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

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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.

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 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

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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 recooling 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 c1.0105. 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

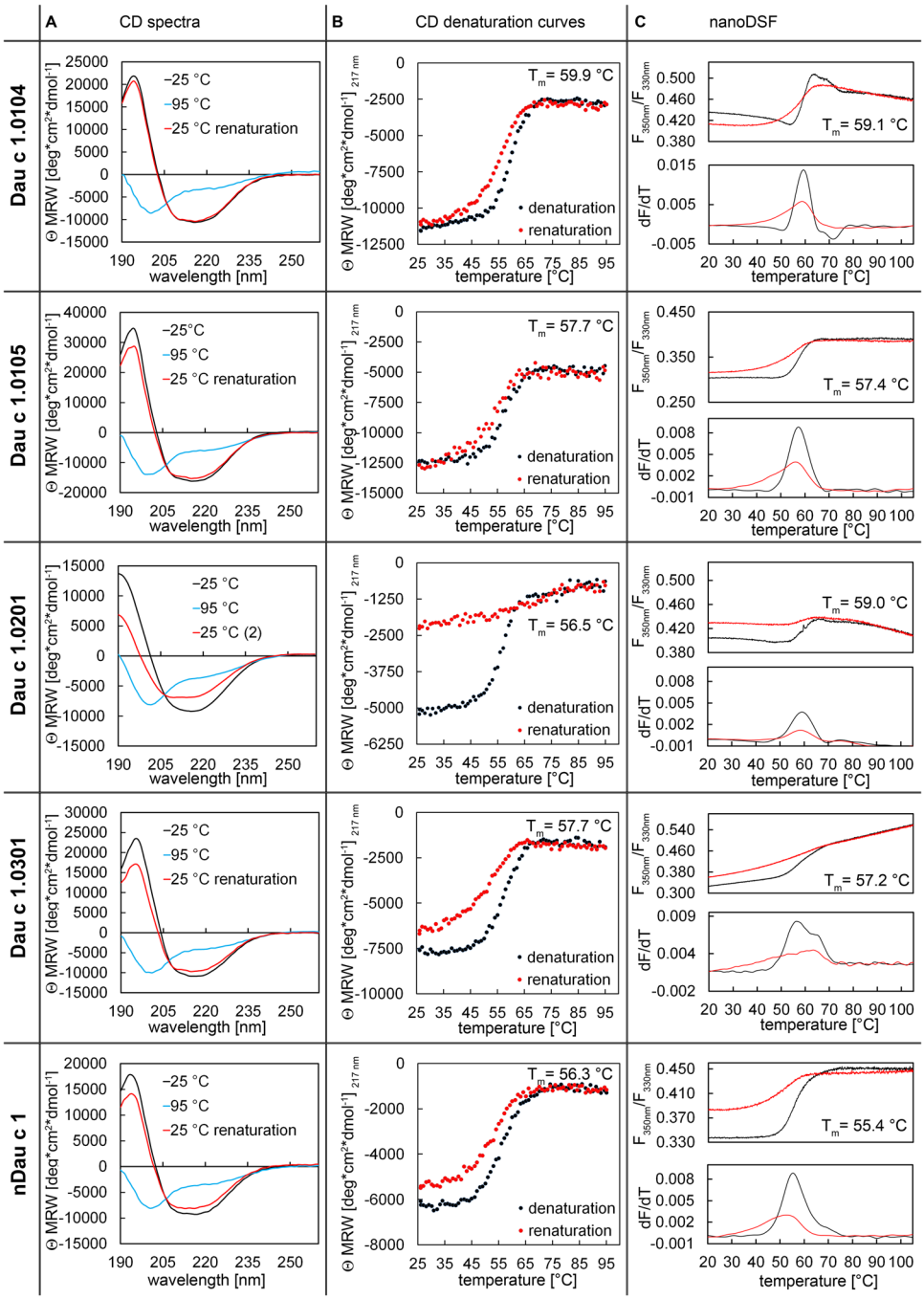

Figure 1. Different refolding capacities of Dau c 1 proteins. A) CD spectra were recorded at 25 °C (black lines), 95 °C (blue lines), and after recooling to 25 °C (red lines). B) CD denaturation (black) and renaturation curves (red). C) NanoDSF denaturation (black) and renaturation curves (red). The 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 ^{15}N-labeled proteins from *Escherichia coli* and recorded [^{1}H, ^{15}N] 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 recooling, 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 recooling, it

