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High pressure effects on allergen food proteins ☆

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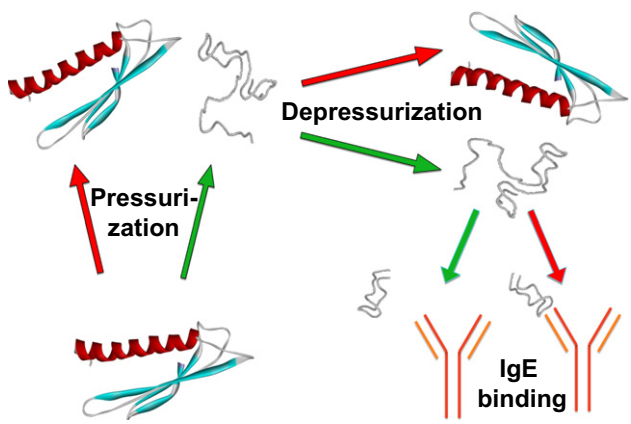
HIGHLIGHTS

- High pressure effect on the structure of allergen proteins is reviewed.
- Effect of pressure treatment on antigenicity of food allergens is reviewed.
- Included food allergens are from: milk, fish, peanut, apple, carrot, and egg.

GRAPHICAL ABSTRACT



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ABSTRACT

There are several proteins, which can cause allergic reaction if they are inhaled or ingested. Our everyday food can also contain such proteins. Food allergy is an IgE-mediated immune disorder, a growing health problem of great public concern. High pressure is known to affect the structure of proteins; typically few hundred MPa pressure can lead to denaturation. That is why several trials have been performed to alter the structure of the allergen proteins by high pressure, in order to reduce its allergenicity. Studies have been performed both on simple protein solutions and on complex food systems. Here we review those allergens which have been investigated under or after high pressure treatment by methods capable of detecting changes in the secondary and tertiary structure of the proteins.

We focus on those allergenic proteins, whose structural changes were investigated by spectroscopic methods under pressure in correlation with the observed allergenicity (IgE binding) changes. According to this criterion we selected the following allergen proteins: Mal d 1 and Mal d 3 (apple), Bos d 5 (milk), Dau c 1 (carrot), Gal d 2 (egg), Ara h 2 and Ara h 6 (peanut), and Gad m 1 (cod).

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1. Introduction

Despite the large number of chemical, biochemical and biophysical investigations performed as a function of temperature, pressure is still less known and rarely used as a thermodynamic parameter. One of the reasons might be the technical difficulty to reach pressures high enough for observable changes. Pressure as a thermodynamic parameter is associated to volume; therefore one can obtain volumetric information from

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pressure studies [1]. The equilibrium of a two-state system with V_1 and V_2 volumes will shift under pressure. The equilibrium constant at T temperature can be written as follows:

$$K = e^{-\frac{\Delta G}{RT}} \quad (1)$$

where K is the ratio of the probabilities of finding the system in states 2 and 1: $K = w_2/w_1$, ΔG is the Gibbs free energy difference between the two states: $\Delta G = G_2 - G_1$. Pressure shifts the equilibrium by favoring the smaller volume state as it could also be expected from the Le Chatelier–Brown principle. The probability of finding the system in state 1 is:

$$\begin{aligned} w_1 &= 1/(1 + \exp(-\Delta G/RT)) \\ &= 1/(1 + \exp(-(\Delta E - T\Delta S + p\Delta V/RT)) \end{aligned} \quad (2)$$

where Δ means difference of the given thermodynamic parameter between the 2nd and 1st states (See the definition of ΔG above); E, S, and R have their usual meaning. It is obvious that the magnitude of ΔV is crucial for the effect of pressure. Most of the chemical and biochemical systems are in liquid phase. In the case of these systems the volume change is almost negligible, since the liquids are almost incompressible (exactly: their compressibility is very low), and there are very few voids in the system which could be filled in order to change the volume. In complex biochemical and biophysical systems, which have certain ordering, however, voids can be observed due to improper packing. Appearance of voids gives the pressure a chance to tune these systems. In the case of spontaneously formed lipid bilayer membranes application of pressure favors the more ordered gel phase instead of the more disordered liquid crystalline phase [2]. In the case of proteins it is the opposite way: in most cases the more ordered native state is destabilized by pressure and the disordered unfolded state is favored. The solution of the paradox is that the ordered native state has packing defects, while the disordered polypeptide chain fits well in the solvent.

The volume of the protein dissolved in an aqueous solution can be written as [1]:

$$V_{\text{protein}} = V_{\text{atom}} + V_{\text{cavities}} + \Delta V_{\text{hydration}}. \quad (3)$$

The last term is due to the higher density of the hydration layer around the protein [3,4]. Since the atoms are practically incompressible in the range of interest for the biological systems, the last two terms can play important roles.

2. Proteins under pressure

Three major effects can be observed if pressure acts on proteins, depending on the magnitude of the pressure. Elastic effects appear already at the smallest pressure values. These are reversible distortions of the primary and secondary bonds. The compression of the primary chemical bonds is very small; their contribution to the volume change of the system is negligible. Compression of the hydrogen bonds can lead to distortion of the conformation, which can reduce the size of the internal cavities in the protein.

If the pressure reaches typically 200 MPa (2 kbar) the intermolecular interactions and the tertiary structure are destabilized [5–7]. This can be explained by the increased molecule surface leading to a larger hydration layer in the monomer state, and by the cavities at the internal surface of the oligomers/aggregates due to improper packing.

Higher pressure can unfold the protein. The typical pressure needed for the unfolding is around 500 MPa but it varies from protein to protein, in the range from 100 MPa to 1 GPa or until even higher pressures in special cases [8].

The pressure induced appearance of the disordered structure is quite unique among the biomacromolecules: nucleic acids are insensitive to pressure [9] while membranes are stabilized by pressure [2,10–12]. Pressure unfolding of proteins is known for a long time and has been reviewed in several papers [1,13–16], however there are some aspects of its mechanism which are still unclear. Although the native structure of the protein seems to be compact, it contains cavities of different sizes. The ones which cannot be filled by water molecules increase the total volume of the protein (V_{cavities}). Loss of these voids can be one of the driving forces of the pressure unfolding [17,18]. On the other hand one should not neglect the effect of the protein on its environment. Protein molecules are in aqueous solution, where the interaction with water via a hydration shell is very important [15,19,20]. Water is known to have a higher density in a thin layer around the protein, which reduces the overall volume of the system [3,4]. Increasing the surface of the polypeptide chain during the unfolding will increase this dense hydration layer, which can contribute to the negative volume change during the pressure unfolding: $\Delta V = V_{\text{unfolded}} - V_{\text{folded}} < 0$ [21]. Also cosolvents can influence the stability of the protein markedly [22–25].

