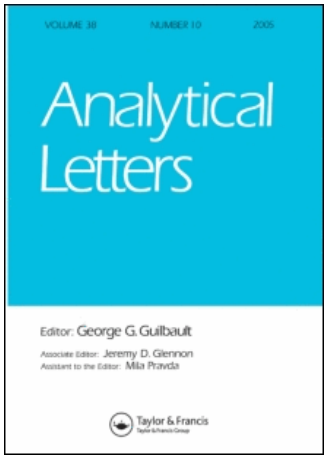

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Construction and Characterization of an Anti-Prion scFv Fusion Protein Pair for Detection of Prion Protein on Antibody Chip

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Abstract: A pair of single chain Fv fragment (scFv) fusion proteins were constructed and characterized. Antibody chips using the pair were designed for sensitive detection of prion protein. Phage displayed antibody library was synthesized by immunizing mice with thioredoxin-mature bovine prion fusion protein (TrxA-bPrP^c). After five rounds of panning against recombinant bovine prion protein (rb-PrP^c) and ELISA test, two positive clones with high affinity to rb-PrP^c, named Z163 and Z186, were obtained. They were conjugated with a linker-streptavidin binding protein (SBP) or human IgG1 constant fragment (Fc) to form the scFv fusion protein pair Z186-L-SBP/Z163-Fc. Western blot experiments showed that the scFv fusion pair specifically interacted with the line epitopes of the protease resistant core region bPrP27-30. Surface plasmon resonance (SPR) sensorgrams revealed that the equilibrium dissociation constants of the interactions with rb-PrP^c were 3.24×10^{-8} M, 8.82×10^{-8}M, and 8.10×10^{-9} M for Z186-L-SBP, Z163, and Z163-Fc, respectively. All binding reactions followed rapid association and slow dissociation kinetics. As a detection pair, Z186-L-SBP functioned as a capture probe and was immobilized on the streptavidin coated slides to form reactive layer of the antibody chip, and Z163-Fc labeled with fluorescence dye Cy3 functioned as a detection probe generating fluorescence signal. The antibody chip could detect existence of rb-PrP^c with detection limit of 1 pg/ml.

Keywords: Bovine prion protein, scFv, phage display, fusion protein, antibody chip

1. INTRODUCTION

Transmissible spongiform encephalopathies (TSE) are a group of rare, 100% fatality rate, and transmissible neurodegenerative diseases. One of the known symptoms of TSE is mad cow disease, which is caused by abnormal form (PrP^{sc}) of the normal host-encoded prion protein (PrP^c) (Prusiner 1982). Since transmission of the prion pathogen from livestock to human has been discovered, the disease has soon become worldwide concern and a big threat to the world livestock trade. To prevent the human being and livestock from infection of the pathogen, it is critical to build sensitive and specific detection means.

PrP^c can be hydrolyzed by the protease K. It is found that when conversion of PrP^c to PrP^{sc} occurs, it leads to a significant increase in β-sheet content and decrease in α-helical content. As a consequence of this conformation change, the prion protein becomes resistant to the protease hydrolysis. A bPrP27-30 fragment of PrP^{sc} is known as the protease-resistance core (Prusiner 1998). So, PrP^c and PrP^{sc} can be discriminated by checking their sensitivity to the protease. So far, the immunoassays of the bovine prion pathogens are mainly based on this mechanism.

Immunoassay of the prion proteins can be performed by either enzyme-linked immunoadsorbent assay (ELISA) or Western blot. The antibodies can be monoclonal antibodies (mAbs) or single chain antibodies. The latter are usually the variable fragments of mAbs (scFvs). mAbs to PrP^{sc} or PrP27-30 were usually generated by hybridoma technique through immunizing PrP-null mice with recombinant PrP (Williamson et al. 1996), while the scFvs could be obtained from phage display. In order to break immunological tolerance, synthetic peptides spanning PrP epitopes were successfully used to produce PrP specific mAbs (Harmeyer et al. 1998). PrP specific antibodies have also been rescued by immunization of animals with PrP DNA (Krasmann et al. 1996). Compared with mAbs, scFvs have large reduction in size and significant decrease in immunogenicity. They can be mass prepared through fermentation of microorganism or cell culture. In addition, scFvs can be easily genetically engineered into various forms, e.g., multivalent antibodies, scFv-Fc body, or fusion with signal molecules, and so on enhancing their flexible applications. ScFvs against murine recombinant prion protein had already been selected from a synthetic human antibody phage display library. The scFvs could recognize a truncated form of murine PrP^c and full-length mouse PrP^c (Leclerc et al. 2000). Ono et al. (2003) constructed an antiprion scFv-Fc fusion protein by recombinant animal cells from a chicken antihuman prion protein mAbs and the Fc region of human IgG1, and the resulting structure showed more stable in vivo protein. A scFv directly antihamster PrP^{sc} was isolated from a synthetic phage antibody library, showing immunoglobulin-like functions using an in vitro procedure, thus overcoming limitations represented by the poor immunogenicity of PrP for conventional monoclonal antibodies preparation (Ascione et al. 2005). Two antiprion scFvs were generated directed against different epitopes, each tagged with different function tags, allowing the study of their specific effects on the synthesis, maturation, and processing of endogenous PrP^c and on PrP^{sc} formation (Cardinale et al. 2005). These recent progresses show the opportunities of using scFvs to set up more efficient detection means.

