

Original article

Immunochemical characterization of Russian thistle (*Salsola kali*) pollen extracts. Purification of the allergen Sal k 1

Background: *Salsola kali* (Russian thistle) is a weed which belongs to the Chaenopodiaceae family. It is widely distributed along the coasts of Europe, North Africa, USA and Australia. The objectives of this study were to study the allergenic composition of *S. kali* pollen and to purify an important allergen from the pollen extracts of this plant.

Methods: A population of 66 individuals with specific IgE-mediated allergic symptoms and positive skin tests to *S. kali* were included in the study. Specific IgE to *S. kali* was determined by direct enzyme-linked immunosorbent assay (ELISA). The antigenic and allergenic profile of *S. kali* was evaluated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focussing (IEF) and immunoblot. Allergen purification was conducted by preparative SDS-PAGE. The allergenicity of the protein was evaluated by skin testing, direct ELISA, ELISA inhibition and immunoblots.

Results: Specific IgE to *S. kali* was detected in 39 of the 66 individuals (59%). An allergen with a molecular weight of approximately 43 kDa was purified. This allergen was termed Sal k 1. A partial sequencing was obtained and no homology was found with other known proteins/allergens. The allergenicity of Sal k 1 was tested *in vitro* and *in vivo*. Of the 39 individuals with a positive specific IgE determination to *S. kali*, 26 (66.6%) had detectable specific IgE to Sal k 1. Twenty of these 39 individuals were skin-prick tested with the purified allergen (0.5 mg/ml) and all of them had a positive skin test to the purified allergen. Ten additional individuals, used as negative controls, had a negative response.

Conclusions: Sal k 1, an important allergen of *S. kali*, is recognized, *in vitro*, by approximately 67% of the patients sensitized to *S. kali*. Twenty patients with a positive skin test to a standardized *S. kali* extract had a positive reaction to the purified allergen.

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The family Chaenopodiaceae contains several genera, such as *Chenopodium* and *Salsola*, which are responsible for allergic sensitization in Europe and North America (1). These plants, especially tumbleweeds, are also abundant in several semi-desertic regions of the United States and central Australia. *S. kali* is a typical plant of salty soils (2), where rainfall is not abundant. The genus *Salsola* is one of the better known genera of the Chaenopodiaceae family and several species have been described, including *S. pestifer*, *S. vermiculata* and *S. soda*. One of the better known species is *S. kali* (Russian thistle). *S. kali* is a summer flowering, annual weed, which as mature plants form spherical bushes. Once they start aging, the plant breaks at the soil line and becomes a tumbleweed (3). In this shape, the plant is blown by the wind, spreading between 20 000 and 50 000 seeds.

In the USA, *Salsola* spp. are found along the coast from the northeast to the west (4). In Europe, it is also very common in coastal areas from the Baltic Sea to the Mediterranean coast. In Spain, *S. kali* is very common in Aragón, Andalucía, Murcia and Levante. In these areas, the pollen load may represent up to 5% of the total pollen, being responsible for many allergic sensitizations (5).

In 1933, Lamson et al. (6) described the first cases of sensitization to this pollen in Arizona. In 1978, Powell et al. (7) described two cases of contact dermatitis after exposure to tumbleweed. Shafiee et al. (8) described nine Iranian patients with hypersensitivity to pollen of *Salsola* spp., who showed positive skin-prick tests to Russian thistle and detectable levels of specific IgE. In some regions of Spain, with a high concentration of Russian thistle pollen, more than 30% of the allergic patients who

inhabit these areas are allergic to this pollen and exhibit a positive skin-prick tests and symptoms upon exposure (5, 9).

The aims of this study were to analyze the antigenic and allergenic profile of *S. kali* extracts, to purify and characterize an important allergen and to evaluate the *in vivo* and *in vitro* responses to this purified allergen.

Material and methods

Extract manufacturing

Salsola kali pollen (Biopol Laboratory Inc., Washington, USA) with a purity >95% was used. Fifty grams of pollen were extracted 1 : 5 (w/v), in phosphate-buffered saline (PBS) 0.01 M (pH 7.4) for 4 h at 4°C. Afterwards, the extract was centrifuged at 16 000 g at 4°C for 30 min, and the supernatant collected. The remaining pollen was reconstituted 1 : 5 (w/v) in PBS 0.01 M (pH 7.4) and extracted again for 18 h. The extract was centrifuged at 16 000 g, the supernatant separated and mixed with the first extract obtained, sterile filtered and dialyzed against twice-distilled water. The extract was sterile-filtered, frozen and freeze-dried. The protein content of the extract was measured by the Lowry-Biuret method (10) (Sigma, St Louis, MO, USA).

