

Purification and Characterization of the Major Oak Pollen Allergen Que a 1 for Component-Resolved Diagnostics Using ImmunoCAP®

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Key Words

Amino acid sequence • Component-resolved diagnostics • Immunoblotting • ImmunoCAP® • Mass spectrometry • Oak pollen • Que a 1 • Two-dimensional gel electrophoresis

Abstract

Background: The aim of this study was to purify the major oak pollen allergen, Que a 1, to perform biochemical and immunological characterization of the allergen and to develop an experimental native (n) Que a 1 ImmunoCAP®. **Methods:** Que a 1 was purified from oak pollen extract using affinity chromatography and characterized by SDS-PAGE, two-dimensional (2D) PAGE, mass spectrometry (MS), N-terminal sequencing and specific IgE inhibition on ImmunoCAP. Samples from 16 subjects sensitized to oak pollen were analyzed by ImmunoCAP for IgE reactivity to nQue a 1, and recombinant (r)Bet v 1 and 2 (profilin). They were also studied in IgE immunoblotting. **Results:** The purity of nQue a 1 was >95%, since a single band was observed on silver-stained SDS-PAGE. The identity was verified by MS analysis, and 2D-PAGE revealed several isoforms. The obtained N-terminal sequence of 50-amino-acid residues from nQue a 1 showed a 58–74% sequence identity with other pathogenesis-related class 10 allergens. Specific IgE inhibition verified a preserved

immunoreactivity (70–92% inhibition). All subjects were sensitized to Que a 1 and Bet v 1, and two to profilin. The IgE antibody levels to nQue a 1 were generally lower than to rBet v 1. The obtained results correlated well with IgE immunoblotting. **Conclusions:** We present a highly purified and extensively characterized preparation of nQue a 1. Que a 1 seems to be an allergen of equal importance in oak pollen as Bet v 1 in birch pollen. An nQue a 1 ImmunoCAP will be useful in component-resolved diagnostics.

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Introduction

Sensitization to tree pollen of the Fagales order (e.g. oak, birch, alder and hazel) is observed in up to 23% of the population [1]. The major allergen in birch pollen, Bet v 1, has been studied in detail. It is a 17-kDa allergen belonging to the pathogenesis-related class 10 (PR-10) protein family [2, 3], and a number of different isoforms of Bet v 1 have been described [3–5]. Studies have shown a prevalence of IgE reactivity to Bet v 1 of >95% in birch-allergic patients from highly exposed areas [6, 7]. Homologous allergens have been identified in pollen from related trees and several vegetables and fruits [8, 9].

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Allergy to oak pollen is a major problem in parts of Europe and Northern America [10, 11]. In Madrid, Spain, 14% of the pollen-allergic patients are sensitized to oak pollen [12], and in Sweden this number is 70–80% of the springtime pollen-allergic patients [13]. Que a 1 is an important oak pollen allergen and belongs to the PR-10 protein family. Similar to other PR-10 allergens from pollen of related trees, it shows high cross-reactivity with Bet v 1 regarding IgE binding [14]. Although several PR-10 allergens have been cloned, sequenced and expressed as recombinant allergens, the knowledge of the biochemical and immunological characteristics of Que a 1 is limited. Ipsen and Hansen [15] purified Que a 1 from pollen extract and presented a 24-residue-long N-terminal amino acid sequence with several reservations. In the present study, we purified native (n) Que a 1 from oak pollen extract using affinity chromatography with a monoclonal antibody, and characterized it biochemically and immunologically. An N-terminal sequence of 50 amino acids is presented.

Component-resolved diagnostics using recombinant or purified native allergens may be relevant in studying sensitization patterns, e.g. when choosing the appropriate pollen vaccine for specific immunotherapy [8]. In the present study, we developed an experimental nQue a 1 ImmunoCAP® and used it in component-resolved diagnostics. The importance of Que a 1 in oak pollen sensitivity was demonstrated in a pilot study including plasma samples from 16 oak-pollen-sensitized individuals.

Materials and Methods

Plasma Samples

Plasma (citrate) samples were obtained from Phadia, Diagnostics Sample Unit, including 15 oak-pollen-sensitized subjects (serological analysis) from the United States (US1–US15) with tree pollen allergy (n = 7, positive anamnesis) or with unknown clinical status (n = 8), and for comparison 1 Swedish oak-pollen-sensitized subject (SW1) and 1 negative control (NC) pool of plasma samples without specific IgE antibodies to any known allergen. For specific IgE inhibition studies, six selected samples from Que-a-1-monosensitized individuals (determined by immunoblotting) from the US and Sweden were used.

Purification

A monoclonal antibody (Bip 1) against PR-10 proteins was coupled to NHS-activated Sepharose HP (GE Healthcare, Uppsala, Sweden) according to the instruction supplied by the manufacturer. The antibody has been described elsewhere [16, 17]. Proteins from oak pollen (*Quercus alba*, Allergon, Ängelholm, Sweden) were extracted for 2 h in 0.1 M Na₂KPO₄, pH 7.6, at 5°C, filtered (0.45 µm) and frozen at –70°C until purification. Oak

pollen extract was thawed and added to the affinity column. Bound Que a 1 was eluted in 0.1 M glycine-HCl, pH 3.0, and immediately neutralized with 1 M K₃PO₄. The protein concentration was determined using the BCA method (BCA protein assay kit, Pierce, Rockford, Ill., USA). The purified allergen was frozen at –20°C until use.

