

Biochemische und immunologische Charakterisierung der PR-10 Allergene Cor a 1 aus Hasel und Dau c 1 aus Karotte

- Liganden, Stabilität, neue Isoallergene

Dissertation

Zur Erlangung des Doktorgrades
der Fakultät für Biologie, Chemie und Geowissenschaften der
Universität Bayreuth

vorgelegt von

Biochemikerin M.Sc.

Thessa Patricia Jacob

aus Münchberg

Bayreuth, 2021

Die vorliegende Arbeit wurde von Juni 2017 bis Oktober 2020 in Bayreuth am Lehrstuhl Biopolymere (Biochemie IV) unter Betreuung von Frau Prof. Dr. Birgitta M. Wöhl angefertigt.

Vollständiger Abdruck der von der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth genehmigten Dissertation zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften (Dr. rer. nat.).

Dissertation eingereicht am: 28.10.2020

Zulassung durch die Promotionskommission: 04.11.2020

Wissenschaftliches Kolloquium: 12.03.2021

Amtierender Dekan: Prof. Dr. Matthias Breuning

Prüfungsausschuss:

Erstgutachterin: Prof. Dr. Birgitta M. Wöhl (Gutachterin)

Zweitgutachter/in: Prof. Dr. Andreas Möglich (Gutachter)

Vorsitzende/r: Prof. Dr. Carlo Unverzagt (Vorsitz)

Weitere Mitglieder: Prof. Dr. Angelika Mustroph

*„Your work is nothing worth unless
your mind is calm and clear and at its best“*

(Tina Dico)

Inhaltsverzeichnis

| | |
|---|-----|
| Zusammenfassung..... | 1 |
| Summary..... | 3 |
| 1 Einleitung | 5 |
| 1.1 Die Häufigkeit allergischer Erkrankungen nimmt zu | 5 |
| 1.2 Ablauf einer allergischen Reaktion | 6 |
| 1.3 Allergenepitope | 7 |
| 1.4 <i>in vivo</i> und <i>in vitro</i> Allergietests..... | 7 |
| 1.5 Die Basis einer Kreuzallergie sind konservierte Epitope..... | 9 |
| 1.6 Behandlung von Allergien..... | 11 |
| 1.7 Merkmale eines Allergens | 12 |
| 1.8 Klassifizierung und Nomenklatur von Allergenen | 13 |
| 1.9 PR-10 Allergene – die größte Bet v 1-Unterfamilie | 15 |
| 1.10 Flavonoide sind natürliche Liganden von PR-10 Allergenen..... | 18 |
| 1.11 Stabilität von PR-10 Allergenen..... | 20 |
| 1.12 Merkmale von Karotten- und Haselallergenen..... | 20 |
| 2 Zielsetzung und Vorgehensweise | 23 |
| 3 Zusammenfassung und Diskussion der Ergebnisse | 24 |
| 3.1 Identifikation eines natürlichen Liganden des Haselallergens Cor a 1 | 24 |
| 3.2 Die Nahrungsmittelzubereitung zerstört nicht die Allergenität des Karottenallergens Dau c 1 | 29 |
| 3.3 Identifizierung eines neuen Isoallergens Dau c 1.0401 in der Karotte | 35 |
| 4 Schlussbetrachtung und Ausblick..... | 40 |
| 5 Abkürzungsverzeichnis | 41 |
| 6 Literaturverzeichnis..... | 42 |
| 7 Publikationsliste | 54 |
| 7.1 Einzelarbeit A | 54 |
| 7.2 Einzelarbeit B | 54 |
| 7.3 Einzelarbeit C | 55 |
| 8 Einzelarbeiten..... | 56 |
| 8.1 Einzelarbeit A | 56 |
| 8.2 Einzelarbeit B | 81 |
| 8.3 Einzelarbeit C | 132 |
| 9 Danksagung..... | 146 |
| 10 Erklärung | 148 |

Zusammenfassung

Pathogenesis related proteins 10 (PR-10 Proteine) in Pflanzen werden oft als Antwort auf Stressstimuli produziert und sind verantwortlich für viele Pollen- und Lebensmittelallergien. Ihre genaue physiologische Funktion ist bisher unklar. In Deutschland sind rund 92 % der Baumpollenallergiker gegen das Hauptbirkenpollenallergen Bet v 1 sensibilisiert. Es existieren zahlreiche zu Bet v 1 homologe Proteine in verschiedenen anderen Pollen und Nahrungsmitteln wie Äpfeln, Nüssen, Steinobst, Sellerie und Karotten, die aufgrund ihrer ähnlichen Struktur bei Birkenpollenallergikern Kreuzallergien auslösen können. PR-10 Allergene liegen meist als Mischung unterschiedlicher Isoallergene und Varianten vor, welche vermutlich unterschiedliche Aufgaben erfüllen. Ihre dreidimensionale Struktur ist hoch konserviert und beinhaltet einen hydrophoben Hohlraum, der kleine Liganden binden kann, und auf eine Funktion als Transport- oder Speicherprotein hinweist.

Ein natürlicher Ligand von Bet v 1.0101 ist das diglykosylierte Flavonoid Quercetin-3-O-Sophorosid (Q3OS). In dieser Arbeit konnte ein natürlicher Ligand des Haselallergens Cor a 1, Quercetin-3-O-(2"-O- β -D-Glukopyranosyl)- β -D-Galaktopyranosid (Q3O-(Glc)-Gal), identifiziert werden. Dieser ist beinahe identisch zu Q3OS und unterscheidet sich nur in der Position der OH-Gruppe am ersten Zucker (Glukose vs. Galaktose). Trotzdem weisen sowohl Cor a 1.0401 als auch Bet v 1.0101 eine sehr hohe Spezifität auf und binden nur ihren eigenen Liganden. Die Struktur von Cor a 1.0401, welche durch multidimensionale NMR-Spektroskopie bestimmt wurde, ermöglichte eine genauere Charakterisierung der Bindungstasche. Dies bietet die Grundlage, um zukünftig die Ursache der Bindungsspezifität zu untersuchen.

Die meisten PR-10 Allergene gelten als instabil gegen Hitze, und der Verzehr gekochter Speisen, die diese Allergene enthalten, wird daher in der Regel als ungefährlich eingestuft. Zu dem Karottenallergen Dau c 1 liegen jedoch widersprüchliche Ergebnisse bezüglich seiner Fähigkeit zur Rückfaltung nach Erhitzen vor. Deswegen wurden in dieser Arbeit erstmalig verschiedene Isoallergene und Varianten des Karottenallergens nach Hitzeeinwirkung bei verschiedenen pH-Werten strukturell und immunologisch analysiert. Darüber hinaus wurde der Einfluss der Karottenmatrix bei Erhitzen berücksichtigt. Die Ergebnisse zeigen, dass die meisten Dau c 1 Isoallergene bei neutralen pH-Werten nach Erhitzen die Fähigkeit zur Rückfaltung besitzen, während sie bei pH 3 hitzeresistente Formen bilden. Erhitzen in Anwesenheit der Karottenmatrix verringert zwar die Stabilität, es scheinen jedoch lineare Epitope erhalten zu bleiben, welche weiterhin eine allergische Reaktion auslösen.

Eine Peptidmassenfingerprintanalyse des gereinigten, natürlichen Karottenallergens gab Hinweise auf die Anwesenheit von mindestens acht bisher noch nicht identifizierten Isoallergenen oder Varianten. In dieser Arbeit konnte ein neues Dau c 1 Isoallergen, Dau c 1.0401, auf mRNA- und auf Proteinebene identifiziert werden. Es weist eine Sequenzidentität von 61 % oder weniger zu den bereits bekannten Isoallergenen auf. Sein allergenes Potential konnte im Mediatorenfreisetzungstest und in Inhibitionsexperimenten bestätigt werden, allerdings war die Reaktion im Immunoblot, Dot Blot und *enzyme-linked immunosorbent assay* (ELISA) sehr schwach. Dies weist darauf hin, dass Dau c 1.0401 nur konformationelle Epitope besitzt, welche offenbar beim Membrantransfer zerstört werden. Dau c 1.0401 scheint daher ein wichtiges Isoallergen zu sein, welches zur Allergenität von Karotten beiträgt. Aufgrund seiner geringen Fähigkeit zur Rückfaltung verliert es jedoch z.B. beim Erhitzen von Karotten während der Nahrungsmittelzubereitung vermutlich sein allergenes Potential.

Summary

Pathogenesis related class 10 (PR-10) proteins in plants are often produced in response to stress stimuli and are responsible for many pollen and food allergies. Their exact physiological function is still unclear. In Germany about 92 % of tree pollen allergy sufferers are sensitized to the major birch pollen allergen Bet v 1. There are numerous proteins homologous to Bet v 1 in various other pollen and foods such as apples, nuts, stone fruit, celery and carrots, which can trigger cross-allergies in birch pollen allergy sufferers due to their similar structure. PR-10 allergens usually consist of a mixture of different isoallergens and variants, which probably fulfill various tasks. Their three-dimensional structure is highly conserved and contains a hydrophobic cavity, which can bind small ligands, indicating a function as transport or storage protein.

A natural ligand of Bet v 1.0101 is the diglycosylated flavonoid quercetin-3-O-sophoroside (Q3OS). In this work a natural ligand of the hazel allergen Cor a 1, quercetin-3-O-(2"-O- β -D-glucopyranosyl)- β -D-galactopyranoside (Q3O-(Glc)-Gal), could be identified. This ligand is almost identical to Q3OS and differs only in the position of the OH group at the first sugar moiety (glucose vs. galactose). Nevertheless, both Cor a 1.0401 and Bet v 1.0101 have a very high specificity and bind only their own ligand. The structure of Cor a 1.0401, which was determined by multidimensional NMR spectroscopy, allowed a more precise characterization of the binding pocket. This provides the basis to investigate the cause of binding specificity in the future.

Most PR-10 allergens are considered unstable to heat, and the consumption of cooked food containing these allergens is therefore generally estimated as harmless. However, contradictory results on the carrot allergen Dau c 1 regarding its ability to refold after heating exist. Therefore, in this work, different isoallergens and variants of the carrot allergen after heat exposure at different pH-values were analyzed structurally and immunologically for the first time. Furthermore, the influence of the carrot matrix on heating was considered. The results show that most Dau c 1 isoallergens possess the ability to refold at neutral pH-values after heating, whereas heat resistant forms can be observed at pH 3. While heating in the presence of the carrot matrix reduces stability, linear epitopes apparently remain, which are still capable of triggering an allergic reaction.

A peptide mass fingerprint analysis of the purified, natural carrot allergen indicated the presence of at least eight previously unknown isoallergens or variants. In this work a new Dau c 1

isoallergen, Dau c 1.0401, could be identified on mRNA and protein level. It shows a sequence identity of 61 % or less to the already known isoallergens. Its allergenic potential could be confirmed in mediator release assays and inhibition assays, however the reaction in the immunoblot, dot blot and enzyme-linked immunosorbent assay (ELISA) was very weak. This indicates that Dau c 1.0401 contains exclusively conformational epitopes, which obviously get destroyed during membrane transfer. Dau c 1.0401 therefore appears to be an important isoallergen, which contributes to the allergenicity of carrots. However, due to its low refolding ability, it probably loses its allergenic potential while heating carrots during food preparation.

1 Einleitung

1.1 Die Häufigkeit allergischer Erkrankungen nimmt zu

Seit über fünfzig Jahren nimmt die Häufigkeit allergischer Erkrankungen in den industrialisierten Ländern zu (Jarvis and Burney, 1998), die Sensibilisierungsraten gegen Allergene liegen bei Schulkindern mittlerweile bei 40 % - 50 % (Pawankar *et al.*, 2013). Da die möglichen Gründe für diese Zunahme vielfältig und bisher noch kaum verstanden sind, ist es schwierig, entgegenzuwirken. Als Hauptrisikofaktoren für allergische Erkrankungen werden genetische Prädisposition (Holloway *et al.*, 2010; Ober & Yao, 2011), epigenetische und pränatale Einflüsse (Martino & Prescott, 2011), die Allergenexposition, ein verändertes Klima (D'Amato *et al.*, 2007) und Umwelteinflüsse wie Schadstoffbelastung (D'Amato, 2000, 2002; D'Amato *et al.*, 2007) diskutiert.

Umweltschadstoffe können auf der Oberfläche von Pollenkörnern haften und so deren Morphologie und ihr allergenes Potential verändern. Darüber hinaus können sie Entzündungen der Atemwege hervorrufen, wodurch die Permeabilität der Membranen erhöht wird. So wird es den Allergenen ermöglicht, die Schleimhautbarriere zu überwinden und allergische Reaktionen auszulösen (D'Amato, 2000, 2002; D'Amato *et al.*, 2007). Die Klimaveränderung beeinflusst weltweit das Wachstum von Pflanzen und somit auch die Pollenverbreitung, was zu einer erhöhten Allergenexposition führen kann (Bergmann, 2016; D'Amato, 2002). 1989 wurde von Strachan die „Hygienehypothese“ aufgestellt, welche die heutzutage stark verbesserte Hygiene, bedingt durch kleinere Familiengrößen, mehr Sauberkeit im Haushalt und verstärkte Körperpflege, als Ursache für den Anstieg allergischer Erkrankungen vorschlägt (Strachan, 1989). Es wird auch angenommen, dass durch den verringerten Kontakt mit Mikroorganismen im Kindesalter das Immunsystem nicht ausreifen kann, und deshalb allergische Erkrankungen begünstigt werden (Romagnani, 2004). Ebenso wird ein Einfluss der veränderten Essgewohnheiten auf die Entstehung von Allergien in Betracht gezogen. Der zunehmende Verzehr von Fertigprodukten soll ebenfalls im Zusammenhang mit der Allergieentwicklung stehen (Wickens *et al.*, 2005). Allerdings konnte bis jetzt keine der vielfältigen Thesen bewiesen werden und möglicherweise findet ein Zusammenspiel unterschiedlicher Faktoren statt.

Der rasante Anstieg von Allergien in den letzten rund 50 Jahren, auch als „Tsunami allergischer Erkrankungen“ bezeichnet, (Campbell & Mehr, 2015), verdeutlicht die Notwendigkeit,

intensive Forschung auf diesem Gebiet zu betreiben, damit die Prävention und die Behandlung von Allergien zukünftig verbessert werden kann.

1.2 Ablauf einer allergischen Reaktion

Bei einer allergischen Reaktion, auch Hypersensitivitätsreaktion vom Typ I (IgE vermittelte Immunreaktion oder Allergie vom Soforttyp) genannt, handelt es sich um das Ergebnis einer fehlgerichteten Immunantwort gegen ubiquitär vorkommende, eigentlich harmlose Proteine (Wills-Karp, 1999; Wills-Karp *et al.*, 2010). Diese Überreaktion löst dann die typischen allergischen Symptome wie Rhinokonjunktivitis, Urtikaria, allergisches Asthma (Averbeck *et al.*, 2007), bis hin zum lebensbedrohlichen anaphylaktischen Schock aus (Simons, 2008). Die molekularen Grundlagen der allergischen Reaktion sind in Abb. 1 schematisch dargestellt.

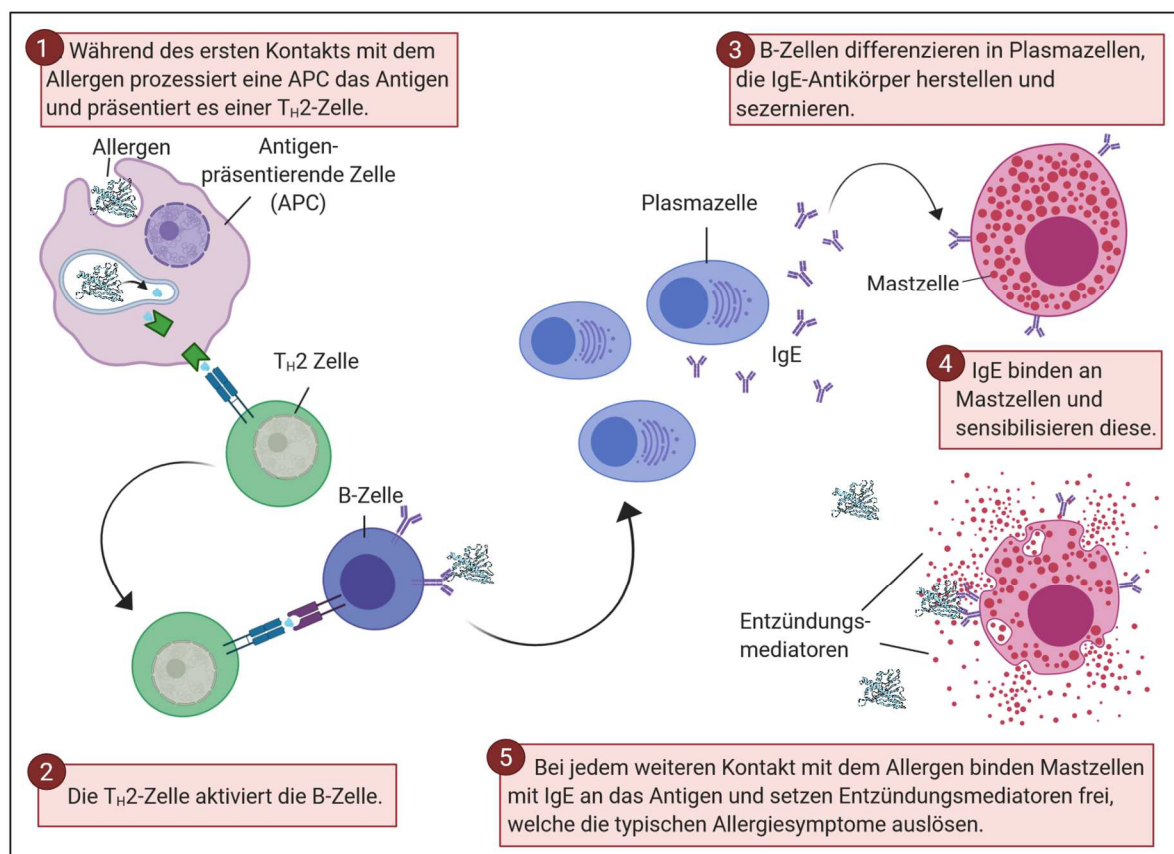


Abbildung 1: Ablauf einer allergischen Reaktion. Beim ersten Kontakt mit dem Allergen präsentiert eine Antigen-präsentierende Zelle (APC) Epitope des Allergens. $CD4^+$ -Helferzellen werden daraufhin in T-Helferzellen vom Typ 2 (T_H2 -Zellen) umgewandelt, welche wiederum B-Zellen aktivieren. Aktivierte B-Zellen differenzieren in Plasmazellen, welche IgE-Antikörper herstellen und sezernieren. IgE binden an Mastzellen. Bei jedem weiteren Kontakt bindet das Allergen an spezifisches IgE, was zur Freisetzung allergieinduzierender Mediatoren aus der Mastzelle führt. Diese lösen die typischen allergischen Symptome aus. (modifiziert nach <https://courses.lumenlearning.com/microbiology/chapter/hypersensitivities/>, dargestellt mit BioRender.com)

Beim ersten Kontakt mit dem Allergen findet eine Sensibilisierung statt, bei der noch keine allergischen Symptome auftreten. Eine antigenpräsentierende Zelle (APC) nimmt das Allergen auf und präsentiert Epitope davon auf ihrer Oberfläche.

Dadurch werden naive CD4⁺-Helferzellen in T-Helfer-Lymphozyten vom Typ 2 (T_H2) umgewandelt (Averbeck *et al.*, 2007), welche ihrerseits die Bildung IgE-produzierender B-Zellen (Abbas *et al.*, 1991) vermitteln. IgE-Antikörper, die dabei entstehen, binden mit hoher Affinität an den IgE Rezeptor FcεRI auf Effektorzellen wie Mastzellen und basophilen Granulozyten (Gould & Sutton, 2008; Metzger *et al.*, 1986).

Beim erneuten Kontakt mit dem Allergen findet die eigentliche allergische Reaktion statt: IgE binden mit der antigenbindenden Region an das Allergen, wodurch eine Quervernetzung der an FcεRI-gebundenen IgE-Antikörper stattfindet. Das löst eine Ausschüttung von Immun-Mediatoren aus, welche die allergischen Symptome hervorrufen.

1.3 Allergenepitope

Eine Bindungsstelle auf einem Antigen, an welche der komplementäre Antikörper bindet, wird als Epitop bezeichnet. Ein Epitop besteht normalerweise aus sechs bis zehn Aminosäuren auf der Oberfläche eines Antigens (Aalberse, 2000). Epitope können von der Tertiärstruktur des Antigens abhängig sein, wenn sie aus Aminosäuren bestehen, welche in der Primärstruktur voneinander entfernt liegen, aber durch die Faltung der Aminosäurekette in räumliche Nähe zueinander treten. Sie werden dann als konformationelle Epitope bezeichnet. Daneben existieren so genannte lineare Epitope, welche aus einer aufeinanderfolgenden Aminosäuresequenz bestehen.

Darüber hinaus wird zwischen B- und T-Zell-Epitopen unterschieden, welche sich in der Art der Epitoperkennung grundlegend unterscheiden: Während T-Zell-Rezeptoren ausschließlich lineare Epitope erkennen, binden B-Zell-Rezeptoren an Oberflächenbereiche auf dem Allergen. Diese können konformationell oder linear sein (Meno, 2011).

1.4 *in vivo* und *in vitro* Allergietests

Die Allergenität eines Proteins oder eines Extrakts kann sowohl *in vivo* als auch *in vitro* nachgewiesen werden. Zu den *in vivo* Tests zählen all jene, die direkt am Körper durchgeführt werden. Dazu gehören beispielsweise Hauttests, verzögerte Hauttests, orale und bronchiale Provokation. Häufig werden sogenannte *Skin Prick* Tests durchgeführt. Hierbei wird ein Tropfen des Allergenextrakts auf die Haut aufgebracht und dann mit einer Nadel durch den

Tropfen hindurch in die Haut gestochen. Eine allergische Reaktion äußert sich in einer lokalen Hautreaktion (Ownby, 1988).

Die meisten *in vitro* Tests sind allergosorbent (-AST). Das zu untersuchende Allergen wird hierbei an eine feste Phase gebunden und im Folgenden mit Patientenserum inkubiert. Wenn das Serum allergenspezifisches IgE enthält, bindet dieses an das Allergen. Nicht bindendes IgE wird zusammen mit dem restlichen Serum heruntergewaschen. Indem die Menge gebundener IgE-Antikörper bestimmt wird, kann die Menge der allergenspezifischen IgEs im Serum abgeschätzt werden. Dafür werden markierte Antikörper gegen humanes IgE eingesetzt. Verschiedene Tests unterscheiden sich in der Art der festen Phase, an welche die Allergene gekoppelt werden und in der Methode, wie die Antikörper gegen humanes IgE markiert werden. Im *radioactive allergosorbent assay* (RAST) sind diese radioaktiv markiert, im *enzyme linked immunosorbent assay* (ELISA) wird eine enzymatische Markierung verwendet, welche die Reaktion eines Substrats in ein Produkt katalysiert. Das Produkt ist typischerweise farbig und kann colorimetrisch quantifiziert werden (Ownby, 1988). Wichtig bei diesen *in vitro* Tests ist, zu beachten, dass sie lediglich die Anwesenheit spezifischer IgE-Antikörper im Serum nachweisen, welche aber nicht zwingend zu körperlichen Symptomen führen müssen. Bei *in vivo* Tests wie dem *Skin Prick* Test, sind auch andere physiologische Faktoren, wie die Bereitschaft der Mastzellen, Mediatoren freizusetzen, ausschlaggebend für die Stärke der Hautreaktion. Ein weiterer Nachteil der *in vitro* Tests ist die Tatsache, dass die Allergene immobilisiert werden. Die Immobilisation kann dazu führen, dass Proteine – zumindest teilweise – denaturieren und konformationelle Epitope zerstört werden. Epitope, die sich in dem Bereich befinden, an dem das Allergen mit der festen Phase fixiert ist, sind ebenfalls nicht zugänglich für die Bindung der IgE-Antikörper (Kaul *et al.*, 2007). Diese *in vitro* Tests weisen zwar nur eine geringe Zahl falsch-positiver, aber eine moderate Menge falsch-negativer Ergebnisse auf (Ownby, 1988).

Eine andere *in vitro* Methode zur Bestimmung der Potenz oder biologischen Aktivität eines Allergens, welche den *in vivo* Tests weitestgehend ähnelt, ist der Mediatorenfreisetzungstest (*mediator release assay*, MRA) (Abb. 2) (Kaul *et al.*, 2007). Er beruht auf der Aktivierung und Mediatorenfreisetzung von Effektorzellen. Hierfür werden gentechnisch veränderte, basophile Leukämiezellen der Ratte (*rat basophilic leukemia*, RBL-2H3 Zellen) verwendet, die den menschlichen hochaffinen IgE-Rezeptor FcεRI (vgl. Kap 1.2) exprimieren. Diese Zellen können mit Allergikerserum sensibilisiert werden. Das IgE bindet hierbei an den FcεRI-Rezeptor. Die Zugabe eines spezifischen Allergens führt zur Quervernetzung der

gebundenen IgE-Antikörper, welche die Freisetzung proinflammatorischer Mediatoren, wie Histamin, Serotonin und β -Hexosaminidase auslöst. Die Mediatorenkonzentration wird stellvertretend im Medium über die Substratumsetzung der β -Hexosaminidase colorimetrisch bestimmt (Kaul *et al.*, 2007).

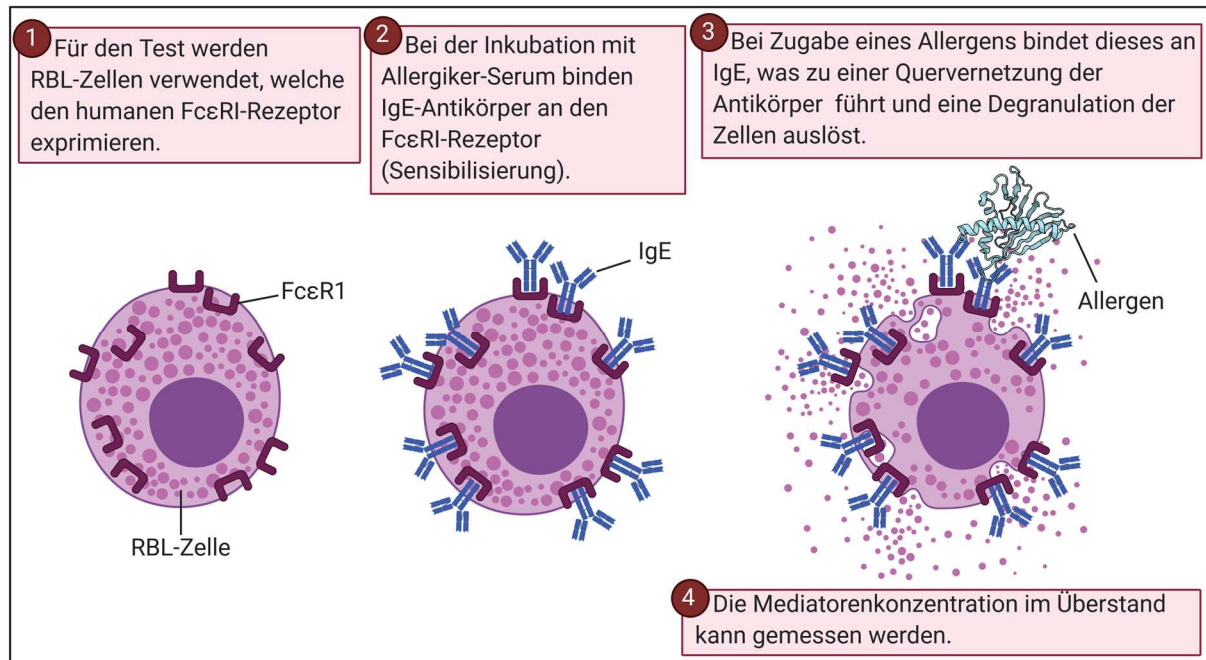


Abbildung 2: Schematische Darstellung des Mediatorenfreisetzungstests nach Vogel *et al.*, 2005.

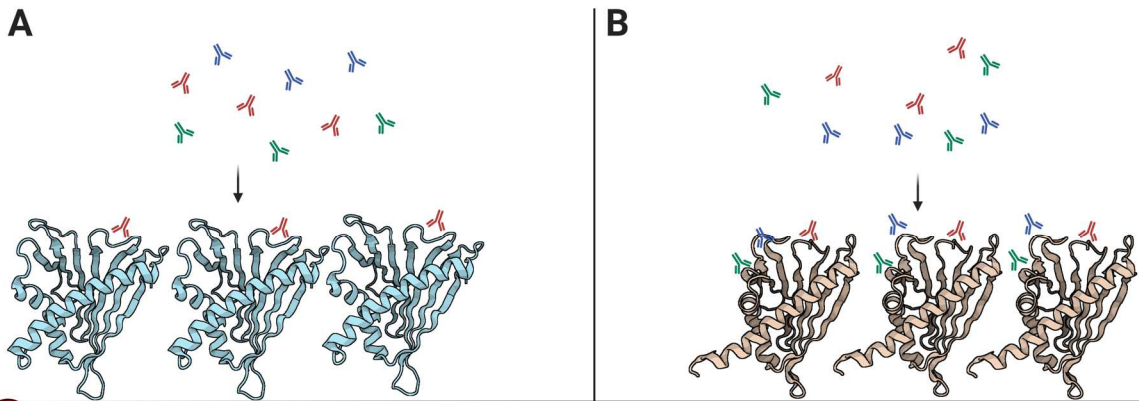
1.5 Die Basis einer Kreuzallergie sind konservierte Epitope

Antikörper, die während der Sensibilisierung gegen ein Allergen gebildet wurden, können ebenfalls an homologe Epitope eines strukturell verwandten Proteins binden und eine sogenannte allergische Kreuzreaktion auslösen. Um Kreuzallergien zu diagnostizieren und das primär sensibilisierende Allergen zu identifizieren, können Kreuzinhibitionsversuche durchgeführt werden (Abb. 3).

Hierbei wird zunächst ein Allergen immobilisiert und mit Allergikerserum, das IgE-Antikörper gegen das Allergen enthält, inkubiert. Die IgE-Bindung an das immobilisierte Allergen kann durch Präinkubation des Serums mit steigenden Konzentrationen eines anderen Allergens inhibiert werden, sofern die beiden Allergene homologe Epitope besitzen. Das sensibilisierende Allergen kann identifiziert werden, da dessen IgE-Bindung durch Präinkubation des Serums mit dem Kreuzallergen meist nur teilweise inhibiert wird, während die Inhibition der IgE-Bindung des Kreuzallergens durch das sensibilisierende Allergen komplett ist. Das hat folgenden Grund: Während der Sensibilisierung wird eine Vielzahl unterschiedlicher

Antikörper produziert. Nur ein Teil dieser Antikörper bindet an das Kreuzallergen, da dieses – verglichen mit dem sensibilisierenden Allergen - nicht alle Epitope besitzt (Mills *et al.*, 2006).

1 Immobilisation jeweils eines der beiden Allergene (A und B) und Inkubation mit Allergikerserum. IgE-Antikörper des Serums binden an die Epitope.



2 Präinkubation des Serums mit dem jeweils anderen Allergen in löslicher Form: Das sensibilisierende Allergen bindet alle IgE-Antikörper und inhibiert so die IgE-Bindung des Kreuzallergens komplett (A). Das Kreuzallergen bindet nur einen Teil der Antikörper und inhibiert die IgE-Bindung des sensibilisierenden Allergens nicht komplett (B).

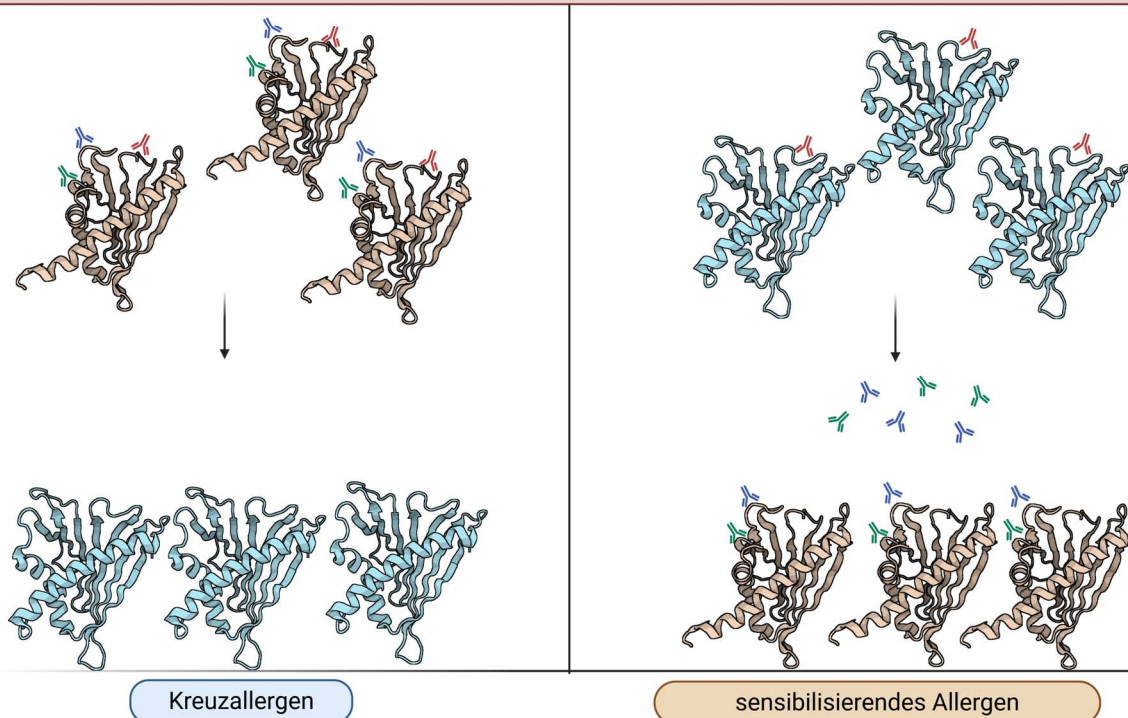


Abbildung 3: Schematische Darstellung der Kreuzinhibition zur Identifizierung von (A) Kreuzallergenen und (B) sensibilisierenden Allergenen. Erstellt mit BioRender.com

Zusätzlich kann die Bindungsaffinität der im Serum enthaltenen IgE-Antikörper gegen Epitope des Kreuzallergens niedriger sein, da diese leichte Unterschiede zu den Epitopen des sensibilisierenden Allergens aufweisen.

1.6 Behandlung von Allergien

1.6.1 Behandlungsstrategien

Die verschiedenen Strategien zur Behandlung von Allergien umfassen die Vermeidung des Kontakts mit dem entsprechenden Allergen, die Einnahme von Medikamenten zur Linderung der Symptome (z.B. Antihistaminika) und die allergenspezifische Immuntherapie (SIT), auch bekannt als Hyposensibilisierung (Berin, 2014).

Letztere ist die einzige bekannte kausale Behandlung allergischer Erkrankungen. Dabei werden dem Patienten wiederholt steigende Mengen an Allergenextrakt zugeführt, um eine klinische Toleranz des symptomauslösenden Allergens zu erreichen. Der zugrunde liegende Mechanismus ist noch nicht vollständig verstanden. Allerdings wurden bei einer erfolgreichen Immuntherapie erhöhte Konzentrationen allergenspezifischer IgG4-Antikörper gefunden (Aalberse, 2011; Aalberse *et al.*, 1983; Ewan *et al.*, 1993; Francis *et al.*, 2008; Gehlhar *et al.*, 1999; Movérare *et al.*, 2001; Shamji *et al.*, 2012). Funktionelle IgG4-Antikörper konkurrieren mit IgE um die Bindung an das Allergen und inhibieren so die allergische Reaktion, da eine IgG-Bindung keine Mediatorenausschüttung der Effektorzellen verursacht (Wachholz *et al.*, 2003). Eine aktuelle Studie konnte nach Injektion spezifischer, monoklonaler IgG4-Antikörper gegen das Katzenallergen Fel d 1 eine Reduktion der allergischen Symptome bei Katzenhaarallergikern demonstrieren (Orengo *et al.*, 2018). Bei dieser passiven Immuntherapie handelt es sich um einen neuen, vielversprechenden Therapieansatz gegen Allergien. Das größte Risiko bei der oben beschriebenen, „aktiven“ Immuntherapie stellen Komplikationen durch eine allergische Reaktion während der Behandlung mit dem Allergenextrakt dar (Lüderitz-Püchel *et al.*, 2001).

Ein Ansatz, um das Risiko für diese Nebenwirkungen zu minimieren, ist die Modifikation der Allergene, um ihr allergenes Potential herabzusetzen. Es konnte gezeigt werden, dass trotz verminderter Allergenität noch eine erfolgreiche Immuntherapie möglich ist. Die Modifikation kann beispielweise durch Erhitzen (Kim *et al.*, 2011; Leonard *et al.*, 2012) und Glykosylierung (Rupa *et al.*, 2014) erreicht werden. Ein weiterer Ansatz ist die Verwendung natürlich vorkommender oder gentechnisch veränderter hypoallergener Varianten (Ferreira *et al.*, 2014).

1.6.2 Rekombinante Allergene in Therapie und Diagnostik

Die Verwendung rekombinanter Allergene für die Therapie und Diagnostik („*component resolved diagnosis*“) von Allergien stellt einen vielversprechenden Ansatz dar. Vorteile gegenüber natürlichen Allergenextrakten sind größere Ausbeuten, höhere Reinheit, die

Abwesenheit von Endotoxinen und die höhere Standardisierung (Chapman *et al.*, 2000). In verschiedenen Studien konnten bereits die Vorteile von rekombinanten im Vergleich zu natürlichen Allergenen in der Immuntherapie, wie die Vermeidung neuer Sensibilisierungen und die Abwesenheit schwerer Nebenwirkungen, bestätigt werden (Cromwell *et al.*, 2011; Pauli *et al.*, 2008; Wild *et al.*, 2007). Die größte Herausforderung bei der Herstellung rekombinanter Allergenprodukte ist die Tatsache, dass viele Allergene im natürlichen Extrakt als Mischung aus unterschiedlichen Isoallergenen und Varianten vorliegen (Pomés *et al.*, 2018). Es ist möglich, dass Allergiker gegen unterschiedliche Varianten reagieren (Ballmer-Weber *et al.*, 2005; Wangorsch *et al.*, 2012). Wenn also das rekombinante Produkt nicht alle natürlichen Varianten beinhaltet, kann in der Diagnostik ein falsch-negatives Ergebnis auftreten. Allerdings kann dies auch bei natürlichen Allergenextrakten passieren, da die Zusammensetzung der Varianten auch dort variieren kann (Erler *et al.*, 2011). Darüber hinaus gibt es sehr instabile Allergene, die in manchen natürlichen, für Diagnostik und Therapie zugelassenen, Allergenextrakten nicht mehr aktiv vorliegen. Aus diesem Grund ist beispielsweise das Bienengiftallergen Api m 10 in vielen therapeutischen Extrakten unterrepräsentiert (Blank *et al.*, 2011; Frick *et al.*, 2016). Die Aufgabe besteht also darin, einen rekombinanten „Allergencocktail“ herzustellen, der dem natürlichen Produkt weitestgehend ähnelt und alle allergenen Komponenten in ihrer aktiven Form enthält (Chapman *et al.*, 1997; Scheiner and Kraft, 1995; Thomas and Smith, 1999; Valenta *et al.*, 1999). Dafür ist allerdings eine genaue Analyse des natürlichen Extrakts notwendig, um die darin enthaltenen Isoallergene und Varianten zu identifizieren und zu charakterisieren.

1.7 Merkmale eines Allergens

Ein grundlegendes Rätsel in der Allergologie ist die Frage, was ein bestimmtes Protein zu einem Allergen macht. Obwohl Allergien weit verbreitet sind, besitzt nur ein sehr kleiner Anteil aller Proteine die Fähigkeit, eine allergische Reaktion auszulösen (Karp, 2010). Warum führen nur wenige, spezifische Proteine zu diesen abweichenden T- und B-Zell-Antworten?

Allergene stammen aus verschiedenen Umweltquellen und unterscheiden sich maßgeblich in ihrer Größe, Funktion, ihrem Glykosylierungsstatus und ihrer Resistenz gegenüber Proteolyse. Auch die T- und B-Zellepitope, welche die allergische Reaktion auslösen (vgl. 1.2 und 1.3), sind sehr vielfältig. Es ist fraglich, ob die alleinige Existenz solcher Epitope ein Allergen ausmacht. Das Enzym Norcochlorinsynthase aus Johanniskraut zeigt z.B. eine hohe strukturelle Ähnlichkeit zu PR-10 Allergenen, aber löst keine allergische Reaktion aus (Berkner *et al.*, 2008).

Die Fähigkeit, das angeborene Immunsystem zu aktivieren, scheint ein wichtiges Merkmal für Allergene zu sein (Deifl & Bohle, 2011). Die enzymatische Aktivität von Allergenen kann die Schleimhautbarrierefunktion zerstören (Bufe, 1998; Herbert *et al.*, 1995) und Zellen des angeborenen und erworbenen Immunsystems aktivieren. An Allergene gebundene Lipide können ebenfalls das angeborene Immunsystem aktivieren (Thomas *et al.*, 2005). Daher ist es von besonderem Interesse, die physiologische Funktion eines Allergens zu kennen, um zu wissen, ob es enzymatische Aktivität besitzt oder welche Liganden es bindet.

1.8 Klassifizierung und Nomenklatur von Allergenen

In der AllFam-Datenbank (*database of allergen families*) sind alle bisher bekannten Allergene aufgelistet und in Proteinfamilien eingeteilt (Radauer *et al.*, 2008). Dort sind insgesamt 1042 Allergene in 151 Familien eingeteilt. 31 % der Allergene können den sechs größten Familien zugeordnet werden. Einige Vertreter der prominentesten Allergenfamilien sind in Abb. 4 dargestellt. Die Allergennomenklatur setzt sich wie folgt zusammen: die ersten drei Buchstaben der Gattung, gefolgt von dem ersten Buchstaben der Spezies, aus dem das Allergen stammt, sowie eine arabische Ziffer, die die Chronologie der Allergenidentifikation anzeigt (Chapman *et al.*, 2007; Pomés *et al.*, 2018). Beispielweise wird das erste aus der gemeinen Hasel (*Coryllus avellana*) identifizierte Allergen als Cor a 1 bezeichnet. Die Nomenklatur schließt darüber hinaus unterschiedliche molekulare Formen des gleichen Allergens mit ein: Isoallergene und Varianten. Isoallergene sind homologe Allergene, welche folgende biochemische Eigenschaften teilen: ähnliche Molekülgröße, ähnliche oder gleiche biologische Funktion (falls bekannt), und mindestens 67 % Identität der Aminosäuresequenz (wobei es sich bei der Sequenzidentität um eine Richtlinie handelt, die nicht immer eingehalten werden muss). Isoallergene können aus mehreren sogenannten Varianten (früher auch als Isoformen bezeichnet) bestehen, welche eine Aminosäuresequenzidentität von über 90 % besitzen. Isoallergene und deren Varianten werden durch weitere Nummern gekennzeichnet, die hinter der oben erläuterten Allergennummer nach einem Punkt folgen: Die ersten zwei Ziffern nach dem Punkt bezeichnen das Isoallergen, und weitere zwei Ziffern Varianten des Isoallergens (Pomás *et al.*, 2018). So existieren für Cor a 1 beispielweise die Isoallergene Cor a 1.01 bis Cor a 1.04. Für das Isoallergen Cor a 1.01 sind bisher insgesamt vier Varianten bekannt,

Cor a 1.0101 bis Cor a 1.0104 (www.allergen.org, Stand am 14.01.2020).

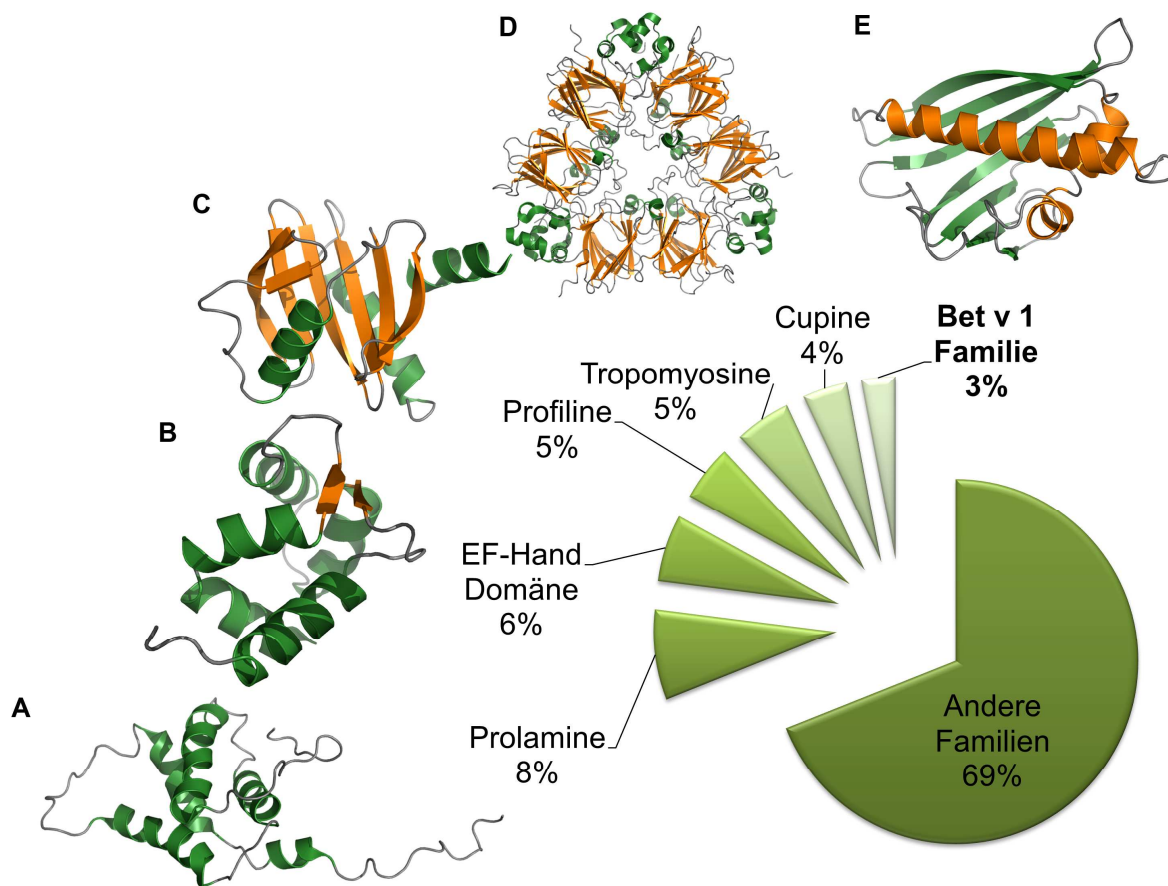


Abbildung 4: Übersicht über die Allergenfamilien. A-E: Bänderdiagramme von Vertretern verschiedener Allergenfamilien. α -Helices sind in grün, β -Faltblätter in orange und Schleifen in grau dargestellt (Software: Pymol, Schrödinger 2010), A: das Prolamin Ara h 6 aus der Erdnuss (pdb: 1w2q), B: das Allergen Bet v 4 aus der Familie der EF-Hand-Domäne aus Birkenpollen (pdb: 1h4b,) C: Das Profilin Bet v 2 aus Birkenpollen (pdb: 1cqa), D: Das Cupin Gly m 6 aus der Sojabohne (pdb: 1fxz), E: Das Hauptallergen der Bet v 1 Familie Bet v 1.0101 aus Birkenpollen (pdb: 4a87). (AllFam, database of allergen families, 24.10.19)

Vertreter der Bet v 1 Familie sind im Pflanzenreich weit verbreitet. Aufgrund von Sequenzähnlichkeiten wurde die Familie in zwei große und einige kleinere Unterfamilien unterteilt (Radauer *et al.*, 2008). Bei der größten Unterfamilie handelt es sich um die PR-10 Unterfamilie (Fernandes *et al.*, 2013). Die Expression der PR-10 Gene wird entweder durch Pathogenangriffe oder abiotischen Stress induziert, doch es gibt auch PR-10 Gene, welche in bestimmten Entwicklungsstadien oder konstitutiv exprimiert werden (Ebner *et al.*, 2001; Fernandes *et al.*, 2013).

1.9 PR-10 Allergene – die größte Bet v 1-Unterfamilie

Obwohl die Bet v 1 Familie nur etwa 3 % aller Allergene beinhaltet (Abb. 4) und damit eine vergleichsweise kleine Gruppe darstellt, kommt ihr dennoch besondere Bedeutung zu. Einerseits deshalb, weil – abhängig von der Region – zwischen 62 % und 98 % aller Baumpollenallergiker gegen das Hauptbirkenpollenallergen Bet v 1 sensibilisiert sind (Movérare *et al.*, 2002). In Deutschland sind es 92 % (Canis *et al.*, 2011). Andererseits, weil Bet v 1 homologe Proteine in zahlreichen Nahrungsquellen wie Äpfeln, Steinobst, Sellerie, Karotten, Nüssen und Sojabohnen vorkommen und Kreuzallergien auslösen können (vgl. 1.5). Tab. 1 gibt einen Überblick über die bisher identifizierten Allergene der PR-10 Familie.

Tabelle 1: Allergene Proteine aus der PR-10 Familie. AllFam-Datenbank: AF069, Stand: Juli 2020, n.d.: keine Angaben bekannt.

| Name | Quelle | Art der Exposition | Isoallergene ¹ | Varianten ¹ |
|--------------|--|------------------------|---------------------------|------------------------|
| Act c 1 | <i>Actinidia chinensis</i> Goldene Kiwi | Verzehr | 1 | 1 |
| Act d 11 | <i>Actinidia deliciosa</i> Grüne Kiwi | Verzehr | 1 | 1 |
| Act d 8 | <i>Actinidia deliciosa</i> Grüne Kiwi | Verzehr | 1 | 1 |
| Aln g 1 | <i>Alnus glutinosa</i> Europäische Erle | Inhalation | 1 | 1 |
| Api g 1 | <i>Apium graveolens</i> Sellerie | Verzehr | 2 | 2 |
| Ara h 8 | <i>Arachis hypogaea</i> Erdnuss | Verzehr | 2 | 2 |
| Bet pl PR-10 | <i>Betula platyphylla</i> Asiatische Weißbirke | Inhalation | n.d. | n.d. |
| Bet v 1 | <i>Betula verrucosa</i> Europäische Weißbirke (<i>Betula pendula</i>) | Inhalation | 3 | 27 |
| Car b 1 | <i>Carpinus betulus</i> Europäische Hainbuche | Inhalation | 3 | 16 |
| Cas s 1 | <i>Castanea sativa</i> Edelkastanie | Inhalation | 1 | 1 |
| Cor a 1 | <i>Corylus avellana</i> Europäische Hasel | Inhalation, Verzehr | 4 | 10 |
| Dau c 1 | <i>Daucus carota</i> Karotte | Verzehr | 3 | 7 |
| Fag s 1 | <i>Fagus sylvatica</i> Europäische Buche | Inhalation | 1 | 1 |
| Fra a 1 | <i>Fragaria ananassa</i> Erdbeere | Verzehr | 1 | 2 |
| Gly m 4 | <i>Glycine max</i> Sojabohne | Verzehr | 1 | 1 |
| Jug r 5 | <i>Juglans regia</i> Englische Walnuss | Verzehr | 1 | 1 |
| Mal d 1 | <i>Malus domestica</i> Apfel | Verzehr | 4 | 24 |
| Mor a PR-10 | <i>Morus alba</i> Weißer Maulbeerbaum | Verzehr | n.d. | n.d. |
| Mor b PR-10 | <i>Morus bombycis</i> Chinesischer Maulbeerbaum | Verzehr | n.d. | n.d. |
| Ost c 1 | <i>Ostrya carpinifolia</i> Europäische Hopfenbuche | Inhalation | 1 | 1 |
| Pru ar 1 | <i>Prunus armeniaca</i> Aprikose | Verzehr | 1 | 1 |
| Pru av 1 | <i>Prunus avium</i> Süßkirsche | Verzehr | 2 | 4 |
| Pru p 1 | <i>Prunus persica</i> Pfirsich | Verzehr | 3 | 3 |
| Pyr c 1 | <i>Pyrus communis</i> Birne | Verzehr | 1 | 1 |
| Que a 1 | <i>Quercus alba</i> Weiße Eiche | Inhalation | 4 | 4 |
| Rub i 1 | <i>Rubus idaeus</i> Rote Himbeere | Verzehr | 1 | 1 |
| Sola l 4 | <i>Solanum lycopersicum</i> Tomate | Verzehr | 2 | 2 |
| Vig r 1 | <i>Vigna radiata</i> Mungbohne | Verzehr | 1 | 1 |
| Vig r 6 | <i>Vigna radiata</i> Mungbohne | Verzehr | 1 | 1 |

¹Die Anzahl der Isoallergene und Varianten wurde www.allergen.org entnommen und umfasst alle aktuell in der WHO/IUIS Allergen Nomenklatur gelisteten PR-10 Allergene (Stand: Juli 2020).

Bet v 1 homologe Allergene sind kleine, globuläre Proteine mit Molekülmassen um 17 kDa und theoretischen isoelektrischen Punkten zwischen 4,4 (Gly m 4.0101 aus Sojabohne) und 6,1 (Cor a 1.0401 aus Haselnuss) (Vieths *et al.*, 2002). Sie besitzen alle eine gemeinsame Grundstruktur, die aus einer langen, C-terminalen Helix, zwei kürzeren, V-förmig angeordneten Helices und einem antiparallelen, siebensträngigen β -Faltblatt besteht. Diese Elemente umgeben einen relativ großen, hydrophoben Hohlraum (Gajhede *et al.*, 1996). Abb. 5A zeigt die Struktur des Birkenpollenallergens Bet v 1.0101. Eine Überlagerung der Strukturen von PR-10 Allergenen aus unterschiedlichen Quellen (Birkenpollen, Erdbeere, Kirsche, Sojabohne, Abb. 5B) veranschaulicht die hohe dreidimensionale Ähnlichkeit, welche die Basis für eine allergische Kreuzreaktion ist (vgl. Kap. 1.5). Die strukturelle sowie sequentielle Ähnlichkeit ist im Bereich der glyzinreichen Schleife (Gly47 - Thr53, Stäbchendarstellung in Abb. 5B) besonders hoch. Diese Schleife hat sequenzielle Ähnlichkeit mit dem *phosphate-binding loop* (p-loop), einem Motiv, welches mit Phosphatbindung assoziiert wird und in vielen nukleotidbindenden Proteinen vorkommt (Saraste *et al.*, 1990). Es konnte für PR-10 Proteine jedoch keine ATP-Bindung nachgewiesen werden (Koistinen *et al.*, 2005) und zusätzlich unterscheidet sich die glyzinreiche Schleife konformationell vom p-loop (Biesiadka *et al.*, 2002). Stattdessen wird vermutet, dass die glyzinreiche Schleife ein Epitop darstellt, welches vermutlich IgE-Antikörper bindet. Diese Interaktion wurde bereits anhand einer Kristallstruktur von Bet v 1.0101 mit dem Fab-Fragment des murinen IgG Antikörpers BV16 gezeigt (Mirza *et al.*, 2000).

Mit etwa 70 % leidet der Großteil aller Birkenpollenallergiker nach Aufnahme von Nahrungsmitteln, die Bet v 1 homologe Proteine enthalten, unter oralen Allergiesymptomen wie Taubheitsgefühl im Mund, Juck- und Niesreiz (Vieths *et al.*, 2002). In einer Studie mit 50 Birkenpollenallergikern zeigten 99 % IgE Bindung zu Mal d 1 aus Apfel, 93 % zu Cor a 1 aus Hasel, 59 % zu Api g 1 aus Sellerie und 38 % zu Dau c 1 aus Karotte (Bohle, 2007).

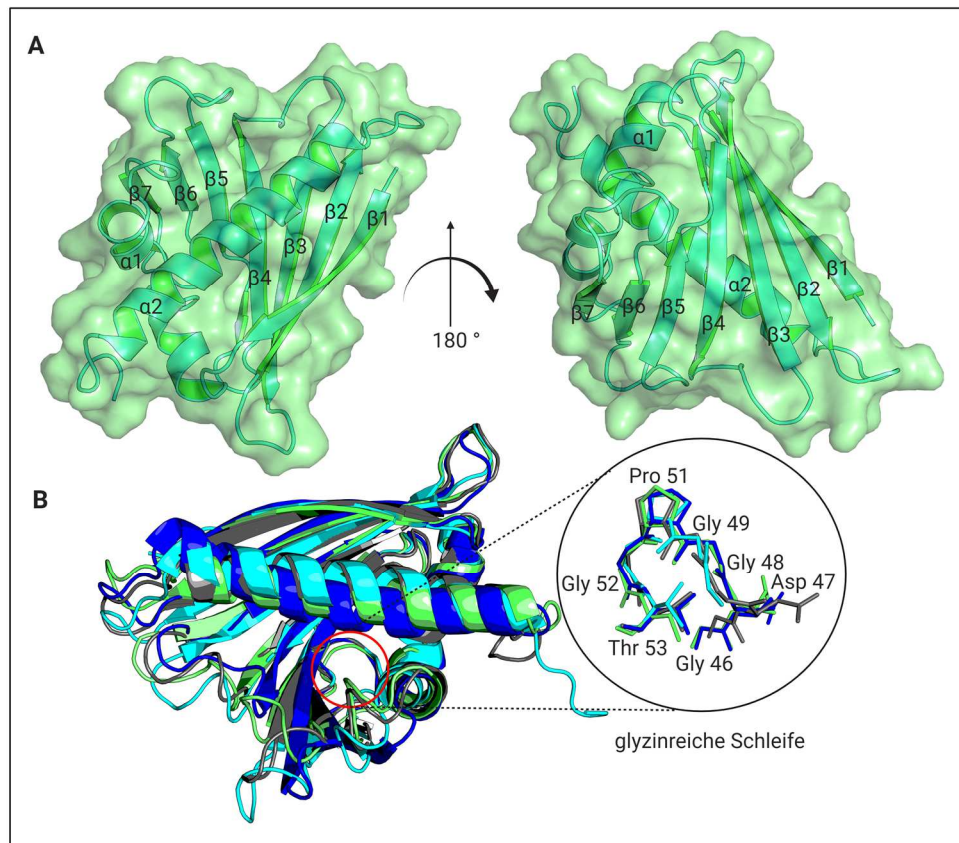


Abbildung 5: Struktur der PR-10 Allergene. **A:** Bänderdiagramm und Oberflächendarstellung des Hauptbirkenpollenallergens Bet v 1.0101 (pdb: 1BV1). Die Sekundärstrukturelemente sind beschriftet. **B:** Vergleich verschiedener PR-10 Allergene. Überlagerung der Strukturen von Bet v 1.0101 aus Birkenpollen (grün, pdb: 1BV1), Pru av 1 aus Kirsche (grau, pdb: 1E09), Fra a 1E aus Erdbeere (türkis, pdb: 2LPX) und Gly m 4 aus Sojabohne (dunkelblau, pdb: 2K7H). Die stark konservierte glyzinreiche Schleife ist vergrößert dargestellt und die entsprechenden Aminosäuren von Bet v 1.0101 angegeben. Die Proteinstrukturen wurden mit Pymol (Schrödinger, 2010) verglichen und dargestellt, die Abbildung wurde mit BioRender.com erstellt.

Ein weiteres Merkmal Bet v 1 homologer Proteine ist ihre Zusammensetzung aus unterschiedlichen Isoallergenen und Varianten (vgl. 1.8, gelistet in Tab. 1), welche sich teilweise erheblich in ihren immunogenen und biochemischen Eigenschaften unterscheiden (Breitenbach *et al.*, 1996; Ferreira *et al.*, 1996). So wurden für Bet v 1 bisher mindestens 18 Isoallergene und Varianten auf Proteinebene identifiziert (Spiric *et al.*, 2015).

Trotz der weiten Verbreitung Bet v 1 homologer Proteine im Pflanzenreich ist deren physiologische Funktion noch weitgehend unklar (Fernandes *et al.*, 2013). Da die Expression vieler PR-10 Gene als Antwort auf biotische oder abiotische Stressstimuli erfolgt (Breda *et al.*, 1996; Crowell *et al.*, 1992) wird angenommen, dass die Proteine eine Rolle im Abwehrsystem der Pflanze einnehmen. Andererseits werden PR-10 Gene auch konstitutiv oder in bestimmten Entwicklungsstadien exprimiert (Sikorski *et al.*, 1999), was sowohl auf eine allgemeinere Funktion als auch auf vielfältige Rollen der unterschiedlichen Isoallergene oder Varianten hinweist.

Der hydrophobe Hohlraum in der Mitte der PR-10 Allergene deutet auf eine Funktion als Transport- oder Speicherprotein hin. Es wurde gezeigt, dass gereinigtes Bet v 1 unterschiedliche Liganden wie Fettsäuren, Cytokine und Flavonoide mit Dissoziationskonstanten im mikromolaren Bereich binden kann (Asam *et al.*, 2014; Kofler *et al.*, 2012; Mogensen *et al.*, 2002), diese wurden allerdings nicht als natürliche Liganden nachgewiesen.

1.10 Flavonoide sind natürliche Liganden von PR-10 Allergenen

In mehreren Studien konnten Flavonoide als physiologisch relevante Bindungspartner von PR-10 Allergenen identifiziert werden (Casañal *et al.*, 2013; Mogensen *et al.*, 2002; Seutter von Loetzen *et al.*, 2014, 2015). Flavonoide sind weit verbreitete Sekundärmetabolite von Pflanzen. Im Grundaufbau handelt es sich um Polyphenole, welche eine oder mehrere Hydroxylgruppen besitzen können, an denen Modifikationen wie Methylierung oder Glykosylierung möglich sind (Kim *et al.*, 2015; Kumar and Pandey, 2013). In Abb. 6A ist Flavon als Grundgerüst der Flavonoide dargestellt. Sie übernehmen vielfältige Aufgaben, wie den Schutz vor UV-Strahlung (Li *et al.*, 1993) und reaktiven Sauerstoffspezies (Fini *et al.*, 2011), die Regulierung der Auxinverteilung und somit auch die Regulierung der Entwicklung (Brunetti *et al.*, 2013), die Regulierung des Pollenschlauchwachstums (Mo *et al.*, 1992), und nicht zuletzt die Pigmentierung von Blüten und Früchten, was die Bestäubung bzw. Verteilung der Samen erleichtert (Bell & Charlwood, 1980).

Die Unterdrückung der Genexpression des Erdbeerallergens Fra a 1 führte zu einer Herabregulation der Flavonoid-Biosynthese, was eine verminderte Rotfärbung der Erdbeeren zur Folge hatte (Muñoz *et al.*, 2010). Es wurde gezeigt, dass Fra a 1 natürliche Flavonoide binden kann (Casañal *et al.*, 2013) und vorgeschlagen, dass dieses Allergen eine Rolle in der Kontrolle des Sekundärmetabolismus spielen könnte, indem es Metaboliten der Flavonoid-Biosynthese bindet (Casañal *et al.*, 2013).

Darüber hinaus konnte das glykosylierte Flavonoid Quercetin-3-O-Sophorosid (Q3OS) als natürlicher Ligand des Birkenpollenallergens Bet v 1 identifiziert werden (Seutter von Loetzen *et al.*, 2014) (Abb. 6B). Q3OS besteht aus einem Flavonoid (Quercetin) und einem Zweifachzucker (Sophorose). Zur physiologischen Bedeutung des Bet v 1/Q3OS Komplexes wurden folgende zwei Hypothesen aufgestellt: Zum einen könnte der Komplex die Birkenpollen-DNA vor UV-Licht schützen. Zum anderen wird Q3OS im Komplex mit Bet v 1 gespeichert, und kann bei der Bestäubung freigegeben werden: Im Plasma vorhandene Glucosyltransferasen können das freigegebene Q3OS deglykosylieren, wobei Quercetin entsteht, welches das Wachstum des Pollenschlauchs induzieren kann (Seutter von Loetzen *et*

al., 2014). Zwar weiß man nicht, wie der natürliche Ligand Q3OS im Protein orientiert ist, aber es existiert eine Kristallstruktur von Bet v 1.0101 im Komplex mit dem Flavonoid Naringenin (Kofler *et al.*, 2012), die verdeutlicht, wie dieses im Protein orientiert ist (Abb. 6C). Ob der Flavonoidrest des natürlichen Liganden ähnlich lokalisiert ist, und wie die Orientierung des Zuckers vorliegt, konnte bisher nicht mit Sicherheit geklärt werden.

