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ORIGINAL PAPER

Pressure and temperature stability of the main apple allergen Mal d1

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Abstract High-pressure Fourier-transform infrared (FTIR) spectroscopy was used to determine the pressure and temperature stability of Mal d1. This study was triggered by contradictory results in the literature regarding the success of pressure treatment in the destruction of the allergen. The protein unfolded at 55°C when heated at normal atmospheric pressure. We also studied the effect exerted on pressure stability by environmental factors, which can be important for the stability of the protein in the apple. The pressure unfolding was measured under different pD conditions, and the effect of sugar mixture similar to that of the apple and the effect of ionic strength were also studied. In all cases the allergen unfolded with a transition midpoint in the range of 150–250 MPa. Unfolding was irreversible and was followed by aggregation of the unfolded protein. Lowering the pD destabilized the protein, while addition of sugar mixture and of KCl had stabilizing effect.

Keywords Unfolding · High pressure · Allergen · FTIR spectroscopy

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Introduction

Allergy has a growing prevalence in industrialized countries (Schafer 2008; Cochrane et al. 2009) and presumably also in the rest of the world (van der Poel et al. 2009). Several natural or artificially produced compounds have been reported to cause allergic hyperreactions. Food allergy has been reported in 5% of young children and 1–2% of adults (Kagan 2003). A number of food constituents and food products can cause allergy, including milk, soy, egg, wheat, rice, kiwi, peanut, fish, shellfish, and also some fruits such as peach, cherry, and apple (Fernandez-Rivas et al. 2006). Here we investigate the Mal d1 allergen, which is the main allergen found in apple (Vanekkrebitz et al. 1995). It can trigger serious reactions in allergic patients, known as oral allergy syndrome, and even anaphylactic shock (Saraswat and Kumar 2005). Apart from its direct allergic effect, allergy to apple fruit often results in sensitization to Bet v 1 from birch pollen (Rouge et al. 2009), which explains why apple allergy can have wider consequences than simply diet.

Mal d1 is a relatively small protein composed of 159 residues with molar mass of 17.6 kDa (Vanekkrebitz et al. 1995). Its three-dimensional structure has not yet been resolved by X-ray crystallography or nuclear magnetic resonance (NMR) methods. Our homology model predictions using the Swissmodel Protein Modeling Server (Arnold et al. 2006) gave predominantly beta sheet structure composed of seven strands (Fig. 1). Two helices were also predicted. These secondary structure features are connected by three short and five longer loops. This structure was in accordance with the one obtained by Ma et al. (2006) using a similar computational method.

High pressure is known to distort the secondary, tertiary, and quaternary structure of proteins, leading in case of

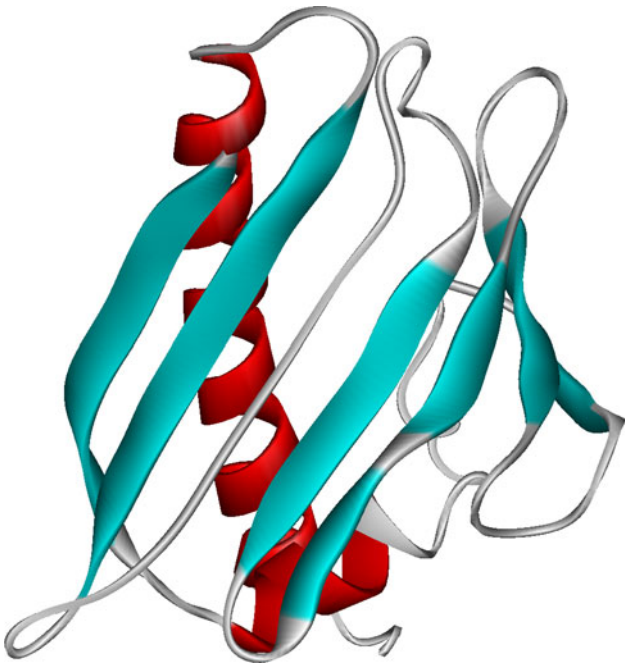

Fig. 1 Three-dimensional structure of the Mal d1 protein obtained from homology modeling Arnold et al. 2006. The figure was constructed by ViewerLite 4.2 (Accelrys, www.accelrys.com)

most proteins to unfolding (Meersman et al. 2006; Smeller 2002; Cordeiro et al. 2006; Silva et al. 2001; Ribo et al. 2006). Several applications of pressure unfolding have been suggested and implemented, from the medical field to food industry, including inactivation of viruses and bacteria (Gaspar et al. 2008). Pressure also has been used for a long time to increase food quality and shelf life (Houska et al. 2006; Rastogi et al. 2007). Analogous to this treatment of foodstuffs, application of high pressure was suggested to eliminate the allergen activity of apple juices (Meyer-Pittroff et al. 2007). Recently, however, more systematic study did not find a decrease in the allergen activity of apple Mal d1 after pressure treatment up to 500 MPa (Houska et al. 2009; Setinova et al. 2008). Both of these studies dealt with pressure-treated samples, whose structural changes were measured only after the pressure cycle under ambient pressure conditions.

