

Screening of Nanobody Specific for Peanut Major Allergen Ara h 3 by Phage Display

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ABSTRACT: Peanut allergy is a major health problem worldwide. Detection of food allergens is a critical aspect of food safety. The VHH domain of single chain antibody from camelids, also known as nanobody (Nb), showed its advantages in the development of biosensors because of its high stability, small molecular size, and ease of production. However, no nanobody specific to peanut allergens has been developed. In this study, we constructed a library with random triplets (NNK) in its CDR regions of a camel nanobody backbone. We screened the library with peanut allergy Ara h 3 and obtained several candidate nanobodies. One of the promising nanobodies, Nb16 was further biochemical characterization by gel filtration, isothermal titration calorimetry (ITC), cocrystallization, and Western blot in terms of its interaction with Ara h 3. Nb16 specifically binds to peanut major allergen Ara h 3 with a dissociation constant of 400 nM. Furthermore, we obtained the Ara h 3-Nb16 complex crystals. Structure analysis shows the packing mode is completely different between the Ara h 3-Nb16 complex crystal and the native Ara h 3 crystal. Structural determination of Ara h 3-Nb16 will provide the necessary information to understand the allergenicity of this important peanut allergen. The nanobody Nb16 may have application in the development of biosensors for peanut allergen detection.

KEYWORDS: nanobody, Ara h 3, peanut allergen, food safety, allergen detection

INTRODUCTION

Food allergy is an adverse immune reaction to food proteins.¹ Based on the results of several studies, Sicherer and Sampson estimated that food allergies affect nearly 8% of children and 5% of adults in the US.² Many studies have shown that the incidence of food allergies has actually increased in the last 20 to 30 years.³ Milk, egg, peanut, tree nuts, shellfish, fish, wheat, and soy triggered the most significant allergies.⁴

Peanuts are cheap and frequently eaten in unprocessed form and are an ingredient in many different prepared foods. Peanut allergy has become a major health concern worldwide, especially in developed countries.⁵ Peanut allergy is the most common cause of fatal food-induced anaphylaxis in the U.S.⁶ Peanuts are designated with 16 allergens by the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee.⁷ Ara h 2, Ara h 6, Ara h 7, Ara h 9, Ara h 16, and Ara h 17 belong to the Prolamin superfamily.^{8–10} Ara h 1 and Ara h 3 belong to the Cupin superfamily, and both of them are important major peanut allergens in specific allergic populations.¹¹ In peanut, Ara h 5 is a Profilin and a pan-allergen. Ara h 8 belongs to the Bet v 1-like superfamily.¹² Ara h 12 and Ara h 13 are categorized as defensins.¹³ Ara h 10, Ara h 11, Ara h 14, and Ara h 15 are categorized as oleosins.¹⁴

Despite intensive clinical studies over the past decade, avoiding peanuts remains the only effective way to prevent

allergic reactions.^{15,16} Therefore, it is very important to establish a reliable peanut allergen detection method.¹⁷ Currently available methods can be classified into two groups: protein-based or DNA-based assays. Because of high sensitivity, high specificity, ease of use, and high sample throughput, ELISA has become the preferred method for allergen detection in the food industry.¹⁸ Another advantage of ELISA over DNA-based methods is that allergen proteins are detected directly, rather than inferred from the presence of peanut DNA. Several immunoassays based on polyclonal antibody for peanut have been developed. In 2004, Schmitt et al. developed antibodies and immunoassays against the major peanut allergens Ara h 1 and 2.¹⁹ In 2011, their group purified recombinant Ara h 3 protein and produced highly specific anti-Ara h 3 antibody, proving it could recognize Ara h 3 in peanuts.²⁰ In 2016, one group developed a double-antibody sandwich immunoassay for peanut and milk using SPR.²¹

Researchers found that certain animals, such as camelids and sharks,^{22,23} produce a unique type of antibody called heavy chain-only antibodies (HCAbs) that are naturally deficient in light chains. The antigen binding domains of the camelid

Received: April 16, 2019

Revised: August 11, 2019

Accepted: August 13, 2019

Published: August 13, 2019

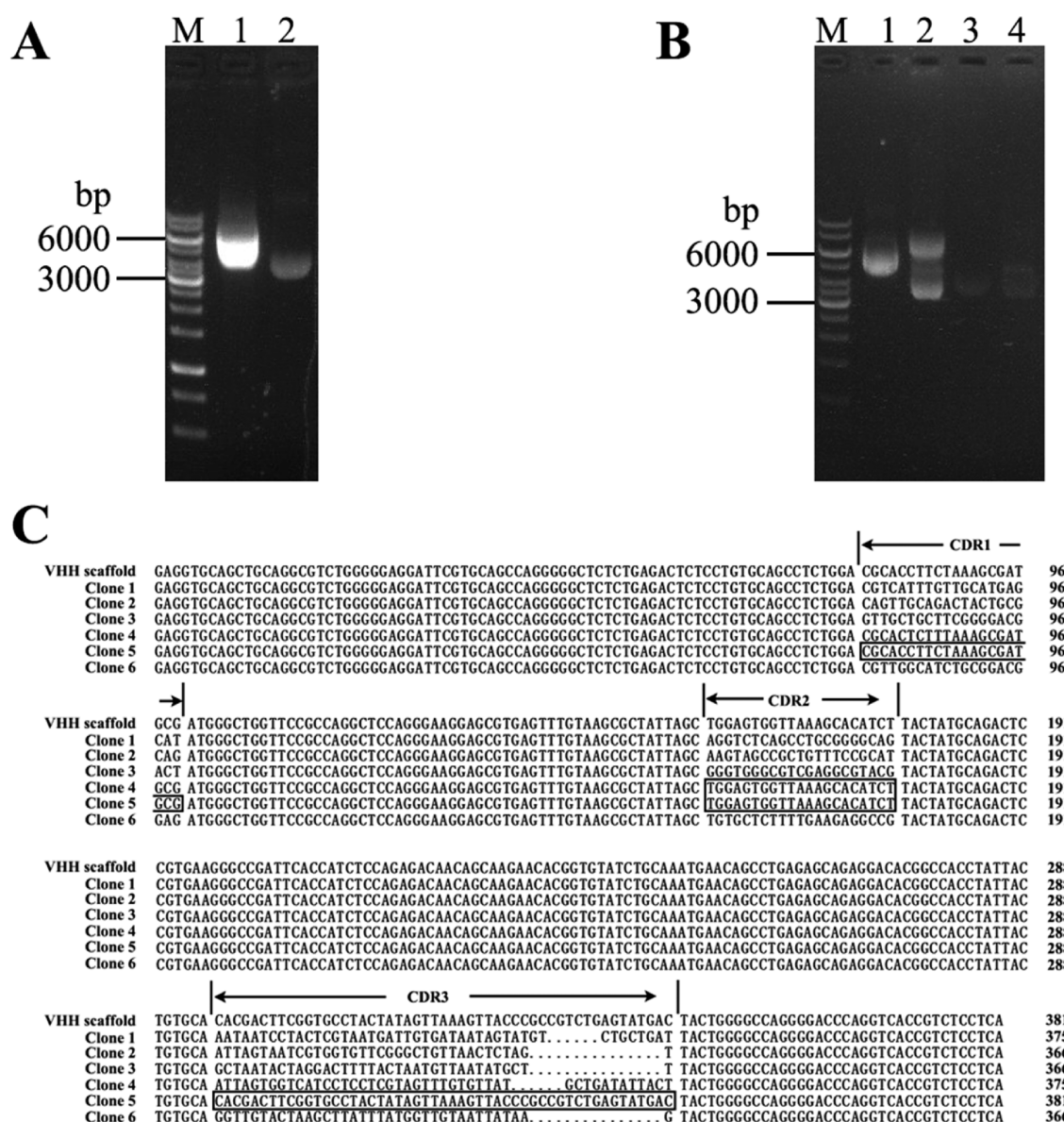


Figure 2. Phage display nanobody library construction. (A) Agarose electrophoresis results of pR2-VHH dsDNA (lane 1) and dU-ssDNA (lane 2). (B) Agarose electrophoresis results of pR2-VHH dsDNA (lane 1), synthesized CCC-dsDNA (lane 2), dU-ssDNA (lane 3) and synthesized negative control without degenerate primers. (C) Sequencing results of 6 CCC-dsDNA transformed clones, 4 of them simultaneously introduced randomization in CDR1–3, CDRs without mutation were indicated in boxes.

