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Thermodynamic characterization of the PR-10 allergens Bet v 1, Api g 1 and Dau c 1 and pH-dependence of nApi g 1 and nDau c 1

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ABSTRACT

Natural and recombinant Bet v 1, the major birch pollen allergen, and homologous allergens, Api g 1 and Dau c 1, from celery and carrot, respectively, were studied by CD spectroscopy under conditions of varying denaturant concentration, pH and temperature to determine fundamental thermodynamic parameters for conformational stability. Thermodynamic studies increase basic knowledge regarding differences between birch pollen-related allergens and are of importance in choosing processing conditions. The conformational stability determined from guanidine hydrochloride denaturation curves was similar for rBet v 1.0101 and rApi g 1.0101. Conformational responses to chaotropic salt were different for recombinant allergens from different species, but were similar for the natural isoform mixtures. The conformational stabilities of nApi g 1 and nDau c 1, were shown to be similar to rBet v 1.2801 at pH > 4.4 [Mogensen, J. E., Ipsen, H., Holm, J., & Otzen, D. E. (2004). Elimination of a misfolded folding intermediate by a single point mutation. *Biochemistry*, 43(12), 3357-3367], but nApi g and nDau c 1 were stable to heating at lower pH-values.

1. Introduction

The major birch pollen allergen, Bet v 1, a pathogenesis-related-10 (PR-10) protein, is known as the primary sensitizer for birch pollen-related food allergy. The structural relationship with homologous proteins in foods is the basis for cross-reactivity (Hoffmann-Sommergruber et al., 1999a, 1999b; Radauer & Breiteneder, 2007). Many birch pollen allergic individuals will develop a birch pollen-related food allergy, in particular to fruits from the *Rosaceae* family, while a smaller group will respond to vegetables, such as carrot and celery from the *Apiaceae* (Hoffmann-Sommergruber & Radauer, 2003). In the case of carrot and celery, the exposure route plays a role, because these foods are most commonly consumed as processed foods, whereas the *Rosaceae* are mostly consumed raw. In general, the PR-10 allergens are characterized as labile proteins, in contrast to most other food allergens (Mills, Sancho, & Moreno, 2007), and cooking could therefore explain reduced IgE binding to Api g 1 from celery and Dau c 1 from carrot proteins (Hoffmann-Sommergruber & Radauer, 2003).

A few physicochemical studies have tested effects of processing on immune reactivity of PR-10 proteins. In several studies it was shown that the IgE binding capacity to Api g 1 of processed celery was almost completely reduced (Ballmer-Weber et al., 2002; Jankiewicz et al., 1997; Luttkopf, Ballmer-Weber, Wuthrich, & Vieths, 2000). Pickled celery, heat-sterilized at a low pH, demonstrated a remarkable reduction of the IgE reactivity (Jankiewicz et al., 1997). Both nonenzymatic and enzymatic browning reactions of rPru av 1 from cherry and of Mal d 1 extracts from apple, caused a remarkable reduction of the IgE reactivity (Garcia-Borrego, Wichers, & Wichers, 2007; Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004). On the other hand, 60 min cooking of different recombinant allergens, Bet v 1, Mal d 1, Api g 1 and Dau c 1, completely abolished IgE binding, but without a reduction of the capacity to activate allergen-specific T-cells (Bohle et al., 2006). Also gastrointestinal digestion destroyed IgE binding, but not T cell activation (Schimek et al., 2005).

The basis of allergen recognition is still unclear and a better understanding is needed of structural dynamics of the allergen. This can be accomplished by studying allergen mutants and by relating structural changes of a protein to changes in IgE binding capacity (Neudecker et al., 2003; Scheurer et al., 1999). Nevertheless, many mutants created are not well characterized and they are different from natural isoforms, due to expression in recombinant systems. This can easily change the IgE binding capacity, resulting in limited relevance for practical situations. Furthermore, allergy research concentrates strongly on the allergen's immune reactivity and its scope is directed to the malfunction of the immune system,

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but is less focused on structural properties of the allergen, which may overlook the impact of these properties on the immune system (e.g. lipid binding (Mogensen et al., 2007)).

Thermodynamic studies can increase basic knowledge of differences between PR-10 allergens and are of importance in choosing experimental conditions during processing. Basic knowledge is obtained in terms of thermodynamic parameters, such as ΔG_{D-N}, T_m, ΔH_{T_m} and ΔC_P, which are derived from the assumption of the reversible two-state model $N \rightleftharpoons D$, were N is the native/folded and D the denatured/unfolded state. The conformational stability, ΔG_{D-N}, is helpful in explaining differences between different PR-10 isoforms, as it is a fundamental measure of the difference of the Gibbs free energy between folded and unfolded molecules (Creighton, 1993). A physicochemical parameter required for the calculation of ΔG_{D-N} is m_{D-N}, which is a measure of the dependence of the free energy on denaturant concentration and reflects the degree of surface area buried in the native state relative to the denatured state (Myers, Pace, & Scholtz, 1995). The midpoint of thermal denaturation, T_m, is a stability parameter that indicates the temperature at which 50% of the protein is unfolded (Creighton, 1993). ΔH_{Tm}, is the enthalpy change required for a $N \rightleftharpoons D$ conversion of 1 mol of protein at T_m. This parameter is needed to calculate the heat capacity change upon unfolding, ΔC_P, which is yet another parameter for measuring stability and it can be used to calculate the conformational stability at any given temperature at constant pressure.

