Pea (*Pisum sativum*) allergy in children: Pis s 1 is an immunodominant major pea allergen and presents IgE binding sites with potential diagnostic value

Jasmin Popp | Valérie Trendelenburg | Bodo Niggemann | Stefanie Randow | Elke Völker | Lothar Vogel | Andreas Reuter | Jelena Spiric | Dirk Schiller | Kirsten Beyer | Thomas Holzhauser

1 Division of Allergology, Paul-Ehrlich-Institut, Langen, Germany
2 Department of Pediatric Pulmonology, Immunology and Intensive Care Medicine, Charité Universitätsmedizin Berlin, Berlin, Germany

Correspondence
Thomas Holzhauser, Division of Allergology, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, Langen 63225, Germany.
Email: Thomas.Holzhauser@pei.de

Abstract

**Background:** Food allergy to pea (*Pisum sativum*) has been rarely studied in children at the clinical and molecular levels.

**Objective:** To elucidate the allergenic relevance and diagnostic value of pea 7S globulin Pis s 1, nsLTP, and 2S albumins PA1 and PA2 in children.

**Methods:** Children with pea-specific IgE ≥ 0.35 kUA/L and clinical evidence of pea allergy or tolerance were included in the study. IgE binding against pea total protein extract, recombinant (r) rPis s 1, rPA1, rPA2, and natural nsLTP was analysed using IgE immunoblot/inhibition. Mediator release potency was investigated in passively sensitized rat basophil leukaemia (RBL) 2H3-cells. IgE binding to synthetic overlapping peptides of Pis s 1 was detected on multipeptide microarrays.

**Results:** 19 pea-sensitized children were included, 14 with doctors’ diagnosed allergy and 5 with tolerance to pea (median age 3.5 and 4.5 years, respectively). 11/14 (78%) pea-allergic and 1/5 (20%) tolerant children were sensitized to Pis s 1. Under the reducing conditions of immunoblot analysis, IgE binding to rPA1 was negligible, sensitization to rPA2 and nsLTP undetectable. Compared to pea total protein extract, rPis s 1 displayed on average 58% IgE binding capacity and a 20-fold higher mediator release potency. Selected Pis s 1-related peptides displayed IgE binding in pea-allergic but not in pea-tolerant children.

**Conclusions and Clinical Relevance:** In this study group, Pis s 1 is a major immunodominant allergen in pea-allergic children. Evidence for sensitization to nsLTP and 2S albumins was low but requires further verification with regard to conformational epitopes. Recombinant Pis s 1 and related peptides which were exclusively recognized by pea-allergic children may improve in vitro diagnosis of pea allergy once verified in prospective studies with larger study groups.

**Keywords**
2S albumin, diagnosis, epitopes, IgE, lipid-transfer protein, pea allergy, Pis s 1, vicilin

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Legumes, including pea (Pisum sativum), are an important source of protein in the human diet. Pea has gained increasing interest in the manufacture of foods and has been advertised as a vegan hypoallergenic source of protein and other dietary components. Food allergy to legumes, such as peanut, soya bean and lupin, has been well documented, as was recently summarized. In foods, the use of these allergenic legumes as ingredients requires mandatory labelling in the EU, in order to enable allergic consumers to immediately identify and avoid these allergens. In addition, peanut and soya bean allergens have been characterized extensively on the molecular level. Especially the globulins and albums of the legume storage proteins have been associated with severe allergic reactions, as was recently summarized. Moreover, sensitization to peanut 2S albumin has been specifically associated with clinical reactivity to peanut.

By contrast, allergy to pea has been characterized to a lesser extent at the clinical and molecular level, and pea has not been assigned as an ingredient for mandatory allergen labelling in the EU. However, severe allergic reactions, including anaphylaxis, after ingestion of pea, have been described in children and adults. In a recent report of Canadian paediatric cases, anaphylaxis was related to unrecognized, so-called hidden pea protein.

While the true prevalence of pea allergy is generally unknown, the rate of sensitization against pea was higher than that for peanut, soya bean, or lupin in a German adult cohort. Hence, allergy to pea and the characterization of pea allergens should gain more attention.

The protein content of pea seeds is on average 22%, and the majority of extractable pea proteins are globulins with an average content of 70% of the total protein. So far, only three pea proteins have been registered as allergens. Pis s 1 and Pis s 2 are globulin storage proteins and have been suggested as potential major pea allergens based on natural purified preparations and serum pools, but only few individual data on IgE binding were shown. Pis s 1 is a 44 kDa vicilin, whereas Pis s 2 is described as a 63 kDa convicilin. Pis s 1 and Pis s 2 have a high sequence identity (~70%) and share serological cross-reactivity to raised antibodies, with the major difference being an N-terminal insertion in the sequence of Pis s 2. Pis s 3 belongs to the prolamin superfamily and is a 9.5 kDa non-specific lipid-transfer protein (nsLTP) that was recently identified and structurally characterized as a putative food allergen with IgE cross-reactivity to Pru p 3, a nsLTP and major peach allergen. Despite IgE cross-reactivity to Pis s 3 in Pru p 3-sensitized subjects, the authors did not provide evidence that the human serum donors had a history of pea allergy.