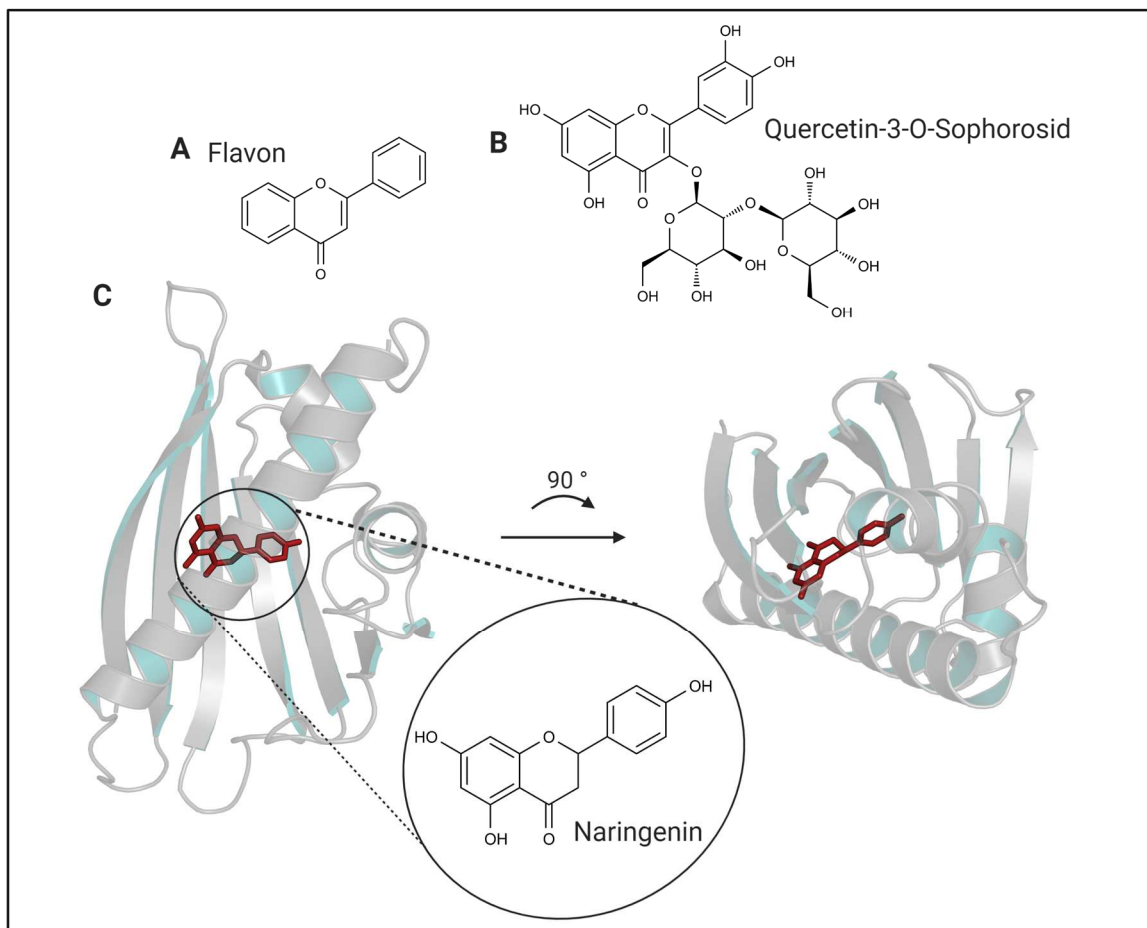


Abbildung 6: Flavonoide als Liganden von PR-10 Allergenen. A: Grundgerüst der Flavonoide (Flavon), B: Natürlicher Ligand des Hauptbirkenpollenallergens Bet v 1.0101, das glykosylierte Flavonoid Quercetin-3-O-Sophorosid (Q3OS), C: Bänderdiagramm der Kristallstruktur von Bet v 1.0101 im Komplex mit dem Flavonoid Naringenin (pdb: 4A87). Proteinstrukturen wurden mit Pymol (Schrödinger, 2010) dargestellt. Die Abbildung wurde mit BioRender.com angefertigt.

Die Ligandenbindung könnte ebenfalls einen Einfluss auf die Allergenität von PR-10 Allergenen haben – entweder, indem sie die Sensibilisierung beeinflusst (Asam *et al.*, 2014), oder indem leichte Strukturänderungen im Zuge einer Ligandenbindung die Epitope verändern. Darüber hinaus konnte gezeigt werden, dass gebundene Liganden die Stabilität eines Allergens gegenüber proteolytischem Abbau oder Hitzedenaturierung und somit die Allergenität indirekt erhöhen können (Soh *et al.*, 2019).

1.11 Stabilität von PR-10 Allergenen

Allergenstabilität kann definiert werden als die Fähigkeit der Proteine, ihre native, dreidimensionale Struktur nach chemischer, physikalischer oder enzymatischer Behandlung beizubehalten (Breiteneder and Mills, 2005; Deller *et al.*, 2016). Allergene, die in Insektengift enthalten sind und intradermal injiziert werden, besitzen meist keine erhöhte Stabilität. Dies ist nicht notwendig, da sie nicht mit Proteasen in Kontakt kommen. Die Anforderungen an potente Inhalationsallergene dagegen sind hohe Löslichkeit in wässrigem Milieu und die passende Partikelgröße, um der Mucusbindung zu entgehen (Pekar *et al.*, 2018; Rimmer *et al.*, 2015). Allergene, welche mit der Nahrung aufgenommen werden und über den Gastrointestinaltrakt sensibilisieren, besitzen eine hohe Stabilität gegenüber Hydrolyse und Proteolyse (Breiteneder and Mills, 2005). Es existieren Nahrungsmittelallergene, welche darüber hinaus eine hohe Hitzeresistenz aufweisen und daher auch beim Verzehr gekochter Nahrungsmittel eine allergische Reaktion auslösen können (Houska *et al.*, 2013). Bei Nahrungsmittelallergenen spielen lineare Epitope, welche im Gegensatz zu konformationellen Epitopen nicht von einer intakten dreidimensionalen Struktur abhängig sind (vgl. 1.3), eine wichtige Rolle, da sie durch Erhitzen und bei der Passage durch den Gastrointestinaltrakt oft erhalten bleiben (Bannon, 2004).

Viele PR-10 Proteine wurden als empfindlich gegenüber Hitze, Denaturierung und Proteolyse beschrieben (Ballmer-Weber *et al.*, 2001, 2002a; Bohle *et al.*, 2006; Gaier *et al.*, 2008; Gruber *et al.*, 2004; Scheurer *et al.*, 2004; Vieths *et al.*, 1997). Nach ihrem Verzehr leiden Allergiker daher oft nur unter dem sogenannten oralen Allergiesyndrom (OAS), das sich auf Jucken und Anschwellen im Mund- und Rachenraum sowie Niesreiz beschränkt, systemische Reaktionen bleiben allerdings meist aus (Ballmer-Weber, 2015; Ballmer-Weber *et al.*, 2002b; Hansen *et al.*, 2009; Mari *et al.*, 2005).

1.12 Merkmale von Karotten- und Haselallergenen

Da in dieser Arbeit mit den PR-10 Allergenen aus der Karotte (*Daucus carota*) und der Hasel (*Coryllus avellana*) gearbeitet wurde, werden sie im folgenden Teil genauer beleuchtet.

1.12.1 Das Karottenallergen Dau c 1

Im Jahr 1993 wurde erstmals die Kreuzreaktivität zwischen Birke, Karotte und Sellerie nachgewiesen (Helbling *et al.*, 1993). Einige Jahre später wurde der cDNA Klon des PR-10 Allergens Dau c 1.0102 aus der Karotte isoliert (Yamamoto *et al.*, 1997). Während in den meisten Fällen einer Kreuzallergie die Symptome mild und auf die Mundhöhle begrenzt sind

(Webber & England, 2010), ist die durch Dau c 1 ausgelöste Nahrungsmittelallergie schwerwiegender, da in 50 % der Fälle systemische Reaktionen auftreten (Ballmer-Weber *et al.*, 2001). Tatsächlich weist Dau c 1, im Gegensatz zu anderen Bet v 1 homologen Allergenen, einige Merkmale eines sensibilisierenden Allergens auf wie hohe Resistenz gegen endolysosomalen Abbau, eine größere T-Zell-aktivierende Region und die Bet v 1 unabhängige Induktion einer Allergie (Moneo *et al.*, 1999; Zulehner *et al.*, 2017). In Kreuzinhibitionsversuchen (vgl. Kap. 1.5) mit natürlichem (n)Dau c 1 konnte die IgE-Bindung durch Bet v 1 nur zu etwa 40 % inhibiert werden – ein Hinweis darauf, dass nDau c 1 einzigartige Epitope besitzt, die nicht kreuzreaktiv mit Bet v 1 sind (Ballmer-Weber *et al.*, 2001). Außerdem wurde in Karotten, bei denen die Produktion der Hauptallergene Dau c 1.0104 und Dau c 1.0201 durch posttranskriptionales *silencing* reduziert wurde, nur teilweise eine Reduktion der Allergenität festgestellt: *Skin Prick* Tests mit dem Extrakt der transgenen Karottenwurzeln fielen zwar weiterhin positiv, aber um 21 – 50 % schwächer aus im Vergleich zum Karottenextrakt aus der Wildtyp-Pflanze. Das deutet auf die Anwesenheit weiterer bedeutender Isoallergene und Varianten hin (Peters *et al.*, 2011). Im Jahr 2012 konnte ein weiteres Isoallergen aus der Karotte, Dau c 1.0301, identifiziert werden (Wangorsch *et al.*, 2012). Stabilitätsuntersuchungen wurden bisher nur mit dem Isoallergen Dau c 1.0103 durchgeführt (Bohle *et al.*, 2006; Bollen, 2009). Dieses gilt mit einer Schmelztemperatur von etwa 45 °C (Bohle *et al.*, 2006; Bollen, 2009) als besonders hitzeempfindlich, jedoch sind die publizierten Ergebnisse bezüglich der Fähigkeit dieses Allergens zur Rückfaltung nach Hitzedenaturierung widersprüchlich: Während Bollen *et al.* in einem pH-Bereich zwischen 5,5 und 9 eine Rückfaltung nach Erhitzen auf 95 °C und Abkühlen beobachteten (Bollen, 2009), wurde diese von Bohle *et al.* bei pH 7 nicht festgestellt (Bohle *et al.*, 2006).

1.12.2 Haselallergene

(Baum-)Nussallergien haben weltweit eine Prävalenz von bis zu 4,9 %, wobei die Haselnussallergie europaweit die häufigste Ursache einer Nussallergie darstellt (McWilliam *et al.*, 2015).

Es existieren unterschiedliche Haselnussallergene aus verschiedenen Allergenklassen, wie PR-10 Allergene (Cor a 1), Lipid-Transfer-Proteine (Cor a 8) (Schocker *et al.*, 2004) und 11S Globuline (Cor a 9) (Beyer *et al.*, 2002), wobei die beiden letztgenannten Vertreter homolog zu Allergenen aus anderen Nüssen sind und für schwere allergische Reaktionen bis hin zum lebensbedrohlichen anaphylaktischen Schock verantwortlich sind. PR-10 Allergene (Cor a 1) lösen in Individuen, die in der Regel gegen Birkenpollen sensibilisiert sind,

allergische Kreuzreaktionen aus (vgl. Kap. 1.5) und führen meistens zu wesentlich mildereren Symptomen. Diese sind allerdings weit verbreitet, in birkenreichen Regionen reagieren bis zu 90 % der erwachsenen Haselnussallergiker gegen Cor a 1.04 (De Knop *et al.*, 2011). Cor a 1 hat sowohl als Inhalationsallergen als auch als Nahrungsmittelallergen Relevanz, da Isoallergene davon sowohl in Haselpollen (Breiteneder *et al.*, 1993; Valenta *et al.*, 1991) als auch in Haselnüssen (Eriksson, 1978; Ortolani *et al.*, 2000) vorkommen (Lüttkopf *et al.*, 2002).

Obwohl PR-10 Allergene aus der Hasel sowohl als Inhalations- als auch als Nahrungsmittelallergene eine hohe klinische Relevanz haben, wurde vor Beginn dieser Arbeit nur aufgrund der Sequenzhomologie davon ausgegangen, dass sie eine für PR-10 Proteine typische Grundstruktur (vgl. Abb. 5B) besitzen. Die genaue dreidimensionale Struktur war jedoch unbekannt.

2 Zielsetzung und Vorgehensweise

In dieser Arbeit sollten folgende Aspekte der PR-10 Allergene genauer beleuchtet werden: die physiologische Funktion, die Stabilität und die Zusammensetzung aus unterschiedlichen Isoallergenen.

Hinweise auf die physiologische Funktion der PR-10 Allergene sollten anhand des Haselallergens Cor a 1 gewonnen werden. Da die dreidimensionale Struktur eines Allergens unerlässlich ist, um Rückschlüsse auf dessen Funktion und Allergenität zu ziehen, sollte zunächst die Struktur der stark IgE-reaktiven Form des Haselallergens, Cor a 1.0401, in dieser Arbeit gelöst werden. Weitere Hinweise auf seine physiologische Funktion liefert die Identifikation eines natürlichen Liganden, die in dieser Arbeit durchgeführt werden sollte. Dafür sollte natürliches (n)Cor a 1 im Komplex mit seinem Bindungspartner aus Haselpollen gereinigt und der Ligand dann extrahiert und identifiziert werden. Anschließend sollten mit dem gereinigten Liganden Bindungsstudien durchgeführt werden, um die Bindung *in vitro* zu bestätigen.

Die Stabilität und Zusammensetzung aus unterschiedlichen Isoallergenen sollte anhand des PR-10 Allergens Dau c 1 aus Karotte näher untersucht werden.

Laut Literaturangaben ist Dau c 1 ein besonders hitzeempfindliches Protein mit einer Schmelztemperatur von nur etwa 45 °C (Bohle *et al.*, 2006; Bollen, 2009). Hinsichtlich seiner Fähigkeit zur Rückfaltung nach Hitzedenaturierung sind widersprüchliche Ergebnisse in der Literatur zu finden. Hinzu kommt, dass die publizierten Stabilitätsuntersuchungen mit nur einer Variante, Dau c 1.0103 durchgeführt wurden. Daher sollte in dieser Arbeit die Stabilität verschiedener Dau c 1 Isoallergene und Varianten bei verschiedenen pH-Werten mit und ohne Hitzeeinwirkung untersucht werden. Hierbei sollte auch der Einfluss der Nahrungsmittelmatrix berücksichtigt werden. Die hitze- und pH-behandelten Proben sollten sowohl auf ihre strukturelle Integrität als auch auf ihre Allergenität untersucht werden.

Auch die Zusammensetzung des Karottenallergens Dau c 1 aus Isoallergenen und Varianten, welche für die Anwendung rekombinanter Allergene in Therapie und Diagnostik von großem Interesse ist (vgl. 1.6.2), sollte mittels *peptide mass fingerprint* (PMF) genau analysiert werden, um die Anwesenheit neuartiger, Dau c 1-typischer Peptide zu prüfen. Vollständige, neuartige Dau c 1-Sequenzen sollten dann auf mRNA-Ebene mittels RNA-Extraktion und RT-PCR nachgewiesen werden. Danach sollte die rekombinante Herstellung des neuen Proteins und dessen biophysikalische und immunologische Charakterisierung erfolgen.

3 Zusammenfassung und Diskussion der Ergebnisse

3.1 Identifikation eines natürlichen Liganden des Haselallergens Cor a 1

Einzelarbeit A: Jacob, T., Seutter von Loetzen, C., Reuter, A., Lacher, U., Schiller, D., Schobert, R., Mahler, V., Vieths, S., Rösch, P., Schweimer, K., Wöhrle, B.M. (2019) Identification of a natural ligand of the hazel allergen Cor a 1. *Scientific Reports*, 9:8714

3.1.1 Die Struktur des Haselallergens Cor a 1.0401

Obwohl rund 52% aller Birkenpollenallergiker unter allergischen Kreuzreaktionen gegen das Haselallergen Cor a 1 leiden (Eriksson *et al.*, 1982) und diesem Allergen daher große klinische Bedeutung zukommt, wurde es bisher strukturell und physiologisch kaum untersucht. Die Aminosäuresequenz zeigt 69 % Sequenzidentität zu Bet v 1.0101 und deutet auf die typische Faltung Bet v 1 homologer Proteine hin (Abb. 5). Die Struktur, welche die Grundlage für die Untersuchung von Interaktionen mit natürlichen Liganden schafft, war jedoch bisher unbekannt. Daher wurde zunächst die Struktur der stark IgE-bindenden Variante Cor a 1.0401 (Lüttkopf *et al.*, 2002) mittels NMR-Spektroskopie gelöst (Abb. 7).

Wie erwartet, konnte die für PR-10 Proteine typische Faltung beobachtet werden. Sie besteht aus einem siebensträngigen, antiparallelen β -Faltblatt, gefolgt von einer langen C-terminalen Helix, die von zwei kürzeren V-förmig angeordneten Helices umgeben wird. Diese Elemente umschließen einen hydrophoben Hohlraum. In den Bereichen Ala35-Thr40 (zwischen Faltblattstrang $\beta 7$ und den zwei kurzen α -Helices) und Thr58-Met68 (zwischen Faltblattsträngen $\beta 5$ und $\beta 6$) konnten keine Resonanzen zugeordnet werden, daher sind dort keine strukturellen Informationen verfügbar (Einzelarbeit A, Abb. 1B, orange gekennzeichnet). Diese Bereiche stellen *loops* dar, welche ein dynamisches Verhalten auf für NMR-Spektroskopie ungünstigen Zeitskalen zeigen. Daher verbreitern sich die Signale und sind nicht mehr detektierbar. In diesen Bereichen kommt es deshalb im berechneten Strukturensamble zu großen Abweichungen. Nichtsdestotrotz konnte durch die Strukturberechnung die hydrophobe Tasche, welche bei der Ligandenbindung eine große Rolle spielt, genau identifiziert werden.

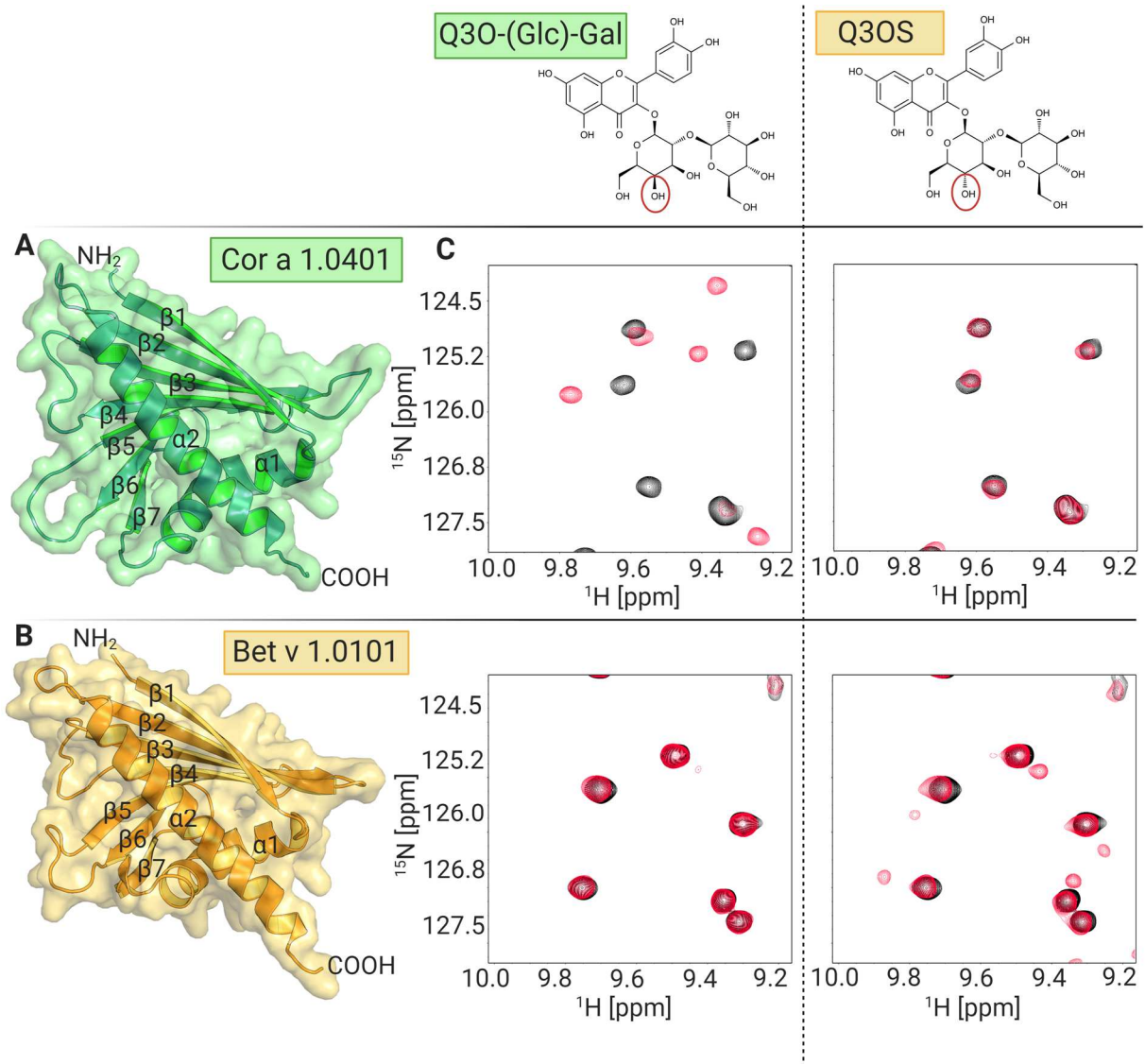


Abbildung 7: Die PR-10 Allergene Cor a 1.0401 und Bet v 1.0101 und ihre natürlichen Liganden. A: NMR-Struktur von Cor a 1.0401 (pdb: 6GQ9), B: NMR-Struktur von Bet v 1.0101 (pdb: 6R3C), C: Spezifität der Ligandenbindung: $^1\text{H},^{15}\text{N}$ HSQCs von Cor a 1.0401 (obere Reihe) und Bet v 1.0101 (untere Reihe) in Abwesenheit (schwarz) und Anwesenheit (rot) der Liganden Q30-(Glc)-Gal (linke Spalte) und Q30S (rechte Spalte) zeigen, dass die Proteine spezifisch an den eigenen Liganden, nicht jedoch an den beinahe identischen Liganden des anderen Allergens binden. Die Strukturen der Liganden sind oberhalb der Spektren abgebildet. Die Orientierung der OH-Gruppe am C4 der ersten Zuckergruppe stellt den einzigen Unterschied der beiden Liganden dar und ist rot eingekreist. Die Proteinstrukturen wurden mit Pymol (Schrödinger, 2010) dargestellt. Die Abbildung wurde mit BioRender.com angefertigt.

3.1.2 Q30-(Glc)-Gal ist ein natürlicher Ligand von Cor a 1

Um natürliche Liganden des Haselallergens Cor a 1 zu identifizieren, sollte der Protein-Liganden-Komplex aus Haselpollen extrahiert und gereinigt werden. Dafür wurde ein nicht-denaturierendes Reinigungsprotokoll verwendet, das von Seutter von Loetzen *et al.* übernommen und leicht modifiziert wurde (Bollen, 2009; Seutter von Loetzen *et al.*, 2014). Während der Reinigung mittels Ammoniumsulfatfällung, hydrophober Interaktionschromatographie (HIC) und Größenausschlusschromatographie (*size exclusion chromatography*, SEC) wurde sowohl die Absorption bei 280 nm (Protein) als auch bei 350 nm (Ligand)

überwacht. Die reine Cor a 1 Probe weist Absorption bei 350 nm auf, was darauf hindeutet, dass ein Ligand gebunden ist, welcher bei dieser Wellenlänge absorbiert (Einzelarbeit A, Abb 2B). Da als letzter Reinigungsschritt eine SEC durchgeführt wurde, in der die Moleküle nach ihrer Größe aufgetrennt werden, konnte eine zufällige Koelution des Proteins mit einem kleinen Molekül ausgeschlossen werden.

Die unterschiedlichen Cor a 1 Isoallergene und Varianten ähneln sich in ihrer Größe, Struktur und Aminosäuresequenz sehr. Daher ist es mit diesem Protokoll nicht möglich sie aufzutrennen, und es wurde eine Mischung unterschiedlicher Cor a 1 Varianten gereinigt. Eine PMF-Analyse (Einzelarbeit A, Tab. 2) bestätigte die Anwesenheit der Varianten Cor a 1.0103 und Cor a 1.0104. Die Varianten Cor a 1.0101 und Cor a 1.0102 besitzen keine einzigartigen Peptide und sind nicht von Cor a 1.0103 und Cor a 1.0104 zu unterscheiden. Daher konnte durch diese Methode ihre Anwesenheit weder bestätigt noch ausgeschlossen werden.

Das gereinigte nCor a 1 wurde lyophilisiert und mit Methanol (MeOH) extrahiert, um gebundene Liganden zu isolieren. Der Methanolextrakt wurde mittels *reversed phase - high performance liquid chromatography* (RP-HPLC) und hochaufgelöster Massenspektroskopie (HR-MS) analysiert. Im Massenspektrum konnte die Anwesenheit eines diglykosylierten Quercetins mit der Masse von 627,155 [M+H]⁺ bestätigt werden (Einzelarbeit A, Abb. S5). Die RP-HPLC Analyse mit einer C18A-Säule ergab einen einzigen Elutionsgipfel bei 350 nm (Einzelarbeit A, Abb. 3A, rote Linie). Es wurde daraufhin Haselpollen-MeOH-Extrakt hergestellt und der Ligand direkt daraus gereinigt, um eine höhere Ausbeute zu erhalten. Diese war ausreichend, um ein ¹³C, ¹H *heteronuclear single quantum coherence* (HSQC) Spektrum des Liganden aufzunehmen, welches bestätigte, dass es sich dabei um das diglykosylierte Quercetin Q3O(-Glc-)Gal, handelte. (Einzelarbeit A, Abb. 3C, Abb. 7). Dieses ist in Haselpollen abundant (Strack *et al.*, 1984). Der Ligand ist beinahe identisch mit dem Liganden des Birkenpollenallergens Bet v 1, Q3OS (Abb 7). Der einzige Unterschied besteht in der Orientierung der OH-Gruppe am C4-Kohlenstoff des ersten Zuckers (Abb. 7, rot eingekreist).

Daraufhin wurden ¹H, ¹⁵N HSQCs der fünf rekombinanten Cor a 1 Proteine Cor a 1.0101, Cor a 1.0102, Cor a 1.0103, Cor a 1.0104 und Cor a 1.0401 in An- und Abwesenheit eines 10-fachen molaren Überschusses an Q3O-Glc-Gal durchgeführt, um die Bindung zu bestätigen. Keine der Cor a 1.01 Varianten interagierte mit dem Liganden, doch es konnte eine Bindung zwischen Cor a 1.0401 und Q3O-Glc-Gal beobachtet werden (Einzelarbeit A, Abb. 5). Dieses Isoallergen wurde allerdings bisher nur in Nüssen, nicht aber in Pollen nachgewiesen (Lüttkopf *et al.*, 2002). Auch in der PMF-Analyse von gereinigtem Cor a 1 und von Haselpollenextrakt

konnte kein Cor a 1.0401 identifiziert werden. Das bedeutet, dass Cor a 1.0401 entweder überhaupt nicht, oder nur in sehr niedrigen Konzentrationen unterhalb der Nachweisgrenze, in Pollen enthalten ist. Die Ergebnisse weisen darauf hin, dass in Haselpollen mindestens noch ein weiteres, bisher nicht identifiziertes Cor a 1-Isoallergen enthalten ist, die den Liganden Q3O-(Glc)-Gal bindet. Diese kann allerdings nicht per PMF bestätigt werden, da ihre Peptide unbekannt sind.

Dennoch könnte auch der Cor a 1.0401/Q3O-(Glc)-Gal Komplex physiologische Bedeutung besitzen. Die Hasel zeichnet sich durch eine ungewöhnliche Reproduktionsbiologie aus: Die Bestäubung findet bereits im Januar oder Februar statt. Der Pollenschlauch wächst dann zur Basis des Blütengriffels, wo sich das noch nicht ausgereifte Ovarmeristem befindet. Daraufhin findet eine relativ lange Ruhephase von vier bis fünf Monaten statt, in der das Ovar heranreift. Danach wächst ein sekundärer Pollenschlauch heran und die Befruchtung findet statt (Olsen, 2013).

Aus der befruchteten Eizelle im Ovar entwickelt sich später die Nuss. Daher ist es möglich, dass Cor a 1.0401 bereits in der Basis der weiblichen Blüte exprimiert wird. Tatsächlich erfolgte der Nachweis der Cor a 1.04-Varianten in unreifen Nüssen (Lüttkopf *et al.*, 2002). Das glykosylierte Flavonoid Q3O-(Glc)-Gal hingegen ist für das Wachstum des Pollenschlauches wichtig (Vogt & Taylor, 1995), wobei das Diglykosid die Speicherform bildet und das Aglykon Quercetin zum Wachstum an sich beiträgt (Miller *et al.*, 1999). Da die Ausbildung des sekundären Pollenschlauches erst Monate nach der Bestäubung stattfindet, könnte der Cor a 1.0401/Q3O-(Glc)-Gal Komplex zur Speicherung des Liganden dienen, welcher kurz vor der Befruchtung freigegeben wird. Dies führt zur Abspaltung der Zuckerreste und das entstehende Aglykon Quercetin kann das Wachstum des Pollenschlauches induzieren.

3.1.3 Bindungsspezifität von Cor a 1.0401 und Bet v 1.0101

Die natürlichen Liganden von Cor a 1.0401 und Bet v 1.0101 sind beinahe identisch und unterscheiden sich nur in der Orientierung einer OH-Gruppe (Abb. 7, rot eingekreist). Es konnte bereits gezeigt werden, dass PR-10 Allergene eine Vielzahl unterschiedlicher kleiner Moleküle mit ähnlichen Dissoziationskonstanten binden (Asam *et al.*, 2014; Kofler *et al.*, 2012; Mogensen *et al.*, 2002), von denen allerdings bis jetzt nicht gezeigt werden konnte, dass sie auch natürliche Liganden sind. Überraschenderweise zeigen jedoch Bet v 1.0101 und Cor a 1.0401 eine hohe Bindungsspezifität gegenüber ihrem eigenen natürlichen Liganden, aber binden den beinahe identischen Liganden des jeweils anderen Allergens nicht. Die molekulare Grundlage dieser Bindungsspezifität ist bisher jedoch ungeklärt. Komplexstrukturen der Allergene mit dem jeweiligen Liganden könnten Hinweise auf die Orientierung des Liganden in der hydrophoben Tasche und somit auf die Ursache der Bindungsspezifität geben.

3.2 Die Nahrungsmittelzubereitung zerstört nicht die Allergenität des Karottenallergens Dau c 1: Einfluss von pH, Temperatur und Nahrungsmittelmatrix

Einzelarbeit B: Jacob, T., Vogel, L., Reuter, A., Wangorsch, A., Kring, C., Mahler, V., Wöhrl, B.M. (2020) Food processing does not abolish the allergenicity of the carrot allergen Dau c 1: Influence of pH, Temperature, and the Food Matrix. *Molecular Nutrition & Food Research*, e2000334

Personen mit Allergien gegen in Nahrungsmitteln vorkommende PR-10 Allergene wird meist empfohlen, auf die entsprechenden rohen Nahrungsmittel zu verzichten. Der Verzehr erhitzter Speisen gilt jedoch mit wenigen Ausnahmen (z.B. Sojaprodukte, (Mills *et al.*, 2009; Mittag *et al.*, 2004)) als unbedenklich, da die meisten PR-10 Allergene offenbar hitzeinstabil sind (Mills *et al.*, 2009).

Diese Annahme galt bisher auch für das Karottenallergen Dau c 1. Allerdings ergaben unterschiedliche Studien zur Hitzestabilität dieses Allergens widersprüchliche Ergebnisse: Während Bohle *et al.* zeigten, dass Dau c 1.0103 nach Erhitzen bei 95 °C bei physiologischem pH nicht zurückfalten konnte, beobachtete M. Bollen in dem pH-Bereich zwischen 6,3 und 9,0 die Fähigkeit zur Rückfaltung (Bohle *et al.*, 2006; Bollen, 2009).

Beide Studien wurden mit kommerziell erhältlichem rDau c 1.0103, welches einen Polyhistidin-Anhang für die Affinitätschromatographie enthält, durchgeführt. Im Gegensatz zu natürlichem (n)Dau c 1, welches eine Schmelztemperatur von etwa 56 °C aufweist (Bollen, 2009), wurde für rDau c 1.0103 eine Schmelztemperatur von nur 45 °C ermittelt. Grund dafür ist vermutlich eine Destabilisierung des Proteins durch den Polyhistidin-Anhang (Bollen, 2009).

Aufgrund der unklaren Datenlage sollte in dieser Arbeit die Hitzestabilität des Karottenallergens umfassend untersucht werden. Dabei sollten unterschiedliche Dau c 1 Isoallergene miteinander verglichen werden. Die Untersuchungen sollten unterschiedliche pH-Bedingungen und die Anwesenheit der Karottenmatrix beinhalten. Zudem sollten sowohl die strukturelle Integrität als auch die Allergenität überprüft werden.

3.2.1 Die meisten Dau c 1 Isoallergene falten nach Erhitzen bei pH 7 zurück

Um ein eindeutigeres und vollständigeres Bild der Stabilität von Dau c 1 bei physiologischem pH-Wert zu erhalten, wurden in dieser Arbeit verschiedene rekombinante Isoallergene und Varianten (Dau c 1.0104, Dau c 1.0105, Dau c 1.0201 und Dau c 1.0301) sowie nDau c 1 mit Hilfe von Circular dichroismus (CD)-Spektroskopie, nano differential scanning fluorimetry (DSF) und 2D NMR-Spektroskopie untersucht. Als Puffer wurde 10 mM Natriumphosphat,

pH 7, gewählt. Die Schmelztemperaturen aller getesteten Proteine lagen zwischen 55,4 °C (nDau c 1) und 59,1 °C (Dau c 1.0104). Alle Proben zeigten bei 25 °C vor Erhitzen das typische Spektrum eines Proteins mit α -Helices und β -Faltblättern und bei 95 °C das Spektrum eines ungefalteten Proteins (Abb. 8, linke Spalte). Nach Erhitzen und zurückkühlen auf 25 °C wiesen alle Proben bis auf Dau c 1.0201 beinahe das identische CD-Spektrum wie vor Erhitzen auf, was auf eine Rückfaltung hinweist. Dau c 1.0201 hingegen faltete kaum zurück.

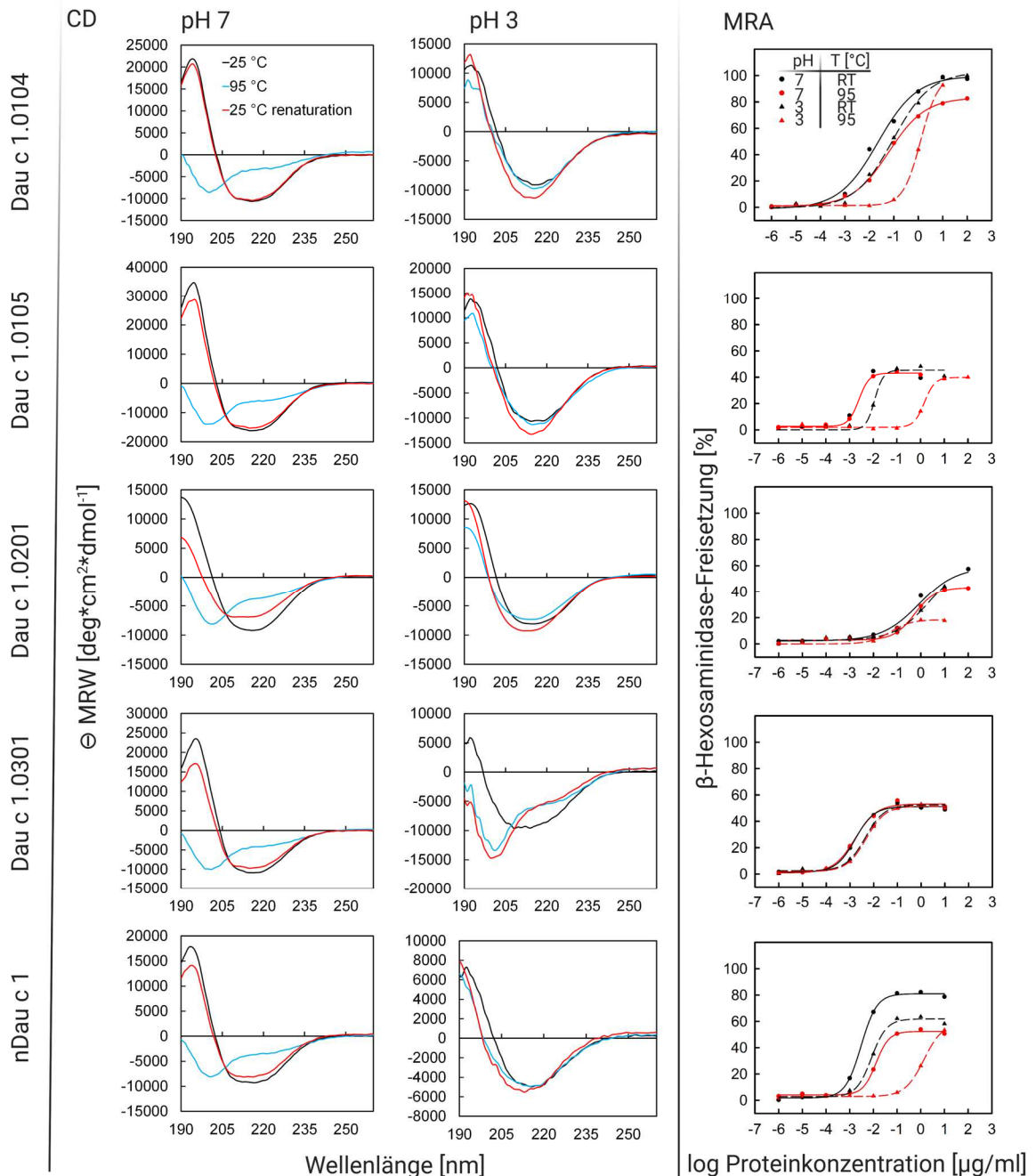


Abbildung 8: Stabilität und Allergenität verschiedener Dau c 1-Isoallergene bei pH 7 und pH 3, mit und ohne Hitzebehandlung: Die linke und mittlere Spalte zeigen CD-Spektren bei 25 °C (schwarz), 95 °C (blau) und nach Abkühlen auf 25 °C (rot) bei pH 7 (links) und pH 3 (Mitte). Die rechte Spalte zeigt die Mediatorenfreisetzung bei pH 7 (Kreise, durchgezogene Linie) und pH 3 (Dreiecke, getrichelte Linie), bei 25 °C (schwarz) und nach 30 min Inkubation bei 95 °C und Abkühlen auf 25 °C (rot).

Diese Ergebnisse wurden exemplarisch für rDau c 1.0105 und rDau c 1.0201 mit Hilfe von ^1H , ^{15}N HSQCs bestätigt, welche genauere Informationen auf Aminosäureebene geben (Einzelarbeit B, Abb. 2).

In dieser Studie wurde Dau c 1.0103 nicht untersucht, allerdings ist diese Variante sehr ähnlich zu Dau c 1.0105, sie unterscheiden sich nur durch vier Aminosäureaustausche (Pro36Thr, Ser67Thr, Thr81Ser, Leu102Met, Einzelarbeit B, Abb. S1). Daher kann davon ausgegangen werden, dass der erhebliche Unterschied in der Schmelztemperatur (ca. 58 °C für rDau c 1.0105, (diese Arbeit) und ca. 45 °C für rDau c 1.0103, (Bohle *et al.*, 2006; Bollen, 2009), wie bereits von Bollen vermutet, im Wesentlichen auf die Anwesenheit des Polyhistidin-Anhangs zurückzuführen ist (Bollen, 2009).

3.2.2 Die Hitzeresistenz von Dau c 1.0105 ist vom pH-Wert abhängig

Die Stabilität von Nahrungsmittelallergenen bei unterschiedlichen pH-Bedingungen ist für Allergiker wichtig, da die Nahrungsmittelzubereitung oft komplex ist und aus mehreren Schritten besteht, in denen sich die pH-Bedingungen erheblich unterscheiden können. Es konnte gezeigt werden, dass ein Absenken des pH-Wertes auf pH 3 – pH 4 vor Erhitzen durch die Zugabe von Zitronensäure oder *Lactobacillae* sowohl die Haltbarkeit als auch den Geschmack von Karottensaft verbesserte (Aguiló-Aguayo *et al.*, 2014). Um die Hitzeresistenz bei verschiedenen pH-Werten (pH 6, 5, 4, 3, 2) zu untersuchen, wurde das Isoallergen Dau c 1.0105 exemplarisch gewählt. Es wurden CD-Spektren vor, während und nach Erhitzen und Abkühlen bei der jeweiligen pH-Bedingung aufgenommen (Einzelarbeit B, Abb. 3). Als Puffer wurde Na-Phosphat (pH 6), Na-Acetat (pH 4 und 5) oder Na-Citrat (pH 3 und 2) gewählt, da diese Puffer auch bei starken Temperaturschwankungen einen konstanten pH-Wert besitzen. Bei pH 6 konnten ähnliche Ergebnisse wie bei pH 7 beobachtet werden. Das Protein lag bei 95 °C entfaltet vor und zeigte nach Rückkühlen auf Raumtemperatur die typischen Sekundärstrukturelemente für PR-10 Allergene. Bei pH 5 hingegen, der nah am isoelektrischen Punkt ($pI=4,61$) dieses Proteins liegt, fiel das Protein bei 95 °C irreversibel aus. Bei pH 4 und pH 3 war das CD-Spektrum vor, während und nach Erhitzen beinahe identisch und deutete auf die Anwesenheit von Sekundärstrukturelementen hin, was auf eine hitzestabile Form hinweist. Bei pH 2 entfaltete Dau c 1.0105 bei 95 °C und faltete nach Abkühlen nicht mehr zurück.

Aufgrund dieser Beobachtungen lassen sich nun auch die widersprüchlichen Ergebnisse bezüglich der Fähigkeit zur Rückfaltung von Dau c 1.0103 erklären. Bohle *et al.*, die keine Rückfaltung beobachteten, verwendeten in ihrer Stabilitätsanalyse Tris/HCl Puffer. Dieser ist sehr hitzesensitiv und sein pH-Wert sinkt bei steigenden Temperaturen. So ist es möglich, dass

bei 95 °C ein tatsächlicher pH-Wert in dem Bereich um den isoelektrischen Punkt ($pI=4,61$) von Dau c 1 vorherrschte, in dem dieses Protein nicht zur Rückfaltung befähigt ist. Um diese Annahme zu bestätigen wurde eine Denaturierungs- und Renaturierungskurve von rDau c 1.0105 in 10 mM Tris/HCl, pH 7 aufgenommen. Im Gegensatz zu den mit Na-Phosphat Puffer erhaltenen Ergebnissen kann Dau c 1.0105 in Tris/HCl Puffer nicht zurückfalten (Einzelarbeit B, Abb. S2).

3.2.3 Die meisten Dau c 1 Proteine sind bei pH 3 hitzeresistent

Nachdem gezeigt wurde, dass Dau c 1.0105 bei pH 3 eine hitzeresistente Form annimmt (vgl. 3.2.2), wurden auch die anderen Isoallergene und Varianten bei pH 3 auf ihre Stabilität untersucht (Abb. 8, mittlere Spalte). Dieser pH-Wert ist relevant, da nach der Nahrungsaufnahme der pH-Wert im Magen auf pH 3-5 ansteigt. Interessanterweise waren bei 25 °C, pH 3 alle Dau c 1-Proteine stabil und zeigten Sekundärstrukturelemente (Abb. 8, mittlere Spalte, schwarze Linien). Die Stabilität bei niedrigen pH-Werten, wie sie im Magen vorherrschen, könnte der Grund dafür sein, dass Dau c 1 auch als sensibilisierendes Allergen fungiert und oft systemische Reaktionen auslöst (Ballmer-Weber *et al.*, 2001). Hitzeresistenz konnte neben Dau c 1.0105 für beinahe alle anderen Dau c 1 Proteine beobachtet werden, und auch bei nanoDSF-Messungen bis 110 °C konnten keine eindeutigen Schmelztemperaturen ermittelt werden (Einzelarbeit B, Abb. 4). Lediglich Dau c 1.0301 zeigte eine Schmelztemperatur von 80,3 °C und konnte bei pH 3 nicht rückfalten.

3.2.4 Dau c 1 Proteine induzieren nach pH- und Hitzebehandlung noch eine allergische Reaktion

IgE-Antikörper erkennen entweder konformationelle oder sequentielle Epitope auf Allergenen und lösen daraufhin die Ausschüttung von Immun-Mediatoren aus. Während für die Erkennung konformationeller Epitope eine intakte dreidimensionale Struktur notwendig ist, werden sequentielle Epitope auch bei einem ungefalteten Allergen erkannt (vgl. 1.3). Sequentielle Epitope spielen daher eine wichtige Rolle bei Nahrungsmittelallergenen (Bannon, 2004), da sie trotz Verlust der dreidimensionalen Struktur des Allergens eine Immunreaktion auslösen können. Es sollte die Allergenität der hitze- und pH-behandelten Dau c 1 Proteine untersucht werden. Dafür wurde ein Mediatorenfreisetzungstest (mediator release assay, MRA) verwendet (Abb. 2). Dabei handelt es sich um einen Zellkulturtest, bei welchem im Wesentlichen der *Skin Prick Test* nachgeahmt wird: Basophile Leukozyten, welche den humanen IgE-Rezeptor exprimieren, werden mit Allergikerserum, das IgE Antikörper gegen das entsprechende Allergen enthält, sensibilisiert. Die Zugabe des Allergens induziert die Kreuzvernetzung der

rezeptorgebundenen IgE-Moleküle, was zur Ausschüttung allergieinduzierender Mediatoren führt (vgl. 1.2). Die Mediatorenfreisetzung kann indirekt über einen colorimetrischen Ansatz quantifiziert werden.

Der Einfluss von alleiniger Hitze- oder pH-Behandlung auf die Mediatorenfreisetzung war relativ gering (Abb 8, rechte Spalte). Interessanterweise führte erst die Kombination von beiden Behandlungen (95 °C, pH 3) zu einer deutlich abgeschwächten Mediatorenfreisetzung. Die einzige Ausnahme dabei stellte Dau c 1.0301 dar: hier war die Mediatorenfreisetzung auch nach Inkubation bei 95 °C und pH 3 vergleichbar mit der unbehandelten Probe.

Die verminderte Allergenität der Hitze- und pH-behandelten Probe könnte an der irreversiblen Bildung von großen Oligomeren (> 75 kDa) liegen, die für Dau c 1.0104 und Dau c 1.0105 nach 30 min Inkubation bei pH 3 und 95 °C mittels SEC nachgewiesen werden konnten (vgl. Einzelarbeit B, Kapitel 2.5, Abb. 5). Bei großen Oligomeren sind nicht mehr alle Epitope zugänglich für IgE-Bindung, daher ist eine höhere Allergenkonzentration für die gleiche Mediatorenfreisetzung nötig. Allerdings werden durch die Bildung von Oligomeren ebenfalls Angriffsstellen für den Proteaseverdau während der Passage durch den Gastrointestinaltrakt unzugänglich. So wird die Proteolyse des Allergens erschwert und das Allergen kann weiterhin allergische Reaktionen auslösen. Das wurde für das Erdnussallergen Ara h 1 beobachtet, welches ein Homotrimer bildet (Maleki *et al.*, 2000).

Dau c 1.0301 stellt das einzige Isoallergen dar, welches bei pH 3 keine hitzestabile Form bildet, sondern irreversibel entfaltet. Hier wurde auch keine verminderte Allergenität bei gleichzeitiger Hitze- und pH-Einwirkung beobachtet. Die mit der unbehandelten Probe vergleichbare Mediatorenfreisetzung deutet auf die Anwesenheit sequentieller Epitope hin, welche auch bei ungefalteten Allergenen erkannt werden.

3.2.5 Die Karottenmatrix reduziert die Hitzestabilität von Dau c 1

Für Karottenallergiker ist es von besonderer Bedeutung, ob Dau c 1 Proteine auch nach der Essenszubereitung noch eine allergische Reaktion auslösen können. Daher sollte die Stabilität dieser Proteine in der Anwesenheit der natürlichen Nahrungsmittelmatrix untersucht werden. Es zeigte sich, dass Karottenextrakt nach Erhitzen noch lösliches Dau c 1 enthält (Einzelarbeit B, Abb. 7A), und immer noch allergenes Potential besitzt (Einzelarbeit B, Abb. 7B). Jedoch war die Mediatorenfreisetzung nach Erhitzen um den Faktor 5 abgeschwächt, wie sich aus den mittleren effektiven Konzentrationen (EC₅₀-Werten) (unbehandelter Extrakt:

0,024 µg/ml, Extrakt nach 40 min Erhitzen: 0,117 µg/ml, Einzelarbeit B, Abb. 7B) ableiten lässt.

Die NMR-Spektroskopie liefert einen einzigartigen Ansatz, die dreidimensionale Stabilität von einzelnen Proteinen auch in Anwesenheit anderer Proteine oder Moleküle zu untersuchen. Dabei wurden die Allergene, welche analysiert werden sollten, rekombinant hergestellt. Die einzige Stickstoffquelle der Bakterien während der Proteinsynthese war $^{15}\text{(NH}_4\text{)}_2\text{SO}_4$. So konnte ^{15}N markiertes rekombinantes Protein hergestellt werden. Durch die Aufnahme von ^1H , ^{15}N HSQCs konnten – auch in Anwesenheit der Nahrungsmittelmatrix - exklusiv die Signale des rekombinanten, markierten Proteins beobachtet werden. Dabei stellte sich heraus, dass Dau c 1.0105 in Anwesenheit der Karottenmatrix zwar nach 20 min Inkubation bei 95 °C noch größtenteils das Spektrum eines gefalteten Proteins zeigte, nach 40 min jedoch aggregierte und ausfiel (Einzelarbeit B, Abb. 7C). Dau c 1.0201 hingegen blieb in Lösung, zeigte allerdings nach 40 min Inkubation bei 95 °C das typische NMR-Spektrum eines ungefalteten Proteins (Einzelarbeit B, Abb 7D).

3.2.6 Zusammenfassende Diskussion

Die Stabilitätsanalysen ergaben, dass Dau c 1 an sich nicht hitzesensitiv ist, sondern seine ursprüngliche Struktur nach Abkühlen wieder annehmen kann. Die Hitzestabilität unterscheidet sich bei den unterschiedlichen getesteten Isoallergenen und ist abhängig vom pH-Wert. Die Karottenmatrix reduziert zwar die Hitzestabilität, jedoch ist in gekochtem Karottenextrakt noch lösliches Dau c 1 vorhanden, welches weiterhin eine Mediatorenfreisetzung auslöst. Aufgrund dieser Ergebnisse können IgE-vermittelte allergische Reaktionen nach dem Verzehr gekochter Karotten nicht ausgeschlossen werden, und Karottenallergikern wird empfohlen, auch auf gekochte Karotten zu verzichten.

3.3 Dau c 1.0401, ein neues Isoallergen aus Karotte: Stabilität, Allergenität und Vergleich mit anderen Dau c 1-Allergenen

Einzelarbeit C: *Jacob, T., *Wangorsch, A., Vogel, L., Reuter, A., Mahler, V., Wöhrl, B.M. (2021). A novel isoallergen Dau c 1.0401 in carrot: stability, allergenicity and comparison with other isoallergens. *Molecular Nutrition & Food Research*, e2001110 *contributed equally

Das Karottenallergen Dau c 1 nimmt unter den PR-10 Allergenen eine Sonderrolle ein, da eine Bet v 1-unabhängige Sensibilisierung beobachtet wurde (Ballmer-Weber *et al.*, 2001) und die IgE-Bindung von nDau c 1 in Kreuzinhibitionsversuchen (vgl. Kap. 1.5) nicht komplett, sondern nur zu etwa 40 % durch Bet v 1 inhibiert werden konnte (Ballmer-Weber *et al.*, 2001; Hoffmann-Sommergruber *et al.*, 1999a). Die IgE-Bindung des Isoallergens Dau c 1.0103 konnte hingegen komplett von Bet v 1 inhibiert werden (Hoffmann-Sommergruber *et al.*, 1999a). Diese Ergebnisse weisen darauf hin, dass Isoallergene/Varianten in nDau c 1 existieren, die keine Kreuzallergene zu Bet v 1 darstellen, sondern einzigartige Epitope besitzen und selbst als sensibilisierende Allergene fungieren (vgl. 1.5). Eine genaue Flüssigkeitschromatographie-Massenspektrometrie (LC-MS^E)-Analyse von nDau c 1 sollte daher Aufschluss über seine Zusammensetzung aus Isoallergenen und Varianten geben.

3.3.1 In natürlichem (n)Dau c 1 sind mindestens acht neuartige Isoallergene/Varianten enthalten

Gereinigtes natürliches (n)Dau c 1 wurde mittels LC-MS^E analysiert, wobei die Probe nicht nur auf die Anwesenheit bereits bekannter und gelisteter Dau c 1 Sequenzen hin untersucht, sondern ebenfalls mit der UniProt-Datenbank mit nicht geprüften Einträgen aus grünen Pflanzen abgeglichen wurde. Hierbei konnten etliche neuartige Peptide identifiziert werden (vgl. Einzelarbeit B, Tab. 1). Eine Einschränkung der Methode ist das sogenannte Protein-Interferenz-Problem. Dieses liegt darin begründet, dass die Probe vor der Analyse tryptisch verdaut wird. Hierbei entstehen einzelne Peptide, deren Zusammengehörigkeit nicht mehr nachvollzogen werden kann (Nesvizhskii & Aebersold, 2005). Daher kann keine genaue Aussage darüber getroffen werden, wie viele unterschiedliche Isoallergene und Varianten tatsächlich in nDau c 1 vorhanden sind. Für das Peptid T55 existieren jedoch insgesamt zehn Variationen, von denen zwei mit den entsprechenden Peptiden für die bereits gelisteten Isoallergene Dau c 1.0103 und Dau c 1.0201 identisch sind. Die übrigen acht Variationen sind neuartigen Isoallergenen oder Varianten zuzuordnen. Daraus kann geschlossen werden, dass mindestens acht neuartige Dau c 1 Proteine vorhanden sind.

Neben der Problematik, die identifizierten Peptide nicht zuordnen zu können, kommt hinzu, dass die Sequenzabdeckung der Datenbankeinträge zwischen 24,0 % und 90,9 % variiert (Einzelarbeit B, Tab. 1). Die Sequenzbereiche, die nicht abgedeckt wurden, könnten sich theoretisch von dem Datenbankeintrag unterscheiden. Um die Anwesenheit einer kompletten Sequenz sicher nachweisen zu können, sollte daher aus RNA, die aus Karotten isoliert wurde, cDNA hergestellt und mittels PCR amplifiziert werden, um das Gen anschließend klonieren und sequenzieren zu können.

3.3.2 Identifizierung des neuen Isoallergens Dau c 1.0401

Die Sequenz des Uniprot-Eintrags A0A161X1M2, welcher von einer *shot gun* Studie des gesamten Karottengenoms abgeleitet ist, wurde mit einer Abdeckung von 87.1 % in der LC-MS^E Analyse detektiert (Einzelarbeit B, Tab. 2). Sie beträgt eine Aminosäure-Sequenzidentität von etwa 60 % zu den anderen bekannten Isoallergenen (Einzelarbeit C, Tab. 1). Somit würde es sich laut Nomenklatur-Regeln (vgl. Kap. 1.8) eigentlich um ein neues Allergen handeln. Eine Modellrechnung der Proteinstruktur (Phyre 2, Kelley *et al.*, 2015) ergab jedoch eine typische PR-10 Faltung (vgl. Einzelarbeit C, Abb. 6B mit Abb. 5), und die Sequenzidentität von 67 % oder mehr zur Klassifizierung als Isoallergen ist eine Richtlinie und keine strikte Vorgabe (Pomés *et al.*, 2018). Daher wurde dieses Allergen als Dau c 1 Isoallergen klassifiziert. Die komplette Sequenz konnte in dieser Arbeit auf mRNA-Ebene identifiziert und die Anwesenheit auf Proteinebene so bestätigt werden (Einzelarbeit C, Abb. S1). Das Gen wurde für *Escherichia coli* (*E. coli*) codonoptimiert, in den Expressionsvektor pET11a kloniert und ohne Affinitätsanhang exprimiert.

3.3.3 Physikochemische Charakterisierung von Dau c 1.04

Nach erfolgreicher rekombinanter Herstellung und Reinigung des neuen Isoallergens und Bestätigung seiner Identität mittels PMF (Einzelarbeit C, Tab. S1) wurde die Struktur und Stabilität von rDau c 1.0401 gründlich analysiert. Das 1D NMR Spektrum weist eine hohe Dispersion auf und ist typisch für ein gefaltetes Protein (Einzelarbeit C, Abb. 2A). Die analytische SEC zeigt einen einzigen Elutionsgipfel bei einem Elutionsvolumen, welches einer Molekülgröße von etwa 24 kDa entspricht (Einzelarbeit C, Abb. 2B). Somit kann davon ausgegangen werden, dass dieses Protein mit einem theoretischen Molekulargewicht von 16,6 kDa als Monomer vorliegt. Die Differenz kommt durch die nicht perfekt globuläre Form und/oder Hydratation des Proteins zustande, welche dazu führt, dass es sich wie ein etwas größeres Protein verhält. Das CD-Spektrum zeigt die für PR-10 Allergene typischen Sekundärstrukturelemente (Einzelarbeit C, Abb. 2 C, schwarze Linie). Bei 95 °C ist das Protein

ungefaltet (Einzelarbeit C, Abb. 2C, blaue Linie). Nach Abkühlen auf 25 °C kann es nur teilweise rückfalten (Einzelarbeit C, Abb. 2C, rote Linie). Die Schmelztemperatur wurde durch CD-Spektroskopie (Abb. 9A) und nanoDSF (Einzelarbeit C, Abb. S3) ermittelt und liegt bei etwa 65 °C. Sie ist somit 5-10 °C höher als bei den anderen Dau c 1 Isoallergenen.

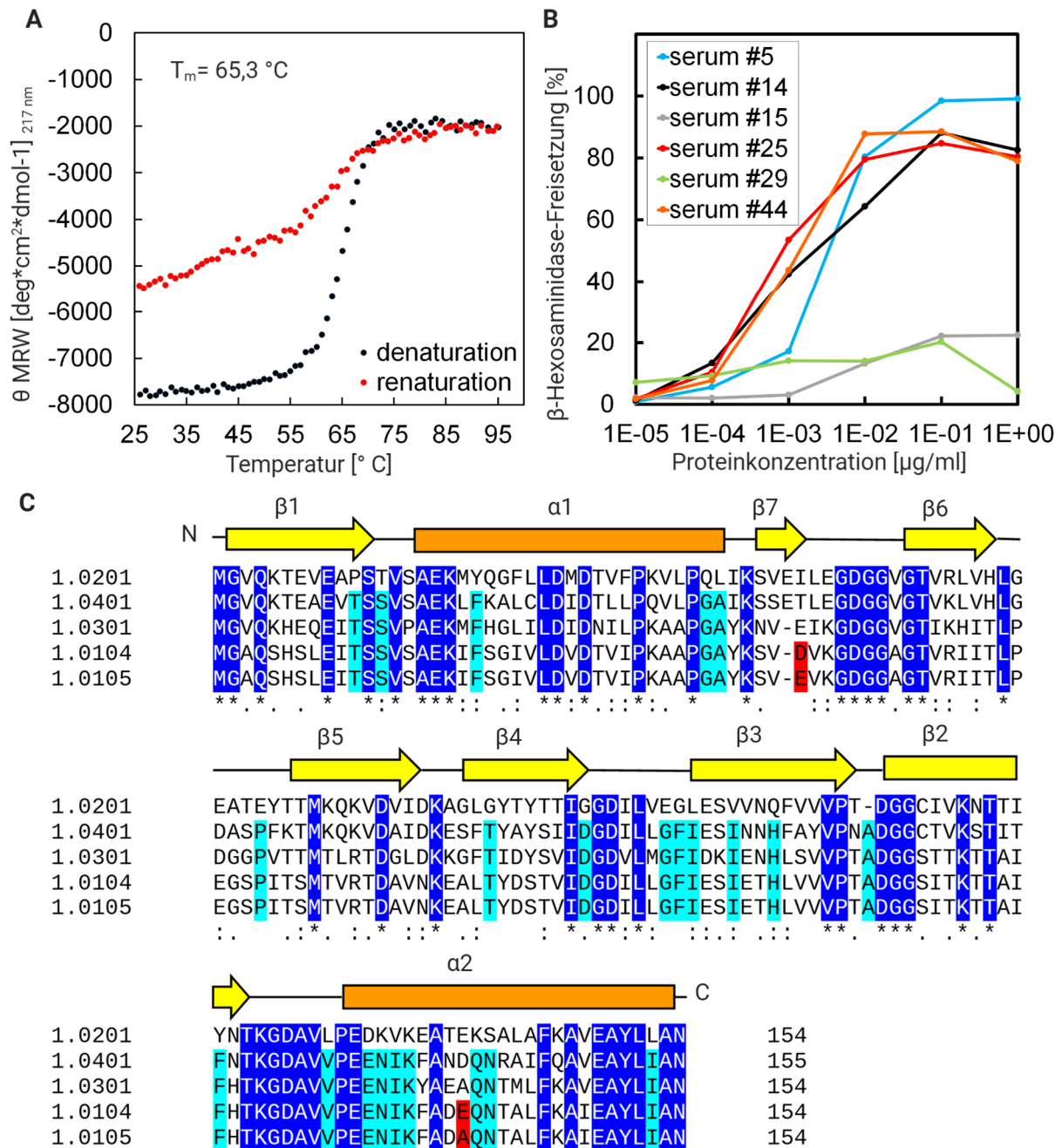


Abbildung 9: Charakterisierung des neuen Isoallergens Dau c 1.0401: A: Schmelzkurve, B: Mediatorenfreisetzungstest mit sechs positiven Seren, C: Sequenzvergleich der verschiedenen Dau c 1-Isoallergene/Varianten. Dunkelblau: konservierte Bereiche, hellblau: konservierte Bereiche zwischen allen Dau c 1-Proteinen mit Ausnahme von Dau c 1.0201, rot: Aminosäureaustausche zwischen Dau c 1.0104 und Dau c 1.0105, Sekundärstrukturelemente wurden wie für Dau c 1.0401 berechnet (Phyre 2, Kelley et al., 2015) angegeben (orange Balken: α-Helices, gelbe Pfeile: β-Faltblätter).

3.3.4 Immunologische Charakterisierung von Dau c 1.04

Dau c 1.0401 zeigt eine für PR-10 Proteine typische Aminosäuresequenz, und in der Modellrechnung wurde eine typische PR-10 Struktur ermittelt. Daher ist es wahrscheinlich, dass dieses Protein eine allergische Reaktion hervorruft. Das Protein wurde deshalb einer umfassenden immunologischen Analyse (Immunoblots, Dot blots, ELISAs, MRAs) unterzogen. Dafür wurden 19 Seren von Karottenallergikern mit milden bis schwerwiegenden klinischen Symptomen verwendet (Einzelarbeit C, Tab. 2). Überraschenderweise konnte im Immunoblot, Dot Blot und ELISA jedoch kaum Allergenität festgestellt werden (Einzelarbeit C, Abb. S4-S6). In Mediatorenfreisetzungstests (vgl. Kap. 1.4 und Abb. 2) konnte dagegen die Allergenität von Dau c 1.04 nachgewiesen werden (Einzelarbeit 3, Abb. 3). Vier der 19 ausgewählten Seren reagierten stark, zwei schwach und die anderen riefen keine Mediatorenfreisetzung hervor (Abb. 9B). Da die gleichen Seren, welche eine Mediatorenfreisetzung ausgelöst hatten, im Immunoblot, Dot Blot und ELISA negativ waren, kann davon ausgegangen werden, dass das Allergen konformationelle Epitope besitzt, welche nach dem Transfer auf die Membran nicht erhalten bleiben. Ähnliche Ergebnisse wurden bereits bei Immunoblots mit Birkenpollenextrakt und den Seren von Birkenpollenallergikern in einer Studie von Cudowska *et al.* beobachtet (Cudowska *et al.*, 2005).

Um die IgE-Reaktivität des Isoallergens Dau c 1.0401, welche im Mediatorenfreisetzungstest demonstriert werden konnte, zweifelsfrei zu bestätigen, wurde ein Kreuzinhibitionsexperiment (vgl. 1.5 und Abb. 3) in Kombination mit Dau c 1.0104 durchgeführt. Eine Voraussetzung für ein positives Ergebnis hierbei ist die Anwesenheit konservierter Epitope zwischen Dau c 1.0104 und Dau c 1.0401. Es wurden die Seren von Karottenallergikern, welche in Kombination mit Dau c 1.0401 im Mediatorenfreisetzungstest positiv waren, ausgewählt. Diese wurden entweder mit Dau c 1.0104 (Selbstinhibition) oder Dau c 1.0401 vorinkubiert, um eine dosisabhängige Inhibition der IgE-Bindung an Dau c 1.0104, welches auf der festen Phase immobilisiert war, durchzuführen. Mit allen der vier getesteten Seren konnte eine Inhibition nach Vorinkubation mit Dau c 1.0401 beobachtet werden (Einzelarbeit C, Abb. 4), womit dessen IgE-Reaktivität zweifelsfrei bestätigt werden konnte.

3.3.5 Vergleich unterschiedlicher Dau c 1 Isoallergene und Varianten

Um die Allergenität des neuen Isoallergens Dau c 1.0401 zu vergleichen, wurden Mediatorenfreisetzungstests mit den gereinigten rekombinanten Dau c 1 Isoallergenen rDau c 1.0104, rDau c 1.0105, rDau c 1.0201, rDau c 1.0301, rDau c 1.0401 und nDau c 1 und fünf verschiedenen Seren von Karottenallergikern durchgeführt (Einzelarbeit C, Abb. 5). Das

natürliche nDau c 1 wurde aus der Karottenwurzel extrahiert und gereinigt und stellt daher eine Mischung verschiedener Dau c 1 Isoallergene und Varianten dar (vgl. 3.3.1). Dau c 1.0103, Dau c 1.0201 und Dau c 1.0401, sowie etliche bisher nicht gelistete, Dau c 1-typische Sequenzen konnten mittels LC-MS^E nachgewiesen werden (vgl. Einzelarbeit B, Tab. 1 und 2), die quantitative Zusammensetzung ist jedoch nicht bekannt. Seren #14, #44, #5 und #25 reagierten mit den Isoallergenen rDau c 1.0104, rDau c 1.0301, rDau c 1.0401 und nDau c 1. Das weist darauf hin, dass diese Isoallergene gemeinsame Epitope haben könnten. Im Gegensatz dazu reagierte nur Serum #44 mit rDau c 1.0105, obwohl diese Variante beinahe identisch zu Dau c 1.0104 ist und sich nur in 2 Aminosäuren unterscheidet (Asp43Glu und Glu139Ala, in Abb. 9B rot markiert). Diese Aminosäuren spielen offenbar bei der IgE-Bindung eine wichtige Rolle.

