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RESEARCH ARTICLE

Identification and characterization of a basic thaumatin-like protein (TLP 2) as an allergen in sapodilla plum (*Manilkara zapota*)

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Scope: Cases of oral allergy syndrome following the ingestion of sapodilla plum (*Manilkara zapota*) have been reported rarely. As the causative allergens are not known, the main objective of this study was to identify and characterize the important allergens in sapodilla.

Methods and results: Allergy to sapodilla was diagnosed by case history, skin prick test, and serum allergen–specific IgE. The allergen was detected by IgE immunoblotting, purified on SP–Sepharose and characterized by native/SDS–PAGE, IEF, MS, and amino acid composition. Several cases of allergy to sapodilla fruit were identified; majority of the sapodilla-allergic subjects (6/7) experienced typical oral allergy syndrome symptoms, and allergen–specific IgE to the purified protein was positive. The allergen has a p$I \geq$9.5 and high contents of arginine, threonine, glycine, and cysteine. Circular dichroism revealed a secondary structure rich in beta sheets/turns. Based on its *N*-terminal sequence of A-T-F-D-I-Q-N-N-C-X-Y-, the allergen (21 578 Da) was identified as a thaumatin-like protein by homology.

Conclusion: The causative allergen in sapodilla plum has been identified and characterized as a highly basic thaumatin-like protein belonging to the pathogenesis-related protein (PR–5) family, which has been recognized as a new family of conserved, cross-reactive plant allergens.

Keywords:
Arginine-rich protein / Fruit allergen / OAS / Sapodilla / Thaumatin-like protein

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Many fresh fruits and raw vegetables cause type I hypersensitivity reactions with symptoms confined to the oropharyngeal area commonly known as oral allergy syndrome (OAS) [1, 2]. A large majority of subjects with OAS (up to 70%) also have sensitization to pollens (pollen food syndrome) with cross-reactive allergens being primarily involved [3]. Multiple fruit and vegetable allergy is also very common among patients with OAS [3, 4]. Symptoms of OAS are generally mild to moderate with itching, burning, tingling, and occasional swelling of the lips, mouth, tongue, and throat. However, it is suggested that close to 9% of subjects with OAS may develop more severe symptoms of food allergy, including anaphylaxis in some cases (~2%) [1, 4]. With the number of fresh foods reported to cause allergy including OAS continually on the rise, studies to precisely identify and characterize the allergens responsible for OAS have become increasingly relevant and important in this area [5, 6].

Sapodilla plum (*Manilkara zapota* van Royen, formerly *Achras zapota*) belonging to the family Sapotaceae, is a

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Abbreviations: CD, circular dichroism; **OAS**, oral allergy syndrome; **PVDF**, polyvinylidene difluoride; **SPT**, skin prick test; **TLP**, thaumatin-like protein

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popular and highly delicious exotic fruit [7, 8]. It is native to Mexico and Central America, but widely cultivated throughout the tropics and in places like Florida in North America. It is a major commercial crop in India, Sri Lanka, Indonesia, and Malaysia; the largest producer of sapodilla in the world is India where it is commonly known as *sapota* or *chikoo*. Sapodilla is consumed throughout the world; it is particularly served as a specialty fruit in European and North American restaurants and stores. The fruits are a rich source of dietary fiber, antioxidants, vitamins A, C, several essential minerals, and has a protein content of 0.7–1.0 g per 100 g pulp [7, 8].

Several cases of oral allergy following ingestion of sapodilla was first reported from India [9], wherein a ~20 kD protein was detected in IgE immunoblots of sapodilla extracts indicating the presence of a putative allergen that might be responsible for OAS in sapodilla-allergic patients. Since sapodilla fruit is widely consumed in India, it appeared likely that more cases of sapodilla allergy could be detected in the Indian population. In order to identify the causative sapodilla allergen, this study was carried out by examining additional cases of allergy to sapodilla, followed by the isolation and detailed characterization of the allergen including its identification by *N*-terminal sequencing and LC-MS/MS.

2 Materials and methods

Ripe sapodilla fruits were procured from the local market. Southern grass pollen mix (#1651) extract was obtained from Bayer (Spokane, WA, USA). Grass pollen mix (#P28) and common weed pollen mix (#P15) extracts were from Greer Laboratories (Lenoir, NC, USA). Maxisorp microtiter ELISA plates were obtained from Nunc, Roskilde, Denmark. Goat anti-human IgE-horseradish peroxidase (HRP) conjugate, mouse monoclonal anti-human IgE-alkaline phosphatase (ALP) conjugate, *o*-phenylenediamine, *p*-nitrophenyl phosphate, TPCK-treated trypsin, laminarin (from *Laminaria digitata*), and laminarinase (from *Trichoderma* sp.; 100–400 units/g solid) were products of Sigma-Aldrich Chemical, St. Louis, MO, USA. Immobilon-P^{SQ} polyvinylidene difluoride (PVDF) membrane was a product of Millipore, Bedford, MA, USA. 2,3,5-Triphenyl-tetrazolium chloride (TTC, A.R. grade) was a product of Reanal, Budapest, Hungary. HPLC-grade solvents used were from Merck India, Mumbai. All other chemicals and reagents were of analytical grade.