Compared with other legumes, such as peanut and soybean, no albumin has yet been described as an allergen in pea. However, it is reported that the albumin fraction might also include allergenic proteins. The albumin fraction of pea contains two low-molecular weight major protein components, the sulphur-rich pea albumin 1 (PA1) and the pea albumin 2 (PA2). PA1 accounts for less than 10% of the pea seed total protein. The 11 kDa PA1 proprotein can be proteolytically cleaved into two mature proteins, PA1a (~6 kDa) and PA1b (~4 kDa) that are not linked by interchain disulfide bonds, thus potentially leading to two separate monomers.

The content of PA2 in pea seeds is comparable to PA1. PA2, that shows high sequence homology with a 2S albumin from lentil, is a homodimer consisting of the two subunits PA2a or PA2b of approx. 26 kDa each that show high sequence identity. The relevance of both pea albums, PA1 and PA2, as potential allergens in pea-allergic patients is still unclear. Besides two studies by Vioque et al, who analysed chickpea-sensitised individuals for their IgE binding to purified natural PA1 and PA2 in dot blot analysis, no IgE binding data are currently available.

Thus, the aim of this study was to further elucidate the relevance of individual pea allergens, in particular the 7S pea allergen Pis s 1, the low-molecular weight 2S albums PA1 and PA2, and pea nsLTP (Pis s 3), in a paediatric population of pea-allergic and pea-sensitized but tolerant subjects. In this work, we describe a clear immunodominance of Pis s 1, with regard to IgE binding capacity and basophil mediator release potency in comparison with pea total protein extract. Further, linear IgE binding areas of Pis s 1 were explored for their potential diagnostic value in comparison with the full-length allergen.

2.1 | Patients and serum controls

Children between one and 18 years of age with pea-specific IgE ≥ 0.35 kU/L (ImmunoCAP, Thermo Fisher Scientific) were included in the study. No skin prick tests were performed. Clinical evidence was determined according to either a convincing history or oral food challenge. A convincing history for a clinically confirmed pea allergy was defined as parent reported clear objective allergic immediate-type reactions after pea consumption of the child whereas a convincing history for pea tolerance was the consumption of pea without symptoms. The oral food challenges were performed using fresh green peas (protein content 5.4 g/100 g) using to a standardized protocol according to PRACtALL guidelines. The cumulative dose was 100 g green peas. The challenge was performed using seven increasing dose steps (0.068 g-0.22g-0.68 g-2.2 g-6.8 g-22 g-68 g) at time intervals of 30 minutes and in case of the absence of allergic reactions, cumulative dose (100 g) was given on another day. Cumulative dose means, that the combined amount of the seven increasing dose steps is given in a single challenge meal. Demographic and clinical data from patients included age, sex, pea-related symptoms and severity of symptoms. Severity was graded on a scale from I to V using a grading system according to Sampson. In microarray analysis, sera from five non-atopic (A-E) [ImmunoCAP fx5 [egg white, milk, fish, wheat, peanut, soya bean] negative; ImmunoCAP SX1 [Dermatophagoides pteronyssinus, cat dander, dog dander, timothy grass, cultivated rye, Cladosporium herbarum, common silver birch, mugwort] negative] and five atopic (F-J) (fx5 negative; SX1 positive) subjects served as controls. All serum samples were collected at the department of paediatric pulmonology, immunology and intensive care medicine, Charité-Universitätsmedizin Berlin with written informed consent. The study was approved by the ethics
committee of the Charité-Universitätsmedizin Berlin (EA2/138/10) and conformed to the standards of the Declaration of Helsinki. Serum from a non-allergic subject served as negative control in immunoblot experiments (PEI231, in-house serum collection). Serum LTP6 of a LTP-sensitized subject with 23.7 kU/L sIgE binding to peach nPru p 3 (ImmunoCAP f420), and to a putative 12 kDa LTP of the legume Lablab purpureus\textsuperscript{26} served for the verification of nLTP in pea total protein extract.

\section*{2.2 Pea total protein extraction}

Dried mature pea seeds of \textit{Pisum sativum} cultivar Regina (Saatzucht Aschersleben) were washed with distilled water, dried overnight, ground to flour under liquid nitrogen and extracted 1:10 (w/v) in phosphate-buffered saline, pH 7.4 (4 hours, 4°C). After centrifugation (4°C, 13 000g, 30 minutes), the supernatant was filtered (0.22 µm pore size, polyethersulfone, Carl Roth GmbH + Co. KG). Protein was determined using Roti\textsuperscript{®}-Nanoquant Bradford reagent (Carl Roth GmbH + Co. KG).

\section*{2.3 Generation and purification of recombinant pea proteins}

The codon-optimized cDNA sequence (GeneArt Strings DNA Fragments, Thermo Fisher Scientific Geneart GmbH) encoding Pis s 1.0101 (Uniprot acc. no. Q702P1) was inserted into vector pET11a and expressed in \textit{Escherichia coli} One Shot\textsuperscript{®} BL21 (DE3) cells. The pea albumins PA1 (Uniprot acc. no. P62927, aa 27-130) and PA2 (Uniprot acc. no. P08688) were codon-optimized (GeneArt Strings DNA Fragments), inserted into vector pPICZaA and expressed in \textit{Pichia pastoris} (X-33). rPis s 1 was purified from inclusion bodies while rPA1 and rPA2 were purified as soluble proteins from culture supernatant (details including full-length sequence and purification in Table S1).

