

Structural Bases for the Allergenicity of Fra a 1.02 in Strawberry Fruits

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Supporting Information

ABSTRACT: Although strawberries are highly appreciated fruits, their intake can induce allergic reactions in atopic patients. These reactions can be due to the patient's previous sensitization to the major birch pollen allergen Bet v 1, by which IgE generated in response to Bet v 1 cross-reacts with the structurally related strawberry Fra a 1 protein family. Fra a 1.02 is the most expressed paralog in ripe strawberries and is highly allergenic. To better understand the molecular mechanisms regulating this allergic response, we have determined the three-dimensional structure of Fra a 1.02 and four site-directed mutants that were designed based on their positions in potential epitopes. Fra a 1.02 and mutants conform to the START fold. We show that the cross-reactivity of all the mutant variants to IgE from patients allergic to Bet v 1 was significantly reduced without altering the conserved structural fold, so that they could potentially be used as hypoallergenic Fra a 1 variants for the generation of vaccines against strawberry allergy in atopic patients.

KEYWORDS: *Fra a 1*, *Fragaria x ananassa*, allergen, PR-10, IgE binding, 3D-structure, hypoallergenic mutants

INTRODUCTION

Strawberries are one of the most economically important fruit crops and are highly appreciated worldwide due to their delicate taste and aroma, as well as for their beneficial effects on human health. However, the consumption of strawberries can elicit allergic responses in atopic patients as happens with other members of the *Rosaceae* family, e.g., apples, cherries, or peaches.^{1,2} The cause of this allergy lies in the presence in the strawberry fruit of three different families of allergens, namely, Fra a 1, Fra a 3, and Fra a 4 (www.allergens.org). Fra a 1 proteins belong to the pathogenesis-related PR-10 family and are structural homologues to Bet v 1, the major birch pollen allergen.³ The Fra a 3 family consists of nonspecific lipid transfer proteins (ns-LTPs), and the Fra a 4 family are profilins.⁴ Allergy to Fra a 1 is the main cause of the adverse reactions to strawberry in Central and Northern Europe,⁵ while allergies to Fra a 3 and 4 are the prevalent ones in the Mediterranean area.⁴

Allergy to Fra a 1 is a type I birch pollen-related food allergy. This allergy to the strawberry Fra a 1, and other related fruit proteins, is caused by a previous sensitization to the major birch pollen allergen Bet v 1. The IgEs generated against Bet v 1 in birch pollen allergic patients cross-react with structural homologue members of the PR-10 family that are present in

fruits.⁶ This cross-reactivity is the reason why more than 70% of the patients with birch pollen allergies in Central and Northern Europe, and in North America, develop allergies to fruits, nuts, vegetables, and legumes;^{7,8} and about the 15–30% of them display allergic reactions after the intake of fresh strawberry fruits.^{3,9} The symptoms are generally mild and in the form of oral allergy syndromes (OAS) coursing with itching and swelling, although, in rare occasions, systemic urticaria or even anaphylaxis has been reported.¹⁰ As pollen-food allergies are mediated by IgE, and the IgE epitopes are predominantly conformational, the allergenic proteins need to display an intact tertiary fold to be recognized.^{11,12}

Allergenic proteins in foods are often identified as a mixture of closely related isoforms.¹³ In the diploid strawberry *Fragaria vesca*, 21 different paralogs of Fra a 1 have been identified in the sequence databases,^{14,15} and up to 39 have been identified in the octoploid, cultivated strawberry (*Fragaria x ananassa*).⁹ These paralogs display differential patterns of expression depending on the tissue, developmental stage, genotype, or

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fruit-processing method.^{5,9,16–18} So, focusing on the fruit, Fra a 1.01 is the most expressed isoform at the green stage, while Fra a 1.02 presents a ripening-induced pattern of expression being the most abundant isoform in the ripe receptacle. The allergenic potential of different Fra a 1 isoforms has also been studied. The analysis of the binding properties of different recombinant Fra a 1 proteins to IgE from birch pollen-allergic patients showed that Fra a 1.01 displays the highest binding capacity, followed by Fra a 1.02,⁹ while in basophil-activation assays, Fra a 1.02 was identified as the most potent allergen in strawberry fruit.⁵ It has been shown that Fra a 1.01, 1.02, and 1.03 bind natural flavonoids.¹⁹ Despite this, the biological function of these proteins in the plants is not yet known.

The main preventative strategy to cope with a fruit allergy is avoidance of that particular fruit, which could eventually lead to vitamin and nutrient deficiencies in the patient. So, the development of antiallergic strategies could improve the quality of life of the patients. Specific immunotherapy (SIT) has been developed against different allergens, and it is based on the injection of increasing amounts of the allergen in order to achieve tolerance.²⁰ However, this treatment also exposes patients to the risk of anaphylactic shock, so the use of hypoallergenic isoforms of the allergens has been tested as a strategy to overcome this detrimental effect.²⁰ In order to be valid for SIT, the hypoallergenic isoforms should present a lower IgE-binding capacity than the allergenic ones but keep a good T-cell antigenicity, thereby offering the possibility of a safer approach to treat immediate-type allergies lowering the risk of anaphylactic shock. Knowledge of the three-dimensional structure of allergens as well as mapping of IgE-binding sites has contributed significantly to the identification of hypoallergenic isoforms of the allergens. The PR-10 family, to which Bet v 1 and other related allergens such as Fra a 1, Pru av 1, and Mal d 1 belong, is very well characterized at the structural level.⁷ In addition, the three-dimensional structure of Bet v 1 in a 1:1 complex with a murine monoclonal IgG, BV16, has been reported (PDB code: 1FSK).¹² As the binding of BV16 to Bet v 1 inhibits its recognition by human IgE, the epitopes found in this study serve as a model for the human IgE-binding ones. This information has allowed the generation of hypoallergenic Bet v 1 isoforms by site-directed mutagenesis²¹ as well as for other food allergens, e.g., Mal d 1 in apples, Pru av 1 in cherries, Api g 1 in celery, or Cor a 1 in hazelnuts.^{22–24}

We have previously reported the crystallographic structures of Fra a 1.01E and Fra a 1.03–catechin complex.^{19,25} Herein, we present the crystal structure of Fra a 1.02 at 2.04 Å resolution. Based on this structural data, we have generated a series of mutant variants that conserve the PR-10 fold but display a lower IgE-binding activity. The results that we have obtained could pave the way for the generation of new vaccines that might be employed in patients with allergies to strawberries.

MATERIALS AND METHODS

Site-Directed Mutagenesis of Fra a 1.02. *Fra a 1.02* coding sequence (GQ148818.1) was cloned into the pETM11 expression vector²⁶ to obtain the construct *F2-pETM11* that included an N-terminal 6x His tag, followed by a tobacco etch virus (TEV) protease cleavage sequence. The *Fra a 1.02* variants were generated by site-directed mutagenesis using *Fra a 1.02* as a template, three of them carrying single mutations at the positions 46 (E46R), 48 (D48R), and 64 (Q64W) and one carrying a double mutation at positions 46 and 48 (E46A/D48A). Such mutations were introduced by overlapping

PCR²⁷ using internal and external primers that included the *NotI* and *EcoRI* restriction sites. The primers used are listed in Table S1. The PCR product was digested by *NotI* and *EcoRI* and cloned into *pETM11* to generate *pETM11 Fra a 1.02 E46R*, *pETM11 Fra a 1.02 D48R*, *pETM11 Fra a 1.02 E46/D48R*, and *pETM11 Fra a 1.02 Q64W*. The constructs were confirmed by sequencing and transformed into *E. coli* One Shot BL21(DE3) competent cells (ThermoFisher Scientific) following standard heat-shock transformation procedures.

Protein Expression and Purification. Protein expression and purification were performed as previously described¹⁹ with some variations. Briefly, *E. coli* BL21 (DE3) cells were transformed with the different *Fra a 1.02* constructs and grown in 1 L LB supplemented with 50 µg/mL of kanamycin at 37 °C to an OD of 0.6–0.8. At this point, incubation temperature was lowered to 20 °C, and after 30 min 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce overnight protein expression. The cells were harvested by centrifugation at 4000 rpm for 20 min at room temperature and kept at –80 °C until protein purification.

The harvested pellets were resuspended at 4 °C in 200 mL of lysis buffer (100 mM Tris pH 7.5, 300 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol, DNaseI (Roche)) and one tablet of protease inhibitor (Roche)) and lysed by sonication (Misonix sonicator S4000). The lysate was cleared by centrifugation at 20 000 rpm at 4 °C for 1 h.

