Molecular Immunology 103 (2018) 89–95

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Pomegranate chitinase III: Identification of a new allergen and analysis of sensitization patterns to chitinases

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ARTICLE INFO

Keywords:
Pomegranate allergen
Chitinase III
Chitinase IV
FABER® test
Food allergy

ABSTRACT

Allergy to pomegranate is often associated with severe symptoms. Two allergens have previously been described: 9k-LTP Pun g 1 and pommaclein Pun g 7. This study describes the isolation of a chitinase III, identified by direct protein sequencing and mass spectrometry. It is a 29-kDa protein showing 69% sequence identity with the latex hevamine and IgE binding in dot blotting, immunoblotting and FABER®test. Chitinase-specific IgE were detected in 69 of 357 patients sensitized to one or more pomegranate allergenic preparations present on the FABER®test. Using this test, 19.2% of the patients sensitized to kiwifruit chitinase IV were also sensitized to pomegranate chitinase III, rather than to latex chitinase I (7.2%) with which it shares the N-terminal hevein-like domain. In conclusion, a new allergen has been identified, contributing to improving food allergy diagnosis. This study reveals the important role of chitinases III and IV as allergy sensitizers and prompts further investigations.

1. Introduction

In recent years the consumption of pomegranate has been greatly increasing because this fruit is considered a functional food with health-promoting properties effective in the risk reduction of diseases such as inflammatory pathologies, cancer and coronary and vascular diseases (Aviram et al., 2008; Kalaycıoğlu and Erim, 2017; Kasimsetty et al., 2010; Pérez-Ramírez et al., 2018; Sumner et al., 2005). Nevertheless, this fruit can be an allergenic source.

The first allergic reaction to pomegranate was reported in 1991 (Igea et al., 1991) in an 85-year-old woman. In 1992, IgE specific for pomegranate fruit were detected by prick test and RAST in a 7-year old girl presenting immediate bronchospasm after pomegranate ingestion (Gaig et al., 1992). In 1999 (Gaig et al., 1999), a 29-kDa allergen was detected by IgE-immunoblotting in the sera of three patients (two 25 year old women and one 3 year old girl) reporting angioedema, urticaria, abdominal pain and anaphylactic shock after pomegranate ingestion. Those patients resulted to be sensitized to the 29-kDa protein component only. Although detected almost twenty years ago, this 29-kDa allergenic protein has not been identified and characterized yet.

The first allergen to be identified in pomegranate was 9k-LTP, that was reported in 2007 and registered by WHO/IUIS as Pun g 1 (Zoccatelli et al., 2007). Later on, different 9k-LTP isoforms displaying different immunological features were detected in this fruit and described (Bolla et al., 2014; Pérez-Ramírez et al., 2018; Zoccatelli et al., 2007). More recently, a 7 kDa allergenic protein (Pun g 7) has been isolated and identified in the pomegranate pulp (Tuppo et al., 2017). This is pommaclein, homologous to the peach allergen peamaclein (Pru p 7) and belonging to the gibberellin-regulated protein family (GASA protein family) (Nahirñak et al., 2012).

The aim of this study has been the isolation, identification and characterization of the 29-kDa protein component reported (Gaig et al., 1999, 1992) to be recognized by specific IgE contained in the sera of patients allergic to pomegranate.

Abbreviations: DB, IgE dot blotting; HLD, hevein-like domain; IB, IgE immunoblotting; LTP, lipid transfer protein; SPT, skin prick test

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https://doi.org/10.1016/j.molimm.2018.09.009
Received 19 July 2018; Received in revised form 30 August 2018; Accepted 10 September 2018

2. Materials and methods

2.1. Purification of chitinase from pomegranate pulp

Pomegranate fruits (*Punica granatum*, accession PR1) were provided by the CRA Fruit Tree Research Unit (Caserta). The fruits were harvested at the commercial ripening stage and the arils contained in each pomegranate were manually separated into pulp and seeds. The red pulp was used to prepare a protein extract, as previously described (Tuppo et al., 2017). Briefly, the red pulp was homogenized in a blender after the addition of 1 M NaCl (1 : 1 w/v or v/w) and stirred at 4 °C for 2 h. Next, the sample was centrifuged at 17,300 x g for 45 min and the supernatant, representing the pomegranate pulp extract, supposed to contain only water-soluble proteins, was collected and analyzed by RP-HPLC and SDS-PAGE. The protein concentration of the pulp extract was 0.035 mg/ml, as determined by the Bradford method BIO-RAD Protein Assay (Biorad, Milan, Italy), using a calibration curve made with bovine serum albumin.

