

Accepted Article

Identification and implication of an allergenic PR-10 protein from walnut in birch pollen associated walnut allergy

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Keywords

Bet v 1-like, *Juglans regia*, Jug r 5, PR-10, walnut allergy

Abbreviations

Received: 12/10/2016; Revised: 21/12/2016; Accepted: 23/12/2016

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/mnfr.201600902](#).

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PR-10, Pathogenesis-related 10; CRD, Component-resolved diagnostics; AA-id, amino acid sequence identity; CD, Circular Dichroism; DBPCFC+, Double Blind Placebo Controlled Food Challenge positive; OPT+, Open Provocation test; SPT+, Skin prick test positive; nsLTP, non-specific Lipid transfer protein; PPV, Positive predicted values; NPV, negative predictive values;

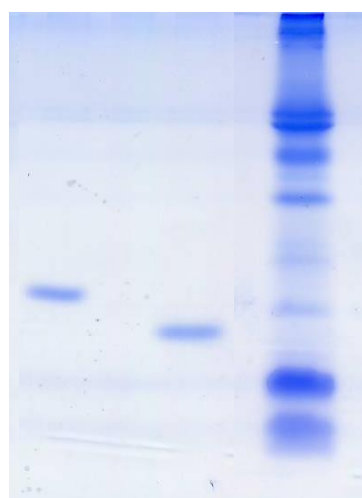
Abstract

Scope: English walnut (*Juglans regia*) belongs to the most important allergenic tree nuts. Co-sensitization with birch (*Betula verrucosa*) pollen has been reported. We aimed to identify a walnut allergen homologous to the major birch pollen allergen Bet v 1.

Methods and Results: A cDNA encoding a Bet v 1-homologous allergen (Jug r 5) in walnut kernels was cloned by RT-PCR. Jug r 5 was expressed in *E.coli*, purified by column chromatography and characterized by CD-spectroscopy. Specific IgE levels to walnut, Bet v 1 and Jug r 5 in birch pollen allergics (n=16) with concomitant walnut allergy were measured by ImmunoCAP: 44% of the patients were tested positive to walnut while 94% were reactive to Jug r 5 and 100% to Bet v 1. Jug r 5 and Bet v 1 allergens showed bi-directional IgE-cross-reactivity by competitive ELISA and were capable of inducing histamine release from effector cells. Immunoblot competition experiments demonstrated the presence of IgE-reactive Jug r 5 in walnut extract, but at low levels.

Conclusion: A Bet v 1-like allergen was identified in walnut. Diagnostic use of Jug r 5 will compensate for the low sensitivity of walnut extract for patients with birch pollen associated walnut allergy.

mnfr.201600902 Graphical abstract - Text

*Bet v 1**Jug r 5**Walnut
extract*

3D structure model overlay
Jug r 5 (yellow) and Bet v 1 (blue)

In walnut extract a Bet v 1-like, PR10-protein (Jug r 5) was identified. Due to high 3D homology of the two proteins, birch pollen allergic patients with concomitant walnut allergy display IgE-cross reactivity with Jug r 5. Diagnostic use of Jug r 5 will compensate for the low sensitivity of walnut extract for patients with birch pollen associated walnut allergy.

Introduction

Walnut trees belong to the botanical order of *Fagales* (family *Juglandaceae*, subfamily *Juglandoideae*). Among several walnut species, *Juglans nigra* (Eastern black walnut) and particularly *Juglans regia* (English-, Common-, California-, or Persian walnut) are the most important nut crops. Walnuts are consumed eaten in a raw dried state or after roasting.

Walnuts (in particular *Juglans regia*) belong to the clinically most important allergenic tree nuts and are known to induce serious and life threatening allergic reactions [1]. The prevalence of self-reported adverse reactions to tree nuts, including walnut, in the US population was estimated to be 0.5% in adults and 1.1% in children [1]. Notably, the prevalence of individual tree nut allergies varied significantly by age of the patients and region. Walnut allergy was the most frequently reported tree nut allergy in the US (37-48% of tree nut allergics) [2, 3]. In Western Europe, hazelnut allergy is a predominant tree nut allergy but, depending on the geographical area, up to 34% of tree nut allergics report reactions to walnut [2]. Among adult Italian food allergic patients, the prevalence of clinically relevant sensitization to walnut was estimated to be 8% [4]. In the general population of eleven European countries, the frequency of sensitization to walnut was highest in France (3.7%) [5]. In line with this, a recent study from Finland showed sensitization to walnut in 41% of subjects with and 11% of subjects without birch pollen sensitization [6]. These results suggest frequent cross-sensitization between birch pollen and walnut and provide evidence for the presence of homologous and cross-reactive allergens in birch and walnut.

IgE sensitization underlying allergic reactivity to walnut can be caused either by direct exposure to walnut or likely by cross-reactive sensitization to other allergens such as peach and birch pollen. Primary walnut sensitization is hallmarked by IgE antibodies to the abundant seed storage proteins and frequently associated with severe and systemic reactions.

