Die physiologische und immunologische Rolle von PR-10 Allergenen

Dissertation

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Zusammenfassung

Die allergische Sofortreaktion vom Typ I ist die am weitesten verbreitete Form der Hypersensitivität. Hierbei wirken prinzipiell harmlose Proteine als Allergene, die von Immunoglobulinen der Klasse E (IgE) erkannt werden und in kürzester Zeit zu allergischen Beschwerden führen. In Nordamerika und Zentraleuropa wird ein Großteil der allergischen Sofortreaktionen von Pollen– und Nahrungsmittel-allergenen der Bet v 1-Superfamilie ausgelöst. Die Bet v 1-homologen Vertreter werden meist in Form von multiplen, beinahe identischen Isoformen durch umweltbedingten Stress induziert und in die *pathogenesis related* 10 Proteinfamilie (PR-10) untergeordnet. Trotz jahrzehntelanger Forschung und der zunehmenden klinischen Relevanz ist die genaue physiologische Funktion, sowie die Anzahl und Lage der Interaktionsflächen (Epitope) von PR-10 Allergenen mit IgE bis heute unbekannt.

In dieser Arbeit wurde durch gezielte Variation von Aminosäuren ein potentielles IgE-Epitop des Hauptbirkenpollenallergens Bet v 1 auf das strukturell homologe, jedoch nicht allergene PR-10 Enzym (s)-Norcoclaurin-Synthase (NCS) übertragen. Nach Bestätigung der strukturellen Integrität mittels Circulardichroismus- (CD) und magnetischer Kernspinresonanz-Spektroskopie (NMR), konnte eine Interaktion der konstruierten NCS-Varianten mit IgE nachgewiesen werden. Dieses System, welches als *epitope grafting* bezeichnet wird, ermöglicht die zukünftige Analyse von PR-10-Epitopen auf molekularer Ebene und könnte zur Herstellung von hypoallergenen Varianten für die Allergen-spezifische Immuntherapie beitragen.

Ein vergleichsweise neues Mitglied der PR-10 Familie ist das Erdbeerallergen Fra a 1. Die in dieser Arbeit mittels NMR-Spektroskopie bestimmte dreidimensionale Struktur der rekombinanten Isoform Fra a 1E zeigt eine für PR-10 Allergene typische Faltung. Im Vergleich zu anderen homologen Allergenen sorgen einige signifikante strukturelle Unterschiede in Fra a 1E jedoch zur Ausbildung eines größeren hydrophoben Innenraums, der zum Transport oder der Lagerung verschiedener Liganden im Biosyntheseweg des Erdbeerfarbstoffes dienen könnte.

Das Hauptbirkenpollenallergen Bet v 1 ist bereits biochemisch, strukturell und immunologisch umfangreich untersucht. Seit Aufklärung seiner dreidimensionalen Struktur vor etwa 20 Jahren wurde jedoch nach einem physiologischen Bindungspartner im hydrophoben Hohlraum des Allergens gesucht. In dieser Arbeit konnte Bet v 1 im Komplex mit seinem natürlichen Interaktionspartner Quercetin-3-O-Sophorosid (Q3OS) aus reifen Birkenpollen extrahiert und gereinigt werden. Anhand von Ultraviolett- (UV), Fluoreszenz- und NMR-Experimenten wurde die hohe Spezifität der Interaktion *in vitro* analysiert und das diglykosylierte Flavonoid als natürlicher Bindungspartner von Bet v 1 bestätigt. Weiterhin zeigen antikörperbasierende Nachweisverfahren, dass die Bindung von Q3OS keinen direkten Einfluss auf das IgE-Interaktionsverhalten von Bet v 1 ausübt. Ein möglicher sensibilisierender oder indirekter Effekt des Bet v 1-Liganden auf das Immunsystem ist jedoch nicht auszuschließen.

Die an diese Erkenntnisse anknüpfenden Experimente beschäftigten sich schließlich mit der Rolle von drei Bet v 1-Isoformen mit unterschiedlichem allergenen Potential. Hierfür wurden die Isoformen

rekombinant produziert, gereinigt und deren Interaktion mit verschiedenen potentiellen Bindungspartnern mittels NMR– und UV-Spektroskopie analysiert. Dabei zeigte sich, dass weitere glykosylierte Flavonoide physiologische Liganden von Bet v 1 darstellen könnten. Jede Isoform weist dabei ein individuelles Bindungsverhalten auf, wobei die Spezifität der Bindung von der Stereochemie und der Anzahl an Zuckerresten der Liganden abhängt. Bedingt durch ihre variablen Absorptionseigenschaften und der hohen UV-Toleranz, dienen Komplexe aus Bet v 1-Isoformen und Flavonoiden vermutlich dem anpassungsfähigen Schutz der Pollen-DNA vor örtlicher Sonnenstrahlung.

Summary

Immediate type I allergies are the most common and widespread form of hypersensitivity reactions. Thereby, mainly proteins from plants and animals are recognized as allergens by Immunoglobulin E (IgE) which results in instant allergic reactions. The main elicitors of type I allergies in North America and Central Europe are pollen and food allergens which belong to the Bet v 1-superfamily. The Bet v 1-homologous members are induced as multiple isoforms upon environmental stress and are further sub-grouped into the family of class 10 pathogenesis related proteins (PR-10). Despite extensive research and rising clinical significance neither the precise physiological function nor the number and location of the interaction surfaces (epitopes) from PR-10 allergens with IgE had been identified yet.

In this work, a putative IgE-epitope of the major birch pollen allergen Bet v 1 was transferred to the structural homologue but non-allergenic PR-10 enzyme (s)-norcoclaurine synthase (NCS) by the variation of specific amino acids. After verifying the structural integrity by circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy, the constructed NCS variants showed significant interaction with IgE. This system will allow analysing epitopes in patients with Bet v 1-related allergies and might help to design hypoallergenic variants for allergen specific immunotherapy.

The major strawberry allergen Fra a 1 was recently identified as a member of the PR-10 family. The three dimensional solution structure of the recombinant isoform Fra a 1E was solved in this work using NMR spectroscopy. While the overall fold is typical for PR-10 allergens, significant structural differences result in a larger hydrophobic cavity in the interior of Fra a 1E compared to other homologous proteins. This cavity might transport or store ligands involved in pigment formation in strawberry plants.

In contrast, the major allergen from birch Bet v 1 has been studied for decades and is well characterized by structural, biochemical and immunological methods. Ever since the determination of its three dimensional structure many attempts have been made to determine a putative physiological ligand in the hydrophobic cavity of Bet v 1. In this work, Bet v 1 was isolated from mature birch pollen in complex with its natural ligand quercertin-3-O-sophoroside (Q3OS). By using ultraviolet (UV), fluorescence and NMR spectroscopy, the binding parameters and the specificity of Q3OS-binding to Bet v 1 were determined *in vitro* and confirm Q3OS as natural ligand of Bet v 1. However, the influence of Q3OS on the allergenicity of Bet v 1 seems to be limited. The obtained data from enzyme linked assays suggest the lack of a direct ligand effect on IgE recognition of Bet v 1, but leave open the possibility of indirect influences such as sensitization.

Subsequent research focused on the physiological properties of three recombinant Bet v 1 isoforms with different allergic potential. The interaction of the isoforms with a variety of binding partners was analysed using UV and NMR spectroscopy. The obtained results suggest that further glycosylated flavonoids, such as quercetin-3-O-sophoroside, are the physiological ligands of the Bet v 1 isoforms. Each isoform shows an individual, highly specific binding behaviour for the ligands. This specificity is

driven by the sugar moieties of the ligands rather than the flavonols. The physiological function of Bet v 1 is probably linked with adaptable DNA protection from solar radiation due to the variable absorption properties and high UV tolerances of the complexes formed by Bet v 1 isoforms and flavonoids.

1 Einleitung

1.1 Allergie und Hypersensitivität

Das adaptive Immunsystem ist ein essentieller Bestandteil der Körperabwehr gegen Infektionen und für den Erhalt der Gesundheit unumgänglich. Allerdings werden adaptive Immunantworten manchmal auch durch Antigene ausgelöst, die nicht mit potentiellen Krankheitserregern zusammenhängen und zu schweren Erkrankungen führen können. Hierbei handelt es sich um prinzipiell harmlose Inhaltstoffe, z. B. aus Pollen, Nahrungsmitteln, Tierhaaren oder Medikamenten, die nach wiederholtem Kontakt eine schädliche Immunantwort hervorrufen. Diese Reaktionen werden allgemein als Hypersensitivität oder Überempfindlichkeit bezeichnet (Murphy *et al.*, 2009). Paul Portier und Charles Richet waren die ersten Wissenschaftler die Anfang des zwanzigsten Jahrhunderts Hypersensitivitätsreaktionen erkannten und beschrieben. Als deutlich wurde, dass konkrete Mechanismen für spezifische Überempfindlichkeiten verantwortlich sind, schlugen die beiden Immunologen Philip Gell und Robert Coombs 1963 ein bis heute gültiges Klassifizierungssystem vor (Gell & Coombs, 1963). Neben der Sofortreaktion vom Typ I, kann noch zwischen drei weiteren Typen der Überempfindlichkeit unterschieden werden (Owen *et al.*, 2013). Aufgrund der weiten Verbreitung und der hohen Anzahl betroffener Patienten, wird jedoch der Begriff der klassischen Allergie heutzutage mit der Sofortreaktion vom Typ I gleichgesetzt.

Die Bezeichnung Allergie wurde zum ersten Mal im Jahr 1906 von dem österreichischen Kinderarzt Clemens von Pirquet erwähnt (Von Pirquet C., 1906). Von Pirquet stellte fest, das sich die Verabreichungen eines Antigens schädlich auf Patienten auswirkt, anstatt eine immunisierende Wirkung zu erzielen. Das aus dem griechischen stammende *allos* (anders oder verschieden) sollte in Kombination mit dem Wort *ergia* (Energie oder Aktion) diese Beobachtung beschreiben (Igea, 2013). Allergien werden von Proteinen hervorgerufen und sind mit Abstand der häufigste Typ von Überempfindlichkeitsreaktionen. Weltweit leiden Millionen von Menschen an den typischen Symptomen wie Heuschnupfen, Asthma, Nahrungsmittelallergien und atopischer Dermatitis. Allein in Nordamerika und Europa sind etwa 50 % der Bevölkerung von der Überempfindlichkeit des Typ I betroffen (Murphy *et al.*, 2009; Owen *et al.*, 2013). Verantwortlich für die Symptome ist das Immunoglobulin der Klasse E (IgE). Diese Antikörper wurden als letzte Klasse der Immunoglobuline von K. Ishizaka und T. Ishizaka zwischen 1960 und 1970 nachgewiesen (Ishizaka *et al.*, 1966; Ishizaka & Ishizaka, 1967). Die eigentliche physiologische Funktion von IgE liegt wahrscheinlich in der Abwehr von Würmern und Parasiten (Erb, 2007; Fitzsimmons *et al.*, 2014). Bei der allergischen Reaktion handelt es sich demnach um eine fehlgeleitete Anti-Parasiten-Antwort des Immunsystems (Artis *et al.*, 2012). Damit es zur Produktion von Allergen-spezifischem IgE kommt, muss zunächst eine Sensibilisierung gegen das entsprechende Allergen stattfinden. Pollen- oder Hausstauballergene können beispielsweise über Diffusion durch die Schleimhautoberfläche in die Epithelzellen von atopischen Individuen eindringen (Grunstein *et al.*, 2005; Renkonen *et al.*, 2009). Beim ersten Kontakt

werden Allergene von verschiedenen Bestandteilen des Immunsystems wie antigenpräsentierenden Zellen (APZ) erkannt und können abhängig von der Allergendosis, dem Präsentationsweg und den ausgeschütteten Zytokininen eine T_H2-Reaktion (Typ-2 T-Helferzellen) auslösen (Abbildung 1; Janeway *et al.*, 2002). Hierbei kommt es zur Differenzierung von naiven allergenspezifischen T-Zellen zu T_H2-Zellen. Dadurch wird die IgE-Produktion von allergenspezifischen B-Zellen stimuliert, die weiterhin durch Zytokinine wie Interleukin 4 und 13 begünstigt wird (Romagnani, 2000). Das gebildete IgE bindet mit sehr hoher Affinität an den IgE-Rezeptor FcεRI auf Effektorzellen wie Mastzellen oder basophile Granulozyten (Gould & Sutton, 2008). Sind IgE-Antikörper einmal als Antwort auf ein Allergen gebildet, kommt es bei erneuter Exposition gegenüber dem Antigen zur Quervernetzung der, an den FcεRI-Rezeptoren gebundenen, polyklonalen Antikörper (Gould & Sutton, 2008). Dies führt zur Degranulierung der Effektorzellen und somit zur Ausschüttung von u. a. Histamin, Serotonin, Proteasen oder Zytokininen (Galli *et al.*, 2002; Kobayashi *et al.*, 2000). Diese proinflammatorischen Mediatoren bewirken direkt oder indirekt eine erhöhte Flüssigkeitssekretion und Peristaltik des Magen-Darm-Trakts sowie eine Erweiterung der Blutgefäße und verringern den Durchmesser der Atemwege. Neben den typischen allergischen Symptomen kann es sogar zu lebensgefährlichen Ganzkörperreaktionen wie dem anaphylaktischen Schock kommen (Müller-Werdan & Werdan, 2011).

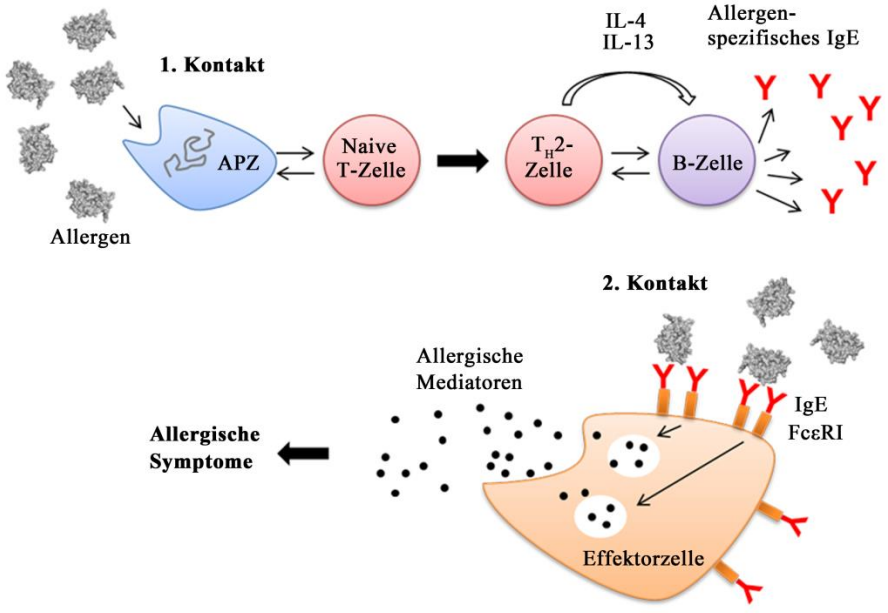

Abbildung 1: Sensibilisierung und schematische Darstellung der allergischen Sofortreaktion vom Typ I.
APZ: Antigenpräsentierende Zelle, IL: Interleukin, T_H2: Typ-2 T-Helferzelle, IgE: Immunoglobulin E, FcεRI: IgE-bindender Rezeptor auf Effektorzellen.

1.2 Klassifizierung von Allergenen

Als Allergene bezeichnet man Proteine, die eine IgE-vermittelte Antwort des Immunsystems hervorrufen (Zhuang & Dreskin, 2013). Eine der bis heute kontrovers diskutierten Fragen in der Allergenforschung lautet: Welche Eigenschaften machen ein Protein zu einem Allergen? Einerseits

wird vermutet, dass prinzipiell jedes Protein zu einer allergischen Reaktion führen kann, wenn es in ausreichender Menge und im richtigen Kontext in Kontakt mit dem Immunsystem von allergischen Individuen tritt (Aalberse, 2006). Andere Thesen behaupten, dass Allergene spezielle Eigenschaften besitzen und nicht jedes Protein als Allergen wirken kann (Radauer *et al.*, 2008b). Viele Informationen die in den letzten Jahren über Allergene gesammelt wurden bestätigen diese Thesen und deuten darauf hin, dass es gemeinsame Eigenschaften unter allergenen Proteinen gibt (Breiteneder & Radauer, 2004; Jenkins *et al.*, 2007; Radauer & Breiteneder, 2006). Seit Anfang der Achtziger Jahre wurden bis heute hunderte von Allergenen identifiziert und deren Sequenzen entschlüsselt. Sämtliche Allergen-spezifischen Informationen werden dabei laufend in verschiedenen *web*-basierenden Datenbanken hinterlegt. Die von der Weltgesundheitsorganisation offiziell anerkannte Nomenklatur (http://www.allergen.org/) benennt Allergene mit den ersten drei Buchstaben der Gattung, gefolgt von dem ersten Buchstaben der Art der Allergenquelle und einer chronologischen Nummer die vom Zeitpunkt der Isolation abhängt (Chapman *et al.*, 2007). Als Beispiel dient in diesem Kontext das Hauptbirkenpollenallergen aus der europäischen Weißbirke (*Betula verrucosa*) Bet v 1. Die Nebenallergene in Birkenpollen stellen Proteine aus anderen Familien dar und werden dementsprechend als Bet v 2, Bet v 4, Bet v 6 und Bet v 7 bezeichnet. Die Datenbank der Organisation Allergome (http://www.allergome.org/) liefert Informationen über Quelle, Sequenz, Anzahl an Isoallergenen, biologische Funktion und IgE-Reaktivität von Allergenen (Mari *et al.*, 2006). Strukturelle Informationen von mittlerweile über 90 Allergenen können von der SDAP (*Structural Database of Allergic Proteins*, Ivanciuc *et al.*, 2003) abgerufen werden. Im Jahr 2007 wurde schließlich das Projekt AllFam gegründet. Diese Datenbank vereinigt Informationen aus der Allergome- und der Pfam-Datenbank (*Protein families*, http://pfam.sanger.ac.uk/; Finn *et al.*, 2006) um Allergene in Proteinfamilien einzuteilen (Radauer *et al.*, 2008b). Tabelle I gibt einen Überblick aus dieser Datenbank über die Quelle und die Art der Exposition aller bekannten Allergensequenzen und -familien.

Tabelle I: **Anzahl an Sequenzen und Proteinfamilien aller bekannten Allergene in der Datenbank AllFam**. (Radauer *et al.*, 2008b), Stand: September 2014.

	Sequenzen	**Familien**	**Familien mit mehr als einem Allergen**
Gesamt	1023	186	113
Quelle			
Pflanzen	466	73	47
Tiere	382	91	58
Pilze	145	66	28
Bakterien	20	12	2
Art der Exposition			
Inhalation	526	137	83
Nahrungsaufnahme	399	71	42
Stich oder Biss	78	21	10
Kontakt	58	39	11
Autoallergen	24	24	0
Iatrogen	16	12	3

Etwa die Hälfte aller bekannten Allergene kommt in Pflanzen vor, wobei die häufigste Art des Kontakts die Nahrungsaufnahme oder Inhalation darstellt. Bei dieser bioinformatischen Analyse stellte sich heraus, dass sich allergene Proteine in eine sehr begrenzte Anzahl an Proteinfamilien mit wenigen definierten physiologischen Funktionen einteilen lassen (Radauer *et al.*, 2008b). Allergene finden sich demnach in nur 186 (1,2 %) aller in der Pfam-Datenbank bekannten 14831 Proteinfamilien (Pfam 27.0). Die Familien mit den meisten Allergenmitgliedern stellen hierbei die Prolamin-Superfamilie (8,0 %), Kalzium-bindende Proteine (6,1 %), Profiline (4,5 %), Tropomyosine (4,5 %), die Cupin-Superfamilie (4,2 %) und die Familie Bet v 1-verwandter Proteine (*Bet v 1-related protein*, 3,0 %) dar. Diese wenigen Proteinfamilien beinhalten damit über 30 %, in Pflanzen allein sogar über 50 %, aller bekannten Allergensequenzen. Weiterhin weisen 73 Familien nur ein einziges Allergenmitglied auf. Abbildung 2 zeigt die dreidimensionalen Strukturen von Vertretern der prominentesten Familien.

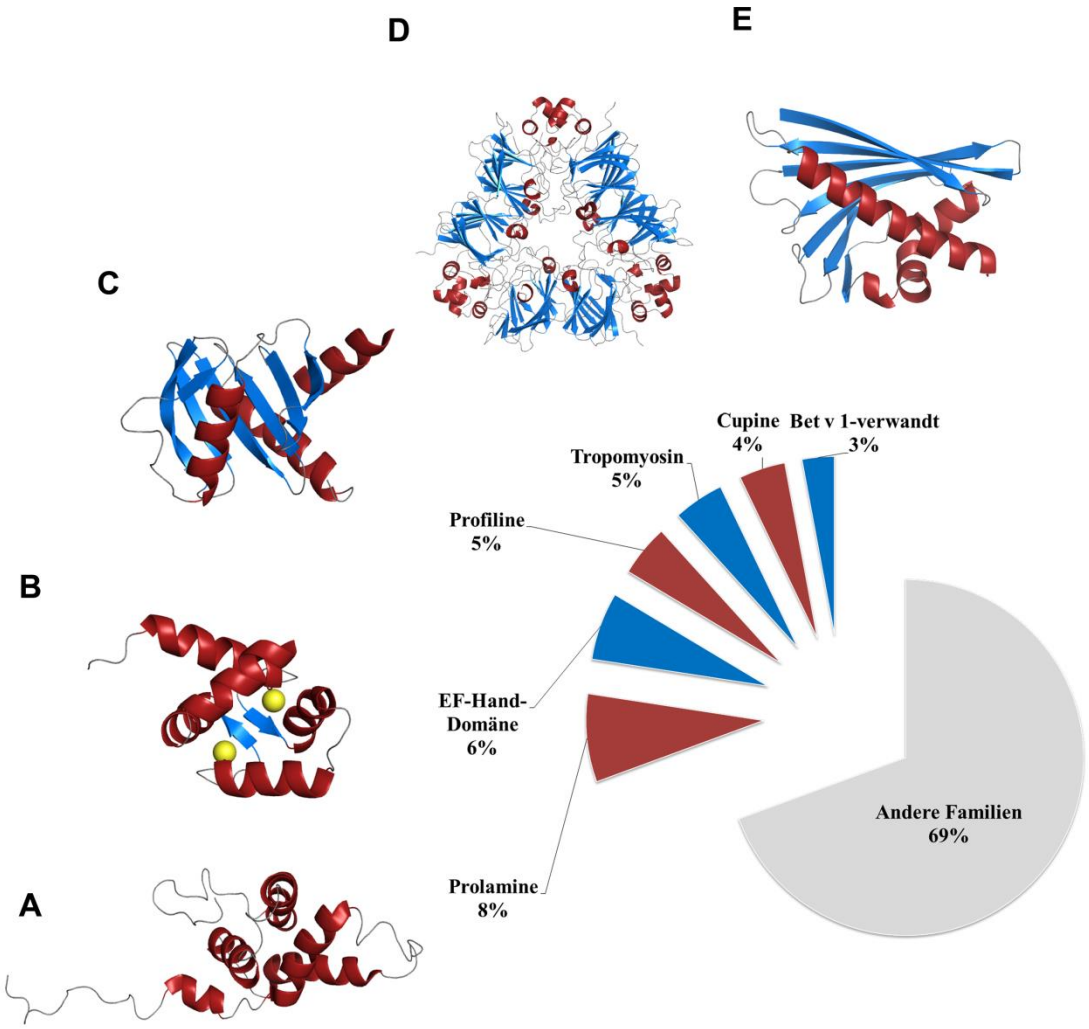

Abbildung 2: Struktur von Allergenen aus den fünf Familien mit den meisten Mitgliedern.
Unstrukturierte Bereiche sind in Grau, α-helikale Bereiche in Rot und β-Faltblätter in Blau dargestellt. (**A**) Das Prolamin Ara h 6 (pdb: 1W2Q), (**B**) Die EF-Hand Domäne Bet v 4 im Komplex mit zwei in Gelb dargestellten Kalzium-Ionen (pdb: 1H4B), (**C**) Das Profilin Bet v 2 (pdb: 1CQA), (**D**) Das Cupin Gly m 6 (pdb: 1FXZ), (**E**) Das Bet v 1-verwandte Sellerieallergen Api g 1 (pdb: 2BK0).

Allergene Proteine finden sich in allen bekannten, durch die SCOP-Datenbank (*Structural Classification of Proteins*) definierten, strukturellen Klassen. Während die Mitglieder der Prolamin-Familie reine α-helikale Strukturen ausbilden (Abbildung 2A), gehören die Profiline, Cupine, EF-

Hand-Domänen und die Bet v 1-verwandten Proteine zu den gemischten α/β-Proteinen (Abbildung 2 B bis E). Proteine aus der Familie der Expansine (2,1 % aller bekannten Allergensequenzen) wie das Graspollenallergen Phl p 3 bilden dagegen reine β-Faltblattstrukturen aus (Schweimer *et al.*, 2008).
Für etwa 75 % der ermittelten Allergensequenzen finden sich in der GOA-Datenbank (*Gene Ontology Annotation*, http://www.ebi.ac.uk/GOA; Ashburner *et al.*, 2000) Einträge über mögliche physiologische Funktionen. Allergene nehmen dabei eine begrenzte Anzahl an molekularen Funktionen, biologischen Prozessen oder zellulären Komponenten ein und wirken u. a. als Hydrolasen und Peptidasen sowie als Metall, Aktin- und Lipidbinder (Radauer *et al.*, 2008b). Während die biologische Rolle von allergenen Proteinen aus den meisten Familien aufgeklärt wurde (Hoffmann-Sommergruber, 2000; Radauer & Breiteneder, 2007; Sinha *et al.*, 2014), ist die Funktion von Allergenen aus der Familie der Bet v 1-verwandten Proteine jedoch weitgehend ungeklärt und wird in der GOA-Datenbank nur sehr allgemein als Abwehrreaktion gegenüber umweltbedingtem Stress beschrieben.

1.3 Bet v 1-homologe Pollen- und Nahrungsmittelallergene

Bet v 1-homologe Proteine werden aufgrund struktureller, sequentieller und funktioneller Eigenschaften in verschiedene Protein-Superfamilien eingeteilt. Aufgrund struktureller Homologien zu Bet v 1 werden mittlerweile elf Proteinfamilien in die Bet v 1 Superfamilie in der SCOP-Datenbank (SCOP:d.129.3, *extended Release* 2.03; Murzin *et al.*, 1995) und 14 Familien aufgrund sequentieller Homologien in den *Clan:Bet v 1 like* (Pfam: CL0209) eingeordnet (Radauer *et al.*, 2008a). Aus funktioneller Betrachtung werden Bet v 1-homologe Proteine dabei in die Familie der *pathogenesis related proteins* (PR) untergeordnet. PR-Proteine sind im Pflanzenreich weit verbreitet und umfassen mittlerweile 17 Klassen, von denen mindestens acht Klassen allergene Protein enthalten (Hoffmann-Sommergruber, 2000; Sinha *et al.*, 2014). Jede dieser Klassen unterscheidet sich dabei in ihrer Primärsequenz, der enzymatischen und serologischen Eigenschaften oder anderer biologischer Funktionen (Sinha *et al.*, 2014). Gemeinsam haben diese Proteine jedoch, dass ihre entsprechenden Gene meistens durch Befall von Pathogenen wie Viren, Bakterien oder Pilzen induziert werden (Lamb *et al.*, 1989; Loon, 1985; Rigden & Coutts, 1988). Das Birkenpollenallergen Bet v 1 und homologe Pollen- und Nahrungsmittelallergene sowie verschiedene nicht-allergene Proteine wie Hauptlatex-proteine, Zytokinin-spezifische Bindungsproteine und das Enzym (s)-Norcoclaurin-Synthase (NCS) werden dabei in die Klasse 10 der PR-Proteine (PR-10, Pfam: PF00407, SCOP:d.129.3.1) eingeordnet (Fernandes *et al.*, 2013). Darunter finden sich beispielsweise Allergene aus Sellerie (Breiteneder *et al.*, 1995), Apfel (Vanekkrebitz *et al.*, 1995), Kirsche (Scheurer *et al.*, 1997), Soja (Crowell *et al.*, 1992), Erdbeere (Karlsson *et al.*, 2004) und aus Pollen verschiedener Bäume der Ordnung *Fagales* (Breiteneder *et al.*, 1989; Breiteneder *et al.*, 1992; Breiteneder *et al.*, 1993). Tabelle II gibt einen Überblick über die bisher identifizierten Allergene aus dieser Familie.

Tabelle II: Allergene Proteine aus der PR-10 Familie. AllFam-Datenbank:AF069, Stand: September 2014.

Name	Quelle	Art der Exposition	Amino-säuren [1]	Sequenzidentität zu Bet v 1.0101 (%)	Uniprot-Eintrag	Bekannte Isoallergene [3]
Act c 8	Kiwi Gold (*Actinidia chinensis*)	Nahrung	158	50	D1YSM4	1
Act d 11	Kiwi Grün (*Actinidia deliciosa*)	Nahrung	149	11	P85524	1
Act d 8	Kiwi Grün (*Actinidia deliciosa*)	Nahrung	156	50	D1YSM5	1
Aln g 1	Erle (*Alnus glutinosa*)	Inhalation	159	81	P38948	1
Api g 1	Sellerie (*Apium graveolens*)	Nahrung	153	41	P49372	2
Ara h 8	Erdnuss (*Arachis hypogaea*)	Nahrung	156	46	Q6VT83	2
Bet ch 1	Chichibubirke (*Betula chichibuensis*)	Inhalation	n.d.[2]	n.d.[2]	C0IVR1	1
Bet co 1	Koreanische Birke (*Betula costata*)	Inhalation	n.d.[2]	n.d.[2]	C0IVP5	1
Bet le 1	Zuckerbirke (*Betula lenta*)	Inhalation	n.d.[2]	n.d.[2]	C0IVQ8	1
Bet n 1	Schwarzbirke (*Betula nigra*)	Inhalation	n.d.[2]	n.d.[2]	C0IVQ4	1
Bet p 1	Asiatische Weißbirke (*Betula platyphylla*)	Inhalation	159	96	Q9AYS2	1
Bet v 1	Weißbirke (*Betula pendula*)	Inhalation	159	100	P15494	18
Car b 1	Hainbuche (*Carpinus betulus*)	Inhalation	159	72	P38949	16
Cas s 1	Kastanie (*Castanea sativa*)	Nahrung/ Inhalation	159	67	Q93YH9	1
Cor a 1	Hasel (*Corylus avellana*)	Nahrung/ Inhalation	159	72	Q08407	10
Cor he 1	Asiatische Hasel (*Corylus heterophylla*)	Inhalation	159	66	A8W7B6	1
Dau c 1	Karotte (*Daucus carota*)	Nahrung	153	37	O04298	7
Fag s 1	Buche (*Fagus sylvatica*)	Inhalation	159	68	Q9ZRU8	1
Fra a 1	Erdbeere (*Fragaria ananassa*)	Nahrung	159	53	Q3T923	7 [4]
Gly m 4	Sojabohne (*Glycine max*)	Nahrung	157	47	P26987	1
Lyc e 4	Tomate (*Lycopersicon esculentum*)	Nahrung	159	44	K4CWC5	1
Mal d 1	Apfel (*Malus domestica*)	Nahrung	158	55	P43211	24
Ost c 1	Europäische Hopfenbuche (*Ostrya carpinifolia*)	Inhalation	159	74	E2GL17	1
Pru ar 1	Aprikose (*Prunus armeniaca*)	Nahrung	159	59	O50001	1
Pru av 1	Kirsche (*Prunus avium*)	Nahrung	159	59	O24248	4
Pru p 1	Pfirsich (*Prunus persica*)	Nahrung	159	59	O2I6V8	1
Pyr c 1	Birne (*Pyrus communis*)	Nahrung	158	57	O65200	1
Que a 1	Weißeiche (*Quercus alba*)	Inhalation	158	58	B6RQS1	4
Rub i 1	Himbeere (*Rubus idaeus*)	Nahrung	n.d.[2]	n.d.[2]	Q0Z8U9	1
Vig r 1	Mungobohne (*Vigna radiata*)	Nahrung	154	44	Q2VU97	1

[1] Anzahl der Aminosäuren unter der Annahmen, dass bei allen Bet v 1-homologen Allergenen das N-terminale Methionin abgespalten wurde (Swoboda *et al.*, 1995).
[2] Anzahl der Aminosäuren und Sequenzidentität nicht definiert, da nur Fragmente der Sequenz hinterlegt wurden.
[3] Anzahl der aktuell mindestens auf mRNA-Ebene nachgewiesenen Isoallergene (http://www.allergen.org/).
[4] Nachgewiesene Isoallergene nach Musidlowska-Persson *et al.*, 2007.

PR-10 Proteine werden aufgrund evolutionärer Genduplikation oder Rekombination von multiplen Genen kodiert und treten als komplexe Mischung verschiedener Isoallergene bzw. Isoformen in Erscheinung (Agarwal & Agarwal, 2014; Schenk *et al.*, 2009). Der Begriff Isoallergen bezeichnet dabei verschiedene, IgE-kreuzreaktive molekulare Formen eines Allergens. In der offiziellen Nomenklatur werden Isoallergene als Allergene einer einzelnen Spezies mit einer Sequenzidentität von mehr als 67 % beschrieben (Chapman *et al.*, 2007). Für das Birkenpollenallergen Bet v 1 sind mittlerweile die entsprechend codierenden Gene für 30 Isoallergene mit putativen Aminosäuresequenzidentitäten von 72,5 % bis 99,4 %, bezogen auf das dominanteste Isoallergen (Bet v 1.0101), nachgewiesen (http://www.allergome.org/). Diese Isoallergene werden durch zwei zusätzliche Nummern von Bet v 1.01 bis Bet v 1.31 unterschieden. Die Bezeichnung Isoform beschreibt des Weiteren polymorphe Varianten eines einzelnen Allergens mit Sequenzidentitäten von über 90 % (Chapman *et al.*, 2007). So werden die 14 Isoformen des Allergens Bet v 1.01 mit zwei weiteren Zahlen als Bet v 1.0101 bis Bet v 1.0114 differenziert.

PR-10 Proteine sind sich in molekularen Eigenschaften wie Größe, Gesamtladung, dreidimensionaler Struktur und im weiteren Sinn auch der Aminosäuresequenz ähnlich. Sie bestehen aus 150 bis 163 Aminosäuren mit entsprechenden molekularen Massen von etwa 15 bis 18 kDa (Agarwal & Agarwal, 2014). Einen hochkonservierten Sequenzbereich stellt die sogenannte Glycin-reiche Schleife oder auch *P-loop* dar. Dieses Motiv umfasst die Sequenzpositionen 47 bis 55 und besteht in den meisten Fällen aus der Konsenssequenz GxGGxGxxK, wobei x für eine variable Aminosäure steht. Die höchste Aminosäurevariabilität findet sich im C-terminalen Bereich der PR-10 Proteine (Fernandes *et al.*, 2013). Bis auf die Abspaltung des N-terminalen Methionins sind keine weiteren posttranslationalen Modifikationen für die allergenen Mitglieder dieser Familie bekannt (Breiteneder *et al.*, 1989; Ipsen & Hansen, 1991; Luttkopf *et al.*, 2002; Swoboda *et al.*, 1995). Die typische PR-10-Faltung besteht aus einem siebensträngigen, antiparallelen β-Faltblatt und zwei kurzen, V-förmig angeordneten α-Helices, welche sich um eine lange C-terminale α-Helix winden (Gajhede *et al.*, 1996; Abbildung 2E). Durch die Anordnung dieser Sekundärstrukturelemente wird ein großer, lösungsmittelzugänglicher hydrophober Hohlraum gebildet.

Trotz jahrzehntelanger Forschung und der Aufklärung von verschiedenen dreidimensionalen Allergenstrukturen ist bis heute die genaue physiologische Funktion sowie die detaillierte Struktur, Anzahl und Lage der Interaktionsoberflächen von PR-10 Allergenen mit IgE (IgE-Epitope) unbekannt. Genau diese Informationen werden jedoch für die Verbesserung der Prävention, Diagnostik und der Therapie von Allergien benötig.

1.3.1 Behandlung von Allergien

Die Allergenforschung liefert trotz steigender Patientenzahlen bis heute keine anerkannte Behandlungsmethode zur Heilung von Allergien. Aktuell werden allergische Symptome entweder durch Allergiekarenz vermieden oder bei unbeabsichtigtem Kontakt mittels Pharmakotherapie (Antiallergika) behandelt (Berin, 2014). Die Verabreichung von Allergie-Medikamenten lindert die Symptome jedoch nur temporär und kann bei einigen Patienten zu Nebenwirkungen wie Kopfschmerzen, Müdigkeit oder Störungen des Magen-Darm-Traktes führen (Dretzke *et al.*, 2013; White *et al.*, 1998). Seit Beginn des zwanzigsten Jahrhunderts befasst sich die spezifische Immuntherapie (SIT) als einzige Therapieform mit der Ursachenbehandlung von Allergien (Akdis & Akdis, 2014; Cavkaytar *et al.*, 2014; Dretzke *et al.*, 2013; Freeman, 1911; Sicherer & Sampson, 2014). Hierbei soll es, vereinfacht ausgedrückt, durch eine steigende Verabreichung zu einer Gewöhnung an ein Allergen kommen, um so eine überschießende Reaktion des Immunsystems im alltäglichen Kontakt mit diesem Allergen zu vermeiden. Der Nachteil der Methode äußert sich durch allergische Nebenreaktionen oder auch, im Extremfall, in lebensbedrohlichen Reaktionen während der Behandlungsdauer (Akdis & Akdis, 2014; Wood *et al.*, 2013). Aktuelle klinische Studien bieten jedoch vielversprechende Ansätze zur Verbesserung der SIT, wie z. B. durch den standardisierten Einsatz von Hypoallergenen oder Allergenfragmenten (Ferreira *et al.*, 2014). Diese Proteine werden

nicht von IgE gebunden und lösen folglich keine oder nur schwache allergische Symptome aus (Ferreira *et al.*, 1996; Ferreira *et al.*, 1998). Ein immunologischer Effekt äußert sich jedoch in der Anregung von Allergen-spezifischen T- und B-Zell, wobei ein Klassenwechsel von IgE hin zu IgG hervorgerufen wird (Purohit *et al.*, 2008; Vrtala *et al.*, 2001). Die IgG-Antikörper konkurrieren mit Allergen-spezifischem IgE um die Bindung an das Allergen und können somit die Ausschüttung von proinflammatorischen Mediatoren wie Histamin blockieren. Allerdings werden neben dem Klassenwechsel noch viele weitere immunologische Faktoren beeinflusst, die im Augenblick noch nicht umfassend verstanden sind und weiterer Forschung bedürfen (Akdis & Akdis, 2014). Bei vielen Patienten zeigt sich trotz jahrelanger Behandlung keine Verbesserung der Symptome oder die Behandlungserfolge sind nicht von Dauer. Wie von Denépoux *et al*. gezeigt, können Allergen-spezifische IgG-Antikörper sogar zu einer Verstärkung der IgE-induzierten Symptome führen (Denépoux *et al.*, 2000). Zur weiteren Verbesserung der SIT besteht daher ein gesteigertes Interesse an der Herstellung von sicheren, hypoallergenen Allergenvarianten. Zusätzlich sind für PR-10 Allergene vieler Pflanzen nur wenige, vorwiegend hyperallergene Isoformen bekannt (siehe Tabelle II), die sich nicht für eine symptomlose SIT eigenen würden.

1.3.2 Molekulare Grundlage der Kreuzallergie zwischen PR-10 Allergenen

Einer der häufigsten Gründe für allergische Symptome in Nord– und Mitteleuropa sowie in Nordamerika stellen Allergene aus Birkenpollen dar (Lin *et al.*, 2002; Stevens *et al.*, 2003). Etwa 98 % aller Birkenpollenallergiker in Österreich, Finnland und Schweden sind gegen das Hauptbirken-pollenallergen Bet v 1 sensibilisiert (Movérare *et al.*, 2002). Typischerweise entwickeln bis zu 70 % der Birkenpollenallergiker auch Kreuzallergien gegen verschiedene Früchte- und Gemüsesorten (Dreborg, 1988; Geroldinger-Simic *et al.*, 2011; Vieths *et al.*, 2002). Dieses Phänomen äußert sich nach dem Nahrungsmittelkontakt durch Jucken oder Anschwellen von Lippen, Zunge oder Hals und wird als orales Allergiesyndrom (o*ral allergy syndrome*) bezeichnet (Ausukua *et al.*, 2009; Mari *et al.*, 2005). Der Grund für diese Effekte wird in der Ähnlichkeit der dreidimensionalen Strukturen von Pollen– und Nahrungsmittelallergenen vermutet. Trotz Sequenzidentitäten von teilweise unter 50 % (Tabelle II), zeigen Bet v 1-homologe Pollen- und Nahrungsmittelallergene eine beinahe identische Tertiärstruktur (Dall'Antonia *et al.*, 2014; Fernandes *et al.*, 2013). Abbildung 3A veranschaulicht dies durch Überlagerung der Allergenstrukturen aus Birke (Bet v 1), Karotte (Dau c 1), Soja (Gly m 4) und Kirsche (Pru av 1).

Das aufgrund der Sensibilisierung gebildete Bet v 1-spezifische IgE bindet strukturelle Epitope auf der Oberfläche des Birkenpollenallergens und erkennt diese strukturellen Muster ebenfalls auf Bet v 1-homologen Allergenen (Geroldinger-Simic *et al.*, 2011). Eine Vorhersage dieser durch IgE erkannten Epitope aufgrund der Allergensequenz ist daher kaum möglich. Bis heute konnte nur ein IgE-Epitop auf dem Hauptbirkenpollenallergen indirekt mittels Co-Kristallisation von Bet v 1.0112 im Komplex mit dem Fab-Fragment (*antigen binding fragment*) des monoklonalen IgG_1 Maus-Antikörpers BV16

identifiziert werden (Mirza *et al.*, 2000). In Abbildung 3B ist die Struktur dieses Antikörper-Allergen-Komplexes dargestellt. Das bestimmte Epitop, welches den Bereich um die hochkonservierte Glycin-reiche Schleife umfasst (Aminosäuren E^{42} bis T^{52}, R^{70}, D^{72}, H^{76}, I^{86} und K^{97}, Abbildung 3C), überlappt vermutlich mit einem IgE-Epitop, da das Maus-Antikörperfragment die Binding von IgE an Bet v 1 teilweise inhibiert (Mirza *et al.*, 2000). In anschließenden Studien wurde der für die Antikörperbindung kritische Rest E^{45} der Allergene Bet v 1 und Pru av 1 mutiert. Beide Varianten zeigten daraufhin eine signifikant niedrigere IgE-Bindung als die nativen Proteine und bestätigten somit die Glycin-reiche Schleife als Teil eines IgE-Epitops von PR-10 Allergenen (Neudecker *et al.*, 2003; Spangfort *et al.*, 2003). Verschiedene Gruppen konnten bereits in den neunziger Jahren zeigen, dass Bet v 1-Isoallergene trotz hoher Sequenzidentitäten erstaunliche Unterschiede in der IgE-Bindung aufweisen (Arquint *et al.*, 1999; Ferreira *et al.*, 1996; Swoboda *et al.*, 1995). Hierbei wurde zwischen stark (hyperallergen), intermediär und schwach (hypoallergen) IgE-bindenden Isoallergenen unterschieden. Durch Sequenzvergleiche und ergänzende Mutationsstudien konnten daraufhin weitere Reste identifiziert werden, die für die IgE-Interaktion mit Bet v 1 oder Mal d 1 wichtig sind (Ferreira *et al.*, 1998; Ma *et al.*, 2006).

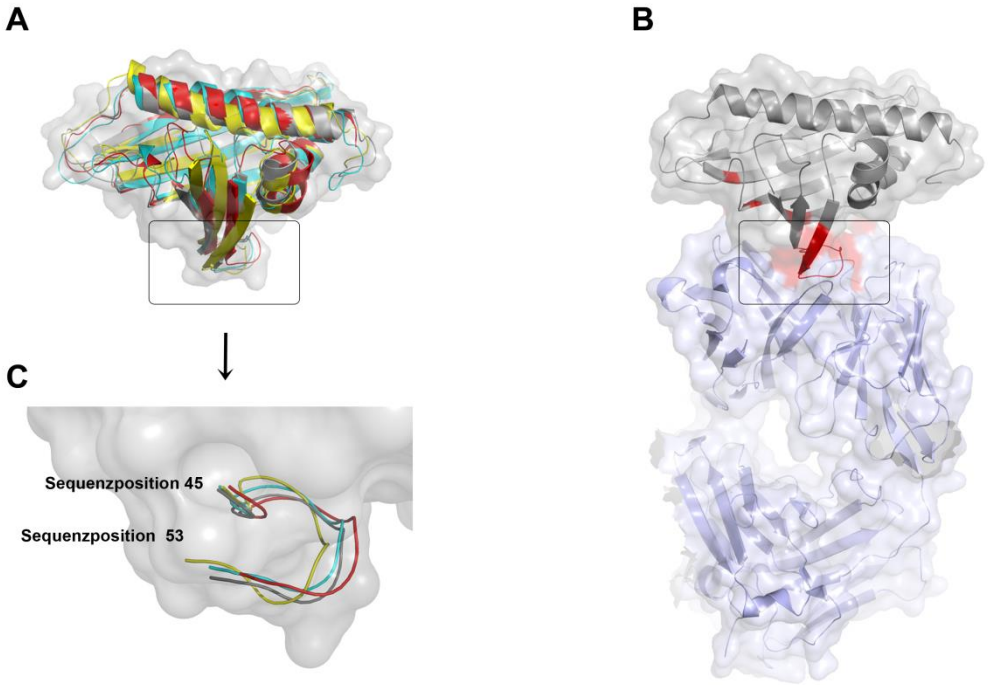

Abbildung 3: Die Glycin-reiche Schleife von Bet v 1 stellt die Interaktionsfläche für den IgG-Antikörper BV16 dar.
(**A**) Überlagerung der dreidimensionalen Strukturen von Bet v 1 (Grau, transparente Oberflächendarstellung in Hellgrau, pdb: 1BV1), Dau c 1 (Rot, pdb: 2WQL), Gly m 4 (Gelb, pdb: 2K7H) und Pru av 1 (Cyan, pdb: 1E09). Der Bereich um die Glycin-reiche Schleife ist eingerahmt. (**B**) Komplexstruktur aus Bet v 1.0112 (grau, transparente Oberflächendarstellung in Hellgrau) und dem Fab-Fragment des BV16-Antikörpers (Blau, transparente Oberflächendarstellung in Hellblau, pdb: 1FSK). Die Epitop-darstellenden Aminosäuren auf Bet v 1 sind in Rot markiert. Der Bereich um die Glycin-reiche Schleife ist eingerahmt. (**C**) Die Glycin-reiche Schleife ist unter Bet v 1-homologen Proteinen sequentiell und strukturell hochkonserviert. Dargestellt ist der Sequenzbereich der Aminosäurepositionen 45 bis 53 für Bet v 1 (grau, transparente Oberflächendarstellung in Hellgrau), Dau c 1 (Rot), Gly m 4 (Gelb) und Pru av 1 (Cyan).

Neben diesen etablierten Methoden stellt das *epitope grafting* eine weitere Möglichkeit der Identifikation von Epitopen dar. Bei dieser speziellen Form des *protein grafting* geht es darum, ein Epitop durch Mutation bestimmter Aminosäuren von einem Protein auf ein zweites strukturell

ähnliches Protein zu übertragen. Für die Charakterisierung von IgE-Epitopen benötigt man dazu ein dem Allergen strukturell ähnliches Protein, das jedoch keine Kreuzreaktivität aufweist (Rösch & Berkner, 2009). Anwendung fand diese Methode bereits bei der Untersuchung von Epitopen, die für Kreuzreaktivitäten zwischen Bet v 1 und Mal d 1 sowie Api g 1 verantwortlich sind (Gepp *et al.*, 2014; Hecker *et al.*, 2012; Holm *et al.*, 2011).

1.3.3 Physiologische Funktion von PR-10 Allergenen

Bei den Mitgliedern der PR-10 Familie handelt es sich um intrazelluläre, zytoplasmatische Proteine, deren Gene durch verschiedene abiotische oder pathogene Stressbedingungen induziert werden können (Liu & Ekramoddoullah, 2006). Für Bet v 1 und einige weitere PR-10 Proteine konnte eine schwache Ribonuklease-Aktivität *in vitro* nachgewiesen werden, die zur Pathogen-Abwehr dienen könnte (Bantignies *et al.*, 2000; Bufe *et al.*, 1996; Park *et al.*, 2004; Swoboda *et al.*, 1996). Die in Bet v 1-homologen Proteinen hochkonservierte Glycin-reiche Schleife wird ebenfalls in Nukleotid-bindenden Proteinen gefunden (Saraste *et al.*, 1990) und wurde in diesem Kontext als Binderegion vorgeschlagen. Daher liegt der Schluss nahe, dass diese Proteine eine schützende Funktion in Pflanzen einnehmen. Andererseits werden einige Vertreter auch konstitutiv in verschiedenen Pflanzen-komponenten exprimiert (Breda *et al.*, 1996; Crowell *et al.*, 1992; Fernandes *et al.*, 2013; Sikorski *et al.*, 1999). Für das Erdbeerallergen Fra a 1 konnte beispielsweise gezeigt werden, dass verschiedene Isoallergene in unterschiedlichen Wachstumsphasen und Pflanzenteilen exprimiert werden (Li *et al.*, 2013; Munoz *et al.*, 2010). Diese Erkenntnisse lassen darauf schließen, dass PR-10 Proteine auch eine generelle biologische Rolle, z. B. während der Pflanzenentwicklung einnehmen könnten (Sinha *et al.*, 2014). Der erste Schritt zur Bestimmung einer definierten physiologischen Funktion dieser Proteine war die Aufklärung der dreidimensionalen Struktur von Bet v 1 im Jahr 1996 (Gajhede *et al.*, 1996). Der hydrophobe Hohlraum im Inneren des Proteins, sowie die strukturelle Ähnlichkeit zu der START-Domäne des humanen Cholesterin-Transportproteins MLN64 lassen auf eine Funktion als Transporter– oder Lagerprotein schließen (Tsujishita & Hurley, 2000). In Abbildung 4A ist dieser hydrophobe Hohlraum schematisch dargestellt. Er ist etwa 30 Å lang, weist ein Volumen von ca. 1500 $Å^3$ auf und ist durch drei Öffnungen lösungsmittelzugänglich (Gajhede *et al.*, 1996). In darauffolgenden Bindungsstudien wurde die Interaktion von einigen hydrophoben und amphiphilen Molekülen mit verschiedenen PR-10 Proteinen analysiert (Bais *et al.*, 2003; Handschuh *et al.*, 2007; Koistinen *et al.*, 2005). Am besten untersucht ist dabei das Birkenpollenallergen Bet v 1. Mogensen *et al*. konnten die Interaktion von physiologisch relevanten Molekülen wie Zytokininen, Flavonoiden, Fettsäuren oder Phospholipiden mit rekombinantem Bet v 1.2801 *in vitro* nachweisen (Mogensen *et al.*, 2002; Mogensen *et al.*, 2007). Weiterhin konnten die Kristallstrukturen von Bet v 1.0107 im Komplex mit zwei Molekülen Deoxycholat (Abbildung 4B) und Bet v 1.0101:Naringenin (Abbildung 4C) aufgeklärt werden (Kofler *et al.*, 2012; Markovic-Housley *et al.*, 2003). Aufgrund der Ähnlichkeit von Deoxycholat zu den Brassinosteroiden und der experimentell bestimmten Interaktion von Pru av 1

mit Homocastasteron (Neudecker *et al.*, 2001) wurde eine Funktion als Hormontransporter für PR-10 Proteine vorgeschlagen. Weiterhin wurde aufgrund von Docking-Simulationen und der teilweisen strukturellen Ähnlichkeit von Bet v 1 zu den Lipocalinen eine Funktion als Eisentransporter angedeutet (Roth-Walter *et al.*, 2014). Diese Hypothese ist jedoch nicht durch experimentelle Daten gestützt. Dem Erdbeerallergen Fra a 1 wurde eine regulatorische Rolle während der Biosynthese des Erdbeerfarbstoffes zugeschrieben, da in Erdbeermutanten eines farblosen Phänotyps, die Produktion von Fra a 1 herunterreguliert ist (Hjerno *et al.*, 2006; Munoz *et al.*, 2010). Casañal *et al*. konnten daraufhin zeigen, dass Fra a-Isoallergene tatsächlich verschiedene physiologische Flavonoide und Flavonoid-Derivate *in vitro* binden können (Casañal *et al.*, 2013b).

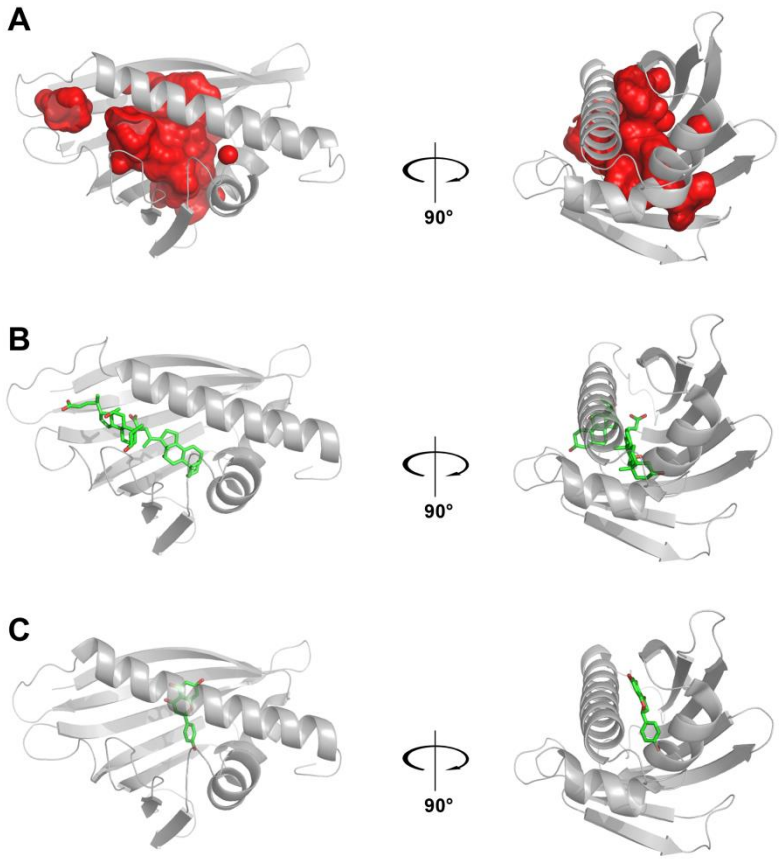

Abbildung 4: Schematische Darstellung des hydrophoben Hohlraums und Bindungsregion von Liganden im Innenraum von Bet v 1.
Bet v 1 Isoallergene sind in Grau dargestellt, Liganden sind in grünen Stäbchen, Sauerstoffe in Rot gezeigt. (**A**) Die Oberfläche des hydrophoben Hohlraums in Bet v 1.0112 (pdb: 1BV1) ist in Rot dargestellt. (**B**) Kristallstruktur des Isoallergens Bet v 1.0107 im Komplex mit zwei Molekülen Deoxycholat (pdb: 1FM4). (**C**) Komplexstruktur von Bet v 1.0101:Naringenin (pdb: 4A87).

Trotz dieser umfassenden Interaktionsstudien konnte jedoch bis heute kein physiologischer Ligand im Komplex mit einem PR-10 Protein aus einer natürlichen Quelle identifiziert werden. Weiterhin ist es aufgrund der Existenz von zahlreichen Isoformen eines Allergens nicht möglich eine exakte Funktion zu definieren. Wegen ihrer weiten Verbreitung im Pflanzenreich und der sich unterscheidenden Anzahl an Isoallergenen zwischen verschiedenen Organismen (Tabelle II) ist anzunehmen, dass PR-10 Proteine eine wichtige, multifunktionale Rolle als Ligand-Transporter im Pflanzenreich einnehmen.

2 Ziele der Arbeit

Der Schwerpunkt dieser Arbeit lag auf der physiologischen Charakterisierung des Hauptbirkenpollenallergens Bet v 1. Seit der Bestimmung der dreidimensionalen Struktur wird vermutet, dass dieses Protein für die Lagerung oder den Transport von verschiedenen Liganden verantwortlich ist. Allerdings konnten bis heute keine physiologischen Interaktionspartner nachgewiesen werden. Durch die Isolation und Reinigung des Allergens direkt aus reifen Birkenpollen sollten gezielt natürliche Liganden identifiziert werden, um auf physiologische Funktionen von Bet v 1 rückzuschließen. Im Anschluss sollte der Einfluss dieser Liganden auf die IgE-Bindungseigenschaften von Bet v 1 analysiert werden, um ein globaleres Bild der allergischen Sofortreaktion zu erhalten. Bis heute ungeklärte ist die Rolle verschiedener Bet v 1 Isoallergene. Hierfür sollten ausgewählte Isoallergene mittels eines bakteriellen Expressionssystem rekombinant hergestellt und die Interaktion mit möglichen natürlichen Liganden charakterisiert werden.

Im Gegensatz zum Birkenpollenallergen ist das Erdbeerallergen Fra a 1 nur sehr eingeschränkt untersucht. Ein wichtiger erster Schritt für die Identifikation der physiologischen Funktion dieses Allergens ist die Bestimmung seiner Struktur. Vor diesem Hintergrund sollte die dreidimensionale Struktur des Erdbeerallergens mittels NMR-Spektroskopie aufgeklärt und die Interaktion mit möglichen natürlichen Bindungspartnern analysiert werden.

Ergänzend zu diesen physiologischen Untersuchungen sollten immunologische Studien zur Analyse der, von IgE erkannten Epitope, von PR-10-Allergenen beitragen. Bis heute ist die genaue Position, Anzahl und klinische Relevanz dieser Epitope ungeklärt. Das Enzym NCS aus der gelben Wiesenraute ist strukturell homolog zu Bet v 1, zeigt jedoch keine Interaktion mit IgE und ist somit geeignet für das sogenannte *epitope grafting*. Hierbei sollte gezielt ein potentielles IgE-Epitop des Birkenpollenallergens Bet v 1 auf die NCS übertagen werden. Mit Hilfe dieses Systems könnte die zukünftige Identifikation weiterer Epitope von Pollen– und Nahrungsmittelallergenen ermöglicht werden.

3 Zusammenfassung und Diskussion der Ergebnisse

3.1 *Epitope grafting* – Ein System zur Epitop-Analyse von PR-10 Allergen

Für die Herstellung von hypoallergenen Varianten für die Behandlung von Allergien (siehe 1.3.1), spielt die Identifikation von IgE-Epitopen eine zentrale Rolle. Die vielversprechendste (jedoch auch anspruchsvolle) Methode zur Epitop-Aufklärung stellt die Kristallisation von Antikörper-Allergen-Komplexen dar. Da die Röntgenstrukturanalyse homogene Proteinproben benötigt, eignet sich polyklonales IgE aus den Blutseren von Allergikern nicht zur Co-Kristallisation mit Allergenen. Folglich stellt die Beschaffung von humanem, monoklonalem IgE das Hauptproblem bei der Einzelanalyse von Epitopen dar (Mirza *et al.*, 2000). Bis heute konnten lediglich IgE-Epitope der Allergene Phl p 2 (Graspollen) und β-Lactoglobulin (Kuhmilch) durch Co-Kristallisation eindeutig bestimmt werden (Niemi *et al.*, 2007; Padavattan *et al.*, 2009). Mit Hilfe der NMR-Spektroskopie konnten vor kurzem einige Reste des Isoallergens Bet v 1.0101 identifiziert werden, die bei der Interaktion mit polyklonalem IgE von Bedeutung sind (Asam *et al.*, 2014). Die exakte Lage und damit die klinische Relevanz von IgE-Epitopen der PR-10 Familie sind jedoch, trotz zahlreicher Studien (Ferreira *et al.*, 1998; Hecker *et al.*, 2012; Ma *et al.*, 2006; Neudecker *et al.*, 2003; Son *et al.*, 1999; Wangorsch *et al.*, 2007) unbekannt.

Im Rahmen von Einzelarbeit A wurde daher ein nicht allergenes Protein mit einer typischen PR-10 Tertiärstruktur verwendet, um durch schrittweise Variation von Aminosäuren eine IgE-Bindung zu induzieren und somit IgE-Epitope auf molekularer Ebene analysieren zu können (Rösch & Berkner, 2009). Ein Vertreter der PR-10 Familie ohne bekannte allergene Eigenschaften ist das Enzym (s)-Norcoclaurin-Synthase (Samanani *et al.*, 2004) aus der gelben Wiesenraute (*Thalictrum flavum*). Trotz vergleichsweiser niedriger Sequenzidentitäten von 25 % bis 38 % zu anderen Bet v 1-homologen Proteinen, weist die NCS die typische PR-10 Faltung auf (Berkner *et al.*, 2007; Berkner *et al.*, 2008; Ilari *et al.*, 2009). Auf Grundlage des mit IgE überlappenden Bet v 1-Epitops des IgG-Antikörpers BV16 (siehe 1.3.2), wurden die zwei Varianten $\Delta 29NCS_{N57/I58E/D60N/V63P}$ (Δ29NCS_4x) und $\Delta 29NCS_{N57/I58E/D60N/V63P/D68K}$ (Δ29NCS_5x) konstruiert. Diese Aminosäurepositionen stimmen mit den entsprechenden Epitop-bildenden Bet v 1-Aminosäuren (N^{43}, E^{45}, N^{47}, P^{50}, K^{55}) überein (Abbildungen 1 und 2A aus Einzelarbeit A). Da IgE konformative Epitope erkennt, wurde eingangs analysiert, ob durch die Aminosäurevariationen strukturelle Änderungen der PR-10-Faltung von Δ29NCS hervorgerufen wurden. Die CD- und NMR-Spektren der beiden Varianten Δ29NCS_4x und Δ29NCS_5x überlagern beinahe vollständig mit dem jeweiligen Referenzspektrum von Δ29NCS (Abbildung 5A, B). Lediglich die Signale der ausgetauschten Aminosäuren und deren unmittelbarer Nachbaren zeigten erwartungsgemäß veränderte Resonanzfrequenzen im ^{1}H-^{15}N-HSQC-Spektrum (*heteronuclear single quantum coherence*). Die Integrität einer Bet v 1-typischen Sekundär- und Tertiärstruktur der Δ29NCS-Varianten wurde somit bestätigt (Abbildungen S1, 2B und C aus Einzelarbeit A). Um die potentiellen IgE-Epitope der Varianten zu analysieren, wurde die Bindung

von polyklonalem IgE aus den Blutseren von 70 Birkenpollenallergikern mit den drei Δ29NCS-Varianten, Bet v 1 und vier weiteren PR-10 Allergenen gemessen und quantifiziert (Einzelarbeit A, Tabelle S1). Erwartungsgemäß konnte in allen Seren eine starke IgE-Interaktion mit Bet v 1 und den kreuzreaktiven Nahrungsmittelallergenen Pru av 1 (Kirsche), Cor a 1 (Haselnuss), Dau c 1 (Karotte) und Api g 1 (Sellerie) detektiert werden. In lediglich 6 % der Seren wurde eine IgE-Interaktion mit Δ29NCS nachgewiesen. Die beiden NCS-Varianten zeigten hingegen eine signifikante IgE-Interaktion mit 36 % (Δ29NCS_4x) bzw. sogar 71 % (Δ29NCS_5x) der verwendeten Seren. Durch die eingeführten Variationen, konnte offensichtlich die IgE-Bindung an ein ursprünglich nicht-allergenes Protein ermöglicht und schrittweise gesteigert werden.

