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

Original article

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Cloning and Expression of Cyclophilin from *Platanus orientalis* Pollens in *Escherichia coli*

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Abstract

Background: Allergy is a clinical disorder affecting the human population with wide geographical distribution. *Platanus orientalis* (*P. orientalis*) trees are planted in many countries and their pollen causes allergic reactions.
Cyclophilin has recently been identified as one of the most important allergens of *P. orientalis* pollen. We aimed to clone and purify this allergen in *Escherichia coli* for further studies and therapeutic and diagnostic purposes for allergy to *P. orientalis.*
Methods: RNA was extracted from *P. orientalis*. A full-length fragment encoding cyclophilin was prepared by polymerase chain reaction amplification of the first-strand cDNA synthesized from *P. orientalis* RNA. The cDNA was inserted into the pET32b (+) vector, and the construct transformed into *E. coli* Top10 and BL21 cells. The expressed protein was purified by the CuSO4 method.
Results*:* The cDNA for the cyclophilin of *P. orientalis* pollen was cloned, and a specific reactivity of recombinant cyclophin was confirmed by immunoblotting using sera from patients allergic to *P. orientalis* pollen.
Conclusion: The recombinant cyclophilin has a potential for immunologic assays for evaluation of allergy to *P. orientalis* pollen**.**

Keywords: Allergy, Cloning, Cyclophilin, *Escherichia coli*, *Platanus orientalis*, Pollen, Recombinant allergen

Introduction

Allergic rhinitis is a global problem, and can be seasonal or perennial. Seasonal allergic rhinitis, also known as hay fever, is most commonly caused by outdoor allergens including grasses, weeds, and tree pollens (1-2). The symptoms of pollen allergy or pollinosis include sneezing, itchy and watery eyes, scratchy throat, runny nose, and skin rashes (3-4).

Oriental Plane trees, such as *Platanus orientalis* (*P. orientalis*), are an important cause of airborne allergens in cities of southwest Asia, southeast Europe, and the USA. In recent decades, Oriental Plane trees (especially *P. orientalis*) have been cultured along streets and in parks of most cities of Iran. The prevalence of allergy to *P. orientalis* pollen has been estimated to be 43.6% in Mashhad, the second biggest city of Iran (5).

Diagnosis of type I allergy essentially depends on the availability of defined allergens, which can be provided by recombinant technology.

One of the newly-defined allergens of *P. orientalis* pollen is cyclophilin, which belongs to the cyclophilin family. Cyclophilins constitute a family of cytosolic proteins that plays a pivotal role in protein folding through enzymatic catalysis of the peptidyl-prolyl cis-trans isomerization reaction (6). These allergen

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Received: Jul 2, 2012; Accepted: Aug 20, 2012

families, formally termed pan-allergens, represent proteins sharing a high degree of sequence homology (7).

Here we report cloning, production, and serological studies of cyclophilin from *P. orientalis* pollen. The cyclophilin gene was cloned in *E. coli* with the aim of establishing an easy system for expression and purification of this 18-kDa allergenic protein, using the pET32b (+) vector. Recombinant cyclophilin may prove to be a useful tool for improving diagnosis, treatment, and basic research in allergy to *P. orientalis* pollen.

Materials and Methods

Pollen collection

Pollens were collected from *P. orientalis* trees during pollination. After removing impurities and dust particles by sieving, the refined pollens were stored in a plastic bag in the freezer.

RNA extraction

Total RNA from *P. orientalis* pollens was extracted by a modified guanidinium isothiocyanate method (Chomczynski) (8). The final pellet was washed with ethanol (70% in DEPC-treated water), air dried, and dissolved in 20 µl of DEPC-treated water.

