approximately 5.6 and 5.0 mg/mL of purified GST-BT22 and GST-BT23, respectively, were obtained.

3.2. Immunological characteristics of GST-BT22 and GST-BT23 assessed by western blotting

The specificities of GST, GST-BT22, and GST-BT23 were assessed by western blotting. Purified samples of GST, GST-BT22, and GST-BT23 were first subjected to SDS–PAGE, transferred electrophoretically to a polyvinylidene fluoride membrane, and incubated first with sera from swine that had been infected with CSFV and then with the secondary antibody. Only GST-BT21 and GST-BT23 reacted with serum antibodies. In contrast, no reaction was observed between GST and serum antibodies (data was not shown).

3.3. Preparation of chip surfaces

The amount of protein that can be immobilized on an SPR chip is affected by the electrostatic attraction [preconcentration; 28] of the protein to the chip surface. To determine the optimal pH for preconcentration of GST-BT22 and GST-BT23, we tested 10 mM sodium acetate solutions, at pH 4.0, 4.5, 5.0, and 5.5, and found that pH 4.5 was optimal solution provided the best preconcentration of GST-BT22 and GST-BT23 (Fig. 2), with the density of immobilized the densities of the immobilized proteins being ~22,000 RU. We then used solutions of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxysuccinimide to activate the chip surface to subsequently couple GST-BT22 and GST-BT23 (each in a 10 mM sodium acetate, pH 4.5 solution) to the surface. Primary amines in GST-BT22 and GST-BT23 were then cross-linked to esters on the chip surface to form stable amide bonds.

The final amounts of coupled proteins corresponded to 8000 RU as detected by Biacore 3000 software. Immobilization sensorgrams for the proteins are shown in Fig. 3. To study specificity, we also immobilized GST (8000 RU) on the same chip in a separate flow cell (data was not shown). GST and the swine serum interacted an order of magnitude less strongly than did GST-BT22 and GST-BT23 (Table 1), which allowed us to conclude that the interactions of BT22 and BT23 with swine serum anti-CSFV antibodies were specific (see below).

3.4. Capture assay and binding kinetics of GST-BT22 and GST-BT23

We used SPR to determine the binding affinities of GST-BT22 and GST-BT23 for antibodies in sera from swine exposed to CSFV. The serum sample was twofold serially diluted (dilutions 1:80–1:1280) with running buffer. The sensorgrams indicated that GST-BT22 and GST-BT23 interacted with serum antibodies and that the SPR signal increased as the serum concentrations increased. As noted above, however, binding was substantially weaker for GST and serum, clearly indicating that the interactions were specific for BT22 and BT23 (Fig. 4).

To obtain the kinetic and equilibrium constants for GST-BT22 and GST-BT23 and the serum anti-CSFV antibodies, we used a 1:1 Langmuir model and the sensorgram data (Table 1). The k_a values indicated a very fast association between serum CSFV-specific antibodies and GST-BT23. The dissociation rate constant also pointed to a relatively stable complex formed by immobilized

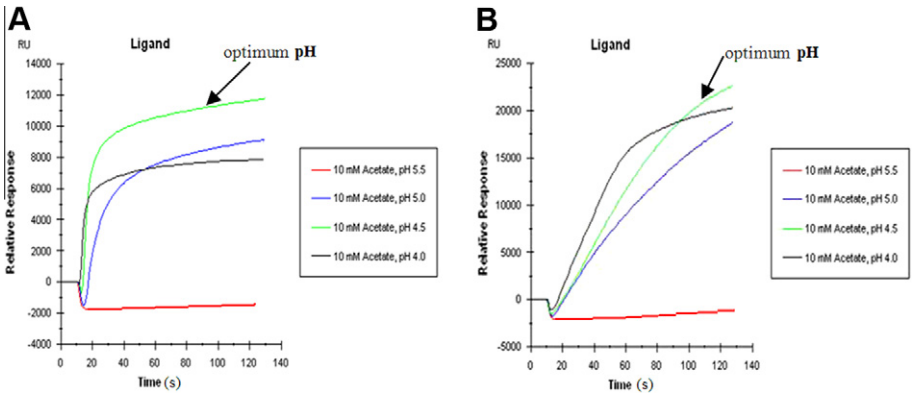

Fig. 2. pH optimization for GST-BT22 and GST-BT23 immobilization on a CM5 sensor chip. The responses for 10 mM sodium acetate solutions adjusted to pH 4.0, 4.5, 5.0, or 5.5 are shown. (A) pH optimization assays for GST-BT22 and (B) pH optimization assays for GST-BT23.