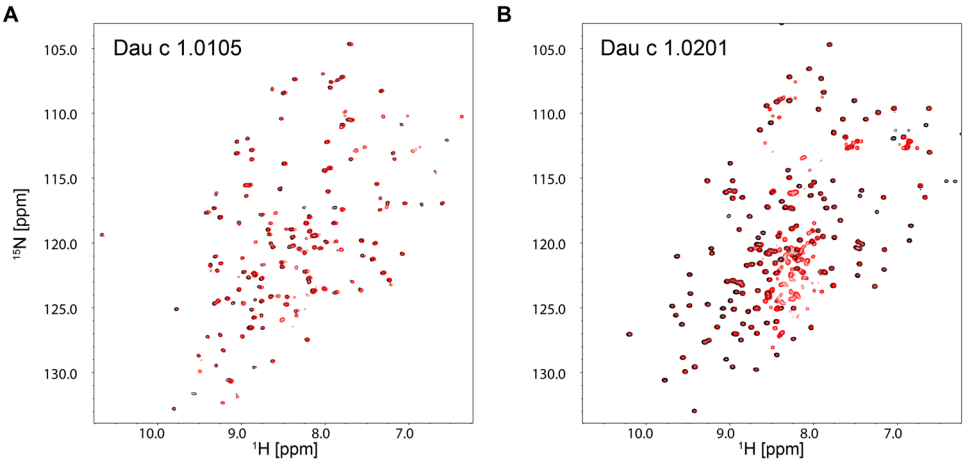

Figure 2. Influence of heat treatment on the protein structure at pH 7. [^{1}H, ^{15}N] HSQC spectra of Dau c 1.0105 (A) and Dau c 1.0201 (B) before (black) and after 1 h at 95 °C (red).

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 ^{1}H 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).

