Food Chemistry 110 (2008) 904–908

Contents lists available at ScienceDirect

Food Chemistry

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

Heat denaturation of Brazil nut allergen Ber e 1 in relation to food processing

Evelien L. van Boxtel [a], Stef J. Koppelman [b], Lambertus A.M. van den Broek [c], Harry Gruppen [a,*]

[a] Wageningen University, Laboratory of Food Chemistry, P.O. Box 8129, 6700 EV Wageningen, The Netherlands
[b] TNO Quality of Life, P.O. Box 360, 3700 AJ Zeist, The Netherlands
[c] WUR-AFSG Biobased Products, P.O. Box 17, 6700 AA Wageningen, The Netherlands

ARTICLE INFO

Article history:
Received 2 October 2007
Received in revised form 2 January 2008
Accepted 27 February 2008

Keywords:
Food allergy
Ber e 1
Brazil nuts
Heat denaturation

ABSTRACT

Ber e 1, a major allergen from Brazil nuts, is very stable to *in vitro* peptic digestion. As heat-induced denaturation may affect protein digestibility, the denaturation behaviour of Ber e 1 was investigated. The denaturation temperature of Ber e 1 varies from approximately 80–110 °C, depending on the pH. Upon heating above its denaturation temperature at pH 7.0, the protein partly forms insoluble aggregates and partly dissociates into its polypeptides, whereas heating at pH 5.0 does neither induce aggregation, nor dissociation of the protein. The denaturation temperature of approximately 110 °C at pH values corresponding to the general pH values of foods (pH 5–7) is very high and is expected to be even higher in Brazil nuts themselves. As a result, it is unlikely that heat processing causes the denaturation of all Ber e 1 present in food products. Consequently, the allergen is assumed to be consumed (mainly) in its native form, having a high stability towards pepsin digestion.

1. Introduction

Food allergens are considered to be able to exert systemic allergic reactions only after they have been absorbed through the intestinal mucosa (Astwood, Leach, & Fuchs, 1996). As a result, the resistance of the IgE binding capacity of an allergen towards digestion in the human gastro-intestinal tract is considered an important factor. Heat processing may affect the digestibility of a protein. Taking into account that most foods are being consumed after some kind of heat processing, the digestibility of pre-heated allergens is an important characteristic as well.

The most important foods and food raw materials causing allergic reactions are cow's milk, eggs, soy, wheat, peanuts, tree nuts, fish, and shellfish. Brazil nuts, belonging to this group of foods and food raw materials, are the seeds of *Bertholletia excelsa* HBK. The nuts can be eaten both raw and roasted (Clark, 2002) and are also used as an ingredient in foods like bakery products (Clemente, Chambers, Lodi, Nicoletti, & Brett, 2004). Allergic reactions in response to Brazil nut consumption can be very severe and even life threatening (Ewan, 1996). The most important allergen from these nuts, Ber e 1, is a member of the seed storage albumin group (Pastorello et al., 1998).

Seed storage albumins usually are products of multigene families. For Ber e 1, so far, 6 different isoforms have been identified (NCBI accession numbers gi/839533, gi/384327, gi/112754, gi/99609, gi/384326, and gi/81557) (Moreno et al., 2004). Ber e 1 is synthesized as a 18 kDa polypeptide, which is post-translationally processed into a 12 kDa polypeptide. This polypeptide is subsequently processed into two polypeptides of approximately 9 and 3 kDa, linked together by 4 disulfide bridges (Altenbach, Pearson, & Sun, 1992). The protein contains 30–47% α-helices, depending on the isoform (Moreno et al., 2004; van Boxtel et al., 2006). One immunodominant conformational epitope has been identified on the large polypeptide of the protein. The epitope corresponds to amino acids 26–63 of the large polypeptide of the protein (Swissprot accession number P04403), which comprises a helix-turn-helix conformation. When unfolded, this epitope binds at least four times less IgE compared with its folded couterpart. Besides the immunodominant conformational epitope, a linear epitope has been identified on the small polypeptide of Ber e 1, corresponding to amino acids 7–20 (QMQRQQMLSHCRMY) (Alcocer et al., 2004).

