Reports of Biochemistry & Molecular Biology
Vol. 1, No. 1, Oct 2012

Original article

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Molecular Cloning and Expression of Cro s 1: an Occupational Allergen from Saffron Pollen (*Crocus sativus*)

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Abstract

Background: The cultivation of saffron is expanding through the southeast of Iran, and allergy to saffron pollen occurs in workers involved in processing this plant. We aimed to clone, sequence and express a major allergen involved in saffron pollen allergy, and to compare the recombinant with the natural allergen.

Methods: The N-terminal amino acid sequence of Cro s 1, an allergen from saffron pollen, was determined after immunoblotting. The cDNA encoding for this allergen was cloned by PCR utilizing a primer based on the N-terminal amino acid sequence. Recombinant Cro s 1 (rCro s 1) was expressed as a soluble protein in *Pichia pastoris* and purified to homogeneity by gel filtration. Inhibition of IgE binding to rCro s 1 by pollen extract was analyzed by ELISA.

Results: The allergen Cro s 1 was identified from saffron pollen extracts and cloned by PCR. Cro s 1 cDNA defined an acidic polypeptide with homology to pollen proteins from *Chenopodium album* and *Ligastrum vulgaris*. The rCro s 1 was expressed in *P. pastoris* at 28 mg/l. Saffron pollen extract inhibited the binding of patient serum IgE to rCro s 1.

Conclusion: We identified and cloned the first *Crocus sativus* pollen allergen. rCro s 1 cDNA shows a very high homology with Che a 1, the major allergen of lamb's-quarter, *Chenopodium album,* Caryophyllales, pollen (97%). Cro s 1 is a useful tool for specific diagnosis and structural studies of occupational allergy to saffron.

Keywords: Allergen, cDNA cloning, Cro s 1, Occupational allergy, Saffron pollen

Introduction

Saffron, one the most expensive and widely sought food condiments, is used for culinary purposes for its intense flavor and color. Saffron is produced from the dried stigmas of saffron flowers (*Crocus sativus*). The stigmas are extracted by hand from freshly picked flowers and air-dried. Stigmas of more than 170 flowers are required to harvest one gram of saffron. *Crocus sativus* belongs to the Genus Spermatophyta, Class Liliopsida and Family Iridaceae (1). In addition to consuming saffron as a food condiment, several other applications have been reported for saffron stigma, including anti-tumor activity, anti-oxidant activity, histological stain, and immunomodulating activity (2-7).

Iran and Spain are the main countries involved in the cultivation, production and exportation of saffron. This plant commonly grows in the center and south of Khorasan, a

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Received: May 20, 2012; Accepted: Aug 24, 2012

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province localized in the northeastern part of Iran. Iran produces annually 170 tonnes of saffron, of which 90 % comes from Khorasan (8). Therefore, saffron has a great economic interest for this region. The majority of people living in this area of Iran are employed in the saffron industry. Symptoms of allergic disease, such as rhinorrhea, sneezing and lower respiratory disorders are very common during saffron flowering season (October-December) in the people of this region. Saffron pollen has been reported to act as an occupational aeroallergen (8-10). The prevalence of allergy to saffron pollen, among saffron workers has been estimated at 24% during the saffron picking season (11).

Six human IgE binding proteins with apparent molecular weight ranging from 13.5 to 85 kDa have been identified and reported in saffron pollen extract (12). It has been difficult to purify small quantities of the native allergen, by biochemical methods. However, the advent of protein engineering methods can allow production of pure and well defined and biologically allergens, which could be used in specific diagnosis and potentially immunotherapy. In this study, we describe the identification of Cro s 1, a saffron pollen IgE binding protein, by N-terminal sequencing and cDNA cloning, and the expression and purification of recombinant Cro s 1 in *Pichia pastoris*.

