

Sal k 5, a Member of the Widespread Ole e 1-Like Protein Family, Is a New Allergen of Russian Thistle (*Salsola kali*) Pollen

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Key Words

Ole e 1-like · *Salsola kali* pollinosis · Amaranthaceae ·
Sal k 5 · Recombinant allergen · Cross-reactivity

Abstract

Background: *Salsola kali* is an Amaranthaceae weed with important repercussions for pollinosis in temperate areas. Ole e 1-like members are relevant allergens in pollen from different species. We aimed to characterize and produce as recombinant allergen *S. kali* Ole e 1-like protein. **Methods:** The natural allergen was purified at homogeneity after three chromatographic steps. Specific cDNA was sequenced and expressed in *Pichia pastoris* yeast. Structural relationships of natural and recombinant forms were carried out by 2D electrophoresis and spectroscopic analyses. Its immunological relevance was analyzed by ELISA and immunoblotting using an IgG antiserum and monoclonal antibodies specific to Ole e 1, as well as sera from 57 allergic patients recruited from two Spanish regions where this pollinosis is frequent. **Results:** The purified allergen, Sal k 5, is an acidic glycoprotein of 151 amino acid residues and 17,628 Da of molecular mass. Its amino acid sequence exhibits 68 and 32% identity with the allergens of Che a 1 and Ole e 1, respectively. The recombinant protein was correctly processed and its structural and immunologic equivalence to the natural form was proven.

A sensitization frequency between 30 and 40% was observed in pollinic patients from the center and east coast of Spain. **Conclusions:** Sal k 5 is a member of the Ole e 1-like protein family which can be considered an important allergen from *S. kali*. Its inclusion in diagnosis protocols would allow the accurate defining of patients allergic to this pollen.

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Introduction

Soil desertification of extensive zones of the Mediterranean area is enhancing the incidence of certain widespread weeds of temperate areas and salt soils, such as Chenopodiaceae species that have been recently included within the Amaranthaceae family [1]. Sensitization to pollen from these species has constituted a severe problem in semi-desert countries such as Iran, Kuwait and Saudi Arabia [2, 3]. Their resistance to drought, use as ornamental decoration or in hedges, as well as their presence as contaminants in culture areas, such as those of olive trees, has made these plants important pollinosis inducers in the Mediterranean region [4–6]. Although Russian thistle (*Salsola kali*) and Lamb's quarter (*Chenopodium album*) are the most representative pollens of the family in terms of allergenic incidence in Spain [6–9],

other species of the Amaranthaceae family such as *S. pes-tifer*, *Amaranthus retroflexus* or *S. oppositifolia* have been reported as allergenic species [10, 11]. Although these weeds have not been considered important sources of allergens because of their reduced counts of pollen grains released during the pollination season, around 40% of allergic individuals with symptoms at the end of summer in several regions of Spain are allergic to these pollens. Nowadays, in Spain, *Salsola* pollen is the third most relevant cause of pollinosis after olive and grasses [6], either in regions where allergy to this pollen is one of the major sensitization causes, for instance Zaragoza and Murcia, or in those regions such as central Spain where the wild growth of this weed is concomitant to olive tree cultivars [4, 12]. Three allergens have been identified and characterized in *C. album* pollen: Che a 1, a member of the Ole e 1-like protein family, the profilin Che a 2 and the pol-calcin Che a 3 [7]. Regarding *S. kali* allergens, Sal k 1, a pectin methylesterase, has been described as a major allergen and specific marker of *S. kali* pollen allergy [9], and the minor allergens reported, Sal k 3, a methionine syn-thase [13] and the profilin Sal k 4 [14, 15]. In addition, sera of patients allergic to *S. kali* contain IgEs able to bind to other proteins in immunoblotting with high frequency, such as 19- or 25-kDa bands that are recognized by a sig-nificant number of patients from several Spanish popu-lations. Based on the presence in most of the pollens of members of the Ole e 1-like family, the most representa-tive member of which is the major allergen from *Olea eu-ropeaea* pollen, Ole e 1 [16], and considering that another member, Che a 1, is the major allergen in *C. album* [17], our aim was focused on identifying and characterizing a homologous allergenic member in *S. kali* pollen. This broad group of allergens has been exclusively expressed in pollens and exceptionally in leaves such as those of *Sambucus nigra* [18]. These cysteine-rich proteins seem to play an essential biological role during hydration/ger-mination and pollinic tube growth [19, 20]. Some of these molecules, Ole e 1, Syr v 1, Lig v 1 and Fra e 1, are major allergens in Oleaceae pollens [9, 21, 22]. Other allergens of the family belonging to taxonomically unrelated spe-cies, such as Lol p 11 from Gramineae and Pla l 1 from Plantaginaceae, have a lower allergenic incidence in the Mediterranean area [23, 24]. Although an allergenic char-acter is associated with this protein family, suggesting that an intrinsic allergenicity could be associated with common structural motives or biochemical capacity, a nonallergenic member has recently been identified in birch [25]. The fact that this family contains important aeroallergens with significant repercussions in allergy

manifestations of many populations makes its identifica-tion essential for diagnostic purposes. The allergenic pan-el from *S. kali* deserves to be elucidated because: (i) the relevance of *Salsola* pollinosis is sharply growing in warm countries; (ii) the qualitative and quantitative differences in the allergenic content among the Amaranthaceae spe-cies extracts of *S. kali* and *C. album* are not clarified [6], and (iii) inaccurate diagnosis of patients is occurring due to the overlapping of allergenic pollens with that of *S. kali* [26]. In this work, we report the isolation and character-ization of an allergenic Ole e 1-like protein from *S. kali* pollen, as well as the analysis of its relevance among pa-tients allergic to this pollen, whose increasing allergenic relevance in South Europe make necessary its inclusion in diagnosis trials. A recombinant form has been pro-duced in *P. pastoris* and validated using the natural form, and, thus, the availability of this protein would allow its use as a tool for diagnosis of allergic patients to this spe-cies widely distributed in semi-desert areas.

