
**Charakterisierung und Identifikation
neuer PR-10-Isoallergene aus Karotte und Hasel
sowie ihrer Liganden**

Dissertation

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Zusammenfassung

Laut dem Robert Koch-Institut sind in Deutschland etwa 15 Mio. Menschen gegen Baumpollen sensibilisiert. Typische Symptome der allergischen Reaktion sind Hautreaktionen, Atemwegs-Beschwerden und in seltenen Fällen Anaphylaxie. Bet v 1 ist das Hauptallergen aus Birkenpollen und gehört zu den sogenannten *pathogenesis related* (PR)-10-ähnlichen Proteinen. Das natürliche (n)Bet v 1 besteht aus einer Mischung verschiedener Isoallergene und Varianten. Isoallergene weisen untereinander eine Aminosäuresequenzidentität von mindestens 67% und Varianten von mindestens 90% auf. Nach erfolgter Sensibilisierung können strukturell ähnliche Bet v 1-homologe Proteine aus anderen Baumpollen (Hasel, Erle, Buche, etc.) und aus botanisch entfernt verwandten Pflanzen (Karotte, Apfel, Erdbeere, etc.) Kreuzallergien auslösen, da gegen Bet v 1 gerichtete IgE-Antikörper mit strukturell ähnlichen Oberflächenstrukturen, sogenannten Epitopen, auf Bet v 1-homologen Proteinen kreuzreagieren können. Welche Epitope genau für die Allergie und Kreuzallergie verantwortlich sind, ist bis heute nicht vollständig geklärt.

In dieser Arbeit wurden die beiden neuen Isoallergene aus der Karotte Dau c 1.0501 und 1.0601, die ebenfalls Bet v 1-homologe Proteine sind, auf mRNA- und Proteinebene identifiziert und rekombinant in *E. coli* hergestellt und allergologisch untersucht. Sie weisen eine Aminosäuresequenzidentität von 54% bis 87% zu den bekannten Isoallergenen Dau c 1.0101 und Dau c 1.0401 auf und zeigten in immunologischen Tests eine starke Reaktion. Eine nicht-allergene Variante Dau c 1-like reagierte kaum, obwohl ihre Aminosäuresequenz mit den neuen Isoallergenen zu >50% identisch ist. Ein Sequenzvergleich zwischen den Dau c 1-Isoallergenen und dem nicht-allergen Dau c 1-like offenbarte bereits bekannte, aber auch mögliche neue konformationelle IgE-Epitope, die auch für Kreuzreaktionen mit gegen Bet v 1 gerichtete IgE Antikörper verantwortlich sein könnten.

Die Hasel kann nicht nur eine Pollenallergie, sondern auch eine pollenassoziierte Nahrungsmittelallergie beim Verzehr der Nüsse auslösen. Die Allergie wird ebenfalls durch eine Kreuzreaktion von ursprünglich gegen Bet v 1 gerichtete IgE ausgelöst. In dieser Arbeit wurden vier neue Isoallergene Cora 1.0501 – 1.0801 in der Hasel identifiziert. Darüber hinaus wurde die Expression aller Isoallergene (Cor a 1.01 – 1.08) in verschiedenen Geweben der Hasel (Kätzchen, Pollen, weibliche Blüte, unreife und reife Nuss) untersucht. Neben den Isoallergenen Cor a 1.01, 1.03, 1.05, 1.06 und 1.07 konnte erstmals auch das Isoallergen Cor a 1.04 in Pollen nachgewiesen werden. Cor a 1.02 und Cor a 1.08 sind die einzigen

Isoallergene, die nicht in Pollen vorkommen. In der weiblichen Blüte, der reifen und unreifen Nuss wurden alle Isoallergene mit sehr unterschiedlichem Expressionslevel nachgewiesen. Die Charakterisierung neuer Isoallergene ist sowohl für die allergologische Diagnostik von Bedeutung. Darüber hinaus liefert die Expressionsanalyse weitere Hinweise auf unterschiedliche Funktionen der Isoallergene in der Pflanze.

PR-10 und PR-10-ähnliche Proteine können in ihrer amphiphilen Tasche kleine, strukturell unterschiedliche Moleküle wie z.B. Flavonoide, Steroide oder Cytokinine unspezifisch binden, weshalb u.a. eine Funktion als Transport- oder Speicherprotein vermutet wird. Ihre genaue physiologische Funktion sowie die Bedeutung der gebundenen Liganden im Allergiegeschehen ist nur wenig erforscht. Am Lehrstuhl Biopolymere wurden erstmals physiologische Liganden von Bet v 1, Quercetin-3-O-Sophorosid (Q3OS), und von Cor a 1, Quercetin-3-O-(2''-O-β-D-Glukopyranosyl)-β-D-Galaktopyranosid (Q3O-(Glc)-Gal) aus Birken- bzw. Haselpollen isoliert. Interessanterweise binden Bet v 1.0101 und Cor a 1.0401 spezifisch nur ihren eigenen Liganden, nicht aber den des anderen Proteins. Dabei unterscheiden sich Q3OS und Q3O-(Glc)-Gal nur in der Stereochemie der 4''-Hydroxylgruppe des ersten Zuckers in Form von Glucose oder Galaktose.

Um weitere physiologische Liganden zu identifizieren und die Hintergründe für die Spezifität der Bindung zu erforschen, wurde nCor a 1, eine Mischung verschiedener Isoallergene und Liganden, aus Haselpollen gereinigt. Mittels *ultra-high-pressure liquid chromatography/quadrupole-time of flight - tandem mass spectrometry* (UHPLC/QTOF-MS/MS) konnten weitere glykosylierte Flavonoide nachgewiesen werden. Neben den bereits bekannten diglykosylierten Flavonoiden Q3OS und Q3O-(Glc)-Gal, wurden die monoglykosylierten Flavonoide Quercetin-3-O-Rhamnosid (Q3OR), Kaempferol-3-O-Rhamnosid (K3OR), Quercetin-3-O-Glykosid (Q3OGlc) und Quercetin-3-O-Galaktosid (Q3OGal) identifiziert. Die Bindung konnte mittels NMR-Spektroskopie und *isothermal titration calorimetry* (ITC) bestätigt werden. Während monoglykosylierte Flavonoide von allen in Pollen vorkommenden Cor a 1-Isoallergenen gebunden wurden, zeigten diglykosylierte Flavonoide eine höhere Spezifität und wurden ausschließlich von Cor a 1.0401, 1.0601 und 1.0701 gebunden. Aus *isotope filtered/edited* NOESY-Experimenten wurden erfolgreich Protonenabstandsinformationen zwischen Protein und Ligand gewonnen. Dies ermöglichte erstmals die Berechnung einer Komplexstruktur, sowohl mit einem monoglykosylierten als auch mit einem diglykosylierten Flavonoid mittels *docking*. Tatsächlich werden

monoglykosylierte Flavonoide und diglykosylierte Flavonoide in unterschiedlicher Orientierung in der amphiphilen Tasche gebunden. Während bei monoglykosylierten Flavonoiden der Zuckerrest im Inneren der Bindungstasche und die Quercetineinheit am Eingang der Tasche lokalisiert ist, befindet sich bei den diglykosylierten Flavonoiden die Quercetineinheit tief im Hohlraum und der Zuckerrest am Eingang der Bindetasche. Die Komplexstruktur von Cor a 1.0401/ Q3O-(Glc)-Gal und erste NMR-Experimente zeigten außerdem, dass die Aminosäure Ile31 einen wichtigen Einfluss auf die Bindungsspezifität hat. Vorläufige Ergebnisse deuten darauf hin, dass der Austausch von Phe31 zu Ile31 bei der Variante Bet v 1.0118 zu einer deutlichen Verschiebung der Spezifität von Q3OS zu Q3O-(Glc)-Gal führt.

Summary

According to the Robert Koch-Institute, around 15 million people in Germany are sensitised to tree pollen. Typical symptoms of the allergic reaction are skin reactions, respiratory complaints and in rare cases anaphylaxis. Bet v 1 is the main allergen from birch pollen and belongs to the so-called pathogenesis related (PR)-10-like proteins. The natural (n)Bet v 1 consists of a mixture of different isoallergens and variants. Isoallergens have an amino acid sequence identity of at least 67% and variants of at least 90%. After sensitisation, structurally similar Bet v 1-homologous proteins from other tree pollen (hazel, alder, beech, etc.) and from botanically distantly related plants (carrot, apple, strawberry, etc.) can trigger cross-allergies as IgE antibodies directed against Bet v 1 can cross-react with structurally similar surface structures, so-called epitopes, on Bet v 1-homologous proteins. However, it is not yet fully understood, which epitopes are responsible for the allergy and cross-allergy.

In this work, the two new isoallergens Dau c 1.0501 and 1.0601 from carrot, which are also Bet v 1-homologous proteins, were identified at the mRNA and protein level and recombinantly expressed in *E. coli* and analysed allergologically. The two newly identified isoallergens show an amino acid sequence identity of 54% to 87% to the known isoallergens Dau c 1.0101 and Dau c 1.0401 and showed a strong reaction in immunological tests. A non-allergenic variant, called Dau c 1-like, hardly reacted at all, although its amino acid sequence is >50% identical to the new isoallergens. A sequence comparison between the Dau c 1 isoallergens and the non-allergenic Dau c 1-like revealed already known, but also possible new conformational IgE epitopes, which could also be responsible for cross-reactions with IgE antibodies directed against Bet v 1.

Hazel not only causes a pollen allergy, but also a pollen-associated food allergy when the nut is consumed. The allergy is also triggered by a cross-reaction of IgE originally directed against Bet v 1. In this work, four new isoallergens Cor a 1.0501 – 1.0801 were identified in hazel. In addition, the expression of all isoallergens (Cor a 1.01 – 1.08) was analysed in different hazel tissues (catkins, pollen, female flower, immature and mature nut). For the first time, the isoallergen Cor a 1.04 could be detected in pollen, in addition to the isoallergens Cor a 1.01, 1.03, 1.05, 1.06 and 1.07. Cor a 1.02 and Cor a 1.08 are the only isoallergens that do not occur in pollen. All isoallergens could be detected in the female flower, the mature and the immature nut, however with different expression levels. The characterisation of new isoallergens is

important for allergy diagnostics. In addition, the expression analysis provides further evidence that the isoallergens fulfil different functions in the plant.

PR-10 and PR-10-like proteins can non-specifically bind small structurally different molecules such as flavonoids, steroids or cytokinins in their amphiphilic pocket, suggesting a function as transport or storage proteins. Their exact physiological function and the role of the bound ligands in the allergic process are still poorly understood. At the Department of Biopolymers, physiological ligands of Bet v 1, quercetin-3-O-sophoroside (Q3OS), and of Cor a 1, quercetin-3-O-(2"-O- β -D-glucopyranosyl)- β -D-galactopyranoside (Q3O-(Glc)-Gal) were isolated for the first time from birch and hazel pollen. Interestingly, Bet v 1.0101 and Cor a 1.0401 specifically bind only their own ligand, but not that of the other protein. Q3OS and Q3O-(Glc)-Gal differ only in the stereochemistry of the 4"-hydroxyl group of the first sugar in the form of glucose or galactose.

To identify additional physiological ligands and to investigate the specificity of binding, nCor a 1, a mixture of different isoallergens and ligands, was purified from hazel pollen. UHPLC/QTOF-MS/MS, was used to detect other glycosylated flavonoids. In addition to the known diglycosylated flavonoids Q3OS and Q3O-(Glc)-Gal, the monoglycosylated flavonoids quercetin-3-O-rhamnoside (Q3OR), kaempferol-3-O-rhamnoside (K3OR), quercetin-3-O-glycoside (Q3OGlc) and quercetin-3-O-galactoside (Q3OGal) were identified. Binding was confirmed by NMR-spectroscopy and ITC. Monoglycosylated flavonoids were bound by all Cor a 1 isoallergens present in pollen, whereas diglycosylated flavonoids showed a higher specificity and were exclusively bound by Cor a 1.0401, 1.0601 and 1.0701. Proton distance information between protein and ligand was successfully obtained from isotope filtered/edited NOESY experiments. Thus, for the first time, complex structures using a monoglycosylated or a diglycosylated flavonoid could be calculated by docking. In fact, they are bound in different orientations in the amphiphilic pocket. The sugar moiety of monoglycosylated flavonoids is located inside the binding pocket and the quercetin moiety at its entrance, whereas the sugar moiety of the diglycosylated flavonoid is situated at the entrance and the quercetin moiety is located deep within the cavity. The complex structure of Cor a 1.0401/ Q3O-(Glc)-Gal and initial NMR experiments also show that the amino acid Ile31 is important for binding specificity. Preliminary results indicate that the Bet v 1.0118 variant shows a significant shift in specificity from Q3OS to Q3O-(Glc)-Gal when Phe31 is substituted with Ile31.

1 Einleitung

1.1 Allergien und ihre Ursachen

In den letzten 100 Jahren ist die Zahl der Menschen, die an einer Allergie leiden, kontinuierlich gestiegen (Murphy et al., 2018; Ring et al., 2010). In Deutschland (Gesamtbevölkerung von 80 Mio.) leiden 20 bis 30 Mio. Menschen an einer Allergie und die Zahl der Erkrankten nimmt weiter zu (Bergmann et al., 2016; Ring et al., 2010). Die häufigsten Allergien richten sich gegen Pollen von Bäumen, Gräsern und Kräutern, gegen Insekten wie Hausstaub- und Vorratsmilben, aber auch gegen Schimmelpilze, Nahrungsmittel und Medikamente (Holzhauser et al., 2020; Murphy et al., 2018; Ring et al., 2010). Die Symptome einer Allergie äußern sich in Form von Hautreaktionen (Juckreiz, Ekzeme), Atemwegsbeschwerden (Husten, Niesen, Schnupfen und Asthma), Verdauungsbeschwerden (Bauchschmerzen, Übelkeit und Erbrechen) aber im schlimmsten Fall auch in Form einer Anaphylaxie (Ring, 2012). Die Gründe für die Zunahme von Allergien in der Bevölkerung sind noch nicht vollständig geklärt es werden jedoch verschiedene Faktoren, wie z.B. Klimawandel, Umweltverschmutzung, übertriebene Hygiene, für die Zunahme von Allergien verantwortlich gemacht (Berger et al., 2021; Ege et al., 2011).

Die IgE-vermittelte Immunantwort ist eigentlich für die Abwehr von parasitären Würmern verantwortlich. So kann z. B. bei einer Infektion mit Helminthen wie *Schistosoma mansoni* ein deutlicher Anstieg von IgE deutlich beobachtet werden (Rihet et al., 1991). Die steigende Lebensqualität und der zunehmende westliche Lebensstil führen jedoch dazu, dass immer weniger Menschen mit parasitären Würmern in Kontakt kommen (Erb, 2007; Fitzsimmons et al., 2014). Zudem führt die bessere Hygiene zu einer geringeren Exposition gegenüber Mikroorganismen. Gerade in der Kindesentwicklung scheint der Kontakt mit Mikroorganismen wichtig für die Entwicklung des Immunsystems zu sein. Kinder, die auf dem Land aufwachsen, sind einem größeren Spektrum verschiedener Mikroorganismen ausgesetzt und haben im Durchschnitt weniger Allergien als Kinder, die in der Stadt aufwachsen (Ege et al., 2011). Auch das veränderte Ernährungsverhalten sorgt für eine erhöhte Zahl an Nahrungsmittelallergikern. Die klassischen Nahrungsmittelallergien richten sich gegen Erdnüsse, Kuhmilch, Eiweiß, Soja, Weizen und Fisch (Bergmann et al., 2016; Longo et al., 2013). Birkenpollenallergiker, deren Allergie sich zunächst nur gegen Birkenpollen richtet, entwickeln später zudem meist eine sogenannte Kreuzallergie (Kapitel 1.5, S. 6) auf Pollen oder Nahrungsmittel verwandter Pflanzen wie Hasel, Karotte, Apfel, Kirsche, Erdbeere, Sellerie, Kiwi etc. (Bergmann et al., 2020, 2016; Biedermann et al., 2019; Henzgen et al., 2008).

1.2 Allergische Reaktion

Wenn von einer Allergie gesprochen wird, ist in der Regel die so genannte Überempfindlichkeit Typ I (IgE-vermittelte Soforttyp-Allergie) gemeint (Murphy et al., 2018). Dabei wird nach erstmaligem Kontakt das Antigen bzw. das Allergen von einer antigen-präsentierenden Zelle (APC, *antigen-presenting cell*) aufgenommen und proteolytisch verarbeitet (Abb. 1). Die Peptide des Antigens werden von der APC auf den *major histocompatibility complex class II* (MHC II) (beim Menschen HLA-System genannt: humanes Leukozyten-Antigen-System) präsentiert. Wenn eine *cluster of differentiation 4* (CD4)⁺ T-Helferzelle (T_H) diesen Komplex erkennt, wird sie aktiviert und transformiert zu einer Effektor-T-Zelle (z. B. T_H1- oder T_H2-Zelle). Damit die B-Zelle zu einer Antikörper sekretierenden Plasmazelle transformieren kann, muss die B-Zelle zum einen Epitope des Antigens mittels des B-Zell-Rezeptors erkennen, zum anderen muss sie durch eine Effektor-T-Zelle aktiviert werden. B-Zellen, welche durch T_H1-Zellen über Interferon-γ aktiviert wurden, produzieren Immunoglobulin (Ig)G. B-Zellen, welche durch T_H2-Zellen über Interleukin 4 und 13 aktiviert wurden, produzieren IgE (Fitzsimmons et al., 2014; Murphy et al., 2018; Rothermel et al., 1991). Der Grund, weshalb Antigene eine IgG- oder IgE-Produktion verursachen, ist noch nicht vollständig geklärt. Während IgG keine Allergie verursachen, kann das IgE jedoch im Gegensatz zu IgG an Mastzellen binden. Ein zweiter Kontakt mit dem Allergen aktiviert die Mastzellen und führt zur Freisetzung von inflammatorischen Mediatoren (Murphy et al., 2018; Wills-Karp, 1999). Die Folge sind typische allergische Symptome wie allergische Rhinokonjunktivitis, Asthma, atopische Dermatitis und Urtikaria, Kontaktekzeme oder im schlimmsten Fall ein anaphylaktischer Schock (Murphy et al., 2018).

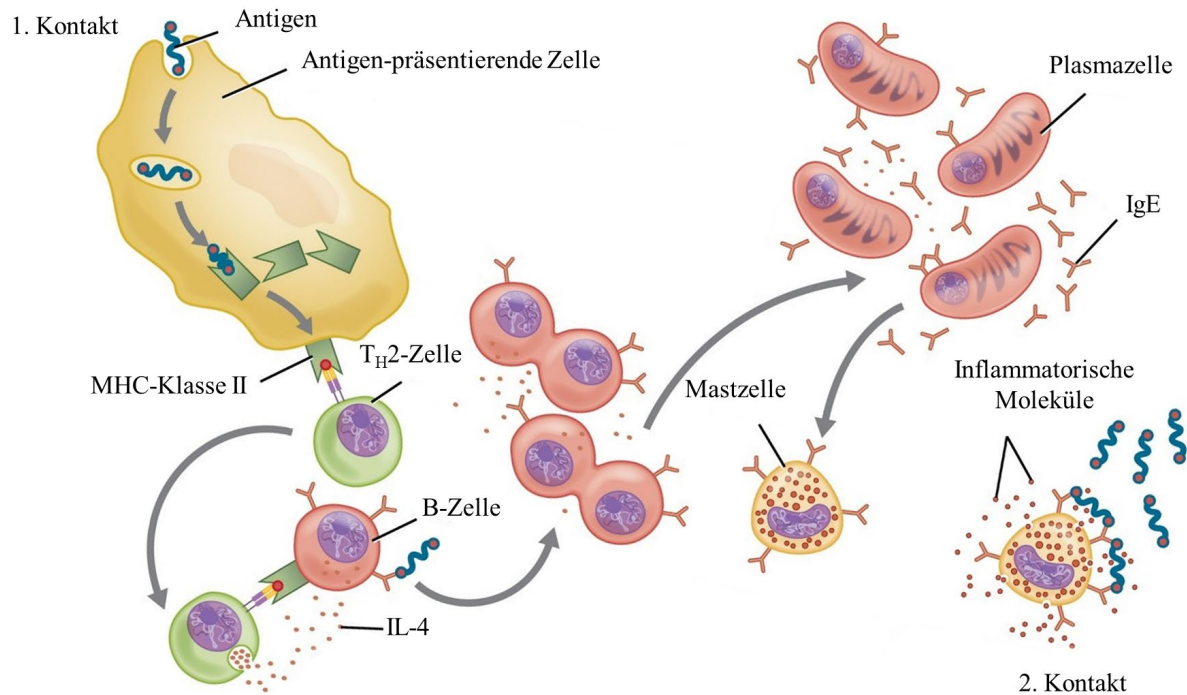


Abb. 1: Schematische Darstellung der Typ I Hypersensitivität. Nach dem ersten Kontakt mit dem Allergen präsentiert die APC der $CD4^+$ -Helferzelle ein Epitop des Allergens, welche darauf zur T_H2 -Zelle transformiert. Die T_H2 -Zelle bindet an die B-Zelle mit einem entsprechenden Rezeptor und schüttet Interleukine aus, wodurch die B-Zelle aktiviert wird. Diese proliferiert und transformiert zur Plasmazelle, welche IgE produziert. Die IgE binden an die *fragment crystallizable* (Fc)-Rezeptoren der Mastzellen, wodurch nach zweitem Kontakt mit dem Allergen inflammatorische Mediatoren freigesetzt werden. Die Darstellung stammt von OpenStax (<https://openstax.org/books/microbiology/pages/19-1-hypersensitivities>; Parker et al., 2016) und wurde modifiziert.

1.3 Proteinfamilien und Nomenklatur der Allergene

Auf der Datenbank AllFam werden Allergene in ihre Proteinfamilien (Abb. 2) eingeordnet (<http://www.meduniwien.ac.at/allfam/> und <http://www.allergen.org/>) (Radauer et al., 2008). 8% der Allergene können der Familie der Prolamine zugeordnet werden (Radauer et al., 2008). Ein bekannter Vertreter ist Ara h 6 aus der Erdnuss, ein starkes Anaphylatoxin (Suhr et al., 2004). Weitere 6% der Allergene weisen eine EF-Hand-Domäne auf, z.B. Bet v 4 aus der Birke (Magler et al., 2010; Radauer et al., 2008). Die Familie der Profilinen wie Bet v 2 und die Familie der Tropomyosine wie Pen a 1, das Hauptallergen der braunen Garnele, haben je einen Anteil von 5% (Radauer et al., 2008; Reese et al., 1997; Rossi et al., 1996). Etwa 4% der Allergene stammen aus der Familie der Cupine, wie z.B. das Sojaallergen Gly m 6 (Radauer et al., 2008; Schiller et al., 2014).

Einer weiteren Proteinfamilie, den sog. PR-10 Proteine (*pathogen-related proteins class 10*) können ca. 3% der Allergene zugeordnet werden (Jain und Kumar, 2015; Walter et al., 1990). PR-10 Proteine werden als Folge von abiotischem und biotischem Stress gebildet (Biedermann et al., 2019; Breiteneder et al., 1989; Breiteneder und Kraft, 2023). Die Unterfamilie der PR-10-ähnlichen Proteine (*PR-10-like*) wird dagegen meist konstitutiv exprimiert und enthält fast alle Bet v 1 homologen Allergene (Breiteneder und Kraft, 2023). Der bekannteste und am besten untersuchte Vertreter ist Bet v 1, das Hauptpollenallergen der Birke (Biedermann et al., 2019; Breiteneder et al., 1989; Breiteneder und Kraft, 2023). Bet v 1-homologe Allergene finden sich sowohl in eng verwandten Bäumen wie Erle, Hasel und Eiche als auch in entfernt verwandten Pflanzen wie Karotte, Sellerie, Apfel, Erdbeere usw. (Biedermann et al., 2019; Breiteneder et al., 1989; Breiteneder und Kraft, 2023). Allerdings sind nicht alle PR-10 Proteine Allergene. Beispielsweise die (s)-Norcoclaurin-Synthase (NCS) aus der Gelben Wiesenraute, die den ersten Schritt der Benzylisoquinolin-Synthese katalysiert, besitzt zwar die typische PR-10 Struktur, ist aber nicht allergen (Lee und Facchini, 2010).

Seit 2007 regelt die *World Health Organization/ International Union of Immunological Societies* (WHO/IUIS) Allergen-Nomenklatur-Sub-Komitee die Benennung von Allergenen (Chapman et al., 2007). Zunächst wird ein Allergen mit den ersten drei Buchstaben der Gattung (*betula*) gefolgt vom ersten Buchstaben der Art (*verrucosa*) benannt. Die nachfolgende Zahl (Bet v 1) gibt in der Regel die numerische Reihenfolge der Allergenidentifikation an. Eine weitere Zahl gruppiert die Allergene in so genannte Isoallergene (Bet v 1.01). Isoallergene haben nahezu das gleiche Molekulargewicht und weisen untereinander eine Aminosäuresequenzidentität von mindestens 67% auf. Die Isoallergene werden wiederum durch eine weitere Nummer in Varianten unterteilt (Bet v 1.0101). Die Varianten weisen untereinander eine Sequenzidentität von mindestens 90% auf (Chapman et al., 2007).

Damit ein Protein offiziell als Allergen anerkannt wird, muss das natürliche oder rekombinante Allergen in gereinigter Form vorliegen und seine Expression auf mRNA- oder Proteinebene nachgewiesen werden. Zudem müssen IgE-Bindungstests an einer ausreichend großen Anzahl klinisch diagnostizierter Patienten durchgeführt werden, wobei das Allergen IgE aus mindestens fünf Seren binden sollte (Pomés et al., 2018).

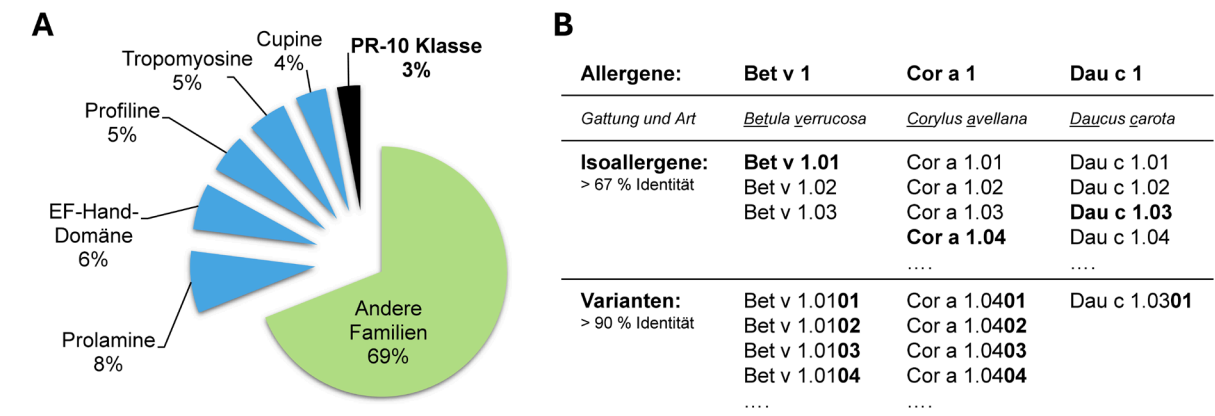


Abb. 2: Proteinfamilien und Nomenklatur der Allergene. (A) Allergenfamilien. Blau: Familie der Prolamine, EF-Hand-Domäne, Profiline, Tropomyosine, Cupine. Grün: Andere Familien. Schwarz: Familie der PR-10 Proteine. (B) Erklärung der Allergennomenklatur anhand von Bet v 1, Cor a 1 und Dau c 1 (<http://www.allergen.org/>) (Radauer et al., 2008).

1.4 Epitope

Epitope sind Oberflächen eines Antigens, die von einem Antikörper gebunden werden (Aalberse, 2000). Welche strukturellen Eigenschaften ein Protein zu einem Allergen machen, ist bis heute nicht vollständig verstanden. Generell unterscheiden man lineare und konformationelle Epitope. Lineare Epitope – auch kontinuierliche Epitope genannt – werden sowohl von T-Zell-Rezeptoren als auch von B-Zell-Rezeptoren gebunden (Kapitel 1.2, Abb. 1, S. 2) und bestehen aus etwa sechs bis zehn aufeinanderfolgenden Aminosäuren (Meno, 2011). Konformationelle Epitope werden ausschließlich von B-Zell-Rezeptoren erkannt. Sie bestehen aus Aminosäuren, die in der nativen dreidimensionalen Struktur des Proteins räumlich nahe beieinander liegen, jedoch in der Primärstruktur weit voneinander entfernt sein können (Meno, 2011). In Bet v 1 wurde eine hochkonservierte Glycin-reiche Schleife (auch „P-Loop“ genannt) zwischen den Aminosäurepositionen 46 und 54 als ein wichtiges konformationelles Epitop identifiziert (Abb. 3 A). Die Co-Kristallisation offenbarte damals die Komplexstruktur von Bet v 1.0112 mit einem Fab-Fragment des monoklonalen IgG₁-Maus-Antikörper BV16 (Mirza et al., 2000). Insbesondere E45 erwies sich als wichtig für die IgE-Erkennung (Abb. 3 B). Im Vogelkirsch-Allergen Pru ac 1 (*Prunus avium*) verringerte die Mutation von E45 zu Tryptophan die IgE-Bindung. Dagegen erhöhte in Api g 1.0101 aus Sellerie (*Apium graveolens*) der Austausch von K44 zu Glutamat die IgE-Bindung (Neudecker et al., 2003). Die Allergenität und die IgE-Bindung von Api g 1.0101 aus Sellerie konnte tatsächlich durch den Aminosäureaustausch K44E (entspricht E46 in Bet v 1) erhöht werden (Neudecker et al., 2003).

Die weitere strukturelle und allergologische Charakterisierung von Allergenen und Identifizierung neuer Epitope ist für das Verständnis der Allergie unverzichtbar.

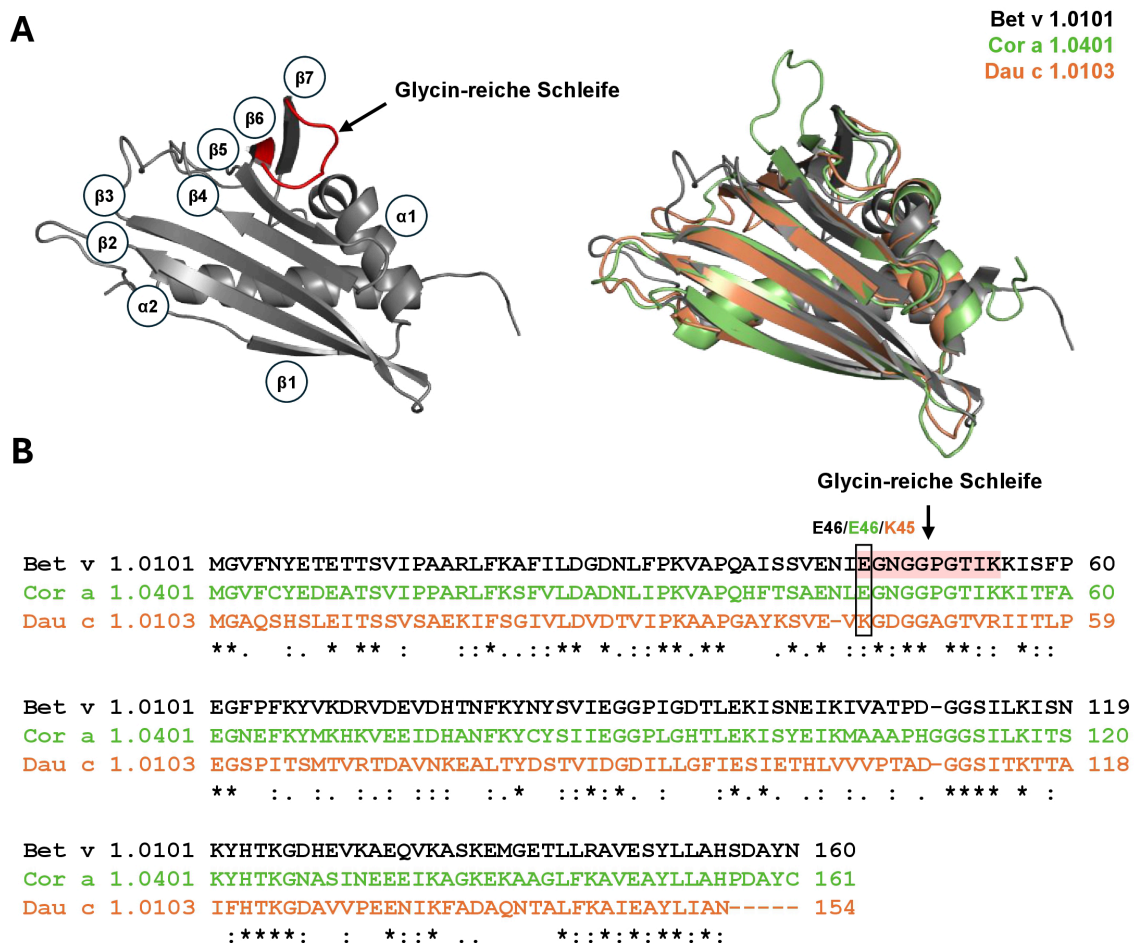


Abb. 3: Struktur von Bet v 1-homologen Allergenen. (A) Struktur von Bet v 1.0101 (PDB:1B6F) und Superposition mit Cor a 1.0401 (PDB:6GQ9) und Dau c 1.0103 (2WQL) (Jacob et al., 2019; Marković-Housley et al., 2009). (B) Alignment von Bet v 1.0101, Cor a 1.0401 und Dau c 1.0103. Die Zeichen unterhalb der Sequenzen bezeichnen stark konservierte (*), konservierte (:) und semikonservierte (.) Aminosäuren. Die schwarze Box markiert E46 in Bet v 1.0101 und Cor a 1.0401 bzw. K45 in Dau c 1.0103.

1.5 Kreuzallergien

Die dreidimensionale Struktur aller Bet v 1-homologen Allergene ist trotz teilweise geringer Sequenzidentität nahezu identisch (Fernandes et al., 2013) (Abb. 3A). Die Struktur besteht aus einem siebensträngigen antiparallelen β -Faltblatt, einer langen C-terminalen α -Helix und einer kurzen V-förmigen α -Helix, die zusammen einen amphiphilen Hohlraum bilden (Gajhede et al., 1996; Jacob et al., 2019; Marković-Housley et al., 2003; Seutter von Loetzen et al., 2012). Dieser Hohlraum ermöglicht es vielen Bet v 1-homologen Proteinen, kleine Moleküle wie

Flavonoide, Steroide, Cytokinine oder Fettsäuren zu binden (Gajhede et al., 1996; Jacob et al., 2019; Marković-Housley et al., 2003; Seutter von Loetzen et al., 2012). Die strukturelle Ähnlichkeit der PR-10 Allergene ist die Ursache für die Entwicklung einer IgE-Kreuzallergie (Brazhnikov et al., 2023). Patienten, die am oralen Allergiesyndrom (OAS) oder *pollen food syndrom* (PFS) in Form von Rhinitis und Kratzen, Juckreiz und Schwellungen in Mund, Rachen oder Lippen leiden, sensibilisieren sich zunächst gegen das Hauptpollenallergen Bet v 1 (Biedermann et al., 2019; Breiteneder und Clare Mills, 2005; Breiteneder und Ebner, 2000; Calamelli et al., 2021). Später entwickeln sich häufig Kreuzallergien gegen Bet v 1-homologe Proteine aus Hasel- und Erlenpollen, aber auch aus Nahrungsmitteln wie Karotte, Apfel, Kirsche, Kiwi oder Pfirsich (Biedermann et al., 2019; Breiteneder und Clare Mills, 2005; Breiteneder und Ebner, 2000; Calamelli et al., 2021). Dabei erkennen IgE-Antikörper, die ursprünglich gegen Bet v 1 gerichtet sind, ähnliche Epitope auf den Bet v 1-homologen Proteinen aus den anderen Pflanzen und verursachen eine allergische Reaktion (Geroldinger-Simic et al., 2011).

1.6 Allergiediagnostik

Zur Diagnose einer Allergie gegen ein bestimmtes Protein stehen verschiedene *in vivo*- und *in vitro*-Testverfahren zur Verfügung. Zu den *in vivo*-Tests zählen insbesondere Hauttests, die eine schnelle und weitverbreitete Methode zur Identifikation von Allergien gegen verschiedene Allergenextrakte darstellen. Beim häufig angewendeten Prick-Test wird eine geringe Menge des Allergens auf die leicht eingeritzte Haut aufgebracht um eine kleine lokale allergische Reaktion zu provozieren (Ownby, 1988; Renz et al., 2009). Zusätzlich können auch nasale, orale und bronchiale Provokationen durchgeführt werden, um eine Allergie zu diagnostizieren. Allerdings besteht bei *in vivo*-Tests stets das Risiko starker allergischer Reaktionen bis hin zur Anaphylaxie (Ownby, 1988).

Ein klassisches *in vitro*-Verfahren zur Allergiediagnose ist der Immunoblot. Dabei werden allergene Proteine zunächst mittels Gelelektrophorese aufgetrennt und anschließend auf eine feste Phase übertragen und fixiert (Western Blot) (Madamanchi und Runge, 2001). Nach Zugabe von Patientenserum kann das im Serum befindliche IgE an das Allergen binden. Zur Detektion werden sekundäre Anti-humane-IgE-Antikörper zugegeben, die an einen enzymatischen Marker wie Meerrettichperoxidase (HRP, *horseradish peroxidase*) oder

alkalische Phosphatase (AP) gekoppelt sind und eine Farbreaktion katalysieren, die kolorimetrisch nachgewiesen werden kann (Ey und Ashman, 1986; Wuhler et al., 2005).

In einem anderen *in vitro*-Test, dem *enzyme-linked immunosorbent assay* (ELISA), wird Allergikerserum zu einer mit dem zu testenden Allergen beschichteten Mikrotiterplatte gegeben. Das Serum-IgE kann an das immobilisierte Allergen binden und über einen enzymgekoppelten sekundären Anti-IgE-Antikörper ähnlich wie beim Immunoblot quantifiziert werden (Konstantinou, 2017). Eine spezielle Form des ELISA ist der *enzyme-allergo-sorbent-test* (EAST), der speziell für die Allergiediagnostik entwickelt wurde und inzwischen den früher verwendeten *radio-allergo-sorbent-test* (RAST) abgelöst hat (Griese und Nicolai, 2010; Ownby, 1988; Renz et al., 2009).

Der ELISA-Kreuzinhibitionsversuch kann Informationen über die Kreuzreaktivität von IgE-Antikörpern zwischen strukturell ähnlichen Allergenen liefern (Abb. 4) (Mills et al., 2006).

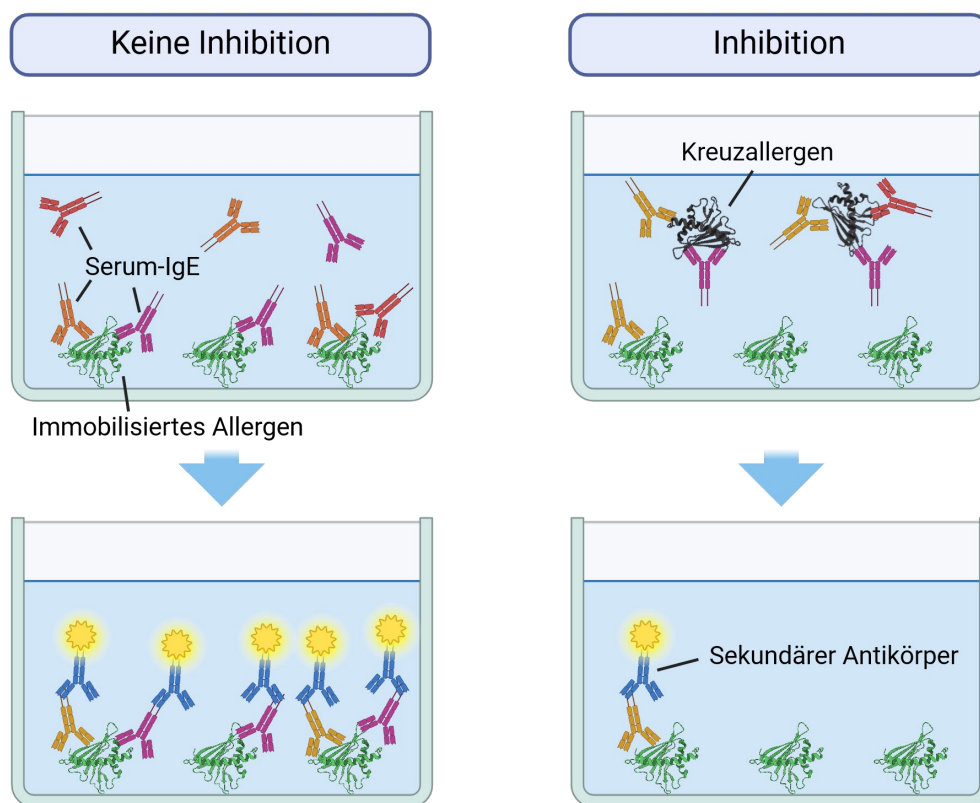


Abb. 4: ELISA-Inhibitionsversuch. Links: IgE aus Allergikerserum bindet an immobilisiertes Allergen. Rechts: Das Allergikerserum wird mit einem potenziellen Kreuzallergen vorinkubiert. Sind kreuzreaktive IgE im Serum enthalten binden diese an das Kreuzallergen und können nicht mehr mit dem immobilisierten Allergen wechselwirken, wodurch weniger immobilisiertes Allergen mit einem sekundären Antikörper nachgewiesen werden kann.

In einem *mediator release assay* (MRA) wird dagegen die Freisetzung bestimmter Mediatoren (z.B. Histamin oder β -Hexosaminidase) aus Mastzellen oder Basophilen nach Zugabe von Allergikerserum und nach Kontakt mit einem Allergen gemessen (Abb. 5). Humanisierte basophile Leukämie-Ratten-Zellen (RBL, *rat basophilic leukemia*), die den humanen hochaffinen IgE-Rezeptor der α -Kette (Fc ϵ R1) exprimieren, werden zunächst mit den im Serum vorhandenen IgE sensibilisiert. Nach Zugabe des Allergens kommt es zu einer Quervernetzung zwischen zwei IgE-Rezeptoren und dem Allergen, wodurch die Freisetzung von Mediatoren ausgelöst wird. Die u.a. freigesetzte β -Hexosaminidase hydrolysiert ein zugegebenes Substrat und kann so indirekt photometrisch bestimmt werden (Kaul et al., 2007; Schwartz et al., 1981; Siraganian et al., 1982; Vogel et al., 2005).

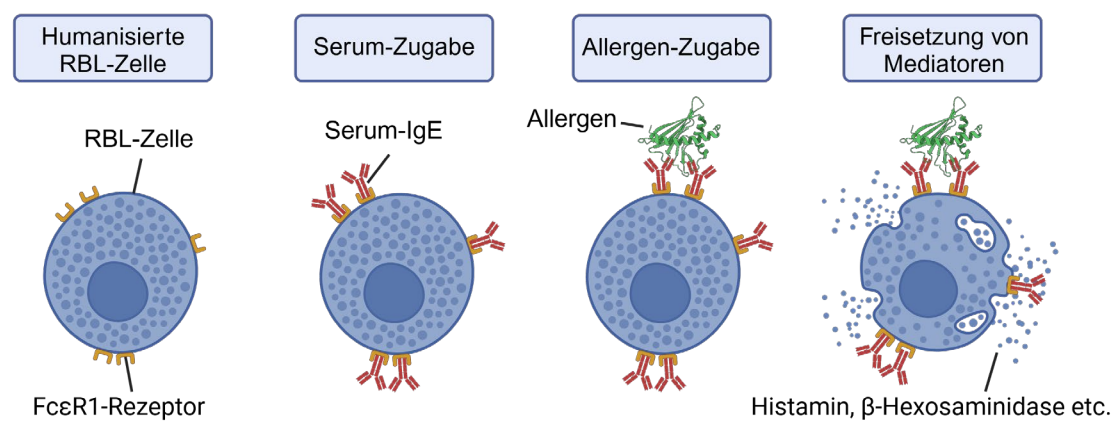


Abb. 5: Mediatorfreisetzungstest. Humanisierte RBL-Zellen, die den humanen Fc ϵ R1-Rezeptor exprimieren, werden mit IgE aus Allergiker-Serum sensibilisiert. Das anschließend zugegebene Allergen bindet an zwei IgE-Antikörper. Durch Quervernetzung kommt es zur Degranulation und zur Freisetzung von Mediatoren, die über die u.a. freiwerdende β -Hexosaminidase stellvertretend nachgewiesen werden können

1.7 Behandlung von Allergien

Neben der medikamentösen Behandlung zur Bekämpfung der allergischen Symptome dient die allergenspezifische Immuntherapie (AIT) dazu, die allergische Erkrankung langfristig zu reduzieren oder zu heilen. Grundsätzlich unterscheidet man zwischen der subkutanen (SCIT, *subcutaneous immunotherapy*) und der sublingualen Immuntherapie (SLIT). Bei der SCIT wird ein niedrig dosierter Allergenextrakt unter die Haut gespritzt, während bei der SLIT der Extrakt in Form von Tabletten oder Tröpfchen unter die Zunge gegeben wird. Ziel ist es dabei, den Körper an eine bestimmte Menge des Allergens zu gewöhnen, um eine starke allergische Reaktion bei

Kontakt zu minimieren oder zu verhindern (Holzhauser et al., 2020; Jutel et al., 2016; Lenzen-Schulte, 2018). Die AIT verringert die T_H2 -Aktivität und erhöht zugleich die T_H1 -Antwort. Die Produktion von IgG4 nimmt zu und führt zu einer Konkurrenz um die Bindungsstellen des Allergens. Infolgedessen können weniger Allergene von IgE gebunden werden und eine allergische Reaktion auslösen (Jutel et al., 2016).

Biologische Allergenextrakte werden sowohl in der Diagnostik (Kapitel 1.6, S. 7) als auch in der Immuntherapie eingesetzt. Eine quantitative und qualitative Analyse dieser Extrakte ist daher unerlässlich, um einheitliche Chargen und eine standardisierte Therapie zu gewähren und im schlimmsten Fall schwerwiegende Nebenwirkungen zu vermeiden. Ein Problem der Extrakte besteht darin, dass die Allergenzusammensetzung aus einer Mischung von Isoallergenen und Varianten besteht, die je nach Umweltbedingungen und Extraktionsverfahren variieren und sich von Hersteller zu Hersteller unterscheiden können. Auch andere unbeabsichtigt extrahierte Moleküle wie Allergenliganden können die Therapie beeinflussen (Holzhauser et al., 2020). Darüber hinaus ist eine Sensibilisierung des Patienten gegen ein neues Protein im Extrakt nicht auszuschließen (Pauli et al., 2008).

Um die Wirksamkeit der Immuntherapie zu erhöhen, wird bereits seit etwa 30 Jahren an der AIT mit rekombinanten Allergenen geforscht. Die Identifizierung und Charakterisierung vieler Allergene einschließlich ihrer DNA- oder Proteinsequenz macht es möglich, diese Allergene rekombinant in Bakterien zu exprimieren und anschließend zu reinigen. Der Patient könnte mit einer individuell abgestimmten Mischung rekombinanter Allergene behandelt werden und spezifisch gegen bestimmte Allergene hypersensibilisiert werden. Da jedoch längst nicht alle Allergene bekannt sind, ist die Identifizierung und Charakterisierung neuer, noch unbekannter Allergene, Isoallergene und Varianten nicht nur unverzichtbar für eine präzise allergologische Diagnostik, sondern auch essenziell, um in Zukunft eine wirkungsvolle und sichere Therapie zu gewährleisten (Holzhauser et al., 2020; Valenta et al., 2011; Zhernov et al., 2019).

1.8 Rolle von PR-10 Proteinen und Flavonoiden

PR-10 Proteine werden in der Pflanze als Reaktion auf abiotischen Stress (z. B. Trockenheit, Verwundungen, UV-Strahlung) und auf biotischen Stress (z. B. Pathogenbefall durch Pilzen, Bakterien oder Viren) vermehrt exprimiert (Agarwal und Agarwal, 2014; Jain und Kumar, 2015). Alle PR-10 Proteine haben eine sehr ähnliche strukturelle Faltung und besitzen eine

amphiphilen Hohlraum, der es ihnen ermöglicht eine Vielzahl verschiedener Liganden zu binden. Daher wird die Funktion der PR-10 Proteine vor allem dem Transport, der Lagerung und der Bindung von Liganden wie Flavonoiden, Steroiden, Cytokinen oder Fettsäuren zugeschrieben (Fernandes et al., 2013; Gajhede et al., 1996; Marković-Housley et al., 2003; Neudecker et al., 2003).

Eine weitverbreitete Stoffgruppe sind die Flavonoide. Flavonoide bestehen aus einem Phenylring A, der an einen heterozyklischen Pyranring C gebunden ist. An die Kohlenstoffposition 2 dieses Pyranrings ist ein weiterer Phenylring B kovalent gebunden (Abb. 6). Flavonoide können weiter in die Klassen der Flavone, Flavonole, Flavanone, Isoflavanone, Chalkone und Anthocyanidine unterteilt werden (Panche et al., 2016). Flavonoide sind häufig an den Positionen 3, 5, 7, 2, 3', 4' und 5' hydroxyliert, seltener methyliert oder acetyliert. Zusätzlich sind viele Flavonoide an den Positionen 3 und 7 glykosyliert, was zum einen der Erhöhung der Löslichkeit und zum anderen der Speicherung von Zucker als Energielieferant dient (Crozier et al., 2006; Jain und Kumar, 2015; Kumar und Pandey, 2013; Miller et al., 1999). Flavonoide dienen z. B. dem Schutz vor UV-Strahlung, aber bestimmen auch die Farbe von Blüten oder das Aroma von Früchten. Anthocyane sind meist für die roten, blauen und violetten Farben von Blüten verantwortlich. Ähnlich wie Cytokine können auch Flavonoide in Pflanzen eine hormonelle Wirkung entfalten. Das Aglykon Kaempferol sorgt beispielsweise für das Wachstum des Pollenschlauchs nach der Befruchtung. Durch Zugabe von Kaempferol konnten Flavonol-defiziente Petunienpollen, die nicht mehr in der Lage waren zu keimen und den Pollenschlauch aufrechtzuerhalten, wieder keimen und einen Pollenschlauch ausbilden (Vogt und Taylor, 1995).

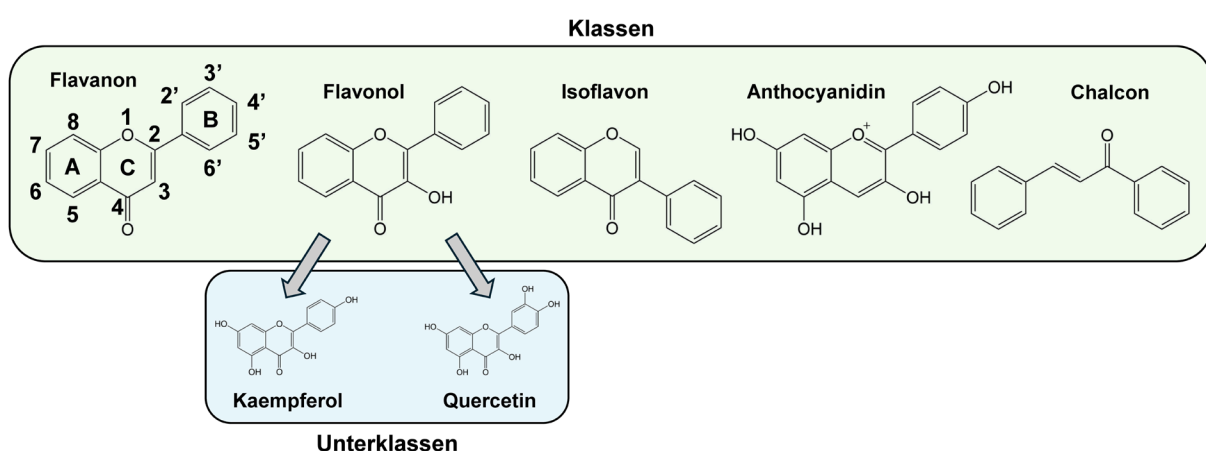


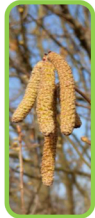
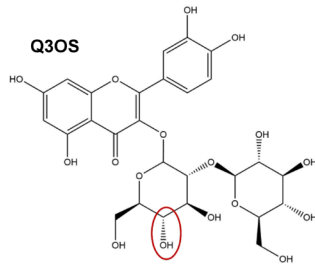
Abb. 6.: Klassen und Unterklassen von Flavonoiden.

Chromatographie (z.B. HPLC), Massen- und NMR-spektroskopie sind wichtige Methoden um kleine Moleküle wie Flavonoide zu identifizieren werden. Bei der *ultra-high-pressure liquid chromatography/ quadrupole-time of flight - tandem mass spectrometry* (UHPLC/QTOF-MS/MS) werden z. B. Extrakte zunächst mittels UHPLC hoch aufgelöst getrennt und anschließend die Ladung und Masse der ionisierten Moleküle detektiert. Eine zusätzliche Fragmentierung der Moleküle kann weiteren Aufschluss über ihre Zusammensetzung geben. Glykosylierte Flavonoide neigen dabei in ihr Glykon und Aglykon zu fragmentieren. Des Weiteren bietet die HPLC die Möglichkeit Moleküle mittels Standards (als Referenz) zu identifizieren, sofern diese kommerziell erhältlich sind. Ist ein Molekül in großen Mengen vorhanden, kann auch NMR-Spektroskopie wertvolle Informationen zur Identifizierung liefern (*Lebensmittelchemie*, 2008).

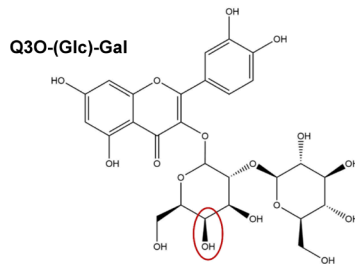
1.9 Spezifische Ligandenbindung von Bet v 1 und Cor a 1

Für Bet v 1.0101 konnte aus Birkenpollen der Ligand Quercetin-3-O-Sophorosid (Q3OS) identifiziert werden (Seutter von Loetzen et al., 2014). Für Cor a 1.0401 wurde Quercetin-3-O-(2''-O-β-D-Glukopyranosyl)-β-D-Galaktopyranosid (Q3O-(Glc)-Gal) als ein physiologischer Ligand aus Haselpollen gereinigt (Jacob et al., 2019) (Abb. 7). Die Bindung der Liganden wurde durch NMR-Spektroskopie gezeigt (Ballmer-Weber et al., 2001; Neudecker et al., 2003). Interessanterweise binden Bet v 1.0101 und Cor a 1.0401 nur ihren eigenen Liganden, nicht aber den des anderen, obwohl Q3OS und Q3O-(Glc)-Gal Stereoisomere sind und sich nur in der Orientierung der 4''-Hydroxylgruppe des Zuckers unterscheiden (Abb. 7) (Ballmer-Weber et al., 2001; Neudecker et al., 2003). Bisher wurde in Haselpollen nur Varianten von Cor a 1.01 nachgewiesen. Cor a 1.0401 dagegen war bislang nur in der Haselnuss nachgewiesen worden, während der Ligand Q3O-(Glc)-Gal aus Pollen isoliert wurde (Lüttkopf et al., 2002). Daher wurde vermutet, dass Cor a 1.0401 – bereits in der weiblichen Blüte exprimiert – den Liganden nach der Bestäubung bis zur Befruchtung speichert und damit das Wachstum des Pollenschlauchs initialisiert (Jacob et al., 2019).

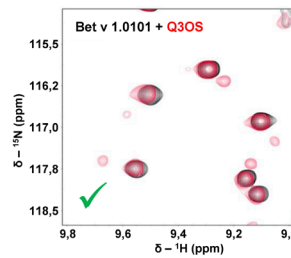
Birkenpollen



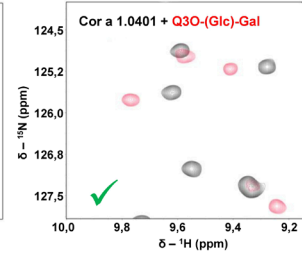
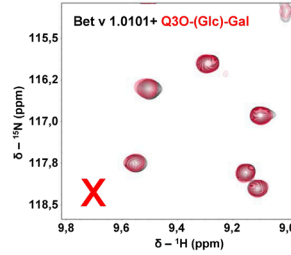
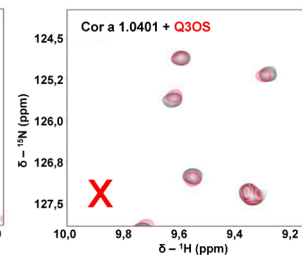
Haselpollen



Bet v 1.0101 (Birke)



Cor a 1.0401 (Hasel)



Seutter von Loetzen et al. (2014)

Jacob et al. (2019)

Abb. 7: Spezifische Ligandenbindung von Bet v 1.0101 und Cor a 1.0401. Links: Birken- und Haselkätzchen. Mitte: Q3OS, der natürliche Ligand von Bet v 1.0101. Q3O-(Glc)-Gal, der natürliche Ligand von Cor a 1 (Jacob et al., 2019; Seutter von Loetzen et al., 2014). Der stereochemische Unterschied zwischen Q3OS und Q3O-(Glc)-Gal ist mit einem roten Kreis markiert. Rechts: Ausschnitte der NMR-Spektren von Bet v 1.0101 und Cor a 1.0101 ohne Ligand (schwarz) und mit Ligand (rot). NMR-Spektren modifiziert nach Seutter von Loetzen et al. (2014) und Jacob et al. (2019) (Jacob et al., 2019; Seutter von Loetzen et al., 2014).

Wie diese beiden Liganden gebunden werden und woher diese Spezifität kommt, ist bisher nicht geklärt. Bisher gibt es nur wenige Komplexstrukturen mit einem physiologisch relevanten Liganden, die diese Frage beantworten könnten. Die Elektronendichte einer Komplexstruktur von Bet v 1 mit Naringenin zeigt zwar, dass der Ligand in der Tasche gebunden wird, gibt aber keinen Aufschluss darüber, wie andere Flavonoide, z. B. glykosylierte Flavonoide, gebunden werden (Kofler et al., 2012). Die NMR-Spektroskopie kann zwar Aminosäuren identifizieren, die direkt oder indirekt an der Bindung beteiligt sind, gibt aber keine Information darüber, in welcher Orientierung der Ligand gebunden wird.

Ein *isotope filtered/edited nuclear overhauser enhancement spectroscopy* (NOESY)-Experiment kann dagegen Aufschluss über eine Komplexstruktur geben (Bax und Grzesiek, 1993; Cavanagh et al., 2007). Beim *nuclear overhauser effect* (NOE) entsteht zwischen zwei räumlich beieinander liegenden Protonen ($<5 \text{ \AA}$) eine magnetische Wechselwirkung (Bax und Grzesiek, 1993; Cavanagh et al., 2007). Diese liefern Informationen über den räumlichen Abstand von Atomkernen. Beim NOESY-Experiment können die Wechselwirkungen zwischen einem ^{13}C ^{15}N -markierten Protein und einem unmarkierten Liganden beobachtet werden,

während intramolekulare NOEs des Proteins oder des Liganden unterdrückt werden (Bax und Grzesiek, 1993; Cavanagh et al., 2007). Die Abstandsinformationen können schließlich für die Erstellung eines Protein-Liganden-Komplexen verwendet werden.

Die Identifizierung neuer Liganden und die Charakterisierung der Bindung ist essentiell, um die physiologische Rolle der PR-10 Proteine in Pflanzen besser zu verstehen und um in Zukunft mögliche Auswirkungen der Liganden auf das Allergiegesehen zu untersuchen.

2 Ziele der Arbeit

Die Charakterisierung neuer Isoallergene und Varianten von PR-10 Allergenen und ihren Liganden ist nicht nur für die medizinische Diagnostik relevant, sondern kann auch den Ursprung der allergenen Eigenschaften eines Proteins klären und Einblicke in seine allgemeine physiologische Funktion geben.

Deshalb sollten zunächst mittels mRNA-Isolation und Reverse Transkriptase Polymerasekettenreaktion (RT-PCR) neue Dau c 1-Isoallergene der Karotte auf mRNA-Ebene identifiziert werden und anschließend die rekombinanten Proteine allergologisch durch Immunoblots, ELISA-Inhibitionsversuchen und MRAs charakterisiert werden. Bislang ist nicht vollständig geklärt, welche strukturellen Epitope der PR-10 Proteine für die Allergenität verantwortlich sind. Daher sollte anschließend ein Aminosäuresequenzvergleich des nicht-allergenigen Dau c 1-like Proteins und der Dau c 1-Isoallergene potenzielle strukturelle Epitope identifiziert werden, die für die IgE-Erkennung wichtig sind.

Auf die gleiche Weise sollten auch neue Isoallergene und Varianten der Hasel identifiziert werden. Darüber hinaus sollte erstmals die Expression aller Cor a 1-Isoallergene in verschiedenen Geweben der Hasel (männliches Kätzchen, Pollen, weibliche Blüte, unreife und reife Nuss) untersucht werden, um Hinweise über ihre physiologische Funktion in der Pflanze zu erhalten.

Bisherige Studien hatten gezeigt, dass PR-10 Proteine in ihrer amphiphilen Tasche etliche, strukturell verschiedene Moleküle unspezifisch binden können (Marković-Housley et al., 2003; Seutter von Loetzen et al., 2012; Jacob et al., 2019; Führer et al., 2021; Gajhede et al., 1996). Für das Birkenpollenallergen Bet v 1 konnte als physiologischer Ligand Q3OS identifiziert werden (Seutter von Loetzen et al., 2014). Für das Haselallergen Cor a 1 wurde Q3O-(Glc)-Gal als physiologischer Ligand festgestellt (Jacob et al., 2019). Bet v 1.0101 und Cor a 1.0401 binden spezifisch nur ihren eigenen Liganden, nicht aber den des anderen Proteins, obwohl sich die Liganden stereochemisch nur in der Orientierung der 4“-Hydroxylgruppe des Zuckers unterscheiden (Jacob et al., 2019; Seutter von Loetzen et al., 2014).

Um die Ligandenbindung von PR-10 Allergenen besser zu verstehen, sollten weitere neue physiologisch relevante Liganden, die von nCor a 1 gebunden werden, identifiziert werden. Dazu sollte in Zusammenarbeit mit Frau Prof. Dr. Baldermann mittels UHPLC/QTOF-MS/MS

nach glykosylierten Flavonoiden, die von nCor a 1 gebunden werden, gesucht werden. Anschließend sollte die Bindung der identifizierten Substanzen an die verschiedenen Cor a 1-Isoallergene mittels NMR-Spektroskopie und ITC nachgewiesen werden. Die aus *isotope filtered/edited* NOESY-Experimenten gewonnenen Abstandsinformationen sollten verwendet werden, um durch *docking*-Analysen Komplexe von Cor a 1-Isoallergenen mit mono- und diglykosylierten Flavonoiden zu generieren, um daraus Aufschluss über die Orientierung und Spezifität der Bindung zu erhalten.

3 Zusammenfassung und Diskussion der Ergebnisse

3.1 Allergenität und IgE-Erkennung neuer Dau c 1-Karottenallergene

Einzelarbeit A: Hendrich, J. M., Wangorsch, A., Rödel, K., Jacob, T., Mahler, V., Wöhrle, B. M., 2023. Allergenicity and IgE Recognition of New Dau c 1 Allergens from Carrot. *Mol. Nutr. Food Res.*, 67 (3), e2200421.