So far, four allergens have been identified in English walnut. Jug r 1 (2S albumin, 16 kDa) [7], Jug r 2 (7S vicilin, 47 kDa) [8], Jug r 3 (nsLTP1, 10 kDa) [9] and Jug r 4 (11S globulin, legumin, 58 kDa) [3] are listed in the allergen database of the International Union of Immunological Societies (IUIS). Jug r 1 and Jug r 3 belong to the prolamin superfamily and Jug r 2 and Jug r 4 to the cupin superfamily [10, 11]. The storage proteins Jug r 1, Jug r 2, and Jug r 4 have been described as major allergens in primary walnut sensitization, with a prevalence of IgE-binding of 75%, 60%, and 57%, respectively, in walnut allergic subjects [3, 7, 8]. Jug r 3 has been described as major allergen in the Mediterranean area, where walnut allergy is considered as clinical manifestation of the LTP syndrome [4], seemingly driven by peach sensitization. In walnut allergic patients from Italy, selected by a positive open food challenge test or with history of severe symptoms, 78% were sensitized to Jug r 3 in walnut extract [9]. The study showed walnut allergic patients without concomitant pollinosis to be mono-sensitized to Jug r 3, whereas patients with pollinosis were reactive to other walnut allergens including yet not identified proteins.

A clinically well-known association exists between birch pollen sensitization and walnut allergy. Such patients typically present a milder form of walnut allergy than those with a primary walnut allergy and often tested negative for IgE to walnut extract. This walnut-birch pollen association strongly suggests the presence of a homologue of the major birch pollen allergen Bet v 1 in walnut, but no such protein has so far been described. The lack of molecular information on this part of the spectrum of walnut allergens prompted an effort to identify a Bet v 1-like protein in walnut and to investigate its allergenic properties.

Materials & Methods

Patients

Sixteen birch pollen allergic patients with concomitant walnut allergy were included in the study (Table 1). All patients showed positive skin prick test to birch pollen extract (Allergopharma, Reinbek, Germany). Walnut allergy was confirmed by double blind placebo controlled food challenge (DBPCFC) or open provocation test (OPT) in 15 patients. Three out of 12 patients who underwent DBPCFC showed no reactions but were positive in OPT. Three of the patients were included by positive OPT and one by a clear history of allergic reaction to walnut. Skin prick test with commercially available walnut extract (Lofarma, Willich, Germany) was performed as described elsewhere [12] and was positive in 44% (7/16) while prick to prick with raw walnut was positive in 19% (3/16) of the patients. Negative skin test results were obtained in nine patients with reported clinical symptoms to walnut. Specific IgE to Bet v 1 (t215) and walnut (f256) was determined in serum of all patients using ImmunoCAP (Thermo Fisher Scientific, Freiburg, Germany), with values ≥ 0.35 kU_A/L being considered positive. The study was approved by the ethical committees KEK (Kantonale Ethikkommission Zuerich; KEK-ZH-Nr. 2012-0519) and EKNZ (Ethikkommission Nordwest- und Zentralschweiz EKNZ-Nr. 2015-368).

cDNA cloning & recombinant expression of a Bet v 1-like protein from walnut

Whole ripe (mature) walnut kernels (*Juglans regia*) were ground under liquid nitrogen. Total RNA was purified by the RNeasy Plant Mini Kit (Qiagen, Hilden Germany). RNA was transcribed to cDNA using the First Strand cDNA Synthesis Kit and NotI(dT)₁₈ primer (GE Healthcare, Freiburg, Germany). For PCR amplification of a Bet v 1-like cDNA from walnut, degenerated 5' and 3' primers were designed based on the cDNA alignment of a pathogenesis related protein 10 (PR-10) from iron walnut (*Juglans sigillata*, Acc.No.: KJ598787) with other PR-10 cDNA sequences from birch, hazelnut, chestnut, apple and cherry. PCR products obtained using primers 5'-ATGGGTGTYTTCACWTWYGARRMYGAG-3' (forward) and 5'-TAGTTGTADGCATCRGRGTTKKGCCWWVA-

3' (reverse) were subcloned into pCR™II-TOPO Vector (Fisher Scientific, Schwerte, Germany) for DNA sequencing (Eurofins, Ebersberg, Germany). The 5' and 3' regions defined by the consensus PCR primers used, were verified by 5' and 3' RACE using the Invitrogen™ 5' RACE System (Fisher Scientific) and a PCR using primers 5'-GACCACGAGATCAAGGAAGAAGACG-3' (forward) and NotI(dT)₁₈ 5'-AACTGGAAGAATTCGCGGCCGCGAGGAATTTTTTTTTTTTTTTTTT-3' (reverse), followed by cloning and sequencing as described above. The full length sequence of the Bet v 1-like cDNA (denominated Jug r 5) from walnut was cloned using primers binding in the 5' and 3' untranslated regions obtained by 5' and 3' RACE. The complete sequence information was used to generate a synthetic gene, codon-optimized for expression in *E. coli* (GeneArt Life Technologies, Regensburg, Germany), which was subcloned into expression vector pET11a (VWR, Darmstadt, Germany) using NdeI and BamHI restriction sites. The Jug r 5/pET11a construct was transformed into BL21(DE3) *E. coli* cells. Protein expression of 3 L *E. coli* culture was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, at an optical density (OD₆₀₀) of 0.7. After 16 hours (h) at 22°C, the culture was harvested and the cells lysed in 20 mM Bis-Tris (pH 6.5) containing 5 M urea for 30 min at 4°C on an end over end rotator (Reax2, Heidolph-Instruments, Schwabach, Germany). Recombinant (r) Jug r 5 was purified from the supernatant by anion exchange chromatography (HiPrep Q HP 16/10, GE Healthcare, Freiburg, Germany) using on-column refolding with a 5-0 M urea gradient followed by a 0-1 M NaCl gradient for elution, both in 20 mM Bis-Tris (pH 6.5). Elution fractions containing rJug r 5 were pooled and applied to size exclusion chromatography (HiLoad 26/600 Superdex 75 pg, GE Healthcare) using 20 mM MOPS, 0.5 mM NaCl (pH 7.3) as running buffer.