Serum #46 reagierte dagegen nur mit rDau c 1.0201, was darauf hindeutet, dass dieses Isoallergen einzigartige Epitope besitzt. Da dieses Serum nicht mit nDau c 1 reagierte, ist die Konzentration von Dau c 1.0201 in der natürlichen Mischung vermutlich relativ niedrig. Abgesehen von Serum 5, das schwache Reaktivität zeigte, reagierte keines der anderen Seren mit rDau c 1.0201. Das deutet darauf hin, dass Epitope existieren, welche bei Dau c 1.0104, Dau c 1.0301 und Dau c 1.0401 konserviert sind, nicht jedoch bei Dau c 1.0201. Die konservierten Aminosäuren aller getesteten Dau c 1 Isoallergene sind in dunkelblau, die konservierten Aminosäuren aller Dau c 1-Isoallergene mit Ausnahme von Dau c 1.0201 in hellblau markiert (Abb. 9C, Einzelarbeit C, Abb. 6B und C).

3.3.6 Dau c 1.0401 ist ein relevantes Isoallergen

In dieser Arbeit wurde ein relevantes neues Dau c 1-Isoallergen identifiziert und charakterisiert, welches vom WHO/IUIS Allergen Nomenklatur Subkommittee als Dau c 1.0401 akzeptiert wurde. Dank seiner Identifikation und Charakterisierung können rekombinante „Dau c 1-Allergencocktails“ für die Verwendung in Immuntherapie und Diagnostik (vgl. 1.6.2) zukünftig verbessert werden. Ungewöhnlich ist die Tatsache, dass die IgE-Bindung von rDau c 1.0401 beim Transfer auf eine Membran beeinträchtigt wird, da hierbei seine Struktur offenbar – zumindest teilweise – verändert wird. Dieses Phänomen, welches zu falsch-negativen Ergebnissen führen kann, sollte zukünftig bei der immunologischen Charakterisierung potentieller neuer Allergene berücksichtigt werden. Da rDau c 1.0401 nach Erhitzen seine ursprüngliche dreidimensionale Struktur nicht wieder annimmt und offenbar nur konformationelle Epitope besitzt, kann davon ausgegangen werden, dass seine Allergenität beim Erhitzen von Karotten abnimmt oder ganz verschwindet.

4. Schlussbetrachtung und Ausblick

Obwohl das Bindungsverhalten der PR-10 Allergene bisher als unspezifisch galt, und gezeigt wurde, dass Bet v 1 eine Vielzahl unterschiedlicher Liganden *in vitro* bindet (Asam *et al.*, 2014; Kofler *et al.*, 2012; Mogensen *et al.*, 2002), wurde sowohl für Bet v 1 als auch für Cor a 1 eine sehr hohe Bindungsspezifität gegenüber ihren physiologisch relevanten Liganden beobachtet: Bet v 1.0101 bindet Q3OS, die Varianten Bet v 1.0118 (Bet v 1m) und Bet v 1.0102 (Bet v 1d) binden diesen Liganden nicht (Seutter von Loetzen *et al.*, 2014, 2015). Der Ligand des Haselallergens Cor a 1, Q3O-(Glc)-Gal, wird nicht von den vier Cor a 1.01-Varianten gebunden, aber von Cor a 1.0401. Zudem erkennen Bet v 1.0101 und Cor a 1.0401 den eigenen Liganden hochspezifisch, und den jeweils anderen Liganden, der beinahe identisch ist (Abb. 7) nicht. Das deutet auf eine hohe funktionelle Diversifikation der PR-10 Allergene hin. Um genauere Hinweise zu den physiologischen Funktionen zu erhalten, sollten weitere natürliche Liganden identifiziert werden (Aglas *et al.*, 2020).

Das Karottenallergen Dau c 1 ist ein besonders interessanter Vertreter der PR-10 Allergene, da es nicht nur als Kreuzallergen zu Bet v 1 fungiert, sondern auch eine Bet v 1-unabhängige Sensibilisierung auslösen kann (Ballmer-Weber *et al.*, 2001; Hoffmann-Sommergruber *et al.*, 1999a; Zulehner *et al.*, 2017). In dieser Arbeit wurde seine Stabilität, Allergenität und Zusammensetzung aus unterschiedlichen Isoallergenen gründlich untersucht.

Zum einen konnte dabei gezeigt werden, dass Dau c 1 gegenüber Hitze und pH-Behandlung stabiler ist als bisher angenommen. Darüber hinaus wurde festgestellt, dass die Dau c 1 Zusammensetzung sich wesentlich von der bisher angenommenen unterscheidet. Ein neuartiges Isoallergen, Dau c 1.0401 wurde auf mRNA- und Proteinebene nachgewiesen und sowohl strukturell als auch immunologisch gründlich analysiert. Seine Allergenität konnte im Mediatorenfreisetzungstest und im Inhibitionstest nachgewiesen werden. Die Identifikation neuer Isoallergene, welche eventuell eigenständige Epitope besitzen, ist für die Anwendung rekombinanter Allergenprodukte in Diagnostik und Therapie von besonderem Interesse. Außerdem könnte ein Dau c 1 Isoallergen identifiziert werden, welches eine Bet v 1-unabhängige allergische Reaktion auslösen kann. Diese Erkenntnisse haben direkte Relevanz für Karottenallergiker und könnten die Handlungsempfehlungen, Diagnose und Behandlung zukünftig verbessern.

5. Abkürzungsverzeichnis

| | |
|-------------------------|---|
| AllFam-Datenbank | Datenbank der Allergenfamilien |
| APZ | Antigenpräsentierende Zelle |
| CD | Circulardichroismus |
| (c)DNA | (komplementäre) Desoxyribonukleinsäure |
| EC ₅₀ | <i>half maximal effective concentration</i> |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| ELISA | <i>Enzyme linked immunosorbent assay</i> |
| HIC | Hydrophobe Interaktionschromatographie |
| HR-MS | Hochaufgelöste Massenspektrometrie |
| HSQC | <i>Heteronuclear single quantum coherence</i> |
| IgE | Immunglobulin der Klasse E |
| IgG(4) | Immunglobulin der Klasse IgG(4) |
| LC-MS ^E | <i>liquid chromatography – tandem mass spectrometry</i> ^{<i>elevated energy</i>} |
| MeOH | Methanol |
| MRA | <i>mediator release assay</i> , Mediatorenfreisetzungstest |
| nanoDSF | <i>nano differential scanning fluorimetry</i> |
| NMR | Magnetische Kernspinresonanz |
| OAS | Orales Allergiesyndrom |
| pdb | <i>protein data bank</i> |
| PMF | <i>peptide mass fingerprint</i> , Peptidmassenfingerabdruck |
| p-loop | <i>phosphate binding loop</i> |
| PR-10 Proteine | <i>pathogenesis related</i> Proteine der Klasse 10 |
| Q3OS | Quercetin-3-O-Sophorosid |
| Q3O-(Glc)-Gal | Quercetin-3-O-(2''-O-β-D-Glucopyranosyl)-β-D-Galaktopyranosid |
| RAST | <i>Radioactive allergosorbent assay</i> |
| RBL-Zellen | <i>Rat basophilic leukaemia</i> -Zellen |
| (m)RNA | (<i>messenger</i>) Ribonukleinsäure |
| RP-HPLC | <i>reversed phase – high performance liquid chromatography</i> |
| RT-PCR | <i>Reverse transcriptase – polymerase chain reaction</i> |
| SDS-PAGE | Natriumdodecylsulfat-Polyacrylamidgelelektrophorese |
| SEC | Größenausschlusschromatographie |
| SIT | Spezifische Immuntherapie |
| T _H 2-Zellen | Typ-2 T-Helferzellen |

6. Literaturverzeichnis

- Aalberse, R.C. (2011). The role of IgG antibodies in allergy and immunotherapy. *Allergy 66 Suppl 95*, 28–30.
- Aalberse, R.C. (2000). Structural biology of allergens. *J. Allergy Clin. Immunol. 106*, 228–238.
- Aalberse, R.C., Dieges, P.H., Knul-Bretlova, V., Vooren, P., Aalbers, M., and van Leeuwen, J. (1983). IgG4 as a blocking antibody. *Clin. Rev. Allergy 1*, 289–302.
- Abbas, A.K., Williams, M.E., Burstein, H.J., Chang, T.L., Bossu, P., and Lichtman, A.H. (1991). Activation and functions of CD4+ T-cell subsets. *Immunol. Rev. 123*, 5–22.
- Aglas, L., Soh, W.T., Kraiem, A., Wenger, M., Brandstetter, H., and Ferreira, F. (2020). Ligand Binding of PR-10 Proteins with a Particular Focus on the Bet v 1 Allergen Family. *Curr. Allergy Asthma Rep. 20*, 25.
- Aguiló-Aguayo, I., Brunton, N., Rai, D.K., Balagueró, E., Hossain, M.B., and Valverde, J. (2014). Polyacetylene levels in carrot juice, effect of pH and thermal processing. *Food Chem. 152*, 370–377.
- Asam, C., Batista, A.L., Moraes, A.H., de Paula, V.S., Almeida, F.C.L., Aglas, L., Kitzmüller, C., Bohle, B., Ebner, C., Ferreira, F., *et al.* (2014). Bet v 1--a Trojan horse for small ligands boosting allergic sensitization? *Clin. Exp. Allergy 44*, 1083–1093.
- Averbeck, M., Gebhardt, C., Emmrich, F., Treudler, R., and Simon, J.C. (2007). Immunologic principles of allergic disease. *J. Dtsch. Dermatol. Ges. 5*, 1015–1028.
- Ballmer-Weber, B.K. (2015). Food allergy in adolescence and adulthood. *Chem. Immunol. Allergy 101*, 51–58.
- Ballmer-Weber, B.K., Wüthrich, B., Wangorsch, A., Fötisch, K., Altmann, F., and Vieths, S. (2001). Carrot allergy: double-blinded, placebo-controlled food challenge and identification of allergens. *J. Allergy Clin. Immunol. 108*, 301–307.
- Ballmer-Weber, B.K., Hoffmann, A., Wüthrich, B., Lüttkopf, D., Pompei, C., Wangorsch, A., Kästner, M., and Vieths, S. (2002a). Influence of food processing on the allergenicity of celery: DBPCFC with celery spice and cooked celery in patients with celery allergy. *Allergy 57*, 228–235.
- Ballmer-Weber, B.K., Scheurer, S., Fritsche, P., Enrique, E., Cistero-Bahima, A., Haase, T., and Wüthrich, B. (2002b). Component-resolved diagnosis with recombinant allergens in patients with cherry allergy. *J. Allergy Clin. Immunol. 110*, 167–173.
- Ballmer-Weber, B.K., Wangorsch, A., Bohle, B., Kaul, S., Kündig, T., Fötisch, K., van Ree, R., and Vieths, S. (2005). Component-resolved in vitro diagnosis in carrot allergy: does the use of recombinant carrot allergens improve the reliability of the diagnostic procedure? *Clin. Exp. Allergy 35*, 970–978.

- Bannon, G.A. (2004). What makes a food protein an allergen? *Curr. Allergy Asthma Rep.* 4, 43–46.
- Bell, E.A., Charlwood, B.V. (eds) (1980). *Secondary Plant Products*. Springer, Heidelberg.
- Bergmann, K.-C. (2016). Weather conditions and climate change have an effect on allergies. *Allergo J. Int.* 25, 131–137.
- Berin, M.C. (2014). Future Therapies for IgE-Mediated Food Allergy. *Curr. Pediatr. Rep.* 2, 119–126.
- Berkner, H., Schweimer, K., Matecko, I., and Rösch, P. (2008). Conformation, catalytic site, and enzymatic mechanism of the PR10 allergen-related enzyme norcochlorogenic acid synthase. *Biochem. J.* 413, 281–290.
- Beyer, K., Grishina, G., Bardina, L., Grishin, A., and Sampson, H.A. (2002). Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions. *J. Allergy Clin. Immunol.* 110, 517–523.
- Biesiadka, J., Bujacz, G., Sikorski, M.M., and Jaskolski, M. (2002). Crystal structures of two homologous pathogenesis-related proteins from yellow lupine. *J. Mol. Biol.* 319, 1223–1234.
- Blank, S., Seismann, H., Michel, Y., McIntyre, M., Cifuentes, L., Braren, I., Grunwald, T., Darsow, U., Ring, J., Bredehorst, R., *et al.* (2011). Api m 10, a genuine *A. mellifera* venom allergen, is clinically relevant but underrepresented in therapeutic extracts. *Allergy* 66, 1322–1329.
- Bohle, B. (2007). The impact of pollen-related food allergens on pollen allergy. *Allergy* 62, 3–10.
- Bohle, B., Zwölfer, B., Heratizadeh, A., Jahn-Schmid, B., Antonia, Y.D., Alter, M., Keller, W., Zuidmeer, L., van Ree, R., Werfel, T., *et al.* (2006). Cooking birch pollen-related food: divergent consequences for IgE- and T cell-mediated reactivity in vitro and in vivo. *J. Allergy Clin. Immunol.* 118, 242–249.
- Bollen, M.A. (2009). Stability of the Bet v 1 cross-reactive allergens Api g 1 and Dau c 1 : a biophysical approach. PhD Thesis. Wageningen University.
- Breda, C., Sallaud, C., el-Turk, J., Buffard, D., de Kozak, I., Esnault, R., and Kondorosi, A. (1996). Defense reaction in *Medicago sativa*: a gene encoding a class 10 PR protein is expressed in vascular bundles. *Mol. Plant Microbe Interact.* 9, 713–719.
- Breitenbach, M., Ferreira, F., Jilek, A., Swoboda, I., Ebner, C., Hoffmann-Sommergruber, K., Briza, P., Scheiner, O., and Kraft, D. (1996). Biological and immunological importance of Bet v 1 isoforms. *Adv. Exp. Med. Biol.* 409, 117–126.

- Breiteneder, H., and Mills, E.N.C. (2005). Molecular properties of food allergens. *J. Allergy Clin. Immunol.* *115*, 14–23.
- Breiteneder, H., Ferreira, F., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1993). Four recombinant isoforms of Cor a I, the major allergen of hazel pollen, show different IgE-binding properties. *Eur. J. Biochem.* *212*, 355–362.
- Brunetti, C., Di Ferdinando, M., Fini, A., Pollastri, S., and Tattini, M. (2013). Flavonoids as antioxidants and developmental regulators: relative significance in plants and humans. *Int. J. Mol. Sci.* *14*, 3540–3555.
- Bufe, A. (1998). The biological function of allergens: relevant for the induction of allergic diseases? *Int. Arch. Allergy Immunol.* *117*, 215–219.
- Campbell, D.E., and Mehr, S. (2015). Fifty years of allergy: 1965-2015. *J. Paediatr. Child Health* *51*, 91–93.
- Canis, M., Gröger, M., Becker, S., Klemens, C., and Kramer, M.F. (2011). Recombinant marker allergens in diagnosis of patients with allergic rhinoconjunctivitis to tree and grass pollens. *Am. J. Rhinol. Allergy* *25*, 36–39.
- Casañal, A., Zander, U., Muñoz, C., Dupeux, F., Luque, I., Botella, M.A., Schwab, W., Valpuesta, V., and Marquez, J.A. (2013). The strawberry pathogenesis-related 10 (PR-10) Fra a proteins control flavonoid biosynthesis by binding to metabolic intermediates. *J. Biol. Chem.* *288*, 35322–35332.
- Chapman, M.D., Smith, A.M., Vailes, L.D., and Arruda, L.K. (1997). Recombinant mite allergens. New technologies for the management of patients with asthma. *Allergy* *52*, 374–379.
- Chapman, M.D., Smith, A.M., Vailes, L.D., Arruda, L.K., Dhanaraj, V., and Pomés, A. (2000). Recombinant allergens for diagnosis and therapy of allergic disease. *J. Allergy Clin. Immunol.* *106*, 409–418.
- Chapman, M.D., Pomés, A., Breiteneder, H., and Ferreira, F. (2007). Nomenclature and structural biology of allergens. *J. Allergy Clin. Immunol.* *119*, 414–420.
- Cromwell, O., Häfner, D., and Nandy, A. (2011). Recombinant allergens for specific immunotherapy. *J. Allergy Clin. Immunol.* *127*, 865–872.
- Crowell, D.N., John, M.E., Russell, D., and Amasino, R.M. (1992). Characterization of a stress-induced, developmentally regulated gene family from soybean. *Plant Mol. Biol.* *18*, 459–466.
- Cudowska, B., Kaczmarek, M., and Restani, P. (2005). Immunoblotting in the diagnosis of cross-reactivity in children allergic to birch. *Rocz. Akad. Med. Białymst.* *50*, 268–273.
- D'Amato, G. (2000). Urban air pollution and plant-derived respiratory allergy. *Clin. Exp. Allergy* *30*, 628–636.

- D'Amato, G. (2002). Urban air pollution and respiratory allergy. *Monaldi Arch. Chest Dis.* 57, 136–140.
- D'Amato, G., Cecchi, L., Bonini, S., Nunes, C., Annesi-Maesano, I., Behrendt, H., Liccardi, G., Popov, T., and van Cauwenberge, P. (2007). Allergenic pollen and pollen allergy in Europe. *Allergy* 62, 976–990.
- De Knop, K.J., Verweij, M.M., Grimmelikhuijsen, M., Philipse, E., Hagendorens, M.M., Britts, C.H., De Clerck, L.S., Stevens, W.J., and Ebo, D.G. (2011). Age-related sensitization profiles for hazelnut (*Corylus avellana*) in a birch-endemic region. *Pediatr. Allergy Immunol.* 22, e139-149.
- Deifl, S., and Bohle, B. (2011). Factors influencing the allergenicity and adjuvanticity of allergens. *Immunotherapy* 3, 881–893.
- Deller, M.C., Kong, L., and Rupp, B. (2016). Protein stability: a crystallographer's perspective. *Acta Crystallogr. F: Struct. Biol. Commun.* 72, 72–95.
- Ebner, C., Hoffmann-Sommergruber, K., and Breiteneder, H. (2001). Plant food allergens homologous to pathogenesis-related proteins. *Allergy* 56 Suppl 67, 43–44.
- Eriksson, N.E. (1978). Allergy to pollen from different deciduous trees in Sweden. An investigation with skin tests, provocation tests and the radioallergosorbent test (RAST) in springtime hay fever patients. *Allergy* 33, 299–309.
- Eriksson, N.E., Formgren, H., and Svenonius, E. (1982). Food hypersensitivity in patients with pollen allergy. *Allergy* 37, 437–443.
- Erler, A., Hawranek, T., Krückemeier, L., Asam, C., Egger, M., Ferreira, F., and Briza, P. (2011). Proteomic profiling of birch (*Betula verrucosa*) pollen extracts from different origins. *Proteomics* 11, 1486–1498.
- Etesamifar, M., and Wüthrich, B. (1998). IgE-vermittelte Nahrungsmittelallergien bei 383 Patienten unter Berücksichtigung des oralen Allergiesyndroms. *Allergologie* 21, 451–457.
- Ewan, P.W., Deighton, J., Wilson, A.B., and Lachmann, P.J. (1993). Venom-specific IgG antibodies in bee and wasp allergy: lack of correlation with protection from stings. *Clin. Exp. Allergy* 23, 647–660.
- Fernandes, H., Michalska, K., Sikorski, M., and Jaskolski, M. (2013). Structural and functional aspects of PR-10 proteins. *FEBS J.* 280, 1169–1199.
- Ferreira, F., Hirtenlehner, K., Jilek, A., Godnik-Cvar, J., Breiteneder, H., Grimm, R., Hoffmann-Sommergruber, K., Scheiner, O., Kraft, D., Breitenbach, M., *et al.* (1996). Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J. Exp. Med.* 183, 599–609.

- Ferreira, F., Wolf, M., and Wallner, M. (2014). Molecular approach to allergy diagnosis and therapy. *Yonsei Med. J.* 55, 839–852.
- Fini, A., Brunetti, C., Di Ferdinando, M., Ferrini, F., and Tattini, M. (2011). Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants. *Plant Signal. Behav.* 6, 709–711.
- Francis, J.N., James, L.K., Paraskevopoulos, G., Wong, C., Calderon, M.A., Durham, S.R., and Till, S.J. (2008). Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. *J. Allergy Clin. Immunol.* 121, 1120–1125.e2.
- Frick, M., Fischer, J., Helbling, A., Ruëff, F., Wiczorek, D., Ollert, M., Pfützner, W., Müller, S., Huss-Marp, J., Dorn, B., *et al.* (2016). Predominant Api m 10 sensitization as risk factor for treatment failure in honey bee venom immunotherapy. *J. Allergy Clin. Immunol.* 138, 1663–1671.e9.
- Gaier, S., Marsh, J., Oberhuber, C., Rigby, N.M., Lovegrove, A., Alessandri, S., Briza, P., Radauer, C., Zuidmeer, L., van Ree, R., *et al.* (2008). Purification and structural stability of the peach allergens Pru p 1 and Pru p 3. *Mol. Nutr. Food Res.* 52 Suppl. 2, S220–229.
- Gajhede, M., Osmark, P., Poulsen, F.M., Ipsen, H., Larsen, J.N., Joost van Neerven, R.J., Schou, C., Løwenstein, H., and Spangfort, M.D. (1996). X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat. Struct. Biol.* 3, 1040–1045.
- Gehlhar, K., Schlaak, M., Becker, W., and Bufe, A. (1999). Monitoring allergen immunotherapy of pollen-allergic patients: the ratio of allergen-specific IgG4 to IgG1 correlates with clinical outcome. *Clin. Exp. Allergy* 29, 497–506.
- Gould, H.J., and Sutton, B.J. (2008). IgE in allergy and asthma today. *Nat. Rev. Immunol.* 8, 205–217.
- Gruber, P., Vieths, S., Wangorsch, A., Nerkamp, J., and Hofmann, T. (2004). Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (*Prunus avium*). *J. Agric. Food Chem.* 52, 4002–4007.
- Hansen, K.S., Ballmer-Weber, B.K., Sastre, J., Lidholm, J., Andersson, K., Oberhofer, H., Lluch-Bernal, M., Ostling, J., Mattsson, L., Schocker, F., *et al.* (2009). Component-resolved in vitro diagnosis of hazelnut allergy in Europe. *J. Allergy Clin. Immunol.* 123, 1134–1141, 1141.e1–3.
- Helbling, A., Lopez, M., Schwartz, H.J., and Lehrer, S.B. (1993). Reactivity of carrot-specific IgE antibodies with celery, apiaceous spices, and birch pollen. *Ann. Allergy* 70, 495–499.

- Herbert, C.A., King, C.M., Ring, P.C., Holgate, S.T., Stewart, G.A., Thompson, P.J., and Robinson, C. (1995). Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. *Am. J. Respir. Cell Mol. Biol.* *12*, 369–378.
- Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W.R., Valent, P., Rohac, M., Rumpold, H., Scheiner, O., and Kraft, D. (1992). Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.* *90*, 927–936.
- Hoffmann-Sommergruber, K., O’Riordain, G., Ahorn, H., Ebner, C., Laimer Da Camara Machado, M., Pühringer, H., Scheiner, O., and Breiteneder, H. (1999a). Molecular characterization of Dau c 1, the Bet v 1 homologous protein from carrot and its cross-reactivity with Bet v 1 and Api g 1. *Clin. Exp. Allergy* *29*, 840–847.
- Hoffmann-Sommergruber, K., Demoly, P., Cramer, R., Breiteneder, H., Ebner, C., Laimer Da Camara Machado, M., Blaser, K., Ismail, C., Scheiner, O., Bousquet, J., *et al.* (1999b). IgE reactivity to Api g 1, a major celery allergen, in a Central European population is based on primary sensitization by Bet v 1. *J. Allergy Clin. Immunol.* *104*, 478–484.
- Holloway, J.W., Yang, I.A., and Holgate, S.T. (2010). Genetics of allergic disease. *J. Allergy Clin. Immunol.* *125*, S81–S94.
- Houska M., Šetinová, I., Kučera, P. (2013). Food allergens and Processing: A Review of Recent Results. In: Yanniotis S., Taoukis P., Stoforos, N., Karathanos V. (eds) *Advances in Food Process Engineering Research and Applications*. Food Engineering Series. Springer, Boston, MA.
- Irvine, G.B. (2001). Determination of molecular size by size-exclusion chromatography (gel filtration). *Curr. Protoc. Cell Biol.* *Chapter 5*, Unit 5.5.
- Jarvis, D., and Burney, P. (1998). The epidemiology of allergic disease. *BMJ* *316*, 607–610.
- Karp, C.L. (2010). Guilt by intimate association: What makes an allergen an allergen? *J. Allergy Clin. Immunol.* *125*, 955–962.
- Kaul, S., Lüttkopf, D., Kastner, B., Vogel, L., Hölz, G., Vieths, S., and Hoffmann, A. (2007). Mediator release assays based on human or murine immunoglobulin E in allergen standardization. *Clin. Exp. Allergy* *37*, 141–150.
- Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., and Sternberg, M.J.E. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols* *10*, 845–858.
- Kim, B.G., Yang, S.M., Kim, S.Y., Cha, M.N., and Ahn, J.-H. (2015). Biosynthesis and production of glycosylated flavonoids in *Escherichia coli*: current state and perspectives. *Appl. Microbiol. Biotechnol.* *99*, 2979–2988.
- Kim, J.S., Nowak-Węgrzyn, A., Sicherer, S.H., Noone, S., Moshier, E.L., and Sampson, H.A. (2011). Dietary baked milk accelerates the resolution of cow’s milk allergy in children. *J. Allergy Clin. Immunol.* *128*, 125-131.e2.

- Kofler, S., Asam, C., Eckhard, U., Wallner, M., Ferreira, F., and Brandstetter, H. (2012). Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen Bet v 1. *J. Mol. Biol.* *422*, 109–123.
- Koistinen, K.M., Soininen, P., Venäläinen, T.A., Häyrinen, J., Laatikainen, R., Peräkylä, M., Tervahauta, A.I., and Kärenlampi, S.O. (2005). Birch PR-10c interacts with several biologically important ligands. *Phytochemistry* *66*, 2524–2533.
- Kumar, S., and Pandey, A.K. (2013). Chemistry and biological activities of flavonoids: an overview. *Sci. World J.* *2013*, 162750.
- Leonard, S.A., Sampson, H.A., Sicherer, S.H., Noone, S., Moshier, E.L., Godbold, J., and Nowak-Węgrzyn, A. (2012). Dietary baked egg accelerates resolution of egg allergy in children. *J. Allergy Clin. Immunol.* *130*, 473-480.e1.
- Li, J., Ou-Lee, T.M., Raba, R., Amundson, R.G., and Last, R.L. (1993). Arabidopsis Flavonoid Mutants Are Hypersensitive to UV-B Irradiation. *Plant Cell* *5*, 171–179.
- Lüderitz-Püchel, U., Keller-Stanislawski, B., and Hausteil, D. (2001). Neubewertung des Risikos von Test- und Therapieallergenen. *Bundesgesundheitsblatt - Gesundheitsforschung - Gesundheitsschutz* *44*, 709–718.
- Lüttkopf, D., Müller, U., Skov, P.S., Ballmer-Weber, B.K., Wüthrich, B., Skamstrup Hansen, K., Poulsen, L.K., Kästner, M., Hausteil, D., and Vieths, S. (2002). Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Mol. Immunol.* *38*, 515–525.
- Machado, Y., Freier, R., Scheibelhofer, S., Thalhamer, T., Mayr, M., Briza, P., Grutsch, S., Ahammer, L., Fuchs, J.E., Wallnoefer, H.G., *et al.* (2016). Fold stability during endolysosomal acidification is a key factor for allergenicity and immunogenicity of the major birch pollen allergen. *J. Allergy Clin. Immunol.* *137*, 1525–1534.
- Maleki, S.J., Kopper, R.A., Shin, D.S., Park, C.W., Compadre, C.M., Sampson, H., Burks, A.W., and Bannon, G.A. (2000). Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J. Immunol.* *164*, 5844–5849.
- Mari, A., Ballmer-Weber, B.K., and Vieths, S. (2005). The oral allergy syndrome: improved diagnostic and treatment methods. *Curr. Opin. Allergy Clin. Immunol.* *5*, 267–273.
- Martino, D., and Prescott, S. (2011). Epigenetics and prenatal influences on asthma and allergic airways disease. *Chest* *139*, 640–647.
- McWilliam, V., Koplin, J., Lodge, C., Tang, M., Dharmage, S., and Allen, K. (2015). The Prevalence of Tree Nut Allergy: A Systematic Review. *Curr. Allergy Asthma Rep.* *15*, 54.
- Meno, K.H. (2011). Allergen structures and epitopes. *Allergy* *66 Suppl.* *95*, 19–21.

- Metzger, H., Alcaraz, G., Hohman, R., Kinet, J.P., Pribluda, V., and Quarto, R. (1986). The receptor with high affinity for immunoglobulin E. *Annu. Rev. Immunol.* *4*, 419–470.
- Miller, K.D., Guyon, V., Evans, J.N., Shuttleworth, W.A., and Taylor, L.P. (1999). Purification, cloning, and heterologous expression of a catalytically efficient flavonol 3-O-galactosyltransferase expressed in the male gametophyte of *Petunia hybrida*. *J. Biol. Chem.* *274*, 34011–34019.
- Mills, C., Wichers, H., and Hoffmann-Sommergruber, K. (2006). *Managing Allergens in Food*. Woodhead Publishing Series in Food Science, Technology and Nutrition.
- Mills, E.N.C., Sancho, A.I., Rigby, N.M., Jenkins, J.A., and Mackie, A.R. (2009). Impact of food processing on the structural and allergenic properties of food allergens. *Mol. Nutr. Food Res.* *53*, 963–969.
- Mirza, O., Henriksen, A., Ipsen, H., Larsen, J.N., Wissenbach, M., Spangfort, M.D., and Gajhede, M. (2000). Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J. Immunol.* *165*, 331–338.
- Mittag, D., Vieths, S., Vogel, L., Becker, W.-M., Rihs, H.-P., Helbling, A., Wüthrich, B., and Ballmer-Weber, B.K. (2004). Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens. *J. Allergy Clin. Immunol.* *113*, 148–154.
- Mo, Y., Nagel, C., and Taylor, L.P. (1992). Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proc. Natl. Acad. Sci. U.S.A.* *89*, 7213–7217.
- Mogensen, J.E., Wimmer, R., Larsen, J.N., Spangfort, M.D., and Otzen, D.E. (2002). The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J. Biol. Chem.* *277*, 23684–23692.
- Moneo, I., Gómez, M., Sánchez-Monge, R., Alday, E., de las Heras, M., Esteban, I., Bootello, A., and Salcedo, G. (1999). Lack of crossreaction with Bet v 1 in patients sensitized to Dau c 1, a carrot allergen. *Ann. Allergy Asthma Immunol.* *83*, 71–75.
- Movérare, R., Vesterinen, E., Metso, T., Sorva, R., Elfman, L., and Haahtela, T. (2001). Pollen-specific rush immunotherapy: clinical efficacy and effects on antibody concentrations. *Ann. Allergy Asthma Immunol.* *86*, 337–342.
- Movérare, R., Westritschnig, K., Svensson, M., Hayek, B., Bende, M., Pauli, G., Sorva, R., Haahtela, T., Valenta, R., and Elfman, L. (2002). Different IgE reactivity profiles in birch pollen-sensitive patients from six European populations revealed by recombinant allergens: an imprint of local sensitization. *Int. Arch. Allergy Immunol.* *128*, 325–335.
- Muñoz, C., Hoffmann, T., Escobar, N.M., Ludemann, F., Botella, M.A., Valpuesta, V., and Schwab, W. (2010). The strawberry fruit Fra a allergen functions in flavonoid biosynthesis. *Mol. Plant* *3*, 113–124.

- Nesvizhskii, A.I., and Aebersold, R. (2005). Interpretation of shotgun proteomic data: the protein inference problem. *Mol. Cell Proteomics* 4, 1419–1440.
- Ober, C., and Yao, T.-C. (2011). The genetics of asthma and allergic disease: a 21st century perspective. *Immunological Reviews* 242, 10–30.
- Olsen, J.L. (2013). Growing Hazelnuts in the Pacific Northwest: Pollination and Nut Development. OSU Extension Catalog 1–4.
- Orengo, J.M., Radin, A.R., Kamat, V., Badithe, A., Ben, L.H., Bennett, B.L., Zhong, S., Birchard, D., Limnander, A., Rafique, A., *et al.* (2018). Treating cat allergy with monoclonal IgG antibodies that bind allergen and prevent IgE engagement. *Nat. Commun.* 9, 1421.
- Ortolani, C., Ballmer-Weber, B.K., Hansen, K.S., Ispano, M., Wüthrich, B., Bindslev-Jensen, C., Ansaloni, R., Vannucci, L., Pravettoni, V., Scibilia, J., *et al.* (2000). Hazelnut allergy: a double-blind, placebo-controlled food challenge multicenter study. *J. Allergy Clin. Immunol.* 105, 577–581.
- Ownby, D.R. (1988). Allergy testing: in vivo versus in vitro. *Pediatr. Clin. North Am.* 35, 995–1009.
- Pauli, G., Larsen, T.H., Rak, S., Horak, F., Pastorello, E., Valenta, R., Valenta, R., Purohit, A., Arvidsson, M., Kavina, A., *et al.* (2008). Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. *J. Allergy Clin. Immunol.* 122, 951–960.
- Pawankar, R., Holgate, S.T., Canonica, G.W., Lockey, R.F., Blaiss, M.S. (Update 2013) The WAO White Book on Allergy: Executive Summary.
- Pekar, J., Ret, D., and Untersmayr, E. (2018). Stability of allergens. *Mol. Immunol.* 100, 14–20.
- Peters, S., Imani, J., Mahler, V., Foetisch, K., Kaul, S., Paulus, K.E., Scheurer, S., Vieths, S., and Kogel, K.-H. (2011). Dau c 1.01 and Dau c 1.02-silenced transgenic carrot plants show reduced allergenicity to patients with carrot allergy. *Transgenic Res.* 20, 547–556.
- Pomés, A., Davies, J.M., Gadermaier, G., Hilger, C., Holzhauser, T., Lidholm, J., Lopata, A.L., Mueller, G.A., Nandy, A., Radauer, C., *et al.* (2018). WHO/IUIS Allergen Nomenclature: Providing a common language. *Mol. Immunol.* 100, 3–13.
- Radauer, C., Bublin, M., Wagner, S., Mari, A., and Breiteneder, H. (2008). Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J. Allergy Clin. Immunol.* 121, 847-852.e7.
- Rimmer, J., Santos, C., Yli-Panula, E., Noronha, V., and Viander, M. (2015). Clinical and laboratory studies of the fate of intranasal allergen. *PLoS ONE* 10, e0127477.

- Romagnani, S. (2004). The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? *Immunology* *112*, 352–363.
- Rupa, P., Nakamura, S., Katayama, S., and Mine, Y. (2014). Effects of ovalbumin glycoconjugates on alleviation of orally induced egg allergy in mice via dendritic-cell maturation and T-cell activation. *Mol. Nutr. Food Res.* *58*, 405–417.
- Saraste, M., Sibbald, P.R., and Wittinghofer, A. (1990). The P-loop--a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* *15*, 430–434.
- Scheiner, O., and Kraft, D. (1995). Basic and practical aspects of recombinant allergens. *Allergy* *50*, 384–391.
- Scheurer, S., Lauer, I., Foetisch, K., San Miguel Moncin, M., Retzek, M., Hartz, C., Enrique, E., Lidholm, J., Cistero-Bahima, A., and Vieths, S. (2004). Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. *J. Allergy Clin. Immunol.* *114*, 900–907.
- Schocker, F., Lüttkopf, D., Scheurer, S., Petersen, A., Cisteró-Bahima, A., Enrique, E., San Miguel-Moncín, M., Akkerdaas, J., van Ree, R., Vieths, S., *et al.* (2004). Recombinant lipid transfer protein Cor a 8 from hazelnut: a new tool for in vitro diagnosis of potentially severe hazelnut allergy. *J. Allergy Clin. Immunol.* *113*, 141–147.
- Schrödinger, LLC. 2010. The PyMOL Molecular Graphics System, Version 1.8
- Seutter von Loetzen, C., Hoffmann, T., Hartl, M.J., Schweimer, K., Schwab, W., Rösch, P., and Hartl-Spiegelhauer, O. (2014). Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. *Biochem. J.* *457*, 379–390.
- Seutter von Loetzen, C., Jacob, T., Hartl-Spiegelhauer, O., Vogel, L., Schiller, D., Spörlein-Güttler, C., Schobert, R., Vieths, S., Hartl, M.J., and Rösch, P. (2015). Ligand Recognition of the Major Birch Pollen Allergen Bet v 1 is Isoform Dependent. *PLoS ONE* *10*, e0128677.
- Seutter von Loetzen, C., Reuter, A., Spiric, J., Schulenburg, T., Bellinghausen, I., Völker, E., Vogel, L., Rösch, P., and Schiller, D. (2019). Quality and potency profile of eight recombinant isoallergens, largely mimicking total Bet v 1-specific IgE binding of birch pollen. *Clin. Exp. Allergy* *49*, 712–723.
- Shamji, M.H., Ljørring, C., Francis, J.N., Calderon, M.A., Larché, M., Kimber, I., Frew, A.J., Ipsen, H., Lund, K., Würtzen, P.A., *et al.* (2012). Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. *Allergy* *67*, 217–226.
- Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* *68*, 850–858.

- Sikorski, M.M., Biesiadka, J., Kasperska, A.E., Kopcińska, J., Łotocka, B., Golinowski, W., and Legocki, A.B. (1999). Expression of genes encoding PR10 class pathogenesis-related proteins is inhibited in yellow lupine root nodules. *Plant Science* *149*, 125–137.
- Simons, F.E.R. (2008). 9. Anaphylaxis. *J. Allergy Clin. Immunol.* *121*, S402-407; quiz S420.
- Soh, W.T., Aglas, L., Mueller, G.A., Gilles, S., Weiss, R., Scheiblhofer, S., Huber, S., Scheidt, T., Thompson, P.M., Briza, P., *et al.* (2019). Multiple roles of Bet v 1 ligands in allergen stabilization and modulation of endosomal protease activity. *Allergy* *74*, 2382–2393.
- Spiric, J., Engin, A.M., Karas, M., and Reuter, A. (2015). Quality Control of Biomedical Allergen Products - Highly Complex Isoallergen Composition Challenges Standard MS Database Search and Requires Manual Data Analyses. *PLoS ONE* *10*, e0142404.
- Strachan, D.P. (1989). Hay fever, hygiene, and household size. *BMJ* *299*, 1259–1260.
- Strack, D., Meurer, B., Wray, V., Grotjahn, L., Austenfeld, F., and Wiermann, R. (1984). Quercetin 3-glucosylgalactoside from pollen of *Corylus avellana*. *Phytochemistry* *23*, 2970–2971.
- Thomas, W.R., and Smith, W. (1999). Towards defining the full spectrum of important house dust mite allergens. *Clin. Exp. Allergy* *29*, 1583–1587.
- Thomas, W.R., Hales, B.J., and Smith, W.-A. (2005). Structural biology of allergens. *Curr. Allergy Asthma Rep.* *5*, 388–393.
- Valenta, R., and Kraft, D. (1996). Type I allergic reactions to plant-derived food: A consequence of primary sensitization to pollen allergens. *J. Allergy Clin. Immunol.* *97*, 893–895.
- Valenta, R., Breiteneder, H., Petternburger, K., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1991). Homology of the major birch-pollen allergen, Bet v I, with the major pollen allergens of alder, hazel, and hornbeam at the nucleic acid level as determined by cross-hybridization. *J. Allergy Clin. Immunol.* *87*, 677–682.
- Valenta, R., Lidholm, J., Niederberger, V., Hayek, B., Kraft, D., and Grönlund, H. (1999). The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin. Exp. Allergy* *29*, 896–904.
- Vieths, S., Jankiewicz, A., Aulepp, H., and Hausteiner, D. (1997). Allergy to heated and processed foods. *Arb. Paul-Ehrlich Inst. Bundesamt Sera Impfstoffe Frankf. a. M.* 250–262.
- Vieths, S., Scheurer, S., and Ballmer-Weber, B. (2002). Current Understanding of Cross-Reactivity of Food Allergens and Pollen. *Annals of the New York Academy of Sciences* *964*, 47–68.
- Vogel, L., Lüttkopf, D., Hatahet, L., Hausteiner, D., and Vieths, S. (2005). Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* *60*, 1021–1028.

- Vogt, T., and Taylor, L.P. (1995). Flavonol 3-O-glycosyltransferases associated with petunia pollen produce gametophyte-specific flavonol diglycosides. *Plant Physiol.* *108*, 903–911.
- Wachholz, P.A., Soni, N.K., Till, S.J., and Durham, S.R. (2003). Inhibition of allergen-IgE binding to B cells by IgG antibodies after grass pollen immunotherapy. *J. Allergy Clin. Immunol.* *112*, 915–922.
- Wangorsch, A., Weigand, D., Peters, S., Mahler, V., Fötisch, K., Reuter, A., Imani, J., Dewitt, A.M., Kogel, K.-H., Lidholm, J., *et al.* (2012). Identification of a Dau c PRPlike protein (Dau c 1.03) as a new allergenic isoform in carrots (cultivar Rodelika). *Clin. Exp. Allergy* *42*, 156–166.
- Webber, C.M., and England, R.W. (2010). Oral allergy syndrome: a clinical, diagnostic, and therapeutic challenge. *Annals of Allergy, Asthma & Immunology* *104*, 101–108.
- Wickens, K., Barry, D., Friezema, A., Rhodius, R., Bone, N., Purdie, G., and Crane, J. (2005). Fast foods - are they a risk factor for asthma? *Allergy* *60*, 1537–1541.
- Wild, C., Wallner, M., Hufnagl, K., Fuchs, H., Hoffmann-Sommergruber, K., Breiteneder, H., Scheiner, O., Ferreira, F., and Wiedermann, U. (2007). A recombinant allergen chimera as novel mucosal vaccine candidate for prevention of multi-sensitivities. *Allergy* *62*, 33–41.
- Wills-Karp, M. (1999). Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* *17*, 255–281.
- Wills-Karp, M., Nathan, A., Page, K., and Karp, C.L. (2010). New insights into innate immune mechanisms underlying allergenicity. *Mucosal Immunol.* *3*, 104–110.
- Yamamoto, M., Torikai, S., and Oeda, K. (1997). A major root protein of carrots with high homology to intracellular pathogenesis-related (PR) proteins and pollen allergens. *Plant Cell Physiol.* *38*, 1080–1086.
- Zulehner, N., Nagl, B., Briza, P., Roulias, A., Ballmer-Weber, B., Zlabinger, G.J., Ferreira, F., and Bohle, B. (2017). Characterization of the T-cell response to Dau c 1, the Bet v 1-homolog in carrot. *Allergy* *72*, 244–251.

7. Publikationsliste

7.1 Einzelarbeit A

Thessa Jacob, Christian Seutter von Loetzen, Andreas Reuter, Ulrike Lacher, Dirk Schiller, Rainer Schobert, Vera Mahler, Stefan Vieths, Paul Rösch, Kristian Schweimer, Birgitta M. Wöhr (2019). Identification of a natural ligand of the hazel allergen Cor a 1. *Scientific Reports*, 9: 8714

Birgitta M. Wöhr und ich verfassten das Manuskript. Paul Rösch und Stefan Vieths initiierten das Projekt und trugen zu dessen Konzeption bei. Birgitta M. Wöhr, Christian Seutter von Loetzen und Kristian Schweimer beaufsichtigten das Projekt und entwarfen Experimente. Christian Seutter von Loetzen und ich exprimierten und reinigten die Proteine. Die NMR-Experimente wurden von Kristian Schweimer, Christian Seutter von Loetzen und mir durchgeführt und ausgewertet. Kristian Schweimer löste die Struktur von Bet v 1.0101. Dirk Schiller klonierte und sequenzierte rCor a 1.0401. Andreas Reuter führte die massenspektrometrischen Analysen von rCor a 1.0401, nCor a 1 und Haselpollenextrakt durch. Ulrike Lacher und Rainer Schobert führten die massenspektrometrischen Analysen des Liganden durch und werteten die Daten aus. Vera Mahler trug zur Analyse und Interpretation der Daten bei. Alle Autoren haben zur Vorbereitung des Manuskripts beigetragen.

7.2 Einzelarbeit B

Thessa Jacob, Lothar Vogel, Andreas Reuter, Andrea Wangorsch, Carolin Kring, Vera Mahler, Birgitta M. Wöhr (2020). Food processing does not abolish the allergenicity of the carrot allergen Dau c 1: influence of pH, temperature and the food matrix. *Molecular Nutrition & Food Research*, e2000334

Die Planung der Experimente und das Verfassen des Manuskripts erfolgte durch Birgitta M. Wöhr und mich. Die verwendeten Proteine wurden von mir exprimiert und gereinigt. Weiterhin wurden SEC, CD und NMR Experimente von mir durchgeführt. Carolin Kring führte die CD-Experimente für Dau c 1.0105 und Dau c 1.0201 durch. Die Daten wurden von Birgitta M. Wöhr und mir ausgewertet. NanoDSF Experimente wurden von Andrea Wangorsch und mir durchgeführt. MRAs wurden von Lothar Vogel durchgeführt, analysiert und bewertet. Andreas Reuter führte alle massenspektrometrischen Analysen durch, interpretierte sie, und verfasste den entsprechenden Teil des Manuskripts. Vera Mahler beteiligte sich an der Analyse und Interpretation der Daten. Alle Autoren haben zur Vorbereitung des Manuskripts beigetragen.

7.3 Einzelarbeit C

*Thessa Jacob ,*Andrea Wangorsch, Lothar Vogel, Andreas Reuter, Vera Mahler, Birgitta M. Wöhr (2021). A novel isoallergen Dau c 1.0401 in carrot: stability, allergenicity and comparison with other isoallergens. *Molecular Nutrition & Food Research*, e2001110

* Beide Autorinnen haben in gleichem Maße zur Arbeit beigetragen.

Die RNA-Extraktion, cDNA-Klonierung, Amplifikation und Identifikation des neuen Isoallergens wurden von mir durchgeführt. Weiterhin klonierte ich das für Dau c 1.0401 kodierende Gen in einen Expressionsvektor und führte die Expression und Reinigung, sowie die CD-Messungen durch. Andrea Wangorsch führte die Immunoblots, ELISAS, Dot Blots, Inhibitionsexperimente sowie die nanoDSF Messung durch. MRAs wurden von Lothar Vogel durchgeführt und analysiert. Andreas Reuter führte die massenspektrometrischen Analysen von nDau c 1 durch. Die Experimente wurden von Birgitta M. Wöhr und mir konzipiert. Birgitta M. Wöhr und ich verfassten das Manuskript. Vera Mahler rekrutierte Karottenallergiker und charakterisierte sie klinisch. Des Weiteren beteiligte sie sich an der Analyse und Interpretation der Daten. Alle Autoren haben zur Vorbereitung des Manuskripts beigetragen.

8. Einzelarbeiten

8.1 Einzelarbeit A

Thessa Jacob, Christian Seutter von Loetzen, Andreas Reuter, Ulrike Lacher, Dirk Schiller, Rainer Schobert, Vera Mahler, Stefan Vieths, Paul Rösch, Kristian Schweimer, Birgitta M. Wöhr (2019). Identification of a natural ligand of the hazel allergen Cor a 1. *Scientific Reports*, 9:8714

SCIENTIFIC REPORTS

OPEN

Identification of a natural ligand of the hazel allergen Cor a 1

Thessa Jacob¹, Christian Seutter von Loetzen¹, Andreas Reuter^{1,3}, Ulrike Lacher⁴, Dirk Schiller³, Rainer Schobert⁴, Vera Mahler³, Stefan Vieths³, Paul Rösch^{1,2}, Kristian Schweimer¹ & Birgitta M. Wöhrl¹

Received: 21 November 2018

Accepted: 22 May 2019

Published online: 18 June 2019

Hazelnut is one of the most frequent causes of food allergy. The major hazel allergen in Northern Europe is Cor a 1, which is homologous to the major birch pollen allergen Bet v 1. Both allergens belong to the pathogenesis related class PR-10. We determined the solution structure of Cor a 1.0401 from hazelnut and identified a natural ligand of the protein. The structure reveals the protein fold characteristic for PR-10 family members, which consists of a seven-stranded antiparallel β -sheet, two short α -helices arranged in V-shape and a long C-terminal α -helix encompassing a hydrophobic pocket. However, despite the structural similarities between Cor a 1 and Bet v 1, they bind different ligands. We have shown previously that Bet v 1 binds to quercetin-3-O-sophoroside. Here, we isolated Cor a 1 from hazel pollen and identified the bound ligand, quercetin-3-O-(2'-O- β -D-glucopyranosyl)- β -D-galactopyranoside, by mass spectrometry and nuclear magnetic resonance spectroscopy (NMR). NMR experiments were performed to confirm binding. Remarkably, although it has been shown that PR-10 allergens show promiscuous binding behaviour *in vitro*, we can demonstrate that Cor a 1.0401 and Bet v 1.0101 exhibit highly selective binding for their specific ligand but not for the respective ligand of the other allergen.

Allergy to hazel is very common in Europe^{1,2} and has even been found to be the most frequent cause of IgE-mediated food allergy^{3–5}. Cor a 1.04, a Bet v 1 homologous allergen, which belongs to the family of pathogenesis-related plant proteins PR-10^{6,7} is the major hazelnut allergen in Northern Europe⁸. About 53% of people allergic to birch pollen suffer from cross reactivity to Cor a 1.04⁹.

PR-10 proteins are part of the plants' immune defence and are mostly induced by attack of different pathogens^{10,11} or abiotic stress stimuli^{12,13}. However, in certain plant tissues that have higher risks of being attacked by insects, fungi or of being damaged by UV-radiation, PR-10 proteins are expressed constitutively¹⁴. They are encoded by multiple genes and therefore occur as a mixture of different isoallergens with >67% sequence identity and variants (formerly also called isoforms) thereof, which share a very high sequence identity of >90%¹⁵.

The molecular role of PR-10 proteins under physiological conditions in different plants remains elusive. Numerous studies on recombinant ligand-free Bet v 1 exist, which show that it binds to a multitude of different ligands *in vitro* like flavonoids, cytokines and fatty acids with dissociation constants in the micromolar range^{16–20}.

Different Cor a 1 isoallergens and variants have been identified in hazel pollen as well as in hazelnut and hazel leaves²¹. In hazel pollen four different variants of Cor a 1.01, termed Cor a 1.0101 to Cor a 1.0104, have been detected²². Cor a 1.02 and Cor a 1.03 isoallergens can be found in mature hazel leaves²³. In hazelnut, four different variants of Cor a 1.04, Cor a 1.0401 to Cor a 1.0404 have been identified⁷. Interestingly, Cor a 1.04 variants show a higher sequence identity to Bet v 1.0101 (66–67%) than to Cor a 1.01 variants from hazel pollen (61–65%)⁷.

Although Bet v 1 has been extensively studied biochemically^{16,24–27} as well as immunologically^{25,28–31}, the exact physiological role of this protein and its homologs derived from different plants remains elusive.

Typically, the structure of PR-10 proteins consists of a seven-stranded, antiparallel β -sheet and a long, C-terminal α -helix which is enclosed by two shorter helices arranged in V-shape. Those elements encompass a large hydrophobic pocket³². Their common structure indicates a more general function, e.g. as storage- or transport-proteins.

To shed light on the physiological role of those proteins, we previously identified the glycosylated flavonoid quercetin-3-O-sophoroside (Q3OS) as a natural ligand of Bet v 1.0101 by co-purification of the

¹Universität Bayreuth, Lehrstuhl Biopolymere, Bayreuth, Germany. ²Forschungszentrum für Bio-Makromoleküle, Universitätsstr. 30, D-95447, Bayreuth, Germany. ³Division of Allergology, Paul-Ehrlich-Institut, D-63225, Langen, Germany. ⁴Universität Bayreuth, Lehrstuhl Organische Chemie I, D-95447, Bayreuth, Germany. Correspondence and requests for materials should be addressed to B.M.W. (email: birgitta.woehrl@uni-bayreuth.de)

protein-ligand-complex from birch pollen³³. Moreover, we found that different Bet v 1.01 variants show different binding behaviour³⁴ and that the binding specificity is driven by the sugar moiety of the ligand³³.

To investigate whether PR-10 proteins from other plants have identical or similar ligands and ligand binding behaviour, we purified Cor a 1 from hazel pollen in the presence of its ligand. We were able to identify quercetin-3-O-(2''-O-β-D-glucopyranosyl)-β-D-galactopyranoside (Q3O-(Glc)-Gal) as a natural ligand. Compared to Q3OS the only difference between the two ligands is the orientation of the C4 OH group in the first sugar moiety.

Most surprisingly, we can demonstrate that although they are known to show promiscuous ligand binding behaviour, the PR-10 allergens Bet v 1.0101 and Cor a 1.0401 exhibit strong binding specificities only for their own, almost identical ligands. Structure determination of Cor a 1.0401 and binding studies with quercetin, as well as with the ligands Q3OS and Q3O-(Glc)-Gal were performed to analyse the binding specificities.

Results and Discussion

Solution structure of Cor a 1.0401. To determine the solution structure of Cor a 1 we used a tagless, 160 amino acids long full length construct of the recombinant variant Cor a 1.0401, expressed in *Escherichia coli* (*E. coli*) and performed multidimensional heteronuclear NMR spectroscopy analyses. The spectra exhibited the large dispersion of chemical shifts typical for a well folded globular protein. The structure of Cor a 1.0401 showed the overall fold typical for PR-10 allergens, consisting of a seven-stranded antiparallel β-sheet followed by a long C-terminal helix, which is enclosed by two shorter helices arranged in V-shape that comprise a hydrophobic pocket (Fig. 1A; Table 1). No resonances could be identified in the NMR spectra for residues in the regions Ala35 - Thr40 (between strand β7 and the two short α-helices) and Thr58 - Met68 (between strand β5 and β6) (Fig. 1B,C). These regions consist of loops which, similarly to the strawberry allergen Fra a 1 probably show dynamic behaviour on unfavorable timescales to broaden NMR signals beyond detection³⁵. As a consequence of missing signals and corresponding lack of structural restraints a structural definition in this regions was not possible, and these loops differ significantly in the calculated structural ensemble (Fig. 1B). We solved the NMR solution structure of the homologous PR-10 allergen Bet v 1.0101 (PDB: 6R3C; see Supplementary Fig. S1, Table S1) in order to compare the two solution structures (Fig. 1D). The overlay of the Cor a 1.0401 solution structure and the high resolution structure of Bet v 1.0101 reveals the high structural similarity of the two proteins. Despite of the lower number of identified restraints for Cor a 1.0401, the overlay indicates that the structure is sufficiently good to show the overall fold of the protein and to identify its hydrophobic cavity, which is important for ligand binding.

Additionally, we performed structural overlays with various PR-10 allergens, Pru a v 1 from cherry, Gly m 4 from soy bean and the strawberry allergen Fra a 1E, which all exhibit highly similar folds (see Supplementary Fig. S2). Only the length of the loops and the orientation and length of the structural elements are slightly different. One common feature of PR-10 allergens is the so-called glycine-rich loop, which is highly conserved in structure and sequence (see Supplementary Fig. S2), however its function has not been identified yet.

Identification of the natural ligand of Cor a 1 isolated from pollen. Recently, we were able to identify a natural ligand of the major birch pollen allergen Bet v 1, Q3OS, by co-purification of the allergen in complex with its ligand from pollen³³. Natural Bet v 1 isolated from pollen consists of a mixture of different Bet v 1 isoallergens and variants thereof. Thus it is difficult to identify which protein(s) bind the ligand. Identification of the natural ligand contributes to the understanding of the function of PR-10 allergens in plants. To determine whether the same ligand can also be found in homologous PR-10 allergens, we purified the natural hazel allergen Cor a 1 from hazel pollen using a similar approach.

Natural Cor a 1 (nCor a 1) was extracted from mature hazel pollen derived from *Corylus avellana* and purified^{33,36}. After each purification step, samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A) (see Supplementary Fig. S3). Size exclusion chromatography (SEC) of pooled nCor a 1 fractions collected from a hydrophobic interaction column revealed two absorption maxima at an elution volume of 24 ml, one at 280 nm (protein) and the other one at 350 nm (ligand) (Fig. 2B). Pure SEC fractions were subjected to SDS-PAGE followed by mass spectrometry to identify the proteins present in the peak fractions.

Similarly to nBet v 1, nCor a 1 is composed of different isoallergens and variants and very likely additional ones will be identified. So far, not all known variants of Cor a 1 are detectable by mass spectrometry. We were able to confirm the presence of the variants Cor a 1.0103 and Cor a 1.0104 on the basis of variant specific tryptic peptides. Moreover, we detected Profilin 4, a member of a different allergen family. The proteins identified in nCor a 1 are summarized in Table 2. The detailed data on variant specific peptides and annotated spectra which unambiguously demonstrate the presence of Cor a 1.0103 and Cor a 1.0104 are displayed in Supplementary Table S2 and Fig. S4. Since the variants Cor a 1.0101 and Cor a 1.0102 do not contain unique tryptic peptides they are indistinguishable from Cor a 1.0103 and Cor a 1.0104. Thus, the presence of these variants can neither be confirmed nor be excluded. However, previous mRNA analyses by Breiteneder *et al.* using RT-PCR indicated their presence in hazel pollen²². The isoallergen Cor a 1.0401 could not be detected in our analysis.

To identify the bound ligand, SEC fractions containing pure nCor a 1 were lyophilised and extracted with methanol. The extract was analysed both by liquid chromatography / mass spectrometry (LC/MS) (see Supplementary Fig. S5) and reversed phase high performance liquid chromatography (RP-HPLC) (Fig. 3A). The total ion current (TIC) chromatogram (see Supplementary Fig. S5) showed one peak apex at a retention time of 2.4 min exhibiting pseudomolecular ions of $m/z = 627.155 [M + H]^+$, which is in good accordance with $m/z = 627.156$ for the putative molecular formula of the most abundant flavonoid in hazel pollen, Q3O-(Glc)-Gal [$C_{27}H_{30}O_{17} + H]^+$ ³⁷. RP-HPLC of the nCor a 1 extract yielded a single peak with a retention time of 14 min (Fig. 3A, red line). To increase the yield of the putative ligand we extracted the ligand directly from hazel pollen, i.e. without purifying the nCor a 1/ligand complex first (Fig. 3A, black line). We performed RP-HPLC and

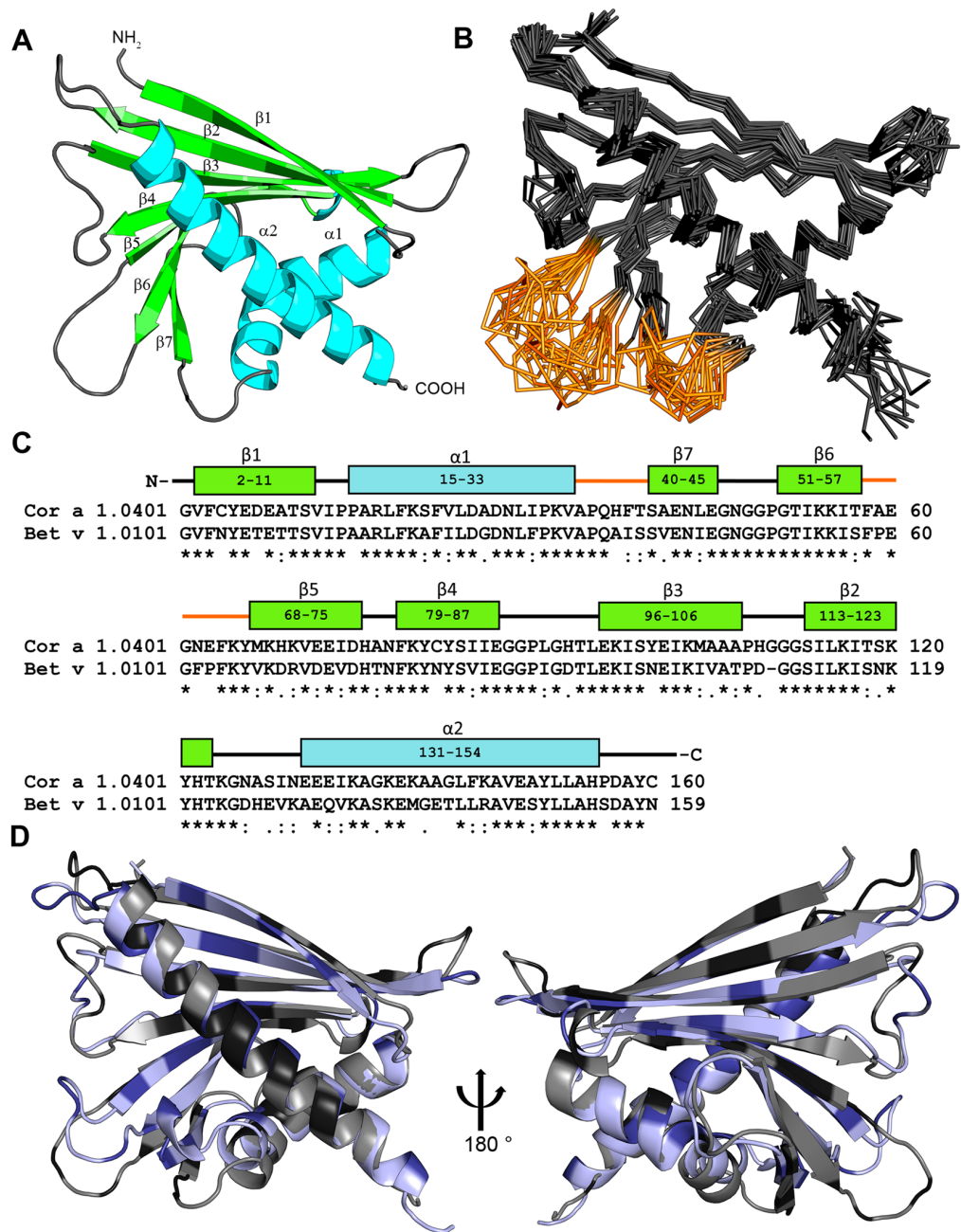


Figure 1. Solution structure of Cor a 1.0401. (A) Cartoon representation of the average of the 20 lowest energy solution structures of Cor a 1.0401 (PDB: 6GQ9) (α -helices, turquoise; β -strands, green; loop-regions, grey). (B) Backbone overlay of the 20 lowest energy solution structures of Cor a 1.0401, with a backbone rmsd value of 0.93 Å and overall rmsd of 1.26 Å. The loop regions Ala35 - Thr40 (between strand $\beta 7$ and the two short α -helices) and Thr58 - Met68 (between strand $\beta 5$ and $\beta 6$) which did not show resonances are highlighted in brown. (C) Amino acid sequence alignment of Cor a 1.0401 and Bet v 1.0101. The α -helices and β strands of Cor a 1.0401 are shown in blue and green, respectively, the two loop regions are highlighted in brown. (D) Overlay of the structures of Cor a 1.0401 (grey) and Bet v 1.0101 (light blue) (PDB: 6R3C). Black and dark blue indicate sequence differences.

collected the peak fractions with the same retention time, and analyzed them further by UV-Vis spectroscopy (Fig. 3B), mass spectrometry, and NMR spectroscopy (Fig. 3C).

The UV-Vis spectrum of the ligand in methanol exhibited absorption maxima at 256, 269, 299 and 357 nm (Fig. 3B), in excellent agreement with UV-Vis spectra of the glycosylated flavonoid Q3O-(Glc)-Gal in methanol

| Experimentally derived restraints | | |
|---|-----------------------|-----|
| distance restraints | | |
| | NOE | 845 |
| | intraresidual | 1 |
| | sequential | 240 |
| | medium range | 176 |
| | long range | 428 |
| | hydrogen bonds | 80 |
| dihedral restraints | | |
| | | 208 |
| restraint violation | | |
| average distance restraint violation (Å) | 0.006020 +/- 0.001643 | |
| distance restraint violation >0.1 Å | 1.5 +/- 1.36 | |
| average dihedral restraint violation (°) | 0.2134 +/- 0.0452 | |
| dihedral restraint violation >1° | 3.00 +/- 1.00 | |
| deviation from ideal geometry | | |
| bond length (Å) | 0.000583 +/- 0.000061 | |
| bond angle (°) | 0.1160 +/- 0.0063 | |
| coordinate precision^{a,b} | | |
| backbone heavy atoms (Å) | 0.93 | |
| all heavy atoms (Å) | 1.26 | |
| Ramachandran plot statistics^c (%) | | |
| | 91.5/7.5/0.3/0.7 | |

Table 1. Solution structure statistics of Cor a 1.0401. ^aThe precision of the coordinates is defined as the average atomic root mean square difference between the accepted simulated annealing structures and the corresponding mean structure calculated for the given sequence regions. ^bCalculated for residues Gly2-Val34, Ser41-Thr58, Lys69-Leu154. ^cRamachandran plot statistics are determined by PROCHECK⁵² and noted by most favored/ additionally allowed/generously allowed/disallowed.

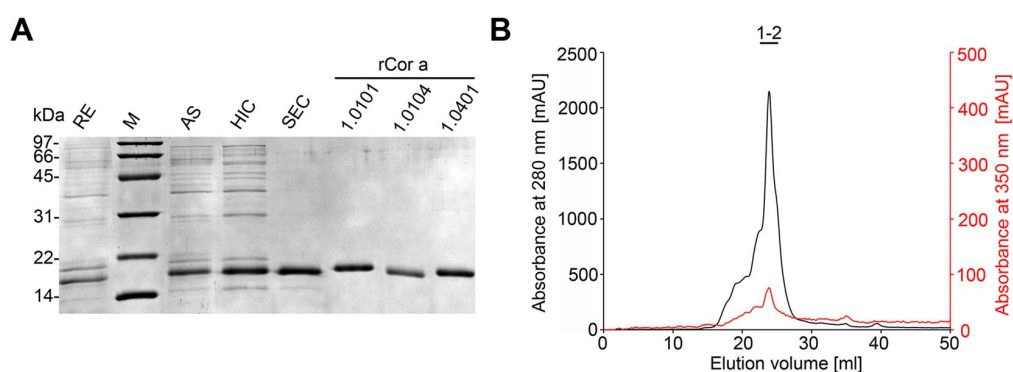


Figure 2. Purification of Cor a 1 from pollen. (A) Analysis of the purification procedure of Cor a 1 from hazel pollen by SDS-PAGE (19% gel). The sample after each purification step is shown: RE, raw extract; M, molecular weight marker (low range, Bio-Rad, Munich, Germany), the corresponding masses are indicated on the left; AS, resuspended pellet after precipitation with 100% ammonium sulfate saturation; HIC, pooled fractions after hydrophobic interaction chromatography; SEC, pure Cor a 1 sample after size exclusion chromatography; rCor a 1 represents the three recombinant purified forms Cor a 1.0101, 1.0104 and 1.0401. (B) SEC of purified nCor a 1. The absorbance at 280 nm (black line, protein) and 350 nm (red line, ligand) was recorded. Fractions 1-2, were pooled and loaded on the SDS-PA gel shown in (A).

reported previously³⁷. The TIC (total ion current) chromatogram of the ligand isolated directly from hazel pollen also confirmed the presence of Q3O-(Glc)-Gal (data not shown).