The denaturation temperature of Ber e 1 has been determined at pH 2.0 to be approximately 83 °C. The denaturation temperature of Ber e 1 at neutral pH is expected to be higher than 110 °C as no change in transition was observed upon heating to 110 °C (Koppelman et al., 2004). Ber e 1, when subjected to *in vitro* peptic hydrolysis, is cleaved into peptides ranging in molecular mass from <1 to approximately 6.5 kDa. The 6.5 kDa fragment contained the region in which the immunodominant conformational IgE epitope (Alcocer et al., 2004) of the allergen is situated (Moreno, Mellon, Wickham, Bottrill, & Mills, 2005). Upon reduction, this 6.5 kDa peptide dissociates into peptides smaller than approximately 3 kDa. It should be noted that after two hours of peptic digestion, which is

* Corresponding author. Tel.: +31 317483211; fax: +31 317484893.
E-mail address: Harry.Gruppen@wur.nl (H. Gruppen).

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doi:10.1016/j.foodchem.2008.02.078

considered as the average gastric transit time (Untersmayr & Jensen-Jarolim, 2006) and generally used as the maximum time of pepsin digestion in *in vitro* tests (Eiwegger et al., 2006; Fu, Abbott, & Hatzos, 2002; Moreno et al., 2005; van Boxtel, van den Broek, Koppelman, & Gruppen, 2007), approximately 25% of the protein was still intact (Moreno et al., 2005). Consequently, it is assumed that the IgE binding capacity of Ber e 1 (partly) remains after peptic digestion (Moreno et al., 2005).

Under the same conditions, peptic digestion of reduced and alkylated Ber e 1, compared to native Ber e 1, results in a fast degradation, with the complete disappearance of the intact polypeptides within 30 s (Koppelman et al., 2004). The structural stability of the native protein thus seems to protect the protein from peptic digestion.

The effects of heat-induced denaturation on the structure and digestibility of Ber e 1 are not known. Therefore, the aim of our research was to study the denaturation behaviour of Ber e 1 in more detail, in order to assess whether heat processing could induce changes in the digestibility of the protein and thereby changes in its IgE binding capacity after digestion.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (Sigma–Aldrich Inc., St. Louis, MO, USA), unless stated otherwise. Unshelled Brazil nuts were purchased from Imko Nut Products (Doetinchem, The Netherlands) and stored at 4 °C under vacuum until use. Ber e 1 was purified from defatted Brazil nuts as described previously (van Boxtel et al., 2006). The purity of the protein was estimated to be >95%, as estimated using a densitometric scan of bands at approximately 9 and 3 kDa on an SDS–PAGE gel, stained with Coomassie Brilliant Blue.

2.2. Brazil nut protein extraction

Brazil nuts were ground using a domestic type mechanical high-speed slicer (Kenwood Corp., Tokyo, Japan) and defatted with hexane using Soxhlet extraction. After drying at room temperature, the partially defatted meal was ground at room temperature using a Waring blender (Waring Products Inc., New Hartford, CT, USA) and was subjected to a second defatting step. Hereafter, the defatted meal was dried at room temperature for 24 h and stored at 4 °C until use. Defatted Brazil nut meal was extracted by stirring in water, at a meal/solvent ratio of 1:100 (w/v), for 1 h at room temperature. Afterwards, the extract was centrifuged (10 min, 22,000*g*, 4 °C). The supernatant was collected and used as Brazil nut extract.

2.3. Protein quantification

Protein concentrations were measured using the Bradford method. Bovine serum albumin (BSA, Sigma) was used as a standard. All assays were performed at least in duplicate.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

Ber e 1 samples were analyzed on a PhastSystem (GE Healthcare), according to the instructions of the manufacturer. High density Phastgels and polypeptide markers (2.5–16.9 kDa; GE Healthcare) were used. Reducing conditions were obtained by adding β-mercaptoethanol to a concentration of 10 mM and heating the samples for 5 min at 100 °C. Gels were stained according to the Coomassie Brilliant Blue procedure provided by the manufacturer.

