

Ber e 1 protein: the versatile major allergen from Brazil nut seeds

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Received: 4 October 2011 / Accepted: 9 December 2011
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Abstract Due mainly to its extremely high content of sulphur amino acids, Ber e 1 protein, the major allergen from Brazil nut, has attracted much scientific and press attention. Ber e 1 was the main target protein in early biotechnology transgenic work, in early processing studies of plant storage proteins, in plant vacuolar targeting studies and as the main protein in early nutritional supplementation experiments. Ber e 1 was also one of the first food allergens to be unintentionally transferred from one plant to another and was involved in the first reported case of systemic allergic reaction caused by a food allergen transferred in semen. In this review, many of the Ber e 1 unique biotechnological and structural functions are discussed with a particular emphasis on its use as model protein for studies of intrinsic allergenicity of food proteins.

Keywords 2S Albumin · Allergenicity · Food allergen · Supplementation

The biology of Ber e 1

Brazil nut (BN) trees (*Bertholletia excelsa* H.B.K.) belong to the Lecythidaceae family in the order Ericales. They are 23–46 m tall trees with branches and fruits located near the top, indigenous to the Amazon region (Mori 1992; Mori and Prance 1990). The mature fruit can weigh up to 2 kg and contains 8–24 seeds. The seeds of BN, which are harvested mostly from wild trees, can take up to 14 months to develop after flowering (Sun et al. 1987). The requirement of particular large-body bees for cross-pollination restricts the production of seeds to pristine forests (Nelson et al. 1985). Although BN plantations have been tried, their production is low and economically challenging (Mori and Prance 1990). The world annual seed production varies but it is estimated to be between 20,000 and 40,000 tons of which Bolivia accounts for about 50, Brazil 40 and Peru 10% (Mori 1992; Mori and Prance 1990). The BN seeds contain 15–17% protein by fresh weight or about 50% protein by dry weight of the defatted flour (Antunes and Markakis 1977). Storage proteins are major components of seeds and are expressed during seed development, stored as protein bodies in endosperm, cotyledons or other tissues of the embryo (Ampe et al. 1986). The total protein of BN can be fractionated into three major classes of proteins, the 11S and 7S globulins and the 2S albumins (Sun et al. 1987). The BN water soluble 2S albumin fraction (sedimentation coefficient of $S_{20,w}$ of two), also known as Ber e

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1, comprise about 30% of the total protein and are exceptionally rich in sulphur amino acids (about 30% methionine and cysteine) (Antunes and Markakis 1977; Sun et al. 1987). In this respect Ber e 1 variants are unique among the plant 2S albumins and are potentially one of the richest food sources of the sulphur containing amino acids, hence, an important target for nutritional studies (Antunes and Markakis 1977).

Ber e 1 and nutrition

In many parts of the world a significant portion of the protein in the diet comes from legumes (Antunes and Markakis 1977). The nutritional value of important grain legumes [e.g. beans (*Phaseolus vulgaris*), broad beans (*Vicia faba*) amongst others] used for human and animal consumption is limited predominantly by its unbalanced essential amino acid composition. In the particular case of legumes, methionine and cysteine are the major limiting essential amino acids (Demidov et al. 2003). Early protein supplementation studies in animals have shown that mixing bean proteins with BN proteins increases the protein efficiency ratio (PER) from 1.53 to 2.42 (2.5 max for casein) (Antunes and Markakis 1977). With the advent of recombinant DNA technology in the 1990s and the need to optimise the plant transgenic approach, two main biotechnology strategies were independently followed by several teams to correct the nutritional value of deficient crops: (i) engineering the protein composition by increasing the proportions of Met-rich endogenous or foreign proteins, and (ii) engineering amino acid biosynthetic pathways leading to increased amounts of total seed methionine (Demidov et al. 2003; Müntz et al. 1998). For the former, the gene encoding methionine-rich 2S storage proteins mainly from BN were transferred to beans (*Phaseolus*) (Aragao et al. 1999), tobacco (*Nicotiana tabacum*) (Altenbach et al. 1989), canola (*Brassica napus*) (Altenbach et al. 1991, 1992b), thale cress (*Arabidopsis thaliana*) (Conceição et al. 1994), soybean (*Glycine max*) (Townsend and Thomas 1994), alfalfa (*Medicago sativa*) (Tabe et al. 1995) lupin (*Lupinus*) (Müntz et al. 1998), potato (*Solanum tuberosum*) (Tu et al. 1998) and field bean (*Vicia nabornensis*) (Pickardt et al. 1995; Saalbach et al. 1994, 1995a, b). In general the reproducibility of the system for stable

transformation and regeneration of the transgenic plants varied greatly amongst cultivars although in some seeds, where the 2S albumin from BN was employed, a methionine content of ~80% of the Food Agriculture Organisation (FAO) standard for nutritionally balanced food protein was achieved (Saalbach et al. 1995a, b). Although precluded by its allergenicity and hence never to become a commercial product (as discussed later), the initial successes obtained with stable transgenic plants expressing Ber e 1 opened new fields of investigation on fundamental processes taking place during maturation, transport and allergenicity of plant storage proteins.