Pressure unfolding of proteins has been detected by a number of experimental techniques, including infrared spectroscopy [25–34], fluorescence spectroscopy [35–37], NMR spectroscopy [38–41], small angle X-ray scattering [42,43], and X-ray crystallography [44,45]. CD spectroscopy is widely used to assay protein structure, but it cannot be used under pressure due to the pressure-induced birefringence of the optical windows. CD can be used only to see effects after the pressure treatment. All the other techniques listed above can be used in situ i.e. to detect the protein structure under high pressure conditions.

Reversibility of pressure unfolding is a very important question. It is well known that unfolding of proteins at high temperature is usually accompanied by an irreversible aggregation of the polypeptide chains. This does not happen under pressure, due to the dissociating effect of pressure. But the question remains whether the protein refolds after the pressure release or not? There are different results in this respect in the literature. Experimental techniques requiring low concentration of the protein (e.g. tryptophan fluorescence [35–37], derivative spectroscopy based on UV–VIS absorption spectroscopy [46]) show reversible unfolding profiles, while aggregation prone intermediate states were often observed [7,13] in infrared spectroscopy, where high concentration of the protein is needed. Since the natural environment of the proteins in the cell or even in the blood plasma is more close to the values used in infrared experiments, one can expect an incomplete refolding or even aggregation after pressure treatment in the biological environment.

3. Allergy and proteins

3.1. Allergy

Allergy has a growing prevalence in the industrialized countries [47,48] and presumably in the rest of the world too [49]. Apart from the series of natural allergens a number of artificially produced compounds have been found to cause allergic reactions.

Food allergy has special importance, nowadays, when the food chain is globalized and the constituents of the food products cannot always be exactly identified. Although there are strict regulations to indicate the presence of the most important known allergenic components, these rules are not always effective as it can be seen from the regularly appearing food scandals. Food allergy is more prevalent in young children (5%) but 3–4% of adults are also involved in some kind of food allergy [50–52]. Several food constituents and food products can cause allergy including milk, egg, soy, peanut, tree nuts, fish, shellfish, wheat, and also some fruits like kiwifruit, peach, cherry, and apple [53,54]. Cow's milk, peanut and eggs have a high impact as allergen sources in young children. In the adult population most of

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the reactions are due to tree nuts, peanut, fish, shellfish and vegetables [51,55].

Allergy also called type I hypersensitivity is the excessive immune response against a protein, which is called allergen [56]. The crucial factor in allergy is the hypersensitivity of the immune system.

The beginning of a type I allergic reaction is the sensitization to an allergen. The initial contact of an allergen with the mucosa of a susceptive organism is followed by a complex series of events, leading to the production of allergen specific IgE. The effector phase of an allergic reaction is initiated upon second exposure to the allergen. Allergen binding to the specific immunoglobulin E (IgE) antibody on the mast cells will cause an inflammatory response due to the release of histamine and other mediators.

Not all allergens are able to sensitize, i.e. induce the production of specific IgE-type antibodies. Allergens which are capable to both sensitize and trigger allergic reactions are called complete allergens. Cross reactive allergens bind to IgE antibodies that are present in the body due to an earlier sensitization by another allergen. Such an example is the allergic reaction caused by the major apple allergen Mal d 1 due to cross reaction with the Bet v 1 allergen of the birch, which earlier sensitized the immune system by producing antibodies. Mal d 1 alone is not able to induce IgE production.

Allergic reactions can range from a simple hay fever to an anaphylactic shock, which can be lethal without an immediate proper treatment.

There are a great number of allergens known, and listed in specialized databases available on the Internet. The *Allergome* database (www.allergome.org) contains data about more than 6000 allergens [57]. Almost 1000 of these allergens are classified in more than 180 families in the *AllFam* (Allergen Families) database (http://www.meduniwien.ac.at/allergens/allfam/) [58].

Although the above description of allergenicity seems to be clear, it has to be noted that allergenicity can have different meaning to the clinical allergists and to the immunologists. While a clinician recognizes allergenicity via the induction of the symptoms or skin reaction, an immunologist focuses on the IgE binding capacity of the antigens [59]. Also the papers reviewed below reflect divergent approaches in detecting allergenicity. The present review will deal with proteins which are food allergens.

3.2. Proteins as allergens

What makes a protein an allergen? This simple question has no straightforward answer in spite of the numerous studies and analyses of the allergen structures [56–62]. Breiteneder and Mills summarized the common biochemical and physicochemical properties of food allergens [62]. They found that thermal stability, resistance to proteolysis, ability to bind ligands, and interactions with lipids are factors which promote the allergenic properties of food proteins. They also mentioned allergens with fully or at least partially unstructured polypeptide chain. Repetitive structures are also common in some type of allergens. Although one or more of these factors are characteristic for the allergen proteins, there is no rule which could predict the allergenicity of a given protein.

A number of allergens were characterized using biochemical and biophysical methods in the recent years, in order to find common structural, functional and biochemical features, which could explain their allergenicity. One of the classifications used a few broad biochemical groups, like enzymes and enzyme inhibitors, transport proteins, regulatory proteins and allergens with activities other than the previous classes [56,61].

Aalberse analyzed the typical folding motifs (*folds*) of allergen proteins [59]. According to his work, most of the allergens can be classified in four structural families: 1) antiparallel β-strands (e.g. immunoglobulins, serine proteases), 2) antiparallel β-sheets associated with at least one α-helix (e. g. lipocalin, profilin families), 3) α + β structures (e.g. lysozyme, lactalbumin) and 4) α-helical proteins (e.g. 2S albumins, insect hemoglobin, fish parvalbumin). His conclusion is, however, that there is no common structural feature, which is characteristic for the antigens.

