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Production of a Single-Chain Variable Fragment Antibody against Fumonisin B1

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The selection of synthetic antibody fragments from large phage libraries has become a common method for the generation of specific antibodies. The technique is particularly valuable when antibodies against small, non-immunogenic molecules (haptens) or highly toxic substances have to be produced. In addition, haptens are usually coupled to protein carriers, bearing the risk that the free hapten is not detectable. Here, a single variable chain antibody (scFv) against the highly toxic mycotoxin fumonisin B1 has been produced. The hapten was coupled via a linker to biotin. Using this conjugate and a naïve scFv library, it was possible to circumvent both the necessity of immunization and the risk of a disguised hapten. The scFv obtained after three panning rounds was found to bind specifically to both free fumonisin B1 and fumonisin−biotin conjugate. Also fumonisin B2 was bound by the scFv. Modeling of both scFv and fumonisin B1 molecule revealed a good fitting of structures. The antibody obtained can potentially be used for developing a rapid and affordable immunoassay for detection of food contamination and can be applied in immunoaffinity chromatography, usually carried out prior to HPLC analysis of mycotoxin-contaminated food and feed.

KEYWORDS: ScFv; fumonisin; recombinant antibody; hapten; mycotoxin

INTRODUCTION

Fumonisin B1 (**Figure 1**) is the principal secondary metabolite produced by the fungus *Fusarium verticillioides.* It is a potent toxin and can be found in fungus-contaminated corn and corn-based food products. Although different related fumonisins are known, fumonisin B1 and B2 are the most common ones. Fumonisin B1 is known to cause cancer in a number of experimental animals and has been linked to human esophageal cancer (*1*). As a consequence, there is a need for routine screening of agricultural commodities used for human and animal consumption. The quality control of food and feed can be achieved by PCR-based screening for toxin-producing fungi (*2*) or by the detection of the toxin itself.

There are different methods for the detection of fumonisin in food and feed, which include thin-layer chromatography (*3*), gas−liquid chromatography, high-performance liquid chromatography (*4*, *5*), and immunoassays (*6*). Antibody-based techniques, such as ELISAs, are widely used for routine screening of possible mycotoxin contamination. Diagnostic assays usually are based on competitive ELISA formats in which the binding of the anti-hapten antibodies to a labeled tracer molecule is inhibited by free antigen from the sample. The decrease of labeled tracer bound to the antibody is then determined (*7*). Alternatively, HPLC-based methods are applied if there is the necessity for a quantitative measurement of a small number of samples. Antibodies become more important in HPLC analysis as well, since immunoaffinity chromatography (IAC) is often used for the clean up of the samples prior to HPLC analysis.

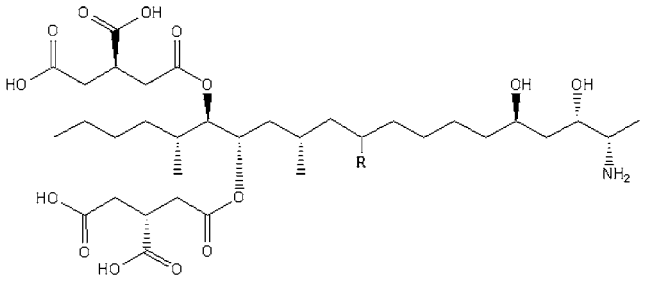

Figure 1. Structure of fumonisin B1 (R═OH), used for conjugation to EZ-Link NHS-LC-biotin, and fumonisin B2 (R═H), used in SPR for testing the specificity of scFv binding to fumonisin (**Figure 4**).