Preparation of walnut extract and Bet v 1

Freshly ground ripe walnut (12 g) was incubated in 25 mL 25 mM Tris-HCl, 150 mM NaCl (pH 7.5) overnight at 4°C on an end over end rotator (see above). The aqueous protein extract was prepared by centrifugation twice for 30 min at 10,000xg and twice for 10 min at 13,000xg, each time applying

the supernatant to a new centrifuge tube, carefully avoiding the fatty phase. After the last centrifugation step, the supernatant was passed through a 0.45 µm filter. Recombinant Bet v 1 was expressed and purified as described elsewhere [13].

Physicochemical characterization of allergens

Recombinant Jug r 5 and Bet v 1 and walnut extract were analyzed by SDS-PAGE (T: 15%, 1 µg/lane for recombinant proteins and 10 µg total protein/lane for walnut extract) under non-reducing conditions using GelCode Blue Safe Protein Stain (Fisher Scientific). Protein concentration of the walnut extract was determined by Roti-Nanoquant 5x Bradford reagent (Carl Roth, Karlsruhe, Germany) and the BCA protein assay (Fisher Scientific) was used for recombinant proteins. Secondary structure elements of the recombinant proteins were analyzed by circular dichroism (CD) spectroscopy as described elsewhere [14].

IgE-reactivity of Jug r 5 and cross-reactivity with Bet v 1

Jug r 5 specific IgE values were determined by experimental ImmunoCAP testing as previously described [15], with values ≥ 0.35 kU_A/L being considered positive. Cross-reactivity between allergens was tested by competitive IgE ELISA inhibition experiments as previously described [14], using Maxisorp™ 96 well plates (Nunc, Fisher Scientific) coated with 2.5 µg/mL rBet v 1 or rJug r 5.

Detection of Jug r 5 in walnuts

For detection of Jug r 5, walnut extract (300 µg/cm) was subjected to SDS-PAGE under non-reducing conditions, transferred to 0.2 µm nitrocellulose membrane (Amersham Protran, GE Healthcare) by semi-dry blotting and stained with Ponceau S (Sigma-Aldrich, Munich, Germany). After blocking with 0.3% Tween 20 in Tris-buffered saline (TBS, 1 h), the membrane was incubated over night with serum (500 µl, diluted 1:8 in TBS, 0.05% Tween20, 0.1% BSA). For inhibition of IgE-binding, rJug r 5, (10 and 50 µg/mL), walnut extract (1 mg/mL) or BSA (500 µg/mL) was added to the serum sample.

Bound IgE was detected as described elsewhere [14]. For detection of natural Jug r 5, a monoclonal antibody (mab) raised against Bet v 1 (mp10, PEI) was applied overnight (500 µl supernatant of hybridoma culture, 1:10 in TBS, 0.05% Tween20, 0.1% BSA). Antibody binding was detected using AP-labelled rat-anti mouse IgE- or IgG-specific antibodies (BD Pharmingen) and the AP substrate reagent kit (Bio-Rad, Munich, Germany). Between each incubation steps the membranes were washed 4 times, 5 min each with TBS, 0.05% Tween20.

Allergenic potency testing of Jug r 5

Histamine release assays using stripped and passively sensitized basophils were performed as previously described [16]. Allergen-induced histamine release levels $\geq 10\%$ of total histamine content of the cells were considered positive.

Results

Cloning of Jug r 5 cDNA

Cloning of a Bet v 1-like protein from walnut resulted in a 569 bp cDNA sequence (GenBank Acc. No. KX034087) containing an open reading frame of 483 bp with 99.9% identity to a PR-10 cDNA (GenBank Acc. No. KJ598787) from leaf tissue of iron walnut (*Juglans sigillata*). The Bet v 1-like cDNA encoded a protein of 160 amino acids (AA) with a theoretical molecular weight of 17.6 kDa and an isoelectric point (pI) of 5.51. The walnut protein, designated Jug r 5, and Bet v 1 (GenBank Acc. No. X15877) share an overall AA sequence identity of 67% AA (Fig. 1A) and 100% identity across the P-loop region (AA 46-55) which is considered to be an important determinant of IgE binding to Bet v 1-like allergens [17]. Further, a stretch comprising a major T cell epitope of Bet v 1 [18] is 67% identical in the Jug r 5 sequence. The AA-identity of Jug r 5 to other Bet v 1-like proteins from legumes (peanut, soybean), tree nuts (hazelnut, chestnut) and fruits (apple, cherry, apricot) ranges from 53% to 74% (Fig. 1B). Moreover, Jug r 5 displays a consensus N-glycosylation motif (Asn-X-Ser/Thr) at AA residue 79 -81 (Asn-Phe-Thr).

Physicochemical characterization of Jug r 5

Recombinant Jug r 5 and Bet v 1 were expressed in *E. coli* as non-fusion (Jug r 5) or N-terminal (His)₆ tagged (Bet v 1) protein and purified by two step chromatographic procedures. Around 13 mg of rBet v 1 and 2 mg of rJug r 5 were obtained from 1 L of *E. coli* culture, respectively. The apparent molecular mass of the purified proteins in SDS-PAGE under non-reducing conditions were 20 kDa and 23 kDa, respectively (Fig. 2A, lane 1: rBet v 1, lane 2: rJug r 5). CD spectroscopy indicated similar secondary structure elements in both proteins, suggesting they were likely properly folded (Fig. 2B).

