A recombinant Sal k 1 isoform as an alternative to the polymorphic allergen from *Salsola kali* pollen for allergy diagnosis

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Short title: Recombinant production of Sal k 1, a sensitization marker of *S. kali*.

Key Words
Sal k 1 · *Salsola kali* pollinosis · Amaranthaceae · Recombinant allergen · Cross-reactivity · Pectin methylesterase

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Conflict of interest

PB and RIM belong to the Research Department of ALK-Abelló S.A. The rest of the authors declare no conflict of interest.

Summary

Background: Amaranthaceae pollen allergy incidence has increased in the last years due to the desertification process occurring in many countries. In some regions of Spain, *Salsola kali* is the main cause of pollinosis at almost the same level than olive and grass pollen. Sal k 1 -the sensitization marker of *S. kali* pollinosis- is actually used in clinical diagnosis, but it is purified at a low yield from pollen. Thus, the aim of the study consists of the production of a recombinant isoform of Sal k 1 able to span the structural and immunological properties of the natural isoforms present in *S. kali* pollen and validate its potential use for diagnosis. ***Methods:*** Sal k 1-encoding cDNA was amplified by PCR, cloned in pET41b vector and used to transform BL21(DE3) *E. coli* cells to produce the recombinant allergen. Immunoblotting, ELISA, basophil activation and skin prick test were used to validate the recombinant protein against Sal k 1 isolated from pollen. Sera and blood cells from *S. kali* pollen sensitized patients and anti-Sal k 1 monoclonal and polyclonal antisera were used. ***Results:*** rSal k 1 was produced in *E. coli* with a final yield of 7.5 mg/L of cell culture. The expressed protein was isolated, purified to homogeneity, and structurally and immunologically validated against the natural form –nSal k 1- isolated from pollen as a useful diagnostic tool. In addition, Sal k 1 exhibited a higher IgE cross-reactivity with plant-derived food extracts such as peanut, almond or tomato than with related and non-related pollen sources such as *P. acerifolia* and Oleaceae members. ***Conclusions:*** rSal k 1 expressed in bacteria maintains its structural and immunological properties intact in comparison to nSal k 1. rSal k 1 spans the immunological properties of most of the natural isoforms found in pollen, and thus, might substitute nSal k 1 in clinical diagnosis.

Introduction

A wide repertory of plant families produces relevant allergenic pollens [1, 2]. Pollens from the Amaranthaceae family are gaining relevance as allergenic inducer in countries where the climate change is causing extensive desertification because these resistant weeds are able to rapidly colonize these areas [3]. The most relevant members of this family in the Mediterranean area are *Salsola kali* and *Chenopodium album* [4, 5].

Despite the allergenic cross-reactivity observed between these members of the Amaranthaceae family [6], about 10 to 30% of the patients are mainly sensitized to *S. kali* [7]. Several allergens from *C. album* (Che a 1 [8], Che a 2 and Che a 3 [9]) and *S. kali* (Sal k 1 [7], Sal k 2 (accession number: Q8L5K9), Sal k 3 [10], Sal k 4 [11, 12], and Sal k 5 [13] pollens have been identified (*for a review* [14]). Sal k 1 is the allergenic marker of *S. kali* pollen able to discriminate between sensitized and non-sensitized patients to this weed [7]. An average prevalence of Sal k 1, a pectin methylesterase (PME), in certain areas of Spain is about 18% among all pollen-sensitized patients, and about 70% in *S. kali* pollen sensitized patients, as revealed by the multi-centre study ("VEGETALIA" and "EXPO") [15]. The same cohort of patients showed a prevalence of 2% to the major allergen of *C. album* (Che a 1) in all pollen sensitized patients [15], and thus confirming the role of Ole e 1-like protein family as sensitization marker of the Amaranthaceae species.

In addition to Sal k 1, PMEs from olive (*Olea europaea*) pollen -Ole e 11-, tomato (*Lycopersicon esculentum*) and kiwi fruit (*Actinia deliciosa*) have also been reported as allergens [16-19]. Sal k 1 shares an amino acid sequence identity of 57, 17 and 23% with olive pollen, tomato and kiwi PMEs, respectively. Despite this low identity observed between PMEs from pollens and plant-derived foods, these proteins possess a catalytic core highly conserved during evolution, containing Ala153, Gln173, Asp174,

Asp195, and Arg253 residues in Sal k 1. This region might enable PME cross-reactivity processes from different sources since a significant IgG recognition could be observed when comparing PMEs from olive pollen and other plant-derived food sources such as avocado, pear or tomato by immunoblotting [16].

Sal k 1 purified from pollen is already being used in commercial diagnostic protocols to determine the specific IgE levels of patients to the sensitization marker of *S. kali* by ImmunoCAP (Thermo Fisher Scientific). However, Sal k 1 is purified from pollen at a very low yield and with a high number of isoforms. Only 100 μg of homogeneous protein can be obtained from 5 g of dried pollen [7]. In addition, a different isoform composition is obtained from batch to batch. Then, the availability of a well characterized recombinant isoform equivalent to those natural allergenic isoforms present in *S. kali* pollen extract should be a useful tool to standardize diagnostic and immunotherapeutic protocols. The recombinant Sal k 1 protein should circumvent batch to batch variability and allergenic protein content concentration problems of the *S. kali* natural protein extracts used in these protocols [20, 21].

