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Enhanced Bovine Herpesvirus Type 1 Neutralization by Multimerized Single-Chain Variable Antibody Fragments Regardless of Differential Glycosylation

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Single-chain variable antibody fragments (scFvs) with a 2-amino-acid linker capable of multimerization as di-, tri-, or tetrabodies that neutralize bovine herpesvirus type 1 (BoHV-1) *in vitro* were constructed and expressed in *Pichia pastoris*. In contrast to the monomeric form, multimeric scFvs had a higher virus neutralization potency, as evidenced by a 2-fold increase in their ability to neutralize BoHV-1 due to avidity effects. Mass spectrum (quadrupole time of flight [Q-TOF]) analyses of multimeric scFv demonstrated extensive heterogeneity due to differential cleavage, variable glycosylation (1 to 9 mannose residues), and the incorporation of minor unidentified adducts. Regardless of the differential glycosylation patterns, the scFvs recognized non-gB or -gE target viral epitopes in the BoHV-1 envelope fraction in a Western blot and also neutralized BoHV-1 in infected Madin-Darby kidney (MDBK) cells *in vitro*. Indirect evidence for the noncovalent multimerization of scFv was the presence of a major peak of multimerized scFv without a His tag (due to differential cleavage) in the Q-TOF profile, unlike monomeric scFv, which copurified with normally His-tagged scFv and recognized the target antigen. Overall, differentially glycosylated recombinant scFvs against BoHV-1 with a short linker (2 amino acids) are capable of assembly into functional multimers that confer high avidity, resulting in increased virus neutralization *in vitro* compared to that of monovalent scFv with a long (18-amino-acid) flexible linker. Overall, recombinant multimerized scFv5-2L potentially provides a high-potency therapeutic and immunodiagnostic reagent against BoHV-1, which is suitable for passive immunization and topical application.

The remarkable specificity to a desired epitope of monoclonal antibodies (Abs) (12) or their variable-region fragments (7) makes these proteins suitable for clinical diagnosis, immunodiagnostics, and therapeutics. A single-chain variable fragment (scFv) is an example of a monovalent Ab fragment where the amino-terminal variable heavy-chain and variable light-chain domains are held together through an artificial flexible peptide linker to form a stable antigen (Ag)-binding site against an epitope. For example, certain viral epitopes provide a suitable target for the binding of scFv to neutralize virus (7). Antibody fragments against human or animal pathogens can be produced economically in plants, mammalian cells, or microorganism-based expression systems such as *Escherichia coli* or *Pichia pastoris*. Previously, our laboratory constructed neutralizing scFvs with 7- and 18-amino-acid linkers against bovine herpesvirus type 1 (BoHV-1) (14, 15). The short linker size (0 to 2 amino acids) has been suggested to form trimers, tetramers, and/or higher multimers (6, 13), as it influences the configuration of the variable domains. The increased avidity as a result of such scFv multimerizations may potentially enhance virus neutralization abilities.

BoHV-1 is an etiological agent of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis in cattle (19, 23). Subsequent to primary viral infection, a lifelong latent infection is established in affected cattle, which can be reactivated during stress (weaning, transport, or crowding) or as a result of immunosuppression. BoHV-1 is also involved in bovine respiratory disease complex (BRDC) or shipping fever, a disease caused by a synergistic effect of viral and bacterial infections leading to severe pneumonia, which may be fatal. The economic losses due to BRDC alone cost the U.S. cattle industry up to 3 billion dollars annually (10). The currently used vaccines do not provide adequate protection against BoHV-1 infection due to associated problems such as viral shedding, abortions, immunosuppression, and reversion to virulence (22). Therefore, passive immunization with neutralizing Abs or their Ag-binding fragments potentially provides an adjunct disease prevention approach for the eradication of BoHV-1 from a herd. In addition, these Ag-binding fragments will provide useful reagents for the specific detection of BoHV-1 in an immunoassay, for example, by immunofluorescence (14, 15).

We demonstrate here the development of a functional multimeric scFv with a 2-amino-acid linker against BoHV-1 with improved *in vitro* virus neutralization potency, as a result of increased avidity via multimerization. The scFvs were expressed in *Pichia pastoris*, a eukaryotic microorganism capable of expressing scFvs in the secreted form and performing posttranslational modifications such as folding, the formation of disulfide bridges, and glycosylation (4). Little is known about the glycosylation of Ab variable regions, although it has been suggested that it may affect antigen recognition negatively or positively (8). The recombinant multimerized scFvs against BoHV-1 described here are heterogeneous proteins due to differential cleavage and glycosylation, but these proteins retain virus recognition and neutralization functions.

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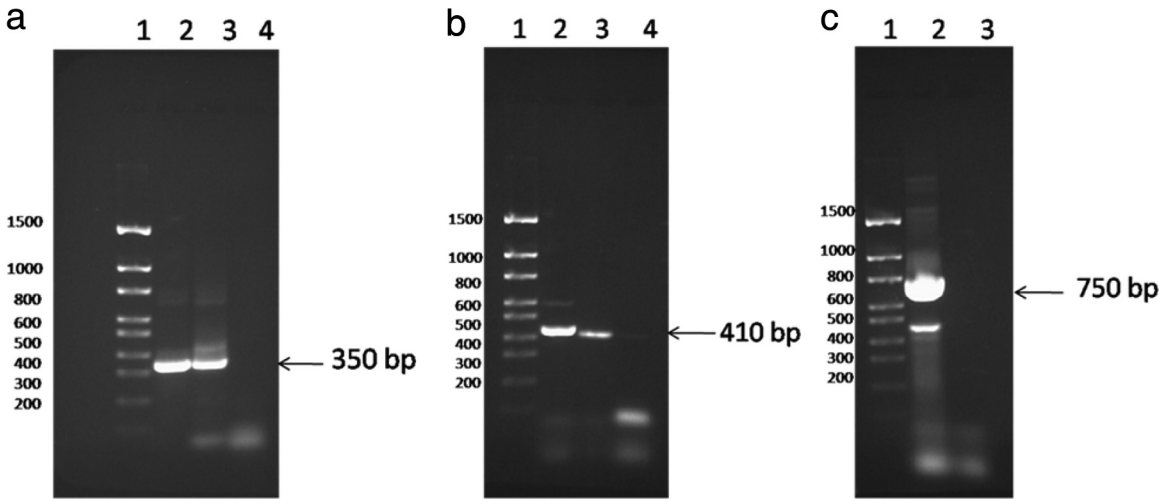


