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

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

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

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

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

1. Introduction

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

To date, three different groups of carrot allergens have been described: Dau c 5, an isoflavone reductase-like protein, Dau c 4, a profiling, and Dau c 1 (www.allergen.org). The latter represents the major carrot allergen responsible for most carrot allergies.[3] Dau c 1 belongs to the class of pathogenesis-related class 10 (PR-10) allergenic proteins and shares homology to the major birch pollen allergen Bet v 1 (36.9% sequence identity between Bet v 1.0101 and Dau c 1.0104). All known PR-10 allergens exhibit common structural features. Bet v 1 as well as Dau c 1 consist of a seven-stranded anti-parallel β-sheet that wraps around a 25 residues long C-terminal amphipathic α-helix. The β-sheet and the C-terminal part of the helix are separated by two short α-helices which are arranged in V-shape. Together with the β-sheet they form a hydrophobic cavity which can bind small ligand molecules.[4,5] Natural ligands for Bet v 1, quercetin-3-O-sophoroside, and for the homologous hazel allergen Cor a 1, quercetin-3-O-(2"-O-β-D-glucopyranosyl)-β-D-"galactopyranoside, have been recently identified.[6,7]

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

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

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

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

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

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

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

2. Experimental Section

2.1. Identification of mRNA

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

2.2. Cloning, Expression, and Purification

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

Expression of different Dau c 1 genes was carried out in *Escherichia coli* BL21 (DE3) harboring the plasmid pET_GB1a with the respective gene.[17] In brief, bacteria were grown in lysogeny broth medium supplemented with 30 µg mL^{-1} kanamycin at 37 °C. At an optical density of 600 nm of 0.6–0.7 the temperature was decreased to 16 °C and overexpression was induced overnight for ca. 18–19 h by the addition of 0.6 mM isopropyl β-D-1-thiogalactopyranoside . After harvesting (6000 x g, 10 min, 4°C), the cell pellet was resuspended in 20 mM Tris–HCl, 10 mM imidazole, 500 mM NaCl, pH 7, supplemented with DNase I (AppliChem GmbH, Darmstadt, Germany) and ¼ EDTA-free protease inhibitor tablet (Roche Diagnostics GmbH, Mannheim, Germany). After lysis (Microfluidics, Newton, USA) the lysate was centrifuged (75 000 x g, 30 min, 4 °C) and the supernatant was applied onto a 5 mL HisTrap HP column (Cytiva Europe GmbH). Elution was carried out using a continuous gradient from 0% to 100% elution buffer (20 mM Tris–HCl, 500 mM imidazole, 500 mM NaCl, pH 7) over 20 column volumes. Fractions containing the protein in question were combined and dialyzed against 20 mM Tris–HCl, 100 mM NaCl, pH 7 (molecular weight cut off (MWCO) 3500 Da) at 4 °C overnight in the presence of TEV protease to cleave off the 6xHis-GB1a fusion protein.

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

2.3. CD Spectroscopy, NanoDSF, and 1D NMR

Circular dichroism (CD) spectra and nano-differential scanning fluorometry (DSF) measurements using a Prometheus NT.48 device were recorded as previously described.[14,15,18] For nanoDSF measurements, the protein concentration was adjusted to 0.5–2 mg mL^{-1}. A continuous gradient from 20 to 110 °C (excitation power 80%, temperature slope 2.0° min^{-1}) was applied. For each isoallergen three independent triplicates were measured. Standard 1D ^{1}H NMR spectra were recorded on a BRUKER Avance 600 MHz spectrometer at 25 °C with WATERGATE solvent suppression.[19] For each measurement 80–330 µM of protein in 10 mM Na-phosphate, pH 7.0, 10 % D_2O was used. All data were processed and visualized with the Bruker software TOP SPIN.

2.4. Patients' Sera

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

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

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

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

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

For the enzyme-linked immunosorbent assays (ELISAs) rDau c 1.0104 (2 µg per well) was coated to Maxisorb plates as described.[14,21] Serum was diluted to reach an $OD_{450\,nm}$ between 0.6 and 0.8. Serial dilutions of purified rDau c 1 isoallergens, or Dau c 1 like, or Dau-NCS were used as inhibitors.