In this context, the aim of this work consisted of the production of a recombinant isoform of Sal k 1 able to span the structural and immunological properties of the natural isoforms present in *S. kali* pollen, and then, validating its use for the diagnosis of *S. kali* allergy. In addition, we have also found that the PME could be implicated in pollen-pollen and plant-derived food-pollen cross-reactivity processes.

Materials and methods

Protein extracts

Pollen from several plant species was obtained from ALK-Abelló (Madrid, Spain). Pollen protein extracts were prepared by saline extraction as described previously [8]. Plant-derived food extracts from fruits and nuts were prepared as previously described [22]. Protein extract concentration was determined using the Bicinchoninic Acid Assay (Pierce Chemical Co., Thermo Fisher Scientific, Rockford, IL, USA).

Cloning strategy

PCR amplification of *S. kali* PME isoform Sal k 1.0103 was performed using the template previously cloned in the pCR2.1 vector (Invitrogen, Groningen, The Netherlands) [7]. Two specific oligonucleotides were designed: a sense primer with an *Nde*I restriction site (underlined), 5'- actcatATGCAGCCGATCCCCCCT -3' that corresponds to the MQPIPP amino acid sequence (the N-terminal end of the mature protein), and an antisense primer, 5'- ttactcgagCACTTTAGGTGGTGGTAG -3' with an *Xho*I restriction site (underlined) that corresponds to LPPPKV amino acids (C-terminal end of Sal k 1). The amplified PCR product was then digested with *Nde*I and *Xho*I restriction enzymes and cloned into the pET41b plasmid (Invitrogen) in phase with a nucleotide sequence codifying for an 8xHis tag. This sequence is included in the expression vector at the C-terminus of the protein.

Expression and purification of rSal k 1

The construct pET41b/Sal k 1.0103 was used to transform *E. coli* BL21(DE3) cells to produce recombinant Sal k 1 according to [11]. BL21(DE3) cultures were centrifuged at 6000 *g* for 20 min and reconstituted in sodium phosphate 20 mM pH 8.0 containing

sodium chloride 0.5 M, imidazole 20 mM and phenylmethane-sulfonylfluoride 1 mM. After bacterial lysis by three cycles of freezing in N_2 and thawing at 42 ºC, the soluble fraction containing rSal k 1 was clarified by centrifugation at 12000 g for 20 min, and subsequently loaded in an AKTA Purifier FPLC system (GE healthcare, Madrid, Spain) with a His-Trap FF crude (GE healthcare) to purify the recombinant protein. The elution of the protein was performed using an isocratic gradient of 0.5 M imidazole in reconstitution buffer according to [23].

Sera and antibodies

Sera from 80 patients suffering from Amaranthaceae pollinosis were collected from three regions of Spain: Zaragoza, Murcia and Alicante (online suppl. Table S1). Written informed consent was obtained from all patients. The non-atopic controls were selected according to a non-allergic history and negative skin prick tests to the usual panel of respiratory allergens.

Rabbit polyclonal antisera (pAb) against the natural PME from *S. kali* pollen was obtained by weekly injections of the protein (100 μg) in complete Freud´s adjuvant. Horseradish peroxidase-labeled goat polyclonal antibody against rabbit IgG was obtained from Bio-Rad (Richmond, Calif). Mouse monoclonal antibody against human IgE and two monoclonal antibodies 1.22.1 (mAb 1) and 1.3.1 (mAb 2) raised against nSal k 1 and belonging to different families were obtained in collaboration with ALK-Abelló (Madrid, Spain). Horseradish peroxidase-labeled goat polyclonal antibody against mouse IgG was purchased from Pierce Chemical Co. Horseradish peroxidase-labeled mouse polyclonal antibody against the His-tag was from Sigma-Aldrich (St Louis, MO, USA).

MS analysis and CD spectroscopy

MS of purified proteins was performed in a Bruker-Reflex IV MALDI-TOF (Bruker-Franzer Analytic, Bremen, Germany). The CD spectra recording and deconvolution of data was obtained in the far-UV, on a Jasco J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) according to [11]. Theoretical percentages of secondary structure were obtained with GOR method [24].

Analytical procedures

SDS-PAGE was performed in 15% polyacrylamide gels. Proteins were stained with Coomassie Blue R-250 (Sigma-Aldrich). Molecular mass determinations were done with protein molecular mass markers SM0431 (Fermentas, Thermo Fisher Scientific). The concentration of the purified proteins was calculated by measuring the absorbance at 280 nm in a DU-7 spectrometer (Beckman, Madrid, Spain) using the theoretical extinction coefficient ($E^{0.1\%}$) of 1.1 of Sal k 1.0103 obtained using the ProtParam Software [25].

Immunological characterization

Immunoblotting procedures were developed as described previously [11] using individual human sera (diluted 1:10), anti-human IgE monoclonal antibody (diluted 1:3000) and horseradish peroxidase-labeled anti-mouse IgG antibody or a rabbit polyclonal antiserum raised against nSal k 1 (diluted 1:50000) followed by horseradish peroxidase-labeled anti-rabbit IgG polyclonal antibody (1:3000). Alternatively, a horseradish peroxidase-labeled mouse polyclonal antibody against His-tag (1:2000) or monoclonal antibodies raised against nSal k 1 (1:200) followed by an horseradish peroxidase-labeled anti-mouse IgG antibody (1:2500) were used. For immunoblotting

inhibition assays an equivolumetric pool of sera (n=8) (diluted 1:10) was alternatively preincubated with 0, 1 and 5 μg of rSal k 1 or, alternatively, nSal k 1. Protein bands were quantified by densitometry using the Multi Gauge Analysis software (Fujifilm, Barcelona, Spain) and referring the inhibition percentage to the control without inhibitor [13].