4. Methods and strategies used in the study of allergen proteins

Unfortunately most of the pressure studies were performed using whole food products, or food extracts (like juices), and only few of them studied the purified allergen itself. We mention these studies only if there is a parallel study focusing on the allergen protein itself, by investigating the structural changes in the protein. Also the majority of the studies investigated the pressure treated product or protein, and only a very few of them followed the structural changes during the pressure treatment. Although from an industrial point of view it is enough to look at the changes which are detectable after the treatment, we believe that in situ experiments, which follow the pressure that caused structural alteration during the whole pressurization cycle, are very important to understand the mechanism of the observed effects. That is why we focus in this review mostly on those proteins for which structural information is also available from pressure studies. Although the methods which are capable to provide information under pressure conditions are quite limited, spectroscopic techniques like FTIR and fluorescence can be used to obtain structural information about the protein under pressure [31,34,63].

In the case of the food allergens digestion is a quite important factor. This is why the question, whether the epitopes remain intact after enzymatic digestion, is also studied widely. A number of papers deal with the enzymatic digestion of allergens after or during high pressure treatment. This method gives also quite indirect structural information. These studies are included only as additional information besides the more exact spectroscopic ones.

Fig. 1. summarizes the research strategies needed to investigate the high pressure treatment of allergen proteins. The first question is: how high is the pressure needed for the unfolding of the protein, which is expected to destroy the epitope? In situ spectroscopic methods are needed to detect the structural changes caused by pressure. The second question is the reversibility of the unfolding, i.e. whether the distortion of the epitope is irreversible. IgE binding studies are needed to confirm the decrease in the allergenicity of the pressure treated allergen protein before or after enzymatic digestion. Only the proper results of all these studies (indicated by green arrows) can guarantee the successful application of the pressure treatment in the reduction of food allergenicity.

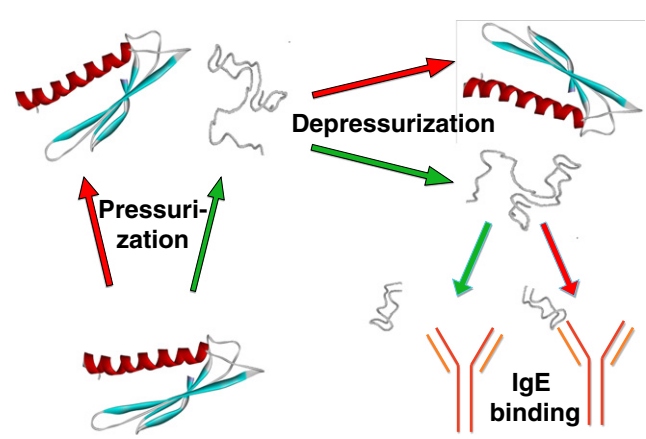

Fig. 1. The scheme of the research strategy for investigation of the high pressure treatment of allergen proteins in order to find conditions for reducing the allergenicity. Spectroscopic methods are needed first to detect the structural changes caused by pressure. The second question is the reversibility of the unfolding. If the structural changes are irreversible, further IgE binding studies are needed to confirm the decrease in the allergenicity before or after enzymatic digestion.

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Table 1
Main structural characteristics of the allergen proteins studied under pressure.

Allergen name	Allergen family	Common name	Size	Structure [PDB code]	Source
Mal d 1	Bet v 1 related		17.6 kDa, 159 AA	β sheet (7 strands) 39%, 2 helices 21%[a]	Apple
Dau c 1	Bet v 1 related		16 kDa, 154 AA	α 28%, β 33% [2WQL]	Carrot
Bos d 5	Lipocalins	Beta-lactoglobulin	18 kDa, 162 AA	β 42% (β-sandwich), α 19% [1B8E, 1CJ5]	Milk
Gal d 2	Serpin	Ovalbumin	43 kDa, 385 AA	α 29%, β 31% [1OVA]	Egg
Ara h 2	Prolamin	Conglutin-7	18 kDa, 151 AA	α 36% + loops[a]	Peanut
Ara h 6	Prolamin	Conglutin	15 kDa, 124 AA	α 53% [1W2Q]	Peanut
Mal d 3	Prolamin		9 kDa, 91 AA	α 38% + loops[a]	Apple
Gad m 1	EF-hand	Parvalbumin	11.4 kDa, 109 AA	α 50% (6 α helices), two Ca^{2+} binding sites	Fish

[a] From our homology modeling using the ExPASy server [108,124,125].

Since the few most allergic foods contain already a number of allergens, we selected those allergens for this review, which were investigated not only by allergenic but also by spectroscopic methods, that able to detect the changes in the molecular structure under pressure or at least after pressure treatment.

5. High pressure effects on allergen proteins

Table 1 contains the known information about the secondary structures of the reviewed proteins. We grouped them according to their structure: Predominantly beta, alpha + beta, and predominantly alpha. Table 2 summarizes the most important data about their behavior under pressure.

5.1. Proteins with predominantly beta structure

5.1.1. Mal d 1

The apple allergens Mal d 1–4 were purified and characterized by Oberhuber et al. [64]. The most important allergens in Europe are the Mal d 1 and Mal d 3. The former one is the major allergen in the Central and Northern European apple allergic population [65]. Mal d 1 is a member of the Bet v 1 related allergen family [66]. Because of the high sequence identity and the structural similarity between the Mal d 1 and Bet v 1 allergens, allergy to apple fruit is mostly a result of sensitization to birch allergen Bet v 1 [67]. These proteins are widely distributed among vascular plants.

Mal d 1 can trigger serious reactions known as oral allergy syndrome in allergenic patients [68]. Mal d 1 is a 17.6 kDa protein, consisting of 159 residues [69], whereas the variant Mal d 1b studied by the Hendrickx's group has 158 residues [70]. In the absence of X-ray and NMR structures the three dimensional structure was determined by homology modeling (Fig. 2a) [71]. Predominantly beta structure has been found; 33 (21%) and 62 (39%) of 159 residues were assigned to the helical and beta-sheet secondary components, respectively. The beta sheet is composed of seven strands. Besides the beta sheet, the molecule has one long helix and one short helix.

A number of studies were devoted to allergenicity of the apple or its allergen proteins under or after pressure treatment. Whole apple slices [72,73], apple juice [74], and the allergen proteins [70,71,74] were also investigated after various pressure treatments. In situ assessment of the secondary structure under pressure was also investigated in the case of Mal d 1 [71].

