Folded or Not? Tracking Bet v 1 Conformation in Recombinant Allergen Preparations

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

Recombinant Bet v 1a (rBet v 1a) has been used in allergy research for more than three decades, including clinical application of so-called hypoallergens. Quantitative IgE binding to rBet v 1a depends on its native protein conformation, which might be compromised upon heterologous expression, purification, or mutational engineering of rBet v 1a.

Objective

To correlate experimental/theoretical comparisons of IgE binding of defined molar ratios of folded/misfolded recombinant Bet v 1a variants and to determine accuracy and precision of immuno- and physicochemical assays routinely used to assess the quality of recombinant allergen preparations.

Methods

rBet v 1a and its misfolded variant rBet v 1a$_{S112P/R145P}$ were heterologously expressed and purified from *Escherichia coli*. Structural integrities and oligomerisation of the recombinant allergens were evaluated by ^{1}H-nuclear magnetic resonance (^{1}H-NMR), circular dichroism (CD) spectroscopy, and dynamic light scattering (DLS). IgE binding of defined combinations of rBet v 1a and rBet v 1a$_{S112P/R145P}$ was assessed using immunoblotting (IB), enzyme-linked immunosorbent assay (ELISA) and mediator release (MR) of humanized rat basophilic leukemia cells sensitized with serum IgE of subjects allergic to birch pollen. Experimental and theoretically expected results of the analyses were compared.

Results

^{1}H-NMR spectra of rBet v 1a and rBet v 1a$_{S112P/R145P}$ demonstrate a native and highly disordered protein conformations, respectively. The CD spectra suggested typical alpha-helical and beta-sheet secondary structure content of rBet v 1a and random coil for rBet v 1a$_{S112P/R145P}$. The hydrodynamic radii (R_H) of 2.49 ± 0.39 nm (rBet v 1a) and 3.1 ± 0.56 nm (rBet v 1a$_{S112P/R145P}$) showed monomeric dispersion of both allergens in solution. Serum IgE of birch pollen allergic subjects bound to 0.1% rBet v 1a in the presence of 99.9% of non-IgE binding rBet v 1a$_{S112P/R145P}$. Immunoblot analysis overestimated, whereas ELISA and mediator release assay underestimated the actual quantity of IgE-reactive rBet v 1a in mixtures of rBet v 1a/rBet v 1a$_{S112P/R145P}$ with a molar ratio of rBet v 1a $\leq$ 10%.

Conclusion

Valid conclusions on quantitative IgE binding of recombinant Bet v 1a preparations depend on the accuracy and precision of physico- and immunochemical assays with which natively folded allergen is detected.

Introduction

The major allergen Bet v 1 is the main cause of birch pollen allergy affecting millions of patients in Central and Northern Europe [1]. The molecular structures of several recombinant Bet v 1 (rBet v 1) isoforms and variants have been resolved via nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography [2–6]. Heterologously expressed and purified Bet v 1 has been used in a variety of research applications. It has been tested in animal models to generate optimized allergen immunotherapy (AIT) antigens [7–10]. Moreover, in a number of studies recombinant Bet v 1 variants harboring single or multiple amino acid substitutions were generated to identify clinically relevant B and T cell epitopes and to analyze cross-reactivity of homologous allergens from plant food [4, 11–17]. Furthermore recombinant Bet v 1 variants and fusion proteins are generated and tested as potential hypoallergenic candidates for birch-specific immunotherapy and vaccination [18–25]. Recombinant Bet v 1 is also used to study cellular aspects of Bet v 1-mediated allergies [26–28]. Studies showing comparable allergenic potential of native and recombinant Bet v 1 and addressing the quality of recombinant Bet v 1 preparations were carried out [29–32]. Threshold levels of serum IgE specific for rBet v 1 have been suggested to predict symptoms of birch pollen allergy [33,34]. Furthermore GMP-produced recombinant Bet v 1 preparations including an unfolded variant of Bet v 1 have been used in clinical trials for AIT and several patents on the production of recombinant Bet v 1 for AIT are registered [35]. Thus protocols for heterologous expression of recombinant Bet v 1 in the host *Escherichia coli* are well established and rBet v 1 is also available as allergen reference standard from the European Directorate for the Quality of Medicines and Health Care, EDQM [36].

Physico- and immunochemical integrity and native-like protein conformation are prerequisites to evaluate suitability of rBet v 1 as an allergen biomarker for diagnosis and therapy of birch pollen-related allergy. In general, allergens of the Bet v 1 protein family mostly bind IgE only in their native protein conformation. However, heterologous expression and purification of rBet v 1 might produce a quantitatively unknown fraction of misfolded and thus non-functional (non-IgE binding) rBet v 1. In this regard it has been recently shown that protein

conformation of rBet v 1 and thus IgE binding capacity is greatly affected by the conditions used for protein purification [37,38]. Ignorance of these phenomena might lead to false estimates and correlations of the IgE binding potency of rBet v 1 preparations. In case of unfolded hypoallergenic Bet v 1 for AIT it has to be ensured that no folded material is present and that the structural modification is stable over time and IgE reactivity is not re-established. Therefore, attempts have been made to develop immunoassays that distinguish between folded and unfolded variants of Bet v 1 [39].

These observations prompted us to systematically analyze the impact of misfolded rBet v 1 on quantitative IgE binding of rBet v 1 preparations. For this purpose we generated and purified rBet v $1a_{S112P/R145P}$, a variant harboring two proline residues which impair proper folding into the Bet v 1-type protein conformation. We used compositional mixtures of rBet v 1a, the major allergen isoform of birch pollen, and its derivative rBet v $1a_{S112P/R145P}$ in defined molar ratios and tested them in immuno- and physicochemical assays routinely used for allergen characterization. Finally we correlated the experimental results with the theoretically expected values for the combinations of the two rBet v 1 proteins in order to i) correlate experimental and theoretically expected data and analytical resolution and to ii) determine the extent of variation depending on biological and technical replicates.

Material and Methods

Study design

Three independent preparations of rBet v 1a and rBet v $1a_{S112P/R145P}$, were made to generate 3 identical compositional mixtures with defined ratios of the two proteins to test both i) the influence of biological replicates and ii) the influence of method variation (technical replicates) on immuno-and physicochemical assays used in allergen characterization. Furthermore we wanted to define the range of quantitative variation within one method to evaluate the correlation of experimental and theoretical data. Finally we compared the resolution of the individual methods to evaluate the performance of the assays with respect to distinguish structural and IgE binding differences in the presence of various ratios of natively folded and unfolded allergen.

Patients

10 allergic patients suffering from rhinoconjunctivitis or asthma to early flowering tree pollen were included as serum donors. Specific sensitization was documented by positive skin prick test responses and by detection of allergen-specific IgE to either rBet v 1a of 13.7–67.3 kU_A/L (patients 1–4) or to birch pollen of 83.7->100 kU_A/L (patients 5–10) as measured by Immuno-CAP[TM] (Thermo Fisher Scientific, Uppsala, Sweden). Patients were recruited at the University Medical Center Mainz, Department of Dermatology, Mainz, Germany, and at the Klinik für Dermatologie, Venerologie und Allergologie, University of Leipzig, Germany. Serum donations were approved by the local ethics committees of the clinical centers, namely the "Ethik-Kommission an der Medizinischen Fakultät der Universität Leipzig" and the "Ethikkomitee der Universitätsmedizin Mainz". Study participants provided written informed consent and ethics approval included consent form and consent procedure. Serum from a non-allergic subject was used as negative control for the specific IgE measurements.

Generation and purification of recombinant Bet v 1a

The open reading frame of Bet v 1a optimized for codon usage of *Escherichia coli* was purchased from Geneart (Life Technologies, Thermo Fisher Scientific, Waltham, MA, U.S.A.) and

cloned into bacterial expression vector pET15b (Novagen, Merck, Darmstadt, Germany) using the Clontech InFusion Kit (Mountain View, CA, U.S.A.). Bet v 1a variant rBet v 1a$_{S112P/R145P}$ was generated with the QuickChange Multi Lighting mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.) using the Bet v 1a ORF in pET15b_Bet v 1a as DNA template and two mutagenic primers Bet v 1$_{S112P}$ (acaccggatggtggtcccattctgaaaattagc) and Bet v 1$_{R145P}$ (ggtgaaaccctgctgcctgcagttgaaagctat) according to manufacturer's instructions. The genes were expressed in *E. coli* strain BL21(DE3) cells, growing at 37°C in 1 liter of LB medium, containing 50 μg/ml carbenicillin to an OD$_{600}$ value of 0.7. A final concentration of 1 mM IPTG was added to induce protein expression. The cells were harvested 4 h after induction by centrifugation (10000 g, 15 min, 4°C), and the cell pellets were stored at −20°C. Upon expression rBet v 1a was found in both cytosolic soluble and in insoluble fractions (inclusion bodies) of bacterial lysates whereas rBet v 1a$_{S112P/R145P}$ was exclusively found in inclusion bodies. To ensure comparability of protein preparation rBet v 1a and rBet v 1a$_{S112P/R145P}$ were purified from inclusion bodies. The proteins were regained from protein pellets after cell lysis with 10 mM potassium phosphate (KP$_i$), pH 7.4, 500 mM NaCl, 8 M urea and 10 mM imidazole, bound to Ni-NTA Superflow (QIAGEN, Hilden, Germany) and refolded by subsequently lowering the urea concentration in 10 mM KP$_i$; pH 7.4, 500 mM NaCl, 10 mM imidazole during liquid chromatography. Proteins were eluted by gradual increase of imidazole to 500 mM. Fractions containing the respective protein were pooled and 6xHis-tag was cleaved off with Thrombin (Sigma Aldrich Chemie GmBH, Steinheim, Germany). Proteins were further purified by size-exclusion chromatography (10 m KP$_i$, pH 7.4), concentrated, frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined with bicinchoninic acid using bovine serum albumin as standard (BCA™ protein assay kit, Thermo Scientific, Rockford, IL, U.S.A.).

