Molecular Nutrition & Food Research
www.mnf-journal.com

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.

1. Introduction

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

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

DOI: 10.1002/mnfr.202001110

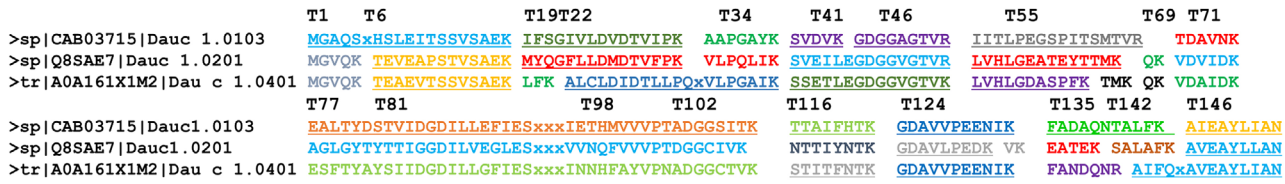

Figure 1. Amino acid sequence alignment of known Dau c 1 isoallergens and the Dau c 1.0401 sequence identified by LC-MS^E. Differing sequence stretches are colour-coded. Peptides which were detected by LC-MS^E are underlined. The positions of the amino acids in the sequence were labeled according to 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 Nomeclature 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 unambigously 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 A0A161×1M2) 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 isoallergens and the Dau c 1.0401 sequence identified previously by LC-MS^E.[20]

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]

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 2**A). 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

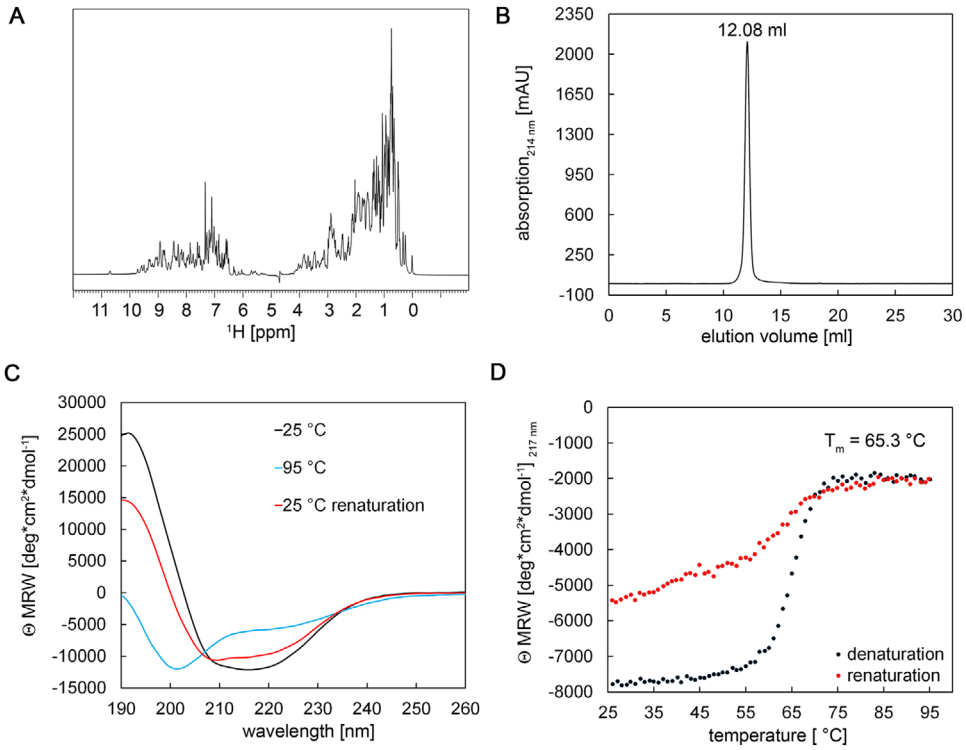

Figure 2. Physicochemical characterization of rDau c 1.0401. A) 1D NMR spectrum B) Analytical SEC using a Superdex 75 GL10/300 column C) CD spectra at 25 °C (black curve), 95 °C (blue curve) and after recooling to 25 °C (red curve), D) denaturation (black) and renaturation (red) curve of Dau c 1.0401.

