

Expression of the Recombinant Major Allergen of *Salsola kali* Pollen (Sal k 1) and Comparison with Its Low-Immunoglobulin E-Binding Mutant

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ABSTRACT

Background: The inhalation of *Salsola kali* pollen is an important cause of pollinosis during summer and early fall throughout desert and semi-desert areas. Sal k 1 has been previously reported as a major allergen of *S. kali* pollen. In this study, we produced the recombinant Sal k 1 and also its low IgE-binding mutant form. We further compared the IgE binding ability of these two recombinant molecules.

Methods: The recombinant Sal k 1 and its low IgE-binding variant, obtained by three amino acid exchanges (R₁₄₂→S, P₁₄₃→A, D₁₄₄→V), were cloned and expressed in *E. coli*, as proteins fused with thioredoxin and His-tags, and then purified by Ni²⁺ affinity chromatography. The IgE-binding capacity of the wild-type and mutated rSal k 1 was compared using immunoblotting, ELISA and inhibition assays by ten sera from *S. kali* allergic patients. Moreover, *in vivo* IgE-reactivity was investigated by the skin prick test.

Results: Both the recombinant and the mutated form of Sal k 1 were expressed in *E. coli* at a relatively high amount and soluble form. All sera recognized rSal k 1 via immunoassay analysis. In addition, inhibition assays demonstrated that the purified rSal k 1 was similar to its counterpart in the crude extract. The mutated rSal k 1 exhibited a reduced IgE-binding capacity against wild-type rSal k 1.

Conclusions: This study demonstrates that purified rSal k 1 is comprised of IgE-epitopes similar to that of its natural counterpart and that the mutated variant showed a reduced IgE-binding capacity based on *in vitro* assays and *in vivo* provocation testing.

KEY WORDS

comparison, expression, mutant, Sal k 1, *Salsola kali*

INTRODUCTION

Salsola is one of the most well known genera in the family of Chaenopodiaceae. The members of this genera, especially *Salsola kali*; also known as *Russian thistle*, and *Salsola incanescens* produce high quantities of pollen grains and are abundant throughout the desert and semi-desert regions of Iran.¹⁻⁴ Moreover, pollens from various genera of Chenopodiaceae are the most common cause of pollinosis in the western United States, Europe and Asia.⁵ In Iran and other

semi-arid countries such as Saudi Arabia and Kuwait, inhalation of these pollens is one of the most typical causes of allergic respiratory symptoms in which the frequency of sensitization ranges from 53% to 76%.^{2,4,6,7}

Recently, a *S. kali* pollen-allergen was identified. Having an apparent molecular weight of 40 kDa, it also displayed pectin methylesterase (PME) properties. This allergen was named Sal k 1 and is considered a major allergen of *S. kali* pollen, as determined by Spanish *salsola* pollen-allergic patients.^{5,8} In addi-

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Table 1 Clinical characteristics, SPT responses and IgE-reactivity of the selected patients with allergy to *S. kali* pollen

Sample No.	Age (years)/sex	Clinical history [†]	Total IgE IU/ml	<i>S. kali</i> extract	
				Skin test [‡]	Specific IgE [§]
Patients					
1.	28/M	AR	1700	37	1.10
2.	22/F	AR, RC	1320	28	0.89
3.	23/F	AR	1200	31	0.90
4.	30/M	AR	2010	33	1.13
5.	27/F	AR, RC	1150	36	1.02
6.	26/F	AR	1980	25	0.80
7.	36/F	AR, RC	2010	32	1.08
8.	27/M	AR	1500	30	0.84
9.	26/M	AR	1820	41	1.07
10.	32/F	AR	1900	22	0.89
Controls					
11.	34/F	AR	700	-	0.10
12.	23/M	AR	550	-	0.10
13.	28/M	-	88	-	0.08
14.	35/F	-	70	-	0.09

[†] AR, allergic rhinitis; RC, rhinconjunctivitis.

[‡] The mean wheal areas are displayed in mm². Histamine diphosphate (10 mg/ml) -positive control; Glycerin-negative control.

[§] Determined in ELISA as OD (optical density) at 450 nm.

tion, more recently, we identified, characterized and submitted two other new allergens from *S. kali* pollen to the WHO/IUIS Allergen Nomenclature Subcommittee: Sal k 3, an approximately 85-kDa protein with methionine synthase properties; and Sal k 4, a profilin protein, (unpublished data). Due to the fact that sufficient quantities of allergens are essential for the development of *in vitro* and *in vivo* diagnosis of pollen-allergic diseases, many allergenic molecules have been cloned and expressed as recombinant proteins. During the last decade, the production of recombinant allergens has in turn allowed scientists to overcome many limitations of cross-reactivity studies and epitope complexities at the molecular level.⁹ Moreover, the availability of purified and well-defined recombinant forms of allergens and low IgE-binding or hypoallergenic variants of common and critical allergens has presented new opportunities both for developing diagnostic assays and therapeutic strategies and for creating specific preventive products.

Natural Sal k 1 has been previously purified⁸ and cloned⁵ as the major allergen of *S. kali* pollen. However, to our knowledge, it has not yet been expressed. For the first time, in this study, we produced the soluble form of the recombinant and hypoallergenic variants of Sal k 1 in *Escherichia coli*. After purification, the allergenic reactivity of these proteins was compared by employing the skin prick test and immunoblotting analysis with the sera from *S. kali* allergic patients.

METHODS

PATIENT POPULATION AND SKIN PRICK TEST (SPT)

Ten patients, six women and four men (mean age, 27.70 ± 4.13 years; age range, 22-36 years), presenting to the Immunology Research Center of Avicenna Research Institute were included in our study on the basis of a past medical history indicating a *S. kali* pollen allergy without asthma and a positive SPT to *S. kali* pollen extract (Table 1).

