## Letter to the Editor

# Isolation and Characterization of Recombinant Variable Domain of Heavy Chain Anti-idiotypic Antibodies Specific to Aflatoxin $B_1$*

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**Some unique subclasses of Camelidae antibodies are devoid of the light chain, and the antigen binding site is comprised exclusively of the variable domain of the heavy chain (VHH). The recombinant VHHs have a high potential as alternative reagents for the next generation of immunoassay. In particular, they might be very useful for molecular mimicry. The present study demonstrated an alpaca immunized with the F(ab')$_2$ fragment of anti-aflatoxin $B_1$ mAb and developed an important anti-idiotypic (anti-Id) responses. Antigen-specific elution method was used for panning private anti-Id VHHs from the constructed alpaca VHH library. The selected VHHs were expressed, renatured, purified, and then identified by a competitive enzyme-linked immunosorbent assay (ELISA). Our findings indicated that the VHH would be an alternative tool for haptens mimicry studies.**

Among the identified aflatoxins, aflatoxin $B_1$ ($AFB_1$) is the most toxic and the most frequently found in contaminated food products. $AFB_1$ can be carcinogenic, hepatoxic, or immunosuppressive and is classified as a group I human carcinogen by the International Agency for Research on Cancer (IARC). In immunological analysis of $AFB_1$, hapten-conjugates connected with a carrier such as bovine serum albumin (BSA), keyhole limpet hemocyanin, or horseradish peroxidase is often necessary, but their synthesis is time-consuming, expensive, and inevitably causes environmental pollution. Therefore, a reliable and nontoxic detection method is imperiously demanded.

Anti-idiotypic antibody (AId or Ab2), first advanced by Jerne in his immune network theory, implies that AId is an antibody raised against the variable region of an original antibody[1]. AIds were classified into four types (α, β, γ, and ε) by Bona[2]. β-AId (Ab2β) binds to idiotopes on the paratope and competes with the binding of the hapten, so it is considered a hapten-inhibitable AId and is called an internal image of the antigen in immunology. AIds have been structurally characterized, as with any conventional antibody, their recognition surfaces are large flat areas composed of all of the six complementarity-determining regions (CDRs) from heavy and light chain variable domains[3], which seriously limit their use for mimicry of small haptens that interact with the cavities or crevices of their target proteins. In camelids, a subset of the immunoglobulins consists of heavy-chain homodimers devoid of light chains, and is thus called heavy-chain immunoglobulin G. Their variable region is the smallest antigen-binding fragment possibly comprising only one polypeptide chain, so it is especially suitable for engineering. Their different binding strategies, longer CDR3 loops protruding from the binding site, and deviation of CDR conformations from the equivalent human or mouse loop structures[4] suggest that VHH may be very useful for the molecular mimicry of haptens.

Alpaca were immunized every 21 days with 1 mg of purified F(ab')$_2$ fragments of anti-$AFB_1$ mAb named 7H2 in Freund's adjuvant (Sigma-Aldrich) at a 1:1 protein to adjuvant ratio for a total of four immunizations. Seven days after each boost, blood samples were collected and the sera were monitored by indirect competitive ELISA to determine whether the anti-Id antibodies recognizing the paratope of the 7H2 were produced. Increasing the amount of injected F(ab')$_2$ fragment caused an increase in inhibition. After four immunizations, as shown by Figure 1A, the anti-Id antibody in the sera (1/1 280 dilution) exhibited an inhibition of 90.8% when the 7H2 was bound to the $AFB_1$-BSA conjugate. The specificity of the serum was also assessed with anti-DON mAb and anti-ZEN mAb, unconspicuous inhibition was observed which indicated that the

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antiserum exhibited well specificity to the 7H2.

