

Development of Monoclonal Antibodies against Pea Globulins for Multiplex Assays Targeting Legume Proteins

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ABSTRACT: Legume proteins are widely used as food ingredients, but only some (soybean, lupin, and peanut) must be declared under consumer safety regulations to protect allergy sufferers. It is not yet mandatory to declare pea proteins as allergens even though they are predicted to be allergenic based on cross-reactivity in sensitized people. The processing of legume proteins can modify their allergenic properties and hence the need for specific and precise methods for the detection of all major legume allergens. There are many commercially available tests for known food allergens but not for ingredients that are yet to be classified as allergenic. We therefore generated sets of pea-specific antibodies targeting globulins to be used in a multiplex assay for the simultaneous detection of soybean, lupin, peanut, and pea proteins. We focused on the 7S globulin family, which is the least conserved among the four legumes, allowing the specific detection of proteins from each species. Having confirmed the specificity and sensitivity of the multiplex assay, we evaluated different processing steps for proteins rich in pea globulins to demonstrate the impact of food processing on antibody binding. Our sensitive multiplex assay provides a fast and reliable method for the specific detection of soybean, lupin, peanut, and pea allergens and is therefore ideal for food safety and authenticity testing applications.

KEYWORDS: legume-derived protein substitutes, 7S globulin, recombinant protein, monoclonal antibodies, multiplexed allergen detection

INTRODUCTION

The growing preference for diets excluding meat and/or dairy products has increased the use of plant-derived protein substitutes, especially flour and isolated proteins from legume species such as soybean (*Glycine max*), lupin (*Lupinus albus*), peanut (*Arachis hypogaea*), and pea (*Pisum sativum*).¹ The EU is not self-sufficient in soybean meal, which is mostly imported from North and South America, so pea protein has been proposed as a more sustainable alternative. Pea protein isolates (PPIs) are typically prepared by selecting for globulins, which are the major protein component of pea seeds. Following alkaline extraction, the solubilized globulins are separated from the residual fiber by centrifugation and precipitated by adjusting the pH to the mean isoelectric point of the major pea globulins (vicilin, legumin, globulin-related proteins, and convicilin).² Pea vicilin and convicilin are considered to be allergenic³ and are therefore designated as Pis s 1 (vicilin) and Pis s 2 (convicilin) according to the Allergen Nomenclature Subcommittee of World Health Organization and International Union of Immunological Societies (WHO/IUIS). Both proteins are 7S globulins, along with Gly m 5 (soybean conglycinin),⁴ Lup an 1 (lupin β -conglutinin),⁵ and Ara h 1 (peanut vicilin-like conarachin).⁶ The resulting isolates are 90–95% protein (based on the dry matter content), and the yield of the process is ~80% of the total protein.⁷ PPIs are used as a gluten-free ingredient in bakery products and for the replacement of animal gelatin during the clarification of wine.⁸

The production and consumption of legume proteins have a lower environmental impact than animal-derived proteins, improve animal welfare, and confer health benefits on

consumers,^{9,10} including the prevention of arteriosclerosis.¹¹ However, the growing popularity of legume proteins has resulted in a greater prevalence of allergies, especially against soybean, lupin, and peanut proteins.^{12,13} Pea proteins are homologous to other legume proteins, but the prevalence of pea allergies is currently very low,^{14–16} and it is not yet mandatory under EU regulations to declare the presence of pea proteins in food.¹⁷ Nevertheless, cross-reactivity with soybean, lupin, and peanut allergens has been reported,¹⁸ and leguminous protein ingredients become more difficult to identify with higher degrees of processing. In order to ensure food safety, it is therefore necessary to develop methods for the species-level detection of legume-derived proteins to protect individuals with legume protein allergies.

Thus far, two key methods have been used for the detection and species-level assignment of food proteins: mass spectrometry (MS) and the enzyme-linked immunosorbent assay (ELISA). MS provides unprecedented accuracy by matching the mass of specific peptides against a database of known protein masses and cleavage patterns and is easier to multiplex¹⁹ but requires expensive equipment and (with few exceptions^{20,21}) lacks sufficient sensitivity for the analysis of highly processed food.²² However, considerable recent

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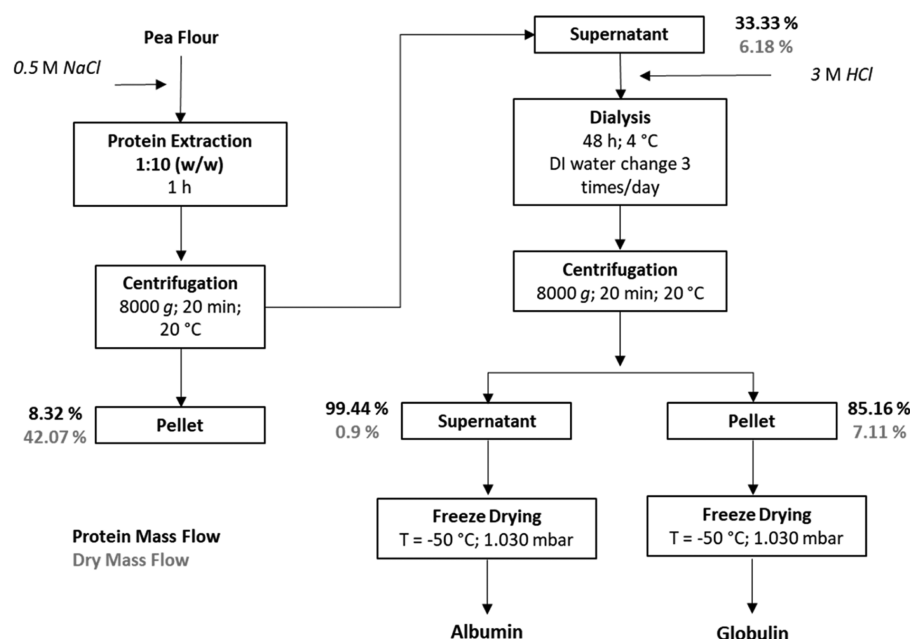


Figure 1. Preparation scheme for pea albumin and globulin extracts. Scheme shows the separation of pea flour resulting in enriched albumin and globulin fractions used to immunize mice for the generation of monoclonal antibodies.

progress has been made to address this challenge.²³ ELISA is quicker and less expensive and can be highly sensitive, but data quality is strongly dependent on the properties of the antibodies. A monoclonal antibody generally binds to an epitope consisting of only 4–5 amino acids, sometimes linear but often conformational. The ability to detect a given protein in food therefore depends on a variety of parameters such as the degree of processing by heating, enzymatic degradation, or chemical hydrolysis, all of which can destroy or modify linear and especially nonlinear epitopes.²⁴

Most ELISAs for the detection of food proteins are based on polyclonal sera produced in rabbits, goats, or other large animals, thus comprising a broad range of uncharacterized antibodies recognizing different epitopes. The precise antibody composition cannot be replicated, and the results achieved with such tests therefore vary between manufacturers and batches, making standardization difficult. Reproducible ELISA methods that can be standardized and harmonized across jurisdictions require monoclonal antibodies, which are well-characterized, homogeneous, and consistent. A mixture of monoclonal antibodies in a defined ratio can fulfil all the functions of older polyclonal ELISAs without the drawbacks.

We previously described monoclonal antibodies specific for soybean proteins, which were successfully used in sets for the specific detection of soybean allergens.^{25,26} We also isolated lupin-specific and peanut-specific antibodies at the same time (unpublished data). Here, we describe monoclonal antibodies raised against selected pea globulins. Monoclonal antibodies have already been raised against pea albumin 1b,²⁷ but this protein shows negligible allergenicity in contrast to other legume 2S albumins.²⁸ Our antibodies against pea globulins showed no cross-reactivity to other legume proteins, allowing their integration into a multiplex legume protein assay for the simultaneous detection of the four most common legume-derived protein ingredients and potential allergens.