2.6. Bioinformatic Tools

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

3. Results and Discussion

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

We recently purified nDau c 1 from carrot root and performed liquid chromatography-mass spectrometry$^{\text{elevated energy}}$ (LC-MS^E) to analyze its composition[15] (**Figure 1**). Surprisingly, we found out that the nDau c 1 composition was more complex than previously thought harboring several additional isoallergens and variants that have not been characterized yet. Dau c 1-related peptides derived from database entries from *Daucus carota* (DAUCS), *Pimpinella brachycarpa* (PIMBR), *Apium graveolens* (APIGR), and *Petrosilium crispum* (PETCR) were identified.[15] Here, we only used the sequence information from *Daucus carota*[15] and thus were able to identify four entries possessing sufficient sequence identity to known Dau c 1 sequences with the following Uniprot accession numbers: A0A164WTA1 (= Dau-NCS), A0A175YPA2, D9ZHP1, and D9ZHP0. In addition, we used the identified peptides to search the carrot genome by BLAST in order to find complete gene sequences. By this approach, four additional sequences were recognized with the following Gene IDs: XP_017215843.1 (Var 1 = Dau c 1.0601), XP_017218034.1 (= Var 2), XP_017220733.1 (Var 3 = Dau c 1-like), XP_017220806.1 (Var 4 = Dau c 1.0501). (Figures 1 and S2, Supporting Information).

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

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

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

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

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

3.3. Biochemical and Biophysical Characterization

Since one of the expressed proteins (Dau-Var2) proved insoluble, only the remaining four purified proteins, Dau c 1.0501, Dau c 1.0601, Dau c 1-like, and Dau-NCS (Table S4, Figure S2, Supporting Information) were subjected to thorough biochemical and biophysical analyses. SDS-PAGE and size exclusion chromatography experiments were performed to clarify the purity and

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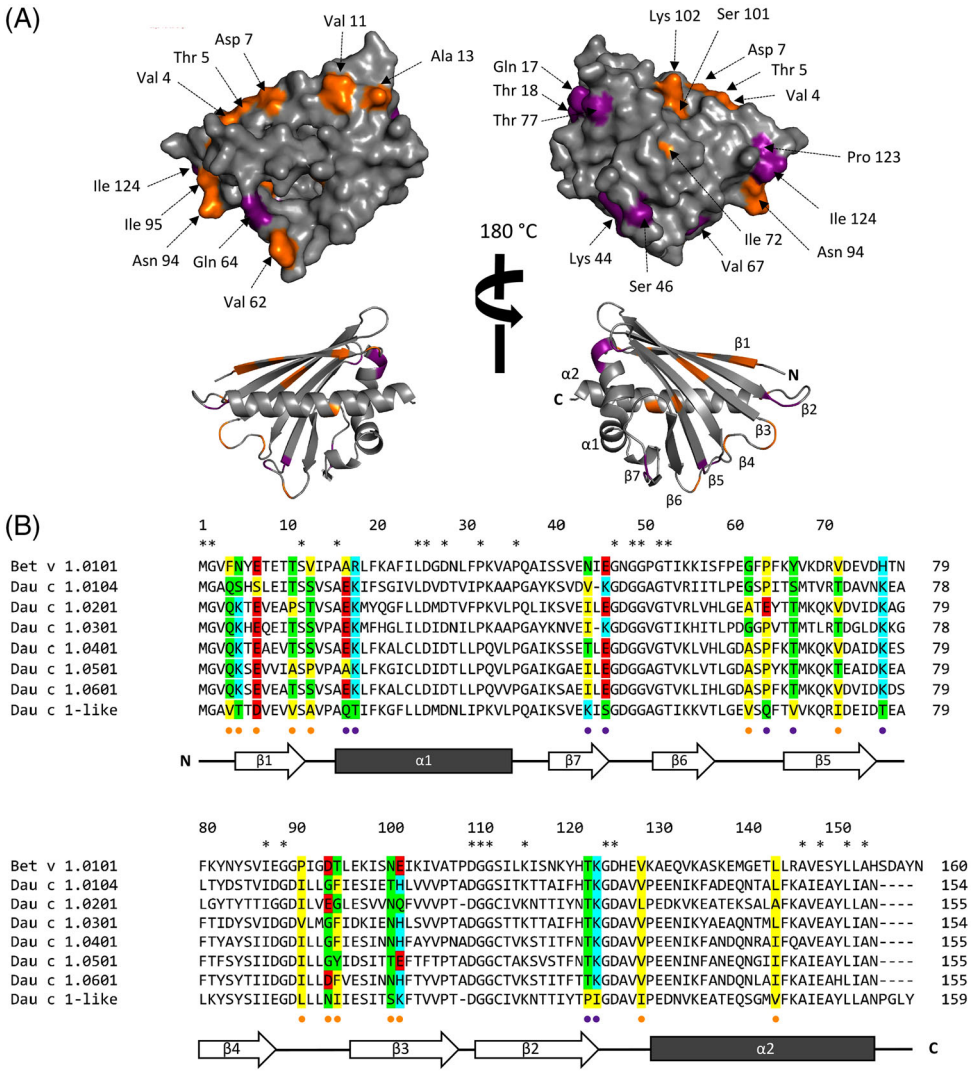

