Food Chemistry 119 (2010) 241–248

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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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ARTICLE INFO

Article history:
Received 3 December 2008
Received in revised form 15 May 2009
Accepted 10 June 2009

Keywords:
Api g 1
Bet v 1
Birch pollen
Dau c 1
Food allergy
Protein stability

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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doi:10.1016/j.foodchem.2009.06.013

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

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