IgE-reactivity of Jug r 5 and cross reactivity with Bet v 1

To determine the frequency of IgE sensitization to Jug r 5 and to analyze co-sensitization with Bet v 1, Jug r 5, Bet v 1 and walnut specific IgE levels were compared in the sera of birch allergic patients with concomitant walnut allergy (Table 1) by ImmunoCAPTM testing (Fig. 3). Results revealed a prevalence of specific IgE to Bet v 1 in 100% (16/16, median: 21.6 kU_A/L) and to Jug r 5 in 94% (15/16, median: 13.2 kU_A/L) of the patients. One patient with a negative ImmunoCAP result to Jug r 5 was also tested IgE negative to walnut extract, despite a positive DBPCFC with walnut. Notably, only 37.5% (6/16, median: 0.16 kU_A/L) of the patients were tested positive for IgE to walnut extract. Low sensitivity of whole walnut extract for IgE testing of birch pollen related sensitization was in accordance with high frequency of negative skin test results by using skin prick test solution (9/16) or prick-to-prick testing (13/16). Finally in 7 patients IgE dependent sensitization could not be confirmed by either skin testing or walnut ImmunoCAP testing. Consequently, we observed a very weak correlation ($R = 0.043$, $p=0.013$) between walnut extract and Jug r 5 specific IgE values (Fig. 3A). In 9 patients without reactivity to walnut extract IgE-reactivity to Jug r 5 was detected. In contrast, we observed a very good correlation ($R = 0.956$, $p=0.0037$) between Bet v 1 and Jug r 5 specific IgE values (Fig. 3B), although almost all patients (except no. 3) revealed higher IgE-levels to Bet v 1. None of 5 sera from non-allergic controls showed IgE-reactivity to any of the tested allergens. According to the definitions of the IUIS Allergen Nomenclature Subcommittee, Jug r 5 fulfills the criteria of a major allergen in walnut and has been added to its official allergen database (www.allergen.org).

Cross-reactivity of IgE between Jug r 5 and Bet v 1 was evaluated by competitive ELISA cross-inhibition experiments using serum of four walnut allergic patients (Fig. 4). The serum samples were incubated with serial dilutions of Bet v 1, Jug r 5, walnut extract and BSA as control. With Bet v 1 on the solid phase, Jug r 5 was able to inhibit IgE binding only up to 60% at the highest inhibitor concentration in 3 out of 4 serum samples (Fig. 4A). Using serum no. 4, Jug r 5 was not at all able to compete with IgE-binding to Bet v 1 (Fig. 4A). Bet v 1 (applied as positive control) was able to inhibit

the IgE binding almost 100% for all tested sera. Walnut extract and BSA as control displayed no inhibitory capacity to Bet v 1 in any of the sera tested (Fig. 4A). With Jug r 5 on the solid phase, both Bet v 1 and Jug r 5 itself were able to outcompete IgE binding to almost 100%, except in serum no. 11 (only up to 70% self-inhibition). IC₅₀ (concentration required to achieve half maximum inhibition) for Bet v 1 was approximately 5-fold (serum no. 16) to almost 100-fold (serum no. 4) lower than for Jug r 5. Notably, walnut extract showed only a modest inhibitory capacity of up to 35% even at the highest concentration applied (Fig. 4B). Again, BSA showed no unspecific inhibitory activity in any of the sera tested.

Detection of Bet v 1-like protein Jug r 5 in walnuts

To confirm the presence of Jug r 5 in walnut, immunoblot experiments using walnut extract on the solid phase were performed. Using the mab mp10 raised against Bet v 1, a band of approximately 20 kDa could be detected (Fig. 5A). Furthermore, IgE immunoblot inhibition experiments were performed (Fig. 5B). Using a serum from a patient (no. 16) positive to Jug r 5 in ImmunoCAP and reactive to a single walnut protein band of approximately 20 kDa in immunoblot, IgE binding to that band was abolished after incubation of serum with rJug r 5 or walnut extract (Fig. 5B). These observations provide strong evidence for the presence of a PR-10 allergen in walnut extract.

Basophil activation capacity of Jug r 5

To evaluate the biological potency of Jug r 5 to activate effector cells, *in vitro* histamine release assays were performed using four sera for passive sensitization of donor basophils and Jug r 5, Bet v 1 and walnut extract as antigens (Fig. 6). Except for serum no. 4, Bet v 1 and Jug r 5 were able to trigger a histamine release of at least 50% (Fig. 6, serum 10, 11 and 16). For all sera tested, Bet v 1 showed slightly stronger biological potency (in terms of antigen concentration eliciting half maximum mediator release) and strongest basophil reactivity (e.g. max release up to 90-100% for sera nos. 10 and 11). Thus, in comparison to Jug r 5, lower concentrations of Bet v 1 are required to

achieve similar mediator release. Walnut extract was only able to induce low levels of histamine release at the highest concentration of 10 µg/mL in two patients tested. BSA did not induce any histamine release in the tested sera.

Discussion

The manifestation of birch pollen associated walnut allergy has been reported in areas with high seasonal exposure to birch pollen [6]. Nevertheless, no pollen-related walnut allergen involved in this birch pollen associated food syndrome has yet been identified. Although there is some evidence for IgE-reactive profilin from walnut (formerly termed Jug r 5), no data regarding its allergenic properties are available [10, 19]. Primary sensitization to Bet v 1, the major allergen of birch pollen, causing IgE cross-reactivity with homologous food allergens (e.g. from fruits, vegetables, peanut, soy and tree nuts) is known to be the most important elicitor of secondary plant food allergies [20]. Therefore, the present study aimed at the identification, molecular cloning and allergenicity assessment of a Bet v 1-like protein predicted to be present in walnut.