Materials and Methods

Protein extraction from saffron pollen

Pollen was extracted from saffron flowers indoors at Novin Saffron (Mashhad, Iran). Stamens were removed by hand from freshly collected saffron flowers and dried at room temperature. The pollen was separated from the rest of the stamen by a collector tissue sieve using gradual screen with a mesh from 860 to 190 µm (Bellco Biotechnology, USA). It is important to eliminate all traces of stigma at this step, because a colored pigment released from the stigma makes the protein extraction difficult. Microscopic examination of the pollen preparation indicated that there were less than 5% impurities (parts of stamen, pollen from other plants…). Pollen (6 g) was defatted by adding 100ml acetone and shaking overnight at 4 °C. The mixture was centrifuged at 5,000 g for 15 minutes at 4 °C. The pellet was dried at room temperature. Carbonate buffer 125 mM pH 8.0 containing 0.02% NaN_3, 50 µM 4-(2-aminoethyl)-benzenesulfonyl fluorides was added to the precipitate. The mixture was shaken overnight using an orbital shaker that, unlike magnet stirring, produces less disruption of pollen. Usually, less than 5% of the pollen in the precipitate was broken when using the orbital shaker. The eluate was centrifuged at 20000 g and the pollen containing pellet was discarded. The supernatant was filtered through a 0.22 µm filter membrane. The protein content was determined by the Bradford method (13).

SDS-PAGE and immunoblot analysis of natural and recombinant Cro s 1

Saffron extracts were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in 5-20% gradient gels under reducing conditions and stained with Coomassie Brilliant Blue R-250 (14). Recombinant Cro s 1 was analyzed by SDS-PAGE, in 8-25% gradient gels under reducing conditions and proteins were detected by silver staining (PhastSystem, Pharmacia Biotech, Uppsala, Sweden).

IgE binding was assessed by immunoblotting. Extracts transferred to nitrocellulose membranes (15) were incubated with serum diluted 5 times in 10 mM Tris, 0.1% Tween-20, 0.9% NaCl, 4% non-fat dry milk, pH 8.0. Five sera from saffron allergic patients (with skin prick test >5mm diameter using 50 µl of crude saffron extract) and 5 sera from non-allergic healthy individuals, among the people coming to the allergy clinic of Bu-Ali Research Institute, were compared. Bound IgE antibody was detected with biotinylated anti-human IgE (Vector, Burlingame, CA), followed by streptavidin-horseradish peroxidase and the luminescent substrate ECL (Pharmacia, Buckinghamshire, UK).

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N-terminal amino acid sequencing
An IgE binding protein corresponding to Cro s 1, identified by immunoblotting, was excised from nitrocellulose membrane, and the N-terminus was sequenced using an Applied Biosystems 477A amino acid sequencer.

PCR-based cloning of Cro s 1 allergen cDNA
Saffron pollen (200 mg) was extracted in 2 ml 10 mM Tris, 0.5 M NaCl, containing 1 mM EDTA and 0.5% SDS, homogenized using a Dounce tissue homogenizer for 10 minutes, and centrifuged for one minute (10000 g). Messenger RNA was purified from the supernatant using a Quick prep Micro mRNA purification kit (Pharmacia). The first cDNA strand was synthesized from 1.2 μg of mRNA using an oligo dT primer and reverse transcriptase (Gibco BRL, Gaithersburg, MD).

A forward oligonucleotide primer, F1, 5´-AGGGACAGGTGTATTGTGAC -3´ and a reverse primer R2 (5'-TCC TTC CTC ATG ATT CCG AG-3') were designed based on the consensus nucleotide sequence for the DNA region with the highest degree of homology among the three allergens Che a 1, Ole e 1 and Lig v 1 which had a high degree of amino acid sequence identity with the N-terminal region of Cro s1 (16-18). A polymerase chain reaction (PCR) was performed using these two primers, cDNA from saffron pollen as template and a Platinum Taq DNA polymerase (Invitrogen, San Diego, CA) (Fig. 1). The PCR consisted of 35 cycles (denaturing step of 30 seconds at 94°C, annealing step of 30 seconds at 55 °C and elongation step of 1 minute at 72 °C), and a final incubation of 7 minutes at 72 °C. The predominant band in the PCR product, corresponding to the expected length of 346 bases from the consensus sequence, was extracted and sequenced. This band showed a high degree of homology (> 95% identity) with Che a 1. Two primers were synthesized based on the Che a 1 sequence, F4 (5'-ATGGCGAAGTGTCAAGC-3') and R4 (5'-TTAATTAGCTTTAACATCATAAAGATCC–3') which allowed amplification of the full length gene encoding Cro s 1 (Fig. 1).

