

Unbiased Immunization Strategy Yielding Specific Nanobodies against Macadamia Allergen of Vicilin-like Protein for Immunoassay Development

Yaozhong Hu,^{||} Sihao Wu,^{||} Yi Wang, Jing Lin, Ying Sun, Chuan Zhang, Jiaxin Gu, Feier Yang, Huan Lv, Xuemeng Ji, Yan Zhang, Serge Muyldermans, and Shuo Wang*



Cite This: <https://doi.org/10.1021/acs.jafc.1c00390>



Read Online

ACCESS |



Metrics & More



Article Recommendations



Supporting Information

ABSTRACT: Macadamia nut contains important food allergens that potentially cause allergic reactions with severe adverse effects in infants and adults. Reliable and accurate detection of macadamia is critical to avoid allergic reactions. However, knowledge on macadamia allergen is scarce and a reliable detection method has not been reported, yet. In this study, an unbiased immunization and selection strategy was employed to select nanobodies (Nbs) recognizing specifically macadamia allergen, as well as to establish a detection method to unveil a macadamia protein contamination. An alpaca was immunized with a crude protein extract of macadamia followed by construction of a Nb library from its lymphocytes. The panning and screening of this immune Nb repertoire resulted in the selection of six target-specific Nbs. Nb-mediated immuno-capturing combined with mass spectrometry allowed us to identify the target as the macadamia vicilin-like antimicrobial peptides 2–3 (MiAMP2), a novel food allergenic protein abbreviated as Mac i 1. Later on, an immunoassay of a heterologous sandwich ELISA method based on the selected Nb-pairs was established, providing a linear response in the range of 0.442–2,800 $\mu\text{g/mL}$ and with a limit of detection of 27.1 ng/mL. The dedicated immunoassay has been verified by detecting the antigen spiked in food samples. Our study provided evidence for the successful application of the unprejudiced strategy to retrieve Nbs against *a priori* undefined macadamia allergen. These target-specific Nbs were used to design a highly reliable and effective immunoassay.

KEYWORDS: macadamia nut, unbiased strategy, nanobody, Mac i 1, sandwich ELISA

INTRODUCTION

A daily intake of tree nuts has been recommended in view of their abundant dietary components, including a high content of lipids and proteins, which are encapsulated to provide essential nutrient resources for the germinating seeds.¹ These recommended tree nuts can be consumed directly, or indirectly when mixed in various food products, such as chocolate, milk, bakery goods, etc. Macadamia has attracted much attention due to its extraordinary organoleptic properties and high nutritional quality. According to the reports from the USDA (<https://www.nal.usda.gov/fnic>), the macadamia nut contains approximately 75.77% of fat and a high level of proteins, up to 7.91%. The high percentages of total fat, especially the high proportion of monounsaturated fat, is assumed to correlate with the promoted healthy state of lower density lipoproteins (LDL).²

The increasing planting area and annual worldwide production of macadamia nuts reflect their popularity and augmented consumption.³ Although the percentage of allergic reactions provoked by the consumption of macadamia nuts is lower in comparison to that of peanut, it cannot be neglected. The steadily growing consumption of macadamia nuts will still potentially increase the anaphylaxis resulting from their allergenic proteins. In infants and adults as well, these proteins might cause severe and sometimes even fatal allergic reactions,

including laryngeal angioedema, generalized urticaria, and hypotonic circulatory reaction.^{4–6}

Apart from the growing consumption of macadamia, the lack of awareness and knowledge on its allergens definitely increases the risks of its exposure. Nonetheless, an allergen of vicilin protein from macadamia nut, referred to as Mac i 1 (molecular mass of around 50 kDa), has been confirmed recently by IUIS/WHO. This finding reinforces the urgent need to promote the investigation and understanding of various macadamia allergens to support the food industry. Currently, no effective therapeutic strategy has been developed to treat the anaphylaxis resulting from tree nut allergens. Therefore, in absence of an effective treatment of allergic reactions, avoiding an unexpected intake of allergens will depend on their sensitive detection in foods.^{7,8}

To date, various analytical methods have been explored and applied to detect allergens, i.e., DNA-based assays (e.g., real-time PCR) and immunoassays (e.g., enzyme-linked immunosorbent assay (ELISA)).^{9,10} The immunoassays based on the

Received: January 20, 2021

Revised: March 18, 2021

Accepted: March 23, 2021

interaction between specific antibodies and allergenic proteins have been developed and are routinely applied as a preferred detection method in the food industry. Traditional immunoassays based on monoclonal or polyclonal antibodies depend heavily on the quality of the purified allergenic proteins and of the antibodies, and failure to provide good-quality reagents will lead inevitably to a low testing efficacy. Moreover, the identification of appropriate conventional antibodies against undetermined allergens is very complicated and alternative affinity reagents should be introduced to improve the allergen analyses.

Camelidae species such as camels, dromedaries, llamas, and alpacas have a distinctive immune response which generates functional heavy chain-only antibodies (HCAbs).¹¹ The antigen binding fragment of these unique HCABs is restricted to one single variable domain, referred to as VHH or nanobody (Nb) with a molecular mass around 14 kDa.¹² It is an attractive immunoreagent with unique and favorable properties. In comparison to conventional antibodies, Nbs can be easily selected and produced in microorganisms. The Nbs also seem to recognize their cognate epitopes with high specificity and affinity, and they can even target epitopes that are less accessible or even cryptic for conventional antibodies.^{13,14} Furthermore, Nbs show excellent solubility in aqueous solutions and possess an intrinsic domain stability even under nonphysiological conditions, such as high temperature, extreme pH, and even chemical denaturants.^{15,16} These eminent properties of Nbs facilitate an extensive application range as a research tool or as diagnostic and therapeutic reagents.^{17–19}

In the current study we identified Nbs against a priority unknown allergen from macadamia based on a crude protein extract, and we designed a practical immunoassay with high sensitivity and specificity (Figure 1). Our approach is

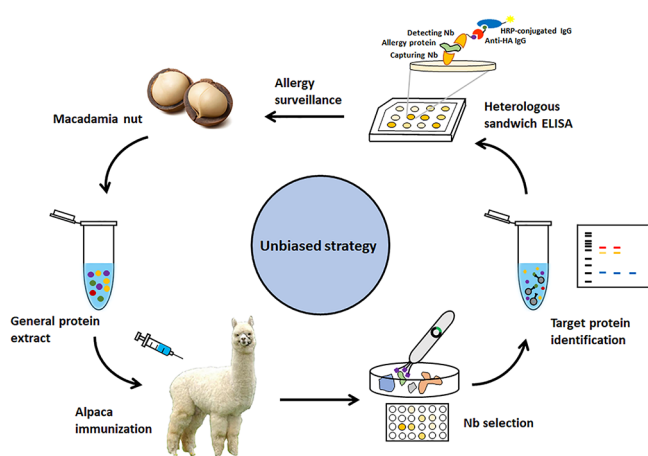


Figure 1. Schematic illustration for the selection of Nbs against Macadamia allergen based on the unbiased immunization strategy and development of the immunoassay.

considered to be unbiased since prior knowledge on target antigen(s) for immunization and Nb selection was not included. Thus, instead of facing difficulties in purification of macadamia antigens for immunization, we immunized an alpaca with the total protein extracts of macadamia nuts and let the immune response within the animal decide which proteins to be antigenic. Then, a large immune Nb repertoire was constructed and used to retrieve Nbs with high specificity and

affinity for their cognate antigen that was subsequently identified as macadamia vicilin-like antimicrobial peptides 2–3 (MiAMP2), or as Mac i 1. This confirms the successful application of the Nb generation technology against allergens. Finally, a heterologous sandwich ELISA has been designed to identify the presence of allergens with good sensitivity and specificity.

EXPERIMENTAL SECTION

Macadamia Protein Extraction. The crude protein extraction from macadamia nut is well established and has been followed according to previous reports.^{20–22} Briefly, 100 g of macadamia nuts (purchased from local markets) was ground. The flour was defatted by adding 500 mL of hexane and stirring for 24 h. After the hexane fraction was decanted, the extracted slurry was left in a fume cabinet to evaporate the residual hexane. The flour was resuspended in water at a 1:50 (w:v) ratio, and the pH was adjusted with 1 N NaOH to reach pH 9.0. After gentle stirring for 4 h, the supernatant was collected after centrifugation, and its pH was adjusted to 4.5 with 1.0 N HCl. Then, the extraction was pelleted after centrifugation and redissolved in water. The final protein solution was lyophilized and stored at -20°C until further usage. For subsequent analysis of the extracted protein, samples were resuspended in phosphate-buffered saline (PBS). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the extracts was performed under reducing and nonreducing conditions and were stained with Coomassie-blue to visualize protein band patterns.

Immunization and Construction of a Nb Library. A healthy young alpaca was immunized with the crude extracted proteins mixed with complete or incomplete Freund's adjuvant to elicit an immune response. The immunization was performed with six injections of 0.5 mg of protein, at weekly intervals.²³ Three days after the last boost of the immunization, 50 mL of anticoagulated blood was collected from the jugular vein. Peripheral blood lymphocytes were isolated using Lymphoprep Density Gradient Medium (STEMCELL Technologies, Canada). The total RNA was extracted from the lymphocytes by TRIzol Reagent (Invitrogen, USA) and used for cDNA synthesis. Then, a two-step nested PCR was performed to amplify VHH fragments by using the cDNA as template. In the first PCR step, primers CALL001 (5'-GTC CTG GCT GCT CTT CTA CAA GG-3') and CALL002 (5'-GGT ACG TGC TGT TGA ACT GTT CC-3') were employed to amplify the region between the leader signal sequence of the variable domain and CH2 domain from the heavy chain of both conventional antibodies and HCAbs. The amplified PCR products were separated on agarose gel, and the fragment with a size around 700 bp (corresponding to HCAB fragments) was extracted by a QIAquick Gel Extraction Kit (QIAGEN, Germany). These fragments were then used as a template for the second PCR step with primers PMCF (5'-CTA GTG CCG CCG CTG AGG AGA CGG TGA CCT GGG T-3') and A6E (5'-GAT GTG CAG CTG CAG GAG TCT GGR GGA GG-3') to produce the amplified VHH fragments with Not I and PstI restriction enzyme sites (underlined in primer sequences). The restriction enzyme digestion of the PCR amplicons was accomplished with Not I and PstI (NEB, USA) to allow their ligation into the phagemid of pMECS cut with the same enzymes. The recombinant plasmids were electro-transformed into *E. coli* TG1 competent cells (Lucigen, USA) that might suppress the amber stop codon between the cloned VHH and gene PIII of M13. The size of the library was determined after serial dilution of a small aliquot of the transformed bacteria and plating on Luria–Bertani (LB) agar supplemented with glucose and ampicillin. The remaining part of the transformed bacteria was plated on large LB agar plates with ampicillin and glucose. The constructed Nb library was prepared after scraping the colonies from the plates and storing them in the presence of 20% glycerol. The percentage of clones with a phagemid containing a VHH insert was assessed by colony PCR with primers of MP57 (5'-TTA TGC TTC CGG CTC GTA TG-3') and GIII (5'-CCA CAG ACA GCC CTC ATA G-3')

on randomly chosen colonies, and agarose-gel electrophoresis was used to visualize the bands corresponding to VHHs.²³