Materials and Methods

Pollen Extracts, S. kali-Sensitized Patients' Sera and Antibodies

S. kali, *C. album*, and *O. europaea* protein extracts were pre-pared using pollen provided by ALK-Abelló (Madrid, Spain) as previously described [9]. Recombinant Che a 1 and Ole e 1 aller-gens were purified according to the methods described by Barderas et al. [17] and Huecas et al. [27]. Sal k 1 was purified according to Barderas et al. [9] and Sal k 4 was obtained as previously reported [15]. Fifty-seven patients sensitized to *S. kali* were recruited from Murcia, an arid coastal region, and Zaragoza, a province from the interior of Spain, fulfilling the following criteria: (1) seasonal rhi-nitis and/or bronchial asthma from May to September, (2) a posi-tive skin prick test to *S. kali* pollen extract and specific IgE values >0.7 kU/l measured by ImmunoCAP System (Thermo Fisher Sci-entific, Waltham, Mass., USA), and (3) no previous *S. kali* immu-notherapy (online suppl. table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000356345). One nonatopic serum was used as a negative control. All patients provided informed con-sent. The Ethical Committee of the Complutense University (Ma-drid, Spain) approved the protocols used for experimental work related to the use of human sera in the study. Rabbit polyclonal antiserum antibodies (pAb) against the *O. europaea* allergen nOle e 1 was used [27]. The use of animals for experimental procedures was supervised for the animal facility of the Fundación Jiménez Díaz (Madrid, Spain). Monoclonal antibodies (mAbs) OL3 and OL7 obtained against nOle e 1 were used in the analysis of the structural integrity of this protein [28]. Horseradish peroxidase-labeled goat polyclonal antibody against rabbit IgG was obtained from Bio-Rad (Richmond, Calif., USA). Mouse monoclonal anti-body against human IgE was kindly donated by ALK-Abelló. Horseradish peroxidase-labeled goat polyclonal antibody against mouse IgG was purchased from Pierce Chemical Co. (Rockford, Ill., USA).

Isolation of Sal k 5

S. kali pollen was homogenized in 0.05 M of ammonium bicarbonate, pH 8, for 3 h and protein extract was obtained after precipitation with ammonium sulfate at 70%. After extensive dialysis of the resuspended precipitate, three chromatographic steps, – a gel permeation onto a Sephacryl S-100 column in 0.2 M of ammonium bicarbonate, pH 8.0, an hydrophobic chromatography on a phenyl-sepharose column eluted with a reverse hydrophobicity gradient (5–0 M) of NaCl, and a reverse-phase HPLC on a Nucleosil C18 column with an acetonitrile gradient (0–60%) in 0.1% trifluoroacetic acid – were performed for the purification of Sal k 5. Fractions containing Sal k 5 were visualized using Coomassie Blue staining or immunostaining with the Ole e 1-specific polyclonal antibody after SDS-PAGE.

Cloning of Sal k 5

Total RNA from *S. kali* pollen was extracted as described [9]. cDNA was synthesized using the SMART RACE cDNA amplification kit (BD Biosciences-Clontech, Madrid, Spain). Specific cDNA was amplified by two rounds of PCR using four specific primers. In the first step two internal primers: 5'-GTNTAYT-GYGAYACNTGYCG-3' (VYCDTCR) and 5'-RTCRTCCTC-RTGCNCCRTC-3' (DGDHEDD), which were designed using the peptide sequences in parentheses obtained from mass spectrometry (MS) analysis, were used to obtain the nucleotide sequence encoding a polypeptide internal fragment of 60 amino acid residues. Afterwards, a second round was performed with the oligonucleotides: antisense 5'-ACCCTCCATCATGGTGCTAAC-3' (VSTMMEGA) and sense 5'-ATGTACACCATCAAAGTCGACGG-3' (MYTIKVDG) primers and the UPM unspecific primer included in the kit to get the whole nucleotide sequence of the protein. Finally, a PCR round with sense 5'-ccgctcgagaaaagaAARG-GNAAYGGNGGNCAYAAAYCT-3' (KGNNGHNL) and antisense 5'-ctgctggccgcTCARTTYTGNGTNACRCTNCCNGG-3' (PGSVTQN) primers corresponding to the N- and C-terminal ends of the mature protein was performed. The sense and the antisense primers contained an *Xho*I and a *Not*I restriction site, respectively (both underlined). The purified DNA was directly cloned into the pCR2.1 plasmid included in the TOPO TA-cloning kit (Invitrogen, Groningen, The Netherlands), used to transform TOP10 *Escherichia coli* cells and sequenced [29]. The DNA fragment corresponding to Sal k 5 was digested with *Xho*I/*Not*I restriction enzymes and subcloned into the same sites of the pPICZαA plasmid using DH5α *E. coli* cells as the hosts.

Expression and Isolation of Recombinant Sal k 5

pPICZαA/Salk5 was linearized with *Sac*I restriction enzyme and used to electroporate the KM71 strain of *P. pastoris* cells. Transformed (His⁺ Mut^s) cells were incubated on YPDS/zeocin at 30°C for 72 h in buffered glycerol complex medium until colonies appeared. The recombinant protein was detected by SDS-PAGE in the extracellular culture medium and after dialysis it was purified as described previously for the natural protein from *S. kali* pollen extract.

Analytical Methods

SDS-PAGE was performed by the method of Laemmli in 15% polyacrylamide gels in the absence or presence of 2-mercaptoethanol (2-ME). Gels were stained with Coomassie Blue R-250 (Sigma-Aldrich, Saint Louis, Mo., USA) or transferred to nitrocellulose

membranes. Molecular mass determinations were done with protein markers MW-SDS-70L (Sigma-Aldrich). N-terminal Edman degradation of the protein was performed using an ABI Procise 494 protein sequencer (Life Technologies, Carlsbad, Calif., USA). The concentration of the purified proteins was calculated by measuring the absorbance at 280 nm in a DU-7 spectrometer (Beckman, Barcelona, Spain) using the extinction coefficient ($\epsilon^{0.1\%}$) of 0.474 according to ProtParam [30].

MS Analysis

MS analysis was carried out on a Bruker-Reflex IV Matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). Peptide mapping fingerprinting and MS/MS sequencing analyses were carried out using the 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems, Carlsbad, Calif., USA) according to previously published methods [9].

Spectroscopic Characterization

Circular dichroism (CD) spectra were obtained in the far UV (190–240 nm) on a Jasco J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) [31]. The protein concentration was 0.20–0.25 mg/ml in 50 mM ammonium bicarbonate, pH 8.0. Mean residue mass ellipticities were calculated based on 113.26 as the average molecular mass/residue, obtained from the amino acid contents of these proteins, and expressed in terms of Θ (degree $\times$ cm² $\times$ dmol⁻¹). CDNN CD spectra deconvolution software (Applied Photophysics, Leatherhead, UK) was used to deconvolute the CD spectra recorded.

Carbohydrate Detection and Deglycosylation Treatment

Carbohydrate detection on protein after SDS-PAGE transferred to nitrocellulose membranes was performed using a biotinylated concanavalin A (Con A) lectin solution [32] followed by horseradish peroxidase-labeled streptavidin diluted 1:5,000. The staining was developed with 0.05% diaminobenzidine/0.03 of 30% H₂O₂ in 0.5 M NaCl, 50 mM Tris, pH 7.5 reaction buffer. Enzymatic deglycosylation was performed with PNGase F endoglycosidase (Boehringer, Mannheim, Germany) as described previously [32]. Glycosylated nOle e 1 and nonglycosylated rSal k 4 allergens were used as controls.

