



Simplified Tracking of a Soy Allergen in Processed Food Using a Monoclonal Antibody-Based Sandwich ELISA Targeting the Soybean 2S Albumin Gly m 8

Elke Ueberham,^{*,†,‡} Holger Spiegel,[‡] Heide Havenith,[‡] Paul Rautenberger,[†] Norbert Lidzba,[†] Stefan Schillberg,[‡] and Jörg Lehmann[†]

[†]Fraunhofer Institute for Cell Therapy and Immunology IZI, Perlickstraße 1, 04103 Leipzig, Germany

[‡]Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Forckenbeckstrasse 6, 52074 Aachen, Germany

Supporting Information

ABSTRACT: Soybean allergens in food samples are currently detected in most cases using enzyme-linked immunosorbent assays (ELISAs) based on antibodies raised against bulk soybean proteins or specifically targeting soybean trypsin inhibitor, conglycinin, or glycinin. The various commercial ELISAs lack standardized reference material, and the results are often inaccurate because the antibodies cross-react with proteins from other legumes. Furthermore, the isolation of allergenic proteins involves laborious denaturing extraction conditions. To tackle these challenges, we have developed a novel sandwich ELISA based on monoclonal antibodies raised against the soybean 2S albumin Gly m 8 and a recombinant Gly m 8 reference protein with native-analogous characteristics. The antibodies do not cross-react with other legume proteins, and the extraordinary stability and solubility of Gly m 8 allows it to be extracted even from complex matrices after processing. The Gly m 8 ELISA therefore achieves greater specificity and reproducibility than current ELISA tests.

KEYWORDS: soy bean, Gly m 8, 2S albumin, recombinant calibrator, ELISA, monoclonal antibodies, allergen detection, processed food

INTRODUCTION

Soy allergy is among the eight most common forms of food allergy, and in severe cases it can trigger life-threatening anaphylaxis. The increasing use of soy flour and soy protein as food additives means that vigilance is increasingly necessary to exclude unintentional entering of soybean (*Glycine max*) allergens from the diet, and this requires accurate and sensitive test methods. Soy allergy can be divided in both mild forms related to food-pollen allergy syndrome mainly caused by the soy allergen Gly m 4 and substantial food allergy resulting from sensitization against the main storage proteins Gly m 5 and Gly m 6 and the minor storage protein Gly m 8. Recently, the estimation of sensitization level against a combination of Gly m 5 and Gly m 8 for diagnosing soy allergy in children was suggested, emphasizing the great significance of these two allergenic soy proteins.¹

Gly m 4 is a PR10 protein, which represents a homologue of birch pollen allergen Bet-v1 and is a water-soluble molecule with weak resistance against heat, acids, and proteases. The physicochemical properties of Gly m 4 drive its loss during manufacturing processes of soy in processed food, for example, roasting, acid precipitation, or pasteurization. Therefore, processed food can scarcely be characterized by Gly m 4 content. Other allergenic proteins of soy include Gly m 1 and Gly m 2, which are hull proteins responsible for severe allergic Barcelona asthma outbreaks.² Peeling of soybeans, however, removes hull proteins in modern food processing. Therefore, they play a minor role in allergen detection of processed food. Other allergenic soy proteins are Gly m 7³ and Gly m Bd28K,⁴ Gly m Bd30K,⁵ and Gly m Bd39K.⁶

Currently, the detection of soy protein in food is realized in most cases using enzyme-linked immunosorbent assays (ELISAs) commonly based on polyclonal antibodies raised against whole soybean protein extracts or isolated components that are particularly stable.⁷ Over decades, intensive effort has been made to detect allergenic soy proteins in food. An impressive number of ELISAs for these components have been published,^{8–15} but all currently commercially available soy ELISA kits use antibodies that detect one of three soybean proteins: trypsin inhibitor (Gly m TI) or the abundant storage proteins conglycinin (Gly m 5) and glycinin (Gly m 6).¹¹ However, often the antibodies are not particularly specific, and false positive results might occur. For example, the Gly m TI antibody is unable to distinguish trypsin inhibitors from soybeans and other bean species, and the Gly m 6 antibody cross reacts with pea (*Pisum sativum*) storage proteins.¹² Furthermore, the extraction of these allergens requires harsh denaturing conditions, which can precipitate the proteins and prevent their detection, resulting in false negative results. Reliable extraction methods are very laborious. That applies to extraction procedures for detection of allergens by mass spectrometry too. Furthermore, mass spectrometry approaches are currently less established compared to allergen detection by ELISA¹⁶ or polymerase chain reaction (PCR).¹⁷ Since PCR targets DNA of the allergen source and not the allergenic protein

Received: April 30, 2019

Revised: July 9, 2019

Accepted: July 12, 2019

Published: July 12, 2019



by itself, PCR based methods are not relevant to assessing highly processed protein isolates and concentrates containing hardly detectable DNA.

Referring to a sophisticated extraction procedure prepared for food proteins, a more suitable target allergen is required for reliable detection by ELISA.

Gly m 8 is a soybean 2S albumin. This protein has not been used before in the context of allergen detection, but it is the best known predictor of severe soy allergy in children.¹⁸ The three-dimensional structure of 2S albumins is considered highly allergenic^{19,20} together with the thermal stability of this protein family and its resistance to complete digestion.²¹ Gly m 8 is also highly soluble in water and low-salt buffers, thus it is easier to extract even from complex food matrices and processed food samples compared to Gly m 5 and Gly m 6, making it particularly suitable for ELISA-based tests. For example, we recently established a sandwich ELISA based on antibodies against Gly m 5,²² but the quantities of native and denatured Gly m 5 extracted from complex matrices and processed food using different extraction methods varied significantly, often underestimating the true Gly m 5 content.

Here, we set out to develop an ELISA based on antibodies specific for Gly m 8 and to confirm the ability of the assay to detect traces of soy proteins in food. We used recombinant Gly m 8 protein as a calibration standard and monoclonal antibodies to ensure the reproducibility of the assay. Detailed characterization of the activity and binding parameters of the antibodies using surface plasmon resonance (SPR) spectroscopy provided effective quality control of the test components. To the best of our knowledge, this is the first time that Gly m 8, which represents a soy storage molecule with a complex maturation cycle,²³ has been used as a target for the ELISA-based detection of soy protein in food products.

MATERIALS AND METHODS

Recombinant Gly m 8 Protein. A synthetic gene coding for the Gly m 8 precursor (UniProt ID P19594) amino acids M1 to D158, including the signal peptide, the pro-peptide, and a 3'-terminal His₆-tag, was codon optimized for *Nicotiana benthamiana* by Geneart (Invitrogen, Carlsbad, CA, USA). The synthetic gene was introduced into the binary plant expression vector pTRAKT-ER²⁴ at the *NcoI*/*BamHI* sites. The final construct pTRAKT_Gly m 8 was verified by sequencing. The pTRAKT_Gly m 8 vector was propagated in *Escherichia coli* DH5α cells (New England Biolabs, Frankfurt/Main, Germany). Plasmid DNA was purified and introduced into electrocompetent *Agrobacterium tumefaciens* cells for transient expression in *N. benthamiana* plants as previously described.²⁵

Purification of Recombinant Gly m 8. The Gly m 8 protein was extracted from leaf tissue and isolated by immobilized metal ion affinity chromatography (IMAC) as previously described.²⁵ The Gly m 8 protein was purified by size exclusion chromatography (SEC) using a Superdex75 16/60 column (GE Healthcare, Freiburg, Germany). The integrity and purity of the recombinant Gly m 8 protein was verified by sodiumdodecylsulfate polyacrylamide electrophoresis (SDS-PAGE) and liquid chromatography/mass spectrometry (LC/MS-MS).

