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ORIGINAL ARTICLE

# Cloning, Expression, Characterization, and Computational Approach for Cross-Reactivity Prediction of Manganese Superoxide Dismutase Allergen from Pistachio Nut

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## ABSTRACT

**Background:** Tree nut allergy is one of the common potentially life-threatening food allergies in children and adults. Recombinant food allergens offer new perspectives to solve problems of clinical and molecular allergology in diagnosis, research, and therapy of food allergies. So far, superoxide dismutase (s) has been identified as a panallergen and studied in different allergenic sources. Manganese Superoxide Dismutase (MnSOD) has also been reported in pistachio that may cause allergic reactions in atopic subjects. The aim of this study was to describe the cloning, expression, and purification of MnSOD from pistachio nut.

**Methods:** The pistachio MnSOD was cloned and expressed in *E. coli* BL21 (DE3) using a vector pET-32b (+). A recombinant protein was purified by metal precipitation. The protein immunoreactivity was evaluated using patients' IgE binding by means of ELISA and immunoblotting assays.

**Results:** The MnSOD gene from pistachio was successfully cloned and expressed in *E. coli*. The purified pistachio MnSOD was recognized by IgE in 10 (40%) out of the 25 sera tested. Our results also showed that this protein might trigger some cross-reactions toward IgE antibodies and thus could be considered as a panallergen.

**Conclusions:** For the first time recombinant manganese superoxide dismutase from nut source was expressed as a possible allergen. This pistachio allergen could be a possible basis for cross-reactivity with MnSOD from other sources.

## KEY WORDS

cloning, cross-reaction, Manganese Superoxide Dismutase (MnSOD), pistachio (*Pistacia vera*), recombinant allergen

## *INTRODUCTION*

Allergic reactions to tree nuts can be serious and life-treating.[1] Pistachio (*Pistacia vera*) belongs to the Anacardiacea family, which also includes mango and cashew. Historically, Iran has been the world's leading pistachio producer and United States of America is the second largest following Iran.[2] Tree nut allergy is considered among the most common food allergies.[1] Allergy to pistachio is fairly common in the province of Kerman which is the main pistachio cultivation region in Iran (unpublished data). Some patients are allergic to the nut; some to the tree pollen, and some others to both. So far, the major allergens have been identified in pistachio nut and characterized as Pis v 1, a 2S albumin; Pis v 2, a 11S globulin

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**Table 1** Clinical characteristics, specific IgE-reactivity and skin prick test responses of the selected patients with allergy to pistachio nut

| Patient No. | Age (years)/sex | Clinical characteristics† | Pistachio nut extract | |
|---|---|---|---|---|
| | | | Specific IgE (OD)‡ | Skin prick test (mm) |
| 1. | 22/M | E, SI | 1.1 | ND |
| 2. | 20/M | OAS, SI, C | 1.2 | 3 |
| 3. | 21/F | OAS, SI | 0.99 | 4 |
| 4. | 36/F | SI | 0.80 | 5 |
| 5. | 36/F | OAS, SI | 0.74 | 4 |
| 6. | 8/M | OAS, C | <0.3 | 3 |
| 7. | 31/M | OAS, SI | 0.68 | 5 |
| 8. | 24/M | G, V, R | 0.56 | - |
| 9. | 50/F | SI, G | 0.43 | ND |
| 10. | 32/F | SI, OAS | 0.57 | 6 |
| 11. | 40/F | SI, C | 0.66 | 5 |
| 12. | 44/M | R, SI | 0.45 | 4 |
| 13. | 35/M | R, SI | 0.57 | ND |
| 14. | 24/F | OAS | 0.32 | ND |
| 15. | 19/F | OAS, SI, C | 0.81 | 6 |
| 16. | 53/F | G, V, C | <0.3 | 3 |
| 17. | 50/M | G, V | <0.3 | - |
| 18. | 20/F | SI, OAS | 0.80 | 3 |
| 19. | 24/M | OAS, R, SI | 0.48 | 4 |
| 20. | 34/F | R, SI | 0.61 | 6 |
| 21. | 25/M | E, R, C | 0.59 | 10 |
| 22. | 21/F | G, OAS | <0.3 | ND |
| 23. | 29/M | G | <0.3 | - |
| 24. | 26/M | OAS | 0.45 | ND |
| 25. | 45/F | OAS, C, SI | 0.77 | ND |

†C, Cough; E, Eczema; R, Rhinitis; G, Gastrointestinal symptoms; SI, Skin itching; OAS, Oral allergy syndrome (OAS; defined as the onset of immediate oral itching with or without angioedema of the lips and oral mucosa); V, Vomiting. ND, Not determined.