NaCl gradient of 0–0.5 M in 100 mL. The peak of Ara h 3 was collected and ammonium sulfate powder was added to the final concentration of 1.5 M. The sample was then loaded onto a 5 mL Phenyl Sepharose hydrophobic interaction column (GE Healthcare) pre-equilibrated with buffer 3 (1.5 M ammonium sulfate, 20 mM Tris–HCl pH 8.0). The bound proteins were eluted with a 95 mL linear gradients of (1.5 M–0 M) ammonium sulfate by mixing buffer 2 and buffer 3. The peak of Ara h 3 was collected and loaded onto a 380 mL Superdex 200 column (GE Healthcare) pre-equilibrated and eluted with buffer 4 (200 mM NaCl, 20 mM Tris–HCl pH 7.0, 5% glycerol).

Sample Vitrification and Cryo-EM Image Acquisition. Ara h 3 was diluted to a final concentration of 2.6 mg/mL in buffer 2 and 3 μ L aliquots were applied to freshly glow discharged Quantifoil R1.2/1.3 grids. Grids were blotted for 5 s at room temperature in 100% humidity and then plunged into liquid ethane using a FEI Vitrobot (FEI Company). Frozen grids were kept in liquid nitrogen. The grids were loaded into a Gatan Cryo-holder and transferred to an FEI Tecnai TF20 electron microscope for sample verification and image acquisition.

Table 2. Enrichment Results after Panning on Ara h 3

rounds	Ara h 3 (μ g)	input phage (pfu)	output phage from blank tube (pfu)	output phage from antigen tube (pfu)
1	400	5×10^{12}	7.81×10^4	6.42×10^4
2	400	1×10^{12}	3.75×10^6	3.33×10^8
3	200	1×10^{12}	8.01×10^5	3.98×10^9
4	200	1×10^{12}	4.75×10^5	1.54×10^{10}
5	200	1×10^{12}	3.03×10^6	1.22×10^{10}

Nanobody Based Phage Display Library Construction.

A highly soluble humanized VHH scaffold³¹ was chosen for phage display synthetic library construction. cDNA of the VHH was synthesized and cloned into pR2 phagemid. The Kunkel mutagenesis technique³² was used to introduce synthetic diversity in the complementarity determining regions (CDRs) to construct the library. Briefly, *E. coli* CJ236 cells (*dut*[−]/*ung*[−] strain) was transformed with recombinant phagemid pR2-VHH and followed by infection with helper phage

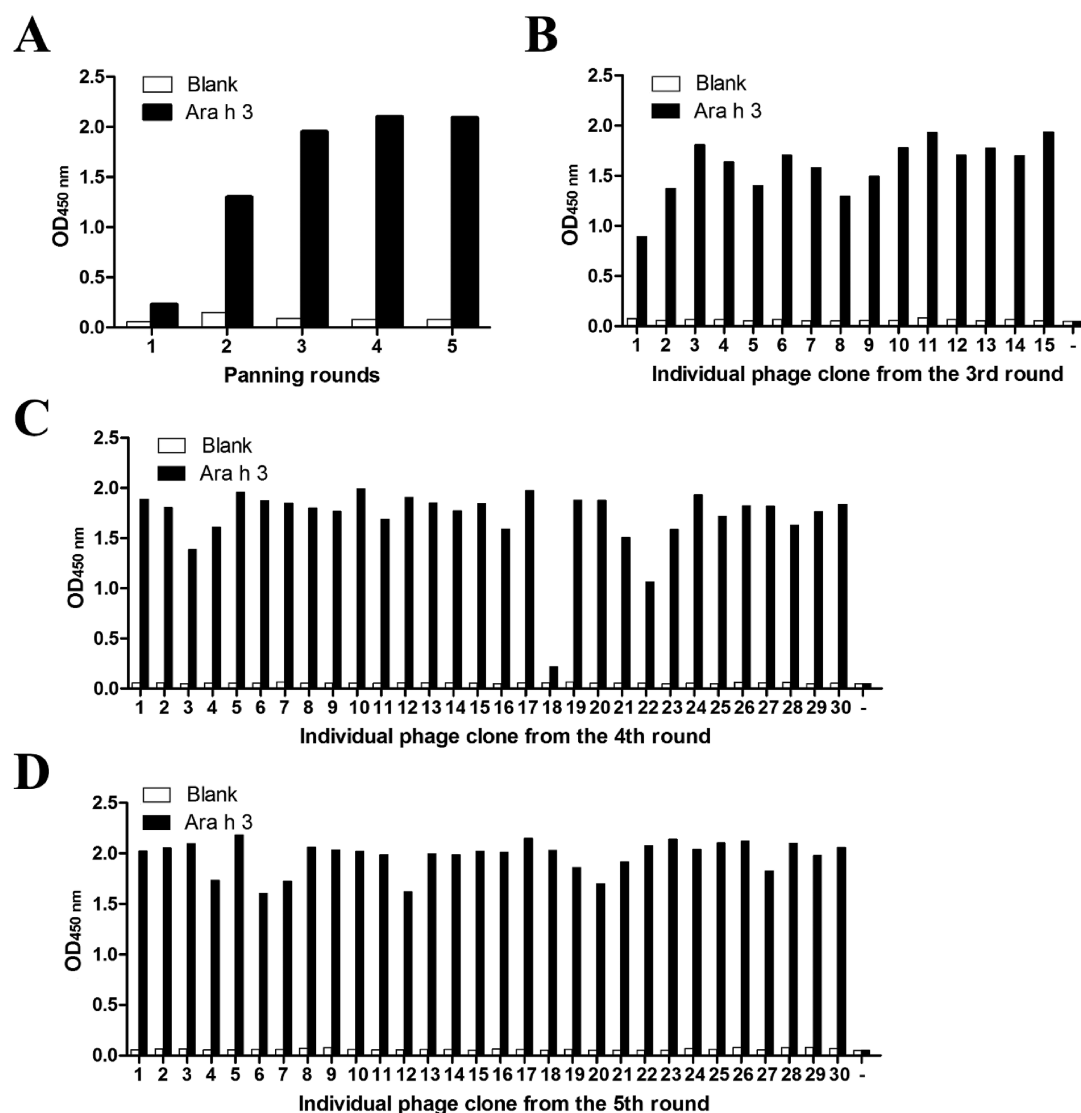


Figure 3. Results of phage ELISA. (A) Results of polyclonal-phage ELISA. The output phages from each rounds of panning were monitored against Ara h 3 and blank control by ELISA. (B–D) Results of monoclonal phage ELISA by using clones from the third, fourth, and fifth round, respectively.

