

# The conformational IgE epitope profile of soya bean allergen Gly m 4

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## Clinical & Experimental Allergy

### Summary

**Background** Birch pollen-related soya allergy is mediated by Gly m 4. Conformational IgE epitopes of Gly m 4 are unknown.

**Objective** To identify the IgE epitope profile of Gly m 4 in subjects with birch pollen-related soya allergy utilizing an epitope library presented by Gly m 4-type model proteins.

**Methods** Sera from patients with ( $n = 26$ ) and without ( $n = 19$ ) allergy to soya as determined by oral provocation tests were studied. Specific IgE (Bet v 1/Gly m 4) was determined by ImmunoCAP. A library of 59 non-allergenic Gly m 4-type model proteins harbouring individual and multiple putative epitopes for IgE was tested in IgE binding assays. Primary, secondary and tertiary protein structures were assessed by mass spectrometry, circular dichroism and nuclear magnetic resonance spectroscopy.

**Results** All subjects were sensitized to Gly m 4 and Bet v 1. Allergen-specific serum IgE levels ranged from 0.94 to  $> 100$  kU<sub>A</sub>/L. The avidities of serum IgE were 5.06 ng (allergic) and 1.8 ng (tolerant) as determined by EC<sub>50</sub> for IgE binding to Gly m 4. 96% (46/48) of the protein variants bound IgE. Model proteins had Gly m 4-type conformation and individual IgE binding clustered in six major surface areas. Gly m 4-specific IgE binding could be inhibited to up to 80% by model proteins harbouring individual IgE binding sites in an epitope-wise equimolar fashion. Receiver operating curve analysis revealed an area under fitted curve of up to 0.88 for model proteins and 0.66 for Gly m 4.

**Conclusion and Clinical Relevance** Serum levels and avidity of Gly m 4-specific IgE do not correlate with clinical reactivity to soya. Six IgE-binding areas, represented by 23 amino acids, account for more than 80% of total IgE binding capacity of Gly m 4. Model proteins may be used for epitope-resolved diagnosis to differentiate birch-soya allergy from clinical tolerance.

**Keywords** allergens and epitopes, basic mechanisms, food allergy, IgE

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### Introduction

About two-thirds of subjects allergic to birch pollen produce IgE against Bet v 1 that cross-reacts with Bet v 1-homologous proteins from plant foods leading to birch pollen-associated food allergies [1]. Homologous allergens from various fruits, nuts and vegetables can cause birch-related food allergy [2]. Food allergy to soya is associated with birch pollinosis in central and northern Europe [3, 4]. The Bet v 1-homologous soya bean allergen, Gly m 4, has been shown to elicit both

mild allergic symptoms and sometimes systemic reactions including anaphylaxis [5]. Several efforts have been made to differentiate the allergen-specific IgE sensitization profiles of birch pollen-allergic patients with and without associated food allergy [6–12]. Even though these studies revealed group differences between patients sensitized to PR-10 allergens and allergic or tolerant to food were detectable, predictions were not possible for the individual patient. Thus except for oral provocation tests (OPT), which are time consuming and might cause severe side-effects, there are no reliable

diagnostic measures available so far to predict birch pollen-associated food allergy [13]. Thus, OPT (especially when performed as double-blind placebo-controlled food challenge/DBPCFC) is still regarded as gold standard in the diagnosis of food allergies.

The interaction of IgE with Bet v 1 and homologous allergens is one critical step in the elicitation of birch-related allergies. However, detailed molecular information on individual amino acids accounting for most of the binding energy to IgE (so-called functional IgE epitopes in [14]) of Bet v 1 and Bet v 1-homologous allergens in foods is very limited. To date, only three IgE epitopes of allergens have been structurally resolved by either cocrystallization or nuclear magnetic resonance (NMR) spectroscopy of IgE-allergen immune complexes. The IgE epitopes of the major allergens of cow's milk ( $\beta$ -lactoglobulin), grass pollen allergen (Phl p 2) and mugwort (Art v 1) give insight into the molecular characteristics of individual conformational IgE epitopes of allergens of different protein families [15–17]. However, detailed structural information on conformational IgE epitope profiles of allergens from the Bet v 1 protein family, which comprises more than 24 allergens from tree pollen and plant foods that are listed by the Allergen Nomenclature Sub-Committee of the WHO (World Health Organization) and IUIS (International Union of Immunological Societies), is lacking.

Several efforts have been made to identify epitopes for IgE. Allergen variants harbouring substitutions of either individual or multiple residues were engineered to either compromise (mutational epitope analysis) or evoke IgE binding (epitope grafting). Such studies have identified specific residues and protein surface areas critical for the interaction of IgE antibodies with Bet v 1 [18–23] and the Bet v 1-related allergens from apple [24, 25], cherry [26, 27] or celery [28]. Furthermore, monoclonal antibodies have been used to localize potential IgE epitopes of Bet v 1 and related allergens [22, 29–31]. The only known epitope of Bet v 1 that has been structurally resolved so far was identified using a complex between BV16, a mouse monoclonal Bet v 1-specific IgG and the major isoform of Bet v 1, Bet v 1.0101 [32]. Binding of BV16 to Bet v 1.0101 reduces serum IgE interactions with the allergen, suggesting competition of IgE and monoclonal IgG for an overlapping binding site of Bet v 1 [33, 34]. Thus, no individual epitope of Bet v 1 for IgE has been structurally solved, and moreover, no epitopes of Bet v 1 and its homologues from other plants and plant foods have been assigned a specific role in birch-associated allergy. We recognize two major reasons for this lack of information: (i) the engineering of recombinant allergen variants to identify amino acids critical for antibody binding can alter the original protein conformation which is essential for proper IgE antibody binding of

Bet v 1-related allergens and (ii) the analysis of individual IgE binding sites of allergens is complicated by the presence of polyclonal and polyspecific serum IgE facilitating multiple antibody–allergen interactions. In this work, we aim to overcome these difficulties by analysing a recombinant model protein with Gly m 4-type protein conformation that (i) does *per se* not bind serum IgE of subjects with birch pollen-associated soya allergy and (ii) that can be modified on the level of individual amino acids to evoke binding of serum IgE.

Here, we present an orthologous norcoclaurine synthase molecule onto which Gly m 4 residues are grafted as a protein model system for a comprehensive and systematic analysis of functional IgE epitopes of Gly m 4. The system is based on a recent proof-of-concept study, in which the non-allergenic protein norcoclaurine synthase proved to be an ideal molecular tool for epitope analyses of PR-10 class allergens [35]. We identified epitope profiles of Gly m 4 for IgE antibodies in patient groups who were either allergic or clinically tolerant to soya and analysed the diagnostic value of model proteins presenting allergenic IgE epitopes.

## Methods

Forty-five patients with sensitization to both Bet v 1 and Gly m 4, who did not receive birch pollen-specific immunotherapy, were included as serum donors. Allergy to soya bean protein was determined by double-blind placebo-controlled food challenge (DBPCFC) or convincing clinical history of anaphylaxis. Patients were recruited at the Allergy Unit, Department of Dermatology, University Hospital Zürich, Switzerland, at the Department of Dermatology, Venerology and Allergology, University of Leipzig, Germany and at the Allergy and Asthma Center, Westend Outpatient Clinic Hanf, Ackermann & Kleine-Tebbe, Berlin, Germany. Serum donations were approved by the local ethics committees of the clinical centres, namely the 'Ethik-Kommission an der Medizinischen Fakultät der Universität Leipzig' and the 'Kantonale Ethikkommission Zürich, Switzerland' of the University Hospital in Zurich. Study participants provided written informed consent. Twenty-six patients exhibited an allergy against soya bean, while 19 patients with sensitization to Bet v 1 and Gly m 4 were tolerant to soya bean. Four sera pools comprising sera of three (pooled sera No. 40) or four (pooled sera No. 41, 43, 44) patients was used. Serum from one non-allergic subject was used as negative control for the specific IgE measurements.

## Determination of allergen-specific IgE and IgG

Specific IgE and IgG levels to recombinant proteins (Bet v 1, t215; Gly m 4, f353; Phadia Thermo Fisher

Scientific, Freiburg, Germany) and soya bean (*Glycine max*, f14; Phadia Thermo Fisher Scientific) extract were determined using ImmunoCAP-FEIA in a Phadia 250 instrument (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions.

#### *Cloning, expression and purification of recombinant variants of $\Delta$ 51NCS and Gly m 4*

The open reading frame of Gly m 4 optimized for codon usage in *Escherichia coli* was purchased from GeneArt (Thermo Fisher Scientific, Waltham, MA, USA) and inserted into the bacterial expression vector pET15b (Novagen, Merck, Darmstadt, Germany).  $\Delta$ 51NCS was generated by truncation of 11 amino acids at both N- and C-termini and two insertions/substitutions namely N42 and P49 compared to pET29b- $\Delta$ 29NCS described elsewhere [35, 36] to adjust the amino acid sequence to a typical Gly m 4-type allergen. Substitutions were sequentially introduced in pET29b- $\Delta$ 51NCS with Quick-Change Lightning Multi Site-Directed Mutagenesis Kit from Agilent Technologies (Waldbronn, Germany). All protein variants were expressed and purified via their His<sub>6</sub>-tag as described [35]. Inclusion body fractions of recombinant Gly m 4,  $\Delta$ 51NCS and  $\Delta$ 51NCS variants were prepared with BugBuster™ Protein Extraction Reagent (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions.

#### *Sequence confirmation of recombinant proteins with mass spectrometry*

The amino acid sequence of the recombinant proteins was confirmed by nano-ultra-performance liquid chromatography–nano-electrospray ionization mass spectrometry (nano-UPLC/nano-ESI-MS) after SDS-PAGE as described elsewhere [37], with slight modifications. Tryptic peptides were eluted with 25 mM NH<sub>4</sub>HCO<sub>3</sub> and 10% acetonitrile (ACN), and the digestion was stopped by adding 5% formic acid. The peptides were analysed using a nanoUPLC system coupled to a nanoESI-MS (nano Acquity UPLC nanoESI Synapt-MS; Waters, Milford, MA, USA) with a ACQUITY UPLC M-Class Symmetry C18 Trap Column, 100 Å, 5 µm, 180 µm × 20 mm ACQUITY UPLC Peptide BEH C18 nanoACQUITY Column 10 K psi, 130 Å, 1.7 µm, 100 µm × 100 mm. A 30 min gradient (3% ACN to 40% ACN at 500 nL/min) after 3 min of trapping (99% water at 5 µL/min) was applied to separate peptides. The instrument was operated in V mode, acquiring MS<sup>E</sup> data and applying standard parameters. Data analysis was performed with ProteinLynx Global Server version 2.4 (Waters), searching an in house database consisting of the UniProt database (as of May 2016, restricted to reviewed entries) and the amino acid sequences of the

variants of  $\Delta$ 29NCS,  $\Delta$ 51NCS and Gly m 4. Proteins hits were accepted at a false-positive rate of < 4%.

The amino acid sequence of the recombinant proteins was confirmed by LC-MS<sup>E</sup> with sequence coverages between 24.3% and 93.8%. The PLGS protein scores were between 611 and 23 692. The average precursor mass error was between 3.6 and 6.7 ppm, respectively. A detailed description of the MS data can be found in the Fig. S1 and Table S1.

#### *Design of recombinant variants of $\Delta$ 51NCS*

Individual amino acids of  $\Delta$ 51NCS were replaced by their respective counterparts of Gly m 4 by site-directed mutagenesis. For this, the three-dimensional models of  $\Delta$ 51NCS and Gly m 4 were overlaid to determine those residues in  $\Delta$ 51NCS that correspond (i.e. similar position and orientation of amino acid in the 3D-modelled structure) to those residues in Gly m 4 in the native protein conformation. Amino acids of  $\Delta$ 51NCS that correspond to amino acids in Gly m 4 are listed in Table S2.

#### *Circular dichroism*

Far UV circular dichroism (CD) spectra of  $\Delta$ 51NCS and  $\Delta$ 51NCS variants were acquired at 293 K using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Gross-Umstadt, Germany) at a band width of 1 nm and a sensitivity of 100 mdeg in a 0.1 cm cell. All proteins were analysed at a concentration of 5–10 µM in 10 mM potassium phosphate, pH 7.4. Each measurement comprised the average of 10 repeated scans between 255 and 185 nm.

#### *Nuclear magnetic resonance spectroscopy*

Nuclear magnetic resonance samples were prepared in 20 mM sodium phosphate buffer, pH 7.0, 0.04% sodium azide and 10% D<sub>2</sub>O containing 50 µM of  $\Delta$ 51NCS or  $\Delta$ 51NCS<sub>E44/N46/K54</sub>. Standard 1D-<sup>1</sup>H spectra with WATERGATE solvent suppression [38] were recorded on a Bruker Avance 600 MHz spectrometer at 298 K. NMR data were processed and visualized with the Bruker spectrometer software TopSPIN (Bruker, Billerica, MA, USA).

#### *Modelling of $\Delta$ 51NCS variants*

Structure predictions of  $\Delta$ 51NCS variants were performed using the Phyre server [39]. The calculated models were based on the structure of wild-type NCS (pdb 2VNE) [40] with a confidence of 100% (according to sequence and secondary structure similarity) and a sequence coverage of 96%. Three-dimensional protein models were visually analysed and illustrated using PyMOL [41].

