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Single-Chain Fv Antibody Fragments Retain Binding Properties of the Monoclonal Antibody Raised Against Peptide P1 of the Human Prion Protein

Nives Škrlj · Vladka Čurin Šerbec · Marko Dolinar

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Abstract Prion diseases are incurable neurodegenerative diseases that affect both humans and animals. The infectious agent is a pathogenic form of the prion protein that accumulates in brain as amyloids. Currently, there is neither cure nor reliable preclinical diagnostics on the market available. The growing number of reports shows that passive immunisation is one of the most promising strategies for prion disease therapy, where antibodies against prions may prevent and even cure the infection. Since antibodies are large molecules and, thus, might not be suitable for the therapy, different antibody fragments are a good alternative. Therefore, we have designed and prepared single-chain antibody fragments (scFvs) derived from the PrP^{Sc}-specific murine monoclonal antibody V5B2. Using a new expression vector pMD204, we produced scFvs in two opposing chain orientations in the periplasm of *Escherichia coli*. Both recombinant antibody fragments retained the specificity of the parent antibody and one of these exhibited binding properties comparable to the corresponding murine Fab fragments with the affinity in nM range. Our monovalent antibody fragments are of special interest in view of possible therapeutic reagents for prion diseases as well as for development of a new generation of diagnostics.

Keywords Prion protein · Recombinant antibody · scFv · *Escherichia coli*

Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
BSA	Bovine serum albumin
CNS	Central nervous system
DEPC	Diethyl pyrocarbonate
ELISA	Enzyme-linked immunosorbent assay

N. Škrlj · V. Čurin Šerbec · M. Dolinar (✉)
Chair of Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Jamova 39, 1000 Ljubljana, Slovenia
e-mail: marko.dolinar@fkkt.uni-lj.si

V. Čurin Šerbec
Department for Production of Diagnostic Reagents and Research, Blood Transfusion Centre of Slovenia, Šlajmerjeva 6, 1000 Ljubljana, Slovenia

Fv	Antibody variable domains (Vl+Vh)
HLL	scFv in Vh-linker-Vl chain arrangement
HRP	Horseradish peroxidase
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LLH	scFv in Vl-linker-Vh chain arrangement
mAb	Monoclonal antibody
PCR	Polymerase chain reaction
PrP	Prion protein
PrP^{C}	Cellular form of the PrP
PrP^{Sc}	Pathogenic form of the PrP
scFv	Single-chain antibody variable domains
TBS	Tris-buffered saline
TMB	3,3′,5,5′-tetramethylbenzidine
Vh	Variable domain of the heavy chain of an antibody
Vl	Variable domain of the light chain of an antibody

Introduction

Prion diseases, also named transmissible spongiform encephalopathies, are lethal neurodegenerative diseases caused by a conformational change of the normal, host-encoded prion protein (PrP^{C}). They affect humans as well as many animal species and include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome, fatal familial insomnia, and kuru in humans, scrapie in sheep and goats, feline spongiform encephalopathy in cats, transmissible mink encephalopathy, chronic wasting disease in deer and elk, bovine spongiform encephalopathy in cattle and encephalopathies of a number of zoo animals [1].

According to the "protein-only hypothesis," a pathological form of the prion protein (PrP^{Sc}) is the disease transmitting infectious particle [2], which accumulates mainly in the brain in the form of insoluble aggregates described as amyloid deposits. Currently, there is neither effective treatment nor reliable preclinical diagnostics available.

Numerous strategies and targets have been proposed for therapy of prion diseases, including passive or active immunization. Therefore, several monoclonal antibodies (mAb) directed against different regions of the prion protein have been developed and some of them efficiently antagonized prion propagation both in vivo and in vitro (for review, see [3]). It was shown that authored scrapie-infected neuroblastoma cells (ScN2a) have been cured by mAb 6H4 [4], antibody fragment 6H4μ successfully prevented scrapie pathogenesis and inhibited prion transport to the central nervous system [5] and mAbs ICSM 35 and ICSM 18 suppressed peripheral prion replication in a murine scrapie model [6]. Unfortunately, none of these antibodies were effective against intracerebral prion challenge. Besides, bivalent mAb may act neurotoxic [7]; therefore, monovalent antibodies like Fab or single-chain antibody fragments (scFv) are preferred for drug development [3, 8]. It was shown in the past that antibody fragments can cure cell cultures from scrapie infection independently of effector functions encoded by constant antibody domains. For instance, Fab fragments D18 and D13 [9] and scFv derived from mAbs 6H4 [10] and D18 [11] have been shown to decrease PrP^{Sc} levels in ScN2a cells.

