

Involvement of Lipid Transfer Proteins in Saffron Hypersensitivity: Molecular Cloning of the Potential Allergens

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■ Abstract

Background: Lipid transfer proteins (LTPs) are relevant allergens, and have recently been proposed as model plant allergens from fruit, vegetables, seeds, and pollens. However, no LTP spice allergen has been characterized to date.

Objectives: To identify and isolate saffron LTPs and to explore their relevance in saffron allergy.

Methods: Six patients with rhinitis and positive skin prick test (SPT) results to saffron extract were selected. Two recombinant LTPs from saffron were isolated, cloned into pPIC9 plasmid, and produced in *Pichia pastoris*. Immunodetection and enzyme-linked immunosorbent assays were performed with the 2 purified allergens and with the major peach allergen Pru p 3.

Results: Full cDNA corresponding to 2 saffron LTP variants was isolated and expressed in *P. pastoris*. The molecular weight of rCro s 3.01 and rCro s 3.02 was 9.15 kDa and 9.55 kDa, respectively. The sequences obtained had a 47% identity with each other and 51% and 43% with Pru p 3. Both proteins were recognized by anti-Pru p 3 antibodies. Specific IgE to the purified allergens was found in 50% of patients for rCro s 3.01 and 33% for rCro s 3.02 and Pru p 3 in the saffron-allergic patients.

Conclusions: Our results indicated that rCro s 3.01 and rCro s 3.02 are minor allergens of saffron, at least in the study patients. To our knowledge, this is the first report on the implication of LTPs in spice allergy.

Key words: Saffron allergy. Lipid transfer protein. Recombinant allergen. Peach Pru p 3.

■ Resumen

Introducción: Las proteínas de transferencia de lípidos (LTPs) son alérgenos relevantes, propuestos recientemente, como modelo de alérgenos en plantas desde frutas, vegetales, semillas y polenes. Hasta la fecha ninguna LTP ha sido caracterizada como alérgeno en especias.

Objetivos: Identificar y aislar LTPs en azafrán y explorar su relevancia en la alergia en dicha especie.

Métodos: Seis pacientes, con síntomas de rinitis principalmente y con prick positivo a extracto de azafrán fueron seleccionados. Dos LTPs recombinantes han sido aisladas, clonadas en pPIC9 y expresadas en *Pichia pastoris*. Inmunodetecciones con sueros de pacientes y el análisis por ELISA directo fueron llevados a cabo utilizando los dos alérgenos purificados y Pru p 3 el alérgeno mayoritario de melocotón.

Resultados: cDNA correspondientes a dos variantes de LTPs en azafrán fueron aisladas y expresadas en *P. pastoris*. El peso molecular de rCro s 3.01 y rCro s 3.02 fue de 9150 Da y 9550 Da, respectivamente. Las secuencias obtenidas mostraron una identidad del 47% entre ellas y un 51% y 43% con respecto a Pru p 3. Ambas proteínas fueron reactivas con anticuerpos anti-Pru p3. El 50% de los pacientes incluidos en el trabajo mostraba una IgE específica a rCro s 3.01 y un 33% a rCro s 3.02 y Pru p 3.

Conclusiones: Nuestros resultados indican que rCro s 3.01 y rCro s 3.02 son alérgenos minoritarios del azafrán al menos en los pacientes incluidos en este trabajo. Este es el primer caso en el que se describe la implicación de las LTPs en alergias a especias.

Palabras clave: Alergia al azafrán, proteínas de transferencia de lípidos, alérgenos recombinantes, Pru p 3.

Introduction

Saffron is a spice derived from the dried stigmas of the *Crocus sativus* flower. It is highly appreciated as a colorant for foodstuffs and for its aromatic and flavoring properties. It is often used in the confectionery and liquor industry.

Saffron spreads vegetatively by means of bulbs called corms. The planting of corms is a difficult task, with bulbs being planted one by one and by hand. Stigmas are also harvested by hand. The conditions of cultivation and handling of saffron facilitate the development of allergies through inhalation and contact [1].

Very few studies describe saffron allergies [1-4]. One of the more relevant articles, published by Feo et al [1], discussed the clinical significance of saffron flower in occupational allergies. Saffron allergy can even produce anaphylaxis. Wüthrich et al [3] reported the case of a German farmer who experienced a severe anaphylactic reaction after eating risotto (an Italian dish containing saffron), due to the presence of high-molecular-weight proteins (40-90 kDa) in the saffron extract. Recently, Martinez et al [4] described a case of occupational airborne contact dermatitis caused by saffron bulbs.