2.5. Differential scanning calorimetry

DSC measurements were performed on a VP-DSC MicroCalorimeter (Microcal Inc., Northampton, MA, USA). Thermograms were recorded from 20 to 130 °C, using a heating rate of 1 °C min^{-1}. Brazil nut extract and purified Ber e 1 (2.0 mg ml^{-1}) were analyzed in water or in buffer of various pH. At pH 2.0 and 3.0, the protein was solubilized in 10 mM sodium phosphate buffer and at pH 4.0 and 5.0 in 10 mM sodium acetate buffer. At pH 6.0 a 10 mM piperazine buffer and at pH 7.0 a 10 mM sodium phosphate buffer was used for solubilization of the protein. All samples were degassed prior to the experiments. Enthalpies were calculated using Origin software (Microcal Inc.), based on integration of the area of the transition. Analyses were performed in duplicate.

2.6. Heating experiments

Ber e 1 solubilized (2.0–5.0 mg ml^{-1}) in 10 mM sodium phosphate buffer, pH 7.0, or 50 mM sodium acetate buffer, pH 5.0, was heated at 110 or 120 °C during various time intervals in kimax tubes. After heating, samples were cooled immediately on ice. Samples were centrifuged at 22,000*g* for 10 min at 4 °C, after which the supernatant was collected and analyzed with size exclusion chromatography and SDS–PAGE.

2.7. Size exclusion chromatography (SEC)

Ber e 1 samples (0.1 and 4 ml) were applied onto a Superdex 75 XK 16/60 column or a Superdex 75 HR 10/30 column (GE Healthcare, Uppsala, Sweden). The columns were equilibrated and eluted with 10 mM sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml min^{-1}, or 50 mM sodium acetate buffer, pH 5.0, at a flow rate of 0.5 ml min^{-1}, respectively, using an Äkta purifier system (GE Healthcare) operated at room temperature. Eluates were monitored at 280 and 214 nm and appropriate fractions were collected.

2.8. Reduction and alkylation of Ber e 1

Lyophilized Ber e 1 (50 mg) was dissolved in 25 mL 6 M guanidinium chloride in 10 mM ammonium hydrogen carbonate, pH 7.8 and heated to 56 °C. Dithiothreitol (DTT) was added to a concentration of 20 mM and the solution was stirred for 60 min. After cooling to room temperature, 2.5 ml 1 M iodoacetamide solution in 100 mM ammonium hydrogen carbonate, pH 7.8, was added. To allow alkylation to occur, the stirred solution was placed for 90 min in the dark. Next, the alkylated protein solution was dialyzed against demineralised water using 2000 MWCO dialysis tubing at 4 °C (Spectrum laboratories Inc., Rancho Dominguez, CA, USA) and the reduced and alkylated (R + A) protein was subsequently lyophilized.

3. Results and discussion

3.1. Denaturation temperature of Ber e 1

The stability of the IgE binding capacity of an allergen towards digestion is considered an important characteristic for food allergens. Unheated Ber e 1, the major allergen from Brazil nuts, is very stable to digestion. As heat-induced denaturation may influence the digestibility of food proteins, the denaturation behaviour of Ber e 1 was investigated.

Purified Ber e 1 solubilized in water showed a transition at approximately 110 °C. The denaturation temperature of purified Ber e 1 in the presence of a Brazil nut extract made in water (pH 7) turned out to be similar, as can be seen from Fig. 1. In both

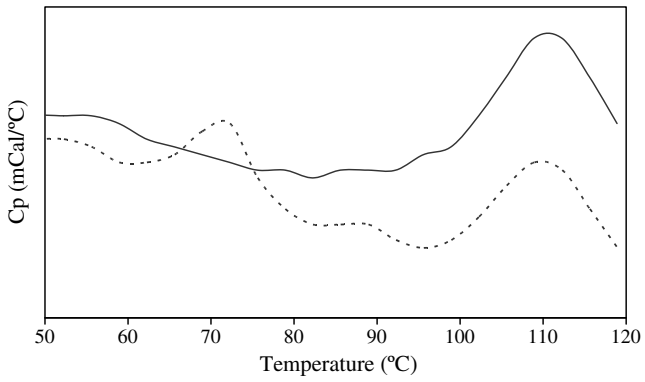

Fig. 1. DSC thermograms of Ber e 1 solubilized in water (solid line) and Ber e 1 solubilized in Brazil nut extract made in water (dashed line).

samples a transition at approximately 110 °C was observed. The additional transition peak at approximately 72 °C in the sample containing Brazil nut extract likely represents the denaturation of other Brazil nut proteins, as this transition peak was also observed when analyzing only Brazil nut extract (data not shown). From these results it could be concluded that components present in Brazil nuts do not influence the denaturation temperature of Ber e 1 in an aqueous system.

