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Screening of High-Affinity scFvs From a Ribosome Displayed Library Using BIAcore Biosensor

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Abstract An experimental protocol was developed to screen high-affinity single-chain Fv antibody fragments (scFvs) from a *Xanthomonas axonopodis* pv. *citri* (Xac) immunized ribosome display library using BIAcore biosensor. The screening methods involved immobilizing antigen [lipopolysaccharides (LPS) of Xac] on sensor chip HPA and then unpurified expression products of scFvs flowing over the immobilized sensor chip. The affinity-improved scFvs were selected based on dissociation rate constants (k_d). Thirty-five enzyme-linked immunosorbent assay-positive scFvs were analyzed by BIAcore, and three of those (scFv A1, B2, and C5) with lower k_d were screened. To demonstrate the accuracy of the screening method, the three scFvs were expressed in *Escherichia coli* HB2151 and purified. The purified scFvs were subsequently further identified according to association rate and affinity constants. The results showed that the three scFvs (A1, B2, and C5) had high affinity for LPS of Xac (3.51×10^{-11}, 1.13×10^{-10}, 5.06×10^{-10} M, respectively). Furthermore, the scFv B2 was highly specific for LPS of Xac and had no any cross-reactions with bovine serum albumin and LPS from Xac-related bacteria. This provided evidence that the information from the BIAcore screening assay could be accurate.

Keywords scFv · Ribosome display · BIAcore · Kinetic selection

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Abbreviations

scFv	single chain antibody Fv fragment
ELISA	enzyme-linked immunosorbent assay
BIAcore	biomolecular interaction analysis
SPR	surface plasmon resonance
k_d	dissociation rate constants
k_a	association rate constants
K_D	equilibrium dissociation constant
RU	response units

Introduction

Screening of specific single chain Fv antibody fragments (scFvs) by ribosome display greatly facilitates determination of specific binders from a large pool of different antibody specificities [1–4]. The source of V-regions can be derived either from immunized [1, 3, 4] or non-immunized individuals [2], the latter providing so-called native antibody libraries. Once an antibody library is screened and evolved by ribosome display, the antigen-positive scFvs in the library will be enriched greatly. Panning of positive scFvs with improved affinity from the enriched library is commonly performed by immunoassays, such as enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA). When screening of scFvs by immunoassays, periplasmic extracts or culture supernatants containing soluble scFvs are used for the experiment, but the expression levels of scFvs are highly variable. ELISA is an equilibrium method, and the results are correlated with the expression levels of scFvs, so it cannot determine the affinity of scFvs, and candidates with high off rates might be lost. Therefore, it needs an advanced technology to select high-affinity scFvs from enriched antibody library.

In recent years, BIAcore (biomolecular interaction analysis) biosensors were developed to select or evaluate antibodies [5–9]. BIAcore is a surface plasmon resonance (SPR)-based system for analyzing biospecific interactions. The protocol involves one interacting molecule that is immobilized to a sensor chip and its counterpart injected into a continuous buffer flow. The interaction is monitored in real time, thereby offering the possibility of calculating the kinetic rate constants for the interaction. The most obvious advantages of BIAcore over ELISA are direct and rapid determination of association and dissociation of binding process, no need for labeling of protein or lipids, and small amounts of sample used in the assay (often nanomolar concentrations of proteins) [10].

In the current study, most of the reports about BIAcore-based antibody experiments were regarded as screening antibodies against proteins from phage libraries or evaluating antibodies [11–17]. During the course of scFv screening, the protein antigens are immobilized onto the most widely used CM5 sensor chip in which carboxymethylated dextran is covalently attached to a gold surface and molecules are coupled to the sensor surface via amine, thiol, aldehyde, or caboxyl groups. There were no reports about screening scFvs against lipopolysaccharides (LPS) which were immobilized on HPA sensors from a ribosome displayed library by BIAcore biosensor. The HPA sensor chip is a flat hydrophobic surface consisting of long-chain alkanethio, and molecules are attached directly to the gold film. The flat hydrophobic sensor surface facilitates adsorption of lipid monolayers for analysis of interactions involving lipid components. Here, an experimental protocol was developed to select high-affinity scFvs from a ribosome

displayed library by BIAcore. It could provide some references for screening antibodies with improved affinity.