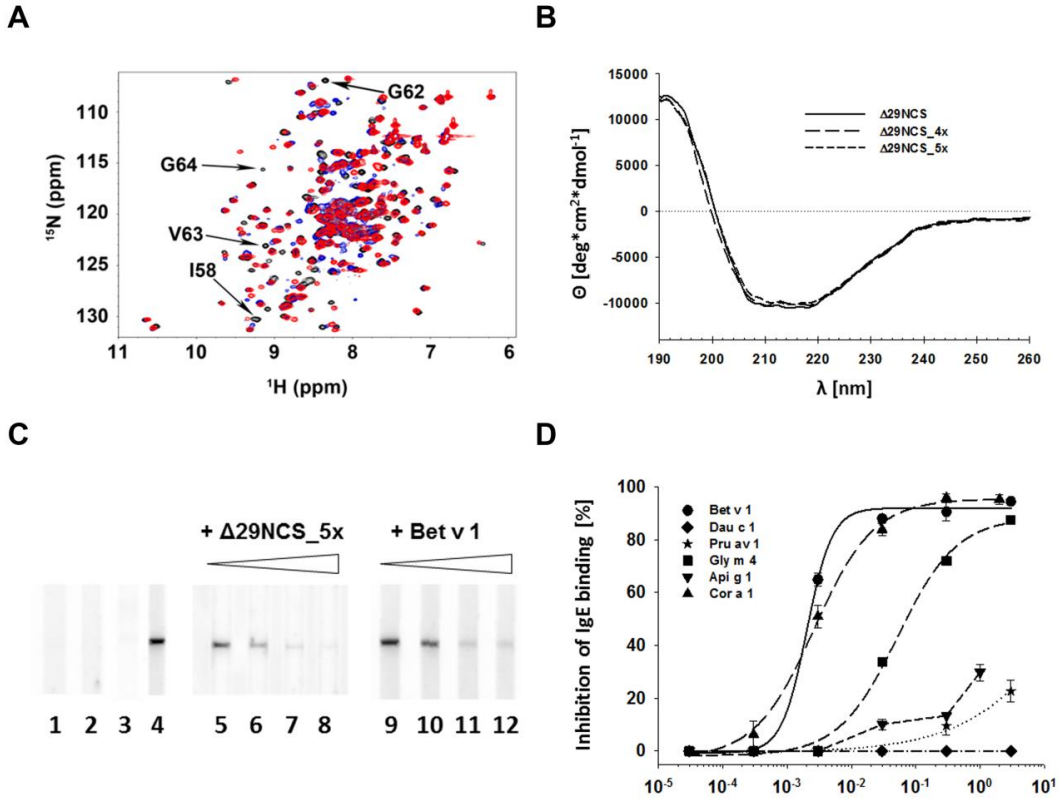

Abbildung 5: Nachweis der strukturellen Integrität und IgE-Interaktionseigenschaften von Δ29NCS_5x.
Abbildung modifiziert aus Einzelarbeit A, Abbildungen 2 und 3 (**A**) Überlagerung der ^{1}H-^{15}N-HSQC-Spektren von Δ29NCS (Schwarz), Δ29NCS_4x (Blau) und Δ29NCS_5x (Rot). Die Pfeile deuten auf Signale von variierten Aminosäuren (bezogen auf Δ29NCS) hin, die in den entsprechenden Varianten erwartungsgemäß unterschiedliche Resonanzfrequenzen aufweisen. (**B**) Überlagerung der CD-Spektren der drei Δ29NCS-Varianten. (**C**) Inhibition der IgE-Bindung an immobilisiertes (0,5 µg) Δ29NCS_5x. Das Δ29NCS_5x-spezifische Serum (Einzelarbeit A, Tabelle S1: Serum 23) wurde in Abwesenheit oder Gegenwart steigender Konzentrationen an Δ29NCS_5x (Bahnen 5 bis 8) oder Bet v 1 (Bahnen 9 bis 12) als primärer Antikörper im Immunblot verwendet. Kontrollexperimente in den Bahnen 1 (kein Serum), 2 (negativ Serum), 3 (0,5 µg Δ29NCS) und 4 (0,5 µg Δ29NCS_5x). Sowohl Bet v 1 als auch Δ29NCS_5x selbst inhibiert die Bindung an immobilisiertes Δ29NCS_5x (**D**) Inhibition der IgE-Bindung an immobilisiertes Δ29NCS_5x in Gegenwart steigender Konzentrationen Bet v 1-homologer Allergene.

Im Folgenden wurde untersucht, ob es sich bei dem konstruierten IgE-Epitop von Δ29NCS_5x um ein Bet v 1-spezifisches oder ein allgemeines PR-10-Epitop handelt. Hierfür wurde zunächst ein Serum (Tabelle S1, Serum 23 aus Einzelarbeit A) mit Bet v 1- und Δ29NCS_5x-spezifischem IgE als primärer Antikörper im Immunoblot verwendet (Abbildung 5C). Die Bindung von IgE an Membran-

gebundenes Δ29NCS_5x wird konzentrationsabhängig durch Bet v 1 und ebenso durch freies Δ29NCS_5x inhibiert. Diese Ergebnisse weisen darauf hin, dass ein gemeinsames Epitop der beiden Proteine von IgE erkannt wird. Die Kreuzreaktivität des Δ29NCS_5x-Epitops zu anderen PR-10 Allergenen wurde mittels kompetitivem IgE-Interaktionstest mit Gly m 4 (Soja), Bet v 1, Pru av 1, Cor a 1, Dau c 1 und Api g 1 analysiert (Abbildung 5D). Die Bindung an IgE wurde von Cor a 1 und Gly m 4 sowie erwartungsgemäß von Bet v 1, inhibiert. Die Allergene Pru av 1, Dau c 1 und Api g 1 konkurrieren dagegen kaum um die Interaktion mit IgE. Das konstruierte Δ29NCS_5x-Epitop stellt offensichtlich kein generelles, sondern vielmehr ein Epitop einer bestimmten Gruppe von PR-10-Allergenen dar.

In weiteren Experimenten konnte gezeigt werden, dass eine strukturelle Überlappung des Δ29NCS_5x–IgE-Epitops und des Bet v 1-BV16-Epitop vorliegt (Abbildung 3C und D aus Einzelarbeit A). Ein struktureller Vergleich des Δ29NCS_5x-Epitops und der entsprechenden PR-10-Allergenoberflächen offenbarte, dass die Bet v 1-Reste N^{43}, E^{45}, N^{47} und K^{55} ebenfalls in Δ29NCS_5x, Gly m 4 und Cor a 1, jedoch nicht in den Strukturen von Dau c 1, Api g 1, Pru av 1 und Δ29NCS gefunden werden (Abbildung 4 aus Einzelarbeit A). Zur experimentellen Bestätigung wurde die IgE/IgG-Interaktion der Variante Bet v $1_{N43I/E45S/N47D/K55A}$ (Bet v 1_4x) untersucht. Bet v 1_4x zeigt ein identisches Bindungsverhalten gegenüber polyklonalem IgE wie Bet v 1, die Affinität zu BV16 wurde jedoch aufgehoben (Abbildung 5 aus Einzelarbeit A). Aus der Gesamtheit der Experimente lässt sich schlussfolgern, dass ein Epitop auf Δ29NCS_5x konstruiert wurde, welches von einer bestimmten Gruppe an PR-10-Allergenen ausgebildet wird. Dabei wird die Interaktion mit kreuzreaktivem IgE/IgG signifikant von den entsprechenden Bet v 1-Aminosäurepositionen (N^{43}, E^{45}, N^{47} und K^{55}) in Δ29NCS_5x (N^{57}, E^{58}, N^{60} und K^{68}) beeinflusst.

Das hier vorgestellte, auf *epitope grafting* basierende System, weist einige Vorteile zur Identifikation von PR10-Epitopen im Vergleich zu anderen Methoden auf. Das „Grundgerüst“ von Δ29NCS zeigt keine Interaktion mit polyklonalem IgE im Vergleich zu Varianten von hyperallergenen Proteinen. Die Induktion einer IgE-Bindung ist somit (vorausgesetzt die strukturelle Integrität wird nicht verändert) auf die variierten Aminosäuren zurückzuführen. Weiterhin kann die Epitop-Analyse mit polyklonalem IgE aus dem Blutserum von Allergikern durchgeführt werden und ist nicht abhängig von rekombinantem monoklonalem IgE. Folglich kann sich, abhängig von den Variationen bestimmter Aminosäuren, ein IgE-Bindungsprofil für Allergene der PR-10 Familie erstellen lassen. Im Idealfall können dabei schrittweise einzelne IgE-Epitope mit polyklonalem IgE identifiziert werden und langfristig hypoallergene Varianten für die individuelle Behandlung von Birken- und Nahrungsmittelallergikern konstruiert werden.

3.2 Strukturelle Besonderheiten des Erdbeerallergens Fra a 1

Das Erdbeerallergen Fra a 1 wurde 2004 von Karlsson *et al*. erstmals in reifen Früchten nachgewiesen und ist damit ein vergleichsweise spät identifiziertes Mitglied der PR-10 Familie (Karlsson *et al.*,

2004). Bis heute konnten sieben Fra a-Isoallergen identifiziert werden. Hierbei handelt es sich um die fünf Isoformen Fra a 1A bis E, die nur sieben variable Aminosäurepositionen aufweisen (Musidlowska-Persson *et al.*, 2007) und zwei zusätzlich identifizierte Isoallergene von Fra a 1, die jedoch entgegen der offiziell anerkannten Nomenklatur, als Fra a 2 und Fra a 3 benannt wurden (79 % und 76 % Sequenzidentität zu Fra a 1E; Munoz *et al.*, 2010). Im Rahmen dieser Arbeit wurden die rekombinanten Isoallergene Fra a 1E, Fra a 2 und Fra a 3 analysiert. Mit Hilfe eines bakteriellen Expressionssystems konnten die drei Proteine produziert und durch verschiedene chromatographische Methoden in hoher Reinheit gewonnen werden. Das Isoallergen Fra a 1E wurde dabei auch in isotopenangereichertem Minimalmedium produziert und gereinigt. Bei dieser Technik werden die sonst nicht oder nur unzureichend detektierbaren Stickstoff– und Kohlenstoffatome des Proteins durch die NMR-aktiven Isotope ^{15}N und ^{13}C ersetzt. Mit Hilfe der daraufhin ermöglichten Tripelresonanz- und NOESY-Experimente (*nuclear overhauser enhancement spectroscopy*) konnte eine experimentelle Struktur von Fra a 1E in Lösung bestimmt werden (pdb: 2LPX, Abbildung 6A). Fra a 1E weist eine beinahe identische Tertiärstruktur wie alle bisher bekannten Strukturen von PR-10 Proteinen auf (Abbildung 4 aus Einzelarbeit B). Ein siebensträngiges antiparalleles β-Faltblatt (β_1 bis β_7) und zwei kurze V-förmig angeordnete α-Helices (α_1) bilden mit einer 23 Aminosäuren umfassenden C-terminalen α-Helix (α_2) einen hydrophoben Hohlraum aus (Abbildung 6A, B). Die größte strukturelle (1,04 Å *root-mean-square deviation* für die Sekundärstrukturelemente) wie auch sequentielle (79,4 %) Ähnlichkeit zu Fra a 1E zeigt das Kirschallergen Pru av 1.

Trotz dieser erwartungsgemäß identischen Tertiärstruktur, weist Fra a 1E einige feine Unterschiede im Vergleich zu anderen PR-10 Strukturen auf. Die β-Faltblattstränge β_5 und β_6 sind auf den Innenraum bezogen nach außen gebeugt (Abbildung 4 aus Einzelarbeit B) und die Aminosäuren E^{131} bis E^{139} der C-terminalen Helix sind zum Lösungsmittel hin und nicht wie in anderen PR-10 Proteinen zum Eingang des hydrophoben Hohlraums orientiert. Diese strukturellen Unterschiede sowie die Anordnung der Schleife von P^{36} bis A^{38} und der flexiblen Schleife von T^{58} bis T^{67} tragen zur Vergrößerung des Volumens und den Zugängen zur hydrophoben Tasche von Fra a 1E im Vergleich zu anderen PR-10 Allergenen bei (Abbildungen 3 und 5 aus Einzelarbeit B). Fra a 1E zeigt mit einem Volumen von etwa 2500 $Å^3$ einen deutlich größeren hydrophoben Hohlraum als Bet v 1, Pru av 1 oder Gly m 4 mit Volumina von jeweils etwa 1600 $Å^3$. Auffällig ist hierbei, dass sich die Volumina der hydrophoben Taschen einiger PR-10 Proteine sogar noch durch die Bindung von Liganden vergrößern (Fernandes *et al.*, 2008; Markovic-Housley *et al.*, 2003). Diese Erkenntnisse lassen darauf schließen, dass Fra a 1E zur Bindung von größeren, sperrigen Liganden oder auch zur Aufnahme von mehreren Molekülen gleichzeitig, wie etwa Pru av 1 aus Kirsche (Neudecker *et al.*, 2001), in der Lage ist.

Da in Erdbeermutanten eines farblosen Phänotyps die Produktion von Fra a 1 herunterreguliert ist (Hjerno *et al.*, 2006; Munoz *et al.*, 2010), wurde nach potentiellen Interaktionspartnern die Vorstufen, Zwischen– oder Endprodukte im Erdbeerfarbstoff-Syntheseweg darstellen, gesucht. Eine enzymatische Beteiligung der Fra a-Allergene bei der Synthese des Erdbeerfarbstoffe ist dabei eher

unwahrscheinlich, da alle Reaktionsschritte bekannt und generell keine strukturelle Ähnlichkeit von Bet v 1-homologen Proteinen zu den beteiligten Enzymen besteht (Munoz *et al.*, 2010). Unklar ist jedoch die Regulation des Synthesewegs in Erdbeeren. Der Erdbeerfarbstoff besteht zu einem großen Teil aus den Anthocyanen Pelargonidin-3-O-Glucosid und Cyanidin-3-O-Glucosid (Abbildung 6C, D; Griesser *et al.*, 2008). Bindungsstudien von verschiedenen Anthocyan-Vorstufen mit Fra a-Isoallergenen und die Kristallstruktur von Fra a 3 im Komplex mit dem Flavanol Catechin untermauern die Theorie für eine Transportfunktion der Allergene im Syntheseweg des Erdbeerfarbstoffes (Casañal *et al.*, 2013a; Casañal *et al.*, 2013b). Durch die Isolation von natürlichem Fra a direkt aus Erdbeeren könnte ein putativer Ligand co-extrahiert und identifiziert werden. Die in dieser Arbeit experimentell bestimmte Struktur von Fra a 1E lieferte jedoch einen wichtigen initialen Beitrag zur physiologischen Charakterisierung des Erdbeerallergens und ermöglicht die weitere Analyse von PR-10-kreuzreaktiven IgE-Epitopen auf molekularer Ebene.

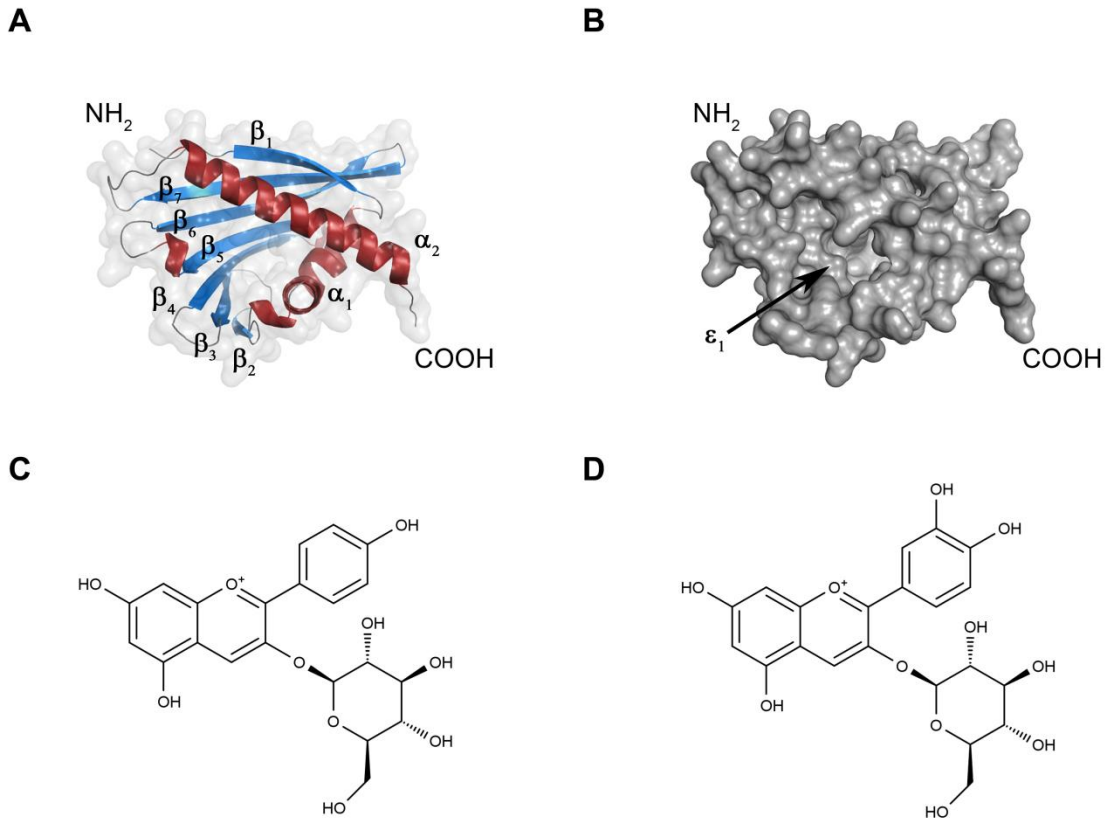

Abbildung 6: Struktur des Erdbeerallergens Fra a 1E und mögliche Bindungspartner.
(**A**) Schematische Darstellung und Markierung der Sekundärstrukturelemente von Fra a 1E (pdb: 2LPX). Unstrukturierte Bereiche sind in Grau, α-helikale Bereiche in Rot und β-Faltblattstränge in Blau gezeigt. Der N-Terminus (NH_2) und der C-Terminus (COOH) sind markiert. Die Oberfläche ist in transparentem Hellgrau angedeutet. (**B**) Graue Oberflächendarstellung von Fra a 1E in derselben Orientierung wie in 6A. Die größte Öffnung zum hydrophoben Hohlraum (ε_1) ist markiert. (**C**) Chemische Strukturen der Hauptkomponenten des Erdbeerfarbstoffes, Pelargonidin-3-O-Glucosid und (**D**) Cyanidin-3-O-Glucosid.

3.3 Das Hauptbirkenpollenallergen Bet v 1

3.3.1 Identifikation des natürlichen Liganden von Bet v 1

Das Konzept zur Isolation des natürlichen Allergens basiert auf der Beobachtung, dass Bet v 1 nach Hydratisierung der Birkenpollen durch Mikrokanäle in den Überstand transportiert wird (Grote *et al.*, 1993). Die Reinigung wurde leicht modifiziert einem etablierten Protokoll entnommen (Bollen *et al.*, 2007). Von Bollen *et al*. nicht erwähnt oder beobachtet, zeigte sich während der in dieser Arbeit beschriebenen Isolation in allen Bet v 1-enthaltenden Fraktionen eine deutliche Gelbfärbung. Das im UV/VIS-Spektrum (sichtbare Ultraviolettstrahlung) ermittelte lokale Intensitätsmaximum von 350 nm wurde anschließend während aller chromatographischen Schritte zur Detektion des farbigen Moleküls verwendet (Abbildung S1 aus Einzelarbeit C). In Abbildung 7A ist das Elutionsprofil von natürlichem Bet v 1 im finalen Reinigungsschritt (Größensausschlusschromatographie, SEC) gezeigt. Trotz deutlich unterschiedlicher Molekülmassen eluieren Bet v 1 (17,5 kDa; $A_{280\ nm}$) und Ligand (ca. 0,6 kDa; $A_{350\ nm}$) gleichzeitig als Komplex. Die entsprechenden Fraktionen zeigen im denaturierenden Gel eine feine Aufspaltung in mindestens drei Proteinbanden mit molekularen Massen von etwa 17 kDa (Abbildung 7B). Durch die Analyse dieser Banden konnten fünf Isoallergene eindeutig identifiziert werden. Aufgrund der hohen Sequenzidentitäten ist jedoch davon auszugehen, dass noch weitere Isoallergene aus den Pollen isoliert wurden. Die Kombination der massenspektrometrischen Daten mit den ^{1}H- und ^{13}C NMR-Spektren der finalen Bet v 1-Fraktionen ermöglichte die Identifikation der gelblichen Substanz als das Flavonol-Diglycosid **Quercetin-3-O-Sophorosid** (Q3OS, Abbildung 7C sowie Abbildungen 2, S2 bis S5 aus Einzelarbeit C). Die seit etwa 20 Jahren offene Frage nach dem physiologischen Interaktionspartner des Birkenpollenallergens konnte somit in dieser Arbeit beantwortet werden.

Die anschließenden Experimente dienten zur Analyse der Affinität, Spezifität und der Q3OS-Interaktionsfläche mit Bet v 1. Hierfür wurde aus Birkenpollen extrahiertes, freies Q3OS und das rekombinante Isoallergen Bet v 1.0101 zur Komplexbildung inkubiert und anschließend, analog zum letzten Reinigungsschritt des natürlichen Allergens, von einer SEC-Säule eluiert (Abbildung 1D, E aus Einzelarbeit C). Das Elutionsprofil zeigte dabei einen beinahe identischen Verlauf wie der natürliche Komplex und bestätigt somit Q3OS als physiologischen Ligand des Isoallergens Bet v 1.0101.

Die mittels Fluoreszenztitration bestimmte Dissoziationskonstante (K_d) von 566±85 nM für Q3OS liegt dabei deutlich unter dem K_d-Wert von 9,2±0,6 µM für Quercetin (Abbildung 3 aus Einzelarbeit C). Offenbar trägt der Sophorose-Rest von Q3OS zu einer gesteigerten Affinität zu Bet v 1 im Vergleich zu dem Aglycon Quercetin bei. Dieser Affinitätsunterschied konnte ebenfalls mittels ^{1}H-^{15}N-HSQC-NMR Spektroskopie beobachtet werden. Bei diesen Experimenten wurden zu ^{15}N-isotopenmarkiertem Bet v 1.0101 Quercetin, Q3OS, Sophorose und Rutin hinzutitriert und die resultierenden Änderungen der Intensitäten, sowie der chemischen Verschiebungen (*chemical shift pertubations*, CSP) aller ^{1}H-^{15}N-Resonanzen gemessen (Abbildung 4 aus Einzelarbeit C).

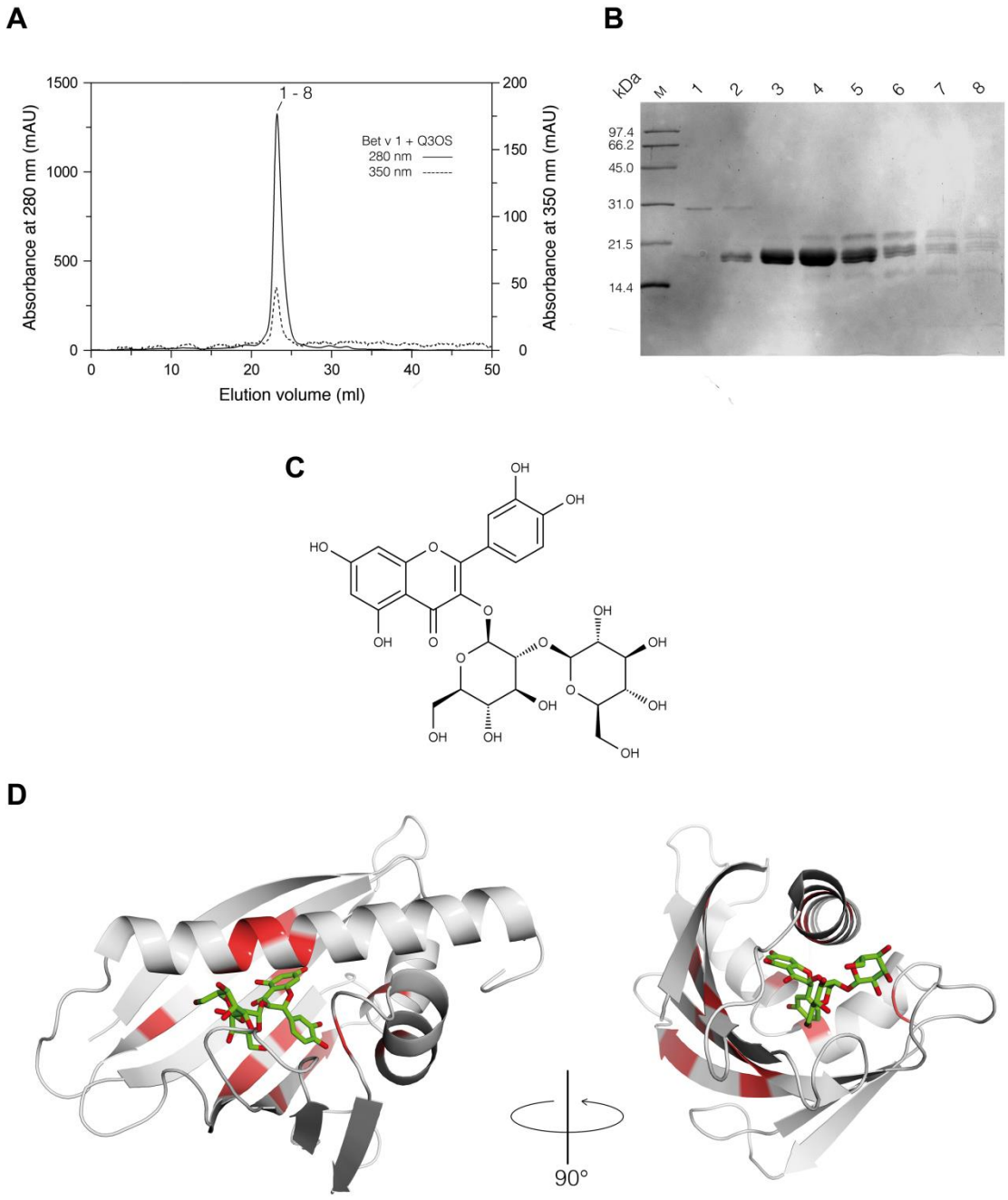

Abbildung 7: Isolation, Identifikation und Interaktion von Q3OS mit Bet v 1.
Abbildung modifiziert aus Einzelarbeit C, Abbildungen 1 und 5. (**A**) Elutionsprofil der Größenausschlusschromatographie als finaler Reinigungsschritt für das aus Birkenpollen extrahierte, natürliche Bet v 1. Die durchgängige Linie repräsentiert die Absorption bei 280 nm, die gestrichelte Linie die Absorption bei 350 nm. (**B**) Entsprechende Analyse der SEC-Fraktionen mittels SDS/PAGE (Natriumdodecylsulfat-Polyacrylamidgelelektrophorese). (**C**) Chemische Struktur des identifizierten Bet v 1-Liganden Quercetin-3-O-Sophorosid. (**D**) Interaktion von Q3OS (grüne Stäbchen, Sauerstoff in Rot) mit Bet v 1.0101 (grau, pdb: 4A87). Die mittels NMR-Spektroskopie nachgewiesenen, mit Q3OS interagierenden Aminosäure-Positionen sind in Rot dargestellt. Q3OS wurde in die hydrophobe Tasche von Bet v 1 modelliert.

In Gegenwart steigender Quercetin-Konzentrationen wurden schrittweise Änderungen der chemischen Verschiebung beobachtet. Dieser Effekt ist charakteristisch für Bindungsaffinitäten im millimolaren bis mikromolaren Bereich und äußert sich durch Komplexbildung im schnellen Austausch bezogen auf die NMR-Zeitskala (Göbl *et al.*, 2014; Marintchev *et al.*, 2007). Aus den Resonanzen, die signifikante Änderungen der chemischen Verschiebung zeigen, konnte die Interaktionsoberfläche von Quercetin in der hydrophoben Tasche im Bereich unterhalb der C-terminalen Helix von Bet v 1 bestimmt werden

(Abbildung 5A aus Einzelarbeit C). Obwohl für Sophorose allein keine Affinität zu Bet v 1 gemessen werden konnte, führt die hohe Affinität von Q3OS gegenüber Bet v 1 im ^{1}H-^{15}N-HSQC-Spektrum des Komplexes zu intermediären bis langsamen Austauschraten (Abbildung 4B, D aus Einzelarbeit C). Dies äußert sich in starker Linienverbreiterung, bzw. im Verschwinden von Signalen (Göbl *et al.*, 2014; Marintchev *et al.*, 2007). Von der Interaktion betroffene Resonanzen wurden im Folgenden auf der Struktur von Bet v 1 markiert (Abbildung 5B aus Einzelarbeit C). Die gesteigerte Affinität von Q3OS im Vergleich zu Quercetin kann durch einen Effekt, der als Enthalpie-Entropie-Kompensation bekannt ist, erklärt werden und wird noch im Detail (siehe 3.3.3) diskutiert.

Eine mögliche Anordnung basierend auf den experimentellen NMR-Daten von Q3OS im Bet v 1-Hohlraum wurde mittels Docking-Simulation analysiert und ist in Abbildung 7D dargestellt. Der Einfluss des Zucker-Rests wurde auch in einer Vergleichstitration von Bet v 1.0101 mit dem natürlich vorkommenden Flavonol-Diglycosid Rutin untersucht. Rutin besteht aus Quercetin und dem Diglycosid Rutinose und unterscheidet sich nur in einer CH_3-Gruppe und der Verknüpfung der Einzelzucker zu dem Q3OS-Zucker Sophorose. Trotz eines hohen molaren Überschusses von Rutin, konnten im ^{1}H-^{15}N-HSQC Spektrum von Bet v 1.0101 nur sehr kleine Änderungen der chemischen Verschiebung und dementsprechend eine sehr schwache Bindung gemessen werden (Abbildung 4C aus Einzelarbeit C). Offenbar spielen die Geometrie und die chemischen Eigenschaften des an Quercetin verknüpften Zuckerrests eine entscheidende Rolle. Zusätzlich scheint die hydrophobe Tasche von Bet v 1 für die Bindung von Zuckern optimiert zu sein.

Vor etwa 20 Jahren setzte die Aufklärung der dreidimensionaler Struktur von Bet v 1 einen Meilenstein in der Allergieforschung (Gajhede *et al.*, 1996). Die Identifikation von Q3OS als natürlicher Ligand von Bet v 1 stellt in diesem Zusammenhang einen weiteren unglaublich wichtigen Schritt zur physiologischen und immunologischen Charakterisierung des Hauptbirkenpollenallergens dar.

3.3.2 Immunologischer Einfluss von Bet v 1-Liganden

Die Erkennung eines Allergens durch IgE stellt zweifelsohne den Schlüsselmoment der allergischen Immunantwort dar. Folglich drängt sich die Frage nach dem Einfluss von Q3OS oder anderer Liganden auf die Interaktion zwischen Bet v 1 und IgE auf. Bereits 1980 konnten Björkstén *et al*. zeigen, dass die IgE-Interaktion des Apfelallergens Mal d 1 entscheidend von phenolischen Molekülen im Apfelextrakt beeinflusst wird (Björkstén *et al.*, 1980). Weiterhin kann die Bindung von Liganden in Komplexstrukturen von Bet v 1-Isoformen zu einem Volumenanstieg der hydrophoben Tasche führen und somit auch eine strukturelle Änderung von Epitopen bewirken (Kofler *et al.*, 2012; Markovic-Housley *et al.*, 2003). Wie eine kürzlich veröffentlichte Studie von Asam *et al*. zeigt, trifft dies nicht auf den physiologisch irrelevanten Ligand Deoxycholat zu. Allerdings übt dieser Ligand einen stabilisierenden Effekt auf Bet v 1 aus und beeinflusst vermutlich dadurch dessen Erkennung durch Zellen des Immunsystems (Asam *et al.*, 2014). Um den Einfluss von physiologisch relevanten

Liganden zu analysieren, wurde in dieser Arbeit (Einzelarbeit D) die Bindung von drei rekombinanten Bet v 1-Isoformen an serologisches IgE mittels indirektem ELISA (*enzyme linked immunosorbent assay*) und β-Hexosaminidase-Freisetzung durch humanisierte RBL-Zellen (*rat basophil leukaemia*) gemessen (Vogel *et al.*, 2005). Hierfür wurden die Isoformen a (Bet v 1.0101), d (Bet v 1.0102) und das Isoallergen Bet v 1m (Bet v 1.0204, 89 % Sequenzidentität zu Bet v 1a) ausgewählt, da diese jeweils einen Vertreter der hyperallergenen (a, e, j), intermediären (b, c, f, m) oder hypoallergenen (d, g, l) Varianten von Bet v 1 repräsentieren (Ferreira *et al.*, 1996; Schenk *et al.*, 2009). Zur Vereinfachung wird das Isoallergen Bet v 1m (bezogen auf Bet v 1a) im Folgenden ebenfalls als Isoform bezeichnet.

In Abwesenheit von Liganden, wurden für die Isoformen a und m vergleichbare IgE-Bindungsaktivitäten detektiert, Bet v 1d hingegen zeigte lediglich eine schwache IgE-Interaktion (Abbildung 4A, C aus Einzelarbeit D). Diese Ergebnisse stimmen mit vorherigen Studien über die IgE-Bindungseigenschaften dieser Bet v 1-Isoformen überein und bestätigen Bet v 1a und m als hyperallergene bzw. intermediäre und Bet v 1d als hypoallergene Isoform (Arquint *et al.*, 1999; Ferreira *et al.*, 1996; Ferreira *et al.*, 1998). Trotz eines fünffachen molaren Liganden-Überschusses, konnte jedoch für keine Isoform ein signifikanter Unterschied der IgE-Interaktion in Gegenwart von Q3OS oder anderer Liganden detektiert werden (Abbildungen S3, 4B, D aus Einzelarbeit D). Diese Ergebnisse verdeutlichen, dass die getesteten Liganden keinen direkten Einfluss auf die Interaktion zwischen Bet v 1 und serologischem IgE ausüben. Allerdings stellt sich hierbei die Frage, ob intakte Bet v 1:Ligand-Komplexe überhaupt in Kontakt mit dem Immunsystem treten.

Wie Mogensen *et al.* zeigten, interagiert Bet v 1 mit Phospholipid-Vesikeln, wobei eine strukturelle Umorientierung hin zu einer α-helikalen Konformation stattfindet (Mogensen *et al.*, 2007). Unter der Annahme, dass diese Effekte auch bei der Interaktion mit Epithelzellmembranen in Lungen- oder Nasenschleimhäuten auftreten, könnten Q3OS oder andere physiologische Liganden bereits beim initialen Bet v 1-Kontakt freigesetzt werden (Golebski *et al.*, 2013). Die freien Liganden könnten dabei eine entscheidende Rolle bei der Initiation zur Produktion von Bet v 1-spezifischem IgE (Sensibilisierung) spielen. Wie von Renkonen *et al*. vorgeschlagen, werden auf den Epithelzell-Membranoberflächen (Bindehaut und Nasenschleimhaut) von Birkenpollenallergikern verstärkt bestimmte Rezeptoren in *lipid rafts* (Lingwood & Simons, 2010; Pike, 2006) und Caveolae (Anderson, 1993) ausgebildet. Mit Hilfe dieser Komplexe kann Bet v 1 in das Gewebe und sogar bis hin zu Epithel-Zellkernen transportiert werden (Joenväärä *et al.*, 2009; Renkonen *et al.*, 2009). In gesunden Individuen scheint eine vergleichsweise niedrige Anzahl dieser Rezeptor-Membrankomplexe exprimiert zu werden und Bet v 1 konnte nicht innerhalb gesunder Epithelzellen nachgewiesen werden. Interessanterweise wird Bet v 1 spezifisch von sensibilisierten Epithelzellen aufgenommen, da Bet v 1-homologe Allergene aus Graspollen nicht von Birkenpollenallergikern erkannt wurden und umgekehrt (Renkonen *et al.*, 2009). Diese Spezifität könnte durch Bet v 1-Liganden wie Q3OS vermittelt werden. Flavonoide lagern sich bevorzugt in *lipid rafts* von Biomembranen ein und können

dort Rezeptoreigenschaften oder Signalkaskaden beeinflussen (Tarahovsky *et al.*, 2014). Bet v 1-gebundene oder auch freie Liganden könnten nach erfolgter Einlagerung in die Epithelmembran einen Einfluss auf die Expression von Bet v 1-spezifischen Rezeptorkomplexen ausüben und damit zur spezifischen Sensibilisierung gegen Bet v 1 beitragen.

Der Einfluss anderer physiologischer Allergen-Liganden auf den Sensibilisierungsprozess konnte bereits nachgewiesen werden (Bublin *et al.*, 2014). Lipide stellen die natürlichen Bindungspartner vieler Allergene wie z. B. Lagerproteine, Lipid-Transferproteine, oder Lipocaline dar und können mit dem angeborenem Immunsystem interagieren oder eine T_H2-vermittelte (Allergie-induzierende) Immunantwort provozieren (Abos-Gracia *et al.*, 2013; Agea *et al.*, 2005; Jyonouchi *et al.*, 2014; Mariani *et al.*, 2007; Scanlon *et al.*, 2011). Vermutlich wirken Liganden eher als „Türöffner" auf das Immunsystem zu Beginn der Sensibilisierung gegen ein Allergen und weniger bei der Interaktion des Allergens mit IgE. Warum diese Effekte nicht bei gesunden Individuen auftreten oder ausgelöst werden bleibt ungeklärt. Die Sensibilisierung gegen ein Allergen stellt einen hochkomplexen immunologischen Vorgang dar, der bis heute nur eingeschränkt verstanden ist. Komponenten wie *pattern recognition receptors* oder Bestandteile von Allergenquellen (Hüllenproteine, Pollenmembranen oder Pathogene) beeinflussen ebenfalls den Sensibilisierungsprozess (Bublin *et al.*, 2014; Thomas, 2014). Zusätzlich ist die Rolle von dendritischen Zellen, Fibroblasten oder der glatten Muskulatur der Atmungswege während der Entstehung einer Immunantwort weitgehend unklar (Holgate *et al.*, 2004; Mariani *et al.*, 2007; Rinia *et al.*, 2007). Der Einfluss von Allergen-Liganden in diesem komplexen Netzwerk ist dabei noch eine weitere Komponente, auf die jeder Mensch sehr wahrscheinlich individuell reagiert (Golebski *et al.*, 2013).

3.3.3 Physiologische Bedeutung von Bet v 1:Ligand-Komplexen

Die Identifikation von Q3OS als natürlicher Ligand von Bet v 1 wirft ebenfalls Fragen zur physiologischen Rolle des Birkenpollenallergens auf. Bis heute ist nicht geklärt, warum Mitglieder der PR-10 Familie als Zusammensetzung von strukturell und sequenziell beinahe identischen Isoformen in Erscheinung treten. Studien des Proteoms von Birkenpollenextrakten offenbarten regionale und Art-abhängige Unterschiede in der Isoformquantität– und Zusammensetzung (Buters *et al.*, 2010; Erler *et al.*, 2011; Schenk *et al.*, 2009). Anscheinend reagieren Birkenbäume damit auf die vorherrschenden Umweltbedingungen. Welche Rolle dabei jedoch der Komplex aus Bet v 1 und Q3OS im Pollenkorn spielt und ob weitere physiologische Liganden für verschiedene Isoformen existieren bleibt ungeklärt. Im Rahmen von Einzelarbeit D wurden die Interaktionen von drei Bet v 1-Isoformen (siehe 3.3.2) mit physiologisch relevanten Liganden analysiert. Zu Beginn wurde die Interaktion zwischen Q3OS und den Bet v 1-Isoformen mittels UV/VIS-Spektroskopie analysiert. Nach Inkubation und anschließender Abtrennung von ungebundenem Q3OS, zeigte sich im UV-Spektrum ein deutliches lokales Absorptionsmaximum bei etwa 360 nm für den Bet v 1a:Q3OS-Komplex, jedoch nicht für die putativen Komplexe Bet v 1d:Q3OS und Bet v 1m:Q3OS (Abbildung 1A aus Einzelarbeit D). Um

diese ersten Hinweisen auf das Isoform-abhängige Bindungsverhalten von Q3OS näher zu untersuchen, wurde die Bet v 1-Interaktion mit einer Reihe von Flavonoiden und Flavonoid-Glykosiden mittels UV/VIS- und NMR-Spektroskopie detailliert untersucht. (Abbildungen S1 und 2 aus Einzelarbeit D). Generell konnten für alle getesteten, nicht-glykosylierten Flavonoide Dissoziationskonstanten im mikromolaren Bereich sowie schnelle Austauschraten in den ^{1}H-^{15}N-HSQC-Experimenten und Bindungsoberflächen im hydrophoben Hohlraum aller Bet v 1-Isoformen ermittelt werden (Tabelle I und Abbildung 2 aus Einzelarbeit D). Hydroxylgruppen scheinen die Affinität der Flavonoide zu erhöhen, wobei deren Lage keinen Einfluss auf die Bindungsregion in den Isoformen ausübt. Offensichtlich ist der hydrophobe Innenraum aller getesteten Bet v 1-Varianten zur *in vitro*-Bindung von kleineren, hydrophoben und amphiphilen Moleküle in der Lage. Die vergleichsweise niedrigen Affinitäten weisen jedoch nicht auf eine entscheidende physiologische Bedeutung dieser Bet v 1:Flavonoid-Komplexen hin.

Die hohe Affinität von Q3OS zu Bet v 1a wird daher offensichtlich durch den Q3OS-Zucker Sophorose vermittelt. Um den Einfluss des Quercetin-Zuckerrests zu analysieren wurde die Interaktion der glykosylierten Flavonoide Quercetin-3-O-Glucosid (Q3OGlc), Quercetin-3-O-Galactosid (Q3OGal) und Q3OS mit den jeweiligen Bet v 1-Isoformen untersucht (Abbildungen 1 und 3 aus Einzelarbeit D). Abbildung 8 zeigt zusammenfassend die von der Interaktion mit den Liganden betroffenen Reste auf den Strukturen der jeweiligen Isoformen.

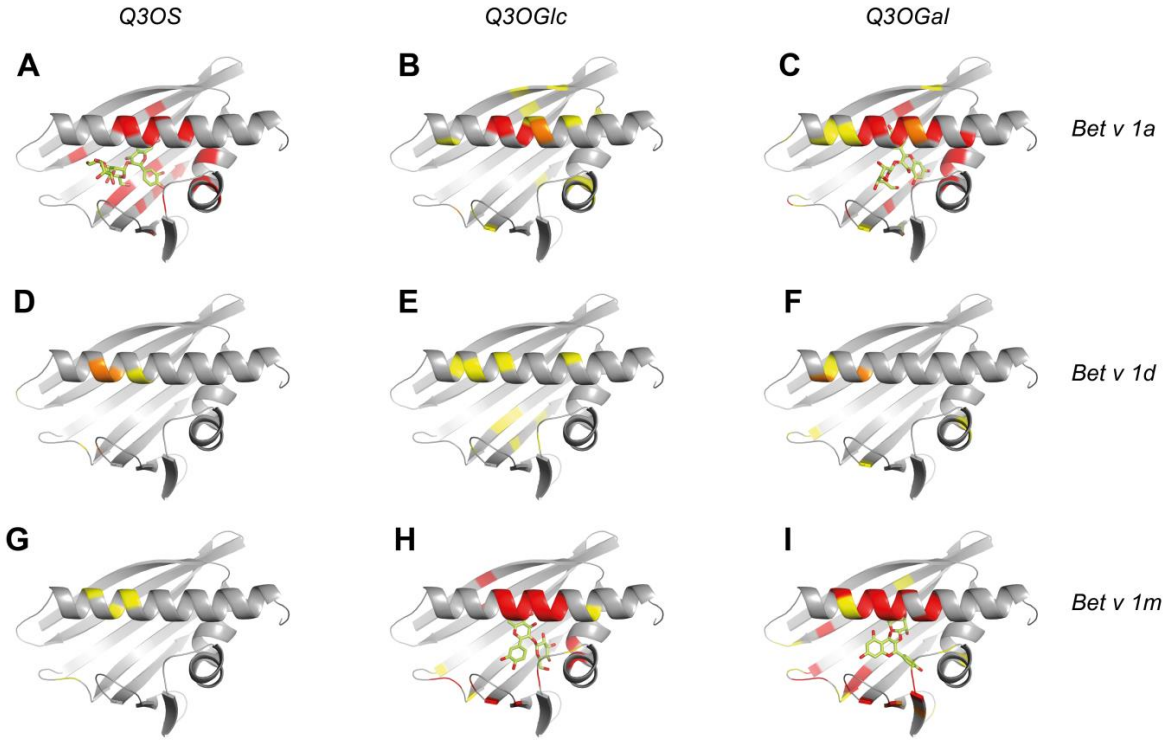

Abbildung 8: Interaktionsflächen von Flavonol-Glykosiden mit den Bet v 1-Isoformen a, d und m.
Abbildung modifiziert aus Einzelarbeit D, Abbildung 3. Von der Interaktion betroffene Reste wurden durch ^{1}H-^{15}N-HSQC-Titrationen von ^{15}N-markierten Bet v 1-Isoformen mit den entsprechenden Liganden identifiziert. Stark interagierende Aminosäurereste sind in Rot, medium interagierend in Orange und schwach interagierend in Gelb auf der Struktur von Bet v 1a (pdb: 1BV1) und den Modellen von Bet v 1d und m dargestellt. In die hydrophoben Taschen modellierte Liganden sind in grünen Sticks und roten Sauerstoffatomen dargestellt. Interaktionsoberflächen von Bet v 1a/d/m mit Q3OS (**A/D/G**), Q3OGlc (**B/E/H**), und Q3OGal (**C/F/I**).

Im Vergleich zu Bet v 1a, zeigen die Isoformen d und m, trotz eines 15-fachen Liganden-Überschusses, keine signifikante Affinität zu Q3OS (Abbildung 8A, D, G). Unter Berücksichtigung der Sequenzidentitäten von Bet v 1d (95,6 %) und Bet v 1m (89,3 %) zu Bet v 1a, scheint die Komplexbildung zwischen Bet v 1a und Q3OS sehr spezifisch zu sein. Verkürzt sich jedoch der an Quercetin verknüpfte Zucker auf einen Glucose-Rest (Q3OGlc), werden Spezifität und Affinität aufgehoben (Abbildung 8B). Interessanterweise wird die Affinität durch eine stereochemische Veränderung des Glucose-Rests zu einem Galaktose-Rest (Q3OGal) wieder hergestellt (Abbildung 8C). Im Gegensatz zu Bet v 1a, zeigt Bet v 1m eine hohe Affinität zu Q3OGlc und Q3OGal (Abbildung 8H und I). Die stereochemischen Unterschiede zwischen den beiden monoglykosylierten Flavonoiden üben offensichtlich keinen spezifischen Einfluss auf die Interaktion mit Bet v 1m aus. Obwohl Bet v 1d generell nicht-glykosylierte Flavonoide bindet (Tabelle I aus Einzelarbeit D), weist diese Isoform nur eine sehr geringe bis keine Affinität zu den monoglykosylierten Flavonoiden auf (Abbildung 8D bis F).

Dieses individuelle Bindungsverhalten gegenüber glykosylierten Flavonoiden kann neben strukturellen oder sterischen Eigenschaften der Isoformen durch einen Effekt, der als Enthalpie-Entropie-Kompensation bekannt ist (Holgersson *et al.*, 2005), erklärt werden. Durch die Bet v 1:Ligand-Komplexbildung werden Wassermoleküle der Hydrathülle des Liganden und der Bet v 1-Bindungstasche verdrängt, einhergehend mit einem Entropiegewinn oder -verlust, abhängig von den ursprünglichen molekularen Wechselwirkungen. Zusätzlich wird die Bindungsenergie von der Anordnung der Hydrathülle des Komplexes, sowie durch den Anstieg oder Abfall an Freiheitsgraden des Liganden und der, an der Bindung beteiligten Bet v 1-Reste, beeinflusst. Zusammenfassend ergibt sich die Gesamtaffinität der Bindung aus der Summe der beschriebenen Effekte und äußert sich in einer energetisch favorisierten (Bet v 1a:Q3OS oder Q3OGal; Bet v 1m:Q3OGlc oder Q3OGal) oder weniger favorisiert Interaktion (Bet v 1a:Q3OGlc) im Vergleich zur Bindung des jeweiligen Aglycons oder sogar in dem Verlust der beobachtbaren Affinität (Bet v 1d:Q3OS, Q3OGlc oder Q3OGal; Bet v 1m:Q3OS). Vergleichbare Effekte wurden bereits in früheren Bindungsstudien beobachtet und scheinen, wie in dieser Arbeit, jeweils individuell für jeden Ligand:Rezeptor-Komplex abzulaufen (Lemieux, 1999; Swaminathan *et al.*, 1998; Toone, 1994).

Die aus diesen Bindungsstudien gewonnen Daten schlagen eine Isoform-spezifische Bindung von glykosylierten Flavonoiden vor, die jeweils entropisch durch den Zuckerrest vermittelt wird. Im Vergleich zum Aglycon kann dessen glykosylierte Verbindung eine bis zu Zehnfach höhere Affinität zu manchen Bet v1-Isoformen aufweisen (Tabelle I aus Einzelarbeit D). Offensichtlich ist das strukturelle Gerüst von Bet v 1-Isoformen für die spezifische Differenzierung zwischen den Zuckeresten von glykosylierten Flavonoiden konstruiert.

Diesen Komplexen könnten unterschiedliche, bzw. sich ergänzende physiologische Funktionen während der Pollenreifung, dem Pollenflug oder der Befruchtung des weiblichen Blütenstands zugewiesen werden. Einige botanische Studien offenbarten, dass Flavonoide generell in Pollen

verschiedener Pflanzen wie Mais, Haselnuss, Petunien oder Bienenragwurz produziert werden und dort hauptsächlich in wasserlöslicher, 3-O-glykosylierter Form vorliegen (Ceska & Styles, 1984; Karioti *et al.*, 2008; Mo *et al.*, 1992; Strack *et al.*, 1984). Es kann davon ausgegangen werden, dass sich ebenfalls in Birkenpollen weitere Flavonoid-Glykoside finden, die mit den vorhandenen Bet v 1-Isoformen spezifische Komplexe bilden (Meurer *et al.*, 1988). Während der Reifung dehydrieren Pollen, wobei deren Wasseranteil auf etwa 20 % abfallen kann (Firon *et al.*, 2012; Pacini, 1994) und viskose, intrazelluläre, Glas-ähnliche Strukturen entstehen (Buitink *et al.*, 1998). In diesem Milieu aus hochkonzentrierten Biomolekülen könnten Flavonoide durch die Komplexbildung mit Bet v 1 vor Abbau oder chemischer Modifizierung geschützt werden.

Eine mögliche Rolle dieser Komplexe könnte in dem Schutz der Pollen-DNA vor UV-Strahlung liegen. Die Produktion von glykosylierten Flavonoiden wie Q3OGlc oder Q3OGal wird beispielsweise in Blättern von Silberbirken oder Petunien durch UV-Strahlung induziert (Brunetti *et al.*, 2013; Lavola *et al.*, 2013; Ryan *et al.*, 1998). Im Vergleich zum Aglycon erhöht die Glykosylierung dabei die Wasserlöslichkeit (Tarahovsky *et al.*, 2014) sowie die Stabilität der Flavonoide gegenüber UV-Strahlung (Cvetkovic *et al.*, 2011; Smith *et al.*, 2000) oder verändert deren Absorptionseigenschaften (Abbildung 1B, Tabelle SI aus Einzelarbeit D). Die beschriebenen Effekte könnten dem dynamischen und anpassungsfähigen UV-Schutz der Pollen-DNA dienen, um gezielt auf die örtliche Sonnenstrahlung zu reagieren. Unter Berücksichtigung des hohen Bet v 1-Anteils von bis zu 30 % des Gesamtproteins in Pollen (Erler *et al.*, 2011) und der starken UV-Absorption von Proteinen im Allgemeinen, liefern die Bet v 1-Komplexe vermutlich einen ausreichenden und noch effektiveren Sonnenschutz als freie Flavonoide. Trotz ihrer hohen UV-Toleranz (Morales *et al.*, 2011), werden Flavonoide allerdings durch dauerhafte und intensive UV-Strahlung destabilisiert und abgebaut (Cvetkovic *et al.*, 2011), was die Komplexbildung zwischen Bet v 1 und Flavonoid auflösen kann. Funktionelle Komplexe könnten deshalb als Qualitätsmerkmal für unbeschädigte Pollen DNA-dienen, wobei beschädigte oder zerstörte Komplexe eine Befruchtung verhindern.

Diese Hypothesen schlagen vor, dass Bet v 1-Isoformen nicht zufällig existieren, sondern jeweils ihre eigenen spezifischen Funktionen erfüllen. Alle Bet v 1-Varianten weisen vermutlich die typische PR-10 Proteinfaltung auf (Gajhede *et al.*, 1996; Kofler *et al.*, 2012; Markovic-Housley *et al.*, 2003). Durch die Variation von wenigen Aminosäuren entsteht jedoch ein von den vorherrschenden Umweltbedingungen abhängiges Netzwerk aus hochspezifischen molekularen Akzeptoren in Birkenpollen, um eine bestimmte Zusammensetzung an glykosylierten Liganden zu komplexieren. Manche Pflanzen wie Birke, Apfel oder Hainbuche (siehe Tabelle II) benötigen einen sehr spezifischen, individuellen Fingerabdruck um in der Natur zu bestehen oder sich fortzupflanzen. In andere Organismen wie Sellerie, Soja, Erdnuss oder Kirsche scheinen die entsprechenden Allergene eine weniger diverse Funktion zu erfüllen. Vereint werden alle Bet v 1-homologen Allergene in ihrer primären Funktion der Lagerung, des Transports oder des Schutzes von pflanzenspezifischen Biomolekülen.