3.1.1 Neue Dau c 1-Isoallergene

Im Gegensatz zu anderen Bet v 1 - homologen Proteinen hat das Karottenallergen Dau c 1 die Fähigkeit Patienten, auch direkt, also Bet v 1 - unabhängig, zu sensibilisieren (Ballmer-Weber et al., 2001). In Kreuzinhibitionsversuchen (Kapitel 1.6, S. 7) konnten zwar die bisher bekannten Dau c 1-Isoallergene durch Zugabe von Bet v 1 vollständig inhibiert werden, das natürliche (n)Dau c 1 (eine Mischung aus verschiedenen Isoallergenen und Varianten) konnte allerdings nicht vollständig inhibiert werden (Ballmer-Weber et al., 2001; Hoffmann-Sommergruber et al., 1999). Dies deutet darauf hin, dass weitere Isoallergene und Varianten (Kapitel 1.3, S. 3) existieren müssen, deren IgE Antikörper nicht mit Bet v 1 kreuzreagieren.

In der Karotte wurde zuletzt das Isoallergen Dau c 1.0401 auf mRNA- und Proteinebene identifiziert (Jacob et al., 2021). Für einen Peptidmassenfingerprint (PMF) wurde nDau c 1 aus Karotten gereinigt, mit Trypsin proteolytisch verdaut und mittels Flüssigchromatographie-Massenspektrometrie (LC-MS, *liquid chromatography-mass spectrometry*) analysiert. Die Peptidsequenzen zeigen, dass es neben den bekannten noch weitere bisher unbekannte Dau c 1-Isoallergene geben muss (Jacob et al., 2020). Um herauszufinden, ob es einzigartige Epitope gibt, die für eine durch Bet v 1 verursachte Kreuzallergie oder eine direkte Sensibilisierung gegen nDau c 1 verantwortlich sind, sollten in dieser Arbeit weitere Dau c 1-Isoallergene und -Varianten identifiziert und allergologisch untersucht werden.

Mit Hilfe von Uniprot Dateneinträge und über *basic local alignment search tool* (BLAST)-Suche konnten diese Peptidsequenzen schließlich unbekannten Dau c 1-Sequenzen zugeordnet werden (Einzelarbeit A, Abb. 1, Tab. S4). Um zu bestätigen, dass die Sequenzen dieser möglichen Isoallergene vollständig, korrekt und tatsächlich in der Karottenwurzel exprimiert werden, wurden diese Gene, wie z.B. das Dau c 1.0401, auf mRNA-Ebene nachgewiesen. So konnte die mRNA von vier neuen Dau c 1-Genen isoliert und nach RT-PCR sequenziert und kloniert werden. Die entsprechenden vier neuen Proteine, genannt Dau c 1.0501, Dau c 1.0601, Dau c 1-like sowie Dau-NCS, wurden schließlich rekombinant in *Escherichia coli* (*E. coli*)

hergestellt und gereinigt. Bis auf Dau-NCS sind diese neuen Proteine sequenziell mit den bekannten Dau c 1-Isoallergen zu 51 – 87% identisch. Obwohl Dau c 1-like 51% Sequenzidentität zu Dau c 1.0101 aufweist, erwies es sich als nicht allergen (Kapitel 3.1.3, S. 19) und wurde daher nur als Dau c 1-like bezeichnet. Dau c 1-NCS ist stimmt auf Aminosäuresequenzebene nur noch bis zu 22% mit den Isoallergenen überein, ist aber zu 53% identisch mit einer NCS aus Kiwi (*Actinia diaphanosticta*) (Sequence-ID PSS32256.1). Obwohl die dreidimensionalen Strukturen der NCS-Proteine den Bet v 1-homologen Proteinen sehr ähnlich sind, sind NCS-Proteine nicht allergen. Außerdem wird ihnen eine enzymatische Funktion in der Biosynthese von Benzylisochinolin nachgesagt (Stadler et al., 1987; Samanani et al., 2004; Lee und Facchini, 2010; Hagel und Facchini, 2013).

3.1.2 Stabilität der neuen Dau c 1-Isoallergene

PR-10 Allergene sind in Lebensmitteln meist nur in roher Form für Allergiker bedenklich. Beim Kochen oder Backen entfalten die Proteine und verlieren in der Regel ihre Allergenität (Mills et al., 2009). Die bisher bekannten Dau c 1-Karottenallergene haben die Besonderheit, dass sie nach Erhitzen bei pH 7 jedoch zurückfalten können und so beim Verzehr weiter allergen sein können. Daher war es von Interesse, das thermische Verhalten der vier neuen Proteine zu untersuchen.

Zunächst wurde durch Gelfiltration und 1D-NMR bestätigt, dass die neu identifizierten, rekombinant hergestellten Dau c 1-Proteine monomer und gefaltet sind (Einzelarbeit A, Abb. S4). Im Circular dichroismus (CD) wurden die vier Dau c 1-Proteine für mindestens 15 min bei 95 °C entfaltet, danach wieder auf 25 °C abgekühlt, wobei sich bis auf Dau-NCS wieder alle Proteine weitgehend zurückfalteten (Einzelarbeit A, Abb. 2). Mittels CD und NanoDSF ergaben die Schmelzkurven von Dau c 1.0501, Dau c 1.0601 und Dau c 1-like ähnlich wie bei den bekannten Dau c 1-Isoallergenen eine Schmelztemperatur (T_m , *melting temperature*) von ca. 60 °C. Dau-NCS hatte einen etwas höheren T_m -Wert von etwa 80 °C. Die bisher bekannten Isoallergene Dau c 1.0104, 0105 und 0301 falten sich fast vollständig zurück, Dau c 1.0601 und Dau c 1-like dagegen nur teilweise. Dau c 1.0501 und Dau-NCS blieben entfaltet. In Nahrungsmitteln mit verarbeiteten Karotten können diese Proteine also möglicherweise noch immer eine allergische Reaktion auslösen. Ob diese vier neuen Proteine überhaupt allergen sind, wurde mit allergologischen Tests untersucht.

3.1.3 Allergenität der neuen Dau c 1-Isoallergene

Die vier neuen Proteine wurden zuerst in Immunoblots (Kapitel 1.6, S. 7) mit 14 Seren von Karottenallergikern mit milden bis schweren Symptomen getestet (Wangorsch et al., 2012). Dau c 1.0501 reagierte schwach auf zwei Seren, wohingegen Dau c 1.0601 mit fünf Seren reagierte (Einzelarbeit A, Abb. S1). Dau c 1-like reagiert mit fünf Seren nur sehr schwach. Dau-NCS zeigte keine Reaktion mit den Seren. Beim Immunoblot liegen die Proteine nach *sodiumdodecylsulfate* (SDS)-Polyacrylamid-Gelelektrophorese (PAGE) und Western-Blot oft entfaltet vor, wodurch nur noch lineare aber keine konformationellen IgE-Epitope mehr erkannt werden können (Jacob et al., 2021); (Okamoto-Uchida et al., 2016).

In einem MRA (Kapitel 1.6, S.7) bindet das freie Allergen an die α -Kette des humanen IgE Rezeptors (Fc ϵ R1) von humanisierten basophilen Leukämiezellen der Ratte (RBL, *rat basophilic leukemia*) und löst die Freisetzung von Mediatoren aus. Die freigesetzte β -Hexosaminidase kann anschließend ein hinzugegebenes Substrat in einer Farbreaktion umsetzen und photometrisch quantifiziert werden (Siraganian et al., 1982; Vogel et al., 2005; Kaul et al., 2007). Das Allergen liegt im Test in nativer Form vor. Sowohl Dau c 1.0501 als auch Dau c 1.0601 zeigten eine starke Freisetzung des Mediators (Einzelarbeit A, Abb. 3). Wohingegen bei Dau c 1-like und Dau-NCS keine Freisetzung beobachtet werden konnte.

In einem ELISA-Inhibitionsversuch (Kapitel 1.6, S. 7) kann speziell die Kreuzreaktivität zwischen Allergenen genauer untersucht werden. Dabei wird ein Allergen wie Dau c 1 immobilisiert und mit Serum inkubiert. Die gebundenen IgE aus dem Serum können über anti-humane Maus-IgE-HRP detektiert werden (Wangorsch et al., 2012). Wird allerdings zuvor das Serum mit einem der neuen Allergene inkubiert und binden die IgE an das neue Allergen, können weniger IgE an das immobilisierte Dau c 1 binden und detektiert werden. Dau c 1.0501 konnte in Inhibitionsversuchen mit fünf verschiedenen Seren Dau c 1.0104 bis zu 80% inhibieren (Einzelarbeit A, Abb. 4). Mit Dau c 1.0601 konnte in drei Inhibitionsversuchen ebenfalls eine Inhibition von 80% erreicht werden und bei einem Serum sogar vollständig inhibieren. Dau c 1.0501 und Dau c 1.0601 wurden folglich offiziell als Allergene von der WHO/IUIS Allergen Nomenklatur Sub-Komitee anerkannt (Pomés et al., 2018). Dau c 1-like und Dau-NCS zeigten auch im Inhibitionsversuch keine Reaktion und werden deswegen nicht als Allergene definiert.

3.1.4 Identifizierung möglicher neuer IgE-Epitope

Die neuen Isoallergene Dau c 1.0501 und Dau c 1.0601 haben zueinander eine Sequenzidentität von etwa 75% und zum bekannten Dau c 1.0101 eine Sequenzidentität von etwa 56 – 58% (Einzelarbeit A, Tab. S4). Interessanterweise ist Dau c 1-like trotz 51% Sequenzidentität zu Dau c 1.0101 nicht allergen.

Möglicherweise sind die IgE-Epitope in Dau c 1-like durch wenige unterschiedliche Aminosäuren verändert und werden von IgE-Antikörper nicht mehr erkannt. Daher wurde in einem Sequenzvergleich zwischen den Dau c 1-Allergenen und dem nicht-allergen Dau c 1-like, nach Aminosäureaustauschen gesucht, die nur im nicht-allergen Dau c 1-like zu finden sind. Für die Identifizierung der Epitope wurden nicht-konservative Austausche als bedeutender als konservative eingestuft, da beim konservativen Austausch die Aminosäureeigenschaft weitgehend unverändert bleibt. Da Dau-NCS zwar wie erwartet nicht allergen ist, sich die Sequenz allerdings zu sehr unterscheidet, ist es ungeeignet, um Epitope zu identifizieren und wurde daher in dem Vergleich nicht mit einbezogen.

Tatsächlich befinden sich fast alle der konservativen und nicht konservativen Austausche an der Proteinoberfläche (Abb. 8 A und B) und bilden zusammen vier neue mögliche Epitope aus. Das erste strukturelle Epitop E1 bildet sich aus den sequenziell entfernten Resten Q17, T18 und T77 und ist bisher in der Literatur nicht beschrieben.

Das zweite Epitop E2 befindet sich in der sog. P-Loop Region und setzt sich aus den Resten K44 und S46 zusammen. Anstatt S46 befinden sich an dieser Position in Bet v 1-homologen Proteinen oft ein Glutamat oder ein Lysin. Diese wurden bereits als wichtig bei der IgE-Bindung beschrieben. Der P-Loop (Kapitel 1.4, S. 5) ist in Bet v 1 homologen Proteinen hoch konserviert und galt daher schon lange als mögliches kreuzreaktives Epitop (Gajhede et al., 1996). In einer Kristallstruktur von Bet v 1 im Komplex mit dem Fab-Fragment eines monoklonalen, murinen IgG1 BC16 bildet der P-Loop mit E46 eine Kontaktfläche mit dem Antikörper aus (Mirza et al., 2000). Beim Allergen Pru ac 1 aus der Vogelkirsche (*Prunus avium*) führte der Austausch des homologen Rests E45 zu Tryptophan zu einer signifikant geringeren IgE-Bindung, ähnlich wie im nicht allergenen Dau c 1-like (Neudecker et al., 2003). Dagegen erhöhte in Api g 1.01 aus Sellerie die Mutation des homologen Rests K44 zu Glutamat die IgE Bindung (Wangorsch et al., 2007). Die Aminosäure S46 in Dau c 1-like ist vermutlich ein Grund, warum Dau c 1-like nicht allergen ist.

Das dritte Epitop E3 wird durch Q64 und V67 gebildet. Das vierte Epitop E4 setzt sich aus N94, P123 und I124 zusammen. Diese beiden Regionen wurden ebenfalls noch nicht als Epitope beschrieben. Insgesamt ist der sequenzielle Vergleich von sich ähnlichen Allergenen und Nicht-Allergenen eine gute Methode, um potenzielle Epitope ausfindig zu machen.

Um zu prüfen, ob diese möglichen Epitope (Abb. 8) wirklich für die IgE-Bindung wichtig sind, wurden Mutationsstudien durchgeführt. Um eine allergene Dau c 1-like Mutante (Dau c 1-like-E1-4) zu erschaffen, wurden die Aminosäuren der Epitope E1-4 durch die Aminosäuren von Dau c 1.0102 ausgetauscht. Der Austausch der Epitope E1-4 in Dau c 1.0102 (Dau c 1.0102-E1-4) mit den denen des Dau c 1-like, sollte dagegen für ein nicht allergenes Protein sorgen. Allerdings waren die Mutanten Dau c 1-like-E1-4 und Dau c 1.0102-E1-4, zu instabil und zeigten daher keine Reaktion im Immunoblot. Um die Stabilität der Mutanten zu erhöhen, wurde in den Mutanten Dau c 1-like-E1 und Dau c 1.0102-E2 nur jeweils ein Epitop ausgetauscht. Während Dau c 1-like-E1 eine erhöhte Reaktion, sollte Dau c 1.0102-E2 eine geringere Reaktion im Immunoblot zeigen. Allerdings konnte in den Immunoblots kein Unterschied zum Wildtyp festgestellt werden. Da jedoch nur wenige Seren zur Verfügung standen, wäre es Ratsam die Mutanten noch an weiteren Seren zu testen.

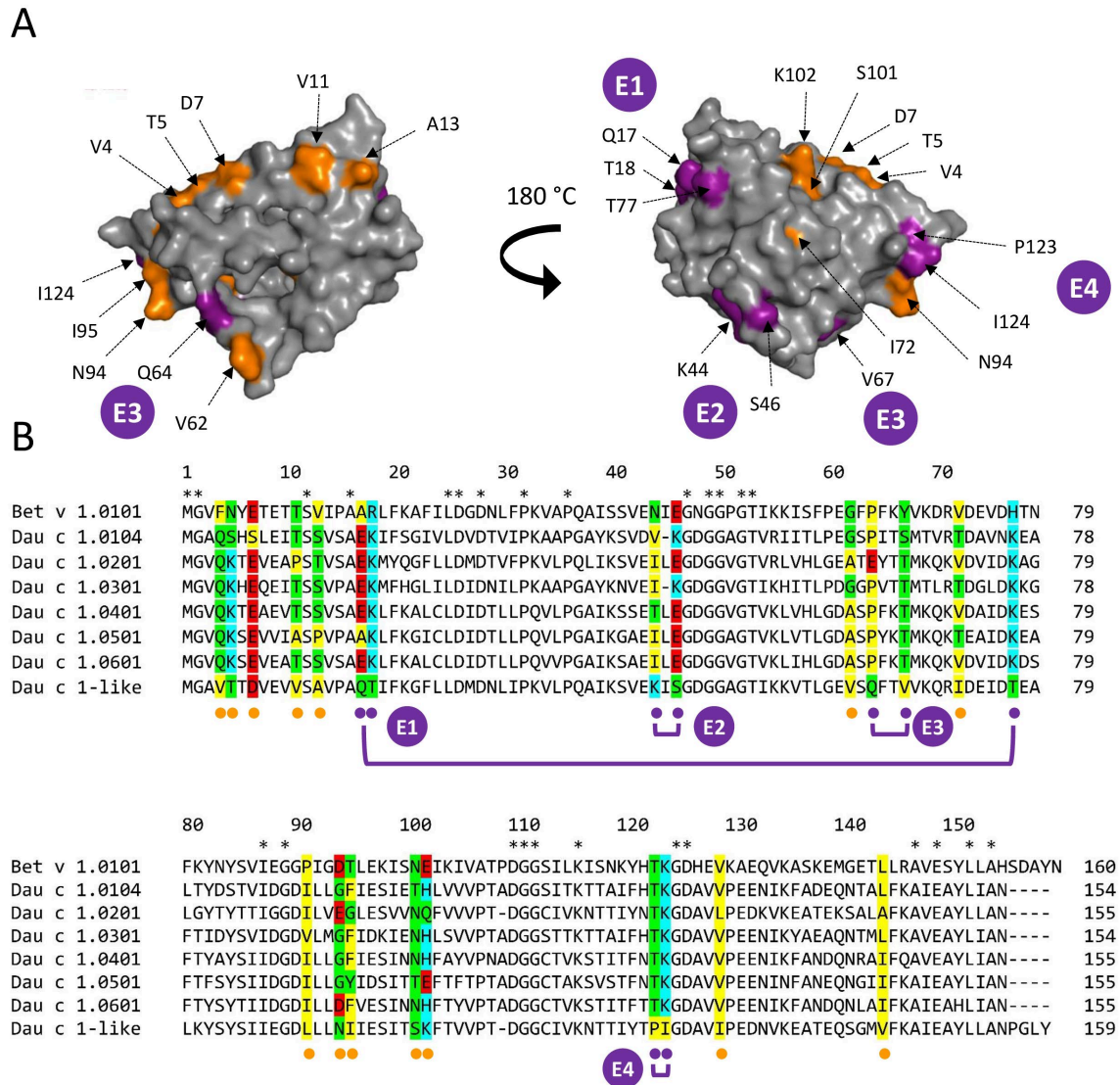


Abb. 8: Nicht allergenes Dau c 1-like offenbart neue mögliche Epitope. (A) Oberflächendarstellung von Dau c 1-like. Struktur wurde mit Phyre2 (Kelley et al., 2015) berechnet. Lila: Für Dau c 1-like einzigartige nicht-konservative Aminosäureaustausche. Orange: Für Dau c 1-like einzigartige konservative Aminosäureaustausche. Epitope 1-4 (E1-4). **(B)** Sequenzalignment von Dau c 1-Isoallergen mit Dau c 1-like. Grün: polar/neutrale Aminosäuren. Gelb: Nicht-polare/hydrophobe Aminosäuren. Cyan: Alkalische Aminosäuren. Rot Saure Aminosäuren. Asterisk: In Dau c 1-Isoallergenen und Dau c 1-like konservierten Aminosäuren. Konservative und nicht-konservative Aminosäureaustausche sind in **(B)** mit einem orangenen oder lila Punkt markiert.

3.2 Allergenität und strukturelle Eigenschaften neuer Cor a 1-Isoallergene aus Hasel, die in verschiedenen Pflanzengeweben identifiziert wurden

Einzelarbeit B: Hendrich, J. M., Reuter, A., Jacob, T., Kara, H., Amer, S., Rödel, K., Wöhrle, B. M., 2024. Allergenicity and Structural Properties of New Cor a 1 Isoallergens from Hazel Identified in Different Plant Tissues. *Sci. Rep.*, 14 (1), 5618.

3.2.1 Neue Cor a 1-Isoallergene in verschiedenen Organen der Hasel

Aus allergologischer Sicht ist die Hasel von Interesse, da Patienten nicht nur den Pollenallergenen ausgesetzt sind, sondern auch mit den Allergenen in der Nuss konfrontiert werden, die in einer Vielzahl von Lebensmitteln verarbeitet wird. (Breiteneder et al., 1993; Lüttkopf et al., 2002). Bisher waren in der WHO/IUIS-Datenbank nur vier Cor a 1-Isoallergene Cor a 1.01 bis Cor a 1.04 vertreten (<http://www.allergen.org/>) (Pomés et al., 2018). Vier Cor a 1.01 Varianten (>90% Identität) wurden bisher nur in Haselpollen identifiziert, während die vier bekannten Varianten von Cor a 1.04 ausschließlich in der Haselnuss gefunden wurden (Breiteneder et al., 1993; Lüttkopf et al., 2002). Die Isoallergene Cor a 1.02 und Cor a 1.03 wurden nur als Gene aus Blatt-DNA nachgewiesen, aber weder auf mRNA- noch auf Proteinebene bestätigt oder allergologisch getestet (Hoffmann-Sommergruber et al., 1997). Ob und in welchen Geweben diese Isoallergene exprimiert werden, war vor Beginn dieser Arbeit nicht umfassend geklärt, kann aber für die Allergenforschung von Bedeutung sein.

In einer Publikation aus dem Jahr 2021 wurde erstmals das Genom der türkischen Hasel „Tombul“ entschlüsselt und offenbarte über BLAST die Präsenz weiterer Cor a 1-Gene (Lucas et al., 2021). In dieser Arbeit wurden deshalb noch nicht bekannte Isoallergene auf mRNA-Ebene identifiziert und ihre Expression in den weiblichen und männlichen Geweben der Hasel (Blüte, unreife Nuss, reife Nuss, Kätzchen und Pollen) untersucht. Dies kann Hinweise darüber geben, ob diese Isoallergene physiologisch unterschiedliche Bedeutungen haben.

Die gesamte RNA wurde aus selbst gesammelten weiblichen Blüten, unreifen und reifen Nüssen, männlichen Blüten (Kätzchen) und Pollen isoliert, die mRNA mittels poly(T)-Primer in cDNA reverse transkribiert und die Cor a 1-Gene mit Isoallergen-spezifischen Primern über eine PCR nachgewiesen (Abb. 9). Vier neue Cor a 1-Isoallergene Cor a 1.0501, 1.0601, 1.0701 und 1.0801 konnten so auf mRNA-Ebene identifiziert werden und zeigten in den o.g. Pflanzengeweben ein unterschiedliches Expressionsverhalten. Die Expressionsanalyse bestätigte, dass, wie bekannt, in Pollen Cor a 1.01 und in der Nuss Cor a 1.04 am häufigsten

vertreten sind. Zudem konnten nun in Pollen aber auch bereits in den Kätzchen eine Expression der Isoallergene Cor a 1.03, 1.04, 1.05, 1.06 und 1.07 nachgewiesen werden. Die Isoallergene Cor a 1.04 – 1.07 sind zwar nur in geringen Mengen zu finden, Cor a 1.01 und 1.03 dagegen wurden am stärksten exprimiert. In Pollen konnten auch auf Proteinebene Cor a 1.01 und 1.03 mit PMF-Analysen bestätigt werden (Einzelarbeit B, Abb. S2). Cor a 1.02 und 1.08 konnten weder in Kätzchen noch in Pollen nachgewiesen werden.

In den weiblichen Geweben wurde neben dem bekannten Cor a 1.04 auch die Expression aller anderen Isoallergene nachgewiesen, wenn auch mit unterschiedlichem Expressionsprofil. Cor a 1.04 und Cor a 1.06 wurden in Blüte, unreifer und reifer Nuss ähnlich stark exprimiert, während Cor a 1.05 und 1.07 am stärksten in der Blüte und in der unreifen Nuss exprimiert werden. Die Expression von Cor a 1.01, 1.02, 1.03 und 1.08 war in der unreifen Nuss am höchsten. Für Cor a 1.03 konnte in der reifen Nuss keine mRNA nachgewiesen werden (Abb. 9)

Generell war außer für Cor a 1.04 und 1.06 nur wenig mRNA in reifen Nüssen nachzuweisen. Daraus kann aber kein Rückschluss auf die Allergenmenge in reifen Nüssen geschlossen werden. Alle Cor a 1-Isoallergene wurden bereits in der Blüte und in der unreifen Nuss exprimiert und können somit als Protein in der reifen Nuss vorliegen. Versuche, diese Isoallergene auch auf Proteinebene in reifer Nuss nachzuweisen, gelangen nur mit geringem Erfolg, da nCor a 1 nur schwer aus den fettreichen, reifen Nüssen isoliert werden konnte. Im PMF konnten so nur wenige Peptidsequenzen von Cor a 1.04 detektiert werden. Die anderen Isoallergene waren nach der Isolation vermutlich in zu geringer Menge vorhanden, um sie im PMF zu detektieren zu können (Einzelarbeit B, Abb. S2).

Das sehr unterschiedlich Expressionsverhalten lässt darauf schließen, dass Isoallergene unterschiedliche Eigenschaften und Funktionen in der Pflanze haben und eine Rolle in der Befruchtung und der Entwicklung der Nuss spielen könnten. Die Expressionsanalyse zeigte auch, dass alle Cor a 1-Isoallergene und auch die neu identifizierten Isoallergene Cor a 1.05, 1.06, 1.07 und 1.08 entweder im Pollen oder in der Nuss zu finden sind und daher für Allergiker eine wichtige Rolle spielen. Die Gene einzelner Varianten dieser Isoallergene, genannt *Cor a 1.0501*, *1.0601*, *1.0701* und *1.0801* die auf mRNA-Ebene nachgewiesen wurden, wurden in Expressionsvektoren kloniert und rekombinant hergestellt (Einzelarbeit B, Abb. 4, Abb. S4; Abb. 9). Zudem konnte erstmals eine neue Variante des Isoallergens Cor a 1.03, genannt

Cor a 1.0302, identifiziert werden. Im Gegensatz zur Variante Cor a 1.0301 konnte Cor a 1.0302 löslich gereinigt werden und wurde in den folgenden Untersuchungen mit einbezogen. Für das Isoallergen Cor a 1.02 konnte keine löslich exprimierbare Variante gefunden werden.

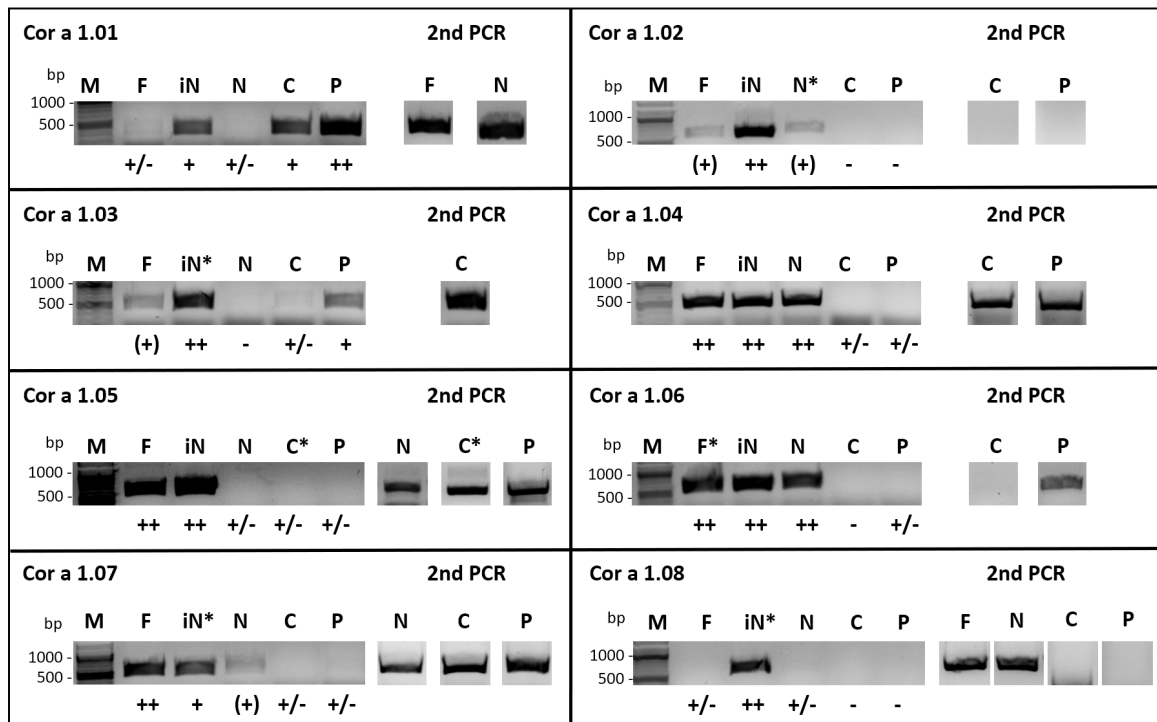


Abb. 9 Expression von Cor a 1-Genen in verschiedenen Haselgeweben. Das Produkt der RT-PCR wurde auf ein 1,5% Agarosegel aufgetragen. Die PCR wurde mit genspezifischen Primern durchgeführt. Wenn nach der ersten PCR keine Bande zu sehen war, wurde das Gelstück ausgeschnitten und die daraus gereinigte DNA für eine zweite PCR verwendet. F: Weiblich; iN: unreife Nuss; N: reife Nuss; C: Pollenkätzchen; P: Pollen; (+/-) sehr geringes, (+) geringes, + moderates, ++ starkes Expressionslevel. Die Gene der neuen Isoallergene wurden aus den mit * markierten Geweben isoliert. M: 100 bp DNA-Standard.

3.2.2 Biochemische und biophysikalische Analyse

Um mehr Einblicke in die Allergenität der neuen Isoallergene Cor a 1.0302, 1.0501, 1.0601, 1.0701 und 1.0801 zu bekommen, wurde die Stabilität der Allergene zunächst mittels CD-Spektroskopie untersucht. Die thermische Stabilität hat, wie bei den Dau c 1-Karottenallergenen bereits beobachtet werden konnte (Kapitel 3.1.2, S. 18), einen großen Einfluss auf die Allergenität eines Allergens.

Alle neuen Isoallergene bis auf Cor a 1.0801 entfalteten sich zwar bei 95 °C, allerdings konnte nach Abkühlung auf 25 °C eine fast vollständige Rückfaltung beobachtet werden (Einzelarbeit B, Abb. 2). Bei einer Schmelzkurve wird der Absorptionsunterschied von links- und rechts-zirkulär polarisiertem Licht zwischen ungefaltetem und gefaltetem Zustand bei einer bestimmten Wellenlänge gemessen. Für Cor a 1.0302, 1.0601 und 1.0701 konnte aus den Schmelzkurven ein T_m -Wert von 54 – 68 °C bestimmt und bis auf Cor a 1.0601 eine nahezu vollständige Rückfaltung beobachtet werden. Cor a 1.0501 weist einen sehr hohen T_m -Wert von fast 85 °C auf und ist damit für ein PR-10 Protein vergleichsweise sehr hitzestabil. Cor a 1.0801 entfaltet sich bei 95 °C kaum, weshalb keine aussagekräftige Schmelzkurve gemessen werden konnte und somit auch kein T_m -Wert ermittelt werden konnte. Vermutlich liegt der T_m -Wert allerdings sogar über dem von Cor a 1.0501 und müsste mit anderen Geräten, die auf über 95 °C heizen können, getestet werden.

Die hohe Stabilität dieser Haselallergene kann für Allergiker zum Problem werden. Vor allem Isoallergene wie Cor a 1.0501 und 1.0801 erwiesen sich als sehr hitzestabil und lösen daher möglicherweise auch nach Hitzebehandlung von Lebensmitteln allergische Reaktionen aus. Generell nimmt die Allergenität eines Allergens mit seiner Stabilität zu (Foo und Mueller, 2021). Stabile Proteine werden schlechter abgebaut und verweilen damit länger in der Natur oder im humanen System (Petersen und Scheurer, 2011; Vissers et al., 2011). Damit steigt auch die Wahrscheinlichkeit einer Exposition und der Entwicklung einer Allergie. Zum Beispiel können Katzenallergene noch Jahre nach Anwesenheit einer Katze nachgewiesen werden und eine Allergie auslösen (Arbes et al., 2004).

3.2.3 Allergenität der neuen Cor a 1-Isoallergene

Ob diese neuen Isoallergene auch wirklich allergen sind, können erst allergologische Untersuchungen bestätigen. Ein Protein wird von der WHO/IUIS Allergen Nomenklatur Sub-Komitee als Allergen anerkannt, wenn ein Allergen in einer relevanten Quelle nachgewiesen wurde, seine Existenz auf mRNA- oder Proteinebene nachgewiesen wurde und seine Allergenität mit mind. fünf Seren nachgewiesen wurde (Pomés et al., 2018).

Um die neuen Isoallergene allergologisch zu testen, wurden 20 Seren von Baumpollen-Allergikern von Dr. med. Angelika Reich (Lungenärztin und Allergologin in Bayreuth) zur

Verfügung gestellt. Zuvor wurden die Seren auf spezifisches IgE gegen eine Mischung von Birken-, Hasel- und Erlenpollenextrakt in einem Immunoblot-basierten Test überprüft. Nur Seren, die einen Wert von $\geq 3,5$ U/ml oder höher aufwiesen und damit mindestens der *enzyme-allergo-sorbent-test* (EAST) Klasse 3 entsprachen, wurden verwendet. Zur Kontrolle wurden im Immunoblot (Abb. 10) und ELISA (Einzelarbeit B, Tab. 2) zusätzlich das rekombinante (r)Bet v 1.0101 sowie die bekannten rCor a 1.0101 und 1.0401 getestet und zeigten mit allen Seren wie erwartet eine starke Reaktion. Da die Patienten sich ursprünglich gegen Birkenpollen sensibilisieren, ist die Reaktion mit rBet v 1.0101 am stärksten.

Die neuen Isoallergene rCor a 1.0302, 1.0501, 1.0601 zeigten wie die Kontrollen in allen Seren ebenfalls eine starke Reaktion (Einzelarbeit B, Tab. 2; Abb. 10). rCor a 1.0302 und 1.0601 sind mit einer Aminosäuresequenzidentität (Einzelarbeit B, Tab. 1) von 69% bzw. 78% dem rBet v 1.0101 noch sehr ähnlich und kreuzreagieren daher gut mit gegen Bet v 1-gerichteten IgE. rCor a 1.0501 hat eine etwas geringere Identität (51%) mit rBet v 1.0101 und zeigte im Test ebenfalls eine starke Reaktion. rCor a 1.0501 ist thermisch sehr stabil ($T_m = 84$ °C) (Einzelarbeit B, Abb. 2) und wird in der unreifen Nuss sehr stark exprimiert, dadurch erhöht sich für Patienten die Wahrscheinlichkeit der Exposition und damit die Entwicklung einer Kreuzallergie. rCor a 1.0701 zeigte im ELISA zwar für alle Seren eine Reaktion, allerdings war im Immunoblot bei vier Seren keine Reaktion mehr nachweisbar (Einzelarbeit B, Tab. 2, Abb. 10). rCor a 1.0701 scheint ein etwas schwächeres Allergen zu sein. Der Grund ist vermutlich die geringere Sequenzidentität von 54% von rCor a 1.0701 zu rBet v 1.0101 verglichen zu rCor a 1.0401 (68%) oder rCor a 1.0601 (78%) zu rBet v 1.0101. Auch die Expression in unreifen Nüssen ist etwas geringer als von *Cor a 1.04* oder *1.06* (Abb. 9). Für rCor a 1.0801 konnten im Immunoblot nur noch mit sieben Seren und im ELISA mit acht Seren eine schwache Reaktion festgestellt werden. Die schwache Reaktion könnte auf die geringere Sequenzidentität von 47% zu rBet v 1.0101 zurückzuführen sein. Darüber hinaus ist rCor a 1.0801 in reifen Haselnüssen nur noch in geringen Mengen vorhanden (Abb. 9) was die Entstehung einer Kreuzallergie unwahrscheinlicher macht.

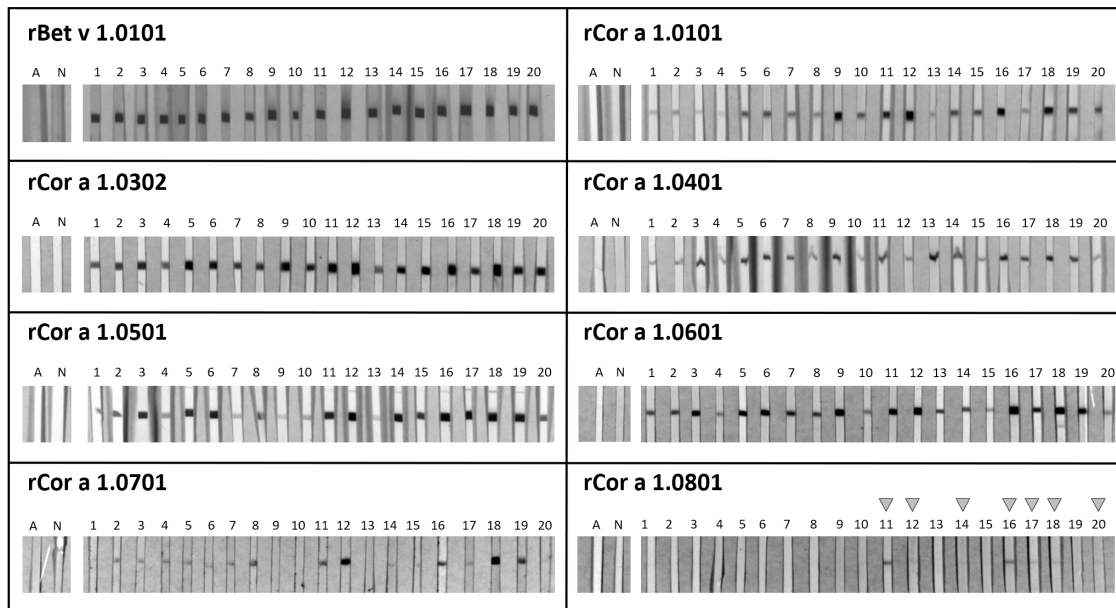


Abb. 10: Immunoblots. rBet v 1.0101 und die rCor a 1-Proteine wurden auf 16% Polyacrylamidgelen aufgetrennt und anschließend auf 0,2 µm Nitrozellulosemembranen übertragen. Außer für rCor a 1.0801 (0,5 µg/cm) wurden 1,5 µg/cm Protein aufgetragen. Die Membranen wurden in Streifen geschnitten und mit Patientenseren inkubiert. Spezifisch gebundene IgE-Antikörper wurden mit einem anti-humanen Maus-IgE-Antikörper gekoppelt mit einer alkalischen Phosphatase und Nitroblau-tetrazoliumchlorid (NBT)/ 5-Bromo-4-chloro-3-indolylphosphat (BCIP) als Substrat gefärbt. Graue Pfeile zeigen schwache IgE-Bindung für rCor a 1.0801. A: Sekundärer Antikörperkontrolle; N: Pferdeserum; 1 – 20: Serum von Baumpollen-Allergikern.

3.2.4 Schlussfolgerung

Mit den neu identifizierten Isoallergenen sind nun insgesamt acht Cor a 1-Isoallergene bekannt. Ihre unterschiedliche Expression in weiblichen und männlichen Geweben der Hasel deutet auf physiologisch unterschiedliche Funktionen hin.

Der Ligand Q3O-(Glc)-Gal wurde aus Haselpollen mit nCor a 1, einer Mischung verschiedener Isoallergene und Varianten, co-gereinigt und die Bindung durch Cor a 1.0401 über NMR bestätigt (Jacob et al., 2019). Allerdings konnte damals Cor a 1.0401 nicht in Pollen, sondern nur in der Haselnuss nachgewiesen (Breiteneder et al., 1993; Jacob et al., 2019; Lüttkopf et al., 2002). In dieser Publikation wurde in Pollen außer Cor a 1.01 auch die Isoallergene Cor a 1.03, 1.04, 1.05, 1.06 und 1.07 nachgewiesen und erklärt wie Q3O-(Glc)-Gal aus nCor a 1 co-gereinigt werden konnte. Zum einen stellt sich nun die Frage, ob Q3O-(Glc)-Gal auch von den neu identifizierten Isoallergenen Cor a 1.03, 1.05, 1.06, 1.07 oder 1.08 gebunden wird. Zum anderen könnten sich neben Q3O-(Glc)-Gal noch weitere bisher unbekannte Liganden in der nCor a 1 Mischung befinden.

3.1 Identifizierung von glykosylierten Flavonoiden als physiologische Liganden des Haselallergens Cor a 1: Unterschiede in der spezifischen Bindung von mono- und diglykosylierten Flavonoiden.

Einzelarbeit C: Hendrich, J. M., Kara, H., Schweimer, K., Jacob, T., Schneider, M., Baldermann S., Wöhrle, B.M., 2025. Identification of glycosylated flavonoids as new physiological ligands of the hazel allergen Cor a 1: Complex structures reveal different binding orientation and specificity of mono- and disaccharide derivatives. *Food Chem.:X*, 29, 102711.

3.1.1 Massenspektroskopische Identifizierung neuer möglicher Cor a 1-Liganden

Noch ist nicht vollständig geklärt, ob und wie stark die Liganden die Allergenität der PR-10 Allergene beeinflussen. Für Bet v 1 konnte beobachtet werden, dass Liganden beispielsweise den proteolytischen Abbau reduzieren und so indirekt durch erhöhte Exposition die Allergenität erhöhen (Petersen und Scheurer, 2011; Soh et al., 2019; Vissers et al., 2011). Ob gebundene Liganden die konformationellen Epitope maßgeblich verändern, oder welchen Effekt die Liganden im menschlichen System haben, bleibt weiter unklar. Flavonoiden werden unter anderem antioxidative und entzündungshemmende sowie hormonelle Wirkungen nachgesagt (Brunetti et al., 2013; Zand et al., 2000).

Liegt die physiologische Funktion der Isoallergene möglicherweise in der Bindung unterschiedlicher Liganden? Bis heute ist nicht verstanden wie genau die Liganden in den PR-10 Proteinen gebunden werden und was die zuletzt beobachtete spezifische Bindung verursacht. Um diesen Fragen entgegenzukommen ist es nötig, die Bindung der Liganden durch PR-10 Proteine näher zu untersuchen.

Wie schon beschrieben, wurden bisher nur die zwei Liganden Q3OS und Q3O-(Glc)-Gal als physiologische Liganden von Bet v 1 bzw. Cor a 1 identifiziert. Beide Proteine binden aber nur spezifisch ihren eigenen Liganden und nicht den Liganden des anderen. Beide Liganden unterscheiden sich nur an der Hydroxylgruppe des 4“-Kohlenstoffs des Zuckers Galaktose bzw. Glukose (Seutter von Loetzen et al., 2014; Jacob et al., 2019) (Kapitel 1.9, Abb. 7, S. 12). Zuvor wurde jedoch angenommen, dass PR-10 Proteine Liganden unspezifisch in ihrer Tasche binden (Fernandes et al., 2013; Gajhede et al., 1996; Marković-Housley et al., 2003; Neudecker et al., 2003; Seutter von Loetzen et al., 2012). Q3OS und Q3O-(Glc)-Gal kommen in großer Menge in Birken- bzw. Haselpollen vor und konnten in früheren Arbeiten unseres Lehrstuhls mit ihrem Bindungspartner über Ammoniumsulfatfällung, HIC und SEC co-gereinigt werden. Die

spezifische Bindung wurde NMR-spektroskopisch untersucht (Seutter von Loetzen et al., 2014; Jacob et al., 2019).

In der Einzelarbeit B (Kapitel 3.2, S.21) wurden bereits weitere neue Isoallergene identifiziert und deren Expression in verschiedenen Haselgeweben nachgewiesen. In Haselpollen sind damit insgesamt sechs Isoallergene, nämlich Cor a 1.01, 1.03, 1.04, 1.05, 1.06 und 1.08 bekannt, aber bisher konnte nur ein physiologischer Ligand Q3O-(Glc)-Gal in der Hasel identifiziert werden (Jacob et al., 2019). Q3O-(Glc)-Gal wird zwar von Cor a 1.04 gebunden, aber z.B. nicht von Cor a 1.01 (Jacob et al., 2019). Zum einen könnte es sein, dass Q3O-(Glc)-Gal noch von noch anderen Isoallergenen gebunden wird. Zum anderen könnte es noch weitere bisher unbekannte Liganden geben, die von Cor a 1.01 oder den anderen Isoallergenen gebunden werden.

Daher wurde in dieser Arbeit nCor a 1 erneut aus selbstgesammelten Haselpollen über Ammoniumsulfatfällung, HIC und SEC gereinigt. Anschließend wurden Liganden mit Methanol extrahiert und in Zusammenarbeit mit Prof. Dr. Susanne Baldermann (Fakultät VII, Professur für Lebensmittelmetabolismus, Universität Bayreuth) über ein UHPLC/QTOF-MS/MS-System analysiert (Einzelarbeit C, Tab. 1; Abb. 11). Dieses System bietet durch Auftrennung via UHPLC zu jedem Elutionszeitpunkt ein UV/Vis sowie ein Massenspektrum mit anschließender Fragmentierung der Substanzen. Die Fragmentierung verrät, um welchen Flavonoidrest es sich handelt, z.B. um Quercetin oder Kaempferol. Aus der Differenz der Gesamtmasse und der Masse des Aglykons kann anschließend die Art des Zuckers ermittelt werden (Einzelarbeit C, Abb. S1). Allerdings können Stereoisomere wie Glucose und Galaktose massenspektroskopisch nicht voneinander unterschieden werden.

In der Messung konnten erneut die bereits bekannten Liganden Q3O-(Glc)-Gal und Q3OS identifiziert werden (Jacob et al., 2019; Seutter von Loetzen et al., 2014). Durch Reinigung mittels HPLC, konnte zudem K3O-(Glc)-Gal erstmals über NMR eindeutig in Haselpollen bestätigt werden. Allerdings konnten K3O-(Glc)-Gal und K3OS präparativ per HPLC nicht voneinander getrennt werden und wurde für weitere Bindungsstudien nicht verwendet (Einzelarbeit C, Abb. S3). Um weitere Komponenten eindeutig zu identifizieren, wurden kommerziell erhältliche Flavonoide als Standard verwendet (Einzelarbeit C, Abb. S2). Dadurch konnten erstmals die glykosylierten Flavonoide Kaempferol-3-O- α -L-Rhamnosid (K3OR), Quercetin-3-O- α -L-Rhamnosid (Q3OR) und Kaempferol-3-O- β -D-Sophorosid (K3OS) eindeutig in Haselpollen identifiziert werden.

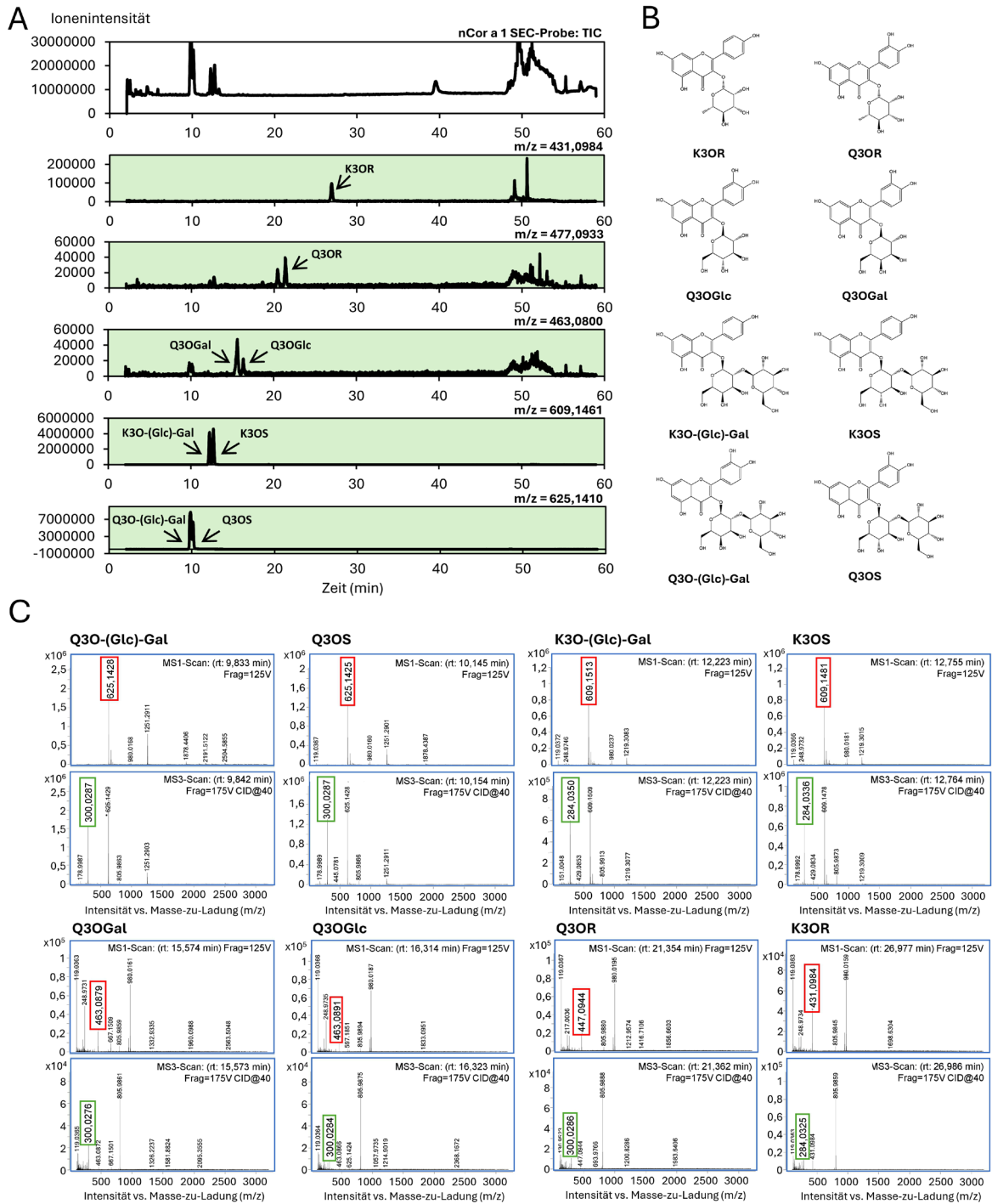


Abb. 11: UHPLC- QTOF-MS. (A) *Total ion count* (TIC) Chromatogramm von nCor a 1 nach SEC und *extracted ion count* (EIC) Chromatogramme der spezifischen Masse der Liganden. (B) Molekülstruktur der identifizierten Liganden. (C) Fragmentationsanalyse des aus nCor a 1/Ligandenkomplexes. Für jede Retentionszeit eines Liganden sind die MS1- (oberes MS-Spektrum) und die MS3-Fragmentierung (unteres MS-Spektrum) dargestellt und beziehen sich auf den EIC des gereinigten nCor a 1 in (A). Für die Fragmentierung wurde eine Spannung von 175 V und eine kollisionsinduzierte Dissoziation (CID, collision-induced dissociation) von 40 eV verwendet. Rote Kästen: Masse des entsprechenden Liganden zur Retentionszeit. Grüne Kästen: Quercetin oder Kaempferol nach Fragmentierung des Liganden.

3.1.2 Isoallergene binden Liganden unterschiedlich

Da das gereinigte nCor a 1 aus Haselpollen noch durch andere Pflanzenproteine verunreinigt war, und die identifizierten Moleküle möglicherweise durch diese mitgereinigt wurden, wurde die Bindung über NMR überprüft. In einem 2D ^1H , ^{15}N -*heteronuclear single quantum correlation* (HSQC) Experiment korrelieren die Resonanzfrequenzen der Amidprotonen mit den direkt verbundenen ^{15}N -markierten Stickstoffatomen. Durch die dreidimensionale Proteinfaltung ist die chemische Umgebung und somit auch die Resonanzfrequenz für jedes Amidproton unterschiedlich, wodurch jedem Amidproton des Proteinrückgrats ein ^1H - ^{15}N -Signal zugeordnet werden kann. Bindet ein Ligand an das Protein, verändert sich dort die chemische Umgebung und somit findet eine sog. chemische Verschiebung der betroffenen Signale statt.

Cor a 1.0501 erwies sich mit einem T_m -Wert von 85 °C als sehr hitzestabil und zeigte einzelne deutliche Signale im ^1H , ^{15}N -HSQC-Spektrum, wodurch 93% der Aminosäurereste den Signalen im ^1H , ^{15}N -HSQC-Spektrum zugeordnet werden konnten (Einzelarbeit C, Abb. S4). Bei den Titrationsen von Cor a 1.0501 mit Q3OR, K3OR, Q3OGlc und Q3OGal sind viele Signale im schnellen Austausch und wandern (Einzelarbeit C, Abb.2, Abb. S6). Andere Signale sind im intermediären Austausch und verschwinden mit Zugabe des Liganden. Beim schnellen Austausch bindet ein Ligand nur schwach an das Protein und die Dissoziationskonstante (K_D) liegt erfahrungsgemäß zwischen $10\text{ }\mu\text{M} \leq 10\text{ mM}$. Beim langsamem Austausch bindet ein Ligand sehr stark und weist einen K_D -Wert von etwa $\leq 30\text{ nM}$ auf (Gemmecker, 2003).

Isotherme Titrationskalorimetrie (ITC, *isothermal titration calorimetry*)-Titrationsen von Cor a 1.0501 mit Q3OR, K3OR, Q3OGlc und Q3OGal bestätigten die Bindungen und ergaben K_D -Werte von 70 – 170 μM , welche mit den Beobachtungen der NMR-Titrationsen übereinstimmt. (Einzelarbeit C, Abb. S5). Bei der Bindung der Liganden in der amphiphilen Tasche des Proteins sind viele Aminosäurereste direkt involviert, wodurch sich viele Signale im Spektrum verändern. Diese Verschiebungen bestätigen zwar eine Bindung des Liganden, allerdings ist weiterhin nicht klar, in welcher Orientierung der Ligand gebunden wird. Interessanterweise zeigte sich in den NMR-Titrationsen, dass Cor a 1.0501 nur monoglykosylierte Flavonoide aber nicht die diglykosylierten Flavonoide Q3OS, K3OS und Q3O-(Glc)-Gal bindet.

Um ein besseres Verständnis zu erhalten, wurden auch die anderen Cor a 1-Isoallergene sowie Bet v 1.0101 in einem Screening mittels NMR untersucht (Einzelarbeit C, Abb. S7; Tab. 1).

Tab. 1: Ligandenbindung von Cor a 1-Isoallergenen.

Liganden	Cor a 1							Bet v 1 0101
	0101	0302	0401	0501	0601	0701	0801	
Q3OGlc								
Q3OGal								
Q3OR								
K3OR								
Q3O-(Glc)-Gal								
Q3OS								
K3OS								

Bindung
 Keine Bindung
(mind. 5-facher Überschuss an Ligand)

Die Daten basieren auf ^1H , ^{15}N -HSQC-Screenings, ^1H , ^{15}N -HSQC-Titrations und ITC-Experimenten.

Die monoglykosylierte Flavonoide werden von fast allen Cor a 1-Isoallergenen und auch von Bet v 1 gebunden. Nur Cor a 1.0801, welches selbst nicht in Pollen, sondern in der Nuss vorkommt, konnte keinen der aus Pollen isolierten Liganden binden. Cor a 1.0701 konnte Q3OR als einziges monoglykosyliertes Flavonoid nicht binden. Das Screening ergab außerdem, dass Q3O-(Glc)-Gal tatsächlich nicht nur von Cor a 1.0401 gebunden wird, sondern auch von Cor a 1.0601 und Cor a 1.0701. Neben Cor a 1.0501 konnten auch Cor a 1.0101 und 1.0301 keines der diglykosylierten Flavonoide binden. Q3OS und K3OS konnten lediglich von Bet v 1.0101 gebunden werden. Wie bereits bekannt, bindet Bet v 1.0101 spezifisch Q3OS aber nicht Q3O-(Glc)-Gal (Jacob et al., 2019; Seutter von Loetzen et al., 2014). Das Screening ergab, dass monoglykosylierte Flavonoide von fast allen Isoallergenen unspezifisch gebunden werden, wohingegen diglykosylierte Flavonoide nur von Cor a 1.0401, 1.0601 und 1.0701 spezifisch gebunden werden.

3.1.3 NOE-Signale gewähren Einblicke in die Komplexstruktur von Cor a 1.0501

Um zu verstehen, wie diese Spezifität zustande kommt und wie die Liganden in der amphiphilen Tasche gebunden werden, wurden NOESY-Experimente mit Cor a 1.0501 und den monoglykosylierten Flavonoide-Liganden durchgeführt. In einem *filtered/edited* NOESY können die Abstände bis zu 5 Å zwischen Protonen eines unmarkierten Liganden und eines

^{15}N ^{13}C markierten Proteins über NOE-Signale beobachtet werden. Können diese NOE-Signale den Protonen des Liganden und den Protonen des Proteins zugeordnet werden, kann auf Grundlage dieser Abstandsinformationen eine Komplexstruktur modelliert werden.

Zunächst wurde als Referenz das *filtered/edited* NOESY Spektrum von Cor a 1.0501 ohne Ligand gemessen (Einzelarbeit C, Abb. 3 A). Nach Zugabe eines Liganden konnten neue Signale beobachtet werden, die durch den Liganden verursacht wurden. Allerdings verbreiterten sich viele Signale im ^1H , ^{13}C -HSQC-Spektrum des Komplexes und verschwanden oder überlagerten mit anderen Signalen, und konnten daher nicht mehr eindeutig zugeordnet werden. Dennoch sind wenige zugeordnete Signale bereits ausreichend, um durch *docking* Experimente mögliche und sinnvolle Komplexstrukturen vorherzusagen. Für den Komplex aus Cor a 1.0501/Q3OGlc konnten drei NOE-Signale und für den Komplex Cor a 1.0501/Q3OR zwei NOE-Signale den Seitenkettenprotonen zugeordnet werden. Sowohl im Komplex mit Q3OGlc als auch mit Q3OR wurde ein starkes NOE-Signal durch das aromatische 5'-Proton des Quercetinrestes und das β -Proton von Ala38 hervorgerufen. Dagegen bildete das 4"-Proton des Zuckerrestes zusammen mit dem β -Proton von Ser137 ein weiteres NOE-Signal. Beim Komplex mit Q3OGlc war zusätzlich ein NOE-Signal zwischen dem 6"-Proton des Zuckerrestes und dem α -Proton von Lys138 zu beobachten (Einzelarbeit C, Abb. 3 A).

3.1.4 Mono- und diglykosylierte Flavonoide binden unterschiedlich

Auf der Basis dieser neuen Abstandsinformationen konnte mit dem HADDOCK 2.4 *webserver* ein Komplexmodell von Cor a 1.0501 mit Q3OR und Q3OGlc berechnet werden. In beiden Modellen (Abb. 12 A und B) ist zu erkennen, dass der 3,4-dihydroxyphenyl-Ring der Quercetineinheit im Eingang der Bindetasche orientiert ist, und das Ala38 das 5'-Proton des 3,4-dihydroxyphenyl-Rings fixiert. Das 4"-Proton des Zuckerrestes wird durch Ser137 fixiert und befindet sich im Inneren der Tasche.

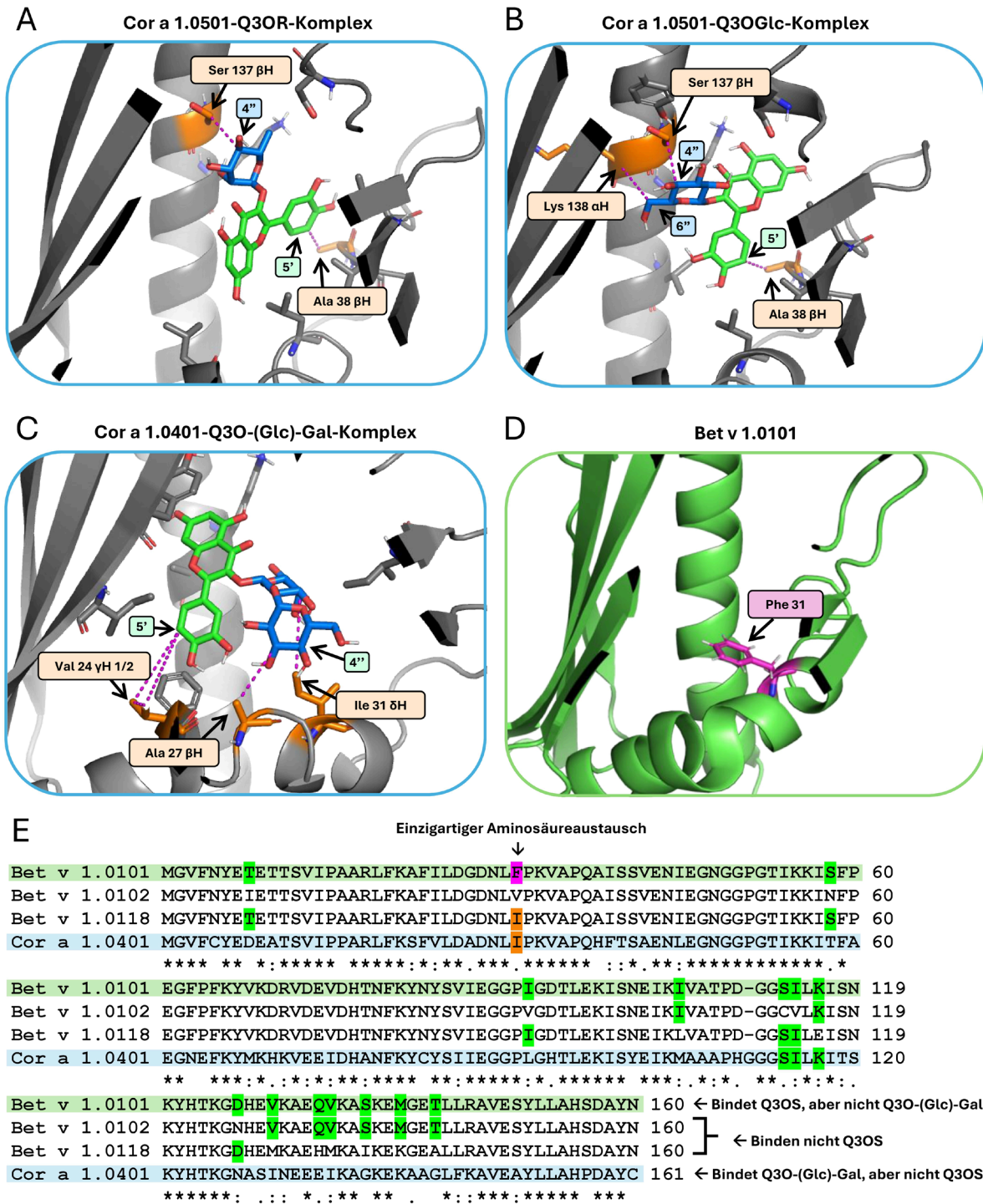


Abb. 12: Komplex von Cor a 1.0501 mit Q3OR und Q3OGlc und Cor a 1.0401-3CS mit Q3O-(Glc)-Gal. (A) Cor a 1.0501/Q3OR. **(B)** Cor a 1.0501/Q3OGlc. **(C)** Cor a 1.0401/Q3O-(Glc)-Gal. **(D)** NMR-Struktur von Bet v 1.0101 (PDB:1B6F) (Schweimer et al., 1999). Der Eingang der Bindungstasche befindet sich räumlich hinter den Liganden. Der Quercetinanteil ist grün und der Zuckeranteil blau hervorgehoben. Die orangefarbenen Aminosäurereste bilden ein NOE-Signal mit den Liganden aus. Die grauen Aminosäurereste befinden sich in unmittelbarer Nähe des Liganden. Magentafarbene Linien verbinden die Kohlenstoffatome, die für die Abstandsbeschränkungen verwendet wurden. **(E)** Aminosäuresequenzalignment von Bet v 1.0101, 1.0102 und 1.0118 sowie Cor a 1.0401. Gleiche Aminosäuren eines Austausches zwischen den Bet v 1-Varianten sind grün markiert. Der Einzigartige Aminosäureaustausch ist lila bzw. orange markiert. Bet v 1-Sequenz ist mit hellgrüner und Cor a 1.0401-Sequenz mit hellblauer Farbe hinterlegt.

