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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.

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); lipoxygenase, 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 acts 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 × *g* for 20 min. The supernatant was treated with 3% polyvinyl polypyrrolidone (PVPP) with continuous stirring for 30 min (to remove interfering phenols), centrifuged at 10000 × *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 $(NH_4)_2SO_4$ between 20 and 80% saturation, and the 80% pellet was solubilized in 30 mL of PBS, centrifuged at 10000 × *g* for 20 min, and the resulting supernatant was dialyzed against PBS containing 1 M $(NH_4)_2SO_4$ for 24 h with two changes of buffer. The retentate was further clarified by centrifugation at 10000 × *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 × 5 cm) previously equilibrated with PBS containing 1 M $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ followed by 0.75, 0.5, 0.25, 0 M $(NH_4)_2SO_4$, 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 (Doğan 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 ~25 μg 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