4 Abkürzungsverzeichnis

APZ	Antigenpräsentierende Zelle
CD	Circulardichroismus
CSP	*chemical shift perturbation*
DNA	Desoxyribonukleinsäure
ELISA	*enzyme linked immunosorbent assay*
Fab	*antigen binding fragment*
GOA	*Gene Ontology Annotation*
HSQC	*heteronuclear single quantum coherence*
IgE	Immunoglobulin der Klasse E
IgG	Immunoglobulin der Klasse G
IgM	Immunoglobulin der Klasse M
IL	Interleukin
K_d	Dissoziationskonstante
LC-DAD	*liquid chromatography diode array detection*
LC-TOF	*liquid chromatography time of flight*
MLN64	*metastatic lymph node 64 protein*
NCS	(s)-Norcoclaurin-Synthase
NMR	Magnetische Kernspinresonanz
NOESY	*nuclear overhauser enhancement spectroscopy*
pdb	*protein data bank*
Pfam	*protein families*
PR-10 Proteine	*pathogenesis related* Proteine der Klasse 10
Q3OGal	Quercetin-3-O-Galactosid
Q3OGlc	Quercetin-3-O-Glucosid
Q3OS	Quercetin-3-O-Sophorosid
RBL	*rat basophil leukaemia*
RNA	Ribonukleinsäure
SCOP	*Structural Classification of Proteins*
SDAP	*Structural Database of Allergic Proteins*
SDS/PAGE	Natriumdodecylsulfat-Polyacrylamidgelelektrophorese
SEC	Größenausschlusschromatographie
SIT	Spezifische Immuntherapie
StAR	*steroidogenic acute regulatory protein*
START	StAR-*related lipid transfer*
T_H2-Zellen	Typ-2 T-Helferzellen
UV/VIS	Sichtbare Ultraviolettstrahlung

5 Literaturverzeichnis

Aalberse RC (2006) Structural features of allergenic molecules. *Chem Immunol Allergy* **91**: 134-146

Abos-Gracia B, del Moral MG, Lopez-Relano J, Viana-Huete V, Castro L, Villalba M, & Martinez-Naves E (2013) Olea europaea pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells. *J Allergy Clin Immunol* **131**: 1393-9.e5

Agarwal P & Agarwal P (2014) Pathogenesis related-10 proteins are small, structurally similar but with diverse role in stress signaling. *Mol Biol Rep* **41**: 599-611

Agea E, Russano A, Bistoni O, Mannucci R, Nicoletti I, Corazzi L, Postle AD, De Libero G, Porcelli SA, & Spinozzi F (2005) Human CD1-restricted T cell recognition of lipids from pollens. *J Exp Med* **202**: 295-308

Akdis M & Akdis CA (2014) Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* **133**: 621-631

Anderson RG (1993) Caveolae: where incoming and outgoing messengers meet. *Proc Natl Acad Sci U S A* **90**: 10909-10913

Arquint O, Helbling A, Crameri R, Ferreira F, Breitenbach M, & Pichler WJ (1999) Reduced in vivo allergenicity of Bet v 1d isoform, a natural component of birch pollen. *J Allergy Clin Immunol* **104**: 1239-1243

Artis D, Maizels RM, & Finkelman FD (2012) Forum: Immunology: Allergy challenged. *Nature* **484**: 458-459

Asam C, Batista AL, Moraes AH, de Paula VS, Almeida FC, Aglas L, Kitzmuller C, Bohle B, Ebner C, Ferreira F, Wallner M, & Valente AP (2014) Bet v 1 - a Trojan horse for small ligands boosting allergic sensitization? *Clin Exp Allergy*

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, & Sherlock G (2000) Gene Ontology: tool for the unification of biology. *Nat Genet* **25**: 25-29

Ausukua M, Dublin I, Echebarria MA, & Aguirre JM (2009) Oral Allergy Syndrome (OAS). General and stomatological aspects. *Med Oral Patol Oral Cir Bucal* **14**: e568-72

Bais HP, Vepachedu R, Lawrence CB, Stermitz FR, & Vivanco JM (2003) Molecular and biochemical characterization of an enzyme responsible for the formation of hypericin in St. John's wort (Hypericum perforatum L.). *J Biol Chem* **278**: 32413-32422

Bantignies B, Seguin J, Muzac I, Dedaldechamp F, Gulick P, & Ibrahim R (2000) Direct evidence for ribonucleolytic activity of a PR-10-like protein from white lupin roots. *Plant Mol Biol* **42**: 871-881

Berin MC (2014) Future Therapies for IgE-Mediated Food Allergy. *Curr Pediatr Rep* **2**: 119-126

Berkner H, Engelhorn J, Liscombe DK, Schweimer K, Wöhrl BM, Facchini PJ, Rösch P, & Matecko I (2007) High-yield expression and purification of isotopically labeled norcoclaurine synthase, a Bet v 1-homologous enzyme, from Thalictrum flavum for NMR studies. *Protein Expr Purif* **56**: 197-204

Berkner H, Schweimer K, Matecko I, & Rösch P (2008) Conformation, catalytic site, and enzymatic mechanism of the PR10 allergen-related enzyme norcoclaurine synthase. *Biochem J* **413**: 281-290

Björkstén F, Halmepuro L, Hannuksela M, & Lahti A (1980) Extraction and properties of apple allergens. *Allergy* **35**: 671-677

Bollen MA, Garcia A, Cordewener JH, Wichers HJ, Helsper JP, Savelkoul HF, & van Boekel MA (2007) Purification and characterization of natural Bet v 1 from birch pollen and related allergens from carrot and celery. *Mol Nutr Food Res* **51**: 1527-1536

Breda C, Sallaud C, el-Turk J, Buffard D, de Kozak I, Esnault R, & Kondorosi A (1996) Defense reaction in Medicago sativa: a gene encoding a class 10 PR protein is expressed in vascular bundles. *Mol Plant Microbe Interact* **9**: 713-719

Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, & Breitenbach M (1989) The gene coding for the major birch pollen allergen Betv1, is highly homologous to a pea disease resistance response gene. *EMBO J* **8**: 1935-1938

Breiteneder H, Ferreira F, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Rumpold H, Kraft D, & Scheiner O (1993) Four recombinant isoforms of Cor a I, the major allergen of hazel pollen, show different IgE-binding properties. *European Journal of Biochemistry* **212**: 355-362

Breiteneder H, Ferreira F, Reikerstorfer A, Duchene M, Valenta R, Hoffman-Sommergruber K, Ebner C, Breitenbach M, Kraft D, & Scheiner O (1992) Complementary DNA cloning and expression in Escherichia coli of Aln g I, the major allergen in pollen of alder (Alnus glutinosa). *J Allergy Clin Immunol* **90**: 909-917

Breiteneder H, Hoffmann-Sommergruber K, O'Riordain G, Susani M, Ahorn H, Ebner C, Kraft D, & Scheiner O (1995) Molecular Characterization of Api g 1, the Major Allergen of Celery (Apium graveolens), and Its Immumological and Structural Relationships to a Group of 17-kDa Tree Pollen Allergens. *European Journal of Biochemistry* **233**: 484-489

Breiteneder H & Radauer C (2004) A classification of plant food allergens. *J Allergy Clin Immunol* **113**: 821-830

Brunetti C, Di Ferdinando M, Fini A, Pollastri S, & Tattini M (2013) Flavonoids as antioxidants and developmental regulators: relative significance in plants and humans. *Int J Mol Sci* **14**: 3540-3555

Bublin M, Eiwegger T, & Breiteneder H (2014) Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol*

Bufe A, Spangfort MD, Kahlert H, Schlaak M, & Becker WM (1996) The major birch pollen allergen, Bet v 1, shows ribonuclease activity. *Planta* **199**: 413-415

Buitink J, Claessens MMAE, Hemminga MA, & Hoekstra FA (1998) Influence of Water Content and Temperature on Molecular Mobility and Intracellular Glasses in Seeds and Pollen. *Plant Physiol* **118**: 531-541

Buters JTM, Weichenmeier I, Ochs S, Pusch G, Kreyling W, Boere AJF, Schober W, & Behrendt H (2010) The allergen Bet v 1 in fractions of ambient air deviates from birch pollen counts. *Allergy* **65**: 850-858

Casañal A, Zander U, Dupeux F, Valpuesta V, & Marquez JA (2013a) Purification, crystallization and preliminary X-ray analysis of the strawberry allergens Fra a 1E and Fra a 3 in the presence of catechin. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **69**: 510-514

Casañal A, Zander U, Muñoz C, Dupeux F, Luque I, Botella MA, Schwab W, Valpuesta V, & Marquez JA (2013b) The Strawberry Pathogenesis-related 10 (PR-10) Fra a Proteins Control Flavonoid Biosynthesis by Binding to Metabolic Intermediates. *Journal of Biological Chemistry* **288**: 35322-35332

Cavkaytar O, Akdis CA, & Akdis M (2014) Modulation of immune responses by immunotherapy in allergic diseases. *Current Opinion in Pharmacology* **17**: 30-37

Ceska O & Styles ED (1984) Flavonoids from Zea mays pollen. *Phytochemistry* **23**: 1822-1823

Chapman MD, Pomes A, Breiteneder H, & Ferreira F (2007) Nomenclature and structural biology of allergens. *J Allergy Clin Immunol* **119**: 414-420

Crowell DN, John ME, Russell D, & Amasino RM (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. *Plant Mol Biol* **18**: 459-466

Cvetkovic D, Markovic D, Cvetkovic D, & Radovanovic B (2011) Effects of continuous UV-irradiation on the antioxidant activities of quercetin and rutin in solution in the presence of lecithin as the protective target. *J Serb Chem Soc* **76**: 973-985

Dall'Antonia F, Pavkov-Keller T, Zangger K, & Keller W (2014) Structure of allergens and structure based epitope predictions. *Methods* **66**: 3-21

Denépoux S, Eibensteiner PB, Steinberger P, Vrtala S, Visco V, Weyer A, Kraft D, Banchereau J, Valenta R, & Lebecque S (2000) Molecular characterization of human IgG monoclonal antibodies specific for the major birch pollen allergen Bet v 1. Anti-allergen IgG can enhance the anaphylactic reaction. *FEBS Lett* **465**: 39-46

Dreborg S (1988) Food allergy in pollen-sensitive patients. *Ann Allergy* **61**: 41-46

Dretzke J, Meadows A, Novielli N, Huissoon A, Fry-Smith A, & Meads C (2013) Subcutaneous and sublingual immunotherapy for seasonal allergic rhinitis: AÂ systematic review and indirect comparison. *J Allergy Clin Immunol* **131**: 1361-1366

Erb KJ (2007) Helminths, allergic disorders and IgE-mediated immune responses: Where do we stand? *Eur J Immunol* **37**: 1170-1173

Erler A, Hawranek T, Krückemeier L, Asam C, Egger M, Ferreira F, & Briza P (2011) Proteomic profiling of birch (Betula verrucosa) pollen extracts from different origins. *Proteomics* **11**: 1486-1498

Fernandes H, Pasternak O, Bujacz G, Bujacz A, Sikorski MM, & Jaskolski M (2008) Lupinus luteus pathogenesis-related protein as a reservoir for cytokinin. *J Mol Biol* **378**: 1040-1051

Fernandes H, Michalska K, Sikorski M, & Jaskolski M (2013) Structural and functional aspects of PR-10 proteins. *FEBS Journal* **280**: 1169-1199

Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger HJ, & Scheiner O (1998) Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* **12**: 231-242

Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breitenbach M, Rheinberger HJ, & Ebner C (1996) Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: Potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* **183**: 599-609

Ferreira F, Wolf M, & Wallner M (2014) Molecular approach to allergy diagnosis and therapy. *Yonsei Med J* **55**: 839-852

Finn RD, Mistry J, Schuster-Böckler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Eddy SR, Sonnhammer ELL, & Bateman A (2006) Pfam: clans, web tools and services. *Nucleic Acids Research* **34**: D247-D251

Firon N, Nepi M, & Pacini E (2012) Water status and associated processes mark critical stages in pollen development and functioning. *Annals of Botany* **109**: 1201-1214

Fitzsimmons CM, Falcone FH, & Dunne DW (2014) Helminth allergens, parasite-specific IgE and its protective role in human immunity. *Frontiers in Immunology* **5**

Freeman J (1911) FURTHER OBSERVATIONS ON THE TREATMENT OF HAY FEVER BY HYPODERMIC INOCULATIONS OF POLLEN VACCINE. *Lancet* **178**: 814-817

Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Joost van Neerven RJ, Schou C, Lowenstein H, & Spangfort MD (1996) X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat Struct Biol* **3**: 1040-1045

Galli S, Wedemeyer J, & Tsai M (2002) Analyzing the Roles of Mast Cells and Basophils in Host Defense and Other Biological Responses. *Int J Hematol* **75**: 363-369

Gell PGH & Coombs RRA (1963) *Clinical aspects of immunology*. Blackwell: Oxford

Gepp B, Lengger N, Bublin M, Hemmer W, Breiteneder H, & Radauer C (2014) Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy. *J Allergy Clin Immunol*

Geroldinger-Simic M, Zelniker T, Aberer W, Ebner C, Egger C, Greiderer A, Prem N, Lidholm J, Ballmer-Weber B, Vieths S, & Bohle B (2011) Birch pollen–related food allergy: Clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. *J Allergy Clin Immunol* **127**: 616-622.e1

Göbl C, Madl T, Simon B, & Sattler M (2014) NMR approaches for structural analysis of multidomain proteins and complexes in solution. *Prog Nucl Magn Reson Spectrosc* **80**: 26-63

Golebski K, Roschmann KI, Toppila-Salmi S, Hammad H, Lambrecht BN, Renkonen R, Fokkens WJ, & van Drunen CM (2013) The multi-faceted role of allergen exposure to the local airway mucosa. *Allergy* **68**: 152-160

Gould HJ & Sutton BJ (2008) IgE in allergy and asthma today. *Nat Rev Immunol* **8**: 205-217

Griesser M, Hoffmann T, Bellido ML, Rosati C, Fink B, Kurtzer R, Aharoni A, Munoz-Blanco J, & Schwab W (2008) Redirection of flavonoid biosynthesis through the down-regulation of an anthocyanidin glucosyltransferase in ripening strawberry fruit. *Plant Physiol* **146**: 1528-1539

Grote M, Vrtala S, & Valenta R (1993) Monitoring of two allergens, Bet v I and profilin, in dry and rehydrated birch pollen by immunogold electron microscopy and immunoblotting. *J Histochem Cytochem* **41**: 745-750

Grunstein MM, Veler H, Shan X, Larson J, Grunstein JS, & Chuang S (2005) Proasthmatic effects and mechanisms of action of the dust mite allergen, Der p 1, in airway smooth muscle. *J Allergy Clin Immunol* **116**: 94-101

Handschuh L, Femiak I, Kasperska A, Figlerowicz M, & Sikorski MM (2007) Structural and functional characteristics of two novel members of pathogensis-related multigene family of class 10 from yellow lupine+. *Acta Biochim Pol* **54**: 783-796

Hecker J, Diethers A, Schulz D, Sabri A, Plum M, Michel Y, Mempel M, Ollert M, Jakob T, Blank S, Braren I, & Spillner E (2012) An IgE epitope of Bet v 1 and fagales PR10 proteins as defined by a human monoclonal IgE. *Allergy* **67**: 1530-1537

Hjerno K, Alm R, Canback B, Matthiesen R, Trajkovski K, Bjork L, Roepstorff P, & Emanuelsson C (2006) Down-regulation of the strawberry Bet v 1-homologous allergen in concert with the flavonoid biosynthesis pathway in colorless strawberry mutant. *Proteomics* **6**: 1574-1587

Hoffmann-Sommergruber K (2000) Plant allergens and pathogenesis-related proteins. What do they have in common? *Int Arch Allergy Immunol* **122**: 155-166

Holgate ST, Holloway J, Wilson S, Bucchieri F, Puddicombe S, & Davies DE (2004) Epithelial-mesenchymal communication in the pathogenesis of chronic asthma. *Proc Am Thorac Soc* **1**: 93-98

Holgersson J, Gustafsson A, & Breimer ME (2005) Characteristics of protein-carbohydrate interactions as a basis for developing novel carbohydrate-based antirejection therapies. *Immunol Cell Biol* **83**: 694-708

Holm J, Ferreras M, Ipsen H, Würtzen PA, Gajhede M, Larsen JN, Lund K, & Spangfort MD (2011) Epitope Grafting, Re-creating a Conformational Bet v 1 Antibody Epitope on the Surface of the Homologous Apple Allergen Mal d 1. *Journal of Biological Chemistry* **286**: 17569-17578

Igea JM (2013) The history of the idea of allergy. *Allergy* **68**: 966-973

Ilari A, Franceschini S, Bonamore A, Arenghi F, Botta B, Macone A, Pasquo A, Bellucci L, & Boffi A (2009) Structural basis of enzymatic (s)-norcoclaurine biosynthesis. *J Biol Chem* **284**: 897-904

Ipsen H & Hansen OC (1991) The NH2-terminal amino acid sequence of the immunochemically partial identical major allergens of Alder (Alnus glutinosa) Aln g I, birch (Betula verrucosa) Bet v I, hornbeam (Carpinus betulus) Car b I and oak (Quercus alba) Que a I pollens. *Mol Immunol* **28**: 1279-1288

Ishizaka K & Ishizaka T (1967) Identification of γE-Antibodies as a Carrier of Reaginic Activity. *The Journal of Immunology* **99**: 1187-1198

Ishizaka K, Ishizaka T, & Hornbrook MM (1966) Physicochemical Properties of Reaginic Antibody: V. Correlation of Reaginic Activity with γE-Globulin Antibody. *The Journal of Immunology* **97**: 840-853

Ivanciuc O, Schein CH, & Braun W (2003) SDAP: database and computational tools for allergenic proteins. *Nucleic Acids Research* **31**: 359-362

Janeway CAj, Travers P, Walport M, & Shlomchik M (2002) *Immunologie.* Spektrum: Heidelberg

Jenkins JA, Breiteneder H, & Mills ENC (2007) Evolutionary distance from human homologs reflects allergenicity of animal food proteins. *J Allergy Clin Immunol* **120**: 1399-1405

Joenväärä S, Mattila P, Renkonen J, Mäkitie A, Toppila-Salmi S, Lehtonen M, Salmi P, Lehti S, Mäkinen J, Sormunen R, Paavonen T, & Renkonen R (2009) Caveolar transport through nasal epithelium of birch pollen allergen Bet v 1 in allergic patients. *J Allergy Clin Immunol* **124**: 135-142.e21

Jyonouchi S, Smith CL, Saretta F, Abraham V, Ruymann KR, Modayur-Chandramouleeswaran P, Wang M-, Spergel JM, & Cianferoni A (2014) Invariant natural killer T cells in children with eosinophilic esophagitis. *Clinical & Experimental Allergy* **44**: 58-68

Karioti A, Kitsaki CK, Zygouraki S, Ziobora M, Djeddi S, Skaltsa H, & Liakopoulos G (2008) Occurrence of flavonoids in Ophrys (Orchidaceae) flower parts. *Flora - Morphology, Distribution, Functional Ecology of Plants* **203**: 602-609

Karlsson AL, Alm R, Ekstrand B, Fjelkner-Modig S, Schiott A, Bengtsson U, Bjork L, Hjerno K, Roepstorff P, & Emanuelsson CS (2004) Bet v 1 homologues in strawberry identified as IgE-binding proteins and presumptive allergens. *Allergy* **59**: 1277-1284

Kobayashi K, Fukuda M, Igarashi D, & Sunaoshi M (2000) Cytokinin-binding proteins from tobacco callus share homology with osmotin-like protein and an endochitinase. *Plant Cell Physiol* **41**: 148-157

Kofler S, Asam C, Eckhard U, Wallner M, Ferreira F, & Brandstetter H (2012) Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen bet v 1. *J Mol Biol* **422**: 109-123

Koistinen KM, Soininen P, Venalainen TA, Hayrinen J, Laatikainen R, Perakyla M, Tervahauta AI, & Karenlampi SO (2005) Birch PR-10c interacts with several biologically important ligands. *Phytochemistry* **66**: 2524-2533

Lamb CJ, Lawton MA, Dron M, & Dixon RA (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* **56**: 215-224

Lavola A, Nybakken L, Rousi M, Pusenius J, Petrelius M, Kellomäki S, & Julkunen-Tiitto R (2013) Combination treatment of elevated UVB radiation, CO2 and temperature has little effect on silver birch (Betula pendula) growth and phytochemistry. *Physiol Plantarum* **149**: 499-514

Lemieux RU (1999) How Water Provides the Impetus for Molecular Recognition in Aqueous Solution. *Acc Chem Res* **32**: 631-631

Li L, Song J, Kalt W, Forney C, Tsao R, Pinto D, Chisholm K, Campbell L, Fillmore S, & Li X (2013) Quantitative proteomic investigation employing stable isotope labeling by peptide dimethylation on proteins of strawberry fruit at different ripening stages. *J Proteomics* **94**: 219-239

Lin RY, Clauss AE, & Bennett ES (2002) Hypersensitivity to Common Tree Pollens in New York City Patients. *Allergy and Asthma Proceedings* **23**: 253-258

Lingwood D & Simons K (2010) Lipid rafts as a membrane-organizing principle. *Science* **327**: 46-50

Liu J & Ekramoddoullah AKM (2006) The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. *Physiol Mol Plant Pathol* **68**: 3-13

Loon LC (1985) Pathogenesis-related proteins. *Plant Mol Biol* **4**: 111-116

Luttkopf D, Muller U, Skov PS, Ballmer-Weber B, Wuthrich B, Hansen KS, Poulsen LK, Kastner M, Haustein D, & Vieths S (2002) Comparison of four variants of a major allergen in hazelnut (Corylus avellana) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Mol Immunol* **38**: 515-525

Ma Y, Gadermaier G, Bohle B, Bolhaar S, Knulst A, Markovic-Housley Z, Breiteneder H, Briza P, Hoffmann-Sommergruber K, & Ferreira F (2006) Mutational Analysis of Amino Acid Positions Crucial for IgE-Binding Epitopes of the Major Apple *(Malus domestica)* Allergen, Mal d 1. *Int Arch Allergy Immunol* **139**: 53-62

Mari A, Ballmer-Weber B, & Vieths S (2005) The oral allergy syndrome: improved diagnostic and treatment methods. *Current Opinion in Allergy and Clinical Immunology* **5**

Mari A, Scala E, Palazzo P, Ridolfi S, Zennaro D, & Carabella G (2006) Bioinformatics applied to allergy: Allergen databases, from collecting sequence information to data integration. The Allergome platform as a model. *Cell Immunol* **244**: 97-100

Mariani V, Gilles S, Jakob T, Thiel M, Mueller MJ, Ring J, Behrendt H, & Traidl-Hoffmann C (2007) Immunomodulatory mediators from pollen enhance the migratory capacity of dendritic cells and license them for Th2 attraction. *J Immunol* **178**: 7623-7631

Marintchev A, Frueh D, & Wagner G (2007) NMR methods for studying protein-protein interactions involved in translation initiation. *Methods Enzymol* **430**: 283-331

Markovic-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, Susani M, Ferreira F, Scheiner O, & Breiteneder H (2003) Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J Mol Biol* **325**: 123-133

Meurer B, Wiermann R, & Strack D (1988) Phenylpropanoid patterns in fagales pollen and their phylogenetic relevance. *Phytochemistry* **27**: 823-828

Mirza O, Henriksen A, Ipsen H, Larsen JN, Wissenbach M, Spangfort MD, & Gajhede M (2000) Dominant Epitopes and Allergic Cross-Reactivity: Complex Formation Between a Fab Fragment of a Monoclonal Murine IgG Antibody and the Major Allergen from Birch Pollen Bet v 1. *The Journal of Immunology* **165**: 331-338

Mo Y, Nagel C, & Taylor LP (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proc Natl Acad Sci U S A* **89**: 7213-7217

Mogensen JE, Wimmer R, Larsen JN, Spangfort MD, & Otzen DE (2002) The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J Biol Chem* **277**: 23684-23692

Mogensen JE, Ferreras M, Wimmer R, Petersen SV, Enghild JJ, & Otzen DE (2007) The Major Allergen from Birch Tree Pollen, Bet v 1, Binds and Permeabilizes Membranes. *Biochemistry* **46**: 3356-3365

Morales LO, Tegelberg R, Brosché M, Lindfors A, Siipola S, & Aphalo PJ (2011) Temporal variation in epidermal flavonoids due to altered solar UV radiation is moderated by the leaf position in Betula pendula. *Physiol Plantarum* **143**: 261-270

Movérare R, Westritschnig K, Svensson M, Hayek B, Bende M, Pauli G, Sorva R, Haahtela T, Valenta R, & Elfman L (2002) Different IgE Reactivity Profiles in Birch Pollen-Sensitive Patients from Six European Populations Revealed by Recombinant Allergens: An Imprint of Local Sensitization. *Int Arch Allergy Immunol* **128**: 325-335

Müller-Werdan U & Werdan K (2011) Anaphylaktischer Schock. In Burchardi H, Larsen R, Marx G, Muhl E, Schölmerich J (eds) pp 1079-1086. Springer Berlin Heidelberg

Munoz C, Hoffmann T, Escobar NM, Ludemann F, Botella MA, Valpuesta V, & Schwab W (2010) The strawberry fruit Fra a allergen functions in flavonoid biosynthesis. *Mol Plant* **3**: 113-124

Murphy KM, Travers P, Walport M, Mahlke K, Seidler L, & HauÃŸer-Siller I (2009) *Immunologie.* Spektrum Akademischer Verlag

Murzin AG, Brenner SE, Hubbard T, & Chothia C (1995) SCOP: A structural classification of proteins database for the investigation of sequences and structures. *J Mol Biol* **247**: 536-540

Musidlowska-Persson A, Alm R, & Emanuelsson C (2007) Cloning and sequencing of the Bet v 1-homologous allergen Fra a 1 in strawberry (Fragaria ananassa) shows the presence of an intron and little variability in amino acid sequence. *Mol Immunol* **44**: 1245-1252

Neudecker P, Lehmann K, Nerkamp J, Haase T, Wangorsch A, Fotisch K, Hoffmann S, Rosch P, Vieths S, & Scheurer S (2003) Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry (Prunus avium) and celery (Apium graveolens): correlating IgE reactivity with three-dimensional structure. *Biochem J* **376**: 97-107

Neudecker P, Schweimer K, Nerkamp J, Scheurer S, Vieths S, Sticht H, & Rösch P (2001) Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1. *J Biol Chem* **276**: 22756-22763

Niemi M, Jylhä S, Laukkanen M, Söderlund H, Mäkinen-Kiljunen S, Kallio JM, Hakulinen N, Haahtela T, Takkinen K, & Rouvinen J (2007) Molecular Interactions between a Recombinant IgE Antibody and the beta-Lactoglobulin Allergen. *Structure* **15**: 1413-1421

Owen JA, Punt J, & Stranford SA (2013) *Kuby Immunology.* W. H. Freeman

Pacini E (1994) Cell biology of anther and pollen development. In Williams E, Clarke A, Knox RB (eds) pp 289-308. Springer Netherlands

Padavattan S, Flicker S, Schirmer T, Madritsch C, Randow S, Reese G, Vieths S, Lupinek C, Ebner C, Valenta R, & Markovic-Housley Z (2009) High-affinity IgE recognition of a conformational epitope of the major respiratory allergen Phl p 2 as revealed by X-ray crystallography. *J Immunol* **182**: 2141-2151

Park CJ, Kim KJ, Shin R, Park JM, Shin YC, & Paek KH (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. *Plant J* **37**: 186-198

Pike LJ (2006) Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res* **47**: 1597-1598

Purohit A, Niederberger V, Kronqvist M, Horak F, Grönneberg R, Suck R, Weber B, Fiebig H, Van Hage M, Pauli G, Valenta R, & Cromwell O (2008) Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives. *Clinical & Experimental Allergy* **38**: 1514-1525

Radauer C, Lackner P, & Breiteneder H (2008a) The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC Evol Biol* **8**: 286

Radauer C & Breiteneder H (2007) Evolutionary biology of plant food allergens. *J Allergy Clin Immunol* **120**: 518-525

Radauer C & Breiteneder H (2006) Pollen allergens are restricted to few protein families and show distinct patterns of species distribution. *J Allergy Clin Immunol* **117**: 141-147

Radauer C, Bublin M, Wagner S, Mari A, & Breiteneder H (2008b) Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J Allergy Clin Immunol* **121**: 847-852.e7

Renkonen J, Mattila P, Lehti S, Mäkinen J, Sormunen R, Tervo T, Paavonen T, & Renkonen R (2009) Birch pollen allergen Bet v 1 binds to and is transported through conjunctival epithelium in allergic patients. *Allergy* **64**: 868-875

Rigden J & Coutts R (1988) Pathogenesis-related proteins in plants. *Trends Genet* **4**: 87-89

Rinia AB, Kostamo K, Ebbens FA, van Drunen CM, & Fokkens WJ (2007) Nasal polyposis: a cellular-based approach to answering questions. *Allergy* **62**: 348-358

Romagnani S (2000) The role of lymphocytes in allergic disease. *J Allergy Clin Immunol* **105**: 399-408

Rösch P & Berkner H (2009) Birke, Kirsche, Sojabohne – das Kreuz mit den Allergenen. *BIOSpektrum* **6**: 618-618

Roth-Walter F, Gomez-Casado C, Pacios LF, Mothes-Luksch N, Roth GA, Singer J, Diaz-Perales A, & Jensen-Jarolim E (2014) Bet v 1 from lipocalin-like protein acting as allergen only when devoid of iron by promoting Th2 lymphocytes. *Journal of Biological Chemistry*

Ryan KG, Markham KR, Bloor SJ, Bradley JM, Mitchell KA, & Jordan BR (1998) UVB Radiation Induced Increase in Quercetin: Kaempferol Ratio in Wild-Type and Transgenic Lines of Petunia. *Photochem Photobiol* **68**: 323-330

Samanani N, Liscombe DK, & Facchini PJ (2004) Molecular cloning and characterization of norcoclaurine synthase, an enzyme catalyzing the first committed step in benzylisoquinoline alkaloid biosynthesis. *Plant J* **40**: 302-313

Saraste M, Sibbald PR, & Wittinghofer A (1990) The P-loop--a common motif in ATP- and GTP-binding proteins. [Review] [31 refs]. *Trends Biochem Sci* **15**: 430-434

Scanlon ST, Thomas SY, Ferreira CM, Bai L, Krausz T, Savage PB, & Bendelac A (2011) Airborne lipid antigens mobilize resident intravascular NKT cells to induce allergic airway inflammation. *J Exp Med* **208**: 2113-2124

Schenk MF, Cordewener JH, America AH, Van't Westende WP, Smulders MJ, & Gilissen LJ (2009) Characterization of PR-10 genes from eight Betula species and detection of Bet v 1 isoforms in birch pollen. *BMC Plant Biol* **9**: 24

Scheurer S, Metzner K, Haustein D, & Vieths S (1997) Molecular cloning, expression and characterization of Pru a 1, the major cherry allergen. *Mol Immunol* **34**: 619-629

Schweimer K, Petersen A, Suck R, Becker WM, Rosch P, & Matecko I (2008) Solution structure of Phl p 3, a major allergen from timothy grass pollen. *Biol Chem* **389**: 919-923

Sicherer SH & Sampson HA (2014) Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *J Allergy Clin Immunol* **133**: 291-307.e5

Sikorski MM, Biesiadka J, Kasperska AE, Kopcińska J, Łotocka B, Golinowski W, & Legocki AB (1999) Expression of genes encoding PR10 class pathogenesis-related proteins is inhibited in yellow lupine root nodules. *Plant Science* **149**: 125-137

Sinha M, Singh RP, Kushwaha GS, Iqbal N, Singh A, Kaushik S, Kaur P, Sharma S, & Singh TP (2014) Current Overview of Allergens of Plant Pathogenesis Related Protein Families. *The Scientific World Journal* **2014**: 19

Smith GJ, Thomsen SJ, Markham KR, Andary C, & Cardon D (2000) The photostabilities of naturally occurring 5-hydroxyflavones, flavonols, their glycosides and their aluminium complexes. *J Photochem Photobiol A* **136**: 87-91

Son DY, Scheurer S, Hoffmann A, Haustein D, & Vieths S (1999) Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *Eur J Nutr* **38**: 201-215

Spangfort MD, Mirza O, Ipsen H, van Neerven, R. J. Joost, Gajhede M, & Larsen J (2003) Dominating IgE-Binding Epitope of Bet v 1, the Major Allergen of Birch Pollen, Characterized by X-ray Crystallography and Site-Directed Mutagenesis. *The Journal of Immunology* **171**: 3084-3090

Stevens W, Ebo D, Hagendorens M, Bridts C, & De Clerck L (2003) Is the prevalence of specific IgE to classical inhalant aeroallergens among patients with respiratory allergy changing? Evidence from two surveys 15 years apart. *Acta Clin Belg* **58**: 178-182

Strack D, Meurer B, Wray V, Grotjahn L, Austenfeld FA, & Wiermann R (1984) Quercetin 3-glucosylgalactoside from pollen of Corylus avellana. *Phytochemistry* **23**: 2970-2971

Swaminathan CP, Surolia N, & Surolia A (1998) Role of Water in the Specific Binding of Mannose and Mannooligosaccharides to Concanavalin A. *J Am Chem Soc* **120**: 5153-5159

Swoboda I, Hoffmann-Sommergruber K, O'Ríordáin G, Scheiner O, Heberle-Bors E, & Vicente O (1996) Bet v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity in vitro. *Physiol Plantarum* **96**: 433-438

Swoboda I, Jilek A, Ferreira F, Engel E, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breiteneder H, Pittenauer E, & Schmid E (1995) Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J Biol Chem* **270**: 2607-2613

Tarahovsky YS, Kim YA, Yagolnik EA, & Muzafarov EN (2014) Flavonoid-membrane interactions: involvement of flavonoid-metal complexes in raft signaling. *Biochim Biophys Acta* **1838**: 1235-1246

Thomas WR (2014) Allergen ligands in the initiation of allergic sensitization. *Curr Allergy Asthma Rep* **14**: 432-014-0432-x

Toone EJ (1994) Structure and energetics of protein-carbohydrate complexes. *Curr Opin Struct Biol* **4**: 719-728

Tsujishita Y & Hurley JH (2000) Structure and lipid transport mechanism of a StAR-related domain. *Nat Struct Biol* **7**: 408-414

Vanekkrebitz M, Hoffmannsommergruber K, Machado MLD, Susani M, Ebner C, Kraft D, Scheiner O, & Breiteneder H (1995) Cloning and Sequencing of Mal d 1, the Major Allergen from Apple (Malus domestica), and Its Immunological Relationship to Bet v 1, the Major Birch Pollen Allergen. *Biochem Biophys Res Commun* **214**: 538-551

Vieths S, Scheurer S, & Ballmer-Weber B (2002) Current understanding of cross-reactivity of food allergens and pollen. *Ann N Y Acad Sci* **964**: 47-68

Vogel L, Lüttkopf D, Hatahet L, Haustein D, & Vieths S (2005) Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* **60**: 1021-1028

von Pirquet C. (1906) Allergie. *Munch Med Wochenschr* **30**: 1457-1458

Vrtala S, Hirtenlehner K, Susani M, Akdis M, Kussebi F, Akdis CA, Blaser K, Hufnagl P, Binder BR, Politou A, Pastore A, Vangelista L, Sperr WR, Semper H, Valent P, Ebner C, Kraft D, & Valenta R (2001) Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1. *FASEB J* **15**: 2045-2047

Wangorsch A, Ballmer-Weber B, Rösch P, Holzhauser T, & Vieths S (2007) Mutational epitope analysis and cross-reactivity of two isoforms of Api g 1, the major celery allergen. *Mol Immunol* **44**: 2518-2527

White P, Smith H, Baker N, Davis W, & Frew A (1998) Symptom control in patients with hay fever in UK general practice: how well are we doing and is there a need for allergen immunotherapy? *Clin Exp Allergy* **28**: 266-270

Wood RA, Sicherer SH, Burks AW, Grishin A, Henning AK, Lindblad R, Stablein D, & Sampson HA (2013) A phase 1 study of heat/phenol-killed, E. coli-encapsulated, recombinant modified peanut proteins Ara h 1, Ara h 2, and Ara h 3 (EMP-123) for the treatment of peanut allergy. *Allergy* **68**: 803-808

Zhuang Y & Dreskin S (2013) Redefining the major peanut allergens. *Immunol Res* **55**: 125-134

6 Publikationsliste

6.1 Einzelarbeit A

Hanna Berkner[*], Christian Seutter von Loetzen[*], Maximilian Johannes Hartl, Stefanie Randow, Michaela Gubesch, Lothar Vogel, Felix Husslik, Andreas Reuter, Jonas Lidholm, Barbara Ballmer-Weber, Stefan Vieths, Paul Rösch, and Dirk Schiller (2014) Enlarging the toolbox for allergen epitope definition: Grafting of a functional epitope on an allergen-related model protein. *PLOS ONE*, **9**: e111691

[*] Beide Autoren haben in gleichem Maße zur Arbeit beigetragen.

Die ursprünglich von Hanna Berkner und Dirk Schiller entworfenen Konstrukte, codierend für die Varianten Δ29NCS_4x und Δ29NCS_5x, wurden von mir exprimiert und zusammen mit Maximilian Johannes Hartl gereinigt. Weiterhin wurden die ^{1}H-^{15}N-HSQC-Spektren der Proteine Δ29NCS, Δ29NCS_4x und Δ29NCS_5x von mir aufgenommen und analysiert. Maximilian Johannes Hartl nahm die CD-Spektren dieser drei Proteine auf. Alle immunologischen Daten wurden von Hanna Berkner, Stefanie Randow, Michaela Gubesch, Lothar Vogel, Felix Husslik und Andreas Reuther aufgenommen und ausgewertet. Die ImmunoCAP-Experimente wurden von Jonas Lidholm, Stefan Vieths und Michaela Gubesch durchgeführt und analysiert. Barbara Ballmer-Weber war an der Beschaffung des Blutserums von Birkenpollenallergikern beteiligt. Das Manuskript wurde von Maximilian Johannes Hartl und Dirk Schiller verfasst.

6.2 Einzelarbeit B

Seutter von Loetzen Christian, Schweimer Kristian, Schwab Wilfried, Rösch Paul, & Hartl-Spiegelhauer Olivia (2012) Solution structure of the strawberry allergen Fra a 1. *Bioscience Reports* **32**: 567-575

Die Klonierung des Fra a 1E-Konstrukts wurde von Wilfried Schwab durchgeführt. Die Expression des für Fra a 1E codierenden Gens und die anschließende Reinigung von unmarkiertem sowie Isotopen-markiertem Fra a 1E wurden von mir durchgeführt. Weiterhin habe ich die CD-Messungen und die analytische Gelfiltration durchgeführt und ausgewertet. Alle NMR-Experimente wurden von mir und Kristian Schweimer gemessen und ausgewertet. Die anschließende Strukturrechnung wurde von Kristian Schweimer mit meiner Unterstützung durchgeführt. Olivia Hartl-Spiegelhauer plante und leitete das Projekt und berechnete weiterhin die Volumen der hydrophoben Taschen der in der Einzelarbeit angegebenen PR-10 Proteine. Das Manuskript wurde von Paul Rösch und Olivia Hartl-Spiegelhauer verfasst.

6.3 Einzelarbeit C

Seutter von Loetzen Christian, Hoffmann Thomas, Hartl Maximillian Johannes, Schweimer Kristian, Schwab Wilfried, Rösch Paul, & Hartl-Spiegelhauer Olivia (2014) Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. *Biochemical Journal* **457**: 379-390

Die Extraktion von natürlichem Bet v 1 aus Birkenpollen sowie die Rekonstitution des Bet v 1:Q3OS-Komplexes wurde von Olivia Hartl-Spiegelhauer durchgeführt. Sämtliche Docking-Simulationen, UV/VIS- und NMR-Titrationen wurden von mir durchgeführt. Unter der Leitung von Maximilian Johannes Hartl führte ich die Fluoreszenztitration mit Olivia Hartl-Spiegelhauer durch. Weiterhin wurden die STD-Experimente unter Leitung von Kristian Schweimer von mir vollzogen. Thomas Hoffmann und Wilfried Schwab identifizierten Q3OS und reinigten den Liganden aus Birkenpollen. Das Projekt wurde von Olivia Hartl-Spiegelhauer und Paul Rösch geplant und geleitet. Das Manuskript wurde von mir, Thomas Hoffmann, Wilfried Schwab, Maximilian Johannes Hartl, Paul Rösch und Olivia Hartl-Spiegelhauer geschrieben.

6.4 Einzelarbeit D

Christian Seutter von Loetzen, Thessa Jacob, Olivia Hartl-Spiegelhauer, Lothar Vogel, Dirk Schiller, Cornelia Spörlein-Güttler, Rainer Schobert, Stefan Vieths, Maximilian Johannes Hartl & Paul Rösch (2014) Isoforms of the Major Birch Pollen Allergen Bet v 1: Allergenicity and Physiological Function. *PLOS ONE, submitted*

Die Klonierung sowie die Expressions- und Reinigungsstrategie für die rekombinanten Isoformen Bet v 1d und m wurden von mir entworfen und experimentell mit Unterstützung von Thessa Jacob umgesetzt. Die UV/VIS- und NMR-Titrationen wurden von Olivia Hartl-Spiegelhauer und mir konzipiert und mit Unterstützung von Thessa Jacob gemessen und ausgewertet. Weiterhin wurde die Zuordnung der ^{1}H-^{15}N-Amidresonanzen von Bet v 1d und m sowie sämtliche Docking-Simulationen von mir durchgeführt bzw. gerechnet. Maximilian Johannes Hartl hat den Bet v 1a:Q3OS-Komplex gereinigt. Die darauffolgenden UV/VIS-Spektren wurden von mir gemessen und ausgewertet. Alle immunologischen Experimente wurden von Lothar Vogel und Dirk Schiller koordiniert und ausgewertet. Cornelia Spörlein-Güttler war unter der Leitung von Rainer Schobert an der Extraktion bzw. der Synthese von Q3OS beteiligt. Das Projekt wurde von Olivia Hartl-Spiegelhauer, Maximilian Johannes Hartl, Stefan Vieths und Paul Rösch geplant und geleitet. Das Manuskript wurde von mir, Paul Rösch, Dirk Schiller und Maximilian Johannes Hartl verfasst.

7 Einzelarbeiten

7.1 Einzelarbeit A

Hanna Berkner[*], Christian Seutter von Loetzen[*], Maximilian Johannes Hartl, Stefanie Randow, Michaela Gubesch, Lothar Vogel, Felix Husslik, Andreas Reuter, Jonas Lidholm, Barbara Ballmer-Weber, Stefan Vieths, Paul Rösch, and Dirk Schiller (2014) Enlarging the toolbox for allergen epitope definition: Grafting of a functional epitope on an allergen-related model protein. *PLOS ONE*, **9**: e111691

[*] Beide Autoren haben in gleichem Maße zur Arbeit beigetragen.

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Enlarging the Toolbox for Allergen Epitope Definition with an Allergen-Type Model Protein

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Abstract

Background: Birch pollen-allergic subjects produce polyclonal cross-reactive IgE antibodies that mediate pollen-associated food allergies. The major allergen Bet v 1 and its homologs in plant foods bind IgE in their native protein conformation. Information on location, number and clinical relevance of IgE epitopes is limited. We addressed the use of an allergen-related protein model to identify amino acids critical for IgE binding of PR-10 allergens.

Method: Norcoclaurine synthase (NCS) from meadow rue is structurally homologous to Bet v 1 but does not bind Bet v 1-reactive IgE. NCS was used as the template for epitope grafting. NCS variants were tested with sera from 70 birch pollen allergic subjects and with monoclonal antibody BV16 reported to compete with IgE binding to Bet v 1.

Results: We generated an NCS variant (Δ29NCS$_{N57/I58E/D60N/V63P/D68K}$) harboring an IgE epitope of Bet v 1. Bet v 1-type protein folding of the NCS variant was evaluated by ^{1}H-^{15}N-HSQC NMR spectroscopy. BV16 bound the NCS variant and 71% (50/70 sera) of our study population showed significant IgE binding. We observed IgE and BV16 cross-reactivity to the epitope presented by the NCS variant in a subgroup of Bet v 1-related allergens. Moreover BV16 blocked IgE binding to the NCS variant. Antibody cross-reactivity depended on a defined orientation of amino acids within the Bet v 1-type conformation.

Conclusion: Our system allows the evaluation of patient-specific epitope profiles and will facilitate both the identification of clinically relevant epitopes as biomarkers and the monitoring of therapeutic outcomes to improve diagnosis, prognosis, and therapy of allergies caused by PR-10 proteins.

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Introduction

Millions of patients with allergies to tree pollen are sensitized (produce IgE antibodies) to the major allergen of birch (*Betula verrucosa*) pollen, Bet v 1 [1]. Bet v 1-binding IgE cross-reacts with Bet v 1-homologous proteins from plant foods, leading to allergy to such foods in a majority of birch pollen-allergic subjects [2]. Despite all efforts so far, a complete IgE epitope profile of Bet v 1 or of a Bet v 1-related allergen is still lacking, although knowledge of clinically relevant IgE binding determinants would benefit diagnosis, therapy, and current understanding of the sometimes puzzling clinical phenomena of pollen-related food allergies. Structural information on IgE epitopes of Bet v 1 and Bet v 1-like allergens in foods is limited. To date only IgE epitopes of allergens Art v 1 (pollen), Phl p 2 (grass), and β-lactoglobulin (milk) were determined from X-ray crystallography and NMR spectroscopy of IgE-allergen complexes [3–5]. Conformational IgE epitopes of allergens from the PR10-protein family, however, are unknown. Allergen variants carrying substitutions of either individual or multiple residues to either attenuate or induce IgE antibody binding (epitope grafting) identified individual residues crucial for IgE recognition by Bet v 1 [6–10] and Bet v 1-type allergens from apple [11,12], cherry [13,14], and celeriac [15]. In addition, monoclonal antibodies have been used to localize potential IgE epitopes of Bet v 1 and related allergens [10,16–19]. The only structural information on an epitope of Bet v 1 to date was obtained from X-ray crystallography of a complex between the monoclonal Bet v 1-specific mouse IgG BV16 and the

major isoform of Bet v 1, Bet v 1 a [20]. Binding of BV16 to Bet v 1 reduces serum IgE interactions, indicating competition of IgE and monoclonal IgG for an overlapping binding site of Bet v 1 [21,22].

IgE-allergen interactions are usually analyzed with polyclonal serum IgE, making it difficult to differentiate individual IgE interaction sites. Engineering of recombinant allergen variants often changes the native protein conformation which is essential for IgE interaction. Thus we suggest to use a non-IgE binding protein with allergen-like conformation as a scaffold that can be modulated on the individual amino acid level to stepwise induce IgE recognition capabilities. For this purpose we recently cloned, expressed, purified, and characterized the enzyme norcoclaurine synthase (NCS) from the meadow rue *Thalictrum flavum* as a recombinant protein variant Δ29NCS [23,24]. As Bet v 1, NCS is a member of the pathogenesis-related protein family (PR-10) sharing the typical Bet v 1 protein fold [25], but has no known allergenic properties. NCS is thus an ideal protein model candidate to study epitopes of PR-10 allergens.

Here we aimed at establishing a recombinant model protein system to specifically analyze epitopes of PR-10 allergens. For this purpose we used the truncated variant Δ29NCS. To study the impact of individual amino acids in IgE binding within NCS we generated variants of Δ29NCS, analyzed their IgE antibody binding with sera of birch pollen allergic subjects and determined the cross-reactivity of suspected IgE epitopes grafted onto NCS.

Methods

Patients

Sixty-nine patients with a convincing history of pollinosis to early flowering tree pollen and specific IgE levels>0.35 kU_A/L to birch pollen measured by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) were included as serum donors. Patients were recruited at the Allergy Unit, Department of Dermatology, University Hospital Zürich, Switzerland, at the Hospital Borkum Riff, Borkum, Germany, and at the Paul-Ehrlich-Institut, Langen, Germany. Study participants provided written informed consent. Ethics approval by the local ethics committee 'Kantonale Ethikkomission Zürich, Switzerland' of the University Hospital in Zürich, Switzerland and the local ethics committee 'Ethik-Kommission, Fachbereich Medizin der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany' of the University Hospital in Frankfurt included consent form and consent procedure. Sera 52–69 were published elsewhere [26]. Serum 70 was purchased from DLab Diagnose GmbH, Hamburg, Germany. Serum from one non-allergic subject was used as negative control for the specific IgE measurements. The positive serum pool (IgE>0.35 kU_A/L against Δ29NCS_5x) comprised sera 11, 14, 20–23, 32, 42, 47, and 70. The negative serum pool (IgE< 0.35 kU_A/L against Δ29NCS_5x) comprised sera 4, 12, 18, 29–31, 33, 40, 43, and 46.

Determination of specific IgE

Specific IgE levels to recombinant proteins were determined by ImmunoCAP in Phadia 100 and 250 instruments (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions. In the case of Bet v 1, Api g 1.01 and Cor a 1.04, commercial ImmunoCAP tests were used, whereas for Pru av 1, Dau c 1.01, NCS and its variants, experimental ImmunoCAPs were manufactured by coupling the recombinant allergens individually to the solid phase, as described elsewhere [27,28].

Cloning, expression and purification of Δ29NCS, Bet v 1 variants, and Bet v 1-related allergens

Mutations leading to the amino acid exchanges/insertion in $\Delta29NCS_{N57/I58E/D60N/V63P}$, $\Delta29NCS_{N57/I58E/D60N/V63P/D68K}$, and Bet v $1_{N43A/E45S/N47A/K55A}$ were sequentially introduced into pET29b-Δ29NCS [23] and pET15b-Bet v 1, respectively, with the QuickChange kit from Stratagene (Heidelberg, Germany). All protein variants were expressed and purified via their His_6-tag as described [23] with minor changes. To yield higher amounts of soluble protein, the synthesis of the protein variants was induced by the addition of 1 mM IPTG at 25°C followed by incubation overnight. A synthetic gene encoding soy allergen Gly m 4 was cloned into pET15b and purified as described above for Bet v 1. The following Bet v 1-related allergens were expressed and purified as described: Pru av 1 (cherry) [29], Dau c 1 (carrot) [26], and Cor a 1.04 (hazelnut) [30]. rApi g 1.01 (celeriac) was purchased from Biomay, Vienna, Austria.

Circular dichroism

Far UV circular dichroism (CD) spectra of the Δ29NCS variants were acquired at 293 K using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Gross-Umstadt, Germany) at a band width of 1 nm and a sensitivity of 100 mdeg in a 0.2 cm cell. All proteins were analyzed at a concentration of 2.5 μM in 5 mM sodium phosphate, pH 7.0. Each measurement comprised the average of 10 repeated scans between 260 and 190 nm.

NMR spectroscopy

NMR samples were prepared by dissolving lyophilized ^{15}N-labeled protein in 20 mM sodium phosphate, pH 7.0, 1 mM DTT, 0.04% sodium azide and 10% D_2O. ^1H-^{15}N-HSQC spectra were recorded on a Bruker Avance 700 MHz spectrometer at 298 K. NMR data were processed using in-house software and were visualized with NMR view [31].

Modelling of NCS variants

Structure predictions of $\Delta29NCS_{N57/I58E/D60N/V63P}$, $\Delta29NCS_{N57/I58E/D60N/V63P/D68K}$, and Bet v $1_{N43I/E45S/N47D/K55A}$ were performed by using the Phyre server [32]. The calculated models were based on the structures of wild type NCS [25] (pdb 2VNE) and Bet v 1a [33] (pdb 1BV1), respectively, with a confidence of 100% and a sequence coverage of at least 85%.

Binding of monoclonal antibody BV16 to Bet v 1 and related allergens

Nunc Maxisorp plates (Fisher Scientific, Schwerte, Germany) were coated overnight at room temperature with 250 ng/100 μl proteins (Δ29NCS_5x; Δ29NCS; Dau c 1; Pru av 1; Bet v 1; Gly m 4; Cor a 1), with 50 ng/100 μl Api g 1, and with 25 ng/100 μl Bet v 1 and Bet v 1_4x in phosphate-buffered saline (PBS). After blocking with PBS containing 2% BSA a dilution series of the murine monoclonal anti-Bet v 1 antibody BV16 [34] was added for 1 h at room temperature in PBS containing 0.05% Tween 20 and 0.1% BSA. Allergen-specific IgG was detected with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (A3673, SigmaAldrich, Taufkirchen, Germany) diluted 1:3000 in PBS containing 0.05% Tween 20 and 0.1% BSA as described for the IgE ELISA below.

Inhibition IgE ELISA

For IgE-ELISA inhibition experiments, Nunc Maxisorp plates (Fisher Scientific, Schwerte, Germany) were coated overnight at room temperature with 300 ng/100 μl Δ29NCS_5x with 10 mM

potassium phosphate-buffered saline (PBS). After blocking with PBS containing 2% BSA, plates were incubated with the human serum pool (dilution 1:10) and increasing concentrations ($3*10^{-5}$–3 μg/well) of recombinant inhibitors (Bet v 1, Bet v 1_4x, Dau c 1.01, Pru av 1, Gly m 4, Cor a 1.04, and Api g 1.01, respectively) for 3 hrs at room temperature with PBS containing 0.05% Tween 20 and 0.1% BSA. Allergen-specific IgE was detected with horseradish peroxidase-conjugated mouse anti-human IgE antibody (Clone B3102E8, Southern Biotech via Biozol, Eching, Germany) diluted 1:1000 with PBS containing 0.05% Tween 20 and 0.1% BSA. The substrate for horseradish peroxidase was 3,3′,5,5′-tetramethylbenzidine (Roth, Karlsruhe, Germany) and the reaction was stopped by addition of 25% H_2SO_4. The absorbance was measured at 450 nm.

For the IgE-ELISA inhibition experiment with monoclonal antibody, ascites fluid of Bet v 1-specific antibody BV16 [34] (diluted: 1:1000; 1:10000, and 1:100000 in PBS containing 0.05% Tween and 0.1% BSA) was pre-incubated with 9 ng/well Bet v 1 as inhibitor for 2 hrs at room temperature. As a negative control, dilution buffer alone was preincubated with Bet v 1. After blocking with PBS containing 2% BSA, rBet v 1 and serum pool IgE diluted 1:10 (final concentration) were incubated on the plate for 3 hrs at room temperature. Human IgE was detected as described below.

Indirect ELISA for IgE binding to Bet v 1 variants

For IgE-ELISA experiments, Nunc Maxisorp plates (Nunc via Fisher Scientific, Schwerte, Germany) were coated overnight at room temperature with 25 ng/100 μl recombinant Bet v 1 and Bet v 1_4x, respectively with phosphate-buffered saline (PBS). Blocking and IgE detection was done as described for IgE ELISA above.

SDS-PAGE and immunoblot analysis

SDS-PAGE was performed with 15% separating gels and 5% stacking gels using a discontinuous buffer system [35]. For immunoblot analysis, 0.5 μg/cm of recombinant protein Δ29NCS_5x was transferred onto 0.2 μm nitrocellulose membranes by semi-dry blotting at 0.8 mA/cm^2 for 1 h [36]. After blocking with Tris-buffered saline (TBS) containing 0.3% Tween 20 blots were cut into strips and incubated overnight at room temperature with 5 μl of human serum pool with or without increasing amounts of recombinant allergens as inhibitors (Δ29NCS_5x and Bet v 1, respectively) with TBS containing 0.05% Tween 20 (TBST 0.05%) and 0.1% BSA (TBST 0.05%–0.1% BSA). After incubation for 1 h with horseradish peroxidase labelled mouse anti-human IgE antibody (Clone B3102E8, Southern biotech via Biozol, Eching, Germany), diluted 1:100000 with TBST 0.05%–0.1% BSA, IgE-binding proteins were visualized by chemiluminescence (LumiGLO Reserve diluted 1:3, KPL via Medac, Wedel, Germany).

Mediator release from humanized Rat Basophil Leukaemia (RBL) cells

The mediator release assay followed an established protocol [37]. Briefly, RBL cells expressing the α-chain of human FcεRI were sensitized overnight with the positive serum pool of human sera (diluted 1:20). After washing, cells were stimulated with serial dilutions of allergens. Degranulation was quantified by photometric measurement of β-hexosaminidase activity in the culture supernatants and was expressed as percent of the total cellular β-hexosaminidase content obtained by lysing the cells with Triton X-100 (Sigma-Aldrich, Steinheim, Germany) after correction for spontaneous release (sensitized cells without allergen).

MS analysis of recombinant protein variants

The identity of recombinant proteins was confirmed by liquid chromatography mass spectrometry (LC-MS). Recombinant proteins were excised from Coomassie stained SDS-PAGE gels and analyzed as described elsewhere [38]. Differing from this, peptides were eluted with 25 mM NH_4HCO_3; 10% aceto nitrile (ACN) and the digestion was stopped by adding 5% formic acid. The peptides were analyzed by using a nano-ultra performance LC system coupled to a nano-ESI- MS (nano Acquity UPLC nanoESI Synapt-MS, Waters, Milford, US) with a 5 μm symmetry 180 μm×20 mm c18 pre-column and a 1.7 μm BEH 130 100 μm×100 mm c18 separation column. After 3 minutes of trapping (99% water at 5 μl/minute), a 30 minutes gradient (3–40% ACN at 500 nl/minute) was applied to separate peptides. MS was operated in V mode, acquiring MSE data and applying standard parameters. Data analysis was performed with protein lynx global server version 2.4 (Waters), searching an in house database consisting of the Uniprot database (as of May 2011, restricted to reviewed entries of eukaryotic organisms) and the amino acid sequences of the recombinant variants Δ29NCS, Δ29NCS_4x and Δ29NCS_5x. The identification of a protein was accepted at a false positive rate of less than 4%; peptide mass accuracy was 9 parts per million (ppm) or better.

Miscellaneous

Amino acid sequence alignments were carried out with ClustalO [39]. 3D protein models were analyzed and illustrated using PyMol [40].

Results

Δ29NCS variants have Bet v 1-type tertiary structure

IgE antibody binding to PR-10 allergens depends on the presence of a native protein fold of the allergen. The crystal structure of recombinant norcoclaurine synthase (NCS, pdb 2VNE) revealed a conformation identical to that of Bet v 1, even though the sequence identity of these proteins is only 25% [25]. We recently optimized purification of a properly folded N-terminally truncated variant of NCS (Δ29NCS), which comprises the complete structurally corresponding amino acid sequence of Bet v 1 [23]. We generated two Δ29NCS variants harboring single amino acids of a Bet v 1 epitope for IgG that competes with IgE [20,22] (Figures 1 and 2A). In addition, Asp68 of NCS was exchanged for Lysine, a corresponding amino acid of Bet v 1 involved in IgE binding [22,41] and located directly adjacent to the epitope. Initially we tested whether the rΔ29NCS variants have the typical Bet v 1 conformation using circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy (Figure 2B, 2C and Figure S1). Both the CD and $^1H^{15}N$-HSQC NMR spectra of $\Delta29NCS_{N57/I58E/D60N/V63P}$ (Δ29NCS_4x) and $\Delta29NCS_{N57/I58E/D60N/V63P/D68K}$ (Δ29NCS_5x) indicated that the amino acid substitutions in the NCS variants did not change the proteins' secondary or tertiary structures and confirmed that the native protein conformation is maintained in the variants (Figure 2D).

Δ29NCS variants selectively bind IgE from sera of birch pollen allergic subjects

To assess potential cross-reactive IgE binding to rΔ29NCS variants, 70 sera of birch pollen allergic subjects were analyzed for IgE binding to a range of PR-10 proteins using ImmunoCAP tests (Table 1). All sera had sIgE to Bet v 1 (from 0.69 to>100 kU_A/L) and a majority to the PR-10 food allergens Pru av 1.01 (cherry, < 0.35 to 58.2 kU_A/L), Cor a 1.04 (hazelnut, <0.35 to>100 $kU_A/$

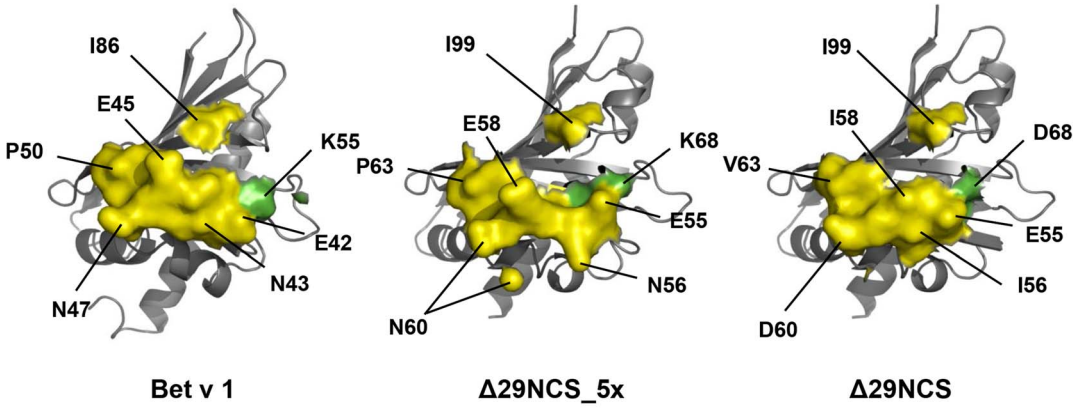

Figure 1. Grafting of an epitope onto Δ29NCS. Left panel: 13 of the 16 amino acids comprising the Bet v 1 epitope (Bet v $1_{E42/N43/I44/E45/G46/N47/G48/G49/P50/G51/T52/R70/D72/H76/I86/K97}$) of mouse monoclonal IgG antibody BV16 (yellow). Six amino acids of the epitope are labeled. Lys55 of Bet v 1 located adjacent to the epitope is highlighted (green). Middle panel: the corresponding 13 residues of the Bet v 1 epitope for BV16 and a lysine corresponding to K55 of Bet v 1 have been grafted onto Δ29NCS to generate Δ29NCS_4x (Δ29NCS $_{N57/I58E/D60N/V63P}$) (not shown) and Δ29NCS_5x (Δ29NCS $_{N57/I58E/D60N/V63P/D68K}$). Right panel: corresponding surface area of Δ29NCS. Protein models were based on the structures of NCS (pdb 2VNE) and Bet v 1a (pdb 1BV1), and modelled with a confidence of 100% and a sequence coverage of at least 85%.
doi:10.1371/journal.pone.0111691.g001

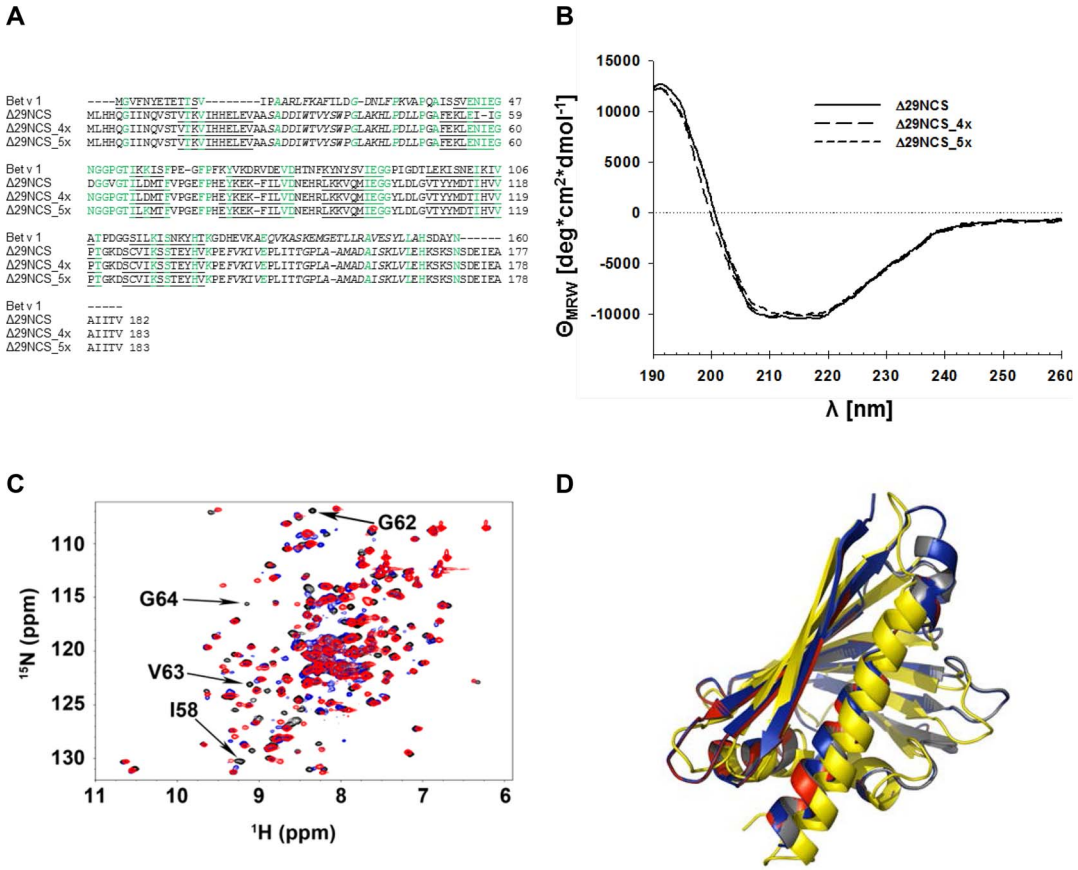

Figure 2. Δ29NCS, Δ29NCS_4x, and Δ29NCS_5x structures are virtually identical to the Bet v 1 structure. (A) Structural sequence alignment. β-strands (underlined), helices (italic), and amino acid identities (green) to Bet v 1 (pdb: 1BV1) are 23.8% (Δ29NCS), 26.8% (Δ29NCS_4x), and 27.5% (Δ29NCS_5x), respectively. (B) Circular dichroism of Δ29NCS variants. (C) Overlay of three ^{1}H-^{15}N-HSQC spectra of Δ29NCS variants. Black arrows, signals numbered and named according to Δ29NCS. Black: Δ29NCS, blue: Δ29NCS_4x, red: Δ29NCS_5x. The arrows highlight signals of selected amino acids that are expected to differ between the three Δ29NCS variants. Labeling is according to Δ29NCS scheme. (D) Overlay of secondary structure topologies of Δ29NCS (grey), Δ29NCS_4x (blue), Δ29NCS_5x (red), and Bet v 1 (yellow).
doi:10.1371/journal.pone.0111691.g002

Table 1. Human sera used in this study.

Patient	Serological data: specific IgE (kU_A/L) and CAP classes (0–6)													
No.	rBet v 1		rPru av 1		rCor a 1.04		Dau c 1.01		rApi g 1.01		Δ29NCS		Δ29NCS_4x $_{N57/I58E/D60N/V63P}$		Δ29NCS_5x $_{N57/I58E/D60N/V63P/D68K}$	
1	73.4	5	32.20	4	78.8	5	5.05	3	5.74	3	0.75	2	3.34	2	11.3	3
2	11.1	3	2.47	2	6.8	3	<0.35	0	<0.35	0	<0.35	0	0.39	1	1.38	2
3	43.3	4	16.1	3	38.3	4	14.1	3	11.8	3	<0.35	0	<0.35	0	2.89	2
4	79.4	5	22.4	4	>100	6	3.36	2	5.95	3	<0.35	0	<0.35	0	<0.35	0
5	48.6	4	12.1	3	57.8	5	6.54	3	6.84	3	<0.35	0	0.99	2	1.75	2
6	89.9	5	34.6	4	>100	6	1.64	2	2.00	2	<0.35	0	<0.35	0	13.8	3
7	69.6	5	18.0	4	55.2	5	9.55	3	10.7	3	<0.35	0	1.74	2	7.34	3
8	>100	6	51.2	5	61.7	5	28.1	4	26.9	4	0.45	1	0.93	2	7.34	3
9	>100	6	41.7	4	71.6	5	45.3	4	43.1	4	<0.35	0	<0.35	0	4.42	3
10	68.4	5	18.7	4	67.3	5	<0.35	0	0.74	2	<0.35	0	<0.35	0	17.4	4
11	>100	6	13.1	3	92.4	5	4.06	3	1.66	2	<0.35	0	<0.35	0	5.15	3
12	5.61	3	1.86	2	4.88	3	<0.35	0	0.39	1	<0.35	0	<0.35	0	<0.35	0
13	54.3	5	2.79	2	25.9	4	1.22	2	1.59	2	<0.35	0	<0.35	0	0.55	1
14	24.2	4	7.71	3	29.3	4	<0.35	0	1.28	2	<0.35	0	3.02	2	10.0	3
15	47.5	4	29.5	4	29.7	4	3.11	2	3.74	3	<0.35	0	1.62	2	2.85	2
16	22.5	4	3.34	2	11.1	3	<0.35	0	1.06	2	<0.35	0	0.63	1	2.56	2
17	73.8	5	12.8	3	69.8	5	33.4	4	38.2	4	<0.35	0	<0.35	0	2.45	2
18	25.6	4	6.60	3	12.0	3	3.76	3	5.91	3	<0.35	0	<0.35	0	<0.35	0
19	17.2	3	5.35	3	18.5	4	1.71	2	2.16	2	<0.35	0	0.40	1	1.77	2
20	37.5	4	9.78	3	37.0	4	6.18	3	6.76	3	<0.35	0	1.03	2	3.27	2
21	31.0	4	9.42	3	28.6	4	5.38	3	5.98	3	<0.35	0	0.85	2	3.36	2
22	35.4	4	11.6	3	21.7	4	<0.35	0	1.01	2	<0.35	0	<0.35	0	5.36	3
23	24.5	4	3.36	2	25.5	4	4.08	3	4.73	3	<0.35	0	0.36	1	4.47	3
24	24.2	4	7.78	3	27.3	4	1.83	2	3.23	2	<0.35	0	<0.35	0	1.82	2
25	77.7	5	6.97	3	15.2	3	6.58	3	7.93	3	<0.35	0	<0.35	0	1.28	2
26	25.4	4	8.16	3	20.9	4	1.58	2	2.00	2	<0.35	0	<0.35	0	2.81	2
27	17.8	4	9.9	3	14.1	3	<0.35	0	<0.35	0	<0.35	0	0.49	1	3.08	2
28	35.4	4	17.8	4	23.4	4	3.51	3	0.39	1	<0.35	0	2.09	2	11.3	3
29	37.1	4	8.82	3	4.99	3	4.99	3	4.57	3	<0.35	0	<0.35	0	<0.35	0
30	41.6	4	4.03	3	34.1	4	16.7	3	16.7	3	<0.35	0	<0.35	0	<0.35	0
31	36.4	4	2.93	2	12.7	3	0.61	1	1.91	2	<0.35	0	<0.35	0	<0.35	0
32	92.4	5	14.2	3	33.7	4	4.21	3	7.11	3	<0.35	0	1.47	2	16.1	3
33	19.0	4	2.16	2	15.9	3	0.35	1	1.62	2	<0.35	0	<0.35	0	<0.35	0

Table 1. Cont.