All commercial antibody-based detection or purification systems for mycotoxins emerged from animal immunization either as polyclonal sera or from hybridoma cell lines, which requires specialized cell culture facilities and usually is time-consuming. Since low molecular weight antigens (haptens) like mycotoxins are not immunogenic, these haptens are conjugated to carrier immunogens, usually a protein. Nevertheless, haptens do not have to be immunogenic (*8*, *9*). Advances in the field of recombinant antibody technology have allowed the production of low-cost antibodies with desirable affinity and specificity by screening of phage display libraries expressing single-chain variable fragments (scFvs). The in vitro selection of antibody fragments is independent of stimulation of an antibody response (*10*, *11*) but requires the immobilization of the hapten. Therefore,

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haptens applied in in vitro selection methods are usually conjugated to a carrier molecule, such as bovine serum albumin (BSA), which allow immobilization of the antigen for panning. However, many antibodies selected from naïve antibody libraries bind to the hapten−BSA conjugate and not to the hapten alone (*12*, *13*).

In the present study, we decided to minimize the possible influence of the carrier molecule on hapten binding by coupling of the fumonisin B1 via a long spacer to biotin, which later could be immobilized using different streptavidin-coated matrixes. Here, we describe the isolation and characterization of a scFv directed against the mycotoxin fumonisin B1 from a human synthetic phage display library.

MATERIALS AND METHODS

Immobilization Procedure. Streptavidin-coated microtiter strips (Nunc, Wiesbaden, Germany) were used for the immobilization of fumonisin B1 (Sigma-Aldrich, Taufkirchen, Germany) via the biotin linker EZ-Link NHS-LC-biotin (Pierce, Rockford, IL). For the coupling procedure, hapten and linker were incubated with a molar ratio of 10:1 (250 μL of 1 mg/mL fumonisin B1 with 15 μL of biotin-linker containing 32.5 nmol and 235 μL of PBS, pH 7) at room temperature for 2 h in a rotator (Rotamix RM1, ELMI, Latvia). Successful coupling was verified by surface plasmon resonance with the conjugate coupled to a sensor chip SA and mAb-anti-fumonisin B1 (R−Biopharm, Darmstadt, Germany) as analyte (data not shown).

Panning. The human synthetic antibody library ETH-2 (*14*) was used for the screening procedure. The panning procedure itself was performed as follows: one well of a Nunc streptavidin plate was coupled with the linker alone (100 μL) for capturing of unspecific phages. To a second well, the conjugate (100 μL of the coupling solution) was bound. After an incubation period of 30 min at 37 °C, the wells were washed three times with PBST (1× phosphate-buffered saline, pH 7.4 (PBS) + 0.05% Tween) for to remove uncoupled hapten, which might still be present. Afterwards, 10^{12} phages (100 μL) were added to the first well and incubated as described before. Then phages were transferred to the second well and kept for the same time and temperature. After that the phage solution was discarded, and the well was washed 15 times with PBST (200 μL). Acidic elution was performed with 100 μL of glycine/HCl, pH 2.8, for 15 min. Neutralization was carried out with 100 μL of 1 M PBS. *Escherichia coli* XL-1 Blue (Stratagene) (10 mL, $OD_{600} = 0.5$ in. 2 × TY (16% (w/v) tryptone, 10% (w/v) yeast extract, 5% (w/v) NaCl, pH 7.5) was infected with 180 μL of scFv phages eluted previously. The remaining 20 μL of scFv phages was used for the titer estimation. The infected bacteria were incubated for 30 min at 37 °C without shaking and then centrifuged for 10 min at 3300*g*. The pellet was resuspended in 1 mL of 2× TY and spread out on 14 cm TYE-glucose agar plates (TY medium plus additional 5.5% (w/v) NaCl, 12% (w/v) agar, 1% glucose, and 100 μg/mL ampicillin). The plates were incubated overnight at 30 °C. The next day 5−10 mL of 2× TY medium, supplemented with 15% glycerol, was added to the plates, and the colonies were loosened with a glass spreader. The suspension was used to inoculate 50 mL of culture in 2× $TY^{Amp-Glu}$ medium with an $OD_{600} = 0.05-0.1$. The culture was grown until $OD_{600} = 0.5$, then 10 mL of culture was infected with Hyperphage (Progen, Heidelberg, Germany). After incubation at 37 °C for 30 min, the culture was centrifuged at 3300*g* for 10 min, and the pellet was resuspended in 100 mL of 2× $TY^{Amp-Glu}$. Cultures were again incubated at 37 °C overnight on a shaker. Amplified scFv phages were purified by PEG precipitation and resuspended in 1 mL of 1× PBS buffer. The estimation of the titer was done as follows: 2× TY media was inoculated with *E. coli* XL-1 Blue and grown at 37 °C up to $OD_{600} = 0.5$. A volume of 50 μL of culture was infected with 10 μL of a serial dilution of the eluted scFv phages each and incubated for 30 min at 37 °C without shaking. The infected bacteria were plated on TYE-agar containing 100 μg/mL ampicillin without glucose. The titer was calculated by the number of colonies grown.