Meyer-Pittroff et al. studied the effect of pressure treatment (up to 600 MPa) on the structure and allergenicity of Mal d 1, using FTIR and CD spectroscopies and prick-to-prick, blinded oral provocation tests [72,73]. Pressure treated samples showed decrease of the helical content and increase of the beta structure as revealed by CD spectroscopy. Infrared spectra showed decrease of both alpha and beta structures after the 600 MPa treatment, but these spectra were recorded on dried films; therefore they should be treated with caution because of the effect of drying on the structure. In spite of the mild secondary structure changes detected by the spectroscopic techniques Meyer-Pittroff et al. reported notable immunological effect of the pressure treatment, namely the decrease of the average wheal area as a function of the pressure of the treatment. 200 MPa resulted already in 80% reduction of the area, but 600 MPa was not enough to eliminate the wheal in the case of all the patients, although there was a great variation from patient to patient.

A subsequent paper questioned the effect of pressure treatment on the allergenicity [74]. This study reported no significant change in basophile activation tests of Mal d 1 solution due to treatment by pressures of up to 500 MPa. They also performed Western blot to

Table 2
Pressure and temperature conditions needed for structural changes in the allergen proteins.

Allergen name	Unfolding		pH (pD)	Method	Remark	Ref.
	p (MPa)	T (°C)				
Mal d 1	260 ± 20	30	(7)	FTIR in situ	Aggregation	[71]
	150 ± 20	30	(3)	FTIR in situ	Aggregation	[71]
	196 ± 12	30	(3)	FTIR in situ	40 mM KCl, aggr.	[71]
	170 ± 10	30	(3)	FTIR in situ	Sugar mixture[a], aggr.	[71]
Bos d 5	160	20	7	SAXS	Radius of gyration ↑	[87]
	160	20	7	FTIR in situ	Irreversible >2% w/w	[87]
	>200	36	2	$^{1}H/^{15}N$ 2D NMR	Intermediates populated	[90]
Gal d 2	575		6.5	ANS-binding	After p treatment	[98]
	415		3	ANS-binding	After p treatment	[98]
	550	30	(7)	FTIR in situ		Somkuti et al. unpublished
Ara h 2 & 6	>700	80	>7.0[b]	CD after p treatm.	Practically no change in the CD spectrum	[70]
Mal d 3	600	80	>7.0[b]	CD after p treatm.	Broad transition, no complete unfolding after p treatment at 800 MPa	[70]
Gad m 1	520	30	(7)	FTIR in situ	Partial unfolding binds one Ca^{2+}	[123]
	1100	40	(7)	FTIR in situ	Complete unfolding	[123]
	890	55	(7)	FTIR in situ	Complete unfolding	[123]

[a] Sugar mixture: fructose, saccharose, and glucose at 5.63%, 2.31%, and 2.06% (w/w) respectively.
[b] Pressure sensitive buffer was used.

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study the IgE binding of the treated Mal d 1. Skin prick to prick tests and double blind placebo controlled food challenge tests were done on the apple juice. A rigorous statistical evaluation was performed to prove the effect of pressure treatment (up to 550 MPa). None of these tests showed any significant difference compared to placebo. It has to be noted that these authors added 3% ascorbic acid during the juice preparation, which could also influence the effectiveness of the pressure treatment. Garcia et al. [75] showed that polyphenol oxidases and peroxidases reduce the allergenicity of Mal d 1, because they contribute to the cross linking of the protein by its tyrosine residues. In presence of ascorbic acid these processes are less effective, which could influence the effectiveness of the pressure treatment too [76].

Fernandez et al. studied the effect of pressure and combined pressure/temperature treatment (up to 800 MPa and 80 °C) of apple extracts [77]. They concluded that high pressure treatment has negligible effects on apple antigenicity and IgE binding.

Hendrickx's group investigated the recombinant Mal d 1b and Mal d 3 [70]. Mal d 1b (Q8L6K9 in UniProt) has only 158 residues and has 90% homology to the Mal d 1 studied by the other groups. Pressure treatments of Mal d 1b at 700 and 800 MPa caused only slight change in the CD spectra. These changes were however not quantified in