Infrared spectroscopy is a suitable tool for determination of the secondary structure content of proteins (Barth 2007; Smeller et al. 1995a), especially for measurements under pressure, where the usual circular dichroism method cannot be applied. Most information can be obtained from analysis of the amide I band, which is situated between 1,600 and 1,700 cm^{-1} in the infrared spectrum. Deconvolution and curve fitting can be used to calculate the amount of the different secondary structure elements (Meersman et al. 2002).

Our aim is to investigate the effect of high-pressure and high-temperature treatment on the level of molecular structures, namely by investigating the secondary and tertiary structure of the protein that acts as the allergen. The central question we try to answer in this paper is whether high-pressure treatment can unfold the protein. In our experimental setup we combined FTIR spectroscopy with a high-pressure diamond anvil cell. This combination has an important advantage over setups used in earlier studies in that it can provide information about the secondary structure of the allergen under pressure (in situ). The effect of pressure in previously published experiments was only measured after the pressure treatment (Meyer-Pittroff et al. 2007; Setinova et al. 2008; Houska et al. 2009).

Materials and methods

Recombinant Mal d1 was obtained from Biomay AG, Wien, Austria. The lyophilized protein was dissolved at concentration of 75 mg/ml in 80 mM pD 7.0 BES D_2O buffer. For the low-pD experiments, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer was used at the same concentration. All solutions contained 80 mM β-mercaptoethanol (unless otherwise stated) to prevent dimer formation via disulfide bridges. Saccharose and fructose were obtained from Reanal (Hungary), and glucose and KCl from Sigma.

A diamond anvil cell (Diacell, Leicester, UK) suitable for high-pressure infrared studies was filled with the above solution. $BaSO_4$ was used to determine the pressure (Wong and Moffat 1989). The pressure cell was placed in a Bruker Vertex80v FTIR spectrometer. The spectrometer was equipped with a beam condenser and a high-sensitivity mercury cadmium telluride (MCT) detector. Five hundred twelve scans were collected at 2 cm^{-1} resolution. During the pressure experiments, spectra were taken at pressures up to 1.2 GPa at 30°C. The pressurizing rate was 5 min/measurement point, corresponding to pressurization rates of 2–12 MPa/min. Lower rate was used in the transition regime, and higher rate only at pressure values well above the transition. Temperature scans were performed in the range from 30°C to 85°C near atmospheric pressure. The heating rate was 0.2 K/min. The temperature was controlled in both (p and T) types of experiments (controller type 2216e; Eurotherm, Durrington, UK) and measured using a thermocouple (OMEGA Engineering, Stamford, CT) attached directly to the gasket of the diamond cell. In one special experiment, high pressure and high temperature were simultaneously used to test the extreme stability of the aggregates.

Spectral evaluation was performed by using ProteIR software (Smeller et al. 1995b), and Microcal Origin 6.0 was used for fitting of sigmoid curves.

For fitting of the pressure- and temperature-induced transitions we used the following curves, which can be derived from the two-state transition model:

$$y(p) = a + bp + \frac{\Delta a + \Delta bp}{1 + \exp\left(\frac{(p-p_{1/2})\Delta V}{RT}\right)} \quad (1)$$

for the pressure transitions, and

$$y(T) = a + bT + \frac{\Delta a + \Delta bT}{1 + \exp\left(\frac{\Delta H}{R}\left(\frac{1}{T} - \frac{1}{T_{1/2}}\right)\right)} \quad (2)$$

for the temperature scans. Here y is the physical parameter to be fitted (e.g., the position of a certain spectral line), a and b are the parameters describing the linear dependence of $y(p)$ or $y(T)$ below the transition, Δa and Δb are the changes of a and b during the transition, $p_{1/2}$ and $T_{1/2}$ are the transition midpoints, and all other symbols have their usual meaning.

Homology modeling was performed by the Swissmodel Protein Modeling Server (Arnold et al. 2006). The method is based on the observation that protein tertiary structure is better conserved than amino acid sequence (Marti-Renom et al. 2000). The template used in the modeling was the structure of the major cherry allergen, Pru av1 (pdb code 1E09). Sequence similarity of 85% was found between the two proteins, which ensures the reliability of the structure prediction.