MATERIALS AND METHODS

Legume Samples. Soybean (*G. max* L. Merr.) seeds were purchased from Naturkost Ernst Weber (Munich, Germany), lupin (*Lupinus angustifolius* cv Boregine) seeds were obtained from Saatzeit Steinach (Steinach, Germany), and pea (*P. sativum* L. cv Navarro) seeds were provided by Norddeutsche Pflanzenzucht Hans-Georg-Lembke (Hohenlieth, Germany). Food containing processed pea protein was purchased from supermarkets, including Edgy Veggie roasted pea (Edgy Food, Birkerød, Denmark) and vegan sausage. A full list is provided in Table S1.

Preparation, Fractionation, and Modification of Pea Proteins. Pea seeds were dehulled and split using an underflow peeler (Streckel & Schrader, Hamburg, Germany) and separated using an airlift system (Alpine Hosakawa, Augsburg, Germany). The split pea seeds were milled in a REKORD A impact mill (Gebrüder Jehmlich, Nossen, Germany) at a maximum peripheral speed (135 m/s) with a 0.5 mm sieve to generate dehulled pea flour (DPF).

PPI was prepared as previously described.²⁹ Briefly, an aqueous alkaline extract (pH 8.0) of the pea flour was prepared in deionized water while stirring constantly for 60 min. The protein extract was adjusted to pH 4.5 for isoelectric precipitation of the proteins. The precipitated proteins were separated, neutralized, pasteurized (70 ± 2 °C) for 2 min, and spray-dried. The dry pea proteins were fractionated into globulins and albumins as previously described³⁰ with the following changes. The flour was suspended 1:10 (w/w) in 0.5 M NaCl for 60 min at room temperature and then centrifuged at 8000g for 20 min at 20 °C. The extract was collected in 12–14 kDa MWCO dialysis tubes (Serva, Heidelberg, Germany) and dialyzed against demineralized water while stirring constantly for 48 h at 4 °C. The water was exchanged three times per day. The contents of the dialysis tubes were then centrifuged as above, and the fractions were freeze-dried. The pellet was described as the globulin fraction, and the supernatant as the albumin fraction (Figure 1).

Food processing conditions were modeled by exposing the precipitated proteins (0.5% w/w in deionized water) to four types of modification: (1) chemical modifications such as acid hydrolysis (PPI-AH) at pH 2 and 90 °C using concentrated HCl (12 M); (2) enzymatic hydrolysis (PPI-EH) using 0.5–2.0% proteolytic enzymes, namely flavourzyme (enzyme 1), papain (enzyme 2), or pepsin (enzyme 3) under optimum reaction conditions; (3) biotechnological modifications such as aerobic fermentation at 30 °C from pH 8 to 7 by inoculation with *Lactobacillus perolens* at a starting concentration of

1×10^8 cfu/mL, with the addition of 0.5% glucose; and (4) physical modifications such as autoclaving at 121 °C for 15 min.

Production of Recombinant Pea Proteins. Synthetic genes encoding pea proteins Pis s 1 (UniProt P02854, provicilin) and pea albumin, which is designated as Pis s Albumin (UniProt P08688, Albumin-2) according to the WHO/IUIS allergen nomenclature, were prepared by GeneArt (Thermo Fisher Scientific, Waltham, MA, USA) and augmented with 3' sequences encoding a His₆ tag for purification by immobilized metal ion affinity chromatography (IMAC).

The Pis s 1 coding region was inserted into vector pET-22b (Merck, Darmstadt, Germany) at the NcoI and BamHI sites and introduced into *Escherichia coli* BL21(DE3) cells (New England Biolabs, Frankfurt/Main, Germany). Recombinant BL21(DE3) cells were cultivated in LB medium, and the expression of Pis s 1 was induced by adding 400 μ M isopropyl β -D-1-thiogalactopyranoside. The cells were harvested by centrifugation, and soluble proteins were extracted using the BugBuster Master Mix (Merck) according to the manufacturer's instructions.

The Pis s Albumin coding region including its native signal peptide was transferred to vector pTRA³¹ and introduced into *Agrobacterium tumefaciens* GV3101:pMP90RK (DSMZ, Braunschweig, Germany) by electroporation. Recombinant bacteria were maintained in YEB medium supplemented with appropriate antibiotics and infiltrated into *Nicotiana benthamiana* plants as previously described.³² Leaf tissues were harvested 5 days post-infiltration. Soluble proteins were extracted by grinding the leaf tissue under liquid nitrogen with a mortar and pestle and resuspending the powder in two volumes (v/w) of phosphate-buffered saline (PBS) containing 0.5 M NaCl and 10 mM sodium metabisulfite. The crude extract was clarified by filtration through Miracloth (Merck) followed by centrifugation (16,000g, 4 °C, 20 min).

Purification of Recombinant Allergens by IMAC. Cleared extracts containing soluble proteins were passed through 0.2 μ m polyethersulfone syringe filters (Carl Roth, Karlsruhe, Germany) and loaded onto a 1 mL HiTrap chelating Sepharose column (GE Healthcare, Freiburg, Germany) charged with Ni²⁺ ions and equilibrated with buffer A (PBS plus 500 mM NaCl) using an ÄKTApurifier device (GE Healthcare). The column was washed with 10% buffer B (PBS plus 500 mM NaCl and 250 mM imidazole) and eluted with a linear gradient of 10–100% buffer B over 30 column volumes. We collected 1 mL fractions in 96-deepwell plates (Carl Roth) and analyzed aliquots by polyacrylamide gel electrophoresis (PAGE) using NuPAGE 4–12% Bis-Tris gels (Invitrogen, Thermo Fisher Scientific) along with PageRuler markers (Thermo Fisher Scientific) followed by staining with Coomassie Brilliant Blue.

Generation of Monoclonal Antibodies. Mouse antipea monoclonal antibodies were generated by immunizing female BALB/c mice (Janvier Labs, Le Genest-Saint-Isle, France) with either the albumin or globulin fraction (described above) under denaturing conditions. The immunization experiments were approved by the State Animal Care and Use Committee (Landesdirektion Sachsen, Leipzig, Germany, V07/14) 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 albumin or globulin proteins and fused to X63.Ag8.653 myeloma cells (ACC 43, DSMZ). Hybridoma supernatants were screened by indirect ELISA on flat-bottom 96-well plates with high protein-binding capacity (Nunc MaxiSorp, Thermo Fisher Scientific). The plates were coated with either the albumin and globulin fraction proteins (10 μ g/mL), recombinant Pis s 1 (1 μ g/mL), commercial pea lectin (MilliporeSigma, St. Louis, MO, USA), or biotinylated peptide sequences matching vicilin (UniProt P13918) and convicilin (UniProt P13919). Cross-reactivity to the total protein extracts from other legumes (soybean, lupin, and peanut) was tested by ELISA using 10 μ g/mL protein extracts.

ELISA. We used three different types of ELISA: (1) an indirect ELISA, in which the protein extract (native or recombinant) was bound to the plate (Nunc MaxiSorp); (2) an indirect ELISA, in which

a biotinylated peptide was bound via streptavidin already coated onto the plate; and (3) a direct sandwich ELISA, in which a specific capture antibody was bound to the plate and the captured protein was detected using a second antibody conjugated to an enzyme that catalyzes a colorimetric reaction. In all three ELISAs, the first step involved the coating of the plate surface followed by the blocking of nonspecific binding sites.