Here, we report a new pair of scFv (Z163 and Z186) to rb-PrP^c, which were screened from the synthetic phage displayed antibodies library. Z186 was conjugated with the streptavidin binding peptide (SBP) to form Z186-L-SBP fusion, and Z163 was fused with the human IgG Fc fragment (Fc) to form Z163-Fc, through gene fusion technology. Bioactivities of all partners of the fusion structures were characterized. An antibody chip was designed using

the scFv fusion pair as capture and detection probes for specific and sensitive detection of prion proteins. The experiment results are presented herein.

2. EXPERIMENTAL

2.1 Materials

E. coli AD494 (DE3) and plasmid pET32a (+) expression vector were from Novagen. *E. coli* TG1 cells (K12 (lac-pro) supE thi hsd 5/F' traD36 (proABlacIq lacZM15)) were provided by Amersham Biosciences, USA, mAb KG9 was donated by Dr. C.R. Birkett and the TSE Resource Center, England. Amersham Biosciences supplied the mouse scFv DNA construction kit; phage-displayed scFv expression and detection kits; anti-M13 tag antibody; and pCANTAB-5E phagemid. Restriction enzymes, mRNA isolation kit, reverse transcriptase, Taq DNA polymerase, and T4 DNA ligase were bought from Takara and Promega. Ampicillin, kanamycin sulfate, bovine serum albumin (BSA), and isopropyl β-D-galactoside (IPTG) were purchased from Sigma Chemical Co.96-well ELISA microtiter plates were bought from Nunc Company, Roskilde, Denmark. POPE101-215 (Yol) vector, donated by Professor Mi-Fang Liang, was used for scFv expression. Dr. Keefe A. D donated plasmid pTAG2K for the experimental study. Meanwhile, rb-PrP^c was purchased from Robosome Company (Germany). Streptavidin coated slides were bought from Xenopore Corp.Cy3 kit was purchased from Amersham Biosciences. BIAcore 3000^{TM} and CM5 sensor chips were both purchased from BIAcore AB (Uppsala, Sweden). All other reagents used were of analytical-reagent grade.

2.2 Cloning and Expression of bPrP^c Gene and bPrP27-30 Encoding Sequence

Two oligonucleotide Primers PrP1 (5′-AGATCTGAAGAAGCGACCAAAA CCTG-3′) and PrP2 (5′-GAATTCTTATTAACTTGCCCCTCGTTGGTA-3′), which span the bovine mature prion sequence, were used to amplify the bovine mature prion sequence (Negro et al. 2000). Another couple of primers PrP90-1 (5′-AATTAGATCTGGGTGGCTGGGGACA-3′) and PrP90 -2 (5′-GGCGGAATTCTTATCTCTGGTACTGGGT-3′), which span the bovine *PrP* gene fragment from position aa90 to aa231 were used to amplify the protease-resistant core sequence (Weiss et al. 1996), using DNA templates generated from bovine white blood cell. Restriction enzymatic sites *Bgl*II and *Eco*RI were introduced to the N- and C-terminal primers, respectively. Polymerase chain reaction (PCR) products were purified by PCR purification minikits, then cloned into the pGEM-T vector and confirmed by sequencing. The bPrP^c and bPrP27-30 DNA fragments

were then inserted to replace the corresponding sequence between *Bgl*II and *Eco*RI in pET32a (+) and yielded the final fusion expression vectors pET32a (+)-bPrP^c and pET32a (+)-bPrP27-30, which were to express fusion proteins TrxA-bPrP^c and TrxA-bPrP27-30, separately. Recombinant strains were grown overnight at 37°C in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin sulfates, then transferred to 400 ml of LB medium and grown until an OD_{600} of 0.5. Fusion proteins, TrxA-bPrP^c, and TrxA-bPrP27-30, were expressed in AD494 (DE3) by inducing with IPTG and purified by Ni^{2+}-chelation affinity chromatography. The concentration of fusion protein was determined using their extinction coefficient at 280 nm (Negro et al. 1997). TrxA-bPrP^c and TrxA-bPrP27-30 were further identified by Western blot (data not shown).

2.3 Production of Murine scFv Antibody Libraries to Bovine Prion Protein by Phage Display

100 μg of recombinant TrxA-bPrP^c proteins were injected subcutaneously with Freund's complete adjuvant and later injection was given every twenty-one days for further five times with Freund's incomplete adjuvant (O'Rourke et al. 1998). Indirect ELISA determined the anti-bPrP^c antibody titer seven days after the last boost. Spleen cells were prepared for mRNA purification using the mRNA kit. Subsequently, the phage-displayed scFvs were generated using a mouse scFv DNA construction kit and an scFv expression kit (Marks et al. 1991). The purified mRNA was transcribed into cDNA using random primers, and the V_H and V_L DNAs were separately amplified through PCR. Gel-purified V_H and V_L DNAs were mixed with linker primers at an equimolar ratio and assembled into scFv DNA. The scFv DNA was then amplified and provided with an *Sfi*I site at the 5′ end and a *Not*I site at the 3′ end. After digestion with restriction enzymes *Sfi*I and *Not*I, the scFv DNA was ligated into the phagemid vector pCANTAB-5E, and the ligated sample was then electroporated into competent cells of *E. coli* TG1 to express phage-displayed scFv (McCafferty et al. 1990).