ScFv is the smallest antibody fragment containing a complete antigen binding site; therefore, it maintains the binding specificity and affinity of the whole antibody. It consists of both light and heavy chain variable domains, covalently joined by a polypeptide linker

[12, 13]. The small size and the ability to produce them in functional form in bacteria made them an interesting tool for protein engineering with potential therapeutic or immunodiagnostic use.

Several attempts have been undertaken to develop antibodies specifically recognizing the pathogenic form of the prion protein. We focused on the mAb V5B2 [14], prepared against peptide P1 from the C-terminus of human PrP, which can discriminate between Creutzfeldt–Jacob's disease-affected and normal brain tissue and, thus, seems promising for development of a diagnostic tool and eventually, after humanization, also as a potential passive vaccine.

Single-chain Fvs, derived from mAb V5B2, were prepared in *Escherichia coli* periplasmic space using a tailor-made expression vector pMD204 [15]. This vector was developed specially for fusion protein construction and its bacterial production. After optimization of recombinant protein production at intermediate temperature, we could easily produce scFvs in two opposing orientations of the heavy and light chain variable domains: heavy-linker-light (HLL) and light-linker-heavy (LLH). Binding properties of purified recombinant antibody fragments were measured by enzyme-linked immunosorbent assay (ELISA) and compared to a corresponding Fab of murine origin. We also determined scFv stability in serum and demonstrated that there was no cross-reactivity with serum proteins.

Materials and Methods

Reagents and Strains

DNA restriction and modification enzymes were from Fermentas, except Pfx50 polymerase (Invitrogen), RNase H (Ambion), and GoTaq polymerase (Promega). Oligonucleotides were from Invitrogen. For DNA purification from agarose gels, we used a reagent kit from Fermentas. Protein molecular weight standards were from Fermentas as well. Synthetic P1 peptide derived from the human prion protein (amino acids 214–226; CITQYERESQAYY) and bovine serum were provided by the Blood Transfusion Centre of Slovenia. Fab fragments of the V5B2 antibody were prepared as described by Colja Venturini et al. [16] and donated by the authors. For all cloning experiments, we used *E. coli* strain DH5α. For expression, BL21[DE3] strain was used.

RNA Isolation

Hybridoma cells producing mAb V5B2 [14] were harvested. Total RNA was isolated as described by Koren et al. [17] and kindly donated by the authors.

cDNA Synthesis

Complete coding regions of both variable domains were obtained by the 5′ rapid amplification of cDNA ends (RACE) technique. First, reverse transcription was performed for first-strand cDNA synthesis using primers zo1_Igk for the kappa light chain and zo1_IgG1 for the heavy chain of immunoglobulin G1 (see Table 1 for primer sequences). Total RNA (2 μg) in DEPC-water was first incubated at 70 °C for 10 min and transferred on ice immediately. After primer addition (1 μg), the mixture was incubated at 42 °C for 1 h and inactivated at 70 °C for 10 min. RNase H was added directly to the mixture and first

Table 1 Oligonucleotides used for the amplification of cDNA of mAb V5B2 variable regions and construction of scFv antibody fragments.

Oligonucleotide	Sequence (5′>3′)
zo1_Igk	TGTTAACTGCTCACTGGATGGTGG
zo1_IgG1	GTTTGATTGGGCCGCAGATCCAGG
IgG1_Hconst	GGAAGATCTATAGACAGATGGGGGTGTCGTTTTGGC
Ig_k_const	GGTGCATGCGGATACAGTTGGTGCAGCATC
d(t)17	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT
scFv_Vh_5F	ATATTCGCGAGCGAGGTGCAGCTTGCTGAGTC
scFv_Vh_5R	GAGGAATTCGCAGAGACAGTGACCAGAGT
scFv_Vl_3F	CACCTCGAGCGACATCCAGATGACTCAGTCT
scFv_Vl_3R	GCGCAAGCTTCCGTTTGATTTCCAGCTTG
scFv_Vl_5F	ACACTCGCGAGCGACATCCAGATGACTCAGTCT
scFv_Vl_5R	GCGGAATTCGCCCGTTTGATTTCCAGCTTG
scFv_Vh_3F	AGACTCGAGCGAGGTGCAGCTTGCTGAGTC
scFv_Vh_3R	GCGCAAGCTTAGAGACAGTGACCAGAGT
scFv_linker_F	AATTCCGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGT GGCGGAAGTGGC
scFv_linker_R	TCGAGCCACTTCCGCCACCGCCAGAGCCACCTCCGCCTGAAC CGCCTCCACCGG

incubated at 37 °C for 30 min and then at 70 °C for 10 min. Complementary DNA was purified and used as a template for terminal deoxynucleotidyl transferase (TdT). Poly(dA) tail was added to the 3′ end of the first strand cDNA using 150 pmol dATP and 30 U of TdT. Again, the reaction was performed at 37 °C for 30 min and terminated by heating to 70 °C for 10 min. The homopolymeric-tailed cDNA was purified and amplified by PCR using primers specific for the CH1 constant domain of the heavy chain IgG1 gene (IgG1_Hconst) or for the constant domain of the κ light chain (Ig_k_const), and one specific for the homopolymeric tail (d(t)17). PCR products were cloned into pJET1/blunt (Fermentas) and sequenced (Macrogen).