Indirect ELISA with Bound Protein Extracts. We coated 96-well plates (Nunc MaxiSorp) with 50 μ L/well of native protein extracts (10 μ g/mL in PBS, pH 8.5) or recombinant protein (1 μ g/mL in PBS, pH 8.5), applied a plate-sealing cover (Candor BioScience, Wangen, Germany), and incubated on a laboratory plate shaker overnight in a humid chamber at 4 °C. The plates were then warmed to ambient temperature, washed three times with PBS containing 154 mM NaCl and 0.05% Tween-20 (PBST), and blocked for 30 min with 200 μ L/well Superblock blocking reagent (Thermo Fisher Scientific). The blocking reagent was removed by tapping the plates, and the antibody-containing hybridoma supernatant or diluted antibody solution (50 μ L/well in 1:1 (v/v) Superblock/PBST) was added and incubated for 30 min. The plates were then washed three times with PBST before applying 50 μ L/well of the goat antimouse IgG horseradish peroxidase (HRP)-conjugated detection antibody (Jackson ImmunoResearch, Westgrove, PA, USA) diluted 1:10,000. After 30 min, the plates were washed three times with PBS-T and incubated for 5 min with the 3,3',5,5'-tetramethylbenzidine (TMBE) substrate (DUNN Labortechnik, Asbach, Germany). The yellow color generated by acidification with 0.5 M sulfuric acid represented the quantity of bound detection antibody and was determined by measuring the optical density at 450 nm (OD₄₅₀) on an ELISA plate reader. All wells containing a nonlegume-specific antibody and with OD₄₅₀ values at least threefold the blank value were considered to be positive.

Peptide-specific Indirect ELISA. Nunc MaxiSorp plates were coated with 50 μ L/well of 1 μ g/mL streptavidin (Thermo Fisher Scientific) in 10 mM carbonate buffer (pH 9.6) overnight at 4 °C. The streptavidin-coated plates were washed three times with PBST, blocked with 200 μ L/mL Superblock reagent, and washed with PBS immediately before coating with peptides. Synthetic peptides specific for vicilin and convicilin³³ were synthesized with an N-terminal biotin-Ahx linker by the Core Unit Peptide Technologies (Medical Faculty, Leipzig University, Leipzig, Germany). The peptide sequences were LSSGDVVFVIPAGHPVAVK (vicilin, P13918.2), LTPGDVVFVIPAGHPVAVR (P02855.1, provicilin), and ENPV-KELTFPGSVQEINR (convicilin, CAB82855.1). Biotinylated peptides (10 μ g/mL in PBS) were applied to the streptavidin plates (50 μ L/well) overnight at 4 °C. The plates were washed three times with PBST and blocked with 200 μ L/well Superblock reagent before adding the antibody-containing hybridoma supernatant or diluted antibody solution (50 μ L/well in 1:1 (v/v) Superblock/PBST) and incubating for 30 min. Further washing and detection with the secondary antibody were carried out as described above.

Sandwich ELISA. Capture antibodies and POD-labeled detection antibodies were used in checkerboard titrations to determine the optimal pairwise combinations. Pea proteins were quantified by sandwich ELISA using different antibody sets. Two sets of antibodies were found to work well in this format: a pea vicilin-specific pair (Izimab Pis s 1-1 and Izimab Pis s 1-2) and a convicilin specific pair (Izimab Pis s 2-1 and Pis s 2-2). The capture antibodies Izimab Pis s 1-1 (pea vicilin) and Izimab Pis s 2-1 (pea convicilin) were immobilized on 96-well plates (Nunc MaxiSorp) in 0.5 M carbonate buffer at 4 °C overnight. The plates were washed three times with PBST and blocked with Superblock reagent for 1 h at room temperature. The plates were then sealed with a Liquid Plate Sealer (Candor BioScience), air-dried, shrink-wrapped, and stored at room temperature.

Extracted samples were incubated for 30 min at room temperature in duplicates. After three washes in PBST, the HRP-conjugated detection antibody Izimab Pis s 1-2 (pea vicilin) or Izimab Pis s 2-2 (pea convicilin) was added, and the plates were incubated for 30 min at room temperature. HRP activity was determined after three further

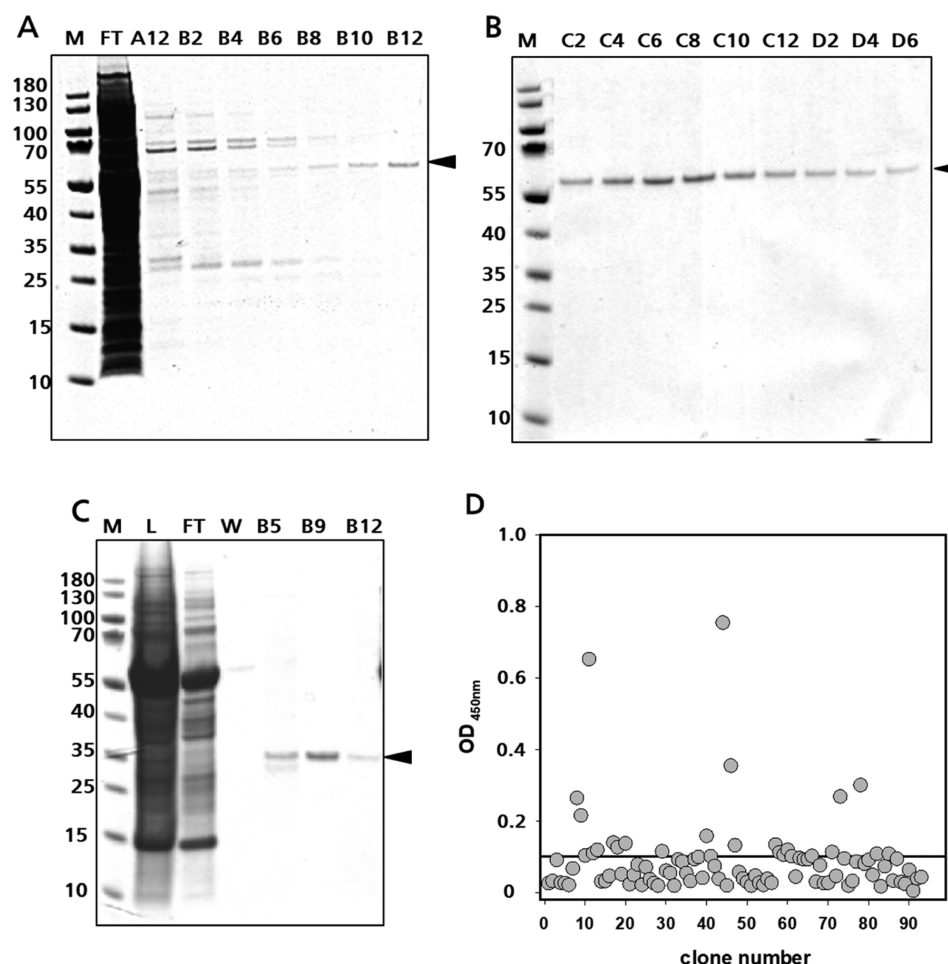


Figure 2. Purification of recombinant Pis s 1 and Pis s Albumin and hybridoma screening on recombinant Pis s 1. (A + B) Purification of recombinant Pis s 1 by IMAC. Aliquots of collected fractions were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. M = molecular weight marker (PageRuler), FT = flow through, A12–D6 = elution fractions with increasing imidazole concentrations. The arrow head points to the recombinant Pis s 1 band with an apparent molecular mass >55 kDa. (C) Purification of recombinant Pis s Albumin by IMAC. Aliquots of collected fractions were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. M = molecular weight marker (PageRuler), L = load, FT = flow through, W = wash, B5–B12 = elution fractions with increasing imidazole concentrations. The arrow head points to the recombinant Pis s Albumin band with an apparent molecular mass of 35 kDa. (D) Screening of 96 antibody-producing hybridoma clones on recombinant Pis s 1. Each dot represents the individual OD₄₅₀ value measured by indirect ELISA. Plates were coated with 1 µg/mL recombinant Pis s 1, blocked, and incubated with diluted supernatants of various clones from the globulin-binding fraction. After washing, the binding of individual IgG antibodies was detected by incubating the plates with a HRP-conjugated antimouse IgG antibody and the peroxidase substrate TMBE followed by acidic stop solution.

washes in PBST by incubation with the TMBE substrate as above, and values were plotted relative to a calibration curve consisting of eight descending concentrations of pea extracts each diluted twice starting with 2000 ng/mL. The standard curve was fitted using the five-parameter logistic functions of Sigmaplot 14 (Systat Software, San Jose, CA, USA) or the Magellan Data Analysis Software (Tecan, Männedorf, Switzerland).

