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Characterization of epitope specificities of reference antibodies used for the quantification of the birch pollen allergen Bet v 1

Short title: Epitope delineation of Bet v 1-specific antibodies

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Abstract

Background: Accurate allergen quantification is needed to document the consistency of allergen extracts used for immunotherapy. Herein, we characterize the epitope specificities of two monoclonal antibodies used in an ELISA for the quantification of the major birch pollen allergen Bet v 1, established as a reference by the BSP090 European project.

Methods: The ability of mAbs 5B4 and 6H4 to recognize Bet v 1 isoforms was addressed by immunochromatography. The capacity of each mAb to compete with patients' IgE for binding to Bet v 1 was measured by ELISA inhibition. Epitope mapping was performed by pepscan analysis, site-directed mutagenesis and hydrogen/deuterium exchange-mass spectrometry.

Results: The 5B4 epitope corresponds to a peptide sequence (I56-K68) overlapping with the binding sites of patients' serum IgEs. Mutation of residues P59, E60 and K65 abolishes 5B4 binding to Bet v 1 and reduces the level of IgE recognition. In contrast, 6H4 recognizes a conformational epitope lying opposite to the 5B4 binding site, involving residues located in segments I44-K55 and R70-F79. Substitution of E45 reduces the binding capacity of 6H4, confirming that it is critical for the interaction. Both mAbs interact with >90% of Bet v 1 content present in the birch pollen extract, while displaying a weak cross-reactivity with other allergens of the PR-10 family.

Conclusions: MAbs 5B4 and 6H4 recognize structurally distinct epitopes present in the vast majority of Bet v 1 isoforms. These results support the relevance as a reference method of the Bet v 1-specific quantitative ELISA adopted by the European Pharmacopoeia.

Key words

5B4; 6H4; Bet v 1 isoforms; ELISA; epitope mapping.

Introduction

More than 90% of patients suffering from allergy to birch pollen have IgEs directed to the major birch pollen allergen Bet v 1 (1). As of today, 27 different Bet v 1 isoforms displaying significant amino acid sequence homology are referenced by the International Union of Immunological Societies (2, 3). Patients with birch pollen allergy often have IgEs cross-reactive with pollen allergens from other Fagales trees, such as alder, chestnut, hazel or hornbeam (4-6), as well as food allergens causing the oral allergy syndrome (7). Such cross-reactivities are mostly due to major allergens of the PR-10 family present in these allergen sources, which display primary and tertiary structures similar to that of Bet v 1 (8-11).

Over the past decade, recombinant forms of Bet v 1 were shown to be as efficient and safe as birch pollen extracts when used for allergen immunotherapy (AIT), establishing this major allergen as a critical component for therapeutic purposes (12-14). To this date, however, only natural extracts are commercially available to treat patients with allergic rhinoconjunctivitis to birch pollen (15, 16). To guarantee the quality and consistency of such therapeutic extracts, the European CREATE Program followed by the Biological Standardization Program 090 have tested and compared multiple immunoassays and selected as a reference the ELISA based on 5B4 and 6H4 monoclonal Abs (mAbs) to quantify Bet v 1 (17-19). In this system, Bet v 1 allergens are first captured by 5B4 and subsequently detected by biotinylated 6H4.

Immunoassays rely upon mAbs which are expected to capture and detect a broad range of naturally occurring isoforms (20-22). In this regard, the in-depth characterization of binding epitopes is important to confirm the suitability of mAbs for ELISAs, and to define their isoform recognition profile. Among the various strategies available, X-ray crystallography and NMR represent the gold standards, but they require significant amounts of time and proteins (23, 24). Recently, Hydrogen/Deuterium eXchange monitored by Mass Spectrometry (HDX-MS) has emerged as a method of choice to rapidly map epitopes (25-28). Compared to X-ray crystallography and NMR, HDX-MS data is of medium resolution (*i.e.*, 8-10 residues), but requires very low quantities of starting material.

Herein, we define the epitope specificities and the recognition profile of the two murine 5B4 and 6H4 anti-Bet v 1 IgG1s using a combination of electrophoretic, chromatographic, pepscan and mass spectrometric approaches, including HDX-MS. Our results confirm the suitability of combining these two mAbs within a sandwich ELISA to achieve a comprehensive quantification of Bet v 1 isoforms (17).

Materials and methods

Allergens and antibodies

Pollens from *Betula verrucosa* (European white birch) were obtained from Allergon (Ängelholm, Sweden) and Stallergenes Greer (Lenoir, NC, USA). Proteins were extracted by incubating pollens (500 mg) in 5 mL of 50 mM ammonium bicarbonate buffer for 6 h at 4°C. Resulting samples were 0.45-µm filtered, and then dialyzed against PBS. To produce anti-Bet v 1 mAbs, Balb/c mice (ByJlco, Charles River, Saint-Germain-sur-l'Arbresle, France) were immunized intraperitoneally with a birch pollen extract. Animal handling was performed according to international regulations. Following selection and cloning, IgG1s produced by hybridomas 5B4 and 6H4 were purified using protein A

affinity chromatography. Detailed procedures for the expression, purification and characterization of rBet v 1 molecules and mAbs are described in supporting Data S1.

Immunochromatography and ELISA

Birch pollen proteins (1 mg) were loaded onto 5 mL NHS columns (GE Healthcare, Vélizy-Villacoublay, France) previously coupled with 15 mg of either 5B4 or 6H4 antibodies. MAb-bound Bet v 1 isoforms were eluted with a glycine buffer pH 3.0, then buffer exchanged on a HiTrap desalting column (GE Healthcare) with PBS. Pollen extract, flow-through and eluate samples were assessed using the Bradford method for protein concentration as well as SDS-PAGE and western blotting, as described in Data S1. The inhibition of patients' IgE binding was assessed by ELISA, as described in Data S1. Human sera from informed blood donors were obtained from the Établissement Français du Sang (La Plaine Saint-Denis, France).

Epitope mapping and label free quantification

Epitope mapping was performed by pepscan analysis and HDX-MS, as described in Data S1, using the recombinant form of Bet v 1.0101. Briefly, the HDX rate of a backbone amide hydrogen (NH) reflects its unique environment within the Bet v 1 3D structure. Upon mAb binding to Bet v 1, NHs located within the epitope recognized become protected from exchange, leading to a decrease of deuterium incorporation due to steric exclusion of the solvent. The MS comparison of the patterns of deuterium incorporation by Bet v 1 alone with that of a Bet v 1-mAb complex, allows to decipher the binding interfaces within the complex. For label-free quantification, proteins from pollen extracts were digested with trypsin and analyzed by LC-MS/MS, as described in Data S1.

Results

MAbs 5B4 and 6H4 interact with most Bet v 1 isoforms

The two monoclonal antibodies, 5B4 and 6H4, were generated after mouse immunization with a birch pollen extract and selected based on their reactivity towards the recombinant form of Bet v 1.0101 (rBet v 1). As measured by surface plasmon resonance, both mAbs bind to rBet v 1 with high affinities ($K_d = 32$ and 0.3 nM for 5B4 and 6H4, respectively) and similar association rates (Table S1). The observed difference in K_d mainly results from the faster dissociation rate of 5B4 compared to 6H4 ($k_{off} = 0.01$ s⁻¹ versus 1.1×10^{-4} s⁻¹, Table S1).

The ability of 5B4 and 6H4 to capture Bet v 1 isoforms was assessed by immunochromatography, after passing three distinct birch pollen extracts (from different geographic locations and years of harvest) through mAb-immobilized columns. As shown in Figure 1A, when analyzing the bound and unbound fractions by SDS-PAGE, only the 18 kDa protein band corresponding to Bet v 1 was captured and eluted from the columns. The other birch pollen allergens, *i.e.*, Bet v 2, Bet v 6 and Bet v 7, were not recognized, confirming the Bet v 1-specificity of mAbs 5B4 and 6H4. In addition, the dramatic reductions in band intensities (96% for 5B4 and 87% for 6H4) for Bet v 1 observed in the unbound fractions suggest that 5B4 and 6H4 recognize most Bet v 1 content encompassing the natural spectrum of isoforms (Fig. 1A, FT lanes). These results were further confirmed by label-free LC-MS/MS experiments performed in order to assess putative affinity differences between 5B4 and 6H4 towards Bet v 1 isoforms. As shown in Figure S1, the two antibodies recognized very efficiently peptides representative of a broad spectrum of Bet v 1 isoforms, with only evidence for a slightly lower reactivity of 6H4 with Bet v 1.02 isoforms when compared to 5B4.

MAbs 5B4 and 6H4 exhibit a limited cross-reactivity with specific Bet v 1 analogs

To assess the cross-reactivity of 5B4 and 6H4 mAbs with Bet v 1 homologs, a western blot analysis was conducted using pollen extracts from either birch (*Betula verrucosa*, positive control), alder (*Alnus glutinosa*), chestnut (*Castanea sativa*), hazel (*Corylus avellana*) or hornbeam (*Carpinus betulus*) trees. As expected, 5B4 and 6H4 showed a high reactivity with the alder pollen allergen, Aln g 1, which shares over 80% amino acid sequence identity with Bet v 1 (Figs 1B,C and S2). In contrast, the two mAbs did not bind the chestnut tree pollen allergen, Cas s 1, which shares only 59% sequence identity with Bet v 1.0101, with a strong divergence in primary sequences in three regions encompassing residues 60-80, 90-105 and 130-145, respectively (Fig. S2). It can thus be inferred that the latter regions contain critical residues for the binding of those mAbs. In addition, 5B4 and 6H4 exhibit only a weak reactivity with Car b 1 or Cor a 1, respectively (Fig. 1B,C), likely due to amino acid substitutions within the epitopes recognized by each of these mAbs within those two allergens, when compared to Bet v 1. Altogether, these data establish that mAbs 5B4 and 6H4 display similar, but limited cross-reactivity with other members of the PR-10 family of pollen allergens from other Fagales.