The phage display library was constructed according to Maass[5] with some modifications. Total RNA was isolated from about $3\times10^7$ lymphocytes collected from the blood of the immunized alpaca and used to synthesize the first-strand complementary DNA (cDNA) employing oligo dT primers. The cDNA served as a template for the first PCR using eTaq (TAKARA) with AlpVh-LD (5'-CTTGGTGGTCCTGGCT GC-3') and CH2-R (5'-GGT ACGTGCTGTTGAACTGTTCC-3') specific primers. Subsequently, the amplified products were used as template DNA for nested PCR using primers specific for the extremities of framework-1 (AlpVh-F1, sense, 5'-tcgcggcccagccggccatggccCAG KTGCAGCTCGTGGAG TCNGGNGG-3') and short/long hinge (AlpVHH-R1/ Alp VHH-R2, anti-sense, 5'-cgagtgcggcc gcGGGGTCTT CGCTGTGGTGCG-3'/5'-cgagtgcggccgcTTGTGGTTTTG GTGTCTTGGG-3'), resulting in an amplified VHH DNA fragment without the CH2 gene segment. These primers also introduced SfiI and Not I restriction sites at the 5' and 3' ends of the VHH sequence, respectively. The nested PCR products were digested with SfiI and Not I (TAKARA) and ligated into similarly digested phagemid pHEN1 at a mole ratio of 4/1, and then incubated at 16 °C for 12 h using 400 U of T4 DNA ligase (NEB). The ligation products were electroporated into *E. coli* TG1 electrocompetent cells. The size of the VHH library was estimated by plating it on LB + ampicillin (100 μg/mL) agar plates. Dilution plating of electro-transformed *E. coli* indicated a total dimension of $2\times10^8$ colonies. It was found that about 93% of 44 clones which were randomly selected from the colonies had VHH inserts. The library was rescued by the helper phage KM13, and the titer of the phage display library was $1\times10^{13}$ colony forming unit/mL.

The phage display library was panned against purified 7H2. Two rounds of panning were carried out adopting specific elution method. About $1\times10^{11}$ phage particles were input for each round of panning, and the titer of the eluted phage was increased in the second round of panning. Phage ELISA was conducted on 48 randomly selected clones from each round of panning. Up to 13 and 33 clones were identified out of the 48 tested phage clones each round showed absorbance higher than that of NC when the capture target was 7H2. The competitive phage ELISA showed that 7 out of the 13 clones acquired from the first round of panning could be inhibited by $AFB_1$ standard when the 7H2 was the capture target, whereas 10 out the of the 33 clones acquired from the second round of panning showed competitive activity with 7H2 while $AFB_1$ standard existed (Figure 1B). Hapten inhibition anti-idiotypic (Ab2β) consisted of 30% and 53%. The phage ELISA and competitive phage ELISA were performed according to Tu et al.[6]. The use of an antigen-specific elution method during the selection of Ab2β from an alpaca VHH library could lead to an

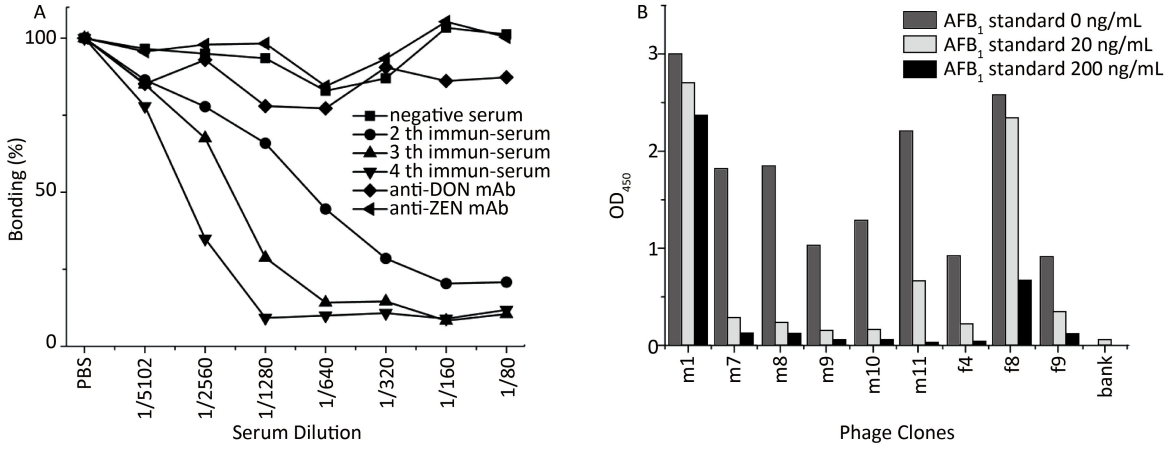


**Figure 1.** (A) Alpaca IgG in serum response against the $F(ab')_2$ fragment of 7H2. Serial dilutions of pre-immunization and post-immunization serum were analyzed by indirect competitive ELISA against 1 μg/mL of $AFB_1$-BSA coated on a ELISA plate with 7H2 ($1:10^4$ dilution). The sera were also monitored by DON-BSA with anti-DON mAb and ZEN-BSA with anti-ZEN mAb. (B) Selected clones from the specific elution protocol. The m1, m7, m8, m9, m10 and m11 were isolated from round 1; f4, f8 and f9 were isolated from round 2. $AFB_1$ standards (20 ng/mL and 200 ng/mL) were used to assess the specificity in competitive phage ELISA.

enrichment of private Ab2β binders, thereby increasing the possibility of selecting mimetic antibodies. This specific selection appears to be a method of choice for small haptens and for macromolecules such as nucleic acids or proteins, as demonstrated by Zarebski et al.[7].