DSC analysis (Fig. 2 and Table 1) of purified Ber e 1 at various pH values showed that the denaturation temperature of Ber e 1 is pH-dependent. At a low pH (pH 2.0) the denaturation temperature is approximately 80 °C and increases with increasing pH to approximately 110 °C at pH 5.0. At pH values between pH 5.0 and pH 7.0 the denaturation temperature is stable around 110 °C. These results are in agreement with those reported previously for pH 2.0 and 7.0 (Koppelman et al., 2004). The broader transitions observed at lower pH values indicate that at lower pH values, the cooperativity of unfolding is lower compared to the cooperativity of unfolding at higher pH values. The higher denaturation temperatures at pH values between 5.0 and 7.0 could be explained because globular proteins generally are most stable to denaturation at pH values close to their pI (Privalov & Khechina, 1974). Ber e 1 isoforms have pI values ranging from pH 5.5 to 8.1. In the protein preparation investigated, the most abundant isoforms had pI values around pH 8 (van Boxtel et al., 2006). At pH 7.0 the enthalpy of unfolding was observed to be highest (Table 1), most likely because this pH value is closest to the most abundant pI value in this protein preparation (van Boxtel et al., 2006).

The denaturation of Ber e 1 at low pH (pH 2.0) appeared to be partly reversible, as can be seen in Table 2. Upon reheating the protein, a transition was observed, although with a lower enthalpy. After a second reheating step again a transition could be observed. These results also correspond with those described in literature, as the denaturation of Ber e 1 at pH 2.0 was previously described to be partly irreversible (Koppelman et al., 2004). At pH 7.0 the denaturation of Ber e 1 appeared to be completely irreversible, as reheating the samples did not show a transition (no further data shown).

3.2. Denaturation and aggregation of Ber e 1

The pH of an aqueous Brazil nut extract was approximately 7, whereas the pH of foods generally ranges between pH 5 and pH 7. Therefore, pH values of 5.0 and 7.0 were used for further investigation of the denaturation behaviour of Ber e 1.

When Ber e 1 was heated at its denaturation temperature (20 min, 110 °C, pH 7.0), no precipitation occurred and on SEC, the protein eluted similarly to the native protein. These results indicate that no changes in the protein's structure had occurred upon heating. When the allergen was heated above its denaturation temperature (20 min, 120 °C, pH 7.0), the protein partly became insoluble. SEC analysis (Fig. 3) of the supernatant after centrifugation showed that the soluble part of the protein had partly dissociated upon heating. Three peaks were present in the chromatogram of the heated protein sample, which were denoted SEC peaks 1, 2, and 3. SEC peak 1 eluted at the same elution volume

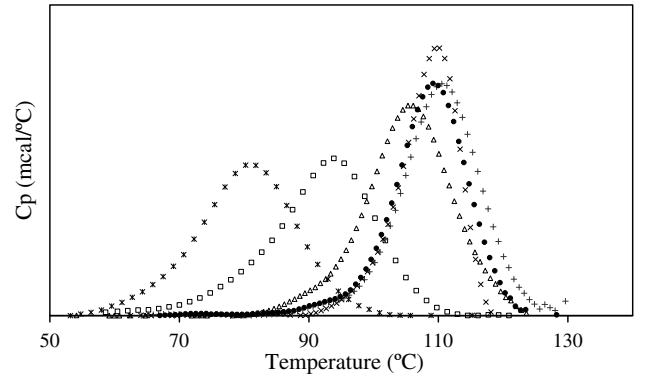

Fig. 2. DSC thermograms of Ber e 1 solubilized in buffer with pH values ranging from 2.0 to 7.0. ⋆, pH 2.0; □, pH 3.0; Δ, pH 4.0; +, pH 5.0; ●, pH 6.0; ×, pH 7.0.