5B4 , but not 6H4, competes with IgEs from allergic patients for binding to rBet v 1

The ability of 5B4 and 6H4 mAbs to inhibit the binding to rBet v 1 of specific serum IgEs from birch pollen allergic patients was assessed by ELISA inhibition. ELISA-plate bound-rBet v 1 was incubated with serial dilutions of each mAb mixed with pools of sera derived from either 16 birch pollen-allergic patients or 13 non-allergic individuals used as a control (data not shown). With the limitation that they were obtained using a pool of sera from birch pollen allergic patients, our results do not

preclude that selected patients may exhibit IgE specificities distinct from the ones detected herein. Notably, 5B4 was confirmed to inhibit the binding of specific IgEs to rBet v 1 in a dose-dependent manner (Fig. 1D). The strong inhibition (up to 50%) observed suggests that the epitope recognized by 5B4 is significantly overlapping with the binding sites of most serum IgEs, as previously observed with the murine BV16 mAb (29). In contrast, 6H4 does not compete with the patients' IgE for binding and displays the same inhibition profile as the Der p 1-specific 5H8 mAb, used as a negative control in those experiments (Fig. 1D). These results provide evidence that 5B4 and 6H4 recognize two distinct epitopes on the Bet v 1 allergen.

MAbs 5B4 and 6H4 recognize structurally distinct epitopes

To map precisely the epitopes recognized by 5B4 and 6H4 mAbs, a set of 16 overlapping linear peptides encompassing the entire amino-acid sequence of Bet v 1.0101 was synthesized, coated onto a solid support, and probed with each of the two mAbs. While 6H4 fails to react with any of the tested peptides, 5B4 exhibits a strong reactivity with the P50-D69 peptide (Fig. 2). Interestingly, peptides S40-P59 and G60-F79, which overlap with the N- and the C-terminal domain of P50-D69, do not interact with 5B4. Thus, the 5B4 mAb recognizes a linear epitope encompassing critical residues in the N- and C-terminus of peptide P50-D69, both needed to allow binding of this mAb.

Whereas pepscan assays provide useful information on linear epitopes with a resolution limited to the size of the tested peptides, they are not appropriate to identify conformational epitopes, as the one likely recognized by 6H4. We thus used HDX-MS to map both linear and conformational epitopes. HDX-MS experiments were conducted with rBet v 1 molecules, either free in solution or bound to each of 5B4 or 6H4 mAbs. The average extent of deuterium uptake was monitored at defined time points using 14 and 11 selected overlapping peptides generated after pepsin or Type XIII protease digestion, respectively (94% sequence coverage, Fig. S3) in order to refine the position of each HDX-MS defined epitope. Results generated after 2 min labeling are summarized in Figure 3. In agreement with the pepscan analysis, binding of 5B4 reduces the deuterium uptake of peptide I56-K68 (C_{term} β 3-loop- N_{term} β 4), thus confirming the latter as encompassing the binding site for this mAb (Fig. 3A). In addition, a slight but significant (*i.e.*, > 0.5 Da) reduction of accessibility is observed with peptide F30-E42 (C_{term} α 2-loop- N_{term} β 2). Mapping of these two segments onto the Bet v 1 crystal structure (Fig. 3B,C) reveals that the HDX-defined 5B4 epitope involves two loops in close vicinity, connecting helix α 2 to strand β 2 and strands β 3 to β 4. Since peptide F30-E42 was not identified as relevant for 5B4 binding by pepscan analysis (Fig. 2), the reduced solvent accessibility observed with F30-E42 likely results from a steric hindrance imposed by the mAb.

The binding of 6H4 on Bet v 1 also induces detectable decreases in deuterium uptake. After 2 min labeling, three peptides including G1-F3 (N_{term} region), R70-F79 (C_{term} $\beta 4$ – loop – $\beta 5$ – loop), and I44-K55 (C_{term} $\beta 2$ – loop – N_{term} $\beta 3$) showed reduced solvent accessibility, with peptide I44-K55 being the most protected (Fig. 3A). Modelling the HDX-MS results onto the 3D structure of Bet v 1 reveals that these three segments are in close proximity with each other, and further, that they are located opposite to the 5B4 epitope (Fig. 3B,C). Thus, the epitope recognized by the 6H4 mAb is conformational, in agreement with the absence of detectable binding to linear peptides observed by pepscan analysis (Fig. 2). Interestingly, the glycine rich loop (*i.e.*, segment I44-K55) connecting strands $\beta 2$ to $\beta 3$ has been previously identified by crystallography as the dominant sequential element within the BV16 mAb epitope (27, 29). Based on these results, we hypothesize that the 6H4 and BV16 epitopes overlap. Altogether, these data confirm that 5B4 and 6H4 recognize structurally distinct epitopes on the Bet v 1 allergen.

Mutations in the HDX-defined epitopes alter Bet v 1 recognition by 5B4 and 6H4 mAbs

To further locate critical residues for mAb binding within HDX-MS defined epitopes, we performed site-directed mutagenesis followed by binding assays. Only those residues which were fully accessible to the solvent and found within these regions where major changes in deuterium uptake occurred were selected for substitutions (Fig. 4A,B). Using these two criteria, 11 surface-exposed amino acids were substituted by alanine, including 5 residues in N43-K55 (*i.e.*, N43, E45, N47, P50, G51) and 6 residues in I56-K68 (*i.e.*, P59, E60, F62, P63, F64, K65). Mutants F62I and E60G-F62L, corresponding to the isoforms Bet v 1.0103 and Bet v 1.0111, respectively, were also produced. The effect of each substitution on the structural integrity of the proteins was determined by CD spectroscopy and local HDX-MS analysis. Only mutants with CD spectra and HDX-behavior identical to that of rBet v 1 were selected for subsequent binding assays. Consequently, we excluded mutants G51A, F62A and F64A as they exhibited altered HDX dynamics and/or structures (Table S2; Fig. S4).

The ability of each selected mutant to interact with 5B4 and 6H4 mAbs was tested by ELISA inhibition. In this approach, mAb-binding to ELISA-plate bound-rBet v 1 is measured in presence or absence of increasing concentrations of each mutant, using rBet v 1 as a positive control. As expected, the substitution of residues located in the HDX-defined 6H4 epitope (*e.g.*, E45) does not affect the binding of the 5B4 mAb (Fig. 4C). In contrast, substitutions of either P59, E60 or K65 to alanine abolishes 5B4 binding. Interestingly, the mutant carrying the F62I substitution retains some ability to interact with 5B4 (Fig. 4C). These data were further confirmed and completed by pepscan analysis using modified versions of peptide P50-D69 (Fig. S5). Specifically, peptides carrying either a E60A,

G61A or F64A substitution completely lose their ability to interact with 5B4, while those containing a F62A or a P63A modification retain some or all their binding capacity, respectively.

By contrast, a single E45A substitution within the HDX-defined epitope affects the binding of the 6H4 mAb to rBet v 1 (Fig. 4D). The amplitude of this inhibition is not as pronounced as the one observed with F62I, but nonetheless confirms that E45 residue is important for the interaction with 6H4. Overall, these observations corroborate our HDX-MS results and highlight specific residues within Bet v 1 critical for the binding of each of the two mAbs.

Residues P59, E60 and K65 within the 5B4 epitope are critical for the binding of patients' IgEs

Since the 5B4 epitope overlaps with the binding sites of most human serum IgEs (Fig. 1D), we further investigated in ELISA inhibition whether mutations impairing the recognition of Bet v 1 by 5B4 also inhibit the binding of allergic patients' IgEs. As shown in Figure 4E, the inhibition of IgE binding to rBet v 1 is not affected by F62I, indicating that, in contrast to 5B4, residue F62 does not contribute to IgE binding. On the other hand, mutants P59A, E60A, K65A and E60G-F62L induce a lower inhibition of IgE binding, when compared to rBet v 1 (Fig. 4E). The reduced inhibition observed with K65A and E60A (~22% and ~18% reduction at the highest concentration, respectively) indicates that both residues are important for IgE binding. The importance of E60 was further confirmed with the double mutant E60G-F62L, in light of the abovementioned evidence that residue F62 is not required for IgE binding. Lastly, the P59A mutation also affects the inhibition of IgE binding to rBet v 1, albeit to a lesser extent (Fig. 4E). Overall, these results confirm that the epitope recognized by mAb 5B4 overlaps with IgE epitopes, with a major contribution of residues P59, E60 and K65 to IgE binding.

Discussion

Proper dosing of allergens is critical to ensure both the safety and efficacy of AIT drug products. According to the European Pharmacopoeia, major allergen content should be determined as part of the standardization of AIT pharmaceutical products, by using suitable immunochemical methods such as ELISA. The accuracy and precision of a quantitative ELISA depends upon the characteristics of the selected mAbs (22, 30). In this study, we used a combination of immunochromatography, pepscan and mass spectrometric methods to delineate the epitopes recognized on Bet v 1 by 5B4 and 6H4 murine mAbs, selected as components of a reference sandwich ELISA to quantify this major allergen in birch pollen extracts (17).

Based on both pepscan and HDX-MS analyses, we determined that the 5B4 epitope is linear and encompasses residues I56-K68, which form a long and flexible loop between strands $\beta 3$ and $\beta 4$ of the Bet v 1 allergen. The 5B4 epitope totally overlaps with that of the recombinant human IgE antibody M0418 which explain the substantial inhibition of human serum IgE binding to Bet v 1 observed with 5B4 (Reference 22, Levin et al). Our mutagenesis results confirm the presence of several critical residues within I56-K68 (*i.e.*, P59, E60, G61, F62, F64 and K65) for 5B4 binding (and also for human serum IgE binding). These data provide, at a molecular level, an explanation for the differences in recognition observed between Bet v 1 analogs. For instance, the absence of cross-reactivity with Cas s 1 and Cor a 1 can be attributed to the substitution of residues P59, G61 (only for Cas s 1) and F64, in those latter allergens, relative to Bet v 1. In addition, the high degree of sequence identity within I56-K68 (~85%) between Bet v 1 and Aln g 1 explains the strong cross-reactivity of 5B4 with those two molecules. In contrast to Bet v 1, the single substitution of residue P59 does not completely abolish 5B4 binding to Car b 1, but dramatically reduces its ability to form stable complexes.