2.4 Panning and ELISA Detection of scFvs

The transformants were grown in 2 × YT medium up to an OD_{600} of 0.5. *E.coli* TG1 cells which were infected with M13K07 helper phage to rescue the phagemid with scFv inserted. The phage particles were precipitated with PEG 8000 and NaCl, then resuspended in 2 × YT medium and stored at 4°C. Five rounds of panning of the phage scFv library were performed against rb-PrP^c, which was coated on 96-well ELISA microtiter plate at a concentration of 5 μg/ml at 4°C overnight. The well was washed with phosphate buffered saline (PBS) and blocked with PBS with 4% nonfat milk powder

(PBSM) for 2 h at room temperature. Unbound phage was removed by twenty time washes with PBS and twenty times with PBS containing 0.1% Tween20 (PBST). rb-PrP^c-binding phages were eluted from the microtiter plate with 100 μl/well of 100 mM tri-ethylamine, followed by neutralization with 50 μl/well of 1 M Tris-HCl (pH 7.5). The eluted phages were then used to infect *E. coli* TG1 cells at 37°C for 1 h, then plated onto a SOBAG plate (Pharmacia, Expression Module) and incubated at 30°C overnight. The recombinant phage antibodies displayed from each clone were assayed for antigen binding by an immunoassay (ELISA).

A 96-well microtiter plate was coated with rb-PrP^c solution (100 μl/well), with TrxA and M13KO7 helper phage as the negative control and the positive control, respectively. The wells were then blocked by PBSM (200 μl/well). Phage particles in PBSM (100 μl) were added into the well, and incubated at 37°C for 2 h. The plate was washed three times with PBST and three times with PBS, and then the mouse anti-M13 antibody (diluted 1:5000) was added (100 μl/well). The bound phages were detected by incubation for 1 h at room temperature with 1:5000 diluted horse radish peroxidase (HRP)-labeled mouse monoclonal antibody directed against major coat protein (gene 8 protein) of M13 (100 μl/well). After washing six times, substrate tetramethylbenzidine (TMB) was added and incubated for 10–30 min at room temperature, then 50 μl H_2SO_4 (1M) was added to each well before color measurement at 450 nm. The phage antibodies were regarded as antigen-positive when OD value at 450 nm was at least 2 times higher as compared to that of the negative control (Long et al. 2000).

2.5 Construction of Expression Vectors of scFv Fusion Proteins

The phagemid pCANTAB-Z186 and pCANTAB-Z163 were digested with *Nco*I and *Not*I and encoding fragment of Z186 and Z163 were recovered, respectively. Two oligonucleotide Primers LSBP1 (5′-ATATAGCGGCCG CTTCGAGCTCAGGAG-3′) and LSBP2 (5′-TGACCGGA TCCTGGTTCA CGTTGACCTT-3′) (Keefe et al. 2001), that span the linker and streptavidin binding peptide sequence, were used to amplify Linker peptide-SBP sequence from the plasmid pTAG2K. Restriction enzymatic sites *Not*I and *BamH*I were introduced to the N- and C-terminal primers, respectively. PCR product was then digested with *Not*I and *BamH*I and Linker peptide-SBP encoding fragment was recovered. Z186 or Z163 and Linker peptide-SBP encoding fragments were then inserted to replace the corresponding sequence between *Nco*I and *BamH*I in pOPE101-215 (Yol), yielding the final fusion expression vectors pOPE-Z186-L-SBP and pOPE-Z163-L-SBP. To generate expression vectors pOPE-Z163-Fc and pOPE-Z1030-Fc, the *scFv* gene was obtained by digesting phagemid pCANTAB-Z163 and pCANTAB-Z1030 using the *sfi*I and *Not*I. The Fc portion of human IgG1

was digested with *Not*I and *sal*I from vector pPICZalfaFc (Powers et al. 2001) and then ligated with the *sfi*I/*sal*I linearized expression vector pOPE101-215 (Yol); yielding the plasmids termed pOPE-Z163-Fc and pOPE-Z1030-Fc. Four express vectors above were to express the fusion proteins Z186-L-SBP, Z163-L-SBP, Z163-Fc, and Z1030-Fc.

2.6 Expression and Purification of Fusion Proteins

The culture inoculated XLblue/pOPE-Z186-L-SBP, XLblue/pOPE-Z163-L-SBP, XLblue/pOPE-Z163-Fc, or XLblue/pOPE-Z1030-Fc was induced with 0.02 mM IPTG at 22°C for 5 h, respectively. The cells were harvested by centrifugation and washed; and were again resuspended in PBS buffer. After being sonicated, the soluble Z186-L-SBP, Z163-L-SBP, Z163-Fc, and Z1030-Fc in the supernatant were purified with $Ni2^{+}$-chelation affinity resin.

2.7 Sandwich ELISA for Selecting scFv Pairs

The wells of the microplates (Nunc Company, Roskilde, Denmark.) were coated with scFv-L-SBP (10 μg/ml in PBS), and then blocked by PBSM (200 μl/well), followed by adding rb-PrP^{c} (10 μg/ml in PBS) and incubation at 37°C for 2 h. Bounded rb-PrP^{c} was detected by incubating with an scFv-Fc at 37°C for 2 h and then HRP-labeled horse antihuman IgG Fc fragment antibody (diluted 1:5000) was added. The color was developed with TMB substrate. OD_{450nm} was measured after 1 h incubation at 37°C. Meanwhile, TrxA was used as negative control in each sandwich pair (Komiya et al. 2004).

2.8 Western Blots

TrxA-bPrP27-30 was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). TrxA was selected as negative control, and then transferred to a nitrocellulose membrane (Bio-RAD) at 15 volt for 20 min. Membranes were blocked with 4% nonfat milk, and then incubated with Z186-L-SBP and Z163-Fc at 37°C for 2 h, respectively. Z186-L-SBP was detected using the streptavidin-alkaline phosphatase (AP)-conjugate. Color development was detected using the AP substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT). Z163-Fc was detected by adding HRP-labeled horse antihuman IgG Fc fragment antibody and TMB as HRP substrate was used for color development (Schaller et al. 1999).