The biochemical characterisation of Ber e 1

The molecular mass of the native 2S sulphur-rich albumin is ~12 kDa when analyzed by gel filtration methods and by sucrose gradient sedimentation (Sun et al. 1987). The purified sulphur rich protein, when reduced by 2-mercaptoethanol and analyzed by SDS-PAGE, produced two low-molecular-mass polypeptides, ~9 and ~3 kDa, suggesting that the polypeptides were associated through disulphide bonds (Ampe et al. 1986; Sun et al. 1987). The 2S albumins are encoded by a multigene family that, when analysed by isoelectric focusing, showed over 20 isoforms. The major polypeptide amounting to about 80% of the total 2S albumin isoforms showed an isoelectric point of pH 6.4 (Sun et al. 1987). Amino acid and nucleotide sequences of several other isoforms have been determined (Altenbach et al. 1992a; Altenbach et al. 1987; Ampe et al. 1986).

The biosynthesis of the mature 2S albumins in the BN seed is developmentally regulated. The larger 18 kDa precursor is detected at earlier stages in the seeds about 8–9 months after flowering. This phase coincides with the disappearance of the liquid endosperm and the filling up of the seed coats with embryos. For BNs, which require 12–14 months for seed development, this time period represents a mid-maturation stage. It differs from the abundant seed proteins in other plants as it is synthesized, extensively post-translationally processed and then stored in protein bodies in the hypocotyls of the embryos rather than in the cotyledons or endosperm (Sun et al. 1987). This highly regulated accumulation of storage proteins is restricted to the latest stages of seed development,

Vacuolar targeting sequences in Ber e 1

such as *Vicia narbonensis* during heterologous expression in plants, further suggested that a vacuolar targeting sequence might be present (Saalbach et al. 1994). In plants, vacuolar targeting information is located in the N- or C-terminal propeptides or within the mature structure of the proteins (Kirsch et al. 1996). By fusing the C-terminal peptide sequence of the BN 2S albumin to the yeast invertase gene and observing the vacuolar deposition of the enzyme in mesophyll cells of transgenic tobacco, it has been shown that in the 2S albumin vacuolar targeting is mediated in vivo by the four amino acids of the C-terminal propeptide and the adjacent 16 amino acids of the mature portion (Kirsch et al. 1996; Saalbach et al. 1994). Deletion of the C-terminal four amino acids (IAGF) resulted in complete secretion of invertase rather than vacuolar retention (Saalbach et al. 1996). In subsequent work, this terminal peptide was shown to interact in vitro specifically with a putative 80 kDa protein vacuole receptor present in fractions enriched in clathrin-coated vesicles from developing pea cotyledons (Kirsch et al. 1996). This receptor does not bind *Arabidopsis* 2S albumin C-terminus suggesting that vacuolar targeting information may reside in different structures even among related proteins (Saalbach et al. 1996). Interestingly no retention has ever been observed inside yeast cells indicating that in yeasts different signals are used for targeting to vacuoles (Saalbach et al. 1996).

The 3D structure of Ber e 1

Early amino acid sequence comparison has shown that plant 2S albumins belong to the large prolamin

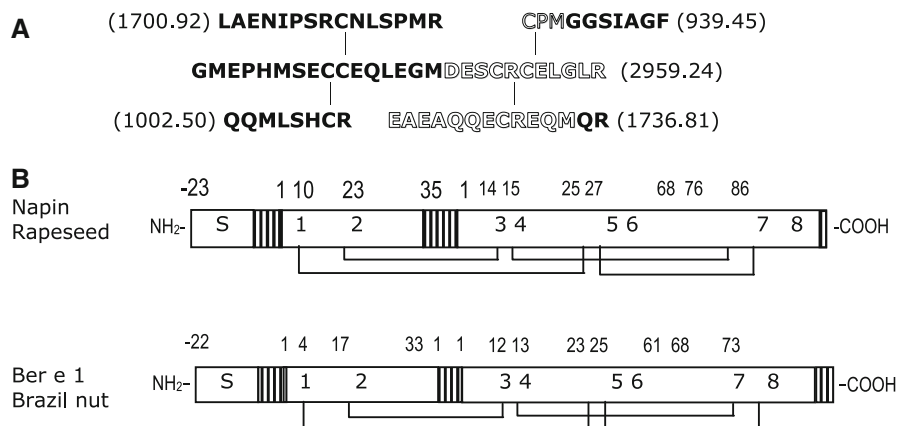
superfamily of proteins and may have evolved from a single ancestral gene consisting of three short related regions with limited sequence homology (Kreis and Shewry 1989; Kreis et al. 1985). Although the members of this large superfamily of storage proteins differ widely in their amino acid sequences, they share a conserved skeleton of eight cysteine residues: C-X_y-C-X_z-CC-X_j-CXC-X_k-C-X_r-C (Egorov et al. 1996; Shewry et al. 2002). This particular skeleton appears as a common and frequently used structure in plant proteins. This framework may therefore serve to evolve different functions from a single sequence scaffold by adding in a combinatorial way specific functional elements (Jose-Estanyol et al. 2004). Despite, or, due to the conserved disulphide connectivity, the 3D structure of 2S albumins share the same structural domain of other protein families such as plant lipid transfer proteins and trypsin-alpha amylase inhibitors (Fig. 2b). In the latest version of Pfam (Pfam.sanger.ac.uk) the combined family *Try-alpha_amyl* (PF00234) comprised 3346 sequences from 266 species and 49 structures. In the protein structural database Scop this family is classified as Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily and are characterised by their conserved structure of antiparallel bundles of four α -helices held together by four disulphide bonds in a distinctive right-handed superhelix fold (Scop). Recently it has been speculated that this particular three-dimensional structure common to all 2S albumins may represent promiscuous protein scaffolds with related function in plant defence (Franco 2011).

