

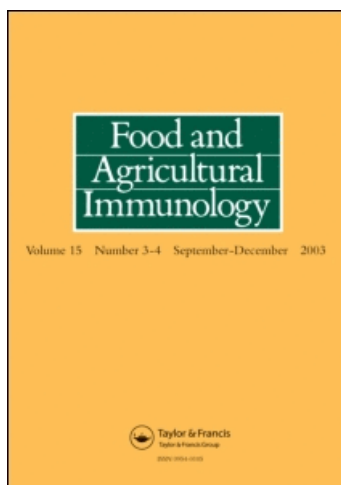
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Samaneh Abedini^a; Mojtaba Sankian^a; Reza Falak^b; Mohsen Tehrani^a; Farideh Talebi^a; Frough Golsaz Shirazi^a; Abdol-Reza Varasteh^a

^a Immunology Research Center, Bu-Ali Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran ^b Immunology Department, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran

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An approach for detection and quantification of fruits' natural profilin: natural melon profilin as a model

Samaneh Abedini^a, Mojtaba Sankian^a, Reza Falak^b, Mohsen Tehrani^a,
Farideh Talebi^a, Frough Golsaz Shirazi^a and Abdol-Reza Varasteh^{a*}

^aImmunology Research Center, Bu-Ali Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran; ^bImmunology Department, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran

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Anaphylactic reactions are of great importance in clinical allergology. Measurement of allergen concentration in foods is helpful to estimate their allergenicity. In this study, a sandwich ELISA for the evaluation of profilin concentration in fruits was designed. A mixture of monoclonal antibodies against saffron profilin was used as a capture antibody. The recombinant melon profilin was expressed in *Escherichia coli* BL-21 and applied as a standard antigen along with different fruit extracts to measure their profilin concentration. Furthermore, a polyclonal antibody against the recombinant melon profilin was produced and applied as the secondary antibody. In this way, the profilin concentration of various fruits including banana, tomato, kiwi, mulberry, cantaloupe, peach, watermelon and zucchini was measured. The results showed that the designed ELISA had a sensitivity threshold of about 1 ng/ml for the melon profilin and it is therefore suggested for determination of profilin concentration in other fruits.

Keywords: allergen; fruit extract; melon; profilin; sandwich ELISA

Introduction

It has been estimated that up to 8% of children and 2% of adults suffer from food allergy (Zuidmeer et al., 2008). The diagnosis of food allergy is routinely based on clinical history and skin prick test (SPT) with allergenic food extracts. These extracts have shown variable sensitivities and specificities in diagnostic and therapeutic procedures (Lidholm, Ballmer-Weber, Mari, & Vieths, 2006). In order to make improved diagnostic tests, it is helpful to determine the concentration of the major allergens in extracts (van Ree, Dorpema, & Vieths, 2005). Several studies discussed standardisation of allergen concentration in extracts by means of sandwich or competitive enzyme-linked immunosorbent assays (ELISAs) (Becker et al., 2006; Becker, Vogel, & Vieths, 2006). Monoclonal antibody (mAb)-based ELISA, in particular, has been reported to be useful in determination of concentration of individual allergens, meanwhile their advantages and disadvantages should be considered (van Ree, 1994).

Plant profilins belong to a highly conserved family of pan-allergens with molecular weights (MWs) ranging from 14 to 15 kDa, where they are vastly found

*Corresponding author. Email: VarastehA@mums.ac.ir

in fruits and pollens (Valenta et al., 1992). The role of these plant proteins in causing allergic cross-reactivity among different foods and pollens, and also their association with mild local symptoms were previously confirmed by several studies (Caballero & Martin-Esteban, 1998; Valenta et al., 1991). In most of these studies, the seroprevalence of specific IgE to food and pollen profilins were reported as 20–30% (Scheurer, Wangorsch, Haustein, & Vieths, 2000; Valenta et al., 1992).

We previously characterised and reported the profilin as a major allergen of melon. It was also produced as a recombinant protein (rCuc m 2) in our lab (Sankian, Varasteh, Pazouki, & Mahmoudi, 2005). Nevertheless, there are few data available on determination of profilin amounts in fruits. In this study, we designed a sandwich ELISA as a quantitative method for assessment of profilins in crude extracts of fruits. This approach was carried out using the natural melon profilin (nCuc m 2) as a fruit allergen model.