‡OD, Optical density at 450 nm.

subunit; Pis v 3, a vicillin; Pis v 4, a manganese superoxide dismutase and Pis v 5; a 11S globulin subunit.[3,4,5]

Superoxide dismutase has been detected in a wide range of living things and implicated as being an essential defense against the potential toxicity of oxygen.[6] Due to the ubiquity of this enzyme family, the allergen could play a role in IgE cross-reactivity and polysensitization.[7] MnSOD was described as an allergen in *A. fumigatus* (Asp f 6) with cross-reactivity with the human MnSOD,[8] and also showed cross-reactivity with MnSODs from yeast, *Drosophila melanogaster and Escherichia coli*.[9] The MnSOD allergen from *M. sympodialis* and *Hevea brasiliensis* have been reported as well.[10,11] Immunolabeling of 1D and 2D membranes also demonstrated IgE-binding to a 23 kDa pistachio MnSOD by 16 out of 27 sera (59%).[5]

Recombinant DNA technology can help to produce pure allergens in large quantity and well-defined reproducibility to be used as diagnostic and therapeutic tools, as well as to design modified molecules that, while maintaining antigenic determinants, display reduced allergenicity.[7]

In this study we described the cloning, expression and purification of the pistachio MnSOD from pistachio nut and the immunological characterization of the recombinant allergen.

## *METHODS*

### PATIENTS AND SKIN PRICK TEST

Twenty five patients, thirteen women and twelve men were included in this study on the basis of a case history of pistachio nut allergy (Table 1). Three subjects who showed negative SPT responses and no specific IgE to pistachio nut extract were considered as negative controls. SPT with a wheal diameter >3 mm were considered positive compare with the results obtained with negative and positive controls. Oral allergy syndrome; gastrointestinal symptoms, and skin itching were the most prominent manifestations on ingesting pistachio nut. The sera were stored at -30℃ until use. A serum pool was obtained by mixing equal volume aliquots of the individual sera, and then was stored in aliquots at -30℃. The study protocol was ap-

proved by the Human Ethics Committee of the Avicenna Research Institute and written informed consent was obtained from all patients.

## PISTACHIO PROTEIN EXTRACT

Defatting was done using cold hexane (1/15 w/v) and shaking for 16 hours. Hexane was removed by suction and then phosphate-buffered saline (PBS, pH: 7.4) was added to the samples (1/10 w/v) and shaken for 8 hours. After centrifuging at 9000 g for 30 minutes, the supernatant of the mixture was dialyzed in PBS for 24 hours. The whole extraction procedure was performed at 4℃. Protein concentration was measured using the Bradford protein assay, with BSA as the standard protein.[12]

## ISOLATION OF RNA, PCR-AMPLIFICATION OF cDNA ENCODING MnSOD FROM PISTACHIO NUT

Pistachio nut was pulverized in the presence of liquid nitrogen with the pre-cooled mortar and pestle. Total RNA was extracted from nut basically by the method of Chomczynski and Sacchi.[13] First-strand cDNA was synthesized from 2 μg total RNA using a first-strand cDNA synthesis Kit (Fermentas, Ontario, Canada) with random hexamer primers. The pistachio MnSOD coding region was amplified with *Pfu* DNA polymerase (Fermentas, Vilnius, Lithuania), using two degenerate primers which were based on the sequence similarity between conserved regions of MnSOD among plants and without the 66 bp at the 5'-terminal coding for the signal peptide: The forward, 5'AAGGCGGCCGCA CACGTCCGTGGAHTKCAGA C-3', mimics the first eight codons and introduces an *Not*I restriction site (underlined); the reverse, 5'-TAT CTCGAG TTCNCYKGCATAYTTCC-3', mimics the last six codons and introduces an *Xho*I restriction site (underlined). PCR products were digested with *Not*I and *Xho*I (both from Fermentas, Vilnius, Lithuania) enzymes and ligated to *Not*I and *Xho*I digested expression vector pET-32b (+) (Novagen, Gibbstown, NJ, USA).

## CLONING PISTACHIO MnSOD IN pET-32b (+)

The predicted 603-bp PCR products were purified from agarose gel using DNA extraction Kit (QIAquick, Germany), digested with *Not*I and *Xho*I enzymes and ligated to *Not*I and *Xho*I digested expression vector pET-32b (+). The recombinant expression vectors provided a sequence coding for a thioredoxin (TrxA) partner, to increase the solubility, and two hexahistidyl (6×His) affinity tags at the 5' and 3' ends of pistachio MnSOD coding sequence. The resulting construct was transformed into TOP10 *E. coli* cells. Colonies were selected and analyzed on ampicillin LB agar. The fidelity of the cloned product was verified by sequencing.

