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Mojtaba Sankian, Forough Glosaze Shirazi, Majid Arafi, Malihe Moghadam, Abdolreza Varasteh

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Production and Characterization of Monoclonal Antibody against Saffron Pollen Profilin, Cro s 2

Mojtaba Sankian, Forough Glosaze Shirazi, Majid Arafi, Malihe Moghadam, Abdolreza Varasteh*

Immunobiochemistry lab, Immunology Research Center, Bu-Ali Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran

ABSTRACT

Background: Allergy to Saffron (*Crocus sativus*) pollen has been described in people involved in processing of saffron flower stamens. Profilins have been identified as a pan-allergen in different plant pollens and foods. This molecule is an actin-binding protein with a molecular weight of 12-16 kDa found in eukaryotic species. **Objective:** The aim of this study was to generate monoclonal antibody against Cro s 2 in order to characterize this major allergen of saffron pollen. **Methods:** BALB/c mice were immunized to obtain adequate humoral response. Splenocytes were prepared from the immunized animals, mixed with the P3-X63-Ag8.653 myeloma cells and fused by means of PEG 1500. After two weeks of culturing in HAT-containing media, the supernatant from those wells growing hybridomas were screened by ELISA using plates coated with Cro s 2. Cells from positive wells were cloned at least 3 times by limiting dilution. Specificity and cross-reactivity of the mAbs were determined by Western blot analysis and sandwich ELISA. **Results:** Two stable hybridoma clones secreting mAbs against Cro s 2 were obtained and expanded. The anti-Cro s 2 mAbs were also found to cross-react with other plant profilins. Isotype of this mAb was identified as μ heavy chain and k light chain. **Conclusion:** The anti-Cro s 2 mAb could be a useful tool for characterization and standardization of many pollen and fruit-derived profilins.

Keywords: Profilin, Allergen, Monoclonal antibody, *Crocus Sativus,* Saffron

INTRODUCTION

Profilin is an actin binding protein of low molecular weight (12-16 KDa) that is present in all eukaryotic cells, from yeast to man (1). At high concentrations, profilin can inhibit actin nucleation and polymerization in vitro. At low concentrations, it catalytically accelerates the rate constant for dissociation of ATP from actin (2, 3). Profilin has

*Corresponding author: Dr. Abdolreza Varasteh, Immunobiochemistry Lab, Immunology Research Center, Bu-Ali Research Institute, Ferdossi Square, Bu-Ali Square, P.O. Box: 9196773117, Mashhad, Iran. Tel: (+) 98 511 7112410, Fax: (+) 98 511 7112616, e-mail: varasteha@mums.ac.ir

also been described as an allergen in different plant sources. The presence of profilin as cross-reacting allergen in various plants provides an explanation why certain patients display type I allergic reactions with pollen from distantly related plants. Due to this broad allergenicity and cross-reactivity, profilin has been described as a pan-allergen (4, 5). Recently profilin encoding genes from several plants have been cloned, and their sequences shown to share a high degree of identity (4, 5, 7). *Crocus sativus* (saffron), is a plant which has important commercial value in food industry in Iran. Allergy to saffron pollen has been described in people involved in the processing of saffron. Sera of saffron allergic patients recognize different proteins in the ranges of 11 to 70 kDa (9). The development of hybridoma technology and the production of monoclonal antibodies have made significant progress in the field of Allergology. Monoclonal antibodies (mAbs) are used for allergen standardizations and improving the quality of allergen extracts used for diagnosis and treatment. They are also used for assessing the relationship between environmental allergen exposure and the onset of allergic symptoms. In both cases, there are clearly several advantages in using mAbs-based assays since mAbs afford the sensitivity, specificity and reproducibility required to quantitate the level of specific allergens (10). The main aim of the present study was to prepare mAbs against saffron pollen profilin that could be used for characterization and standardization of pollen and fruit-derived profilins.