Recombinant protein work

In order to study the three dimensional structure of *Ber e 1*, the expression of the recombinant 2S albumin as fusion product was directed to the secretory pathway of the methylotrophic yeast *Pichia pastoris* (Alcocer et al. 2002). The recombinant protein was secreted at high levels and showed similar fold and thermo/chemical resistance to the wild-type natural protein as shown by its circular dichroism (CD) profile (Alcocer et al. 2002; Murtagh et al. 2003). The protein fold was further confirmed by Q-ToF MS disulphide mapping using sequences produced from protease and cyanobromide-digested r*Ber e 1* (Fig. 2a) (Alcocer et al. 2002). The sequencing connectivity showed perfect agreement with the disulphide map originally described in other species (Egorov et al. 1996) (Fig. 2).

Through the *Pichia* expression system, ¹³C- and ¹⁵N-labelled recombinant proteins have been produced and the backbone ¹H-, ¹³C- and ¹⁵N-NMR resonances of *Ber e 1* have been completely assigned for all residues. Chemical shift index, NOE data, ³J_{HNH α} coupling constants obtained from HNHA experiments and backbone torsion angles have been determined. The ¹H, ¹³C and ¹⁵N chemical shift values of *Ber e 1* have been deposited at the BioMagResBank database (<http://www.bmrb.wisc.edu>) under accession number 6529 (Tengel et al. 2005). Based on the disulphide connectivity and NOE constraints a preliminary experimental model with the following secondary elements were predicted: helix 1 (residues 6–14), helix 2 (residues 17–30), helix 3 (residues

Fig. 2 **a** *Ber e 1* disulphide mapping showing the amino acid sequences and their connectivity after protease and cyanobromide digestion (Q-ToF MS) (Alcocer et al. 2002). **b** Comparison of the patterns of disulphide bonds formed by 2S albumins. Cysteine residues are numbered 1–8 (Egorov et al. 1996; Kolivas and Gayler 1993; Rico et al. 1996)



44–56), helix 4 (residues 63–75) and helix 5 (residues 81–101).

A model of Ber e 1 (Fig. 3) using SFA-8, a 2S albumin from sunflower (SFW) seeds (PDB entry 1s6d), as a template was created by Swiss-model (Schwede et al. 2003). The model shows good agreement with the disulphide pattern of 2S albumins and the secondary elements of Ber e 1 identified by the earlier NMR study (Tengel et al. 2005). A high resolution solution structure of Ber e 1, together with the protein backbone dynamics, extracted from ^{15}N -spin relaxation, is in its final analysis stage and will be submitted for publication soon.

Biophysical properties of Ber e 1

pH, chemical and thermal resistance

The main biophysical characteristics of plant 2S albumins, discussed later in the “[The allergenicity controversy](#)” section, are their proteolytic, thermal, acid and chemical stabilities. Ber e 1 is no exception. In a series of articles the native and recombinant Ber e 1 proteins have been compared under stringent conditions and the CD spectra showed very similar profiles at different pHs (Murtagh et al. 2002, 2003). Temperatures over 62°C were necessary to start

unfolding both *Ber e 1* proteins at pH 2.2, further demonstrating the high resistance of these proteins to acidic pH. At 95°C, both recombinant and native proteins were significantly destabilised although the transition was not complete. Upon cooling, the denaturation was largely reversible. The native and recombinant proteins were also very resistant to chemical-induced unfolding. No changes in the CD secondary structure were observed up to 4.5 M guanidinium chloride and the protein still exhibited a significant amount of secondary structure in 6 M guanidinium chloride (Murtagh et al. 2002, 2003).

Proteolytic resistance

Regarding stability to proteases, and when compared to the total proteins extracted from the BN seed, Ber e 1 is the water-soluble protein least susceptible to digestion by simulated gastric fluid (SGF). In the model employed, the Ber e 1 fraction from BN total protein extracts persisted for up to 30 min, whereas other protein components of BN were completely hydrolysed by pepsin after a few seconds (Murtagh et al. 2003) (Fig. 4). In a complementary approach, native Ber e 1 before and after heating (100°C/20 min) was subjected to a gastric and duodenal digestion system. Neither prior heating of Ber e 1 nor the presence of the physiological surfactant phosphatidylcholine