2D Electrophoretic Analysis

2D electrophoresis was developed in a PROTEAN IEF Cell (Bio-Rad). Isoelectric focusing was achieved under reducing conditions in the presence of 3 mM of tributylphosphine using 7-cm long, pH 3–10 linear ReadyStrip IPG gels (Bio-Rad). After isoelectric focusing, proteins were separated by SDS-PAGE in 15% polyacrylamide gels under reducing conditions in the presence of 50 mM of dithiothreitol and 3.7% iodoacetamide. 2D gels were alternatively stained with silver reagent or transferred to nitrocellulose membranes.

Immunologic Analysis

Immunodetection on membranes was achieved using two Ole e 1-specific mAbs (diluted 1:5,000), a pAb raised against nOle e 1 (diluted 1:5,000), individual sera or a pool of sera from patients sensitized to *S. kali* pollen (diluted 1:10) as described elsewhere [15]. The IgG binding was alternatively detected with a horseradish peroxidase-labeled goat anti-rabbit IgG diluted 1:3,000 (Bio-

Rad), or a horseradish peroxidase-labeled goat anti-mouse IgG diluted 1:5,000 (Pierce Chemical Co.), and the binding of human IgE was detected with a mouse anti-human IgE antibody diluted 1:5,000 (ALK-Abelló) followed by horseradish peroxidase-labeled goat anti-mouse IgG (diluted 1:5,000) [9]. For immunoblotting inhibition assays, 0.5 µg of purified protein or 50 µg of pollen extract were incubated with a pool of sera (diluted 1:10) previously incubated with 500 µg of pollen extracts from *S. kali*, *C. album* and *O. europaea*, or 5 µg of purified proteins. The signal was detected using ECL Western blotting reagent (Amersham Biosciences, Barcelona, Spain). IgE-binding quantification was performed by ELISA in microtiter plates coated with 100 ng/well of nSal k 5 or rSal k 5, or 500 ng/well for bromelain [33]. Plates were incubated with sera from allergic patients (diluted 1:10). The binding of human IgE was detected as above. IgG binding by ELISA was detected with monoclonal antibodies against Ole e 1 followed by a horseradish peroxidase-labeled goat anti-mouse IgG (diluted 1:5,000) or, alternatively, with a polyclonal antiserum against Ole e 1 followed by a horseradish peroxidase-labeled goat anti-rabbit IgG (diluted 1:3,000). OD values >0.1 arbitrary units (mean + 3 × SD of the OD of the negative control) were considered positive. For ELISA inhibition assays the plates coated with 100 ng of purified protein were incubated with a pool of sera (diluted 1:10) previously incubated with different concentrations of purified allergens or *C. album*, *S. kali* and *O. europaea* pollen extracts as inhibitors, and the signal was developed as above. Serum from a nonatopic individual was used as a negative control in the experiments.

Results

Identification of a 19-kDa Protein Band with IgE-Binding Reactivity

Twenty-eight patients allergic to *S. kali* pollen from Murcia and 29 from Zaragoza were recruited to study the allergenic profile of *S. kali* protein extract (fig. 1a). Immunoblots obtained with individual sera showed protein bands from 10–70 kDa. A high number of sera recognized a band with a molecular mass of approximately 40 kDa, of which 18 and 16 sera, respectively, were positive to the pectin methylesterase allergen Sal k 1 (online suppl. table S1) [9]. In addition, other IgE-reactive proteins with apparent sizes of 13–15, 19 and 25–27 kDa were observed. A protein band of 19 kDa was clearly visible in 14 sera (4, 5, 11, 19, 21, 22 and 23 in the upper panel, and 2, 6, 9, 11, 15, 26 and 27 in the bottom panel; fig. 1a). The molecular mass fitted well to those of the Ole e 1-like protein family. The putative presence of a homologue in *S. kali* pollen was analyzed by immunostaining of the *S. kali* extract under reducing conditions with an antibody raised against Ole e 1 on a 2D electrophoresis (fig. 1b). A set of IgG-binding spots confirmed this protein as an Ole e 1 homologue and showed the polymorphic character of this pro-

tein. To determine if it has any IgE cross-reactivity to Ole e 1-like proteins and considering that the cross-reactivity is restricted to the phylogenetic-related species, inhibition assays were performed with Che a 1, an Amaranthaceae member of this family, already established as the major allergen from *C. album*, and Ole e 1, an Oleaceae allergen, using an equivolumetric pool of the positive sera (fig. 1c). The abolishment of 80% of the IgE-binding signal with Che a 1 was observed after quantifying the bands of the blot by densitometry, indicating that this protein shares IgE epitopes with its Ole e 1-like counterpart Che a 1. However, Ole e 1 was unable to inhibit the IgE-binding signal.

Isolation and Molecular Characterization of the nSal k 5 Allergen

Three chromatographic steps, one gel permeation step by Sephacryl S-100, one hydrophobicity separation in a phenyl-sepharose column and one reverse-phase HPLC, were carried out to purify the protein at homogeneity. A profile of the last chromatographic step is shown in figure 2a. MS analysis rendered a broad peak with a maximum at 17,628 Da (fig. 2a inset). The protein migrated as a single band of 19 kDa after SDS-PAGE analysis and the glycosylated character was assessed by Con A lectin staining (fig. 2b). This allergen was approved and included as Sal k 5 following the criteria of the Allergy Nomenclature Sub-Committee of the Immunological Societies. The IgG-binding ability of Sal k 5 using Ole e 1-specific pAb and two mAbs (OL3 and OL7), as well as a pool of sera were assayed by immunoblotting (fig. 2c). From the mAbs tested, only OL3 directed against a continuous epitope was able to recognize the protein under reducing conditions. On the other hand, the IgG binding from pAb was visible under nonreducing conditions and the signal was more intense when 2-ME was added. When a pool of sera from patients sensitized to *S. kali* pollen was used, a marked decrease of the IgE-binding signal was observed in the presence of 2-ME. Therefore, the disruption of the disulfide bonds seems to affect the IgE binding and the IgG reactivity of Sal k 5 in a different manner. The pattern of spots of the purified protein obtained after 2D electrophoretic analysis and immunostaining with the pAb (data not shown) was equivalent to that obtained with the *S. kali* extract (fig. 1b). On the other hand, Edman degradation of purified Sal k 5 rendered a single N-terminal sequence of KGN. The peptide mass fingerprinting and MS/MS sequencing analyses obtained by proteomic analysis of purified Sal k 5 led to sequences VYCDTCR, TMMEGATVSIKCR,