Three preparations each of rBet v 1a and rBet v 1a$_{S112P/R145P}$ were generated to yield 3 individual rBet v 1a/rBet v 1a$_{S112P/R145P}$ combinations which were analyzed with 1 (immunoblot, CD), 2 (MR assay), and 3 (ELISA) technical replicates each.

Circular dichroism spectroscopy

Far UV circular dichroism (CD) spectra of the rBet v 1a 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 0.2 cm cell. All proteins were analyzed at a concentration of 10 μM in 10 mM KP$_i$, pH 7.4. Each measurement comprised the average of 10 repeated scans between 255 and 185 nm.

The mean estimates of residual ellipticities ($\Delta\Theta_{experimental}$) of rBet v 1a and rBet v 1a$_{S112P/R145P}$ at wave lengths characteristic for secondary structure (193 and 222 nm for α-helix [40], 195 and 218 nm for β-sheet [41], and about 200 nm for random coil [42]) were determined according to the following equation:

$$\Delta\Theta_{experimental} = \Theta_{x\%\ Betv1a} - \Theta_{100\%\ Betv1a(S112P/R145P)} \qquad (1)$$

Where $\Theta_{x\%Bet\ v1a}$ is the residual mean ellipticity of the *rBet v 1a/rBet v 1a$_{S112P/R145P}$ mixture with 100, 80, 60, 40, 20, 10, 1, 0.1, 0.01, and 0% of rBet v 1a, respectively* and $\Theta_{100\%Bet\ v1a(S112P/R145P)}$ is the residual mean ellipticity of the non-folded rBet v 1a variant. Theoretical mean estimates of residual ellipticities ($\Delta\Theta_{theoretical}$) were obtained by multiplication of $\Delta\Theta_{experimental}$ determined with 100% rBet v 1a with 1.25, 1.67, 2.5, 5, 10, 100, 1000, or 10000 representing 80, 60, 40, 20, 10, 1, 0.1, 0.01, and 0.001% of rBet v 1a in the protein mixtures, respectively. The following formula was used:

$$\Delta\Theta_{theoretical} = \Delta\Theta_{experimental\ (100\%\ rBetv1a)} * x \qquad (2)$$

where $\Delta\Theta_{\text{experimental (100\% rBet v 1a)}}$ is the experimentally determined mean residual ellipticity of 100% rBet v 1a and x is the respective multiplication factor as described above. The experimental/theoretical comparisons for each rBet v 1a/rBet v $1_{\text{S112P/R145P}}$ mix were calculated by dividing $\Delta\Theta_{\text{experimental}}$ by the corresponding $\Delta\Theta_{\text{theoretical}}$.

Dynamic light scattering

Dynamic light scattering analysis was used to determine hydrodynamic radii (R_H) in nm using a Zetasizer Nano ZS (Malvern, Herrenberg, Germany). 10 µM of recombinant Bet v 1a and Bet v $1a_{\text{S112P/R145P}}$ in 10 mM KP_i, pH 7.4 were analyzed at 25°C. Three individual measurements (3 x 10 runs per measurement) per protein were carried out. Data were analyzed by the proprietary software of the DLS instrument where the hydrodynamic radius (R_H) is calculated from the diffusion coefficient (D) which is obtained by measurement of fluctuations of intensities of scattered light over time and fitting of the respective correlation curve to an exponential function from which the diffusion coefficient can be calculated. R_H is then calculated by the Stokes-Einstein Eq (3):

$$R_H = \frac{kT}{3\pi\eta D} \qquad (3)$$

Where R_H is the hydrodynamic radius, k is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, and D is the diffusion coefficient.

NMR spectroscopy

NMR samples were prepared in 20 mM sodium phosphate buffer, pH 7.0, 0.04% sodium azide and 10% D_2O containing 30 µM of rBet v 1a or rBet v $1a_{\text{S112P/R145P}}$. Standard 1D ^1H spectra with WATERGATE solvent suppression [43] were recorded on a Bruker Avance 700 MHz spectrometer at 298 K. NMR data were processed and visualized with the Bruker spectrometer software TopSpin.

SDS-PAGE and immunoblot analysis

SDS-PAGE was performed with 15% separating gels and 5% stacking gels using a discontinuous buffer system [44]. For immunoblot analysis, 0.5 µg/cm (total protein) of recombinant Bet v 1a/Bet v $1_{\text{S112P/R145P}}$ was transferred onto 0.2 µm nitrocellulose membranes by semi-dry blotting at 0.8 mA/cm² for 1h [45]. After blocking with Tris-buffered saline (TBS) containing 0.3% Tween 20, blots were incubated overnight at room temperature with 10 µl of human serum pool in 1 ml TBS containing 0.05% Tween 20 and 0.1% BSA. After extensive washing, blotting membranes were incubated for 1h with horseradish peroxidase labelled mouse anti-human IgE antibody (Clone B3102E8, Southern biotech via Biozol, Eching, Germany), diluted to 1:100000. IgE-binding proteins were visualized by chemiluminescence (LumiGLO Reserve diluted 1:3, KPL via Medac, Wedel, Germany). For detection of IgG binding to rBet v 1a variants on nitrocellulose membranes, Bet v 1-specific polyclonal rabbit IgG (ALK, Hørsholm, Denmark) was diluted 1:10000 in TBS containing 0.05% Tween 20 and 0.1% BSA and incubated for 1 h at room temperature. Bound IgG was detected with goat anti-rabbit IgG conjugated with horseradish peroxidase (Southern biotech via Biozol, Eching, Germany) (1:10000 dilution) as described above.

IgE bound to rBet v 1 in Western blot analyses was analyzed by densitometry (Fusion Fx, Vilber Lourmat, Eberhardzell, Germany) and evaluated using ImageJ (Rasband, NIH, Bethesda, MD, U.S.A.) Theoretical mean estimates ($me_{\text{theoretical}}$) of IgE signals after 100 ms exposure were calculated by setting the IgE signal of rBet v 1a to 100% and the IgE signals of

the rBet v 1a/rBet v 1a$_{S112P/R145P}$ combinations to 80, 60, 40, 20, 10, 1, 0.1, 0.01, 0.001, and 0% according to the respective molar content of rBet v 1a in the protein mixtures. The following equation was used:

$$me_{theoretical} = me_{experimental\ (100\%\ rBet\ v\ 1\ a)} * \frac{x}{100} \qquad (4)$$

where me$_{exp(100\%\ Bet\ v\ 1a)}$ is the densitometric IgE signal of 100% rBet v 1a, x is mol% (i.e. 80, 60, 40, 20, 10, 1, 0.1, 0.01., 0.001, or 0%) of rBet v 1a in the respective rBet v 1a/rBet v 1a$_{S112P/R145P}$ mixture, and me$_{theoretical}$ is the resulting calculated mean estimate of the IgE signal in dependence of rBet v 1a content. The experimental/theoretical comparisons for each rBet v 1a/rBet v 1a$_{S112P/R145P}$ mix was calculated by dividing the experimentally derived mean estimate (me$_{experimental}$) by the respective me$_{theoretical}$ and listed in S2 Table. Immunoblot data were statistically analyzed assuming a linear quantitative correlation of IgE signal and amount of protein blotted.

Inhibition IgE ELISA

For IgE-ELISA inhibition experiments, Nunc Maxisorp plates (Fisher Scientific, Schwerte, Germany) were coated overnight at room temperature with 50 ng Bet v 1/100 μl of 10 mM potassium phosphate-buffered saline (PBS). After blocking with PBS containing 2% BSA, plates were incubated with the human serum pool (dilution 1:80) and increasing molar ratios of rBet v 1a/rBet v 1a$_{S112P/R145P}$ for 3 hrs at room temperature in PBS containing 0.05% Tween 20 and 0.1% BSA. Allergen-specific human IgE was detected with a horseradish peroxidase-conjugated mouse anti human IgE antibody (Clone B3102E8, Southern biotech via Biozol, Eching, Germany) diluted 1:1000. As substrate 3,3',5,5'-tetramethylbenzidine (Roth, Karlsruhe) was used for the horseradish peroxidase and the absorbances at 450 nm and 630 nm were measured after stopping the reaction with 25% H$_2$SO$_4$. For all data points absorbances at 630 nm were substracted from absorbances at 450 nm. Inhibition of IgE binding was calculated with the following equation:

$$\left(1 - \frac{abs_{sample}}{abs_{max}} \right) * 100\% \qquad (5)$$

Where abs$_{sample}$ is the absorbance of the respective sample and abs$_{max}$ is the absorbance without inhibitor. Experimental half-maximal inhibition (EC$_{50\ experimental}$) of IgE binding to rBet v 1a (ELISA) was calculated using a four parameter sigmoid curve fit with the restrictions that slope, minimum and maximum asymptote had to be the same for all curves to ensure parallelism among curves. The following equation was used:

$$F(x) = \frac{a-d}{1 + \left(\frac{x}{c} \right)^b} + d \qquad (6)$$

where F(x) is the inhibition of IgE binding (%), a is the minimum asymptote, b is the slope, c is the EC$_{50}$, and d is the maximum asymptote. Theoretical half-maximal inhibition (EC$_{50\ theoretical}$) was obtained by multiplication of EC$_{50\ experimental}$ obtained by 100% rBet v 1a with factors 1.25, 1.67, 2.5, 5, 10, 100, 1000, 10000 representing 80, 60, 40, 20, 10, 1, 0.1, 0.01, and 0.001% of rBet v 1a in the protein mixtures, respectively. The following equation was used:

$$EC_{50\ theoretical} = EC_{50\ experimental\ (100\%\ rBetv1a)} * x \qquad (7)$$

where EC$_{50\ experimental\ (100\%\ rBet\ v\ 1a)}$ is the experimentally derived half maximal effective concentration with 100% rBet v 1a and x is the respective multiplication factor as described above.