FIG 1 PCR amplification of V_λ-J_λ (a), V_H-D_H-J_H (b), and overlap V_λ-J_λ-2L-V_H-D_H-J_H (c) from cDNA from HB9907 xenogeneic hybridomas secreting IgG1 against BoHV-1. (a) Lane 1, molecular weight (in thousands) ladder; lane 2, HB9907 hybridoma $V_\lambda J_\lambda$ amplicon; lane 3, HB9908 $V_\lambda J_\lambda$ positive control; lane 4, negative control. (b) Lane 1, molecular weight ladder; lane 2, HB9907 $V_HD_HJ_H$ amplicon; lane 3, HB9908 $V_HD_HJ_H$ positive control; lane 4, negative control. (c) Lane 1, molecular weight ladder; lane 2, $V_\lambda J_\lambda$–2-codon-linker–$V_HD_HJ_H$ amplicon; lane 3, negative control.

MATERIALS AND METHODS

Hybridoma. A mouse × cattle xenogeneic hybridoma, HB9907 (ATCC, Rockville, MD) (17), secreting monoclonal IgG1 antibody against BoHV-1, provided cDNA, the genetic source of rearranged $V_HD_HJ_H$ and $V_\lambda J_\lambda$ genes. The hybridomas were grown in RPMI 1640 (Gibco Canada) supplemented with 10% horse serum, 5 mM sodium pyruvate, 0.5 M minimal essential medium (MEM) nonessential amino acids, 1 mM L-glutamine, gentamicin (50 μg/ml), and 5 μM 2-mercaptoethanol (14).

cDNA synthesis, PCR, and cloning. First-strand cDNA was synthesized by using SuperScript II reverse transcriptase (Invitrogen) from 5 μg of total RNA (TRIzol; Invitrogen) isolated from HB9907 hybridomas. The V_λ-J_λ recombinations were PCR amplified from cDNA using a sense primer corresponding to framework 1 (FR-1) with a built-in SfiI restriction site (underlined) (sVJ-s [5′-GTGGCCCAGCCGGCCCAGGCTGTG CTGACTCAG-3′]) and an antisense primer corresponding to FR-4 with the designed flexible linker (VJL-as [5′-AGAACCTAGGGACGGTCAGT GTGGTCCCGCT-3′]) (Mobix, McMaster University, Hamilton, Ontario, Canada). Platinum *Taq* high-fidelity polymerase (Invitrogen) was used to amplify the targeted cDNA using PCR conditions that included hot-start denaturation at 95°C for 30 s followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min. Similarly, $V_HD_HJ_H$ recombinations were amplified by using sense (LVDJ-s [5′-ACACTGACCGTCCTAGGTTCTCAGGTGCAGCTGCG-3′]) and antisense (VDJS-as [5′-CTGGCCGGCTTGGCCACTAGTGGAGGAGACG-GTGACCAG-3′]) primers (Mobix, McMaster University, Hamilton, Ontario, Canada) designed from the linker and FR-1, and FR-4 with an SfiI restriction site (underlined), respectively. The PCR conditions were similar to those used for $V_\lambda J_\lambda$ amplifications except for annealing at 68°C for 30s. The cDNA derived from HB9908 hybridomas (ATCC, Rockville, MD) (17) provided the positive control for both $V_\lambda J_\lambda$ and $V_HD_HJ_H$ amplification reactions.

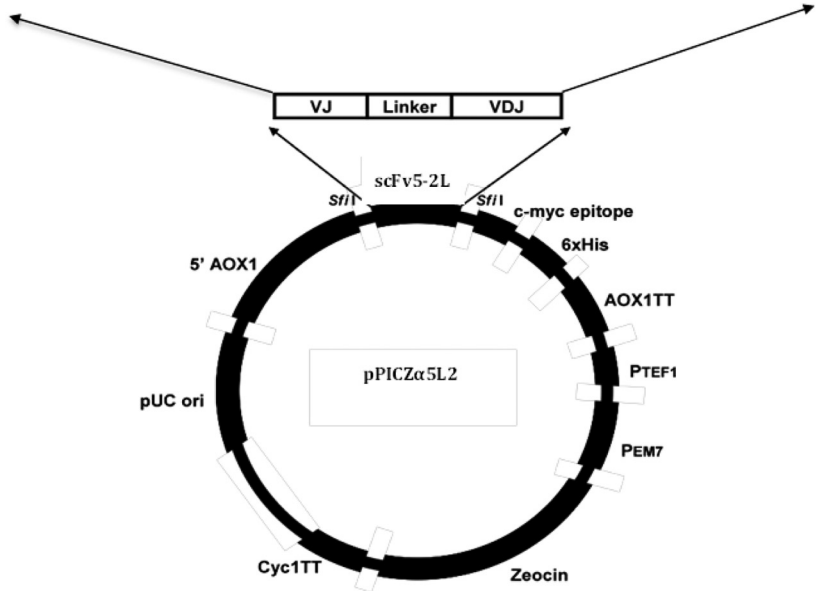
The purified $V_\lambda J_\lambda$ and $V_HD_HJ_H$ amplicons were combined in an overlap PCR (9, 14) including the nucleotide sequence encoding the 2-amino-acid linker (glycine-serine), using sense (SVJ-s) and antisense (VDJS-as) primers. The PCR conditions included a hot start at 94°C for 30 s followed by 25 cycles of denaturation at 94°C for 15 s, annealing at 65°C for 15 s, and extension at 68°C for 2 min, followed by a final extension step at 68°C for 30 min. The overlap SfiI-digested $V_\lambda J_\lambda$-2L-$V_HD_HJ_H$ amplicon was ligated into the SfiI-digested and dephosphorylated (calf intestinal alkaline phosphatase; Invitrogen) pPICZα expression vector. The ligate was used to transform *E. coli* One Shot TOP10 cells (Invitrogen), and plasmids isolated from zeocin-resistant colonies (QIAprep miniprep; Qiagen Inc.) were sequenced (Mobix, McMaster University, Hamilton, Ontario, Canada).