MATERIALS AND METHODS

Protein Extraction from Saffron Pollen and Purification of Profiling. 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 graded 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 protein extraction in the following step difficult. The homogenicity of the pollen was checked by a light microscope. An impurity of less than 5% (parts of stamen or pollen from other plants) was usually obtained. Pollen (6 g) was defatted by adding 100 ml acetone and shaking overnight at 4°C. The mixture was centrifuged at 5,000 g for 15 minutes at 4°C. The supernatant was discarded and the pellet dried at room temperature. Diffusates of the pollens were prepared by thoroughly mixing dry pollen (1g) with 10 ml phosphate buffered saline (PBS) by continuous stirring for 16 h at 4 °C. The mixture was centrifuged at 16,000 g for 10 min. The supernatant was dialyzed against 50 mM phosphate-buffered saline (pH 8) and filtered through a 0.22-µm membrane (Millipore Corp., Bedford, MA, USA). The filtrate was then lyophilized and stored at -20°C in sterile vials. The protein content was determined by the Bradford method (11). Saffron pollen profilin was purified from the crude extract by size exclusion chromatography using an AKTA-FPLC system (Amersham Biosciences, Freiburg, Germany) over a HR200 Superdex column.

Fractions were analyzed on 4 to 20% Tris-Glycine gels (Invitrogen) under reducing and non-reducing conditions by Coomassie staining and western blot analysis. Proteins were electrophoretically transferred to PVDF membranes. After blocking with 2% BSA for 16 hr at 4°C, the blot was incubated for another 16 hr at 4°C with rabbit polyclonal anti-Cuc m 2 (melon profilin). After washing, blots were incubated with biotin conjugated anti-rabbit IgG (BioRad, 1:10000 diluted). Upon incubating the membranes with horse-

radish peroxidase streptavidin (1:2000 diluted, BioRad Laboratories, Hercules, California, USA), profilin binding antibodies were detected by exposure to ECL Hyperfilm (Amersham Pharmacia Biotech, Uppsala, Sweden.

Immunization and Screening of Immunized Animals. BALB/c mice (8 to ten weeks old) were immunized intraperitoneally and subcutaneously with 100 µl saline containing 5 µg high-performance liquid chromatography purified Saffron pollen profilin emulsified in complete Freund's adjuvant. The same amount of antigen and incomplete Freund's adjuvant were given at days 14 and 21. Ten days after the third injection, sera were screened by direct ELISA for the presence of antibodies against profilin using this antigen to coat microplate wells. A final intravenous dose of 2 µg antigen in phosphate buffered saline was administered without an adjuvant, and the mice were sacrificed 3 days later.

The screening ELISA used for the presence of antibody against profilin was optimized as follows: different dilutions of Saffron profilin (1.5, 0.75, 0.375, 0.187, 0.093, 0.046 µg/well in carbonate buffer, pH 9.6) were coated onto wells of polystyrene microplates (NUNC, Denmark) and blocked with 2% BSA. Immunized mouse sera were added to each well and incubated for 1 hour at room temperature. After 5 washes with PBS (150 mM, pH 7.4), an affinity purified anti-mouse IgG-HRP (Sigma, USA) was added in different dilutions (1/100, 1/300, 1/600, 1/1000, 1/2000, 1/5000) to a volume of 100 µl/well and incubated at 37 °C for 1 hour. Wells were then washed for 3 times with PBS followed by the addition of substrate solution (3,3,5,5 Tetra methylbenzidine and hydrogen peroxide) to each well at room temperature. After 20 min, the absorbances were measured at 450 nm using an ELISA microplate reader.

Hybridoma Preparation. Three days after final immunization, mice were sacrificed and spleens were aseptically removed. Single-cell suspensions were obtained by gently teasing the spleen into 5 ml 0.09% NaCl containing 2% FCS. The tubes were centrifuged at 90 g (1000 rpm) for 3 minutes and the supernatant discarded. Ten milliliters of PBS were added to the cell pellet and centrifuged under the same conditions. The cells were then suspended in RPMI 1640 medium and the lymphocytes were counted using a Neubauer hemocytometer.