Assembly of scFvs

The regions coding for light chain variable domain (Vl) and heavy chain variable domain (Vh) of V5B2 were amplified by PCR using specific primers (Table 1): scFv_Vh_5F and scFv_Vh_5R for Vh of HLL, scFv_Vl_3F and scFv_Vl_3R for Vl of HLL, scFv_Vl_5F and scFv_Vl_5R for Vl of LLH, and scFv_Vh_3F and scFv_Vh_3R for Vh of LLH. Restriction sites *Nru*I and *Eco*RI were introduced at the 5′ end and at the 3′ end of variable domains for the N-terminus of the scFv fusion (Vh in HLL and Vl in LLH), respectively. *Xho*I and *Hin*dIII sites were added at each end of variable domain for the C-terminus of the scFv fusion (Vl in HLL and Vh in LLH). Each variable region was inserted into expression vector pMD204 between two appropriate restriction sites (Fig. 1). To obtain the 15-amino acid $(Gly_4Ser)_3$ linker, a pair of oligonucleotides (scFv_linker_F and scFv_linker_R) was synthesized, phosphorylated, and hybridized. This cassette was ligated into *Eco*RI/*Xho*I cleaved pMD204. The final vectors pMD204-HLL and pMD204-LLH (Fig. 1) were checked by DNA sequencing and used for scFv production.

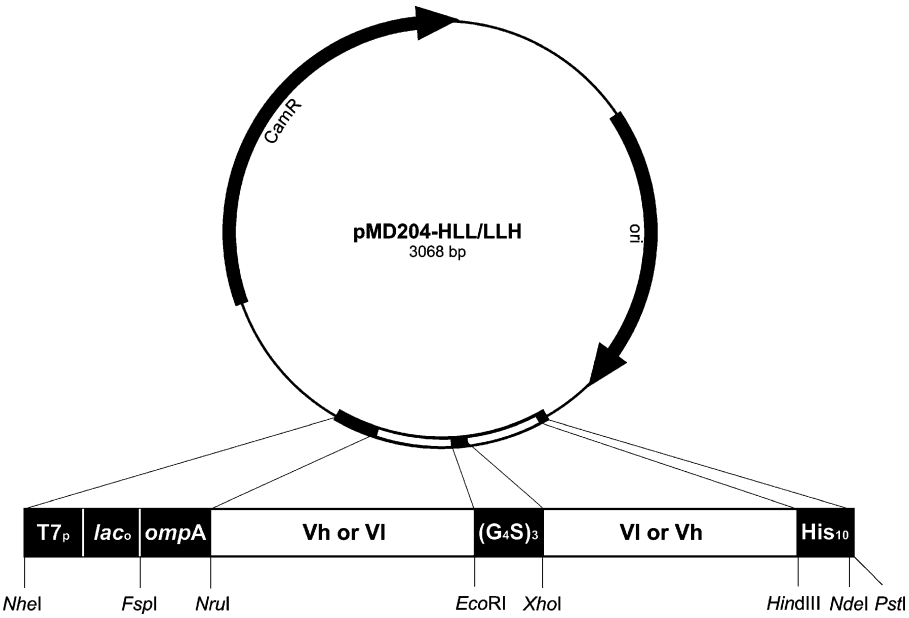

Fig. 1 Schematic representation of the expression vectors pMD204-HLL and pMD204-LLH and of the scFv gene assembly. V5B2 single-chain Fv was constructed both in Vh-Vl and in Vl-Vh arrangement, connected by a linker peptide $(Gly_4Ser)_3$. Each scFv was fused to *omp*A signal sequence at the 5′ end and His10 tag at the 3′ end. Periplasmic expression was under the control of T7 promoter ($T7_p$) and *lac* operator (lac_o). Unique restriction sites are indicated