Materials and methods

Production and purification of rCuc m 2

We previously produced rCuc m 2 in *Escherichia coli* BL-21 (DE3) strain (Novagen, WI, USA), lyophilised and kept at -30°C (Sankian et al., 2005). Herein, rCuc m 2 was re-expressed as described previously and purified by immobilised metal affinity chromatography. Briefly, a lyophilised bacterial clone was subcultured in Luria-Bertani (LB) broth medium containing $1\text{ }\mu\text{g/ml}$ ampicillin at 37°C ; after reaching an OD_{600} of 0.4, the production of the recombinant protein was induced by adding 0.1 mM isopropyl-beta-thio galactopyranoside (IPTG). Four hours later, the culture media was centrifuged and the pellet was suspended in lysis buffer containing 0.05 M Tris-HCl (pH 8), 0.15 M imidazole, 0.1 M NaCl, 10% glycerol and 0.5% Triton X-100, and subjected to three freezing and thawing cycles using liquid nitrogen. Following a re-centrifugation, the recombinant profilin, which was produced as a 6His-tagged fusion protein, was purified from the supernatant using a column of Ni-NTA agarose (Invitrogen, San Diego, CA, USA) according to the manufacturers' instruction. Purified rCuc m 2 were then dialysed against 10 mM potassium phosphate buffer (pH 7.5) and lyophilised.

Production of polyclonal and monoclonal antibodies against profilin

Polyclonal anti-serum against rCuc m 2 was produced in rabbits. Briefly, rCuc m 2 was dissolved in sterile distilled water and mixed with equal volume of Freund's complete or incomplete adjuvant. Each rabbit was immunised with 1 mg of rCuc m 2 emulsified in complete Freund's adjuvant as the first injection. Four booster injections were performed with $500\text{ }\mu\text{g}$ of the recombinant protein and incomplete Freund's adjuvant, every other week. After a total of five intradermal injections, sera from the sensitised rabbits were collected and the anti-rCuc m 2 antibody levels were evaluated by an indirect ELISA.

Three anti-Cro s 2 secreting clones were previously produced and identified as IgM (Sankian, Glosaz-Shirazi, Araf, Moghadam, & Varasteh, 2008). They were also confirmed to be specifically reactive against native and denatured forms of saffron recombinant and natural profilins as determined by ELISA and western blotting. In

this study, those clones were retrieved and subcultured in RPMI-1640 supplemented with 10% foetal calf serum. The fresh supernatants were collected as a source of mAbs.

Purification of polyclonal and monoclonal antibodies

Protein-A column chromatography was used for the isolation of total rabbit serum IgG. Briefly, 5 ml of protein-A sepharose gel (Pharmacia, Uppsala, Sweden) was packed in a column and equilibrated with phosphate buffered saline (PBS, pH 7). Rabbit sera were diluted 1:1 in PBS and applied on the column. The unbound proteins were removed by several washings of the column with PBS. Finally, IgG was eluted by adding 0.2 M glycine-HCl buffer (pH 3). The collected fractions were neutralised with hydroxymethyl-aminomethan (Tris) powder and dialysed against PBS.

Purification of mAbs from cell culture supernatants was performed in two steps. Firstly, the globulins were precipitated by steadily adding of ammonium sulfate crystals to cell culture supernatant up to 45% saturation. After 2 h of gentle agitation, the mixture was centrifuged at 9000g for 20 min. The supernatant was discarded and the pellet was dissolved in PBS at 1:20 of its initial volume. Monoclonal IgM was purified using an IgM purification kit (Pierce, Rockford, IL, USA) according to the manufacturer's instruction.

Preparation of fruit extracts

Crude extracts from nine common allergenic fruits including Persian melon, cantaloupe, kiwi, peach, tomato, banana, mulberry, zucchini and watermelon were prepared as described by Moller, Kayma, Vieluf, Paschke, and Steinhart (1998) with some modifications. The edible part of each fruit was removed and homogenised in a blender. The blends were then suspended in 1:10 w/v in potassium phosphate buffer (100 mM, pH 8) containing 2% (w/v) polyvinylpyrrolidone (PVPP) and 10 mM ethylene diamine tetracetic acid (EDTA). The mixture was shaken for 5 h at 4°C. The slurry was then centrifuged at 9000g for 30 min at 4°C. The supernatant was dialysed against potassium phosphate buffer (10 mM, pH 8) for 18 h at 4°C. After lyophilisation, they were kept at -20°C until use. Total protein content of the each extract was measured using the Bradford's (1976) method.