Stability measurements by circular dichroism (CD) have been performed previously with rBet v 1.2801 and its mutant Y120 W and also with rMal d 1, which showed relatively low values of ΔG_{D-N} and ΔC_P (Mogensen, Ipsen, Holm, & Otzen, 2004). By isolating the allergens from their natural source under mild conditions, isoform mixtures can be obtained (Bollen et al., 2007), which are closer to practical situations than are recombinant proteins. These mixtures can be studied for overall stability. The objective of this investigation was to study the thermodynamic stability of recombinant and natural Bet v 1, Api g 1 and Dau c 1 by determining the conformational stability, ΔG_{D-N} by guanidine hydrochloride (GuaHCl) denaturation, using CD measurements. Also, the effect of pH on thermal stability, on nApi g 1 and nDau c 1, was studied to determine T_m, ΔH_{Tm} and ΔC_P to increase general knowledge of the stability of natural isoform mixtures and their pH-dependence.

2. Materials and methods

2.1. Allergens

The natural allergens, nBet v 1, nApi g 1 and nDau c 1, from birch pollen, celery tuber and carrot, respectively, were purified as isoform mixtures, as described previously (Bollen et al., 2007). Briefly, Bet v 1 was purified from birch pollen of *Betula pendula* 'Youngii', Api g 1 from celery tuber purchased from a supermarket and Dau c 1 from *Daucus carota* 'Narbonne'. Ammonium sulphate precipitation, with the protein extracts, was followed by hydrophobic interaction and size exclusion chromatography. The purified allergens were identified as isoform mixtures, using Q-TOF MS/MS. The single recombinant allergen isoforms, rBet v 1a (further referred to as rBet v 1.0101), rApi g 1.0101 and rDau c 1.2 (further referred to as rDau c 1.0103), were purchased from Biomay (Vienna, Austria). All allergens were dissolved in 10 mM potassium phosphate buffer, pH 7.0, buffer exchanged and concentrated on a Microsep 3 K centrifugal device (Pall Life Sciences, Ann Arbor, MI, USA). Protein concentrations were determined using the Micro-BCA™ Protein Assay (Pierce, Rockford, IL, USA) with BSA as a standard.

2.2. GuaHCl denaturation curves

Guanidine hydrochloride (GuaHCl) denaturation experiments were carried out with natural and recombinant Bet v 1, Dau c 1 and Api g 1 at a protein concentration of 10 μM in 10 mM potassium phosphate buffer (pH 7.0). A 6 M GuaHCl stock solution was prepared in 10 mM potassium phosphate buffer and diluted into 4, 2, 1 and 0.5 M solutions. After filtering the solution with a 0.2 μm syringe filter (Schleicher & Schuell, Dassel, Germany), the final GuaHCl concentrations were determined from refractive index measurements according to Nozaki (1972), as calculated from Eq. (1):

$$[\text{GuaHCl}] = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3 \quad (1)$$

[GuaHCl] is given in mol/l and ΔN is the difference between the refractive index of the GuaHCl solution and the 10 mM phosphate buffer.

Circular dichroism (CD) spectra were recorded at 20 °C on a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) after allowing the GuaHCl–protein solutions to equilibrate for 2 h. Far-UV spectra were recorded from 210 to 260 nm with a quartz cuvette of 1 mm path length, by accumulating 10 scans at a scanning speed of 50 nm/min, using a 0.2 nm step width and 2.0 nm band width. Ellipticity values at 222 nm were corrected for buffer/GuaHCl background and plotted against the GuaHCl concentration to display protein denaturation curves. The raw CD data were converted into the mean residue weight ellipticity, $[\theta]_{MRW}$ (units in deg cm^2 dmol^{-1}), by using the following equation:

$$[\theta]_{MRW} = \frac{100 \times \theta_{obs}}{C \times l \times n} \quad (2)$$

θ_{obs} is the observed signal in degrees, C is the concentration in mol/l, l is the path length of the cuvette in cm and n is the number of amino acids of the protein.

From the denaturation plots, the conformational stability, i.e. the free energy of unfolding $\Delta G^{H_2O}_{D-N}$ of the protein in water was estimated, by assuming a two-state mechanism with a linear dependence of the pre- and post-transition baselines (Pace, 1986; Tanford, 1970). Eq. (3) was fitted to the data (Clarke & Fersht, 1993), by using non-linear least squares regression with the programme TableCurve (Jandel Scientific, Erkrath, Germany). Y_{obs} is the observed signal and [GuaHCl] the chaotropic salt concentration. The other six estimated parameters, from Eq. (3), include

$$Y_{obs} = \frac{\alpha_N + \beta_N[\text{GuaHCl}] + (\alpha_D + \beta_D[\text{GuaHCl}]) \times \exp\left\{\frac{m_{D-N}([\text{GuaHCl}] - [\text{GuaHCl}_{50\%}])}{RT}\right\}}{1 + \exp\left\{\frac{m_{D-N}([\text{GuaHCl}] - [\text{GuaHCl}_{50\%}])}{RT}\right\}} \quad (3)$$

the slopes and intercepts of the baselines of the native (β_N) and denatured (β_D) states with the ellipticity values of the native (α_N) and denatured (α_D) state at 0 M GuaHCl. [GuaHCl$_{50\%}$] is the midpoint of denaturation from the transition state where 50% of the protein is denatured. The m_{D-N} value (in kJ mol^{-1} M^{-1}) is a parameter correlating with the degree of protein surface exposed to the solvent upon unfolding. The proteins started to unfold already at low [GuaHCl] resulting in a imprecise estimate of the parameter, β_N, for the slope of the baseline for the native protein. Therefore, a change was made in the fitting procedure by fixing this parameter to zero, resulting in similar standard errors from the regression analysis for all GuaHCl denaturation curves, showing that β_N is redundant here.

From the estimated values of m_{D-N} and [GuaHCl$_{50\%}$], the conformational stability, $\Delta G^{H_2O}_{D-N}$, of the allergen in water, is calculated by using (Pace, 1986):

$$\Delta G_{D-N} = \Delta G^{H_2O}_{D-N} - m_{D-N}[\text{GuaHCl}] \quad (4)$$

The apparent free energy difference, ΔG_{D-N}, is the value of $\Delta G^{H_2O}_{D-N}$ in the absence of denaturant and is 0 at [GuaHCl$_{50\%}$].

