Molecular Imprint of Enzyme Active Site by Camel Nanobodies

RAPID AND EFFICIENT APPROACH TO PRODUCE ABZYMES WITH ALLIINASE ACTIVITY

Background: The generation of catalytic antibodies (abzymes) by the anti-idiotypic antibody approach was hampered by their incompatible CDR structure with the active site of the enzyme.

Results: The high incidence of anti-idiotypic abzymes with alliinase activities was produced by the anti-idiotypic nanobody approach.

Conclusion: The anti-idiotypic nanobodies are efficient natural enzyme mimics.

Significance: This study provided a new approach for producing abzymes that could be broadly applied to antibody-catalyzed prodrug activation.

Screening of inhibitory Ab1 antibodies is a critical step for producing catalytic antibodies in the anti-idiotypic approach. However, the incompatible surface of the active site of the enzyme and the antigen-binding site of heterotetrameric conventional antibodies becomes the limiting step. Because camelid-derived nanobodies possess the potential to preferentially bind to the active site of enzymes due to their small size and long CDR3, we have developed a novel approach to produce abzymes with alliinase activities by exploiting the molecular mimicry of camel nanobodies. By screening the camelid-derived variable region of the heavy chain cDNA phage display library with alliinase, we obtained an inhibitory nanobody VHHA4 that recognizes the active site. Further screening with VHHA4 from the same variable domain of the heavy chain of a heavy-chain antibody library led to a higher incidence of anti-idiotypic Ab2 abzymes with alliinase activities. One of the abzymes, VHHC10, showed the highest activity that can be inhibited by Ab1 VHHA4 and alliinase competitive inhibitor penicillamine and significantly suppressed the B16 tumor cell growth in the presence of alliin in vitro. The results highlight the feasibility of producing abzymes via anti-idiotypic nanobody approach.

Since the initial reports of Pollack et al. (1) and Tramontano et al. (2) regarding the catalytic activity of antibodies, a great number of catalytic antibodies (abzymes) were developed (3). A common basis for the principles and methods to elicit catalytic abzymes is to produce haptons that mimic transition state features in catalytic reactions. Despite the remarkable success in the generation of anti-transition state analogs abzymes, this approach still has drawbacks, including weak activities and inefficient mimicking of target-specific natural enzymes (4–6).

An alternative approach to generate catalytic antibodies is to produce catalytic anti-idiotypic antibodies by using natural enzyme as the original antigen to obtain catalytic abzymes that have activities similar to natural enzymes (7, 8). Based on the concept by Jerne (9), relying on the internal image properties of anti-idiotype antibodies, enzymatic active sites can thus be mimicked by successive complementary interactions. A first antibody (idiotype, Ab1) is raised against the active site of the enzyme, and a second antibody (anti-idiotypic, Ab2) that is complementary to Ab1 may display features of the enzymatic sites. This approach was proven to allow the characterization of efficient abzymes with esterase mAb 9A8 (10, 11) and amidase mAb 9G4H9 activities using acetylcholinesterase and β-lactamase (12, 13), respectively, as the model enzymes. Using a similar approach, other groups obtained efficient abzymes with carboxypeptidase A-like activity (14) and, more recently, the target-specific parent antigen hydrolysis activities, such as anti-idiotypic mAb 68E12 with amidase and protease activity (15, 16).

With the feasibility of anti-idiotypic approach proven, the generation of such abzymes is rather difficult, and only a few abzymes exist. In producing anti-idiotypic catalytic antibodies (17), it is a critical step of successful generation and screening of the idiotype Ab1, i.e. possession of inhibitory activity against the antigen (enzyme) via the anti-idiotypic approach. The antibody repertoire is widely recognized as a source that is capable of generating a molecular imprint of virtually any natural or synthetic compound; however, the number of conventional antibodies (i.e. heterotetramers of two light chains and two heavy chains) acting as competitive enzyme inhibitors remains scarce. One of the reasons is incompatible surface topography of the enzyme’s active site and the antigen-binding site of conventional antibodies. The analysis of a panel of enzyme structures reveals that the active site is found almost exclusively in
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The total serum IgG from the immunized camel at day 70 was measured by ELISA as described (31). Briefly, 96-well microplates were coated with alliinase (10 μg/mL). After blocking, plates were incubated for 1 h at 37 °C with 100 μL of camel serum at different dilutions to measure total IgG. The bound camel antibodies were detected with a rabbit anti-camel antisera (prepared in our laboratory, see Ref. 31) and an HRP-conjugated goat anti-rabbit IgG (Southern Biotech).