With the exception of E60 and F62, all residues essential for 5B4 binding (*i.e.*, P59, G61, F64 and K65) are conserved among the different Bet v 1 isoforms (Fig. 5). F62 represents one of the most diverse residues found within the 5B4 epitope and as such, may play a critical role in the recognition profile of the antibody. The modification of F62 into alanine or isoleucine was found to be compatible with binding confirming that the two isoforms carrying the single F62I modification (*i.e.*, Bet v 1.0103 and 1.0116) would be detected by 5B4 (although with less reactivity compared to Bet v 1.0101). However, the effect of F62L (*i.e.*, Bet v 1.0112) remains to be determined since the absence of binding to 5B4 observed with E60G-F62L can be attributed to the substitution of either E60 alone or both residues. However, it is important to note that Bet v1.0112 is the only isoform carrying this single substitution (Figure S5). In addition, the F62S substitution, found in all Bet v 1.02 isoallergens, is not expected to prevent 5B4 binding, since this mAb strongly cross-reacts with Aln g 1 (Figs. 1B, S2). This result is further corroborated by our label free MS data that shows that 5B4 efficiently interact with Bet v 1.02 isoforms (*i.e.*, 5B4 depletes the *quasi* totality of the pool of Bet v 1.02 isoforms present in the three pollen extracts, as shown in Figure S1). Finally, our results provide evidence that, although 5B4 and M0418 target the same epitope, they likely interact with Bet v 1 in a distinct way. Indeed, as described by Levin et al. (Reference 22), M0418 is expected to recognize most Bet v 1 isoforms with the exception of all members of the Bet v 1.02 isoallergen group carrying the F62S substitution. Therefore, compared to the M0418 recombinant antibody, 5B4 displays a much broader recognition profile and should bind to the majority of Bet v 1 isoforms (with the exception of Bet v 1.0111 and potentially Bet v 1.0112).

Converging evidence establishes that the 6H4 mAb recognizes an epitope on Bet v 1 distinct from the one involved in 5B4 binding, as requested for a pair of mAbs used for a sandwich ELISA. Pepscan experiments failed to identify a linear epitope for the 6H4 antibody, whereas HDX-MS pointed to an interaction with segments R70-F79, G1-F3 and I44-K55 containing the glycine rich loop (Fig. 5). Thus, 6H4 recognizes a conformational epitope lying opposite to the 5B4 epitope. Of note, the I44-K55 region corresponds to the dominant element of the BV16 epitope, suggesting that 6H4 and BV16 binding sites at least partially overlap. Interestingly, the effects of the single E45A substitution on 6H4 binding are not as pronounced as those described with BV16 (Reference 36). While E45A completely impairs BV16 binding to Bet v 1, 6H4 retains some capabilities to interact with the allergen (Fig 4D). Therefore, the continuous region I44-K55 of Bet v 1 does not appear to participate in the majority of 6H4 interactions. The glycine-rich loop region represents one of the most conserved regions among the various Bet v 1 isoforms (Fig. 5).

Consequently, any antibody targeting this region should be (in theory) capable to interact with all isoforms. Interestingly, our label free MS data reveals that 6H4 bind less efficiently.

Similarly to 5B4, 6H4 also display significant cross-reactivity with Aln g 1, whereas a weak affinity is observed for Cor a 1. As for 5B4, our immunochromatography and MS experiments, confirm that the 6H4 mAb recognizes most Bet v 1 content. Although our data establish that both antibodies react efficiently with a broad spectrum of natural Bet v 1 isoforms, we cannot exclude that some low abundance isoforms are poorly recognized..

Our study also sheds light on the molecular motifs within Bet v 1, which are recognized by human IgEs. Interestingly, 5B4 recognizes the same epitope as two recently characterized Bet v 1-specific monoclonal human IgEs (24). In agreement with our own results, the latter study also reported a limited cross-reactivity between allergens of the PR-10 family for IgE antibodies recognizing this epitope. In addition, the 5B4 epitope partially overlaps with that of mAb2, a murine mAb competing with human IgE for binding to Bet v 1 (31). Our study and others (24) also highlight the importance of region I56-K68 in the binding of patient's IgEs. In addition, Gieras et al. have identified a surface-exposed patch on Bet v 1 containing major IgE epitopes, comprising segments G49-F58, E73-G88 and G88-K103 (31). This patch includes residues confirmed to be important for IgE-binding by site-directed mutagenesis studies (32), as well as competition experiments with well-characterized mAbs such as BV16 (29, 33). The IgE binding area, defined in the present study using 5B4, partially overlaps with the G49-Y66 region predicted to contain discontinuous IgE epitopes (34-36). Hence, the significant reduction of Bet v 1-specific IgE reactivity, due to single alanine substitutions of residues P59, E60 and K65, confirms that region I56-K68 is an additional epitope recognized by patients' IgEs.

In conclusion, based on the evidence that i) 5B4 and 6H4 antibodies recognize distinct epitopes within Bet v 1 and ii) they both interact with the vast majority of Bet v 1 content present in birch pollen, we conclude that such reagents are suitable for the accurate and comprehensive quantification of Bet v 1 by ELISA.

Conflicts of interest

This work was funded by Stallergenes Greer. S.B., M.L.M., K.J., C.L., F.P., V.B., E.N. and P.M. are employees of Stallergenes Greer. CK declares no conflicts of interest.

Author contributions

SB designed and performed HDX-MS experiments and wrote the manuscript. KJ performed WB experiments, ELISA, CD and helped in the preparation of samples for HDX-MS. MLM performed immunochromatography and LC-MS/MS analyses. CL and FP expressed rBet v 1 molecules. CK performed SPR measurements. VB and LM supervised rBet v 1 expression. EN and PM supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1 Patterns of reactivity of 5B4 and 6H4 monoclonal antibodies. (A) MAbs 5B4 and 6H4 capture most Bet v 1 content. Birch pollen extracts (SM: source material) were passed through mAb-immobilized columns and the bound (E: eluate) and unbound (FT: flow through) fractions were analysed by SDS-PAGE. Results in Figure 1A are representative of three different birch pollen batches. (B, C) Cross-reactivity profiles of mAb 5B4 (B) and 6H4 (C). Western blot analyses were performed using pollen extracts from birch (Bet v 1), alder (Aln g 1), hornbeam (Car b 1), chestnut tree (Cas s 1) and hazel (Cor a 1) (lanes 1 to 5, respectively). (D) The binding of specific serum IgEs derived from a pool of 16 birch-allergic patients to rBet v 1 was assessed by ELISA inhibition in the presence of increasing concentrations of either 5B4 or 6H4, or a house dust mite Der p 1 allergen-specific control mAb. Each value corresponds to the mean and standard deviation of three independent replicates.

Figure 2 Epitope mapping by pepscan analysis. (A) Results generated with the EpiFlag technology (Innobiochips). Each peptide was printed in triplicate on three distinct biochips. The binding of mAbs 5B4 and 6H4 was revealed with an anti-mouse IgG-rhodamine conjugate using a microarray scanner. Florescence values correspond to the mean and standard deviation of three independent replicates. (B) Cartoon and surface representations of the Bet v 1 allergen (pdb entry 1BV1 (37)) showing the position of the P50-D69 peptide detected by mAb 5B4.

Figure 3 Mapping of the 5B4 and 6H4 epitopes by HDX-MS. (A) Changes in deuterium uptake measured in the rBet v 1 allergen upon binding to 5B4 or 6H4. Uptake difference values were calculated after 2 min labeling by subtracting the deuterium level of the free allergen from that of the complex. Negative values indicate mAb-induced protection. Each data point represents the average of three independent HDX-MS experiments. The cumulative error of measuring the deuterium incorporation was +/- 0.40 Da; changes larger than 0.50 Da (blue dashed lines) were considered as significant. (B, C) Mapping of HDX-MS results onto the cartoon and surface representations of the Bet v 1 allergen (pdb entry 1BV1 (37)). Major (> 1.0 Da) and minor (0.5-1.0 Da) changes in deuterium uptake upon mAb binding are colored in red and orange, respectively. No changes (uptake differences of 0.0-0.5 Da) are colored in cyan.

Figure 4 Identification of residues critical for binding of 5B4, 6H4 and patients' IgEs. (A) Sequence alignment of residues 30 to 80 of the 27 reported Bet v 1 isoforms (IUIS nomenclature). Shaded bold residues in light gray, light blue and black correspond to highly similar, weakly similar and non-conserved residues, respectively. The two regions where major changes in deuterium uptake occur upon mAb binding are reported on the Bet v 1.0101 sequence and colored in light orange (I44-K55, mAb 6H4) and red (I56-K68, mAb 5B4). Residues selected for mutagenesis are indicated by a black arrow. Contact residues identified in the Bet v 1 – BV16 Fab' complex are underlined (38). (B) Cartoon representation of Bet v 1 showing the position of 11 surface-exposed residues selected for mutagenesis. (C, D) ELISA inhibition assays. The effect of substitutions on the binding of (C) 5B4 and (D) 6H4 to coated rBet v 1 was measured in presence of increasing concentrations of each mutated protein. (E) Effects of selected substitutions on the binding of patients' IgEs to rBet v 1. Each value in (C, D, E) corresponds to the mean and standard deviation of three individual replicates.

Figure 5 (A) Surface representations of the HDX-defined epitopes recognized by 5B4 and 6H4 mAbs within Bet v 1. Major (> 1 Da) and minor (> 0.5 Da) reduction of solvent accessibility upon mAb binding are reported. (B) Varying (yellow) or conserved (cyan) amino acids between the 27 referenced Bet v 1 isoforms are represented for comparison with (A). Residues critical for binding of mAbs 5B4 and 6H4 are specified.

