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A pectin methylesterase as an allergenic marker for the sensitization to Russian thistle (*Salsola kali*) pollen

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Clinical and Experimental Allergy

Summary

Background Chenopodiaceae pollen is considered the main cause of pollen allergy in desert countries and its incidence is world-wide increasing by the desertization of extensive zones. Although the correlation between the sensitization to *Chenopodium album* and *Salsola kali* pollens of patients suffering from allergy to Chenopodiaceae pollens is high, a significant number of patients exhibited IgE sensitivity exclusively towards *S. kali*.

Objective To analyse this differential reactivity and to purify, clone and characterize the putative responsible allergen.

Methods Immunoblotting was used to analyse the IgE binding to pollen extract for *S. kali* and *C. album*. The protein was isolated by two chromatographic steps and characterized by Edman degradation, mass spectrometry, finger print analysis and Concanavalin A lectin staining. Specific cDNA was amplified by polymerase chain reaction, cloned in *Escherichia coli* and sequenced. Immunologic characterization was performed by immunoblotting, enzyme-linked immunoassay detection and inhibition experiments using sera from 11 patients allergic to *S. kali* pollen.

Results cDNA codifies for a mature protein of 339 amino acids plus a putative signal peptide of 23 residues and it belongs to the plant pectin methylesterase (PME) family. It is a mildly basic and polymorphic protein and was recognized by the IgE from all the patients allergic to *S. kali* included in the study, and was called Sal k 1. The protein was not recognized in the *C. album* pollen extract using the sera of these patients.

Conclusion Sal k 1 is a protein from the PME family with a high allergenic relevance. Considering this allergen as responsible for the different sensitization between *S. kali* and *C. album* pollen, it may be a useful marker to classify patients allergic to Chenopodiaceae allowing a safer and more specific immunotherapy.

Keywords Chenopodiaceae pollinosis, pectin methylesterase, Sal k 1, *Salsola kali*

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Introduction

Pollens from the Chenopodiaceae family have been reported as important sources of pollinosis in Western United States [1] and temperate European countries [2]. Chenopodiaceae sensitization is a severe problem in semi-desertic countries such as Saudi Arabia, Iran and Kuwait where these weeds are the main cause of sensitization probably due to their recent use as in greening programmes or ornamental plants [3, 4]. As regards the allergenic character, the most representative members of this family are Lamb's quarter (*Chenopodium album*) and

Russian thistle (*Salsola kali*), although other pollens of this family have been described as allergy sensitizers, such as sugar beet (*Beta vulgaris*) and frebush (*Kochia scoparia*) [5].

Cross reactivity within the Chenopodiaceae family is a common feature of patients suffering from pollinosis to these pollens [6]. In fact, high cross reactivity between *C. album* and *S. kali*, and considering other species taxonomically less related as members of the Amaranthaceae family like *Amaranthus retroflexus*, has been reported for patients suffering sensitization to Chenopodiaceae pollens [6]. Correlations of the sensitivity against

Table 1. Characteristics of the population

Patient	Age/sex	Symptoms	Chenopodium album extract		Salsola kali extract		Sal k 1	Other sensitizations
			Skin test	Specific IgE*	Skin test1	Specific IgE*	Specific IgE*	
1	46/M	RC	–	0.04	+	1.20	0.39	O, Phl, Art, Par, Cup
2	35/F	RC, A	–	0.07	+	1.95	0.32	–
3	38/F	RC	–	0.07	+	1.40	0.25	–
4	4/F	RC, A	–	0.02	+	0.30	0.36	O, Cat, Dog
5	26/M	RC	–	0.03	+	0.65	0.25	O, Phl, Art, Pla, Ros
6	42/F	RC	–	0.01	+	0.12	0.30	–
7	32/M	RC	–	0.04	+	1.35	0.26	O, Phl, Par
8	25/F	RC	–	0.01	+	0.70	0.22	O, Phl
9	20/F	RC	–	0.06	+	0.95	0.32	Ros
10	35/F	RC	–	0.01	+	0.35	0.19	–
11	35/F	RC	–	0.04	+	0.70	0.26	–

Age in years.

*Determined in ELISA as optical density at 492 nm.

ELISA assays were used to measure the specific IgE to *C. album* and *S. kali* pollen extracts and purified Sal k 1 allergen. They are shown as OD values. RC, rhinoconjunctivitis; A, asthma; M, male; F, female; O, olive; Phl, *Phleum*; Art, *Artemisia*; Par, *Parietaria*; Cup, *Cupressus*; Pla, *Plantago*; Ros, *Rosaceae*.

C. album in patients sensitized to *S. kali* have been reported to vary between 70–96% and 70–75% by skin prick test (SPT) or RAST, respectively [7–9].