Multiplex Assay. Selected sets of pea-specific antibodies were tested in a multiplex legume allergen detection assay (LADA) based on a Luminex (Austin, TX, USA) platform according to the manufacturer's recommendations. Briefly, carboxy-functionalized standard MagPlex microspheres (Luminex) were analyzed on a FLEXMAP 3D flow analyzer system (Luminex). Capture antibodies (anti-Gly m 5, anti-Lup an 1, anti-Pis s 1, and anti-Ara h 1) were conjugated to four different MagPlex bead regions (14, 15, 19, and 20, respectively) using the xMAP Antibody Coupling Kit according to the manufacturer's recommendations for two-step carbodiimide coupling. The corresponding detection antibodies were labeled with biotin using the EZ-Link NHS-PEG12-Biotin Kit (Thermo Fisher Scientific). Free biotin was removed on Zeba Spin Desalting Columns

(Thermo Fisher Scientific). All incubations were carried out at room temperature in the absence of light.

The capture antibody-conjugated microspheres were combined and diluted to 2500 microspheres per 50 µL assay buffer in PBST and incubated for 1 h on a rotator as a preliminary blocking step. We then added 50 µL of the blocked microspheres to each well of a 96-well low-binding plate and added 50 µL of diluted sample and incubated for 1 h on an orbital shaker. The plates were then washed three times with 200 µL of PBST on a magnetic plate washer to remove the unbound sample. We then added a combined detection mixture of biotinylated detection antibodies diluted in assay buffer (100 µL per well). After incubation for 1 h as above, the plates were washed three times to remove unbound detection antibodies. Streptavidin-conjugated phycoerythrin (BioLegend, San Diego, CA, USA) was diluted 1:2000 in assay buffer, and 100 µL was added per well to the sandwich complex and agitated for 1 h. The reaction was terminated by washing the wells three times with PBST and filling with 120 µL of assay buffer. The median fluorescence intensity per 100 beads of each region was captured by the FLEXMAP 3D system. Accuracy and precision were determined by intra-assay and interassay comparisons, and the coefficient of variation (CV) was calculated from six replicates

for the calibration curve in a 1:2 dilution series (soybean and pea = 64 to 1 ppm; lupin and peanut = 128 to 2 ppm). The CV upper acceptance limit was set at 20% for the intra-assay and interassay experiments. The lower limit of detection was defined as the lowest concentration tested which was three times the blank value.

Sample Extraction. Milled legume crops, protein, and commercial food samples (50 mg) were extracted by homogenizing in 1500 μ L of 50 mM Tris (pH 8.5) containing 2% sodium dodecylsulfate (SDS) (SERVA Electrophoresis, Heidelberg, Germany) and 400 mM NaCl and shaking for 30 min at 60 $^{\circ}$ C.

Western Blot. Samples were separated by SDS-PAGE on TruPage 4–20% precast gradient gels (MilliporeSigma) and blotted onto PVDF membranes in Power Blotter PVDF regular Transfer Stacks (PB5340) using the Power Blotter System according to the manufacturer's instructions (Thermo Fisher Scientific). Membranes were blocked with 5% skimmed milk powder in PBST and incubated overnight with monoclonal antibodies at 4 $^{\circ}$ C. After washing in PBST, membranes were incubated with the peroxidase-conjugated goat antimouse IgG (Fc-specific) detection antibody (Dianova, Hamburg, Germany) for 1 h at room temperature.

RESULTS

We fractionated pea proteins to simulate the industrial manufacturing process for PPIs (Figure 1). We used the resulting globulin and albumin fractions to immunize mice and isolated a large panel of monoclonal anti-pea protein antibodies. We matched potential allergenic proteins to specific antibodies in proof-of-principle experiments using two recombinant proteins (Figure 2A–C) and synthetic peptides for screening by indirect ELISA (Figures 2D and S1).

Production of Recombinant Allergens. Recombinant Pis s 1 was produced in *E. coli* as a fusion protein with an N-terminal pelB leader and C-terminal His₆ sequence. The recombinant allergen was purified by IMAC to near homogeneity, as demonstrated by PAGE analysis of the elution fractions (Figure 2A). The fusion protein migrated with an apparent molecular mass of >55 kDa, indicating the production and purification of an intact full-length protein ($MW_{\text{theor}} = 52$ kDa). Recombinant Pis s Albumin was produced by transient expression in *N. benthamiana* plants with a native N-terminal signal peptide and C-terminal His₆ and tag54 sequences. It was extracted from infiltrated leaf tissues and purified by IMAC (Figure 2C). The recombinant allergen migrated with an apparent molecular mass of 35 kDa, indicating the production and purification of an intact full-length protein ($MW_{\text{theor}} = 29$ kDa). The purity of both recombinant proteins was judged sufficient for ELISA-based screening of monoclonal antibodies produced by hybridoma lines.

Selection of Monoclonal Antibodies for Sandwich ELISA. More than 600 individual clones binding specifically to either the pea globulin or albumin fractions were isolated and cryostored for further analysis. The collection with specificity for the water-soluble albumin fraction included both albumin-specific and several lectin-specific clones, as shown by their ability to bind recombinant albumin and native commercial lectin (data not shown). Most of these clones have not been characterized in detail and remain in the cryostorage for further studies. Clones with specificity for the globulin fraction were tested for cross-reactivity to other legume proteins (peanut, lupin, soybean, faba bean, and various other beans). Those specific for pea globulins were used to assemble several sandwich ELISAs for the detection of pea vicilin or pea convicilin to make a suitable pair for integration into the multiplex assay. Accordingly, the globulin-specific antibodies

were assigned either by indirect ELISA to the recombinant Pis s 1 protein expressed in *E. coli* (Figure 2D) or to biotinylated pea vicilin/convicilin-specific peptides³³ (Figure S1). The pea vicilin sandwich assay was assembled by using one of the antibodies binding to recombinant Pis s 1 as the capture antibody (Izimab Pis s 1–1). The pea convicilin sandwich assay was assembled by using an antibody that was bound to the convicilin-specific peptide ELTFPGSVQEINR as the capture antibody (Izimab Pis s 2–1). Suitable detection antibodies (Izimab Pis s 1–2 for vicilin and Izimab Pis s 2–2 for convicilin) were chosen by testing potential globulin binders in checkerboard assays.