To remove possible DNA contamination, the extracted sample was subjected to DNase and purified by the phenol-chloroform method. The quality of purified RNA was evaluated by gel electrophoresis using 1% agarose. The RNA concentration and purity were determined by spectrophotometry at 260 and 280 nm (A 260/A 280).

cDNA synthesis and PCR amplification of cDNA encoding cyclophilin

The cDNA was synthesized from 2 µg of total RNAusing a first-strand cDNA synthesis Kit (Fermentas, Lithuania) with a random hexamer as the primer.

The cyclophilin coding region was amplified with *Pfu* DNA polymerase (Fermentas, Lithuania), using specific primers: forward primer, 5′-AACCC(T/C)AAGGT(C/T/G)TTCTT(C/T)GA -3’, and reverse primer, 5′-GAG(T/C)TGACC(A/G)CAGTCGGC(G/A)A -3′. These primers were designed based on reported protein sequences of cyclophilin.

The PCR was hot-started at 95 °C for 5 min followed by 5 cycles, each consisting of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, then 35 cycles, each consisting of 45 sec at 95 °C, 45 sec at 57 °C, and 45 sec at 72 °C, and finally, 3 min at 72 °C.

Adenosine was added to the 3' end of the PCR-amplified products by incubating the PCR product with 2 units of *Taq* polymerase at 72 ºC for 30 min.

PCR samples were visualized using fluorescence photomicrography of an ethidium bromide-stained 2% agarose gel. After electrophoresis of the PCR product on low-melting gel 1% agarose, the PCR product was purified using the *AccuPrep*™ Plasmid Mini Extraction kit (Bioneer, Korea)

Cloning

The PCR product was cloned into PTZ 57 R/T. The resulting construct was transformed into competent TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA) with Transformation kit (Fermentas). The plasmids were sequenced and, based on the obtained sequence, new primers for cloning in pET32b+ plasmid were designed. The forward primer was 5’-ATTAGCGGCCGCAACCCTAAGGTTTTCT TCGACA -3’ and the reverse primer was5’-ATACTCGAGGAGCTGACCGCAGTCGGC AA -3’. The new PCR utilized*Pfu* polymerase and the PTZ 57 construct containing cyclophilin as the DNA template.

The PCR was hot-started at 95 °C for 5 min followed by 35 cycles, each consisting of 45 sec at 95 °C, 45 sec at 59 °C, and 45 sec at 72 °C, and finally, 10 min at 72 °C.

The PCR product and pET32b+ were sequentially digested with *Xho* I and *Not* I restriction enzymes and purified. The digested and purified PCR product and plasmid were ligated. The ligation was transformed into competent *E. coli* Top10 cells. After confirmation of correct constructs with sequencing and restriction digestion analysis, constructs were transformed

into expressing host cells, *E. coli* BL21 (DE3) pLysS.

Expression and purification of cyclophilin

For expression of cyclophilin, protein synthesis was induced by adding 0.2 mM IPTG (Isopropyl β-D-thiogalactoside) to transformed cells in LB medium at 37 °C. Subsequently, the cells were harvested by centrifugation (3000 x g, 15 min) at 4 °C, and purification of cyclophilin was performed by the precipitation method using CuSO4 (9).

Immunoblotting

Purified cyclophilin was subjected to reducing 12% (w/v) SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA).

After blocking with 2% BSA, the blots were exposed for 3 h at room temperature to sera (diluted 1:5 in PBS) from individual patients allergic to *P. orientalis*, confirmed by the skin prick test. Pooled serum from non-allergic patients was used as the negative control. Using anti-human IgE biotin-conjugated goat antibody (Sigma, USA) (1:1000 diluted in BSA 1%) and horseradish peroxidase-streptavidin (1:20000 diluted), the observed antigen-antibody reactions were documented by G-BOX Chemi-Doc (Syngene, Cambridge, UK) after exposure.

Results

RNA extraction

Gel electrophoresis showed good-quality RNA, with visible 25S and 18S bands, and no DNA (Fig. 1). The A260/A280 ratio was 1.95. The final RNA concentration was 2000 µg/mL, and60.3 µg of RNA was obtained from 0.1 g of pollen.