2.1 Allergic subjects

Allergic subjects who had a clear history of food allergy to the ingestion of ripe sapodilla fruits were included in the study. Detailed case histories are described in Supporting Information Table S1. The study was undertaken after clearance by the Institutional Human Ethics Committee (Permission # IHEC–07–04). Informed consent was obtained from all the allergic and normal subjects.

2.2 Preparation of sapodilla juice and extract

Sapodilla juice was obtained by blending ripe sapodilla pulp for ~5 min and filtering through Whatman number 1 filter paper. Sapodilla extract was prepared by blending the pulp in cold PBS (pH 7.4) containing 0.2 mM PMSF, 3 mM EDTA, and 0.2% sodium azide, and filtering to obtain a clear solution. Protein content was determined by the dye-binding method of Bradford.

2.3 Allergy skin tests

Skin prick tests (SPT) were performed with fresh sapodilla juice (protein content, 0.45 mg/mL). For prick-by-prick testing, the pulp portion of a freshly opened, ripe sapodilla was pricked with a sterile lancet; the subject's skin was then pricked with the same lancet. The diameter of wheal/flare reaction was read after 15 min. Sterile glycerinated (50%) PBS, pH 7.4, served as a negative control, and histamine.2HCl, 1.66 mg/mL (equivalent to 1 mg/mL histamine base), served as a positive control. SPTs were also done using the common grass pollen mix, and common weed pollen mix extracts. A wheal diameter of 3 mm greater than the negative control was considered as positive.

2.4 ELISA for allergen-specific IgE

Allergen-specific IgE was detected by ELISA. Briefly, Maxisorp microtiter wells were coated with sapodilla extract in 0.1 M carbonate-bicarbonate buffer, pH 9.6 (50 μg protein/well); this was followed by incubation with sapodilla-allergic or normal serum (1:3 dilution). The secondary antibody used was either goat anti-human IgE–HRP conjugate or mouse monoclonal anti-human IgE-ALP conjugate (at 1:1000 dilution). The wells were washed between steps with PBS containing 0.05% Tween-20. Color development was done by adding 100 μL/well of either 0.5 mg/mL *o*-phenylenediamine in 0.2 M phosphate buffer, pH 7.0 (for HRP) or 1 mg/mL *p*-nitrophenyl phosphate in 1% diethanolamine buffer, pH 9.8 (for ALP). Reaction was stopped by adding 3 M NaOH solution (40 μL/well), and the absorbance at 492 nm (for HRP) or 405 nm (for ALP) was read in a microplate reader.

2.5 IgE–immunoblot analysis

Sapodilla extract was subjected to SDS–PAGE (reducing); after electrophoresis, the gel was stained either with Coomassie brilliant blue R-250 or silver stain. For immunoblot analysis, the proteins separated by SDS–PAGE were blotted by electrotransfer on to nitrocellulose or PVDF membrane. After transfer, protein bands were transiently visualized using Ponceau S stain (1% in glacial acetic acid). Blocking was done using 0.5% gelatin in Tris-buffered saline, pH 7.4 (TBS), containing 0.5% Tween-20. Membrane was washed between steps using TBS containing 0.05% Tween-20 (wash buffer, TBS-T). The membrane, after blocking, was probed with normal or

sapodilla-allergic serum diluted 1:3 in wash buffer, at 4°C for 16 h. Next, it was incubated with mouse monoclonal anti-human IgE–ALP conjugate, diluted 1:1000 in wash buffer, at 37°C for 2 h. Finally, immunospecific IgE-binding protein bands were visualized using BCIP/NBT substrate solution.

2.6 Allergen purification by chromatography of sapodilla extract on SP-Sepharose

The allergen was isolated by cation-exchange chromatography on SP-Sepharose FF (fast flow) under acidic conditions (pH 4) using sodium acetate buffer for binding and increasing salt gradient for separation and elution. Sapodilla extract (50% w/v) was dialyzed against 25 mM sodium acetate buffer, pH 4. This was subjected to cation-exchange chromatography on SP-Sepharose FF column (1.6 × 10 cm) pre-equilibrated with the same buffer, at a flow rate of 12 mL/h, at 25°C. After washing the column with four volumes of starting buffer, proteins were eluted using increasing concentration of NaCl in a gradient fashion (0 to 0.6 M; total volume 120 mL). Two milliliter fractions were collected and the elution was monitored by absorbance at 280 nm. Fractions containing the partially enriched allergen as determined by ELISA were pooled and further separated by re-chromatography at pH 3. The purity of the isolated protein was assessed by SDS–PAGE (reducing and nonreducing conditions) analysis.