Biopanning and Selection of Nbs. Three rounds of biopanning were performed according to the established protocol for the enrichment of antigen-specific Nbs.²⁴ Briefly, an aliquot of the Nb library was used to inoculate $2 \times$ TY medium supplemented with glucose and ampicillin to bring the cells into the exponential growth phase. Subsequently, M13K07 helper phages were added to the shake flask to infect TG1 cells and to rescue their Nbs by expression on phage particles. After overnight incubation, phage particles with surface-expressed Nbs were collected from the supernatant after centrifugation to remove TG1 cells and then were precipitated with polyethylene glycol (PEG)/NaCl to allow the final resuspension in 1 mL of sterile PBS. Wells of microtiter plates (Corning, USA) were coated with 100 μ L of extracted protein antigen at a concentration of 50 μ g/mL, overnight at 4 °C, and empty wells were used as a negative control. After the remaining protein binding sites were blocked with 200 μ L of 3% (w/v) skim milk, around 1×10^{11} pfu of Nb-displaying phages were added to the antigen coated and noncoated wells and incubated at room temperature (RT) for 1 h. Unbound phages were removed by stringent washing steps with PBST (PBS containing 0.05% Tween-20) for 10 times in the first round of panning and for 20 times in the second and third rounds of panning. The remaining phage particles were eluted with 100 μ L of TEA solution (100 mM triethylamine, pH 11.0) and neutralized with 100 μ L of Tris-HCl (1.0 M, pH 7.4) immediately after collection. Next, 10 μ L of the harvested phages were serially diluted and subsequently added into wells with 90 μ L of TG1 cells for enrichment analysis. The remaining phage particles were incubated with TG1 cells to allow the infection and to proceed to the next round of panning after preparing fresh phage particles.

Screening of Specific Nbs. For the screening of Nbs with specificity against macadamia extracted proteins, individual colonies obtained after three-rounds of biopanning were randomly selected and cultured in 100 μ L of $2 \times$ TY medium supplemented with 10% (w/v) glycerol, 2% (w/v) glucose, and 100 μ g/mL ampicillin in 96-well culture plates (Corning, USA). After overnight incubation at 37 °C without shaking, 10 μ L of each culture was inoculated into deep well plates (Axygen, USA) with $2 \times$ TY medium supplemented with 0.1% (w/v) glucose and 100 μ g/mL ampicillin and cultured while shaking at 37 °C for 3–4 h until the exponential growth phase was reached. The induction and periplasmic expression of Nbs with His- and Hemagglutinin (HA)-tags was accomplished overnight after adding isopropyl β -D-1-thiogalactopyranoside (IPTG), reaching a concentration of 1 mM in the wells. Then, a total protein extract including an enriched periplasmic fraction containing Nbs (periplasmic enriched, PE) was obtained after freeze–thaw cycles to evaluate the binding capacity of the released Nbs to coated macadamia proteins in a PE-ELISA.²⁵ Wells coated with PBS instead of the macadamia protein extracts were utilized as a negative control to estimate the background signal of Nbs. The antigen bounded Nbs were detected with mouse anti-HA IgG (Invitrogen, USA) and alkaline phosphatase (AP) conjugated goat antimouse IgG (Invitrogen, USA) as the primary and secondary antibodies, respectively. The colonies providing at least a 2-fold higher signal in wells with macadamia extract compared to that of noncoated wells were considered as positive, and these clones were chosen for the subsequent phagemid extraction and nucleotide sequencing of their inserted VHHs.

Expression and Purification of Selected Nbs. The pMECS vectors containing the genes of the selected Nbs were prepared and electro-transformed into *E. coli* WK6 cells for expression of their Nbs with HA-tag and His-tag at the C-terminus. The WK6 cells were cultured in shake flasks containing 330 mL of Terrific Broth (TB) medium supplemented with 0.1% (w/v) glucose, 100 μ g/mL ampicillin, and 2 mM $MgCl_2$ at 37 °C until an $OD_{600\text{ nm}}$ of around 0.6 was reached. Induction of Nb expression was performed with 1 mM IPTG, leading to accumulation of Nbs in the periplasm after shaking the cultures overnight at 28 °C. Bacteria were then pelleted by centrifugation and exposed to an osmotic shock to release the Nbs

residing in the periplasm. Immobilized metal affinity chromatography (IMAC) on HisPur Ni-NTA Resin (Thermo Scientific, USA) was performed to purify the Nbs. After the nonspecific contaminants were washed off, His-tagged protein bounded tightly to Ni^{2+} in the resin was eluted with an elution buffer containing 0.5 M imidazole. The elution fractions were collected and applied to size exclusion chromatography (SEC) on a HiLoad 16/600 Superdex 75 pg preppacked column (GE Healthcare, USA) for further purification and buffer exchange. The purity and identity of the prepared Nbs was analyzed by SDS-PAGE and Western blot. The concentration of Nbs was determined by measuring $OD_{280\text{ nm}}$ and using the theoretical extinction coefficient of the Nb as calculated from its amino acid content. Eventually, the purified Nbs were stored at a concentration of 1 mg/mL for further investigation.

Binding Capacity and Cross-Reactivity of Nbs. The binding capacity and cross-reactivity of selected Nbs were confirmed by indirect ELISA against total protein extracts from macadamia, lupine, peanut, hazelnut, and almond. Briefly, protein extracts at a concentration of 50 μ g/mL were coated overnight at 4 °C in wells of microtiter plates. Residual protein binding sites were blocked by incubation with 3% skim milk for 1 h at RT. After being washed with PBST, Nbs (1 μ g/mL) were added to the wells and incubated for 1 h at RT. After being rinsed with PBST, mouse anti-His IgG and HRP-conjugated goat antimouse IgG were incubated successively for 1 h at RT. Fresh TMB (3,3',5,5'-tetramethylbenzidine) substrate was added and incubated for color development. The reaction was terminated with 2 M H_2SO_4 solution, and the optical density at 450 nm was read with a microplate reader (Tecan, Switzerland).

Antigen Specificity of Nbs. A Western blot was performed as a first identification step of the target protein of the purified Nbs. Total macadamia protein extract was separated by SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare, USA) for the Western blot analysis. The membrane was cut into parallel strips after blocking with 3% skim milk and with further incubation with 1 μ g/mL of Nb in sterile PBS at RT. The blank control was set up with incubation of milk PBS instead of Nbs. After the washing steps with PBST, mouse anti-His-tag IgG and HRP-conjugated goat antimouse IgG were then incubated sequentially as the primary and secondary antibody, respectively. The binding of Nbs to antigen was visualized after developing with mixed HRP-substrate staining solutions (solution A: 6 mL of methanol with 18 mg of chloro-1-naphthol; solution B: 30 mL of TPA solution (500 mL: 14.63 g of NaCl, 1.4 g of Trizma base, pH 7.5) with 19 μ L of H_2O_2). The same analytical method was applied to determine the binding of Nbs to the heat-processed macadamia proteins (extraction from roast macadamia (150 °C for 30 min)).

Thermal Stability of Nbs. Thermal stability of Nbs was assessed by thermofluor in a CFX Connect Real-Time PCR System (Bio-Rad, USA).²⁶ Generally, the prepared Nbs were concentrated and adjusted to a final concentration of 2.5 mg/mL. The reaction vial of 30 μ L contained 15 μ L of Nb, 5 μ L of 100 $\times$ diluted SYPRO Orange Protein Gel Stain (Sigma-Aldrich, USA), and 10 μ L of PBS and was prepared in wells of a microtiter plate. PBS with SYPRO Orange Protein Gel Stain was employed for baseline control. The temperature raising program was set from 25 to 95 °C at a rate of 0.5 °C/min to record the fluorescence signal every 0.5 °C. All samples were measured in triplicate. Data analysis was performed with OriginPro 8 software by employing nonlinear fitting to determine the melting temperature (T_m) of the Nbs.

Affinity of Nbs for Their Target. The apparent affinity of Nbs against the targeted proteins was inferred by determining the binding of the selected Nb to the crude macadamia protein extract in an ELISA. Briefly, protein extract at a concentration of 50 μ g/mL was coated overnight at 4 °C in wells of microtiter plates. After blocking of the residual protein binding sites with 3% skim milk, serial dilution of Nbs at 100000, 10000, 1000, 100, 10, 1, 0.1, 0.01, 0.001, and 0.001 nM were added and incubated for 1 h at RT. Additional wells were supplemented with PBS instead of Nbs and used as a negative control for baseline evaluation. After incubation with mouse anti-His-tag IgG and HRP-conjugated goat antimouse IgG, TMB chromogenic

solution was used for color reaction. The absorbance was detected by a microplate reader at a wavelength of 450 nm. The apparent affinity of the Nbs was estimated as the concentration of Nb yielding half of the maximum signal.

Immuno-Capturing and Antigen Identification by LC-MS/MS. To identify the antigen protein targeted by the selected Nbs, we first captured the antigen out of the complex macadamia protein extract with the Nbs. The coimmunoprecipitate (Co-IP) was applied on SDS-PAGE to confirm the Western blot data and was then analyzed by LC-MS/MS. Briefly, 250 μ L of macadamia protein extract at a concentration of 1 mg/mL was incubated with 5 μ g of Nbs overnight at 4 °C with shaking. Afterward, 10 μ L of HisPur Ni-NTA Magnetic Beads (Thermo Scientific, USA) was added to the mixture to capture the Nb-antigen complex with a head-over-end rotator for 30 min. After collection on a magnetic stand, the beads were washed with washing buffer (PBST containing 50 mM imidazole; pH 8.0) and the supernatant was discarded. After the washing steps, 25 μ L of elution buffer (PBS containing 250 mM imidazole; pH 8.0) was incubated with the beads for 10 min to elute the Ni²⁺ binding Nb-antigen complex. Then, the eluted fraction was collected and applied for SDS-PAGE and stained with Coomassie-blue to visualize the bands corresponding to the target antigen.

The bands, corresponding to the target antigen confirmed by SDS-PAGE and Western blot, were excised from gel and submitted to the peptide preparation protocol and used in LC-MS/MS analysis. Briefly, the excised gel material was fully destained to remove the Coomassie-blue and then digested with trypsin to produce peptides for the analysis with LC-MS/MS (Waters Corporation, USA).²⁷ For peptide identification, data were generated and analyzed in the Proteome Discoverer 1.4 (Thermo Scientific, USA) with the protein database generated from NCBI and Uniprot. Peptide matches were filtered using the *q*-value and Posterior Error Probability calculated by the Percolator algorithm ensuring a false detection rate below 5%.

Expression and Purification of Nbs with His-tag Only. Based on the established protocol to develop heterologous sandwich ELISA, the Nbs with His-tag only was proposed to provide the capturing antibody candidates. Therefore, the genes encoding the selected Nbs were subcloned into the pHEN6c expression vector and transformed into *E. coli* WK6 cells for periplasmic expression. After induction of the His-tagged Nb expression by adding 1 mM IPTG to cells in TB medium, bacteria were collected and the PE was prepared by an osmotic shock. The same purification strategies, including IMAC and SEC, were applied as described above to prepare the Nbs with HA and His-tag. The purity and identity of the obtained Nbs were confirmed by SDS-PAGE and Western blot, accompanied by an indirect ELISA for Nb-antigen binding assay.