Two grams of the *Salsola kali* defatted pollen with a purity of more than 95% (purchased from Greer Laboratories, USA) were extracted as described previously.⁴ The protein content of the extract was measured by the Bradford's method.¹⁰ Skin prick tests were performed with *S. kali* pollen extract and also with purified wild-type and mutated recombinant Sal k 1 (at concentrations of 5, 10, 15 and 20 µg/ml) on the inner left forearms. After 15 minutes, the mean diameter of the wheal reaction in every patient was measured and compared with negative (50% Glycerin) and positive (histamine diphosphate, 10 mg/ml) controls. SPT with a wheal diameter >3 mm were considered positive compared to results obtained with negative and positive controls. Informed written consent was obtained from each patient and the study protocol was approved by the Human Ethics Committee of the Avicenna Research Institute.

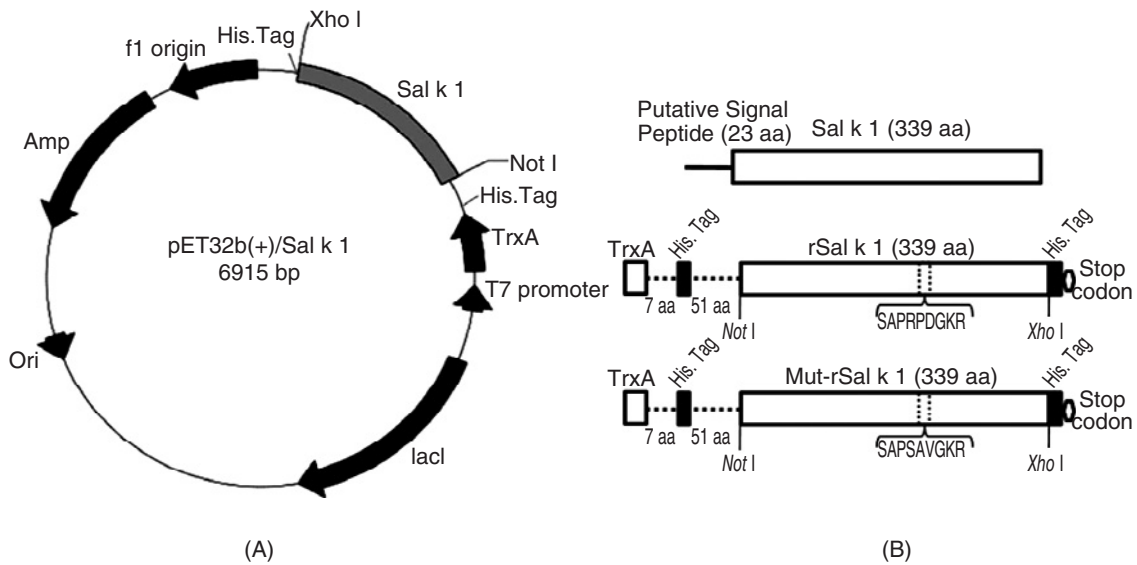


Fig. 1 (A) Schematic representation of expression vector, pET-32b (+)/Sal k 1, with T7 promoter, a fusion partner of thioredoxin (TrxA), two hexahistidyl (6 × His) affinity tag at the 5' and 3' ends of the Sal k 1 coding sequence and ampicillin resistance gene. (B) Schematic view of unprocessed natural Sal k 1 with putative signal peptide (*upper*), TrxA-rSal k 1 (*middle*) and TrxA-Mut-rSal k 1 (*lower*). The mutated amino acid residues are shown in bold.

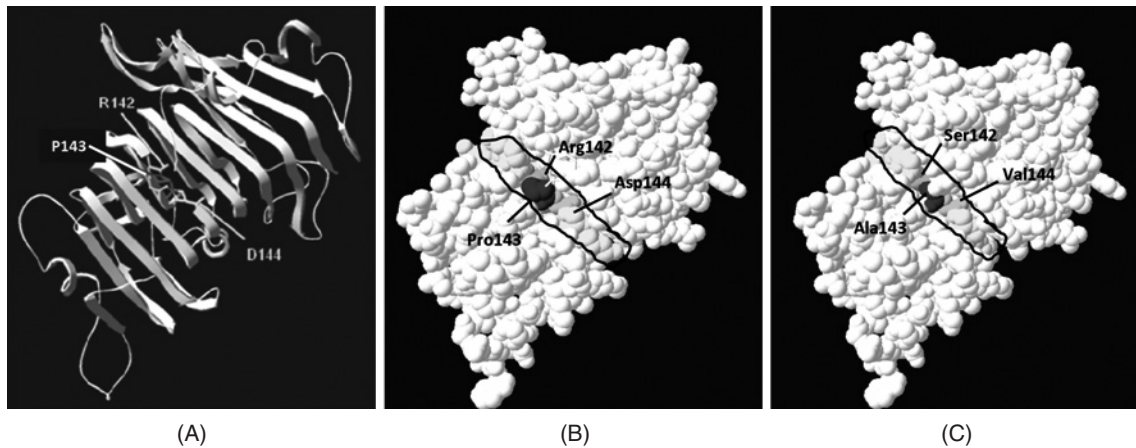


Fig. 2 Three-dimensional view of Sal k 1. (A) Ribbon style of Sal k 1 with the three amino acid residues corresponds to predicted B-cell epitope. The conformation of the amino acid residues are *trans* (R142, P143 and D144). (B) Space-filling model of Sal k 1 with predicted B-cell epitope (amino acids 135-150, are shown with closed line) and critical amino acids residues (Arg142, Pro143 and Asp144). (C) Mutated Sal k 1 (Mut-Sal k 1) with three point mutations (Arg₁₄₂ → Ser, Pro₁₄₃ → Ala and Asp₁₄₄ → Val).