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 Tm is 5–10 °C higher than the Tm 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 1.0401 could be observed, whereas IgE-binding to the rDau c 1.0104 contol, 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.	Clinical symptoms to carrot	Provocation	Skin test	CAP [kU_A L^{-1}] (CAP class)					
Patient	symptoms	OFC	Carrot	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.

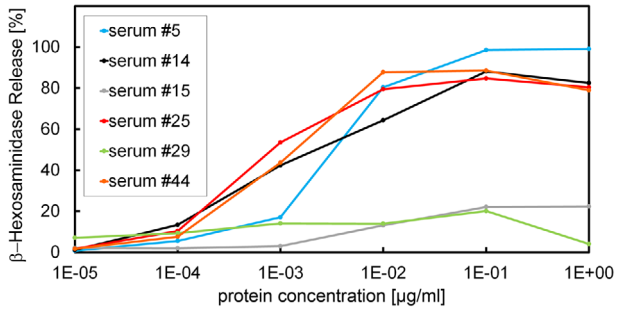

Figure 3. MRAs of rDau c 1.0401 with sera of six carrot-allergic patients. Patient sera (serum #5, blue lines; #14, black lines;#15, grey lines; #25, red lines; #29, green lines; #44, brown lines) were incubated with increasing amounts of rDau c 1.0401.

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,

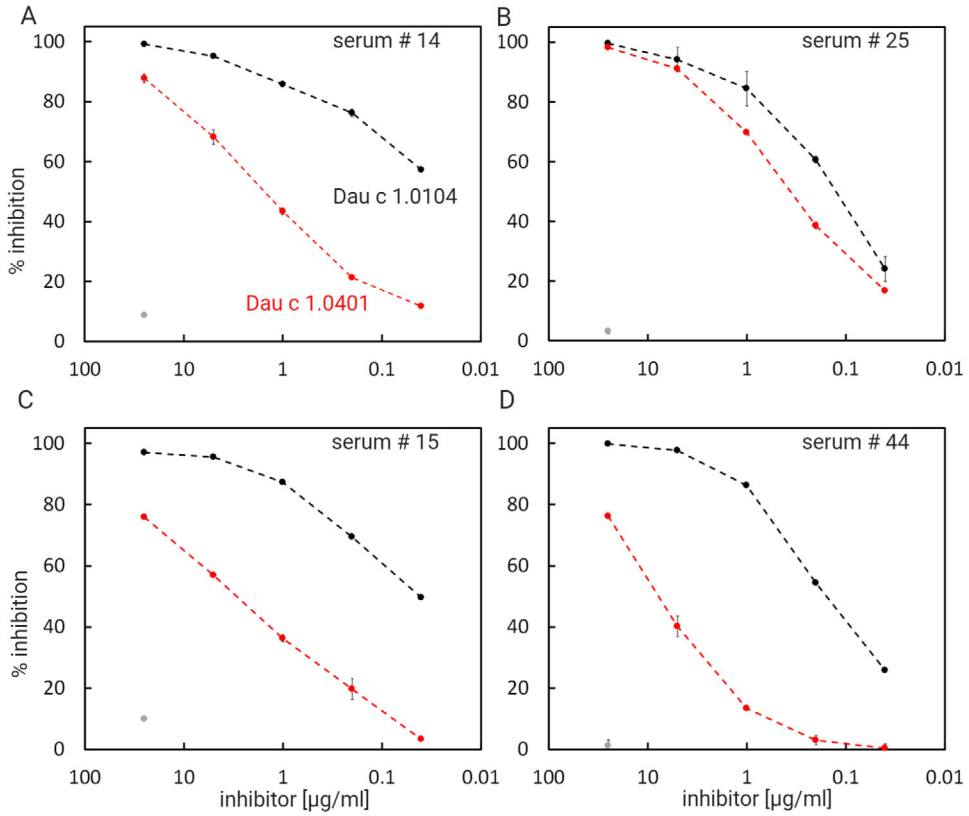

Figure 4. Inhibition of IgE-binding to rDau c 1.0104. Sera from carrot allergic patients (as indicated) were incubated with increasing amounts of either rDau c 1.0104 (black curves, self inhibition) or rDau c 1.0401(red curves). The ELISAs were performed with rDau c 1.0104 on the solid phase. IgE binding of rDau c 1.0104 after pre-incubation with 25 μg 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 6**A,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).