## RECOMBINANT PROTEIN EXPRESSION AND PURIFICATION

For expression of pistachio MnSOD, the pET-32b (+)/pistachio MnSOD constructs were transformed in *E. coli* (DE3) competent BL21 Star cells (Invitrogen, USA) and protein synthesis was induced with 0.2 mM IPTG (isopropyl β-D-thiogalactoside) for 5 hours at 37℃. Bacteria were harvested by centrifugation (9000 g, 20 minutes, and 4℃). The pellet from a 100 mL bacterial culture was resuspended in lysis buffer A (50 mM Tris-HCl; pH 8.0, 10 mM NaCl, and 5 mM EDTA) and also 16 μl of 50 mg/ml lysozyme per gram of cells and placed in 37℃ water bath until solution became viscous, then subjected to three freeze-thaw cycles using liquid nitrogen (frozen 3 times in liquid nitrogen). After centrifugation (9000 g for 20 min at 4℃), the harvested pellet was resuspend in buffer B (20 mM $Na_2HPO_4$ pH 7.2; 20 mM NaCl, and 5 mM EDTA). Inclusion body pellet was recovered by centrifugation (9000 g, 10 min). The inclusion body pellet was washed two times with washing buffer (2M Urea, 100 mM Tris-HCl, pH 7. 0.5 mM EDTA, 2% (w/v) Triton-X100). The final wash conducted with washing buffer without Triton X-100. Washed inclusion bodies were dissolved in 8M Urea. Protein purification was conducted according to Ni-NTA purification system using denaturing condition of the commercial kit procedure (Invitrogen, USA). The purity of the recombinant protein was evaluated by SDS-PAGE at 12% acrylamide concentration followed by Coomassie brilliant blue staining. The protein concentration was evaluated by the method of Bradford.[12]

## PROTEIN REFOLDING

Denatured, unfolded protein samples in concentrated denaturant solution were dialyzed against a refolding buffer; hence, exposed to descending concentration of the denaturant. Denaturant concentration decreases with time to the concentration of refolding solvent. As the concentration of denaturant is decreased, the rate of folding into the intermediate and native structures increases.[14] Dialysis against descending concentration of denaturant was used. The unfolded protein sample at high denaturant concentration (6 M urea) is placed in dialysis bag and equilibrium with 4 M urea concentration. The PBS buffer (refolding solvent) was pumped into the dialyzing solvent with the rate of 20 ml per min. When the dialyzing solvent reached to 100 mM urea, the dialysis bag immersed in PBS and dialysis continued for another 8 hours.

## IMMUNOBLOTTING ASSAYS

Purified recombinant pistachio MnSOD (3 μg protein/well) and total extract of pistachio nut (30 μg protein/well) were subjected to reducing 12% (w/v) SDS-PAGE.[15] Separated protein bands by electropho-

resis were electro-transferred to Polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, MA, US).[16] After blocking with 2% BSA for 16 hours at 4℃, the blots were incubated for 16 hours at 4℃ with the sera of allergic patients individually. Several dilutions of human sera were tested to determine an optimal dilution for the assay. In an effort to increase sensitivity and decrease background, a 1 : 6 v/v dilution was chosen for this study. The blots were then incubated with anti-human IgE biotin-conjugated goat antibody (KPL, USA) (1 : 1000 diluted in PBS) for 2 hours at room temperature. After each step, washing was done 3 times with PBS along with shaking following one hour incubation with horseradish peroxidase streptavidin (BD Biosciences, MD, USA) (1 : 40000 diluted in PBS). Blots were incubated with Supersignal West Pico Chemiluminescent Substrate Kit (Pierce, USA) for 5 min, and binding antibodies were then visualized by chemiluminescence using G-Box gel documentation system (Syngene, Cambridge, UK). For inhibition experiment, 150 µl of a pooled serum consisting of 5 sera (Patient #1, 2, 3, 5, and 18) with IgE reactivity to pistachio nut extract, were mixed with 40 µl of recombinant pistachio MnSOD (containing 70 µg protein) and shook at room temperature for 3 hours. Incubated serum was used to assess the reactivity on PVDF membrane blotted with total extract of pistachio nut as described above.

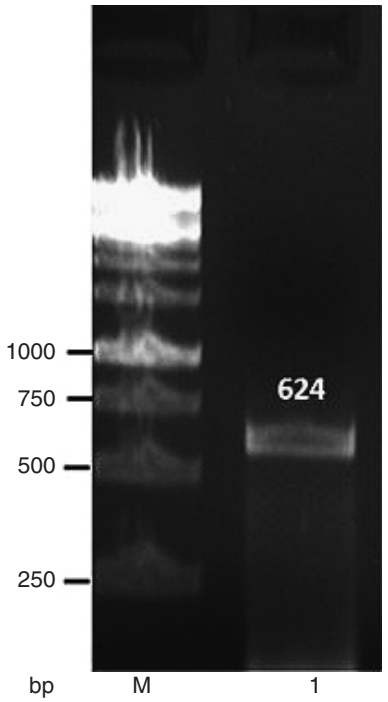


**Fig. 1** PCR products were subjected to agarose gel electrophoresis. Electrophoresis of the PCR-amplification of cDNA encoding pistachio MnSOD from nut. M: PCR marker (1 Kb, Fermentas, Lithuania), 1: PCR product.

### IgE ELISA

Specific ELISA was performed as described previously[17] with minor modifications. Polystyrene plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 0.1 ml/well of a coating buffer (15 mM Na2CO3 and 35 mM NaHCO3, pH 9.6) containing 10 µg/ml of recombinant pistachio MnSOD at 4℃ for 16 h. The amount of antigen to be used for plate coating was determined on the basis of preliminary titration experiments. The plates were washed with phosphate buffer saline (PBS) pH 7.2 containing 0.05% (v/v) Tween 20 (PBS-T). Remaining reactive sites on the solid phase were saturated with 200 µL 2% BSA (overnight at 4℃). After washing, prediluted 1 : 5 human sera were incubated on the plates overnight at room temperature. The plates were than washed with PBS-T and then incubated with biotinylated anti-human IgE (KPL, USA) (1 : 2000 v/v in PBS) for two hours at room temperature. The unbound antibodies were removed from the wells by washing, which was followed by incubation with HRP-linked streptavidin (BD Biosciences) (1 : 20000 v/v in PBS) for 45 minutes at room temperature. After visualization of the enzymatic activity with tetramethylbenzidine (TMB) as a substrate at 37℃ for 15 minutes, the reaction was stopped by the addition of 100 µl of 3N HCl, and absorption was measured at 450 nm. The assays were performed in duplicate.