AMPLIFICATION OF Sal k 1 cDNA BY RT-PCR AND DETERMINATION OF NUCLEOTIDE SEQUENCE

Coding sequences of Sal k 1, without putative signal sequence, were amplified using degenerate primers designed according to reported nucleotide sequences of Sal k 1 (AY776248, AY776249, AY590141): sense primer, 5'-CAA/GCC/TA/GATC/TCCA/CCCA/C/T AATCC-3'; and anti-sense primer, 5'-CACC/TTTA/

C/TGGC/TGGC/TGGA/TA/GGTAGCCA-3'. A PCR product of 1017 bp length was cloned into the pTZ57R/T vector (Fermentas, Vilnius, Lithuania) and transformed into a non-expression host, *E. coli* cells strain TOP10 (Invitrogen, San Diego, CA, USA). The isolated plasmids (pTZ57R/T-Sal k 1) were sequenced by the dideoxy method and analyzed by an automatic sequencer (SeqLab, Göttingen, Germany).

CONSTRUCTION OF PROKARYOTIC EXPRESSION PLASMID CARRYING THE Sal k 1 GENE

The obtained nucleotide sequence allowed for the designing of these two non-degenerate primers according to the 5' and 3' ends of the Sal k 1 cDNA: the sense primer, 5'-TCCGCGGCCGCACAGCTGATTCC CCCTAATCC-3' (with the *Not I* site underlined); and the anti-sense primer, 5'-TCTCTCGAGCACTTTCGG TGGTGGAGGTA-3' (with the *Xho I* site underlined). The amplified product was digested with *Not I* and *Xho I* (both from Fermentas, Vilnius, Lithuania) and ligated into the pET-32b(+) expression vector (Novagen, Gibbstown, NJ, USA) that was restricted with the same enzymes. The resulting expression construct, pET-32b(+)/Sal k 1 (Fig. 1), was transformed into competent BL21 (DE3) *E. coli* cells (Novagen, Gibbstown, NJ, USA) and the obtained positive clones were confirmed by restriction digestion analysis and sequencing.

MODELING, B-cell EPIOTOPE PREDICTION AND SITE-DIRECTED MUTAGENESIS OF Sal k 1

Homology model of Sal k 1 was generated using the coordinates from the pectin methylesterase of *Erwinia chrysanthemi* (PDB number: 1QJV) by the Internet server Swiss Model (<http://swissmodel.expasy.org/SWISS-MODEL.html>), as previously reported (Fig. 2A).

We used ABCpred, B cell epitope prediction software based on trained recurrent neural network (RNN), and Bcepred, a method for prediction of continuous B-cell epitopes in antigenic sequences using physico-chemical properties, for prediction of IgE-binding epitopes.^{11,12}

We generated the Sal k 1 mutant (R₁₄₂→S, P₁₄₃→A, D₁₄₄→V) by using the Phusion™ Site-Directed Mutagenesis Kit (FINNZYMES, Espoo, Finland) according to the manufacturer's instructions and by employing the mutagenic oligonucleotide (5'-CTCCAAGcgCAGtTGGA AAAA-3', with the mutant nucleotides indicated in lowercase). The expression construct pET-32b(+)/Sal k 1 was used as a template. The sequences of the mutated clones were confirmed by sequencing.

EXPRESSION AND PURIFICATION OF WILD-TYPE AND MUTATED rSal k 1 (Mut-rSal k 1)

The *E. coli* expression strain BL21 (DE3)pLysS (Invitrogen, San Diego, CA, USA) was transformed by recombinant plasmids harboring the wild-type or Mut-rSal k 1 genes. It was cultured in LB medium containing 50 µg/mL of ampicillin and incubated at 37°C. Subsequent to reaching an OD₆₀₀ of 0.4, protein expression was induced by the addition of isopropyl-β-D-thiogalactosidase (IPTG) to a final concentration of 0.2 mM. Following induction, in order to improve the solubility of recombinant proteins, the cultures were incubated at 18°C and cells were grown for 12 hours,

after induction. The cells were then disrupted by freezing in liquid nitrogen followed by thawing at 37°C. This freeze-thaw cycle was repeated three times. Insoluble materials were removed from the cell lysate by centrifugation at 12000 g for 20 minutes at 4°C. The supernatant containing expressed rSal k 1 or Mut-rSal k 1 was transferred to new tubes and stored at -20°C until use.

The purification of rSal k 1 and Mut-rSal k 1 was performed according to the IMAC (Immobilized Metal Affinity Chromatography) column using Ni-NTA agarose (Invitrogen) and by following the manufacturer's instructions. Protein concentration was determined according to the Bradford's method.¹⁰

TOTAL AND DIRECT ELISA

Total serum levels of IgE were measured in duplicate by a commercial ELISA kit (RADIM, Pomezia, Italy) according to the manufacturer's instructions. To determine the specific IgE reactivity of *S. kali* pollen extract, all ten sera were evaluated by ELISA. The four subjects who showed negative SPT responses to *S. kali* pollen extract (Table 1, sample numbers 11-14) were also used as negative controls. Ninety-six-well ELISA microplates (Nunc Maxisorb, Roskilde, Denmark) were coated with *S. kali* pollen extract (10 µg/well in 0.1 M sodium bicarbonate, pH 9.6), rSal k 1 or Mut-rSal k 1 (5 µg/well in 0.1 M sodium bicarbonate, pH 9.6) and incubated overnight at 4°C. Plates were then washed four times with PBS (pH 7.4, containing 0.05% Tween-20) and blocked with PBS/1% bovine serum albumin (BSA) for one hour at 37°C. After washing, 100 µl of the diluted patients' sera (1 : 2 in PBS/0.05 Tween-20/0.3% BSA) were added to each well and the plates were incubated overnight at room temperature. Plates were washed again and then incubated with biotinylated anti-human IgE (KPL, MD, USA) (1 : 1000 v/v in 1% BSA) for two hours at room temperature. The unbound antibodies were removed from the wells by washing, which was followed by incubation with 1 : 40000 v/v in BSA1% HRP-linked streptavidin (BD Biosciences, MD, USA) for 45 minutes at room temperature. After visualization of the enzymatic activity with tetramethylbenzidine (TMB) as a substrate at 37°C for 15 minutes, the reaction was stopped by the addition of 100 µl of 3N HCl, and absorption was measured at 450 nm. Optical density (OD₄₅₀) greater than four times the median values of negative controls were considered as positive.