\section*{2.4 Confirmation of pea protein sequences by mass spectrometry}

The identity of the recombinant proteins rPA1, rPA2 and rPis s 1, and the presence of nPA1 and nPA2 in pea total protein extract was confirmed by liquid chromatography-mass spectrometry (LC-MS\textsuperscript{5}) after separation using SDS-PAGE as described earlier\textsuperscript{27} with slight modifications (details in Appendix S1).

\section*{2.5 Physicochemical characterization of recombinant pea proteins}

Secondary structure of rPis s 1, rPA1 or rPA2 was analysed by circular dichroism (CD) spectroscopy. Spectra were recorded between 255 and 185 nm at a bandwidth of 1 nm. Hydrodynamic radii ($R_d$) of either rPis s 1, rPA1 or rPA2 were determined at 25°C using dynamic light scattering (DLS) (details in Appendix S1).

\section*{2.6 SDS-PAGE and IgE immunoblot analysis}

rPis s 1, rPA1 and rPA2 were separated under reducing conditions using pre-cast NuPAGE\textsuperscript{™} 4%-12% Bis-Tris gels and either stained with Coomassie Brilliant Blue G250 for visualization, or semi-dry blotted onto nitrocellulose membranes for immunoblot analysis. For blotting, rPA1 (12 587 Da) and rPA2 (27 553 Da) were combined and separated in one lane because of lack of cross-reactivity. Strips were blocked (2% powdered milk), incubated overnight with diluted patients’ sera and detected using horseradish peroxidase labelled mouse anti-human IgE (SouthernBiotech, via Biozol) and chemiluminescent substrate LumiGLO Reserve\textsuperscript{™} (KPL). Chemiluminescence was recorded (luminescence imager), and Pis s 1-specific IgE (kU/\textit{L}) quantified densitometrically, based on calibrated signal intensity. Cut-off for positive results was defined as two times the densitometric volume of the non-allergic control serum, that is on average 6.0 kU/L (details in Appendix S1).

\section*{2.7 IgE immunoblot inhibition analysis}

A total of 20 µg/cm pea extract protein were separated by SDS-PAGE under reducing conditions and blotted as described above. Patients’ sera were pre-incubated with 50 µg of inhibitor protein, that is rPis s 1, rPA1 or nPru p 3, or buffer for 2 hours and subsequently overnight on blocked blot strips. Immunodetection was done as described above. Signal intensity was recorded densitometrically. After subtraction of signal intensity of non-allergic negative control serum, per cent inhibition was calculated for every patient as follows:

\begin{equation}
100 - \frac{\text{Signal intensity of serum inhibited by rPis s 1}}{\text{Signal intensity of serum uninhibited (buffer)}} \times 100.
\end{equation}

\section*{2.8 Mediator release from humanized rat basophilic leukaemia (RBL) 2H3-cells}

The mediator release assay was carried out as previously described.\textsuperscript{28} Briefly, RBL-2H3 cells expressing the α-chain of the human IgE receptor FceRI were sensitized overnight with a 1:10 diluted serum pool of pea-allergic patients (nos. 1, 3, 4, 8, 11). Cells were stimulated with serial dilutions of either rPis s 1, pea extract or BSA as negative control. Total release was determined by lysing the passively sensitized cells with 1% Triton X-100 (Sigma Aldrich Chemie GmbH). Specific mediator release was determined photometrically on the basis of β-hexosaminidase activity in cell culture supernatant. Results were expressed as per cent of total mediator release after correction for spontaneous release.
2.9 | Generation and immunodetection of Pis s 1 multipeptide microarrays

101 Celluspot™ peptides (15 amino acids, 4 amino acids offset) covering the entire primary structure of Pis s 1.0101 (Uniprot number Q702P1) were synthesized, as previously described, on cellulose support, and spotted in quadruplicate onto microscope slides (Intavis AG). DMSO solvent was multiply spotted as a control for background signal. Blocked array slides were incubated overnight with 45 µL of defatted serum sample (Table 1). Bound IgE was detected on hyperfilm using horseradish peroxidase labelled mouse anti-human IgE (SouthernBiotech) and LumiGLO Reserve™ substrate (details in Appendix S1).

2.10 | Microarray data analysis

Developed films were scanned as 16-bit greyscale TIF files, and the inverted signal intensity of each spot was analysed using TIGR Spotfinder 3.2.1 software. Signal intensities were transformed into Z-scores, as previously described with some modifications. Briefly, the median signal intensity (Si) was calculated for all