### COMPUTATIONAL ANALYSIS AND HOMOLOGY MODELING OF PISTACHIO MnSOD

The deduced amino acid sequence of pistachio MnSOD was blasted in GenBank within the BLAST similarity search. Conserved residues between pistachio MnSOD and the other MnSOD proteins in *Hevea brasiliensis* (CAC13961), *Vitis vinifera* (ABX 79342), *Homo sapiens* (AAP36352), *Malasezia sympodialis* (CAD68071) and *Aspergillus fumigatus* (XP_ 752824) were mapped with sequence alignment using the ClustalW software.[18] Isoelectric pH, amino acid composition, and potential glycosylation sites of pistachio MnSOD were analyzed using GeneRunner software (http:www.generunner.com).

The homology model of pistachio MnSOD was generated using the coordinates from the MnSOD of *Homo sapiens* (PDB Number: 2ADQ) by the Internet server Swiss Model (http://swissmodel.expasy.org/SWISS-MODEL.html), as previously described.[17] The MnSOD of *Homo sapiens* has 58% sequence identity with the pistachio MnSOD.

Solvent-accessibilities were calculated on the final three-dimensional model of the MnSOD with the program NetSurfP.[19]

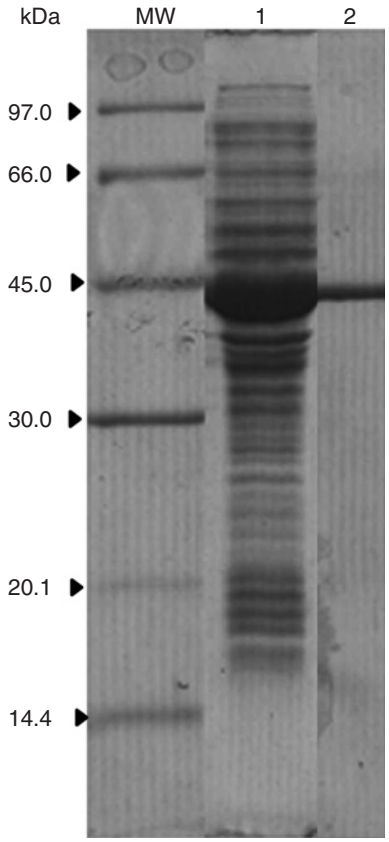


**Fig. 2** SDS-PAGE (12%) for analyzing of pistachio MnSOD expression in bacteria lysate with Coomassie blue staining, low molecular weight marker (MW) (Amersham, UK), an expressed 42 kDa band is seen after IPTG induction (lane 1), sample of purified recombinant pistachio MnSOD (lane 2).

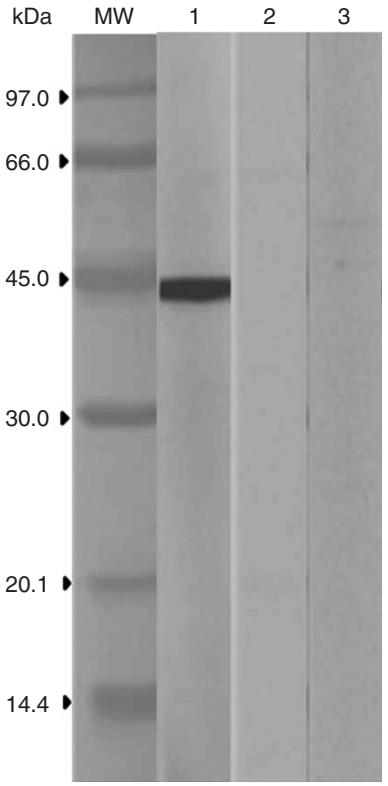


**Fig. 3** Immunoblotting of pistachio MnSOD of a pool of 5 representative sera positive to pistachio MnSOD (lane 1); negative controls were represented by a non atopic serum on pistachio MnSOD (lane 2), and by the pistachio MnSOD-positive pool on a bacterial lysate obtained from the BL21 strain with pET32b+ but without pistachio MnSOD, containing all of the tags (lane 3). Low molecular weight marker (MW), (Amersham).

## *RESULTS*

### AMPLIFICATION, CLONING AND SEQUENCING OF cDNA CLONING FOR PISTACHIO MnSOD

The sequence of the pistachio MnSOD has been deposited in GenBank under the accession number: EF 470980. Amplification of pistachio nut cDNA produced a main fragment of approximately 603 bp (Fig. 1). The sequence of inserted fragment in recombinant plasmid was identical with the pistachio MnSOD sequence reported in GenBank.

### EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF RECOMBINANT PISTACHIO MnSOD

Following proliferation of the clones selected, plasmids were extracted and successfully transformed into *E. coli* strain BL21 (DE3) for expression. The expression of the pistachio MnSOD region was carried under the control of the T7 promoter. A fusion form of pistachio MnSOD was expressed in *E. coli* BL21 (DE3) after induction using IPTG. SDS-PAGE analysis showed that the fusion protein was expressed mainly in an insoluble form, with a molecular weight around 42 kDa, as determined by coomassie brilliant

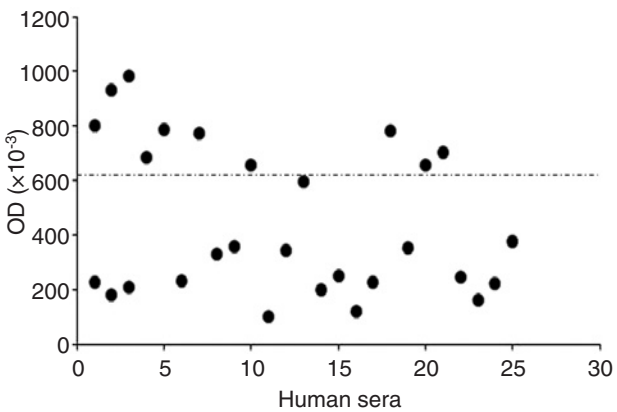


**Fig. 4** ELISA IgE screening of 25 pistachio-allergic subjects on the pistachio MnSOD.

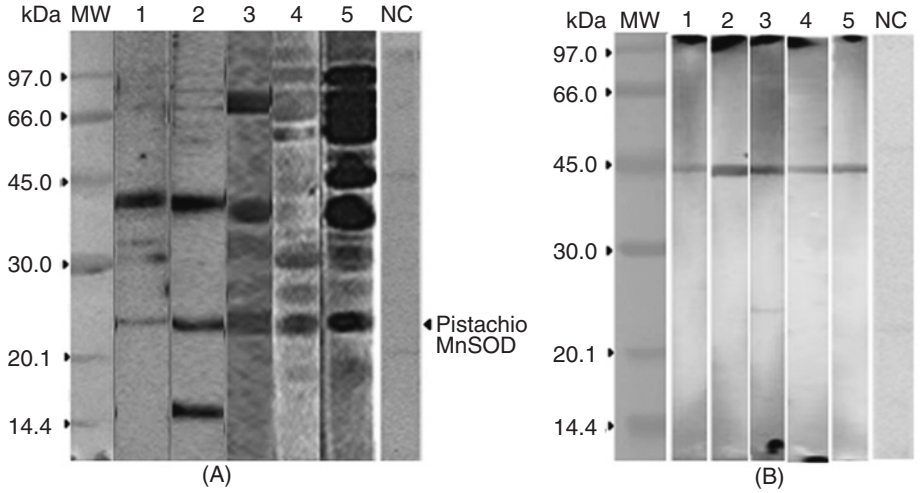


**Fig. 5** (**A**) IgE-Immunoblot of pistachio MnSOD in total extract of pistachio nut using pistachio allergic patients' sera; Low molecular weight marker (MW) (Amersham); Probed with individual pistachio allergic patients' sera (lanes 1-5); negative control (lane NC); (**B**) IgE-immunoblotting analysis of purified recombinant pistachio MnSOD using 5 pistachio allergic patients. Low molecular weight marker (Amersham) (MW); blotted recombinant pistachio MnSOD probed with individual pistachio allergic patients' sera (lanes 1-5); negative control (lane NC).

blue-stained SDS-PAGE (Fig. 2). After metal precipitation, recombinant pistachio MnSOD showed a major single band with an apparent molecular mass of 42 kDa of the recombinant allergen. The final concentration of the purified recombinant protein was about 1.8 mg per mL (Fig. 2). Pooled sera from five allergic patients to pistachio nut were tested for IgE reactivity to purified recombinant pistachio MnSOD after SDS-PAGE along with bacterial lysate obtained from the BL21 strain with pET-32b (+) and without pistachio MnSOD (Fig. 3). Immunoblotting assessment of bacterial lysate obtained from the BL21 strain with pET $32b^{+}$ and without pistachio MnSOD by the same pooled serum as used for the immunobot of pistachio MnSOD, confirmed the presence of tags at the ends of recombinant protein had not reactivity with IgE.