Figures

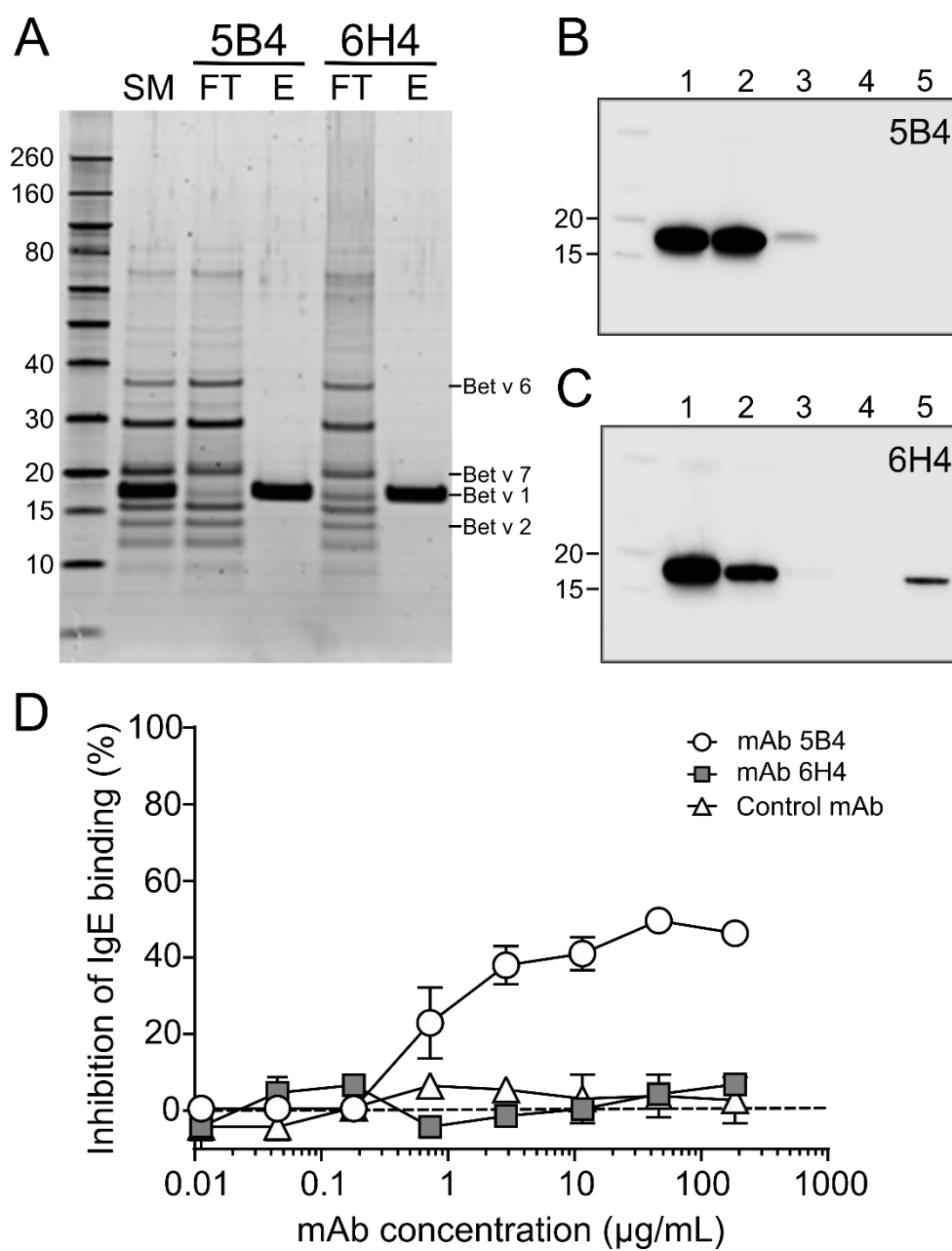


Figure 1

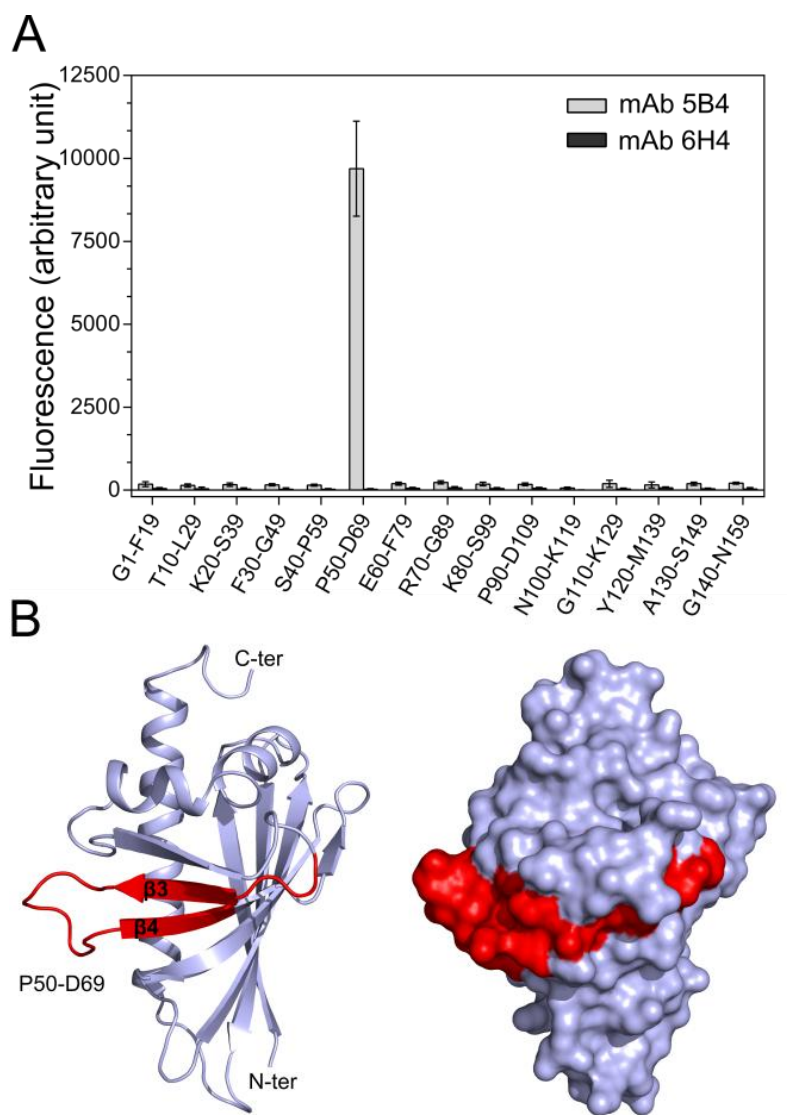


Figure 2

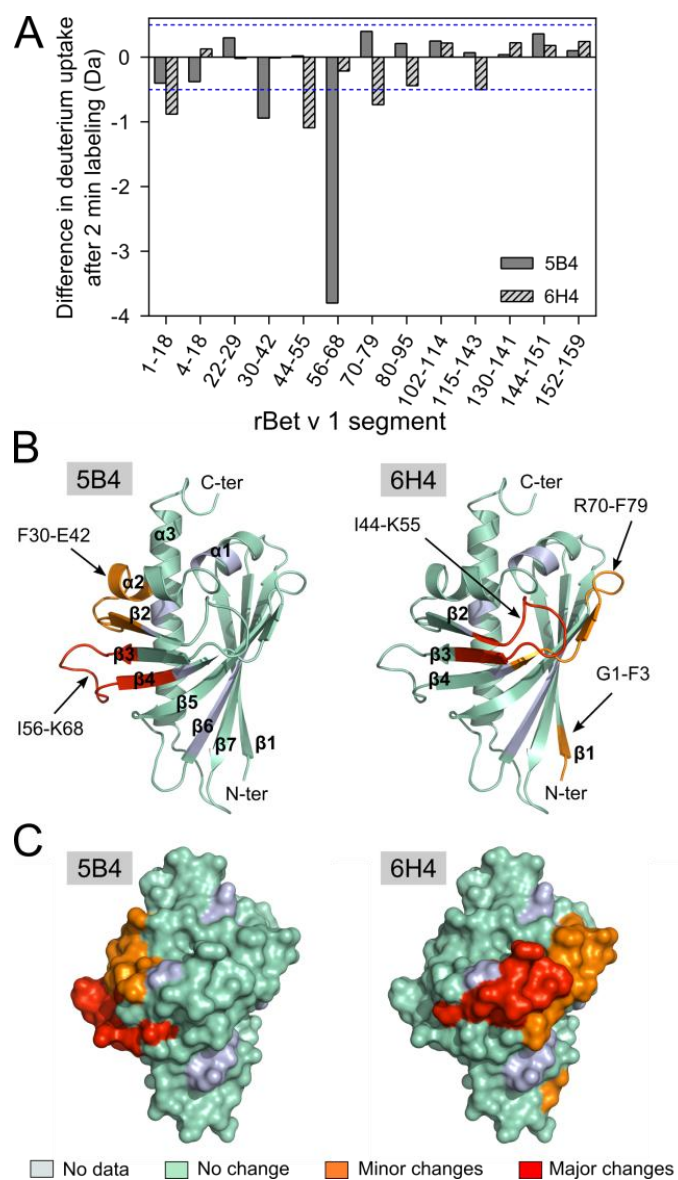


Figure 3

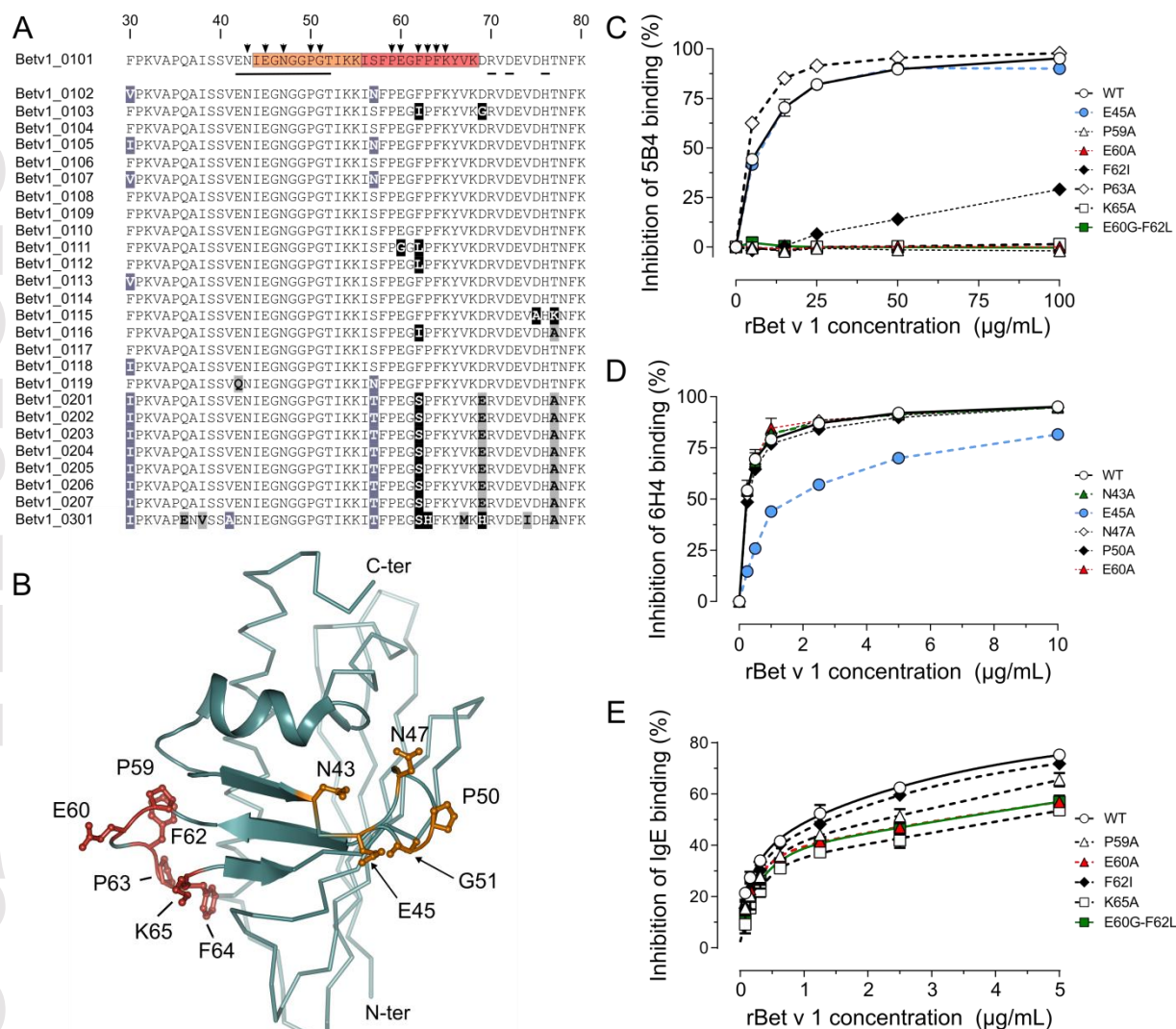


Figure 4

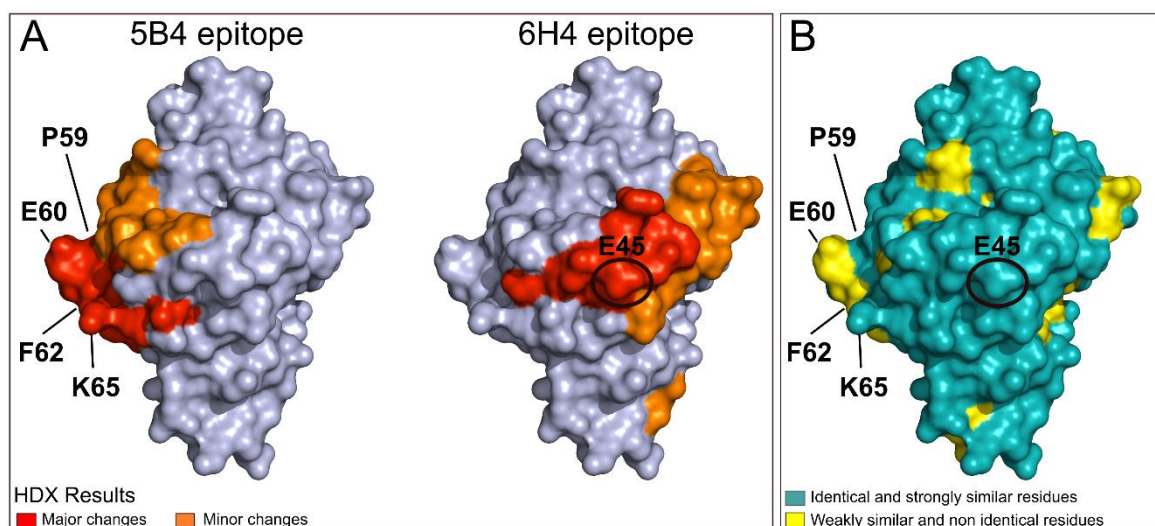


Figure 5

Characterization of epitope specificities of reference antibodies used for the quantification of the birch pollen allergen Bet v 1

Short title: Epitope specificities of Bet v 1-specific monoclonal antibodies

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Material and methods

Expression and purification of recombinant His-tagged Bet v 1 proteins (rBet v 1)

Recombinant Bet v 1.0101 (thereafter named rBet v 1) and mutants were expressed in *E. coli* BL21 (DE3) cells (Agilent) with a N-terminal 6-His tag. Briefly, cultures of transformed cells were grown at 37 °C to an OD₆₀₀ of 0.5-0.6 in LB medium supplemented with 25 µg/mL kanamycine and induced for 4 h with 1 mM isopropyl-β-D-thio-galactoside. Cells from 1 L cultures were resuspended in 20 mL lysis buffer [(20 mM Na₂HPO₄, 250 mM NaCl, 25 mM imidazole, pH 8.0, containing a cocktail of protease inhibitors (Complete EDTA free, Roche)] and disrupted by three rapid freezing/thawing cycles in liquid nitrogen followed by three 1-min sonication cycles. The solution was then heated for 10 min at 70°C (1), centrifuged for 10 min at 18,000 x g at 4°C and the supernatant was 0.45 µm-filtered.

Purifications were performed at room temperature (RT) on an AKTA purifier system (GE Healthcare) equipped with a fraction collector. Supernatants were loaded onto a 1 mL HisTrap Crude FF column (GE Healthcare) previously equilibrated in lysis buffer. Bound His-tagged Bet v 1 proteins were eluted with a step gradient of imidazole (250 mM). Column fractions (0.5 mL) containing the purified proteins were analyzed by SDS-PAGE, pooled and desalted by size exclusion chromatography (5 mL HiTrap desalting column, GE Healthcare). The presence and position of each substitution was confirmed by mass spectrometry (MS). Protein sample concentrations were determined by reverse phase chromatography and infra-red spectroscopy (Direct Detect spectrometer, Millipore). The effects of each substitution on the structural integrity of the protein were investigated by circular dichroism and local HDX-MS experiments using rBet v 1.0101 as reference.

Coding sequences for Bet v 1.0101 and a series of 11 alanine scanning variants and 2 naturally occurring variations (see table below) with N-terminal MKH₆Q histidine tag were synthesized *in vitro* and cloned into pET24 a(+) vector by LifeTechnologies.

Amino acid position	Mutation
43	Asn/Ala
45	Glu/Ala
47	Asn/Ala
50	Pro/Ala
51	Gly/Ala
59	Pro/Ala
60	Glu/Ala

62	Phe/Ala
62	Phe/Ile
63	Pro/Ala
64	Phe/Ala
65	Lys/Ala
60; 65	Glu/Gly ; Phe/Leu

Characterization of mAbs by Surface Plasmon Resonance

Surface plasmon resonance experiments were performed using a Biacore T200 instrument (GE Healthcare) with CMD500 (CM5 type) sensor chips (Xan Tec Bioanalytics) according to the manufacturer's instructions. Briefly, proteins were immobilized by the amine-coupling method, to reach 100, 370 and 400 Response Units (RU) of immobilized of Bet v 1, 6H4 and 5B4 antibodies, respectively. Blocking of the reference flow cell was performed by activation followed by immediate quenching, as for the active flow cell. A running buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl and Tween (0.05% v/v) was used in all experiments, using a flow rate of 10 to 30 μ L/min. Two types of experiments were achieved, including either Single Cycle Kinetics (SCK) with regeneration (glycine 10 mM, pH 2.5) or Multi Cycle Kinetics (MCK, steps of association: 200 s and dissociation: 400-1200s) without regeneration. For immobilized Bet v 1, MCK protocols were applied by injecting 6H4 or 5B4 mAbs at concentrations ranging from 0.073 to 37 nM. For immobilized mAbs (6H4 or 5B4), SCK protocols (steps of association: 200 s and dissociation: 400-1200s) were applied by injecting Bet v 1 at concentrations ranging from 0.0045 nM to 20 nM and 0.012 to 106 nM for mAbs 6H4 and 5B4, respectively. Sensorgrams were obtained by measuring the response in the active flow cell after subtracting the signal obtained in the reference cell.

Characterization of Bet v 1 proteins by circular dichroism (CD) spectroscopy

Recombinant Bet v 1 proteins were diluted to $\sim$ 300 μ g/mL in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.0. CD measurements were conducted on a Jasco J-815 CD spectrometer using square (21-Q-1/CD) 1-mm light path quartz cuvettes (Starna Scientific, Hainault, UK). CD spectra were recorded at 20°C from 200 to 260 nm collecting data with a bandwidth of 2 nm, an integration time of 1 sec and a scan rate of 100 nm/min (61 data points per spectrum). Each spectrum represents the arithmetic mean of six determinations corrected for buffer absorption.

SDS-PAGE and western blotting

Two µg of pollen extracts from birch (*Betulla verrucosa*), hazel (*Corylus avellana*), alder (*Alnus glutinosa*), hornbeam (*Carpinus betulus*) and chestnut tree (*Castanea sativa*) were resolved on a NuPAGE 4-12% Bis-Tris SDS-PAGE gel (Invitrogen) under reducing conditions. Proteins were subsequently transferred to nitrocellulose membrane by dry-blotting (iBlot system, Invitrogen) and the membrane was blocked overnight at room temperature (RT) with 1% milk in PBS buffer. After blocking, proteins were detected using mAbs diluted at 0.8 µg/mL in Duplecco's PBS + 0.1% Tween 20 (DPBS-T). Bound mAbs were detected with horse radish peroxidase-linked goat mAb anti-mouse IgG (Sigma-Aldrich) diluted 1/1000 in DPBS-T.

ELISA inhibition assays

Patients with birch pollen allergy were selected on the basis of both clinical history, positive *in vivo* skin prick test and *in vitro* IgE detection. Sera from 16 patients with IgE titers greater than class 3 were pooled and the final birch pollen-specific IgE level (66.9 kU/L) determined by ImmunoCAP. A pool of sera from 13 healthy individuals was included for control purposes.

Inhibition of patients' IgE binding. The ability of mAb 5B4, mAb 6H4, and rBet v 1 mutants to inhibit the binding of birch pollen allergic patients' IgE to rBet v 1 was investigated by ELISA inhibition. Briefly, ELISA plates (Costar) were coated with 0.25 µg rBet v 1 (100 µL per well; c = 2.5 ng/µL) in PBS buffer overnight at 4°C. The plates were washed three times with DPBS-T and blocked with 1% BSA in PBS buffer for 2 h 30 at RT. After blocking, plates were washed three times in DPBS-T. Diluted sera from birch pollen allergic patients (1/16) were mixed with either 4-fold dilution series of mAb or 2-fold dilution series of rBet v 1 mutants and transferred to the coated plates. All dilutions were performed in triplicate in buffer A [PBS, 0.1% BSA, 0.05% Brij L23 (30%, w/v)]. After 2 h incubation at RT, plates were washed three times in DPBS-T and bound IgE Abs were detected with HRP-linked goat monoclonal anti-human IgE (Kirkegaard & Perry Laboratories) diluted 1/2000 in buffer A. OD at 450 nm (reference filter at 620 nm) was determined with a Sunrise microplate reader (TECAN). Two negative controls were carried out in parallel with a pool of sera derived from 13 non-allergic patients and the anti-mite Der p 1-specific 5H8 IgG1 mAb (Indoor Biotechnologies).