Indirect ELISA was performed as described [26] in 96-well plates coated with 100 ng of purified recombinant protein, 20 μg of pollen extract or 500 ng of pineapple stem bromelain [27] (Sigma-Aldrich). OD values >0.1 arbitrary units (mean + 3 x SD of the OD of the negative control) were considered positive. Inhibition assays were performed with an equivolumetric pool of human sera (n=8) (diluted 1:10), the anti-nSal k 1 polyclonal antiserum (diluted 1:50000) or monoclonal antibodies raised against nSal k 1 (1:10000), previously adsorbed to different concentrations of inhibitors ranging from 1 ng to 5 μg for purified proteins and 20 or 200 μg for the pollen and plant-derived food extracts. Binding of human IgE was detected by anti-human IgE monoclonal antibody (diluted 1:5000) followed by horseradish peroxidase-labeled anti-mouse IgG antibody (diluted 1:2500). The binding of anti-nSal k 1 polyclonal antiserum was detected with the horseradish peroxidase-labeled anti-rabbit IgG antibody (1:3000) and binding of mouse monoclonal antibodies with the horseradish peroxidase-labeled antibody (1:2500). The inhibition percentage was calculated according to the formula: inhibition (%) = [1-($Abs_{492\ nm}$ with inhibitor/$Abs_{492\ nm}$ without inhibitor)] x 100 [13].

Basophil activation test (BAT)

This test was performed as previously described [28]. After blood-cell separation, 50 μL of each patient's cell suspension were incubated with 50 μL of indicated protein concentration. To evaluate background basal values without stimulation (negative

control), we added 50 μL of stimulation buffer (cRPMI), containing IL-3 (2 ng/mL) in the cell suspension. As a positive control, a monoclonal anti-IgE anti-receptor antibody was used at a final concentration of 1 μg/mL. A positive response was concluded for values ≥15%, and stimulation index (antigen-specific response/basal level) ≥2.

Results

Cloning, recombinant production and purification of rSal k 1

We previously reported the polymorphic character of Sal k 1 allergen and the sequencing of three different isoforms: Sal k 1.0101 (AY590141), Sal k 1.0102 (AY776249) and Sal k 1.0103 (AY776248), which showed 94 to 99% identity in their amino acid sequences [7].

Here, we aimed to express, purify and characterize a recombinant isoform of Sal k 1, which might be used as a replacement of nSal k 1 in diagnostic protocols. To this end, we amplified by PCR the cDNA-encoding mature Sal k 1.0103 without the nucleotide sequence codifying for the signal peptide, and cloned into the pCR2.1 vector. The cDNA was then subcloned into the expression vector pET41b, and the construct subsequently used to transform BL21(DE3) cells to produce Sal k 1 as a protein tagged at the C-terminus with a 8xHis sequence.

Different temperatures and IPTG-induction times were surveyed and the supernatants and pellets analyzed by Coomassie Blue staining to determine the optimal conditions to produce rSal k 1. The highest yield of soluble recombinant protein was observed at 16 ºC and 48 h of induction with 1 mM IPTG (fig. 1a).

After affinity chromatography, fractions containing the recombinant protein purified to homogeneity were pooled, dialyzed, concentrated and directly used in subsequent experiments. rSal k 1 was obtained with yields of approximately 7.5 mg/L of cell culture. The recombinant protein integrity and purity was verified by Coomassie Blue staining after SDS-PAGE and by immunostaining after transference to membranes with (i) a pool of sera from allergic patients to *Salsola kali,* (ii) two specific monoclonal antibodies (mAb 1 and mAb 2), (iii) one polyclonal antiserum raised against nSal k 1,

and (iv) a polyclonal antibody directed against the His-tag (fig. 1b). These experiments assessed the high purity of the recombinant allergen.

Then, we proceeded to structurally and immunologically characterize rSal k 1 in comparison to nSal k 1.

Structural characterization of rSal k 1

We first performed CD analyses using rSal k 1 and nSal k 1 at a concentration of 200 μg/ml (fig. 2a). Similar CD spectra were obtained for both proteins. We observed a higher α-helix content in rSal k 1 (15.7% vs 8.6% for nSal k 1), and minor differences in the β-sheet, β–turn and non-periodic structure content (fig. 2b).

Immunological characterization

We analyzed the antigenic properties of both recombinant and natural Sal k 1 by ELISA and WB using two different mouse monoclonal antibodies and a polyclonal antiserum raised against nSal k 1. By WB under denaturing conditions, we observed that the ability of nSal k 1 and rSal k 1 to bind IgG from the monoclonal antibodies and the polyclonal antisera was also equivalent (fig. 2c). ELISA titration of mAb 1, mAb 2 and the polyclonal antisera with rSal k 1 and nSal k 1 showed that IgG-binding ability of both proteins was equivalent, suggesting that the antibodies recognized the epitopes in nSal k 1 and rSal k 1 in a similar manner (fig. 2d).