2.9 Binding Kinetic Analysis Using SPR

Binding affinities of Z186-L-SBP, Z163, and Z163-Fc were analyzed by surface plasmon resonance (SPR) analyzer. BIAcore 3000 is a dual channel analyzer that has two flow cells. rb-PrP^c was covalently attached onto the surface of one of the flow cells of a CM5 sensor chip through amine-coupling, giving an increased resonance unit (RU) of 2140. ScFv antibodies were resuspended in the solution (10 mM Tris, 150 mM NaCl (pH 7.4)) at different concentrations from 0 to 320 nM, 0 to 720 nM, and 0 to 150 nM for Z186-L-SBP, Z163, and Z163-Fc, respectively. They were subsequently injected into the flow cell at a flow rate of 30 μl/min. Another flow cell was as negative control, which had no immobilized rb-PrP^c, but was injected with antibodies. Both flow cells were injected with buffer solution as blank control. Negative control and blank control were subtracted to each measurement according to the operation manual. Data management and calculation of kinetics were performed using the BIA evaluation 4.1 software (Biacore) and 1:1 (Langmuir) binding model (Yau et al. 2003). Similar experiment protocol was applied for kinetic analysis of affinity of SBP fusion partner of Z186-L-SBP to streptavidin, where streptavidin modified sensor chip was used as the immobilized phase for capturing the flowing ligand, with concentrations ranging from 11.3 to 180 nM.

2.10 Cy3-labeling of Z163-Fc

Z163-Fc was labeled with Cy3 mono-reactive dye following the recommendations of the manufacturer. Briefly, the analytes were labeled in 0.1 M sodium carbonate (pH 9.3) at a molar ratio of 1:1 between protein and dye for 30 min at room temperature. Next, the mixtures were loaded to a PD-10 column (10 ml of bed of Sephadex G-25M), which had been pre-equilibrated by PBS. After washing the column with PBS (2 × 1 ml), the labeled protein was eluted by adding 2 ml of water to the column top. Another 10 ml of water was added to elute all unbound dye, and the column was regenerated with 20 ml of PBS (Renberg et al. 2005).

2.11 Preparation of Antibody Chip

The Z186-L-SBP solution at a concentration of 10 μg/ml was arrayed to the slide, coated with streptavidin at 0.1 μl/dot and incubated in a humid chamber at room temperature for 1 h, allowing site-directed immobilization of Z186 through its fusion partner SBP interacting with the streptavidin on the surface. After removal of the solution, the dots of the array were blocked with 4% PBSM, washed four times using PBS, sequentially. The antibody chip (scFv chip) was then ready for prion protein detection in sandwich format.

2.12 Assay of rb-PrP^c and Natural Mouse PrP^c (mPrP^c) Using the Antibody Chip

A solution of rb-PrP^c (or mPrP^c) in PBS was incubated with the Z186-L-SBP for 1 h, followed by a wash with PBST and PBS. 1 μg/ml Cy3-labeled Z163-Fc was spotted onto the dots. After the unbound Cy3-labeled Z163-Fc were removed, a GenePix 4000B (Axon Instrument) fluorescence scanner with sensitivity of 0.1 fluorophore/μm^2 for Cy3 was used to obtain the Cy3 fluorescence images of each spot using the fixed circle method. Each data point presented the mean value of three replicates after subtracting local background. All images were analyzed using GenePix Pro 4.0 analysis software (Axon Instruments) (Bi et al. 2003). The detection limit was defined as the lowest concentration of antigen at which the mean fluorescence signal intensity provided a signal-to-noise ratio (S/N) value of 3 (Cretich et al. 2004).

3. RESULTS AND DISCUSSION

3.1 Construction of Anti rb-PrP^c scFv Library and Selection of the Positive Clones

Purified TrxA-bPrP^c was used to immunize the mice. ELISA assayed the immune sera. The titer of the prepared antiserum against rb-PrP^c was as high as 1.28×10^5. Immunohistochemical detection indicated that PrP^c antigen was highly expressed in both mice and bovine brains. It is found that there is about 90% homology between mice and bovine PrP^c proteins (Harmeyer et al. 1998). Directly immunizing mice with PrP^c resulted in poor production of antibodies because of the immunological tolerance in mice to the bovine PrP^c protein. The problem can be overcome by using PrP^c-deficient mouse (PrP$^{0/0}$). As an alternative, in this study, we constructed a fusion protein TrxA-bPrP^c and used it to immunize the mice. Using all these means, the antibody titer reached a high level, which satisfied to construct a phage-displayed library.

mRNA was extracted from the spleen cells of two immune mice and used for constructing recombinant antibody library. After cDNA synthesis, the V_H coding sequence (340 bp) and V_L coding sequence (320 bp) were amplified and assembled into scFv coding sequence (about 750 bp) and then cloned into the phagemid vector pCANTAB-5E. The pCANTAB-scFv was cleaved by enzyme *sft*I and *Not*I to form the antibody coding sequence pool, which will be expressed in *E. coli* as the anti-bPrP^c scFv library. The size of the library was estimated to contain 1.0×10^6 clones. After five rounds of panning, ten antigen-positive phage clones were selected from 500 preselected phage clones. There are three out of the ten, which showed stronger positive signal and were named as Z163, Z186, and Z1030.