Results

Infrared spectrum and structure of native Mal d1

Figure 2 shows the section of the infrared spectrum of Mal d1 near atmospheric pressure containing the most characteristic vibrations. This is the region of the amide I and II bands of the protein and the ring vibration of the tyrosine residue. The antisymmetric stretching vibration of the COO^- group of the aspartic and glutamic acids also falls in this region (Barth 2007).

The amide I band comes from the vibration of the protein backbone; therefore, it is sensitive to the secondary structure. The majority of the energy of vibration is associated to the C=O stretching of the carbonyl group (Barth 2007). The strength of hydrogen bonding to other segments of the polypeptide chain in ordered structures influences the vibration frequency. These frequency shifts provide the opportunity to estimate the secondary structure from analysis of the most complex amide I band.

The position of the amide I band of Mal d1 is at 1,637 cm^{-1}, which is characteristic for beta-sheet-type structure. The evaluation of the amide I band profile by Fourier self-deconvolution and curve-fitting results in a

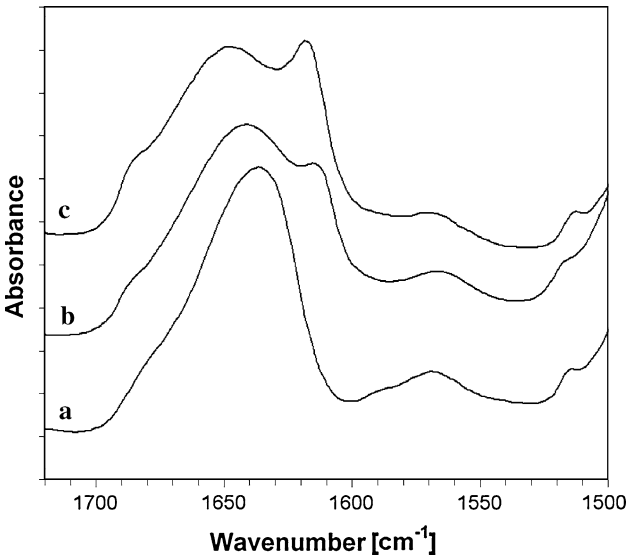

Fig. 2 Infrared spectrum of Mal d1 in the region of the most important amide I and II bands and of the tyrosine and carboxylic vibration bands at ambient conditions: before pressure treatment (**a**), at 1 GPa at 30°C (**b**), and near atmospheric pressure at 80°C (**c**)

14% alpha and 33% beta structure. This is in accordance with the predicted three-dimensional structure of Mal d1 we obtained using the Swissmodel Protein Modeling Server (Arnold et al. 2006). Homology modeling predicted predominantly beta structure, assigning 33 (20.7%) and 62 (39%) of 159 residues to the helical and beta-sheet secondary component, respectively. Taking into account that infrared spectroscopy only detects residues involved in intrahelix or intrasheet hydrogen bonds, the agreement is even better: 25 and 47 residues are involved in such intrastructure hydrogen bonds, which gives 15.7% and 29.6% for alpha and beta structures, respectively.

Whereas the amide I band is sensitive to the secondary structure, the energy of the amide II vibration has major contribution from N–H in-plane bending; therefore, the vibration frequency shifts significantly upon deuteration of the protein, giving information about the flexibility of the tertiary structure. Normally, the amide II band appears at 1,545 cm^{-1}, but in the case of deuteration the band is called amide II' and appears at 1,450 cm^{-1}. Generally the solvent-exposed amino acids exchange quickly (within minutes), whereas the interior of the protein can be reached only when the protein unfolds or at least the tertiary structure is loosened. In case of Mal d1, practically no amide II band (at 1,545 cm^{-1}) was detectable at the beginning of the measurement, because the sample was allowed to exchange for 8 h before the measurement. The lack of the amide II band means that the protein is totally exchanged. This suggests that Mal d1 does not have any rigid core that could have resisted deuteration under atmospheric pressure.

Since the protein contains 10 tyrosine residues, the ring vibration of the phenol ring can be clearly observed in the spectrum at 1,514 cm^{-1}. The symmetric COO^- stretching vibration of aspartic and glutamic acid side-chains gives an absorption band around 1,568 cm^{-1}.