Around 7% of food allergies in adults are attributed to spices. Patients with a high risk of developing this type of allergy are young adults who are mainly sensitive to birch and *Artemisia* pollen. Cross-sensitization to other plant allergens is common [5]. Lipid transfer proteins (LTPs), which have been identified as relevant allergens in several foods and pollens, [6,7] are highly resistant to digestion and heat treatment, and seem to be linked to severe and systemic symptoms. High levels of sensitization to this allergen family occur in the Mediterranean area. Surprisingly, to date, no LTPs have been associated with spice allergy. This study was designed to determine whether LTPs occur in saffron and their possible role in saffron allergy.

Methods

Patients and Skin Tests

The study population comprised 40 consecutive patients living in an important saffron crop area in Ciudad Real province (200 km south of Madrid). They had all attended the allergy department from December 2007 to November 2008 and answered the standardized questionnaire on saffron handling (October-November), degree of exposure, development of sensitization during the rest of the year, and personal and family history of allergy. Sera from 6 patients showing positive skin prick test (SPT) results to saffron extract and serum-specific immunoglobulin (Ig) E (sIgE) determined by enzyme-linked immunosorbent assay (ELISA) were selected (see below). Their characteristics are shown in the Table.

SPTs were carried out using an in-house saffron extract (500 mg/mL of protein) and date palm profilin (Pho d 2; ALK-Abelló, Madrid, Spain) (50 µg/mL) following standard procedures and using 0.9% NaCl, 50% glycerol, and 0.4% phenol as diluent. Histamine hydrochloride (10 mg/mL) and saline solution were used as positive and negative controls,

Table. Patient Data and Results of the In Vivo Test, and Determination of Total Serum IgE, Saffron-Specific IgE, and Purified rCro s 3.01, rCro s 3.02, and Pru p3

Patients	Age, y/Sex	Symptoms	IgE (kU _A /L)	SPT response, mm		Specific IgE, OD units		
				Saffron extract	Pho d 2	Sensitization to pollens	Saffron Extract	rCro s 3.01
1	9/M	R, A	334	6 × 5	Negative	Gr, Ol, Pn, Ar, Ch	0.27	0.23
2	32/M	Rh, A	230	4 × 4	Negative	Gr, Ol, Pn, Ar, Ch, Pg, Sa	0.79	—
3	61/M	Rh, A	953	6 × 5	6 × 5	Gr, Ol, Ch, Pg, Sa	0.30	—
4	17/M	Rh	311	7 × 5	5 × 5	Gr, Ol, Cu, Pn, Ar, Ch, Pg, Sa	0.24	—
5	29/M	Rh, A	1206	3 × 3	Negative	Gr, Ol, Ch, Pg, Sa	0.40	0.43
6	26/M	Rh, A	2293	7 × 7	Negative	Gr, Ol, Ar, Ch, Pg, Sa	0.68	0.72
							0.60	0.60
							0.96	0.96

Abbreviations: A, asthma; R, rhinitis; Rh, rhinoconjunctivitis; Gr, grass; Ol, olive; Pg, Plantago; Cu, Cupressus; Sa, Salsola; Ch, Chenopodium; Sá, Salsola; Ar, Artemisia; Ph, Platanus; Pg, Cupressus; Pg, Plantago. —, OD < 0.20.

respectively. Duplicates of each sample were assayed. A mean wheal diameter of 3 mm or greater 15 minutes after puncture was considered a positive response. All patients gave their written informed consent, and the Ethics Committee of Hospital General de Ciudad Real (Ciudad Real, Spain) approved the study.

Saffron Extract

The extract was made using dried and ground stigmas that were defatted with acetone ($2 \times 1:5$ w/v, 1 h, 4°C), dried, and extracted using phosphate-buffered saline (PBS) (0.1 mol/L sodium phosphate [pH 7.0], 0.15 mol/L NaCl). After centrifugation (16 300g, 30 min, 4°C), the supernatant was dialyzed against H₂O and freeze-dried. The protein concentration was determined according to Bradford [8].