The allergenic protein profile of the Chenopodiaceae family has not been extensively studied, mainly due to the limited allergenic relevance of these pollens in developed countries. However, the allergenic incidence of these weeds has been lately going up due to the desertization of extensive zones in Europe. In 1981, two allergenic proteins with molecular weight of 39.0 and 42.0 were reported by Shafiee et al. [10] in the *Salsola pestifer* pollen. In the last years, four allergens belonging to *C. album* and *S. kali* pollen have been isolated and characterized. Our group has identified and characterized Che a 1, member of the Ole e 1-like protein family, Che a 2 (profilin) and Che a 3 (polcalcin) from *C. album* [11–13]. In addition, Sal k 1 from *S. kali*, whose partial sequence did not permit the authors to find similarities with other known proteins of the Database banks, has also been reported [14]. The high prevalence of sensitization to some of these allergens in many patients allergic to these weeds could explain the cross reactivity. In fact, Che a 2 and Che a 3 belong to protein families of panallergens and are responsible for cross reactivity with both taxonomically related and non-related pollens [15, 16]. In addition, it is well established that the taxonomically related allergens from the Ole e 1-like family are involved in cross reactivity [17] and, thus, the involvement of Che a 1 as a cross-reactive allergen within the Chenopodiaceae family should not be ruled out.

Even though the concomitant sensitization to *S. kali* and *C. album* could be attributed to the presence of homologous allergens with closely related protein sequences, the fact that a number of patients, ranging

between 10% and 30%, are sensitized exclusively to *S. kali* led us to search for the specific allergen that could be responsible for this differential sensitization. In this study, we aimed to analyse the different reactivity to these pollens in a population of patients suffering from sensitization to *S. kali* but not to *C. album* pollen and find the allergenic markers for this differential sensitization.

Materials and methods

Pollens, extracts and sera

C. album and *S. kali* pollens were purchased from ALK-Abelló (Madrid, Spain). Pollen extracts were prepared according to Barderas et al. [13]. Serum samples were obtained from 11 patients sensitized to *S. kali* from Hospital Virgen de la Arrixaca (Murcia, Spain). These patients were polysensitized to other pollens such as *Cupressus*, *Parietaria*, *Artemisia* (Table 1) and were non-reactive by SPT to *C. album* pollen extract. A non-atopic subject was used as negative control. All patients and control subjects provided written informed consent.

Electrophoresis and immunological analyses

SDS-PAGE was performed in 15% polyacrylamide gels by the method of Laemmli [18]. Proteins (100 µg of pollen extracts and 2 µg of purified protein) were stained with Coomassie blue or electrophoretically transferred (1 µg of purified protein) to nitrocellulose membranes. Immunodetection on the membranes was achieved by using a pool of serum samples ($n = 11$) from the selected patients (diluted 1:10) or the individual sera. The binding of human IgE was detected by mouse anti-human IgE

antibodies (ALK-Abelló) (diluted 1 : 5000) followed by horseradish peroxidase (HRP)-labelled goat anti-mouse IgG (diluted 1 : 5000) (Pierce Chemical Co., Rockford, IL, USA). The reaction was developed using a Western Blotting reagent (ECL; Amersham Biosciences, Barcelona, Spain) [19].

The IgE-binding quantification was performed by ELISA in microtitre plates coated with 40 µg of protein extract or 0.1 µg of purified protein per well. Plates were incubated with allergic sera (diluted 1 : 10). After incubation with the mouse anti-human IgE antibody and HRP-labelled goat anti-mouse IgG described above, the reaction was developed with *o*-phenylenediamine, and the optical density was measured at 492 nm [11]. Each value was calculated as mean of two determinations after blank subtraction. Each OD values <0.1 were considered negative responses.

For immunoblotting inhibition assays, the electrophorized and blotted *S. kali* pollen extract (100 µg) was immunostained with the same pool of sera mentioned above, although previously incubated with PBS as negative control or, alternatively, with 500 µg of *S. kali* or *C. album* pollen extract as inhibitors. The percentage of inhibition was calculated by densitometric analysis of protein bands using the program UVISoft-UVIb (UVI Tech, Cambridge, UK).

Protein isolation and molecular analysis

Two purification steps were performed to isolate the allergen. *S. kali* pollen extract was loaded onto a Sephacryl S-100 size-exclusion chromatography column in 0.2 M ammonium bicarbonate, pH 8.0. Fractions containing the protein were pooled and lyophilized to be loaded onto an ionic exchange column of fast protein liquid chromatography (FPLC), Mono Q HR 5/5, the elution was carried out with a continuous gradient of NaCl 0–0.5 M. Fractions were tested by Coomassie blue staining, and IgE-binding measuring with a pool of allergic sera by immunoblotting. The purified allergen was lyophilized and stored at –20 °C until use.