Analytical RP HPLC analysis was carried out using a Zorbax C8 column (DuPont, Wilmington, DE, USA; 4.6 × 150 mm, 5 μm) in a Shimadzu LC-6A HPLC system. The sample was eluted using a binary gradient of solvents A (0.1% TFA) and B (70% ACN in 0.05% TFA) at a flow rate of 0.7 mL/min.

2.7 Native PAGE and zymography for β–1,3–glucanase activity

Native PAGE (7.5%) was performed at alkaline pH (pH 9.5) using Tris-glycine buffer system (also called 'Davis system') [10]. Gels were stained with Coomassie brilliant blue R-250.

β-1,3-Glucanase activity on a native polyacrylamide gel was carried out according to Stahmann et al. [11]. After the native PAGE run, the gel was washed three times with distilled water and equilibrated in 0.5 M sodium acetate buffer, pH 4.5. The gel was then incubated in the same buffer containing laminarin (6.7 mg/mL) at 37°C for 2 h. After washing three times in distilled water, the gel was immersed in a solution containing 0.15 g TTC in 100 mL of 1 M NaOH, and placed in a boiling water bath until red bands on a clear background appeared. The gel was then immediately transferred to a solution of 7.5% acetic acid to conserve the clear background.

2.8 *p*I determination by 2D–GE

2D–GE analysis of the purified protein was carried out with IEF in the first dimension and SDS-PAGE in the second dimension. For IEF, gels were cast in SE 2D tube gel adapter (Pharmacia, Uppsala, Sweden) and performed in tube gels using an ECPS unit 3000/150 according to manufacturer's instructions (Pharmacia) on 5% polyacrylamide gel with pH 3.5–9.5 range ampholyte mixture at 500 V/400 mA for 4 h. After IEF, the tube gel was layered on to an SDS–PAGE gel (10% separating gel) and run in the second dimension using Laemmli's buffer system. The 2D gel was stained using Coomassie brilliant blue R-250.

2.9 MALDI-TOF MS

The molecular mass of the purified protein was determined by MALDI–TOF MS in an analytical SEQ MALDI–TOF mass spectrometer (Kompact, Kratos, UK), using a nitrogen laser of 337 nm wavelength and 5 ns pulse width. The laser beam was focused on to the sample spot (10–30 μm size) at an angle of 45°. Ions were accelerated to energy of 3 kV before they entered the TOF mass spectrometer. At the detector, ions were postaccelerated to a maximum kinetic energy of up to 30 kV for more efficient detection.

2.10 LC-MS/MS analyses of tryptic digest of sapodilla basic allergen

An LC-MS/MS analysis of sapodilla basic allergen was performed following trypsin digestion. In-gel digestion of Coomassie-stained bands was performed according to Shevchenko et al. [12]. The bands were destained, reduced, alkylated with iodoacetamide, and digested with TPCK-treated trypsin. Peptide masses were obtained on Bruker Daltonics ultrafleXtreme instrument (Bruker, Billerica, MA, USA). The results were then fed into MASCOT MS/MS Ions Search (http://www.matrixscience.com) for protein identification.

2.11 Circular dichroism (CD)

The far-UV circular dichroism (CD) spectrum (190–240 nm) of the purified sapodilla allergen was recorded on a JASCO J720 spectropolarimeter (Jasco, Maarssen, The Netherlands) at 25°C in 10 mM sodium phosphate buffer, pH 7.4 using 1 mm quartz cell. The spectrum represents mean molar residue ellipticity [θ] averaged from four scans.

2.12 Amino acid composition

Purified allergen from SDS-PAGE gel was transferred on to Immobilon-P^{SQ} PVDF membrane by electroblotting using 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer, pH 11, containing 10% methanol as the transfer buffer. The allergen band cut out from the PVDF blot was hydrolyzed using 5.8 M HCl in vacuo at 110°C for 24 h. Amino acid analysis was performed by precolumn derivatization with

phenylisothiocyanate. The phenylthiocarbamyl (PTC) amino acids were analyzed by RP–HPLC (UV detection at 254 nm) as described in the literature [13]. The mole percent of each amino acid was calculated by comparing the peak area for each amino acid with that of standard amino acids.

2.13 *N*-terminal amino acid sequencing

The purified allergen was subjected to SDS–PAGE and electrotransferred on to Immobilon-P^{SQ} PVDF membrane. The protein was detected transiently by staining with 0.2% Ponceau-S. The allergen band was cut out and subjected to *N*-terminal amino acid sequencing by automated Edman degradation using Procise Protein Sequencing System, model LC 491 (Applied Biosystems, Foster City, CA, USA).