Patient	Serological data: specific IgE (kU_A/L) and CAP classes (0–6)													
No.	rBet v 1		rPru av 1		rCor a 1.04		Dau c 1.01		rApi g 1.01		Δ29NCS		Δ29NCS_4x $_{N57/I58E/D60N/V63P}$		Δ29NCS_5x $_{N57/I58E/D60N/V63P/D68K}$	
34	44.2	4	4.3	3	18.3	4	0.39	1	<0.35	0	<0.35	0	0.93	2	2.95	2
35	>100	6	58.2	5	98.5	5	15.9	3	20.5	4	<0.35	0	<0.35	0	4.65	3
36	78.3	5	11.5	3	34.5	4	1.89	2	0.89	2	0.46	1	<0.35	0	5.58	3
37	17.1	3	11.4	3	18.1	4	5.74	3	4.73	3	<0.35	0	1.23	2	4.01	3
38	17.2	3	5.56	3	12.4	3	4.22	3	5.16	3	<0.35	0	<0.35	0	0.94	2
39	94.2	5	17.1	3	30.9	4	9.13	3	12.1	3	0.46	1	0.74	2	1.19	2
40	19.0	4	9.86	3	13.0	3	3.02	2	4.03	3	<0.35	0	<0.35	0	<0.35	0
41	6.9	3	1.27	2	6.61	3	<0.35	0	<0.35	0	<0.35	0	<0.35	0	0.63	1
42	51.8	5	13.8	3	22.2	4	0.67	1	0.77	2	<0.35	0	1.15	2	6.09	3
43	14.2	3	4.36	3	15.9	3	5.82	3	6.51	3	<0.35	0	<0.35	0	<0.35	0
44	18.6	4	1.87	2	6.84	3	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0
45	4.01	3	2.18	2	3.59	3	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<035	0
46	3.51	3	<0,35	0	1.22	2	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0
47	16.1	3	3.00	2	9.48	3	1.91	2	4.85	3	<0.35	0	3.80	3	5.88	3
48	32.2	4	8.73	3	14.0	3	1.43	2	2.40	2	<0.35	0	<0.35	0	0.72	2
49	17.4	3	2.99	2	7.05	3	2.72	2	2.23	2	<0.35	0	<0.35	0	1.64	2
50	21.2	4	15.4	3	23.3	4	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0
51	28.8	4	5.82	3	13.8	3	0.89	2	1.01	2	<0.35	0	<0.35	0	0.40	1
52	18.4	4	5.69	3	9.34	3	0.48	1	0.56	1	<0.35	0	<0.35	0	<0.35	0
53	19.0	4	2.02	2	8.52	3	3.58	3	3.81	3	<0.35	0	0.40	1	1.92	2
54	0.90	2	<0.35	0	0.58	1	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0
55	13.8	3	7.73	3	10.0	3	0.49	1	2.28	2	<0.35	0	0.49	1	2.39	2
56	5.47	3	1.49	2	4.04	3	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0
57	19.0	4	10.6	3	16.0	3	0.84	2	0.58	1	<0.35	0	1.55	2	4.3	3
58	0.69	1	0.35	1	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0
59	52.3	5	14.8	3	34.9	4	6.61	3	9.04	3	<0.35	0	<0.35	0	1.49	2
60	1.58	2	0.41	1	1.27	2	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0
61	77.8	5	23.5	4	57.3	5	12.6	3	11.5	3	<0.35	0	4.52	3	9.2	3
62	50.8	5	13.3	3	27.6	4	0.4	1	0.94	2	<0.35	0	<0.35	0	<0.35	0
63	17.0	3	4.73	3	8.27	3	<0.35	0	<0.35	0	<0.35	0	<0.35	0	0.60	1
64	8.46	3	2.28	2	4.69	3	0.54	1	<0.35	0	<0.35	0	<0.35	0	<0.35	0
65	2.80	2	0.40	1	1.45	2	0.35	1	<0.35	0	<0.35	0	<0.35	0	<0.35	0
66	6.85	3	2.56	2	6.23	3	0.52	1	1.09	1	<0.35	0	<0.35	0	0.41	1

Table 1. Cont.

Patient	Serological data: specific IgE (kU_A/L) and CAP classes (0–6)														
No.	rBet v 1		rPru av 1		rCor a 1.04		Dau c 1.01		rApi g 1.01		Δ29NCS		Δ29NCS_4x $_{N57/I58E/D60N/V63P}$		Δ29NCS_5x $_{N57/I58E/D60N/V63P/D68K}$	
67	33.1	4	5.37	3	18.8	4	0.45	1	1.02	2	<0.35	0	<0.35	0	1.6	2
68	31.5	4	8.82	3	15.7	3	0.41	1	0.68	1	<0,35	0	1.02	2	4.87	3
69	3.03	2	<0.35	0	2.61	2	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0
70*	67	-	n.d.	-	33	-	<0.3	-	<0.3	-	n.d.	-	n.d.	-	n.d.	-
71**	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0	<0.35	0

*sIgE determined by ISAC. **Non-allergic control. Sera 52–69 were published elsewhere [40].
Summary of specific Immunoglobulin E against recombinant Bet v 1 and Δ29NCS variants.
doi:10.1371/journal.pone.0111691.t001

L), Dau c 1.01 (carrot, <0.35 to 98.5 kU_A/L), and Api g 1.01 (celeriac, <0.35 to 43.1 kU_A/L), respectively. In contrast, most of the sera (66/70, 94%) tested negative (<0.35 kU_A/L) to Δ29NCS, whereas 25/70 (36%) showed significant IgE binding (0.35 to 3.34 kU_A/L, $p < 0.0001$) to Δ29NCS_4x and 50/70 (71%) to Δ29NCS_5x (0.35 to 17.4 kU_A/L, $p<0.0001$). Thus, gradual substitution of certain amino acids of the low-IgE binding protein Δ29NCS with those of Bet v 1 at the corresponding positions induced significant binding of serum IgE from subjects with birch pollinosis.

Δ29NCS_5x presents an IgE epitope that cross-reacts with a subset of Bet v 1-homologous allergens

Since the majority of the patients' sera IgE bound Δ29NCS_5x, we asked whether this interaction was due to a Bet v 1-specific IgE epitope presented by the Δ29NCS variant. Therefore we used serum with sIgE to both Bet v 1 and Δ29NCS_5x and tested its IgE interaction with the Δ29NCS_5x variant in the presence of Bet v 1 (Figure 3). As expected, no IgE interaction of the serum pool with Δ29NCS was observed (Figure 3A). In contrast, IgE binding to Δ29NCS_5x was inhibited by Δ29NCS_5x itself and Bet v 1 in a dose-dependent manner, suggesting the presence of an IgE epitope shared by the two proteins.

Next, we asked whether the IgE epitope presented by Δ29NCS_5x is cross-reactive with PR-10 food allergens. Thus, we performed competitive IgE binding experiments (ELISA) with the PR-10 food allergens Cor a 1.04, Gly m 4, Dau c 1.01, Api g 1.01, and Pru av 1, respectively (Figure 3B). Bet v 1 as well as Cor a 1.04 and Gly m 4 competed for serum IgE binding to Δ29NCS_5x, whereas only very low inhibition of IgE binding to Δ29NCS_5x was observed with Api g 1, Pru av 1, and Dau c 1, respectively. We conclude that the IgE epitope offered by Δ29NCS_5x is not ubiquitous but specific for a subgroup of PR-10 allergens.

The IgE cross-reactive epitope of Δ29NCS_5x overlaps with an IgG binding site

Δ29NCS harbors 47% and Δ29NCS_5x 76% of the structurally resolved IgG epitope (total of 16 amino acids) of Bet v 1, as defined earlier [20]. We therefore asked whether the IgE binding epitope of Δ29NCS_5x overlaps with the binding site of BV16 on Bet v 1. Thus, we tested serum IgE binding of immobilized Δ29NCS_5x in the presence of both Bet v 1 and increasing amounts of BV16 (Figure 3C). In the absence of BV16, IgE binding to Δ29NCS_5x was inhibited to 84% by Bet v 1. The observed Bet v 1-induced inhibition of IgE-Δ29NCS_5x interaction could, however, been abolished by the monoclonal antibody BV16 in a dose-dependent manner. This result suggests a structural overlap between the individual cross-reactive IgE epitope of Δ29NCS_5x and the Bet v 1-specific IgG epitope recognized by monoclonal antibody BV16. Furthermore, when we tested BV16 binding only Bet v 1, Gly m 4, Cor a 1.04, and Δ29NCS_5x interacted markedly with the monoclonal antibody, whereas Dau c 1.01, Api g 1.01 1, Pru av 1, and Δ29NCS did not (Figure 3D).

Subtle structural differences define the IgE cross-reactivity pattern of Bet v 1-homologous allergens

The differential interaction of Δ29NCS_5x and Pr-10 allergens as above with both serum IgE and the Bet v 1-specific mouse monoclonal BV16 let us to hypothesize that Δ29NCS_5x displays an epitope shared only with Bet v 1, Cor a 1.04, and Gly m 4, but not with Dau c 1.01, Api g 1.01, and Pru av 1. Thus, we structurally aligned the epitope grafted onto Δ29NCS_5x and the

corresponding surface areas of cross-reactive and non-cross-reactive Bet v 1-related proteins (Figure 4). Residue by residue comparison of the epitope revealed that N43, E45, N47, and K55 of Bet v 1 are shared with Δ29NCS_5x, Gly m 4, and Cor a 1.04, but not with Pru av 1, Api g 1.01, and Dau c 1.01, thus mirroring the IgE/IgG inhibition results (cf. Figure 3) and suggesting that they are critical determinants of IgE cross-reactivity and BV16 binding. To challenge this hypothesis we investigated the substitutional variant Bet v 1_4x (Bet v $1_{N43I/E45S/N47D/K55A}$). As for Δ29NCS_5x and Δ29NCS_4x, the amino acid substitutions did not alter the protein conformation according to CD and $^{1}H^{15}N$-HSQC NMR spectroscopy (Figure S2). Serum IgE binding to Bet v 1_4x was virtually identical to that of wild type Bet v 1 (Figure 5A). However, inhibition of IgE binding to Δ29NCS_5x was strongly reduced by Bet v 1_4x as compared to Bet v 1 (Figure 5B), and interaction of monoclonal BV16 with Bet v 1_4x was virtually abolished (Figure 5C). The capacity of Bet v 1_4x to induce mediator release in humanized rat basophil leukemia cells *via* allergen mediated cross-linking of receptor bound human IgE was only slightly reduced as compared to wild type Bet v 1, suggesting the presence of further IgE epitopes compensating for the loss of one IgE epitope in Bet v 1_4x (Figure 5D). As expected, the Δ29NCS variants did not induce mediator release due to the lack of a second IgE epitope needed for allergen-mediated IgE cross-linking. Since Bet v 1_4x could not compete with Δ29NCS_5x for binding to serum IgE, we conclude that we have identified an epitope in a subset of PR-10 allergens that binds cross-reactive IgE and IgG.

Discussion

To analyze epitopes of allergens it is common to genetically engineer specific allergen variants and test their antibody binding capacity in comparison to the respective wild type allergen. A residual response, however, can be misleading if the epitope to be analyzed is conformational and proper binding of the antibody is dependent on the native fold of the allergen. In this case any observed compromised antibody-allergen interaction may well be due to an altered protein conformation of the allergen variant rather than due to the exchange of amino acids crucial for direct antibody binding. Furthermore, the analysis of epitopes in allergens is often hampered by the fact that the polyclonal serum antibody repertoire makes it technically challenging to measure and assign the functionality of single epitopes in the background of the overall multiple antibody binding to the allergen. As the

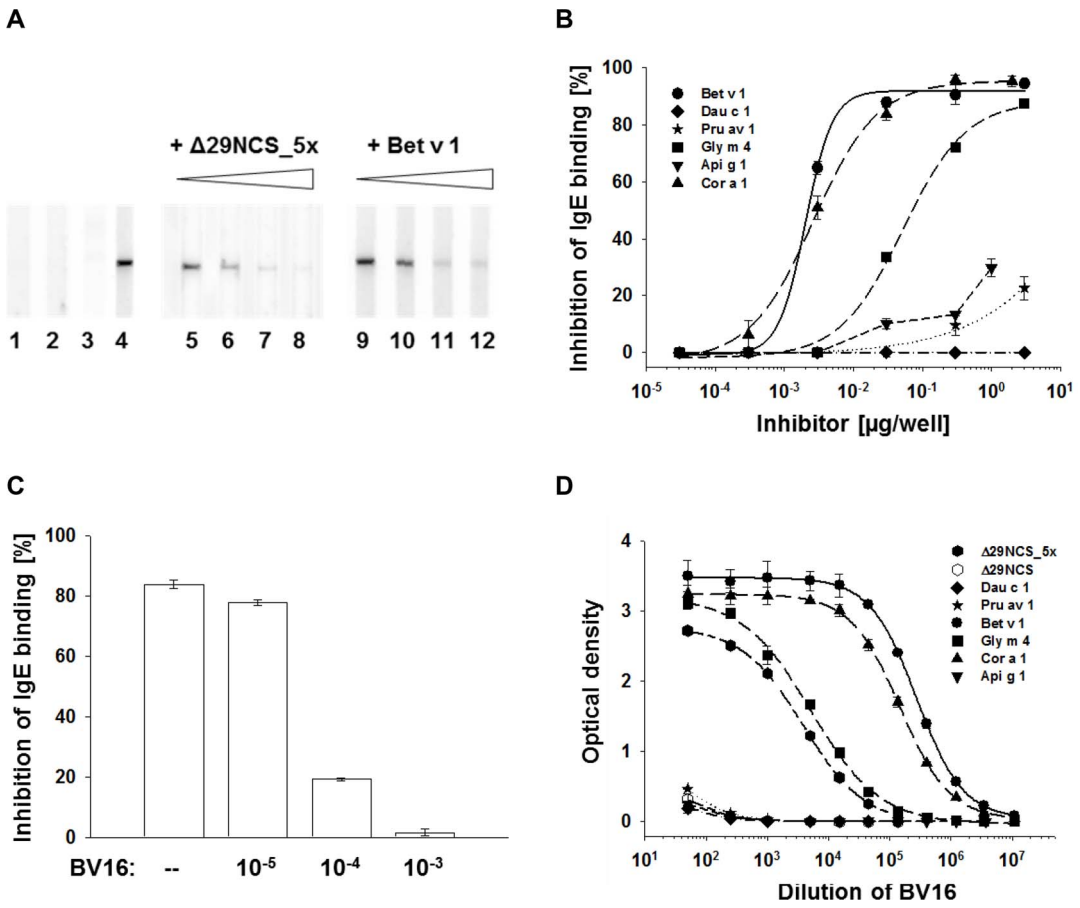

Figure 3. Δ29NCS_5x presents a cross-reactive Ig epitope. (A) Inhibition of IgE binding to Δ29NCS_5x. Binding of serum IgE to Δ29NCS (lane 3) and to Δ29NCS_5x (lane 4) in the presence of increasing amounts of inhibitors Δ29NCS_5x (lanes 5–8) or Bet v 1 (lanes 9–12). No serum (lane 1) and negative serum pool (lane 2) served as control. (B) Dose-dependent inhibition of IgE binding to surface-coated Δ29NCS_5x in the presence of increasing concentrations of PR-10 allergens in ELISA. (C) Inhibition of IgE binding to surface-coated Δ29NCS_5x in the presence of both Bet v 1 and serial dilutions of Bet v 1-binding monoclonal mouse IgG antibody BV16 (–: no BV16; 10^{-5} to 10^{-3}: dilutions of BV16) in ELISA. (D) Binding of mouse monoclonal IgG antibody BV16 to surface-coated Bet v 1-related proteins. 250 ng of protein (50 ng of Api g 1) was coated and incubated with dilution series of the monoclonal BV16 in ELISA.
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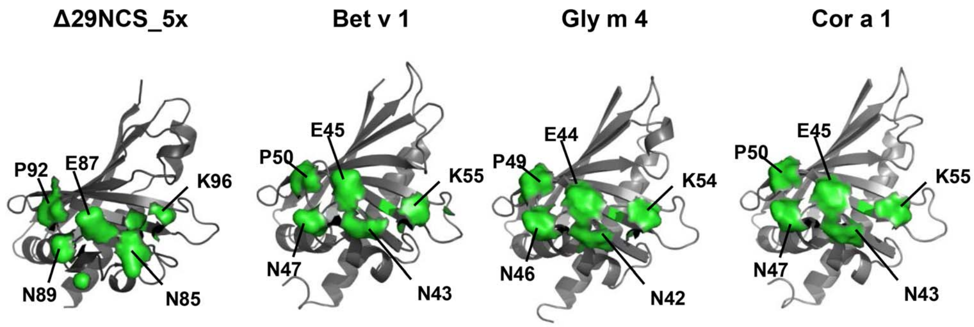
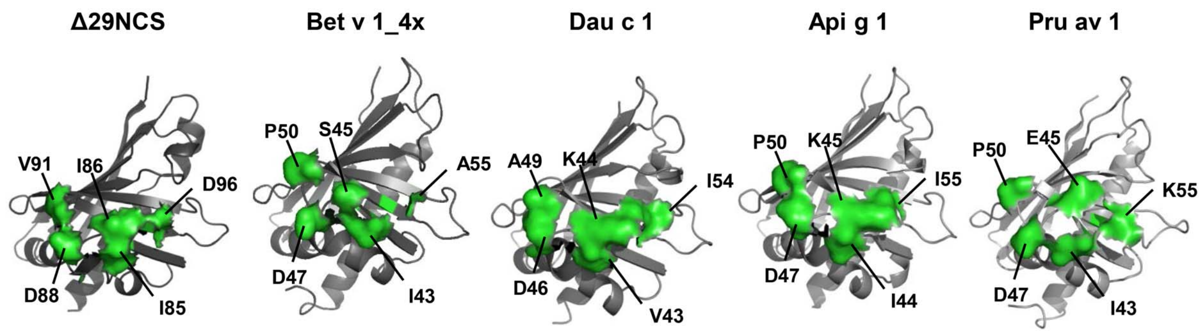


Δ29NCS_5x	E84	**N85**	I86	**E87**	G88	**N89**	G90	G91	**P92**	G93	T94	**K96**	K111	I113	N117	I127	Y138
Bet v 1	E42	**N43**	I44	**E45**	G46	**N47**	G48	G49	**P50**	G51	T52	**K55**	R70	D72	H76	I86	K97
Gly m 4	E41	**N42**	V43	**E44**	G45	**N46**	G47	G48	**P49**	G50	T51	**K54**	K69	E71	E75	V85	K96
Cor a 1	E42	**N43**	I44	**E45**	G46	**N47**	G48	G49	**P50**	G51	T52	**K55**	K70	E72	H76	I86	K97



Δ29NCS	E84	**I85**	**I86**	---	G87	**D88**	G89	G90	**V91**	G92	T93	**D96**	K111	I113	N117	I127	Y138
Bet v 1_4x	E42	**I43**	I44	**S45**	G46	**D47**	G48	G49	**P50**	G51	T52	**A55**	R70	D72	H76	I86	K97
Dau c 1	E42	---	**V43**	**K44**	G45	**D46**	G47	G48	**A49**	G50	T51	**I54**	R69	D71	K75	I85	S96
Api g 1	E43	---	**I44**	**K45**	G46	**D47**	G48	G49	**P50**	G51	T52	**I55**	R70	D72	K76	I86	S97
Pru av 1	E42	**I43**	L44	**E45**	G46	**D47**	G48	G49	**P50**	G51	T52	**K55**	K70	D72	K76	I86	K97

Figure 4. Structural alignment of the Bet v 1 epitope forBV16 and amino acids critical for Ig cross-reactivity in PR-10 allergens. Upper panel: model structures of Δ29NCS_5x and PR-10 allergens showing IgE cross-reactivity with Δ29NCS_5x. The amino acids comprising the BV16-binding epitope and lysine 55 of Bet v 1 and the corresponding residues of Δ29NCS_5x, Gly m 4, and Cor a 1 are listed. Residues of the epitope and of position 55 of Bet v 1 that are critical for Ig cross-reaction are highlighted in green. Lower panel: model structures of Δ29NCS and allergens that do not show IgE cross-reactivity with Δ29NCS_5x. The amino acids corresponding to the epitope in the cross-reactive proteins are listed and highlighted in green.
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majority of epitopes of inhalative allergens is thought to be conformational and the IgE repertoire of allergic patients to a specific allergen is polyclonal, we circumvented these issues by employing a model protein that i) does not interact with patients' IgE, ii) has a stable allergen-like protein fold, and iii) allows the analysis of individual antibody binding by amino acid substitutions.

Here we analyzed a non IgE-binding variant of the PR-10 enzyme Δ29NCS of the common meadow rue with Bet v 1-type protein structure [25]. We showed that amino acid insertion/substitution did not alter the Bet v 1-like protein fold of Δ29NCS, although it induced significant binding of IgE from sera of birch pollen allergic subjects. IgE that bound to the epitope presented by the model protein Δ29NCS_5x, cross-reacted with a subgroup of PR-10 allergens. As analyzed with a Bet v 1 variant with exchanged residues that mediate antibody binding to the epitope, the observed IgE cross-reaction depended on a subset of amino acids not ubiquitously present in PR-10 allergens. The functional IgE epitope identified in this study overlaps with the known binding site of the Bet v 1-specific murine monoclonal antibody BV16.

The native protein conformation of Bet v 1 and highly likely other PR-10 allergens is critical for IgE interaction since i) neither sequential IgE binding peptides of Bet v 1 nor IgE binding truncated variants of Bet v 1 have been described so far [42] ii) a folding variant of Bet v 1 with native primary structure, but permanently altered secondary structure has very low IgE reactivity [43]. iii) several Bet v 1 variants with amino acid substitutions that induced an altered protein conformation showed reduced binding of serum IgE [7,44]. Thus we thoroughly studied the secondary and tertiary structure of our model protein by CD and $^{1}H^{15}N$-HSQC NMR spectroscopy. The CD spectra of the Δ29NCS variants were virtually identical and typical for PR-10 proteins. Furthermore, the $^{1}H^{15}N$-HSQC spectra of the Δ29NCS variants were superimposable for the majority of resonances, and the spectra are in agreement with the notion of only minor local

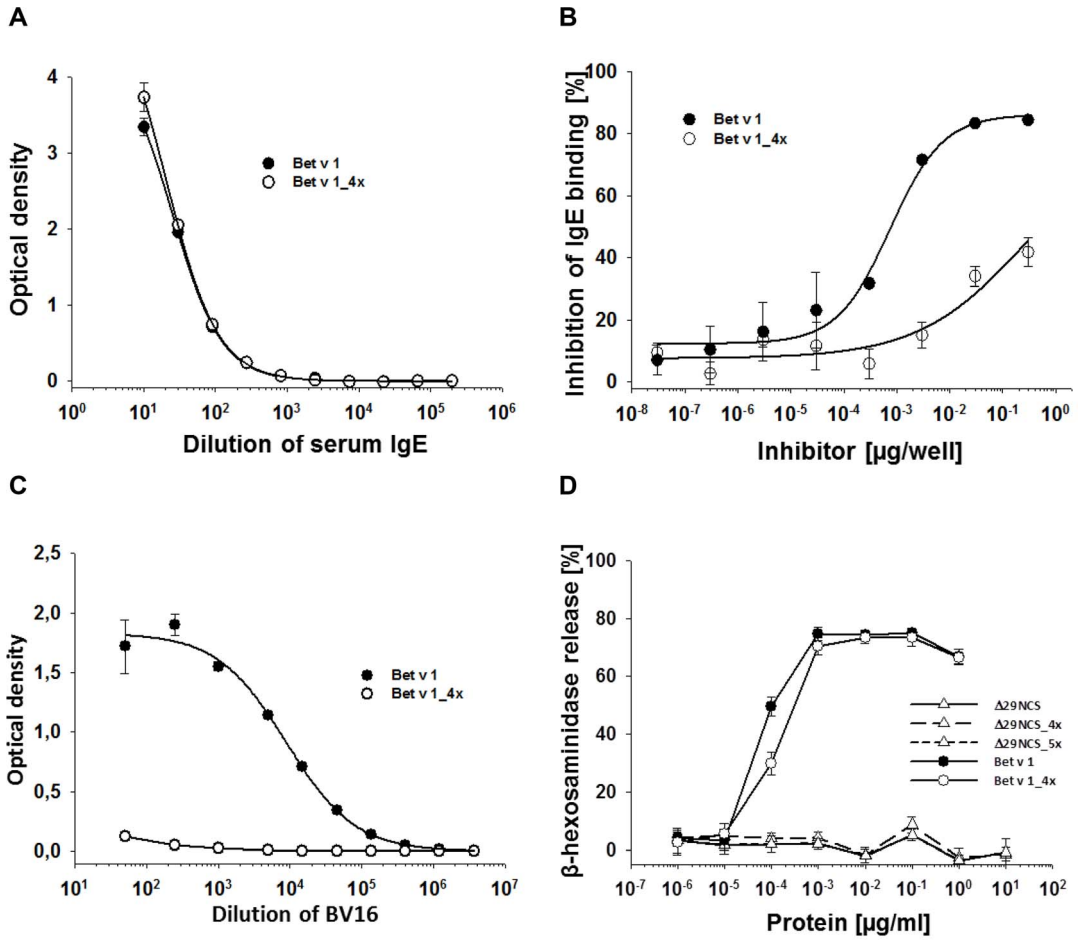

Figure 5. Amino acids critical forIgE and IgG cross-reactivity in a subgroup of Bet v 1-related proteins. (A) Binding of serial dilutions of pool serum IgE to surface-coated equimolar amounts of Bet v 1 and Bet v 1_4x (Bet v $1_{N43I/E45S/N47D/K55A}$) in ELISA. (B) Inhibition of IgE binding to surface-coated Δ29NCS_5x in the presence of either Bet v 1 or Bet v 1_4x in ELISA. (C) Binding of serial dilutions of monoclonal BV16 to surface-coated equimolar amounts of Bet v 1 and Bet v 1_4x in ELISA. (D) Mediator release induced by recombinant NCS and Bet v 1 variants. Humanized RBL cells were sensitized with a pool of human birch-specific sera. Cross-linking of membrane-bound human IgE by IgE-protein interaction and subsequent release of β-hexosaminidase was determined with serial dilutions of the proteins.
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differences caused by the amino acid substitutions (cf. Figure 2C), but otherwise identical protein conformation of the Δ29NCS variants. Hence, the Δ29NCS variants have the typical PR-10 protein structure and fulfill the essential requirement for molecular epitope analysis.

When we tested the impact of specific amino acids on serum IgE binding, we found that amino acid substitutions in Δ29NCS with structurally corresponding residues of Bet v 1 induced a significant increase in IgE interaction of the model protein to up to four CAP classes (cf. Table 1). As the protein folds of the Δ29NCS variants were essentially identical, we concluded that the observed serological IgE interaction of the model protein was caused solely by the residues substituted. This hypothesis was further confirmed by the allergen variant Bet v $1_{N43I/E45S/N47D/K55A}$ (Bet v 1_4x) which showed only little competition for serum IgE binding to Δ29NCS_5x and did not bind the Bet v 1-reactive murine monoclonal antibody BV16. Interestingly, residue E45 of Bet v 1 has already been identified as being critical for both IgE and BV16 interaction [17,22]. Thus we confirmed the existence of a functional overlapping Bet v 1 epitope for human IgE and murine IgG, with the majority (71%) of our study population having serum IgE against the epitope displayed by Δ29NCS_5x.

Since Bet v 1-specific IgE cross-reacts with PR-10 food allergens [2], we analyzed whether the functional epitope presented by Δ29NCS_5x is also relevant for IgE cross-reactivity. Binding of patients' IgE to Δ29NCS_5x could be inhibited by Bet v 1, Gly m 4, and Cor a 1.04, respectively, suggesting that Δ29NCS_5x displays an epitope shared by these allergens. Interestingly, the PR-10 allergens Pru av 1, Dau c 1.01, and Api g 1.01 did not cross-react with the NCS variant. Since the observed IgE reactivity of the PR-10 allergens we studied correlated well with their binding to the monoclonal antibody BV16, we assumed subtle structural differences within the epitope between the allergens. A sequential/structural alignment of the BV16 epitope indeed revealed that five amino acids are identical and oriented in a similar fashion among the cross-reactive proteins, but not in the non-cross-reactive proteins. To confirm our hypothesis, we generated a corresponding Bet v 1 variant to address these sequential differences and found that the protein variant behaved like the non-cross-reactive Bet v 1-homologous allergens with regard to binding IgG (BV16) and competing with serum IgE for Δ29NCS_5x.

Whereas the structural epitope of an antigen usually spans a molecular surface area of about 400–1000 $Å^2$ and comprises

10–25 amino acids, only a small subset of these residues dominates the energetics of the antibody-antigen interaction and thus constitutes the functional (active) epitope [45,46]. Our model system is ideal for the analysis of functional epitopes, since even low-affinity and individual antibody binding might be detected due to the lack of multiple antibody binding sites in our model protein.

Some information on functional IgE epitopes of Bet v 1 and other PR-10 allergens has emerged in recent years. These studies analyzed the interaction of IgE with either native Bet v 1 isoforms or recombinant allergen variants [8,9,12–16,22,47]. Even though great care had been taken to ensure a Bet v 1-like conformation of the studied protein variants, they were inevitably targeted by a polyclonal IgE antibody response, making it difficult to define the importance and architecture of any single epitope structure. Moreover, bioinformatic approaches are emerging that use either peptide:IgE interaction or structure-based comparison of allergen surfaces to localize Ig epitopes [18,26,48–50]. We believe that our approach to induce measurable antibody interaction of allergen-like protein variants is ideal to identify individual residues critical for antibody interaction. However, the truncated version of NCS used in this study still carries N- and C-terminal extensions that might locally interfere with epitope analysis. Thus our future research aims to further optimize our model protein system and adapt it to the average number of amino acids of PR-10 allergens.

No mediator release in humanized rat basophil leukemia cells sensitized with sera of birch pollen allergic subjects has been observed with the Δ29NCS variants, indicating that no additional IgE epitope that would mediate IgE receptor cross-linking is displayed in the variants (cf. Figure 5D). In contrast, Bet v 1_4x induced full wild type-like mediator release indicating that the lack of one particular epitope is easily compensated by polyclonal serum IgE. Thus our allergen model system also allows the investigation of the role of epitopes of different affinities to IgE antibodies in the IgE-mediated activation of basophils and mast cells, a crucial step of Type I allergic reactions.

In summary, we have established a protein model system which enables the specific analysis of functional IgE and IgG epitopes and their homologies among PR-10 allergens. Upon single amino acid substitutions of the non-allergenic protein variant Δ29NCS, individual epitopes are identified in a residue-specific manner by detection of newly generated immunoglobulin interactions of the model protein. Our system allows the evaluation of patient-specific epitope profiles and will facilitate both the identification of clinically relevant epitopes as biomarkers and the monitoring of therapeutic outcomes to improve diagnosis, prognosis, and therapy of allergies caused by PR-10 proteins.

Supporting Information

Figure S1 ^{1}H-^{15}N-HSQC spectra of different NCS variants. Spectra of (**A**) 400 μM Δ29NCS (black) (**B**) 250 μM $\Delta29NCS_{N57/I58E/D60N/V63P}$ (Δ29NCS_4x) (blue), and (**C**) 400 μM $\Delta29NCS_{N57/I58E/D60N/V63P/D68K}$ (Δ29NCS_5x) (red). The proteins were measured in 20 mM sodium phosphate, pH 7.0, 1 mM DTT, 0.04% sodium azide and 10% D_2O at a 700 MHz spectrometer at 298 K. Due to its solubility limit $\Delta29NCS_{N57/I58E/D60N/V63P}$ (Δ29NCS_4x) was measured at a lower protein concentration.
(TIF)

Figure S2 Folding and structural integrities of Bet v 1 and Bet v 1_4x variant. (A) Circular dichroism of Bet v 1 and Bet v 1_4x. (B) ^{1}H-^{15}N-HSQC spectra of Bet v 1 variants. Overlay of the spectra of 100 μM Bet v 1 (petrol) and 100 μM Bet v 1_4x (purple). The proteins were measured in 20 mM sodium phosphate, pH 7.0, 1 mM DTT, 0.04% sodium azide and 10% D_2O at a 700 MHz spectrometer at 298 K.
(TIF)

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Author Contributions

Conceived and designed the experiments: HB PR SV MH DS. Performed the experiments: HB MH CSvL SR MG LV. Analyzed the data: HB CSvL MH SR MG LV FH AR SV PR DS. Contributed reagents/materials/analysis tools: SV MG JL. Wrote the paper: MH DS. Recruitment of patients and clinical evaluation of data: BB-W.

References

1. D'Amato G, Cecchi L, Bonini S, Nunes C, Annesi-Maesano I, et al. (2007) Allergenic pollen and pollen allergy in Europe. Allergy 62: 976–990.
2. Vieths S, Scheurer S, Ballmer-Weber B (2002) Current understanding of cross-reactivity of food allergens and pollen. Ann N Y Acad Sci. 964: 47–68.
3. Niemi M, Jylhä S, Laukkanen ML, Söderlund H, Mäkinen-Kiljunen S, et al. (2007) Molecular interactions between a recombinant IgE antibody and the beta-lactoglobulin allergen. Structure 15: 1413–1421.
4. Padavattan S, Flicker S, Schirmer T, Madritsch C, Randow S, et al. (2009) High-affinity IgE recognition of a conformational epitope of the major respiratory allergen Phl p 2 as revealed by X-ray crystallography. J Immunol 182: 2141–2151.
5. Razzera G, Gadermaier G, de Paula V, Almeida MS, Egger M, et al. (2010) Mapping the interactions between a major pollen allergen and human IgE antibodies. Structure 18: 1011–1021.
6. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, et al. (1998) Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. FASEB J 12: 231–242.
7. Holm J, Gajhede M, Ferreras M, Henriksen A, Ipsen H, et al. (2004) Allergy vaccine engineering: epitope modulation of recombinant Bet v 1 reduces IgE binding but retains protein folding pattern for induction of protective blocking-antibody responses. J Immunol 173: 5258–5267.
8. Holm J, Ferreras M, Ipsen H, Würtzen PA, Gajhede M, et al. (2011) Epitope grafting, re-creating a conformational Bet v 1 antibody epitope on the surface of the homologous apple allergen Mal d 1. J Biol Chem 286: 17569–17578.
9. Klinglmayr E, Hauser M, Zimmermann F, Dissertori O, Lackner P, et al. (2009) Identification of B-cell epitopes of Bet v 1 involved in cross-reactivity with food allergens. Allergy 64: 647–651.
10. Hecker J, Diethers A, Schulz D, Sabri A, Plum M, et al. (2012) An IgE epitope of Bet v 1 and fagales PR10 proteins as defined by a human monoclonal IgE. Allergy 67: 1530–1537.
11. Son DY, Scheurer S, Hoffmann A, Haustein D, Vieths S (1999) Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. Eur J Nutr 38: 201–215.
12. Ma Y, Gadermaier G, Bohle B, Bolhaar S, Knulst A, et al. (2006) Mutational analysis of amino acid positions crucial for IgE-binding epitopes of the major apple (Malus domestica) allergen, Mal d 1. Int Arch Allergy Immunol 139: 53–62.
13. Scheurer S, Son DY, Boehm M, Karamloo F, Franke S, et al. (1999) Cross-reactivity and epitope analysis of Pru a 1, the major cherry allergen. Mol Immunol 36: 155–167.
14. Neudecker P, Lehmann K, Nerkamp J, Haase T, Wangorsch A, et al. (2003) Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry (Prunus avium) and celery (Apium graveolens): correlating IgE reactivity with three-dimensional structure. Biochem J 376: 97–107.
15. Wangorsch A, Ballmer-Weber BK, Rosch P, Holzhauser T, Vieths S (2007) Mutational epitope analysis and cross-reactivity of two isoforms of Api g 1, the major celery allergen. Mol Immunol 44: 2518–2527.

16. Wiche R, Gubesch M, König H, Fötisch K, Hoffmann A, et al. (2005) Molecular basis of pollen-related food allergy: identification of a second cross-reactive IgE epitope on Pru av 1, the major cherry (Prunus avium) allergen. Biochem J 385: 319–327.
17. Schirmer T, Hoffmann-Sommergruber K, Susani M, Breiteneder H, Markovic-Housley Z (2005) Crystal structure of the major celery allergen Api g 1: molecular analysis of cross-reactivity. J Mol Biol 351: 1101–1109.
18. Gieras A, Cejka P, Blatt K, Focke-Tejkl M, Linhart B, et al. (2011) Mapping of conformational IgE epitopes with peptide-specific monoclonal antibodies reveals simultaneous binding of different IgE antibodies to a surface patch on the major birch pollen allergen, Bet v 1. J Immunol 186: 5333–5344.
19. Levin M, Davies AM, Liljekvist M, Carlsson F, Gould HJ, et al. (2014) Human IgE against the major allergen Bet v 1 - defining an epitope with limited cross-reactivity between different PR-10 family proteins. Clin Exp Allergy 44: 288–299.
20. Mirza O, Henriksen A, Ipsen H, Larsen JN, Wissenbach M, et al. (2000) Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. J Immunol 165: 331–338.
21. Spangfort MD, Mirza O, Svensson LA, Larsen JN, Gajhede M et al. (1999) Crystallization and preliminary X-ray analysis of birch-pollen allergen Bet v 1 in complex with a murine monoclonal IgG Fab′ fragment. Acta Crystallogr D Biol Crystallogr 55: 2035–2036.
22. Spangfort MD, Mirza O, Ipsen H, Van Neerven RJ, Gajhede M, et al. (2003) Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. J Immunol 171: 3084–3090.
23. Berkner H, Engelhorn J, Liscombe DK, Schweimer K, Wöhrl BM, et al. (2007) High-yield expression and purification of isotopically labeled norcoclaurine synthase, a Bet v 1-homologous enzyme, from Thalictrum flavum for NMR studies. Protein Expr Purif 56: 197–204.
24. Berkner H, Schweimer K, Matecko I, Rosch P (2008) Conformation, catalytic site, and enzymatic mechanism of the PR10 allergen-related enzyme norcoclaurine synthase. Biochem J 413: 281–290.
25. Ilari A, Franceschini S, Bonamore A, Arenghi F, Botta B, et al. (2009) Structural basis of enzymatic (S)-norcoclaurine biosynthesis. J Biol Chem 284: 897–904.
26. Ballmer-Weber BK, Wangorsch A, Bohle B, Kaul S, Kündig T, et al. (2005) Component-resolved in vitro diagnosis in carrot allergy: does the use of recombinant carrot allergens improve the reliability of the diagnostic procedure? Clin Exp Allergy 35: 970–978.
27. Axen R, Drevin H, Kober A, Yman L (1988) A new laboratory system applied to allergy testing. N Engl Reg Allergy Proc 9: 503.
28. Marknell DeWitt A, Niederberger V, Lehtonen P, Spitzauer S, Sperr WR, et al. (2002) Molecular and immunological characterization of a novel timothy grass (Phleum pratense) pollen allergen, Phl p 11. Clin Exp Allergy 32: 1329–1340.
29. Scheurer S, Metzner K, Haustein D, Vieths S (1997) Molecular cloning, expression and characterization of Pru a 1, the major cherry allergen. Mol Immunol 34: 619–629.
30. Lauer I, Alessandri S, Pokoj S, Reuter A, Conti A, et al. (2008) Expression and characterization of three important panallergens from hazelnut. Mol Nutr Food Res 52 Suppl 2: S262–S271.
31. Johnson BA and Blevins RA (1994) NMR View: A computer program for the visualization and analysis of NMR data. J Biomol NMR 4: 603–614.
32. Kelley LA and Sternberg MJ (2009) Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4: 363–371.
33. Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, et al. (1996) X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. Nat Struct Biol 3: 1040–1045.
34. Larsen JN, Casals AB, From NB, Stroman P, Ipsen H (1993) Characterization of recombinant bet vI, the major pollen allergen of Betula verrucosa (white birch), produced by fed-batch fermentation. Int Arch Allergy Immunol 102: 249–258.
35. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
36. Kyhse-Andersen J (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 10: 203–209.
37. Vogel L, Luttkopf D, Hatahet L, Haustein D, Vieths S (2005) Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. Allergy 60: 1021–1028.
38. Albrecht M, Alessandri S, Conti A, Reuter A, Lauer I, et al. (2008) High level expression, purification and physico- and immunochemical characterisation of recombinant Pen a 1: a major allergen of shrimp. Mol Nutr Food Res 52 Suppl 2: S186–S195.
39. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7: 539.
40. DeLano WL (2002) The PyMOL Molecular Graphics System.
41. Subbarayal B, Schiller D, Möbs C, de Jong NW, Ebner C, et al. (2013) Kinetics, cross-reactivity, and specificity of Bet v 1-specific IgG4 antibodies induced by immunotherapy with birch pollen. Allergy 68: 1377–1386.
42. Lebecque S, Dolecek C, Laffer S, Visco V, Denépoux S, et al. (1997) Immunologic characterization of monoclonal antibodies that modulate human IgE binding to the major birch pollen allergen Bet v 1. J Allergy Clin Immunol 99: 374–384.
43. Kahlert H, Suck R, Weber B, Nandy A, Wald M, et al. (2008) Characterization of a hypoallergenic recombinant Bet v 1 variant as a candidate for allergen-specific immunotherapy. Int Arch Allergy Immunol 145: 193–206.
44. Wallner M, Hauser M, Himly M, Zaborsky N, Mutschlechner S, et al. (2011) Reshaping the Bet v 1 fold modulates T(H) polarization. J Allergy Clin Immunol 127: 1571–1578.
45. Dall′Acqua W, Goldman ER, Lin W, Teng C, Tsuchiya D, et al. (1998) A mutational analysis of binding interactions in an antigen-antibody protein-protein complex. Biochemistry 37: 7981–7991.
46. Jenkins JA, Griffiths-Jones S, Shewry PR, Breiteneder H, Mills EN (2005) Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an in silico analysis. J Allergy Clin Immunol 115: 163–170.
47. Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, et al. (1996) Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. J Exp Med 183: 599–609.
48. Mittag D, Batori V, Neudecker P, Wiche R, Friis EP, et al. (2006) A novel approach for investigation of specific and cross-reactive IgE epitopes on Bet v 1 and homologous food allergens in individual patients. Mol Immunol 43: 268–278.
49. Dall′Antonia F, Gieras A, Devanaboyina SC, Valenta R, Keller W (2011) Prediction of IgE-binding epitopes by means of allergen surface comparison and correlation to cross-reactivity. J Allergy Clin Immunol 128: 872–879.
50. Jimenez-Lopez JC, Gachomo EW, Ariyo OA, Baba-Moussa L, Kotchoni SO (2012) Specific conformational epitope features of pathogenesis-related proteins mediating cross-reactivity between pollen and food allergens. Mol Biol Rep 39: 123–130.

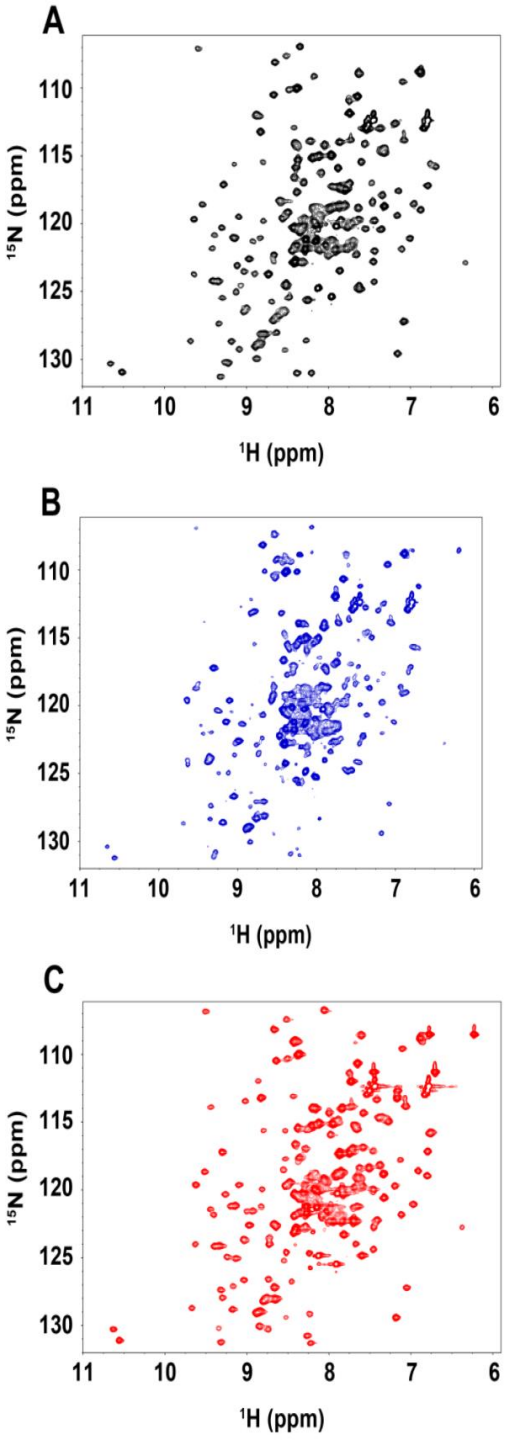

Figure S1. ^{1}H-^{15}N-HSQC spectra of different NCS variants.
Spectra of (**A**) 400 µM Δ29NCS (black) (**B**) 250 µM $\Delta29NCS_{N57/I58E/D60N/V63P}$ (Δ29NCS_4x) (blue), and (**C**) 400 µM $\Delta29NCS_{N57/I58E/D60N/V63P/D68K}$ (Δ29NCS_5x) (red). The proteins were measured in 20 mM sodium phosphate, pH 7.0, 1 mM DTT, 0.04% sodium azide and 10% D_2O at a 700 MHz spectrometer at 298 K. Due to its solubility limit $\Delta29NCS_{N57/I58E/D60N/V63P}$ (Δ29NCS_4x) was measured at a lower protein concentration.

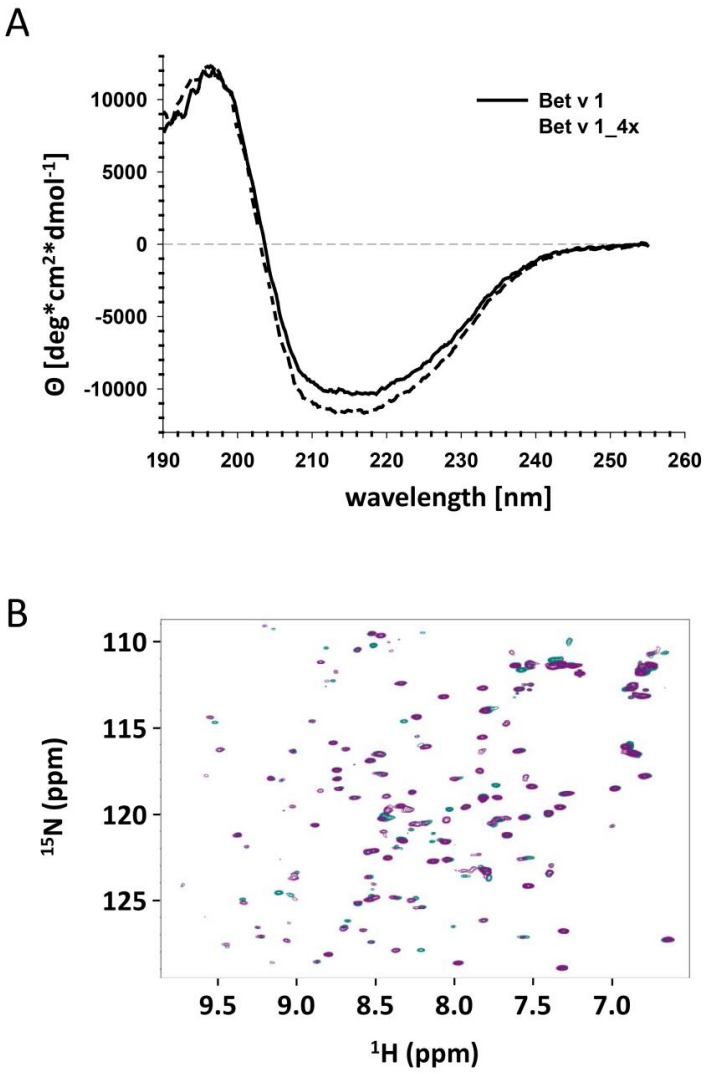

Figure S2 Folding and structural integrities of Bet v 1 and Bet v 1_4x variant.
(A) Circular dichroism of Bet v 1 and Bet v 1_4x. (B) ^{1}H-^{15}N-HSQC spectra of Bet v 1 variants. Overlay of the spectra of 5µM Bet v 1 (petrol) and 5µM Bet v 1_4x (purple). The proteins were measured in 20 mM sodium phosphate, pH 7.0, 1 mM DTT, 0.04% sodium azide and 10% D_2O at a 700 MHz spectrometer at 298 K.

7.2 Einzelarbeit B

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Solution structure of the strawberry allergen Fra a 1

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Synopsis

The PR10 family protein Fra a 1E from strawberry (*Fragaria x ananassa*) is down-regulated in white strawberry mutants, and transient RNAi (RNA interference)-mediated silencing experiments confirmed that Fra a 1 is involved in fruit pigment synthesis. In the present study, we determined the solution structure of Fra a 1E. The protein fold is identical with that of other members of the PR10 protein family and consists of a seven-stranded antiparallel β-sheet, two short V-shaped α-helices and a long C-terminal α-helix that encompass a hydrophobic pocket. Whereas Fra a 1E contains the glycine-rich loop that is highly conserved throughout the protein family, the volume of the hydrophobic pocket and the size of its entrance are much larger than expected. The three-dimensional structure may shed some light on its physiological function and may help to further understand the role of PR10 proteins in plants.

Key words: Bet v 1 superfamily, Fra a 1, *Fragaria x ananassa*, NMR structure, pathogenesis-related protein

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INTRODUCTION

In North and Central Europe as well as in North America approximately 15–20% of the population are allergic to pollen, with birch pollen being the main cause of those allergies [1,2]. Over 90% of the birch pollen allergic patients show sensitization to the major birch pollen allergen Bet v 1 [3]. In total 50–90% of the patients who are sensitized to Bet v 1 show IgE-mediated allergic cross reactions to food allergens such as those contained in fruits, vegetables and nuts [4,5]. These cross reactions are often based on the similarity of the three-dimensional allergen structures [6–9].

A series of Bet v 1 homologous food allergens have been identified and summarized in the superfamily of Bet v 1-like proteins, such as Api g 1 from celery [10], Mal d 1 from apple [11], Pru av 1 from cherry [12] and Gly m 4 from soya bean [13]. The members of the Bet v 1 superfamily are 153–160 amino acid proteins with molecular mass ~17 kDa and theoretical isoelectric points between pH 4.4 and 6.1. The sequence identities with Bet v 1 are 40–70%, and all these proteins show similar conformations with identical arrangements of secondary structure elements that correlate with the cross-reactivity.

The pollen and food allergens belong to the class 10 of pathogenesis-related proteins, protein family PR10, a subdivision of the Bet v 1-like superfamily. The PR10 proteins are conserved and ubiquitous in the plant kingdom, with roles in plant development [17] and immune defence induced by microbial attack [18], fungal elicitors [18,19], wounding [20] or stress stimuli [21]. The PR10 family contains, for example, the yellow lupine proteins LIPR-10.2A and LIPR-10.2B [14,15] and the St. John's Wort protein Hyp1 [16]. The classification of this protein group is based on the 3D (three-dimensional) structure. All structurally studied proteins show the same overall fold. However, their biological function is still not well recognized.

As an additional member of the Bet v 1 superfamily, a strawberry (*Fragaria x ananassa*) protein was detected in red fruited genotypes and named Fra a 1 [22]. A hypoallergenic white fruited genotype was almost devoid of the protein. Subsequently, five potential Fra a 1 isoforms (Fra a 1A–Fra a 1E) were identified with only seven variable amino acid positions, and it could be shown that Fra a 1A is recognized by IgE antibodies from strawberry allergic patients [23,24]. Two additional Fra a isoforms with greater variability were identified and named Fra a 2 and Fra a 3 [25]. Whereas *Fra a 1e* transcripts are expressed predominantly in roots with decreasing expression levels

Abbreviations used: LB, Luria–Bertani; NOESY, nuclear Overhauser enhancement spectroscopy; rmsd, root mean square deviation; RNAi, RNA interference.

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during fruit ripening, *Fra a 2* expression increases in ripe fruits and *Fra a 3* expression is constant in all stages. Transient RNAi (RNA interference)-mediated silencing of the *Fra a* genes in strawberries leads to reduced levels of anthocyanins and upstream metabolites as well as down-regulation of chalcone synthase (*FaCHS*) and *PAL* (phenylalanine ammonia lyase) genes [25], clearly indicating a key role of Fra a isoforms in the pigment synthesis pathway.

These different appearances of the members of the Bet v 1 superfamily of proteins, combined with the observation of divergent expression patterns in different species and different isoforms within a single species, renders it difficult to assign clear physiological functions to these proteins. One of the possible approaches to clarify the role of the Bet v 1 proteins in Nature is the study and comparison of their conformations in detail, an approach we here continue with the presentation of the solution structure of Fra a 1 in atomic resolution.

MATERIALS AND METHODS

Expression of recombinant *Fra a 1e*

The *Fra a 1e* gene was cloned into the expression vector pQE70 (Qiagen) by using the restriction sites SphI and BamHI. The protein contained a His_6 tag at the C-terminus. The recombinant plasmid was transformed together with the vector pREP4 (Qiagen) into *Escherichia coli* BL21 (DE3). *Fra a 1e* was expressed in the host strain in LB (Luria–Bertani) medium at 20°C over 4 h. To obtain ^{15}N- and $^{15}N/^{13}C$-labelled protein overexpressing *E. coli* were grown on M9 minimal medium with ^{15}N enriched $(^{15}NH_4)_2SO_4$ or ^{15}N enriched $(^{15}NH_4)_2SO_4$/[U-^{13}C]glucose respectively.

Protein purification

Crude cell extracts were prepared in 50 mM Hepes buffer (pH 7.0) with 300 mM NaCl for purification. Soluble His-tagged Fra a 1E was purified by immobilized metal-ion affinity chromatography [Ni-NTA (Ni^{2+}-nitrilotriacetate) column with a bed volume of 1 ml; Qiagen] using a step gradient from 20 to 300 mM imidazole. Fractions were analysed by SDS/19 % PAGE, and pure protein fractions were pooled and dialysed against 20 mM Hepes buffer, pH 7.0, containing 150 mM NaCl. This buffer was used as equilibration and elution buffer of a size-exclusion chromatography to remove residual impurities (Superdex S75 GL 10/300 column, total bed volume: 24 ml; GE Healthcare). Two litres of cell culture from LB and M9 minimal medium yielded approximately 11.5 and 7.6 mg of protein respectively. The purity of recombinant Fra a 1E exceeded 95 % as estimated from SDS/PAGE on 19 % gels. The DC protein assay (BioRad) as well as a molar absorption coefficient of $\varepsilon_{280} = 14900\ M^{-1} \cdot cm^{-1}$ were used to calculate protein concentrations. Samples of Bet v 1.0101 were prepared as described previously [26].

Size-exclusion chromatography

In order to determine the oligomerization state and the molecular mass of recombinant Fra a 1E, size-exclusion chromatography was performed with a Superdex S75 GL 10/300 column (total bed volume: 24 ml; GE Healthcare). Potassium phosphate buffer, 10 mM, pH 7.0, containing 150 mM NaCl was used as sample buffer as well as for column equilibration and for isocratic protein elution. Molecular mass calibration of the column was performed with albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) and cytochrome *c* (12.4 kDa).

CD spectroscopy

Far ultraviolet CD measurements were recorded on a Jasco J-810 spectropolarimeter (Japan Spectroscopic) equipped with a 1-mm quartz cuvette (Hellma). Spectra of 10 μM Fra a 1E and 7.0 μM Bet v 1.0101 were obtained at 20°C in 1 mM Hepes buffer, pH 7.0, containing 5 mM NaCl. At least 10 scans in the range between 260 and 190 nm with 50 nm/min scanning speed were averaged for each measurement, and the resulting spectrum was smoothed and normalized using the following formula to calculate the mean residue molar ellipticity:

$$[\Theta]_{MRW} = \Theta / c \cdot d \cdot N$$

where Θ is the measured ellipticity, MRW is the mean residue mass, *c* is the protein concentration, *d* is the path length and *N* is the number of amino acids.

Computational methods

Multiple and pairwise sequence alignments were performed with ClustalW (EMBL-EBI) [27] and LALIGN (EMBL-EBI) [28], and homologous protein structures were aligned with the program Coot [29]. The cavity volumes of the Bet v 1 homologous proteins were calculated with CASTp with default parameters [30]. For NMR structures, the cavity volumes were determined for every single structure of the NMR bundle. The quoted volumes are means $\pm$ S.D. Figures of protein structures were generated using the program PyMOL v1.0 (2009, DeLano Scientific LLC).

NMR spectroscopy

All NMR experiments were performed at 298 K on a Bruker Avance 800 MHz spectrometer with cryogenically cooled triple resonance probes equipped with pulsed field-gradient capabilities, and standard double- and triple-resonance through-bond correlation. NMR spectra to assign chemical shifts [31] were obtained with a 700 μM ^{15}N, ^{13}C-labelled Fra a 1E sample in 20 mM Hepes, pH 7.0, 150 mM NaCl and 10 % ^2H_2O. Three-dimensional ^{13}C and ^{15}N edited NOESY (nuclear Overhauser enhancement spectroscopy) experiments (mixing times 120 ms) were recorded for derivation of distance restraints. NMR data were processed using in-house software and visualized with NMRView [32].

Table 1 Comparison of Bet v 1 homologous proteins

Protein	PDB code (method)	Sequence identity to Fra a 1E (%)	Sequence similarity to Fra a 1E (%)	Backbone rmsd values (Å)	Backbone rmsd values for secondary structures (Å)	Cavity size (Å^3)	Reference
Fra a 1E	2LPX (NMR)	100	100	–	–	2468 ± 440	Present study
Pru av 1.0101	1E09 (NMR)	79.4	95.0	1.82	1.04	1643 ± 280	[6]
Bet v 1.0101	1BTV (NMR)	53.8	81.2	2.33	1.71	1633 ± 152	[45]
	1BV1 (X-ray)	53.8	81.2	1.85	1.32	1527	[45]
Gly m 4.0101	2K7H (X-ray)	52.5	78.1	2.32	1.71	1621 ± 242	[9]
LIPR10.2B	2QIM (X-ray)	51.9	80.6	1.24	1.93	2181.7	[41]
Bet v 1.1001	1FM4 (X-ray)	51.2	80.6	1.28	1.93	2359	[38]
LIPR10.2A	1XDF (X-ray)	50.9	78.6	1.42	1.80	329.3	[46]

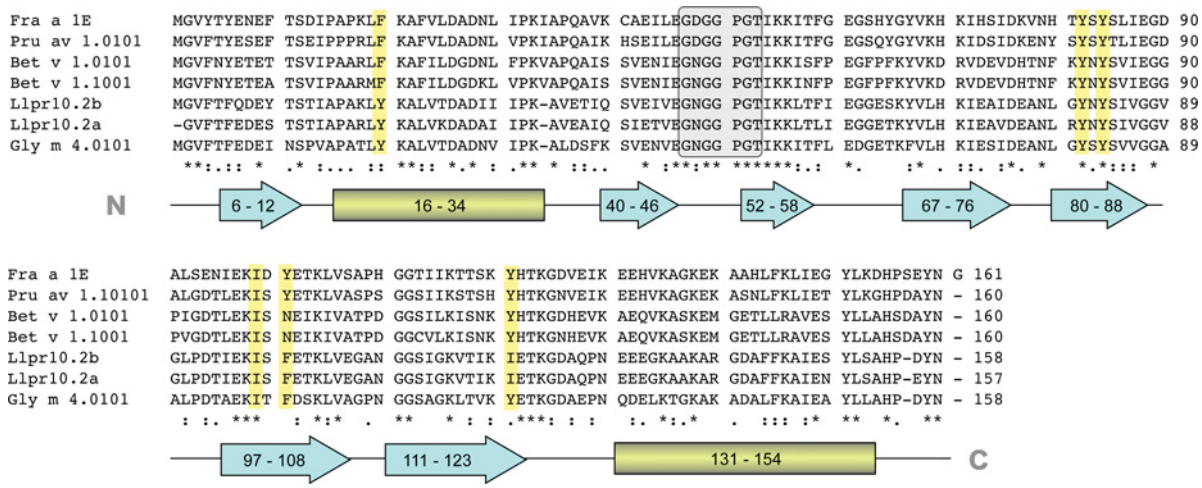

Figure 1 Sequence alignment of Bet v 1 homologous proteins with known structure
Multiple sequence alignment of Bet v 1 homologous proteins as performed with ClustalW: Fra a 1E (*Fragaria x ananassa*, strawberry), Pru av 1 (*Prunus avium*, cherry), Bet v 1 (*Betula verrucosa*, birch), LIPR-10.2A (*Lupinus luteus*) and Gly m 4 (*Glycine max*, soya bean). Amino acids are marked with asterisks (identical), colons (conserved) and dots (semi-conserved). The highly conserved glycine-rich loop region is highlighted by a grey box. Conserved cavity residues are highlighted in yellow. Secondary structure elements are indicated as present in Fra a 1E (bottom line: β-sheets, blue; α-helices, green).

Structure calculation

NOESY cross peaks were classified according to their relative intensities and converted into distance restraints with upper limits of 3.0 Å (strong), 4.0 Å (medium), 5.0 Å (weak) and 6.0 Å (very weak) (where 1 Å = 0.1 nm). For ambiguous distance restraints, the r^{-6} summation over all assigned possibilities defined the upper limit. Dihedral restraints were taken from analysis of chemical shifts by the TALOS software package [33]. Hydrogen bonds were included for backbone amide protons in regular secondary structure, if the amide proton did not show a water exchange cross peak in the ^{15}N-edited NOESY spectrum. Structures were calculated with the program XPLOR-NIH 1.2.1 [34] using a three-step simulated annealing protocol with floating assignment of prochiral groups including a conformational database potential.

The 20 structures showing the lowest values of the target function excluding the database potential were further analysed with XPLOR-NIH 1.2.1 [34], MolMol [35] and PROCHECK 3.5.4 [36]. The structure coordinates were deposited in the protein data bank (pdb accession number 2LPX), and chemical shift assignments were deposited in the BioMagResBank (BMRB accession code 18281).