We also evaluated the allergenic properties of both proteins by ELISA and WB. The prevalence of both natural and recombinant proteins was tested using sera of *S. kali* sensitized-patients from three different regions of Spain with significant differences in *S. kali* pollen counts (fig. 3). Recognition frequency was almost equivalent for both proteins: nSal k 1 (52%) and rSal k 1 (56%). Glycoprotein bromelain with a

carbohydrate very similar to that of plant glycoproteins was tested to identify those sera able to recognize the carbohydrate moiety of glycoallergens. Interestingly, only six out of the eighty patients tested possessed IgE reactivity against the glycan of bromelain (fig. 3a), indicating that the global contribution of the carbohydrate moiety to the IgE reactivity of Sal k 1 was not relevant in this case. Correlation values obtained when the IgE-binding ability of nSal k 1 and rSal k 1 was compared were quite low for all patients (r^2=0.45), especially for the population of Zaragoza. Surprisingly, the regression analysis of the data seemed to indicate a slightly better IgE recognition of rSal k 1 by the sensitized patients than for nSal k 1 in all three populations of sensitized patients studied (data not shown).

Finally, we analyzed the IgE-binding ability of both proteins by WB under denaturing conditions to determine the role of the conformational epitopes in the IgE reactivity of the sera. We used a random set of twenty of the previously identified ELISA-positive sera (fig. 3b). The correlation obtained (r^2=0.94), indicated that the IgE binding for the two proteins under denaturizing conditions was almost equivalent.

Collectively, these data indicate that rSal k 1 is able to span the antigenic and allergenic properties of Sal k 1 isoforms found in pollen.

Inhibition assays

We then analyzed the IgG- and IgE-equivalence of nSal k 1 and rSal k 1 by ELISA and WB inhibition experiments (fig. 4).

The IgG binding to rSal k 1 was equivalent to that of nSal k 1 when using two monoclonal antibodies, whereas the experiments performed with the polyclonal antisera indicated that rSal k 1 was not able to completely inhibit the IgG binding to nSal k 1 (fig. 4a-c).

Regarding the IgE-binding analysis by ELISA, it was observed a 30% reduction in the IgE-binding ability of rSal k 1 with respect to nSal k 1 in native conditions. Furthermore, WB inhibition analyses of nSal k 1 and rSal k 1 using both proteins as inhibitors showed in all cases similar inhibition values, and thus indicating that both proteins possessed similar IgE-inhibition capacity under denaturing conditions (fig. 4d).

Basophil activation test

Three patients (two allergic and one non-atopic control) were selected to perform basophil activation test (fig. 5). First, we tested the natural and the recombinant proteins by ELISA. A good correlation was observed for rSal k 1 and nSal k 1 (fig. 5a). Then, we observed that rSal k 1 and nSal k 1 performed similarly in basophil activation test (fig. 5b). At 20 µg/ml of rSal k 1 or nSal k 1, we observed similar results for IgE-positive patients, whereas at 5 µg/ml one patient showed higher activation of basophils. In contrast, the non-atopic control was non-responder at all concentrations and allergen tested (fig. 5b).

IgE cross-reactivity

We tested a panel of pollen and plant-derived food extracts with anti-Sal k 1 polyclonal antiserum to identify those extracts able to potentially cross-react with rSal k 1 (fig 6a). Then, we surveyed the extracts described to contain allergenic PME by ELISA inhibition experiments to rSal k 1 using a randomly selected pool of 8 sera from *S. kali* sensitized patients (fig. 6b).

Regardless the complete inhibition observed with *S. kali* pollen protein extract, *Platanus acerifolia* (plane tree) pollen protein extract showed the highest IgE-inhibition capacity, reaching 25% of inhibition using 200 µg of extract. Other IgG-positive PME pollen protein extracts-, *Ligustrum vulgare* (wild privet), S*yringa vulgaris* (common

lilac) were also able to inhibit the IgE-binding to Sal k 1, reaching 24 and 20% of inhibition, respectively. A scarce inhibition was observed with *Olea europaea* (olive) or with the Amaranthaceae counterpart, *Chenopodium album* (Lamb´s quarter). Regarding PME plant-derived food extracts, nuts from *Prunus dulcis* (almond), *Arachis hypogea* (peanut), *Pistacia vera* (pistachio) and fruits from *Solanum esculentum* (tomato), *Solanum tuberosum* (potato) and *Pyrus communis* (pear) were also shown to cross-react better with Sal k 1 with inhibition percentages of 40, 38, 30, 23, 22 and 18%, respectively.

Discussion

The role of Amaranthaceae pollens as important sources of allergy has special relevance in countries with a desert climate, high temperatures and dry soil [18], and in extensive areas of the south of Europe which have suffered from an increase in the desertification process in the last years [4, 5]. Indeed, in south-eastern and central areas of Spain, *Salsola kali* pollen has become one of the first causes of seasonal allergy at almost the same level than olive and grass pollinosis [4, 5].

To date, five allergens from *S. kali* have been reported: the protein kinase homologue Sal k 2 (accession number: Q8L5K9), the methionine synthase Sal k 3 [10], the profilin Sal k 4 [11, 12], the Ole e 1-like protein Sal k 5, and the here studied major allergen and *S. kali* specific marker -Sal k 1- [7].