3 Results

3.1 Detection of a 21 kDa allergen in sapodilla pulp

Case histories of the sapodilla-allergic subjects indicated that all of them had immediate allergic symptoms to the ingestion of ripe sapodilla fruit (see Supporting Information Table S1). All the allergic subjects, with the exception of case 6, had symptoms typical of OAS. Case 6 had generalized urticaria after sapodilla ingestion. SPT with fresh sapodilla juice was positive in all the sapodilla-allergic subjects (Table 1). Results of SPT with sapodilla were negative (0–1/0 mm) in 12 normal subjects. Sera from all the seven sapodilla-allergic subjects showed higher ELISA units as compared with control serum, ranging from 1.4- to 4.4-fold increase over control, indicating the presence of serum allergen specific IgE to proteins in sapodilla extract (Table 1).

SDS-PAGE analysis of sapodilla extract revealed several protein bands in the molecular mass range 15 to 90 kDa; only three major bands corresponding to relative molecular weights (M_r) of 37, 28, and 21 kDa were seen (Fig. 1A). IgE immunoblots for sapodilla-allergic cases 1–3 detecting the ~21 kDa molecular weight protein were described in our previous report [9]. The result of IgE immunoblot using the sera of sapodilla-allergic subjects 4–7 is shown in Fig. 1B. With the exception of serum from case 5, all the other sera specifically detected the ~21 kDa protein band.

3.2 Purification of sapodilla allergen

The chromatographic profile of sapodilla extract on SP-Sepharose at pH 3 is shown in Fig. 1C. The SDS-PAGE analysis of fractions showed that the ~21 kDa major band (allergen) eluted as a separate peak (fractions 49–52) at a NaCl concentration of 0.36 M (Fig. 1D). These fractions containing the purified allergen were pooled, dialyzed against water, lyophilized, and used for further studies. Based on protein estimation by Bradford assay, the purified allergen was obtained in a yield of ~4 mg/kg ripe sapodilla pulp.

3.3 Allergen purity

The purified sapodilla allergen eluted as a single component upon RP HPLC on Zorbax C8 indicating a purity of 97% (Fig. 2A). The protein preparation upon analysis by SDS-PAGE appeared as a single band under both nonreducing (without β-ME) and reducing (with β-ME) conditions (Fig. 2B). However, there was considerable decrease in its mobility under reducing condition compared to nonreducing condition. The purified protein appeared as a single band in native PAGE at alkaline pH (Fig. 2C) that further established the purity of the sample. The purified allergen, when examined for the presence of β-1,3-glucanase activity by zymography using laminarin as substrate, did not show any detectable activity (data not shown).

Table 1. Results of SPT and allergen-specific IgE to sapodilla extract and purified allergen

Subject[a)]	Age (year)/ sex	SPT[b)]	Other foods/pollens to which SPT is positive[c)]	Allergen-specific IgE to sapodilla extract (A_{405})[d)]	Allergen-specific IgE to purified sapodilla allergen (A_{405})[e)]
Case 1	20/ F	9.5/>30	-	0.358	0.650
Case 2	23/ F	4/20	-	0.301	0.413
Case 3	23/ M	5/20	-	0.312	0.398
Case 4	65/ F	3/25	B, E, W, G	0.692	0.552
Case 5	48/ F	4/25	B, T, C, CF, E, W	0.362	0.220
Case 6	55/ M	3.5/25	-	0.448	0.425
Case 7	23/ F	4/30	E	0.958	0.821
CS	n.a.	n.a.	n.a.	0.220	0.210

a) Cases 1–3 refer to cases previously reported [9]; CS, control serum (pooled sera from five normal subjects); n.a.: not applicable.
b) Histamine dihydrochloride, 1.66 mg/mL (equivalent to 1 mg/mL histamine base) produced wheal/flare diameter of 6–8/20->30 mm in these subjects; glycerinated-PBS produced wheal/flare diameter of 0–1/0 mm in these subjects. The SPT data for cases 1–3 is reproduced from our earlier study [9] with permission from Elsevier, ©2002.
c) G: grass pollen mixes; W: common weed pollen mix; B: banana; C: cucumber; CF: citrus fruits; E: eggplant (brinjal); T: tomato.
d) Mean of triplicate determinations; secondary antibody: mouse monoclonal anti-human IgE-ALP conjugate.
e) Mean of triplicate determinations; coating antigen: 4 μg purified sapodilla allergen per microtiter well.