### *SDS-PAGE and immunoblot analysis with $\Delta 51\text{NCS}$ variants from inclusion bodies*

SDS-PAGE was performed with 15% separating gels and 5% stacking gels using a discontinuous buffer system [42]. For immunoblot analysis, 0.5  $\mu\text{g}/\text{cm}$  of inclusion body  $\Delta 51\text{NCS}$  variants was transferred onto 0.2  $\mu\text{m}$  nitrocellulose membranes by semidry blotting at 0.8  $\text{mA}/\text{cm}^2$  for 1 h [43]. After blocking with Tris-buffered saline (TBS) containing 0.3%, Tween 20 blots were incubated overnight at room temperature with 1 : 10 dilution of human sera with TBS containing 0.05% Tween 20 (TBST 0.05%) and 0.1% BSA (TBST 0.05–0.1% BSA). After incubation for 1 h with horseradish peroxidase labelled mouse anti-human IgE antibody (Clone B3102E8; Southern biotech via Biozol, Eching, Germany), diluted 1 : 100 000 with TBST 0.05–0.1% BSA, IgE-binding proteins were visualized by chemiluminescence (LumiGLO Reserve diluted 1 : 3; KPL via Medac, Wedel, Germany) using a Luminescence imager (Fusion Fx; Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany) and signal intensities higher than control ( $\Delta 51\text{NCS}_{\text{N42/P49}}$ ) were considered positive.

### *Binding of serum IgE to purified $\Delta 51\text{NCS}$ variants*

Dot blotting with purified recombinant  $\Delta 51\text{NCS}$  variants was performed according to immunoblot analysis described above, but without prior SDS-PAGE and semidry blotting. Recombinant proteins were spotted directly on nitrocellulose membrane. Chemiluminescence was detected with horseradish peroxidase labelled mouse anti-human IgE antibody (Clone B3102E8; Southern biotech via Biozol), diluted 1 : 100 000 with TBST 0.05%, 0.1% BSA. IgE-binding proteins were visualized by chemiluminescence (LumiGLO Reserve diluted 1 : 3; KPL via Medac). Densitometric analysis was performed with IMAGEJ (Rasband; NIH, Bethesda, MD, USA). Each spot was quantified at three different exposure times and mean value as well as standard deviation was calculated. Intensities of each variant were correlated to mean values of  $\Delta 51\text{NCS}$  model protein plus three times standard deviation which was set to 1. IgE signals obtained with patients' sera were identified as positive, if mean values minus standard deviation were above ( $> 1$ ) the signal of  $\Delta 51\text{NCS}$  model protein plus three times standard deviation.

### *Depletion of serum IgE with recombinant Gly m 4 and $\Delta 51\text{NCS}$ variants*

Serum IgE was depleted by incubation of either Gly m 4,  $\Delta 51\text{NCS}$ ,  $\Delta 51\text{NCS}$  variants or BSA with a 300  $\mu\text{L}$  dilution (1 : 10) of patients' sera for 3 h at 22°C followed by precipitation of allergen–antibody

complexes with Ni-beads (Ni-NTA Superflow; Qiagen, Hilden, Germany). Thereafter, supernatants containing unbound IgE antibodies were added to Nunc Maxisorp microtiter plates (Fisher Scientific, Schwerte, Germany) coated with 200 ng/100  $\mu\text{L}$  Gly m 4 in phosphate-buffered saline (PBS) and blocked with PBS containing 2% BSA for 2 h at 22°C. Gly m 4-specific IgE was detected with horseradish peroxidase labelled mouse anti-human IgE antibody (Clone B3102E8; Southern biotech via Biozol), diluted 1 : 1000 with PBS 0.05% Tween20, 0.1% BSA. The substrate for horseradish peroxidase was 3,3',5,5'-tetramethylbenzidine (Roth, Karlsruhe, Germany), and the reaction was stopped by addition of 25%  $\text{H}_2\text{SO}_4$ . The absorbance was measured at 450 nm.

### *IgE inhibition ELISA*

For IgE inhibition experiments, Nunc MaxiSorp plates (Fisher Scientific) were coated overnight at room temperature with 50 ng/100  $\mu\text{L}$  Gly m 4 or 300 ng/100  $\mu\text{L}$   $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  in 10 mM potassium PBS, pH 7.4. After blocking with PBS containing 2% BSA, plates were incubated with the human sera pool (dilution 1 : 10) and increasing concentrations of recombinant inhibitors (Gly m 4 or Gly m 4<sub>N42I/E44S/N46D/K54A</sub>) for 3 h at room temperature with PBS containing 0.05% Tween 20 and 0.1% BSA. Gly m 4- or  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$ -specific IgE was detected as described above.

### *Receiver operating curve analysis*

Receiver operating curve (ROC) analysis was carried out according to Eng J. ROC analysis: web-based calculator for ROC curves. Baltimore: Johns Hopkins University (updated 2014 March 19). Available from: <http://www.jrocf.it.org>. As input data, the quantified IgE binding of  $\Delta 51\text{NCS}$  variants ( $\text{NCS}_1$  to  $\text{NCS}_{\text{VI}}$ ) and of Gly m 4 from sera of subjects allergic or tolerant to soya was used.

### *Miscellaneous*

Amino acid sequence alignment was carried out with EMBOSS Needle Pairwise Alignment tool [44].

## **Results**

### *$\Delta 51\text{NCS}$ – a Gly m 4-type model protein for analysis of specific IgE epitopes*

Recently, we established a non-IgE binding Bet v 1-homologous model protein (norcochlorine synthase,  $\Delta 29\text{NCS}$ ) which presents an IgE/IgG epitope of Bet v 1 after epitope grafting and displays cross-reactivity with Gly m 4 [35]. Here, we optimized our

model protein by truncations of the C- and N-terminal extensions to generate the Gly m 4-type variant  $\Delta 51\text{NCS}$  (Fig. 1a). To introduce a recently confirmed IgE antibody binding site of Gly m 4 [35] to  $\Delta 51\text{NCS}$ , we also generated and purified the variant  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  and tested binding of serum IgE to the  $\Delta 51\text{NCS}$  variants offered either as purified protein or as inclusion body fractions (Fig. 1b). Whereas  $\Delta 51\text{NCS}$  did not bind serum IgE, the variant  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  bound IgE under both conditions. We subsequently performed CD and NMR spectroscopy of both  $\Delta 51\text{NCS}$  variants to verify that the optimization process of  $\Delta 29\text{NCS}$  did not abolish the original NCS fold and that the Gly m 4 residues E44, N46 and K54 are actually part of a conformational epitope recognized on  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$ . The spectra of  $\Delta 51\text{NCS}$  and  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  are virtually identical and characteristic for folded proteins with a high degree of resonance dispersion resulting from secondary structural elements such as  $\alpha$ -helices and  $\beta$ -sheets (Fig. 1c) [45, 46], as well as positive maximum ellipticity at approximately 195 nm [47] and negative maximum ellipticity at 217 nm (Fig. 1d) [48, 49]. These results indicated that the amino acid substitutions in  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  did not change the structural arrangement of  $\Delta 51\text{NCS}$ . As  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  is recognized by IgE that was originally directed against the structure of Bet v 1, we conclude that both  $\Delta 51\text{NCS}$  variants still show the original PR-10 class fold. To test whether the new IgE epitope presented by  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  is recognized by IgE directed against Gly m 4, we performed an IgE inhibition ELISA with  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  at the solid phase and wild-type Gly m 4 as inhibitor which clearly showed dose-related inhibition (Fig. 1e). Moreover, we generated a variant of Gly m 4 in which the amino acids corresponding to the epitope were exchanged (Gly m 4<sub>N42I/E44S/N46D/K54A</sub>) showing drastically reduced inhibition capacity in this assay system (Fig. 1e). Physicochemical analyses indicated that Gly m 4<sub>N42I/E44S/N46D/K54A</sub> has a typical monomeric Gly m 4 protein conformation (Fig. S2). From this dataset, we concluded that  $\Delta 51\text{NCS}$  is an ideal Gly m 4-type model protein for a comprehensive systematic analysis of IgE epitopes of Gly m 4 (Fig. 1f).

#### *Birch pollen-allergic subjects sensitized to Gly m 4 and with or without allergy to soya*

All patients ( $n = 45$ ) included in the study were sensitized to both Bet v 1 and Gly m 4 and also possessed IgG to Gly m 4 (Tables 1 and S3). Gly m 4-sensitized subjects with clinically confirmed allergy ( $n = 21$ ) or tolerance ( $n = 13$ ) to soya had Gly m 4-specific IgE

levels between 2.7 and  $> 100 \text{ kU}_\text{A}/\text{L}$  (median  $9.8 \text{ kU}_\text{A}/\text{L}$ ) and 0.9 to  $> 100 \text{ kU}_\text{A}/\text{L}$  (median  $5.4 \text{ kU}_\text{A}/\text{L}$ ), respectively. Only serum of patient 4 had low levels of specific IgE against the soya allergens Gly m 5 and Gly m 6, where all other patients had no detectable IgE against these allergens. IgG levels against Gly m 4 were comparable in both groups with median values of 8.2 and  $9.1 \text{ mg}_\text{A}/\text{L}$  for the soya allergic and non-allergic group. Thus, no major differences regarding the serological level between the allergic and the non-allergic group of our study population were observed. We wondered whether the avidities of Gly m 4-specific IgE would differ in sera of subjects with (potentially higher avidity) or without (potentially lower avidity) allergy to soya. We therefore pooled the sera of allergic or non-allergic subjects, tested IgE binding to immobilized Gly m 4 in the presence of increasing concentrations of Gly m 4 and determined  $\text{EC}_{50}$ , the amount of Gly m 4 needed for half-maximum inhibition of IgE-Gly m 4 binding (Fig. 2). The  $\text{EC}_{50}$  values of 5.1 ng (soya allergic) and 1.8 ng (soya tolerant) rGly m 4 were quite similar and did not support our hypothesis, as there was an apparently higher avidity in patients clinically tolerant to soya.

#### *Generation of a $\Delta 51\text{NCS}$ library harbouring IgE epitopes of Gly m 4*

We generated a protein library of 48 recombinant  $\Delta 51\text{NCS}$  variants harbouring 1–5 substitutions of amino acids of Gly m 4 potentially critical for IgE binding (Fig. S3). A rational protein design was chosen considering mainly surface accessible polar, charged or aromatic residues of Gly m 4. Furthermore, amino acids of putative epitopes of Gly m 4 were considered, based on a recent bioinformatic approach, mapping phage-displayed IgE binding peptides onto the molecular surface of Gly m 4 to display potential antibody binding sites [50]. All  $\Delta 51\text{NCS}$  variants were heterologously expressed in *E. coli*, and purified inclusion bodies were screened by IgE Western blotting with sera from all patients to obtain a preliminary patient-specific profile of putative IgE binding sites (Table S4). In total, 96% (46/48) of the NCS variants bound serum IgE. 48% (22/46) of the identified putative functional IgE epitopes were homologous to Bet v 1, while 52% (24/46) represented amino acids that were specific for Gly m 4. On average 21 (12–32) and 18 (14–26), NCS variants were recognized by sera of subjects allergic or tolerant to soya, respectively. We compared the serum IgE binding frequencies of all NCS variants (Fig. 3). Four NCS variants bound all sera, and 21 model proteins were recognized by at least 50% of all sera tested. Interestingly, we observed no major differences in IgE binding between the groups allergic or tolerant to soya.

