

RESEARCH ARTICLE

Molecular and immunological characterization of wheat Serpin (Tri a 33)

Hamza Mameri^{1,2}, Sandra Denery-Papini¹, Manon Pietri¹, Olivier Tranquet¹, Colette Larré¹, Martine Drouet³, Evelyne Paty⁴, Anne-Marie Jonathan⁵, Etienne Beaudouin⁶, Denise-Anne Moneret-Vautrin⁶, Thierry Moreau⁷, Pierre Briozzo² and Jean-Charles Gaudin¹

¹INRA, UR 1268 Biopolymères Interactions Assemblages, Nantes, France

²INRA, UMR 1318 Institut Jean-Pierre Bourgin, Versailles, France

³CHU d'Angers, Unité d'Allergologie Générale et de Pneumologie, Angers, France

⁴Service de Pneumologie et d'Allergologie Pédiatrique, Groupe Hospitalier Necker, Paris, France

⁵AH-HP, Groupe Hospitalier Cochin, Service de Pathologies Professionnelles, Université Paris Descartes, Paris, France

⁶Université de Nancy I, Service d'Allergologie, CH Epinal, Epinal, France

⁷INSERM U1100, Pathologies respiratoires: protéolyse & aérosolthérapie, Tours, France

Scope: Several wheat proteins are responsible for food and respiratory allergies. Due to their large polymorphism, the allergenic potential of a number of them has not yet been precisely established. The aim of this work was to perform a thorough assessment of serpin (Tri a 33) allergenicity.

Methods and results: Recombinant wheat Serpin-Z2B isoform (rSerpin-Z2B) was expressed in *Escherichia coli*. Synchrotron radiation circular dichroism data indicated that the recombinant serpin contains slightly more β -strands than α -helix structures. IgE reactivity of sera from 103 patients with food allergy and 29 patients with Baker's asthma was evaluated using ELISA, a model of basophil activation and linear epitope mapping (Pepscan). Twenty percent of patients with food allergy to wheat and 31% of those with Baker's asthma displayed rSerpin-Z2B-specific IgE in ELISA. The protein was able to induce IgE-dependent basophil degranulation. The Pepscan experiment identified four regions involved in IgE binding to serpin. Heating the protein induced its irreversible denaturation and impaired IgE binding, revealing the predominance of conformational epitopes.

Conclusion: This study confirms wheat serpin allergenicity and shows that recombinant serpin may be a marker of a broad spectrum of sensitization to wheat proteins.

Received: April 26, 2012
Revised: July 24, 2012
Accepted: September 5, 2012

**Keywords:**

Allergen / Baker's asthma / Food allergy / Serpin / Wheat

1 Introduction

Allergic reactions to wheat can occur by several routes of exposure: inhalation, ingestion, or skin contact. Baker's asthma

Correspondence: Dr. Jean-Charles Gaudin, INRA, UR 1268 Biopolymères Interactions Assemblages, F-44316 Nantes Cedex 3, France

E-mail: jean-charles.gaudin@nantes.inra.fr

Fax: +33-0-2-40675025

Abbreviations: AEDS, atopic-eczema dermatitis syndrome; A/G, albumins and globulins; BA, Baker's asthma; EIA, exercise-induced anaphylaxis; FAW, food allergy to wheat; GI, gastro-intestinal; HisTag, 6-histidine tag; RBL, rat basophilic leukemia; SPT, skin prick test; SRCD, synchrotron radiation circular dichroism

(BA) and, more generally, respiratory allergy to wheat are frequent pathologies found among bakery workers and millers, with the incidence varying from 0.1 to 1% of the subjects exposed to flour [1, 2]. Allergic rhinitis and asthma are the main symptoms. The prevalence of food allergy to wheat (FAW) has been estimated by oral challenge tests to be in the range of 0.2–0.5% of children. In adults, sensitization to wheat was estimated between 0.4 and 3.6% [3, 4]. FAW induces a wide range of symptoms: asthma, atopic-eczema dermatitis syndrome (AEDS), urticaria, gastro-intestinal (GI) disorders, and anaphylaxis (including exercise-induced anaphylaxis (EIA)).

Wheat proteins are classically separated into two groups according to their solubility: the salt-soluble proteins referred to albumins and globulins (A/G), and the gluten fraction corresponding to gliadins and glutenins. Both gluten and A/G

fractions are involved in allergies to wheat [5]. Numerous constituents of the A/G fraction such as IgE-binding proteins have been reported following 2D-immunoblotting (allergenomics) approaches in both BA and FAW [6–10]. Among the allergens from the A/G fraction common to food and respiratory allergies to wheat, members of the α -amylase/trypsin inhibitors (Tri a 15, 28–30) and LTP1 (Tri a 14) have been characterized in detail. Their recognition by patient IgE has been studied using ELISA, the rat basophilic leukemia (RBL) cell model, human basophil activation tests, skin prick tests (SPT) [9, 11, 12], and epitope identification [13, 14]. Wheat serpins (Tri a 33) have also been shown to be involved in both food and respiratory wheat allergies, mainly using allergenomics [6–10, 15] and, more recently, with ImmunoCAPs [16]. However, with the exception of Sander et al. [16], studies were carried out on only a few sera and neither biological activity nor IgE-binding epitopes were characterized. Wheat serpins belong to the superfamily of SERine Protease INhibitors (referred to as serpins) and have been identified in almost all organisms including plants. Most of them possess inhibitory functions resulting from large conformational transitions between native serpin and the protease-serpin complex (generally proteases of the chymotrypsin family) [17]. It can be mentioned that a serine proteinase-like inhibitor of 9.9 kDa has been cloned and characterized as a wheat allergen in BA [18]. However, this serpin-like allergen does not belong to the defined classical serpin superfamily [17]; it instead belongs to the potato inhibitor I family.

Wheat serpins were first described by Rosenkrands et al. [19], but their low abundance in wheat grain (approximately 1.6% of the weight of the A/G fraction) [20] and their genetic polymorphism (about 15 isoforms) [21] make it very difficult to study them at the molecular level. Five isoforms (Fig. 1) of wheat serpin have been produced as recombinant proteins in *Escherichia coli* (*E. coli*) [16, 22]. They act as suicide substrate inhibitors of chymotrypsin and cathepsin G [22]. No 3D structure of a cereal serpin has yet been established. The closest serpin (~50% protein sequence identity) with a known crystal structure is that of *Arabidopsis thaliana* [23].

The aim of this work was to make a thorough assessment of serpin allergenicity in both food and respiratory wheat allergies. For this purpose, we expressed and characterized a recombinant wheat serpin (rSerp-Z2B), and studied IgE binding to this protein using ELISA, RBL cell line activation, and epitope mapping using a large collection of patient sera.