Bet v 1, used in the inhibition study, was purified from birch pollen extract using the same affinity method as described for nQue a 1.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on NuPAGE 4–12% Bis-Tris Gel from Invitrogen (Carlsbad, Calif., USA). Oak pollen extract and purified nQue a 1 was treated with NuPAGE LDS sample buffer, and for reducing conditions NuPAGE sample reducing agent, and heated at 97°C for 5 min. The gel was loaded with 0.5 µg reduced oak pollen extract and 0.25 µg nQue a 1 (reduced and non-reduced). The molecular weight calibration of the gel was achieved using Mark 12 unstained standard (Invitrogen). Electrophoresis was performed in MES running buffer following the manufacturer's instruction. The gel was silver stained according to Blum et al. [18], in a Hoefer Processor Plus (GE Healthcare).

The glycosylation status of Que a 1 was determined from SDS-PAGE, where 5 and 10 µg of nQue a 1 and 5 µg horseradish peroxidase (positive control) were run on the gel under reducing and non-reducing conditions. The gel was stained using a glycoprotein detection kit (Sigma-Aldrich, St. Louis, Mo., USA) based on the periodic acid-Schiff (PAS) method.

Two-Dimensional PAGE

Isoelectric focusing (IEF) was carried out using Immobiline DryStrips, 7 cm, pH 3–10 non-linear, IPG buffer, pH 3–10 non-linear, and IPG cover fluid (all purchased from GE Healthcare). Three micrograms of oak pollen extract or 0.5 µg nQue a 1 were added to the rehydration solution that contained 8 M urea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.002% (w/v) bromophenol blue (BPB), 100 mM dithiothreitol (DTT, freshly added) and 0.5% (v/v) IPG buffer. The IPG strips were incubated in the sample-containing rehydration solution for at least 10 h at 20°C before IEF. IEF was performed in an IPGphor system (GE Healthcare) under the following conditions: 500 V for 30 min, 1,000 V for 30 min and 5,000 V for 100 min. After IEF, the strips were equilibrated for 2 × 15 min in SDS equilibration buffer with 100 mM DTT and 50 mM iodoacetamide, respectively. The SDS equilibration buffer was composed of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) BPB. NuPAGE gels were used to perform the second-dimension SDS-PAGE step. The focused IEF strips were placed on top of the SDS-PAGE gel and sealed with 0.5% (w/v) agarose and 0.002% (w/v) BPB. Electrophoresis was performed in MES running buffer at 20 mA for 15 min, followed by 40 mA for 105 min. The gels were extensively rinsed in distilled water to remove residual ampholytes and reduce background staining. Silver staining was performed as described above.

In-Gel Digestion

Protein spots of interest were cut out of the gel and destained in 30 µl of 15 mM K₃Fe(CN)₆, 50 mM Na₂S₂O₃ for 30 min. They were then washed in 50 mM NH₄HCO₃, 50% EtOH for 30 min fol-

lowed by 15 min in 100% EtOH and run in a SpeedVac vacuum centrifuge (Savant, Farmingdale, N.Y., USA). The gel pieces were rehydrated in digestion buffer containing 12.5 ng/ μ l trypsin (Promega, Madison, Wisc., USA) in 25 mM NH_4HCO_3 and incubated at 37°C overnight.

Mass Spectrometry Analysis

Peptide samples (0.5 μ l supernatant after overnight digestion) were applied onto a stainless-steel 384 MALDI target (Bruker Daltonic, Bremen, Germany) followed by application of an equal volume of matrix solution containing saturated α -cyano-4-hydroxy cinnamic acid, 50% acetonitrile, 0.1% trifluoroacetic acid and 10 mM $\text{NH}_4\text{H}_2\text{PO}_4$ for suppression of matrix signals [19]. All mass spectrometry (MS) analyses were performed using a Bruker Autoflex II (Bruker Daltonic). MALDI-TOF MS spectra were recorded in positive reflector ion mode, calibrated with peptide calibration standard covering ~1–4 kDa (Bruker Daltonic) using the Flex Control software. The obtained MS spectra were also internally calibrated with two trypsin autolytic peptides with mass-over-charge ratios (m/z) of 842.51 and 2,211.104 Da using the Flex Analysis software. Fragment spectra were recorded by MALDI-TOF/TOF MS/MS in LIFT mode. De novo sequencing of the obtained MS/MS spectra was performed using the software Mascot Distiller (Matrix Science, London, UK). Putative sequences retrieved were used for homology searching using BLAST [20].

MALDI-TOF MS characterization of intact nQue a 1 was performed by mixing 0.2 mg/ml purified protein sample 1:1 with saturated 3,5-dimethoxy-4-hydroxy cinnamic acid (sinapinic acid) dissolved in 25% (v/v) acetonitrile, 0.05% trifluoroacetic acid, 5 mM $\text{NH}_4\text{H}_2\text{PO}_4$. The protein/matrix suspension (0.5 μ l) was applied onto the stainless-steel 384 MALDI target. Data were recorded in positive linear ion mode and the instrument was calibrated with protein calibration standard I covering ~3–25 kDa (Bruker Daltonic).