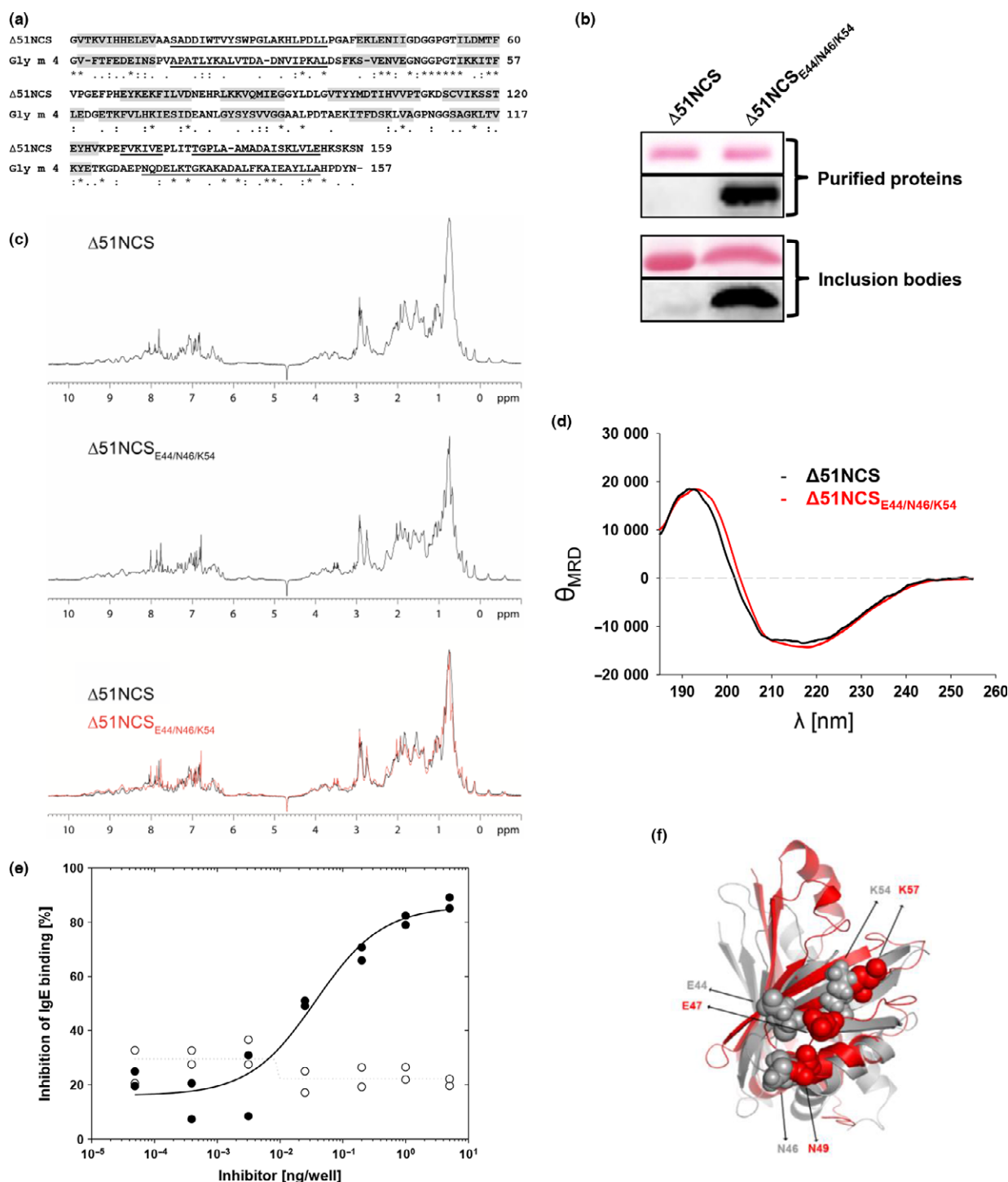


Fig. 1.  $\Delta 51\text{NCS}$  has Gly m 4-type structure and allows presentation of Gly m 4-specific epitopes. (a) Structural sequence alignment of  $\Delta 51\text{NCS}$  and Gly m 4. The position of secondary structure elements of Gly m 4 were obtained from pdb: 2K7H [55] and putative positions of  $\Delta 51\text{NCS}$  from the modelled variant.  $\beta$ -strands (highlighted in grey) and  $\alpha$ -helices (underlined) are shown. Amino acid identity is 20.4%. (b) Binding of human IgE to purified (upper panel) or inclusion bodies of (lower panel)  $\Delta 51\text{NCS}$  and  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  with a serum of a birch pollen-allergic subject sensitized to Gly m 4. Ponceau S stain (top) and Western blot (bottom) of each subfigure. (c) One-dimensional  $^1\text{H}$ -NMR of  $\Delta 51\text{NCS}$  and  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$ . (d) Circular dichroism of  $\Delta 51\text{NCS}$  (black) and  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  (red) presenting a Gly m 4-specific IgE epitope. (e) Dose-dependent inhibition of IgE binding to surface-coated  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  in the presence of increasing concentrations of Gly m 4 (filled circles) or Gly m 4<sub>N42I/E44S/N46D/K54A</sub> (open circles). Inhibition was determined in duplicate. (f) Structural overlay of Gly m 4 (pdb 2K7H in grey) and a model of  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$  in red. The putative functional epitope comprising amino acids of Gly m 4 E44/N46/K54 shared by both proteins (E47, N49 and K57 in  $\Delta 51\text{NCS}_{\text{E44/N46/K54}}$ ) is illustrated in spheres. NMR, nuclear magnetic resonance.

Table 1. Allergen-specific IgE/IgG of human sera of subjects being allergic or tolerant (sensitized without subsequent symptoms) to soya

| Study group           | sIgE (kU <sub>A</sub> /L) |         |         |            | tIgG (mg <sub>A</sub> /L) |
|-----------------------|---------------------------|---------|---------|------------|---------------------------|
|                       | Gly m 4*                  | Gly m 5 | Gly m 6 | Bet v 1*   |                           |
| Allergic (n = 21)     |                           |         |         |            |                           |
| Median                | 9.84                      | 0       | 0.01    | 40.1       | 8.2                       |
| Min/max               | 2.68/> 100                | 0/0.47  | 0/0.77  | 7.48/> 100 | 2.9/36.9                  |
| Non-allergic (n = 13) |                           |         |         |            |                           |
| Median                | 5.42                      | 0.04    | 0.03    | 30.6       | 9.1                       |
| Min/max               | 0.65/> 100                | 0/0.17  | 0/0.26  | 6.07/> 100 | 4.5/61.3                  |

\*The significances of differences in the Ig levels between the allergic and non-allergic group were as follows:  $P = 0.51$  (sIgE Bet v 1),  $P = 0.16$  (sIgE Gly m 4) and  $P = 0.31$  (tIgG Gly m 4).

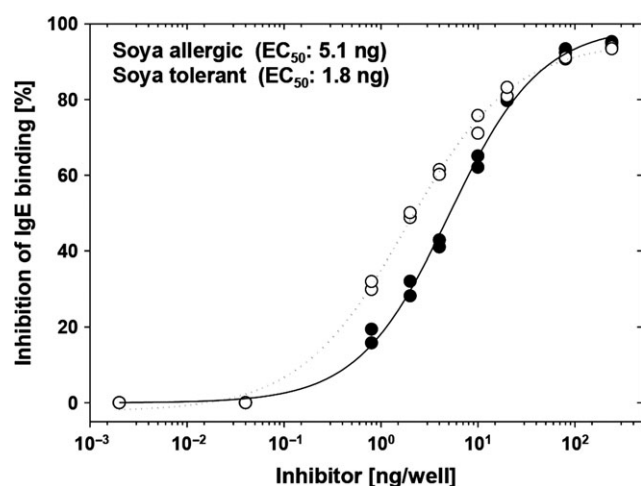


Fig. 2. Avidity of serum IgE of Gly m 4-sensitized subjects. Pooled sera of subjects allergic (closed symbols) and tolerant (open symbols) to soya were analysed. Binding of serum IgE antibodies to immobilized Gly m 4 in the presence of increasing concentrations of Gly m 4 was determined. The EC<sub>50</sub> for both patient groups is shown.

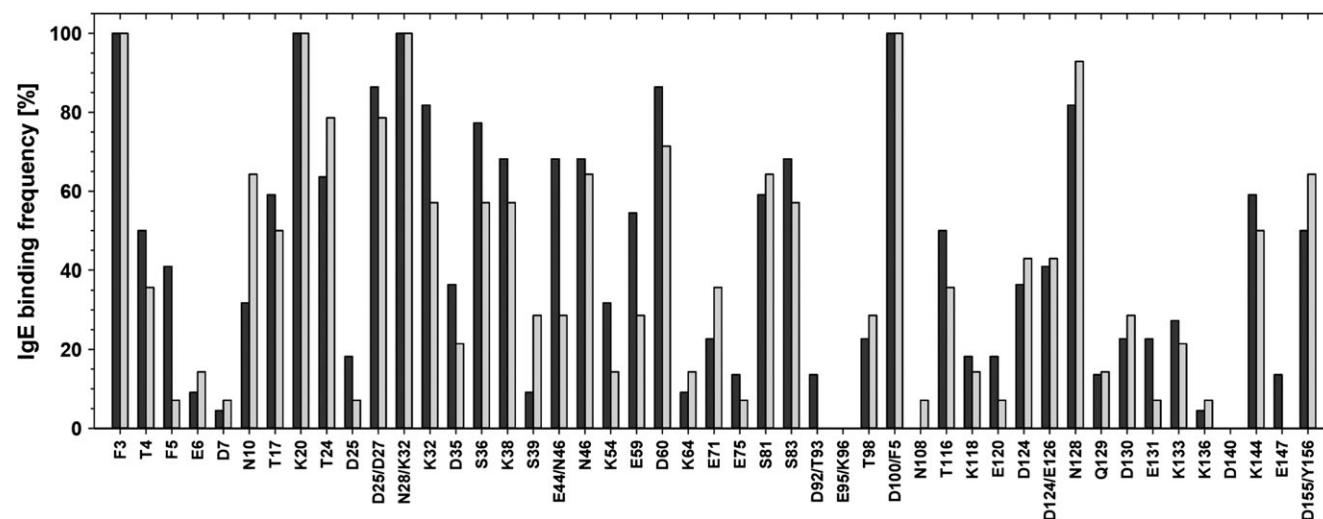


Fig. 3. IgE binding frequencies of Gly m 4-type NCS variants. IgE binding frequencies of sera of birch allergic subjects with clinically confirmed allergy (dark grey) or tolerance (light grey) to soya to inclusion body  $\Delta 51$ NCS variants, presenting single or double amino acids of Gly m 4.

### Polyclonal IgE binding from individual patients might cluster in six surface areas of Gly m 4

We clustered the identified amino acids whose side chains are approximately in cis-conformation to a virtual molecular surface plane of 1000 Å<sup>2</sup> to six putative IgE-binding areas as this spatial arrangement results in a maximum number of virtual epitopes on the molecular surface of Gly m 4 that would enable simultaneous antibody binding to at least two different areas (Fig. 4), respectively. Amino acids at the intersection of two virtual plane surface areas might represent residues belonging to at least two of six hypothetical IgE binding sites. The putative functional IgE epitopes (I–VI) are represented by 3–10 amino acids each and cover molecular surface areas between 311 and 967 Å<sup>2</sup> (3–11% of total protein surface, respectively) of Gly m 4. According to individual NCS variants most frequently recognized by serum IgE, the six IgE binding areas (I–VI) were bound by 100%, 59%, 100%, 86%, 68% and 100% and 100%, 50%, 93%, 71%, 36% and 100% of sera of subjects allergic or tolerant to soya, respectively.

### $\Delta 51$ NCS variants presenting Gly m 4-specific IgE epitopes

To verify the data obtained in the initial screen, we purified and characterized  $\Delta 51$ NCS variants presenting multiple Gly m 4-specific amino acids selected from the putative IgE binding areas I–VI (Fig. S3, cf. Fig. 4). NCS variants with a Gly m 4-type secondary structure were analysed for individual serum IgE binding in dot blot analysis to generate both a patient-specific (Fig. 5) and a group-specific epitope profile (Fig. 6a).  $\Delta 51$ NCS variants bound serum IgE, however with patient-specific

GVFTFEDEINSPVAPATLYKALVTDADNVIPKALDSFKSVENVEGNGGPGTIKKITFLED 60  
 GETKFLVHKIESIDEANLGYSSVVGGAALPDTAEKITFD SKLVAGFPNGGSAGKLT VKYE 120  
 TKGDAEFPNQDELKTGKAKADALFKAI EAYLLAHPDYN 157

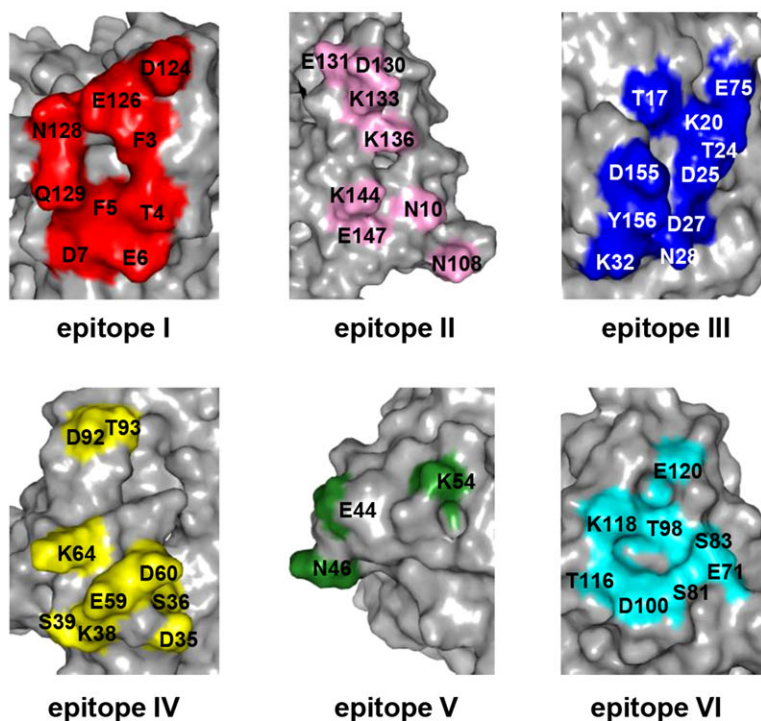
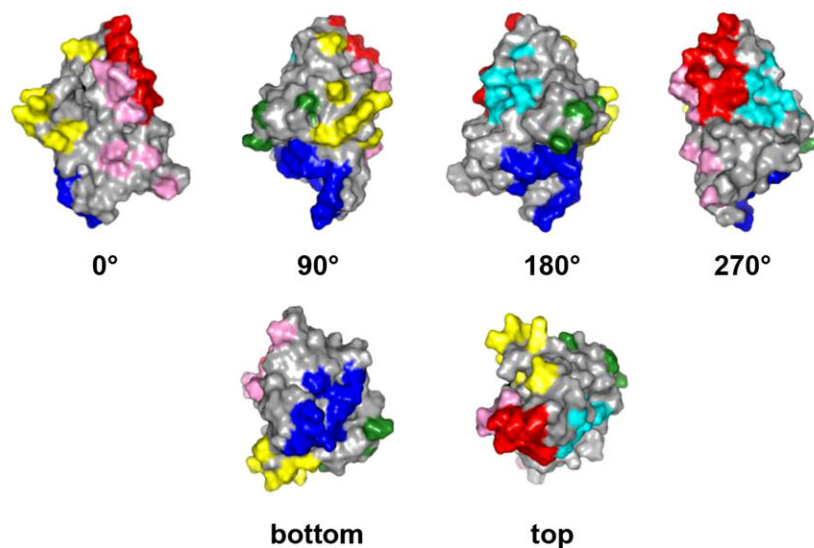


