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Original Article

Identification of protease-resistant proteins from allergenic nuts using two-dimensional gel electrophoresis and mass spectrometry

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Abstract

Nuts are one of the most common sources of allergies in individuals of all ages. In order for a particular protein to render an allergic reaction, it must resist proteolytic digestion by intestinal enzymes. In this study, three well-known allergenic nuts, almonds, cashew nuts, and peanuts, were used as samples, and enzyme digestion with Bacillus protease and porcine pepsin was tested. A proteomic approach using two-dimensional gel electrophoresis and an MS/MS analysis was applied to visualize and identify the proteins that were resistant to enzyme digestion. Among the 150 protein spots tested, 42 proteins were assigned functions. Due to the lack of genomic databases, 41% of the identified proteins were grouped as hypothetical. However, 12% of them were well-known allergens, including AraH. The remainder were grouped as storage, enzymes, and binding proteins.

Keywords : nut proteins, protease resistant protein, allergen, pepsin, MS/MS

Introduction

Nuts can provide a significant portion of protein, fiber, folic acid, and vitamin E in the human diet, making them a “super food”. Yet, despite their high nutritional value, nuts can pose risks to some individuals in the form of allergies. Nut allergies are the second most common food allergy (Katz, 2012), where nuts from both leguminous and tree sources can produce allergic reactions. Peanuts are most likely to cause anaphylaxis and death, with estimates of one death for every 200 cases of anaphylaxis. Studies have also shown that individuals with peanut allergies are also most likely to have tree nut allergies (Roux et al., 2002). In the registry maintained by the United States-based Food Allergy and Anaphylaxis Network, it was reported that cashew and almonds affected 20% and 15% of the respondents, respectively (Teuber *et al*, 2002). Meanwhile, in Australia, IgE-mediated food allergies are an emerging epidemic with an incidence of 1 in 10 infants, and the trend is increasing (Prescott and Allen, 2011).

Many food allergens have been found to be stable under conditions stimulating human gastrointestinal digestion. Thus, food allergens are usually stable under heat and acidic conditions and relatively resistant to proteolytic digestion (Kopper *et al*, 2004). However, the digestive stability of allergenic food proteins relative to nonallergenic proteins has not yet been systematically established (Akkerdaas et al., 2005).

While clinical studies have shown that young children are the most prone to food allergies, where the occurrence is often associated with eczema and rashes in early infancy, little is known about the causes of nut allergies. The most effective way of reducing or preventing food allergies is eliminating the allergy before it begins and acquiring tolerance to allergens (Katz, 2012). Thus, understanding the biochemical features of nut proteins can provide ways of preventing the prevalence of nut allergies. In addition to exploring the allergenic potential of nuts, the significance of protease-resistant proteins as fiber is another important area for investigation, as dietary fiber has been described as a substance impervious to degradation by human endogenous enzymes (East wood et al., 2005).

Accordingly, this study attempted to identify the proteins in nuts that are resistant to enzymatic digestion. The samples used included peanuts (*Arachis hypogea*), as a representative of leguminous nuts, almonds (*Prunus dulcis*), which is often consumed as an ingredient in chocolate bars and pastries, and cashews (*Anacardium occidentale*), which based on previous studies is the most common cause of strong allergic reactions. Thus, SDS-PAGE electrophoresis and tandem mass spectrometry were both used to identify the proteins from well-known allergenic nuts that are resistant to enzymatic digestion.

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Materials and Methods

Nut Protein Extraction

The nut samples (cashews, almonds, peanuts, and soybeans) were crushed and the proteins from each sample solubilized with a 0.1X Phosphate buffered saline solution (PBS) by incubation at 4℃ for 2 hours. The supernatant was then collected at 10000 rpm, 20 minutes.

Digestion with Bacillus Protease

The soluble proteins were treated with the protease enzyme from *Bacillus* (Sigma, St. Louis, MO, USA) and incubated overnight (18 hours) at 37℃ with agitation. The supernatant fractions were obtained after high-speed centrifugation (15000 g), desalted using a PD 10 column (BioRad, Herculus, CA, USA), and precipitated using 10% trichloroacetic acid (TCA). The precipitate was then washed with ethanol, dried, and dissolved in a minimum volume of a rehydration buffer. Thereafter, the two-dimensional electrophoresis was conducted. Protein spots were picked and excised from the gels, washed three times with deionized water, and destained by vigorous vortexing in 50 mM ammonium bicarbonate/ acetonitrile (6:4). The gel pieces were dried using a speed vacuum, and then digested with 10 ng/ul Trypsin. The peptides were extracted using 0.1% TFA in 60% acetonitrile and concentrated to a final volume of 10 uL using the speed vacuum. Finally, the peptides were identified using an MS/MS analysis.