Protein concentration was quantified by Lowry method [20] of protein extracts and purified samples by amino acid analysis after acid hydrolysis with 5.7 N HCl. Samples were analysed on a 6300 amino acid analyzer (Beckman Coulter, Fullerton, CA, USA).

Isoelectric focusing was achieved by using strip gels containing an immobilized pH gradient from 3 to 10 (BioRad Laboratories, Hercules, CA, USA), using the Pro-tean Isoelectrofocusing (IEF) system (BioRad) as described [21]. Standard proteins from Bio-Rad (IEF standards, pI range 3.6–9.3) were used to calibrate the strips. After the isoelectrofocusing, proteins were separated by SDS-PAGE in 15% polyacrylamide gels, transferred to membranes and immunostained with a pool of the allergic sera.

Amino acid sequencing of the N-terminal region of the polypeptides was performed by Edman degradation using a 494 sequencer (Applied Biosystems, Foster City, CA, USA). Prediction of the N-terminal cleavage of the signal peptide was performed by using the Signal IP 3.0 program (<http://www.cbs.dtu.dk/services/SignalP/>) [22].

The model of the three-dimensional (3D) structure was obtained by using the coordinates of the *Erwinia chrysanthemi* pectin methylesterase (PME) (Accession number 1QJV) in the Protein Data Bank and the SWISS MODEL software (<http://swissmodel.expasy.org/>) [23].

Carbohydrate detection

Carbohydrate detection was performed on proteins transferred to nitrocellulose membranes after bidimensional SDS-PAGE and transference to nitrocellulose membranes, using a biotinylated Concanavalin A solution followed by the procedure as described [24]. The staining was developed by horseradish peroxidase reaction with 0.05% 3, 3'-diaminobenzidine/0.03% of 30% H₂O₂ as substrate in Tris-HCl/NaCl, pH 7.5, reaction buffer.

Mass spectrometry and Edman degradation analyses

Mass spectrometry (MS) was performed according to [24]. In order to obtain the peptide fingerprint, bands of interest were manually excised from polyacrylamide gels, alkylated and digested with trypsin [21]. Matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) MS analyses were performed in a Voyager-DETMSTR instrument (PerSeptive Biosystems, Framingham, MA, USA). All mass spectra were externally calibrated using a standard peptide mixture (Sigma-Aldrich, St Louis, MO, USA). Peptides from the auto-digestion of trypsin were used for the internal calibration. MS/MS sequencing analyses were carried out using the MALDI-tandem-TOF MS spectrometer 4700 Proteomics Analyzer (Applied Biosystems).

Native protein (1–1.8 nmol) was digested with sequence grade tosylphenyl-alanine-chloromethane-treated trypsin and soluble peptides were fractionated by RP-HPLC on a Nucleosil C-18 column. Edman degradation of peptides were performed using an Applied Biosystem model 494 sequencer (Applied Biosystems-PE Corp.) [25].

Cloning and sequence of *Sal k 1*

Total RNA from *S. kali* pollen was extracted as described [26]. cDNA was synthesized from RNA using the SMART RACE cDNA amplification kit (BD Biosciences-Clontech, Madrid, Spain). *Sal k 1*-specific cDNA was amplified by PCR in a three-step procedure. A sense 5'-TTYCARGA YACNGTNTGYGAY and an antisense 5'-AARGTYCTR TGNCANACRCTR degenerate primers which hybridized with a DNA whose sequence derived from that of the