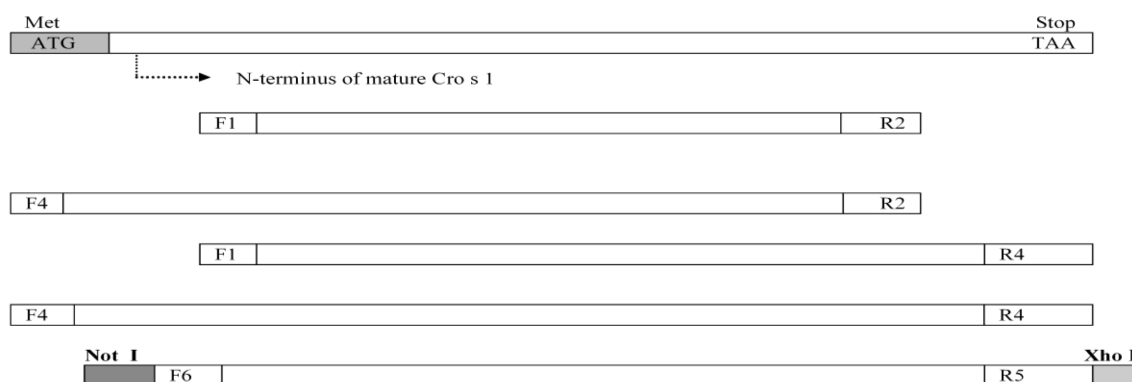

Fig. 1. Scheme showing the relative position of primers used for cloning the Cro s 1 gene. Shaded box of ATG represent the beginning of the open reading frame

DNA Sequencing and analysis
DNA was sequenced using an ABI Prism 100 Model 377 automated DNA sequencer (Biomolecular Research Facility, University of Virginia). BLAST software was used to search databases for DNA homologies. Sequence alignments and restriction site map were made with the on line software Clustal w (www2.eb.ac.uk/clustalw/) and Bioweb.uwlax.edu site, respectively. The Generunner program was used to analyze the deduced amino acid sequence of Cro s 1 and its biochemical properties.

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Production of recombinant Cro s 1 in Pichia pastoris

Cro s 1 cDNA was subcloned into the *Xho I* and *Not* sites of the pGAPZαC vector for constitutive expression of the allergen in *Pichia pastoris*. The resulting plasmid was linearized with *Avr II* restriction, and electroporated into *Pichia pastoris* GS115 strain cells prepared for electroporation as described by Invitrogen (Carlsbad, CA). Five colonies were selected and re- streaked on YPDS plates containing100 µ/ ml zeocin From each plate a colony was picked, grown in YPD as previously described, and the media was tested for expression of Cro s 1 by SDS-PAGE[19]. Recombinant Cro s 1 was purified from a 4 day-old 1 L culture supernatant by high performance chromatography using a Sephacryl 100 gel filtration column. Elution was carried out at a flow rate of 0.5 ml/min using 500 mM phosphate buffer containing 500 mM NaCl pH 7.2. Protein concentration was determined using Advance Protein Assay kit (Cytoskeleton, Denver, CO) and the purity established by SDS-PAGE.

Inhibition of IgE antibody binding to rCro s 1 with natural Cro s 1 from saffron pollen extracts

Assays were carried out using a pool of sera from five patients with positive skin prick test to saffron pollen extract. All steps were carried out at room temperature and after each step five times washes were performed. Microtiter plate wells were coated overnight with 100 µl of rCro s 1 (1 µg/ml). After blocking with a 1% BSA-PBS solution (1 hour), each well was incubated with 50 µl total saffron pollen extract (diluted serially) and 50 µl of the human serum pool (1:2 dilution in blocking buffer) for one hour at room temperature. Biotinylated anti-human IgE diluted in 1% BSA-PBS (1:1000) was added and incubated for one hour at room temperature. Streptavidin-HRP (Sigma, USA) diluted in 1% BSA-PBS (1:2000) was added and incubated for 1 hour. The peroxidase substrate tetramethylbenzidine (TMB) (100 µl) was added to each well and incubated in dark for 30 minutes. Reactions were stopped by addition of 20 µl of 3 M HCl, and optical density at 450 nm was measured using an ELISA plate reader.