Analysis of Capture and Detection Antibodies by Western Blot. All capture and detection antibodies to be used in the multiplex assay were tested for their ability to bind all four legume extracts under denaturing conditions with and without the chemical reduction of disulfide bridges, revealing exclusive binding to the specific legume protein extracts used for immunization. Western blots with the monoclonal capture antibody Izimab Pis s 1–1 and the detection antibody Izimab Pis s 1–2, which were used in the ELISA and multiplex bead assay, generated the band pattern anticipated for Pis s 1, a multimeric protein consisting of homologous polypeptides formed by post-translational proteolysis to generate 55 and 33 kDa products as well as smaller polypeptides of ~ 19 kDa (Figure 3). Such multimeric forms are common among the legume vicilin family, which are 7S globulins.³⁴

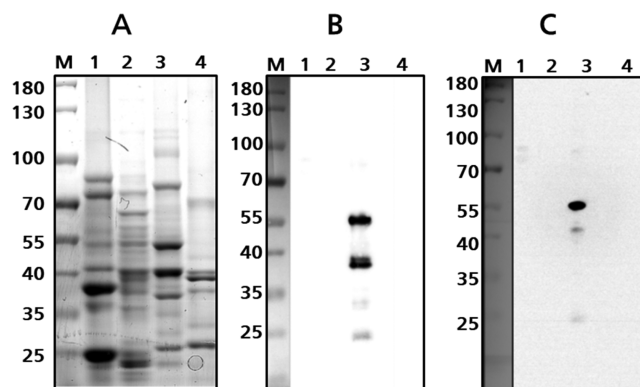


Figure 3. Western blot analysis of legume proteins using monoclonal Pis s 1 antibodies. The four legume extracts tested in the multiplex assay were separated by SDS PAGE (lanes 1–4: soybean, lupin, pea, and peanut) and blotted onto PVDF membranes. (A) Typical band pattern of legume extracts in Coomassie staining of SDS-PAGE is shown (M-molecular weight marker, 1-soybean extract, 2-lupin-extract, 3-pea extract, and 4-peanut extract). The membranes were incubated with Pis s 1 capture (B) or Pis s 1 detection (C) antibodies, which were in turn detected using a HRP-conjugated antimouse IgG antibody and a chemiluminescent peroxidase substrate.

ELISA Quantification of Various Processed Pea Fractions. The suitability of vicilin-specific antibodies in a sandwich format, which corresponds to the final format of the planned multiplex assay, was tested in several preliminary assays. By using the pea-specific antivicalin antibodies, we investigated the impact of the extraction, fractionation, and modification steps on antibody binding (Figure 4). Compared to the crude pea flour, the isoelectric isolation of the proteins (referring to the PPI sample in Figure 4) led to a slight increase in antibody binding to Pis s 1 and (to a greater extent) Pis s 2 (Figure S2). This may reflect the efficient extraction and

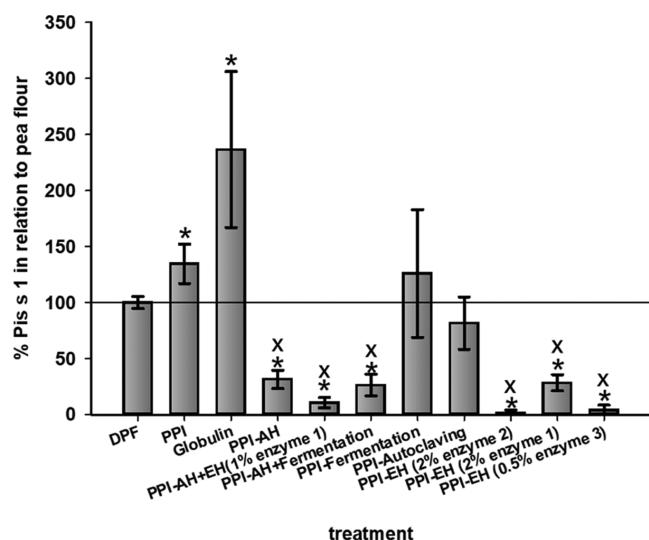


Figure 4. Relative quantification of Pis s 1 using newly developed monoclonal antibodies in sandwich assays. Monoclonal antibodies in sets (capture and detection antibodies) can be used in a sandwich ELISA format as in-process control assays during the technological refinement of protein preparations. The relative amount of the appropriate protein preparations is determined by comparison with the original substance (DPF) set to 100% as shown in the first column. Processing steps, namely globulin precipitation (globulin), precipitation at pH 4.5 to generate pea protein isolates (PPIs), AH of PPIs (PPI-AH), and enzymatic hydrolysis of PPIs (PPI-EH, where enzyme 1 = Flavourzyme, enzyme 2 = papain, and enzyme 3 = pepsin) were compared pairwise to DPF and PPIs, if PPIs were the starting material for the corresponding processing step. The content of immunoreactive Pis s 1 in the different protein extracts was compared pairwise to the control DPF. Treatments led to significant changes ($p < 0.01$ paired t -test) indicated with an asterisk. Protein extracts derived from PPIs were also compared pairwise with PPIs, and significant changes ($p < 0.01$ paired t -test) are indicated with a cross.

precipitation of the two globulins and depletion of the albumin fraction during protein precipitation.

Fermentation has been proposed to degrade the mature vicilin protein, reducing the intensity of bands detected by SDS-PAGE.²⁹ However, we were unable to confirm a drop in signal intensity in different fermentation runs (Figure 4). Autoclaving can also partially reduce the immunoreactivity of pea albumins,³⁵ but the decrease in signal intensity observed in one process run (Figure S2) was not reproducible (Figure 4) when comparing untreated crude pea flour to a thermal treatment in which the protein precipitate was dispersed in aqueous solution before autoclaving. All hydrolysis treatments (chemical and enzymatic) consistently resulted in a statistically significant loss of immunoreactivity in the sandwich assay (Figure 4).

Acid or enzymatic hydrolysis was more efficient for the degradation of Pis s 1 than PPI and DPF, respectively, and to a lesser extent, this was also the case for Pis s 2. These treatments involved the extensive cleavage of peptide bonds (AH) or proteolytic digestion (enzymatic hydrolysis). The combination of AH with subsequent microbial fermentation or enzymatic hydrolysis further reduced the antibody binding activity.

Multiplex Assay. The multiplex assay required substantial optimization, including the appropriate combination of paired sets of antibodies, the amount of capture antibody conjugated

to the beads, and both the biotinylation of the detection antibodies and the amount of detection antibody used in the assay (Table 1, Figure 5). Several combinations of capture and

Table 1. Optimized Parameters Used in the LADA

target 7S protein	microsphere region	amount of capture antibody per 10 ⁶ beads	detection antibody concentration/biotin excess
Gly m 5	14	Izimab Gly m 5-4 5 μ g	Izimab Gly m 5-3 2 μ g/mL/20
Lup an 1	15	Izimab Lup an 1-1 5 μ g	Izimab Lup an 1-2 2 μ g/mL/20
Ara h 1	19	Izimab Ara h 1-1 1 μ g	Izimab Ara h 1-2 2 μ g/mL/20
Pis s 1	20	Izimab Pis s 1-1 5 μ g	Izimab Pis s 1-2 8 μ g/mL/20

detection antibodies without mutual cross-reactivity were assembled into optimized sets for the specific detection of different legume species (Figure 6). Each of the capture antibodies was tested against all four detection antibodies. In the optimization protocol, several individual antibodies were replaced to achieve final panels that showed no cross-reactivity between the capture and detection sets. The optimal concentrations of capture antibodies (1–5 μ g/mL) and detection antibodies (2–8 μ g/mL) were determined empirically (Figure 5A,B), and the optimal labeling reaction included a 20-fold excess of biotin (Table 1). These conditions were used to generate the optimal standard curve range shown in Figure 5C. The standard curve was fitted using the five-parameter logistic functions of Sigmaplot 14 (Systat Software) or the Bioplex Manager Software. The four different sets of legume-specific anti-7S globulin antibodies were used to build a multiplex assay with lower limits of quantification for target extracts of 0.9 ppm (soybean), 0.6 ppm (lupin), 0.5 ppm (peanut), and 0.2 ppm (pea). LADA analysis revealed high reproducibility and repeatability for all four legume sets, with intra-assay and interassay CVs below 20%, as determined by measuring seven independent dilution series in four independent experiments (Figure 5D,E).