Materials and Methods

Preparation of Materials

Xanthomonas axonopodis pv. *citri* (Xac) strain Gxo4 was isolated from infected leaves of sweet orange and cultured using potato dextrose agar medium at 28 °C for 72 h and then stored in the Genetic Engineering Center of Chongqing University. LPS were isolated and purified according to Albrecht [18]. Binding experiments were performed using BIAcore X optical biosensors (Biacore, Piscataway, NJ, USA). Sensor chip HPA, sensor chip CM5, and amine-coupling reagents (EDC, NHS, and sodium ethanolamine HCl, pH 8.5) were obtained from Biacore (Biacore). Anti-E antibody, vector pCANTAB 5E, *Escherichia coli* TG1, and *E. coli* HB2151 were purchased from Amersham UK.

Preparation of Ribosome Displayed Antibody Library

The scFv library against Xac were constructed from spleen messenger RNA of immunized mice in Genetic Engineering Center of Chongqing University, and the library was enriched and evolved three rounds by ribosome display according to Yuan [19]. The enriched library DNA was cloned into *E. coli* TG1, and single clone was isolated for expression using the vector pCANTAB 5E. The soluble periplasmic scFvs were extracted with TES buffer (0.2 M Tris pH 8.0, 0.5 M sucrose, 1 mM EDTA) and detected by ELISA according to Yuan [19], and then the ELISA-positive scFvs were used for the next BIAcore analysis.

Immobilization LPS on HPA Sensor Chip

Binding of scFv to LPS of Xac was assessed with a BIAcore X system. LPS were immobilized on a hydrophobic HPA sensor chip. The chip was pre-cleaned and conditioned by washing twice with 40 mM noctyl glucoside (Sigma). Then, 120 μl of LPS preparations (1 mg/ml) was dispersed in 100 mM NaCl containing 10 mM HEPES buffer (pH 7.4) and passed across the chip's surface for 1 h at a flow rate of 2 μl/min. Residual unbound LPS were washed away with 0.1 M HCl and 0.1 M NaOH. Bovine serum albumin (BSA; 0.1 mg/ml) was used to detect nonspecific binding on the LPS-coated surface.

scFv Screening

ScFv screening was performed at 25 °C. The screening buffer (HEPES buffer) was prepared and vacuum-filtered and then degassed immediately before use. The soluble periplasmic scFvs were super-filtrated to remove the high concentration iron in TES buffer and reduce the effects of refraction of iron. Then, the filtrated scFvs were diluted with HEPES buffer and subsequently centrifuged at 14,000 rpm for 5 min at 4 °C. Each scFv was injected (2-min association, 5-min dissociation) at a flow rate of 20 μl/min over the LPS-immobilized surface, and the expression products of blank plasmid were used as control. Each binding was done in triplicate, and the chip was regenerated between trials by washing it with 0.1 M HCl and 0.1 M NaOH. The dissociation of scFv to LPS was

measured by observing the change in the SPR angle, and the dissociation rate constants (k_d) was calculated by evaluation software. As the dissociation constant had no correlation with the concentration of scFvs, the affinity of each scFv could be preliminarily determined according to the k_d.