RESULTS AND DISCUSSION

General comparison, CD spectroscopy and size-exclusion chromatography

According to sequence alignment with PR10 proteins with known structure, Fra a 1E is most similar to the major cherry allergen Pru av 1 with a sequence identity of 79.4% [6] (Figure 1 and Table 1). The glycine-rich loop motif, residues 46–51, is highly conserved throughout the Bet v 1 superfamily.

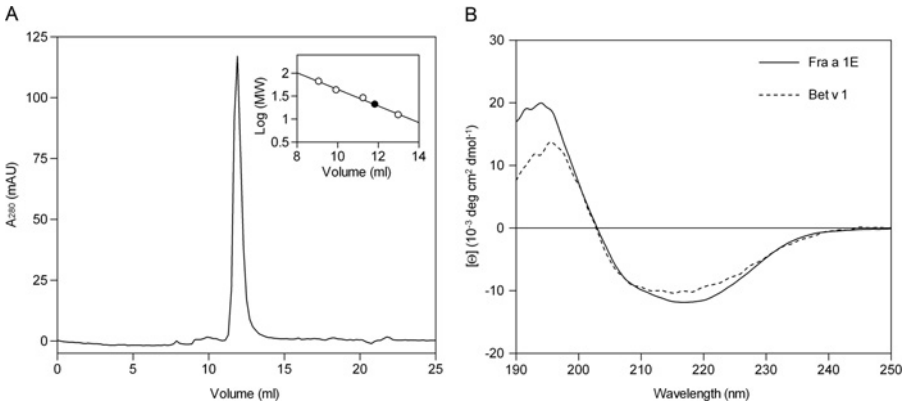

Figure 2 Biophysical properties of Fra a 1E

(**A**) Size-exclusion chromatography of Fra a 1E with a Superdex S75 GL 10/300 column. The run was performed with 0.35 mg of Fra a 1E in 10 mM potassium phosphate buffer, pH 7.0 and 150 mM NaCl. The fit to the data obtained for the molecular masses of the standard proteins (inset, open circles) was used for the determination of the molecular mass of Fra a 1E (closed circle). (**B**) Far UV CD spectra of Fra a 1E (continuous line) and Bet v 1.0101 (dotted line) were acquired at 20 °C with a bandwith of 1 nm, a sensitivity of 100 mdeg and a data density of 10 points/nm in a 1 mm quartz cuvette with 10 μM Fra a 1E in 1 mM Hepes buffer, pH 7.0, containing 5 mM NaCl, and 7 μM Bet v 1.0101 in identical buffer.

Size-exclusion chromatography of Fra a 1E revealed a single peak corresponding to a molecular mass of 20.8 kDa, in good agreement with the theoretical mass of 19.0 kDa (Figure 2A). No additional peaks could be detected, indicating that under the present conditions Fra a 1E is monomeric.

The CD spectrum of Fra a 1E displays characteristic features of a folded protein (Figure 2B), but differs from the Bet v 1 CD spectrum in several respects: the minima wavelengths (Fra a 1E: 217 nm; Bet v 1: 215 nm) as well as the maxima wavelengths (Fra a 1E: 194 nm; Bet v 1: 196 nm) are slightly different, the Bet v 1 CD 196 nm has lower amplitude in maximum as well as minimum ellipticity.

NMR spectroscopy

The NMR spectra of Fra a 1E show signal dispersion characteristics of a well-folded protein, and the application of double- and triple-resonance NMR experiments yielded nearly complete assignments of all ^{1}H, ^{13}C and ^{15}N resonances (see Supplementary Figures S1 and S2 at http://www.bioscirep.org/bsr/032/bsr0320567add.htm). In total 1601 experimentally derived restraints were obtained for the structure calculation. The resulting ensemble of 20 structures shows good stereochemical properties and only low violation of experimental and geometrical restraints, it is well defined with an overall backbone rmsd (root mean square deviation) of 0.92 Å for residues Gly^{2}–Asp^{154} and 0.54 Å for the regions with defined secondary structure only. More than 90 % of the residues are located in the most favoured regions of the Ramachandran map (see Table 2).

Description of the structure

The solution structure (Figure 3A) of Fra a 1E consists of a seven-stranded antiparallel β-sheet (residues Tyr^{6}–Ser^{12}, Lys^{40}–Glu^{46}, Gly^{52}–Thr^{58}, Tyr^{67}–Asp^{76}, His^{80}–Glu^{88}, Glu^{97}–Ala^{108} and

Table 2 Statistics for structure calculations

NOE, nuclear Overhauser effect.

Experimental restraints	Value
Distance restraints	
NOE	1270
Intraresidual	52
Sequential	287
Medium range	340
Long range	591
Hydrogen bonds	122
Dihedral restraints	209
Restraint violation	
Average distance restraint violation	0.0027 ± 0.001 Å
Maximum distance restraint violation	0.13 Å
Average dihedral restraint violation	0.15 ± 0.07°
Maximum dihedral restraint violation	3.5°
Deviation from ideal geometry	
Bond length	0.00053 ± 0.00002 Å
Bond angle	0.11 ± 0.004°
Co-ordinate precision*	
Val^{2}–Asp^{154}	
Backbone heavy atoms	0.91 Å
All heavy atoms	1.42 Å
Defined secondary structure†	
Backbone heavy atoms	0.54 Å
All heavy atoms	1.06 Å
Ramachandran plot statistics‡	90.3 %, 7.9 %, 0.7 %, 1.1%

*The precision of the coordinates is defined as the average atomic rmsd between the accepted simulated annealing structures and the corresponding mean structure calculated for the given sequence regions.

†The region of defined secondary structure is composed of Val^{2}–Ser^{11}; Ala^{16}–Ile^{34}; Lys^{40}–Glu^{46}; Gly^{53}–Thr^{58}; Tyr^{67}–Asp^{76}; His^{80}–Glu^{88}; Glu^{97}–Ala^{108}; Gly^{111}–Thr^{123}; Lys^{130}–Lys^{153}.

‡Ramachandran plot statistics are determined by PROCHECK and noted by most favoured/additionally allowed/generously allowed/disallowed.

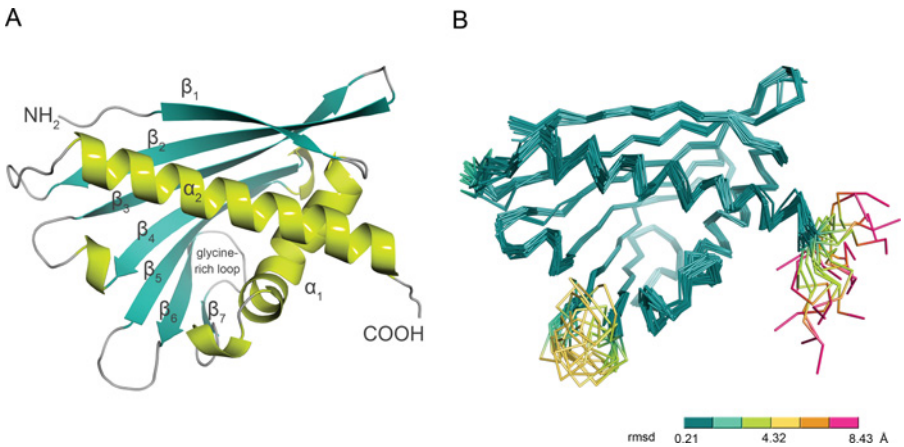

Figure 3 **The overall structure of Fra a 1E**
(**A**) Cartoon representation of the lowest energy solution structure of Fra a 1E (α-helices, green; β-sheets, blue; loop-regions, grey). (**B**) Backbone overlay of the 20 lowest energy solution structures of Fra a 1E, with backbone rmsd values (0.21–1.58 Å, blue; 1.58–2.95 Å, dark green; 2.95–4.32 Å, light green; 4.32–5.69 Å, yellow; 5.69–7.06 Å, orange; 7.06–8.43 Å, pink). Higher rmsd values generally correspond to higher structural flexibility.

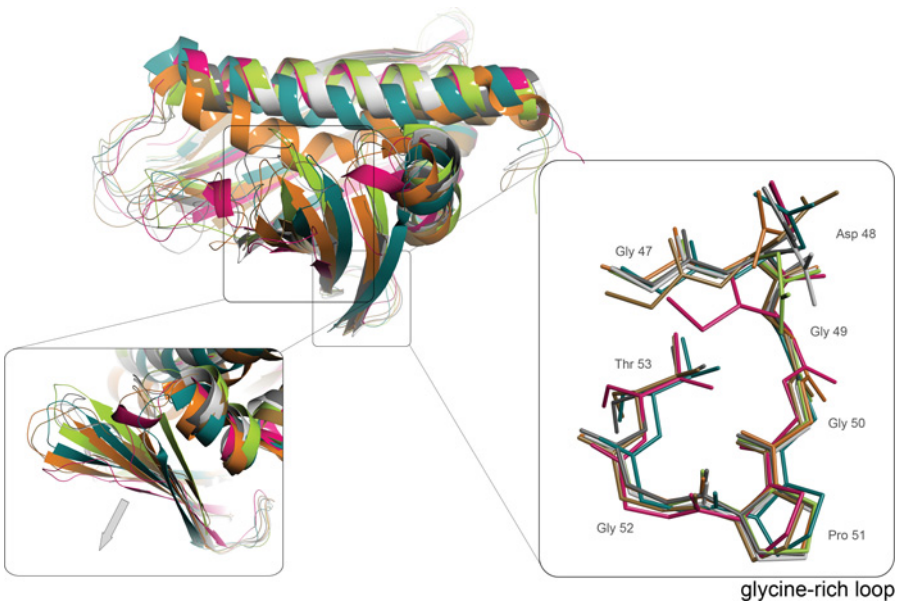

Figure 4 **Comparison of Fra a 1E with homologous proteins**
Overlay of the average Fra a 1E structure (2LPX, pink) with the structures of Pru av 1 (1E09, brown), Bet v 1.0101 (1BV1, dark grey), the hypoallergenic Bet v 1 isoform Bet v 1.1001 (1FM4, light grey), Gly m 4 (2K7H, dark green), LIPR-10.2A (1XDF, orange) and LIPR-10.2B (2QIM, light green). The structures are displayed in cartoon representation. The insets show the glycine-rich loop region, residues Gly^{47}–Thr^{53}, in stick representation and the region around β-strands 5 and 6 in cartoon representation, colours as above.

Gly^{111}–Thr^{123}) that is closed by a long C-terminal (residues Glu^{131}–Asp^{154}) and a short V-shaped α-helix (residues Ala^{16}–Ile^{34}) with a kink at Asp^{26}. The helices and the β-strands encompass a very large hydrophobic cavity, as is typical for Bet v 1 homologous proteins. A comparison of the secondary structure elements of Fra a 1E with the structures listed in Table 1 reveals backbone atomic rmsd values between 1.04 and 1.93 Å, with the most closely related structure being that of Pru av 1 [6] which also has the highest sequence homology to Fra a 1E. Whereas these proteins show the same overall fold, there detailed structures contain subtle differences.

For example, strands β5 and β6 are bent away from the hydrophobic pocket (Figure 4, inset, grey arrow) in Fra a 1E, and the orientation of the C-terminal helix differs between the homologous proteins; both variations influence size and shape of the hydrophobic cavity.

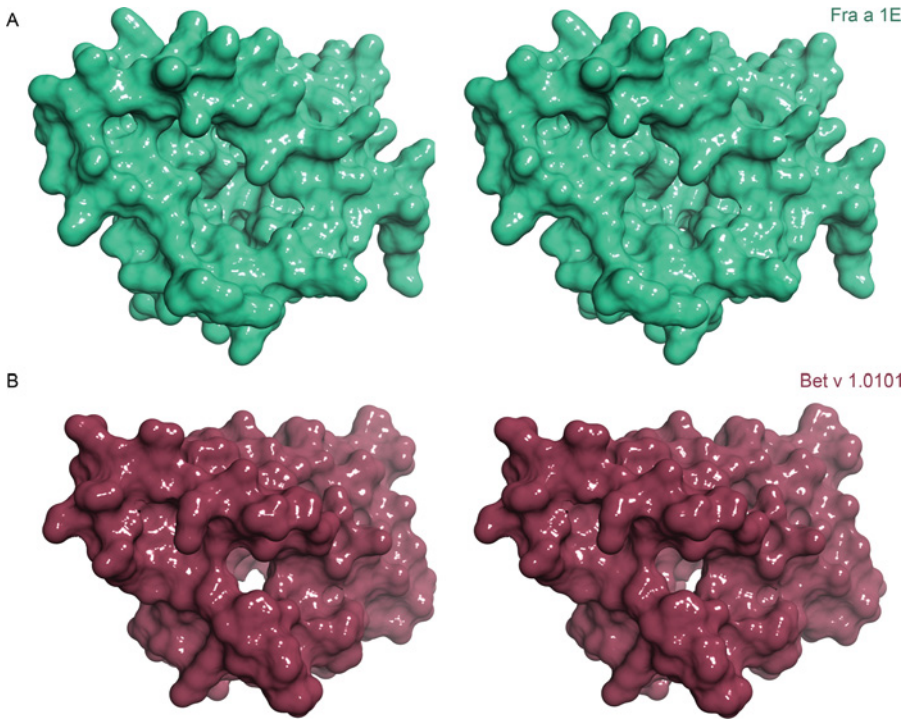

Figure 5 **The hydrophobic pocket**
Stereoscopic view of surface representations of (**A**) Fra a 1E (green) and (**B**) Bet v 1 (red), with orientation close to Figure 3 displaying the large entrance to the hydrophobic cavity.

Several studies were focused on ligand binding to this hydrophobic pocket. Binding, transport or storage are obvious possibilities for the physiological function of these proteins [6,37–40]. A range of ligands that might be of physiologic relevance could be identified in an ANS (8-anilinonaphthalene-1-sulfonic acid) displacement assay with Bet v 1.0101, and compounds such as flavonoids, fatty acids and cytokinins bind with low micromolar affinities to the protein [37]. Interestingly, the cytokinin kinetin seems to have a binding site alternative to the hydrophobic cavity. Structural studies revealed that two or three ligand molecules can bind inside the pocket, mainly stabilized by hydrophobic and a few hydrogen-bonding interactions [38,41,42].

The structural differences are also reflected in the differences of the CD spectra. Fra a 1E contains 32% α-helical, 39% β-sheet and 29% unstructured regions, while Bet v1 shows 24% α-helical, 39% β-sheet and 37% unstructured regions.

The hydrophobic cavity

Like in other family members, the cavity of Fra a 1E is accessible via three structural gaps, between the long C-terminal α-helix and β1, close to the glycine-rich loop, and between C-terminal α-helix and the loop from Thr^{58}–Tyr^{67}.

The first gap is not present in all homologues as its presence depends on exact shape and arrangement of the C-terminal helix. The second gap is quite small, and its size of approximately 4–9 Å depends on the nature of the amino acid side chains in different homologues (in Fra a 1E: 5 Å). The glycine-rich loop between β2 and β3, from Gly^{47}–Thr^{53}, has the consensus sequence GXGGXGT which is highly conserved throughout the Bet v 1 superfamily (rmsd of 0.35 Å for the alignment of all loop atoms, Figure 4, inset). This loop was suggested to be a binding region for kinetin [39] and to play an essential role in IgG and IgE binding [6,7,43,44], making it a general IgE binding epitope responsible for cross reactions between Bet v 1 homologous proteins [4].

The third gap, between the C-terminal α-helix and the loop from Thr^{58}–Tyr^{67}, is the largest. Compared with Bet v 1 and Pru av 1 [6,45], this loop is less well defined in Fra a 1E (Figure 3B). The loop flexibility is strongly related to the cavity volume, as can be judged from the volume difference of approximately 200 $Å^3$ between the Fra a 1E structures with the highest rmsd difference of the loops.

The major difference between other members of the Bet v 1 superfamily and Fra a 1E is the large volume of its hydrophobic pocket of roughly 2500 $Å^3$. The cavities of Bet v 1, Pru av 1 or Gly m 4 are only approximately 1600 Å, and the cavity of LIPR-10.2A is even smaller at 330 $Å^3$, due to a strong inward kink of the C-terminal helix. The flexible orientation of the loop Thr^{58}–Tyr^{67} as well as the displacements of strands β5 and β6 in Fra a 1E also contributes to the remarkable size of its cavity. Compared with the homologous structures, the main cavity entrance is much larger in Fra a 1E (Figures 5A and 5B) due to the different orientation of the C-terminal helix and the rearrangement of amino acids in

the N-terminus of the helix. This helix aligns well with those of Pru av 1 and LIPR-10.2B (Figure 4) and, in addition, in Fra a 1E the helix residues Glu[131]–Glu[139] are shifted away from the hydrophobic core, moving the larger amino acids such as Lys[130], Glu[131], His[132], Lys[135] and Glu[139] away from the cavity entrance. The increased size of this gap is also due to the movement of loop Pro[36]–Ala[38] away from the entrance on the opposite side of the C-terminal helix, so that the protein backbone and Gln[37] are not restrictive.

The residues inside the Fra a 1E cavity are very similar to those of homologous proteins, particularly Pru av 1 ([6], Figure 1). The hydrophobic residues Phe[20], Tyr[82], Tyr[84], Ile[99], Tyr[101] and Tyr[121] are widely conserved and adopt similar conformations in the different structures, pointing to similar functions. Indeed, these amino acids were suggested to establish van der Waals interactions with ligands [6,38,40].

Structural relatives

In spite of the overall folds being highly similar, the structural alignment of the homologous proteins reveals several differences, and, although Fra a 1E may well play a role in substrate binding, storage, or transport, the substrate class may be different from the other Bet v 1 proteins. LIPR-10.2A and LIPR-10.2B from yellow lupine are rather flexible proteins that can undergo conformational adaptations upon ligand binding. This might explain the large variations of the cavity volumes observed for LIPR-10.2A, LIPR-10.2B, Bet v 1.0101 and Bet v 1.1001 (Table 1). LIPR-10.2B binding of three *trans*-zeatin molecules inside the pocket leads to an increase of the cavity volume by 1800 Å^3 [41,42,46] when compared with LIPR-10.2A in the empty state. The Bet v 1.1001 cavity volume increases by 700 Å^3 when two molecules of deoxycholate are bound [38]. Hence, the 2500 Å^3 cavity of Fra a 1E provides for accommodation of more copies of a single ligand or, alternatively, a larger ligand.

As Fra a 1E is expressed in different plant compartments, it may fulfil various functions [25]. In addition to all the ligand-binding studies it was observed that Bet v 1 is able to bind and permeabilize membranes, and ligand binding may facilitate translocation [47]. One may thus speculate that Fra a 1E is involved in the transport of hydrophobic ligands into plant vacuoles for storage, or that it may bind hydrophobic compounds necessary for cell growth, such as plant hormones or flavonoids. To contribute to ending these speculations, the current focus of our studies is the identification of physiological ligands of the Bet v 1 superfamily proteins.

AUTHOR CONTRIBUTION

Olivia Hartl-Spiegelhauer designed the experiments and, together with Paul Rösch, co-ordinated the study. Christian Seutter von Loetzen performed the majority of the experiments together with Kristian Schweimer, who designed the NMR experiments. Wilfried Schwab provided reagents and conceptual input for the completion of the paper. Paul Rösch and Olivia Hartl-Spiegelhauer wrote the paper. All authors read and approved the manuscript.

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REFERENCES

1 Wuthrich, B., Schindler, C., Leuenberger, P. and Ackermann-Liebrich, U. (1995) Prevalence of atopy and pollinosis in the adult population of Switzerland (SAPALDIA study). Swiss Study on Air Pollution and Lung Diseases in Adults. Int. Arch. Allergy Immunol. **106**, 149–156
2 Vieths, S., Scheurer, S. and Ballmer-Weber, B. (2002) Current understanding of cross-reactivity of food allergens and pollen. Ann. N. Y. Acad.Sci. **964**, 47–68
3 Ipsen, H. and Lowenstein, H. (1983) Isolation and immunochemical characterization of the major allergen of birch pollen (*Betula verrucosa*). J. Allergy Clin. Immunol. **72**, 150–159
4 Dreborg, S. (1988) Food allergy in pollen-sensitive patients. Ann. Allergy **61**, 41–46
5 Geroldinger-Simic, M., Zelniker, T., Aberer, W., Ebner, C., Egger, C., Greiderer, A., Prem, N., Lidholm, J., Ballmer-Weber, B. K., Vieths, S. and Bohle, B. (2011) Birch pollen-related food allergy: clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. J. Allergy Clin. Immunol. **127**, 616–622.e1
6 Neudecker, P., Schweimer, K., Nerkamp, J., Scheurer, S., Vieths, S., Sticht, H. and Rösch, P. (2001) Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1. J. Biol. Chem. **276**, 22756–22763
7 Neudecker, P., Lehmann, K., Nerkamp, J., Haase, T., Wangorsch, A., Fotisch, K., Hoffmann, S., Rösch, P., Vieths, S. and Scheurer, S. (2003) Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry (*Prunus avium*) and celery (*Apium graveolens*): correlating IgE reactivity with three-dimensional structure. Biochem. J. **376**, 97–107
8 Schirmer, T., Hoffimann-Sommergrube, K., Susani, M., Breiteneder, H. and Markovic-Housley, Z. (2005) Crystal structure of the major celery allergen Api g 1: molecular analysis of cross-reactivity. J. Mol. Biol. **351**, 1101–1109
9 Berkner, H., Neudecker, P., Mittag, D., Ballmer-Weber, B. K., Schweimer, K., Vieths, S. and Rösch, P. (2009) Cross-reactivity of pollen and food allergens: soybean Gly m 4 is a member of the Bet v 1 superfamily and closely resembles yellow lupine proteins. Biosci. Rep. **29**, 183–192
10 Breiteneder, H., Hoffmann-Sommergruber, K., O'Riordain, G., Susani, M., Ahorn, H., Ebner, C., Kraft, D. and Scheiner, O. (1995) Molecular characterization of Api g 1, the major allergen of celery (*Apium graveolens*), and its immunological and structural relationships to a group of 17-kDa tree pollen allergens. Eur. J. Biochem. **233**, 484–489
11 Vanek-Krebitz, M., Hoffmann-Sommergruber, K., Laimer da Camara Machado, M., Susani, M., Ebner, C., Kraft, D., Scheiner, O. and Breiteneder, H. (1995) Cloning and sequencing of Mal d 1, the major allergen from apple (*Malus domestica*), and its immunological relationship to Bet v 1, the major birch pollen allergen. Biochem. Biophys. Res. Commun. **214**, 538–551
12 Scheurer, S., Metzner, K., Haustein, D. and Vieths, S. (1997) Molecular cloning, expression and characterization of Pru a 1, the major cherry allergen. Mol. Immunol. **34**, 619–629
13 Kleine–Tebbe, J., Vogel, L., Crowell, D. N., Haustein, U. F. and Vieths, S. (2002) Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1- related PR-10 protein in soybean, SAM22. J. Allergy Clin. Immunol. **110**, 797–804

14 Sikorski, M. M. (1997) Expression of *Lupinus luteus* cDNA coding for PR10 protein in *Escherichia coli*: purification of the recombinant protein for structural and functional studies. Acta Biochim. Pol. **44**, 565–578
15 Handschuh, L., Femiak, I., Kasperska, A., Figlerowicz, M. and Sikorski, M. M. (2007) Structural and functional characteristics of two novel members of pathogenesis-related multigene family of class 10 from yellow lupine. Acta Biochim. Pol. **54**, 783–796
16 Bais, H. P., Vepachedu, R., Lawrence, C. B., Stermitz, F. R. and Vivanco, J. M. (2003) Molecular and biochemical characterization of an enzyme responsible for the formation of hypericin in St. John's wort (*Hypericum perforatum* L.). J. Biol. Chem. **278**, 32413–32422
17 Hoffmann-Sommergruber, K. (2000) Plant allergens and pathogenesis-related proteins. What do they have in common? Int. Arch. Allergy Immunol. **122**, 155–166
18 Somssich, I. E., Schmelzer, E., Bollmann, J. and Hahlbrock, K. (1986) Rapid activation by fungal elicitor of genes encoding "pathogenesis-related" proteins in cultured parsley cells. Proc. Natl. Acad. Sci. U.S.A. **83**, 2427–2430
19 Walter, M. H., Liu, J. W., Grand, C., Lamb, C. J. and Hess, D. (1990) Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens. Mol. Gen. Genet. **222**, 353–360
20 Warner, S. A. J., Scott, R. and Draper, J. (1992) Characterisation of a wound-induced transcript from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins. Plant Mol. Biol. **19**, 555–561
21 Walter, M. H., Liu, J. W., Wunn, J. and Hess, D. (1996) Bean ribonuclease-like pathogenesis-related protein genes (Ypr10) display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression. Eur. J. Biochem. **239**, 281–293
22 Karlsson, A. L., Alm, R., Ekstrand, B., Fjelkner-Modig, S., Schiott, A., Bengtsson, U., Bjork, L., Hjerno, K., Roepstorff, P. and Emanuelsson, C. S. (2004) Bet v 1 homologues in strawberry identified as IgE-binding proteins and presumptive allergens. Allergy **59**, 1277–1284
23 Hjerno, K., Alm, R., Canback, B., Matthiesen, R., Trajkovski, K., Bjork, L., Roepstorff, P. and Emanuelsson, C. (2006) Down-regulation of the strawberry Bet v 1-homologous allergen in concert with the flavonoid biosynthesis pathway in colorless strawberry mutant. Proteomics **6**, 1574–1587
24 Musidlowska-Persson, A., Alm, R. and Emanuelsson, C. (2007) Cloning and sequencing of the Bet v 1-homologous allergen Fra a 1 in strawberry (*Fragaria ananassa*) shows the presence of an intron and little variability in amino acid sequence. Mol. Immunol. **44**, 1245–1252
25 Munoz, C., Hoffmann, T., Escobar, N. M., Ludemann, F., Botella, M. A., Valpuesta, V. and Schwab, W. (2010) The strawberry fruit Fra a allergen functions in flavonoid biosynthesis. Mol. Plant. **3**, 113–124
26 Boehm, M. and Rösch, P. (1997) Expression in *Escherichia coli*, purification, and spectroscopic characterization of two mutant Bet v 1 proteins. Biol. Chem. **378**, 687–695
27 Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G. and Thompson, J. D. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. **31**, 3497–3500
28 Huang, X. and Miller, W. (1991) A time-efficient, linear-space local similarity algorithm. Adv. Appl. Math. **12**, 337–357
29 Emsley, P. and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D: Biol. Crystallogr. **60**, 2126–2132
30 Dundas, J., Ouyang, Z., Tseng, J., Binkowski, A., Turpaz, Y. and Liang, J. (2006) CASTp: computed atlas of surface topography of proteins with structural and topographical mapping of functionally annotated residues. Nucleic Acids Res. **34**, W116–W118
31 Sattler, M., Schleucher, J. and Griesinger, C. (1999) Herteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients. Prog. NMR Spectrosc. **34**, 93–158
32 Johnson, B. A. and Blevins, R. A. (1994) NMRView: A computer program for the visualization and analysis of NMR data. J. Biomol. NMR **4**, 603–614
33 Cornilescu, G., Delaglio, F. and Bax, A. (1999) Protein backbone angle restraints from searching a database for chemical shift and sequence homology. J. Biomol. NMR **13**, 289–302
34 Schwieters, C. D., Kuszewski, J. J., Tjandra, N. and Clore, G. M. (2003) The Xplor-NIH NMR molecular structure determination package. J. Magn. Reson. **160**, 66–74
35 Koradi, R., Billeter, M. and Wuthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. J. Mol. Graph. **14**, 51–55
36 Laskowski, R. A., Rullmann, J. A. C., MacArthur, M. W., Kaptein, R. and Thornton, J. M. (1996) AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR. J. Biomol. NMR **8**, 477–486
37 Mogensen, J. E., Wimmer, R., Larsen, J. N., Spangfort, M. D. and Otzen, D. E. (2002) The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. J. Biol. Chem. **277**, 23684–23692
38 MarkovicHousley, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O. and Breiteneder, H. (2003) Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J. Mol. Biol. **325**, 123–133
39 Koistinen, K. M., Soininen, P., Venalainen, T. A., Hayrinen, J., Laatikainen, R., Perakyla, M., Tervahauta, A. I. and Karenlampi, S. O. (2005) Birch PR-10c interacts with several biologically important ligands. Phytochemistry **66**, 2524–2533
40 Pasternak, O., Bujacz, G. D., Fujimoto, Y., Hashimoto, Y., Jelen, F., Otlewski, J., Sikorski, M. M. and Jaskolski, M. (2006) Crystal structure of Vigna radiata cytokinin-specific binding protein in complex with zeatin. Plant Cell **18**, 2622–2634
41 Fernandes, H., Pasternak, O., Bujacz, G., Bujacz, A., Sikorski, M. M. and Jaskolski, M. (2008) Lupinus luteus pathogenesis-related protein as a reservoir for cytokinin. J. Mol. Biol. **378**, 1040–1051
42 Fernandes, H., Bujacz, A., Bujacz, G., Jelen, F., Jasinski, M., Kachlicki, P., Otlewski, J., Sikorski, M. M. and Jaskolski, M. (2009) Cytokinin-induced structural adaptability of a Lupinus luteus PR-10 protein. FEBS J. **276**, 1596–1609
43 Mirza, O., Henriksen, A., Ipsen, H., Larsen, J. N., Wissenbach, M., Spangfort, M. D. and Gajhede, M. (2000) Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. J. Immunol. **165**, 331–338
44 Spangfort, M. D., Mirza, O., Ipsen, H., Joost van Neerven, R. J., Gajhede, M. and Larsen, J. N. (2003) Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. J. Immunol. **171**, 3084–3090
45 Gajhede, M., Osmark, P., Poulsen, F. M., Ipsen, H., Larsen, J. N., Joost van Neerven, R. J., Schou, C., Lowenstein, H. and Spangfort, M. D. (1996) X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. Nat. Struct. Biol. **3**, 1040–1045

46 Pasternak, O., Biesiadka, J., Dolot, R., Handschuh, L., Bujacz, G., Sikorski, M. M. and Jaskolski, M. (2005) Structure of a yellow lupin pathogenesis-related PR-10 protein belonging to a novel subclass. Acta Crystallogr. D: Biol. Crystallogr. **61**, 99–107

47 Mogensen, J. E., Ferreras, M., Wimmer, R., Petersen, S. V., Enghild, J. J. and Otzen, D. E. (2007) The major allergen from birch tree pollen, Bet v 1, binds and permeabilizes membranes. Biochemistry **46**, 3356–3365

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online data

Original Paper

SUPPLEMENTARY ONLINE DATA

Solution structure of the strawberry allergen Fra a 1

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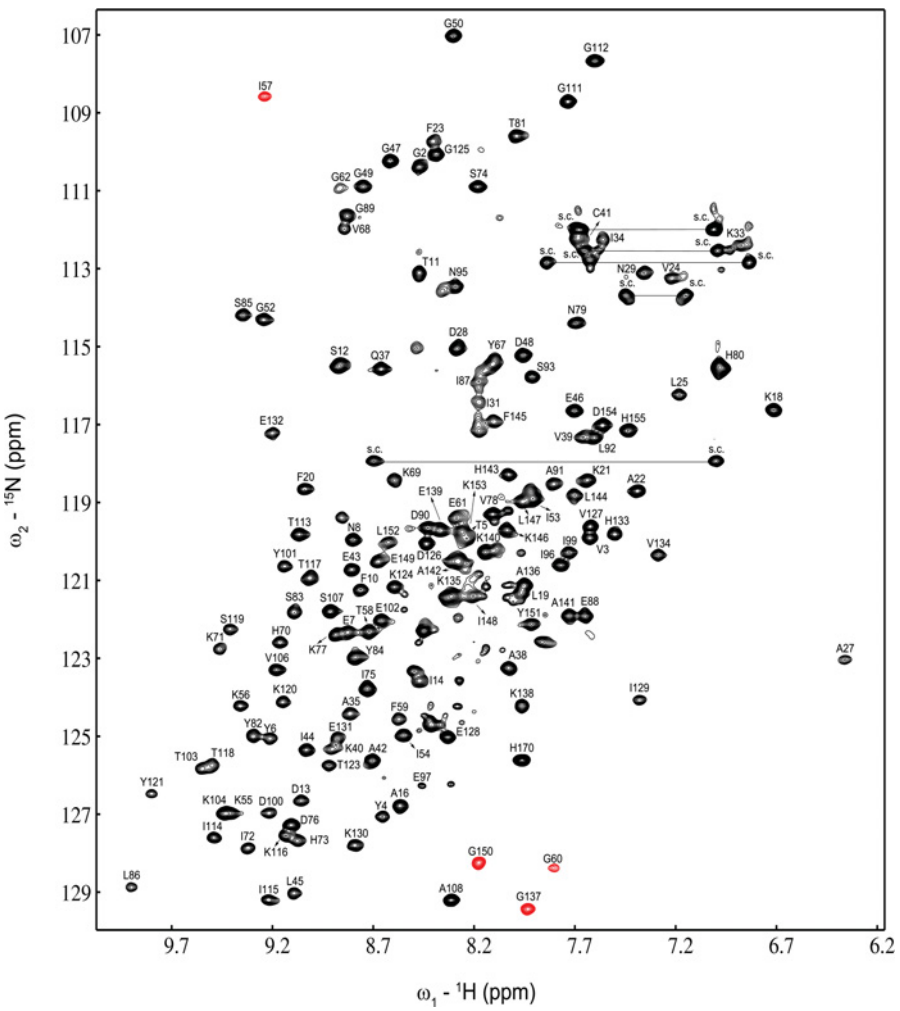

Figure S1 **Backbone assignment of Fra a 1E**
[^{15}N]-HSQC NMR spectrum of ^{15}N, ^{13}C-labelled Fra a 1E (400 μM), measured at 298 K and 800 MHz. Assigned backbone amide resonances are labelled with amino acid type in one letter code and residue number; s.c., side-chain amide resonance.

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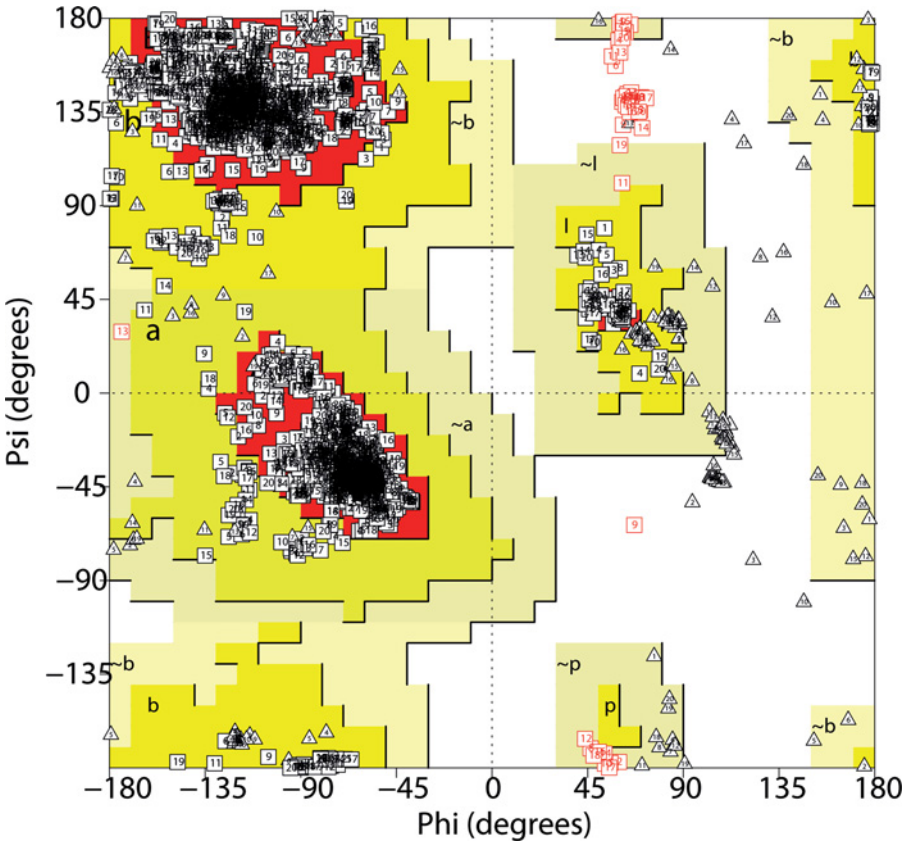

Figure S2 **Ramachandran plot of the 20 NMR solution structures**
Squares, all residues except glycine and proline residues; triangles, glycine residues. Model numbers are shown inside each data point. PROCHECK-NMR analysis showed that 90.3% of the residues are located in the most favoured regions (A, B, L), 7.9% are located in additional allowed regions (a, b, l, p), 0.7% are located in generously allowed regions (~a, ~b, ~l, ~p) and only 1.1% are located in disallowed regions of the Ramachandran map.

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7.3 Einzelarbeit C

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Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand

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The major birch pollen allergen Bet v 1 is the main elicitor of airborne type I allergies and belongs to the PR-10 family (pathogenesis-related proteins 10). Bet v 1 is the most extensively studied allergen, and is well characterized at a biochemical and immunological level; however, its physiological function remains elusive. In the present study, we identify Q3OS (quercetin-3-*O*-sophoroside) as the natural ligand of Bet v 1. We isolated Q3OS bound to Bet v 1 from mature birch pollen and confirmed its binding by reconstitution of the Bet v 1–Q3OS complex. Fluorescence and UV–visible spectroscopy experiments, as well as HSQC (heteronuclear single-quantum coherence) titration, and the comparison with model compounds, such as quercetin, indicated the specificity of Q3OS binding. Elucidation of the binding site by NMR combined with a computational model resulted in a more detailed understanding and shed light on the physiological function of Bet v 1. We postulate that the binding of Q3OS to Bet v 1 plays an important, but as yet unclear, role during the inflammation response and Bet v 1 recognition by IgE.

Key words: allergen, Bet v 1, NMR, quercetin-3-*O*-sophoroside (Q3OS).

INTRODUCTION

The major birch pollen allergen Bet v 1 from *Betula verrucosa* (European white birch) [1] probably exceeds 20 % (w/w) of the total protein content of mature pollen and is an elicitor of airborne type I allergies [2,3]. Up to 20 % of the population of the temperate climate zone of the northern hemisphere are affected by pollen allergies, and 50–90 % of these patients are sensitized to Bet v 1 [4–7]. Up to 90 % of the latter individuals also exhibit IgE-mediated allergic cross-reactions (oral allergy syndrome) to Bet v 1 homologous food allergens contained in fruits, vegetables and nuts [8,9].

On the basis of amino acid sequence similarities and the protein 3D structures, Bet v 1 as well as homologous pollen and food allergens belong to the plant PR-10 proteins (pathogenesis-related proteins 10) family within the Bet-v-1-like superfamily. The PR-10 family is ubiquitous in Nature, and proteins of this family can be found in virtually all plants studied [10]. They are part of the immune defence mostly induced by microbial attacks [11,12], fungal elicitors [13,14], wounds [15] or abiotic stress stimuli [16]. Additionally, they are expressed constitutively in different plant compartments during various developmental stages. PR-10 family proteins are encoded by multiple genes and thus occur as a mixture of different isoforms [17]. Although several PR-10 proteins are well characterized, their particular functions are still elusive.

Even as the most extensively studied allergen, the physiological role of Bet v 1 is unknown, although this protein was analysed extensively with biochemical [1,18–22] as well as immunological [1,23–27] methods, and speculations about its physiological role vary widely [28–30]. The crystal and NMR structures of the most abundant Bet v 1 isoform, Bet v 1a (Bet v 1.0101) [21], show a globular protein comprising a seven-stranded anti-parallel β-sheet closed by a 25-residue C-terminal α-helix that is clasped by two short α-helices in a V-shape. The protein contains a glycine-rich loop motif (GXGGXG) that is highly conserved throughout the PR-10 family. All structural elements form a huge hydrophobic pocket [21], a feature that suggested Bet v 1 to function as a carrier or a storage protein. Bet v 1a is able to bind to different ligands *in vitro*, including flavonoids, cytokinins and fatty acids, with micromolar dissociation constants, and the hydrophobic pocket was implied as the binding region [28]. Interestingly, two deoxycholate molecules, aligned in tandem and nearly completely protected from the solvent, were found inside the Bet v 1l hydrophobic pocket [29]. The Bet v 1 homologue PR-10c [31] interacts with cytokinin, flavonoid glycosides, sterols and emodin, and the hydrophobic cavity was again suggested as a binding epitope [32]. Crystal structures of Bet v 1a and Bet v 1j (Bet v 1.0801) [19] in complex with a set of ligands including ANS (1-anilino-8-naphthalene sulfonate), deoxycholate, kinetin and naringenin revealed that these ligands occupy different regions within the hydrophobic pocket and that differences in ligand binding exist between the different isoforms [33].

In spite of all these studies, no physiological ligand of Bet v 1 has been identified as yet. In the present study, we show the isolation of Bet v 1 from mature birch pollen naturally containing Q3OS (quercetin-3-*O*-sophoroside) as an initial hint to the function of this protein and other pollen and food allergens.

EXPERIMENTAL

Isolation of Bet v 1 from birch pollen

The isolation of Bet v 1 from mature birch pollen is on the basis of the observation that the protein could be detected in

Abbreviations: CSP, chemical shift perturbation; DAD, diode array detection; HSQC, heteronuclear single quantum coherence; PR-10, pathogenesis-related proteins 10; Q3OS, quercetin-3-*O*-sophoroside; STD, saturation transfer difference; TIC, total ion chromatogram; UPLC, ultra-performance liquid chromatography; UV/VIS, UV-visible spectroscopy.

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the aqueous supernatant of rehydrated pollen [34]. The extraction was conducted as published previously [35] with minor changes. As the basic material, we used 5 g of birch pollen (Allergon) that was incubated in 100 ml of extraction buffer [10 mM potassium phosphate, 1 mM EDTA, 0.1 % ascorbic acid, 4 mM DTT and one Complete™ protease inhibitor cocktail tablet (EDTA-free, Roche), pH 7.0] at 4 °C overnight. The supernatant was precipitated with ammonium sulfate, and the Bet-v-1-containing fractions were pooled. HIC (hydrophobic interaction chromatography) (Octyl Sepharose 4 Fast Flow, 5 ml bed volume, GE Healthcare) was used to further purify and concentrate the samples according to the manufacturer's protocol, and the remaining impurities were removed by gel-filtration chromatography after concentrating the Bet v 1 fractions to a volume of 500 μl with VIVASPIN 20 concentrators (molecular-mass cut-off 3000 Da, Sartorius Stedim Biotech). Subsequently, the protein was loaded on to two sequentially connected Superdex 75 10/300 GL columns (24 ml bed volume each, GE Healthcare), equilibrated with 10 mM potassium phosphate and 300 mM NaCl buffer, pH 7.0, and then the Bet v 1 fractions were pooled and concentrated to a volume of 500 μl. This purification step was then repeated. The columns were calibrated with albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) and ribonuclease A (13.7 kDa). The final protein fractions were freeze-dried and further analysed by MS. All purification steps were followed by UV/VIS (UV–visible spectroscopy) and SDS/PAGE (19 % gel). The most prominent protein bands in the range of 17 kDa were excised and analysed by tryptic digestion followed by LC-MS (Bioanalytics/Center for Molecular Medicine, University of Cologne, Germany) to confirm their identity with Bet v 1.

Pollen extraction for HPLC analysis

Birch pollen was extracted with methanol at room temperature (21 °C) overnight. After centrifugation, the supernatant was concentrated until dry under reduced pressure (40 °C), redissolved in methanol and centrifuged before HPLC analysis.

Preparative RP18–HPLC–UV/ESI–MS^n

Purification of Q3OS was performed using a preparative HPLC–UV/ESI–MS^n system. The Jasco HPLC system consisted of two Jasco PU-2087 Plus pumps connected to a Jasco UV-2075 Plus variable wavelength detector set at 370 nm, an Advantec CHF122SC fraction collector (Tokyo Seisakusho Kaisha) and an Agilent LC/MSD Trap XCT mass spectrometer (Agilent). The column was a Synergi 4u Fusion-RP 80, 25 cm × 21.5 mm (Phenomenex). The HPLC solvents were 0.1 % formic acid in water (A), 0.1 % formic acid in methanol (B) and 0.1 % formic acid in acetonitrile (C). For separation of Q3OS, gradients from 0 % B and 100 % A for 2 min, then to 100 % B in 28 min, 5 min under these conditions, returning to 100 % A and 0 % B at a flow rate of 9.5 ml/min were used. Approximately 2 % were diverted to the mass spectrometer and 9.3 ml fractions were collected. The fraction containing Q3OS was further purified by gradients from 0 % C and 100 % A for 2 min, then to 30 % C and 70 % A in 28 min, followed by 5 min at 100 % C, finally returning to 100 % A. Ionization parameters were as follows: the voltage of the capillary was -4000 V and the end plate was set to -500 V. The temperature of the dry gas (N_2) was 330 °C at a flow of 9 l/min. The full scan mass spectra of the metabolites were measured from *m/z* 50 to 800 until the ICC (ion charge control) target reached 70000 or 200 ms, whichever was reached first. MS^2 was performed using helium as the collision gas, and the collision energy was set at 1.0 V. The target mass for MS^2 spectra was set to *m/z* 625. Mass spectra were acquired in the negative ionization mode. Data analyses were performed using the ChromPass Version 1.9.302.1124 software (Jasco) and the 6300 Series Trap Control Version 6.2 software (Bruker).

UPLC (ultra-performance liquid chromatography)–TOF-MS

High-resolution mass spectra of Q3OS were measured on a Waters Synapt G2 HDMS mass spectrometer (Waters) coupled with an Acquity UPLC core system (Waters) consisting of a binary solvent manager, sample manager and column oven. Aliquots (1 μl) of samples (2 μl) were injected into the UPLC–TOF-MS system equipped with a BEH C_{18} column (2 mm × 150 mm, 1.7 μm, Waters). Operated with a flow rate of 0.3 ml/min at a temperature of 40 °C, the following gradient was used for chromatography: starting with a mixture (35:95, v/v) of acetonitrile (0.1 % HCOOH) and aqueous formic acid (0.1 % HCOOH), the acetonitrile content was increased to 95 % within 3 min and then kept constant for 1 min. Scan time for the MSE method (centroid) was set to 0.1 s. Measurements were performed using negative ESI and the resolution mode consisting of the following ion source parameters: capillary voltage -2.0 kV, sampling cone 20, extraction cone 4.0, source temperature 150 °C, desolvation temperature 450 °C, cone gas 30 l/h and desolvation gas 850 l/h. Data processing was performed by using MassLynx 4.1 SCN 779 (Waters) and the elemental composition tool for determining the exact mass. All data were lock mass corrected on the pentapeptide leucine enkephalin (YGGFL, *m/z* 554.2615, $[M-H]^-$) in a solution (2 ng/μl) of acetonitrile/0.1 % formic acid (1:1, v/v). Scan time for the lock mass was set to 0.3 s, an interval of 15 and three scans to average with a mass window of $\pm$0.3 Da. Calibration of the Synapt G2 apparatus in the range *m/z* 50–600 was performed using a solution of sodium formate (5 mmol/l) in 2-propanol/water (9:1, v/v).

NMR spectroscopy of Q3OS

^{1}H-NMR and ^{13}C spectroscopy of Q3OS was performed on an Avance III 500 MHz spectrometer with a TCI Cryo Probe (Bruker). Chemical shifts were referenced to TMS (tetramethylsilane). Data processing was performed with Topspin version 1.3 (Bruker) and MestReNova version 5.2.3 software (Mestrelab Research).

Expression and purification of Bet v 1a

The Bet v 1a gene was cloned into the bacterial expression vector pET-11a (Novagen) with the restriction enzymes NdeI and BpU 1102I (Fermentas). The gene was expressed in *Escherichia coli* strain BL21(DE3) cells growing at 37 °C in 1 litre of LB medium, supplemented with 100 μg/ml ampicillin to a D_{600} value of 0.6. A final concentration of 1 mM IPTG was added to the medium to induce the protein expression. To obtain ^{15}N-labelled Bet v 1a, overexpressing *E. coli* were grown on M9 minimal medium with ^{15}N-enriched $(^{15}NH4)_2SO_4$. The cells were harvested 4 h after induction by centrifugation (5500 ***g***, 10 min, 4 °C), and the cell pellets were stored at -20 °C.

For protein purification the frozen cells were resuspended in 20 mM Tris buffer, pH 8.0, containing one Complete™ protease inhibitor cocktail tablet (EDTA-free, Roche), lysozyme and DNase. The cells were stirred on ice for 30 min followed by lysis using a microfluidizer. The lysate was centrifuged at 19000 ***g*** for 30 min at 4 °C and PEI (polyethylenimine) was added to the supernatant to a final concentration of 0.35 % to precipitate nucleic acids. The solution was incubated for 15 min at room

temperature and the emerging precipitate was removed by centrifugation (19000 ***g***, 30 min, 4 °C). The clarified protein solution was loaded on to a 10-ml Q XL Sepharose column (HiTrap Q XL, GE Healthcare) and was eluted using a stepwise gradient including 10, 15, 20 and 100 % (v/v) elution buffer (20 mM Tris buffer, pH 8.0, and 300 mM NaCl). Fractions were then analysed by SDS/PAGE (19 % gel). Bet v 1-containing fractions were pooled and dialysed for at least 4 h at 4 °C against Millipore water and finally the protein was freeze-dried and stored at 4 °C.

Cell culture (1 litre) from LB and M9 minimal medium yielded approximately 32 and 18 mg of Bet v 1 respectively. Protein concentrations were determined by the DC protein assay (Bio-Rad Laboratories) and UV/VIS using the molar extinction coefficient $\varepsilon_{280} = 10430\ M^{-1} \cdot cm^{-1}$.

Reconstitution of Bet v 1a–Q3OS

For reconstitution we used final concentrations of 500 μM Bet v 1a and excess amounts of Q3OS in 10 mM sodium phosphate buffer, pH 7.0, in a total volume of 500 μl. The sample was loaded on to two sequentially connected Superdex 75 10/300 GL columns (24 ml bed volume each, GE Healthcare) equilibrated with sample buffer containing 300 mM NaCl. The isocratic protein elution was carried out at a flow rate of 0.4 ml/min. In addition, a sample of Q3OS with identical concentrations and volumes was loaded separately for comparison.

UV/VIS

Quercetin (Sigma–Aldrich) and Q3OS were dissolved in 50 mM sodium phosphate, 50 mM NaCl and 10 % (v/v) DMSO buffer, pH 7.0, to a final concentration of 20 μM. The solutions (500 μl) were used for the titration with Bet v 1a in a 1 cm quartz cuvette (Hellma Analytics) at 25 °C. The titrations were performed by adding small amounts of a 2 mM Bet v 1a stock solution prepared in the same buffer. Absorption spectra from 200 to 800 nm were recorded using an Agilent 8453 UV-visible spectrophotometer. Changes of absorption at 320 nm for quercetin and 358 nm for Q3OS were plotted against Bet v 1a concentration. Before curve fitting, absorbance data were corrected for dilution. If possible, the equilibrium dissociation constant (K_d) was determined by non-linear regression analysis of the data with GraFit-5 (version 5.0, Erithacus Software) using eqn (1):

$$\Delta A = \frac{\Delta A_{max}}{2Q}\left\{(B + Q + K_d) - \left[(B + Q + K_d)^2 - (4BQ)^{0.5}\right]\right\} \quad (1)$$

where ΔA_{max} is the maximum change in absorbance at specific wavelengths; B is Bet v 1a concentration; and Q is total flavonoid concentration.

In all titration experiments a suitable amount of DMSO was present to avoid solubility problems and therefore to disable unwanted side effects of the flavonoids. With HSQC (heteronuclear single quantum coherence) NMR measurements of Bet v 1a, we could exclude aggregation or partial unfolding of the protein in the presence of increasing DMSO concentrations (see Supplementary Figure S6). Additionally, titration experiments at different DMSO concentrations indicate that there is only a very small increase in the K_d value at rising DMSO concentrations.

Fluorescence spectroscopy

For fluorescence equilibrium titrations the intrinsic fluorescence of protein-bound Q3OS was used. The measurements were carried out with a Horiba Fluoromax fluorimeter at 25 °C in fluorescence buffer [50 mM sodium phosphate buffer, pH 7.0, containing 50 mM NaCl and 10 % (v/v) DMSO] in a total volume of 1 ml using a 10 mm × 4 mm quartz cuvette (Hellma Analytics). Q3OS was provided at a concentration of 500 nM and Bet v 1a was added in 10 μl steps from a 50 μM stock solution. The excitation wavelength was 340 nm, and emission spectra were measured from 410 to 430 nm. Slit widths were set at 6 nm for excitation and emission. Data were analysed by plotting the relative change of fluorescence intensity at 416 nm against increasing concentrations of Bet v 1a corrected for dilution. The equilibrium dissociation constant (K_d) was determined from data fitted to eqn (2) by non-linear regression using GraFit-5 software (version 5.0, Erithacus Software):

$$\Delta F_{norm} = \frac{\Delta F_{max} \cdot \left[(Q + B + K_d) - \sqrt{(Q + B + K_d)^2 - (4QB)}\right]}{2Q} \quad (2)$$

where ΔF_{norm} is the normalized change in fluorescence; B is Bet v 1a concentration; Q is total concentration flavonoid; and ΔF_{max} is maximum fluorescence change.

Protein NMR spectroscopy

All NMR experiments were performed at 298 K in 50 mM sodium phosphate buffer, 50 mM NaCl, pH 7.0, and 10 % ^2H_2O with ^{15}N uniformly labelled Bet v 1a using Bruker Avance 700 MHz and Avance 800 MHz spectrometers with cryogenically cooled triple-resonance probes equipped with pulsed field-gradient capabilities. NMR data were processed using in-house software and visualized with NMRViewJ [36]. The sequence-specific assignments of the amide resonances of Bet v 1a are reported elsewhere [22].

For the different titration experiments quercetin and rutin stock solutions were prepared in deuterated DMSO and Q3OS and sophorose stock solutions were prepared in 50 mM sodium phosphate and 50 mM NaCl buffer, pH 7.0, containing 100 μM Bet v 1a. Rutin and Q3OS were added in a single step, whereas quercetin and sophorose were added stepwise to a final excess of 1:15 to the protein sample; final DMSO concentrations did not exceed 13.5 % (v/v). CSPs (chemical shift perturbations) caused by increasing DMSO concentrations within the measurements of quercetin and rutin were identified by titrating DMSO in comparable steps. CSPs were calculated using eqn (3):

$$\Delta\delta_{norm} = \sqrt{(\Delta\delta_{HN})^2 + (0.1 \cdot \Delta\delta_N)^2} \quad (3)$$

where $\Delta\delta_{HN}$ and $\Delta\delta_N$ are chemical shift differences in amide proton and nitrogen resonances respectively in p.p.m. The K_d value for quercetin binding was determined with NMRViewJ [36]. CSPs from amino acid residues that were unaffected by increasing DMSO concentrations were fit to a quadratic binding curve with default setting, and an average K_d (app) was calculated.

For chemical shift mapping of quercetin and Q3OS binding, different experimental setups were used. Quercetin was added to a 15-fold excess to 100 μM Bet v 1a solution containing 10 % (v/v) DMSO to ensure a constant DMSO concentration. The CSPs were mapped on to the Bet v 1a structure (PDB code 1BV1). Titration experiments with Q3OS were performed by stepwise addition of Bet v 1a to a 680 μM sample of Q3OS to Q3OS/Bet v 1a ratios of 15:1 to 2.4:1. Only amino acids in which signals disappear following Q3OS binding were labelled in the Bet v 1a structure.

For the displacement experiment, 100 μM Bet v 1a and 25 μM quercetin were used in the same buffer without DMSO. Q3OS was added from a stock solution to a final excess of 1:10 over Bet v 1a.

STD (saturation transfer difference) NMR spectroscopy

All experiments were performed on a Bruker Avance 600 MHz spectrometer equipped with a standard triple resonance probe. NMR samples were prepared in 550 μl of 99.85 % ^2H_2O buffer containing 50 mM sodium phosphate and 50 mM NaCl, pH 7.0, with 2 mM of Q3OS (and 4 % d_6-DMSO) or sophorose respectively. Bet v 1a was added from a stock of 3.42 mM to a final concentration of 225 μM.

For the STD NMR experiments, a saturation period with on/off resonance continuous wave saturation of 4 s was applied. The on-resonance frequency was set to 0.7 p.p.m. and the off-resonance frequency was set to 20 p.p.m. Protein signals were suppressed by using a spinlock pulse of 50 ms.

Docking simulation

We used AutoDockVina [37] to dock Q3OS into the hydrophobic pocket of Bet v 1a. The allergen listed under PDB code 4A87 served as a template for Bet v 1a. The PDB file for Q3OS was created with ProDrg [38]. Furthermore, input files for Bet v 1a and Q3OS were generated with AutoDockTools [39]. The grid box ($20\,\text{Å} \times 24\,\text{Å} \times 28\,\text{Å}$ or $13\,440\,\text{Å}^3$) (1 Å = 0.1 nm) was centred over the hydrophobic pocket of Bet v 1a and AutoDockVina was run with default settings. Affinity scores were given by AutoDockVina as binding energies (ΔG), which were subsequently used to calculate an equilibrium dissociation constant (K_d) by eqn (4) with $R = 0.001968\,\text{kcal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ and $T = 298.15$ K:

$$K_d = e^{\frac{-\Delta G}{R \cdot T}} \quad (4)$$

The docking simulations resulted in nine different possible orientations of Q3OS in the Bet v 1a pocket. Seven models show similar binding for the quercetin moiety, whereas the binding situation for the sugar moiety is less defined. The model in best agreement with our experimental NMR data was chosen to illustrate Q3OS binding to Bet v 1.

RESULTS

Isolation of Bet v 1 from mature birch pollen

Extraction of Bet v 1 from mature birch pollen [35] yielded SEC (size exclusion chromatography) fractions containing Bet v 1 that were brightly yellow coloured, and the colour persisted during protein concentration (Supplementary Figure S1 at http://www.biochemj.org/bj/457/bj4570379add.htm). UV/VIS absorption spectra of these fractions had a maximum at 350 nm, which was subsequently used to detect the protein-bound ligand during LC. The protein (280 nm) and the coloured ligand (350 nm) both eluted from the SEC column at a volume of 23 ml as almost pure compounds (Figure 1A). The protein fractions exhibit a minimum of three intense and closely spaced protein bands in the SDS/PAGE (Figure 1B), corresponding to molecular masses of approximately 17 kDa. UPLC–TOF-MS identified Bet v 1a (Bet v 1.0101), Bet v 1d (Bet v 1.0401), Bet v 1f (Bet v 1.0601), Bet v 1g (Bet v 1.0701) and Bet v 1m (Bet v 1.1401) as components of the protein fractions. The mixture of isoforms will be termed Bet v 1 for simplicity in the present paper.

Identification of the bound ligand

The biochemically purified Bet v 1 protein was freeze-dried. The yellowish residue was extracted with water/methanol (1:1), centrifuged and the supernatant analysed by LC-DAD (diode array detection)-MS. The UV trace (200–600 nm) and the TIC (total ion chromatogram) showed only one single compound (Figure 2, lower two traces), exhibiting UV maxima at 252 and 358 nm as well as pseudomolecular ions of *m/z* 625 $[M-H]^-$ and 649 $[M+Na]^+$ in the negative and positive ion mode respectively (Supplementary Figures S2A, S2B and S2D at http://www.biochemj.org/bj/457/bj4570379add.htm). The fragmentation patterns indicated the presence of up to two glucose residues in the molecule (Supplementary Figures S2C and S2E). The molecular mass (626 g/mol) of the metabolite was confirmed by LC-TOF analysis and provided a putative sum formula of $C_{27}H_{29}O_{17}$ for the isolated product (Supplementary Figure S3 at http://www.biochemj.org/bj/457/bj4570379add.htm), suggesting the presence of a quercetin dihexoside. Since the amount of the material isolated from the Bet v 1 protein was too low for NMR analysis we purified a larger quantity from birch pollen by preparative LC. The comparison of the ^{1}H- and ^{13}C-NMR spectra (Supplementary Figures S4 and S5 at http://www.biochemj.org/bj/457/bj4570379add.htm) of the isolated metabolite with those of different flavonol diglycosides yielded Q3OS (Figure 1C, and Supplementary Table S1 at http://www.biochemj.org/bj/457/bj4570379add.htm) as the structure for the natural ligand of Bet v 1. Although the birch pollen extract contained numerous phenolic glycosides (Figure 2, upper two traces) only Q3OS was co-purified with the Bet v 1 protein (Figure 2, lower two traces).

Reconstitution of Bet v 1a–Q3OS

We reconstituted Bet v 1–Q3OS by adding the ligand to recombinantly produced protein. SEC analysis yielded an elution diagram basically identical to that of the natural complex (Figures 1D and 1E). A minor amount of ligand eluted at a volume of 35 ml as identified by SEC analysis of Q3OS alone (Figure 1D, grey line).

UV/VIS/fluorescence titrations

To determine equilibrium dissociation constants (K_d), we followed the titration of Q3OS and quercetin with Bet v 1a by UV/VIS (Figures 3A–3D). The spectra of the titration of quercetin with Bet v 1a show isosbestic points indicating a two-state binding process for the flavonoid (Figure 3A) with $K_d = 9.2 \pm 0.6$ μM (Figure 3B).

For the Q3OS titration with Bet v 1 only small changes in the absorbance spectrum could be observed (Figure 3C), and we could not obtain analysable data, although isosbestic points indicated complex formation (Figure 3D).

We performed a fluorescence titration in tandem with the UV/VIS titrations to determine the K_d value for Q3OS binding to Bet v 1 (Figure 3E). In contrast with the UV/VIS experiment, the fluorescence data were analysable and yielded $K_d = 566 \pm 85$ nM (Figure 3F).

NMR spectroscopy

To study the binding behaviour of quercetin, Q3OS, rutin and sophorose to Bet v 1 and to identify ligand binding sites on Bet v 1 we employed CSP measurements with ^{1}H-^{15}N HSQC NMR spectroscopy. Addition of the compounds to ^{15}N-labelled Bet v 1a in protein/ligand ratios of 1:0.1 to 1:15 led to clearly observable compound-specific changes in the ^{1}H-^{15}N HSQC spectra (Figure 4). Since DMSO is indispensable in the measurement to ensure the solubility of quercetin we had to take into account the effect of increasing DMSO concentrations on the CSPs (Supplementary Figure S6 at http://www.biochemj.org/bj/457/bj4570379add.htm).

