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Emerging food allergens: Identification of polyphenol oxidase as an important allergen in eggplant (*Solanum melongena* L.)

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ABSTRACT

Although many allergens have been detected in eggplant (*Solanum melongena* L.), their identity have not been elucidated. The aim of this study was to investigate whether polyphenol oxidase (PPO), an important eggplant enzyme, acts as an allergen. The proteins of eggplant peel extract were separated on phenyl-Sepharose (PS), and analyzed by skin prick test (SPT), ELISA and IgE-immunoblotting; the components were analyzed for PPO activity, presence of protein-bound copper, and recognition by rabbit polyclonal anti-sweet potato PPO antiserum. LC–MS/MS and *in silico* analysis were employed to identify the separated allergens and prediction of IgE epitopes. Eggplant allergens were separated into 5 components (PS1–PS5), of which component PS2 exhibited high specific PPO activity. SPT and ELISA with PPO-rich pool (PS2) were positive in all 6 eggplant-allergic subjects; the 43, 64 and 71 kDa proteins displayed strong IgE-binding ability. The 64 and 71 kDa IgE-binding proteins show PPO activity, presence of copper, and recognition by anti-sweet potato PPO antiserum, clearly identifying them as PPOs; the 43 kDa protein appears to be a degradation product of the 64 or 71 kDa proteins based on enzymic activity and recognition by PPO antiserum. The 64 kDa protein upon further resolution by SDS–PAGE displayed two components (identified as eggplant PPO1 and PPO4 by LC–MS/MS). Based on bioinformatics approaches, PPO4 has been identified as an allergen since it harbors an IgE epitope. This study clearly demonstrates that the 64 and 71 kDa allergens in eggplant peel are PPOs based on enzymic activity and recognition by PPO antiserum; the 64 kDa copper-containing protein is identified as one of the several eggplant allergens (Sola m PPO4). This is the first instance of polyphenol oxidase being identified as a new food allergen.

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1. Introduction

Food allergens are proteins that invoke IgE-mediated immune reactions. The members of the nightshade family can provoke immediate hypersensitivity reactions in susceptible individuals and several allergens are identified from potato, tomato and bell pepper with the exception of eggplant (*Solanum melongena* L.). Eggplant, also known as aubergine or brinjal, is an economically important vegetable largely consumed in Asia, Europe and other

parts of the world mostly in cooked form (Daunay, 2008; Lawande and Chavan, 1998). However, in recent years, there has been an increase in the number of reports on food allergy to eggplant, especially from Asian and European countries (Bansal, 2013; Dey et al., 2014; Gubesch et al., 2007; Harish Babu and Venkatesh, 2009; Harish Babu et al., 2008; Lee et al., 2004; Pramod and Venkatesh 2004). Further, a fairly good number of IgE-binding proteins varying in molecular mass have been detected in eggplant with predominance in the eggplant peel (Harish Babu and Venkatesh, 2009).

It is well established that most of the allergenic proteins identified in plant foods are known to have essential physiological roles in the plant system. For instance, some of the allergens reported from plant foods are storage proteins (vicilins, legumins, 2S albumins, patatin), enzymes (α -amylase, cysteine protease), involved in plant defense like pathogenesis-related proteins (PR-proteins like glucanases, chitinases, peroxidase) and perform varied biological functions (Clare Mills et al., 2009; Ebner et al., 2001). The major potato allergen patatin (Sola t 1) has shown lipid acyl hydrolase (Andrews et al., 1988) and phospholipase activity (Hirschberg et al.,

Abbreviations: ARP, allergen representative peptide; 4-MC, 4-methyl catechol; PPO, polyphenol oxidase; PS, phenyl-Sepharose; SmePPO4, *Solanum melongena* PPO4; SPT, skin prick test.

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2001). Four minor allergens of potato (Sola t 2–Sola t 4) are known to be trypsin inhibitors (Seppälä et al., 2001). The major tomato allergen (Sola l 2) has β -fructofuranosidase activity (Westphal et al., 2003), while the minor allergens are known to possess polygalacturonase and pectinesterase activity involved in fruit ripening (Kondo et al., 2001). Similarly, some of the cross-reactive allergens from bell pepper are identified as profilin, β -1,3-glucanase and L-ascorbate peroxidase enzymes in the plant system (Palomares et al., 2005; Wagner et al., 2004; Willerroider et al., 2003).

Although eggplant is known to contain a good number of allergenic proteins, the identification of eggplant allergens has remained a challenging task due to very low protein content (1% of fresh weight) and multitude of proteins (Harish Babu and Venkatesh, 2009). Among the eggplant proteins, only the 6 kDa protease inhibitor has been studied structurally in detail (Richardson, 1979), while a few other eggplant proteins have been studied in relation to their function in some moderate detail – polyphenol oxidase (PPO), 56 kDa (Mishra et al., 2012); lipoxigenase, 97 kDa (Pérez-Gilabert et al., 2001); and linoleate hydroperoxide isomerase, 298 kDa (three 97 kDa subunits) (Grosman et al., 1983). Since eggplant PPO has been studied in relation to browning and wounding of eggplant (Mishra and Gautam, 2016; Shetty et al., 2011), it appeared interesting to investigate whether PPO in eggplant can act as an allergen causing food allergy. In the present study, we have made an attempt to separate the proteins present in eggplant peel and analyze PPO-enriched pools biochemically and immunochemically for allergenic activity.