Patient population

A total of 66 patients from the Zaragoza area (Spain) were entered in the study. All patients suffered from IgE-mediated intermittent allergic rhinoconjunctivitis and/or allergic asthma and met the criteria outlined by Johansson et al. (11). All patients were skin-prick tested with a standardized extract of *S. kali* (100 HEP; CBF LETI, S.A. Madrid, Spain). Patients with a wheal diameter >3 mm were considered positive (12) and were asked to donate a serum sample. Serum samples of each patient were stored at -20°C.

Direct ELISA

All 66 sera were evaluated by direct enzyme-linked immunosorbent assay (ELISA) to determine specific IgE binding against whole *S. kali*. Each serum was diluted 1 : 2 in PBS 0.01 M and incubated for 2 h in plastic microtiter plates previously coated with 10 µg of protein/ml of a *S. kali* extract (Immulon IV, Dynex Technologies, Chantilly, USA). Three individual sera from non-allergic patients were used as negative controls. Optical densities ≥4 times the median values of negative control were considered to be positive. A *S. kali* serum pool was prepared with the sera of 39 positive individuals. The sera of 39 patients were further evaluated by direct ELISA to determine specific IgE binding to the purified allergen.

SDS-PAGE, and immunoblot analyses of the *S. kali* extract

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and IEF analyses were used to determine the antigenic profile of the whole *S. kali* extract. The samples were run in SDS-PAGE gels with 2.67% C, 15% T acrylamide/bisacrylamide as described by Laemmli (13). Fifty micrograms of protein was loaded on the gels. The gel was electrophoretically transferred to P-Immobilon membrane (PVDF) (Millipore, Bedford, USA) and dried at room temperature for 4 h. The

membrane was incubated overnight with a pool of sera diluted 1 : 5 (v/v) in PBS 0.01 M Tween 2%. The antigenic and allergenic profiles were studied with the Scanner Sharp JX-330 and analyzed with Image Master 1-D Elite v 2.01 (Pharmacia Biotech, Uppsala, Sweden). Based on specific IgE binding, a protein of approximately 43 kDa was selected.

Protein purification

The 43-kDa molecule was purified by gel filtration chromatography and continuous elution electrophoresis. The freeze-dried *S. kali* extract was diluted in PBS 0.01 M and separated by gel filtration chromatography in a fast performance liquid chromatography (FPLC) system (Pharmacia). Sephadex G-75 was used as matrix and the sample was eluted with PBS 0.01 M at a constant flow rate of 1.5 ml/min in a 1 m × 3 cm column. A chromatogram was obtained representing separated proteins and pigments. All the fractions corresponding to absorption peaks of proteins were mixed and freeze-dried. The pigments were eliminated. Further purification was conducted using a 491 Prep Cell (BioRad, Hercules, CA), adapting the suggested method to our specific condition. A large gel tube (37 mm of diameter) was used, with a total separating gel length of 10 cm. The gel composition for separating gel was 10% T of acrylamide/bisacrylamide and for the stacking gel length was 2.5 cm and 2.67% T of acrylamide/bisacrylamide. Five milligrams of *S. kali* extract, diluted in sample buffer under non-reducing conditions were loaded onto the gel. The elution was made with SDS buffer elution at 1 ml/min. The fraction containing the purified protein was dialyzed in a 10 kDa dialysis membrane against twice-distilled water, sterile filtered and freeze-dried. The freeze-dried sample was reconstituted in twice-distilled water at 40 mg/ml and stored at -20°C. A skin-prick test solution of the purified allergen at a concentration of 0.5 mg/ml was also prepared. A total of 20 patients and 10 controls were skin tested with this purified allergen.