Nb-based Heterologous Sandwich ELISA. Nbs with His-tag only and Nbs with both tags (His-tag and HA-tag) were used as the capturing and detecting Nbs, respectively, to identify the best performing Nb pair for the development of the heterologous sandwich ELISA. Briefly, 100 μ L of Nbs with His-tag only (10 μ g/mL) were coated in wells of microtiter plates for 1 h at RT. After several washing steps with PBST, the residual protein binding area was blocked with 3% skim milk at RT for 1 h. Then, 100 μ L of macadamia protein at a concentration of 50 μ g/mL was added and incubated. After washing, 100 μ L of Nbs with His- and HA-tags (10 μ g/mL) were incubated to associate with the captured antigen. Then, mouse anti-HA IgG and HRP-conjugated goat antimouse IgG were employed as the primary and secondary antibodies, respectively. TMB substrate was used for the color development, and the optical density at 450 nm was recorded by a microplate reader. The Nb-based sandwich ELISA was performed repeatedly to assess the contribution of variables, such as the selected Nb pair employed for the development of sandwich ELISA, the determination of the optimized concentration of the selected capturing and detecting Nbs through a checkerboard titration with a series of dilution, and the confirmation of the established sandwich ELISA against the macadamia protein under the optimized conditions. The calibration curve and linear standard curve was provided with the natural logarithm of the macadamia protein concentration and OD_{450 nm} value.

Cutoff Value, LOD, and LOQ. After the development and optimization of the sandwich ELISA, the cutoff value was determined by testing blank wells with PBS only (*n* = 10). The limit of detection (LOD) and the limit of quantitation (LOQ) were defined as the protein concentration corresponding to the mean of the cutoff value plus 3 or 10 times the standard deviations (SD), respectively.²⁸

Analysis of Spiked Sample. The dairy product of skim milk was purchased from local supermarkets to validate the effectiveness of the established Nb-based sandwich ELISA. The spiked sample was prepared by supplementing the milk with various amounts of crude macadamia protein extract. Then, the spiked samples were centrifuged to remove the cream layer and the pellet. The upper supernatant was then collected and diluted 1000 times in PBS before testing. This sample was used in the sandwich ELISA to determine the recovery rate and coefficient of variation (CV) for assessing the precision and reproducibility of the sandwich ELISA, respectively.

Statistical Analysis. The statistical data were obtained by GraphPad Prism 8 software. The data and error bars represent the mean $\pm$ SD. The experiments were carried out at least three times for *in vitro* assays.

RESULTS

Crude Extract of Macadamia Proteins. A crude extract of macadamia proteins was obtained following a well-established protocol, yielding an acceptable efficiency above 90%, which facilitated its lyophilization and prolonged storage. The extracted proteins revealed after SDS-PAGE and Coomassie staining (Figure 2) a range of molecular masses

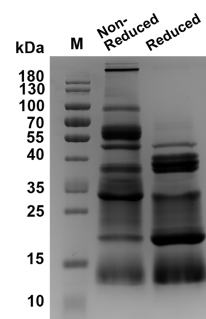


Figure 2. SDS-PAGE analysis of the crude macadamia protein extract. The left lane is the size marker. The next lane contains the macadamia proteins separated under nonreducing conditions, while the right lane is the same sample run under reducing conditions.

from 10 to 70 kDa, which corresponded well to previous results.²⁹ A significant difference was noticed when polypeptides were separated by SDS-PAGE under reducing or nonreducing conditions. The nonreduced electrophoretic profile revealed the presence of proteins with large molecular weight above 180 kDa (possibly hexamers and trimers of globulins) and a variety of bands of intermediate molecular masses around 50–100 kDa. Conversely, the proteins separated under reducing conditions revealed the disappearance of proteins with high molecular masses (>50 kDa) and an increased intensity of bands corresponding to a size of 10–35 kDa. It all reflected the high content of natural interpeptide disulfide bonds and a hetero-oligomeric composition of the nonreduced proteins in the macadamia extract. A similar protein band distribution was also noticed for crude protein extracts of other tree nuts like walnut and hazelnut.^{30,31} The proteins from extracts of these nuts have been identified as seed storage proteins like legumins (11S hexameric globulins composed of 30–40 kDa acidic peptides and 15–20 kDa basic

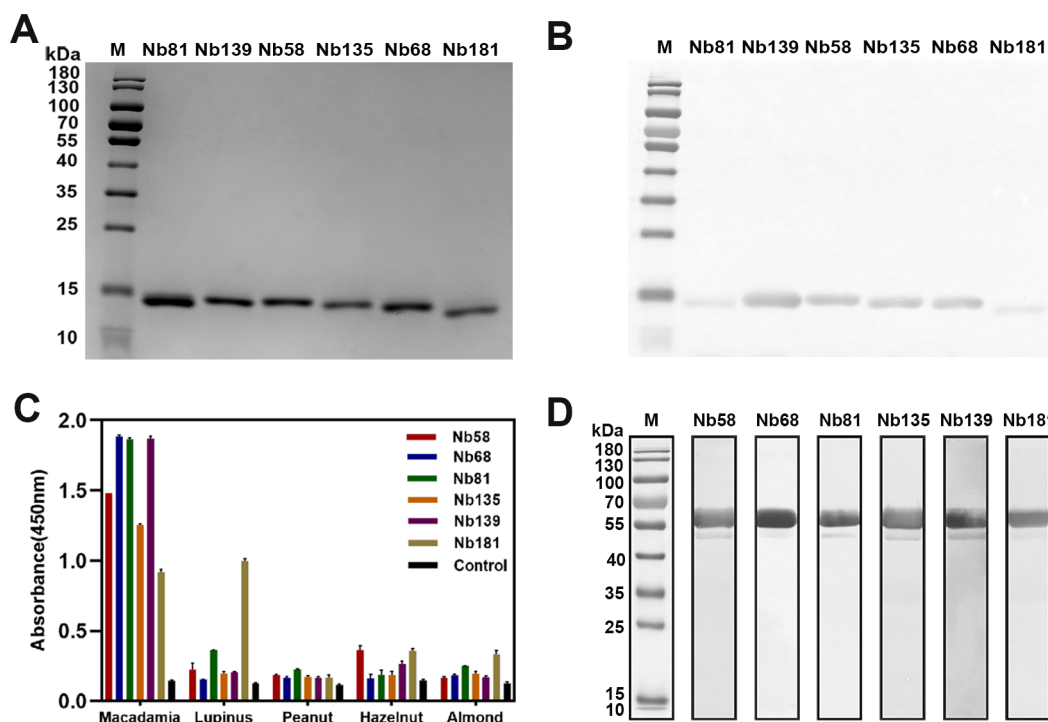


Figure 3. Identification of antigen-specific Nbs. (A) SDS-PAGE and (B) Western blot analysis of the His- and HA-tagged Nbs after expression and purification by IMAC and SEC. (C) Indirect ELISA was performed to detect the antigen-Nb binding capacity and cross-reactivity with protein extracts from other origins (lupinus, peanut, hazelnut, and almond). (D) Antigen specificity of the Nbs. Antigen proteins were separated under nonreducing conditions by SDS-PAGE, transferred to nitrocellulose and probed separately with each of the six Nbs. All data are expressed as the mean $\pm$ SD ($n = 3$). Every test was repeated at least 3 times.

peptides) and vicilins (7S trimeric globulins comprising about 50 kDa subunits).^{32,33}

Construction of an Antimacadamia Immune Nb Library. A young alpaca was immunized to elicit HCABs against macadamia proteins. The repertoire of the antigen binding domains of these HCABs (VHH) was ligated in a phage display vector and transformed in *E. coli*. The VHH sequence was amplified from the cDNA of blood lymphocytes after a two-step nested PCR. After the first PCR, two fragments with the sizes of 900 bp and 700 bp were obtained, reflecting the amplicons corresponding to the VH–CH1–CH2 regions and to the VHH–CH2 regions of heavy chains from conventional and from HCABs, respectively (Figure S1A). After eluting the 700 bp fragment from agarose gels and performing the second PCR, VHH regions with the size of 400 bp were amplified (Figure S1B). The restriction enzyme digestion of these VHH amplicons were ligated into the pMECS phage display vector cut with the same enzymes. The ligated material was transformed in TG1 *E. coli* cells yielding 1.44×10^7 individual transformants. This number is larger than the estimated amount of B cells from which we started, indicating that the entire VHH repertoire within this blood sample was represented in the library. Furthermore, 24 colonies were randomly chosen for colony PCR analysis to assess the presence of an insert with the size of a VHH in the pMECS phagemid. Apparently, 75% of the clones contained an insert reflecting the gene size of a VHH (Figure S1C). In conclusion, these criteria are in line with a high-quality immune Nb library containing sufficient Nb diversity to retrieve successfully target-specific Nbs.

Biopanning and Screening of Nbs. A representative aliquot of the immune Nb library was cultured and the Nbs

were expressed at the tip of M13 virions, where they are available for selection during biopanning on macadamia crude proteins. The enrichment of putative macadamia protein recognizing Nbs during consecutive rounds of panning was followed by taking the ratio of the number of colonies originating from virions eluted from wells with or without immobilized macadamia protein. The ratio of these number of virions increased from 1.7 to 84.3-fold, indicating a good enrichment of target specific Nbs during the consecutive rounds of panning (Figure S2A).

To identify the target-specific Nbs within this enriched fraction, 190 individual colonies (48 colonies from the first round, 107 colonies from the second round, and 35 colonies from the third round of panning) were randomly chosen and the encoded Nb was expressed in the periplasm from where it was extracted and tested in a PE-ELISA. The results showed 27 colonies for which the extracted Nbs gave at least a 2-fold higher signal in wells with coated macadamia proteins compared to wells without these proteins (Figure S2B). The identified positive binders were sent for nucleotide sequencing. The *in silico* translated amino acid sequence was then classified according to Nb families, based on the CDR3 loop length and amino acid sequence. Six Nb families were identified (Figure S2C), and these were named Nb58, Nb68, Nb81, Nb135, Nb139, and Nb181.

Expression and Purification of Antigen-Specific Nbs.