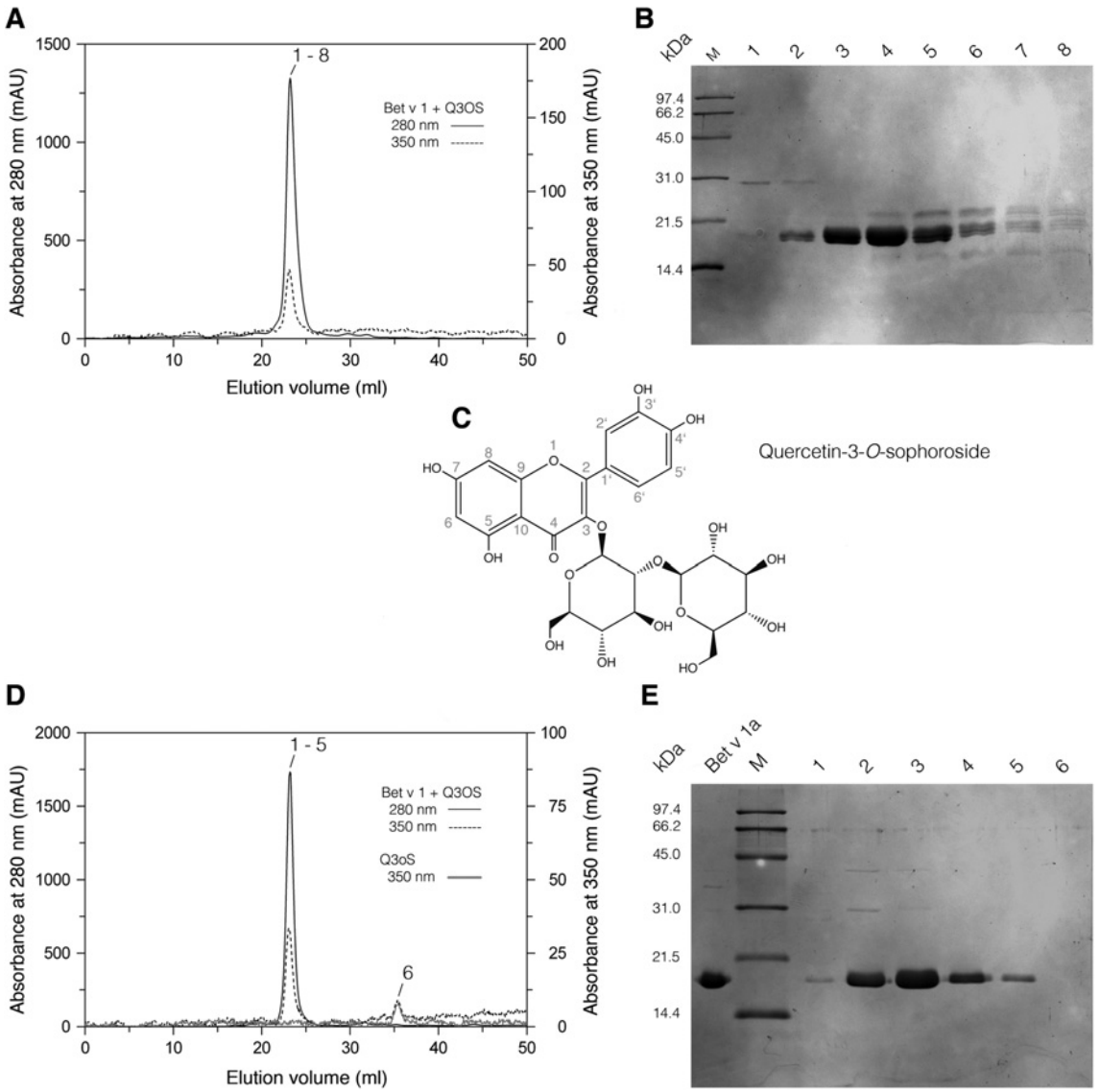

Figure 1 Identification of Q3OS as a ligand of Bet v 1

(**A**) SEC of Bet v 1 isolated from mature birch pollen with two sequentially connected Superdex S75 10/300 GL columns performed in 10 mM phosphate buffer, pH 7.0, and 300 mM NaCl. The traces correspond to absorbance at 280 nm (continuous line) and 350 nm (broken line). (**B**) Corresponding analysis of the SEC fractions by SDS/PAGE (19 % gel). M, molecular-mass standard (SDS/PAGE Molecular Weight Standards, Low Range, Bio-Rad Laboratories). Lanes 1–8, proteins eluted within the 20 and 25 ml elution volumes. (**C**) Structure of the identified ligand Q3OS. (**D**) SEC analysis of the reconstitution of recombinant Bet v 1a–Q3OS. The continuous and broken black traces correspond to the run of Bet v 1–Q3OS with absorbance measured at 280 and 350 nm respectively. The grey line corresponds to a run of Q3OS alone with absorbance measured at 350 nm. (**E**) Analysis of the SEC fractions of Bet v 1–Q3OS by SDS/PAGE (19 % gel). Bet v 1a, recombinantly purified protein. M, molecular-mass standard (SDS/PAGE Molecular Weight Standard, Low Range, Bio-Rad Laboratories). Lanes 1–6, Bet v 1a–Q3OS eluted within the 20 and 25 ml elution volumes.

For quercetin, gradual CSPs in the ^{1}H-^{15}N HSQC spectra could be observed which are characteristic for complex formation in the fast-exchange regime of the NMR time scale (Figure 4A, and Supplementary Figure S7 at http://www.biochemj.org/bj/457/bj4570379add.htm). The K_d value was determined from CSPs of amino acids that are not affected by DMSO as 33 ± 2 μM, in agreement with the UV/VIS measurements. Titration of Bet v 1a with Q3OS led to ^{1}H-^{15}N HSQC spectra with peaks of several residues disappearing completely during titration, which is characteristic for intermediate exchange rates (Figure 4B, and Supplementary Figure S8 at http://www.biochemj.org/bj/457/bj4570379add.htm). This does not allow a proper line shape analysis for determination of the dissociation constant. However, this observation is in agreement with the K_d value gained from the fluorescence titration.

Very small CSPs even at higher rutin concentrations indicated very weak rutin binding to Bet v 1a (Figure 4C, and Supplementary Figure S9 at http://www.biochemj.org/bj/457/bj4570379add.htm). No CSPs and thus no conclusive evidence of Bet v 1a interaction could be observed during sophorose titration (Figure 4D and Supplementary Figure S10 at http://www.biochemj.org/bj/457/bj4570379add.htm). In addition, we performed STD NMR experiments with Bet v 1a and sophorose, a technique used to investigate very weak interactions between proteins and ligands. Still, we were not able to detect any interactions between the two molecules. Remarkably, quercetin can be partially displaced from Bet v 1a by addition of Q3OS in competition ^{1}H-^{15}N HSQC experiments with ^{15}N-labelled Bet v 1a, quercetin and added Q3OS (Supplementary Figure S11 at http://www.biochemj.org/bj/457/bj4570379add.htm).

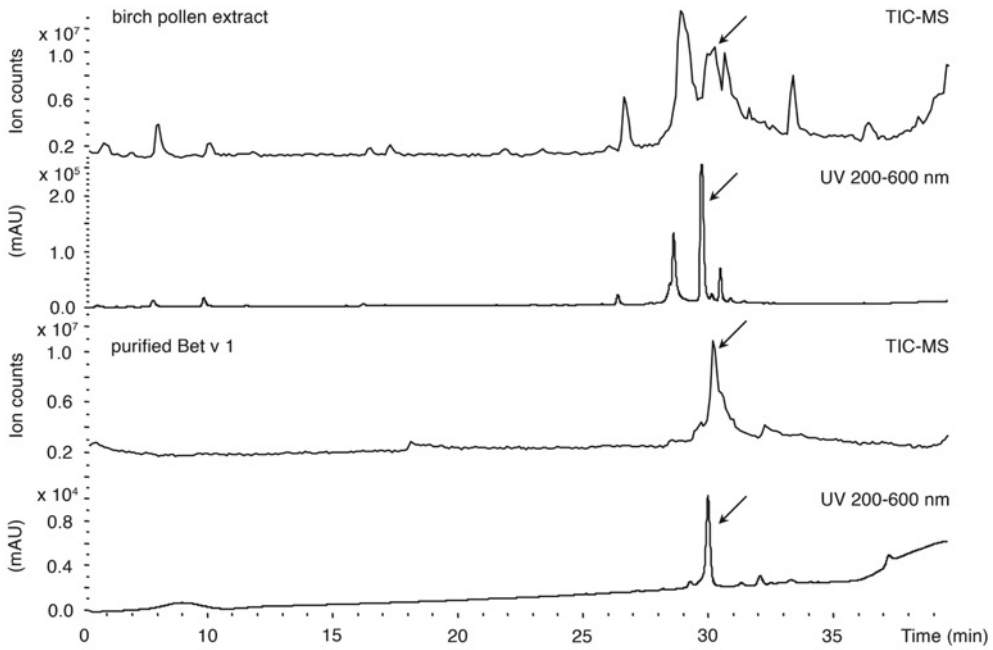

Figure 2 Co-purification of Q3OS with the Bet v 1 protein

LC-DAD-MS analysis of a birch pollen extract and the purified Bet v 1 protein obtained by ammonium sulfate precipitation followed by hydrophobic interaction and SEC. The TIC acquired in the negative mode (TIC-MS) and the UV trace (200–600 nm) show different metabolites of the birch pollen extract (upper two traces), whereas Q3OS (arrow) is highly enriched in the purified Bet v 1 protein (lower two traces).

In different ^{1}H-^{15}N HSQC experiments, we analysed the interaction surfaces of quercetin and Q3OS with Bet v 1. Quercetin was added to ^{15}N-labelled Bet v 1a containing 10 % (v/v) DMSO to neutralize the effects of DMSO addition on the CSPs (Supplementary Figure S12 at http://www.biochemj.org/bj/457/bj4570379add.htm). Thus we could safely map residues showing CSPs larger than 0.04 p.p.m. following addition of quercetin on to the structure of Bet v 1a (Figure 5A). Most residues that showed significant CSPs (e.g. Thr^{10}, Lys^{115} and Lys^{137}–Leu^{143}) were found in the region around the C-terminal helix. To study Q3OS binding, the protein was added stepwise to a concentrated sample of Q3OS (Supplementary Figure S13 at http://www.biochemj.org/bj/457/bj4570379add.htm). Amino acids with disappearing signals were mapped on the Bet v 1 structure (Figure 5B). Surprisingly, most of these amino acids were also located around the C-terminal helix, indicating that the glycosylation does not severely change the binding properties of the quercetin moiety. A structural model confirmed the binding situation of the Bet v 1a–Q3OS complex and provided an estimated K_d value of 1.7 μM (Figure 5D). Although the docking simulation demonstrates that the Bet v 1a pocket is, in general, large enough to easily allow Q3OS binding, the exact binding geometry of the disaccharide moiety still remains elusive.

DISCUSSION

The physiological role of PR-10 and, therefore allergenic, proteins in this family is poorly understood. However, the biological function is important even for the understanding of allergenicity of proteins, as it is still unknown whether structural features as such are responsible for the immune response or whether additional functional properties are also important factors [40].

Identification of the natural ligand of Bet v 1

All Bet-v-1-containing fractions that were obtained during the purification process displayed a yellow colour and even during SEC the colour could be used as an indicator for spotting the samples with the allergen (Figure 1A). LC-DAD-MS analyses confirmed the co-purification of a single UV active compound, which was also detected in the initial birch pollen extract among numerous other metabolites (Figure 2). The natural ligand was unambiguously identified as Q3OS according to its LC-DAD-MS and NMR data. Sophorose [glucosyl(1-2)-β-glucose] was first isolated from *Sophora japonica* in which it occurs as kaempferol-3-*O*-sophoroside. Subsequent studies showed that the sugar is attached to anthocyanins and flavones isolated from many plant sources [41], and pollen seem to be particularly rich in 3-*O*-sophorosides of kaempferol and quercetin [42,43]. Although from prior binding studies, it was expected that flavonols may be natural ligands of Bet v 1, it was surprising to find a glycosylated compound as natural Bet v 1 ligand.

Binding of quercetin and Q3OS

To investigate Q3OS–Bet v 1 interaction we used the recombinantly purified isoform Bet v 1a. Q3OS was not only identified as a ligand of Bet v 1 purified from mature birch pollen, but also bound strongly to recombinant Bet v 1a in a reconstitution experiment (see Figure 1). The identical elution profiles of the Bet v 1a–ligand complexes confirm that Q3OS is indeed a physiological binding partner of Bet v 1.

We performed various NMR experiments with Q3OS and its quercetin and sugar moiety in order to locate the binding site of Q3OS on Bet v 1. In an initial experiment with quercetin, significant CSPs in the protein could be determined and mapped on to the Bet v 1a structure (Figure 5A). Most of the affected residues lie in a region around the centre of the long C-terminal

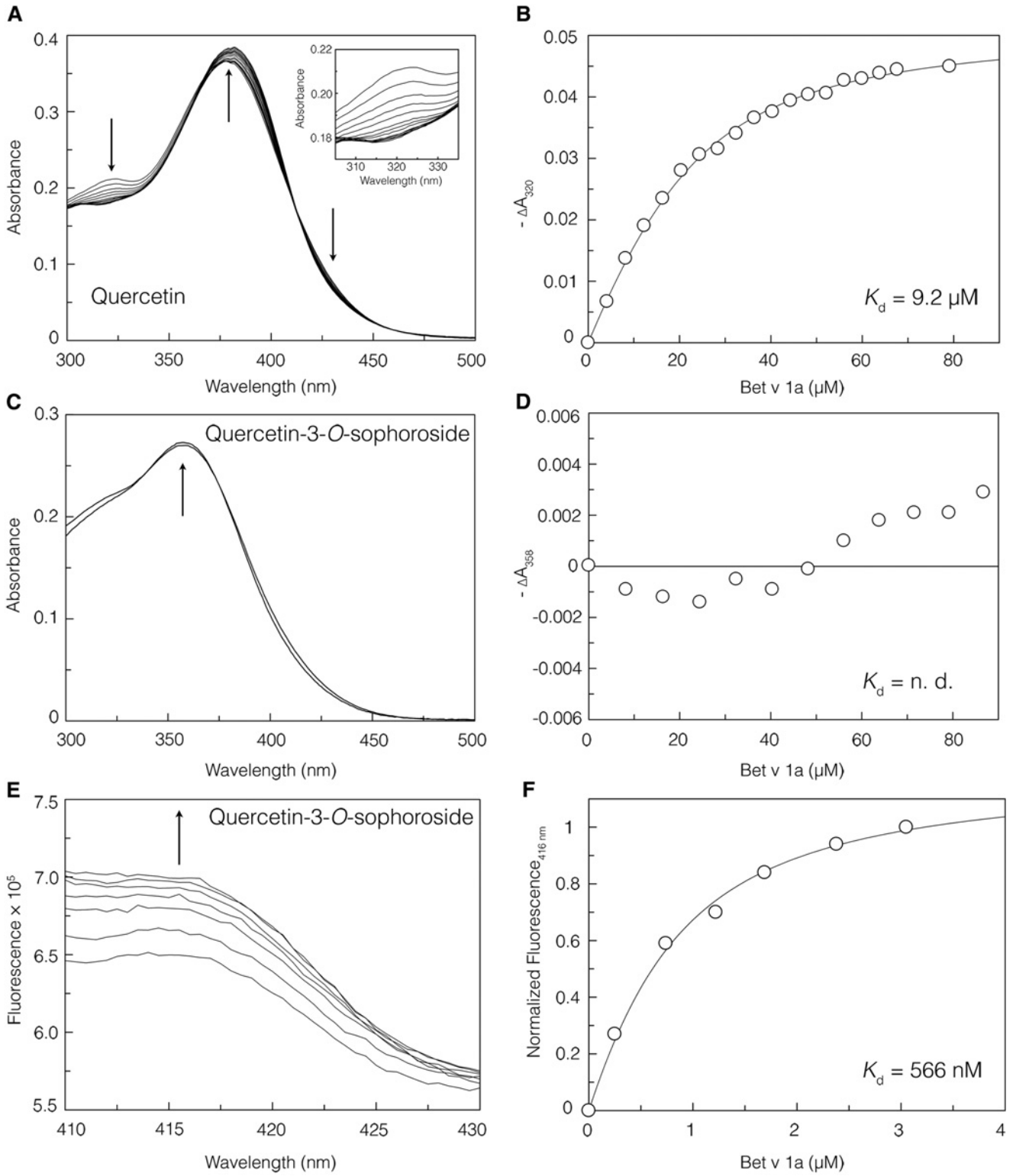

Figure 3 Binding interactions of Bet v 1 with quercetin and Q3OS

Measurements were carried out in 50 mM sodium phosphate buffer, pH 7.0, containing 50 mM NaCl and 10 % (v/v) DMSO at 25 °C. (**A**) UV/VIS spectra of the equilibrium titration of 20 μM quercetin with increasing concentrations of Bet v 1a. (**B**) Absorbance changes at 320 nm against Bet v 1a concentrations for the data as shown in (**A**). The curve shows the best fit to eqn (1). (**C**) Initial and final spectrum of the UV/VIS equilibrium titration of 20 μM Q3OS with increasing concentrations of Bet v 1a. (**D**) Absorbance changes at 358 nm against Bet v 1a concentrations for the data shown in (**C**). (**E**) Fluorescence spectra of the equilibrium titration of 500 nM Q3OS with Bet v 1a. (**F**) Fluorescence changes at 416 nm against Bet v 1a concentrations for the data shown in (**E**). The curve shows the best fit to eqn (2).

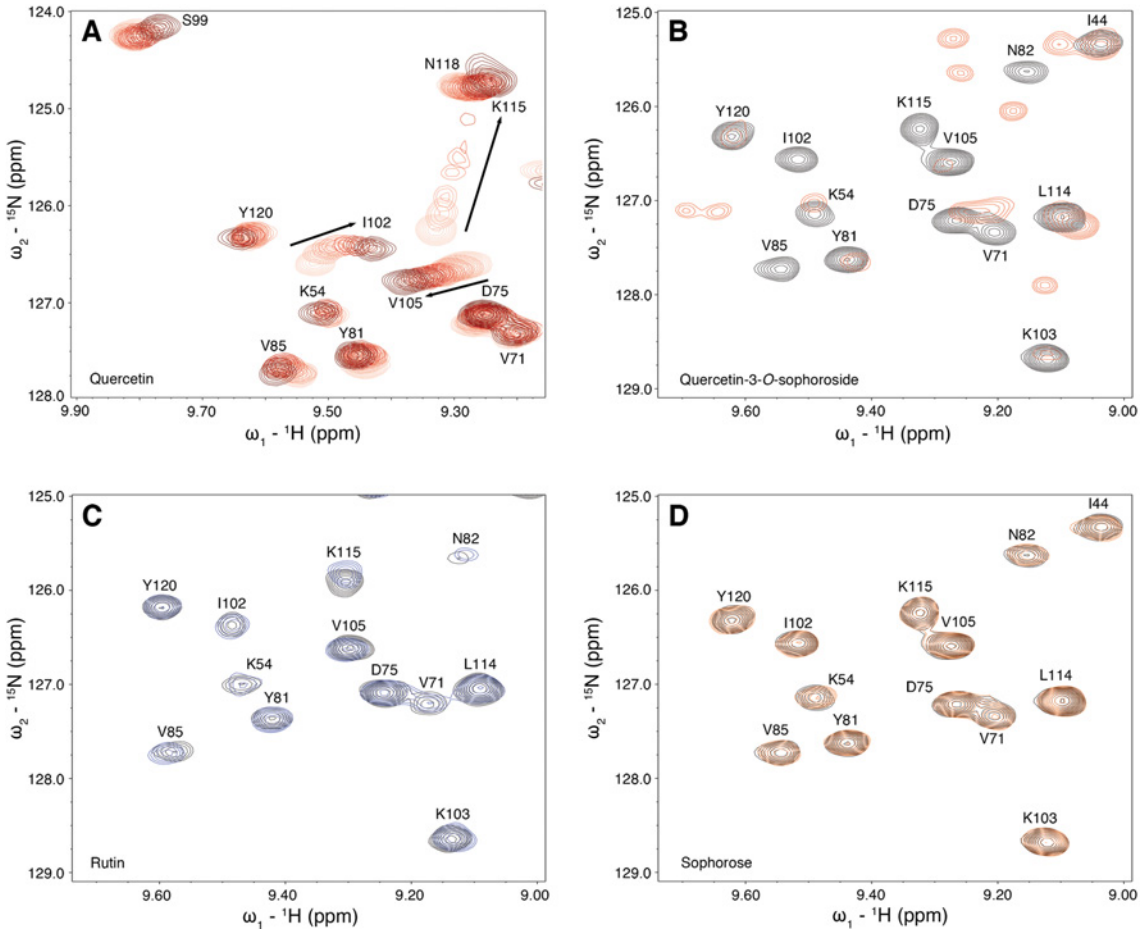

Figure 4 NMR equilibrium titration experiments of Bet v 1a and different compounds

All experiments were performed with 100 μM ^{15}N-uniformly labelled Bet v 1a at 298 K in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and 10 % $^{2}H_2O$ with Bruker Avance 700 MHz and Avance 800 MHz spectrometers. (**A**) Overlay of ten ^{1}H-^{15}N HSQC spectra of Bet v 1a in the presence of increasing quercetin (DMSO) concentrations, from light to dark red. Quercetin was added from a stock solution prepared in deuterated DMSO to a final excess of 1:15 over Bet v 1a and a final DMSO concentration of 13.5 % (v/v). ^{1}H-^{15}N chemical shift changes (indicated with arrows) of residues Ile^{102}, Val^{105} and Lys^{115} indicate complex formation in the fast-exchange regime. (**B**) Overlay of two ^{1}H-^{15}N HSQC spectra of Bet v 1a in the absence (black) and presence of a 15-fold excess of Q3OS (red). Several ^{1}H-^{15}N resonances (Asn^{82}, Val^{85}, Ile^{102} and Lys^{115}) disappear completely upon addition of Q3OS. Consequently, appearing resonances cannot be identified. (**C**) Overlay of two ^{1}H-^{15}N HSQC spectra of Bet v 1a in the absence (black) and presence (blue) of a 15-fold excess of rutin. In the region shown only residues Asn^{82}, Val^{85} and Lys^{115} show marginal shift changes following addition of rutin. (**D**) Overlay of two ^{1}H-^{15}N HSQC spectra of Bet v 1a in the absence (black) and presence (orange) of a 15-fold excess of sophorose. The addition of sophorose did not result in chemical shift changes of Bet v 1a resonances.

α-helix, in agreement with a recently published structure of a Bet v 1a–naringenin complex [33] (Figure 5C). Naringenin, which lacks the 3′-OH and the 3-OH groups of quercetin (Figure 4), is co-ordinated by hydrogen bonding and hydrophobic interactions between the C-terminal α-helix and the N-terminal β-strand. Contrary to expectations that two or more copies of a small molecule would occupy the large cavity in Bet v 1 [29], a single naringenin molecule is bound and fills only a small fraction of the hydrophobic pocket. Assuming that quercetin is bound in an orientation identical with that of naringenin, the quercetin 3-OH group would point towards the pocket interior which still would provide enough space to accommodate the sophorose residue of Q3OS. As we were not able to assign all residues of Bet v 1 in the presence of Q3OS, we decided to simply use signals with intense signal broadening (‘disappearing’ signals) for chemical shift mapping (Figure 5B). Interestingly, the result is very similar to that for the quercetin titration. This led us to propose that the disappearing signals arise from the quercetin moiety of Q3OS that is bound in the same region as quercetin alone. Hence these data support the notion that quercetin both in its free and glycosylated form binds in naringenin-like fashion.

Additional information on the complex geometry was gained from fluorescence titration and NMR measurements with Q3OS. Not unexpectedly, Q3OS in solution exhibits only marginal fluorescence, as flavonols (like quercetin or Q3OS) containing a 5-OH group (see Figure 1) are generally non-fluorescent due to an intramolecular hydrogen bond between the carbonyl oxygen (position C4) and the 5-OH group that facilitates non-radiative de-excitation [44]. The appearance of a fluorescence signal of Q3OS following Bet v 1a binding (Figure 3E) may indicate that this intramolecular hydrogen bond is distorted and that either the carbonyl oxygen or the 5-OH group or both are somehow involved in the binding process.

The Bet v 1a–naringenin complex structure and our NMR data support this idea. On titration of quercetin to Bet v 1a, several residues (e.g. Phe^{30}, Tyr^{83}, Ile^{102}, Ser^{136} or Glu^{141}) show strong CSPs in the HSQC spectra. These residues are identical with those that are involved in direct hydrogen bonds and hydrophobic contacts with naringenin, except for residue Asn^{118}. In the Bet

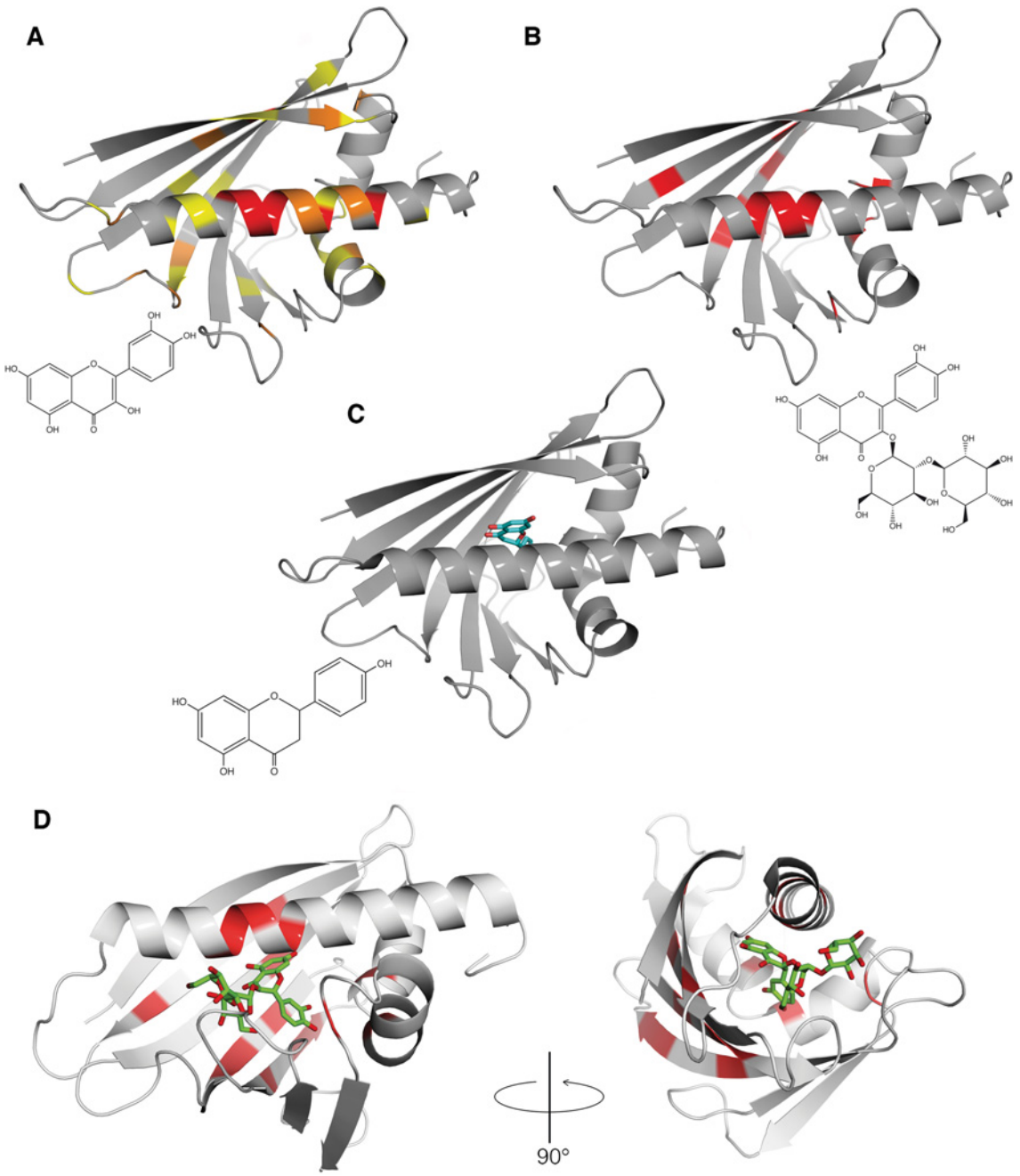

Figure 5 Comparison of the solution structure models of Bet v 1–quercetin, Bet v 1–Q3OS and the crystal structure of the Bet v 1a–naringenin

(**A**) Chemical shift changes ($\Delta\delta$) upon quercetin binding mapped on a cartoon representation of Bet v 1a (PDB code 1BV1; grey); 0.04 p.p.m.$\leqslant\Delta\delta\leqslant$0.08 p.p.m. shown as yellow; 0.08 p.p.m.$<\Delta\delta\leqslant$0.12 p.p.m. shown as orange; and 0.12 p.p.m.$<\Delta\delta$ shown as red. (**B**) as (**A**), with Q3OS. Amino acids whose signals disappear during titration, red. (**C**) Cartoon representation of the crystal structure of Bet v 1a–naringenin (PDB code 4A87). Grey, Bet v 1a; blue sticks, naringenin; red, oxygen. (**D**) Bet v 1a–Q3OS model based on AutoDockVina (based on PDB code 4A87). Grey, Bet v 1a; spectral changes upon Q3OS coded as in (**B**). Green sticks, Q3OS; red, oxygen.

v 1a–naringenin complex the carbonyl oxygen of Asn[118] forms a sodium bridge with the carbonyl oxygen and the 5-OH group of naringenin [33]. This salt bridge may explain the fact that Asn[118] does not show CSPs in our HSQC measurements following quercetin binding, but nevertheless is involved in quercetin binding according to the fluorescence data.

These data strongly support the idea that quercetin, the quercetin moiety of Q3OS, and naringenin bind to the same Bet v 1 region near the C-terminal helix and are oriented similarly. Although the sophorose moiety of Q3OS could not be located experimentally, docking simulations (Figure 5D) were in agreement with the experimental results.

Specificity of Q3OS binding

Next, we asked whether the binding of Q3OS to Bet v 1a is specific. We thus tested the binding characteristics of an additional set of compounds. Rutin was used as an alternative glycosylated

flavonoid as it was shown to interact with the Bet v 1 homologue birch PR-10c [32]. Rutin is a naturally occurring compound composed of the flavonol quercetin and the disaccharide rutinose [α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose], and it can be found in more than 70 plant species [45]. The difference between Q3OS and rutin is the composition and therefore the geometry of the disaccharide. Our data show only weak interaction between rutin and Bet v 1a (Figure 4C), thus we conclude that the nature of the glycosylation plays an important role and that the Bet v 1 interaction is very specific for Q3OS.

Therefore we studied sophorose binding to the protein. Interestingly, even with a 10- or 15-fold excess of the disaccharide we were not able to detect STD signals or CSPs (Figure 4D) respectively, indicating no or very weak binding. Low affinities for proteins and carbohydrates is a well-reported phenomenon resulting from a balance of a favourable negative enthalpy difference caused by hydrogen bonding, van der Waals or hydrophobic interactions, and a counteracting entropy term caused, e.g. by the restricted carbohydrate geometry or solvation effects [46–50]. This enthalpy–entropy compensation is a general characteristic of weak intermolecular interactions [51]. The different dissociation constants for quercetin (9.2–33 μM), sophorose (no binding) and Q3OS (566 nM) show that the disaccharide in Q3OS causes an up to 60-fold higher affinity of the flavonol, although the carbohydrate alone shows no or only weak binding. Thus the enthalpy of the sophorose binding to Bet v 1 may be too low to sufficiently counteract the loss in entropy resulting in a dissociation constant that cannot safely be determined with the applied experiments. In contrast, on Q3OS binding its sophorose moiety is forced into binding by the high affinity of the quercetin moiety and each additional protein–ligand contact co-operatively lowers the dissociation constant. Alternatively, the change in order of the solvent may simply be different for sophorose and Q3OS as compared with their Bet v 1 complexes [52]. A similar effect of glycosylation on affinities could be observed with the HIV protein gp120 and the human CD4 receptor [53]. Therefore we assume that the architecture of the binding pocket of Bet v 1a is specific for Q3OS and is optimized for carbohydrate binding.

The amino acids variable among the Bet v 1 isoforms are uniformly distributed among the sequences, however, many of these residues are spatially located in the peripheral region of the sophorose moiety given the prediction of the Q3OS binding geometry. This may be the structural basis for the observation that specificity for a certain ligand is isoform dependent [33].

Physiological relevance

The enzymatic activity of various allergenic proteins such as Der p 1 from house dust mite has been suggested to promote airway inflammation and therefore influence sensitization [54–56]. Although for Bet v 1a a transport or storage function without enzymatic activity has been proposed [21], its physiological function may nevertheless influence its allergenicity as ligands may induce unspecific inflammatory responses or will affect the immunological shift towards an allergic reaction [40]. Bet v 1–ligand interaction generally leads to an increase of the volume of the hydrophobic pocket [29,33], altering the protein surface and thus the IgE-binding epitopes. However, whether or not these structural changes influence allergenicity currently remains unanswered.

As the physiological ligand of Bet v 1 is a glycosylated flavonol, Bet v 1 may be linked with various activities in plants. Flavonoids contribute to the formation of pigments in flowering plants, they act as signals for pollinators, they are involved in plant hormone signalling, they facilitate pollen-tube germination and they protect plants from UV radiation [57,58]. Pollen from different plants, such as petunia, maize or *Ophrys*, have been studied, and flavonol glycosides with diverse flavonoid and sugar moieties identified [59,60]. At least ten glycosides of kaempferol, quercetin and isorhamnetin were found in maize pollen [61]. Interruption of kaempferol synthesis in petunia and maize resulted in self-sterile plants due to a failure in the production of functional pollen tubes. Even though flavonols in pollen only occur as sugar conjugates, pollen tube formation could be rescued by the artificial addition of flavonol aglycones. This indicates that the flavonoids are stored as glycosylated precursors and at some point, probably during rehydration of the pollen grain, are processed into their active form by pollen glycosyltransferases [57,59-63]. At this time point the lipid- or glycolipid-binding capability of Bet v 1 may lead to the displacement of Q3OS from the Bet v 1–Q3OS complex making Q3OS accessible for deglycosylation. Therefore Bet v 1 binding to lipids may not only play an important role in its uptake by nasal and conjunctival epithelial cells during allergic inflammatory response, but also be a physiological function as well [28,30,64,65]. Generally, the vast majority of pollen flavonoids are present as sugar-conjugated highly water-soluble flavonol-3-*O*-glycoside [61,66]. Synthesis of the flavonol is catalysed in the tapetal cells, and glycosylation is the final step after the non-polar molecule has entered the pollen grain [63,67–69].

Flavonoids like quercetin-3-*O*-glycosides are expressed upon UV-B radiation stress in petunia leaves (in contrast with, e.g. kaempferol-3-*O*-glycosides) [70], where they absorb UV radiation and have antioxidant capacities [58]. Thus Q3OS together with the high Bet v 1 content may be a means to protect birch pollen DNA from UV damage.

We thus hypothesize that after entering birch pollen quercetin is glycosylated into Q3OS to enhance its solubility. Bet v 1 stores Q3OS until pollen germination. The Bet v 1–Q3OS complex protects the birch pollen DNA from UV damage, and functional Q3OS may serve as an important signal for unharmed pollen DNA. On contact of the pollen with the stigma, glycosidase activity is induced and Q3OS is transformed into quercetin, which in turn enables the pollen to germinate. The known mixture of Bet v 1 isoforms in the pollen may match the diversity of flavonol glycosides, possibly providing an individual fingerprint preventing self-pollination.

AUTHOR CONTRIBUTION

Christian Seutter von Loetzen performed the UV/VIS and NMR titrations as well as the computational docking. Thomas Hoffmann and Wilfried Schwab identified Q3OS and isolated the compound from birch pollen. Maximilian Hartl designed the fluorescence measurements and performed them together with Christian Seutter von Loetzen and Olivia Hartl-Spiegelhauer. Kristian Schweimer and Christian Seutter von Loetzen conducted the STD measurements and NMR assignments. Olivia Hartl-Spiegelhauer performed the extraction of Bet v 1 from birch pollen and the reconstitution of Bet v 1a with Q3OS. Paul Rösch and Olivia Hartl-Spiegelhauer designed and supervised the project. Thomas Hoffmann, Wilfried Schwab, Paul Rösch, Maximilian Hartl and Olivia Hartl-Spiegelhauer wrote the paper, which was read and approved by all authors.

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REFERENCES

1 Ipsen, H. and Lowenstein, H. (1983) Isolation and immunochemical characterization of the major allergen of birch pollen (*betula verrucosa*). J. Allergy Clin. Immunol. **72**, 150–159
2 Schenk, M. F., Cordewener, J. H., America, A. H., Van't Westende, W. P., Smulders, M. J. and Gilissen, L. J. (2009) Characterization of PR-10 genes from eight betula species and detection of Bet v 1 isoforms in birch pollen. BMC Plant. Biol. **9**, 24
3 D'Amato, G., Spieksma, F. T., Liccardi, G., Jager, S., Russo, M., Kontou-Fili, K., Nikkels, H., Wuthrich, B. and Bonini, S. (1998) Pollen-related allergy in europe. Allergy **53**, 567–578
4 Wuthrich, B., Schindler, C., Leuenberger, P. and Ackermann-Liebrich, U. (1995) Prevalence of atopy and pollinosis in the adult population of switzerland (SAPALDIA study). Swiss study on air pollution and lung diseases in adults. Int. Arch. Allergy Immunol. **106**, 149–156
5 Vieths, S., Scheurer, S. and Ballmer-Weber, B. (2002) Current understanding of cross-reactivity of food allergens and pollen. Ann. N.Y. Acad. Sci. **964**, 47–68
6 Jarolim, E., Rumpold, H., Endler, A. T., Ebner, H., Breitenbach, M., Scheiner, O. and Kraft, D. (1989) IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*. Allergy **44**, 385–395
7 Moverare, R., Westritschnig, K., Svensson, M., Hayek, B., Bende, M., Pauli, G., Sorva, R., Haahtela, T., Valenta, R. and Elfman, L. (2002) Different IgE reactivity profiles in birch pollen-sensitive patients from six european populations revealed by recombinant allergens: an imprint of local sensitization. Int. Arch. Allergy Immunol. **128**, 325–335
8 Dreborg, S. (1988) Food allergy in pollen-sensitive patients. Ann. Allergy. **61**, 41–46
9 Geroldinger-Simic, M., Zelniker, T., Aberer, W., Ebner, C., Egger, C., Greiderer, A., Prem, N., Lidholm, J., Ballmer-Weber, B., Vieths, S. and Bohle, B. (2011) Birch pollen-related food allergy: clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. J. Allergy Clin. Immunol. **127**, 616–622
10 Liu, J. and Ekramoddoullah, A. K. M. (2006) The family 10 of plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses. Physiol. Mol. Plant Pathol. **68**, 3–13
11 Breda, C., Sallaud, C., el-Turk, J., Buffard, D., de Kozak, I., Esnault, R. and Kondorosi, A. (1996) Defense reaction in medicago sativa: a gene encoding a class 10 PR protein is expressed in vascular bundles. Mol. Plant Microbe Interact. **9**, 713–719
12 Robert, N., Ferran, J., Breda, C., Coutos-Thévenot, P., Boulay, M., Buffard, D. and Esnault, R. (2001) Molecular characterization of the incompatible interaction of *Vitis vinifera* leaves with *Pseudomonas syringae* pv. *pisi*: expression of genes coding for stilbene synthase and class 10 PR protein. Eur. J. Plant Pathol. **107**, 249–261
13 Somssich, I. E., Schmelzer, E., Bollmann, J. and Hahlbrock, K. (1986) Rapid activation by fungal elicitor of genes encoding "pathogenesis-related" proteins in cultured parsley cells. Proc. Natl. Acad. Sci. U.S.A. **83**, 2427–2430
14 Walter, M. H., Liu, J. W., Grand, C., Lamb, C. J. and Hess, D. (1990) Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens. Mol. Gen. Genet. **222**, 353–360
15 Warner, S. A. J., Scott, R. and Draper, J. (1992) Characterisation of a wound-induced transcript from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins. Plant Mol. Biol. **19**, 555–561
16 Walter, M. H., Liu, J. W., Wunn, J. and Hess, D. (1996) Bean ribonuclease-like pathogenesis-related protein genes (Ypr10) display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression. Eur. J. Biochem. **239**, 281–293
17 Hoffmann-Sommergruber, K. (2000) Plant allergens and pathogenesis-related proteins. what do they have in common? Int. Arch. Allergy Immunol. **122**, 155–166
18 Ferreira, F. D., Hoffmann-Sommergruber, K., Breiteneder, H., Pettenburger, K., Ebner, C., Sommergruber, W., Steiner, R., Bohle, B., Sperr, W. R. and Valent, P. (1993) Purification and characterization of recombinant Bet v I, the major birch pollen allergen. immunological equivalence to natural Bet v I. J. Biol. Chem. **268**, 19574–19580
19 Swoboda, I., Jilek, A., Ferreira, F., Engel, E., Hoffmann-Sommergruber, K., Scheiner, O., Kraft, D., Breiteneder, H., Pittenauer, E. and Schmid, E. (1995) Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. J. Biol. Chem. **270**, 2607–2613
20 Faber, C., Lindemann, A., Sticht, H., Ejchart, A., Kungl, A., Susani, M., Frank, R. W., Kraft, D., Breitenbach, M. and Rösch, P. (1996) Secondary structure and tertiary fold of the birch pollen allergen Bet v 1 in solution. J. Biol. Chem. **271**, 19243–19250
21 Gajhede, M., Osmark, P., Poulsen, F. M., Ipsen, H., Larsen, J. N., Joost van Neerven, R. J., Schou, C., Lowenstein, H. and Spangfort, M. D. (1996) X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. Nat. Struct. Biol. **3**, 1040–1045
22 Schweimer, K., Sticht, H., Nerkamp, J., Boehm, M., Breitenbach, M., Vieths, S. and Rösch, P. (1999) NMR spectroscopy reveals common structural features of the birch pollen allergen Bet v 1 and the cherry allergen Pru a 1. Appl. Magn. Reson. **17**, 449–456
23 Ferreira, F., Ebner, C., Kramer, B., Casari, G., Briza, P., Kungl, A. J., Grimm, R., Jahn-Schmid, B., Breiteneder, H., Kraft, D. et al. (1998) Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. FASEB J. **12**, 231–242
24 Krebitz, M., Wiedermann, U., Essl, D., Steinkellner, H., Wagner, B., Turpen, T. H., Ebner, C., Scheiner, O. and Breiteneder, H. (2000) Rapid production of the major birch pollen allergen Bet v 1 in *Nicotiana benthamiana* plants and its immunological *in vitro* and *in vivo* characterization. FASEB J. **14**, 1279–1288
25 Mirza, O., Henriksen, A., Ipsen, H., Larsen, J. N., Wissenbach, M., Spangfort, M. D. and Gajhede, M. (2000) Dominant epitopes and allergic cross-reactivity: complex formation between a fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. J. Immunol. **165**, 331–338
26 Spangfort, M. D., Mirza, O., Ipsen, H., Joost van Neerven, R. J., Gajhede, M. and Larsen, J. N. (2003) Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. J. Immunol. **171**, 3084–3090
27 Mittag, D., Batori, V., Neudecker, P., Wiche, R., Friis, E. P., Ballmer-Weber, B. K., Vieths, S. and Roggen, E. L. (2006) A novel approach for investigation of specific and cross-reactive IgE epitopes on Bet v 1 and homologous food allergens in individual patients. Mol. Immunol. **43**, 268–278
28 Mogensen, J. E., Wimmer, R., Larsen, J. N., Spangfort, M. D. and Otzen, D. E. (2002) The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. J. Biol. Chem. **277**, 23684–23692
29 Markovic-Housley, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O. and Breiteneder, H. (2003) Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J. Mol. Biol. **325**, 123–133
30 Mogensen, J. E., Ferreras, M., Wimmer, R., Petersen, S. V., Enghild, J. J. and Otzen, D. E. (2007) The major allergen from birch tree pollen, Bet v 1, binds and permeabilizes membranes. Biochemistry **46**, 3356–3365
31 Koistinen, K. M., Kokko, H. I., Hassinen, V. H., Tervahauta, A. I., Auriola, S. and Kärenlampi, S. O. (2002) Stress-related RNase PR-10c is post-translationally modified by glutathione in birch. Plant Cell Environ. **25**, 707–715
32 Koistinen, K. M., Soininen, P., Venalainen, T. A., Hayrinen, J., Laatikainen, R., Perakyla, M., Tervahauta, A. I. and Karenlampi, S. O. (2005) Birch PR-10c interacts with several biologically important ligands. Phytochemistry **66**, 2524–2533
33 Kofler, S., Asam, C., Eckhard, U., Wallner, M., Ferreira, F. and Brandstetter, H. (2012) Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen Bet v 1. J. Mol. Biol. **422**, 109–123
34 Grote, M., Vrtala, S. and Valenta, R. (1993) Monitoring of two allergens, Bet v I and profilin, in dry and rehydrated birch pollen by immunogold electron microscopy and immunoblotting. J. Histochem. Cytochem. **41**, 745–750
35 Bollen, M. A., Garcia, A., Cordewener, J. H., Wichers, H. J., Helsper, J. P., Savelkoul, H. F. and van Boekel, M. A. (2007) Purification and characterization of natural bet v 1 from birch pollen and related allergens from carrot and celery. Mol. Nutr. Food Res. **51**, 1527–1536
36 Johnson, B. A. and Blevins, R. A. (1994) NMRview: a computer program for the visualization and analysis of NMR data. J. Biomol. NMR **4**, 603–614
37 Trott, O. and Olson, A. J. (2010) AutoDock vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. **31**, 455–461
38 Schüttelkopf, A. W. and van Aalten, D. M. (2004) PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. Acta Crystallogr., Sect. D: Biol. Crystallogr. **60**, 1355–1363
39 Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S. and Olson, A. J. (2009) AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J. Comput. Chem. **30**, 2785–2791
40 Bufe, A. (1998) The biological function of allergens: relevant for the induction of allergic diseases? Int. Arch. Allergy Immunol. **117**, 215–219
41 Harborne, J. B., Baxter, H. and Moss, G. P. (1999) Phytochemical Dictionary: a Handbook of Bioactive Compounds From Plants, CRC Press, London
42 Ross, S. A., ElSohly, M. A., Sultana, G. N., Mehmedic, Z., Hossain, C. F. and Chandra, S. (2005) Flavonoid glycosides and cannabinoids from the pollen of cannabis sativa L. Phytochem. Anal. **16**, 45–48
43 Arraez-Roman, D., Zurek, G., Bässmann, C., Almaraz-Abarca, N., Quirantes, R., Segura-Carretero, A. and Fernandez-Gutierrez, A. (2007) Identification of phenolic compounds from pollen extracts using capillary electrophoresis-electrospray time-of-flight mass spectrometry. Anal. Bioanal. Chem. **389**, 1909–1917
44 Falkovskaia, E., Sengupta, P. K. and Kasha, M. (1998) Photophysical induction of dual fluorescence of quercetin and related hydroxyflavones upon intermolecular H-bonding to solvent matrix. Chem. Phys. Lett. **297**, 109–114

45 Ghiasi, M., Taheri, S. and Tafazzoli, M. (2010) Dynamic stereochemistry of rutin (vitamin P) in solution: theoretical approaches and experimental validation. Carbohydr. Res. **345**, 1760–1766
46 Bundle, D. R. and Young, N. M. (1992) Carbohydrate–protein interactions in antibodies and lectins. Curr. Opin. Struct. Biol. **2**, 666–673
47 Toone, E. J. (1994) Structure and energetics of protein-carbohydrate complexes. Curr. Opin. Struct. Biol. **4**, 719–728
48 García-Hernández, E., Zubillaga, R. A., Rojo-Domínguez, A., Rodríguez-Romero, A. and Hernández-Arana, A. (1997) New insights into the molecular basis of lectin-carbohydrate interactions: a calorimetric and structural study of the association of hevein to oligomers of N-acetylglucosamine. Proteins: Struct., Funct., Bioinf. **29**, 467–477
49 García-Hernández, E. and Hernández-Arana, A. (1999) Structural bases of lectin-carbohydrate affinities: comparison with protein-folding energetics. Protein Sci. **8**, 1075–1086
50 Dam, T. K. and Brewer, C. F. (2002) Thermodynamic studies of lectin–carbohydrate interactions by isothermal titration calorimetry. Chem. Rev. **102**, 387–430
51 Dunitz, J. D. (1995) Win some, lose some: enthalpy–entropy compensation in weak intermolecular interactions. Chem. Biol. **2**, 709–712
52 Holgersson, J., Gustafsson, A. and Breimer, M. E. (2005) Characteristics of protein–carbohydrate interactions as a basis for developing novel carbohydrate-based antirejection therapies. Immunol. Cell Biol. **83**, 694–708
53 Wilhelm, D., Behnken, H. N. and Meyer, B. (2012) Glycosylation assists binding of HIV protein gp120 to human CD4 receptor. ChemBioChem **13**, 524–527
54 Robinson, B. W., Venaille, T. J., Mendis, A. H. and McAleer, R. (1990) Allergens as proteases: an *Aspergillus fumigatus* proteinase directly induces human epithelial cell detachment. J. Allergy Clin. Immunol. **86**, 726–731
55 Herbert, C. A., King, C. M., Ring, P. C., Holgate, S. T., Stewart, G. A., Thompson, P. J. and Robinson, C. (1995) Augmentation of permeability in the bronchial epithelium by the house dust mite allergen der p1. Am. J. Respir. Cell Mol. Biol. **12**, 369–378
56 Hassim, Z., Maronese, S. E. and Kumar, R. K. (1998) Injury to murine airway epithelial cells by pollen enzymes. Thorax **53**, 368–371
57 Miller, K. D., Guyon, V., Evans, J. N., Shuttleworth, W. A. and Taylor, L. P. (1999) Purification, cloning, and heterologous expression of a catalytically efficient flavonol 3-O-galactosyltransferase expressed in the male gametophyte of petunia hybrida. J. Biol. Chem. **274**, 34011–34019
58 Brunetti, C., Di Ferdinando, M., Fini, A., Pollastri, S. and Tattini, M. (2013) Flavonoids as antioxidants and developmental regulators: relative significance in plants and humans. Int. J. Mol. Sci. **14**, 3540–3555
59 Karioti, A., Kitsaki, C. K., Zygouraki, S., Ziobora, M., Djeddi, S., Skaltsa, H. and Liakopoulos, G. (2008) Occurrence of flavonoids in ophrys (orchidaceae) flower parts. Flora **203**, 602–609
60 Mo, Y., Nagel, C. and Taylor, L. P. (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. Proc. Natl. Acad. Sci. U.S.A. **89**, 7213–7217
61 Ceska, O. and Styles, E. D. (1984) Flavonoids from *Zea mays* pollen. Phytochemistry **23**, 1822–1823
62 Taylor, L. P. and Miller, K. D. (2002) The use of a photoactivatable kaempferol analogue to probe the role of flavonol 3-O-galactosyltransferase in pollen germination. Adv. Exp. Med. Biol. **505**, 41–50
63 Vogt, T. and Taylor, L. P. (1995) Flavonol 3-O-glycosyltransferases associated with petunia pollen produce gametophyte-specific flavonol diglycosides. Plant Physiol. **108**, 903–911
64 Mattila, K. and Renkonen, R. (2009) Modelling of Bet v 1 binding to lipids. Scand. J. Immunol. **70**, 116–124
65 Golebski, K., Röschmann, K. I., Toppila-Salmi, S., Hammad, H., Lambrecht, B. N., Renkonen, R., Fokkens, W. J. and van Drunen, C. M. (2013) The multi-faceted role of allergen exposure to the local airway mucosa. Allergy **68**, 152–160
66 Strack, D., Meurer, B., Wray, V., Grotjahn, L., Austenfeld, F. A. and Wiermann, R. (1984) Quercetin 3-glucosylgalactoside from pollen of *Corylus avellana*. Phytochemistry **23**, 2970–2971
67 Xu, P., Vogt, T. and Taylor, L. P. (1997) Uptake and metabolism of flavonols during *in vitro* germination of *Petunia hybrida* (L.) pollen. Planta **202**, 257–265
68 Zerback, R., Bokel, M., Geiger, H. and Hess, D. (1989) A kaempferol 3-glucosylgalactoside and further flavonoids from pollen of petunia hybrida. Phytochemistry **28**, 897–899
69 Taylor, L. P. and Hepler, P. K. (1997) Pollen germination and tube growth. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**, 461–491
70 Ryan, K. G., Markham, K. R., Bloor, S. J., Bradley, J. M., Mitchell, K. A. and Jordan, B. R. (1998) UVB radiation induced increase in quercetin: kaempferol ratio in wild-type and transgenic lines of *Petunia*. Photochem. Photobiol. **68**, 323–330

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online data

SUPPLEMENTARY ONLINE DATA

Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand

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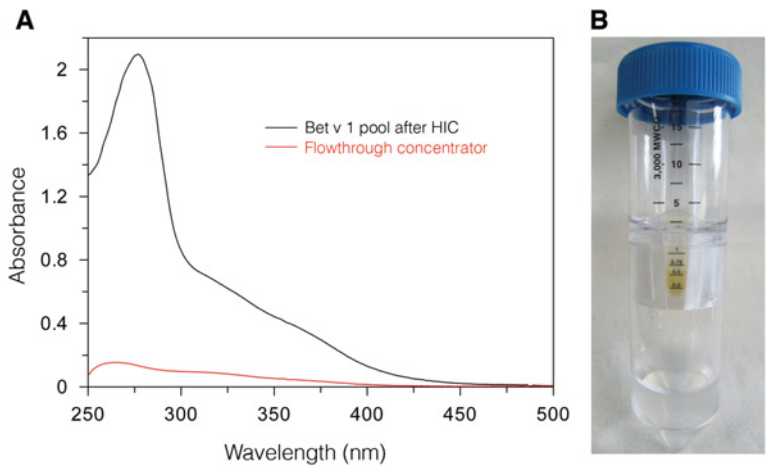

Figure S1 Concentration of isolated Bet v 1 fractions from mature birch pollen after hydrophobic interaction chromatography

(**A**) UV/VIS spectra of pooled Bet v 1 fractions after the hydrophobic interaction chromatography column, before concentration (black line) and the flow-through after concentration (red line). Conditions: 10 mM potassium phosphate buffer, pH 7.0, and ~1.5 M $(NH_4)_2SO_4$. Absorption spectra were recorded using an Agilent 8453 UV-visible spectrophotometer. (**B**) Picture of the concentrator (VIVASPIN 20 concentrator, molecular-mass cut-off 3000 Da, Sartorius Stedim Biotech) after concentration.

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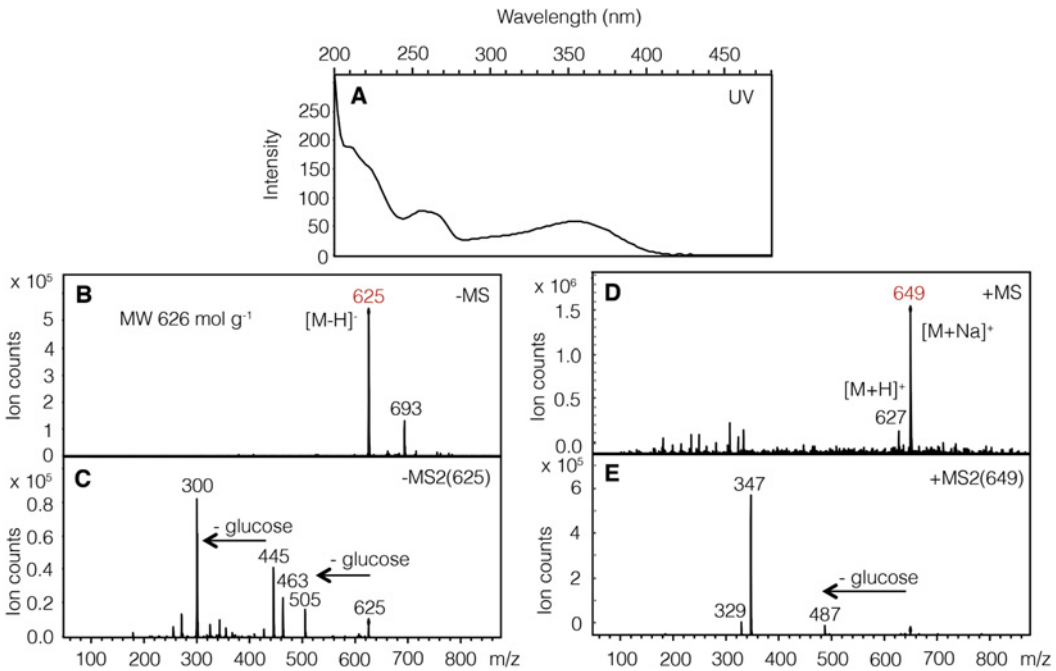


Figure S2 LC-DAD-MS analysis of Q3OS

UV maxima at 252 and 358 nm were determined (**A**). Pseudomolecular ions $[M-H]^-$ (**B**) and $[M+Na]^+$ (**D**) were observed in the negative and positive mode respectively. Product ion spectra of *m/z* 625 (**C**) and 649 (**E**) show the loss of two and one glucose residues respectively.

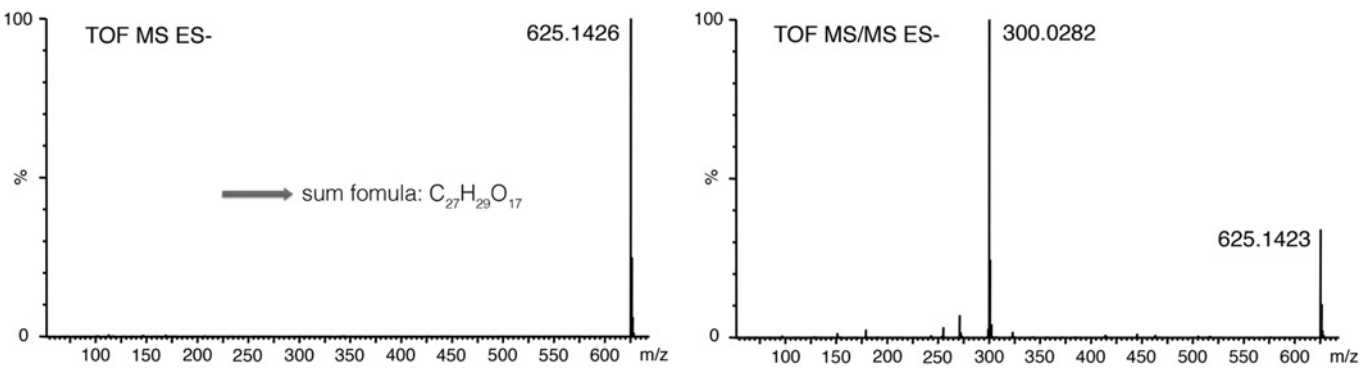


Figure S3 LC-TOF mass spectrum and MS^2 of Q3OS

LC-TOF mass spectrum (left-hand panel) and MS^2 (right-hand panel) of Q3OS. Spectra were acquired in the negative mode.