***P. pastoris* transformation.** Electrocompetent *P. pastoris* strain KM71H (Mut^s Arg^+; Invitrogen) cells were prepared according to the manufacturer's instructions for EasySelect. For transformation, 80 μl of *P. pastoris* was mixed with 5 μl SacI-linearized recombinant plasmid (~1 μg/μl) and incubated for 5 min at 0°C in a cuvette (0.2-cm gap; Bio-Rad). Electroporation conditions were a voltage of 1.5 kV, a field strength of 7.5 kV/cm, a capacity of 25 μF, a resistance (pulse controller) of 400 Ω, and a time constant of 8.1 ms (Genepulser; Bio-Rad). After the cells were pulsed, 1 ml 1 M sorbitol was added, followed by incubation at 30°C for 1.5 h. The transformants were plated onto 2% yeast extract-peptone-dextrose (YPD) agar supplemented with 1 M sorbitol and 100 μg zeocin/ml and incubated for 4 days at 30°C. Single colonies were grown at 30°C in buffered minimal glycerol complex (BMGY) medium for 18 to 24 h to an optical density at 600 nm (OD_{600}) of >2.0, and protein expression was induced in buffered minimal methanol complex (BMMY) medium containing 0.5% methanol. Methanol (0.5%, vol/vol) was added every 24 h to maintain induction until supernatant collection at 96 h postinduction.

scFv purification. The His-tagged recombinant protein secreted by *P. pastoris* KM71H was purified on a nickel-charged affinity column (ProBond; Invitrogen) under native conditions, as described previously (14). The purified recombinant protein was concentrated by using a centrifugal 10,000 molecular-weight cutoff (MWCO) filter device (Millipore, Bedford, MA), and protein concentrations were determined by the use of a Bio-Rad protein assay kit.

Western immunoblotting. Purified scFv was fractionated in 12% SDS-PAGE gels (15a) and stained with Coomassie blue (0.15%, wt/vol) (Brilliant Blue R-250; Fisher). The recombinant protein was electrophoretically transferred onto a nitrocellulose membrane (Protran; Schleicher & Schuell Inc.) and immunodetected by using a mouse anti-His antibody conjugated with alkaline phosphatase (AP) (1:2,000; Invitrogen) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)–nitroblue tetrazolium (NBT) chromogen (Promega). The negative methodological control included the pPICZα (without insert)-transformed *P. pastoris* KM71H (P4 clone) supernatant that did not express the recombinant protein, according to the manufacturer's protocol.

5′ CGGCCCAGGCTGTGCTTACTCATCTGGAAGCAGCGCCGTCCTCCGTGTCCGGCTCCCTG
GGCAGAGGGTCTCCATCACCTGCAGCAACATCGGTAGATATGGTGTGGGCTGGTACCAACAG
GTCCCAGGATCGGGCCTCAGAAGGATCATATATGGTAGTGTCAGTCGACCCTCGGGGGTCCC
CGTCCGATTCTCCGGCTCCAAGTCTGGCGACACAGCCACCCTGACCATCAGCTCGCTCCAGG
CTGAGGACGAGGCGGATTATTTCTGTGCAACTGCTGACTACACTAGTAGTCCTGTTCTTTTC
GGCAGCGGGACCACACTGACCGTCCTA**GGTTCT**CAGGTGCAGCTGCGGGAGTCGGGCCCCAG
CCTGGTGAAGCCCTCACAGACCCTGTCCCTCACCTGCACGGTCTCTGGATTCTCATTAAGCG
GTAATAGTGTAGGCTGGGTCCGCCAGACTCCAGGAAAGGCGCTGGAGTGGCTCGGTAACATG
GATGGTATAGGAACCACAGACTATAACCCAGCCCTGAAATCCCGGCTCAGCATCACCAAGGA
CAACTCCAAAAGCCAAGTCTCTCTATCACTGAGCAGCGTAACAACTGAGGACACGGCCACAT
ACTATTGTGCGAAGTGTACTGGTGCTTATTGCTGGAGGTTTGATGACGCTTATGGTTATGAT



GACTGGGGCCAAGGACTCCTGGTCACCGTCTCCTCCACTAGTGGCCAAGC 3′

FIG 2 Nucleotide sequence of the scFv5-2L variable-region construct in the pPICZα vector. Complementarity-determining regions (CDRs) are underlined according to the IMGT numbering system (22, 23), and the linker is shown in boldface type.

Mass spectrometry. Samples of purified and desalted recombinant protein were run on a Waters/Micromass Ultima quadrupole time of flight (Q-TOF) mass spectrometer operated in the positive-ion mode (McMaster Regional Centre of Mass Spectrometry, Hamilton, Ontario, Canada).

Virus neutralization assay. A plaque reduction assay was performed with recombinant scFv to determine its BoHV-1 virus neutralization ability (14). Recombinant scFv3-18L (15) and bovine serum positive for neutralizing antibodies against BoHV-1 (titer, 1:48; Animal Health Laboratory, Ontario Veterinary College) were used as positive controls. The negative controls included fetal bovine serum (FBS), bovine serum albumin (BSA), phosphate-buffered saline (PBS), and Dulbecco's minimal essential medium (DMEM). To determine if the recombinant protein caused a significant plaque reduction (≥50%), a one-sample *t* test (two-tailed *P* value) was performed (GraphPad Prism, version 4.03; GraphPad Software).