| TABLE 1 | Characteristics of pea-allergic and sensitized but clinically tolerant childrena |
|---|---|
| No. | Age (years)d | Clinical evidence | Clinical relevance/ outcome OFC | Threshold dose of OFC (g) | Pea-related symptoms | Severity gradinge | Total IgE (kU/L), cut-off 0.35 (ImmunoCAP) | Pea sIgE (kU/L), cut-off 0.35 (ImmunoCAP) | rPis s 1 sIgE (kU/L), cut-off 6.0 (ImmuNBlot) | % IgE inhibition of pea extract by rPis s 1 (ImmuNBlot) |
| 1 | 3/m | History | Yes | n.a. | F, PU | I | 2239 | 92.3 | 42.7 | 39.1 |
| 2 | 15/m | History | Yes | n.a. | Em, N | II | nd | 5.5 | 11.8 | 19.1 |
| 3 | 1/f | History | Yes | n.a. | U, AE | II | 104 | 32.1 | 29.8 | 96.6 |
| 4 | 2/m | History/ OFC | Yes/positive | 6.8 | F/GU | I/II | 226 | 54.7 | 45.6 | 60.6 |
| 5 | 3/m | OFC | Yes/positive | 22 | GU | II | nd | 2.0 | 8.0 | n.a |
| 6 | 12/f | History | Yes | n.a. | AE, Em | II | nd | 1.5 | 8.4 | n.a |
| 7 | 2/m | History | Yes | n.a. | AE | II | nd | 1.2 | (5.2) | n.a |
| 8 | 4/m | DBPCFC | Yes/positive | 100 | PU, P, R | III | nd | 45.3 | 23.7 | 57.9 |
| 9 | 1/f | OFC | Yes/positive | 0.22 | F, C, R | III | nd | 0.9 | (5.0) | 26.1 |
| 10 | 6/m | History/ OFC | Yes/positive | 100 | PU, R, IT | II/III | nd | 8.9 | 16.1 | 81.6 |
| 11 | 6/m | History | Yes | n.a. | Em, GU, W, D | IV | 141 | 23.1 | 22.5 | 98.2 |
| 12 | 7/f | History | Yes | n.a. | Nb | II | 459 | 5.1 | (5.6) | 0 |
| 13 | 4/m | History | Yes | n.a. | AE | II | nd | 2.1 | 6.3 | n.a |
| 14 | 2/m | History | Yes | n.a. | Em, SA | II | 2748 | 6.5 | 6.2 | 99.9 |
| 15 | 2/m | History | No | n.a. | None | n.a. | 2813 | 1.3 | (5.2) | n.a |
| 16 | 12/m | OFC | No/ negative | n.a. | PR, SAc | n.a. | nd | 4.0 | 6.9 | 65.6 |
| 17 | 7/m | DBPCFC | No/ negative | n.a. | None | n.a. | 152 | 1.0 | (5.6) | 0 |
| 18 | 2/m | History | No | n.a. | None | n.a. | 64.6 | 0.4 | (5.1) | n.a |
| 19 | 8/m | History | No | n.a. | None | n.a. | 7663 | 3.6 | (5.2) | n.a |

Note: sIgE values in parenthesis are below method cut-off.

Abbreviations: AE, angioedema; C, conjunctivitis; D, dyspnoea; DBPCFC, double-blind placebo-controlled food challenge; Em, emesis; f, female; F, flush; GU, generalized urticaria; IT, itching throat; m, male; N, nausea; n.a., not applicable; n.d., not determined; OFC, open food challenge; P, pruritus; PE, perioral erythema; PR, perioral redness; PU, perioral urticaria; R, rhinitis; SA, stomach ache; U, urticaria; W, wheezing.

aThe severity grading is based on the documented or reported symptoms as well as on their documented or reported severity of the specific symptoms (ie mild localized flushing (severity grade I) vs. generalized marked flushing (severity grade II)).

bThe patient had at a later occasion another accidental reaction after consumption of processed pea protein resulting in itching throat and angioedema.

cMild perioral redness as well as stomach ache occurred to the 7th dose of OFC. Due to these inconclusive symptoms, the cumulative dose of 100 g pea was given on another day, which was tolerated without any symptoms. Hence, the challenge was judged as negative.

dAge at time of blood sampling.

eSeverity grading according to the grading system developed by Sampson.24
quadruplicate peptide signals within the same array. The constant mean (m) of the DMSO spots and its standard deviation (s) were determined. A Z-score was calculated for each Pis s 1.0101-derived peptide.

Non-specifically detected peptides were removed by subtracting the maximum Z-score of the controls (serum A-J) of the respective peptides. IgE binding to a peptide with a Z-score > 2 was defined as positive. For specificity analysis using two serum pools ( sera 1/4/8 and 3/10/11/13) and rPis s 1 as inhibitor, Z-scores were calculated for each serum pool with/without rPis s 1 inhibition (details in Appendix S1).

### 2.11 Selection of peptides exclusively recognized by pea-allergic children

Peptides exclusively recognized by serum IgE of pea-allergic children were included with a median Z-score > 5 to ensure sufficiently high signal intensity. Further, a minimal inhibition of 30% of peptide IgE binding by rPis s 1 in at least one of the used serum pools was required to confirm Pis s 1 specificity. Between two selected candidate peptides, an offset of at least 4 peptides was applied to ensure that the peptides display non-overlapping amino acid sequences. In the case of multiple peptide hits, those with a high frequency of patient recognition were preferred. If several peptides showed identical frequency of recognition by rPis s 1 in at least one of the used serum pools was required to confirm Pis s 1 specificity. Between two selected candidate peptides, an offset of at least 4 peptides was applied to ensure that the peptides display non-overlapping amino acid sequences. In the case of multiple peptide hits, those with a high frequency of patient recognition were preferred. If several peptides showed identical frequency of recognition, such peptides with the highest median Z-score were selected. Of these preselected peptides, those that most frequently bound IgE (median frequency of preselected peptides) were finally selected.