2 Materials and methods

2.1 Serpin-Z2B cloning

Total RNA was extracted from wheat grain (Neepawa cultivar) using a total RNA midi spin columns kit (Macherey-Nagel GmbH, Düren, Germany). Mature serpin cDNA was cloned by RT-PCR as described in [24] with the following degenerated primers: 5'-CAT-ATG-GCA-ACC-ACC-

CTY-GCC-ACY-GAC-G-3' (SERP-1) and 5'-GGA-TCC-TTA-CRA-AGA-TMR-GAG-GGG-ATT-GAC-C-3' (SERP-2). cDNA was cloned into pGEMT-Easy plasmid (Promega, Madison, USA) and sequenced by Sanger method. Full-length mature Serpin-Z2B cDNA was amplified with the following specific primers: 5'NNN-NCA-TAT-GGC-AAC-CAC-CCT-CGC-C-3' (SERP-F1) and 5'NNN-NGG-ATC-CGA-GGG-GAT-TGA-CCA-C-3' (SERP-R1) and cloned into the *Nde*I and *Bam*HI restriction site of pET-21b (Novagen, Merck, Darmstadt, Germany). Primers were originally designed to use the natural stop codon of the protein, but since we subsequently decided to produce the protein with a C-terminal 6-histidine tag (HisTag) sequence, a linker encoding the HisTag sequence was inserted immediately downstream of the last codon of Serpin-Z2B cDNA with *Bam*HI and *Eco*RI.

2.2 Expression and purification of rSerp-Z2B

Recombinant expression was done in the *E. coli* BL21-DE3 strain (Invitrogen, Life Technologies, Carlsbad, USA) as described in [22]. After cell lysis, the soluble fraction or the inclusion bodies solubilized in 20 mM Tris-HCl, pH 8, 200 mM NaCl, 20 mM imidazole, and 8 M urea were applied on a HisTrap TMFF column (GE Healthcare, Chalfont St Giles, UK) for Ni^{2+} affinity chromatography. Elution was performed with a 50 mM–300 mM imidazole gradient. The purity was assessed by SDS-PAGE. The very pure fractions (> 95%) of rSerp-Z2B were pooled and dialyzed against a 20 mM Tris-HCl, pH 8, 100 mM NaCl buffer at 4°C for 48 h. The protein concentration was determined with the bicinchoninic acid (BCA) reagent kit (Pierce, Fisher Scientific, Strasbourg, France) protein assay using BSA as a standard. rSerp-Z2B_{sf} was obtained from the soluble fraction and rSerp-Z2B_{ib} from inclusion bodies.

2.3 Patients

Sera were obtained from 132 patients allergic to wheat, including 103 patients who suffered from FAW and 29 from BA. All BA patients were adults and presented rhinitis and/or asthma. The FAW group contained 21 adults and 82 children whose symptoms included AEDS, EIA, anaphylaxis, urticaria, and gastro-intestinal disorders (Table 1). Sera from 12 nonatopic subjects and ten patients with grass pollen allergy were used as controls. Sensitization to wheat proteins was looked for in patients using SPT to wheat flour (Moulins Soufflet), to gluten and to rye extracts (ALK-Abello, Varennes en Argonne, France) and/or using specific IgE assay to wheat flour, gluten and ω 5-gliadins (Phadia ImmunoCAP, Uppsala, Sweden). Wheat allergy was confirmed by a positive challenge or evident effect of wheat avoidance. The double-blind placebo-controlled food challenge and inhalation challenge with wheat flour are described in [13] and [25]. Blood collection, SPT, and challenges were done with the informed

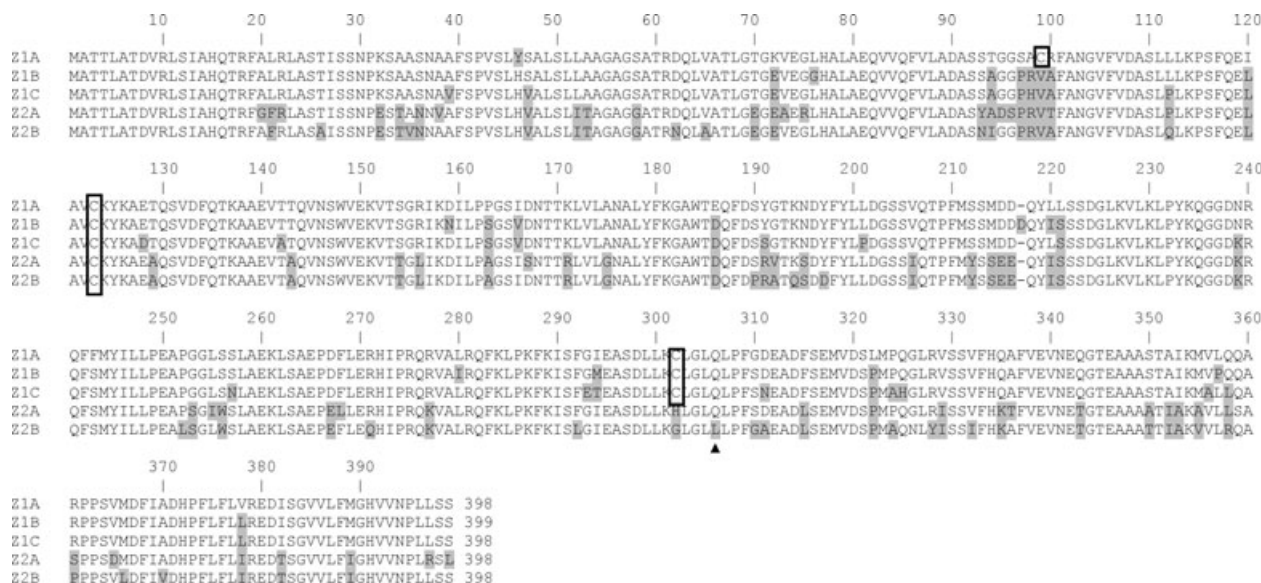


Figure 1. Alignment of wheat "prototype" Serpin-Z1A (UniProtKB ID Q41593), -Z1B (UniProtKB ID P93693), -Z1C (UniProtKB ID Q9ST58), -Z2A (UniProtKB ID Q9ST57), and -Z2B (UniProtKB ID P93692) sequences. Divergent residues compared to Serpin-Z1A as reference are in gray. Cysteine residues are boxed. Point mutation: L₃₀₅G between Serpin-Z2B reference sequence (UniProtKB ID P93692) and rSerpin-Z2B (GenBank ID JN903908) is marked by a black arrow.

consent of the patients or their parents, and after approval by the Ethics Committee of Ile de France III and AFSSAPS (authorization number 2008-A01565–50).