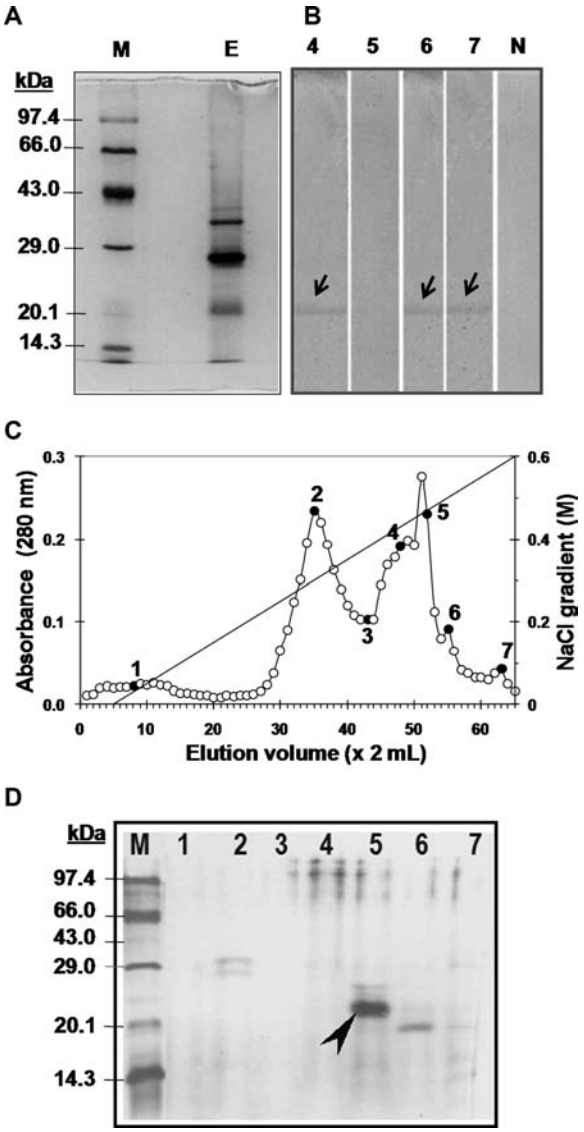

Figure 1. Analysis of 50% w/v sapodilla extract by electrophoresis and IgE immunoblotting. (A) 12% SDS–PAGE (silver staining): lane M, molecular weight markers; lane E, sapodilla extract (protein load, ~10 μg). (B) IgE immunoblot: lanes 4–7, sera from sapodilla–allergic cases 4–7, respectively; lane N, control serum (pooled sera of normal subjects). The IgE-reactive band is indicated by an arrow. (C) Chromatography of sapodilla extract on SP-Sepharose (1.6 × 10 cm) at pH 3. A gradient of 0–0.6 M NaCl in 25 mM sodium acetate buffer, pH 3 was used for elution (flow rate: 12 mL/h). (D) SDS-PAGE profile (12%, reducing) of the numbered fractions indicated as filled circles in panel (C); M, mol. wt. markers; the numbers on the left refer to kDa. The arrow indicates the 21.5 kDa protein.

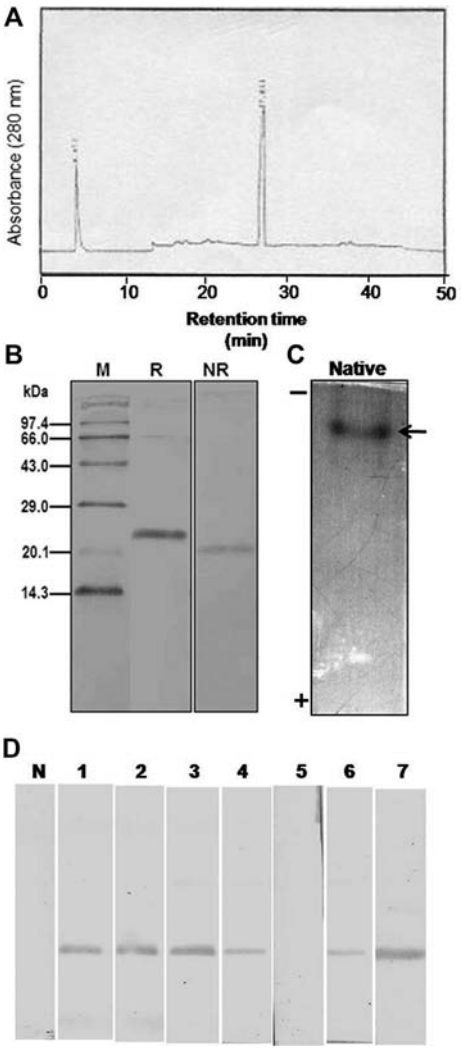

Figure 2. Purity of sapodilla basic allergen. (A) RP–HPLC profile of purified sapodilla allergen on Zorbax C8 column (4.6 × 150 mm, 5 μm). The purified protein showed a retention time of 27.611 min; the peak at 4.452 min is the solvent peak; purity: ~97%. (B) SDS-PAGE (15%) of purified sapodilla allergen. R, reducing (with β-mercaptoethanol); M, mol. wt. markers; NR, nonreducing (without β-mercaptoethanol). (C) Native PAGE (7.5%) at alkaline pH of the purified sapodilla basic allergen (indicated by an arrow). (D) IgE immunoblot of purified sapodilla 21 kDa allergen: lane N, control serum (pooled sera of normal subjects); lanes 1–7, sera from sapodilla-allergic cases 1–7, respectively.