### 2.12 Modelling of Pis s 1

The structure of natural Pis s 1 (UniProt acc. Q702P1), that is without start codon, thrombin cleavage site, linker or His<sub>6</sub>-tag, was modelled using SWISS-MODEL Workspace (https://swissmodel.expasy.org/). The beta subunit of soya bean beta-conglycinin (PDB ID 1uij.1.A), having a sequence identity of 59%, was used as template. The modelled Pis s 1 structure was visualized using The PyMOL Molecular Graphics System (Version 1.5.0.4 Schrödinger, LLC).

### 3 RESULTS

#### 3.1 Study population

Sera from nineteen children (79% male, median age 4 years (range 1-15 years)) with pea-specific IgE ≥ 0.35 kUa/L with clinical evidence on pea allergy or tolerance were included into this study. Clinical characteristics are summarized in Table 1. Fourteen of these children had confirmed doctor’s diagnosed pea allergy, whereas five were clinically tolerant. Pea allergy was diagnosed by oral food challenge (5/14 children) or according to convincing history alone (9/14 children) at the time of indicated patient’s age. Although in two of the oldest patients (nos 2 and 6) with pea allergy the specific time-point of recurrent and/or reproductive symptoms (accidental reactions to pea) could not be evaluated from the patient chart, the allergist in the outpatient clinic of our tertiary care centre diagnosed the patient with clinical relevant pea allergy at the given time-point.

Most pea-allergic patients experienced allergy symptoms ranging from mild to moderate (i.e., urticaria, emesis, angioedema or rhinitis) severity, while one patient reported on two severe reactions with wheezing and dyspnoea. Concerning the youngest patients between 1 and 3 years of age (nos. 1, 3, 7, 14), symptoms were reported without specific time-points or at 2 years of age (no. 7). Concerning patients between 4 and 7 years of age (nos. 11, 12, 13), symptoms were reported between 2 and 12 months before the doctor’s appointment. Concerning the oldest patients (nos. 2 and 6), recurrent or reproductive symptoms were reported during the doctor’s appointment without a specific time-point. Tolerance was diagnosed by oral food challenge in two of the five children and in the remaining three according to convincing history. Pea-specific IgE ranging from 0.9 to 92.3 kUa/L was elevated in the pea-allergic group (median 6.0 kUa/L) compared with 0.4 to 4.0 kUa/L in the tolerant group (median 1.3 kUa/L). About 64% of pea-allergic patients (9/14) had a doctor’s diagnosed allergy to other legumes in parallel, including peanut (n = 7), lentils (n = 2), soy (n = 1), lupin (n = 1) and/or beans (n = 1).

#### 3.2 Expression, purification and physicochemical characterization of recombinant proteins

Expression of rPA1 and rPA2 in Pichia pastoris and of rPis s 1 in Escherichia coli resulted in full-length pea proteins. The his-tagged proteins were purified by immobilized metal affinity chromatography and, if necessary, by size-exclusion chromatography. Identity of full-length proteins was confirmed by mass spectrometry (Table S2a,b). In Coomassie-stained SDS-PAGE (Figure S1a), purified preparations of recombinant rPis s 1, rPA1 and rPA2 displayed molecular masses of approximately 50, 14 and 28 kDa, respectively. Secondary structure was analysed using CD spectroscopy (Figure S1b). rPis s 1 showed spectral characteristics that are comparable to published data of the homologous soy 7S globulin Gly m 5,32 indicating a native-type secondary structure of rPis s 1. The CD spectrum of rPA1 showed characteristics, comparable to those of peanut 2S albumin Ara h 2,33 and typical for α-helical proteins. By contrast, rPA2 displayed high content of unstructured protein.

The hydrodynamic radii <i>R</i><sub>hi</sub> indicated multimeric rPis s 1 and rPA2, in contrast to monomeric rPA1 (details in Appendix S1, inset Figure S1b).

#### 3.3 Pis s 1 is a major allergen in pea-allergic children

In Immunoblot analysis of pea total protein extract, the paediatric sera showed a diverse pattern of protein bands that bound serum IgE (Figure 1A, uninhibited lane). For 3 of the pea-allergic children
(patients nos. 5, 6, 7) and 3 of the pea-tolerant children (patients nos. 15, 18, 19), no serum IgE binding to pea total protein extract was detectable on immunoblots in contrast to the more sensitive pea ImmunoCAP (Table 1).