Amino Acid Sequencing

Five micrograms of nQue a 1 were diluted in 1 ml 6 M guanidine-HCl, 0.1 M Tris, pH 8, and incubated with 5 μ l 0.5 M DTT for 2 h at 37°C. After cooling the solution to room temperature (RT), 20 μ l 0.5 M iodoacetamide were added and the solution was incubated in the dark for 15 min. N-terminal protein sequencing (50 cycles) was then performed using the Edman degradation method in a G1000A sequencer (Hewlett-Packard, Palo Alto, Calif., USA). The obtained sequence was compared with other known PR-10 allergens using BLAST. Sequence homology between nQue a 1, Cas s 1 (from chestnut), Fag s 1 (beechnut), Cor a 1 (hazel), Mal d 1 (apple), Bet v 1 (birch), Pru av 1 (cherry), Aln g 1 (alder) and Car b 1 (hornbeam) was visualized using T-Coffee [21].

Specific IgE Inhibition

Patient samples from seemingly Que-a-1-monosensitized individuals (3 from the US and 3 from Sweden) were diluted to <12 kU_A/l oak- and birch-pollen-specific IgE in ImmunoCAP sample diluent (Phadia, Uppsala, Sweden) and used in specific IgE inhibition to study the immunoreactivity of affinity-purified nQue a 1. Purified nQue a 1 and nBet v 1 were diluted in Dulbecco's phosphate buffered saline to 50, 10 and 2 μ g/ml, and 400, 80 and 16 ng/ml. The six nQue a 1 and nBet v 1 dilutions and Dulbecco's

phosphate buffered saline only (non-inhibited sample) were mixed 1:4 with diluted patient samples and incubated at RT for 2 h with gently mixing. The concentrations of free specific IgE antibodies in the samples were determined using both extract-based oak and birch pollen ImmunoCAP (code t7 and t3, respectively) and recombinant (r)Bet v 1 ImmunoCAP (t215), and the standard specific IgE FEIA method on ImmunoCAP 100 instrument (Phadia). Results are expressed as percent free specific IgE (kU_A/l) of non-inhibited sample.

Component-Resolved Diagnostics

Experimental ImmunoCAP with nQue a 1 was developed by MIAB (Uppsala, Sweden). The allergen was coupled to CNBr-activated ImmunoCAP matrix at 0.5–50 μ g/ml following the standard method of Phadia. The optimal coupling concentration was determined using plasma samples with Que-a-1-specific IgE antibodies at various concentrations. The presence of contaminating panallergens on nQue a 1 ImmunoCAP was tested using mouse monoclonal and/or polyclonal antibodies derived against rBet v 2 and rBet v 4 (representing profilin and polcalcin, respectively) and an in-house method for mouse IgG antibodies for ImmunoCAP 100. The specificity of nQue a 1 ImmunoCAP was also confirmed using samples from oak-pollen-sensitized individuals with IgE reactivity <0.35 kU_A/l to the Que a 1 homologue rBet v 1, but with IgE reactivity to rBet v 2 ($\leq$ 20 kU_A/l) and/or rBet v 4 ($\leq$ 22 kU_A/l) on ImmunoCAP.

Plasma samples from 16 subjects with known sensitization to oak pollen and a NC plasma pool were analyzed by ImmunoCAP for IgE reactivity to nQue a 1, rBet v 1, rBet v 2 (profilin) and cross-reactive carbohydrate determinants (CCD)/MUXF³ using the standard specific IgE FEIA method. ImmunoCAP with rBet v 1, rBet v 2 and CCD/MUXF³ are commercially available (Phadia).

Immunoblotting

Freeze-dried extracts of oak pollen (*Q. alba*) were dissolved in sample buffer (50 mM Tris, 1% SDS, 1 mM EDTA- Na_2 , 0.01% BPB and 520 mM DTT) and heated for 5 min at 97°C before separation by SDS-PAGE (~28 μ g protein/cm) on ExcelGel SDS homogeneous 12.5% on Multiphor II (GE Healthcare). The molecular weight calibration of the gel was achieved using the low-molecular-weight calibration kit for SDS electrophoresis (GE Healthcare). Directly after SDS-PAGE, the proteins were transferred to a nitrocellulose membrane by semidry electroblotting (Multiphor II), as described by the manufacturer, using a discontinuous buffer system. Unspecific binding was blocked for 1 h with blocking buffer (43 mM NaH_2PO_4 , 7.3 mM KH_2PO_4 , 154 mM NaCl, 0.3% dextran and 0.1% Tween 20, pH 7.4). The nitrocellulose membrane was cut into 4-mm strips and incubated overnight with patient samples diluted to about 2 kU_A/l in blocking buffer. Control strips were incubated with an NC plasma pool. The strips were washed 3 times for 5 min each in 0.9% NaCl solution with 0.5% Tween 20 (washing buffer). Bound IgE antibodies were detected by incubation with in-house ¹²⁵I-conjugated anti-human IgE monoclonal antibodies in blocking buffer for 4 h at RT. After 3 washes with washing buffer and 2 washes with distilled water, the strips were dried and exposed to a storage phosphor screen for 19 h at RT. Scanning of the screen was performed with a Typhoon TM 9410 (GE Healthcare).