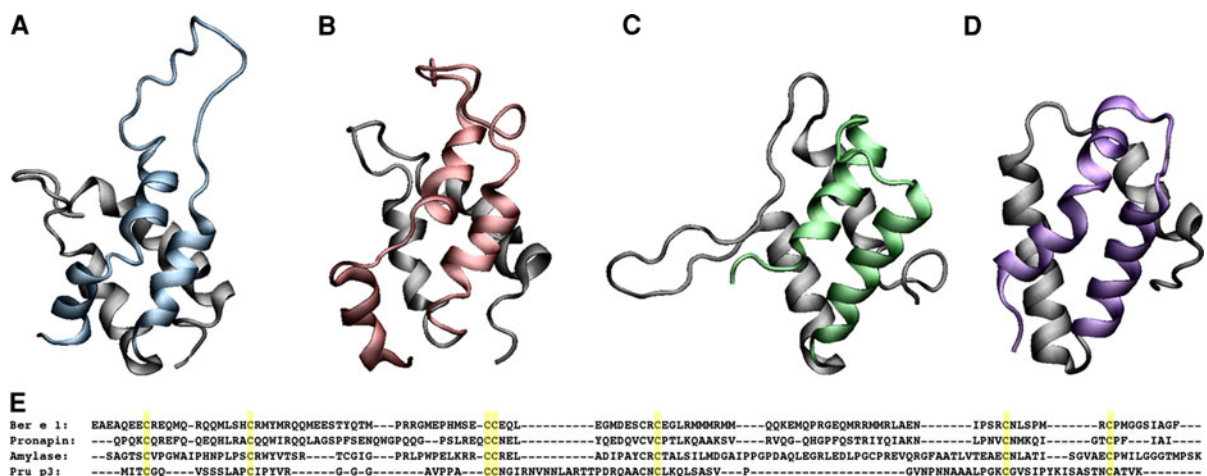


Fig. 3 Three-dimensional representations of **a** a model of Ber e 1 generated by Swiss-Model (Schwede et al. 2003) using SFA-8 (PDB accession number 1s6d) as a template. **b** NMR solution structure of pronapin, a 2S albumin from rapeseed (PDB accession number 1SM7). **c** NMR structure of bifunctional hageman factor/amylose inhibitor from maize (PDB: 1BEA).

d Crystal structure of Peach Pru p 3, a nonspecific lipid transfer protein (PDB: 2B5S) The unstructured *N*- and *C*-terminal sequences in the structures in **a–d** have been shortened in all structures for clarity. **e** Primary structure alignment of the proteins from **a–d**

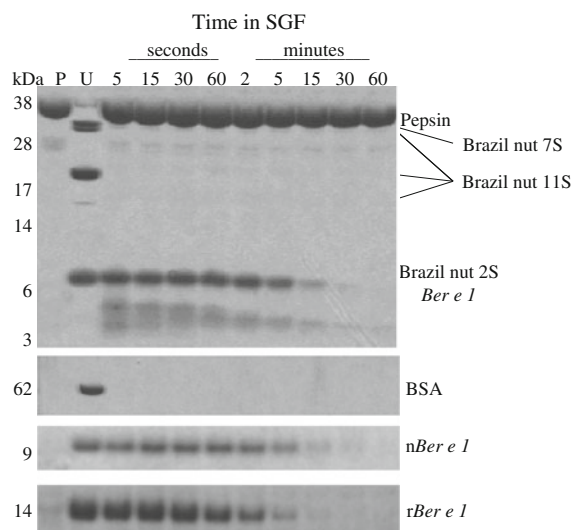


Fig. 4 In vitro digestibility of native and recombinant proteins in SGF. *P* SFG alone, *U* protein sample untreated with SGF

affected the pattern of proteolysis (Moreno et al. 2004, 2005; Moreno 2006). After extensive in vitro digestion the resulting small structures still contained potentially active B- and T-cell epitopes (Moreno et al. 2005). By reducing and alkylating the two subunits, a complete proteolytic digestion has been achieved, within 30 s (Koppelman et al. 2005). This further demonstrated the importance of the tight disulphide skeleton to the protein stability. Overall, these results are in agreement with similar results obtained with other members of the 2S albumin family as discussed elsewhere (Moreno 2006; Murtagh et al. 2003). Altogether they suggest that Ber e 1 and its polymorphic variants are likely to be the only intact BN proteins that come into contact with cells of the immune system found in the gut mucosa and that the protease resistance properties can be mimicked by the recombinant protein in vitro.

Lipid, metal-binding properties

The conserved disulphide connectivity and compact 3D structures of 2S albumins share the same structural domain of plant lipid transfer proteins. Our preliminary work on lipid binding activity has established that Ber e 1 can interact with the hydrophobic fluorescent probe ANS (unpublished work). In addition, NMR spectra of ^{15}N -labelled Ber e 1 show chemical shift perturbations after addition of a