Specific IgE binding was further investigated using the recombinant pea proteins rPis s 1, rPA1 and rPA2. 11/14 (78%) pea-allergic children showed serum IgE binding to rPis s 1 (Figure 1B), considering an average cut-off for positivity of 6.0 kUA/L. Natural Pis s 1 undergoes post-translational proteolytic cleavage resulting in subunits of M₉, 36 to 12.5 kDa. In recombinant rPis s 1, degradation products with molecular masses between 20 kDa and 30 kDa were also detected with serum IgE. In pea-allergic patients’ nos. 1, 3, 4, 8, 10, and 11, rPis s 1 showed the strongest IgE binding capacity. Only 1/5 (20%) of pea-tolerant children (Figure 1B) displayed

![Figure 1](image)

**Figure 1** A, IgE immunoblot of sera 1-19 (Table 1) to pea extract. Serum samples were pre-incubated with rPis s 1 (+) or untreated (−). Arrows indicate position of full-length Pis s 1 and its proteolytic subunits. B, IgE immunoblot of sera 1-19 to rPis s 1 (49.1 kDa). Filled triangle, full-length rPis s 1; open triangles, degradation products of rPis s 1. C, IgE immunoblot of sera 1-19 to rPA1 (12.6 kDa, open triangle) and rPA2 (27.6 kDa, filled triangle). (M, low-molecular weight marker; P, total protein stained with PonceauS; N, non-allergic serum control; N1 or N2, non-allergic serum control on membrane 1 or 2)
weak sIgE binding to rPis s 1 (Table 1, Figure 1B). In the pea-allergic children, rPis s 1-specific IgE levels were elevated, ranging from 6.2 to 45.6 kU/L (Table 1, median 16.1 kU/L). This finding correlates well ($R^2 = 0.8346$, linear regression of rPis s 1 sIgE vs. pea sIgE) with the elevated sIgE levels against pea extract in pea-allergic children (Figure 2A, Table 1). Except for patient no. 1, none of the patients showed IgE binding to rPA1 and rPA2 (Figure 1C) in IgE immunoblot analysis under reducing conditions. The relevance of this finding is addressed in detail in the discussion.

3.4 High IgE binding capacity of Pis s 1 compared to pea total protein

To further address the relevance of Pis s 1 in this study population, the IgE inhibition capacity of rPis s 1 against pea total protein extract was determined densitometrically on immunoblots (Figure 1A, Table 1). On average, rPis s 1 inhibited 58% of the IgE binding capacity of pea total protein extract in the evaluable pea-allergic subjects (nos. 1, 2, 3, 4, 8, 9, 10, 11, 12, 14). For the remaining subjects, as serum IgE binding to pea extract protein was undetectable (nos. 5, 6, 7) or comprised by high background signal (no. 13), calculation of inhibition was not applicable. In four pea-allergic children (patients nos. 3, 10, 11, 14), rPis s 1 inhibited IgE binding to the extract almost completely, in other pea-allergic children (patients nos. 1, 2, 4, 8, 9) and one pea-tolerant child (16) partially. No inhibition could be observed in pea-allergic patient 12 and in pea-tolerant patient 17. rPis s 1 was able to inhibit IgE binding in the lower molecular weight (LMW) range (Figure 1A, patient nos. 3, 4, 11, 14) where mass spectrometry confirmed the presence of PA1 isoforms and PA2 (Figure S2, Table S2b). In addition, PA1 was confirmed as 11 kDa full-length proprotein instead of 6 and 4 kDa proteolytic fragments (data not shown). IgE binding to LMW proteins was also related to post-translational proteolytic fragments of Pis s 1 that were fully or partially inhibited by rPis s 1 (Figure 1A). Moreover, rPis s 1 could also inhibit IgE binding to a protein located at 70-80 kDa (Figure 1A, patients no. 3, 10 and 11) which could resemble either a multimer of Pis s 1 fragments or possibly Pis s 2 (convicilin). No inhibition in this molecular weight range (>66 kDa) could be observed for patients 1 and 4 (allergic) and 16 (tolerant). Only pea-allergic patients nos. 1, 4 and 8 and pea-tolerant patient 16 showed, after the excess addition of rPis s 1, uninhibited IgE binding to pea proteins in the LMW range < 30 kDa that could potentially constitute 2S albumins or nsLTP. However, only patient 1 showed IgE binding to recombinant PA1. In two subjects, pea-allergic patient 1 and pea-tolerant patient 16, an IgE binding protein band at or below 14 kDa remained after rPis s 1 inhibition (Figure 1A, Figure S3a). Using nPru p 3 as inhibitor (Figure S3b), or natural peach nsLTP (nPru p 3) that was purified from peach peals as described previously16 (Figure S3c), the IgE binding to this protein remained. In addition, serum LTP6 of a LTP-sensitized donor showed IgE binding to this 14 kDa protein and to an additional protein below (Figure S3c).

Using nPru p 3, as inhibitor, only the IgE binding below 14 kDa disappeared. The results indicate that the pea nsLTP, presumably Pis s 3, is present in the pea total protein extract. Despite the verified presence of PA1 and PA2, and strong evidence for the presence of pea nsLTP in our pea extract, none of the pea-allergic or tolerant patients displayed detectable IgE binding to these proteins in pea total protein extract using IgE immunoblot analysis under reducing conditions.