Fig. 4. Six IgE binding areas of Gly m 4. Upper panel: 47 Gly m 4-specific amino acids shown to evoke IgE binding of  $\Delta$ 51NCS cluster into six potential IgE binding surface areas of Gly m 4. Secondary structure elements are indicated as  $\beta$ -strands (highlighted in grey) and  $\alpha$ -helices (underlined) according to pdb 2K7H [55]. Middle panel: Molecular surface presentation. Lower panel: The following amino acids represent six different potential IgE binding areas (I-VI) of Gly m 4 with I (red: F3, T4, F5, E6, D7, D124, E126, N128, Q129), II (pink: N10, N108, D130, E131, K133, K136, K144, E147), III (blue: T17, K20, T24, D25, D27, N28, K32, E75, D155, Y156), IV (yellow: D35, S36, K38, S39, E59, D60, K64, D92, T93), V (green: E44, N46, K54) and VI (cyan: E71, S81, S83, T98, D100, T116, K118, E120).

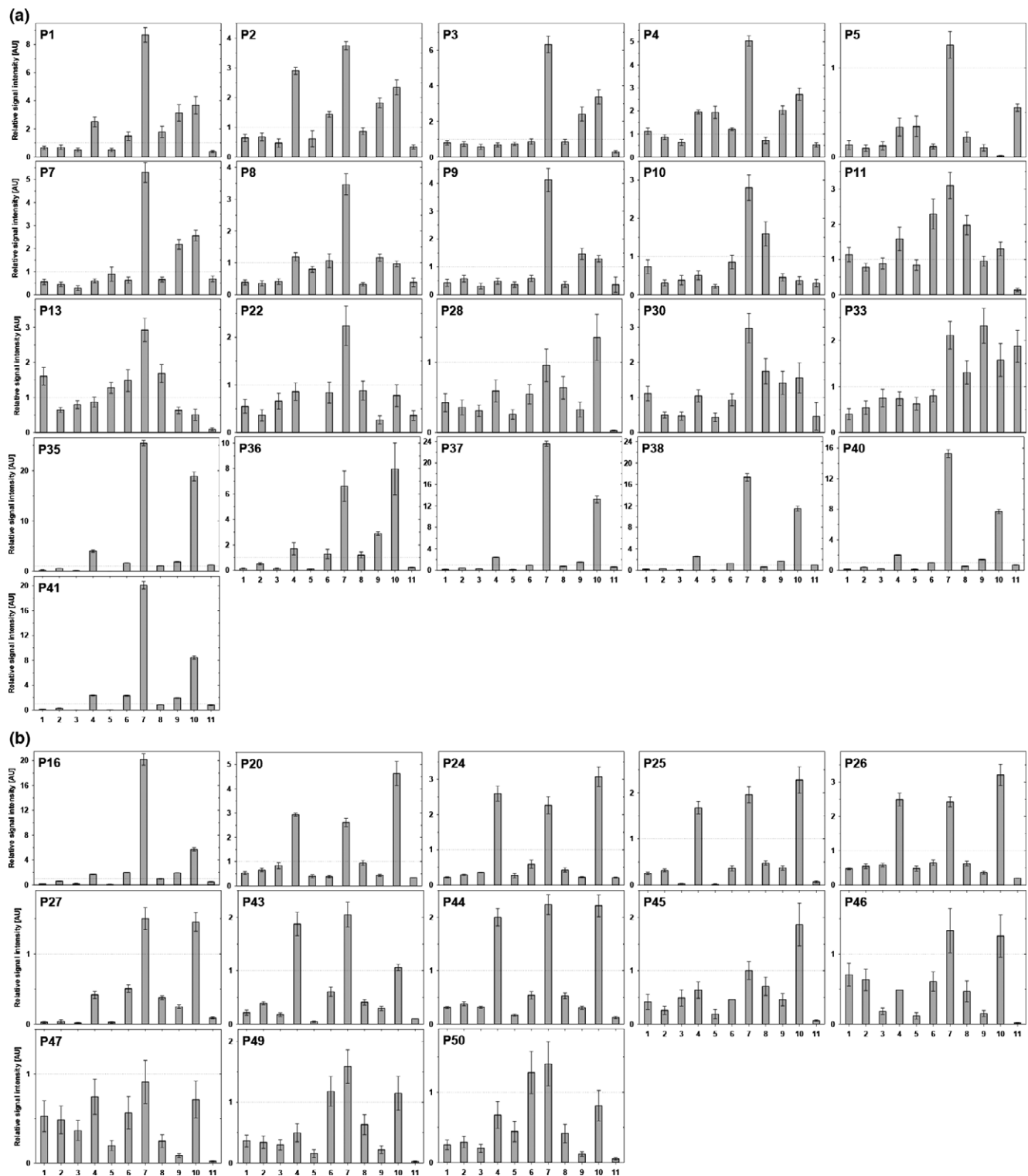


Fig. 5. Serum IgE binding to recombinant  $\Delta 51$ NCS variants. Patient-specific serum IgE binding to purified recombinant NCS variants presenting single and multiple putative IgE epitopes of Gly m 4 was determined in dot blot analysis. Antibody binding was quantified densitometrically. Sera of subjects with clinically confirmed birch pollen-related soya allergy (a) and sera of subjects sensitized to Gly m 4 but tolerant to soya (b) were analysed. Chemiluminescence signal for  $\Delta 51$ NCS not presenting a Gly m 4-specific epitope was set to 1 (dotted line). 1–11: NCS<sub>I</sub>, NCS<sub>III</sub>, NCS<sub>IV</sub>; NCS<sub>V</sub>, NCS<sub>II</sub>, NCS<sub>III/IV/V/V1</sub>\*, NCS<sub>I/IV</sub>, NCS<sub>I/III/IV/V1</sub>, NCS<sub>I/III/V/V1</sub>, NCS<sub>I/II/III/IV/V1</sub> and NCS<sub>I/II/III/IV/V1</sub>.

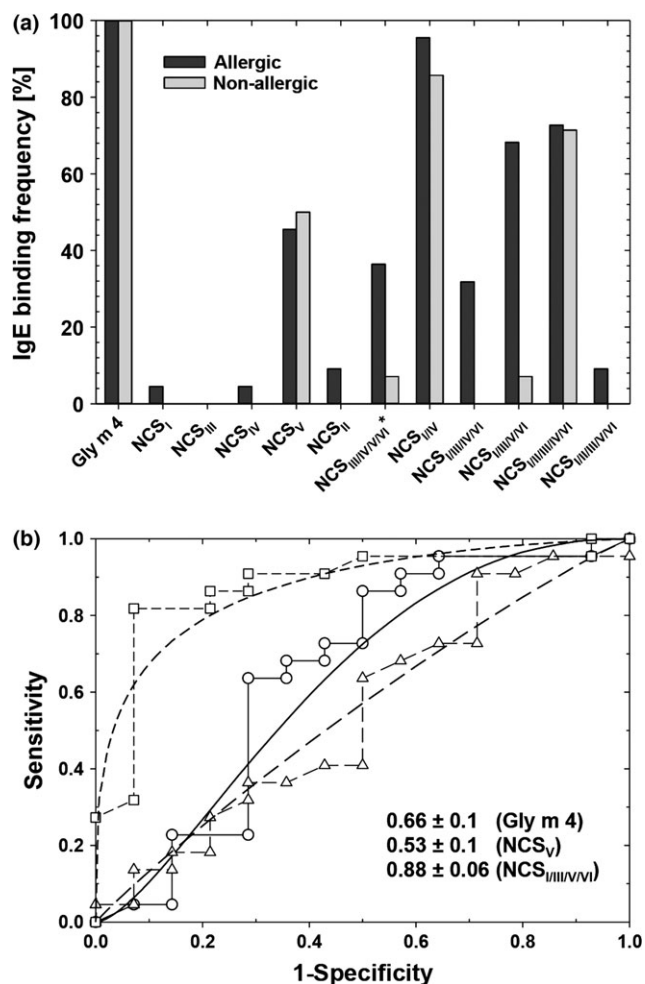


Fig. 6. Frequencies of serum IgE binding to recombinant Gly m 4 and  $\Delta$ 51NCS variants and receiver operating curve (ROC) analysis. (a) Percentages of IgE binding to recombinant  $\Delta$ 51NCS variants were determined for subjects with (dark grey bars) and without (light grey bars) allergy to soya. (b) Receiver operating analysis showing sensitivity and specificity of serum IgE binding to Gly m 4 (circles, straight line), to NCS<sub>V</sub> (triangles, long-dashed line), and to NCS<sub>III/IV/V/VI</sub> (squares, short-dashed line). Area under fitted curve (AUC) with estimated standard error for each protein is indicated. AUC for remaining  $\Delta$ 51NCS variants were as follows: NCS<sub>I</sub>: no significance, NCS<sub>III</sub>:  $0.69 \pm 0.09$ , NCS<sub>IV</sub>:  $0.64 \pm 0.09$ , NCS<sub>II</sub>:  $0.69 \pm 0.09$ , NCS<sub>III/IV/V/VI</sub>\*:  $0.78 \pm 0.08$ , NCS<sub>I/IV</sub>:  $0.83 \pm 0.07$ , NCS<sub>I/III/IV/V/VI</sub>:  $0.78 \pm 0.08$ , NCS<sub>I/II/III/IV/V/VI</sub>:  $0.55 \pm 0.1$  and NCS<sub>I/II/III/IV/V/VI</sub>:  $0.86 \pm 0.06$ .

binding preferences (Fig. 5). 82% (9/11) and 45% (5/11) of the NCS variants bound serum IgE of allergic and tolerant subjects, respectively. None of the proteins was recognized by all sera, but the variants NCS<sub>V</sub>, NCS<sub>I/IV</sub> and NCS<sub>III/IV/V/VI</sub> were most frequently recognized by serum IgE in both patient groups. The most striking difference however was observed with NCS<sub>III/IV/V/VI</sub> representing four putative IgE binding areas. NCS<sub>III/IV/V/VI</sub> was bound by 68% and 7% of the soya allergic and tolerant subjects, respectively. We performed a ROC analysis to

identify the best diagnostic marker among the recombinant model proteins used (Fig. 6b). Whereas Gly m 4 and NCS<sub>V</sub> had area under fitted curve (AUC) of  $0.63 \pm 0.1$  and  $0.55 \pm 0.1$ , respectively, NCS<sub>III/IV/V/VI</sub> had an AUC of  $0.88 \pm 0.06$  and turned out to be most suitable candidate for a diagnostic marker that would distinguish soya allergic from tolerant subjects in our study population.

To test the total binding capacity of Gly m 4-specific IgE of the generated NCS variants, we used a combination of six model proteins to deplete Gly m 4-specific serum IgE. As controls, the  $\Delta$ 51NCS and Gly m 4 were used (Fig. 7).  $80 \pm 5.3\%$  and  $70 \pm 5.3\%$  could be depleted by the mixture of NCS variants compared to  $97.3 \pm 2.1\%$  and  $92.4 \pm 2.4\%$  by Gly m 4 when used in epitope-wise equimolar amounts in sera of allergic and tolerant subjects, respectively. In contrast, only  $20 \pm 7.7\%$  and  $21.7 \pm 7.8\%$  of Gly m 4-specific IgE could be depleted by the native NCS control. These results suggested that the vast majority of IgE epitopes of Gly m 4 are covered by our model protein-based recombinant Gly m 4-specific epitope library containing a total number of 23 amino acid substitutions (i.e. F3, T4, F5, T17, T24, K32, S36, K38, E44, N46, K54, D60, K64, E75, S81, S83, D100, K118, D124, E126, N128, K133, K144) towards Gly m 4.