Generation of Monoclonal Antibodies. Mouse anti-Gly m 8 monoclonal antibodies were generated by immunizing female BALB/c mice (Janvier Laboratories, Le Genest-Saint-Is, France) with the recombinant Gly m 8 protein described above. The immunization experiments were approved by the State Animal Care and Use Committee (Landesdirektion Sachsen, Leipzig, Germany, V 07/14) and were carried out in accordance with the European Communities Council Directive (86/609/EEC) for the Care and Use of Laboratory Animals. Splenocytes were isolated from the mouse with the highest antibody titer specific for Gly m 8 and were fused to X63.Ag8.653 myeloma cells (ACC 43, DSMZ, Braunschweig, Germany). Hybridoma

supernatants were screened by indirect ELISA on flat-bottom high protein-binding capacity 96-well ELISA plates (Nunc MaxiSorp, Thermo Fisher Scientific, Darmstadt, Germany) coated with either recombinant protein (2 µg/mL) or whole soy extract (10 µg/mL). Cross-reactivity to total protein extracts from other legumes, namely peas, lupin (*Lupinus albus*), peanuts (*Arachis hypogaea*), and different beans and nuts, as indicated in Figure 2B, was tested by indirect ELISA using 10 µg/mL seed protein extracts.

SPR Spectroscopy. Eleven IgG-positive clones were selected for SPR analysis on covalently coupled purified recombinant Gly m 8 protein using a Biacore T200 SPR biosensor instrument (GE Healthcare) as previously described, based on an Fc-specific antibody capture system.²⁶ The most promising Gly m 8-specific monoclonal antibodies anti-Gly m 8-3 (mAb3) and anti-Gly m 8-8 (mAb8) were used for calibration and further testing as described below.

Calibration-Free Concentration Analysis (CFCA). CFCA²⁷ was used to determine the active concentration of recombinant Gly m 8 using a Biacore T200 instrument and a CM5-S-Series sensor chip with recombinant Protein A prepared as previously described.²⁸ The measurements were performed at 25 °C in HBS-EP running buffer (10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 150 mM NaCl, 3 mM EDTA, 0.005% (w/v) Tween 20). The surface was regenerated by pulsing with 30 mM HCl for 1 min.

To ensure a rapid initial binding rate, 2500 response units (RU) of mAb3 were captured in each assay step. Purified recombinant Gly m 8 was used at three different dilutions (1/3000, 1/4500, and 1/6000) to ensure an initial binding rate (IBR) between 0.5 and 5 RU/s at a flow rate of 5 µL/min. The IBR was measured at 5 and 100 µL/min using double referencing. The antigen-specific antibody concentration was determined using the CFCA module of the Biacore T200 Evaluation Software (GE Healthcare). The binding model was based on a molecular weight of 16 000 kDa and a diffusion coefficient of 9.16×10^{-11} m²/s.

Kinetic Analysis. The kinetic properties of mAb3 and mAb8 were determined using the Biacore T200 instrument. We captured 500 RU of mAb8 on an CM5 chip prepared with a mouse antibody capture kit (GE Healthcare), whereas mAb3 was captured on a Protein A surface prepared as described elsewhere.²⁸ To determine the kinetic binding constants, purified recombinant Gly m 8 was injected at a flow rate of 30 µL/min for 150 s (mAb8) or 180 s (mAb3), followed by dissociation for 900 s (mAb8) or 400 s (mAb3). Gly m 8 was used at CFCA-based concentrations of 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 nM. Between measurements, the surface was regenerated by pulsing for 1 min with 10 mM glycine/HCl. Buffer injections were used for double referencing. Binding curves were evaluated based on a 1:1 binding model using the Biacore T200 Evaluation Software.

To confirm the simultaneous binding of mAb3 and mAb8, mAb3 was captured on a Protein A-functionalized surface and saturated with recombinant Gly m 8. Then, mAb8 was injected to confirm the binding of mAb8 to Gly m 8 captured by mAb3.

ELISA. Gly m 8 was quantified by sandwich ELISA using mAb3 and mAb8. The capture antibody (mAb3) was immobilized onto 96-well plates (Nunc MaxiSorp) in 0.5 M carbonate buffer at 4 °C overnight. The plates were washed three times with phosphate-buffered saline (PBS) containing 154 mM NaCl and 0.05% Tween-20 (PBS-T) and blocked with Superblock blocking reagent (Thermo Fisher Scientific) for 1 h at room temperature. The plates were then sealed (Candor BioScience, Wangen, Germany), air-dried, shrink-wrapped, and stored at room temperature.

Both extracted samples and recombinant Gly m 8 standards were incubated for 10 min at room temperature in duplicate in PBS-T, Superblock mixture (ThermoFisher, Massachusetts). After three washes in PBS-T, the horseradish peroxidase (HRP)-conjugated detection antibody (mAb8) was added, and the plates were incubated for 10 min at room temperature. HRP activity was determined after three further washes in PBS-T by incubating the plate with 3,3',5,5'-tetramethylbenzidine (TMB-E) substrate (DUNN Labortechnik, Asbach, Germany). The yellow color generated by acidification with 0.5 M sulfuric acid represented the quantity of bound detection antibodies and was measured at 450 nm relative to a calibration curve consisting of

eight known concentrations of pure Gly m 8. The ELISA was verified according to AOAC guidelines, Appendix M,²⁹ and DIN ISO11843-5.

The limit of detection (LOD) was determined by measuring eight different concentrations of purified recombinant Gly m 8 in extraction buffer. Recovery was calculated by spiking five different concentrations of recombinant Gly m 8 into three different matrices relevant for processed soy: almond-wheat muffin, rice cookie, and minced boiled sausage. The LOD and limit of quantification (LOQ) were calculated by checking sensitivity and specificity using the methodology for linear and nonlinear calibration (ISO 11843-5:2008). The ability of the anti-Gly m 8 sandwich ELISA to resist changes in results due to minor deviations in the experimental procedure was tested by deviations in time (two times $\pm$ 10% of recommended time of 10 min), volume (two volumes $\pm$ 10% of set volume 100 μ L), and temperature (ambient temperature, 20, 28, and 37 $^{\circ}$ C). Furthermore, two different individuals performed the test on three different days.

The specificity of the antibodies was tested by spiking the ELISA with recombinant Gly m 4,³⁰ Gly m TI (Fraunhofer IME), and native Gly m 6, Gly m 5, and Kunitz inhibitor (Kunitz, Sigma-Aldrich, Deisenhofen, Germany). The selectivity of the antibodies (the extent to which they can bind the antigen in complex mixtures without interference) was tested using the three different matrices described above. Samples (3.3 mg/mL, Supporting Information Table 1) were extracted by homogenizing and mixing for 30 min in 10 mM Tris at pH 9.0 and 0.5% sarcosyl at room temperature.