## SPECIFIC IgE REACTIVITY

In order to evaluate whether the recombinant protein pistachio MnSOD was able to react with human IgE, an ELISA screening was performed on a sample population of 25 pistachio nut allergic subjects. The results showed that 10 out of the 25 sera tested (40%) recognized recombinant pistachio MnSOD (Fig. 4). In this screening, a positivity cut-off value (OD 0.6) was calculated on the basis of the mean of OD values obtained with 3 normal sera plus 3-fold its standard deviation. To further characterize the pattern of IgE recognition, IgE immunoblotting was performed. Immunoblotting was tested using purified recombinant pistachio MnSOD after SDS-PAGE. Pistachio MnSOD 42 kDa protein was recognized by MnSOD

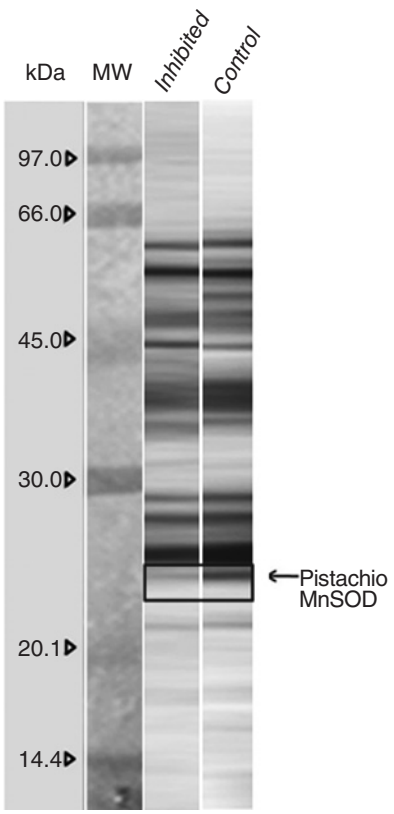


**Fig. 6** IgE reactivity inhibition of pooled serum of allergic subjects to pistachio MnSOD. Blotting of pistachio nut extract on PVDF membrane showed IgE reactivity of allergic sera with 23 kDa protein was inhibited after incubation with pistachio MnSOD in total extract of pistachio nut.