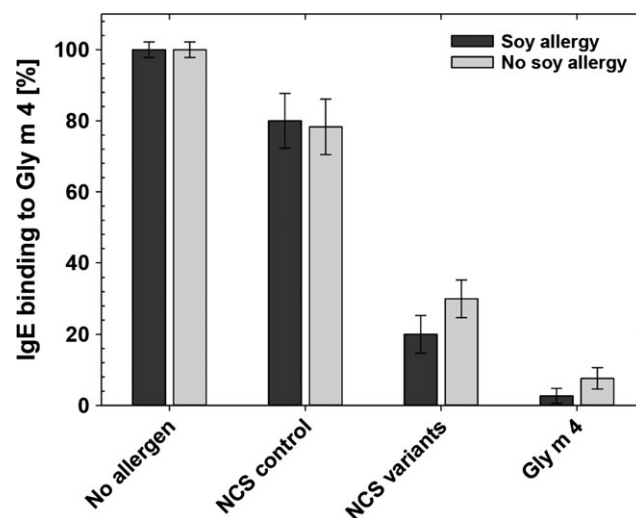


Fig. 7. Depletion of Gly m 4-specific IgE with recombinant  $\Delta$ 51NCS variants. Pooled sera of subjects with (dark grey bars) and without (light grey bars) birch-related allergy to soya were incubated with  $10 \mu\text{M}$  of either  $\Delta$ 51NCS control, a pool of  $\Delta$ 51NCS variants NCS<sub>V</sub>, NCS<sub>I/IV</sub>, NCS<sub>III/IV/V/VI</sub>, NCS<sub>I/III/IV/V/VI</sub> and NCS<sub>I/II/III/IV/V/VI</sub> ( $10 \mu\text{M}$  each), Gly m 4, or no allergen as control. Following pull-down of protein-IgE complexes, the supernatants were analysed for residual IgE binding to Gly m 4 in ELISA. Percentages of Gly m 4-specific depletions of IgE for the allergic and tolerant group in each experiment were  $0 \pm 2.2$  (no allergens),  $20 \pm 7.7$  and  $21.7 \pm 7.8$  (NCS control),  $80 \pm 5.3$  and  $70 \pm 5.3$  (NCS variants) and  $93.4 \pm 2.1$  and  $92.4 \pm 3$  (Gly m 4).

## Discussion

In this study, we intended to generate for the first time comprehensive structural data of unique conformational IgE epitopes of a Bet v 1-related food allergen. Gly m 4 from soya bean was selected as a model allergen due to its known high clinical relevance including the potential to elicit severe food allergic reactions [5].

For this, (i) we engineered recombinant variants of a non-allergenic Gly m 4 (Bet v 1)-type model protein (norcochlorine synthase, NCS) harbouring Gly m 4-specific amino acid substitutions, (ii) confirmed correct folding of the engineered proteins by spectroscopic methods, and (iii) analysed serum IgE binding to NCS variants. On the basis of IgE binding data presented here, we suggest six potential IgE binding areas of Gly m 4 representing approximately 80% of the total IgE binding capacity of Gly m 4.

In our molecular analysis of IgE epitopes of Gly m 4, we followed the concept of functional (active) epitopes in which only a subset of amino acids within an epitope dominates the energetics of allergen-antibody binding [14]. Thus, the incorporation of very few Gly m 4-specific amino acids into our non-IgE binding model protein NCS might mimic an actual partial antibody binding site of Gly m 4 that can be localized by specific binding of serum IgE. In a recent proof-of-concept study, we showed that such minor alterations of few surface-exposed amino acid residues of a non-IgE-reactive model protein led to the presentation of a cross-reactive binding site for serum IgE antibodies [35]. Therefore, NCS qualifies for the intended epitope analysis presented here.

For our initial screen of potential IgE binding sites, we chose to analyse inclusion bodies of expressed NCS variants with only 1–2 amino acid substitutions in immunoblot. This procedure provided useful data based on a crude (but fast) purification of the recombinant NCS from host cell proteins. Furthermore, as IgE binding to Bet v 1-type allergens requires native protein conformation, we detected only those NCS variants which were able to refold stably into allergen-like conformation upon denaturing SDS-PAGE and subsequent transfer to nitrocellulose membrane. Subsequently, some differences in serum IgE binding between the inclusion body and dot blot/ELISA analyses of purified multiple NCS variants occurred which could be due to differing quantitative epitope presentations in the assays.

Grafting of putative antibody binding sites on allergens has been performed by interexchange of amino acids between homologous allergens. While this approach might be well suited to preserve the allergen-like conformation and reduce binding to single conformational epitopes of the analysed protein, it is affected

by the high polyclonality of the IgE response resulting in relative low reduction in the IgE binding capacity of the engineered allergen compromising the intended epitope analysis [18, 23]. As we observed no/very low IgE binding to our NCS template protein, we consider the grafting of conformational epitopes into a non-allergenic matrix as an invaluable advantage of our model system.

Our serum donors consisted of 45 patients with sensitization to both Bet v 1 and Gly m 4 with clinically relevant soya allergy ( $n = 26$ ) or tolerance to soya bean ( $n = 19$ ). One potential reason for a latent and clinically irrelevant sensitization to Bet v 1 homologues could be weaker IgE binding, that is lower avidity in patients clinically tolerant to food, or the presence of blocking IgG antibodies. To test these hypotheses, we analysed the avidity of Gly m 4-specific IgE in the two groups by dose-related inhibition experiments and the levels of IgE and IgG to Gly m 4, but were unable to identify any correlation to the presence and severity of birch pollen-related food allergy to soya bean.

In general, specific IgE does not indicate clinical relevance of a sensitization [51]. However, whether allergen-specific IgE levels correlate directly with clinical symptoms is under debate. Whereas in peanut allergy the severity of symptoms concurs with the level of allergen-specific IgE, no such correlation was found in birch-related allergy to apples [6, 52, 53]. In our study population, we found, however, not statistically significant slightly higher median levels of Gly m 4- (9.3 vs. 4.5 kU<sub>A</sub>/L,  $P = 0.1606$ ) and Bet v 1-specific (37.0 vs. 29.6 kU<sub>A</sub>/L,  $P = 0.5141$ ) IgE in subjects allergic as compared to those individuals tolerant to soya. In the patient groups, sIgE levels ranged from 0 to > 100 kU<sub>A</sub>/L for both Bet v 1 and Gly m 4, respectively. Moreover, 70% of soya allergic subjects had severe symptoms after soya bean consumption with sIgE levels not correlating quantitatively with the severity of allergic symptoms. Patients with high levels of sIgE against Gly m 4 (46.3 kU<sub>A</sub>/L, patient 2) had mild symptoms such as oral allergy syndrome (OAS), whereas subjects with a relatively low amount of Gly m 4-specific IgE (4.0 kU<sub>A</sub>/L, patient 33) exhibited severe reactions such as face swelling and throat tightness. These observations suggested that Gly m 4-specific IgE levels *per se* might not be a proper diagnostic tool for clinical birch-related soya allergy. In contrast, in our ROC analysis, the NCS variant NCS<sub>I/III/V/VI</sub> presenting two Gly m 4-specific amino acids (T4 and T17) and two Gly m 4/Bet v 1-specific amino acids (N46/N47 and S83/S84, respectively) had an AUC of 0.88 with 71% sensitivity and 92% specificity and hence a higher diagnostic potential to predict soya allergy than Gly m 4, indicating a potential advantage of epitope-resolved over current component-resolved diagnosis of birch-soya allergy.

Even though our study population did not receive allergen-specific immunotherapy (AIT), we observed Gly m 4-specific serum IgG. The presence of allergen-specific IgG antibodies in particular following AIT is often accompanied with a reduction in allergic symptoms upon allergen exposure due to, for example, blocking of IgE-mediated allergic responses by serum IgG [54]. However, similar to our observations with IgE, we did not observe a quantitative correlation of Gly m 4-specific IgG levels and severity of symptoms as patients with mild or severe symptoms had both high and low levels of allergen-specific IgG, respectively. Furthermore, we did not find a negative impact of Gly m 4-specific IgG on the avidity of Gly m 4-specific IgE in ELISA even though a 428- and 862-fold molar excess of Gly m 4-specific IgG over IgE was present in sera of allergic and tolerant subjects, respectively. Thus, the analysis of specific IgE and IgG levels to Gly m 4 was not suitable as a biomarker for the allergic phenotype of birch pollen-related food.

However, when we analysed the diagnostic potential of our model proteins, we observed that IgE binding to particular NCS variants presenting specific amino acids of Gly m 4 in four putative IgE binding areas were apparently better suitable to discriminate allergic and tolerant subjects than Gly m 4 itself. Even though we did not observe IgE binding areas unequivocally indicating soya allergy, we hypothesize that an epitope-resolved *in vitro* diagnosis might bear the potential to

provide an improved correlation to the clinical phenotype. However, further studies and larger study populations are required to prove this hypothesis.

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### Conflict of interest

BBW has received fees for speaking from Thermo Fisher. RT received funding for research on birch-related soya allergy by the German Federal Ministry of Education and Research (FK 01KG0911). TH had consultant arrangements with 'Institut für Produktqualität', received fees from 'Die Akademie Fresenius' for speaking and organizing education and received reimbursement for attending symposia from European Academy of Allergology and Clinical Immunology, World Allergy Organization, Deutscher Allergie- und Asthmabund e.V., International Life Sciences Institute/Health and Environmental Sciences Institute, International Association for Food Protection, AOAC International, SAG Süderelbe Projektgesellschaft AG & Co. KG. FH, JN, CSvL, TM, JKT, JCS, SR, EV, AR, PR, SV, DS declare no conflict of interest.

### References

- Vieths S, Scheurer S, Ballmer-Weber B. Current understanding of cross-reactivity of food allergens and pollen. *Ann N Y Acad Sci* 2002; **964**:47–68.
- Breiteneder H, Mills C. Structural bioinformatic approaches to understand cross-reactivity. *Mol Nutr Food Res* 2006; **50**:628–32.
- Treudler R, Werner M, Thiery J *et al*. High risk of immediate-type reactions to soy drinks in 50 patients with birch pollinosis. *J Invest Allergol Clin Immunol* 2008; **18**:483–4.
- Treudler R, Simon J. Schwere Sojaallergie im Erwachsenenalter. Hilft eine spezifische Immuntherapie? *Hautarzt* 2012; **63**:307–12.
- Kleine-Tebbe J, Vogel L, Crowell DN *et al*. Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1-related PR-10 protein in soybean, SAM22. *J Allergy Clin Immunol* 2002; **110**:797–804.
- Ebo DG, Bridts CH, Verweij MM *et al*. Sensitization profiles in birch pollen-allergic patients with and without oral allergy syndrome to apple. Lessons from multiplexed component-resolved allergy diagnosis. *Clin Exp Allergy* 2010; **40**:339–47.
- Tolkki L, Alanko K, Petman L *et al*. Clinical characterization and IgE profiling of birch (*Betula verrucosa*)-allergic individuals suffering from allergic reactions to raw fruits and vegetables. *Journal Allergy Clin Immunol Pract* 2013; **1**:623–31.e1.
- Le T, Bublin M, Breiteneder H *et al*. Kiwifruit allergy across Europe. Clinical manifestation and IgE recognition patterns to kiwifruit allergens. *J Allergy Clin Immunol* 2013; **131**:164–71.
- Ballmer-Weber BK, Skamstrup Hansen K, Sastre J *et al*. Component-resolved in vitro diagnosis of carrot allergy in three different regions of Europe. *Allergy* 2012; **67**:758–66.
- Hansen KS, Ballmer-Weber BK, Sastre J *et al*. Component-resolved in vitro diagnosis of hazelnut allergy in Europe. *J Allergy Clin Immunol* 2009; **123**:1134–41, 1141.e1–3.
- Reuter A, Lidholm J, Andersson K *et al*. A critical assessment of allergen component-based in vitro diagnosis in cherry allergy across Europe. *Clin Exp Allergy* 2006; **36**:815–23.
- Ballmer-Weber BK, Wangorsch A, Bohle B *et al*. Component-resolved in vitro diagnosis in carrot allergy: does the use of recombinant carrot allergens improve the reliability of the diagnostic procedure? *Clin Exp Allergy* 2005; **35**:970–8.
- Niggemann B. When is an oral food challenge positive? *Allergy* 2010; **65**:2–6.
- Dall'Acqua W, Goldman ER, Lin W *et al*. A mutational analysis of binding interactions in an antigen-antibody protein-protein complex. *Biochemistry* 1998; **37**:7981–91.
- Niemi M, Jylhä S, Laukkanen M *et al*. Molecular interactions between a recombinant IgE antibody and the