Purification of Native Gly m 8 Antigen by Immunoprecipitation. Extracts of hexane-defatted soy flakes (Fraunhofer IVV; prepared as previously described)³¹ were preincubated with 0.5 mL of Protein G Sepharose 4 Fast Flow (90- μ m particle size, GE Healthcare) for 1 h at room temperature. Afterward, the Protein G Sepharose was removed by filtration through a 30- μ m polyethylene filter (Thermo Fisher Scientific). Preadsorbed protein extract was incubated with the capture antibody (mAb3) for 1 h at room temperature on a Stuart Tube Rotator SB3 (Cole-Parmer, Wertheim, Germany) before adding 1 mL of Protein G Sepharose 4 Fast Flow as above and mixing for another 1 h at room temperature. The mixture was filtered as above, forming a column matrix by gravity flow. This column was washed 10 times with 10 mL of PBS and eluted with 0.5 mL of 0.1 M glycine-HCl (pH 3.6). The eluate was neutralized with 50 μ L of 1 M Tris (pH 9.0), and the proteins were separated by polyacrylamide gel electrophoresis using 16% (w/v) tricine gels.³² The bands were analyzed by LC-MS/MS as previously described.³³

RESULTS

Plant Expression of Recombinant Protein. Recombinant Gly m 8 was produced in *N. benthamiana* by *Agrobacterium*-mediated transient expression. The native Gly m 8 sequence, including the N-terminal signal peptide, prepropeptide, and a C-terminal His₆ tag, was codon-optimized for expression in *N. benthamiana* and transferred to an expression cassette in the binary plant expression vector pTRAKT-ER (Figure 1A). After proteolytic cleavage of the signal peptide and prepropeptide, the mature Gly m 8 protein consisted of two subunits joined by a disulfide bridge (Figure 1B). Transient expression of Gly m 8 and subsequent purification by IMAC and SEC yielded a highly pure recombinant protein (Figure 1C). During expression in *N. benthamiana*, Gly m 8 underwent a complete maturation cycle as demonstrated by the presence of two bands representing the processed subunits on reducing gels and one band representing the 14 kDa complex of the two covalently linked subunits on nonreducing gels (Figure 1D). The molecular weight of the protein bands determined in the SDS-PAGE appears higher compared to the theoretical molecular weight. This is the case for both the recombinant protein (Figure 1) and the native protein represented by immunoprecipitation (Supporting Information Figure 1). Deviations between the calculated and the apparent molecular weight determined by SDS-PAGE is a

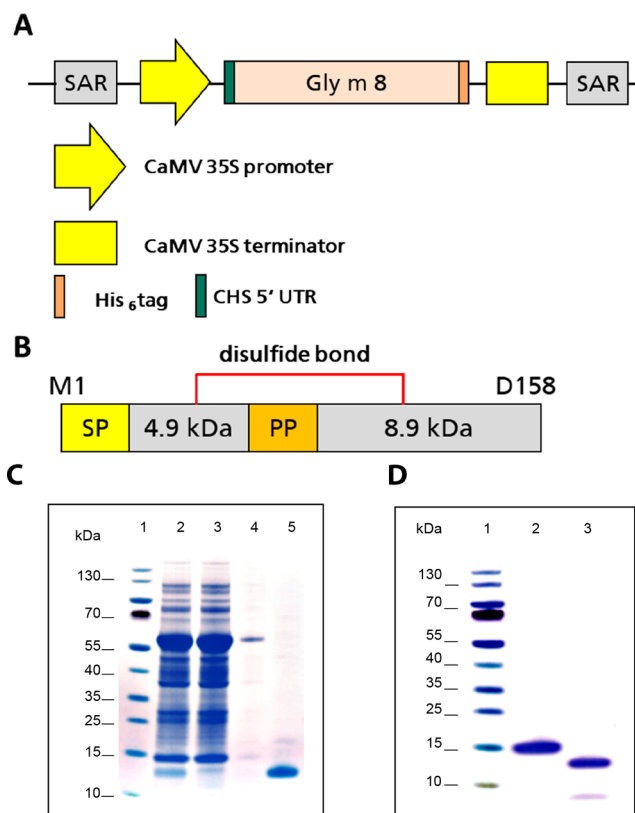


Figure 1. Plant expression construct and purity and integrity of recombinant Gly m 8. (A) Schematic presentation (not to scale) of the expression cassette Gly m 8. SAR, scaffold attachment region; CaMV 35S promoter and terminator, promoter with duplicated enhancer and terminator of the *Cauliflower mosaic virus* (CaMV) 35S gene; 5' untranslated region, 5'-UTR of the chalcone synthase gene from *Petroselinum crispum* (CHS 5' UTR); Gly m 8, coding sequence for Gly m 8, UniProt ID 19594; His₆ tag, six histidine residues (affinity purification tag). (B) Schematic presentation (not to scale) of the Gly m 8 protein, including signal peptide (SP), propeptide (PP), and disulfide bond. (C) Analysis of purification of expressed recombinant Gly m 8 by SDS-PAGE under reducing conditions. Lane 1 = molecular weight marker. Crude filtered extracts of *N. benthamiana* leaves (lane 2) were loaded onto IMAC columns, and both the flow-through and wash-out samples were collected (lanes 3 and 4, respectively). In the eluate (lane 5), a protein band with the expected size of $\sim$ 12 kDa, representing the large subunit of Gly m 8 under reducing conditions, was detected. The small unit, with a molecular weight of $\sim$ 5 kDa, ran within the running front of the gel but was separately displayed in D. (D) SDS-PAGE analysis of SEC-purified Gly m 8 under nonreducing (lane 2) and reducing (lane 3) conditions. A 99% pure recombinant Gly m 8 protein was purified by SEC, which separates under reducing conditions into two subunits.

common observation, since many different factors, besides incomplete processing or unexpected post-translational modifications, influence the running behavior of a protein in SDS-PAGE. The buffer system, the SDS concentration, and the pH in combination with the specific protein sequence may lead to such deviations.³⁴ Since the experimental data suggest successful processing of the pre-propeptide as well as the formation of disulfide bridges, the MW deviations are most likely such artifacts. Similar deviations have been described for various proteins, for example, for PyMsp1-19, a *Plasmodium yoelii* surface protein, which runs between 17 and 19 kDa in SDS-

PAGE, while the calculated as well as MS-derived MW is around 12 kDa³⁵

Monoclonal Antibodies. Immunization of mice with recombinant Gly m 8 protein led to the recovery of antibodies that bound with high affinity to both the native and recombinant protein. Indirect ELISA (Figure 2) captured the native protein

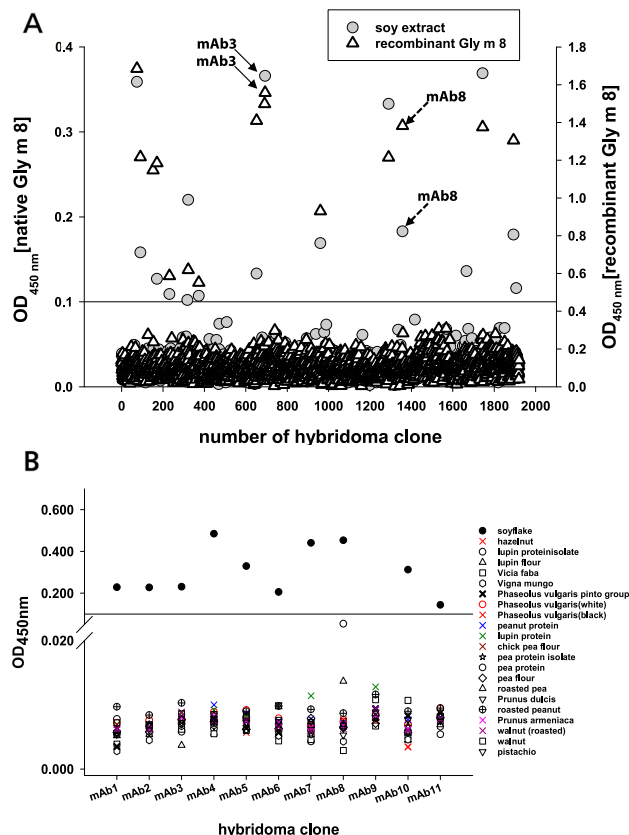


Figure 2. Screening of antibody-producing hybridoma clones by indirect ELISA using plates coated with soy extract (native) or recombinant Gly m 8 and extracts of legumes and nuts (A). Supernatants of hybridoma cultures were tested for the presence of Gly m 8-specific IgG antibodies which bound to both native soy extracts (filled circle) and recombinant Gly m 8 (triangle) using an indirect ELISA. Binding of antibodies to the Gly m 8 antigen resulted in a high OD_{450nm} signal as shown in the scatter plot of 2000 hybridoma clones. Read-outs higher than 0.1 OD identified high-affinity anti-Gly m 8 antibodies. Clones producing high-affinity antibodies were cryopreserved, and antibody-containing supernatants were collected for further analysis. The arrows (solid line mAb3 and dotted line mAb8) are tag specific signals for the clones finally used in ELISA. (B) Supernatants of selected hybridoma cultures (mAb1 to mAb11) which were tested for the presence of Gly m 8-specific IgG antibodies which bound to both native soy extracts using an indirect ELISA (Figure 2) were rescreened on both native soy extract (filled circle) and legume and nut extracts as indicated. OD values above 0.1 were assessed as positive according to signal-to-noise ratios above 10 in the appropriate ELISA.