Digestion with Pepsin

The conditions of the human stomach were mimicked to determine the resistance or digestibility of the nut proteins by the pepsin enzyme. The nut proteins were digested using simulated gastric fluid containing 0.03 M sodium chloride, 0.32% pepsin, and hydrochloric acid, resulting in a solution

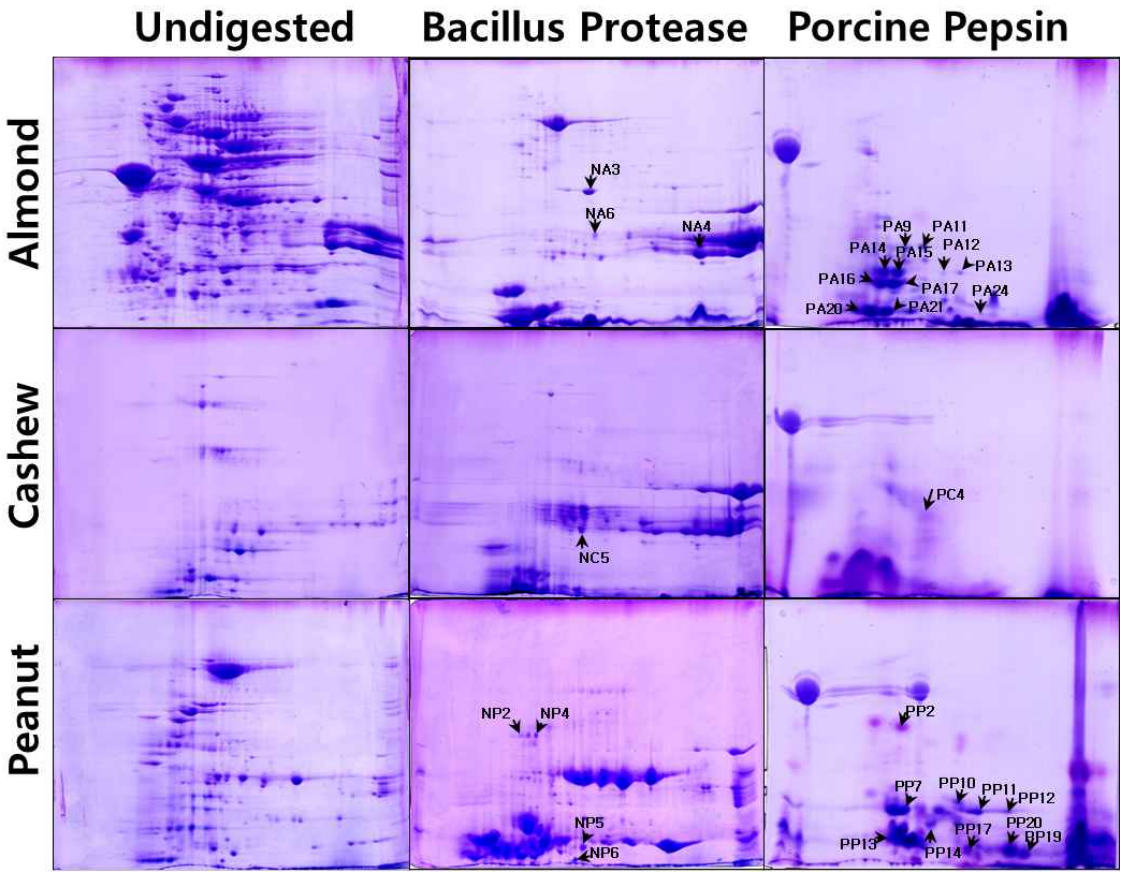

Figure 1. Proteins from well-known allergenic nuts are resistant to proteases

2-D gel images of proteins from almonds, cashew nuts, and peanut were obtained after overnight digestion with *Bacillus Protease* and *Pepsin* from porcine gastric mucosa. Proteins were separated by 17-cm IPG strips, pH 3-10 and gels stained with Coomassie Brilliant Blue R-250. Protein spots indicated by arrows were excised and trypsinized for identification by MS/MS analysis.

pH of 1.2. For the almonds, the protein extracts were digested for 30 minutes and one hour, while the soy proteins were digested for 1, 2, and 4 hours. The reactions were stopped using 1M sodium hydroxide. Thereafter, the samples digested with pepsin were desalted using a PD10 column and the filtrate lyophilized, dissolved in a rehydration buffer, and subjected to two-dimensional SDS-PAGE electrophoresis and an MS/MS analysis. Similar procedures (protease digestion) were carried out for the peptide preparation and extraction.