Analysis of Selected Clones. Recovered clones were first checked for full-length inserts by colony PCR. Plasmids from full-length clones were isolated and used for transformation of the *E.coli* strain TB1. Transformants were checked for scFv expression via SDS−PAGE of supernatants and immunostaining with anti-FLAG antibody, which is directed against the C-terminal FLAG-tag (*15*), and goat anti-mouse alkaline phosphatase (Sigma-Aldrich). SDS−PAGE, Western blotting, and immunostaining were performed as described (*16*, *17*). The nitrocellulose membranes were blocked using blotto (5% Uelzena nonfat milk, Germany) in 1× PBS + 0.05% Tween + 0.02% NaN_3). The NBT/BCIP system was used for the detection during Western blots (*18*). Positively identified clones were sequenced. Soluble scFvs were produced by overnight induction of 100 mL of cultures in TB medium supplemented with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 24 °C. Supernatant was applied to protein A Sepharose columns. ScFv from the library can be purified by protein A since all scFvs contain a V_H DP-47 germline segment (*19*). Eluted fractions from protein A columns were analyzed via SDS−PAGE and silver staining for purity. Positively identified fractions were pooled and dialyzed against HBS-EP buffer (Biacore, Uppsala, Sweden). A gelfiltration step for separating the monomeric from the dimeric form of the scFv was not necessary since the monomeric form of scFv BFB1-02 amounts to more than 93% (data not shown). Concentration of scFv was determined by A_{280} measurement.

Binding Kinetics. Affinity was determined by surface plasmon resonance (SPR) using a Biacore 3000 (Biacore, Uppsala, Sweden). The fumonisin B1-biotin linker was coupled to one flow cell on a sensor chip SA. A second flow cell was coupled with the biotin-linker only as a negative control. Measurement parameters were as follows: 3 min injection time of analyte at a flow rate of 30 μL/min, followed by a dissociation time of 10 min. After each cycle the chip was regenerated by a 30 s pulse of regeneration solution (100 mM NaOH, 0.1% SDS). The affinity of the scFvs for fumonisin B1 was assessed by injecting the appropriate scFv at a concentration ranging from 0 to 500 nM. Except for the 0 nM sample, all steps were performed twice. A mass transfer experiment was performed to exclude any influence of steric hindrance on the binding data obtained. For the competition experiments with free fumonisin B1, 500 nM scFv was incubated with free fumonisin B1 in concentrations from 0 nM to 5 μM of mycotoxin. To exclude an effect of DMSO (dimethyl sulfoxide) on binding kinetics, DMSO was added to the sample without fumonisin B1 as well. Injection time was only 2 min at a flow rate of 30 μL/min. Competition experiments using free fumonisin B2 or *N*-acetyl-D-sphingosine were performed at a concentration of 500 nM mycotoxin. Methanol was used for the solubilization of the mycotoxins. As described above, samples without mycotoxins were supplemented with methanol. Injection time and flow rates were the same as within the fumonisin B1 competition experiments. Response curves were generated by subtracting the control flow cell, with the linker only, from the flow cell with the bound conjugate. Biacore sensorgram curves were evaluated in BIA-Evaluation 3.2 RC1 using the 1:1 Langmuir binding model for response curves of various antibody concentrations. Dissociation constants (K_D) were calculated using the standards of the program.