Sal k 1 is a very polymorphic molecule with multiple protein spots identified by two-dimensional electrophoresis (online suppl. Fig. S1), with pI ranging from 5.9 to 7.7 and different glycosylation patterns [7]. Although this heterogeneity hampers the development of a suitable purification procedure to obtain a consistent and reproducible batch-to-batch protein, a recombinant approach is especially helpful for this protein. Thus, our main concern about the production of Sal k 1 consisted of the obtaining of a recombinant protein able to be recognized by all sensitized patients to *S. kali*. As previously reported, non-glycosylated PMEs like that of *Diospyros kaki* conserved their 3D-folding and its enzymatic activity when comparing with other glycosylated PME [29]. Thus, we expressed Sal k 1-specific cDNA in bacteria because glycosylation should not be critical for the 3D-folding of PMEs [29] and it has also been successfully used to express other glycosylated allergens, which maintained their structural and immunological properties intact [30, 31].

Remarkably, we performed an extensive characterization of rSal k 1 produced in *E. coli* in comparison to nSal k 1 to verify that the recombinant protein possessed similar structural and immunological properties than the natural protein isolated from pollen. Although we observed slight differences in the secondary structure between rSal k 1 and nSal k 1, we determined that the most general structural features such as the percentage of lamin β remained in rSal k 1. On the other hand, the antigenic behavior of rSal k 1 was almost equivalent to nSal k 1 pointing out that the structural similarity between both proteins was very high. No significant differences were observed in the IgE-binding of both proteins under denaturing conditions, while in ELISA we observed that IgE binding was similar when both proteins were tested individually, biased towards a slightly better recognition to nSal k 1 (about 30% of reduced inhibition ability of rSal k 1 with respect to nSal k 1) when tested in inhibition experiments. However, the differences observed cannot be attributed to a wrong folding of the recombinant protein since 37 individual serums gave a better recognition to rSal k 1 and 15 to nSal k 1.

To determine if these differences in the IgE binding to rSal k 1 and nSal k 1 could be attributed to the glycosydic moieties -absent in rSal k 1-, or to conformational epitopes absernt in the selected isoform, we also tested bromelain –a protein containing CCD-. Only a 6.6% of the patients showed specific IgE directed to the glycosydic fractions found in bromelain, and only one patient (1.1% of the population) showed correlation between the IgE reactivity to glycosydic moiety and a better recognition of the natural protein. Then, we could confirm that the contribution of the glycan moiety to the whole IgE-reactivity of nSal k 1 was not relevant in the assayed population, as it has also been reported for CCDs in a clinical context [32, 33].

Collectively, our results seemed to indicate that differences in the allergenic recognition with some bias towards nSal k 1 should be attributed to amino acid changes inside of the conformational epitopes due to the polymorphism of the natural protein

which are able to induce changes in the affinity and specificity of the IgE antibodies. Finally, we further confirmed the equivalence of rSal k 1 and nSal k 1 by BAT. rSal k 1 was able to induce basophil activation from blood of *S. kali* pollen-sensitized patients at a similar level than nSal k 1, correlating BAT values with the IgE-levels to rSal k 1 and nSal k 1 of this selected population of sensitized patients. Remarkably, these data confirmed the usefulness of rSal k 1 for *in vitro* diagnosis.

We also investigated the potential role of Sal k 1 in cross-reactivity because several facts pointed out to a direct association of Sal k 1 with these processes: i) the biochemical function of this protein family is associated to a well-conserved catalytic amino acid core across species [7, 16], which might be an immunogenic region target of IgE from sensitized patients, ii) the existence of allergenic PMEs in pollen -Ole e 11 with prevalence ranging from 55.9% to 75.6% in three different olive pollen allergic populations [16]-, and in plant-derived foods like tomato [17, 18] or kiwi (Act d 7) with 32% of prevalence [19], and iii) because a higher prevalence of Sal k 1 has been recently published in patients sensitized to plant-derived food and pollen than in patients only sensitized to pollen [15]. Here, we have confirmed the role of Sal k 1 in cross-reactivity as deduced from the inhibition experiments performed with a panel of plant-derived food and pollen extracts, which showed percentages of inhibition ranging from 9% to 24% with allergenic pollens and 18% to 40% with plant-derived food extracts.

In summary, this work shows that rSal k 1 i) was almost equivalent to nSal k 1 regarding its physicochemical properties, ii) spanned most of the allergenic properties of the natural isoforms present in *S. kali* pollen, and iii) was almost equivalent to nSal k 1 at allergenic level as demonstrated through immunological approaches and by histamine release. Accordingly, the herein presented data provide evidences that rSal k 1 might substitute nSal k 1 in the diagnostic protocols actually used in clinics, and could be

considered for a future use in immunotherapy protocols in *S. kali* pollen sensitized patients.

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Legend to the figures

Fig. 1. Recombinant expression and purification of Sal k 1. **a** SDS-PAGE analysis of the time-course of the recombinant expression of Sal k 1 in *E. coli* BL21(DE3) cells at indicated temperatures and times of induction with IPTG. **b** SDS-PAGE of the purification process of rSal k 1 and immunostaining of nitrocellulose-blotted proteins with: 1, an equivolumetric pool of allergic patients sera; 2 and 3, monoclonal antibodies (mAb 1 and mAb 2, respectively); 4, the polyclonal antiserum raised against nSal k 1; and 5, the polyclonal antibody against His-tag. FT, flow-through; P, purified rSal k 1. Molecular mass markers are shown.

Fig. 2. Structural and antigenic properties of rSal k 1 in comparison to nSal k 1. **a** Circular Dichroism spectra of nSal k 1 and rSal k 1 at a concentration of 200 ng/µl. **b** Percentages of secondary structure of both proteins were obtained by de-convolution with GOR method. **c** SDS-PAGE and immunoblot of nitrocellulose-blotted 200 ng of natural and recombinant Sal k 1 with the two monoclonal antibodies (mAb 1 and mAb 2) and the polyclonal antisera (pAb) raised against nSal k 1. Molecular mass markers are shown. **d** Titration of the mAb 1, mAb 2 and the pAb. ELISA plates were coated with 100 ng/well of rSal k 1 or nSal k 1.