To further characterize the purified ligand, ¹H and ¹³C chemical shifts were obtained from a ¹H, ¹³C heteronuclear single quantum coherence (HSQC) spectrum in deuterated DMSO (d₆-DMSO) (Fig. 3C). The NMR resonances agree well with published data³⁷ and confirmed the identity of this glycosylated flavonoid in the β-glycosidic form. Ligand identification was reproducible using three different pollen batches.

Interestingly, this ligand is very similar to the epimeric Bet v 1 ligand Q3OS. The only difference is the orientation of the hydroxyl group at the C4 of the first sugar moiety resulting in a glucose moiety in Q3OS vs a galactose moiety in Q3O-(Glc)-Gal linked to quercetin (Fig. 4).

| internal | Genebank | UniProt | Description | S | SC | E | DP | MP |
|-------------------------------------|----------|---------|---------------------------------|------|------|-----|----|----|
| (a) purified nCor a 1 in gel | | | | | | | | |
| PEI127 | X70998 | Q08407 | Cor a 1 0104, Corylus avellana | 7180 | 61.0 | 5.8 | 8 | 1 |
| PEI126 | X70997 | Q08407 | Cor a 1 0103, Corylus avellana | 5860 | 57.2 | 6.3 | 7 | 0 |
| PEI124 | X70999 | Q08407 | Cor a 1 0101, Corylus avellana | — | — | — | — | — |
| PEI125 | X71000 | Q08407 | Cor a 1 0102, Corylus avellana | — | — | — | — | — |
| n.a. | n.a. | A4KA45 | Profilin 4, Corylus avellana | 1723 | 40.6 | 7.2 | 3 | 0 |
| (b) rCor a 1.0401 in gel | | | | | | | | |
| PEI 128 | n.a. | n.a. | rCor a 1 0401, Corylus avellana | 8489 | 88.8 | 6.0 | 15 | 1 |
| (c) rCor a 1.0102 in gel | | | | | | | | |
| PEI 163 | X71000 | Q08407 | Cor a 1.0102, Corylus avellana | 1847 | 81.9 | 1.8 | 18 | 4 |
| (d) rCor a 1.0103 in gel | | | | | | | | |
| PEI 161 | X70997 | Q08407 | Cor a 1.0103, Corylus avellana | 2963 | 81.3 | 1.3 | 15 | 3 |

Table 2. Summary of MS data on natural and recombinant Cor a 1. n.a. not applicable; S: Score, SC: sequence coverage; E average precursor mass error; DP: number of detected tryptic peptides; MP: number of detected modified peptides.

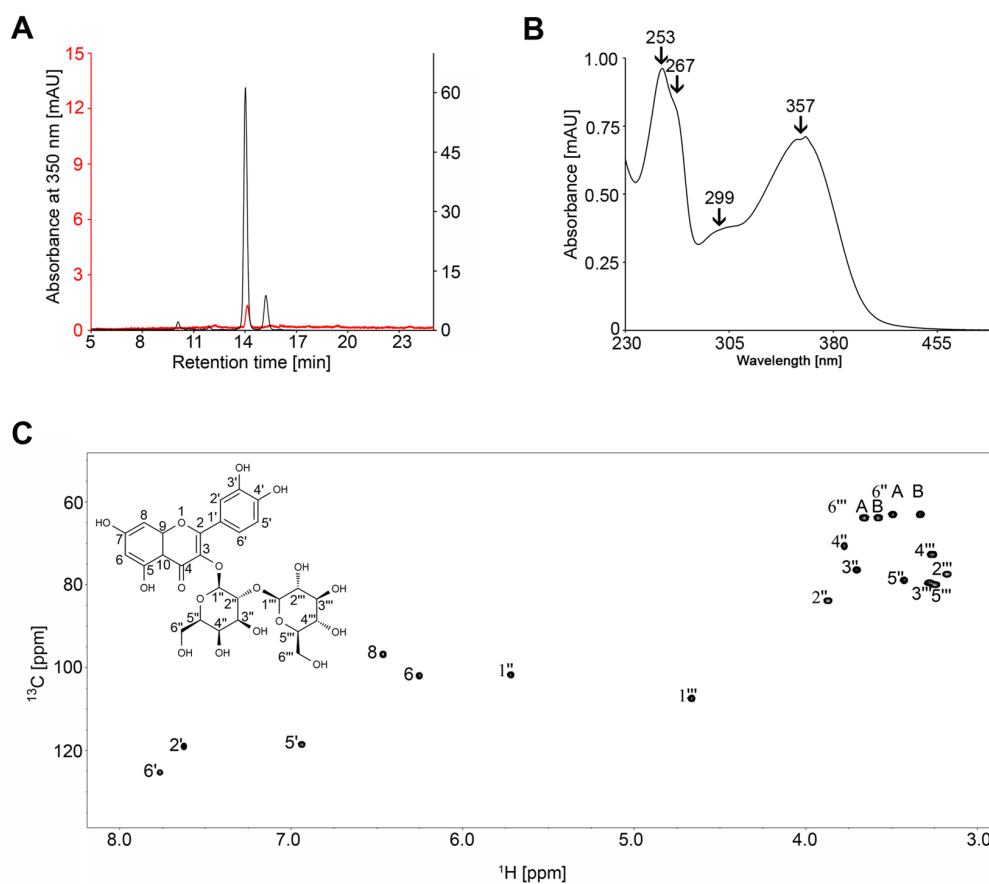


Figure 3. Identification of the natural ligand Q3O-(Glc)-Gal. (A) RP-HPLC chromatogram of hazel pollen extract (black line) and the ligand of nCor a 1 isolated from pure SEC fractions (red line) using a C18A column. (B) UV/Vis spectrum of the ligand purified from pollen. Absorption maxima at 253 nm, 267 nm, 299 nm, and 357 nm are indicated. (C) ^1H , ^{13}C HSQC spectrum of the ligand purified from pollen in d_6 -DMSO. The structure is shown on the top left. A and B indicate the two different hydrogen atoms at positions 6'' and 6''', respectively.

Binding of rCor a 1 isoallergens to Q3O-(Glc)-Gal. To learn more about the binding properties of the ligand we analysed its interaction with different rCor a 1 proteins. The high sensitivity of the chemical shift to structural changes makes NMR spectroscopy a powerful tool to investigate ligand binding to a protein as binding

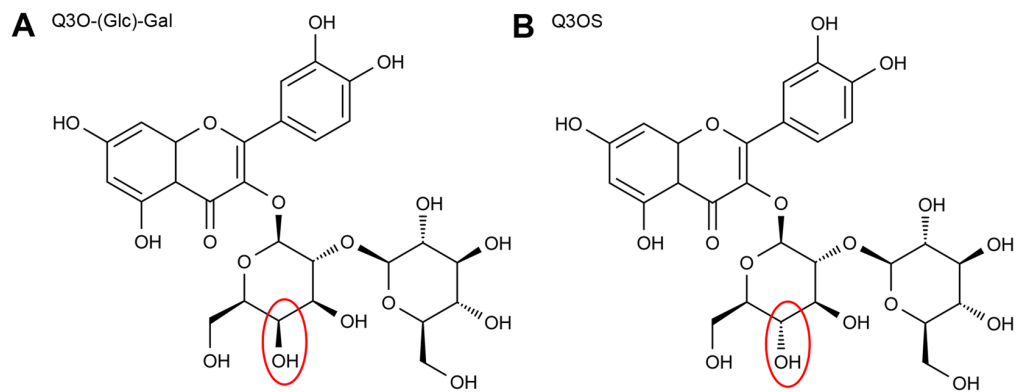


Figure 4. Structural comparison of Q3OS and Q3O-(Glc)-Gal.

of a ligand causes structural changes at least in the binding region. This can be easily observed by comparison of NMR spectra of the protein before and after addition of the ligand.

We decided to further analyse four known Cor a 1 variants previously detected in hazel pollen and one detected in hazel nut⁷. Thus, we purified the ¹⁵N labelled variants rCor a 1.0101, rCor a 1.0102, rCor a 1.0103, rCor a 1.0104 (pollen), and rCor a 1.0401 (nut) from *E. coli* to perform binding experiments by two-dimensional protein NMR spectroscopy. ¹H, ¹⁵N HSQC spectra of the proteins were recorded before and after the addition of a ten-fold molar excess of Q3O-(Glc)-Gal. The spectra of the four Cor a 1 variants from pollen did not show changes even with a ten-fold excess of ligand, indicating no binding. This is exemplary shown for rCor a 1.0101 and rCor a 1.0104, (Fig. 5A,B). In contrast, binding could be observed to rCor a 1.0401 (Fig. 5C) corroborating Q3O-(Glc)-Gal as a ligand.

Cor a 1.0401 was previously shown to be present in hazelnut⁷. To determine whether Cor a 1.0401 is also present in pollen, crude pollen extracts were analyzed by mass spectrometry to avoid loss of individual Cor a 1 isoallergens or variants, which may have occurred during purification of nCor a 1. However, the results matched the ones of purified nCor a 1. We found Cor a 1.0104 and Cor a 1.0103 to be accompanied by Profilin 4 and 5, and several other non allergenic proteins, but not by Cor a 1.0401 (Table S3). Our results indicate that Cor a 1.04 variants are either absent in hazel pollen or they are only present at concentrations below the detection limit. Since rCor a 1.0401 binds Q3O-(Glc)-Gal with high specificity other still unknown Cor a 1 isoallergens may be present in hazel pollen as well as in purified nCor a 1 and bind to the ligand.

Contrariwise, HPLC analyses of hazelnut skin extracts were negative for Q3O-(Glc)-Gal (see Supplementary Fig. S6). This is in line with earlier experiments that introduced 3-O-(2''-O-beta-D-glucopyranosyl)-beta-D-galactopyranoside conjugates of kaempferol and quercetin as a pollen-specific class of glycosylated flavonoids^{38,39}.

Binding of Q3O-(Glc)-Gal to Cor a 1.0401. To investigate binding of quercetin, which lacks the sugar moiety, to the hydrophobic pocket of Cor a 1.0401, a titration was performed and ¹H, ¹⁵N HSQC spectra were recorded after each step (Fig. 6A). While some signals (F146, L92) disappeared, indicating binding in the intermediate or slow exchange regime, others shifted with each titration step (Y152, R18, K21). This is characteristic for binding in the fast exchange resulting in the observation of population-averaged chemical shifts between ligand-bound and unbound protein. The residues which disappeared and therefore were affected most by binding were mapped on the solution structure of Cor a 1.0401 (Fig. 6B). Remarkably, binding of quercetin to Bet v 1.0101 revealed similar, but not identical binding interfaces³³.

To further investigate binding of Q3O-(Glc)-Gal to Cor a 1.0401, a titration of Q3O-(Glc)-Gal to ¹⁵N, ¹³C labeled protein was performed and after each step, ¹H, ¹⁵N HSQC as well as ¹H, ¹³C HSQC spectra were recorded. Figure 6C,D show overlays of the ¹H, ¹⁵N and ¹H, ¹³C HSQC spectra, respectively, in the absence of ligand and after the addition of the highest ligand concentration.

During titration, about 80% of all signals disappeared and reappeared somewhere else in the spectrum, which is characteristic for binding in the slow exchange regime. Upon binding of the ligand to the protein, the affected nuclear spins change between at least two states (bound and unbound state) with different chemical shifts. The effect of the exchange on the spectrum depends on the relation between the off rate and the magnitude of the chemical shift difference in Hertz. Observation of spectra in the slow exchange regime implies that the off rate is smaller than the chemical shift difference. A small off rate means slow dissociation of the ligand from the protein, indicating rather strong binding with dissociation constants typically below 5 μM⁴⁰. However, it was not possible to determine exact dissociation constants. As about 80% of all signals were strongly affected by binding, it was also impossible to determine a binding interface. This extreme change in the spectrum could either point towards a structural rearrangement of Cor a 1.0401 upon ligand binding or a rearrangement of the side chains, which influence the chemical environment of the nuclear spins.

Confirmation of specific ligand binding by two-dimensional NMR experiments. It is often stated that PR-10-proteins show promiscuous binding behaviour^{16,18,19}. To investigate binding specificities, a set of two

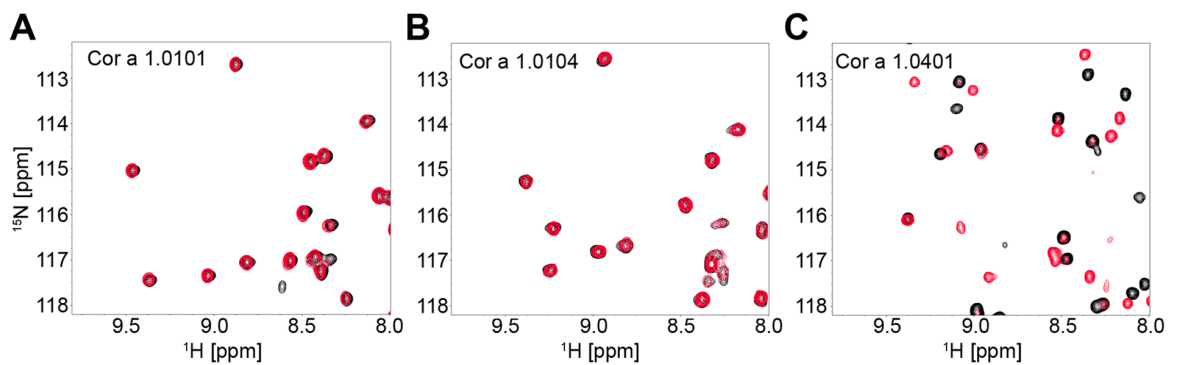


Figure 5. Binding of Q3O-(Glc)-Gal to different Cor a 1 isoallergens. Overlay of two ^1H - ^{15}N spectra of $60\ \mu\text{M}$ of Cor a 1 isoallergens in 10 mM sodium phosphate, pH 7.0 in the absence (black) or presence of a ten-fold molar excess of Q3O-(Glc)-Gal (red) recorded at a Bruker Avance 700 MHz spectrometer at 298 K (A) Cor a 1.0101, (B) Cor a 1.0104, and (C) Cor a 1.0401. Upon ligand addition no significant shifts can be observed for Cor a 1.0101 and Cor a 1.0104, implying no or weak binding, whereas with Cor a 1.0401, the signal intensity of many residues decreases and new signals appear, indicating binding.

dimensional NMR-experiments was performed with Bet v 1.0101 and Cor a 1.0401 in the presence and absence of the corresponding natural ligands (Fig. 7).

Most surprisingly, Cor a 1.0401 only binds to Q3O-(Glc)-Gal (Fig. 7A,B), whereas Q3OS binding is specific for Bet v 1.0101 (Fig. 7C,D), even though the amino acid identity of the two proteins is rather high (67.3%) and the only difference between the two ligands is the orientation of the OH-group in the respective sugar moiety (glucose vs. galactose). However, the physiological roles and functions of the different binding specificities remain to be elucidated.

The high binding specificities of Bet v 1 and Cor a 1 variants allow the discrimination between the natural ligand and a highly similar, epimeric ligand of the homologous allergen (Fig. 7). A similar substrate specificity can be observed in glycosyl transferases that are responsible for glycosylation and deglycosylation of flavonoids in pollen³⁸. A single point mutation of the UDP-galactose galactosyltransferase from *Aralia cordata* (H374Q) changed the preferential donor from galactose to glucose⁴¹.

Ligand discrimination might already take place at the entrance to the hydrophobic pocket. The largest opening is located between T58 and Y67. In this region there are three amino acid exchanges between Bet v 1.0101 and Cor a 1.0401, P59A, L62N and P63E, which might play a role in ligand discrimination. However, to explain the molecular basis of the highly selective ligand binding, complex structures of both proteins are needed.

Conclusions

Despite the high clinical relevance of the hazel allergen Cor a 1, physiological and structural knowledge is scarce. The solution structure of the variant Cor a 1.0401, which we present here, provides the basis for future immunological and physiological studies since Cor a 1.0401 is one of the strong IgE binding variants⁷.

Moreover, we identified Q3O-(Glc)-Gal as a natural ligand of Cor a 1.0401. Our findings demonstrate binding of rCor a 1.0401 usually found in hazelnut to the pollen specific ligand Q3O-(Glc)-Gal. This might be explained by the unusual reproduction biology of hazel. Within 4–7 days after pollination in January and February the pollen grows to the base of the style, which connects the stigma with the ovary. Here, the tip of the tube enters a long resting period of several months. The ovary grows over ca. 5 months until it becomes mature and contains egg cells. The resting sperm becomes activated and after growth of secondary pollen tubes, fertilization takes place. This results in rapid growth of the kernel over a period of 6 weeks⁴².

Glycosylated flavonoids comprise a storage form, whereas the aglycons are functionally active and are indispensable for the formation of the pollen tube^{38,43}. Thus, we propose that upon first contact of hazel pollen with the stigma, Q3O-(Glc)-Gal from pollen and Cor a 1.0401 from the stigma might form a complex persisting throughout the maturation of the ovules. Formation of the complex is necessary to prevent premature deglycosylation of Q3O-(Glc)-Gal to quercetin by glycosyltransferases. After maturation, the ligand is released and converted into quercetin, which might then assist the formation of the secondary pollen tube^{43,44}. A prerequisite of this hypothesis is, of course, that Cor a 1.0401 is already present in the stigma and is not only expressed during kernel formation. However, this has not been tested yet.

Nevertheless, a yet unidentified Cor a 1 isoallergen bound to Q3O-(Glc)-Gal is present in hazel pollen, since SEC showed that the ligand (627 Da) and nCor a 1 (17 kDa) co-purify in one peak and moreover, we were able to isolate the ligand Q3O-(Glc)-Gal from highly purified nCor a 1. Although peptide mass fingerprint by LC-MS^E is a powerful method to identify already known proteins, uncharacterized Cor a 1 isoallergens will not be discovered by this method. It is highly probable that there are more Cor a 1 isoallergens to be found in the future. For Bet v 1, which is the PR-10 allergen investigated most thoroughly, 18 isoallergens have been unambiguously identified so far⁴⁵.

The binding specificity of Bet v 1.0101 and Cor a 1.0401 for their natural ligands, Q3OS and Q3O-(Glc)-Gal, respectively, as well as the fact that the other Cor a 1 variants rCor a 1.0101 and rCor a 1.0104 do not bind

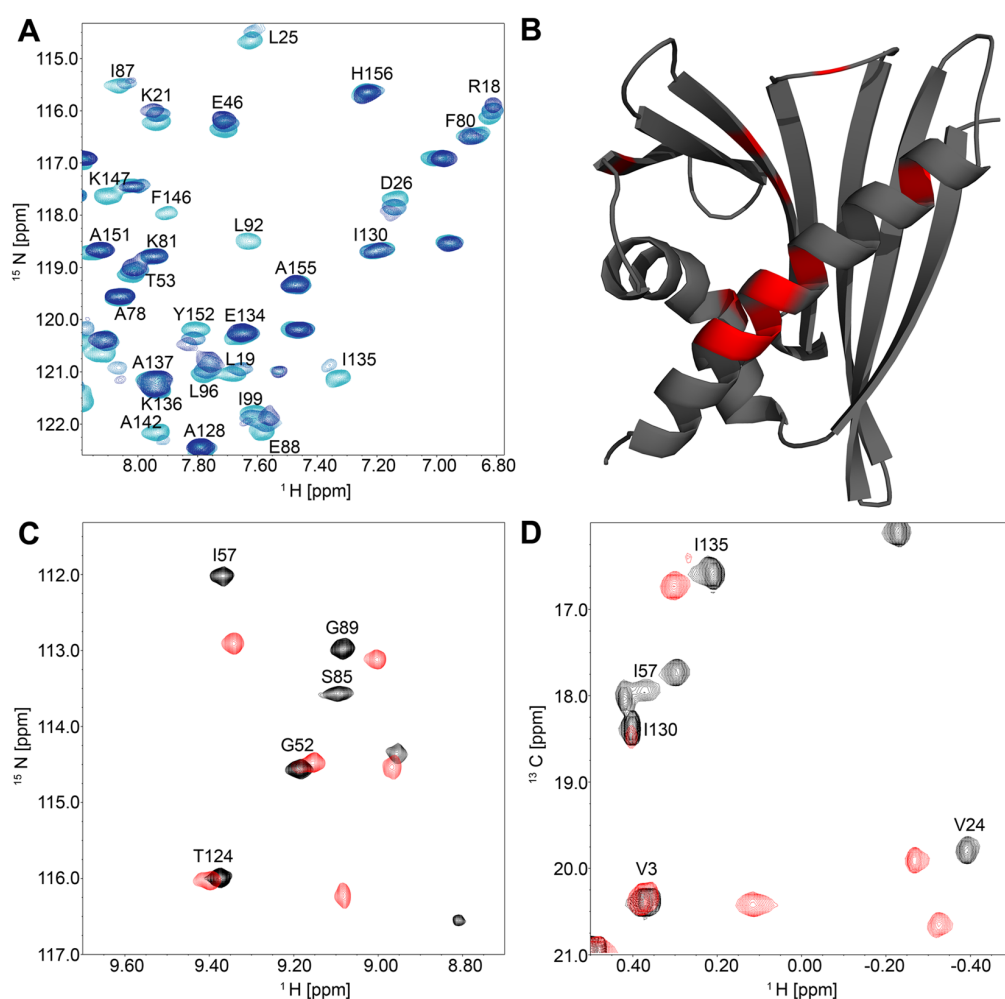


Figure 6. NMR titration of Cor a 1.0401 with quercetin and Q3O-(Glc)-Gal. All experiments were performed with a Bruker Avance 700 MHz spectrometer at 298 K with 60 to 100 μ M Cor a 1.0401 uniformly labelled with 15 N and 13 C. **(A)** Overlay of three 1 H- 15 N HSQC spectra of Cor a 1.0401 in the presence of increasing quercetin concentrations, from light to dark blue; ligand to protein ratio: 0, 0.5 and 1. The quercetin stock solution was prepared in d_6 -DMSO to obtain a final DMSO concentration of 2.2% (v/v). **(B)** Cartoon representation of Cor a 1.0401. The amino acids that were strongest affected by quercetin binding are highlighted in red. **(C)** Section of an overlay of two 1 H, 15 N HSQC spectra of Cor a 1.0401 in the absence (black) and presence (red) of an 8-fold excess of Q3O-(Glc)-Gal. **(D)** Section of an overlay of two 1 H, 13 C HSQC spectra of Cor a 1.0401 in the absence (black) and presence (red) of an 8-fold excess of Q3O-(Glc)-Gal.

Q3O-(Glc)-Gal suggests that despite their high sequence identity and structural similarity, Bet v 1 homologous proteins and variants bind to different ligands and might even fulfil different physiological functions. This might be the reason why a precise function of Bet v 1 homologous proteins could not be identified so far.

Materials and Methods

Polyphenols. Quercetin was purchased in analytical grade from Sigma-Aldrich (St. Louis, USA), and Q3OS from Phytolab (Vestenbergsgreuth, Germany). Q3O-(Glc)-Gal was purified from hazel pollen extracts (Allergon, Ängelholm, Sweden). To extract the flavonoids, pollen was dissolved in H_2O (500 mg dry wt/10 ml H_2O), stirred at room temperature for 3 h and centrifuged (20 min, 4 $^{\circ}C$, 10000 \times g). The supernatant was collected and the pellet was redissolved in 10 ml H_2O , and treated as described above. The procedure was repeated and the extract stirred over night. H_2O extracts were evaporated to dryness *in vacuo* and the pellets were resuspended in 1 ml of 61% solvent A (2% v/v acidic acid) and 39% solvent B (0.5% v/v acidic acid, 50% v/v acetonitrile), centrifuged and filtered through a nylon filter (45 μ m; Phenomenex, Aschaffenburg, Germany). The compounds were purified by HPLC by isocratic elution with 61% solvent A and 39% solvent B using a C18 column (SP 250/21 Nucleosil 100-7; Macherey-Nagel, Düren, Germany) at a flow rate of 10 ml/min. Fractions containing ligands were evaporated to dryness *in vacuo* afterwards. The purified compounds were stored at 4 $^{\circ}C$ in the dark. Q3O-(Glc)-Gal

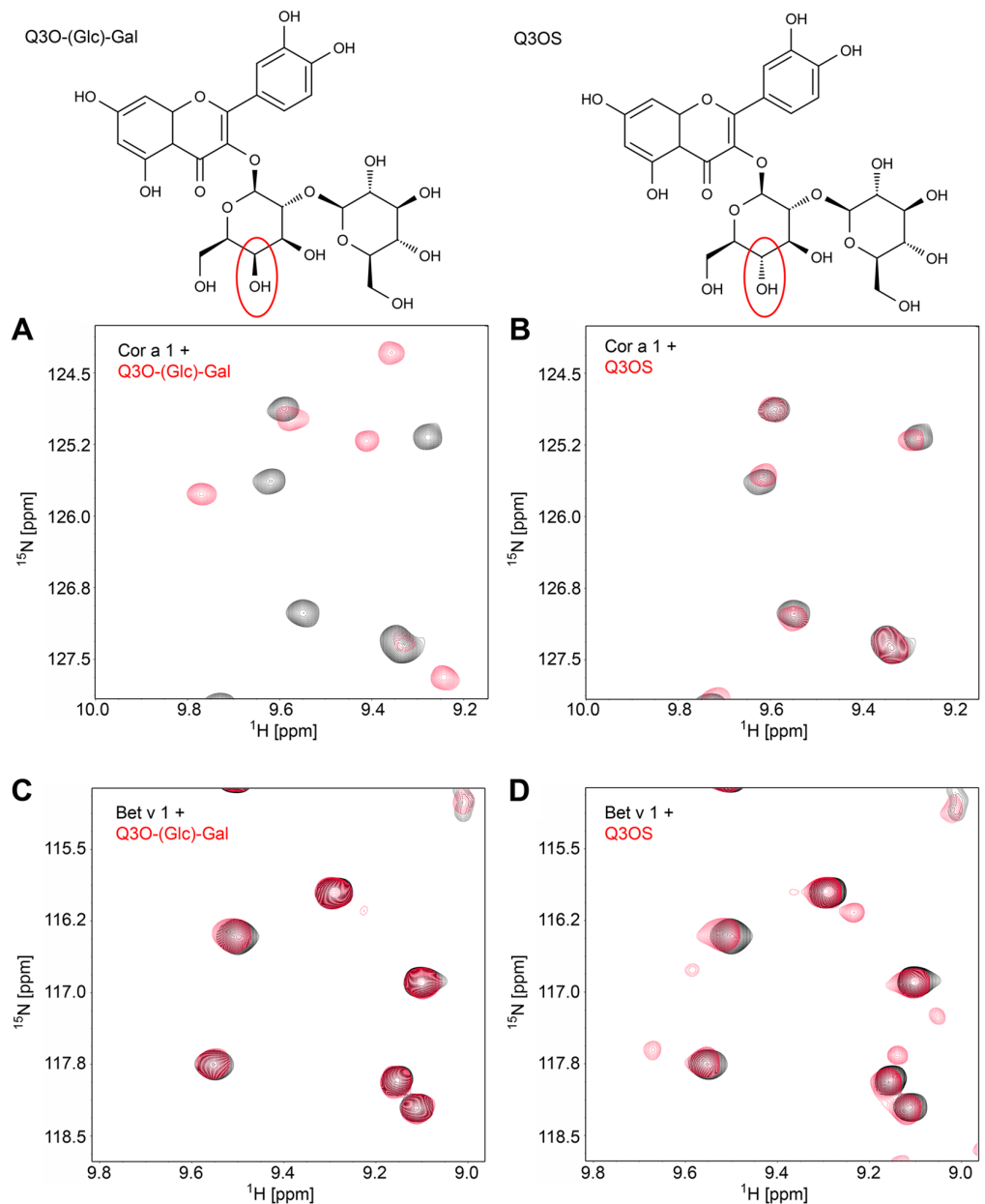


Figure 7. Binding specificity of Bet v 1.0101 and Cor a 1.0401. The structures of Q3O-(Glc)-Gal (left) and Q3OS (right) are shown on top of the panels. Section of overlays of ^1H - ^{15}N HSQC spectra of Cor a 1.0401 or Bet v 1.0101 in the absence or presence of ligand: Cor a 1.0401 with (A) Q3O-(Glc)-Gal or (B) Q3OS; Bet v 1.0101 with (C) Q3O-(Glc)-Gal or (D) Q3OS.

content was measured by the absorbance at 350 nm, for quantitative calculations the extinction coefficient of Q3OS ($\epsilon_{350} = 13500 \text{ M}^{-1} \text{ cm}^{-1}$) was used.

For the analysis of polyphenols from hazelnut skin, the skins were removed from the nuts (type “Barcelona”, origin: USA) with a scalpel and dried overnight at 50°C . Afterwards, they were shock frozen and grinded using a porcelain mortar. 250 mg of the grinded hazelnut skins was then extracted with 10 ml 100% methanol over night, the extract centrifuged, the supernatant dried *in vacuo* and redissolved in 2 ml of 90% solvent A (2% v/v acidic acid) and 10% solvent B (0.5% v/v acidic acid, 50% v/v acetonitrile). After centrifugation and filtration through a $45 \mu\text{M}$ nylon filter (Phenomenex, Aschaffenburg, Germany), the extract was analysed by RP-HPLC using a gradient from 10% B to 100% B within 35 min.

Cloning, expression and protein preparation. Synthetic genes coding for Cor a 1.0101; Cor a 1.0104 (Genescript, Piscataway, New Jersey, USA) and Cor a 1.0401 (optimized for codon-usage in *E. coli*) were cloned

via NdeI, BamHI into the expression vector pET11a (Novagen-Merck, Germany). To obtain Cor a 1.0102 and Cor a 1.0103, plasmids pET11a Cor a 1.0104 and pET11a Cor a 1.0101, respectively, were used as templates for site-directed mutagenesis according to the QuickChange Method Cornell iGEM 2012³³ protocol (http://2012.igem.org/wiki/images/a/a5/Site_Directed_Mutagenesis.pdf).

Gene expression for all unlabelled, ¹⁵N, and ¹⁵N, ¹³C labelled allergens was performed as described previously for pET11a_Bet v 1a (Bet v 1.0101)³³ using (¹⁵NH₄)₂SO₄ and ¹³C-glucose. An amino acid sequence alignment of the Cor a 1 and Bet v 1 proteins used in this study is shown in Supplementary Fig. S7. The protein bank entry accession numbers and a sequence identity matrix of the proteins are listed in Supplementary Table S4.

Purification of recombinant proteins. Protein purification for Cor a 1.0101, Cor a 1.0401, and Bet v 1.0101 was performed as described for Bet v 1a (Bet v 1.0101)³³ with the following modifications: For Cor a 1.0101, streptomycin sulfate was added to a final concentration of 1% to precipitate nucleic acids. 1.5 M (NH₄)₂SO₄ was then added to the protein solution, and the solution was loaded on to a 5 ml octylsepharose column (Octylsepharose 4 Fast Flow; GE Healthcare, Munich, Germany) equilibrated with 10 mM sodium phosphate, pH 7.0, 1.5 M (NH₄)₂SO₄, and eluted using a gradient from 0 to 60% elution buffer (10 mM sodium phosphate, pH 7.0) followed by a step to 100% elution buffer.

Cor a 1.0102, Cor a 1.0103, or Cor a 1.0104 containing cell extracts were centrifuged (19000 g, 30 min, 4 °C) and the pellet was resuspended in 50 mM sodium phosphate, pH 7.8, 500 mM NaCl and 8 M urea. The denatured protein was refolded by stepwise lowering the urea concentration during dialysis to 4 M, 2 M, 1 M for 1 h and dialysis for 4 h and over night in 10 mM sodium phosphate, pH 7.0.

To remove remaining contaminants identified by ¹H, ¹⁵N HSQC spectra, purified Bet v 1.0101, was unfolded using 8 M urea, 50 mM sodium phosphate pH 7.0, 50 mM NaCl for 1.5 h at room temperature. After centrifugation of the sample in a Vivaspin concentrator 20 (MWCO 10 kDa, Sartorius Stedim Biotech, Göttingen, Germany), 20 ml of buffer without urea was added to the remaining solution. After centrifugation the procedure was repeated and the sample was then concentrated to 1 ml. The pure proteins were either shock frozen and stored at -80 °C or dialysed against Milli Q H₂O, lyophilised and stored at 4 °C.

Protein analysis. Standard methods were used to analyse purity (SDS-PAGE), oligomeric state (SEC) and structural integrity (1D-NMR, ¹H-¹⁵N HSQC spectroscopy for the ¹⁵N labelled proteins) of all variants. The proteins were stored as described above.

Purification of nCor a 1 from pollen of *Corylus avellana* and ligand isolation. nCor a 1 was purified from hazel pollen (Allergom, Ängelholm, Sweden) as described previously for the isolation of Bet v 1 from birch pollen^{33,36} with minor changes, using ammonium sulfate precipitation (50%, 60% and 100% (NH₄)₂SO₄ saturation), followed by hydrophobic interaction chromatography (HIC) as described above for the purification of recombinant Cor a 1.0101 but with only one purification step with 100% elution buffer. To remove remaining impurities, Cor a 1-containing HIC-fractions were concentrated (Vivaspin 20 concentrator, molecular-mass cut-off 3000 Da) to a final volume of 500 µl and loaded on to two consecutively connected Superdex 75 10/300 GL columns (24 ml bed volume each; GE Healthcare, Penzberg, Germany), equilibrated with a buffer containing 10 mM sodium phosphate pH 7.0, and 300 mM NaCl. If required, another HIC chromatography using a step gradient was performed. The nCor a 1 fractions were pooled, concentrated and lyophilised.

Subsequently, the prominent protein band at 17 kDa on an SDS polyacrylamide gel was excised and the protein was analysed by tryptic digestion followed by liquid chromatography – mass spectrometry (LC-MS^E) to confirm its identity.

For ligand isolation pure Cor a 1 fractions were lyophilised and redissolved in methanol. The methanol extract was dried *in vacuo*, redissolved in 50 µl 2.5% methanol and analyzed by RP- HPLC using a 150 × 4 mm 5 µm C18A vertex plus column with a pore size of 100 Å (Knauer, Berlin, Germany), equilibrated with solvent A (2% acetic acid) followed by a gradient from 10% to 90% solvent B (0.5% acetic acid, 50% acetonitrile) within 35 min, and analysed by mass spectrometry.

Mass spectrometric confirmation of Cor a 1 isoallergens and variants. The isoallergen and variant composition of purified nCor a 1 and the identity of purified rCor a 1.0102, rCor a 1.0103 and rCor a 1.0401 were determined by nano-UPLC (ultra performance liquid chromatography) nano-ESI MS^E (electron spray ionisation mass spectrometry)⁴⁶ after SDS-PAGE separation and in gel digestion⁴⁷ as published previously⁴⁵ using an in-house database consisting of reviewed entries of the UniProt database (as at January 2016) (Table 2). The isoallergen and variant composition of crude hazel pollen extract was determined by nano-UPLC nano ESI MS^E⁴⁶ after in solution tryptic digestion as published previously²⁴, except that we used the above mentioned in-house database for analyses. Three different crude extracts were obtained by extracting proteins from hazel pollen following an extraction protocol applied previously on birch pollen⁴⁵. Differing from this, three buffers were used. Sample (a): sodium phosphate buffer³³, (b): 100 mM NH₄HCO₃-buffer⁴⁵ and (c): 8 mM Tris and 10 mM (NH₄)₂B₁₀O₁₆·8H₂O.

Mass spectrometry of the ligand Q30-(Glc)-Gal. For MS analysis, the purified ligand extracted from hazel pollen was loaded on a HPLC RP-C18 column (Phenomenex Inc. USA, Kinetex 5 µm EVO C18, 100 Å, 30 × 2.1 mm) which was connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) with a hybrid quadrupole orbitrap mass analyzer (maximum mass range 50–6000 Da, resolution 140.000 @ m/z = 200), using a gradient from 20–95% acetonitrile within 10 min. Mass spectra were acquired after (positive mode) electrospray ionisation (ESI pos) in full scan mode (70–1050 amu) recording the TIC.

To confirm the presence of the ligand of the purified nCor a 1, the methanol extract of nCor a 1 after SEC was loaded onto a C18 column (Accucore RP-MS, 2.6 μm , 150 \times 2.1 mm) connected to the Q Exactive mass spectrometer. Isocratic elution was performed with 50% acetonitrile within 20 min. Mass spectra were acquired as described above.

NMR experiments. All NMR-experiments were performed at 298 K on Bruker Avance spectrometers with proton resonance frequencies of 600, 700, 900 and 1000 MHz, the latter three equipped with cryogenically cooled triple resonance probes.

NMR-spectroscopy of ligands. ^1H -NMR and ^{13}C NMR spectroscopy of about 3 mM Q3O-(Glc)-Gal in d_6 -DMSO was performed at 600 MHz ^1H frequency. Chemical shifts were referenced to tetramethylsilane. Data was processed using Topspin version 3.2 (Bruker, Karlsruhe, Germany).

Determination of the solution structure of Cor a 1.0401 and Bet v 1.0101. Resonance assignments were done with standard double- and triple-resonance through-bond correlation experiments. Threedimensional NMR experiments of Cor a 1.0401 were recorded using non-uniform sampling (NUS) with 25% data amount. NMR-spectra to assign chemical shifts were obtained with a 600 μM [^1H , ^{13}C , ^{15}N] Cor a 1.0401 sample in 10 mM sodium phosphate, pH 7.0, 10% (v/v) D_2O , 0.03% NaN_3 and 2 mM DTT. Three-dimensional ^{13}C and ^{15}N edited nuclear Overhauser enhancement spectroscopy (NOESY) experiments (mixing times 120 ms) were recorded for derivation of distance restraints. NMR data were processed using in-house software and visualized with NMRViewJ (OneMoon Scientific, Inc.). Iterative soft thresholding was applied for processing NUS NMR experiments⁴⁸.

NOESY cross peaks were classified according to their relative intensities and converted into distance restraints with upper limits of 3.0 \AA (strong), 4.0 \AA (medium), 5.0 \AA (weak) and 6.0 \AA (very weak). Dihedral restraints were taken from analysis of chemical shifts by the TALOS software package⁴⁹. Structures were calculated using the programme XPLOR-NIH^{50,51}. The 20 structures showing the lowest overall energy were analysed with XPLOR-NIH and PROCHECK-NMR⁵².

Binding experiments. To investigate the binding interface of Cor a 1.0401 upon binding of quercetin, quercetin was dissolved in DMSO and added in different concentrations to 60 $\mu\text{M}^{13}\text{C}^{15}\text{N}$ Cor a 1.0401, up to an equimolar concentration. The DMSO concentration of the final NMR sample was always 2.2%. To identify chemical shift perturbations caused by DMSO, a sample was prepared with 60 $\mu\text{M}^{13}\text{C}^{15}\text{N}$ Cor a 1.0401 and 2.2% DMSO. ^1H , ^{15}N HSQCs of each sample were recorded. Where binding in the fast exchange rate occurred, chemical shift perturbations (CSPs) resulting from ligand binding were calculated based on equation (1):

$$\Delta\delta_{\text{norm}} = \sqrt{(\Delta\delta_{\text{HN}})^2 + (0.1 \Delta\delta_{\text{N}})^2}$$

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

Where binding in the slow exchange rate occurred, the relative intensities of each signal were compared to the corresponding signal in the reference spectrum without ligand.

For titration of Q3O-(Glc)-Gal to Cor a 1.0401, a 9.9 mM stock solution of Q3O-(Glc)-Gal in 10 mM sodium phosphate, pH 7.0 was prepared and added stepwise to 100 μM of ^{13}C , ^{15}N Cor a 1.0401 up to an 8-fold molar excess. After each titration step, a ^1H , ^{15}N HSQC and a ^1H , ^{13}C HSQC spectrum was recorded.

Binding specificity of Bet v 1.0101, Cor a 1.0401, Cor a 1.0101 and Cor a 1.0104 was investigated by recording ^1H , ^{15}N HSQCs of 60 μM of the respective ^{15}N -labelled protein in the absence and in the presence of a ten-fold molar excess of Q3OS or Q3O-(Glc)-Gal.

Computational methods. Figures of protein structures were generated and homologous protein structures were aligned using the programme PyMOL Molecular graphics System, Version 1.5.0.4. Theoretical isoelectric points, extinction coefficients and molecular weight of the different PR-10 proteins were determined using the ProtParam Tool⁵³. For the calculation of the cavity volumes, the programme CastP⁵⁴ was used with default parameters. They were determined for every single structure of the NMR bundle and are given as means \pm S.D. Multiple and pairwise sequence alignments were performed with ClustalOmega⁵⁵ and EMBOSS Needle⁵⁶.

Data deposition. Coordinates and restraints for structure calculation of Cor a 1.0401 and Bet v 1.0101 were deposited in the Protein Data Bank (PDB) under the accession codes 6GQ9 and 6R3C, respectively. Chemical shift assignments were deposited in the BioMagResBank: accession numbers 34281 (Cor a 1.0401) and 34383 (Bet v 1.0101).

References

- Datema, M. R. *et al.* Hazelnut allergy across Europe dissected molecularly: A EuroPrevall outpatient clinic survey. *J. Allergy Clin. Immunol.* **136**, 382–391 (2015).
- Ortolani, C. & Pastorello, E. A. Food allergies and food intolerances. *Best Pract. Res. Clin. Gastroenterol.* **20**, 467–483 (2006).
- Burney, P. *et al.* Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy* **65**, 1182–1188 (2010).
- Burney, P. G. J. *et al.* The prevalence and distribution of food sensitization in European adults. *Allergy* **69**, 365–371 (2014).
- Etesamifar, M. & Wüthrich, B. IgE-vermittelte Nahrungsmittelallergien bei 383 Patienten unter Berücksichtigung des oralen Allergiesyndroms. *Allergologie* **21**, 451–457 (1998).
- Breiteneder, H. & Radauer, C. A classification of plant food allergens. *J. Allergy Clin. Immunol.* **113**, 821–830 (2004).
- Lüttkopf, D. *et al.* Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Mol. Immunol.* **38**, 515–525 (2002).

8. Lauer, I. *et al.* Expression and characterization of three important panallergens from hazelnut. *Mol. Nutr. Food Res.* **52**(Suppl 2), S262–271 (2008).
9. Eriksson, N. E., Formgren, H. & Svenonius, E. Food hypersensitivity in patients with pollen allergy. *Allergy* **37**, 437–443 (1982).
10. Breda, C. *et al.* Defense reaction in *Medicago sativa*: a gene encoding a class 10 PR protein is expressed in vascular bundles. *Mol. Plant. Microbe Interact.* **9**, 713–719 (1996).
11. Robert, N. *et al.* Molecular Characterization of the Incompatible Interaction of *Vitis Vinifera* Leaves With *Pseudomonas Syringae* Pv. Pisi: Expression Of Genes Coding For Stilbene Synthase And Class 10 PR Protein. *Eur. J. Plant Pathol.* **107**, 249–261 (2001).
12. Jain, S. & Kumar, A. The Pathogenesis Related Class 10 proteins in Plant Defense against Biotic and Abiotic Stresses. *Adv. Plants Agric. Res.* **2**, 1–10 (2015).
13. Walter, M. H., Liu, J. W., Grand, C., Lamb, C. J. & Hess, D. Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens. *Mol. Gen. Genet.* **222**, 353–360 (1990).
14. Ebner, C., Hoffmann-Sommergruber, K. & Breiteneder, H. Plant food allergens homologous to pathogenesis-related proteins. *Allergy* **56**(Suppl 67), 43–44 (2001).
15. Hoffmann-Sommergruber, K. Plant allergens and pathogenesis-related proteins. What do they have in common? *Int. Arch. Allergy Immunol.* **122**, 155–166 (2000).
16. Mogensen, J. E., Wimmer, R., Larsen, J. N., Spangfort, M. D. & Otzen, D. E. The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J. Biol. Chem.* **277**, 23684–23692 (2002).
17. Marković-Housley, Z. *et al.* Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J. Mol. Biol.* **325**, 123–133 (2003).
18. Koistinen, K. M. *et al.* Birch PR-10c interacts with several biologically important ligands. *Phytochemistry* **66**, 2524–2533 (2005).
19. Kofler, S. *et al.* Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen bet v 1. *J. Mol. Biol.* **422**, 109–123 (2012).
20. Asam, C. *et al.* Bet v 1—a Trojan horse for small ligands boosting allergic sensitization? *Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol.* **44**, 1083–1093 (2014).
21. Hirschwehr, R. *et al.* Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.* **90**, 927–936 (1992).
22. Breiteneder, H. *et al.* Four recombinant isoforms of Cor a I, the major allergen of hazel pollen, show different IgE-binding properties. *Eur. J. Biochem.* **212**, 355–362 (1993).
23. Hoffmann-Sommergruber, K. *et al.* Genomic characterization of members of the Bet v 1 family: genes coding for allergens and pathogenesis-related proteins share intron positions. *Gene* **197**, 91–100 (1997).
24. Spiric, J. *et al.* Model for Quality Control of Allergen Products with Mass Spectrometry. *J. Proteome Res.* **16**, 3852–3862 (2017).
25. Ipsen, H. & Löwenstein, H. Isolation and immunochemical characterization of the major allergen of birch pollen (*Betula verrucosa*). *J. Allergy Clin. Immunol.* **72**, 150–159 (1983).
26. Swoboda, I. *et al.* Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J. Biol. Chem.* **270**, 2607–2613 (1995).
27. Faber, C. *et al.* Secondary structure and tertiary fold of the birch pollen allergen Bet v 1 in solution. *J. Biol. Chem.* **271**, 19243–19250 (1996).
28. Ferreira, F. *et al.* Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J.* **12**, 231–242 (1998).
29. Krebitz, M. *et al.* Rapid production of the major birch pollen allergen Bet v 1 in *Nicotiana benthamiana* plants and its immunological *in vitro* and *in vivo* characterization. *FASEB J.* **14**, 1279–1288 (2000).
30. Mirza, O. *et al.* Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J. Immunol.* **165**, 331–338 (2000).
31. Spangfort, M. D. *et al.* Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. *J. Immunol.* **171**, 3084–3090 (2003).
32. Gajhede, M. *et al.* X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat. Struct. Biol.* **3**, 1040–1045 (1996).
33. Seutter von Loetzen, C. *et al.* Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. *Biochem. J.* **457**, 379–390 (2014).
34. Seutter von Loetzen, C. *et al.* Ligand Recognition of the Major Birch Pollen Allergen Bet v 1 is Isoform Dependent. *PLoS One* **10**, e0128677 (2015).
35. Seutter von Loetzen, C., Schweimer, K., Schwab, W., Rösch, P. & Hartl-Spiegelhauer, O. Solution structure of the strawberry allergen Fra a 1. *Biosci. Rep.* **32**, 567–575 (2012).
36. Bollen, M. A. *et al.* Purification and characterization of natural Bet v 1 from birch pollen and related allergens from carrot and celery. *Mol. Nutr. Food Res.* **51**, 1527–1536 (2007).
37. Strack, D. *et al.* Quercetin 3-galactosylgalactoside from pollen of *Corylus avellana*. *Phytochemistry* **23**, 2970–2971 (1984).
38. Miller, K. D., Guyon, V., Evans, J. N., Shuttleworth, W. A. & Taylor, L. P. Purification, cloning, and heterologous expression of a catalytically efficient flavonol 3-O-galactosyltransferase expressed in the male gametophyte of *Petunia hybrida*. *J. Biol. Chem.* **274**, 34011–34019 (1999).
39. R. Markham, K. & Campos, M. 7- And 8-O-methylherbacetin-3-O-sophorosides from bee pollens and some structure/activity observations. *Phytochemistry* **43**, 763–767 (1996).
40. Roberts, G. C. K. BioNMR in drug research. (Wiley-VCH, 2003).
41. Kubo, A., Arai, Y., Nagashima, S. & Yoshikawa, T. Alteration of sugar donor specificities of plant glycosyltransferases by a single point mutation. *Arch. Biochem. Biophys.* **429**, 198–203 (2004).
42. Olsen, J. L. Growing Hazelnuts in the Pacific Northwest: Pollination and Nut Development. *OSU Ext. Cat.* 1–4 (2013).
43. Mo, Y., Nagel, C. & Taylor, L. P. Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proc. Natl. Acad. Sci. USA* **89**, 7213–7217 (1992).
44. Vogt, T. & Taylor, L. P. Flavonol 3-O-glycosyltransferases associated with *petunia* pollen produce gametophyte-specific flavonol diglycosides. *Plant Physiol.* **108**, 903–911 (1995).
45. Spiric, J., Engin, A. M., Karas, M. & Reuter, A. Quality Control of Biomedicinal Allergen Products - Highly Complex Isoallergen Composition Challenges Standard MS Database Search and Requires Manual Data Analyses. *PLoS One* **10**, e0142404 (2015).
46. Silva, J. C. *et al.* Quantitative proteomic analysis by accurate mass retention time pairs. *Anal. Chem.* **77**, 2187–2200 (2005).
47. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–858 (1996).
48. Hyberts, S. G., Milbradt, A. G., Wagner, A. B., Arthanari, H. & Wagner, G. Application of iterative soft thresholding for fast reconstruction of NMR data non-uniformly sampled with multidimensional Poisson Gap scheduling. *J. Biomol. NMR* **52**, 315–327 (2012).
49. Shen, Y., Delaglio, F., Cornilescu, G. & Bax, A. TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J. Biomol. NMR* **44**, 213–223 (2009).
50. Schwieters, C. D., Kuszewski, J. J., Tjandra, N. & Clore, G. M. The Xplor-NIH NMR molecular structure determination package. *J. Magn. Reson.* **160**, 65–73 (2003).

51. Schwieters, C. D., Kuszewski, J. J. & Clore, G. M. Using Xplor–NIH for NMR molecular structure determination. *Prog. Nucl. Magn. Reson. Spectrosc.* **48**, 47–62 (2006).
52. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R. & Thornton, J. M. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* **8**, 477–486 (1996).
53. Gasteiger, E. *et al.* Protein Identification and Analysis Tools on the ExpASY server. in *The Proteomics Protocols Handbook* 571–607 (Humana Press, 2005).
54. Dundas, J. *et al.* CASTp: computed atlas of surface topography of proteins with structural and topographical mapping of functionally annotated residues. *Nucleic Acids Res.* **34**, W116–118 (2006).
55. Sievers, F. *et al.* Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539 (2011).
56. Rice, P., Longden, I. & Bleasby, A. EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet.* **16**, 276–277 (2000).

Acknowledgements

The authors thank Ramona Heissmann, Andrea Hager, Luisa Schwaben, and Elke Völker for excellent technical assistance. This work was supported by the University of Bayreuth and the Paul-Ehrlich-Institut.

Author Contributions

B.M.W. and T.J. wrote the manuscript. P.R. and S.V. initiated the project and provided conceptual input. C.S.v.L., K.S. and B.M.W. supervised the project and designed experiments. C.S.v.L. and T.J. carried out the expression and purification experiments. C.S.v.L., K.S. and T.J. performed the NMR experiments and evaluated the data. K.S. solved the solution structure of Bet v 1.0101. D.S. cloned and sequenced rCor a 1.0401, A.R. performed the MS analyses of rCor a 1.0401, nCor a 1 and hazel pollen extract. U.L. and R.S. performed the mass spectrometry of the ligand and evaluated the data. V.M. contributed to the analysis and interpretation of data. All authors contributed in preparing the manuscript.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-019-44999-2>.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



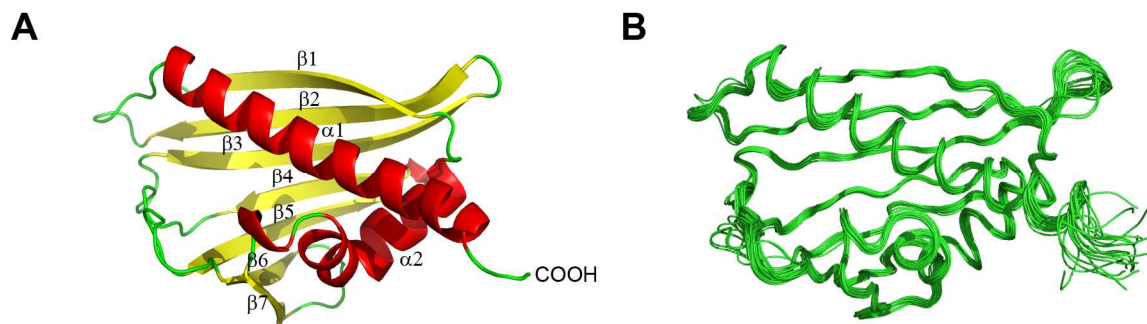
Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019

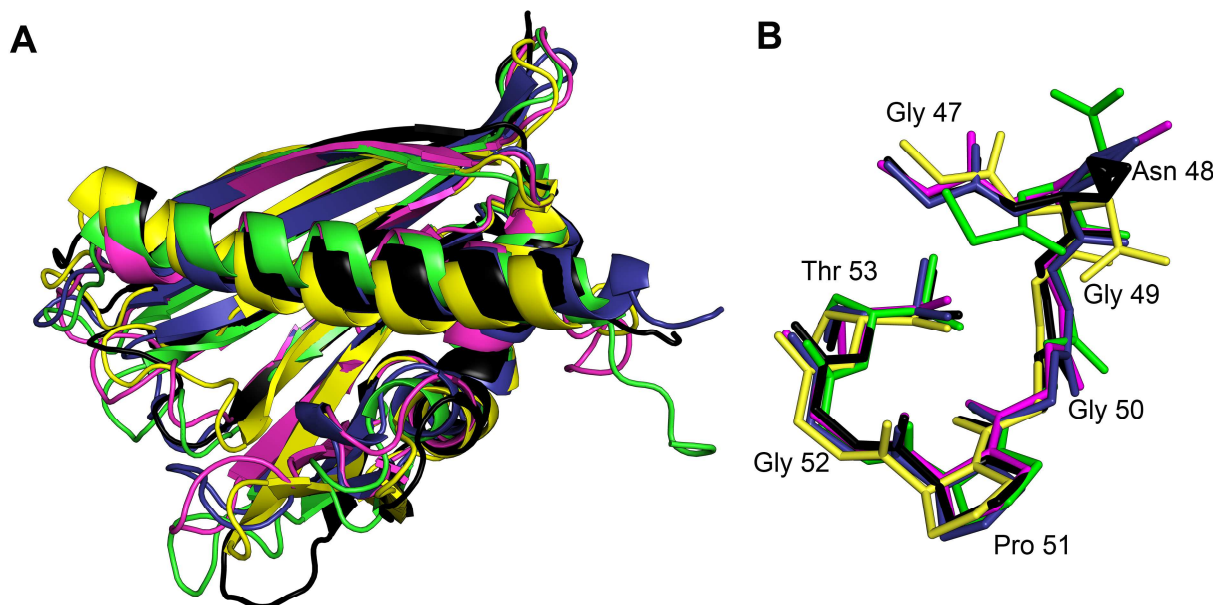
Identification of a natural ligand of the hazel allergen

Cor a 1

Thessa Jacob¹, Christian Seutter von Loetzen¹, Andreas Reuter³, Ulrike Lacher⁴, Dirk Schiller³, Rainer Schobert⁴, Vera Mahler³, Stefan Vieths³, Paul Rösch^{1,2}, Kristian Schweimer¹, Birgitta M. Wöhrl^{1#}

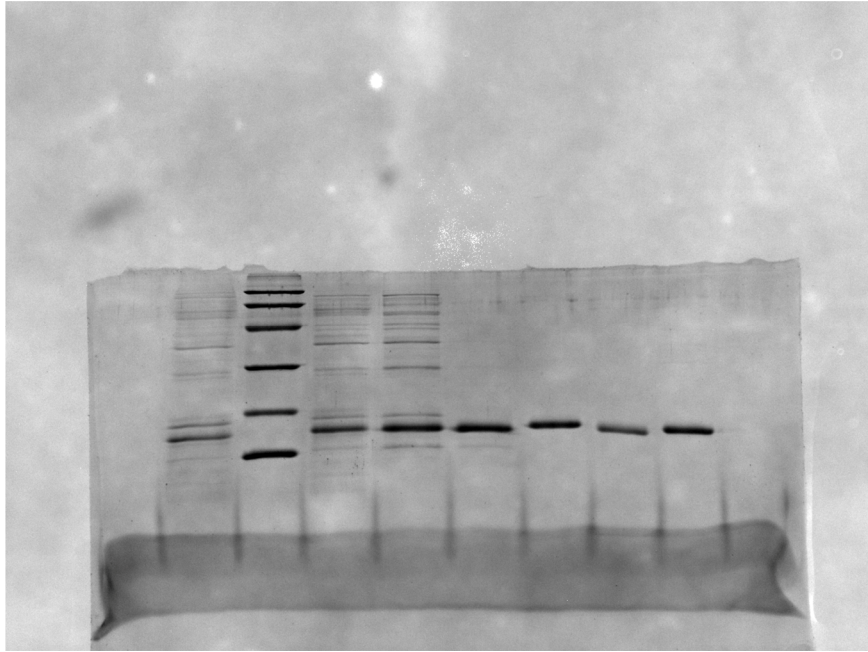


Supplementary Figure S1: Solution structure of Bet v 1.0101. (A) Cartoon representation of the average of the 20 lowest energy solution structures of Bet v 1.0101 (PDB: 6R3C). α -helices: red; β -strands, yellow; loop-regions, green. (B) Backbone overlay of the 20 lowest energy solution structures of Bet v 1.0101, with a backbone rmsd value of 0.46 Å and overall rmsd of 0.89 Å.

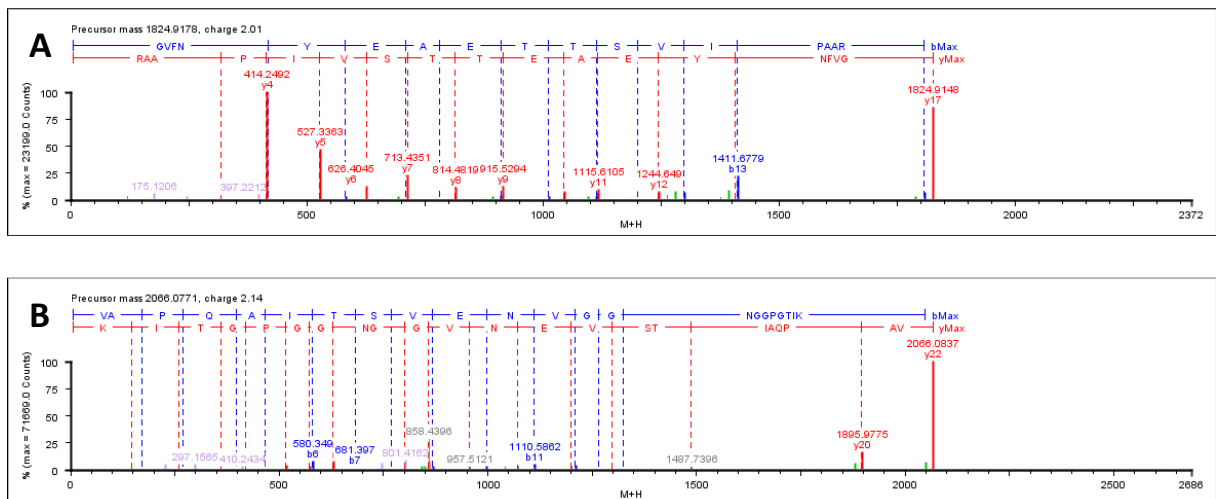


| allergen | source | sequence identity to Cor a 1.0401 [%] | RMSD _{total} [Å] | RMSD _{g-rich loop} [Å] | reference | PDB entry |
|---------------------|--------------|---------------------------------------|---------------------------|---------------------------------|--|-----------|
| Cor a 1.0401 | hazelnut | 100 | - | - | actual work | 6GQ9 |
| Bet v 1.0101 | birch pollen | 67.3 | 1.682 | 0.675 | actual work | 6R3C |
| Fra a 1E | strawberry | 62.2 | 1.971 | 0.643 | Seutter von Loetzen <i>et al.</i> , 2012 | 2LPX |
| Gly m 4 | soy bean | 57.3 | 2.088 | 0.344 | Berkner <i>et al.</i> , 2009 | 2K7H |
| Pru av 1 | cherry | 65.4 | 2.179 | 0.417 | Neudecker <i>et al.</i> , 2001 | 1E09 |

Supplementary Figure S2: Structure of Cor a 1.0401 in comparison with homologous PR-10 allergens. (A) Overlay of the cartoon representation of Cor a 1.0401 (PDB: 6GQ9, black) with Pru av 1 (1E09, pink), Bet v 1.0101 (6R3C, blue), Gly m 4 (2K7H, yellow) and Fra a 1E (2LPX, green). **(B)** Overlay of the glycine-rich loop (residues Gly 47 – Thr 53) in stick-representation. Colors as in **(A)**.



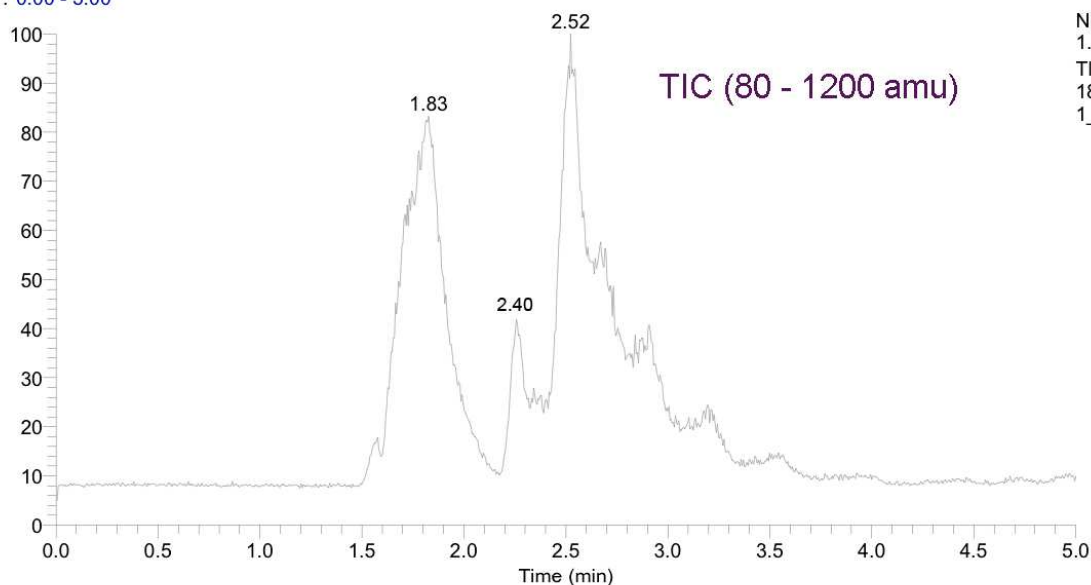
Supplementary Figure S3: Purification of Cor a 1 from pollen. Analysis of the purification procedure of Cor a 1 from hazel pollen by SDS-PAGE (19 % gel). (Uncropped gel from Fig. 2)



Supplementary Figure S4: Annotated spectra of the variant specific peptides of Cor a 1.01 from natural purified Cor a 1. Panel **A**: N-terminal peptide specific for Cor 1.0103. Panel **B**: Internal peptide specific for Cor a 1.0104.

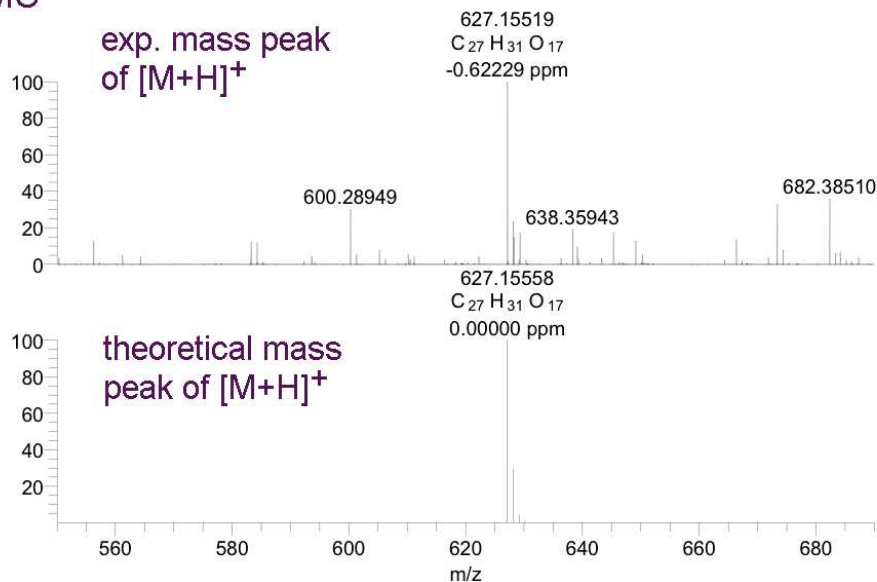
Chromatogram

RT: 0.00 - 5.00



NL:
1.93E9
TIC MS
180903cora
1_4

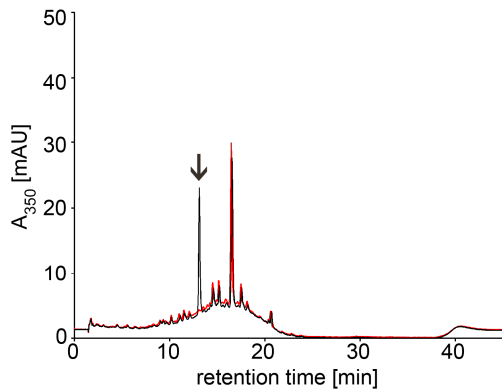
MS



NL:
2.53E5
180903cora1_4#412-440 RT:
2.33-2.48 AV: 29 SB: 70
2.13-2.27 , 2.60-2.83 T: FTMS
+ p ESI Full ms
[80.00-1200.00]

NL:
7.15E5
 $C_{27}H_{30}O_{17} + H:$
 $C_{27}H_{31}O_{17}$
pa Chrg 1

Supplementary Figure S5: Identification of Q3O-(Glc)-Gal extracted from nCor a 1. The upper panel shows the TIC of nCor a 1 MeOH extract. The second panel shows the mass spectrum at a retention time of 2.33 – 2.48 minutes with the experimental mass peak of 627.1552 m/z and the third panel the theoretical mass peak of Q3O-(Glc)-Gal $[M+H]^+$ (627.1555 m/z).