KM13. After several hours of culture in uridine supplemented LB broth, the phage uracil-containing single strand DNA (dU-ssDNA) was propagated and secreted into the medium. M13 Isolation Kit (OMEGA biotek) was used to extract and purify the dU-ssDNA. Using the dU-ssDNA as template, we designed five degenerated oligonucleotides, two of which contain approximately 20 bases complementary to the 5' and 3' regions of the CDR1 and CDR2 region respectively, and the other three with different CDR length are complementary to the CDR3 region. "NNK" codons were used for "hard" randomization for CDRs, and the five degenerate oligonucleotides sequences were shown in Table 1. Oligonucleotides mixture was phosphorylated at their 5' end and annealed to 20 μ g of the dU-ssDNA. The molar ratio of each oligonucleotide to template is 3:1. Covalently closed circular double-stranded heteroduplex DNA (CCC-dsDNA) was synthesized by a fill-in reaction with T7 DNA polymerase and T4 DNA ligase in vitro and purified by using five columns from Cycle Pure Kit (OMEGA biotek).

Library Phage Preparation. Electrocompetent *E. coli* TG1 cells (dut⁺/ung⁺ strain) were transformed by using the BTX ECM 399 Electroporation System (Harvard Apparatus) with the following setting: 2.5 kV, 6 ms. 0.25 μ g of CCC-dsDNA was transformed into 100 μ L of electrocompetent *E. coli* TG1 cells in 0.1 cm gap electroporation cuvette each time. Each electroporation was resuspended with 3 mL of warm SOC medium and incubated at 37 °C, 220 rpm for 45 min. Serial dilution was performed to measure the number of

transformants. The remaining bacterial was spread on three 150 mm TYE agar plates supplemented with 2% glucose and 100 μ g/mL ampicillin, followed by culturing overnight at 37 °C. The colonies were scraped from the plates with liquid 2 $\times$ TY and stored in the presence of 20% glycerol at –80 °C. After all transformations were finished, all stored transformants were thawed on ice, mixed together thoroughly, and restored at –80 °C in 1 mL aliquots at OD₆₀₀ of 75. The size of the library was calculated. To make nanobody displaying phage repertoire, 1 mL of frozen library was thawed on ice, diluted into 250 mL of 2 $\times$ TY medium supplemented with 2% glucose and 100 μ g/mL ampicillin, and cultured at 37 °C, 220 rpm until OD₆₀₀ to 0.5. KM13 helper phages (1×10^{12} pfu) were added to the culture and incubated at 37 °C water bath for 45 min. After centrifugation, cell pellets were resuspended in 500 mL of 2 $\times$ TY medium supplemented with 0.1% glucose, 100 μ g/mL ampicillin, and 50 μ g/mL kanamycin. The cell suspension was grown for 16 h at 25 °C, 220 rpm to propagate the library phage. After spinning, the culture supernatant was collected and the phage particles were precipitated by using Poly Ethylene Glycol (PEG) precipitation. The phage particle pellets were dissolved in 12 mL of PBS, then filtered through a 0.45 μ m filter, and stored in the presence of 20% glycerol at –80 °C in 1 mL aliquots. Phage titer was estimated according to the following empirical formula: phage/mL = OD₂₆₀ $\times$ 100 $\times$ 22.14 $\times$ 10¹⁰.

Panning. Five rounds of biopanning were performed in Immuno MaxiSorb Tubes (Nunc). The tubes were coated with 4 mL of Ara h 3

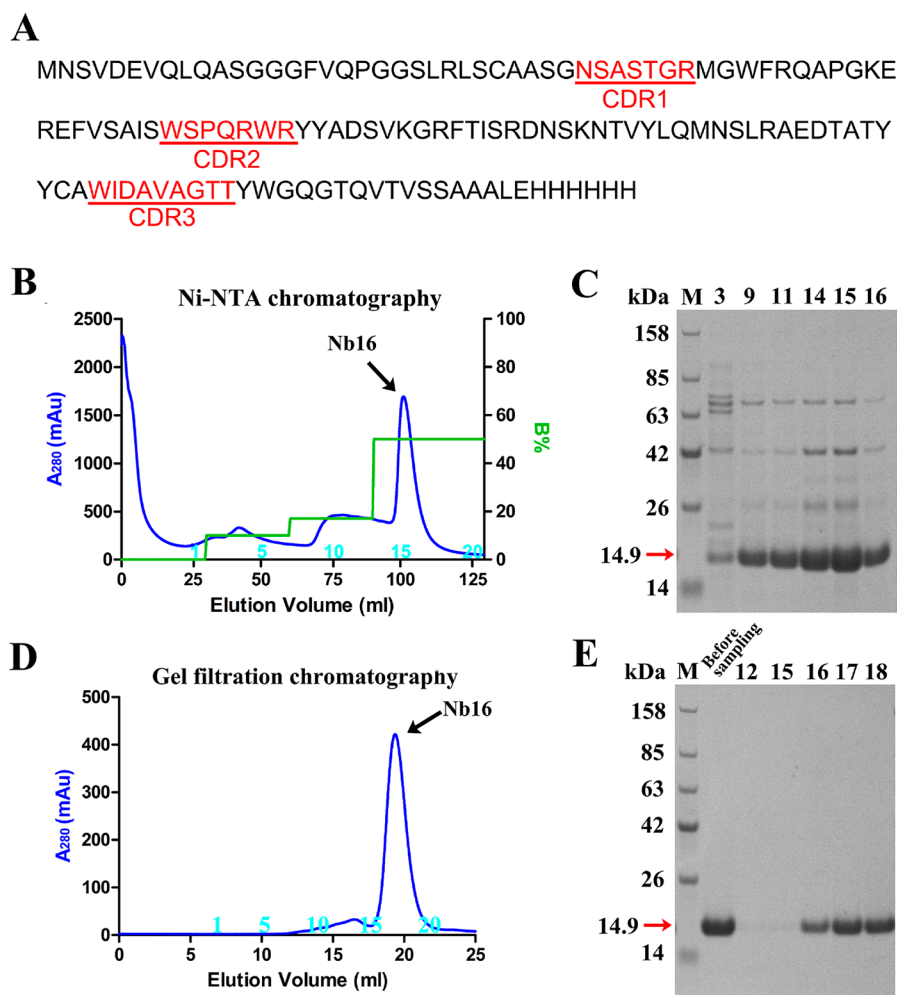


Figure 4. Identification and purification of Nb16. (A) Amino acid sequences of Nb16 was identified. (B) Purification of Nb16 with Ni-NTA chromatography. (C) SDS-PAGE of Ni-NTA chromatography analysis shown in (B). (D) Superdex 200 gel filtration chromatography of Nb16. (E) SDS-PAGE of gel filtration chromatography analysis shown in (D).

solution (100, 100, 50, 50, and 50 $\mu\text{g/mL}$ in the first, second, third, fourth, and fifth round, respectively) and blocked with buffer 5 (200 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 8.0) supplemented with 5% Marvel milk powder (w/v). Negative control tubes without antigen coating were used in parallel in every round of panning. After 3 times washing with 4.5 mL of buffer 6 (200 mM NaCl, 2 mM EDTA, 0.1% Tween 20, 20 mM Tris-HCl pH 8.0), 5×10^{12} pfu of library phages diluted to 4 mL of buffer 5 supplemented with 5% Marvel milk powder (w/v) were incubated with the immunotubes for 1 h at room temperature rotating using an under-and-over turntable. The tubes were washed for 10 times with buffer 6 to remove unbound phages. Bound phages were eluted by 1 h of digestion using 500 μL of 0.5 mg/mL trypsin at room temperature with rotating. The eluted phages were used to infect *E. coli* TG1 for titer determination. Meanwhile, infected TG1 cells were rescued using KM13 helper phage for the next round of panning. The amount of input phage of the next four rounds was 1×10^{12} pfu, and the washing time was 20 times. The output phage titer of every round of panning was measured.