Immunodetection of the BoHV-1 epitope. BoHV-1 (Cooper strain)-infected MDBK cells were grown in DMEM (4% FBS and 1 mM L-glutamine) until approximately a 90 to 100% cytopathic effect was achieved. The clarified supernatant was spun at 115,460 × *g* for 1 h at 4°C (Optima L-80XP ultracentrifuge; Beckman Coulter) on 30% sucrose in a TNE-buffer layer (10 mM Tris, 100 mM NaCl, 1 mM EDTA [pH 7.4]), and the viral pellet was resuspended in Tris-EDTA (TE) buffer (pH 8.0).

Purified BoHV-1 was separated into envelope and capsid protein fractions (6a) and subjected to immunodetection by recombinant scFv in a Western blot. Briefly, BoHV-1 (250 μg) was resuspended in 250 μl viral lysis buffer (10 mM Tris buffer [pH 7.5] [Fisher Scientific], 1% Igepal-630 [Sigma]) and incubated at 20°C for 1 h, followed by centrifugation at 13,148 × *g* for 90 min at 4°C. The pellet comprising the capsid fraction was washed with viral lysis buffer and resuspended in loading buffer (100 mM Tris Cl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). The envelope protein fraction in the supernatant was precipitated with trichloroacetic acid (TCA) (100%, wt/vol) for 10 min at 4°C, and the pellet was washed three times with 200 μl cold acetone, dried, and resuspended in loading buffer. The samples were boiled for 10 min prior to fractionation on 12% SDS-PAGE gels and transferred onto a nitrocellulose membrane (Protran; Schleicher & Schuell Inc.). The nitrocellulose membrane was washed (0.05% Tween 20 in PBS) and blocked (2.5% Tween 20 in PBS), and the viral epitope was immunodetected by using recombinant scFv (5 μg/ml), followed by mouse anti-His-AP antibody (1:2,000; Invitrogen) and NBT-BCIP chromogen (Promega).

BoHV-1 gB and gE enzyme-linked immunosorbent assays (ELISAs). gB and gE enzyme immunoassays (Herdcheck IBRgB; Idexx, Switzerland) for the detection of BoHV-1-specific antibodies were used to determine whether recombinant scFv recognized the gB or gE immunodominant epitopes of BoHV-1. Briefly, 50 μl of phosphate wash buffer (Idexx, Switzerland) was added to each well coated with BoHV-1 antigen, followed by 50 μl of scFv3-18L (40 μg/ml) or scFv5-2L (40 μg/ml). The controls included negative BoHV-1 gB/gE (negative) and positive BoHV-1 gB/gE (positive) sera. The plates were incubated for 2 h at 37°C, followed by the incubation of BoHV-1 gB- or BoHV-1 gE-specific monoclonal antibody conjugated to horseradish peroxidase (HRP) for 1 h at 20°C. After the plates were washed, TMB substrate solution (3,3′,5,5′-tetramethylbenzi-

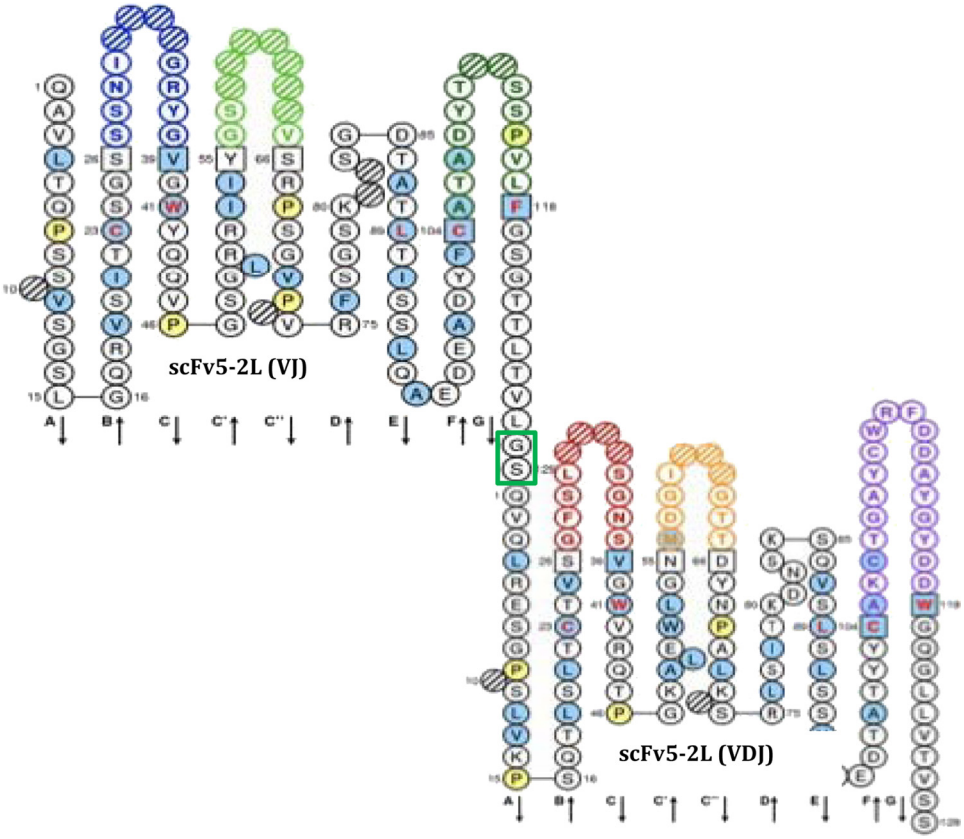


FIG 3 Two-dimensional IMGT Collier de Perles of the amino acids (shown as one-letter abbreviations) of the V_L-linker-V_H region of recombinant scFv5-2L expressed in *Pichia pastoris*. Hydrophobic amino acids (I, V, L, F, C, M, A, and W) in β-strands are shown in blue; proline (P) is shown in yellow. The loops are limited by amino acids shown in squares (anchor positions). Arrows indicate the direction of the β-strands. Blue, light green, and dark green loops represent CDR1L, CDR2L, and CDR3L, respectively; red, yellow, and purple loops represent CDR1H, CDR2H, and CDR3H, respectively; and hatched circles correspond to missing positions according to the IMGT numbering system for V and V-like domains (11, 16). The linker composed of glycine and serine amino acids is shown in the green box.

dine and H_2O_2) (Idexx, Switzerland) was added, and the reaction was stopped with 1 M HCl after 10 to 15 min. The absorbance was measured at 650 nm (Titertek Multiscan). The blocking of the gB and gE dominant epitopes was calculated as a percentage of the absorbance by the sample relative to the average absorbance value of the negative control (NC_{av}): $[(NC_{av} - OD_{sample})/NC_{av}] \times 100\%$. For the detection of the gB and the gE dominant epitopes, blocking percentages of <45% and <30%, respectively, were considered negative.