Supplementary Figure S6: HPLC chromatogram of hazelnut skin extracts.

Analysis of methanol extracts from hazelnut skin by RP-HPLC. Hazelnut skin extract before (red) and after the addition of 100 ng Q3O-(Glc)-Gal from hazel pollen (black). The additional peak in the black spectrum representing Q3O-(Glc)-Gal is indicated by an arrow.

| | | |
|--------------|--|-----|
| Cor a 1.0401 | GVFCYEDEATSVIPPARLFKSFVLDADNLIKVPVAPQHFTSAENLEGNGGPGTIKKITFAE | 60 |
| Bet v 1.0101 | GVFNJETETTSVIPAARLFKAFILDGDNLFKVPVAPQAISSVENIEGNGGPGTIKKISFPE | 60 |
| Cor a 1.0104 | GVFNIEVETPSVISAARLFKSYVLDGDKLIPKVPVAPQAITSVENVGGNGGPGTIKNITFGE | 60 |
| Cor a 1.0103 | GVFNIEAETTSVIPAARLFKSYVLDGDKLIPKVPVAPQAITSVENVEGNGGPGTIKNITFGE | 60 |
| Cor a 1.0101 | GVFNIEVETPSVIPAARLFKSYVLDGDKLIPKVPVAPQAITSVENVEGNGGPGTIKNITFGE | 60 |
| Cor a 1.0102 | GVFNIEVETPSVIPAARLFKSYVLDGDKLIPKVPVAPQAITSVENVEGNGGPGTIKNITFGE | 60 |
| | *** ** *: *** *****::**.*:***** :*:** : *****:*** * | |
| Cor a 1.0401 | GNEFKYMKHKVVEEIDHANFKYCYSIEGGPLGHTLEKISYEIKMAAAPHGGGSILKITSK | 120 |
| Bet v 1.0101 | GFPFKYVKDRVDEVDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATP-DGGSILKISNK | 119 |
| Cor a 1.0104 | GSRYKYVKERVDEVDNTNFKYSYTVIEGDVLGDKLEKVCSELKIVAAP-GGGSTLKISSK | 119 |
| Cor a 1.0103 | GSRYKYVKERVDEVDNTNFTYSYTVIEGDVLGDKLEKVCHELKIVAAP-GGGSILKISSK | 119 |
| Cor a 1.0101 | GSRYKYVKERVDEVDNTNFTYSYTVIEGDVLGDKLEKVCHELKIVAAP-GGGSILKISSK | 119 |
| Cor a 1.0102 | GSRYKYVKERVDEVDNTNFKYSYTVIEGDVLGDKLEKVCSELKIVAAP-GGGSILKISSK | 119 |
| | * :**.*:***:***:***.* **:***.* :*.***. **:***.* ** ***:** * | |
| Cor a 1.0401 | YHTKGNASINEEIKAGKEKAAGLFKAVEAYLLAHPDAYC | 160 |
| Bet v 1.0101 | YHTKGDHEVKAEQVKASKEMGETLLRAVESYLLAHSADAYN | 159 |
| Cor a 1.0104 | FHAKGDHEINAEMKGAKEMA EKLLRAVETYLLAHSAEYN | 159 |
| Cor a 1.0103 | FHAKGDHEINAEEIKGAKEMA EKLLRAVETYLLAHSAEYN | 159 |
| Cor a 1.0101 | FHAKGDHEINAEMKGAKEMA EKLLRAVETYLLAHSAEYN | 159 |
| Cor a 1.0102 | FHAKGDHEINAEMKGAKEMA EKLLRAVETYLLAHSAEYN | 159 |
| | :**.*: .:: *:*..** . **:***:****** * | |

Supplementary Figure S7: Amino acid sequence alignments of Cor a 1 and Bet v 1 proteins used

Supplementary Table S1: Solution structure statistics of Bet v 1.0101

| Experimentally derived restraints | |
|---|--------------------------|
| distance restraints | |
| NOE | 3643 |
| intraresidual | 1092 |
| sequential | 1001 |
| medium range | 572 |
| long range | 978 |
| hydrogen bonds | 64 |
| dihedral restraints | 212 |
| restraint violation | |
| average distance restraint violation (Å) | 0.004798 +/- 0.000564 |
| distance restraint violation > 0.1 Å | 3.15 +/- 1.24 |
| average dihedral restraint violation (°) | 0.0561 +/- 0.0162 |
| dihedral restraint violation > 1° | 0.0 +/- 0.0 |
| deviation from ideal geometry | |
| bond length (Å) | 0.000576 +/- 0.000034 |
| bond angle (°) | 0.1068 +/- 0.0048 |
| coordinate precision^{a,b} | |
| backbone heavy atoms (Å) | 0.46 |
| all heavy atoms (Å) | 0.89 |
| Ramachandran plot statistics^c (%) | |
| | 92.7/7.0/0.3/0.0 |

^a The precision of the coordinates is defined as the average atomic root mean square difference between the accepted simulated annealing structures and the corresponding mean structure calculated for the given sequence regions.

^b calculated for residues 2-154

^c Ramachandran plot statistics are determined by PROCHECK ⁵² and noted by most favored/additionally allowed/generously allowed/disallowed.

Supplementary Table S2: Summary of MS data on specific peptides of Cor a 1.01 isoforms variants

| PM | PE | S | Peptide Amino Acid Sequence | F | B- and Y- Fragments | FE |
|---------------------|------|-----|-----------------------------|----|--|-----|
| Cor a 1.0103 | | | | | | |
| 1824.9178 | 2.9 | 8.3 | GVFNIEAETTSVIPAAAR | 39 | b4b5b5*b6b6*b6°b7b7*b7°b8b8*b8°b9b9°b10b10°b11 b11°b12b12°b13b13°b17b17°y3y4y5y6y7y8y9y10y10°y1 1y11°y12y13y15°y17 | 9.2 |
| Cor a 1.0104 | | | | | | |
| 2066.0771 | -5.1 | 8.4 | VAPQAITSVENVGGNGGPGTIK | 53 | b2b3b4b5b5*b6b6*b7b7°b8b8°b9b9°b10b10°b11b11°b 12b12°b13b13°b14b22*y1y2y3y4y5y5°y6y6°y7y7°y8*y9 y9*y9°y10y10°y10°y11y11*y12y12*y13y13*y14y16y16* y20y20*y22y22* | 7.6 |

PM: precursor mass in dalton; PE: precursor mass error in ppm; S: PLGS peptide score; F: number of y-series and b-series fragments; B- and Y-Fragments: specific y- and b- series fragments matched; FE: mean fragment mass error in ppm

Supplementary Table S3: Summary of MS data on unpurified extract from hazel pollen.

| internal | Genebank | UniProt | Description | | S | SC | E | DP | MP |
|-------------------------------|----------|---------|--------------|--|-------|------|------|----|----|
| crude extract buffer a | | | | | | | | | |
| PEI127 | X70998 | Q08407 | Cora_Cora1 | Cor a 1 0104, Corylus avellana | 13119 | 62.9 | 5.8 | 12 | 1 |
| PEI126 | X70997 | Q08407 | Cora_Cora1 | Cor a 1 0103, Corylus avellana | 6270 | 58.5 | 6.1 | 10 | 0 |
| n.a. | n.a. | A4KA45 | PROF4_CORAV | Profilin 4, Corylus avellana | 7259 | 42.1 | 4.1 | 5 | 2 |
| n.a. | n.a. | O65002 | O65002_BETPN | Isoflavone reductase homolog Bet v 6 0101 Fragment | 599 | 14.3 | 4.3 | 6 | 0 |
| n.a. | n.a. | P21568 | CYPH_SOLLC | Peptidyl prolyl cis trans isomerase, Solanum lycopersicum | 481 | 23.4 | 16.2 | 7 | 3 |
| n.a. | n.a. | P34887 | CYPH_ALLCE | Peptidyl prolyl cis trans isomerase, Allium cepa | 468 | 22.0 | 6.4 | 3 | 1 |
| n.a. | n.a. | A2XGP6 | SODC1_ORYSI | Superoxide dismutase Cu Zn 1, Oryza sativa subsp indica | 413 | 11.2 | 16.8 | 2 | 0 |
| n.a. | n.a. | Q38936 | FK152_ARATH | Peptidyl prolyl cis trans isomerase FKBP15 2, Arabidopsis thaliana | 345 | 22.7 | 5.8 | 3 | 0 |
| n.a. | n.a. | P42739 | UBIQP_ACEPE | Polyubiquitin Fragment, Acetabularia peniculus | 203 | 8.0 | 6.8 | 4 | 0 |
| n.a. | n.a. | P29409 | | Phosphoglycerate kinase chloroplastic Fragment, Spinacia oleracea | 175 | 8.5 | 23.2 | 3 | 0 |
| crude extract buffer b | | | | | | | | | |
| PEI127 | X70998 | Q08407 | Cora_Cora1 | Cor a 1 0103, Corylus avellana | 10884 | 57.2 | 7.3 | 8 | 0 |
| PEI126 | X70997 | Q08407 | Cora_Cora1 | Cor a 1 0104, Corylus avellana | 10199 | 62.9 | 7.1 | 11 | 1 |
| n.a. | n.a. | A4KA45 | PROF4_CORAV | Profilin 4, Corylus avellana | 15652 | 47.4 | 5.8 | 6 | 1 |
| n.a. | n.a. | P25816 | PROF1_BETPN | Profilin 1, Betula pendula | 13179 | 33.1 | 5.8 | 5 | 1 |
| n.a. | n.a. | A2XKU9 | COSA_ORYSI | Costars family protein, Oryza sativa | 2081 | 14.9 | 1.7 | 1 | 0 |
| n.a. | n.a. | O65002 | O65002_BETPN | Isoflavone reductase homolog Bet v 6 0101 Fragment, Betula pendula | 1756 | 24.7 | 5.3 | 8 | 0 |
| n.a. | n.a. | Q8L5T1 | Q8L5T1_BETPN | Peptidyl prolyl cis trans isomerase, Betula pendula | 1202 | 42.8 | 8.2 | 7 | 1 |

| | | | | | | | | | |
|-------------------------------|--------|--------|-------------|---|-------|------|------|----|---|
| n.a. | n.a. | Q38936 | FK152_ARATH | Peptidyl prolyl cis trans isomerase FKBP15 2, Arabidopsis thaliana | 1201 | 8.6 | 0.0 | 1 | 0 |
| n.a. | n.a. | P86354 | VIRE2_HELVI | Viresin Fragment, Heliothis virescens | 838 | 31.8 | 12.2 | 1 | 0 |
| n.a. | n.a. | P52578 | IFRH_SOLTU | Isoflavone reductase homolog, Solanum tuberosum | 481 | 10.4 | 9.1 | 5 | 0 |
| n.a. | n.a. | P09678 | SODC_BRAOC | Superoxide dismutase Cu Zn, Brassica oleracea | 480 | 6.6 | 4.1 | 2 | 0 |
| n.a. | n.a. | A2WN93 | CALM1_ORYSI | Calmodulin 1, Oryza sativa subsp indica | 477 | 47.0 | 15.4 | 6 | 0 |
| n.a. | n.a. | A4KA41 | PROF5_CORAV | Profilin 5, Corylus avellana | 325 | 33.6 | 3.9 | 2 | 0 |
| n.a. | n.a. | P29409 | PGKH_SPIOL | Phosphoglycerate kinase chloroplastic Fragment, Spinacia oleracea | 283 | 8.5 | 17.0 | 3 | 0 |
| n.a. | n.a. | P35131 | UBC8_ARATH | Ubiquitin conjugating enzyme E2 8, Arabidopsis thaliana | 242 | 22.3 | 19.3 | 2 | 0 |
| n.a. | n.a. | P60399 | RSMH_SPIKU | Ribosomal RNA small subunit methyltransferase H, Spiroplasma kunkelii | 181 | 8.0 | 3.5 | 1 | 0 |
| n.a. | n.a. | P0C030 | RUB1_ORYSJ | Ubiquitin NEDD8 like protein RUB1, Oryza sativa | 177 | 18.3 | 4.8 | 3 | 0 |
| n.a. | n.a. | Q1QEI9 | UBIG_PSYCK | Ubiquinone biosynthesis O methyltransferase, Psychrobacter cryohalolentis | 134 | 13.2 | 13.0 | 2 | 0 |
| n.a. | n.a. | P37943 | LEPP_BACNA | Signal peptidase I P, Bacillus subtilis | 125 | 29.0 | 1.6 | 3 | 0 |
| n.a. | n.a. | P48495 | TPIS_PETHY | Triosephosphate isomerase cytosolic, Petunia hybrida | 110 | 15.4 | 7.7 | 2 | 0 |
| n.a. | n.a. | P57550 | PPID_BUCAI | Peptidyl prolyl cis trans isomerase D, Buchnera aphidicola | 65 | 3.5 | 8.7 | 4 | 1 |
| crude extract buffer c | | | | | | | | | |
| PEI127 | X70998 | Q08407 | | Cor a 1 0104, Corylus avellana | 13181 | 70.4 | 6.6 | 12 | 1 |
| PEI126 | X70997 | Q08407 | | Cor a 1 0103, Corylus avellana | 9470 | 52.8 | 7.1 | 8 | 0 |
| n.a. | n.a. | A4KA45 | | Profilin 4, Corylus avellana | 8513 | 57.1 | 6.5 | 7 | 2 |
| n.a. | n.a. | P25816 | | Profilin 1, Betula pendula | 7512 | 57.1 | 6.9 | 7 | 2 |
| n.a. | n.a. | A4KA45 | | Profilin 4, Corylus avellana | 8513 | 57.1 | 6.5 | 7 | 2 |
| n.a. | n.a. | A2XKU9 | | Costars family protein, Oryza sativa | 2077 | 23.0 | 10.7 | 2 | 0 |
| n.a. | n.a. | Q8L5T1 | | Peptidyl prolyl cis trans isomerase, Betula pendula | 1157 | 38.7 | 7.5 | 6 | 1 |

| | | | | | | | | |
|------|------|--------|--|------|------|------|---|---|
| n.a. | n.a. | O49886 | Peptidyl prolyl cis trans isomerase, <i>Lupinus luteus</i> | 1128 | 26.2 | 11.6 | 6 | 0 |
| n.a. | n.a. | O65002 | Isoflavone reductase homolog Bet v 6 0101 Fragment, <i>Betula pendula</i> | 1081 | 29.3 | 6.6 | 8 | 1 |
| n.a. | n.a. | P86354 | Viresin Fragment, <i>Heliothis virescens</i> | 864 | 31.8 | 7.7 | 1 | 0 |
| n.a. | n.a. | A4KA41 | Profilin 5, <i>Corylus avellana</i> | 505 | 33.6 | 6.7 | 3 | 0 |
| n.a. | n.a. | P09678 | Superoxide dismutase Cu Zn, <i>Brassica oleracea</i> | 425 | 16.4 | 24.2 | 3 | 0 |
| n.a. | n.a. | P35131 | Ubiquitin conjugating enzyme E2 8, <i>Arabidopsis thaliana</i> | 394 | 29.7 | 25.1 | 3 | 0 |
| n.a. | n.a. | O31535 | Uncharacterized protein YetH, <i>Bacillus subtilis</i> | 320 | 18.3 | 7.4 | 3 | 0 |
| n.a. | n.a. | Q41188 | Cold shock protein 2, <i>Arabidopsis thaliana</i> | 311 | 22.7 | 7.8 | 2 | 1 |
| n.a. | n.a. | P0C030 | Ubiquitin NEDD8 like protein RUB1, <i>Oryza sativa</i> | 235 | 19.0 | 3.1 | 3 | 0 |
| n.a. | n.a. | A1USC7 | 50S ribosomal protein L7 L12, <i>Bartonella bacilliformis</i> | 159 | 22.0 | 7.8 | 3 | 0 |
| n.a. | n.a. | P49310 | Glycine rich RNA binding protein GRP1A, <i>Sinapis alba</i> | 156 | 13.9 | 3.8 | 2 | 0 |
| n.a. | n.a. | A1W5A4 | Thymidylate synthase, <i>Acidovorax</i> sp | 82 | 5.1 | 31.2 | 3 | 0 |
| n.a. | n.a. | Q1PER6 | L ascorbate peroxidase 2 cytosolic, <i>Arabidopsis thaliana</i> | 75 | 8.0 | 4.5 | 1 | 0 |
| n.a. | n.a. | Q5F3T9 | UDP glucose 6 dehydrogenase, <i>Gallus gallus</i> | 66 | 7.1 | 24.6 | 3 | 1 |
| n.a. | n.a. | A4R017 | Probable zinc metalloprotease MGG 02107, <i>Magnaporthe oryzae</i> | 64 | 3.8 | 18.7 | 5 | 0 |
| n.a. | n.a. | Q4JV77 | Protein RecA, <i>Corynebacterium jeikeium</i> | 60 | 3.2 | 4.8 | 3 | 0 |

n.a. not applicable; S: score, SC: sequence coverage; E average precursor mass error; DP: number of detected tryptic peptides; MP: number of detected modified peptides.

Supplementary Table S4: Sequence identity matrix of recombinant allergens used in this work

| | Cor a 1.0401 | Bet v 1.0101 | Cor a 1.0104 | Cor a 1.0103 | Cor a 1.0101 | Cor a 1.0102 | Protein bank entry |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------------|
| Cor a 1.0401 | 100.00 | 67.30 | 62.26 | 64.78 | 63.52 | 64.15 | AAD48405 |
| Bet v 1.0101 | 67.30 | 100.00 | 71.07 | 72.96 | 72.33 | 72.96 | CAA33887 |
| Cor a 1.0104 | 62.26 | 71.07 | 100.00 | 94.97 | 96.86 | 98.11 | CAA50326 |
| Cor a 1.0103 | 64.78 | 72.96 | 94.97 | 100.00 | 98.11 | 96.86 | CAA50325 |
| Cor a 1.0101 | 63.52 | 72.33 | 96.86 | 98.11 | 100.00 | 98.74 | CAA50327 |
| Cor a 1.0102 | 64.15 | 72.96 | 98.11 | 96.86 | 98.74 | 100.00 | CAA50328 |

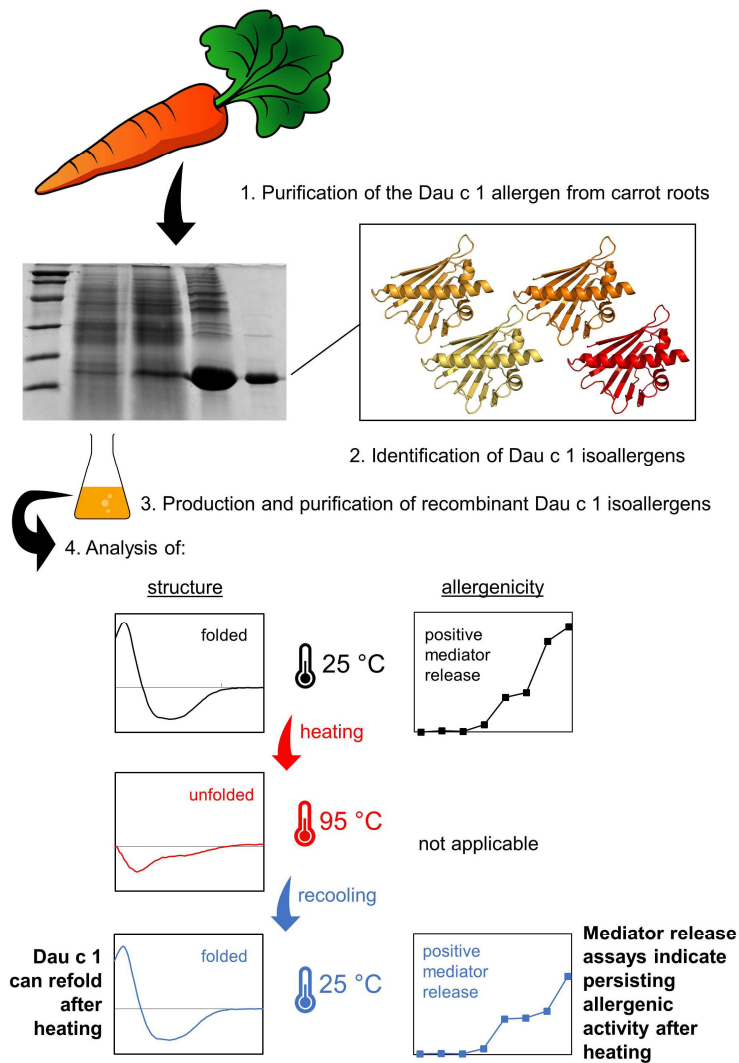
8.2 Einzelarbeit B

Thessa Jacob, Lothar Vogel, Andreas Reuter, Andrea Wangorsch, Carolin Kring, Vera Mahler, Birgitta M. Wöhrl (2020). Food Processing Does Not Abolish the Allergenicity of the Carrot Allergen Dau c 1: Influence of pH, Temperature, and the Food Matrix. *Molecular Nutrition & Food Research*, e2000334

Molecular Nutrition Food Research

18 | 20





The carrot allergen Dau c 1, which comprises a mixture of different molecular forms (isoallergens), was purified from carrot roots and its composition was analysed. Single isoallergens were recombinantly expressed in *E. coli* and purified. Analysis of Dau c 1 after heating to 95 °C revealed that most isoallergens regain their structure and provoke mediator release, indicating persisting allergenic activity. Hence, carrot allergic patients should avoid even processed carrot containing foodstuff.



Food Processing Does Not Abolish the Allergenicity of the Carrot Allergen Dau c 1: Influence of pH, Temperature, and the Food Matrix

Thessa Jacob, Lothar Vogel, Andreas Reuter, Andrea Wangorsch, Carolin Kring, Vera Mahler, and Birgitta M. Wöhrl*

Scope: The major carrot allergen Dau c 1 belongs to the group of pathogenesis related class 10 (PR-10) proteins and is homologous to the birch pollen allergen Bet v 1. In contrast to most other PR-10 allergens, Dau c 1 can elicit Bet v 1 independent sensitization. Although Dau c 1 is considered heat labile, allergic reactions against cooked carrots are possible.

Methods and results: The pH and temperature stability as well as the allergenic potential before and after treatment of purified natural (n) Dau c 1 and different recombinant (r) isoallergens is investigated: rDau c 1.0104, rDau c 1.0105, rDau c 1.0201, rDau c 1.0301. All proteins except rDau c 1.0201 are able to refold at physiological pH. pH conditions around the pI (4.4–5.5) or the presence of the carrot matrix reduce the refolding capacity. Below the pI, most isoallergens are heat resistant and still able to cause mediator release, indicating allergenicity. Moreover, cooked carrot extract is still able to provoke mediator release due to remaining soluble Dau c 1.

Conclusion: Patients allergic to carrots should avoid processed carrot containing foodstuff because heating or pH treatment do not completely abolish the allergenicity of Dau c 1.

pathogenesis related class 10 (PR-10) proteins. Due to its cross-reactivity to Bet v 1, Dau c 1 often elicits allergic symptoms in people allergic to Bet v 1. In contrast to most PR-10 allergens, Dau c 1 can also cause Bet v 1 independent sensitization, which is often more severe, with systemic reactions in about 50% of patients.^[1]

Bet v 1-homologous allergens generally exist as a mixture of highly similar isoallergens (more than 67% sequence identity) and variants thereof (more than 90% sequence identity).^[2] A study in Central Europe revealed that about 98% of people allergic to carrots react to the Dau c 1.0104 variant. Another isoallergen, Dau c 1.0201, was recognized by IgE antibodies of 65% of the study population.^[3]

For patients allergic to PR-10 food allergens, it is important to know whether they can eat certain allergen-containing foods after they were processed. Several Bet v 1 homologous proteins are

structurally labile with respect to heating, denaturation, and proteolysis.^[1, 4–7] Every process that modifies the structure of an allergen might affect its ability to be recognized by antibodies and subsequently its allergic properties.^[8] Thermal processing may cause protein deterioration.^[9] Heating can reduce the allergenicity of PR-10 proteins, for example, in hazelnut, apple, or celeriac.^[4, 10, 11] We have shown previously that recombinant (r)Api g 1.0101 is heat resistant in vitro. However, the pH and the celery matrix influence the stability of rApi g 1.0101.^[12]

Dau c 1 is believed to be particularly heat sensitive. rDau c 1.0103 was not able to refold after heating to 95 °C.^[5] Contrariwise, another study with rDau c 1.0103 showed refolding, but a very low melting temperature (45 °C) compared to natural (n)Dau c 1 (56 °C) was determined.^[13]

Here, we demonstrate that the heat sensitivity of Dau c 1 is isoallergen-specific and pH-dependent. Natural (n)Dau c 1 isolated from carrots was heat-stable suggesting a prevalence of heat-stable isoallergens in carrot roots. In addition, pH- and heat-treated nDau c 1 samples still exhibited allergenic activity in mediator release assays (MRAs), albeit at a lower level compared to untreated nDau c 1. MRAs also showed that cooked carrot extract is still able to provoke mediator release. Our results provide evidence that the majority of Dau c 1 isoallergens and variants is heat

1. Introduction

The major carrot allergen in Central Europe, Dau c 1, is homologous to the birch pollen allergen Bet v 1 and belongs to the

T. Jacob, C. Kring, Prof. B. M. Wöhrl
Biochemie IV - Biopolymere, Universität Bayreuth
Universitätsstr. 30, Bayreuth 95447, Germany
E-mail: birgitta.woehrl@uni-bayreuth.de

Dr. L. Vogel, Dr. A. Reuter, Prof. V. Mahler
Division of Allergology
Paul-Ehrlich-Institut
Langen 63225, Germany

Dr. A. Wangorsch
Molecular Allergology
Paul-Ehrlich-Institut
Langen 63225, Germany

The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/mnfr.202000334>

© 2020 The Authors. Published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

DOI: 10.1002/mnfr.202000334

Table 1. Summary of MS data on natural purified Dau c 1.

| Sample | Acc. Nr. | Description | S | P | SC | PE | F | FE |
|----------|--------------|--------------|-------|----|------|-----|-----|------|
| nDau c 1 | A0A175YPA2 | DAUCS | 46868 | 12 | 90.9 | 2.1 | 442 | 7.6 |
| | CAB03715 | Dau c 1.0103 | 36403 | 12 | 90.9 | 1.9 | 326 | 7.3 |
| | A0A175YQC3 | DAUCS | 24478 | 9 | 62.3 | 2.0 | 243 | 8.1 |
| | A0A164SJC3 | DAUCS | 17675 | 3 | 75.8 | 1.3 | 108 | 7.0 |
| | A0A161 × 1M2 | DAUCS | 11730 | 7 | 58.1 | 1.2 | 179 | 7.7 |
| | D9ZHP1 | DAUCS | 9845 | 9 | 77.9 | 2.6 | 194 | 8.3 |
| | A0A164WTA1 | DAUCS | 8568 | 7 | 74.4 | 1.0 | 164 | 10.0 |
| | D9ZHP0 | DAUCS | 7403 | 8 | 64.9 | 1.6 | 150 | 8.4 |
| | A0A175YRQ3 | DAUCS | 7266 | 4 | 43.7 | 3.5 | 67 | 8.8 |
| | P49372 | Api g 1 | 6582 | 6 | 35.7 | 5 | 129 | 9 |
| | A0A175YR25 | DAUCS | 5459 | 7 | 69.4 | 2 | 140 | 10 |
| | Q40795 | PETCR | 5453 | 4 | 32.3 | 2 | 91 | 7 |
| | O81640 | PIMBR | 4817 | 4 | 24.0 | 2 | 102 | 7 |
| | P27538 | PETCR | 2528 | 6 | 36.1 | 2 | 108 | 12 |
| | Q8SAE7 | Dau c 1.0201 | 2231 | 6 | 48.7 | 13 | 76 | 10 |

S, PLGS protein score; SC, sequence coverage [%]; PE, mean peptide mass error [ppm]; P, number of tryptic peptides identified; F, number of fragments detected; FE, mean fragment mass error [ppm].

resistant. Thus, the allergenicity cannot be entirely abolished by food-processing, suggesting that Dau c 1-allergic patients should completely avoid Dau c 1-containing food stuff.

2. Results and Discussion

2.1. Most Dau c 1 Isoallergens Refold after Heating at Physiological pH

Previous stability studies with the his-tagged variant Dau c 1.0103 showed a melting temperature of only 45.1 °C, whereas for nDau c 1, a melting temperature of 55.7 °C was determined.^[13] Bohle et al.^[5] found that rDau c 1.0103 was not able to refold after heating to 95 °C at physiological pH, and Bollen^[13] observed refolding in a pH range between 6.3 and 9.0.

Thus, we investigated the thermo-resistance and refolding capacity of different isoallergens and variants at physiological pH using circular dichroism (CD), nano differential scanning fluorimetry (nanoDSF), and 2D NMR-spectroscopy. An overview of the experiments performed with the different Dau c 1 isoallergens is listed in Table S2, Supporting Information.

The isoallergen/variant composition of nDau c 1 purified from carrot extract was analyzed by liquid chromatography-mass spectrometry (LC-MS) (Table 1). Apart from Dau c 1.0103 and Dau c 1.0201, at least nine additional Dau c 1 related sequences were found, indicating that the composition of nDau c 1 is more complex than previously thought (Table S3, Supporting Information). For CD and nanoDSF measurements, we chose to compare all known Dau c 1 isoallergens as well as nDau c 1. For Dau c 1.01, five different variants which comprise a sequence identity of more than 95% to each other are known so far. We chose Dau c 1.0104, as this variant was shown to induce allergic reactions in 98 % of carrot allergic patients^[1] and another variant, Dau c 1.0105, to find out if we can see differences despite a very high

amino acid sequence identity (98.7%). For Dau c 1.02 and Dau c 1.03, only one representative (Dau c 1.0201 and Dau c 1.0301, respectively) is known so far.

The CD spectra of all proteins at pH 7 before heat treatment (Figure 1, panel A, black lines) are typical for a protein harboring alpha-helices (minima at 210 and 222 nm) and beta-strands (minimum at 217 nm). At 95 °C (Figure 1, panel A, light blue lines), the spectra indicate an unstructured protein with a minimum between 195 and 205 nm. After recoiling to 25 °C (Figure 1A, red line), the spectra of all isoallergens except rDau c 1.0201 are similar to the ones before heating, implying that they regained their structure. Comparison of denaturation and renaturation curves of rDau c 1.0301 implies that refolding is not completely achieved. This is also true for nDau c 1 because it comprises a mixture of different isoallergens.

In contrast, the isoallergen rDau c 1.0201 is unable to refold. Similar results were obtained recording CD denaturation and renaturation (Figure 1, panel B) or nanoDSF spectra (Figure 1, panel C) confirming the reliability of the experiments.

Both CD and nanoDSF provided melting temperatures of ≈59.1 and 59.9 °C for rDau c 1.0104, and 57.7 and 57.4 °C, respectively, for rDau c 1.0105. In a recent publication, the melting temperature determined for the variant rDau c 1.0103 was 45.1 °C.^[5,13] As rDau c 1.0105, rDau c 1.0104 (our results), and rDau c 1.0103 are almost identical (six and four amino acid exchanges, respectively; Figure S1, Supporting Information), the significantly lower melting temperature of rDau c 1.0103 determined by Bollen is most likely due to the use of a his-tagged protein.^[13]

2.2. Structural Changes Detected by NMR

To observe structural changes of the proteins after heat treatment, we performed 2D NMR-experiments, which allow for a more

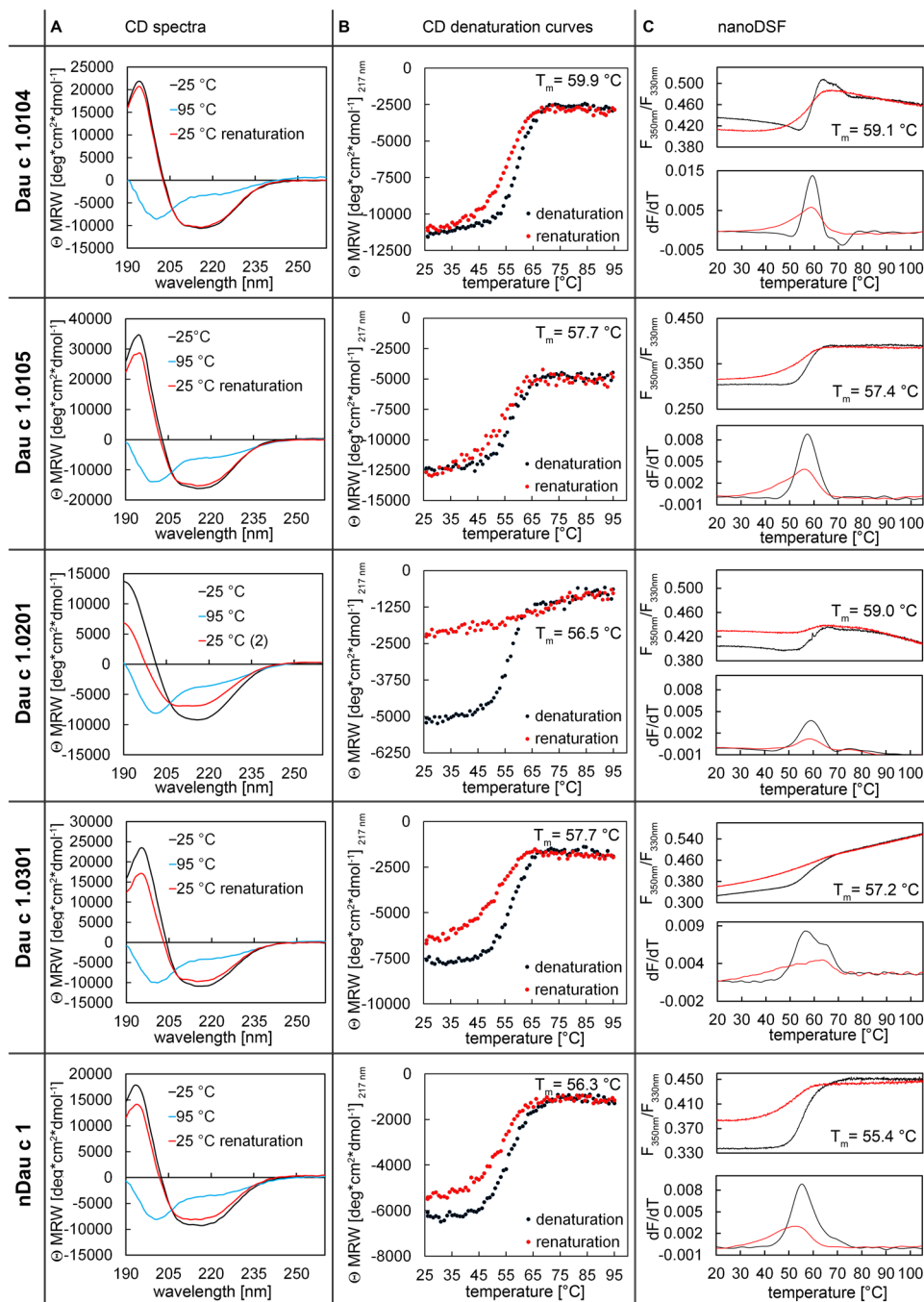


Figure 1. Different refolding capacities of Dau c 1 proteins. A) CD spectra were recorded at 25 °C (black lines), 95 °C (blue lines), and after recoiling to 25 °C (red lines). B) CD denaturation (black) and renaturation curves (red). C) NanoDSF denaturation (black) and renaturation curves (red). The upper panels show the fluorescence ratio of 350 nm:330 nm, the lower panels show the first derivative thereof.

detailed information on the amino acid level. We chose one isoallergen, which is still able to refold after heating, that is, Dau c 1.0105, and another one, which shows a poor refolding capacity, that is, Dau c 1.0201 (Figure 1). We purified ¹⁵N-labeled proteins from *Escherichia coli* and recorded [¹H, ¹⁵N] heteronuclear single quantum coherence (HSQC)-spectra in 10 mM Na-phosphate (pH 7.0), before heating (black spectra) and after 60 min at 95 °C (red spectra). The spectra before heat treatment showed

the broad signal dispersion typical for a well-folded protein (Figure 2, black). This is in good agreement with the CD spectra (Figure 1, panel A). After 60 min at 95 °C and recoiling, rDau c 1.0105 displayed nearly the same spectrum with similar signal intensities, indicating that the majority of Dau c 1.0105 regained its original conformation (Figure 2A, red).

In contrast, rDau c 1.0201 was more sensitive toward temperature increase. After 60 min at 95 °C followed by recoiling, it

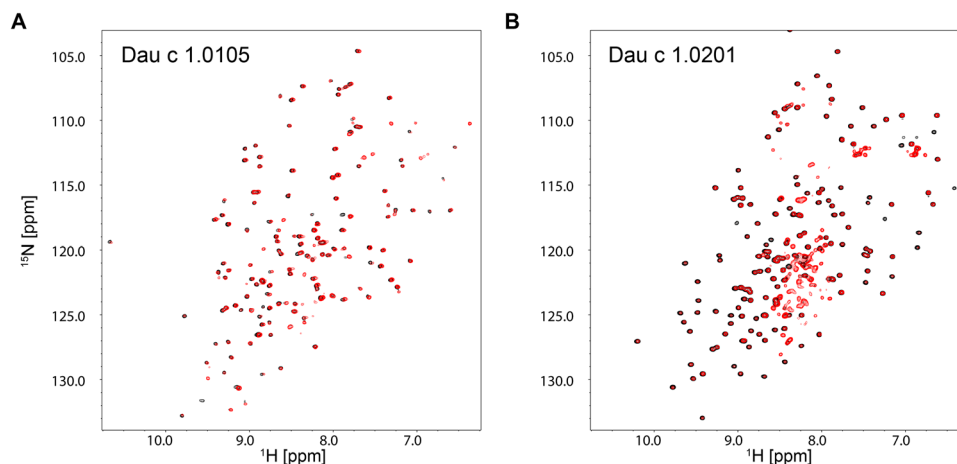


Figure 2. Influence of heat treatment on the protein structure at pH 7. [^1H , ^{15}N] HSQC spectra of Dau c 1.0105 (A) and Dau c 1.0201 (B) before (black) and after 1 h at 95 °C (red).

still showed the signals of the folded protein, however, at a much lower intensity (around 35% of the intensity before heating) (Figure 2B). Moreover, new signals in the ^1H range of 7.5 to 8.5 ppm appeared, typical for an unfolded protein. These results are in line with the CD spectra and nanoDSF measurements described above (Figure 1).

2.3. Thermo-Resistance of Dau c 1.0105 is pH Dependent

The stability of food allergens at different pH-values is another property important for allergic persons. Food processing is often very complex and consists of multiple steps, which are not reproducible if food is not industrially processed. It was shown that during the production of carrot juice, the addition of citric acid or *Lactobacillae* resulting in a pH around 3–4 before pasteurization increased storage life and taste of the product.^[14] Salad dressings often include vinegar, which also lowers the pH. Furthermore, vegetable broth can reach pH values as low as pH 5.^[15]

To exemplify the heat stability of Dau c 1, we chose one of the proteins, which is stable at pH 7, Dau c 1.0105, and investigated its properties at different pH values ranging from pH 2 to pH 6. We recorded CD spectra of rDau c 1.0105 before, during, and after heating to 95 °C to test its pH stability (Figure 3). At pH 6, similar result as for pH 7 (Figure 1), that is, refolding, was observed. At pH 5.0, which is very close to its pI = 4.61, rDau c 1.0105 precipitated almost instantly. This finding might explain the previously observed heat sensitivity of Dau c 1.0103 at pH 7 by Bohle et al.^[5] We assumed that the use of Tris buffer, which is known to decrease its pH significantly at elevated temperatures (AppliChem, Biological Buffers 2008), was responsible for the heat sensitivity observed by Bohle et al.^[5] Since Dau c 1.0103 and Dau c 1.0105 differ by only four amino acid exchanges (Figure S1, Supporting Information), we repeated the CD measurements with Dau c 1.0105 in 20 mM Tris, pH 7, and heating to 95 °C. Under these conditions, Dau c 1.0105 was not able to refold (Figure S2, Supporting Information), thus confirming that not temperature increase alone but simultaneous pH decrease was responsible for the denaturation of Dau c 1.0105.

In contrast, below the pI, at pH 4 and pH 3, the secondary structure elements of Dau c 1.0105 were retained, indicating that the protein might still be allergenic. Only at pH 2, the protein was not heat resistant. It has been shown that salt bridges and cation- π interactions enhance thermoresistance.^[16–18] At pH 2, the high positive net charge of rDau c 1.0105 (pI = 4.6) might hinder those interactions. Our results demonstrate that the thermo-resistance of rDau c 1.0105 is strongly pH dependent.

2.4. Most Dau c 1 Proteins are Heat-Stable at pH 3

The structural state of Dau c 1 proteins at pH 3 appears relevant since after food uptake the pH in the stomach increases and is around pH 3–5.^[19] Thus, we analyzed the stability of all Dau c 1 proteins at pH 3 at 25 and at 95 °C using CD spectroscopy and nanoDSF (Figure 4). Remarkably, all proteins were stable at pH 3 and 25 °C, indicating that the acidic pH of the stomach does not impair Dau c 1 stability. This might be the reason why Dau c 1 can also act as a sensitizing allergen.^[1]

Moreover, all tested allergens except Dau c 1.0301 were heat resistant (Figure 4, CD spectra). The CD spectrum of Dau c 1.0301 at 95 °C (Figure 4, CD spectrum, blue line) is typical for an unstructured protein, and after recooling, no refolding was detected (Figure 4, CD spectrum, red line). Dau c 1.0301 exhibits the highest pI (= 5.1, Table S1, Supporting Information) of all tested isoallergens and therefore possesses a higher positive net charge at pH 3 than the other isoallergens. This might result in a lower stability.

Using nanoDSF, the melting temperature (T_m) for Dau c 1.0301 was 80.3 °C, whereas for the other proteins, no T_m -values could be determined in a temperature range from 20 to 110 °C. However, the first deviation (Figure 4, nanoDSF, dF/dT, lower graph), revealed two distinct minima at ≈ 38 and 51 °C for Dau c 1.0104 and one minimum at 38 °C for Dau c 1.0201 indicating minor structural changes, which could not be detected by CD. No changes in backreflection could be observed for any Dau c 1 isoallergen, suggesting that heating did not result in aggregation (data not shown).

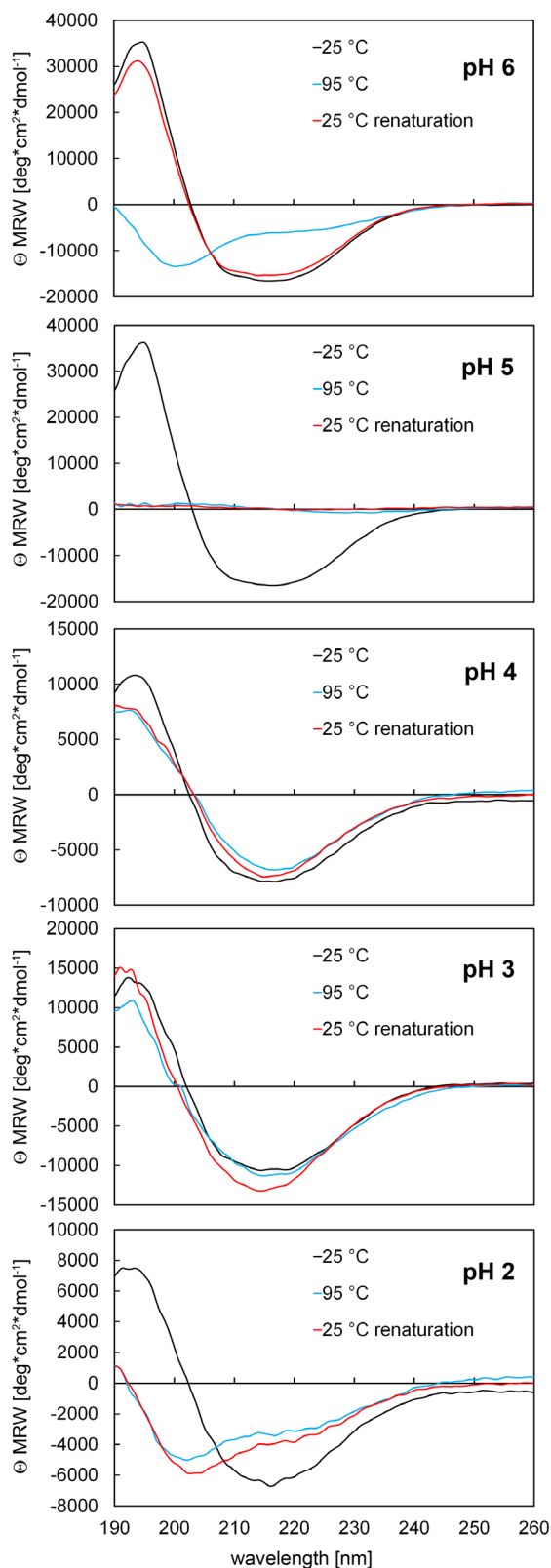


Figure 3. Stability of Dau c 1.0105 at different pH values. CD spectra of Dau c 1.0105 from pH 2 to pH 6 as indicated, before (25 °C, black line) and after heating to 95 °C (95 °C, blue line), and after recooling to 25 °C (25 °C renaturation, red line).

2.5. Dau c 1.0104 Forms Oligomers upon Heat Treatment at pH 3

To investigate whether the minor structural changes, observed by nanoDSF with rDau c 1.0201 and rDau c 1.0104 at pH 3 during heating, could be due to the formation of oligomers, size exclusion chromatography (SEC) was exemplarily performed with rDau c 1.0104 after incubation at pH 3 for 30 min at different temperatures. As a control, we confirmed that the protein remains monomeric after incubation for 30 min at pH 7 (Figure S3A, Supporting Information). SEC was carried out either at pH 3 or after dialysis in 10 mM Na-phosphate buffer, pH 7 (Figure 5 and Figure S3, Supporting Information). The latter procedure mimics physiological conditions because, after food uptake, Dau c 1 is first exposed to the acidic pH of the stomach (pH 2–4), whereas in the small intestine a pH of 7 prevails.

Incubation and SEC at pH 3 resulted in >95% oligomeric protein (Figure 5, lilac bars) and a small amount of degradation products <16 kDa at all temperatures tested (Figure 5, blue bars). However, SEC at pH 7 exhibited >50% of rDau c 1.0104 monomers and dimers at room temperature, 40 °C, and 55 °C (Figure 5 brown and green bars, respectively), showing clearly that the oligomeric state of the protein obtained at pH 3 is partly reversible at neutral pH. This implies that in raw carrots, the protein remains allergenic throughout its passage through the stomach and small intestine. However, after incubation at 95 °C, only a small proportion of monomer could be detected, meaning that the oligomeric state was also preserved at pH 7. The formation of stable oligomers at 95 °C might contribute to a somewhat reduced allergic reaction after cooking carrots due to the inaccessibility of certain epitopes.

2.6. Dau c 1 Proteins Still Provoke Mediator Release after pH- and Heat Treatment

To investigate whether the Dau c 1 proteins still exhibit allergenic potential after pH and heat treatment, MRAs were performed. Controls of each protein, incubated at room temperature and pH 7, were tested. Samples were analyzed after incubation for 30 min either at room temperature and pH 3, or at 95 °C and pH 7, or at 95 °C and pH 3. Neither heat nor pH treatment completely abolished mediator release (Figure 6). It varied, however, for the different proteins, since the strength of the reaction depends on the individual IgE composition present in the serum used. In comparison to the control, all proteins, apart from rDau c 1.0301, showed a reduced reaction for all conditions tested. Remarkably, mediator release for Dau c 1.0301 was similar before and after heating at pH 7 and pH 3 (Figure 6D), even though CD and nanoDSF measurements indicated that at pH 3 it unfolded irreversibly upon heating to 95 °C (Figure 4). We speculate that sequential epitopes might be present that cause mediator release. As during the passage through the gastrointestinal tract proteins get denatured leading to a loss of conformational epitopes, sequential or linear epitopes play an important role in food allergy.^[20] All other Dau c 1 proteins exhibited lower mediator release after heating to 95 °C, pH 3, although the CD spectra recorded under these conditions showed that the secondary structures were retained (Figure 4). This implies that minor changes in the 3D structure and/or

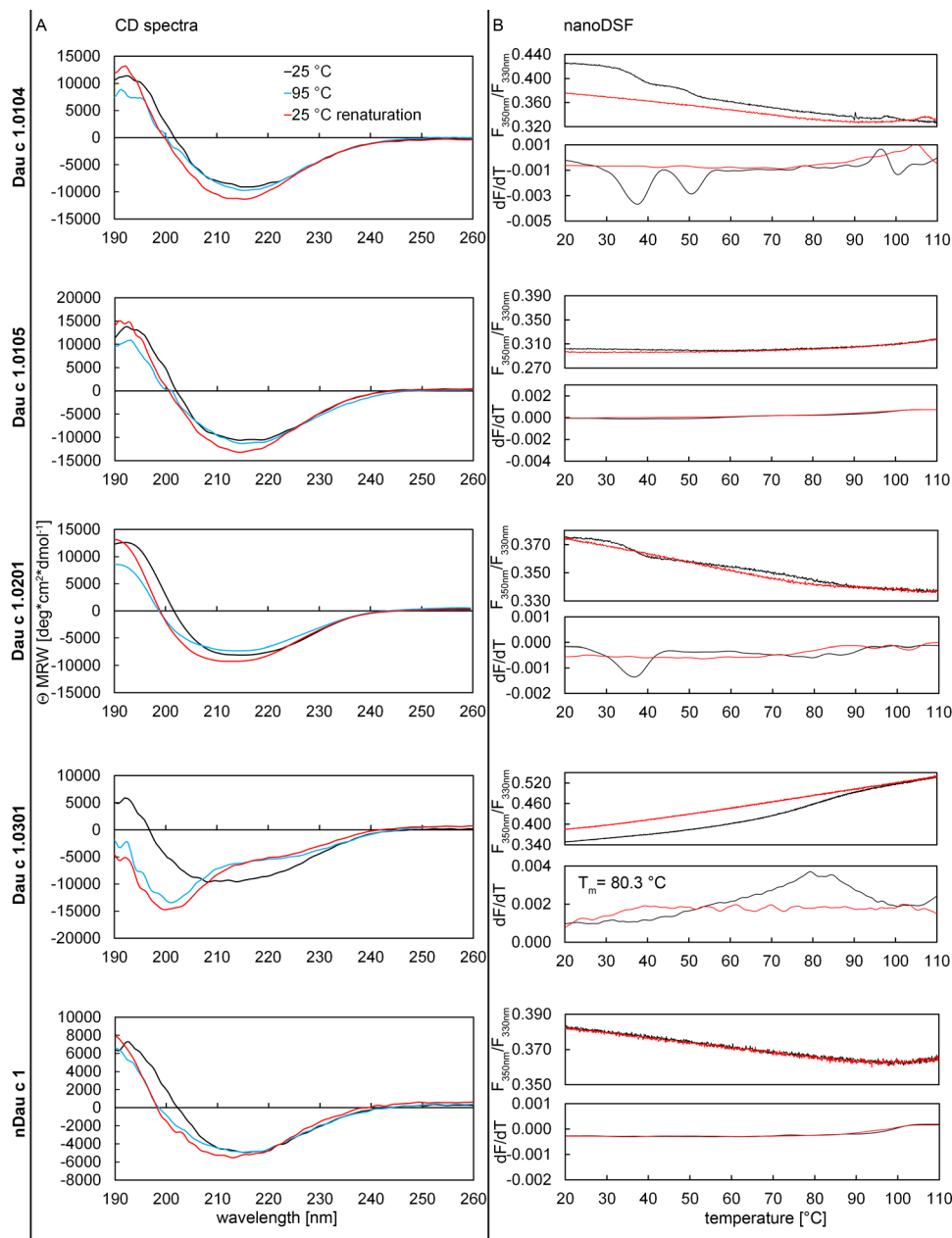


Figure 4. Stability of Dau c 1 proteins at pH 3. CD (left panel) and nanoDSF spectra (right panel). CD spectra were recorded at 25 °C (black), 95 °C (blue), and after recooling to 25 °C (red). NanoDSF denaturation (black) and renaturation (red) curves are presented. The upper parts show the fluorescence ratio of 350 nm:330 nm and the lower parts show the first derivative thereof.

oligomerization occur that are not visible in the CD spectra but might influence the allergenic potential.

2.7. The Carrot Matrix Reduces Dau c 1 Heat Stability

For patients with carrot allergy, it is important to know whether the allergens can still cause an allergic reaction when processed carrots are eaten. To investigate the stability of Dau c 1 proteins in the presence of the natural food matrix, we incubated carrot extract (25 mg mL⁻¹ in water) for 30 min at 95 °C or room

temperature and analyzed the supernatant after centrifugation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 7A). Although weaker than in the untreated sample (room temperature, RT) a band at about 16 kDa, corresponding to the molecular weight of nDau c 1, was still present after heat treatment, indicating incomplete denaturation.

Preparation of carrot extract at pH 3 (25 mg mL⁻¹ in 10 mM Na-citrate, pH 3), and incubation at room temperature resulted in denaturation of most carrot proteins since there were only a few bands visible in the gel, one of them around 16 kDa. After heat treatment, no band could be detected anymore

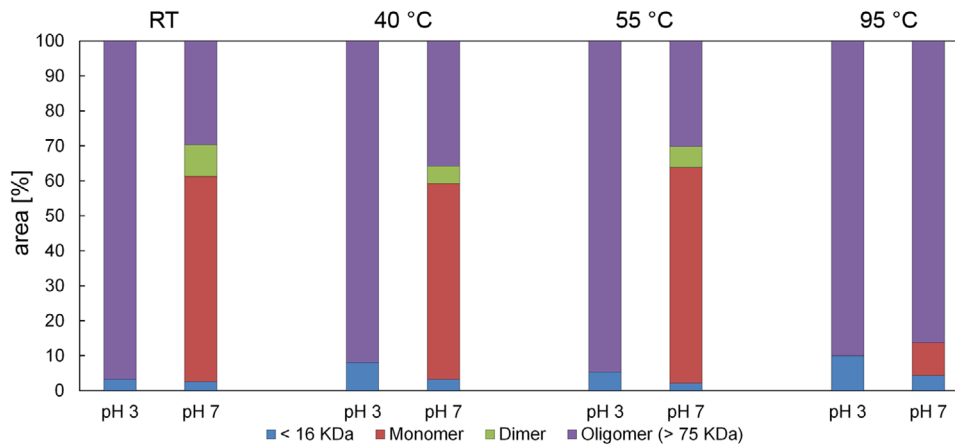


Figure 5. Oligomerization of Dau c 1.0104 upon heat and pH treatment. Dau c 1.0104 samples were incubated at room temperature (RT), 40 °C, 55 °C, or 95 °C in 10 mM Na-citrate, pH 3, for 30 min and then either loaded directly onto a Superdex S75 GL10/300 column equilibrated with 5 mM Na-citrate, pH 3, 300 mM NaCl, or dialyzed against 10 mM Na-phosphate, pH 7, and loaded onto the column equilibrated with 10 mM Na-phosphate, pH 7, 300 mM NaCl. Runs were performed with the corresponding equilibration buffer. Calibration curves served to determine the molecular mass of the proteins in peak fractions. The areas of corresponding peaks in the SEC-chromatograms were determined and the relative distribution of Dau c 1.0104 monomers (brown), dimers (green), and oligomers (lilac) is illustrated by bars. Protein fragments smaller than 16 kDa are shown in blue.

(Figure 7A). The 16 kDa bands of all samples (even though no distinct band was visible) were excised, and in-gel digestion followed by LC-MS^E was performed (Table 2). Dau c 1 proteins could be detected in all samples, even at pH 3 after heating (Table 2 and Table S4, Supporting Information). However, after heating and/or pH treatment, both the number of detected Dau c 1 sequences and the sequence coverage was reduced: In the H₂O extract before heating, at least nine different isoallergens and variants were identified (Table 2); eight of them have also been detected in purified nDau c 1 (Table 1). The presence of Dau c 1.0105, which was absent in purified nDau c 1, could also be confirmed in this extract. Additionally, eight isoallergens/variants which were present in nDau c 1 could not be detected in carrot extract. The different composition of Dau c 1 proteins in carrot extract compared to purified nDau c 1 can be explained by different extraction conditions, the potential loss of isoallergens/variants during purification, and the use of a different carrot batch. After heating, seven isoallergens/variants were still detectable. At pH 3, five isoallergens/variants could be verified before heating and two afterward (Table 2). Although mass spectroscopy is useful to determine the composition of a protein mixture, it is not possible to make a statement about the quantity of each protein detected. Attempts to separate Dau c 1 isoallergens present in nDau c 1 by HPLC using a reversed phase C8 column in order to quantify them were not successful (data not shown).

MRAs were performed after 0, 10, 20, and 40 min at 95 °C to test the allergenic potential of heat-treated carrot extract (Figure 7B). Interestingly, cooked carrot extract still provoked mediator release; however, the EC50 values show that a three- to fivefold higher dose was needed to obtain the same β -hexosaminidase level as in the untreated sample (0 min).

Moreover, we tested by NMR spectroscopy whether the carrot matrix influences the stability of Dau c 1 proteins. [¹H, ¹⁵N] HSQC spectra of purified rDau c 1.0105 and rDau c 1.0201 at 95 °C and pH 7 showed that they can be used as examples for a stable or an unstable protein, respectively (Figure 2). Similarly,

[¹H, ¹⁵N] HSQC spectra of the ¹⁵N-labeled proteins were now recorded in the presence of unlabeled, dissolved carrot powder. With this approach, we can exclusively observe the structural state of the labeled rDau c 1 proteins in their natural environment, that is, with all other (unlabeled and thus invisible) proteins present in the carrot matrix.

The [¹H, ¹⁵N] HSQC spectrum of Dau c 1.0105 before heat treatment (Figure 7C, black spectrum) is very similar to the spectrum of purified Dau c 1.0105 in 10 mM Na-phosphate buffer, pH 7 (Figure 2A). After 20 min at 95 °C, signals indicating the presence of a folded protein were still clearly visible (Figure 7C, red spectrum). However, new signals in the ¹H-range of 8 ppm appeared, which are typical for an unfolded protein. After 40 min at 95 °C, the protein was completely precipitated since no signals were detectable anymore. Precipitation was confirmed by SDS-PAGE analysis of the precipitate (data not shown). Dau c 1.0201 (Figure 7D) was also folded before heat treatment (black spectrum). After 20 min at 95 °C, most of the protein was unfolded (red spectrum), and after 40 min at 95 °C, the protein was completely unfolded. Unlike Dau c 1.0105, however, it was not precipitated, as indicated by the increase of signals around 8 ppm in the ¹H range (blue spectrum). Thus, the presence of the carrot matrix contributes to heat denaturation of Dau c 1 proteins. However, putative linear epitopes of the unfolded protein could still induce mediator release.

3. Conclusions

In this work, we could show that, depending on the pH conditions, different Dau c 1 isoallergens are intrinsically heat-resistant or able to restore their natural conformation. Obviously, incubation at pH 3 and room temperature, which mimics the conditions in the stomach, does not result in denaturation or hydrolyzation (Figure 4), and mediator release could still be observed (Figure 6). Additionally, even after unfolding of Dau c 1.0201 at pH 7 (Figure 1) or of Dau c 1.0301 at pH 3 (Figure 4),

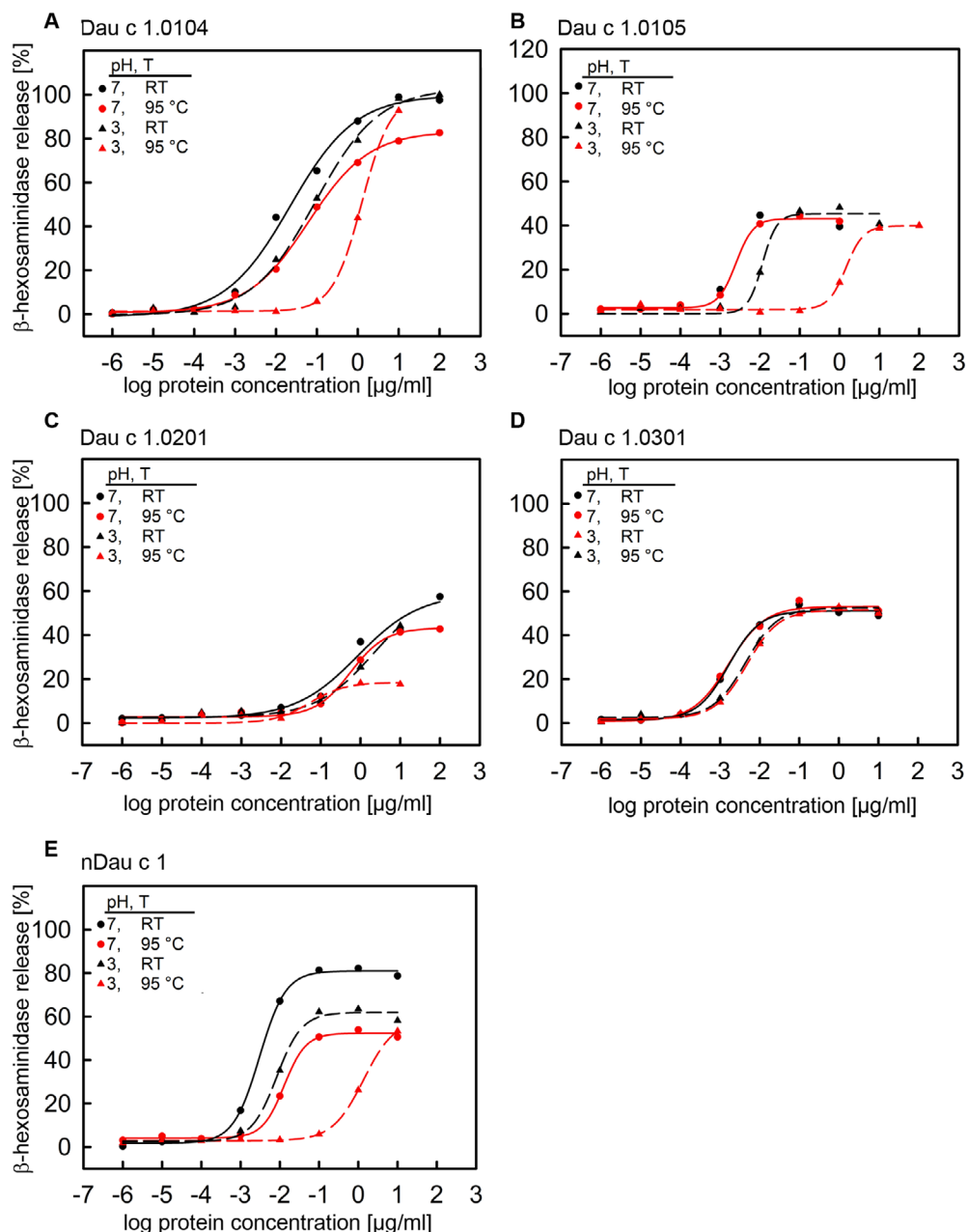


Figure 6. Allergenicity of pH- and heat-treated Dau c 1. MRAs of Dau c 1.0104 (A), Dau c 1.0105 (B), Dau c 1.0201 (C), Dau c 1.0301 (D), and nDau c 1 (E) at pH 7 (circles) and pH 3 (triangles) after 30 min incubation at room temperature (RT) (black) or 95 °C (red). Regression curves were performed with the 4-parameter logistic model (sigma plot, version 14). No curve could be fitted to the data points of Dau c 1.0105 at pH 7, RT (black circles) (B) with this model.

mediator release could be triggered (Figure 6). Our results imply that sequential epitopes might be present which cannot be destroyed upon heat denaturing even at low pH. Although the presence of the food matrix reduced Dau c 1 heat stability, a significant amount of soluble Dau c 1 remained, which clearly induced allergenic activity in MRAs (Figure 7).

Hence, it is not safe to assume that after processing of carrots, IgE-mediated allergic reactions can be totally excluded, suggesting that carrots should be completely avoided by allergic persons.