Results

Immunochemical detection and N-terminal sequencing of natural Cro s 1

A 19.5 kDa protein with IgE binding activity (Cro s 1) was identified by immunoblot analysis using a serum pool from saffron pollen allergic subjects. The band in the Coomassie-stained SDS-PAGE which aligned with the IgE reactive band in the immunoblot was transferred to a nitrocellulose membrane, excised and used for N-terminal sequencing. The following 13-residues were identified: DQHFKVQGRVYxDTx, while two residues (x) were not identified. A data base search for homologous sequences revealed extensive amino acid identity with Che a 1, Ole e 1 and Lig v 1. Given the high degree of homology among the N-terminus of Che a 1, Ole a 1, Lig v 1 and Cro s 1, two consensus sequences were used to design the forward and reverse primers based on the sequences of these three proteins.

cDNA cloning and sequence analysis of Cro s 1

Messenger RNA extracted from saffron pollen was used for a first strand synthesis of cDNA. A PCR product of 346 bp was obtained by PCR amplification of cDNA using primers F1 and R2. This fragment showed more than 98% homology to the DNA sequence of Che a 1 (16-20). Another set of primers (F4 & R4) was designed according to the Che a 1 sequence to obtain the full sequence from a 507 bp fragment encoding for the allergen.

The amino acid sequence of Cro s 1 showed 97% identity to Che a 1, 51% to Ole e 1, 45% to LAT 52, a protein from tomato (21), and 37% to Lig v 1 (18) (Fig. 2). Cro s 1 contained 142 amino acid residues with a theoretical MW of 15,787 Da and a theoretical isoelectric point of 5.07. The amino acids D1, Q2 and R9 in the N-terminus sequenced from natural Cro s 1 corresponded to E1, N2 and M9, respectively, in the cDNA sequence. Two residues in positions 12 and 15, whose identity was unknown by N-terminal amino acid sequencing of native Cro s 1, were identified as cysteines in

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the cDNA. One putative N-glycosylation site was identified at amino acid position 64-66 (NIT) (Fig. 2). There is an Ole e 1 motif at the position 33-42 (22). This sequence has been deposited to the GenBank under the accession number AY923864. The name Cro s 1 has been approved by the WHO/IUIS Subcommittee for Allergen Nomenclature (http://www.allergen.org/).

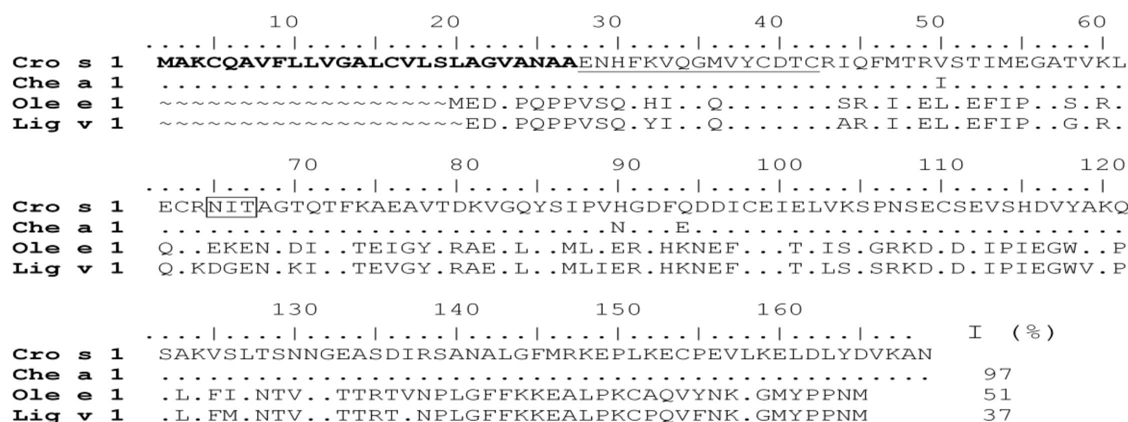

Fig. 2. Multiple alignment of the amino acid sequence of Cro s 1 with the homologous allergens Che a 1 (from *Chenopodium album*), Lig v 1 (from privet), and Ole e 1 (from olive tree). Their GenBank accession numbers are AY923864, AY049012, CAA54819 and X76395, respectively. The signal peptide and n-terminal sequence are shown in bold and underline, respectively. The open box corresponds to putative N-glycosylation site. Percentage of identity to Cro s 1 in indicated (I).