To prepare VHH phage display libraries, cDNA was first synthesized by reverse transcription from immunized camel lymphocytes total RNA using oligo(dT) primers. VHH coding domain and part of the hinge region DNA were amplified from camel cDNA with the following specific primers: sense primer P1-SfiI(CATGCCATGACTCGGGGCCCAGCCGGCCGTCCGGCTGCCTCTCTTCTACAAGG) annealing at the leader sequence, antisense primers P2-IgG2-SfiI(CATGCCATGACTCGGGCCTCGGGGCCCAGCCGGCCGTCCGGCTGCCTCTCTTCTACAAGG), and P3-IgG3-SfiI(CATGCCATGACTCGGGCCTCGGGGCCCAGCCGGCCGTCCGGCTGCCTCTCTTCTACAAGG), which was mini-modified by substitution of a NotI cutting sequence (GGCGCCGCG) with the SfiI sequence (GGCGCCGCG). The VHH repertoire expressed on phage after infection with M13K07 helper phages (GE Healthcare), which was mini-modified by substitution of a NotI cutting sequence (GGCGCCGCG) with the SfiI sequence (GGCGCCGCG). The VHH repertoire expressed on phage after infection with M13K07 helper phages (GE Healthcare).

Selection of Phage-displayed VHH Library—Library size was calculated by planting aliquots on 2×YT ampicillin agar. Fifty clones were used for plasmid DNA preparation and sequenced for diversity analysis. Transformants were grown in 2× YT medium with 100 μg/ml ampicillin for 3 h at 37 °C (OD 0.5–0.6) and incubated with 20-fold excess of M13K07 helper phage for 1 h at 37 °C. Infected cells were harvested by centrifugation, resuspended in 500 ml of 2× YT with 100 μg/ml ampicillin and 50 μg/ml kanamycin, and incubated overnight at 30 °C with agitation. Phage particles were precipitated from culture supernatant with 4% PEG 6000, 0.5 M NaCl, resuspended in sterile PBS, and used for panning.

During panning, specific VHHs against the alliinase were enriched by three consecutive rounds of in vitro selection on 96-well microtiter plates coated with antigen (1 μg/well). Bound phage particles were eluted by incubation with 100 mM glycine HCl (pH 2.2) for 10 min, followed by neutralization with 2 M Tris-HCl (pH 8.0). Eluted phages were titrated and subjected to two other rounds of panning following the same procedure. The titers of phage particles in input and output at all steps were estimated by enumeration of ampicillin-resistant colonies obtained from TG1 cells infected with different phage dilutions. Following the third panning, the individual colony was picked and expressed as soluble periplasmic protein induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). The recombinant VHH extracted from the periplasm was tested for antigen recognition by ELISA.

Expression and Purification of Selected VHH Clones—Selected VHHs were amplified from the phage vector, subcloned into an IPTG-inducible pET30a vector, and expressed in Esch-
erichia coli BL21 (DE3) cells at 25 °C via standard cloning techniques. E. coli expression of VHHs was purified by immobilized metal affinity chromatography (nickel-nitrotriacetic acid, FastFlow, GE Healthcare) and analyzed by an SDS-PAGE (12% acrylamide). The purity of recombinant VHHs was evaluated by Coomassie Brilliant Blue stain.