4. Experimental Section

Cloning, Gene Expression, and Protein Purification: The codon usage adapted rDau c 1.0105 gene (Uniprot: O04298) (Genscript, Piscataway, NJ, USA) and the genes coding for rDau c 1.0104 (Uniprot: O04298),^[3] rDau c 1.0201 (Uniprot: Q8SAE7),^[3] and rDau c 1.0301 (Uniprot: D9ZHN9)^[21] were cloned into the vector pET-11a without a tag (Merck (Novagen), Darmstadt, Germany). (n)Dau c 1 was extracted from carrot roots, as described previously, with minor modifications.^[22] Briefly, 800 g carrot roots were peeled and blended in a mixer in 500 mL 10 mM Na-phosphate, pH

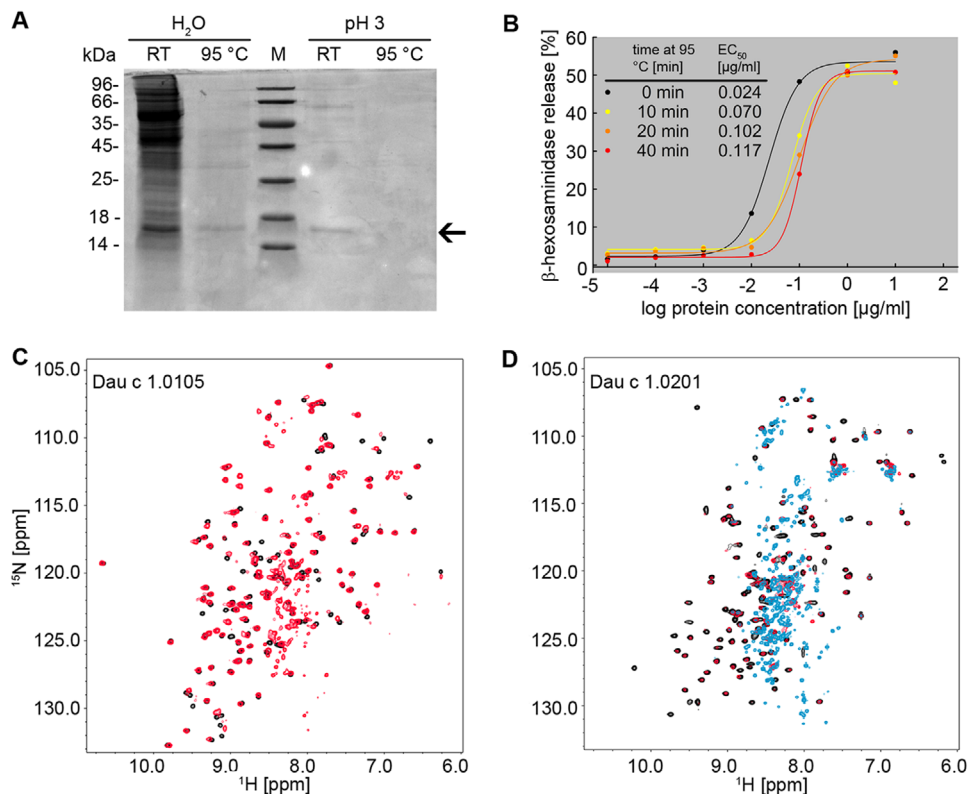


Figure 7. Dau c 1 stability in the presence of carrot matrix. A) SDS-PAGE (19% gel) of carrot extract (30 mg mL⁻¹) in water before and after heating for 30 min at 95 °C. M, molecular weight standard (Biorad, low range); RT, room temperature. B) MRA of carrot extract before and after incubation for 10, 20, and 40 min at 95 °C as indicated. Regression curves were performed with the 4-parameter logistic model (sigma plot, version 14). [1H, 15N] HSQC spectra of Dau c 1.0105 (C) and Dau c 1.0201 (D) were recorded in the presence of carrot matrix before (black) and after 20 min (red) or 40 min (light blue) incubation at 95 °C.

7.0, 4 mM DTT, 0.1% ascorbic acid, and 1 tablet/l EDTA-free protease inhibitor (Roche, Mannheim, Germany), followed by filtration through a linen cloth and centrifugation (19 000 × g, 1 h, 4 °C). The supernatant was used for protein purification.

Gene expression and cell lysis was performed as described previously.^[23] After extraction of nDau c 1 from carrots, (NH₄)₂SO₄ was added to the supernatant to 40% saturation. Bacterial cells expressing rDau c 1.0301 or rDau c 1.0104 were lysed and (NH₄)₂SO₄ was added to 40% (rDau c 1.0301) or 60% (rDau c 1.0104) saturation. The solutions were centrifuged at 4 °C and 10 960 × g. (NH₄)₂SO₄ was added to the supernatants until saturation was achieved. After centrifugation at 4 °C and 10 960 × g, the pellet was resuspended in 10 mM Na-phosphate, pH 7.0, and dialyzed against the same buffer (MWCO 3500 Da) overnight at 4 °C.

rDau c 1.0105 and rDau c 1.0201 were isolated from bacterial inclusion bodies as described for Bet v 1 m.^[24] However, 10 mM Na-phosphate, pH 7.0, with a stepwise reduced urea concentration was used for refolding. After centrifugation (10 960 × g, 30 min, 4 °C), (NH₄)₂SO₄ was added to all Dau c 1 samples to a final concentration of 1 M before loading them on a 5 mL column (Octylsepharose 4 Fast Flow; GE Healthcare, Munich, Germany) equilibrated with 10 mM Na-phosphate, pH 7.0, 1 M (NH₄)₂SO₄. Elution was carried out by either using a gradient from 0 to 60% elution buffer (10 mM Na-phosphate, pH 7) followed by a step with 100% elution buffer (rDau c 1.0104 and rDau c 1.0301) or by using steps with 50% and 100% elution buffer (rDau c 1.0105 and rDau c 1.0201). nDau c 1 was eluted with 100% elution buffer to avoid any loss of isoallergens.

Dau c 1-containing fractions were dialyzed against 10 mM Na-phosphate, pH 7.0, 300 mM NaCl and concentrated in Vivaspin

concentrators (MWCO 5000 Da; Sartorius Stedim Biotech, Göttingen, Germany) to a final volume of ≈2 mL. SEC was performed with all Dau c 1 proteins as described previously.^[12]

Fractions containing pure Dau c 1 were shock frozen and stored at 80 °C. Protein concentration was determined via the A₂₈₀ using the molar extinction coefficients derived from the EXPASY Server (Table S1, Supporting Information).^[25] For nDau c 1, the molecular weight and extinction coefficient for Dau c 1.01 variants were used.

Mass Spectrometry: The identity of purified nDau c 1 and the presence of nDau c 1 isoallergens in heat- and pH-treated carrot root extracts were confirmed using nano ultra-performance liquid chromatography nano electrospray ionization mass spectrometry. Heat- and pH-treatment of carrot extracts were performed by adding water to 150 g of peeled, diced carrots to a final volume of 300 mL, and shredding in a mixer. 1 mL of the suspension was then mixed with 1 mL of water (H₂O-extract) or with a stock solution of Na-citrate to obtain a final concentration of 10 mM Na-citrate, pH 3. The samples were incubated at room temperature or 95 °C for 30 min followed by centrifugation at 25 °C at 16 100 g for 10 min. 30 µL of the supernatant was mixed with 10 µL of 4 × SDS sample buffer (Roti-Load1, Roth), and 25 µL was loaded on 19% gels. 16 kDa bands were excised for in-gel digestion.^[26] Especially, the identification and manual verification of sequence variants were done as described in this study. Differing from this, a UniProt database restricted to unreviewed entries of green plants (as of 2019.09) was used for MS/MS database search.

pH and Heat Treatment of Dau c 1: Dau c 1 samples were dialyzed (MWCO 3500 Da) against ultrapure water (18.2 MΩ) overnight at 4 °C. Subsequently, the desired pH was adjusted by 100 mM Na-citrate (pH 2, pH 3) or Na-acetate (pH 4) stock solutions to a final concentration of

Table 2. Summary on MS data of carrot extract.

| Sample | Acc. Nr. | Description | S | P | SC | PE | F | FE | |
|---------------------|------------------------|--------------|--------------|-------|------|------|-----|-----|---|
| RT/H ₂ O | CAB03715 | Dau c 1.0103 | 54148 | 16 | 94.8 | 1.9 | 612 | 6.0 | |
| | CAB06416 | Dau c 1.0105 | 35177 | 14 | 90.9 | 2.2 | 484 | 7.0 | |
| | A0A164SJC3 | DAUCS | 34379 | 3 | 75.8 | 1.5 | 129 | 6.0 | |
| | A0A175YPA2 | DAUCS | 33567 | 16 | 94.8 | 1.9 | 513 | 7.2 | |
| | A0A161 × 1M2 | DAUCS | 21792 | 12 | 87.1 | 1.7 | 327 | 7.9 | |
| | Q8SAE7 | Dau c 1.0201 | 18805 | 15 | 64.9 | 1.8 | 364 | 7.5 | |
| | Q40795 | PETCR | 5760 | 6 | 39.4 | 10.4 | 100 | 8.4 | |
| | A0A175YQC3 | DAUCS | 3067 | 4 | 26.8 | 0.8 | 66 | 6.8 | |
| | O81640 | PIMBR | 2841 | 7 | 31.2 | 4.7 | 108 | 5.9 | |
| | 95 °C/H ₂ O | CAB03715 | Dau c 1.0103 | 19448 | 18 | 94.8 | 2 | 464 | 9 |
| CAB06416 | | Dau c 1.0105 | 16476 | 12 | 65.6 | 2 | 283 | 6 | |
| A0A175YPA2 | | DAUCS | 12626 | 11 | 83.8 | 3 | 242 | 8 | |
| A0A161 × 1M2 | | DAUCS | 9071 | 7 | 74.8 | 1 | 135 | 9 | |
| A0A164SJC3 | | DAUCS | 7476 | 2 | 45.2 | 2 | 52 | 6 | |
| Q8SAE7 | | Dau c 1.0201 | 5402 | 12 | 59.1 | 3 | 147 | 8 | |
| O81640 | | PIMBR | 872 | 3 | 11.0 | 2 | 39 | 9 | |
| RT/pH 3 | | CAB03715 | Dau c 1.0103 | 20412 | 17 | 94.8 | 2 | 437 | 7 |
| | | CAB06416 | Dau c 1.0105 | 19620 | 14 | 90.9 | 2 | 345 | 7 |
| | | A0A175YPA2 | DAUCS | 18614 | 13 | 90.9 | 2 | 320 | 7 |
| | Q8SAE7 | Dau c 1.0201 | 14157 | 16 | 93.5 | 4 | 275 | 7 | |
| | A0A161 × 1M2 | DAUCS | 10224 | 7 | 45.8 | 1 | 159 | 7 | |
| | A0A164SJC3 | DAUCS | 548 | 1 | 24.2 | 1 | 16 | 8 | |
| 95 °C/pH 3 | CAB03715 | Dau c 1.0103 | 2291 | 9 | 70.8 | 2 | 105 | 9 | |
| | Q8SAE7 | Dau c 1.0201 | 277 | 2 | 18.2 | 2 | 13 | 10 | |

Supernatant of carrot extract in water (H₂O) or 10 mM Na-citrate, pH 3 (pH 3) after incubation for 30 min at room temperature or 95 °C and centrifugation for 10 min at room temperature, 16 100 g. The supernatant was loaded onto a 19% SDS gel and the 16 kDa band was excised and analyzed by LC-MS^E. S, PLGS protein score; SC, sequence coverage [%]; PE, mean peptide mass error [ppm]; P, number of tryptic peptides identified; F, number of fragments detected; FE, mean fragment mass error [ppm].

10 mM. For pH 5, 6, and 7, aliquots were directly dialyzed against the desired buffer (pH 5:10 mM Na-acetate, pH 6 and 7:10 mM Na-phosphate). For heat-treatment, the pH of the samples was adjusted, followed by incubation at 95 °C for the time indicated, recooling to room temperature and centrifugation at 25 °C, 16 100 × g for 10 min.

CD Spectroscopy: CD spectra were recorded as described previously,^[12] but with 20 scans. For temperature scans, 2.5 μm protein in 10 μm Na-phosphate, pH 7.0, in a 1 cm quartz cuvette was used. A step-scan procedure was applied, during which samples were heated at a rate of 1 °C min⁻¹ from 25 to 95 °C and cooled back to 25 °C with the same rate. Temperature was held ±0.03 °C for 5 s before measurement. Signals at a wavelength of 217 nm were monitored with a step resolution of 1 °C.

Size Exclusion Chromatography: SEC was carried out as described previously.^[12] Dau c 1.0104 (1 mg mL⁻¹) was either incubated in 10 mM Na-phosphate, pH 7.0, or 10 mM Na-citrate, pH 3 for 30 min at room temperature or 95 °C and dialyzed against 10 mM Na-phosphate, pH 7. Subsequently 100 μg was loaded onto a column equilibrated with 10 mM Na-phosphate, pH 7, and 300 mM NaCl. Dau c 1.0401 at pH 3 was directly loaded onto a column equilibrated with 5 mM Na-citrate, pH 3, and 300 mM NaCl. Blue dextran was used to determine the void volume.

NMR-Spectroscopy: [¹H, ¹⁵N] HSQC spectra of 100–200 μm uniformly ¹⁵N-labeled Dau c 1.0105 and Dau c 1.0201 in 10 mM Na-phosphate, 10% D₂O, with or without 20 mg mL⁻¹ of resuspended carrot powder were recorded on a Bruker Avance 600 MHz spectrometer at 25 °C. For preparation of carrot powder, carrot roots were diced, shock frozen, and freeze-dried (Alpha 2–4 LDplus freeze dryer, Christ). Freeze-dried samples were ground in a mortar at –80 °C. Samples were incubated for

0 min, 20 min, 40 min, or 1 h at 95 °C. All NMR data were processed using in-house software and were visualized with NMR ViewJ (OneMoon Scientific, Inc.).

Mediator Release Assays: For the preparation of carrot extract, 65 mg mL⁻¹ carrot powder was stirred for 30 min in 50 mM Na-phosphate, pH 8.0, at room temperature. The carrot extract was heated to 95 °C for 10, 20, or 40 min, followed by centrifugation at 16 100 × g for 10 min (25 °C). The total protein content of the supernatant was measured at 280 nm. The different Dau c 1 allergens were prepared as described above in the section “pH and heat treatment of Dau c 1.” Briefly, 1 mg mL⁻¹ stock solutions in 10 mM Na-P, pH 7, or 10 mM citrate, pH 3 were prepared and incubated at room temperature or 95 °C for 30 min. After recooling and centrifugation, no precipitation or change in protein concentration was observed.

MRA were essentially performed as described previously.^[27] At least 100-fold dilutions with Tyrodes’s buffer guaranteed a physiological pH 7.4 also for the samples, which were previously incubated at pH 3, a prerequisite for IgE-binding. A pH around 7 is also present in the small intestine, where allergic reactions of food allergens take place. Mediator release was quantified by photometrically measuring the β-hexosaminidase activity in culture supernatants with 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich, Taufkirchen, Germany) as a substrate. Total β-hexosaminidase content was determined by lysing the cells with 1% Triton X-100. Allergen-specific release was expressed as percentage of total release after correction for spontaneous release (cells incubated with serum, but without allergen). Sera from carrot allergic patients were obtained after patient written consent and approval of the ethics committee (Faculty of Medicine, University of Erlangen-Nuremberg, No. 3494).

nanoDSF: For thermal unfolding experiments, the proteins were diluted to a final concentration of 1 mg mL⁻¹. 10 µL of sample was loaded into UV capillaries (NanoTemper Technologies, Munich, Germany) and sealed at the ends to prevent evaporation at elevated temperatures. Triplicates were generated for each condition. Experiments were carried out on a Prometheus NT.48. The temperature gradient was set to 2 °C min⁻¹ from 20 to 110 °C and subsequently back to 20 °C. Protein unfolding was measured by detecting the temperature-dependent change in tyrosine fluorescence at emission wavelengths of 330 and 350 nm. Moreover, aggregation of the protein during heating was monitored by changes in backreflection.

Melting temperatures were determined using the software PR.ThermControl (NanoTemper Technologies, Munich, Germany) by detecting the maximum of the first derivative of the fluorescence ratios (F350/F330).

Supporting Information

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

Acknowledgements

The authors thank Ramona Heißmann, Andrea Hager, and Ulrike Persau (University of Bayreuth) for excellent assistance, Stefan Knauer for help with protein analyses (University of Bayreuth), Anna Maria Engin (Paul-Ehrlich-Institute) for LC-MS analysis, and Maria Strobl (Paul-Ehrlich-Institute) for support with nanoDSF analysis. The support of Marco Schwieder (NanoTemper) and Franziska Toppel (NanoTemper) in using the nanoDSF software is greatly acknowledged. The authors thank the University of Bayreuth for financial support.

Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

B.M.W. and T.J. wrote the manuscript and designed experiments. T.J. carried out the expression and purification experiments, SEC, CD, and NMR analyses and evaluated the data together with B.M.W. L.V. performed mediator release assays and analyzed and evaluated the data. A.R. designed, analyzed, and interpreted the mass spectrometry data and wrote the corresponding part of the manuscript. A.W. and T.J. performed and evaluated the nanoDSF experiments. C.K. performed CD spectrometry of Dau c 1.0201 and Dau c 1.0105. V.M. contributed to the analysis and interpretation of data. All authors contributed in preparing the manuscript.

Keywords

allergenicity, circular dichroism, mass spectrometry, nano differential scanning fluorimetry, NMR, PR-10 allergen Dau c 1, protein stability

Received: April 6, 2020

Revised: June 18, 2020

Published online:

- [1] B. K. Ballmer-Weber, B. Wüthrich, A. Wangorsch, K. Fötisch, F. Altmann, S. Vieths, *J. Allergy Clin. Immunol.* **2001**, *108*, 301.
- [2] A. Pomés, J. M. Davies, G. Gadermaier, C. Hilger, T. Holzhauser, J. Lidholm, A. L. Lopata, G. A. Mueller, A. Nandy, C. Radauer, S. K. Chan, U. Jappe, J. Kleine-Tebbe, W. R. Thomas, M. D. Chapman, M. van Hage, R. van Ree, S. Vieths, M. Raulf, R. E. Goodman, WHO IUIS Allergen Nomenclature Sub-Committee, *Mol. Immunol.* **2018**, *100*, 3.
- [3] B. K. Ballmer-Weber, A. Wangorsch, B. Bohle, S. Kaul, T. Kündig, K. Fötisch, R. V. Ree, S. Vieths, *Clin. Exp. Allergy* **2005**, *35*, 970.
- [4] B. K. Ballmer-Weber, A. Hoffmann, B. Wüthrich, D. Lüttkopf, C. Pompei, A. Wangorsch, M. Kästner, S. Vieths, *Allergy* **2002**, *57*, 228.
- [5] B. Bohle, B. Zwölfer, A. Heratizadeh, B. Jahn-Schmid, Y. D. Antonia, M. Alter, W. Keller, L. Zuidmeer, R. van Ree, T. Werfel, C. Ebner, *J. Allergy Clin. Immunol.* **2006**, *118*, 242.
- [6] P. Gruber, S. Vieths, A. Wangorsch, J. Nerkamp, T. Hofmann, *J. Agric. Food Chem.* **2004**, *52*, 4002.
- [7] S. Vieths, A. Jankiewicz, H. Aulepp, D. Hausteiner, *Arb. Aus. Dem. Paul-Ehrlich-Inst. Bundesamt Sera Impfstoffe Zu Frankf. AM* **1997**, 250.
- [8] K. Thomas, C. Herouet-Guichenev, G. Ladics, G. Bannon, A. Cockburn, R. Crevel, J. Fitzpatrick, C. Mills, L. Privalle, S. Vieths, *Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc.* **2007**, *45*, 1116.
- [9] M. Amigo-Benavent, J. M. Silván, F. J. Moreno, M. Villamiel, M. D. Del Castillo, *J. Agric. Food Chem.* **2008**, *56*, 6498.
- [10] L. J. Masthoff, R. Hoff, K. C. M. Verhoeckx, H. van Os-Medendorp, A. Michelsen-Huisman, J. L. Baumert, S. G. Pasmans, Y. Meijer, A. C. Knulst, *Allergy* **2013**, *68*, 983.
- [11] E. N. C. Mills, A. I. Sancho, N. M. Rigby, J. A. Jenkins, A. R. Mackie, *Mol. Nutr. Food Res.* **2009**, *53*, 963.
- [12] C. Rib-Schmidt, P. Riedl, V. Meisinger, L. Schwaben, T. Schulenburg, A. Reutter, D. Schiller, C. Seutter von Loetzen, P. Rösch, *Mol. Nutr. Food Res.* **2018**, *62*, 1700886.
- [13] M. A. Bollen, *PhD Thesis*, Wageningen University **2009**.
- [14] I. Aguiló-Aguayo, N. Brunton, D. K. Rai, E. Balagueró, M. B. Hossain, J. Valverde, *Food Chem.* **2014**, *152*, 370.
- [15] T. Jacob, B. M. Wöhr, unpublished.
- [16] I. Jelesarov, A. Karshikoff, *Methods Mol. Biol.* **2009**, *490*, 227.
- [17] S. Kumar, R. Nussinov, *J. Mol. Biol.* **1999**, *293*, 1241.
- [18] F. Pucci, M. Rooman, *Philos. Transact. A Math. Phys. Eng. Sci.* **2016**, *374*, 20160141.
- [19] M. Koziolok, F. Schneider, M. Grimm, C. Modeß, A. Seekamp, T. Roustom, W. Siegmund, W. Weitschies, *J. Controlled Release* **2015**, *220*, 71.
- [20] G. A. Bannon, *Curr. Allergy Asthma Rep.* **2004**, *4*, 43.
- [21] A. Wangorsch, D. Weigand, S. Peters, V. Mahler, K. Fötisch, A. Reuter, J. Imani, A. M. Dewitt, K.-H. Kogel, J. Lidholm, S. Vieths, S. Scheurer, *Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol.* **2012**, *42*, 156.
- [22] M. A. Bollen, A. Garcia, J. H. G. Cordewener, H. J. Wichers, J. P. F. G. Helsper, H. F. J. Savelkoul, M. A. J. S. van Boekel, *Mol. Nutr. Food Res.* **2007**, *51*, 1527.
- [23] C. Seutter von Loetzen, T. Hoffmann, M. J. Hartl, K. Schweimer, W. Schwab, P. Rösch, O. Hartl-Spiegelhauer, *Biochem. J.* **2014**, *457*, 379.
- [24] C. Seutter von Loetzen, T. Jacob, O. Hartl-Spiegelhauer, L. Vogel, D. Schiller, C. Spörlein-Güttler, R. Schobert, S. Vieths, M. J. Hartl, P. Rösch, *PLoS One* **2015**, *10*, e0128677.
- [25] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M. R. Wilkins, R. D. Appel, A. Bairoch, in *Proteomics Protoc. Handb* (Ed: J. M. Walker), Humana Press, Totowa, NJ **2005**, p. 571.
- [26] J. Spiric, A. M. Engin, M. Karas, A. Reuter, *PLoS One* **2015**, *10*, e0142404.
- [27] L. Vogel, D. Lüttkopf, L. Hatahet, D. Hausteiner, S. Vieths, *Allergy* **2005**, *60*, 1021.

Supplementary Figures

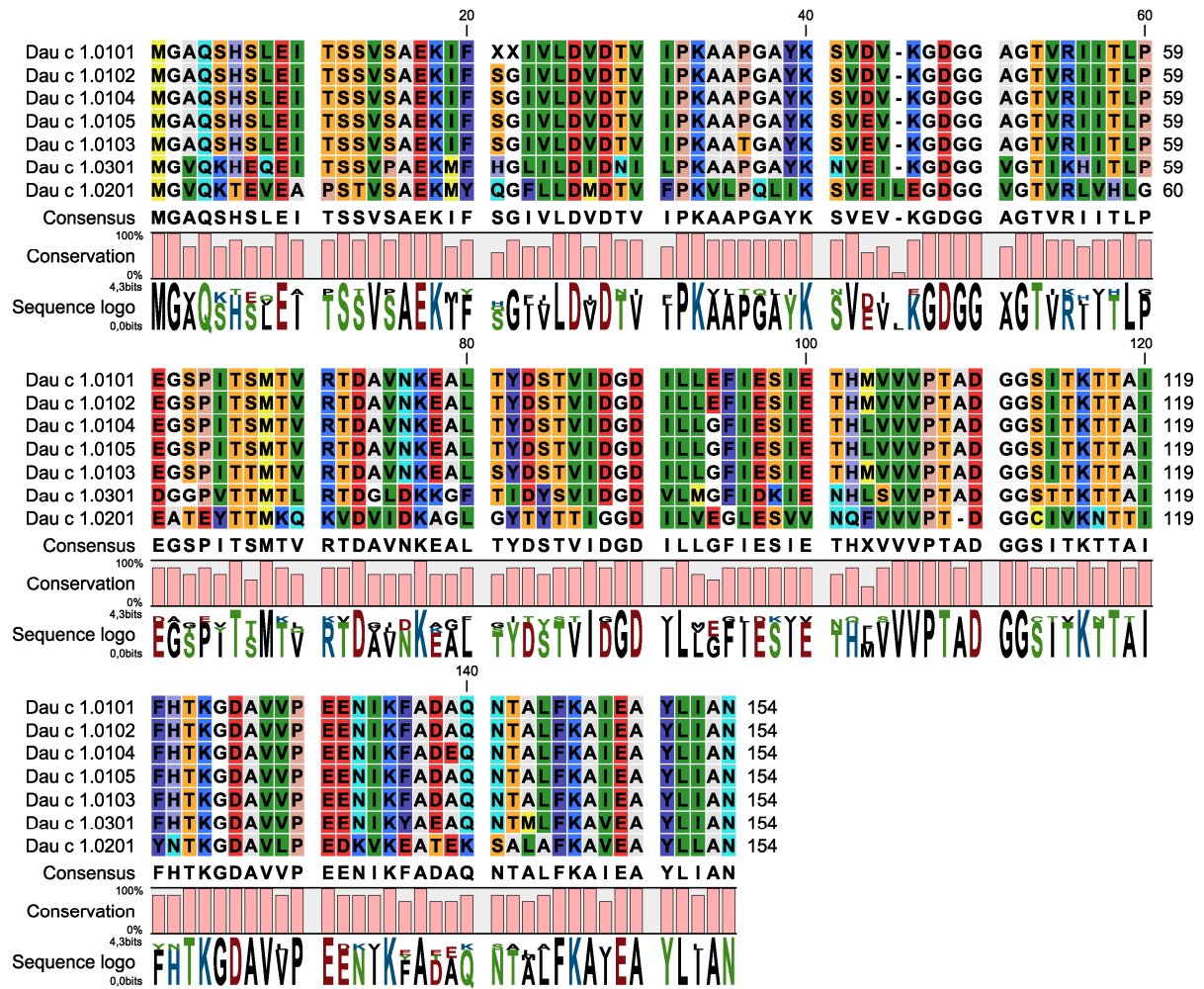


Figure S1: Alignment of all Dau c 1 isoallergens and variants listed in the IUIS data bank.

The alignment was performed using the program CLC Protein Workbench 5.

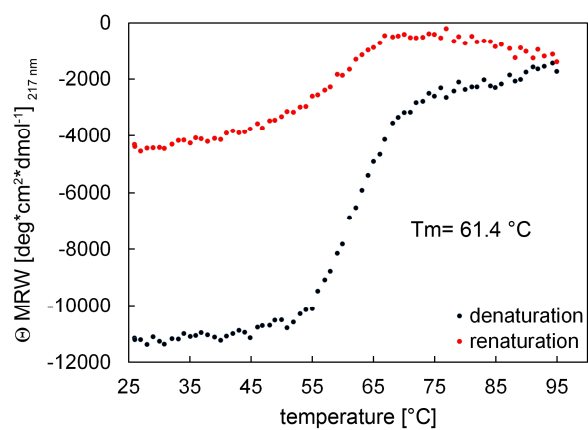


Figure S2. Stability of Dau c 1.0105 in Tris-HCl buffer. CD denaturation (black) and renaturation (red) curve of Dau c 1.0105 in 20 mM Tris-HCl buffer, adjusted to pH 7 at 25 °C.

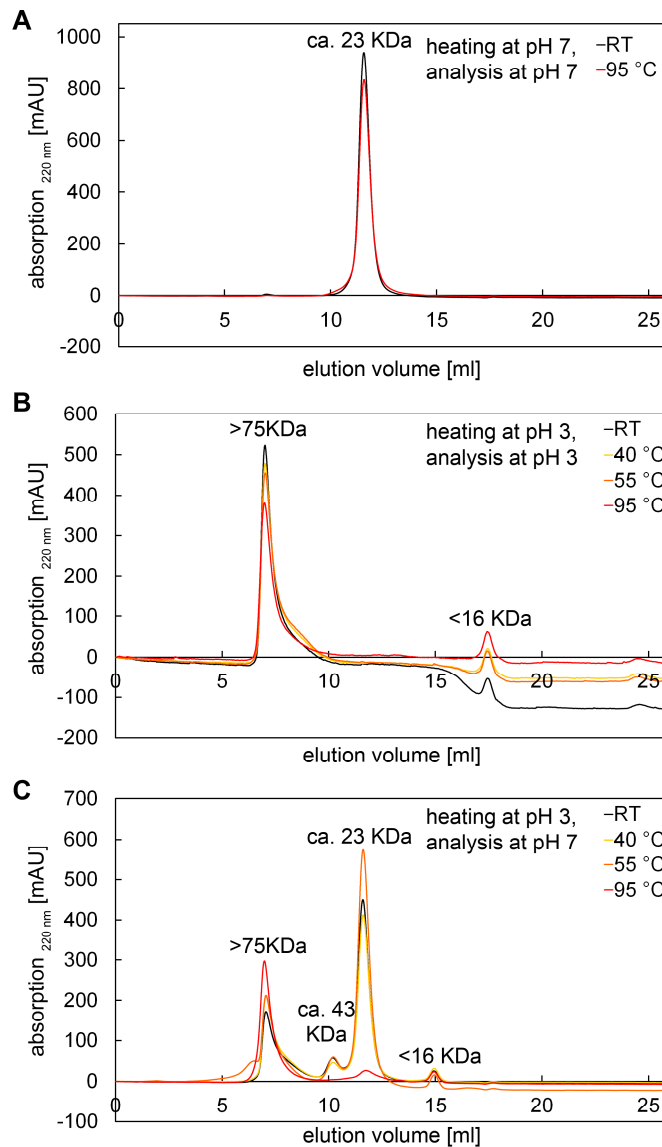


Figure S3. Oligomerisation of pH and heat-treated Dau c 1.0104. (A): SEC chromatograms of Dau c 1.0104 after 30 min at RT (black line) or 95 °C (red line) at pH 7. SEC run was performed at pH 7. **(B) and (C):** SEC chromatogramms of Dau c 1.0104 after 30 min at RT (black line), 40 °C (yellow line), 55 °C (orange line) and 95 °C (red line). Samples were either directly loaded and run at pH 3 **(B)** or dialysed against 10 mM Na-phosphate, pH 7 and run at pH 7 **(C)**.

Supplementary Tables

Table S1: Biophysical data of Dau c 1 variants/isoallergens

| Dau c 1 isoallergen/variant | ϵ_{280} [$M^{-1} cm^{-1}$] | Theoretical pI | Molecular weight [Da] |
|--------------------------------|---------------------------------------|----------------|-----------------------|
| Dau c 1.0104 | 4470 | 4.55 | 16093 |
| Dau c 1.0105 | 4470 | 4.63 | 16049 |
| Dau c 1.0201 | 8940 | 4.64 | 16517 |
| Dau c 1.0301 | 5960 | 5.11 | 16486 |

Table S2: Overview of experiments on Dau c 1 stability at different conditions.

| Condition | Experiment/investigated property | | Figure/Table | rDau c 1.0104 | rDau c 1.0105 | rDau c 1.0201 | rDau c 1.0301 | nDau c 1 | Carrot extract | |
|----------------|--|---------------------------------|-----------------------|-----------------------------------|--------------------|--------------------|---------------|----------------|----------------|--|
| pH 7 | T _m (°C) | CD | Fig. 1 | 59.9 °C | 57.7 °C | 56.5 °C | 57.2 °C | 56.3 °C | | |
| | | nanoDSF | Fig. 1 | 59.1 °C | 57.4 °C | 59.0 °C | 57.7 °C | 55.4 °C | | |
| | Refolding | | Fig. 1 | yes | yes | no | partial | partial | | |
| | Mediator release | RT | Fig. 6 | yes | yes | yes | yes | yes | | |
| | | 30 min @95 °C | Fig. 6 | yes | yes | yes | yes | yes | | |
| | Oligomeric state | RT | Fig. 5 | Monomer | - | - | - | - | - | |
| | | 30 min @95 °C | Fig. 5 | Monomer | - | - | - | - | - | |
| | NMR | RT | Fig. 2 | - | folded | folded | - | - | - | |
| 1h @95 °C | | Fig. 2 | - | folded | partially unfolded | - | - | - | | |
| pH 3 | T _m (°C) | CD | Fig. 4 | n.d. > 95 °C | n.d. > 95 °C | n.d. > 95 °C | - | n.d. > 95 °C | | |
| | | nanoDSF | Fig. 4 | n.d. > 110 °C | n.d. > 110 °C | n.d. > 110 °C | 80.3 °C | n.d. > 110 °C | | |
| | Refolding | | Fig. 4 | heat resistant | heat resistant | heat resistant | no | heat resistant | | |
| | Mediator release | RT | Fig. 6 | yes | yes | yes | yes | yes | | |
| | | 30 min @95 °C | Fig. 6 | yes | yes | yes | yes | yes | | |
| | Oligomeric state | RT | Fig. 5 | Monomer, Oligomer (>75 kDa) | - | - | - | - | - | |
| 30 min @95 °C | | Fig. 5 | Oligomer (>75 kDa) | - | - | - | - | - | | |
| Carrot extract | NMR | RT | Fig. 7 | - | folded | folded | - | - | | |
| | | 20 min @95 °C | Fig. 7 | - | partially unfolded | partially unfolded | - | - | | |
| | | 40 min @95 °C | Fig. 7 | - | aggregated | unfolded | - | - | | |
| | Soluble isoallergens identified in carrot extract (number) | H ₂ O, RT | Fig. 7, Table 2 | - | - | - | - | 9 | | |
| | | H ₂ O, 30 min @95 °C | Fig. 7, Table 2 | - | - | - | - | 7 | | |
| | | pH 3, RT | Fig. 7, Table 2 | - | - | - | - | 6 | | |
| | | pH 3, 30 min @95 °C | Fig. 7, Table 2 | - | - | - | - | 2 | | |
| | Mediator release of carrot extract | RT | Fig. 7 | - | - | - | - | - | yes | |
| | | 10 min @95 °C | Fig. 7 | - | - | - | - | - | yes | |
| 20 min @95 °C | | Fig. 7 | - | - | - | - | - | yes | | |
| 40 min @95 °C | | Fig. 7 | - | - | - | - | - | yes | | |

RT, room temperature; n.d., not determinable in the temperature range tested for CD (25 °C - 95 °C), or nanoDSF (25 °C - 110 °C);

Table S3: Summary of mass spectrometric fragmentation data of nDau c 1. Red colour highlights peptides which are specific for the protein indicated on the left. If only one specific peptide was detected for an individual protein, an annotated spectrum is shown directly below.

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|-------------------------------|-----------|------|-----|---|-----|----|---|------|
| 100 CAB03715 - Dauc 1.0103 | 1730.857 | 0.9 | 9.6 | GAQSHSLEITSSVSA EK | | 61 | b2b3*b4b4*b4*b5b5*b6b6*b7b7*b7*b8b8*b9b9*b9*b10b10*b11b11*b12b12*b13b13*b14b14*b15b15*b16b16*b17b17*y1y2y3y3*y4y4*y5y6y6*y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y13*y14y14*y15y15*y16 | 5.4 |
| | 1615.9329 | 1.5 | 9.6 | IFSGIVLDVDTVIPK | | 44 | b1b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*b9b9*b10b10*b11b11*b12b12b13b13*b14b14*y1y2y3y4y5y6y6*y7y8y8*y9y10y10*y11y11*y12y12*y13y14 | 3.2 |
| | 1317.673 | -2.1 | 7.8 | SVDVKDGGGAGTV R | | 11 | b2b4*y1y2y4y6y7y8y10y11y12 | 7.8 |
| | 547.3087 | 0.2 | 8.6 | SVDVK | | 7 | b2b3y1y2y3y4y5 | 8.1 |
| | 789.3842 | -0.9 | 8.1 | GDDGAGTVR | | 9 | b2y1y2y3y4y4*y5y6y7 | 10.3 |
| | 1714.9434 | 1.5 | 9.3 | IITLPEGSPITSMTVR | | 54 | b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*b9b9*b10b10*b11b11*b12*b13b13*b14b14*b15b15*b16b16*y1y2y3y3*y4y4*y5y5*y6y6*y7y7*y8y8*y9y9*y10y10*y11*y12y13y13*y14y14*y15*y16 | 7.4 |
| | 4179.0957 | 0.4 | 7.2 | EALTYDSTVIDGDILL EFIESIETHMVVVPT ADGGSITK | | 27 | b18b23*b24*b25b25*b26*b29b29*b30*b31b35*b36b37b37*b39*y18*y23y23*y26*y28y29y30y31y33*y34y34*y35 | 13.9 |
| | 918.5028 | -1.7 | 8.7 | TTAIFHTK | | 18 | b1b2*b3*b6b6*b7y1y2y2*y3y3*y4y4*y5y5*y6*y7y8 | 6.4 |
| | 1170.6024 | 2.0 | 9.2 | GDAVVPEENIK | | 27 | b2b2*b3b3*b4b4*b5b5*b8b10b11b11*y1y2y3y3*y4y4*y5y5*y6y6*y7y8y8*y9 | 6.2 |
| | 1226.6107 | 4.5 | 6.8 | FADAQNTALFK | D/N | 5 | b2b3b4b5b10° | 13.2 |
| | 1225.6228 | 1.4 | 8.8 | FADAQNTALFK | | 36 | b2b3b4b5b5*b5*b6b6*b7b7*b7*b8b8*b8*b9b9*b9*y1y2y3y4y5y6y6*y6*y7y7*y7*y8y8*y8*y9y9*y9*y10*y10° | 4.4 |
| | 977.5329 | 2.8 | 9.0 | AIEAYLIAN | | 25 | b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*y1y1*y2y2*y3y3*y4y4*y5y5*y6*y7 | 5.9 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|-----------|------|-------------|---|-----|--|---|------|
| A0A175YPAZ – DAUCS | 1730.857 | 0.9 | 9.6 | GAQSHSLEITSSVSA EK | | 61 | b2b3*b4b4*b4*b5b5*b6b6*b7b7*b7*b8b8*b9b9*b9*b10b10*b11b11*b12b12*b13b13*b14b14*b15b15*b16b16*b17b17*y1y2y3y3*y4y4*y5y6y6*y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y13*y14y14*y15y15*y16 | 5.4 |
| | 1615.9329 | 1.5 | 9.6 | IFSGIVLDVDTVIPK | | 44 | b1b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*b9b9*b10b10*b11b11*b12b13b13*b14b15*y1y2y3y4y5y6y6*y7y8y8*y9y10y10*y11y11*y12y12*y13y14 | 3.2 |
| | 1331.6887 | -2.0 | 8.3 | SVDVKGDDGAGTIR | | 28 | b2b2*b3b3*b6b7b9b9*y1y2y3y3*y4y4*y5y5*y7y7*y8y8*y9y9*y10y10*y11y12y12*y13 | 7.4 |
| | 547.3087 | 0.2 | 8.6 | SVDVK | | 7 | b2b3y1y2y3y4y5 | 8.1 |
| | 803.3991 | -1.8 | 8.4 | GDGGAGTIR | | 15 | b2b3b3*b9y1y2y3y4y4*y5y5*y6y6*y7y7 | 8.8 |
| | 1732.9149 | 0.0 | 8.6 | TITLPEGSPITMTV R | O/M | 25 | b1b2b2*b3b3*b4b4*b5b6b7b8b8*b12y1y2y3y4y5y6y7y8*y10y11y12*y16 | 6.3 |
| | 1716.9241 | 2.4 | 9.5 | TITLPEGSPITMTV R | | 54 | b1b2b2*b3b3*b4b4*b5b5*b6b7b7*b8b8*b9b10b10*b11b11*b12b13b13*b14b14*b15b15*b16y1y2y3y3*y4y4*y5y5*y6y6*y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y13*y14y14*y15 | 7.1 |
| | 4107.078 | 1.2 | 9.2 | EALTYDSTVIDGDILL GFIESIETHMVVVPT ADGGSITK | | 75 | b1b2*b3b3*b4b4*b5b5*b6b7b8b9b9*b11b13b14b14*b15b15*b16b17b17*b18*b21b24*b25*b27b28b29b30*b32*b35 b37b39b39*y1y2y2*y4y5y6y7y8y9y10y10*y11y12y13y14y17y18y18*y19y19*y20y21y21*y22y23y23*y24y24*y25y26*y29y30y31y33y34y35*y36y37y37*y38 | 11.2 |
| | 918.5028 | -1.7 | 8.7 | TTAIFHTK | | 18 | b1b2*b3*b6b6*b7y1y2y2*y3y3*y4y4*y5y5*y6*y7y8 | 6.4 |
| | 1170.6024 | 2.0 | 9.2 | GDAVVPEENIK | | 27 | b2b2*b3b3*b4b4*b5b5*b8b10b11b11*y1y2y3y3*y4y4*y5y5*y6y6*y6*y7y8y8*y9 | 6.2 |
| 1283.6292 | 2.0 | 9.0 | FADEQNTALFK | | 39 | b2b3b3*b4b4*b5b5*b6b6*b6*b7b7*b7*b8b8*b9b9*b11b11*y1y2y3y4y5y5*y6y6*y6*y7y7*y7*y8y8*y9y9*y9*y10y10*y10 | 6.7 | |
| 977.5329 | 2.8 | 9.0 | AIEAYLIAN | | 25 | b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*y1y1*y2y2*y3y3*y4y4*y5y5*y6*y7 | 5.9 | |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|-----------------------|-----------|-------|-----|---------------------|-----|----|---|------|
| Q8SAF7 - Dau c 1.0201 | 1347.6643 | 0.4 | 7.2 | TEVEAPSTVSAEK | | 17 | b1b2b3b4b4°b5y2°y3y4y4°y6y7y8y9y10y10°y11 | 10.2 |
| | 1804.8094 | -30.7 | 5.9 | MYQGFLDMDTVF PK | | 5 | b4*b12°b13*b15y14° | 17.5 |
| | 1487.773 | 2.0 | 7.3 | SVEILEGDDGGVGT R | | 20 | b2b3b4b5b12b14y3y4y5y6y7y7°y8y8°y9y9°y10y11y12y15 | 8.31 |
| | 1592.7986 | -0.2 | 7.0 | LVHLGEATEYTTMK | | 19 | b3b4b5b6b7b8b9°b10b10°y2y3y4y5y5°y6y6°y10y11y12° | 11.3 |
| | 943.469 | -4.3 | 5.4 | GDAVLPEDK | | 5 | b8°y2°y5y6y7 | 12.6 |
| | 963.5156 | 1.1 | 8.0 | AVEAYLLAN | | 10 | b2b4b5b7b8y1y2*y4y4*y8 | 5.08 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

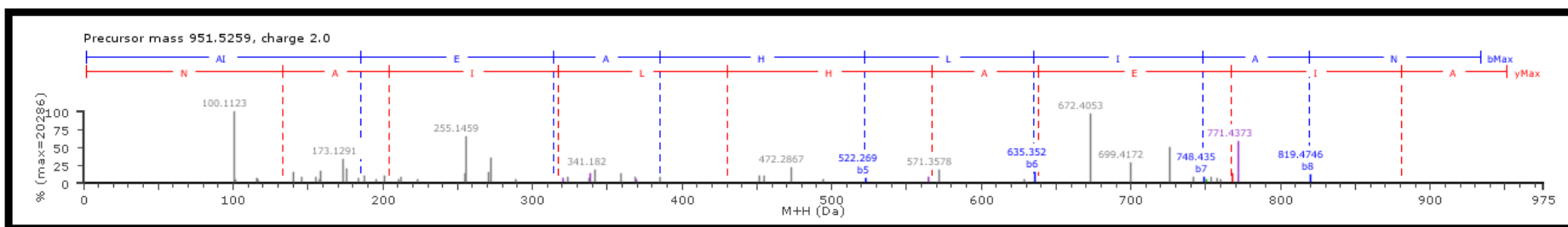
102

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|-----------|------|-----|-------------------------|-----|----|--|-----|
| A0A164SJC3 - DAUCS | 1323.6283 | 0.6 | 8.5 | SEVEATSSVSAEK | | 27 | b2b2°b3b4b4°b5b5°b6b6°b9b12y1y2y2°y3y4y5y6y7y7°y8y9y9°y10y10°y11°y13 | 5.9 |
| | 2036.1458 | -0.2 | 8.5 | ALCLDIDTLLPQVVP GAIK | C/C | 33 | b2b3b4b5b5°b6b7b7°b8b8°b9b9°b10b10°b11b14*b15°y2y3y4y5y6y7y8y8*y9y9*y10y11y12y13y14*y16° | 8.3 |
| | 1431.7356 | 2.1 | 8.8 | SAEILEGDDGGVGT K | | 47 | b2b2°b3b3°b4b4°b5b5°b6b6°b7b8b8°b9b10b10°b11b11°b12b13b14b14°b15°y1y2y3y3°y4y4°y5y5°y6y6°y7y7°y8y9y9°y10y10°y11y11°y12y12°y13y13°y14 | 6.7 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------------|-----------|------|-----|-------------|-----|----|---|-------|
| 081640 - PIMBR | 803.3991 | -1.8 | 8.4 | GDGGAGTIR | | 15 | b2b3b3°b9y1y2y3y4y4°y5y5°y6y6°y7y7° | 8.826 |
| | 918.5028 | -1.7 | 8.7 | TTAIFHTK | | 18 | b1b2°b3°b6b6°b7y1y2y2°y3y3°y4y4°y5y5°y6°y7y8 | 6.42 |
| | 1283.6292 | 2.0 | 9.0 | FADEQNTALFK | | 39 | b2b3b3°b4b4°b5b5°b6b6°b6°b7b7°b7°b8b8°b9b9°b11b11°y1y2y3y4y5y5°y6y6°y6°y7y7°y7°y8y8°y9y9°y9°y10y10°y10° | 6.694 |
| | 951.5259 | 0.1 | 8.0 | AIEAHLIAN | | 23 | b2b3b3°b4b4°b5b5°b6b6°b7b8y1y1°y2y2°y3y4y5y5°y6y7y7°y8 | 5.976 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]



| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|-----------|------|-----------|----------------------------|-----|--|--|------|
| A0A175YQC3 - DAUCS | 1267.7279 | 1.8 | 9.0 | SEVVIASPVPAAK | | 35 | b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8°b9b9°b11b12b13y1y2y3y4y5y6y7y7°y8y8°y9y9°y10y10°y11y11°y12° | 4.3 |
| | 2036.1454 | -0.3 | 8.9 | GICLDIDTLLPQVLP GAIK | C/C | 38 | b2b3b4b5b6b7b8b8°b9b9°b10b10°b11b12b13b14b17b19y1y2y3y4y5y6y7y8y9y9°y10y11y12y13y14y15y15°y15°y17y19 | 4.8 |
| | 1373.6927 | 1.4 | 8.9 | GAEILEGDDGGAGTV K | | 51 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°b10b10°b11b11°b12b12°b13b13°b14b14°b15b15°y1y2y3y3°y4y4°y5y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y14y14°y15 | 5.4 |
| | 883.4524 | 0.5 | 7.6 | SVSTFNTK | | 9 | y1y2y3y3°y4y5y5°y6y6° | 11.8 |
| | 2418.1946 | 0.4 | 8.2 | GDAVVPEENINFAN EQNGIIFK | | 25 | b2b3b3°b5b10°b11b17°b18°y1y5y6y6°y7y8°y9y9°y10y12y12°y16y17y17°y18y19°y20 | 10.0 |
| | 2419.1816 | 1.7 | 8.4 | GDAVVPEENINFAN EQNGIIFK | D/N | 35 | b2b3b3°b5b8b10b11b12b18b19°b20y1y5y6y7y7°y8y8°y10y11y11°y11°y12y12°y12°y13y14y16y17y17°y18y18°y19y20°y21 | 12.4 |
| | 2419.1843 | 2.8 | 7.7 | GDAVVPEENINFAN EQNGIIFK | D/N | 13 | b3b19°b20b21°y5y7y12y14°y17y17°y18y18°y19° | 7.4 |
| 977.5329 | 2.8 | 9.0 | AIEAYLIAN | | 25 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°y1y1°y2y2°y3y3°y4y4°y5y5°y6°y7 | 5.9 | |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

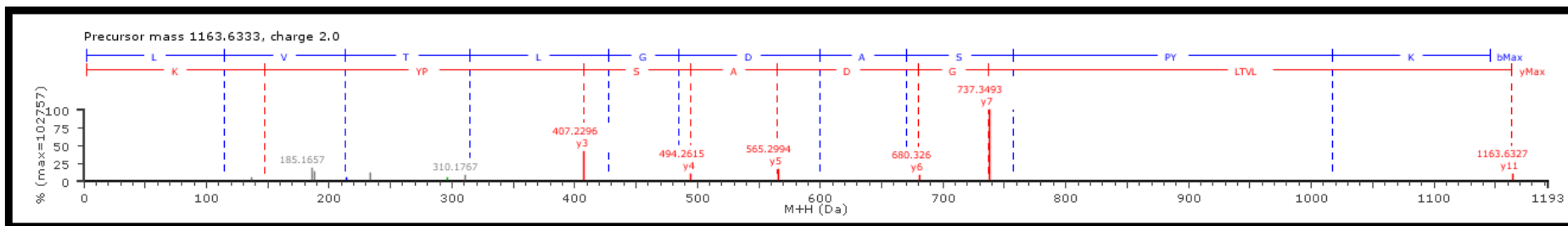
104

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|-----------|------|-----|-------------------------|-----|----|--|------|
| A0A161X1M2 - DAUCS | 1337.643 | -0.1 | 8.4 | TEAEVTSSVSAEK | | 39 | b1b2b2°b3b3°b4b4°b5b5°b7b7°b8b8°b9b9°b10b10°b11°b12y1y2y3y3°y4y5y5°y6y7y7°y8°y9y9°y10y10°y11y11°y12y12°y13 | 5.7 |
| | 2050.1602 | -0.7 | 7.2 | ALCLDIDTLLPQVLP GAIK | C/C | 16 | b2b3b5b6b8b8°b9b9°b10b10°b11°b12b13b14°b15°b17 | 10.1 |
| | 1435.6921 | 0.7 | 8.9 | SSETLEGDDGGVGT K | | 47 | b2b2°b3b3°b4b4°b5b5°b6b6°b7°b8b8°b9b9°b10b10°b11°b12b12°b13b13°b14b14°b15b15°y1y2y3y4y4°y5y7y7°y8y8°y9y9°y10y10°y11y12y12°y13y13°y14y14° | 3.9 |
| | 1183.6482 | 1.0 | 8.7 | LVHLGDASPFK | | 17 | b1b3b4b5b6b6°b7b8b8°b10y3y5y6y7y8y9y10 | 6.6 |
| | 911.4821 | -1.2 | 7.4 | STITFNTK | | 5 | y2y2°y3y5y6 | 3.2 |
| | 1170.6024 | 2.0 | 9.2 | GDAVVPEENIK | | 27 | b2b2°b3b3°b4b4°b5b5°b8b10b11b11°y1y2y3y3°y4y4°y5y5°y6y6°y7y8y8°y9 | 6.2 |
| | 1422.7606 | -1.5 | 8.2 | AIFQAVEAYLIAN | | 25 | b2b3b4b5b5°b6b7b7°b8b9b10b11b12y1y1°y2y2°y3y3°y4y6°y7y7°y8y12 | 12.0 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------------|-----------|------|-----|---|-----|----|---|------|
| Q40795 - PETCR | 2050.1602 | -0.7 | 7.2 | ALCLDIDTLLPQVLP GAIK | C/C | 16 | b2b3b5b6b8b8 ^b b9b9 ^b b10b10 ^b b11 ^b b12b13b14 ^b b15 ^b b17 | 10.1 |
| | 1163.6333 | 2.2 | 8.0 | LVTLGDA SPYK | | 27 | b1b2b3b3 ^b b4b4 ^b b5b5 ^b b6b6 ^b b7b7 ^b b8b9 ^b b10b10 ^y y1y3y4y5y5 ^y y6y6 ^y y7y7 ^y y8 ^y y11 | 7.5 |
| | 1170.6024 | 2.0 | 9.2 | GDAVVPEENIK | | 27 | b2b2 ^b b3b3 ^b b4b4 ^b b5b5 ^b b8b10b11b11 ^y y1y2y3y3 ^y y4y4 ^y y5y5 ^y y6y6 ^y y6 ^y y7y8y8 ^y y9 | 6.2 |
| | 963.5156 | 1.1 | 8.0 | AVEAYLLAN | | 10 | b2b4b5b7b8y1y2 ^y y4y4 ^y y8 | 5.1 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]



| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------------|-----------|------|-----|--------------------------|-----|----|--|------|
| D9ZHP1 - DAUCS | 1454.712 | -0.1 | 8.3 | HEQEITSSVPAEK | | 32 | b1b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°y1y2y2°y3y3°y4y4°y5°y6y6°y7y7°y8y9y11 | 8.0 |
| | 1386.755 | -2.7 | 6.9 | NVEIKGDGGVGTIK | | 15 | b11y2y4y4°y6y7y8y9y9°y10y10°y11y12y12°y13 | 6.7 |
| | 1722.9186 | -1.2 | 8.4 | HITLPEGGPVTTMT LR | | 38 | b1b2b3b3°b4b4°b5b6b6°b7b8b8°b9b10b12°b15y1y2y3y4y4°y5y6y6°y7y7°y8y8°y9y10y10°y11y11°y12y12°y13y14y14 | 6.9 |
| | 2353.0688 | -1.6 | 6.8 | NCTIDYSYIDGDILM GFIDK | C/C | 6 | b2y2y3y5y7y11 | 12.7 |
| | 1838.925 | 0.4 | 7.9 | IENHLSVVPNADGG STTK | | 28 | b4°b5b6°b7b7°b7°b8b8°y1y2y2°y3y3°y4y6y6°y7y7°y8y9y9°y9°y10y10°y10°y11y12y13 | 8.4 |
| | 918.5028 | -1.7 | 8.7 | TTAIFHTK | | 18 | b1b2°b3°b6b6°b7y1y2y2°y3y3°y4y4°y5y5°y6°y7y8 | 6.4 |
| | 1170.6024 | 2.0 | 9.2 | GDAVVPEENIK | | 27 | b2b2°b3b3°b4b4°b5b5°b8b10b11b11°y1y2y3y3°y4y4°y5y5°y6y6°y6°y7y8y8°y9 | 6.2 |
| | 1315.6376 | 1.9 | 6.9 | YAEAQNTMLFK | | 13 | b1b2b3b3°b4b5°b6°b11°y2y9y9°y10y11 | 16.0 |
| | 963.5156 | 1.1 | 8.0 | AVEAYLIAN | | 10 | b2b4b5b7b8y1y2°y4y4°y8 | 5.1 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

106

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------------|-----------|------|-----|-----------------------|-----|----|---|------|
| D9ZHP0 - DAUCS | 1454.712 | -0.1 | 8.3 | HEQEITSSVPAEK | | 32 | b1b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°y1y2y2°y3y3°y4y4°y5°y6y6°y7y7°y8y9y11 | 8.0 |
| | 1386.755 | -2.7 | 6.9 | NVEIKGDGGVGTIK | | 15 | b11y2y4y4°y6y7y8y9y9°y10y10°y11y12y12°y13 | 6.7 |
| | 1708.9025 | -1.5 | 6.9 | HITLPDGGPVTTMT LR | | 11 | b4°b7b8b11b12°y11y11°y12y12°y14y14° | 16.0 |
| | 1825.9316 | 1.4 | 7.7 | IENHLSVVPADGG STTK | | 25 | b3b3°b4b4°b4°b5b6b6°b7b7°b8b8°b10°b17y2y2°y6y7y8y8°y9y10y10°y15y17° | 8.5 |
| | 918.5028 | -1.7 | 8.7 | TTAIFHTK | | 18 | b1b2°b3°b6b6°b7y1y2y2°y3y3°y4y4°y5y5°y6°y7y8 | 6.4 |
| | 1170.6024 | 2.0 | 9.2 | GDAVVPEENIK | | 27 | b2b2°b3b3°b4b4°b5b5°b8b10b11b11°y1y2y3y3°y4y4°y5y5°y6y6°y6°y7y8y8°y9 | 6.2 |
| | 1355.685 | 0.7 | 7.5 | YAEEQNTLLFK | | 12 | b2b3b4y3y4y5y6y8y8°y9y10y11 | 10.1 |
| | 963.5156 | 1.1 | 8.0 | AVEAYLIAN | | 10 | b2b4b5b7b8y1y2°y4y4°y8 | 5.1 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|-----------|------|-----|----------------------|-----|----|---|-----|
| A0A175YRQ3 - DAUCS | 1728.8772 | 0.6 | 8.3 | GAQSHVLEISSVSA EK | | 40 | b5b5°b6b6°b7b7°*b7°b8b8°*b8°b9b9°b10b10°*b10°b11°b12b12°b13b13°b14b14°b15°*b16°b17 b17°y8y8°y9y9°y10y10°y11y11°y12y13y14y15y15°y17 | 6.7 |
| | 547.3087 | 0.2 | 8.6 | SVDVK | | 7 | b2b3y1y2y3y4y5 | 8.1 |
| | 829.4131 | -3.8 | 7.5 | GDGGPGTIR | | 16 | b2b3b3°b6°b8y1y2y3y3°y4y4°y5y6y7y7°y8 | 6.3 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|-----------------------|-----------|-------|-----|---------------------|-----|----|---|------|
| 107 P49372 - APIGR | 1714.941 | 0.2 | 7.9 | IITLPDGGPITMTL R | O/M | 29 | b2b3b3°b4b4°b6b6°b7°b9b11b15°b16y1y3°y8y8°y9y9°y10y10°y11y11°y12y12°y13y13°y14y14°y15 | 11.8 |
| | 918.5028 | -1.7 | 8.7 | TTAIFHTK | | 18 | b1b2°b3°b6b6°b7y1y2y2°y3y3°y4y4°y5y5°y6°y7y8 | 6.4 |
| | 1170.6024 | 2.0 | 9.2 | GDAVVPEENIK | | 27 | b2b2°b3b3°b4b4°b5b5°b8b10b11b11°*y1y2y3y3°*y4y4°y5y5°y6y6°*y6°y7y8y8°y9 | 6.2 |
| | 1299.6211 | -0.3 | 7.2 | YANEQNTALFK | D/N | 9 | b2b3b6b7b8b9b9°b11°*y11 | 13.1 |
| | 1298.6136 | -18.5 | 6.1 | YANEQNTALFK | | 3 | y3y6°y7° | 16.5 |
| | 977.5329 | 2.8 | 9.0 | ALEAYLIAN | | 25 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°y1y1°*y2y2°*y3y3°*y4y4°*y5y5°*y6°*y7 | 5.9 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|-----------|------|-----|--|-----|----|--|------|
| A0A175YR25 - DAUCS | 1430.7467 | -1.3 | 7.6 | QRIDEIDTEALK | | 25 | b2b2*b3b3*b4b4*b4*b5b5*b5*b6*b7b8*b10b10*b12y1y2y3y4y5y5*y6y6*y7 | 9.8 |
| | 1146.5898 | 0.9 | 6.8 | IDEIDTEALK | | 7 | b2b2*b3y2y3y5*y6 | 10.1 |
| | 1392.7203 | 0.8 | 7.4 | FTVVPTDGGCIVK | C/C | 16 | b1b2b2*b3b3*b4y2y3y4y5y6y7y8y9*y11y13 | 6.0 |
| | 3267.6 | -2.7 | 6.4 | NTTIYTPIGDAVIPE DNVKEATEQSGMV FK | | 7 | b2*b9*b9*y7y12*y17y24 | 15.3 |
| | 2060.0557 | 0.5 | 8.0 | NTTIYTPIGDAVIPE DNVK | | 44 | b1b2b2*b2*b3b3*b4*b5*b6*b7b7*b10b10*b11b11*b12b12*b13b13*b17b19y1y2y3y4y5y5*y6y6*y7y7*y8y8*y9y10y11y11*y12*y13y13*y14y15y16y19 | 11.4 |
| | 1226.576 | 3.2 | 7.3 | EATEQSGMVFK | | 17 | b1b1*b2b2*b3*b4b4*b8*b9*b10y2y3y4y5y6y9y11 | 11.1 |
| | 1407.7529 | 0.8 | 7.3 | AIEAYLLANPLY | | 20 | b2b3*b4b5b6b7b8b9b9*b10b12y1y2y4y5y6y7y8y9y11 | 9.8 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

108

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------------|-----------|------|-----|----------------|-----|----|---|------|
| P27538 - PETCR | 1375.7303 | 1.0 | 6.8 | GFLLDMDNIIPK | | 13 | b2b6b9b9*b10b10*b10*y6y7y8y9y9*y12 | 11.4 |
| | 768.4965 | -1.6 | 7.0 | VLPQAIK | | 6 | b5y3y4y4*y5y6 | 9.7 |
| | 1534.8818 | -1.4 | 7.1 | KVTLGEVSQFTVVK | | 26 | b1b2b3*b4b5b6b8b8*b9b9*b11b11*b13y1y2y3y5y6y6*y7y7*y7*y8y8*y11*y13* | 13.8 |
| | 1406.7909 | 1.4 | 7.3 | VTLGEVSQFTVVK | | 22 | b2b2*b4*b5b10b10*b11b11*b12y1y2y4y6y7y8y9y10y10*y11y11*y12y13 | 13.4 |
| | 1392.7203 | 0.8 | 7.4 | FTVVPTDGGCIVK | C/C | 16 | b1b2b2*b3b3*b4y2y3y4y5y6y7y8y9*y11y13 | 6.0 |
| | 1226.576 | 3.2 | 7.3 | EATEQSGMVFK | | 17 | b1b1*b2b2*b3*b4b4*b8*b9*b10y2y3y4y5y6y9y11 | 11.1 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|-----------|------|-----|---------------------------------|-----|----|---|------|
| AOA164WTA1 - DAUCS | 2447.2305 | -0.2 | 6.4 | MAGSVSCELVINAP ASSVWDLIK | C/C | 4 | y1y6y13y18 | 19.7 |
| | 1671.8933 | -1.2 | 8.2 | LAAAIEESFTHLIEK | | 38 | b1b2b3b4b5b6b8b11b12b12°b13b14b15y1y2y2°y3y4y4°y5y5°y6y6°y7y8y8°y9y9°y10y10°y11y11°y12y12°y13y13°y14°y15 | 5.7 |
| | 1587.825 | 1.6 | 7.6 | IDAVGDGSGVTELN IK | | 17 | b2b13b14b16b16*y2y3y4y8y9y10y12y12°y13y13°y14y15 | 5.7 |
| | 1433.7446 | -1.3 | 6.9 | FKPGTVPFSSYYK | | 5 | b3y4y5y6y10 | 10.2 |
| | 1850.7894 | -0.4 | 7.9 | ENEVFEGGYCDLGF SK | C/C | 25 | b2b2°b3b3°b4b5b6*b8°b11y2y2°y3y3°y4y5y6y6°y7y8y9y10y11y11°y12y13 | 16.2 |
| | 954.4768 | -1.1 | 7.5 | ATTEYELK | | 15 | b2b2°b3°b4b6°y1y2y3y3°y4y5y5°y6y7y7° | 8.6 |
| | 2657.327 | -0.2 | 8.4 | ADADPNLASLVSVD QMMGVNLNLAANK | | 52 | b2b2°b3b4b5b6b7b7*b8b9°b10b10°b11b11°b12b12°b13b13*b15b15*b16b16*b18b18*b19b19°b20b22b26y2y2*y3y4y4*y5y6y6*y7y8y9y10y11y12y13y13°y15y17y19y22y22*y23°y25° | 9.4 |

MH+: molecular mass of singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

Table S4: Summary of MS data on specific peptides of Dau c 1.01 variants from carrot extract. Supernatant after incubation for 30 min at RT or 95 °C and centrifugation for 10 min at RT, 16100 g; 16 kDa band, excised from a 19 % SDS gel (Fig 6A).

Table S4-1: Summary of mass spectrometric fragmentation data of carrot extract in H₂O at ambient temperature. Red colour indicates peptides which are specific for the protein indicated on the left. If only one specific peptide was detected for an individual protein, an annotated spectrum is shown directly below. Additionally, spectra are provided for A0A175YQC3 - DAUCS and For O81640 - PIMBR even though two specific peptides were detected in each case. However, the quality of the spectra for A0A175YQC3 - DAUCS was limited and the second peptide for O81640 - PIMBR (GAVVPEENIK) was a pass two match and represents very likely a false positive.