Results

The yield of the affinity-purified nQue a 1 was 0.73 mg per gram pollen. The purity was >95% as determined from SDS-PAGE and silver staining, since a single band at ~17 kDa was observed under reducing conditions (fig. 1). Non-reduced nQue a 1 migrated to a slightly lower position on the gel. Sugar-specific PAS-staining analysis showed that nQue a 1 was not glycosylated (results not shown).

The purified nQue a 1 was composed of several isoforms as resolved and visualized by two-dimensional (2D) PAGE (fig. 2). By comparing the protein pattern of oak pollen extract and purified nQue a 1, it seems that most major isoforms of Que a 1 present in the extract were also included in the purified fraction (fig. 2). At least five isoforms were observed.

The purified nQue a 1 was further analyzed with mass spectrometry. The exact molecular weight of nQue a 1 was determined using MALDI-TOF. The spectrum showed a single peak representing a molecular weight of 17,147 Da (fig. 3a). MALDI-TOF MS generated a peptide mass fingerprint of trypsin-digested Que a 1 extracted from the 2D-PAGE gel (fig. 3b). MALDI-TOF/TOF MS/MS of a selected peptide (m/z : 1,829.0) combined with de novo sequencing and homology searches using the BLAST search engine resulted in a sequence highly homologous to Cas s 1 from chestnut and Que a 1 from oak (fig. 3c). This peptide was found to be present in all of the spots marked by the box in figure 2, indicating that these spots represent different isoforms of nQue a 1.

The sequence obtained by MS/MS was verified by N-terminal sequencing. This uncovered an extended N-ter-

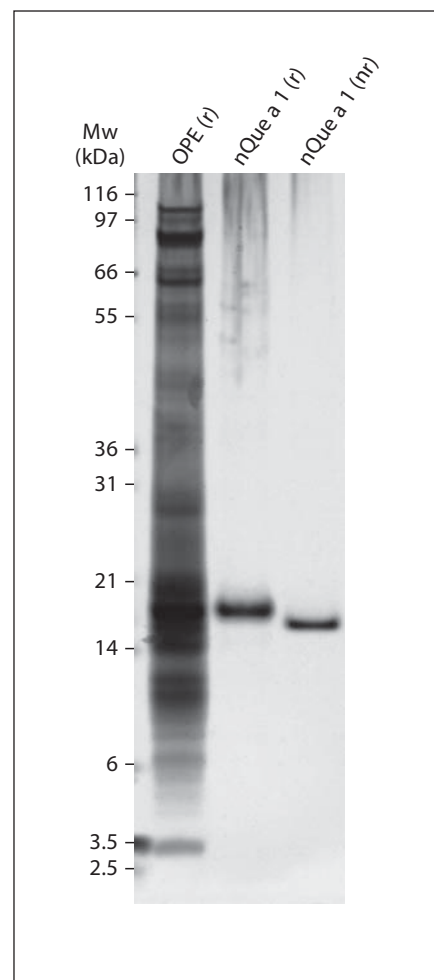
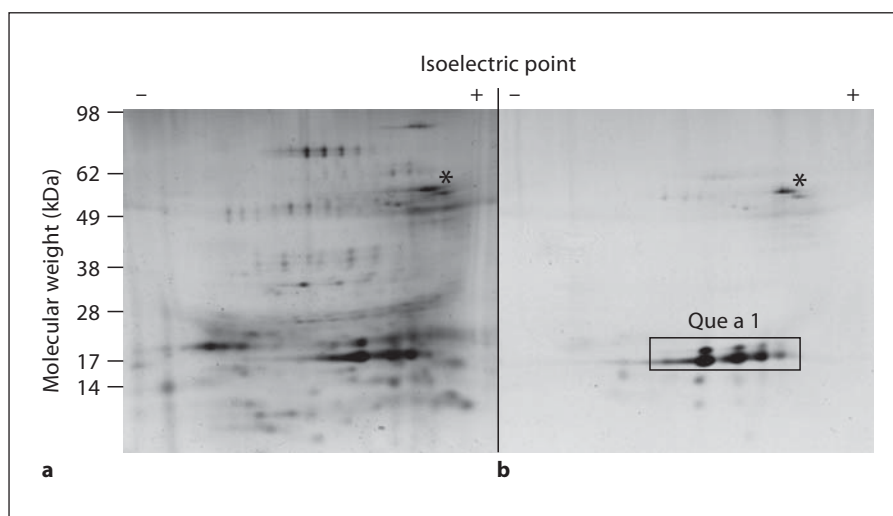


Fig. 1. SDS-PAGE of oak pollen extract (OPE) and affinity-purified nQue a 1 under reducing (r) and non-reducing (nr) conditions after silver staining.

Fig. 2. Two-dimensional PAGE. **a** 3 μ g of oak pollen extract. **b** 0.5 μ g of affinity-purified nQue a 1. Protein spots inside the box all represent Que a 1 as identified by MS analysis. Asterisks indicate an artifact frequently observed in 2D PAGE performed in our laboratory.