Temperature- and pressure-induced unfolding of Mal d1 at ambient pH

Upon heating, the protein loses its secondary structure, as seen from the shift (Fig. 3a) and broadening of the amide I band (data not shown). The shift of the amide I peak position was fitted by a sigmoid curve, assuming a two-state model. The midpoint of the transition was 55.5 ± 0.1°C. The initial and final positive slopes of the curve are associated to the temperature-induced weakening of the intraprotein hydrogen bonds. (There is a transient negative slope before the unfolding between 43°C and 51°C.) Above 60°C the position of the amide I band is characteristic for the unordered (unfolded) structure. The width of the amide I band follows also a sigmoid curve, showing marked broadening at the same temperature (55.3 ± 0.1°C), where the maximum position changes. The position of the tyrosine peak is shifted by 0.7 cm^{-1} during a similar sigmoid transition at 55.4 ± 0.1°C (Fig. 3b).

A marked feature of the spectrum at high temperature is the appearance of a strong side-band at 1,616 cm^{-1}, accompanied by a smaller one at 1,685 cm^{-1} (Fig. 2c). These bands were associated to intermolecular beta-sheet-like aggregated structures (Ismail et al. 1992; Meersman and Heremans 2003). The 1,616 cm^{-1} band was evaluated, because the intensity of this peak was always higher. Its integrated area was plotted against temperature and fitted with a sigmoid curve (Fig. 3c). The transition point was 57.7 ± 0.1°C. All these changes are irreversible, as can be seen from Fig. 3. The midpoint of the transition of the 1,616 cm^{-1} peak differs slightly but significantly from the others (Fig. 5a). It is also clear that the tyrosine transition regime is definitely broader than the width of the other transitions.

During pressure treatment performed at 30°C, the conformation-sensitive amide I band showed a cooperative transition with clear sigmoid character. The position of the peak shifts to the wavenumber characteristic of the unordered structure (Fig. 2b). Simultaneous broadening of the amide I band supports the denaturation of the allergen. The position of the band as a function of pressure can be seen in Fig. 4a. The midpoint of the unfolding transition was found at $p_{1/2} = 260 \pm 20$ MPa. The absence of the amide II band, even at ambient conditions, suggests a very flexible structure, which is in accordance with the low-pressure stability of the protein. The absorption band of the tyrosine residues shows a transition similar to that of the amide I band (Fig. 4b). The sigmoid curve in Fig. 4b is superimposed on a linear shift, which is a result of the elastic compression of the chemical bonds involved in the tyrosine ring vibration. Appearance of the side-bands (1,616 and 1,685 cm^{-1}) follows the unfolding transition with a slight delay (at 280 ± 5 MPa), similarly to the temperature experiment (Fig. 4c). Above the sigmoid transition the protein conformation is not affected further. The transition is not reversible; after returning to atmospheric pressure, the amide I band corresponds to the one measured at high pressure. It is dominated by a broad feature with position of 1,644 cm^{-1} characteristic for the unfolded structure. The