Figure 5. Comparison of allergenic Dau c 1 isoallergens with the non-allergenic Dau c 1-like protein. **A)** Structural model of Dau c 1-like protein. The model of Dau c 1-like was calculated by Phyre2[24] Purple: non-conservative amino acid exchanges, unique for Dau c 1-like; orange: conservative amino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

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

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

Refolding CD-experiments indicated that Dau c 1.0501 as well as Dau-NCS were not able to refold (Figure 2B, black dotted

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                          T1    T6              T19 T22          T32/34  T41    T46      T55             T69 T71
Dau-NCS                   MAGSVSCELVINAPASSVWDLIK GLK LAAAIEESFTHLIEK  IDAVGDGSxVGTELNIK FK PGTVPFSYYK  ER FTK VDNESMVK
Dau c 1.0201              -MGVQK TEVEAPSTVSAEK  MYQGFLLDMDTVFPK  VLPQLIK SVEILExGDGGVGTVR LVHLGEATEYTTMK  QK  VDVIDK
Dau-Var2                  -MGAITxTNVEVESSV-PAQTIYK GFLLDMDNIIPK  ILPQAIK RVEIISxGDGGIGTIK QITLGEVSQFTIVK  QRIDEIDREGLK
Dau c 1-like              -MGAVTxTDVEVVSAV-PAQTIFK GFLLDMDNLIPK  VLPQAIK SVEKISxGDGGAGTIK K VTLGEVSQFTVVK QRIDEIDTEALK
Dau c 1.0401              -MGVQK TEAEVTSSV-SAEK LFK ALCLDIDTLLPQxVLPGAIK SSETLExGDGGVGTVK LVHLGDASPFK TMK QK VDAIDK
Dau c 1.0601              -MGVQK SEVEATSSV-SAEK LFK ALCLDIDTLLPQxVVPGAIK SAEILExGDGGVGTVK LIHLGDASPFK TMK QK VDVIDK
Dau c 1.0501              -MGVQK SEVVIASPV-PAAK LFK GICLDIDTLLPQxVLPGAIK GAEILExGDGGAGTVK LVTLGDASPYK TMK QK TEAIDK
Dau c 1.0103              -MGAQSxHSLEITSSV-SAEK IFSxGIVLDVDTVIPK AATGAYK SVE-VK GDGGAGTVR IITLPEGSPITxTMTxVR TDAVNK
Dau c 1.0101              -MGAQSxHSLEITSSV-SAEK IFXxXIVLDVDTVIPK AAPGAYK SVD-VK GDGGAGTVR IITLPEGSPITxSMTxVR TDAVNK
Dau c 1.0102              -MGAQSxHSLEITSSV-SAEK IFSxGIVLDVDTVIPK AAPGAYK SVD-VK GDGGAGTVR IITLPEGSPITxSMTxVR TDAVNK
Dau c S-A0A175YPA2        -MGAQSxHSLEITSSV-SAEK IFSxGIVLDVDTVIPK AAPGAYK SVD-VK GDGGAGTIR TITLPEGSPITxTMTxVR TDAVNK
Dau c 10104               -MGAQSxHSLEITSSV-SAEK IFSxGIVLDVDTVIPK AAPGAYK SVD-VK GDGGAGTVR IITLPEGSPITxSMTxVR TDAVNK
Dau c 10105               -MGAQSxHSLEITSSV-SAEK IFSxGIVLDVDTVIPK AAPGAYK SVE-VK GDGGAGTVR IITLPEGSPITxSMTxVR TDAVNK
Dau c S-D9ZHP1            -MGVQK HEQEITSSV-PAEK MFHxGLILDIDNVLPK AAPGAYK NVE-IK GDGGVGTIK HITLPEGGPVTxTMTxLR TDGLDK K
Dau c S-D9ZHP0            -MGVQK HEQEITSSV-PAEK MFHxGLILDIDNILPK AAPGAYK NVE-IK GDGGVGTIK HITLPDGGPVTxTMTxLR TDGLDK K
Dau c 1.0301              -MGVQK HEQEITSSV-PAEK MFHxGLILDIDNILPK AAPGAYK NVE-IK GDGGVGTIK HITLPDGGPVTxTMTxLR TDGLDK K