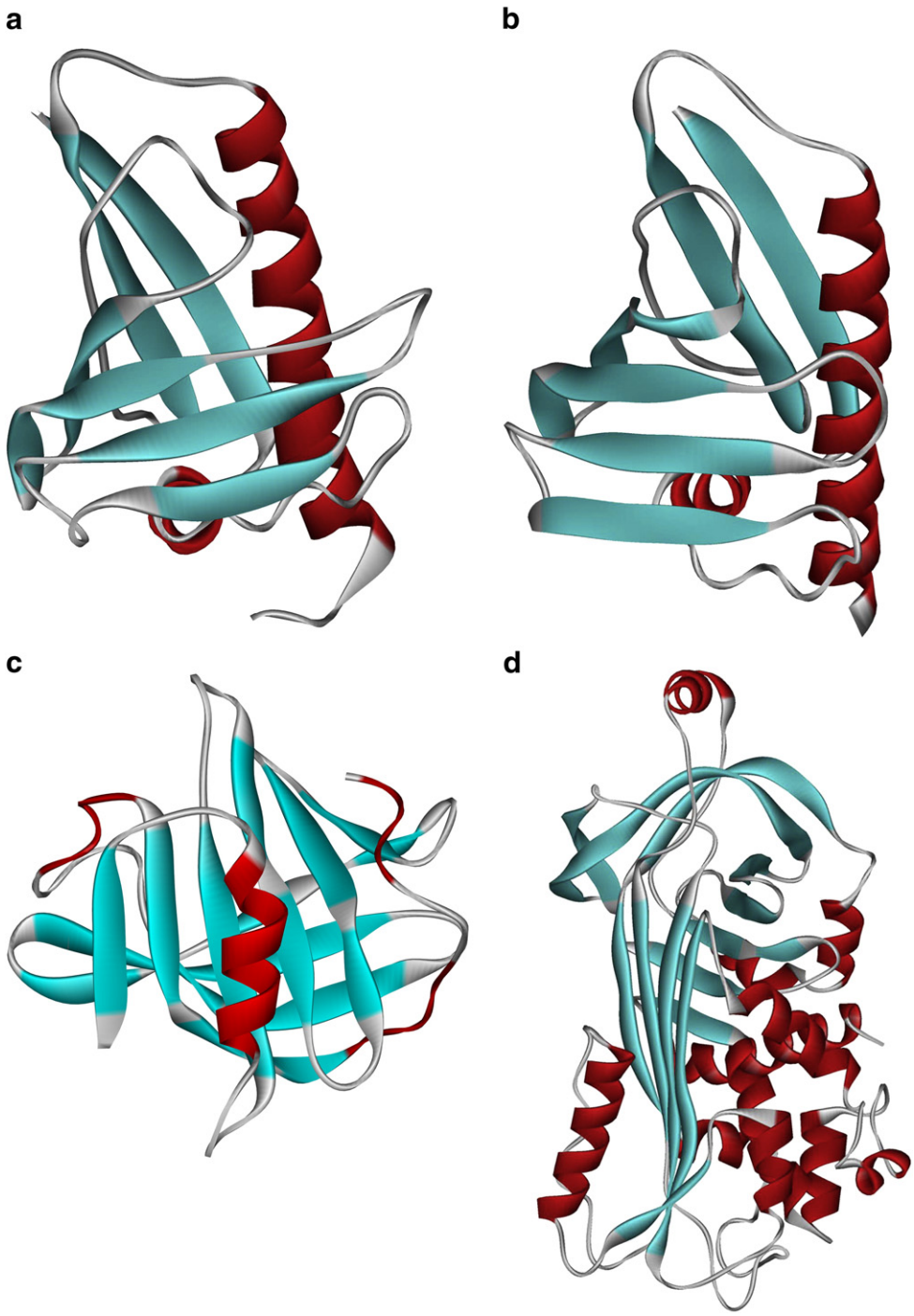

Fig. 2. 3D structures of the allergen proteins discussed in this review. a) Mal d 1; b) Dau c 1 [2WQL]; c) Bos d 5: beta-lactoglobulin [1B8E]; d) Gal d 2: ovalbumin [1OVA]; e) Ara h 2; f) Ara h 6 [1W2Q]; g) Mal d 3; h) Gad m 1: parvalbumin.The PDB identifiers of known 3D structures used in the figures are given in [brackets]. Where no PDB identifier is given, the displayed structure is based on homology modeling [71,108,123–125].

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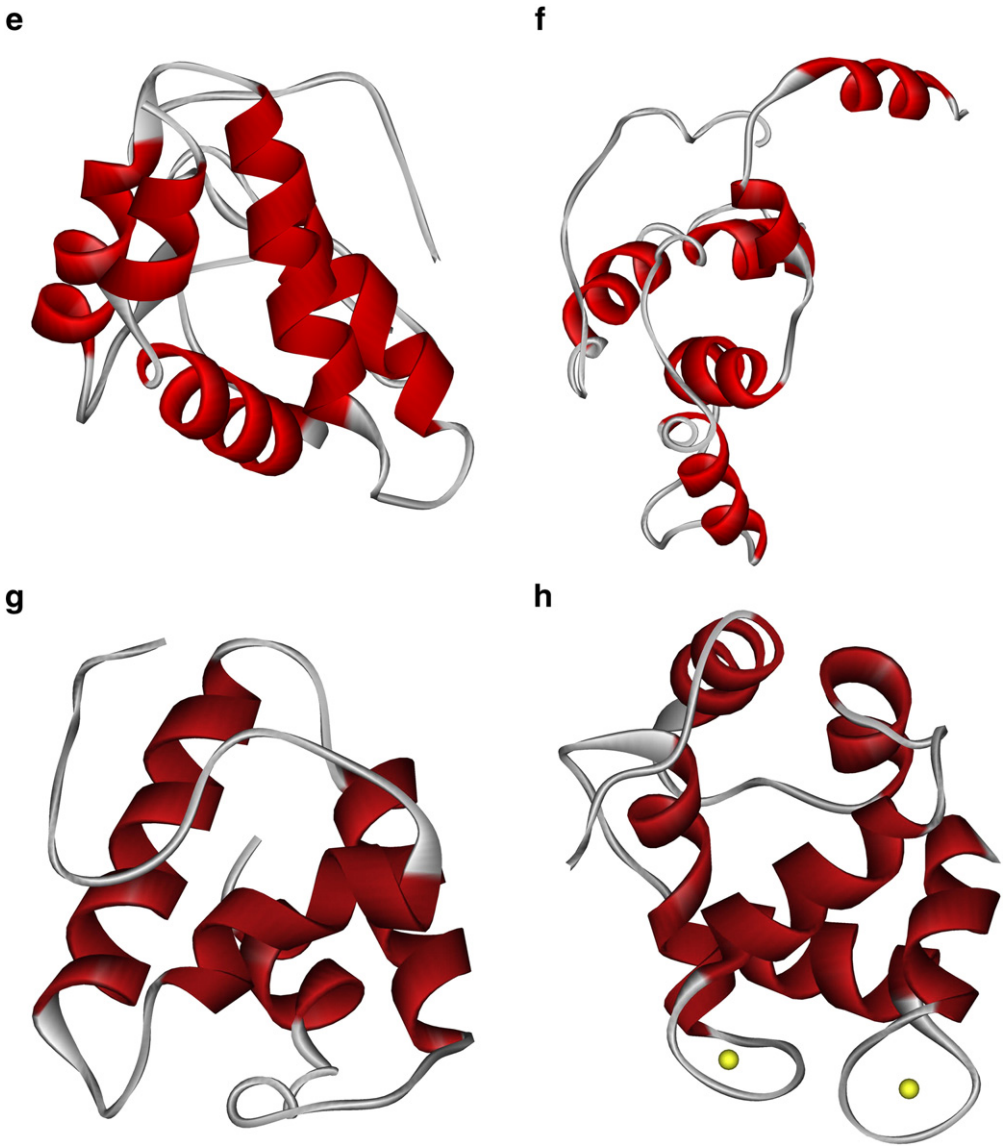

Fig. 2 (*continued*).

terms of secondary structure content. Increase of the treatment temperature to 80 °C slightly increased the effect.