Expression and characterization of rCro s 1 in yeast Pichia pastoris

Recombinant Cro s 1 was expressed in *Pichia pastoris* using the pGAPZαC vector and purified by gel filtration HPLC (Fig. 3 Left, A). A yield of 28.5 mg rCro s 1 was obtained per liter of *Pichia pastoris* culture supernatant. rCro s 1 was analyzed for molecular weight by SDS-PAGE (8-25%) with silver staining (Fig. 3 Left, B). The rCro s 1 has a MW of 20 kDa, as calculated by the relative mobility value of the molecular weight marker in SDS-PAGE.

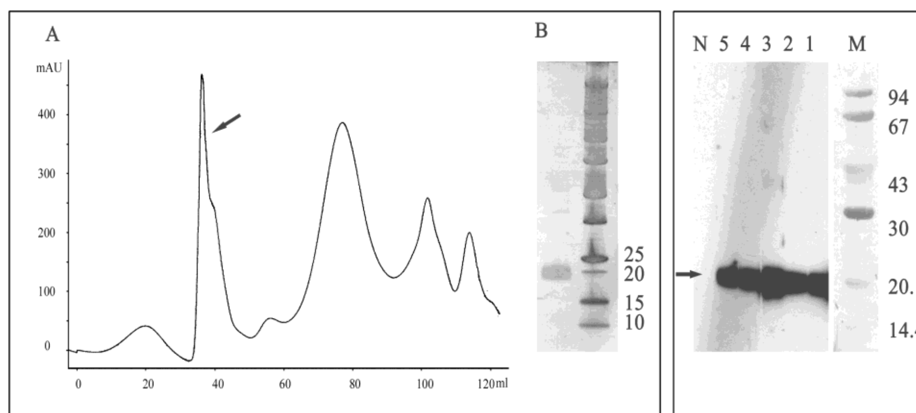

Fig. 3. Left) Purification of rCro s 1 expressed in *P. pastoris* by size-exclusion HPLC: A) Absorbance profile at OD 280 nm to monitor elution of rCro s 1 from the column.
The arrow indicates the rCro s 1 containing fraction. B) SDS-PAGE analysis of the purified rCro s 1 (lane 1) and a marker (lane 2) silver stained. **Right)** Immunoblot analysis of the IgE reactivity of patients allergic to saffron pollen with rCro s 1. Arrow shows the 19.7 kD IgE reactive band corresponding to rCro s 1. Lanes 1-5 correspond to saffron allergic patients, and last lane is a negative control using sera from non- allergic patient.

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Recombinant Cro s 1 reactivity with serum IgE from saffron pollen sensitized patients
Western blot analysis was performed to test whether rCro s 1 could bind IgE from patients with positive skin prick test to saffron pollen extract. Recombinant Cro s 1 was recognized by 5/5 sera from saffron allergic patients who showed IgE reactivity to 19.5 kDa band of total extract, whereas no positive signal was obtained with pooled sera from five non-allergic healthy individuals (Fig. 3 Right). To confirm that IgE reactivity was due to specific IgE to Cro s 1, a competitive ELISA was performed. Total saffron pollen extract significantly inhibited IgE binding to immobilized rCro s 1 in a dose dependent manner whereas albumin did not (Fig. 4).

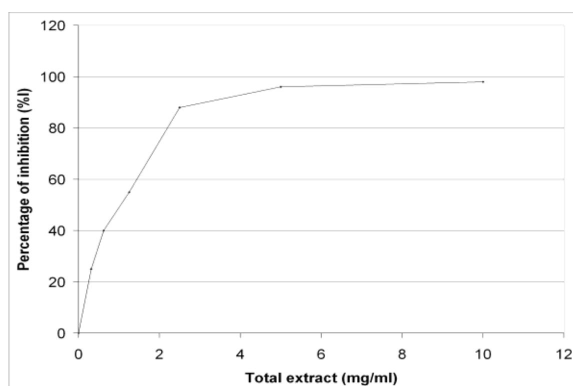

Fig. 4. Inhibition of IgE binding to recombinant Cro s 1 by saffron pollen extract. ELISA plates were coated with rCro s 1 and assayed for binding of IgE antibodies from patients sensitive to saffron pollen.