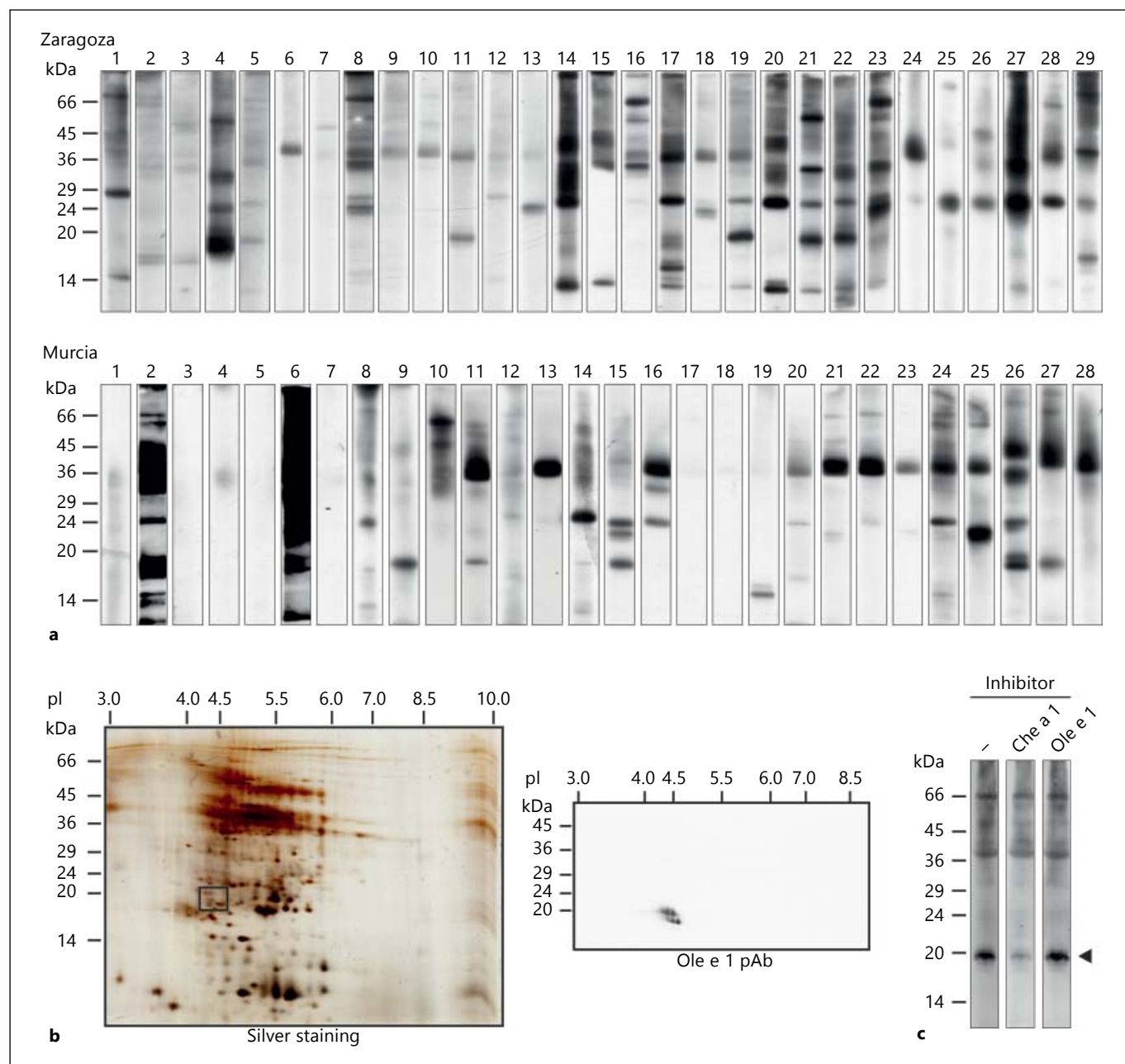


Fig. 1. Identification of a 19-kDa protein in *S. kali* pollen protein extract as an allergenic molecule belonging to the Ole e 1-like family. **a** Analysis of the IgE-binding from individual allergic patients to *S. kali* pollen extract from two Spanish populations (Zaragoza, $n = 29$, and Murcia, $n = 28$). **b** Silver staining of the protein extract (100 µg of

total protein) after 2D electrophoresis, and immunoblotting with Ole e 1-specific IgG after transference to nitrocellulose membranes. **c** Immunoblotting inhibition assay of the specific binding of a pool of sera to *S. kali* pollen extract using 5 µg of Che a 1 and Ole e 1 allergens as inhibitors, or without an inhibitor. Molecular markers are shown.

VDGDHEDDLCKLVLVK and ANALGFLK. The alignment of these sequences with proteins included in the GenBank® database revealed homology with members of the Ole e 1-like protein family, and especially to Che a 1.

Immunological Properties of nSal k 5 in Comparison to Ole e 1-Like Homologues

A prevalence of 40% in Murcia and 32% in Zaragoza was obtained by ELISA using the sera included in the study and the purified nSal k 5. To assess the cross-reac-

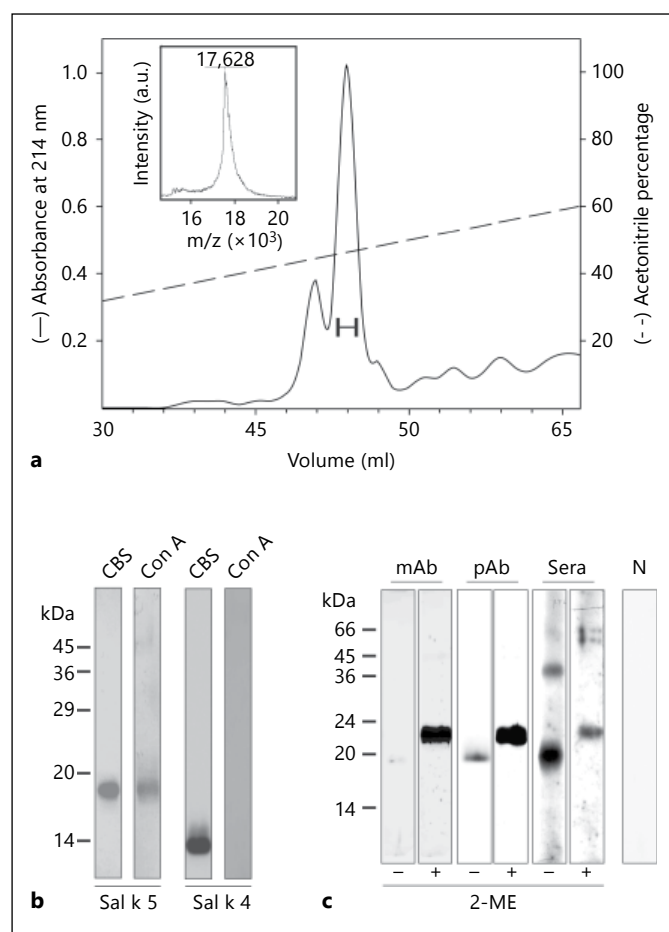


Fig. 2. Purification and characterization of the natural allergen nSal k 5 from *S. kali*. **a** Reverse-phase HPLC was the last step in the isolation of nSal k 5. The horizontal bar represents peak fractions where Sal k 5 eluted. **Inset** MS of the purified protein. a.u. = Arbitrary units. **b** Electrophoretic mobility of nSal k 5 and carbohydrate detection. CBS = Coomassie Blue staining; Con A = concanavalin A staining. rSal k 4 nonglycosylated protein was used as a negative control. **c** Immunoblotting analysis under nonreducing (–) and reducing (+) conditions with 2-ME of the IgG binding by monoclonal OL3 (mAb) and pAb antibodies raised against Ole e 1, and IgE-binding with a pool of sera and a nonatopic control (N). Molecular markers are shown.