Immunodetection Assays

Samples (20 µg of saffron extract, 5 µg of purified allergens) were fractionated using sodium dodecyl-polyacrylamide gel electrophoresis before being electrotransferred onto nitrocellulose membranes as described by Towbin et al [9]. After blocking with 5% bovine serum albumin (BSA) in PBS buffer, membranes were incubated overnight with a serum pool from saffron-allergic patients (1:4 dilution in 0.5% BSA, 0.05% Tween-20 in PBS), washed with 0.05% Tween-20 in PBS, and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid (Alk-Abelló, Madrid, Spain; 1:3000 dilution) for 2 hours [10]. After washing, a rabbit anti-mouse IgE peroxidase-conjugate antibody (DAKO A/S, Glostrup, Denmark; 1:5000 dilution) was added for 1 hour. IgE-binding components were detected by enhanced chemiluminescence following the manufacturer's instructions (ECL-Amersham Biosciences, Little Chalfont, UK).

Alternatively, replica gels were electrotransferred onto nitrocellulose membranes, immunodetected with rabbit polyclonal antibodies to Pru p 3 (Alk-Abelló, Madrid, Spain; 1:1000 dilution), treated with peroxidase-conjugated anti-rabbit IgG (Sigma, St Louis, Missouri, USA; 1:5000 dilution), and revealed as previously reported [11].

Isolation and Heterologous Expression of Saffron LTPs

In an ongoing project using fresh stigmas, we isolated 2 expressed sequence tags (ESTs) which correspond to nonspecific LTPs. RNA from fresh stigmas was used to synthesize the 5' and 3' ends of the first-strand of cDNA in a SMART RACE cDNA Amplification Kit (Clontech-Takara-Europe, Nucliber, Madrid, Spain) and specific primers were designed from the 2 ESTs sequences. The full-length clones rCro s 3.01 and rCro s 3.02 were amplified from the cDNA with the following primer sequences: rCro s 3.01 forward primer, 5'-TCAGCTGCAGCACTGTGGCCT-3', and rCro s 3.01 reverse primer 5'-TCATGCCACCTTAGTGCAGTCAGTG-3'; and rCro s 3.02 forward primer, 5'-ATCACGTGCGGCACGGTCGT-3', and rCro s 3.02 reverse primer 5'-TCAGCCTTCAATTGGAGCAGT-3'. The PCR product was purified, digested with Xho I/EcoR I

restriction enzymes, and inserted into the same sites of the pPIC9 plasmid to transform *Escherichia coli* JM109 cells. The DNA from several clones was sequenced to confirm the proper arrangement of the construct by allowing extracellular expression of rCro s 3.01 and rCro s 3.02 with an extra glutamic acid residue at the N-terminus. The pPIC9/Cro s 3.01 and pPIC9/Cro s 3.02 DNA were digested with a Sac I restriction enzyme, and the larger purified fragments were used to transform GS115 *P pastoris* cells by electroporation. Positive clones were cultured in buffered minimal glycerol medium, and expression of recombinant allergens was induced by resuspension of collected cells in a buffered minimal methanol medium using conditions described elsewhere for r Pru p 3 [12]. The recombinant saffron LTPs were purified from the supernatant of the corresponding *P pastoris* culture medium. After dialysis against 100 mM ammonium acetate and freeze-drying, the preparation (6 mg of protein) was fractionated by means of gel filtration on a Superdex HR 75 16/26 column (Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted with 100 mM ammonium acetate (1 ml/min). LTP-enriched fractions were dialyzed against H₂O and freeze-dried, before being further separated by reverse-phase high-performance liquid chromatography (HPLC) on a Nucleosil 300-C4 column (8 × 250 mm; particle size 5 µm; Scharlau Science, Barcelona, Spain) and eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (10% in 5 min and 10–100% in 150 min; 1 mL/min).

N-terminal amino acid sequencing and matrix-assisted laser desorption/ionization mass spectrometry (MALDI) analysis were carried out using standard methods with an Applied Biosystems 477A gas-phase sequencer (Foster City, California, USA) and a Biflex III spectrometer with delayed extraction (Brucker-Franzen Analytik, Bremen, Germany), respectively.

Specific IgE Determination

Specific IgE binding to recombinant LTPs of saffron and purified Pru p 3 was carried out as previously described [12] using a 1:3 dilution of individual sera from saffron-allergic patients (Table) and 5 µg/mL of each purified allergen. The PBS buffer with 1% BSA was tested as a negative control, as was a serum pool from patients allergic to dust mite but not to plant foods or pollens. Specific IgE levels of >0.20 OD (mean [OD] + 3 × SD to the negative serum pool from patients allergic to dust mite) were considered positive. All tests were performed in triplicate.