2. Material and methods

2.1. Preparation of eggplant peel extract

All steps were carried out at 4 °C unless stated specifically. Freshly obtained Mysore green type eggplant (*Solanum melongena* var. *serpentinum*) having a slender long appearance was used due to its frequent consumption in this region. Eggplant peel extract was prepared by blending 200 g of pre-chilled eggplant peel (obtained from 2200 g eggplants) for 5 min using equal volumes of 10 mM sodium phosphate buffer, pH 7.4 containing 140 mM NaCl (phosphate-buffered saline, PBS), 2 mM phenylmethane-sulfonyl fluoride (PMSF), 100 mM L-ascorbic acid (to prevent enzymic browning reaction), and centrifuged at $5300 \times g$ for 20 min. The supernatant was treated with 3% polyvinyl pyrrolidone (PVPP) with continuous stirring for 30 min (to remove interfering phenols), centrifuged at $10000 \times g$ for 15 min, and filtered through Whatman No. 1 filter; this was designated as 'eggplant peel extract'. Similarly, 40 g eggplant peel obtained from the same batch of eggplant was used separately for preparation of peel extract, which was subjected to acetone precipitation as described in our previous study on eggplant allergens (Harish Babu and Venkatesh, 2009), and after resolubilization was designated as 'eggplant peel concentrate'; this was used for skin prick test (SPT), ELISA and IgE-immunoblot analysis since it is devoid of PMSF and other small molecules.

2.2. Fractionation of eggplant proteins on phenyl-Sepharose (PS)

The eggplant peel extract obtained was treated with $(\text{NH}_4)_2\text{SO}_4$ between 20 and 80% saturation, and the 80% pellet was solubilized in 30 mL of PBS, centrifuged at $10000 \times g$ for 20 min, and the resulting supernatant was dialyzed against PBS containing 1 M $(\text{NH}_4)_2\text{SO}_4$ for 24 h with two changes of buffer. The retentate was further clarified by centrifugation at $10000 \times g$ for 30 min and the supernatant was filtered through Whatman No. 1 filter. The filtrate was loaded onto phenyl-Sepharose HP (Sigma-Aldrich Chemical Co., St Louis, MO, USA) column (1 $\times$ 5 cm) previously equilibrated

with PBS containing 1 M $(\text{NH}_4)_2\text{SO}_4$ at 4 °C, and washed with two column volumes of this buffer. The bound proteins were eluted in a step-wise manner starting with 1 M $(\text{NH}_4)_2\text{SO}_4$ followed by 0.75, 0.5, 0.25, 0 M $(\text{NH}_4)_2\text{SO}_4$, and finally with double-distilled water. PPO activity was monitored at 25 °C in the peak fractions of all the step-eluted components, and the fractions having PPO activity were pooled, dialyzed against three changes of double-distilled water at 4 °C for 24 h, and concentrated by lyophilization. Protein concentration was determined by Bradford's dye-binding assay using bovine serum albumin (BSA) as a standard (Bradford, 1976).

2.3. PPO activity determination

PPO activity was determined in triplicate at 25 °C as described (Dogān et al., 2002). The PPO activity assay mixture consisted of 2.7 mL of 10 mM sodium phosphate buffer, pH 6.5/140 mM NaCl, 0.2 mL of 0.1 M 4-methyl catechol (4-MC) and 100 μL of sample [eggplant peel extract or concentrate or phenyl-Sepharose (PS) chromatographic components] in a 1-cm path length cuvette. The change in absorbance at 420 nm was monitored for 3 min using Shimadzu UV-vis spectrophotometer. One unit of the enzyme activity is defined as the amount of enzyme that causes an increase in absorbance of 0.001 per min per mL at 25 °C. The fractions exhibiting high PPO specific activity were pooled and designated as PPO-rich pool (PS2).

2.4. Electrophoresis and immunoblotting

SDS-PAGE was performed according to Laemmli (1970). Ten micrograms of protein sample from eggplant peel concentrate or PS chromatographic components was diluted in sample buffer containing 5% β -mercaptoethanol (β -ME), boiled at 100 °C for 10 min and run on SDS-PAGE gels (reducing PAGE). Similarly, SDS-PAGE was performed under native conditions (without β -ME in the electrophoresis sample buffer and without boiling). Since SDS has been shown to activate many latent plant PPOs (Gandía-Herrero et al., 2005), SDS was used in sample buffer with eggplant peel concentrate and PPO-rich pool. After electrophoresis, the gels were fixed for 45 min and then silver stained. The size of the protein bands mentioned in SDS-PAGE hereafter refers to their relative molecular mass (M_r).

Western blot (Towbin et al., 1979) was performed with both eggplant peel concentrate and PPO-rich pool after the proteins were separated by SDS-PAGE (12%, reducing). Following electrophoresis, the proteins were transferred onto nitrocellulose membrane; after blocking, the membrane was incubated with rabbit polyclonal anti-sweet potato PPO antiserum 1: 2000 dilution in Tris-buffered saline containing 1% BSA and 0.05% Tween-20 (TBS-T) at 37 °C for 2 h. The secondary antibody used was alkaline phosphatase (ALP)-conjugated goat anti-rabbit IgG (Bangalore Genei, Bengaluru, India) at 1:2000 dilution. The immunoblots were developed using the insoluble substrate BCIP-NBT (1: 5 dilution in TBS-T without BSA).

2.5. PPO activity staining on polyacrylamide gel

For enzymic activity staining on polyacrylamide gel, 12% SDS-PAGE was performed at 4 °C under native condition using $\sim 25 \mu\text{g}$ of protein sample from eggplant peel concentrate and 10 μg of PPO-rich pool (PS2). Similarly, PPO-rich pool (10 μg) incubated with 2 mM tropolone, a specific inhibitor of PPO (Espin and Wichers, 1999) for 90 min was used as control for inhibition of PPO activity. Electrophoresis was carried out at a constant voltage of 50 V for 4 h. After electrophoresis, the gel was immediately blotted *in situ* (at ambient temperature) for PPO activity by pressing it onto the top of a dried 4-MC-immobilized paper without adding buffer solution as per the method of Cheng et al. (2007). The gel was rinsed

with water, immersed in 1 mM L-ascorbic acid for 2 min to stop the reaction, soaked overnight in distilled water, and finally stored in 30% ethanol. The protein bands having PPO activity appeared as purple bands against a clear background on the gel, and as dark brown imprints on 4-MC-immobilized paper.