tivity degree with Ole e 1-like allergens belonging to phylogenetically related or unrelated families, an immunoblotting inhibition analysis of the IgE binding to purified nSal k 5 was carried out using the same pool of allergic patients' sera and Che a 1 and Ole e 1 allergens from Amaranthaceae and Oleaceae families, respectively. A complete inhibition (100%) of the IgE binding was observed with 500 µg of total protein of the *S. kali* pollen extract and 5 µg of purified Sal k 5, whilst 80 and 95% was obtained with *C. album* extract and Che a 1, respectively

(fig. 3a). Lack of inhibition was observed when the olive pollen extract and Ole e 1 were used as inhibitors. These results were further confirmed by ELISA inhibition using the same proteins and extracts as inhibitors (fig. 3a). No significant correlation was observed between the individual IgE response to Sal k 5 and Ole e 1 ($r^2 = 0.0002$) with the 57 sensitized patients to *S. kali* pollen (fig. 3b). However, Sal k 5 and Che a 1 showed a correlation of $r^2 = 0.8555$, assessing the high cross-reactivity between the Ole e 1-like allergens of the Amaranthaceae family (fig. 3c).

PCR Cloning and Sequence Analysis

cDNA was synthesized from total RNA from *S. kali* pollen and used as the template for amplification of Sal k 5 by PCR using oligonucleotides designed from internal peptide sequences derived from the peptide fingerprinting analysis of the natural allergen. Two overlapping fragments corresponding to the 5' and 3' ends were used to get the Sal k 5-nucleotide sequence including the signal peptide in two rounds of PCR (fig. 4a). Finally, a PCR performed with primers designed from the C- and N-terminal ends of the mature protein rendered the whole encoding sequence of the allergen. The open reading frame of this protein contains 176 amino acids including a signal peptide of 25 residues according to the N-terminal sequence obtained by Edman degradation (accession No.: GQ427676). The theoretical molecular mass was 16,448.6 Da and a isoelectric point, pI, of 5.02 was obtained without the signal peptide. Sal k 5 contains a putative N-glycosylation consensus site at position 43, this feature being associated with the structural relationships among members of the Ole e 1-like family from phylogenetically related species. The sequence identity of Sal k 5 with Che a 1 was 68%, but only 42, 26 and 32% with Phl p 11, Pla l 1 and Ole e 1, respectively (fig. 4b).

cDNA Expression and Purification of rSal k 5

The cDNA encoding the mature sequence of Sal k 5 was subcloned into the pPICZaA expression vector. The construction pPICZaA/Sal k 5 was used to transform *P. pastoris* KM71 cells to produce the secreted allergen by means of the AOX1 promoter induction with a yield of 40 mg/l of culture of the extracellular medium. A major band of approximately 21 kDa was visualized by SDS-PAGE. After dialysis of the culture medium, a three-step procedure, involving ionic exchange (DEAE-cellulose), size exclusion (Sephadex G-75) and RP-HPLC chromatographies, was performed to purify the recombinant protein (fig. 5a).

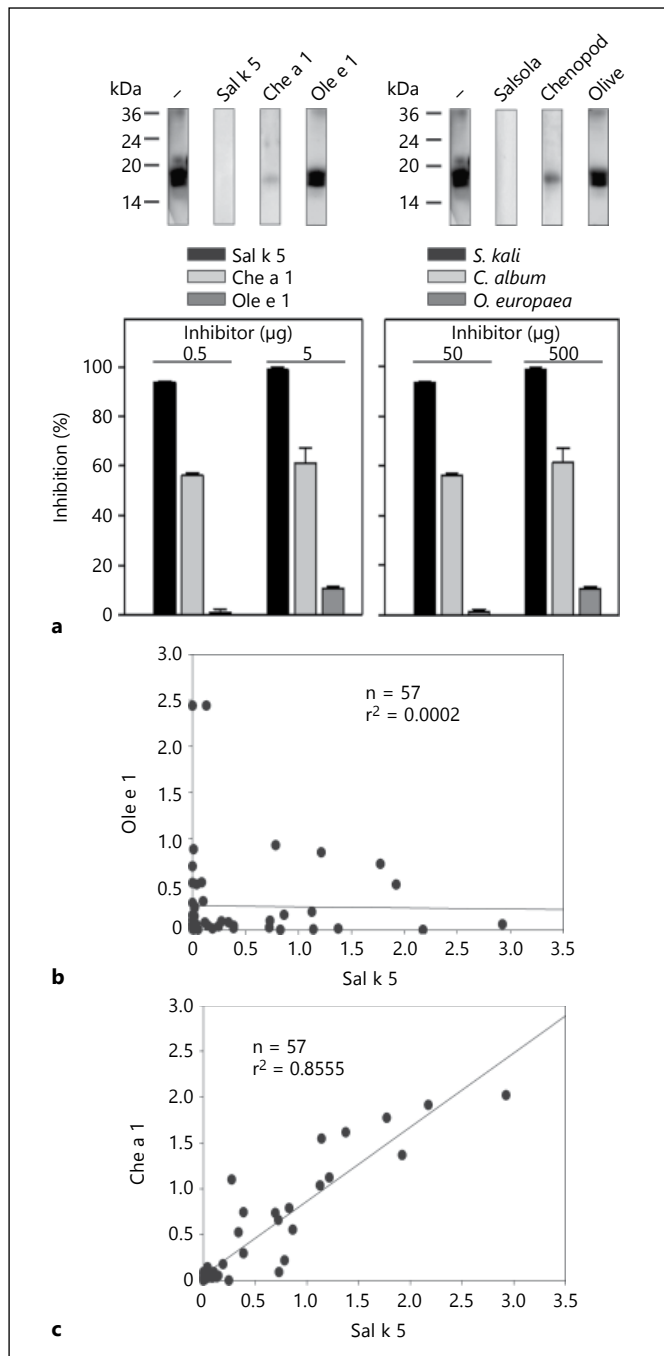


Fig. 3. IgE cross-reactivity between allergens from the Ole e 1-like family. **a** Top, immunoblotting inhibition assays of the IgE binding to nSal k 5 with purified proteins, or pollen extracts from *S. kali* (Salsola), *C. album* (Chenopod) and *O. europaea* (Olive). Molecular markers are shown. Bottom, ELISA inhibition assays of the IgE binding to nSal k 5 with 0.5 and 5 µg of purified allergens, or 50 and 500 µg of *S. kali*, *C. album* and *O. europaea* pollen extracts. **b**, **c** Regression plot of the specific IgE values of the 57 patients' sera measured by ELISA to nSal k 5 in comparison with those of Ole e 1 or Che a 1. The calculated correlations (r^2) observed for nSal k 5 versus Ole e 1 and nSal k 5 versus Che a 1 were included.