Hence, in 11/14 (78.6%) pea-allergic children, Pis s 1 is a major allergen with a high IgE binding capacity that directly correlates to that of total pea protein. LMW proteins < 30 kDa in pea total protein extract, that are not related to Pis s 1 fragments, did not seem to play a major role in the IgE binding capacity of pea-allergic children.
3.5 | High functional mediator release potency of rPis s 1

RBL 2H3 cells were passively sensitized with an IgE serum pool of five pea-allergic children (patients’ nos. 1, 3, 4, 8, 11) that showed high levels of IgE to Pis s 1 as compared to total pea protein (Table 1). rPis s 1 and pea extract induced a comparably high basophil degranulation with maximal mediator release of around 64% relative to total release. The amount of protein required for half-maximal mediator release (EC_{50}) was approximately 20-fold higher for pea extract (2.3 ng/mL) than for rPis s 1 (0.1 ng/mL).

3.6 | Apparent differences in IgE binding to Pis s 1 peptides between pea-allergic and tolerant children

Signal intensity of individual peptide IgE binding to synthetic overlapping peptides, that constitute the entire primary sequence of Pis s 1, was expressed as Z-score. Figure 3 displays the IgE binding frequency to each Pis s 1 peptide (Z-score > 2) of pea-allergic in comparison with pea-tolerant children.

14/14 (100%) pea-allergic children and 4/5 (80%) pea-tolerant children showed IgE binding (Z-score > 2) to at least one peptide of Pis s 1 (Table S3). IgE of pea-allergic children bound more peptides (median 31) than of pea-tolerant children (median 1) and consequently showed a higher IgE binding diversity (Table S3).

3.7 | Pis s 1 peptides potentially indicative for pea allergy

IgE of pea-allergic children recognized various peptide stretches that were not recognized by the IgE of tolerant children (Figure 3: peptides 3-5, 14-29, 31-33, 38-47, 49-51, 53-54, 58-68, 73-79, 97-100). Thereof, eleven peptides fulfilled the selection criteria as described in the methods section (peptides 3, 14, 20, 28, 44, 53, 64, 74, 78, 93, 100) (Figure 3, Table S3). Their respective amino acid sequences and position on the modelled molecular surface of Pis s 1 are shown in Figure S4. Candidate peptides are distributed over large areas of the Pis s 1 primary protein sequence and molecular surface with no predominant location. 13/14 (93%) pea-allergic children showed an IgE binding to at least one of the eleven selected candidate peptides (Table S3). Among these 13 pea-allergic children, the number of recognized candidate peptides ranged from 1 to 11 (median 6), whereas serum IgE from pea-tolerant children did not bind to any of these peptides.

4 | DISCUSSION

We investigated the relevance of individual pea allergens in relation to pea total protein in a paediatric population of pea-allergic and pea-tolerant subjects.

The recombinant 2S albumins rPA1 and rPA2 were expressed in P pastoris for reasons of protein folding and a simple purification from cell culture supernatant. The 7S globulin rPis s 1 was not successfully expressed in P pastoris (data not shown) but in E coli. Purified rPis s 1 and rPA1 presented characteristic secondary structural elements related to allergens of the 7S and 2S storage proteins, such as soya bean Gly m 5 and peanut Ara h 2.12,33 However, rPA2 displayed a high content of unstructured protein.

Sanchez-Monge et al 12 first described Pis s 1 as a major pea allergen on the basis of IgE inhibition of pea extract with the lentil 7S vicilin Len c 1 using a serum pool. In this study, for the first time the pea 7S globulin allergen Pis s 1 was investigated for its IgE binding capacity on the basis of purified recombinant allergen and individual serum analysis (Figure 1B). Here, serum IgE binding in 11/14 (78%) pea-allergic children confirmed rPis s 1 as a major pea allergen. Moreover, the recombinant 2S albumins rPA1 and rPA2 did not bind IgE from sera of pea-allergic children, except for rPA1 and one serum. Although the results may suggest that the 2S albumins rPA1 and rPA2 are not relevant allergens in pea, we like to stress that possible conformational epitopes of the 2S albumins may have been overlooked. Reasons are the experimental conditions of electrophoretic separation prior to immunoblot analysis and the observation of partially unstructured rPA2. While the rPA1 appeared to be in good shape, the lack of IgE binding possibly carries less weight of evidence than positive results, as in the case of rPis s 1.

The relevance of Pis s 1 and other pea allergens was further investigated in densitometric IgE immunoblot inhibition experiments of native pea total protein extract with rPis s 1 as inhibitor. rPis s 1 showed a high IgE binding capacity that resulted on average in 58% inhibition of IgE binding to pea total protein (Figure 1A, Table 1). In some patients, rPis s 1 completely abolished IgE binding to pea total protein extract, even at distinct low-molecular weight (LMW) bands. This observation was in accordance with reported post-translational proteolytic cleavage that results in subunits.12,34
of comparable molecular weight. In all but two patients (nos. 1, 16), IgE binding to these LMW proteins was almost completely inhibited by rPis s 1 (Figure 1A). Although the presence of PA1 and PA2 in pea total protein extract was shown using mass spectrometry (Figure S2, Table S2b), and PA1 was also found as a proprotein, our recombinant preparation of rPA1 did not inhibit IgE that was bound to LMW extract proteins (Figure S3). Again, the results suggest that PA1 is not a relevant allergen in pea. However, in the case of natural PA1, which is also proteolytically cleaved into two subunits, it cannot be fully excluded that the electrophoretic conditions caused potential subunit separation that may result in loss of serum IgE binding to possibly relevant conformational epitopes.