ELISA INHIBITION

ELISA inhibition assays were performed as above, with the exception of a pooled serum (1 : 2 v/v) from *S. kali* allergic patients (Nos. 1, 2, 4, 6, 7, 9 and 10) which were preincubated for one hour at room temperature with 0.01, 0.1, 1, 10 and 100 µg of each inhibitor or with BSA as a negative control. Assays were performed in duplicate. The percentage of inhi-

ACO34813	-----	-----	---QLIPPNP	AEELESWFQGA	VKPVSEQKGL	EPSVVQAESG	GVETIEVRQD	GSG--KFKTI	55
AAX11262	-----	-----	---P....						55
AAX11261	-----	-----	---P....						55
AAT99258	MEEHVSMMLV	GFVLINIAFT	SIA.....			T..			78
P83218	-----	-----	-----	-----	-----	-----QS.T	VTPNVV.AA.	-DY..V	22
P07863	-----	-----	-----	-----MLKTI	SGTLALSLLI	AA..H..QAA	TTYNAV.SKS	S.DGKT....	45
ACO34813	SDAVKHVKVG	NTKRVIITIG	PGEYREKVKI	ERLHPYITLY	GIDPKNRPTI	TFAGT-----	-AAEFGTVDS	ATLIVESDYF	129
AAX11262									129
AAX11261				G.....					129
AAT99258								V.....S	152
P83218	.E..AAPED	SKT.YV.R.K	A.V...N.DV	PKKKKN.MFL	.DGRSTIIT	ASKNV-----	-QDGST.FN.	..VAAVGAG.	96
P07863	A..IASAPA	S.P-FV.L.K	N.V.N.RLT.	T.N--NLL.K	.ESRNGAVIA	AATAAGTLKS	DGSKW..AG.	S.ITISAKD.	122
ACO34813	VGANLIVSN-	-----SA	PRPDGKRKGA	QASALRIS--	GDRAAFYNCK	FTGFDDTVCD	DKGNHLFKDC	YIEGTVFIF	198
AAX11262		-----		R.....					198
AAX11261		-----		A.....				L..	198
AAT99258	...H...T.-	-----		.G.....			F.T..	.T.....	221
P83218	LARDITFQ.-	-----T.	G-----AAKH	.V...VG--	S.LS...R.D	ILAY..SLYV	HSNRQF.IN.	F.A.....	160
P07863	SAQS.TIR.D	FDFPANQAKS	DSDSS.I.DT	.V..YVTKS	...Y.KDVS	LV.Y..LYV	SG.RSF.S..	R.S.....	202
ACO34813	GEARSLYLNT	ELHVVP----	-GDPMAMITA	HARKNADGVG	GYSFVHCKVT	GTGG-----	--TALLGRAW	FEARVVFSY	265
AAX11262		-----				-----	-----		265
AAX11261		-----				-----	-----		265
AAT99258								D.....	288
P83218	.N.AVVLDQC	DI.ARRPG--	-SGQKN.V..	QG.TDPNQNT	.IVIQKSRIQ	A.SDLQPVQS	SEPTY...P.	K.YS.T.VMQ	237
P07863	.DGTA.FN.C	D.VSRYRADV	KSGNVSGYLT	APST.INQKY	.LVITNSR.I	RESDSVP---	AKSYG...P.	HPTTTFSDGR	279
ACO34813	CNLSDAVKP-	-----	-EGWSDNNK-	PAAQKTIFFG	-----EYK	NTGPGAAADK	RVPYT---KQ	LTEADAKTFT	322
AAX11262		-----							322
AAX11261		-----							322
AAT99258	A.-	-----		.E...L..		P..	.A.....		345
P83218	SSITNVIN.-	-----	-A..FPWDG-	NF.LD.LYY.	-----Q...	A...TSG	..TWKGFVKI	TSSTE.QG..	297
P07863	YADPN.IQGT	VFLNLSMDNH	IY..DKMSGK	DKNGN..W.N	PEDSRFF...	SY.A...VS.	DRR-----	..D.Q.AEY.	353
ACO34813	SLEYIEAAKW	LPPPPKV---	--	339	%Identity	%Similarity			
AAX11262		.L.....	--	339	99	99			
AAX11261			--	339	98	98			
AAT99258		.L.....	--	362	95	96			
P83218	PGSF.AGGS.	.KATTFPFSL	GL	319	31	47			
P07863	QSKVLG--D.	T.TL-----	--	366	29	42			

Fig. 3 The comparison of Sal k 1 amino acid sequence (ACO34813) (without signal peptide) with three different isoforms previously deposited in database banks (AAX11262, AAX11261, AAT99258) and pectin methylesterases from *Daucus carota* (carrot) (P83218) and *Erwinia chrysanthemi* (a gram-negative bacillus) (P07863). The full-length of Sal k 1 consists of 362 amino acids including a putative signal peptide of 23 residues. The cleavage position of signal peptide is between Ala23 and Gln24. The catalytic residues Gln151, Gln173, Asp174, Asp195 and Arg253 are shaded in gray. Numbers at right side showed the length of each sequence. Percentage of sequence identity and similarity with respect to Sal k 1 (ACO34813) are included at the bottom right of the figure.

bition was calculated using the following formula: (OD of sample without inhibitor - OD of sample with inhibitor/OD of sample without inhibitor) $\times$ 100.