Validation of rSal k 5 with its Natural Counterpart nSal k 5

Structural and immunological comparisons of nSal k 5 and rSal k 5 were carried out to assess the integrity of the recombinant form. To that end, CD analyses of both forms were performed in the far UV. The ellipticity of nSal k 5 with rSal k 5 was almost equivalent, giving very similar values of secondary structure elements (fig. 5b). MS analysis of rSal k 5 rendered a major peak of 18,561.4 Da, and several minor peaks with differences of 160–162.5 Da, which would correspond to different mannose residue content (fig. 5c). rSal k 5 was treated with PNGase F endoglycosidase to assess its N-glycosylation character. Treated and untreated samples were subjected to SDS-PAGE (fig. 5d). No deglycosylation of the natural protein was achieved, which suggests the presence of fucose monosaccharides in the glycan structure as also occurs with Ole e 1, where fucose-containing oligosaccharide are not removed by PNGase F. However, a partially deglycosylated recombinant form was observed from a mannose-rich glycan. A comparative analysis of the IgG- and IgE-binding activities of rSal k 5 and nSal k 5 was performed by immunoblotting and ELISA. IgG binding of mAb and pAb to both proteins were similar and, therefore, no significant differences could be attributed to the polymorphism of the natural protein (data not shown). The 18 sera, which resulted positive to purified nSal k 5 were tested for IgE binding by ELISA (fig. 6a) and immunoblotting (fig. 6b) to both natural and recombinant proteins, showing a close correlation between both forms ($r^2 = 0.78$). Nine sera better recognized rSal k 5 and 9 better recognized nSal k 5 by ELISA. IgE binding was also analyzed by means of ELISA inhibition with an equivolumetric pool of 5 sera. nSal k 5 and rSal k 5 were coated in ELISA plates and 5 µg of both allergenic variants were used as inhibitors (fig. 6c). The shape of the two curves was similar when rSal k 5 was coated in the wells, whereas rSal k 5 was not able to reach a total inhibition of the IgE binding to nSal k 5 coated in the wells, indicating the contribution of the specific glycosidic group of nSal k 5 to the IgE binding, or, alternatively, the loss of some of the epitopes on the recombinant form.

Discussion

The geographic distribution of pollen from different species is strongly influenced by geo-climatic conditions and thus contributes to the sensitization profile of the allergic patients living in a particular area [34]. In the south-

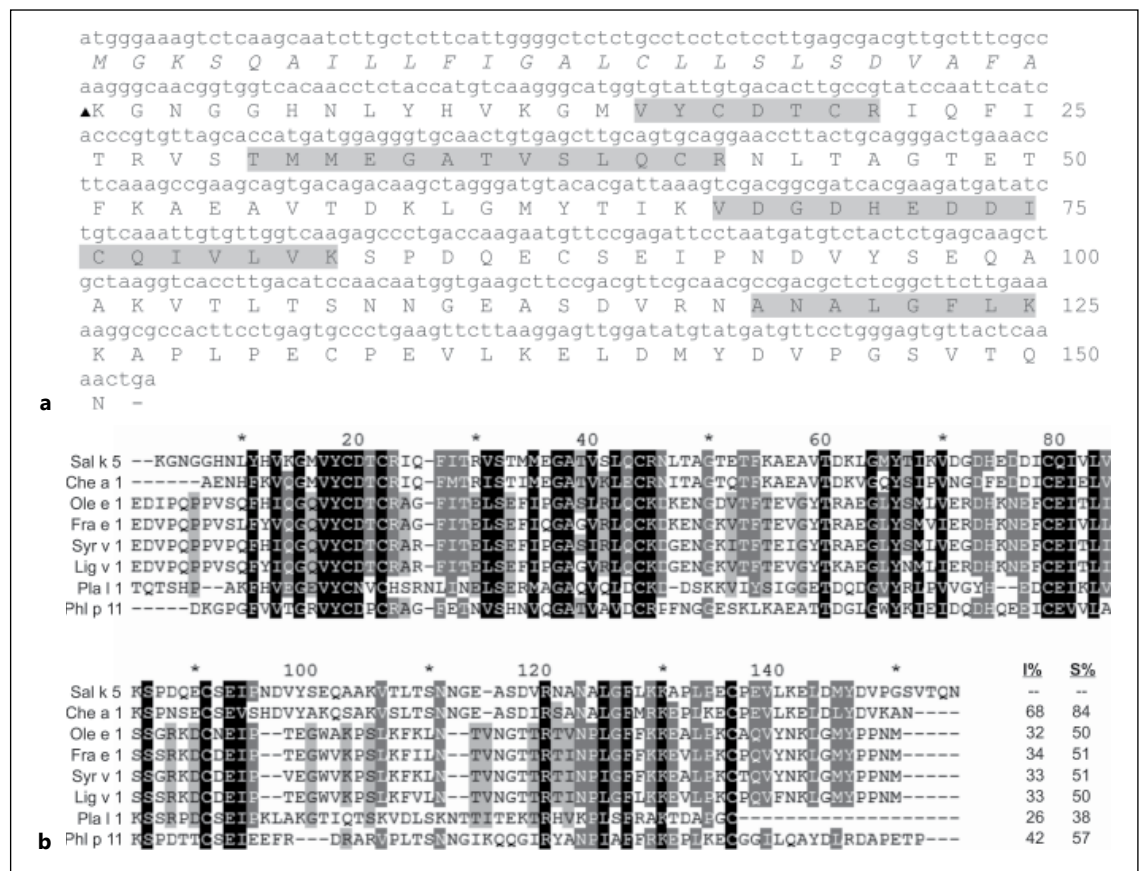


Fig. 4. Amino acid and nucleotide sequences of Sal k 5. **a** Nucleotide sequence and deduced amino acid sequence of the cDNA encoding Sal k 5. Peptide sequences showing homology to peptides obtained by MS are highlighted in gray. The amino acid sequence in italics corresponds to the signal peptide; the arrowhead indicates the processing site. **b** Amino acid sequence alignment of Sal

k5 and other members of the Ole e 1-like family: Sal k 5 (GQ427676), Che a 1 (Q8LGR0), Ole e 1 (C53806), Fra e 1 (AY652744), Syr v 1 (S43242), Lig v 1 (O82015), Pla l 1 (AJ313166) and Phl p 11 (Q8H6L7). Percentages of identity (I%) and similarity (S%) using Sal k 5 as reference are shown.