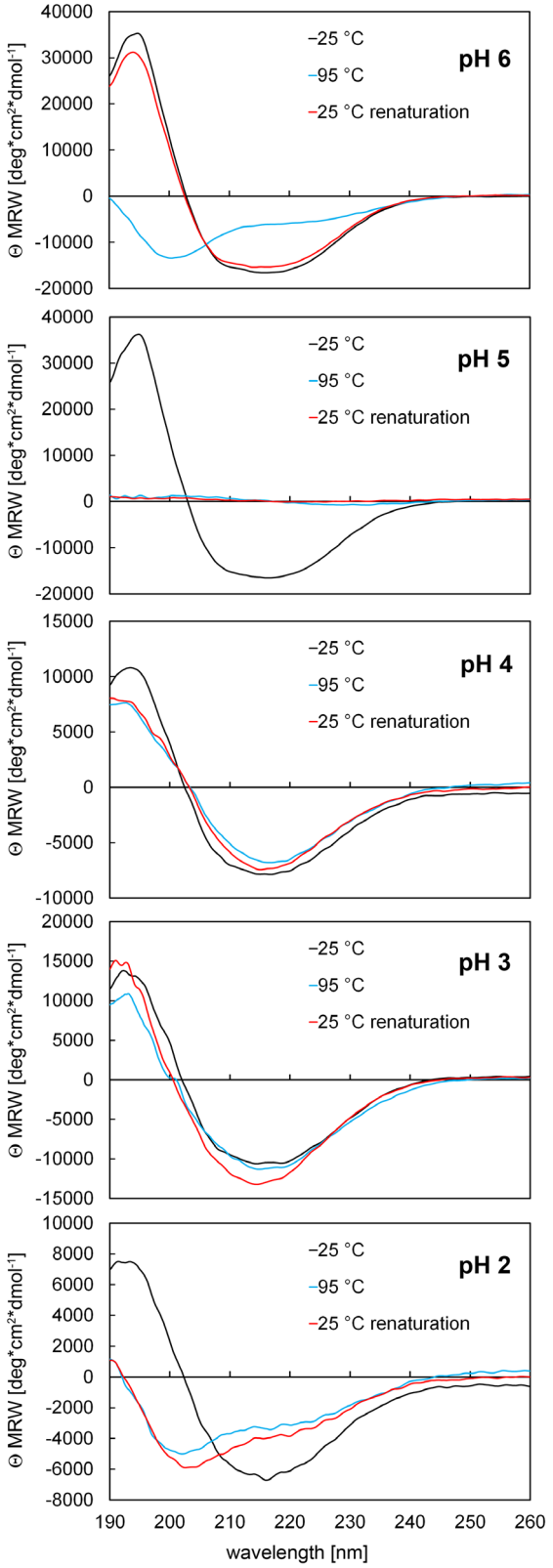

Figure 3. Stability of Dau c 1.0105 at different pH values. CD spectra of Dau c 1.0105 from pH 2 to pH 6 as indicated, before (25 °C, black line) and after heating to 95 °C (95 °C, blue line), and after recooling to 25 °C (25 °C renaturation, red line).