2.4 Fluorimetric ELISA

The concentrations in specific-IgE for rSerpin-Z2B, heated rSerpin-Z2B, and the A/G fraction in-patient sera were measured as described in [7]. rSerpin-Z2B and the A/G fraction extracted from the cultivar Recital were solubilized in PBS buffer at 5 µg.mL⁻¹ and coated on white 384-well plates (MaxiSorp, NUNC™, Fischer Scientific) for 2 h at room temperature in carbonate buffer. After washing with PBS-Tween 0.1% (PBS-T), plates were saturated with PBS-T-gelatin 0.5% w/v (PBS-TG) for 1 h at 37°C and incubated at 37°C overnight with 1:10 and 1:40 diluted sera. After washing, alkaline phosphatase-conjugated goat anti-human IgE (Sigma, Saint Quentin-Fallavier, France) diluted to 1:500 in PBS-TG was added for 2 h at 37°C. Plates were washed with PBS-T prior the addition of 4-MUP substrate (4-methylumbelliferyl phosphate, Sigma) diluted to 1:5 in Tris-HCl 1 M pH 9.8 for 90 min at room temperature away from light. Fluorescence was measured with FLX800 plate reader (BIO-TEK instruments, Colmar, France) at 440 nm (excitation 360 nm). All determinations were carried out in triplicate. In order to quantify specific IgE, a range of standard IgE (Human Serum Immunoglobulin E: NIBSC ref 75/502) from 0.8 ng.mL⁻¹ up to 160 ng.mL⁻¹ was performed by sandwich ELISA, under the same conditions, using a rabbit anti-human IgE (DAKO,

Trappes, France) as capture antibody. The specific IgE concentrations in the different sera were calculated after adjusting the sigmoid from the standard curve.

The Mann-Whitney nonparametric test was used for comparisons between the groups of patients with respect to specific IgE concentration. GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com) was used. *p* values less than 0.05 were considered to be statistically significant.

2.5 Activation of RBL cells by rSerpin-Z2B

The activation of RBL cells was carried out with the SX38 P4B7 clone expressing the human FcεRI α,β and γ-chains as described in [11]. Activation was measured by β-hexosaminidase enzyme release assay. Three concentrations (0.02, 0.2, and 2 µg.mL⁻¹) of the rSerpinZ2B_{SF} or proteins from the A/G fraction were used. BSA was used as a negative control. Sera were diluted to 1:10. Additionally, a rabbit anti-human IgE was used as a positive control. RBL cell degranulation was measured in duplicate and expressed as the percentage of total β-hexosaminidase release calculated using the following formula: (optical density (OD) sample release–OD cell spontaneous release/OD total release–OD spontaneous release) × 100. Total release of β-hexosaminidase was obtained by cell lysis with triton X-100. A positive threshold of 11% degranulation was determined from control sera response (nonatopic subjects or grass pollen allergy subjects); it corresponded to the mean + 3 SD.

Table 1. Demographic and clinical data on patients studied with FAW and BA. Data on all sera are on the top, data on serpin-positive sera are on the bottom of the table. Total and specific IgE concentrations are expressed in ng.mL⁻¹, both were determined by F-ELISA

All sera	Food allergy to wheat		Baker's asthma	Nonatopic	Grass pollen allergy
	Adults (n = 21)	Children (n = 82)	Adults (n = 29)	n = 12	n = 10
Gender (% of men)	57%	70%	73%	12%	83%
Age (mean ± SD)	38 ± 18	7 ± 6	41 ± 13	38 ± 19	34 ± 24
Symptoms (%)	57% EIA or AS 14% AEDS 14% U 24% GI	9% EIA or AS 51% AEDS 29% U 15% GI	69% rhinitis 61% asthma		
Total IgE (ng/mL)					
Mean	1904	2526	2067	147	549
SD	2615	2257	2158	115	484
A/G-positive sera	n = 8 (38%)	n = 73 (89%)	n = 21 (72%)	n = 1 (8%)	n = 4 (40%)
A/G-specific IgE (mean)	12.5	77	23	2	10
LMW-positive sera	n = 11 (52%)	n = 52 (63%)	n = 11 (38%)	n = 3 (25%)	n = 1 (10%)
LMW-specific IgE (mean)	12	20	3	0.36	2
LTP1-positive sera	n = 8 (38%)	n = 45 (55%)	n = 15 (52%)	n = 1 (8%)	n = 3 (30%)
LTP1-specific IgE (mean)	3	22	3	0.01	0.03
Total gliadins-positive sera	n = 11 (52%)	n = 65 (79%)	n = 19 (66%)	n = 2 (17%)	n = 3 (30%)
Total gliadins-specific IgE (mean)	21	49	2	0.67	6
ω5-gliadin-positive sera	N = 12 (57%)	n = 37 (45%)	n = 4 (14%)	n = 1 (8%)	n = 2 (20%)
ω5-gliadin-specific IgE (mean)	33	12	0.14	0.01	5
Serpin-positive sera	n = 2 (9.5%)	n = 19 (23%)	n = 9 (31%)	n = 1 (8%)	n = 0 (0%)
Serpin-specific IgE (mean)	6.5	25	7	1.2	
Age (mean)	55	7	41	46	
Symptoms	50% AEDS 50% U	5% AS 58% AEDS 21% U 16% GI	50% rhinitis 83% asthma		
Total IgE (mean ng/mL)	5694	4501	2113	128	
A/G-specific IgE (mean)	18	164	18	0	
LMW-GS-specific IgE (mean)	14	59	5	0.1	
LTP1-specific IgE (mean)	1	52	5	0	
Total gliadin-specific IgE (mean)	21	144	2	0	
ω5-gliadin-specific IgE (mean)	12	35	0	0.1	

2.6 IgE binding to solid-phase synthetic peptides (Pepscan)

Peptides of 14 residues, overlapping on 11 residues, spanning a full-length Serpin-Z2B sequence (195 amino acids – UniProtKB accession Q9ST57) covalently immobilized by their C-terminus on a cellulose membrane (Abimed) were used (5–10 nmol of peptide per spot). Revelation of IgE binding was performed as described in [13]. After washing for 5 min with methanol and three times for 5 min with Tris-HCl 50 mM pH 8.0 buffer containing 0.05% Tween 20 v/v (TBS-T), membranes were blocked with 2.5% w/v skimmed dried milk and 5% w/v sucrose in TBS-T for 1 h, washed with TBS-T and incubated overnight with sera from patients diluted 1:20 (or 1:40 for serum 901) in blocking buffer. Each step was performed at room temperature under agitation. After further washing, HRP-conjugated anti-human IgE antibodies (SouthernBiotech, Fisher Scientific, Strasbourg, France) were added in blocking buffer at 1:10 000 for 1 h. The revelation was made with the Super Signal West Dura Extended Duration Substrate kit (Pierce, Fisher Scientific) containing

luminol as a substrate. Chemiluminescent IgE-binding detection was performed using a camera (Fujifilm Luminescent Image Analyzer LAS 3000, Raytest, La Défense, France) allowing semi-quantitative analysis. Spots intensities were estimated using Multi-Gauge v3.0 software (Fujifilm, La Défense, France).

The membranes were regenerated by incubation in 50 mM Tris-HCl pH 6.7 containing 100 mM β-mercaptoethanol and 2% w/v SDS for 30 min at room temperature and in 50 mM glycine pH 2.8 for 15 min at 50°C.