**Enzymatic Activities**—The carbon-sulfur (C–S) bond lyase activity of alliinase and catalytic VHHs was assayed using two different methods. First, enzymatic activities were measured with alliin (Sigma) as substrate by the pyruvate method based on the detection of the pyruvate product described previously (32). In brief, the standard reaction mixture was composed of 80 mM NaH2PO4 (pH 7.0), 20 μM pyridoxal 5’-phosphate, 16 μM alliin and the enzyme, in a total volume of 250 μL. After incubation for 5 min at 35 °C, the reaction was stopped by adding of 2 ml of 10% (mass/volume) trichloroacetic acid, and 10 μL of 2,4-dinitrophenylhydrazine was added after that and kept for 5 min, followed by adding 5 μL of 2.5 M NaOH and kept for 10 min. Pyruvic acid was estimated colorimetrically by conversion to its thio-semicarbazone at 520 nm. When 1 μM pyruvate/min was catalyzed by alliinase at 35 °C, the activity of alliinase was defined as 1 unit. Second, alliinase activity was measured using the recently proposed method of Rabinkov and co-workers (33), where the oxidation of 4-mer-captopyridine (4-MP) by the enzymatically formed disulfide-S-monoxides was monitored spectrophotometrically. The present assay of alliinase activity is based on the assessment of the production rate of alliin, using 4-MP. The molar consumption of 4-MP in its reaction with alliin is equivalent to the production of pyruvic acid. The reaction mixture (1.0 ml) contained 4-MP (1 mM) in 50 mM sodium phosphate, 2 mM EDTA, 0.02 mM PLP (pH 7.2), and alliin (10 mM). One enzyme unit was defined as the amount of enzyme-converting alliin into pyruvic acid at a rate of 1 μmol/min. Enzymatic activity started upon addition of an aliquot of alliinase at 35 °C. The initial rate of the decrease in absorbance at 324 nm was measured, and the enzymatic activity was calculated using ε324 = 19,800 M−1 cm−1. In terms of depleting the endogenous alliin, alliinase and catalytic antibodies were pre-treated with 10 mM 4-MP for 30 min in an ice bath and were diluted prior to the assay of enzyme activity.

**Inhibition of Alliinase Activity by VHHs and Penicillamine**—The inhibitory capacity of VHHs and penicillamine on alliinase was determined by preincubating alliinase with various concentrations of VHHs and penicillamine for 30 min at 35 °C in a 96-well plate. Alliinase activity was determined by adding alliin (100 μM) as substrate and measured via the pyruvate method as mentioned above at 520 nm. The 50% inhibitory concentrations (IC50) were obtained by plotting alliinase residual activity as a function of increasing antibody fragment concentration.

**Determination of Enzyme Kinetics**—The kinetics data of alliinase and catalytic VHHs (Kmmax, Vmax, kcat, and kcat/Km) were determined from Lineweaver-Burk plots and were based on the formation of pyruvate during catalytic turnover. Analyses were conducted in vitro with alliin at concentrations between 0 and 13.0 mM. The enzymatic activities of alliinase or catalytic VHHs were assayed by pyruvate method as described above.

**Alliinase Binding of Ab1 Evaluated by Competitive ELISA**—Alliinase (10 μg/ml) was coated onto the wells of microtiter plates at 4 °C overnight. BSA was used at the same concentration as a negative control. Wells were blocked with PBS-skimmed milk 3% (200 μg/well) and incubated with dilutions of the Ab1 antibody VHH A4 in 1% skimmed milk/PBS. After three washes, the bound VHHs were detected with anti-His tag mouse mAb and a goat anti-mouse IgG-HRP. Peroxidase enzyme activity was determined following the addition of tetramethylbenzidine as substrate and measuring at 450 nm. Competitive ELISA was performed to assess specific binding of VHH A4 to alliinase. Alliinase (10 μg/ml) was coated onto the wells of microtiter plates at 4 °C overnight and then incubated with serial dilutions of the VHH A4 and penicillamine (0.1 μM) for 90 min. Unbound VHH domains were washed with 0.05% Tween/PBS, and bound VHH domains were detected as described above.

**Cell Proliferation Assay**—B16 F10 (B16) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM: Invitrogen) containing 10% (v/v) fetal calf serum and 2 mM L-glutamine in a humidified atmosphere at 37 °C under 5% CO2. The cells were initially seeded at 5 × 104 cells/well in 96-well tissue culture plates, and the adhered cells were incubated under appropriate conditions overnight. For cell proliferation assay, cells were incubated with VHH C10 (10 μg/ml), alliinase (10 μg/ml), alliin (20 μg/ml), and PLP (25 μM) for 24 h. Native allicin (1 μg/ml, Jiansu Chia-Tai Tianqing Pharmacy, China) was used as a positive control. Cell viability was assessed by incubating the cells with 25 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) for another 4 h. The MTT-formazan produced by viable cells was dissolved in dimethyl sulfoxide, and the absorbance of the converted dye was measured at a wavelength of 590 nm with background subtraction at 650 nm with a microtiter plate reader (Bio-Rad). The untreated wells containing the proliferated cells were used as the control group. The ratio of cell proliferation inhibition was calculated as follows: (average value of A590 of untreated group – average value of A590 of treated group)/average value of A590 of treated group) × 100%. At least three independent experiments were performed.