The supernatant was loaded onto a 5 mL HisTrap HP column (Amersham Biosciences) pre-equilibrated with lysis buffer and connected to an ÄKTA Prime Plus purifier (GE Healthcare). The column was washed with lysis and washing (100 mM Tris pH 7.5, 300 mM NaCl, 30 mM imidazole, 1 mM β-mercaptoethanol) buffers. Recovery of the *Fra* proteins was performed by an imidazole gradient (30–250 mM) used with elution (100 mM Tris pH 7.5, 300 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol) and washing buffers. The purified protein was cleaved overnight to remove the His tag with 1 mg of TEV protease in a dialysis cassette (Slide-A-Lyzer 3.5 K MWCO, 30 mL, ThermoFisher) submerged in 5 L of dialysis buffer (100 mM Tris pH 7.5, 300 mM NaCl, 15 mM imidazole, 1 mM β-mercaptoethanol) at 4 °C. The cleaved samples were loaded onto 5 mL HisTrap HP columns to remove uncleaved proteins, TEV protease, and other contaminants. An Amicon ultracentrifuge-15 (10 kDa cutoff) was later used to concentrate the eluted fraction to 40 mg/mL of protein. Size exclusion chromatography was performed loading the protein solution onto a Superdex75 column (GE Healthcare) previously equilibrated in gel filtration buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM β-mercaptoethanol). The protein was concentrated again to 40 mg/mL, frozen in liquid nitrogen, and kept at –80 °C. All expression and purification steps, as well as the correct size of the recombinant *Fra a 1.02* proteins, were monitored by SDS-PAGE.

Crystallization, X-ray Data Collection, and Structure Refinement. All crystallization and crystal processing experiments were performed as previously described²⁸ and done at the High Throughput Crystallization Laboratory of the EMBL Grenoble Outstation (<https://htxlab.embl.fr/>) with the assistance of the Crystallization Information Management System (CRIMS, www.crim-project.eu). Initial crystallization-screening experiments were carried out using the sitting-drop vapor-diffusion method at 20 °C with commercially available screens (Crystal screen I and II (Qiagen), Index screen, Crystal screen light, Crystal Screen PEG-Ion, Crystal screen Ammonium Sulfate, Screen Malonate, QuickScreen, Peg 6K screen, MPD screen, Peg-LiCl screen (Hampton), sodium formate and PEG MME 5000 screens (HTX), Wizard I + II (Emerald)). Droplets (200 nL) with a 1:1 protein:precipitant ratio were set up in the CrystalDirect plates^{29,30} containing 45 µL of reservoir solution and using a Cartesian PixSys 4200 robot (Cartesian Technologies). Microseeding with crystals from A141F (PDB code: 5AMW)³⁰ was necessary for crystal growth of both *Fra a 1.02* wild-type as well as the rest of the mutants. A141F microcrystal seeds were prepared by collecting the crystals and breaking them first manually using a glass hyssop and then with Seed Bead kits (Hampton Research). The

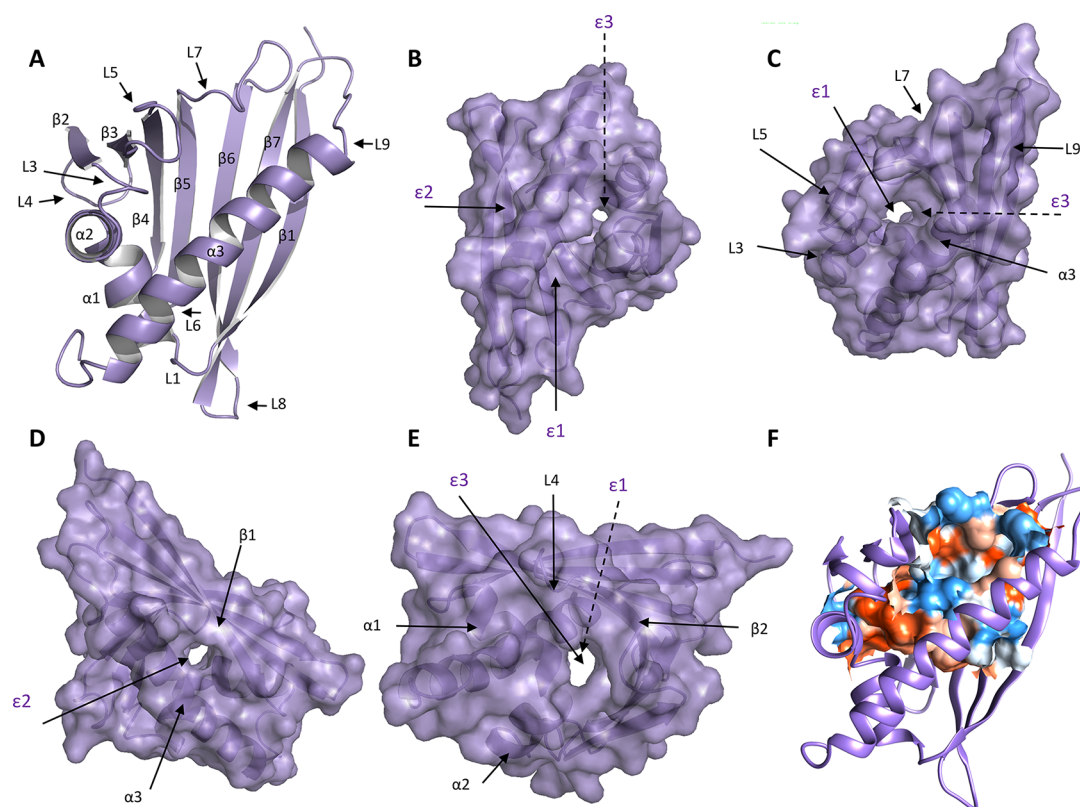


Figure 1. Three-dimensional structure of Fra a 1.02. (A) Shown in ribbon representation. (B, C, D, and E) Surface representation from different perspectives to show the three entrances to the cavity ($\epsilon 1$, $\epsilon 2$, and $\epsilon 3$). Black arrows indicate the entrances to the cavity. Dashed lines point to the entrances to the cavity which are in the posterior side of the representation. (F) Ribbon representation highlighting the cavity. The hydrophobic residues in the cavity are colored in white, the negatively charged in red, and the positively charged in blue.

crystallization assays were carried out with 100 nL of protein sample, 70 nL of the reservoir solution, and 30 nL of the A141F seeds in commercial (Index, SaltRX (Hampton) Wizard I + II (Rigaku), JCSG (Molecular dimension)), and refinement plates using a Mosquito-LCP robot (TTP Labtech). Plates were stored at 20 °C and monitored with a Rock Imager 1000 system (Formulatrix). The crystals were harvested and cryocooled automatically using the CrystalDirect technology.^{30,31}

X-ray diffraction experiments were carried out at the European Synchrotron Radiation Facility (ESRF) ID29, ID30a-3, and ID30a-1 beamlines and processed, scaled, and merged with autoPROC³² using anisotropy correction.³³ Initial phases were obtained by molecular replacement using PHASER³⁴ within the Collaborative Computational Project Number 4 (CCP4)³⁵ and Fra a 1.01E (PDB code: 9C9I).¹⁹ Refmac³⁶ and BUSTER³⁷ were employed for the refinement, COOT³⁸ for manual model building, and Molprobity³⁹ for the validation of the structures.

Induced Fit Docking. Molecular docking of myricetin into the cavity of Fra a 1.02 was performed using Induced Fit Docking (IFD) method in the Schrödinger software suite.⁴⁰ The input model was the crystal structure of the wild-type Fra a 1.02 protein prepared using Protein Preparation Wizard. All crystallographic waters of the model were removed due to lack of data that could classify them as conserved waters. The possible binding cavity for myricetin was defined based on the crystal structure of Fra a 3 complexed with catechin (PDB code: 4C94),¹⁹ a compound structurally related to myricetin. Twenty conformational poses were calculated, and the most favorable one based on the IFD score was visualized.

Serum Samples and Determination of the Immunological Activity of Fra a 1.02 Wild-Type and Mutant Variants. To determine the potential allergenicity of the samples, an ELISA-type assay was performed using a pool sera of 20 birch pollen-allergic patients from Milan (Italy). Skin prick tests (SPT) to a battery of allergens, vegetables, fruits, and nuts were performed on the 20

patients, all of them being positive to Bet v 1 (Supporting Information Table 2). Sera collection and the study were performed with the approval of the local ethics committees and with consent of the patients.

The ELISA plates were coated with 50 μ L of Fra a 1.02 wild-type and mutant variants at different concentrations (0, 2.5, 5, and 10 μ g/mL) in PBS for 2 h at 37 °C. Afterward, the plates were washed four times with PBST (PBS + Tween 0.05%) and blocked for 1 h at room temperature with 200 μ L of casein (Sigma-Aldrich). The pool of sera was incubated overnight at 4 °C, and the plates were washed four times with PBST. To detect binding of human IgE antibodies, the plates were incubated with anti IgE-HRP (ThermoFisher) for 1 h at room temperature and washed four times (PBS Tween 0.04%). The HRP activity was measured using the TMB substrate kit (Pierce), and the absorbance was measured at 405 nm. All the samples were tested three times.

Alignments and Statistical Analysis. Sequence alignment was performed using Clustal Omega (EMBL-EBI), and the figure was generated by ESPrpt 3.0.⁴¹ Representations of the three-dimensional structures were generated with PyMOL.⁴² Surface and volume of the cavity of Fra a 1.02 were determined using ProteinsPlus (<http://proteins.plus/>). Color code for polarity and hydrophobicity of the cavity was performed using UCSF Chimera.⁴³ Statistical analysis and graphs were performed with the Prism 6 software (<https://www.graphpad.com/scientific-software/prism/>).