Expression of Soluble Antibody Fragments in HB2151

Soluble recombinant antibodies could be expressed either in TG1 or HB2151 cells, but the yield of soluble antibodies would be much higher in *E. coli* HB2151 cells due to these non-suppressor cells producing only soluble antibodies. Once a positive scFv with improved affinity had been isolated using BIAcore biosensor, the corresponding TG1 recombination clone is infected with M13KO7 helper phage to rescue the phagemid with its scFv gene insert, and then the phage is used to infect log phase *E. coli* HB2151 cells for the purpose of large-scale production of soluble antibodies. Soluble functional scFv fragments bearing a 13-amino acid peptide tag (E-tag) were expressed in *E. coli* HB2151. Briefly, 5 ml of overnight culture was added to 50 ml of freshly prepared 2× YT medium containing 100 μg/ml ampicillin and 2% (*w*/*v*) glucose, and the cells were incubated for 1 h at 30 °C with shaking at 250 rpm. Then, the cells were pelleted and resuspended in 50 ml of freshly prepared 2× YT containing 100 μg/ml ampicillin, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubated for 5 h at 30 °C with shaking at 250 rpm. The soluble periplasmic scFvs were extracted with TES buffer. The expressed scFvs were purified with HiTrap anti-E tag affinity column according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA).

Western Blot

The TCA-precipitated soluble periplasmic scFvs were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% gel), and the gel was blotted onto polyvinylidene fluoride (PVDF) membrane using a semi-dry electroblotter (Bio-Rad). After blocking with 5% skim milk in phosphate-buffered saline, the membrane was incubated for 1 h at room temperature with anti-E tag antibody (Amersham, UK). A secondary antibody that was conjugated to an alkaline-phosphatase-labeled goat anti-mouse IgG was used to detect the bound anti-E tag antibody. Detection occurred after addition of NBT/BCIP substrate (Sigma, Poole, UK).

Affinity Analysis

The LPS was immobilized on HPA sensor chip according to the above process steps. ScFv binding was measured by observing the change in SPR angle of the LPS bound to the chip as 30 μl of purified scFv (2, 4, 8, 16 nM) flowed over the LPS at a rate of 20 μl/min. These scFv solutions were also prepared in HEPES buffer. Each binding study was done in triplicate, and the chip was regenerated between trials by washing it with 0.1 M HCl and 0.1 M NaOH. The affinity constant (equilibrium dissociation constant, K_D) was calculated by evaluation soft and Scatchard plots analysis.

Specificity Analysis

The purified scFv was diluted in 10 mM sodium acetate buffer (pH 4.5) and immobilized on CM5 sensor chips (Biacore, Inc.) using amine coupling kit obtained from manufacturer.

The surface with immobilized scFv was instable over time and was therefore freshly prepared for each set of experiments. The LPS of Xac, *X. oryzae* pv. *oryzae* (Xoo), *X. campestris* pv. *campestris* (Xcc), *X. oryzae* pv. *oryzicola* (Xoc) were extracted and diluted in HBS buffer (10 mM HEPES with 3 mM EDTA, 0.005% surfactant P20, and 150 mM NaCl, pH 7.4), respectively. BSA (0.1 mg/ml) was used as negative control. The prepared 30 μl of LPS (0.1 mg/ml) of each bacterium flowed over the scFv-immobilized chip at a rate of 20 μl/min, respectively, and the association and dissociation were measured. Surface regeneration was done by washing the chip with 10 mM glycine, pH 2.5.

Result

Immobilization of LPS on HPA Sensor Chip

The constructed scFv library was enriched and evolved three rounds by ribosome display. The library DNA from three rounds of display was cloned into *E. coli* TG1, and single clone was cultured for expression. Soluble functional scFv fragments were expressed in periplasmic space of TG1 cells. Before scFv screening by BIAcore, ELISA was first used for selecting antigen-positive scFvs. Then, SPR-based BIAcore system was used for panning scFvs with improved affinity from the ELISA-positive individuals. The first step of BIAcore assay was antigen immobilization. The LPS of Xac were immobilized on the sensor chip HPA, and the total response units (RU) generated for the immobilization was more than 1,000. Loosely bound molecules were washed away by short pulse injection of 100 mM NaOH. Injection of 0.1 mg/ml BSA prepared in the HEPES buffer were passed