Bisher wurde bereits vermutet, dass die Zuckerreste der glykosylierten Flavonoide die Spezifität verursachen (Jacob et al., 2019; Seutter von Loetzen et al., 2015, 2014). Das *screening* (Tab. 1) und die Komplexmodelle (Abb. 12 A und B) bestätigen diese Vermutung. Interessanterweise steht das 4“-Proton des Zuckers in direkter Nähe zum Ser137. Gleichzeitig unterscheiden sich die beiden Stereoisomere Glucose und Galaktose nur in der Orientierung der 4“-Hydroxylgruppe. Ist dieses Ser137 möglicherweise der Grund dafür, dass Q3OS nur von Bet v 1 gebunden wird während Q3O-(Glc)-Gal nur von Cor a 1 gebunden werden kann? Tatsächlich findet sich bei allen Cor a 1-Isoallergenen bis auf Cora 1.0501 an dieser Position statt einem Serin- ein Alanin- oder Glycinrest. Bet v 1.0101 besitzt dagegen wie Cor a 1.0501 an dieser Position ein Serin. Die Mutation zu Alanin in Bet v 1.0101-S137A führt allerdings zu einer Destabilisierung der zweiten α -Helix, wodurch keine vollständige Faltung mehr möglich ist (Kapitel 1.5, Abb. 3 A, S. 6). In der Folge konnte auch keine Bindung beobachtet werden. Cor a 1.0401-G138S war zwar vollständig gefaltet, allerdings war in ^1H , ^{15}N -HSQC-Titrations keine Veränderung der Bindungsspezifität der Liganden Q3OS bzw. Q3O-(Glc)-Gal zu beobachten (Einzelarbeit C, Abb. S11)

Unter Verwendung der aus den NOESYs gewonnenen Abstandsinformationen konnte Q3O-(Glc)-Gal in Cor a 1.0501 nicht in der gleichen Position und Orientierung wie die monoglykosylierten Flavonoide Q3OR oder Q3OGlc gedockt werden. Dies deutet darauf hin, dass eine Bindung von Q3O-(Glc)-Gal in der gleichen Orientierung wie monoglykosylierte Flavonoide sterisch nicht möglich ist und anders gebunden werden. Daher wurde das NOESY-Experiment auch mit dem Komplex aus Cor a 1.0401-3CS und Q3O-(Glc)-Gal durchgeführt (Abb. 12 C). Um Dimerisierung während des Experimentes zu vermeiden, wurden an der Oberfläche von Cor a 1.0401 drei Cysteine durch Serine ersetzt. Das β -Proton und die beiden γ -Protonen von Val24 bilden mit der Quercetineinheit deutliche NOE-Signale aus. Das γ -Proton von Val24, das α -Proton und β -Proton von Ala27 und das δ -Proton von Ile31 bilden dagegen starke NOE-Signale mit dem Disaccharid-Rest aus (Einzelarbeit C, Abb. 3 C). Diese Ergebnisse zeigen eine völlig andere Bindung für das diglykosylierte Flavonoide. Die aromatische Quercetineinheit liegt tief im Hohlraum, während der Zucker-Rest am Eingang der Tasche lokalisiert ist (Abb. 12 C). Folglich ist der Rest Ser138 nicht an der Bindung beteiligt und erklärt warum, die Mutanten Bet v 1.0101-S137A und Cor a 1.0401-G138S keine Änderung im Bindeverhalten zeigten.

Das neue Komplexmodell von Cor a 1.0401/Q3O-(Glc)-Gal zeigt, dass die 4“-Hydroxylgruppe der Galaktose-Einheit räumlich in Richtung des Aminosäurerests Ile31 gerichtet ist (Abb. 12 C). Die 4“-Hydroxylgruppe ist genau die Hydroxylgruppe in der sich Q3OS und Q3O-(Glc)-Gal stereochemisch unterscheiden. Erstaunlicherweise befindet sich in Bet v 1.0101 genau an dieser Stelle ein Phenylalanin (Phe31). Dieser aromatische Rest könnte der Grund sein, warum Bet v 1.0101 das Stereoisomer Q3O-(Glc)-Gal nicht binden kann, aber Q3OS. Die Orientierung der 4“-Hydroxylgruppe des Q3OS würde sterisch an der Position 31 einen aromatischen Rest erlauben, die stereochemische Orientierung der 4“-Hydroxylgruppe des Q3O-(Glc)-Gal dagegen nicht (Abb. 12 C und D). Interessanterweise ergaben *docking*-Experimenten von Cor a 1.0401 und Q3OS nicht die gleiche Orientierung wie Q3O-(Glc)-Gal und bekräftigt die Annahme einer hochspezifisch Bindungstasche.

3.1.5 Ursache für die spezifische Bindung von Q3OS und Q3O-(Glc)-Gal

In einer früheren Veröffentlichung unseres Lehrstuhls konnte über NMR gezeigt werden, dass im Gegensatz zu Bet v 1.0101 die Varianten Bet v 1.0102 (Bet v 1d) und Bet v 1.0118 (Bet v 1m) Q3OS nicht binden können (Seutter von Loetzen et al., 2015). Ein Sequenzvergleich (Abb. 12 C und D) der Bet v 1-Varianten offenbart, dass in Bet v 1.0101 an Position 31 ein Aminosäureaustausch von Phenylalanin zu Valin (F31V) bzw. Isoleucin (F31I) stattgefunden hat. Sowohl bei Cor a 1.0401 als auch bei Bet v 1.0118 findet sich an Position 31 ein Isoleucin. Beide Proteine binden Q3OS nicht. Dieser Sequenzvergleich bekräftigt die Annahme, dass der Aminosäurerest an Position 31 wichtig für die spezifische Unterscheidung zwischen Q3OS und Q3O-(Glc)-Gal ist.

Diese neuen Ergebnisse deuten zum einen darauf hin, dass mono- und diglykosylierte Flavonoide grundlegend unterschiedlich gebunden werden. Monoglykosylierte Flavonoide sind etwas kleiner und binden deshalb möglicherweise unspezifischer, da sie eine geringere sterische Hinderung aufweisen. Diglykosylierte Flavonoide dagegen können auf Grund ihrer Größe sterisch nur in einer anderen Orientierung in der Tasche gebunden werden. Wahrscheinlich ist nur ein Aminosäurerest für die spezifische Unterscheidung zweier Stereoisomere entscheidend ist. Um ein vollständiges Bild über die Ligandenbindung von PR-10 Proteinen zu erhalten, sollten in Zukunft auch andere PR-10 Protein/Liganden-Komplexe analysiert werden.

4 Schlussbetrachtung und Ausblick

Dau c 1-Allergene aus der Karotte können nicht nur eine Kreuzallergie auslösen, sondern wie Bet v 1 auch direkt sensibilisieren (Ballmer-Weber et al., 2001; Hoffmann-Sommergruber et al., 1999). Daher ist die Entdeckung und Charakterisierung weiterer bisher unbekannter Isoallergene aus der Karotte von großem Interesse. In der vorliegenden Arbeit konnten auf mRNA- und Protein-Ebene weitere neue Isoallergene, genannt Dau c 1.0501 und 1.0601, identifiziert werden. Ihre Allergenität wurde durch Immunoblots, ELISA-Inhibitionsversuchen und MRA bestätigt. Ein weiteres Protein, Dau c 1-like, erwies sich als nicht allergen, obwohl es den anderen Isoallergenen sequenziell sehr ähnlich ist. Bis heute ist nicht vollständig geklärt, welche strukturellen Epitope der PR-10 Proteine von IgE erkannt werden und dem Protein seine allergene Wirkung verleihen. Die Kombination sequenzieller und allergologischer Charakterisierung der allergenen Dau c 1-Isoallergene und des nicht-allergen Dau c 1-like offenbarten vier plausible strukturelle Epitope, die für die IgE-Erkennung wichtig sein könnten. Der sequenzielle Vergleich eines allergenen und nicht-allergen Proteins mit gleichzeitig hoher Sequenzidentität ist ein vielversprechender Ansatz, um potenzielle Epitope zu identifizieren. In Zukunft könnten durch Mutationsstudien diese neuen Epitope weiter charakterisiert werden. Beispielsweise könnten die neu identifizierten Epitope auf Bet v 1 so verändert werden, dass ein nicht-allergenes Bet v 1 entsteht.

Die Haselpollenallergene lösen nicht nur durch Inhalation der Pollen, sondern auch durch den Verzehr der Nüsse eine allergische Reaktion aus. In dieser Arbeit wurden vier neue Cor a 1-Isoallergene, genannt Cor a 1.0501 – 1.0801, identifiziert und allergologisch charakterisiert. Erstmals wurde auch die Expression aller Cor a 1-Isoallergene in Pollen, Kätzchen, weiblichen Blüten, unreifer und reifer Nuss vollständig aufgeklärt. Nicht nur die Proteinstabilität, die Sequenzähnlichkeit zum sensibilisierenden Bet v 1 und die Epitope bestimmen die Allergenität, sondern auch die Menge, in der ein Allergen vorkommt. Das schwächere Isoallergen Cor a 1.0801 ist in der reifen Nuss nur in geringen Mengen vorhanden und verursacht unter anderem daher wahrscheinlich nur bei wenigen Patienten eine Kreuzallergie. Zukünftig ist die Identifizierung und Charakterisierung neuer Isoallergene und Varianten in anderen Quellen von entscheidender Bedeutung, um die allergologischen Diagnostik zu verbessern und der Entwicklung einer AIT mit rekombinanten Allergenen einen Schritt näher zu kommen (Holzhauser et al., 2020; Valenta et al., 2011; Zhernov et al., 2019).

Der Grund für die spezifische Bindung der Liganden Q3OS bzw. Q3O-(Glc)-Gal durch Bet v 1.0101 bzw. Cor a 1.0401 blieb bisher unklar (Jacob et al., 2019; Seutter von Loetzen et al., 2014). In dieser Arbeit wurden weitere natürliche Liganden (Q3OR, K3OR, Q3OGlc und Q3OGal) von nCor a 1 identifiziert. Mit Hilfe von NOESY- und *docking*-Experimenten wurden erstmals Komplexstrukturen von Cor a 1-Isoallergenen mit monoglykosylierten Flavonoiden (Q3OR und Q3OGlc) und dem diglykosylierten Flavonoid Q3O-(Glc)-Gal gelöst. Die Komplexstrukturen Cor a 1.0501/Q3OR, Cor a 1.0501/Q3OGlc sowie Cor a 1.0401/Q3O-(Glc)-Gal offenbaren nicht nur, dass mono- und diglykosylierte Flavonoide in völlig unterschiedlicher Orientierung gebunden werden, sondern auch welche Aminosäure für die spezifische Bindung von Q3O-(Glc)-Gal wichtig ist: Die 4“-Hydroxylgruppe von Q3O-(Glc)-Gal befindet sich unmittelbarer Nähe von Ile31. In Bet v 1.0101 ist an dieser Position ein Phenylalanin und verhindert somit sterisch die Bindung von Q3O-(Glc)-Gal, eine Bindung von Q3OS ist jedoch möglich, da hier die 4“-Hydroxylgruppe von Q3OS in die entgegengesetzte Richtung zeigt und sterisch nicht mit dem Phe31 kollidieren kann. In einer früheren Arbeit unseres Lehrstuhls konnte für die Variante Bet v 1.0118 (Bet v 1m), die interessanterweise an der Position 31 ein Isoleucin statt einem Phenylalanin aufweist, keine Bindung von Q3OS beobachtet werden (Seutter von Loetzen et al., 2015). Vorläufige Ergebnisse mit der Variante Bet v 1.0118 bestätigen die Bedeutung dieser Aminosäure: erste ¹H, ¹⁵N-HSQC-Titrationsexperimente (Abb. 13) mit Bet v 1.0118 zeigten, dass sich, anders als bei Bet v 1.0101, die ¹H, ¹⁵N-Signale durch Zugabe von Q3OS kaum ändern, d.h. es findet keine Bindung mehr statt. Allerdings konnte bei Zugabe von Q3O-(Glc)-Gal eine Verbreiterung oder das Verschwinden vieler Signale beobachtet werden, was auf eine Bindung dieser Substanz hindeutet. In dieser Variante hat folglich eine Verschiebung der Spezifität von Q3OS hin zu Q3O-(Glc)-Gal stattgefunden.

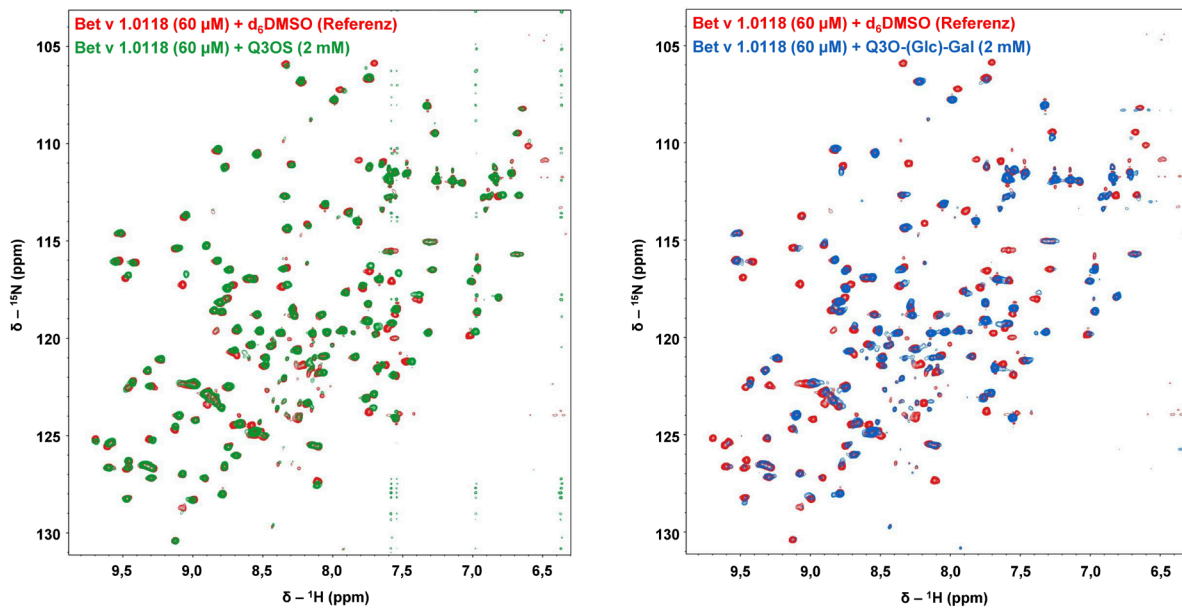


Abb. 13: ^1H , ^{15}N -HSQC von Bet v 1.0118 mit Q3OS und Q3O-(Glc)-Gal. Zu Bet v 1.0118 (60 μM) wurde Q3OS (links in grün) oder Q3O-(Glc)-Gal (rechts in blau) in einer Endkonzentration von 2 mM hinzugegeben. Die Liganden wurden in 100% (v/v) deuteriertem Dimethylsulfoxid ($\text{d}_6\text{-DMSO}$) gelöst. Als Referenz wurde 100% (v/v) $\text{d}_6\text{-DMSO}$ titriert (rot). NMR-Puffer: 10 mM Na-Phosphat pH 7,0, 50 mM NaCl und 10% $^2\text{H}_2\text{O}$. ^1H , ^{15}N -HSQC-Spektren wurden mit einem 900 MHz-NMR-Spektrometer bei 298 K gemessen.

In Zukunft könnten auf die gleiche Weise neue Liganden in anderen Allergenquellen wie Karotte, Sellerie, Apfel etc. identifiziert werden und ihre Bindung NMR-spektroskopisch über NOESY-Experimente untersucht werden, um einen tieferen Einblick in die Funktionsweise der PR-10 Allergene zu erhalten.

5 Abkürzungsverzeichnis

AIT	<u>a</u> llergenspezifische Immun <u>t</u> herapie
AP	<u>a</u> lkalische <u>P</u> hosphatase
APC	<u>a</u> ntigen- <u>p</u> resenting <u>c</u> ell
BLAST	<u>b</u> asic <u>l</u> ocal <u>a</u> lignment <u>s</u> earch <u>t</u> ool
bp	Basenpaare
CD	<u>C</u> ircular <u>d</u> ichroismus
CD4	<i>cluster of differentiation 4</i>
cDNA	<i>complementary DNA</i>
d ₆ -DMSO	deuteriertes Dimethylsulfoxid
EAST	<u>e</u> nzyme- <u>a</u> llergo- <u>s</u> orbent- <u>t</u> est
EIC	<u>e</u> xtracted <u>i</u> on <u>c</u> ount
ELISA	<u>e</u> nzyme- <u>l</u> inked <u>i</u> mmuno <u>s</u> orbent <u>a</u> ssay
<i>E. coli</i>	<u>E</u> scherichia <u>c</u> oli
Fc	<u>f</u> ragment <u>c</u> rystallizable
HIC	Interaktionschromatographie
HRP	<u>h</u> orser <u>a</u> dish <u>p</u> eroxidase
HSQC	<u>h</u> eteronuclear <u>s</u> ingle <u>q</u> uantum <u>c</u> oherence
UHPLC/QTOF-MS/MS	<u>u</u> ltra- <u>h</u> igh- <u>p</u> ressure <u>l</u> iquid <u>c</u> hromatography/ <u>q</u> uadrupole- <u>t</u> ime <u>o</u> f <u>f</u> light - tandem mass spectrometry
Ig	Immunoglobulin
ITC	<u>i</u> sothermal <u>t</u> itration <u>c</u> alorimetry
IUIS	<i>International Union of Immunological Societies</i>
K _D	Dissoziationskonstante
K3OR	Kaempferol-3-O-Rhamnosid
LC-MS	<u>l</u> iquid <u>c</u> hromatography- <u>m</u> ass <u>s</u> pectrometry
n	natürlich

NCS	(s)-Norcocclaurin-Synthase
NMR	<i><u>n</u>uclear <u>m</u>agnetic <u>r</u>esonance</i>
NOESY	<i>nuclear Overhauser and exchange spectroscopy</i>
MRA	<i><u>m</u>ediator <u>r</u>elease <u>a</u>ssay</i>
PAGE	Polyacrylamid-Gelelektrophorese
PCR	<i><u>p</u>olymerase <u>c</u>hain <u>r</u>eaction</i>
PMF	Peptidmassenfingeraabdruck
PR-10	<i>class 10 of pathogenesis related proteins</i>
Q3OGal	Quercetin-3-O-Galaktosid
Q3OGlc	Quercetin-3-O-Glykosid
Q3OR	Quercetin-3-O-Rhamnosid
Q3OS	Quercetin-3-O-Sophorosid
Q3O-(Glc)-Gal	Quercetin-3-O-(2''-O-β-D-Glukopyranosyl)- β-D-Galaktopyranosid
r	rekombinant
RAST	<i><u>r</u>adio-<u>a</u>llergo-<u>s</u>orbent-<u>t</u>est</i>
RBL	<i><u>r</u>at <u>b</u>asophilic <u>l</u>eukemia</i>
RT	Reverse Transkriptase
SCIT	<i><u>s</u>ub<u>c</u>utaneous <u>i</u>mmuno<u>t</u>herapy</i>
SDS	<i>sodiumdodecylsulfate</i> , Natriumdodecylsulfat
SEC	<i>size exclusion chromatography</i> , Größenausschlusschromatographie
SLIT	<i><u>s</u>ublingualen <u>i</u>mmun<u>t</u>herapie</i>
TIC	<i><u>t</u>otal <u>i</u>on <u>c</u>ount</i>
T _H	T-Helferzelle
T _m	<i><u>m</u>elting <u>t</u>emperature</i> , Schmelzpunkt
WHO	<i>World Health Organization</i>

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7 Publikationsliste

7.1 Einzelarbeit A

Hendrich, J. M., Wangorsch, A., Rödel, K., Jacob, T., Mahler, V., Wöhr, B. M., **2023**. Allergenicity and IgE Recognition of New Dau c 1 Allergens from Carrot. *Mol. Nutr. Food Res.*, 67 (3), e2200421.

Birgitta M. Wöhr, Andrea Wangorsch, Thessa Jacob und ich planten die Experimente. Thessa Jacob, Katharina Rödel und ich isolierten die mRNAs, klonierten die Gene und führten die Expression und Aufreinigung, CD-Messungen und NMR-Spektroskopie durch. Birgitta M. Wöhr und ich evaluierten die Experimente. Die Immunoblots, ELISAs, Inhibitionsversuche, MRAs und nanoDSF wurden von Andrea Wangorsch und mir durchgeführt und zusammen mit Birgitta M. Wöhr ausgewertet. Vera Mahler stellte Sera klinisch charakterisierter Karottenallergiker zur Verfügung und half bei der Interpretation der Daten. Birgitta M. Wöhr, Thessa Jacob und ich schrieben das Manuskript. Alle Autoren haben an der endgültigen Fassung des Manuskripts mitgewirkt, es gelesen und genehmigt.

7.2 Einzelarbeit B

Hendrich, J. M., Reuter, A., Jacob, T., Kara, H., Amer, S., Rödel, K., Wöhr, B. M., **2024**. Allergenicity and Structural Properties of New Cor a 1 Isoallergens from Hazel Identified in Different Plant Tissues. *Sci. Rep.*, 14 (1), 5618.

Birgitta M. Wöhr leitete und koordinierte das Projekt. Andreas Reuter entwarf, analysierte und interpretierte die massenspektroskopischen Daten und schrieb den entsprechenden Teil des Manuskriptes. Birgitta M. Wöhr und ich entwarfen alle anderen Experimente und werteten die Daten aus. Thessa Jacob plante die ersten Experimente, reinigte Cor a 1.0101 und 1.0401 und lieferte konzeptionelle Beiträge. Sherine Amer reinigte Cor a 1.0302 und 1.0801. Hatice Kara klonierte und reinigte Cor a 1.0701. Katharina Rödel reinigte Cor a 1.0601 und führe CD-Experimente durch. Ich führte RNA-Extraktionen, PCR-Reaktionen, CD-Experimente, Immunoblots und ELISAs durch. Birgitta M. Wöhr und ich verfassten den Artikel. Alle Autoren haben an der endgültigen Fassung des Manuskripts mitgewirkt, es gelesen und genehmigt.

7.3 Einzelarbeit C

Hendrich, J. M., Kara, H., Schweimer, K., Jacob, T., Schneider, M., Baldermann S., Wöhlrl, B.M., **2025**. Identification of glycosylated flavonoids as new physiological ligands of the hazel allergen Cor a 1: Complex structures reveal different binding orientation and specificity of mono- and disaccharide derivatives. *Food Chem.:X*, 29, 102711.

Birgitta M. Wöhlrl leitete und koordinierte das Projekt. Thessa Jacob plante die ersten Experimente, reinigte Cor a 1.0101 und 1.0401 und leistete konzeptionelle Beiträge. Thessa Jacob, Maïke Schneider und ich reinigten das Q3O-(Glc)-Gal aus Haselpollen. Kristian Schweimer, Thessa Jacob, Maïke Schneider und ich ordneten die NMR-Signale dem Cor a 1.0401 im Komplex mit Q3O-(Glc)-Gal zu und führten erste NOESY-Experimente damit durch. Hatice Kara führte einen Teil der NMR-Titrationen sowie das NMR-Screening mit Cor a 1.0601 und 1.0701 durch und ordnete mit mir die NMR-Signale dem Komplexproben mit Cor a 1.0501 zu. Die restlichen NMR-Titrationen und NMR-Screenings sowie NOESY-Experimente an den Cor a 1.0501-Komplex-Proben wurden von mir durchgeführt. Kristian Schweimer half bei der Durchführung und Interpretation aller NMR-Experimente. Susanne Baldermann und ich entwarfen und führten die massenspektroskopischen Experimente durch und werteten die massenspektroskopischen Daten aus. Alle anderen Experimente wurden von Birgitta M. Wöhlrl, Kristian Schweimer und mir konzipiert und ausgewertet. Der Artikel wurde von Birgitta M. Wöhlrl, Kristian Schweimer, Susanne Baldermann und mir verfasst. Alle Autoren haben an der Endfassung des Manuskripts mitgewirkt, es gelesen und genehmigt.

8 Einzelarbeiten

8.1 Einzelarbeit A

Hendrich, J. M., Wangorsch, A., Rödel, K., Jacob, T., Mahler, V., Wöhrle, B. M., **2023**. Allergenicity and IgE Recognition of New Dau c 1 Allergens from Carrot. *Mol. Nutr. Food Res.*, 67 (3), e2200421.

Allergenicity and IgE Recognition of New Dau c 1 Allergens from Carrot

Julian M. Hendrich, Andrea Wangorsch, Katharina Rödel, Thessa Jacob, Vera Mahler, and Birgitta M. Wöhrl*

Scope: Carrot (*Daucus carota*) allergy is caused by the major carrot allergen Dau c 1, which is a mixture of several isoallergens and variants with sequence identities of >67% or >90%, respectively. However, little is known about the qualitative and quantitative composition of natural Dau c 1.

Methods and results: Mass spectrometry of isolated natural Dau c 1 reveals the existence of several yet unknown Dau c 1-like proteins. The study expresses four Dau c 1-like proteins in *Escherichia coli*. Two of the purified proteins, designated Dau c 1.0501 and 1.0601, exhibit sequence identities to Dau c 1.0101 and 1.0401 between 54% and 87%. They possess allergenic potential and are accepted as new isoallergens. One protein, designated as Dau c 1-like is >50% identical with the new isoallergens but exhibits no allergenicity. Sequence and structural comparisons of this protein with the known Dau c 1 isoallergens offer relevant clues about putative structural IgE epitopes.

Conclusion: Identification of new isoallergens and the identification of IgE epitopes may contribute to a more refined component resolved diagnosis and may lay ground for further epitope mapping and personalized targeted treatment approaches of carrot allergy in preclinical and clinical studies.

1. Introduction

About 25% of food-allergic patients in Central Europe suffer from carrot (*Daucus carota*) allergy,^[1] which is often associated with hypersensitivities to other plants of the family *Apiacea*, as well as to birch or mugwort pollen.^[2] The basis of this so-called pollen-related food-allergy is caused by IgE cross reactivity between proteins from pollen allergens and the food source, sharing a high structural and sequential homology.

To date, three different groups of carrot allergens have been described: Dau c 5, an isoflavone reductase-like protein, Dau c 4, a profiling, and Dau c 1 (www.allergen.org). The latter represents the major carrot allergen responsible for most carrot allergies.^[3] Dau c 1 belongs to the class of pathogenesis-related class 10 (PR-10) allergenic proteins and shares homology to the major birch pollen allergen Bet v 1 (36.9% sequence identity between Bet v 1.0101 and Dau c 1.0104).

All known PR-10 allergens exhibit common structural features. Bet v 1 as well as Dau c 1 consist of a seven-stranded anti-parallel β -sheet that wraps around a 25 residues long C-terminal amphipathic α -helix. The β -sheet and the C-terminal part of the helix are separated by two short α -helices which are arranged in V-shape. Together with the β -sheet they form a hydrophobic cavity which can bind small ligand molecules.^[4,5] Natural ligands for Bet v 1, quercetin-3-O-sophoroside, and for the homologous hazel allergen Cor a 1, quercetin-3-O-(2"-O- β -D-glucopyranosyl)- β -D-galactopyranoside, have been recently identified.^[6,7]

In most cases, Bet v 1 acts as the sensitizing allergen. However, cross-allergies to other PR-10 proteins from different sources like apple, hazelnut, stone fruit, carrot, celery or pollen from other *Fagales* can occur due to their high structural homology to Bet v 1.^[8] In addition, it is known that Dau c 1 can also function as a sensitizing allergen, independent of Bet v 1.^[9]

Most PR-10 allergens comprise a mixture of several isoallergens (at least 67% sequence identity) and variants (at least 90% sequence identity) which differ in abundance and immunogenicity (www.allergen.org).^[10]

For natural (n)Bet v 1 isolated from birch pollen, 3 different isoallergens and 27 variants have been characterized so far

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(www.allergen.org), and it is well known that the most abundant (50–70% of total nBet v 1) and most immunogenic variant is Bet v 1.0101.^[11–13] Moreover, a quality and potency profile of eight recombinant isoallergens, largely mimicking total nBet v 1-specific IgE binding of birch pollen, exists.^[13] This precise knowledge of the qualitative and quantitative nBet v 1 composition is of great importance for the use of recombinant allergens in component-resolved diagnosis and therapy.

Although Dau c 1 is an important food allergen, information on the composition of nDau c 1 isolated from carrot is scarce. In addition, little is known about the abundance and immunogenicity of the different Dau c 1 isoallergens and variants. So far, four different Dau c 1 isoallergens, Dau c 1.01, 1.02, 1.03, and 1.04, have been identified. Dau c 1.01 comprises five different variants: Dau c 1.0101 to Dau c 1.0105 (www.allergen.org). We have previously reported that the qualitative composition of nDau c 1 is unexpected, with at least eight isoallergens or variants that have not been characterized yet.^[14]

We recently identified and analyzed one of those novel isoallergens and designated it Dau c 1.0401.^[15] It shows an unusual feature: while the soluble protein clearly exhibits IgE-binding with serum of carrot-allergic patients, IgE-binding is impaired if the protein is immobilized on a membrane, i.e., during immunoblot procedures,^[15] indicating that structural IgE epitopes are destroyed.

To get a more complete picture, the qualitative composition of nDau c 1 was further investigated in more detail in this work.

2. Experimental Section

2.1. Identification of mRNA

Isolation and identification of mRNA was performed as previously described^[15] using specific primers in the 3'- and 5'-UTR region of the different Dau c 1 genes based on their hypothetical mRNA (Table S1, Supporting Information).

2.2. Cloning, Expression, and Purification

The Dau c 1 genes amplified from cDNA (see above) were cloned into the bacterial expression vector pET_GB1a (G. Stier, EMBL) by circular polymerase extension cloning (CPEC)^[16] using the primers listed in Table S2, Supporting Information. The resulting proteins carried an N-terminal 6xHis-GB1a fusion protein, and a tobacco etch virus (TEV) protease cleavage site for removal of the tag.

Expression of different Dau c 1 genes was carried out in *Escherichia coli* BL21 (DE3) harboring the plasmid pET_GB1a with the respective gene.^[17] In brief, bacteria were grown in lysogeny broth medium supplemented with 30 µg mL⁻¹ kanamycin at 37 °C. At an optical density of 600 nm of 0.6–0.7 the temperature was decreased to 16 °C and overexpression was induced overnight for ca. 18–19 h by the addition of 0.6 mM isopropyl β-D-1-thiogalactopyranoside. After harvesting (6000 x g, 10 min, 4 °C), the cell pellet was resuspended in 20 mM Tris–HCl, 10 mM imidazole, 500 mM NaCl, pH 7, supplemented with DNase I (AppliChem GmbH, Darmstadt, Germany) and ¼ EDTA-free protease inhibitor tablet (Roche Diagnostics GmbH, Mannheim,

Germany). After lysis (Microfluidics, Newton, USA) the lysate was centrifuged (75 000 x g, 30 min, 4 °C) and the supernatant was applied onto a 5 mL HisTrap HP column (Cytiva Europe GmbH). Elution was carried out using a continuous gradient from 0% to 100% elution buffer (20 mM Tris–HCl, 500 mM imidazole, 500 mM NaCl, pH 7) over 20 column volumes. Fractions containing the protein in question were combined and dialyzed against 20 mM Tris–HCl, 100 mM NaCl, pH 7 (molecular weight cut off (MWCO) 3500 Da) at 4 °C overnight in the presence of TEV protease to cleave off the 6xHis-GB1a fusion protein.

The dialysate was again loaded on a 5 mL HisTrap HP column. The flow through fractions containing the tagless Dau c 1 protein (or Dau c 1-like, or Dau-norococlaurine synthase (NCS)) were combined and dialyzed at 4 °C over night against 10 mM Na-phosphate, 300 mM NaCl, pH 7, and concentrated (Vivaspin MWCO 5000 Da). The protein was further purified by size exclusion chromatography (SEC) using two consecutive Superdex 75 10/300 GL columns (Cytiva Europe GmbH). Fractions containing pure protein were combined and dialyzed against 10 mM Na-phosphate, pH 7 at 4 °C over night. The concentrated dialysate (Vivaspin MWCO 5000 Da) was shock frozen in liquid nitrogen and stored at –80 °C.

2.3. CD Spectroscopy, NanoDSF, and 1D NMR

Circular dichroism (CD) spectra and nano-differential scanning fluorometry (DSF) measurements using a Prometheus NT.48 device were recorded as previously described.^[14,15,18] For nanoDSF measurements, the protein concentration was adjusted to 0.5–2 mg mL⁻¹. A continuous gradient from 20 to 110 °C (excitation power 80%, temperature slope 2.0° min⁻¹) was applied. For each isoallergen three independent triplicates were measured. Standard 1D ¹H NMR spectra were recorded on a BRUKER Avance 600 MHz spectrometer at 25 °C with WATERGATE solvent suppression.^[19] For each measurement 80–330 µM of protein in 10 mM Na-phosphate, pH 7.0, 10 % D₂O was used. All data were processed and visualized with the Bruker software TOP SPIN.

2.4. Patients' Sera

Sera from carrot-allergic patients that were recently used to test their reactivity against nDau c 1 and to characterize the isoallergen Dau c 1.0401 were applied in this study.^[15] The sera were obtained after written consent and approval of the ethics committee (Faculty of Medicine, Friedrich-Alexander-University of Erlangen-Nuremberg, No. 3494).^[15] Characterization of the sera and their use in the various assays described here was shown in Table S3, Supporting Information.

2.5. Immunoblots, Mediator Release Assays and IgE Inhibition by ELISA

Mediator release assays (MRAs) and IgE immunoblots were essentially performed as previously described.^[14,15,20,21] For

	T1	T6	T19	T22	T32/34	T41	T46	T55	T69	T71
Dau-NCS	MAGSVSCVLINAPASSVWDLIK	GLK	LAAAEIESFTHLIEK	IDAVGDGSxVGTENIK	FK	PGTVPFSSYYK	ER	FTK	VDNESMVK	
Dau c 1.0201	-MGVQK	<u>TEVEAPSTVSAEK</u>	<u>MYGGFLDDMTVFPK</u>	VLPQLIK	<u>SVEILExGDGGVGTVR</u>	<u>LVLHGEATEYTTMK</u>	QK	VDVIDK		
Dau-Var2	-MGAITxTNVEVESSV-PAQTIIK	<u>GFLLDMDNIIPK</u>	ILPQAIIK	RVEIISxGDGGIGTIK	QITLGEVSQFTIVK	QRIDEIDREGLK				
Dau c 1-like	-MGAVTxTDVEVVSxAV-PAQTIFK	<u>GFLLDMDNIIPK</u>	VLPQAIIK	SVEKISxGDGGAGTIK	<u>K VTLGEVSQFTIVK</u>	<u>QRIDEIDTEALK</u>				
Dau c 1.0401	-MGVQK	<u>SEVEATSSV-SAEK</u>	LFK	<u>ALCLDIDITLLPQxVLPQAIIK</u>	<u>SSETILExGDGGVGTVR</u>	<u>LVLHGDASPFK</u>	TMK	QK	VDAIDK	
Dau c 1.0601	-MGVQK	<u>SEVEATSSV-SAEK</u>	LFK	<u>ALCLDIDITLLPQxVLPQAIIK</u>	<u>SAEILExGDGGVGTVR</u>	<u>LIHLGDASPFK</u>	TMK	QK	VDVIDK	
Dau c 1.0501	-MGVQK	<u>SEVVIASPV-PAEK</u>	LFK	<u>GICLDIDITLLPQxVLPQAIIK</u>	<u>GAEILExGDGGAGTIK</u>	<u>LVTGLDASPFK</u>	TMK	QK	TEAIDK	
Dau c 1.0103	-MGAQxHSLEITSSV-SAEK	<u>IFSxGIVLDVDTVIPK</u>	AATGAYK	SVE-VK	<u>GDGGAGTIR</u>	<u>IITLPEGSPITxTMTxVR</u>			TDAVNK	
Dau c 1.0101	-MGAQxHSLEITSSV-SAEK	<u>IFSxGIVLDVDTVIPK</u>	AAPGAYK	SVD-VK	<u>GDGGAGTIR</u>	<u>IITLPEGSPITxSMTxVR</u>			TDAVNK	
Dau c 1.0102	-MGAQxHSLEITSSV-SAEK	<u>IFSxGIVLDVDTVIPK</u>	AAPGAYK	SVD-VK	<u>GDGGAGTIR</u>	<u>IITLPEGSPITxSMTxVR</u>			TDAVNK	
Dau c S-A0A175YPA2	-MGAQxHSLEITSSV-SAEK	<u>IFSxGIVLDVDTVIPK</u>	AAPGAYK	SVD-VK	<u>GDGGAGTIR</u>	<u>IITLPEGSPITxTMTxVR</u>			TDAVNK	
Dau c 1.0104	-MGAQxHSLEITSSV-SAEK	<u>IFSxGIVLDVDTVIPK</u>	AAPGAYK	SVD-VK	<u>GDGGAGTIR</u>	<u>IITLPEGSPITxSMTxVR</u>			TDAVNK	
Dau c 1.0105	-MGAQxHSLEITSSV-SAEK	<u>IFSxGIVLDVDTVIPK</u>	AAPGAYK	SVE-VK	<u>GDGGAGTIR</u>	<u>IITLPEGSPITxSMTxVR</u>			TDAVNK	
Dau c S-D9ZHP1	-MGVQK	<u>HEQEITSSV-PAEK</u>	MFXxGLILDIDNVLK	AAPGAYK	NVE-IK	<u>GDGGVGTIK</u>	<u>HITLPEGGPVTxTMTxLR</u>		TDGLDK	K
Dau c S-D9ZHP0	-MGVQK	<u>HEQEITSSV-PAEK</u>	MFXxGLILDIDNVLK	AAPGAYK	NVE-IK	<u>GDGGVGTIK</u>	<u>HITLPEGGPVTxTMTxLR</u>		TDGLDK	K
Dau c 1.0301	-MGVQK	<u>HEQEITSSV-PAEK</u>	MFXxGLILDIDNVLK	AAPGAYK	NVE-IK	<u>GDGGVGTIK</u>	<u>HITLPEGGPVTxTMTxLR</u>		TDGLDK	K
Dau-NCS	ENEVFEGGYCDLGFSK	YFVR	FEIVK K	DENSICIAK	ATTEYELK	ADADPNLASLVSDQMMGVNLNAANK	VVNGSK			
Dau c 1.0201	AGLGTYTTIGDILVEGLESxVVNQFVVVPTx	DDGGCIVK	NTTIYNTK	<u>GDAVLEEDK VK</u>	EATEK	<u>SALAFK</u>	<u>AVEAYLLAN</u>			
Dau-Var2	YSYSIIEGDLMLGIESIASK	FTVVPTxKxxxEGGCIVK	NTAVYTPixGDAVPEENxVK	<u>EATEQSGMVFK</u>	QIEAYLLANPAAY					
Dau c 1-like	YSYSIIEGDLMLGIESIASK	FTVVPTxKxxxEGGCIVK	NTTIYTPixGDAVPEENxVK	<u>EATEQSGMVFK</u>	<u>QIEAYLLANPGLY</u>					
Dau c 1.0401	ESFTYAYSIIIDGILLGFIESxINNHFAYVPNADGGCTVK	STITFNTK	<u>GDAVPEENxIK</u>	FANDQNR	<u>AIFQxAVEAYLLAN</u>					
Dau c 1.0601	DSFTYSYTIIDGILLDFVESxINNHFYVPTADGGCTVK	STITFTTK	<u>GDAVPEENxIK</u>	FANDQNLx	<u>AIFK</u>	<u>AIEAYLLAN</u>				
Dau c 1.0501	EAFTFYSYTIIDGILLGYIDSxITTEFTFTPTADGGCTAK	SVSTFNTK	<u>GDAVPEENxINx</u>	FANEQNGx	<u>IIFK</u>	<u>AIEAYLLAN</u>				
Dau c 1.0103	EALSYDSTVIDGILLGFIESxIETHMVVPTADGGCITK	TTAIFHTK	<u>GDAVPEENxIK</u>	FADAQNTx	<u>ALFK</u>	<u>AIEAYLLAN</u>				
Dau c 1.0101	EALTYDSTVIDGILLGFIESxIETHMVVPTADGGCITK	TTAIFHTK	<u>GDAVPEENxIK</u>	FADAQNTx	<u>ALFK</u>	<u>AIEAYLLAN</u>				
Dau c 1.0102	EALTYDSTVIDGILLGFIESxIETHMVVPTADGGCITK	TTAIFHTK	<u>GDAVPEENxIK</u>	FADAQNTx	<u>ALFK</u>	<u>AIEAYLLAN</u>				
Dau c S-A0A175YPA2	EALTYDSTVIDGILLGFIESxIETHMVVPTADGGCITK	TTAIFHTK	<u>GDAVPEENxIK</u>	FADEQNTx	<u>ALFK</u>	<u>AIEAYLLAN</u>				
Dau c 1.0104	EALTYDSTVIDGILLGFIESxIETHLVVPTADGGCITK	TTAIFHTK	<u>GDAVPEENxIK</u>	FADEQNTx	<u>ALFK</u>	<u>AIEAYLLAN</u>				
Dau c 1.0105	EALTYDSTVIDGILLGFIESxIETHLVVPTADGGCITK	TTAIFHTK	<u>GDAVPEENxIK</u>	FADAQNTx	<u>ALFK</u>	<u>AIEAYLLAN</u>				
Dau c S-D9ZHP1	x	TTAIFHTK	<u>GDAVPEENxIK</u>	YAEQNTx	<u>LLFK</u>	<u>AVEAYLLAN</u>				
Dau c S-D9ZHP0	xGFTIDYSVIDGDVLMGFIDK	LENHLSVVPPTADGGSTTK	TTAIFHTK	<u>GDAVPEENxIK</u>	YAEQNTx	<u>LLFK</u>	<u>AVEAYLLAN</u>			
Dau c 1.0301	xGFTIDYSVIDGDVLMGFIDK	LENHLSVVPPTADGGSTTK	TTAIFHTK	<u>GDAVPEENxIK</u>	YAEQNTx	<u>LLFK</u>	<u>AVEAYLLAN</u>			

Figure 1. Sequence alignment of Dau c 1 peptides identified by LC-MS^E. Amino acid sequences of Dau c 1-like proteins from *Daucus carota* were aligned. Corresponding peptides detected by LC-MS^E are underlined. Differing sequence stretches are color coded. Gaps indicate trypsin cleavage sites. An "x" indicates a missing trypsin cleavage site and was used to adjust the sequence alignment. Sections highlighted in grey represent already known Dau c 1 sequences. The positions of the amino acids in the sequence were labeled according to the following scheme: e.g., T6 means that the corresponding peptide is released after "T"-(Trypsin) cleavage and starts at amino acid position 6. The corresponding amino acid identity matrix is presented in Table S4, Supporting Information.

immunoblotting 0.8 µg non-reduced protein cm⁻¹ was used for the SDS-polyacrylamide gel electrophoresis (PAGE). Transfer of the putative allergens onto nitrocellulose membranes was controlled by Ponceau S staining (Figure S1A, Supporting Information). The membranes were cut into stripes which were incubated with sera listed in Table S3, Supporting Information. The serum numbers are shown on top of the immunoblots (Figure S1B, Supporting Information). Nitrocellulose membrane stripes using rDau c 1.0101 were prepared identically (Figure S1C, Supporting Information) and applied as a positive control using serum #44 (Figure S1B, Supporting Information).

For the MRAs huRBL-1B2 cells were sensitized with serum (diluted 1:10) and then cross-linked with 1 µg mL⁻¹ rDau c 1 isoallergen. The following controls were performed: serum negative control (sensitization with serum, but no cross-linking with allergen), non-allergenic control (sensitization with serum of non-allergic patient), Bovine serum albumin (BSA) control (cross-linking with BSA, instead of an allergen), human (hu) IgE control (sensitization with huIgE and crosslinking with anti-huIgE). To measure the maximal release, which could occur by cross-linking polyclonal IgE on the huRBL-1B2 cells, huIgE was used for sensitization and anti-huIgE for cross-linking. The β-hexosaminidase release was normalized to the release of this IgE-control.^[20]

For the enzyme-linked immunosorbent assays (ELISAs) rDau c 1.0104 (2 µg per well) was coated to Maxisorb plates as described.^[14,21] Serum was diluted to reach an OD_{450 nm} between

0.6 and 0.8. Serial dilutions of purified rDau c 1 isoallergens, or Dau c 1 like, or Dau-NCS were used as inhibitors.

2.6. Bioinformatic Tools

Pairwise sequence alignments were performed with EMBOSS needle and multiple sequence alignments with Clustal Omega^[22] and by SnapGene software (from Insightful Science; available at snapgene.com). Similar sequences in the carrot genome were found with the Basic Local Alignment Search Tool (BLAST).^[23] The model of Dau c 1-like (Figure 5) was calculated by Phyre2^[24] and visualized with PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

3. Results and Discussion

3.1. Unexpected Composition of nDau c 1: Identification of Novel Putative Dau c 1 Genes

We recently purified nDau c 1 from carrot root and performed liquid chromatography-mass spectrometry^{elevated energy} (LC-MS^E) to analyze its composition^[15] (Figure 1). Surprisingly, we found out that the nDau c 1 composition was more complex than previously thought harboring several additional isoallergens and variants that have not been characterized yet. Dau c 1-related peptides derived from database entries from *Daucus carota* (DAUCS),

Pimpinella brachycarpa (PIMBR), *Apium graveolens* (APIGR), and *Petroselinum crispum* (PETCR) were identified.^[15] Here, we only used the sequence information from *Daucus carota*^[15] and thus were able to identify four entries possessing sufficient sequence identity to known Dau c 1 sequences with the following Uniprot accession numbers: A0A164WTA1 (= Dau-NCS), A0A175YPA2, D9ZHP1, and D9ZHP0. In addition, we used the identified peptides to search the carrot genome by BLAST in order to find complete gene sequences. By this approach, four additional sequences were recognized with the following Gene IDs: XP_017215843.1 (Var 1 = Dau c 1.0601), XP_017218034.1 (= Var 2), XP_017220733.1 (Var 3 = Dau c 1-like), XP_017220806.1 (Var 4 = Dau c 1.0501). (Figures 1 and S2, Supporting Information).

As will be described thoroughly in the following sections our data show that two of the proteins (Var 1 and Var 4) proved to have allergenic potential and thus were recognized as Dau c 1 isoallergens by the WHO/IUIS Allergen Nomenclature Sub-Committee and given the designations Dau c 1.0601 for Var 1 and Dau c 1.0501 for Var 4, respectively.

3.2. Expression of the Putative Dau c 1 Genes in Carrot Roots

To confirm that the sequences of the putative Dau c 1 genes are correct and complete and that they are in fact expressed in carrot roots we isolated RNA from the roots and identified the corresponding genes on the mRNA level by RT-PCR. Specific primers from the 3' and 5' UTR regions based on the *Daucus carota* genome^[25] (GenBank Assembly Accession Number: GCA 001625215.1) were used for cDNA amplification. The DNA obtained was subsequently cloned into an expression vector and sequenced (Figure S2, Supporting Information).

Due to their high similarity to Dau c 1.0104 or Dau c 1.0301 (aa-identity of >95 %) (Table S4, Supporting Information), identification on the mRNA level was not considered for the sequence entries D9ZHP1, D9ZHP0, and A0A175YPA2 since we assumed that their biochemical and immunological properties would be comparable to the already well characterized isoallergens Dau c 1.0104 and Dau c 1.0301.^[14,15]

The remaining five novel Dau c 1-related sequences were expressed in *E. coli*. Four of them (Var 1–Var 4), Dau c 1.0501 (= Var4), Dau c 1.0601 (= Var1), Dau-var2 (= Var2), Dau c 1-like (= Var3) exhibited an amino acid identity of 59–87% to the already known Dau c 1 allergens (Table S4, Supporting Information). The fifth one, namely A0A164WTA1, was a rather unusual hit with only about 22% sequence identity to any of the other Dau c 1-related sequences. However, it showed 53% amino acid identity with S-norococlaurine synthase (NCS) from Kiwi plant (*Actinidia chinensis*) (Sequence ID PSS32256.1). NCS catalyzes the first committed step in the biosynthesis of benzylisoquinoline and is present in different plants.^[26] Thus, we tentatively named the protein Dau-NCS, although we do not know yet whether it has enzymatic activity and included it in our studies to reveal its biochemical and immunological properties.

3.3. Biochemical and Biophysical Characterization

Since one of the expressed proteins (Dau-Var2) proved insoluble, only the remaining four purified proteins, Dau c 1.0501,

Dau c 1.0601, Dau c 1-like, and Dau-NCS (Table S4, Figure S2, Supporting Information) were subjected to thorough biochemical and biophysical analyses. SDS-PAGE and size exclusion chromatography experiments were performed to clarify the purity and oligomerization status of the proteins. The gels exhibit one pronounced band indicating that all four proteins are highly pure (ca. 95%) (Figure S3, Supporting Information). This is confirmed by the size exclusion chromatography, which shows only one distinct peak for each protein. The retention volumes of Dau c 1.0501, Dau c 1.0601, and Dau-NCS were around 12 mL, Dau c 1-like eluted at 11 mL. This corresponds to apparent molecular masses of 15.2–15.7 kDa, revealing that all four proteins are monomeric (Figure S4A, Supporting Information). ¹H NMR spectroscopy revealed a large dispersion of amide protons (7–9.5 ppm) and upfield shifted methyl resonances (below 0.6 ppm) indicating the structural integrity and three-dimensional folding of all purified proteins (Figure S4B, Supporting Information).

In addition, the CD spectra (Figure 2A) at 25 °C (Figure 2A, blue lines) show a broad minimum around 215 nm typical for proteins consisting of α and β secondary structures. The low ellipticity above 210 nm and the minimum around 200 nm (Figure 2A, red lines) demonstrate that all proteins get denatured when heated to 95 °C. Except for Dau-NCS complete or at least partial refolding is possible after heating to 95 °C and cooling to 25 °C (Figure 2A, green lines). In order to determine the protein stability, melting point (T_m) experiments were performed by CD spectroscopy and nanoDSF (Figure 2B,C, Table S5, Supporting Information). For each protein the two methods resulted in similar T_m -values (Figure 2B,C, red lines). However, whereas the T_m -values of the new Dau c 1 isoallergens 1.0501 and 1.0601 and the Dau c 1-like protein were similar, ranging from ca. 57 to 63 °C, that of Dau-NCS was strikingly higher with values of 79.7 °C (CD) and 72.2 °C (nanoDSF), indicating a much higher thermal stability (Table S5, Supporting information).

Refolding CD-experiments indicated that Dau c 1.0501 as well as Dau-NCS were not able to refold (Figure 2B, black dotted lines) after a longer time period at temperatures above the T_m , whereas the other proteins were able to fold back, albeit incompletely (Figure 2B). The differences as compared to Figure 2A are probably due to the extended time period at high temperatures when determining the T_m (ca. 15 min at 95 °C above the melting point during recording of CD-spectra (Figure 2A) vs 46–74 min above the melting point during refolding experiments (Figure 2B)). Similarly, none of the proteins is capable of refolding in nanoDSF experiments (Figure 2C), probably because here the protein solution is heated to 110 °C. The comparison of the experiments shown in Figure 2A–C indicates that the time period of the temperature above T_m affects the refolding ability.

In summary, Dau-NCS shows the greatest variation in its properties compared to the other Dau c 1 proteins. It appears to be more resistant towards higher temperatures. This is probably mainly attributable to the considerable differences in its amino acid composition (Figure 1).

3.4. IgE Binding

We previously showed with Dau c 1.0401 that the immune response can be attenuated if the allergen is partially or fully denatured as is the case with immunoblots.^[15] This might be due

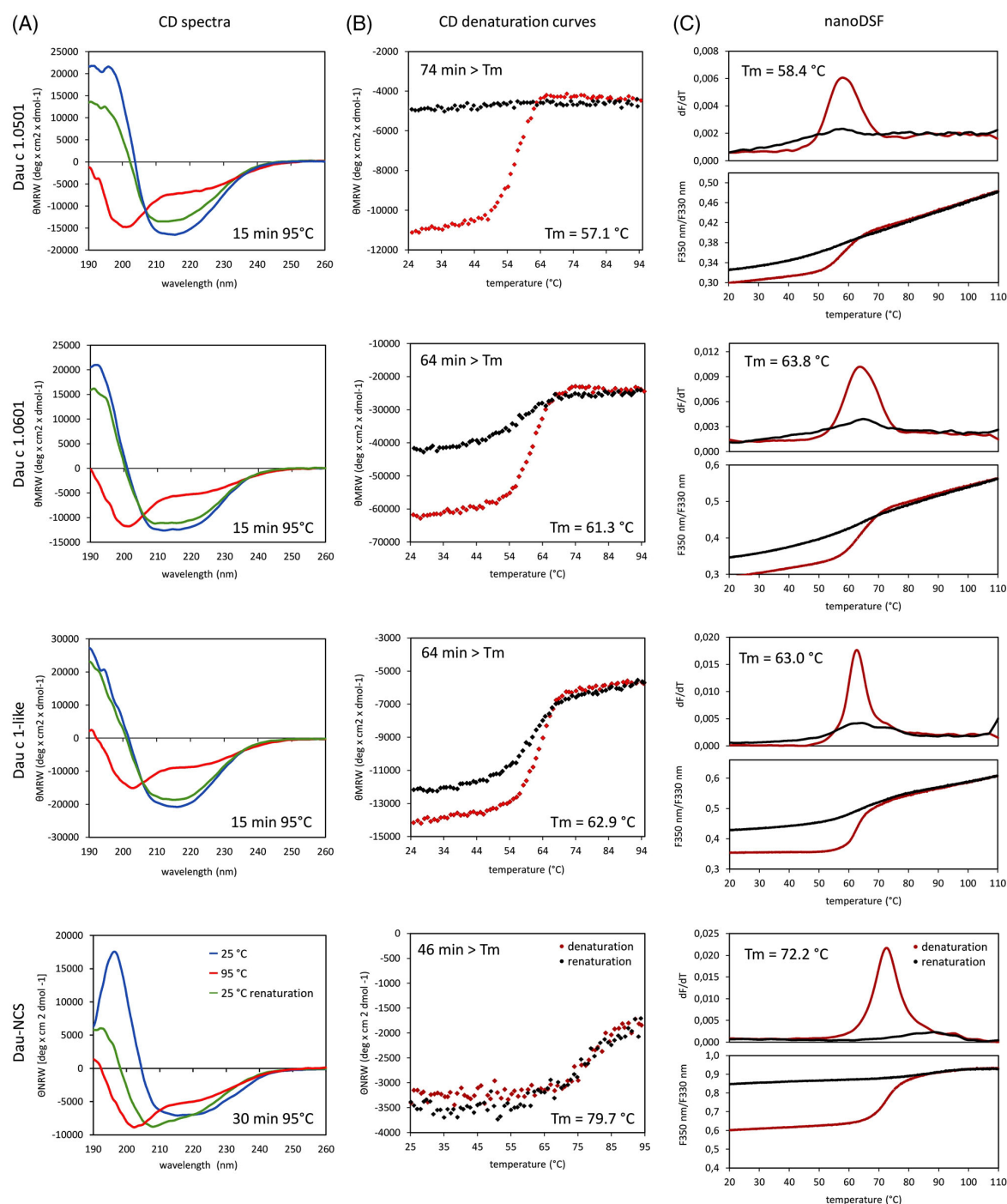


Figure 2. CD spectra and nanoDSF analyses. CD spectra (panel A) at 25 °C, after heating at 95 °C for 15 min (or 30 min for Dau-NCS), followed by stepwise cooling to 25 °C (green) are shown. CD (panel B) and nanoDSF (panel C) denaturation (red) and renaturation (black) curves are shown. The duration of incubation above the T_m is displayed at the top left of each spectrum in B). The CD curves (panel B) were recorded at 217 nm. The upper segments of the nanoDSF graphs (panel C) show the fluorescence ratio of 350 nm:330 nm, the lower segments show the first derivative thereof. Buffer for CD-measurements: 10 mM Na₂HPO₄/NaH₂PO₄ pH 7.0. Buffer for nanoDSF: 10 mM K₂HPO₄/KH₂PO₄ pH 7.0. An overview of the melting points is shown in Table S5, Supporting Information.

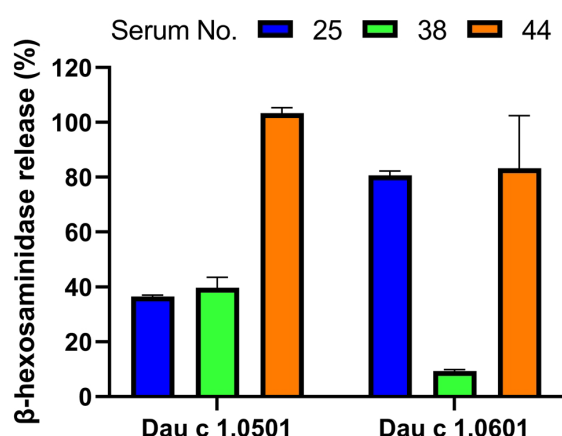


Figure 3. Mediator release assays with huRBL-TB2 cells. For sensitization a 1:10 dilution of the sera was used. Cross-linking was performed with $1 \mu\text{g mL}^{-1}$ Dau c 1.0501 or Dau c 1.0601. 4-nitrophenyl N-acetyl- β -D-glucosaminide was used as a substrate. The β -hexosaminidase release was normalized to the human IgE-control.

to the disintegration of structural epitopes that are recognized by IgE antibodies. Thus, we performed different immunological experiments in which the protein in question was either present in its native or denatured form.

We used sera from 14 different patients with known carrot allergy that exhibited mild to severe clinical symptoms^[15] (Table S3, Supporting Information) and performed immunoblots using non-reducing conditions for the SDS-PAGE (Figure S1, Supporting Information). Previous experiments showed that during the blotting procedure the proteins get at least partially denatured resulting in weaker reactivity.^[15] Dau c 1.0501 reacted weakly with #14 and #44. Dau c 1.0601 reacted with five sera (#14, #31, #38, #40, #44), however a strong reaction is only visible with #44. Dau c 1-like showed weak bands with #25, #39, and #40, and stronger reactions with #14 and #44, whereas Dau-NCS exhibited no IgE binding (Figure S1B, Supporting Information). These results imply that Dau-NCS is either non-allergenic or comprises no structural epitopes recognized by IgE antibodies originating from carrot allergy. Similarly, Dau c 1-like also appeared to be only weakly allergenic. However, since these results might be due to the denaturing conditions of the immunoblot, MRAs were conducted.

3.5. Mediator Release Assays

MRAs were performed with rat basophilic leukemia cells (RBLs) that recombinantly express the human high affinity IgE receptor α -chains. In response to IgE receptor binding and IgE cross-linking, the release of the mediator β -hexosaminidase can be measured.^[20] In contrast to the immunoblots described above, the protein under investigation remains in solution and consequently is present in its native conformation.

Although in this experiment all available sera were tested (Table S3, Supporting Information), significant mediator release was only detectable with Dau c 1.0601 and Dau c 1.0501 using the sera #25, #38, and #44 (Figure 3), whereas Dau c 1-like and Dau-NCS did not induce mediator release (data not shown). Since

two different IgE epitopes must be present on the allergen in order to achieve cross-linking of IgE antibodies in this assay, we assume that some sera harbor only one specific type of IgE directed against one single epitope. Consequently, inhibition assays were performed, which do not require the presence of two different epitopes.

3.6. Inhibition of IgE Binding to Dau c 1.0104 by ELISA

To investigate the cross-reactivity and epitope diversity of IgE antibodies, inhibition assays were performed, which detect whether IgE binding to an immobilized Dau c 1 protein can be inhibited by preincubation of the serum with one of the new Dau c 1 proteins (Figure 4). A prerequisite for inhibition is that the immobilized protein shares one or more epitopes with the novel Dau c 1 proteins. We resorted to Dau c 1.0104 as the immobilized protein as it had been used successfully in inhibition assays previously.^[15] Only five sera were chosen for this experiment, since they displayed high reactivity in ImmunoCAP assays with the known recombinant Dau c 1 proteins Dau c 1.0101, 1.0201, and 1.0301 (Table S3, Supporting Information). The extent of inhibition of IgE binding to Dau c 1.0104 differed among the five sera used. Dau c 1.0501 (Figure 4, green circles) and Dau c 1.0601 (Figure 4, blue circles) showed strong inhibition using the sera #14, #25, and #28, whereas no inhibition was achieved with Dau c 1-like and Dau-NCS. Serum #29 was less reactive in general, even when self-inhibited with Dau c 1.0104 (Figure 4, grey triangles). However, at high concentrations (10 and $100 \mu\text{g mL}^{-1}$) inhibition could be detected with Dau c 1.0501. BSA used as a negative control in all experiments caused no inhibition at all (Figure 4, brown squares). These experiments confirmed that Dau-NCS and Dau c 1-like exhibit only very weak or no allergenic potential. However, Dau c 1.0501 and Dau c 1.0601 met the requirements of the WHO/IUIS Allergen Nomenclature Sub-Committee to be recognized as Dau c 1 isoallergens.

In summary, the immunological assays (Figures 3, 4, and S1, Supporting Information) also indicate that the IgE repertoire of patients allergic to carrots is highly individual since the sera exhibit differences in their reactivity towards the proteins tested.

4. Discussion

Sequence comparisons showed that Dau c 1.0501 and 1.0601, exhibit sequence identities to Dau c 1.0101 of 54.55% and 58.44%, and to Dau c 1.0401 of 74.84% and 87.1% respectively, indicating new isoallergens (Table S4, Supporting Information, bold numbers). Our immunological assays confirmed the allergenicity of these proteins, thus they are considered new Dau c 1 isoallergens, which were only recently accepted by the WHO/IUIS Allergen Nomenclature Sub-Committee.

Dau c 1.0501 shares 74.84 % sequence identity with Dau c 1.0601. Surprisingly, the non-allergenic Dau c 1-like protein still possesses a rather high sequence identity between 50% and 55% to the major allergen Dau c 1.0101 as well as to 1.0501 and 1.0601 (Table S4, Supporting Information, bold numbers).

We thus used sequence and structure comparisons to detect putative epitope differences between the known isoallergens and the non-allergenic Dau c 1-like protein. Amino acid

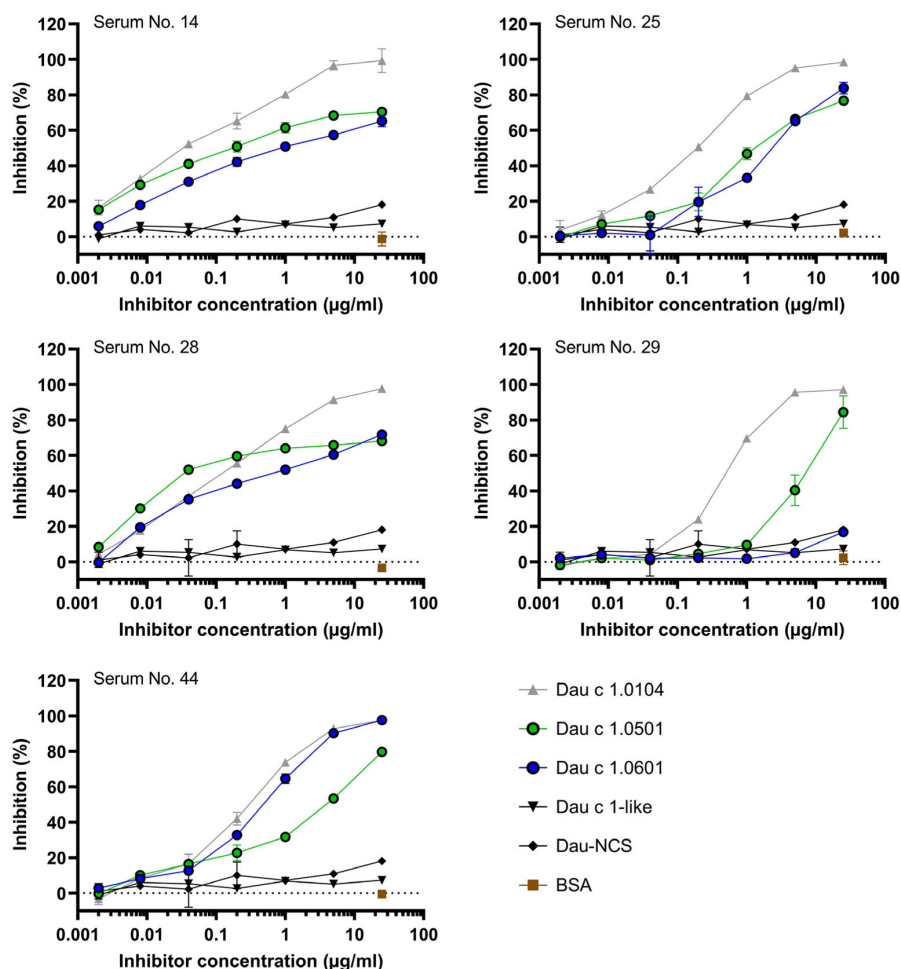


Figure 4. Inhibition of IgE binding to Dau c 1.0104. ELISA Inhibition was performed as previously described.^[21] Microtiter plates were coated with 2 µg Dau c 1.0104 per well. Serial dilutions of the putative rDau c 1 protein were added to the well, followed by the addition of serum. Bound specific IgE was quantified using horse-radish-peroxidase-labeled mouse anti-human IgE and 3,3',5,5'-Tetramethylbenzidine as a substrate. After stopping the reaction, the OD at 450/630 nm was determined. The Dau c 1 proteins added for inhibition are presented in different colors as indicated on the bottom right.

sequence alignments were performed to identify amino acid exchanges which are only present in Dau c 1-like but not in all the other known isoallergens Dau c 1.01 to Dau c 1.06 (**Figure 5**). A structural model of Dau c 1-like was calculated highlighting the conservative and non-conservative amino acid exchanges in the structure (**Figure 5A**). In particular, the non-conservative exchanges were considered important for epitope recognition by IgE antibodies. (**Figure 5B**, purple dots below alignment). These non-conservative exchanges are found in different structural elements of the protein (α -helix, β -strand, and loop structures), forming putative epitopes, most of which are accessible to antibodies (**Figure 5A**). These data show that there exist several distinct sites, which are identical and common to the known isoallergens but have been exchanged exclusively in Dau c 1-like. We hypothesize that these sites might be part of IgE epitopes in the isoallergens which are destroyed, once a particular amino acid is exchanged, as is the case in Dau c 1-like. The exchange makes

the epitope unrecognizable for antibodies. Further investigations need to be conducted to confirm this hypothesis.