Phage ELISA. The output phages from each round of panning were monitored against Ara h 3 and blank control by polyclonal phage ELISA. Polyclonal phage ELISA was carried out using PEG precipitated phage from the end of each round of panning. 100 μL of diluted phage mixture (diluted 1:100 with 5% buffer 5 supplemented with 5% Marvel milk powder (w/v)) was added to each well of a MaxiSorb 96-well plate coated with 100 μL of 2 $\mu\text{g/mL}$ of antigen and incubated for 1 h at room temperature. The solution was discarded, and the wells washed 5 times with buffer 6. The plate was incubated with 100 μL of anti-M13-HRP

antibody (diluted 1:10 000 with buffer 5 supplemented with 5% Marvel milk powder (w/v) in each well for 1 h at room temperature. After 5 times washing with buffer 6, 100 μL of TMB (Beyotime) was added to each well. The plate was incubated in the dark for 2 min at room temperature, and the chromogenic reaction was stopped with 50 μL of 1 M sulfuric acid. OD_{450} was measured. Monoclonal phage ELISA was carried out by using the phage in the culture medium of the individual phage-infected TG1 that was rescued by KM13 helper phage infection. 100 μL of diluted phage mixture (diluted 1:4 with buffer 5 supplemented with 5% Marvel milk powder (w/v)) was tested by using phage ELISA as above. Subsequently, the phagemid extracted from those positive phages was sent for sequencing.

In addition, the specificity of the nanobodies which have sufficient quantity for subsequent studies was further detected with six irrelevant proteins (Fag e 1, SPV II, 2S albumin, ZIKV MTase, phiYY P4, and hUbc13) by monoclonal phage ELISA, the concentration for coating was 20 $\mu\text{g/mL}$. For detection of the sensitivity of the nanobodies, Ara h 3 at different concentration (0.25–4 $\mu\text{g/mL}$) was coated on to an ELISA plate and monoclonal phage ELISA performed as described above.

Nanobody Expression and Purification. After phagemid extraction, the VHH was amplified by PCR using specific primers (forward: 5'-ATAGTCGACGAGGTGCAGCTGCAGGCGTCTGG-3', reverse: 5'-AATAATGCGGCCGCTGAGGAGACGGT-GACCTGGGTCC-3'). The amplified VHH gene was cloned into pET30a vectors with a C terminal hex-histidine (6-his) tag using the *SaII*/Not I restriction site. The construct was transferred into *E. coli* BL21 (DE3). Soluble His-tagged nanobodies were purified on a

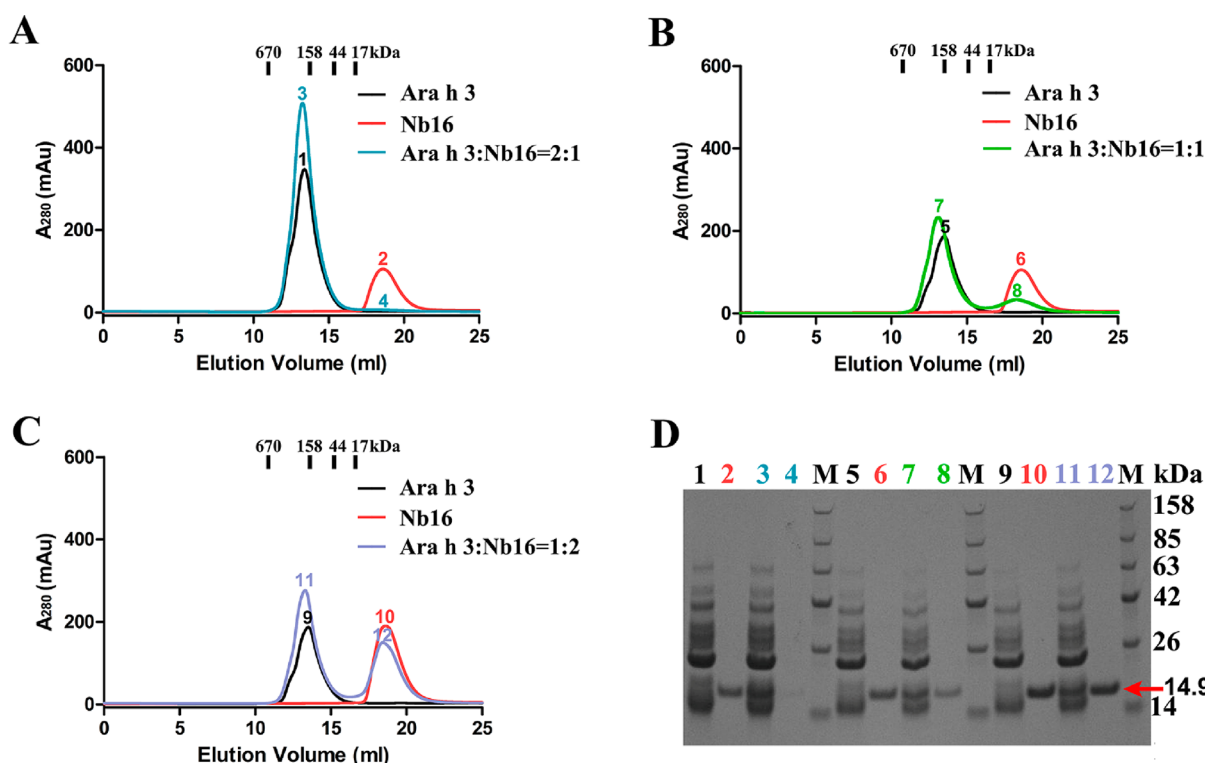


Figure 5. Gel filtration analysis of the interaction between Nb16 and Ara h 3. (A) 0.088 mM Ara h 3 and 0.044 mM Nb16 were mixed. (B) 0.044 mM Ara h 3 and 0.044 mM Nb16 were mixed. (C) 0.044 mM Ara h 3 and 0.088 mM Nb16 were mixed. (D) SDS-PAGE of the individual peaks of the gel filtration analysis shown in (A–C).

Ni-NTA Superflow Sepharose column in buffer 7 (200 mM NaCl, 20 mM Tris-HCl pH 7.0, 14 mM imidazole and 5% glycerol) and was eluted with buffer 8 (500 mM NaCl, 20 mM Tris-HCl pH 7.0, 400 mM imidazole and 5% glycerol). The protein was further purified by gel filtration chromatography with buffer 4. The purity of the eluted proteins was assessed by 15% SDS-PAGE.