Culture conditions were optimized using different pH values and different serum volumes. The cell line was cultured in RPMI 1640 medium, pH 7.4 supplemented with 20% (v/v) fetal bovine serum and 10µg/ml Tylosin at 37°C in a humidified, CO2-controled (5%) incubator. Cells were centrifuged at 90g for 3 minutes. Precipitated cells were suspended in PBS and centrifuged under the same condition. Cells were then suspended in RPMI 1640 medum and were counted using a Neubauer hemocytometer. The viability of the cells was checked using Trypan blue (0.25% in PBS).

The suspension of isolated spleen cells was mixed with P3-X63-Ag8.65 (Ag8) at a ratio of 3:1 and 2:1 and fused at 37°C with 1 ml of 50% (w/v) polyethylene glycol (Sigma). They were then diluted with 15 mL of RPMI, centrifuged, and rediluted into a complete (20% fetal calf serum) selective medium containing 100 µM hypoxanthine, 16 µM thymidine, 0.4 µM aminopterin (Hybri-Max, Sigma-Aldrich, USA) at 2×10^6 cells/ml. The cell suspension was added in 0.1 ml aliquots to 96 well plates and incubated at 37°C in 5% CO2. Culture plates were pre-seeded with peritoneal resident macrophages from non-immunized BALB/c mice as feeder cells at a density of $5x10^4$cells per well one day prior to transfer of fused cells. Culture medium was replaced weekly in all wells.

Screening of Antibody Secreting Hybridoma Cells. The supernatant from those wells growing hybridomas were screened by ELISA (as described above) on day 15. Cells

from positive wells were cloned 3 times by limiting dilution. Hybridomas were cultured and then stored frozen in liquid nitrogen at -80°C.

Appropriate monoclonal antibody was produced by hybridoma cell culture in RPMI 1640 medium supplemented with 20% (v/v) fetal bovine serum. In order to reach to the maximum concentration of mAbs in the supernatant, incubation was continued until the death of all cells. The supernatant was concentrated using 45-50 %saturated ammonium sulfate followed by dialysis against PBS. The monoclonal antibodies were stored frozen at -20°C.

Monoclonal antibody Ig class and subclass determinations were performed using mouse monoclonal isotyping Kit (Serotec Ltd, Oxford, UK).

Immunoblot Analysis of Anti-Cro s 2 Monoclonal Antibodies. The specificity of the anti-Cro s 2 was also confirmed by an immunoblotting procedure. Saffron pollen profilin was separated by SDS-PAGE according to Laemmli in 12.5% polyacrylamide gels under reducing conditions (12) and were electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA). After blocking with 2% BSA for 16 hour at 4°C, the blot was incubated for 16 hour at 4°C with the hybridoma culture supernatant. The blots were then incubated with anti-mouse immunoglobulin biotin-conjugated goat antibody (Sigma, USA) (1:500 diluted). Following incubation with horseradish peroxidase-streptavidin (1:20000 diluted), profilin binding antibodies were detected by ECL hyperfilm after exposure for 5 min.

Assessment of Anti-Cro s 2 Cross-Reactivity with the Other Allergenic Profilins by a Sandwich ELISA. To evaluate capability of anti-Cro s 2 mAbs as a tool for characterization and standardization of pollen and food-derived profilins, a sandwich ELISA was developed to measure profilin content of *Cynodon dactylon* pollen, *Poa pratense* pollen, peach, cantaloupe and tomato extracts and a solution containing recombinant melon profilin that was produced and purified as described previously (13).

Microplates were coated overnight at 4 $^{\circ}$ C with 100 µl of a mixture of anti-Cro s 2 mAbs in coating buffer (0.1 M bicarbonate buffer pH 9.6) at a concentration of 10 µg/ml. Plates were saturated with 2% BSA for 1 h at 37 $^{\circ}$C. Then 100 µl of serially diluted Saffron profilin (2.3-150 ng/well) or the total extract were added and incubated for 2 h at room temperature. After 5 washes with PBS containing 0.05% Tween, 100 µl of mouse anti-profilin IgG (diluted 1/40000 in 1% BSA) were added to each well and the plates were incubated for 1 h at room temperature. After adding 100 µl biotinylated goat anti-mouse Ig (diluted 1:10000 in 1% BSA–Tween), the plates were incubated at room temprature for 1 h. The plates were washed again and developed by incubating with 100 µl streptavidin–HRP conjugate diluted 1:10000 in 1% BSA–Tween for 1 h at room temperature. The plates were then washed again and developed by incubating with 200 µl/well of a solution of TMB. The reaction was stopped by adding 20 µl of 3 M HCl and the absorbances were measured at 450 nm. A standard curve was generated with serial two-fold dilution of purified Saffron profilin. The concentrations of profilin were interpolated from the linear portion of the standard curve.