Table 1
Denaturation temperatures (T_d) and calorimetric enthalpies of unfolding of Ber e 1 at various pH's, with standard deviations

pH	T_d (°C)	ΔH (kJ mol^{-1})
2.0	81.1 ± 0.1	148 ± 5
3.0	94.3 ± 0.2	146 ± 3
4.0	105.7 ± 0.2	122 ± 1
5.0	110.3 ± 0.0	111 ± 3
6.0	109.1 ± 0.1	131 ± 4
7.0	109.9 ± 0.0	196 ± 8

Table 2
Calorimetric enthalpy (ΔH) of Ber e 1 during three subsequent heating experiments at pH 2.0

Heating step	ΔH (J g^{-1})	ΔH (kJ mol^{-1})
1	12	148
2	11	133
3	6	73

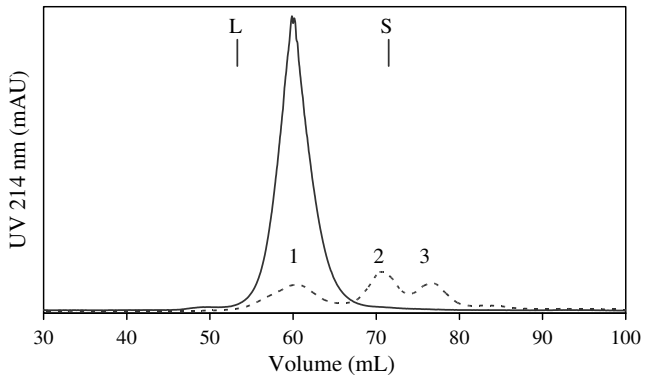

Fig. 3. Size exclusion chromatograms of soluble Ber e 1 in buffer pH 7.0 before (solid line) and after (dashed line) heating at 120 °C for 20 min. Indicated are the elution volumes of the reduced and alkylated large (L) and small (S) polypeptides of the protein.

as unheated Ber e 1, thus pointing towards this peak to represent intact Ber e 1. The other two peaks eluted at later elution volumes, indicating that these peaks contained proteins or peptides with lower molecular masses compared with intact Ber e 1.

Fractions corresponding to the three SEC peaks were collected and analyzed on SDS–PAGE. Fractions under SEC peak 1 showed two protein bands on reducing SDS–PAGE, at approximately 3 and 9 kDa, thus pointing towards this peak to indeed represent intact Ber e 1. SDS–PAGE of the other two SEC peaks showed protein bands of approximately 3 kDa. This molecular mass coincides with the molecular mass of the small polypeptides of Ber e 1 (~3 kDa). Under non-reducing conditions, SEC peak 1 on SDS–PAGE showed a protein band at approximately 12 kDa, as well as a protein band at a molecular mass of approximately 9 kDa (data not shown). SEC peaks 2 and 3 on non-reducing SDS–PAGE showed similar results as the results obtained under reducing conditions (data not shown).

The results from non-reducing SDS–PAGE of SEC peak 1 indicated that next to intact Ber e 1, this (slightly asymmetric) SEC peak also represented the dissociated large (~9 kDa) polypeptides of the allergen. In order to verify this, reduced and alkylated Ber e 1 was applied to the same column, as can be seen in Fig. 3. The reduced and alkylated large polypeptides (~9 kDa) of Ber e 1 eluted before the intact protein (the elution volume indicated with L in Fig. 3), whereas the reduced and alkylated small polypeptides (~3 kDa) eluted at an elution volume of approximately 70 ml (the elution volume indicated with S in Fig. 3). Considering a mass gain of 348.4 Da by alkylation of 6 cysteines, the reduced and alkylated large polypeptides were not expected to elute earlier than the parental protein on SEC. However, as SEC is dependent on hydrodynamic volume, the hydrodynamic volume of the reduced and alkylated large polypeptides of Ber e 1 could have caused them to behave differently from the compact, native structure of Ber e 1 (Alcocer et al., 2002). Apparently, the large polypeptides, which dissociated from the Ber e 1 complex upon heating, had a slightly more compact structure in comparison with the reduced and alkylated polypeptides, as they eluted later from the SEC column, at a comparable elution volume to that of intact Ber e 1 protein.