2.3. pH stability experiments

pH stability experiments were performed with natural Api g 1 and Dau c 1 by recording far-UV CD spectra, as in the GuaHCl denaturation experiment, and the thermal denaturation CD curves at different pH-values. Changes in ellipticity were followed at 222 nm by heating to 95 °C at a rate of 1 °C/min using a band width of 1.0 nm with 10 μM protein in different 50 mM buffers with the pH set at room temperature, as in the method used by Mogensen et al. (2004): 100 mM HCl at pH 1.0, glycine at pH 2.0–3.5, sodium acetate at pH 4.0–5.3, MES at pH 5.5–6.5, MOPS at pH 6.5–7.5, and Tris at pH 8.0–9.0. The thermal denaturation curves were fitted using a non-linear least squares fit method (Yadav & Ahmad, 2000) from the software programme TableCurve according to the relationship derived from the van't Hoff equation:

$$\theta = \frac{\alpha_N + \beta_N T + (\alpha_D + \beta_D T) \times e^{-\left[\Delta H_{T_m}\left(1-\frac{T}{T_m}\right)\right]/RT}}{1 + e^{-\left[\Delta H_{T_m}\left(1-\frac{T}{T_m}\right)\right]/RT}} \quad (5)$$

Six parameters were estimated from fitting Eq. (5), which includes the slopes and intercepts of the baselines of the native (β_N) and denatured (β_D) states with the ellipticity values (intercepts) for the folded (α_N) and unfolded (α_D) state. The other derived parameters were T_m, the temperature at the midpoint of denaturation, and ΔH_{T_m} the enthalpy of unfolding at T_m. After heating, the protein was cooled to room temperature to study the refolding properties of the protein. The programme CDNN was used to deconvolute the secondary structure of measured CD spectra (Bohm & Jaenicke, 1992).

From the linear relationship between ΔH_{T_m} and T_m at varied pH, the specific heat capacity, ΔC_P, can be obtained from the slope of the graph (Becktel & Schellman, 1987), making the assumption that ΔC_P does not depend on pH and temperature in the thermal transition range. With the value of ΔC_P, the Gibbs energy change of unfolding at any temperature, ΔG_T, is calculated by using the Gibbs–Helmholtz equation at 298.15 K (Yadav & Ahmad, 2000).

$$\Delta G_T = \Delta H_{T_m}\left(1 - \frac{298.15}{T_m}\right) - \Delta C_P\left[(T_m - 298.15) + 298.15 \ln\left(\frac{298.15}{T_m}\right)\right] \quad (6)$$

Gibbs energy values for protein stability are obtained by taking the value of ΔH_{T_m} and T_m at pH 7.0. Values thus calculated can be compared with $\Delta G^{H_2O}_{D-N}$ values of protein stability curves.

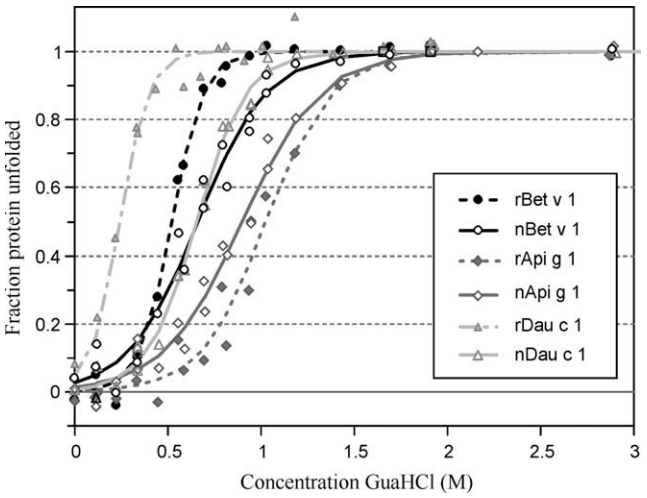

Fig. 1. CD denaturation curves of natural and recombinant allergens by increasing the GuaHCl concentration. The individual measured points are displayed with the stability curves fitted using Eq. (3).

3. Results

3.1. Protein stability estimated from denaturation with guanidine hydrochloride

Conformational stability of proteins can be determined by measuring the unfolding curves in aqueous solutions with increasing concentrations of chaotropic salts such as guanidine hydrochloride (GuaHCl) (Pace, 1986). The parameters derived from the fit of these graphs resulted in a value for the change in Gibbs free energy, ΔG_{D-N}, in water ($\Delta G^{H_2O}_{D-N}$). Protein stability curves were measured with GuaHCl denaturation for recombinant and natural Bet v 1, Api g 1 and Dau c 1. The resulting graphs are shown in Fig. 1 with the individual data points as a fraction of the unfolded protein versus the increasing GuaHCl concentration and the corresponding denaturation curves fitted with Eq. (3). The estimated parameters, m_{D-N} and [GuaHCl$_{50\%}$], are given in Table 1 with the standard deviations of the fits and the calculated $\Delta G^{H_2O}_{D-N}$ from Eq. (4).

The stability curves of Fig. 1 and the $\Delta G^{H_2O}_{D-N}$ values in Table 1 clearly showed a low stability of rDau c 1.0103, which started to unfold already at low concentrations of GuaHCl, which incidentally resulted in a larger fitting error (Table 1). The sharp transition from native to denatured state was reflected in a larger value of m_{D-N} for rDau c 1.0103. The conformational stability in terms of Gibbs free energy change was calculated from the two parameters, [GuaHCl$_{50\%}$] and m_{D-N}. The generally observed trend was a lower m_{D-N} value for the natural allergens and a higher $\Delta G^{H_2O}_{D-N}$ for the recombinant Bet v 1 and Api g 1. Apart from rDau c 1.0103, the [GuaHCl$_{50\%}$] values of natural and recombinant Api g 1 were 0.24–0.51 M higher than Dau c 1 and Bet v 1.

