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Characterization of mutants of a highly cross-reactive calcium-binding protein from *Brassica* pollen for allergen-specific immunotherapy

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ABSTRACT

The major turnip (*Brassica rapa*) pollen allergen, belongs to a family of calcium-binding proteins (*i.e.*, two EF-hand proteins), which occur as highly cross-reactive allergens in pollen of weeds, grasses and trees. In this study, the IgE binding capacity and allergenic activity of three recombinant allergen variants containing mutations in their calcium-binding sites were analyzed in sensitized patients with the aim to identify the most suitable hypoallergenic molecule for specific immunotherapy.

Analysis of the wildtype allergen and the mutants regarding IgE reactivity and activation of basophils in allergic patients indicated that the allergen derivative mutated in both calcium-binding domains had the lowest allergenic activity. Gel filtration and circular dichroism experiments showed that both, the wildtype and the double mutant, occurred as dimers in solution and assumed alpha-helical fold, respectively. However, both fold and thermal stability were considerably reduced in the double mutant. The use of bioinformatic tools for evaluation of the solvent accessibility and charge distribution suggested that the reduced IgE reactivity and different structural properties of the double mutant may be due to a loss of negatively charged amino acids on the surface. Interestingly, immunization of rabbits showed that only the double mutant but not the wildtype allergen induced IgG antibodies which recognized the allergen and blocked binding of allergic patients IgE.

Due to the extensive structural similarity and cross-reactivity between calcium-binding pollen allergens the hypoallergenic double mutant may be useful not only for immunotherapy of turnip pollen allergy, but also for the treatment of allergies to other two EF-hand pollen allergens.

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Introduction

The genus *Brassica* comprises a diverse group of more than 100 species including important crop plants, grown as vegetables, as sources of vegetable oil, as spices and increasingly also as sources of biodiesel. With the expansion of cultivation of *Brassica* crops their potential as allergen sources has become evident (Singh et al. 1995; Hemmer et al. 1997; Focke et al. 1998). Overall the prevalence of IgE-mediated allergy to *Brassica* species in pollen allergic patients seems to be low (7%, Hemmer et al. 1997), but among personal occupationally exposed to *Brassica* (*e.g.* plant breeders) the prevalence of sensitization was found to rise up to 44% (Hermanides et al. 2006).

The best characterized *Brassica* pollen allergens (Toriyama et al. 1995; Rozwadowski et al. 1999; Okada et al. 1999, 2000) belong to a protein family of highly conserved, cross-reactive calcium-binding allergens, which are not only contained in pollen of the genus *Brassica*, but also in pollen of taxonomically unrelated plants like trees, grasses and weeds (Valenta et al. 1998; Radauer and Breiteneder 2006). These allergens are small water-soluble proteins with a molecular weight of 8–9 kDa, which are characterized by the presence of two highly conserved Ca^{2+}-binding domains, termed EF-hands. Each of these calcium binding motifs consists of two alpha-helices connected by a loop which coordinates the calcium ions (Kawasaki and Kretsinger 1994).

Even though these calcium-binding allergens are recognized only by relatively few (*i.e.*, 10–20%) pollen-sensitized patients (Rossi et al. 2001; Mari 2003) their importance has been demonstrated by their potential to elicit strong allergic reactions (Niederberger et al. 1999) and their extensive cross-reactivity causing a phenotype of apparent polysensitization in allergic patients (Twardosz-Kropfmüller et al., 2010).

With the aim to develop vaccine candidates for immunotherapy we studied three *Brassica* two EF-hand allergen mutants regarding their IgE binding capacities and allergenic activities in patients sensitized to calcium-binding allergens and identified the mutant most suitable for specific immunotherapy. In addition a detailed characterization of the physicochemical and structural properties of the wildtype allergen and the double mutant and their immunogenicity was performed.