RESULTS

Saffron Profilin Purification. Figure 1A shows the SDS-PAGE of crude saffron pollen profilin before and after separation on a Superdex HR200 FPLC column. Peak 2 in the

chromatogram below contained a purified 14 kDa protein. In Figure 1B, western blot with a rabbit polyclonal anti-profilin confirmed that fraction 2 contained saffron pollen profilin.

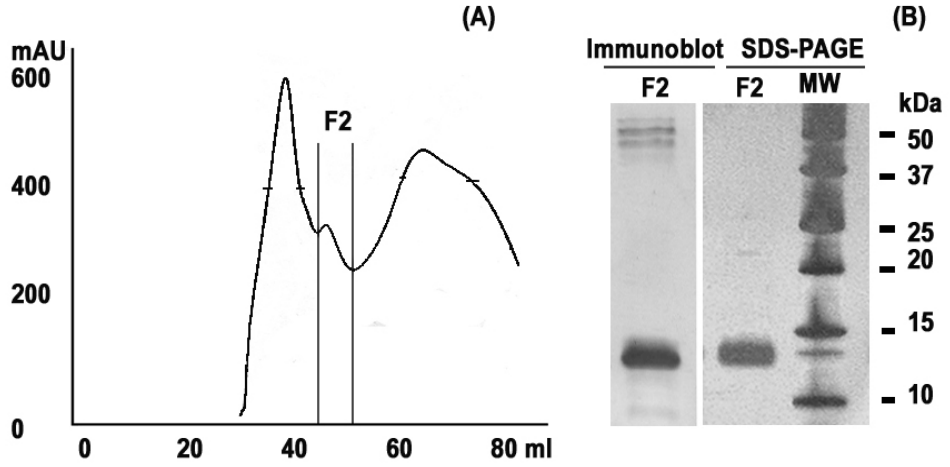

Figure 1. Purification of saffron pollen profilin by FPLC and, SDS-PAGE and the immunoblot analysis of purified profilin. (A) Chromatography of saffron pollen proteins on Superdex HR200 FPLC column. Profilin containing fraction was collected in the second peak (F2), (B) SDS-PAGE and immunoblot analysis of purified profilin containing fraction is shown in the F2 lanes. MW; Molecular weight.

Monoclonal Antibody Production. Two hybridomas were obtained by fusion of immunized murine spleen cells with myeloma cells. Hybridoma secreting antibodies against saffron profilin were screened by ELISA. Antibody-producing hybridomas were cloned by limiting dilution. Two of the twenty clones were stable and expanded. Monoclonal antibodies obtained from both clones showed binding to profilin by immunoblotting and ELISA. Amount of profilin for ELISA test was optimized using different dilutions. The optimal dilution of profilin (as coating antigen), anti-mouse immunoglobulin biotin-conjugated secondary antibody and streptavidin-HRP used for indirect ELISA were 1/800 dilution (1.8 ng/µl), 1/5000 dilution and 1/2000 dilution, respectively. Immunoglobulin isotype was determined by mouse mAb isotyping kit (Serotec). It was typed as IgM protein, with k light chains.

Immunoblot and ELISA Analysis of Anti-Cro s 2 Monoclonal Antibodies. The selected antibodies were further evaluated by western blot analysis. Monoclonal antibodies recognized an approximately 14 kDa protein that was also recognizable with saffron allergic patient sera. Antigenic cross-reactivity among profilin allergens in saffron pollen profilin and recombinant melon profilin was detected with the generated mAb (Fig. 2).