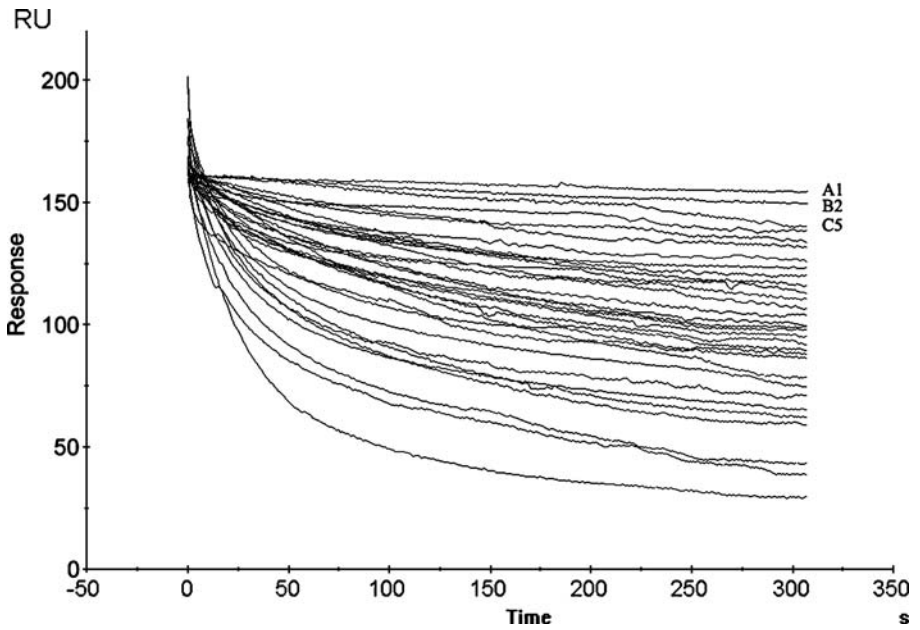

Fig. 1 The dissociation phase of the interaction between immobilized LPS and soluble periplasmic scFvs from displayed ribosome library

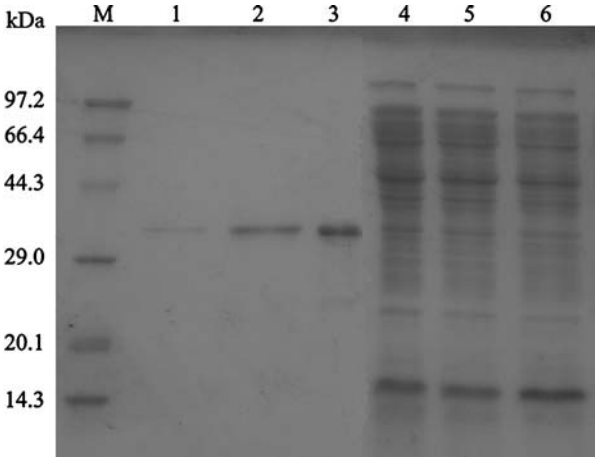

Fig. 2 SDS-PAGE analysis of the three selected scFvs (A1, B2, and C5). Lane *M*: Low-molecular-weight calibration kit; lanes *1–3*: purified scFv A1, B2, and C5; lanes *4–6*: the unpurified soluble periplasmic scFv C5, B2, and A1

across the LPS-coated surfaces for a 5-min period. It showed an increase of less than 50 RU, whereas the same amount of BSA pass across the uncoated, *n*-octyl glucoside washed surface showed an increase of roughly 1,000 RU. This demonstrated that the surface of the chip was fully covered by LPS.