2.6. Specific detection of copper metalloproteins on polyacrylamide gels

The presence of bound copper in the polypeptides on polyacrylamide gels can be detected using copper-protein stain or cyanide-tetrazolium stain (Gould and Karolus, 1975). Briefly, 18 mL of 60 mM KCN, pH 8 and 10 mL of Tris-HCl buffer, pH 8 were mixed with ~0.5 g of polyvinylpyrrolidone (PVP) in a stoppered 50-mL Erlenmeyer flask. Within an hour of use, 50 mg of *p*-nitrotetrazolium blue chloride (NBT) was dissolved in 10 mL distilled water, and added to the flask. Phenazine methosulfate (PMS; 3 mg/mL in distilled water) can be made up ahead of time and stored at 4 °C for about a week, but it must be added (0.6 mL) to the cyanide-tetrazolium solution just before use. The proportions of Tris-HCl buffer, pH 8: KCN: NBT: PMS should be kept close to 5: 9: 5: 0.3; this is designated as cyanide-tetrazolium stain. After performing native PAGE (12%) of eggplant peel concentrate (40 µg protein) and PPO-rich pool (10 µg protein), each gel lane was sliced and placed in a stoppered glass tube. The stain was thoroughly mixed and poured over the gel strips, tubes were stoppered, inverted several times, and placed in the dark at 25 °C for 30–40 min. The copper-protein complex appears as pink to orange colored bands occasionally edged with yellow color. After visualization of the bands, the gels were thoroughly rinsed thrice in tap water at 15 min intervals, and finally the gels were washed and stored in double distilled water.

2.7. SPT and serum collection

This study was undertaken following approval by the Institutional Ethics Committee (approval number: IHEC-07-04). The work described here has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. All participants (subjects with eggplant allergy and non-atopic subjects) gave written informed consent and patient anonymity was preserved using methods approved by the IEC. SPT was performed as per the standard procedure (Sanico et al., 2002) using sterile prick lancetter (Bayer Pharmaceutical Division, Spokane, WA, USA). All samples used for SPT were sterile-filtered by passing through 0.22-µ membrane filters (Millipore, Billerica, MA, USA) and glycerinated to a final concentration of 50% (v/v) and the protein content adjusted to 0.5 mg/mL. SPT was performed with eggplant peel concentrate, PPO-rich pool (PS-2), and other phenyl-Sepharose pools (PS1, PS3, PS4 and PS5). Histamine.2HCl equivalent to 1 mg/mL histamine base and 50% glycerinated PBS were used as positive and negative controls, respectively. A wheal diameter of >3 mm compared to the negative control was considered as positive. Blood was collected from 6 subjects selected from our previous studies (Harish Babu et al., 2008; Harish Babu and Venkatesh, 2009) having a clinical case history indicative of hypersensitivity to eggplant, positive SPT, and positive eggplant-specific IgE. Pooled sera from non-atopic subjects (without any history of eggplant allergy) were used to ascertain the specificity.

2.8. Detection of specific IgE to PPO protein bands and IgE-immunoblotting

Detection of allergen-specific IgE by ELISA (Hamilton and Adkinson, 2003) was carried out using sera from six eggplant-

allergic subjects as described earlier (Pramod and Venkatesh, 2004; Harish Babu et al., 2008; Harish Babu and Venkatesh, 2009). When the A₄₀₅ values were two-fold higher than that of non-atopic subjects' sera (serum pooled from 5 non-atopic subjects), the ELISA was considered positive. Eggplant peel concentrate was used as a positive control.

IgE-immunoblotting of PPO-rich pool (PS2) along with eggplant peel concentrate using pooled eggplant-allergic sera was carried out as described previously (Pramod and Venkatesh, 2004; Harish Babu et al., 2008; Harish Babu and Venkatesh, 2009). The pooled serum from 5 non-atopic subjects (without any history of eggplant allergy) was used as a negative control.

2.9. LC-MS/MS analysis

The putatively identified PPO bands (71 and 64 kDa) were subjected to tryptic digestion followed by LC-MS/MS analysis of peptides using Bruker Daltonics ultrafleXtreme instrument (Bruker, Billerica, MA, USA). The Coomassie-stained bands were destained, reduced, alkylated with iodoacetamide, and digested with TPCK-treated trypsin (Shevchenko et al., 2006). The peptide masses obtained were then compared with NCBI non-redundant protein database using MASCOT MS/MS Ions Search (<http://www.matrixscience.com>).

2.10. Bioinformatic approaches for prediction of structure and IgE epitopes

The prediction of structure and IgE epitopes of eggplant PPO was performed using bioinformatics methods similar to those employed in the case of sapodilla acidic thaumatin-like protein (Ashok Kumar and Venkatesh, 2014). The full-length sequences of six eggplant PPO precursors (PPO1 – ACT22523.1; PPO2 – ADG56700.1; PPO3 – ADY18409.1; PPO4 – ADY18410.1; PPO5 – ADY18411.1; PPO6 – ADY18412.1) were retrieved from the NCBI database and submitted for structure and IgE epitope prediction by bioinformatics approaches. The 3D structure of eggplant PPO4 was predicted by homology modelling *ab initio* using HHPRED-MODELLER. The predicted model was used to obtain the characteristics of an allergen using PDBSUM, CFSSP, Predict Protein, DAS server and ProtScale.

The IgE epitope prediction of the identified immunoreactive eggplant PPOs was performed using an algorithm, AlgPred server (www.imtech.res.in/raghava/algpred) (Saha and Raghava, 2006). The propensity of eggplant PPO1-PPO6 as an allergen was analyzed by FARRP, Allermatch and Evaller. The FAO/WHO criterion was adhered to predict the allergenicity of eggplant PPOs. AlgPred employs a four method hybrid approach to predict allergens: (i) MEME/MAST motif search, (ii) SVM (support vector machine)-based classification of allergens and non-allergens by single amino acid composition, (iii) SVM-based classification of allergens and non-allergens by dipeptide composition, and (iv) BLAST search against allergen representative peptides (ARP). The B-cell epitopes were predicted using BCPREDS, BepiPred, EPITOPIA and ELLIPRO.