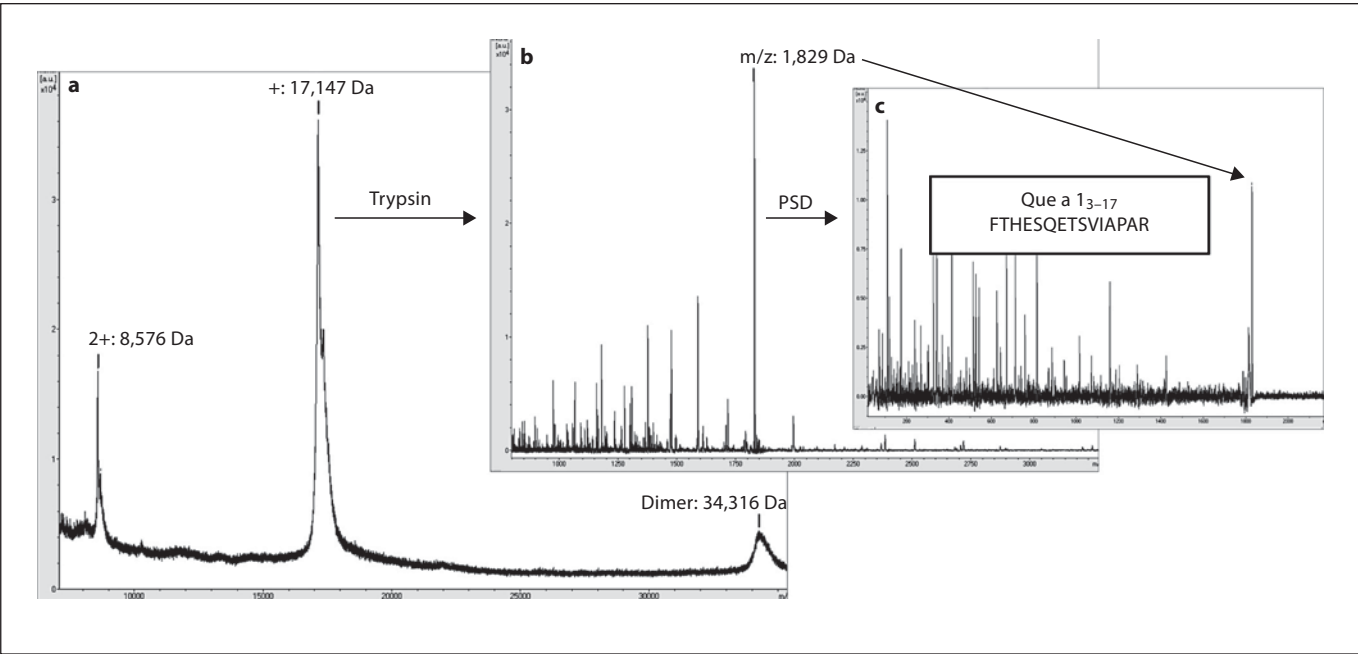


Fig. 3. MS analysis of affinity-purified nQue a 1. **a** MALDI-TOF in linear positive ion mode for molecular weight determination, m/z : 17,147 Da. The other labeled peaks represent a dimeric form of nQue a 1 (m/z : 34,316 Da) and doubly charged nQue a 1 (m/z : 8,576 Da). **b** Peptide mass fingerprint of in-gel trypsin-digested nQue a 1 generated by MALDI-TOF. **c** Spectrum of the peptide m/z : 1,829.0 after post source decay (PSD) fragmentation generated by MALDI-TOF/TOF. The displayed sequence was obtained from de novo sequencing and verified as Que a 1.

Table 1. N-terminal amino acid sequence of nQue a 1 and comparison with other PR-10 allergens

CLUSTAL FORMAT for T-COFFEE Version_1.41, CPU=13.69 sec, SCORE=63, Nseq=9, Len=60

	1	11	21	31	41	51	
Que a 1 (P85126)	-GVFTHE	TSVIAP	KALFL DSNL	IQ KVLPQAIK	STEII	EGNGG	P-----
Cas s 1 (Q9S8Q4)	-GVFTHE	TSVIPP	KAFVL-----	-----	-----	-----	88% identity
Fag s 1 (Q9ZRU8)	MGVFTY	TSVIPP	KAFVLDADNL	IPKVAPQSIK	STETLEGDGG	PGTIKKITFG	74% identity
Cor a 1 (Q39454)	MGVFN	TSVIPP	KRFVLDADNL	IPKVAPKAIK	STETLEGDGG	PGTIKKICFD	74% identity
Mal d 1 (Q4VPH9)	MGVFTY	TSVIPP	NAFVLDADNL	IPKIAPQAVK	STEILEGDGG	VGTIKKINFG	69% identity
Bet v 1 (P15494)	MGVFN	TSVIAP	KAFILDGDNL	FPKVAPQAIS	SVENIEGNGG	PGTIKKISFP	68% identity
Pru av 1 (O24248)	MGVFTY	TSEIPP	KAFVLDADNL	VPKIAPQAIK	HSEILEGDGG	PGTIKKITFG	66% identity
Aln g 1 (P38948)	MGVFN	PSVIPA	KAFILDGDKL	LPKVAPQAVS	SVENIEGNGG	PGTIKKITFP	60% identity
Car b 1 (P38949)	MGVFN	PSVIPA	KSYVLDGDKL	IPKVAPQVIS	SVENVGGNGG	PGTIKNITFA	58% identity
	***.:::	* *...*	: .**.*:	. *: *: :	* : *.*		

Unmatched amino-acid residues in nQue a 1 are bold and underlined. The underlined residue in position 49 (G, glycine) could be asparagine (N) according to the N-terminal sequencing.
 Clustal format for T-Coffee version_1.41, CPU = 13.69 s, score = 63, Nseq = 9, Len = 60.

minimal sequence of the first 50-amino-acid residues, which was compared to other known PR-10 allergens (table 1). The comparison shows a sequence identity of 88% with Cas s 1 and 58–74% with other PR-10 allergens. The obtained N-terminal sequence has been submitted to the

UniProtKB database with the assigned accession No. P85126.