Finally, we examined samples of processed food containing pea protein according to the manufacturers' labeling. This revealed that our new anti-pea antibodies can detect pea proteins in roasted food with a mean recovery rate of 80%. Accordingly, a burger patty with a stated pea protein content of 17% was quantified with a pea protein content of 14% in our assay. All the analyzed samples showed reasonable recovery rates except for one in which the measured quantity of pea protein was six times higher than declared (Table S1). These data support the quantification of Pis s 1 in the model-processed foods (Figure 4), which showed little change during heating. This is important because sausages and cold meat products are heated during the production process. However, the precise pea protein content is difficult to determine without further information because it is unclear whether the tested products were fortified with pea protein sources such as pea isolate (80–90% protein) or a pea protein concentrate (25–80% protein). Furthermore, the total globulin content can vary from 50 to 80% of total pea protein.² Accordingly, further validation steps will be necessary to extrapolate the vicilin content to the total pea protein content, for example, by using more than one set of antibody pairs per legume species.

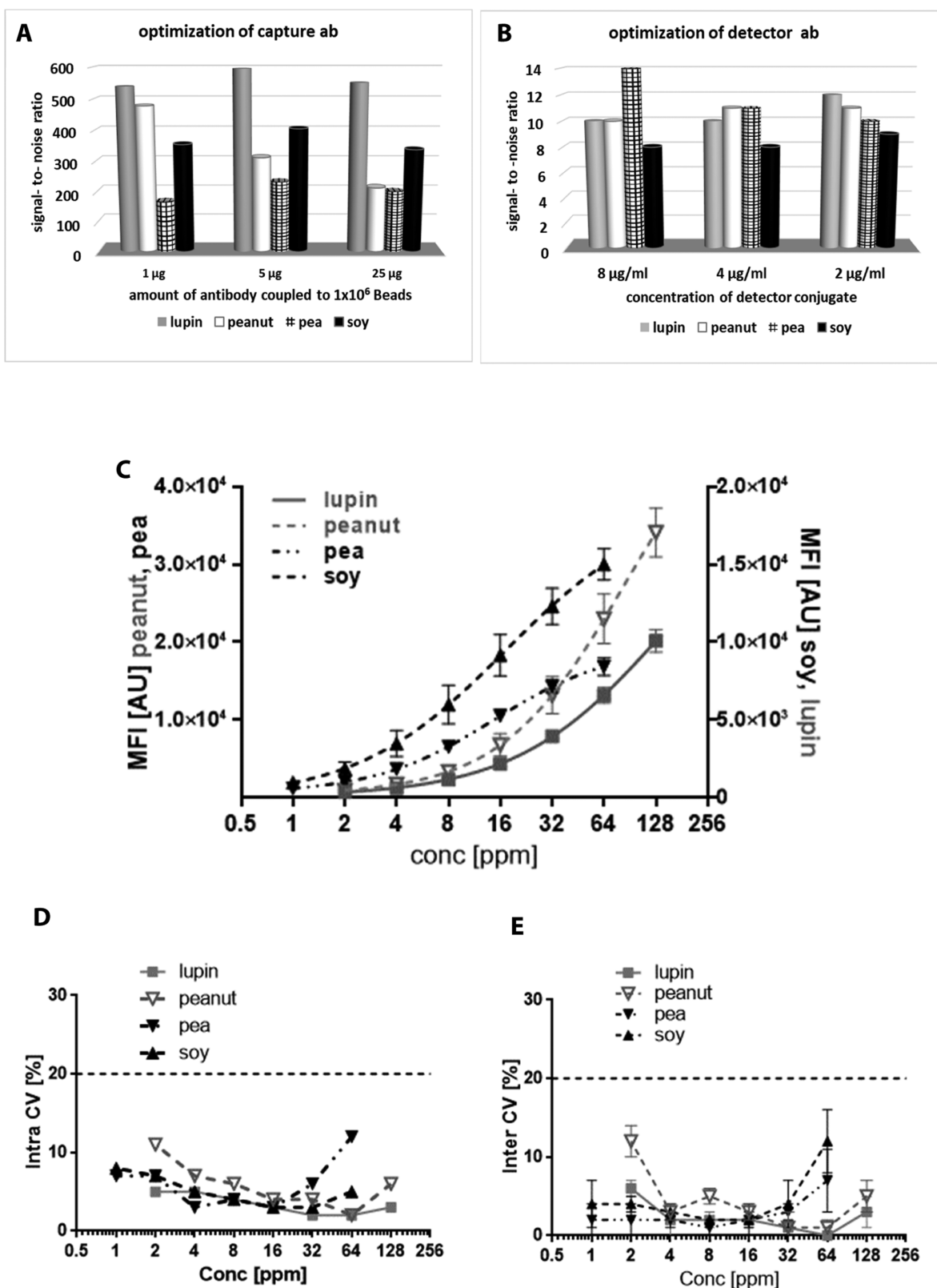


Figure 5. Features of the LADA. Different amounts of capture antibodies were coupled (per 10^6 beads), and the amount with the highest signal-to-noise ratio was selected for the final assay format (A). The signal-to-noise ratio at different concentrations of biotinylated detection antibodies was used to estimate the optimal detection antibody concentration for each set (B): The determined amounts and concentrations were used to establish calibration curves which were plotted in Sigmaplot 14 or using Bioplex Manager Software. Characteristic trends and ranges of calibration curves for soybean, lupin, pea, and peanut were expressed as arbitrary units [AU] of the fluorescence signal versus concentration in ppm (C). Intra-assay repeatability (expressed as the CV) (D) and interassay repeatability (E) were determined using seven independent dilution series in four independent experiments.