from completely aqueous soy extracts. Clones with signal-to-noise ratios of OD 450 nm ≥ 10 corresponding to an OD of >0.1 were considered to be positive. Eleven antibodies showing no cross-reactivity against protein extracts isolated from legumes peas, peanuts, and lupins as well as various beans and nuts (Figure 2B) were preselected to develop a sandwich ELISA. Ranking the antibodies according to their binding affinity and stability on recombinant Gly m 8 covalently conjugated to the

surface of Biacore CM 5 chips and proving their compatibility with respect to pair production in a sandwich led to the selection of mAb3 and mAb8 (Figure 3). The kinetic analysis of these

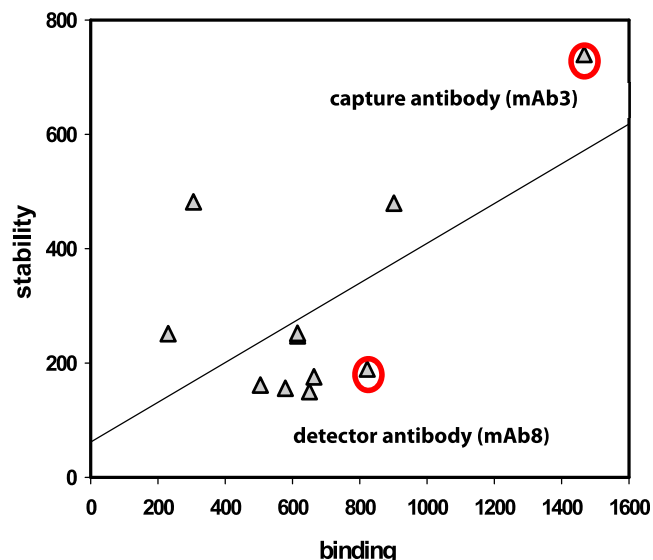


Figure 3. Ranking of anti-Gly m 8 antibodies. Binding and stability of selected anti-Gly m 8 antibodies (mAb1 to mAb11) tested using recombinant Gly m 8 conjugated onto the surface of a CM5 chip with the SPR biosensor instrument Biacore T200. Response units (RU) indicate specific binding of the antibody to the recombinant Gly m 8 covalently coupled to the chip at the late association phase (binding) and late dissociation phase (stability). The plot shows these response units from the late association phase (binding) and late dissociation phase (stability) of 11 selected antibodies on a Gly m 8 surface in order to choose appropriate capture antibodies. The binding and stability are related to both the association and dissociation rates of the interaction. The red encircled antibodies were used in the sandwich ELISA as the capture (mAb3) and detection (mAb8) antibodies.

antibodies (Figure 4) revealed K_d values in the subnanomolar range: 3.92×10^{-10} for mAb3 and 1.57×10^{-10} for mAb8 (Figure 4 and Supporting Information Table 2). Furthermore, the captured mAb3 was characterized by the immunoprecipitation of native soy extract with Protein G Sepharose, because mAb3 does not bind to denatured protein under western-blot conditions. Analysis of the precipitate by polyacrylamide gel electrophoresis revealed two major protein species with molecular weights of 25 kDa and 15 kDa under nonreducing conditions (Supporting Information Figure 1). LC-MS/MS analysis confirmed the 25-kDa protein was the mouse kappa light chain from mAb3 (score 331, UniProt P01837), and the 15-kDa protein was Gly m 8 (score 896.03, UniProt ID P19594). Under reducing conditions, the 15-kDa band was converted into two bands of ~ 11 kDa and 5 kDa (Supporting Information Figure 1) representing the linkage of the two subunits by disulfide bonds. The LC-MS/MS data confirmed the specific binding of mAb3 to Gly m 8. In addition, no cross-reactivity was observed for mAb3 and mAb8 either by indirect (screening) ELISA (Figure 2B) or by sandwich ELISA when tested against extracts derived from *Triticum aestivum*, *Apium graveolens*, *Brassica nigra*, *Brassica juncea*, *Sinapis alba*, *Vigna angularis*, *Vigna mungo*, *Phaseolus vulgaris*, *Phaseolus vulgaris* Pinto Group, lupin beans, peanuts, peas, and field beans (*Vicia faba*).

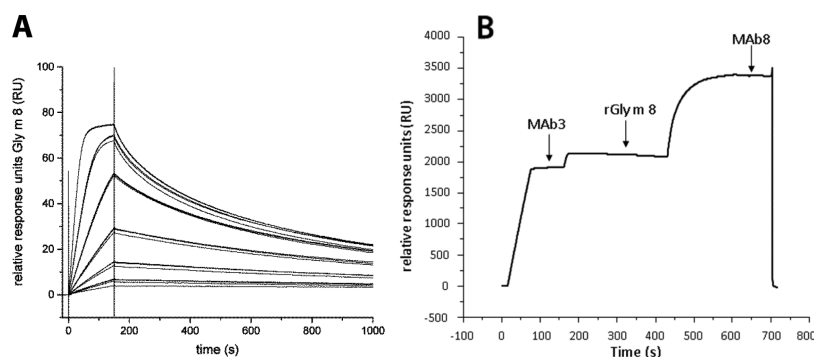


Figure 4. Representative SPR sensorgrams for the kinetic analysis of the Gly m 8-specific mAb8 and simultaneous binding of mAb3 and mAb8 to recombinant Gly m 8. (A) The affinity of mAb8 for recombinant Gly m 8 was determined by SPR spectroscopy. For each cycle, purified mAb8 was captured onto a Protein G-coated surface (500 response units (RUs)). Subsequently, recombinant Gly m 8 was injected at concentrations of 5, 2.5, 1.25, 0.625, 0.3125, or 0.15625 nM for 150 s to determine the on-rate (k_a) and dissociation was observed for 900 s to determine the off-rate dissociation (k_d). The k_D values were estimated by fitting the data to interaction models using the Biacore T200 evaluation software, applying the 1:1 Langmuir fit model. (B) Because mAb8 is an IgG isotype IgG1, it binds only weakly to Protein A, whereas mAb3 (IgG2A) can be efficiently captured on a Protein A functionalized CM5 sensor surface. Therefore, it was possible to illustrate the compatibility of the two antibodies with a sandwich ELISA format in the context of an SPR experiment. The figure shows the subsequent injection of mAb3 (captured onto a Protein A surface), followed by recombinant Gly m 8 and finally mAb8. The comparable response unit (RU) levels for the two antibodies (1500–1700 RU) indicate that each molecule of recombinant Gly m 8 can be simultaneously recognized by both antibodies, confirming the suitability of the antibody combination for the development of a sandwich ELISA for the quantification of Gly m 8.