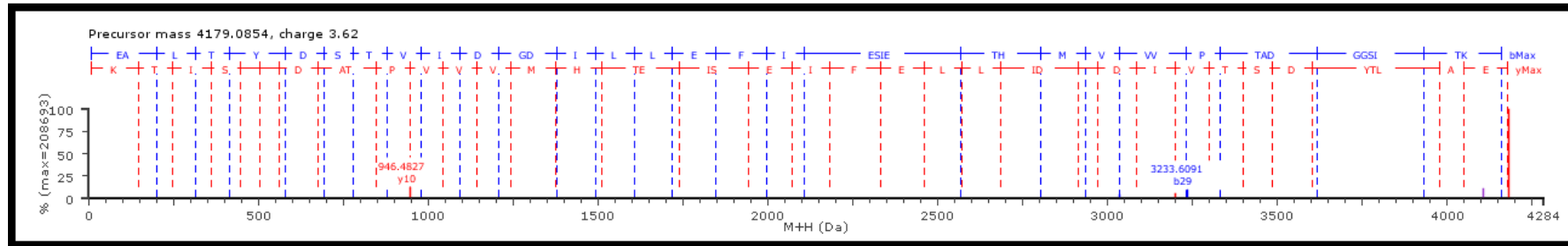
| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|------|-----|---|-----|----|---|-----|
| 1730.855 | 0.9 | 9.5 | GAQSHSLEITSSVSAEK | | 49 | b5b5°b6b6°b7b7°b7°b8b8°b9b9°b10b10°b11b11°b12b12°b13°b14b14°b16°b17b17°y2y2°y3y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y11°y12y12°y14y14°y15y15°y15°y16y16° | 5.1 |
| 1615.931 | 1.1 | 9.7 | IFSGIVLDVDTVIPK | | 45 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b9b9°b10b10°b11b11°b12b12°b13b13°b14b15b15°y1y2y3y4y5y6y6°y7y8y8°y9y10y10°y11y11°y12y13y13°y14y14° | 3.9 |
| 1317.674 | -0.1 | 9.1 | SVDVKDGGGAGTVR | | 42 | b2b2°b3b3°b4b4°b5b5°b6b7b8b9b9°b10b10°b11b11°b12b13b13°b14°y3y3°y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y14 | 5.9 |
| 789.3858 | 1.0 | 8.8 | GDGGAGTVR | | 16 | b2b2°b3b3°b4b4°b5b5°b6°b7b7°b8b8°y1y2y5° | 3.1 |
| 1730.936 | 1.4 | 9.6 | IITLPEGSPITSMTVR | O/M | 51 | b1b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b10b11b11°b13b13°b14b14°b15b15°b16b16°y1y2y3y3°y4y5y6y6°y7y7°y8y8°y9y10y10°y11y11°y12y12°y13y13°y14y14°y15y15° | 4.6 |
| 1714.943 | 2.2 | 9.5 | IITLPEGSPITSMTVR | | 53 | b1b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9°b10b10°b11b11°b12b12°b13b13°b14b14°b15b15°y1y2y3y3°y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y13y13°y14y14°y16 | 3.5 |
| 4807.403 | -1.9 | 7.9 | TDAVNKEALTYDSTVI DGDILLEFIESIETHMV VVPTADGGSITK | | 30 | b2b3b8b9b10b11°b12°b13b14b14°b15°b16°b18b20b21°b29b32b33b34b35b35°b37°b43b44°b45b45°y35°y40y42y45 | 7.5 |
| 4179.086 | -2.0 | 9.6 | EALTYDSTVIDGDILLE FIESIETHMVVVPTAD GGSITK | | 90 | b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°b10b10°b11b13b13°b14b14°b15b15°b16b17b17°b18b18°b19b22°b23b23°b25b25°b26b27b27°b28°b29b29°b30b33b36°b37b39y1y2y2°y3y4y5y6y7y7°y9y10y10°y11y12y13y14y15y16°y17y17°y19y19°y20y21y22y23y23°y24y25y25°y27y28y29y30y31y32y33y34y34°y37y38y38°y39 | 5.3 |
| 4195.078 | -2.6 | 8.1 | EALTYDSTVIDGDILLE FIESIETHMVVVPTAD GGSITK | O/M | 31 | b1b14b15b17b18b19b22°b26b27b27°b28b28°b29b29°b32b32°b33b35°b37y11y17y22°y23°y25y28y29y32y33y34y34°y36 | 5.9 |
| 918.5038 | -0.6 | 8.9 | TTAIFHTK | | 22 | b2°b3b3°b4°b6b6°b7b7°b8b8°y1y2y3y3°y4y4°y5y5°y6y6°y7y7° | 3.8 |

110

CAB03715 - Dau c 1.0103

| | | | | | | | |
|----------|------|-----|------------------------|-----|--|---|-----|
| 2070.078 | -4.0 | 8.8 | TTAIFHTKGDVVPEE NIK | 43 | b2b2°b3°b5°b10b10°b11b11°b12b12°b13b16b16°b17b18b18°b18°b19y2y3y3*y4y4°y5°y6y6*y6°y7y7°y8y9y10y10*y11y12y12*y13y13°y14y14°y15y16y17° | 12.2 | |
| 1170.602 | 2.3 | 9.2 | GDAVVPEENIK | 33 | b2b2°b3b3°b4b4°b5b5°b6°b7b8b8°b10b10°b11b11°y2y3y3*y4y4°y5y5°y6y6*y6°y7y7*y7°y8y8°y9y10° | 7.1 | |
| 1225.624 | 2.8 | 9.0 | FADAQNTALFK | 40 | b2b3b4b5b5°b5°b6b6°b6°b7b7°b7°b8b8°b9b9°b9°b11b11*y1y2y3y4y5y5°y6y6*y6°y7y7*y7°y8y8*y8°y9y9*y9°y10y10*y10° | 3.7 | |
| 1226.61 | 4.3 | 8.0 | FADAQNTALFK | D/Q | 11 | b2b3b6°b11°y2y3y4y5y6y9y11 | 9.8 |
| 977.531 | 1.2 | 9.1 | AIEAYLIAN | | 25 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8°b9y1y1*y2y2*y3y3*y4y5y5*y6y7y9* | 4.9 |
| 978.5145 | 0.6 | 9.0 | AIEAYLIAN | D/N | 18 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b9y1y2y5y6y7y8 | 4.4 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]



| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|------|-----|---|-----|----|--|------|
| 1730.855 | 0.9 | 9.5 | GAQSHSLEITSSVSAEK | | 49 | b5b5°b6b6°b7b7°b8b8°b9b9°b10b10°b11b11°b12b12°b13°b14b14°b16°b17b17°y2y2°y3y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y11°y12y12°y14y14°y15y15°y15°y16y16° | 5.1 |
| 1615.931 | 1.1 | 9.7 | IFSGIVLDVDTVIPK | | 45 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°b10b10°b11b11°b12b12°b13b13°b14b14°b15b15°y1y2y3y4y5y6y6°y7y8y8°y9y10y10°y11y11°y12y13y13°y14y14° | 3.9 |
| 1331.688 | -1.2 | 8.2 | SVEVKDGGGAGTVR | | 24 | b2°b4°b6b9b10°y1y2y3y4y4°y5y6y7y8y8°y9y9°y10y10°y11y11°y12y12°y13 | 8.0 |
| 789.3858 | 1.0 | 8.8 | GDGGAGTVR | | 16 | b2b2°b3b3°b4b4°b5b5°b6°b7b7°b8b8°y1y2y5° | 3.1 |
| 1714.943 | 2.2 | 9.5 | IITLPEGSPITSMTVR | | 53 | b1b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9°b10b10°b11b11°b12b12°b13b13°b14b14°b15b15°y1y2y3y3°y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y13y13°y14y14°y16 | 3.5 |
| 1730.936 | 1.4 | 9.6 | IITLPEGSPITSMTVR | O/M | 51 | b1b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b10b11b11°b13b13°b14b14°b15b15°b16b16°y1y2y3y3°y4y5y6y6°y7y7°y8y8°y9y10y10°y11y11°y12y12°y13y13°y14y14°y15y15° | 4.6 |
| 4089.111 | -1.3 | 7.1 | EALTYDSTVIDGDILLG FIESIETHLVVVPTADG GSITK | | 16 | b17°b20b22b27b28b29b29°y14y21°y25y29y29°y31y32y33y38° | 12.6 |
| 2070.078 | -4.0 | 8.8 | TTAIFHTKGDVAVPEENIK | | 43 | b2b2°b3°b5°b10b10°b11b11°b12b12°b13b16b16°b17b18b18°b18°b19y2y3y3°y4y4°y5°y6y6°y6°y7y7°y8y9y10y10°y11y12y12°y13y13°y14y14°y15y16y17° | 12.2 |
| 918.5038 | -0.6 | 8.9 | TTAIFHTK | | 22 | b2°b3b3°b4°b6b6°b7b7°b8b8°y1y2y3y3°y4y4°y5y5°y6y6°y7y7° | 3.8 |
| 1170.602 | 2.3 | 9.2 | GDVAVPEENIK | | 33 | b2b2°b3b3°b4b4°b5b5°b6°b7b8b8°b10b10°b11b11°y2y3y3°y4y4°y5y5°y6y6°y6°y7y7°y7°y8y8°y9y10° | 7.1 |
| 1225.624 | 2.8 | 9.0 | FADAQNTALFK | | 40 | b2b3b4b5b5°b5°b6b6°b6°b7b7°b7°b8b8°b9b9°b9°b11b11°y1y2y3y4y5y5°y6y6°y6°y7y7°y7°y8y8°y8°y9y9°y9°y10y10°y10° | 3.7 |
| 1226.61 | 4.3 | 8.0 | FADAQNTALFK | D/Q | 11 | b2b3b6°b11°y2y3y4y5y6y9y11 | 9.8 |
| 978.5145 | 0.6 | 9.0 | AIEAYLIAN | D/N | 18 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b9y1y2y5y6y7y8 | 4.4 |
| 977.531 | 1.2 | 9.1 | AIEAYLIAN | | 25 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8°b9y1y1°y2y2°y3y3°y4y5y5°y6y7y9° | 4.9 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|-------------------|----------|-----|-----|-------------------------|-----|----|--|-----|
| A0A164SJ3 - DAUCS | 1323.627 | 0.3 | 8.7 | SEVEATSSVSAEK | | 35 | b2b2°b3b3°b4b4°b5b5°b6b6°b7b8°b9b9°b10b10°b11b11°b12y2y3y3°y4y4°y5y6°y7y7°y8y8°y9y9°y10°y12°y13 | 6.9 |
| | 2036.144 | 0.8 | 8.9 | ALCLDIDTLLPQVVPG AIK | C/C | 34 | b3b4b5b5°b6b6°b7b7°b8b8°b9b9°b10b13b14b15°b17°y1y4y5y6y7y8y8*y9y10y11y12y13y14y15y15°y17y19 | 6.3 |
| | 1431.732 | 0.1 | 9.6 | SAEILEGDGGVGTVK | | 51 | b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b10b10°b11b11°b12b12°b13b13°b14b14°b15y1y2y3y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y13y13°y14y14° | 2.3 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|------|-----|---|-----|----|--|------|
| 1730.855 | 0.9 | 9.5 | GAQSHSLEITSSVSAEK | | 49 | b5b5°b6b6°b7b7°b7°b8b8°b9b9°b10b10°b11b11°b12b12°b13°b14b14°b16°b17b17°y2y2°y3y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y11°y12y12°y14y14°y15y15°y15°y16y16° | 5.1 |
| 1615.931 | 1.1 | 9.7 | IFSGIVLDVDTVIPK | | 45 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°b10b10°b11b11°b12b12°b13b13°b14b14°b15b15°y1y1°y2y2°y3y3°y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y13y13°y14y14° | 3.9 |
| 1331.69 | -0.2 | 8.6 | SVDVKDGGGAGTIR | | 37 | b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°b10b10°b11b11°b12b12°b13b13°y3y3°y4y4°y5y5°y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y13y13°y14 | 7.1 |
| 803.4018 | 1.5 | 7.7 | GDGGAGTIR | | 11 | b2b2°b3b3°b4b4°b5b5°b7°y1y2 | 2.7 |
| 1716.92 | 1.1 | 9.3 | TITLPEGSPITMTVR | | 54 | b1b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°b10b10°b11b11°b12b12°b13b13°b14b14°b15b15°b16y16°y2y2°y3y3°y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y13y13°y14y14°y15y15°y16 | 6.0 |
| 1732.914 | 0.8 | 9.4 | TITLPEGSPITMTVR | O/M | 48 | b1b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°b11b11°b12b12°b13b13°b14b14°b15b15°y1y1°y2y2°y3y3°y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y13y13°y14y14°y16 | 4.8 |
| 4735.381 | -2.0 | 7.0 | TDAVNKEALTYDSTVI DGDILLGFIESIETHM VVVPTADGGSITK | | 12 | b17°b18°b18°b32°b38b43b44y1y32y34y36°y45 | 17.2 |
| 4123.055 | -3.1 | 8.7 | EALTYDSTVIDGDILLG FIESIETHM VVVPTAD GGSITK | O/M | 58 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9°b10°b12°b13b14b15b16b18b23b27b28b28°b29b30b30°b32y2y2°y3y3°y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y13y13°y14y14°y15y15°y16y16°y17y17°y18y18°y19°y20y21y22y25y25°y28°y30y34y35y36y39 | 8.7 |
| 2070.078 | -4.0 | 8.8 | TTAIFHTKGDVVPEE NIK | | 43 | b2b2°b3°b5°b10b10°b11b11°b12b12°b13b13°b16b16°b17b17°b18b18°b18°b19y2y3y3°y4y4°y5°y6y6°y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12°y13y13°y14y14°y15y15°y16y16°y17° | 12.2 |
| 918.5038 | -0.6 | 8.9 | TTAIFHTK | | 22 | b2°b3b3°b4°b6b6°b7b7°b8b8°y1y2y3y3°y4y4°y5y5°y6y6°y7y7° | 3.8 |
| 2435.203 | -0.2 | 7.7 | GDAVVPEENIKFADE QNTALFK | | 8 | b8b21°y11°y12y17y17°y18y22 | 11.7 |
| 1170.602 | 2.3 | 9.2 | GDAVVPEENIK | | 33 | b2b2°b3b3°b4b4°b5b5°b6°b7b7°b8b8°b10b10°b11b11°y2y3y3°y4y4°y5y5°y6y6°y6°y7y7°y7°y8y8°y9y10° | 7.1 |
| 1284.614 | 3.2 | 8.0 | FADEQNTALFK | D/Q | 14 | b2b3b5b5°b11°y2y3y4y5y6y6°y9y10y10° | 7.1 |
| 1283.627 | 1.1 | 8.1 | FADEQNTALFK | | 19 | b4b5b5°b6b6°b7b7°b8b8°b8°b9°b11y8y9y9°y9°y10y10°y10° | 3.7 |
| 978.5145 | 0.6 | 9.0 | AIEAYLIAN | D/N | 18 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b9y1y2y5y6y7y8 | 4.4 |
| 977.531 | 1.2 | 9.1 | AIEAYLIAN | | 25 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8°b9y1y1°y2y2°y3y3°y4y4°y5y5°y6y6°y7y7° | 4.9 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|------|-----|---|-----|----|--|------|
| 1337.643 | 0.2 | 8.9 | TEAEVTSSVSAEK | | 38 | b1b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b9b11b11°b12b13y1y2y2°y3y4y4°y5y6y6°y7y7°y8y9y9°y10y10°y11y11°y12y12° | 5.7 |
| 2050.16 | 0.9 | 8.4 | ALCLDIDTLLPQVLPG AIK | C/C | 25 | b3b5b7b8b8°b9b9°b10b10°b11°b13b14b14°b15b19y5y6y7y8y9y10y11y13y15y19 | 7.9 |
| 1435.691 | 0.3 | 9.3 | SSETLEGDGGVGTVK | | 52 | b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°b10b10°b11b11°b12b12°b13b13°b14b14°b15b15°y1y2y3y3°y4y4°y5y5°y6y6°y7y8y9y9°y10y10°y11y11°y12y12°y13y13°y14y14° | 2.5 |
| 1183.645 | -0.6 | 8.9 | LVHLGDASPFK | | 23 | b3b4b5b6b7b7°b8b8°b9°b10y3y4°y5y5°y6y7y7°y8y8°y9y10y10°y11 | 9.9 |
| 4952.387 | -2.1 | 7.3 | VDAIDKESFTYAYSIID GDILLGFIESINNHFAV VPNADGGCTVK | C/C | 22 | b3b9b14°b15°b16°b27b29°b33b34b35b43y5y6y10y11y21y23y26°y28y32y33y45 | 12.2 |
| 4311.045 | -3.1 | 6.9 | ESFTYAYSIIDGDILLGF IESINNHFAVVPNADG GCTVK | C/C | 9 | b1°b11b13b26b28b32y22°y28y31 | 17.4 |
| 2063.066 | 2.0 | 8.8 | STITFNTKGDVVPEE NIK | | 42 | b2b3b3°b4b4°b5°b7°b9°b11b12b12°b12°b13b13°b17°b19y2y3y3°y4y4°y5y6y6°y7y7°y8y9y11y11°y12y13y13°y14y14°y14°y15y16y16°y17y17°y18 | 6.5 |
| 2064.053 | 3.4 | 7.4 | STITFNTKGDVVPEE NIK | D/N | 11 | b13b13°b16b17y2y10°y14y16y17y17°y19 | 12.9 |
| 911.4831 | -0.2 | 8.4 | STITFNTK | | 21 | b2b2°b3°b4°b5°b6°b7b7°y1y2y2°y3y4y4°y5y5°y6y6°y7y7°y8 | 5.8 |
| 1170.602 | 2.3 | 9.2 | GDAVVPEENIK | | 33 | b2b2°b3b3°b4b4°b5b5°b6°b7b8b8°b10b10°b11b11°y2y3y3°y4y4°y5y5°y6y6°y6°y7y7°y7°y8y8°y9y10° | 7.1 |
| 1423.748 | 1.4 | 7.3 | AIFQAVEAYLIAN | D/N | 10 | b5b6b7b8b9b10°b13y1y6°y13 | 8.6 |
| 1422.764 | 1.3 | 8.7 | AIFQAVEAYLIAN | | 32 | b2b3b4b4°b5b5°b6b7b8b8°b9b9°b9°b10b10°b11b12y1y1°y2y2°y3y3°y4y5y6y7y7°y8y8°y9y10 | 7.1 |

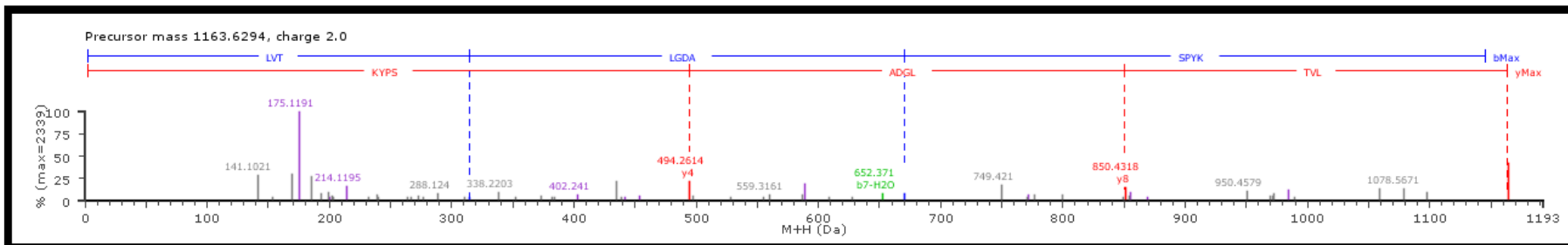
MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|------|-----|----------------------|-----------------|----|---|------|
| 1347.664 | 1.0 | 9.0 | TEVEAPSTVSAEK | | 45 | b1b2b2°b3b3°b4b4°b5b5°b6b6°b7b8b8°b9b9°b11b11°b12b12°b13b13°y1y2y2°y3y3°y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y12° | 4.0 |
| 1820.857 | -0.1 | 8.6 | MYQGFLDMDTVFP K | O/M | 27 | b3b4b5b5°b6b6°b7°b8b8°b11b12b12°b13y1y2y3y6y7y8y9y10y11y12y13y13°y13°y15 | 8.4 |
| 1820.86 | 1.2 | 7.8 | MYQGFLDMDTVFP K | O/M | 18 | b2b3b4b5b6b10b12b12°y2y4y5y6y7y8y10y10°y12y13 | 10.6 |
| 1836.854 | 0.8 | 8.5 | MYQGFLDMDTVFP K | O/M , O/M | 22 | b2b3b4b5b5°b6b7b12b12°b13y1y2y3y6y7y8y8°y9y10y11y13y15 | 6.7 |
| 1804.864 | 0.6 | 7.6 | MYQGFLDMDTVFP K | | 13 | b3b6b7°b11°b12°b13y3y5y8y9y10°y12y13* | 14.2 |
| 810.544 | -1.1 | 7.9 | VLPQLIK | | 13 | b2b4b5b5°b6b7y1y2y3y4y4°y6y7 | 5.1 |
| 1487.771 | 1.1 | 9.2 | SVEILEGDDGGVGTVR | | 47 | b2b3b3°b4b4°b5b5°b6b6°b7b8b8°b9b9°b10b10°b11b11°b12b12°b13b13°b14b14°b15y2y3y4y4°y5y5°y6y7y7°y8y9y9°y10y10°y11y11°y12y12°y13y13°y14y14° | 3.4 |
| 1608.793 | 0.6 | 8.8 | LVHLGEATEYTTMK | O/M | 37 | b1b2b3b4b5b6b7b8b8°b9°b10b10°b11b12b13b14b14°y1y2y3y3°y4y4°y5y5°y6y6°y7y7°y8y8°y9y10y11y11°y12y14 | 6.1 |
| 1592.798 | 0.5 | 9.0 | LVHLGEATEYTTMK | | 38 | b1b2b3b4b5b6b7b8b8°b9b9°b11b11°b12b12°b13°y1y2y3y4y4°y5y5°y6y6°y7y7°y8y8°y9y9°y10y10°y11y11°y12y13y14 | 4.1 |
| 944.5391 | -2.1 | 8.3 | QKVDVIDK | | 23 | b2b2°b3b3°b4b4°b5b5°b5°b6°b7°b7°b8y1y2y2°y3y4y5y6y7y8 | 3.9 |
| 1878.944 | 1.5 | 7.5 | NTTIYNTKGDALPED K | | 22 | b1b3b3°b5°b6b7°b7°b8b8°b11°b12°b13b13°b17y2y4y5y9y12y13y14y17 | 14.0 |
| 955.471 | -2.2 | 7.2 | NTTIYNTK | D/N | 8 | b5°b5°b8y2°y3y4y4°y5 | 9.1 |
| 1170.636 | 0.0 | 8.4 | GDAVLPEDKVK | | 19 | b2b3b3°b5b9°b10b10°b11y1y2y3y5y6y6°y7y7°y8y9y9° | 9.7 |
| 964.5003 | 1.8 | 7.1 | AVEAYLLAN | D/N | 7 | b2b3°b5b6b7y1y5* | 14.2 |
| 963.5153 | 1.1 | 8.4 | AVEAYLLAN | | 23 | b2b3b3°b4b5b5°b6b6°b7b7°b8b9b9°y1y1°y2y2°y3y4y4°y5y6y8 | 4.9 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

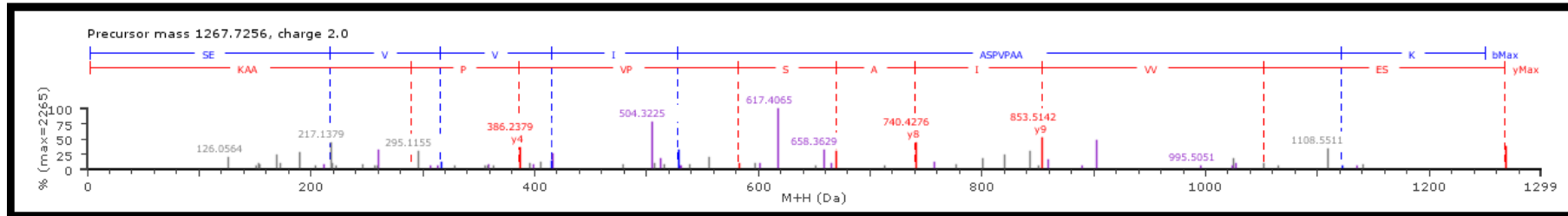
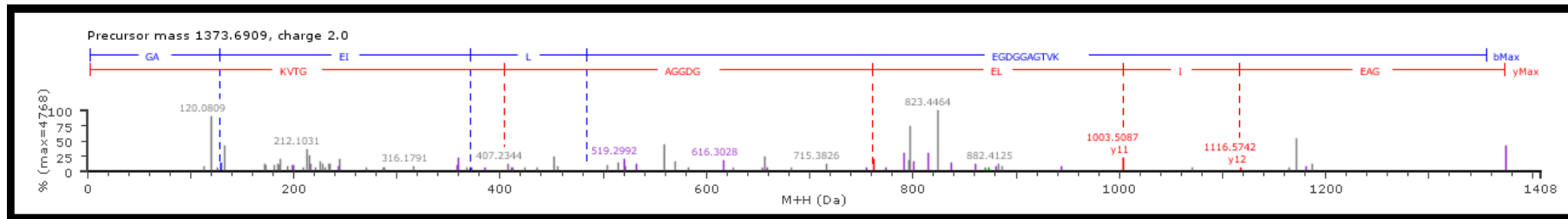
| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------------|----------|-------|-----|-------------------------|-----|----|--|------|
| Q40795 - PECTR | 2050.16 | 0.9 | 8.4 | ALCLDIDTLLPQVLPG AIK | C/C | 25 | b3b5b7b8b8°b9b9°b10b10°b11°b13b14b14°b15b19y5y6y7y8y9y10y11y13y15y19 | 7.9 |
| | 1163.629 | -0.8 | 6.9 | LVTLGDASPYK | | 7 | b3b3°b7b7°y4y8y11 | 3.7 |
| | 1170.602 | 2.3 | 9.2 | GDAVVPEENIK | | 33 | b2b2°b3b3°b4b4°b5b5°b6°b7b8b8°b10b10°b11b11°y2y3y3*y4y4°y5y5°y6y6*y6°y7y7*y7°y8y8°y9y10° | 7.1 |
| | 1323.635 | -25.2 | 6.0 | FANDQNNLIFK | | 5 | b1b3b5*b9°y3 | 18.4 |
| | 964.5003 | 1.8 | 7.1 | AVEAYLLAN | D/N | 7 | b2b3°b5b6b7y1y5* | 14.2 |
| | 963.5153 | 1.1 | 8.4 | AVEAYLLAN | | 23 | b2b3b3°b4b5b5°b6b6°b7b7°b8b9b9*y1y1*y2y2*y3y4y4*y5y6y8 | 4.9 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]



| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|----------|-----|-----|------------------|-----|----|---|-----|
| A0A175YQC3 - DAUCS | 1267.726 | 0.4 | 7.3 | SEVVIASPVPAAK | | 14 | b2b3b4b5b12y3y4y6y7y8y9y* ⁹ y11y13 | 9.5 |
| | 1373.691 | 0.8 | 6.6 | GAEILEGDDGGAGTVK | | 9 | b2b4b5y3* ⁴ y4y9y10* ⁹ y11y12 | 9.4 |
| | 978.5145 | 0.6 | 9.0 | AIEAYLIAN | D/N | 18 | b2b3b3*b4b4*b5b5*b6b6*b7b7*b9y1y2y5y6y7y8 | 4.4 |
| | 977.531 | 1.2 | 9.1 | AIEAYLIAN | | 25 | b2b3b3*b4b4*b5b5*b6b6*b7b7*b8*b9y1y1*y2y2*y3y3*y4y5y5*y6y7y9* | 4.9 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]



| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------------|----------|------|-----|-------------|-----|----|---|------|
| O81640 - PIMBR | 803.4018 | 1.5 | 7.7 | GDGGAGTIR | | 11 | b2b2°b3b3°b4b4°b5b5°b7°y1y2 | 2.7 |
| | 918.5038 | -0.6 | 8.9 | TTAIFHTK | | 22 | b2°b3b3°b4°b6b6°b7b7°b8b8°y1y2y3y3°y4y4°y5y5°y6y6°y7y7° | 3.8 |
| | 1112.61 | 13.8 | 5.2 | GGAVVPEENIK | | 2 | b8°y4 | 16.2 |
| | 1284.614 | 3.2 | 8.0 | FADEQNTALFK | D/Q | 14 | b2b3b5b5°b11*y2y3y4y5y6y6*y9y10y10° | 7.1 |
| | 1283.627 | 1.1 | 8.1 | FADEQNTALFK | | 19 | b4b5b5°b6b6°b7b7°b8b8°b8°b9°b11y8y9y9*y9°y10y10*y10° | 3.7 |
| | 951.5248 | -1.0 | 7.8 | AIEAHLIAN | | 22 | b2b3b3°b4b5b6b7b8b8°y1y1*y2y2*y3y3*y5y5*y6y7y7*y9y9* | 5.0 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

119

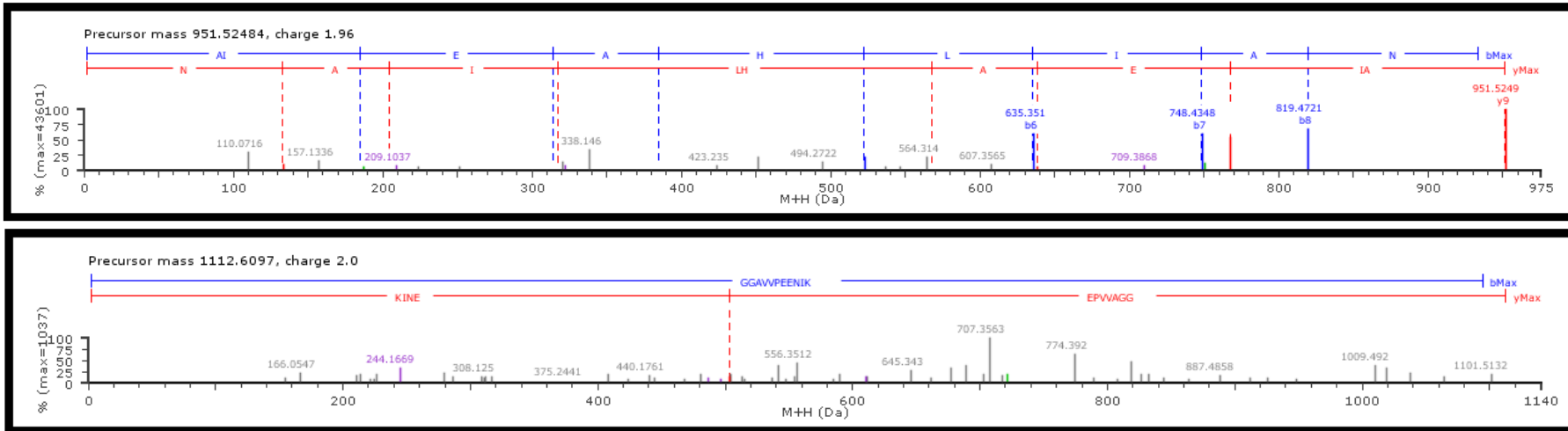


Table S4-2: Summary of mass spectrometric fragmentation data of carrot extract in H2O at 95°C. Red colour indicates peptides which are specific for the protein indicated on the left. No annotated spectra were displayed because no deviating or additional protein sequences were detected compared to the results at ambient temperature and pH 7.

| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|------|-----|--|-------------|----|--|------|
| 1730.851 | -2.5 | 8.1 | GAQSHSLEITSSVSAEK | | 26 | b3*b5b6b7b7*b8b8*b8*b9b10b13*y2y2*y3y4y5y5*y6y7y7*y8y10*y12y14y15y17 | 8.6 |
| 1615.929 | -0.8 | 8.7 | IFSGIVLDVDTVIPK | | 27 | b2b4*b5b5*b6b6*b7b8b12b12*b13b13*b15y2y3y5y6y7y8y9y10y10*y11y12y13y13*y14 | 4.9 |
| 1317.671 | -3.3 | 8.2 | SVDVKDGGGAGTVR | | 34 | b2b2*b3b3*b6b7b8b9b9*b10b11b13b14y3y3*y4y4*y5y6y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y13*y14 | 6.2 |
| 789.3848 | -0.2 | 7.2 | GDGGAGTVR | | 11 | b2b3b3*b4b4*b5b5*b7*b8*y1y2 | 7.1 |
| 1730.935 | -0.5 | 9.0 | IITLPEGSPITSMTVR | O/M | 51 | b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*b9b9*b10b10*b11b11*b12*b13b13*b14b15b15*b16b16*y1y2y3y3*y4y4*y5y6y6*y7y7*y8y8*y9y10y10*y11y11*y12y12*y13y14y14*y16 | 4.5 |
| 1714.939 | -1.0 | 8.7 | IITLPEGSPITSMTVR | | 34 | b2b3b3*b4b4*b5b6b7b7*b8b8*b10b11b13b14y1y3y4y4*y5y6y6*y7y8y8*y9y10y11y11*y12y12*y13y14y14* | 6.7 |
| 4824.397 | 1.3 | 7.2 | TDAVNKEALTYDSTVIDGDILLEFIES IETHMVVPTADGGSITK | D/N, O/M | 21 | b4b9b14*b19b20b21b21*b26*b32b34b38*b38*b39*b43*y9*y10*y16y16*y17y31y37* | 15.2 |
| 4808.4 | 0.7 | 7.2 | TDAVNKEALTYDSTVIDGDILLEFIES IETHMVVPTADGGSITK | D/N | 23 | b3b29b34b34*b35*b35*b36b37b39b40*b41b44b45y4y15*y17y25y29*y32*y34y34*y42y43 | 9.9 |
| 4807.401 | -2.4 | 6.9 | TDAVNKEALTYDSTVIDGDILLEFIES IETHMVVPTADGGSITK | | 25 | b5b5*b9b13b15b15*b15*b16b17*b18b19*b27*b28*b30b31*b35b41*y1y2y21*y25*y29*y33*y36*y45 | 19.2 |
| 4823.398 | -1.8 | 6.8 | TDAVNKEALTYDSTVIDGDILLEFIES IETHMVVPTADGGSITK | O/M | 11 | b1b8b12b15*b17b26b35b37*b42b44*y12* | 11.4 |
| 4179.088 | -1.4 | 8.3 | EALTYDSTVIDGDILLEFIESIETHMV VVPTADGGSITK | | 42 | b4b5b5*b6b8b8*b9b9*b10*b13b14b15b17*b18b22b25b27b28b28*b29b39*y5y6y7y7*y8y10y10*y11y12y14y17*y19y19*y20y21y22y24y27*y33y35y39 | 7.1 |
| 4195.082 | -1.7 | 7.9 | EALTYDSTVIDGDILLEFIESIETHMV VVPTADGGSITK | O/M | 28 | b4b4*b5b5*b6b8*b13b16b17b27b27*b28b29b31*y6y10y11y12y17y17*y18y19y19*y22y23*y24y34y39 | 9.9 |
| 918.503 | -1.5 | 8.1 | TTAIFHTK | | 17 | b2*b3*b4*b6b6*b7y1y2y3y3*y4y4*y5y5*y6*y7y8 | 4.6 |
| 1170.6 | -0.1 | 8.9 | GDAVVPEENIK | | 29 | b2b2*b3b3*b4b4*b5b5*b6b8b9b10*b11b11*y1y2y3y3*y4y4*y5y5*y6y6*y6*y7y7*y8y9 | 5.3 |
| 1225.622 | 0.5 | 8.7 | FADAQNTALFK | | 37 | b2b3b4b5b5*b5*b6*b7b7*b7*b8b8*b9b9*b11b11*y1y2y3y4y5y5*y6y6*y6*y7y7*y7*y8y8*y8*y9y9*y9*y10y10*y10* | 4.6 |
| 1226.61 | 3.9 | 6.4 | FADAQNTALFK | D/N | 3 | b5*y6y9 | 10.4 |
| 977.5295 | -0.7 | 8.5 | AIEAYLIAN | | 20 | b2b3b4b4*b5b5*b6b6*b7b7*b8b9y1y1*y2*y3y4y5y6y7 | 6.7 |
| 978.5126 | -1.6 | 8.0 | AIEAYLIAN | D/N | 12 | b2b3b4b5b5*b6b6*b7b8y1y2y6* | 6.5 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

120
CAB03715 - Dauc 1.0103

| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|------|-----|-------------------|-----|----|--|------|
| 1730.851 | -2.5 | 8.1 | GAQSHSLEITSSVSAEK | | 26 | b3*b5b6b7b7°b8b8*b8°b9b10b13*y2y2°y3y4y5y5°y6y7y7°y8y10°y12y14y15y17 | 8.6 |
| 1615.929 | -0.8 | 8.7 | IFSGIVLDVDTVIPK | | 27 | b2b4°b5b5°b6b6°b7b8b12b12°b13b13°b15y2y3y5y6y7y8y9y10y10°y11y12y13y13°y14 | 4.9 |
| 1331.685 | -4.9 | 6.7 | SVEVKDGGGAGTVR | | 5 | y7y8y12y12°y13 | 12.7 |
| 789.3848 | -0.2 | 7.2 | GDGGAGTVR | | 11 | b2b3b3°b4b4°b5b5°b7°b8°y1y2 | 7.1 |
| 1730.935 | -0.5 | 9.0 | IITLPEGSPITSMTVR | O/M | 51 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b9°b10b10°b11b11°b12°b13b13°b14b15b15°b16b16°y1y2y3y3°y4y4°y5y6y6°y7y7°y8y8°y9y10y10°y11y11°y12y12°y13y14y14°y16 | 4.5 |
| 1714.939 | -1.0 | 8.7 | IITLPEGSPITSMTVR | | 34 | b2b3b3°b4b4°b5b6b7b7°b8b8°b10b11b13b14y1y3y4y4°y5y6y6°y7y8y8°y9y10y11y11°y12y12°y13y14y14° | 6.7 |
| 918.503 | -1.5 | 8.1 | TTAIFHTK | | 17 | b2°b3°b4°b6b6°b7y1y2y3y3°y4y4°y5y5°y6°y7y8 | 4.6 |
| 1170.6 | -0.1 | 8.9 | GDAVVPEENIK | | 29 | b2b2°b3b3°b4b4°b5b5°b6b8b9b10°b11b11*y1y2y3y3*y4y4°y5y5°y6y6*y6°y7y7°y8y9 | 5.3 |
| 1225.622 | 0.5 | 8.7 | FADAQNTALFK | | 37 | b2b3b4b5b5*b5°b6°b7b7*b7°b8b8°b9b9°b11b11*y1y2y3y4y5y5°y6y6*y6°y7y7*y7°y8y8*y8°y9y9*y9°y10y10*y10° | 4.6 |
| 1226.61 | 3.9 | 6.4 | FADAQNTALFK | D/N | 3 | b5*y6y9 | 10.4 |
| 977.5295 | -0.7 | 8.5 | AIEAYLIAN | | 20 | b2b3b4b4°b5b5°b6b6°b7b7°b8b9y1y1*y2*y3y4y5y6y7 | 6.7 |
| 978.5126 | -1.6 | 8.0 | AIEAYLIAN | D/N | 12 | b2b3b4b5b5°b6b6°b7b8y1y2y6* | 6.5 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|----------|------|-----|------------------|-----|----|--|-----|
| A0A164SJC3 - DAUCS | 1323.627 | -0.5 | 7.4 | SEVEATSSVSAEK | | 13 | b2b2°b3b4y4y5y7y8y9y9°y10y10°y13 | 7.3 |
| | 1431.731 | -1.1 | 8.3 | SAEILEGDDGGVGTVK | | 38 | b2b2°b3b3°b4b4°b5b5°b6b6°b9b11b12b12°b13b13°b14b14°b15y2y3y4y4°y5y5°y6y7y7°y8y9y9°y10y10°y11y11°y12y12°y13 | 6.1 |

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|---------------------------|----------|------|-----------|---|-----|-----------------------------|---|------|
| 122 A0A175YPA2 - DAUCS | 1730.851 | -2.5 | 8.1 | GAQSHSLEITSSVSAEK | | 26 | b3°b5b6b7b7°b8b8°b8°b9b10b13*y2y2°y3y4y5y5°y6y7y7°y8y10°y12y14y15y17 | 8.6 |
| | 1615.929 | -0.8 | 8.7 | IFSGIVLDVDTVIPK | | 27 | b2b4°b5b5°b6b6°b7b8b12b12°b13b13°b15y2y3y5y6y7y8y9y10y10°y11y12y13y13°y14 | 4.9 |
| | 1331.687 | -3.6 | 7.7 | SVDVKGDDGGAGTIR | | 26 | b2b2°b3b3°b7b8y1y2y3y4y4°y5y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y13y14 | 8.9 |
| | 1732.914 | -0.7 | 8.5 | TITLPEGSPITMTVR | O/M | 41 | b2b2°b3b3°b4b4°b5b5°b6°b7b7°b8b8°b9°b10b11b12b13b15b15°b16°y1y2y3y4y5y6y6°y7y8y8°y10y10°y11y11°y12y12°y13y14y14°y16 | 5.3 |
| | 1716.919 | -1.0 | 8.1 | TITLPEGSPITMTVR | | 22 | b2b3b3°b4b4°b8b8°b9°b11b13°b16y1y4y5y6y8y9y10y12y12°y13y14 | 6.0 |
| | 4123.049 | -4.6 | 6.8 | EALTYDSTVIDGDILLGFIESIETHMV VVPTADGGSITK | O/M | 13 | b8b10°b22b27b29b34°b37y7y9y11y21y28°y39 | 14.1 |
| | 4107.083 | 2.5 | 6.6 | EALTYDSTVIDGDILLGFIESIETHMV VVPTADGGSITK | | 6 | b11b14°b27b28y29y39 | 10.7 |
| | 918.503 | -1.5 | 8.1 | TTAIFHTK | | 17 | b2°b3°b4°b6b6°b7y1y2y3y3°y4y4°y5y5°y6°y7y8 | 4.6 |
| | 1170.6 | -0.1 | 8.9 | GDAVVPEENIK | | 29 | b2b2°b3b3°b4b4°b5b5°b6b8b9b10°b11b11°y1y2y3y3°y4y4°y5y5°y6y6°y6°y7y7°y8y9 | 5.3 |
| | 977.5295 | -0.7 | 8.5 | AIEAYLIAN | | 20 | b2b3b4b4°b5b5°b6b6°b7b7°b8b9y1y1*y2*y3y4y5y6y7 | 6.7 |
| 978.5126 | -1.6 | 8.0 | AIEAYLIAN | D/N | 12 | b2b3b4b5b5°b6b6°b7b8y1y2y6* | 6.5 | |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|----------|------|-----|--|-----|----|---|------|
| A0A161X1M2 - DAUCS | 1337.642 | -0.7 | 7.7 | TEAEVTSSVSAEK | | 15 | b2b4b5b5°b11°y2°y4y5y6y7y8y9y10y11y11° | 7.4 |
| | 1435.69 | -0.9 | 8.5 | SSETLEGGGGVTVK | | 44 | b2b3°b4b4°b5b5°b6b7b7°b8b9°b10°b11b11°b12b12°b13b13°b14b14°b15b15°y1y2y3y4y4°y5y6y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y13y14y14° | 6.6 |
| | 1183.646 | -1.1 | 7.0 | LVHLGDASPFK | | 8 | b3b6b7y3y4°y7y8°y10 | 10.3 |
| | 4952.403 | 1.2 | 6.8 | VDAIDKESFTYAYSIIIDGDILLGFIESI NNHFAYVNPADGGCTVK | C/C | 15 | b5b12°b15°b25b34b35y5°y6y7y10y15°y23y24y42*y42° | 14.7 |
| | 911.4817 | -1.7 | 7.2 | STIFNTK | | 13 | b2b3°b4°b5°y2°y3y4y5y5*y5°y6y6° | 10.1 |
| | 2063.062 | -1.5 | 6.9 | STIFNTKGDVVPEENIK | | 7 | b2b5°b13b16*y6y7y19 | 9.4 |
| | 1422.761 | -1.0 | 6.6 | AIFQAVEAYLIAN | | 4 | b7b9b12y1 | 12.9 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|-----------------------|----------|------|-----------|------------------|-----|-------------------------|---|------|
| Q8SAE7 - Dau c 1.0201 | 1347.663 | -0.6 | 7.9 | TEVEAPSTVSAEK | | 23 | b1b2b3b3°b4b4°b5b5°b9b9°b11b12y2y3y4y6y6°y7y8y9°y10y10°y13 | 6.4 |
| | 810.5435 | -1.5 | 7.2 | VLPQLIK | | 11 | b2b4b5b6y1y2y3y4y4*y6y7 | 5.8 |
| | 1487.771 | 0.5 | 8.3 | SVEILEGGGVGTVR | | 41 | b2b3b3°b4b4°b5b5°b6b6°b7b8°b9b10b10°b11b11°b12b13b14b14°b15y1y2y3y3°y4y4°y5y6y7y7°y8y9y9°y10y10°y11y11°y12y12°y13 | 4.6 |
| | 1608.791 | -1.5 | 7.2 | LVHLEATEYTTMK | O/M | 15 | b2b3b4b6b7b8b8°y1y2y3y4y5y6y12y12° | 9.9 |
| | 1592.794 | -3.3 | 6.1 | LVHLEATEYTTMK | | 7 | b3b5b6b8°y4°y6y10 | 12.3 |
| | 944.5366 | -4.8 | 5.8 | QKVDVIDK | | 5 | b4*y2y5y6y8 | 8.8 |
| | 1878.943 | -0.6 | 6.0 | NTTIYNTKGDALPEDK | | 6 | b2b4°b7b17°y5°y14° | 18.2 |
| | 954.4919 | 2.9 | 5.9 | NTTIYNTK | | 4 | b2*b3°b7y5* | 13.4 |
| | 943.4703 | -2.9 | 6.7 | GDAVLPEDK | | 3 | y3°y4y7 | 6.2 |
| | 1170.635 | -1.5 | 6.0 | GDAVLPEDKVK | | 5 | b2b3b6y1y8 | 13.4 |
| | 636.3722 | 1.1 | 6.8 | SALAFK | | 7 | b2b2°b4°b5y4y5y6 | 4.8 |
| 963.513 | -1.7 | 7.3 | AVEAYLLAN | | 10 | b4b5b7b8b9y1y1*y3*y4y6* | 5.3 | |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------------|----------|------|-----|-----------|-----|----|--|------|
| O81640 - PIMBR | 918.503 | -1.5 | 8.1 | TTAIFHTK | | 17 | b2°b3°b4°b6b6°b7y1y2y3y3°y4y4°y5y5°y6°y7y8 | 4.6 |
| | 951.5242 | -1.6 | 6.8 | AIEAHLIAN | | 16 | b2b3b5b6b6°b7b8b8°y1y2y2*y5y6y7y7*y8 | 6.7 |
| | 952.5078 | -2.1 | 5.8 | AIEAHLIAN | D/N | 6 | b3b6b7b8y6y7° | 17.7 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

Table S4-3: Summary of mass spectrometric fragmentation data of carrot extract in 10mM sodium citrate, pH 3 at ambient temperature. Red colour indicates peptides which are specific for the protein indicated on the left. No annotated spectra were displayed because no deviating or additional protein sequences were detected compared to the results at ambient temperature and pH 7.

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--|----------|------|-----|---|-------------|----|---|------|
| | 1730.856 | 0.5 | 7.6 | GAQSHSLEITSSVSAEK | | 13 | b2b3*b4*b9b12*y1y3y3*y4y7y7*y8y9 | 8.0 |
| | 1615.93 | -0.2 | 9.0 | IFSGIVLDVDTVIPK | | 34 | b2b3b3*b4b4*b5b5*b6b6*b7b8b10b11b12b12*b13b13*b15y1y2y3y5y6y6*y7y8y8*y9y10y10*y11y12y13y14 | 5.2 |
| | 1317.672 | -2.8 | 8.3 | SVDVKGDDGAGTVR | | 38 | b2b2*b3b3*b4b4*b6b7b8b9b9*b10b11b13b14y3y3*y4y4*y5y5*y6y6*y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y13*y14 | 5.9 |
| | 789.3849 | -0.1 | 7.8 | GDGGAGTVR | | 15 | b2b2*b3b3*b4b4*b5b5*b6*b7b7*b8b8*y1y2 | 7.2 |
| | 1730.936 | 0.3 | 9.3 | IITLPEGSPITSMTVR | O/M | 55 | b1b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*b9b9*b10b10*b11b11*b13b13*b14b14*b15b15*b16*y1y2y3y3*y4y4*y5y6y6*y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y13*y14y14*y15y15*y16 | 3.4 |
| | 1714.939 | -1.2 | 8.8 | IITLPEGSPITSMTVR | | 49 | b1b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*b9b9*b10b10*b11b11*b12b12*b15y1y2y3y3*y4y5y5*y6y6*y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y14y14*y15y16 | 6.8 |
| | 4824.391 | -0.1 | 6.9 | TDAVNKEALTYDSTVIDGDILLEFIES IETHMVVVPTADGGSITK | D/N, O/M | 16 | b13b14b16*b17*b19b31b35*b38*b40*y1y16*y19*y30y30*y40° | 14.4 |
| | 4807.402 | -2.0 | 6.8 | TDAVNKEALTYDSTVIDGDILLEFIES IETHMVVVPTADGGSITK | | 16 | b2*b6b15b15*b26b26*b29b34b34*b35*b38b38*y25*y27y29*y45 | 13.7 |
| | 4195.082 | -1.6 | 8.0 | EALTYDSTVIDGDILLEFIESIETHMV VVPTADGGSITK | O/M | 23 | b6b9*b10b10*b18b26b27b28b29b31*y6y8y10y11y16y20y21y22y24y25y33y37y39 | 7.5 |
| | 4179.082 | -2.9 | 7.9 | EALTYDSTVIDGDILLEFIESIETHMV VVPTADGGSITK | | 36 | b4*b5b5*b7*b8b9b9*b13b15b16*b26b26*b27b27*b28*b29b29*b35b39*y6y7y9y10y11y12y13*y18y19y21y22*y23*y24y30y31y33y39 | 9.5 |
| | 918.5032 | -1.3 | 8.4 | TTAIFHTK | | 18 | b3*b6b6*b7b7*b8b8*y2y3y3*y4y4*y5y5*y6y6*y7y7° | 3.2 |
| | 2070.077 | -4.6 | 7.0 | TTAIFHTKGDVVPEENIK | | 4 | b3*b19y3y6 | 5.0 |
| | 1170.6 | -0.2 | 8.9 | GDAVVPEENIK | | 35 | b2b2*b3b3*b4b4*b5b5*b6b8b8*b9*b9*b10*b11b11*b11*y2y3y3*y4y4*y5y5*y6y6*y7y7*y8y8*y9y9*y9*y10 | 7.6 |
| | 1225.622 | 0.2 | 8.7 | FADAQNTALFK | | 41 | b2b3b4b5b5*b5*b6b6*b6*b7b7*b7*b8b8*b8*b9b9*b9*b11b11*y1y2y3y4y5y5*y6y6*y6*y7y7*y7*y8y8*y8*y9y9*y9*y10*y10° | 4.5 |
| | 1226.61 | 4.1 | 7.0 | FADAQNTALFK | D/N | 11 | b3b8b9*y1y5y5*y6y7y7*y9y10* | 12.4 |
| | 977.5304 | 0.1 | 8.6 | AIEAYLIAN | | 20 | b2b3b3*b4b4*b5b5*b6b6*b7b7*b8y1y1*y2y2*y3y4y5y7 | 4.1 |
| | 978.5134 | -0.8 | 8.0 | AIEAYLIAN | D/N | 11 | b2b3b4b4*b6b6*b7b8y1y2y7 | 6.6 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|------|-----|---|-----|----|---|------|
| 1730.856 | 0.5 | 7.6 | GAQSHSLEITSSVSAEK | | 13 | b2b3*b4*b9b12*y1y3y3*y4y7y7*y8y9 | 8.0 |
| 1615.93 | -0.2 | 9.0 | IFSGIVLDVDTVIPK | | 34 | b2b3b3*b4b4*b5b5*b6b6*b7b8b10b11b12b12*b13b13*b15y1y2y3y5y6y6*y7y8y8*y9y10y10*y11y12y13y14 | 5.2 |
| 1331.687 | -3.6 | 7.8 | SVEVKDGGGAGTVR | | 14 | b2*b10y1y2y4y5y6y7y8y10y11y12y12*y13 | 8.0 |
| 789.3849 | -0.1 | 7.8 | GDDGGAGTVR | | 15 | b2b2*b3b3*b4b4*b5b5*b6*b7b7*b8b8*y1y2 | 7.2 |
| 1730.936 | 0.3 | 9.3 | IITLPEGSPITSMTVR | O/M | 55 | b1b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*b9b9*b10b10*b11b11*b13b13*b14b14*b15b15*b16*y1y2y3y3*y4y4*y5y6y6*y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y13*y14y14*y15y15*y16 | 3.4 |
| 1714.939 | -1.2 | 8.8 | IITLPEGSPITSMTVR | | 49 | b1b2b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*b9b9*b10b10*b11b11*b12b12*b15y1y2y3y3*y4y5y5*y6y6*y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y14y14*y15y16 | 6.8 |
| 4089.134 | 4.3 | 7.0 | EALTYDSTVIDGDILLGFIESIETHLV VVPTADGGSITK | | 10 | b16b27b28b29y17*y19y20y30y31y39 | 10.9 |
| 918.5032 | -1.3 | 8.4 | TTAIFHTK | | 18 | b3*b6b6*b7b7*b8b8*y2y3y3*y4y4*y5y5*y6y6*y7y7* | 3.2 |
| 2070.077 | -4.6 | 7.0 | TTAIFHTKGDVVPEENIK | | 4 | b3*b19y3y6 | 5.0 |
| 1170.6 | -0.2 | 8.9 | GDAVVPEENIK | | 35 | b2b2*b3b3*b4b4*b5b5*b6b8b8*b9*b9*b10b11b11*b11*y2y3y3*y4y4*y5y5*y6y6*y6*y7y7*y8y8*y9y9*y9*y10 | 7.6 |
| 1225.622 | 0.2 | 8.7 | FADAQNTALFK | | 41 | b2b3b4b5b5*b5*b6b6*b6*b7b7*b7*b8b8*b8*b9b9*b9*b11b11*y1y2y3y4y5y5*y6y6*y6*y7y7*y7*y8y8*y8*y9y9*y9*y10y10*y10* | 4.5 |
| 1226.61 | 4.1 | 7.0 | FADAQNTALFK | D/N | 11 | b3b8b9*y1y5y5*y6y7y7*y9y10* | 12.4 |
| 977.5304 | 0.1 | 8.6 | AIEAYLIAN | | 20 | b2b3b3*b4b4*b5b5*b6b6*b7b7*b8y1y1*y2y2*y3y4y5y7 | 4.1 |
| 978.5134 | -0.8 | 8.0 | AIEAYLIAN | D/N | 11 | b2b3b4b4*b6b6*b7b8y1y2y7 | 6.6 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|----------|------|-----|-----------------|-----|----|--|------|
| A0A164SJC3 - DAUCS | 1431.731 | -1.4 | 6.1 | SAEILEGDDGGVGTK | | 16 | b2b3b4b5b9b12y3y4°y8°y9y9°y10y10°y11y12y15 | 8.22 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|------|-----|---|-----|----|--|------|
| 1730.856 | 0.5 | 7.6 | GAQSHSLEITSSVSAEK | | 13 | b2b3*b4*b9b12*y1y3y3*y4y7y7*y8y9 | 8.0 |
| 1615.93 | -0.2 | 9.0 | IFSGIVLDVDTVIPK | | 34 | b2b3b3*b4b4*b5b5*b6b6*b7b8b10b11b12b12*b13b13*b15y1y2y3y5y6y6*y7y8y8*y9y10y10*y11y12y13y14 | 5.2 |
| 1331.687 | -3.5 | 8.1 | SVDVKDGGGAGTIR | | 33 | b2b2*b3b4*b6b7b8b8*b9b9*b10b11b12b14y3y3*y4y4*y5y7y7*y8y8*y9y9*y10y10*y11y11*y12y12*y13y14 | 6.9 |
| 803.4014 | 1.0 | 7.0 | GDGGAGTIR | | 12 | b2b3b3*b4b4*b5b5*b6*b7*b8*y1y2 | 7.3 |
| 1732.915 | -0.2 | 9.0 | TITLPEGSPITMTVR | O/M | 47 | b2b2*b3b3*b4b4*b5b5*b6b6*b7b7*b8b8*b9b9*b10b11b12b13b14b15b15*y1y2y3y4y4*y5y6y6*y7y8y8*y9y10y10*y11y11*y12y12*y13y13*y14y14*y15y16 | 4.9 |
| 1716.92 | -0.2 | 8.9 | TITLPEGSPITMTVR | | 41 | b2b2*b3b3*b4b4*b5b5*b6b7b7*b8b8*b9b9*b10b11b11*b12b13b15y1y2y3y4y5y5*y6y6*y8y8*y9y10y11y12y12*y13y14y14*y15y16 | 7.1 |
| 4123.055 | -3.2 | 7.3 | EALTYDSTVIDGDILLGFIESIETHMV VVPTADGGSITK | O/M | 15 | b10*b13b18*b28b34y7y10y11y15*y17y17*y19y24y36y39 | 14.5 |
| 918.5032 | -1.3 | 8.4 | TTAIFHTK | | 18 | b3*b6b6*b7b7*b8b8*y2y3y3*y4y4*y5y5*y6y6*y7y7° | 3.2 |
| 2070.077 | -4.6 | 7.0 | TTAIFHTKDAVVPEENIK | | 4 | b3*b19y3y6 | 5.0 |
| 1170.6 | -0.2 | 8.9 | GDAVVPEENIK | | 35 | b2b2*b3b3*b4b4*b5b5*b6b8b8*b9*b9*b10*b11b11*b11*y2y3y3*y4y4*y5y5*y6y6*y6*y7y7*y8y8*y9y9*y9*y10 | 7.6 |
| 1283.626 | -0.5 | 7.8 | FADEQNTALFK | | 20 | b4b5b5*b6b6*b7b7*b7*b8b8*b9*b11y8y8*y8*y9y9*y10y10*y10° | 5.9 |
| 977.5304 | 0.1 | 8.6 | AIEAYLIAN | | 20 | b2b3b3*b4b4*b5b5*b6b6*b7b7*b8y1y1*y2y2*y3y4y5y7 | 4.1 |
| 978.5134 | -0.8 | 8.0 | AIEAYLIAN | D/N | 11 | b2b3b4b4*b6b6*b7b8y1y2y7 | 6.6 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------|----------|------|-----|-------------------|-----|----|--|-------|
| | 1337.641 | -1.3 | 8.1 | TEAEVTSSVSAEK | | 21 | b1b2b2°b4b4°b5b5°b6°b7b10y2y2°y3y4y5y7y8y9y10y11y13 | 5.542 |
| | 1435.69 | -0.5 | 8.7 | SSETLEGDGGVGTVK | | 48 | b2b2°b3°b4b4°b5b5°b6b6°b7b7°b8b9b10b10°b11b11°b12b12°b13b14b14°b15b15°y1y2y3y4y4°y5y5°y6y7y7°y8y8°y9y9°y10y10°y11y11°y12y12°y13y13°y14y14° | 4.415 |
| A0A1G1X1M2 - DAUCS | 1183.645 | -2.0 | 7.7 | LVHLGDASPFK | | 10 | b3b4b6b10y3y5y7y8y10y11 | 5.103 |
| | 2063.064 | -0.9 | 7.6 | STIFNTKGDVVPEENIK | | 17 | b4°b5°b7°b12b12°b13y3y3*y5y6y9y11y11*y15y17y18y19 | 7.85 |
| | 911.4805 | -3.1 | 7.4 | STIFNTK | | 17 | b2b2°b3°b4°b5°b6°b8y2y2°y3y4y4*y5y5°y6y6°y7° | 5.04 |
| | 1170.6 | -0.2 | 8.9 | GDAVVPEENIK | | 35 | b2b2°b3b3°b4b4°b5b5°b6b8b8°b9°b9°b10°b11b11*b11°y2y3y3*y4y4°y5y5°y6y6*y6°y7y7°y8y8°y9y9*y9°y10 | 7.604 |
| | 1422.762 | -0.4 | 6.6 | AIFQAVEAYLIAN | | 8 | b5b7b8y2y2*y6*y9y13* | 13.74 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

| MH+ | PE | S | Sequence | Mod | F | BY | FE |
|----------|-------|-----|--|-------------|----|--|------|
| 1347.664 | 0.1 | 8.8 | TEVEAPSTVSAEK | | 41 | b1b2b2°b3b3°b4b4°b5b5°b6b6°b7b7°b8b8°b9b11b11°b12b12°b13b13°y1y2y2°y3y3°y4y4°y6y7y8y8°y9y9°y10y10°y11y11°y12y12° | 4.3 |
| 1836.854 | -0.4 | 7.6 | MYQGFLDMDTVFPK | O/M, O/M | 13 | b2b3b4b5b6y2y3y8y9y10y12y13y15* | 7.5 |
| 1820.854 | -3.1 | 6.6 | MYQGFLDMDTVFPK | O/M | 8 | b4°b5b12b14b15y3y7y8 | 10.9 |
| 1804.832 | -18.3 | 6.5 | MYQGFLDMDTVFPK | | 3 | b4°b8b13° | 11.4 |
| 810.5439 | -1.1 | 7.8 | VLPQLIK | | 12 | b2b4b5b5°b6y1y2y3y4y4°y6y7 | 2.0 |
| 1487.771 | 0.4 | 9.0 | SVEILEGGVGTVR | | 45 | b2b3b3°b4b4°b5b5°b6b6°b7b7°b8b9b9°b10b10°b11b11°b12b12°b13b13°b14b14°b15y1y2y3y4y4°y5y6y7y7°y8y9y9°y10y10°y11y11°y12y12°y13y14 | 2.7 |
| 1608.791 | -1.8 | 8.5 | LVHLGEATEYTTMK | O/M | 28 | b2b3b4b5b6b7b8b8°b9b9°b10b13y1y2y3y3°y4y5y6y6°y7y7°y8y8°y11y12y12°y14 | 6.2 |
| 1592.798 | -0.8 | 8.4 | LVHLGEATEYTTMK | | 27 | b3b4b5b6b6°b7b8b8°b9b9°b10b11y1y2y3y4y4°y5y6y6°y7y8y10y11y11°y12y14 | 7.2 |
| 944.5394 | -1.9 | 7.2 | QKVDVIDK | | 11 | b2°b3°b4°b4°b5°y2y2°y3y5y6y8 | 5.1 |
| 3942.014 | 1.3 | 6.5 | AGLGYTYTTIGGDILVEGLESVVNQF VVVPTDGGCIVK | D/N, C/C | 6 | b8b22°b27b28y6y13 | 11.0 |
| 1878.942 | -1.1 | 6.9 | NTTIYNTKGDVLPEDK | | 9 | b4°b6°b7y4y11y14y14°y15y17 | 13.6 |
| 1170.635 | -0.9 | 8.2 | GDAVLPEDKVK | | 13 | b2b3b6b10y1y2y3y5y6y7y7°y8y9 | 4.6 |
| 943.4722 | -0.9 | 7.5 | GDAVLPEDK | | 6 | y3y3°y4y5y6y7 | 8.3 |
| 636.3715 | 0.0 | 7.6 | SALAFK | | 10 | b2b2°b3b3°b4°b5b5°y2y4y5 | 7.6 |
| 963.5147 | 0.2 | 8.2 | AVEAYLLAN | | 19 | b2b3b3°b4b5b5°b6b6°b7b8b8°b9b9°y1y1°y4y4°y5y7 | 7.5 |
| 964.4976 | -1.0 | 7.3 | AVEAYLLAN | D/N | 7 | b2b4b5b6b7b8y1* | 12.7 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

Table S4-4: Summary of mass spectrometric fragmentation data carrot extract in 10 mM sodium citrate, pH 3, 95°C. Red colour indicates peptides which are specific for the protein indicated on the left. No annotated spectra were displayed because no deviating or additional protein sequences were detected compared to the results at ambient temperature and pH 7.

131

| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|------------------------|-----------|------|-----|---|-----|----|--|------|
| CAB03715 - Dauc 1.0103 | 1615.929 | -1.0 | 6.3 | IFSGIVLDVDTVIPK | | 7 | b3b13y2y8y9y10y13 | 10.4 |
| | 1730.9312 | -2.7 | 6.8 | IITLPEGSPITSMTVR | O/M | 12 | b2b3°b4°b8y3y6y7y8y12y12°y13y14 | 4.7 |
| | 1714.9366 | -2.4 | 6.5 | IITLPEGSPITSMTVR | | 9 | b3b3°y3y5y6y8y9y10y12 | 7.6 |
| | 4179.0825 | -2.8 | 6.4 | EALTYDSTVIDGDILLEFIESIETHMVVVPTADGGSITK | | 13 | b4b25°b28b28°b29b39°y6y10y11y12y21y29y34 | 11.3 |
| | 4195.076 | -3.0 | 6.3 | EALTYDSTVIDGDILLEFIESIETHMVVVPTADGGSITK | O/M | 7 | b28b37°y10y11y15y22y39 | 9.2 |
| | 918.5016 | -3.0 | 6.0 | TTAIFHTK | | 7 | b2°b3y4y4°y5y6y6° | 12.0 |
| | 1170.5986 | -1.3 | 7.2 | GDAVVPEENIK | | 19 | b2b3b4b5b9b9°y1y2y3y3°y4y4°y6y6°y6°y7y8y9y11 | 10.5 |
| | 1225.6202 | -0.7 | 6.7 | FADAQNTALFK | | 17 | b2b3b9°y1y2y3y5y6y6°y7y7°y8y8°y9°y9°y10°y11 | 8.2 |
| | 977.5297 | -0.6 | 7.1 | AIEAYLIAN | | 14 | b2b3b4b5b6b6°b7b8y1y2°y3°y4y5y9° | 8.2 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

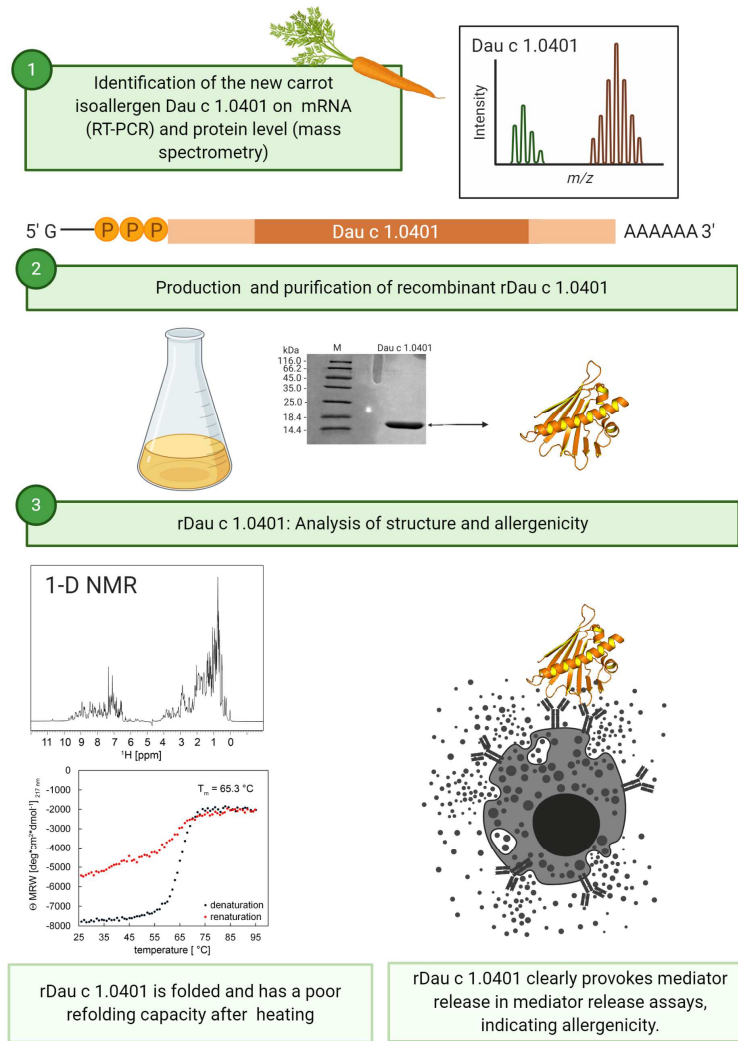
| | MH+ | PE | S | Sequence | Mod | F | BY | FE |
|--------------------------|-----------|-----|-----|------------------|-----|---|------------------------|------|
| Q8SAE7 - Dau c 1.0201 | 1347.6672 | 2.5 | 5.4 | TEVEAPSTVSAEK | | 4 | y8y9y10y13 | 5.1 |
| | 1487.7701 | 0.1 | 5.7 | SVEILEGDDGGVGTVR | | 9 | b3b5b9y7y9y10y11y12y15 | 11.7 |

MH+: molecular mass of singly protonated & singly charged precursor [Da] S: PLGS peptide score; PE: peptide mass error [ppm]; Mod: variable and fixed modifications. D/Q & D/N indicate deamidation of asparagine & glutamine residues, O/M indicates a oxidation of methionin and C/C a carbamidomethylation of cystein residues F: indicates the number of fragments detected, FE: mean fragment mass error [ppm]

8.3 Einzelarbeit C

*Thessa Jacob, *Andrea Wangorsch, Andreas Reuter, Lothar Vogel, Vera Mahler, Birgitta M. Wöhr (2021). A novel isoallergen Dau c 1.0401 in carrot: stability, allergenicity and comparison of isoallergens. *Molecular Nutrition & Food Research*, e2001110

**contributed equally*



A new carrot isoallergen, designated Dau c 1.0401 was identified by RT-PCR with mRNA isolated from carrots using primers specific for the 5' and 3' untranslated mRNA regions. Mass spectrometry confirmed the presence of the isoallergen in natural Dau c 1. The recombinant protein was characterized by NMR and CD spectroscopy. Mediator release assays demonstrated its allergenicity.