RESULTS AND DISCUSSION

Crystallization and Structural Characterization of Fra a 1.02. Since Fra a 1.02 is the most expressed allergen in the ripe strawberry fruit and has also been identified as highly allergenic,^{5,9,16} it is considered as the main responsible allergen causing type I pollen-related allergies to strawberry. The

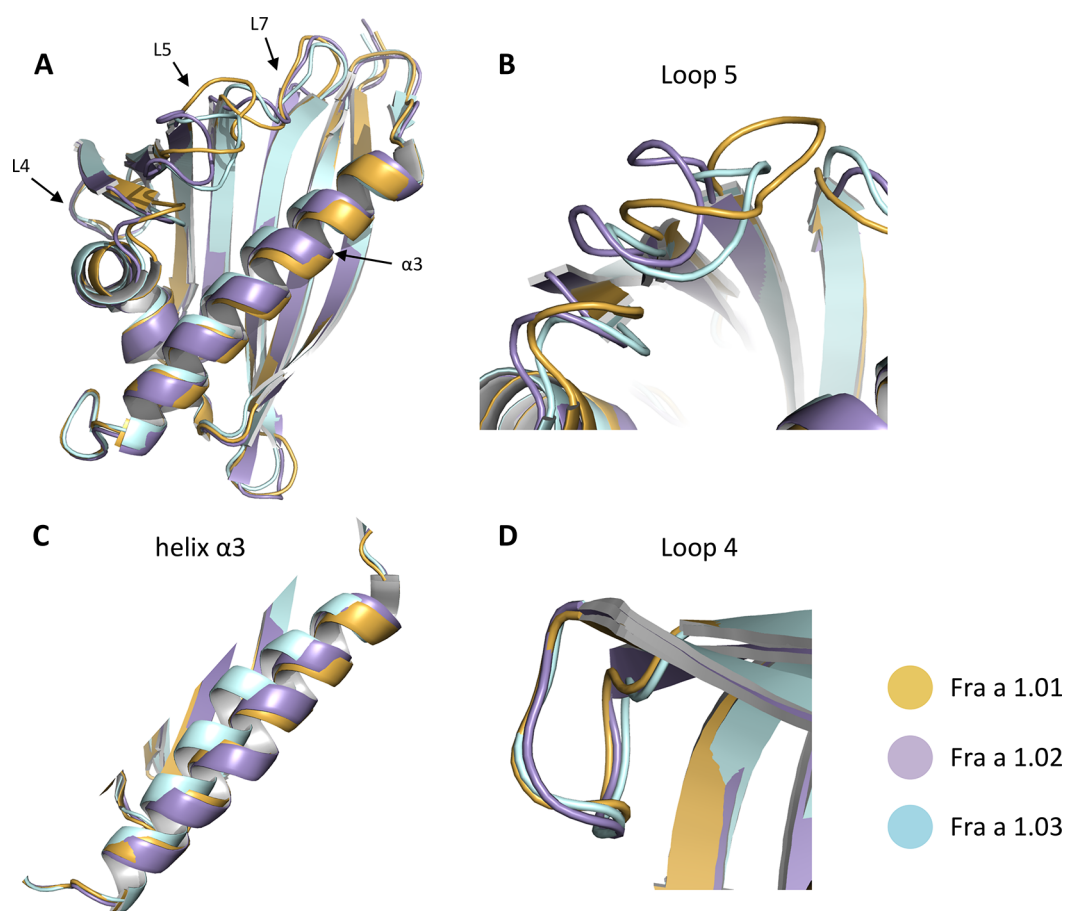


Figure 2. Structural superposition of Fra a 1.01E, Fra a 1.02, and Fra a 1.03. (A) Structures of Fra a 1.01E, Fra a 1.02, and Fra a 1.03 are shown in ribbon representation. Details of the structural superposition are shown in (B) loop 5, (C) helix $\alpha 3$, and (D) loop 4. The color codes of the different paralogs are indicated in the figure.

structural analysis of Fra a 1.02 could contribute to elucidate the residues involved in IgE binding and help generate strategies to eliminate or minimize the allergenic potential of the fruits. Although the structures of the close Fra a 1.02 paralogs Fra a 1.01E and Fra a 1.03 had been successfully determined,^{19,44} previous crystallization attempts of Fra a 1.02 wild-type were not fruitful despite the high sequence identity shared by the three Fra a 1 proteins (Supporting Information Figure 1A–B).

Fra a 1.01E, 1.02, and 1.03 bind different natural metabolites of the flavonoid pathway in their hydrophobic cavity¹⁹ (Supporting Information Figure 1C), and Fra a 1.02 specifically binds myricetin. In order to identify the key residues of Fra a 1.02 involved in its interaction with this ligand, we generated a series of Fra a 1.02 mutants and carried out crystallization assays in both the presence and absence of myricetin. While no crystals could be obtained for the wild-type and most of the mutants, one of these variants produced crystals very efficiently in the absence of myricetin.³⁰ In this variant, Ala141 was substituted by Phe (A141F, PDB code: SAMW). Ala 141 is located in the predicted α -helix 3 (Supporting Information Figure 1A) which, based on the structure of Fra a 1.01E and Fra a 1.03, faces the cavity.¹⁹ An X-ray diffraction data set was obtained with the A141F crystals to a resolution of 1.9 Å, and its three-dimensional structure was determined by the molecular replacement method using Fra a 1.01E as an input model (PDB 4C9C¹⁹). Analysis of the

electron density map of A141F confirmed that the structure indeed corresponded to the A141F Fra a 1.02 mutant (see below).

We then used Fra a 1.02 A141F crystals to seed crystallization experiments of the wild-type Fra a 1.02 protein, which produced crystals successfully that diffracted at a resolution of 2.04 Å (Supporting Information Tables 3 and 4). Initial phases for Fra a 1.02 were obtained by the molecular replacement method, using A141F as a starting model. The structure of apo-Fra a 1.02 shows the characteristic star-related lipid transfer domain (START) fold (β - α - α - β x6- α)⁴⁵ that is conserved in PR-10 proteins⁷ (Figure 1A). It consists of three α -helices, the longest at the C-terminal end of the protein ($\alpha 3$) and two short consecutive ones near the N-terminus ($\alpha 1$ and $\alpha 2$), and a seven-stranded antiparallel β -sheet ($\beta 1$ – $\beta 7$). The helices and strands are connected by a total of nine loops (Figure 1A, Supporting Information Figure 1A). This structure encloses a large internal cavity that can be reached by three openings ($\epsilon 1$ – $\epsilon 3$, following the nomenclature in ref 46, Figure 1B–E). The main entrance to the cavity, $\epsilon 1$, is delimited by the N-terminal end of helix $\alpha 3$ and by loops L3, L5, L7, and L9 (Figure 1C). This entrance leads to a tunnel that connects two smaller openings, $\epsilon 2$ and $\epsilon 3$. $\epsilon 2$ is delimited by $\alpha 3$ and $\beta 1$ (Figure 1D), and $\epsilon 3$ is shaped by helices $\alpha 1$ and $\alpha 2$, $\beta 2$, and L4 (Figure 1E).

The cavity is overall hydrophobic but has polar residues pointing into its lumen (Figure 1F). It has an internal surface