IgE-IMMUNOBLOTTING AND IMMUNOBLOTTING INHIBITION

The *S. kali* pollen extract was fractionated by the means of SDS-PAGE in accordance with the Laemmli method¹³ on Bio-Rad Miniprotein II System gels (12.5% polyacrylamide) and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes, as previously explained.⁴ Moreover, 5 μ g/cm gel of purified rSal k 1 or mutated rSal k 1 (Mut-rSal k 1) was separated by the SDS-PAGE, and PVDF strips containing equal amount of blotted rSal k 1 or Mut-rSal k 1 was then exposed to sera from the patients.

The IgE-immunoblot inhibition experiment was performed as described previously,⁴ except that the pooled sera (1 : 5 v/v) from *S. kali* allergic patients

were preincubated for 3 hours at room temperature with equal volumes of purified inhibitors or with PBS-BSA as negative control.

RESULTS

PCR AMPLIFICATION AND CLONING OF Sal k 1 cDNA AND SEQUENCING OF PCR PRODUCTS

Total RNA was obtained from *S. kali* pollen, followed by the synthesis of the first cDNA strand and PCR using a pair of degenerate primers designed according to the reported nucleotide sequences of Sal k 1 (AY776248, AY776249, AY590141).⁵ The size of the PCR product was approximately 1017 bp. The nucleotide sequence of Sal k 1 was deposited in GenBank/NCBI databases under the accession number of FJ821453. The sequence analysis of the mature (processed) form of Sal k 1 showed an open-reading frame of 1017 bp length coding for 339 amino acid residues with a predicted molecular mass of 36.793 kDa, a cal-

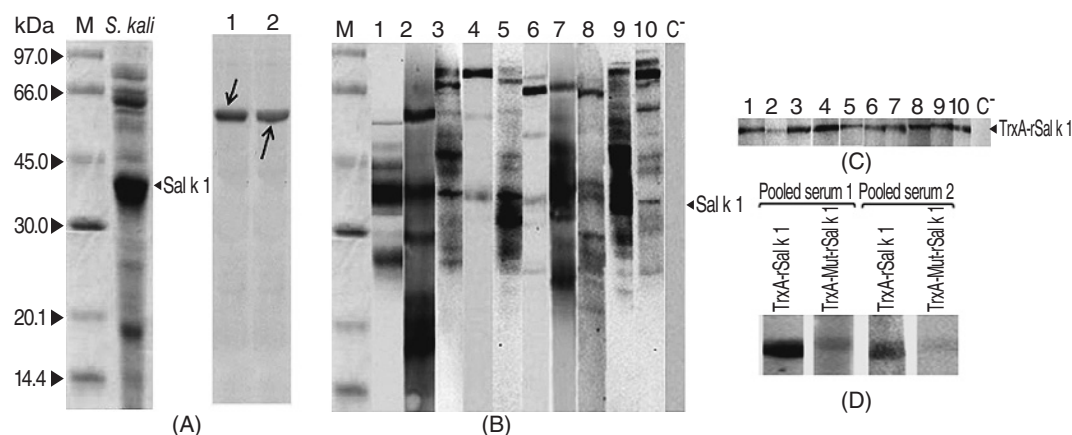


Fig. 4 (A) Reducing SDS-PAGE of *S. kali* pollen crude extract, TrxA-rSal k 1 and TrxA-Mut-rSal k 1 on 12.5% acrylamide gel (Coomassie staining). Lane M: low molecular weight (Amersham, Buckinghamshire, UK); Lane 1: Purified TrxA-rSal k 1; Lane 2: Purified TrxA-Mut-Sal k 1; (B) IgE-Immunoblot of *S. kali* pollen extract using allergic patients' sera; lane M, Low molecular weight (Amersham); lanes 1-10: Probed with individual allergic patients' sera; lane C: negative control; (C) IgE-immunoblotting analysis of rSal k 1 using 10 allergic patients. Lane M: low molecular weight (Amersham); lane 1-10: blotted rSal k 1 probed with individual *S. kali* -allergic patients' sera; lane C: negative control. (D) IgE-immunoblotting analysis of wild-type and mutated rSal k 1; equal amounts of purified rSal k 1 and Mut-rSal k 1 were subjected to SDS-PAGE, blotted onto a PVDF membrane and probed with two pooled sera consisting of *S. kali* allergic patients 1-5 (pooled serum 1) and patients 6-10 (pooled serum 2).

culated pI of 7.7, and a potential N-glycosylation site at Asn-267. According to an earlier study,⁵ the unprocessed protein consists of 362 amino acids including a putative signal peptide of 23 residues. Moreover, the suggested cleavage position for processing of mature Sal k 1 is located between Ala23 and Gln 24.⁵

The amino acid sequence comparisons of the deposited Sal k 1 (ACO34813) (without signal peptide of 23 amino acids) with those of previously reported isoforms of Sal k 1 (AAX11262, AAX11261, AAT99258), and with pectin methylesterases from *Daucus carota* (carrot) (P83218) and a gram-negative bacillus, *Erwinia chrysanthemi* (P07863), are showed in Figure 3.

EXPRESSION AND IMMUNOREACTIVITY OF NATURAL AND RECOMBINANT Sal k 1

The pET-32b(+)/Sal k 1 clone was successfully constructed and confirmed by digestion with *Not* I and *Xho* I restriction enzymes and sequencing. This recombinant plasmid containing TrxA, to increase the solubility, and his-tags, for Ni-NTA purification, was expressed in *E. coli* strain BL21 (DE3)pLysS as an approximately 56-kDa recombinant protein (TrxA-rSal k 1). According to the Bradford's method,¹⁰ concentration of the purified recombinant protein (rSal k 1) and the Mutated rSal k 1 (Mut-rSal k 1) were approximately 1.1 and 1.3 grams per 1 liter of expression medium, respectively (Fig. 4A).