Sixteen birch pollen allergics with concomitant walnut allergy were included in the study. In agreement with a former study [21], we observed a discrepancy between both walnut specific IgE testing and skin testing using commercial walnut extracts in comparison to positive food challenge tests. The results suggest that certain walnut allergens are not adequately represented in aqueous protein extracts, probably due to limited accumulation in walnuts and/or low solubility, likely due to interference with the lipid-containing food matrix. We speculate that a substantial fraction of the allergen maybe present in the lipophilic fraction. In line with this, Ara h 8, the Bet v 1 homologue from peanut, was efficiently isolated by means of chloroform/methanol extraction under alkaline conditions [22]. Although walnut extract shows low sensitivity in birch pollen allergics with concomitant nut allergy, this was not the case for commercial extracts of almond, hazelnut and peanut [21]. Since patients did not even react to fresh walnuts by prick-to-prick techniques, the authors speculated that walnuts might not contain a Bet v 1-like protein and thought that DBPCFC in

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birch pollen allergics would be required to determine whether walnut is truly a birch pollen-related allergenic food [21]. The discrepancy of negative skin testing (SPT & PTP) and positive oral provocation test in some patients in the present study might have several explanations: (1) The overall amount of allergens which is exposed to the patients likely is different between both methods. (2) While oral food challenge tests are performed with whole walnuts, skin prick testing uses aqueous walnut extract which does not necessarily comprise a full representation of the proteins present in intact walnuts (e.g. by lacking lipophilic allergens), potentially affecting the diagnostic sensitivity of the procedure. (3) In oral food challenge tests, allergens are administrated to the oral and gastrointestinal mucosa which in some food allergic patients may have a higher immunoreactivity than the skin.

In the present study, walnut allergy was confirmed by DBPCFC or OPT in all birch pollen allergic patients who underwent provocation tests (n=15). Noteworthy, food challenge tests are the gold standard for allergy diagnosis rather than sensitization tests such as SPT and *in vitro* IgE testing methods. We were able to identify a Bet v 1-related allergen, termed Jug r 5, in walnuts by recombinant cloning of a Bet v 1 homologous gene and at the level of natural protein extract. Notably, we identified the almost identical sequence of Jug r 5 in the edible nut as in leaf of *Juglans sigillata* indicating the protein is highly conserved among different cultivars and organs. Although natural Jug r 5 was very scarcely represented in self-prepared walnut extract, we were able to demonstrate its presence by immunoblot inhibition. We conclude that Jug r 5 comprises only a very minor constituent in walnuts or to be present in the lipophilic fraction, which would explain the low diagnostic sensitivity obtained with aqueous walnut extract. In contrast, walnut allergens belonging to the legumin, vicilin and 2S albumin families are predominant seed storage protein constituents [11]. By using recombinant Jug r 5 for IgE testing with an experimental ImmunoCAP assay, we found 15 out of 16 patients to be IgE-reactive, despite a negative walnut test in 9 of these patients. These data demonstrated a clearly increased diagnostic sensitivity in comparison to whole walnut extract.

Utilization of Jug r 5 for component-resolved diagnostics (CRD) in walnut allergy is of particular importance for detection of patients with mono-sensitization to Jug r 5. Although in the present study, several patients were sensitized to multiple walnut allergens (data not shown), mono-sensitization to Jug r 5 appeared to be present in at least one patient (no. 16).

An association of walnut allergy with pecan [6, 23] and peanut allergy [24], as well as cross-reactivity of IgE between walnut and mustard [25], hazelnut and brazil nut [26] has been reported. Our data clearly showed a bi-directional IgE cross-reactivity between Jug r 5 and Bet v 1, indicating common IgE-binding epitopes on the two allergens. As demonstrated also for several other Bet v 1 related food allergens [27], Jug r 5 showed weaker IgE inhibition capacity than Bet v 1, and in one patient (no. 4) Jug r 5 was not at all able to compete IgE-binding to Bet v 1. Since walnut and birch belong to the botanical order of *Fagales* we speculate an IgE cross-reactivity of Jug r 5 with conserved allergens from other members of the same taxonomical family. In line with this, it was reported that in hazelnut allergics recruited from across Europe, IgE reactivity to walnut strongly correlated with sensitization to hazelnut LTP (Cor a 8) and storage proteins (Cor a 9 and Cor a 14) but not with the Bet v 1-homologous Cor a 1 although substantial number of patients were sensitized to birch pollen [28]. Considering the results of the present study the lack of correlation with Cor a 1 probably was due not to a lack of cross-reactivity between the PR-10 allergens of walnut and hazelnut but rather to the scarcity of Jug r 5 in walnut extract, causing underestimation of Jug r 5 reactive IgE or failure to even detect its presence in serum samples tested.

We found Jug r 5 to display allergenic activity as it triggered histamine release from effector cells in a dose-dependent manner. Considering the higher Bet v 1-specific IgE values, stronger inhibition capacity and higher allergenic potency of Bet v 1, the results provide compelling evidence for primary sensitization to Bet v 1 in cases of birch pollen associated walnut allergy.