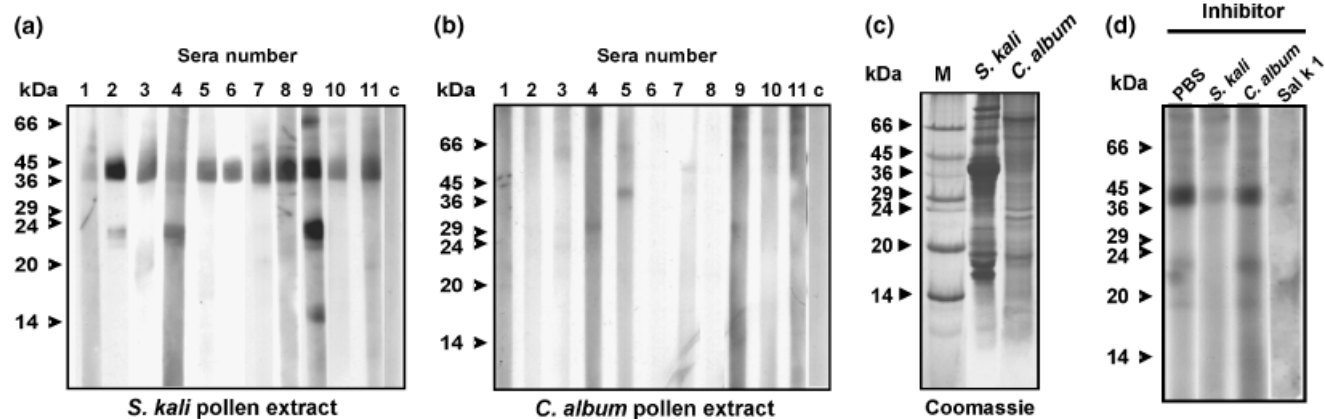


Fig. 1. IgE-immunoblotting of *Salsola kali* (a) and *Chenopodium album* (b) pollen extracts after incubation with the sera from 11 patients and a non-atopic control (c). Coomassie blue staining after SDS-PAGE of both extracts (c). Inhibition of the IgE binding to *S. kali* pollen extract by using PBS, *S. kali* or *C. album* pollen extracts (500 µg), and Sal k 1 allergen (5 µg), as inhibitors (d). M: molecular mass markers.

internal sequence FQDTVCD of the protein determined by the Edman reagent, together with the non-specific primer included in the kit, were used in the first two rounds of PCR. Using these degenerate primers, the sequences of the 5' and 3' ends of the Sal k 1-encoding region were obtained. A third PCR round, now with the sense primers, 5'-cgtctcgagCARCCNATHCCNCCNAAYCC and 5'-cgtctcgagATGGARGARCAYGTNAGY, which hybridized with the 5' (with and without the signal peptide) end of the Sal k 1-encoding sequence, and the antisense primer, 5'-cgtccgcggTTANACYTTNGGNGGNGG, which hybridized with the 3' end, was performed to amplify the complete cDNA sequence encoding Sal k 1. The purified DNA was cloned directly in the pCR2.1 plasmid included in the TOPO TA-cloning kit (Invitrogen, Groningen, the Netherlands), and used to transform TOP10 *Escherichia coli* cells. Several DNA clones were sequenced by standard procedures.

Results

Allergenic profile for patients allergic to *Salsola kali*

IgE immunostaining of *S. kali* and *C. album* pollen extracts blotted onto nitrocellulose membranes after SDS-PAGE was performed with the 11 sera included in this study (Fig. 1a and b). IgE-binding assays for *S. kali* and *C. album* in ELISA with the same patients' sera are included in Table 1. The protein composition of both extracts was analysed by Coomassie blue staining (Fig. 1c). The IgE-binding profile of *S. kali* is mainly composed of a major reactive band of 40 kDa, which is recognized by 100% of the patients' sera, and two allergenic components of 23 and 14 kDa, whose frequencies of sensitization were 27% ($n = 3$) and 9% ($n = 1$), respectively. These bands were not detected in *C. album* pollen, which would be in line with the data obtained by SPT and ELISA (Table 1).

The molecular mass of the main IgE-reactive protein from *S. kali* corresponds to the mobility of the most abundant protein in this extract (Fig. 1c).

Aiming to assess the involvement of the 40 kDa protein in the IgE differential reactivity to *S. kali* and *C. album* pollens shown by these patients, an IgE-binding inhibition assay was performed using a pool of the 11 patients' serum samples, with *S. kali* as coating extract and *S. kali* and *C. album* pollen extracts as alternative inhibitors (Fig. 1d). After densitometric analysis of the immunoblotting, a 74% of reduction ($74 \pm 4\%$) in the response of the IgE-reactive band was obtained when *S. kali* extract was used as inhibitor, whereas $<4\%$ inhibition was obtained with *C. album* pollen extract.

Isolation of a 40 kDa-allergenic protein: molecular and immunologic properties

Purification of a component of 40 kDa of apparent molecular mass was performed by means of two chromatographic steps consisting of a size-exclusion chromatography on a Sephacryl-S100 column, and an ion-exchange separation using a MonoQ column (Fig. 2a).

No sequence was obtained from the N-terminal end of the 40 kDa protein band by Edman degradation, indicating blockage of the first amino acid of the polypeptide chain. Sequences of internal segments of the allergen were determined by Edman degradation (LHPYITLYGI, FTGFQDTVCD, SLYP/LNTELHVVPD and TFTSLEYIEA) and MALDI-TOF-MS (VVITIGPGEYR, AWFD/EAAR and TFTSLEYIEA) after digestion of the protein with trypsin (Table 2). The comparison of the sequencing data from this table with the protein sequences of the data banks allowed us to detect homology with plant PMEs.