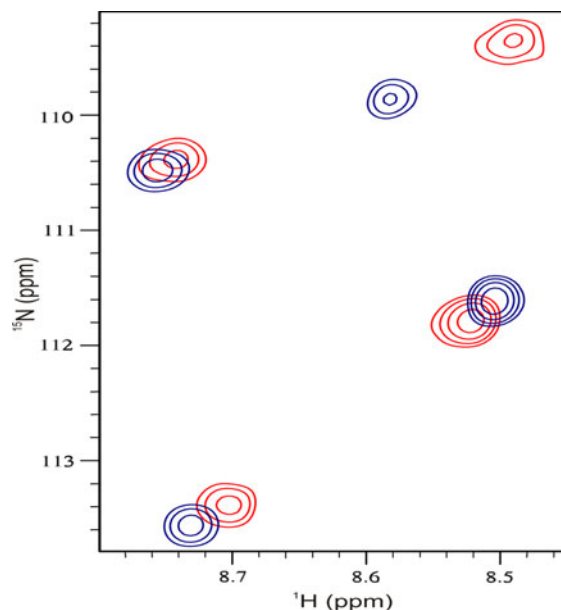


Fig. 5 Expanded region of ^1H - ^{15}N correlated NMR spectra in absence (*blue*) and presence (*red*) of BN lipids. Each cross peak represents one amino acid of Ber e 1. The perturbations of the NMR peaks in presence of lipids imply a direct interaction with one or several of the lipids from BN

mixture of BN lipids (Fig. 5). This strongly suggests that one or several lipid types from the BN show the ability to directly interact with Ber e 1. As discussed later, the observation that the purified Ber e 1 protein is unable to elicit an allergic response in mice and needs a lipid component of the BN seed for the activity (Dearman et al. 2007) suggests that this putative lipid binding property may be important for the allergenicity of Ber e 1. However, Ber e 1 is not the only 2S albumin shown to be able to interact with lipids. Sin a 1, a 2S albumin from mustard seeds, has also been shown to display affinity for a spectrum of different phospholipids (Onaderra et al. 1994). Interestingly and fulfilling the putative storage function of the 2S albumins, Ber e 1 is not only a sulphur, nitrogen and carbon source for the germinating seed but may also store metals. Although the specific case for Ber e 1 has not been made, it has been reported that soluble proteins from BN seeds bind Fe^{2+} , Mn^{2+} and Zn^{2+} (Naozuka and Oliveira 2007). Furthermore, during expression of recombinant Ber e 1 in large fermenters it has been noticed that supplementary metal ions do bind Ber e 1 (A. Brans, University of Liege, Belgium, personal communication).

Immunoreactivity and allergenicity of Ber e 1

B and T cell epitopes

IgG originating from rabbits immunized with recombinant Ber e 1 using normal Freund's adjuvant regime reacted positively both with the native and corresponding recombinant protein. Low or no cross reactivity with related proteins has been observed (Murtagh et al. 2003). Patients with a positive skin-prick tests (SPT) and positive labial/oral challenge to BN reacted strongly with Ber e 1 (Murtagh et al. 2003) confirming early work (Arshad et al. 1991; Nordlee et al. 1996; Pastorello et al. 1998) that the 2S albumin (Ber e 1) is the major allergen in this nut. In addition, human-specific IgE ELISAs with these selected patients showed little discrimination between recombinant and native Ber e 1. ELISA results largely reflected the clinical RAST measurements (Murtagh et al. 2003). Taken together these results corroborate the potential value of employing purified recombinant proteins for diagnostic purposes. The immune recognition of Ber e 1 by IgE from sensitised patients has been accessed by several different techniques and summarised in Fig. 6.

Regarding epitopes, sera from BN allergic subjects were screened by ELISA and dot blot assay against 14 aminoacids long peptides, offset by seven, encompassing the entire Ber e 1 mature protein sequence. Only one linear peptide derived from the Ber e 1 sequence bound IgE antibodies consistently by both

ELISA and dot-blot assay. This sequence, (QMQRQ QMLSHCRMY), starts at amino acid 11 within helix 1 of the Ber e 1 small subunit (Fig. 6) (Alcocer et al. 2004). Using recombinant chimeric proteins, where the domains of Ber e 1 have been replaced by domains from related 2S albumins, and sera from 18 well-characterised BN allergic patients, the structural IgE epitope of Ber e 1 was mapped to a helix3-turn4-helix4 region (Alcocer et al. 2004). The same structural region has been previously reported as the immunodominant region in related 2S albumins from different sources using different techniques. The cumulative data demonstrate that in the majority of BN-sensitive individuals Ber e 1-specific IgE is predominantly directed against either longer or non-linear epitopes. Therefore, for Ber e 1, a linear epitope appears to be immunologically of minor importance (Alcocer et al. 2004).

Although experimental determination of T-cell epitope peptides of Ber e 1 from sensitised BN allergic individuals is not yet available, potential T-cell epitopes from random donors have been determined (Stickler et al. 2003). This in vitro assay employed dendritic cells co-cultivated with synthetic peptides and CD4(+) T cells from random donors. T-Cell proliferation was then measured and immunodominant CD4(+) T-cell epitopes of Ber e 1 mapped (Stickler et al. 2003). Bearing in mind the large patient variation and different methodologies, the structural and linear immunodominant regions of Ber e 1, as shown in Fig. 6, do seem to overlap.