Expression of Recombinant scFvs

E. coli BL21[DE3] cells were transformed with pMD204-HLL or pMD204-LLH and grown overnight in M9 minimal medium supplemented with 2% glucose, 0.5% peptone, and chloramphenicol (50 μg/ml) at 37 °C and 250 rpm. Dilutions (1/20) of the overnight cultures were grown as 500-ml cultures in 2-l shake flasks at 37 °C and 250 rpm until OD_{550} reached 1.0. Then, IPTG was added to a final concentration of 0.1 mM and cell growth was continued for 4 h at 37 °C or 8–20 h at 16 °C and 200 rpm. Cell samples were collected prior to and after IPTG induction for analysis of total bacterial proteins. Cell lysates and soluble and insoluble fractions were prepared as described [15]. Periplasmic fraction was isolated essentially as described [18]. Briefly, cells were harvested and resuspended in 20 mL of ice-cold TS buffer (100 mM Tris-HCl, pH 9.0, 25% sucrose). After incubation on ice for 15 min with occasional shaking, cells were collected and resuspended in 20 ml 10 mM Tris/HCl, pH 8.0, and incubated on ice for another hour with occasional stirring. Spheroplasts were pelleted by centrifugation at 8,000×*g* for 20 min and 4 °C, and supernatant was collected as periplasmic fraction. Freshly prepared periplasmic fraction was concentrated using OM10 membranes (Pall Life Science) and dialyzed against 50 mM sodium phosphate, pH 8.0, 0.3 M NaCl for immobilized metal ion affinity chromatography (IMAC). Before binding to immobilized nickel ions, periplasmic fraction was clarified by passing through a 0.2-μm filter (Sartorius).

Protein concentrations of functional recombinant antibody fragments were determined by Bradford assay [19] using bovine serum albumin (BSA) as a standard. SDS-PAGE

analyses ware performed on 15% polyacrylamide gels according to Laemmli [20] under reducing condition.

Single-Chain Antibody Fragment Purification

IMAC was performed at room temperature using 250 µl of His-Select Nickel Affinity Gel (Sigma). After equilibration with start buffer (50 mM sodium phosphate, pH 8.0, 0.3 M NaCl), the periplasmic fraction was loaded onto the column and washed with the washing buffer (50 mM sodium phosphate, pH 8.0, 0,3 M NaCl, 20 mM imidazole) until the absorbance at 280 nm of the effluent dropped below 0.01. The recombinant scFv was liberated from the column with the elution buffer (50 mM sodium phosphate, pH 8.0, 0.3 M NaCl, 250 mM imidazole). Collected fractions were analysed by SDS-PAGE, Western blot, and ELISA.

Western Blot

Samples of scFv were separated by SDS-PAGE under reducing conditions and electrophoretically transblotted onto 0.45 µm nitrocellulose membrane (Whatman) at 200 mA for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, 20% MeOH) using Bio-Rad Mini Trans Blot cell. The membrane was then blocked in 5% nonfat milk/TBS for 2 h, washed with TBS buffer (10 mM Tris/HCl, pH 7.6, 150 mM NaCl), and incubated for 1 h with primary anti-His_6 antibodies (0.5 µg/ml, Roche Diagnostics) dissolved in 1% nonfat milk/TBS. After washing with TBS, the membrane was incubated with the solution of goat anti-mouse IgG secondary antibodies, conjugated to horseradish peroxidase (HRP; 1:5,000 in 1% nonfat milk/TBS, Jackson ImmunoResearch) for 1 h and washed as before. Chromogenic peroxidase substrate 3,3′,5,5′-TMB (Sigma) was used for immunoblot detection.

scFv Specificity

The antigen-binding activity of scFvs was determined by ELISA. Microtiter plates were coated overnight at 4 °C with 50 µl of 5 µg/ml peptide P1 or 10% bovine serum in carbonate/bicarbonate buffer (pH 9.6). The wells were blocked for 30 min at 37 °C with 1% BSA in 10 mM phosphate buffer (pH 7.2) before adding the purified antibody fragments. After incubation at 37 °C for 1 h, scFvs were detected using primary mouse anti-His_6 antibodies and secondary goat anti-mouse IgG antibodies conjugated with HRP. Incubation with each antibody was performed at 37 °C for 1 h. For V5B2 Fab fragments used for comparison, only secondary antibodies were used. Peroxidase activity was detected using the peroxidase substrate ABTS. After 20-min incubation at 37 °C, absorbance at 405 nm was determined. The absorbance signal was plotted versus total concentration of scFv added to the wells. Apparent dissociation constants K_D were estimated by fitting the experimental values to equation $S = S_{max} * Ab/(Ab + K_D)$. In the equation, Ab is the antibody concentration added to each well and $K_D = [Ab][Ag]/[AbAg]$, where [Ab], [Ag], and [AbAg] are the concentrations of the free antibody, free antigen, and of the complex AbAg, respectively.

Stability in Serum

Purified scFv was incubated in 10% bovine serum at 37 °C for 20 and 40 h at a concentration of 30 µg/ml. Samples were then assayed for binding to its specific antigen

(P1) using ELISA as described above, except that scFvs and anti-His_6 antibodies were incubated for 2 h at 16 °C instead of 1 h at 37 °C.