2.7 Synchrotron radiation circular dichroism

rSerpin-Z2B_{5F} and rSerpin-Z2B_{1B} were solubilized in 10 mM phosphate buffer, pH 8, containing 100 mM Na₂SO₄. Measurements were carried out on the DISCO beamline at the SOLEIL synchrotron facility (Gif-sur-Yvette, France), Scans using wavelengths ranging from 170 to 280 nm with 1 nm steps were measured four times for each sample. Spectra of dialysis buffer were subtracted from those of

the corresponding samples. The 263–270 nm region was set to zero, and resulting spectra were calibrated with d-10-camphorsulfonic acid using the CDtool software [26]. Spectra were normalized to protein concentrations. Ratios of secondary structures were determined using ContinLL program in Dichroweb [27] and using SP175 as the reference set [28, 29]. For all measurements, spectra were analyzed down to 175 nm; this corresponded to a photomultiplier high tension remaining below half of its total variation.

Normalized root-mean-square deviations (NRMSD) provided the most accurate fit for each piece of data. For the thermostability experiment, rSerp-Z2B_{SF} was heated. The cell temperature was directly controlled on the beamline using a Peltier effect with 5°C steps up to 85°C (15 min. incubation for each step) and cooled down in the same way. Synchrotron radiation circular dichroism (SRCD) spectra were recorded at each step.

2.8 Modeling

Homology modeling was performed using I-TASSER [30] an Internet service system for protein structure and function predictions (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>). The quality of the obtained model was evaluated with Procheck [31]. Procheck score of -0.15 confirmed the good stereochemistry of the model (for good quality score has to be > -0.5).

3 Results

3.1 Cloning of Serpin-Z2B

Out of the five serpins described in [22] Serpin-Z1A, -Z1B, -Z1C, -Z2A, and -Z2B (Fig. 1), all except Serpin-Z2A were

cloned. Serpin-Z2B was chosen for recombinant expression because peptides corresponding to its sequence were found in 2D-electrophoresis spots recognized by sera from patients allergic to wheat [7]. Moreover, it contains a unique cysteine that reduces the possibility of protein oxidation and inappropriate disulfide bond formation during recombinant protein production and purification. Compared to the Serpin-Z2B reference sequence (UniProtKB ID P93692), rSerp-Z2B (GenBank ID JN903908) had one point mutation: L₃₀₅G (Fig. 1).

3.2 Expression, purification, and structural characterization of rSerp-Z2B

rSerp-Z2B was produced in *E. coli* as a fusion protein with a C-terminal HisTag. The expressed protein was found in both the soluble fraction and inclusion bodies. Its purity and integrity were confirmed by SDS-PAGE (Fig. 2A), Western blot (Fig. 2B), and mass spectrometry. Nano-LC-MS/MS analysis resulted in 71% (overlapped) sequence coverage with peptides covering N-terminal extremity which led to a confident determination of rSerp-Z2B (Supporting Information Fig. S1). The presence of N-terminal histidines was confirmed by Western blot using an anti-HisTag antibody (Fig. 2B). In vitro kinetics experiments showed that rSerp-Z2B_{SF} inhibited 80% of human cathepsin G activity and 60% of chymotrypsin activity (not shown). rSerp-Z2B_{IB} had 20% less inhibitory activity.

Far-UV SRCD spectra (Fig. 2C) showed that rSerp-Z2B_{IB} and rSerp-Z2B_{SF} were folded and contained predominantly β -strand structures. However, rSerp-Z2B_{SF} contains more helical structures than rSerp-Z2B_{IB}. Secondary structure analysis using Dichroweb [27] with the SP175 reference dataset [28] revealed 26% α -helix, 32% β -strands, 11% turns, and 31% coiled structures for rSerp-Z2B_{SF} with good NRMSD values (0.15 or less) between the experimental data

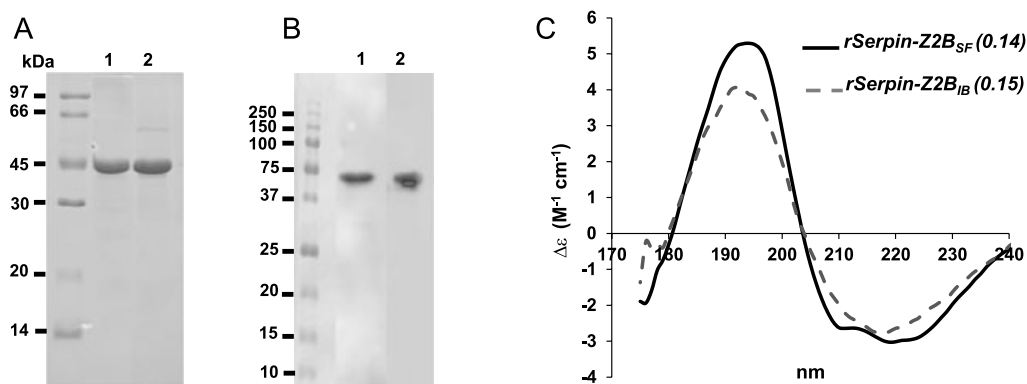


Figure 2. (A) Coomassie brilliant blue-stained SDS-PAGE 15% containing purified recombinant Serpin-Z2B produced in soluble fraction (rSerp-Z2B_{SF}) (lane 1) and in inclusion bodies (rSerp-Z2B_{IB}) (lane 2). (B) Western blotting of the two forms of recombinant Serpin-Z2B (an anti-HisTag antibody was used). Lane 1: rSerp-Z2B_{SF}; lane 2: rSerp-Z2B_{IB}. (C) SRCD spectra of rSerp-Z2B_{SF} and rSerp-Z2B_{IB} (NRMSD values are given in parentheses).

and the derived spectra; and 20% α -helix, 33% β -strands, 12% turns, and 35% for rSerpIn-Z2B_{IB}.

3.3 Characterization of patient sera

Characteristics of the wheat allergic patients and control subjects are presented in Table 1 (upper part). Total IgE and specific IgE to a wheat A/G fraction and to other wheat proteins (from the cultivar Recital) were measured by F-ELISA. Patients with FAW were divided into two age subgroups. Children ($n = 82$) mainly presented symptoms of AEDS (51% of the patients) and urticaria (29%); 89% of these sera contained IgE specific to A/G. In contrast, most adults with FAW ($n = 21$) suffered from anaphylaxis or EIA (57% of the cases) and their sera displayed IgE specific to A/G less often (38%). Children with FAW ($n = 82$) had significantly ($p = 0.005$) higher content in A/G-specific IgE (mean concentration 77 ng.mL^{-1}) than adults with FAW ($n = 21$) (mean concentration 12.5 ng.mL^{-1}). Variations were also observed for other proteins. Among patients with BA, 72% possessed IgE specific to A/G, (mean concentration 23 ng.mL^{-1}). Mean concentrations in total IgE from these three groups of patients allergic to wheat were comparable and far higher than those of the control groups. One nonatopic person and four patients allergic to grass pollen had very weak concentrations in wheat A/G-specific IgE.