Fig. 3. Specific IgE binding of rSal k 1 and nSal k 1. **a** Determination of the IgE-binding ability of 80 individual sera from three regions of Spain to rSal k 1 (100 ng/well), nSal k 1 (100 ng/well) or bromelain (500 ng/well). Correlation coefficient (R^2) among rSal k 1 and nSal k 1 IgE levels is also shown for each population. **b** Analysis of the specific IgE-binding of 20 sera to 200 ng of nitrocellulose-blotted rSal k 1 or nSal k 1 after SDS-PAGE under reducing conditions. Molecular mass markers are shown.

Fig. 4. Immunological characterization and validation of rSal k 1 in comparison with nSal k 1. **a-b** mAb 1 and mAb 2; **c** a polyclonal antiserum; and **d** an equivolumetric pool of eight randomly selected sera were tested by ELISA and immunoblotting to determine the IgE-binding inhibition between rSal k 1 and nSal k 1. In the ELISA experiments 100 ng/well of protein were coated and indicated amounts of each protein as inhibitor were used. In the immunoblotting assays under reducing conditions, 0.5 µg of each protein were blotted onto nitrocellulose membranes and 5 µg of inhibitor were

used. Percentage of inhibition was calculated by densitometry of protein bands. (I%), inhibition percentage; (-), no inhibition; (r), rSal k 1; (n), nSal k 1.

Fig. 5. Sal k 1 specific IgE values and basophil activation assays. **a** IgE levels quantified by ELISA of the two *S. kali* pollen sensitized patients and the non-atopic control. **b** Stimulation index of basophil activation test of patients allergic to *S. kali* pollen and the non-atopic control are shown.

Fig. 6. Sal k 1 cross-react with PMEs from pollen and plant-derived foods. **a** Detection of PMEs from different pollen and plant-derived food sources by immunoblotting using the pAb raised against Sal k 1. 20 µg of different pollen and plant-derived food protein extracts were blotted onto nitrocellulose membranes and immunostained with the pAb obtained against nSal k 1. Molecular mass markers are shown. **b** The same pollen and plant-derived food protein extracts were tested by ELISA inhibition experiments using a pool of sera from allergic patients to *S. kali* against 0.1 µg/well of rSal k 1. 200 µg of *S. kali* and *Betula verrucosa* pollen protein extracts were used as positive and negative controls, respectively.

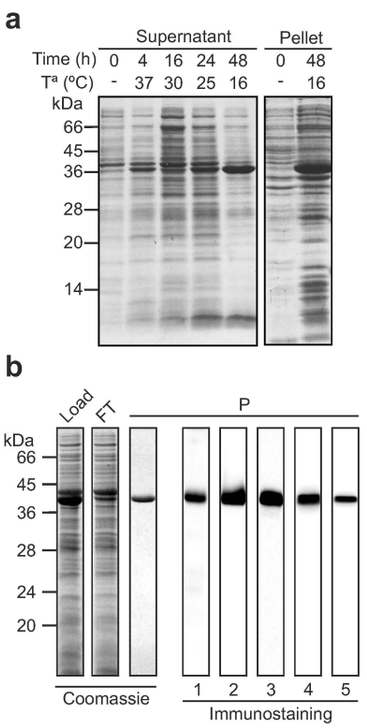
a
Supernatant
Pellet
Time (h) 0 4 16 24 48 0 48
Ta (°C) - 37 30 25 16 - 16
kDa
66
45
36
28
20
14
b
Load
FT
P
kDa
66
45
36
28
24
20
1 2 3 4 5
Coomassie
Immunostaining

Fig. 1.

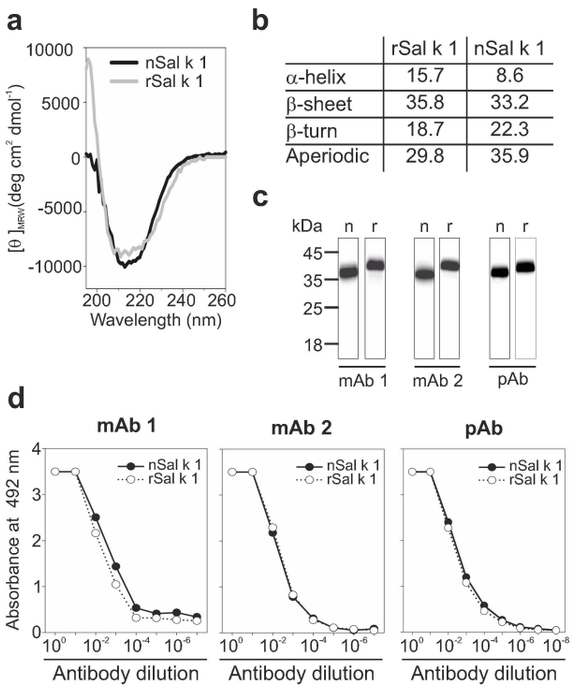
a
10000
5000
0
-5000
-10000
nSal k 1
rSal k 1
[θ]MRW (deg cm2 dmol-1)
200 220 240 260
Wavelength (nm)
b
rSal k 1 | nSal k 1
α-helix | 15.7 | 8.6
β-sheet | 35.8 | 33.2
β-turn | 18.7 | 22.3
Aperiodic | 29.8 | 35.9
c
kDa
n r n r n r
45
35
25
18
mAb 1
mAb 2
pAb
d
mAb 1
mAb 2
pAb
Absorbance at 492 nm
nSal k 1
rSal k 1
Antibody dilution

Fig. 2.