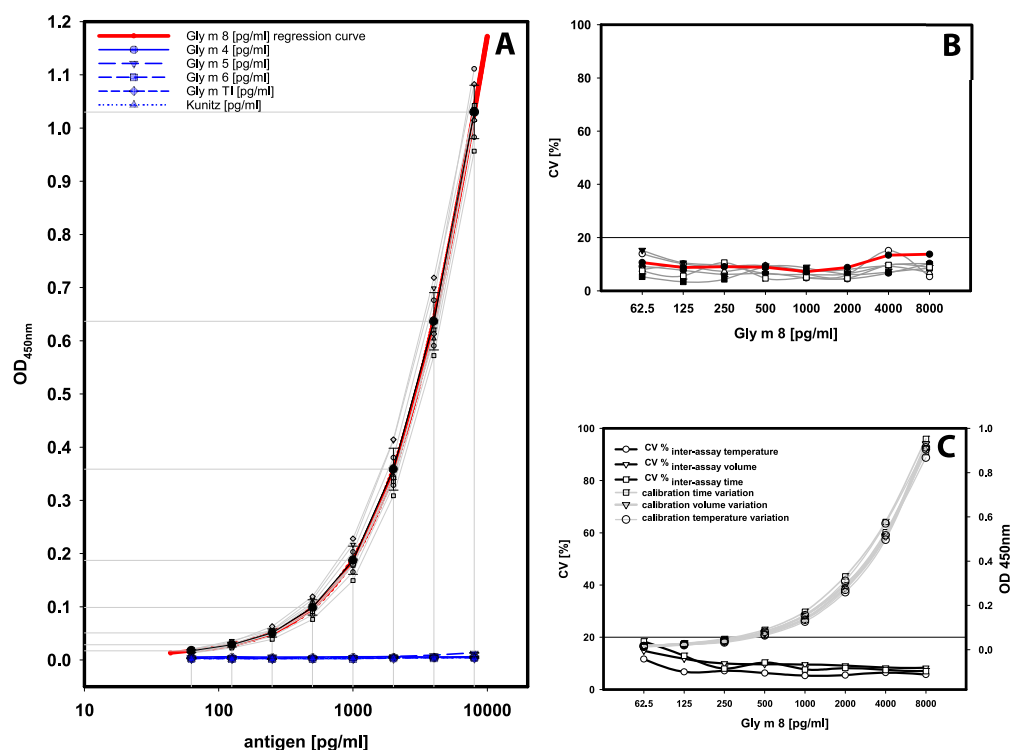


Figure 5. Calibration curve, precision profile, and robustness testing of the Gly m 8 ELISA. (A) Representative calibration curves of the Gly m 8 sandwich ELISA are depicted in gray with the regression curve fitted by a four-parameter logistic model in red (A). LOD and LOQ as functions of the analytical specificity of Gly m 8 ELISA were determined by the linear and nonlinear calibration methods on the basis of calibration curve (ISO 11843–5:2008). The blue curves represent the reaction of antibodies with potential interfering proteins naturally present in whole soy extracts, namely, recombinant proteins produced in *N. benthamiana* Gly m 4 and Gly m T1 and commercially purified native proteins Gly m 5, Gly m 6, and Kunitz (Sigma-Aldrich). Two different operators performed the ELISA on three different days. (B) Precision profile shows the repeatability calculated by coefficients of intra-assay variance (gray lines) and intermediate precision calculated by coefficients of inter-assay variance (red line). (C) Representative calibration curves of the sandwich ELISA obtained by measuring recombinant Gly m 8 at three different temperatures (20, 28, and 37 °C; gray curves, circles), using two different incubation volumes ($\pm 10\%$; gray curves, triangles), and using an incubation time variation ($\pm 10\%$, gray curves, rectangles). The right axis of the ordinate presents the corresponding absorbance values (OD_{450nm}). The corresponding precision profiles are depicted in the same coordinate system related to the left axis of the ordinate.

Sensitivity, Specificity, and Robustness of ELISA. The new Gly m 8 sandwich ELISA achieved a LOD > 10 pg/mL Gly

m 8 (determined from the average of 10 matrix blanks plus three standard deviations) and a LOQ of 65 pg/mL Gly m 8

(determined as the lowest concentration of spiked Gly m 8 in three different matrices or buffer that is still reliably detectable). As described in the [Materials and Methods](#) section, a regression curve fitted by a four-parameter logistic model was used as the nonlinear equation for the estimation of the lower quantification limit. The LOQ represents the lowest Gly m 8 concentration in picograms per milliliter, which is measurable with a coefficient of variance below 20% ([Figure 5B](#)). The interassay variance (robustness) was determined by analyzing the same samples on three different days by two different operators ([Figure 5C](#)). The precision of the assay was confirmed by processing 10 technical replicates of three different samples ([Figure 5B](#)). When spiking recombinant Gly m 8 into three different matrices, we achieved recovery rates of 98–109% ([Table 1](#)). Gly m 8 was detected in

Table 1. Recovery of Recombinant Gly m 8 at Five Different Concentration Levels in Three Different Matrices^a

spiked Gly m 8 [pg/mL]	recovery in almond muffin matrix in pg/mL and [%]	recovery in minced boiled sausage matrix pg/mL and [%]	recovery in rice cookie matrix pg/mL and [%]	recovery in extraction buffer pg/mL and [%]
5000	5206 ± 228 [104 ± 4.5]	5409 ± 230 [108 ± 4.6]	5507 ± 612 [110 ± 12.2]	5077 ± 296 [101 ± 5.9]
2500	2546 ± 115 [101 ± 4.6]	2620 ± 167 [104 ± 6.7]	2749 ± 298 [109 ± 11.9]	2621 ± 236 [109 ± 15.0]
500	497 ± 39 [99 ± 7.8]	540 ± 30 [108 ± 5.9]	534 ± 67 [106 ± 13.4]	545 ± 75 [109 ± 15.1]
100	95 ± 15 [95 ± 14.7]	99 ± 9 [99 ± 9.0]	98 ± 18 [98 ± 18.4]	103 ± 16 [103 ± 16.1]
65	63 ± 7 [97 ± 10.3]	68 ± 7 [105 ± 10.9]	64 ± 13 [99 ± 20.4]	70 ± 16 [107 ± 23.2]

^aMatrices produced by extraction of indicated processed food were spiked with recombinant Gly m 8 protein, and the recovery rates (percent of the spiked amount) were measured by Gly m 8 sandwich ELISA.

all three of the food matrices we tested. In complex processed food matrices, Gly m 8 is easily detectable by the sandwich antibodies below 1 ppm in soy protein and soy milk and below 10 ppm in tofu and texturized vegetable protein, which is often a challenge to detect. In roasted soy material, it is more poorly detectable, but this is rather due to limitations of whole protein isolation than a specific matter of Gly m 8 isolation, e.g., the higher the roasting degree, the lower the protein content of the extract ([Supporting Table 3](#)).

DISCUSSION

The detection of allergens in food products by ELISA depends on efficient protein isolation during the preparation of samples from complex food matrices.³⁶ The limited solubility of globulins in legume extracts, which is often a desired techno-functional characteristic of protein isolates, concentrates, and extruded material,^{37–40} reduces the reliability of ELISA results, as previously shown for the two main soybean storage proteins Gly m 5 and Gly m 6.¹⁴ Soluble proteins such as albumins are therefore preferable targets because they are protease resistant and thermostable, and they retain their native protein structure. In addition, there is less cross-reactivity among the albumins of different legume species, whereas the more strongly conserved globulins such as Gly m 6 lead to false positive results.¹² Phylogenetic analysis of the legume 2S albumins²¹ suggests that only lupin δ -conglutin and peanut Ara h6 should cross-react with Gly m 8, but the monoclonal antibodies we selected (mAb3 and

mAb8) showed no cross-reactivity to extracts of lupin and peanut. Compared to the storage protein glycinin (Gly m 6), the other main storage protein, the 7S globulin Gly m 5, does not share sufficient sequence identity to its equivalents in other legumes to cause cross-reaction, but the detection of Gly m 5 requires labor-intensive heat extraction, which makes the assay more cumbersome.^{41,42}

A key advantage of the Gly m 8 sandwich ELISA presented herein is the high affinity of the antibodies mAb3 and mAb8, which therefore bind the Gly m 8 antigen at very low concentrations. Detailed characterization by SPR analysis allowed us to perform stringent quality control of both antibodies and the recombinant Gly m 8 reference protein. Further Gly m 8-specific antibodies are available that could be used together with mAb3 and mAb8 to detect other epitopes either individually or together, thus further increasing the sensitivity of the assay.