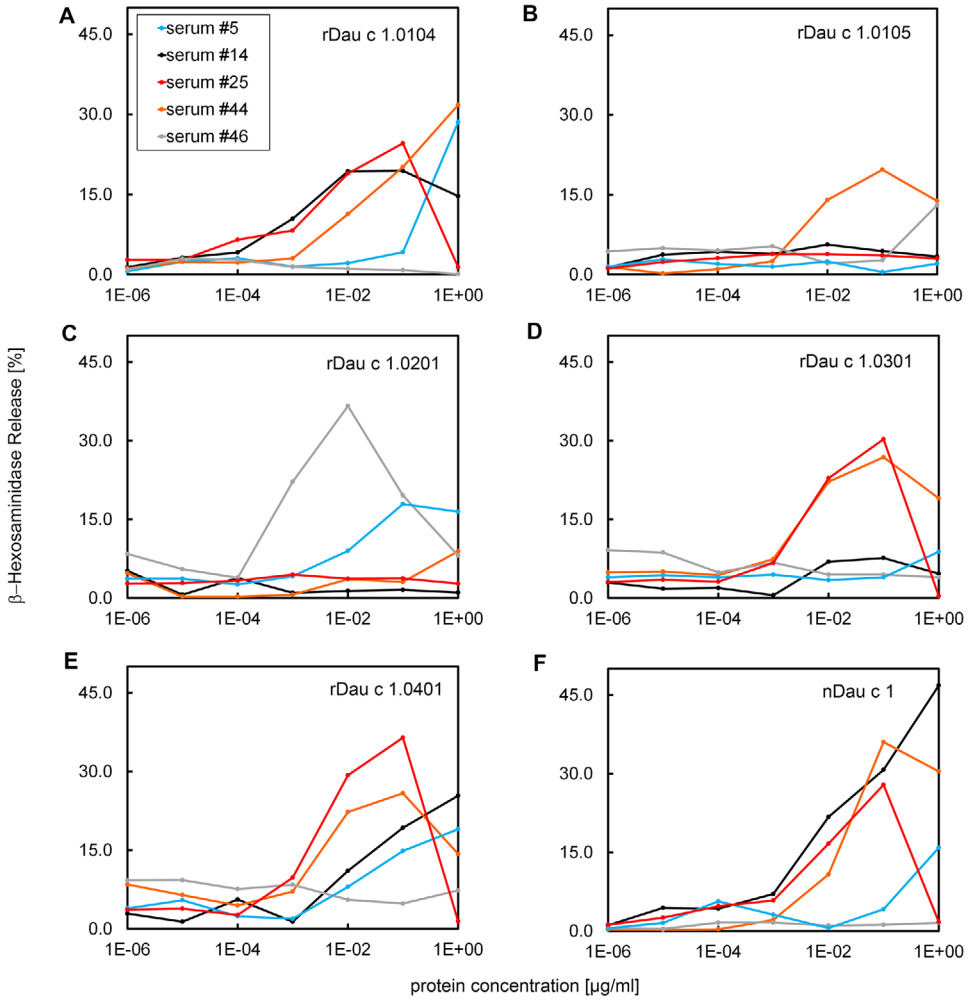

Figure 5. MRAs with different Dau c 1 proteins and sera of five carrot-allergic patients. Patient sera (serum #5, blue lines; #14, black lines; #25, red lines; #44, brown lines; #46, grey lines) were incubated with increasing amounts of Dau c 1 proteins.