ScFv Screening

Recombination clones were isolated from the three rounds of displayed antibody library by ribosome display and expressed in *E. coli* TG1. Thirty-five ELISA-positive periplasmic scFvs were prepared and flowed over the LPS-immobilized sensor chip HPA. The results

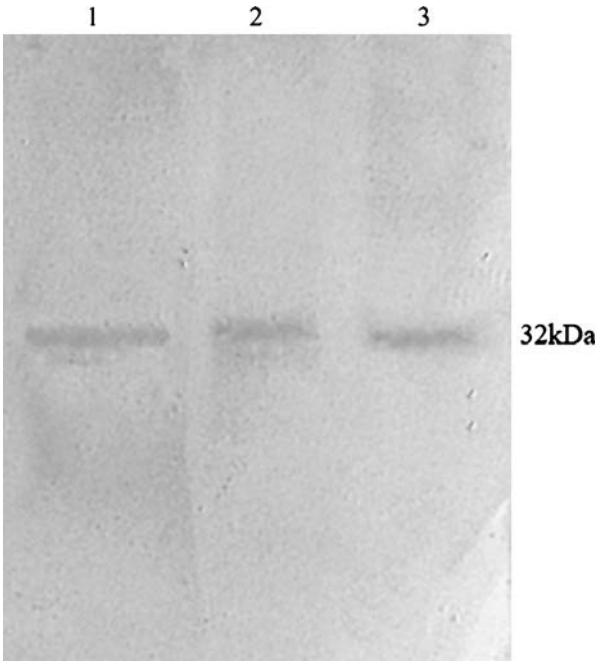

Fig. 3 Detection of soluble scFvs in periplasmatic extracts by Western blot. The scFvs were detected with an anti-E Tag antibody and alkaline phosphate conjugated antibody (anti-mouse IgG) using BCIP/NBT as substrate. Lanes *1–3*: periplasmatic extracts derived from clones A1, B2, and C5