Molecular Modeling. The analysis of scFv BFB1-02 was performed using the WAM algorithm (*20*, *21*). Parameters were as follows: dead-end elimination method was used for side chain building, and RMS deviation was used to screen for the final model. For visualization of pdb files, the program 3DMol viewer (Informax Inc.) was used. Measurement of distances was carried out with the same software package.

The analysis of the fumonisin B1 molecule was conducted based on elaborate designs in an interface of molecular graphics, being carried out at first geometry optimization with a molecular mechanics force field MM2 (*22*). The quantum calculations were made at semiempirical level using PM3, AM1, MINDO/3, and MNDO formalism with the MOPAC v.6 program. PM3 was the selected calculus to illustrate. The ZINDO/S method like the modified version of INDO was used to illustrate the molecular orbitals (*23*).

RESULTS

A synthetic antibody phage display library was used for the selection of scFvs against the mycotoxin fumonisin B1. The

Table 1. Overview of Selection of Antibodies against FB-1[a]

	panning round		
	1	2	3
phages applied	1×10^{12}	2×10^{10}	2×10^{10}
phages eluted	1.75×10^{6}	8.0×10^{5}	1.69×10^{8}

[a] Three rounds of screening were performed (1–3). Bound phages were eluted with glycine–HCl after 15 washing steps using PBST. For details about the panning procedure, see Materials and Methods. During the panning procedure, the number of phages applied decreases but the number of bound phages increases.

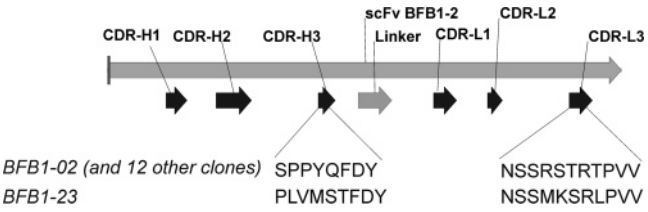

Figure 2. All scFv genes share the same sequence, except the CDR-3s of light and heavy chains. CDR regions of both heavy (CDR-H1 to CDR-H3) and light (CDR-L1 to CDR-L3) are indicated as arrows below the bar, representing the scFv gene. The linker connects heavy and light chain. After the final panning round, 13 out of 14 scFv-expressing clones were found to be identical in their nucleic acid sequence. From this group, clone BFB1-02 was used for further experiments. Only clone BFB1-23 showed different sequences in CDR-3 of heavy and light chain. The sequence variation between clone BFB1-02 and BFB1-23 in the region of the CDR-3s are outlined at the bottom of the figure.

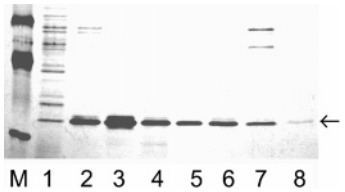

Figure 3. Silver-stained SDS–PAGE with different fractions from protein A Sepharose columns, which were used for the purification of the scFvs. Fractions with purified scFv were pooled and used for binding analysis. Lane M, Bio-Rad SDS-Marker Low; lane 1, washing fraction; lanes 2–8, eluate was fractionized in 900 μL fractions 1–7. Fractions 2–4 (lanes 3–5) were pooled for SPR analysis. Arrow indicates scFv BFB1-02 band.

antigen was bound as a biotin linker-mycotoxin conjugate to streptavidin coated ELISA wells. Three rounds of panning with decreasing amounts of scFv phages applied were performed. The titer of the eluted phages droped after the second round (**Table 1**) and increased strongly after the third. After the third panning round, 100 colonies resulting from the phage rescue were screened for full-length single-chain fragments. Sixty-one out of 100 clones contained a full-length scFv gene. These clones were subcloned into the host strain *E. coli* TB1 for expression as a soluble antibody.

