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Cloning, expression, and purification of recombinant major mango allergen Man i 1 in *Escherichia coli*

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ABSTRACT

In recent years, the number of people around the world who suffer from fruit allergies has increased. Mango can induce anaphylaxis, and two major mango allergens have been identified – Man i 1 and Man i 2. Apart from their molecular weights and pI values, no other information about them is known. This work identifies the DNA and amino acid sequences of Man i 1 and constructs an expression system for recombinant Man i 1 (rMan i 1). Firstly, 3′ and 5′ RACE assays were used to identify the cDNA fragment of Man i 1. Subsequently, the full length of Man i 1 cDNA was inserted into a pET-21a(+) vector, and the inserted plasmid was transformed to *Escherichia coli* BL21 (DE3) to express rMan i 1. The conditions for the expression of rMan i 1, including IPTG concentration, induction temperature, and induction time, were optimized. The highest amount of soluble rMan i 1 was obtained after induction with 0.1 mM IPTG at 16 °C for 20 h. The His-tagged rMan i 1 was purified using Ni-NTA agarose and its identity was verified using an anti-histidine antibody and the serum of a mango-allergic person. Additionally, rMan i 1 was identified as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and shared 86.2% identity in amino acid sequence of GAPDH from wheat. Finally, an *E. coli* expression system of rMan i 1 was established, with the potential to be used in immunotherapy against mango allergy or the development of assays for detecting the residues of mango allergens.

1. Introduction

Mango (*Mangifera indica* L.) is an economically favorable fruit that is planted in the tropical and subtropical areas of Asia and Africa. Mango is often processed into a variety of products, such as juice, shakes, ice cream, jams, pickles, dried goods, cookies or cakes [1]. The demand for mango has recently increased possibly owing to its bright golden color, sweet taste, favorable flavor, and high nutritional value. For instance, mango contains high levels of polyphenols, carotenoids, and ascorbic acid. In particular, β-carotene accounts for 48–84% of mango flesh by dry weight [2]. Although mango is a popular fruit globally, it is also an important allergic source and can induce a life-threatening anaphylactic reaction [3,4]. Some people are allergic to not only mango flesh, but also its pollen and seeds [5,6]. Regarding prevalence, mango-allergic populations constitute 0.3%, 6%, and 16% of all food-allergic patients in Switzerland, France, and Thailand, respectively [7–9]. This variation in the prevalence of mango allergy may be associated with differences of cultivars, eating habits, and heredity.

The symptoms of mango allergy usually present in the gastrointestinal, skin and respiratory systems, and include diarrhea, itchy mouth or throat, swollen lips, erythema, urticaria, rhinoconjunctivitis, cough and dyspnea [10,11]. Mango-allergic reactions can be generally identified as manifesting immediate hypersensitivity and delayed hypersensitivity. The former occur with a few minutes to 30 min of ingestion, while the latter may occur after 8–12 h of exposure, with the allergic reactions reaching a peak at 24–72 h, before slowly decaying [12]. Mango proteins of 14, 16, 18, 20, 25, 27, 30, 37, 40, 43, 50, 60, and 67 kDa have been recognized using sera from mango allergic patients, and two have been identified as major allergens, named Man i 1 (40 kDa) and Man i 2 (30 kDa) [11,13–15]. The amino acid sequences of Man i 1 or Man i 2 have not been determined.

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Escherichia coli, an enteric bacterium, is extensively used as an expression host for the production of recombinant protein, because its genetic and physiological characteristics are well known. Additionally, the expression system of *E. coli* has several favorable characteristics for producing large-scale recombinant proteins, including inexpensive carbon sources for the growth, rapid bacterial growth and easy handling [16]. However, the *E. coli* expression system is associated with such issues as a lack of post-translational modification and the degradation of native protein [17]. Moreover, the expressed proteins are sometimes aggregated as inclusion bodies, which must undergo complicated processes, such as unfolding, dialysis, and refolding, to yield soluble and active proteins. Therefore, optimizing entire isolation process from inclusion bodies takes time and the process commonly does not provide high yields [18,19]. Thus, an efficient way is to optimize the expression conditions to obtain directly a soluble and active recombinant protein, instead of a denatured recombinant protein form the inclusion bodies.

This study determines the amino acid sequence of Man i 1 and optimizes the expression and purification system for obtaining a recombinant Man i 1 (rMan i 1). Firstly, the full-length cDNA sequence of Man i 1 was determined using the 3′ and 5′ RACE assays, cloned into the pET-21a(+) vector; and transformed to *E. coli* BL21 (DE3) for expression. To obtain a soluble rMan i 1, the expression conditions of temperature, induction time, and IPTG concentrations were optimized. Finally, the soluble rMan i 1 was purified using Ni-NTA resins and identified using immunoassays with an anti-histidine antibody and a serum from mango-allergic patient.