A Novel Isoallergen Dau c 1.0401 in Carrot: Stability, Allergenicity, and Comparison with Other Isoallergens

Thessa Jacob, Andrea Wangorsch, Lothar Vogel, Andreas Reuter, Vera Mahler, and Birgitta M. Wöhrl*

Scope: Around 25% of food allergic persons in Central Europe suffer from carrot allergy caused by the major carrot allergen Dau c 1. Three different isoallergens, Dau c 1.01, Dau c 1.02 and Dau c 1.03 are identified. However, information about the qualitative and quantitative composition of natural (n)Dau c 1 is scarce.

Methods and Results: The new carrot allergen Dau c 1.0401 is identified on the mRNA and protein level by RT-PCR and mass spectrometry. It displays only around 60% sequence identity to the other known Dau c 1 isoallergens. NMR and CD-spectra are typical for a well-folded protein containing both α -helices and β -strands. It showed a poor refolding capacity after incubation at 95 °C. IgE-binding is impaired in immunoblots, whereas in inhibition assays IgE binding to soluble Dau c 1.0401 is detected and it clearly provoked a response in mediator release assays.

Conclusion: Dau c 1.0401 is a new isoallergen which contributes to the allergenicity of carrots. The absence of immunoreactivity in immobilized assays indicates that IgE binding is impaired when the protein is blotted on a solid phase. Altogether, the results point out that its allergenicity can be reduced upon carrot processing.

Survey^[1] and around 24% of food-allergic persons in Central Europe suffer from allergy to carrot,^[2,3] which is one important type of pollen-related food allergies.^[4-6] The major carrot allergen in Central Europe is Dau c 1, a Bet v 1-homologous allergen.^[7] Both allergens belong to the family of pathogenesis related proteins 10 (PR-10).^[8] Usually, patients allergic to Bet v 1-homologous allergens are sensitized to the major birch pollen allergen Bet v 1, but show allergic cross-reactions after the consumption of other fruits, nuts or vegetables, e.g. celery, apple or hazelnut.^[2,4,5,9,10] Bet v 1-homologous allergens share a high structure and sequence similarity.^[11] Certain IgE antibodies against Bet v 1 thus, recognize homologous allergens, causing cross-reactivity. Allergic symptoms to Dau c 1 are very often limited to the oral cavity (oral allergy syndrome, OAS),^[12] but in many cases, systemic reactions were also observed.^[7,13] In contrast to the other Bet

v 1-homologous allergens, Dau c 1 can induce food allergy independently from previous sensitization by Bet v 1.^[14]

To date, three different Dau c 1 isoallergens (Dau c 1.01, Dau c 1.02 and Dau c 1.03) are known. Dau c 1.01 includes five variants, Dau c 1.0101 to Dau c 1.0105. Dau c 1.0104 is a highly IgE-reactive variant and elicits allergic reactions in 98% of patients allergic to carrots in Central Europe, whereas 65% of the study population react to the Dau c 1.0201 isoallergen.^[15] An interesting study showed a reduced, but not completely abolished, allergenicity of transgenic carrot plants in which the Dau c 1.01 and Dau c 1.02 genes were silenced, indicating that additional isoallergens must be present.^[16] In 2012, the identification of the novel isoallergen Dau c 1.0301 was published.^[17]

For Bet v 1, 18 different isoallergens and variants have been unambiguously identified so far^[18] and recently, a quality and potency profile of eight recombinant isoallergens which largely mimic the total Bet v 1 specific IgE binding of birch pollen has been established.^[19] Most likely, other PR-10 allergens also consist of many isoallergens and variants which have not been identified yet.

So far, no studies exist on the qualitative and quantitative composition of Dau c 1 isoallergens and variants present in carrot. However, a detailed analysis of the allergen composition in carrots is necessary in order to improve recombinant allergen formulations for diagnosis and therapy.

1. Introduction

The prevalence of sensitization to carrot was identified to be at 3.6% in the European Community Respiratory Health

T. Jacob, Prof. B. M. Wöhrl
Biochemie IV - Biopolymere
Universität Bayreuth
Universitätsstr. 30, Bayreuth D-95447, Germany
E-mail: birgitta.woehrl@uni-bayreuth.de

Dr. A. Wangorsch
Molecular Allergology
Paul-Ehrlich-Institut
Langen D-63225, Germany

Dr. L. Vogel, Dr. A. Reuter, Prof. V. Mahler
Division of Allergology
Paul-Ehrlich-Institut
Langen D-63225, Germany

 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/mnfr.202001110>

© 2021 The Authors. Molecular Nutrition & Food Research published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

DOI: 10.1002/mnfr.202001110

```

T1   T6           T19T22         T34   T41   T46           T55           T69 T71
>sp|CAB03715|Dauc 1.0103  MGAQSxHSLEITSSVSAEK  IFSGIVLDVDTVIPK  AAPGAYK  SVDVK  GDGGAGTVR  IITLPEGSPITSMTVR  TDAVVK
>sp|Q8SAE7|Dauc 1.0201  MGVQK  TEVEAPSTVSAEK  MYQGFLLDMDTVFPK  VLPQLIK  SVEILEGDDGGVGTVR  LVHLGEATEYTTMK  QK  VDVIDK
>tr|A0A161X1M2|Dau c 1.0401  MGVQK  TEAEVTSVSAEK  LFK  ALCLDIDTLLPQxVLPGAIK  SSETLEGDDGGVGTVK  LVHLGDASPFK  TMK  QK  VDVIDK
T77   T81           T98   T102         T116   T124         T135 T142   T146
>sp|CAB03715|Dauc1.0103  EALTYDSTVIDGDILLEFIESxxxIETHMVFVPTADGGSITK  TTAIFHTK  GDAVVPEENIK  FADAQNTALFK  AIEAYLIAN
>sp|Q8SAE7|Dauc1.0201  AGLGYTYTTIGGDILVEGLSxxxVNVQFVVVPTDGGCIVK  NTTIYNTR  GDAVLPEDK  VK  EATEK  SALAFK  AVEAYLIAN
>tr|A0A161X1M2|Dau c 1.0401  ESFTYAYSIIIDGDIILGFIESxxxINNHFAYVNPADGGCTVK  STITFNTR  GDAVVPEENIK  FANDQNR  AIFQxAVEAYLIAN

```

Figure 1. Amino acid sequence alignment of known Dau c 1 isoallergens and the Dau c 1.0401 sequence identified by LC-MS^E. Differing sequence stretches are colour-coded. Peptides which were detected by LC-MS^E are underlined. 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. Gaps indicate trypsin cleavage sites. An X indicates a missing trypsin cleavage site and was used to adjust the alignment.

Peptide mass fingerprints (PMF) of purified natural (n)Dau c 1 isolated from carrot revealed that the protein mixture contains at least eight isoallergens/variants that have not been identified previously.^[20] This study describes the detection and biochemical, biophysical and immunological characterization of a new Dau c 1 isoallergen, designated and accepted by the WHO/IUIS Allergen Nomenclature Sub-Committee as Dau c 1.0401.

2. Results and Discussion

2.1. Cloning and Sequence Analysis of the New Dau c 1.0401 Isoallergen

Recently, we thoroughly investigated the stability of nDau c 1 isolated from carrot roots and of several recombinant (r)Dau c 1 isoallergens at different pH values and temperatures.^[20] In the course of this project, LC-MS^E was performed to identify the components of nDau c 1.^[20] Surprisingly, the isoallergen/variant composition of nDau c 1 was more complex than previously thought. Nevertheless, the known isoallergens Dau c 1.0103 and Dau c 1.0201 could be unambiguously identified in the purified nDau c 1 mixture (**Figure 1**). Numerous Dau c 1-like peptides showed sequence similarity to known Dau c 1 isoallergens but could not be assigned to any of them. However, using this method we were able to confirm the existence of the novel Dau c 1.0401 protein with a sequence coverage of 81% (Figure 1).^[20] The hypothetical protein was already deposited (UniProt entry A0A161x1M2) and derived from a whole shotgun entry of carrot (EMBL:KZM86183.1). To confirm the complete sequence, we isolated RNA from carrot roots, and performed RT-PCR using a polyT primer to obtain cDNA derived solely from mRNA. Using the shotgun entry data set, 5' and 3' primers complementary to the untranslated regions (UTRs) of the putative Dau c 1.0401 mRNA were designed and used for amplification (Figure S1, Supporting Information). The resulting DNA fragment was cloned into the pCRBlunt vector and sequenced (Figure S1, Supporting Information). Amino acid sequence comparison with the protein from the shotgun entry exhibited 100% sequence identity; thus, confirming the presence of the molecule on the mRNA level. Dau c 1.0401 has an amino acid identity of approximately 60% to Dau c 1.0301, Dau c 1.01 variants and Dau c 1.0201 (**Table 1**). Dau c 1.0401 comprises 155 amino acids, has a deduced molecular weight of 16,620 Da and a theoretical pI of 4.66. Figure 1 shows the alignment of known Dau c 1

Table 1. Amino acid identity matrix of all known and listed Dau c 1 isoallergens and variants and the new isoallergen Dau c 1.0401.

| Dau c 1 | 0.0301 | 0.0103 | 0.0104 | 0.0105 | 0.0102 | 0.0201 | 0.0401 |
|---------|--------|--------|--------|--------|--------|--------|--------|
| .0301 | 100.0 | 70.1 | 70.1 | 71.4 | 69.5 | 49.0 | 61.0 |
| .0103 | 70.1 | 100.00 | 96.1 | 97.4 | 96.7 | 51.0 | 58.4 |
| .0104 | 70.1 | 96.1 | 100.0 | 98.7 | 98.0 | 51.0 | 58.4 |
| .0105 | 71.4 | 97.4 | 98.7 | 100.0 | 98.0 | 51.0 | 59.1 |
| .0102 | 69.5 | 96.7 | 98.0 | 98.0 | 100.0 | 51.0 | 57.8 |
| .0201 | 49.0 | 51.0 | 51.0 | 51.0 | 51.0 | 100.0 | 59.7 |
| .0401 | 61.0 | 58.4 | 58.4 | 59.1 | 57.8 | 59.7 | 100.0 |

Sequences with less than 67% identity are highlighted in dark blue, between 67 and 90% in light blue and above 90% in cyan. Data are based on the LC-MS^E data from.^[20]

isoallergens and the Dau c 1.0401 sequence identified previously by LC-MS^E.^[20]

2.2. Physicochemical Characterization of rDau c 1.0401

We have previously shown by analysing recombinant proteins that the protein stability of different Dau c 1 isoallergens/variants varies significantly. Some of them, i.e., rDau c 1.0104 or rDau c 1.0105, regain their three-dimensional structure after heating at pH 7, whereas rDau c 1.0201 does not.^[20] To analyze the thermostability of rDau c 1.0401, we expressed the tagless, recombinant (r) protein in *E. coli* and purified it to homogeneity (Figure S2, Supporting Information). Its identity was confirmed by LC-MS^E (Table S1).

Proper 3D-folding of rDau c 1.0401 was demonstrated by a 1D-NMR spectrum, which reveals the signal dispersion typical for a well-folded protein (**Figure 2A**). Analytical size exclusion chromatography (SEC) showed one single peak indicating homogeneity of the purified protein. The determined molecular weight of approx. 24 kDa (Figure 2B) implies that rDau c 1.0401 is a monomer and does not form oligomers under the experimental conditions. The higher molecular weight compared to the theoretical (16.6 kDa) and the molecular weight determined by SDS-PAGE (16 kDa, Figure S2) is a consequence of the shape of the protein and/or hydration by solvent molecules.

To investigate the refolding capacity of the protein upon heating we recorded CD-spectra of rDau c 1.0401. The spectrum at 25 °C (Figure 2C, black curve) is typical for a protein with α -helices and β -strands and closely resembles the spectra of the other known Dau c 1 isoallergens.^[20] At 95 °C, the protein was unfolded (Figure 2C, blue curve) and after recooling to 25 °C, it

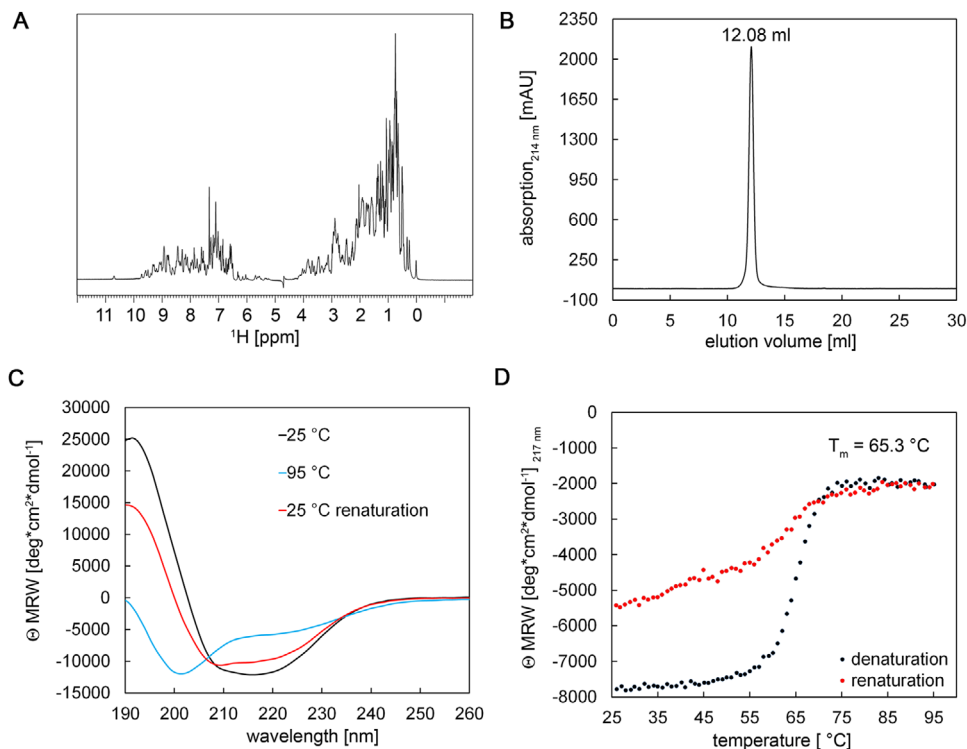


Figure 2. Physicochemical characterization of rDau c 1.0401. A) 1D NMR spectrum B) Analytical SEC using a Superdex 75 GL10/300 column C) CD spectra at 25 °C (black curve), 95 °C (blue curve) and after recooling to 25 °C (red curve), D) denaturation (black) and renaturation (red) curve of Dau c 1.0401.

only partially regained secondary structure elements (red curve). The denaturation and renaturation curves confirmed the partial refolding. A melting temperature of 65.3 °C could be determined (Figure 2D) which was verified by nanoDSF (Supplementary Figure S3). The T_m is 5–10 °C higher than the T_m values obtained previously for other known Dau c 1 isoallergens.^[20] For Bet v 1.0101, it could be demonstrated that increased fold stability of the allergen increases its allergenicity.^[21]

2.3. IgE Binding to rDau c 1.0401

To investigate binding of IgE antibodies to rDau c 1.0401 we carried out several experiments with 19 sera of carrot-allergic patients that exhibited mild to severe clinical symptoms. Sensitization to carrot was either tested by open food challenge, skin prick testing or prick-to-prick testing (carrot cv *Rodelika*) (Table 2). IgE immunoblots using non-reducing and reducing (30 mM DTT) conditions for the SDS-PAGE were performed. Interestingly, using the sera listed in Table 2, no bands or only very weak bands (#29, reducing conditions; #14 both conditions) could be detected in the immunoblot (Figure S4). Similar results were obtained when we carried out dot blots with purified rDau c 1.0401 (Supplementary Figure S5). Here the protein is directly applied to the nitrocellulose membrane without prior denaturing.

Additionally, we performed enzyme-linked immunosorbent assays (ELISAs) with rDau c 1.0401 (Supplementary Figure S6, A and B, respectively). The isoallergen rDau c 1.0104 was used as a positive control since this isoallergen was shown to possess

the strongest IgE-binding capacity.^[15] Weak IgE-binding to Dau c 1.0104 could be observed, whereas IgE-binding to the rDau c 1.0104 control, was at least 10-fold higher. Thus, we postulated that the protein either does not effectively bind IgE at all or irreversibly changes its structure when applied to solid phases, which in turn impairs IgE-binding or renders epitopes inaccessible to IgE antibodies. This corresponds to the results from CD spectra (Figure 2C) which showed incomplete refolding of the denatured protein after heating and cooling.

2.4. rDau c 1.0401 Triggers Mediator Release

To investigate whether rDau c 1.0401 is yet a functional allergen, we performed mediator release assays (MRAs) with 19 sera of carrot-allergic patients (Table 2). In contrast to the experiments described above, here, rDau c 1.0401 is kept in solution. Six of the sera tested (#5, #14, #15, #25, #29, #44) induced mediator release upon rDau c 1.0401 addition (Figure 3). Four of them exhibited a strong reaction, proving the allergenicity of rDau c 1.0401.

2.5. rDau c 1.0401 Inhibits IgE-Binding of rDau c 1.0104

To confirm the results obtained with the MRAs (Figure 3) and to unambiguously verify the IgE-binding properties of rDau c 1.0401, we resorted to inhibition assays, which show whether soluble rDau c 1.0401 preincubated with serum can thereby inhibit IgE-binding of immobilized rDau c 1.0104. Of course,

Table 2. Sera used in this study.

| No. Patient | Clinical symptoms to carrot symptoms | Provocation OFC | Skin test Carrot | CAP [kU _A L ⁻¹] (CAP class) | | | | | |
|----------------|--------------------------------------|--------------------|---------------------|--|----------------|----------------|----------------|---------------|----------|
| | | | | Carrot (F31) | r Bet v 1.0101 | r Dau c 1.0104 | r Dau c 1.0201 | rDau c 1.0301 | rDau c 4 |
| 4 | OAS, CU | Pos | Pos | 0.10 (0) | 6.62 (3) | 0.00 (0) | 2.04 (2) | 0.04 (0) | 0.01 (0) |
| 5 | OAS | Nd | Pos | 2.76 (2) | >100 (6) | 5.82 (3) | 17.00 (3) | 6.13 (3) | 0.05 (0) |
| 14 | OAS (mild) | Pos | Pos | 0.07 (0) | 43.10 (4) | 5.25 (3) | 4.32 (3) | 9.75(3) | 2.11 (2) |
| 15 | OAS | Pos | Pos | 2.82 (2) | 35.90 (4) | 3.70 (3) | 4.32(3) | 3.57 (3) | 0.09 (0) |
| 21 | OAS | Nd | Pos | 0.79 (2) | 7.56 (3) | 1.17 (2) | 1.43 (2) | 1.38 (2) | 0.02 (0) |
| 22 | U, D | Pos | Pos | 0.71 (2) | 2.58 (2) | 0.02 (0) | 0.17 (0) | 0.09 (0) | 0.05 (0) |
| 25 | OAS | Pos | Pos | 5.20 (3) | 55.80 (5) | 10.50 (3) | 4.90 (3) | 7.93 (3) | 1.48 (2) |
| 26 | Q, D, U | Nd | Nd | 0.05 (0) | 7.14 (3) | 0.85 (2) | 0.20 (0) | 0.67 (1) | 0.03 (0) |
| 27 | GI, Q | Nd | Neg | 0.25 (0) | 16.60 (3) | 0.82 (2) | 0.40 (1) | 3.12 (2) | 0.05 (0) |
| 28 | OAS | Pos | Pos | 2.20 (2) | 9.28 (3) | 2.53 (2) | 1.55 (2) | 2.61 (2) | 0.88 (2) |
| 29 | OAS | Pos | Pos | 2.51 (2) | 11.90 (3) | 1.95 (2) | 0.77 (2) | 0.92 (2) | 5.31 (3) |
| 30 | OAS | Pos | Pos | 0.62 (1) | 5.35 (3) | 0.643 (1) | 0.91 (2) | 0.73 (2) | 0.05 (0) |
| 31 | OAS, D | Pos | Pos | 0.44 (1) | 3.16 (2) | 0.59 (1) | 0.45 (1) | 0.48 (1) | 0.22 (0) |
| 38 | OAS | Pos | Pos | 1.05 (2) | 10.50 (3) | 0.69 (1) | 1.06 (2) | 1.06 (2) | 0.04 (0) |
| 39 | OAS | Nd | Nd | 0.13 (0) | 0.00 (0) | 0.00 (0) | 0.01 (0) | 0.03 (0) | 0.00 (0) |
| 40 | OAS, GI | Pos | Neg | 0.42 (1) | 29.40 (4) | 1.29 (2) | 2.28 (2) | 1.28 (2) | 0.03 (0) |
| 44 | OAS | Pos | Pos | 24.00 (4) | >100 (6) | 28.40 (4) | 16.60 (3) | 28.40 (4) | 0.54 (1) |
| 46 | OAS | Pos | Pos | 8.16 (3) | 0.06 (0) | 4.86 (3) | 14.40 (3) | 6.92 (3) | 0.18 (0) |
| 47 | OAS | n.d. | Neg | 0.02 (0) | 0.05 (0) | 0.02 (0) | 0.03 (0) | 0.01 (0) | 0.06 (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.

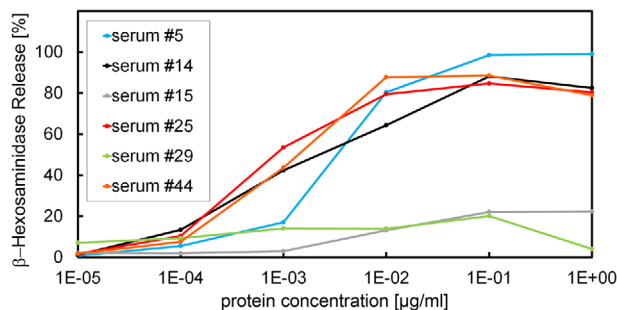


Figure 3. MRAs of rDau c 1.0401 with sera of six carrot-allergic patients. Patient sera (serum #5, blue lines; #14, black lines; #15, grey lines; #25, red lines; #29, green lines; #44, brown lines) were incubated with increasing amounts of rDau c 1.0401.

a prerequisite for a positive result is the presence of common epitopes between the two tested isoallergens.

We tested sera from four carrot allergic patients (sera #14, #15, #25, #44) that induced mediator release upon rDau c 1.0401 addition (Figure 3). They were preincubated either with rDau c 1.0104 (self inhibition) or rDau c 1.0401 to perform dose-dependent IgE competition assays (Figure 4) in which rDau c 1.0104 was used on the solid phase. Pre-incubation with each of the two isoallergens resulted in IgE inhibition. After self inhibition 100% inhibition of IgE-binding was achieved at 10 µg

of rDau c 1.0104 in the solution. For rDau c 1.0401 a similar inhibitory effect occurs only with serum #25, whereas the other three sera show less inhibition. Our data indicates that IgEs of serum #25 recognize the same or similar epitopes of rDau c 1.0104 and rDau c 1.0401, whereas in sera #14, #15, and #44 additional types of IgE antibodies are present that recognize solely epitopes on rDau c 1.0104 but not on rDau c 1.0401. As IgE-binding of rDau c 1.0401 is impaired when blotted on a membrane (Supplementary Figure S4-S6), it was not possible to perform inhibition assays in the other direction, i.e., using immobilized rDau c 1.0401.

In summary, these results suggest that rDau c 1.0401 harbors epitopes that are modified, denatured or not accessible when the allergen is bound to solid membranes, but can be recognized by IgEs if the native allergen is used in inhibition assays or MRAs. We assume that this behavior can occur also with other PR-10 allergens and this needs to be considered if the allergenicity of new isoallergens is determined.

2.6. Comparison of Dau c 1 Isoallergens

To compare the allergenicity of the new allergen rDau c 1.0401 with other known Dau c 1 isoallergens, we performed MRAs with purified different recombinant Dau c 1 isoallergens using five different sera (Figure 5). Intriguingly, serum #14 (black), #44 (brown), #5 (blue) and #25 (red) reacted with rDau c 1.0104,

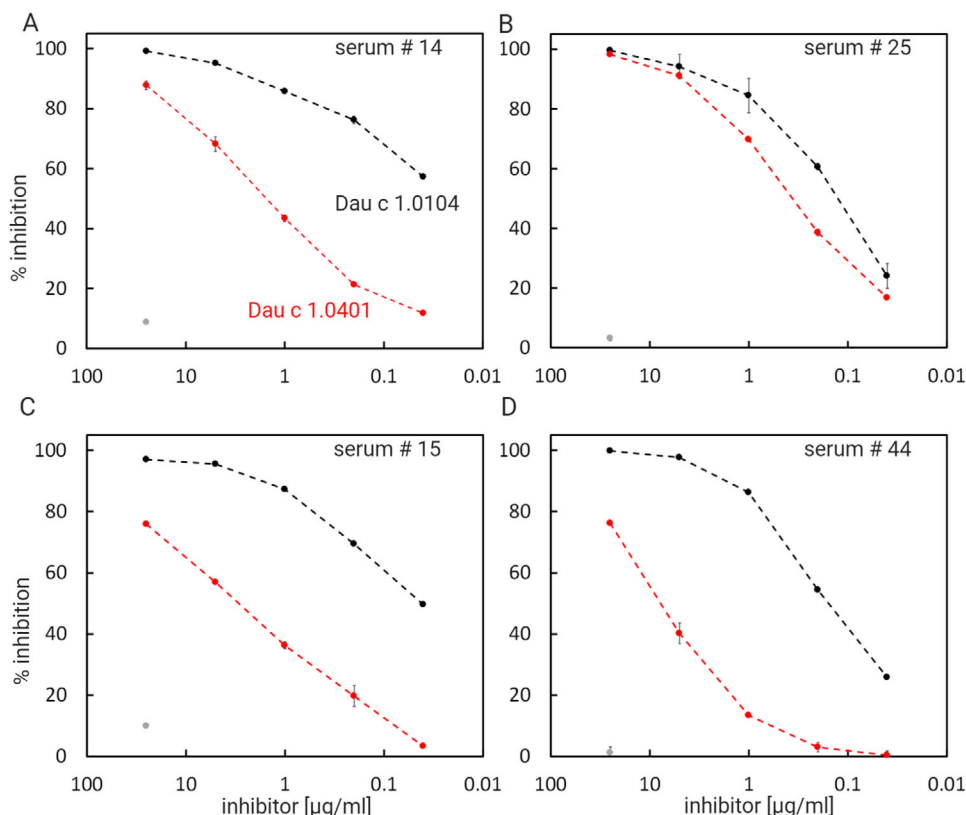


Figure 4. Inhibition of IgE-binding to rDau c 1.0104. Sera from carrot allergic patients (as indicated) were incubated with increasing amounts of either rDau c 1.0104 (black curves, self inhibition) or rDau c 1.0401 (red curves). The ELISAs were performed with rDau c 1.0104 on the solid phase. IgE binding of rDau c 1.0104 after pre-incubation with 25 $\mu\text{g mL}^{-1}$ BSA as a negative control is shown as a grey data point.

rDau c 1.0301, rDau c 1.0401 and nDau c 1, suggesting that those isoallergens possess common epitopes. Remarkably, serum #44 (brown lines) was the only one that reacted with rDau c 1.0105, although this variant only differs in two amino acids from Dau c 1.0104 (D43E and E139A, depicted in red in **Figure 6A,C**), implying that those amino acids might be crucial for epitope recognition.

Serum #46 (Figure 5 grey lines) reacted exclusively with rDau c 1.0201, indicating that this isoallergen contains unique epitopes. Since serum #46 (grey lines) exhibited no reaction with nDau c 1, we assume that the concentration of Dau c 1.0201 in purified nDau c 1 is too low to induce a reaction. Apart from serum #5 (blue lines), which shows weak reactivity, none of the other sera tested reacted with rDau c 1.0201.

We suggest that epitopes may be present which are conserved between rDau c 1.0104, rDau c 1.0201 and rDau c 1.0301, but not rDau c 1.0201. Figure 6 shows an amino acid sequence comparison of all tested Dau c 1 isoallergens. Homologous amino acid stretches exist that are present in all rDau c 1 isoallergens except rDau c 1.0201 (cyan) and thus might form epitopes which are not present in rDau c 1.0201.

3. Concluding Remarks

Dau c 1.0401 was accepted by the WHO/IUIS Allergen Nomenclature Sub-Committee as a new Dau c 1 isoallergen. Our results

prove that Dau c 1.0401 functions as an allergen as shown by MRAs and inhibition assays. The fact that IgE-binding is inhibited when rDau c 1.0401 is applied to a solid phase is rather unusual and implies that false negative results have to be considered if IgE-binding of novel potential allergens is solely analyzed with methods that require the protein to be fixed onto a solid phase. Our data imply that after membrane binding of Dau c 1.0401 most epitopes are no longer accessible or are denatured. As CD spectra demonstrate that the 3-D structure of rDau c 1.0401 is lost when heated and is not completely recovered when cooled down to room temperature, Dau c 1.0401 concomitantly will lose its allergenic potential upon heating. However, other thermostable isoallergens present in nDau c 1 can still provoke an allergic reaction. The knowledge of new isoallergens and their biophysical and immunological behavior is key to the improvement of component resolved diagnosis of carrot allergy and to the generation of potential hypoallergens for food allergy immunotherapy.

4. Experimental Section

Identification of Dau c 1.0401 mRNA: RNA was isolated from approx. 200 mg of freeze-dried, ground carrots using the RNA plant extraction mini kit (Qiagen, Hilden, Germany). Subsequently, total cDNA was prepared using the SuperScript IV First-Strand Synthesis System with ezDNase Enzyme (Thermo Fisher, Schwerte, Germany).

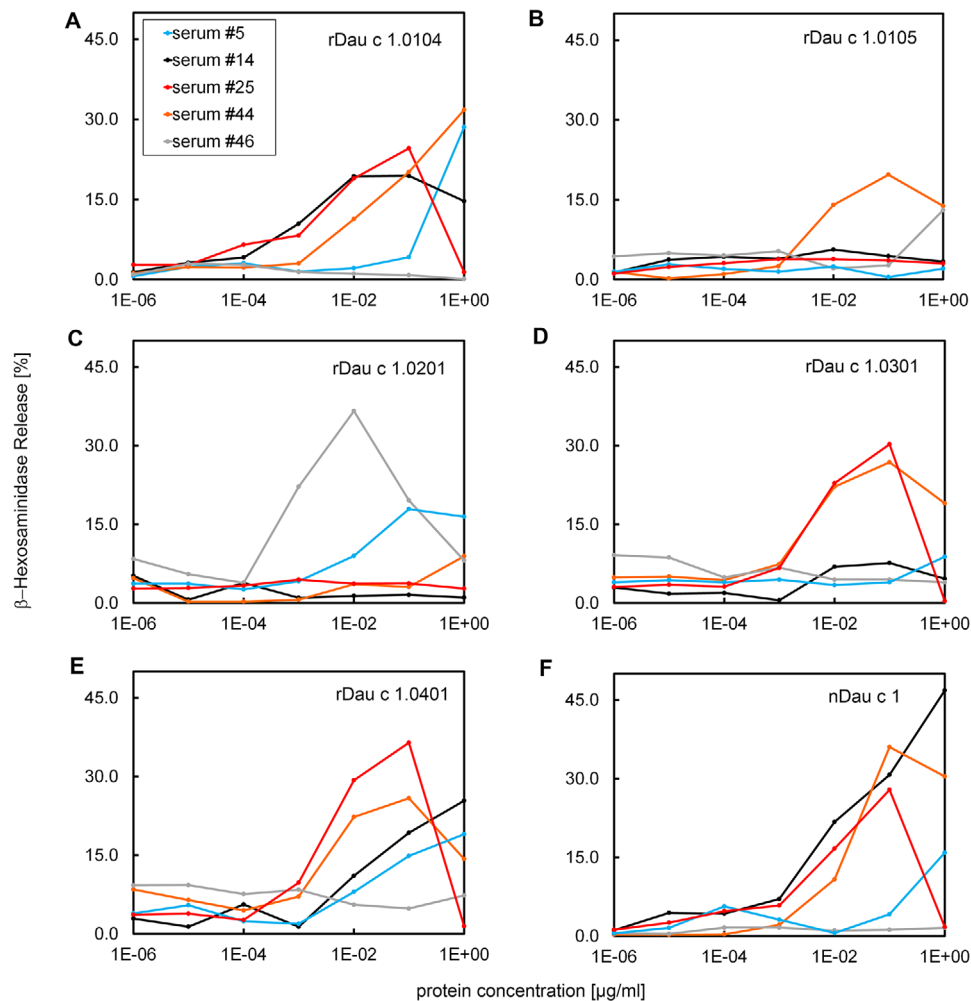


Figure 5. MRAs with different Dau c 1 proteins and sera of five carrot-allergic patients. Patient sera (serum #5, blue lines; #14, black lines; #25, red lines; #44, brown lines; #46, grey lines) were incubated with increasing amounts of Dau c 1 proteins.

Primers in the 5' UTR (5' -CACAGCATTCTTGATAAGCTC, forward) and 3' UTR (5' -CCATCCAGGTCTCACGAACA, reverse) region of the Dau c 1.0401 protein (Uniprot Accession number: A0A161X1M2) were designed based on its hypothetical mRNA (https://www.ncbi.nlm.nih.gov/nucore/XM_017360362.1). Dau c 1.0401 cDNA was amplified by PCR and cloned into the pCR-Blunt vector using the Zero Blunt PCR Cloning Kit (Thermo Fisher). The Dau c 1.0401 sequence of isolated plasmids was confirmed by Sanger Sequencing (Eurofins, Ebersberg, Germany).

Cloning, Expression and Purification of rDau c 1.0401: A codon-optimized Dau c 1.0401 gene strand was purchased (Eurofins) and cloned into the bacterial expression vector pET11a (Merck (Novagen), Darmstadt, Germany). Recombinant gene expression and cell lysis were performed as described for Bet v 1.0101.^[22] After cell lysis, $(\text{NH}_4)_2\text{SO}_4$ precipitation in two steps (40% and 100% saturation) was performed at 4 °C as described previously for nDau c 1.^[20] The sample was then subjected to hydrophobic interaction chromatography (HIC) or anion exchange chromatography. HIC was performed as described for Dau c 1.0301.^[20] Anion exchange chromatography was performed using a HiTrap QXL column (GE Healthcare) by stepwise increasing the concentration of elution buffer (20 mM Tris/HCl, pH 8.0, 1 M NaCl). Dau c 1-containing elution fractions were pooled and size exclusion chromatography (SEC) was performed.^[20] After SEC, Dau c 1.0401 was dialyzed against 10 mM Na-phosphate, pH 7.0 overnight at 4 °C, shock-frozen in liquid nitrogen and stored at -80 °C.

Patients' Sera: Sera from carrot allergic patients were obtained after patient written consent and approval of ethics committee (Faculty of Medicine, University of Erlangen-Nuremberg, No. 3494).

CD Spectroscopy, nanoDSF, MRAs, IgE Immunoblots and IgE Inhibition by ELISA: Circular dichroism (CD) spectroscopy, nano differential scanning fluorimetry (nanoDSF), MRAs and IgE immunoblots were performed as described previously.^[17,20,23] For the ELISAs rDau c 1.0104 was coated overnight to Maxisorp plates as described.^[17]

Dot Blots: Two or 4 μg protein was dotted to 0.45 μm nitrocellulose membranes, dried for 30 min and blocked using TBS + 0.3% Tween, 2 \times 30 min, followed by incubation with 500 μL /strip patient's serum 1:10 in TBS + 0.05% Tween (TBST 0.05%) + 0.1% BSA overnight at RT on a shaker. IgE detection was performed after 4x washing with TBST 0.05%, incubation with anti human IgE-AKP (mouse mAb, Pharmingen 555859) 1:750 in TBST 0.05%, 1 mL/strip, 4 h at RT on a shaker, 5x washing (as above), NBT/BCIP staining.

Mass Spectrometry of rDau c 1.0401: Mass spectrometry was performed in commercial service (Department of Biochemistry, University of Bayreuth). The method is therefore only described in so far as it serves the understanding. The band of the putative Dau c 1.0401 was cut from a 19% SDS-gel (Figure S2) and trypsinized.^[24] LC-ESI-MS/MS of 5 μL of the sample was performed on an LTQ-XL mass spectrometer (Thermo Scientific) coupled with an EASY-nLC II chromatographic system (Thermo Scientific) using an in-house packed column with ReproSil-Pur

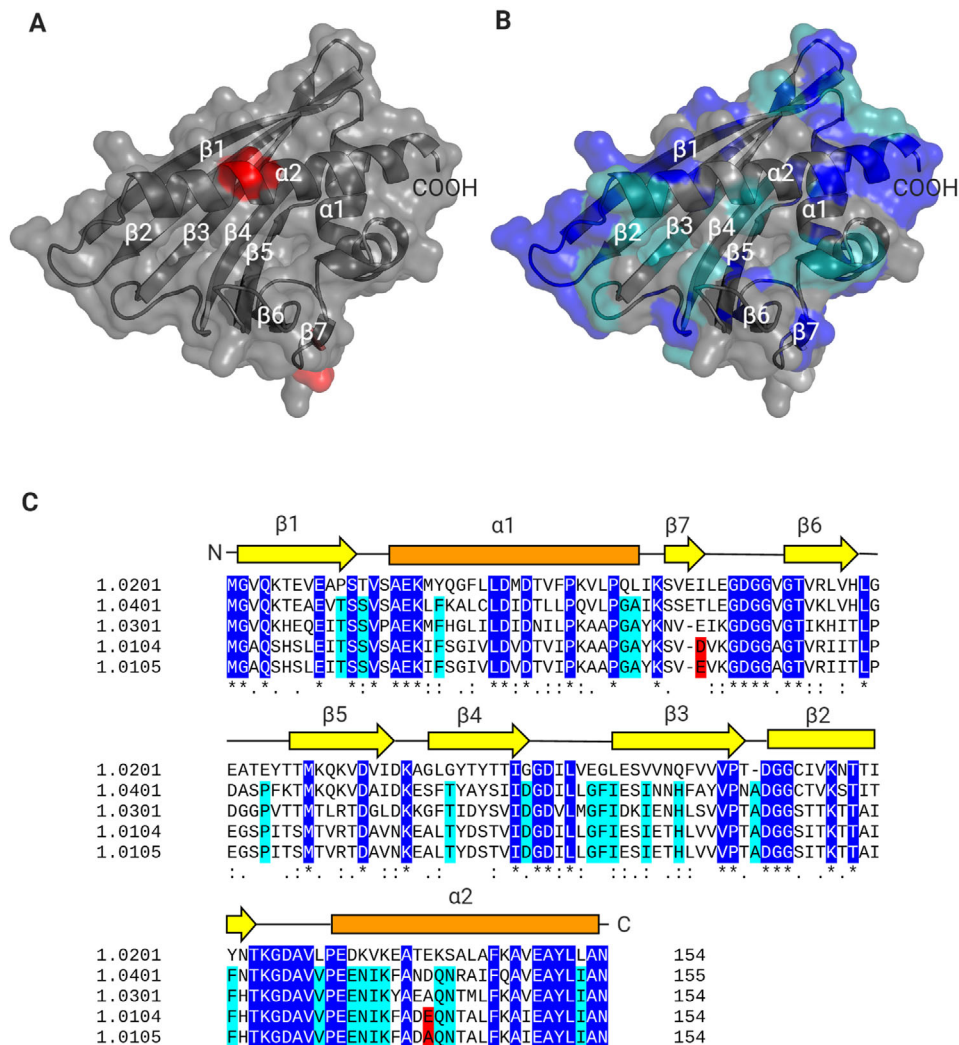


Figure 6. Sequence comparison of the tested Dau c 1 isoallergens. A) Cartoon representation of the Dau c 1.0105 model (Phyre 2). The two amino acid exchanges between Dau c 1.0104 and Dau c 1.0105 (D43E and E139A) are highlighted in red. B) Cartoon representation of the Dau c 1.0401 model (Phyre 2). Conserved surface patches of all tested isoallergens are highlighted in dark blue, and conserved surface patches of all isoallergens except Dau c 1.0201 in cyan. C) Amino acid sequence alignment of all tested isoallergens. Colours are used as in A) and B). Secondary structure elements (yellow: β -strands, orange: α -helices) are depicted above the sequence as determined for Dau c 1.0401.

C18-AQ 3 μ m beads (Dr. Maisch GmbH, Ammerbuch, Germany). Protein identification was performed using the Bionic software (Protein Metrics).

Modelling of Dau c 1 Isoallergens: Dau c 1.0105 and Dau c 1.0401 were modelled using the Phyre 2 server V 2.0.^[25]

Supporting Information

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

Acknowledgements

The authors thank Ramona Heißmann, Ulrike Persau and Andrea Hager from the University of Bayreuth and Renate Höppner, Paul-Ehrlich-Institut, for excellent technical assistance, and Dr. Kristian Schweimer for his support recording NMR spectra.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

T.J. and A.W. contributed equally to this work. B.M.W. and T.J. designed the experiments. T.J. isolated the mRNA, cloned the Dau c 1.0401 gene and performed the expression and purification, CD measurements and NMR spectroscopy. B.M.W. and T.J. evaluated the experiments. A.W. carried out and evaluated the immunoblots, ELISAs, inhibition assays and nanoDSF. L.V. performed and evaluated the MRAs. A.R. designed MS experiments and analyzed and interpreted MS data. V.M. recruited and clinically characterized carrot allergic patients and contributed to the interpretation and analysis of the data. B.M.W. 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, mass spectrometry, protein stability, RT-PCR

Received: November 19, 2020

Revised: January 5, 2021

Published online: March 3, 2021

-
- [1] P. Burney, C. Summers, S. Chinn, R. Hooper, R. van Ree, *J. Lidholm Allergy* **2010**, 65, 1182.
- [2] N. E. Eriksson, H. Formgren, E. Svenonius, *Allergy* **1982**, 37, 437.
- [3] M. Etesamifar, B. Wüthrich, *Allergologie* **1998**, 21, 451.
- [4] K. Hoffmann-Sommergruber, G. O'Riordain, H. Ahorn, C. Ebner, M. Laimer Da Camara Machado, H. Pühringer, O. Scheiner, H. Breiteneder, *Clin. Exp. Allergy* **1999**, 29, 840.
- [5] R. Valenta, D. Kraft, *J. Allergy Clin. Immunol.* **1996**, 97, 893.
- [6] S. Vieths, S. Scheurer, B. Ballmer-Weber, *Ann. N. Y. Acad. Sci.* **2002**, 964, 47.
- [7] B. K. Ballmer-Weber, B. Wüthrich, A. Wangorsch, K. Fötisch, F. Altmann, S. Vieths, *J. Allergy Clin. Immunol.* **2001**, 108, 301.
- [8] C. Radauer, M. Bublin, S. Wagner, A. Mari, H. Breiteneder, *J. Allergy Clin. Immunol.* **2008**, 121, 847.e7.
- [9] R. Hirschwehr, R. Valenta, C. Ebner, F. Ferreira, W. R. Sperr, P. Valent, M. Rohac, H. Rumpold, O. Scheiner, D. Kraft, *J. Allergy Clin. Immunol.* **1992**, 90, 927.
- [10] K. Hoffmann-Sommergruber, P. Demoly, R. Cramer, H. Breiteneder, C. Ebner, M. Laimer Da Camara Machado, K. Blaser, C. Ismail, O. Scheiner, J. Bousquet, G. Menz, *J. Allergy Clin. Immunol.* **1999**, 104, 478.
- [11] H. Fernandes, K. Michalska, M. Sikorski, M. Jaskolski, *FEBS J.* **2013**, 280, 1169.
- [12] C. M. Webber, R. W. England, *Ann. Allergy Asthma Immunol.* **2010**, 104, 101.
- [13] K. Foetisch, S. Scheurer, S. Vieths, K.-M. Hanschmann, J. Lidholm, V. Mahler, *J. Allergy Clin. Immunol.* **2013**, 131, 1711.
- [14] N. Zulehner, B. Nagl, P. Briza, A. Roulias, B. Ballmer-Weber, G. J. Zlabinger, F. Ferreira, B. Bohle, *Allergy* **2017**, 72, 244.
- [15] B. K. Ballmer-Weber, A. Wangorsch, B. Bohle, S. Kaul, T. Kündig, K. Fötisch, R. V. Ree, S. Vieths, *Clin. Exp. Allergy* **2005**, 35, 970.
- [16] S. Peters, J. Imani, V. Mahler, K. Foetisch, S. Kaul, K. E. Paulus, S. Scheurer, S. Vieths, K.-H. Kogel, *Transgenic Res.* **2011**, 20, 547.
- [17] A. Wangorsch, D. Weigand, S. Peters, V. Mahler, K. Fötisch, A. Reuter, J. Imani, A. M. Dewitt, K.-H. Kogel, J. Lidholm, S. Vieths, S. Scheurer, *Clin. Exp. Allergy* **2012**, 42, 156.
- [18] J. Spirc, A. M. Engin, M. Karas, A. Reuter, *PLoS One* **2015**, 10, e0142404.
- [19] C. Seutter von Loetzen, A. Reuter, J. Spirc, T. Schulenburg, I. Bellinghausen, E. Völker, L. Vogel, P. Rösch, D. Schiller, *Clin. Exp. Allergy* **2019**, 49, 712.
- [20] T. Jacob, L. Vogel, A. Reuter, A. Wangorsch, C. Kring, V. Mahler, B. M. Wöhr, *Mol. Nutr. Food Res.* **2020**, 64, 2000334.
- [21] Y. Machado, R. Freier, S. Scheibhofer, T. Thalhamer, M. Mayr, P. Briza, S. Grutsch, L. Ahammer, J. E. Fuchs, H. G. Wallnoefer, A. Isakovic, V. Kohlbauer, A. Hinterholzer, M. Steiner, M. Danzer, J. Horejs-Hoeck, F. Ferreira, K. R. Liedl, M. Tollinger, P. Lackner, C. M. Johnson, H. Brandstetter, J. Thalhamer, R. Weiss, *J. Allergy Clin. Immunol.* **2016**, 137, 1525.
- [22] C. Seutter von Loetzen, T. Hoffmann, M. J. Hartl, K. Schweimer, W. Schwab, P. Rösch, O. Hartl-Spiegelhauer, *Biochem. J.* **2014**, 457, 379.
- [23] L. Vogel, D. Lüttkopf, L. Hatahet, D. Hausteiner, S. Vieths, *Allergy* **2005**, 60, 1021.
- [24] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, *Anal. Chem.* **1996**, 68, 850.
- [25] L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass, M. J. E. Sternberg, *Nat. Protoc.* **2015**, 10, 845.

A novel isoallergen Dau c 1.0401 in carrot: stability, allergenicity and comparison with other isoallergens

*Thessa Jacob, *Andrea Wangorsch, Lothar Vogel, Andreas Reuter, Vera Mahler and Birgitta

M. Wöhrl[#]

* contributed equally

[#]corresponding author, correspondence to: birgitta.woehrl@uni-bayreuth.de

Supplementary information

```

CCG AGA TGG AAT TCC CCC CGG GGG GTT TTT GAA AAA ACC CCC CCC TTT TCC GGA GGG AAA
AAG GGG GCC CCG TTT TCC GAT AGG GGG GGG TGA AAC CGG GAG CCC CCG GGG GGT CTT CCG
GGG GAA ACC CCT GGT TTC TTT TAA TTC CCT TGG GGT TTC CCC CCT TTG AAC TGA GCG TCG
ATT TTT GGG ATC TTT GTC AGG GGG GCG GAG CCT TTG GAA AAA CGC CCA GCA ACC CGG CCT
TTT TAC GGT TCC TGG CCT TTT GCT GGC CTT TTG CTC ACA TGT TCT TTC CTG CGT TAT CCC
CTG ATT CTG TGG ATA ACC GTA TTA CCG CCT TTG AGT GAG CTG ATA CCG CTC GCC GCA GCC
GAA CGA CCG AGC GCA GCG AGT CAG TGA GCG AGG AAG CGG AAG AGC GCC CAA TAC GCA AAC
CGC CTC TCC CCG CGC GTT GGC CGA TTC ATT AAT GCA GCT GGC ACG ACA GGT TTC CCG ACT
GGA AAG CGG GCA GTG AGC GCA ACG CAA TTA ATG TGA GTT AGC TCA CTC ATT AGG CAC CCC
AGG CTT TAC ACT TTA TGC TTC CGG CTC GTA TGT TGT GTG GAA TTG TGA GCG GAT AAC AAT
TTC ACA CAG GAA ACA GCT ATG ACC ATG ATT ACG CCA AGC TAT TTA GGT GAC GCG TTA GAA
TAC TCA AGC TAT GCA TCA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTA ACG GCC GCC AGT
GTG CTG GAA TTC AGG CAC AGC ATT CCT TGA TAA GCT CTT AGT ACT TTT CTC AGT TTC TGT
TTC AAT AGA ATT ATG GGT GTC CAA AAG ACT GAA GCC GAA GTC ACT TCC TCT GTA TCA GCA
- - - - M G V Q K T E A E V T S S V S A
GAG AAA CTG TTC AAG GCC TTG TGC CTC GAC ATC GAT ACA CTT CTT CCT CAG GTT CTC CCT
E K L F K A L C L D I D T L L P Q V L P
GGT GCT ATC AAG AGT TCC GAG ACT CTT GAG GGC GAT GGT GGA GTT GGA ACC GTC AAG CTT
G A I K S S E T L E G D G G V G T V K L
GTC CAT CTT GGC GAT GCG AGC CCC TTC AAG ACA ATG AAG CAA AAG GTG GAT GCC ATT GAC
V H L G D A S P F K T M K Q K V D A I D
AAA GAG TCC TTC ACA TAC GCT TAC AGC ATC ATC GAC GGA GAC ATT CTT CTG GGA TTT ATT
K E S F T Y A Y S I I D G D I L L G F I
GAA TCC ATC AAC AAT CAC TTC GCG TAT GTC CCT AAT GCT GAT GGT GGC TGC ACT GTC AAG
E S I N N H F A Y V P N A D G G C T V K
AGT ACC ATC ACT TTC AAC ACC AAA GGC GAT GCT GTC GTC CCT GAA GAG AAC ATT AAG TTC
S T I T F N T K G D A V V P E E N I K F
GCT AAT GAC CAG AAC CGC GCC ATT TTC CAG GCT GTT GAG GCT TAT CTC ATT GCC AAC TAA
A N D Q N R A I F Q A V E A Y L I A N -
GTA TGA TTA TAT AAT AGT ATG TTC GTG AGA CCT GGA TGG CCT GAA TTC TGC AGA TAT CCA
TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT AGA TAC TGA

```

Figure S1: cDNA sequence of *Dau c 1.0401* in the pCRBlunt vector. The sequences complementary to the 5' and 3' UTR primers are underlined, the coding sequence and the corresponding amino acid sequence are depicted in bold letters.

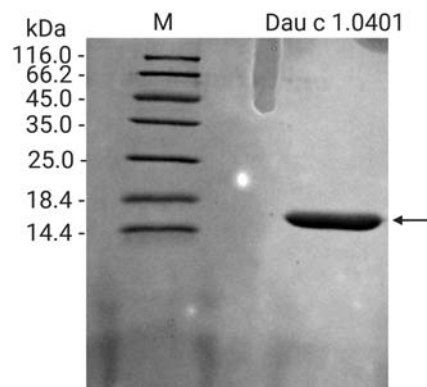


Figure S2: 19 % SDS-Gel of *Dau c 1.0401*; M, marker (unstained protein molecular weight marker, Thermo Scientific). A molecular weight of 16 kDa was determined for *Dau c 1.0401* using the software Image Lab 5.2.1 (BioRad). The arrow marks the *Dau c 1.0401* band, which was confirmed by LC-MS^E.

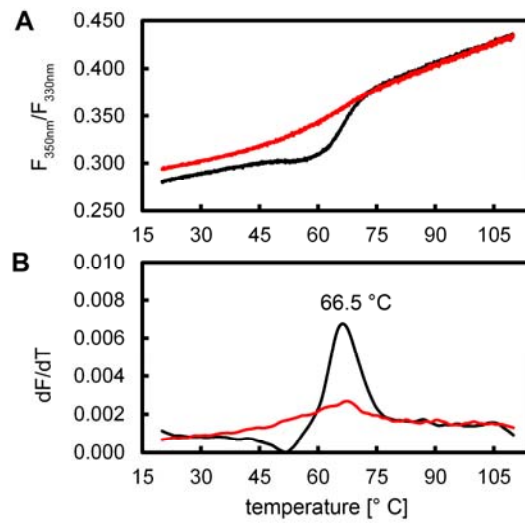


Figure S3: nanoDSF measurement of rDau c 1.0401 (2.9 mg/ml protein in 10 mM NaP, pH 7); **A**, fluorescence ratio of 350 nm:330 nm; **B**, first derivative thereof

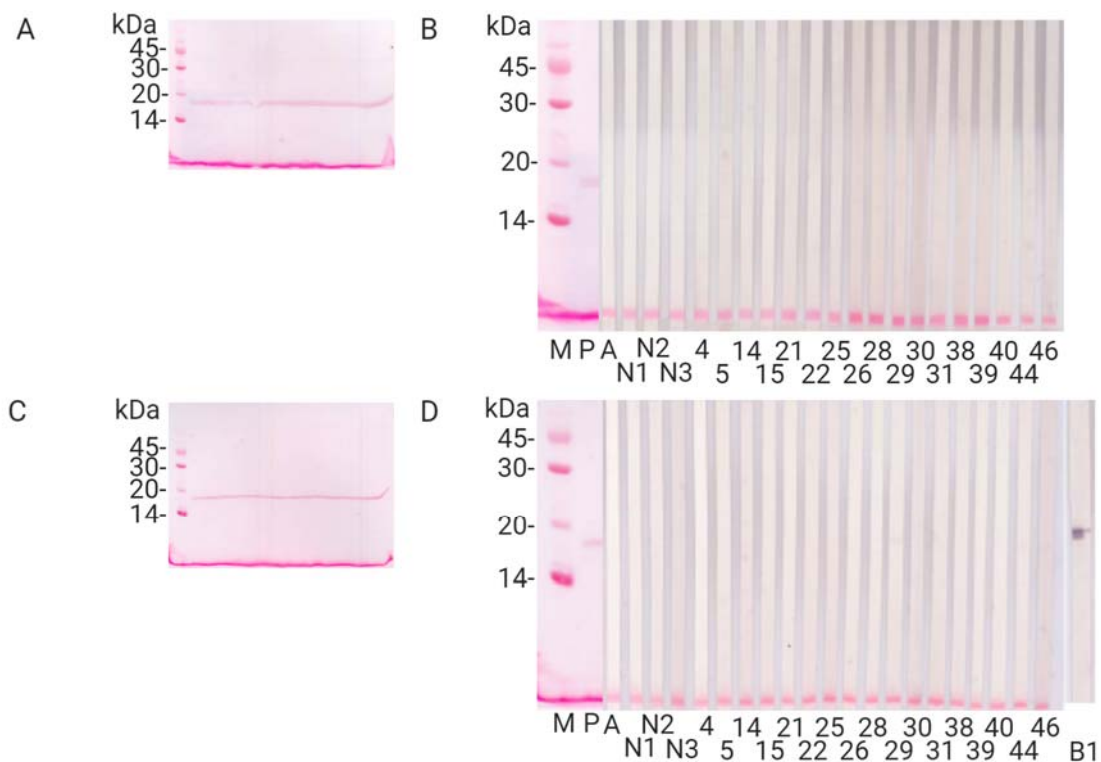


Figure S4. Immunoblots of Dau c 1.0401. The immunoblots were performed with sera of carrot-allergic patients. SDS-PAGE with Dau c 1.0401 was carried out under non-reducing (A, B) and reducing conditions by addition of DTT (C, D), followed by protein transfer onto a nitrocellulose membrane (A, C). Blotted Dau c 1.0401 was visualized by PonceauS staining; (B, D). M, molecular weight marker (LMW, GE Healthcare); P, PonceauS staining of blotted protein; A, secondary antibody control; N1-N3, sera of non-allergic control patients; 4-46, sera of carrot-allergic patients, B1, positive control (rBet v 1.0101 + serum of Bet v 1-allergic patient.)

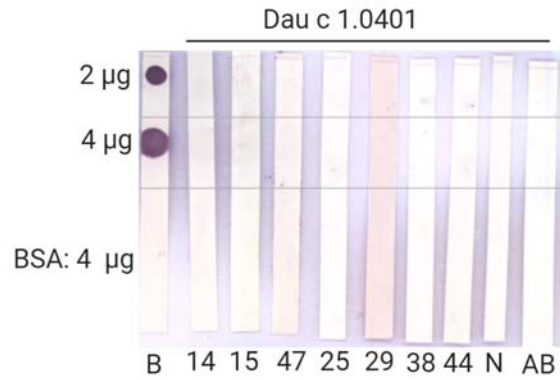


Figure S5: Dot blot of Dau c 1.0401 with sera of carrot-allergic patients. B, positive control (Bet v 1.0101 and serum of a Bet v 1 allergic patient); 14-44: Blotted Dau c 1.0401 and respective sera of carrot-allergic patients; N, Dau c 1.0401 plus serum of a non-allergic subject; AB, Dau c 1.0401 secondary antibody control

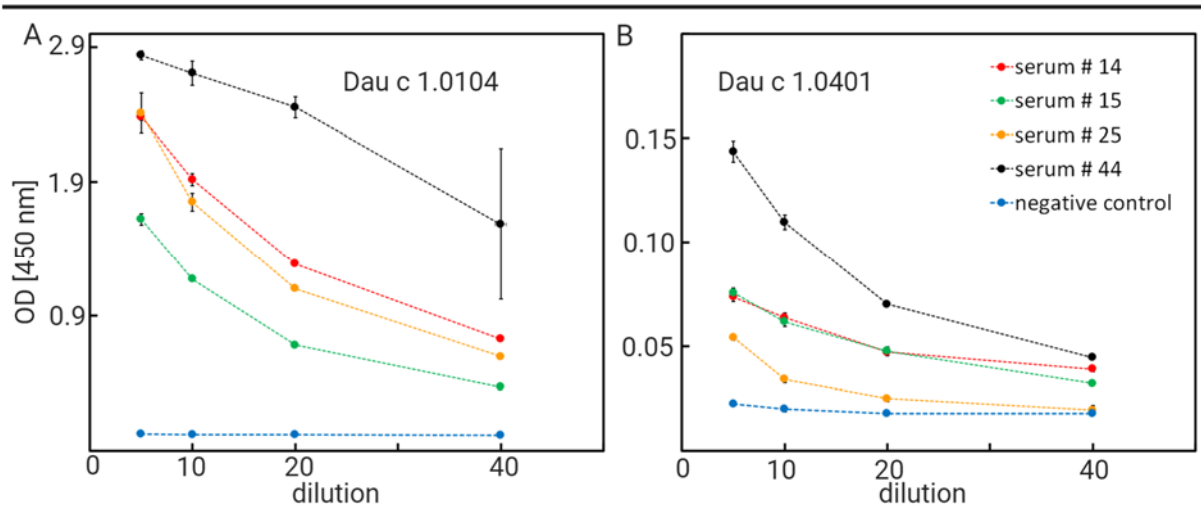


Figure S6: ELISA of Dau c 1.0104 (A) and Dau c 1.0401 (B) with sera of carrot-allergic patients. Negative control, serum of a non-allergic patient.

Table S1: Summary of mass spectrometric fragmentation data of rDau c 1.0401.

| Total Intensity | # of spectra | # of unique peptides | # of mod peptides | Coverage [%] | # AA's |
|-----------------|--------------|----------------------|-------------------|--------------|--------|
| 154343826.0 | 531 | 30 | 14 | 99.35 | 155 |

9. Danksagung

Ohne die Unterstützung und Mitwirkung zahlreicher Personen wäre diese Arbeit nicht möglich gewesen.

Zunächst möchte ich mich herzlich bei meiner Betreuerin, **Prof. Dr. Birgitta Wöhrl** bedanken. Sie hat mich mit vielfältigen Vorschlägen und großem Engagement in der Entwicklung meiner Arbeit hervorragend unterstützt. Gleichzeitig hat sie mir den Freiraum gegeben, eigene Ideen zu verfolgen und hatte jederzeit ein offenes Ohr und konstruktive Lösungsvorschläge bei auftretenden Problemen. Ganz besonders bedanken möchte ich mich für ihre beispiellose Unterstützung darin, Familie und Promotion zu vereinbaren.

Mein Dank gilt ebenfalls **Dr. Christian Seutter von Loetzen**, der dieses Projekt früher geleitet hat und hervorragende Vorarbeit geleistet hat, auf die ich aufbauen konnte. Danke vielmals für die zahlreichen Ratschläge und die Hilfsbereitschaft, auch über die Zeit der Projektleitung hinaus.

Vielen Dank an **Dr. Kristian Schweimer** für die Hilfe bei allen NMR-Experimenten und an **Dr. Stefan Knauer** für hilfreiche Diskussionen und Ratschläge.

Vanessa Boritzki, Philipp Zuber und **Benjamin Dudenhöffer** möchte ich für die stets sehr angenehme Atmosphäre und die vielen netten und hilfreichen Gespräche danken. Großer Dank gilt auch **allen Studierenden**, die durch ihre praktische Tätigkeit im Labor zum Gelingen meiner Doktorarbeit beigetragen haben.

Rainer Hofmann hat sich jederzeit hervorragend um alle IT-Angelegenheiten gekümmert. **Anja Groh** hat alle administrativen Angelegenheiten hervorragend erledigt und war immer sehr aufmunternd und hilfsbereit.

Andrea Hager, Ramona Heißmann und **Ulrike Persau** haben mich nicht nur im Labor exzellent unterstützt, sondern auch jederzeit für ein angenehmes und fröhliches Arbeitsklima gesorgt. Danke Andy für den leckeren Kaffee und Uli für den tollen Roller ☺!

Vielen herzlichen Dank an meine KooperationspartnerInnen vom Paul-Ehrlich-Institut in Langen, **Dr. Lothar Vogel, Dr. Andrea Wangorsch, Dr. Andreas Reuter** und **Prof. Dr. Vera Mahler** für die große Hilfsbereitschaft und die sehr angenehme, konstruktive Zusammenarbeit!

Zuletzt möchte ich mich herzlich bei meiner Familie bedanken, die mich jederzeit unterstützt hat, vor allem durch die liebevolle Betreuung meiner Kinder, um mir die Anfertigung dieser

Arbeit zu ermöglichen, aber auch in allen anderen Angelegenheiten. Besonderer Dank gilt meinen Eltern **Gudrun** und **Martin**, meinen Geschwistern **Thies** und **Rica** und meinen Schwiegereltern **Birgit** und **Heinz**.

Ganz besonderer Dank gilt meinem Mann **Andy** für sein Verständnis, seine unendliche Geduld und Unterstützung, und meinen Töchtern **Mara** und **Mila** für ihr fröhliches Kinderlachen und dafür, dass sie es auch nach harten Arbeitstagen innerhalb kürzester Zeit immer schaffen, mich erfolgreich abzulenken!

Vielen Dank auch **allen Freunden**, die mich in dieser Zeit moralisch unterstützt haben!

10. Erklärung

(Eidesstattliche) Versicherungen und Erklärungen

(§ 8 Satz 2 Nr. 3 PromO Fakultät)

Hiermit versichere ich eidesstattlich, dass ich die Dissertation selbstständig verfasst habe und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe (vgl. Art. 64 Abs. 1 Satz 6 BayHSchG).

(§ 8 Satz 2 Nr. 3 PromO Fakultät)

Hiermit erkläre ich, dass ich die Dissertation nicht bereits zur Erlangung eines akademischen Grades anderweitig eingereicht habe und dass ich nicht bereits diese oder eine gleichartige Doktorprüfung nicht bestanden habe.

(§ 8 Satz 2 Nr. 4 PromO Fakultät)

Hiermit erkläre ich, dass ich Hilfe von gewerblichen Promotionsberatern bzw. -vermittlern oder ähnlichen Dienstleistern weder bisher in Anspruch genommen habe noch diese künftig in Anspruch nehmen werde.

(§ 8 Satz 2 Nr. 7 PromO Fakultät)

Hiermit erkläre ich mich einverstanden, dass die elektronische Fassung meiner Dissertation unter Wahrung meiner Urheberrechte und des Datenschutzes einer gesonderten Überprüfung unterzogen werden kann.

(§ 8 Satz 2 Nr. 8 PromO Fakultät)

Hiermit erkläre ich mich einverstanden, dass bei Verdacht wissenschaftlichen Fehlverhaltens Ermittlungen durch universitätsinterne Organe der wissenschaftlichen Selbstkontrolle stattfinden können.

Ort, Datum, Unterschrift