Results

Immunoblotting of the in-house Pru p 3 extract revealed a band of approximately 9 kDa, indicating the presence of LTP in the saffron extract (Figure 1A).

The results of the in vivo tests and total serum IgE determinations are shown in the Table. Patients 3, 4, 5, and 6 had a clinical history of occupational asthma caused by inhalation of saffron in the workplace. In contrast, patients 1 and 2 reported both asthma and rhinitis, while patient 2 also

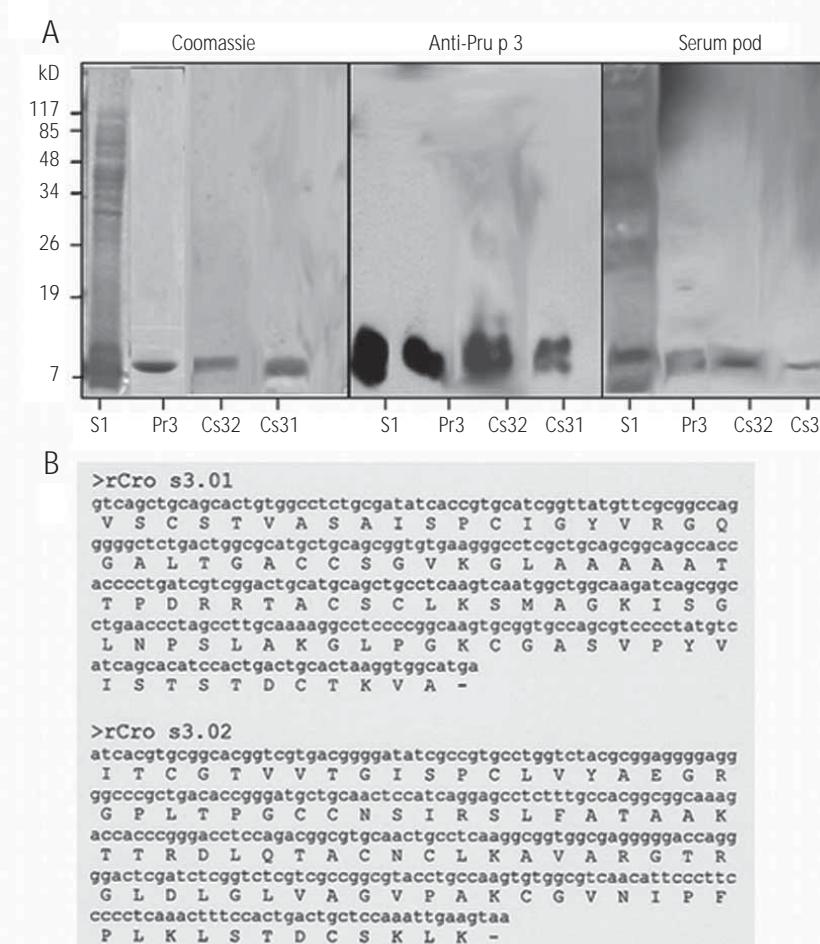


Figure. A, Protein staining (Coomassie), immunodetection with polyclonal antibodies against Pru p 3 (Anti-Pru p 3) and with a serum pool from saffron-allergic patients of the following samples separated by SDS-PAGE: Saffron extract (Sf; 20 µg), purified recombinant allergens rCro s 3.01 (Cs31), rCro s 3.02 (Cs32), and Pru p 3 (Pp3; 5 µg each). B, Nucleotide sequences and deduced amino acid sequences of the cDNA clones encoding rCro s 3.01 and rCro s 3.02. SDS-PAGE indicates sodium dodecyl-polyacrylamide gel electrophoresis.

reported rhinoconjunctivitis during saffron pollination, although they tolerated its ingestion. All patients had an in vivo response (SPT, ranging from 3×3 to 7×7 mm) to saffron extract (500 extract µg/mL). Patients 3 and 4 in the Table had a positive SPT result to Pho d 2 (a marker of profilin sensitization), while the others did not show any reaction to this allergen.

IgE immunoblotting performed under reducing conditions with pooled sera from saffron-allergic patients revealed 2 main IgE-binding low-molecular-mass components (Figure 1A). One band had an apparent molecular weight of approximately 9 kDa and the other of approximately 7 kDa.