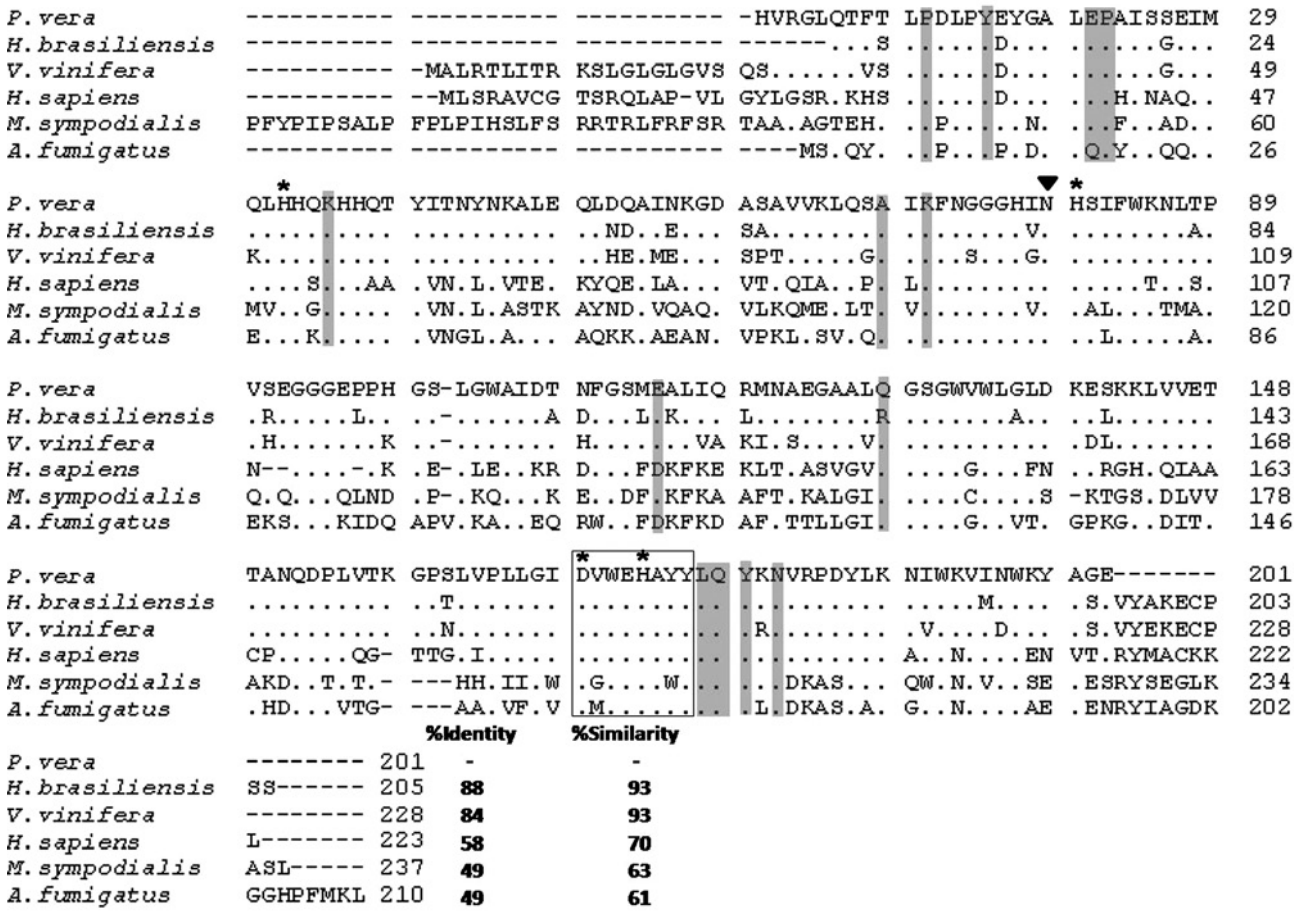


**Fig. 7** The comparison of cloned pistachio MnSOD deduced amino acid sequence (without signal peptide) with five other MnSOD from different species previously deposited in database banks. Hevea brasiliensis (CAC13961), Vitis vinifera (ABX 79342), Homo sapiens (AAP36352), Malasezia sympodialis (CAD68071) and Aspergillus fumigatus (XP_752824). The amino acid sequence identity and similarity of cloned pistachio MnSOD with other MnSODs are indicated at the end of each amino acid sequence. The full-length of pistachio MnSOD consists of 230 amino acids (ABX79342) including a putative signal peptide of 22 residues. The potential N-glycosylation site (N79) is indicated by arrowhead. The residues involved in binding to ligand (manganese) (His32, His80, Asp169, and His173) are showed with asterisks. The conserved residues (Pro11, Tyr15, Glu21, Pro22, Lys35, Ala69, Lys71, Glu114, Gln128, Leu178, Gln179, Tyr180 and Asn182) which were suggested as IgE-binding sites of MnSODs are shaded in gray (10). The consensus features of MnSOD corresponding to the peptide 169-176 are boxed.

reactive sera from five individual pistachio allergic patients (Fig. 5).

Total extract of pistachio nut was separated by SDS-PAGE and blotted onto PVDF. IgE-binding of a pooled serum, including five pistachio-allergic patients was studied after pre-incubation with purified recombinant pistachio MnSOD (inhibitor). The inhibition experiment showed a considerable decrease in reactivity of IgE in around 23 kDa protein after inhibition with recombinant pistachio MnSOD in total extract of pistachio nut (Fig. 6).

## COMPUTATIONAL ANALYSIS OF PISTACHIO MnSOD SEQUENCE

The deduced amino acid sequence pistachio MnSOD data was subjected to a BLAST similarity search and the percentage identities were determined by comparison of the amino acid sequences after multiple sequence alignment carried out using the ClustalW software. Sequence alignment reveals pistachio MnSOD shares sequence homology with MnSOD from *H. brasiliensis A*, *H. sapiens*, *M. sympodialis* and *A. fumigatus* which have been already identified as allergens, It has highly sequence identity (84%) with MnSOD from *Vitis Vinifera* as well (Fig. 7). Furthermore, sequence alignment reveals some amino acids residues that are conserved between MnSOD from different sources.

The cloned pistachio MnSOD from the present study encodes a protein spanning 201 amino acid residues with an isoelectric point of approximately pH 6.61. A single potential N-glycosylation site was

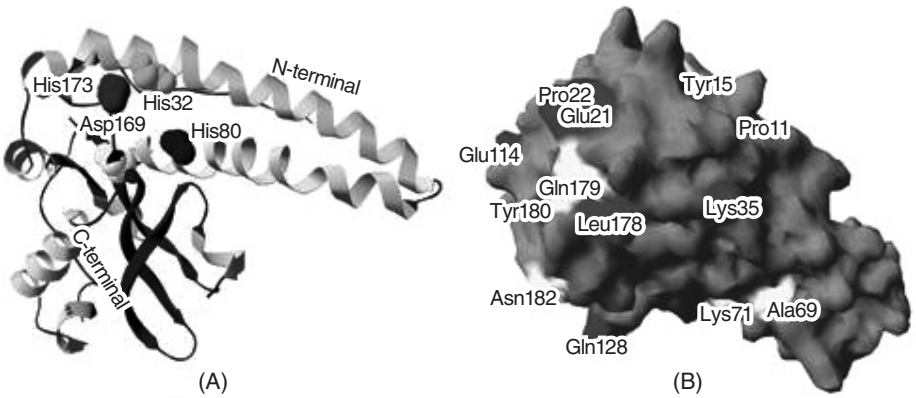


**Fig. 8** Homology model of pistachio MnSOD (**A**), Ribbon representation of the structure of the pistachio MnSOD modeled according to the three-dimensional structure of the MnSOD of Homo sapiens (PDB number: 2AADQ). The Manganese ligands are His32, His80, Asp169 and His173 (**B**). The molecular surface of pistachio MnSOD showing highly conserved potential IgE-binding amino acid residues. Solvent exposed amino acid residues are Pro11, Glu21, Pro22, Lys35, Glu114, Gln128, Leu178, and Tyr180; Moreover, those are not recognized as solvent exposed amino acid residues are Tyr15, Ala69, Lys71, Gln179 and Asn182.

found at N-79 (NHSI). In addition, Manganese ligand-binding sites (His-32, His-80, Asp-169, and His-173) corresponding to the MnSOD of cloned pistachio were all well conserved in the aligned MnSOD sequences and consensus features of MnSOD (DVWEHAYY) was observed from residues 169-176. The results demonstrated that residues K35, A69, K71, E21, P22, E114, Q128, L178, Q179, Y180, N182, P11, and Y15, which have been suggested as IgE-binding sites of MnSOD,[11] were conserved in the cloned pistachio MnSOD.