**Statistical Analysis**—Results are shown as the mean values ± S.D. The results are average data of triplicate experiments. The Student’s t test and analysis of variance were used to assess statistical significance. p values <0.05 were deemed statistically significant.

**RESULTS**

**Identification and Production of Alliinase Inhibitory Nanobodies (Ab1)**—The whole procedure of producing abzymes with alliinase activity is illustrated in Fig. 1. In the whole process, the preparation of inhibitory antibody is the critical step for producing anti-idiotypic catalytic antibodies.

**Library Construction and Isolation of Alliinase-specific VHHs**—For construction of camel VHH library, the natural alliinase was purified from garlic cloves with size exclusion and affinity chromatography and was homogeneous in SDS-PAGE (supplemental Fig. S1). A Bactrian camel was immunized with the purified alliinase over a period of 10 weeks. The total IgG titer of immunized serum raised against alliinase reached 1:12,800 in the last immunization (supplemental Fig. S2). The antigen-
binding gene fragments of the heavy-chain antibodies from the immunized camel were cloned. 1.5 × 10^7 lymphocytes were isolated and used as the starting materials. The constructed phage display VHH library contained 2.8 × 10^5 individual transformants of which about 91% of clones had an insert of 400 bp or so as determined by colony PCR (supplemental Fig. S3). Fifty randomly selected clones were sequenced, and all represented distinct VHH sequences and indicated the diversity and high quality of the initial library. The VHH repertoire was expressed on phages after rescuing with M13K07 helper phage. Selection following three rounds of panning resulted in a strong enrichment of clones binding to the alliinase, which indicated enriched high affinity antibody fragments. The number of clones eluted in relation to the size of the input after each round of panning is shown in Table 1.

**Sequence Alignment of Alliinase-specific VHHs**—After the third round of panning against alliinase, 36 colonies were randomly selected for sequencing. The VHH clones derived from the library were named VHH-Ax. The 36 colonies represent 16 distinct individuals, in which 11 clones encoded VHH A4, 8 clones encoded VHH A8, and 4 clones encoded VH A14 as well. The other 13 clones encoded the single VHH gene separately. The deduced amino acid sequences of the 16 alliinase-specific antibody fragments were aligned against the human VH consensus sequence (Fig. 2). Regarding the VHHs retrieved from the library, it is very clear that almost all but VH A14 and VH A28 clones were derived from heavy-chain antibody-specific VHH germ line genes, as they all contain the hallmark amino acid substitutions in framework 2 (V42Y/V42F, G49E, L50R, W52X) based on the IMGT numbering system (34). The CDR3 length of VHHs varies from 8 to 30 amino acids with an average of 18, which is longer than those in human and mouse VH (12 and 9 amino acids, respectively) (24).

**Binding Characteristics of Antigen-specific VHHs**—According to the deduced amino acid sequences, 16 clones specific to alliinase were selected and subcloned into an expression vector (pET30a). The induced recombinants were expressed in *E. coli* and purified from bacterial periplasmic extracts by immobilized nickel-nitrilotriacetic acid-affinity chromatography resulting in the >95% pure protein as deduced from Coomassie staining of the SDS-PAGE (Fig. 3A). The specific binding of VHHs to alliinase was confirmed via Western blotting (Fig. 3B) and ELISA analysis (Fig. 3C), in which A4 showed the highest binding activity and therefore was chosen for further investigation.

**TABLE 1**

| Round of panning | Input phages | Output phages | Output phages/input phages |
|------------------|--------------|---------------|---------------------------|
| Round 1          | 10^10 pfu    | 9.9 × 10^6 pfu| 1.25 × 10^-3             |
| Round 2          | 10^10 pfu    | 1.25 × 10^5   | 1.25 × 10^-3             |
| Round 3          | 1.25 × 10^6  | 150           | 1.2 × 10^-3              |

**FIGURE 2.** Alignment of VHH-Ax sequences with human VH consensus sequence (hVH). The hypervariable regions are shown in **boldface type**. The VHH hallmark amino acids (V42Y/V42F, G49E, L50R, W52X) and the Cys residues involved in an intra-domain (C23 and C104) disulfide bond are **underlined** and **highlighted in gray**, respectively.