Inhibition of 5B4 mAb binding by rBet v 1 mutants. ELISA plates were coated, blocked, washed and read as described above. A fixed concentration of mAb 5B4 (1.3 µg/mL) was mixed with a series of 2-fold dilutions of rBet v 1 mutants and transferred to coated plates. All dilutions were performed in triplicate in buffer A. After 1 h 30 incubation at RT, plates were washed three times in DPBS-T and

bound mAbs were detected with HRP-linked goat monoclonal anti-mouse IgG (Sigma-Aldrich) diluted 1/6000 in buffer A.

Label-free MS quantification

Proteins (2 µg) from pollen extracts and flow through samples were diluted at 25 ng/µL in 75 µL of 50 mM ammonium bicarbonate, pH 8 supplemented with 0.025% ProteaseMAX (Promega, Charbonnières-les-Bains, France). Disulfide bridges were reduced with 2 µL of 100 mM dithiothreitol (DTT) for 30 min at 56°C. Subsequently, 1 µL of 500 mM iodoacetamide (IAM) was added and alkylation was performed at room temperature for 30 min in the dark. The excess of IAM was quenched by adding 3 µL of 100 mM DTT. Proteins were then digested by adding 25 µL of a trypsin solution at 4 ng/µL. Samples were incubated for 3 h at 37°C. The enzymatic reaction was stopped with 1 µL of 100% formic acid (FA). Lastly, samples were centrifuged for 15 min at 14,000 g, and the supernatants were transferred into new LC-MS vials (Waters, Guyancourt, France) after a 3-fold dilution with 5% acetonitrile (ACN) / 0.1 % FA.

LC-MS/MS analyzes were performed using an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific) in-line connected to an Impact HD mass spectrometer (Bruker, Wisssembourg, France) equipped with a CaptiveSpray source. Fifteen ng of digests were loaded onto a Acclaim PepMap 100 C18 trap column (Thermo Fisher Scientific, Villebon-sur-Yvette, France) equilibrated at 50°C with 0.1% trifluoroacetic acid (TFA) and desalted for 10 min with a flow rate of 15 µL/min. Peptides were eluted and separated on an Acclaim PepMap RSLC C₁₈ column (75 µm x 25 cm, 2 µm particle size) with a linear gradient from 99% solvent A (0.1% FA) and 1% solvent B (0.1% FA in 80% acetonitrile) to 35% solvent B over 30 min at a flow rate of 450 nL/min.

The capillary voltage and the dry gas temperature of the mass spectrometer were set to 1400 V and 150°C, respectively. MS spectra were acquired in positive mode over the *m/z* range 50–2000 with a scan rate of 2 Hz. MS/MS spectra were acquired using the Intensity Dependent Acquisition Speed (IDAS) mode with a MS-MS/MS cycle time fixed at 2 s. Singly charged ions were excluded from triggering MS/MS events. The calibration was performed using an internal lockmass calibration at 1221.9906 amu.

Label-free MS quantification was performed using Progenesis QI software (Waters). MS data were loaded as centroid data with a resolution power instrument and a filter strength set at 30000 and 1.2, respectively. LC-MS/MS analyzes of pollen extract samples were chosen as a reference run for the alignment step. Sensitivity parameters for MS detection were set as default value. Only peptides eluted between 15 and 49 min with a peak width higher than 0.1 min were considered. The normalization

step was based on peptides issued from proteins not impacted by the immunochromatography (Bet v 2, Bet v 4, Bet v 6, copper/zinc-superoxide dismutase, glutathione S-transferase and peptidyl-propyl cis-trans isomerase). Only peptides with abundances $> 2 \times 10^5$ were used for the normalization factor calculation.

Tandem mass spectra were processed by Progenesis QI and exported to PEAKS 7 software (Bioinformatics Solutions Inc., Waterloo, Canada) for protein/peptide identification. Searches were performed against the *Betula pendula* protein database available at Uniprot.org supplemented with all Bet v 1 allergen sequences referenced by the International Union of Immunological Societies (IUIS). Peptide identification was performed with the following parameters: peptide tolerance of 10 ppm (0.05 Da for fragment ions), non-specific cleavage not allowed, two missed cleavages permitted, carbamidomethylation of cysteine as a fixed modification, variable modifications (deamidation of glutamine and asparagine, oxidation of methionine), allowing a maximum of 5 variable post-translational modifications. Peptide identifications were confirmed with the application of a 0.1% peptide-spectrum match false discovery rate (FDR, estimated with the decoy fusion method)(2).

Epitope mapping by HDX-MS: MALDI ToF-ToF analysis and HDX-MS data processing

Immobilization of the 5B4 and 6H4 mAbs. ~200 μ L of protein G beads were prepared following the supplier's instructions and incubated with 1.5 mg of mAbs in PBS 1X buffer at 4°C overnight while mixing. After extensive washing with PBS buffer, 4.1 mM DSS was added to covalently attached antibodies to protein G beads. The cross-linking reaction was carried out at RT using a final dimethyl sulfoxide concentration of ~ 8.0%. After 2 h 30 incubation, the reaction was quenched with 2 mL of Tris-HCl 1M (pH 7.4) for 30 min at RT. Beads were extensively washed with PBS and then with a solution of 100 mM Tris-HCl, 300 mM NaCl, pH 2.0, to eliminate non-covalently bound antibodies. Lastly, antibody beads were transferred into empty Micro Bio-Spin chromatography columns, equilibrated with an excess of 5 mM NaP buffer, 120 mM NaCl, pH 8.0, and stored in ~200 μ L aliquots at 4°C until use.

Recombinant Bet v 1 binding to antibody beads and to protein G alone (unspecific binding control) were assessed by pull-down assays and SDS-PAGE analysis. These experiments revealed that rBet v 1 does not bind nonspecifically to protein G alone, even under conditions in which the allergen was in large excess (data not shown).

Sample preparation for epitope mapping. The mAb/rBet v 1 complexes were formed by adding 3.2 nmoles of rBet v 1 to ~200 μ L immobilized mAb beads equilibrated with 5 mM NaP buffer, 120 mM

NaCl, pH 8.0. After 20 min incubation at RT, beads were washed with an excess of 5 mM NaP buffer, 120 mM NaCl, pH 8.0 to remove unbound allergens. All labeling reactions were performed at 4°C unless specified. The labeling was initiated by adding 600 µL of deuterated buffer (5 mM NaP, pH 8.0). At predetermined time points (ranging from 2 min to 1 h), aliquots of beads (55 µL for 5B4; 15 µL for 6H4) were removed and quenched with an equal volume of ice-cold acidic quench buffer (200 mM NaP, pH 2.4) to dissociate the allergen-antibody complex and to lower the pH to 2.4. The quenched aliquots were immediately centrifuged for 20 sec to remove the immobilized antibody and the supernatant containing the free labeled allergen was flash frozen in ethanol/dry ice and stored at -80°C for less than 24 h.

A control experiment without antibody was performed by diluting 860 pmoles of rBet v 1 with 10-fold deuterated NaP buffer (pH 8.0) at 4°C. For each deuteration point, 10 µL of labeled rBet v 1 were removed and quenched with 10 µL of ice-cold acidic quench buffer. Samples were immediately flash frozen in ethanol/dry ice and stored at -80°C for less than 24 h. A fully deuterated control was carried out as described above and incubated for 3 h at 70°C followed by 12 h at 37°C.

HDX-MS data processing. Mass spectra were acquired on a MALDI ToF-ToF Autoflex Speed mass spectrometer (Bruker Daltonics) equipped with the SmartbeamTM-II laser and LIFTTM technologies. Spectra were obtained in reflectron mode by accumulating an average of 3,000 shots at a 1 GHz repetition rate. The calibration was performed in positive mode before each acquisition using the peptide calibration standard II mixture (Bruker Daltonics).

Frozen samples were defrosted quickly and immediately subjected to enzymatic digestion with either 20 µL of immobilized pepsin or type XIII protease from *Aspergillus sato*i (protein/enzyme ratio: 1/10, w/w). After 5 min incubation on ice, digested samples were loaded onto a C₁₈ ZipTip (Millipore) and desalted with ice-cold 0.1% TFA. Deuterated peptides were directly eluted from the ZipTip on the MALDI plate using 2 µL of ice-cold alpha-cyano-4-hydroxycinnamic acid matrix at 10 mg/mL in 50% acetonitrile, 25% ethanol, 0.1% TFA, and spots were dried under nitrogen flow. After drying, the MALDI plate was transferred as quickly as possible to the mass spectrometer. The time from sample spotting to data collection (~ 3 min) was kept constant for each analysis. Sequences of each peptide generated after pepsin or type XIII digestion were confirmed using the LIFTTM technology (3).

MALDI raw data were processed with FlexAnalysis 3.3 and Biotoools 3.2 software (Bruker Daltonics). The excel-based program HX-Express was used to extract the centroid mass of each peptic peptide as a function of the labeling time (4). The deuterium level (D) of each peptide was corrected for back-exchange using the method described by Zhang and Smith (5)

$$D = \frac{(m_t - m_{0\%})}{(m_{100\%} - m_{0\%})} \times N$$

200 where m_t is the centroid mass value of the peptide at a specified exchange time t , and $m_{0\%}$ and $m_{100\%}$
201 correspond to the centroid mass of the undeuterated and the fully deuterated control, respectively. N
202 represents the number of exchangeable backbone amide hydrogens and is calculated by subtracting the
203 first two N-terminal residues (assuming they cannot retain deuterons (6)) and the number of proline
204 residues to the total number of amide hydrogens present in the peptide.

205

Supplementary Figures

Table S1 Analysis of the interaction between rBet v 1 and either 5B4 or 6H4 mAbs by surface plasmon resonance measurements. K_d , equilibrium dissociation constant; k_{on} , association rate constant; k_{off} , dissociation rate constant

Antibody	K_d , (M) $\times 10^{-9}$	k_{on} , ($M^{-1}.s^{-1}$) $\times 10^5$	k_{off} , (s^{-1})
mAb 5B4	32.0	3.2	0.01
mAb 6H4	0.3	5.0	1.1×10^{-4}

Table S2 Effect of each amino acid substitution on the structural integrity of rBet v 1 probed by local HDX-MS analysis. Proteins were labeled for 30 sec on ice and the deuterium content measured by mass spectrometry. Peptides showing obvious altered incorporation values (i.e., > 1.0 Da) compared to Bet v 1 WT are highlighted.