Dau-NCS was also detected in purified nDau c 1. However, it was not included in our structural comparisons since it exhibited a much higher sequence identity of 53% to the NCS enzyme from Kiwi plant (*A. chinensis*) (Sequence ID PSS32256.1) as compared to only 22% to any of the known Dau c 1 isoallergens. Furthermore, it also differs considerably from the Dau c 1-like protein (21.71% identity) (Table S4). The enzyme NCS (EC 4.2.1.78) catalyzes the condensation of 3,4-dihydroxyphenylethylamine (dopamine) and 4-hydroxyphenylacetaldehyde (4-HPAA) as the first committed step in the biosynthesis of benzyloquinoline alkaloids in plants.^[27–29] We have not yet determined whether Dau-NCS in fact exhibits enzymatic activity. Structural information on NCS enzymes is only available for the NCS from the common meadow rue *Thalictrum flavum*. CD and NMR spectroscopic data indicated a high structural similarity of the NCS from

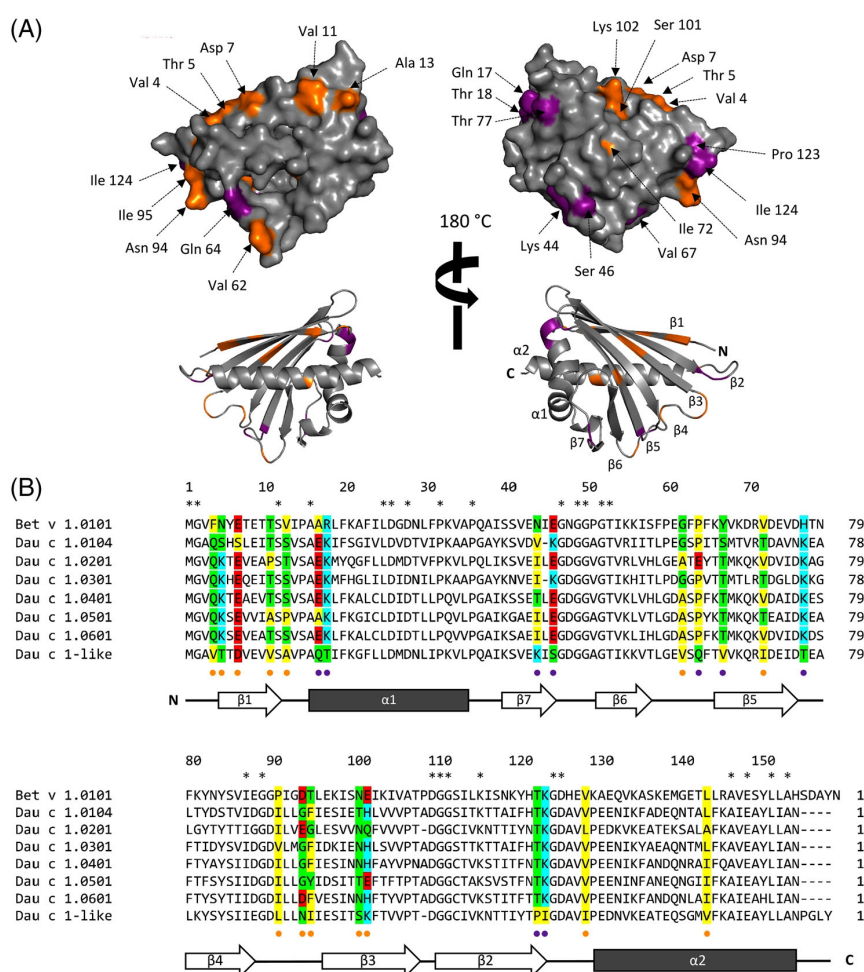


Figure 5. Comparison of allergenic Dau c 1 isoallergens with the non-allergenic Dau c 1-like protein. **A)** Structural model of Dau c 1-like protein. The model of Dau c 1-like was calculated by Phyre2^[24]. Purple: non-conservative amino acid exchanges, unique for Dau c 1-like; orange: conservative amino acid exchanges, unique for Dau c 1-like. **B)** Alignment of Dau c 1 isoallergen sequences. Green: polar/neutral amino acids; yellow: non-polar/hydrophobic amino acids; cyan: alkaline amino acids; red: acidic amino acids; asterisk: conserved amino acids in all known Dau c 1 isoallergens and Dau c 1-like; Conservative and non-conservative amino acid exchanges shown in **B)** are marked by orange or purple dots, respectively.

T. flavum with the major birch pollen allergen Bet v 1 even if the sequence identity is rather low (12–15% identity).^[30] Crystal structures confirm the high structural homology to proteins of the PR-10 family and identify the catalytic residues of NCS.^[31,32] Like PR-10 proteins, NCS possesses an accessible hydrophobic cleft located between a seven-stranded antiparallel β -sheet and three α -helices. Interestingly, despite its structural similarity with PR-10 allergens, *T. flavum* NCS is also not allergenic and does not bind to IgE antibodies.^[30,33] This corresponds to our data with Dau-NCS, which also proved to be non-allergenic.

5. Concluding Remarks

Identification of new isoallergens and comparison with a non-allergenic Dau c 1-like protein may contribute to a more refined component resolved diagnosis and may lay ground for fur-

ther epitope mapping and personalized targeted treatment approaches of carrot allergy in preclinical and clinical studies. Additionally, the knowledge of IgE epitopes can help to determine why some PR-10 proteins function as allergens whereas other similar proteins do not.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

B.M.W., J.M.H., A.W., and T.J. designed the experiments. J.M.H., T.J., and K.R. isolated the mRNAs, cloned the genes and performed the expression and purification, CD measurements, and NMR spectroscopy. B.M.W. and J.M.H. evaluated the experiments. J.M.H. and A.W. carried out the immunoblots, ELISAs, inhibition assays, MRAs, and nanoDSF. J.M.H., A.W., and B.M.W. evaluated the data. V.M. recruited and clinically characterized carrot-allergic patients and contributed to the interpretation of the data. B.M.W., J.M.H., and T.J. wrote the manuscript. All authors contributed to, read, and approved the final version of the manuscript.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

Keywords

allergenicity, Dau c 1 isoallergen, IgE cross reactivity, IgE epitopes, mediator release assay

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Supplementary information

Allergenicity and IgE recognition of new Dau c 1 allergens from carrot

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Tables

Table S1. Primers used for the amplification of different Dau c 1 genes from cDNA

Amplified genes	Sequence of primer pairs
Dau c 1.0501 (XM_017365317.1; XP_017220806.1)	fw: 5'- CAC TCA ATC TTT CTT TTC TAC ATC TCT TCT TTA GC - 3' rev: 5'- GCA AAC AAA ATT ATG AAC TCG CAA CTT GTA CAA AAG - 3'
Dau c 1.0601 (XM_017360354.1; XP_017215843.1)	fw: 5'- CCA GTC TCA CAA CAT CTA TAA TAT TCA CCC - 3' rev: 5'- CAC ACG CGC ACA CAT TAT TGA TTA TTC TC - 3'
Dau c 1-like (XM_017365244.1; XP_017220733.1)	fw: 5'- CAT TTC CAT ATA GCT TAT TAA GTT TTA GCT TGC TAG C - 3' rev: 5'- CTG AAA GAT AAC TTG AGA AAT TCA AGC ATA CAA AAT ATA GC - 3'
Dau-Var 2 (XM_017362545.1; XP_017218034.1)	fw: 5'- CAG CTA GAT AGC ACA AGC AAC TCC AGT TTC - 3' rev: 5'- GGC TCC TGG GGA AGT TTG CTA TAT AAA TG - 3'
Dau-NCS (XP_017255601.1; XM_017400112.1; A0A164WTA1)	fw: 5'- AAT CCA CCT TCG AAC AAG TCA - 3' rev: 5'- TCA AAA CCC AAG TGC AGA AAC TA - 3'

The amplified fragments were cloned into the vector pCR-Blunt using the Zero Blunt Cloning Kit (Thermo Fisher).

Table S2. Primers used for cloning of Dau c 1 genes into pET-GB1a

Primer	Sequence of primer pairs
Vector amplification Dauc1.0601	fw: 5'- GCT CAT CTC ATT GCA AAC TAA GGT ACC GGA TCC GAA TTC - 3' rev: 5'- CAC TCT TTT GGA CAC CCA TGC CCT GAA AAT AAA GAT TCT CAG - 3'
Insert amplification Dauc1.0601	fw: 5'- CTG AGA ATC TTT ATT TTC AGG GCA TGG GTG TCC AAA AGA GTG - 3' rev: 5'- GAA TTC GGA TCC GGT ACC TTA GTT TGC AAT GAG ATG AGC - 3'
Vector amplification Dau- Var2	fw: 5'- CAA ATC CTG CTG CCT ATT GAG GTA CCG GAT CCG AAT TC - 3' rev: 5'- CAT TAG TGG TGA TAG CAC CCA TGC CCT GAA AAT AAA GAT TCT CAG - 3'
Insert amplification Dau-Var2	fw: 5'- CTG AGA ATC TTT ATT TTC AGG GCA TGG GTG CTA TCA CCA CTA ATG - 3' rev: 5'- GAA TTC GGA TCC GGT ACC TCA ATA GGC AGC AGG ATT TG - 3'
Vector amplification Dauc1- like	fw: 5'- GCA AAT CCT GGT CTC TAT TGA GGT ACC GGA TCC GAA TTC - 3' rev: 5'- ACA TCA GTA GTA ACA GCA CCC ATG CCC TGA AAA TAA AGA TTC TCA - 3'
Insert amplification Dauc1- like	fw: 5'- CTG AGA ATC TTT ATT TTC AGG GCA TGG GTG CTG TTA CTA CTG ATG T - 3' rev: 5'- GAA TTC GGA TCC GGT ACC TCA ATA GAG ACC AGG ATT TGC - 3'
Vector amplification Dauc1.0501	fw: 5'- GGC TTA CCT CAT TGC AAA CTA AGG TAC CGG ATC CGA ATT C - 3' rev: 5'- GCT CTT TTG GAC ACC CAT GCC CTG AAA ATA AAG ATT CTC AG - 3'
Insert amplification Dauc1.0501	fw: 5'- CTG AGA ATC TTT ATT TTC AGG GCA TGG GTG TCC AAA AGA GC - 3' rev: 5'- GAA TTC GGA TCC GGT ACC TTA GTT TGC AAT GAG GTA AGC C - 3'
Vector amplification Dau- NCS	fw: 5'- GGT AGT CAA TGG GTC GAA ATA AGG TAC CGG ATC CGA ATT C - 3' rev: 5'- GAC ACA CTT CCC GCC ATG CCC TGA AAA TAA AGA TTC TCA G - 3'
Insert amplification Dau-NCS	fw: 5'- CTG AGA ATC TTT ATT TTC AGG GCA TGG CGG GAA GTG TGT C - 3' rev: 5'- GAA TTC GGA TCC GGT ACC TTA TTT CGA CCC ATT GAC TAC C - 3'

Table S3. Characterization of patients' sera.

Patient number	Assays performed in this study	Clinical symptoms to carrot	Provocation OFC	Skin test Carrot	CAP [kUA L-1] (CAP class)					
					Carrot (F31)	rBet v 1.0101	rDau c 1.0101	rDau c 1.0201	rDau c 1.0301	rDau c 4
2	MRA	OAS (mild)	Nd	Pos	0.52 (1)	0.06 (0)	0.02 (0)	0.01 (0)	0.00 (0)	0.02 (0)
4	MRA, IB	OAS, CU	Pos	Pos	0.10 (0)	6.62 (3)	0.00 (0)	2.04 (2)	0.04 (0)	0.01 (0)
14	MRA, Inh, IB	OAS (mild)	Pos	Pos	0.07 (0)	43.1 (4)	5.25 (3)	4.32 (3)	9.75 (3)	2.11 (2)
15	MRA, IB	OAS	Pos	Pos	2.82 (2)	35.9 (4)	3.7 (3)	4.32 (3)	3.57 (3)	0.09 (0)
16	MRA	OAS (mild)	Nd	Pos	0.19 (0)	13.0 (3)	1.01 (2)	0.04 (0)	0.87 (2)	0.04 (0)
21	MRA	OAS, Q	Nd	Pos	0.79 (2)	7.56 (3)	1.17 (2)	1.43 (2)	1.38 (2)	0.02 (0)
22	MRA, IB	U GI	Pos	Pos	0.71 (2)	2.58 (2)	0.02 (0)	0.17 (0)	0.09 (0)	0.05 (0)
25	MRA, Inh, IB	OAS	Pos	Pos	5.20 (3)	55.8 (5)	10.5 (3)	4.9 (3)	7.93 (3)	1.48 (2)
26	MRA, IB	Q, D, U	Nd	Nd	0.05 (0)	7.14 (3)	0.85 (2)	0.20 (0)	0.67 (1)	0.03 (0)
27	MRA	GI, Q	Nd	Neg	0.25 (0)	16.6 (3)	0.82 (2)	0.40 (1)	3.12 (2)	0.05 (0)
28	MRA, Inh, IB	OAS	Pos	Pos	2.20 (2)	9.28 (3)	2.53 (2)	1.55 (2)	2.61 (2)	0.88 (2)
29	MRA, Inh, IB	OAS	Pos	Pos	2.51 (2)	11.9 (3)	1.95 (2)	0.77 (2)	0.92 (2)	5.31 (3)
30	MRA, IB	OAS	Pos	Pos	0.62 (1)	5.35 (3)	0.64 (1)	0.91 (2)	0.73 (2)	0.05 (0)
31	MRA, IB	OAS, D	Pos	Pos	0.44 (1)	3.16 (2)	0.59 (1)	0.45 (1)	0.48 (1)	0.22 (0)
32	MRA	Q, D	Nd	Neg	0.37 (1)	69.8 (5)	0.71 (2)	2.15 (2)	2.29 (2)	0.08 (0)
33	MRA	OAS (mild)	Nd	Neg	0.02 (0)	>100 (6)	0.02 (0)	3.03 (2)	0.09 (0)	0.03 (0)
34	MRA	OAS	Nd	Pos	1.43 (2)	>100 (6)	12 (3)	0.77 (2)	8.57 (3)	0.09 (0)
37	MRA	OAS, D	Nd	Nd	0.68 (1)	7.39 (3)	1.5 (2)	1.21 (2)	0.94 (2)	0.04 (0)
38	MRA, IB	OAS	Pos	Pos	1.05 (2)	10.5 (3)	0.69 (1)	1.06 (2)	1.06 (2)	0.04 (0)
39	IB	OAS	Nd	Nd	0.13 (0)	0.00 (0)	0.00 (0)	0.01 (0)	0.03 (0)	0.00 (0)
40	MRA, IB	OAS, GI	Pos	Neg	0.42 (1)	29.4 (4)	1.29 (2)	2.28 (2)	1.28 (2)	0.03 (0)
42	MRA	GI. (raw carrots only)	Nd	Pos	11.4 (3)	0.64 (1)	0.27 (0)	0.34 (0)	0.40 (1)	0.38 (1)
44	MRA, Inh, IB	OAS	Pos	Pos	24.0 (4)	>100 (6)	28.4 (4)	16.6 (3)	28.4 (4)	0.54 (1)
45	MRA	OAS	Nd	Pos	0.61 (1)	6.32 (3)	0.58 (1)	0.47 (1)	0.76 (2)	0.05 (0)
46	MRA	OAS	Pos	Pos	8.16 (3)	0.06 (0)	4.86 (3)	14.4 (3)	6.92 (3)	0.18 (0)

CU, contact urticaria; D, dyspnea; GI, gastrointestinal symptoms; Nd, not determined; OAS, oral allergy syndrome; Q, Quincke's edema; U, urticaria. Nineteen carrot-allergic patients, selected by positive case history (OAS or systemic reactions) of carrot allergy were included in the study. Sensitization to carrot was tested by prick-to-prick testing (carrot cv Rodelika), and open food challenge (OFC). Specific IgE against carrot extract, the PR-10 allergens rBet v 1.0101, rDau c 1.0104, rDau c 1.0201, rDau c 1.0301 and the profilin rDau c 4 were determined in experimental ImmunoCAP measurements and assigned to the respective CAP class. MRA: mediator release assays with rat basophilic leukemia cells. Inh: inhibition assay. IB: immunoblot.

Table S4. Amino acid identity matrix of database hits detected in nDau c 1

Accession number		Dau-NCS	1.0201	Var2	Dau c 1-like	1.0401	Dau c 1.0601	Dau c 1.0501	1.0103	1.0101	1.0102	AOA175YPA2	1.0104	1.0105	D9ZHP1	D9ZHP0	1.0301
AOA164WTA1	Dau-NCS (Var5)	100.00	20.81	21.05	21.71	21.33	21.33	20.67	22.67	22.00	22.67	22.67	22.67	22.00	19.33	20.00	20.00
	1.0201	20.81	100.00	56.49	59.74	59.74	60.39	53.25	50.98	50.33	50.98	50.98	50.98	50.98	49.02	49.67	48.37
XP_017218034.1	Var2	21.05	56.49	100.00	84.18	50.00	50.65	50.00	49.67	48.37	49.02	50.98	50.33	50.33	49.02	50.33	50.33
XP_017220733.1	Dau c 1-like (Var3)	21.71	59.74	84.18	100.00	53.25	52.60	54.55	51.63	50.98	51.63	52.94	52.29	52.29	47.71	47.71	47.71
	1.0401	21.33	59.74	50.00	53.25	100.00	87.10	74.84	58.44	57.79	57.79	58.44	58.44	59.09	61.04	61.04	61.04
XP_017215843.1	1.0601 (Var1)	21.33	60.39	50.65	52.60	87.10	100.00	74.84	58.44	58.44	58.44	58.44	58.44	59.09	58.44	59.74	59.74
XP_017220806.1	1.0501 (Var4)	20.67	53.25	50.00	54.55	74.84	100.00	100.00	55.84	54.55	55.19	56.49	56.49	55.84	55.84	57.14	57.14
	1.0103	22.67	50.98	49.67	51.63	58.44	58.44	55.84	100.00	95.45	96.75	96.10	96.10	97.40	70.78	69.48	70.13
	1.0101	22.00	50.33	48.37	50.98	57.79	58.44	54.55	95.45	100.00	98.70	95.45	96.75	96.75	69.48	68.18	68.83
	1.0102	22.67	50.98	49.02	51.63	57.79	58.44	55.19	96.75	98.70	100.00	96.75	98.05	98.05	70.13	68.83	69.48
AOA175YPA2		22.67	50.98	50.98	52.94	58.44	58.44	56.49	96.10	95.45	96.75	100.00	97.40	96.10	71.43	71.43	70.78
	1.0104	22.67	50.98	50.33	52.29	58.44	58.44	56.49	96.10	96.75	98.05	97.40	100.00	98.70	70.78	70.78	70.13
	1.0105	22.00	50.98	50.33	52.29	59.09	59.09	56.49	97.40	96.75	98.05	96.10	100.00	100.00	72.08	70.78	71.43
D9ZHP1		19.33	49.02	49.02	47.71	61.04	58.44	55.84	70.78	69.48	70.13	71.43	70.78	72.08	100.00	94.16	95.45
D9ZHP0		20.00	49.67	50.33	47.71	61.04	59.74	57.79	69.48	68.18	68.83	71.43	70.78	70.78	100.00	98.70	98.70
	1.0301	20.00	48.37	50.33	47.71	61.04	59.74	57.14	70.13	68.83	69.48	70.78	70.13	71.43	95.45	98.70	100.00

Sequences with less than 67 % identity are highlighted in yellow, between 67 and 90 % (isoallergens) in orange and above 90 % (variants) in pink. Data taken from [14] Bold numbers are mentioned in the main text.

Table S5. Melting point (T_m) of new isoallergens and Dau c 1-like.

Isoallergen	CD (T _m)	*nanoDSF (T _m)
Dau c 1.0501	57.1 ± 0.1 °C	58.4 ± 0.1 °C
Dau c 1.0601	61.3 ± 0.1 °C	63.8 ± 0.4 °C
Dau c 1-like	62.9 ± 0.1 °C	63.0 ± 0.3 °C
Dau-NCS	79.7 ± 1.1 °C	72.2 ± 0.6 °C

*Mean values and standard deviation of three independent measurements

Figures

Dau c 1.0501: XM_017365317.1; XP_017220806.1

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taa cgg ccg cca gtg tgc tgg aat tca ggc act caa tct ttc ttt tct aca tct ctt ctt
tag ctc ctt cat ttc aac tgt ata atg ggt gtc caa aag agc gaa gta gtt atc gcc tct
- - - - - - - - M G V Q K S E V V I A S
cct gtc cca gca gct aag ctg ttc aaa ggc ata tgc ctc gac atc gat acc ctc ctt ccc
P V P A A K L F K G I C L D I D T L L P
cag gtt ctt ccc gga gcc atc aag ggt gct gaa atc ctt gaa ggt gac ggt gga gct gga
Q V L P G A I K G A E I L E G D G G A G
acc gtc aag ctc gtc act ctc gga gat gct agc ccc tac aag acc atg aag caa aag act
T V K L V T L G D A S P Y K T M K Q K T
gag gcc atc gac aag gaa gct ttc aca ttc tca tac agc atc atc gac gga gac att ctt
E A I D K E A F T F S Y S I I D G D I L
ctg gga tac atc gac tcc atc acc acc gaa ttc aca ttt aca ccc acc gct gac gga ggc
L G Y I D S I T T E F T F T P T A D G G
tgc acc gcc aaa agc gta tgc acc ttc aac acc aaa ggc gat gct gtg gtt ccg gaa gag
C T A K S V S T F N T K G D A V V P E E
aat att aac ttc gcc aat gag caa aac ggc atc att ttc aag gct att gag gct tac ctc
N I N F A N E Q N G I I F K A I E A Y L
att gca aac taa ggg atc agt ttc gac aga gct tga aga aac ctt ttg tac aag ttg cga
I A N - - - - - - - - - - - -
gtt cat aat ttt gtt tgc cct gaa ttc tgc aga tat cca tca cac tgg ccg ccg ctc gag

```

Dau c 1.0601: XM_017360354.1; XP_017215843.1

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gaa ttc agg cca gtc tca caa cat cta taa tat tca ccc ctg ctc tcc tgc att tgc tct
tat ttt ctc agt ttc tgt atc atg ggt gtc caa aag agt gaa gtc gaa gcc act tct tct
- - - - - - - M G V Q K S E V E A T S S
gtg tca gca gag aaa ctg ttc aag gcc ttg tgc ctc gac atc gat aca ctt ctt ccc cag
V S A E K L F K A L C L D I D T L L P Q
gtt gtt ccc ggt gct atc aag agt gcc gaa atc ctt gag ggt gac ggt gga gtt gga acc
V V P G A I K S A E I L E G D G G V G T
gtc aag ctt att cat ctt ggc gat gct agc ccc ttc aag aca atg aag caa aag gtt gat
V K L I H L G D A S P F K T M K Q K V D
gtc att gac aaa gac tcc ttc acc tac tct tac acc atc atc gac gga gac att ctt cta
V I D K D S F T Y S Y T I I D G D I L L
gat ttt gtg gaa tcc atc aac aac cat ttc acc tac gtc cct act gct gat gga ggc tgc
D F V E S I N N H F T Y V P T A D G G C
act gtc aag agt acc atc act ttc acc acc aaa ggc gat gct gtc gtc cct gaa gag aac
T V K S T I T F T T K G D A V V P E E N
atc aag ttc gct aat gac cag aac ctc gcc att ttc aag gct att gag gct cat ctc att
I K F A N D Q N L A I F K A I E A H L I
gca aac taa tta tga tca tgc tat att ttg atg agg gcg att gag aat aat caa taa tgt
A N - - - - - - - - - - - -
gtg cgc gtg tgc ctg aat tcc agc aca ctg gcg gcc gtt act agt gga tcc gag ctc ggt

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Dau c 1-like: XM_017365244.1; XP_017220733.1

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tca ggc att tcc ata tag ctt att aag ttt tag ctt gct agc aca agt gaa atc aac ttc
ttt tgt ata tca aca cat aaa agt ttt ttc ttt ttt tta cca taa att atg ggt gct gtt
- - - - - - - - - - - - M G A V
act act gat gtt gag gtt gtg tcc gct gtc cct gca caa aca ata ttc aag ggc ttt ctc

```

T T D V E V V S A V P A Q T I F K G F L
ctt gac atg gat aac ctt att cct aag gtt ttg ccc cag gct att aag agt gtc gag aaa
L D M D N L I P K V L P Q A I K S V E K
att tcc gga gat ggc gga gct gga acc atc aag aaa gtc act ctt gga gaa gtg agc caa
I S G G D G G A G T I K K V T L G E V S Q
ttc acc gtg gtg aag cag agg atc gac gaa atc gac acc gag gca ttg aaa tac tcc tac
F T V V K Q R I D E I D T E A L K Y S Y
agc ata atc gaa ggc gat ctc cta ctg aac ata att gag tcc atc act agt aag ttc aca
S I I E G D L L L N I I E S I T S K F T
gtg gtg cct act gat gga gga tgc atc gtg aag aac act acg att tat acg ccg atc ggt
V V P T D G G C I V K N T T I Y T P I G
gat gcc gtg atc ccg gaa gat aat gtc aag gaa gcc act gaa caa tca gga atg gtg ttc
D A V I P E D N V K E A T E Q S G M V F
aag gca att gaa gct tac ctt ctg gca aat cct ggt ctc tat tga ttt gct ata ttt tgt
K A I E A Y L L A N P G L Y - - - - -
atg ctt gaa ttt ctc aag tta tct ttc agc ctg aat tct gca gat atc cat cac act ggc

Dau-Var2: XM_017362545.1; XP_017218034.1

ttg gta ccg agc tcg gat cca cta gta acg gcc gcc agt gtg ctg gaa ttc agg cag cta
gat agc aca agc aac tcc agt ttc aaa tga tca tac tcc atg ggt gct atc acc act aat
- - - - - - - - - - - M G A I T T N
ggt gag gtt gaa tcc tca gtg cca gca caa aca att tac aag gga ttt ctc ctt gac atg
V E V E S S V P A Q T I Y K G F L L D M
gat aac atc att cct aag att ctc ccg cag gct att aag cgt gtc gag atc att tcc gga
D N I I P K I L P Q A I K R V E I I S G
gac ggc ggg atc gga acc atc aag cag atc act ctc gga gaa gtg agc caa ttc acc atc
D G G I G T I K Q I T L G E V S Q F T I
gta aag caa agt atc gat gaa ata gac agg gag ggc ttg aaa tac agt tac agc ata att
V K Q R I D E I D R E G L K Y S Y S I I
gaa ggt gat cta cta atg ggc ata att gag tct ata gca agt aaa ttc acc gtc gtg cct
E G D L L M G I I E S I A S K F T V V P
acg gag gga ggg tgc att gtc aag aac acg gcc gtt tat act ccg atc ggt gat gcc gtg
T E G G C I V K N T A V Y T P I G D A V
atc cca gag gag aat gtc aag gaa gct act gaa caa tca ggg atg gta ttc aag caa att
I P E E N V K E A T E Q S G M V F K Q I
gag gct tac ctt cta gca aat cct gct gcc tat tga ttt cta cac tat aca ttt ata tag
E A Y L L A N P A A Y - - - - -
caa act tcc cca gga gcc cct gaa ttc tgc aga tat cca tca cac tgg cgg ccg ctc gag

Dau-NCS: A0A164WTA1

atc tgc aga att cgc cct taa tcc acc ttc gaa caa gtc ata atc aca aat cga tat tta
ctc aac ata aga aag cat act aca gaa cta cat tga aga gag atg gct ggg tca gtt agc
- - - - - - - - - - - M A G S V S
tgt gaa tta gtg atc aat gca cca gca agc tcg gtg tgg gat ctg atc aaa ggt ctt aag
C E L V I N A P A S S V W D L I K G L K
tta gcg gct gct att gaa gaa tca ttc act cat ctt atc gag aaa atc gac gcc gtc ggt
L A A A I E S F T H L I E K I D A V G
gat ggt tcc gtc ggt aca gaa ctc aat atc aag ttc aaa cca ggc aca gtt ccc ttt tct
D G S V G T E L N I K F K P G T V P F S
tac tac aaa gag agg ttt acc aag gtt gac aac gag agc atg gtg aaa gag aat gag gtt
Y Y K E R F T K V D N E S M V K E N E V
ttt gag ggc ggt tat tgt gat ttg gga ttc agc aaa tac ttt gtt cga ttc gag atc gta
F E G G Y C D L G F S K Y F V R F E I V
aag aag gat gag aat agc tgc att gca aaa gct aca act gag tat gaa ctt aag gca gat
K K D E N S C I A K A T E Y E L K A D
gct gat cct aat ctc gct tcc tta gtt tca gtt gat caa atg atg ggt gtt ctc aac ctt
A D P N L A S L V S V D Q M M G V L N L
gct gct aac aaa gtc gta aac gga tcc aag taa cta att agc ctc tga ttt agt ttc tgc
A A N K V V N G S K - - - - -
act tgg gtt ttg aaa ggg cga att cca gca cac tgg cgg ccg tta cta gtg gat ccg agc

Figure S1. cDNA sequences of Dau c 1.0501, Dau c 1.0601, Dau c 1-like, Dau-Var2, and Dau-NCS. The sequences used for designing the 5' and 3' UTR primers are underlined, the coding sequences and the corresponding amino acid sequences are depicted in bold letters.

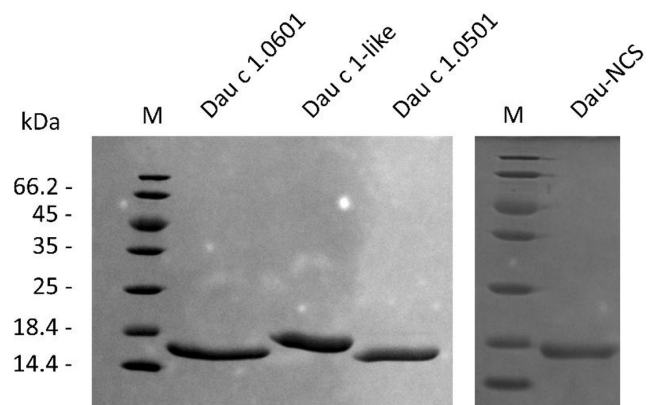


Figure S2. SDS-PAGE of purified proteins. 5 μ g of the purified proteins were subjected to electrophoreses on 19% SDS polyacrylamide gels, followed by Coomassie staining. M: molecular weight marker.

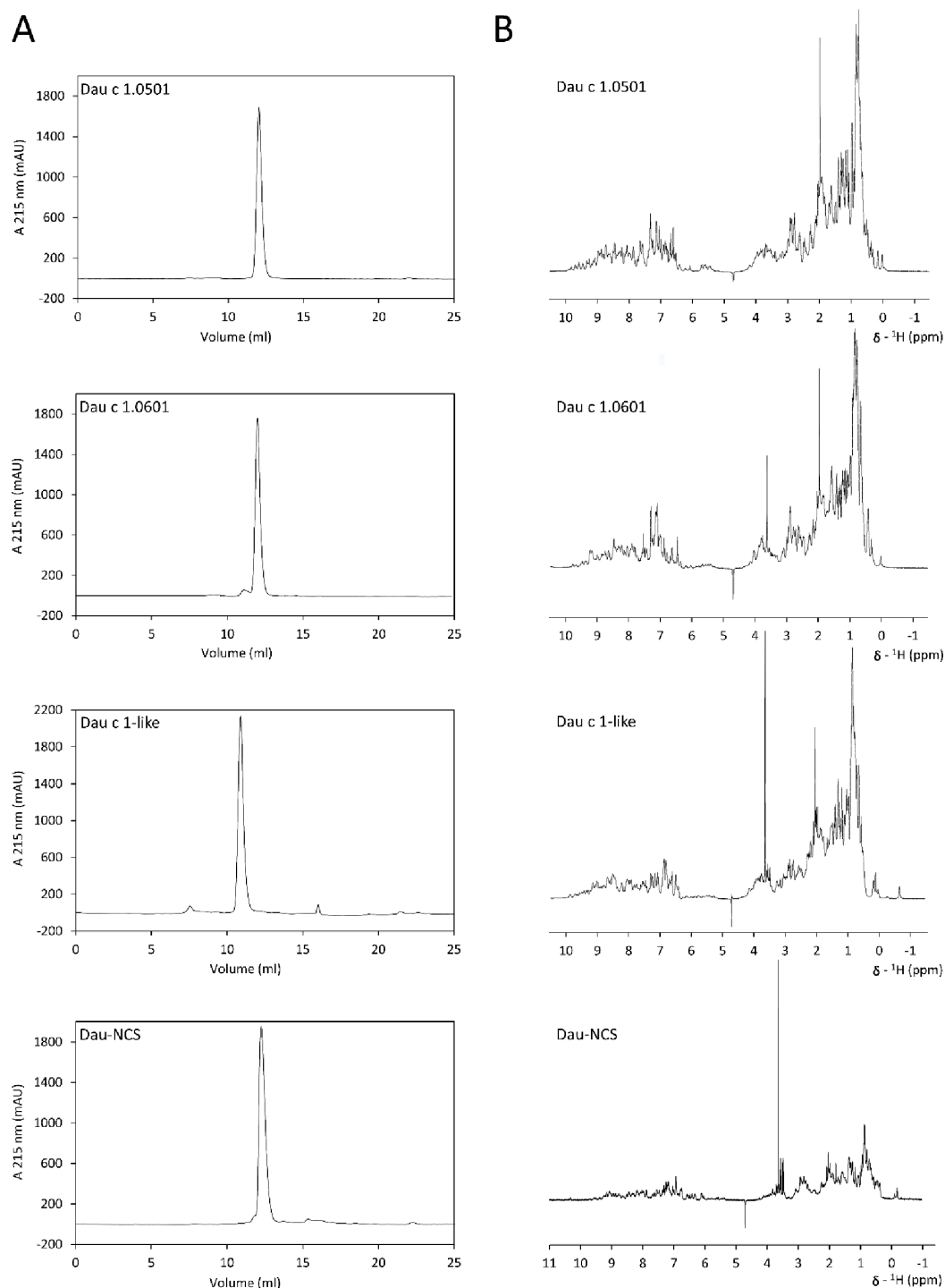
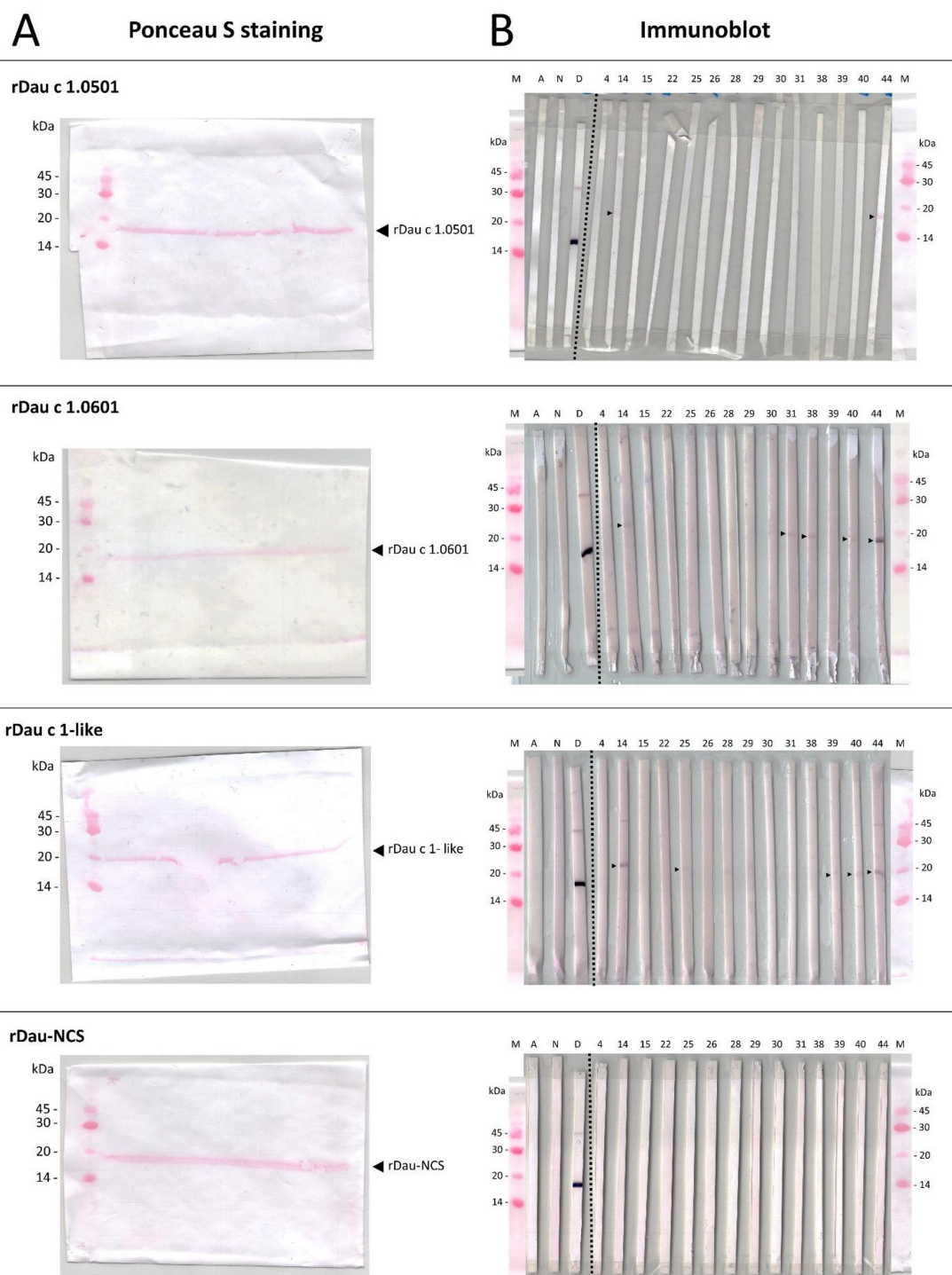


Figure S3. Size exclusion chromatography and NMR spectroscopy. **A)** Analytical SEC. 300 μg of rDau c 1.0501, rDau c 1.0601, rDau c 1-like or 100 μg rDau-NCS were loaded on a Superdex 75 Increase 10/300 GL column. The retention volumes of Dau c 1.0501, Dau c 1.0601 and Dau-NCS were ca. 12 ml, Dau c 1 like eluted at 11 ml. The calculated apparent molecular masses were between 15.2 and 15.7 kDa, indicating monomeric proteins. **B)** ^1H -NMR-spectroscopy. Spectra of rDau c 1.0501 (212 μM), rDau c 1.0601 (165 μM), rDau c 1-like (330 μM), and rDau-NCS (81 μM) were recorded in a Bruker Avance 600 MHz spectrometer at 295 K in 10% (v/v) D_2O , 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH7.0.



C Ponceau S staining

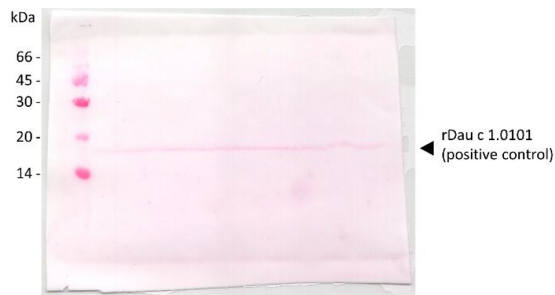


Figure S4: Ponceau S staining and immunoblots. A) Ponceau S staining of the new proteins. SDS-PAGE (16 % PA gels) was performed with the Dau c 1 proteins shown, followed by blotting onto a 0.2 μ m nitrocellulose membrane. Successful protein transfer was visualized by Ponceau S staining. **B)** The membranes produced in **A)** were cut into stripes and incubated with patients' sera. Bound specific IgE antibodies were detected using a mouse-anti-human IgE antibody coupled to alkaline phosphatase and nitroblue tetrazoliumchloride (NBT) /5-Bromo-4-chloro-3-indolylphosphate (BCIP) staining. The black arrows indicate successful IgE binding. No positive reaction is visible for Dau-NCS. Labels on top of the immunoblots: M: Ponceau S staining of low molecular weight marker. A: Secondary antibody control. N: Serum of non-allergic control patient. D: Positive control (rDauc1.0101 testet with serum 44). 4-44: sera of carrot allergic patients.

C) Ponceau S staining of the control protein rDau c 1.0101. The nitrocellulose membrane of the blotted and stained protein rDau c 1.0101 was cut into stripes and used as a positive control (labelled D) in **B)**.

8.2 Einzelarbeit B

Hendrich, J. M., Reuter, A., Jacob, T., Kara, H., Amer, S., Rödel, K., Wöhrle, B. M., **2024**. Allergenicity and Structural Properties of New Cor a 1 Isoallergens from Hazel Identified in Different Plant Tissues. *Sci. Rep.*, *14* (1), 5618.

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OPEN Allergenicity and structural properties of new Cor a 1 isoallergens from hazel identified in different plant tissues

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The hazel allergen Cor a 1 is a PR-10 protein, closely related to the major birch pollen allergen Bet v 1. Hazel allergies are caused by cross-reactive IgE antibodies originally directed against Bet v 1. Despite the importance of PR-10 proteins in allergy development, their function and localization in the plant remain largely elusive. Therefore, the presence of Cor a 1 mRNA and proteins was investigated in different tissues, i.e., the female flower, immature and mature nuts, catkins, and pollen. Four yet unknown Cor a 1 isoallergens, i.e., Cor a 1.0501–1.0801, and one new Cor a 1.03 variant were discovered and characterized. Depending on the isoallergen, the occurrence and level of mRNA expression varied in different tissues, suggesting different functions. Interestingly, Cor a 1.04 previously thought to be only present in nuts, was also detected in catkins and pollen. The corresponding Cor a 1 genes were expressed in *Escherichia coli*. The purified proteins were analysed by CD and NMR spectroscopy. Immunoblots and ELISAs to determine their allergenic potential showed that the new proteins reacted positively with sera from patients allergic to birch, hazel and elder pollen and were recognized as novel isoallergens/variants by the WHO/IUIS Allergen Nomenclature Sub-Committee.

Keywords PR-10 protein, Bet v 1, Cor a 1 isoallergens, Gene expression, LC-MS^E, Hazel genome, *Corylus avellana*, Immunoblot, ELISA, RT-PCR, T_m -values

Anemophilous or wind-driven pollen from certain trees can trigger allergic diseases and therefore impose serious health problems. An estimated 10–30% of the global population are afflicted by seasonal allergies and asthma caused by pollen, and the number continues to rise¹. Changes in life style, environmental pollution or dietary habits as well as climate change appear to play a role². One of the best studied allergens is the major birch pollen allergen Bet v 1, which is the founding member of the Bet v 1-like superfamily of proteins. This superfamily has representatives in all three domains of life, archaea, bacteria and eukaryotes³, and is comprised of 11 subfamilies. Bet v 1 itself belongs to the subfamily of the pathogenesis related proteins 10 (PR-10)-like family of proteins^{3–5}. Most of the Bet v 1 homologous allergens from pollen and plants, including the Cor a 1 allergen from hazel, belong to this subfamily. Whereas “real” PR-10 proteins, which have been detected in various plants are only induced in pathological stress situations, suggesting a role in plant defence responses, PR-10-like proteins are constitutively expressed, indicating a more general role in plant development^{3,6,7}.

In most cases allergic reactions to Bet v 1 and homologous proteins are not life threatening and usually result in mild symptoms like rhinitis, burning and itching of the tongue and oral mucosa, which are described as the so-called oral allergy syndrome (OAS) or pollen food syndrome (PFS). However, allergy induced asthma can also occur^{4,8–10}. Most pollen allergy sufferers are primarily sensitized to Bet v 1¹¹. Over time, many patients develop cross-reactivity of IgE antibodies originally directed against Bet v 1 proteins to homologous PR-10 allergens, which are found in almost all angiosperms and have at least 50% sequence identity amongst them⁶. Thus, birch pollen allergic individuals frequently experience IgE mediated allergic reactions upon contact with various plant food products harbouring homologous PR-10 proteins, i.e. fruits like apple, cherry or peach, vegetables and roots such as celeriac or carrots, and nuts, e.g. almonds and hazelnuts^{4,12}.

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The structural similarity of PR-10 allergens is the basis of the development of cross-reactive IgE antibodies¹³. The three-dimensional structure of many PR-10 and PR-10-like proteins is known and is almost identical⁶. They exhibit conserved, common structural elements, suggesting a general and maybe indispensable function in plants. They consist of a seven-stranded, antiparallel β -sheet, a long C-terminal α -helix and two short helices arranged in V-shape, which together form a predominantly hydrophobic or amphiphilic pocket, being composed of a combination of hydrophilic and hydrophobic residues, that can bind small ligands^{14–18}. A variety of small molecules were shown to bind to different PR-10 allergens, e.g. flavonoids, steroids or cytokinins, suggesting that they are involved in transport and/or storage of secondary plant compounds, UV protection and germination^{15,17,19–23}. However, for most PR-10 allergens the precise functions in the plant remain elusive.

IgE antibodies of Bet v 1 allergic individuals can also cross-react with the corresponding Cor a 1 allergen from hazel (*Corylus avellana*)^{24,25}. Consequently, contact with hazel pollen can trigger an allergic reaction similar to that described above, and in addition, hazelnut is a common cause of food allergy^{10,25–28}.

Due to the high relevance for the development of cross-allergies, it is imperative to obtain a more complete picture of the occurrence of known and novel Cor a 1 isoallergens in different hazel tissues. So far, four Cor a 1 isoallergens (> 67% amino acid sequence identity), termed Cor a 1.01 to Cor a 1.04, have been identified^{29,30}. Four different Cor a 1.01 variants (> 90% identity), named Cor a 1.0101 to Cor a 1.0104, have been found in hazel pollen²⁹. Furthermore, four variants of the isoallergen Cor a 1.04 have been detected in nuts³⁰. In contrast, Cor a 1.02 and Cor a 1.03 have been suggested to occur in leave tissue, however in that work the corresponding genes were only detected in the genome, but no expression analyses, neither on the mRNA nor on the protein level, were performed³¹. Thus, the presence of those isoallergens in different hazel tissues and in pollen has not yet been confirmed.

A recent study produced a fully assembled genome sequence and annotation for the hazelnut species *C. avellana* cv ‘Tombul’, an important Turkish variety. In addition to the known Cor a 1 genes, several new Cor a 1 related sequences were detected, some of which with only 40–50% identity to Cor a 1³². The study implies that there are many more than the already known Cor a 1 isoallergens and variants. However, similar to most other PR-10 allergens, the exact functions and localization of Cor a 1 proteins in the plant are not known. Some of the Cor a 1 proteins appear to be involved in transport and/or storage of flavonoids. We have previously identified a natural ligand of the Cor a 1.04 isoallergen, quercetin-3-O-(2⁶-O- β -D-glucopyranosyl)- β -Dgalactopyranoside (Q3O-(Glc)-Gal), and suggested a function of the protein/ligand complex in fertilization¹⁷.

As mentioned above, some Cor a 1 proteins have been shown to be localized in pollen or nuts²⁹. However, no comprehensive study has yet been conducted to demonstrate which Cor a 1 proteins are present in different plant tissues. Therefore, we wanted to identify new Cor a 1 isoallergens and analyse their biochemical and biophysical properties as well as the allergenic potential to contribute to a better understanding of hazel allergy.

Results and discussion

Identification and mRNA expression profiles of Cor a 1 isoallergens

There appear to be more Cor a 1 isoallergens and variants present in hazel than have been described so far, as indicated by similarities of genes found in the hazel genome³². Although the presence of the isoallergens Cor a 1.01 and Cor a 1.04 in pollen and kernel, respectively, has been shown, the expression and localization of the other known isoallergens Cor a 1.02 and Cor a 1.03 in different tissues has not yet been clarified.

To shed more light on the occurrence and distribution of Cor a 1 isoallergens, we determined the presence of Cor a 1 mRNA in different hazel plant tissues and in pollen. Since the fully assembled and annotated genome sequence of a European hazel species, *C. avellana* cv ‘Tombul’ was recently published³² it was possible to search specifically for novel Cor a 1 genes and their expression in different hazel tissues.

Total RNA was isolated from the female flower, immature and mature nuts, male catkins, and pollen. After cDNA production with an mRNA-specific poly(T) primer, PCR was performed using specific primers binding either to the 5' or 3' end of the gene (only Cor a 1.0302) or to the 5' and 3' untranslated regions (UTRs). Since the UTR regions of variants of a certain isoallergen are rather similar or even identical³², it was not possible to distinguish different variants using this method. Thus, only the existence of a specific isoallergen, possibly consisting of several variants, could be determined in a specific plant material (Figs. 1, S6). However, this procedure allowed us to use the PCR sample for cloning and for sequencing the complete amplified gene for the identification of new isoallergens (Table S1). To verify that the amplicates contained Cor a 1 DNA, all PCR probes were subjected to DNA sequencing. To detect even extremely low specific mRNA levels, a 2nd PCR amplification step was carried out in such cases with the gel purified DNA of the first PCR.

So far, the Cor a 1.01 protein has been detected in hazel pollen²⁹. However, the mRNA expression analyses presented here indicate that Cor a 1.01 is, in addition, expressed in minimal amounts in the female flower and in the mature nut as indicated by the 2nd PCR amplification step. Significantly stronger specific mRNA levels could be detected in the immature nut as well as in catkins and pollen.

Cor a 1.02 mRNA was present in the female flower and the mature nut, and high mRNA concentrations were visible in the immature nut, but no specific mRNA was discovered in the male tissues. Moreover, a new, putative Cor a 1.02 variant, designated Cor a 1.02- Cav01g11820 was detected via sequencing of the PCR product (Figure S1). However, since it was not possible to purify the protein from *E. coli* to determine its allergenic properties, this variant was not considered further in our studies.

Cor a 1.03 mRNA was present in all tissues with the exception of the mature nut even though high mRNA levels were detected in the immature nut. Although we could detect a very faint band after the first PCR round of the mature nut, sequencing of the amplicate of the 2nd PCR revealed no Cor a 1.03 gene sequence (Fig. 1). In addition, using LC-MS^E (liquid chromatography–mass spectrometry^{elevated energy}) variants of Cor a 1.01 and Cor a 1.03 could also be discovered on the protein level by LC-MS^E in pollen extract (Figure S2).

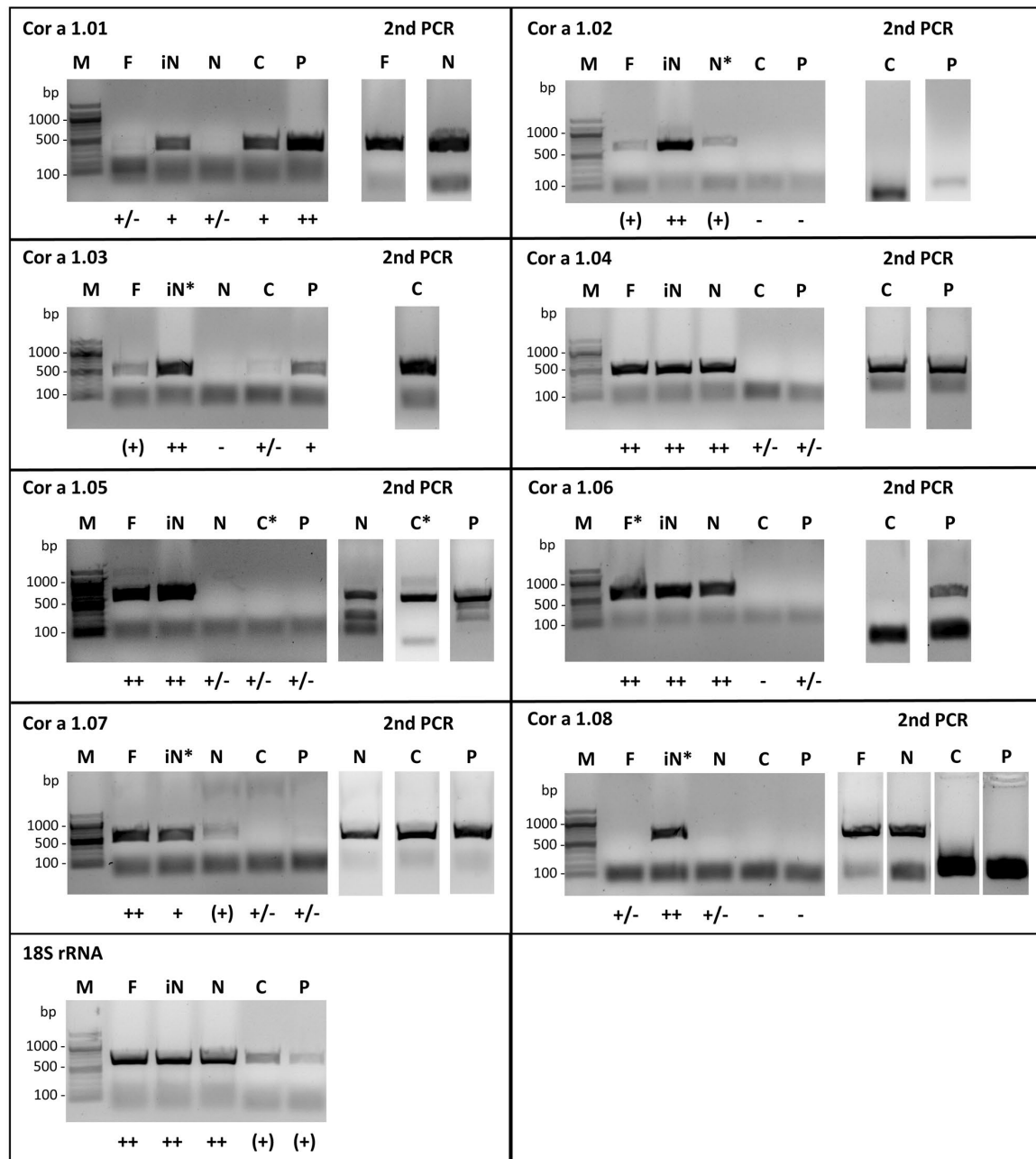


Figure 1. Expression of Cor a 1 genes in different hazel tissues. The RT-PCR products using gene specific primers to determine Cor a 1 mRNA expression were loaded on 1.5% agarose gels. In case no band was visible, a gel slice at the corresponding height was cut out. The purified mRNA was then used for the 2nd PCR. F: female flower; iN: immature nut; N: mature nut; C: catkin; P: pollen; ((+)) very low, (+): low, +: moderate, ++: strong expression levels. The RT-PCR samples used for cloning of the genes into expression vectors are marked with *M: 100 bp DNA standard (New England Biolabs; Frankfurt, Germany). 18S rRNA isolated in a similar fashion was used as a control.

It has been shown previously that Cor a 1.04 is present in nuts³⁰. Since Cor a 1.04 was found to bind to a ligand isolated from pollen extracts it was suggested that it might play a role in fertilization¹⁷. Thus, it was interesting to determine whether Cor a 1.04 is localized in other tissues as well. Surprisingly, the 2nd PCR amplification step

followed by sequencing revealed the presence of Cor a 1.04 specific mRNA also in catkins and pollen, albeit at very low amounts (Fig. 1). Thus, we show for the first time that Cor a 1.04 expression also occurs in the male tissues. This result provides a reasonable explanation why a ligand isolated from pollen extracts bound specifically to Cor a 1.0401 which was thought to be only present in the nut.

In addition, Cor a 1.04 mRNA could also be detected in the female flower and the immature nut (Fig. 1). Furthermore, LC-MS^E data showed the presence of the Cor a 1.04 protein in extracts of the mature nut (Figure S2). However, only a few peptides could be confirmed, suggesting that protein isolation from nuts might be less efficient than mRNA extraction. In addition, no amplification step is performed before the MS analysis. This might also explain why other isoallergens (Fig. 1) could not be identified at the protein level by LC-MS^E. Moreover, it is also possible that protein amounts in nuts are lower and do not necessarily correspond to mRNA levels.

Cor a 1.05 mRNA was found in the female flower and in the immature nut, however, in the mature nut, as well as in catkins and pollen specific mRNA was only visible after the 2nd PCR procedure (Fig. 1).

Cor a 1.06 mRNA was detected in the female flower, as well as in the immature and mature nut, and was also visible in pollen after the 2nd PCR. Cor a 1.07 mRNA levels were very low in all tissues. In contrast, Cor a 1.08 mRNA concentration was high only in the immature nut, whereas it was weak in the female flower and mature nut, and no expression could be verified in the male tissues (Fig. 1).

It is important to note, that our intention was to identify new expressed Cor a 1 genes by detecting the corresponding mRNAs in different tissues and to clone the genes. Our data represent only one specific time point, which means that Cor a 1 isoallergens that were not visible in our experiments might nevertheless be present at an earlier or later stage of development.

The observation that the mRNA expression of the individual isoallergens differs so strongly implies different properties and functions in the plant, e.g. during fertilization and kernel development, that still need to be explored. Different roles of Cor a 1 isoallergens have already been suggested as only the Cor a 1.04 isoallergen but not Cor a 1.01 binds the flavonoid derivative Q3O-(Glc)-Gal¹⁷. In summary, the data show that there are more Cor a 1 isoallergens than previously known and that their presence in mature nuts and pollen could play a role in hazelnut allergy (see below).

Identification of additional variants

In order to identify new isoallergens and variants, the amplified DNA products from specific tissues were cloned into the pCR-Blunt vector (Thermo Fisher, Schwerte, Germany) and sequenced. It turned out that several highly similar variants of a certain isoallergen could be detected in the different tissues tested (Figures S1, and S3). For some of them, only gene fragments could be amplified (data not shown). Due to the high similarity of the variants only one variant per isoallergen was selected and included in our studies to verify the biochemical and biophysical properties as well as the allergenicity (Figure S1). Furthermore, we identified variants of several isoallergens that could not be detected in the genome of the Turkish cultivar “Tombul”³² by BLAST analyses, indicating genetic differences of *C. avellana* used in our studies. These genes are labelled with a GenBank accession number only, whereas a Cav01 number represents genes also found in the “Tombul cultivar”. Our data indicate that for each isoallergen additional potential variants are present (Figure S3). However, due to their high similarity we did not include different variants of a certain isoallergen in our study.

As described above, we were able to identify four new isoallergens, namely Cor a 1.0501, Cor a 1.0601, Cor a 1.0701 and Cor a 1.0801 on the mRNA level (Figure S1). In addition to those isoallergens a new Cor a 1.03 variant, designated Cor a 1.0302 was detected. These proteins were included in biophysical and immunological studies. Due to their allergenic potential (see below), the four new isoallergens were recognized by the WHO/IUIS Allergen Nomenclature Sub-Committee and named as described.

Amino acid sequence alignments and the identity matrix of Cor a 1 isoallergens and variants revealed that the new proteins possess sequence identities to the already known isoallergens of 46.88 – 84.38%. The new Cor a 1.0302 variant is 96.23% identical to Cor a 1.0301 (Table 1).

Biochemical and biophysical analyses

The identified genes were cloned into the expression vectors pETGB1a or pET11a (only Cor a 1.0302) and expressed in *E. coli* (Table S2). The SDS polyacrylamide gel of the purified proteins exhibits apparent molecular masses of ca. 16 kDa to 18 kDa (Figure S4).

To determine the refolding capacity and stability of the proteins, CD spectra were recorded (Fig. 2, panel A, B). At 25 °C the spectra of the recombinant (r)Cor a 1 isoallergens are characteristic for proteins harboring α - and β -like secondary structures (Fig. 2, panel A). After heating to 95 °C, rCor a 1.0302, 1.0501, 1.0601, and 1.0701 unfolded as indicated by the minimum at 200 nm. In contrast, rCor a 1.0801 appeared to be more stable. The curve after heating to 95 °C (Fig. 2, panel A, red curve) changed only slightly and there was no discrete minimum at 200 nm, signifying that only a few sections of the protein became unfolded. After cooling to 25 °C, rCor a 1.0302, 1.0501, 1.0601 and 1.0701 almost completely refolded whereas rCor a 1.0801 was still folded and the proportion of unfolded protein moieties decreased as compared to the spectrum at 95 °C (Fig. 2, panel A, green curves).

rCor a 1.0302, 1.0601 and 1.0701 revealed relatively similar T_m -values of 68.29, 54.08 or 62.68 °C. Cor a 1.0501 exhibited the highest T_m -value of 84 °C (Fig. 2, panel B). rCor a 1.0801 displayed a biphasic melting behaviour during heating to 95 °C. However, complete denaturation could not be achieved. Due to the small differences of the denaturation and renaturation curves, the noise in the measurement of the melting curve of Cor a 1.0801 is quite high (Fig. 2, panel B).

Cor a 1.0801 shows a lower sequence identity to Bet v 1.0101 and also to most of the other Cor a 1 isoallergens (Table 1). This difference could be the reason for its higher thermostability. Previously, the structure of the

Identity matrix		Bet v 1.0101	Cor a 1								
			0101	0201	0301	0302	0401	0501	0601	0701	0801
Bet v 1.0101		100.00	72.50	83.12	71.25	69.18	67.50	51.25	78.12	54.38	46.88
Cor a 1	0101	72.50	100.00	75.00	65.00	64.78	63.13	48.75	78.12	50.00	46.88
	0201	83.12	75.00	100.00	71.25	69.18	70.00	50.63	84.38	53.12	50.00
	0301	71.25	65.00	71.25	100.00	96.23	71.25	48.75	69.38	58.12	53.12
	0302	69.18	64.78	69.18	96.23	100.00	71.07	50.31	68.55	59.12	54.09
	0401	67.50	63.13	70.00	71.25	71.07	100.00	52.50	68.12	60.62	51.25
	0501	51.25	48.75	50.63	48.75	50.31	52.50	100.00	48.75	51.88	55.62
	0601	78.12	78.12	84.38	69.38	68.55	68.12	48.75	100.00	51.25	47.50
	0701	54.38	50.00	53.12	58.12	59.12	60.62	51.88	51.25	100.00	46.88
	0801	46.88	46.88	50.00	53.12	54.09	51.25	55.62	47.50	46.88	100.00

Table 1. Identity matrix of Cor a 1 isoallergens and variants. Numbers indicate the amino acid sequence identity in %

hypothetical protein TTHA0849 from the thermophilic bacterium *Thermus thermophilus* had been determined, which also shows the typical PR-10 fold of Bet v 1³³. However, sequence comparisons revealed no evidence of conserved regions in Cor a 1.0801 and TTHA0849 that might explain its thermostability. Despite the similar three-dimensional structures, the sequence identity between Cor a 1.08 and TTHA0849 is only 20.98%, which is even lower than that of all the other Cor a 1 isoallergens to TTHA0849, except Cor a 1.05 (20.42%) (Figure S7).