Discussion

The present studies identify and characterize an allergen from saffron pollen (*Crocus sativus*) involved in occupational allergy. The recombinant allergen shows an apparent molecular weight of 20 kDa in SDS-PAGE, whereas the apparent molecular weight of the natural Cro s 1 is approximately 1KD lower, probably due to glycosylation differences. A cDNA encoding Cro s 1 was PCR amplified, cloned and expressed in *Pichia pastoris*. The N-terminal amino acid sequence deduced from the cDNA showed 95% identity (97% homology) with the N-terminal amino acid sequence of native Cro s 1. The small difference between both N-terminus could reflect the existence of at least two Cro s 1 isoforms. The natural allergen bound IgE from sera of patients with positive skin prick tests to saffron pollen extract (data not shown). IgE antibody binding to the recombinant allergen was inhibited by saffron pollen extract, indicating that the natural and recombinant allergens share similar IgE binding epitopes. The difference between the apparent MW of Cro s 1 estimated by SDS-PAGE and the MW based on the deduced amino acid sequence (15.8 kDa) could be explained by glycosylation of the allergen on at least one identified N-glycosylation site, acidic or basic pI and high proline content (23).

The high degree of amino acid identity between Cro s 1 and the lamb's quarters allergen Che a 1 (97%) was surprising given the phylogenetic difference between these plants (monocotyledons versus eucotyledons, respectively). We considered if contamination with lamb's quarters pollen was possible during the preparation of the cDNA library. However, it is very unlikely because there is a different pollination time for both plants: saffron grows at the end of autumn and *Chenopodium* in the summer. Additionally, the presence of lamb's quarters pollen would have been visible in the pollen preparation under the microscope due to the difference in grain size between both species.

Saffron pollen allergy has reported as an occupational disease (10). It is assumed that the limitation of this allergy to saffron workers could be related to: a) the saffron stigma collection method, and b) the large size of the saffron pollen. Saffron flowers are collected early in the morning and their stigmas are manually extracted indoors by all members of the family during the evening (because of the low temperature during the saffron flowering season). The majority of patients displaying clinical symptoms of sensitivity indoors during the evening, when they are in contact with the saffron flower, but do not show these symptoms during the day in the saffron farm. The large size of saffron pollen (100 μm, approximately five times bigger than mountain cedar and *Chenopodium* pollens) and the high humidity

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early in the morning that make the pollen too heavy for good aerodynamic spread. In spite of this fact, some of the allergic individuals did not have a history of industrial saffron pollen exposure, yet have a positive skin prick test. Our clinical data (unpublished) also indicated that most of the saffron pollen allergic patients also suffer simultaneously from allergy to lamb's quarter (*Chenopodium album).* This could be explained by the high degree of aa sequence identify between homology of Cro s 1 (98% homology) to Che a 1, an allergen from *Chenopodium album*. In addition, *Chenopodium album* is a widely spread grass in Iran. The cross-reactivity between *Crocus sativus* and *Chenopodium album* is under further study.

Pollen is one of the major causes of allergic disease worldwide. Pollen allergens can cause an immediate hypersensitivity (type I, IgE-mediated) response in susceptible individuals (24-26). Diagnosis based on recombinant allergen molecules (versus allergen extract-based diagnostic) enables analysis of complex reactivity profile of allergic patients and precise identification of allergens for each patient. The cloning and expression of rCro s 1 should provide a new tool for diagnosis of occupational allergy to saffron.

Acknowledgments

This work was supported financially by the Vice Chancellor of Research, Mashhad University of Medical Sciences and by Indoor Biotechnologies, Inc.