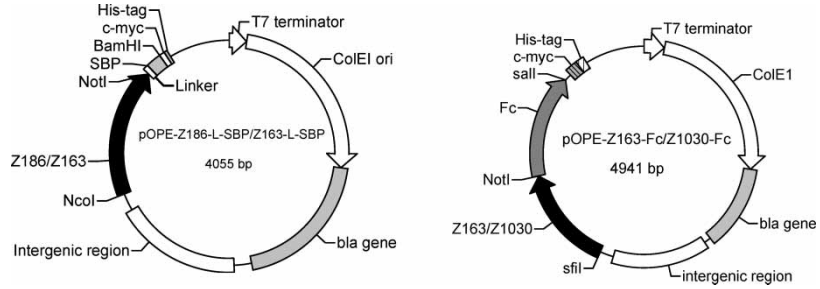

Figure 1. Construction of expression vectors of the scFv fusion proteins Z186-L-SBP, Z163-L-SBP, Z163-Fc and Z1030-Fc.

3.2 Expression and Purification of scFv Fusion Proteins

Fig. 1 shows the vectors constructed by gene splicing for expression of the scFv fusion proteins Z186-L-SBP, Z163-L-SBP, Z163-Fc, and Z1030-Fc. Maximum amounts of the expressions in *E. coli* cells were found at 22°C with IPTG induction and no "inclusion body" problem was found under this condition. High purity of the fusion proteins was obtained with His-tag affinity chromatography.

3.3 Selection of scFv Fusion Pairs by Double Antibody Sandwich ELISA

To constitute a pair of capture/signal scFv fusion proteins, scFv fusions were mated as three pairs: Z186-L-SBP/Z163-Fc, Z186-L-SBP/Z1030-Fc, and Z163-L-SBP/Z1030-Fc. Each candidate pair contained an scFv-L-SBP fusion and an scFv-Fc fusion. The former was the candidate of the capture probe and coated on the microplate wells, and the latter was the candidate of the detection probe. In ELISA experiment, rb-PrP^c sample was captured by the scFv-L-SBP, followed by binding of the scFv-Fc. It was observed

Table 1. Evaluation of the scFv fusion pairs against rb-PrP^c by double antibody sandwich ELISA

ScFv fusion pair	$OD_{450\,nm}$ readout[a]	Ratio of signal to negative control
Z186-L-SBP/Z163-Fc	0.381 ± 0.034	4.2
Z186-L-SBP/Z1030-Fc	0.117 ± 0.011	1.3
Z163-L-SBP /Z1030-Fc	0.090 ± 0.008	1.1

[a]Average of three duplicate measurements.

that an HRP-labeled horse antihuman IgG Fc fragment antibody bonded the complex. This four-layer was thus termed as "double sandwich ELISA". HRP catalyzed enzymatic color reaction were read out at OD_{450} nm using TrxA sample as a negative control. The pair Z186-L-SBP/Z163-Fc displayed obviously positive results. The data were summarized in Table 1. There is a clear cut between Z186-L-SBP/Z163 and other two pairs. Therefore, the Z186-L-SBP/Z163-Fc pair was selected for the following experiments.

3.4 Binding Characteristics of scFv and scFv Fusion Pair Through SPR Sensorgrams

Binding affinities of Z163, Z186-L-SBP/Z163-Fc pair to rb-PrP^c were analyzed by SPR. Fig. 2 shows SPR sensorgrams, where the equilibrium dissociation constants (K_D values) were 3.24×10^{-8} M, 8.82×10^{-8}M, and 8.10×10^{-9} M for Z186-L-SBP, Z163, and Z163-Fc, respectively (Table 2). They all displayed rapid association and slow dissociation kinetics. The affinity of Z163-Fc to rb-PrP^c was obvious higher than those of the structures containing no Fc fusion partner.

SPR experiment showed that the fusion structures retained their antibody activities against rb-PrP^c. Generally, affinity of the single chain antibody affinity is one or two orders lower than that of the parent antibody, which usually leads to lower the detection sensitivity. Using the antibody engineering this problem may be solved. Z163-Fc is a fusion structure of an scFv and a human IgG Fc region, which displayed an obvious increased affinity to rb-PrP^c.

Fig. 3 shows the SPR sensorgram of Z186-L-SBP which reacted with the streptavidin-modified surface. The figure gives the residue activity of SBP fusion partner of Z186-L-SBP to streptavidin. Also, the response and washing curves indicate a rapid association and slow dissociation kinetics. K_D was 5.97×10^{-9} M, which is higher than that of SBP (2.5×10^{-9} M) (Keefe et al. 2001), implying SBP as a fusion partner, which lost about 50% of its original activity. SPR analysis revealed that the SBP fusion partner of Z186-L-SBP retained its binding activity to streptavidin. Thus it is able to apply the SBP-streptavidin interaction mechanism for site-directed immobilization of Z186-L-SBP to prepare the antibody chip with high-homogeneity reaction surface. The high residual affinity activities of all these fusion structures are largely due to the insertion of linker peptide between the fusion partners. The linker peptide (repeat serine and glycin sequence) is a rigid and hydrophilic α-helices chain in solution (Shi et al. 2004), which plays a spacer to help to correct folding of each fusion partners. The linker peptide function had been demonstrated in our previous studies and many other investigations (Freund et al. 1993; Zhou et al. 2001; Shao et al. 2001) and worked well again in this study.