Materials and methods

Characterization of patients

Sera and blood samples from seven patients with a positive case history of IgE-mediated allergy to pollen from various unrelated plant species, IgE reactivity to commercially available extracts of rape (*Brassica napus*), timothy grass (*Phleum pratense*), birch (*Betula verrucosa*) and mugwort (*Artemisia vulgaris*) pollen, as determined by ImmunoCAP measurements (Phadia, Uppsala, Sweden), were analyzed in this study for initial IgE binding studies and basophil histamine release experiments after informed consent was obtained. For control purposes, blood samples from a non-allergic individual were analyzed. Demographic, clinical and serological data of these individuals are given in Table 1. Blood samples from additional 5 patients (A1–A5) containing IgE antibodies against two EF-hand pollen allergens and three non-allergic individuals (N1–N3) were used for comparing the IgE reactivity of the N- and C-terminally his-tagged wildtype and double mutant and for cellular experiments.

Expression and purification of recombinant proteins

Mutations were introduced into the cDNA of the allergen, which was originally designated Bra r 1 (DDBJ/EMBL/GenBank accession number D63153; Toriyama et al. 1995) and now has been renamed according to the Allergen Nomenclature subcommittee Bra r 5.0101 in the first (mu1), in the second (mu2) and in both calcium-binding sites (muW) as described (Okada et al. 1998; Fig. 1) using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The wildtype allergen and the mutated proteins mu1, mu2 and muW (double mutant) were expressed with a N-terminal hexa-histidine tag in *Escherichia coli* M15 and purified by Ni^{2+}-affinity chromatography (QIAGEN GmbH, Hilden, Germany). For large scale expression in *E. coli*, genes coding for the wildtype allergen and the double mutant with a codon-usage optimized for *E. coli* expression were synthesized (GenScript, Piscataway, USA) and inserted into the *NdeI*/*EcoRI* sites of plasmid pET-27b (Novagen, Darmstadt, Germany). The genes contained sequences coding for a C-terminal hexa-histidine tag. Their DNA sequences were confirmed by restriction analysis and sequencing of both DNA strands. *E. coli* BL21(DE3) (Stratagene, La Jolla, CA) were transformed with the plasmid constructs and grown in LB medium containing 30 μg/mL kanamycin at 37 °C under continuous shaking until an $OD_{600\,nm}$ of 0.6 was reached and protein expression was induced by addition of isopropyl-β-thiogalactopyranoside (Calbiochem, Merck, Darmstadt, Germany) to a final concentration of 0.5 mM for another 4 h. After harvesting of cells by centrifugation, recombinant proteins were isolated by Nickel affinity chromatography under denaturing conditions according to the manufactures protocol (QIAGEN). Purified proteins were soluble in PBS, their concentration was determined by Micro-BCA analysis (Pierce, Rockford, IL) and their purity was determined by SDS polyacrylamide gels (SDS-PAGE) and Coomassie blue staining under reducing and non-reducing conditions (Laemmli 1970).

Recombinant Aln g 4 and Phl p 7 were expressed in *E. coli* BL21(DE3) and purified by DEAE anion exchange chromatography

Table 1
Clinical and serological characterization of individuals.

Individual	Age (y)	Sex	Allergen sources	Symptoms	Therapy	Total serum IgE (kU/L)	Specific IgE (kUA/L) to			
							Rape *Brassica napa*	Timothy grass *Phleum pratense*	Birch *Betula verrucosa*	Mugwort *Artemisia vulgaris*
1	27	m	g, t, w, m	rc, ad	ah	141	0.78	27.1	7.4	4.0
2	26	m	g, t, w	rc	ah	140	2.32	25.9	5.2	4.4
3	34	f	g, t, w, a	rc, u	IT	168	3.88	66.6	10.8	14.9
4	45	m	g, t, w, m, a	rc, as	c	401	27.80	52.8	29.3	9.2
5	28	m	g, t, w, f	rc, as	ah	144	3.66	39.3	22.1	3.1
6	28	m	g, t, w, a	rc, as, u	β, IT	543	6.30	>100.0	33.3	5.7
7	23	m	g, t, w, a, f	rc, ad, as	ah, IT	315	6.18	>100.0	38.1	3.2
8[a]	25	f	–	–	–	<100	<0.3	<0.3	<0.3	<0.3

g, grasses; t, trees; w, weeds; m, mites; a, animals; f, food; rc, rhinoconjunctivitis; ad, atopic dermatitis; u, urticaria; as, asthma; ah, antihistamines; IT, immunotherapy with grass pollen extract; c, corticosteroids; β, beta-sympathomimetica.

[a] Non-atopic individual.