3.4 Determination of patients' reactivity toward Serpin-Z2B

The concentration in rSerpIn-Z2B-specific IgE was measured by indirect F-ELISA (Fig. 3). In preliminary assays, both rSerpIn-Z2B_{SF} and rSerpIn-Z2B_{IB} were tested with a small number of sera. In all cases, the protein purified from the soluble fraction was able to bind higher amounts of IgE than the form obtained from the insoluble bodies (data not shown). rSerpIn-Z2B_{SF} was therefore chosen for the following analysis. Control sera were negative in ELISA with the exception of one serum from a nonatopic subject that reacted weakly against rSerpIn-Z2B_{SF}. Since serpins belong to the A/G fraction, the reactivity of sera to the A/G extract was used as a reference in this work. All rSerpIn-Z2B_{SF}-positive sera were positive toward the A/G fraction and the median of A/G-specific IgE concentration in the children-FAW group (37 ng.mL^{-1}) differed significantly ($p < 0.05$) from that of the adults-FAW group (10 ng.mL^{-1}).

The percentage of serpin-positive sera was higher in the BA population (31%) than in the FAW population (20%). Among the FAW population, 23% of the children and 9% of the adults were positive (Table 1). In the group of patients with BA serpin-positive patients suffered more frequently from asthma. Among children with FAW, those positive to serpin displayed higher mean concentrations in total IgE and

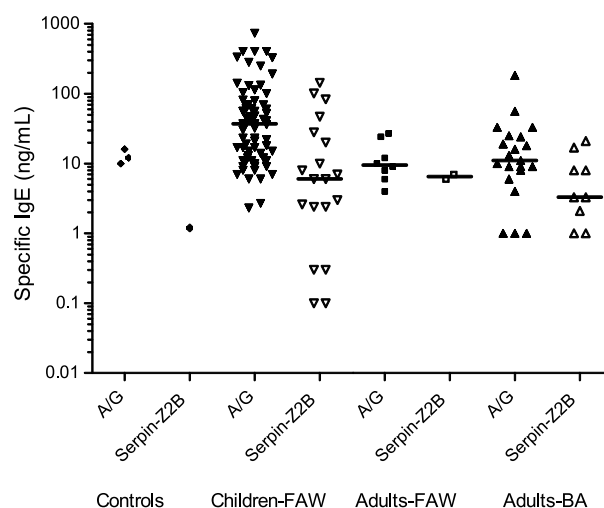


Figure 3. Specific IgE assay for sera from 132 allergic patients and 22 control patients to recombinant rSerpIn-Z2B_{SF} (SerpIn-Z2B lanes) and the A/G fraction (A/G lanes) by ELISA. Patients had FAW ($n = 103$, 21 adults and 82 children) or BA ($n = 29$, adults only). Only positive values are reported and horizontal bars indicate medians.

specific IgE for the A/G fraction, gliadins, glutenins, and LTP1 ($p < 0.05$).

We compared the median concentrations of serpin-specific IgE of serpin-positive sera in the following groups: children-FAW (6 ng.mL^{-1}), adults-FAW (7 ng.mL^{-1}) and adults-BA (3.3 ng.mL^{-1}). No significant difference was observed. Mean age and symptom distribution were similar between serpin-positive and serpin-negative children. No patient suffering from EIA had serpin-specific IgE.

3.5 Effect of heating on rSerpIn-Z2B_{SF} structure and IgE binding

Figure 4A shows the dichroic signals of rSerpIn-Z2B monitored with increasing temperatures. Spectra showed an isodichroic point at 207 nm. A decrease of positive (195 nm) and negative (220 nm) peak intensity was observed when heating, implying the conversion of α -helices and β -strands into random coil structures. The denaturation remained limited up to 65°C , and increased when heated at 80°C . These structure changes were irreversible when the temperature was lowered.

IgE binding to the rSerpIn-Z2B_{SF} heated up to 85°C was analyzed by ELISA and compared to IgE binding to the non-heated protein using 13 serpin-positive sera from patients with BA ($n = 3$) or FAW ($n = 10$) (Fig. 4B). For ten sera, protein heating induced a decrease of IgE binding. A decrease of more than 50% of the value was obtained for six of them. Nevertheless, for the three remaining sera, heating of rSerpIn-Z2B had no effect.

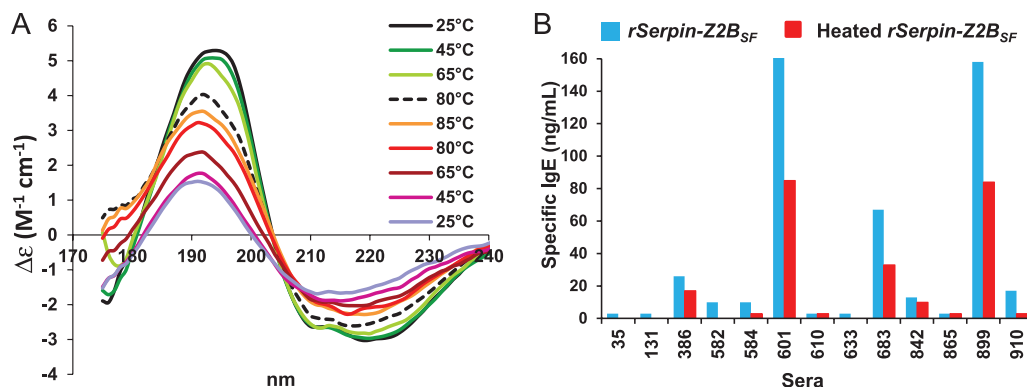


Figure 4. Thermostability of rSerp-Z2B_{SF}. (A) Dichroic signals of heated rSerp-Z2B_{SF}. A 5°C gradient step was applied from 25 to 85°C (go/back). (B) Specific IgE assay with rSerp-Z2B_{SF} and heated-rSerp-Z2B_{SF}.

3.6 Activation of RBL cell by rSerp-Z2B_{SF}

The allergenicity of rSerp-Z2B_{SF} in solution was evaluated by its capacity to induce degranulation of RBL cells in the presence of specific IgE and compared with the triggering potential of the A/G fraction (Fig. 5). Twelve sera from wheat-allergic patients were selected on the basis of their serpin-specific IgE concentrations and available volumes. As expected, no significant degranulation occurred either with BSA or when RBL cells were incubated with serpin or the A/G fraction and sera from four nonatopic subjects. The A/G fraction induced RBL cell degranulation when incubated with 11 sera from wheat-allergic patients. Nine of them elicited more than 25% degranulation. The rSerp-Z2B_{SF} induced RBL cell degranulation when incubated with seven sera (all from patients with FAW). Three of them induced more than 25% degranulation. For most sera, the degranulation elicited by serpin and the A/G fraction occurred in the same range of protein concentrations (0.02 μg.mL⁻¹); for the others it occurred at 2 μg.mL⁻¹. For one serum (#899), the degranulation

obtained with rSerp-Z2B_{SF} was higher than that observed with the A/G fraction.