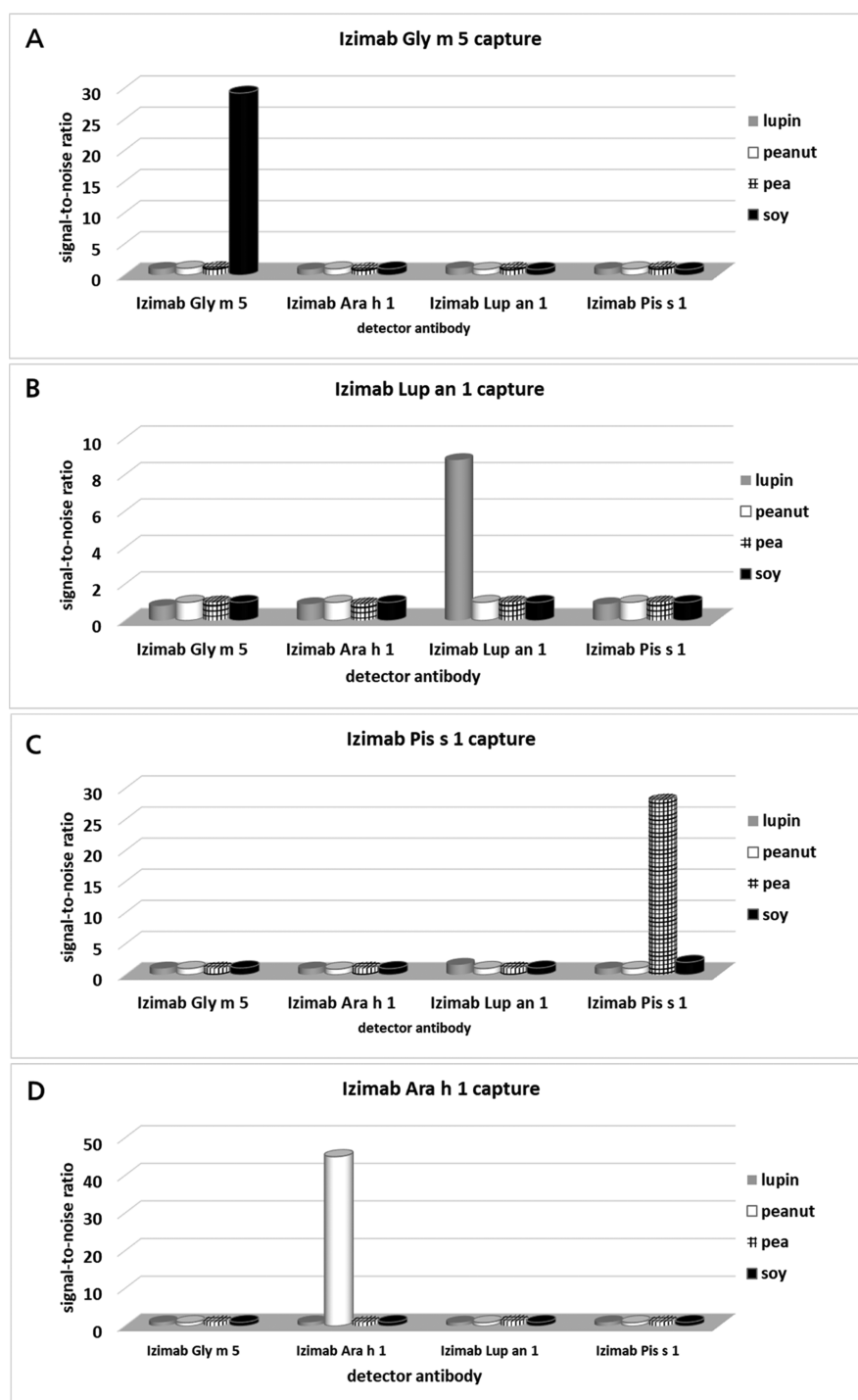


Figure 6. Intra-assay cross-reactivity of the LADA. The LADA bead assay requires four sets of different antibody pairs that interact specifically with the corresponding target protein. Each panel represents a specific capture antibody: (A) Izimab Gly m 5-4; (B) Izimab Lup an 1-1; (C) Izimab Pis s 1-1; and (D) Izimab Ara h 1-1. The final sets are displayed, confirming that the final sets do not cross-react with each other. All four capture antibodies show signal-to-noise ratios above a threshold of 3 exclusively with their corresponding extract and detection antibody.

DISCUSSION

The development of monoclonal antibodies specific for allergenic proteins is laborious and time-consuming, but companies are generally reluctant to commit significant resources to the detection of allergens using antibody-based methods. For example, we previously showed that immunization with a purified or recombinant protein such as soybean Gly m 5 or Gly m 8 generates several hundred clones that must

be characterized.^{25,26} Furthermore, most antibodies produced by immunization with a single pure protein often bind only to the unprocessed native protein. This may be advantageous for Gly m 8 and other small 2S proteins²⁶ but is more likely to be disadvantageous for 7S globulins such as Gly m 5, which is only sparingly soluble after technical processing and requires harsh conditions for solubilization. We therefore selected a complex approach to generate monoclonal antibodies specific for several proteins in pea seeds with the potential to cross-

react with allergenic proteins from other legumes. In this manner, we generated a large pool of antibodies that bind to processed pea proteins, allowing the development of protein-class-specific sandwich assays to detect pea proteins in a variety of food products. The preparation scheme initially separates pea flour into water-soluble and saline-soluble fractions with different swelling properties (Figure 1). We focused on vicilin, the pea 7S globulin, intending to complete a set of legume-specific antibodies detecting the 7S globulins of the most commonly used legumes in convenience foods (soybean, peanut, lupin, and pea). Another reason to focus on pea 7S proteins is that they represent the protein class most likely to be allergenic in sensitized people.³⁶ In this context, our ELISAs showed that only the hydrolytic treatments, which shorten the vicilin polypeptide chains, result in a significant drop of immunoreactivity. Thermal treatment was able to reduce the detectable amount of Pis s 1 (vicilin) and Pis s 2 (convicilin) (Figure S2), but this effect was not reproducible (Figure 4) and needs further investigation. The thermal treatment of legumes can modify allergenicity in both directions; for example, the roasting of whole peanuts enhances their IgE-binding capacity, whereas boiling has the opposite effect.^{34,37} Furthermore, the low residual binding activity of the specific monoclonal antibody sets following a combination of AH and subsequent microbial fermentation or enzymatic hydrolysis (Figure 4) suggests that these methods could be suitable as in-process-quality control for the production of pea protein formulations with lower levels of the allergenic protein Pis s 1 detectable by ELISA, as already shown for other legume proteins.³⁸

We used monoclonal antibodies to detect four different legume 7S proteins in a sandwich format, which is compatible with state-of-the-art multiplexing.³⁹ However, sandwich formats require two accessible epitopes in the protein and/or peptide analyte. The monoclonal antibodies developed in this study could also be used in inhibitory assays to detect the smallest fragments of legume 7S proteins in complex matrices. Nevertheless, the test settings should be carefully selected according to the purpose of the assay. The sandwich format is the preferred option to detect intact units of allergenic proteins or at least larger fragments of allergenic proteins in food ingredients that are still able to cause allergic reactions because allergic reactions require the interconnection of two receptor-bound IgE molecules to initiate the signaling cascade within a sensitized individual.⁴⁰ This interconnection can only be produced if two neighboring epitopes of a protein or protein fragment can bind to the neighboring receptor-bound IgE molecules on a mast cell or another responsive cell. Even so, the only definitive, evidence-based test for reduced allergenicity is a human study (prick testing or preferably oral provocation).

The newly developed antibodies could be used in competitive assays, comparable to MS-based methods, to detect traces of legume proteins (even if hydrolyzed) in highly processed food, for example, to evaluate on-site cleaning practices, to reveal unintentional mixing of legume products during the production of protein meal, or to detect fraudulent and intentional adulteration. In the latter case, if a single peptide were detected carrying a unique epitope, the likelihood of allergenicity would be low due to the absence of the second epitope which is strictly necessary for signaling within the responder cell. Furthermore, recommendations on the allergenic potential of plant-based ingredients could be

developed if the results generated using the antibody set presented herein are corroborated by clinical studies of allergenicity in humans.

Only a few multiplex assays have been developed thus far for the detection of allergenic proteins in food.^{39,41} In contrast to these assays, which are based on commercially available pairs of antibodies to determine cross-reactivity among food ingredients, we aimed to develop a multiplex assay that can distinguish between the four legumes used most widely as substitutes for animal-based ingredients. Because the antibodies used in the multiplex assay are specific for the 7S globulins, whereas food product labels usually declare the total protein content, further work is necessary to refine the calibration standards including the reference material and validation of the multiplex assay by proficiency testing. A large pool of antibodies covering nearly the entire spectrum of pea proteins was stored, making it possible to extend the spectrum of analytical targets to include 11S globulins or albumins.

A multiplex approach is desirable because it reduces the cost of testing, allowing a comprehensive panel of allergens to be identified and quantified in one assay. Our LADA format is the first step toward the general multiplexing of all allergenic proteins in one assay and will be complemented in the future with sets of antibodies specific for milk, egg, and nut proteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.0c07177>.

Indirect screening ELISA with the convicilin-specific peptide ENPVKELTFPGSVQEINR (PDF)

Relative quantification of Pis s 1 and Pis s 2 using sets of monoclonal antibodies in sandwich assays (PDF)

Processed foods containing pea protein analyzed in this study (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; IMAC, immobilized metal ion affinity chromatography; LOQ, lower limit of quantification; mAb, monoclonal antibody; SDS-PAGE, sodium dodecylsulfate polyacrylamide electrophoresis; LC/MS–MS, liquid chromatography coupled with tandem mass spectrometry; PBS, phosphate-buffered saline; TMBE, 3,3',5,5'-tetramethylbenzidine ELISA substrate; MFI, median fluorescence intensity; AU, arbitrary unit; PPI, pea protein isolate; AH, acid hydrolysis; EH, enzymatic hydrolysis