The isolated protein was recognized by the pool of 11 sera in immunoblotting (Fig. 2a), and it is able to almost completely inhibit the IgE binding to the 40 kDa band. The

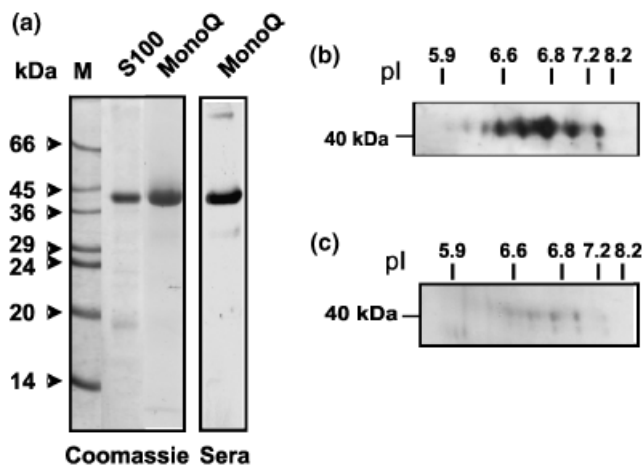


Fig. 2. Coomassie blue staining of the purification steps and immunostaining with a pool of sera of purified protein transferred to membranes after SDS-PAGE (a). M, molecular mass markers. Two-dimensional-electrophoresis analysis of purified Sal k 1 after transferred to nitrocellulose membranes: staining with the pool of serum samples used in this study (b), and with Concanavalin A lectin (c).

analysis by ELISA with the individual sera from the 11 patients showed specific IgE antibodies against the purified allergen (Table 1).

A potential polymorphism of the protein was further investigated by means of two-dimensional (2D)-electrophoresis in polyacrylamide gels and immunodetection of the transferred proteins with the mixture of sera. The existence of several dots (Fig. 2b) indicated that the protein possessed a number of microheterogeneities in a range of pI from 6.5 to 7.7. These results were also supported by MS analysis which rendered a broad peak between 35 000 and 38 000 Da, with a main maximum at 37 214 Da of molecular mass (data not shown).

The Concanavalin A staining of the 2D-electrophoresis membranes also gave multiple glycosylated forms (Fig. 2c).

Cloning and sequence of pectin methylesterase from *Salsola kali*: identification as Sal k 1

The allergen-encoding DNA was amplified by means of PCR using a cDNA synthesized from *S. kali* pollen RNA as template, and degenerate oligonucleotides designed from amino acid sequences obtained by Edman degradation and by sequencing of the DNA fragments from the first-round PCR amplification.

The cDNA encoding the complete sequence consists of 1086 bp and the deduced amino acid sequence rendered a polypeptide of 362 residues in length, including a putative signal peptide of 23 amino acids (Fig. 3). We applied the rules of Bendtsen et al. [22] to detect the cleavage position of signal peptides, obtaining the processing site of this protein between Ala-23 and Gln-24. The sequence from

Table 2. Amino acid sequences of peptides from *Salsola kali* allergen

Sequences	Method	Position
VVITIGPGEYR	MS/MS	70–80
LHPYITLYGI	ED	88–97
FTGFQDTVCDD	ED	169–179
SLYP/LNTELHVVPD ...*	ED	203–216
AWFD/EAAR*	ED and MS/MS	254–260
TIMFGEYK	MS/MS	288–296
TFTSLEYIEA	ED	320–331

ED, Edman degradation; MS/MS, mass spectrometry/mass spectrometry.

*Double sequence in the positions indicated.

this Gln-24 was used to design a PCR primer and amplify the complete sequence encoding the native protein as it is described in 'Materials and methods' section. Three clones were obtained, two of them encoding the putative N-terminal end of the native protein and one including the whole sequence of the protein with the signal peptide.

The comparison of the complete amino acid sequence of the allergen from *S. kali* pollen with proteins from the database banks confirmed that it belongs to the PME family and the sequence contains the essential amino acids reported for the enzymatic activity of this protein. The weak but significant similarity of the sequence of this PME with peptides derived from Sal k 1 [14] reported to the database banks led to the naming of this allergen as Sal k 1, following the indications from the Allergen Nomenclature Subcommittee of the IUIS. Sal k 1 is the first allergenic member of PME protein family from a pollen tissue whose sequence is reported.

Sal k 1 is a polymorphic, mildly basic protein with a molecular mass ranging from 36.595 to 36.804 Da and pI from 6.74 to 7.75 deduced from the various sequenced polypeptides. Differences of 619 Da between molecular masses of the sequence deduced from the clone (36 595 Da) and that of the natural protein obtained from MS (37 214 Da) could support the presence of a small glycan moiety.

The deduced amino acid sequences of the three clones are shown in Fig. 4a. The identity among the different clones is higher than 88%, and the alignment with PMEs stored under the database banks gives a sequence identity of 10%, 22%, 16% and 50% with those from *E. chrysanthemi*, *Daucus carota*, *Cochliobolus carbonum* and *Arabidopsis thaliana*, respectively. The catalytic residues Gln151, Gln173, Asp174, Asp195, and Arg253 were conserved in the protein sequence (Fig. 4a). The modelling of Sal k 1 – faces anterior and posterior – using the coordinates of the PME from *E. chrysanthemi* is also shown (Fig. 4b). The 3D structure modelling revealed a parallel β -sheet coiled into a large, right-handed cylinder, conserved in the enzyme of *S. kali* pollen.