Purification of natural melon profilin (nCuc m 2)

Immunoaffinity chromatography was carried out to purify the nCuc m 2. Briefly, an immunosorbent column was prepared by coupling the rabbit anti-rCuc m 2 with cyanogen bromide (CNBr)-activated sepharose 4B gel (Pharmacia, Uppsala, Sweden) according to the manufacturer's instruction. Melon extract was passed through the column and the unbound proteins were removed by frequent washing with PBS. nCuc m 2 was then eluted by adding 0.2 M glycine-HCl buffer (pH 2.5) and the collected fractions were immediately neutralised with Tris powder. The neutralised fractions were pooled and dialysed repeatedly against 10 mM potassium phosphate buffer (pH 8) and lyophilised. The purity and concentration of the

purified nCuc m 2 were assessed by the SDS-PAGE and Bradford's assay, respectively.

Western blotting

Western blotting was performed to evaluate the immunoreactivity of the purified monoclonal and polyclonal antibodies with the crude melon extract and the purified nCuc m 2. Briefly, 5 µg of the purified nCuc m 2 along with 10 µg of the crude melon extract were subjected to a 12.5% SDS-PAGE. The resolved proteins were electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore Corp., Bedford, MA, USA) at 300 mA for 15 min in a solution containing 20% methanol, 0.192 M glycine and 0.025 M Tris base (pH 8.0). The membrane was then cut into strips and blocked in 2% bovine serum albumin (BSA) in PBS (4°C, overnight). The strips were gently washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with 1:1000 dilution of polyclonal anti-Cuc m 2 in 1% BSA for 2 h at room temperature. Subsequently, the blots were washed three times for a total of 15 min with PBS-T. As a secondary antibody, HRP-conjugated goat anti-rabbit antibody (Kirkegaard & Perry Laboratories, MD, USA) was diluted 1:3000 in 1% BSA and added to strips. After 1 h incubation at room temperature, the strips were washed thoroughly with PBS-T and incubated with the Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) for 3 min. The binding of the secondary antibodies visualised by chemiluminescence G-Box gel documentation system (Syngene, Cambridge, UK) using GeneSnap Version 6.08 software (SynGene Ltd.). All incubations were performed on a rocker.

ELISA

In order to measure the natural profilin concentration in fruit extracts, we developed a sandwich ELISA. Using different concentrations of nCuc m 2 as a known antigen, and rCuc m 2 as a standard, optimal dilution of coating and capturing antibodies were primarily determined by checkerboard titration. Subsequently, ELISA was carried out as follows. Maxisorp immunoplates (Nunc, Fisher Scientific, Pittsburg, PA, USA) were coated with 0.1–10 µg/ml of a mixture of three anti-saffron profilin mAbs in 0.1 M bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Wells were then aspirated and washed five times, each time with 300 µl of PBS-T.

Each well was then blocked with 300 µl of 2% BSA for 1 h at 37°C. Following another washing step, 100 µl of serially diluted concentrations of rCuc m 2 (4.6–150 ng/well) along with nCuc m 2 (10–100 ng/well) were added to each well in triplicate and incubated for 2 h at room temperature. The plates were then washed with PBS-T and added with 100 µl of the purified rabbit anti-profilin IgG (1:2000–1:180000 dilution) and incubated for 1 h at room temperature. The plates were washed again as above and added with 100 µl of 1:10000 biotinylated goat anti-rabbit antibody (Bio-Rad, MD, USA) and incubated for 2 h at room temperature. After another wash, the plates were added with 100 µl of 1:10,000 HRP-conjugated streptavidin (Serotec, NC, USA). The plates were incubated for 1 h at room temperature and washed. Chromogenic substrate was made by adding 50 µl of 3,5,3',5'-tetramethylbenzidine (TMB) solution (6 mg/ml in dimethyl sulfoxide) and 1.5 µl of 3% H₂O₂ to 2.5 ml of 0.1 M sodium acetate (pH 5.5) solution. One hundred microlitres of the chromogenic

substrate were added to each well. After 15 min of incubation in the dark, colour development was stopped by addition of 100 μ l of 3 M hydrochloric acid. The absorbance was read at 450 nm using an ELISA reader (StatFax-2100, Awareness Technology, Palm City, FL, USA).

The standard curve was plotted with two-fold serial dilutions of the purified rCuc m 2. Profilin concentrations in nine fruit extracts were then measured by the established sandwich ELISA, as mentioned above. In order to check the reproducibility of the established ELISA, the assay was performed several times with rCuc m 2.

Notably, all of the recombinant protein, crude extract and polyclonal/monoclonal antibody dilutions were prepared using BSA 1% in PBS.