Primers in the 5′ UTR (5′ -CACAGCATTCCTTGATAAGCTC, forward) and 3′ UTR (5′-CCATCCAGGTCTCACGAACA, reverse) region of the Dau c 1.0401 protein (Uniprot Accession number: A0A161×1M2) were designed based on its hypothetical mRNA (https://www.ncbi.nlm.nih.gov/nuccore/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, $(NH_4)_2SO_4$ 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 × 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

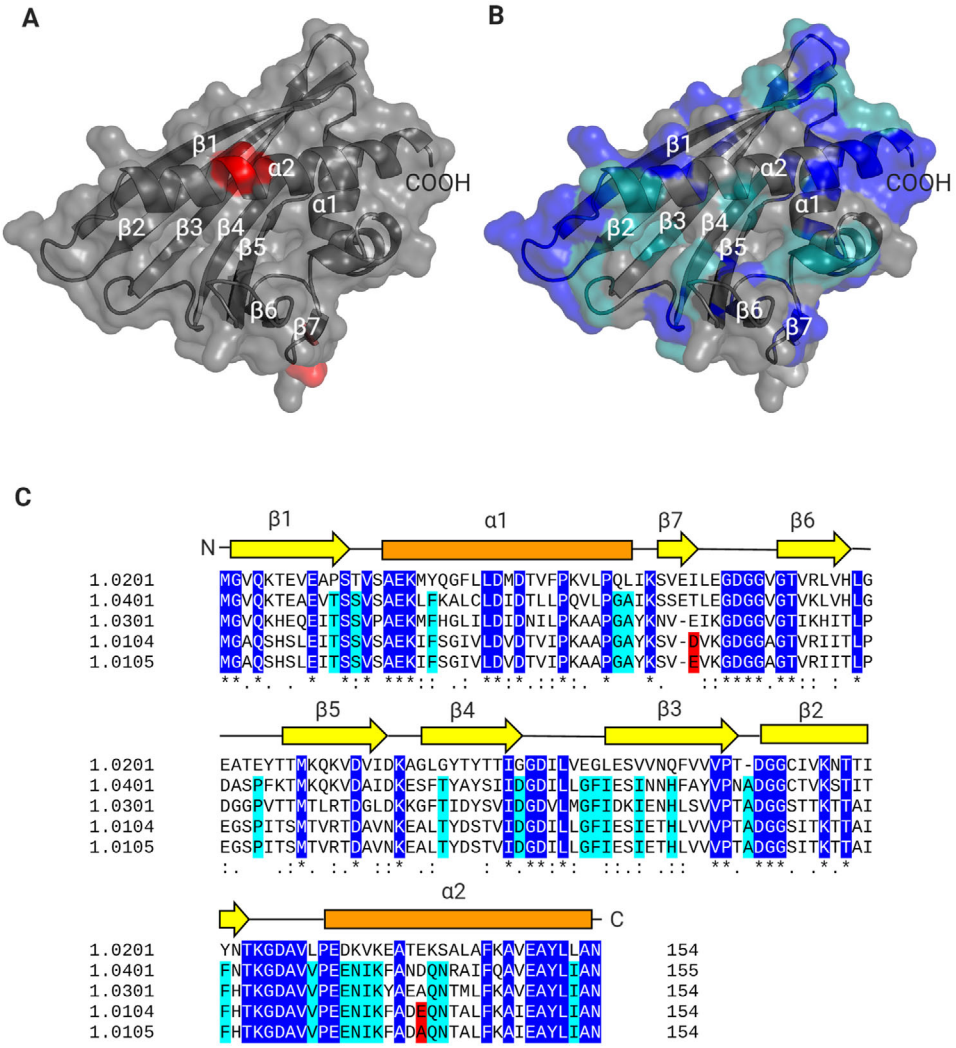

Figure 6. Sequence comparison of the tested Dau c 1 isoallergens. A) Cartoon representation of the Dau c 1.0105 model (Phyre 2). The two amino acid exchanges between Dau c 1.0104 and Dau c 1.0105 (D43E and E139A) are highlighted in red. B) Cartoon representation of the Dau c 1.0401 model (Phyre 2). Conserved 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 μm beads (Dr. Maisch GmbH, Ammerbuch, Germany). Protein identification was performed using the Byonic 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. Crameri, 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. Spiric, A. M. Engin, M. Karas, A. Reuter, *PLoS One* **2015**, *10*, e0142404.
[19] C. Seutter von Loetzen, A. Reuter, J. Spiric, T. Schulenborg, 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. Wöhrl, *Mol. Nutr. Food Res.* **2020**, *64*, 2000334.
[21] Y. Machado, R. Freier, S. Scheiblhofer, 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. Haustein, 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.