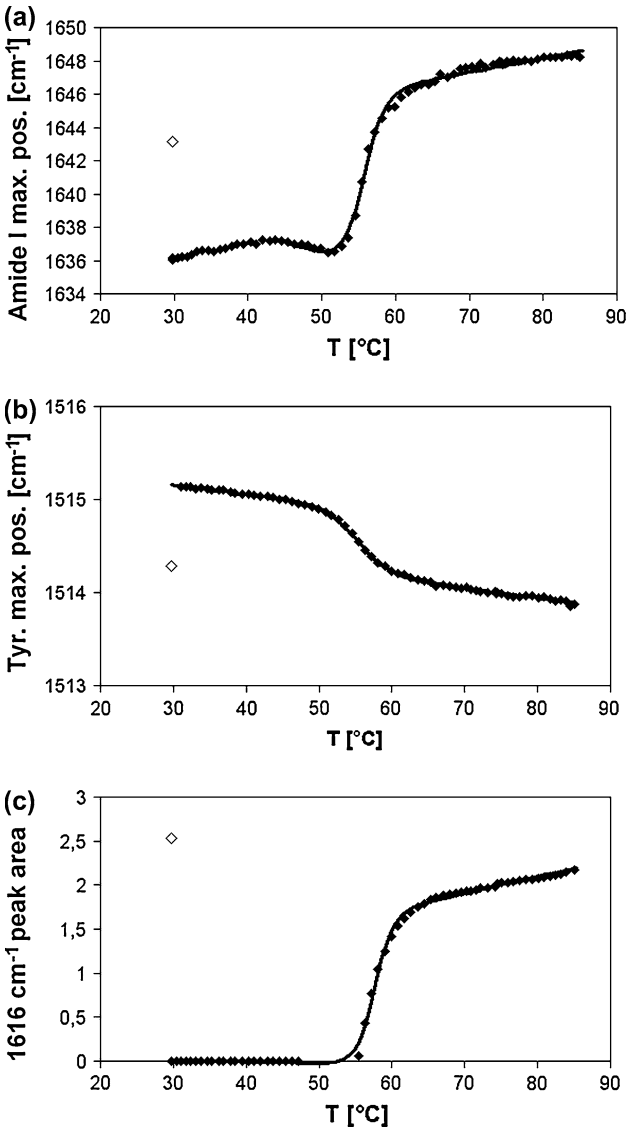

Fig. 3 Temperature dependence of the maximum position of the amide I band (**a**) and of the tyrosine band (**b**), and the temperature dependence of the integrated area of the side-band at 1,616 cm^{-1} (**c**). The *open symbol* corresponds to the sample cooled down after the temperature cycle. The *lines* are sigmoid curves fitted according to Eq. 2

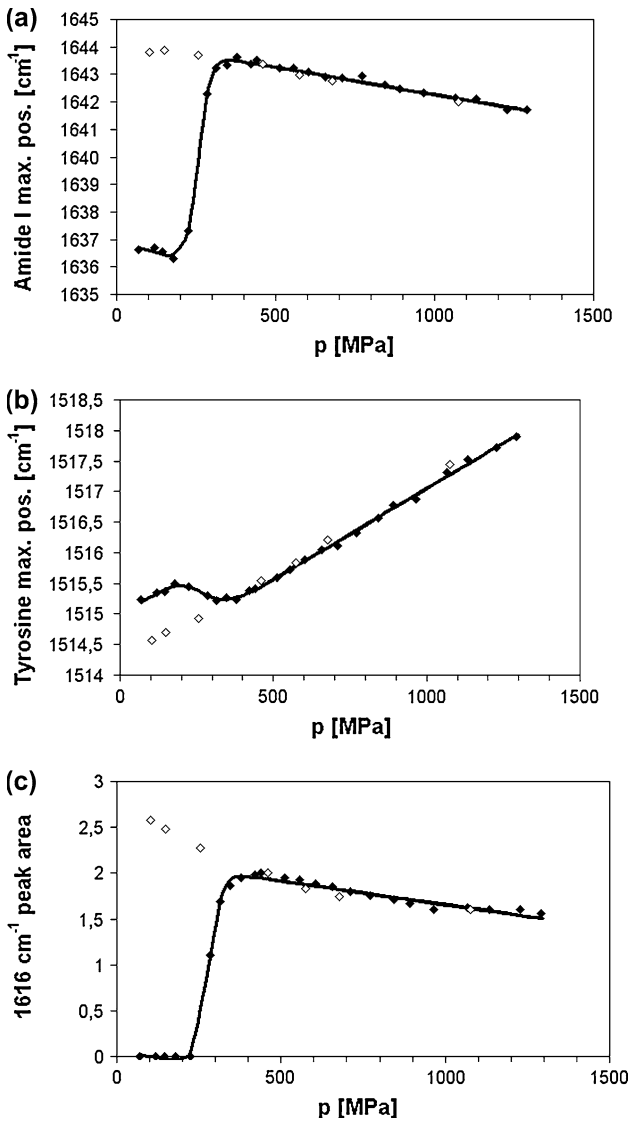

Fig. 4 Pressure dependence of the maximum position of the amide I band (**a**) and of the tyrosine band (**b**), and the pressure dependence of the integrated area of the side-band at 1,616 cm^{-1} (**c**). The *open symbols* were measured during the release of the pressure. The *lines* are sigmoid curves fitted according to Eq. 1

aggregation-specific band pair also remains visible in the spectrum. The amplitudes of the aggregation peaks increase during the lowering of the pressure; the area under the 1,616 cm^{-1} peak almost doubles during the release of the pressure. These peaks did not disappear during a second pressurizing cycle. The only way to dissociate the aggregate was to heat the aggregate under 1.2 GPa pressure up to 70°C, which resulted in the integrated area of the aggregation peak decreasing to 10% of its maximum value, but the dissociation was reversible, and the aggregation peak reappeared as the temperature was decreased.

The sigmoid curves obtained for the maximum position of the amide I and tyrosine peaks (Fig. S1) as well as for the area of the side-peak at 1,616 cm^{-1} were compared. A systematic difference can be seen between the unfolding and aggregation transitions. Aggregation appears slightly after the unfolding transition in both the pressure and temperature experiments. The transition reported by the vibration of the tyrosine residues is less cooperative, presumably because of the inhomogeneous distribution of the tyrosine residues: seven of them are in beta, one in alpha, and two of them in unordered structure; six are on the surface, and four are buried in the protein interior.

The pressure experiment was performed with and also without mercaptoethanol. No significant difference was found between the two cases. The temperature scan was carried out without mercaptoethanol. In all the following experiments, mercaptoethanol was used.