The in-house extract was difficult to manage due to the presence of nonprotein components, such as the apocarotenoid compounds responsible for the yellow-orange color of saffron. Therefore, we decided to isolate genes coding LTPs instead of

purifying native LTPs from the saffron extract. RNA from fresh stigmas was used to synthesize the 5' and 3' ends of the first-strand of cDNA, along with specific primers designed from 2 sequences of ESTs corresponding to putative saffron LTPs (Gómez-Gómez, unpublished results). The coding region of the 2 LTPs (Accession nos. FJ997554 and FJ997555) (Figure 1B) was amplified from the cDNA, and PCR products were cloned into pPIC9 and expressed in *P pastoris*. Maximum extracellular expression of the recombinant allergens was achieved 4 days after induction with methanol. The 2 LTPs were isolated from the supernatant of the transformed *P pastoris* culture medium by gel-filtration chromatography followed by reverse-phase HPLC (not shown). The sequences obtained from the two LTPs had 47% identity with each other and 51% and 43% identity with peach Pru p 3, the model LTP food allergen.

The purified recombinant proteins rCro s 3.01 and rCro s 3.02 showed a single band on SDS-PAGE, bound IgE from the serum pool from saffron allergic patients, and were recognized by anti-Pru p 3 antibodies (Figure 1A). Proper expression and purification were confirmed by N-terminal sequencing (EVSCSTVASA) for rCro s 3.01 and (EITCGTVVTG) rCro s 3.02 and MALDI analysis, which revealed the molecular weights of rCro s 3.01 and rCro s 3.02 to be 9.158 kDa and 9.562 kDa, respectively. The data obtained from these analyses fully matched those obtained from the nucleotide sequences of the cDNA clones.

Specific IgE-binding extract of total proteins and purified rCro s 3.01, rCro s 3.02, and Pru p 3 to saffron was tested (Table). The results obtained from the ELISA analyses showed positive reactive IgE to saffron extract for all the sera tested. Specific IgE to purified allergens was found in 50% of individual sera from saffron-allergic patients for rCro s 3.01 and in 33% for rCro s 3.02 and Pru p 3. Three sera (50%) showed no recognition of any LTP allergen tested.

Discussion

In the last decade, significant efforts have been made to determine the role of LTPs in food allergies [6,12-15]. However, few studies have focused on spice allergy [16-18]. The aim of the present work was to determine the involvement of nonspecific plant LTPs in saffron allergy. Six patients were selected for study on the basis of clinical symptoms suggestive of a type I hypersensitivity reaction to saffron, and with positive SPT responses and specific IgE to saffron extract.

Two recombinant LTPs were produced in *P pastoris* cells transformed with cDNA isolated by PCR amplification using specific primers for saffron LTPs. Identification of the 2 purified allergens as members of the LTP family was confirmed by their reaction to peach LTP (Pru p 3)-antibodies, and the determination of their N-terminal amino acid sequences and molecular weights (MALDI analysis), both of which are typical characteristics of LTPs.

rCro s 3.01 and rCro s 3.02 shared 47% identity with each other and 51% and 43% with peach Pru p 3. Several nonconservative amino acid changes were detected in the regions corresponding to the sequential IgE epitopes defined for the peach allergen [19,20].

Both the purified saffron allergens and Pru p 3 were recognized in IgE immunodetection assays by the pool of sera from saffron-allergic patients. Fifty percent of individual sera from saffron-allergic patients had specific IgE to rCro s 3.01, while only 33% were positive to rCro s 3.02 and Pru p 3. Interestingly, Pru p 3 was clearly recognized by IgE from the saffron-allergic patients in immunodetection assays and by some individual sera in ELISA. It seems reasonable to consider that some patients included in this study might suffer from cross-allergy to peach fruit, since sera from 3 patients reacted with Pru p 3; however, further experiments are needed to confirm this.

To date, 1 allergen—the profilin Cro s 2—has been isolated and characterized from saffron pollen both in vitro and in vivo [2]. Over 70% of sera from patients with allergy to this spice

had specific IgE to Cro s 2 and positive SPT test responses. It appears that sensitization to saffron profilin is associated with underlying pollinosis. No profilin allergen was detected in our extract, probably because the in-house extract was made from dried stigmas.

In summary, rCro s 3.01 and rCro s 3.02 have been characterized as minor saffron allergens, with this being the first report on the potential involvement of LTPs in spice allergy. In addition, 2 allergenic members of the LTP family with a limited amino acid sequence identity (under 50%) were uncovered in a single plant source.

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