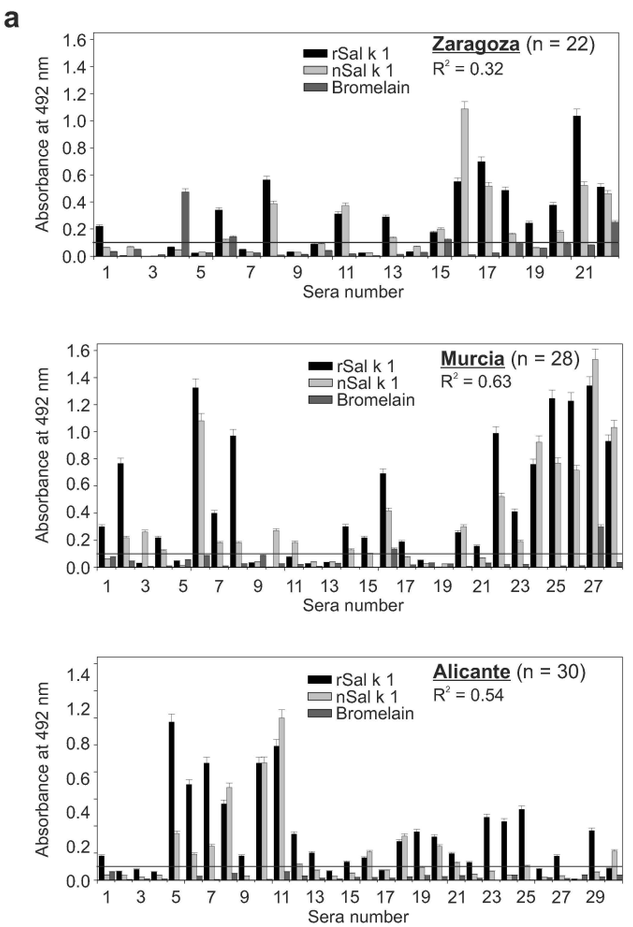
a
Zaragoza (n = 22)
R² = 0.32
rSal k 1
nSal k 1
Bromelain
Absorbance at 492 nm
Sera number
Murcia (n = 28)
R² = 0.63
Alicante (n = 30)
R² = 0.54
b
Sera number
kDa
45
35
25
18
rSal k 1
nSal k 1

Fig. 3.

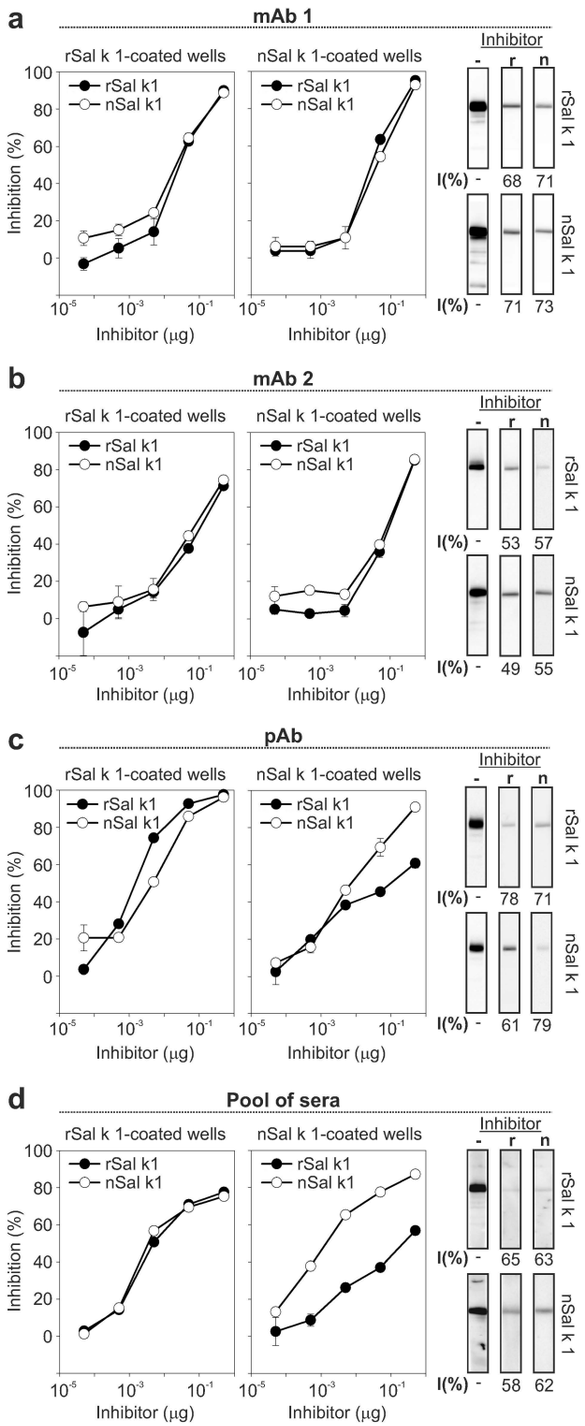
a
mAb 1
rSal k 1-coated wells
nSal k 1-coated wells
rSal k1
nSal k1
Inhibition (%)
Inhibitor (μg)
Inhibitor
-
r
n
rSal k 1
nSal k 1
I(%) - 68 71
I(%) - 71 73
b
mAb 2
I(%) - 53 57
I(%) - 49 55
c
pAb
I(%) - 78 71
I(%) - 61 79
d
Pool of sera
I(%) - 65 63
I(%) - 58 62

Fig. 4.