The experimental/theoretical comparisons for each rBet v 1a/rBet v 1a$_{S112P/R145P}$ mix was calculated by dividing EC$_{50\ experimental}$ by the respective EC$_{50\ theoretical}$. For statistical analysis the EC$_{50}$ values with 95% Confidence Intervals were estimated for all curves showing a significant regression and/or having values measured at least from minimum asymptote up to the inflection point of the curve. P values for significant horizontal shifts of the curves, i.e. significant different EC$_{50}$ values were calculated (P values were not adjusted for multiple comparisons due to the exploratory character of the study).

β-hexosaminidase release from humanized rat basophil leukaemia (RBL) cells

The mediator release assay was performed as described [46]. Briefly, RBL cells expressing the α-chain of the high affinity receptor FcεRI for human IgE were sensitized overnight with a sera pool of birch pollen allergic subjects (diluted 1:40). After washing, cells were stimulated with serial dilutions of rBet v 1a/rBet v 1a$_{S112P/R145P}$ compositional mixtures of defined ratios. Degranulation was quantified by photometric measurement of β-hexosaminidase activity in the culture supernatants. The percentage of β-hexosaminidase activity relative to cells lysed with Triton X-100 (Sigma-Aldrich, Steinheim, Germany) was calculated and corrected for spontaneous release (sensitized cells without allergen). Experimental and theoretical half maximal β-hexosaminidase releases (EC$_{50\ experimental}$ and EC$_{50\ theoretical}$) were calculated in a similar fashion as described for the ELISA above using the similar formula:

$$F(x) = \frac{a-d}{1 + \left(\frac{x}{c}\right)^b} + d \qquad (8)$$

Where F(x) is the observed mediator release induced be the respective rBet v 1a/rBet v 1a$_{S112P/R145P}$ mixture. Fit of dose-response curves and statistical analysis was done in a similar fashion as described for 'Inhibition IgE ELISA' above.

Mass spectrometry analysis of recombinant Bet v 1a

We expressed and purified rBet v 1a and rBet v 1a$_{S112P/R145P}$ to homogeneity from *Escherichia coli*. The amino acid sequence of rBet v 1a and rBet v 1a$_{S112P/R145P}$ was confirmed by LC_MS with sequence coverage of 72% and 78% and a score of 11137 and 3957, respectively. The peptides harboring either S112 and R145 or S112P and R145P were individually double checked and detected with a mass error of 1.2 ppm and 2.5 ppm, or 2.4 ppm or 1.9ppm, respectively. The identity of rBet v 1a$_{S112P/R145P}$ was confirmed by liquid chromatography mass spectrometry (LC-MS) after SDS PAGE separation as described elsewhere [47] with slight modifications. Peptides were eluted with 25 mM NH$_4$HCO$_3$; 10% aceto nitrile (ACN) and the digestion was stopped by adding 5% formic acid. The peptides were analysed by using a nano-ultra performance LC system coupled to a nano-ESI-MS (nano Acquity UPLC nanoESI Synapt-MS, Waters, Milford, US) with a 5 μm symmetry 180 μm x 20 mm c18 pre-column and a 1.7 μm BEH 130 100 μm x 100 mm c18 separation column. A 30 minutes gradient (3% to 40% ACN at 500 nl/minute) after 3 minutes of trapping (99% water at 5 μl/minute) was applied to separate peptides. MS was operated in V mode, acquiring MSE 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 2011, restricted to reviewed entries of eukaryotic organisms) and the amino acid sequences of recombinant Bet v 1a and Bet v 1a$_{S112P/R145P}$. Protein hits were accepted at a false positive rate of less than 4%.

Results

Recombinant Bet v 1aS112P/R145P lacks Bet v 1a-type protein conformation

To generate a rBet v 1a variant unable to fold into the native Bet v 1-type conformation we substituted prolines for serine 112 and arginine 145, respectively to create rBet v 1a$_{S112P/R145P}$ (Fig 1a). The S112P substitution causes a significant change in the secondary and tertiary structure of Bet v 1a and reduces binding of serum IgE as described previously [48]. Additionally we chose the exchange R145P to prevent a potential formation of the Bet v 1a-typical long extended C-terminal alpha-helix that might contribute to IgE binding of the allergen. When we analyzed the secondary structure of the two proteins by circular dichroism (CD) spectroscopy, we found typical Bet v 1-like spectrum for rBet v 1a, indicating high content of β-pleated sheets and α-helices, whereas rBet v 1a$_{S112P/R145P}$ showed very low ellipticity below 200 nm as characteristic for unstructured protein (Fig 1b). Next we recorded 1D-^{1}H-NMR spectra to detect differences in signal dispersion of rBet v 1a versus rBet v 1a$_{S112P/R145P}$ (Fig 1c). rBet v 1a showed its characteristic spectrum with a high degree of resonance dispersion resulting from secondary structural elements like α-helices and β-sheets [49,50]. In contrast, the spectrum of rBet v 1a$_{S112P/R145P}$ exhibited typical random coil shifts close to those found in conformationally disordered peptides [51–53]. We concluded that the amino acid substitutions S112P and R145P abolish the original protein fold of rBet v 1a. To analyze dispersity and potential aggregation of rBet v 1a and rBet v 1a$_{S112P/R145P}$ in solution, we determined the hydrodynamic radii (R_H) in dynamic light scattering (DLS) and the ^{15}N spin relaxation rates of rBet v 1a in NMR. The R_H of 2.49 ± 0.39 nm (rBet v 1a) and 3.1 ± 0.56 nm (rBet v 1a$_{S112P/R145P}$) as well as the relaxation rates (S1 File) suggested that both proteins were mono-disperse in solution and did not aggregate.

Recombinant Bet v 1a$_{S112P/R145P}$ does not bind serum IgE of subjects allergic to birch pollen

Next we tested antibody binding of the allergen variants in immunoblot analysis (Fig 2). Both proteins bound polyclonal Bet v 1-specific IgG from rabbit serum, showing that antibody binding *per se* is not compromised in rBet v 1a$_{S112P/R145P}$. In contrast no binding of human IgE to rBet v 1a$_{S112P/R145P}$ as opposed to rBet v 1a could be detected, supporting the general view that rBet v 1a binds human IgE only in its native protein conformation. We conclude that lack of IgE binding to rBet v 1a$_{S112P/R145P}$ is due to an irreversible change of the Bet v 1-like protein fold.