RESULTS

Construction of anti-BoHV-1 scFv with a 2-amino-acid linker. The variable lambda light- and heavy-chain rearrangements amplified from HB9907 hybridoma cDNA were combined in an overlap PCR (Fig. 1) together with a 2-codon (GGT TCT) linker and cloned into the pPICZα vector in the V_L-V_H configuration with a built-in c-myc epitope and His tag targeted for expression under the influence of the AOX1 promoter. Upon the screening of recombinant clones, the scFv2L5 clone was isolated and analyzed for nucleotide sequence (Fig. 2). The deduced protein sequence and secondary structure, presented as IMGT Collier de Perles in Fig. 3, demonstrated the characteristic strands, loops, and turns typical of variable domains. The supernatant of *P. pastoris* (KM71H strain) transformed with the scFv2L5 plasmid, expressing the recombinant protein after 96 h of induction with 0.5% methanol, was subjected to immunochemical analysis. The recombinant protein secreted by *P. pastoris*, designated scFv5-2L, demonstrated the presence of an approximately 34-kDa protein (Fig. 4) in a Western blot, consistent with the expected theoretical molecular mass of 30.3 kDa. These experiments demonstrated the stable expression of scFv with a 2-amino-acid linker in a V_L-linker-V_H configuration in *P. pastoris* that could be purified via a His tag on nickel-charged affinity resin.

Differential glycosylation in recombinant scFvs expressed in *P. pastoris*. Mass spectrum (Q-TOF) analysis of scFv5-2L showed multiple peaks at around 27 kDa and weaker signals at around 30.3 kDa (Fig. 5a). A careful analysis of the data, taking into consideration a 5-Da margin for readout accuracy (see Table S1 in the supplemental material), showed differential cleavage that resulted in a loss of the His tag on scFvs. Nevertheless, these scFvs without a His tag copurified on Ni-chelating resin with properly expressed scFvs with a His tag via noncovalent multimerization. This consistent observation provides indirect evidence for scFv5-2L multimerization *per se* as a result of the 2-amino-acid linker. The addition of 1 to 9 mannose residues and other unidentified small adducts (Fig. 5a) seemed to provide further scFv heterogeneity.

Mass spectrum analyses of monomeric scFv3-18L showed the most prominent signals at around 31.7 kDa (Fig. 5b; see also Table S1 in the supplemental material), where recombinant protein heterogeneity was due to the differential cleavage of the α-signal se-

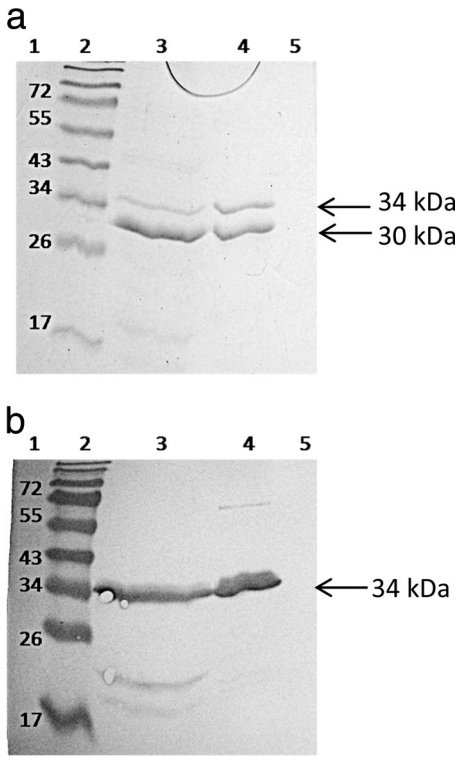


FIG 4 Coomassie blue-stained 12% SDS-PAGE gel (a) and Western immunoblot (b) of recombinant protein scFv5-2L. Lane 1, negative control (PBS); lane 2, molecular mass marker (kDa); lane 3, scFv5-2L supernatant (10× concentrate); lane 4, purified scFv5-2L protein; lane 5, negative-control recombinant *P. pastoris* P4 (without insert) supernatant.

quence by the Ste13 endoprotease together with a lack of glycosylation (31.552 kDa) or variable 1- to 8-mannose-residue (31.712 to 32.844 kDa) additions. The possible cleavage and posttranslational modification sites of recombinant scFv5-2L expressed in *P. pastoris* are diagrammatically shown in Fig. 6. As would be expected, His tag cleavage was not observed in the mass spectrum of recombinant scFv3-18L, consistent with its monomeric form.

Multimerized scFvs have higher BoHV-1-neutralizing potency. The virus-neutralizing ability of scFv5-2L was determined by a plaque reduction assay and evaluated in comparison to that of scFv3-18L. Recombinant scFv5-2L inhibited BoHV-1 replication in MDBK cells in a dose-dependent manner (Fig. 7). An scFv5-2L concentration as low as 1 μM neutralized (>50%) BoHV-1 replication in MDBK cells *in vitro* ($P < 0.05$). In contrast, a 2-fold amount (2 μM) of monomeric scFv3-18L was needed to achieve a comparable level of virus neutralization. Clearly, the *in vitro* virus neutralization potency of scFv5-2L is much higher (75%) than that of monomeric scFv3-18L (59%) at a 10 μM concentration (Fig. 7). While no nonspecific plaque reduction was observed with a heterologous protein, bovine serum albumin (BSA), anti-BoHV-1 bovine serum significantly inhibited BoHV-1 replication (65%; dilution, 50×). High-titer serum with anti-BoHV-1 antibodies achieved 100% plaque reduction in virus neutralization assays (data not shown). These observations suggest a 2-fold-higher BoHV-1 neutralization potency of multimerized scFv5-2L than that of monomeric scFv3-18L, apart from a higher degree of virus neutralization ability.