The utilization of Gly m 8 as an antigen for the detection and quantification of soy allergens in food has a second important advantage because this protein is currently the best predictor of severe allergic reactions in children.^{18,1} However, the allergenicity of Gly m 8 has been assessed using different methods in different studies.^{18,43,44} Ebisawa and colleagues tested native Gly m 8 coupled to an immunocap device,¹⁸ whereas the other study used a recombinant Gly m 8 protein produced in *E. coli*⁴³ or microarrays with overlapping peptides representing solely linear epitopes, thus not reflecting the three-dimensional structure of the protein.⁴⁴ The Gly m 8 ELISA is also advantageous because it provides information about the allergen content of processed soy proteins. The commercial ELISA kits are reliable if the samples of processed food achieve recovery rates of 50–150%.²⁹ The new Gly m 8 ELISA would therefore be particularly suitable for the detection of soy ingredients in chocolate, which often contains texturized vegetable protein and native soy protein. Moreover, we were able to detect soy in highly processed food such as roasted soy beans and minced boiled sausages with the newly developed Gly m 8 ELISA, even though there are some limitations regarding the quantification. Minced boiled sausage material of proficiency testing 2017 (LVU, Germany) clearly tested positive, though quantification was not exactly possible because the value was below the LQL of the ELISA ([Supporting Information Table 3](#)). Furthermore, the available material was difficult to quantify, and only 9 out of 34 participants released a quantitative statement with very high deviations according to the evaluation report (LVU, Germany).

Every 1 g of total soy protein contains 300–600 mg of Gly m 5 and Gly m 6^{45,46,8} and 60 mg Gly m TI⁸ but only 1.1 mg of Gly m 8. Nevertheless, the new Gly m 8 ELISA was able to detect minimal amounts of soy protein in both rice cookies and minced boiled sausage using extraction conditions avoiding heating and denaturation, which produced negative results using antibodies to the other allergens. This indicates that the new Gly m 8 ELISA has an unprecedented sensitivity.

In summary, the Gly m 8 ELISA combines the advantages of monoclonal antibodies (which can be produced in unlimited quantities) and a robust, highly purified recombinant protein standard that can be used as reference material to ensure uniform and stable quality. The simple sample preparation method that is the effortless extraction method will also allow the antibodies to be used as an on-site test system compatible with food swabs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jafc.9b02717](https://doi.org/10.1021/acs.jafc.9b02717).

Supporting Figure 1: Analysis of native Gly m 8 isolated from soy extracts by immunoprecipitation with mAb3 followed by PAGE (PDF)

Supporting Table 1: Soy-containing foods and food ingredients (PDF)

Supporting Table 2: Kinetic parameters derived from SPR-based interaction analysis (PDF)

Supporting Table 3: Amount of Gly m 8 measured in processed food (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49 341 355 36 1290. E-mail: elke.ueberham@izi.fraunhofer.de.

ORCID

Elke Ueberham: [0000-0002-2121-0627](https://orcid.org/0000-0002-2121-0627)

Funding

This work was funded by the Fraunhofer Zukunftsstiftung as part of the joint research project FoodAllergen 883126.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Mrs. Ulrike Scholz and Mr. Leander Zitzmann (Fraunhofer IZI, Leipzig, Germany) for their help with the production of monoclonal antibodies and the development of the sandwich ELISA. We are grateful to Pia Meinlschmidt and Isabel Murany (Fraunhofer IVV, Freising, Germany) for providing soy flakes, soy flour, and protein isolates and Martin Röder (Institut für Produktqualität, Berlin, Germany) for providing the model cake and chocolate. We thank Andreas Pich, MHH Institute of Toxicology, Core Unit Proteomics (Hannover, Germany) for the LC-MS/Ms analysis. We thank Richard M. Twyman for manuscript editing.

■ ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; CFCA, calibration-free concentration analysis; IBR, initial binding rate; IMAC, immobilized metal ion affinity chromatography; LOD, limit of detection; LOQ, limit of quantification; mAb, monoclonal antibody; RU, response unit; SEC, size exclusion chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; LC/MS-MS, liquid chromatography coupled with tandem mass spectrometry; PBS, phosphate-buffered saline; TMB-E, 3,3',5,5'-tetramethylbenzidine ELISA substrate

■ REFERENCES

- (1) Maruyama, N.; Sato, S.; Cabanos, C.; Tanaka, A.; Ito, K.; Ebisawa, M. Gly m 5/Gly m 8 fusion component as a potential novel candidate molecule for diagnosing soya bean allergy in Japanese children, *Clinical and experimental allergy. Clin. Exp. Allergy* **2018**, *48*, 1726–1734.
- (2) Maggio, P.; Monso, E.; Baltasar, M.; Morera, J. Occupational asthma caused by soybean hull. A workplace equivalent to epidemic asthma. *Allergy* **2003**, *58*, 350–351.
- (3) Riascos, J. J.; Weissinger, S. M.; Weissinger, A. K.; Kulis, M.; Burks, A. W.; Pons, L. The Seed Biotinylated Protein of Soybean (Glycine max). A Boiling-Resistant New Allergen (Gly m 7) with the Capacity

To Induce IgE-Mediated Allergic Responses. *J. Agric. Food Chem.* **2016**, *64*, 3890–3900.

(4) Tsuji, H.; Bando, N.; Hiemori, M.; Yamanishi, R.; Kimoto, M.; Nishikawa, K.; Ogawa, T. Purification of characterization of soybean allergen Gly m Bd 28K. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 942–947.

(5) Ogawa, T.; Tsuji, H.; Bando, N.; Kitamura, K.; Zhu, Y.-L.; Hirano, H.; Nishikawa, K. Identification of the Soybean Allergenic Protein, Gly m Bd 30K, with the Soybean Seed 34-kDa Oil-body-associated Protein. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 1030–1033.

(6) Xiang, P.; Baird, L. M.; Jung, R.; Zeece, M. G.; Markwell, J.; Sarath, G. P39, a novel soybean protein allergen, belongs to a plant-specific protein family and is present in protein storage vacuoles. *J. Agric. Food Chem.* **2008**, *56*, 2266–2272.

(7) Tukur, H. M.; Lallès, J.-P.; Plumb, G. W.; Mills, E. N. C.; Morgan, M. R. A.; Toullec, R. Investigation of the Relationship between in Vitro ELISA Measures of Immunoreactive Soy Globulins and in Vivo Effects of Soy Products. *J. Agric. Food Chem.* **1996**, *44*, 2155–2161.

(8) Brandon, D. L.; Friedman, M. Immunoassays of Soy Proteins. *J. Agric. Food Chem.* **2002**, *50*, 6635–6642.

(9) Geng, T.; Stojšin, D.; Liu, K.; Schaalje, B.; Postin, C.; Ward, J.; Wang, Y.; Liu, Z. L.; Li, B.; Glenn, K. Natural Variability of Allergen Levels in Conventional Soybeans. Assessing Variation across North and South America from Five Production Years. *J. Agric. Food Chem.* **2017**, *65*, 463–472.

(10) Liu, B.; Teng, D.; Wang, X.; Wang, J. Detection of the soybean allergenic protein Gly m Bd 28K by an indirect enzyme-linked immunosorbent assay. *J. Agric. Food Chem.* **2013**, *61*, 822–828.