The nQue a 1 showed preserved immunoreactivity in inhibition of Que-a-1-specific IgE antibodies in samples from 6 sensitized individuals (fig. 4a). The highest con-

Fig. 4. Specific IgE inhibition with affinity-purified allergen. **a** nQue a 1 and oak pollen ImmunoCAP. **b** nQue a 1 and birch pollen ImmunoCAP. **c** nBet v 1 and oak pollen ImmunoCAP. **d** nBet v 1 and birch pollen ImmunoCAP. Six individual plasma samples from Que-a-1-monosensitized subjects from the US (closed symbols) and Sweden (open symbols) were used. Two of the samples (closed triangles, US5, and closed circles, US15) are represented in the component-resolved diagnostics study (table 2 and fig. 5). Results are expressed as specific IgE binding in percent non-inhibited sample.

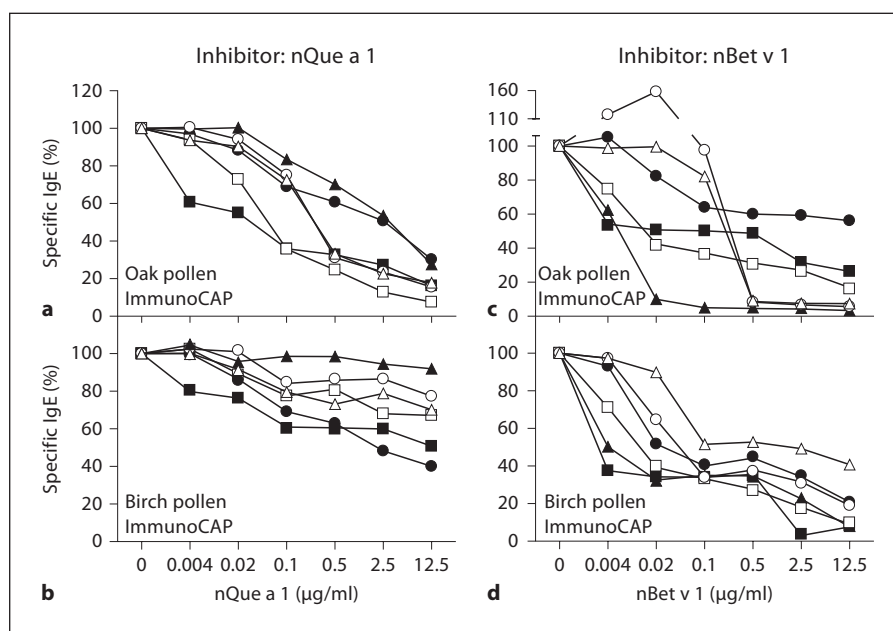


Table 2. Results from component-resolved diagnostics using ImmunoCAP®

Sample ID	Specific IgE antibodies, $\text{kU}_\text{A}/\text{l}$				
	oak pollen extract	nQue a 1	rBet v 1	rBet v 2 (profilin)	CCD (MUXF ³)
US1	72.9	90.5	>100	<0.35	<0.35
US2	1.8	2.5	14.6	<0.35	<0.35
US3	38.6	24.1	22.0	59.7	<0.35
US4	2.9	6.0	43.9	<0.35	<0.35
US5	6.7	12.6	27.8	<0.35	<0.35
US6	1.6	1.5	5.3	<0.35	<0.35
US7	3.1	6.1	36.3	<0.35	<0.35
US8	56.2	64.2	52.3	<0.35	0.5
US9	19.4	24.2	49.7	<0.35	<0.35
US10	10.2	1.2	1.6	3.6	<0.35
US11	16.6	20.2	10.6	<0.35	0.6
US12	12.5	15.3	21.0	<0.35	<0.35
US13	35.1	53.3	>100	<0.35	<0.35
US14	4.6	4.9	16.9	<0.35	1.3
US15	41.9	63.3	74.0	<0.35	<0.35
SW1	86.8	>100	>100	<0.35	<0.35
NC	<0.35	<0.35	<0.35	<0.35	<0.35

centration of nQue a 1 rendered an inhibition of 70–92% of the IgE antibodies to oak pollen extract.

Que a 1 only partly inhibited Bet-v-1-specific IgE antibodies to birch pollen extract (fig. 4b) and rBet v 1 (data not shown). Purified nBet v 1, however, was generally a

potent inhibitor of specific IgE to both oak and birch pollen, i.e. to Que a 1 and Bet v 1, respectively (fig. 4c, d). No major difference between the samples from the US and Sweden was observed.