The supernatant of small-scale overnight cultures were analyzed via SDS−PAGE, Western Blot, and immunostain. Fourteen clones expressing scFv were sequenced. Out of these clones, 13 were found to have the same nucleic acid sequence (**Figure 2**). From these identical clones, BFB1-02 was chosen for further analysis. Additionally, the clone BFB1-23, which was different from all others clones, was further analyzed as well. Since all scFvs contain a V_H DP-47 germline segment (*19*), the soluble scFvs could be isolated using protein A Sepharose columns (**Figure 3**). Eluted fractions containing

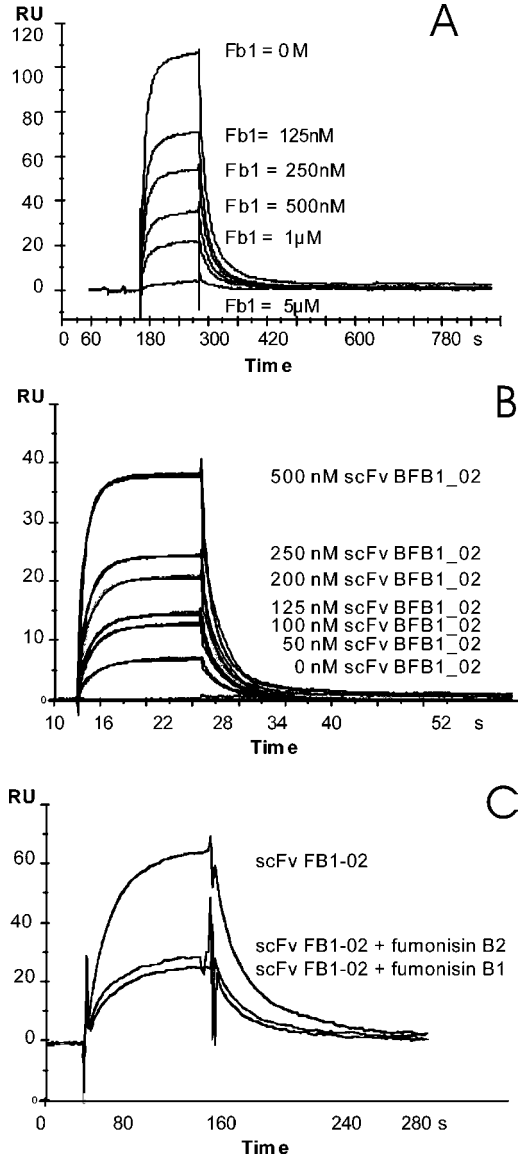

Figure 4. Binding analysis of scFv BFB1-02 using surface plasmon resonance. (**A**) Dose-dependent binding of scFv BFB1-02 by SPR. Purified scFvs were injected at concentrations indicated over a flow cell coated with fumonisin B1-biotin conjugate. (**B**) Competition between fumonisin B1-biotin conjugate and free fumonisin B1 in various concentrations as indicated. 500 nM of scFv BFB1-02 was used. (**C**) SPR analysis using scFv BFB1-02 with either fumonisin B1 or the closely related fumonisin B2 (**Figure 1**) at concentrations of 500 nM shows that both mycotoxins can be detected by scFv BFB1-02. Values for all figures shown have been corrected for binding to the linker-coated flow cell.

purified scFvs were pooled and applied in binding analysis using SPR.

Both scFvs were tested for their ability to bind the fumonisin B1−biotin conjugate using SPR. No high affinity binding was detectable for BFB1-23 (data not shown). The scFv BFB1-02 revealed good binding kinetics with a $K_D = 4.08 \times 10^{-7}$ M, as shown in **Figure 4A**. To exclude possible artifacts by mass transfer, some runs were carried out with identical parameters but different flow rates. These experiments revealed no influence of any mass transfer on binding kinetics measured before. After analysis of binding toward the mycotoxin−biotin conjugate, we performed competition experiments with free fumonisin B1. 500 nM scFv BFB1-02 was incubated with increasing concentrations of fumonisin B1 from 0 nM up to 5 μM (**Figure 4B**).