In this study, a sandwich ELISA method was set up to measure plant profilin using solid-phase coated mAbs. This method showed cross-reactivity of anti-Cro s 2 monoclonal antibodies with the natural profilins of *Cynodon dactylon* pollen, *Poa pratense* pollen, cantaloupe and tomato extracts**,** and recombinant profilin of melon (rCuc m 2). The estimated profilin content of rCuc m 2 solutions were close to those determined with Bradford`s method. The respective profilin content of *Cynodon dactylon* pollen, *Poa pratense* pollen, cantaloupe and tomato extracts were estimated as 0.018, 0.030, 0.0147, and 0.15 percent of their total protein by comparison with 2.3-150 ng/well

of serially diluted Saffron profilin as standard. No reactivity was observed with the profilin of the peach extract.

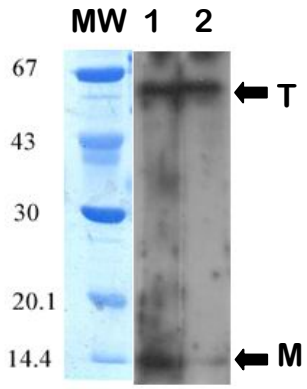

Figure 2. Immunoblot analysis of profilin-specific monoclonal antibodies. Lane 1; Saffron pollen profilin. Lane 2; melon recombinant profilin. Reactivity of mAb with the monomeric and tetrameric forms of profilins are indicated with M and T, respectively. MW; Molecular weight.

DISCUSSION

Allergic extracts used in clinical applications contain various components and many factors contribute to the batch-to-batch variability in allergen extracts. The preparation of these reagents can be improved by carefully identifying, characterizing and standardizing major allergens in the extracts. Profilin has been identified as a major allergen in several plants. Monoclonal antibodies provide an excellent standard tool for the analysis of the molecular basis of cross-reactivity between proteins and glycoproteins from different allergenic sources.

To further characterize the panallergen profilin, we produced mAbs against saffron pollen profilin. Monoclonal antibodies were generated by immunization of BALB/c mice with saffron pollen purified profilin and after screening by ELISA, two antiprofilin mAbs producing hybridomas were obtained.

The specificity of these mAbs was analyzed by immunoblotting after SDS-PAGE and they demonstrated binding to profilin in denaturing conditions. Those mAbs could bind to profilin both in native form (ELISA) and after denaturation (SDS-PAGE). Produced mAbs recognized a protein of 14 kDa molecular weight which corresponded to the molecular mass of profilin. Nevertheless, our mAb also recognized a protein with an apparent molecular mass of 56 kDa. Aggregation of profilin is common and was reported first by Babich et al (14,15), who showed that silver stained SDS-PAGE of poly-L-proline purified profilin detected profilin (14.8 kDa) and higher protein bands (primarily 30 kDa and 58 kDa). The oligomrization of profilin may increase its allergenicity, as was recently shown by Vrtala et all (16). Other studies also demonstrated that oligomerization occurs with natural pollen profilins and is not an artifact resulting from its recombinant production and the purification process (17). Previously, human profilin purified from platelets was also shown to exist in solution as dimer and tetramer forms (15). It has been demonstrated that oligomerization promotes the reactivity of recombinant profilins with rabbit anti-profilin antibody, in accordance with the notion that oli-

gomeric forms would be more antigenic than monomers because larger molecules are likely to have additional epitopes (18).
Although the sandwich ELISA was developed using anti-Cro s 2 monoclonal antibody, it was also found capable of detecting *Cynodon dactylon* pollen, *Poa pratense* pollen, and cantaloupe and tomato profilins that share common epitopes with the saffron profilin. The estimation of the profilin content of the allergenic crude extracts was probably based on the existence of similar but not identical epitopes in all of them. Therefore, the estimation of profilin content in a sample may vary according to the type of profilin used as a standard (19).
In conclusion, we have produced a monoclonal antibody specific for an allergen that is an important component of plant pollens. This mAb could be employed in a highly reliable assay for the detection and standardization of pollens and fruit-derived profilins.

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