2. Materials and methods

2.1. Materials

Mangos of different varieties (Irwin, Yuwen, Jinhuang, Keitt, and Tu) were bought from local markets in Taiwan. Bovine serum albumin (BSA), β-Nicotinamide adenine dinucleotide (NAD^+), DL-Glyceraldehyde 3-phosphate, phenyl methyl sulfonyl fluoride (PMSF), isopropyl β-D-1-thiogalactopyranoside (IPTG), Triton X-100, Tween 20, chloroform, β-mercaptoethanol, diethylpyrocarbonate (DEPC), and Coomassie Brilliant Blue R250 (CBB) were purchased from the Sigma-Aldrich Corporation (USA). Ethylenediaminetetraacetic acid (EDTA), triethanolamine, polyvinyl polypyrrolidone (PVPP), pET-21a(+) vector, and *E. coli* BL21 (DE3) were purchased from the Merck Millipore Corporation (Germany). TRIzol®reagent, pCR™II-TOPO® Vector, 5′ and 3′ rapid-amplification of cDNA ends (RACE) kit, a cDNA synthesis kit, and Taq DNA polymerase were purchased from Invitrogen (USA). The restriction enzymes and T4 DNA ligase were bought from New England Biolabs (USA). A mango allergic patient was recruited by National Taiwan University Hospital (NTUH) and the other three patients were recruited by Taipei Veterans General Hospital (TVGH) and the sera used in this study were obtained therefrom. Luria-Bertain (LB) broth and agar were purchased from Acumedia Manufacturers, Inc. (USA).

2.2. Mango protein extract

The method of Singh et al. for extracting mango protein was slightly modified for use in this study [20]. Briefly, mango fruits were cut into small pieces, frozen with liquid nitrogen, and immediately ground using a grinding apparatus (IKA, Germany). The fine mango powders were mixed using the extraction buffer (20 mM sodium phosphate buffer, 20 mM EDTA, 20 mM cysteine, 0.5% Triton X-100, 0.1% β-mercaptoethanol, 1 mM PMSF, and 66 mg PVPP, pH 7.4) at 4 °C for 20 min with gentle stirring. After being centrifuged twice at 8,000 rpm and 4 °C for 20 min, the supernatant of the extraction solution was dialyzed overnight against 10 mM sodium phosphate buffer (pH 7.4). The protein concentration of the extraction sample was measured using the Bio-Rad protein assay with BSA as the standard.

2.3. SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed as described by Laemmli [21]. Each protein sample was mixed with 5X sample buffer (10% SDS, 50% glycerol, 0.25 M Tris–HCl pH 6.8, β-mercaptoethanol, and 0.05% bromophenol blue) prior to the denaturation in boiling water for 5 min. The protein samples were analyzed by SDS-PAGE with a 5% (w/v) stacking gel and a 12% (w/v) resolving gel at a constant voltage of 100 V. Proteins in the gel were detected by staining with CBB, and then destained using a destaining solution that contained 10% acetic acid and 25% methanol.

2.4. Dot blot and Western blot

Dot blot and Western blot were conducted as described elsewhere with some modifications [22,23]. One microgram of mango extract was spotted on each PVDF membrane and dried for 10 min at 60 °C for dot blotting. In the Western blot, proteins were separated by SDS-PAGE analysis and then transferred to the PVDF membrane. For both assays, the PVDF membrane was blocked with 5% skimmed milk powder in phosphate buffer saline with 0.1% Tween 20 (PBST, pH 7.4) for one hour at 37 °C. To investigate the specific interaction between sample proteins and the serum of a mango-allergic patient, the membrane was firstly incubated overnight with a ten-fold dilution of the patient serum (1:10 in 0.3% BSA, PBST) at 4 °C. After it had been washed with PBST three times, the membrane was incubated with goat anti-human IgE antibody (1:2500 in PBST; KPL, USA) at 37 °C for one hour. After it had been washed with PBST three times, biotin-labeled anti-goat antibody (1:5000 in PBST; Abcam, USA) was added and incubated at 37 °C for one hour. The membrane was washed three times with PBST, and then incubated with peroxidase-labeled streptavidin (1:10000 in PBST, KPL, USA) at 37 °C for 30 min. The results were obtained by staining with ECL reagents and analyzing using a Luminescence Image System (Hansor, Taiwan). To study the specific interaction between the protein sample and the anti-histidine antibody, the blocked membrane was incubated overnight with anti-histidine antibody (1:1000 in PBST; Miniport, Germany) at 4 °C, and then reacted for one hour at RT with anti-mouse antibody that was conjugated with horseradish peroxidase (1:5000 in PBST; GeneTex, USA). After the membrane had been washed, the target protein was detected with ECL reagents, as described above.

2.5. LC/MS/MS and MALDI-TOF analysis

For the peptide mapping of the target, the protein sample was firstly separated out using SDS-PAGE analysis and stained with CBB. The band that contained Man i 1 (40 kDa) was cut out and digested by trypsin in the gel of SDS-PAGE. The peptide mapping of the trypsinized sample was carried out by LC/MS/MS (LC: 1200 Series Binary Pump, Agilent; Mass Spectrometry: LTQ-XL, Thermo Scientific) analysis or a MALDI-TOF assay (Voyager DE PRO, Applied Biosystems).

2.6. RNA extraction and cDNA synthesis

Mango fruit was frozen in liquid nitrogen and immediately