Since nut allergens are difficult to purify or not adequately represented in natural protein extracts, application of CRD in the diagnostic workup for walnut allergy will rely on the use of recombinant walnut allergens. Serological testing combining walnut storage proteins and the Bet v 1 homologue Jug r 5 will help to discriminate between primary and secondary food allergy to walnut. Molecular diagnostic test results may also indicate possible cross-reactivity with tree nuts or pollen allergens and therefore potentially predict the risk for more severe systemic reactions (sensitization to storage proteins or LTP) or mild reactions (sensitization to pollen-related allergens such as Jug r 5). Finally, CRD will improve the management of walnut allergy because it is likely that patients with mono-sensitization to Jug r 5 will tolerate walnut after heating.

Author contributions

SV and SS designed and supervised the study with input by JL. AW and AJ performed molecular cloning, expression, purification and immunological characterization of Jug r 5. BBW, NG, and CL planned and conducted the clinical part of the study. JL was responsible for the preparation and quality testing of the experimental ImmunoCAP testing. The manuscript was written by AW and SS and all co-authors reviewed the manuscript.

Acknowledgement

The clinical part of the study was funded by the Ulrich Müller-Gierok-Foundation.

Conflict of interest statement

Jonas Lidholm is an employee of Thermo Fisher Scientific. Barbara Ballmer-Weber has received honoraria for talks in regard to this study. All other authors declare no conflict of interest.

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Table 1

Clinical and serological data of birch pollen allergic patients with concomitant walnut allergy

patient #	age	sex	DBPCFC/OPT clinical symptoms	symptoms by history	birch			Provocation type	ImmunoCAP™ [kU _A /L] (CAP class)		
					SPT	SPT	PTP		Bet v 1 (t215)	walnut (f256)	Jug r 5
1	34	M	OAS, R	OAS	+	+	+	DBPCFC	0,93 (2)	0,40 (1)	0,93 (2)
2	21	F	OAS, N, thorax pressure	OAS, chest pressure	+	+	+	DBPCFC neg/OPT pos	23,1 (4)	50,7 (5)	20,8 (4)
3	23	F	R, OAS, Co, throat tightness	OAS	+	+	+	DBPCFC	8,75 (3)	3,96 (3)	10,8 (3)
4	15	F	OAS, R	OAS, F, R, C	+	-	-	DBPCFC	47,4 (4)	0,08 (0)	26,0 (4)
5	52	F	OAS	OAS, AE, Dph	+	+	-	DBPCFC	14,4 (3)	0,06 (0)	12,9 (3)
6	33	M	OAS	OAS, R	+	+	-	DBPCFC neg/OPT pos	21,1 (4)	1,13 (2)	17,9 (4)
7	48	M	OAS	OAS, Dph, heart burn	+	+	(+)	DBPCFC	12,9 (3)	0,12 (0)	7,15 (3)
8	31	F	OAS	not known	+	+	-	DBPCFC neg/OPT pos	28,3 (4)	0,00 (0)	13,5 (3)
9	35	F	OAS	OAS, U	+	-	-	DBPCFC	1,46 (2)	0,10 (0)	0,25 (0)
10	27	F	OAS, thorax tightness	OAS, AE	+	-	-	OPT	>100 (6)	3,37 (2)	>100 (6)
11	44	M		OAS, D	+	-	-	history	87,4 (5)	0,13 (0)	58,8 (5)
12	28	M	OAS, enoral swelling, wheals scalp	OAS	+	-	-	DBPCFC	83,6 (5)	8,30 (3)	57,0 (5)
13	40	M	OAS, thightness throat, itch face	OAS	+	-	-	DBPCFC	6,76 (3)	0,00 (0)	5,30 (3)
14	34	F	OAS	OAS	+	-	-	DBPCFC	21,5 (4)	0,00 (0)	7,54 (3)
15	32	M	OAS, Itching	OAS	+	-	-	OPT	21,7 (4)	0,13 (0)	11,8 (3)
16	46	F	OAS	OAS	+	-	-	OPT	55,8 (5)	0,01 (0)	49,2 (4)

Abbreviation: DBPCFC, double-blind placebo-controlled food challenge; OPT, open provocation test; SPT, skin prick test; PTP, prick to prick; OAS, oral allergy syndrome; R, rhinitis; N, nausea; Co, cough; F, flush; C, conjunctivitis; AE, angioedema; Dph, dysphagia; U, urticarial; D, dyspnea.

Figure Legends

Figure 1: Amino acid sequence comparisons. (A) Amino acid (AA) alignment of Jug r 5 with Bet v 1, the major allergen from birch. The P-loop region (AA 46-55) and the T-cell epitope (AA 142-156) of Bet v 1 are underlined and indicated in bold. **(B)** AA sequence identities (AA-id, %) of Jug r 5 with allergenic PR-10 proteins from a variety of pollens (P) and foods (F).