Circular dichroism of rBet v 1a/rBet v 1a$_{S112P/R145P}$ combinations

Next we analyzed the CD spectra of mixtures of rBet v 1a and rBet v 1a$_{S112P/R145P}$ in defined molar ratios from 100 to 0.01% rBet v 1a to observe the corresponding quantitative changes in ellipticities in particular at 193, 195, 200, 218, and 222 nm, because these wave lengths are most prominent for protein secondary structures (Fig 3). When we compared the experimentally determined mean residual ellipticities with those we theoretically expected, we observed that the experimental-theoretical comparisons correlated well with those calculated for molar combinations from 100% to 10% rBet v 1a (S1 Table). Below 10% of rBet v 1a, however, low linear correlations of the experimental and theoretical ellipticities were observed. According to the 95% confidence interval for each mean residual ellipticity we found that rBet v 1a mixtures containing 60% of rBet v 1a$_{S112P/R145P}$ could be resolved from 0% of the Bet v 1 folding variant at wave lengths 193, 195, 200, and 218 nm, respectively, whereas the ellipticities of the rBet v
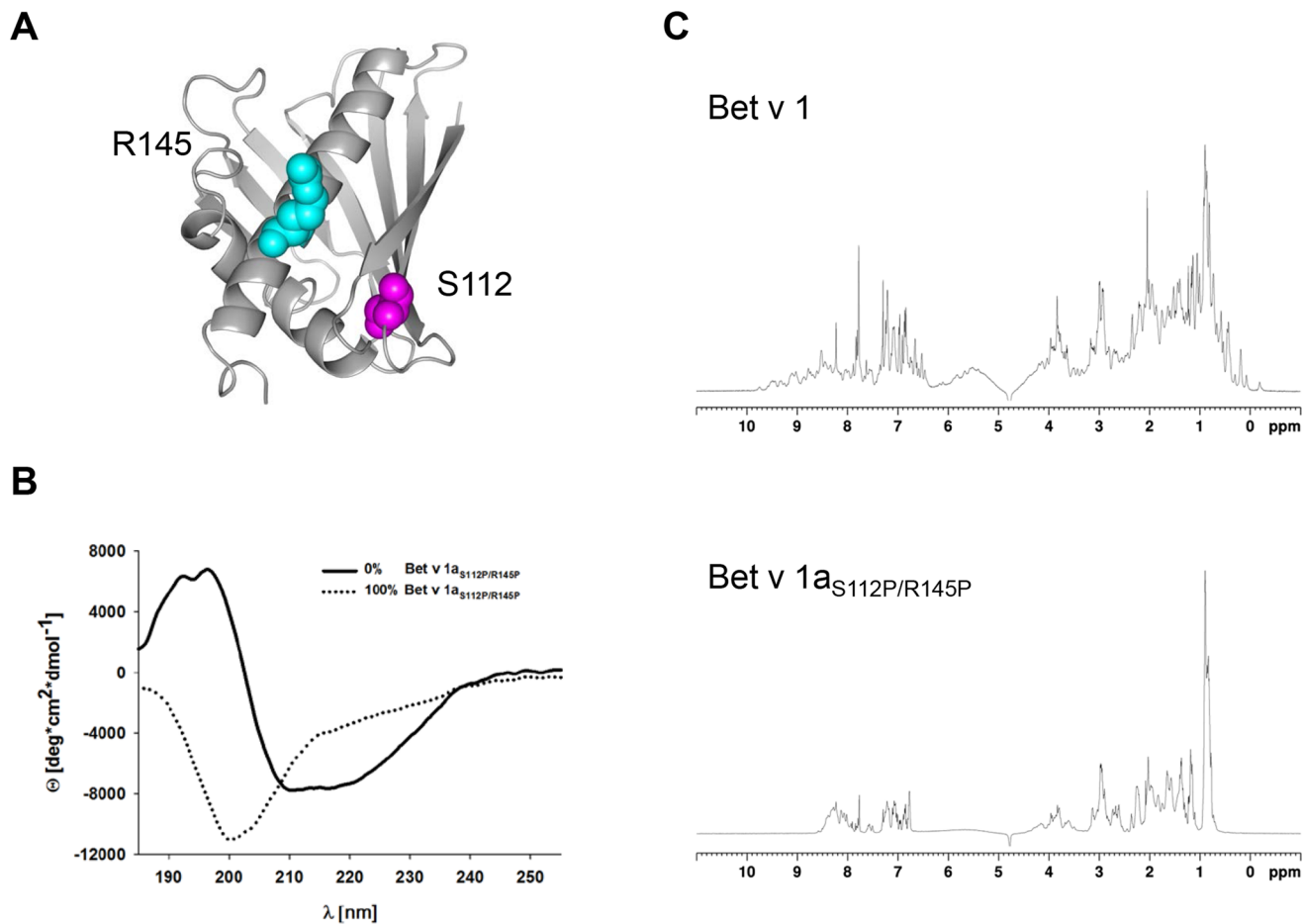
A

B

C

Fig 1. Structural integrity of recombinant Bet v 1a and Bet v 1a$_{S112P/R145P}$. A) Secondary structure topology of Bet v 1a (pdb: 1BV1). The amino acids S112 (purple) of β-strand 7 and R145 (cyan) of α-helix 3 were exchanged for proline. B) Circular dichroism of rBet v 1a and rBet v 1a$_{S112P/R145P}$. C) ^{1}H-NMR spectra of 30 μM of rBet v 1a (upper panel), 30 μM rBet v 1a$_{S112P/R145P}$ (middle panel), and the overlay rBet v 1a in red and rBet v 1a$_{S112P/R145P}$ in black (lower panel). 3D-model of Bet v 1a was visualized with PyMOL [54].

doi:10.1371/journal.pone.0132956.g001

1a/rBet v 1a$_{S112P/R145P}$ combinations at 222 nm clearly separated 40% of rBet v 1a$_{S112P/R145P}$ from 0% rBet v 1a$_{S112P/R145P}$ (Fig 3F).

Immunochemical analyses of rBet v 1a/rBet v 1a$_{S112P/R145P}$ combinations

To correlate the physicochemical data in the previous section with the IgE binding of defined ratios of rBet v 1a and rBet v 1a$_{S112P/R145P}$ we performed a series of immunochemical analyses. First we tested the signal intensity of serum IgE from birch pollen allergic subjects bound to the rBet v 1a protein mixtures in immunoblot (Fig 4). As expected, we observed decreasing IgE signal intensities with increasing molar fraction of unfolded rBet v 1a$_{S112P/R145P}$ in the rBet v 1a/rBet v 1a$_{S112P/R145P}$ combinations. Experimental-theoretical comparison of the IgE signal, however, ranged from 0.94 to 13.16 (80% to 0.01% Bet v 1a), thus over-estimating the quantity of IgE bound to rBet v 1a $\leq$ 1% in the protein mixtures blotted (S2 Table).

Next we tested inhibition of IgE antibody binding to immobilized rBet v 1a with rBet v 1a/rBet v 1a$_{S112P/R145P}$ protein combinations in ELISA (Fig 5). Since only rBet v 1a is the IgE-reactive component, a rBet v 1a concentration-dependent shift of half-maximal inhibition (EC 50)
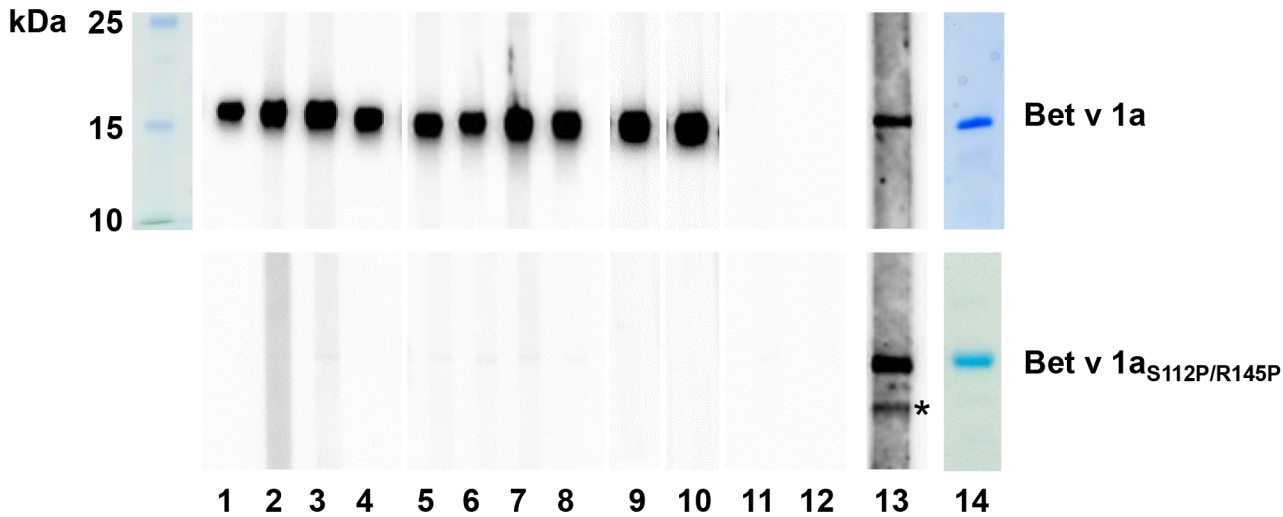
Fig 2. Immunoglobulin binding of rBet v 1a and rBet v 1a$_{S112P/R145P}$. Binding of IgE from sera of subjects allergic to birch pollen bound to purified (lane 14, Coomassie stain) rBet v 1a (upper panel) and rBet v 1a$_{S112P/R145P}$ (lower panel) (lanes 1–10). Binding of rabbit anti-Bet v 1 IgG to the rBet v 1a proteins is shown (lane 13). A minor degradation product of rBet v 1a$_{S112P/R145P}$ detected by IgG is shown (*). As controls, serum of non-allergic subject (lane 11) and buffer control (HRP-conjugated anti human IgE antibody only) (lane 12) were used.
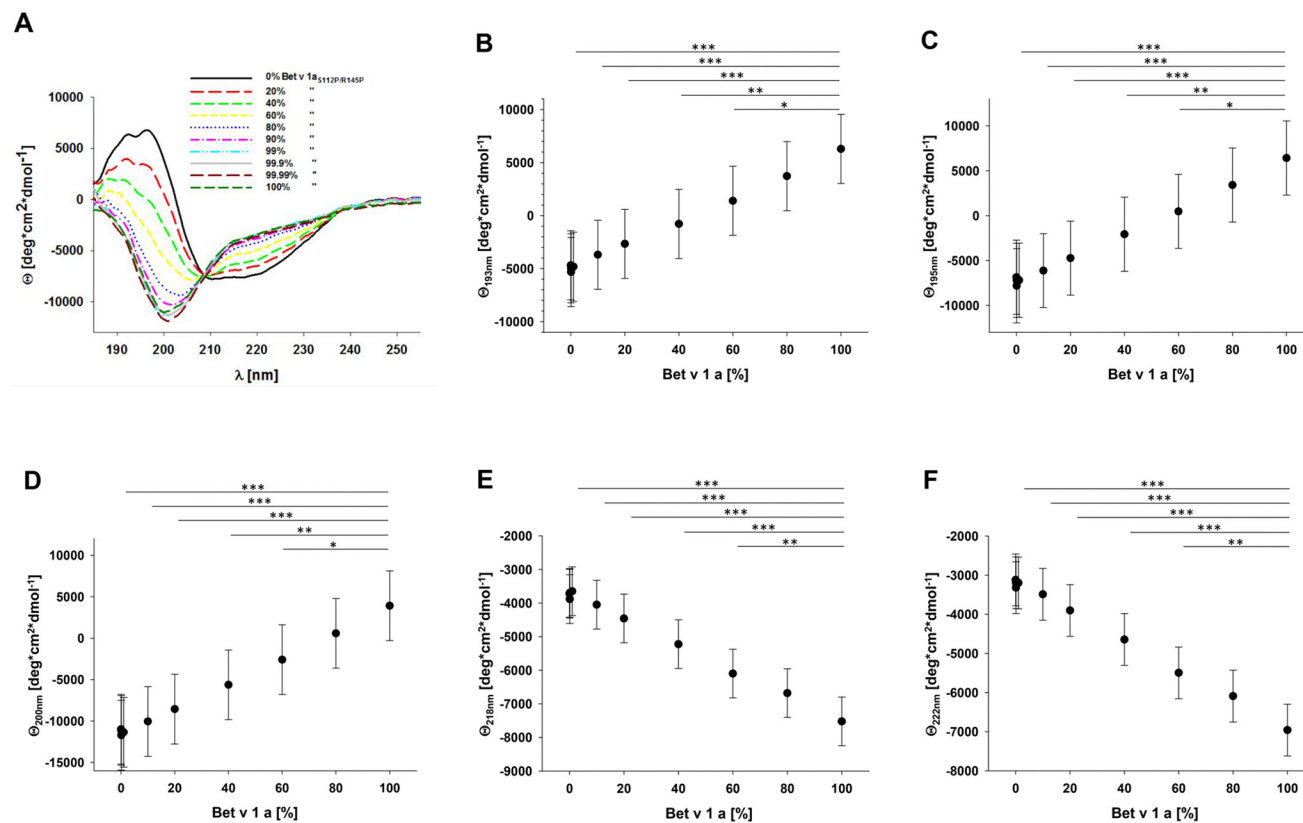
doi:10.1371/journal.pone.0132956.g002