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

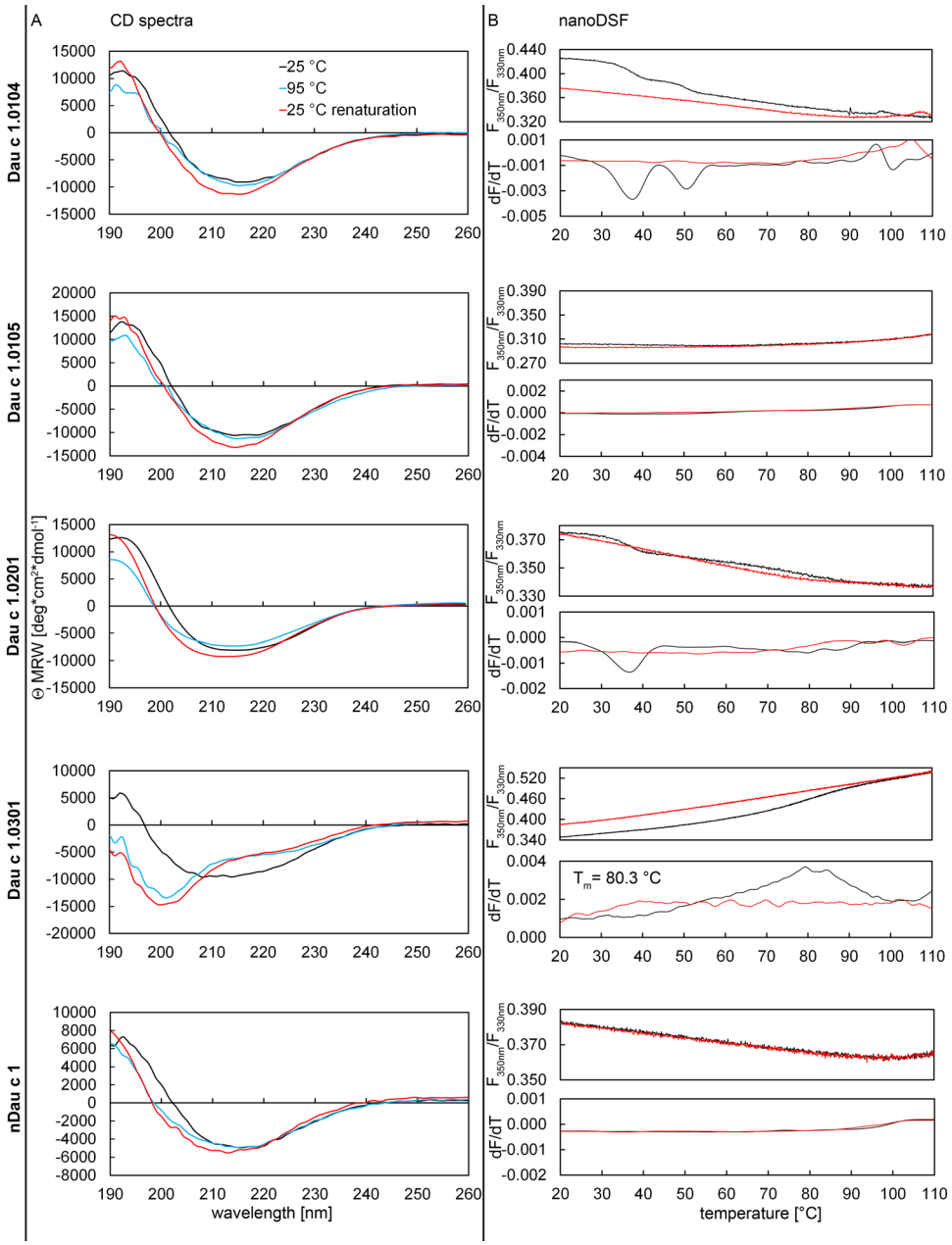

Figure 4. Stability of Dau c 1 proteins at pH 3. CD (left panel) and nanoDSF spectra (right panel). CD spectra were recorded at 25 °C (black), 95 °C (blue), and after recooling to 25 °C (red). NanoDSF denaturation (black) and renaturation (red) curves are presented. The upper parts show the fluorescence 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 7**A). 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

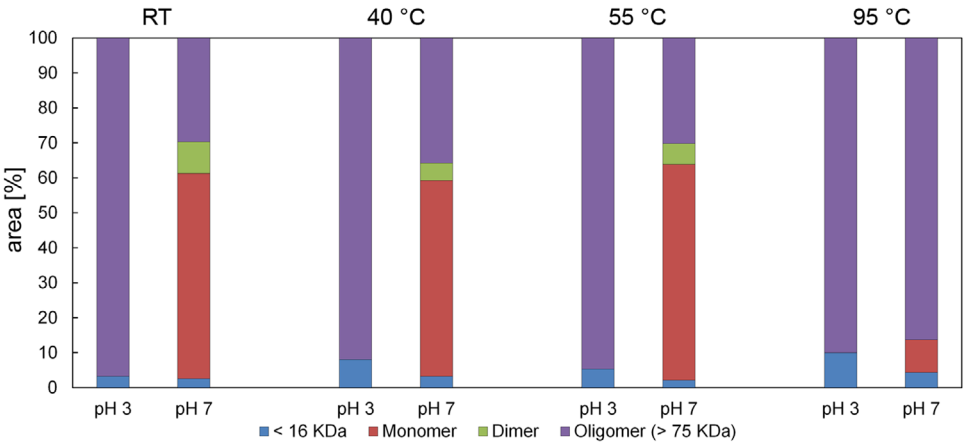

Figure 5. Oligomerization of Dau c 1.0104 upon heat and pH treatment. Dau c 1.0104 samples were incubated at room temperature (RT), 40 °C, 55 °C, or 95 °C in 10 mM Na-citrate, pH 3, for 30 min and then either loaded directly onto a Superdex S75 GL10/300 column equilibrated with 5 mM Na-citrate, pH 3, 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 H_2O extract before heating, at least nine different isoallergens and variants were identified (Table 2); eight of them have also been detected in purified nDau c1 (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. [^{1}H, ^{15}N] 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, [^{1}H, ^{15}N] HSQC spectra of the ^{15}N-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 und thus invisible) proteins present in the carrot matrix.