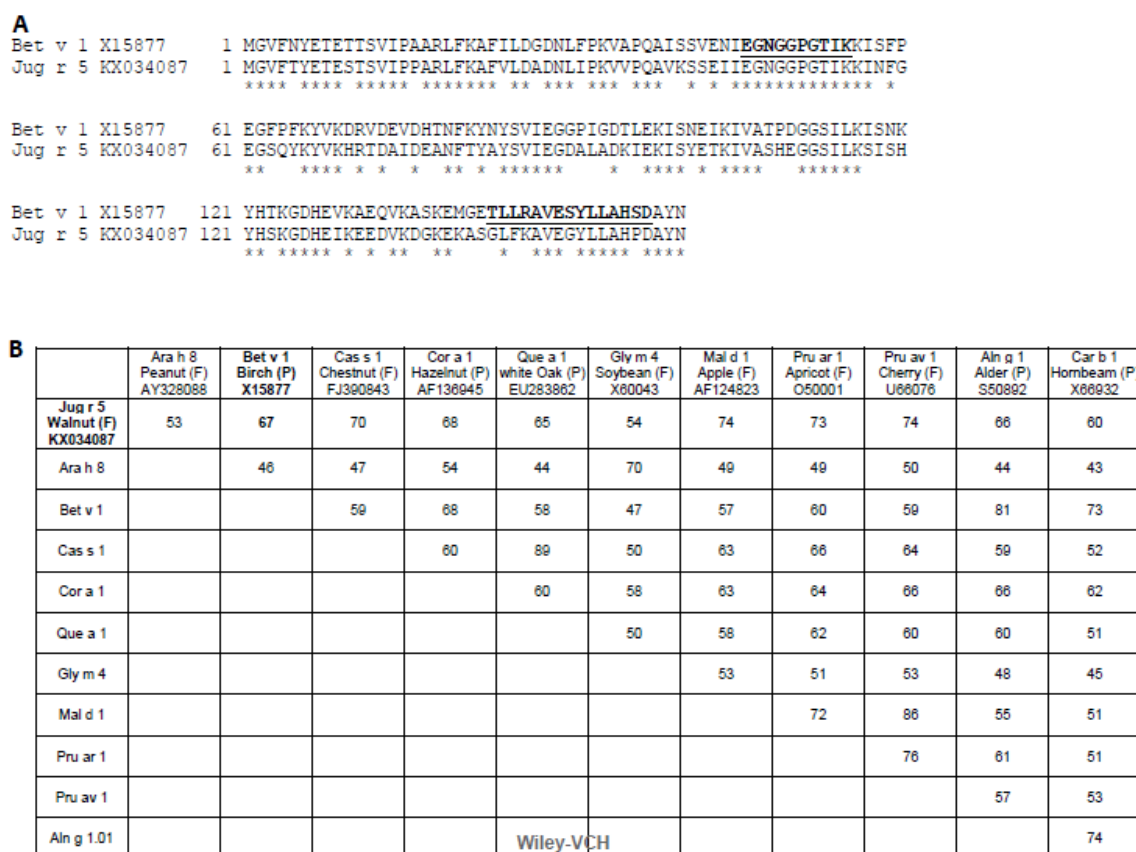


Figure 2: Physicochemical characterization of Jug r 5. (A) SDS-PAGE and Coomassie staining of rBet v 1 (1 µg, lane 1), rJug r 5 (1 µg, lane 2) and walnut extract (10 µg, lane 3) under non-reducing conditions; M: molecular weight marker. **(B)** Circular dichroism spectroscopy of rJug r 5 in comparison to rBet v 1.

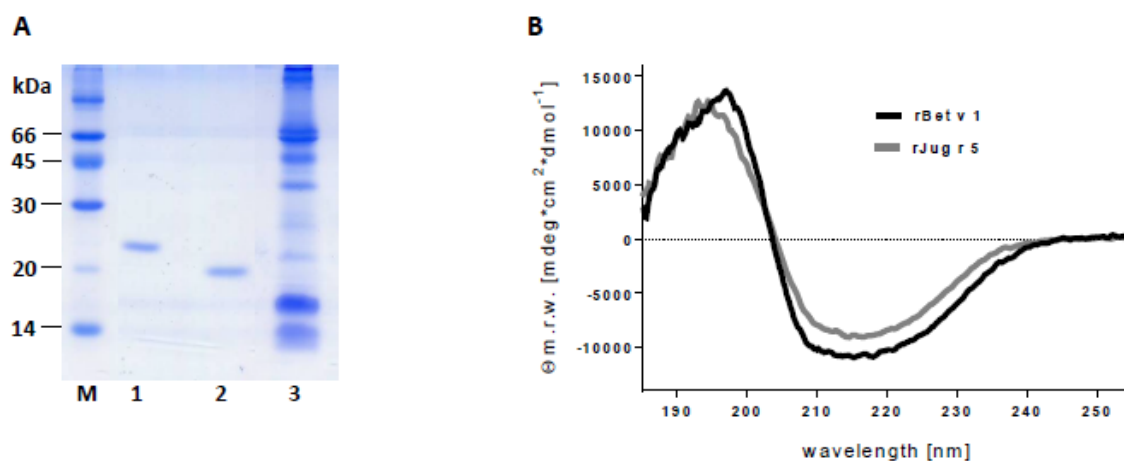


Figure 3: IgE-reactivity of walnut allergic patients. Comparison of IgE-binding to **(A)** rJug r 5 and walnut extract, and **(B)** rJug r 5 and rBet v 1 in sera from walnut allergic patients with birch pollen associated walnut allergy (n=16) by ImmunoCAP testing.