Proteins	Incorporated deuterium after 30 sec labeling at 4°C														
	Peptic peptides														
	Nter-3	4-18	10-18	11-18	22-42	43-69	59-79	66-79	70-79	80-95	102-114	115-143	130-141	145-151	152-156
WT	1.6	2.3	0.5	0.4	1.3	4.0	4.3 (a)	/	1.7	1.9	1.4	6.0	1.7	0.1	1.2
N43A	1.5	2.6	0.5	0.5	/	4.0	/	/	1.7	2.1	1.6	6.0	1.6	0.3	0.9
E45A	1.4	2.7	0.5	0.5	1.1	4.6	/	/	1.6	2.3	1.5	5.6	/	0.4	1.2
N47A	1.6	3.0	0.7	0.5	1.1	5.0	/	/	1.7	2.8	1.7	6.2	1.4	0.5	1.4
P50A	1.6	3.0	0.8	0.6	1.2	4.9	/	/	1.9	/	1.6	6.6	/	0.4	1.5
G51A	1.4	5.3	2.4	1.8	0.1	9.9	/	/	2.5	/	3.5	9.7	5.8	2.3	0.9
P59A	1.6	2.2	0.4	0.3	1.1	/	3.0	/	1.5	1.6	1.3	6.0	/	0.1	1.6
E60A	1.8	2.6	0.5	0.4	1.0	3.8	/	/	1.7	1.6	1.3	6.0	1.5	0.1	1.1
F62A	1.0	1.8	0.3	0.3	0.5	/	/	/	1.0	0.8	0.9	3.9	1.2	0.0	0.6
F62I	1.6	2.5	0.5	0.5	1.1	4.0	/	/	1.6	2.3	1.5	5.4	1.5	0.4	1.2
P63A	1.6	2.5	0.5	0.5	1.1	/	/	/	1.6	/	1.3	5.6	1.7	0.2	1.1
F64A	1.7	4.0	1.5	1.2	2.0	7.1	/	/	2.1	/	2.5	7.8	2.7	1.0	1.1
K65A	1.7	2.2	0.5	0.4	0.9	/	/	1.5	1.6	/	1.2	5.4	1.2	0.2	1.1
E60G-F62L	1.7	2.9	0.5	0.5	1.2	4.4	/	/	1.7	1.2	1.5	5.7	1.6	0.2	1.0

- (a) The MS signal recovered after 30 sec labeling is of poor quality and does not allow an accurate measurement of the deuterium content by HX Express. This peptide was not considered for this analysis.

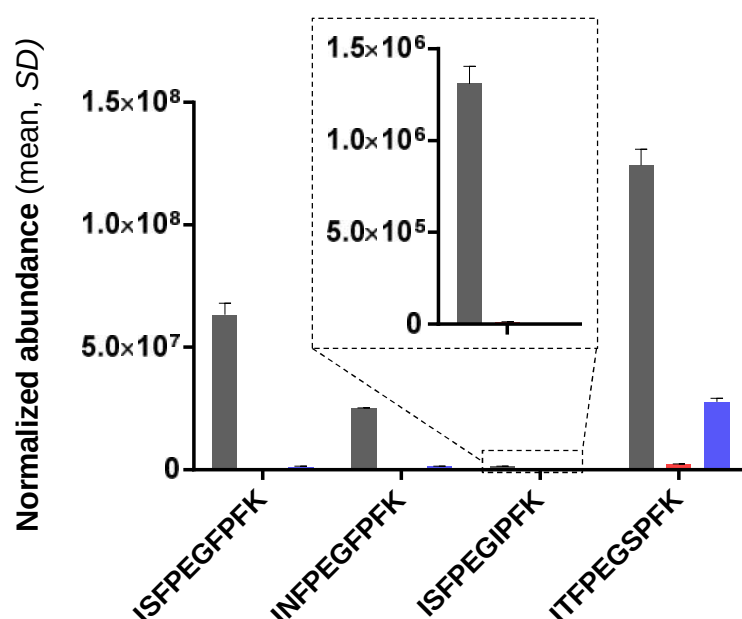


Figure S1 Bet v 1 immunocapture by 5B4 and 6H4 monoclonal antibodies. Birch pollen extracts (back bars) were subjected to chromatography with immobilized 5B4 (red bars) or 6H4 (blue bars) and resulting products were analyzed by MS. Relative abundances of peptides 56-65 from different isoallergens were measured by MS following trypsin digestion in pollen extract and 5B4/6H4 flow-through fractions. Each value corresponds to the mean and standard deviation calculated from two label-free MS analyzes of a unique tryptic digest. Peptide ISFPEGFPFK encompasses Bet v 1.0101, 0104, 0106, 0108, 0109, 0110, 0113, 0114, 0115, 0117 and 0118; peptide INFPEGFPFK encompasses Bet v 1. 0102, 0105, 0107, 0119; ISFPEGIPFK encompasses Bet v 1.0112, 0103 and 0116; peptide ITFPEGSPFK encompasses Bet v 1.0201, 0202, 0203, 0204, 0205, 0206 and 0207. Representative of 3 different batches of birch pollen tested.

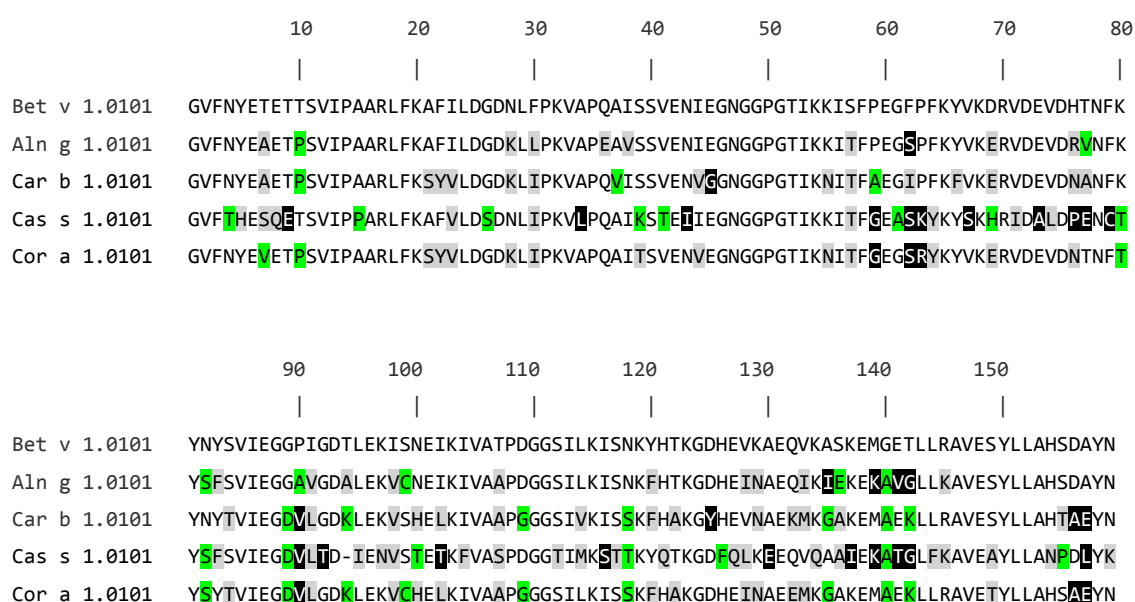


Figure S2 Sequence alignment of Aln g 1.0101, Car b 1.0101, Cas s 1.0101 and Cor a 1.0101 against Bet v 1.0101. For comparison purposes, strongly similar, weakly similar and non-conserved residues are highlighted in grey, green and black, respectively.

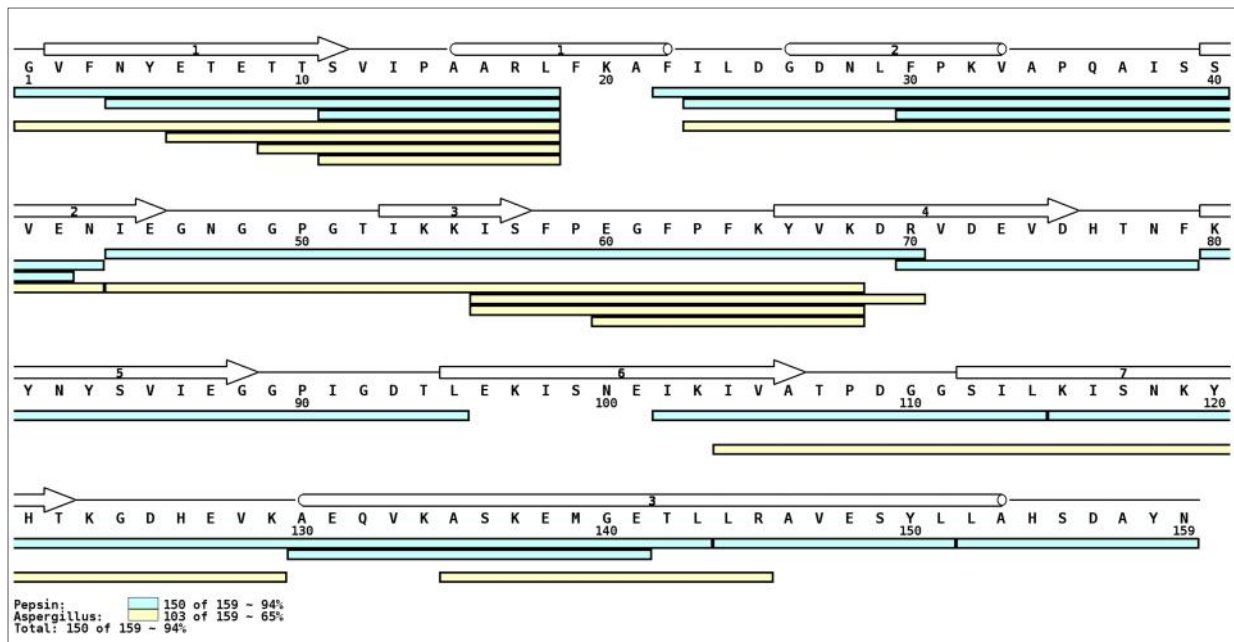


Figure S3 Protein sequence of rBet v 1 showing the pepsin- (blue) and type XIII- (yellow) cleaved fragments used for HDX-MS analysis (final sequence coverage of 94%). The use of type XIII does not increase the final sequence coverage but allows higher sequence resolution in region 40-70. Figure prepared with MS-tool (7).