Immunoblot analysis revealed that all selected patients were reactive to natural Sal k 1 pollen (Fig. 4

B). Moreover, all ten *S. kali* allergic patients' sera reacted to recombinant Sal k 1, in contrast to the healthy control sera which exhibited no reaction (Fig. 4C).

IgE-binding reactivity of the purified TrxA protein was evaluated by immunoblotting and ELISA assays and showed no reaction with the pooled sera from *S. kali* allergic patients (data not shown).

Inhibition experiments were performed to investigate the IgE-binding potential of the purified rSal k 1 in comparison with its natural counterpart. The results showed that pre-incubation of serum samples with the native crude extract of *S. kali* pollen (Fig. 5, lane 2) or rSal k 1 (Fig. 5, lane 3) completely inhibited IgE binding to natural Sal k 1. However, preincubation of the pooled serum with PBS-BSA (Fig. 5, lane 1) or the purified TrxA protein (data not shown) did not affect IgE binding. These results demonstrated that the recombinant protein allergen (rSal k 1) had a high degree of immunological identity with its natural counterpart.

Moreover, the specific IgE values from the *S. kali* pollen extract (Table 1) and purified rSal k 1 were determined using ten individual patients' sera. (Table 2). All patients exhibited significantly elevated specific IgE when tested with the *S. kali* pollen extract or with the purified rSal k 1.

Mut-rSal k 1 SHOWS REDUCED IgE-BINDING REACTIVITY

The results of B-Cell epitope prediction using

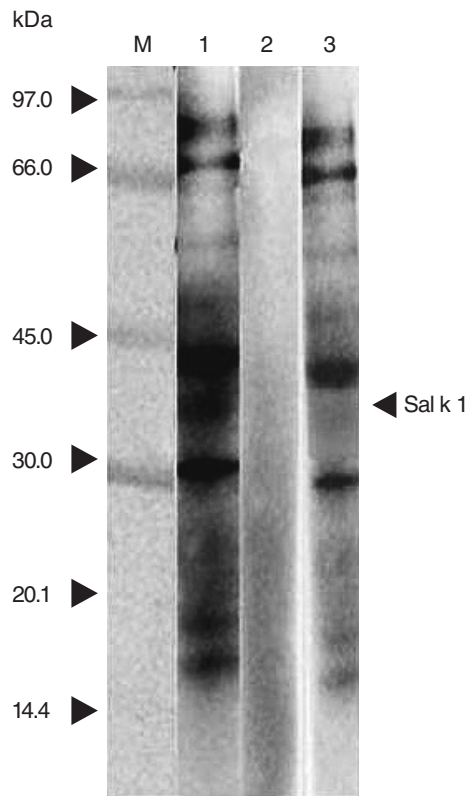


Fig. 5 IgE-immunoblotting inhibition analysis. The pollen extract of *S. kali* was separated by SDS-PAGE and blotted onto PVDF. IgE-binding of a pooled serum, including that of four *S. kali* pollen allergic patients, Nos. 1, 3, 4 and 8, were studied after preincubation with PBS-BSA (lane 1), *S. kali* extract (50 µg/ml) (lane 2) and purified rSal k 1 (50 µg/ml) (lane 3). Lane M, low molecular weight (Amersham).

ABCpred, a predictive software based on artificial recurrent neural network (RNN), indicated that the peptide corresponding to amino acids 135-150 was one of the most dominant B-cell epitopes of Sal k 1 (Fig. 2B, C). Three other dominant B-cell epitopes were the peptides corresponding to amino acids 52-67, 274-289 and 214-229.

Mut-r Sal k 1 was expressed in *E. coli* strain BL21 (DE3)pLysS and purified by Ni-NTA agarose according to procedures described earlier for wild-type rSal k 1.

The evaluation of the IgE-binding reactivity of Mut-rSal k 1 with three amino acid substitution compared to that of wild-type rSal k 1 was performed by immunoblot assays using two pooled sera from ten allergic patients. Figure 4D shows the IgE-binding reactivity of two pooled sera from allergic patients to wild-type and Mut-rSal k 1. Both of the two pooled sera displayed low IgE-binding reactivity to Mut-rSal k 1 compared to the wild-type rSal k 1. Therefore, it appeared that the amino acid substitutions were effective in reducing the IgE-binding capacity of Sal k 1.

The direct ELISA assay with Mut-rSal k 1 indicated that the IgE-binding capacity of this mutated recombinant protein was significantly lower than that of wild-type rSal k 1 ($p < 0.001$) (Table 2). Also, using a pooled serum from the allergic patients, ELISA inhibition assays were performed to evaluate the inhibition of IgE-binding to immobilized wild-type rSal k 1 by its mutated counterpart. The results showed that the Mut-rSal k 1 exhibited a much lower inhibition capacity than the wild-type recombinant protein (Fig. 6).

Table 2 Skin prick tests and specific ELISA with rSal k 1 and Mut-rSal k 1

Patients	Skin prick test [†]								Specific IgE [‡]			
	TrxA-rSal k 1 (µg/ml)				TrxA-Mut-rSal k 1 (µg/ml)				Histamine	Glycerin	TrxA-rSal k 1	TrxA-Mut-rSal k 1
	5	10	15	20	5	10	15	20				
1.	5	15	20	30	3	8	10	15	15	0	0.90	0.52
2.	5	10	15	25	0	0	8	10	13	0	0.79	0.41
3.	4	6	15	28	2	4	8	13	14	0	0.85	0.45
4.	10	18	25	31	4	9	15	20	20	0	0.95	0.49
5.	10	12	20	32	5	9	12	18	18	0	0.92	0.51
6.	3	5	12	19	0	0	5	12	12	0	0.75	0.38
7.	5	12	25	28	2	6	12	15	12	0	0.88	0.40
8.	4	15	20	25	1	8	12	17	15	0	0.80	0.40
9.	12	20	25	40	6	9	15	23	16	0	0.96	0.58
10.	4	10	13	18	0	0	4	10	10	0	0.78	0.36
Mean (±SD)	6.2 (3.1)	12.3 (4.8)	19.0 (5.0)	27.6 (6.4)	2.3 (2.1)	5.3 (3.9)	10.1 (3.8)	15.3 (4.2)	14.5 (2.9)	0 (0)	0.85 (0.75)	0.45 (0.71)

[†] The mean wheal areas are displayed in mm². Histamine diphosphate (10 mg/ml) -positive control; Glycerin-negative control. The wheals induced by Mut-rSal k 1 were significantly smaller than the ones obtained with rSal k 1 ($p < 0.001$) using student's *t* test.