3. Results

3.1. Extraction and partial purification of eggplant PPOs

The specific activity of PPO in 50% w/v eggplant peel extract was found to be 227 units/mg, and 251 units/mg in 20–80% (NH₄)₂SO₄ precipitate. The elution profile of eggplant peel extract on phenyl-Sepharose HP is presented in Fig. 1, and shows five distinct components labeled PS1-PS5 in the order of elution. PPO activity was present mainly in 3 components (PS1, PS2, and PS4) with the bulk of the PPO activity concentrated in component PS2 eluted

Table 1
SPT results of eggplant peel concentrate and its phenyl-Sepharose components on eggplant-allergic subjects.

Sample	Wheal/flare diameter (mm)						
	NS ^a	A1	A2	A3	A4	A5	A6
Negative control ^b	1/0	1/0	1/0	1/0	1/0	2/0	1/0
PS1 pool	1/0	4/12	4/20	4.5/16	4/12	4/16	4/20
PS2 pool (PPO-rich pool)	1/0	4.5/16	4/12	5/20	4.5/12	5/20	5/0
PS3 pool	1/0	3.5/0	4/12	4/0	4/0	3.5/0	4/0
PS4 pool	1/0	5/12	4.5/0	4/12	4/12	4.5/20	4/0
PS5 pool	1/0	3.5/0	4.5/0	3.5/0	4/0	4/0	3.5/0
Eggplant peel concentrate (50%, w/v)	1–2/0	5/20	4.5/16	4.5/20	4/20	6/25	5/16
Positive control ^c	5/20	5/25	5/20	5/20	5/25	6/25	5/25

^a NS, Non-atopic subjects; A1–A6: eggplant-allergic subjects.

^b Negative control: 50% glycerinated PBS.

^c Positive control: histamine.2HCl at 1.66 mg/mL (equivalent to 1 mg/mL histamine base) in 50% glycerinated PBS.

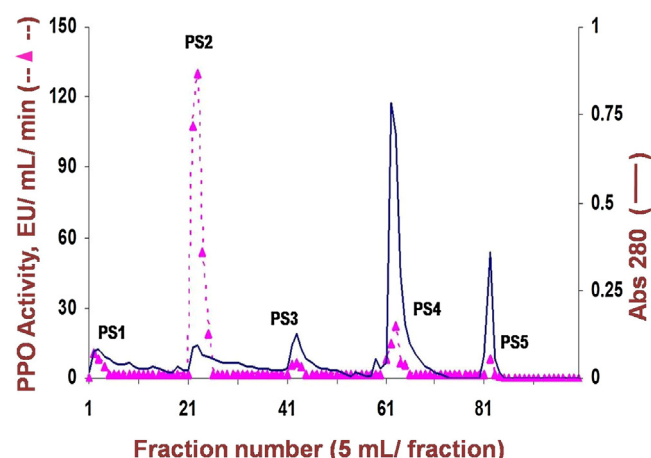


Fig. 1. Chromatographic profile of eggplant peel extract on phenyl-Sepharose HP. The column (1 × 5 cm) was equilibrated with PBS containing 1 M (NH₄)₂SO₄. Sequential step-elution was carried out using various concentration of (NH₄)₂SO₄ in PBS: 0.75 M (fractions 1–20, PS1); 0.5 M (fractions 21–40, PS2); 0.25 M (fractions 41–60, PS3); 0 M (fractions 61–80, PS4), or distilled water (fractions 81–100, PS5). PPO activity (–▲–) and A280 nm (—) of the chromatographic fractions are indicated. PPO activity was measured using 4-methyl catechol as the substrate.

with 0.5 M (NH₄)₂SO₄ (Fig. 1). The SDS-PAGE (reducing) pattern of the separated chromatographic components (Fig. 2) revealed that components PS2 and PS4 showed similar protein profile with respect to 2 major bands of 64 and 71 kDa (Fig. 2, panel a, lanes 3 and 5). The specific activity of PPO was highest in component PS2 (813 units/mg) followed by component PS1 (114 units/mg).

Although PS1 has fairly good specific activity of PPO, the protein amount was very low in component PS1 as indicated by the faint protein bands in SDS-gel profile (Fig. 2, panel a, lane 2). Therefore, the fractions eluted under PS2 were pooled together and designated as the PPO-rich pool.

Component PS3 (PPO specific activity: 69 units/mg) displays 7 protein bands in the region of 10–50 kDa (Fig. 2, panel a, lane 4). However, components PS4 and PS5 (PPO specific activity: 30 units/mg) showed many protein bands in the region of 10–80 kDa (Fig. 2, panel a, lanes 5 and 6). Although component PS4 exhibited moderately high PPO activity, the specific activity of PPO was very low (38 units/mg). Due to the multitude of protein bands in components PS3, PS4 and PS5, they were not considered for subsequent analyses. Further separation of proteins from the PPO-rich pool (PS2 component) on cation-exchange column (SP-Sepharose) was unsuccessful due to precipitation of proteins at the top of the chromatographic bed (data not shown).

3.2. Characterization of proteins present in fractionated eggplant peel extract

The results of SPT analysis with PS chromatographic components on 6 eggplant-allergic subjects are presented in Table 1. All the six subjects showed positive SPT with all the 5 components, namely, PS1–PS5. The wheal/flare response with PPO-rich pool (PS2) was comparable to that of eggplant peel concentrate.