- beta-lactoglobulin allergen. *Structure* 2007; 15:1413–21.
- 16 Padavattan S, Flicker S, Schirmer T *et al.* High-affinity IgE recognition of a conformational epitope of the major respiratory allergen Phl p 2 As revealed by X-ray crystallography. *J Immunol* 2009; 182:2141–51.
  - 17 Razzera G, Gadermaier G, de Paula V *et al.* Mapping the interactions between a major pollen allergen and human IgE antibodies. *Structure* 2010; 18:1011–21.
  - 18 Holm J, Ferreras M, Ipsen H *et al.* Epitope grafting, re-creating a conformational Bet v 1 antibody epitope on the surface of the homologous apple allergen Mal d 1. *J Biol Chem* 2011; 286:17569–78.
  - 19 Ferreira F, Ebner C, Kramer B *et al.* Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* 1998; 12:231–42.
  - 20 Holm J, Gajhede M, Ferreras M *et al.* Allergy vaccine engineering: epitope modulation of recombinant Bet v 1 reduces IgE binding but retains protein folding pattern for induction of protective blocking-antibody responses. *J Immunol* 2004; 173:5258–67.
  - 21 Klinglmayr E, Hauser M, Zimmermann F *et al.* Identification of B-cell epitopes of Bet v 1 involved in cross-reactivity with food allergens. *Allergy* 2009; 64:647–51.
  - 22 Hecker J, Diethers A, Schulz D *et al.* An IgE epitope of Bet v 1 and fagales PR10 proteins as defined by a human monoclonal IgE. *Allergy* 2012; 67:1530–1537.
  - 23 Gepp B, Lengger N, Bublin M *et al.* Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy. *J Allergy Clin Immunol* 2014; 134:188–94.
  - 24 Son DY, Scheurer S, Hoffmann A *et al.* Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *Eur J Nutr* 1999; 38:201–15.
  - 25 Ma Y, Gadermaier G, Bohle B *et al.* Mutational analysis of amino acid positions crucial for IgE-binding epitopes of the major apple (*Malus domestica*) allergen, Mal d 1. *Int Arch Allergy Immunol* 2006; 139:53–62.
  - 26 Scheurer S, Son DY, Boehm M *et al.* Cross-reactivity and epitope analysis of Pru a 1, the major cherry allergen. *Mol Immunol* 1999; 36:155–67.
  - 27 Neudecker P, Lehmann K, Nerkamp J *et al.* Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry (*Prunus avium*) and celery (*Apium graveolens*): correlating IgE reactivity with three-dimensional structure. *Biochem J* 2003; 376:97–107.
  - 28 Wangorsch A, Ballmer-Weber BK, Rösch P *et al.* Mutational epitope analysis and cross-reactivity of two isoforms of Api g 1, the major celery allergen. *Mol Immunol* 2007; 44:2518–27.
  - 29 Wiche R, Gubesch M, König H *et al.* Molecular basis of pollen-related food allergy: identification of a second cross-reactive IgE epitope on Pru av 1, the major cherry (*Prunus avium*) allergen. *Biochem J* 2005; 385:319–27.
  - 30 Schirmer T, Hoffmann-Sommergrube K, Susani M *et al.* Crystal structure of the major celery allergen Api g 1: molecular analysis of cross-reactivity. *J Mol Biol* 2005; 351:1101–9.
  - 31 Gieras A, Cejka P, Blatt K *et al.* Mapping of conformational IgE epitopes with peptide-specific monoclonal antibodies reveals simultaneous binding of different IgE antibodies to a surface patch on the major birch pollen allergen, Bet v 1. *J Immunol* 2011; 186:5333–44.
  - 32 Mirza O, Henriksen A, Ipsen H *et al.* Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J Immunol* 2000; 165:331–8.
  - 33 Spangfort MD, Mirza O, Holm J *et al.* The structure of major birch pollen allergens-epitopes, reactivity and cross-reactivity. *Allergy* 1999; 54 (Suppl. 50):23–6.
  - 34 Spangfort MD, Mirza O, Svensson LA *et al.* Crystallization and preliminary X-ray analysis of birch-pollen allergen Bet v 1 in complex with a murine monoclonal IgG Fab' fragment. *Acta Crystallogr D Biol Crystallogr* 1999; 55:2035–6.
  - 35 Berkner H, Seutter von Loetzen C, Hartl M *et al.* Enlarging the toolbox for allergen epitope definition with an allergen-type model protein. *PLoS ONE* 2014; 9:e111691.
  - 36 Berkner H, Engelhorn J, Liscombe DK *et al.* High-yield expression and purification of isotopically labeled norcoclaurine synthase, a Bet v 1-homologous enzyme, from *Thalictrum flavum* for NMR studies. *Protein Expr Purif* 2007; 56:197–204.
  - 37 Albrecht M, Alessandri S, Conti A *et al.* High level expression, purification and physico- and immunochemical characterisation of recombinant Pen a 1. A major allergen of shrimp. *Mol Nutr Food Res* 2008; 52 Suppl 2:S186–195.
  - 38 Piotto M, Saudek V, Sklenár V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J Biomol NMR* 1992; 2:661–5.
  - 39 Kelley LA, Sternberg MJE. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* 2009; 4:363–71.
  - 40 Ilari A, Franceschini S, Bonamore A *et al.* Structural basis of enzymatic (S)-norcoclaurine biosynthesis. *J Biol Chem* 2009; 284:897–904.
  - 41 DeLano WL. The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.
  - 42 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
  - 43 Kyhse-Andersen J. Electrophoretic transfer of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 1984; 10:203–9.
  - 44 Rice P, Longden I, Bleasby A. EMBOSS: the European Molecular Biology Open Software suite. *Trends Genet* 2000; 16:276–7.
  - 45 Williamson MP. Secondary-structure dependent chemical shifts in proteins. *Biopolymers* 1990; 29:1423–31.
  - 46 Wishart DS, Sykes BD, Richards FM. Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. *J Mol Biol* 1991; 222:311–33.
  - 47 Holzwarth G, Doty P. The ultraviolet circular dichroism of polypeptides. *J Am Chem Soc* 1965; 87:218–28.
  - 48 Greenfield N, Fasman GD. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* 1969; 8:4108–16.

- 49 Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc* 2006; 1:2876–90.
- 50 Mittag D, Batori V, Neudecker P *et al*. A novel approach for investigation of specific and cross-reactive IgE epitopes on Bet v 1 and homologous food allergens in individual patients. *Mol Immunol* 2006; 43:268–78.
- 51 Asero R, Ballmer-Weber BK, Beyer K *et al*. IgE-mediated food allergy diagnosis: current status and new perspectives. *Mol Nutr Food Res* 2007; 51:135–47.
- 52 Sampson HA, Ho DG. Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents. *J Allergy Clin Immunol* 1997; 100:444–51.
- 53 Sampson HA. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J Allergy Clin Immunol* 2001; 107:891–6.
- 54 Tomičić S, Norrman G, Fälth-Magnusson K *et al*. High levels of IgG 4 antibodies to foods during infancy are associated with tolerance to corresponding foods later in life. *Pediatr Allergy Immunol* 2009; 20:35–41.
- 55 Berkner H, Neudecker P, Mittag D *et al*. Cross-reactivity of pollen and food allergens. Soybean Gly m 4 is a member of the Bet v 1 superfamily and closely resembles yellow lupine proteins. *Biosci Rep* 2009; 29:183.

## Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Mass spectrometric sequence confirmation of recombinant  $\Delta 51\text{NCS}$ ,  $\Delta 29\text{NCS}$  and Gly m 4 variants.

**Figure S2.** CD and  $R_{\text{H}}$  of Gly m 4 and Gly m 4<sub>N42I/E44S/N46D/K54A</sub>.

**Figure S3.** Amino acids of Gly m 4 selected for mutational epitope analysis.

**Figure S4.** Recombinant  $\Delta 51\text{NCS}$  variants. **Table S1.** Parameter of mass spectrometric sequence confirmation.

**Table S2.** Candidate amino acids of Gly m 4 and their corresponding counterparts of  $\Delta 51\text{NCS}$ .

**Table S3.** Human sera used in this study.

**Table S4.** IgE binding of Gly m 4-type NCS variants.

## Supporting information

### The conformational IgE epitope profile of soybean allergen Gly m 4

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**Table E1 Parameter of mass spectrometric sequence confirmation**

| description                   | S     | SC   | P* | E   |
|-------------------------------|-------|------|----|-----|
| Δ51NCS                        | 10802 | 93.8 | 15 | 5.8 |
| Δ51NCS; E45/N47/K55           | 6196  | 24.3 | 5  | 5.7 |
| rGly m 4; N42I/E45S/N46D/K54A | 12742 | 78.3 | 12 | 4.1 |
| Δ51NCS I                      | 611   | 58.1 | 7  | 5.4 |
| Δ51NCS III                    | 7287  | 76.2 | 20 | 6.7 |
| Δ51NCS IV                     | 20427 | 81.2 | 17 | 6.3 |
| Δ51NCS II                     | 8844  | 83.1 | 13 | 6.5 |
| Δ51NCS I/IV                   | 7101  | 60.2 | 10 | 5.8 |
| Δ51NCS I/III/IV/VI            | 23692 | 81.8 | 13 | 4.9 |
| Δ51NCS I/III/V/VI             | 19358 | 79.0 | 11 | 5.8 |
| Δ51NCS I/II/III/V/VI          | 8772  | 24.9 | 6  | 3.6 |
| Δ29NCS III/IV/V/VI            |       |      | 24 |     |

S: PLGS protein score; SC: sequence coverage in %; P: number of identified tryptic peptides\* includes modified and unmodified peptides i.e. oxidized and not oxidized versions; E: mean precursor mass error in ppm

**Table E2 Candidate amino acids of Gly m 4 and their corresponding counterparts of  $\Delta 51$ NCS**

| No. | Gly m 4 | $\Delta 51$ NCS | No. | Gly m 4 | $\Delta 51$ NCS |
|-----|---------|-----------------|-----|---------|-----------------|
| 1   | F3      | K4              | 28  | S81     | K84             |
| 2   | T4      | V5              | 29  | S83     | Q86             |
| 3   | F5      | I6              | 30  | D92     | L95             |
| 4   | E6      | H7              | 31  | T93     | G96             |
| 5   | D7      | H8              | 32  | E95     | T98             |
| 6   | N10     | E11             | 33  | K96     | Y99             |
| 7   | T17     | D18             | 34  | T98     | M101            |
| 8   | K20     | T21             | 35  | D100    | T103            |
| 9   | T24     | P26             | 36  | N108    | K111            |
| 10  | D25     | G27             | 37  | T116    | S119            |
| 11  | D27     | A29             | 38  | K118    | E121            |
| 12  | N28     | K30             | 39  | E120    | H123            |
| 13  | K32     | D34             | 40  | D124    | E127            |
| 14  | D35     | G38             | 41  | E126    | V129            |
| 15  | S36     | A39             | 42  | N128    | V132            |
| 16  | K38     | E41             | 43  | Q129    | E133            |
| 17  | S39     | K42             | 44  | D130    | P134            |
| 18  | N42     | --              | 45  | E131    | L135            |
| 19  | E44     | I47             | 46  | K133    | I136            |
| 20  | N46     | D49             | 47  | K136    | T138            |
| 21  | P49     | V52             | 48  | D140    | A142            |
| 22  | K54     | D57             | 49  | K144    | D146            |
| 23  | E59     | P62             | 50  | E147    | S149            |
| 24  | D60     | G63             | 51  | D155    | S157            |
| 25  | K64     | H67             | 52  | Y156    | S159            |
| 26  | E71     | I74             |     |         |                 |
| 27  | E75     | N78             |     |         |                 |