[‡] Determined in ELISA as OD (optical density) at 450 nm. The OD values of Mut-rSal k 1 is statistically lower than the ones with rSal k 1 ($p < 0.001$).

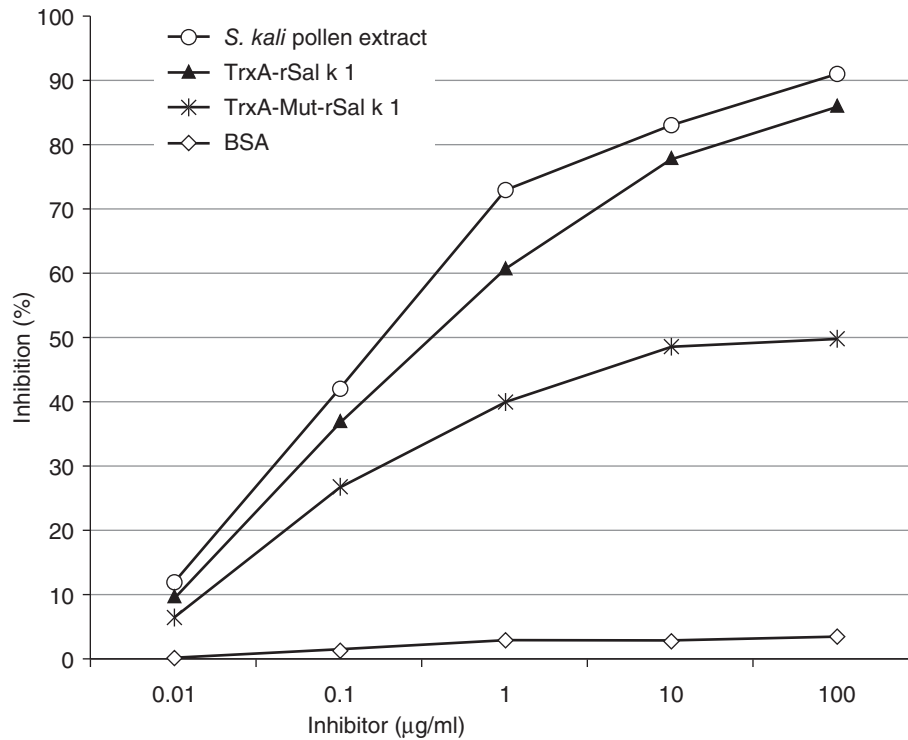


Fig. 6 Inhibition of IgE-binding to rSal k 1 by ELISA using *S. kali* pollen extract, rSal k 1, and -Mut-rSal k 1. Control experiments were performed with BSA.

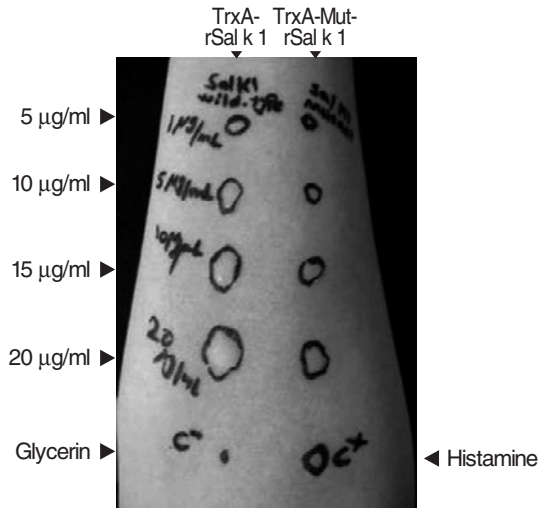


Fig. 7 Ability of purified rSal k 1 and Mut-rSal k 1 to elicit immediate skin reactions. The results of skin prick test on a *S. kali* allergic patient (patient No. 9) with different concentrations (5, 10, 15 and 20 µg/ml) of rSal k 1 and Mut-rSal k 1 are presented. Histamine diphosphate (10 mg/ml) (Histamine) and glycerin were used as positive and negative control, respectively. The wheal reactions were marked with a ball-point pen.

Mut-rSal k 1 HAS A REDUCED ABILITY TO ELICIT IMMEDIATE CUTANEOUS REACTIONS

The capacity of the wild-type Sal k 1 and Mut-rSal k 1 to induce immediate skin reactions were investigated in ten *S. kali* allergic patients by *in vivo* SPTs (Table 2). All of the allergic patients showed a positive cutaneous reaction to wild-type rSal k1 at different concentrations (5, 10, 15 and 20 µg/ml). Following the application of Mut-rSal k 1 on the inner left forearm of allergic patients, three patients (Nos. 2, 6 and 10) showed negative SPT at concentrations of 5 and 10 µg/ml. The mean wheal areas induced by different concentrations of Mut-rSal k 1 were significantly smaller than those obtained with rSal k 1 ($p < 0.001$) (Table 2, Fig. 7). In control patients, no skin reactions were elicited with the application of purified rSal k 1 or Mut-rSal k 1.