The phagemid encoding macadamia recognizing Nbs were purified from TG1 cells and transformed into WK6 *E. coli* cells for the periplasmic expression of the Nb protein after induction with IPTG. An osmotic shock was used to extract the soluble Nbs, which were purified to homogeneity using a two-step protocol encompassing IMAC and SEC. The final

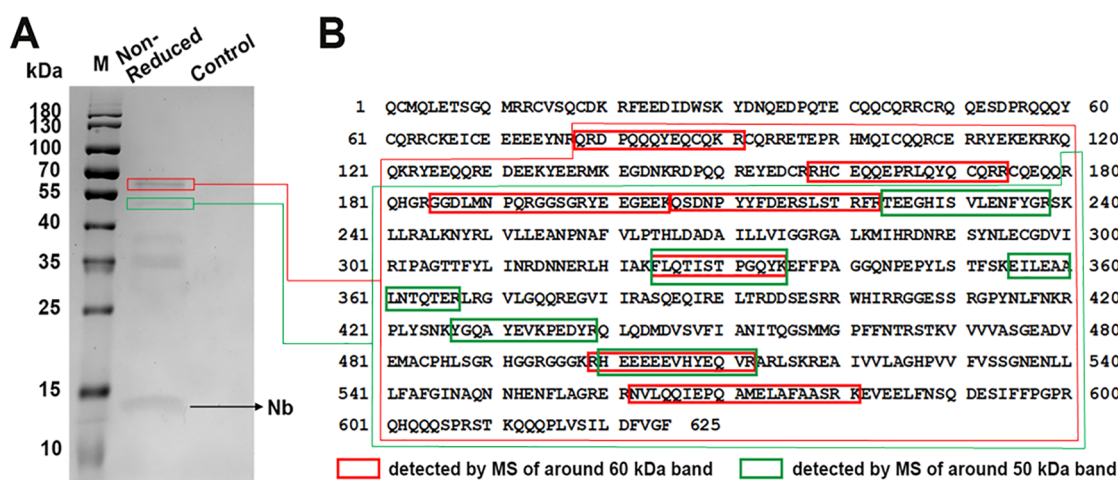


Figure 4. Nb-mediated identification of the target antigen in Macadamia protein to be the vicilin-like antimicrobial peptides 2–3. (A) The Co-IP material from macadamia protein extract captured by Nb81 via Ni-NTA magnetic beads. The antigenic protein and Nb were separated and analyzed by SDS-PAGE under nonreducing conditions. Two bands with a size of around 60 kDa and 50 kDa that were also visible in Western blot (Figure 3D) were identified as the target antigens and (B) subsequently analyzed through LC-MS/MS. The peptides of around 60 kDa and 50 kDa band detected by MS analysis are highlighted by red and green boxes, respectively. Both of the proteins are part of a 7S seed storage protein called vicilin-like antimicrobial peptides 2–3 (UniProt sequence accession number: Q9SPL3).

yields of Nbs ranged from 5 to 10 mg/L in TB medium in shake flasks. The purity of the Nbs was evaluated by SDS-PAGE. The Coomassie staining revealed the presence of a single band for each sample with a molecular mass around 15 kDa, as expected from the Nb amino acid sequence (Figure 3A). The Western blot of these proteins probed with anti-His antibodies confirmed that the proteins in these bands contained the His-tag (Figure 3B).

Antigen Recognition and Potential Cross-Reactivity of Nbs. After preparing the selected Nbs, an indirect ELISA was performed to evaluate and confirm their interaction with macadamia crude protein extracts, as well as to test the possible cross-reactivity with protein extracts from other tree nuts, such as lupinus, peanut, hazelnut, and almond. The ELISA results confirmed the target specificity of the selected Nbs by the high absorbance values obtained for wells containing the macadamia proteins relative to control wells without such proteins (Figure 3C). Moreover, the interaction of our Nbs with protein extracts from lupinus, peanut, hazelnut, and almond demonstrated a significant cross-reactivity of Nb181 with lupine proteins, a slight interaction of Nb181 with hazelnut and almond proteins, and a cross-reaction of Nb58 with hazelnut proteins. Whereas, the remaining Nbs did not show a significant interaction with the lupinus, peanut, hazelnut, or almond proteins.

Antigen Identification of Our Nbs. The antigen specificity of our Nbs was first analyzed by Western blot after separating the extracted macadamia proteins under reducing or nonreducing conditions on SDS-PAGE. For the nonreduced samples, all our Nbs reacted strongly to a protein with a size around 60 kDa, and a relatively low staining was also observed against a protein of around 50 kDa (Figure 3D). In contrast, no bands were revealed for antigen samples separated under reducing conditions. This demonstrated that the selected Nbs recognize conformational epitopes on their cognate antigen. The targeting of the selected Nbs to the heat-processed macadamia protein was determined by Western blot, which reflected the reduced ability of Nb181 to target the heat-

processed antigen, whereas no significant variation of the binding capacity was observed for other Nbs (Figure S3).

Thermal Stability and Antigen Affinity of Nbs. The stability of Nbs is an important characteristic as it is correlated to the shelf life and robustness of the Nb reagent in nonphysiological test conditions. The stability of Nbs is conveniently measured by a thermofluor assay. As shown in Table S1, Figure S4, and Figure S5, the stability of selected Nbs was verified and indicated T_m values ranging from 59 to 82 °C, of which Nb81 was confirmed to possess the highest T_m value. The obtained data demonstrated that the selected Nbs possessed a high thermal stability.

The apparent affinity of Nbs against their cognate antigen was estimated via saturated ELISA binding studies against immobilized macadamia crude protein extracts. Generally, a high apparent affinity was observed for the selected Nbs. A relatively higher binding capacity was noticed for Nb68, Nb81, and Nb139 with IC₅₀ values of 7.5 nM, 11.1 nM, and 9.2 nM, respectively. A comparatively lower affinity was recorded for Nb58, Nb135, and Nb181 with IC₅₀ values of 120 nM, 142 nM, and 86 nM, respectively.

LC-MS/MS Identification of the Antigen Targeted by Nb81. To identify the cognate antigen of Nb81, we first captured its target protein by a co-immunoprecipitation (Co-IP). The captured proteins were then analyzed by LC-MS/MS. Since all Nbs recognize apparently the same protein in Western blot, we hypothesized that it would not matter which Nb we used. The Nb81 was preferred as it is a robust Nb with high affinity for its antigen, therefore suitable for immunocapturing of the antigen from the macadamia crude protein extract by utilizing Ni-NTA magnetic beads. Thereafter, the captured proteins were separated via SDS-PAGE. Two main additional bands with a size of around 60 kDa and 50 kDa were observed in comparison with the control. These bands corresponded to the bands revealed after Western blot using the Nbs as a probe. Also, a band with a size of 15 kDa was noticed, but this is most likely the Nb81 itself (Figure 4A). The major bands of interest were excised from gel and digested with trypsin for the peptide analysis by LC-MS/MS. A search

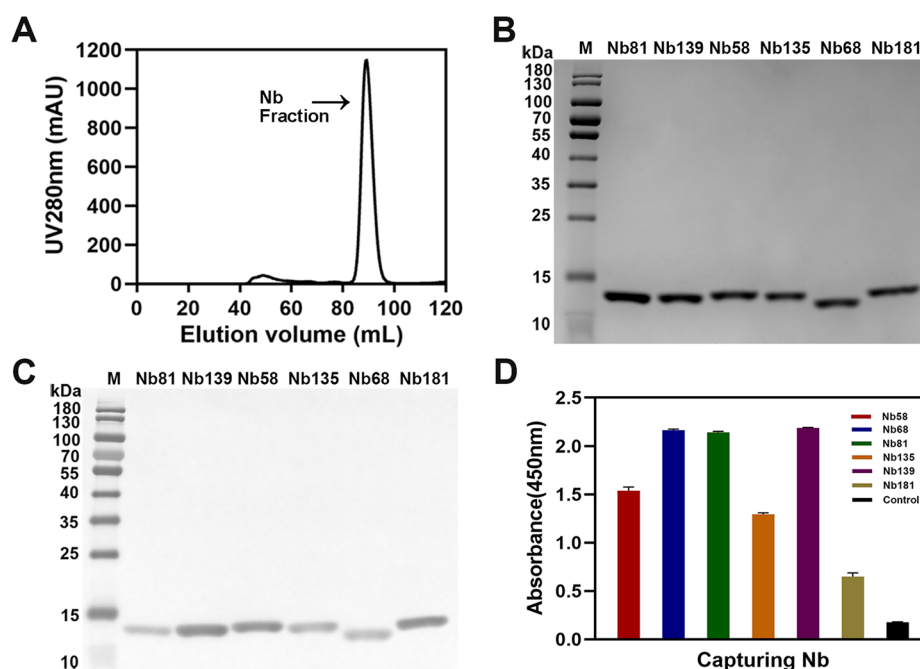


Figure 5. Characterization of antigen capturing Nbs with His-tag (without HA-tag). (A) SEC. (B) SDS-PAGE and (C) Western blot analysis of the His-tagged Nbs after expression and purification by IMAC and SEC. (D) Indirect ELISA for detecting antigen capturing Nb binding capacity. The wells were coated with macadamia protein extract and subsequently bound by Nbs, which were detected by anti-His antibodies as a probe. All data are expressed as mean $\pm$ SD ($n = 3$). Each test was repeated at least three times.

of the unique peptides in the Protein database indicated several peptides that could be aligned with the sequence of MiAMP2 (UniProt sequence accession number: Q9SPL3) (see Figure 4B and Table S2). MiAMP2 are a kind of 7S vicilin precursors belonging to the seed storage protein family.³⁴ They contain hydrophilic segments of 199 aa and paired “CXXXC” motifs in their N-terminal, also observed as isolated peptides with known antimicrobial activities and referred to as MiAMP2b-2d.^{34,35} They also include the vicilin 7S globulin invariantly represented regions containing 426 aa with two conservative Cupin domains in their C-terminal.³⁶ 7S vicilin proteins are regularly recognized as food allergens in tree nuts, such as Ana o 1 in cashew, Cor a 11 in hazelnut, Ara h 1 in peanut, Jug r 2 in walnut, etc.^{8,33} More importantly, MiAMP2 shares a high level of sequence homology up to 49% to Jug r 2 in walnut and 45% to Car i 2 in pecan, which is highly suggestive that the antigen of our Nbs is also a major allergen of macadamia.²⁹ The material shown on SDS-PAGE as a band of 60 kDa probably corresponded to the protein fragment between R77-F625, whereas the band of 50 kDa was predicted to be the R180-F625 fragment.³⁴ Remarkably, the protein of around 50 kDa has been identified recently as a new food allergen referred to as Mac i 1 in the IUIS/WHO allergen database (<http://www.allergen.org>). Therefore, our Nbs could be a potential tool to detect the presence of allergenic macadamia proteins in food supplies.

The 625 aa macadamia vicilin precursor is processed into smaller fragments upon seed imbibition, and proteins with a molecular mass of around 60 kDa and 50 kDa are expected to be formed and present on SDS-PAGE. In this view, the other visualized protein bands with the size between 35 and 40 kDa on the gel after immunocapturing by Nb81 could well be the remaining released precursor components.³⁴

Expression and Purification of Nbs with His-tag Only. MiAMP2 vicilin-like protein accounts for almost 20% of the

protein abundance in macadamia.²⁹ Thus, it is frequently employed for the detection of macadamia residuals.^{9,37} We therefore aimed to develop a sandwich ELISA detection strategy with the allergen-specific Nbs against vicilin-like proteins of macadamia. Thus, Nbs with His-tag only (as opposed to HA- and His-tag) were generated to serve as the antigen capturing Nbs. The Nb genes from pMECS (HA-His tagged) were transferred to pHEN6 expression vectors (for His-tag only expression). As described above, Nbs with His-tag only were purified by IMAC and SEC yielding about 20 mg/L culturing medium (Figure 5A). The purity and identity of the Nbs were analyzed by SDS-PAGE (Figure 5B) and Western blot (Figure 5C). Thereafter, an indirect ELISA was performed to confirm the high binding capacity of the purified only His-tagged Nbs (Figure 5D).