                          T77                 T98      T102     T116     T124          T135   T142  T146
Dau-NCS                   ENEVFEGGYCDLGFSK YFVR FEIVK K   DENSCIAK ATTEYELK ADADPNLASLVSVDQMMGVLNLAANK VVNGSK
Dau c 1.0201              AGLGYTYTTIGGDILVEGLESxVVNQFVVVPTxDGGCIVK NTTIYNTK GDAVLPEDK VK EATEK SALAFK AVEAYLLAN
Dau-Var2                  YSYSIIEGDLLMGIIESIASK FTVVPTxxxxxEGGCIVK NTAVYTPIxGDAVIPEENxVK EATEQSGMVFK  QIEAYLLANPAAY
Dau c 1-like              YSYSIIEGDLLLNIIESITSK FTVVPTxxxxxDGGCIVK NTTIYTPIxGDAVIPEDNxVK EATEQSGMVFK  AIEAYLLANPGLY
Dau c 1.0401              ESFTYAYSIIDGDILLGFIESxINNHFAYVPNADGGCTVK STITFNTK GDAVVPEENxIK FANDQNR AIFQxAVEAYLIAN
Dau c 1.0601              DSFTYSYTIIDGDILLDFVESxINNHFTYVPTADGGCTVK STITFTTK GDAVVPEENxIK FANDQNLxAIFK AIEAHLIAN
Dau c 1.0501              EAFTFSYSIIDGDILLGYIDSxITTEFTFTPTADGGCTAK SVSTFNTK GDAVVPEENxINxFANEQNGxIIFK AIEAYLIAN
Dau c 1.0103              EALSYDSTVIDGDILLGFIESxIETHMVVVPTADGGSITK TTAIFHTK GDAVVPEENxIK FADAQNTxALFK AIEAYLIAN
Dau c 1.0101              EALTYDSTVIDGDILLEFIESxIETHMVVVPTADGGSITK TTAIFHTK GDAVVPEENxIK FADAQNTxALFK AIEAYLIAN
Dau c 1.0102              EALTYDSTVIDGDILLEFIESxIETHMVVVPTADGGSITK TTAIFHTK GDAVVPEENxIK FADAQNTxALFK AIEAYLIAN
Dau c S-A0A175YPA2        EALTYDSTVIDGDILLGFIESxIETHMVVVPTADGGSITK TTAIFHTK GDAVVPEENxIK FADEQNTxALFK AIEAYLIAN
Dau c 1.0104              EALTYDSTVIDGDILLGFIESxIETHLVVVPTADGGSITK TTAIFHTK GDAVVPEENxIK FADEQNTxALFK AIEAYLIAN
Dau c 1.0105              EALTYDSTVIDGDILLGFIESxIETHLVVVPTADGGSITK TTAIFHTK GDAVVPEENxIK FADAQNTxALFK AIEAYLIAN
Dau c S-D9ZHP1            x                                        TTAIFHTK GDAVVPEENxIK              AVEAYLIAN
Dau c S-D9ZHP0            xGFTIDYSVIDGDVLMGFIDK IENHLSVVPTADGGSTTK TTAIFHTK GDAVVPEENxIK YAEEQNTxLLFK AVEAYLIAN
Dau c 1.0301              xGFTIDYSVIDGDVLMGFIDKxIENHLSVVPTADGGSTTK TTAIFHTK GDAVVPEENxIK YAEAQNTxMLFK AVEAYLIAN
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Figure 1. Sequence alignment of Dau c 1 peptides identified by LC-MS^E. Amino acid sequences of Dau c 1-like proteins from *Daucus carota* were aligned. Corresponding peptides detected by LC-MS^E are underlined. Differing sequence stretches are color coded. Gaps indicate trypsin cleavage sites. An "x" indicates a missing trypsin cleavage site and was used to adjust the sequence alignment. Sections highlighted in grey represent already known Dau c 1 sequences. The positions of the amino acids in the sequence were labeled according to the following scheme: e.g., T6 means that the corresponding peptide is released after "T"-(Trypsin) cleavage and starts at amino acid position 6. The corresponding amino acid identity matrix is presented in Table S4, Supporting Information.