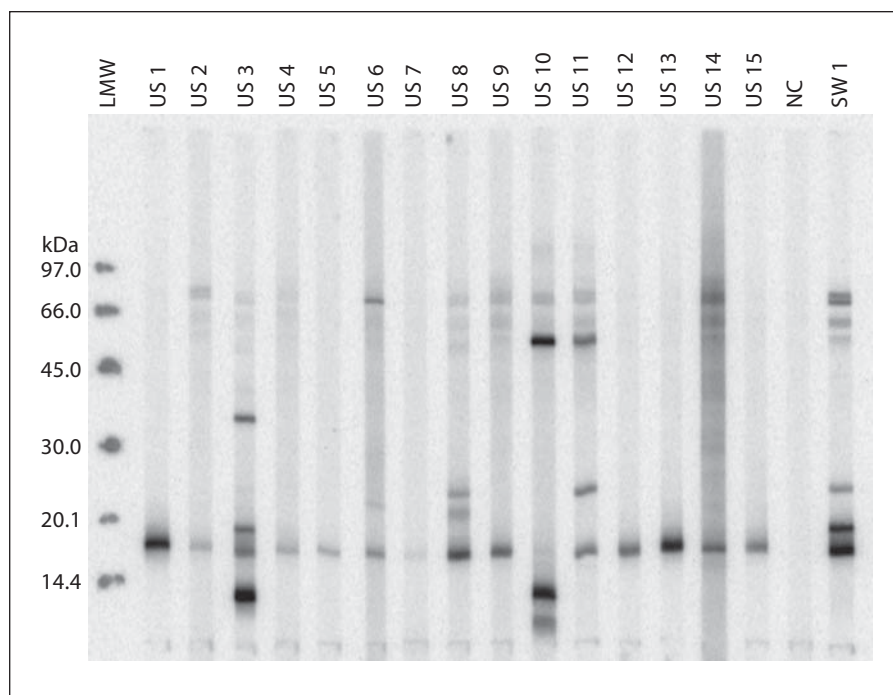
Optimal coupling concentration of nQue a 1 to ImmunoCAP was found to be around $50 \mu\text{g/ml}$. No binding of profilin- or polyclonal-reactive human IgE or mouse monoclonal/polyclonal IgG antibodies was observed, confirming the specificity of the test (data not shown).

Component-resolved diagnostics using ImmunoCAP showed a prevalence of IgE reactivity to nQue a 1 of 100% in 16 oak-pollen-sensitized individuals. All Que-a-1-sensitized subjects also had IgE antibodies to rBet v 1, in most cases at higher levels than to nQue a 1 (table 2). Two subjects had IgE reactivity to profilin (rBet v 2). Three subjects had low concentrations of CCD-specific IgE antibodies ($0.5\text{--}1.3 \text{ kU}_\text{A}/\text{l}$). The obtained results correlated well with the results from immunoblotting (fig. 5). Besides IgE reactivity to Que a 1 at 17 kDa, several subjects had weak IgE reactivity against high-molecular-weight proteins, especially to a protein around 75 kDa.

Discussion

In the present study we purified nQue a 1. The allergen was characterized and subsequently coupled to ImmunoCAP for use in component-resolved diagnostics. Que a 1 is a major oak pollen allergen that belongs to the PR-10

Fig. 5. Specific IgE immunoblotting with oak pollen extract and samples from 16 sensitized subjects from the US and Sweden (SW). NC consists of a plasma pool from non-allergic subjects.



protein family together with a number of other important allergens from tree pollen (e.g. Bet v 1, Aln g 1, Cor a 1 and Car b 1), fruits (e.g. Mal d 1, Pru av 1 and Pyr c 1) and vegetables (e.g. Api g 1 and Dau c 1) [8, 9, 22]. Previous studies have shown extensive cross-reactivity between PR-10 allergens regarding binding of specific IgE antibodies [14, 22] and monoclonal antibodies [17, 23].

We purified nQue a 1 from oak pollen extract by affinity chromatography using a monoclonal antibody developed against Bet v 1 [16]. A commonly used argument against purified native allergens is that the purity may differ between preparations. Small amounts of contaminating allergens can give rise to false-positive results and incorrect determinations of specific IgE antibody concentrations. The present one-step purification resulted in highly purified nQue a 1. No contaminating proteins were observed on silver-stained SDS-PAGE. Furthermore, 2D-PAGE and MS analysis strengthened our purity estimation as only one protein species could be detected.

The identity of the affinity-purified nQue a 1 was verified by MS analysis and N-terminal sequencing. We obtained a sequence of 50-amino-acid residues that showed 58–74% sequence identity with other known PR-10 allergens. A comparison with the 24-amino-acid residues of the N-terminal sequence of Cas s 1 from chestnut resulted in an even higher identity (88%) [24]. This is somewhat

higher than previously reported for nQue a 1 (50–56% identity with other PR-10 allergens) [15]. However, the previous comparison covered only 24 residues of Que a 1, and the N-terminal sequence contained several uncertain amino acids. The sequence presented here covers about one third of the whole Que a 1 sequence, assuming a similar number of amino acids as common PR-10 proteins.