Nb-based Sandwich ELISA. A sandwich ELISA platform typically requires a pair of analyte-specific capturing and detecting antibodies that recognize (preferably) different epitopes on the targeted antigen. Therefore, the best pair of Nbs for the proposed heterologous sandwich ELISA was determined by using Nbs with His-tag only for antigen capturing and Nbs with both tags (HA and His) as the antigen-detection antibody. The P/N ratio, i.e. the positive (P, OD_{450 nm} of sample with macadamia protein) to negative (N, OD_{450 nm} of sample without macadamia protein) signal was used to reflect the detection response.³⁸ As shown in Figure 6A, the highest P/N ratio was obtained using Nb139H as the capturing agent and Nb68HA as the detection agent.

Next, we optimized the concentrations of the selected Nb pair for providing the most sensitive sandwich ELISA and using a minimal amount of Nb material. The P/N value giving the highest detection signal and lowest background was determined. The best conditions used 6 μ g/mL for Nb139H and 8 μ g/mL Nb68HA, as they produced the highest response signal and lowest background (Figure 6B).

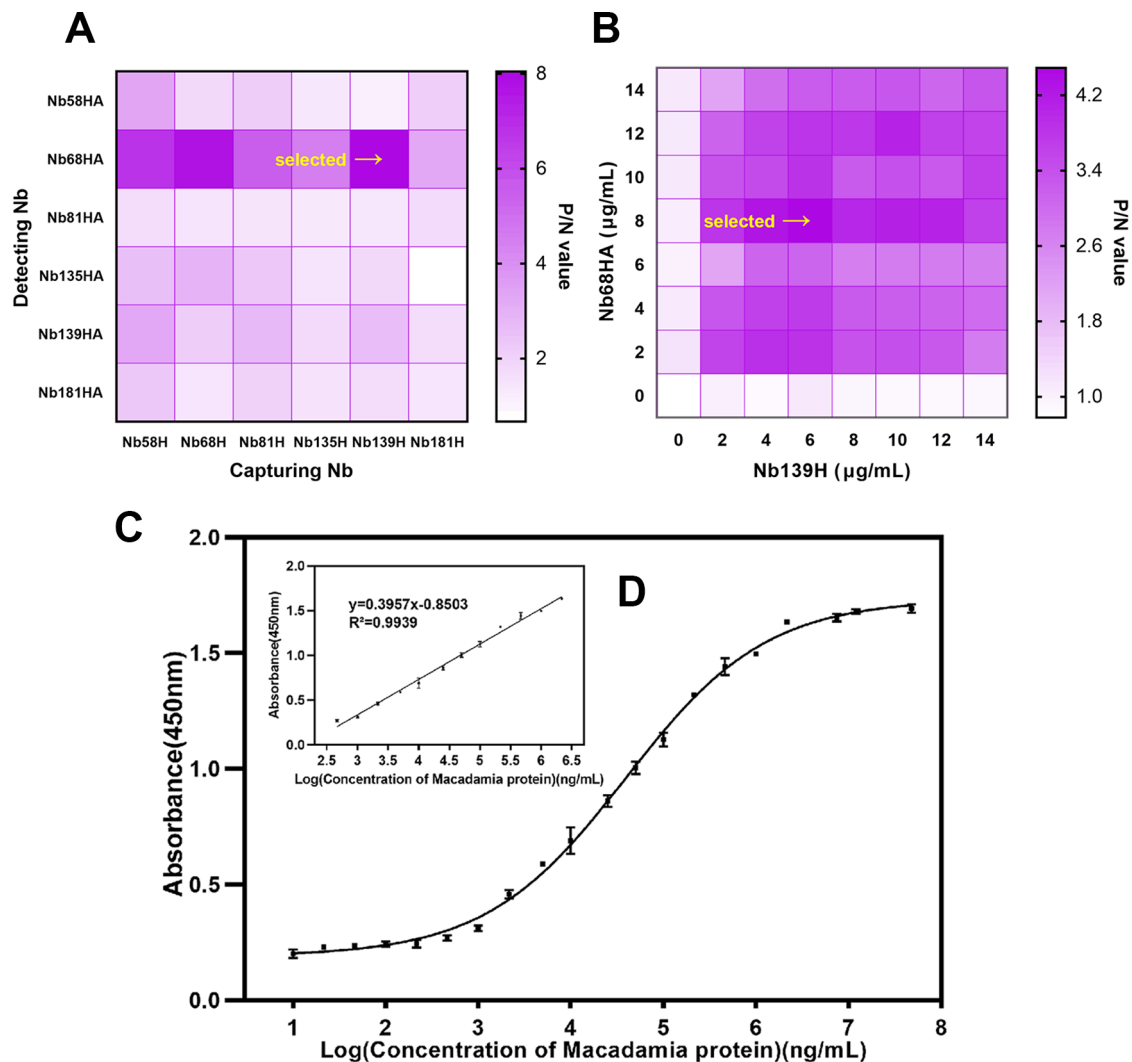


Figure 6. A sandwich ELISA method developed for macadamia protein detection. (A) Selection of the best pair of target capturing Nb and detection Nb according to P/N value. The Nb139H and Nb68HA pair was chosen as the best antigen capturing and detection Nb, respectively. (B) Optimization of the concentration of capture Nb139H and detection Nb68HA according to P/N value. Concentrations of 6 µg/mL and 8 µg/mL were chosen for capturing Nb139H and detection Nb68HA, respectively. (C) Calibration curve and (D) linear standard curve of optimized sandwich ELISA for macadamia protein detection. All data are expressed as the mean ± SD ($n = 3$). Each test was repeated at least three times.

Using these conditions, we evaluated the linear response and the LOD and LOQ of our Nb-based sandwich ELISA in detecting the macadamia allergen. A titration curve was generated for crude antigen preparations at concentrations between 10 to 4.8×10^7 ng/mL (Figure 6C). The fitted equation of the standard curve was $y = 0.3957 \log(x) - 0.8503$, with a correlation coefficient of 0.9939 (R^2 ; $n = 3$) (Figure 6D). The linear range of the assay was from 0.442 to 2800 µg/mL of crude protein extract, yielding a calculated LOD and LOQ of 27.1 ng/mL and 164.1 ng/mL, respectively. In summary, a novel immunoassay based on selected Nbs was established for the broad range quantification of macadamia allergen protein.

Analysis of Spiked Samples. The applicability of the established detection approach was evaluated by spiking various amounts of macadamia protein extracts in milk samples. The spiked samples were centrifuged to remove the cream layer and the pellet. The upper supernatant was collected and diluted 1000 times in PBS before testing in ELISA. As shown in Table 1, the recovery of the antigen was determined to reach between 87% to 91.9%, which provided

Table 1. Recoveries of Macadamia Protein in Food Sample Model Tested by Sandwich ELISA

food sample	spiked concentration (mg/mL)	detected concentration (mg/mL)	recovery ^a (%)	CV ^b (%)
skimmed milk	0	not detectable	—	—
	1	0.87 ± 0.11	87.0	11.0
	2	1.76 ± 0.21	88.0	10.5
	5	4.37 ± 0.34	87.4	6.8
	10	9.19 ± 0.63	91.9	6.3

^aEach assay was repeated three times; the result for recovery was the average of three replicates. ^bCV was the ratio of the standard deviation to the mean.

evidence for the reliability of the established Nb-based sandwich ELISA to detect macadamia allergen in a complex food matrix. Thus, the selected Nbs are applicable for the surveillance of residual macadamia components by detecting the allergen components of vicilin-like proteins.

DISCUSSION

The current study employed a strategy that we describe as an unbiased method to immunize and select target specific Nbs against crude proteins from macadamia. The capacity of the alpaca immune system to raise HCABs against the most immuno-dominant proteins was exploited. A similar technique was already successfully used to identify Nbs that recognized particular antigens on dendritic cells, *Brucella*, trypanosomes, and glioblastoma and also *Arabidopsis* seed proteins.^{39–41} The challenge with this technique is in the identification of the antigen. For this purpose, the easiest method is to use the Nbs to associate with their antigen and then to capture the Nb::antigen complex via the His-tag of the Nbs on Ni²⁺-NTA magnetic beads followed by SDS-PAGE and trypsinization of the captured proteins that are finally identified by LC-MS/MS. Actually, the unbiased approach was expected to generate Nbs against multiple targets within the crude protein extract. However, under the conditions we used, we only obtained binders to the most abundant proteins within the crude extract. By size fractionation of the crude macadamia extract and panning on the different fractions it should be possible to identify new binders to additional targets.^{29,42} More importantly, for macadamia protein extracts this unbiased immunization and selection strategy was anticipated to facilitate the identification of potential allergens from the general protein extract, which could be used in analytical detection methods to screen for allergen contaminations in foods.

After a successful construction of an immune Nb library, target-specific Nbs have been retrieved by phage display panning, and the cognate antigen of all the Nbs has been identified as an allergen protein from macadamia nut. Two bands enriched by Nb-based Co-IP were identified as MiAMP2. The vicilin protein is abundant in macadamia nuts serving as a seed storage protein, and it is often associated with allergic reactions described in the literature.^{35,43} One of the Nb target proteins has been identified recently as a novel food allergen named Mac i 1. Hence, MiAMP2 has the potential to serve as a marker for macadamia nut contamination. Meanwhile, the structural analysis and epitope mapping of the macadamia vicilin-like protein can further facilitate the interpretation of its preferential immune response after injection of the crude extract. Whereas, more research needs to be performed to select specific Nbs against the crude extract of other tree nuts to verify the relationship between the immunogenicity and protein structure.

Currently, the detection of macadamia allergen mainly depends on the PCR-based methods, and no immunoassay has been reported for the surveillance of the macadamia allergen of vicilin-like protein. Torricelli et al., designed primers for the *vicilin precursor* gene as the target to develop a fast real-time PCR. The practical LOD was determined as 0.006%.⁹ Ito et al. also reported a PCR-based detection technique with a detection limit of 1 pg of macadamia DNA.¹⁰ However, the residual level of vicilin-like protein is difficult to estimate by PCR-based detection results, due to the dynamic distribution of the contaminants. Generally, an immunoassay can facilitate the surveillance of the traceable macadamia allergen contamination in a food matrix with the marked characteristics of being straightforward, sensitive, and accurate when compared with the PCR-based methods.³⁴ In the current study, an immunoassay of heterologous sandwich ELISA has been

developed based on the retrieved Nbs. Whereas the determined parameters of the sandwich ELISA are based on the analysis against the crude protein extract, a more accurate description of the immunoassay could be provided by investigating the ELISA with purified macadamia vicilin-like proteins. The detection of antigen spiked in food samples has been performed to confirm the detection capacity in a complex food matrix. The current study on the design of sandwich ELISA for macadamia allergen reveals a general strategy to identify target-specific Nbs that could facilitate the further development of alternative, simple, and sensitive immune detection strategies for allergen surveillance.