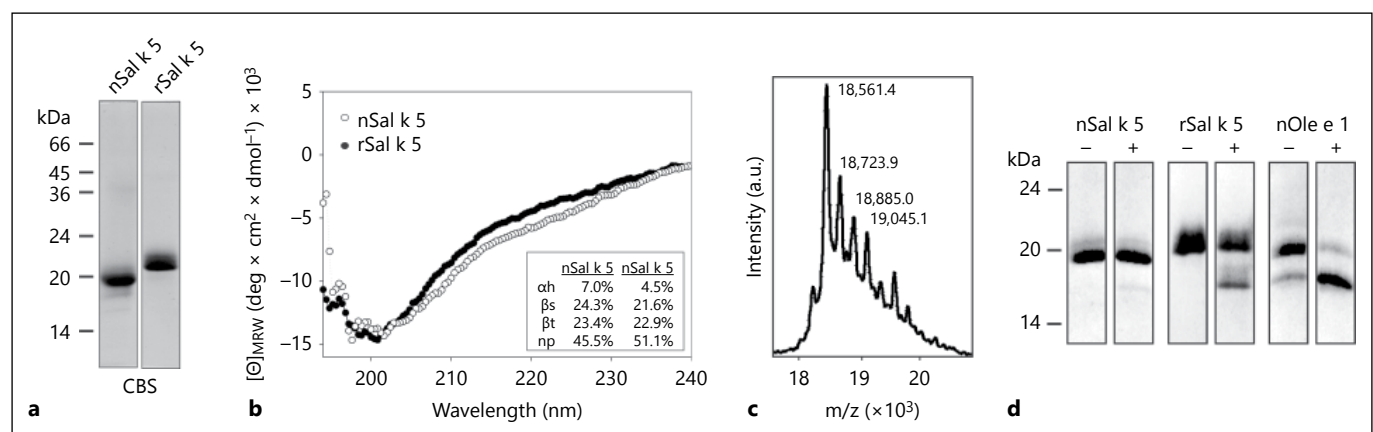


Fig. 5. Molecular comparison of recombinant and natural purified Sal k 5. **a** Coomassie Blue staining (CBS) after SDS-PAGE. **b** Far UV CD spectra. αh = Alpha helix; βs = beta sheet; βt = beta turn; np = nonperiodic conformation.

c MS of rSal k 5. a.u. = Arbitrary units. **d** Deglycosylation of both forms compared to nOle e 1 by endoglycosidase F. Molecular markers are shown.

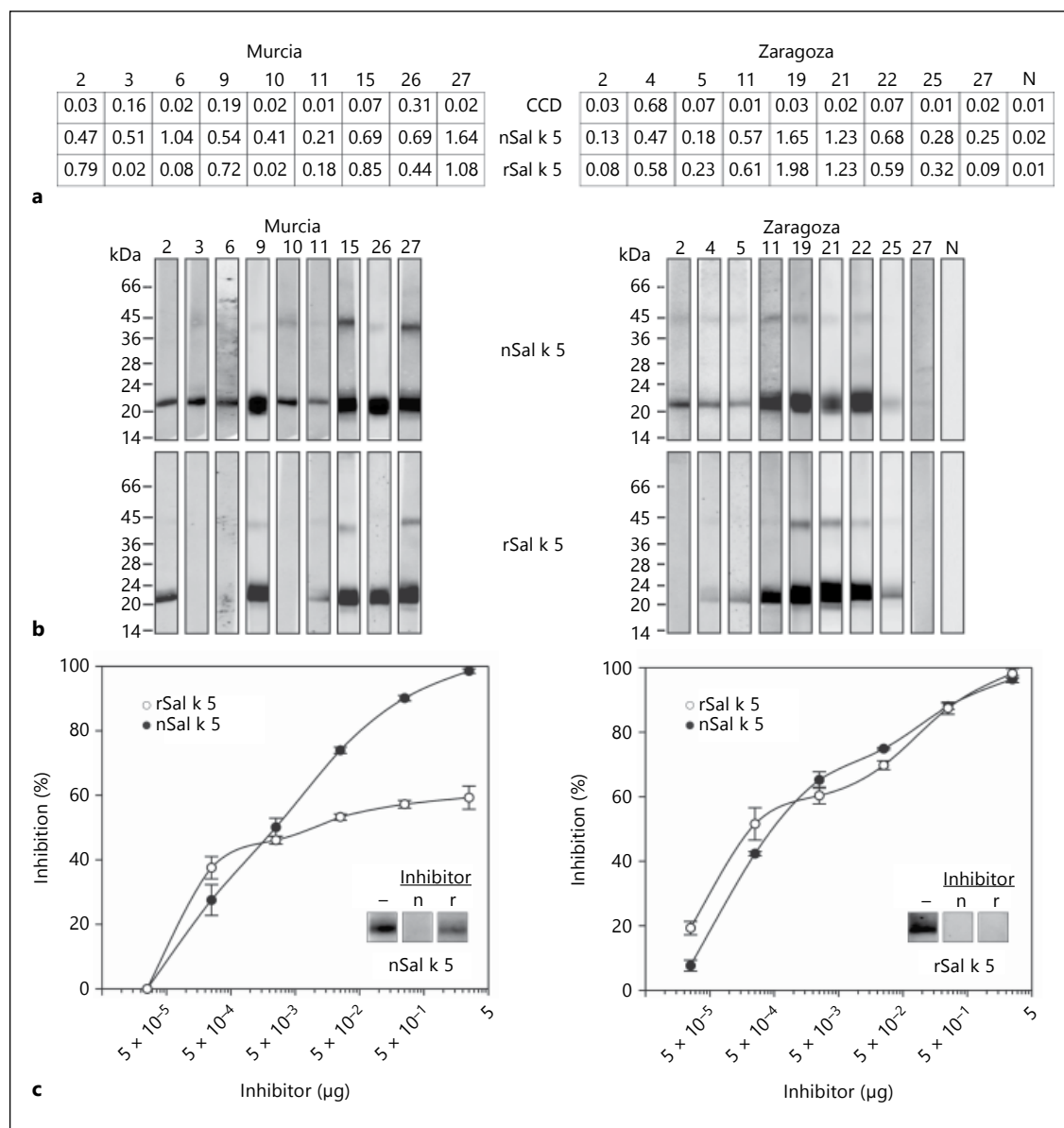


Fig. 6. Analysis of the IgE binding to natural and recombinant Sal k 5 of 18 sera from allergic patients. **a** Specific IgE-binding values measured by ELISA to CCD (Bromelain), nSal k 5 and rSal k 5. **b** Immunoblotting staining; a nonatopic serum (N) was used as a negative control. **c** IgE ELISA inhibition assays with nSal k

5-coated wells (left panel) and rSal k 5-coated wells (right panel), using a pool of sera and the same proteins as inhibitors; the same inhibition experiment of each panel performed by immunoblotting are also shown.