MALDI-Q-TOF MS/MS analysis

The protein spots in the 2D SDS-PAGE were excised and similar

Table 1. Identification of protease resistant proteins from nuts

No	Accession No.	Score	Species	Protein Name	Function
PA4	Q9MV55_9CONI	47	*Pseudolarix amabilis*	Maturase	enzyme
PA9	Q7Y1C0_ARAHY	65	*Arachis hypogea*	*Allergen Ara h 2 isoform*	*allergen*
PA11	T47467	56	*Arabidopsis thaliana*	hypothetical protein	hypothetical
PA12	ABA99086	53	*Oryza sativa*	DP000011 NID	hypothetical
PA13	T50002	47	*Arabidopsis thaliana*	hypothetical protein	hypothetical
PA14	Q8LES9_ARATH	64	*Arabidopsis thaliana*	Hypothetical protein	hypothetical
PA15	Q5JJX6_ORYSA	62	*Oryza sativa*	Pr1-like protein	protein folding
PA16	Q8GV20_ARAHY	50	*Arachis hypogea*	*Allergen Ara h2 isoform*	*allergen*
PA17	Q6PSU1_ARAHY	66	*Arachis hypogea*	*Seed storage protein*	*allergen*
PA20	Q7XY71_9ROSI	65	*Kandelia candel*	Cyclophilin	protein folding
PA21	ABF95015	66	*Oryza sativa*	DP000009 NID	hypothetical
PA24	Q5JKW6_ORYSA	41	*Oryza sativa*	Hypothetical protein	hypothetical
PP2	Q6TBE7_9ROSI	51	*Calophyllum soulattri*	PHYC (Fragment)	transduction
PP7	Q6J603_SOYBN	60	*Glycine max*	LRR-kinase protein	enzyme
PP8	S31705	54	*Glycine max*	4-coumarate-CoA ligase	enzyme
PP10	Q7XLL5_ORYSA	77	*Oryza sativa*	hypothetical protein	hypothetical
PP11	Q5NAM4_ORYSA	57	*Oryza sativa*	Hypothetical protein	hypothetical
PP12	Q6AUR2_ORYSA	47	*Oryza sativa*	P-II nitrogen sensing protein	transcription
PP13	Q6ZG74_ORYSA	79	*Oryza sativa*	Hypothetical protein	hypothetical
PP14	Q6ZL68_ORYSA	39	*Oryza sativa*	Hypothetical protein	hypothetical
PP17	Q5Z5M6_ORYSA	45	*Oryza sativa*	Hypothetical protein	hypothetical
PP19	Q75LJ7_ORYSA	50	*Oryza sativa*	Putative RNA binding protein	protein binding
PP20	T01086	85	*Arabidopsis thaliana*	serine/threonine protein kinase	enzyme
PC4	Q5K496_SILDI	39	*Silene dioica*	calcium dependent protein kinase	enzyme
NA3	Q43607_PRUDU	120	*Prunus dulcis*	Prunin precursor	storage
NA4	S51942	70	*Prunus dulcis*	prunin 2 precursor	storage
NA6	ABF95817	66	*Oryza sativa*	DP000009 NID	hypothetical
NC5	gi|9558423	52	*Oryza sativa*	Hypothetical protein	hypothetical
NP2	S24044	52	*Arachis hypogea*	Lectin precursor	galactose binding
NP4	Q5I6T2_ARAHY	55	*Arachis hypogea*	Arachin Ahy-4	storage
NP5	Q6T2T4_ARAHY	55	*Arachis hypogea*	Storage protein	storage
NP6	Q8LKN1_ARAHY	55	*Arachis hypogea*	*Allergen Arah3/Arah4*	*allergen*

procedures to the protease digestion were carried out for peptide preparation and extraction. The peptides were then subjected to a MALDI-Q-TOF tandem mass spectrometry analysis using Protein Lynx™ Browser (Waters, Milford, MA, USA) for identification.