(11) Pedersen, M. H.; Holzhauser, T.; Bisson, C.; Conti, A.; Jensen, L. B.; Skov, P. S.; Bindsløv-Jensen, C.; Brinch, D. S.; Poulsen, L. K. Soybean allergen detection methods—a comparison study. *Mol. Nutr. Food Res.* **2008**, *52*, 1486–1496.

(12) Lacorn, M.; Dubois, T.; Siebeneicher, S.; Weiss, T. Accurate and Sensitive Quantification of Soy Proteins in Raw and Processed Food by Sandwich ELISA. *Food Sci. Technol.* **2016**, *4*, 69–77.

(13) Morishita, N.; Kamiya, K.; Matsumoto, T.; Sakai, S.; Teshima, R.; Urisu, A.; Moriyama, T.; Ogawa, T.; Akiyama, H.; Morimatsu, F. Reliable enzyme-linked immunosorbent assay for the determination of soybean proteins in processed foods. *J. Agric. Food Chem.* **2008**, *56*, 6818–6824.

(14) Scharf, A.; Kasel, U.; Wichmann, G.; Besler, M. Performance of ELISA and PCR methods for the determination of allergens in food. An evaluation of six years of proficiency testing for soy (Glycine max L.) and wheat gluten (Triticum aestivum L.). *J. Agric. Food Chem.* **2013**, *61*, 10261–10272.

(15) Holzhauser, T.; Franke, A.; Treudler, R.; Schmiedeknecht, A.; Randow, S.; Becker, W.-M.; Lidholm, J.; Vieths, S.; Simon, J.-C. The BASALIT multicenter trial. Gly m 4 quantification for consistency control of challenge meal batches and toward Gly m 4 threshold data. *Mol. Nutr. Food Res.* **2017**, *61*, 1600527.

(16) Nitride, C.; Lee, V.; Baricevic-Jones, I.; Adel-Patient, K.; Baumgartner, S.; Mills, E. N. C. Integrating Allergen Analysis Within a Risk Assessment Framework. Approaches to Development of Targeted Mass Spectrometry Methods for Allergen Detection and Quantification in the iFAAM Project. *J. AOAC Int.* **2018**, *101*, 83–90.

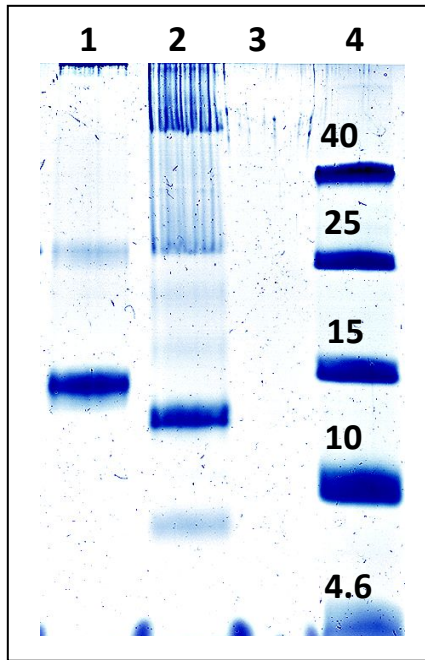
(17) Cucu, T.; Jaxsens, L.; De Meulenaer, B. de Analysis to support allergen risk management. Which way to go? *J. Agric. Food Chem.* **2013**, *61*, 5624–5633.

(18) Ebisawa, M.; Brostedt, P.; Sjölander, S.; Sato, S.; Borres, M. P.; Ito, K. Gly m 2S albumin is a major allergen with a high diagnostic value in soybean-allergic children. *J. Allergy Clin. Immunol.* **2013**, *132*, 976–978.e5.

(19) Offermann, L.; Perdue, M.; He, J.; Hurlburt, B.; Maleki, S.; Chruszcz, M. *Structural Biology of Peanut Allergens*; JCI, 2015.

(20) Han, Y.; Lin, J.; Bardina, L.; Grishina, G. A.; Lee, C.; Seo, W. H.; Sampson, H. A. What Characteristics Confer Proteins the Ability to Induce Allergic Responses? IgE Epitope Mapping and Comparison of the Structure of Soybean 2S Albumins and Ara h 2. *Molecules* **2016**, *21*, E622 DOI: [10.3390/molecules21050622](https://doi.org/10.3390/molecules21050622).