While CD spectra can be used to determine secondary structures, 1D NMR spectroscopy can reveal three-dimensional folding of a protein. The 1D NMR spectra showed dispersion of the amide proton signals and high-field shifted signals of the methyl groups, which confirmed a three-dimensional folding for all isoallergens (Fig. 2, panel C).

These data suggest that the high stability and refolding capacity of the proteins could have an impact on their allergenicity. Heating might not completely abolish the allergenicity in hazel nut containing food stuff as Cor a 1 proteins could refold if the food is cooled down.

IgE binding

The rCor a 1 proteins purified from *E. coli* were further used for immunological studies in order to determine their allergenicity. Sera from 20 tree-pollen allergic individuals that had been tested positive for specific IgE (Spez. IgE Kit from Gold Standard Diagnostics, Kassel, Germany) against a mixture of birch, hazel and alder pollen were used for immunoblot analyses with the recombinant isoallergens. Only sera reaching values ≥ 3.5 U/ml corresponding to the enzyme-allergo-sorbent-test (EAST) class 3 or higher were selected. As expected, all sera reacted strongly with the Bet v 1 control from birch since this is usually the sensitizing agent⁴ (Fig. 3, Figure S5). Furthermore, all sera reacted with rCor a 1.0101, albeit with varying intensities. Similarly, IgE binding could be detected with all sera employing rCor a 1.0302, 1.0401, 1.0501, and 1.0601, indicating that all of them are potent allergens. Using rCor a 1.0701, serum #1, #9, #10, and #13 displayed no IgE binding. rCor a 1.0801 proved to be the weakest allergen and showed IgE binding only with seven sera, namely #11, #12, #14, #16, #17, #18, and #20. Obviously, the sera responded better to those Cor a 1 isoallergens which possess a high identity with the sensitizing Bet v 1.0101 protein (Table 1). The weak allergens Cor a 1.0701 and Cor a 1.0801 have only 54.38 and 46.88% identity with Bet v 1.0101, respectively.

Since we have previously described that denaturation of an allergen during the blotting procedure can impair IgE binding³⁴, ELISAs were chosen as an additional method to confirm the allergenicity of the isoallergens (Table 2). Here, all sera reacted with rCor a 1.0101 to rCor a 1.0601. With the exception of serum #12, all sera appeared to be less reactive using Cor a 1.0701. However, in contrast to the Western Blots, rCor a 1.0701 exhibited

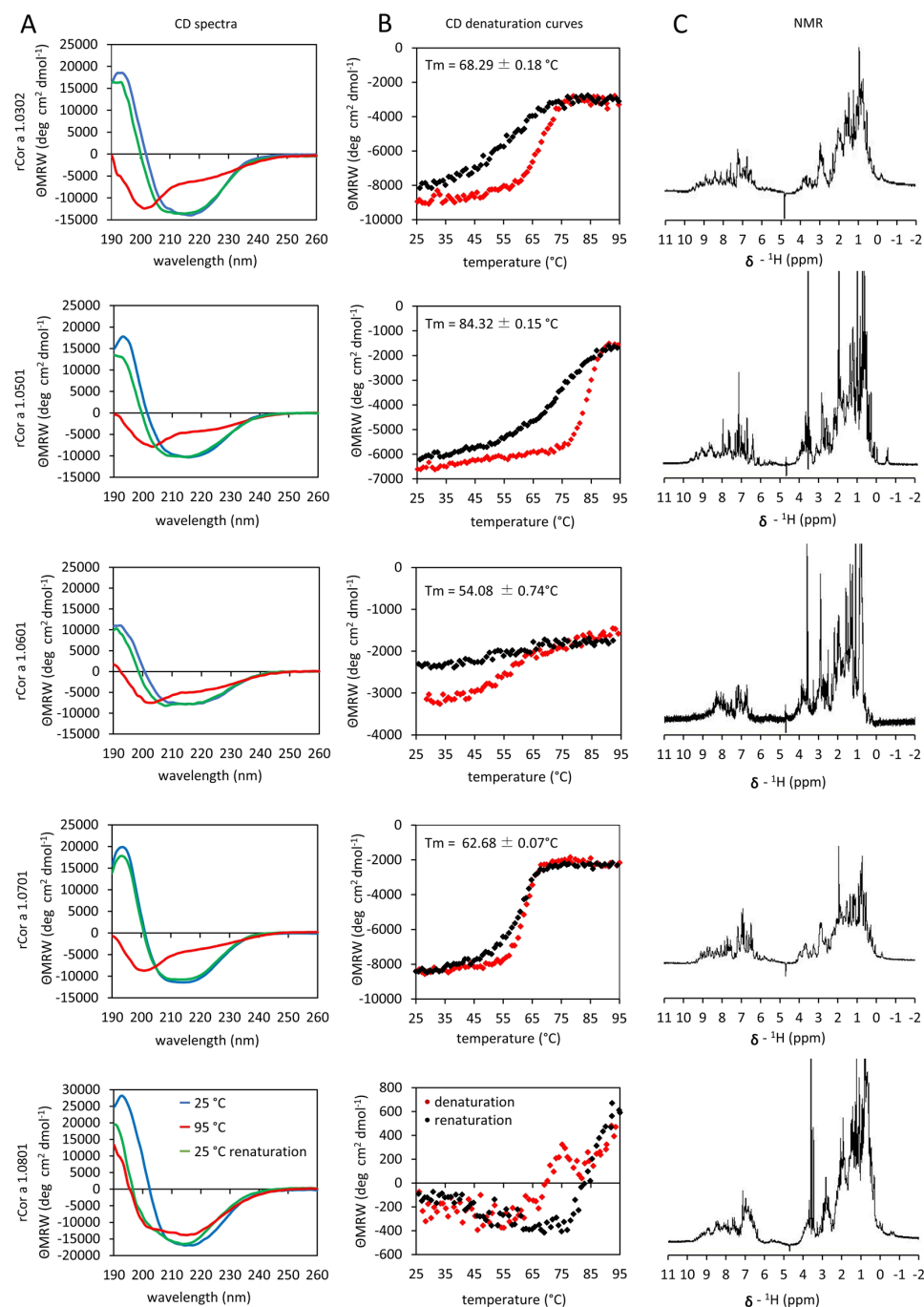


Figure 2. Physicochemical characterization of Cor a 1 isoallergens. (A) CD spectroscopy. CD spectra at 25 °C (blue curve), after heating to 95 °C (red curve), followed by stepwise cooling to 25 °C (green curve). (B) CD melting curves. CD denaturation (red) and renaturation (black) curves were recorded at 217 or 220 nm in 10 mM Na₂HPO₄/NaH₂PO₄ pH 7.0. The T_m value is displayed on top of each spectrum. No T_m could be calculated for Cor a 1.0801. (C) ¹H-NMR-spectroscopy. Spectra of Cor a 1.0302 (160 μM), Cor a 1.0501 (200 μM), Cor a 1.0601 (60 μM), Cor a 1.0701 (190 μM) and Cor a 1.0801 (70 μM) were recorded in a Bruker Avance 600 MHz spectrometer at 295 K in 10% (v/v) D₂O, 10 mM Na₂HPO₄/NaH₂PO₄, pH7.0.

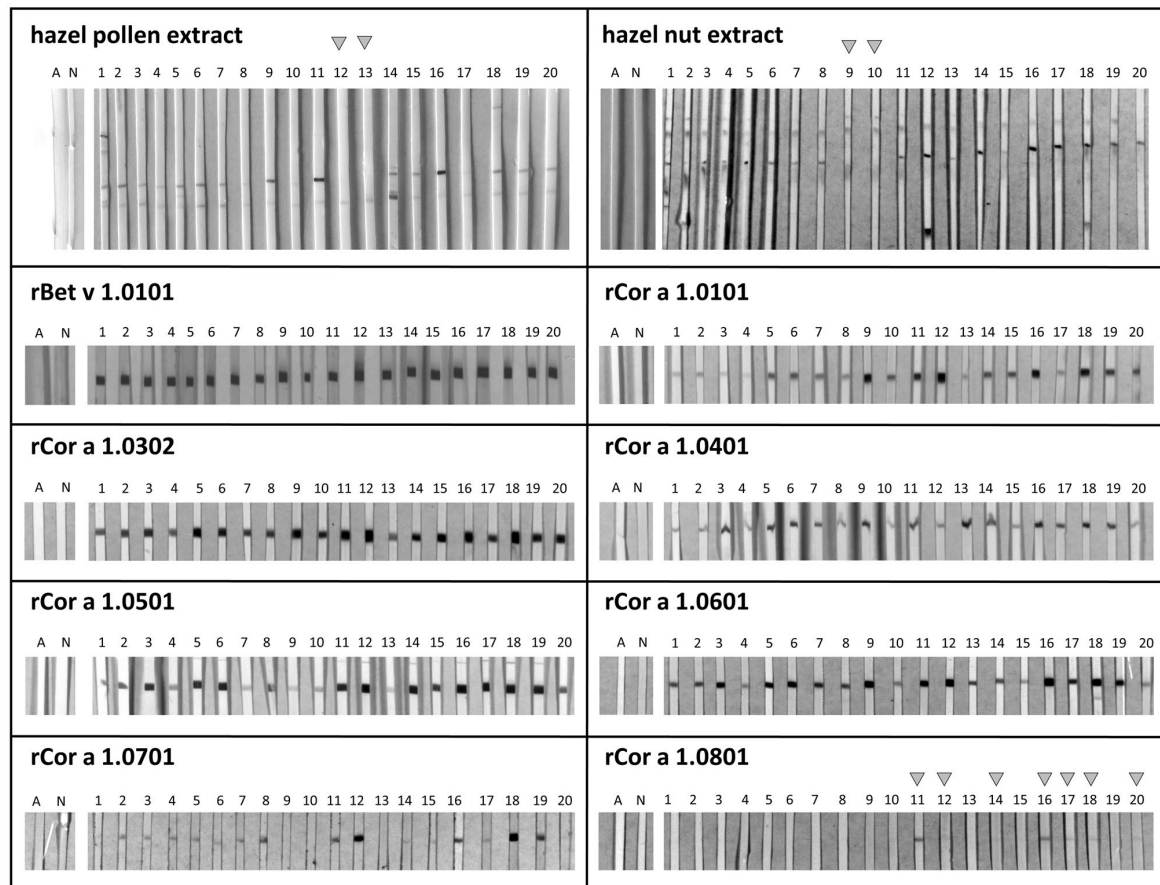


Figure 3. Immunoblots. SDS-PAGE (16% PA gels) was performed with the Cor a 1 proteins, followed by blotting onto a 0.2 μ m nitrocellulose membrane as indicated on top of the immunoblots. 1.5 μ g/cm protein was blotted except for Cor a 1.0801 with 0.8 μ g/cm. The membranes were cut into stripes and incubated with patients' sera. Bound specific IgE antibodies were detected using a mouse-anti-human IgE antibody coupled to alkaline phosphatase followed by nitroblue tetrazoliumchloride (NBT)/5-Bromo-4-chloro-3-indolylphosphate (BCIP) staining. The grey arrows indicate weak IgE binding for nCor a 1 from hazel pollen and hazel nut extract and for rCor a 1.0801. Labels on top of the immunoblots: A: secondary antibody control; N: horse serum; 1–20: sera of patients allergic against tree pollen.

IgE binding with all sera, indicating that denaturation of the antigen in the Blots could play a role. Furthermore, the ELISA might be more sensitive than the Blot. Interestingly serum #12 was highly reactive with all isoallergens other than Cor a 1.0801. In addition to the sera reacting with Cor a 1.0801 in the above Western Blot, Cor a 1.0801 also showed weak IgE binding using serum #19. These results confirm that Cor a 1.0801 is a weaker allergen than the others but nevertheless several individuals appear to possess reactive IgE antibodies.

Concluding remarks

Our data show that there exist more isoallergens and variants of Cor a 1 than previously known, e.g., four potential variants of the newly identified isoallergen Cor a 1.05 as well as three additional variants of Cor a 1.03 could be detected (Figs. 4, S1, S3). We have already shown that Cor a 1.0401 binds to the specific natural ligand Q3O-(Glc)-Gal¹⁷. To obtain further information on ligand binding, we are currently conducting experiments to show whether the newly identified isoallergens have different ligand binding affinities.

Since little was previously known about the expression of Cor a 1 in different plant tissues the results presented here (Fig. 1, Table 1) show for the first time, that the expression of the isoallergens studied is dependent on the plant tissue, e.g. Cor a 1.01 could be found in all tissues, whereas Cor a 1.08 was only present in the female tissues, albeit at different mRNA expression levels. Obviously, the localization of a certain isoallergen has an impact on its expression level (Fig. 1). These differences suggest different functions of the isoallergens, e.g., during plant development, fertilization and germination, that remain to be elucidated in the future.

The mRNA levels of Cor a 1.01 and Cor a 1.03 are high in pollen (Fig. 1) and thus the proteins appear to play an important role as triggers of pollen allergy. In contrast, the other isoallergens, present in mature nuts, might be relevant for allergic reactions caused after intake of hazel nut containing foodstuff (Fig. 1).

Serum no.																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Isoallergen																				
rBet v 1.0101	6.69 (±0.65)	7.78 (±1.03)	17.86 (±2.14)	10.14 (±0.03)	24.28 (±0.18)	10.79 (±0.27)	7.72 (±0.02)	6.10 (±0.14)	25.05 (±0.21)	9.27 (±0.48)	21.59 (±1.62)	38.16 (±1.30)	12.02 (±1.14)	14.85 (±0.00)	23.33 (±1.69)	22.67 (±2.32)	16.50 (±1.18)	27.70 (±0.55)	8.19 (±0.13)	10.12 (±0.48)
rCor a 1.0101	2.29 (±0.05)	3.90 (±0.11)	2.39 (±0.10)	1.65 (±0.02)	7.38 (±0.10)	3.76 (±0.00)	2.58 (±0.11)	1.92 (±0.06)	5.86 (±0.22)	2.81 (±0.05)	5.50 (±0.14)	29.41 (±1.73)	2.35 (±0.24)	2.60 (±0.11)	6.75 (±0.08)	8.14 (±0.14)	4.68 (±0.08)	6.01 (±0.02)	2.44 (±0.03)	2.26 (±0.03)
rCor a 1.0302	3.60 (±0.05)	5.59 (±0.21)	6.48 (±0.66)	4.58 (±0.41)	18.14 (±0.61)	10.47 (±0.14)	8.75 (±0.23)	7.25 (±0.36)	19.94 (±0.06)	9.41 (±0.46)	18.08 (±0.61)	33.24 (±5.29)	7.94 (±0.43)	11.28 (±0.01)	17.28 (±0.55)	12.23 (±0.34)	9.31 (±0.28)	21.27 (±0.35)	7.35 (±0.36)	10.53 (±0.25)
rCor a 1.0401	5.52 (±0.31)	3.71 (±0.12)	17.23 (±0.25)	5.15 (±0.28)	21.02 (±1.61)	9.10 (±0.16)	10.05 (±1.61)	5.35 (±0.04)	14.17 (±0.69)	2.94 (±0.13)	15.63 (±0.33)	38.42 (±1.65)	12.45 (±0.19)	13.01 (±0.57)	6.14 (±0.41)	23.94 (±0.31)	16.59 (±0.87)	25.11 (±0.13)	10.57 (±0.55)	5.69 (±0.21)
rCor a 1.0501	2,07 (±0.11)	2,10 (±0.12)	5,34 (±0.66)	1,45 (±0.01)	6,88 (±0.88)	5,14 (±0.66)	1,30 (±0.00)	3,76 (±0.50)	1,16 (±0.03)	1,40 (±0.00)	5,49 (±0.29)	27,01 (±0.20)	1,13 (±0.02)	5,57 (±0.01)	2,76 (±0.14)	10,38 (±0.33)	9,34 (±0.51)	10,02 (±0.16)	6,00 (±0.69)	1,87 (±0.05)
rCor a 1.0601	1,29 (±0.05)	1,09 (±0.02)	1,75 (±0.06)	1,16 (±0.03)	3,74 (±0.66)	2,24 (±0.12)	1,42 (±0.21)	2,01 (±0.02)	1,74 (±0.06)	1,29 (±0.02)	1,79 (±0.10)	19,02 (±0.22)	3,60 (±0.13)	1,35 (±0.03)	1,30 (±0.09)	2,47 (±0.11)	2,68 (±0.03)	12,38 (±0.17)	1,47 (±0.03)	1,25 (±0.07)
rCor a 1.0701	2.89 (±0.21)	3.98 (±0.06)	4.05 (±0.08)	2.79 (±0.04)	6.22 (±0.15)	1.95 (±0.16)	3.22 (±0.18)	2.94 (±0.19)	2.58 (±0.03)	1.12 (±0.03)	8.56 (±0.05)	30.55 (±1.00)	1.37 (±0.01)	1.64 (±0.17)	3.05 (±0.02)	5.90 (±0.44)	3.54 (±0.12)	9.01 (±0.20)	3.24 (±0.24)	1.42 (±0.08)
rCor a 1.0801	1.00 (±0.00)	0.96 (±0.02)	0.99 (±0.00)	0.95 (±0.01)	0.99 (±0.02)	0.98 (±0.00)	0.97 (±0.00)	0.97 (±0.00)	0.95 (±0.01)	0.98 (±0.00)	1.23 (±0.01)	1.30 (±0.02)	0.98 (±0.00)	1.02 (±0.02)	1.00 (±0.01)	1.11 (±0.02)	1.11 (±0.00)	1.08 (±0.02)	1.03 (±0.00)	1.06 (±0.03)

Table 2. ELISA with purified rCor a 1 isoallergens and sera of tree pollen allergic patients. The values represent the ratio of the measurements at 450/620 nm normalized to the 450/620 ratio of the negative control (horse serum). Serum dilution 1:10. Two technical replicates were performed. Light yellow: 1–1.5, yellow: 1.5–5, orange: 5–10, red: 10–20, purple: >20.

[illegible]

Figure 4. Amino acid sequence alignment of identified cor a 1 genes expressed in *Corylus avellana*. The newly identified isollargens are highlighted in green and are shown in comparison with the known Cor a 1 sequences. * conserved amino acids; : conservative exchanges; . semi-conservative exchanges.

The allergenic potential of Cor a 1 proteins is likely to be enhanced by the folding stability of the isoallergens (Fig. 2). All isoallergens regained their secondary structure elements after short heating to 95 °C and recooling (Fig. 2, panel A). Similar results have already been shown for Bet v 1.0101 as well as for the homologous carrot allergen Dau c 1 (Jacob u. a. 2020; Machado u. a. 2016; Hendrich u. a. 2023). For Bet v 1.0101 it has been demonstrated that the increased fold stability of the protein has an impact on its allergenicity³⁶. The Dau c 1.0105

isoallergen from carrot exhibits a pronounced heat stability which can be reduced but not completely abolished after heating of the food matrix^{35,37}.

The immunoblots and ELISAs suggest that different Cor a 1 isoallergens are recognized by different pollen allergic patients. It depends on the individual immune system against which isoallergen the IgE antibodies are directed. Consequently, pollen allergic patients need to test whether it is safe to consume hazel nuts that have been baked, roasted or even untreated.

Methods

Identification of Cor a 1 genes in the hazel genome

Identification of Cor a 1 homologous genes was performed via BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the known isoallergen sequences of Cor a 1 on the nucleotide and protein level. At least four unknown potential isoallergens were identified in the genome of *Corylus avellana* cv Tombul (GenBank assembly accession number: GCA_901000735.2)³², with the following GenBank accession numbers: OQ450370 (Cor a 1.0302), OQ230635 (Cor a 1.0501), OQ450371 (Cor a 1.0601), OQ230636 (Cor a 1.0701) and OQ230637 (Cor a 1.0801). The program Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>) was used to create multiple sequence alignments and to calculate sequence identities.

Primer design

Based on the *C. avellana* genome, primers were designed derived from the 5' and 3' untranslated regions or the 5' and 3' ends of the genes. Primers used are listed in Table S1.

Expression analyses by mRNA isolation from different plant tissues and cDNA production

Immature and mature hazelnuts, pollen, hazel catkins, and female flowers were collected from hazel trees (*C. avellana*) on the Campus of the University of Bayreuth, in accordance with the Ethics Committee of the University of Bayreuth and with relevant institutional, national, and international guidelines and legislation. Female flowers were harvested early in January 2021 before the onset of the pollen flight. Pollen was shaken off from mature blooming catkins and cleaned through a fine metal sieve. The different plant materials were stored at -80°C . As previously described³⁴, total RNA was isolated from ~200 mg plant material using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), followed by reverse transcription with a poly(T) primer to obtain cDNA from mRNA using the SuperScript IV First-Strand Synthesis System with ezDNase enzyme (Thermo Fisher Scientific, Germany) and the designed primer pairs (Table S1). 18S rRNA and the specific PCR products thereof (Table S1) were obtained similarly and used as a control to determine the efficiency of RNA isolation.

Cloning procedures, sequencing and plasmid preparation

PCR amplicates using Cor a 1 specific primers (Table S1) were loaded onto 1.5% agarose gels to visualize gene expression and purified via gel extraction (Qiaquick Gel Extraction Kit, Qiagen, Germany). In case no band could be detected on the gels, slices were cut out from the gel at the corresponding heights and also purified via gel extraction. 5 μl of the extracted sample were used as a template for a second PCR using the same conditions as described above for the first PCR. To determine whether the expected Cor a 1 gene was present in the PCR amplicate, the PCR products were either sequenced directly using the amplification primers and/or they were cloned into the pCR-Blunt vector using the Zero Blunt PCR Cloning Kit (Thermo Fisher Scientific Germany) as previously described³⁴. Sequences were analyzed by Sanger sequencing of the isolated plasmids or by colony sequencing (Microsynth AG, Lindau, Germany). Four to ten clones were subjected to sequencing for each Cor a 1 gene detection.

The genes of Cor a 1.0501, 1.0601, 1.0701 and 1.0801 identified by sequencing of the pCRBlunt plasmids were cloned into the bacterial expression vector pET-GB1a (G. Stier, EMBL) via Gibson cloning³⁸ using the primers shown in Table S2. Thus, the proteins contain an N-terminal 6His-GB1a fusion which can be cleaved off by tobacco etch virus (TEV) protease. All cleaved off Cor a 1 proteins start N-terminally with the amino acid sequence GMGV. The Cor a 1.0302 gene was ordered in the pET11a expression vector (Eurofins Genomic, Ebersberg, Germany), resulting in a tagless protein starting with a methionine.

LC-MS^E analysis

LC-MS^E analysis of pollen extracts was performed applying published protocols^{17,34,35}. Differing from this a Uniprot database was used for MS database search. The database consisted of reviewed entries of all species as of 2022.09 and amino acid sequences translated from nucleic acid sequences mentioned above. To obtain hazelnut extract for LC-MS^E analysis, procedures were similar to those previously described²⁴. The LC-MS^E analysis was performed as described above for pollen extracts. In brief, mature hazelnuts (Seeberger GmbH, Ulm, Germany) from the supermarket were crushed in a blender, ground in liquid nitrogen, and stirred overnight at 4°C in 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ pH 7.0, 2% (w/v) polyvinylpyrrolidone, 2 mM EDTA, and 2 mM sodium azide. After centrifugation (20 min, 6000 g, 4°C), the supernatant was passed through a paper filter (Rotilabo-folded filters, type 113P, Carl Roth GmbH, Karlsruhe, Germany). After dialysis against 5 L 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ pH 7.0 overnight, the protein sample was separated on a 19% SDS-PA gel. A protein band of the size of ca. 18 kDa corresponding to nCor a 1 was cut out and prepared for LC-MS^E as described³⁴.

Protein purification of Cor a 1 proteins recombinantly expressed in *E. coli*

Purification of rCor a 1.0501, 0601, 0701 and 0801 was performed as previously described³⁷ omitting the size exclusion chromatography. Elution of the fusion proteins was performed on a 5 ml HisTrap HP column (Cytiva

Europe GmbH, Freiburg, Germany) by a stepwise increase (15%, 25%, 50%, 70%, 100%) of the imidazole concentration.

pET11a_Cor a 1.0302 was expressed in BL21(DE3). Bacteria were grown in lysogeny broth (LB) supplemented with 100 µg/ml ampicillin at 37 °C. At an optical density at 600 nm of 0.6–0.7, the temperature was decreased to 18 °C and overexpression was induced overnight for ca. 18 h with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After harvesting (6000 × g, 10 min, 4 °C), the cell pellet was resuspended in 20 mM TrisHCl pH 8.0, supplemented with DNase I and EDTA-free protease inhibitor and lysed as previously described³⁷. The supernatant was purified by ion exchange chromatography using a 5 ml HiTrap Q XL column (Cytiva Europe GmbH) in 20 mM TrisHCl pH 8.0. rCor a 1.0302 was eluted using a stepwise increase (15%, 25%, 50%, 75%, 100%) of the elution buffer (20 mM TrisHCl, 500 mM NaCl pH 7.0). Fractions containing the Cor a 1.0302 protein were pooled. After the addition of 1 M (NH₄)₂SO₄ the protein was further purified by hydrophobic interaction chromatography with a 5 ml HiTrap Octyl FF column (Cytiva Europe GmbH). After washing with 10 mM Na₂HPO₄/NaH₂PO₄ buffer containing 1 M ammonium sulfate, Cor a 1.0302 was eluted with 10 mM Na₂HPO₄/NaH₂PO₄.

CD und NMR spectroscopy

Circular dichroism (CD) spectra and standard 1D ¹H NMR spectra were recorded as previously described³⁷.

Patients' Sera

Sera were obtained from patients after informed consent and approval by the Ethics Committee of the University of Bayreuth. All experiments were performed in accordance with relevant guidelines and regulations. Sera from patients diagnosed with seasonal (early spring) rhinitis, cough and breathing difficulties were tested for specific IgE against a tree pollen mixture including birch, hazel and alder pollen (Spez. IgE Kit from Gold Standard Diagnostics, Kassel, Germany). Only sera reaching values ≥ 3.5 U/ml corresponding to the enzyme-allergo-sorbent-test (EAST) class 3 or higher were selected and tested further against Bet v 1.0101 (used as a control), as well as hazel pollen extract and an extract from mature hazel nuts to confirm the presence of specific IgE antibodies against Cor a 1 proteins. Those sera were used in this study to detect new Cor a 1 isoallergens.

IgE immunoblots and IgE ELISAs

IgE immunoblots and ELISAs were performed similarly to previously described procedures^{37,39}. Mouse anti-human IgE coupled to alkaline phosphatase (BD Bioscience, Heidelberg, Germany) and AP Conjugate Substrate Kit (Bio-Rad, Germany) was used for immunoblots. Horse serum was used as a negative control and a commercially available mixture of sera from birch pollen and hazel pollen allergic patients (Gold Standard Diagnostics Kassel GmbH) as a positive control.

For ELISAs 2 µg/ml antigen (Cor a 1 proteins or Bet v 1.0101) was coated on high binding ELISA plates F (Sarstedt, Nümbrecht, Germany) in coating buffer (50 mM Na₂CO₃/NaHCO₃ pH 9.6) overnight at 4 °C. After washing three times with PBS-T (phosphate buffered saline, 0.05% Tween 20) and blocking with blocking buffer (PBS-T containing 2% BSA) for 1 h at room temperature (RT), sera (diluted 1:10 with blocking buffer) were added and incubated overnight at RT. After washing four times with PBS-T, bound specific IgE was detected with horse radish peroxidase (HRP) labeled mouse-anti-human IgE-Fc-HRP (SouthernBiotech, Birmingham, USA) (diluted 1:5000 with blocking buffer). After 2 h at RT and washing with PBS-T, 100 µl substrate solution of the KPL TMB Microwell Peroxidase Substrate System (2-C) (Sera care Life Science, MA, USA) was added. The color reaction was stopped with 25% sulfuric acid (50 µl/well) and absorbance was read at 450 nm and 620 nm as a reference.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files). *Accession numbers of proteins:* Cor a 1.0302: *Acc. no.* OQ450370; Cor a 1.0501: *Acc. no.* OQ230635; Cor a 1.0601: *Acc. no.* OQ450371; Cor a 1.0701: *Acc. no.* OQ230636; Cor a 1.0801: *Acc. no.* OQ230637.

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Author contributions

B.M.W. supervised and coordinated the project. A.R. designed, analysed, and interpreted the mass spectrometry data and wrote the corresponding part of the manuscript. B.M.W. and J.M.H. designed all other experiments and evaluated the data. T.P.J. designed initial experiments, purified Cor a 1.0101 and 1.0401 and provided conceptual input. S.A. purified Cor a 1.0302 and 1.0801. H.K. cloned and purified Cor a 1.0701. K.R. purified Cor a 1.0601 and performed CD experiments with the protein. J.M.H. performed RNA extractions, PCR reactions, CD experiments, immunoblots and ELISAs. B.M.W. and J.M.H. wrote the paper. All authors contributed to, read and approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Supplement

Tables

Table S1. Primers used for isoallergen identification at the mRNA level

Primer	Sequence of primer pairs (5' > 3')	localization
Cor a 1.01	fwd: CCTCTTCCCATATTCTTAATTCCTTAGATC rev: GGGCGAAGACACAAGACGAGG	UTR
Cor a 1.02	fwd: CCTCTACTACGTACTCCTCTTCATATTCTTCC rev: GAAGATGATGATCACAATTCACAACCGC	UTR
Cor a 1.03	fwd: CCTCTAGTCCTCATATATCTCCCAACCTC rev: CCTTAACACGTGATGGCGGAAATAAGC	UTR
Cor a 1.03	fwd: ATGGGTGTGTTCACTTATGAAACCG rev: TTAATACTCATTAGGGGTGCGCAAGAGG	Start, stop codon
Cor a 1.04	fwd: ATGGGTGTTTTCTGCTACGAGG rev: CTAACAGTAGGCATCAGGGTGTG	Start, stop codon
Cor a 1.05	fwd: GTTGTGCACCATTCGAATCAGTCCTCC rev: GCGCCACAAAGCAAAGACATAGAACCTC	UTR
Cor a 1.06	fwd: CCAACTTTGATCAATCCTACTACTCCTCG rev: CATTACTGGGCGAAGACACAATATATATATAAGAC	UTR
Cor a 1.07	fwd: GGCAAGTACGTTCTCACATCTCCACAAC rev: GAAGGCCTTAAGACAGTTTTGACCAGACAAATC	UTR
Cor a 1.07	fwd: ATGGGTGTTTACACATATGAGAATG rev: TTAGTTGTAGGCTTCAGGATTG	Start, stop codon
Cor a 1.08	fwd: GCCTAGCTAGTTTCATTAATCCTCAAGAAAGG rev: CACCATCACTATTCACTAGTGATTGAGACTTATTAT	UTR
18S rRNA	fwd: CTGGTCTTAATTGGCCGGGT rev: CAGGCTGAGGTCTCGTTC	

Table S2. Primers used for cloning of Cor a 1 genes into pET-GB1a via Gibson cloning.

	Amplification	Sequence of primer pairs (5' > 3')
Cor a 1.0501	Vector	fwd: CCTGAGGCCTATGCTTGAGGTACCGGATCCGAATTC rev: CACTTAGAGTGTGAACACCCATGCCCTGAAAATAAAGATTCTCAG
	Insert	fwd: CTGAGAATCTTTATTTTCAGGGCATGGGTGTTCACTCTAAGTG rev: GAATTCGGATCCGGTACCTCAAGCATAGGCCTCAGG
Cor a 1.0601	Vector	fwd: CGCATAGTGCTGAGTACAATAAGGTACCGGATCCGAATTC rev: CGTAGTTAAACACGCCCATGCCCTGAAAATAAAGATTCTCAG
	Insert	fwd: CTGAGAATCTTTATTTTCAGGGCATGGGCGTGTAACTACG rev: GAATTCGGATCCGGTACCTTAGTTGTACTIONCAGCACTATGCG
Cor a 1.0701	Vector	fwd: CAAATCCTGAAGCCTACAATAAGGTACCGGATCCGAATTC rev: CATTCTCATATGTGTAAACACCCATGCCCTGAAAATAAAGATTCTCAG
	Insert	fwd: CTGAGAATCTTTATTTTCAGGGCATGGGTGTTACACATATGAGAATG rev: GAATTCGGATCCGGTACCTTAGTTGTAGGCTTCAGGATTTG
Cor a 1.0801	Vector	fwd: CTGGCGCCTATGCTTAAGGTACCGGATCCGAATTC rev: TGTGTAAGTGATTACGCCCATGCCCTGAAAATAAAGATTCTCAG
	Insert	fwd: CTGAGAATCTTTATTTTCAGGGCATGGGCGTAATCACTTACACA rev: GAATTCGGATCCGGTACCTTAAGCATAGGCGCCAG

Figures

Figure S1

Cora1.02-Cav01g11820 (<https://www.hardwoodgenomics.org/>)

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- - - - - - - - - - - - - - - - - -
atc atc atg ggt gtt ttc aat tac gag gct gag acc acc tcc gtt att cca gca gct agg
- - M G V F N Y E A E T T S V I P A A R
ctg tct aag gcc ttt atc ctt gat ggc aat aac ctc atc cca aag gtt gca cct caa gct
L S K A F I L D G N N L I P K V A P Q A
gtt agc agt gtt gaa aac gtt gaa gga aat gga ggg cca gga aca atc aag aag atc acc
V S S V E N V E G N G G P G T I K K I T
ttt tcc gaa ggc agt cct ttc aag tac gtg aag gag agg gtt gaa gag gtt gac cac aca
F S E G S P F K Y V K E R V E V D H T
aac ttc aaa tac agc tac acc gtc atc gag ggt ggt ccc gtg gga gac aaa gtg gag aag
N F K Y S Y T V I E G G P V G D K V E K
atc tgt aac gag ata aag att gtg gca gcc cct gat gga gga tcc atc ttg aag atc tcc
I C N E I K I V A A P D G G S I L K I S
aac aag tac cac acc aaa ggt gac cat gag gtg gat gca gag cat att aag ggt ggc aaa
N K Y H T K G D H E V D A E H I K G G K
gaa aag gtt gag ggt ctt ttc agg gcg gtt gag gcc tac ctc ttg gca cac tct gct gaa
E K V E G L F R A V E A Y L L A H S A E
tac aac taa aaa cct taa ttg tct ttt ggc ttt cat ttt gtc ttt aat ttc ggc ttg cta
Y N - - - - - - - - - - - -
... ... ... gcg gtt gtg aat tgt gat cat cat ctt c
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Cora1.0302 (GenBank accession number: OQ450370). Cav01g11830, Cav01g11840
(<https://www.hardwoodgenomics.org/>)

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K S F V L D S D N L I P K V A P K A I K
agc att gaa atc atc gaa gga aat gga ggt ccc gga acc att aag aag atc tgc ttc gat
S I E I I E G N G G P G T I K K I C F D
gaa ggc agc cca ttc aac tac ata aag caa aag gtt gaa gag att gac caa gca aac ttt
E G S P F N Y I K Q K V E E I D Q A N F
tca tat cgc tac agt gtg att gaa ggc gat gct ttg tcc gac aaa ctg gag aaa atc aat
S Y R Y S V I E G D A L S D K L E K I N
tac gag atc aag ata gtg gca tcc cct gat gga gga tct atc ttg aag agc atc agc aag
Y E I K I V A S P D G G S I L K S I S K
tac cac acc ata gga gac cat gaa ctc aag gac gag cag att aag gct gga aaa gag aag
Y H T I G D H E L K D E Q I K A G K E K
gcc tca gga ctt ttc aaa gct gtt gag ggc tac ctc ttg gca cac cct aat gag tat taa
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F K A L I L D A D N L L P K L L P Q A I
aag agt atc gag acg att gag ggc gat gga ggg cct gga acc atc aag aag atc act atc
K S I E T I E G D G G P G T I K K I T I
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A E G T H I K H L K H R I D A V E E E K
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Y T V I E G G V V G D K L E K I C N E L
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Cora1.0701: (GenBank accession number: OQ230636)

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-

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Cora1.0801: (GenBank accession number: OQ230637). Cav01g11530
(<https://www.hardwoodgenomics.org/>)

```

cct agc tag ttt cat taa tcc tca aga aag gat atc atg ggc gta atc act tac aca gat
- - - - - - - - - - - M G V I T Y T D
gag tac acc tct cct atc cca cca gct aga ttg ttc aaa gcc ttg gtc att gat gct cac
E Y T S P I P P A R L F K A L V I D A H
atc ctc atc cca aaa ctc ctc cca cag gct gtt aag agc att gaa atc att caa ggc gat
I L I P K L L P Q A V K S I E I I Q G D
gga ggg gct gga agc atc agg caa atc act ttt gcc gaa ggt agc caa ttt agt act gtc
G G A G S I R Q I T F A E G S Q F S T V
aag aac cga att gat gag ctg aat gaa aaa act tat tac tac aag tac acg gtg atc gaa
K N R I D E L N E K T Y Y Y K Y T V I E
ttc gaa ggt gat gcc ttg gct gac aag ctt gaa ttg att gtt cat gag gtt caa ttt gag
F E G D A L A D K L E L I V H E V Q F E
gca acg gct gaa ggt ggg agt aaa aat aag atg aca acc aag tac cat acc aag gat gac
A T A E G G S K N K M T T K Y H T K D D
gta gtg atc aag gaa gag gaa atc aag gct ggc aag gaa aag gtg ctg ggt atg tac aaa
V V I K E E E I K A G K E K V L G M Y K
gtt gtg gaa ggc tac ctc atc cag aac cct ggc gcc tat gct taa tta atg cta atc tcc
V V E G Y L I Q N P G A Y A - - - - -
aat cag tct ctt ttg tct act gtt taa tgt tgt ggc ttt tac ctt tgt ctt tga ata atc
- - - - - - - - - - - - - - -
gcc tta atg tgg tgt gat aat aag tct caa tca cta gtg aat agt gat ggt g
- - - - - - - - - - - - - - -

```

Figure S1: Coding sequences of Cor a 1 isoallergens and flanking regions used for the selection of PCR primers. The coding sequences and the corresponding amino acid sequences are depicted in bold letters. Underlined sequences represent the primer sequences used for PCR amplification. They are based on the genome sequences of the Turkish cultivar “Tombul” (Lucas u. a. 2021). Dotted sections indicate regions that were not covered by sequencing.

Figure S2

Hazel pollen extract:

Cor a 1.0101	1	19	22	23	30	34	38	41	56	57	65	67	70			
MGVENEVETPSVIPPAR	LFK	S--YVLDGDK	LIPK	VAPQ-AIT-SVENVEGNGGPGTIK	N--ITFEGGSR	YK	YK	ER								
MGVENEVETPSVISAR	LFK	S--YVLDGDK	LIPK	VAPQ-AIT-SVENVEGNGGPGTIK	N--ITFEGGSR	YK	YK	ER								
MGVENEVETTSVIPPAR	LFK	R	FVLDSDN-LIPK	VAPK	AIK	STEIIEGNGGPGTIK	K	ICFDEGSP-FN-YIK	QK							
MGVFETETTSVIPPAR	LFK	S--FVLDSDN-LIPK	VAPK	AIK	STEIIEGNGGPGTIK	K	ICFDEGSP-FN-YIK	QK								
Cor a 1.0302	72	82	84	96	99	105	117	121	125	131	133	136	139	141	144	147
VDEVNTNFT-YS-YTVIEGDVLGDK	LFK	VCHEIK	IVAPBGGSILK	ISSK	FHAK	GDHEIN-AE-EMK	GAK	EM-AEK	LIR	AVEGYLLAHSAEYN						
VDEVNTNFK	YS-YTVIEGDVLGDK	LFK	VCSEIK	IVAPBGGSILK	ISSK	FHAK	GDHEIN-AE-EMK	GAK	EM-AEK	LIR	AVEGYLLAHSAEYN					
VEEIDQANFS-YR	YSVIEGDALSDK	LFK	INYEIK	IVASPHGGSILK	SISK	YHTI-GDHEIK	DE-QIK	AGK	EK	ASG-LFK	AVEGYLLAHSDAYN					
VEEIDQANFS-YR	YSVIEGDALSDK	LFK	INYEIK	IVASPDGGSILK	SISK	YHTI-GDHEIK	DE-QIK	AGK	EK	ASG-LFK	AVEGYLLAHSDAYN					

Mature hazel nut (in-gel digestion):

Cor a 1.0403	1	19	22	34	56	57	67	70			
	<u>MGVFCEDEATSVIPPAR</u>	LFEK	<u>SFVLADADNLIIPK</u>	VAPQHTGAENLENGGPGTIK	K	ITFAEGSEFK	YMK	HK			
Cor a 1.0403	72	82	99	105	118	122	126	137	140	142	148
	VEEIDHANFK	XCYSITIEGGLGHTTEK	ISYEIK	MAAAPHGGGSILK	ITSK	YHTK	GNASISEEIEIK	AGK	EK	AAG-LFK	AVEAYILLAHDPDYCC

Figure S2: Amino acid sequences of Cor a 1 peptides identified in extracts of hazel pollen and mature hazel nut by LC-MS^E. Corresponding peptides detected by LC-MS^E are underlined. Differing sequence stretches are color coded. Identical colors indicate identical amino acid sequences of peptides, black stretches indicate peptides which were not detected. Underlined stretches indicate unambiguously confirmed peptides. A dashed underline indicates a C-terminal peptide which is indistinguishable due to a possible deamidation N→D. The positions of the amino acids in the sequence were labeled according to the following scheme: e.g., T19 means that the corresponding peptide is released after "T"-(Trypsin) cleavage and starts at amino acid position 19.

Figure S3

Cor a 1.01

Cor a 1.01- Cav01g11790 (flower):

CCTCTTCCCATATTCTTAATTCCTTAGATCATCATCATCATGGGTGTTTTCAATTACGAGGTCGAGACCCCTCCGTT
ATCCCAGCGGCAAGGCTGTTCAAGTCCTATGTCCTTGATGGCGATAAGCTCATCCCAAAGGTTGCACCTCAAGCT
ATTACCAGCGTTGAAAACGTTGGAGGAAATGGAGGGCCTGGAACCATCAAGAATATCACCTTTGGCGAAGGCA
GCCGTTACAAGTACGTGAAGGAGAGGGTTGATGAGGTTGACAACACAACTTCAAATATAGCTACACCGTGATC
GAGGGTGATGTCCTGGGTGACAAGCTGGAGAAGGTCTGCAGCGAGCTGAAGATAGTGGCAGCCCCTGGTGGG
GGATCCATCTTGAAGATCAGCAGCAAGTTCACGCCAAAGGCGACCATGAGATTAATGCAGAGGAGATGAAGG
GTGCCAAAGAAATGGCCGAGAACTTTTAAGGGCGGTTGAGACCTACCTATTGGCACACTCTGCTGAATACAAC
TAAATATCGTCTTGCTCTTCGCCC

Cor a 1.01-like variant 1 (GenBank accession number: OQ569792) (pollen):

CCTCTTCCCATATTCTTAATTCCTTAGATCATCATCATCATGGGTGTTTTCAATTACGAGGCTGAGACCACCTCTGTT
ATCCCTGCGGCAAGGCTGTTCAAGTCCTATGTCCTTGATGGCGATAAGCTCATCCCAAAGGTTGCACCTCAAGCTA
TTACCAGCGTTGAAAACGTTGGAGGAAATGGAGGGCCTGGAACCATCAAGAATATCACCTTTGGCGAAGGCAG
CCGTTACAAGTACGTGAAGGAGAGGGTTGATGAGGTTGACAACACAACTTACATACAGCTACACCGTGATCG
AGGGTGATGTCCTGGGTGACAAGCTGGAGAAGGTCTGCCACGAGCTGAAGATAGTGGCAGCCCCTGGTGGAG
GATCCATCTTGAAGATCAGCAGCAAGTTCACGCCAAAGGTGACCATGAGATTAATGCAGAGGAGATGAAGGGT
GCCAAAGAAATGGCCGAGAACTTTTAAGGGCGGTTGAGACCTACCTATTGGCACACTCTGCTGAATACAATA
AACCTCGTCTTGCTCTTCGCCC

Cor a 1.01-like variant 2 (GenBank accession number: OR326622) (mature nut):

CCTCTTCCCATATTCTTAATTCCTTAGATCATCATCATCATGGGTGTTTTCAATTACGAGGCTGAGACCACCTCTGTT
ATCCCTGCGGCAAGGCTGTTCAAGTCCTATGTCCTTGATGGCGATAAGCTCATCCCAAAGGTTGCACCTCAAGCTA
TTACCAGCGTTGAAAACGTTGGAGGAAATGGAGGGCCTGGAACCATCAAGAATATCACCTTTGGCGAAGGCAG
CCGTTACAAGTACGTGAAGGAGAGGGTTGATGAGGTTGACAACACAACTTACATACAGCTACACCGTGATCG
AGGGTGATGTCCTGGGTGACAAGCTGGAGAAGGTCTGCCACGAGCTGAAGATAGTGGCAGCCCCTGGTGGAG
GATCCATCTTGAAGATCAGCAGCAAGTTCACGCCAAAGGTGACCATGAGATTAATGCAGAGGAGATGAAGGGT
GCCAAAGAAATGGCCGAGAACTTTTAAGGGCGGTTGAGACCTACCTATTGGCACACTCTGCTGAATACAATA
AACCTCGTCTTGCTCTTCGCCC

Cor a 1.02

Cor a 1.02-like variant (GenBank accession number: OQ569793) (flower):

ATGGGTGTTTTCAATTACGAGGCTGAGACCACCTCCGTTATTCCAGCAGCTAGGCTGTTAAGGCCTTATCCTT
GATGGCAATAACCTCATCCCAAAGGTTGCACCTCAAGCTGTTAGCAGTGTTGAAAACGTTGAAGGAAATGGAG
GGCCAGGAACAATCAAGAAGATCACCTTTTCCGAAGGCAGTCCTTTCAAGTACGTGAAGGAGAGGGTTGAAG
AGGTTGACCACACAACTTCAAATACAGCTACACCGTCATCGAGGGTGGTCCCGTGGGAGACAAAGTGGAGA
AGATCTGTAACGAGATAAAGATTGTGGCAGCCCCTGATGGAGGATCCATCTTGAAGATCTCGAACAAAGTACCA
CACCAAAGGTGACCATGAGGTGGATGCAGAGCATATTAAGGGTGGCAAAGAAAAGGTGAGGGTCTTTTCAG
GGCGGTTGAGGCCTACCTCTTGGCACACTCTGCTGAATACAATAA

Cor a 1.03

Cor a 1.03-Cav01g11870 (immature nut):

CTCTAGTCCTCATATATCTCCCAACCTCAAAAACCTCTCCACTTAATTATTATCATTCTCTCTCACATCACTTCCAAA
GTCTTAGCTCATCATGGGTGTGTTCACTTATGAAACCGAGACCACTTCAGTTATCCCTCCCGCTAGGCTGTTCAA
GAGCTTTGCTCTAGATTCCGACAACCTCATCCCAGAGGTTGCTCCAAAGGCCATCAAGAGCATTGAAATCATCG
AAGGAAATGGAGGTCCCGGAACCTTAAGAAGATCTGCTTCGATGAAGGCAGCCCAATCAGCTACATAAAGC
AAAAGGTTGATGAGATTGACCAAGCAAACCTTTTCATATCGCTACAGTGTGATTGATGGCGATGCTTTGTCCGAC
AAACTGGAGAAAATCAATTGCGAGATCAAGATAGTGGCATCCCCTGATGGAGGATCTATCATGAAGAGCATCA
GCAAGGACCACACCATAGGAGACCATGAGCTCGAGGAAGAGCAGATTAAGGCTGGAAAAGAGATGGCCTCA
GGACTTTTCAAAGCTGTTGAGGACTACCTCTTGGCACACCCTAATGAGTATTAATTAAGGCTAATTGTGTGGTG
GTTATATTAATTTGCTTATTTCCGCCATCACGTGTTAAGG

Cor a 1.03-like variant 1 (GenBank accession number: OQ569794) (immature nut):

CCCAACCTCAAAAACCTCTCCACTTAATTATTATCATTCTCTCTCACATCACTTCCAAAGTCTTAGCTCATCATGGG
TGTGTTCACTTATGAAACCGAGACCACTTCAGTTATCCCTCCCGCTAGGCTGTTCAAGAGCTTTGCTCTAGATT
CGACAACCTCATCCCAGAGGTTGCTCCAAAGGCCATCAAGAGCATTGAAATCATCGAAGGAAATGGAGGTCCC
GGAACCTTAAGAAGATCTGCTTCGATGAAGGCAGCCCAATCAGCTACATAAAGCAAAGGTTGATGAGATTG
ACCAAGCAAACCTTTTCATATCGCTACAGTGTGATTGATGGCGATGCTTTGTCCGACAAACTGGAGAAAATCAAT
TGCGAGATCAAGATAGTGGCATCCCCTGATGGAGGATCTATCATGAAGAGCATCAGCAAGGACCACACCATA
GGAGACCATGAGCTCGAGGAAGAGCAGATTAAGGCTGGAAAAGAGATGGCCTCAGGACTTTTCAAAGCTGTT
GGGACTACCTCTTGGCACACCCTAATGAGTATTAATTAAGGCTAATTGTGTGGTGGTTATATTAATTTGCTTA
TTTCGCCATCACGTGTTAAGG

Cor a 1.03-like variant 2 (GenBank accession number: OR326623) (catkin):

ATGGGTGTGTTCACTTATGAAACCGAGACCACTTCAGTTATCCCTCCCGCTAGGCTGTTCAAGAGCTTTGCTCT
AGATTCCGACAACCTCATCCCAAAGGTTGCTCCAAAGGCCATCAAGAGCATTGAAATCATCGAAGGAAATGGA
GGTCCCGGAACCTTAAGAAGATCTGCTTCGATGAAGGCAGCCATTCAACTACATAAAGCAAAGGTTGAAG
AGATTGACCAAGCAAACCTTTTCATATCGCTACAGTGTGATTGAAGGCGATGCTTTGTCCGACAAACTGGAGAA
AATCAATTACGAGATCAAGATAGTGGCATCCCCTGATGGAGGATCTATCATGAAGAGCATCAGCAAGGACCAC
ACCATAGGAGACCATGAGCTCAAGGAAGAGCAGATTAAGGCTGGAAAAGAGATGGCCTCAGGACTTTTCAA
GCTGTTGAGGACTACCTCTTGGCACACCCTAATGAGTATTAATTAAGGCTAATTGTGTGGTGGTTATATTAATTTGCTTA

Cor a 1.04

Cor a 1.04-like variant 1 (GenBank accession number: OQ569795) (immature nut):

CCTCTACTACGTACTCCTCTTCATATTCTTCCATTTCCATAGATCATCATCATCATCATCATCATGGGTGTTTT
CTGCTACGAGACTGAGACCACCTCCGTTATCCCTCCCGCTAGGCTGTTCAAGTCCTTTGCTCTAGATGCCGACA
ACCTCATTCCCAAGGTTGCTCCTCAGCACTTCACCGGCGCTGAAAACCTCGAAGGAAATGGCGGGCCTGGAAC
CATCAAGAAGATCACCTTCGCCGAAGGCAGCGAATTCAAGTACATGAAGCACAAAGGTTGAGGAGATCGACCA
CGCAAACCTTCAAATACTGCTACAGCATCATCGAGGGAGGTCCATTGGGGCACACACTGGAGAAGATCTTTAC
GAGATCAAGATGGCGGCAGCCCCCTCATGGAGGAGGATCCATCTTGAAGATCACCAGCAAGTACCACACCAAG
GGCAACGCTTCAATCAGTGAGGAGGAGATCAAGGCTGGCAAAGAGAAGGCCCGGCACTTTTCAAGGCTGTT
GAGGCTTACCTCTTGGCACACCCTGATACCTACTGTTAGAGTAGCTAACCCACGTACAACCTAAAGCTTGTGT
TGTGTTGTGTGGTCCCTCAATAATGAGTTGTTACTGCTTGATTTTGGTTTGCTAATAAAGGAGTTTGCGGTTGT
GAATTGTGATCATCATCTTC

Cor a 1.05

Cor a 1.05-like variant 1 (GenBank accession number: OQ569796) (catkin):

GTTGTGCACCATCCAATCAGTCCTGCATTTTCTACCATCTCTAAATTCTGAATATATTCGATCATGGGTGTTCA
ACTCTAAGTGATGAGGTCAGTCCCATCCCAGCACCAAAGCTGTTCAAGGCCTTGATCCTTGATGCTGACAA
CCTCCTTCCCAAGCTCCTGCCTCAGGCCATTAAGAGTATCGAGACAGTTGAGGACAATGGAGGGCCTGGAACC
ATCAAGAAGATCACCATCGCTGAAGGTACCCACATCAAGCACTGAAGCATAGGATTGATGCAGTAGACGAAG
AGAAATTGACATACAGTTACACACTGATTGAGGGTGATGATTTGCTGGACAAGTTTGAATCAATTTCTTATGAG
ATTAAGTTTGAGTCTTCTCCTGATGGGGGAGCCAAATGTACAAATTTAGCAAGTACCATCCTAAACCAGGGGC
CCAGATCAATGAAGAGGAAATGAAGGCAAGCAAGGAAAAGGGCTTGGCTGTTTACAGAGCTATGGAAGCCTA
CCTCTTGGCCAATCCTGAAGCCTATGCTTGATGTTTCTTATTGAGACACTATATATGTATCCTGTGTTCTCTCAA
GTGATTGAGGTTCTATGTCTTTGCTTTGTGGCGC

Cor a 1.05-like variant 2 (GenBank accession number: OR232687) (mature nut)

CTAATTCTGATATATTCGATCATGGGTGTTCACTCTAAGTGATGAGTTCACTAGCCCCATCCCAGCACCAA
GCTGTTCAAGGCCTTGATCCTTGATGCTGACAACCTCCTTCCCAAGCTCCTGCCTCAGGCCATTAAGAGTATCG
AGACGGTTGAGGGCGATGGAGGGCCTGGAACCATCAAGAAGATCACTATCGCTGAAGGTACCCATATCAAGC
ACTGAAGCATAGGATTGATGCAGTAGAGGAAGAGAAATTGACATACAGTTACACACTGATTGATGGTGATG
ATTTGCTGGACAAGTTTGAATCAATTTCTTATGAGATTAAGTTTGAGTCTCTCCTGATGGGGGAGCCAAATGT
ACAAATCTTAGCAAGTACCATCCTAAACCAGGGGTCCAGATCAATGAAGAGGAAATCAAGGCAAGCAAGGAA
AAGGGCATGGCTGTTTACAGAGCCGTGGAAGCCTTCTCTTGGCCAATCCTGAGGCCTATGCTTGATGCTTCCT
TGTTGAGACACTATTATATGTATCTCGTGTTCTCAAAGTTTATTGAGGTTCTATGTCTTTGCTTKGTGGCGCAA
AA

Cor a 1.05-Cav01g11940 (flower):

GTTGTGCACCATCCAATCAGTCCTCCATTTTCTACCATTTTCTAAATTCTGAATATATTCGATCATGGGTGTTCA
CACTCTAAGTGATGAGTTCACTAGCCCCATCCCAGCACCAAAGCTGTTCAAGGCCTTGATCCTTGATGCTGACA
ACCTCCTTCCCAAGCTCCTGCCTCAGGCCATTAAGAGTATCGAGACGGTTGAGGGCGATGGAGGGCCTGGAAC
CATCAAGAAGATCACTATCGCTGAAGGTACCCACATCAAGCACTGAAGCATAGGATTGATGCAGTAGAGGAA
GAGAAATTGACATACAGTTACACACTGATTGAGGGTGATGATTTGCTGGACAAGTTTGAATCAATTTCTTATGA
GATTAAGTTTGAGTCTCTCCTGATGGGGGAGCCAAATGTACAAATCTTAGCAAGTACCATCCTAAACCAGGG
GTCCAGATCAATGAAGAGGAAATCAAGGCAAGCAAGGAAAAGGGCATGGCTGTTTACAGAGCCGTGGAAGC
CTTCTCTTGGCCAATCCTGAGGCCTATGCTTGATGCTTCCTTGTGAGACACTATTATATATATCTCGTGTTCT
CAAAGTTTATTGAGGTTCTATGTCTTTGCTTTGTGGCGC

Cor a 1.05-Cav01g11520 (immature nut):

AAATTTCCCTCTCTAATTCTGAATATATTCGATCATGGGTGTTCACTCTAAGTGATGAGGTCAGTCCCAT
CCCAGCACCAAAGCTGTTCAAGGCCTTGATCCTTGATGCTGACAACCTCCTTCCCAAGCTCCTGCCTCAGGCCAT
TAAGAGTATCGAGACAGTTGAGGGCAATGGAGGGCCTGGAACCATCAAGAAGATCACCATCGCTGAAGGTAC
CCACATCAAGCACTGAAGCATAGGATTGATGCAGTAGACGAAGAGAAATTGACATACAGTTACACACTGATT
GAGGGTGATGATTTGCTGGACAAGTTTGAATCAATTTCTTATGAGATTAAGTTTGAGTCTTCTCCTGATGGGGG
AGCCAAATGTACAAATTTAGCAAGTACCATCCTAAACCAGGGGCCAGATCAATGAAGAGGAAATGAAGGC
AAGCAAGGAAAAGGGCTTGGCTGTTTACAGAGCTATGGAAGCTACCTCTTGGCCAATCCTGAAGCCTATGCT
TGATGTTTCTTATTGAGACACTATCTATGTATCCTGTGTTCTCAAAGTGATTGAGGTTCTATGTCTTTGCTTT
GTGGCGC

Cor a 1.07

Cor a 1.07-like variant 1 (GenBank accession number: OR232688) (catkin)

TCCTCTTCTTCTTCTTCTTCTAGTCTTCATAGCTAGATATCATCATGGGTGTTTTACATATGAGAATGAGG
TCACCTCCCCCTCCCTCCATCGAAGTTGTTCAAGGCCTTCGTCTTGATGCCGACAACCTTGTCCTCAAGATT
ACAAGCACGGCATAAATGACCTGAACGTGGAATCCTTGAAGGCCATGGAGGGCTGGAACCATCAAAAAGT
TTACGTTTCATGAAGGCGGCCACTTGAAATTTTTGAAGCACAAGGTTGATGTGCTAGACAAGGAACACTTCAC
ATACAATTACAGCGTGTTGAAGGTGGTCTTTGTCGGAGACACTCGAGAAAGTCTCATTGAGACCAAGTTG
GTGGCTTCCCCAGATGGAGGAACCATCTTCAAGAGCACCGGCAAGTATTACAAAAGATCACGCTGAGATAA
ATGAAGAGAAAAATCAAGGCTGAAGATGAAAAGGCCACGGGGGTGTTCAAGGCAGTTGAAGGCTACCTCTTG
GCAATCCTGAAGCCTACAATAAGCTGCTTGATTGTTGCATGTATATCTGATTGTCTGGTCAAAACTGTCTT
AAGGCCTC

Cor a 1.07-like variant 2 (GenBank accession number: OR232689) (pollen)

TCTCTTCTTCTTCTTCTTTGATCATCTTCTAGTACGCTAGATATCATCATGGGTGTTTTACATATGAGAAT
GAGGTCACCTCCCCCTCCCTCCATCGAAGTTGTTCAAGGCCTTCGTCTTGATGCCGACAACCTTGTCCTCAAG
ATTTACAAGCACGGCATAAATGACCTGAACGTGGAATCCTTGAAGGCCATGGAGGGCTGGAACCATCAAA
AAGTTTACGTTTCATGAAGGCGGCCACTTGAAATCTTGAAGCACAAGGTTGATGTGCTAGACAAGGAACACT
TCACATACAATTACAGCGTGTTGAAGGTGGTCTTTGTCGGAGACACTCGAGAAAGTCTCATACGAGACCAA
GTTGGTGGCTTCCCAGACGGAGGAACCATCTTCAAGAGCACCGGCAAGTATTACAAAAGATCACGCTGAG
ATCAATGAAGAGCAAATCAAGGCTGAAGATGTAAAGGCCACAGGGGTGTTCAAGGCAGTTGAAGGTTACCTC
TTGGCAAATCCTGAAGCCTACAATAAGCTGCTTGATTGTTGCATGTGTATCTGATTGTCTGGTCAAAACTGT
CTTAA

Cor a 1.08

Cor a 1.08-like variant 1 (GenBank accession number: OR232690) (flower)

GCCTAGCTAGTTTCATTAATCCTCAAGAAAGGATATCATGGGCGTAATCACTTACGTAGACGAGTACACCTCCC
CTATTCCACCAGCCAGATTGTTCAAAGCCTTGGTCATTGATGCTCACATCCTCATCCCCAAACTCCTCCACAGG
CTGTTAAGAGCATTGAAATCATTCAAGGCGATGGAGGGGCTGGAAGCATCAGGCAAACCACTTTTGCCGAAG
GTAGCCAATTTAGTACTGTCAAGAACC GAATTGATGAGCTGAATGAAAAAACTTATTACTACAAGTACACGGT
GATCGAATTCGAAGGTGATGCCTTGGCTGACAAGCTTGAATTGATTGTTTCATGAGGTTCAATTTGAGGCAACG
GCTGAAGGTGGGAGTAAAAATAAGATGACAACCAAGTACCATACCAAGGATGACGTAGTGATCAAGGAAGA
GGAAATCAAGGCTGGCAAGGAAAAGGTGCTGGGTATGTACAAAGTTGTGGAAGGCTACCTCATCCAGAACCC
TGGCGCCTATGCTTAATTAATGCTAATCTCCAATCAGTCTCTTTGTCTACTGTTTAATGTTGTGGCTTTACCTT
TGCTTTGAATAATCGCCTAATGTGGTGTGATAATAAGTCTCAATCACTAGTGAATAGTGATGGTG

Cor a 1.08-like variant 2 (GenBank accession number: OR232691) (mature nut)

GCCTAGCTAGTTTCATTAATCCTCAAGAAAGGATATCATGGGCGTAATCACTTACACAGATGAGTACACCTCTC
CTATCCCACCAGCTAGATTGTTCAAAGCCTTGGTCATTGATGCTCACATCCTCATCCCCAAACTCCTCCACAGG
CTGTTAAGAGCATTGAAATCATTCAAGGCGATGGAGGGGCTGGAAGCATCAGGCAAACCACTTTTGCCGAAG
GTAGCCAATTTAGTACTGTCAAGAACC GAATTGATGAGCTGAATGAAAAAACTTATTACTACAAGTACACGGT
GATCGAATTCGAAGGTGATGCCTTGGCTGACAAGCTTGAATTGATTGTTTCATGAGGTTCAATTTGAGGCAACG
GCTGAAGGTGGGAGTAAAAATAAGATGACAACCAAGTACCATACCAAGGATGACGTAGTGATCAAGGAAGA
GGAAATCAAGGCTGGCAAGGAAAAGGTGCTGGGTATGTACAAAGTTGTGGAAGGCTACCTCATCCAGAACCC
TGGCGCCTATGCTTAATTAATGCTAATCTCCAATCAGTCTCTTTGTCTACTGTTTAATGTTGTGGCTTTACCTT
TGCTTTGAATAATCGCCTAATGTGGTGTGATAATAAGTCTCAATCACTAGTGAATAGTGATGGTG

Figure S3: Sequences of additional putative Cor a 1 variants identified at the mRNA level in different hazel tissues. Sequences labeled with a Cav01 number are present in the “Tombul”

cultivar (Lucas et al. 2021). Variants labeled with a Genbank accession number are genes identified by us which were not found in the “Tombul cultivar”. Grey: coding sequence.