A ratio from the GuaHCl and urea denaturation data could be calculated from the m_{D-N}-values of rBet v 1.0101 of our data and of rBet v 1.2801 of Mogensen et al. (2004). This m(GuaHCl)/m(urea) ratio is 2.8 and describes the unfolding of Bet v 1 as polar compared to ratios of 1.6–2.3 for unfolding of non-polar sidechains (Greene & Pace, 1974). Myers et al. (1995) showed a linear relationship between changes in the accessible surface area (ΔASA) of a protein and the m_{D-N} values measured by GuaHCl or urea denaturation. ΔASA is defined as the difference between the solvent-accessible surface area of the native protein and the modelled denatured protein. From the values of ΔASA, calculated from the GuaHCl data m_{D-N} (Table 2), an estimate could be made of the theoretical number of amino acid residues, ΔC_P and m_{D-N} of urea denaturation. The

Table 1
Thermodynamic parameters of the recombinant and natural allergens of Bet v 1, Api g 1 and Dau c 1 determined from GuaHCl denaturation[a] and the pH experiment.[b]

	m_{D-N} (kJ/mol M)	[GuaHCl$_{50\%}$] (M)	$\Delta G^{H_2O}_{D-N}$ (kJ/mol)	ΔC_P (kJ/mol K)	ΔG_T (kJ/mol)	pH optimum for T_m	pH transition at 20 °C
rBet v 1.0101	28.7 ± 2.6	0.52 ± 0.01	15.0	–	–	–	–
nBet v 1	13.4 ± 2.1	0.66 ± 0.03	8.8	–	–	–	–
rApi g 1.0101	14.2 ± 2.4	1.03 ± 0.04	14.6	–	–	–	–
nApi g 1	11.7 ± 1.4	0.90 ± 0.03	10.5	5.5	19.9	6.5	4.75
rDau c 1.0103	31.4 ± 6.2	0.24 ± 0.02	7.6	–	–	–	–
nDau c 1	18.5 ± 1.6	0.66 ± 0.01	12.1	4.7	17.4	6.3–6.5	4.42
rBet v 1.2801[c]	10.1 ± 1.3	2.43 ± 0.07	24.4	5.0	18.8	6.5–7.0	~3.5
rBet v 1.2801 Y120W[c]	11.0 ± 1.1	2.46 ± 0.03	27.0	–	–	–	–
rMal d 1[c]	11.6 ± 1.3	2.61 ± 0.03	30.5	–	–	–	–

[a] All conditions for GuaHCl denaturation are at 25 °C and pH 7.0. Errors reported are standard deviations from regression analysis. m_{D-N} and [GuaHCl$_{50\%}$] values were estimated from the fits of Fig. 1 and were used to calculate the conformational stability in water, $\Delta G^{H_2O}_{D-N}$, from Eq. (4).
[b] The value of the heat capacity change, ΔC_P, was derived from the slopes of Fig. 4 and was used to calculate the conformational stability, ΔG_T, at 25 °C for pH 7.0 from Eq. (6). The pH optimum for reversible unfolding and the pH transition of the midpoint of the fit at 20 °C were both determined from Fig. 3.
[c] Urea denaturation and pH experimental data adapted from Mogensen et al. (2004).

Table 2
Theoretical derived parameters from the linear relationship of m_{D-N} values and the changes in accessible surface area.

	ΔASA[a] (Å^2)	Number of AA residues[b]	ΔC_P[c] (kJ/mol K)	Urea m_{D-N}[d] (kJ/mol)
Theoretical protein[e]	13,880	159	9.4	7.9
rBet v 1.0101	27,459	305	17.6	12.7
nBet v 1	10,641	124	7.4	6.0
rApi g 1.0101	11,550	134	8.0	6.4
nApi g 1	8823	105	6.3	5.3
rDau c 1.0103	30,186	334	19.3	13.8
nDau c 1	16,095	183	10.7	8.2

All parameters were calculated from the m_{D-N} values of GuaHCl denaturation (Table 1) from the empirically established linear correlations described by Myers et al. (1995). N.B. All linear correlations are given in Cal.
[a] The change in accessible surface area, ΔASA, was calculated from: $m_{D-N(GuaHCl)} = 859 + 0.22(\Delta ASA)$.
[b] The number of amino acid residues was calculated from; $\Delta ASA = -907 + 93(\#res)$.
[c] The heat capacity change, ΔC_P, was calculated from: $\Delta C_P = -336 + 0.66(m_{D-N(GuaHCl)})$.
[d] The theoretical value for urea denaturation, urea m_{D-N}, was calculated from: $m_{D-N(GuaHCl)} = -110 + 2.3(m_{D-N(urea)})$.
[e] A theoretical protein is included in the table with the parameters calculated for 159 amino acid residues.

number of amino acid residues was overestimated for rBet v 1.0101 for the GuaHCl experiment, whereas the number of amino acid residues for nBet v 1 and nApi g 1 were underestimated, due to lower m_{D-N} values as a result of isoform mixtures. The m_{D-N} value of rBet v 1.0101, calculated for urea denaturation, corresponded to the m_{D-N} value of Mogensen et al. (2004) and was slightly higher, because the linear relationship did not correct for polar unfolding.