Results

Cloning

After RNA isolation from hybridoma cells, antibody transcripts had to be amplified to obtain coding sequences of variable regions. Since mouse immunoglobulin genes are highly diverse in sequence at their 5′ ends, degenerate primers are usually used for gene amplification [21]. As we wished to determine the complete nucleotide sequence of variable regions including 5′ termini, we used 5′ RACE [22]. After PCR with primers binding to antibody constant region at 5′ end and to poly(dA) tail at 3′ end of the first strand cDNA, PCR products of 600 and 500 bp for Vh and Vl were obtained, respectively. Each PCR product was inserted into the cloning vector pJET1/blunt, and DNA sequencing was preformed. The complete cDNA sequence of variable regions, including leader sequences, of PrP^{Sc}-specific mAb V5B2 was determined.

For V5B2-derived scFv production in *E. coli*, we used expression vector pMD204, especially designed for easy cloning of fusion segments connected by a linker [15]. Two constructs with opposing orientation of the light and heavy chain were prepared. Both HLL and LLH scFv constructs were introduced into pMD204 by stepwise insertion of PCR-amplified Vh and Vl regions and the $(Gly_4Ser)_3$ linker encoded by a synthetic cassette. The final 744 bp long scFv cDNA was fused in the vector with *E. coli ompA* leader sequence for periplasmic expression at the 5′ end and His10 tag for affinity purification at the 3′ end (Fig. 1). Final vectors pMD204-HLL and pMD204-LLH with confirmed DNA sequence were used for periplasmic expression of antibody fragments under the control of the T7 promoter and *lac* operator.

Expression and Purification of scFvs

As we were interested in the importance of scFv domain order on protein expression and antigen binding properties, two mirror forms of V5B2 scFv, named HLL and LLH, were produced. *E. coli* BL21[DE3] cells were transformed with pMD204-HLL or pMD204-LLH and used for expression of the encoded antibody fragments. We initially tested expression levels after IPTG induction at 37 °C for 4 h and 16 °C for 16 h. Despite lower overall yields, higher binding activity was recovered from cells grown at 16 °C. Lowering the incubation temperature had improved the production of soluble antibody fragments in the bacterial periplasm (data not shown), especially after shortening of the induction time to 8 h (Fig. 2).

The relative amounts of HLL and LLH scFvs were estimated by SDS-PAGE and immunoblot analysis of cell lysates, soluble and insoluble fractions, and periplasmic extracts (Figs. 3 and 4). After induction, an additional band of approximately 30 kDa, which corresponded to the calculated size of the mature scFv, was observed in all analyzed fractions. The yield of scFv recovered from the periplasmic fraction was similar for both forms, although slightly higher amounts of recombinant protein could be observed in the cell lysate after IPTG induction for the HLL construct (Fig. 4a, lane +).

Since His10 tag was added to the 3′ end of scFvs, nickel affinity chromatography was used to purify HLL and LLH from periplasmic fractions. Most of contaminating periplasmic

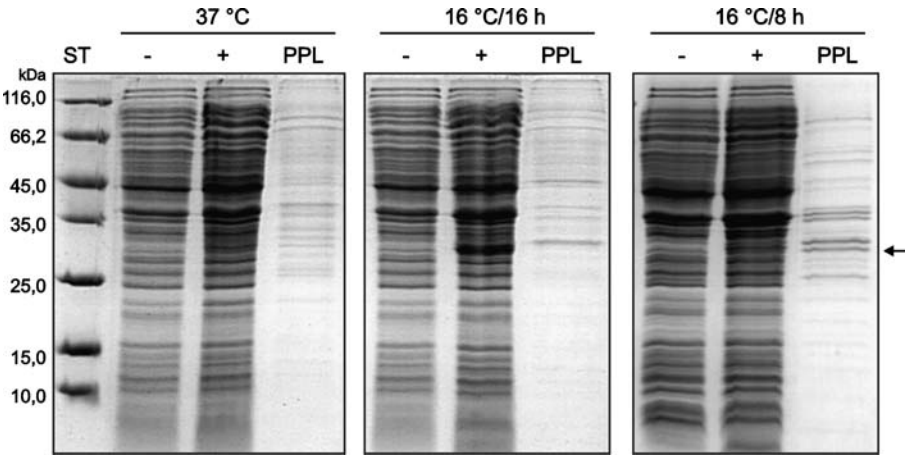

Fig. 2 SDS-PAGE analysis of LLH scFv expressing bacterial cell lysates before (−) and after (+) IPTG induction and periplasmic fractions (*PPL*) after expression at 37 °C for 4 h (*left*), at 16 °C for 16 h (*middle*), and at 16 °C for 8 h (*right*). scFv is indicated by an *arrow*. Molecular masses (kDa) of protein standards are indicated on the left (*ST*)