Small subunit:

QEECREQMQRQQMLSHCRMYMRQQMEEStyqtm
QMQRQQMLSHCRMY (1)
RQQMLSHCRMDMRQQ (2)

Large subunit:

RRGMPEPHMSECCEQLEGMDSECRCEGLRMMMRMMQKEMQPRGEQMRRMMRLAENIPSCRRCRGMEPHMSECCEQ
LEGMDSECRCEGLR (2)
LRMMMRMMQKEMQ (2)
EGLRMMMRMMQKEMQPRGEQMRRMMRLAENIPSRCNL (5)
CRCEGLRMMMRMQ (4)
MQPRGRQMRRMMRLA (2)
MRLAENIPSRCNL (3)

Fig. 6 Ber e 1 primary aminoacid sequence of the linear and structural IgE epitopes. The primary Ber e 1 sequence is in **bold**, peptide sequence in italic and T-cell peptides underlined. (1) Recognised by IgE-ELISA and dotblot, 18 patients (Alcocer et al. 2004); (2) potential CD4(+) T-cell epitope, from 91

random patients, underlined (Stickler et al. 2003); (3) recognised by IgE-dotblot only, one patient (Alcocer et al. 2004); (4) recognised by IgE-ELISA and dotblot, one patient (Alcocer et al. 2004). (5) structural epitope (Alcocer et al. 2004)

Dendritic cell (DC) and T-cell work

The intrinsic allergenic properties of recombinant Ber e 1 were investigated by monitoring cell surface phenotype and cytokine production in co-culture of murine DC and T cells (Kean et al. 2006) the central orchestrators of the immune system. By comparing the major allergen Ber e 1 with a “weak” or non allergen from the same 2S albumin family (SFA8 from SFW) it has been shown that murine DCs exposed to SFA8, not Ber e 1, induced the production of cytokines (IL-12 p40 and TNF- α) found mostly in T cell type 1 interactions (Th1). Interestingly, these Th1 interactions were triggered by a mechanism independent of recognition by Toll receptors (TLRs) as shown by cells from animals that have had their essential Toll transducer gene (MyD88) knocked out (Kean et al. 2006). Depending on the mouse strain used, increased expression of surface markers such as MHC class II and co-stimulatory molecules such as CD40, CD80, and CD86 were associated with exposure to the non allergen, but not Ber e 1. Hence, unexpectedly, the major allergen Ber e 1 has shown an immature DC phenotype in both mouse strains. In co-culture experiments with activated T cell, mouse DCs exposed to both proteins induced T cells to produce Th1 cytokine (IFN- γ) but only Ber e 1 could induce significant production of Th2 cytokines (IL-4 and IL-5). Likewise, analysis of key transcription factors that modulate a Th1/Th2 interaction shows increased T-bet (a Th1 type) with respect to both allergens, but also GATA-3 (a Th2) with respect to Ber e 1 (Kean et al. 2006).

Altogether these results corroborate epidemiological observations that countries with high consumption of SFE seeds have low prevalence of SFA8 allergic cases whereas Ber e 1 cases are widely reported with consumption of BN. The data also suggest that the ability of food antigens to induce IL-12 production may contribute to the absence of IgE-specific Abs. Furthermore, these data suggest that Ber e 1 acts as an allergen, at least in part, due to its failure to induce a mature co-stimulatory DC phenotype and pro-inflammatory cytokine production. Within this environment DCs exposed to Ber e 1 are unable to promote the development of a polarized Th1 response as do SFA8. Rather, Ber e 1 acts to promote an immature Th2 environment and, potentially, the development of an allergic response (Kean et al. 2006). How atopic and

non atopic individuals will deal with this Th2 environment and the role of other potential immunomodulator cells remain to be clarified.

Clinical history

Since the late 1920s, there have been reports of manual workers in the oil milling industry (castor and cotton seed) or in the baking industry (“baker’s asthma”) becoming sensitised by inhalation of air-borne particles (Atkins et al. 1988; Spies et al. 1951; Spies 1974). During the 1970–1980s, the major allergens implicated in those allergies were characterised as belonging to the family of the 2S albumin/amylase–protease inhibitor proteins (Spies 1974; Youle and Huang 1981) and references therein. Regarding BN specifically, Hide reported four cases of anaphylaxis to BN (Hide 1983). The same group, together with Arshad and co-workers reported 12 cases of allergy in a selected atopic population from the Isle of Wight (UK) and warned of the potent antigenic components in BN seeds (Arshad et al. 1991).

First allergen transferred from one plant to another

Although the case for the allergenicity of the 2S family of proteins and BN in particular has been made, it was not until the methionine rich Ber e 1 was engineered into soybean cultivars and sensitised patients presented positive SPT that alerted media attention. Historically therefore, Ber e 1 was the first food allergen transferred by transgenic techniques from one plant to another, triggering strong international debate (Nordlee et al. 1996). In their landmark paper, Nordlee et al. (1996) showed that 8 out of 9 sera from BN allergic patients recognised 2S albumin as a major IgE binding protein. The IgE from these patients also bound to Ber e 1 from transgenic soybeans. SPT with an extract of transgenic soybean were positive in three allergic subjects while the soybean background remained non-responsive.

In subsequent clinical work using western blot, BN allergic patients, who manifested an anaphylactic reaction when compared against a group who showed no symptoms, Ber e 1 was unequivocally shown to be the allergen underlying the clinical anaphylactic reactions to BN seeds (Pastorello et al. 1998).