Effect of low pH/pD on pressure stability of Mal d1

Since the pH of the apple is considerable lower than neutral (Houska et al. 2009) and fruit products also have lower pH or the pH can be lowered during processing, we also investigated the effect of low pD. The pH of the apple fruit is between 3 and 4, therefore we chose pD 3 for our experiments.

Figure 5a shows the amide I maximum position versus pressure at pD 3. A similar cooperative transition occurs as was observed at neutral pD; however, the transition point is considerably lowered at pD 3. The sigmoid fit resulted in $p_{1/2} = 150 \pm 20$ MPa, compared with 260 ± 20 MPa at pD 7. The broadening of the amide I band and the appearance of the aggregation-specific band pair were also observed. Similarly to the neutral pD case, the integrated area of the 1,616 cm^{-1} band has a slightly higher transition pressure ($p_{1/2} = 168 \pm 10$ MPa) than that of the amide I maximum. The tyrosine band could not be fitted, due to the fact that this transition is broader than the one of the amide I, therefore we did not have enough points below the transition pressure for the fit.

Effect of sugars and ionic strength on pressure stability

To mimic the environment of the allergen in the apple, we added sugars in the same concentration as they are found in the fruit. Fructose, saccharose, and glucose were added at 5.63%, 2.31%, and 2.06% (w/w), respectively, giving total sugar concentration of 10% (Scherz and Senser 2000). The presence of the sugar mixture stabilized the protein against pressure unfolding, increasing the unfolding midpoint by 20 MPa ($p_{1/2} = 170 \pm 10$ MPa), as determined from the position of the amide I band (Fig. 5b). The broadening of the amide I band could also be fitted with a sigmoid ($p_{1/2} = 188 \pm 10$ MPa), as well as the position of the tyrosine band ($p_{1/2} = 175 \pm 16$ MPa). The 1,616 cm^{-1}

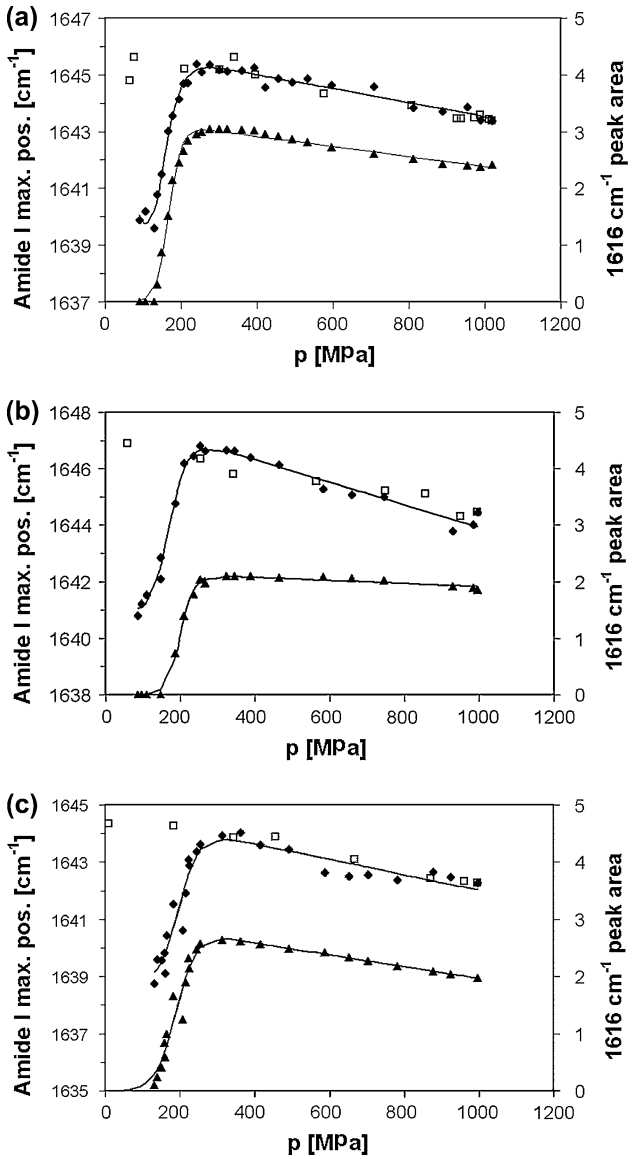

Fig. 5 Pressure dependence of the maximum position of the amide I peak of Mal d1: at pD 3 (**a**), at pD 3 with sugar mixture (**b**), and at pD 3 with 40 mM KCl (**c**). *Diamonds* indicate pressurizing, *open squares* indicate pressure release. The integrated area of the 1,616 cm^{-1} peak is represented by *triangles*

side-peak appears again after the conformational transition: $p_{1/2} = 200 \pm 5$ MPa for the integrated area of the aggregation peak.