In summary, the current study provided convincing evidence for the selection of specific Nbs against allergen protein after immunization and selection strategies using crude and complex proteins. The acquisition and identification of macadamia allergen protein-targeted Nbs demonstrated the successful application of an unbiased immunization and selection strategy. Nbs categorized in six families were retrieved, and all of them seem to recognize the same antigen, although with variable affinity. This confirmed the dominant allergen properties of the antigen. The cognate target antigen was identified via Co-IP, SDS-PAGE, and LC-MS/MS to coincide with the MiAMP2. A specific Nb-based sandwich ELISA was developed to provide a promising tool for the sensitive and specific detection of macadamia allergen, even when present in a complex food matrix. Furthermore, it is also imperative to focus on the development of a biosensor for a more rapid and accurate detection of macadamia allergen. Further studies should be undertaken to identify the exact binding epitope and on the structural characterization of the macadamia antigen Mac i 1.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c00390>.

(Table S1) Properties of selected Nbs; (Table S2) identification of the target antigen with the size around 60 kDa by LC-MS/MS; (Table S3) identification of the target antigen with the size around 50 kDa by LC-MS/MS; (Figure S1) construction of the immune Nb library; (Figure S2) target-specific VHH retrieved from the immune Nb library; (Figure S3) targeting of the Nbs to the heat-processed macadamia proteins; (Figure S4) thermal stability of selected Nbs; and (Figure S5) apparent binding affinity of selected Nbs (PDF)

AUTHOR INFORMATION

Corresponding Author

Shuo Wang – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China; orcid.org/0000-0003-0910-6146; Phone: +86 22 85358445; Email: wangshuo@nankai.edu.cn

Authors

Yaozhong Hu – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China; orcid.org/0000-0002-6228-9220

Sihao Wu – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China

Yi Wang – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China

Jing Lin – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China

Ying Sun – College of Food Science and Engineering, Tianjin University of Science & Technology, Tianjin 300457, China

Chuan Zhang – College of Food Science and Engineering, Tianjin University of Science & Technology, Tianjin 300457, China

Jiaxin Gu – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China

Feier Yang – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China

Huan Lv – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China

Xuemeng Ji – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China

Yan Zhang – Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China; orcid.org/0000-0003-3374-0868

Serge Muyldermans – Cellular and Molecular Immunology, Vrije Universiteit Brussel, 1050 Brussels, Belgium

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acs.jafc.1c00390>

Author Contributions

[†]These authors (Y. Hu and S. Wu) contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (31901779).

REFERENCES

- (1) Clarke, A.; Jones, J.; Sirirat, R.; LeBeau, K.; Heskey, C.; Oda, K.; Rajaram, S.; Sabaté, J. Daily Macadamia Nut Intake and Its Effect on Macronutrient Intake and Nutrient Displacement in Overweight and Obese Adults. *Curr. Dev. Nutr.* **2020**, *4*, 1622–1622.
- (2) Griel, A. E.; Cao, Y.; Bagshaw, D. D.; Cifelli, A. M.; Holub, B.; Kris-Etherton, P. M. A Macadamia Nut-Rich Diet Reduces Total and LDL-Cholesterol in Mildly Hypercholesterolemic Men and Women. *J. Nutr.* **2008**, *138*, 761–767.
- (3) INC (International Nut and Dried Fruits). Nuts and Dried Fruits. *Statistical Yearbook* 2019/2020.
- (4) McWilliam, V.; Koplin, J.; Lodge, C.; Tang, M.; Dharmage, S.; Allen, K. The Prevalence of Tree Nut Allergy: A Systematic Review. *Curr. Allergy Asthma Rep.* **2015**, *15*, 54.
- (5) Yoshida, K.; Shirane, S.; Kinoshita, K.; Furukawa, M.; Omura, Y.; Kawaguchi, E.; Matsushita, S.; Toda, M.; Nakajima-Adachi, H.; Akasawa, A. Clinical features and allergen analysis in five children with macadamia nut allergy. *J. Allergy Clin. Immunol.* **2018**, *141*, AB252.

(6) Robert-Sullerot, I.; Moneret-Vautrin, D. A. Anaphylaxie à la noix de macadamia (*Macadamia integrifolia*): à propos de deux cas. *Rev. Fr. Allergol.* **2013**, *53*, 639–641.

(7) Sicherer, S. H.; Sampson, H. A. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *J. Allergy Clin. Immunol.* **2014**, *133*, 291–307.

(8) Crespo, J. F.; James, J. M.; Fernandez-Rodriguez, C.; Rodriguez, J. Food allergy: nuts and tree nuts. *Br. J. Nutr.* **2006**, *96*, S95–102.

(9) Torricelli, M.; Pierboni, E.; Rondini, C.; Altissimi, S.; Haouet, N. Sesame, Pistachio, and Macadamia Nut: Development and Validation of New Allergenic Systems for Fast Real-Time PCR Application. *Foods* **2020**, *9*, 1085.

(10) Ito, M.; Mizota, T.; Kitaguchi, T.; Ohno, K.; Ohba, T.; Tanaka, M. Simultaneous detection of eight species of tree nut in foods using two tetraplex polymerase chain reaction assays. *Biosci., Biotechnol., Biochem.* **2018**, *82*, 1985–1991.

(11) Hassanzadeh-Ghassabeh, G.; Devoogdt, N.; De Pauw, P.; Vincke, C.; Muyldermans, S. Nanobodies and their potential applications. *Nanomedicine* **2013**, *8*, 1013–1026.

(12) Muyldermans, S. A guide to: generation and design of nanobodies. *FEBS J.* **2021**, *288*, 2084–2102.

(13) Mitchell, L. S.; Colwell, L. J. Comparative analysis of nanobody sequence and structure data. *Proteins: Struct., Funct., Genet.* **2018**, *86*, 697–706.

(14) Muyldermans, S.; Baral, T. N.; Retamozzo, V. C.; De Baetselier, P.; De Genst, E.; Kinne, J.; Leonhardt, H.; Magez, S.; Nguyen, V. K.; Revets, H.; Rothbauer, U.; Stijlemans, B.; Tillib, S.; Wernery, U.; Wyns, L.; Hassanzadeh-Ghassabeh, G.; Saerens, D. Camelid immunoglobulins and nanobody technology. *Vet. Immunol. Immunopathol.* **2009**, *128*, 178–83.

(15) Ingram, J. R.; Schmidt, F. I.; Ploegh, H. L. Exploiting Nanobodies' Singular Traits. *Annu. Rev. Immunol.* **2018**, *36*, 695–715.

(16) Muyldermans, S. Nanobodies: Natural Single-Domain Antibodies. *Annu. Rev. Biochem.* **2013**, *82*, 775–797.

(17) De Meyer, T.; Muyldermans, S.; Depicker, A. Nanobody-based products as research and diagnostic tools. *Trends Biotechnol.* **2014**, *32*, 263–70.

(18) Salvador, J. P.; Vilaplana, L.; Marco, M. P. Nanobody: outstanding features for diagnostic and therapeutic applications. *Anal. Bioanal. Chem.* **2019**, *411*, 1703–1713.

(19) Khodabakhsh, F.; Behdani, M.; Rami, A.; Kazemi-Lomedasht, F. Single-Domain Antibodies or Nanobodies: A Class of Next-Generation Antibodies. *Int. Rev. Immunol.* **2018**, *37*, 316–322.

(20) Carrillo, W.; Lara, D.; Vilcacundo, E.; Carrillo, C.; Silva, M.; Alvarez, M.; Carpio, C. Obtention of protein concentrate and polyphenols from Macadamia (*Macadamia integrifolia*) with aqueous extraction method. *Asian J. Pharm. Clin. Res.* **2017**, *10*, 138.

(21) Ghorbani, M.; Morgan, M. R. A. Protein characterisation and immunochemical measurements of residual macadamia nut proteins in foodstuffs. *Food Agric. Immunol.* **2010**, *21*, 347–360.

(22) Bora, P. S.; Ribeiro, D. Note: Influence of pH on the Extraction Yield and Functional Properties of Macadamia (*Macadamia Integrifolia*) Protein Isolates. *Food Sci. Technol. Int.* **2004**, *10*, 263–267.

(23) Pardon, E.; Laeremans, T.; Triest, S.; Rasmussen, S. G. F.; Wohlfkönig, A.; Ruf, A.; Muyldermans, S.; Hol, W. G. J.; Kobilka, B. K.; Steyaert, J. A general protocol for the generation of Nanobodies for structural biology. *Nat. Protoc.* **2014**, *9*, 674–693.

(24) Desmyter, A.; Spinelli, S.; Roussel, A.; Cambillau, C. Camelid nanobodies: killing two birds with one stone. *Curr. Opin. Struct. Biol.* **2015**, *32*, 1–8.

(25) Kazemi-Lomedasht, F.; Behdani, M.; Habibi-Anbouhi, M.; Shahbazzadeh, D. Production and Characterization of Novel Camel Single Domain Antibody Targeting Mouse Vascular Endothelial Growth Factor. *Monoclonal Antibodies Immunodiagn. Immunother.* **2016**, *35*, 167–171.

(26) Reinhard, L.; Mayerhofer, H.; Geerlof, A.; Mueller-Dieckmann, J.; Weiss, M. S. Optimization of protein buffer cocktails using

Thermofluor. *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.* **2013**, *69*, 209–214.

(27) Shevchenko, A.; Tomas, H.; Havli, J.; Olsen, J. V.; Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **2006**, *1*, 2856–2860.

(28) *Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics*, 2nd ed.; Magnusson, B.; Örnemark, U., Eds.; 2014.

(29) Rost, J.; Muralidharan, S.; Lee, N. A. A label-free shotgun proteomics analysis of macadamia nut. *Food Res. Int.* **2020**, *129*, 108838.

(30) Ribeiro, M.; Costa, J.; Mafra, I.; Cabo, S.; Silva, A. P.; Goncalves, B.; Hillion, M.; Hebraud, M.; Igrejas, G. Natural Variation of Hazelnut Allergenicity: Is There Any Potential for Selecting Hypoallergenic Varieties? *Nutrients* **2020**, *12*, 2100.

(31) Kong, X.; Zhang, L.; Lu, X.; Zhang, C.; Hua, Y.; Chen, Y. Effect of high-speed shearing treatment on dehulled walnut proteins. *Lwt* **2019**, *116*, 108500–108510.

(32) Sathe, S. K.; Liu, C.; Zaffran, V. D. Food Allergy. *Annu. Rev. Food Sci. Technol.* **2016**, *7*, 191–220.

(33) Roux, K. H.; Teuber, S. S.; Sathe, S. K. Tree nut allergens. *Int. Arch. Allergy Immunol.* **2003**, *131*, 234–44.

(34) Marcus, J. P.; Green, J. L.; Goulter, K. C.; Manners, J. M. A family of antimicrobial peptides is produced by processing of a 7S globulin protein in *Macadamia integrifolia* kernels. *Plant J.* **1999**, *19*, 699–710.