3.2. Effect of pH on thermal stability of nApi g 1 and nDau c 1

A more practical situation is represented by the variety of isoforms present in different food extracts as compared to single recombinant isoforms. Therefore, the thermal stabilities of natural isoform mixtures of Api g 1 and Dau c 1 were determined under various pH-conditions by heating to 95 °C, followed by cooling, using CD spectrometry at 222 nm (Fig. 2A). The thermal unfolding curves, determined for each pH in the range 2.0–9.0, showed similar patterns for nDau c 1 (Fig. 2A) and nApi g 1 (not shown) and could be divided into four different groups: (1) stable to heating between pH 2.0–4.0; (2) unfolding, but no refolding, due to aggregation, in the pH range 4.4–5.6 for nDau c 1 and 4.4–6.0 for nApi g 1; (3) unfolding with a double transition, at approximately 60 and 85 °C at pH 6.0 for nDau c 1 and pH 6.3 for Api g 1; (4) unfolding and refolding between pH 6.3–9.0 for nDau c 1 and 6.5–9.0 for nApi g 1.

Thermal stability was expressed in terms of T_m, the temperature values where 50% of the protein is unfolded and ΔH_{Tm}, the enthalpy of unfolding at T_m. Reproducibility of these two parameters was confirmed by duplicate measurements of thermal denaturation curves at pH 4.9 and by determining the differences between the two buffers, MES and MOPS, at pH 6.5, which were minimal (with %RSD $\leqslant$1% for T_m-values and $\leqslant$10% for ΔH_{Tm}). Differences were observed between 10 mM potassium phosphate and 50 mM MOPS buffers at pH 7.0, with approximately 5 °C lower T_m-values for the potassium phosphate buffer. These differences may be caused by changes in buffer pH at higher temperature, but also by differences in ionic strength.

Although nApi g 1 and nDau c 1 proved to be heat-stable in the pH range 2.0–4.0 at 222 nm, changes did occur in the CD spectrum at other wavelengths. A representative example of nDau c 1 CD spectra at 20, 95 and 20 °C after cooling at pH 4.0 is displayed in Fig. 2B, together with the CD spectrum of the unheated allergen at pH 7.0. A clear difference in shape is visible between the spectra of the unheated allergen at pH 7.0 and pH 4.0, which was apparent from the estimates of decreasing helical structure (from 33.4% to 20.9%) and an increase in β-sheet content (from 21.7% to 27.2%). Heating at pH 4.0 did not change the CD-signal between 210 and 260 nm, but a shift occurred in the spectrum on the horizontal axis to a lower wavelength. Upon cooling, the protein did not refold to its starting signal and the signal at 222 nm decreased, showing a minimum at 217 nm in the full spectrum. The programme CDNN, for secondary structure deconvolution, could not determine a difference between the protein spectra at 95 °C and the cooled protein but, by comparing heated and unheated protein, decreases were found for the helical (from 20.9% to 18.6%) and β-sheet contents (from 27.2% to 25.1%). For pH 3.5 and below, cooling after a 95 °C treatment resulted in an increase in the helical content and a decrease in β-sheet (data not shown).

Differences between nApi g 1 and nDau c 1 were observed by plotting $[\theta]_{MRW,222nm,}$ from the thermal denaturation curves of Fig. 2A at four different temperatures, against the pH (Fig. 2C and D). To show the similar transitions at pH 6.0, different tempera-