**Table E3 Human sera used in this study**

| Patient | Symptoms                    | sIgE [kU <sub>A</sub> /L] and CAP class (1-6) |          |          |          | tIgG [mg <sub>A</sub> /L] |
|---------|-----------------------------|-----------------------------------------------|----------|----------|----------|---------------------------|
|         |                             | Bet v 1                                       | Gly m 4  | Gly m 5  | Gly m 6  | Gly m 4                   |
| 1       | OAS, TT                     | 90.4 (5)                                      | 8.64 (3) | 0.02 (0) | 0.03 (0) | 14.5                      |
| 2       | OAS                         | >100 (6)                                      | 46.3 (4) | 0.08 (0) | 0.08 (0) | 7.3                       |
| 3       | OAS                         | 67.8 (5)                                      | 14.5 (3) | 0.00 (0) | 0.01 (0) | 27.6                      |
| 4       | OAS, F, C                   | >100 (6)                                      | >100 (6) | 0.47 (1) | 0.77 (2) | 13.6                      |
| 5       | OAS, RH, TT, H              | 33.5 (4)                                      | 14.2 (3) | 0.01 (0) | 0.01 (0) | 5.4                       |
| 7       | OAS, TT, H                  | 28.3 (4)                                      | 8.69 (3) | 0.00 (0) | 0.00 (0) | 3.9                       |
| 8       | F, OAS, B                   | 31.3 (4)                                      | 9.84 (3) | 0.01 (0) | 0.02 (0) | 2.9                       |
| 9       | OAS                         | 14.6 (3)                                      | 3.93 (3) | 0.00 (0) | 0.00 (0) | 5.2                       |
| 10      | n.d.                        | 7.48 (3)                                      | 3.34 (2) | 0.00 (0) | 0.00 (0) | 6.6                       |
| 11      | OAS                         | 84.1 (5)                                      | 22.6 (4) | 0.00 (0) | 0.01 (0) | 36.9                      |
| 13      | OAS                         | >100 (6)                                      | 23.7 (4) | 0.06 (0) | 0.08 (0) | 16.0                      |
| 22      | OAS                         | 52.9 (5)                                      | 3.85 (3) | 0.00 (0) | 0.02 (0) | 8.2                       |
| 28      | DY, HU                      | 42.7 (4)                                      | 9.98 (3) | 0.00 (0) | 0.00 (0) | 8.0                       |
| 30      | FS, DY, HU                  | 48.8 (4)                                      | 11.4 (3) | 0.01 (0) | 0.02 (0) | 6.5                       |
| 33      | FS, OAS, TT                 | 8.02 (3)                                      | 4.00 (3) | 0.00 (0) | 0.00 (0) | 8.2                       |
| 35      | FS, OAS, DY, DR, GD, NS     | 25.3 (4)                                      | 7.09 (3) | 0.00 (0) | 0.00 (0) | 9.7                       |
| 36      | FS, OAS, TT, HU, DR         | 33.9 (4)                                      | 11.2 (3) | 0.00 (0) | 0.01 (0) | 7.8                       |
| 37      | FS, OAS, TT, HU, GD         | 13.8 (3)                                      | 2.68 (2) | 0.00 (0) | 0.00 (0) | 35.8                      |
| 38      | FS, OAS, TT                 | 58.1 (5)                                      | 22.9 (4) | 0.03 (0) | 0.02 (0) | 5.7                       |
| 40      | Pool of 3 sera (a, b, c)    | 40.1 (4)                                      | 7.76 (3) | 0.01 (0) | 0.02 (0) | 13.1                      |
| a       | FS, OAS, TT, DR, GD         |                                               |          |          |          |                           |
| b       | FS, OAS, TT                 |                                               |          |          |          |                           |
| c       | FS                          |                                               |          |          |          |                           |
| 41      | Pool of 4 sera (a, b, c, d) | 22.8 (4)                                      | 5.95 (3) | 0.02 (0) | 0.02 (0) | 9.9                       |
| a       | FS, OAS, HU, DR             |                                               |          |          |          |                           |
| b       | FS, OAS, TT                 |                                               |          |          |          |                           |
| c       | FS, OAS, TT, DY             |                                               |          |          |          |                           |
| d       | FS, OAS, TT                 |                                               |          |          |          |                           |
| 16      | non-allergic to soy         | 49.5 (4)                                      | 7.18 (3) | 0.00 (0) | 0.00 (0) | 36.9                      |
| 20      | "                           | 28.5 (4)                                      | 5.42 (3) | 0.01 (0) | 0.03 (0) | 26.5                      |
| 24      | "                           | 10.9 (3)                                      | 3.66 (3) | 0.00 (0) | 0.00 (0) | 15.3                      |
| 25      | "                           | 7.64 (3)                                      | 1.22 (2) | 0.18 (0) | 0.21 (0) | 61.3                      |
| 26      | "                           | >100 (6)                                      | >100 (6) | 0.08 (0) | 0.10 (0) | 14.7                      |
| 27      | "                           | >100 (6)                                      | 55.9 (5) | 0.16 (0) | 0.24 (0) | 6.1                       |
| 43      | " (Pool of 4 sera)          | 30.6 (4)                                      | 6.04 (3) | 0.17 (0) | 0.26 (0) | 9.1                       |

|           |                    |          |          |          |          |      |
|-----------|--------------------|----------|----------|----------|----------|------|
| <b>44</b> | " (Pool of 4 sera) | 18.3 (4) | 0.65 (1) | 0.07 (0) | 0.14 (0) | 6.6  |
| <b>45</b> | "                  | 6.07 (3) | 1.32 (2) | 0.00 (0) | 0.03 (0) | 7.5  |
| <b>46</b> | "                  | 8.89 (3) | 0.94 (2) | 0.00 (0) | 0.00 (0) | 4.5  |
| <b>47</b> | "                  | 47.4 (4) | 17.7 (4) | 0.00 (0) | 0.00 (0) | 7.3  |
| <b>49</b> | "                  | >100 (6) | 3.31 (2) | 0.04 (0) | 0.02 (0) | n.d. |
| <b>50</b> | "                  | >100 (6) | 21.1 (4) | 0.04 (0) | 0.02 (0) | n.d. |

Allergen-specific levels of IgE and IgG were determined by ImmunoCAP. Sera 1-5, 7-11, 13, 22, 28, 30, 33-38, 40-41 were taken from patients allergic to soy bean as confirmed by DBPCFC. Sera 16, 20, 24-27, 43-50 were taken from patients non-allergic to soy. Abbreviations of clinical symptoms: B – blisters; C – conjunctivitis; DR – drowsiness, vascular dysregulation; DY – dyspnea; F – flush; FS – face swelling; GD – gastrointestinal discomfort, vomiting; H – hoarseness; HU – hives, urticaria; NS – nasal secretion, nasal congestion; OAS – oral allergy syndrome; RH – rhinitis; TT – throat tightness, swallowing discomfort; n.d. – not determined. Sera were published elsewhere: 2, 7-8 [1,2]; 3-5 [3]; 28, 30, 33, 35-38, 40-41, 43-44 [4].

**Table E4 IgE binding of Gly m 4-type NCS variants**

|                   |    | F3 | T4 | F5 | E6 | D7 | N10 | T17 | K20 | T24 | D25 | D25/D27 | N28/K32 | K32 | D35 | S36 | K38 | S39 | E44/N46 | N46 | K54 | E59 | D60 | K64 | E71 | E75 | S81 | S83 | D92/T93 | E95/K96 | T98 | D100/F5 | N108 | T116 | K118 | E120 | D124 | D124/E126 | N128 | Q129 | D130 | E131 | K133 | K136 | D140 | K144 | E147 | D155/Y156 | control |  |  |  |  |  |
|-------------------|----|----|----|----|----|----|-----|-----|-----|-----|-----|---------|---------|-----|-----|-----|-----|-----|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|---------|---------|-----|---------|------|------|------|------|------|-----------|------|------|------|------|------|------|------|------|------|-----------|---------|--|--|--|--|--|
| Allergic subjects | 1  |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 2  |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 3  |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 4  |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 5  |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 7  |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 8  |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 9  |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 10 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 11 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 13 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 22 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 28 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 30 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
| 33                |    |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
| 35                |    |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
| 36                |    |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
| 37                |    |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
| 38                |    |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
| 40                |    |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
| 41                |    |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
| Tolerant subjects | 16 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 20 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 24 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 25 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 26 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 27 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |
|                   | 43 |    |    |    |    |    |     |     |     |     |     |         |         |     |     |     |     |     |         |     |     |     |     |     |     |     |     |     |         |         |     |         |      |      |      |      |      |           |      |      |      |      |      |      |      |      |      |           |         |  |  |  |  |  |

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NCS variants presenting Gly m 4-specific amino acids were heterologously expressed in *Escherichia coli*. Purified inclusion bodies were tested for serum IgE binding by IgE western blotting. Grey shading indicates a positive IgE signal according to a subjective ranking of signals (higher chemiluminescence than control ( $\Delta 51$ NCS)). Amino acids identical in Gly m 4 and Bet v 1 are highlighted in light grey.

**Figure E1 Mass spectrometric sequence confirmation of recombinant  $\Delta 51$ NCS,  $\Delta 29$ NCS and Gly m 4 variants**

Bold letters indicate amino acid substitutions compared to wild type NCS or wild type Gly m 4, respectively. Peptides detected by MS are underlined.

**Figure E2 CD and  $R_{(H)}$  of Gly m 4 and Gly m 4<sub>N42I/E44S/N46D/K54A</sub>**

Circular dichroism of Gly m 4 (dotted line) and Gly m 4<sub>N42I/E44S/N46D/K54A</sub> (straight line) recorded at a 10  $\mu$ M protein concentration in 10 mM potassium phosphate pH 7.4 at 25°C. Each measurement comprised the average of 10 repeated scans between 255 and 185 nm. Inset: hydrodynamic radii ( $R_{(H)}$ ) of Gly m 4 ( $2.2 \pm 0.2$  nm) and Gly m 4<sub>N42I/E44S/N46D/K54A</sub> ( $2.5 \pm 0.3$  nm) are shown.

**Figure E3 Amino acids of Gly m 4 selected for mutational epitope analysis**

Upper panel: Amino acid sequence of Gly m 4. 51 amino acids were selected for functional IgE epitope analysis. Amino acids are colored according to their positive charge (blue, 9 residues), negative charge (orange, 21 residues), polarity (yellow, 17 residues), and hydrophobicity (cyan, 3 residues). Secondary structures are indicated as  $\beta$ -strands (highlighted in grey) and  $\alpha$ -helices (underlined). Lower panel: Molecular surface presentation of Gly m 4 and distribution of amino acids selected for IgE epitope analysis.

**Figure E4 Recombinant  $\Delta 51$ NCS variants**

(a) Purified recombinant  $\Delta 51$ NCS variants presenting individual or multiple amino acids of the six potential IgE epitopes (I–VI, cf. Figure 4) of Gly m 4 were analyzed in SDS-PAGE and Coomassie-stained. Lanes 1 to 11 represent the following  $\Delta 51$ NCS variants:

- 1: NCS<sub>I</sub>: NCS<sub>F3/F5/E6/D124/E126</sub>
- 2: NCS<sub>III</sub>: NCS<sub>D25/D27/N28/V29/K32/A152/D155/Y156</sub>
- 3: NCS<sub>IV</sub>: NCS<sub>K38/S39/E59/D60/K64</sub>

- 4: NCS<sub>V</sub>: NCS<sub>E44/N46/K54</sub>
- 5: NCS<sub>II</sub>: NCS<sub>N10/D130/E131/K133/K136/K144/E147</sub>
- 6: NCS<sub>III/IV/V/VI</sub>\*: NCS<sub>K32/E44/N46/K54/D60/E120/E126</sub>
- 7: NCS<sub>I/IV</sub>: NCS<sub>F3/K38/D60/N128</sub>
- 8: NCS<sub>I/III/IV/VI</sub>: NCS<sub>T24/S36/S81/D124</sub>
- 9: NCS<sub>I/III/V/VI</sub>: NCS<sub>T4/T17/N46/S83</sub>
- 10: NCS<sub>I/II/III/IV/VI</sub>: NCS<sub>F5/T17/T24/S36/K64/S81/K118/D124/K133</sub>
- 11: NCS<sub>I/II/III/IV/VI</sub>: NCS<sub>T4/T17/K32/N46/E75/S83/D100/D124/E126/K144</sub>

Arrow heads (◄) indicate purified protein. \*based on Δ29NCS<sub>N57/I58E/D60N/V63P/D68K</sub> which is published elsewhere [5]. (b) CD spectra of Δ51NCS and eleven corresponding NCS variants thereof each recorded at a 10 μM protein concentration in 10 mM potassium phosphate pH 7.4 at 25°C. Each measurement comprised the average of 10 repeated scans between 255 and 185 nm.