3.4 Immunoreactivity of the purified allergen with sapodilla-allergic sera

The IgE-binding activity of the purified allergen was tested by direct ELISA using the sera of subjects allergic to sapodilla. The results indicated that six out of seven subjects' sera showed IgE binding to the purified 21 kDa protein (Table 1), with an increase in ELISA units over control serum by 1.9- to 3.9-fold. Serum from case 5 showed IgE binding to sapodilla extract, but not with the purified 21 kDa protein. However, this subject's serum IgE did not show binding to any other protein in immunoblotting (Fig. 1B) indicating that the allergen in this case may be a small molecule or a protein present in minute amounts in sapodilla extract. The results of IgE immunoblotting with the purified 21 kDa allergen is shown in Fig. 2D. It is seen that, with the exception of serum from

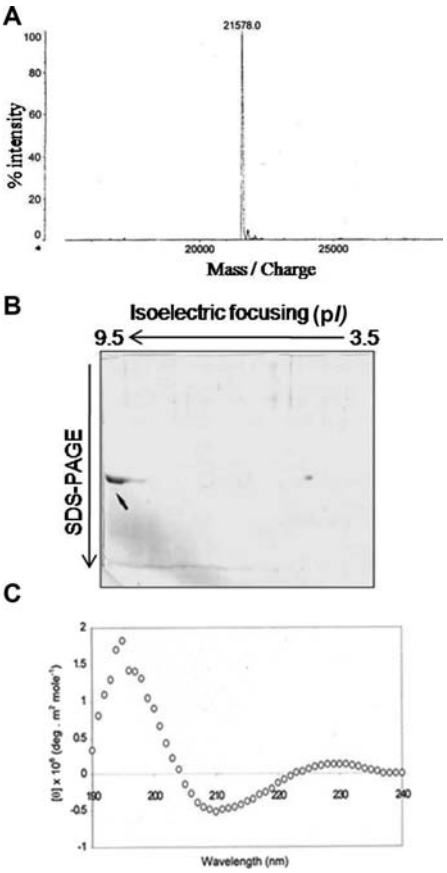

Figure 3. Characterization of purified sapodilla basic allergen. (A) MALDI-TOF mass spectrum. (B) 2D–GE: IEF in the first direction, and SDS-PAGE in the second direction. The arrow near the extreme of basic pH indicates the sapodilla allergen. (C) Far–UV CD spectrum of the purified sapodilla allergen. Spectrum represents mean molar residue ellipticity [θ] of four replicate recordings.

case 5, all the other sapodilla-allergic serum reacted with the purified protein. Overall, the results show that the 21 kDa protein is the IgE-binding allergen in most of the sapodilla-allergic subjects examined and hence, has been detected as the causative allergen.

3.5 Characterization of the sapodilla allergen

The MALDI–TOF MS of the purified sapodilla allergen showed the presence of a single mass signal at 21 578 Da (Fig. 3A). The purified protein was subjected to 2D–GE with IEF in the pH range of 3.5–9.5 (Fig. 3B). The protein spot moved close to the basic end of 9.5, and is likely to have a p*I* >9.5 indicating its highly basic nature. The far–UV CD spectrum of the purified allergen was analyzed for its secondary structures (Fig. 3C). The CD spectral profile showed an increase in [θ] around 195 nm followed by a single negative peak with the negative maximum at 210 nm suggesting a secondary structure low in α-helices, but predominantly consisting of β-sheets and β-turns.

Table 2. Amino acid composition of the purified sapodilla allergen[a)]

Amino acid	Mole percent (experimental)	Mole percent (normalized)[b)]
Asp (D)[c)]	14.39	13.15
Glu (E)[d)]	6.11	5.58
Ser (S)	4.46	4.08
Gly (G)	11.67	10.67
His (H)	0.48	0.44
Arg (R)	13.50	12.34
Thr (T)	16.46	15.05
Ala (A)	6.41	5.86
Pro (P)	2.78	2.54
Tyr (Y)	2.05	1.87
Val (V)	3.07	2.81
Met (M)	0.23	0.21
Cys (C)[e)]	0.58	8.00[e)]
Ile (I)	2.10	1.92
Leu (L)	3.84	3.51
Phe (F)	4.41	4.03
Lys (K)	7.08	6.47
Trp (W)[f)]	0.00	1.50[f)]

a) Based on acid hydrolysis (mean of two determinations).
b) The experimental mole % is normalized, taking into account that there are 16 conserved cysteines and approximately 200 amino acid residues in most plant TLPs.
c) Represents the sum of Asp (D) + Asn (N).
d) Represents the sum of Glu (E) + Gln (Q).
e) Mole percent of cysteine is 8 since all TLPs contain 16 cysteines in a polypeptide chain length of ~200 residues.
f) Trp (W) is destroyed under acid hydrolysis conditions; most TLPs contain, on the average, approximately three tryptophans per molecule.