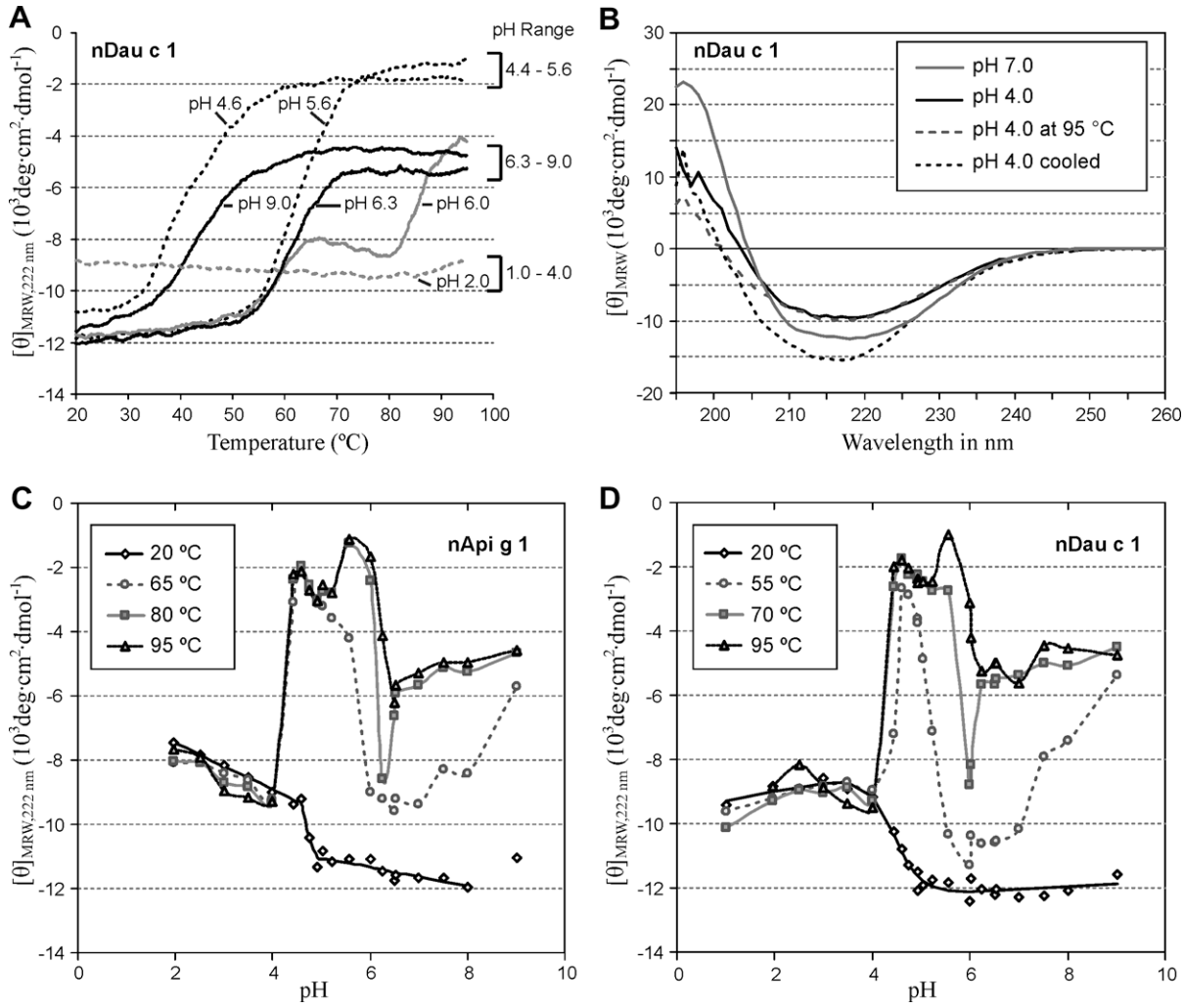

Fig. 2. Effect of pH on thermal stability of nDau c 1 and nApi g 1 in the range 20–95 °C. (A) Thermal denaturation curves of nDau c 1 can be divided into four different groups as indicated in the graph: (1) heat-stable from pH 1.0–4.0; (2) unfolding but no refolding capacity from pH 4.4–5.6; (3) both unfolding and refolding capacity from pH 6.3–9.0; (4) a double transition at pH 6.0. (B) CD spectra of nDau c 1 at pH 7.0 and 4.0. The CD spectra at pH 4.0 were measured at 20, 95 and at 20 °C after cooling. (C and D) CD-signal at 222 nm at different pH-values at four different temperatures, 20, 65, 80 and 95 °C, for nApi g 1 and 20, 55, 70 and 95 °C for nDau c 1. The CD-signal at 20 °C is represented with a fit to the data points at different pH-values.

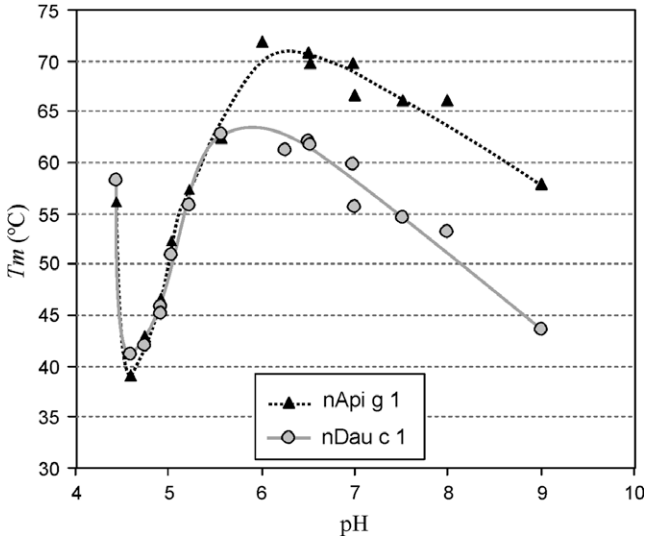

Fig. 3. pH stability of nApi g 1 and nDau c 1 measured by CD. The pH is plotted for the T_m-values, as determined from the fits of the thermal denaturation curves of each buffer. The lines are shown to guide the eye.

tures were used for nApi g 1 and nDau c 1, as the T_m-values for nApi g 1 were approximately 10 °C higher than those for nDau c 1. Both plots at 20 °C showed a transition region near their pI, at 4.4 for nDau c 1 and 4.75 for nApi g 1, but a difference in the shape of the fits. The smaller $[\theta]_{MRW,222\ nm}$-change in the transition region for nApi g 1 as compared to nDau c 1 was a result of the continuously decreasing CD-signal of nApi g 1, whereas the CD-signal of nDau c 1 was relatively unchanged in the pH ranges 2.0–4.0 and 6.0–8.0. The graphs also showed that $[\theta]_{MRW,222\ nm}$ did not change upon heating in the pH range 2.0–4.0. The steep slope for the graphs at 95 °C between pH 5.6 and 6.5 was at a point where irreversible unfolding changed into reversible folding in the higher pH range.

Other differences in thermal stability between nApi g 1 and nDau c 1 were seen by plotting T_m versus pH for each thermal denaturation curve (Fig. 3). The highest T_m value found for nApi g 1 was 71.9 °C at pH 6.0 and 62.8 °C for nDau c 1 at pH 5.6. The thermal denaturation curves with a double transition region at pH 6.3 and 6.0 for nApi g 1 and nDau c 1, respectively, were not taken into account. At these pH-values the allergens were more stable to heating, as shown by an intermediate state visible after the first transition (Fig. 2A, pH 6.0), which continued unfolding at a