With some fatalities already reported by ingestion of BN (Bock et al. 2001), most of the work on Ber e 1

towards the end of the 1990s seemed to suggest that this major allergen from BN was a high risk protein to individuals potentially sensitised with BN seeds (see also the allergenicity controversy at the end of this section).

First case of systemic allergic reaction caused by food allergen transferred in semen

The high risk to sensitised individuals is illustrated further by a clinical case in which a 20-year-old woman allergic to BN developed widespread urticaria and mild dyspnoea after intercourse with her boyfriend who had earlier consumed BNs. SPT with the boyfriend's semen after BN consumption confirmed significant reactivity whereas a sample before nut consumption was negative. This is the first case of a systemic allergic reaction caused by food allergens transferred in semen (Bansal et al. 2007).

The diagnosis of BN allergy

BN allergy occurs in highly atopic individuals of any age with a strong family history of atopy. Although double-blind, placebo-controlled, food challenge (DBPCFC) is considered to be the 'gold standard' method for diagnosis of food allergens, generally this method is time consuming, costly and may introduce an unwanted risk of a serious anaphylactic reaction. The diagnosis of BN allergy therefore is usually made on the basis of history and examination at the time of a reaction, with confirmation by investigative procedures such as SPT, measurement of serum-specific IgE and challenge procedures (Ridout et al. 2006).

By analysing children and adults ($n = 56$) with a history of an allergic reaction to BN and comparing questionnaire, SPT, specific IgE and oral challenges, Ridout et al. (2006), have concluded that a combination of history, SPT and serum-specific IgE was adequate in achieving a diagnosis in the majority (77%) patients with suspected BN allergy. However, a doubtful history with SPT (skin wheal size between 1 and 5 mm), or a low serum-specific IgE (less than 3.5 kU/l) may require an oral challenge to help determine the risk of a BN allergic reaction (Ridout et al. 2006). These results complement earlier findings by other groups (Pastorello et al. 1998) and suggest that serum determination of specific Ber e 1 antibodies

may help on the diagnosis. Table 1 show a summary of most of the clinical tests involving Ber e 1 or BN extracts.

Biotechnology and diagnosis

The move towards miniaturization of diagnostic testing is a popular one, not just for economic but also functional reasons. In the past 10 years, the ability to produce microarrays has provided the technology for monitoring, on a solid based media of the size of microscope slide, protein functionality, protein interactions and enzymatic activity amongst others (Renault et al. 2007). Essentially protein microarray represents a multi-analyte solid-phase immunoassay, where proteins are immobilised onto the solid surface and micro-quantities of the serum samples are then incubated under standard conditions (Harwanegg et al. 2003, Renault et al. 2011). When diagnosis is based on pure allergen molecules produced either by recombinant expression of allergen encoding cDNA or by purification from natural sources, this technology is termed component resolved diagnostic (CRD) (Valenta et al. 2007). CRD as a concept was greatly aided by recent availability of pure proteins and is becoming increasingly important for identification of allergenic reactivity profile of a patient along with their potential cross-reactivity interactions. This is particularly important for establishment of geographic patterns of sensitisation and the development of strategies for allergen immunotherapy. Whether Ber e 1 could be used in immunotherapy or as marker of severity of allergic disease as recently described in other CRD systems (Nicolaou et al. 2010) remains to be demonstrated.

The allergenicity controversy

Although treated as a well established fact, in reality the allergenicity of Ber e 1 is also unusual and should be further explored. There are, however, many angles for this debate. From the structural point of view, extensive studies on plant 2S albumin family of proteins have agreed that their compact, α -helical, disulphide-bridged rigid structure confers the high chemical and proteolytical stability of the family as shown for Ber e 1. Consequently, these proteins are

Table 1 Summary of the clinical tests involving Ber e 1 or BN extracts

Extract format	<i>n</i>	Main allergy	Test	Age	Remarks	References
BN extract	4	BN	Rast	1–3	First report acute reaction to BN and suggestion BN as potent allergen	Hide (1983)
BN extract	12	BN			Seven anaphylaxis	Arshad et al. (1991)
	62		History, SPT	1–53		Ewan (1996)
Transgenic soybean expressing Ber e 1	3	BN	SPT		3/3 SPT positive reaction to soybean expressing Ber.	Nordlee et al. (1996)
	9		Western blot		Soybean only = negative	
	9		Rast		8/9 positives to Ber e 1	
BN extract	1	BN	SPT, Rast		vomiting, diarrhoea and loss of consciousness caused by oral contact with the BN, SPT+ to BN, Rast Class 4 to BN	Bartolomé et al. (1997)
BN extract	1	BN	SPT rast	11	Oral syndrome of allergy followed by laryngeal angioedema, SPT+ (15 × 22 mm), Rast 0.86 kU/l	Dutau et al. (1998)
BN extract	21 (11)	Ber e 1	SPT, CAP Oral chall	5–54	CAP+, 11 anaphylaxis and 10 asymptomatic BN allergic patients. Ber e 1 marker for severity.	Pastorello et al. (1998)
TN	54	BN			4/54 nut allergic were BN specific	Sicherer et al. (1998)
BN extract	1	BN, AN, SFW, HN	SPT, rast, Western blot	31	Pharyngeal itching, lip swelling, dysphonia, dyspnea, wheezing, and generalized macular exanthema. BN Rast Class 2, HN + SFW class 3	Borja et al. (1999)
BN, PN, HZ	731		CAP	1–65	Similar pattern of specificity to the PN and hazelnut	Pumphrey et al. (1999)
BN extract	5		SPT, CAP, Total IgE	<6	Most of SPT negative for other nuts	Bourrier et al. (2001)
BN extract	1	BN	SPT Western blot	15	Sensitised to a non 2S BN protein	Asero et al. (2002)
	1000	PN, TN	SPT		162/1,000 PN or nut allergic patients (16%) showed their strongest reaction to BN	Clark and Ewan (2003)
PN extract	8	BN, PN	Western blot ELISA	1–19	Rast >2 to PN Cross reaction PN, BN, HZ, CN	de Leon et al. (2003)