Figure S4

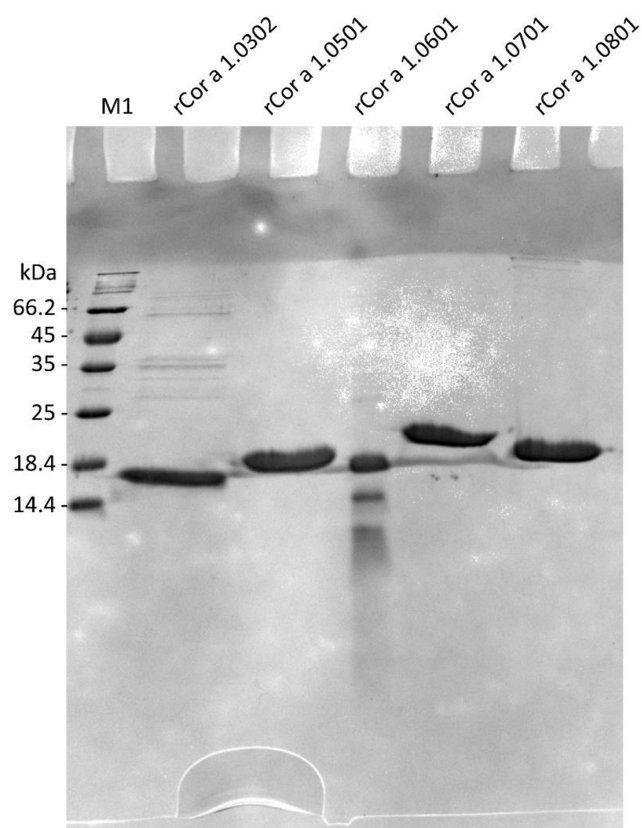
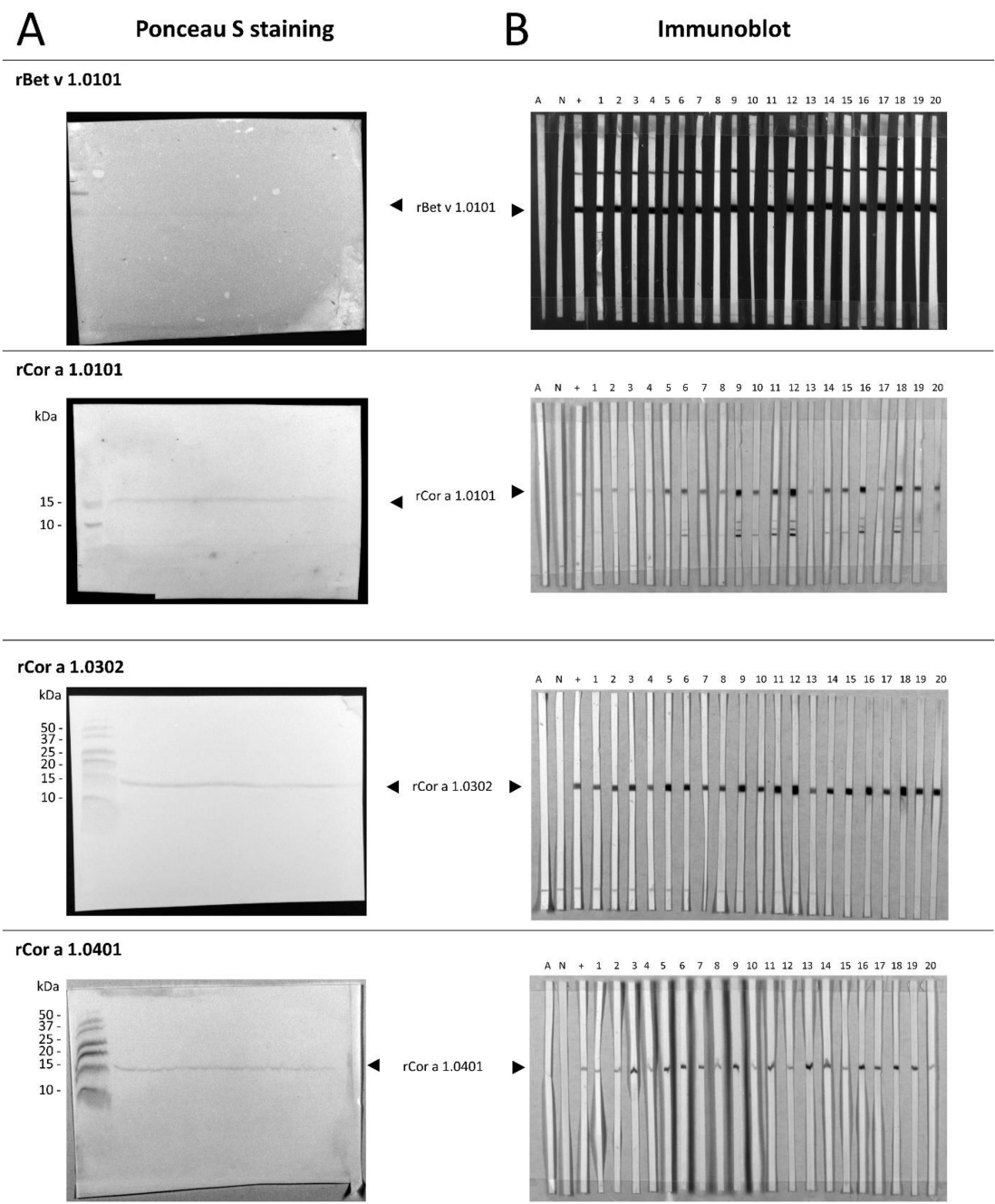


Figure S4. Coomassie stained SDS-polyacrylamide gel showing Cor a 1 proteins purified from *E. coli*. 5 μ g of the purified proteins were applied on a 19 % SDS polyacrylamide gel and subjected to electrophoresis, followed by Coomassie staining. M1 molecular weight marker. The sizes of the marker proteins are indicated on the left.

Figure S5



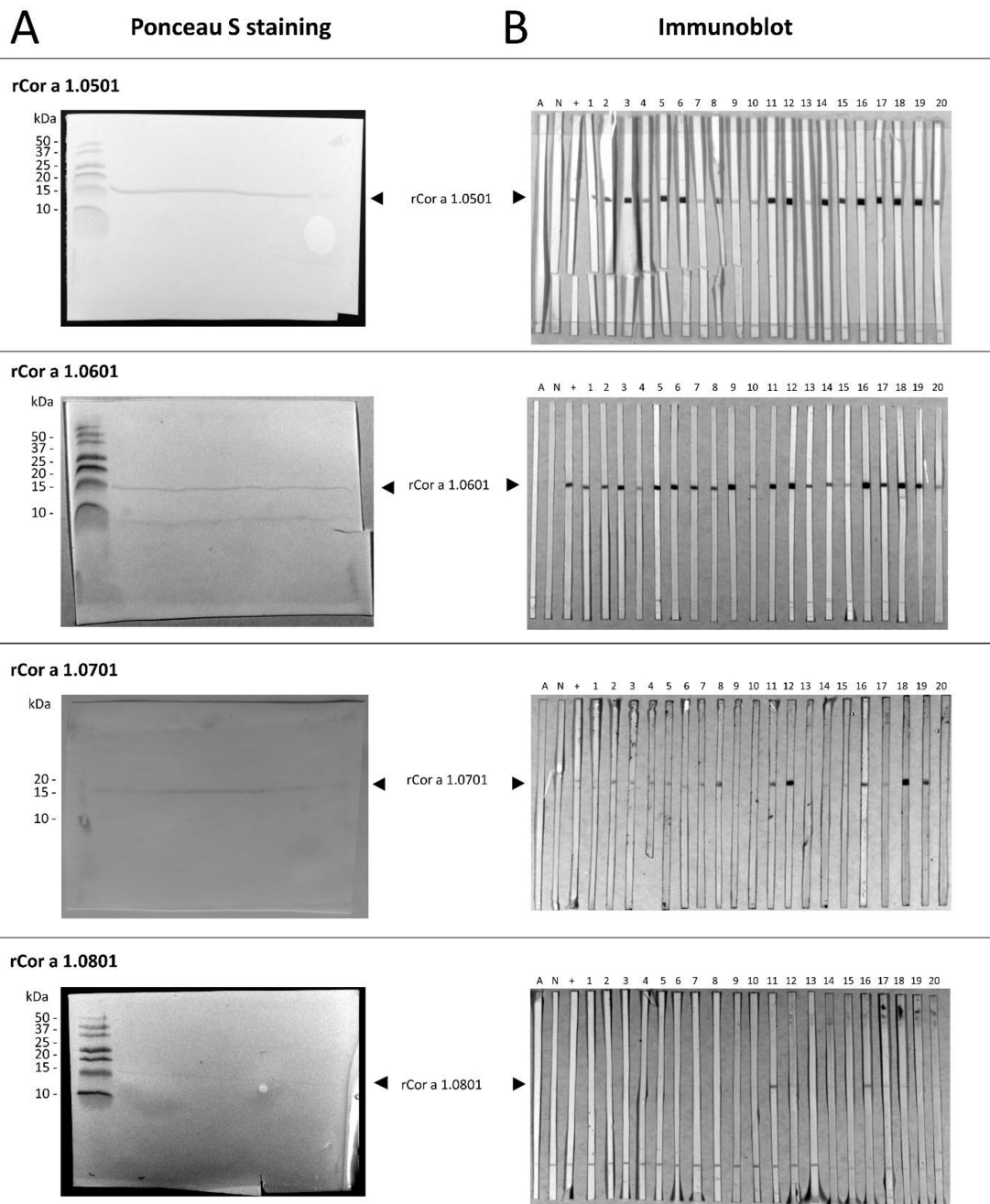
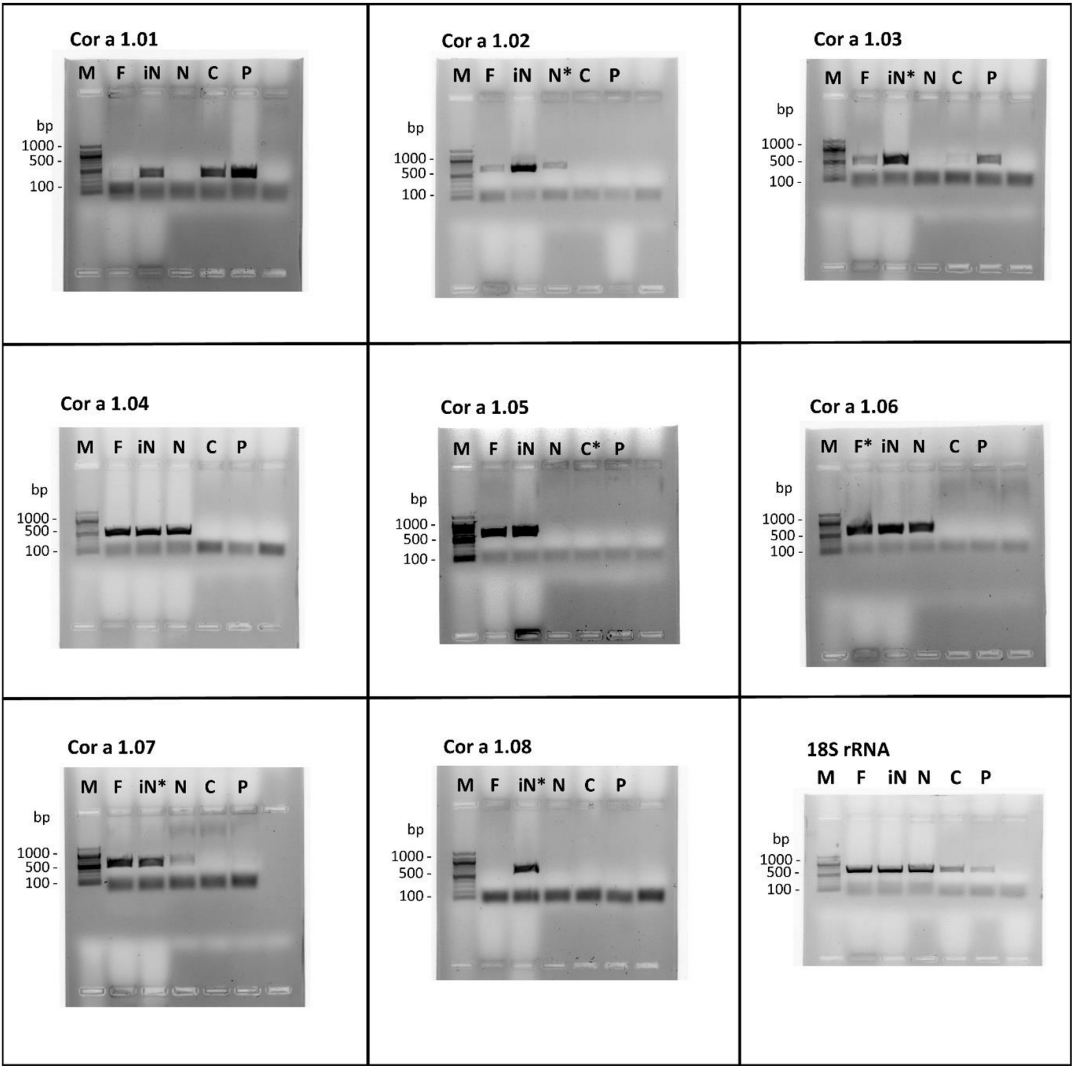


Figure S5: Immunoblots of Cor a 1 isoallergens with sera from tree pollen allergic individuals. A) Ponceau S staining. SDS-PAGE (16 % PA gels) was performed with 1.5 µg/cm or 0.8 µg/cm (Cor a 1.0801) of the Cor a 1 proteins shown, followed by blotting onto 0.2 µm nitrocellulose membranes. Successful protein transfer was visualized by Ponceau S staining. **B) Immunoblots.** The membranes produced in A) were cut into stripes and incubated with patients' sera. Bound specific IgE antibodies were detected using a mouse-anti-human IgE antibody coupled to alkaline phosphatase followed by nitroblue tetrazoliumchloride (NBT) /5- Bromo-4-chloro-3-indolylphosphate (BCIP) staining. Labels on top of the immunoblots: A, secondary antibody control; N, horse serum; +, positive control, mixture of sera from birch pollen and hazel pollen allergic persons (purchased from Gold Standard Diagnostics Kassel GmbH); 1-20, sera of patients allergic to tree pollen.

Figure S6

A



B

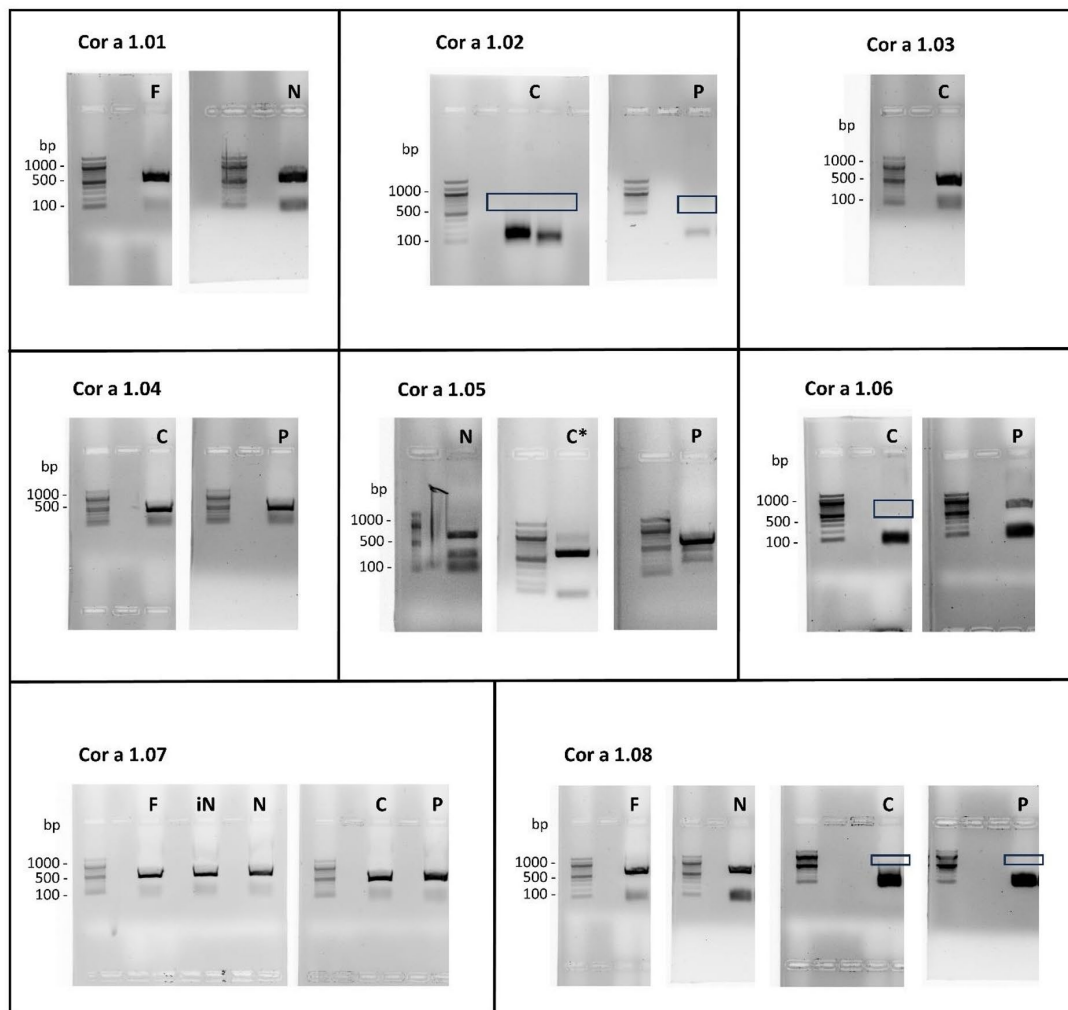


Figure S6: Expression of Cor a 1 genes in different hazel tissues. Full-length gels presented in Figure 1. The results of the 1st PCR assays are shown in A), the gels of the 2nd PCR assays are presented in B. The boxes indicate the regions where DNA products of the corresponding sizes were expected.

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TTHA0849      --MPEVRAERYIPAPPERVYRLAK-DLEGLKP--YLKEVESLEVAREGARTSRWVAVA 55
Cor a 1.0801  MGVTYTYTDEYTSPIPPARLFKALVIDAHILIPKLLPQAVKSIEIIQDGGGAGSIRQITFA 60
               :      *      * * * * : : :      * . * *      : * : * : * : : : * .      * : : . *

TTHA0849      MGKKVRWLEEEWDDENLRNRFFSPEGDFDRYEGTWVFLPEGEGTRVVLTL---TYELTI 112
Cor a 1.0801  EGSQFSTVKNR-IDELNEKTYYY-----KYT---VIEFEGDALADKLELIVHEVQFEA 109
               * . . .      : : : .      * : * : . : :      : *      * :      * : .      * *      : :