References

1. Saxena RB. Botany, Taxonomy and Cytology of *Crocus sativus* series. Ayu. 2010 Jul;31(3):374-81.
2. Abdullaev FI. Biological effects of saffron. Biofactors. 1993 May;4(2):83-6.
3. Abdullaev FI. Inhibitory effect of crocetin on intracellular nucleic acid and protein synthesis in malignant cells. Toxicol Lett. 1994 Feb 1;70(2):243-51.
4. Fornasier VL, Protzner K, Zhang I, Mason L. The prognostic significance of histomorphometry and immunohistochemistry in giant cell tumors of bone. Hum Pathol. 1996 Aug;27(8):754-60.
5. Martinez-Tome M, Jimenez AM, Ruggieri S, Frega N, Strabbioli R, Murcia MA. Antioxidant properties of Mediterranean spices compared with common food additives. J Food Prot. 2001 Sep;64(9):1412-9.
6. Thatte U, Bagadey S, Dahanukar S. Modulation of programmed cell death by medicinal plants. Cell Mol Biol (Noisy-le-grand). 2000 Feb;46(1):199-214.
7. Nair SC, Pannikar B, Panikkar KR. Antitumour activity of saffron (*Crocus sativus*). Cancer Lett. 1991 May 1;57(2):109-14.
8. Varasteh A, Rahimzadeh M, Farid R. Determination of saffron (*Crocus sativus*) allergenicity. Iranian Journal of Basic Medical Sciences. 2000 Jun;3(6):33-7.
9. Farid R, Eskoi M, Varasteh A, Farid F. Allergy to Saffron (*Crocus Sativus*). ACIN. 1994;Supplement 2:304.
10. Feo F, Martinez J, Martinez A, Galindo PA, Cruz A, Garcia R, et al. Occupational allergy in saffron workers. Allergy. 1997 Jun;52(6):633-41.
11. Fereidouni M, Sankian M, Varasteh AR. The prevalence of saffron pollen allergy in saffron workers of Khorasan (Iran) in 2002. Journal of Kerman University of Medical Sciences,. 2005;12:7-13.
12. Varasteh AR, Kaghazian H, Tayebi D, Moghadam M, Sankian M. Immunoblot analysis of saffron pollen proteins recognized by human IgE antibodies. Iranian journal of Basic Medical Sciences. 2005;7:33-7.
13. Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-day binding. Anal Biochem. 1976;72:248-54.
14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5.

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15. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A. 1979 Sep;76(9):4350-4.

16. Asturias JA, Arilla MC, Gomez-Bayon N, Martinez J, Martinez A, Palacios R. Cloning and expression of the panallergen profilin and the major allergen (Ole e 1) from olive tree pollen. J Allergy Clin Immunol. 1997 Sep;100(3):365-72.

17. Barderas R, Villalba M, Lombardero M, Rodriguez R. Identification and characterization of Che a 1 allergen from Chenopodium album pollen. Int Arch Allergy Immunol. 2002 Jan;127(1):47-54.

18. Batanero E, Gonzalez De La Pena MA, Villalba M, Monsalve RI, Martin-Esteban M, Rodriguez R. Isolation, cDNA cloning and expression of Lig v 1, the major allergen from privet pollen. Clin Exp Allergy. 1996 Dec;26(12):1401-10.

19. Barderas R, Villalba M, Rodriguez R. Che a 1: recombinant expression, purification and correspondence to the natural form. Int Arch Allergy Immunol. 2004 Dec;135(4):284-92.

20. Pomes A, Vailes LD, Helm RM, Chapman MD. IgE reactivity of tandem repeats derived from cockroach allergen, Bla g 1. Eur J Biochem. 2002 Jun;269(12):3086-92.

21. Twell D, Wing R, Yamaguchi J, McCormick S. Isolation and expression of an anther-specific gene from tomato. Mol Gen Genet. 1989 Jun;217(2-3):240-5.

22. Villalba M, Batanero E, Lopez-Otin C, Sanchez LM, Monsalve RI, Gonzalez de la Pena MA, et al. The amino acid sequence of Ole e I, the major allergen from olive tree (Olea europaea) pollen. Eur J Biochem. 1993 Sep 15;216(3):863-9.

23. Hames BD, Rickwood D. Gel electrophoresis of proteins. New York: Oxford, Tokyo: IRL Press; 1990.

24. Heiss S, Mahler V, Steiner R, Spitzauer S, Schweiger C, Kraft D, et al. Component-resolved diagnosis (CRD) of type I allergy with recombinant grass and tree pollen allergens by skin testing. J Invest Dermatol. 1999 Nov;113(5):830-7.

25. Hyde HA. Atmospheric pollen and spores in relation to allergy. I. Clin Allergy. 1972 Jun;2(2):153-79.

26. Solomon WR. Airborne pollen: a brief life. J Allergy Clin Immunol. 2002 Jun;109(6):895-900.

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