The pressure induced conformational changes in Mal d 1 (UniProt code: Q9SYW3) were investigated under pressure by Somkuti et al. [71]. They determined the temperature and pressure unfolding conditions of the protein by in situ high pressure FTIR spectroscopy. A diamond anvil cell was used to generate high pressure [78]. In these experiments Mal d 1 unfolded at 260 MPa at 30 °C at pD 7 (pD was given instead of pH, because heavy water was used for the infrared spectroscopy). Interestingly, this unfolding was accompanied by aggregation of the protein, which is highly unusual under pressure [7]. This could be explained by the relatively low unfolding pressure, which was not able to prevent aggregation, and by the relatively high concentration of the protein, which is needed for the FTIR spectroscopy. Although high protein concentration is not unusual in biological systems, including food, the effect of concentration and molecular crowding desires further investigation. To mimic the conditions in the apple sample pD was adjusted to 3 and sugars and potassium (KCl) were added. Lowering the pD decreased the pressure stability of the protein to 150 MPa, but the addition of a sugar mixture (fructose, saccharose and glucose) corresponding to the apple composition increased pressure stability (transition pressure = 170 MPa). Similar stabilization effect (to ≈200 MPa) was observed when KCl was added (Fig. 3) [71].

In summarizing the effect of pressure on Mal d 1, we have to emphasize the effect of the environment of the protein. A consistent picture arises from the diverse experimental results: the protein unfolds mainly reversibly in the range of 150–260 MPa depending on the solvent conditions. In the case of high concentration and presence of possible partners it can aggregate, but in the low-concentration experiments (e.g. CD) the unfolding is reversible. In the case of food matrix, special biochemical processes, like the cross-linking by polyphenol oxidases, do modify the reversibility and presumably the allergenicity.

5.1.2. Dau c 1

Another member of the Bet v 1 family is the Dau c 1, which is a major allergen of carrot. It is quite well characterized, both the sequence and the X-ray structure are known (O04298 in UniProt and 2WQL in PDB) [79]. Its 3D structure (Fig. 2b) shows a striking similarity with the one of the Mal d 1 although the 3D structure of Mal d 1 was determined by homology modeling, another protein, Dau c 1, was used as a template in the calculations. Dau c 1 is a 16 kDa ribonuclease consisting of 154 residues [80]. The three helices give 28% of the structure, while the beta content is 33%.

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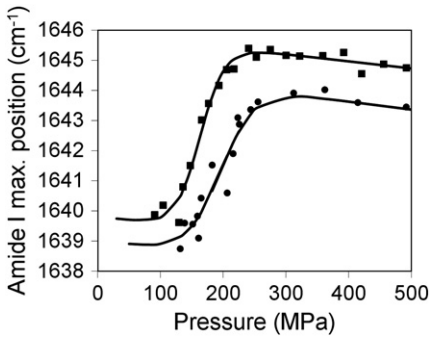

Fig. 3. Unfolding of Mal d 1 as seen from the shift of the amide I band of the infrared spectrum. Sample conditions: Mal d 1 (75 mg/ml) in pD 3 buffer, without (squares) and with 40 mM KCl (circles).
Adapted from [71] with kind permission from Springer Science and Business Media.

Heroldova et al. studied the effect of pressure treatment (500 MPa at 30, 40 and 50 °C) on the secondary structure and on the IgE binding [81]. CD spectroscopy showed very little irreversible changes in the secondary structure even after 10 min 500 MPa treatment at 50 °C. Neither basophile activation test nor Western blot studies could show any significant reduction in the IgE binding using sera of allergic patients. Unfortunately the pH of the solution is not given, which would be important, because marked effect on the temperature stability of Dau c 1 was reported [82]. Heroldova et al. studied the carrot juice also with similar negative results [81].

5.1.3. Bos d 5: beta-lactoglobulin

Beta-lactoglobulin is one of the major allergens of milk. This 18 kDa protein consist of 162 residues (P02754 in UniProt). Its three dimensional structure has been determined by both X-ray crystallography [83,84] and NMR technique [85]. The secondary structure is mainly a beta sheet type; 42% of the amino acid residues are classified for this structure. These sheets are arranged in a beta sandwich type fold (Fig. 2c). Among the beta-sheets an alpha helical segment and three short helices are formed by 19% of the residues [84]. The protein belongs to the lipocalin family; its original function could be transport of small hydrophobic molecules. It was shown to be capable to bind a number of molecules, like retinol and fatty acids [86].

Pressure effect on the structure of beta-lactoglobulin was studied by Winter's group using FTIR and SAXS [87] at 20 °C, pH 7. They found an increase of the radius of gyration between 150 and 170 MPa, which is indicative of the unfolding of the molecule. Simultaneously FTIR spectroscopy showed a decrease in the beta sheet and alpha helical structures, while the unordered component increased in the pressure range of 130–200 MPa. Hydrogen-deuterium exchange studies [88] revealed tertiary structural changes after pressure treatments in the 0–200 MPa region. Although FTIR spectroscopy [87] could not show any further changes above 200 MPa, the complete unfolding was not achieved even at 1 GPa. They also mention that the pressure unfolding was irreversible above 2% w/w protein concentration. The refolding after the pressure treatment at different temperature and pH conditions was investigated by Belloque et al. using NMR spectroscopy [89]. They found faster refolding when the protein was subjected to 200 MPa compared to the 400 MPa treatment. These results suggest further structural changes in the 200–400 MPa range. The 0–200 MPa region was also studied by $^{1}H/^{15}N$ two-dimensional NMR spectroscopy [90]. This study revealed two intermediate conformers with locally unfolded conformation either in the hydrophobic core or at the side of the molecule.

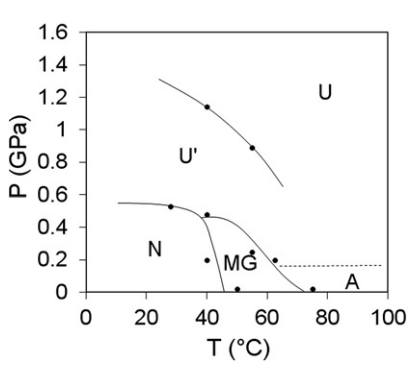

Fig. 4. Pressure–temperature phase diagram of Gad m 1. Abbreviations: N, native; MG, molten globule; A, aggregated; U, unfolded; U′, partially unfolded.
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Zeece et al. [91] investigated the in vitro digestibility of the beta-lactoglobulin by pepsin after a 10 minute treatment at 20 °C and 200, 400, 600 and 800 MPa pressures. The 400 MPa treatment already slightly increased the speed of the hydrolysis, but the samples treated at 600 MPa or higher pressures were hydrolyzed almost completely already within the first minute. This finding is in agreement with the above suggestion that the unfolding of beta-lactoglobulin is not complete at 200 MPa, but there is a remaining core which can serve as a template for the refolding. Fast hydrolysis would need an open unfolded conformation, which did not refold when reducing the pressure after the pressure treatment. In the same time aggregation could also protect the protein from the pepsin digestion. Such effect has been observed for fibrous aggregates, which are formed in several conformational diseases [92,93]. The peptides formed after the enzymatic digestion of the high pressure treated beta lactoglobulin [91] were remarkably short, only 7–10 residues long. The authors assume that these short peptides contain only one epitope, preventing them to bind to several antibodies, which is known to be required for triggering the allergenic reaction [94]. This way the high pressure treatment can be expected to lower the allergenic effect.