Results

Purification of rCuc m 2 and polyclonal anti-rCuc m 2 antibody

rCuc m 2 was successfully purified by metal affinity chromatography and used as a standard antigen for development of a sandwich ELISA. SDS-PAGE revealed a single protein band with an apparent MW of 15.5 kDa (Figure 1). The concentration of the purified rCuc m 2 was calculated as about 1 mg/ml by the Bradford's method. Western blot showed the reactivity of the purified rCuc m 2 with both polyclonal and monoclonal anti-Cuc m 2 antibodies (Figure 2).

Purification of nCuc m 2 and polyclonal anti-nCuc m 2 antibody

nCuc m 2 was reasonably purified by immunoaffinity chromatography and used as a positive control antigen for development of the sandwich ELISA. A protein band with an apparent MW of 16 kDa was revealed by SDS-PAGE (Figure 2). Furthermore, a polymeric form of the nCuc m 2 was detected with a MW between 60 and 70 kDa. Using the Bradford's method, the concentration of purified nCuc m 2 was calculated as about 0.1 mg/ml. Both polyclonal and monoclonal anti-Cuc m 2 antibodies showed reactivities to the purified nCuc m 2 via immunoblotting (Figure 2).

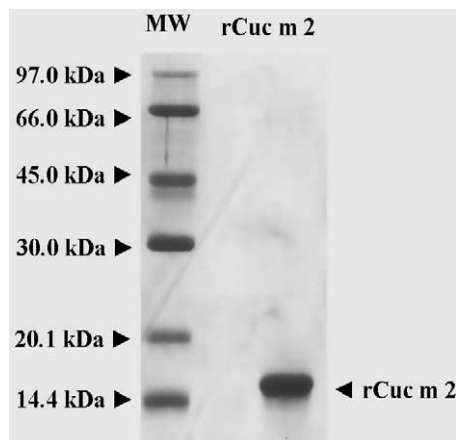


Figure 1. SDS-PAGE analysis of recombinant melon profilin. The gel was stained by coomassie blue. MW: low molecular weight marker (Amersham, USA).

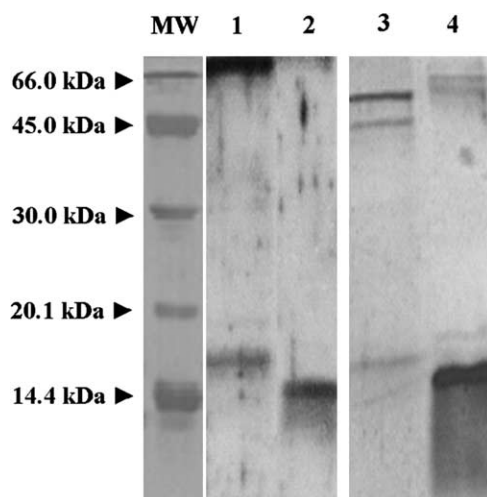


Figure 2. Immunoblotting of nCuc m 2 and rCuc m 2. Lane MW: low MW marker (Amersham, USA); Lane 1: monomer and tetramer forms of nCuc m 2 reacted with polyclonal anti-Cuc m 2 antibody; Lane 2: rCuc m 2 reacted with polyclonal anti-Cuc m 2 antibody; Lane 3: monomer and polymer forms of nCuc m 2 reacted with monoclonal anti-Cuc m 2 antibody; and Lane 4: rCuc m 2 reacted with monoclonal anti-Cuc m 2 antibody.

Measurement of profilin concentrations of fruits by ELISA

ELISA was developed using rCuc m 2 as standard and nCuc m 2 as a control allergen. Optimum amount of the capturing antibody was determined as 1 μ g/well. Furthermore, optimum dilution of the polyclonal detecting antibody was determined as 1:40,000. Using a serial dilution of nCuc m 2, the sensitivity threshold of the ELISA was calculated as 1 ng/ml of melon profilin. Thereafter, profilin concentrations of eight fruit extracts were measured by ELISA. By means of a typical standard curve (Figure 3), the concentration of profilin in banana, tomato, mulberry, cantaloupe and zucchini was calculated as 373.24, 48.00, 25.16, 11.83 and 2.50 ng/g weight of the fruit pulp, respectively. However, it was measured as very low or non-detectable concentrations in peach, watermelon and kiwi (Table 1).

Discussion

In this study, a sandwich ELISA was established to quantify profilin concentration in various fruits. The notable advantages offered by the present method are the following: firstly, a mixture of three previously produced mAbs against different epitopes of the saffron profilin was employed as the capture antibody to increase the possibility of binding to profilin molecules from different fruits. Secondly, in order to increase the binding of the detection antibody to the captured antigens, a polyclonal antibody against rCuc m 2 was produced which was used as a secondary antibody. Finally, a serial dilution of rCuc m 2 that was comparable with its natural counterpart was served as standard.