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1  ATGGAGGAGCAGCTAAGCATGTTATTTGGTAGGTTTGTCTTATTATATAGCT
   M E E H V S M L L V G F V L I N I A
55  TTCACGTCAATAGCACAACTAATCCACCAATCCAGCTGAACCTGAATCTTGG
   F T S I A Q L I P P N P A E L E S W
109  TTTCAGGCGCTGTCAAACCTGTCCAGCGAACAAAAGGATTGGAGCCGAGTGA
   F Q G A V K P V S E Q K G L E P S V
163  GTACAAACAGAAAGCGGAGGAGTAGAAACAATTGAAGTACGACAAGATGGAAGT
   V Q T E S G G V E T I E V R Q D G S
217  GGAAGTTTAAAGACAATTTCAGATGCTGTGAAGCATGTTAAAGTAGGGAATACT
   G K F R K T I S D K A V K H V K V G N T
271  AAACGTGTGATTATAACAATTGGTCCAGGAGAATACAGAGAAAAGTAAATAAT
   K R V I I T I G P G E Y R E K V K I
325  GAAAGATTACACCCATACATTACTTTATACGGAATAGACCTAAATAAGACCA
   E R L H P Y I T L Y G I D P K N R P
379  ACTATACACATTGTGGGACAGCTGCTGAATTTGGAAGTGTGGACAGTGCACAA
   T I T F A G T A A E F G T V D S A T
433  GTGATTGTAGAGTCTGATTACTCTGTGGGCGCTCACCTTATCGTGACGAAGTCT
   V I V E S D Y S V G A H L I V T N S
487  GCTCCAAGGCGAGATGGAAGAAAGGAGGACAGGCGCGGTGTGAATA
   A P R P D G K R D K G A [Q] A G A L R I
541  TCTGTTGATAGACAGCAATCTATACTGTAAATTCACAGGTTTTCAGACACC
   S G D R A A F Y N C K F T G F [Q] [D]
595  GTTTGTGATGACAAGGGCAACCATTTCTTACAGACTGTATACCTGAAGGACT
   V C D D K G N H F F T D C Y T E G T
649  GTTGATTTTATTTTGGTGAAGCCAGATCTCTATATTTGAACACAGAATTCAT
   V [D] F I F G E A R S L Y L N T E L H
703  GTTGTCGGGTGATCCATGGCAATGATACTGCACATGCAAGGAAGACGCA
   V V P G D P M A M I T A H A R K N A
757  GATGTTGTTGGTGGATACCTCTCGTGCAATGCAAGGTCAACGGAACTGGTGGT
   D G V G G Y S F V H C K V T G T G G
811  ACAGCCCTCTTAGGTAGAGCTTGGTTGATGCTGCTGCGTGTGTTTCTCTTAC
   T A L L G [R] A W F D A A R V V F S Y
865  TGTAACTTGAGCGATGCTGCTAAACCCGAGGGTTGGTCTGATAACACAAAGCCA
   C [N] L S D A A K P E G W S D N N K P
919  GAAGCCCAAAAGACAATACTCTTTGGAGAGTACAAGAACTGGTCCAGGTGCA
   E A Q K T I L F G E Y K N T G P G A
973  GCTCCTGATAAAGAGCTCCATACAAAGCAACTTACAGAAGCAGATGCTAAA
   A P D K R A P Y T K Q L T E A D A K
1027  ACATTCACTAGTCTGAGTACATTGAGGCGAGTAAATGGCTACTACCGCGCGCG
   T F T S L E Y I E A A K W L L P P P
1081  AAGGTGTAA
      K V *
339

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Fig. 3. Sal k 1.0101-nucleotide sequence and its deduced amino acid sequence. The amino acid sequences used for designing the primers for cloning are underlined. The signal peptide is in italics. The catalytic residues are shown in squares. The N-glycosylation potential site is circled. Numbers at right indicate the position of residues in the deduced amino acid sequence excluding those of the signal peptide.

Discussion

In this report, we aimed to present the characterization of an allergenic member of the protein family of PME with an important role in the IgE sensitization of patients allergic to *S. kali* pollen. Several pollen allergens, such as polymethylgalacturonases (Jun a 2 or Cry j 2) [27], pectate lyases (Jun a 1 or Cry j 1) [28], 1,3- β -glucanases (Ole e 9) [25], and PMEs (Bet v 8) [29] are involved in the carbohydrate metabolism of plants. Although the members of the first three groups of proteins have been deeply characterized, this is not the case of the PME from pollen tissue. Pectins comprise heterogeneous polymers composed mainly of galacturonic acid residues joined by 1,4- α -linkages, and are main components of the plant cell wall, constituting up to 35% of the plant's dry weight. Wall-associated PMEs (EC.3.1.1.11) catalyse the deesterification of these components, releasing acidic polymers and methanol. They have been found in both plant tissues and some plants cell wall-degrading micro-organisms, and their enzymatic activity has been involved in a number of processes, including cell wall extension, fruit ripening, senescence and pathogenesis [30–32].