Fig 3. Circular dichroism of rBet v 1a/rBet v 1a$_{S112P/R145P}$ combinations. A) Circular dichroism of defined molar ratios of rBet v 1a/rBet v 1a$_{S112P/R145P}$. 5 μM total rBet v 1a was measured with increasing fractions of rBet v 1a$_{S112P/R145P}$ from 0% to 100%. Mean residual ellipticities with 95% confidence interval and statistical evaluations at wave lengths 193 nm (B), 195 nm (C), 200 nm (D), 218 nm (E), and 222 nm (F) are shown.

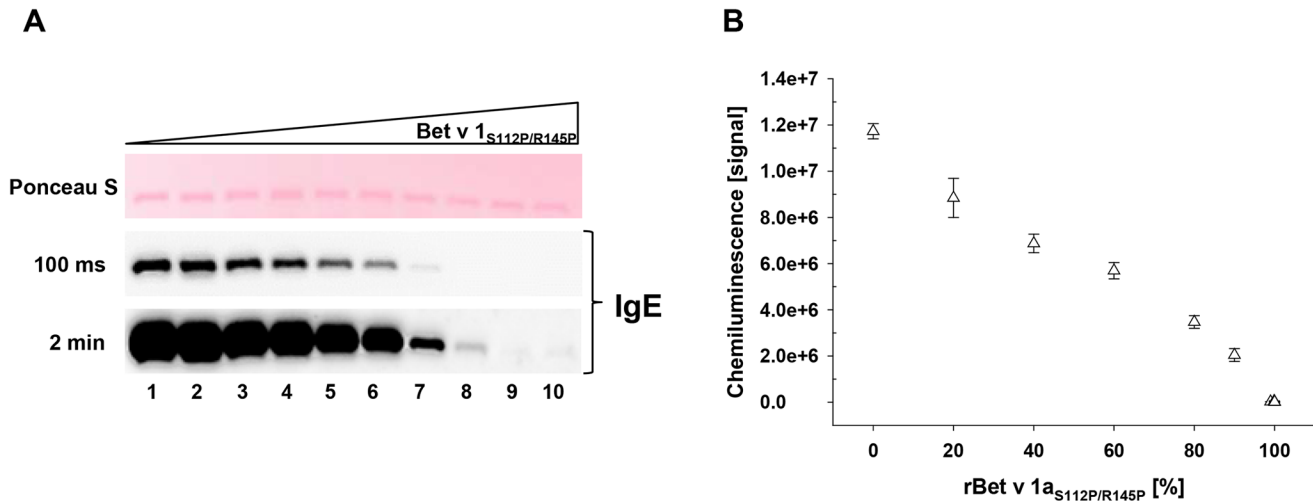doi:10.1371/journal.pone.0132956.g003

A

B

Fig 4. IgE Immunoblot of rBet v 1a/rBet v 1a$_{S112P/R145P}$ mixtures. A) 1 μg of total rBet v 1a with increasing fractions of rBet v 1a$_{S112P/R145P}$ 0% to 100% were transferred onto nitrocellulose and stained with Ponceau S. Binding of sera pool IgE to rBet v 1a combinations was determined by chemiluminescence after 100 milliseconds. To visualize IgE signals with molar ratios of rBet v 1a$_{S112P/R145P}$ from 99% to 100% (lanes 7–10) a 2 min exposure is shown (left). B) Chemiluminescence was quantified densitometrically and plotted against the molar fraction of rBet v 1a$_{S112P/R145P}$ (right).

doi:10.1371/journal.pone.0132956.g004

of IgE binding was expected. We fitted the inhibition curves with a 4-parameter logistic model assuming sigmoidal curve fit with same lower and higher asymptote for all curves. Estimated common slope was 0.91 with a 95% confidence interval of 0.85–0.96. As opposed to rBet v 1a the unfolded rBet v 1a$_{S112P/R145P}$ could not inhibit IgE-Bet v 1a complex formation. The experimental-theoretical values for half-maximal inhibition ranged from 1.09 with 80% of rBet v 1a
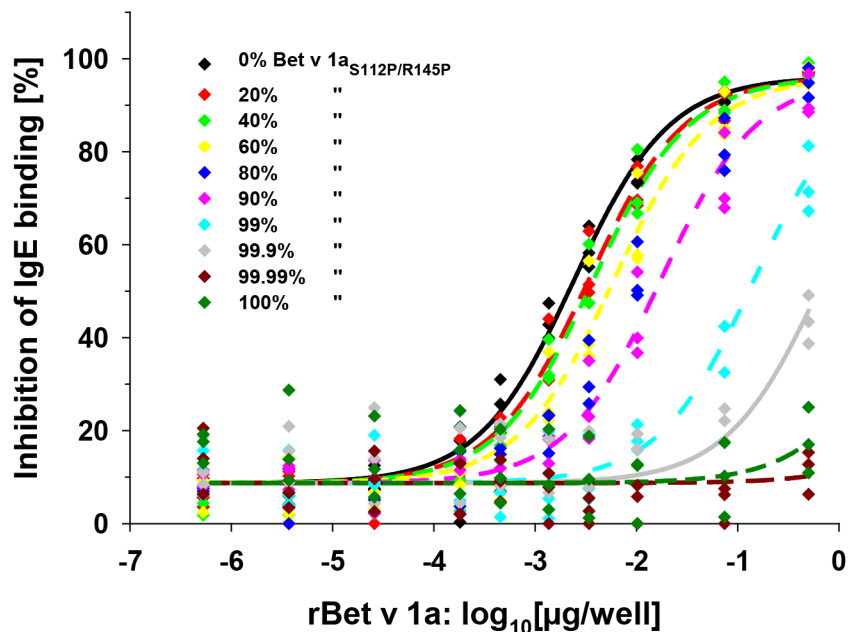

Fig 5. Inhibition of IgE binding to immobilized rBet v 1a. Dose-dependent inhibition of IgE binding to surface-coated rBet v 1a by rBet v 1a/rBet v 1a$_{S112P/R145P}$ mixtures in the presence of increasing molar ratios of rBet v 1a$_{S112P/R145P}$ in ELISA. Curves were fitted in parallel with a 4-parameter, logistic sigmoidal curve fit with same slope, lower and upper asymptote for all curves.

doi:10.1371/journal.pone.0132956.g005

to 0.29 with 0.1% of rBet v 1a in the protein mix with lower content of IgE-reactive rBet v 1a (<0.01%) yielding non-evaluable inhibition curves (S2 Table). Only half-maximal inhibitions observed with 80% and 99.9% of rBet v 1a$_{S112P/R145P}$ differed statistically significant (p = 0.039 and p = 0.002) from EC$_{50}$ obtained for 100% rBet v 1a.