**FIGURE 1.** Schematic procedure for production of abzymes with alliinase activity.
Inhibiting Property of VHNA on Alliinase Activity—The inhibitory capacity of the isolated VHVs was measured by residual enzymatic activity in an enzyme/VHH mixture. VH A4 antibody was shown to significantly inhibit the enzymatic activity of alliinase at a low concentration. The IC50 value obtained from the measurements was 0.2 μM for A4 (Fig. 4A). To identify whether A4 interacts with the active site of alliinase, the binding capacity of A4 was measured in a competitive ELISA. A4 was found not to bind to alliinase in the presence of penicillamine as an alliinase competitive inhibitor. Apparently, over 50% of the VH A4 was displaced upon addition of penicillamine (Fig. 4B) (35). The evidence suggests that A4 is a competitive enzyme inhibitor that interacts with the active site of alliinase.

Screening and Production of Anti-idiotypic Ab2 from the VH Phage Display Library, Isolation and Identification of VH A4-specific VHVs(Ab2)—To obtain alliinase “internal image” anti-idiotypic antibody, the inhibitory antibody VH A4 was used as the Ab1 in the idiotypic network system to re-screen the camel VH phage display library. The same library for the retrieval of anti-alliinase VHVs was used to screen the VH A4 binders. The library with 2.8 × 107 individual transformants was rescued by M13K07 helper phages so that the virions were amplified to 1013 and used in the first round of panning. The VH clones derived from the library by rescoring with VH A4 were named VHH Cx. The result of PCR with 49 different clones showed that only 21 clones had proper VHH fragment (supplemental Fig. S4). DNA sequencing from these 21 clones identified 16 distinct VHH A4 binders (the other five failed to yield any positive results in sequencing for unknown reasons) (Fig. 5). According to the deduced amino acid sequences, two of clones (VH C9 and VH C15) were derived from VH germ line genes, and the rests were from VHH derived from VH germ line genes. It was recognized that all selected VH clones showed a long length in CDR3 with 18 amino acids on average. To evaluate the alliinase activities, seven A4 binders (C1, C10, C11, C12, C13, and C17) with longer CDR3 amino acid sequences, two of clones (VH C9 and VH C15) were selected from the VHH library with lysozyme as antigen. The VHHA4 available in the VHH library was evaluated in the idiotypic network system to identify VHHA4 binders. Microtiter plates were coated with alliinase at a concentration of 10 μg/mL. The plates were incubated with serial dilutions of the VH A4 and penicillamine (0.1 μM). VHHA4 over penicillamine ratio is 1:1, 1:10, and 1:100, respectively. Mouse anti-His tag mAb was subsequently incubated and detected with a goat anti-mouse IgG-HRP. Each column corresponds to the mean value of triplicate measures.

High Incidence of Catalytic Behavior in Anti-idiotypic Ab2—To evaluate the alliinase activities, seven A4 binders (C1, C10, C11, C12, C13, and C17) with longer CDR3 amino acid composition were selected, expressed, and purified in E. coli. The purified His-tagged recombinant protein was nearly homogeneous as examined by Coomassie staining of the SDS-PAGE (Fig. 6A). The pyruvate method was used to evaluate their enzymatic activities. The values of catalytic activities of VHVs are shown in Table 2. Of seven VHs, four had obvious catalytic activity (57%) and exhibited a high incidence of catalytic behavior in anti-idiotypic Ab2. In all of Ab2 VHs, VH C10 had the highest C–S bond lyase activity (25.08 units/mM), which was nearly 6-fold lower compared with the natural alliinase activity (153.93 units/mM), and was chosen for further study in terms of its catalytic properties.

Catalytic Properties of the Anti-idiotypic Ab2—To determine the specific nature of the nanobody catalysis, 4-MP method was
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used to ensure the alliinase activity of VHH C10 using alliin as substrate at different concentrations. The results showed that lysis of the Cys sulfoxide bond of alliin, which was catalyzed by VHH C10, followed the Michaelis-Menten equation with $K_m$ and $V_{\text{max}}$ values as follows: 1.521 mM and 42.03 units/mg, respectively. A typical hyperbolic saturation curve is shown in Fig. 7, and values of $K_m$ and $V_{\text{max}}$ were 1.418 mM and 41.67 units/mg, respectively. The IC_{50} values of the two inhibitors were 0.1 and 0.05 $\mu M$, respectively. The inhibition modes of VHH A4 and penicillamine on VHH C10 were similar to their inhibiting native alliinase.