Gel Filtration Chromatography. Apparent molecular weights of Nb16, Ara h 3 and their complexes were estimated with an ENrich SEC 650 column (10/300 GL, BIO RAD). Briefly, samples or molecular-weights standards were applied onto an ENrich SEC 650 column at a flow rate of 0.6 mL/min and eluted with buffer 4. In order to obtain the stereochemistry of binding, different antigen/antibody ratio was tested, namely 0.088:0.044 mM or 2:1 molar ratio for group 1, 0.044:0.044 mM or 1:1 molar ratio for group 2, and 0.044:0.088 mM or 1:2 molar ratio for group 3. The protein mixtures in all three sets were incubated at 4 °C for 3 h prior to analysis.

Isothermal Titration Calorimetry (ITC). Proteins were dialyzed in buffer 4 and measured using an ITC-200 titration calorimeter (GE Healthcare) at 25 °C. The cell volume was filled with the protein solution (Ara h 3). The injection syringe was filled with the ligand solution (Nb16). At specified time intervals, Nb16 protein (~2 µL per injection) at a concentration of 150 µM in the syringe was titrated into an Ara h 3 solution at 20 µM in the cell (~280 µL). Control experiments were performed under identical conditions by titration with Nb16 into buffer alone. The ITC data were subsequently fitted to a single-site model using Origin 7.0 (OriginLab).

Crystallization. The Ara h 3 and Nb16 complex collected from gel filtration was further supplemented with 10% excess of Nb16. The complex was concentrated to an A_{280} to 2.2 for crystallization screen using the hanging-drop vapor-diffusion method with the PEG/Ion 2 Screen HR2–098 kit (Hampton Research, Aliso Viejo, CA, USA). For each drop, 0.7 µL protein solution was mixed with 0.7 µL crystallization solution and sealed against 0.25 mL crystallization solution in one well of a 24-well Limbro plate. Crystallization plates were incubated at 18 °C. Sizable single crystals were obtained with a condition containing 0.2 M sodium formate pH 7.0 and 20% w/v

PEG3350. Additional ethylene glycol (20%) and glucose (10%) in the crystallization buffer were used as Cryo-protectants.

Western Blot, Two-Dimensional Liquid Chromatography Multistage Mass Spectrometer (2DLC-MS) and Dot Blot.

To verify the presence of His-tagged Nb16 in the Ara h 3-Nb16 complex crystals, single crystals were manually picked by nylon loops and washed in crystallization buffer. The crystals were dissolved in distilled water and analyzed by SDS-PAGE. After transferring, the nitrocellulose membrane was blocked with 5% skim milk. The membrane was incubated with mouse anti-His antibody (BBI Life Sciences) and then incubated with HRP-conjugated goat antimouse IgG antibody (BBI Life Sciences). The bands were visualized using an ECL kit. The band corresponding to the Nb16 in the complex crystal lane was excised and its identity was further confirmed by a two-dimensional liquid chromatography multistage mass spectrometer (2DLC-MS; ThermoFisher Scientific).

For dot blot analysis, a polyvinylidene fluoride membrane was blotted with peanut extract, sesame extract and 1 µg of Ara h 3, respectively. After air-dry for 10 min and blocking with 5% (w/v) skim milk, the membrane was incubated with Nb16 at a final concentration of 9 µg/mL. The membrane was incubated with mouse anti-His antibody (BBI Life Sciences) and then incubated with HRP-conjugated goat antimouse IgG antibody (BBI Life Sciences). The membrane were visualized using an ECL kit.

X-ray Diffraction and Crystallographic Characterization.

X-ray diffraction data were collected at the Shanghai synchrotron radiation facility (SSRF) beamline BL-19U1. A data set of 402 one-degree images were collected at 12.695 keV with 0.2 s exposure. The diffraction data were processed using the HKL-2000 suite of programs,³³ and the space group was determined to be P1. An initial structural solution was obtained by molecular replacement (MR) calculations using the program Phaser.^{34,35} A hexameric Ara h 3 structure (PDB code 3C3 V) was used in the molecular replacement calculation with Phaser.

RESULTS AND DISCUSSION

Purification of Ara h 3. In the gel filtration step of the purification, Ara h 3 was eluted at 220 mL as a single peak with

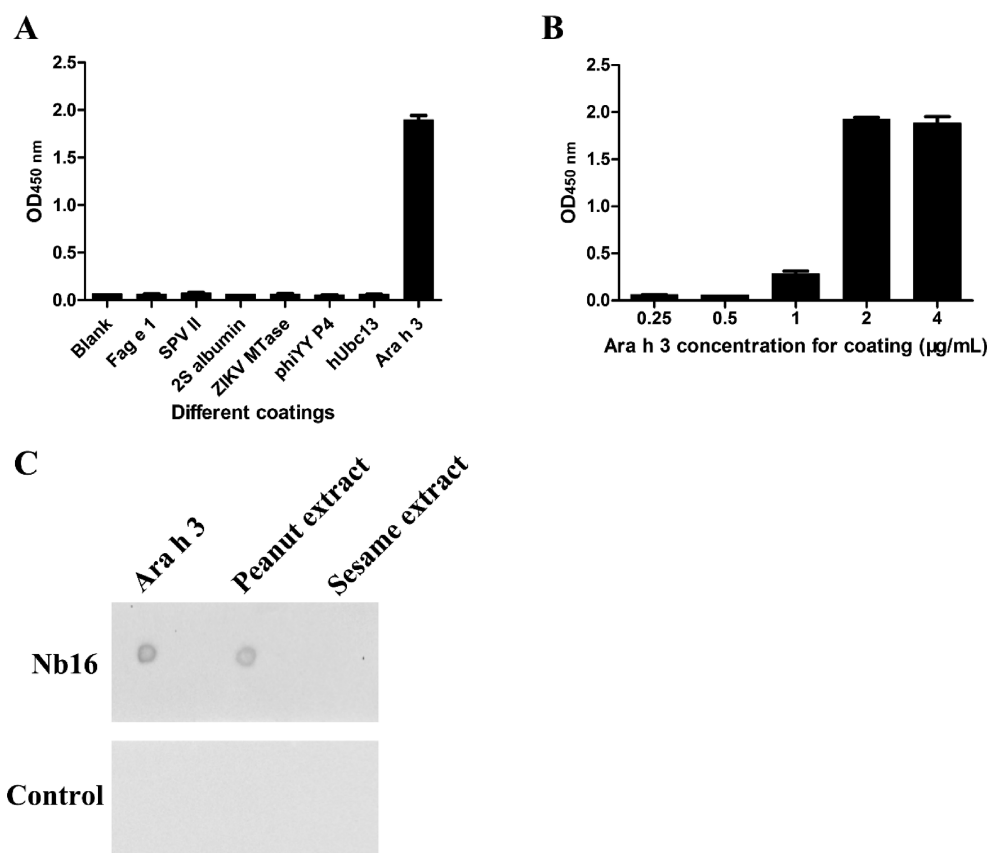


Figure 6. Characterization of the interaction between Nb16 and Ara h 3. (A) Detection of the specificity of Nb16 by phage ELISA. Fag e 1 is a buckwheat allergen. SPV II is the shake parvalbumin. 2S albumin is sesame 2S albumin. ZIKV MTase is ZIKV nonstructural protein NS5MTase. phiYY P4 is the bacteriophage phiYY P4. hUbc13 is the recombinant human Ubc13 protein, which is the ubiquitin E2 conjugating enzyme in K63-linked polyubiquitination. The assay was performed in two replicates and error bar represents for mean $\pm$ standard deviation. (B) Detection of the sensitivity of Nb16 by phage ELISA. Purified Ara h 3 allergen was coated directly onto an ELISA plate at designated concentration. The assay was performed in two replicates and error bar represents for mean $\pm$ standard deviation. (C) Detection of the specificity of Nb16 by dot blot. The control experiment did not incubate Nb16.

an apparent molecular weight of ~ 350 kDa, indicating that it exists as a hexamer in solution (monomer molecular weight is ~ 58 kDa). It was separated into more than one band on reducing SDS-PAGE, consistent with reports in the literature^{30,36} (Figure 1A–D).