Sequencing of Sal k 1 by mass spectrometry

The N-terminal of the protein was blocked. The protein was fragmented with trypsin to obtain internal peptide sequences. The protein was run in a SDS-PAGE gel under non-reducing conditions. Slices were cut from the gel and washed in 200 mmol/l ammonium carbonate (pH 8.9) for 30 min and hydrated with 200 mmol/l ammonium carbonate (pH 8.9) 0.02% Tween 20. Enzymatic digestion was accomplished by addition of trypsin [1 : 4 (w/w)] using a slightly modified procedure (14).

An aliquot of 0.5 µl was analyzed by MALDI-TOF mass spectrometry by a process based on Konecny et al. (15). Analysis by MALDI-TOF mass spectrometry were performed using a Kompact Probe instrument (Kratos-Shimadzu, Manchester, UK).

ELISA inhibition

The *S. kali* extract and the purified protein were tested by ELISA inhibition to evaluate the allergenic potency. Plastic microtiter plates (Immulon IV) were coated with *S. kali* extract (1 µg of protein/well). Several dilutions were made from whole extract and from the purified protein. Each dilution was incubated with the serum pool [diluted 1 : 5 (v/v)] for 2 h at room temperature. Afterwards, the dilutions were transferred to the *S. kali*-coated plates and incubated overnight. Allergenic activity was evaluated by establishing the 50% of inhibition point.

Results

Protein and allergenic profile

The protein content of whole extract was 815 µg of protein/mg of freeze-dried material. SDS-PAGE from pollen extract of *S. kali* showed several proteins with a molecular weight (MW) between 8 and 97 kDa. The most prominent band had a MW of approximately 43 kDa. Other predominant bands were identified at 15, 17, 18, 29, 58, 69, 74 and 97 kDa (Fig. 1A). The allergenic profile was studied by immunoblot. Bands that were more frequently recognized had MWs between 36 and 90 kDa, especially those with MWs of 43, 57, 69 and 81 kDa (Fig. 1B).

The serum pool recognized just one band when incubated with the purified extract (Fig. 1B). The purified protein was visualized as a single band of approximately 43 kDa (Fig. 1A) and showed six bands within the pI range of 5.5 and 7 (data not shown).

Direct ELISA to the total extract and to Sal k 1

The sera of 66 patients were evaluated for specific IgE binding to the whole *S. kali* extract and to the purified protein; 39 patients (59.1%) had detectable specific IgE levels against the total *S. kali* extract. Of these 39 patients, 26 (66%) had specific IgE against the purified protein.

ELISA inhibition

The purified allergen and the whole *S. kali* extract were tested by ELISA inhibition to investigate the biological activity of Sal k 1. The two curves had a correlation

coefficient (r value) >0.95 and passed the test for parallelism using the whole *S. kali* extract as a reference. The experiments revealed that IgE binding to the whole *S. kali* extract was greater than to the purified allergen; 1.12 µg of protein were necessary to reach 50% inhibition for the whole extract, whereas 2.08 µg were needed for Sal k 1 (Fig. 2). It was estimated, that the content of Sal k 1 represents approximately 24% of the total protein in the whole extract by scanning densitometry (16).

Skin tests

Of the 39 individuals with a positive specific IgE determination to *S. kali*, 20 individuals were further skin-prick tested with Sal k 1. All of the positive patients recognized the whole *S. kali* extract and the purified protein. The geometric mean of the wheal size were 44.9 mm² (range 7–127) for the whole extract and 70.9 mm² (range 17–195) for the purified protein. A box-plot view of the results is shown in Fig. 3. The statistical analysis (Wilcoxon signed rank test) showed significant differences ($P < 0.01$) in the wheal sizes induced by both skin test reagents. The correlation

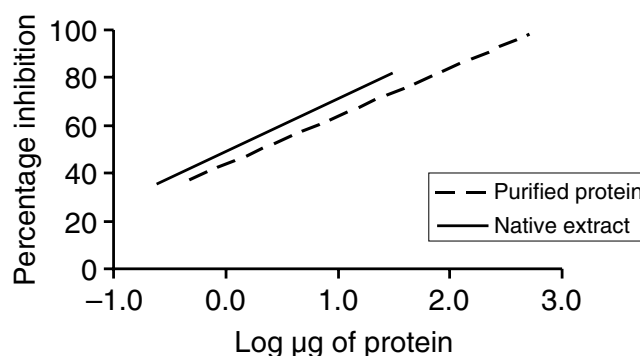


Figure 2. ELISA inhibition of the whole *Salsola kali* extract (in the solid phase) and Sal k 1. Both curves had a coefficient of correlation >0.95 and passed the test of parallelism.