Amplification and cloning of cDNA coding for cyclophilin

A 509-bp fragment was obtained after PCR amplification of *P. orientalis* pollen cDNA (Fig. 2).

After cloning this PCR product into the pET32b+ vector, analysis of the purified plasmids on gel agarose showed the expected size of 6408 bp. Restriction digestion confirmed the integrity of the plasmid construct. Consistency of cloning was determined by sequencing (result not shown).

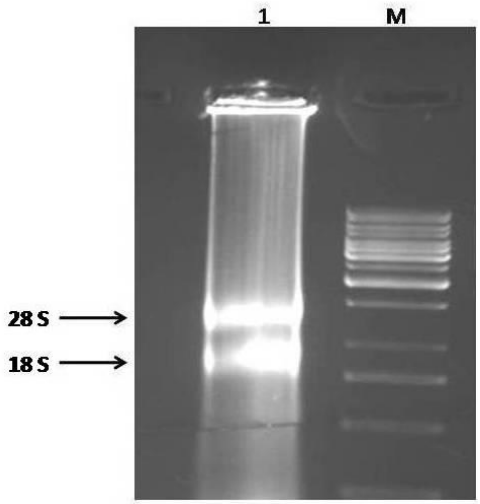

Fig. 1. Agarose gel electrophoresis of total RNA extracted from *P. orientalis* pollen. Lane 1: *P. orientalis* pollen RNA; Lane M: 1 Kb ladder (Fermentas).

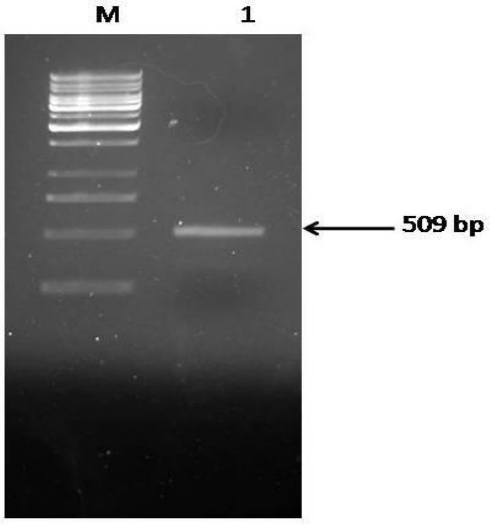

Fig. 2. Agarose gel electrophoresis of cyclophilin PCR product. Lane 1: PCR product of cyclophilin-specific primers and *P. orientalis* pollen cDNA; Lane M: 1 Kb ladder (Fermentas).

Expression and purification of recombinant cyclophilin

Following proliferation of the clones selected, plasmids were extracted and transformed into *E. coli* strain BL21 (DE3) for expression. SDS-PAGE analysis showed that the fusion protein was expressed mainly in soluble form, with a molecular weight of 35 kDa, as determined by Coomassie Brilliant Blue-stained SDS-PAGE (Fig. 3).

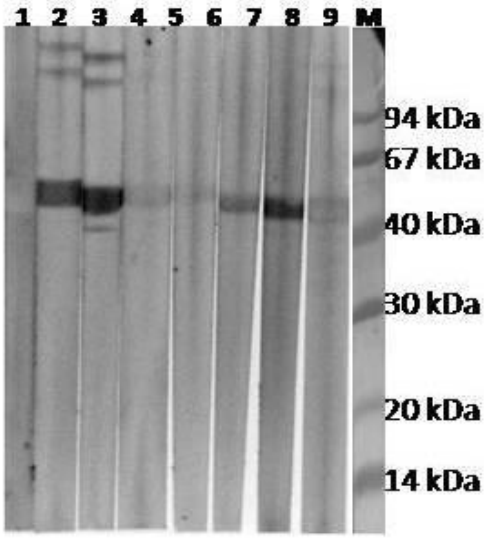

Fig. 3. SDS-PAGE of purified recombinant cyclophilin. Purified protein was electrophoresed by SDS-PAGE in 12% acrylamide gel and stained with Coomassie Brilliant Blue. Lane 1: purified cyclophilin; Lane M: protein marker.