ELISA was performed only with PPO-rich pool (PS2 component) as well as eggplant peel concentrate using individual eggplant-allergic serum (Table 2), while components PS1, PS2, PS3, PS4 and PS5 were not assessed due to limited availability of eggplant-

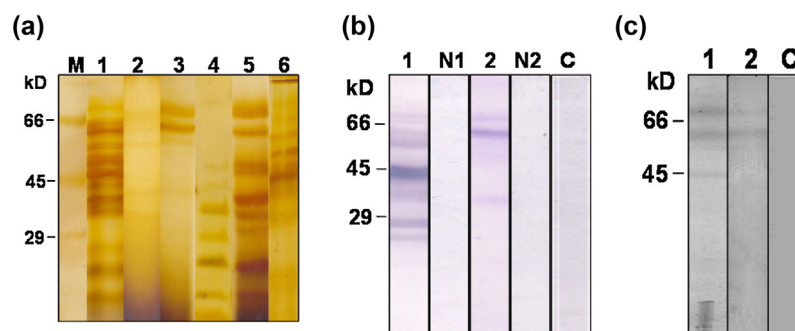


Fig. 2. Panel (a): SDS-PAGE (12%, reducing) pattern of eggplant peel extract and phenyl-Sepharose chromatographic pools (PS1–PS5). Lane 1: eggplant peel extract; lanes 2–6: chromatographic pools PS1–PS5, respectively; M: molecular weight markers. Panel (b): IgE-immunoblot of partially purified eggplant allergens probed with pooled eggplant-allergic sera. Lane 1: eggplant peel concentrate; lane 2: PPO-rich pool (PS2 component); lanes N1 and N2: eggplant peel concentrate and PPO-rich pool probed with pooled sera from non-atopic subjects, respectively; lane C: eggplant peel concentrate probed directly with the secondary antibody conjugate (murine monoclonal anti-human IgE-ALP) without the use of human sera to demonstrate the absence of non-specificity in the immunoblots. Panel (c): IgG-immunoblot of PS2 component using rabbit polyclonal anti-sweet potato PPO antiserum. Lane 1: eggplant peel concentrate; lane 2: PPO-rich pool (PS2 component); lane C: BSA and OVA probed directly with rabbit polyclonal anti-sweet potato PPO antiserum to demonstrate the absence of non-specificity in the immunoblots.

Table 2

Results of specific IgE to eggplant peel concentrate and PPO-rich pool (PS2 component).

Serum	ELISA units (A_{405}) ^a	
	Eggplant peel concentrate	PPO-rich pool (PS2) ^b
A1	0.433	0.402
A2	0.616	0.544
A3	0.594	0.481
A4	0.473	0.440
A5	0.482	0.454
A6	0.516	0.469
NS ^c	0.196	0.171

^a Mean of triplicate determinations; amount of protein coated per well: 25 μ g (eggplant peel concentrate), 5 μ g (PPO-rich pool); serum dilution, 1:3; secondary antibody dilution, 1: 1000.

^b PPO-rich pool (PS2 component).

^c NS, pooled sera from non-atopic subjects.

allergic sera; moreover, these 4 components revealed the presence of several proteins in them. Allergen-specific IgE values (A_{405}) for both eggplant peel concentrate as well as PPO-rich pool were comparable in all the 6 eggplant-allergic subjects and were significantly higher (>2-fold) than that of pooled normal sera ($p < 0.05$) (Table 2).

3.3. IgE-immunoblot and immunoreactivity of eggplant-allergic sera to PPO components

IgE-immunoblot was performed with eggplant peel concentrate and PPO-rich pool after the samples were subjected to 12% SDS-PAGE under reducing (Fig. 2, panel a) and native conditions (Fig. 3, panel a). When IgE-immunoblot was performed following SDS-PAGE (reducing), the pooled eggplant allergic serum detected three IgE-binding protein bands (43, 64 and 71 kDa) in the PPO-rich pool (Fig. 2, panel b, lane 2), whereas nine IgE-reactive protein bands in the 26–75 kDa range were detected in the eggplant peel concentrate (Fig. 2, panel b, lane 1). However, in the IgE-immunoblot after SDS-PAGE (native), only two IgE-binding protein bands (64 and 71 kDa) were detected in PPO-rich pool (Fig. 3, panel d, lane 2), while 5 major IgE-binding proteins in the 10–75 kDa region were found in the case of eggplant peel concentrate (Fig. 3, panel d, lane 1).

When rabbit polyclonal anti-sweet potato PPO antiserum was used to check its immunoreactivity with eggplant peel concentrate and PPO-rich pool (PS2), three proteins (43, 64 and 71 kDa) were detected in eggplant peel concentrate (Fig. 2, panel c, lane 1) whereas only the 64 and 71 kDa bands were detected in PPO-rich pool (Fig. 2, panel c, lane 2) in IgG-immunoblotting.

3.4. PPO activity staining and detection of proteins containing copper on PAGE

PPO activity was observed in 3 protein bands (43, 64 and 71 kDa) in both eggplant peel concentrate and PPO-rich pool (Fig. 3, panel b, lanes 1 and 2) by zymography under native conditions using 4-methyl catechol as the substrate. However, no enzymic activity bands were observed on the gel when the PPO-rich pool was previously incubated with tropolone, a known specific inhibitor of PPO (Fig. 3, panel b, lane 3). The cyanide-tetrazolium stain used for specific detection of copper-containing proteins on gels has revealed the presence of bound copper in the 64 and 71 kDa protein bands (Fig. 3, panel c) in both eggplant peel concentrate and PPO-rich pool.

3.5. LC-MS/MS analysis indicates that the 64 kDa protein is a PPO

The separation of eggplant extract on a 10% reducing long gel revealed that the putatively identified PPO bands (71 and 64 kDa)

each consists of two protein components (Fig. 3, panel e). The tryptic peptides of the two 71 kDa bands did not match with the known eggplant PPO sequences though it is not clear why this is so.

The MS/MS ions search carried out on the tryptic digests of 64 kDa resulted in significant matches for 'a' and 'b' bands (Fig. 4). A comprehensive MASCOT search against the NCBI nr database showed a significant identity of two peptide sequence match with *Solanum melongena* PPO1 (Accn. No. ACT22523; Fig. 4, panel a) for protein band 'a' and also with *Solanum melongena* PPO4 (Accn. No. ADY18410; Fig. 4, panel b) for protein band 'b' with a significant identity. This confirmed that the 64 kDa PPO band, in fact, contains two protein components, viz., eggplant PPO1 and PPO4.

3.6. Structure and IgE epitope prediction of eggplant PPOs

The predicted 3D structure of eggplant PPO4 showed that its secondary structure comprises of 65% β -sheets. Eggplant PPO4 (501 aa residues; pI: 5.77) was predicted to be localized in chloroplasts and has 4 predicted substrate-binding sites. BCPRED predicted 15 overlapping B-cell epitopes in eggplant PPO4.