So far the immunoassays above addressed only serological IgE binding to rBet v 1a combinations. To analyze the biological activity of the recombinant allergens in a cellular system we carried out mediator release in humanized rat basophil leukemia cells sensitized with pooled sera IgE (Fig 6). We evaluated the mediator release assays in the same fashion as we did for the ELISA above and found that rBet v 1a/rBet v 1a$_{S112P/R145P}$ protein mixtures induced mediator release up to a rBet v 1a content of 1% with experimental-theoretical values of half maximal mediator releases of 0.86 to 1.32 (20–99% rBet v 1a$_{S112P/R145P}$). However lower contents of IgE binding rBet v 1a (<0.1%) did not allow valid determination of half-maximal mediator release. No significant differences could be observed between the EC$_{50}$ values for 100% rBet v 1a and all rBet v 1a/rBet v 1a$_{S112P/R145P}$ compositional protein preparations. However, according to a confidence interval of 95%, only the rBet v 1a/rBet v 1a$_{S112P/R145P}$ protein combination containing 1% rBet v 1a resolved analytically from the mediator release obtained with 100% rBet v 1a.

Finally we compared the experimental/theoretical comparisons of all assays used to analyze rBet v 1a/rBet v 1a$_{S112P/R145P}$ protein combinations with up to 90% of unfolded rBet v 1a$_{S112P/R145P}$ (Fig 7). Whereas the experimental/theoretical values for CD$_{218nm}$ and ELISA were 0.86 and 0.72, respectively, 1.74 and 0.3 were determined for immunoblot and RBL mediator release. The experimental/theoretical comparisons of all assays scattered largely for Bet v 1a/rBet v 1a$_{S112P/R145P}$ protein combinations with molar content of rBet v 1a < 10%.

Discussion

Recombinant Bet v 1a is used in basic research as well as in in vitro allergy diagnosis and is available as chemical reference standard from the European Directorate for the Quality of Medicines and Health Care (EDQM). In particular for diagnostic purposes, recombinant Bet v 1 needs to bind IgE from sera of subjects allergic to birch pollen in a similar fashion as the native allergen since clinically relevant IgE interaction of Bet v 1 requires a native protein conformation. On the other hand recombinant unfolded Bet v 1 has been used in allergen immunotherapy trials and in this scenario refolding needs to be avoided to ensure the safety of study participants.

The major goal of this study was to correlate changes of secondary/tertiary structure of recombinant variants of Bet v 1a with quantitative IgE binding of protein mixtures comprising defined molar fractions of IgE-reactive natively folded rBet v 1a and non IgE-binding artificially stably unfolded rBet v 1a$_{S112P/R145P}$. We applied physico- and immunochemical assays that are routinely used to judge quality and IgE binding of rBet v 1a preparations according to two quantitative parameters, namely i) the accuracy with which the actual IgE-binding protein moiety is determined and ii) the precision according to the confidence intervals of 95% chosen with which the actual IgE-binding protein moiety is determined resolved quantitatively by the respective analytical method.

All assays showed experimental-theoretical comparisons (0.26–1.74) up to a molar fraction of 10% rBet v 1a in the rBet v 1a/rBet v 1a$_{S112P/R145P}$ protein mixtures. However with molar ratios of rBet v 1a < 10% we found that all assays performed with large deviations from expected values. As opposed to ELISA and cellular mediator release, immunoblot analysis overestimated the actual amount of rBet v 1a-specific bound serum IgE.
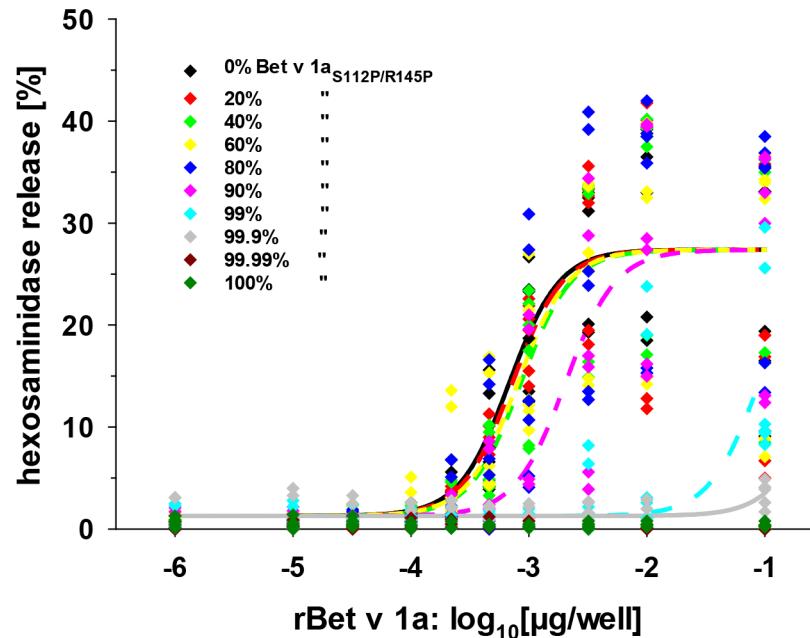
Fig 6. Mediator release of humanized rat basophils. β-hexosaminidase release of humanized rat basophil leukemia cells sensitized with a pool of human sera of donors allergic to birch pollen. Cross-linking of membrane-bound human IgE by IgE-Bet v 1 interaction and subsequent release of β-hexosaminidase was determined in the presence of defined molar ratios of rBet v 1a/rBet v $1a_{S112P/R145P}$. The legend shows molar ratios (in %) of rBet v $1a_{S112P/R145P}$ in the rBet v 1a/rBet v $1a_{S112P/R145P}$ combinations analyzed.

doi:10.1371/journal.pone.0132956.g006

Considering the accuracy, precision and resolution according to 95% confidence intervals of the individual assays we found that circular dichroism and ELISA could differentiate a 1.67-fold reduction (i.e. 60% of rBet v 1a in a rBet v 1a/rBet v $1a_{S112P/R145P}$ mixture) of the total IgE binding content of rBet v 1a (i.e. 100% rBet v 1a) with an accuracy of 1.03 (CD_{222nm}) and 0.93 (ELISA), respectively (Table 1). In this regard the immunoblot and cellular mediator release assays performed lower as they could reliably differentiate only a 5-fold (20% mol ratio of rBet v 1a) and 100-fold (1% mol ratio of rBet v 1a) reduction of the IgE binding recombinant allergen, respectively. These findings did not necessarily correlate with the statistical analyses where we found that significant reduction of both IgE binding and native-like Bet v 1a secondary structure in the rBet v 1a/rBet v $1a_{S112P/R145P}$ mixtures was observed with molar ratios of rBet v 1a of 80% (IB), 20% (ELISA), and 60% (CD_{222nm}). No statistical significance was obtained for the cellular in vitro mediator release assay, because of large variation of the results in this biological assay. Our analyses revealed that experimental/theoretical comparisons of physicochemical and immunoassays with physically integer and IgE-reactive rBet v 1a in the range of 100–10% molar content lies within 0.26–1.74, with CD and ELISA correlating best with the theoretical values.

In the European Pharmacopeia Monograph on Allergen Products (01/2010:1063) the total allergenic activity of an allergen product as assayed by inhibition of binding capacity of specific IgE may range from 50–150%. Considering that total IgE binding of a birch pollen extract is mainly caused by Bet v 1, we cover at least the 50–100% range which is represented by accuracies of > 0.48 (MR) to < 1.21 (IB) at 40% rBet v 1a to 1.0 at 100% rBet v 1a (cf. Fig 7). Thus, deviations of the content of the actual active (i.e. IgE binding) component(s) in the allergen product of down to a factor of 0.5 are detected with reasonable accuracy of all assays used in this study.
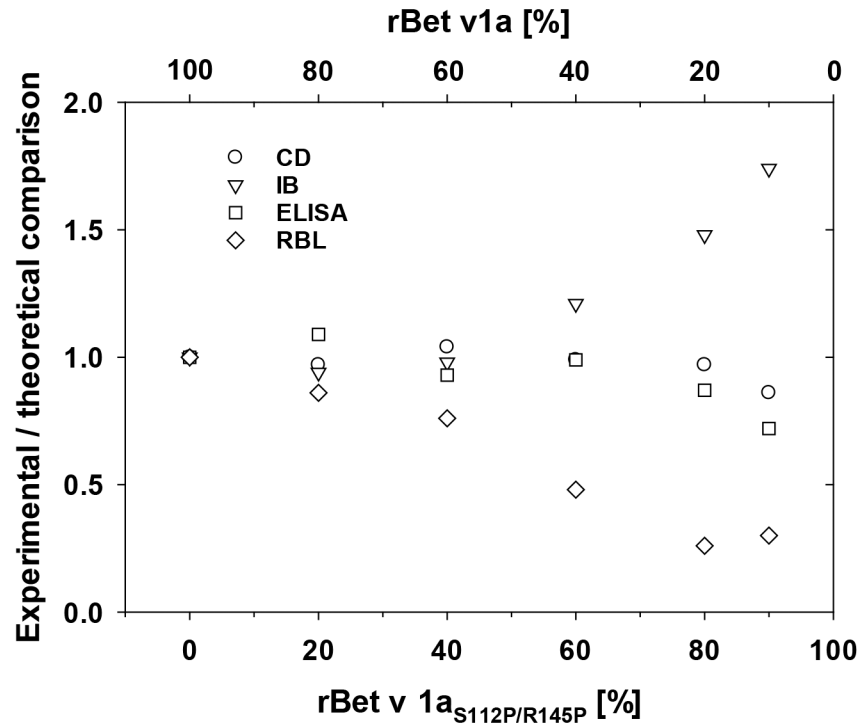
Fig 7. Experimental/theoretical comparison of CD, IB, ELISA and RBL mediator release. The experimental/theoretical comparisons of the individual experiments shown in Figs 3–6 for molar fractions of rBet v 1a$_{S112P/R145P}$ from 0% to 90% in rBet v 1a/rBet v 1a$_{S112P/R145P}$ combinations analyzed are shown.

doi:10.1371/journal.pone.0132956.g007

Recombinant Bet v 1 has been generated as low IgE-binding (hypoallergenic) variants to test therapeutic potential (reviewed by Grönlund and Gafvelin, 2010). Reduced IgE binding capacity in *in vitro* assays is one parameter that qualifies a rBet v 1a variant as potential hypoallergenic candidate molecule for AIT. In this regard the immunoassays used in this study are well suited to screen for potential therapeutics as they easily detected IgE responses of ≤ 1% of total IgE binding.