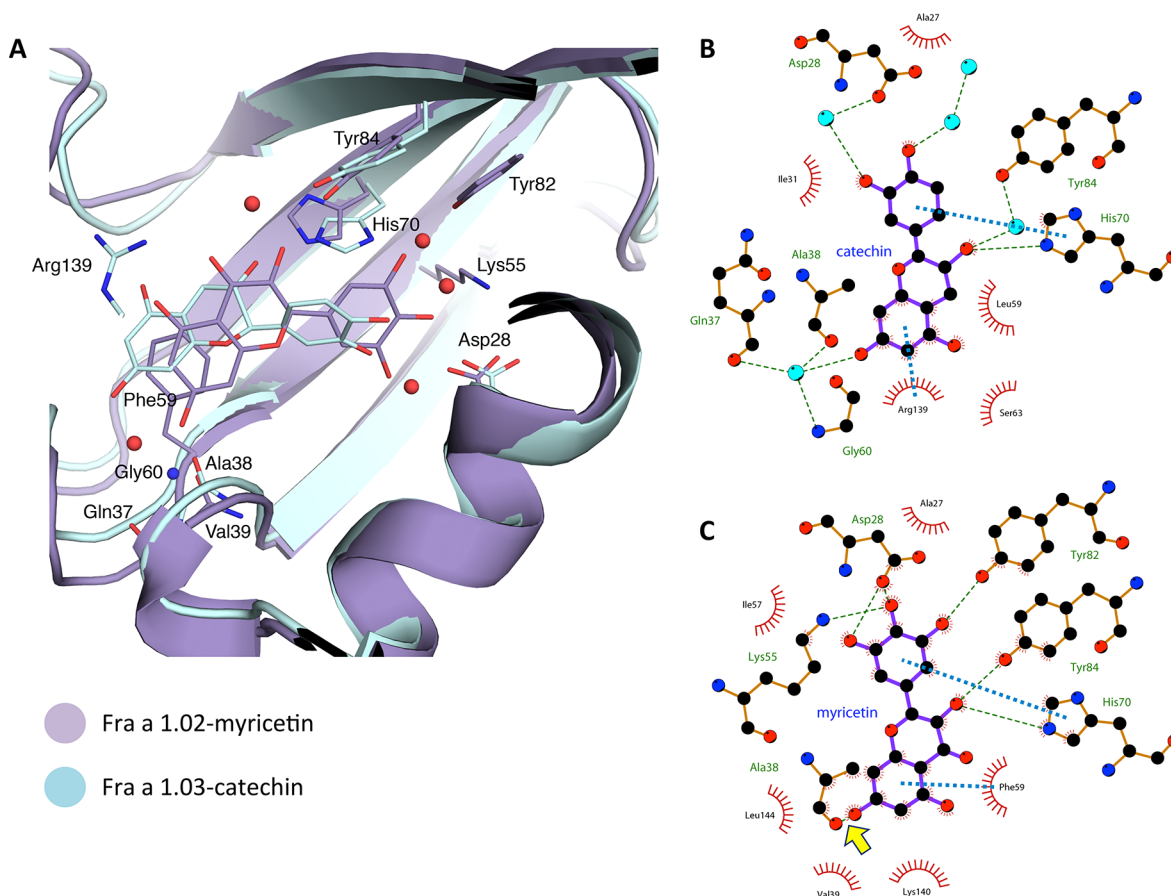


Figure 3. Docking of myricetin to Fra a 1.02. (A) Stick representation of the superimposition of the active site of Fra a 1.03 with catechin and Fra a 1.02 bound to myricetin. Red spheres represent water molecules. Amino acids involved in the binding are labeled. The color codes of the different paralog complexes are indicated in the figure. (B, C) Ligplot representations of the molecular interactions between Fra a 1.03 and catechin (B) and Fra a 1.02 and myricetin (C). Green dashed lines represent hydrogen bonds, red semicircles indicate hydrophobic interactions, and blue dashed lines π - π show stacking interactions.

of 1807.88 Å² with a depth of 25.62 Å, generating a volume of 1671.30 Å³. This volume is in the range of other PR-10 proteins, where significant differences (of more than 2000 Å³) in the volume of the cavity in close homologues have been described.⁷ When comparing the volume of the cavity of Fra a 1.02 with that calculated for other Fra a 1 proteins (2204.8 Å³ for Fra a 1.01E and 1646.4 Å³ for Fra a 1.03¹⁹) it is closer to Fra a 1.03, and this could explain their selectivity toward the binding of different flavonoids. Fra a 1.01E, Fra a 1.02, and Fra a 1.03 bind quercetin-3-*O*-glucuronide, myricetin, and catechin, respectively, in the low micromolar range,¹⁹ and while myricetin and catechin represent oxidized variants of the flavan nucleus with expected similar sizes, quercetin-3-*O*-glucuronide additionally includes a glycosyl moiety that makes this molecule larger (Supporting Information Figure 1C). Thus, the different volumes of the cavities could contribute to the binding specificities of distinct ligands for the three Fra a 1 proteins.

Structure superposition of Fra a 1.02 with that of Fra a 1.01E and Fra a 1.03 illustrates the high structural similarity between these proteins, all showing the characteristic START fold (Figure 2A). However, some conformational changes can be observed in specific areas of the proteins. The main differences between the three structures are in the area of the flexible loops lining the entrance to the cavity and in helix α 3 (Figure 3A–C). From these, the highest structural variability between the

three Fra a 1 proteins is found in loop 5 (L5, residues 60–67, Figure 2B), which is of special interest as it shows a high level of conformational flexibility adopting a closed conformation in ligand-bound structures and an open or variable conformation in ligand-free structures.^{19,47} Accordingly, it can be observed that L5 shows flexibility in the crystallized apo form of Fra a 1.01E and Fra a 1.02, while in the structure of the Fra a 1.03–catechin complex this loop closes over the ligand entrance.¹⁹ Thus, L5 is probably acting as a gating loop controlling the main entrance to the cavity, as is the case in the structurally related ABA receptors,⁴⁷ and playing a role in ligand recognition.

Helix α 3 also shows differences in the three Fra a 1 proteins. While α 3 closely overlaps in Fra a 1.01E and Fra a 1.02, it is displaced toward the cavity in Fra a 1.03 (Figure 2A and C). Catechin binding to Fra a 1.03 promotes a closer conformation of α 3 toward the ligand adopting a more compact structure, likely caused by the conformational change in loop 5. The closer overlap in α 3 observed in isoforms 1.01E and 1.02 of Fra a can be due to both being crystallized in their apo form, in contrast with Fra a 1.03, which is in its ligand-bound form. This compaction of the 3D structure and rigidification of the loops upon ligand binding observed in the Fra a 1 paralogs is in concordance to that observed by NMR diffusion measurements in Bet v 1 when comparing ligand-bound and ligand-free forms of this protein.⁴⁸ It is still unknown if the changes that

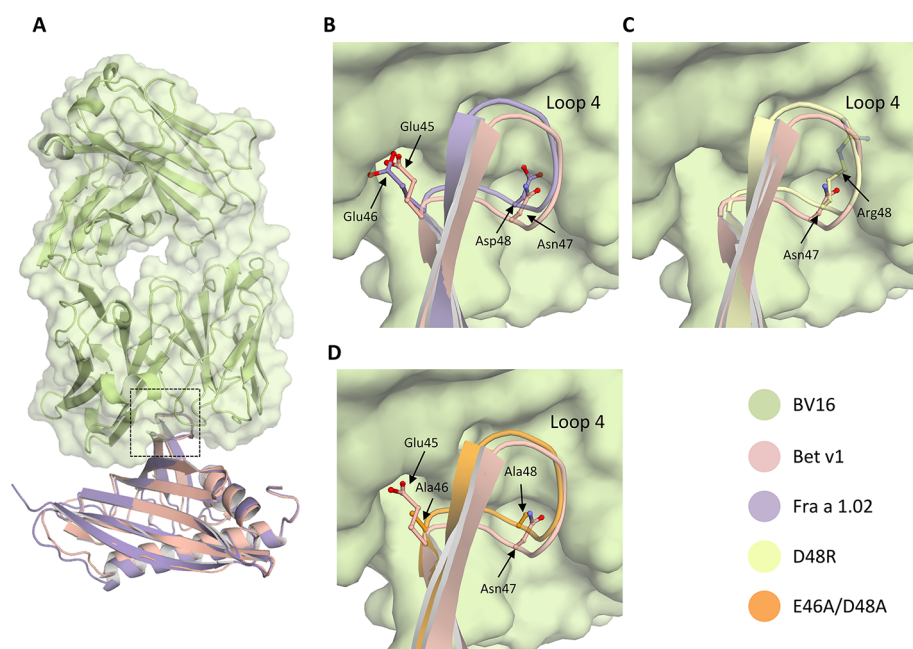


Figure 4. Structural superposition of Bet v 1-IgE and Fra a 1.02. (A) Structural superposition of Fra a 1.02 with the Bet v 1–BV16 complex. The structure of IgE is shown in ribbon and surface representation, and Bet v 1 and Fra a 1.02 are shown in ribbon representation. Loop L4 is highlighted in the box. (B) Caption of the squared area in panel A corresponding to L4. (C–D) Captions of loop L4 in the superposition of Bet v 1 with Fra a 1.02 D48R (C) and Fra a 1.02 E46A/D48A within the Bet v 1–BV16 complex. The color codes of the different proteins and mutants are indicated in the figure.

we observed in the 3D structure of the Fra a 1 paralogs upon ligand binding have an effect in their interactions with other proteins, or in their interactions with IgE, and hence in their allergenicity.

It is known that not all PR-10 proteins have similar entrances.⁷ We have found that $\epsilon 1$ and $\epsilon 3$ openings are present in the three Fra a 1 proteins, $\epsilon 1$ being the one showing the highest differences in shape and diameter between the three paralogs (Supporting Information Figure 2A). Interestingly, opening $\epsilon 2$, which has been proposed as an entrance to the cavity in the related Mal d 1 protein from apples,⁴⁶ is absent in Fra a 1.01E (Supporting Information Figure 2B). The $\epsilon 2$ opening is precisely delimited by $\alpha 3$; however, whether the differences among the Fra a 1 proteins in the conformation around this region are determinant in their ligand-binding affinity cannot be concluded here.

Loop 4 (L4, residues 47–53, Figure 1, Supporting Information Figure 1) is a Gly-rich loop that has been identified as the most rigid element of the PR-10 fold.⁷ The overlap of three Fra a 1 structures at L4 is almost perfect (Figure 2D), supporting the rigidity of this loop in the strawberry allergens. Interestingly, this loop L4 in Bet v 1 and in other related food allergens has been reported to be a major allergenic epitope.^{21–23}

In Silico Docking Studies of Myricetin and Fra a 1.02.