FARRP analysis showed that eggplant PPO4 has a low propensity for cross-reactivity with known allergens. The FAO/WHO criterion was adhered to predict allergenicity of eggplant PPO. AlgPred employs a four method hybrid approach to predict allergens: MEME/MAST motif search, SVM-based classification of allergens and non-allergens by single amino acid composition and by dipeptide composition, and BLAST search against allergen representative peptides (ARP). The AlgPred analysis of eggplant PPO4 predicted the presence of a single IgE epitope (PKPGMGTIEN, residues 231–240) matching the allergenic IgE epitope (LKPGVDTIEN) existing in the AlgPred database (Fig. 4, panel b). The prediction of IgE epitope mapping for eggplant PPO1–6 using AlgPred is summarized in Table 3. It is interesting to note here that the allergenic IgE epitope (PKPGMGTIEN, residues 229–238) of eggplant PPO6 was identified by IgE epitope prediction by AlgPred, although eggplant PPO6 turned out to be a non-allergen by overall analyses and immunochemical analyses.

4. Discussion

Although eggplant contains very little protein (~1% on fresh weight basis), the presence of large number of protein bands on SDS-PAGE indicated the complexity of the protein profile of eggplant (Harish Babu and Venkatesh, 2009). A few reports on eggplant allergy have appeared in recent years especially from Asian and European countries (Pramod and Venkatesh, 2004; Lee et al., 2004; Gubesch et al., 2007; Harish Babu et al., 2008; Dey et al., 2014; Harish Babu and Venkatesh, 2009; Bansal, 2013), and several allergenic proteins were detected having preponderance in eggplant peel (Harish Babu and Venkatesh, 2009). Recently, two heat-stable allergens of 15 and 35 kDa have been detected in the Kamer variety of eggplant causing eggplant allergy in Iran (Hoseini-Alfatemi et al., 2015). Among the eggplant allergens in the 20–75 kDa region described from our earlier studies (Pramod and Venkatesh, 2004; Harish Babu and Venkatesh, 2009), it appeared to us that PPO could be a putative allergen in eggplant.

Among the enzymes present in eggplant, PPO has been studied extensively in terms of its enzymic activity (Dogān et al., 2002; Roudsari et al., 1981; Fujita and Tono, 1988; Shetty et al., 2011). PPOs are implicated in plant defense (Yoruk and Marshall, 2003; Mishra and Gautam, 2016), in a similar way to that of the allergenic proteins of PR family viz., chitinases, glucanases and peroxidases (Ebner et al., 2001; Clare Mills et al., 2009). Prior to 2012, eggplant PPO has been purified only partially whereas PPOs from potato and tomato have been purified to homogeneity (Pérez-Gilabert and

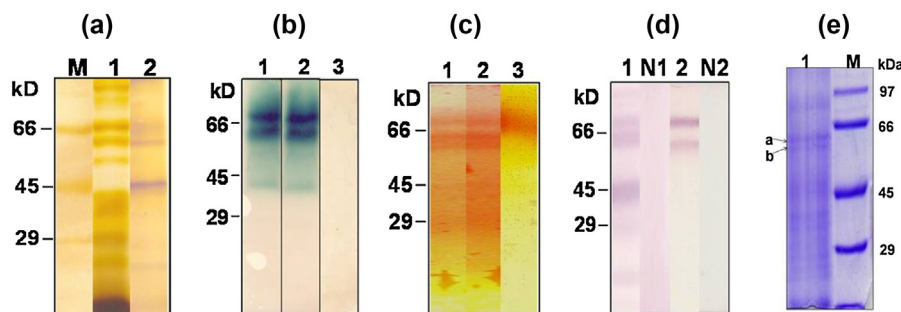


Fig. 3. Panel (a): 12% SDS-PAGE of PPO-rich pool under partially denaturing condition (silver staining). The numbers on the left indicate molecular weight in kDa. Lane 1: eggplant peel extract; lane 2: PPO-rich pool (PS2 component); M: molecular weight markers. Panel (b): PPO activity staining on partially denaturing SDS-PAGE (12%). Lane 1: eggplant peel concentrate; lane 2: PPO-rich pool; lane 3: PPO-rich pool incubated with 2 mM tropolone, a known inhibitor of PPO. Panel (c): Detection of copper-protein complex on partially denaturing SDS-PAGE (12%). Lane 1: eggplant peel concentrate; lane 2: PPO-rich pool; lane 3: hemocyanin cross-linked molecular weight marker. Panel (d): IgE-immunoblot of PPO-rich pool probed with pooled eggplant-allergic sera. Lane 1: eggplant peel concentrate; lane 2: PPO-rich pool; lanes N1 and N2 correspond to eggplant peel concentrate and PPO-rich pool probed with pooled sera from non-atopic subjects, respectively. Panel (e): SDS-PAGE (12%, reducing) pattern of eggplant peel extract on a long gel. The 64 kDa band resolves further into 'a' and 'b' bands.



MASCOT Search Results

Panel (a) – 64 kDa _ PPO band 'a'

Search title : PPO_64kDa
Database : NCBIInr
Top Score : 92 for gi|251851953
Chloroplast polyphenol oxidase precursor [*Solanum melongena*]
Theoretical MW : 68256
Theoretical pI : 7.22
Matched peptides shown in *bold red*.