Table 1. Accuracy, precision and resolution of methods to distinguish rBet v 1a conformation-dependent IgE binding and secondary structure of rBet v 1a/rBet v 1a$_{S112P/R145P}$ mixtures.

Method	experimental/theoretical comparison	95% CI	p-value	resolution[1]: %	resolution[1]: factor
CD$_{222nm}$	1.03	-6157 to -4835 [Θ][2]	0.0039	60	1.67
IB	1.48	1.94*10^6–6.21*10^6 [au][3]	<0.0001	20	5
ELISA	0.93	3.05–4.62 [EC$_{50}$][4]	0.412	60	1.67
RBL	1.32	44.4–174.8 [EC$_{50}$][5]	0.682	1	100

[1]analytical resolution between 100% and x% Bet v 1a in Bet v 1a/Bet v 1a$_{S112P/R145P}$ mixtures

[2]in deg*cm^2*dmol^{-1}

[3]arbitrary units

[4]inhibitor concentration (ng/well) for half-maximum inhibition of IgE binding

[5]protein concentration (ng/well) for half-maximum mediator release

CD$_{222nm}$: mean residual ellipticiy at 222 nm; IB: immunoblot; ELISA: Inhibition-Enzyme-linked immunosorbent assay; RBL: cellular mediator release with humanized rat basophilic leukemia cells

doi:10.1371/journal.pone.0132956.t001

Considering the potential use of recombinant Bet v 1a variants for AIT or as diagnostic reagents, the variants rBet v 1a and rBet v $1_{S112P/R145P}$ used in this study could be used as quality reference standards. However other attempts to assess Bet v 1-type protein conformation and thus potential allergenicity could also be considered to evaluate the quality of recombinant Bet v 1 preparations. The recently identified natural ligand of Bet v 1a and the determined structures of Bet v 1 isoforms with bound ligands could be employed for the development of quantitative ligand binding assays with clear-cut correlation to allergen conformation und thus IgE binding activity [5,55]. Such applications would clearly advance the evaluation of allergen folding-dependent IgE binding of rBet v 1 preparations.

The list of methods to analyze Bet v 1 conformation and correlated IgE interaction is not complete in this study. Surface plasmon resonance, infrared spectroscopy, isothermal titration calorimetry and other methods are also valuable additions to be included in a set of assays to assess the quality of recombinant Bet v 1-type allergens. However, lower IgE binding might not always be necessarily related to loss of Bet v 1-type conformation. Additional preparation/storage-dependent phenomena like modifications of single amino acid side chains (e.g. deamidation, oxidation and others) while maintaining native protein fold may also contribute to decreased IgE binding due to decreased antibody affinity on the epitope level.

Taken together the set of physico- and immunochemical assays used here to correlate rBet v 1a protein conformation and IgE binding revealed that ELISA and Circular Dichroism could accurately and precisely determine lower rBet v 1a contents ($\leq 60\%$) in the context of absolute allergen concentration whereas immunoblot and cellular mediator release needed larger deviations of rBet v 1a ratios, $\geq 20\%$ and $\geq 1\%$, respectively, to resolve the content of IgE-binding rBet v 1a in recombinant protein preparations. The principal findings in this study should be considered when employing methods for quality assurance of allergen preparations used in *in vivo* diagnostics and allergen-specific immunotherapies.

Supporting Information

S1 File. ^{15}N spin relaxation of rBet v 1a.
(PDF)

S1 Table. Summary of circular dichroism of rBet v 1a protein combinations.
(PDF)

S2 Table. Experimental and theoretical values of quantitative IgE immunoassays.
(PDF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: FH AK CSvL KS TH DS. Performed the experiments: FH AK CSvL KS LV AR EV SR. Analyzed the data: FH KMH CSvL KS AR SV PR TH DS. Contributed reagents/materials/analysis tools: LV, SV, PR. Wrote the paper: DS CSvL. Recruitment of patients and clinical evaluation: IB, RT, JCS.

References

1. D'Amato G, Cecchi L, Bonini S, Nunes C, Annesi-Maesano I, Behrendt H, et al. Allergenic pollen and pollen allergy in Europe. Allergy 2007; 62: 976–990. PMID: 17521313

2. Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Joost van Neerven RJ, et al. X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. Nat Struct Biol 1996; 3(12):1040–5. PMID: 8946858

3. Markovic-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, Susani M, et al. Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J Mol Biol 2003; 325(1):123–33. PMID: 12473456

4. Holm J, Gajhede M, Ferreras M, Henriksen A, Ipsen H, Larsen JN, 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: 5258–5267. PMID: 15470071

5. Kofler S, Asam C, Eckhard U, Wallner M, Ferreira F, Brandstetter H. Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen bet v 1. J Mol Biol 2012; 422(1):109–23. doi: 10.1016/j.jmb.2012.05.016 PMID: 22634284

6. Kofler S, Ackaert C, Samonig M, Asam C, Briza P, Horejs-Hoeck J, et al. Stabilization of the dimeric birch pollen allergen Bet v 1 impacts its immunological properties. J Biol Chem 2014; 289(1):540–51. doi: 10.1074/jbc.M113.518795 PMID: 24253036

7. Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. J Exp Med 1996; 183: 599–609. PMID: 8627171

8. Wiedermann U, Jahn-Schmid B, Bohle B, Repa A, Renz H, Kraft D, et al Suppression of antigen-specific T- and B-cell responses by intranasal or oral administration of recombinant bet v 1, the major birch pollen allergen, in a murine model of type I allergy. J Allergy Clin Immunol 1999; 103(6):1202–10. PMID: 10359907

9. Winkler B, Baier K, Wagner S, Repa A, Eichler HG, Scheiner O, et al. Mucosal tolerance as therapy of type I allergy: intranasal application of recombinant Bet v 1, the major birch pollen allergen, leads to the suppression of allergic immune responses and airway inflammation in sensitized mice. Clin Exp Allergy 2002; 32(1):30–6. PMID: 12002733

10. Siebeneicher S, Reuter S, Krause M, Wangorsch A, Maxeiner J, Wolfheimer S, et al. Epicutaneous immune modulation with Bet v 1 plus R848 suppresses allergic asthma in a murine model. Allergy 2014; 69: 328–37 doi: 10.1111/all.12326 PMID: 24329861

11. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. FASEB J 1998; 12: 231–242. PMID: 9472988

12. Holm J, Ferreras M, Ipsen H, Würtzen PA, Gajhede M, Larsen JN, 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: 17569–17578. doi: 10.1074/jbc.M110.194878 PMID: 21454600

13. Jahn-Schmid B, Radakovics A, Lüttkopf D, Scheurer S, Vieths S, Ebner C, et al. Bet v 1142–156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. J Allergy Clin Immunol 2005; 116(1): 213–9. PMID: 15990797

14. Klinglmayr E, Hauser M, Zimmermann F, Dissertori O, Lackner P, Wopfner N, et al. Identification of B-cell epitopes of Bet v 1 involved in cross-reactivity with food allergens. Allergy 2009; 64(4): 647–51. doi: 10.1111/j.1398-9995.2008.01844.x PMID: 19154550

15. Hecker J, Diethers A, Schulz D, Sabri A, Plum M, Michel Y, 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. doi: 10.1111/all.12045 PMID: 23066955

16. Gepp B, Lengger N, Bublin M, Hemmer W, Breiteneder H, Radauer C. 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(1): 188–94. doi: 10.1016/j.jaci.2013.12.1073 PMID: 24529686

17. Berkner H, Seutter von Loetzen C, Hartl M, Randow S, Gubesch M, Vogel L, et al. Enlarging the toolbox for allergen epitope definition with an allergen-type model protein. PLoS One 2014; 2014 Oct 30; 9(10):e111691. doi: 10.1371/journal.pone.0111691 PMID: 25356997

18. Bohle B, Breitwieser A, Zwölfer B, Jahn-Schmid B, Sára M, Sleytr UB, et al. A novel approach to specific allergy treatment: the recombinant fusion protein of a bacterial cell surface (S-layer) protein and the major birch pollen allergen Bet v 1 (rSbsC-Bet v 1) combines reduced allergenicity with immunomodulating capacity. J Immunol 2004; 172(11): 6642–8. PMID: 15153479