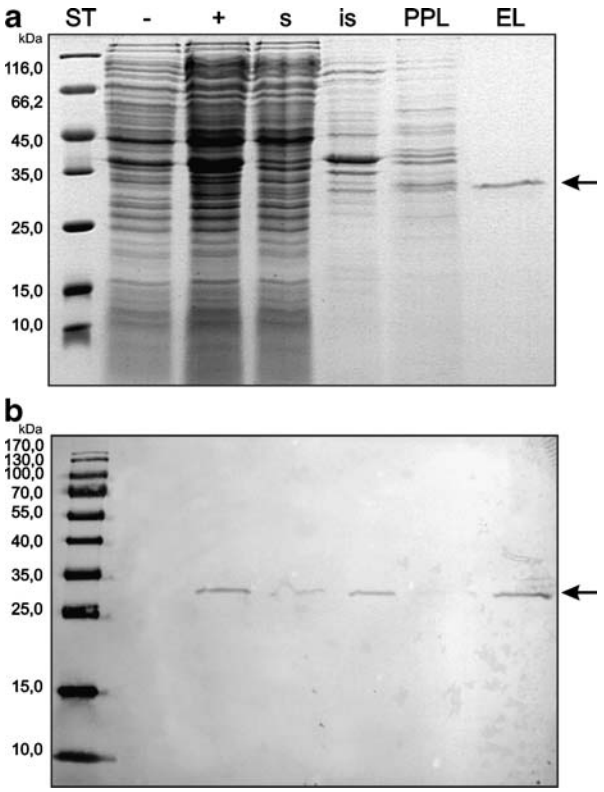

Fig. 3 SDS-PAGE (**a**) and Western blot (**b**) analyses of V5B2 scFv LLH. Cell lysates were analyzed before (−) and after (+) IPTG induction, along with soluble (*s*), insoluble (*is*), and periplasmic fractions (*PPL*). Purified scFv is shown in the last lane (*EL*). Molecular masses (kDa) of protein standards are indicated on the left (*ST*)

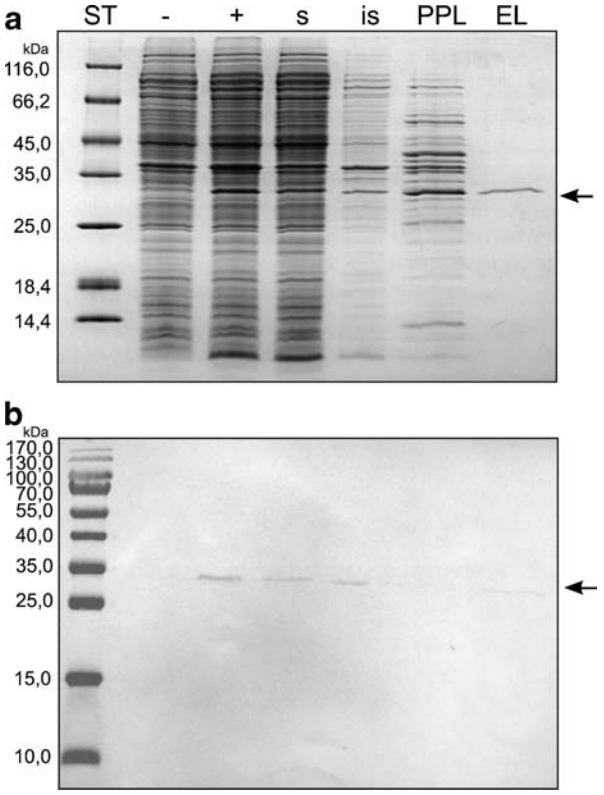

Fig. 4 SDS-PAGE (**a**) and Western blot (**b**) analyses of V5B2 scFv HLL. Cell lysates were analyzed before (−) and after (+) IPTG induction, along with soluble (*s*), insoluble (*is*), and periplasmic fractions (*PPL*). Purified scFv is shown in the last lane (*EL*). Molecular masses (kDa) of protein standards are indicated on the left (*ST*)

proteins were eliminated by washing with a buffer containing 20 mM imidazole. Both scFvs were eluted from the column after increasing imidazole concentration to 250 mM. The final yield of purified scFvs was estimated at 0.5–1 mg from 1 l of bacterial culture with a purity greater than 90%, since no contamination was seen on Coomassie-stained SDS-PAGE gels (Figs. 3a and 4a, lane EL). Purified antibody fragments were used for ELISA.