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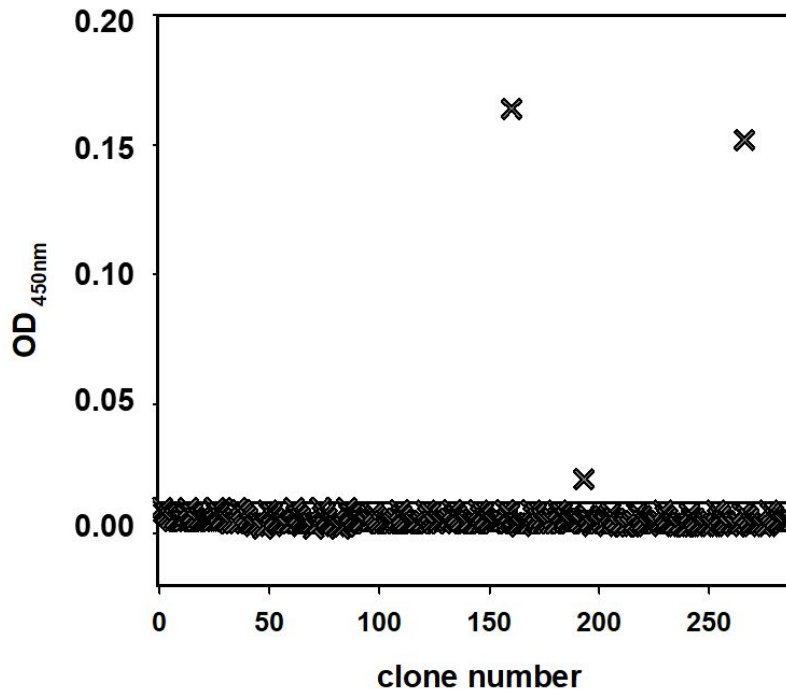
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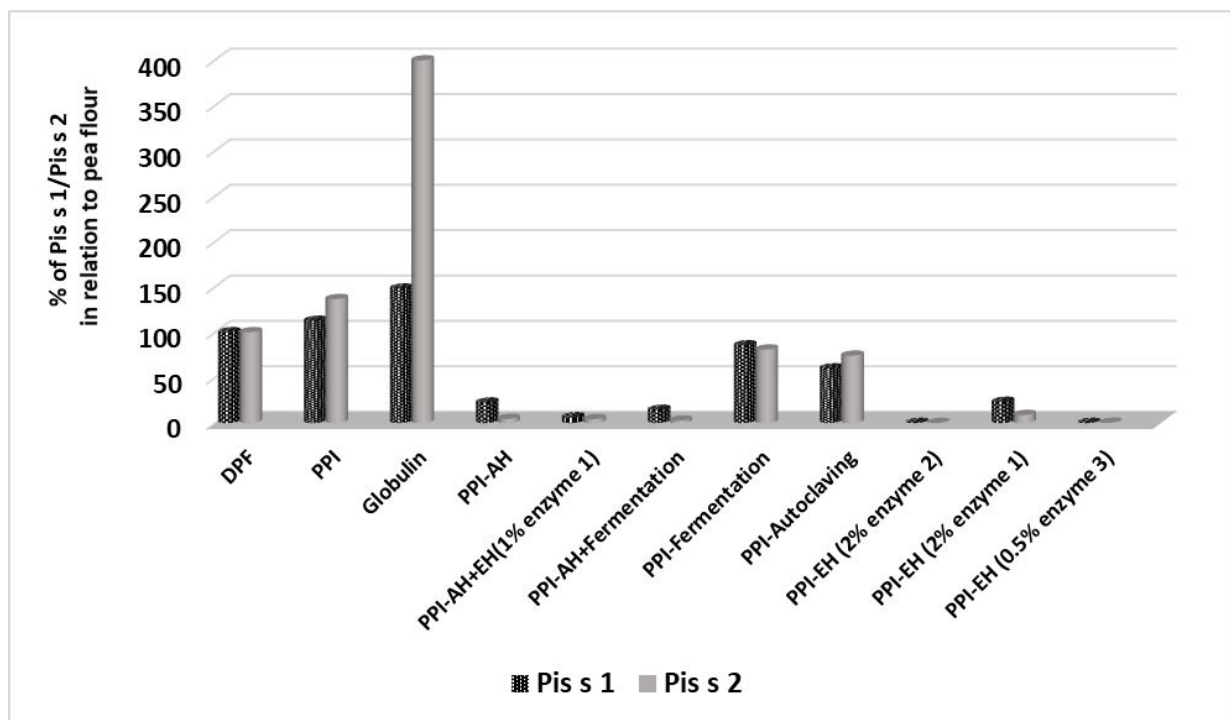
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Supporting Figure 1: Indirect screening ELISA with the convicilin-specific peptide ENPVKELTFPGSVQEINR

The convicilin-specific peptide ENPVKELTFPGSVQEINR, with an N-terminal biotin attached via an Ahx linker for better accessibility, was applied to streptavidin-coated 96-well plates. After washing, the antibody-containing hybridoma supernatant (represented by the clone number) was added, and antibodies binding to the peptide remained on the solid phase after washing and were detected by the binding of a secondary HRP-conjugated goat anti-mouse IgG after further washing. The HRP converts the substrate to a colored end product, which is detected by measuring the OD₄₅₀. Clones with OD₄₅₀ values three-fold higher than the background were designated positive, and were selected for further characterization. A representative 300 clones are shown, each indicated by X.



Supporting Figure 2: Relative quantification of Pis s 1 and Pis s 2 using sets of monoclonal antibodies in sandwich assays

Monoclonal antibodies in sets (capture and detection antibodies) were used in a sandwich ELISA format and various protein preparations were quantified to determine the content of immunoreactive Pis s 1 and Pis s 2. The plot shows a unique ELISA illustrating the trend changes. The relative amount of the appropriate protein preparations was determined by comparison with the original substance (dehulled pea flour, DPF) set to 100% as shown in the first column (black = Pis s 1-ELISA, gray = Pis s 2-ELISA). Further processing steps: globulin precipitation (globulin, Figure 1), precipitation at pH4.5 resulting in pea protein isolate (PPI), acid hydrolysis of PPI (PPI-AH), and enzymatic hydrolysis of PPI (EH-PPI, enzyme 1 = Flavourzyme, enzyme 2 = papain and enzyme 3 = pepsin).

Supporting Table 1. Processed foods containing pea protein analyzed in this study.

Product	Company	Declaration	Basis of recovery calculation [%]	MW ppm	STD ppm	%	Recovery pea protein	Recovery soybean protein
Edgy Veggie roasted pea	Edgy Food, Birkerød, Denmark	100% Roasted green pea	100	791730.4	375604.4	79.2	79.2	
Vegan burger pattie	K-take it veggie (Kaufland trademark, Neckarsulm, Germany)	17% Pea protein	17	139747.8	30080.4	14.0	82.4	
Vegan pea balls	K-take it veggie (Kaufland trademark, Neckarsulm, Germany)	17% Pea protein	17	44070.7	35688.8	4.4	25.9	
Vegetarian Schinkenwurst	Berief Food, Beckum, Germany	Protein isolates (13% soybean, 50% pea)	6.5	29899.4	8027.3	3.0	46.0	
Vegetarian cold meat Schinkenart	veggyfriends, Hilden, Germany	4% Pea and potato protein	2	18624.0	4049.3	1.9	93.1	
Vegetarian cold meat Schinkenspiker	Rügenwalder Mühle, Bad Zwischenahn, Germany	2% Pea protein	2	135975.2	57305.4	13.6	679.9	
Vegetarian pork sausage (Fleischwurst)	Wiesenhof Geflügel, Visbek, Germany	4% Pea and potato protein	2	18528.7	2769.4	1.9	92.6	
Vegetarian Schinkenwurst	Berief Food, Beckum, Germany	Protein isolates (13% soybean, 50% pea)	6.5	248062.2	37731.1	24.8		381.6

The specified processed foods containing pea protein as declared were measured with the LADA multiplex assay and the listed recovery rates were determined. Soybean protein was determined as listed in gray. In all cases no lupin or peanut was detected. Samples were extracted with buffer containing 2% SDS for 30 min at 60 °C. Every sample was measured several times, at least on two different days in duplicate.