eastern and central areas of Spain, where extensive irrigation is turning grass areas into semi desert, *Salsola* is the first cause of seasonal allergy in parallel to olive or grass pollinosis [4, 6, 35]. Amaranthaceae IgE-binding profiles are complex. The immunoblotting of *S. kali* protein extracts with individual sera from selected patients allergic to Amaranthaceae pollens rendered many IgE-reactive bands, the identities of which have not been completely

elucidated. Different IgE-binding profiles were detected from individual blots of both populations used in this study. Patients from Zaragoza recognized a higher number and variety of IgE-reactive bands than those from Murcia, whereas the latter ones preferentially recognized bands of 19, 28 and 40 kDa. One of the proteins detected at 40 kDa is Sal k 1, which has been considered a specific marker to the primary sensitization to *S. kali* [9]. How-

ever, significant inter- and intraspecies cross-reactivity has been attributed to different allergens already described in this family, such as Che a 2 and Sal k 4 (profilins) or Che a 3 (polcalcin) [7, 8, 15, 36]. In this report, we have identified a new allergen from *S. kali* pollen belonging to the widespread Ole e 1-like family, which can explain the IgE cross-reactivity with other Amaranthaceae members. Interestingly, the cross-reactivity between Ole e 1-like members has always been associated with those phylogenetically related allergens [17, 37–39]. The reason for these IgE-binding differences is based on their amino acid sequence, which is determinant for the interaction between the IgE immunoglobulin and the allergen. Thus, the identities among Oleaceae members – Ole e 1, Lig v 1, Syr v 1 and Fra e 1 – are around 85%, and among Amaranthaceae allergens – Che a 1 and Sal k 5 – are 68%. However, a high sequence variability exists between Sal k 5 and the Ole e 1-like allergens from phylogenetically nonrelated species – Ole e 1, Pla l 1 or Phl p 11 – the identity degree being 32, 26 and 42%, respectively. These low values notably reduce the possibility of IgE cross-reactivity among these allergens [40]. In fact, the important amino acid sequence differences observed between Ole e 1 and Sal k 5 could explain the low IgG- and IgE-reactivities between these two allergens and the fact that epitopes hidden in the native structure of the allergen are exposed to the IgG binding only when the unfolding of the molecule is produced. These results were also observed when Ole e 1 and Che a 1 were immunologically compared [17]. Here, we observed similar findings pointing to the high cross-reactivity between phylogenetically related allergens and an opposite situation for phylogenetically nonrelated allergens. The IgE binding to Sal k 5 using a pool of sera rendered a complete inhibition when Sal k 5 and *S. kali* extract were used as inhibitors, and a nearly complete inhibition was reached with Che a 1 and *C. album* extract. Neither Ole e 1 nor *O. europaea* extract were able to inhibit the signal. Finally, prevalence higher than 30% for natural Sal k 5 purified from pollen was reached in the two populations of sera herein analyzed from patients allergic to *S. kali*, indicating the importance of Sal k 5. The yeast *P. pastoris* has been selected as the expression system to produce rSal k 5. Stability, glycosylation and disulfide bond formation are structural features to consider when choosing the expression system to produce a protein. The success of this selection is herein simplified by the fact that many members of this family had been efficiently produced in this eukaryotic system [17]. Sal k 5 was secreted to the extracellular medium of the yeast cells. The recombinant protein displayed the overall fold of these

protein families, which corresponds to that of unstructured proteins as determined by spectroscopic analysis and was also glycosylated. However, the molecular mass difference between the natural and the recombinant protein is assumed due to a bigger size of the oligosaccharide incorporated in the N-glycosylation consensus site of Sal k 5. This should be composed of a core of N-acetylglucosamine and a group of 10 mannose residues for the higher intensity peak, and 1 extra mannose of 160 Da for each additional peak for rSal k 5 (fig. 5c). This is in contrast to nSal k 5, where fucose monosaccharides were present in the glycan structure, as also occurs in Ole e 1 glycosylated molecules. However, the recombinant allergen with a putative rich-mannose glycan component added by the yeast is partially deglycosylated by the enzyme. The glycosylation consensus site in these proteins seems to be coincident in all members of the same botanical family. Thus, a common N-glycosylation consensus site at Asn43 or Asn39 for Sal k 5 and Che a 1, respectively, was observed. However, a different site for the Oleaceae proteins, at Asn110 in Ole e 1, Syr v 1, Fra e 1 and Lig v 1, or Gramineae proteins, at Asn24 in Lol p 11 or Phl p 11, has been identified. Interestingly, the birch member of this family, which is neither glycosylated nor contains a consensus site in its amino acid sequence, is the only Ole e 1-like member described so far which lacks the IgE-binding ability [25]. Another structural feature that defines this family of allergens is their polymorphism, which was also observed for Sal k 5 by 2D-PAGE, although significantly lower than that observed for Ole e 1 with more than 10 different isoform sequences identified [41]. An essential requisite to include a recombinant allergen in a diagnosis protocol is its accurate validation of structural and immunologic parameters, mainly its IgE-binding capacity, and taking as reference the natural molecule. However, in most cases this comparison is exclusively made using the complete pollen extract where the natural allergen is expressed but in so low amounts that its isolation is a hard task. Herein, the availability of both forms of Sal k 5 allowed us to accurately perform these analyses. The spectroscopic data as well as the immunologic inhibition assays confirmed the integrity of the recombinant protein and the presence of most of the B cell epitopes. The lack of a total inhibition of the IgE binding to nSal k 5 using rSal k 5 as the inhibitor suggests a possible involvement of the glycan moiety in the IgE reactivity, as occurs with Ole e 1, since a different carbohydrate component is incorporated in the N-glycosylation site by the yeast. This different glycosylation could be one of the reasons for the different IgE response between natural and recombinant

allergens in some of the patients (No. 3 and 23, Murcia). Another possibility could be the absence of certain B epitopes, present in this polymorphic natural protein, in the recombinant isoform. By contrast, rSal k 5 could be better recognized by the IgE of those patients sensitized to the isoform selected for recombinant production (No. 2, Murcia), which would be diluted in the mixture of isoforms constituting the natural allergen. In summary, *S. kali* pollen extract could be enough for the diagnosis and treatment of most representative Amaranthaceae pollen allergies, since it contains a specific and major allergen (Sal k 1) and is able to inhibit the IgE binding of all the other already known allergens from *C. album* pollen. Moreover, the availability of Sal k 5, together with Sal k 1 [9] and panallergens Sal k 4 [14, 15] and polcalcin as molecular tools to establish a diagnostic algorithm, could enable the stratification of patients depending on their sensitization: Sal k 1 as a marker of allergy to *S. kali* pollen

[9], Sal k 5 as an index to Amaranthaceae sensitization, and finally Sal k 4 [14, 15] and polcalcin as markers of polysensitization. Furthermore, the prevalent presence of these species in warm and dry areas as well as the relevance of this allergenic Ole e 1-like family makes the new member of *S. kali* pollen a suitable candidate to optimize diagnosis protocols.

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