Similar results have been obtained by Chicon et al. [95] using enzymatic hydrolysis by trypsin and chymotrypsin.

Lopez-Exposito et al. compared hydrolysates of beta-lactoglobulin produced with chymotrypsin at atmospheric pressure with those produced at 400 MPa with chymotrypsin and with pepsin [96]. In this experiment the hydrolysis was performed under pressure, and it was stopped after the pressure treatment. The hydrolysates were tested on orally sensitized mice, which showed no anaphylactic syndromes. The absence of allergic reaction was explained by the fact that hydrolysates lost their ability to cross link the IgE antibodies, which was explained by the assumption that only one epitope is present in the peptides. Although neither the high pressure nor the atmospheric pressure hydrolysates triggered allergic reactions, the enzymatic process under high pressure was remarkably faster (5–10 min instead of 48 h).

In summary, the structure of beta lactoglobulin was mainly lost at around 200 MPa as seen by spectroscopic methods, but there are strong evidences that there is a core resisting higher pressures. Presence or absence of this core has a marked effect on the reversibility of the high pressure treated protein. This core is unable to facilitate the refolding if the treatment was above 400 MPa, but the complete disordered structure cannot be reached at 1 GPa. Enzymatic digestion is enhanced by prior pressure treatment, leading to peptides, which may contain epitopes, but they are not able to crosslink IgE antibodies. The lack of crosslinking could reduce the allergenicity of the pressure treated food.

5.2. Proteins with alpha and beta domains

5.2.1. Gal d 2: ovalbumin

More than half (58% W/W) of the egg white is ovalbumin, which is also one of the major allergens of an egg (Gal d 2). It is a glycoprotein (P01012) with 385 residues which give a molecular mass of 43 kDa. The secondary structure has been determined by X-ray crystallography

(PDB code: 1OVA) [97]. 29% of the residues are assigned to helices, and 31% builds beta sheets (Fig. 2d). The structure is stabilized by a disulphide bond. Ovalbumin has three tryptophan residues, one of them completely, while two of them almost buried in the interior of the molecule. This makes the protein suitable for fluorescence experiments.

Our unpublished FTIR spectroscopic results show that the protein unfolds during a broad transition with a midpoint of 550 MPa (30 °C). The loosening of the tertiary structure starts above 400 MPa and the unfolding process completes around 700 MPa. Smith et al. studied the secondary structure changes of ovalbumin caused by high pressure treatment [98]. CD, FTIR and UV–VIS absorption spectroscopies and fluorescence-quenching were used, unfortunately not during the pressure treatment, but before and after pressurization. Infrared spectroscopy showed reduction in the intramolecular beta structure and increase in the intermolecular beta structure after 400, 600 and 800 MPa treatments at pH 6.5. Since the spectroscopic measurements were done after the treatment, the possibility of refolding during depressurizing could not be excluded. Such competitive refolding and aggregation were shown for a number of proteins after pressure treatment [7,28,29]. Tryptophan fluorescence showed reduced intensity after treatments at 600 and 800 MPa, but no spectral shift was observed which could be a sign of unfolding. This indicates again refolding of the protein. The loss of intensity can be the result of aggregation. The most pronounced changes were measured in the ANS binding to ovalbumin. The observed increased ANS binding indicates the distortion of the tertiary structures, and exposure of hydrophobic surfaces available for binding. The midpoint of the transition was at 575 and 415 MPa at pH values of 6.5 and 3 respectively.

Lopez-Exposito et al. investigated the effect of high pressure on digestibility and antigenicity of ovalbumin [99]. Only high pressure treatment at 400 MPa was investigated. Pepsin digestion for 10 min at 400 MPa (pH 2.5, 37 °C) resulted in a complete hydrolysis, in contrast to 0.1 MPa, where intact proteins remained even after 24 h hydrolysis in spite of the higher enzyme/substrate ratio (1/5 and 1/20). It is important to notice that the hydrolysis itself was performed at high pressure, i.e. the porcine pepsin was also pressurized together with the target protein. The distribution of the fragment sizes was different at high and low pressure digestion, indicating different mechanisms. The RP-HPLC results showed significantly bigger fragments if the hydrolysis was conducted at high pressure. This is most probably due to the unfolding of ovalbumin, which can be expected from the above mentioned spectroscopic results [98]. Opening the structure can facilitate the first step of the digestion; however the hydrolysis of the larger fragments seems to be slower even under pressure. It has to be noted, that there are no data about the effect of pressure on the pepsin itself. The hydrolysates had lower antigenicity, although residual IgE and IgG binding was retained. The authors also analyzed the fragments obtained by pepsin digestion. Although the fragments contained known epitopes, each of them had only one epitope, which made them impossible to crosslink IgE-s. The authors argue that the hydrolysates can be more easily tolerated by the immune system.

In summary, ovalbumin loses its structure above 400 MPa pressure. Although it will partially refold after the pressure treatment, aggregation of the protein after the pressure treatment cannot be explained. The high pressure pepsin digestion is very fast, but gives larger fragments. This can be explained by the assumption that the different cleavage sites have different activation volumes. Those with negative activation volume have accelerated hydrolysis under pressure, while hydrolysis of others with positive activation volume is slowed down. These fragments have reduced allergenicity.