Several studies measured the concentration of allergenic proteins in nuts such as peanuts, cashews and walnuts (Niemann, Taylor, & Hefle, 2009; Pomes et al., 2003;

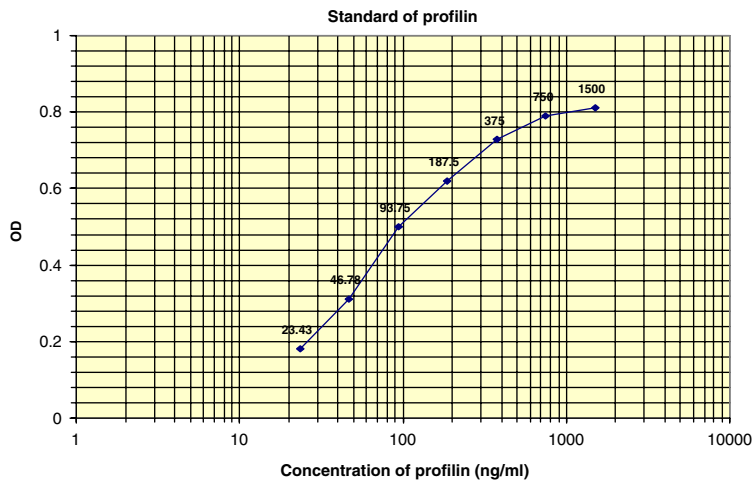


Figure 3. Typical standard curve of the developed ELISA. Recombinant Cuc m 2 was used as standard of profilin.

Wei, Sathe, Teuber, & Roux, 2003), as well as in fruits like kiwi, cherry and apple (Gavrovic-Jankulovic et al., 2008; Zuidmeer et al., 2005) using sandwich or competitive ELISAs. Asturias et al. (1999) have recently established a sandwich ELISA to determine the profilin concentration in various pollen extracts. They produced several mAbs against a recombinant profilin from the sunflower pollen (Hel a 2) and used them to quantify the profilin contents of the other pollen extracts with a sensitivity threshold of 0.5 ng/ml for the sunflower profilin.

In the present study, for the first time, the profilin concentrations of various fruit extracts were measured by the developed sandwich ELISA. The natural profilin of melon (nCuc m 2) was also purified and used for evaluation of the ELISA sensitivity. A serial dilution of known quantities of nCuc m 2 were used in three independent repeats of experiment to determine the sensitivity threshold of the ELISA, which resulted in a threshold of 1 ng/ml of melon profilin.

After establishment of ELISA, the profilin content of different fruit extracts including banana, tomato, kiwi, mulberry, cantaloupe, peach, watermelon and

Table 1. Concentration of natural profilin in the different fruits using mAb-based sandwich ELISA method.

Extract	Total protein (µg/ml) of fruit extract	Profilin concentration (ng/ml) in fruit extracts	Profilin concentration (ng/g) in weight of fruits
Banana	128	256.6	373.24
Tomato	18	27.27	48
Kiwi	52	< Treshhold	< Treshhold
Mulberry	141	12	25.16
Cantaloupe	80	11.8	11.83
Peach	80	< Treshhold	< Treshhold
Watermelon	30	< Treshhold	< Treshhold
Zucchini	45	5.95	2.5

zucchini were measured. The profilin concentrations of banana and tomato extracts were higher than the other fruits (373 and 48 ng/g of the fruit pulp, respectively). Concurrent with our results, profilin has been shown as a major allergen of both banana and tomato (Reindl et al., 2002; Willerroider et al., 2003). The profilin concentrations of the cantaloupe and zucchini were also calculated as 11.83 and 2.5 ng/g of fruit weight, respectively.

In peach, watermelon and kiwi, the profilin concentration was below the measurable threshold. This may be due to a low content of profilin in these fruits. However, it could be resulted from lower binding ability of profilins of these fruits with the capturing and/or detection antibodies. In consistent with this notion, our previous study showed that immuno-reactivity of melon profilin corresponds to the highly conserved conformational epitopes, which is not predictable by the sequence similarity (Sankian et al., 2005).

It is suggested that application of a developed ELISA for measurement of allergen concentration in fruits should be validated by using a purified natural or recombinant allergen from the same fruit as standard antigen. In future, this method may be helpful to prepare standard allergenic extracts from fruits in which the profilin is considered as a major allergen.

Acknowledgements

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