Allergy to Chenopodiaceae weeds has a special relevance in countries with a desert climate, high temperatures and a dry soil [3]. However, sensitization to these pollens has been extended to the centre and the Mediterranean area of Spain: Albacete, Zaragoza, Murcia and Alicante, probably due to the increased desertization of the south of Europe. Additional aspects like the use of these weeds as ornamental plants have been added to this environmental problem, enhancing the allergenic relevance of these species. Although pollens of this family seem to be indistinguishable from a morphological and immunological point of view, in such a way that they have been used indistinctly so far in the diagnostic analysis, significant differences have been found in the IgE sensitization of many patients to these pollens. The response was, in general, more frequent and stronger to *S. kali* than to other members of this family [3]. These discrepancies pointed to the involving of specific proteins in the sensitization to different Chenopodiaceae species. Three different allergens were found in *C. album* pollen, Che a 1, and the panallergens Che a 2 (profilin) and Che a 3 (polcalcin). Most of the patients sensitized against to both *C. album* and *S. kali* pollens recognize any of these proteins, thus concluding that these three allergens are present in both allergenic sources, and explaining the cross reactivity shown by these patients [6, 33]. However, the exclusive sensitization of a population to *S. kali* seems to point to the presence of a specific allergen in this pollen, which would be responsible for this different reactivity.

Eleven patients from Murcia, a dried coastal region of Spain, were included in this study because they were allergic to *S. kali*, being not sensitized to *C. album*, and their IgE sera were used to search for this specific allergen. The presence of a band of 40 kDa of molecular mass was detected in all the IgE blots from these patients. Following the isolation of this allergen, the purified 40 kDa-protein was subjected to ELISA assays with the individual sera, and was recognized by the IgE from all of them. The protein belongs to the PME family, and allergenic counterparts of this protein were not found in the databases from other vegetable sources. This protein possesses some of the features that define the members of this family, such as neutral or mildly alkaline isoelectric points, a glycosylated nature and a high polymorphism [34]. A certain number of heterogeneities were detected after sequencing different clones, which would be in agreement with the data observed by means of 2D-electrophoretic analysis of the *S. kali* extract where up to six different spots corresponding to the same protein were obtained. This supports the idea, already established, that these enzymes typically occur as multigenic isozymes [35].

The prediction of the N-terminal residue was performed by means of EXPASY.CH/TOOLS using the clone sequence that includes the signal peptide. Glutamine is predicted to be the amino acid residue located at the N-terminal end of