Next, the extract (about 14 mg) was dialyzed against 10 mM Tris-HCl, pH 7.2, and loaded on a DE52 (Whatman, Brentford, UK) column (28 x 150 mm), equilibrated in the same buffer. The bound proteins were eluted with 10 mM Tris-HCl, pH 7.2, containing 0.5 M NaCl. During the purification procedure, the 29-kDa protein was monitored by RP-HPLC or SDS-PAGE. The fractions eluted from the DE52 column containing the protein (about 7 mg) were dialyzed against 10 mM Tris-HCl, pH 8.0 (buffer A). Next, the sample was loaded on a Q-Sepharose (Amersham Biosciences, Uppsala, Sweden) column (17 x 90 mm), equilibrated in the same buffer. The elution was carried out with a linear gradient from 0% to 100% of buffer B (20 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl). The fractions containing the 29-kDa protein were pooled and concentrated with a centrifugal vacuum concentrator (Savant Speedvac Plus SC110 A, Ramsey, Minnesota, USA). A further purification step was performed on a Superdex 75 HR 10/30 column (Amersham-Pharmacia), using an FPLC system (Amersham-Pharmacia, Uppsala, Sweden), equilibrated in 10 mM phosphate buffer, pH 7.4, containing 0.25 M NaCl. The elution was carried out at a flow rate of 0.5 ml/min, monitoring the absorbance at 280 nm and collecting 0.5 ml fractions. Afterwards, the fractions containing the 29-kDa protein were pooled, and dialyzed against 2 mM phosphate buffer and concentrated by the centrifugal vacuum concentrator. The protein concentration was estimated on the basis of the molar extinction coefficient, at 280 nm ($48735\ M^{-1}\ cm^{-1}$), of the isoform from pomegranate seeds (UniProt accession number G1UH28) calculated using the ProtParam tool.

2.2. Analysis by RP-HPLC and mass spectrometry

The extract and the purified protein were analyzed by RP-HPLC using a Beckman System Gold apparatus (Fullerton, CA, USA) as already described (Tuppo et al., 2017). Briefly, the samples were loaded on a Vydac (Deerfield, IL, USA) C8 column (4.6 x 250 mm) equilibrated in 99% of eluent A (0.1% TFA) and 1% of eluent B (0.08% TFA in acetonitrile). Elution was made by a multistep linear gradient of eluent B in eluent A at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm. The eluted peaks were collected and analyzed by SDS-PAGE and by N-terminal amino acid sequencing.

The molecular mass of the purified protein was determined on a QSTAR® Elite System equipped with a Nano-electroSpray® Source (Applied Biosystems, San Diego, CA, USA). Spectral deconvolution and mass reconstruction were performed using the algorithm “Mass Reconstruction” in the Analyst QS software.

2.3. Identification of the purified protein by amino acid sequencing

The protein purified from the pomegranate pulp was identified by N-terminal amino acid sequencing using 300 pmol loaded on a Procise 492 automatic sequencer (Applied Biosystems, Foster City, CA, USA). The obtained amino acid sequence was used to search the UniProtKB (www.uniprot.org) and Allergome (www.allergome.org/) databases.

Table 1
Immunological and clinical data of patients.

Patients	Sera	Gender	Years	Symptoms	Pun g [Pulp] (SPT)	Pun g 14 (IgE DB)	Pun g 14 (IgE IB)
1	34254	M	12	Ang	nd	positive	positive
2	57010	F	9	OAS	nd	negative	negative
3	58372	F	38	OAS, U, C	1	negative	negative
4	64925	M	10	Ang, OAS	nd	positive	positive
5	66653	F	27	OAS	nd	positive	positive
6	BH63	F	21	ne	1	negative	positive
7	EE10	F	23	ne	1	positive	positive
8	69024	M	8	Ang, U	nd	negative	positive
9	68730	F	30	ne	nd	positive	positive
10	67646	M	9	ne	1	negative	positive

Pun g [Pulp], in-house produced pomegranate pulp extract; Ang, angioedema; Asth, asthma; C, conjunctivitis; OAS, oral allergic syndrome; U, urticaria; nd, not determined; ne, not eaten; IgE DB, dot blotting; IgE IB, immunoblotting.

2.4. SDS-PAGE and electroblotting

Extracts and purified proteins were subjected to reducing 15% SDS-PAGE on a Bio-Rad Mini Protean apparatus (Biorad, Segrate, Italy).

Following electrophoresis, proteins were either stained with Coomassie Brilliant Blue or transferred onto PVDF membranes. For N-terminal amino acid sequence analysis, transferred proteins were stained with Coomassie Brilliant Blue, and the protein bands, after excision, were subjected to automated Edman degradation. For immunoblotting analysis, the transferred proteins were stained with ponceau red to check electrotransfer of proteins.

2.5. IgE dot blotting and immunoblotting

To evaluate the IgE binding of the native and denatured forms, purified pomegranate chitinase was analyzed by dot blotting (DB) and immunoblotting (IB), respectively, using the sera listed in the Table 1. The experiments were carried out following the already reported procedures (Tuppo et al., 2014). A mouse monoclonal anti-IgE conjugated to alkaline phosphatase was used (Becton Dickinson Biosciences, San Jose, CA, USA) as the secondary antibody.

2.6. Solutions for skin prick test (SPT)

SPT were carried out using the in-house pulp extract from pomegranate prepared as described above. The protein extract was mixed with sterile glycerin in a 1:1 ratio and sterilized by membrane filtration through a 0.22-μm filter (Millex; Millipore, Bedford, MA, USA), in a sterile horizontal laminar flow hood. The final protein concentration was 0.04 mg/ml. Each SPT was performed and recorded as weal area using a standard methodology as already reported (Bernardi et al., 2011).

2.7. Specific IgE detection by FABER® testing system

FABER® (ADL s.r.l., Latina, Italy) is an in vitro serological test that allows the detection of IgE antibodies produced by allergic subjects and specifically recognizing the allergens spotted on the biochip (Alessandri et al., 2017). The FABER version used to perform the present study (FABER 244-122-122) bears 244 allergenic preparations, representing 122 purified molecular allergens and 122 multiple protein allergenic extracts. This multiplex diagnostic test allows the detection of specific IgE to each of the 244 allergenic preparations in a single run. The FABER allergen list includes preparations from many different