Myricetin was described as a ligand for Fra a 1.02 through isothermal titration calorimetry (ITC) experiments with a K_d of 19.5 μ M and a stoichiometry of 1:1.¹⁹ In order to determine if such binding was compatible with the three-dimensional structure obtained for Fra a 1.02, myricetin was docked in the obtained apo form of Fra a 1.02 using Induced Fit Docking method from the Schrödinger software suite.⁴⁰ The binding site was defined based on the available crystal structure of Fra a 1.03 protein bound to catechin (PDB: 4C94)¹⁹ as the sequence identity of Fra a 1.02 and 1.03 is 81.13%, and

catechin is structurally related to myricetin (Supporting Information Figure 1B and C). The results from the in silico docking experiment indicate that the binding pose of myricetin into the Fra a 1.02 cavity is similar to that observed for catechin in the Fra a 1.03 structure; however, myricetin is slightly moved toward the interior of the cavity (Figure 3A). This could be due to the slightly outward conformation of helix $\alpha 3$ in Fra a 1.02 as compared to that of Fra a 1.03 (Figure 2C), leading to a somewhat larger cavity. On the other hand, it could also be possible that ligand binding to Fra a 1.02 induces a rearrangement of helix $\alpha 3$ adopting a configuration closer to that of the complex of Fra a 1.03 with catechin, which might alter the position of the ligand. The interactions of the dihydroxyphenyl group of the catechin with Tyr84, Asp28, and His70 are mediated by water molecules (Figure 3B) while the equivalent trihydroxyphenyl group of myricetin forms direct interactions with Tyr82, Asp28, and Lys55 (Figure 3C). In addition, both groups are engaged in π – π stacking interactions with His70 (Figure 3B–C). The hydroxyl group in position 3 of the chromane ring of catechin is involved in hydrogen bonding directly with His70 and mediated by water molecule with Tyr84. The corresponding hydroxyl group of chromone ring in myricetin directly interacts with the same side chains of His70 and Tyr84. Finally, a series of hydrogen bonds are formed to stabilize the hydroxyl group in position 8 of catechin chromane ring through the interaction with water molecule that is coordinated by backbone atoms of Gln37, Ala38, and Gly60. For the analogous hydroxyl group in position 8 of chromone ring of myricetin, only one hydrogen bond with the carbonyl atom of Ala38 is predicted. Both chromone and chromane rings are participating in π – π stacking interaction with guanidinium group of Arg139 and phenyl group of Phe59, respectively. The binding of catechin is strengthened by hydrophobic interactions of Ala27, Ile31, Leu59, and Ser63

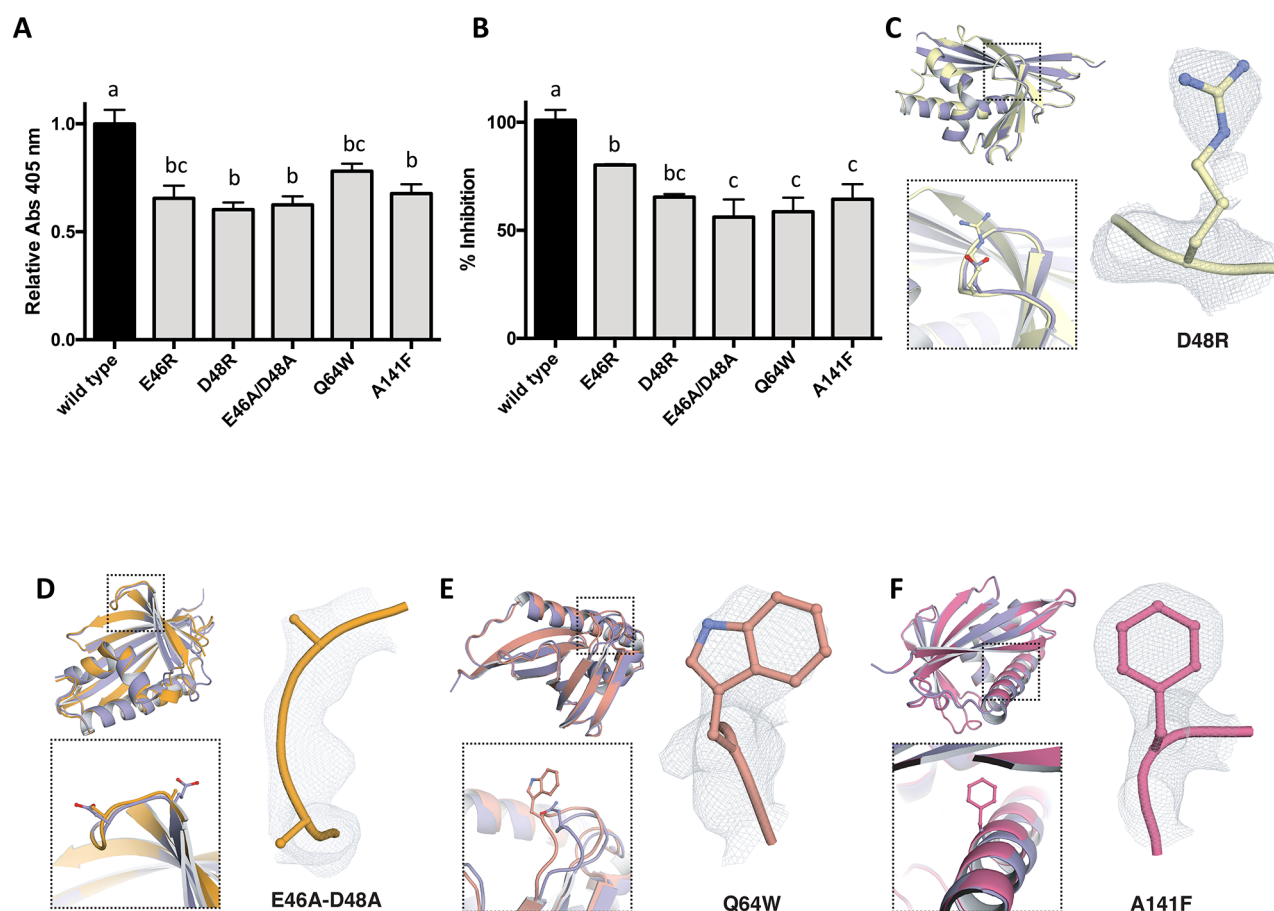


Figure 5. IgE-binding capacity of Fra a 1.02 reduced in the mutant variants. (A) Relative IgE binding of Fra a 1.02 and the indicated mutants assayed with pooled sera from 20 patients allergic to Bet v 1. Bars represent average $\pm$ SD of the relative values of IgE binding obtained by IgE ELISA assay normalized to the absorbance of Fra a 1.02 wild-type in three technical replicates. Protein concentration was 5 μ g/mL. Letters b and c indicate a significant effect of the mutation on IgE binding compared to the wild-type protein, with letter a (one-way ANOVA with Tukey's multiple comparison test, $p < 0.05$). (B) Percentage of inhibition of the different mutants compared to the wild-type. The pooled sera was incubated individually with 10 μ g/mL of the different proteins. ELISA plates were coated with 5 μ g/mL of native Fra a 1.02. The bars represent the average $\pm$ SD of the percentage of inhibition of each of the proteins in three technical replicates. Letters b and c indicate a significant effect of the mutation on IgE binding compared to the wild-type protein, with letter a (one-way ANOVA with Tukey's multiple comparison test, $p < 0.05$). (C–F) Structural superposition of Fra a 1.02 and its indicated mutant. In each panel the structural superposition of Fra a 1.02 and the indicated mutant, a detail of this superposition around the mutated amino acid, and the electron density map of the mutated amino acid are represented. All the structures are shown in ribbon representation, and the mutated residues are shown in ball and stick representation. Sigma values for the electron density maps are 1.0 for E46A-D48A, D48R, and A141F and 0.6 for Q64W. The color codes of the wild-type and mutants are indicated in the figure.

and in the case of myricetin of Ala27, Ile57, Leu144, Val39, and Lys140.

Rational Design of Fra a 1.02 Variants with Reduced Allergenic Potential through Structure-Based Analysis.

To identify residues potentially involved in the allergic response against Fra a 1.02, we compared the structure of Fra a 1.02 with that of Bet v 1 in complex with a IgG FAB fragment (BV16) (PDB code: 1FSK,^{12,21} Figure 4), and based on that, we generated a series of Fra a 1.02 mutants by site-directed mutagenesis. In Bet v 1, the stretch between amino acids 42 and 52 that spans the end of β 2 and L4 (Supporting Information Figure 1A) is important for antibody binding and accounts for the 80% of the contact surface with BV16.¹² Within this stretch, Glu45 and Asn47, located just at the end of β 2 (Figure 4B, Supporting Information Figure 1A), have been shown to play a key role in the recognition of Bet v 1 by IgE and interact through hydrogen bonds with BV16.^{12,21} The corresponding amino acids were also shown to be involved in IgE binding in a number of different pollen food

allergens.^{22,23,49,50} The structural superposition of Fra a 1.02 and Bet v 1 in the Bet v 1 - BV16 complex shows conservation in the spatial orientation of this stretch and that both Glu46 and Asp48 in Fra a 1.02 (corresponding to Glu45 and Asn47 in Bet v 1, Supporting Information Figure 1A) are compatible with the interaction with BV16 (Figure 4B). Therefore, we hypothesized that the Glu46 and Asp48 residues of Fra a 1.02 could also be important determinants of the allergenic cross-reactivity properties of this protein. To test this hypothesis, we substituted Glu46 by an Arg (mutant E46R) expecting that such a mutation would impair IgE binding and lead to decreased cross-reactivity.