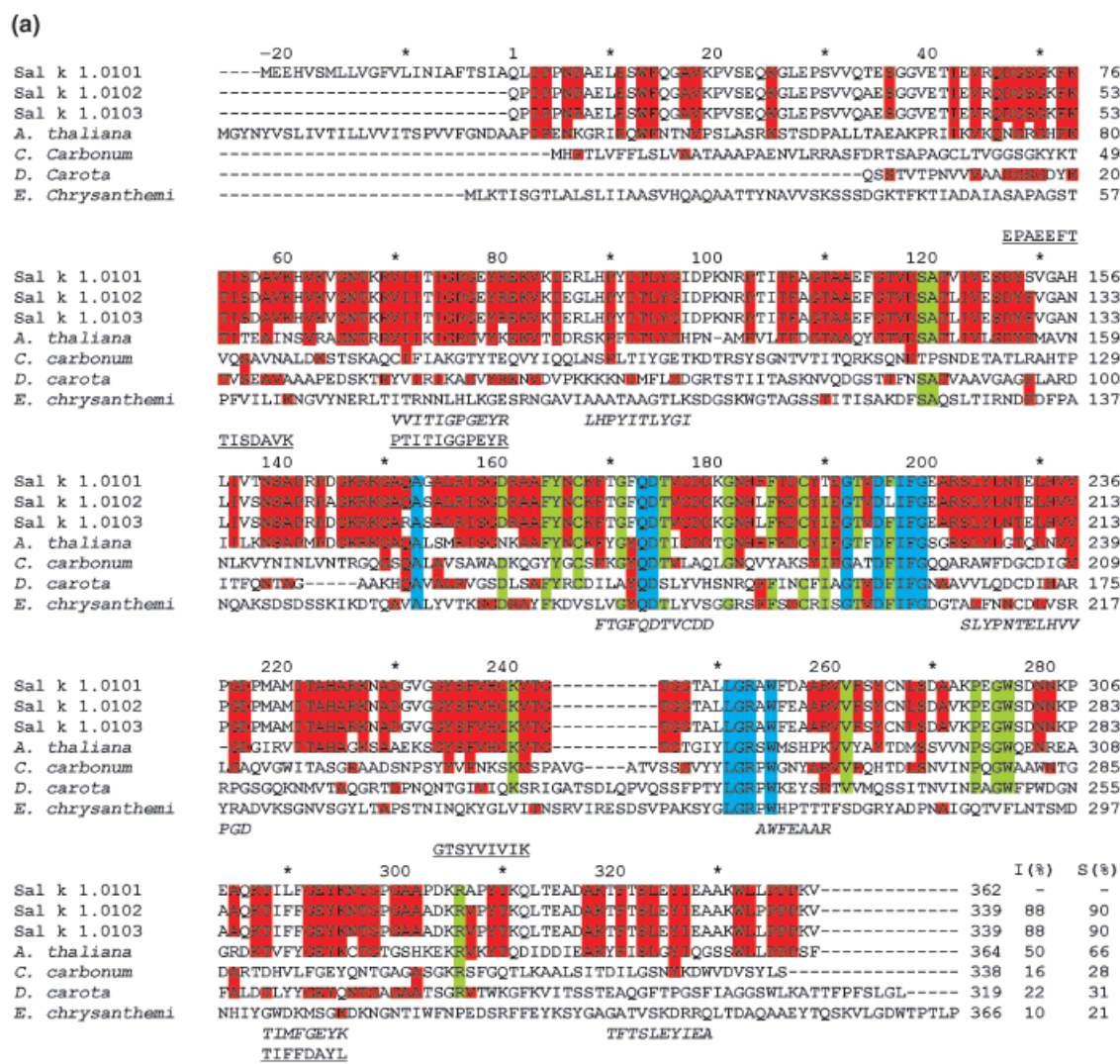


Fig. 4. Alignment of the three amino acid sequences deduced from Sal k 1 clones with those of pectin methylesterases (PMEs) from *Arabidopsis thaliana*, *Cochliobolus carbonum*, *Daucus carota* and *Erwinia chrysanthemi* (a). Peptides obtained by Edman degradation and mass spectrometry/mass spectrometry (MS/MS) by our group (italic) and those obtained by Carnés et al. [14] (underlined; Accession number P83181) are also shown. Accession numbers: Sal k 1.0101 (AY590141), Sal k 1.0102 (AY776248), Sal k 1.0103 (AY776249), *A. thaliana* (Q9FKF3), *C. carbonum* (Q9Y881), *D. carota* (P83218) and *E. chrysanthemi* (P07863). Numbers at right side indicate the length of each sequence. Percentages of identity (I) and similarity (S) are shown. Numbers located at the top of the sequence indicate the positions taking the Sal k 1.0101 isoform as a reference. (b) Three-dimensional (3D) modelling of Sal k 1.0102 using the coordinates from the PME of *E. chrysanthemi* (PDB number:1QJV) is shown (b).

the protein. This residue is frequently cyclized to pyro-Glu, which would be in agreement with the lack of reactivity of the isolated protein to Edman reagent.

A low sequence identity among PME enzymes from different vegetable species was found, although the catalytic residues were conserved. The molecular model of Sal k 1 was built using the parameters obtained from the X-ray crystal structure of the phytopathogenic bacterial enzyme produced by *E. chrysanthemi* (PDB number 1QJV) [35]. The structural features of the PME are common to all bacterial and fungal pectic enzymes: pectate lyases, pectin lyases, polygalacturonidases. The structure of this family is similar to that of the PMEs identified in phytopathogenic organisms (bacteria, fungi) [36]. Comparison of PME action patterns between pollen, during pollen tube growth and phytopathogenic bacteria, during plant infection, show that both PMEs were involved in plant cell wall soaking [37].

In order to assess the relevance of the different allergens common to the Mediterranean area, epidemiological surveys using panels of allergen molecules, including Sal k 1 and Che a 1, are being used. The initial data coming from these studies ('VEGETALIA' and 'EXPO'), with the involvement of more than 40 Clinical Groups indicate that the prevalence of Sal k 1 in several areas of the south-east of Spain is, in average, more than 70%, and thus support that *S. kali* pollen is a main cause of allergic pollinosis in these areas. Interestingly, for the same populations, Che a 1 prevalence is much lower thus confirming the leading role of *S. kali* and its linked allergen Sal k 1 in the Chenopodiaceae pollinosis and made it a potential tool in diagnosis of allergic patients in these particular areas.

In conclusion, a new allergen of *S. kali* pollen has been identified, isolated, and sequenced. This allergenic protein is a PME and has been named Sal k 1. It could be considered as an allergenic marker to discriminate among patients allergic to Chenopodiaceae pollens, taking into account that it constitutes a major allergen for patients sensitized to *S. kali* pollen and non-sensitized to *C. album* pollen.

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