5.3. Proteins with predominantly alpha helical structure

5.3.1. Ara h 2 and Ara h 6

Ara h 2 is the most problematic allergen of peanut. It has 59% sequence identity with another peanut allergen Ara h 6 [100]. They are both 2S albumins, and they have high degree of structural similarity. Ara h 2 is a 18 kDa protein containing 151 residues. Ara h 6 is shorter by 26 residues, giving a molar mass of only 15 kDa. These proteins have predominantly alpha helical structure as shown by CD spectroscopy [101–103] and homology modeling (Fig. 2e, f). Additionally for Ara h 6 the 3D structure is known from NMR spectroscopy (PDB code: 1W2Q) [104]. In situ spectroscopic pressure experiments have not yet been performed on these proteins, but Johnson et al. investigated the effect of pressure treatment on the mixture of Ara h 2 and Ara h 6 [70]. They are remarkably temperature stable as it can be seen from the CD spectrum, which was practically unchanged up to 100 °C. The pressure treatment did not result in any significant irreversible change. The structure was practically unchanged after 700 MPa treatments at 20 and 80 °C. This might be due to the four and five disulphide bridges present in these proteins (see Q6PSU2 and Q647G9 in UniProt) [104,105]. It has to be mentioned that this group used phosphate buffer (pH 7.0), which is very pressure sensitive; a pressure of approximately 300 MPa can shift the pH by an entire unit [106,107]. These proteins need further investigation under pressure both from structural and from allergenic point of view.

5.3.2. Mal d 3

Mal d 3 is a nonspecific lipid transfer protein, and belongs to the prolamin family [66]. It is an important apple allergen in Europe. Allergy against Mal d 3 is more prevalent in Southern Europe [65]. Only the primary structure is known, which has 91 residues giving a mass of 9 kDa. CD and FTIR spectra of Mal d 3 both showed predominantly alpha helical secondary structure content for the protein [64]. Homology modeling using the SWISS-MODEL Protein Modeling Server [108] resulted in a structure with four helices of different lengths (Fig. 2g).

Hendrickx's group investigated also the Mal d 3 [70]. The secondary structure changes for Mal d 3 were only pronounced if the treatment was performed at 80 °C. It has to be noted that the temperature treatment at atmospheric pressure resulted in the loss of secondary structure at 80 °C. Antibody recognition of Mal d 3 was decreased for the samples pressure treated at 80 °C. This effect was more pronounced above 400 MPa, where the immunoreactivity decreased to less than 50% of that of the pressure untreated sample.

5.3.3. Gad m 1: fish parvalbumin

The major fish allergen is parvalbumin, which is found in the white muscle of the fish [109] and it is responsible for the allergic reaction in 95% of the fish allergic patients [110,111]. The structure of parvalbumins is stabilized by two Ca^{2+} ions. Ca^{2+} binding is the main role of the protein; it serves as a calcium buffer in the fast twitch muscle, and this way it is responsible for the relaxation of the muscle [112]. In the absence of bound Ca^{2+} the protein was suggested to belong to the family of the intrinsically unordered proteins [113]. This is however questionable, because recently three dimensional structures of Ca^{2+}-free proteins were reported for some species (other than fish) [114]. On the other hand increased IgE-binding has been reported for Ca^{2+}-bound carp parvalbumin compared to the apo-form [115]. This underlines the role of the Ca^{2+}-binding in the allergenicity. The native structure of parvalbumin (in the presence of the two Ca^{2+} ions) has been determined by X-ray crystallography for parvalbumins from several species, like carp, whiting, pike, and silver hake [116–119] (Fig. 2h). A comparison of the parvalbumin allergens from different sources was performed by Ma et al. [120].

There are a very limited number of pressure studies on fish allergens [121–123]. Liu et al. did not observe any allergenicity change of silver carp protein extract after pressure treatments up to 300 MPa [121]. Similar negative result was reported by the same group for largemouth bass allergens [122]. It has to be noted that both experiments were done on simple protein extracts, and not on purified allergen protein samples.

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A systematic study on the pressure–temperature phase behavior of cod parvalbumin (Gad m 1 allergen) was performed by Somkuti et al. [123]. The 11.4 kDa protein consist of 109 amino acid residues. Although the three dimensional structure of cod parvalbumin is not known from crystallographic or NMR studies, homology modeling has been performed [123], which resulted in 6 helical segments, arranged in three EF-hand motifs. Two of these motifs correspond to the two Ca^{2+} binding sites.

The structural changes caused by a combination of high pressure and temperature in cod parvalbumin were detected by high pressure FTIR and fluorescence spectroscopies [123]. From the analysis of the amide I band of the infrared spectrum 50% helical, 34% turn and loop and 16% disordered structures were obtained in accordance with the homology modeling. To adopt this structure the presence of the two Ca^{2+} ions was necessary. During pressurization at 30 °C the protein underwent a broad transition with a midpoint of 520 MPa. At this transition the native structure was disrupted, but the completely unfolded state was not achieved. The antisymmetric stretching modes of the glutamic acid residues show that one Ca^{2+} ion is still bound to this partially unfolded conformation. To unfold the protein completely, 40 °C and more than 1.1 GPa was needed. Below 400 MPa a molten globule structure was found above 45 °C. The existence of the molten globule was proved by detecting the hydrogen/deuterium exchange in the FTIR spectrum and also by using tryptophan fluorescence spectroscopy. In this state the two Ca^{2+} ions are still bound to the protein. The protein unfolded and aggregated at high temperatures (at 75 °C at atmospheric pressure). This kind of aggregation was not observed above 200 MPa. The phase diagram of cod parvalbumin was found to be quite complex (Fig. 4).

Due to technical limitations allergenicity test was only possible for samples treated under lower pressure. ELISA test was performed on the samples heat treated (80 °C) at 300 MPa pressure, but the IgE binding ability was not significantly different from that of the control samples.

In summary, Gad m 1 is an extremely pressure stable protein. Its stability can be mainly attributed to the two Ca^{2+} binding sites. To change its allergenicity can only be hoped using combined harsh pressure and temperature conditions.

6. Summary and conclusions

The pressure stability of allergen proteins varies highly according to their secondary structure and the presence of additional stabilizing factors, like disulfide bridges and Ca^{2+}-binding. The question whether high pressure reduces the allergenicity of the proteins does not have a simple answer. Comparison of the results published in the literature emphasizes the role of the environment, which is usually very crowded in the food matrix. Also presence of different additives, cosolvents, or enzymes has been proven to affect the results of the pressure treatment. The reversibility of the unfolding can also be affected by the presence of high concentration of proteins, which could lead to aggregation, or by the crowded environment. Although at present there is no accepted method to reduce the allergenicity of foods, the combination of the above factors with pressure treatment could be a successful strategy to reach hypoallergenization. To reach this we have to collect more information about the pressure behavior of these proteins at various environmental conditions and in presence of possible food additives. There is a definite need for further studies at basic science level, which can report the effect of pressure on the 3D structure of the allergenic proteins.

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