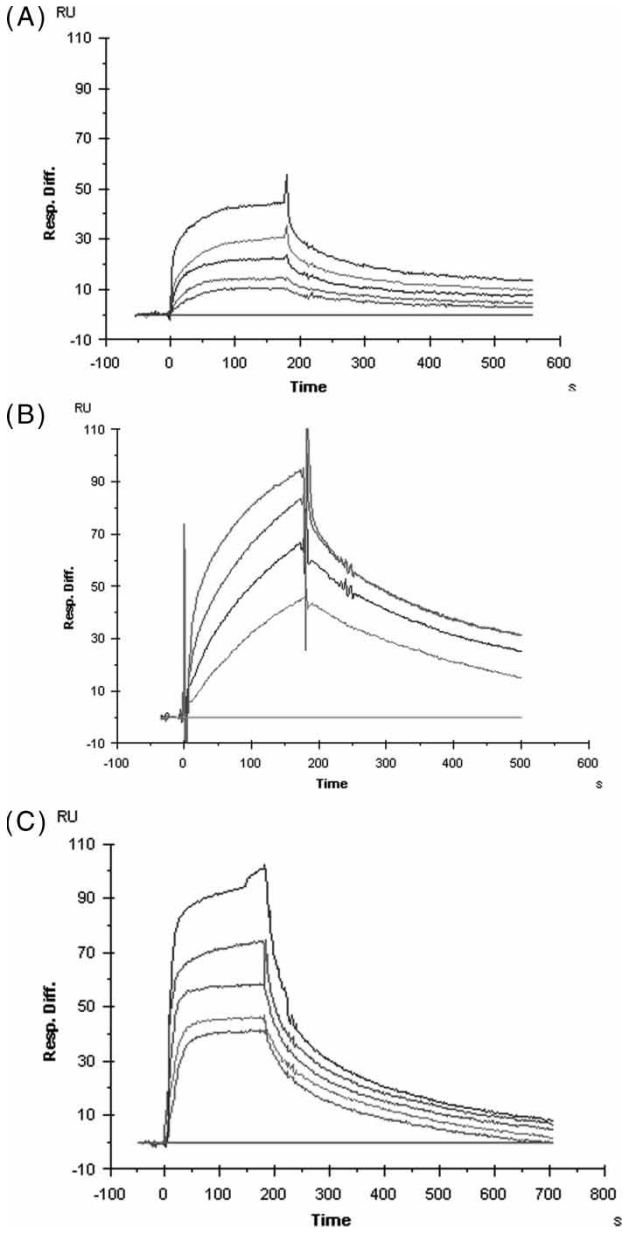

Figure 2. Overlay of sensorgrams showing the binding of scFv and scFv fusion protein pair with various concentrations to the immobilized rb-PrP^{c}. (A) Z186-L-SBP was injected at concentrations of 0, 20, 40, 80, 160, 320 nM from bottom to top respectively; (B) Z163 was injected at concentrations of 0, 90, 180, 360, 720 nM from bottom to top respectively; (C) Z163-Fc was injected at concentrations of 0, 9.4, 18.8, 37.5, 75.0, 150.0 nM from bottom to top respectively.

Table 2. Kinetic data of SPR analysis

Binding interaction	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_A (M^{-1})	K_D (M)
Z186-L-SBP to rb-PrP^c	6.82×10^4	2.21×10^{-3}	3.09×10^7	3.24×10^{-8}
Z163 to rb-PrP^c	4.93×10^4	4.35×10^{-3}	1.13×10^7	8.82×10^{-8}
Z163-Fc to rb-PrP^c	5.42×10^5	4.39×10^{-3}	1.24×10^8	8.10×10^{-9}
Z186-L-SBP to streptavidin	6.56×10^4	3.92×10^{-4}	1.67×10^8	5.97×10^{-9}

3.5 Recognition of bPrP27-30 Line Epitopes of the Fusion Pair by Western Blot

bPrP27-30 corresponds to amino acids 90-231 of $bPrP^c$, which is a protease resistant core region found in $bPrP^{sc}$. As TrxA-bPrP27-30 and bPrP27-30 share the same linear epitope, the TrxA-bPrP27-30 was prepared and used to probe the fusion pairs in the Western blot experiment. Fig. 4 shows that both Z186-L-SBP and Z163-Fc could bind to 29 kDa fragment, and no band was observed in the control. The results indicate that both the Z186-L-SBP and Z163-Fc recognize the linear epitopes localized at bPrP27-30. Considering the positive results of the double sandwich ELISA, Z186-L-SBP and Z163-Fc must have different recognition sites or linear epitopes in the region of bPrP27-30.

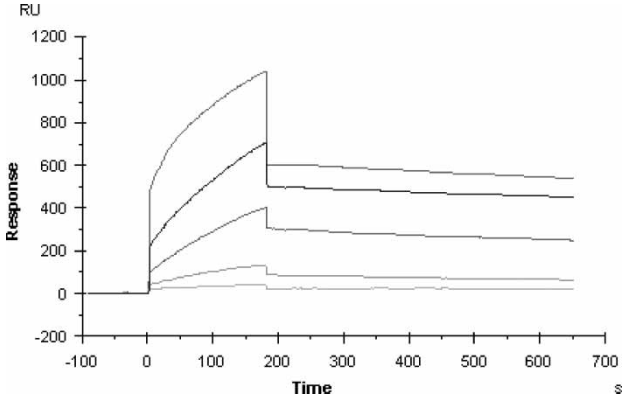

Figure 3. Overlay of sensorgrams showing the binding of Z186-L-SBP with various concentrations to the immobilized streptavidin. Z186-L-SBP was respectively injected at concentrations of 11.3, 22.5, 45.0, 90.0, 180.0 nM from bottom to top respectively.