- (21) Moreno, F. J.; Clemente, A. 2S Albumin Storage Proteins. What Makes them Food Allergens? *Open Biochem. J.* **2008**, *2*, 16–28.
- (22) Meinlschmidt, P.; Ueberham, E.; Lehmann, J.; Schweiggert-Weisz, U.; Eisner, P. Immunoreactivity, sensory and physicochemical properties of fermented soy protein isolate. *Food Chem.* **2016**, *205*, 229–238.
- (23) Lin, J.; Fido, R.; Shewry, P.; Archer, D. B.; Alcocer, M. J. C. The expression and processing of two recombinant 2S albumins from soybean (*Glycine max*) in the yeast *Pichia pastoris*. *Biochim. Biophys. Acta, Proteins Proteomics* **2004**, *1698*, 203–212.
- (24) Sack, M.; Paetz, A.; Kunert, R.; Bomble, M.; Hesse, F.; Stiegler, G.; Fischer, R.; Kättinger, H.; Stoeger, E.; Rademacher, T. Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. *FASEB J.* **2007**, *21*, 1655–1664.
- (25) Feller, T.; Thom, P.; Koch, N.; Spiegel, H.; Addai-Mensah, O.; Fischer, R.; Reimann, A.; Pradel, G.; Fendel, R.; Schillberg, S.; Scheuermayer, M.; Schinkel, H. Plant-based production of recombinant Plasmodium surface protein pf38 and evaluation of its potential as a vaccine candidate. *PLoS One* **2013**, *8*, No. e79920.
- (26) Schräml, M.; Biehl, M. Kinetic screening in the antibody development process. *Methods Mol. Biol. (N. Y., NY, U. S.)* **2012**, *901*, 171–181.
- (27) Pol, E.; Roos, H.; Markey, F.; Elwinger, F.; Shaw, A.; Karlsson, R. Evaluation of calibration-free concentration analysis provided by Biacore systems. *Anal. Biochem.* **2016**, *510*, 88–97.
- (28) Boes, A.; Spiegel, H.; Delbrück, H.; Fischer, R.; Schillberg, S.; Sack, M. Affinity purification of a framework 1 engineered mouse/human chimeric IgA2 antibody from tobacco. *Biotechnol. Bioeng.* **2011**, *108* (12), 2804–2814, DOI: [10.1002/bit.23262](https://doi.org/10.1002/bit.23262).
- (29) Abbott, M.; Hayward, S.; Ross, W.; Godefroy, S. B.; Ulberth, F.; van Hengel, A. J.; Roberts, J.; Akiyama, H.; Popping, B.; Yeung, J. M.; Wehling, P.; Taylor, S. L.; Poms, R. E.; Delahaut, P. Validation procedures for quantitative food allergen ELISA methods. Community guidance and best practices. *J. AOAC Int.* **2010**, *93*, 442–450.
- (30) Havenith, H.; Kern, K.; Rautenberger, P.; Spiegel, H.; Szardenings, M.; Ueberham, E.; Lehmann, J.; Buntru, M.; Vogel, S.; Treudler, R.; Fischer, R.; Schillberg, S. Combination of two epitope identification techniques enables the rational design of soy allergen Gly m 4 mutants. *Biotechnol. J.* **2017**, *12*, 1600441.
- (31) Meinlschmidt, P.; Sussmann, D.; Schweiggert-Weisz, U.; Eisner, P. Enzymatic treatment of soy protein isolates. Effects on the potential allergenicity, technofunctionality, and sensory properties. *Food Sci. Nutr.* **2016**, *4*, 11–23.
- (32) Schagger, H.; von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *166*, 368–379.
- (33) Meinlschmidt, P.; Brode, V.; Sevenich, R.; Ueberham, E.; Schweiggert-Weisz, U.; Lehmann, J.; Rauh, C.; Knorr, D.; Eisner, P. High pressure processing assisted enzymatic hydrolysis – An innovative approach for the reduction of soy immunoreactivity. *Innovative Food Sci. Emerging Technol.* **2017**, *40*, 58–67.
- (34) Rabilloud, T. Variations on a theme. Changes to electrophoretic separations that can make a difference. *J. Proteomics* **2010**, *73*, 1562–1572.
- (35) Ma, C.; Wang, L.; Webster, D. E.; Campbell, A. E.; Coppel, R. L. Production, characterisation and immunogenicity of a plant-made Plasmodium antigen—the 19 kDa C-terminal fragment of Plasmodium yoelii merozoite surface protein 1. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 151–161.
- (36) Amponsah, A.; Nayak, B. Evaluation of the efficiency of three extraction conditions for the immunochemical detection of allergenic soy proteins in different food matrices. *J. Sci. Food Agric.* **2018**, *98*, 2378.
- (37) Hager, D. F. Effects of extrusion upon soy concentrate solubility. *J. Agric. Food Chem.* **1984**, *32*, 293–296.
- (38) Liu, K.; Hsieh, F.-H. Protein-protein interactions during high-moisture extrusion for fibrous meat analogues and comparison of protein solubility methods using different solvent systems. *J. Agric. Food Chem.* **2008**, *56*, 2681–2687.
- (39) Lee, K. H.; Ryu, H. S.; Rhee, K. C. Protein solubility characteristics of commercial soy protein products. *J. Am. Oil Chem. Soc.* **2003**, *80*, 85–90.
- (40) Jiang, J.; Xiong, Y. L.; Chen, J. pH Shifting alters solubility characteristics and thermal stability of soy protein isolate and its globulin fractions in different pH, salt concentration, and temperature conditions. *J. Agric. Food Chem.* **2010**, *58*, 8035–8042.
- (41) Pavlicevic, M.; Stanojevic, S.; Vucelic-Radovic, B. Influence of extraction method on protein profile of soybeans. *Hem. Ind.* **2013**, *67*, 687–694.
- (42) Lin, J.; Alcocer, M. J. C. *Food Allergens. Methods and Protocols*; Humana Press: New York, 2017.
- (43) Lin, J.; Shewry, P. R.; Archer, D. B.; Beyer, K.; Niggemann, B.; Haas, H.; Wilson, P.; Alcocer, M. J. C. The potential allergenicity of two 2S albumins from soybean (*Glycine max*). A protein microarray approach. *Int. Arch. Allergy Immunol.* **2006**, *141*, 91–102.
- (44) Han, Y.; Lin, J.; Bardina, L.; Grishina, G. A.; Lee, C.; Seo, W. H.; Sampson, H. A. What Characteristics Confer Proteins the Ability to Induce Allergic Responses? IgE Epitope Mapping and Comparison of the Structure of Soybean 2S Albumins and Ara h 2. *Molecules* **2016**, *21*, E622 DOI: [10.3390/molecules21050622](https://doi.org/10.3390/molecules21050622).
- (45) Nielsen, N. C.; Dickinson, C. D.; Cho, T. J.; Thanh, V. H.; Scallan, B. J.; Fischer, R. L.; Sims, T. L.; Drews, G. N.; Goldberg, R. B. Characterization of the glycinin gene family in soybean. *Plant Cell* **1989**, *1*, 313–328.
- (46) Shuttuck-Eidens, D. M.; Beachy, R. N. Degradation of -Conglycinin in Early Stages of Soybean Embryogenesis. *Plant Physiol.* **1985**, *78*, 895–898.



Supporting Figure 1: Analysis of native Gly m 8 isolated from soy extracts by immunoprecipitation with mAb3 followed by polyacrylamide gel electrophoresis.

Soy extract was incubated with mAb3 and the antibody was captured by protein G Sepharose, washed and eluted by reducing the pH to 3.6. The eluate was collected and the proteins were separated on a 16% (w/v) tricine gel. Lanes 1 and 2 show the protein bands under non-reducing conditions and reducing conditions, respectively. Lane 3 is empty and lane 4 shows the Spectra Multicolor Low Range Protein Ladder as a calibration standard to estimate the molecular weight of the protein bands.

- 1 **Supporting Table 1: List of Soy-containing foods and food ingredients.**
- 2 Commercial soy products, samples of previous proficiency tests and in-house soy
- 3 processed food were used as samples.

Sample	Manufacturer/provider
Soy flake	In-house flakes prepared at Fraunhofer IVV from untoasted soybeans (<i>G. max</i> Merr.) from Naturkost Ernst Weber (Munich, Germany)
Soy protein isolate	Wilcon G100, Wilmar, Singapore
Texturized vegetable protein	Different manufacturers, e.g. GutBio (France) 43-49% protein
TOFU	Different manufacturers, e.g. Alnatura (Bickenbach, Germany) and Tofu Life (Hillersheim, Germany), 12.5-16.7% protein
Minced boiled sausage	LVU Durchführung von Labor-vergleichsuntersuchungen (Herbolzheim, Germany)
Rice cookies	LVU Durchführung von Labor-vergleichsuntersuchungen (Herbolzheim, Germany)
Roasted soy bean	Seeberger (Ulm, Germany)
Model chocolate containing 1% (w/v) soy	Ifp Institut für Produktsicherheit, Berlin, Germany, in-house production
Model cookie containing 1% (w/v) soy	Ifp Institut für Produktsicherheit, Berlin, Germany, in-house production
Soy milk	Alpro soya light 2.1% (v/v) protein (Wevelgem, Belgium)

Supporting Table 2: Kinetic parameters derived from SPR-based interaction analysis.

Kinetic parameters k_a , k_d , and K_D were derived by analyzing binding curves at six concentrations of recombinant Gly m 8. A representative sensorgram is shown in Figure 4.

Sample	k_a [M ⁻¹ s ⁻¹]	$SE(k_a)$ [M ⁻¹ s ⁻¹]	k_d [s ⁻¹]	$SE(k_{di})$ [s ⁻¹]	K_D [M]
mAb8	3.76x10 ⁷	1.6x10 ⁵	5.89x10 ⁻³	2.5x10 ⁻⁵	1.57x10 ⁻¹⁰
mAb3	1.74x10 ⁷	1.1x10 ⁵	6.7x10 ⁻³	2.5x10 ⁻⁵	3.92x10 ⁻¹⁰

Supporting Table 3: Performance of Gly m 8 ELISA in processed food as indicated.

Determinable ppm amounts of indicated processed soy products by ELISA were calculated by the maximal dilution factor, which gives a detectable signal and the soy protein content of the product. Provided that the complete initial sample weight is 1×10^6 ppm (1 kg soy / kg solid substance) and known percentage of protein in the sample the ppm amount results from the equation:

$$\text{Ppm}_{(\text{measurable})} = \frac{1 \times 10^6 \text{ ppm} \times \% \text{ protein}}{100 \% \times \text{dilution factor}}$$

Food/food ingredient	Maximal dilution factor	Protein content [%]	Minimal measurable ppm
Soy flake	512,000	45	0.87890625
Soy protein isolate	5,120,000	80	0.15625
Texturized vegetable protein	128,000	43	3.359375
TOFU	16,000	13.3	8.3125
Minced boiled sausage *	5	0.001	2
Rice cookies	5	0.002	4
Roasted soy bean	2,000	45	225
Model chocolate containing 1% soy	2,000	1	5
Model cookie containing 1% soy	500	1	20
Soy milk	156,250	3	0.192

*the value of minced boiled sausage was below the LQL and could not exactly determined as absolute Gly m 8 value in pg/ml.