**FIGURE 5.** Alignment of VHHCx sequences with human VH consensus sequence (hVH). The hypervariable regions are shown in boldface type. The VHH hallmark amino acids and the Cys residues involved in an intradomain (Cys-23 and Cys-104) disulfide bond are underlined and highlighted in gray, respectively.

**TABLE 2**

| Catalyst | Units/mg | Units/mM |
|----------|----------|----------|
| VHH C1   | 528 ± 1.24 | 13.20 ± 0.12 |
| VHH C10  | 1005 ± 1.35 | 25.08 ± 0.17 |
| VHH C11  | 58 ± 0.12  | 1.45 ± 0.06 |
| VHH C12  | 65 ± 0.22  | 1.63 ± 0.02 |
| VHH C13  | 81 ± 0.30  | 2.03 ± 0.09 |
| VHH C16  | 163 ± 0.92 | 4.07 ± 0.12 |
| VHH C17  | 984 ± 2.22 | 24.6 ± 1.62 |
| Native alliinase | 2989 ± 3.20 | 153.93 ± 2.50 |

**FIGURE 6.** Identification of nanobody VHHC10 with alliinase activity. A, SDS-PAGE analysis of the purity of VHH C10. B, inhibition curve of the alliinase activity of VHH C10 by enzymatic inhibitors penicillamine and VHH A4. VHH C10 (100 $\mu M$) was preincubated for 10 min with VHH A4 (A) and penicillamine (B) (0–0.5 $\mu M$). The residual activity was measured upon adding 100 $\mu M$ alliin to the reaction mixture. The values are the means of three independent determinations.

**DISCUSSION**

In this study, we demonstrated that cameld-derived nanobodies mimic the enzyme active center via transferring the catalytic interface from the inhibitory idiotypic to the catalytic anti-idiotypic antibody. Anti-idiotypic antibodies have been structurally characterized (37–39) like any conventional antibody, and their recognition surfaces are large flat areas composed of all six complementarity determining regions from heavy- and light-chain variable domains, which seriously limits their usage of mimicry of epitopes in the cavities or crevices in the active center of the enzymes. By taking advantage of cameld single domain antibodies, we successfully generated the antibodies with catalytic activities. To the best of our knowledge, this is the first report of the nanobodies with enzymatic activities.

To avoid possible contamination of antibody preparations by enzymes, efficient protocol for purification of the VHH abzyme
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TABLE 3
Comparison of catalytic parameters for enzyme activity of alliinase and VHH C10 by alliin.

| Method of assay | Alliinase | VHH C10 |
|-----------------|-----------|----------|
|                 | $k_{cat}$ | $K_m$    | $k_{cat}/K_m$ |
| Pyruvic acid    | 0.888 ± 0.25 | 1.493 ± 0.51 | 0.595 ± 0.04 |
|                 | 0.453 ± 0.17 | 1.521 ± 0.32 | 0.331 ± 0.04 |
| 4-MP            | 0.170 ± 0.11 | 1.418 ± 0.35 | 0.110 ± 0.05 |
|                 | 0.111 ± 0.04 | 1.320 ± 0.27 | 0.084 ± 0.02 |

FIGURE 7. Michaelis-Menten plot of VHHC10 enzymatic kinetic reaction. Conditions of the reaction are described under “Experiment Procedures.” The reaction used natural alliin as substrate at concentrations between 0 and 13.0 mM. Enzymatic activities were assayed by the pyruvate method. The experiments were repeated three times, and the results are expressed as mean values. Inset, Lineweaver-Burk plot for determination of kinetic data. The $V_{max}$ and $K_m$ values were estimated using nonlinear fitting plots with the GraphPad Prism 5.0.

FIGURE 8. Inhibition of B16 F10 cell proliferation by VHH C10 in the presence of alliin. Cell viability was assayed by the MTT method. The effect of VHH C10 treated on B16 cells was presented as inhibitory ratio compared with the untreated group. The bars represent the average of six determinations ± S.D. In contrast to natural alliinase (indicated as $E$ in the figure) and alliin, VHH C10 (indicated as $C10$ in the figure) abzyme can efficiently inhibit the growth of B16 cells in the presence of alliin in vitro. The inhibiting rates of C10 and alliinase on tumor cells were 55 and 70%, respectively. PLP can accumulate abzyme and alliinase activity.

was developed. All VHH abzymes used in the assay were over 95% pure in the SDS-PAGE examination. This is consistent with the network theory that the parental Ab1 antibody VHHA4 inhibits the catalytic activity of the abzyme VHHC10 (Fig. 6B). No evidence of contamination of antibody preparations with either alliinase or other C-S bond lyases was found.