In the Cryo-electron image, Ara h 3 exhibits as highly stable triangular shape particles (Figure 1E), consistent with the shape of the hexameric Ara h 3 crystal structure.

Construction of a Synthetic Phage Display Nanobody Library. The method used for library construction here was based on the classical oligonucleotide-directed mutagenesis method developed by Kunkel et al.³² A large phage-displayed antibody repertoires ($>10^{10}$ members) can be constructed efficiently by this method. In order to construct a highly diversified phage display nanobody library for efficient antigen target screening, a highly soluble humanized VHH was chosen as a scaffold, and diversity was restricted to positions within the CDRs. Five diversified oligonucleotides (Table 1) are used to modify the CDRs. Each position of CDRs was randomized using degenerate codons “NNK”. A constant length of seven amino acids was randomized for CDR1 and CDR2. A large spectrum of Camelidae VHH CDR3 length is naturally observed and this loop is known to contribute strongly to antigen binding selectivity. Thus, we chose to use three different lengths of CDR3 to cover this spectrum (9, 12, and 15 amino acids) and introduced “NNK” randomization at each position. VHH DNA was synthesized

and cloned into a phagemid vector pR2. After several attempts, we purified approximately $60 \mu\text{g}$ of high-purity pR2-VHH dU-ssDNA (Figure 2A), which is essential for successful library construction. Using the $20 \mu\text{g}$ of the dU-ssDNA as template, about $32 \mu\text{g}$ of CCC-dsDNA was synthesized by using the five degenerate oligonucleotides. Agarose gel electrophoresis results showed that the reaction product contained three bands (Figure 2B), and the band with the highest electrophoretic mobility represents the correctly extended and ligated CCC-dsDNA. The CCC-dsDNA was transformed into *E. coli* DH5 α in small scale, and six clones were picked randomly for sequencing. Results showed four of them simultaneously introduced randomization in three CDRs (Figure 2C). Consequently, the CCC-dsDNA was used to transform electrocompetent *E. coli* TG1 cells to make the library. Electroporation was performed in 100 sets, transformation efficiency was measured after each electroporation, and a VHH library with the size around 3×10^9 was obtained. VHH displaying phage library was prepared after KM13 helper phage infection and PEG precipitation.

Biopanning of Synthetic Library against Ara h 3.

To produce Ara h 3 specific nanobodies, five successive rounds of biopanning reactions against Ara h 3 were performed. Input and output phage titers from each round are shown in Table 2. The output phage titers were increased from first to fourth round. Comparing the fourth round to the first round, the output phage titers were increased about 240 000-fold. The output phage

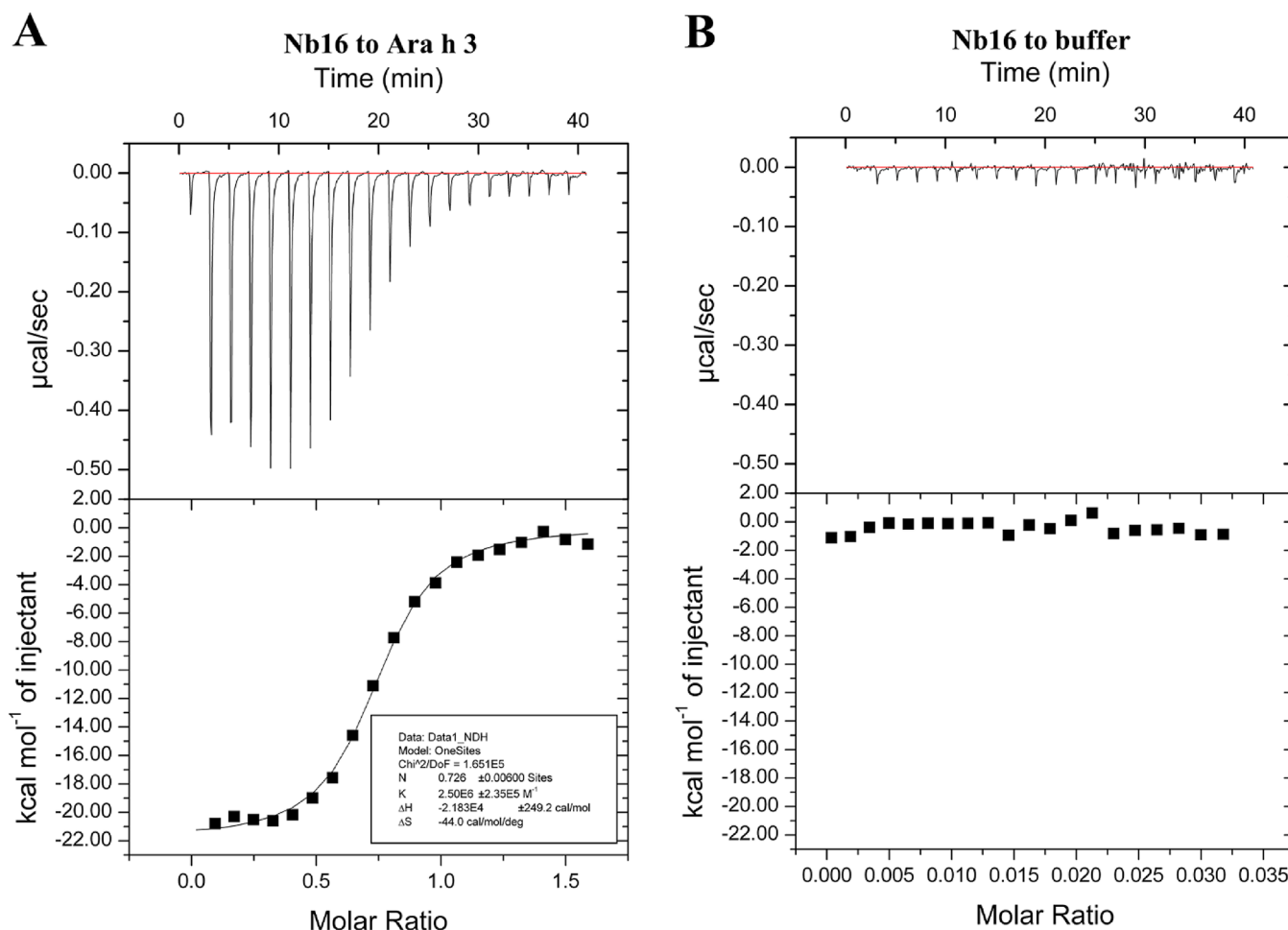


Figure 7. ITC-based measurements of the binding affinity between Ara h 3 and Nb16. Nb16 was titrated with Ara h 3 (A) and the Nb16 was titrated with buffer (B).

titers of fifth and fourth were very close. These indicated that the Ara h 3-bound phages were enriched effectively with the progress of panning. This indication was confirmed by polyclonal phage ELISA. As showed in Figure 3A, eluted phages from the second to the fifth round of panning showed their specific binding abilities to Ara h 3. Phages from the third, fourth, and fifth round exhibited similar absorbance value, indicating that sufficient high-affinity antibodies could be screened after 3 rounds of panning.