The presence of an nsLTP in pea total protein extract, likely the registered allergen Pis s 3, was identified in IgE immunoblot analysis using an additional LTP-sensitized subject and was further confirmed by IgE inhibition using Pru p 3, the natural peach nsLTP. However, the detected and inhibited IgE binding protein had a lower molecular weight than that detected by serum IgE of the pea-allergic or pea-sensitized and tolerant children. In summary, pea nsLTP, and the 2S albums PA1 and PA2 were present in pea total protein extract but we had little evidence of IgE binding in this study group of pea-allergic children under the applied experimental conditions.

This is in contrast to other legumes such as soybean or peanut where the 2S albums Gly m b35,36 and Ara h 27,37,38 are reported as major allergens, in the case of Ara h 2 even as one of the most important peanut allergens. Bogdanov et al15 described the pea nsLTP Pis s 3 on the basis of serological cross-reactivity to Pru p 3 sensitized “food allergic” individuals. We were able to confirm this IgE cross-reactivity between Pru p 3 and pea nsLTP. However, we did not confirm pea nsLTP as an allergen in our paediatric study group.

Further, the IgE immunoblot analysis of pea protein extract revealed only some sera with IgE binding to proteins at molecular weight above that of Pis s 1 (44 kDa). The pea convicilin Pis s 2, which was first described as an IgE binding protein by Sanchez-Monge,12 was reported with a molecular weight of 63 kDa. Assuming that uninhibited higher molecular weight proteins with IgE binding capacity constitute Pis s 2, this allergen would likely be relevant in only two pea-allergic children (nos. 1, 4) and, thus, present a minor allergen in this study. However, we cannot exclude that additional uninhibited IgE binding protein bands at or below that of Pis s 1 constitute Pis s 2 or fragments thereof.

In addition to a high IgE binding capacity of Pis s 1, the ability to cross-link specific IgE on effector cells was investigated. Hence, humanized RBL cells were passively sensitized with IgE of a serum pool of pea-allergic children. The amount of protein required for half-maximal mediator release was approximately one order of magnitude higher for pea extract compared to rPis s 1 (Figure 2B). Even though the exact ratio of vicilin to total protein in this extract preparation is unknown, a major portion of the allergenic potency of pea total protein extract appears to be related to Pis s 1, when taking into account the relative position of both curves and the fact that vicilin constitutes between 26% and 52% of total extractable pea protein.11 Considering the high frequency of sensitization to Pis s 1 and its high allergenic potency, Pis s 1 was analysed in depth for its linear IgE binding areas making use of overlapping synthetic peptides to represent the primary sequence. Spotted onto multipeptide microarrays, the IgE binding peptides were resolved for each individual serum of pea-allergic and sensitized but tolerant children. Special emphasis was on the identification of potential diagnostic peptides. We are aware that the study groups of pea-allergic (n = 14) and pea-sensitized but tolerant (n = 5) children are fairly small. Thus, the calculation of diagnostic metrics, such as receiver operating characteristic (ROC) curve analysis, was omitted. Nonetheless, eleven peptides which exclusively bound serum IgE from children in the pea-allergic group were identified. These peptides should be further investigated for their diagnostic value in an independent prospective study with larger study groups.

Our study was principally based on immunoblots that were performed under reducing conditions and on IgE binding to synthetic peptides. Consequently, the study focused rather on IgE binding to sequential than conformational epitopes. The RBL cell assay could have been useful to study the importance of the conformational epitopes for those pea proteins, such as the 2S albums PA1 and PA2, and the nsLTP Pis s 3, for which conformation might be critical for their allergenic potency. Owing to the fact that rPis s 3 was not available to this study and that the serum donations were too limited to investigate all allergen preparations in mediator release experiments, our results are mainly based on immunoblot analysis which revealed an IgE immunodominance of Pis s 1 when compared to pea total protein. Nonetheless, additional investigations with a focus on conformational epitopes should clarify whether our findings of limited IgE binding to the 2S albums PA1 and PA2, and to nsLTP were due to conformational epitopes and thus potentially underestimated. Such studies can include, for example, the investigation of properly folded purified natural 2S albums and nsLTP of pea under native conditions such as provided in IgE-ELISA or mediator release experiments.

Another limitation of our analysis was the use of different methods and thus reporting units for pea extract (ImmunoCAP fluorescence), Pis s 1 (immunoblot densitometry) and Pis s 1 peptides (microarray densitometry). Subsequent prospective analyses should make use of one diagnostic method, such as ImmunoCAP, in order to obtain comparable reporting units for the calculation of diagnostic metrics.

In conclusion, we were able to show that Pis s 1 is an immunodominant major allergen in pea-allergic children and presents distinct IgE binding sites with a potential diagnostic value. Low-molecular weight proteins, such as 2S albums or nsLTP, did not show major IgE binding capacity in this study group of pea-allergic children. However, these findings should be refined using experimental conditions that in particular allow for the investigation of conformational IgE epitopes in these protein families.

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CONFLICT OF INTEREST
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ORCID
Dirk Schiller https://orcid.org/0000-0002-6315-9161
Thomas Holzhauser https://orcid.org/0000-0002-7818-7261

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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