Antigen-Binding Properties of scFvs

The ability of the purified scFv to recognize its target molecule was confirmed by ELISA. Both scFvs recognized the epitope located at the C-terminal part of PrP, which was initially used as antigen for the preparation of the parent mAb, whereas no signal was detected when the wells were coated with bovine serum (Fig. 5). We, thus, concluded that the specificity of the parent antibody V5B2 was maintained. As shown in Fig. 5, LLH retained strong binding to peptide P1 with K_D of 30 nM, which compared favorably with the 5 nM estimated for the Fab V5B2 in a parallel assay. With much lower apparent affinity, HLL seemed to have lost some binding affinity for the antigen. Since direct ELISA was used for Fab and indirect ELISA for scFv binding quantification, ELISA signal values could not be directly compared. Nevertheless, positive ELISA results indicated that both antibody fragments had correctly folded antigen binding site that efficiently bound the synthetic peptide based on the C-terminal sequence of the prion protein. Such recombinant antibody fragments could, thus, be a useful substitute for disease-specific monoclonal antibody.

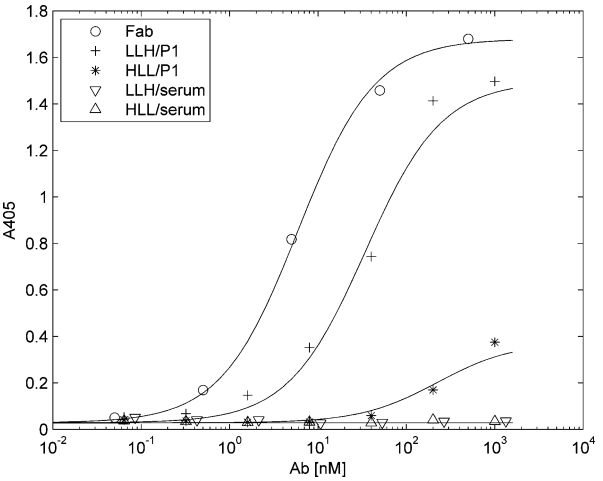

Fig. 5 ELISA analysis of antigen-binding efficiency of two V5B2 scFvs with opposing arrangement of variable domains and the corresponding Fab. The absorbance at 405 nm is plotted against the concentration of antibody fragment added to the wells coated with the P1 peptide or with 10% bovine serum. The data were fitted as described in Materials and Methods

Stability of scFv in Serum

The stability of scFv was examined by incubating the purified LLH scFv at 37 °C up to 40 h in bovine serum. The antigen-binding activity of incubated scFv was then assayed by ELISA. As shown in Fig. 6, LLH retained strong binding to peptide P1 even after incubation at 37 °C for 40 h, suggesting excellent stability of recombinant antibody fragment.

Discussion

Single-chain antibodies (scFvs) are recombinant antibody fragments consisting of light-chain (Vl) and heavy-chain variable region (Vh), where carboxyl terminus of one domain is connected to the amino terminus of the other variable domain by a polypeptide linker. Since the whole antigen binding site is retained, they are expected to exhibit the same binding

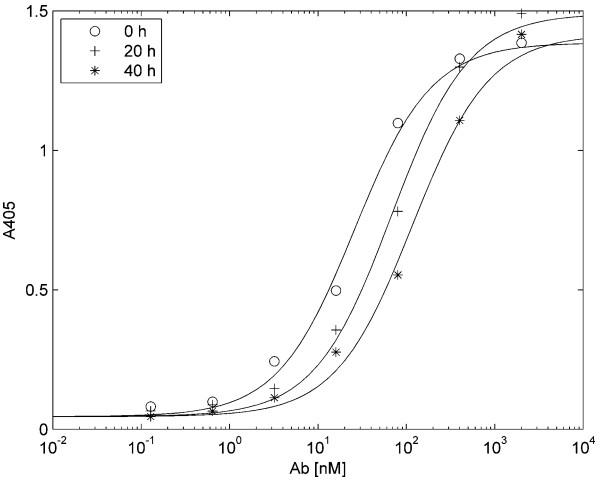

Fig. 6 LLH stability at 37 °C in 10% bovine serum. Samples were incubated at 37 °C for up to 40 h and then assayed for binding activity by ELISA

specificity and affinity as the parent antibody. The most widely used linkers consist of Gly and Ser residues which lack a well-ordered 3-dimensional structure [23]. Since they are less immunogenic, they are the most appropriate for construction of scFvs with potential therapeutic applications. Thus, a flexible linker of 15 amino acid residues with sequence $(Gly_4Ser)_3$ was used in both forms of scFv derived from V5B2.