Regarding Asn47 in Bet v 1, residues equivalent to that have been found to be important for IgE binding in allergens such as in Pru av 1 in cherries.²³ The superposition of Fra a 1.02 and Bet v 1 shows high structural similarity at position Asp48 of Fra a 1.02 and Asn47 in Bet v 1 (Supporting Information Figure 1A, Figure 4C). Therefore, we generated a second

mutant, D48R, in which the Asp48 of Fra a 1.02 was substituted by Arg.

We also disrupted this potential Fra a 1.02 interaction site with IgE by introducing a double mutation in which both Glu46 and Asp48 were replaced by Ala (mutant E46A/D48A), whose small nonpolar side chain would preclude the formation of hydrogen bonds with the IgE (Figure 4D).

In addition to these mutations that should disrupt the interaction of Fra a 1.02 with the IgE generated in response to Bet v 1 based on the PDB 1FSK model, we generated another mutant to test whether distal loops from the L4 region could contribute to IgE-binding activity. To that, we chose the flexible L5 loop, which is potentially involved in flavonoid binding, knowing that in Bet v 1, flavonoid binding sites overlap with some epitopes. As the conformation of loop L5 changes between the apo and ligand-bound Fra a 1.02 isoforms, we wanted to determine if this change in conformation could play a role in IgE recognition. As we could not obtain crystals of Fra a 1.02 with myricetin, we introduced a mutation that could alter the conformation of the L5 loop. Thus, Gln64 (Supporting Information Figure 1A) was substituted by Trp (Q64W). The Trp side chain at this position would likely affect the conformation of L5 in the native protein.

Finally, the amino acid region comprised between residues 142 and 156 has also been identified as the major T-cell epitope in patients allergic to birch pollen.⁵¹ Ala141 in Fra a 1.02 is located in $\alpha 3$ (that comprises residues 131–156) (Supporting Information Figure 1A) facing the hydrophobic cavity and in close proximity to this allergenic stretch. As we already generated the A141F mutant, designed to preclude the ligand-binding interaction of Fra a 1.02 with myricetin and that microseeded the crystallization experiments with wild-type Fra a 1.02, we decided to test this mutant protein for IgE binding as well.

The IgE-Binding Capacity of the Structure-Based Fra a 1.02 Mutant Variants Is Reduced Compared to That of the Wild-Type Protein. In order to determine if the residues mutated in our Fra a 1.02 variants are key part of epitopes involved in the allergic reaction to strawberry, we expressed and purified the recombinant proteins to assay their IgE-binding capacity by ELISA experiments. For this, we used pooled human sera obtained from 20 atopic patients with confirmed allergies to the major birch pollen allergen Bet v 1 (Supporting Information Table 2). We assayed three different Fra a 1.02 concentrations (2.5, 5.0, and 10 $\mu\text{g/mL}$) by triplicates (Supporting Information Figure 3). As the maximum IgE binding for Fra a 1.02 wild-type was achieved at 5 $\mu\text{g/mL}$, the IgE binding to the mutants was analyzed at this concentration and normalized to that of the wild-type protein (Figure 5A). In all cases, the mutant variants showed a significant reduction of 30–40%, compared to that of the wild-type Fra a 1.02, in the binding to the IgE generated against Bet v 1. The mutant versions in the 42–52 region, E46R, D48R, and E46A/D48A, as well as A141F, showed the lowest binding capacity, this being reduction in IgE binding in the same range to that observed for the E45S Bet v 1 mutant.²¹ The Q64W variant also showed a significant reduction in IgE binding. In this case, the modified residue is in the gating loop L5, at a position that shows sequence variability between the three Fra a 1 proteins (Supporting Information Figure 1A). This could partially explain the previously reported different allergenic potential of the Fra a 1 proteins.^{5,9}

The effect of the different mutations on the IgE binding was also addressed in IgE-inhibition assays using the same pool of sera. The pooled sera was incubated with three different concentrations (2.5, 5.0, and 10 $\mu\text{g/mL}$) of each of the proteins and then hybridized in ELISA plates coated with 5 $\mu\text{g/mL}$ of native Fra a 1.02. There were no statistical differences between the inhibition found using 5 and 10 $\mu\text{g/mL}$ of the mutant proteins, with the exception of mutant E46R, indicating that we reached saturation and that the lower inhibition was not due to a lack of protein (Supporting Information Figure 4). Because of mutant E46R, we analyzed the inhibition incubating the sera with 10 $\mu\text{g/mL}$ of the proteins (Figure 5B). In all cases, the mutants showed a significant reduction in the IgE inhibition between 55 and 80%, compared to that of the wild-type, set as 100%.

Together, these data confirm that the binding to IgE generated against Bet v 1 is reduced in our mutants compared to the native Fra a 1.02 protein.

The Overall Fold of the Fra a 1.02 Mutant Variants Is Not Affected. As all of the mutant versions that we generated showed a reduced IgE binding from patients allergic to Bet v 1 compared to that of the wild-type, we proceeded to check whether the overall structure of Fra a 1.02 was affected in the mutants. Therefore, we performed crystallization assays with all the mutant variants, following the same microseeding strategy used for Fra a 1.02. We were able to obtain crystals for variants D48R, E46AD48A, and Q64W, which diffracted to 1.97, 2.19, and 2.27 Å resolution, respectively (Supporting Information Tables 2 and 3). Structures were resolved by the molecular replacement method using Fra a 1.02 A141F as starting model. While we obtained for crystals for E46R, they were of poor resolution, so we were not able to determine if the crystals corresponded to the actual E46R mutant or to A141F (which was the mutant used for seeding), or if they corresponded to a mixture of both proteins. Thus, its structure was not analyzed.

The analyses of the structures of the mutant variants of Fra a 1.02 are shown in Figure 5C–F. As can be observed, the mutations did not affect the overall fold of the modified Fra a 1.02 proteins, and, in all cases, the presence of the mutated amino acid could be confirmed in some of the asymmetric units. Mutations in the 42–52 region could directly affect the hydrogen bonds that sustain the Bet v 1–IgE interaction, either due to the change of size and charge of the residue at position 48 (D48R) (Figure 5C) or the substitution of the negatively charged amino acids Glu46 and Asp48 for the small nonpolar Ala (E46A/D48A) (Figure 5D), and therefore explain the reduced IgE-binding capacity of these variants (Figure 5A). In the case of Q64W, it can be observed how the large side chain of the Trp by which the Gln was substituted affects the local conformation of the L5 loop (Figure 5E). As for the A141F mutation, located in helix $\alpha 3$, the change of Ala141 for Phe also reduced the IgE binding of the mutated protein (Figure 5A). Helix $\alpha 3$ has been reported as highly allergenic in Bet v 1, and some amino acids in this region have already been identified as T-cell epitopes.^{51,52} However, since Ala141 is located inside the cavity, where it is probably not easily accessible by the IgE, it suggests that its role in IgE recognition may be indirect. The fold of the protein around this position in helix $\alpha 3$ is altered in the mutant variant (Figure 5F), and this could consequently have altered the IgE-binding affinity of A141F.

The strategy followed herein has allowed the generation of new isoforms of Fra a 1.02 that retain their overall fold and

display a lower IgE-binding capacity. Further studies to decrease their IgE-binding even more could make these mutants potential candidates to be eventually tested as vaccines against strawberry allergy, following standardized protocols established for vaccine development. This strategy was successful in Bet v 1, where isoforms generated by site-directed mutagenesis that retained the overall fold but displayed a lower IgE-binding capacity stimulated the T-cell response and induced the production of IgGs, which inhibited IgE binding.^{21,53} The production of rationally designed protein variants described herein with lower allergenic potential could contribute to the development of safer treatments of immediate type allergies like the one to strawberry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.9b05714>.

Supplemental experimental procedures; tables including list of primers, results of the SPT, crystallization conditions, and crystallographic data collection and statistics; figures including identities of Bet v 1 and Fra a 1.01, 1.02, and 1.03 and molecular structures of myricetin, (+)catechin, and quercetin-3-O-glucuronide, structures of Fra 1.01, 1.02, and 1.03, cross-reactivity of Fra a 1.02 and mutants, and inhibition of IgE binding by Fra a 1.02 mutants (PDF)

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Notes

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SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

Skin Prick Testings (SPT)

The SPTs were performed according to the guidelines of the European Academy of Allergy and Clinical Immunology (EAACI) as described in van Kampen et al (2013) using commercial extracts of the different allergens.