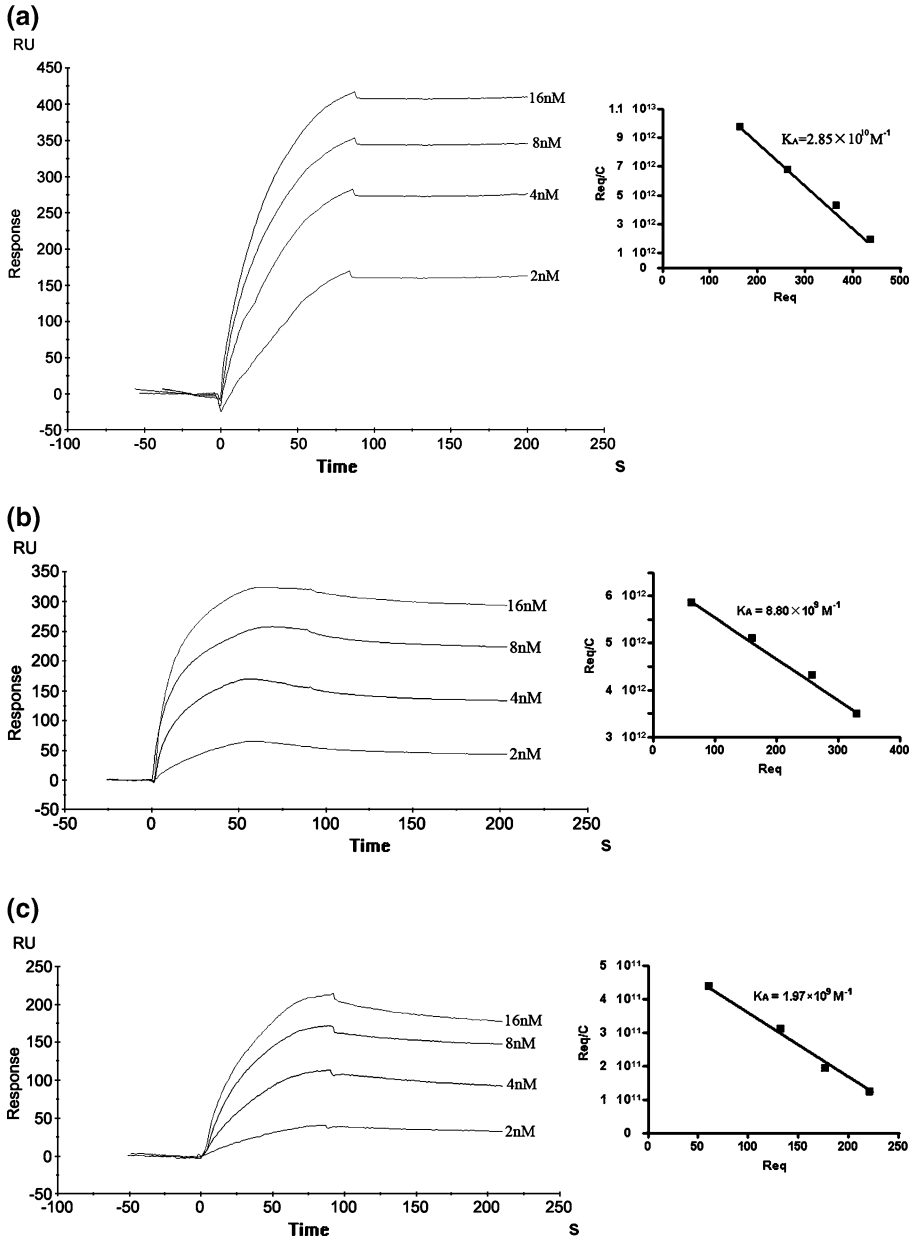

Fig. 4 Analysis of the interaction between different scFv concentrations and LPS of Xac by BIAcore. The scFv concentrations were 2, 4, 8, and 16 nM, respectively. **a** scFv A1; **b** scFv B2; **c** scFv C5. The *insets* are Scatchard plots of the data: R_{eq} is the response in response units when the binding reaches equilibrium, and C is the concentration of scFv. A plot of R_{eq} versus R_{eq}/C has a slope of $-K_A$

showed that 31 of those had an antigen-binding activity, and the other four scFvs had no binding with the LPS antigen. The response unit of the 31 scFvs changed around 100–300, but the other four scFvs and the expression products of blank plasmid only had an increase of under 20 RU. The dissociations of the 31 scFvs are shown in Fig. 1, and the calculated

Table 1 The association rate constant (k_a), dissociation rate constant (k_d), and the equilibrium constant (K_D) were determined by BIAcore analysis for A1, B2, and C5, respectively.

scFv	k_a ($M^{-1}\cdot s^{-1}$)	k_d (s^{-1})	k_a/k_d (K_A) M^{-1}	k_d/k_a (K_D) M
A1	2.82×10^{6}	9.89×10^{-5}	2.85×10^{10}	3.51×10^{-11}
B2	2.12×10^{6}	2.41×10^{-4}	8.80×10^{9}	1.13×10^{-10}
C5	1.24×10^{6}	6.28×10^{-4}	1.97×10^{9}	5.06×10^{-10}

dissociation rate constants by evaluation soft were around in 10^{-2}–10^{-5}. Three scFvs of those (A1, B2, and C5) with the least k_d were selected for further analysis.

Expression and Purification

To confirm that the selected clones contained the target scFv antibodies, the corresponding phages were used to infect HB2151 cells for the purpose of large-scale production of soluble antibodies. The soluble scFvs could be produced in culture supernatant or periplasmic extract. With prolonged IPTG induction (up to 24 h), the yield of soluble scFvs in culture supernatant would increase. In our study, the selected three scFvs were expressed by IPTG induction for 5 h, and the produced soluble scFvs existed mainly in periplasmic space of HB2151 cells. Purification of soluble scFvs was performed with HiTrap anti-E tag affinity column. The purified scFvs were run on a denaturing polyacrylamide gel, and protein bands of approximately 32 kDa were visualized by Coomassie brilliant blue staining (Fig. 2). In addition, the TCA-concentrated periplasmic extracts on the denaturing polyacrylamide gel were transferred to PVDF membrane for Western blot analysis and 32-kDa bands were apparent (Fig. 3). It indicated that the selected clones contained the target scFv antibodies and it had no false-positive results during the course of scFv screening by BIAcore.