Table 1 continued

Extract format	<i>n</i>	Main allergy	Test	Age	Remarks	References
BN extract	10	Ber e 1	SPT ELISA Rast oral			Murtagh et al. (2003)
TN	1	BN, HN, WN	SPT, rast, Western blot	11	Cross reaction BN, HN, WN	Asero et al. (2004)
BN extract	56	Ber e 1	SPT (53) CAP (54) Oral (19)	4–83	Weal >6 mm or >class 3 confirm clinical reactivity. Less require oral challenge for diagnostic.	Ridout et al. (2006)
Semen	1	BN	SPT	20	Widespread urticaria and mild dyspnoea	Bansal et al. (2007)

BN Brazil nut, PN peanut, TN tree nut, SPT skin-prick test, HN Hazelnut, SFW sunflower, WN walnut, AN almond

expected to be stable in the harsh conditions of the gastrointestinal tract and able to cross the gut mucosal barrier to sensitize the mucosal immune system and/or elicit an allergic response (Moreno and Clemente 2008). Although the current annotation of modern databases supports this view, a quick look at the epidemiology of food allergies suggests otherwise. The plant 2S albumin and its family are ubiquitously represented in the plant kingdom (266 species—Pfam, 2011). Important food crops consumed by millions of people worldwide possess one or more members of the 2S albumin family, hence a high exposure to the family is observed. Nevertheless, comparatively, very few cases of food allergy linking 2S albumins have been reported. Soybean (*Glycine max*), for instance, is one of the largest world crops and possesses at least two 2S albumins. These two 2S proteins have been characterised and contain the distinctive disulphide structure of the class, are non-glycosylated and are very stable to temperature and chemical denaturants (Lin et al. 2004, 2006) as expected and similar to Ber e 1. However, despite the large exposure, proteins other than 2S albumins have been demonstrated as major allergens (www.allergen.org) in this species (Lin et al. 2006).

Another interesting and closely related example to Ber e 1 is SFA8, the 2S albumin from SFW seed. Countries with high consumption of SFW seeds, such as Spain (pipas), Germany or Greece, have a very low prevalence of SFA8 specific allergic cases (Kean et al. 2006). In the few reported cases, SFW seed

sensitization has been suggested to occur via inhalation rather than ingestion as found with the ownership of caged birds fed with SFW seeds and with high pollen counts in the workplace. As consistently shown, none of the intrinsic properties such as compactness, chemical stability, resistance to digestion, can differentiate Ber e 1 from SFA8 (Alcocer et al. 2002, 2004; Murtagh et al. 2003). Nevertheless, DCs were clearly able to drive the polarisation of the immune system making the distinction between the 2S proteins (Kean et al. 2006). These data suggest that other primary structural features that might affect DC uptake and processing may take priority on the determination of the intrinsic allergenicity of proteins. Current ongoing work on murine/human DC uptake, processing and up-regulation of the genes involved in this process may shed some light on this mechanism.

From the sensitisation point of view, it is clear from the early transgenic work for nutritional supplementation that Ber e 1 alone was the main protein recognised by allergic patients sensitised to BN proteins. However, so far it has not been shown that the purified Ber e 1 alone is able to sensitise naïve individuals. Early experiments in animals concluded that Ber e 1, when injected intradermally, did not induce specific IgG1 or IgE and when administered orally it induced tolerance (Melo et al. 1994). Indeed, the purified native or recombinant proteins are very poor immunogens and poor allergens in animal model systems (Dearman et al. 2007). Further, complex lipid components from the BN seed need to be present for

the sensitisation and IgE production in naive animals (Dearman et al. 2007). Preliminary and yet non-published work in mouse and human cells suggest that NKTs and in particular the involvement of CD1 receptor activation potentiate the immature Th2 environment created by Ber e 1 (M. Alcocer, unpublished results).

In summary, Ber e 1 is a small and unique plant allergenic protein with a long track record of unexpected results and of attracting media and scientific attention. Acting as a model protein it has featured in plant transgenic work, processing studies, in vacuolar targeting studies, nutritional supplementation experiments, food allergenicity studies and as a food allergen transferred in semen. Would the further understanding of the intrinsic allergenicity of Ber e 1 bring new insight into the allergenicity of common food proteins or based on its track record, bring yet again some more unexpected results?

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