The [^{1}H, ^{15}N] 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 ^{1}H-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 ^{1}H 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),

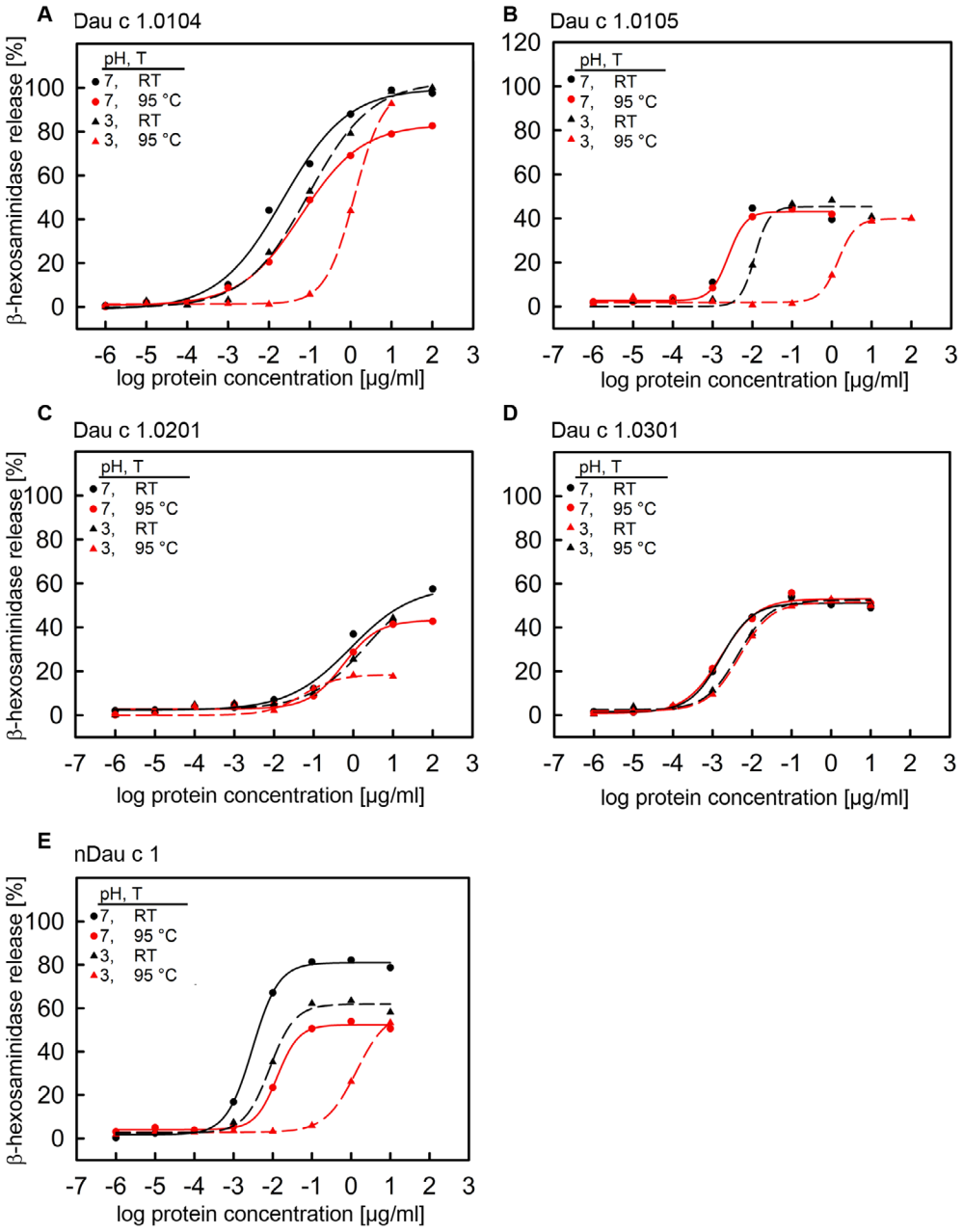

Figure 6. Allergenicity of pH- and heat-treated Dau c 1. MRAs of Dau c 1.0104 (A), Dau c 1.0105 (B), Dau c 1.0201 (C), Dau c 1.0301 (D), and nDau c 1 (E) at pH 7 (circles) and pH 3 (triangles) after 30 min incubation at room temperature (RT) (black) or 95 °C (red). Regression curves were performed with 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