After metal precipitation, recombinant cyclophilin showed a major single band with an apparent molecular mass of 35 kDa.

Immunoblotting

IgE reactivity of serum from individual patients allergic to *P. orientalis* pollen was tested against purified recombinant cyclophilin after SDS-PAGE. The specific reactivity was confirmed by immunoblotting (Fig. 4).

Discussion

P. orientalis (Oriental Plane) is found in the streets of most cities in Iran (10-11). Platanus pollen has been defined as a major contributor to pollinosis symptoms during March and April (10). The prevalence of positive skin prick tests to *P. orientalis* pollen extract has been identified as about 43% in Mashhad, Iran (11).

Allergens of *P. orientalis* pollen were identified using immunoblotting with sera of allergic subjects and spectrometry. Nine proteins with molecular weights ranging from 12-68 kDa have been detected. Pla or 1 (18 kDa), Pla or 2 (42 kDa), Pla or 3 (12 kDa), Pla or 4 (28 kDa) and cyclophilin from *P. orientalis* were introduced as the main allergens (5, 11-12).

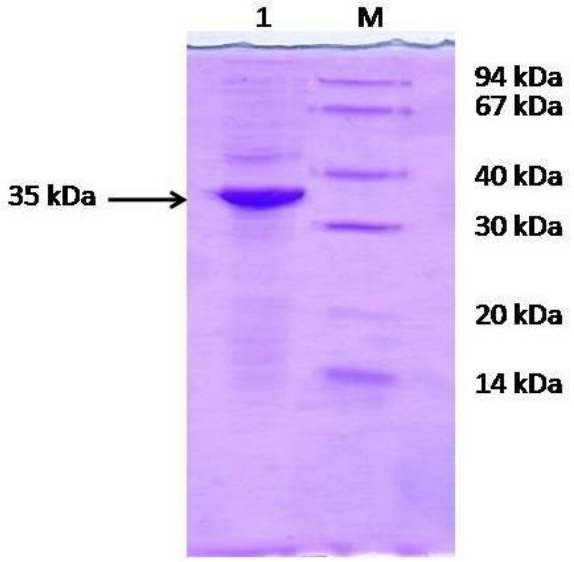

Fig. 4. Western blot analysis of purified cyclophilin. Purified protein was electrophoresed by SDS-PAGE in 12% acrylamide gel and subjected to Western blot analysis. Lane M: protein marker; Lane 1: negative control immunoblotted with pooled sera from non-allergic subjects. Lanes 2 to 9: immunoblotted with serum from individual patients allergic to cyclophilin

In recent studies, a *P orientalis* pollen 18-kDa IgE-binding component of was identified by mass spectrometry. Peptide mass fingerprinting revealed that the allergenic protein component at 18 kDa belongs to the cyclophilin family (12). Cyclophilins have also been identified as allergens in other plants and moulds such as *Betula verrucosa*, carrot, *Psilocybe cubensis*, pumpkin, and *Aspergillus fumingatus*(6-7, 12-15).

In this study, due to fusion of cyclophilin gene to histidine and thioredoxin tags, the size of the produced protein was increased. These tags had no apparent effect on immunoreactivity of patients' sera with cyclophilin.

Recently, purified native and recombinant allergens have proved to be safe and reliable tools in the diagnosis and therapy of allergic disorders (16-17).

In this research, cyclophilin from *P. orientalis* pollen was cloned and an 18-kDa cyclophilin protein was purified.

Acknowledgements

This study was supported from the Research Council of Mashhad University of Medical Sciences (MUMS), Mashhad, Iran.

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