As already mentioned, SEC peaks 2 and 3 both showed single protein bands of approximately 3 kDa on SDS–PAGE. The elution volume of the first of the two peaks corresponded to the elution volume of the reduced and alkylated small polypeptides of Ber e 1, as can be seen in Fig. 3. This peak thus likely represented the dissociated small Ber e 1 polypeptides. The presence of the third SEC peak, containing peptides with molecular masses of approximately 3 kDa, could not be explained.

Heating of Ber e 1 at pH 5.0 (20 min, 120 °C), in contrast to the results at pH 7.0, did not show any changes in the protein. No insolubility was observed upon heating and on SEC the protein eluted similar to the native protein (Fig. 4). Also after prolonged heating (till 4 h), no changes in the SEC elution profile between native protein and heated protein could be observed (data not shown). As Ber e 1 isoforms have iso-electric pH values of 5.5 and higher, with the most predominant isoforms having pI values of around 8 in the used protein preparation (van Boxtel et al., 2006), at pH 5.0 all isoforms are expected to have a net positive charge. At pH 7.0, which is closer to the (main) pI of the protein preparation, the Ber e 1 isoform pool likely has a low net charge. These apparent differences in charge could have caused the differences in aggregation behaviour, as more charges are expected to induce more repulsion and thereby less aggregation and precipitation. Thus, although the denaturation temperatures of the protein at pH 5.0 and pH 7.0 are comparable and approximately 110 °C, the effects of heating on the aggregation behaviour of Ber e 1 differ greatly with the pH value.

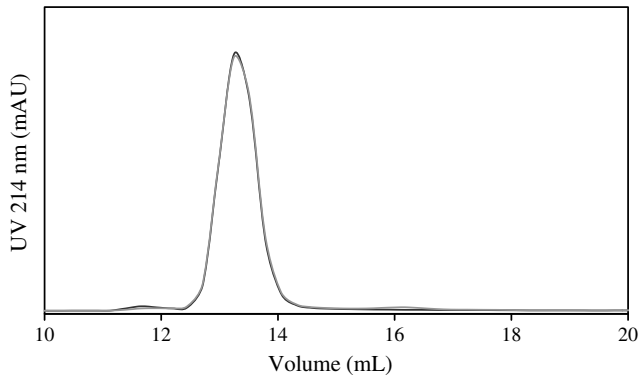

Fig. 4. Size exclusion chromatograms of soluble Ber e 1 in buffer pH 5.0 before (black line) and after (gray line) heating at 120 °C for 20 min.

When Brazil nuts are used as an ingredient in, for example, cakes, the (crushed) nuts are usually mixed with the dough and distributed over the whole food product. The baking of such products generally implies heating temperatures ranging from 180 °C to 230 °C. Despite these relatively high temperatures, due to the water content in the crumb of these products, the temperature inside these crumbs will not exceed 100 °C. As a result, Ber e 1 situated only at the outside of a food product can possibly denature during heat processing. The majority of Ber e 1, however, will be present in the crumb of the products, where the lower temperature will not induce the protein to denature.

In conclusion, in aqueous solutions with pH values between 5.0 and 7.0, a temperature exceeding 110 °C is needed to denature Ber e 1. The denaturation temperature of Ber e 1 in Brazil nuts could, because of the low water content, is even higher than the temperature measured (Gekko & Timasheff, 1981). When using common heat processing methods, the largest part of foods normally do not reach temperatures > 100 °C. This implies that Ber e 1 will always be consumed (mainly) in its native structure, having an intact immunodominant conformational epitope, and a high stability to pepsin digestion (Moreno et al., 2005). As a result, common heat processing techniques are not likely to affect the digestibility of Brazil nuts and its concomitant IgE binding capacity after digestion.

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