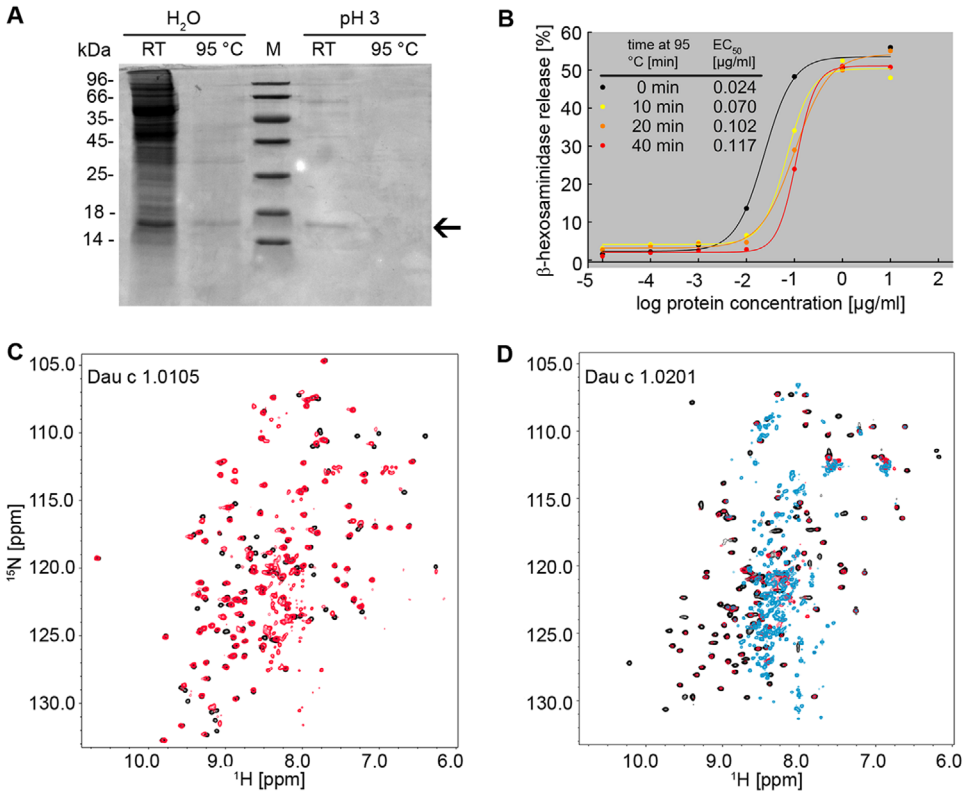

Figure 7. Dau c 1 stability in the presence of carrot matrix. A) SDS-PAGE (19 % gel) of carrot extract (30 mg mL^{-1}) in water before and after heating for 30 min at 95 °C. M, molecular weight standard (Biorad, low range); RT, room temperature. B) MRA of carrot extract before and after incubation for 10, 20, and 40 min at 95 °C as indicated. Regression curves were performed with the 4-parameter logistic model (sigma plot, version 14). [^{1}H, ^{15}N] 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, $(NH_4)_2SO_4$ was added to the supernatant to 40% saturation. Bacterial cells expressing rDau c 1.0301 or rDau c 1.0104 were lyzed and $(NH_4)_2SO_4$ 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*. $(NH_4)_2SO_4$ 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), $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$. 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 A_{280} 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 (H_2O-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_2O	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_2O	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 (H_2O) 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: [^{1}H, ^{15}N] HSQC spectra of 100–200 µM uniformly ^{15}N-labeled Dau c 1.0105 and Dau c 1.0201 in 10 mM Na-phosphate, 10% D_2O, 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.

MRAs 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.

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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

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