### POTENTIAL CROSS-REACTIVE IgE BINDING EPITOPES

Three-dimensional structure of the pistachio MnSOD is shown in Figure 8. The models were evaluated in terms of stereochemical and geometric parameters such as bond lengths, bond angles, torsion angles, G-factor and packing environment, and it was found to satisfy all stereochemical and geometric criteria. No residue was located in the disallowed regions of the Ramachandran map. After energy minimization of the models, the overall conformational energy of comparative models of the pistachio MnSOD was -1120 kcal/mol. Main-chain Cα atoms of 2ADQB and the pistachio MnSOD superimpose with an RMS deviation of 0.40Å.

In this study, we used NetSurfp software to predict the solvent accessibility of amino acid residues of pistachio MnSOD.[19] The results showed there were eight solvent-exposed amino acids corresponding to positions P11, E21, P22, K35, E114, Q128, L178, and Y180 in this protein (Fig. 8B).

## *DISCUSSION*

For the first time recombinant manganese superoxide dismutase from a nut source was expressed as a possible allergen. Pistachio MnSOD has been identified as a major pistachio allergen.[5] The pistachio MnSOD gene, like other MnSODs, starts with a signal peptide,[20] showing a consensus sequence typical for transport peptides. As this study is focused on the IgE-binding capacity of allergens, we expressed the MnSOD starting from the sequence encoding the putative mature protein. The selected coding sequence was cloned and recombinant protein expressed by pET-32b (+)/pistachio MnSOD vector in *E. coli* BL21. Expression of recombinant MnSOD from *Nelumbo nucifera*, also reported by pET-32a vector which is similar to pET-32b (+).[21,22] The successful recombinant bacterial expression of insoluble proteins requires denaturation and subsequent refolding in order to obtain the correct conformational, which is especially important for the IgE-binding reactivity of recombinant allergens. In order to confirm that the purified MnSOD was correctly folded and bound to IgE as the natural counterpart in pistachio extract, specific ELISA and immunoblotting assays were carried out.

While previous study showed that IgE-binding frequency with MnSOD pistachio was 59%,[5] our screening revealed 40% of pistachio nut allergic patients were positive against MnSOD using ELISA screening. The difference between the results might be related to different population patients under these different studies. Therefore, the results showed that the

produced recombinant protein had the IgE reactivity to patients' sera, as well. Furthermore, a nearly complete inhibition of IgE-binding to natural MnSOD in pistachio extract, around 23 kDa, suggesting the presence of monomer of this protein in pistachio while both *Aspergillus fumigatus* and *Homo sapiens* MnSODs were suggested as a tetrameric structure with high similarity in the overall.[23] Structure analysis of all known MnSOD revealed the metal ion is bound by four invariant protein ligands: one aspartate and three histidines.[23] In the present study, the conserved motif is also found in MnSOD isolated from pistachio showing similar protein structure. Moreover, sequence alignment of the MnSODs from pistachio together with the MnSODs from *Hevea brasiliensis*, *Homo sapiens*, *Malasezia sympodialis* and *Aspergillus fumigatus* revealed 62 identical amino acids between theses known allergens. Results of other study showed sequence identities of *Hevea brasiliensis* MnSOD with *A. fumigatus* and *Homo sapiens* MnSODs (48.5% and 58.3%, respectively), meanwhile, the IgE-binding of patient's serum sensitized to *Hevea brasiliensis* MnSOD recognized *A. fumigatus* as well as *Homo sapiens* MnSOD.[24] This could be due to the structural model of the different MnSODs, which displays the conserved residues scatter over the surface of the MnSOD molecules.[25] The availability of the crystal structure of the human MnSOD[26] allowed defining in more details the possible residues likely to be involved in IgE-mediated cross-reactivity by molecular modeling (Fig. 8). The three-dimensional structures of *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD were also modeled using homology-based approaches.[25]

The sequence similarities between MnSODs of different organisms to assign identical residues as IgE binding candidates showed that P11, Y15, E21, P22, K35, A69, K71, E114, Q128, L178, Q179, Y180, and N182 residues were well conserved.[11] Our findings also confirmed that these residues were conserved in the pistachio MnSOD as well. However, only identical amino acids of the different MnSODs exposed to the solvent are expected to play a key role for binding of cross-reactive IgE antibodies.[25] The results of solvent accessibility of pistachio MnSOD using NetSurfp software demonstrated only P11, E21, P22, K35, E114, Q128, L178, Y180, and N182 residues exposed to solvent and could be considered as potential IgE binding sites. Taken together, this might be an explanation for the cross-reaction capability of pistachio MnSOD with the MnSODs from unrelated organisms. More experimental work will be required to characterize the epitopes involved in cross-reactivity among MnSODs. Due to the widespread presence of this enzyme in the living cells, its presence is also expected in other nuts. On the other hand, highly sequence identity (84%) and the conserved IgE-reactive sites between MnSOD from pistachio and *V. Vinifera* suggests that this protein can be considered as a new allergen in the later one. Further studies need to verify the allergenicity of MnSOD in *V. Vinifera*.

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