1	MASVCHTSTA	TLKSSFIPSP	NSLGSTPKPS	QLFLHGKRNO	AFKVSCKVTN
51	NNGDONQNVV	DTNSVDRNV	LLGLGGLYGV	ANAIPLAASA	TPIAPNAPS
101	CGTATISDGP	EVPTCCPPG	MPEDIEKILY	YKFPSATKLR	IRQPAHAVDE
151	ELIAKYNLAI	SKMRELDITD	HFSPPLAFKQ	ANIHCAVCNG	AYKIGGKELQ
201	VHNSWLFEPF	HRWLYFYER	ILGKLIDPPT	FALPYWNWDH	PKGMRLPPIF
251	DRQGTALYDE	RRSTQVRNGT	VMDLGSFGDK	VQTTQLQLMS	NNLTIMYRQM
301	VINAPCLPMI	LWRTLRSEI	TLKPQETVEV	IPHIPVHIWV	GTARGSKFPD
351	GSTSYGEDMG	NEFSAGLDPV	FYCHHSNVDV	MWNEWKQIGG	KRRDISQRDW
401	LNSEFFFFDE	NKNPYRVRVR	DCLDTKTMGY	DYAPMPTPWR	NFKPKTKASS
451	GKANTSAPPP	ASQVYPLAKM	DKVITFSIKR	PASSRTQKEK	NEKEEMITFN
501	NIKYDNREYV	RFDVFLNVDN	NVNANELDKA	EFAGSYTSLP	HVHRASQTDH
551	VATATLQLAI	TELLEDIGLE	DEDTIAVTLV	PKKGGEGISI	EGVEISLADC

Panel (b) – 64 kDa _ PPO band 'b'

Search title : PPO_64kDa
Database : NCBIInr
Top Score : 84 for gi|324105298
Chloroplast polyphenol oxidase Precursor, partial [*Solanum melongena*]
Theoretical MW : 66973
Theoretical pI : 6.29
Matched peptides shown in *bold red*.

1	SSSTLPLCNS	KSLFFSFCNS	PFLPQPSKLF	LQRTSRQRFK	VSCNANNVGE
51	HDKNLDAVDR	RNVILGLGGL	YGAANLAPLA	ANAAPIPPPE	LKTCGRVVVN
101	DTTGELVYYS	CCPPIPDID	SVPIYKFPSP	TKLRIRPPAH	AVDEEYIAKY
151	QLATSQMREL	DKDPFGPIGF	KQQANIHCAV	CNGAYKAGKG	ELQVHFSWLF
201	FPFHRWLYLF	YERILGSLIN	DPTFALPYWN	WDHPKGMRLP	PMFDVEGSSL
251	YDAKRNSHR	NGKIIDLGF	GQETETTELO	TMTNNLTLMY	RQMTVNAPCP
301	LLFFGNPYPL	GTDPKPGMG	IENLPHTPVH	IWTGDSRQRP	NGEDMGNFYS
351	AGLDPVFYCH	HANVDRMWNE	WKAIGGKRRD	LADKDWLNSE	FFFYDENRNP
401	FKVKVRDCLD	SKKMGFDYAP	MPTPWRNFKE	VRRTTSGKAN	TRSIPPASKV
451	FPTCETRQSD	FIFHRQTSFV	KDSKAEKNEQ	EGDTNIRIQY	YDSSQYVRFD
501	VFLNVDKTVK	ALELDQPEFA	GSYTSPLPHVH	GDKDRAPVTF	KLAITELLED
551	NNLEDEESIV	ITLIPKAGGD	GISIONAVID	LVDG	

Fig. 4. LC-MS/MS analysis of eggplant PPO (64 kDa band). Panel (a): 64 kDa band 'a' indicating the matched tryptic peptide sequences (in red) of eggplant PPO1. The mature protein sequence starts from residue 91 (510 residues, molecular mass of mature unglycosylated protein = 58,291 Da (average); has 3 potential glycosylation sites). Panel (b): 64 kDa band 'b' indicating the matched tryptic peptide sequences (in red) of eggplant PPO4. The mature protein sequence starts from residue 84 (501 residues, molecular mass of mature unglycosylated protein = 57,322 Da (average); has 1 potential glycosylation site). The signal peptides in both the sequences are underlined (...). The individual ions score > 50 indicates identity or extensive homology ($p < 0.05$). The predicted IgE epitope of *Solanum melongena* PPO4 by using AlgPred algorithm is highlighted in box in panel (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

García-Carmona, 2000; Partington and Bolwell, 1996; Kwon and Kim, 1996; Casado-Vela et al., 2005). Only recently, eggplant PPO has been purified to homogeneity in a yield of 0.37 mg/kg eggplant (cv. bangi kotary) (Mishra et al., 2012); the native eggplant PPO was found to be a homodimer of two 56 kDa subunits. Since many plant PPOs have been purified using hydrophobic interaction chro-

matography (Partington and Bolwell, 1996), purification of PPO from eggplant peel was attempted using phenyl-Sepharose. The majority of the PPO activity was found in the pool eluted with 0.5 M $(\text{NH}_4)_2\text{SO}_4$; however, it proved very difficult to further purify any of the other proteins from PS2 (PPO-rich pool) due to low amounts of protein in conjunction with protein lability during the purifica-

Table 3
In silico IgE epitope mapping analyses of eggplant PPO1–6 using AlgPred.

Mode of analysis	PPO1	PPO2	PPO3	PPO4	PPO5	PPO6
Mapping of IgE epitopes and PID	–	–	–	+	–	+
MEME/MAST motif ^a	–	–	–	–	–	–
SVM module based on amino acid composition	–	–	+	+	+	–
SVM module based on dipeptide composition	–	–	+	+	–	–
BLAST search on allergen representative peptides (ARPs)	–	–	–	–	–	–
Hybrid approach (SVMc + IgE epitope + ARPs BLAST + MAST)	–	–	–/+	+	–/+	+/-

+ indicates allergen; – indicates non-allergen.

^a Multiple Em for Motif Elicitation/Motif Alignment & Search Tool.

tion. Similar observations have been made during the purification of a glycosylated PPO from potato (Kwon and Kim, 1996).

Comparable magnitude of wheal/flare response observed in SPT with PPO-rich pool and eggplant peel concentrate with all the 6 eggplant-allergic subjects obviously demonstrate the ability of PPO-rich pool to cause activation of mast cells bearing specific IgE. Detection of moderate levels of specific IgE to PPO-rich pool and eggplant peel concentrate in eggplant-allergic serum samples compared to pooled non-atopic serum clearly indicates that PPO is an important allergen in eggplant.