DNA sequencing was conducted on the 17 positive clones from the two rounds of selection containing 8 different coding sequences. Among these clones, m1, m7, m8, m9, m10, and m11 were panning from the first round, and f4, f8 and f9 were from the second round. The m1 and f9 were the same sequence acquired from both panning rounds. All the sequences were similar in framework region and CDR, except for some differences in amino acid residues. Clones m10 and m11 had the longest CDR3 with 14 amino acid residues, while the others all had 13 amino acid residues. Furthermore, clones m1, m7, f4 and f8 had the same amino acid residues in CDR3; differences only existed in FRS, CDR1, and CDR2 (Table 1). These identified camelid IGHV genes belong to the subgroup IGHV1, which corresponds to the human IGHV3 subgroup. Sequence analysis of the clones revealed that these amino acid sequences displayed a high level of homology with each other, especially in CDR3, whether acquired from round 1 or round 2. These results suggest that only a few different structural solutions are needed to create a specific binder against a given idiotope, as previously shown by Zarebski et al.[7].

One of the VHH clones with specificity to 7H2 named m7 was chosen to express in pET 22b vector. For expression, the *Nco* I/*Not* I digested VHH fragments were cloned into the pET 22b vector containing a 6HIS tag. *E. coli* BL21 (DE3) transformed with the expression vector were grown in 100 mL of LB medium supplemented with 100 μg/mL ampicillin and 0.1% (w/v) glucose. When the cells were induced at 30 °C with 0.4 mmol/L isopropyl-β-D-thiogalactopyranoside for 4 h, proteins m7 were expressed as inclusion bodies. The

**Table 1.** Comparison of the Deduced Amino Acid Sequences of the Nine Anti-Id VHHs Specific to $AFB_1$

| | FR1 | CDR1 | FR2 | CDR2 |
|---|---|---|---|---|
| | 1 25 26 33 34 50 51 60 | | | |
| m1 | QLQLVESGGGLVQPGGSLRLSCAAS GFTLDYYV TGWFRQAPGKEREGVSC ISRS--GTST | | | |
| m7 | QLQLVESGGGLVQAGGSLRLSCVAS GVTLDYYA IIFFRQAPAKEREGVAC IRGS--G-ST | | | |
| m8 | QLQLVESGGGMVQPGESLTLSCEFS GYNP—YA MGWFRQAPGKEREGVSC ISRS--GGST | | | |
| m9 | QVQLVESGGGLVQAGGSLRLSCAAS GGTLDYYA IAWFRQAPGKEREGVSC ISST--GDT | | | |
| m10 | QLQLVESGGDLVRPGESLRLSCVVS GISLHYTT LGWFRQAPGKEREGVAC IS--GSRT | | | |
| m11 | QLQLVESGGGSVQSGGSLRLSCVGS TFSLNSYA VGWFRRAPGKEREEIAC ISSE--GYKT | | | |
| f4 | QLQLVESGGGLVQPGGSLRVSCAVS GSTLEHYA IGWLRQAPGKEREGVAC ISSRSLGGAT | | | |
| f8 | QLQLVESGGDSVQPGGSLRLSCTSS GITLENYA VAWFRQAPGKEREGISC ISSK--TSST | | | |
| f9 | QLQLVESGGGLVQPGGSLRLSCAAS GFTLDYYV TGWFRQAPGKEREGVSC ISRS--GTST | | | |