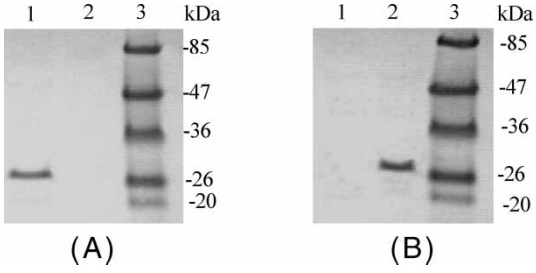

Figure 4. Western blot analysis showing scFv fusion proteins recognize recombinant bovine PrP27-30 (TrxA-bPrP27-30) linear epitopes. (A) Z186-L-SBP and (B) Z163-Fc. The TrxA-bPrP27-30 was separated in a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane, and reacted with Z186-L-SBP and Z163-Fc. (A) Lane 1, purified TrxA-bPrP27-30; Lane 2, TrxA; Lane 3, prestained protein molecular weight marker. (B) Lane 1, TrxA; Lane 2, purified TrxA-bPrP27-30; Lane 3, prestained protein molecular weight marker.

PrP^{c} is known sensitive to protease K, while PrP^{sc} is resistant to the hydrolysis of the enzyme. PrP27–30 lacking 67aa at the N-terminal end of PrP^{sc} represents the protease resistant core in TSE prion preparations associated in amyloid-like rods or scrapie associated fibrils (SAF). This protease-resistant property of PrP27–30 has been used to identify PrP^{sc} from PrP^{c} in a number of immunological analytical methods (Grathwohl et al. 1997; Grassi et al. 2000). The mechanism had been applied to develop the

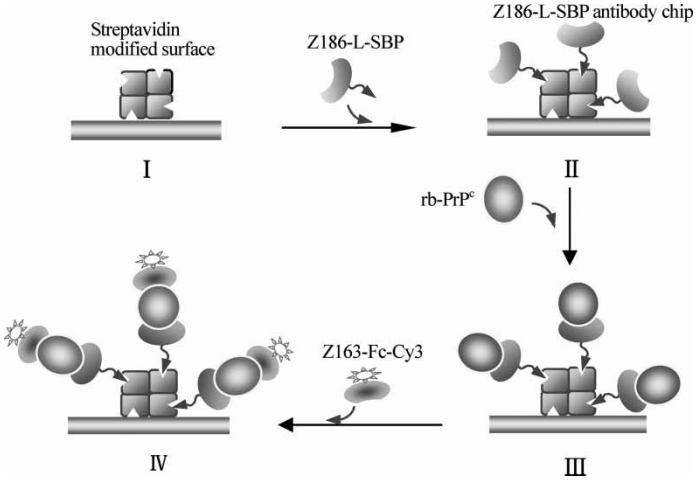

Figure 5. Schematic diagram of detection of rb-PrP^{c} using the scFv fusion pair-based antibody chip. I) streptavidin modified chip; II) preparation of the antibody chip by immobilization of Z186-L-SBP through SBP-streptavidin interaction; III) capture of rb-PrP^{c} by the antibody chip; IV) binding of detection antibody Z163-Fc-Cy3 to the captured rb-PrP^{c}, producing fluorescent signal.

standard detection method, called conformation-dependent immunoassay (CDI) (Safar et al. 2005). Since the recombinant bovine PrP27-30 has the same amino-acid sequence with the protease-resistant domain bPrP27-30, they should have the same linear epitope. scFv-Z186-SBP and scFv-Z163-Fc could recognize and bind recombinant bPrP27-30 linear epitope in Western blots, and thus are able to recognize PrP^{sc}.