**Figure E1**

|                                              | 1                 | 11                  | 21                | 31                | 41                | 51                | 61                 | 71                |
|----------------------------------------------|-------------------|---------------------|-------------------|-------------------|-------------------|-------------------|--------------------|-------------------|
| $\Delta 51\text{NCS}$                        | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTKVIHHE</u> | <u>LEVAASADDI</u> | <u>WTVYSWPGLA</u> | <u>KHLPDLLPGA</u> | <u>FEKLENIIGD</u>  | <u>GGPGTILDMT</u> |
| $\Delta 51\text{NCS}_{\text{E45/N47/K55}}$   | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTKVIHHE</u> | <u>LEVAASADDI</u> | <u>WTVYSWPGLA</u> | <u>KHLPDLLPGA</u> | <u>FEKLENIIGN</u>  | <u>GGPGTILKMT</u> |
| $\Delta 51\text{NCS}_{\text{I}}$             | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTFVFEHE</u> | <u>LEVAASADDI</u> | <u>WTVYSWPGLA</u> | <u>KHLPDLLPGA</u> | <u>FEKLENIIGD</u>  | <u>GGPGTILDMT</u> |
| $\Delta 51\text{NCS}_{\text{III}}$           | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTKVIHHE</u> | <u>LEVAASADDI</u> | <u>WTVYSWDGLD</u> | <u>NVLPKLLPGA</u> | <u>FEKLENIIGD</u>  | <u>GGPGTILDMT</u> |
| $\Delta 51\text{NCS}_{\text{IV}}$            | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTKVIHHE</u> | <u>LEVAASADDI</u> | <u>WTVYSWPGLA</u> | <u>KHLPDLLPGA</u> | <u>FKSLENIIGD</u>  | <u>GGPGTILDMT</u> |
| $\Delta 51\text{NCS}_{\text{II}}$            | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTKVIHHE</u> | <u>LNVAASADDI</u> | <u>WTVYSWPGLA</u> | <u>KHLPDLLPGA</u> | <u>FEKLENIIGD</u>  | <u>GGPGTILDMT</u> |
| $\Delta 51\text{NCS}_{\text{I/IV}}$          | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTFVIHHE</u> | <u>LEVAASADDI</u> | <u>WTVYSWPGLA</u> | <u>KHLPDLLPGA</u> | <u>FKKLENIIGD</u>  | <u>GGPGTILDMT</u> |
| $\Delta 51\text{NCS}_{\text{I/III/IV/VI}}$   | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTKVIHHE</u> | <u>LEVAASADDI</u> | <u>WTVYSWTGLA</u> | <u>KHLPDLLPGS</u> | <u>FEKLENIIGD</u>  | <u>GGPGTILDMT</u> |
| $\Delta 51\text{NCS}_{\text{I/III/V/VI}}$    | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTKTIHHE</u> | <u>LEVAASADTI</u> | <u>WTVYSWPGLA</u> | <u>KHLPDLLPGA</u> | <u>FEKLENIIGN</u>  | <u>GGPGTILDMT</u> |
| $\Delta 51\text{NCS}_{\text{I/II/III/V/VI}}$ | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTKTIHHE</u> | <u>LEVAASADTI</u> | <u>WTVYSWPGLA</u> | <u>KHLPKLLPGA</u> | <u>FEKLENIIGN</u>  | <u>GGPGTILDMT</u> |
| $\Delta 29\text{NCS}_{\text{III/IV/V/VI}^*}$ |                   | <u>M LHHQGIINQV</u> | <u>STVTKVIHHE</u> | <u>LEVAASADDI</u> | <u>WTVYSWPGLA</u> | <u>KHLPKLLPGA</u> | <u>FEKLENIIGN</u>  | <u>GGPGTILKMT</u> |
| rGlym4 <sub>N42I/E44S/N46D/K54A</sub>        | <u>MGSSHHHHHH</u> | <u>SSGLVPRGSH</u>   | <u>MGVTFFEDEI</u> | <u>NSPVAPATLY</u> | <u>KALVTDADNV</u> | <u>IPKALDSFKS</u> | <u>VEIVSGDGGP</u>  | <u>GTIKAITFLE</u> |
|                                              | 81                | 91                  | 101               | 111               | 121               | 131               | 141                | 151               |
| $\Delta 51\text{NCS}$                        | <u>FVPGEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVQMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKPEFV</u>  | <u>KIVEPLITTG</u> |
| $\Delta 51\text{NCS}_{\text{E45/N47/K55}}$   | <u>FVPGEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVQMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKPEFV</u>  | <u>KIVEPLITTG</u> |
| $\Delta 51\text{NCS}_{\text{I}}$             | <u>FVPGEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVQMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKDDEEV</u> | <u>KIVEPLITTG</u> |
| $\Delta 51\text{NCS}_{\text{III}}$           | <u>FVPGEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVQMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKPEFV</u>  | <u>KIVEPLITTG</u> |
| $\Delta 51\text{NCS}_{\text{IV}}$            | <u>FVEDEFPKEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVQMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKPEFV</u>  | <u>KIVEPLITTG</u> |
| $\Delta 51\text{NCS}_{\text{II}}$            | <u>FVPGEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVQMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKPEFV</u>  | <u>KIVDELKTTK</u> |
| $\Delta 51\text{NCS}_{\text{I/IV}}$          | <u>FVPDEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVQMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKPENV</u>  | <u>KIVEPLITTG</u> |
| $\Delta 51\text{NCS}_{\text{I/III/IV/VI}}$   | <u>FVPGEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKSVMIE</u>  | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKDEFV</u>  | <u>KIVEPLITTG</u> |
| $\Delta 51\text{NCS}_{\text{I/III/V/VI}}$    | <u>FVPGEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVSMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKPEFV</u>  | <u>KIVEPLITTG</u> |
| $\Delta 51\text{NCS}_{\text{I/II/III/V/VI}}$ | <u>FVPGEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVSMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYHVKPDFFE</u> | <u>KIVEPLITTG</u> |
| $\Delta 29\text{NCS}_{\text{III/IV/V/VI}^*}$ | <u>FVPDEFPHEY</u> | <u>KEKFILVDNE</u>   | <u>HLRKKVQMIE</u> | <u>GGYLDLGVTY</u> | <u>YMDTIHVVP</u>  | <u>GKDSCVIKSS</u> | <u>TEYEVKPEFE</u>  | <u>KIVEPLITTG</u> |
| rGlym4 <sub>N42I/E44S/N46D/K54A</sub>        | <u>DGETKFVLHK</u> | <u>IESIDEANLG</u>   | <u>YSYSVVGGA</u>  | <u>LPDTAEKITF</u> | <u>DSKLVAGPNG</u> | <u>GSAGKLTVKY</u> | <u>ETKGDAEPNQ</u>  | <u>DELKTGKAKA</u> |
|                                              | 161               | 171                 | 181               |                   |                   |                   |                    |                   |
| $\Delta 51\text{NCS}$                        | <u>PLAAMADAIS</u> | <u>KLVLEHKS</u>     | <u>SKS</u>        | <u>N</u>          |                   |                   |                    |                   |
| $\Delta 51\text{NCS}_{\text{E45/N47/K55}}$   | <u>PLAAMADAIS</u> | <u>KLVLEHKS</u>     | <u>SKS</u>        | <u>N</u>          |                   |                   |                    |                   |
| $\Delta 51\text{NCS}_{\text{I}}$             | <u>PLAAMADAIS</u> | <u>KLVLEHKS</u>     | <u>SKS</u>        | <u>N</u>          |                   |                   |                    |                   |
| $\Delta 51\text{NCS}_{\text{III}}$           | <u>PLAAMADAIS</u> | <u>KLVLAHKDKY</u>   | <u>N</u>          |                   |                   |                   |                    |                   |
| $\Delta 51\text{NCS}_{\text{IV}}$            | <u>PLAAMADAIS</u> | <u>KLVLEHKS</u>     | <u>SKS</u>        | <u>N</u>          |                   |                   |                    |                   |
| $\Delta 51\text{NCS}_{\text{II}}$            | <u>PLAAMAKAIE</u> | <u>KLVLEHKS</u>     | <u>SKS</u>        | <u>N</u>          |                   |                   |                    |                   |
| $\Delta 51\text{NCS}_{\text{I/IV}}$          | <u>PLAAMADAIS</u> | <u>KLVLEHKS</u>     | <u>SKS</u>        | <u>N</u>          |                   |                   |                    |                   |
| $\Delta 51\text{NCS}_{\text{I/III/IV/VI}}$   | <u>PLAAMADAIS</u> | <u>KLVLEHKS</u>     | <u>SKS</u>        | <u>N</u>          |                   |                   |                    |                   |

Husslik *et al.*, The conformational IgE epitope profile of soybean allergen Gly m 4. Supporting information.

|                                              |                                                          |
|----------------------------------------------|----------------------------------------------------------|
| $\Delta 51\text{NCS}_{\text{I/III/V/VI}}$    | <u>PLAAMADAIS</u> <u>KLVLEHKSKS</u> N                    |
| $\Delta 51\text{NCS}_{\text{I/II/III/V/VI}}$ | <u>PLAAMAKAIS</u> <u>KLVLEHKSKS</u> N                    |
| $\Delta 29\text{NCS}_{\text{III/IV/V/VI}^*}$ | <u>PLAAMADAIS</u> <u>KLVLEHKSKS</u> NSDEIEAII TVLEHHHHHH |
| rGlym4 <sub>N42I/E44S/N46D/K54A</sub>        | <u>DALFKAIEAY</u> <u>LLAHPDYN</u>                        |

**Figure E2**

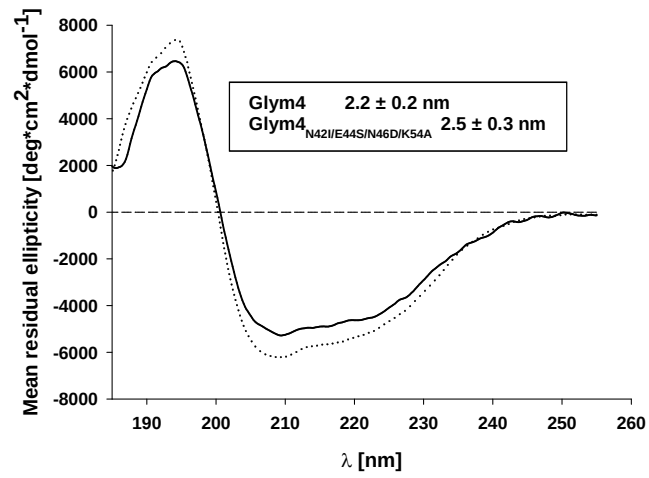


**Figure E3**

GVFTFEDEINSPVAPATLYKALVTDADNVIPKALDSFKSVENVEGNGGPGTIKKITFLED 60  
 GETKFVLHKIESIDEANLGYSSVVGGAALPDTAEKITFDSKLVAGPNNGGSAGKLTVKYE 120  
 TKGDAEPNQDELKTGKAKADALFKAFAYLLAHPDYN 157

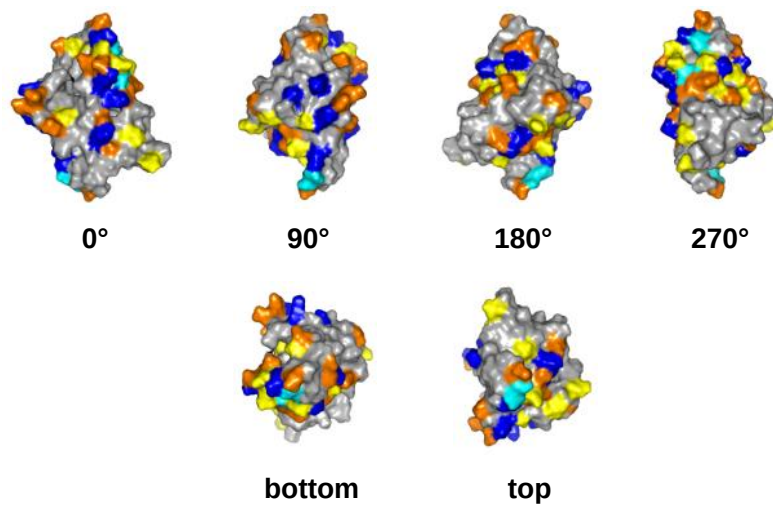
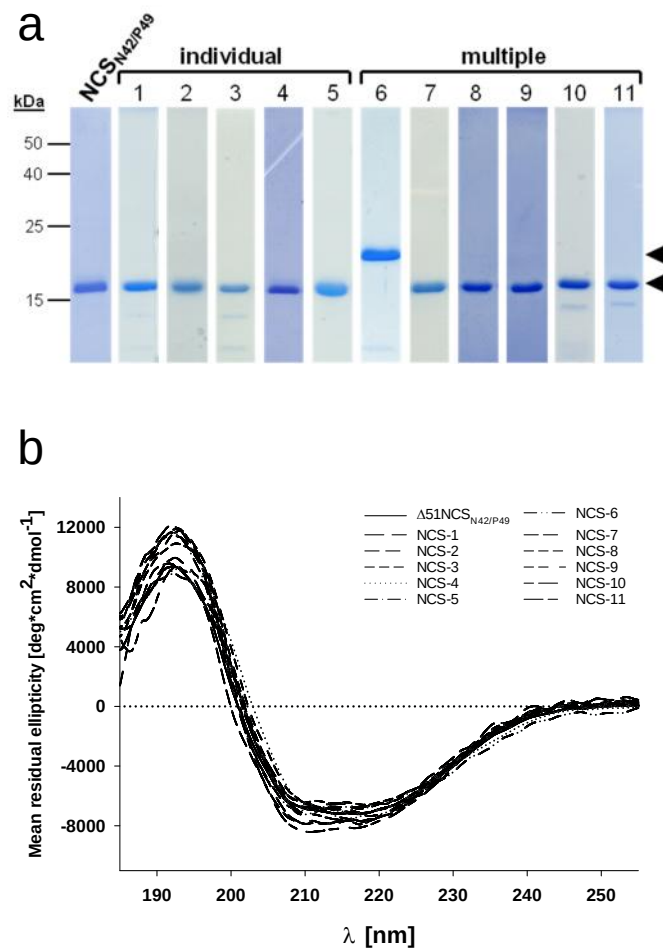


**Figure E4**



## References

1. Ballmer-Weber BK, Holzhauser T, Scibilia J, Mittag D, Zisa G, Ortolani C, Oesterballe M, Poulsen LK, Vieths S, Bindslev-Jensen C. Clinical characteristics of soybean allergy in Europe: a double-blind, placebo-controlled food challenge study. *J Allergy Clin Immunol.* 2007 Jun;119(6):1489-96. Epub 2007 Mar 26.
2. Berkner H, Neudecker P, Mittag D, Ballmer-Weber BK, Schweimer K, Vieths S, Rösch P. Cross-reactivity of pollen and food allergens: soybean Gly m 4 is a member of the Bet v 1 superfamily and closely resembles yellow lupine proteins. *Biosci Rep.* 2009 Jun;29(3):183-92.
3. Mittag D, Vieths S, Vogel L, Becker WM, Rihs HP, Helbling A, Wüthrich B, Ballmer-Weber BK. Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens. *J Allergy Clin Immunol.* 2004 Jan;113(1):148-54.
4. Kleine-Tebbe J, Vogel L, Crowell DN *et al.* Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1- related PR-10 protein in soybean, SAM22. *The Journal of allergy and clinical immunology* 2002;**110**:797–804.
5. Berkner H, Seutter von Loetzen C, Hartl M *et al.* Enlarging the Toolbox for Allergen Epitope Definition with an Allergen-Type Model Protein. *PLoS ONE* 2014;**9**:e111691.