The presence of intense PPO activity in the 64 and 71 kDa protein bands by zymography in the case of both PPO-rich pool and eggplant peel concentrate indicated the presence of at least two different PPOs in eggplant peel, similar to the reported observations for PPOs from potato (60 and 69 kDa), tomato (50 and 52 kDa) and other plants (Partington and Bolwell, 1996; Kwon and Kim, 1996; Casado-Vela et al., 2005). The appearance of a weak PPO activity band at 43 kDa could be due to proteolytic degradation of the PPO isoform(s) during extraction and/or purification similar to those observed for other plant PPOs (Ganesa et al., 1992). Since many plant PPOs including eggplant are known to exist in the genome as multigene families, they are generally expressed as different gene products (Shetty et al., 2011; Partington and Bolwell, 1996; Kwon and Kim, 1996; Casado-Vela et al., 2005; Ganesa et al., 1992; Yoruk and Marshall, 2003; Marusek et al., 2006; Mishra and Gautam, 2016). A wide variety of behavior has been described for plant PPOs which indicate the existence of heterogeneity in their enzymic characteristics, number of enzymic forms and molecular masses (Yoruk and Marshall, 2003; Marusek et al., 2006; Mishra and Gautam, 2016). The abolition of PPO activity bands in the PPO-rich pool incubated with tropolone (a specific inhibitor of PPO) further confirms that the observed bands by zymography are indeed PPOs.

The presence of two copper atoms in the protein is a characteristic feature of all PPOs (Kuwabara and Katoh, 1999; Yoruk and Marshall, 2003; Marusek et al., 2006; Flurkey and Inlow, 2008). The cyanide-tetrazolium staining, which is very specific for detection of copper-protein complex on gels, clearly shows that the 64 and 71 kDa proteins contain copper, a characteristic marker to infer that the 64 and 71 kDa proteins in eggplant peel extract and PPO-rich pool are polyphenol oxidases. However, the reason for the non-detection of 43 kDa protein band on the gels with copper-protein stain is not clear. In addition, rabbit polyclonal anti-sweet potato PPO antiserum shows immunoreactivity to 64 and 71 kDa proteins clearly demonstrating that, among the proteins present in PPO-rich pool, the 64 and 71 kDa proteins are PPOs since an extensive sequence homology exists among the plant PPOs (Shetty et al., 2011; Kuwabara and Katoh, 1999; Yoruk and Marshall, 2003; Marusek et al., 2006; Flurkey and Inlow, 2008). It is obvious from the IgE-immunoblots of PPO-rich pool that the 64 and 71 kDa PPOs display strong IgE-binding with pooled eggplant-allergic serum, thus providing unequivocal evidence for the identification of the 64 and 71 kDa allergens in eggplant peel as PPOs. The 71 kDa band might represent one or more of eggplant PPO precursors (pre-

dicted molecular mass of 66–68 kDa for unglycosylated PPO1–PPO6 precursors) and is consistent with the earlier observation of similar mass for eggplant extracts (Shetty et al., 2011). In addition, the 43 kDa protein is also recognized in IgE-immunoblots. The reported molecular masses of PPO from potato, tomato, broad bean, spinach, banana and other plants are in the range of 60–65 kDa and 40–45 kDa (Partington and Bolwell, 1996; Kwon and Kim, 1996; Casado-Vela et al., 2005; Ganesa et al., 1992; Yoruk and Marshall, 2003; Marusek et al., 2006; Flurkey and Inlow, 2008). Further, the 64 and 71 kDa proteins are known to be glycoprotein allergens in eggplant peel having long persistence in simulated gastric fluid (Harish Babu and Venkatesh, 2009). It is evident from the literature that many plant PPOs are glycoproteins as in the case of potato, tomato and other plants (Partington and Bolwell, 1996; Kwon and Kim, 1996; Casado-Vela et al., 2005; Ganesa et al., 1992).

The analysis of tryptic peptides of eggplant 64 kDa protein bands showed significant similarity in peptide matches to the eggplant PPO sequences available in the database. Eggplant PPO is known to exist as a multigene family encoded by *SmePPO1–SmePPO6* genes (Shetty et al., 2011). The Mascot search showed two peptide sequence similarity with eggplant PPO1 and PPO4. AlgPred server predicted the allergenicity potential of the eggplant PPO by matching the computationally predicted IgE epitopes against the known IgE epitopes available in the database. AlgPred analysis predicted the presence of a single IgE epitope in eggplant PPO4; the predicted IgE epitope was found to share 78% sequence similarity with the IgE epitope of a cross-reactive 48 kDa latex allergen identified as enolase (Hev b 9) from *Hevea brasiliensis* (Wagner et al., 2000). So far, only one copper protein has been identified as a cross-reactive allergen (30 kDa L-ascorbate peroxidase) from bell pepper involved in latex-fruit syndrome (Wagner et al., 2004). Although only one IgE epitope has been predicted in *SmePPO4*, it is probable that one IgE epitope binds to specific IgE and the adjacent IgE molecule binds to another IgE epitope of a second PPO4 molecule, since eggplant PPO is known to exist as a dimer under native conditions (Mishra et al., 2012).

5. Conclusions

Six cases of eggplant allergy were confirmed by positive SPT and presence of allergen-specific IgE. Eggplant peel extract was fractionated on phenyl-Sepharose into 5 components (PS1–PS5) of which PS2 component exhibited high PPO specific activity. Although the PS2 component contained several allergens by immunoblot, a 64 kDa protein was confirmed as an allergen (Sola m PPO4) based on the presence of bound copper, detection by polyclonal sweet potato PPO antiserum and bioinformatics analysis including prediction of IgE epitope. To the best of our knowledge, this is the first description of identification of any of the allergens reported from the multi-allergenic vegetable – eggplant – as well as the first report identifying plant PPO as an allergen.

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The sponsor (DBT, New Delhi) did not have any involvement in the study design, collection, analysis and interpretation of data, in writing the report and in the decision to submit the article for publication.

Conflicts of interest

None.

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