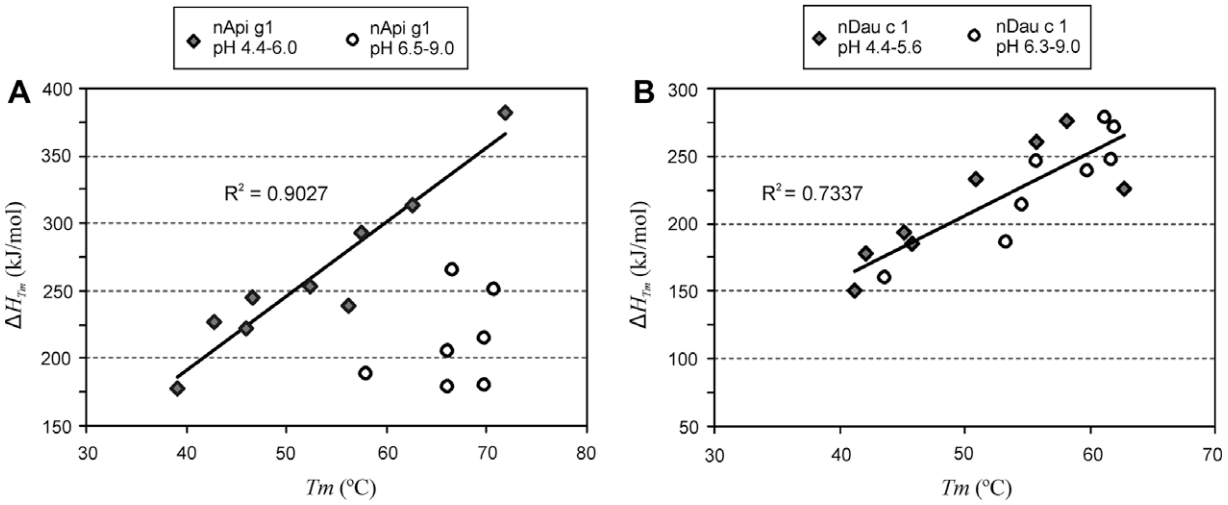

Fig. 4. Relationship of enthalpy of unfolding, ΔH_{Tm}, and T_m of nApi g 1 (A) and nDau c 1 (B). ΔH_{Tm} and T_m were determined from the fits of the thermal denaturation curves between pH 4.4 and 9.0. The data points are split into two series; (1) irreversible unfolding (low pH range, diamond shapes), (2) reversible unfolding (higher pH range, open circles). For nApi g 1, a linear fit is shown through the data points at low pH (diamond shapes) and, for nDau c 1, a fit is shown through all data points. The heat capacity change ΔC_P was derived from the slopes of the linear fits.

temperature above 80 °C. Optimal pH stability, with ability to refold completely, was observed at pH 6.5 and 6.3–6.5 for nApi g 1 and nDau c 1, respectively. Furthermore, the T_m-values were lowest at pH 4.6: 39.1 °C for nApi g 1 and 41.2 °C for nDau c 1. T_m increased very rapidly below pH 4.6, resulting in a higher T_m value at pH 4.4 for both nApi g 1 and nDau c 1. At a pH below 4.4, no thermal denaturation occurred, as judged from the $[\theta]_{MRW,222\ nm}$ signal, and consequently no parameters could be calculated.

The conformational stability of the allergens, as determined from the GuaHCl stability curves for pH 7.0, could be derived from the proposed linear relationship of T_m and ΔH_{Tm} at different pH-values (Fig. 4), as was empirically established by Privalov (1979). For this approach, the calculation of ΔG_T from Eq. (6) required the specific heat capacity, ΔC_P, as a parameter. ΔC_P is defined as the change in enthalpy with temperature and could be derived from the slope of the linear regression of ΔH_{Tm} versus T_m. All calculated ΔH_{Tm} and T_m parameters were calculated from the different buffers by assuming two-state unfolding kinetics. However, protein, between pH 4.4 and 6.0, did not follow two-state unfolding kinetics, as shown by irreversible unfolding. Therefore, these data points were visually divided in the plots from the pH range 6.5–9.0 for reversible unfolding. All data points of nDau c 1 showed a clear linear relationship but, for nApi g 1, a linear relationship was only observed for the irreversible unfolding range. Therefore, the value of ΔC_P (Table 1) was derived from the linear slopes through the data points at pH 6.0 and below for nApi g 1 and all data points for nDau c 1. The ΔG_T values were calculated at 25 °C by using the T_m and ΔH_{Tm} values of nApi g 1 (T_m = 66.5 °C; ΔH_{Tm} = 265.1 kJ/mol) and nDau c 1 (T_m = 55.7 °C; ΔH_{Tm} = 247.0 kJ/mol) from thermal denaturation curves at pH 7.0 (Table 1).

4. Discussion

This study describes differences in conformational stability of the major birch pollen allergen, Bet v 1, and the vegetable allergens, Api g 1 and Dau c 1, from celery and carrot, respectively. Conformational stability was determined from thermodynamic parameters, derived from CD measurements. The characterization of thermodynamic parameters is helpful in explaining differences between the folded and unfolded states of PR-10 proteins and could form a basis for explaining the severity of the allergic response to different isoforms. The recombinant single isoforms, rBet v 1.0101, rApi g 1.0101 and rDau c 1.0103, were compared with isoform mixtures, purified from their natural source (Bollen et al., 2007). In general, the conformational stabilities of the measured allergens were low and differences observed between natural and recombinant and between the vegetable and birch protein showed how important this thermodynamic characterization could be.

In the first part of this study, differences in conformational stability were clearly visible between the recombinant PR-10 allergens. Naturally occurring globular proteins, in general, have a $\Delta G^{H_2O}_{D-N}$ between 21 and 42 kJ/mol (Creighton, 1993), but the values found by GuaHCl denaturation were below 15 kJ/mol for all allergens. By using a linear extrapolation method, the lowest estimate for $\Delta G^{H_2O}_{D-N}$ is obtained, but this method, in general, should give the best agreement with $\Delta G^{H_2O}_{D-N}$ values from urea denaturation curves (Pace, 1986). In particular rDau c 1.0103 showed an extremely low $\Delta G^{H_2O}_{D-N}$, resulting from a 3 times lower [$GuaHCl_{50\%}$], as for nDau c 1. This commercially available allergen was purchased with an attached his-tag, which likely decreased protein stability. Although similar conformational stabilities were observed for rBet v 1.0101 and rApi g 1.0101, different properties were observed in the presence of denaturant shown from the m_{D-N} and [$GuaHCl_{50\%}$] values. A point of attention is that the parameters derived for the natural allergens were averages of a mixture, whereas the parameters determined for recombinant allergens were based on a single isoform. This resulted in reduced slopes in the transition regions for the natural allergens and therefore lower estimates of the conformational stability in GuaHCl denaturation experiments. However, [$GuaHCl_{50\%}$] values were similar between the recombinant and natural allergens of Bet v 1. For Api g1, both [$GuaHCl_{50\%}$] and m_{D-N} values were similar between natural and recombinant allergen preparations.