3.7 IgE epitope mapping by Pepscan

Eighteen sera from allergic patients (13 with FAW and five with BA) were used in Pepscan experiments, 12 previously used in RBL assays and four additional also selected on the basis of ELISA data and availability. No response was detected for eight sera from wheat allergic patients as well as for four sera from nonatopic subjects. The other ten sera from allergic patients displayed IgE binding to a large number of peptides, generally with low-binding intensities (data not shown). We did not observe any difference in peptide recognition according to the type of allergy (FAW versus BA). Several overlapping peptides were recognized with higher frequencies and intensities. They covered four regions of the Serpin-Z2B sequence (Fig. 6A): region 364–384 recognized by five sera, region 271–293 recognized by four sera, regions

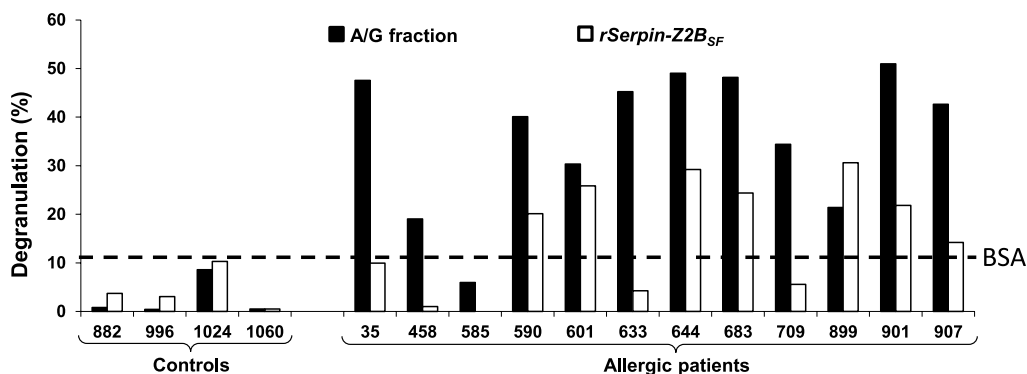


Figure 5. Degranulation of RBL cells. RBL were cells incubated with IgE from patients ($n = 12$) or with control sera ($n = 4$) and the A/G fraction (2 μg.mL⁻¹) or rSerp-Z2B_{SF} (2 μg.mL⁻¹). Among the allergic patients, three had BA (#35, #458, and #633) and the others had FAW. The result is expressed as the percentage of degranulation obtained by the β-hexosaminidase release assay. BSA line: BSA was used as a negative control ($n = 16$). The line indicates the mean of degranulation + 3*SD.

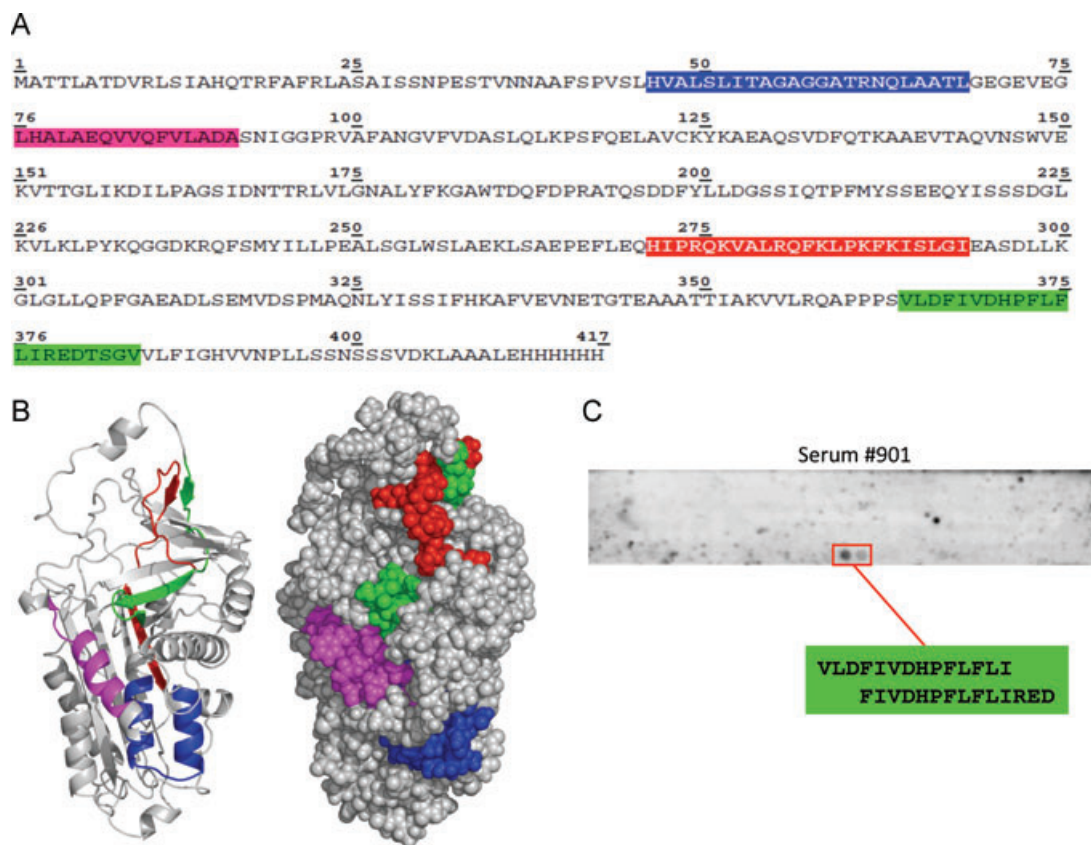


Figure 6. Localization of major IgE-binding regions identified by Pepsan (A) on the Serpin-Z2B sequence and (B) on the three-dimensional Serpin-Z2B model (cartoon (left) and spheres (right)). Epitopes are shown in blue (46–68), magenta (76–92), red (271–293), and green (364–384). In (C) an example of Pepsan result is shown (serum #901). This serum recognizes two peptides spanning VLDFIVDHPFLFLIRED sequence, part of epitope 364–384 (green).

46–68, and 76–92 recognized by two sera. Even though weak responses were observed, the binding to these sequences was confirmed in two independent experiments. An example of Pepsan result is shown in Fig. 6C.

4 Discussion

Due to the complexity of gluten and the A/G fractions in wheat, most of their constituents have not yet been assessed for their allergenic potential. In fact, purifying them to homogeneity with yields compatible with most characterization methods and allergenicity studies is very difficult. Wheat serpins are present as several isoforms in the A/G fraction. In this work, we evaluated the involvement of the Serpin-Z2B isoform in food and respiratory allergies to wheat using several different approaches. We chose to clone and express Serpin-Z2B isoform in *E. coli* in order to use this recombinant protein to evaluate serpin involvement in wheat allergy. The major purpose of this production was to obtain a well-folded bioactive serpin with the features of a native one. The high protease inhibitor activity of rSerp-Z2B_{SF} indicates that the protein purified from the soluble fraction is correctly folded.

Nevertheless, more than 90% of the rSerp-Z2B produced was entrapped in inclusion bodies. Compared to rSerp-Z2B_{SF}, rSerp-Z2B_{IB} displayed 20% less inhibitory activity, and differences in SRCD dichroic signals were observed, suggesting that rSerp-Z2B_{IB} is less folded. Compared to rSerp-Z2B_{SF}, rSerp-Z2B_{IB} was less recognized by patient IgE.