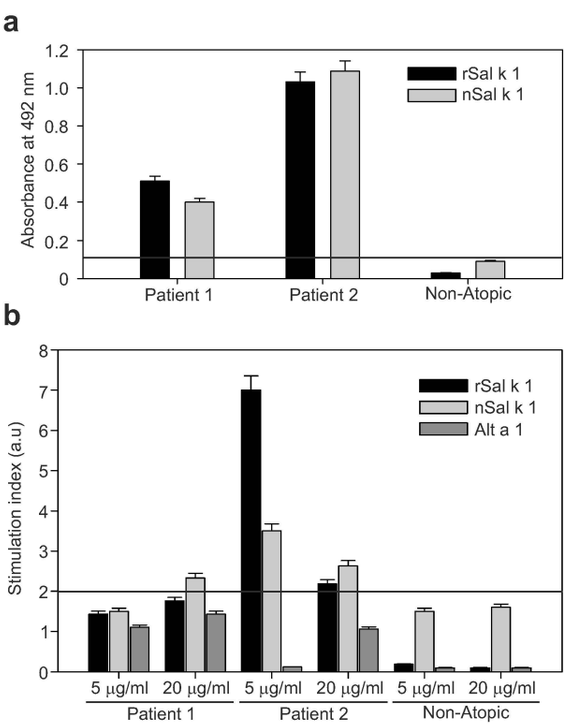
a
Absorbance at 492 nm
1.2
1.0
0.8
0.6
0.4
0.2
0
rSal k 1
nSal k 1
Patient 1
Patient 2
Non-Atopic
b
Stimulation index (a.u)
8
7
6
5
4
3
2
1
0
rSal k 1
nSal k 1
Alt a 1
5 μg/ml
20 μg/ml
5 μg/ml
20 μg/ml
5 μg/ml
20 μg/ml
Patient 1
Patient 2
Non-Atopic

Fig. 5.

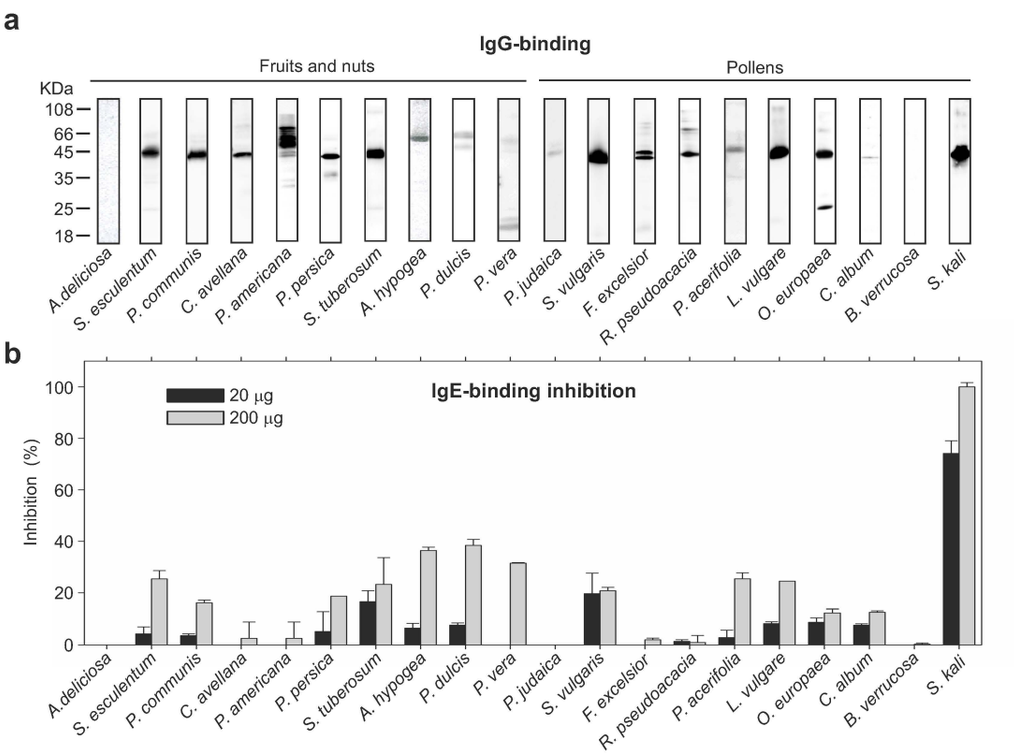
a
IgG-binding
Fruits and nuts
Pollens
KDa
108
66
45
35
25
18
A.deliciosa
S. esculentum
P. communis
C. avellana
P. americana
P. persica
S. tuberosum
A. hypogea
P. dulcis
P. vera
P. judaica
S. vulgaris
F. excelsior
R. pseudoacacia
P. acerifolia
L. vulgare
O. europaea
C. album
B. verrucosa
S. kali
b
IgE-binding inhibition
20 μg
200 μg
Inhibition (%)
100
80
60
40
20
0

Fig. 6.