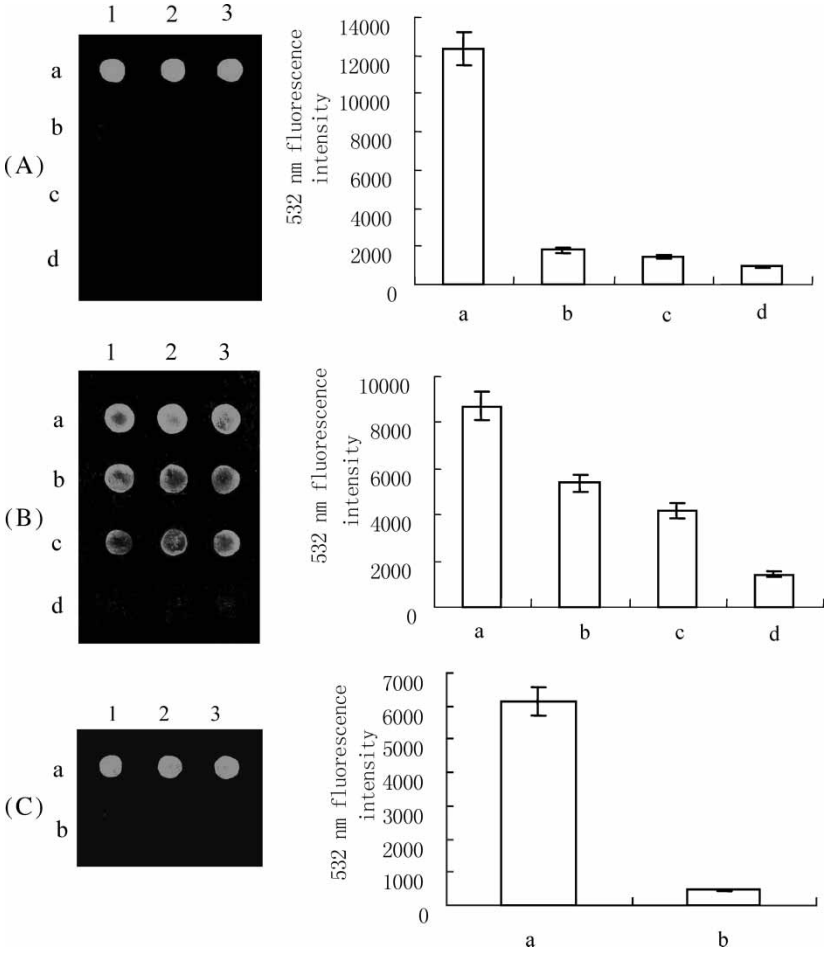

Figure 6. Detection of PrP^{c} using scFv fusion pair-based antibody chip. Left, signal images of the antibody chips, each loaded as three duplicated with sample size of 0.1 μL/spot; Right, fluorescence intensity at 532 nm corresponding to the images. (A) Detection of rb-PrP^{c}. a1-a3, rb-PrP^{c} (10 ng/mL); b1-b3, TrxA (10 ng/mL); c1-c3, BSA (10 ng/mL); d1-d3, OVA (10 ng/mL). (B) Detection limit to rb-PrP^{c}. a1-a3, 100 pg/mL; b1-b3 10 pg/mL; c1-c3, 1 pg/mL; d1-d3, negative control (100 pg/mL TrxA). (C) Detection of murine PrP^{c}. a1-a3, murine brain PrP^{c}; b1-b3, TrxA, 10 ng/mL.

3.6 Detection of rb-PrP^c and mPrP^c Using scFv Fusion Pair on Antibody Chip

Fig. 5 shows the working principle of the antibody chip, which was based on using the scFv fusion pair. To prepare the antibody chip, the Z186-L-SBP was immobilized on the streptavidin-modified slide through SBP-streptavidin interaction. rb-PrP^c in sample solution was recognized and captured by the scFv fusion partner of the Z186-L-SBP. The detection of scFv fusion Z163-Fc-Cy3 was then added to bind the captured rb-PrP^c. Fluorescent signal generated from Cy3 was the indication of existence of the prion protein. Fig. 6a shows the response signals of the antibody chip to rb-PrP^c, TrxA, BSA, and ovalbumin (OVA). The latter three proteins were used as negative control, which the fluorescence intensities were taken as the noise level. Concentrations of all four samples were 10 ng/ml. Response signal to noise level ratios (S/N) of rb-PrP^c/TrxA, rb-PrP^c/BSA, and rb-PrP^c/OVA were 6.8, 8.5, and 13.3, respectively. Fig. 6b shows the response signals of the antibody chip to rb-PrP^c at different concentrations. S/N for the concentrations of 100 pg/ml, 10 pg/ml, and 1 pg/ml were 6.1, 3.8, and 3.1, respectively. The detection limit was about 1 pg/ml ($S/N \geq 3$). The antibody chip was also applied to detect natural murine prion protein (mPrP^c). A sample of homogenate of mouse brain containing mPrP^c was loaded onto the chip. S/N value was 13 (Fig. 6c), an indication of an obvious positive detection.

4. CONCLUSION

A pair of scFv fusion structures recognizing the recombinant bovine prion protein were constructed and characterized with multi techniques (immunizing mice, phage display, antibody engineering, SPR analysis, ELISA, and Western blot). They were used to construct the antibody chip. As the scFv fusion pair could recognize and bind recombinant bPrP27-30, the antibody chip would be able to detect the PrP^{sc}. The proposed detection system is competitive with the existing detection methods in terms of sensitivity, rapidity, easy to perform, and cost. The reasons are as follows:

(a) Mass production and purification of the scFv fusion pair is easy using high efficient expression system and affinity chromatography, and the cost of detection can be largely reduced.
(b) The capture scFv can be immobilized on the chip surface with controlled orientation, which is crucial to obtain homogeneous protein/antibody chips (Shi et al. 2004). In this study, the capture probe Z186-L-SBP was site-directed immobilized on the chip surface by self assembling through SBP-streptavidin interaction, leaving the scFv fusion partner to open to the bulk solution (Fig. 5), and thus giving reproducible

responses. Whereas, conventional immobilization of antibody by chemical coupling is a random adsorption that usually results in an uncontrolled and heterogeneous surface.

(c) The detection probe can be either scFv or scFv-Fc fusion based. Both forms are mono-body against rb-PrP^c, but scFv-Fc gained a K_D value about 10 times higher than that of scFv. The high affinity of scFv-Fc to rb-PrP^c obviously increases the specificity and sensitivity of the detection. In fact, the detection limit using the method was as low as 1pg/ml, which is comparable to the literature data, where the detection limits were reported to be 25–50 ng PrP 27-30 per lipid class by high-performance liquid chromatography (HPLC; Klein et al. 1998), approximately 20 pg/ml recombinant hamster PrP by mAb sandwich ELISA (Yang et al. 2005), less than 50 pg normal and disease-associated isoforms of prion protein (PrP) by time-resolved dissociation-enhanced fluorescence mAb sandwich ELISA (Yang et al. 2005) and 6 or 80 ng/ml bovine recombinant prion protein by capillary electrophoresis-based noncompetitive or competitive fluorescence immunoassay (Barnard et al. 2000; Völkel et al. 2001).

In conclusion, a pair of scFv fusion structures recognizing the recombinant bovine prion protein was constructed and characterized with multi techniques (immunizing mice, phage display, antibody engineering, SPR analysis, ELISA, and Western blot). The performance of the fusion pair in detection of prion protein was demonstrated by an antibody chip format using the recombinant bovine prion protein and the natural murine prion protein as the targets. Real sample application is expected when available.

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