The apple contains also considerable amount of minerals, mostly potassium (145 mg/100 g) (Scherz and Senser 2000). We investigated the effect of KCl on the pressure unfolding of the allergen at pD 3. In presence of 40 mM KCl, the protein became more stable against pressure denaturation, with midpoint of 196 ± 12 MPa (Fig. 5c). The broadening of the amide I band and the shift of the tyrosine vibration frequency were similar to what we observed in the previous experiments. The midpoint of the appearance of the aggregation peak was 190 ± 4 MPa. All the changes were irreversible.

Discussion

Using FTIR spectroscopy, the secondary structure of Mal d1 was determined to be of predominantly beta sheet type. Our spectral analysis supports the predictions of homology modeling performed by us and others.

Our FTIR experiments show clear pressure unfolding of the apple allergen protein in the range of 150–260 MPa, depending on the environmental conditions, indicating that it belongs to the class of proteins with relatively low pressure stability, such as *Staphylococcus* nuclease (Snase) (Panick et al. 1999), phosphoglycerate-kinase (Osvath et al. 2009), and the 23-kDa protein of photosystem II (Tan et al. 2005). Most proteins conserve their secondary structures up to 500–700 MPa, and some of them even up to more than 1 GPa, such as the 16.5-kDa small heat-shock protein from *Methanococcus jannaschii* (Tolgyesi et al. 2004). The temperature stability of Mal d1 is also quite low, compared with globular proteins, which denature usually above 70°C (Meersman et al. 2002).

The most interesting and unique feature is the appearance of the aggregation of the protein under pressure above the pressure unfolding point. Generally, pressure causes dissociation of protein oligomers (Santos et al. 2008) and aggregates (Kornblatt and Kornblatt 2002; Smeller et al. 1999). Although there are several reports in the literature describing pressure-induced aggregation, most of them report aggregate formation only after pressure treatment. Either the measurements were done only after pressure treatment (Dumay et al. 1994; Vancamp and Huyghebaert 1995; Defaye and Ledward 1995) or, if the protein was monitored during the entire pressure cycle, aggregation was observed only after pressure treatment, during depressurization, typically below 200 MPa (Smeller et al. 1999; Meersman et al. 2002; Meersman and Heremans 2003). Although some papers call these aggregates "pressure induced," we suggest that they should be called "pressure treatment induced." Aggregation found *after* pressure treatment is therefore common, but protein aggregation *under* pressure (true pressure-induced aggregation) is very rare. Seefeldt et al. (2005) observed aggregation of recombinant human interleukin-1 receptor antagonist (IL-1ra) under pressure, using turbidity measurement; however, IL-1ra is a very pressure-labile protein, with unfolding pressure under 200 MPa. IL-1ra aggregates were formed in the range of 180–220 MPa, but they did not measure the stability of the aggregates.

In our experiments, the aggregates were formed under pressure, above the unfolding pressure (even above 250 MPa

in some cases). They also exhibited exceptional stability, not dissociating even above 1 GPa (at 30°C). Only simultaneous high-pressure and high-temperature conditions were able to cause 90% dissociation. These aggregates are considerably more stable than the ones found earlier for other proteins such as myoglobin, lysozyme (Smeller et al. 2006) or apo-horseradish peroxidase (Smeller et al. 2003). The appearance of such strong pressure-resistant intermolecular antiparallel beta-sheet-type aggregates may be a consequence of the high beta content of the native protein.

The aggregation arises in all the experiments slightly after the change of the amide I band. We conclude that the pressure unfolding precedes the aggregation; i.e., a considerable amount of unfolded protein is needed for the start of aggregation. It should also be noted, however, that the aggregation is a kinetic process, which generally occurs on a longer time scale compared with the unfolding of a single molecule. One might assume that the higher midpoint of the aggregation in the pressure and temperature experiments is due to the slower aggregation and not because a higher temperature or pressure is needed for the aggregation. This can be ruled out, however, by the following consideration. The pressure and temperature shifts between the amide I and the aggregation (1,616 cm^{-1}) transition curves correspond to 8 and 11 min measurement time delay, respectively. If the separation of the amide I and aggregation transition were caused by kinetic effects, one would expect shorter time shift at higher temperature. Since this is not the case, we can rule out that kinetics plays a major role; consequently, at least partial unfolding is a prerequisite for aggregation.