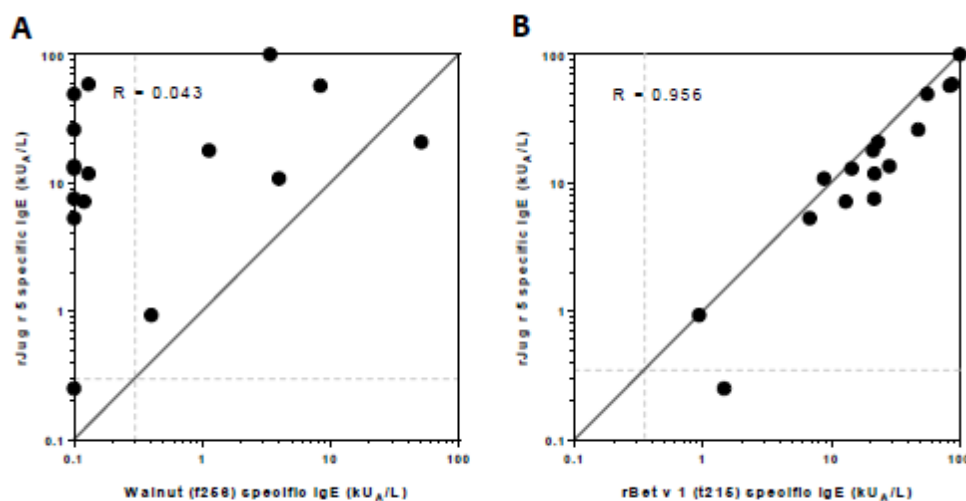


Figure 4: IgE cross-reactivity between Jug r 5 and Bet v 1. IgE cross-inhibition ELISA was performed using (A) rBet v 1 and (B) rJug r 5 on the solid phase. IgE-binding of sera (n=4) from birch pollen allergics with associated walnut allergy was determined upon dose-dependent inhibition with rBet v 1, rJug r 5, walnut extract and BSA as control.

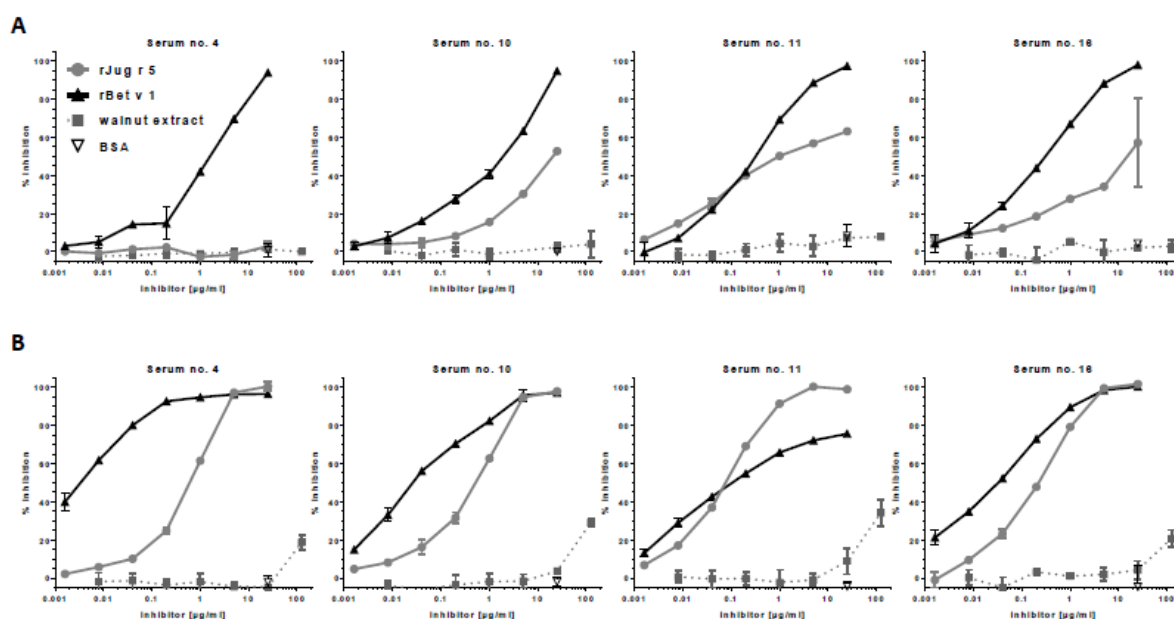


Figure 5: Detection of a Bet v 1-like protein in walnut extract. (A) IgG binding of mab (mp10) raised against Bet v 1 to walnut extract. M: Molecular marker; PonceauS staining (lane 1); mab (mp10) (lane 2); secondary antibody control (lane 3). (B) IgE binding to walnut extract (lane 2) was inhibited by pre-incubation of serum from a Jug r 5 mono-sensitized patient (no. 16) with rJug r 5 (10 and 50 µg/mL, lanes 3 + 4), walnut extract (1 mg/mL, lane 5) and BSA as control (50 µg/mL, lane 6). M: Molecular marker; Ponceau S staining (lane 1); secondary antibody control (lane 7).

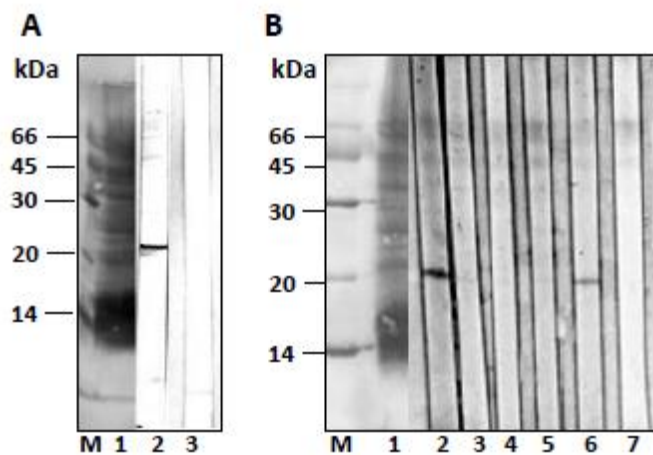


Figure 6: Allergenic potency testing of Jug r 5. Allergenic activity of rJug r 5 and rBet v 1 was determined by *in vitro* mediator release assay using sera from patients with birch pollen associated walnut allergy. Walnut extract and BSA served as positive and negative controls, respectively.