Recombinant scFv against BoHV-1 recognizes an epitope on the 32-kDa envelope protein. In a Western blot, recombinant scFv5-2L recognized an approximately 32-kDa protein in the envelope fraction of BoHV-1 (Fig. 8). Such an immunodetection of the 32-kDa protein in the BoHV-1 envelope is specific, as it was absent in the capsid fraction (Fig. 8). The scFvs with 7- and 18-amino-acid linker sizes are known to specifically recognize BoHV-1 antigen in infected but not uninfected MDBK cells in an immunofluorescence assay (14, 15). Furthermore, two immunodominant envelope glycoproteins, gB and gE, of BoHV-1 were not recognized by either multimeric scFv5-2L or monomeric scFv3-18L in a blocking ELISA (Fig. 9). These findings suggest that neutralizing recombinant scFvs against BoHV-1 likely recognize a non-gE or -gB immunodominant epitope on the viral envelope.

DISCUSSION

With the objective to enhance the BoHV-1 neutralization function of an scFv by multimerization to increase avidity, a recombinant scFv with a 2-amino-acid linker (scFv5-2L) was constructed and expressed in *P. pastoris* under the influence of the AOX1 promoter. The scFv was expressed as a secreted fusion protein with c-myc and His tags for subsequent purification and immunodetection. The presence of additional residues of a vector origin or the 2-amino-acid linker did not affect Ag recognition or hinder the multimerization required to form an Ag-binding site (6, 13). The functionality of scFv5-2L was confirmed by its BoHV-1 neutralization ability *in vitro* and antigen recognition in a Western blot. The scFv with a linker shorter than 12 amino acids cannot fold to form a functional domain but needs to pair with another scFv in order to form a functional Ag-binding site (2, 13). In addition to the linker size, scFv multimerization is influenced by factors such as the V_LJ_L-$V_HD_HJ_H$ interface stability, concentration, and ionic strength (1, 5). The noncovalent formation of multivalent multimers clearly increased the *in vitro* BoHV-1 neutralization potency, as it was achieved at a lower molar concentration (1 μM) by scFv5-2L, similarly to scFv1-7L (14) but in contrast to monovalent scFv3-18 (2 μM). Whether the formation of trimers, tetramers, or higher multimers generates only functional Ag-binding sites is not known, but the increased avidity of such multimers likely enhances the virus neutralization potency. Evidence for scFv5-2L multimerization leading to increased avidity, however, is largely circumstantial, relying on mass spectrum analyses and scFv functionality *per se*. Direct evidence for scFv5-2L multimerization using structural approaches, e.g., electron microscopy or crystallography, is needed to confirm the degree of multimerization in the context of valency. The *in vivo* protective ability of the scFvs is being experimentally tested in rabbit animal models and bovine calves.

The recombinant protein scFv5-2L, including monomeric scFv3-18L (data not shown), recognized the 32-kDa protein present in the envelope fraction of BoHV-1. The neutralizing epitope on BoHV-1 recognized by monoclonal IgG (HB9907) or the derived scFvs has not yet been identified. Based on theoretical molecular modeling (14), a probable carbohydrate moiety in the BoHV-1 epitope is predicted to be recognized by scFv5-2L or scFv3-18L present on the envelope glycoprotein. However, both scFv5-2L and scFv3-18L did not recognize immunodominant