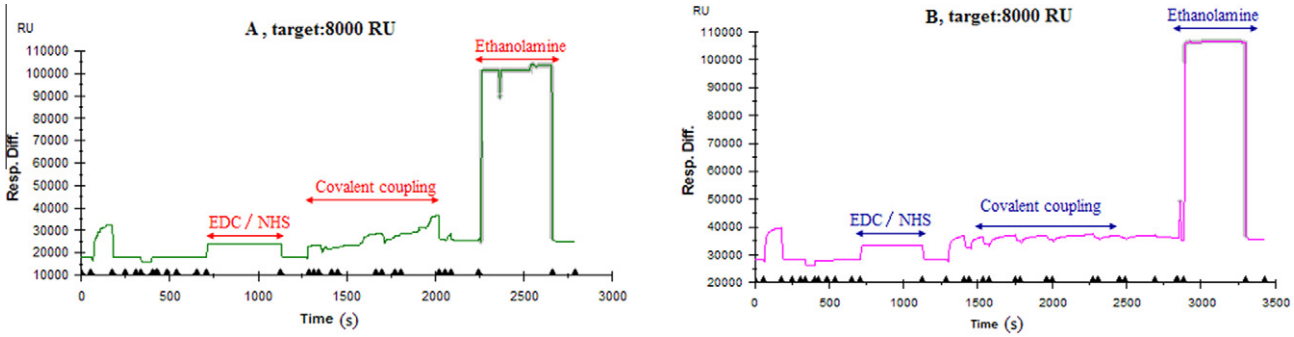

Fig. 3. Sensorgrams showing the immobilization of GST-BT22 and GST-BT22 on the CM5 sensor chip surface. (A) GST-BT22 coupling and (B) GST-BT23 coupling.

Table 1
Kinetic and equilibrium constants for the interaction of GST-BT22, GST-BT23, and GST with antibodies in serum from swine infected with CSFV.

Ligand	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_a (k_a k_d, M^{-1})
GST-BT22	6.72×10^5	1.56×10^{-3}	4.31×10^8
GST-BT23	5.96×10^6	1.19×10^{-3}	5.01×10^8
GST	1.02×10^3	5.36×10^{-3}	1.91×10^7

Kinetic and equilibrium constants were determined by fitting the initial association and dissociation rates and the individual sensorgrams to a 1:1 Langmuir-binding model. k_a, k_d: association and dissociation rate constants, respectively; K_a, equilibrium association constant (K_A).

GST-BT23 and serum antibodies. The K_a value measured for GST-BT22 was 4.31×10^8 M^{-1}, and that for GST-BT23 was 5.01×10^8 M^{-1}. Overall, our data clearly demonstrated a very high affinity and specific interactions between BT22 and BT23 and components of the sera of swine exposed to CSFV, and this epitope-combination may be multi-epitope candidate for genetically engineered CSFV vaccines.

4. Discussion

Peptides containing multiple copies of epitopes are usually considered the best vaccine options because they are safe, multivalent, result in effective presentation of the antigen, and are easy to delivery. A mixture of five peptides that contain overlapping sequences between residues 693 and 777 of E2 has been proposed as a possible CSFV-specific marker vaccine [13]. Animal trials indicated that the polypeptider GST-4E, which contains four copies of the E2-epitope TAVSPTTLR, is more immunogenic than the peptide containing a single copy of the epitope and effectively induces protective immunity against CSFV infection of swine [14]. Another study, involving the multi-epitope construct rE2-ba, which contains the rE2-a and rE2-b epitopes from E2 (residues 844–865 and 693–716, respectively), demonstrated that this construct confers greater protection against CSFV infection than does rE2-a or rE2-b alone [21]. These studies highlight the potential of multiple-epitope proteins to act as novel marker vaccines.

Not all epitopes can be used as vaccines. Seasonal outbreaks of influenza remain a major public health concern, and manufacture of traditional egg-based vaccine precludes rapid production of large vaccine quantities, which may be especially problematic when confronting pandemic strains, e.g., H5N1 [22], and prompts the development of vaccines that can be produced more efficiently. However, rapid and simple analytical tools are also required [23,24]. SPR biosensors may address these needs by providing prophylactic and therapeutic approaches to control viral diseases such as influenza and CSF.

In the study reported herein, the multi-epitope BT22 was designed to contain two tandemly arranged copies of the epitopes containing residues 693–704, 770–780, and 826–843 from the CSFV protein E2 (Fig. 1A). BT23 was designed to contain BT23 coupled C-terminally with residues 1446–1460 from the CSFV nonstructural protein NS2-3, with (for both proteins) the linker sequence GGSSGG inserted between the epitopes to preserve the independent immunological activities of the epitopes [25–27]. A protein containing tandemly arranged epitopes involving residues 693–777 from CSFV E2 protected swine from a lethal challenge by CSFV only when coupled to a large carrier protein [28,29].