19. Mahler V, Vrtala S, Kuss O, Diepgen TL, Suck R, Cromwell O, et al. Vaccines for birch pollen allergy based on genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1. Clin Exp Allergy 2004; 34(1): 115–22. PMID: 14720271

20. Gafvelin G, Thunberg S, Kronqvist M, Grönlund H, Grönneberg R, Troye-Blomberg M, et al. Cytokine and antibody responses in birch-pollen-allergic patients treated with genetically modified derivatives of the major birch pollen allergen Bet v 1. Int Arch Allergy Immunol 2005; 138(1): 59–66. PMID: 16103688

21. Daniel C, Repa A, Wild C, Pollak A, Pot B, Breiteneder H, et al. Modulation of allergic immune responses by mucosal application of recombinant lactic acid bacteria producing the major birch pollen allergen Bet v 1. Allergy 2006; 61(7): 812–9. PMID: 16792578

22. Pree I, Reisinger J, Focke M, Vrtala S, Pauli G, van Hage M, et al. Analysis of epitope-specific immune responses induced by vaccination with structurally folded and unfolded recombinant Bet v 1 allergen derivatives in man. J Immunol 2007; 179(8): 5309–16. PMID: 17911617

23. Purohit A, Niederberger V, Kronqvist M, Horak F, Grönneberg R, Suck R, et al. Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives. Clin Exp Allergy 2008; 38(9): 1514–25. doi: 10.1111/j.1365-2222.2008.03042.x PMID: 18564326

24. Meyer W, Narkus A, Salapatek AM, Häfner D. Double-blind, placebo-controlled, dose-ranging study of new recombinant hypoallergenic Bet v 1 in an environmental exposure chamber. Allergy. 2013; 68(6): 724–31. doi: 10.1111/all.12148 PMID: 23621350

25. Pichler U, Asam C, Weiss R, Isakovic A, Hauser M, Briza P, et al. The fold variant BM4 is beneficial in a therapeutic Bet v 1 mouse model. Biomed Res Int 2013; 2013: 832404. doi: 10.1155/2013/832404 PMID: 24175303

26. van Hage-Hamsten M, Johansson E, Roquet A, Peterson C, Andersson M, Greiff L, et al. Nasal challenges with recombinant derivatives of the major birch pollen allergen Bet v 1 induce fewer symptoms and lower mediator release than rBet v 1 wild-type in patients with allergic rhinitis. Clin Exp Allergy 2002; 32(10): 1448–53. PMID: 12372124

27. Smole U, Balazs N, Hoffmann-Sommergruber K, Radauer C, Hafner C, Wallner M, et al. Differential T-cell responses and allergen uptake after exposure of dendritic cells to the birch pollen allergens Bet v 1.0101, Bet v 1.0401 and Bet v 1.1001. Immunobiology 2010; 215(11): 903–9. doi: 10.1016/j.imbio.2009.11.003 PMID: 20005001

28. Kitzmüller C, Wallner M, Deifl S, Mutschlechner S, Walterskirchen C, Zlabinger GJ, et al. A hypoallergenic variant of the major birch pollen allergen shows distinct characteristics in antigen processing and T-cell activation. Allergy 2012; 67(11): 1375–82. doi: 10.1111/all.12016 PMID: 22973879

29. Godnic-Cvar J, Susani M, Breiteneder H, Berger A, Havelec L, Waldhör T, et al. Recombinant Bet v 1, the major birch pollen allergen, induces hypersensitivity reactions equal to those induced by natural Bet v 1 in the airways of patients allergic to tree pollen. J Allergy Clin Immunol 1997; 99(3):354–9. PMID: 9058691

30. Hoffmann-Sommergruber K, Susani M, Ferreira F, Jertschin P, Ahorn H, Steiner R, et al. High-level expression and purification of the major birch pollen allergen, Bet v 1. Protein Expr Purif 1997; 9(1): 33–9. PMID: 9116499

31. Tresch S, Holzmann D, Baumann S, Blaser K, Wüthrich B, Crameri R, et al. In vitro and in vivo allergenicity of recombinant Bet v 1 compared to the reactivity of natural birch pollen extract. Clin Exp Allergy 2003; 33(8): 1153–8. PMID: 12911792

32. Himly M, Nony E, Chabre H, Van Overtvelt L, Neubauer A, van Ree R, et al. Standardization of allergen products: 1. Detailed characterization of GMP-produced recombinant Bet v 1.0101 as biological reference preparation. Allergy 2009; 64(7): 1038–45. doi: 10.1111/j.1398-9995.2009.01957.x PMID: 19183416

33. De Amici M, Mosca M, Vignini M, Quaglini S, Moratti R. Recombinant birch allergens (Bet v 1 and Bet v 2) and the oral allergy syndrome in patients allergic to birch pollen. Ann Allergy Asthma Immunol 2003; 91(5): 490–2. PMID: 14692434

34. Ciprandi G, Fenoglio G, Kalli F, De Amici M, Leonardi S, Miraglia Del Giudice M, et al. Patients with oral allergic syndrome to apple have intense proliferative response to BET V 1. J Biol Regul Homeost Agents 2012; 26(1 Suppl): S113–7. PMID: 22691258

35. Grönlund H, Gafvelin G. Recombinant Bet v 1 vaccine for treatment of allergy to birch pollen. Hum Vaccin 2010; 6(12): 970–7. PMID: 21157177

36. Vieths S, Barber D, Chapman M, Costanzo A, Daas A, Fiebig H, et al. Establishment of recombinant major allergens Bet v 1 and Phl p 5a as Ph. Eur. reference standards and validation of ELISA methods for their measurement. Results from feasibility studies. Pharmeur Bio Sci Notes 2012; 2012: 118–34. PMID: 23327896

37. Kahlert H, Suck R, Weber B, Nandy A, Wald M, Keller W, et al. Characterization of a hypoallergenic recombinant Bet v 1 variant as a candidate for allergen-specific immunotherapy. Int Arch Allergy Immunol 2008; 145: 193–206. PMID: 17912007

38. Wallner M, Hauser M, Himly M, Zaborsky N, Mutschlechner S, Harrer A, et al. Reshaping the Bet v 1 fold modulates T(H) polarization. J Allergy Clin Immunol 2011; 127: 1571–1578. doi: 10.1016/j.jaci.2011.01.064 PMID: 21420160

39. Fiebig H, Kahlert H, Weber B, Suck R, Nandy A, Cromwell O. Antibody-based methods for standardization of allergoids and recombinant hypoallergens. Arb Paul Ehrlich Inst Bundesinstitut Impfstoffe Biomed Arzneim Langen Hess. 2009; 96: 71–82. PMID: 20799447

40. Holzwarth G, Doty P. The ultraviolet circular dichroism of polypeptides. J Am Chem Soc 1965; 87: 218–228. PMID: 14228459

41. Greenfield N, Fasman GD. Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry 1969; 8: 4108–4116. PMID: 5346390

42. Venyaminov S, Baikalov IA, Shen ZM, Wu CS, Yang JT. Circular dichroic analysis of denatured proteins: inclusion of denatured proteins in the reference set. Anal Biochem 1993; 214: 17–24. PMID: 8250221

43. Piotto M, Saudek V, Sklenar V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. J Biomol NMR 1992; 2: 661–665. PMID: 1490109

44. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–685. PMID: 5432063

45. Kyhse-Andersen J. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J Biochem Biophys Methods 1984; 10: 203–209. PMID: 6530509

46. Vogel L, Luttkopf D, Hatahet L, Haustein D, Vieths S. Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. Allergy 2005; 60: 1021–1028. PMID: 15969682

47. Albrecht M, Alessandri S, Conti A, Reuter A, Lauer I, Vieths S, 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–S195. doi: 10.1002/mnfr.200700424 PMID: 18727010

48. Son DY, Scheurer S, Hoffmann A, Haustein D, Vieths S. 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(4): 201–15. PMID: 10502033

49. Williamson MP. Secondary-structure dependent chemical shifts in proteins. Biopolymers 1990; 29: 1423–1431. PMID: 2375792

50. Wishart DS, Sykes BD, Richards FM. Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. J Mol Biol 1991; 222: 311–333. PMID: 1960729

51. Braun D, Wider G, Wuethrich K. Sequence-corrected 15N "random coil" chemical shifts. J Am Chem Soc 1994; 116: 8466–8469.

52. Merutka G, Dyson HJ, Wright PE. 'Random coil' 1H chemical shifts obtained as a function of temperature and trifluoroethanol concentration for the peptide series GGXGG. J Biomol NMR 1995; 5: 14–24. PMID: 7881270

53. Schwarzinger S, Kroon GJ, Foss TR, Chung J, Wright PE, Dyson HJ. Sequence-dependent correction of random coil NMR chemical shifts. J Am Chem Soc 2001; 123: 2970–2978. PMID: 11457007

54. DeLano WL (2002) The PyMOL Molecular Graphics System.

55. Seutter von Loetzen C, Hoffmann T, Hartl MJ, Schweimer K, Schwab W, Rösch P, et al. Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. Biochem J 2014; 457(3): 379–90. doi: 10.1042/BJ20130413 PMID: 24171862