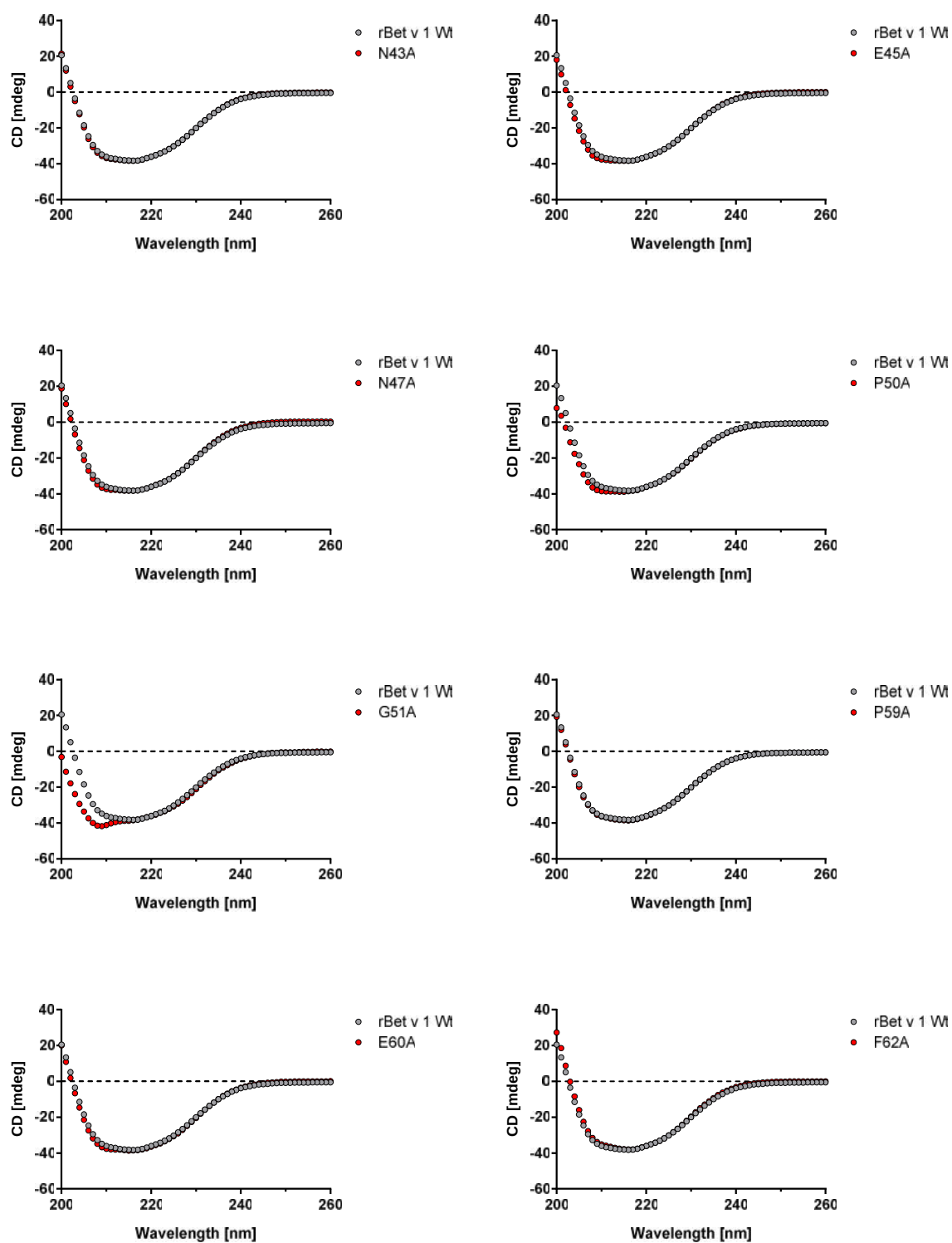
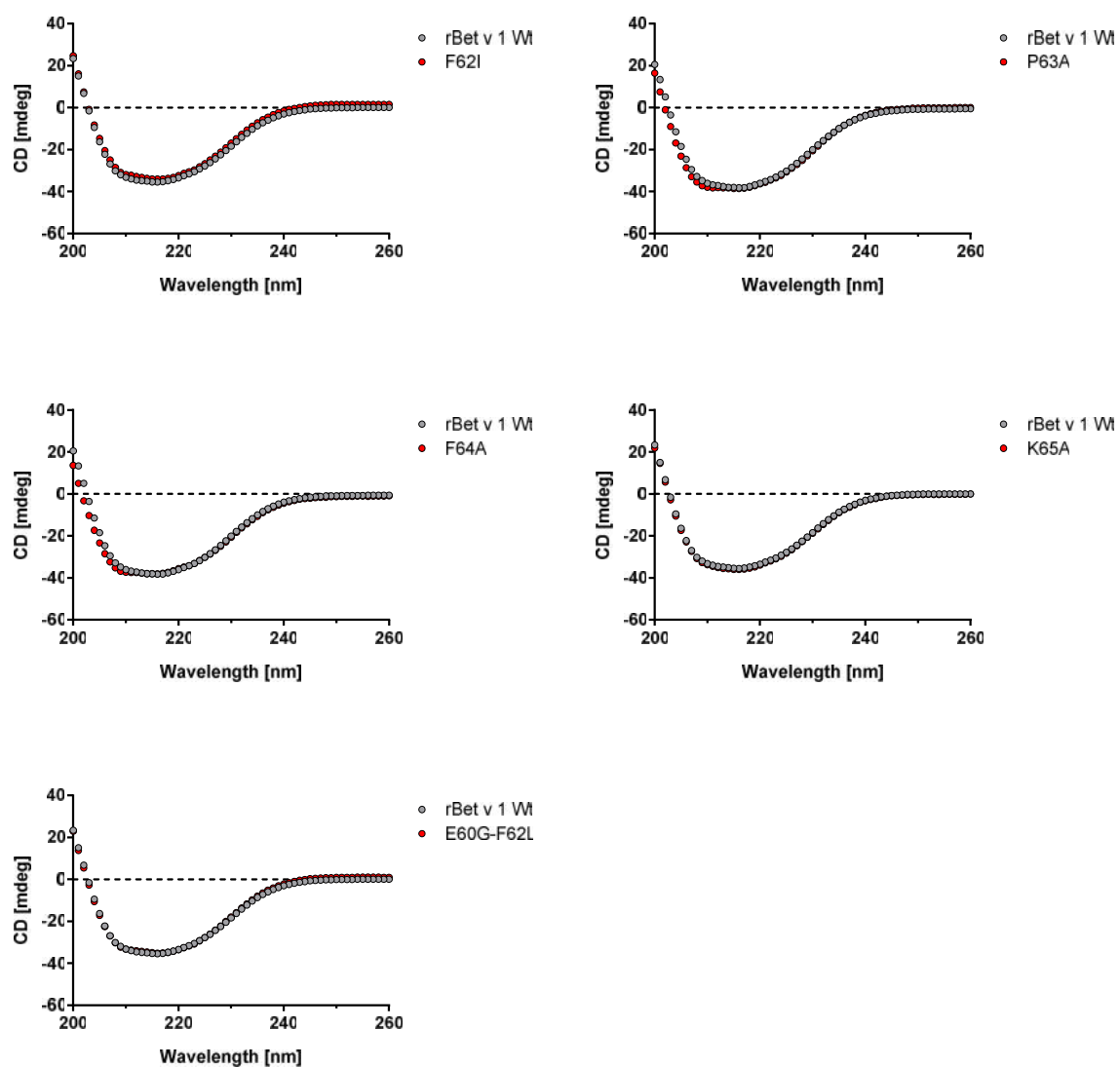


Figure S4. CD spectra of all rBet v 1 proteins used in this study. Wild type and mutated proteins were prepared in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.0 to a final concentration of ~ 0.3 mg/mL. Spectra were acquired at 20°C on a Jasco J-815 Cd Spectrometer between 260 and 200 nm.

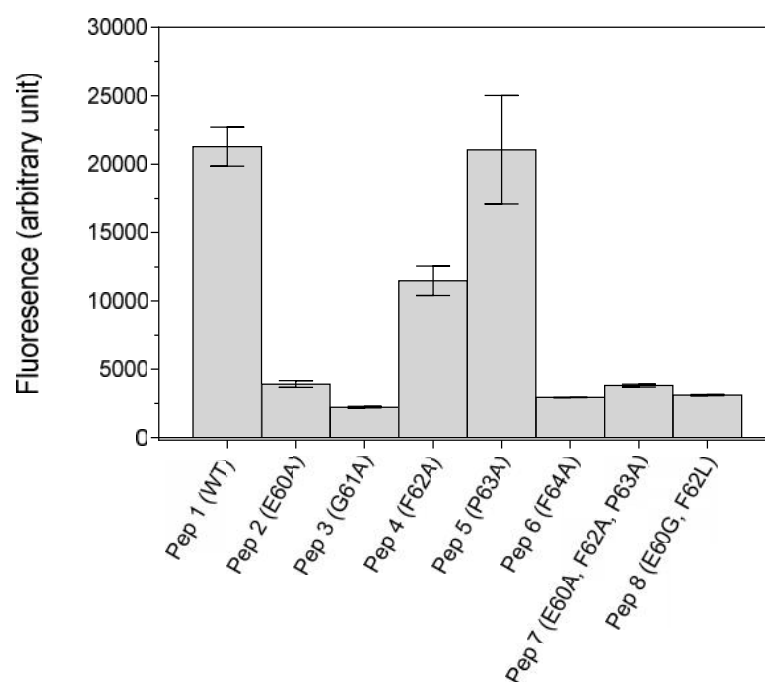
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267 Figure S4 (continued)

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270 **Figure S5** Identification by pepscan analysis of critical residues for 5B4 binding. Each peptide encompasses
 271 residues P50-D69 of the Bet v 1.0101 allergen. The single alanine substitution of residues E60, G61 and F64 is
 272 sufficient to completely abolish the binding of 5B4. Peptide 4 carrying the F62A modification retains some
 273 capacity to interact with the mAb. No effect is observed with Pep 5 (P63A).

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