The pressure unfolding of the allergen destroys the secondary and tertiary structure of the protein. The conformational transition is not reversible; the structure of the Mal d1 protein did not recover even several hours after the pressure cycle. Consequently, if intact secondary structure is important for allergen recognition, we have to expect that pressure treatment has an effect on the allergenicity of the protein. As mentioned at the beginning of the article, the literature is not unequivocal in this regard. There are several possibilities, e.g., the physicochemical environment of the apple stabilizes the allergen, or the epitope contains only very small or no folded secondary structure elements.

To investigate the role of the physicochemical environmental factors of the apple, we studied the allergen at low pD in the presence of sugar and KCl. None of these factors, nor the combination of low pD and sugar or KCl, changed the stability radically. At 500 MPa, which was the treatment pressure in the literature studies, the protein was already well above the unfolding transition. Lowering the pD decreased the pressure stability of the apple allergen. This effect is well known for many other proteins. Low pH is frequently used by investigators with access to limited pressure range. In this case the low pH serves as a predenaturant, shifting the pressure unfolding into the measurable range.

Addition of sugars has a slight stabilizing effect, namely increasing the unfolding pressure by 20 MPa. The effect of different cosolvents on the pressure stability of proteins was investigated by Herberhold et al. (2004) in case of Snase. They found a stabilization of 230 ± 30 MPa/M for saccharose. Our total sugar concentration was around 0.5 M, which would result in an increase of the unfolding pressure by 115 ± 15 MPa. Our experimental conditions, like the pD, are also different, and we used a mixture of sugars while the Snase study used only saccharose. If we take only the saccharose concentration into account, the expected stabilization is 15 ± 2 MPa, which is closer to our experimental value. Presumably fructose and glucose, which contribute to the total sugar concentration significantly, do not have stabilizing effects comparable to that of saccharose, probably because of their smaller molecule size and monomeric nature.

Addition of KCl also strengthened the protein, with 40 mM KCl causing an almost 50 MPa increase of the unfolding point. This effect, however, is still too small, and the stability of the allergen is still well below 500 MPa.

The question remains whether intact secondary structure is needed for allergic reaction, or whether partial refolding of the secondary structure fragments is enough. Ma et al. (2006) tried to identify the fragment responsible for the antigen–antibody binding, using mutational analysis, but no binding site could be identified unequivocally. They conclude that conserved secondary structure is a prerequisite for IgE binding, but this assumption was not justified, since they did not perform unfolding–refolding studies.

It has to be pointed out that not only the physicochemical but also the biochemical environment in the apple is quite complex. The presence of other enzymes (e.g., polyphenol oxidases) may also influence the unfolding behavior of the allergen protein. As could be seen, Mal d1 has high affinity to aggregate, probably not only with itself but also to coaggregate with other proteins in the apple and apple juice. These aggregates can also change the allergenic properties. Chung et al. (2005) observed reduction of allergenicity as a result of crosslinking catalyzed by polyphenol oxidase. In this respect the earlier experiments by Meyer-Pittroff et al. (2007) and Houska et al. (2009) were different, since Houska used ascorbic acid to prevent the polyphenol oxidases from oxidizing the apple juice. This could be the reason for the preserved allergenicity. Meyer-Pittroff did not use ascorbic acid, so polyphenol oxidases could decrease the allergenicity in their experiments. In our experiment the role of such factors is naturally excluded, because we used pure protein solution.

It cannot be excluded that a small part of the protein preserves its secondary structure even in the pressure-unfolded state. If this fragment plays a role in the allergic reaction, the allergenicity can resist pressure unfolding. Alternatively, one can imagine that the epitope, the part of the molecule which is significant in the immune recognition, is still recognizable in the unfolded state.

Although several allergens can survive drastic physical conditions (e.g., some of them survive cooking), Mal d1 is known to be less resistant (Soler-Rivas and Wichers 2001). More specific information about the nature of the epitope is needed to understand the effect of pressure unfolding on allergenicity more deeply.

We believe that our results have an impact on the prospects for production of hypoallergenic food. Further studies are, however, necessary to clarify the optimal parameters and effectiveness of possible pressure treatment of the Mal d1 apple allergen.

Conclusions

The main apple allergen Mal d1 unfolds at pressures of 150–250 MPa, depending on the environmental conditions. The unfolding was irreversible in all cases, and the unfolded protein aggregated. Mimicking the apple environment by lowering the pD and by addition of a sugar mixture and KCl did not increase the pressure stability of the protein above 260 MPa. This suggests that destruction of the allergen by pressure is possible under certain conditions, but more information is needed about the connection between the structure of the allergen and its allergenic effect before practical application of such pressure treatment.

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