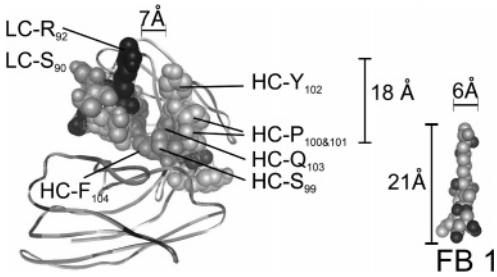

Figure 5. Computer-based molecular modeling of scFv (**left**) and fumonisin B1 (**right**). Amino acid residues are colored according to their hydrophobicity. Only amino acids involved in binding toward fumonisin B1 are shown as space fill representation. Labeling relates to amino acid numbering of HC gene, starting from M_1, and LC after the synthetic linker, starting at E_1, which is amino acid 136 of the whole scFv protein. Fumonisin was modeled as described in the text. Bars indicate size of fumonisin B1 and the cavity of the scFv.

Competition was observed with increasing concentrations of fumonisin B1, which shows that the scFv BFB1-02 recognizes free fumonisin B1. Finally the specificity of binding was tested. As described above, the scFv BFB1-02 was incubated with either fumonisine B1 or fumonisin B2 (**Figure 4C**). No significant difference in binding was found, indicating that both forms are detectable by the scFv BFB1-02, whereas the same amount of *N*-acetyl-D-sphingosine added to the scFv showed no effect on binding (data not shown).

The modeling of the scFv according to Whitelegg and Rees (*20*) revealed that CDR-3 of both light and heavy chains are part of the binding cavity (**Figure 5**). The size and shape of the cavity fits well with the fumonisin B1 molecule.

DISCUSSION

The vast majority of antibodies produced against mycotoxins were obtained by immunization of animals with the molecule coupled to a carrier protein (fumonisin B1 (*24*), aflatoxin B1 (*25*), or diacetylnivalenol (*26*)). All but one scFvs directed against mycotoxins, described up to now, originated from cloning of monoclonal mouse hybridoma cell lines or from phage display (*24*, *25*, *27*, *28*). Only one report described the production of scFvs against the mycotoxin aflatoxin B1 starting from a naïve scFv library (*12*). There have been only a few reports of isolation of a scFv against other low molecular weight haptens from a naive phage antibody library as well. Most authors described the use of small molecules coupled to a carrier protein, usually BSA for immobilization to a matrix (e.g., phenyloxazolone−BSA (*19*, *29*), iodohydroxynitrophenyl acetate−BSA (*19*), progesterone−BSA, testosterone−BSA, digoxigenin−BSA, and estradiol−BSA (*30*)). Since BSA may influence the accessibility of the hapten, many of these antibodies directed against the hapten−BSA conjugate did not bind the soluble form of the hapten (*13*). In some of our experiments, we used a fumonisin B1−peroxidase conjugate in SPR analysis of scFv BFB1-02. As expected, the affinity of the scFv toward the fumonisin B1−peroxidase conjugate was lower than toward fumonisin B1−biotin or free fumonisin B1 (data not shown). Furthermore, many of the haptens used in the model studies mentioned above are not readily soluble in aqueous solutions. There are only very few reports of scFvs characterized for binding to soluble unconjugated hapten (*13*).

To circumvent these problems, we decided to couple the fumonisin B1 via a linker to a modified biotin. We have chosen this strategy because the linker is commercially available with different spacer lengths and does not limit the accessibility of the scFv toward the fumonisin B1. Biotin binds with very high affinity to streptavidin and withstands pH values as low as 2.0. Finally, the conjugate can be used for both the panning procedure in streptavidin-coated ELISA plates and the analysis via SPR.

During the panning procedure, titer of eluted phages dropped after the second round, which was probably caused by low amplification titers after the first panning round. We used 2×10^{10} phages for the second and third panning round (**Table 1**). The strong increase after the third round indicates the enrichment of specific binding phages. This observation proved true, since out of 14 scFv-expressing clones after the third panning 13 clones were found to be identical in their nucleotide sequence. The acidic elution took place for 15 min in order to elute the strong binding scFv phages (*31*). The antigen-specific elution may lead to the isolation of binders more specific to the free hapten but raises the risk to select low-affinity binders (*31*).