| | FR3 | CDR3 | FR4 |
|---|---|---|---|
| | 61 98 99 112 113 123 | | |
| m1 | NYADSVKGRFTISRDNAKNTVYLQMNSLLPEDTAVYYC ALDKSCSG-SFKDY WGQGTQVTVSS | | |
| m7 | RYADDVKGRFTISRDNAKNTVYLQMNSLLPEDTAVYYC ALDKSCSG-SFKDY WGQGTQVTVSS | | |
| m8 | NYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYC AADKSCSG-SFKDY WGQGTQVTVSS | | |
| m9 | YAADSVKDRFTISRNNAKNTVYLQMNSLKPEDTAVYYC AADKVYAC-SRPDY WGQGTQVTVSS | | |
| m10 | NYAEFAKGRFTISRDNAKNTVYLQMDNLKPEDTAVYYC AADRTRGCRVGMDY WGKGILVTVSS | | |
| m11 | YSLNSVKDRFQISRDNDKNTVYLQMNSLKPEDTAVYYC AADRTRGCRVGMDY WGKGTLVTVFS | | |
| f4 | NVVDSVKDRFTIGEDDGKNTVWLQMNSLKPEDTAVYYC ALDKSCSG-SFKDY WGQGTQVTVSS | | |
| f8 | RYSGSVEGRFTISRDNAKNTIYLQMNSLEPEDTAVYYC ALDKSCSG-SFKDY WGQGTQVTVSS | | |
| f9 | NYADSVKGRFTISRDNAKNTVYLQMNSLLPEDTAVYYC ALDKSCSG-SFKDY WGQGTQVTVSS | | |

***Note.*** The FRs and CDRs are Determined according to Francois et al.[8]

renaturation and purification of the inclusion bodies were performed as previously[9]. Finally, about 18 kD proteins were obtained, as shown in Figure 2 inset. To prove that the expression of VHH is capable of effectively binding 7H2, we performed inhibition assays. ELISA plates were coated with 10 μg/mL anti-$AFB_1$ mAb (100 μL/well) at 37 °C, and then washed and blocked with 3% BSA. Proteins (m7) were diluted and mixed with an equal volume of analytical standard $AFB_1$ or other mycotoxins (such as $AFB_2$, $AFM_1$, DON, OTA, and ZEN) with different concentrations in the plates. Bound proteins were determined with anti-6HIS HRP conjugate (CoWin Biotech Co., Ltd., China). Figure 2 displays the corresponding calibration plots, the binding rate of m7 with the 7H2 was proportional to $AFB_1$ concentration over the range from 1.0 to 20.0 ng/mL, with the $IC_{50}$ of 6.3 ng/mL. The linear equation was $B=-52.90\ lgC_{AFB1}+92.22$ with a correlation coefficient of 0.9975 ($R^2$). The cross-reactivity of the 7H2 to related mycotoxins with the m7 as a competitor was 0.25% to $AFB_2$, 2.20% to $AFM_1$ and no cross-reactivity to OTA, DON and ZEN.

Camelid heavy-chain antibodies form a novel concept in both immunology and biotechnology because of their unique structural features, including absence of immunoglobulin light chains and CH1 constant domain. Their variable region, VHH, have lots of advantages such as nontoxicity, easy production and greater stability. In the present work, the VHH antibody, m7, mimics the reaction of $AFB_1$ in ELISA and, thus, can serve as a surrogate for $AFB_1$-conjugates. This strategy avoids the synthesis of $AFB_1$-conjugates, which is usually time-consuming, expensive, and inevitably causes environmental pollution. The results of this work indicate that anti-idiotypic VHH would be an excellent tool for developing environment- friendly immunoassays.

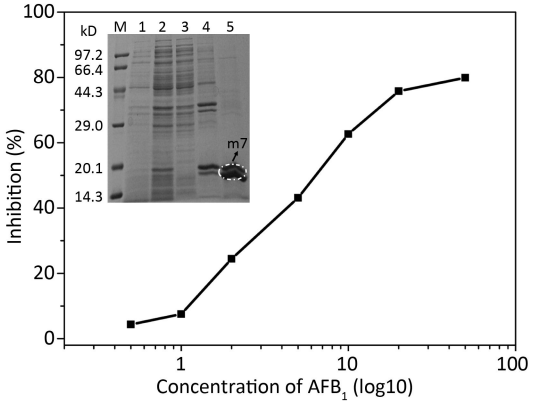


**Figure 2.** Inhibition of binding to 7H2 by purified anti-Id VHHs m7. The purified protein m7 was incubated with different concentrations of $AFB_1$ standards with 7H2 in ELISA plates for the inhibition assays. Insert: The expressions were analyzed by SDS-PAGE. Band 1: Aliquots of uninduced whole cells; band 2: induced whole cells; band 3: soluble fraction of induced cells; band 4: inclusion body fraction of induced cells; band 5: purified protein of m7.

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