(35) Zhang, J.; Payne, C. D.; Pouvreau, B.; Schaefer, H.; Fisher, M. F.; Taylor, N. L.; Berkowitz, O.; Whelan, J.; Rosengren, K. J.; Mylne, J. S. An Ancient Peptide Family Buried within Vicilin Precursors. *ACS Chem. Biol.* **2019**, *14*, 979–993.

(36) Dall'antonia, F.; Pavkov-Keller, T.; Zangger, K.; Keller, W. Structure of allergens and structure based epitope predictions. *Methods* **2014**, *66*, 3–21.

(37) Brežná, B.; Piknová, L.; Kuchta, T. A novel real-time polymerase chain reaction method for the detection of macadamia nuts in food. *Eur. Food Res. Technol.* **2009**, *229*, 397–401.

(38) Schocker, F.; Scharf, A.; Kull, S.; Jappe, U. Detection of the Peanut Allergens Ara h 2 and Ara h 6 in Human Breast Milk: Development of 2 Sensitive and Specific Sandwich ELISA Assays. *Int. Arch. Allergy Immunol.* **2017**, *174*, 17–25.

(39) Abbady, A. Q.; Al-Daoude, A.; Al-Mariri, A.; Zarkawi, M.; Muyldermans, S. Chaperonin GroEL a *Brucella* immunodominant antigen identified using Nanobody and MALDI-TOF-MS technologies. *Vet. Immunol. Immunopathol.* **2012**, *146*, 254–63.

(40) Jovcevska, I.; Zupanec, N.; Kocevar, N.; Cesselli, D.; Podergajs, N.; Stokin, C. L.; Myers, M. P.; Muyldermans, S.; Ghassabeh, G. H.; Motaln, H.; Ruaro, M. E.; Bourkoulou, E.; Turnsek, T. L.; Komel, R. TRIM28 and beta-actin identified via nanobody-based reverse proteomics approach as possible human glioblastoma biomarkers. *PLoS One* **2014**, *9*, e113688.

(41) De Meyer, T.; Eeckhout, D.; De Rycke, R.; De Buck, S.; Muyldermans, S.; Depicker, A. Generation of VHH antibodies against the *Arabidopsis thaliana* seed storage proteins. *Plant Mol. Biol.* **2014**, *84*, 83–93.

(42) Li, Z.; Pinto Torres, J. E.; Goossens, J.; Vertommen, D.; Caljon, G.; Sterckx, Y. G.; Magez, S. An Unbiased Immunization Strategy Results in the Identification of Enolase as a Potential Marker for Nanobody-Based Detection of *Trypanosoma evansi*. *Vaccines (Basel, Switz.)* **2020**, *8*, 415.

(43) Ehlers, A. M.; Rohwer, S.; Otten, H. G.; Brix, B.; Le, T. M.; Suer, W.; Knulst, A. C. IgE-binding to vicilin-like antimicrobial peptides is associated with systemic reactions to macadamia nut. *Clin. Transl. Allergy* **2020**, *10*, 55.

Supporting Information

Unbiased immunization strategy yielding specific Nanobodies against Macadamia allergen of vicilin-like protein for immuno-assay development

Yaozhong Hu^{1, #}, Sihao Wu^{1, #}, Yi Wang¹, Jing Lin¹, Ying Sun², Chuan Zhang², Jiaxin Gu¹, Feier Yang¹, Huan Lv¹, Xuemeng Ji¹, Yan Zhang¹, Serge Muyldermans³, Shuo Wang^{1, *}

1. Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China.
2. College of Food Science and Engineering, Tianjin University of Science & Technology, Tianjin 300457, China.
3. Cellular and Molecular Immunology, Vrije Universiteit Brussel, 1050 Brussels, Belgium.

[#]: These authors (Y. Hu and S. Wu) contribute equally to this work.

^{*}: Corresponded to Shuo Wang (wangshuo@nankai.edu.cn)

Table S1 Properties of selected Nbs

	Nb58	Nb68	Nb81	Nb135	Nb139	Nb181
MW/kDa	16.05	14.43	15.73	15.75	15.91	15.67
pI	7.65	8.65	9.25	7.14	8.20	7.66
Tm value/°C	67.49 ± 0.06	66.75 ± 0.07	82.03 ± 0.16	60.12 ± 0.15	67.82 ± 0.06	59.11 ± 0.05
Affinity/nM	119.8 ± 23.6	7.531 ± 3.11	11.05 ± 4.57	142.3 ± 12.6	9.23 ± 4.82	86.05 ± 24.95

Results are represented as mean ± SD (n = 3). Repeated at least 3 times for every test.

Table S2 Identification of the target antigen with the size around 60 kDa by LC-MS/MS

Sequence	PSMs	Protein groups	Protein group accessions	XCorr	Charge	MH+ [Da]	Δ M [ppm]	RT [min]
FLQTISTPGQYK	1	3	AAD54246.1; AAD54245.1; AAD54244.1	3.57	2	1382.72576	-4.14	117.18
HcEQQEPRQLQYQcQRR	1	1	AAD54246.1	2.66	3	2215.99765	-9.94	118.07
qRDPQQQYEQcQKR	1	1	AAD54246.1	2.60	2	1933.88494	-4.37	117.58
rHcEQQEPRQLQYQcQR	1	1	AAD54246.1	2.52	3	2258.00992	-9.00	118.64
rHEEEEEVHYEQVR	1	1	AAD54246.1	2.13	2	1910.85564	-3.75	119.42
qSDNPYYFDERSLSTRFR	1	2	AAD54246.1; AAD54245.1	2.06	2	2323.08135	3.22	119.31
RHcEQQEPRQLQYQcQR	1	1	AAD54246.1	2.05	2	2216.00273	-7.65	119.29
gGDLINPQRGGSGRYEEGEEK	1	1	AAD54245.1	2.73	3	2290.07114	0.69	117.57
nVLQQIEPQAmELAFAPRK	1	1	AAD54245.1	2.62	3	2312.21006	1.39	119.94

Note:

PSMs: peptide sequence matches.

MH+ [Da]: protonated molecular weight.

Δ M [ppm]: mass shift.

RT [min]: Retention time.

Table S3 Identification of the target antigen with the size around 50 kDa by LC-MS/MS

Sequence	PSMs	Protein groups	Protein group accessions	XCorr	Charge	MH+ [Da]	ΔM [ppm]	RT [min]
EILEAALNTQTER	1	1	AAD54246.1	4.21	2	1487.77654	4.34	118.22
FLQTISTPGQYK	1	2	AAD54246.1; AAD54245.1	2.91	2	1382.72222	-6.70	117.10
HEEEEEVHYEQVR	3	1	AAD54246.1	2.66	3	1712.75791	3.96	82.29
YGQAYEVKPEDYR	1	2	AAD54246.1; AAD54245.1	2.41	2	1617.76323	5.46	114.36
TEEGHISVLENFYGR	1	2	AAD54246.1; AAD54245.1	2.41	2	1750.84416	2.63	118.79

Note:

PSMs: peptide sequence matches.

MH+ [Da]: protonated molecular weight.

ΔM [ppm]: mass shift.

RT [min]: Retention time.

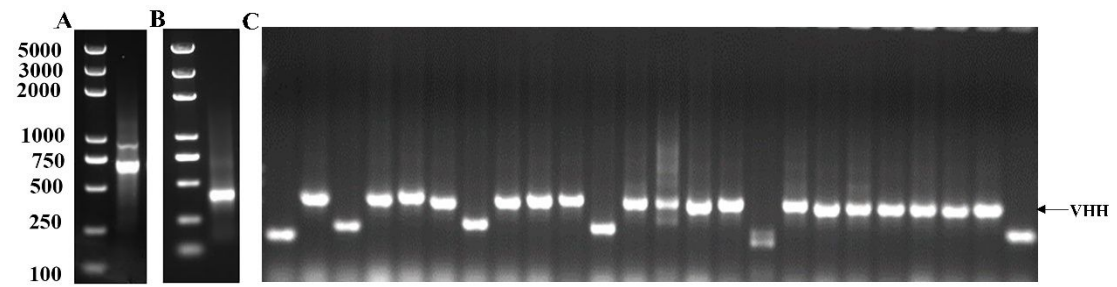


Figure S1. Construction of the immune Nb library.

(A) Fragments amplified after 1st PCR. (B) VHH fragments amplified after 2nd PCR.

(C) Proper insert percentage of VHH was determined via colony PCR.

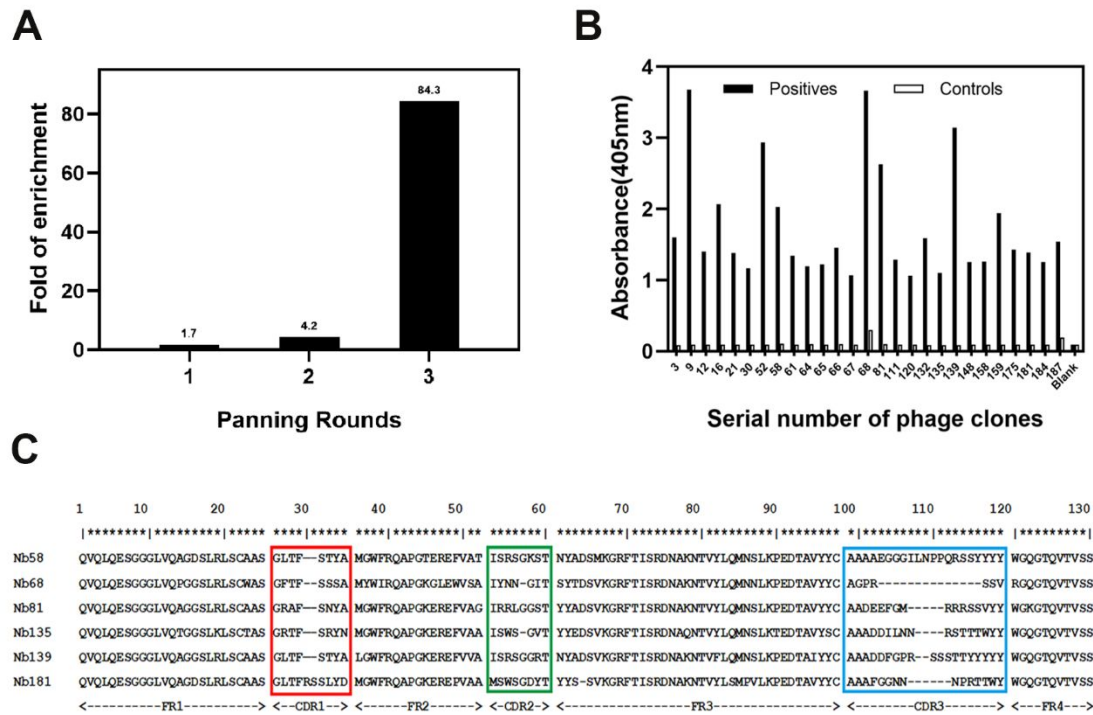


Figure S2. Target-specific VHH retrieved from the immune Nb library.