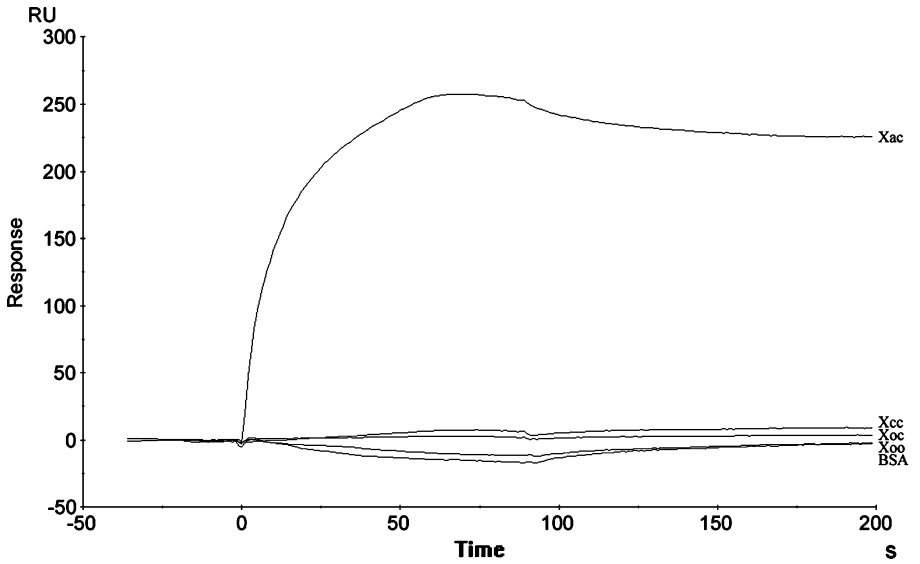

Fig. 5 Analysis of the specificity of scFv B2 against LPS of Xac, Xcc, Xoc, and Xoo by BIAcore biosensor

Affinity Analysis

The affinities of the three scFvs were determined using surface plasmon resonance analysis. Injection of LPS from Xac was stopped when the RU reached 1,000. BSA in HEPES buffer (0.1 mg/ml) was passed across the LPS-coated HPA surface for a 5-min period, and it showed an increase of around 50 RU. This indicated that the surface of the chip was fully covered by LPS. When different scFv concentrations between 2 to 16 nM were run across the surface of HPA, the binding studies suggested that the three scFvs showed significant specific binding activity to LPS. The affinity constant (K_D) were determined independently by global fit and Scatchard analysis. The calculated K_D for scFv A1, B2, and C5, as determined by both analytical methods, were 3.51×10^{-11}, 1.13×10^{-10}, and 5.06×10^{-10} M, respectively. The on and off rate for scFvs are shown in Fig. 4 and Table 1.

To demonstrate that BIAcore-based assay for screening was more accurate than ELISA, the unpurified scFv A1, B2, and C5 in periplasmic extracts were tested three times by ELISA, and the absorbances at OD_{405} were 0.73, 0.85, and 0.62. Then, the different concentrations of purified scFv A1, B2, and C5 were assayed in triplicate for their affinity by competition ELISA. The results showed that the affinities of those scFvs were 3.41×10^{-11}, 1.08×10^{-10}, and 5.02×10^{-10} M, respectively, which were accorded with the results of BIAcore. It indicated that ELISA results were correlated with the expression levels of scFvs and could not determine the affinity of scFvs.

Specificity Analysis

Binding kinetics was determined by SPR using BIAcore X biosensor system. The surfaces of CM5 sensor chips were immobilized with the purified scFv A1, B2, and C5, respectively, using amine coupling kit. The total RU generated for the immobilization reaction units of scFv A1, B2, and C5 were 5,200, 5,050, and 5,010. When the LPS of Xac, Xcc, Xoc, Xoo, and BSA were run across the scFv-coated chip, scFv A1, B2, and C5 gave a substantial binding to the LPS of Xac and no binding to BSA. Moreover, scFv B2 had no cross-reactions with the LPS from related bacteria Xcc, Xoc, and Xoo (Fig. 5). It showed that the BIAcore biosensor method not only could be used for screening scFv with high affinity but also could be used for analyzing the specificity of scFv.