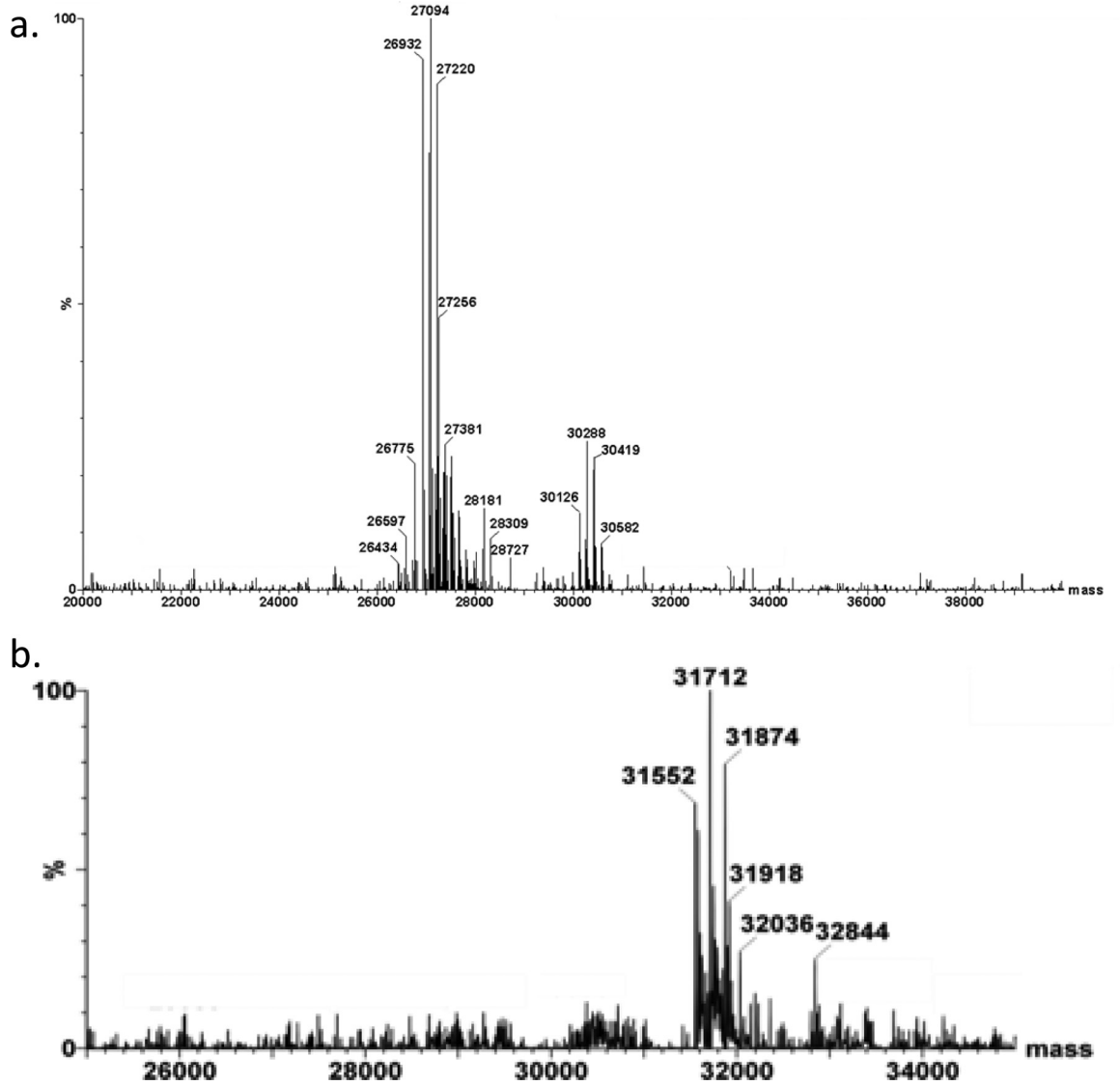


FIG 5 Mass spectra of recombinant scFv5-2L (a) and scFv3-18L (b) expressed in *P. pastoris*. The expected molecular mass for properly processed and unglycosylated scFv5-2L is 30,261 Da, and that for scFv3-18L is 31,555 Da. A detailed description of separate peaks is provided in Table S1 in the supplemental material.

epitopes on essential gB and nonessential gE envelope proteins (20). Experiments aimed at an analysis of surface-accessible neutralizing epitopes on the envelope protein, essential for viral replication, are in progress.

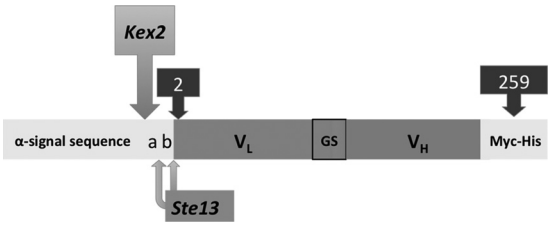


FIG 6 Schematic representation of possible cleavage and posttranslational modification sites of recombinant scFv5-2L expressed in *P. pastoris*. The signal sequence necessary for the secreted protein is cleaved by the Kex2 and Ste13 endoproteases; scFv5-2L amino acid positions 2 and 259 are likely sites of cleavage by an unidentified protease. Yellow boxes represent the sequence originating from the pPICZα vector. GS, glycine-serine (2-amino-acid linker).

Mass spectrum analysis revealed multiple scFv5-2L species at around 27 and 30.3 kDa but none within a 5-Da range of the expected mass of 30.261 kDa. Several peaks close to the expected molecular mass can be explained by the differential cleavage of the signal sequence and variable glycosylation. Some of the peaks representing molecular masses of around 27 kDa were found to be compatible with cleavage at position 259 of scFv5-2L, which would result in the loss of the c-myc and His tags, consistent with Coomassie blue-stained SDS-PAGE and Western blot observations. Purified His-tag-cleaved recombinant scFv5-2L, revealed during the mass spectrum analysis, could exist only because of noncovalent multimerization permitting copurification on Ni-chelating resin with properly His-tagged scFv5-2L. As the built-in expression vector His tag site resides outside the variable region of the heavy and light chains of the parental Ab, it does not interfere with the formation of the functional Ag-binding site. Such a cleavage of the His tag in some scFvs is consistent with the presence of a single but the absence of a separate protein band in the Western

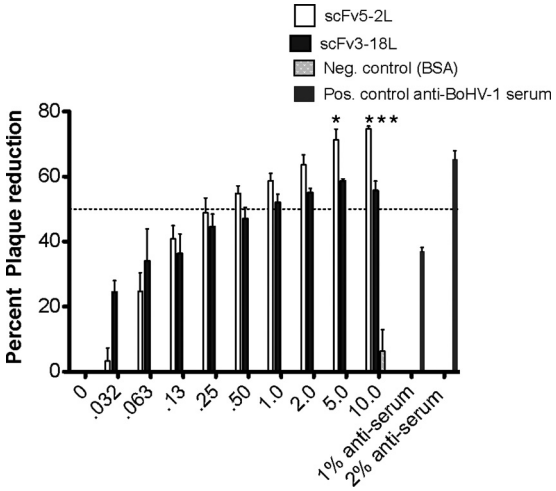


FIG 7 Virus neutralization potency of recombinant scFv5-2L and scFv3-18L. BoHV-1 neutralization is indicated by the >50% plaque reduction in 3 to 8 individual experiments. Note that multimerized scFv5-2L significantly inhibits plaque formation by BoHV-1 compared to monomeric scFv3-18L at concentrations of 5 μM ($P < 0.05$) and 10 μM ($P < 0.001$). Various controls included 100 PFU BoHV-1 with media including 2% FBS, BSA (negative), and 1% or 2% anti-BoHV-1 serum (positive). Bars indicate means ± standard errors (*, $P < 0.05$ [significant]; ***, $P < 0.001$ [extremely significant] [determined by an unpaired *t* test, with a two-tailed *P* value]).