Single-chain Fv can be made in either HLL or LLH orientation. The linker of the same length is more strained in the case of LLH, since the distance between the C-terminus of Vl and N-terminus of Vh is longer than between the C-terminus of Vh and N-terminus of Vl [24]. Even though the antigen binding site should retain the same structure in both orientations, HLL and LLH form of scFv only rarely exhibit the same expression levels and antigen binding properties. In our case, LLH was found to be better suited for further work, since it retained a strong antigen binding affinity, even though the expression level was slightly lower than in the case of HLL. Similar observations have been published for some other scFvs (e.g., [25]).

Single-chain Fvs have a much simpler structure than complete antibodies; besides, they do not require glycosylation so they can be successfully expressed in bacteria [26]. In *E. coli*, they can be produced in the cytoplasm as inclusion bodies or in the periplasmic space, where oxidizing environment enables disulfide formation and correct protein folding. Although much has been learned in the recent years about factors influencing antibody expression in *E. coli*, it is still not possible to determine universal conditions that can guarantee high yields. Primary structure of antibody variable domains remains the most important factor that influences the successful production of soluble antibody fragments [27]. Even after expression system improvement, the levels of protein recovery from the periplasm are usually below 1 mg/l bacterial culture [26]. In our experiments, both forms of V5B2 scFv have been successfully produced in bacterial periplasm with the reasonable yield of 0.5–1 mg functional protein per liter of bacterial culture after the optimization of expression. The ratio of soluble to insoluble recombinant protein was improved by lowering incubation temperature to 16 °C and shortening the incubation time from 16 to 8 h.

To make cloning easier and faster, the novel expression vector pMD204 was used [15]. In vectors pMD204-HLL and pMD204-LLH, parts that constitute scFv are separated by unique restriction sites; therefore, each of them can be easily exchanged. For example, with a simple exchange of the linker, scFv multimers with increased avidity could be produced [28]. Alternatively, by signal sequence excision, scFvs could be produced in bacterial cytoplasm as inclusion bodies. In addition, purification tag or even regulatory regions could be replaced if optimization of expression or purification is required.

Antibody fragments, produced in this study, were derived from mAb V5B2, prepared against a peptide P1, chosen from the C-terminus of the human prion protein. The monoclonal antibody V5B2 has the ability to discriminate the pathological (PrP^{Sc}) from the celullar form (PrP^{C}) of the prion protein [14]. Only a few antibodies distinguishing PrP^{Sc} from PrP^{C} have been reported to date, including mAb 15B3 [29] and mAb 8G8 [30, 31]. As we have shown by ELISA, both forms of V5B2 scFv retained the specificity of the parent mAb and efficiently recognized the epitope derived from the C-terminal part of PrP. Whereas the LLH form exhibited binding properties comparable to Fab V5B2 with affinity in nM range, the HLL form showed weaker binding in comparison with the Fab fragment. Similar results have been previously observed with other antibodies, where recombinant scFv in LLH orientation showed better binding properties (e.g., [32]), whereas others have found that the HLL exhibited both higher expression and better binding than LLH (e.g. [25]). However, binding weaker to antigen could be compensated by engineering the scFv to increase affinity or avidity.

As a part of a separate project, we have shown that our recombinant single-chain antibody fragment not only recognizes P1 peptide in ELISA but also specifically recognizes PrP^{Sc} aggregates in tissue samples of CJD patients (Škrlj, Vranac et al., manuscript in preparation).

We additionally assessed the question of specificity by assaying scFv binding to a mixture of antigens, such as bovine serum. No binding was detected, suggesting that there are no nonspecific targets in the broad spectrum of serum proteins. Moreover, we found in the literature no report that would corroborate the idea that preparation of scFv would expand or modify the specificity of the parent antibody.

Sufficient stability is one of the main requirements for proteins used in medical and biotechnological applications [33]. Therapeutic and diagnostic reagents need to retain their activity for several hours at 37 °C in serum or buffered solutions. Although many scFv antibody fragments are not stable for longer periods under such conditions (e.g. [34, 35]), V5B2 scFv was demonstrated to be highly stable in bovine serum at physiological temperatures.

Since there is no therapy or reliable premortem diagnostics for prion diseases available at the moment, numerous strategies and targets have been tested, including immunotherapy. The development of an effective anti-prion compound is challenging because the drug has to penetrate into the CNS. Many compounds are effective in a cell culture system but not in vivo, since they are not able to cross the blood–brain barrier. Moreover, a reliable preclinical test for diagnosing the disease as early as possible is a great challenge, so treatment at an early stage of the disease could be initiated. Since scFvs are much smaller than whole antibody molecules and could be easily produced and modified, new variations or conjugates with the ability to enter the CNS could be developed. It was already shown that effector functions encoded by antibody constant domains are unnecessary for their anti-prion activity [10]. Therefore, PrP^{Sc}-specific scFv V5B2 described here may be further modified into valuable diagnostic or therapeutic tools for prion disorders.

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