DISCUSSION

Sal k 1 is an allergenic member of the pectin methylesterase superfamily. Several allergens from this superfamily, such as Bet v 8 and a 34-kDa-protein of tomato^{14,15} have already been identified. In this study, we produced wild-type rSal k1 as a major allergen of *S. kali* pollen, along with a low IgE-binding variant of rSal k 1 (Mut-rSal k 1), both in *E. coli*. In addition, it was demonstrated that generating a low IgE-binding rSal k 1 variant by the exchange of amino acid residues via a site-directed mutagenesis is possi-

ble.

Baraderas *et al.* showed that the purified natural Sal k 1 like the one in the total extract reacted to all eleven allergic patients' sera.⁵ Concurrently, in the present study, natural Sal k 1 in total extract of *S. kali* pollen was recognized by all of the patients' sera.

This study evaluated the IgE-binding ability of purified rSal k 1 to the sera from ten *S. kali* allergic patients by immunoblotting assays in order to confirm that the recombinant protein was equivalent to its natural counterpart and correctly folded. The results revealed that, similar to natural Sal k 1 in total extract of *S. kali* pollen, all sera reacted with rSal k 1.

In addition, complete inhibition of IgE-binding to natural Sal k 1 was obtained after preincubation of pooled serum with purified recombinant Sal k 1. Moreover, the results of the ELISA inhibition experiments were entirely consistent with those obtained from immunoblotting assays. Regarding these results, it appears that rSal k 1 is comprised of IgE-epitopes, similar to that of its natural counterpart.

Among four defined allergens from *S. kali* pollen (Sal k 1, Sal k 2, Sal k 3 and Sal k 4, submitted to WHO/IUIS), the study selected Sal k 1 to generate a low IgE-binding variant. This was for two reasons. First, Sal k 1 is the most abundant protein in *S. kali* pollen extract.⁸ Second, the results, in accordance with previous studies, showed that almost 100% of *S. kali* pollen allergic patients reacted to Sal k 1.^{5,8} However, several studies indicated that in addition to Sal k 1, there are a number of other allergenic components in the total extract of *S. kali* pollen, which are frequently recognized by specific IgE.^{1,4,5,8,16}

SDS-PAGE results showed that the pattern of migration of the Sal k 1 was not changed under both non-reducing and reducing conditions (data not shown). This was in accordance with the previous study⁸ and suggests that five cysteine residues of Sal k 1 may be associated with intrachain disulfide bonds.

Several studies have demonstrated that it is possible that a few changes in critical residues from the predicted epitope region for antibody binding drastically decrease the IgE-binding capacity of the epitope.¹⁷⁻²¹ Accordingly, the strategy to generate rSal k 1 with a low IgE-binding capacity (Mut-rSal k 1) was based on the exchange of the three amino acid residues (Arg₁₄₂→Ser, Pro₁₄₃→Ala, Asp₁₄₄→Val) of the dominant predicted B cell epitope located around the middle sequence of this recombinant protein. It is noteworthy that, based on Bcepred, a continuous B-cell epitope prediction software,¹² all three residues are among the most accessible, flexible and hydrophilic residues in Sal k 1; in addition, the residue Asp 144 has the highest antigenic propensity.

Using the B cell epitope prediction software, all the possible exchanges of one or more amino acid residues were evaluated to select the most appropriate

amino acid substitutions to reduce the antigenic propensity of the peptide corresponding to amino acids 135-150. Nevertheless, further epitope mapping experiments are necessary to demonstrate the effect of exchange of each residue on decreasing the IgE-binding capacity of the predicted B cell epitope. Moreover, Takai *et al.* showed that replacement of proline with alanine may be an efficient strategy for reducing of IgE-binding activity of Der f 2.²²

Based on the previous studies, it is possible to reduce the IgE-binding activity of an allergen by a few point mutations without considerably alteration of the overall structural fold of the molecule.^{23,24} Moreover, analysis of Sal k 1 and Mut-Sal k 1 molecular models by PROCHECK²⁵ and evaluation of predicted conformational B cell epitopes with CEP (<http://bioinformatics.in/cep.htm>) and SEPPA (<http://lifecenter.sgsc.cn/seppa/submit.php>) software seem not to significantly alter the similarity exhibited between the two models.

To exchange critical residues and modify one of dominant predicted B cell epitopes of rSal k 1, *in vitro* site-directed mutagenesis was used. This method has been effectively performed in several studies for decreasing or abolishing the IgE-binding capacity of allergenic recombinant proteins.^{18,19,24,26}

The findings of the study are in line with several previous studies which demonstrated that manipulated allergen variants are shown to elicit a reduced IgE reactivity.^{23,24,26}

The IgE-binding reactivity of allergenic proteins is usually assessed by SPT.^{17,27,28} Thus, this study employed SPT to compare the ability of wild-type rSal k 1 with its mutated variant (Mut-rSal k 1) to elicit IgE-mediated reactions. Indeed, SPT was used to imitate early cutaneous allergic reactions (characterized by a local edema known as wheal). The results showed a reduction in the IgE-binding reactivity of Mut-rSal k 1 compared to that of wild-type rSal k 1. However, the findings of SPTs revealed variations among allergic patients in immediate cutaneous reactions to both wild-type and mutated rSal k 1. This is in accordance with previous studies.^{18,29}

Taken together, the results of *in vitro* and *in vivo* assays indicate that amino acid positions 142, 143 and 144 may play an important in the IgE-binding reactivity of the Sal k 1 allergen.

In conclusion, this study demonstrates that purified rSal k 1 is comprised of IgE-epitopes similar to that of its natural counterpart and that the mutated variant (Mut-rSal k 1) showed a reduced IgE-binding capacity based on *in vitro* assays and *in vivo* provocation testing. All together, these recombinant proteins could be effective candidates for further studies on cross-reactivity and the development of diagnostic and therapeutics approaches.

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