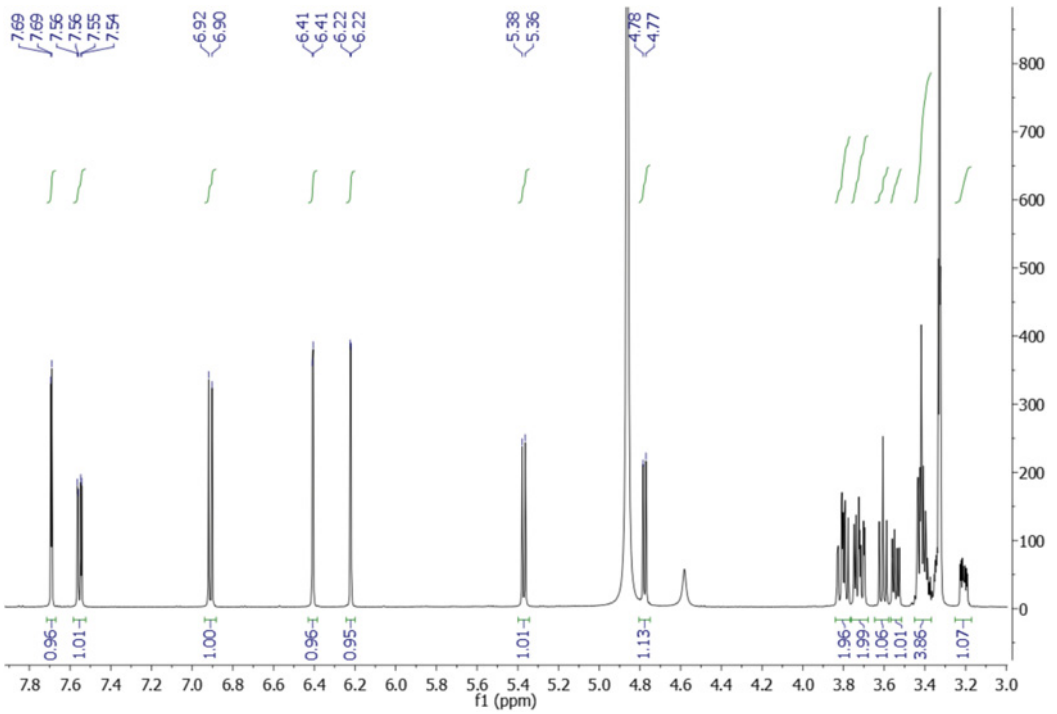


Figure S4 ^{1}H-NMR spectrum of Q3OS

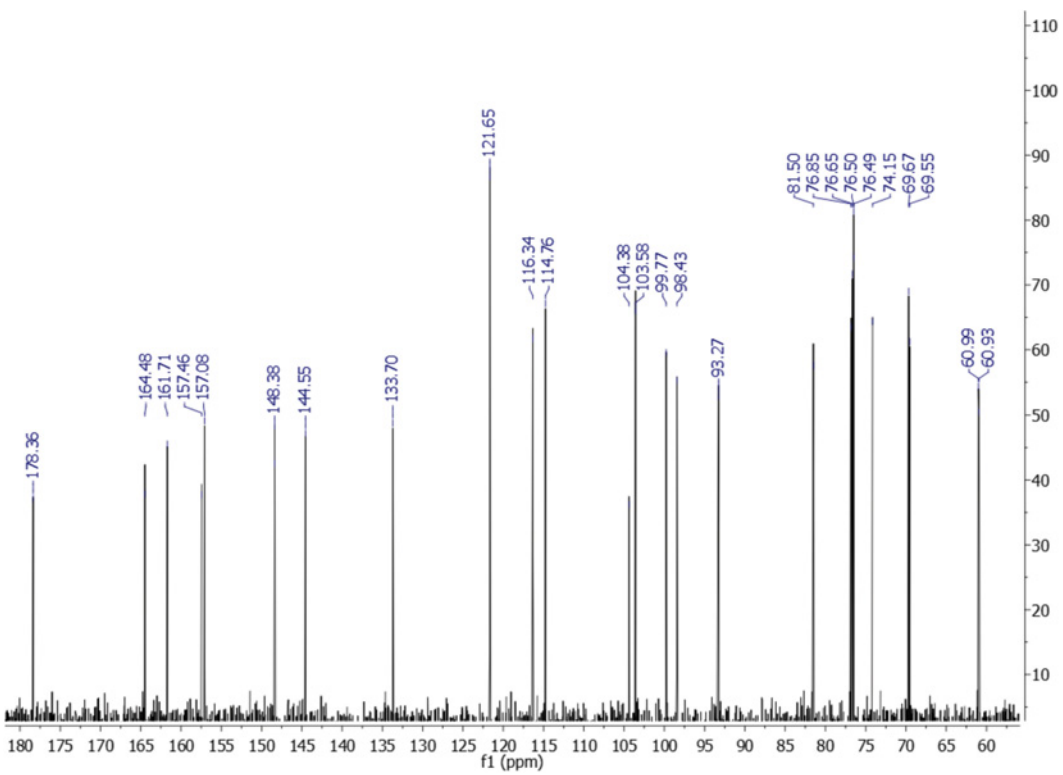


Figure S5 ^{13}C-NMR spectrum of Q3OS

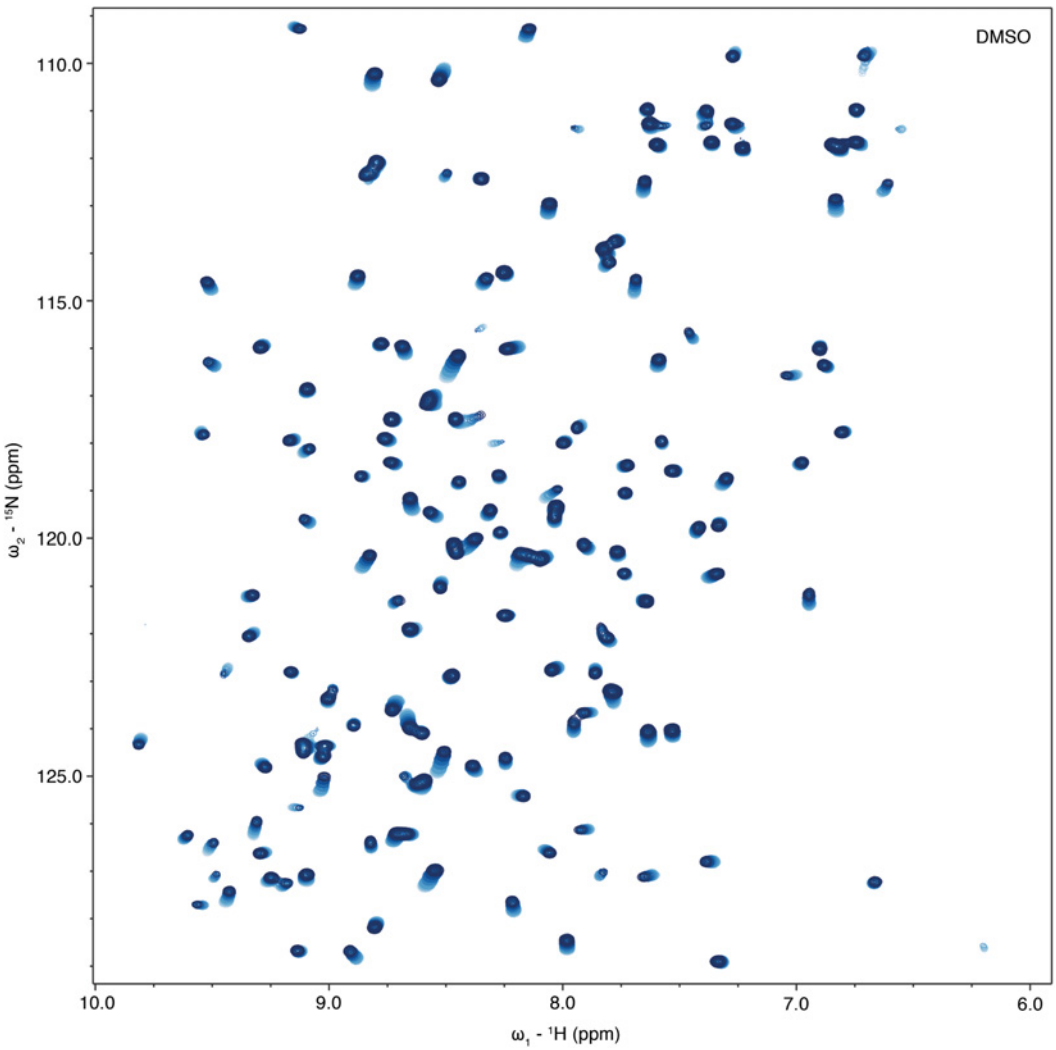


Figure S6 Overlay of eight ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled Bet v 1a at increasing DMSO concentrations shown from light to dark blue

Bet v 1a (100 μM) was stepwise titrated with deuterated DMSO to a final concentration of 8.3 %. The experiment was performed in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and 10 % $^{2}H_2O$ at 298 K.

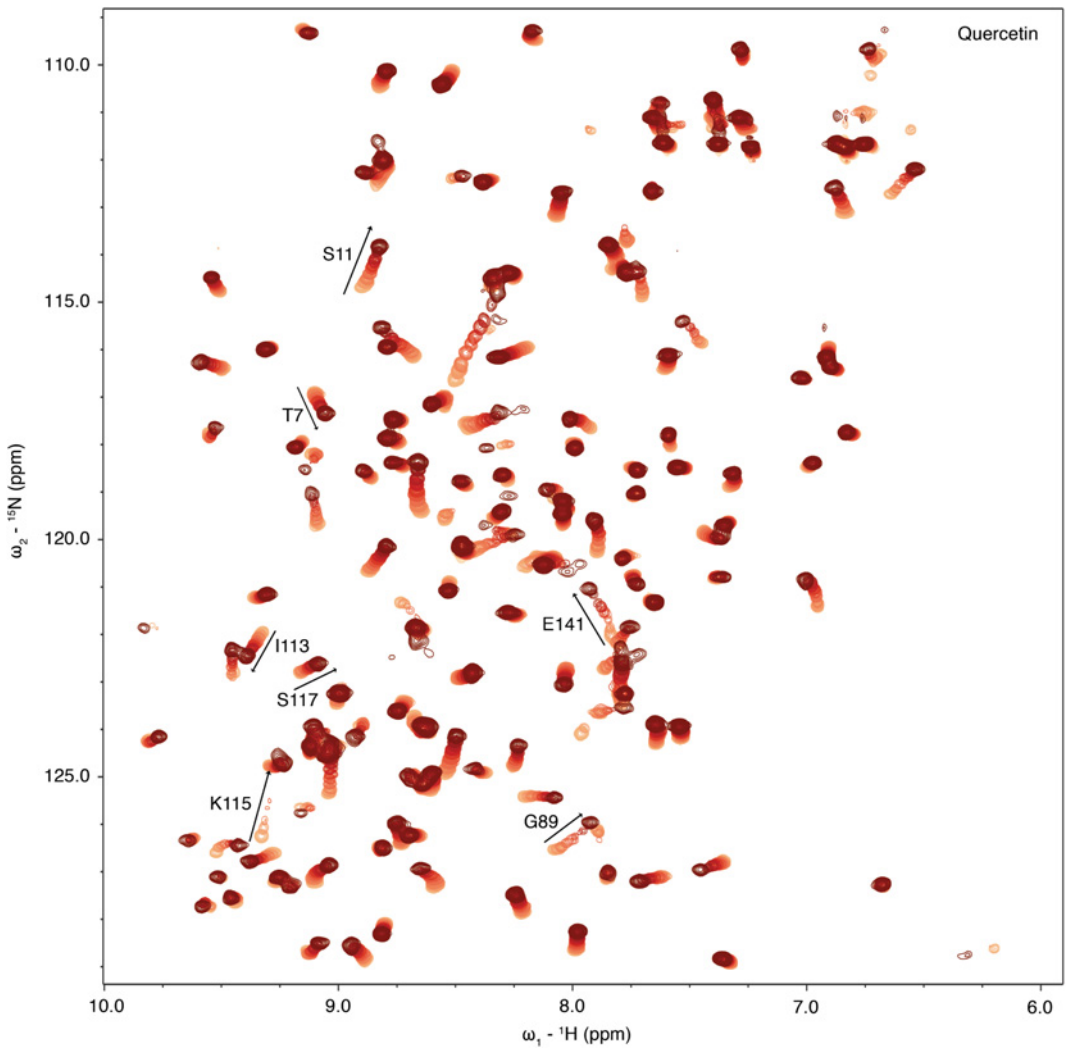


Figure S7 Overlay of ten ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled Bet v 1a at increasing quercetin concentrations shown from light to dark red

Bet v 1a (100 μM) was stepwise titrated with a quercetin stock solution prepared in deuterated DMSO to a final excess of 1:15 over Bet v 1a and final a DMSO concentration of 13.5 % (v/v). The arrows indicate the residues, which were not influenced by the increasing DMSO concentrations and thus were used for K_d determination. The experiment was performed in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and 10 % $^{2}H_2O$ at 298 K.

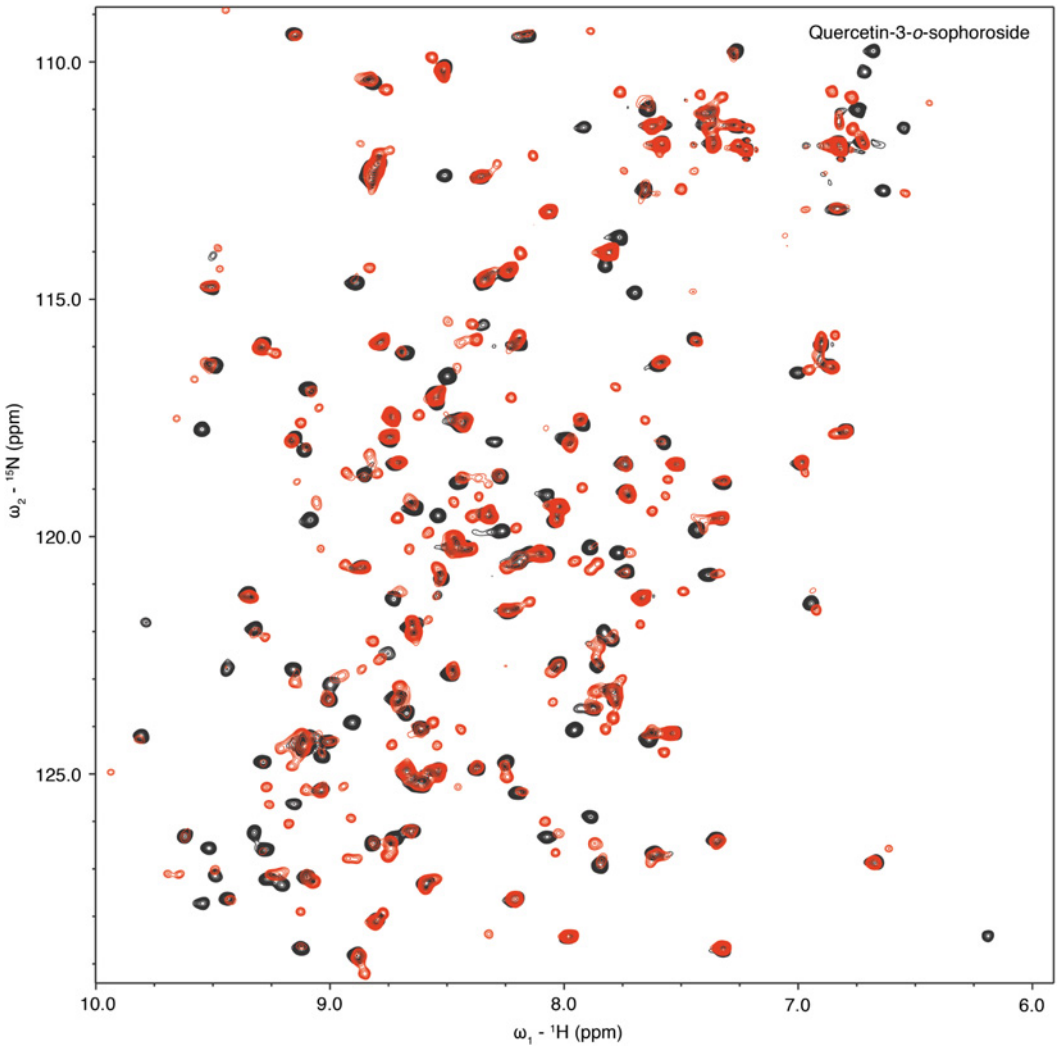


Figure S8 Overlay of two ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled Bet v 1a in the absence (black) and presence (red) of a 15-fold excess of Q3OS

The experiment was performed in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and 10 % $^{2}H_2O$ at 298 K.

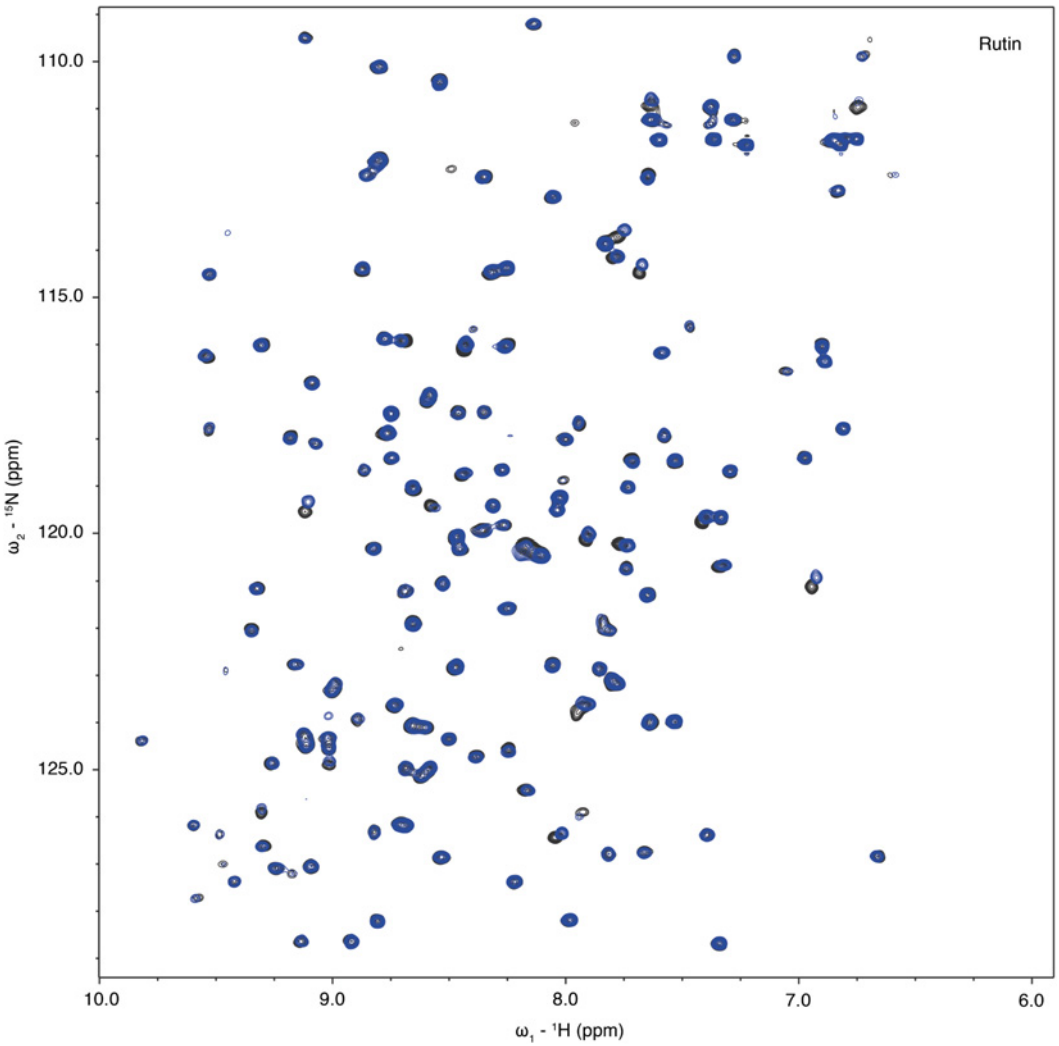


Figure S9 Overlay of two ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled Bet v 1a in the absence (black) and presence (blue) of a 15-fold excess of rutin

Rutin was added from a stock solution prepared in DMSO resulting in a final DMSO concentration of 10 % (v/v). The experiment was performed in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and 10 % $^{2}H_2O$ at 298 K.

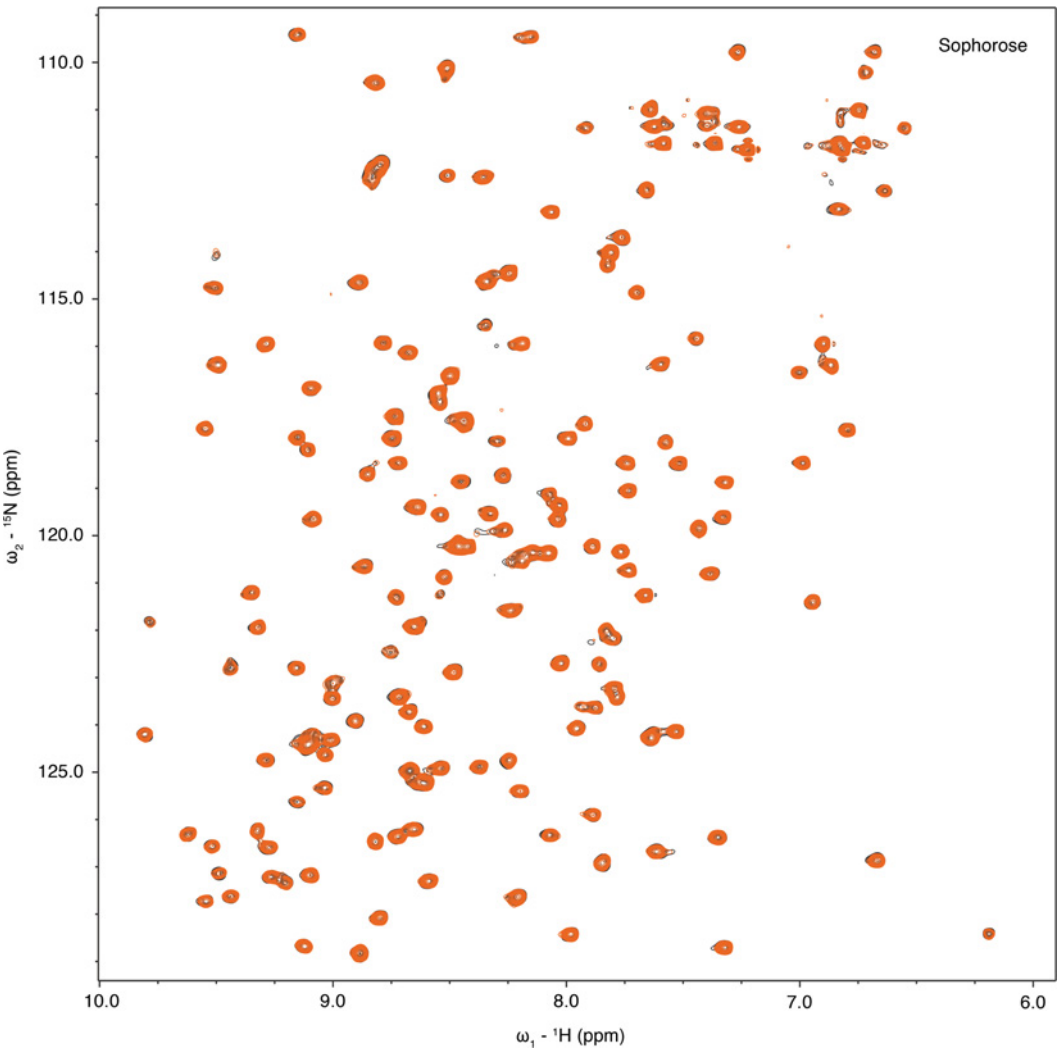


Figure S10 Overlay of two ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled Bet v 1a in the absence (black) and presence (orange) of a 15-fold excess of sophorose

The experiment was performed in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and 10 % $^{2}H_2O$ at 298 K.

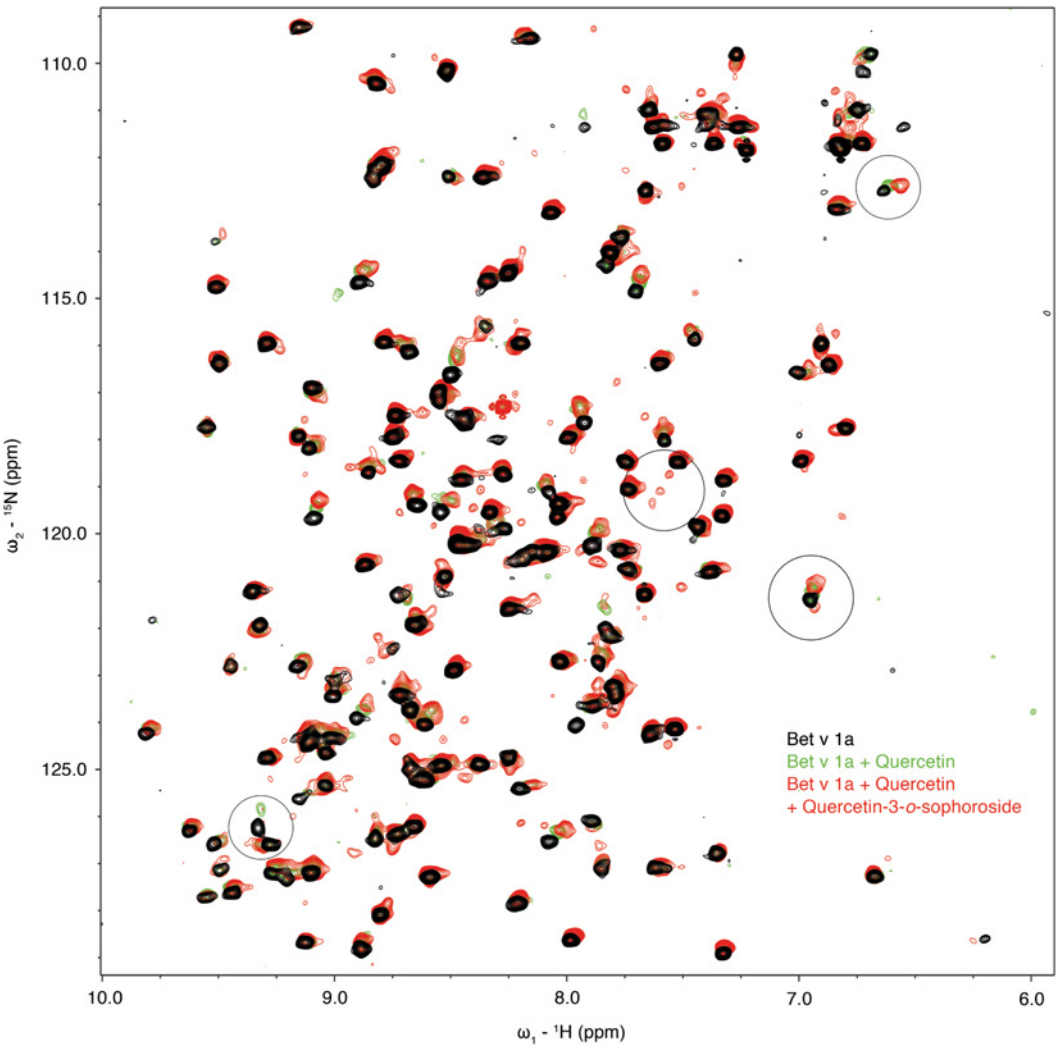


Figure S11 Displacement of quercetin bound to Bet v 1a by Q3OS

Overlay of three ^{1}H-^{15}N HSQC spectra of 100 μM ^{15}N-labelled Bet v 1a (black), 100 μM ^{15}N-labelled Bet v 1a + 25 μM quercetin (green) and 100 μM ^{15}N-labelled Bet v 1a + 25 μM quercetin + 1 mM Q3OS. The circles highlight regions in which the replacement can be seen clearly. The experiment was performed in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and 10 % $^{2}H_2O$ at 298 K.

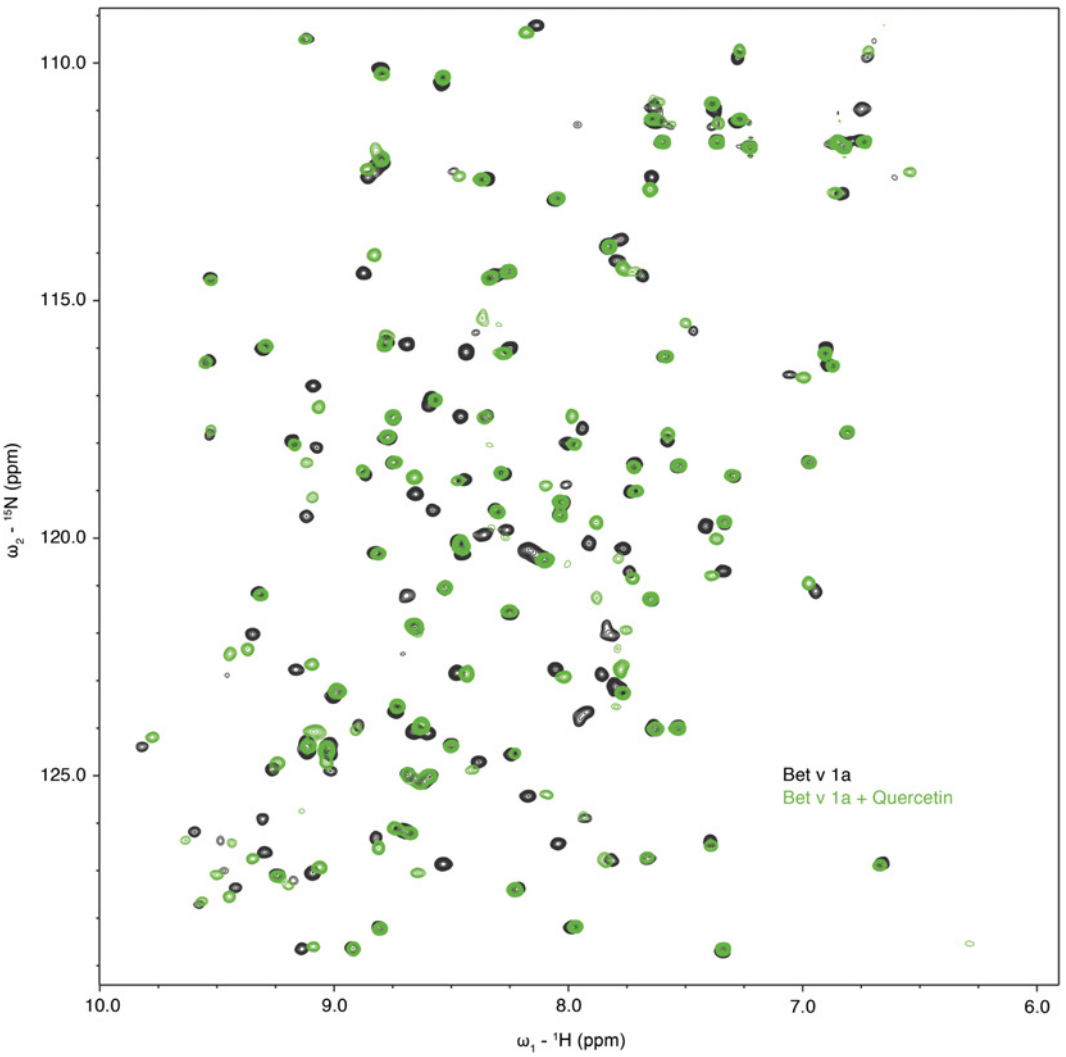


Figure S12 Overlay of two ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled Bet v 1a in 10 % (v/v) DMSO in the absence (black) and presence (green) of a 15-fold excess of quercetin

Quercetin was added from a stock solution prepared in DMSO resulting in an unchanged DMSO concentration of 10 % (v/v). The experiment was performed in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and 10 % $^{2}H_2O$ at 298 K.

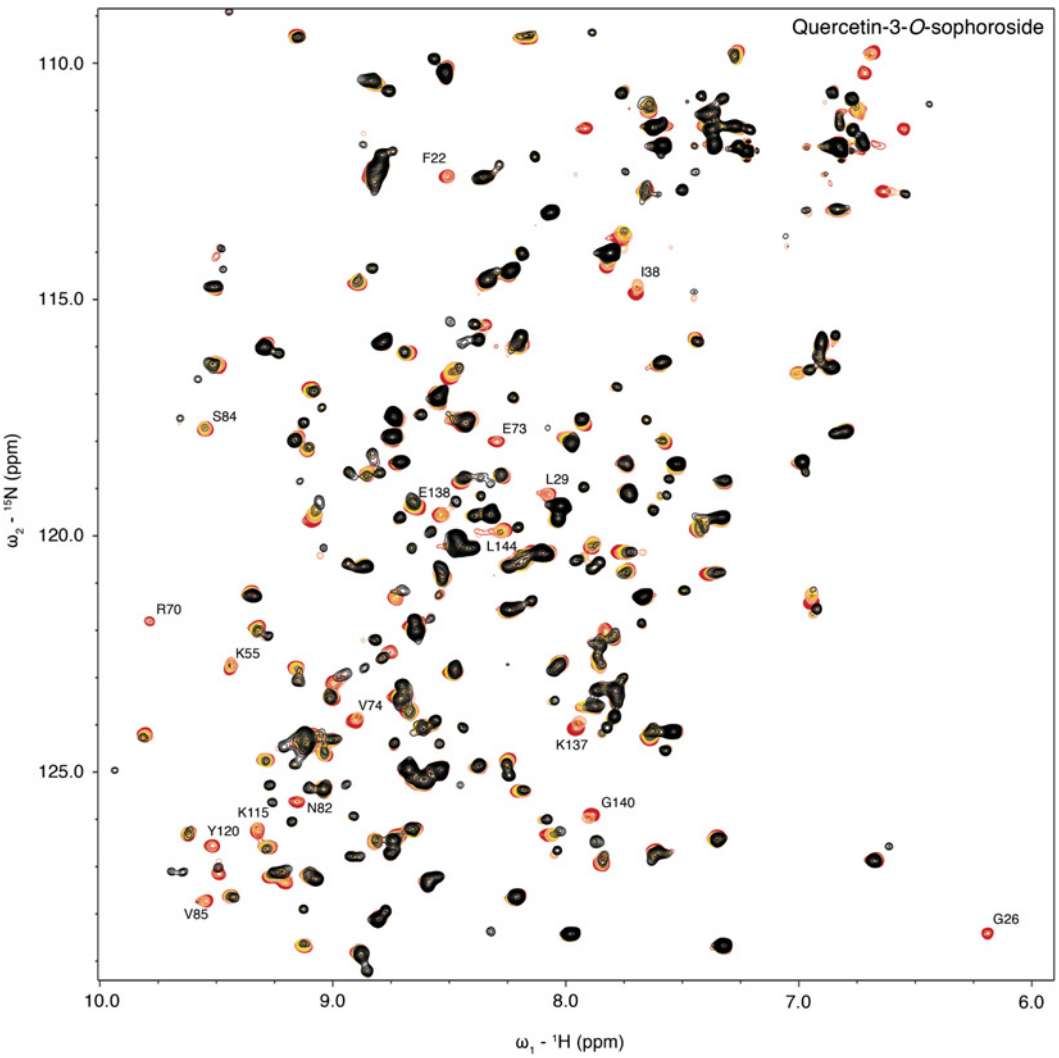
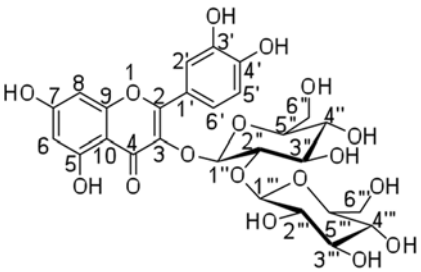


Figure S13 Effect of Q3OS on the ^{1}H-^{15}N chemical shifts of ^{15}N-labelled Bet v 1a (red)

The protein was added stepwise to 680 μM Q3OS in ratios (Q3OS/Bet v 1a) of 15:1 (black), 10:1 (grey), 5:1 (yellow) and 2.4:1 (light red). Labelled residues indicate those with disappearing signals. The experiment was performed in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and 10 % $^{2}H_2O$ at 298 K.

Table S1 ^{1}H- and ^{13}C-NMR data of Q3OS

Atom Reference	^{13}C [1]	^{1}H [1]	^{13}C [2]	^{1}H [2]	^{13}C [3]	^{13}C The present study	^{1}H The present study
							
2	156.7				154.98	157.5	
3	133.5				132.45	133.7	
4	177.9				176.90	178.4	
5	161.6				160.72	161.7	
6	98.5	6.17 d (2.4)	98.9	6.18 d (2.1)	97.98	98.4	6.22 d (2.1)
7	164.4				163.45	164.5	
8	93.8	6.39 d (1.8)	93.7	6.41 d (1.5)	92.87	93.3	6.41 d (2.1)
9	156.0				155.68	157.1	
10	104.5				103.39	104.4	
1′	122.2				120.58	121.7	
2′	116.5	7.56 d (2.3)	115.6	7.55 d (2.4)	115.51	116.4	7.69 d (2.2)
3′	145.1				144.27	144.6	
4′	148.8				147.95	148.4	
5′	115.8	6.87 d (7.8)	116.2	6.87 d (8.5)	115.27	114.8	6.91 d (8.4)
6′	121.6	7.45 m	122.0	7.60 dd (2.1;8.5)	121.30	121.7	7.55 dd (2.2;8.4)
Sugar at C^{3}							
1″	99.0	5.67 d (7.2)	98.2	5.69 d (7.0)	97.45	99.8	5.37 d (7.5)
2″	83.1	3.52 t (8.4)	82.9		82.20	81.5	
3″	76.9	3.49 t (8.4)	76.8		75.99	76.5	
4″	69.9	3.15 t (8.4)	69.7		69.39	69.7	
5″	77.8	3.08 m	77.7		76.98	76.9	
6″	61.0	3.28 m	60.8		60.08	61.0	
		3.29 dd (6.0; 12.0)					
Sugar at C$^{2''}$							
1‴	104.4	4.60 d (7.2)	104.4	4.60 d (7.9)	103.66	103.6	4.78 d (7.2)
2‴	74.6	3.17 t (8.4)	74.6		73.85	74.2	
3‴	76.8	3.20 t (8.4)	76.7		75.99	76.5	
4‴	69.8	3.10 t (8.4)	69.7		69.04	69.6	
5‴	77.1	3.14 m	77.0		75.99	76.7	
6‴	60.9	3.54 m	60.8		60.14	60.9	
		3.47 m					

REFERENCES

1 Nørbaek, R. and Kondo, T. (1999) Flavonol glycosides from flowers of *Crocus speciosus* and *C. antalyensis*. Phytochemistry **51**, 1113–1119

2 Gluchoff-Fiasson, K., Fiasson, J. L. and Waton, H. (1997) Quercetin glycosides from European aquatic *Ranunculus* species of subgenus *Batrachium*. Phytochemistry **45**, 1063–1067

3 Price, K. R., Casuscelli, F., Colquhoun, I. J. and Rhodes, M. J. C. (1998) Composition and content of flavonol glycosides in broccoli florets (*Brassica olearacea*) and their fate during cooking. J. Sci. Food Agric. **77**, 468–472

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7.4 Einzelarbeit D

Christian Seutter von Loetzen, Thessa Jacob, Olivia Hartl-Spiegelhauer, Lothar Vogel, Dirk Schiller, Cornelia Spörlein-Güttler, Rainer Schobert, Stefan Vieths, Maximilian Johannes Hartl* & Paul Rösch (2014) Isoforms of the Major Birch Pollen Allergen Bet v 1: Allergenicity and Physiological Function. *PLOS ONE, submitted*

Isoforms of the Major Birch Pollen Allergen Bet v 1: Allergenicity and Physiological Function

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Abstract

Each spring millions of patients suffer from allergies when birch pollen is released into the air. In most cases, the major pollen allergen Bet v 1 is the elicitor of the allergy symptoms. Bet v 1 comes in a variety of isoforms that share virtually identical conformations, but their relative concentrations are plant-specific. Glycosylated flavonoids, such as quercetin-3-O-sophoroside, are the physiological ligands of Bet v 1, and here we found that different isoforms show an individual, highly specific binding behaviour for the different ligands. This specificity is driven by the sugar moieties of the ligands rather than the flavonols. While the influence of the ligands on the allergenicity of the Bet v 1 isoforms may be limited, the isoform and ligand mixtures add up to a complex and thus individual fingerprint of the pollen. We suggest that this mixture is not only acting as an effective chemical sunscreen for pollen DNA, but may also play an important role in recognition processes during pollination.

Introduction

Allergies are a major health problem worldwide. In particular, type I or immediate type allergies (Gell & Coombs, 1963) that involve proteins as causative agents are very widespread and potentially severe. The major birch pollen allergen Bet v 1 from the European white birch (*Betula verrucosa*) alone (Ipsen & Lowenstein, 1983) affects an estimated 100 million people (Vrtala *et al.*, 2001). Although birch pollen contain a variety of allergens from different protein families, more than 60 % of all birch pollen-allergic patients react exclusively to Bet v 1 (Jarolim *et al.*, 1989). Up to 90 % of the Bet v 1-sensitized patients also exhibit IgE-mediated allergic cross-reactions (oral allergy syndrome) to Bet v 1-homologous food allergens, with fruits, vegetables, and nuts as the most important elicitors of the allergy (Dreborg, 1988; Geroldinger-Simic *et al.*, 2011).

On the basis of sequence similarities and the protein three-dimensional structures, Bet v 1 and related pollen and food allergens belong to the family of class 10 pathogenesis-related proteins (PR-10) within the Bet v 1 superfamily. It was suggested that proteins in this family are involved in plant defence mechanisms, since expression of the respective genes is induced upon attacks of pathogens and by environmental stress (Liu & Ekramoddoullah, 2006). However, the physiological roles of PR-10 proteins seem to extend beyond stress and pathogen response. Thus, the PR-10 strawberry allergen Fra a 1 is involved in controlling flavonoid biosynthesis and this protein is capable of binding different metabolic intermediates (Casañal *et al.*, 2013). In general, PR-10 proteins often co-occur with flavonoids *in vivo* (Bollen *et al.*, 2007; Breiteneder *et al.*, 1993; Grote *et al.*, 1993; Hostetler *et al.*, 2013; Leja *et al.*, 2013; Meurer *et al.*, 1988; Strack *et al.*, 1984) and interact with flavonoids *in vitro* (Casañal *et al.*, 2013; Koistinen *et al.*, 2005), as clearly evidenced, for example, for Bet v 1 (Mogensen *et al.*, 2002; Seutter von Loetzen *et al.*, 2014). Why many, if not all, PR-10 proteins appear as mixtures of isoforms, however, remains elusive (Agarwal & Agarwal, 2014; Hoffmann-Sommergruber, 2000; Lebel *et al.*, 2010).

The first Bet v 1 isoform described on the DNA level was Bet v 1a (Breiteneder *et al.*, 1989) followed by the identification of numerous other isoform sequences (Schenk *et al.*, 2006; Swoboda *et al.*, 1995a; Swoboda *et al.*, 1995b). At least 18 Bet v 1 variants found in pollen on the mRNA or protein level (Erler *et al.*, 2011; Schenk *et al.*, 2009; Swoboda *et al.*, 1995a) are officially listed as isoallergens (http//www.allergen.org). Studies on the proteomic profile of birch pollen extracts of different origin or species revealed significant differences of isoform composition and quantity (Erler *et al.*, 2011; Schenk *et al.*, 2009). For example, Bet v 1 constitutes up to 30 % of the total protein content in Swedish pollen and 12 % in Austrian pollen. In all cases so far, the most abundant isoform is Bet v 1a (50 % to 70 %), followed by Bet v 1d (20 %), Bet v 1b (3 % to 20 %), Bet v 1f (2 % to 8 %), and Bet v 1j (~1 %) (Erler *et al.*, 2011).

Bet v 1a is well characterized by biochemical (Ferreira *et al.*, 1993; Ipsen & Lowenstein, 1983; Mogensen *et al.*, 2002) and structural (Faber *et al.*, 1996; Gajhede *et al.*, 1996; Kofler *et al.*, 2012) studies. The large hydrophobic pocket formed by the secondary structure elements of Bet v 1

suggested that this allergen acts as storage or carrier protein (Fernandes *et al.*, 2013; Gajhede *et al.*, 1996; Radauer *et al.*, 2008). Previous research work focused on trial-and-error approaches or docking simulations to test various ligands for binding to recombinant Bet v 1 (Kofler *et al.*, 2012; Mogensen *et al.*, 2002; Roth-Walter *et al.*, 2014). We recently purified Bet v 1 in complex with its natural ligand quercetin-3-O-sophoroside (Q3OS) directly from mature birch pollen and confirmed binding by reconstitution of the Bet v 1a:Q3OS complex from its recombinant protein and synthetic ligand component (Seutter von Loetzen *et al.*, 2014). We hypothesized that this complex may be involved in UV-protection of the pollen DNA and that Q3OS may stimulate pollen tube formation upon rehydration of the pollen. We then asked why different isoforms exist and whether there are physiological ligands other than Q3OS. Although it is tempting to believe on the basis of the high sequence identities of 87.4 % - 99.4 % to Bet v 1a that all isoforms specifically interact with Q3OS, Bet v 1 isoforms are strikingly different in their immunological and allergenic properties (Ferreira *et al.*, 1996) and, although allergenicity is mainly correlated with binding epitopes at the surface of allergens (Ferreira *et al.*, 1998) it has always been speculated that Bet v 1 proteins as such are only part of the story, and that IgE binding needs to be tested in complex with their natural binding partners to arrive at meaningful results (Kofler *et al.*, 2012).

In order to characterize serological IgE binding as a measure for allergenicity as well as the physiological function of Bet v 1, we thoroughly studied ligand- and antibody-binding behaviour of the Bet v 1 isoforms a, d, and m. While none of the ligands significantly alters the allergenicity of Bet v 1, surprisingly, ligand binding to the different isoforms is diverse and highly dependent on the composition of the ligands' sugar moieties.

Results and Discussion

Bet v 1:Q3OS interaction is isoform-dependent

We were asking whether isoforms a, d, and m form identical complexes with the Bet v 1a natural ligand Q3OS (Seutter von Loetzen *et al.*, 2014). In an initial experiment we noticed that Q3OS exhibits slightly different shades of yellow when incubated with these Bet v 1 isoforms. After incubation we removed excess Q3OS with a G25 column and recorded UV/VIS absorption spectra of the protein fractions (Figure 1A). In the presence of Bet v 1a, the UV/VIS spectrum of Q3OS shows a clear shoulder around 360 nm, while this is not the case for Bet v 1 isoforms d or m. These absorbance differences suggest that the Bet v 1d:Q3OS and Bet v 1m:Q3OS complexes are different from the Bet v 1a:Q3OS complex.

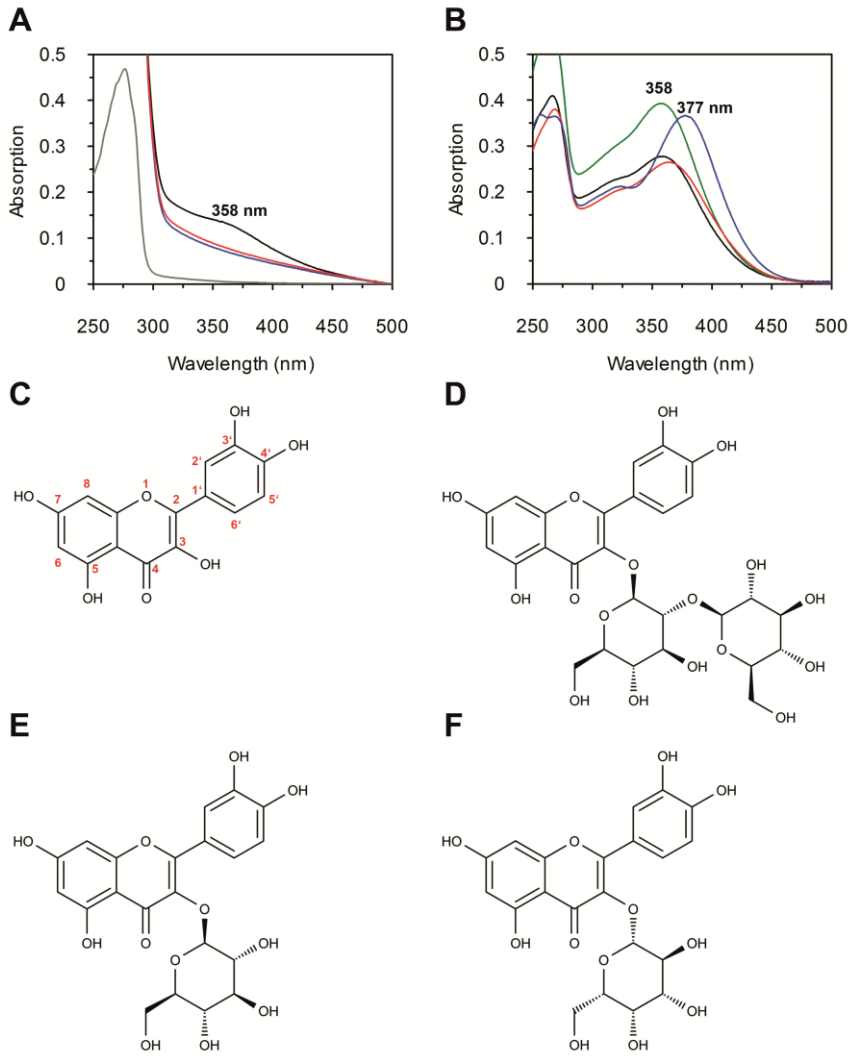

Figure 1: UV/VIS spectroscopy and structures of flavonoids.
All spectra were recorded at 298 K with 50 mM sodium phosphate, 50 mM NaCl at pH 7.0 and 10 % DMSO as sample buffer. (**A**) Binding of Q3OS to Bet v 1 isoforms. UV/VIS spectra of 20 μM Bet v 1a (-) and Q3OS incubated with Bet v 1a (-), Bet v 1d (-) and Bet v 1m (-) concentrated and subsequently eluted from a G25 column. (**B**) UV/VIS spectra of 20 μM Q3OS (-), quercetin (-), Q3OGlc (-) and Q3OGal (-) reveal differences in absorption maxima and intensities. (**C**) Chemical structures of quercetin, (**D**) Q3OS, (**E**) Q3OGlc and (**F**) Q3OGal.

Binding of unglycosylated flavonoids to Bet v 1 isoforms

Since the determination of the three-dimensional structure of Bet v 1a in 1996 (Gajhede *et al.*, 1996) it has been suggested that the protein functions as a carrier or storage protein. The existence of various highly similar, structurally almost identical isoforms could be evidence for a complex network of different acceptors, targeted to bind chemically similar ligands. Hitherto, there is only limited comparable information available about differences in ligand binding behaviour between Bet v 1 isoforms. Recent approaches used indirect methods (ANS replacement assay, Mogensen *et al.*, 2002) or analysed ligand binding in protein crystals (Kofler *et al.*, 2012; Markovic-Housley *et al.*, 2003). We now used UV/VIS and NMR spectroscopy to systematically analyze and compare binding of physiologically relevant ligands to different Bet v 1 isoforms in solution, with a focus on flavonoids.

A set of seven different flavonoids was used to analyze the influence of number and position of hydroxyl groups of the flavonoid moiety during binding to Bet v 1 isoforms (Figure S1). UV/VIS and chemical shift perturbation (CSP) measurements with ^{1}H-^{15}N HSQC NMR spectroscopy were performed to study affinities and binding sites of various flavonoids. The UV/VIS spectra from the titration experiment of naringenin and Bet v 1a show isosbestic points indicating a two-state binding process with a K_d of roughly 60 μM (Figure 2A, B). In the ^{1}H-^{15}N HSQC spectra of ^{15}N-Bet v 1a with increasing concentration of naringenin, the G^{140} resonance was in the intermediate exchange regime, but gradual CSPs were observed for the majority of affected resonances (Figure 2C), from which a K_d value of approximately 30 μM could be estimated (Figure 2D, E). The CSP mapping on the Bet v 1a:naringenin structure (pdb code 4A87, Kofler *et al.*, 2012) agreed well with the results from X-ray crystallography (Figure 2F).

The K_d values of all tested flavonoids were in the micromolar range (Table I). We observed shifts of the UV/VIS absorption maxima upon Bet v 1 addition for all flavonoids and isoforms (Table SI), with the highest K_d generally for the non-hydroxylated flavone. The significant CSPs obtained during titration were generally spread over the sequence of each isoform, making it difficult to predict a precise binding site for flavone. Due to hydrophobic interactions, flavone seems to bind more flexibly and somewhat more weakly inside the hydrophobic pocket. While the position of hydroxylation seems insignificant, additional hydroxyl groups lead to a decrease of K_d. The interaction surfaces of all flavonoids are located inside the hydrophobic pocket of Bet v 1 and vary only slightly (affected amino acid positions T^{7}, E^{8}, T^{9}, T^{10}, S^{11}, I^{23} to N^{28}, F^{64}, G^{89} to I^{91}, K^{115} to N^{118} corresponding to Bet v 1a). The most significant CSPs are found in the region of the C-terminal helix between residues K^{137} and R^{145}. Most likely, flavonoids enter the hydrophobic pocket via one of the two gaps formed by the mostly nonpolar residues F^{62}, P^{63}, F^{64}, P^{90}, Q^{132}, A^{135}, S^{136}, and M^{139} (entrance 1) or by residues I^{23}, L^{24}, D^{25}, D^{27}, T^{52}, K^{54}, Y^{81}, and I^{102}. The third gap, Y^{5}, T^{7}, V^{133}, and K^{137}, with a diameter of ~6 Å, is probably too small for flavonoids to enter the cavity (Kofler *et al.*, 2012). The Bet v 1 cavity has a volume of approximately 1500 Å^3 (Gajhede *et al.*, 1996; Seutter von Loetzen *et al.*, 2012) and can accommodate

two ligands simultaneously (Kofler *et al.*, 2012; Markovic-Housley *et al.*, 2003; Neudecker *et al.*, 2001).

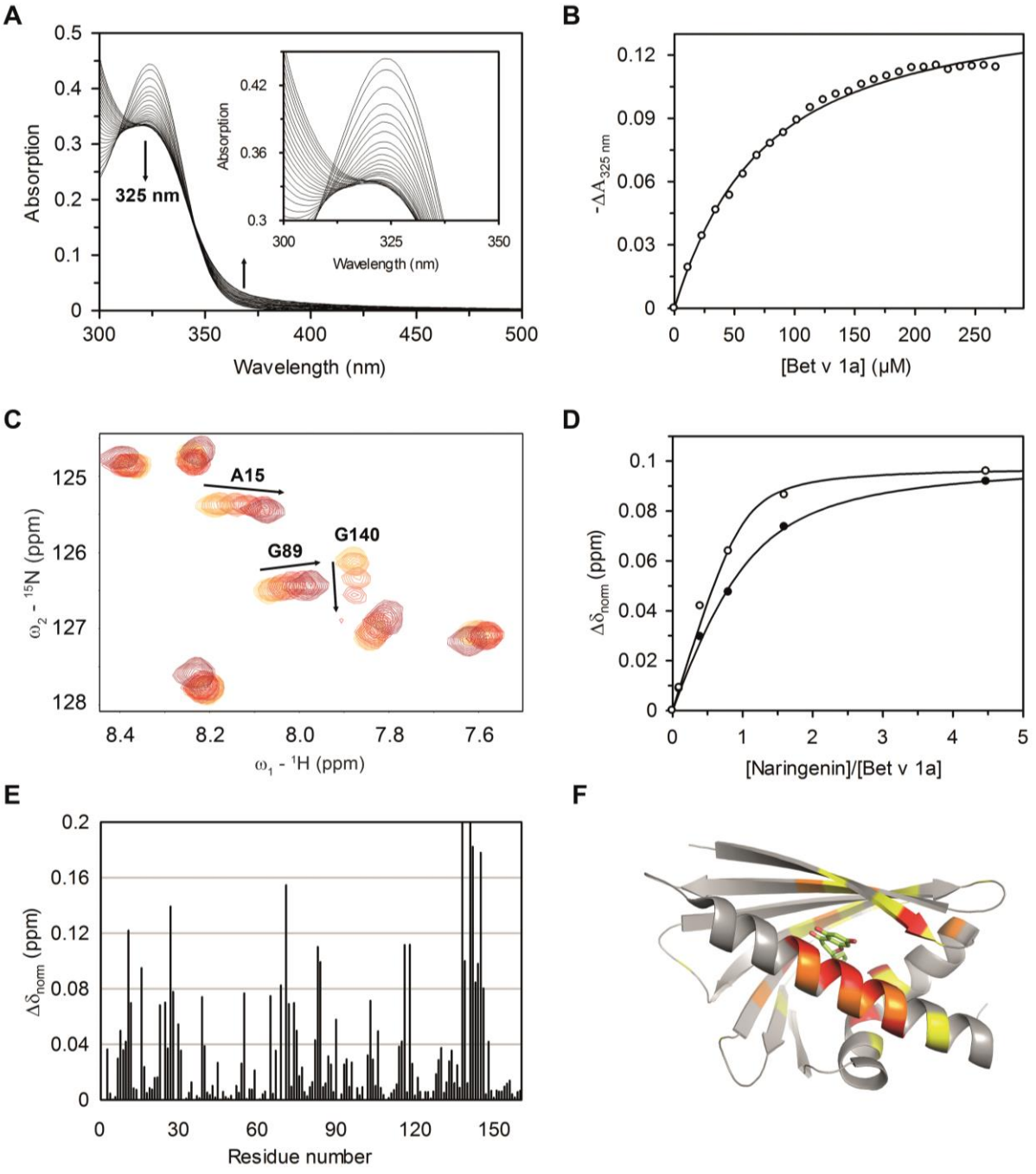

Figure 2: Binding interactions of naringenin with Bet v 1a.
(**A**) UV/VIS spectra of the equilibrium titration of 20 μM naringenin with Bet v 1a. All spectra were recorded at 298 K with 50 mM sodium phosphate, 50 mM NaCl at pH 7.0 and 10% DMSO as sample buffer. (**B**) Absorbance changes at 325 nm plotted against the Bet v 1a concentration as shown for the data in (**A**). The curve represents the best fit to equation (1) resulting in a K_d value of 60.6 ± 3.2 μM. (**C**) Overlay of six ^{1}H-^{15}N HSQC spectra of 100 μM Bet v 1a in the presence of increasing naringenin concentrations from light to dark red. The experiments were performed with a Bruker Avance 700 MHz spectrometer in 50 M sodium phosphate, 50 mM NaCl, pH 7.0 and 10% $^{2}H_2O$ at 298 K. Naringenin was added from a stock prepared in deuterated DMSO to a final excess of 1:4.5 over Bet v 1a and a final DMSO concentration of 10%. (**D**) Chemical shift changes ($\Delta\delta_{norm}$) calculated with equation (2) for residues A^{15} (○) and G^{89} (●) plotted against the ration of naringenin:Bet v 1a during titration. The curves represent the best fit to a quadric binding equation from the analysis software of NMRViewJ (Johnson & Blevins, 1994) resulting in K_d values of 7.4 ± 4.0 μM for A^{15} and 32.5 ± 4.2 μM for G^{89}. (**E**) Calculated $\Delta\delta_{norm}$ values upon naringenin addition plotted against the Bet v 1a amino acid sequence and (**F**) mapped on a cartoon representation of the complex structure of Bet v 1a:naringenin (pdb code: 4A87) with 0.04 ppm ≤ Δδ ≤ 0.08 ppm shown as yellow; 0.08 ppm ≤ Δδ ≤ 0.12 ppm shown as orange; and 0.12 ppm < Δδ shown as red. Bet v 1a in grey, naringenin in green sticks, oxygen in red.

Table I: Dissociation constants for Bet v 1 isoform interaction with flavonoids and sugars.
K_d values from UV/VIS titration experiments were determined by non-linear regression analysis. The error represents the standard error of the best fit according to equation 1. The dissociation constants determined with NMR spectroscopy represent an averaged $K_{d\,app}$ value of all analysable residues showing CSPs > 0.08 ppm with the corresponding standard deviation. K_d ranges from docking simulation were obtained from binding energies for ligands docked inside the hydrophobic pocket of Bet v 1a and Bet v 1m.

		Dissociation constant K_d (μM)		
Flavonoid	**Method**	**Bet v 1a**	**Bet v 1m**	**Bet v 1d**
Flavone	UV/VIS	n.a.	n.a.	-
	NMR	67.1±12.1	213.3±36.6	69.9±14.8
Naringenin	UV/VIS	60.6±3.2	28.1±0.8	37.7±6.4
	NMR	30.0±7.0	22.1±5.5	-
Apigenin	UV/VIS	19.5±0.6	5.4±0.5	4.3±0.2
	NMR	26.2±4.5	-	-
Fisetin	UV/VIS	14.9±1.2	n.a.	13.9±2.1
	NMR	37.2±6.5	85.1±21.7	-
Kaempferol	UV/VIS	n.a.	n.a.	n.a.
	NMR	60.7±14.6	62.8±9.8	-
Quercetin	UV/VIS	9.2±0.6	26.5±1.5	10.2±1.0
	NMR	31.4±10.3	65.8±8.2	<10
Myricetin	UV/VIS	4.2±0.7	n.a.	1.2±0.2
	NMR	14.6±6.5	99.3±19.4	-
Q3OGlc	UV/VIS	n.a.	n.a.	-
	NMR	288.4±24.0	<1	No binding
	Docking	-	0.2-6.1	-
Q3OGal	UV/VIS	n.a.	n.a.	-
	NMR	<1	<1	No binding
	Docking	3.2-14.8	0.4-10.4	-
Q3OS	UV/VIS	n.a.	n.a.	-
	NMR	<1	No binding	No binding
	Docking	0.1-1.7	-	-

n.a. : not analysable; - : not measured

Obviously, the hydrophobic cavity of Bet v 1 is a promiscuous acceptor of small hydrophobic and amphiphilic molecules *in vitro*. The low binding affinities, however, are not necessarily indicative of a major physiological importance of these complexes.

Binding of glycosylated flavonoids is governed by the sugar moiety

As no specific Bet v 1:flavonoid binding pattern could be derived, we focused on the sugar moiety of the quercetin glycosides quercetin-3-O-sophoroside (Q3OS, Figure 1D), quercetin-3-O-glucoside (Q3OGlc, Figure 1E), and quercetin-3-O-galactoside (Q3OGal, Figure 1F) as binding partners of Bet v 1 isoforms. UV/VIS absorption spectra show maxima of different intensity at physiological pH for Q3OGlc at 364 nm, and for Q3OGal and Q3OS at 358 nm (Figure 1B), but the spectral changes on

Bet v 1 binding were too small to be analysed with confidence. Thus we resorted to ^{1}H-^{15}N HSQC spectroscopy for further studies.

Titration of Bet v 1a with Q3OS resulted in a change of resonance positions on the intermediate to slow exchange limit on the NMR time-scale, with a resulting K_d of 566 ± 85 nM (Seutter von Loetzen *et al.*, 2014; Figure 3A, B). Surprisingly, titration with Q3OS did not lead to significant CSPs for Bet v 1d or m, even at 15-fold ligand excess (Figure 3E, F, I, J), suggesting that Bet v 1a:Q3OS formation is highly specific.

Although Q3OGlc is rather similar to Q3OS and simply shortened by a single glucose moiety, the Bet v 1a:Q3OGlc K_d of 288 μM is three orders of magnitude higher than that of Bet v 1a:Q3OS (Figures 3C, S2A). In contrast, Bet v 1a:Q3OGal has a K_d < 1 μM, with resonances of 15 residues in intermediate exchange and CSPs > 0.04 ppm for resonances of residues F^{22}, I^{23}, G^{26}, K^{54}, F^{64}, R^{70}, E^{73}, D^{93}, K^{115}, S^{136} to E^{141}, and L^{144} (Figures 3D, S2B). According to docking simulations, Q3OGal binds in the hydrophobic pocket of Bet v 1a, with the sugar moiety either completely inside or at the opening of the pocket (entry ε1, Kofler *et al.*, 2012) at the flexible loop connecting β7 with α3. Affinity scores of the models resulted in K_d values from 3.2 μM to 14.8 μM (Table I). Obviously, stereochemical changes in the sugar moiety of flavonol glycosides can strongly influence the affinity to Bet v 1a.

In contrast to Bet v 1a, Bet v 1m binds to Q3OGlc with K_d < 1 μM, with resonances of 20 residues in intermediate exchange and CSPs > 0.04 ppm for resonances of residues I^{23}, G^{26}, I^{38}, T^{57},F^{64}, Y^{66}, G^{89} to G^{92}, I^{98}, I^{136} to L^{144} (Figures 3K, S2E). The docking simulation suggested Q3OGlc to bind in the hydrophobic pocket of Bet v 1m with K_d values of 0.4 μM to 10.4 μM. Bet v 1m also shows high affinity for Q3OGal (K_d < 1 μM, 22 intermediate exchanging residues) with the most significant CSPs on residues I^{38}, S^{39}, T^{57}, F^{64}, Y^{66}, M^{85} to E^{87}, G^{89} to G^{92}, E^{96}, K^{134}, I^{136} to E^{141}, L^{143}, L^{144} (Figure 3L, S2F) and K_d values from 0.2 μM to 6.1 μM (Table I).

Although Bet v 1d generally binds flavonoids (Table I), it shows only very weak affinity for the glycosylated flavonoids that we have analysed here. As with Q3OS, a 15-fold excess of Q3OGlc or Q3OGal (Figures 3G, 3H, S2C, S2D) did not produce significant CSPs for Bet v 1d.

Bet v 1d shows seven amino acid variations (T7I, F30V, S57N, I91V, S112C, I113V, and D125N) compared to Bet v 1a. Thus, strong specific binding and virtual lack of such is achieved by variation of just seven or even fewer amino acids. None of those seven variable residues, however, is directly involved in Q3OS or Q3OGal binding in Bet v 1a or is part of the amino acids which form the potential entrances. T^{7} is part of the third opening in Bet v 1a, which is presumably too small for glycosylated flavonoids. The loss of affinity might be explained by a slightly different structural arrangement of Bet v 1d, which could result in variations in the openings to the hydrophobic pocket. In contrast to Bet v 1d, Bet v 1m shows four variations in entrance 1 (F62S, P90A, Q132H, and S136I compared to Bet v 1a) which are likely to directly block the access route for Q3OS, but not for Q3OGlc and Q3OGal, into the hydrophobic pocket.