IgE inhibition assays

200 µL of the pooled sera (diluted 1:3 in PBS pH 7.4) were incubated for 3 h at 4 °C in a rotating wheel with different concentrations (0, 2.5, 5, and 10 µg/mL) of the native Fra a 1.02 and the mutant proteins. ELISA plates were coated with 50 µL of 5 µg/mL of Fra a 1.02 in PBS for 2 h at 37 °C. Afterwards, the plates were washed four times with PBST (PBS + Tween 0,05%) and blocked for 1 h at room temperature with 200 µL of casein (Sigma-Aldrich). Then the ELISA plates were incubated overnight with the pooled sera at 4 °C. The plates were washed four times with PBST. To detect binding of human IgE antibodies the plates were incubated with anti IgE-HRP (ThermoFisher) for 1 h at room temperature and washed 4 times (PBS Tween 0,04%). The HRP activity was measured using the TMB substrate kit (PierceTM) and the absorbance was measured at 405 nm. All the samples were tested three times. To calculate the percentage of inhibition, the value of the blank obtained was subtracted and the value obtained by the Fra a 1.02 wild type was taken as 100% activity.

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Van Kampen, V.; De Blay, F.; Folletti, I.; Kobierski, P.; Moscato, G.; Olivieri, M.; Quirce, S.; Sastre, J.; Walusiak-Skorupa, J.; Raulf-Heimsoth, M. EAACI position paper: skin prick testing in the diagnosis of occupational type I allergies. *Allergy (Oxford, U. K.)*. **2013**, 68(5), 580-584.

SUPPLEMENTARY TABLE 1. List of the primers used in this study. The triplet corresponding to the mutant amino acid is underlined.

Primers	Sequence (5' - 3')
External primer forward (FD42)	GGGCCATGGCGGGTGTGTTCACTTATGAAACCG
External primer reverse (FD43)	CCCGAATTCTTAACAGTATTCATTAGGATTGGC
E46R Internal primer forward (E46R For)	GTTAAGTGTGCTGAAATCATT <u>CGCG</u> GAGACGGAGGCGTAGGCACC
E46R Internal primer reverse (E46R Rev)	CTACGCCTCCGTCTCC <u>GCG</u> AATGATTTCAGCACACTTAACTGCTTG
D48R Internal primer forward (D48R For)	GTGCTGAAATCATTGAAGGAC <u>GCG</u> GAGGCGTAGGCACCATCAAG
D48R Internal primer reverse (D48R Rev)	GGTGCCTACGCCTCC <u>GCG</u> TCCTTCAATGATTTCAGCACACTTAAC
E46/D48A Internal primer forward (E46/D48A For)	GTTAAGTGTGCTGAAATCATT <u>GCGGGAGCGG</u> GAGGCGTAGGCACCATCAAG
E46/D48A Internal primer reverse (E46/D48A Rev)	GGTGCCTACGCCTCC <u>GCG</u> TCC <u>GCG</u> CAATGATTTCAGCACACTTAACTGCTTG

Q64W Internal primer forward (Q64W For)	CCTTTGGCGAAGGCAGCT <u>TGG</u> TCGGCTCAGTGACCCACAAGATC
Q64W Internal primer reverse (Q64W Rev)	GTGGGTCACTGAGCCGAAC <u>CAG</u> CTGCCTTCGCCAAAGGTGATC

40 **SUPPLEMENTARY TABLE 3. Crystallization conditions**

	WT	E46R	D48R	E46AD48A	Q64W
Method	Sitting-drop vapor-diffusion method Random Microseed Matrix Screening (A141 seeds)				
Temperature (K)	293				
Plate type	CrystalDirect (Mitegen)				
Protein concentration (mg mL ⁻¹)	10	40	40	40	10
Buffer composition of protein solution	50 mM Tris pH 7.5, 150 mM NaCl 1 mM β - mercaptoethanol	50 mM Tris pH 7.5 150mM NaCl 1 mM β - mercaptoethanol	50 mM Tris pH 7.5 150 mM NaCl 1 mM β - mercaptoethanol	50 mM Tris pH 7.5 150 mM NaCl 1 mM β - mercaptoethanol	50 mM Tris pH 7.5 150 mM NaCl 1 mM β - mercaptoethanol
Composition of the reservoir solution:	100mM Bis-Tris pH5.5 140mM Ammonium Sulfate 33% PEG3350	100mM Bis-Tris pH5.5 200mM Ammonium Sulfate 31% PEG3350	100mM Bis-Tris pH5.5 200mM Ammonium Sulfate 34% PEG3350	1.6M sodium citrate tribasic	100mM Bis-Tris pH5.5 200mM Magnesium chloride 26.5% PEG3350

Volume and ratio of the drops	200 nL (100 nL protein + 70 nL reservoir solution + 30 nL seeds)
Volume of the reservoir (μL)	45

41

42 **SUPPLEMENTARY TABLE 4. Crystallographic data-collection statistics**

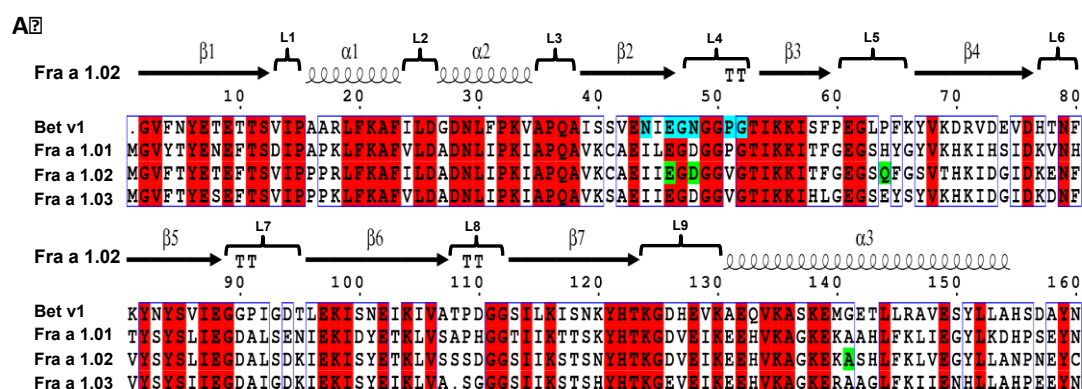
	WT	D48R	E46A/D48R	Q64W
Data collection	ESRF-ID29	ESRF-ID30a1	ESRF-ID30a3	ESRF-ID30a1
Space group	C2	C2	P212121	P212121
Cell dimensions				
a, b, c (Å)	105.92, 41.73, 91.35	105.58, 41.83, 91.79	58.14, 75.43, 83.11	41.84, 59.52, 131.22
α, β, γ (°)	90, 117.14, 90	90, 116.15, 90	90, 90, 90	90, 90, 90
Resolution (Å)	52.45 -2.04 (2.27-2.04)	82.40-1.97 (2.14-1.97)	55.86-2.19 (2.40-2.19)	54.20-2.27 (2.53-2.27)
R_{meas}	8.5 (95.7) ^a	7.1 (134.1) ^a	149 (1572) ^a	140 (1341) ^a
$I/\sigma(I)$	8.7 (1.1) ^a	12.6 (1.2) ^a	11.0 (1.4) ^a	8.4 (1.4) ^a
$CC_{1/2}$	99.8 (56.0) ^a	99.8 (45.1) ^a	99.8 (69.9) ^a	99.7 (61.5) ^a
Spherical completeness (%)	65.7 (12.1) ^a	75.9 (17.5) ^a	77.7 (17.2) ^a	66.4 (12.1) ^a
Ellipsoidal completeness (%)	88.1 (49.6)	91.6 (59.5)	92.2 (45.1)	90.5 (56.5)
Redundancy	3.2 (2.7) ^a	4.7 (5.4) ^a	7.3 (7.7) ^a	5.0 (7.0) ^a
Refinement				
Resolution (Å)	47.13-2.04	82.40-1.97	55.86-2.19	23.34-2.27
No. reflections	15169	19595	14981	10525
$R_{\text{work}} / R_{\text{free}}$	0.216/0.278	0.201/0.249	0.213/0.256	0.202/0.252

No. atoms	2658	2667	2470	2484
Protein	2477	2495	2385	2330
Water	181	171	85	54
<i>B</i> factors				
Protein	51.05	51.15	49.17	52.44
Water	48.30	50.02	46.10	42.93
R.m.s. Deviations				
Bond lengths (Å)	0.010	0.010	0.010	0.010
Bond angles (°)	1.26	1.2	1.12	1.30
Ramachandran outliers (%)	0	0	0	0
Ramachandran preferred (%)	96.89	97.83	98.04	96.82
Ramachandran allowed (%)	3.11	2.17	1.96	3.18