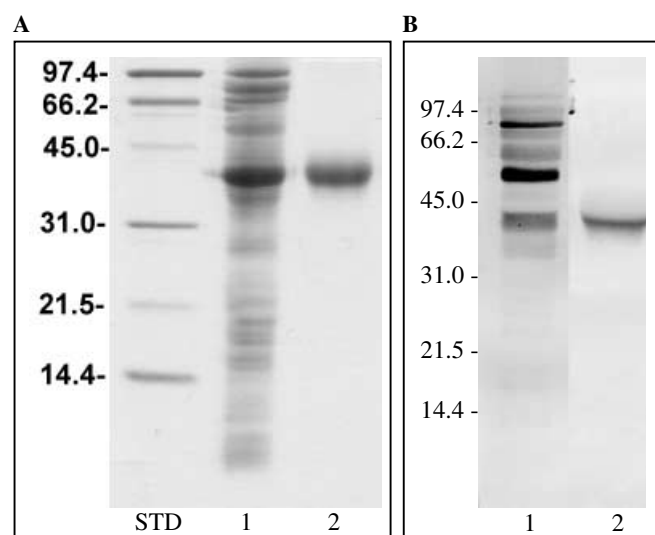


Figure 1. SDS-PAGE and immunoblot of the whole *Salsola kali* extract and of the purified protein. (A) SDS-PAGE: lane STD, standard low molecular weight (BioRad); lane 1, whole *S. kali* extract; lane 2, Sal k 1. (B) Immunoblot: lane 1, whole *S. kali* extract; lane 2, Sal k 1.

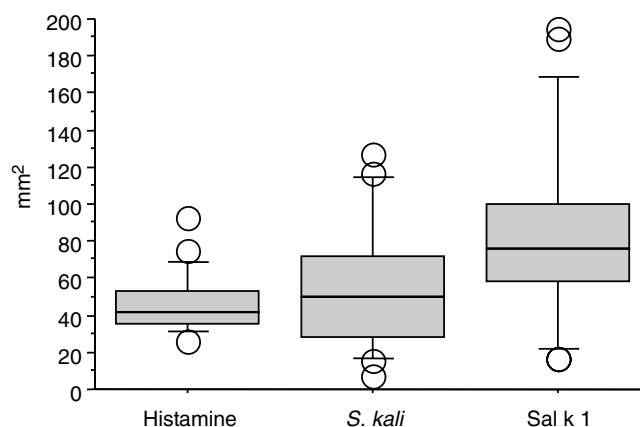


Figure 3. Box-plot view of the wheal sizes induced by the whole *Salsola kali* extract, by Sal k 1 and by histamine (10 mg/ml).

Table 1. Aminoacid sequences of four fragments obtained by MALDI-TOF after digestion of Sal k 1 with trypsin

Purified protein	Amino acid sequences
Fragment 1	... PTITIGGPEYR ...
Fragment 2	... TIFFDAYL ...
Fragment 3	... GTSYVIVIK ...
Fragment 4	... EPAEEFTTISDAVK ...

coefficient between both prick tests was $r = 0.73$; 10 individuals were used as negative controls and all had a negative skin test with the whole extract and the purified protein.

Partial amino acid sequencing of the purified protein

Four peaks were eluted and sequenced. The amino acid sequences of four internal regions were obtained (Table 1). The sequence of the four fragments did not show any homology with other known proteins. The accession number at the SWISS-PROT data bank is P83181 and the allergen has been registered as Sal k 1 in the allergen data bank.