The choice of the adequate idiotypic Ab1 is critical for accurate reproduction of the functional internal image of enzyme via the idiotypic network. By taking advantage of the simple structure of heavy chain only nanobodies from camel, the VHH A4 (Ab1) was selected from a camel VHH single domain antibody phage display library. The antibody only bears the VHH of heavy chain and is designated as nanobody, and it exhibited strong inhibitory activity against alliinase with IC_{50} of 0.2 μM. The mechanism of the nanobody preferentially inhibiting the enzymatic activities is mostly related to their convex paratope predominantly formed by the long H3 loop (22), which is not usually found at the antigen-combing site in conventional antibody. Therefore, it is inferred that VHH A4 accommodates inside the enzyme active site via its long CDR3 (18 amino acids) and guides the immune system toward generation of the functional internal image of the parental enzyme.

Single domain antibody libraries are by far more easily generated than heterodimeric single chain antibody libraries (40). Because the intact antigen-binding fragment is encoded in a single gene fragment (the VHH), small libraries of only $10^6$–$10^7$ VHH individual clones have routinely caused the isolation of single-domain proteins with nanomolar affinity for their antigen (41). In our work, anti-alliinase VHHs were retrieved easily from the relatively small library (2.8×10^6). The production of anti-idotypic abzymes (Ab2) in using this approach is straightforward by screening the same VHH library based on the theory of the idiotypic network using the Ab1 VHHA4 as an antigen. The occurrence of anti-Id antibodies with alliinase activity in the library was rather high (57%). With a single round screening, four of seven anti-idotypic nanobodies (C1, C10, C16, and C17) of purified A4 (Ab1) binders possess relatively high alliinase activities. In comparison with the conventional antibody-based anti-idotypic approach for abzymes, the occurrences of catalytic activity of Ab2 were very low, varying from 6.9 to 11.1% (42, 43). This suggests that our nanobody-based anti-idotypic procedure is an efficient and straightforward approach for abzymes.

VHH C10 was chosen for further study due to its relatively high alliinase activity. The primary structure of VHH C10 showed no significant homology with any element of the primary structure of alliinase. As an abzyme, VHH C10 efficiently mimicked natural alliinase and C10, and alliinase can be inhibited by idotypic Ab1 VHHA4 and small molecular inhibitor penicillamine. In addition, the enzymatic activities of C10 and alliinase depend on cofactor PLP. Conversely, the catalytic interface of anti-Id catalytic antibodies may form the cleft structure according to the network hypothesis (44). Such a structure mode can be seen in conventional antibody-derived abzymes such as 6B8-E12 (16), an anti-idotypic catalytic antibody with serine proteases activities. It has a large cleft between the VH and the VL complementarity determining regions. It
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would be advantageous to examine whether and where the cleft exists in the nanobody VHHC10 to explain the mechanism of the enzymatic mimic. Because six complementarity determining regions exist in the Fab region, it is difficult to find the correlation that highlights the fidelity of transfer of the enzyme active center site by the idiotypic mimic. The smaller size of camel single domain antibodies facilitates the steps of recombinant production and structural studies. Moreover, their particular binding strategy allows us to select anti-idiotypic mimetics carrying this capacity in their long CDR3s, allowing the use of synthetic peptides or peptide mimetics for the design of binders. In this regard, our current studies identified the molecular basis of mimicry of alliinase by a single domain anti-idiotypic antibody.

In the proposal by Miron et al. (45) of antibody-directed enzyme prodrug therapy (ADEPT) using the alliinase-mAb conjugate, N87 and CB2 tumor cells were effectively killed by converting the nontoxic alliin precursor alliin into the cytotoxic allicin. Taking into consideration the immunogenicity of the alliinase-mAb conjugate, the ADEPT approach is frustrating and should be limited in the clinical application. In contrast to the ADEPT, we are proposing a straightforward way of converting allin into allin catalyzed by the enzyme VH C10. Our study suggests that nanobody VH C10 with authentic alliinase activity can effectively inhibit tumor cell growth in vitro.

In conclusion, by taking advantage of the mimicry property of cameld-derived nanobodies, we successfully produced the abzymes with alliinase activities. The technique we developed will provide a new approach for producing abzymes, which could be broadly applied to antibody-catalyzed prodrug activation.

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