43

44

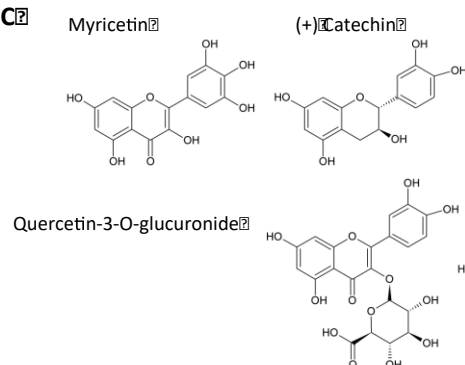
45 **SUPPLEMENTARY FIGURES**



B

	Bet v 1	Fra a 1.01	Fra a 1.02	Fra a 1.03
Bet v 1	100	53,46	58,86	58,49
Fra a 1.01		100	79,87	76,25
Fra a 1.02			100	81,13
Fra a 1.03				100

C



46

47 **Supplementary Figure 1. (A)** Multiple sequence alignment of Bet v 1, Fra a 1.01, Fra a 1.02, and Fra a 1.03. The secondary structural elements correspond to Fra a 1.02. Main
48 residues related to Bet v 1 allergenic potential are highlighted in cyan. The Fra a 1.02
49 residues mutated by site-directed mutagenesis are highlighted in green. **(B)** Identities
50 at the amino acid level shared by Bet v 1, Fra a 1.01, Fra a 1.02, and Fra a 1.03
51 (Accession numbers: P15494.2, CAJ85645.1, ACX47057.1, and ACX47058.1,
52 respectively) **(C).** Molecular structures of Myricetin, (+)-Catechin, and Quercetin-3-O-
53 glucuronide, ligands of Fra a 1.01, Fra a 1.02, and Fra a 1.03, respectively.
54

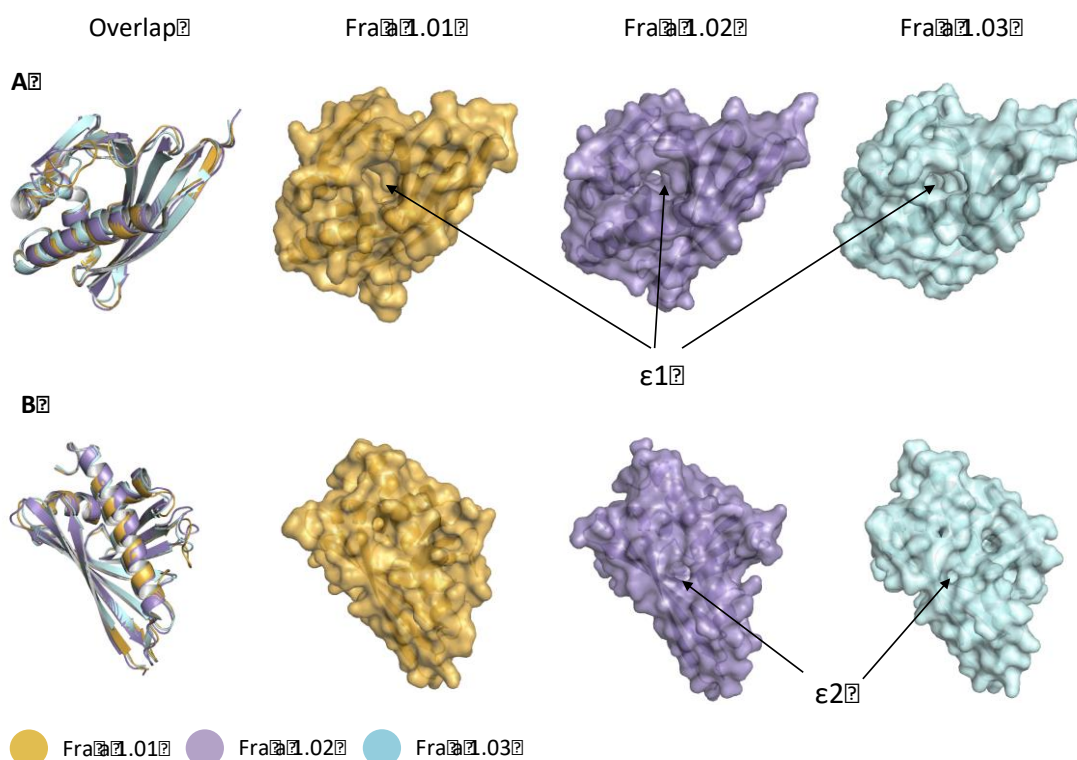


Figure S2. Structures of Fra a 1.01, 1.02 and 1.02 showing the $\epsilon 1$ and $\epsilon 2$ entrances to the cavity. Ribbon representation of the superimposition of Fra a 1.01, 1.02 and 1.03, and surface representation of the three of them showing the $\epsilon 1$ (A) and $\epsilon 2$ (B) entrances to the cavity. The color codes of the different paralogs are indicated in the figure.

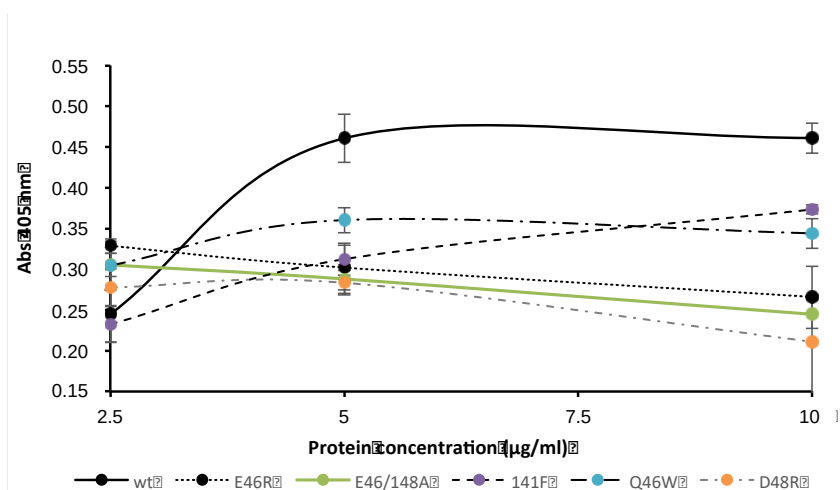


Figure S3. Cross-reactivity of Fra a 1.02 and mutants with a pool of sera from patients allergic to Bet v1. IgE binding capacity of Fra a 1.02 and the different mutant variants measured by standard ELISA using a pool of sera of 20 patient allergic to Bet v 1. IgE binding was quantified as absorbance at 405 nm. The assays were performed at three protein concentrations (2.5, 5, and 10 µg/mL). Each value represents the mean ±SD of three technical replicates. The color codes of the wild-type and mutants are indicated in the figure.

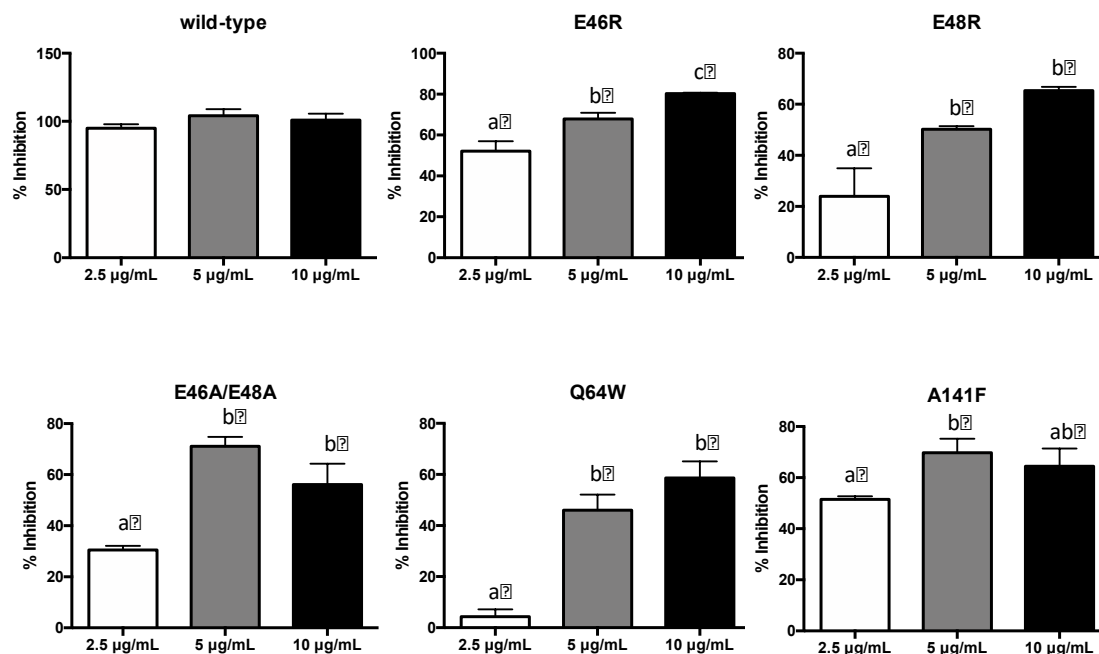


Figure S4. Inhibition of the IgE binding by Fra a 1.02 mutants. Three different concentrations of Fra a 1.02 wild type and mutants were incubated individually with the pooled sera. Bars represent the percentage of inhibition in each concentration compared to that of the wild type protein ±SD. Letters indicate a significant effect on the inhibition by the different concentrations (One-way ANOVA with Tukey's multiple comparison test, $p < 0.05$).