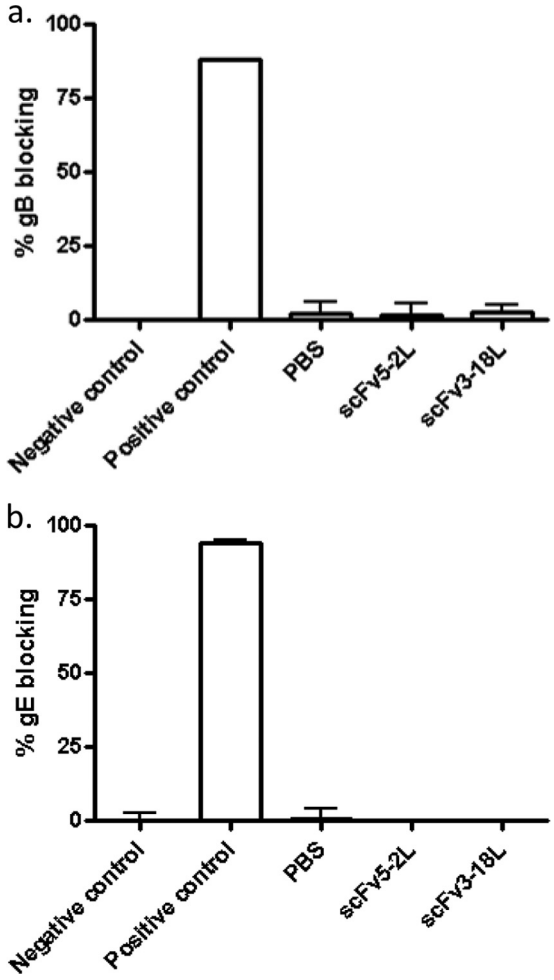


FIG 9 Blocking by recombinant scFv5-2L and scFv3-18L of the BoHV-1 envelope immunodominant epitope on gB using anti-gB-HRP monoclonal Ab (a) and on gE using anti-gE-HRP Ab (b) in an ELISA. Negative controls included bovine serum without gB or gE antibodies against BoHV-1 or phosphate-buffered saline (PBS), while bovine serum with a positive antibody titer for gB or gE provided the positive control.

blot. Mass spectrum analyses of scFv3-18L also showed protein heterogeneity with molecular masses of around 31.7 kDa containing a peak of 31.552 kDa, which seems to represent the expected theoretical molecular mass of 31.555 kDa. The observed scFv3-18L heterogeneity is likely because of differential cleavage, glycosylation, and the addition of minor adducts. The second cluster of peaks is absent as His-tag-cleaved recombinant scFv3-18L, as it will not be purified on nickel-charged affinity resin, confirming its monomeric form *per se*. Another research group (21) previously showed a similar cleavage of an scFv at the C terminus, resulting in the loss of the c-myc and His tags. Such a cleavage at the C terminus is probably caused by an unidentified protease that is not affected by the presence of the proteinase inhibitor Casamino Acids.

The scFv5-2L and scFv3-18L glycosylations related to a mass of 1 to 9 mannoses may reflect O-linked glycosylation, since a significantly higher mass would be expected if it were due to N-linked glycosylation. Since O-linked glycosylation has been described for *P. pastoris* on threonine and serine, these residues could also compete for the glycosylation of potential N-glycosylation sites (Asn-X-Ser/Thr). O-glycosylation is seen in both mammals and yeast, but glycosylation in *P. pastoris* can occur at sites not necessarily glycosylated in the native protein (4). Furthermore, the phosphorylation of mannose residues is common and could account for some of the unknown minor adducts (3). The relatively lower level of glycosylation of proteins expressed in *P. pastoris* together with reduced immunogenicity, in comparison to *Saccharomyces cerevisiae*, make *P. pastoris* a preferred expression system for proteins requiring posttranslational modifications (18). Little is known about the effect of glycosylation on the variable Ab regions, but it can positively or negatively influence Ag binding (8). Furthermore, glycosylation has been suggested to play an important role in the prevention of protein degradation (4). The experiments outlined here demonstrate that the differential glycosylation of scFvs (scFv5-2L and scFv3-18L) did not affect their antigen recognition or virus neutralization function. Furthermore, the inhibition of scFv glycosylation by the growth of *P. pastoris* in the presence of tunicamycin did not affect antigen recognition (data

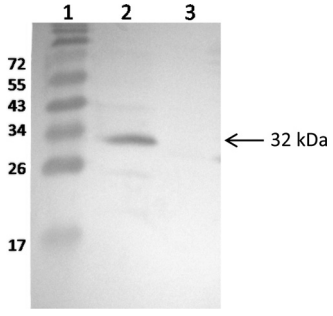


FIG 8 Western immunoblot of BoHV-1 envelope and capsid protein fractions immunodetected by recombinant scFv5-2L. Lane 1, molecular mass marker; lane 2, BoHV-1 envelope fraction; lane 3, BoHV-1 capsid fraction.

not shown). These observations together with mass spectrometry data suggest an O-glycosylation of recombinant scFv that is not inhibited by tunicamycin treatment. This is consistent with the absence of an N-glycosylation site on recombinant scFv. Nevertheless, scFv heterogeneity due to posttranslational modifications deserves important consideration in the development of standardized therapeutics and immunodiagnostics. Future experiments should aim at the development of growth conditions to prevent the heterogeneous glycosylation or enzymatic deglycosylation of recombinant proteins. The effect of the differential glycosylation of Ag-binding sites on scFv function needs to be examined.

The recombinant scFvs against BoHV-1 potentially provide an adjunct immunotherapeutic, together with the available vaccines against BoHV-1, especially during transportation, when latent BoHV-1 becomes activated as a result of stress, leading to "shipping fever" and bovine respiratory disease complex. Recombinant scFv may be used as an intranasal spray in vaccinated cattle during transportation to prevent BoHV-1 infection by blocking viral transmission. These immunotherapeutics may also be used to prevent the spread of BoHV-1 infection by artificial insemination by treating high-quality semen to inhibit BoHV-1 replication. Furthermore, these immunotherapeutics may be used for local application to treat infectious pustular vulvovaginitis caused by BoHV-1. While antibody-based therapy currently may be costly, technological advances, e.g., new expression systems, optimized antibody fragments, or combination therapies, are likely to make these therapies cost-effective in the future. Apart from their therapeutic significance, recombinant scFvs may be used for the rapid, specific, and sensitive immunodetection of BoHV-1 infection, for example, by immunofluorescence (14, 15). Overall, multimerized scFv5-2L has a high virus neutralization efficacy due to increased valency and is potentially suitable for passive immunization and topical application.

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