TTHA0849      PIFGGLLRKLVQKLMQENV----ESLLKGLEERVLAASS----- 147
Cor a 1.0801  TAEGGSKNKMTTKYHTKDDVVIKEEEIKAGKEKVLGMYKVVEGYLIQNPGAYA 162
               **      . * : . *      : :      * . : * . : * : * .      .

```

Identity matrix	TTHA0849	Cor a 1								
		0101	0201	0301	0302	0401	0501	0601	0701	0801
TTHA0849	100	21.68	23.08	23.78	23.08	22.22	20.42	25.87	22.38	20.98

Figure S7: Sequence comparison and identity matrix of TTHA0849 and Cor a 1 proteins. (*) conserved amino acids; (:) conservative exchanges; (.) semi-conservative exchanges. Numbers in the identity matrix indicate the amino acid sequence identity in %.

8.3 Einzelarbeit C

Hendrich, J. M., Kara, H., Schweimer, K., Jacob, T., Schneider, M., Baldermann S., Wöhl, B.M., **2025**. Identification of glycosylated flavonoids as new physiological ligands of the hazel allergen Cor a 1: Complex structures reveal different binding orientation and specificity of mono- and disaccharide derivatives. *Food Chem.:X*, 29, 102711.



Identification of glycosylated flavonoids as new physiological ligands of the hazel allergen Cor a 1: Complex structures reveal different binding orientation and specificity of mono- and disaccharide derivatives

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ABSTRACT

The global production of hazelnuts is rising, but hazelnut allergies remain common and severe. The PR-10-like allergen Cor a 1, found in hazel pollen and nuts, consists of different isoallergens with over 67 % sequence identity. Cor a 1 binds plant secondary metabolites like the diglycosylated flavonoid Q3O-(Glc)-Gal. Understanding the ligand binding of Cor a 1 isoallergens is crucial to understand their physiological and allergenic properties.

We identified physiological mono- and diglycosylated flavonoids from hazel pollen by UHPLC/QTOF-MS and NMR spectroscopy and investigated their binding to Cor a 1 isoallergens by NMR HSQC experiments and ITC titrations. Screening analyses revealed different binding properties of isoallergens. Whereas the isoallergen Cor a 1.0501 bound only monoglycosylated flavonoids, Cor a 1.0401 also bound diglycosylated flavonoids. Protein/ligand complex structures based on NMR NOESY and computational docking experiments revealed different orientations of mono- vs. diglycosylated flavonoids in the amphiphilic pocket and specific binding of disaccharide derivatives.

1. Introduction

Class 10 pathogenesis-related (PR-10) proteins are central to the plant's immune defence protecting plants against biotic and abiotic stress as well as pathogens (Jain & Kumar, 2015; Walter et al., 1990). The subfamily of PR-10-like proteins includes different allergy causing proteins from pollen and plants. The best studied member is the major birch pollen allergen Bet v 1 (Breiteneder & Kraft, 2023). PR-10-like proteins are known to trigger rhinitis and burning and itching of the tongue and oral mucosa. In general, these symptoms are not life threatening and are referred to as pollen-food syndrome (PFS) or oral

allergy syndrome (OAS). However allergy induced asthma has also been described (Biedermann et al., 2019; Breiteneder & Clare Mills, 2005; Breiteneder & Ebner, 2000; Calamelli et al., 2021).

Most patients allergic to tree pollen are primarily sensitized to Bet v 1 (Lin et al., 2002). However, the IgE antibodies originally directed against Bet v 1 are known to cross-react with other PR-10 like proteins in fruits, e.g. apple, cherry, pear, vegetables and roots such as carrot or celeriac and nuts like hazel nut or stone fruits like almonds (Biedermann et al., 2019; Popescu, 2015). Cross-reaction of IgE antibodies can occur because these proteins are homologous to Bet v 1 and have a sequence identity of at least 50 % and a high structural similarity (Brazhnikov

Abbreviations: *E. coli*, *Escherichia coli*; EIC, extracted ion count; HIC, hydrophobic interaction chromatography; HSQC, heteronuclear single quantum coherence; ITC, isothermal titration calorimetry; K_D , dissociation constant; n, natural; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; PR-10, pathogenesis related class 10; r, recombinant; SEC, size exclusion chromatography; Q3OS, quercetin-3-O- β -D-sophoroside; K3OS, kaempferol-3-O- β -D-sophoroside; Q3O-(Glc)-Gal, quercetin-3-O-(2"-O- β -D-glucopyranosyl)- β -D-galactopyranoside; K3O-(Glc)-Gal, kaempferol-3-O-(2"-O- β -D-glucopyranosyl)- β -D-galactopyranoside; Q3OGlc, quercetin-3-O- β -D-glucopyranoside; Q3OGal, quercetin-3-O- β -D-galactopyranoside; Q3OR, quercetin-3-O- α -L-rhamnoside; K3OR, kaempferol-3-O- α -L-rhamnoside; RMSD, root mean square deviation; TIC, total ion count; UHPLC/QTOF-MS, ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry.

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et al., 2023; Fernandes et al., 2013).

PR-10 like proteins possess a conserved three-dimensional structure consisting of a seven-stranded antiparallel β -sheet, a long C-terminal α -helix and two short helices arranged in V-shape. Together, they form a hydrophobic or amphiphilic cavity, which harbours hydrophobic as well as hydrophilic residues that can bind small molecules (Chruszcz et al., 2021; Fernandes et al., 2013; Gajhede et al., 1996; Jacob et al., 2019; Marković-Housley et al., 2003; Seutter von Loetzen et al., 2012; Seutter von Loetzen et al., 2014). PR-10 allergens were shown to bind to different chemical compounds, e.g. flavonoids, steroids or cytokinins, indicating a role in UV-protection, germination or transport of small molecules (Chruszcz et al., 2021; Jacob et al., 2019; Kofler et al., 2012; Mogensen et al., 2002; Seutter von Loetzen et al., 2014).

An important member of the PR-10 allergens is the major hazel allergen Cor a 1 from *Corylus avellana*, which also contributes to PFS since IgE antibodies against Bet v 1 can cross-react with Cor a 1. Natural PR-10 allergens like Bet v 1 and Cor a 1 exist as a mixture of different isoallergens (> 67 % amino acid sequence identity) and variants (> 90 % amino acid sequence identity) (Pomés et al., 2018). So far eight Cor a 1 isoallergens, termed Cor a 1.01 to Cor a 1.08 as well as three Bet v 1 isoallergens have been identified (Breiteneder et al., 1993; Lüttkopf et al., 2002; Hendrich et al., 2024;) (WHO/IUIS Allergen Nomenclature Sub-Committee, <https://allergen.org>). Understanding the structural characteristics of PR-10 allergens like Cor a 1 and their interactions with various ligands is critical for explaining their biological roles in plants and their allergenic properties in humans. While the major allergenic IgE epitopes on Cor a 1 are conformational, ligand binding could change the structural stability of the proteins or could induce conformational changes that modulate IgE recognition.

LC-MS profiles of methanolic extracts of the male flowers of *C. avellana* revealed a variety of different compounds, including diarylheptanoids as well as glycosylated flavonoids (Cerulli et al., 2018; Masullo et al., 2016). Research has shown that phenolic compounds like flavonoids are abundant in different hazel tissues, including kernels and flowers. Many of them are thought to have biological activities, such as antioxidants, UV protecting or antimicrobial agents, suggesting an important role in human nutrition and health. They are also involved in the plant's defence reactions that depend on the different sugar residues. However, to date none of these compounds have been identified as ligands of Cor a 1 proteins (Bottone et al., 2019).

Although different small molecules are bound by PR-10 allergens in vitro, so far only two natural ligands have been identified: the glycosylated flavonoid derivative quercetin-3-O-sophoroside (Q3OS) was identified as a natural ligand of the isoallergen Bet v 1.0101, and quercetin-3-O-(2'-O- β -D-glucopyranosyl)- β -D-galactopyranoside (Q3O-(Glc)-Gal) was shown to be a natural ligand of the Cor a 1 isoallergen Cor a 1.0401 (Jacob et al., 2019; Seutter von Loetzen et al., 2014). Interestingly, the isoallergens Bet v 1.0101 and Cor a 1.0401 each selectively bind their own ligand, even though the two ligands differ only in the orientation of one OH-group in the sugar moiety (i.e. glucose vs. galactose) (Jacob et al., 2019; Seutter von Loetzen et al., 2014). Furthermore, several Bet v 1.01 variants (> 90 % amino acid sequence identity) do not bind Q3OS (Seutter von Loetzen et al., 2015). Since non-glycosylated flavonoids bind rather promiscuously to PR-10 allergens, it has been suggested that the sugar moieties are responsible for the specificity of binding (Chruszcz et al., 2021).

In a recent study we identified new Cor a 1 isoallergens and determined their localization in different plant tissues (Hendrich et al., 2024). In this work, we identified new glycosylated flavonoids extracted from natural Cor a 1 and from hazel pollen by high resolution mass spectrometry and nuclear magnetic resonance (NMR) experiments and analysed the binding properties of these compounds to the known Cor a 1 isoallergens. $^{13}\text{C}/^{15}\text{N}$ labelled Cor a 1.0401 and Cor a 1.0501 were used to identify relevant amino acid residues of the proteins for ligand binding by NMR filtered/editing nuclear Overhauser enhancement spectroscopy (NOESY) experiments.

2. Materials and methods

2.1. Cor a 1 purification

Expression and purification of recombinant (r)Cor a 1 proteins from *Escherichia coli* (*E. coli*) and purification of natural (n)Cor a 1 from hazel pollen extract was performed as previously described (Hendrich et al., 2024). The genes for Cor a 1.0401-G138S and Bet v 1-S137A were received from Eurofins Genomics (Ebersberg, Germany) and cloned into the expression vector pET-GB1a (Gunter Stier, EMBL, Heidelberg, Germany) via Gibson cloning, resulting in an N-terminal 6His-GB1a fusion of the proteins which can be cleaved off by tobacco etch virus (TEV) protease. Expression and purification was done as described for the other Cor a 1 proteins (Hendrich et al., 2024).

To obtain the protein Cor a 1.0401-3CS harbouring three amino acid exchanges: Cys5 > Ser, Cys83 > Ser and Cys161 > Ser, the corresponding wild type gene of plasmid pET11a-Cor a 1.0401 was subjected to site directed mutagenesis. The protein was purified as described (Fig. S1) (Jacob et al., 2019).

To obtain recombinant ^{15}N or $^{15}\text{N}/^{13}\text{C}$ labelled proteins, *E. coli* strains were grown in M9 minimal medium supplemented with $(^{15}\text{NH}_4)_2\text{SO}_4$ (1.5 g/L), or $(^{15}\text{NH}_4)_2\text{SO}_4$ and ^{13}C -glucose (2 g/L) (Jacob et al., 2019; Seutter von Loetzen et al., 2014) and purified accordingly. A purity of >95 % for the recombinant labelled and unlabelled proteins and of around 80 % for nCor a 1 was achieved (Fig. S1).

Purification of nCor a 1 was performed as previously described for nBet v 1 and nCor a 1 (Hendrich et al., 2024) For further analysis the fraction obtained by size exclusion chromatography (SEC) containing the nCor a 1/ligand complex was desalted using a PD-10 desalting column (Merck, Germany) and lyophilised. The sample was resolved in methanol, centrifuged and analysed by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS).

2.2. UHPLC/QTOF-MS

The flavonoid glycosides were extracted as previously described (Harbart et al., 2023) with the following modification that the samples were redissolved in 50 μL methanol/water (1:9, v/v).

The hazel pollen extracts were separated by an Agilent 1260 Infinity II HPLC equipped with an Ascentis® Express F5 column (150 mm $\times$ 4.6 mm, 5 mm, Supelco, Sigma Aldrich Chemical Co., St Louis, MO, USA). Compounds were eluted using solvent A: 0.5 % acetic acid and solvent B: acetonitrile in gradient mode. The flavonoid glycosides were detected using a photodiode array detector at wavelength 370 nm as well as an Agilent 5646 LC/Q-ToF (Agilent Technologies, Waldbronn, Germany). The QTOF system was equipped with a DUAL AJS ESI source (Agilent Technologies, Waldbronn, Germany) and the ions were detected in negative polarity (m/z 70–3200), vaporized at 350 °C at a drying gas flow of 8 L min^{-1} and nebulizer at 35 psi. The Vcap voltage was set to 3500 V and the fragmentor 175.

Additionally, to confirm the presence of quercetin 3-O- β -D-galactopyranoside (Q3OGal) in the hazel pollen extract, quercetin 3-O- β -D-glucopyranoside (Q3OGlc), Q3OGal, and the pollen extract were analysed using a Vanquish HPLC coupled to an Orbitrap Exploris 120 instrument (Thermo Fisher Scientific GmbH, Bremen) equipped with a H-EIS ion source. The chromatographic conditions were unchanged, and the source parameters were as follows: neg ionisation mode (m/z 100–1000, Res. 60,000), 2550 V, sheath gas 60 Arb, Aux gas 15 Arb, sweep gas 2 Arb and the ion transfer tube as well as the vaporizer temperatures were set to 350 °C.

2.3. Preparative purification of Q3O-(Glc)-Gal, K3OS and K3O-(Glc)-Gal by reversed phase HPLC

To obtain the potential ligands hazel pollen was extracted similarly

to the procedure previously described for the extraction of flavonoids (Jacob et al., 2019). In brief, pollen was dissolved in 19 % acetonitrile, 1 % acetic acid and mixed by vortex mixer. After centrifugation at 17900 g for 10 min, 100 μ L of the supernatant was isocratically purified by reversed phase (RP)-HPLC using an SP260/21 Nucleosil 100–7 C18 column (87 mL) (Macherey-Nagel, Düren, Germany) at a flow rate of 10 mL/min. The elution profile was analysed using the absorbance values at 255, 283 and 350 nm. Fractions containing Q3O-(Glc)-Gal or kaempferol-3-O- β -D-sophoroside (K3OS) and kaempferol-3-O-(2"-O- β -D-glucopyranosyl)- β -D-galactopyranoside (K3O-(Glc)-Gal) were lyophilized and resolved in 100 % d_6 DMSO. 1D 1 H NMR spectra and 13 C- heteronuclear single quantum coherence (HSQC) spectra were recorded on a 700 MHz NMR BRUKER Avance spectrometer at 25 °C to identify the molecules.

For ligand identification in the hazel pollen extract by UHPLC/QTOF-MS, for isothermal titration calorimetry (ITC), and for NMR experiments commercially purchased quercetin-3-O- α -L-rhamnoside (Q3OR), kaempferol-3-O- α -L-rhamnoside (K3OR), Q3OGlc, K3OS (BIOZOL Diagnostica in Eching, Germany) and Q3OGal (Avantor VWR) was used.

2.4. NMR spectroscopy

All NMR spectra were recorded at on Bruker Avance II+ 600 MHz, Avance IIIHD 700 MHz, Avance IIIHD 900 MHz, and Avance IIIHD 1000 MHz spectrometers, the latter three equipped with cryogenically cooled triple-resonance probes.

Small molecules were analysed by one-dimensional 1 H and two-dimensional 1 H, 13 C HSQC experiments in 2 H₂O or d_6 -DMSO. Proteins for NMR spectroscopy were dissolved in 10 mM Na-phosphate pH 7.5, 50 mM NaCl and 10 % 2 H₂O (NMR buffer).

For ligand screening a ligand excess of at least fivefold was chosen, as binding affinities in the range of approximately 100–200 μ M were expected. According to the law of mass action, the following eq. $AB = A_0 \times B / (K_D + B)$, with A_0 = total protein = 60 μ M; B = ligand = 300 μ M; was used and a K_D = 100 μ M for the ligand was assumed. Solving the equation for AB , at a dissociation constant (K_D) of 100 μ M, 45 μ M or 75 % of the protein is in complex with the ligand, meaning that chemical shift changes should be visible.

For protein resonance assignment standard double and triple resonance through-bond experiments were recorded (Sattler et al., 1999). Chemical shift perturbations were detected by 1 H, 15 N HSQC experiments during stepwise titration of potential ligands (30 mM stock solution in d_6 -DMSO) to uniformly 15 N labelled protein samples in NMR buffer. Reference titrations by adding d_6 -DMSO (without ligand) to protein samples were used to observe chemical shift perturbations caused by DMSO only. Chemical shift changes of amino acid residues being in the fast exchange upon ligand titration were used to determine K_D -values by fitting the two-state binding model to the changes of chemical shifts during the titration using in house written MATLAB routines.

Intermolecular NOEs were observed with 15 N/ 13 C-labelled protein (300 μ M) samples in 2 H₂O based NMR buffer with 10–15 fold excess of potential ligand using three-dimensional 13 C-edited/ 13 C-filtered NOE experiments (Zwahlen et al., 1997). To identify intraprotein NOE cross signals resulting from incomplete 13 C labelling or incomplete 13 C filtering the experiment was also recorded using a protein sample without ligand. NMR data were processed using TopSpin (Bruker) or in-house routines and visualised with TopSpin or NMRViewJ (Johnson & Blevins, 1994) (OneMoon Scientific, Inc.).

2.5. Isothermal titration calorimetry

ITC experiments were carried out on a NanoITC (TA Instruments, USA). Proteins were dialysed against 10 mM Na₂HPO₄/NaH₂PO₄, 50 mM NaCl, pH 7.5. Ligands were dissolved in the same buffer at the

following final concentrations of the stock solutions: Q3OR, 3.3 mM; K3OR, 1.5 mM; Q3OGlc, 1.3 mM, and Q3OGal, 1.3 mM. All samples were centrifuged at 17900 g for 10 min and degassed. The initial protein concentration in the calorimeter cell was 320–340 μ M in a volume of 400 μ L. The thermogram was recorded at 25 °C using 2.0 μ L injection volume of the ligand solution, an injection interval of 200 s and a stirring rate of 300 rpm. Buffer titrations were used as a blank and subtracted from the measurements. To determine K_D -values, curves to the data sets were fitted with an independent binding model using the program NanoAnalyze Data Analysis Version 4.0.0.4 (TA Instruments, USA).

2.6. Bioinformatic tools

Pairwise sequence alignments were performed with EMBOSS needle and multiple sequence alignments with Clustal Omega (Madeira et al., 2019) and by SnapGene software (from Insightful Science; available at snapgene.com).

2.7. Complex structure model

The HADDOCK 2.4 web server was used to dock Q3OR and Q3OGlc to Cor a 1.0501 and Q3O-(Glc)-Gal to Cor a 1.0401 (Honarato et al., 2021, 2024). The structure and PDB file of Cor a 1.0501 were generated by AlphaFold2 (Jumper et al., 2021). For Cor a 1.0401 the PDB file 6GQ9 was used. The SMILES codes of the ligands were generated by ChemDoodle 6.0 (Todsén, 2014) and their PDB files were generated with NovoPro (NovoPro Bioscience Inc). The structure of Q3OR, Q3OGlc and Q3O-(Glc)-Gal (Table S1) was energy optimised with the program Avogadro 1.2.0 (Hanwell et al., 2012). For Cor a 1.0501 the amino acid residues 23, 24, 31, 35, 38, 57, 59, 61, 63, 68, 70, 82, 84, 101, 117, 121, 137, 138, 140, 144, were used. For Cor a 1.0401 the residues 141, 145, 84, 101, 103, 70, 86, 56, 36, 40, 42, 59, 37, 138, 139, 31, 142, 24, 23, 57, 116, 105, 82, 118 were selected as active residues, as these form the binding pocket and its entrance. For distance restraints used for HADDOCK (Table S2), the carbon atoms of the corresponding amino acid residues and the corresponding protons of the ligands derived from the NOESY experiments were used and set between 5 and 8 Å. For clustering the RMSD value was used with a cutoff of 2.5 Å.

3. Results and discussion

3.1. Identification of potential Cor a 1 ligands by mass spectrometry

We have recently shown that there exist a lot more Cor a 1 isoallergens and variants than previously known (Hendrich et al., 2024). So far eight Cor a 1 isoallergens have been identified which could be detected in different plant tissues (Breiteneder et al., 1993; Hendrich et al., 2024; Lüttkopf et al., 2002). In addition, the glycosylated flavonoid Q3O-(Glc)-Gal was found to be a natural ligand for the isoallergen Cor a 1.0401 (Jacob et al., 2019). Moreover, it has been demonstrated that the binding of flavonoids to Bet v 1.01 is variant dependent (Seutter von Loetzen et al., 2015). Bound ligands are thought to stabilize allergens and to enhance the immune reaction (Chruszcz et al., 2021). Therefore, identifying potential interaction partners for isoallergens is crucial for understanding and potentially mitigating the allergenic properties of Bet v 1 and Cor a 1.

To further elucidate the binding properties of Cor a 1 isoallergens to glycosylated flavonoids, we set out to identify new ligands and characterise their binding specificities. Thus, natural (n)Cor a 1, which is in principle a mixture of different ligand-bound Cor a 1 isoallergens, was isolated from hazel pollen extract and analysed by UHPLC/QTOF-MS. For comparison, the same procedure was performed with hazel pollen extract, i.e. without prior nCor a 1 purification, which showed the presence of the same potential ligands. Among other substances, such as caffeoyl-spermidine (data not shown), several glycosylated flavonoid derivatives were identified (Fig. 1; Fig. S2, S3) and their binding

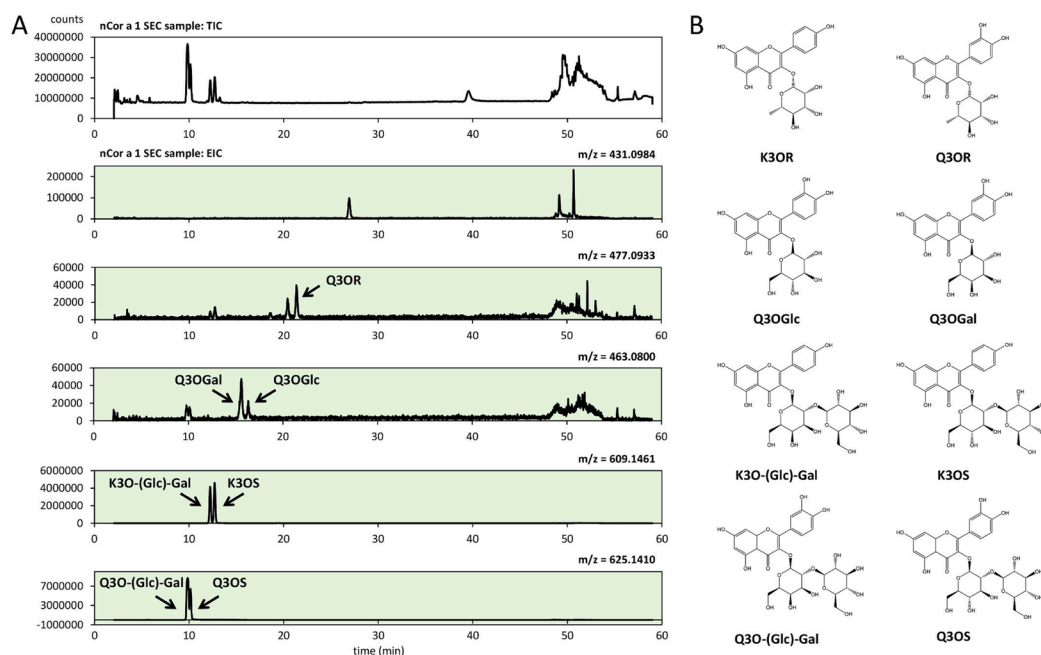


Fig. 1. UHPLC/QTOF-MS. **A)** Total ion count (TIC) chromatogram of nCor a 1 after SEC and extracted ion count of ligand specific calculated mass (green background). **B)** Corresponding molecular structure of identified ligands. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

properties further analysed. Determination of the accurate masses, as well as characteristic fragmentation patterns and chromatographic behaviour was used for identification are summarized in Table 1.

To confirm the identity of the compounds the extracted ion count (EIC) for the specific mass $[M-H]^-$ of the corresponding commercially available flavonoid derivatives was used. Q3O-(Glc)-Gal was purified as described (Jacob et al., 2019). K3O-(Glc)-Gal was not commercially available. Preparative purification from the pollen extract by reversed-phase HPLC yielded a mixture of the two compounds K3O-(Glc)-Gal and K3OS, which were clearly identifiable by NMR with a 1H , ^{13}C HSQC (Fig. S4). Since separation of the two compounds failed, only K3OS was used in our studies. The monosaccharide flavonoid derivatives K3OR, Q3OR and the disaccharide flavonoid derivatives K3OS, as well as the already known binding ligands (Jacob et al., 2019; Seutter von Loetzen et al., 2014; Strack et al., 1984) Q3OS and Q3O-(Glc)-Gal were clearly identifiable (Fig. 1, Table 1; Fig. S2, S3).

The EIC and fragmentation patterns (Fig. 1, Fig. S2, S3) indicated the presence of Q3OGlc ($m/z = 463.08$) or quercetin-3-O- β -D-galactopyranoside (Q3OGal) ($m/z = 463.08$), since a quercetin fragment ($m/z =$

300.02) is formed during the fragmentation. Since glucose and galactose have identical masses, resulting in the same m/z of glycosylated quercetin, they cannot be distinguished based on their spectra. However, additional analyses with Q3OGlc, Q3OGal, and the hazel pollen extract using a Vanquish HPLC coupled to an Orbitrap confirmed the presence of Q3OGal in the extract (Fig. S3-F1, S3-F2). In addition, Q3OGlc was previously shown to be present in male hazel flowers and nut (Cerulli et al., 2018; Masullo et al., 2016).

3.2. Chemical shift assignment of Cor a 1.0501 and titrations with potential ligands

Structural changes induced by the binding of polyphenols/flavonoids can alter the allergenic potential and modulate the immune response as has been shown in previous (model) studies for allergenicity e.g. of the birch pollen allergen Bet v 1, peanuts or milk proteins (Chruszcz et al., 2021; Plundrich et al., 2017; Wu et al., 2018). Therefore, binding studies are necessary for understanding allergenicity and potentially provide a basis for reducing the allergenicity of foods.

Table 1
Corresponding physical data from Fig. 1.

Compound	Formula	Calculated $[M-H]^-$ (m/z)	Measured $[M-H]^-$ (m/z)	Error (ppm)	Retention time (min)	Qualifier fragment ion (m/z)
Q3O-(Glc)-Gal	$C_{27}H_{30}O_{17}$	625.14102	625.1429	3.007321	9.851	300.0287, 178.9987
Q3OS	$C_{27}H_{30}O_{17}$	625.14102	625.1429	3.007321	10.149	445.0781, 300.0287, 178.9989
K3O-(Glc)-Gal	$C_{27}H_{30}O_{16}$	609.14611	609.1457	-0.673073	12.240	429.0853, 284.0350
K3OS	$C_{27}H_{30}O_{16}$	609.14611	609.1457	-0.673073	12.714	429.0834, 284.0336, 178.9992
Q3OGal	$C_{21}H_{20}O_{12}$	463.0882	463.0888	1.295650	15.604	300.0276
Q3OGlc	$C_{21}H_{20}O_{12}$	463.0882	463.0893	2.375357	16.332	300.0284
Q3OR	$C_{21}H_{20}O_{11}$	447.0933	447.0935	0.447334	21.332	300.0286
K3OR	$C_{21}H_{20}O_{10}$	431.0984	431.0981	-0.695897	26.943	284.0325

Circular dichroism (CD) melting curves performed previously exhibited that Cor a 1.0501 is a very stable protein with a high melting point ($T_m = 84.32^\circ\text{C}$) (Hendrich et al., 2024). Thus, this isoallergen was chosen for backbone assignment by NMR analysis followed by titrations with the putative ligands. An NMR spectrum correlating resonance frequencies of amide protons and directly bonded ^{15}N -labelled nitrogen atoms (2D ^1H , ^{15}N HSQC, heteronuclear single quantum correlation) allows the individual detection of peptide backbone amide signals. Since each signal represents a single amino acid of the peptide chain, such a spectrum provides information about the folding state of a protein. The position of each signal in the spectrum depends on the chemical environment, which is different for each amino acid in a folded protein. The corresponding ^1H , ^{15}N HSQC spectrum (Fig. S5) of Cor a 1.0501 shows that the residues are well dispersed (6.9–9.7 ppm) indicating a folded protein with a defined structure. 93 % of the residues could be assigned (Fig. S5A, B). Formation of secondary structures in proteins causes systematic deviations of backbone chemical shifts from random coil values in folded proteins. The direction of deviation of the chemical shifts relative to the random coil values is expressed as Chemical Shift Index (CSI) and allows the determination of sequence specific secondary structure elements. Upfield chemical shift changes (lower resonance frequencies, lower ppm values) of the α -protons (HA; negative CSI values) and downfield chemical shift changes for the α - and carbonyl carbons (CA, CO, positive CSI value) can be observed for helical secondary structures, whereas β -strands show the opposite direction of chemical shifts. The CSI values of Cor a 1.0501 show secondary structure elements whose arrangement corresponds well to that of other PR-10-like proteins (Fig. S5C). Furthermore, a 3D model of Cor a 1.0501 was created with AlphaFold 2 (Jumper et al., 2021) (Fig. S5D). The model structure obtained is consistent with known structures of PR-10 proteins (Chruszcz et al., 2021; Fernandes et al., 2013; Gajhede et al., 1996; Jacob et al., 2019; Seutter von Loetzen et al., 2012; Seutter von Loetzen et al., 2014). The amphiphilic cavity formed by the structural elements is large enough to accommodate small ligands such as glycosylated flavonoids. (Jacob et al., 2019).

Titration of a ligand results in chemical shift changes of affected amino acids since ligand binding changes their chemical environment. The putative ligand Q3OR was titrated to Cor a 1.0501 and ^1H , ^{15}N HSQC

spectra were recorded for each titration step. An overlay of the ^1H , ^{15}N HSQC spectra shows the chemical shift changes induced by binding of Q3OR (Fig. 2A).

To reveal the residues involved in ligand binding, the 3D model of Cor a 1.0501 created with AlphaFold2 (Fig. S5D) was used (Fig. 2B). The residues affected by ligand binding and showing intermediate or fast exchange in the NMR titration experiments are highlighted in red and orange, respectively (Fig. 2B).

Titrations with the monosaccharide derivatives K3OR, Q3OGal, and Q3OGlc, as well as the disaccharide derivatives Q3OS, K3OS and Q3O-(Glc)-Gal were performed (Fig. S6). While the monosaccharide flavonoids showed binding, none of the disaccharide derivatives tested bound to Cor a 1.0501. Moreover, the binding affinities of the monosaccharide flavonoids were determined by measuring the K_D -values of several amino acid residues undergoing fast exchange during ligand titration. The average values of these measurements for each ligand were in a range of $86\ \mu\text{M}$ to $348\ \mu\text{M}$ (Fig. S6). Since only the amino acid residues being in the fast exchange can be measured, less accurate and usually higher K_D -values are expected than if a method that considers the overall binding affinity is used.

To confirm ligand binding, isothermal titration calorimetry (ITC) was carried out (Fig. S7). Although this method is not ideal for K_D -values above $100\ \mu\text{M}$, it was used because fluorescence titrations could not be performed due to the lack of tryptophan in the protein. Measurements were performed using the same pH conditions as for the NMR experiments. The K_D -values determined with the four compounds, Q3OR, K3OR, Q3OGlc and Q3OGal, are all quite similar, ranging from ca. 70 to $170\ \mu\text{M}$ and comparable to the average values obtained by NMR (Fig. S6, S7).

3.3. Ligand screening with Cor a 1 isoallergens

Previous studies have shown that the isoallergen Cor a 1.0401 binds of Q3O-(Glc)-Gal specifically (Jacob et al., 2019). To gain deeper insight into the binding specificities of Cor a 1 isoallergens, the ^{15}N -labelled Cor a 1 isoallergens 1.0101, 1.0302, 1.0401, 1.0501, 1.0601, 1.0701 and 1.0801 were purified from *E. coli* as already described (Hendrich et al., 2024). Cor a 1.02 could not be expressed in a soluble form in *E. coli*

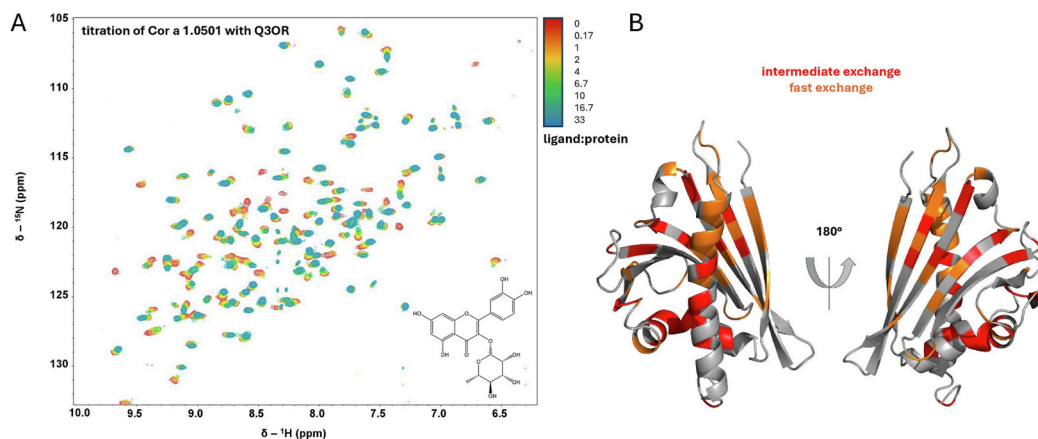


Fig. 2. Binding of Cor a 1.0501 to Q3OR. A) ^1H , ^{15}N HSQC spectrum of the titration of ^{15}N -labelled Cor a 1.0501 with Q3OR. Colour coding indicates the ligand: protein ratio as shown by the bar at the bottom right of the ^1H , ^{15}N HSQC spectrum. Measurement was performed with $60\ \mu\text{M}$ ^{15}N -Cor a 1.0501 at 298 K in 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, 50 mM NaCl at pH 7.5, 10 % $^2\text{H}_2\text{O}$ with Bruker Avance 900 MHz spectrometer. (The titrations of the other ligands are shown in the supplement in Fig. S6) B) 3D Model of Cor a 1.0501 created with Alpha Fold 2. The residues that showed intermediate or fast exchange during the titration are highlighted in red and orange, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Hendrich et al., 2024) and could therefore not be included in the analyses. For initial screening experiments only one ligand concentration was added to 60 μM of a ^{15}N -labelled isoallergen to find chemical shift events in the ^1H , ^{15}N HSQC spectra indicative of binding (Table 2, Fig. S8). Based on the results obtained above (see Figs. S6 and S7), a ligand excess of at least fivefold was chosen, as binding affinities in the range of approximately 100 μM to 200 μM were expected. According to the law of mass action, at a K_D of 100 μM , 75 % of the protein is in complex with the ligand, meaning that chemical shift changes should be visible.

The ^1H , ^{15}N HSQC spectra revealed binding of flavonoid monosaccharide derivatives by all Cor a 1 isoallergens except Cor a 1.0801. However, this is not surprising, as the potential ligands were isolated from pollen and Cor a 1.0801 was shown to be present only in immature nuts, and not in pollen (Hendrich et al., 2024). All the other isoallergens bound to Q3OGlc, Q3OGal, and K3OR. Only Cor a 1.0701 was not able to bind Q3OR (Table 2, Fig. S8). The flavonoid disaccharides exhibited greater differences in binding, indicating a higher specificity: Binding of Q3O-(Glc)-Gal to Cor a 1.0401 has previously been shown (Jacob et al., 2019). In addition, it was also bound by Cor a 1.0601 and 1.0701. These isoallergens are weakly expressed in hazel pollen (Hendrich et al., 2024).

None of the Cor a 1 isoallergens bound to the flavonoid sophorosides Q3OS and K3OS (Table 2; Fig. S8). In contrast, these compounds were bound by Bet v 1.0101 from birch pollen, which was used as a control (Table 2; Fig. S8). As already known, Bet v 1 binds specifically to the sophoroside Q3OS but not to Q3O-(Glc)-Gal. (Jacob et al., 2019).

To further investigate the binding properties and to identify specific residues responsible for ligand binding Cor a 1.0501 was selected for further analysis because we had shown in a previous publication that it is a particularly stable protein (Hendrich et al., 2024), with clear, easily assigned signals in the HSQC spectrum (Fig. S5).

3.4. Isotope edited/filtered nuclear Overhauser enhancement spectroscopy

To gain a better understanding of the different binding behaviours of mono- and diglycosylated flavonoids, further NMR binding studies were conducted. Although chemical shift changes in the protein can be used to show which amino acid residues are affected by ligand binding, this information was not sufficient to determine the orientation of the ligand by modelling. Since the ligands bind in the amphiphilic pocket of the protein, many chemical shift changes are due to general conformational changes of the protein and thus the affected amino acid residues do not directly interact with the ligand.

Thus, to obtain a complex structure of Cor a 1.0501 and to demonstrate the orientation of a ligand in the amphiphilic cavity, isotope edited/filtered NOESY experiments were performed with Q3OGlc. Intermolecular NOE interactions are used for characterizing interfaces.

For two spatially close protons (distance less than 5 Å) an NOE interaction can be detected. In an isotope filtered/edited NOESY experiment the magnetic transfer of a ^{13}C bound proton of the protein to a ^{12}C bound proton of the ligand can be observed and gives information about the interaction of the protein with the ligand.

Using ^{13}C -labelled Cor a 1.0501 in complex with the ligand Q3OR two NOE signals, and with the ligand Q3OGlc, three NOE signals could be assigned to the ligand and to amino acid side chains of the protein (Fig. 3; Fig. S9). The aromatic 5' protons of Q3OR and of Q3OGlc show an NOE signal to the β protons of Ala 38, whereas the 4' protons of the sugar moiety of Q3OR and Q3OGlc show an NOE signal with the β proton of Ser 137. For Q3OGlc, also the 6' proton of the sugar moiety forms an NOE signal with the α proton of Lys138. In addition, ^1H , ^{13}C HSQC spectra of the complex were performed to confirm the signals derived from the ligand (Fig. S10).

This distance information of the NOESYs was then used to define distance restraints for docking the ligands Q3OR and Q3OGlc into the model structure (Fig. 4A, B) of Cor a 1.0501 using the HADDOCK 2.4 webserver (Honarato et al., 2021, 2024). The structure models of the Cor a 1.0501/Q3OR and Cor a 1.0501/Q3OGlc complexes (Fig. 4A, B) represent the interactions obtained by the NOE signals. The data show that the 3,4-dihydroxyphenyl ring of the quercetin moiety (labelled in green, Fig. 4A, B) of Q3OR and Q3OGlc is located at the entrance of the cavity with Ala 38 fixing the 5' proton of the 3,4-dihydroxyphenyl ring, whereas the sugar moiety (labelled in blue, Fig. 4A, B) points into the cavity, and its 4' protons interact with Ser 137 (Fig. 4A, B).

Since it was suggested that the sugar moieties of the flavonoid derivatives may be important for binding specificity (Chruszcz et al., 2021), and the NOESY and docking experiments indicated that Ser 137 is relevant for sugar binding, we mutated the corresponding residues in Cor a 1.0401 (G138S) and Bet v 1.0101 (S137A) (Fig. S11), as these two proteins exhibit different binding preferences for Q3O-(Glc)-Gal, which binds only to Cor a 1.0401, and Q3OS, which binds only to Bet v 1.0101 (Jacob et al., 2019). However, HSQC titrations with the ligands showed that Cor a 1.0401-G138S and Bet v 1-S137A were still able to bind to their ligands (Fig. S12).

In addition, docking experiments were performed with Cor a 1.0501 and Q3O-(Glc)-Gal, which was not bound by the protein in the experiments shown above, to determine the cause of this observation. Using binding of Ser 137 to the galactose moiety as a restraint, Q3O-(Glc)-Gal could not dock to Cor a 1.0501 in the same position and orientation as the monosaccharide derivatives Q3OR or Q3OGlc, suggesting that this is sterically impossible.

Since in contrast, Cor a 1.0401 and Bet v 1.0101 bind disaccharide flavonoid derivatives (Jacob et al., 2019; Seutter von Loetzen et al., 2014), but did show no effect of the mutations introduced above to disrupt binding, we speculated that mono- and disaccharide derivatives might bind in different orientations. Thus, we used the partial amino acid side chain assignment of Cor a 1.0401 in complex with the

Table 2
Summary of Cor a 1 isoallergens and ligand binding.

Ligands	Cor a 1							Bet v 1 0101
	0101	0302	0401	0501	0601	0701	0801	
Q3OGlc								
Q3OGal								
Q3OR								
K3OR								
Q3O-(Glc)-Gal								
Q3OS								
K3OS								

binding no binding (at least 5-fold excess of ligand).

Data are based on HSQC screening (Fig. S7) and are consistent with HSQC titrations (Fig. S6) and ITC experiments (Fig. S7).

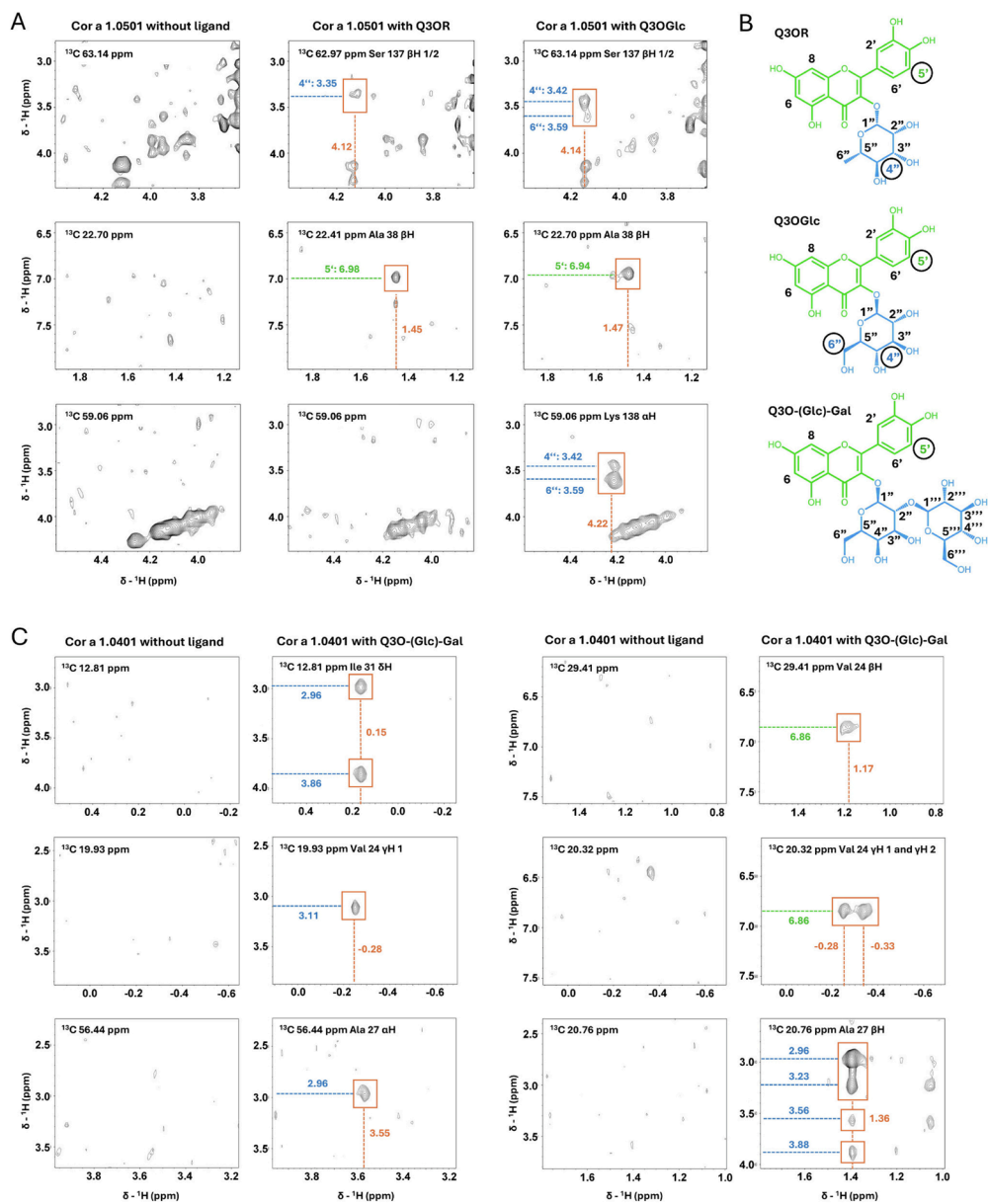


Fig. 3. Filtered/edited NOESYs of Cor a 1.0501 in complex with Q3OR or Q3OGlc, and Cor a 1.0401 in complex with Q3O-(Glc)-Gal. **A)** Filtered/edited NOESY of Cor a 1.0501 without ligand (left column), with Q3OR (middle column), and with Q3OGlc (right column). Spectra show a cross section in the corresponding ^{13}C level. NOE signals caused by the ligands are marked by orange boxes. The green and blue lines show the ^1H signals caused by the quercetin and sugar moieties of the ligand. The orange lines show the ^1H signals caused by the amino acid, respectively. **B)** Molecular structures of Q3OR, Q3OGlc and Q3O-(Glc)-Gal. The green and circled numbers represent the protons that build up the NOE with the protein (green and blue lines in A and C). **C)** Filtered/edited NOESY Cor a 1.0401 without ligand (left column), with Q3O-(Glc)-Gal (right column). Spectra show a cross section in the corresponding ^{13}C level. NOE signals caused by the ligands are marked by orange boxes. The green and blue lines show the ^1H signals caused by the quercetin and sugar moiety of the ligand. The orange lines show the ^1H signals caused by the amino acid, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

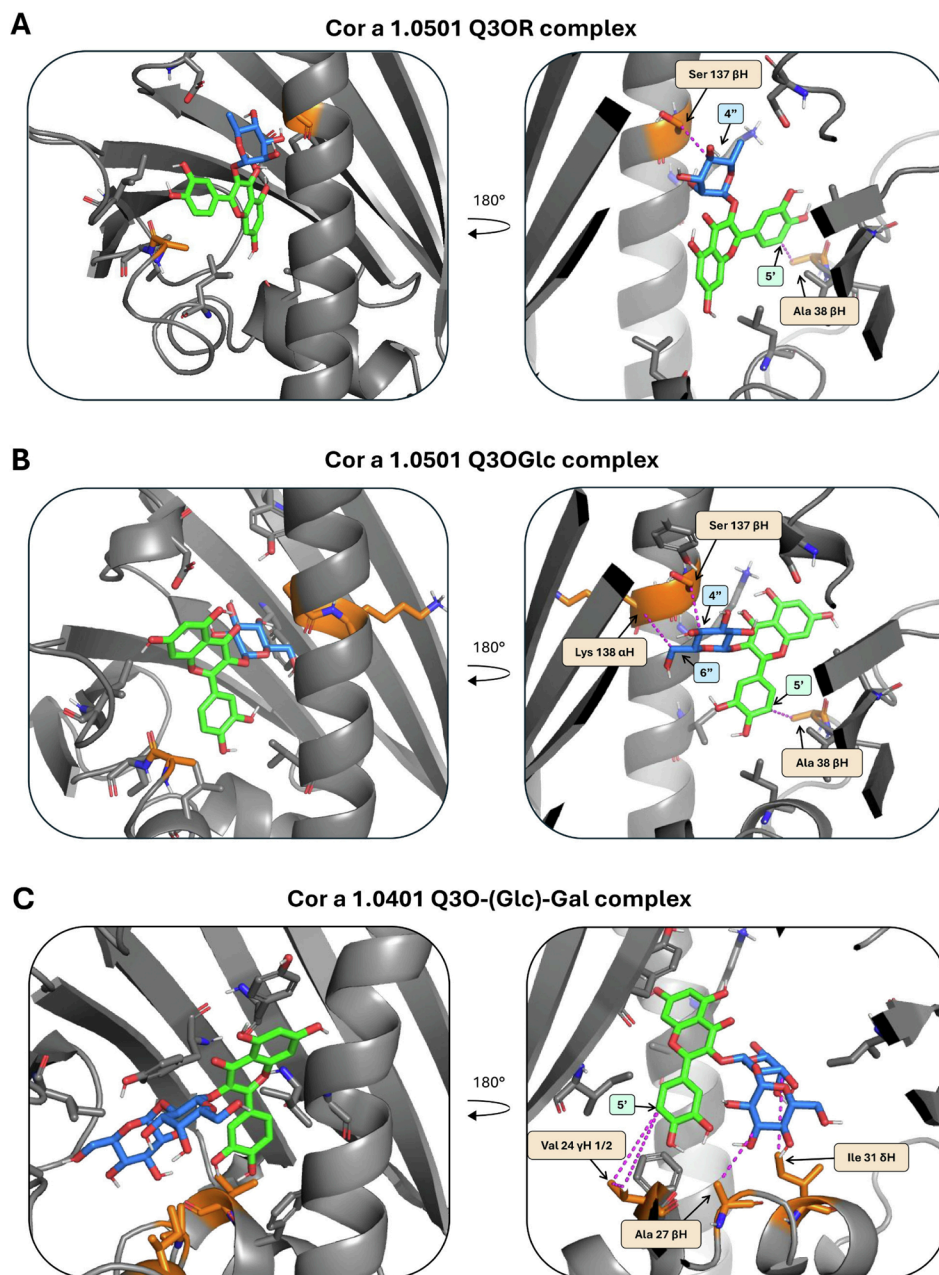


Fig. 4. Docking and Complex Structures.

Complex of Cor a 1.0501 with Q3OR (A) and Q3OGlc (B) and Cor a 1.0401-3CS with Q3O-(Glc)-Gal (C). The left side shows the entrance to the binding pocket and the right side shows the binding from the opposite side. The quercetin moiety is highlighted in green and the sugar moiety in blue. The orange amino acids form a NOE signal to the ligand. The grey amino acids are located in close proximity to the ligand. Magenta coloured lines connect the carbon atoms used for distance restraints. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

disaccharide flavonoid derivative Q3O-(Glc)-Gal (Table S3) and performed isotope edited/filtered NOESY experiments to clarify whether differences in binding could be detected. Since Cor a 1.0401 is prone to dimerisation, the mutant protein Cor a 1.0401-3CS, in which three surface accessible cysteines were replaced by serines, was used for the measurements. Partial assignment of the protein in complex with Q3O-(Glc)-Gal was performed to identify interacting amino acid side chains.

The NOEs obtained indicate interaction of the three protons of the Val24 side chain (Val 24 β -proton, and Val 24 γ -protons 1/2) of the protein with the quercetin moiety, and of four protons of the amino acid side chains Val24, Ala27 and Ile31 (Val 24 γ -proton; Ala 27 α -proton and β -proton; and Ile 31 δ -proton) with the disaccharide moiety of Q3O-(Glc)-Gal (Fig. 3C). These results strongly suggest a completely different binding mode for the disaccharide flavonoid, i.e. here the aromatic quercetin moiety is located deep in the cavity and the sugar moiety is situated at the entrance of the pocket (Fig. 4C).

4. Conclusion

Various glycosylated flavonoids could be identified by UHPLC/QTOF-MS and NMR analyses in pollen extracts and bound to nCor a 1, indicating that they are natural ligands. Screening analyses using ^1H , ^{15}N HSQCs revealed that Cor a 1 isoallergens exhibit different binding properties, suggesting different functions in the plant. Interestingly, Cor a 1.0801, which is not found in pollen but in the nut or flower does not bind any of the glycosylated flavonoids. Whereas Cor a 1.0501 was only able to bind to monosaccharide flavonoid derivatives, Cor a 1.0401 was also able to bind to disaccharide flavonoids. NMR NOESY experiments revealed that the sugar bound covalently to the flavonoid moiety is responsible for specific binding. Although Ser 137 located in the amphipathic cavity of the protein interacts with the 4' protons of the sugar when monosaccharide flavonoids like Q3OR or Q3OGlc are used, this is not the case when Q3O-(Glc)-Gal is bound. Docking experiments based on the data obtained by NMR NOESY analyses revealed for the first time that the orientation of the monosaccharide flavonoids in the complex is completely different to that of the bound disaccharide flavonoids: The sugar moiety of the monosaccharide flavonoid protrudes into the amphiphilic cavity, whereas in the case of the disaccharide flavonoid the sugar moieties are located at the entrance of the pocket. These findings provide valuable information on how to determine ligand binding specificity for different isoallergens. In the long term, these results may provide an important basis for elucidating the different functions of isoallergens and their corresponding ligands in plants and thus may help to reduce the allergenic potential of foods.

CRedit authorship contribution statement

Julian M. Hendrich: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Hatice Kara:** Validation, Investigation, Formal analysis. **Kristian Schweimer:** Writing – original draft, Formal analysis, Conceptualization. **Thessa P. Jacob:** Validation, Investigation, Formal analysis. **Mailke Schneider:** Validation, Investigation, Formal analysis. **Susanne Baldermann:** Writing – original draft, Validation, Supervision, Formal analysis, Conceptualization. **Birgitta M. Wöhr:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2025.102711>.

Data availability

NMR data are available from the Biomolecular Magnetic Resonance Data Bank (BMRB): BMRB ID: 52769 (Cor a 1.0401-3CS), 52770 (Cor a 1.0501-Q3OR), 52771 (Cor a 1.0501-Q3OGlc), 52772 (Cor a 1.0501).

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Appendix A. Supplementary Data

Figures

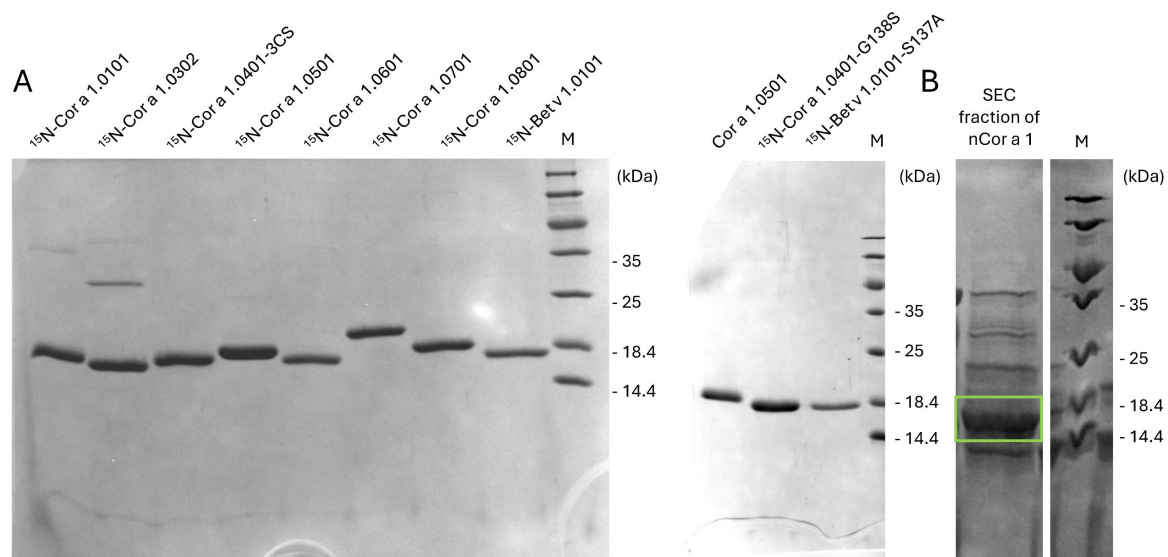


Figure S1. SDS polyacrylamide gel electrophoresis of purified proteins. A) Purified nonlabelled and labelled recombinant Cor a 1 and Bet v 1 proteins. B) Purified nCor a 1 used for fragmentation analysis. The band representing nCor a 1 is highlighted by a green box. Lanes M, molecular weight standard; the apparent molecular weights (kDa) are indicated on the right.

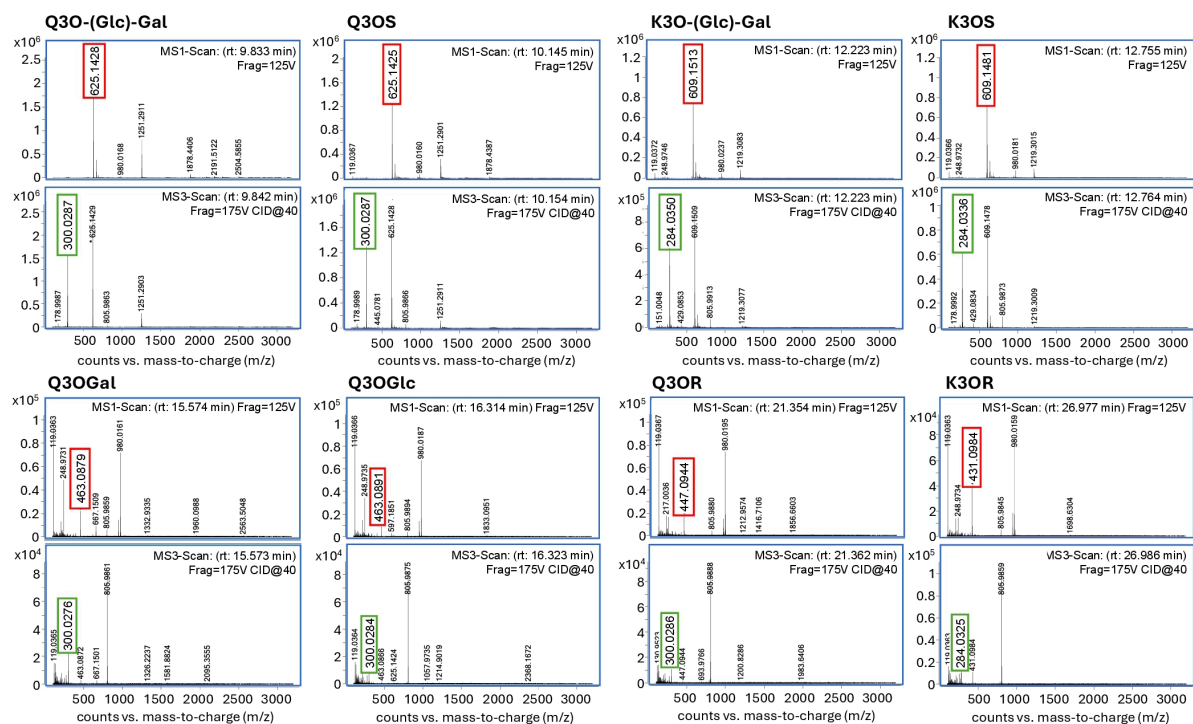


Figure S2. Fragmentation analysis of the nCor a 1/ligand complex. For each retention time of a ligand, the MS1 (upper MS spectrum) and the MS3 fragmentation (lower MS spectrum) are shown and refer to the EIC of purified nCor a 1 in Fig 1. For fragmentation a voltage of 175V and collision-induced dissociation (CID) of 40 eV was used. Red boxes: Mass of the corresponding ligand at its retention time. Green boxes: Quercetin or kaempferol after fragmentation of the ligand.

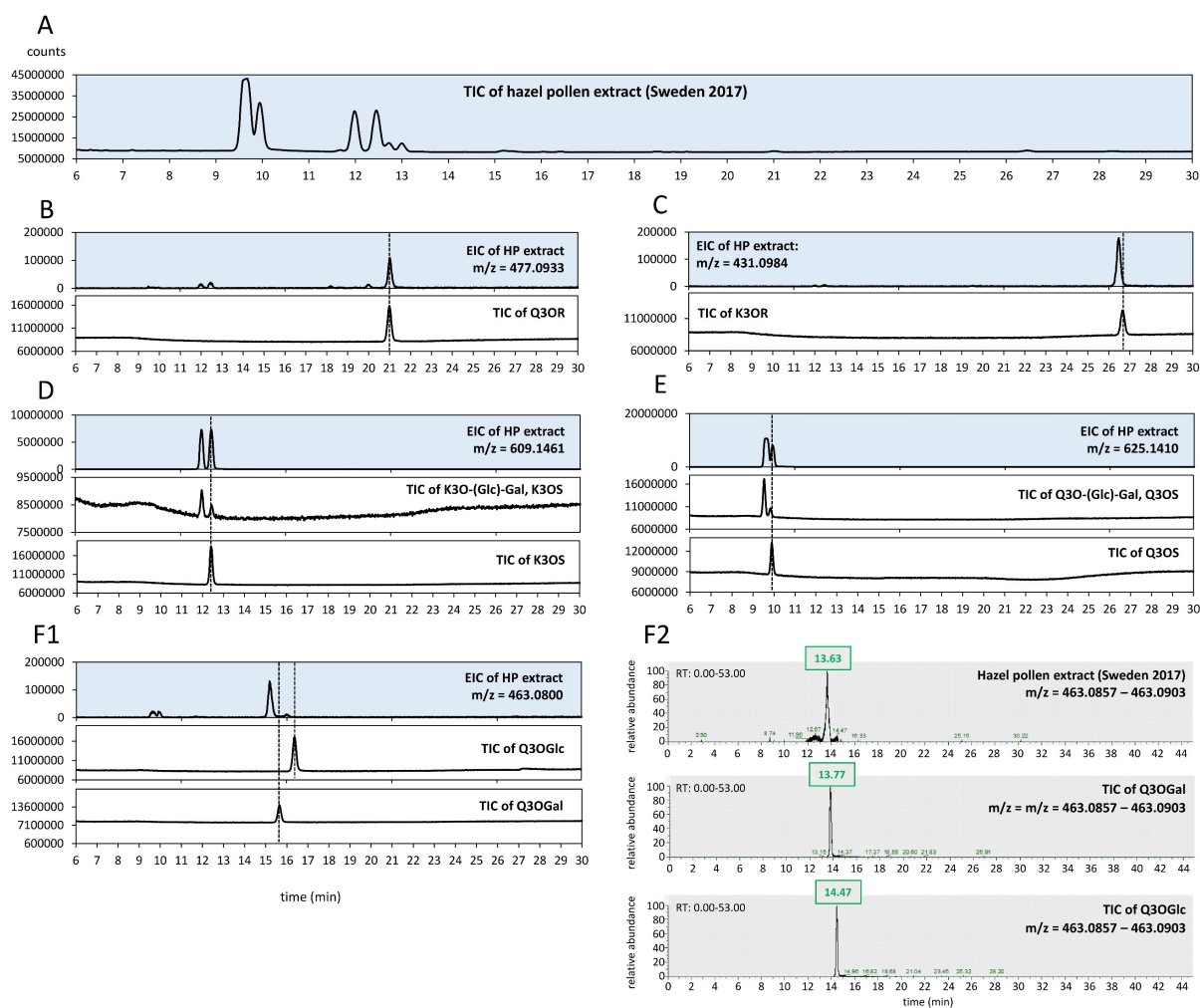


Figure S3. Identification of flavonoids. **A)** Chromatogram of the total ion count (TIC) of hazel pollen extract separated by UHPLC. **B)-F1)** Chromatograms of the extracted ion count (EIC) for the specific calculated mass $[M-H]^-$ of the individual ligands from the hazel pollen extract (upper chromatograms) aligned with the TIC of the pure ligands (lower chromatograms). The pure ligands were similarly separated by UHPLC and used as a standard to identify the ligands in the hazel pollen extract. **D) and E)** The mixture of K3O-(Glc)-Gal/ K3OS and of Q3O-(Glc)-Gal/ Q3OS was purified from hazel pollen by preparative HPLC. K3OR, Q3OR, Q3OGlc, Q3OGal, K3OS and Q3OS were purchased. **F2)** For ligand identification, hazel pollen extract, Q3OGlc and Q3OGal were additionally measured on a Vanquish HPLC coupled to an Orbitrap Exploris 120 instrument.

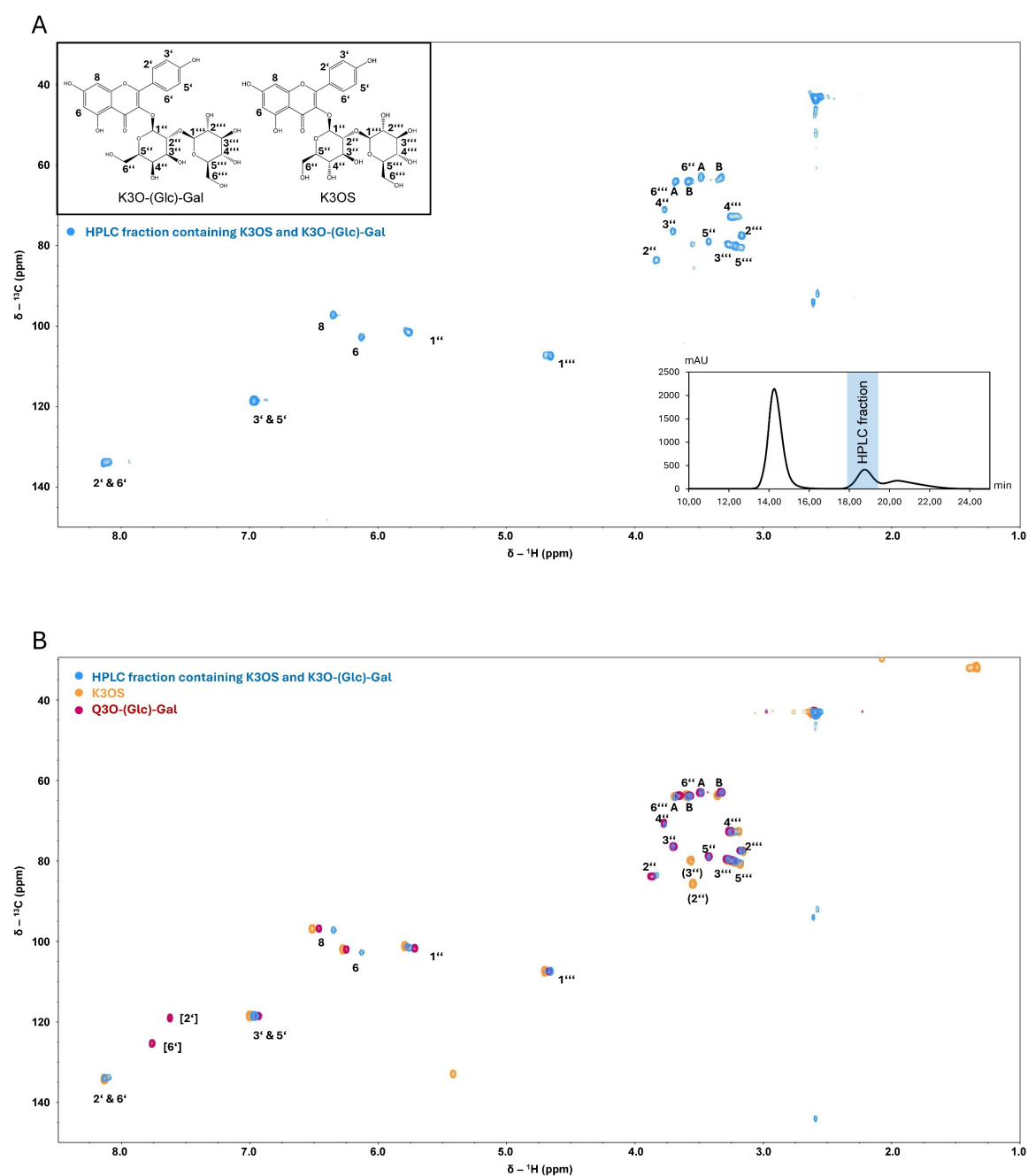


Figure S4. ^1H , ^{13}C -HSQC NMR spectrum of a purified HPLC fraction containing K3OS and K3O-(Glc)-Gal. The HPLC peak (blue box) was fractionated, lyophilised and resolved in d_6DMSO . **A)** The ^1H , ^{13}C -HSQC of that HPLC fraction (blue signals) was measured at 298K on a 700 MHz spectrometer. **B)** K3OS and K3O-(Glc)-Gal were identified by superimposing these spectra with the spectra of purchased K3OS (orange signals) and Q3O-(Glc)-Gal (red signals) purified from hazel pollen. Numbers [2'] and [6'] mark the peaks of Q3O-(Glc)-Gal, numbers (3'') and (2'') refer to the peaks of K3OS.

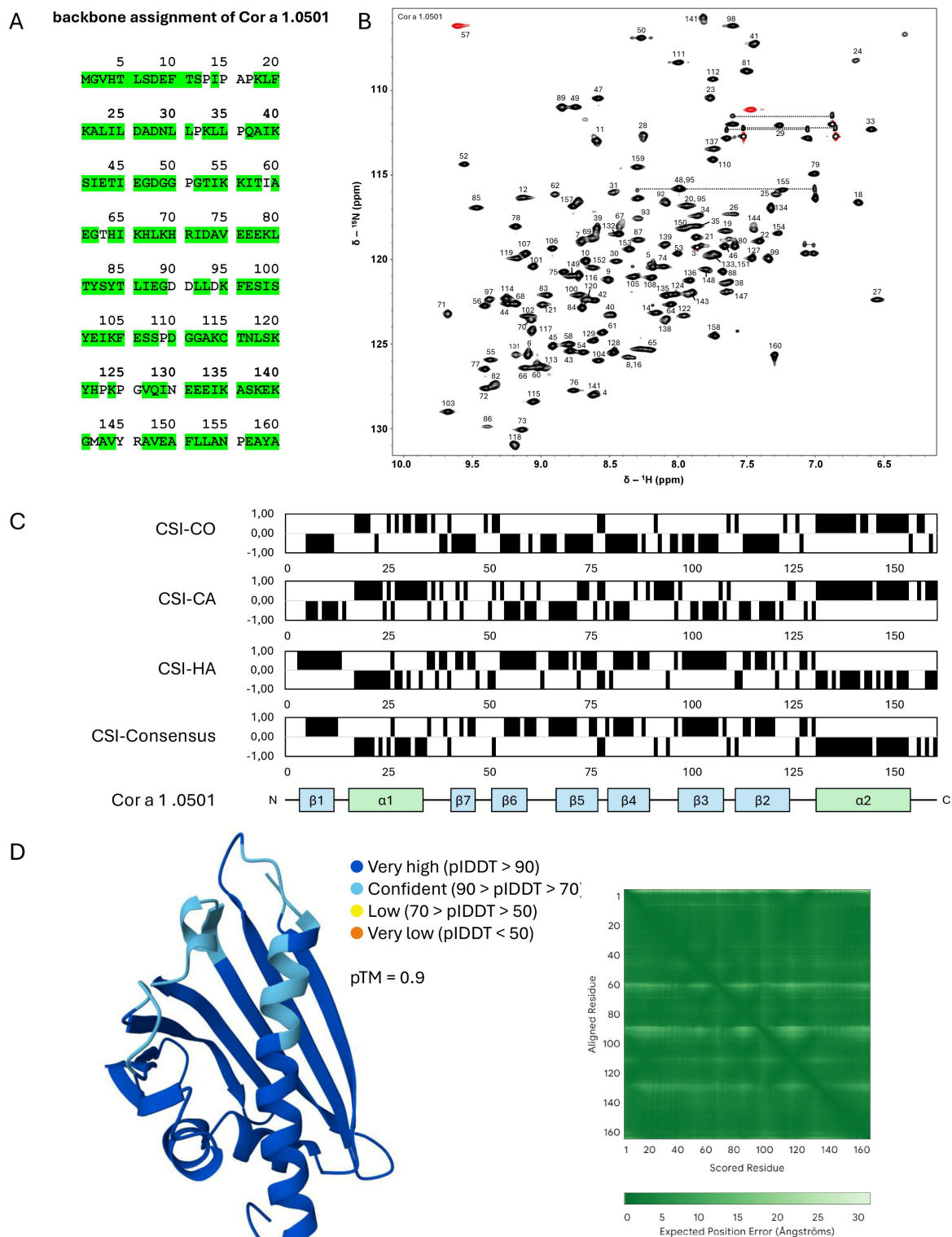
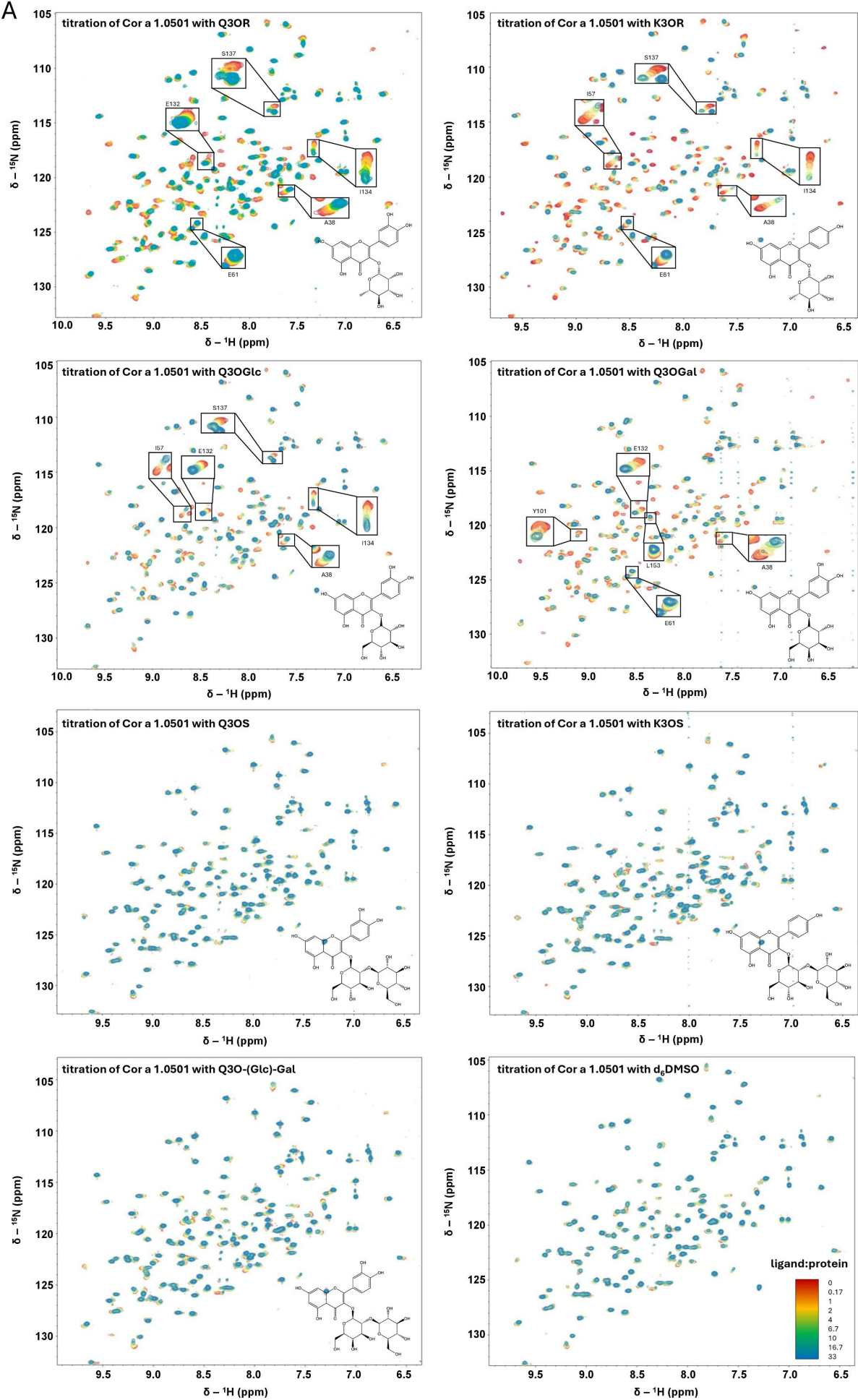


Figure S5. Backbone assignment of Cor a 1.0501 and structure prediction. **A)** Amino acid sequence of Cor a 1.0501. Assigned residues are highlighted in green. **B)** ¹H, ¹⁵N HSQC of 500 μ M uniformly ¹⁵N-labelled Cor a 1.0501. Amide proton resonances are labelled according to their residue numbers. Measurement was performed at 298 K in 10 mM Na₂HPO₄/NaH₂PO₄ buffer at pH 7.5, 10% ²H₂O, 1 mM DTT on a Bruker Avance 900 MHz spectrometer. **C)** Secondary chemical shift indices (CSI) for H α , C α , and CO nuclei, along with the consensus CSI combining all three nuclei. β -strands are represented by blue boxes and α -helical regions by green

boxes. **D)** AlphaFold 2 structural prediction. Dark blue and light blue highlight regions with very high and high confidence, respectively, based on the predicted local Distance Difference Test (pLDDT) scores.

A



B

		K_D (μ M)			
NMR	amino acids	ligands			
		K3OR	Q3OR	Q3OGal	Q3OGlc
	A38	110	202	406	526
	I57	85	-	-	209
	E61	72	202	448	-
	Y101	-	-	267	153
	E132	-	176	175	152
	I134	109	176	-	205
	S137	54	120	-	118
	153	-	-	445	-
ITC	$\emptyset$	86	175	348	272
	$\emptyset$	162	125	75	126

C

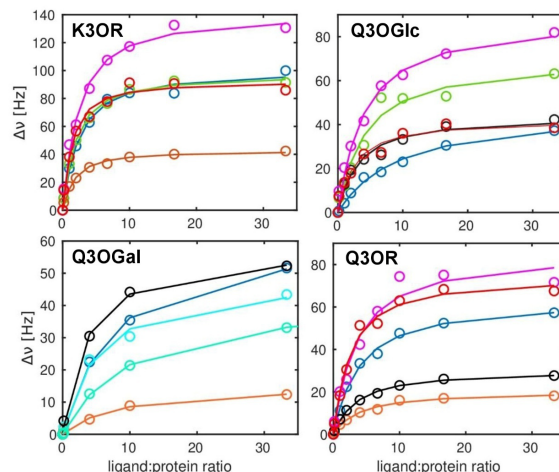


Figure S6. Titration of Cor a 1.0501 with putative ligands. **A)** ^1H , ^{15}N HSQC spectra of a titration of Cor a 1.0501 (60 μM) with the corresponding compounds dissolved in 100 (v/v) d_6DMSO . A reference titration was performed using 100 % (v/v) d_6DMSO . The boxes shown in the titration spectra with Q3OR, K3OR, Q3OGlc and Q3OGal show blow-ups of the chemical shift changes used for the determination K_D -values. NMR buffer: 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ pH 7.5, 1 mM EDTA 50 mM NaCl and 10 % $^2\text{H}_2\text{O}$. Data were recorded on a 900 MHz or 1GHz NMR spectrometer at 298K. **B)** Summary of K_D -values for individual amino acid residues under fast exchange conditions determined by NMR titrations. Average K_D -values of the amino acid residues analysed by NMR titrations for each protein are shown in green. For comparison the K_D -values obtained by ITC measurements presented in Figure S7 are depicted in blue. Colour coding of the dots corresponds to the colours of the curves shown in C. **C)** Fit of the curves to the data obtained by NMR titrations. Chemical shift perturbations ($\Delta\nu$) are plotted against the ligand:protein ratio for each ligand.

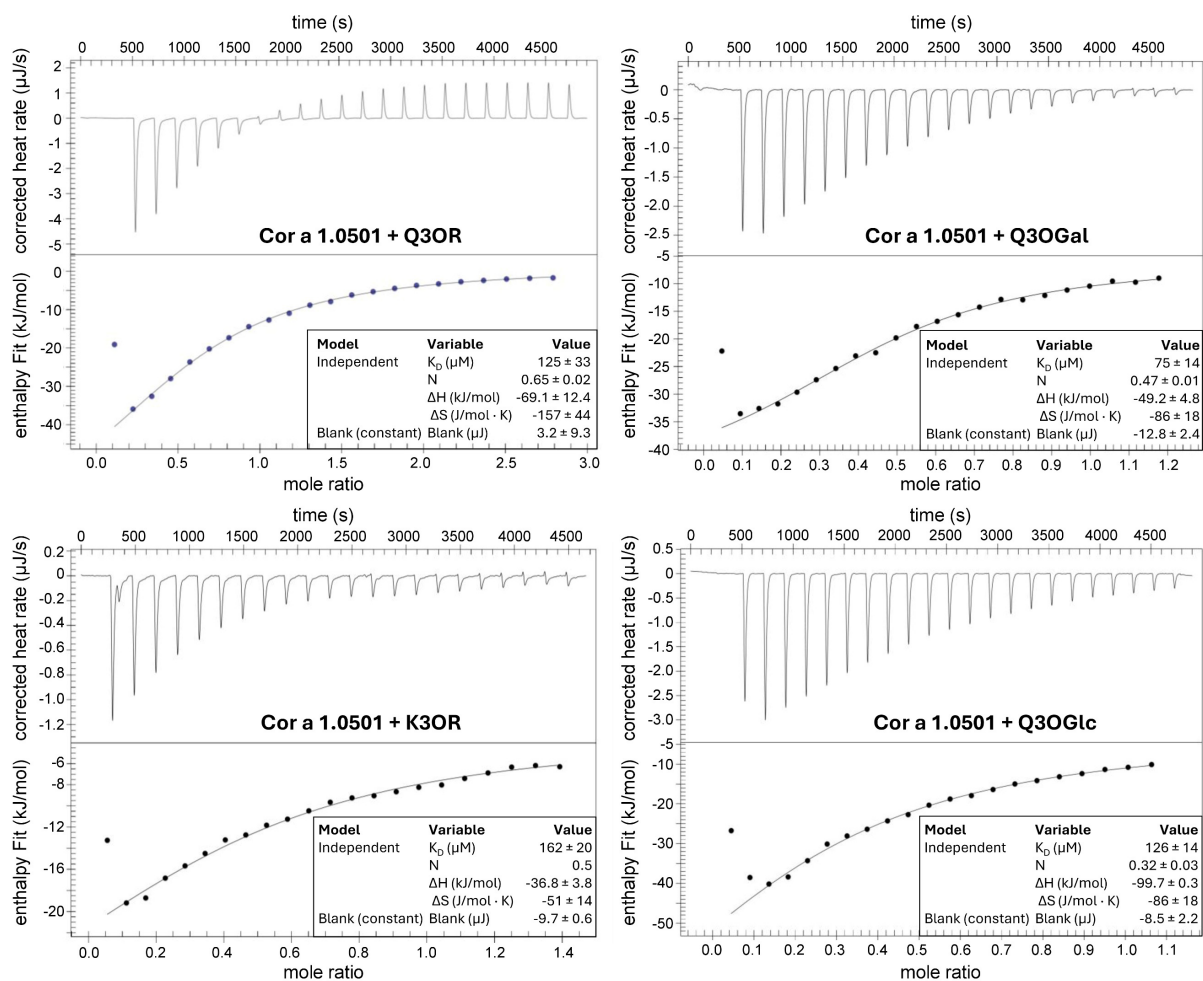
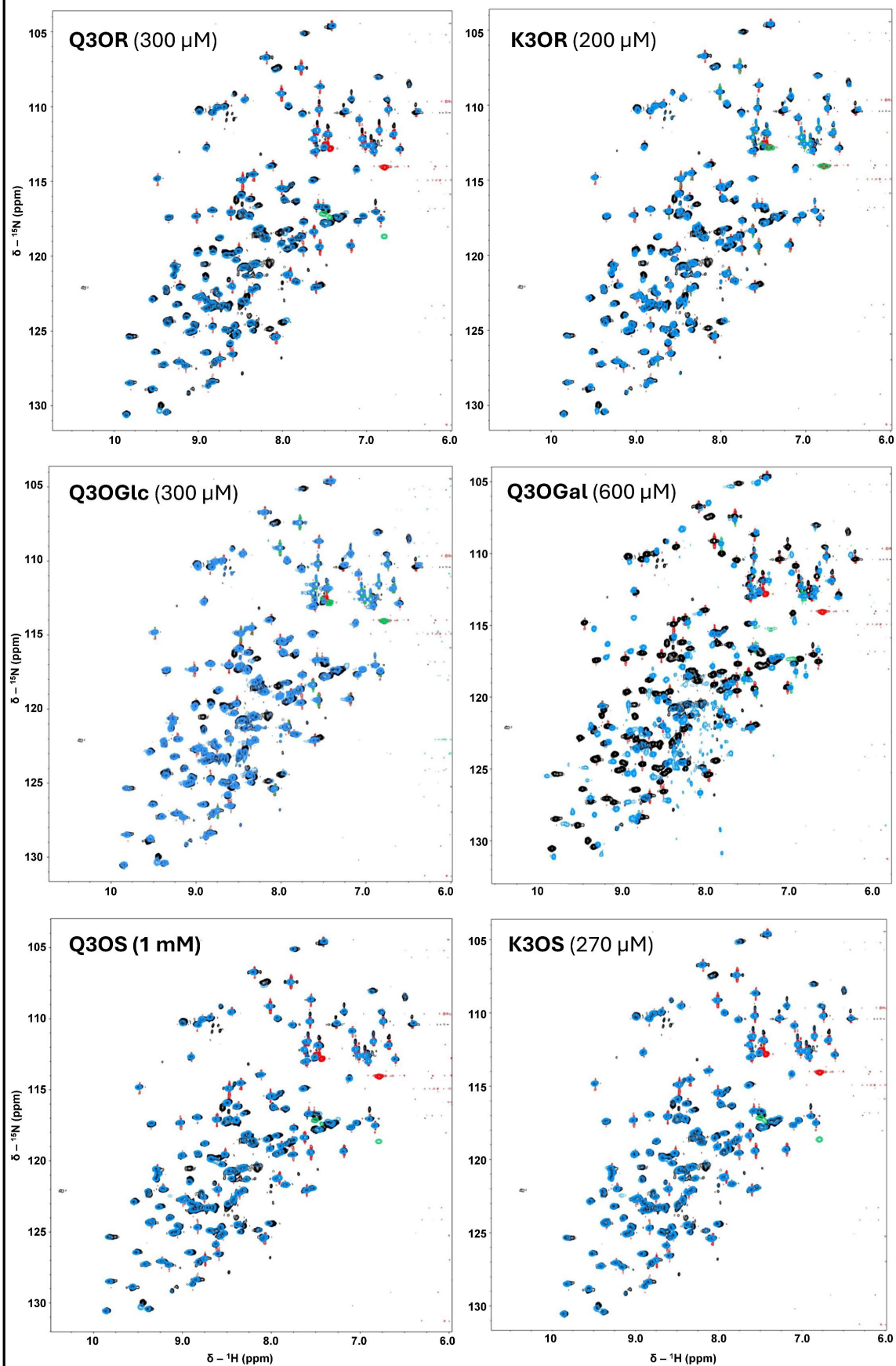
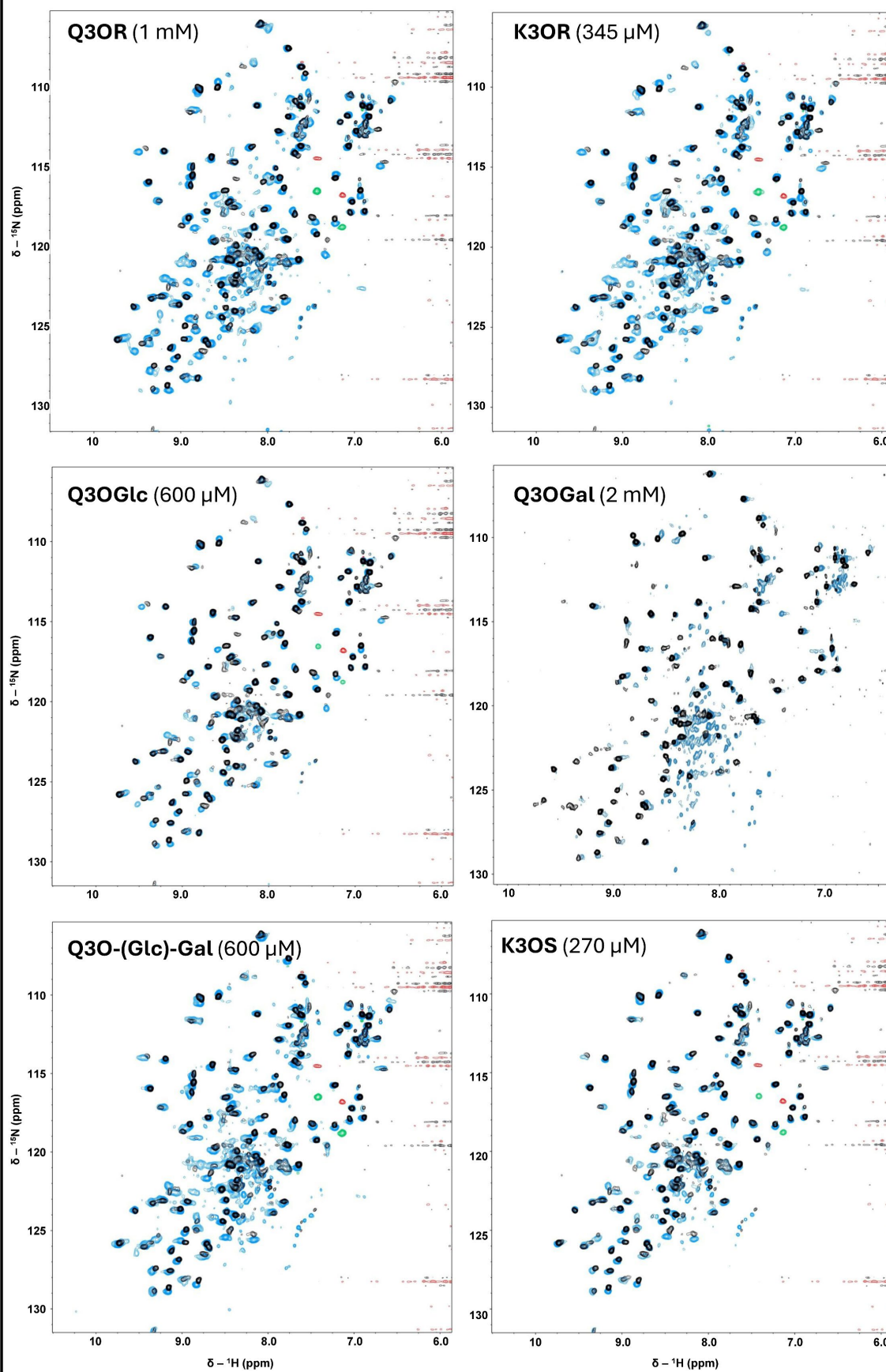


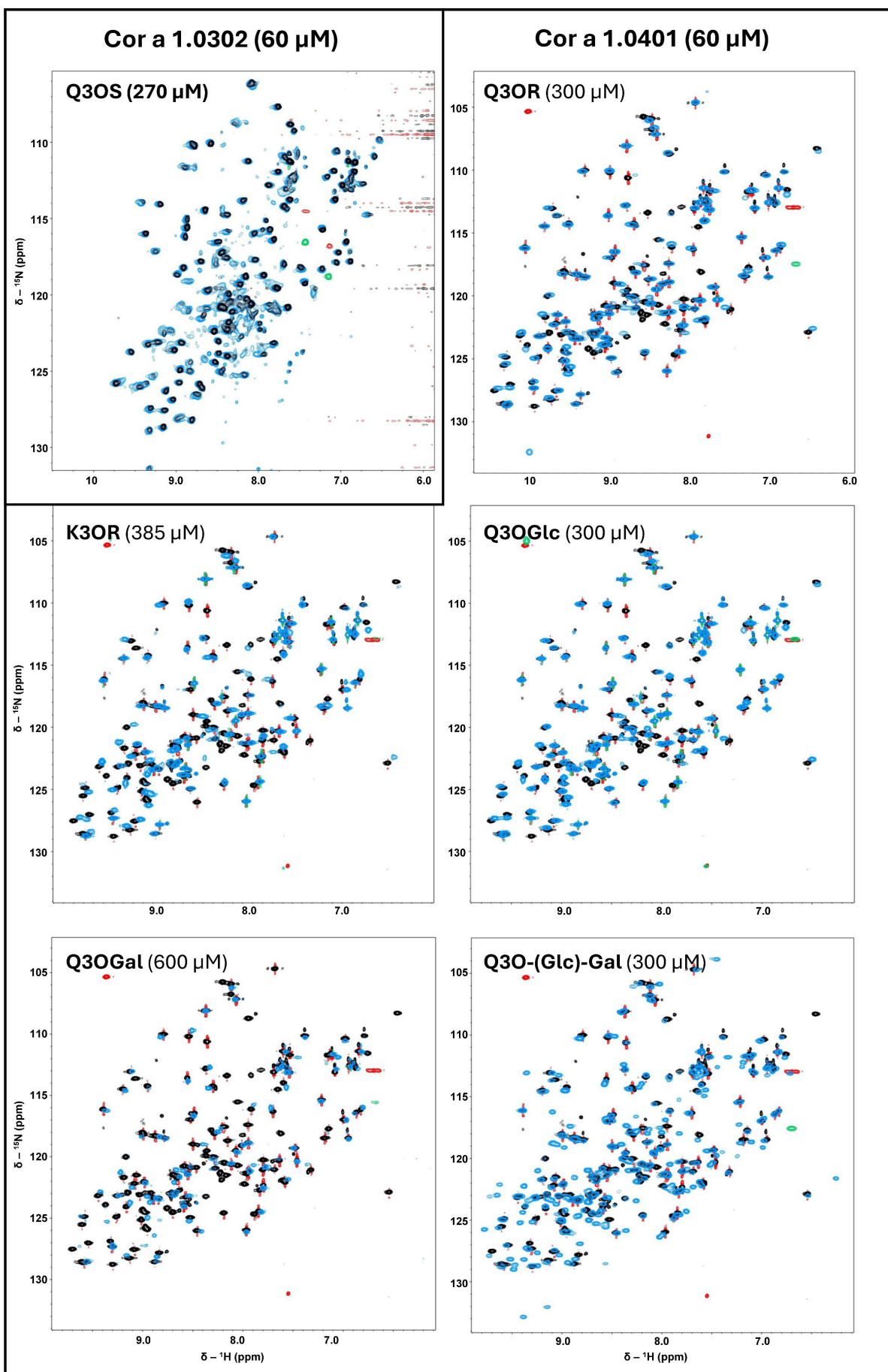
Figure S7. Isothermal titration calorimetry. Titration of Cor a 1.0501 (320 – 340 μM) with Q3OR (3.3 mM), K3OR (1.5 mM), Q3OGlc (1.3 mM) and Q3OGal (1.3 mM). Buffer: 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 50 mM NaCl, pH 7.5 at 298 K. The heat rate was corrected with a buffer titration as a blank. Measurements were made in triplicate, with K3OR measurements were carried out in duplicate. Curves were fitted to the data with an independent binding model using NanoAnalyze Data Analysis Version 4.0.0.4 (TA Instruments, USA). The first two data points were not used for fitting.

Cor a 1.0101 (60 μ M)

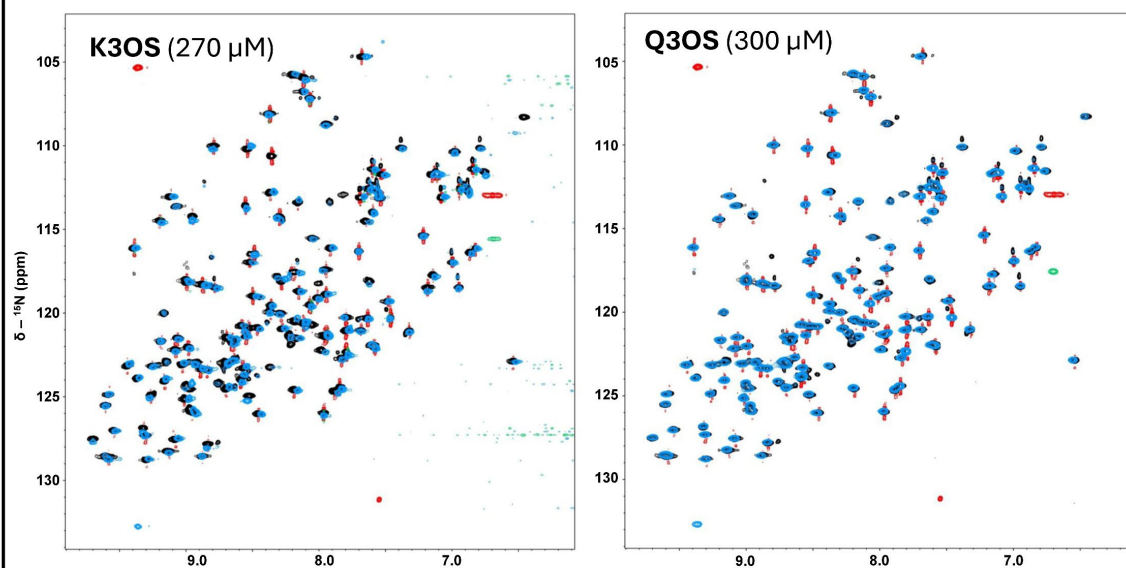


Cor a 1.0302 (60 μ M)

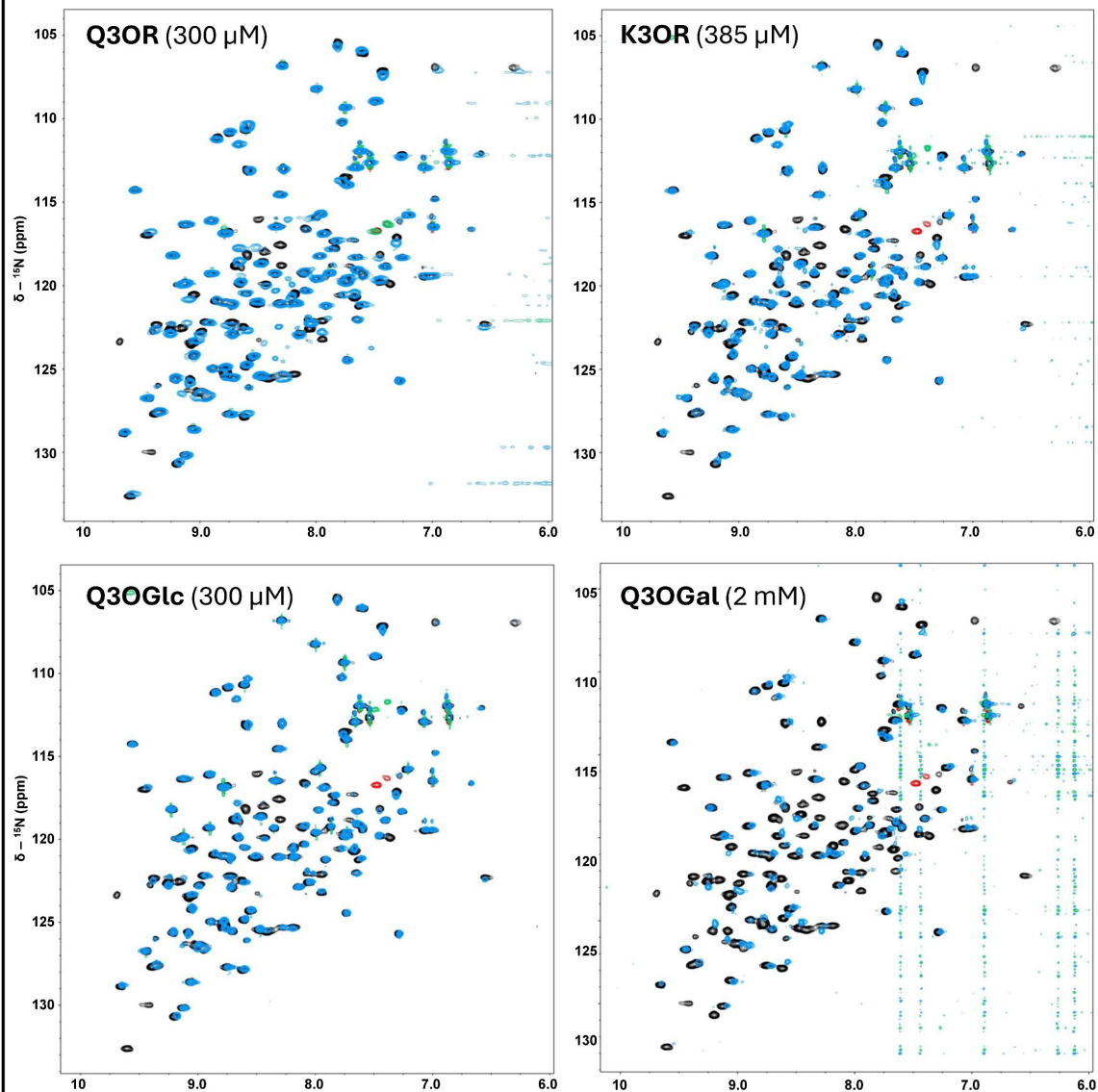




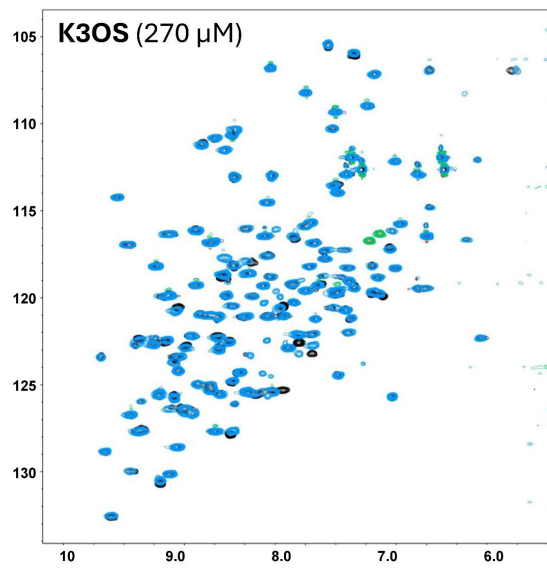
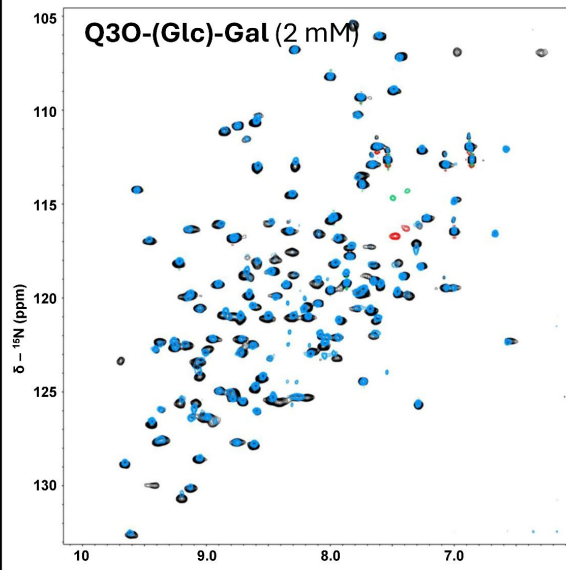
Cor a 1.0401 (60 μ M)



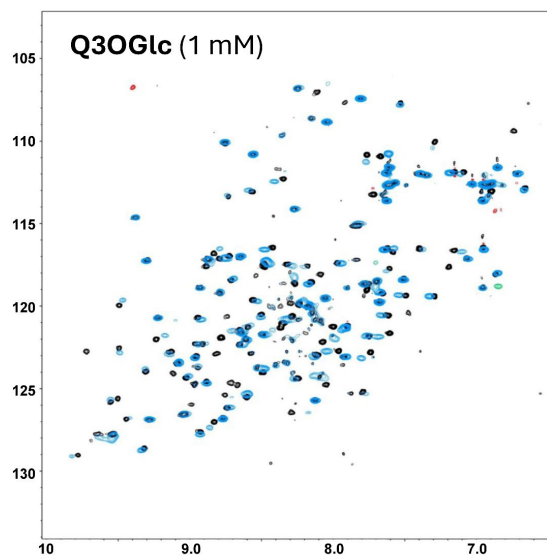
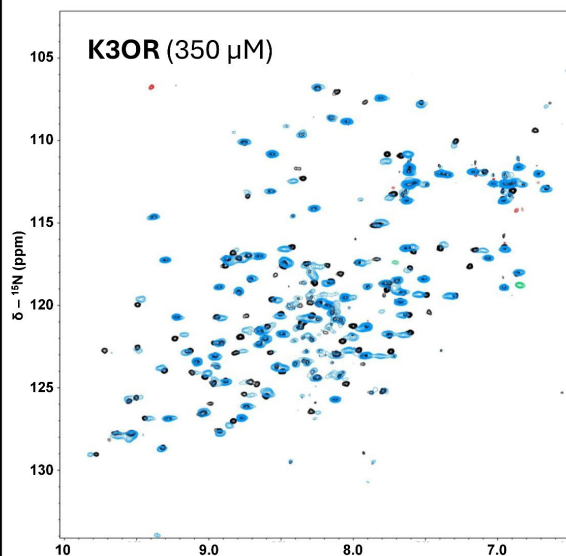
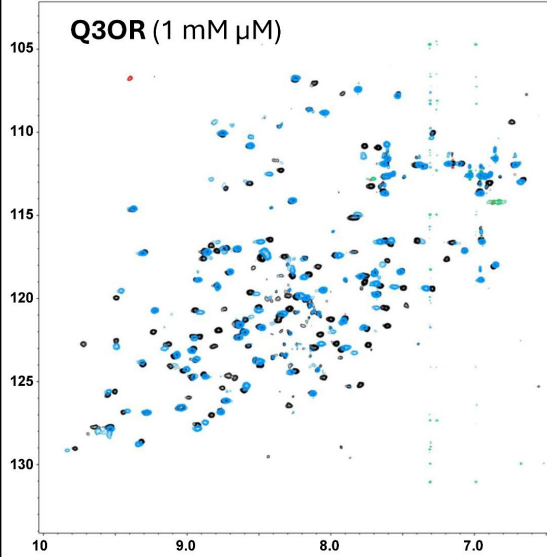
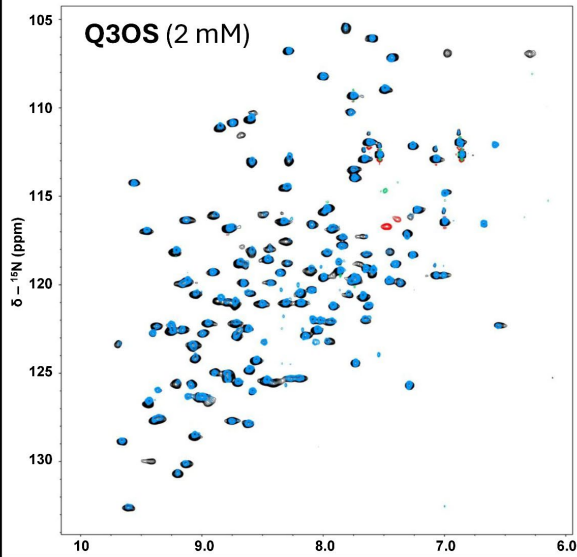
Cor a 1.0501 (60 μ M)



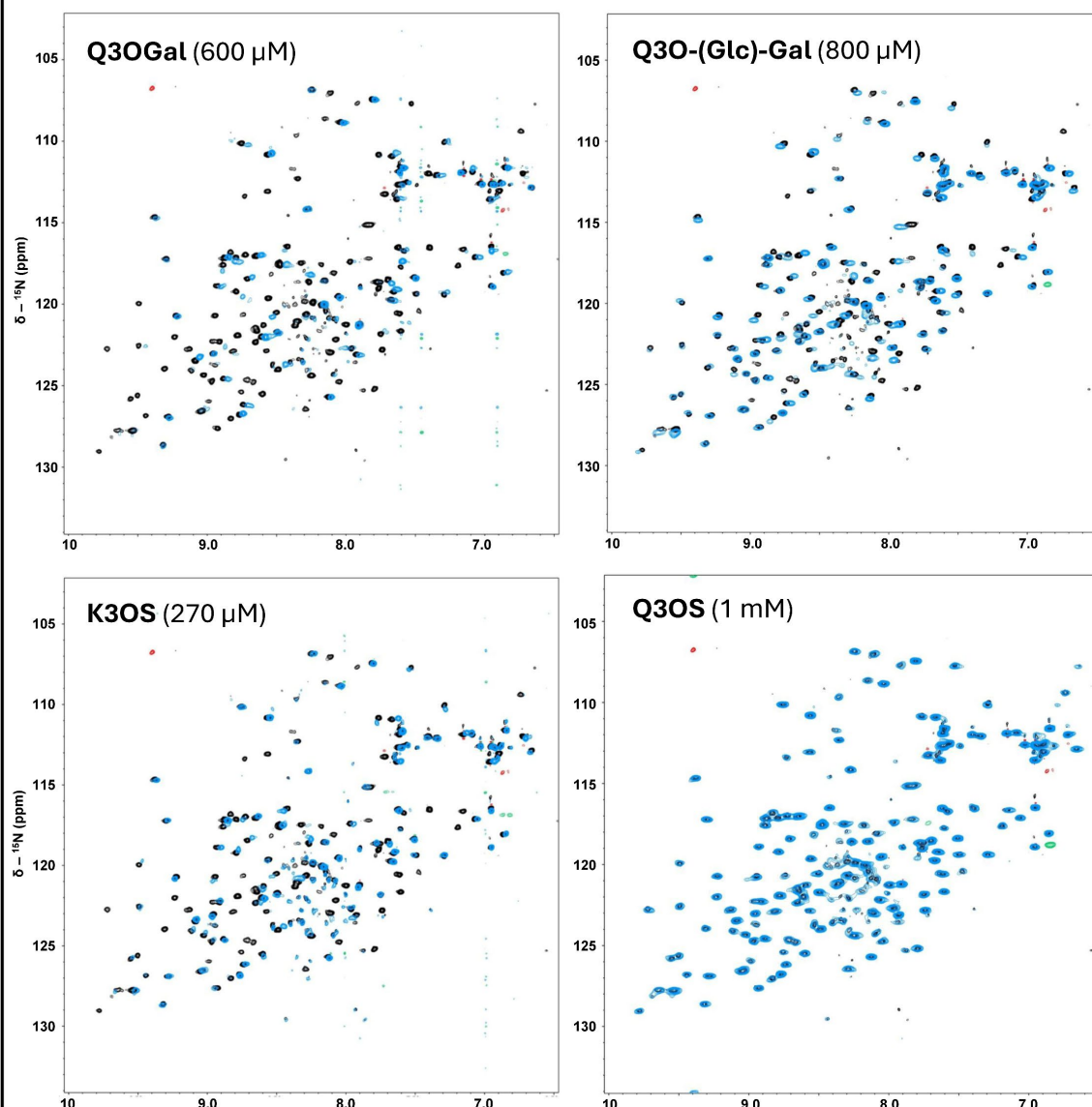
Cor a 1.0501 (60 μ M)



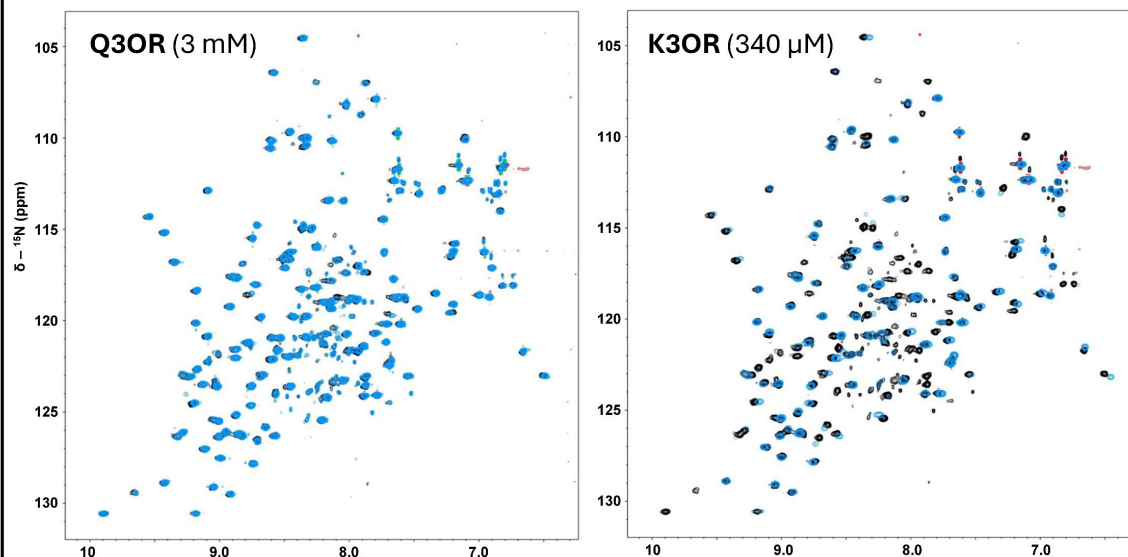
Cor a 1.0601 (60 μ M)



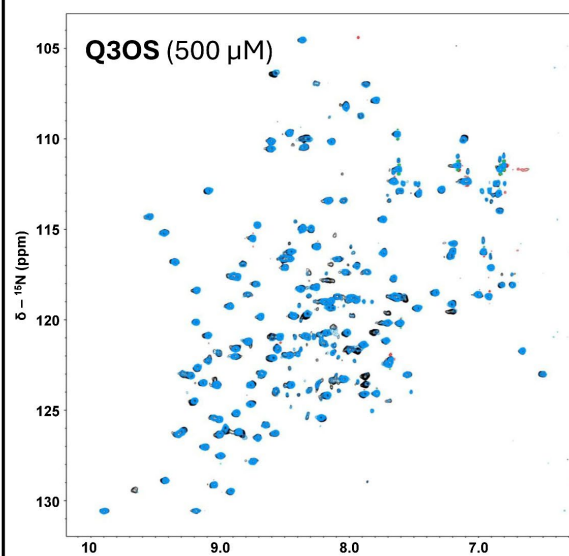
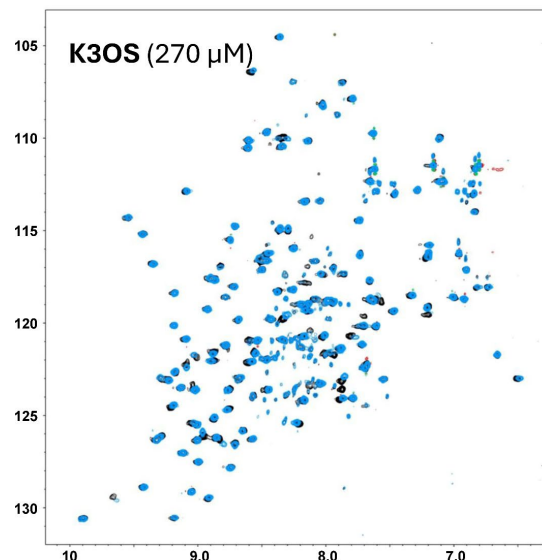
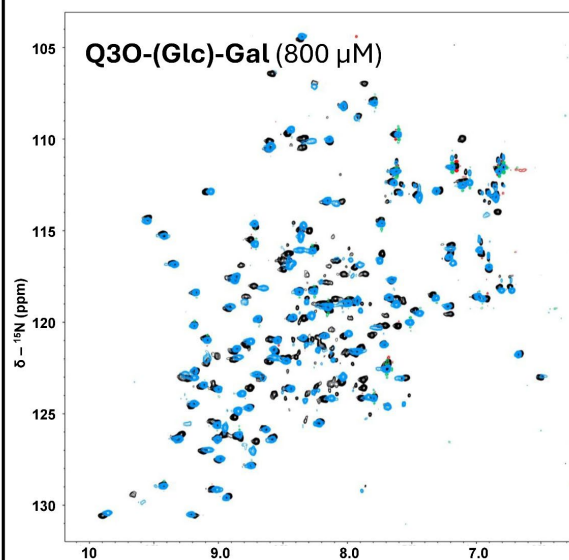
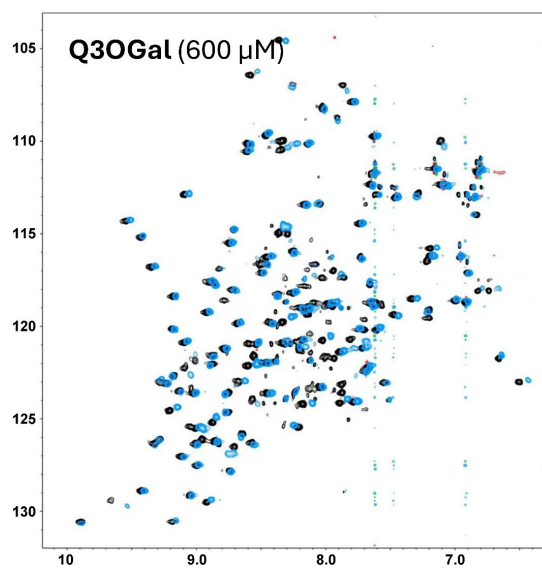
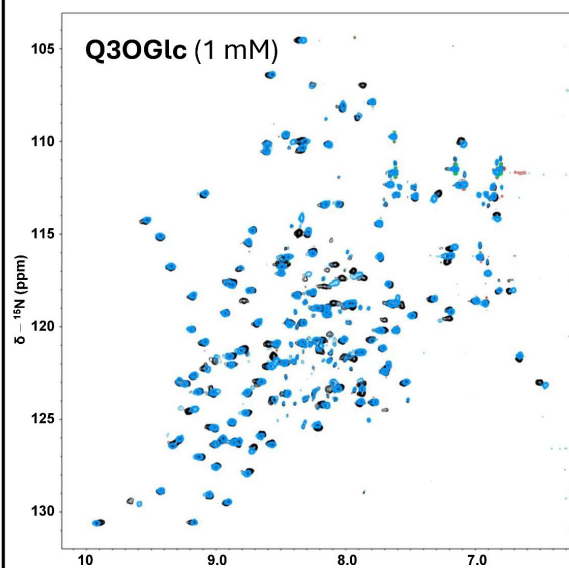
Cor a 1.0601 (60 μ M)



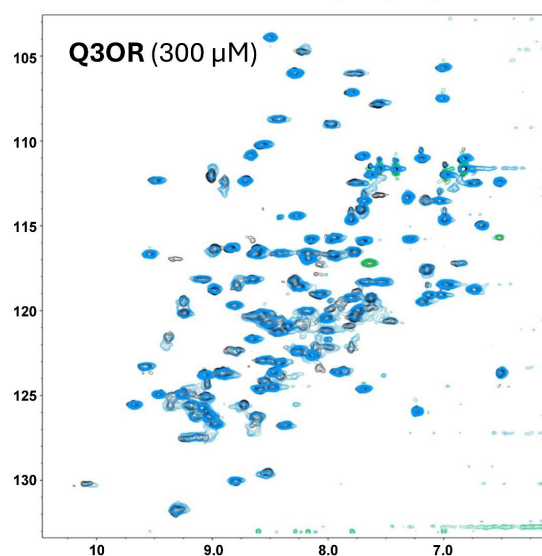
Cor a 1.0701 (60 μ M)



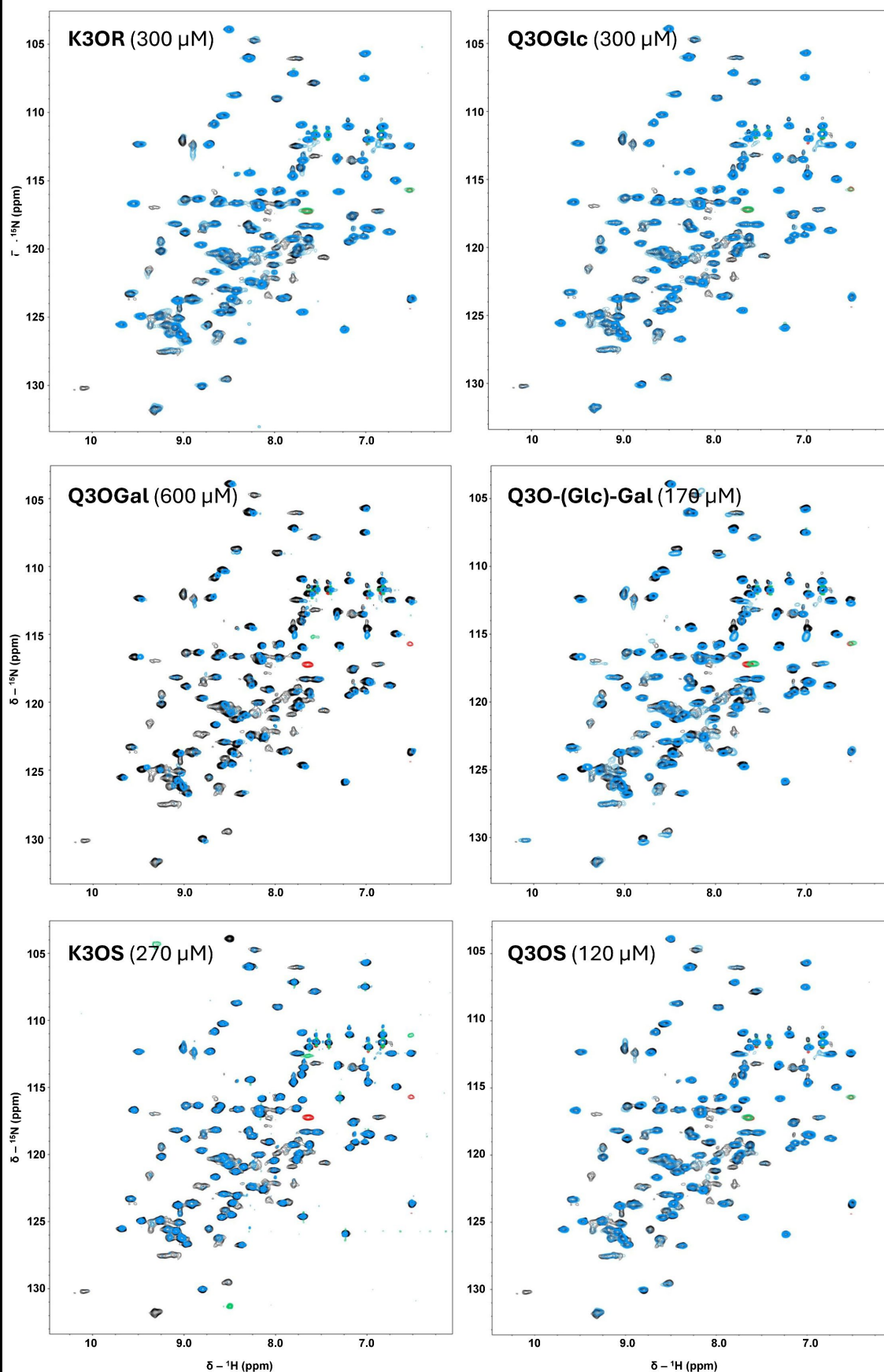
Cor a 1.0701 (60 μ M)



Cor a 1.0801 (60 μ M)



Cor a 1.0801 (60 μ M)



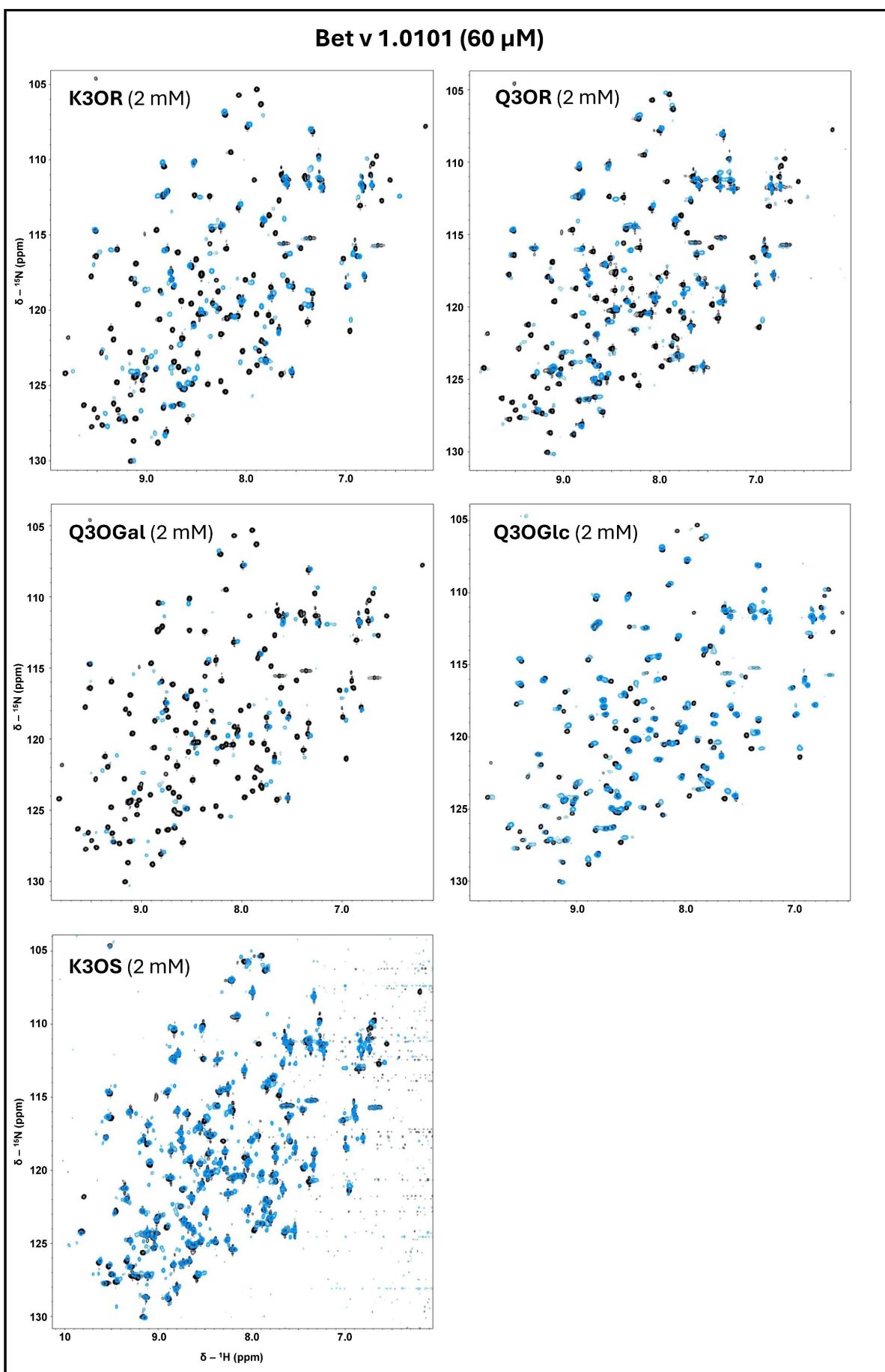
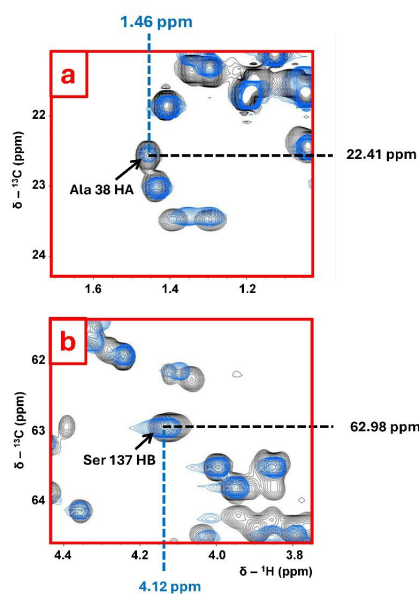
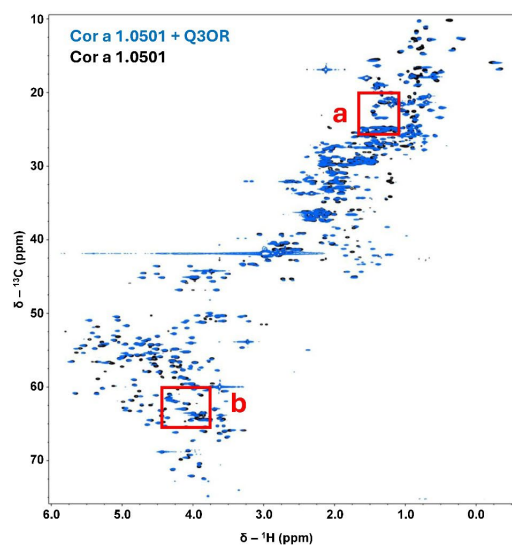
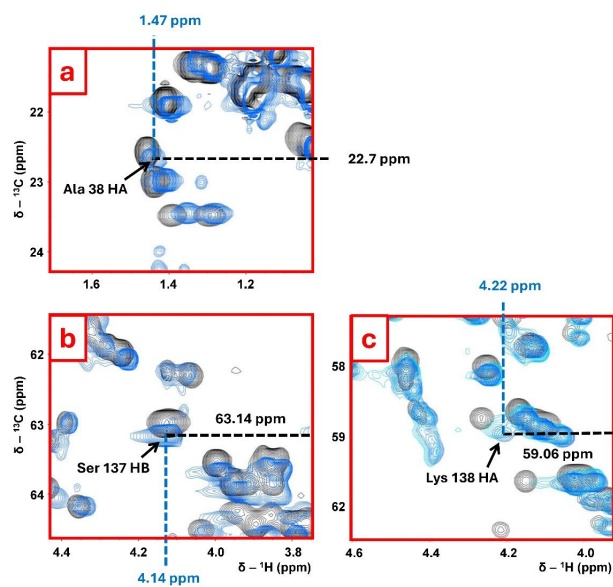
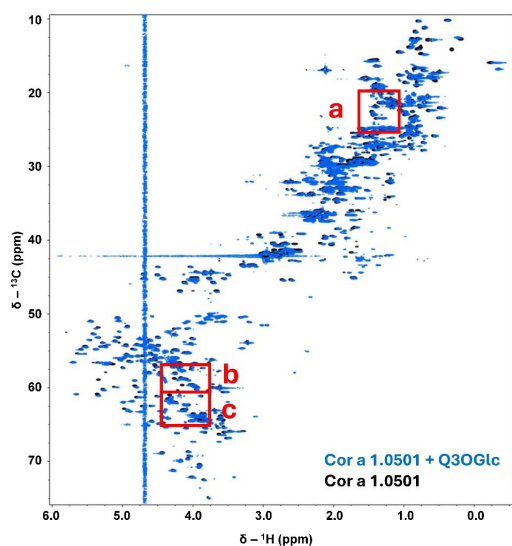


Figure S8: Ligand screening with different Cor a 1 isoallergens using ^1H , ^{15}N HSQC spectra. Superimposed spectra of Cor a 1 isoallergens (60 μM) without ligand (black/red) and at least a 5-fold excess of the putative ligand (blue/green). Buffer: 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 50 mM NaCl , 10% $^2\text{H}_2\text{O}$ pH 7.5. Data were recorded on a Bruker Avance 900 MHz or 1GHz NMR spectrometer at 298 K.

A



B



C

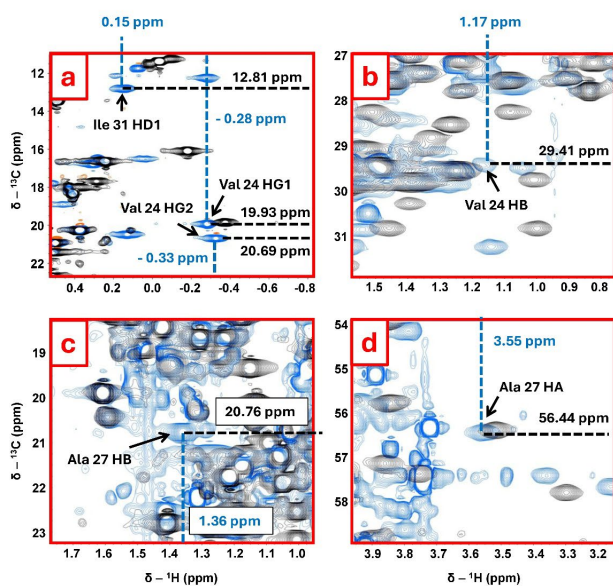
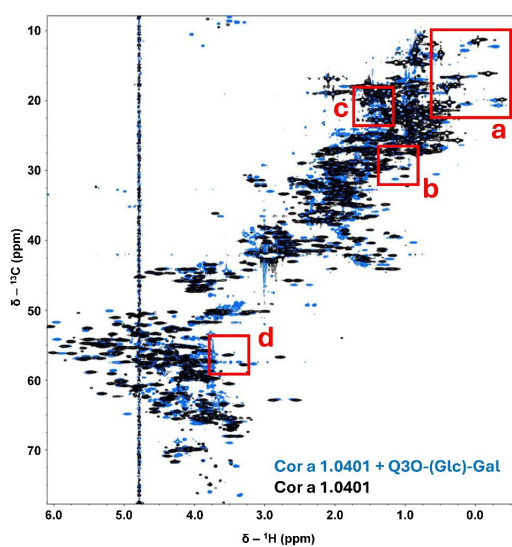


Figure S9: Assignment of the filtered/edited NOESY signals to the side chain protons of Cor a 1.0501 in complex with Q3OR or Q3OGlc. ^1H , ^{13}C HSQC spectrum of Cor a 1.0501 (black) in complex (blue) with **A)** Q3OR, **B)** Q3OGlc. and **C)** Q3O-(Glc)-Gal. The red boxes **a**, **b** and **c** show blow up sections of the spectra. The black arrows point to the signal of the assigned side chain proton. Measurements were performed at 298 or 308 K in 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, 50 mM NaCl at pH 7.5, 10% $^2\text{H}_2\text{O}$, 1 mM DTT on a Bruker Avance 900 MHz or 1 GHz spectrometer.

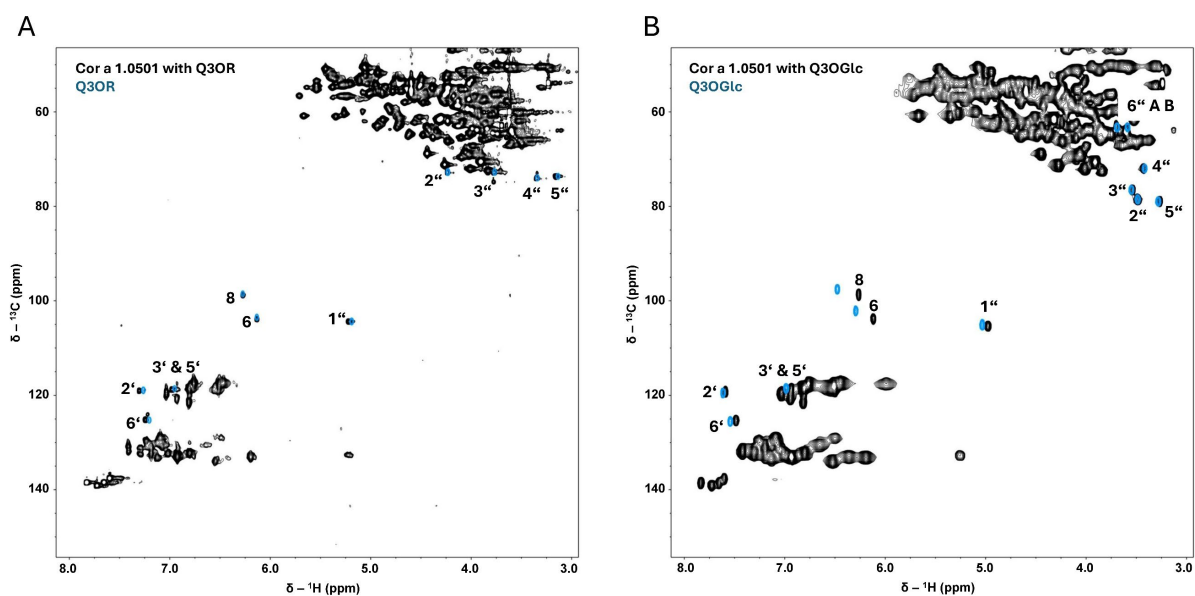


Figure S10: ^1H , ^{13}C -HSQC spectra of Q3OR and Q3OGlc bound to Cor a 1.0501. Signals of the ligand in the protein/ligand complex sample (black) were identified by superimposing spectra of the ligand (blue) in the same NMR buffer (10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, 50 mM NaCl at pH 7.5, 10% $^2\text{H}_2\text{O}$) measured on a Bruker Avance 900 MHz or 1 GHz spectrometer at 308 K.

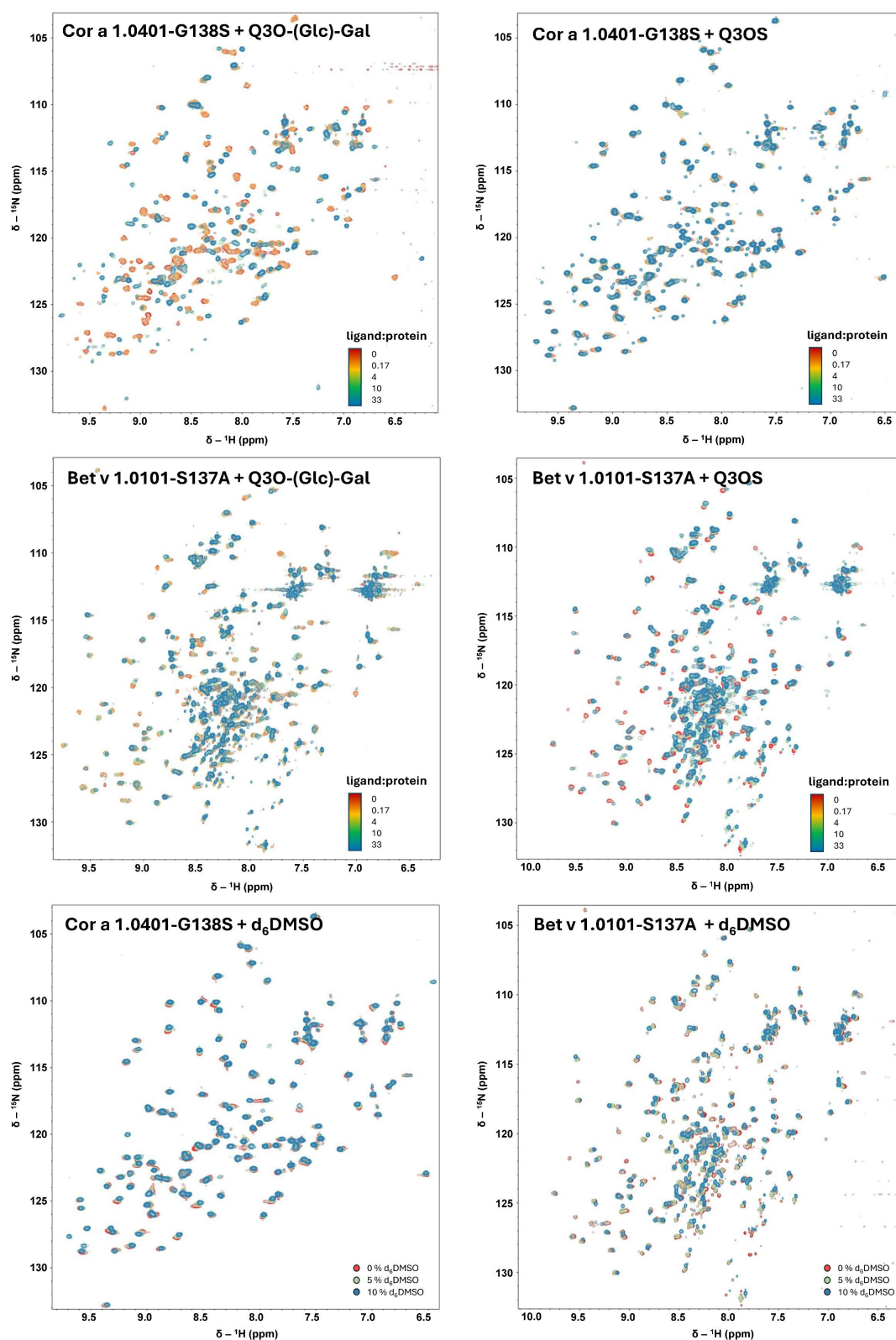


Figure S12: ^1H , ^{15}N HSQC spectra of Cor a 1.0401-G138S and Bet v 1.0101-S137A. Titration of Cor a 1.0401-G138S (60 μM) and Bet v 1.0101-S137A (60 μM) with Q3O-(Glc)-Gal or Q3OS as indicated. Compounds were dissolved in 100 (v/v) d_6DMSO . As a reference a 100 % (v/v) d_6DMSO was titrated to achieve final d_6DMSO

concentrations of 5 and 10 % respectively. NMR buffer: 10 mM Na-phosphate pH 7.5, 1 mM EDTA 50 mM NaCl and 10 % $^2\text{H}_2\text{O}$. ^1H , ^{15}N HSQC were recorded on a 900 MHz or 1GHz NMR spectrometer at 298K.

Tables

Table S1: PDB code of Q3OR, Q3OGlc and Q3O-(Glc)-Gal

Energy optimised PDB code of Q3OR:

HETATM	1	O1	UNL	1	1.935	-0.075	4.605	1.00	0.00	O
HETATM	2	C1	UNL	1	0.848	0.662	4.227	1.00	0.00	C
HETATM	3	C2	UNL	1	0.376	1.654	5.093	1.00	0.00	C
HETATM	4	C3	UNL	1	-0.727	2.414	4.719	1.00	0.00	C
HETATM	5	O2	UNL	1	-1.227	3.393	5.525	1.00	0.00	O
HETATM	6	C4	UNL	1	-1.360	2.199	3.501	1.00	0.00	C
HETATM	7	C5	UNL	1	0.219	0.430	2.994	1.00	0.00	C
HETATM	8	C6	UNL	1	-0.885	1.207	2.642	1.00	0.00	C
HETATM	9	O3	UNL	1	-1.567	1.061	1.462	1.00	0.00	O
HETATM	10	C7	UNL	1	0.670	-0.604	2.036	1.00	0.00	C
HETATM	11	O4	UNL	1	1.636	-1.336	2.229	1.00	0.00	O
HETATM	12	C8	UNL	1	-0.108	-0.731	0.768	1.00	0.00	C
HETATM	13	C9	UNL	1	-1.168	0.075	0.580	1.00	0.00	C
HETATM	14	C10	UNL	1	-1.999	0.021	-0.639	1.00	0.00	C
HETATM	15	C11	UNL	1	-2.186	1.188	-1.402	1.00	0.00	C
HETATM	16	C12	UNL	1	-2.966	1.167	-2.561	1.00	0.00	C
HETATM	17	C13	UNL	1	-3.560	-0.022	-2.953	1.00	0.00	C
HETATM	18	O5	UNL	1	-4.317	-0.046	-4.089	1.00	0.00	O
HETATM	19	C14	UNL	1	-2.620	-1.172	-1.047	1.00	0.00	C
HETATM	20	C15	UNL	1	-3.390	-1.184	-2.206	1.00	0.00	C
HETATM	21	O6	UNL	1	-4.001	-2.322	-2.656	1.00	0.00	O
HETATM	22	O7	UNL	1	0.244	-1.714	-0.142	1.00	0.00	O
HETATM	23	C16	UNL	1	1.564	-1.538	-0.673	1.00	0.00	C
HETATM	24	O8	UNL	1	1.500	-1.069	-2.030	1.00	0.00	O
HETATM	25	C17	UNL	1	0.885	-1.999	-2.929	1.00	0.00	C
HETATM	26	C18	UNL	1	0.845	-1.345	-4.306	1.00	0.00	C
HETATM	27	C19	UNL	1	2.341	-2.868	-0.567	1.00	0.00	C
HETATM	28	O9	UNL	1	3.739	-2.708	-0.884	1.00	0.00	O
HETATM	29	C20	UNL	1	1.760	-3.896	-1.539	1.00	0.00	C
HETATM	30	O10	UNL	1	2.599	-5.066	-1.583	1.00	0.00	O
HETATM	31	C21	UNL	1	1.652	-3.327	-2.958	1.00	0.00	C
HETATM	32	O11	UNL	1	0.991	-4.288	-3.789	1.00	0.00	O
HETATM	33	H1	UNL	1	2.235	0.182	5.491	1.00	0.00	H
HETATM	34	H2	UNL	1	0.864	1.832	6.045	1.00	0.00	H
HETATM	35	H3	UNL	1	-0.703	3.443	6.341	1.00	0.00	H
HETATM	36	H4	UNL	1	-2.221	2.801	3.223	1.00	0.00	H
HETATM	37	H5	UNL	1	-1.717	2.123	-1.102	1.00	0.00	H
HETATM	38	H6	UNL	1	-3.105	2.067	-3.151	1.00	0.00	H
HETATM	39	H7	UNL	1	-4.616	-0.974	-4.164	1.00	0.00	H
HETATM	40	H8	UNL	1	-2.494	-2.077	-0.461	1.00	0.00	H

HETATM	41	H9	UNL	1	-3.741	-3.059	-2.077	1.00	0.00	H
HETATM	42	H10	UNL	1	2.129	-0.762	-0.143	1.00	0.00	H
HETATM	43	H11	UNL	1	-0.153	-2.172	-2.622	1.00	0.00	H
HETATM	44	H12	UNL	1	1.856	-1.119	-4.662	1.00	0.00	H
HETATM	45	H13	UNL	1	0.309	-0.391	-4.257	1.00	0.00	H
HETATM	46	H14	UNL	1	0.348	-1.984	-5.042	1.00	0.00	H
HETATM	47	H15	UNL	1	2.294	-3.274	0.449	1.00	0.00	H
HETATM	48	H16	UNL	1	4.101	-2.079	-0.231	1.00	0.00	H
HETATM	49	H17	UNL	1	0.774	-4.233	-1.200	1.00	0.00	H
HETATM	50	H18	UNL	1	3.514	-4.720	-1.514	1.00	0.00	H
HETATM	51	H19	UNL	1	2.657	-3.180	-3.373	1.00	0.00	H
HETATM	52	H20	UNL	1	1.427	-5.141	-3.590	1.00	0.00	H
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CONNECT	7	2	8	10						
CONNECT	8	6	7	9						
CONNECT	9	8	13							
CONNECT	10	7	11	12						
CONNECT	11	10								
CONNECT	12	10	13	22						
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CONNECT	25	24	26	31	43					
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CONNECT	27	23	28	29	47					
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CONNECT	29	27	30	31	49					
CONNECT	30	29	50							
CONNECT	31	25	29	32	51					
CONNECT	32	31	52							
CONNECT	33	1								

CONNECT	34	3
CONNECT	35	5
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Energy optimised PDB code of Q3OGlc:

HETATM	1	C1	UNL	1	2.064	-0.812	5.783	1.00	0.00	C
HETATM	2	C2	UNL	1	1.081	0.163	5.920	1.00	0.00	C
HETATM	3	C3	UNL	1	0.461	0.668	4.778	1.00	0.00	C
HETATM	4	C4	UNL	1	0.827	0.199	3.512	1.00	0.00	C
HETATM	5	C5	UNL	1	1.812	-0.777	3.389	1.00	0.00	C
HETATM	6	C6	UNL	1	2.434	-1.288	4.528	1.00	0.00	C
HETATM	7	O1	UNL	1	2.218	-1.280	2.179	1.00	0.00	O
HETATM	8	C7	UNL	1	1.666	-0.753	1.021	1.00	0.00	C
HETATM	9	C8	UNL	1	0.687	0.171	0.998	1.00	0.00	C
HETATM	10	C9	UNL	1	0.197	0.708	2.289	1.00	0.00	C
HETATM	11	O2	UNL	1	-0.707	1.540	2.338	1.00	0.00	O
HETATM	12	O3	UNL	1	0.150	0.709	-0.153	1.00	0.00	O
HETATM	13	C10	UNL	1	-1.046	0.023	-0.564	1.00	0.00	C
HETATM	14	O4	UNL	1	-0.739	-0.725	-1.739	1.00	0.00	O
HETATM	15	C11	UNL	1	-1.874	-1.410	-2.293	1.00	0.00	C
HETATM	16	C12	UNL	1	-2.914	-0.380	-2.743	1.00	0.00	C
HETATM	17	C13	UNL	1	-3.325	0.466	-1.538	1.00	0.00	C
HETATM	18	C14	UNL	1	-2.112	1.100	-0.857	1.00	0.00	C
HETATM	19	O5	UNL	1	-2.590	1.754	0.331	1.00	0.00	O
HETATM	20	O6	UNL	1	-4.242	1.485	-1.972	1.00	0.00	O

HETATM	21	O7	UNL	1	-4.076	-1.009	-3.287	1.00	0.00	O
HETATM	22	C15	UNL	1	-1.388	-2.271	-3.465	1.00	0.00	C
HETATM	23	O8	UNL	1	-0.617	-3.368	-2.966	1.00	0.00	O
HETATM	24	C16	UNL	1	2.232	-1.341	-0.207	1.00	0.00	C
HETATM	25	C17	UNL	1	2.205	-2.736	-0.387	1.00	0.00	C
HETATM	26	C18	UNL	1	2.719	-3.317	-1.549	1.00	0.00	C
HETATM	27	C19	UNL	1	3.267	-2.503	-2.527	1.00	0.00	C
HETATM	28	C20	UNL	1	3.341	-1.126	-2.339	1.00	0.00	C
HETATM	29	C21	UNL	1	2.815	-0.536	-1.197	1.00	0.00	C
HETATM	30	O9	UNL	1	3.964	-0.337	-3.264	1.00	0.00	O
HETATM	31	O10	UNL	1	3.702	-3.067	-3.692	1.00	0.00	O
HETATM	32	O11	UNL	1	-0.499	1.623	4.975	1.00	0.00	O
HETATM	33	O12	UNL	1	2.699	-1.336	6.870	1.00	0.00	O
HETATM	34	H1	UNL	1	0.786	0.539	6.895	1.00	0.00	H
HETATM	35	H2	UNL	1	3.204	-2.049	4.441	1.00	0.00	H
HETATM	36	H3	UNL	1	-1.406	-0.657	0.222	1.00	0.00	H
HETATM	37	H4	UNL	1	-2.289	-2.079	-1.527	1.00	0.00	H
HETATM	38	H5	UNL	1	-2.500	0.271	-3.523	1.00	0.00	H
HETATM	39	H6	UNL	1	-3.888	-0.146	-0.823	1.00	0.00	H
HETATM	40	H7	UNL	1	-1.693	1.887	-1.496	1.00	0.00	H
HETATM	41	H8	UNL	1	-1.818	1.925	0.914	1.00	0.00	H
HETATM	42	H9	UNL	1	-4.335	2.083	-1.201	1.00	0.00	H
HETATM	43	H10	UNL	1	-4.748	-0.298	-3.342	1.00	0.00	H
HETATM	44	H11	UNL	1	-0.745	-1.686	-4.132	1.00	0.00	H
HETATM	45	H12	UNL	1	-2.221	-2.689	-4.037	1.00	0.00	H
HETATM	46	H13	UNL	1	-0.134	-3.741	-3.722	1.00	0.00	H
HETATM	47	H14	UNL	1	1.759	-3.379	0.369	1.00	0.00	H
HETATM	48	H15	UNL	1	2.664	-4.392	-1.695	1.00	0.00	H
HETATM	49	H16	UNL	1	2.870	0.542	-1.080	1.00	0.00	H
HETATM	50	H17	UNL	1	4.751	-0.835	-3.553	1.00	0.00	H
HETATM	51	H18	UNL	1	3.517	-2.412	-4.389	1.00	0.00	H
HETATM	52	H19	UNL	1	-0.859	1.889	4.100	1.00	0.00	H
HETATM	53	H20	UNL	1	2.352	-0.912	7.673	1.00	0.00	H
CONNECT	1	2	6	33						
CONNECT	2	1	3	34						
CONNECT	3	2	4	32						
CONNECT	4	3	5	10						
CONNECT	5	4	6	7						
CONNECT	6	1	5	35						
CONNECT	7	5	8							
CONNECT	8	7	9	24						
CONNECT	9	8	10	12						
CONNECT	10	4	9	11						
CONNECT	11	10								
CONNECT	12	9	13							

[illegible]

Energy optimised PDB code of Q3O-(Glc)-Gal:

HETATM	1	C1	UNL	1	-2.017	2.355	1.797	1.00	0.00	C
HETATM	2	C2	UNL	1	-2.993	1.693	2.548	1.00	0.00	C
HETATM	3	C3	UNL	1	-2.643	0.558	3.261	1.00	0.00	C
HETATM	4	C4	UNL	1	-1.336	0.085	3.241	1.00	0.00	C
HETATM	5	C5	UNL	1	-0.352	0.762	2.527	1.00	0.00	C
HETATM	6	C6	UNL	1	-0.685	1.896	1.766	1.00	0.00	C
HETATM	7	C7	UNL	1	0.316	2.616	0.939	1.00	0.00	C
HETATM	8	C8	UNL	1	1.220	1.982	0.163	1.00	0.00	C
HETATM	9	C9	UNL	1	2.138	2.804	-0.668	1.00	0.00	C
HETATM	10	O1	UNL	1	3.005	2.315	-1.387	1.00	0.00	O
HETATM	11	C10	UNL	1	1.966	4.260	-0.614	1.00	0.00	C
HETATM	12	C11	UNL	1	2.757	5.124	-1.381	1.00	0.00	C
HETATM	13	C12	UNL	1	2.562	6.502	-1.317	1.00	0.00	C
HETATM	14	C13	UNL	1	1.574	7.007	-0.478	1.00	0.00	C
HETATM	15	C14	UNL	1	0.784	6.159	0.292	1.00	0.00	C
HETATM	16	C15	UNL	1	0.982	4.780	0.221	1.00	0.00	C
HETATM	17	O2	UNL	1	0.171	3.995	0.996	1.00	0.00	O
HETATM	18	O3	UNL	1	1.344	8.347	-0.380	1.00	0.00	O
HETATM	19	O4	UNL	1	3.743	4.684	-2.223	1.00	0.00	O
HETATM	20	O5	UNL	1	1.329	0.606	0.132	1.00	0.00	O
HETATM	21	C16	UNL	1	1.132	0.001	-1.168	1.00	0.00	C
HETATM	22	C17	UNL	1	0.599	-1.426	-0.863	1.00	0.00	C
HETATM	23	C18	UNL	1	0.027	-2.118	-2.115	1.00	0.00	C
HETATM	24	C19	UNL	1	-0.786	-1.167	-3.006	1.00	0.00	C
HETATM	25	C20	UNL	1	-0.001	0.122	-3.249	1.00	0.00	C
HETATM	26	O6	UNL	1	0.245	0.763	-1.989	1.00	0.00	O
HETATM	27	C21	UNL	1	-0.766	1.120	-4.120	1.00	0.00	C
HETATM	28	O7	UNL	1	0.077	2.229	-4.430	1.00	0.00	O
HETATM	29	O8	UNL	1	-2.029	-0.922	-2.318	1.00	0.00	O
HETATM	30	O9	UNL	1	-0.873	-3.176	-1.718	1.00	0.00	O
HETATM	31	O10	UNL	1	1.717	-2.137	-0.276	1.00	0.00	O
HETATM	32	C22	UNL	1	1.499	-3.423	0.310	1.00	0.00	C
HETATM	33	C23	UNL	1	0.418	-3.383	1.408	1.00	0.00	C
HETATM	34	C24	UNL	1	0.265	-4.772	2.018	1.00	0.00	C
HETATM	35	C25	UNL	1	0.045	-5.821	0.921	1.00	0.00	C
HETATM	36	C26	UNL	1	1.186	-5.746	-0.116	1.00	0.00	C
HETATM	37	O11	UNL	1	1.283	-4.422	-0.669	1.00	0.00	O
HETATM	38	C27	UNL	1	0.966	-6.715	-1.288	1.00	0.00	C
HETATM	39	O12	UNL	1	1.415	-8.026	-0.970	1.00	0.00	O
HETATM	40	O13	UNL	1	-0.046	-7.103	1.543	1.00	0.00	O
HETATM	41	O14	UNL	1	-0.854	-4.802	2.921	1.00	0.00	O
HETATM	42	O15	UNL	1	0.765	-2.427	2.439	1.00	0.00	O
HETATM	43	O16	UNL	1	-1.064	-1.069	3.925	1.00	0.00	O

HETATM	44	O17	UNL	1	-3.599	-0.102	3.977	1.00	0.00	O
HETATM	45	H1	UNL	1	-2.311	3.235	1.227	1.00	0.00	H
HETATM	46	H2	UNL	1	-4.017	2.054	2.566	1.00	0.00	H
HETATM	47	H3	UNL	1	0.674	0.413	2.564	1.00	0.00	H
HETATM	48	H4	UNL	1	3.184	7.155	-1.921	1.00	0.00	H
HETATM	49	H5	UNL	1	0.017	6.572	0.942	1.00	0.00	H
HETATM	50	H6	UNL	1	1.960	8.817	-0.968	1.00	0.00	H
HETATM	51	H7	UNL	1	3.788	3.703	-2.161	1.00	0.00	H
HETATM	52	H8	UNL	1	2.116	-0.062	-1.651	1.00	0.00	H
HETATM	53	H9	UNL	1	-0.188	-1.327	-0.108	1.00	0.00	H
HETATM	54	H10	UNL	1	0.840	-2.573	-2.691	1.00	0.00	H
HETATM	55	H11	UNL	1	-1.038	-1.669	-3.947	1.00	0.00	H
HETATM	56	H12	UNL	1	0.964	-0.093	-3.726	1.00	0.00	H
HETATM	57	H13	UNL	1	-1.079	0.664	-5.063	1.00	0.00	H
HETATM	58	H14	UNL	1	-1.646	1.518	-3.605	1.00	0.00	H
HETATM	59	H15	UNL	1	0.443	2.541	-3.581	1.00	0.00	H
HETATM	60	H16	UNL	1	-2.616	-0.417	-2.908	1.00	0.00	H
HETATM	61	H17	UNL	1	-1.735	-2.734	-1.563	1.00	0.00	H
HETATM	62	H18	UNL	1	2.459	-3.649	0.793	1.00	0.00	H
HETATM	63	H19	UNL	1	-0.553	-3.068	1.014	1.00	0.00	H
HETATM	64	H20	UNL	1	1.142	-5.030	2.625	1.00	0.00	H
HETATM	65	H21	UNL	1	-0.919	-5.630	0.434	1.00	0.00	H
HETATM	66	H22	UNL	1	2.139	-6.001	0.366	1.00	0.00	H
HETATM	67	H23	UNL	1	1.524	-6.379	-2.169	1.00	0.00	H
HETATM	68	H24	UNL	1	-0.093	-6.755	-1.569	1.00	0.00	H
HETATM	69	H25	UNL	1	1.076	-8.620	-1.661	1.00	0.00	H
HETATM	70	H26	UNL	1	-0.677	-6.989	2.282	1.00	0.00	H
HETATM	71	H27	UNL	1	-0.742	-4.039	3.519	1.00	0.00	H
HETATM	72	H28	UNL	1	1.352	-1.779	1.990	1.00	0.00	H
HETATM	73	H29	UNL	1	-0.257	-1.469	3.534	1.00	0.00	H
HETATM	74	H30	UNL	1	-3.119	-0.839	4.405	1.00	0.00	H
CONNECT	1	2	6	45						
CONNECT	2	1	3	46						
CONNECT	3	2	4	44						
CONNECT	4	3	5	43						
CONNECT	5	4	6	47						
CONNECT	6	1	5	7						
CONNECT	7	6	8	17						
CONNECT	8	7	9	20						
CONNECT	9	8	10	11						
CONNECT	10	9								
CONNECT	11	9	12	16						
CONNECT	12	11	13	19						
CONNECT	13	12	14	48						
CONNECT	14	13	15	18						

CONNECT	15	14	16	49	
CONNECT	16	11	15	17	
CONNECT	17	7	16		
CONNECT	18	14	50		
CONNECT	19	12	51		
CONNECT	20	8	21		
CONNECT	21	20	22	26	52
CONNECT	22	21	23	31	53
CONNECT	23	22	24	30	54
CONNECT	24	23	25	29	55
CONNECT	25	24	26	27	56
CONNECT	26	21	25		
CONNECT	27	25	28	57	58
CONNECT	28	27	59		
CONNECT	29	24	60		
CONNECT	30	23	61		
CONNECT	31	22	32		
CONNECT	32	31	33	37	62
CONNECT	33	32	34	42	63
CONNECT	34	33	35	41	64
CONNECT	35	34	36	40	65
CONNECT	36	35	37	38	66
CONNECT	37	32	36		
CONNECT	38	36	39	67	68
CONNECT	39	38	69		
CONNECT	40	35	70		
CONNECT	41	34	71		
CONNECT	42	33	72		
CONNECT	43	4	73		
CONNECT	44	3	74		
CONNECT	45	1			
CONNECT	46	2			
CONNECT	47	5			
CONNECT	48	13			
CONNECT	49	15			
CONNECT	50	18			
CONNECT	51	19			
CONNECT	52	21			
CONNECT	53	22			
CONNECT	54	23			
CONNECT	55	24			
CONNECT	56	25			
CONNECT	57	27			
CONNECT	58	27			
CONNECT	59	28			

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CONNECT 60 29
CONNECT 61 30
CONNECT 62 32
CONNECT 63 33
CONNECT 64 34
CONNECT 65 35
CONNECT 66 36
CONNECT 67 38
CONNECT 68 38
CONNECT 69 39
CONNECT 70 40
CONNECT 71 41
CONNECT 72 42
CONNECT 73 43
CONNECT 74 44
MASTER      0 0 0 0 0 0 0 0 0 74 0 74 0
END

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Table S2: Distance restraints used for HADDOCK

Distance restraints for Cor a 1.0501 and Q3OR:

```

ASSIGN (segid A and resid 38 and name CB) (segid B and resid 1 and name C12) 7.0 7.0 0.0
ASSIGN (segid A and resid 137 and name CB) (segid B and resid 1 and name C21) 7.0 7.0 0.0

```

Distance restraints for Cor a 1.0501 and Q3OGlc:

```

ASSIGN (segid A and resid 38 and name CB) (segid B and resid 1 and name C18) 7.0 7.0 0.0
ASSIGN (segid A and resid 138 and name CA) (segid B and resid 1 and name C15) 5.0 5.0 0.0
ASSIGN (segid A and resid 137 and name CB) (segid B and resid 1 and name C12) 6.0 6.0 0.0

```

Distance restraints for Cor a 1.0401 and Q3O-(Glc)-Gal:

```

ASSIGN (segid A and resid 24 and name CG2) (segid B and resid 1 and name C12) 5.0 5.0 0.0
ASSIGN (segid A and resid 27 and name CG2) (segid B and resid 1 and name C19) 5.0 5.0 0.0
ASSIGN (segid A and resid 31 and name CG2) (segid B and resid 1 and name C24) 5.0 5.0 0.0

```


Table S3: Partial amino acid side chain assignment of Cor a 1.0401-3CS in complex with Q3O-(Glc)-Gal

Amino acid number	Amino acid	Proton type	Number of protons	Hetero nuclei	δ-1H (ppm)	δ-13C, δ-15N (ppm)
3	VAL	HA	1	C	4.832	61.401
3	VAL	HB	1	C	1.618	33.583
3	VAL	HG1*	3	C	0.374	20.240
3	VAL	HG2*	3	C	0.491	20.937
4	PHE	HN	1	N	8.912	128.662
5	SER	HN	1	N	8.354	118.769
6	TYR	HN	1	N	9.274	123.221
7	GLU	HN	1	N	8.527	121.258
8	ASP	HN	1	N	9.143	123.395
10	ALA	HB*	3	C	1.412	22.899
11	THR	HA	1	C	5.054	59.920
11	THR	HB	1	C	4.265	71.755
11	THR	HG2*	3	C	1.211	21.716
12	SER	HN	1	N	8.370	112.414
13	VAL	HN	1	N	8.226	120.824
13	VAL	HA	1	C	4.380	62.795
13	VAL	HB	1	C	2.258	31.843
13	VAL	HG1*	3	C	0.622	21.316
13	VAL	HG2*	3	C	0.700	21.834
14	ILE	HN	1	N	8.346	127.504
14	ILE	HD1*	3	C	0.784	10.634
17	ALA	HN	1	N	8.336	117.169
17	ALA	HB*	3	C	1.540	17.934
18	ARG	HN	1	N	6.865	116.352
19	LEU	HN	1	N	7.865	121.978
20	PHE	HN	1	N	9.104	118.729
22	SER	HN	1	N	6.997	110.507
23	PHE	HN	1	N	8.247	121.701
24	VAL	HA	1	C	3.700	63.382

24	VAL	HB	1	C	1.167	29.408
24	VAL	HG1*	3	C	-0.282	19.935
24	VAL	HG2*	3	C	-0.326	20.692
25	LEU	HN	1	N	7.571	114.772
26	ASP	HN	1	N	7.084	118.519
27	ALA	HN	1	N	6.258	121.611
27	ALA	HA	1	C	3.554	56.439
27	ALA	HB*	3	C	1.359	20.764
28	ASP	HN	1	N	8.123	113.696
29	ASN	HN	1	N	7.155	112.134
30	LEU	HN	1	N	8.329	120.270
30	LEU	HA	1	C	3.966	57.668
30	LEU	HB1	1	C	1.336	43.457
30	LEU	HB2	1	C	1.502	43.428
30	LEU	HG	1	C	1.072	26.685
30	LEU	HD1*	3	C	0.449	26.769
30	LEU	HD2*	3	C	0.516	23.026
31	ILE	HD1*	3	C	0.146	12.812
33	LYS	HN	1	N	6.694	113.170
34	VAL	HG1*	3	C	0.953	19.106
34	VAL	HG2*	3	C	1.069	21.356
42	ALA	HN	1	N	8.697	124.708
42	ALA	HA	1	C	5.078	51.605
42	ALA	HB*	3	C	1.240	22.871
43	GLU	HN	1	N	8.453	118.752
44	ASN	HN	1	N	9.124	122.922
45	LEU	HN	1	N	9.254	127.495
45	LEU	HB1	1	C	1.626	42.351
45	LEU	HB2	1	C	1.401	42.083
45	LEU	HG	1	C	1.580	26.848
45	LEU	HD1*	3	C	0.812	25.457
45	LEU	HD2*	3	C	0.756	22.547
46	GLU	HN	1	N	7.676	116.450

47	GLY	HN	1	N	8.471	110.413
48	ASN	HN	1	N	8.220	114.149
49	GLY	HN	1	N	8.800	110.258
50	GLY	HN	1	N	8.008	126.507
52	GLY	HN	1	N	9.115	114.511
53	THR	HN	1	N	7.634	118.075
53	THR	HA	1	C	4.554	64.484
53	THR	HB	1	C	3.949	69.886
54	ILE	HN	1	N	8.366	125.703
54	ILE	HA	1	C	4.606	60.592
54	ILE	HG2*	3	C	0.813	17.516
54	ILE	HD1*	3	C	0.858	12.682
55	LYS	HN	1	N	9.483	128.540
56	LYS	HN	1	N	9.334	124.046
57	ILE	HN	1	N	9.093	111.182
57	ILE	HA	1	C	4.721	59.611
57	ILE	HB	1	C	1.218	39.618
57	ILE	HG11	1	C	0.432	18.126
57	ILE	HG12	1	C	0.432	18.126
57	ILE	HG2*	3	C	0.432	18.126
57	ILE	HD1*	3	C	-0.277	12.233
70	HIS	HN	1	N	9.343	122.830
72	VAL	HN	1	N	9.221	128.774
72	VAL	HA	1	C	3.871	64.299
72	VAL	HB	1	C	2.170	32.118
72	VAL	HG1*	3	C	1.026	20.706
72	VAL	HG2*	3	C	0.929	20.500
75	ILE	HN	1	N	8.756	123.995
75	ILE	HA	1	C	4.331	62.088
75	ILE	HG2*	3	C	0.853	17.479
75	ILE	HD1*	3	C	0.879	13.422
76	ASP	HN	1	N	9.445	128.040
78	ALA	HN	1	N	8.048	119.370

78	ALA	HA	1	C	3.787	54.156
78	ALA	HB*	3	C	1.302	18.501
79	ASN	HN	1	N	7.069	111.412
80	PHE	HN	1	N	7.212	117.362
81	LYS	HN	1	N	7.995	117.030
84	TYR	HN	1	N	8.750	121.498
85	SER	HN	1	N	9.409	113.060
86	ILE	HN	1	N	9.812	125.902
88	GLU	HN	1	N	7.666	122.327
89	GLY	HN	1	N	9.002	113.167
92	LEU	HN	1	N	7.342	119.349
92	LEU	HB1	1	C	1.581	39.751
92	LEU	HB2	1	C	1.602	39.757
92	LEU	HG	1	C	1.702	26.858
92	LEU	HD1*	3	C	0.678	25.245
92	LEU	HD2*	3	C	0.564	22.822
93	GLY	HN	1	N	7.459	124.909
95	THR	HA	1	C	4.454	62.811
95	THR	HB	1	C	4.319	70.184
95	THR	HG2*	3	C	1.096	22.067
97	GLU	HN	1	N	9.216	121.024
98	LYS	HN	1	N	8.050	118.173
99	ILE	HN	1	N	7.682	122.669
99	ILE	HA	1	C	4.782	60.763
99	ILE	HB	1	C	1.223	42.656
99	ILE	HG11	1	C	1.454	27.634
99	ILE	HG12	1	C	1.070	27.644
99	ILE	HG2*	3	C	0.034	16.508
99	ILE	HD1*	3	C	0.825	14.773
102	GLU	HN	1	N	8.532	121.142
103	ILE	HA	1	C	5.066	60.763
103	ILE	HB	1	C	0.881	40.304
103	ILE	HG11	1	C	1.310	29.530

103	ILE	HG12	1	C	1.042	29.481
103	ILE	HG2*	3	C	0.779	18.781
103	ILE	HD1*	3	C	0.776	14.747
104	LYS	HN	1	N	8.548	125.674
105	MET	HN	1	N	9.050	123.638
106	ALA	HA	1	C	4.714	50.460
106	ALA	HB*	3	C	1.414	22.525
107	ALA	HN	1	N	8.792	123.298
107	ALA	HA	1	C	4.319	52.373
107	ALA	HB*	3	C	1.415	18.384
108	ALA	HN	1	N	8.010	126.329
108	ALA	HA	1	C	4.313	50.262
108	ALA	HB*	3	C	1.183	18.153
112	GLY	HN	1	N	8.336	128.774
113	GLY	HN	1	N	8.079	127.807
114	SER	HN	1	N	9.025	118.837
115	ILE	HN	1	N	9.234	122.899
115	ILE	HD1*	3	C	0.852	14.091
116	LEU	HN	1	N	9.280	128.006
118	ILE	HN	1	N	9.094	127.886
119	THR	HA	1	C	5.337	61.945
119	THR	HB	1	C	4.176	69.979
119	THR	HG2*	3	C	1.173	21.595
122	TYR	HN	1	N	9.565	124.628
123	HIS	HN	1	N	8.704	123.505
124	THR	HN	1	N	9.398	116.018
124	THR	HA	1	C	5.005	60.395
124	THR	HB	1	C	4.424	70.830
124	THR	HG2*	3	C	1.200	22.944
128	ALA	HN	1	N	7.818	122.563
128	ALA	HA	1	C	4.368	52.989
128	ALA	HB*	3	C	1.607	19.876
130	ILE	HD1*	3	C	0.588	13.561

131	ASN	HN	1	N	8.614	124.278
133	GLU	HN	1	N	8.403	118.707
135	ILE	HD1*	3	C	0.073	11.740
137	ALA	HA	1	C	4.202	55.017
137	ALA	HB*	3	C	1.500	17.954
138	GLY	HN	1	N	8.113	127.487
143	ALA	HA	1	C	4.202	55.017
143	ALA	HB*	3	C	1.518	18.140
147	LYS	HN	1	N	7.921	116.696
149	VAL	HN	1	N	8.928	117.271
149	VAL	HA	1	C	3.430	66.820
149	VAL	HB	1	C	1.541	30.839
149	VAL	HG1*	3	C	0.462	23.068
149	VAL	HG2*	3	C	0.130	20.487
152	TYR	HN	1	N	7.890	120.550
153	LEU	HN	1	N	8.567	120.744
153	LEU	HA	1	C	4.016	57.577
153	LEU	HD1*	3	C	0.951	22.318
153	LEU	HD2*	3	C	0.729	27.442
154	LEU	HN	1	N	8.353	120.100
155	ALA	HA	1	C	4.104	52.747
155	ALA	HB*	3	C	1.219	18.570
156	HIS	HN	1	N	7.189	115.568
158	ASP	HN	1	N	8.625	114.253
159	ALA	HN	1	N	7.848	125.111
161	SER	HN	1	N	7.518	118.666

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