Most striking were the relatively high values of m_{D-N} for rBet v 1.0101 and rDau c 1.0103, as compared to other denaturation studies (Clarke & Fersht, 1993; Creighton, 1993; Greene & Pace, 1974; Myers et al., 1995; Saito & Wada, 1983). The theoretically derived values for the number of amino acid residues (Table 2) showed the possibility that rBet v 1.0101 and rDau c 1.0103 do not follow two-state unfolding, whereas rApi g 1.0101 does. It has been shown that rBet v 1.0101 is able to form dimers (and oligomers) in solution (Schöll et al., 2005), which have an effect on unfolding kinetics. In folding experiments by Mogensen et al. (2004), a misfolded

intermediate state was detected for rBet v 1.2801 whereas Mal d 1 folded without intermediates.

The second part of this study described the pH-dependence of nApi g 1 and nDau c 1 as another method for estimating the conformational stability, which could be compared with a similar study with rBet v 1.2801 (Mogensen et al., 2004). According to this method, the conformational stabilities of nApi g 1 and nDau c 1 were similar to rBet v 1.2801 but higher than the conformational stability determined in the GuaHCl denaturation experiment. However, the effect of pH on the thermal stabilities of nApi g 1 and nDau c 1 was different from that of rBet v 1.2801.

For the thermal denaturation curves, two-state folding kinetics were assumed, which is a completely reversible process, meaning that the unfolded protein is able to refold completely. For both nApi g 1 and nDau c 1, a pH range was observed without two-state kinetics (simplest representation; $N \rightleftharpoons D$ aggregation) showing irreversible unfolding from pH 4.4–6.0 and pH 4.4–5.6, respectively. However, a linear relationship was observed for nDau c 1 for both reversible and irreversible unfolding data points fitted to a two-state equation, showing more correlation than for rBet v 1.2801 (Mogensen et al., 2004). For nApi g 1, a linear relationship was only visible for the data points corresponding to irreversible unfolding (pH 4.4–6.0). The linear relationship between ΔH_{Tm} and T_m was likely affected by non-two-state kinetics and not by the presence of multiple isoforms in the measured solution.

Nevertheless, a similar behaviour in unfolding was observed for the natural allergens, which allowed us to study the overall protein stability. The slopes of the linear fits of nApi g 1 and nDau c 1 from our study showed ΔC_P values, similar to those of rBet v 1.2801 observed by Mogensen et al. (2004). The parameter ΔC_P is related to m_{D-N} and gives indications for exposure of the non-polar accessible surface area by unfolding. The low values of ΔC_P, found in this experiment, indicated that the protein is not strongly hydrophobic, which corresponds to polar unfolding established by m_{D-N} (Creighton, 1993). By using the pH-dependence as a model system for determining the conformational stability instead of GuaHCl denaturation, the influence of the isoform mixture could be averaged, resulting in better estimates of ΔG.

Besides differences in thermodynamic parameters, differences were also observed in the pH stability of the natural allergens, Api g 1 and Dau c 1, and rBet v 1.2801 (Mogensen et al., 2004). A midpoint for a pH transition, similar to Fig. 2C and D at 20 °C, was visible at pH 3.5 for rBet v 1.2801 which was approximately 1 pH unit lower than for nApi g 1 and nDau c 1 and 2 units lower than Bet v 1's pI. The optimal pH value of approximately pH 6.4, with the highest T_m, was similar for the three allergens.

The most remarkable difference was the observation that both nApi g 1 and nDau c 1 were stable to heating below pH 4.4 whereas rBet v 1.2801 showed unfolding at pH 3.5–4 (Mogensen et al., 2004). In a different study, it was shown that rBet v 1.0101 was completely unfolded at pH 2.2 (Schöll et al., 2005), whereas nApi g 1 and nDau c 1 are folded. Moreover, the Bet v 1 homologue, rPru p 1 from peach, showed reversible unfolding when heated to 95 °C but, at pH 3, rPru p 1 was completely unfolded at room temperature (Gaier et al., 2008). The natural allergens Api g 1 and Dau c 1 in our study therefore contradict the general statements that Bet v 1 homologues are more susceptible to denaturation at low pH than at neutral pH (Gaier et al., 2008). The pH-dependence properties of nApi g 1 and nDau c 1 are similar to those of patatin, a soluble protein from potato tuber also known as the allergen Sol t 1, showing no unfolding at lower pH and irreversible unfolding at pH 6 (Pots, de Jongh, Gruppen, Hessing, & Voragen, 1998).

The few studies, reporting on the physicochemical stability of PR-10 proteins and the effect on immune reactivity, in terms of IgE binding, have characterized these types of allergens as labile (Mills et al., 2007). Experimental evidence, for the reduced IgE binding capacity by food processing, is available, but a primary cause of this reduction is unknown (e.g. Garcia-Borrego et al., 2007; Gruber et al., 2004). However, only a few studies show the fundamental, thermodynamic, properties of the isolated allergens. Most of the food matrix effects are shown for the commercially available recombinant isoforms and are only directed to changes in IgE reactivity and not to changes in conformational stability. For aqueous solutions, it seems that the PR-10 allergens are able to bind IgE antibodies, as long as they are able to refold after physicochemical treatment (unpublished data).

In conclusion, our study reports the effect of denaturant, pH and temperature on different PR-10 allergens in aqueous solutions, showing different properties between Bet v 1 and Dau c 1 and Api g 1. When each isoform of the natural mixture is studied individually, differences will be observed in thermodynamic parameters. It is important to find the relationship between the thermodynamic and physicochemical properties and the immunological response of these types of allergens to better understand how processing influences the isoform variety of PR-10 allergens.

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