SDS-PAGE analysis under reducing conditions showed a single protein band at ~17 kDa. The protein migrated to a slightly lower position in non-reducing SDS-PAGE, probably due to some degree of preserved secondary structure. MALDI-TOF analysis showed that nQue a 1 had a molecular weight of 17,147 Da. This is comparable with other PR-10 allergens that generally have theoretical molecular weights of 16–18 kDa.

Several allergens have been cloned and expressed as recombinant allergens. Even if many of them have shown excellent performance in in vitro assays for specific IgE measurements, purified native allergens may be good alternatives. In some cases native allergens even show better performances than their recombinant counterparts. An advantage of native allergens is that they often exhibit a higher immunoreactivity than recombinant allergens produced in bacteria [25, 26]. The reason might be that the appropriate folding of recombinants can be difficult to achieve in bacterial expression systems. In addi-

tion, preparations of native allergens often consist of a panel of different isoforms and isovariants that may express different IgE binding epitopes [27, 28]. The preserved immunoreactivity of nQue a 1 was shown by specific IgE inhibition, and 2D PAGE indicated that our nQue a 1 preparation was composed of at least five different isoforms.

Experimental nQue a 1 ImmunoCAP was developed. No binding of monoclonal or polyclonal mouse antibodies directed to profilins or calcium-binding polcalcins was observed. Similarly, human samples without IgE antibodies to Bet v 1 but with IgE-reactivity to other allergens, including the panallergens profilin and polcalcin, were all negative on nQue a 1 ImmunoCAP, confirming the specificity of the test.

It is well known that IgE reactivity to allergenic glycoproteins may be due to CCDs. The clinical relevance of IgE antibodies to CCD has been questioned, and the fact that recombinant allergen produced in bacteria lacks glycosylation has been in favor of the use of recombinant allergens in *in vitro* diagnostics [29]. The well-characterized PR-10 allergen Bet v 1 has a putative N-glycosylation site, but in common with other members of the PR-10 protein family it does not seem to be glycosylated [5]. In this study, nQue a 1 was confirmed to be non-glycosylated, since no carbohydrates were detected by the PAS staining method. Nevertheless, we used a newly developed CCD ImmunoCAP to test for CCD reactivity in our component-resolved diagnostics study.

Our study showed that Que a 1 is an important allergen in oak pollen. All 16 oak-pollen-sensitized subjects included in our study had IgE antibodies to nQue a 1. Similar prevalence figures have been shown for Bet v 1 sensitization in birch-pollen-allergic patients [6, 7, 30]. However, the prevalence of IgE reactivity to Bet v 1 differs between countries, probably due to differences in pollen exposure [7]. It can be assumed that the prevalence of Que a 1 sensitization differs between oak-pollen-sensitized populations, too. IgE reactivity to profilin (rBet v 2) and CCDs was uncommon in the present population. In the present study all but 1 oak-pollen-sensitized individual were from the US. A majority of the subjects had higher specific IgE levels to rBetv 1 than to nQue a 1. It seems likely that these subjects have been exposed predominantly to birch pollen and that the Que a 1 sensitization is the result of cross-reactive IgE antibodies originally developed against Bet v 1 [13, 14, 31]. Our cross-inhibition study using nQue a 1 and nBet v 1 supports the Bet v 1 cross-reactivity theory of acquiring Que a 1 sensitization in the 6 US and Swedish subjects studied. A major part of

the IgE binding to Que a 1 in oak pollen extract was abolished by nBet v 1 in 5 of the samples, while nQue a 1 only gave partial inhibition of the IgE binding to Bet v 1. However, even if birch trees are common in both the US and Sweden, there are also areas where oak pollen might be of great importance for the development of sensitization to tree pollen allergens.

The clinical significance of cross-reactive IgE antibodies to Que a 1 is not clear. Oak pollen allergy can be difficult to diagnose since the pollination seasons for different plants often overlap, and provocation with pollen extracts in the disease organs are generally not performed. However, from Japan it has been reported that mainly birch-pollen-allergic individuals might have symptoms during the oak pollen season due to cross-reactivity between the species [32].

The results from the component-resolved diagnostics using ImmunoCAP were confirmed using immunoblotting. A previously published immunoblotting study with American patients found that 71% of the 30 sera tested contained IgE antibodies against a 17-kDa band, probably Que a 1 [33]. The same frequency of IgE reactivity was also seen for an allergen at 74–78 kDa. We also noticed frequent IgE reactivity to an allergen in that molecular-weight range in the present immunoblot. This allergen is at present unknown.

Que a 1 ImmunoCAP offers a practical way to study IgE reactivity to the major oak pollen allergen. The preserved immunoreactivity, the presence of several isoforms and the high purity of our affinity-purified nQue a 1 preparation makes it suitable for *in vitro* assays of Que-a-1-specific IgE antibodies. Component-resolved diagnostics using nQue a 1 ImmunoCAP may be relevant in studying sensitization patterns, *i.e.* when choosing an appropriate pollen vaccine for specific immunotherapy [8]. In the future, pollen allergy may be treated with specific immunotherapy using vaccines containing recombinant allergens instead of the crude pollen extracts used today. This may lead to a more efficient treatment and a reduced risk of acquiring new sensitizations to irrelevant allergens in the pollen extract [34]. Component-resolved diagnostics using major allergens, such as Que a 1, and cross-reactive panallergens may be helpful to identify the adequate allergens to be used in the vaccine for each individual.

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