The amino acid composition of sapodilla allergen (Table 2) revealed that the protein is rich in threonine (15%), glycine (11%), and arginine (12.5%). The presence of high percentage of arginine residues together with significant percentage of lysine residues (6.5%) is reflected in its highly basic nature (p*I* ≥9.5). The high percentage of glycine (11%) further supports a secondary structure predominant in β-sheets.

3.6 Identification of the sapodilla basic allergen as a thaumatin-like protein (TLP) by *N*-terminal sequencing and MS/MS analysis

The *N*-terminal sequence of sapodilla allergen was determined to be Ala-Thr-Phe-Asp-Ile-Gln-Asn-Asn-Cys- (ATFDIQNNC-, UniProt KB accession number B3EWE5). Although the tenth amino acid residue could not be identified, the 11th amino acid residue was found to be tyrosine (Y; data not shown). The results of BLAST homology search for the *N*-terminal sequence (nine residues) of the sapodilla basic allergen are shown in Supporting Information Table S2. The *N*-terminal sequence alignment with the top 12 matches obtained from the BLAST search is shown in Supporting Information Fig. S1. The analysis clearly indicated the identity of the sapodilla basic allergen as a thaumatin-like


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                    1    5     10   14
Man z TLP2          ATFDIQNNC-Y---
Act d 2.0101        ATFNIINNCPFTVW
Cap a 1.0101        ATFEVRNNCPYTVW
Nic t Osmotin       ATIEVRNNCPYTVW
Cry j 3.1           ATFDITNQCPYTVW
Cry j 3.2           ATFDITNQCPYTVW
Pru d 2             AKMSFKNNCPYTVW
Pru p 2             AKITFTNKCSYTVW
Pru p 2.0101        AKITFTNKCSYTVW
Vit v TLP           ATSTILNKCTYTVW
                    *.   . *:*.:***
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Figure 4. Alignment of *N*-terminal sequence of sapodilla basic TLP (Man z TLP2) with known basic TLPs currently identified as pollen and food allergens. The allergens are indicated by their allergen nomenclature as retrieved from Allergome database, and their descriptions are given in Supporting Information Table S2. Alignments were created using T-Coffee multiple sequence alignment. Symbols: (*), complete identity; (:), strongly similar; (.), weakly similar; no symbol, different.

protein (TLP) (belongs to PF00314 thaumatin family and AF060 thaumatin-like protein allergen family).

LC-MS/MS analysis of tryptic digest of sapodilla basic TLP by MS/MS ion search resulted in two significant matches (Supporting Information Fig. 2). A MASCOT search result against the SwissProt database identified a 13-residue peptide match (residues 136–148 in the mature polypeptide) from kiwi TLP (*Actinidia deliciosa*; Act d 2), and is shown in panel A. Additionally, a MASCOT search result against the NCBInr database identified a 26-residue peptide match (residues 93–118 in the mature polypeptide) from cocoa (*Theobroma cacao*) osmotin-like protein (panel B). It is interesting to note that both these significant matches are basic proteins of PR-5 family with 201 amino acid residues.

3.7 Comparison of sapodilla basic TLP with known pollen and food allergenic TLPs

Currently, there are nine basic TLPs identified as allergens from foods and pollens (Supporting Information Table S3) as retrieved from Allergome database – www.allergome.org; these range in p*I* values from 7.53 to 9.15. The similarity of *N*-terminal residues of sapodilla basic TLP with those of all the allergenic basic TLPs known at present is examined by alignment (Fig. 4). It is seen that residues 1, 7, 9, 12–14 are highly conserved in all the TLPs examined here. Further, the residues at positions 5 and 11 are strongly similar among the TLPs. The occurrence of Cys at position 9 and Trp at position 14 is a characteristic feature of all TLPs.

4 Discussion

Sapodilla allergen has been purified to homogeneity by ion-exchange chromatography on SP-Sepharose; the purified allergen was found to react with six out of seven sapodilla-allergic sera by ELISA and IgE immunoblot. The purified sapodilla allergen has a molecular mass of 21 578 Da. Sapodilla allergen showed characteristically high percentage of threonine, glycine, and arginine in amino acid analysis. The content of arginine is approximately twice that of lysine suggesting a strongly basic nature; this was confirmed by 2D-GE wherein the purified protein was found to have a p$I \geq 9.5$. Sapodilla basic TLP appears to be the most basic TLP among the basic TLP allergens identified so far. The mobility difference of the allergen under reducing and nonreducing conditions of SDS-PAGE indicated that the protein is very rich in disulfides, as has been shown in the case of kiwi TLP [14]; this seems to agree well with the presence of 16 highly conserved cysteines that form eight disulfide bonds and stabilize the structure in majority of the TLPs. The unique secondary structure of the purified allergen consisting mainly of β-sheet and β-turn structures as shown by the far-UV CD spectrum is characteristic of thaumatin structure that is known to utilize two building motifs: a folded β sheet, or a flattened β "barrel", and the β ribbons and small loops stabilized by disulfide bonds [15].