(A) Macadamia protein specific VHHs were enriched after each round of panning in comparison to control wells without antigen coated. (B) PE-ELISA for 190 colonies after three-rounds of panning. Only the colonies are shown whose absorbance signal is at least 2-fold higher than the signal of negative controls (no antigen coated in wells). (C) Six different amino acid sequences of macadamia protein-specific VHHs were identified. The amino acid sequences of the nanobodies were aligned and their antigen-binding loops or complementarity determining regions (CDR) and framework regions (FR) are indicated.

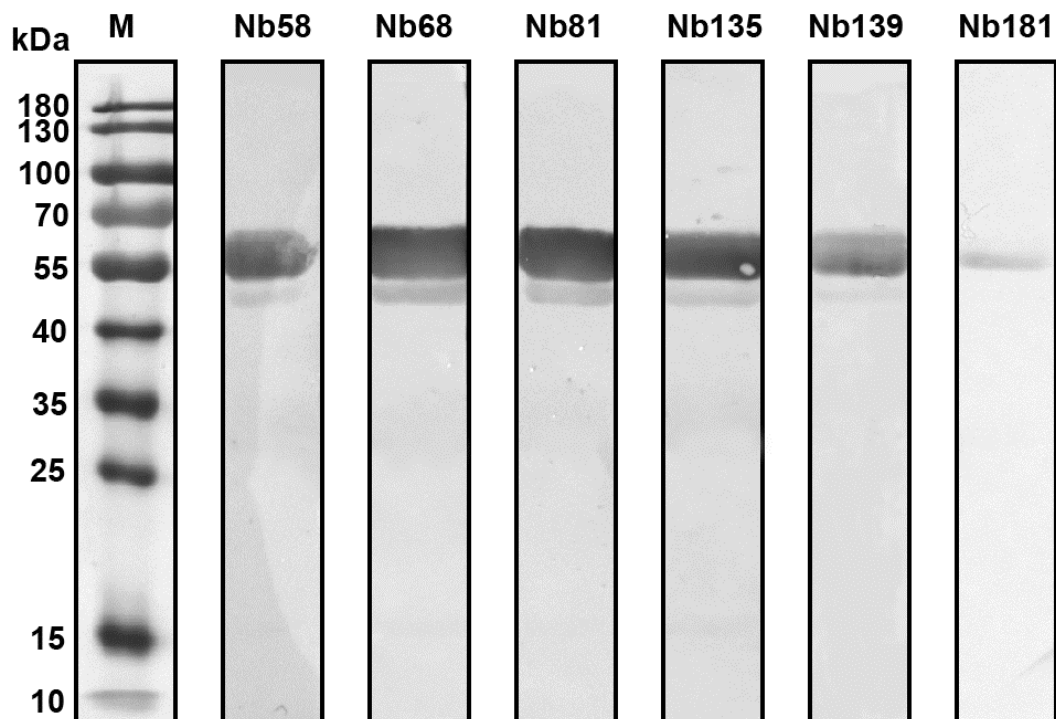


Figure S3. Targeting of the Nbs to the heat-processed macadamia proteins.

Antigen proteins undergoing heat-processing were separated under non-reducing conditions by SDS-PAGE, transferred to nitrocellulose and probed separately with each of the six Nbs.

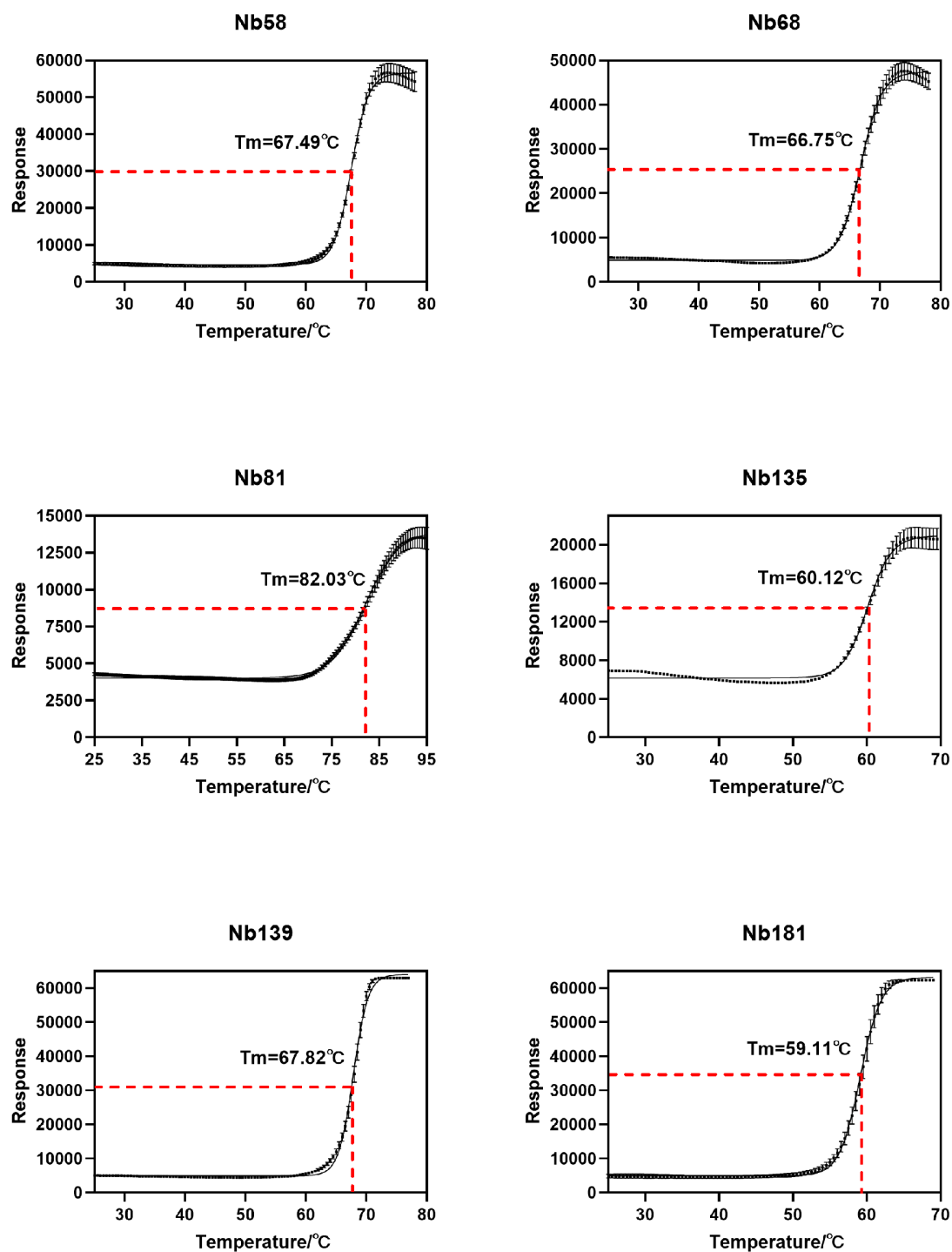


Figure S4. Thermal stability of selected Nbs.

All data plotted are expressed as mean $\pm$ SD ($n = 3$). Repeated at least 3 times for every test.

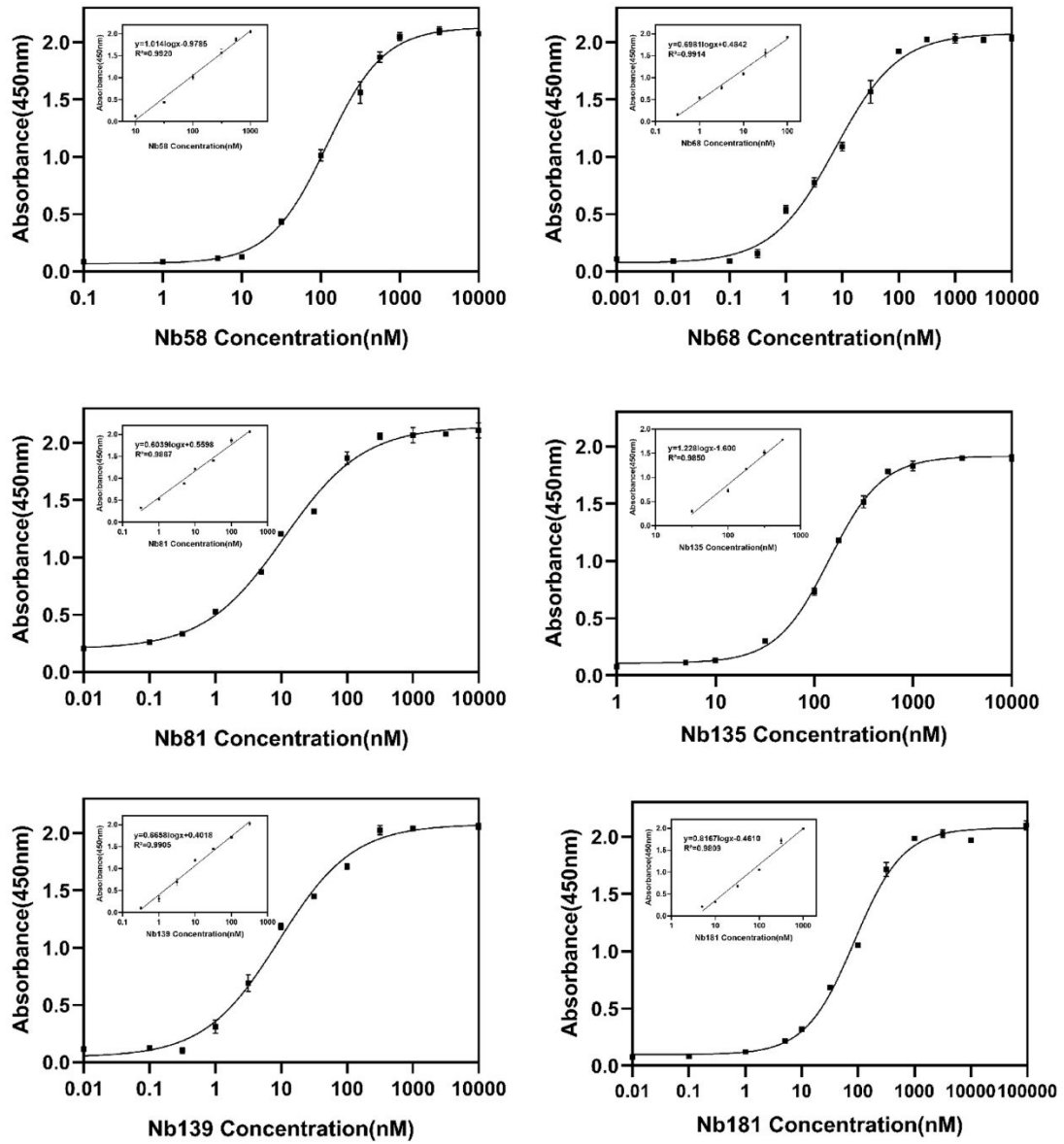


Figure S5. Apparent binding affinity of selected Nbs.

All data plotted are expressed as mean $\pm$ SD ($n = 3$). Repeated at least 3 times for every test.