Until now, the involvement of serpins in wheat allergies was suggested based on allergenomic studies in both BA [8] and FAW [7, 9, 15] individuals. However, it was not possible to draw firm conclusions about the real implication of serpin in wheat allergy on the basis of this approach because a single spot rarely contains a single protein. Using ELISA on a panel of 132 sera, this work shows, for the first time, that serpins were involved in both FAW and BA patients since 20 and 31% of these patients produced specific IgE. According to these recognition frequencies, Serpin-Z2B cannot be considered as a major wheat allergen. The recognition frequency we observed in BA patients was significantly higher than that observed by Sander et al. [16] who found, using ImmunoCap, that recombinant Serpin-Z2A expressed as a fusion protein with the maltose-binding protein was recognized by IgE in about 5% of a German BA population.

Several hypotheses can explain this difference: (i) the populations were geographically different; (ii) the two recombinant serpins may contain different epitopes despite their strong sequence identity (89%) and/or could be differently folded; (iii) in ImmunoCAP and ELISA, proteins could be presented differently; and (iv) the fusion with maltose-binding protein could modify epitope accessibility.

The highest concentrations in serpin-specific IgE were found in the children of the FAW group and were linked to A/G fraction-specific IgE concentrations. Among children with FAW, serpin-positive sera also showed high total IgE concentration and strong IgE reactivity to other wheat allergens (A/G fraction, gliadins). For these patients, the IgE response to serpin may be associated with a more diverse and intense response to all wheat allergens. Mono-sensitization to serpin was never observed in our population of patients. Such a mono-sensitization is sometimes observed for wheat allergens (e.g. LTP1, ω 5-gliadins). Serpin may therefore be considered as a marker of a broad and strong sensitization to wheat in children with FAW.

rSerp-Z2B was able to induce RBL cell activation with protein concentrations in the range of $0.02\text{--}2\text{ }\mu\text{g.mL}^{-1}$, as was previously observed for other natural wheat allergens [11]. This is the first demonstration of the ability of this protein to elicit a biological reaction that confirms the allergenicity of wheat serpins.

The contrast between low serpin-specific IgE concentrations in ELISA and good elicitation potential in the RBL cell assay suggests that serpin epitopes could be more effectively presented in solution than in solid phase.

Using Pepscan, several IgE-binding peptides were revealed on the Serpin-Z2B sequence. However, nearly half of the sera tested were negative when this technique was used (e.g., despite a high serpin-specific IgE concentration of 83 ng.mL^{-1} , serum #899 was negative in Pepscan). The others displayed low intensity responses. Using the same approach, Wu et al. [21] were not able to map epitopes for a monoclonal anti-serpin antibody. As already observed for LTP1 [13], Pepscan (synthetic peptides) is probably not the best technique for epitope mapping in the case of very structured proteins for which most of the antigenicity is carried by conformational epitopes. Nevertheless, four epitopic regions bound by several sera were identified. No distinction could be made in regions detected by IgE from patients with FAW or with BA. As already observed for LTP1, common epitopic areas were observed, even though sensitization occurred via different routes (ingestion versus inhalation) [13, 14].

In the case of respiratory allergy, allergens are probably presented to the immune system in their native state, whereas in food allergy, it is assumed that they are denatured during heating, digestion, etc. It was therefore interesting to analyze serpin behavior after heating. SRCD data obtained from heated rSerp-Z2B_{SF} showed that the protein irreversibly loses its native structure by heating above 80°C . For most sera, this caused a decrease of IgE binding. This result clearly demonstrates the importance of serpin confor-

mation in its recognition by IgE. It could explain why rSerp-Z2B_{IB} (less folded) is less recognized by IgE than rSerp-Z2B_{SF}. Accordingly, some sera that were negative in the Pepscan experiment may mainly recognize conformational epitopes. However, the fact that serpin reactivity is observed in the case of FAW, i.e. after heating of the protein during cooking, suggests that thermal denaturation does not eliminate antigenicity.

The four linear epitopic regions identified by Pepscan were positioned on a structural model of Serpin-Z2B (Fig. 6B) constructed on the basis of the high sequence identity (49%) between Serpin-Z2B and AtSerp1 from *A. thaliana*, whose crystal structure is known [23] (PDB code 3LE2). This model contains 32% α and 25% β secondary structures, in accordance with values calculated from SRCD spectra of rSerp-Z2B_{SF} (32% α and 26% β). The two most IgE-reactive regions (residues 271–293 and 364–384) were located on β strands and loops. These epitopes were partially exposed on the surface of the structure model of the allergen, suggesting that they are available for IgE binding. On the model, it appears that the regions 271–293 (red on Fig. 6B) and 364–384 (green) are very close, so that they could be found within the same conformational epitope.

In conclusion, the present work establishes that wheat serpins are allergens that primarily contain conformational epitopes. rSerp-Z2B is recognized by about a quarter of the patients with FAW or BA. It could be considered as a complementary tool in the diagnosis of wheat allergy in children as a marker of a broad and strong sensitization. rSerp-Z2B could be representative of the members of a family of about 15 serpin isoforms that are not fully characterized and probably closely equivalent in terms of IgE reactivity and of the relative amount in the A/G fraction.

This work was funded by the French National Research Agency (ANR-08-ALIA-14 Predexpitope). We would like to thank the following people: Dr. Frank Wien (DISCO beamline at the SOLEIL synchrotron facility, Gif-sur-Yvette, France) for his invaluable help during SRCD experiments, and Yann Gohon for CD data processing; Véronique Solé for the use of the protein purification facilities; the BIBS mass-spectrometry platform (INRA Nantes, France); T. Mothes (University Hospital, Leipzig, Germany) for the supply of Pepscan membranes; Chantal Brossard for help in statistical analyses. Florence Pineau and Laurence Lavenant for their technical assistance.

The authors have declared no conflict of interest.

5 References

- [1] Salcedo, G., Quirce, S., Diaz-Perales, A., Wheat allergens associated with Baker's asthma. *J. Invest. Allergol. Clin. Immunol.* 2011, 21, 81–92.
- [2] Rosenberg, N., in: INRS (Ed.), 90 2002, pp. 189–196.