N-terminal sequence of sapodilla allergen up to nine residues indicated a strong homology to TLPs belonging to the pathogenesis-related protein family, PR-5 [16]. Most plant TLPs have Tyr at the 11th position [16]. All the allergenic basic TLPs including sapodilla basic TLP from the current study have Tyr at position 11 with the exception of Act d 2.0101 (p*I* 7.91) which has Phe. LC-MS/MS analysis of sapodilla basic TLP indicated a significant match with kiwi TLP (Act d 2) and cocoa osmotin-like protein, both of which are basic proteins having molecular masses of 21 614.2 and 21 795.3 Da, respectively. It is highly likely that the matched peptide sequences of these two proteins are present in sapodilla basic TLP.

Thaumatin is an intensely sweet protein first isolated from the seed arils of the tropical plant *Thaumatococcus daniellii*, native to West Africa [17]. Proteins sharing close sequence similarity with thaumatins but without a defined biological activity are usually referred to as TLPs [18]. Proteins of PR-5 family share remarkable homology with thaumatins and were one of the first identified TLPs [19]. Despite the structural similarities with thaumatins, TLPs are not sweet-tasting proteins [19, 20]. Some PR-5 family proteins are induced by osmotic stress (osmotins) or by biotic/abiotic stress in leaves and roots. Several other TLPs, however, are not pathogenesis related, but are developmentally regulated and accumulate in developing seeds, flower tissues, or ripening fruits [20]. In fact, several TLPs are known to accumulate at high levels in conjunction with sugar accumulation during fruit ripening [19–21]. It should be noted that, all the allergic cases described in this study were due to the ingestion of ripe sapodilla fruit; more so, the TLP allergen was purified from ripe fruit pulp. Generally, the basic TLPs tend to be in vacuoles or other vesicles, while the acidic ones are likely secreted into the extracellular spaces [22].

During the course of our investigation on basic TLP, it became apparent to us that another form of TLP exists in sapodilla fruit that is acidic in nature and with a slightly higher molecular mass (22 kDa). The additional purified TLP was identified as an acidic TLP based on native PAGE, IEF analysis, and *N*-terminal sequence (UniProt accession B3EWX8) [23]. Sequence analysis of the genomic clone of the acidic TLP gene (GenBank ID: G5DC91.1) revealed that it is intronless; the deduced protein lacks an *N*-glycosylation site [23]. Evolutionary relatedness to olive, grape, and kiwi fruit allergenic TLPs were highly evident by phylogenetic analysis. Based on this independent study, an acidic TLP (TLP 1) was identified as a new allergen in sapodilla causing OAS. Sapodilla TLP 1 is a single polypeptide (207 residues) belonging to the thaumatin family of the GH64-TLP-SF superfamily.

TLPs from kiwi fruit [14], apple [22], sweet cherry [24, 25], bell pepper [26], grape [27], almond [28], and peach [29] have been identified as food allergens and studied. Interestingly, TLP from olive fruits has been shown to cause occupational asthma in an oil-mill worker [30]. Wheat TLP has been found to be the causative allergen for baker's respiratory allergy in some cases [31]. Overall, allergenic TLPs have been recognized as a new family of cross-reactive allergens or pan allergens [32]. In the present study also, we have seen sapodilla-allergic subjects (cases 4, 5, and 7) are also allergic to other foods and pollens based on case histories and SPT, although detailed cross-reactivity studies with the purified allergen have not been carried out.

TLPs from several plant sources namely, cherry, banana, tomato, and tobacco have been crystallized and their three-dimensional structures elucidated [24, 33–36]. However, the precise biological function of TLPs in plants remains to be established. At least some of them are believed to be involved in plant defense with demonstrated antifungal activity [37] and β-1,3-glucanase activities [38, 39]. Sapodilla basic TLP does not exhibit β-1,3-glucanase activity with laminarin as substrate. Some TLPs have also been shown to bind to β-1,3-glucans despite lack of β-1,3-glucanase activity [40].

Due to the importance of exotic fruits in the western diet as well as in developing countries, awareness about allergic reactions and causative allergens in such foods is warranted. Food allergy to other exotic fruits/vegetables such as fig, lychee, pineapple, cassava (manioc), and raspberry have been reported in the literature [41–46]. The present study on food allergy to sapodilla is one of such select few in which food allergy to an exotic fruit has been investigated in detail. Although allergic reactions to sapodilla plum are rarely reported, the identified TLP allergen is a well-known plant defense protein and a panallergen in certain pollens and other foods including fruits and vegetables [32, 46]. Further research is needed to fully understand the possible cross-reactivity of sapodilla basic TLP with other foods and pollens. In conclusion, a 21.5 kDa arginine-rich TLP (Man z TLP2) has been identified as an important allergen in sapodilla fruit responsible for OAS in sapodilla-allergic subjects.

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