lines) after a longer time period at temperatures above the T_m, whereas the other proteins were able to fold back, albeit incompletely (Figure 2B). The differences as compared to Figure 2A are probably due to the extended time period at high temperatures when determining the T_m (ca. 15 min at 95 °C above the melting point during recording of CD-spectra (Figure 2A) vs 46–74 min above the melting point during refolding experiments (Figure 2B)). Similarly, none of the proteins is capable of refolding in nanoDSF experiments (Figure 2C), probably because here the protein solution is heated to 110 °C. The comparison of the experiments shown in Figure 2A–C indicates that the time period of the temperature above T_m affects the refolding ability.

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

3.4. IgE Binding

We previously showed with Dau c 1.0401 that the immune response can be attenuated if the allergen is partially or fully denatured as is the case with immunoblots.[15] This might be due to the disintegration of structural epitopes that are recognized by IgE antibodies. Thus, we performed different immunological experiments in which the protein in question was either present in its native or denatured form.

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

3.5. Mediator Release Assays

MRAs were performed with rat basophilic leukemia cells (RBLs) that recombinantly express the human high affinity IgE receptor α-chains. In response to IgE receptor binding and IgE cross-linking, the release of the mediator β-hexosaminidase can be

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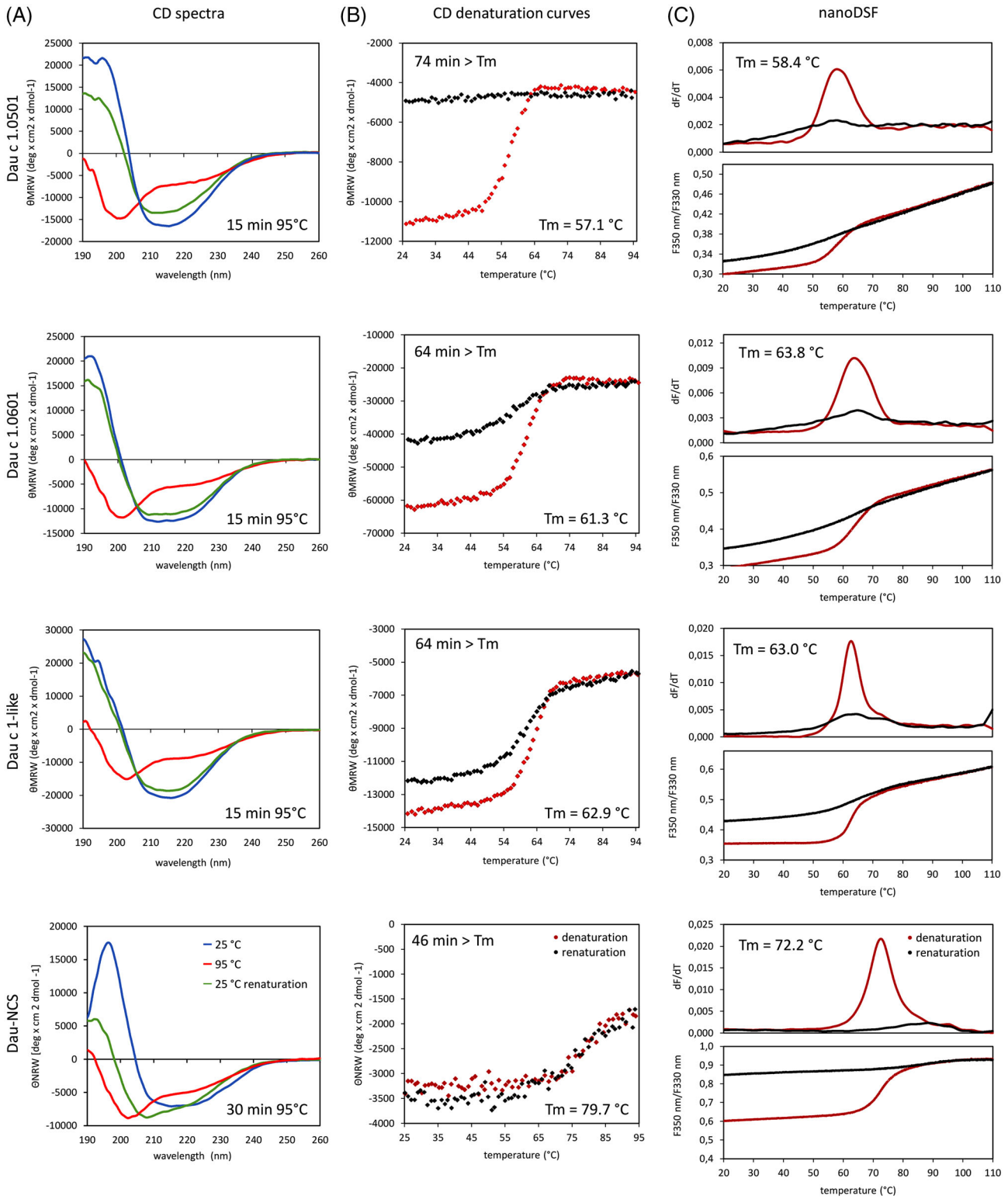

Figure 2. CD spectra and nanoDSF analyses. CD spectra (panel **A**) at 25 °C, after heating at 95 °C for 15 min (or 30 min for Dau-NCS), followed by stepwise cooling to 25 °C (green) are shown. CD (panel **B**) and nanoDSF (panel **C**) denaturation (red) and renaturation (black) curves are shown. The duration of incubation above the T_m is displayed at the top left of each spectrum in **B)**. The CD curves (panel **B**) were recorded at 217 nm. The upper segments of the nanoDSF graphs (panel **C**) show the fluorescence ratio of 350 nm:330 nm, the lower segments show the first derivative thereof. Buffer for CD-measurements: 10 mM Na_2HPO_4/NaH_2PO_4 pH 7.0. Buffer for nanoDSF: 10 mM K_2HPO_4/KH_2PO_4 pH 7.0. An overview of the melting points is shown in Table S5, Supporting Information.