SPR is a surface-sensitive technique that is ideal for studying interactions between immobilized biomolecules and solution-phase analytes because the kinetic constants (k_a and k_d) are directly obtained from sensorgrams and labeling of reagents is not

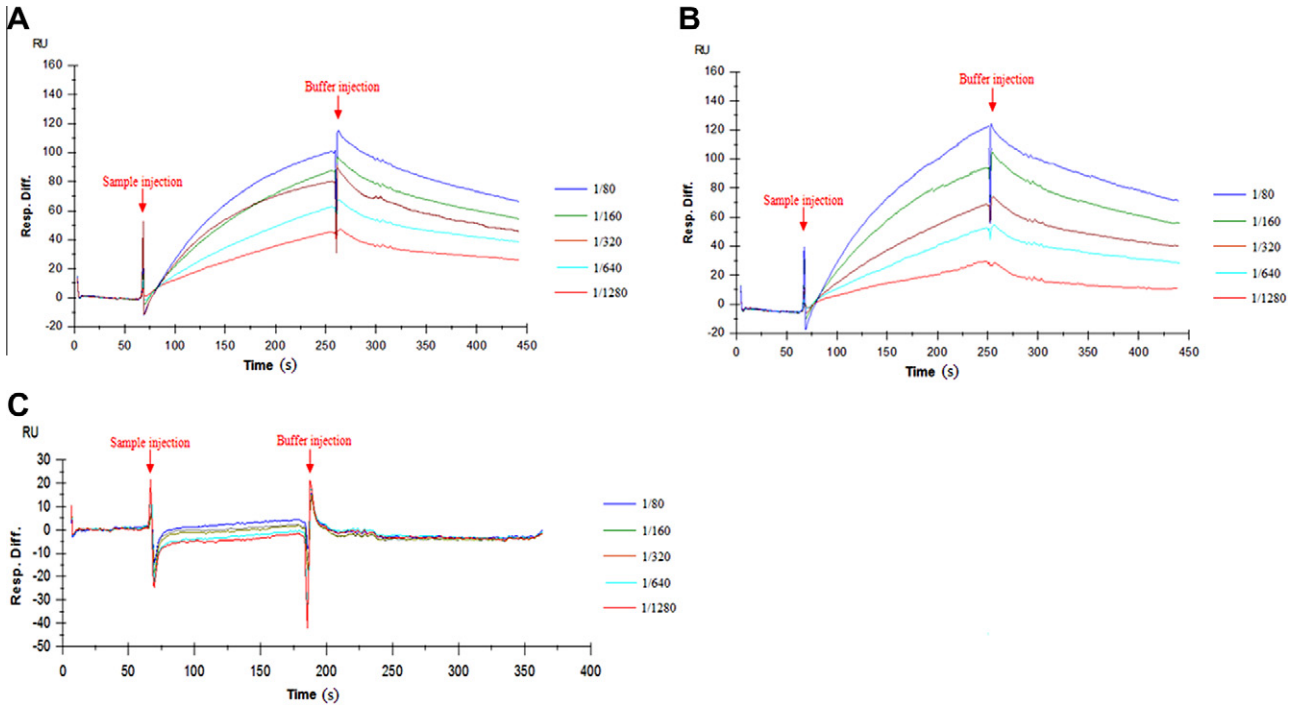

Fig. 4. Kinetics of the association and dissociation of GST-BT22 and GST-BT23 with components of sera from swine that with CSFV. Sensorgrams for (A) GST-BT22, (B) GST-BT23, and (C) GST. CSFV-positive serum was diluted 1:80, 1:160, 1:320, 1:640, and 1:1280.

needed; thus, observation of the reactions is made in real time [30]. Our SPR study demonstrates that serum components bind GST-BT22 and GSTBT23 and confirms the value of SPR for efficient characterization of antigen–antibody interactions. Our results suggest that GST-BT23 and GST-BT23 may be promising diagnostic reagents to detect CSFV infection of swine, even in populations that have been vaccinated against the virus.

For the study reported herein, we subcloned the genes encoding the multi-epitope proteins BT22 and BT23 into pGEX-6p-1 vectors and obtained high yields of soluble, bacterially expressed GST-BT22 and GST-BT23. Western blotting demonstrated that the proteins are highly reactive with antibodies in the sera of swine that infected with CSFV.

We then used SPR to quantitatively evaluate the interactions of GST-BT22 and GST-BT23 with serum components. This surface-sensitive technique is ideal for studying interactions between immobilized biomolecules and solution-phase analytes because the kinetic constants (k_a and k_d) are directly obtained from sensorgrams and labeling of reagents is not needed; thus, observation of the reactions is made in real time [31,20]. Our SPR study demonstrates that serum components bind GST-BT22 and GSTBT23 and confirms the value of SPR for efficient characterization of antigen–antibody interactions.

We have successfully constructed an efficient system for the expression and purification of GST-BT22 and GST-BT23 in *E. coli*. Western blotting and SPR demonstrated the high reactivity of the multiple-epitope peptide BT22 and BT23 with components of sera from swine infected with CSFV, suggesting that GST-BT23 and GST-BT23 may be promising diagnostic reagents to detect CSFV infection of swine, even in populations that have been vaccinated against the virus.

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