Discussion

In the present study we describe the characterization, purification and partial sequencing of an important allergen of *S. kali*, a weed which is responsible for a great number of sensitization in endemic areas (5). Sensitization produced by *S. kali* is not well documented, and there are not many studies about the allergenicity of this plant. Studies by Gómez et al. (17) in Texas, USA, attempted to characterize the antigenic and allergenic profiles of *S. kali* using SDS-PAGE, IEF and immunoblotting, describing numerous bands in the MW range of 8–90 kDa. Similar results were obtained in our study. In another study, the most commonly recognized band had a MW of 40 kDa and the most representative antigens focussed between pIs 5 and 5.55 (8). Würtzen et al. (18), while evaluating the allergenic composition of four weeds using crossed radio immuno electrophoresis (CRIE) and SDS-PAGE, detected four important allergens in pollen extracts of *S. pestifer*. Among them there was a 43-kDa molecule. In 1981, Shaffie et al. (19) attempted the characterization of *S. kali* allergens. The authors purified two allergens of *S. kali* with MWs of approximately 39 and 41 kDa. However, none of these previous studies partially sequenced, or assigned a name to any of the identified allergens.

The preparative SDS-PAGE purification method used in our study was selected because of the high recovery yields and minimal modifications induced to the purified proteins (20, 21). In our purification scheme, we incorporated a preliminary step, in which the pigments of the extract were removed by gel filtration. This fraction,

containing a large proportion of pigments and other irrelevant material, was tested by skin testing and ELISA inhibition, showing negative results. At the end of the purification process, a protein was obtained which retained specific IgE recognition, as shown by *in vivo*, and *in vitro* tests. As its amino terminal was blocked, trypsin digestion had to be performed to obtain internal peptides to be sequenced by mass spectrometry. Four peptides, without homology to other known proteins, were obtained. IEF studies revealed six isoforms at the MW of Sal k 1 (43 kDa).

The inhalation of pollen grains of *S. kali* induces allergic sensitization (22) in human beings in areas where these plants are abundant. Significant cross-reactivity has also been described between *Salsola* and *Chenopodium* spp. (23) and with foods, such as Swiss Chard (24, 25). Cross-reactivity with other plants, which are similar in distribution and biological characteristics, such as *Mercurialis annua*, has also been described (26). In this study we included a total of 66 patients residing in the area of Zaragoza, Spain, a region where sensitization to *S. kali* is common. Maximal pollination levels are reached at the end of the summer (27). The patients were selected based on a positive skin-prick test with a standardized extract of *S. kali* and a clinical history strongly suggestive a *S. kali*-induced allergic pollinosis. Thirty-nine patients (59%) of the 66 individuals had a positive specific IgE determination to the whole *S. kali* extract. Skin tests solutions were prepared with the purified protein and 20 *S. kali*-sensitive patients as well as 10 negative controls were tested. It was estimated that Sal k 1 represents approximately 24% of the total protein in the extract. Therefore, we selected a concentration of 0.5 mg of Sal k 1 and the whole extract contained 2 mg/ml of *S. kali* extract. All these 20 individuals (100%) had a positive skin test to this allergen, while *S. kali*-negative patients had negative results. In general, the size of the reaction to the purified protein was consistently bigger than to the whole extract, which shows the importance of this molecule in the total allergenicity of *S. kali* extracts.

Its specific IgE binding capacity was tested by immunoblot, direct ELISA and ELISA inhibition. Approximately 66% of the patients with positive specific IgE tests to the total extract had a positive specific IgE determination to Sal k 1. The immunoblot results demonstrated the IgE binding capacity of Sal k 1 using the pooled sera of the ELISA-positive patients. The serum pool recognized several bands in the *S. kali* extract, while Sal k 1 was recognized as a single band. The ELISA inhibition experiments showed that 2 µg of Sal k 1 were needed to obtain 50% of inhibition. These aspects demonstrate that Sal k 1 plays an important role in the allergenicity of the *S. kali* pollen and could be considered as a main allergen in *Salsola* extracts. There is an apparent contradiction between the *in vivo* and *in vitro* data for Sal k 1, which seems to be more potent *in vivo* than *in vitro*. A possible explanation is that skin testing is a more sensitive method

than ELISA inhibition to evaluate allergenicity. Another explanation is that the concentration of Sal k 1 was overestimated and, therefore, the skin tests were conducted with concentrations of Sal k 1 greater than those present in the original extract. Further studies can now evaluate major allergen content in *S. kali* extracts from different commercial sources and the batch to batch consistency of *S. kali* allergen extracts (28).

In conclusion, in this study we have characterized the antigenic and allergenic pattern of *S. kali* extracts. Our

results confirm previous studies which suggested that an allergenic molecule of approximately 43 kDa is an important allergen of *S. kali*.

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