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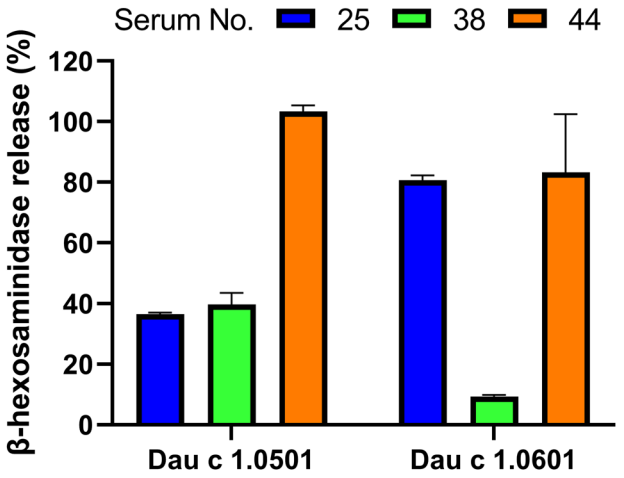

Figure 3. Mediator release assays with huRBL-1B2 cells. For sensitization a 1:10 dilution of the sera was used. Cross-linking was performed with 1 µg mL^{-1} Dau c 1.0501 or Dau c 1.0601. 4-nitrophenyl N-acetyl-β-D-glucosaminide was used as a substrate. The β-hexosaminidase release was normalized to the human IgE-control.

measured.[20] In contrast to the immunoblots described above, the protein under investigation remains in solution and consequently is present in its native conformation.

Although in this experiment all available sera were tested (Table S3, Supporting Information), significant mediator release was only detectable with Dau c 1.0601 and Dau c 1.0501 using the sera #25, #38, and #44 (**Figure 3**), whereas Dau c 1–like and Dau-NCS did not induce mediator release (data not shown). Since two different IgE epitopes must be present on the allergen in order to achieve cross-linking of IgE antibodies in this assay, we assume that some sera harbor only one specific type of IgE directed against one single epitope. Consequently, inhibition assays were performed, which do not require the presence of two different epitopes.

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

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

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

4. Discussion

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

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

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

Dau-NCS was also detected in purified nDau c 1. However, it was not included in our structural comparisons since it exhibited a much higher sequence identity of 53% to the NCS enzyme from Kiwi plant (*A. chinensis*) (Sequence ID PSS32256.1) as compared to only 22% to any of the known Dau c 1 isoallergens. Furthermore, it also differs considerably from the Dau c 1-like protein (21.71% identity) (Table S4). The enzyme NCS (EC 4.2.1.78) catalyzes the condensation of 3,4-dihydroxyphenylethylamine (dopamine) and 4-hydroxyphenylacetaldehyde (4-HPAA) as the first committed step in the biosynthesis of benzylisoquinoline