The screening of 100 colonies by colony PCR revealed that 61% of checked clones contained a full-length scFv. Similar observation was also made by other groups (*31*). Interestingly only 14 out of the 61 clones with full-length insert showed detectable expression. This can be explained by mutations leading to frameshifts (*32*). Out of the 13 identical clones identified, we chose clone BFB1-02 for further analysis and clone BFB1-23, which contained CDR-3s without any sequence homology toward the other clones (**Figure 2**). No high-affinity binding toward fumonisin B1 could be detected for BFB1-23 (data not shown). For SPR analysis, soluble scFvs were purified via protein A Sepharose. A further gelfiltration step to remove dimeric scFvs was not necessary: the heavy chain used for the construction of the library belongs to the VH3 germline family. As part of an scFv, they exhibit several superior properties concerning yield and stability, they can be purified via protein A, and they show a stable monomeric state (*33*). The SPR analysis, which followed, covered at least four different aspects of binding analysis (**Figure 4**): first, binding to the fumonisin B1−biotin conjugate was analyzed, and then the detailed analysis of scFv BFB1-02 binding toward fumonisin B1 was analyzed against free hapten. For the clone BFB1-02, a K_D of 4.08×10^{-7} M was determined using a Biacore 3000. This affinity is in the range of affinities described for scFvs, which are directed against haptens (*34*). Kinetic studies using large analytes such as antibodies are prone to generate steric hindrance and mass-transport artifacts that affect true kinetic data (*35*). To exclude any influence of mass transport effects, SPR was performed at a constant concentration of scFvs. Sensorgrams, recorded at different flow rates, showed no changes. Therefore, an influence of mass transfer effects could be ruled out. Finally, the application of the structural closely related fumonisin B2 results in a strong decrease of the signal, showing that BFB1-02 can be used for the detection of both fumonisins. *N*-Acetyl-D-sphingosine could not compete binding of the scFv BFB1-02 toward fumonisin B1−biotin (data not shown), indicating that the detection of fumonisin is a specific feature of this antibody fragment. The capability for the detection of both fumonisin B1 and B2 is advantageous since the sensitivity toward fumonisin B2 of most commercially available ELISA-based detection systems is only 40−50% as compared to fumonisin B1.

One prominent feature of the scFv phage library used is that only three antibody germline gene segments (DP-47 for the heavy chain, DPK-22 and DPL-16 for the light chain; *36*) were utilized. The large repertoire was produced by appending short variable CDR-3s onto them. Since the binding clone BFB1-02

differs from nonbinding clone BFB1-23 only in the CDR-3 of light and heavy chain (**Figure 2**), the binding moiety for fumonisin B1 must be located within the cavity built up by these two CDR-3s **(Figure 5)**. The shape and size of the binding sites correspond well to the modeled structure of fumonisin B1 (**Figure 5**): the cavity has a diameter of about 7 Å as compared to 6 Å for fumonisin B1. The linker bound nitrogen of fumonisin B1 (length about 21 Å) sticks out of the cavity (18 Å), the CH_2-chain aligning to the rigid CH_2-chain of Arg 92 of the light chain. The other end of the fumonisin B1 molecule is larger in diameter. This is also true for the base of the scFv cavity, which is wider than the upper moiety. The model presented here will facilitate the further improvement of the scFv BFB1-02 toward its use in a cost-effective and rapid immunoassay for mycotoxin detection in food and beverage. The in vitro screening technology enables a cheaper antibody production, especially, when plants are used as production system (*37*). This opens up new vistas, like the use in affordable immunoaffinity chromatography, which is used prior to highly sensitive analytical systems.

ABBREVIATIONS USED

BSA, bovine serum albumin; PBS, phosphate-buffered saline, pH 7.4; TY, tryptone yeast medium; CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay; scFv, single variable chain fragment; SPR, surface plasmon resonance; V_H, variable heavy chain.

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