Monoclonal Phage ELISA Selection. After five rounds of panning, 15 clones from the third round, 30 clones from the fourth round, and 30 clones from the fifth round were picked randomly and employed for monoclonal phage ELISA, respectively. As showed in Figure 3B–D, except for one clone (clone 18) from the fourth round exhibited low absorbance value, all other clones from the third–fifth round were positive. This result corresponds the above results. 56 of the positive clones from the fourth–fifth round were sent for sequencing, among them 17 were unique clones.

Expression of Nanobody in *E. coli*. The 17 unique clones were subcloned into pET30a vector. After purification test, only 2 of the 17 nanobodies, namely Nb4–16 and Nb4–21, have sufficient quantity for subsequent studies. The amino acid sequences of Nb4–16 (abbreviated as Nb16) were deduced (Figure 4A). After purification by Ni-NTA chromatography and gel filtration chromatography, Nb16 was migrated as a single

band of 14.9 kDa in the SDS-PAGE, which is in agreement with its expected molecular weight (Figure 4 B–E).

Characterization of the Interaction between Nb16 and Ara h 3. Nb21 was not bound to Ara h 3 (data not show). To test whether Nb16 was bound to Ara h 3 in solution, we performed the gel filtration analysis with 2:1, 1:1, and 1:2 molar ratio, and the results showed the Nb16 bound to Ara h 3 nearly by 1:1 molar ratio (Figure 5A–C). For example, binding analysis with 1:1 molar ratio with gel filtration is shown in Figure 5B. The mixture of Ara h 3 and Nb16 showed a decreased peak height (peak 8) compared to Nb16 alone (peak 6) and a peak shift (peak 7) compared to Ara h 3 alone (peak 5), indicating Ara h 3 and Nb16 form complex. Moreover, the presence of both Ara h 3 and Nb16 in peak 7 was verified by SDS-PAGE analysis (Figure 5D). In addition to its binding activity in solution, the specificity of Nb16 was evaluated against six irrelevant proteins namely Fag e 1, SPV II, 2S albumin, ZIKV MTase, phiYY P4, and hUbc13 by phage ELISA. As shown in Figure 6A, Nb16 only binds to Ara h 3, indicating Nb16 has high recognition specificity for Ara h 3. Due to the lack of a capture antibody for Ara h 3, we were unable to do a sandwich ELISA for the direct detection of Ara h 3. Instead, to characterize the sensitivity of Nb16, phage ELISA was performed against Ara h 3 coated at different concentrations. As shown in Figure 6B, when the coating concentration of Ara h 3 was 1 μg/mL, the ELISA still showed positive signal, which indicates that the Ara h 3 sensitivity of Nb16 is at least 1 μg/mL, considering the coating efficiency may be low.

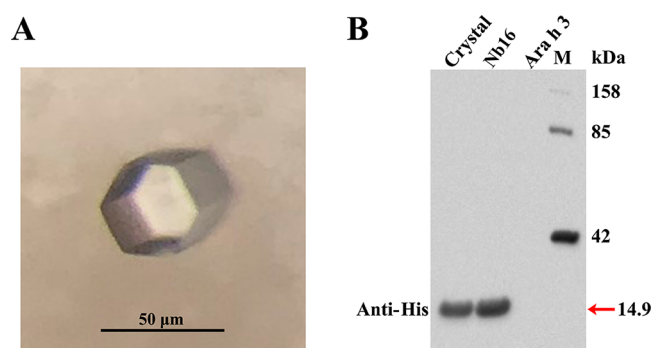


Figure 8. Crystallization of Ara h 3-Nb16 complex. (A) Single crystal of Ara h 3-Nb16 obtained by vapor diffusion using the hanging-drop method. (B) Anti-His Western blot analysis of complex crystal.

Furthermore, in the dot blot, as shown in Figure 6C, the Nb16 can detect peanut crude extract and Ara h 3, without binding to sesame crude extract. The membrane coated with the same antigens but without incubating with the Nb16 was used as a control to rule out the possible binding of the secondary antibody. The above results demonstrate the specificity of NA16 nanobody and its potential usefulness in peanut allergen detection.

Affinity Measurement by ITC. ITC can be used to measure the free energy, enthalpy, heat capacity and stoichiometry of binding reaction of modest affinity. To further characterize Ara h 3-Nb16 interactions, we designed ITC experiments after optimizing the buffer conditions. The results showed that Ara h 3 interacts with Nb16 with a dissociation constant of 400 nM and a $\Delta H \approx -21$ kcal/mol (Figure 7).

Crystallization of Ara h 3-Nb16 Complex. After optimization, disc-like single crystals of $\sim 50 \times 50 \mu\text{m}$ were obtained using this condition (Figure 8A). To further confirm the presence of Nb16 in crystals, crystals were pick manually, washed with crystallization solution and dissolved in distilled water. After SDS-PAGE and transferred to nitrocellulose membrane, the presence of Nb16 in the crystals was confirmed with anti-His antibody (Figure 8B, Lane Crystal). Lanes loaded with Nb16 alone and Ara h 3 alone were used as positive and negative controls. Furthermore, our mass spectrometry analysis showed the coverage of 69% and the peptide-spectrum match scores (PSMs) of 204 for Nb16 (Table 3), confirming that the apparent ~ 15 kDa band in the crystal is Nb16. Combining the results of Western blot and mass spectrometry, we confirmed

Table 4. X-ray Data Collection and Refinement Parameters for Ara h 3-Nb16 Complex

data collection	
space group	<i>P1</i>
unit cell (<i>a</i> , <i>b</i> , <i>c</i>) (Å)	95.438, 186.335, 186.508
(α, β, γ) (deg)	59.996, 90.022, 89.939
resolution (Å)*	50.00–6.30 (6.52–6.30)
no. of reflections	788169 (2365)
redundancy*	4.6 (4.6)
completeness (%)*	98.6 (95.9)
<i>I</i> / σ (<i>I</i>)*	12 (1.8)
R-meas*	0.170 (0.630)
<i>R</i> _{pim}	0.077 (0.285)
CC(1/2)*	NA (0.887)
structure determination	
solvent content (%)	43.93%
Phaser Z-score	10.01
LLG	139.821

that Nb16 is present in the crystal, in other words, we grew complex crystals.

Crystallographic Characterization of Ara h 3-Nb16 Complex. Ara h 3-Nb16 complex crystals are nice looking and are well separated; nevertheless, the crystals diffracted poorly at synchrotron. The best data set was scaled to 6.30 Å, with *I*/ σ = 1.8 at its highest resolution shell (6.52–6.30 Å) and a corresponding *R*_{pim} = 0.285 and CC (1/2) = 0.887. In order to solve its structure, a hexameric Ara h 3 structure (PDB code 3C3V) was used in the molecular replacement calculation with Phaser. This led to a solution with three Ara h 3 hexamers in an asymmetric unit and TFZ score of 10.01. Manual inspection of the electron density map failed to identify any additional Ara h 3 hexamer. While MR calculation with nanobody did not produce reasonable solutions, it is probably due to the poor resolution and phase error. Assuming there are three Ara h 3 hexamers and 18 nanobodies in one asymmetric unit, the corresponding Mathew's coefficient is 2.19 and solvent content is 43.93% (Table 4).