Discussion

BIAcore biosensor had been utilized throughout the different steps of generating antibodies by genetic engineering, i.e, screening, selection, characterization, and epitope mapping [5–17, 20]. In our study, the scFvs anti-LPS of Xac were screened by BIAcore biosensor from a ribosome displayed library. Here, traditional methods such as ELISA or RIA could also be used for panning, but the immunoassay was an equilibrium method; thus, candidates with high off rates might be lost. BIAcore biosensors had a number of advantages over immunoassays. First, BIAcore technology could be used to measure complex formation without labeling the reactants. Seconds, complex formations could be monitored in real time, providing detailed information about the reaction kinetics and equilibrium dissociation constants (affinities). Third, samples from crude preparations might be analyzed. Of course, there were limits to this technology, most of which could be minimized by careful assay design and experimental execution. For example, it was important to design the antibody assay properly to avoid misinterpretation of the reaction

data. Using BIAcore biosensor, bacterial culture supernatants could be directly injected into the biosensor without any further purification, and the concentration of antibody fragments need not be known if we based the selection on dissociation rate constants (k_d). This had been demonstrated by Hawkins and Duenas [11, 13]. In our study, scFvs were expressed in *E. coli* TG1 and the periplasmic extracts contained soluble scFvs were collected. To eliminate the effects of high concentration of iron in periplasmic extracts on BIAcore experiment, the solution TES containing scFvs were changed with HEPES buffer by membrane filtration.

During LPS immobilization process, it was important to cover the sensor chip surface as completely as possible. Maximum responses usually reached in the region of 1,000–1,500 RU. If the response was significantly lower, this might be an indication that surface coverage was inadequate. The tendency of the hydrophobic surface on sensor chip HPA to bind proteins indiscriminately was largely eliminated when the surface was covered by a lipid monolayer. To get an indication of surface coverage, performing a 5-min injection of BSA was used to estimate the effect of immobilization. When the sensor chip was saturated with LPS antigen, injection of BSA would not bind to the chip and the RU would not increase. Injecting BSA or another irrelevant protein before the assay could help to reduce unwanted binding of sample components to the surface by blocking any exposed hydrophobic areas on the sensor chip.

After the LPS of Xac were immobilized on the sensor chip HPA, the scFvs were subsequently injected for analyzing of antigen–antibody interactions. Here, 35 ELISA-positive soluble periplasmic scFvs were tested using BIAcore. The results showed that only 31 of those had binding activity with LPS of Xac, and their binding RUs (response units) were around 100–300, while those of the other four scFvs and the blank plasmid control were under 20. To clarify this point, the four scFvs were further purified and analyzed in triplicate by ELISA and BIAcore again. The results showed that they had no any binding activity to antigen. It indicated that the four scFvs were false positive in ELISA screening assay and suggested that Biacore assay for screening scFvs was more accurate than ELISA. When BIAcore assay was over, the dissociation rate (k_d), but not the association rate (k_a) of the 31 scFvs, were used to preliminary evaluation their affinity. Comparison of the k_{ds} of the 31 scFvs, three of those with lower k_d, were selected for further research by expression and purification. The SDS-PAGE and Western blot results indicated that the expression and purification products of the three scFvs were the aim proteins. A serial-diluted scFvs were assayed by BIAcore, and the three selected scFvs had significant affinity with the LPS of Xac. Furthermore, one of those (scFv B2) showed a high specificity to LPS of Xac, and no cross-reactions were observed with the LPS of Xac-related bacteria (Xcc, Xoo, Xoc). It indicated that the protocol of scFv screening by biacore was feasible and reasonable.

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