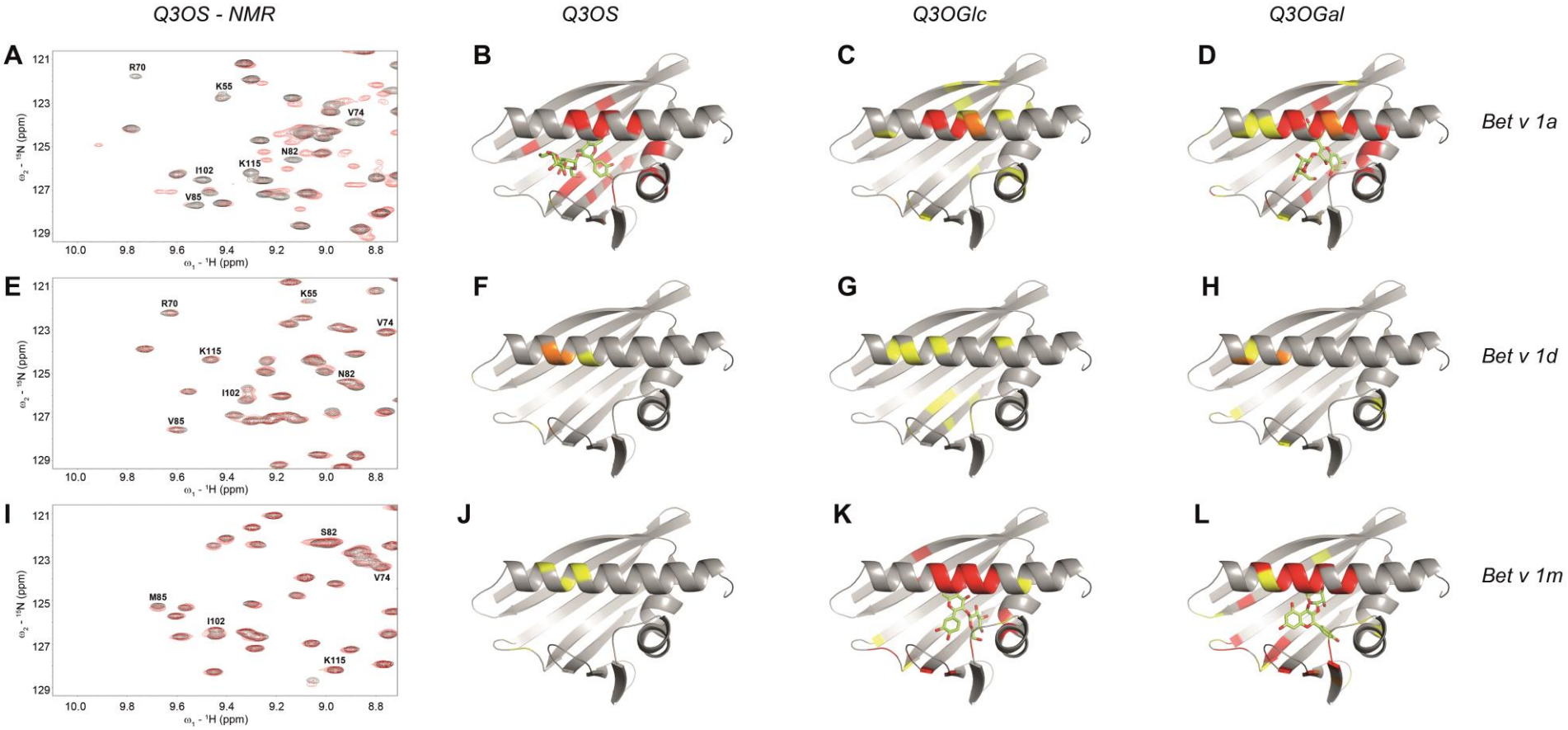

Figure 3: Binding of quercetin glycosides to Bet v 1 isoforms.
All experiments were performed with 50 µM (Q3OS) or 100 µM (Q3OGlc, Q3OGal) 15N-uniformly labelled Bet v 1 isoforms at 298 K in 50 mM sodium phosphate buffer, 50 mM NaCl at pH 7.0, and 10 % $^{2}H_2O$ with Bruker Avance 700 MHz and Avance 800 MHz spectrometers. Chemical shift changes were mapped on Bet v 1a (pdb code: 1BV1, grey) or models of Bet v 1d and Bet v 1m as in Figure 2F. Models of Bet v 1d and Bet v 1m were created using the Phyre server (Kelley & Sternberg, 2009). Docked ligands (Trott & Olson, 2010) are illustrated in green sticks, oxygen in red. (**A**) Overlay of two ^{1}H-^{15}N HSQC spectra of Bet v 1a in the absence (black) and presence of a 15-fold excess of Q3OS (red). Several ^{1}H-^{15}N resonances (K^{55}, R^{70}, V^{74}, N^{82}, V^{85}, I^{102} and K^{115}) disappear completely upon addition of Q3OS. (**B**) Disappearing resonances after addition of Q3OS mapped on Bet v 1a in red. Q3OS is docked inside the hydrophobic pocket (Seutter von Loetzen et al., 2014). (**C**) Mapping of chemical shift changes of (weak) Q3OClc or (**D**) (strong) Q3OGal interaction on Bet v 1a. (**E**) Overlay of two ^{1}H-^{15}N HSQC spectra of Bet v 1d in the absence (black) and presence of a 15-fold excess of Q3OS (red) and (**F**) occurring chemical shift changes mapped on a model of Bet v 1d. Weak affinity is observed for interaction of Bet v 1d with (**G**) Q3OGlc of (**H**) Q3OGal. (**I**) Overlay of two 1H-15N HSQC spectra of Bet v 1m in the absence (black) and presence of a 15-fold excess of Q3OS (red) and (**J**) occurring chemical shift changes mapped on a model of Bet v 1m. High affinity is observed for the interaction of Bet v 1m with (**K**) Q3OGlc and (**L**) Q3OGal. Regions of the ^{1}H-^{15}N HSQC spectra during titration of Bet v 1d or Bet v 1m with Q3OGlc and Q3OGal are provided in the supplementary figure S3.

Substitutions of amino acids in the C-terminal helix (S136I, M139K, and T142A) could contribute to an increased affinity towards Q3OGlc as compared to Bet v 1a as the C-terminal helix determines size and character of the hydrophobic cavity in PR-10 proteins (Fernandes *et al.*, 2013).

In addition to structural aspects, a phenomenon known as enthalpy-entropy compensation (Holgersson *et al.*, 2005) can explain the binding behaviour of the isoforms to glycosylated flavonoids. Upon Bet v 1 isoform–ligand complexation, water molecules that form the hydration shell of the sugar moiety and the binding cavity will tend to escape to the bulk with a concomitant decrease or increase in entropic energy, depending on the pre-existing molecular interactions. This event is accompanied by the increase or decrease of degrees of freedom for the ligand and the residues forming the binding site. The setup of solvent clusters on the surface of the protein-ligand complex also contributes to the overall binding affinity with enthalpy/entropy gains (Bet v 1a:Q3OS or Q3OGal; Bet v 1m:Q3OGlc or Q3OGal), penalties (Bet v 1a:Q3OGlc) or even complete abolishment of observable binding (Bet v 1d:Q3OS, Q3OGlc or Q3OGal; Bet v 1m:Q3OS). Similar effects have been reported and seem to be generally characteristic for each ligand/receptor involved (Lemieux, 1999; Swaminathan *et al.*, 1998; Toone, 1994).

In summary, our results firmly suggest that Bet v 1:ligand binding is isoform-specific and that the binding specificity is entropically driven by the sugar moiety. Glycosylation of quercetin can thereby increase the affinity more than 10-fold compared to the aglycon (Table I). The hydrophobic pockets formed by Bet v 1 isoforms are obviously designed for specific discrimination between the sugar moieties of glycosylated flavonoids.

Allergenicity of Bet v 1 isoforms is unaffected by ligands

Bet v 1 isoforms can be grouped into three classes with molecules showing high (isoforms a, e and j), intermediate (isoforms b, c, and f), and low/no IgE-binding activities (d, g, and l; Ferreira *et al.*, 1996). A study on the modulation of IgE reactivity by site-directed mutagenesis revealed a limited number of crucial amino acid positions (residues F^{30}, S^{57}, S^{112}, I^{113}, and D^{125} corresponding to Bet v 1a) that strongly influence IgE binding (Ferreira *et al.*, 1998). Although Bet v 1d, g, and l are highly similar in sequence to Bet v 1a, (95.6 %, 95.0 %, 94.3 % identity), those hypoallergenic isoforms show variations in each of these positions. A small subset of critical amino acids can drastically modulate the binding of IgE to an epitope and consequently change the allergenicity of Bet v 1 isoforms as exemplified by Bet v 1a and Bet v 1d (Arquint *et al.*, 1999; Ferreira *et al.*, 1996). In the absence of ligands, we observed comparable IgE interactions (Figure 4A) and mediator release activities (Figure 4C) for isoforms a and m measured by indirect ELISA and β-hexosaminidase release from humanized rat basophil leukaemia (RBL) cells. Sequence and allergenicity of Bet v 1m and the intermediate IgE-binding isoform Bet v 1b are nearly identical (Bet v 1.0201, 98.1 % identity; Swoboda *et al.*, 1995a). The IgE-binding capacity of Bet v 1d is only marginal in the ELISA, and consequently an approximately 10-fold shift to a higher Bet v 1d concentration is needed for half-maximum release of

β-hexosaminidase in comparison to the other isoforms (Figure 4A, C). Hence, our results are in good agreement with previous experiments on the allergenicity of the respective Bet v 1 isoforms (Arquint *et al.*, 1999; Ferreira *et al.*, 1996; Ferreira *et al.*, 1998).

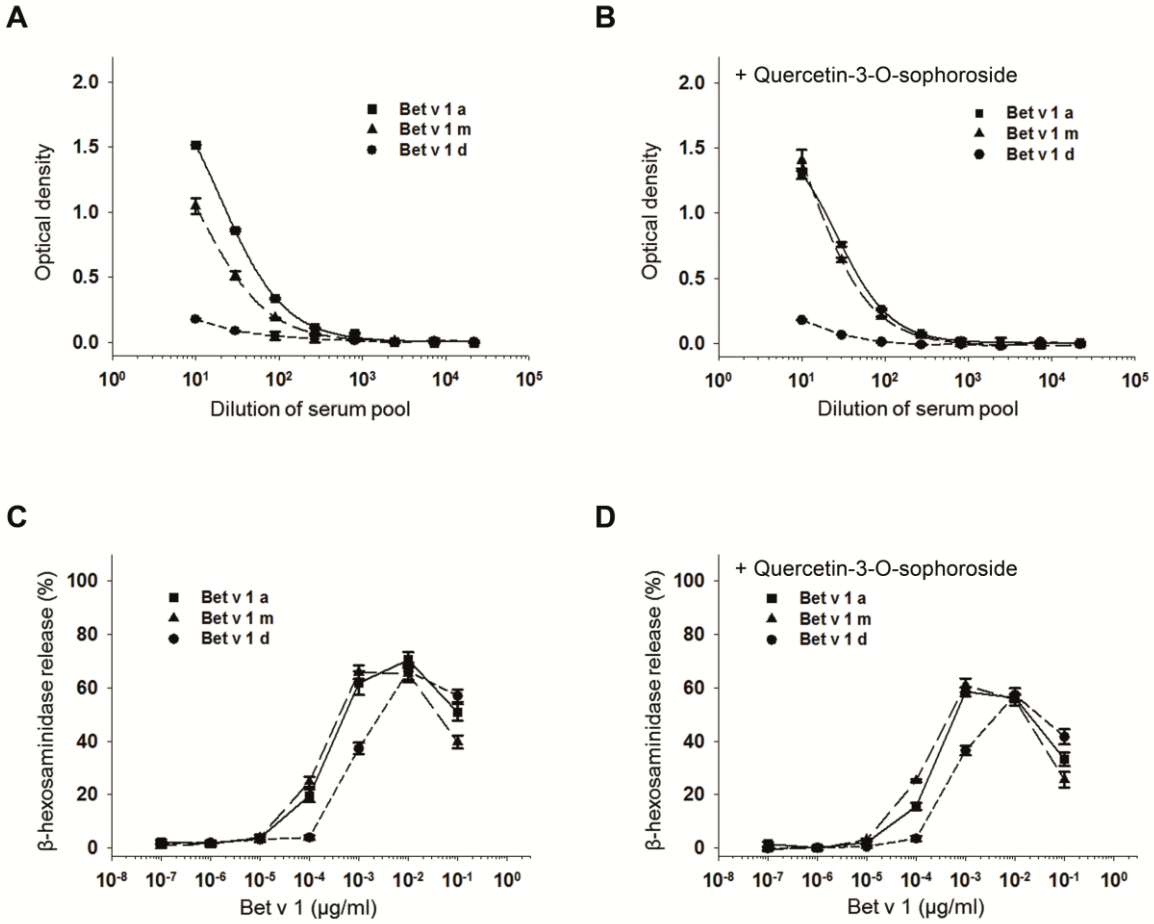

Figure 4: Interaction of Bet v 1 isoforms with IgE in the absence and presence of Q3OS.
(**A**) Binding of serial dilutions of pool serum IgE to equimolar amounts of surface-coated Bet v 1a, Bet v 1d, and Bet v 1m without and (**B**) with a 5-molar excess of Q3OS. (**C**) Mediator release induced by recombinant Bet v 1 isoforms. Humanized RBL cells were sensitized with a pool of human birch-specific sera. Cross-linking of membrane-bound human IgE by IgE-Bet v 1 isoform interaction and subsequent release of β-hexosaminidase was determined with serial dilutions of Bet v 1 a, d and m without and (**D**) with a 5-molar excess of Q3OS.

X-ray crystallography revealed that Bet v 1:ligand interaction could lead to an increase in volume of the hydrophobic pocket, thus altering the protein surface (Kofler *et al.*, 2012; Markovic-Housley *et al.*, 2003) an effect that was hypothesized to influence IgE epitopes. Our results, however, do not indicate any significant influence of high-affinity ligands on the IgE binding properties of Bet v 1. Presence of a 5-fold molar excess of Q3OS does not significantly influence the interaction of IgE with any of the three isoforms (Figure 4B, D), and rutin, quercetin, Q3OGlc, Q3OGal, and sophorose did not modify IgE-binding of the Bet v 1 isoforms either (Figure S3). Although recognition of an allergen by IgE is the key step in the allergic response, numerous other factors such as functional activity, presence of infective agents or chemical substances can induce non-specific inflammatory responses or will augment the immunological shift towards an allergic reaction (Bufe, 1998). We suggest the lack of a direct ligand effect on IgE recognition of Bet v 1, but leave open the possibility of indirect influences.

Indeed, flavonoids influence the inflammatory pathway in human cells (Chirumbolo, 2010), and their uptake by the human body may be facilitated by Bet v 1 (Golebski *et al.*, 2013; Mogensen *et al.*, 2007).

Bet v 1:flavonol-glycosides - adaptable sunscreens for birch pollen DNA?

The Bet v 1:Q3OS complex was suggested to protect pollen DNA from UV-damage, and the mixture of different isoforms was suggested to provide an individual fingerprint to prevent self-pollination (Seutter von Loetzen *et al.*, 2014). Indeed, glycosylated flavonoids are common in plant pollen. Flavonol-3-O-glycosides, e. g. were found in pollen from hazelnut, petunia, maize, and ophrys (Ceska & Styles, 1984; Karioti *et al.*, 2008; Mo *et al.*, 1992; Strack *et al.*, 1984) and quercetin-3-O-glycosylgalactoside was identified in pollen from *Betula verrucosa* (Meurer *et al.*, 1988) along with the Bet v 1a ligand Q3OS. Interactions of glycosylated flavonoids with different Bet v 1 isoforms in combination with variations in the production and composition of isoforms during maturation of pollen are probably dependent on a set of parameters like climate, location, and solar radiation, as the Bet v 1 levels in pollen are not constant over time (Buters *et al.*, 2010) show variable IgE reactivity (Schenk *et al.*, 2009) and vary geographically (Buters *et al.*, 2008; Erler *et al.*, 2011). Upon UV-B radiation flavonoids (mostly quercetin derivatives) are produced to protect the DNA from radiation damage (Lavola *et al.*, 2013) and glycosylation increases the UV tolerance of a flavonoid compared to the aglycon (Cvetkovic *et al.*, 2011; Smith *et al.*, 2000). As we observed a shift of the absorption maximum of quercetin depending on the sugar moiety (Figure 1B) and the absorption maxima of different unglycosylated flavonoids shift towards higher (apigenin, naringenin) or lower (myricetin, quercetin, fisetin) wavelengths during UV/VIS titration with Bet v 1 isoforms (Table SI), Bet v 1 complex formation combined with variation of isoform composition in pollen may be a means to expand or to optimize the absorption spectrum for sunlight-emitted UV-A radiation.

After maturation and before dispersing into the environment, the pollen dehydrate (Firon *et al.*, 2012) to reduce their water content to 20 % (Pacini, 1994), thus forming highly viscous intracellular glass-like structures (Buitink *et al.*, 1998). In this milieu of highly concentrated biomolecules, glycosylated flavonoids may be protected from degradation or chemical modulation by complex formation with Bet v 1.

Although flavonoids are considered most effective UV-B screening compounds because of their strong absorbance in the UV region (Morales *et al.*, 2011) continuous UV-irradiation leads to degradation (Cvetkovic *et al.*, 2011). Existence of functional complexes of glycosylated flavonoids and Bet v 1 in high concentration may serve as an important signal for unharmed pollen DNA as UV-damage of the flavonoid moiety may undo the complex and prevent pollination. The pollen–pistil interaction before fertilization comprises a series of complex cellular interactions involving a continuous exchange of signals between pollen and the pistil of the stigma (Heslop-Harrison, 1975; Hiscock & Allen, 2008). Upon contact, birch pollen get rehydrated, and the Bet v 1-ligand complexes are released onto the

stigma surface (Firon *et al.*, 2012; Grote *et al.*, 1993) with the specific mixture of the isoforms and ligands possibly serving as molecular fingerprints to prevent self-pollination. This means that Bet v 1 isoforms do not simply just exist, but have been selected through evolution with each isoform fulfilling an own function. Isoforms from other Bet v 1 homologs like Fra a 1 (Casañal *et al.*, 2013) or Api g 1 (Breiteneder *et al.*, 1995; Hoffmann-Sommergruber *et al.*, 2000) seem to have a less diverse function *in vivo* without the necessity to provide an individual fingerprint. In those cases, the amount of genetically available and actually expressed isoforms are significantly lower than observed for Bet v 1 in birch pollen.

Materials and Methods

Flavonoids

All nonglycosylated and monoglycosylated flavonoids were purchased from Sigma-Aldrich with analytical grade. Q3OS was obtained from ALNuMed or AApin Chemicals Limited (UK).

Protein preparation

The genes coding for Bet v 1d (Bet v 1.0102; UniProt P43177) and Bet v 1m (Bet v 1.0204; UniProt P43186) were purchased from GeneScript and cloned into the bacterial expression vector pET11a (Novagen) using the restriction enzymes NdeI and BamHI-HF (New England Biolabs). The expression for all isoforms was performed as previously described for Bet v 1a (Bet v 1.0101, UniProt P15494, Seutter von Loetzen *et al.*, 2014) with minor modifications. For purification, isoforms Bet v 1d and Bet v 1m were regained from protein pellets after cell lysis with 50 mM sodium phosphate, pH 7.4, 200 mM NaCl and 8 M urea and refolded by subsequently lowering the urea concentration during dialysis in 20 mM Hepes buffer, pH 8.0 and 500 mM NaCl at 4 °C (Bet v 1d) or 20 mM Hepes buffer, pH 8.0 at RT (Bet v 1m).

Refolded Bet v 1d was further purified via hydrophobic interaction chromatography on a 4 ml octyl sepharose column (HiTrap, Octyl Fast flow, GE Healthcare) equilibrated with loading buffer (20 mM Hepes, pH 8.0, 1 M ammonium sulphate) and eluted stepwise with elution buffer (20 mM Hepes, pH 8.0). Refolded Bet v 1m was loaded on a 25 ml Q sepharose column (Q sepharose Fast flow, GE Healthcare) equilibrated with loading buffer (20 mM Hepes, pH 8.0) followed by elution with 20 mM Hepes, pH 8.0, 300 mM NaCl. Bet v 1a was purified as previously described (Seutter von Loetzen *et al.*, 2014). Fractions containing the respective Bet v 1 isoform were pooled and dialyzed at 4 °C against 50 mM sodium phosphate, pH 7.0, 50 mM NaCl, concentrated and stored at -80 °C. Protein concentrations were determined by the DC protein assay (BioRad) and UV/VIS spectroscopy using the molar extinction coefficient ε_{280} =10430 M^{-1} cm^{-1}. Standard methods were used to analyze purity (SDS/PAGE), oligomeric state (size exclusion chromatography) and signal dispersion (^{1}H-^{15}N HSQC spectroscopy) of all isoforms (Figure S4).

UV/VIS spectroscopy

All flavonoids and Bet v 1 isoforms were dissolved in 50 mM sodium phosphate, 50 mM NaCl, 10 % (v/v) DMSO, pH 7.0, to a final concentration of 10 to 20 µM in 500 μl. Absorption spectra from 200-800 nm were recorded using a 8453 UV-visible spectrophotometer (Agilent) at 25 °C in a 1 cm quartz cuvette (Hellma GmbH).

To observe binding of Q3OS to the isoforms Bet v 1a, d and m, 20 μM Q3OS were initially incubated with 20 μM of the corresponding isoform in buffer without DMSO for 30 min at room temperature

(RT) in a total volume of 550 μl. Samples were concentrated to a final volume of 100 μl using a Vivaspin concentrator (Sartorius, molecular mass cut off 10.000 Da). The concentrated samples where loaded on a G25 spin trap column (GE Healthcare) and eluted as described in the manual. Absorption spectra of the eluted fractions were normalized at 280 nm and set to zero at 700 nm.

To further characterize flavonoid binding to Bet v 1, titration experiments were performed by adding small amounts of concentrated Bet v 1a, d or m to different flavonoids. Changes of flavonoid absorption occurring at specific wavelengths were plotted against the protein concentration. Prior to curve-fitting, absorbance data were corrected for dilution. If possible, the equilibrium dissociation constant (K_d) was determined by non-linear regression analysis of the data with GraFit-5 (Version 5.0, Erithacus Software, UK) using the following equation (1):

$$\Delta A = \frac{\Delta A_{max}}{2Q}[(B + Q + K_d) - ((B + Q + K_d)^2 - (4BQ)^{0.5})] \quad (1)$$

ΔA_{max}, maximum change in absorbance at specific wavelengths; *B*, Bet v 1a concentration; *Q*, total flavonoid concentration.

NMR spectroscopy

All NMR experiments were performed at 298 K in 50 mM sodium phosphate buffer, 50 mM NaCl, pH 7.0, 10 % deuterium oxide (^2H_2O) with ^{15}N-uniformly labelled Bet v 1 isoforms using Bruker Avance 700 MHz and Avance 800 MHz spectrometers with cryogenically cooled triple-resonance probes equipped with pulsed field-gradient capabilities. NMR data were processed using NMRPipe (Delaglio *et al.*, 1995) and visualized with NMRViewJ (Johnson & Blevins, 1994). Three-dimensional ^{15}N-edited NOESY (nuclear Overhauser enhancement spectroscopy, mixing times 120 ms) experiments to assign chemical shifts were obtained with 500 μM ^{15}N-labeled samples of Bet v 1d or m and yielded 91% of assigned residues for Bet v 1d and 89% for Bet v 1m. The sequence-specific assignments of the amide resonances of Bet v 1a are reported elsewhere (Schweimer *et al.*, 1999).

Interaction of Q3OS with the Bet v 1 isoforms was measured by incubating 700 μM Q3OS with 50 μM of each ^{15}N-labeled Bet v 1 isoform in 50 mM sodium phosphate, 50 mM NaCl buffer, pH 7.0.

For titration experiments all other flavonoids were dissolved in deuterated DMSO and titrated stepwise to a final excess of up to 17-fold to protein samples (ca. 100 μM). Final DMSO concentrations did not exceed 10 % (v/v). Chemical shift perturbations caused by increasing DMSO concentrations within the measurements were identified by titrating DMSO in comparable steps. CSPs for ligand binding were calculated based on equation (2):

$$\Delta\delta_{norm} = \sqrt{(\Delta\delta_{HN})^2 + (0.1 \cdot \Delta\delta_N)^2} \quad (2)$$

$\Delta\delta_{HN}$ and $\Delta\delta_{N}$, chemical shift differences in amide proton and nitrogen resonances, respectively, in ppm.

K_d values for flavonoid binding were determined with NMRViewJ (Johnson & Blevins, 1994). All analysable amino acid residues showing CSPs > 0.08 ppm were fitted to a quadratic binding curve with default setting, and an average $K_{d\ app}$ was calculated. The CSPs were mapped either on models of Bet v 1d and Bet v 1m or on the Bet v 1a structure (pdb code 1BV1; Gajhede *et al.*, 1996).

Modelling and docking simulation

Models of Bet v 1d and Bet v 1m were created using the Phyre2 server (Kelley & Sternberg, 2009). The calculated models are based on the structural fold of PR-10 proteins with a confidence of 99 % and a coverage of 92 % (Bet v 1d) and 87 % (Bet v 1m) compared to the template sequence. We used AutoDockVina (Trott & Olson, 2010) to dock ligands into the hydrophobic pocket of Bet v 1a and the model of Bet v 1m. The PDB files for Q3OGlc and Q3OGal were created with ProDrg (Schüttelkopf & van Aalten, 2004). Furthermore, input files for Bet v 1a (pdb code 1BV1), the model of Bet v 1m, Q3OGlc and Q3OGal were generated with AutoDockTools (Morris *et al.*, 2009). The grid box (20 Å×24 Å×28 Å or 13440 $Å^3$) (1 Å=0.1 nm) was centred over the hydrophobic pocket of the isoforms and AutoDockVina was run with default settings. Affinity scores were given by AutoDockVina as binding energies (ΔG), which were subsequently used to calculate an equilibrium dissociation constant (K_d) by equation (3) with R = 0.001968 kcal·mol^{-1}·K^{-1} and T = 298.15 K:

$$K_d = e^{\frac{-\Delta G}{RT}} \quad (3)$$

Ligand docking was performed only if more than five strong interacting amino acids ($\Delta\delta \geq 0.12$ or intermediate exchange rates) were observed during NMR titration. The output of the docking simulation lists up to nine energetically most favourite orientations of the respective ligand in the Bet v 1 pocket. The models in best agreement with our experimental NMR data were chosen to illustrate ligand binding to Bet v 1a or m.

Sera used in the study

Fifteen sera of birch pollen-allergic subjects were collected, tested and pooled according to the guideline of the European Medicines Agency (EMEA/CHMP/BWP/304831/2007). The serum pool is routinely used for batch-release testing of birch pollen-derived allergenic products at the Paul-Ehrlich-Institut. The same serum pool was used for both, ELISA and mediator release assays.

Indirect ELISA for IgE binding to Bet v 1 isoforms

For IgE-ELISA experiments, Maxisorp plates (Nunc via Fisher Scientific) were coated overnight at room temperature with 50 ng/100 μl recombinant Bet v 1a, d or m with and without a 5-molar excess of quercetin-3-O-sophorose, rutin, quercetin, quercetin-3-O-glucoside, quercetin-3-O-galactoside, or sophorose, respectively, in phosphate-buffered saline (PBS). After blocking with PBS containing 2 % bovine serum albumin (BSA) the plates were incubated with a dilution series of a serum pool of birch-pollen allergic subjects for 1 h at room temperature with PBS containing 0.05 % Tween 20 and 0.1 % BSA. Allergen-specific human IgE was detected with a horseradish peroxidase-conjugated mouse anti-human IgE antibody (Clone B3102E8, Southern biotech via Biozol, Eching, Germany) diluted 1:1000. 3,3′,5,5′-tetramethylbenzidine (Roth, Karlsruhe) was used as substrate for horseradish peroxidase and the absorbance at 450 nm was measured after stopping the reaction with 25 % H_2SO_4.

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

The mediator release assay was performed according to an established protocol (Vogel *et al.*, 2005). Briefly, RBL cells expressing the α-chain of the high-affinity receptor for human IgE were sensitized overnight at 37 °C (5 %CO_2) with a serum pool of birch pollen-allergic subjects (diluted 1:40 in culture medium). After washing, cells were stimulated with serial dilutions of Bet v 1 a, d, or m in Tyrode's buffer containing 50 % ^2H_2O, respectively. For complex formation, the Bet v 1 isoforms were incubated overnight with a 5-molar excess of Q3OS, rutin, quercetin, Q3OGlc, Q3OGal, or sophorose, respectively, before stimulating the cells. Degranulation was quantified by photometric measurement of β-hexosaminidase activity in the culture supernatants. The percentage of β-hexosaminidase activity relative to cells lysed with Triton X-100 (Sigma-Aldrich, Steinheim, Germany) was calculated and corrected for spontaneous release (sensitized cells without allergen).

Supplement

Figures

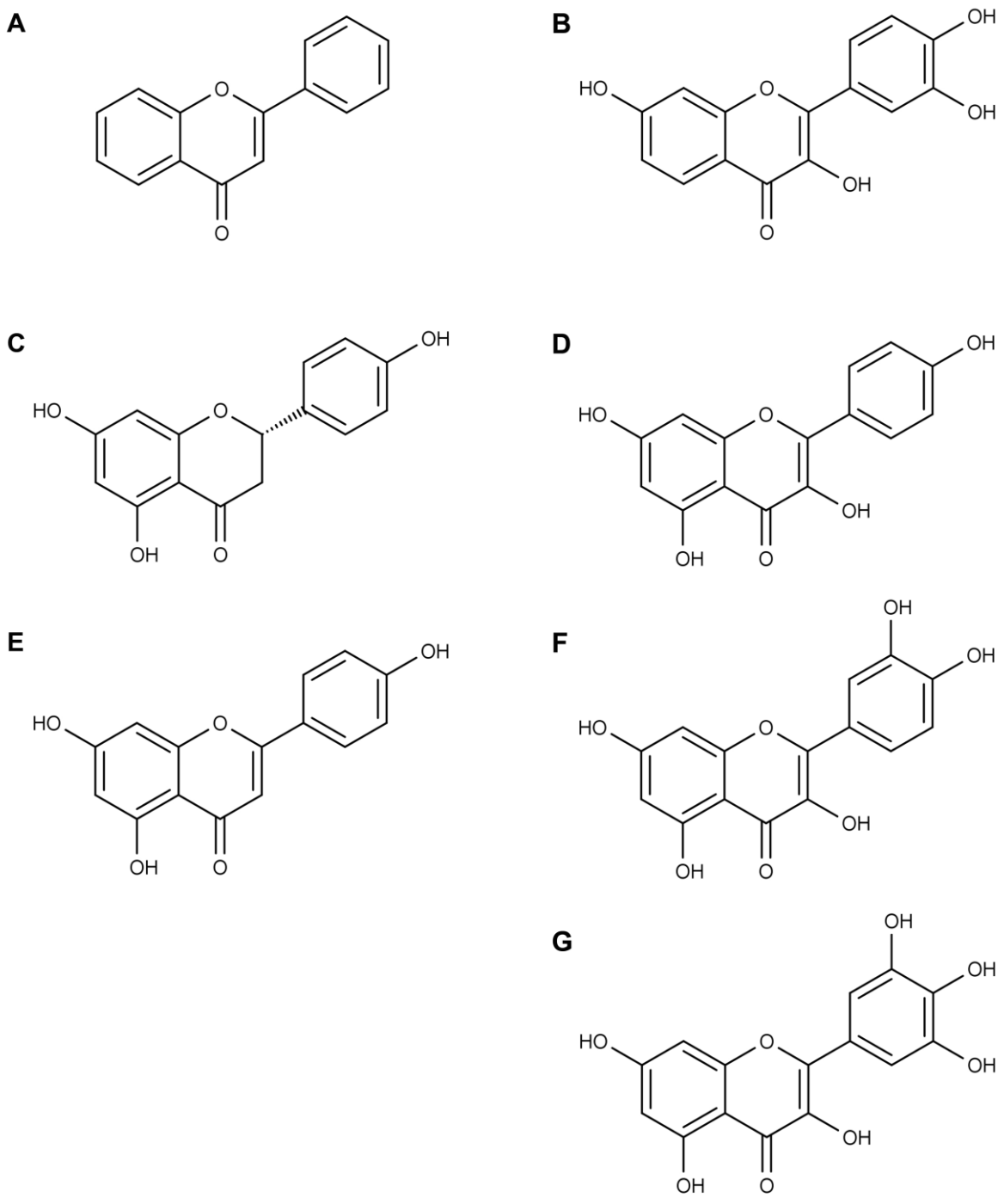

Figure S1: Unglycosylated flavonoids.
Chemical structures of (**A**) flavone, (**B**) fisetin, (**C**) naringenin, (**D**) kaempferol, (**E**) apigenin, (**F**) quercetin, (**G**) myricetin.

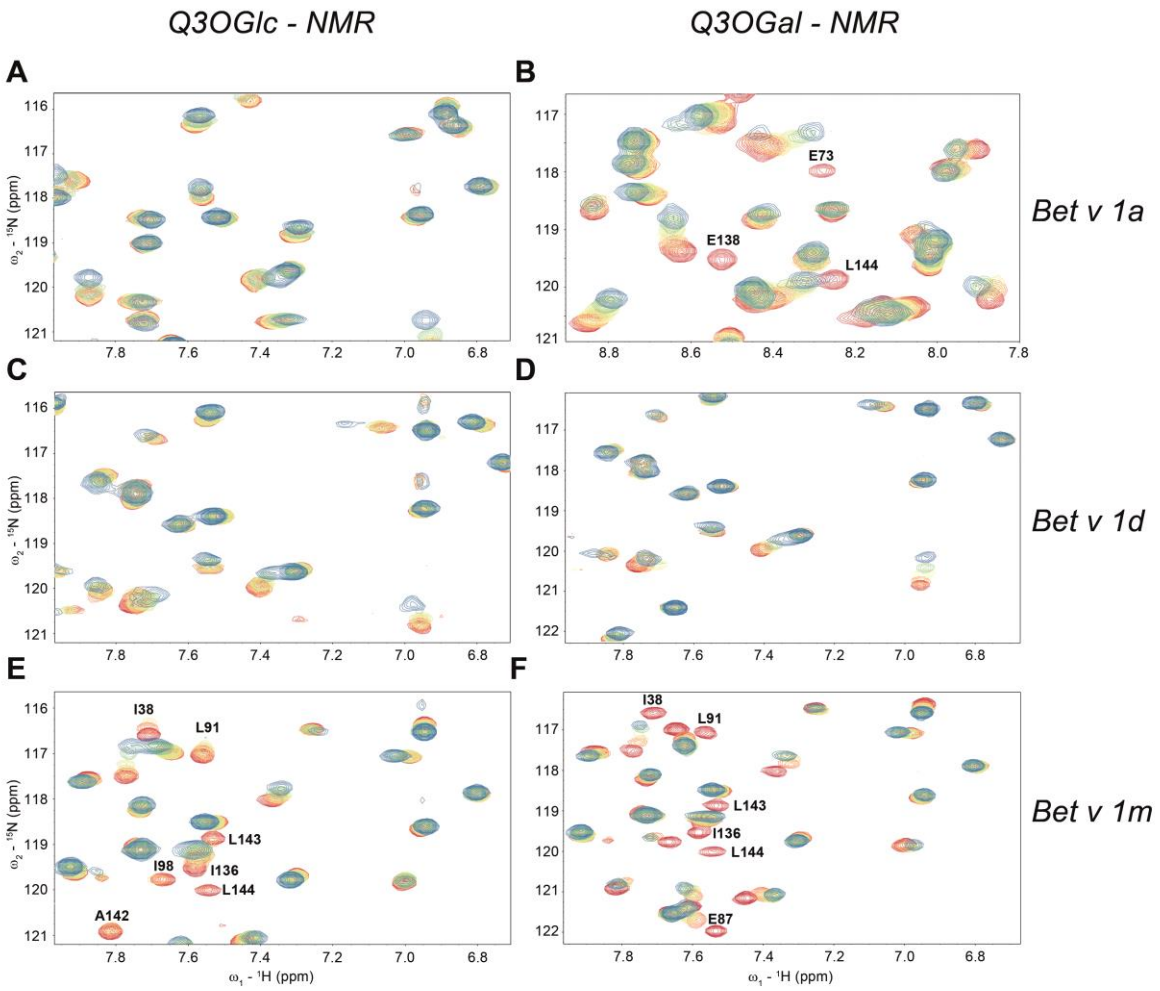

Figure S2: NMR titration experiments of Q3OGlc and Q3OGal with the Bet v 1 isoforms.
The experiments were performed with 100 μM ^{15}N-uniformly labelled Bet v 1 isoforms at 298 K in 50 mM sodium phosphate buffer, 50 mM NaCl at pH 7.0, and 10 % $^{2}H_2O$ with Bruker Avance 700 MHz and Avance 800 MHz spectrometers. Q3OGlc and Q3OGal were dissolved in deuterated DMSO and titrated stepwise to a final excess of up to 1:17 to protein samples. Final DMSO concentrations did not exceed 10 % (v/v). Spectra are illustrated in a divergent colour scheme from red (absence of ligand) to blue (final excess of ligand). Intermediate exchanging residues are labelled. Titration experiments of Bet v 1a with (**A**) Q3OGlc and (**B**) Q3OGal, Bet v 1d with (**C**) Q3OGlc, (**D**) Q3OGal and Bet v1m with (**E**) Q3OGlc and (**F**) Q3OGal.

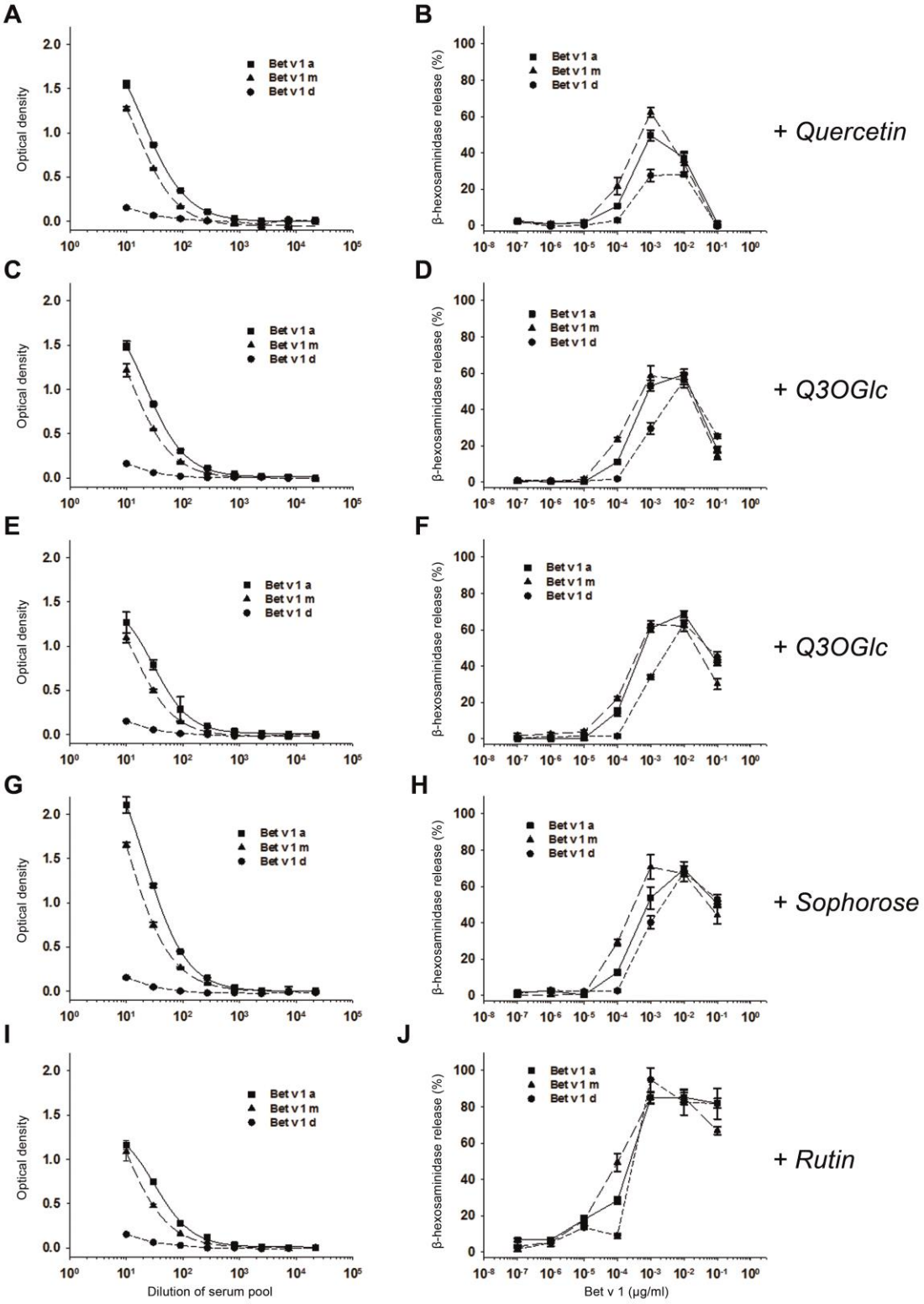

Figure S3: Interaction of Bet v 1 isoforms with IgE in the presence of different flavonoids and sophorose.
The left panel shows binding of serial dilutions of serum IgE to equimolar amounts of surface-coated Bet v 1a (■), Bet v 1d (●), and Bet v 1m (▲) with 5-molar excess of (**A**) quercetin, (**C**) Q3OGlc, (**E**) Q3OGal, (**G**) sophorose, and (**I**) rutin respectively. Mediator release induced by recombinant Bet v 1 isoforms is illustrated in the right panel. Humanized RBL cells were sensitized with a pool of human birch-specific sera. Cross-linking of membrane-bound human IgE by IgE-Bet v 1 isoform interaction and subsequent release of β-hexosaminidase was determined with serial dilutions of Bet v 1a (■), Bet v 1d (●), and Bet v 1m (▲) with 5-molar excess of (**B**) quercetin, (**D**) Q3OGlc, (**F**) Q3OGal, (**H**) sophorose, and (**J**) rutin respectively.

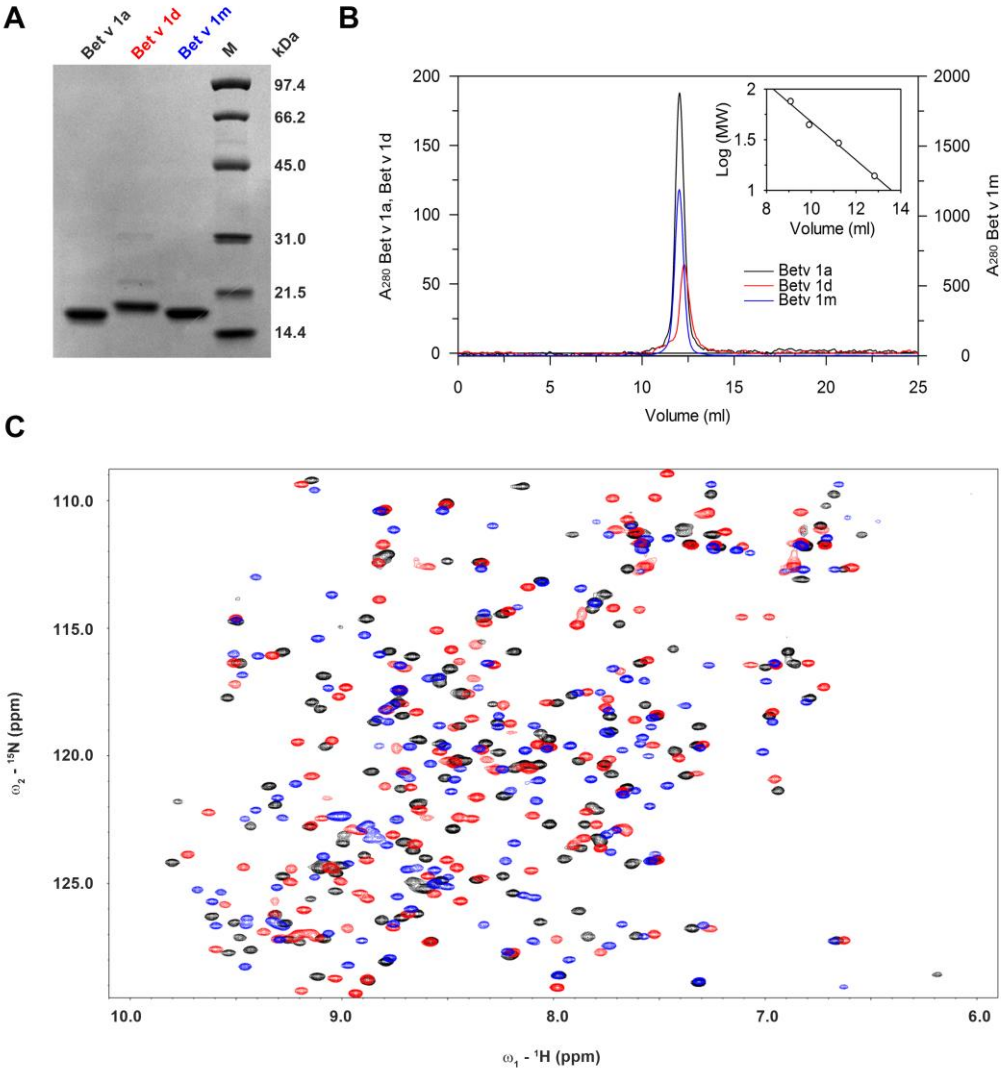

Figure S4: Protein analytics.
(**A**) SDS/PAGE on 19 % gels of ca. 1 μg Bet v 1 isoforms (MW 17.4 kDa) after purification. M, molecular-mass standard (Low Range, Bio-Rad Laboratories). (**B**) SEC of the isoforms performed with a Superdex S75 GL 10/300 column (total bed volume: 24 ml; GE Healthcare) in 50 mM sodium phosphate, 50 mM NaCl, pH 7.0 at RT. Column calibration was performed with conalbumin (75.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) and ribonuclease (13.7 kDa). The elution profile of 0.5 mg Bet v 1a is shown in black, 0.25 mg of Bet v 1d in red and 2.4 mg of Bet v 1m in blue. The peaks correspond to monomeric proteins with molecular masses of 19.66 kDa (Bet v 1a), 17.63 kDa (Bet v 1d) and 19.74 kDa (Bet v 1m). (**C**) ^{1}H-^{15}N HSQC spectra of 100 μM Bet v 1a (black), Bet v 1d (red) and Bet v 1m (blue) in 50 mM sodium phosphate, 50 mM NaCl, pH 7.0 and 10 % $^{2}H_2O$ at 298 K.

Tables

Table SI: Absorption maxima of unglycosylated flavonoids and their Bet v 1-complexes.

	Absorption maxima (nm)			
	Flavonoid	**Bet v 1a:Flavonoid**	**Bet v 1m:Flavonoid**	**Bet v 1d:Flavonoid**
Naringenin	325	325	322	325
Apigenin	351	349	347	349
Fisetin	368	372	370	371
Quercetin	377	382	381	382
Myricetin	378	387	379	382

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Author Contributions

CSvL and TJ performed expression and purification of recombinant Bet v 1d and Bet v 1m. CSvL conducted the UV/VIS and NMR titrations, assisted by TJ and OHS. CSvL performed the assignment of the Bet v 1d and Bet v 1m amide resonances and the computational docking. MJH and CSvL performed the purification of the Bet v 1a:Q3OS complex. LV conducted and evaluated the mediator release assays. DS coordinated and analysed the ELISA and mediator release assays. CSG contributed to the synthesis of Q3OS. PR, OHS and MJH designed and supervised the project. CSvL, DS, SV, PR and MJH, wrote the manuscript, which was read and approved by all authors.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

Agarwal P & Agarwal P (2014) Pathogenesis related-10 proteins are small, structurally similar but with diverse role in stress signaling. *Mol Biol Rep* **41**: 599-611

Arquint O, Helbling A, Crameri R, Ferreira F, Breitenbach M, & Pichler WJ (1999) Reduced in vivo allergenicity of Bet v 1d isoform, a natural component of birch pollen. *J Allergy Clin Immunol* **104**: 1239-1243

Bollen MA, Garcia A, Cordewener JH, Wichers HJ, Helsper JP, Savelkoul HF, & van Boekel MA (2007) Purification and characterization of natural Bet v 1 from birch pollen and related allergens from carrot and celery. *Mol Nutr Food Res* **51**: 1527-1536

Breiteneder H, Hoffmann-Sommergruber K, O'Riordain G, Susani M, Ahorn H, Ebner C, Kraft D, & Scheiner O (1995) Molecular characterization of Api g 1, the major allergen of celery (Apium graveolens), and its immunological and structural relationships to a group of 17-kDa tree pollen allergens. *Eur J Biochem* **233**: 484-489

Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, & Breitenbach M (1989) The gene coding for the major birch pollen allergen BetvI, is highly homologous to a pea disease resistance response gene. *EMBO J* **8**: 1935-1938

Breiteneder H, Ferreira F, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Rumpold H, Kraft D, & Scheiner O (1993) Four recombinant isoforms of Cor a I, the major allergen of hazel pollen, show different IgE-binding properties. *European Journal of Biochemistry* **212**: 355-362

Bufe A (1998) The biological function of allergens: relevant for the induction of allergic diseases? *Int Arch Allergy Immunol* **117**: 215-219

Buitink J, Claessens MMAE, Hemminga MA, & Hoekstra FA (1998) Influence of Water Content and Temperature on Molecular Mobility and Intracellular Glasses in Seeds and Pollen. *Plant Physiol* **118**: 531-541

Buters JTM, Kasche A, Weichenmeier I, Schober W, Klaus S, Traidl-Hoffmann C, Menzel A, Huss-Marp J, Krämer U, & Behrendt H (2008) Year-to-Year Variation in Release of Bet v 1 Allergen from Birch Pollen: Evidence for Geographical Differences between West and South Germany. *Int Arch Allergy Immunol* **145**: 122-130

Buters JTM, Weichenmeier I, Ochs S, Pusch G, Kreyling W, Boere AJF, Schober W, & Behrendt H (2010) The allergen Bet v 1 in fractions of ambient air deviates from birch pollen counts. *Allergy* **65**: 850-858

Casañal A, Zander U, Muñoz C, Dupeux F, Luque I, Botella MA, Schwab W, Valpuesta V, & Marquez JA (2013) The Strawberry Pathogenesis-related 10 (PR-10) Fra a Proteins Control Flavonoid Biosynthesis by Binding to Metabolic Intermediates. *Journal of Biological Chemistry* **288**: 35322-35332

Ceska O & Styles ED (1984) Flavonoids from Zea mays pollen. *Phytochemistry* **23**: 1822-1823

Chirumbolo S (2010) The role of quercetin, flavonols and flavones in modulating inflammatory cell function. *Inflammation & Allergy-Drug Targets* **9**: 263-285

Cvetkovic D, Markovic D, Cvetkovic D, & Radovanovic B (2011) Effects of continuous UV-irradiation on the antioxidant activities of quercetin and rutin in solution in the presence of lecithin as the protective target. *J Serb Chem Soc* **76**: 973-985

Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, & Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* **6**: 277-293

Dreborg S (1988) Food allergy in pollen-sensitive patients. *Ann Allergy* **61**: 41-46

Erler A, Hawranek T, Krückemeier L, Asam C, Egger M, Ferreira F, & Briza P (2011) Proteomic profiling of birch (Betula verrucosa) pollen extracts from different origins. *Proteomics* **11**: 1486-1498

Faber C, Lindemann A, Sticht H, Ejchart A, Kungl A, Susani M, Frank RW, Kraft D, Breitenbach M, & Rosch P (1996) Secondary structure and tertiary fold of the birch pollen allergen Bet v 1 in solution. *J Biol Chem* **271**: 19243-19250

Fernandes H, Michalska K, Sikorski M, & Jaskolski M (2013) Structural and functional aspects of PR-10 proteins. *FEBS Journal* **280**: 1169-1199

Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger HJ, & Scheiner O (1998) Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* **12**: 231-242

Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breitenbach M, Rheinberger HJ, & Ebner C (1996) Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* **183**: 599-609

Ferreira FD, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, Steiner R, Bohle B, Sperr WR, & Valent P (1993) Purification and characterization of recombinant Bet v I, the major birch pollen allergen. Immunological equivalence to natural Bet v I. *J Biol Chem* **268**: 19574-19580

Firon N, Nepi M, & Pacini E (2012) Water status and associated processes mark critical stages in pollen development and functioning. *Annals of Botany* **109**: 1201-1214

Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Joost van Neerven RJ, Schou C, Lowenstein H, & Spangfort MD (1996) X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat Struct Biol* **3**: 1040-1045

Gell PGH & Coombs RRA (1963) *Clinical aspects of immunology*. Blackwell: Oxford

Geroldinger-Simic M, Zelniker T, Aberer W, Ebner C, Egger C, Greiderer A, Prem N, Lidholm J, Ballmer-Weber B, Vieths S, & Bohle B (2011) Birch pollen–related food allergy: Clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. *J Allergy Clin Immunol* **127**: 616-622.e1

Golebski K, Roschmann KI, Toppila-Salmi S, Hammad H, Lambrecht BN, Renkonen R, Fokkens WJ, & van Drunen CM (2013) The multi-faceted role of allergen exposure to the local airway mucosa. *Allergy* **68**: 152-160

Grote M, Vrtala S, & Valenta R (1993) Monitoring of two allergens, Bet v I and profilin, in dry and rehydrated birch pollen by immunogold electron microscopy and immunoblotting. *J Histochem Cytochem* **41**: 745-750

Heslop-Harrison J (1975) Incompatibility and the Pollen-Stigma Interaction. *Annu Rev Plant Physiol* **26**: 403-425

Hiscock SJ & Allen AM (2008) Diverse cell signalling pathways regulate pollen-stigma interactions: the search for consensus. *New Phytol* **179**: 286-317

Hoffmann-Sommergruber K (2000) Plant allergens and pathogenesis-related proteins. What do they have in common? *Int Arch Allergy Immunol* **122**: 155-166

Hoffmann-Sommergruber K, Ferris R, Pec M, Radauer C, O'Riordain G, Laimer da CM, Scheiner O, & Breiteneder H (2000) Characterization of Api g 1.0201, a New Member of the Api g 1 Family of Celery Allergens. *Int Arch Allergy Immunol* **122**: 115-123

Holgersson J, Gustafsson A, & Breimer ME (2005) Characteristics of protein-carbohydrate interactions as a basis for developing novel carbohydrate-based antirejection therapies. *Immunol Cell Biol* **83**: 694-708

Hostetler GL, Riedl KM, & Schwartz SJ (2013) Effects of food formulation and thermal processing on flavones in celery and chamomile. *Food Chem* **141**: 1406-1411

Ipsen H & Lowenstein H (1983) Isolation and immunochemical characterization of the major allergen of birch pollen (Betula verrucosa). *J Allergy Clin Immunol* **72**: 150-159

Jarolim E, Rumpold H, Endler AT, Ebner H, Breitenbach M, Scheiner O, & Kraft D (1989) IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of Betula verrucosa. *Allergy* **44**: 385-395

Johnson BA & Blevins RA (1994) NMRview: A computer program for the visualization and analysis of NMR data. *J Biomol NMR* **4**: 603-614

Karioti A, Kitsaki CK, Zygouraki S, Ziobora M, Djeddi S, Skaltsa H, & Liakopoulos G (2008) Occurrence of flavonoids in Ophrys (Orchidaceae) flower parts. *Flora - Morphology, Distribution, Functional Ecology of Plants* **203**: 602-609

Kelley LA & Sternberg MJ (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* **4**: 363-371

Kofler S, Asam C, Eckhard U, Wallner M, Ferreira F, & Brandstetter H (2012) Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen bet v 1. *J Mol Biol* **422**: 109-123

Koistinen KM, Soininen P, Venalainen TA, Hayrinen J, Laatikainen R, Perakyla M, Tervahauta AI, & Karenlampi SO (2005) Birch PR-10c interacts with several biologically important ligands. *Phytochemistry* **66**: 2524-2533

Lavola A, Nybakken L, Rousi M, Pusenius J, Petrelius M, Kellomäki S, & Julkunen-Tiitto R (2013) Combination treatment of elevated UVB radiation, CO2 and temperature has little effect on silver birch (Betula pendula) growth and phytochemistry. *Physiol Plantarum* **149**: 499-514

Lebel S, Schellenbaum P, Walter B, & Maillot P (2010) Characterisation of the Vitis vinifera PR10 multigene family. *BMC Plant Biology* **10**: 184

Leja M, KamiÅ„ska I, Kramer M, Maksylewicz-Kaul A, Kammerer D, Carle R, & Baranski R (2013) The Content of Phenolic Compounds and Radical Scavenging Activity Varies with Carrot Origin and Root Color. *Plant Foods for Human Nutrition* **68**: 163-170

Lemieux RU (1999) How Water Provides the Impetus for Molecular Recognition in Aqueous Solution. *Acc Chem Res* **32**: 631-631

Liu J & Ekramoddoullah AKM (2006) The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. *Physiol Mol Plant Pathol* **68**: 3-13

Markovic-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, Susani M, Ferreira F, Scheiner O, & Breiteneder H (2003) Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J Mol Biol* **325**: 123-133

Meurer B, Wiermann R, & Strack D (1988) Phenylpropanoid patterns in fagales pollen and their phylogenetic relevance. *Phytochemistry* **27**: 823-828

Mo Y, Nagel C, & Taylor LP (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proc Natl Acad Sci U S A* **89**: 7213-7217

Mogensen JE, Wimmer R, Larsen JN, Spangfort MD, & Otzen DE (2002) The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J Biol Chem* **277**: 23684-23692

Mogensen JE, Ferreras M, Wimmer R, Petersen SV, Enghild JJ, & Otzen DE (2007) The Major Allergen from Birch Tree Pollen, Bet v 1, Binds and Permeabilizes Membranes. *Biochemistry* **46**: 3356-3365

Morales LO, Tegelberg R, Brosché M, Lindfors A, Siipola S, & Aphalo PJ (2011) Temporal variation in epidermal flavonoids due to altered solar UV radiation is moderated by the leaf position in Betula pendula. *Physiol Plantarum* **143**: 261-270

Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, & Olson AJ (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* **30**: 2785-2791

Neudecker P, Schweimer K, Nerkamp J, Scheurer S, Vieths S, Sticht H, & Rösch P (2001) Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1. *J Biol Chem* **276**: 22756-22763

Pacini E (1994) Cell biology of anther and pollen development. In Williams E, Clarke A, Knox RB (eds) pp 289-308. Springer Netherlands

Radauer C, Lackner P, & Breiteneder H (2008) The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC Evol Biol* **8**: 286

Roth-Walter F, Gomez-Casado C, Pacios LF, Mothes-Luksch N, Roth GA, Singer J, Diaz-Perales A, & Jensen-Jarolim E (2014) Bet v 1 from lipocalin-like protein acting as allergen only when devoid of iron by promoting Th2 lymphocytes. *Journal of Biological Chemistry*

Schenk MF, Cordewener JH, America AH, Van't Westende WP, Smulders MJ, & Gilissen LJ (2009) Characterization of PR-10 genes from eight Betula species and detection of Bet v 1 isoforms in birch pollen. *BMC Plant Biol* **9**: 24

Schenk M, Gilissen L, Esselink G, & Smulders M (2006) Seven different genes encode a diverse mixture of isoforms of Bet v 1, the major birch pollen allergen. *BMC Genomics* **7**: 168

Schüttelkopf AW & van Aalten DM (2004) PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr D Biol Crystallogr* **60**: 1355-1363

Schweimer K, Sticht H, Nerkamp J, Boehm M, Breitenbach M, Vieths S, & Rösch P (1999) NMR Spectroscopy Reveals Common Structural Features of the Birch Pollen Allergen Bet v 1 and the Cherry Allergen Pru a 1. *Appl Magn Reson* **17**: 449-456

Seutter von Loetzen C, Schweimer K, Schwab W, Rösch P, & Hartl-Spiegelhauer O (2012) Solution structure of the strawberry allergen Fra a 1. *Biosci Rep* **32**: 567-575

Seutter von Loetzen C, Hoffmann T, Hartl MJ, Schweimer K, Schwab W, Rösch P, & Hartl-Spiegelhauer O (2014) Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. *Biochemical Journal* **457**: 379-390

Smith GJ, Thomsen SJ, Markham KR, Andary C, & Cardon D (2000) The photostabilities of naturally occurring 5-hydroxyflavones, flavonols, their glycosides and their aluminium complexes. *J Photochem Photobiol A* **136**: 87-91

Strack D, Meurer B, Wray V, Grotjahn L, Austenfeld FA, & Wiermann R (1984) Quercetin 3-glucosylgalactoside from pollen of Corylus avellana. *Phytochemistry* **23**: 2970-2971

Swaminathan CP, Surolia N, & Surolia A (1998) Role of Water in the Specific Binding of Mannose and Mannooligosaccharides to Concanavalin A. *J Am Chem Soc* **120**: 5153-5159

Swoboda I, Jilek A, Ferreira F, Engel E, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breiteneder H, Pittenauer E, & Schmid E (1995a) Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J Biol Chem* **270**: 2607-2613

Swoboda I, Scheiner O, Heberle-Bors E, & Vicente O (1995b) cDNA cloning and characterization of three genes in the Bet v 1 gene family that encode pathogenesis-related proteins. *Plant Cell and Environment* **18**: 865-874

Toone EJ (1994) Structure and energetics of protein-carbohydrate complexes. *Curr Opin Struct Biol* **4**: 719-728

Trott O & Olson AJ (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* **31**: 455-461

Vogel L, Lüttkopf D, Hatahet L, Haustein D, & Vieths S (2005) Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* **60**: 1021-1028

Vrtala S, Hirtenlehner K, Susani M, Akdis M, Kussebi F, Akdis CA, Blaser K, Hufnagl P, Binder BR, Politou A, Pastore A, Vangelista L, Sperr WR, Semper H, Valent P, Ebner C, Kraft D, & Valenta R (2001) Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1. *FASEB J* **15**: 2045-2047

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9 Erklärung

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Hiermit erkläre ich, dass keine Tatsachen vorliegen, die mich nach den gesetzlichen Bestimmungen über die Führung akademischer Grade zur Führung eines Doktorgrades unwürdig erscheinen lassen.

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