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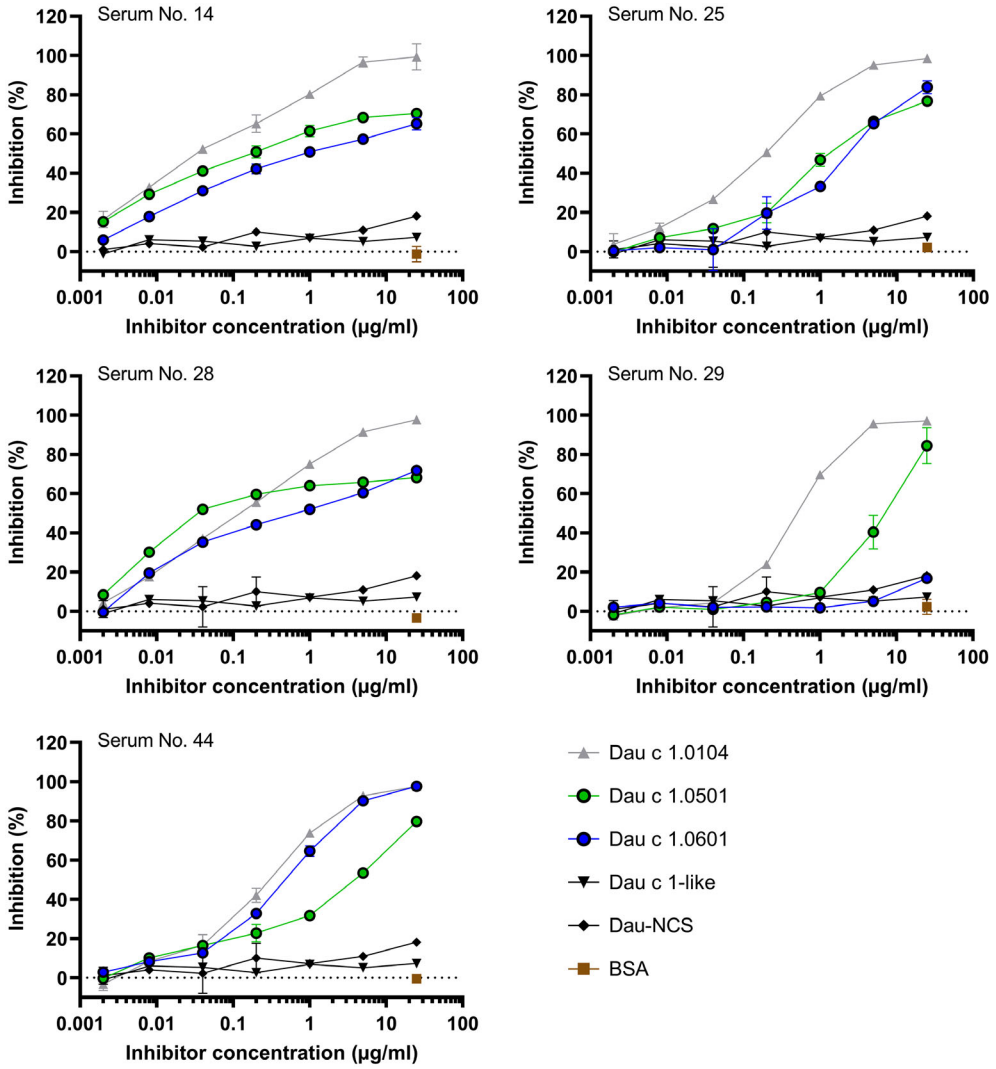

Figure 4. Inhibition of IgE binding to Dau c 1.0104. ELISA Inhibition was performed as previously described.[21] Microtiter plates were coated with 2 µg Dau c 1.0104 per well. Serial dilutions of the putative rDau c 1 protein were added to the well, followed by the addition of serum. Bound specific IgE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

alkaloids in plants.[27–29] We have not yet determined whether Dau-NCS in fact exhibits enzymatic activity. Structural information on NCS enzymes is only available for the NCS from the common meadow rue *Thalictrum flavum*. CD and NMR spectroscopic data indicated a high structural similarity of the NCS from *T. flavum* with the major birch pollen allergen Bet v 1 even if the sequence identity is rather low (12–15% identity).[30] Crystal structures confirm the high structural homology to proteins of the PR-10 family and identify the catalytic residues of NCS.[31,32] Like PR-10 proteins, NCS possesses an accessible hydrophobic cleft located between a seven-stranded antiparallel β-sheet and three α-helices. Interestingly, despite its structural similarity with PR-10 allergens, *T. flavum* NCS is also not allergenic and does not bind to IgE antibodies.[30,33] This corresponds to our data with Dau-NCS, which also proved to be non-allergenic.

5. Concluding Remarks

Identification of new isoallergens and comparison with a non-allergenic Dau c 1-like protein may contribute to a more refined component resolved diagnosis and may lay ground for further epitope mapping and personalized targeted treatment approaches of carrot allergy in preclinical and clinical studies. Additionally, the knowledge of IgE epitopes can help to determine why some PR-10 proteins function as allergens whereas other similar proteins do not.

Supporting Information

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

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

Keywords

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

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