- [3] Zuidmeer, L., Goldhahn, K., Rona, R. J., Gislason, D. et al., The prevalence of plant food allergies: a systematic review. *J. Allergy Clin. Immunol.* 2008, **121**, 1210–1218.
- [4] Poole, J. A., Barriga, K., Leung, D. Y. M., Hoffman, M. et al., Timing of initial exposure to cereal grains and the risk of wheat allergy. *Pediatrics* 2006, **117**, 2175–2182.
- [5] Tatham, A. S., Shewry, P. R., Allergens to wheat and related cereals. *Clin. Exp. Allergy*. 2008, **38**, 1712–1726.
- [6] Akagawa, M., Handoyo, T., Ishii, T., Kumazawa, S. et al., Proteomic analysis of wheat flour allergens. *J. Agric. Food Chem.* 2007, **55**, 6863–6870.
- [7] Larre, C., Lupi, R., Gombaud, G., Brossard, C. et al., Assessment of allergenicity of diploid and hexaploid wheat genotypes: Identification of allergens in the albumin/globulin fraction. *J. Proteomics* 2011, **74**, 1279–1289.
- [8] Sander, I., Flagge, A., Merget, R., Halder, T. M. et al., Identification of wheat flour allergens by means of 2-dimensional immunoblotting. *J. Allergy Clin. Immunol.* 2001, **105**, 907–913.
- [9] Šotkovský, P., Hubálek, M., Hernychová, L., Novák, P. et al., Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients. *Proteomics* 2008, **8**, 1677–1691.
- [10] Pastorello, E. A., Farioli, L., Conti, A., Pravettoni, V. et al., Wheat IgE-mediated food allergy in European patients: alpha-amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins – Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *Int. Arch. Allergy Immunol.* 2007, **144**, 10–22.
- [11] Bodinier, M., Brossard, C., Triballeau, S., Morisset, M. et al., Evaluation of an in vitro mast cell degranulation test in the context of food allergy to wheat. *Int. Arch. Allergy Immunol.* 2008, **146**, 307–320.
- [12] Armentia, A., Sanchez-Monge, R., Gomez, L., Barber, D., Salcedo, G., In vivo allergenic activities of eleven purified members of a major allergen family from wheat and barley flour. *Clin. Exp. Allergy* 1993, **23**, 410–415.
- [13] Denery-Papini, S., Bodinier, M., Pineau, F., Triballeau, S. et al., Immunoglobulin-E-binding epitopes of wheat allergens in patients with food allergy to wheat and in mice experimentally sensitized to wheat proteins. *Clin. Exp. Allergy* 2011, **41**, 1478–1492.
- [14] Tordesillas, L., Pacios, L. F., Palacin, A., Quirce, S. et al., Molecular basis of allergen cross-reactivity: non-specific lipid transfer proteins from wheat flour and peach fruit as models. *Mol. Immunol.* 2009, **47**, 534–540.
- [15] Šotkovský, P., Sklenář, J., Halada, P., Cinová, J. et al., A new approach to the isolation and characterization of wheat flour allergens. *Clin. Exp. Allergy* 2011, **41**, 1031–1043.
- [16] Sander, I., Rozynek, P., Rihs, H. P., van Kampen, V. et al., Multiple wheat flour allergens and cross-reactive carbohydrate determinants bind IgE in Baker's asthma. *Allergy* 2011, **66**, 1208–1215.
- [17] Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C. et al., The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. *J. Biol. Chem.* 2001, **276**, 33293–33296.
- [18] Constantin, C., Quirce, S., Grote, M., Touraev, A. et al., Molecular and immunological characterization of a wheat serine proteinase inhibitor as a novel allergen in Baker's asthma. *J. Immunol.* 2008, **180**, 7451–7460.
- [19] Rosenkrands, I., Hejgaard, J., Rasmussen, S. K., Bjørn, S. E., Serpins from wheat grain. *FEBS Lett.* 1994, **343**, 75–80.
- [20] Dupont, F., Vensel, W., Tanaka, C., Hurkman, W., Altenbach, S., Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry. *Proteome Sci.* 2011, **9**, 10.
- [21] Wu, M. J., McKay, S., Howes, N., Chin, J., Hegedus, E., Identification of novel serpin isoforms and serpin polymorphisms among Australian wheat cultivars. *J. Cereal Sci.* 2012, **55**, 202–209.
- [22] Ostergaard, H., Rasmussen, S. K., Roberts, T. H., Hejgaard, J., Inhibitory serpins from wheat grain with reactive centers resembling glutamine-rich repeats of prolamin storage proteins. *J. Biol. Chem.* 2000, **275**, 33272–33279.
- [23] Lampl, N., Budai-Hadrian, O., Davydov, O., Joss, T. V. et al., Arabidopsis AtSerp1, crystal structure and in vivo interaction with its target protease responsive to desiccation-21 (RD21). *J. Biol. Chem.* 2010, **285**, 13550–13560.
- [24] Gaudin, J. C., Breuils, L., Haertle, T., Mouse orthologs of human olfactory-like receptors expressed in the tongue. *Gene* 2006, **381**, 42–48.
- [25] Bensefa, L., Villette, C., Tabka, F., Causse-Sounillac, E. et al., Rye flour induces a stronger early bronchial response than wheat flour in occupational asthma. *Allergy* 2004, **59**, 833–838.
- [26] Lees, J. G., Smith, B. R., Wien, F., Miles, A. J., Wallace, B. A., CDtool-an integrated software package for circular dichroism spectroscopic data processing, analysis, and archiving. *Anal. Biochem.* 2004, **332**, 285–289.
- [27] Whitmore, L., Wallace, B. A., DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucl. Acids Res.* 2004, **32**, 668–673.
- [28] Lees, J. G., Miles, A. J., Wien, F., Wallace, B. A., A reference database for circular dichroism spectroscopy covering fold and secondary structure space. *Bioinformatics* 2006, **22**, 1955–1962.
- [29] van Stokkum, I. H. M., Spoelder, H. J. W., Bloemendal, M., van Grondelle, R., Groen, F. C. A., Estimation of protein secondary structure and error analysis from circular dichroism spectra. *Anal. Biochem.* 1990, **191**, 110–118.
- [30] Roy, A., Kucukural, A., Zhang, Y., I-TASSER: a unified platform for automated protein structure and function prediction. *Nature Protoc.* 2010, **5**, 725–738.
- [31] Laskowski, R. A., MacArthur, M. W., Moss, D. S., Thornton, J. M., PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* 1993, **26**, 283–291.

Mameri et al. Supporting Information

MATTLATDVRLSIAHQTRFAFRLASAISSNPESTVNNAAFSPVSLHVALSLITAGAGGATR
NQLAATLGEGEVEGLHALAEQVVQFVLADASNIGGPRVAFANGVFVDASLQLKPSFQELAVCK
YKAEAQSVDFQTKAAEVTAQVNSWVEKVTTGLIKDILPAGSIDNTTRLVLGNALYFKGAWTD
QFDPRATQSDDFYLLDGSSIQTPFMYSSEEQYISSSDGLKVLKLPYKQGGDKRQFSMYILLP
EALSGLWSLAEKLSAEPEFLEQHIPRQKVALRQFKLPKFKISLGIEASDLLKGLGLLLPFGA
EADLSEMVDSPMAQNLYISSIFHKAFVEVNETGTEAAATTIAKVVLRQAPPPSVLDFIVDHP
FLFLIREDTSGVVLFIGHVVPNLLSSVDKLAAALEHHHHHH

Supporting Information Figure S1: Coverage of rSerpín-Z2B sequence by NanoLC-MS/MS analysis. Identified sequence is in bold and underlined.