Furthermore, the electron density map showed a clear protein–solvent boundary and comparable quality with its 6.30 Å resolution. However, no direct interaction interface was observed between Ara h 3 molecules in the unit cell, and the closest distance is 17.6 Å between Ara h 3 hexamers in the crystals (Figure 9B). In addition, the packing mode is completely

Table 3. Identification of Nb16 by 2DIC-MS

accession	description	exp. <i>q</i> -value: sequest HT	coverage [%]	no. peptides	no. PSMs	no. unique peptides
cf20190618	Nb16	0	69	17	204	17
P01009	Alpha-1-antitrypsin OS = <i>Homo sapiens</i> GN = SERPINA1 PE = 1 SV = 3	0	20	7	56	7
P02751	Fibronectin OS = <i>Homo sapiens</i> GN = FN1 PE = 1 SV = 4	0	2	2	15	2
P02647	Apolipoprotein A-I OS = <i>Homo sapiens</i> GN = APOA1 PE = 1 SV = 1	0	18	4	5	4
P49064	Serum albumin OS = <i>Felis catus</i> GN = ALB PE = 1 SV = 1	0	2	1	2	1
P36955	Pigment epithelium-derived factor OS = <i>Homo sapiens</i> GN = SERPINF1 PE = 1 SV = 4	0	4	1	1	1
A0A0D3QNP8	Quinolone resistance protein (Fragment) OS = <i>Escherichia coli</i> GN = qnrS1 PE = 4 SV = 1	0.091	7	1	1	1
Q6JYY7	Proline permease (Fragment) OS = <i>Escherichia coli</i> GN = putP PE = 3 SV = 1	0.143	12	1	1	1
A7K7H6	Transposase OS = <i>Comamonas testosteroni</i> CNB-1 GN = tnpA PE = 4 SV = 1	0.133	4	1	1	1
D5FL09	MrkF OS = <i>Escherichia coli</i> GN = mrkF PE = 4 SV = 1	0.111	10	1	1	1

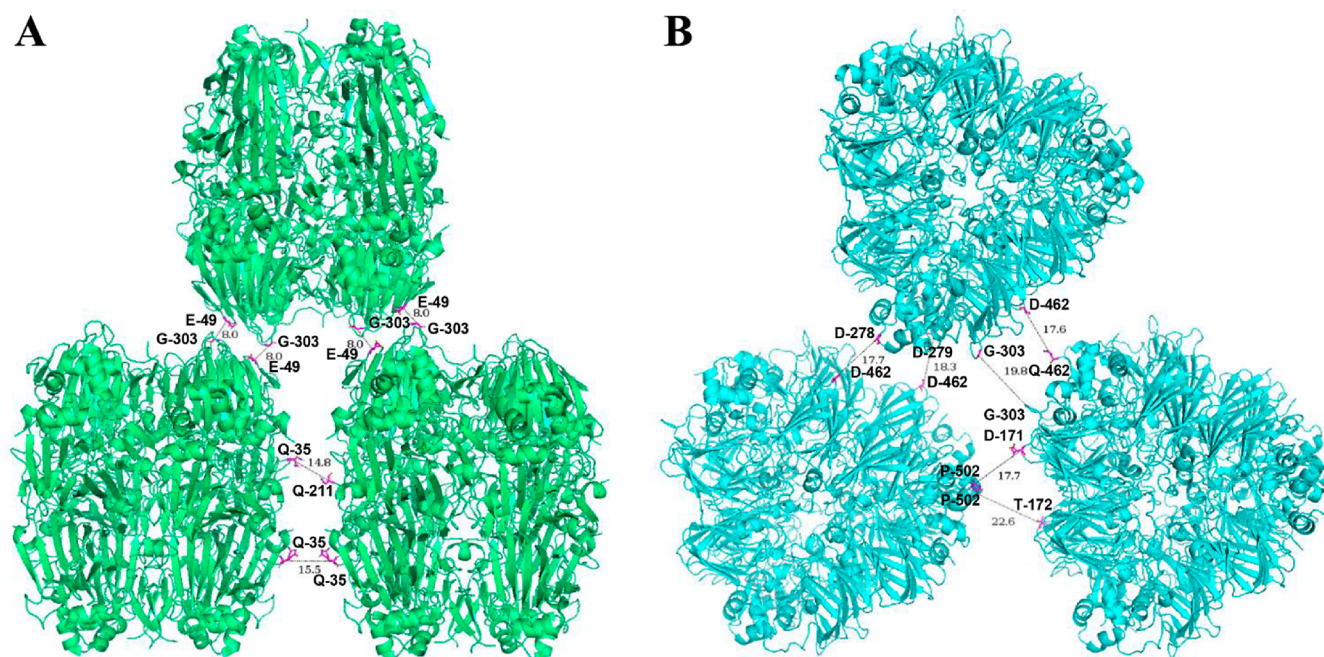


Figure 9. Packing interface comparison between native Ara h 3 crystal and the Ara h 3-Nb16 complex crystal. (A) Packing mode of native Ara h 3 in 3C3 V. (B) Ara h 3 hexamer is separated apart in the Ara h 3-Nb16 complex crystal.

different between the Ara h 3-Nb16 complex crystal and the native Ara h 3 crystal. The Ara h 3 hexamers lie in approximately in the same plane, while the hexamers are not in the same plane for the native Ara h 3 structure solved before.³⁰ Both of the different packing mode and Western blot results of crystals favor the presence of Nb16 nanobody in the crystals. Currently, crystal optimization is under way to obtain higher quality diffraction data. Structural determination of Ara h 3-Nb16 will provide information required to understand the allergenicity of this important peanut allergen.

In summary, in this study, by designing a phage display library with randomized CDR sequences in a humanized camel nanobody scaffold, the Nb16 nanobody specific to the peanut major allergen Ara h 3 was obtained. Biochemical characterization by gel filtration, ITC, Western blot, as well as cocrystallization experiments revealed the binding properties of Nb16 to Ara h 3. Additional work is needed to improve the affinity and further explore its application in the food safety industry.

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Author Contributions

F.C. and H.M. contributed equally to the manuscript. T.J. and X.F. participated in study design, data analysis, and extensively reviewed the manuscript. F.C. and H.M. performed the experiments, analyzed the data, and drafted the manuscript. Other authors participated in the experiments or reviewed the manuscript.

Funding

T.J. is supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDB29030104), the National Natural Science Fund (Grant No.: 31870731 and U1732109), the Fundamental Research Funds for the Central Universities and the 100 Talents Program of The Chinese Academy of Sciences. X.F. is supported by the National Natural Science Fund for Young Scholars (Grant No.: 31800639) and the Fundamental Research Funds for the Central Universities (Grant No.: WK2070000110).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to thank the staff at the BL17U1, BL18U, and BL19U1 beamline at the Shanghai Synchrotron Radiation Facility (SSRF) for the assistance during data collection. We also want to show our appreciation to the staff at the core facility in the school of life science, USTC for their support of doing ITC and 2DIC-MS.

ABBREVIATIONS USED

HCabs; heavy chain-only antibodies; VHH; variable domain of heavy chains of HCabs; Nb; nanobody; ITC; isothermal titration calorimetry; CDRs; complementarity determining regions; CCC-dsDNA; covalently closed circular double-stranded heteroduplex DNA; dU-ssDNA; uracil-containing single strand DNA; PEG; poly(ethylene glycol); IPTG; isopropyl- β -D-thiogalactoside; 2DIC-MS; two-dimensional liquid chromatography multistage mass spectrometer; MR; molecular replacement; PSMs; peptide-spectrum match scores

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