Results and Discussion

Protease-resistant proteins

Proteomic approaches were used to delineate the proteins that remained resistant to digestion after enzymatic action. Figure 1 shows the two-dimensional SDS-PAGE analysis of the nut samples after digestion with the protease enzymes (Bacillus Protease). When compared with the gel image of the nut samples without enzymatic digestion (Undigested) in Figure 1, several discrete spots were visible, even after protease digestion, suggesting that these spots were proteins that were resistant to protease digestion. An MS/MS analysis of 150 spots showed that these proteins included the widely known allergen Arah3/Arah4 and some storage proteins, such as prunins and arachins (12%) (Table 1). Both prunins and arachins are hexamer proteins, wherein each subunit is composed of an acidic and basic chain derived from a single precursor and linked by a disulfide bond. The resistance of a protein to digestion by the harsh conditions of the gastro-intestinal tract is attributed to its compact three-dimensional structure, ligand-binding, disulphide bonds, and glycosylation (Mills and Breiteneder, 2005). Thus, the disulfide bonds of prunins and arachins make them resistant to protease digestion.

Pepsin-resistant proteins

Simulated gastric fluid was used to determine the resistance of the proteins to pepsin digestion, which mimics the conditions of the human stomach. Figure 1 (Porcine Pepsin) shows two-dimensional gel images of the almonds, cashews, and peanuts after pepsin digestion. In the images, the pepsin appears as a dominant spot located in the acidic pH region. Plus, there are a number of proteins that are undigested by the pepsin, as shown by the spots on the SDS-PAGE gels. These proteins were resistant to digestion in the gastric model (simulated gastric fluid), making them potential allergens. This data is consistent with the hypothesis that food allergens must exhibit sufficient gastric stability to reach the intestinal mucosa where absorption and sensitization (development of atopy) can occur. Moreover, the data in this study falls into the range of known food allergens that have molecular weights between 10 and 70 kDa, stimulate the immune response (induce the production of allergen-specific IgE), and are stable molecules that are resistant to processing, cooking, and digestion.

For identification, the enzyme-resistant proteins were excised from the 2-D gels and processed for an MS/MS analysis. Table 1 shows the list of the identified proteins. The undigested proteins were classified as storage proteins, allergens, metabolic enzymes, and binding and transcription factors (Figure 2). As the genomic database for nut species is not yet well established, several proteins were identified as hypothetical proteins with unknown functions. This was also why the searches were not restricted only to nuts.

In this study, the majority of the identified allergens were obtained from the almonds. Previous studies have identified Arah1 as a major allergen in peanuts. Similarly, most of the identified allergens were *Arah* isoforms, such as *Arah2* and *Arah3/4* (Nicolaou and Custovic, 2011). These allergens have already been shown to be stable under conditions simulating human gastrointestinal digestion. Resistance to digestion is still considered by many a relevant parameter for assessing the allergenic potential of proteins (Fu *et al*, 2002). Another storage protein, seed storage protein *SSP2* (*Fragment*) was also previously identified as an allergen from almonds (Teuber et al., 2002). Most groups of proteins belonging to a storage or structural protein classification are inherently more resistant to proteolysis in cellular environments than other types of proteins, such as enzymes, making them a highly probable allergen candidate. The identification of these allergens could have a significant impact in further studies based on generating recombinant allergens for future immunotherapeutic approaches.

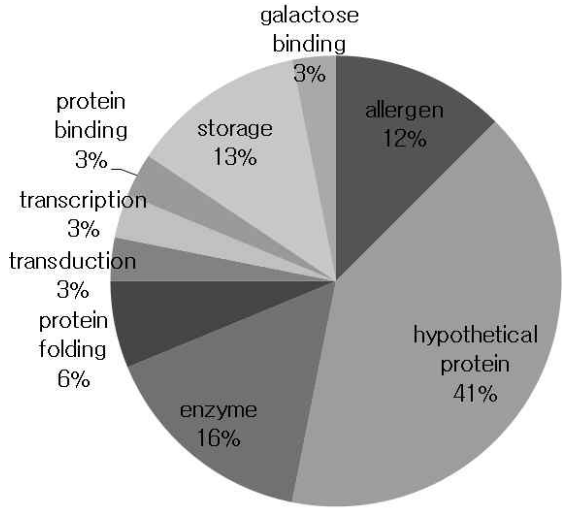

Figure 2. Distribution of identified nut proteins into their respective functional class

One probable function of such undigested proteins is as dietary fiber. Indigestible proteins are considered part of the dietary fiber composite. The charged groups in these proteins would be expected to be involved in binding. Among the identified proteins, *putative RNA binding protein* and *lectin precursor*, which function on protein and galactose binding, respectively, could qualify as a component of dietary fiber. Indigestible proteins may play a significant role in the observed physiological effects of dietary fiber in the lower digestive tract (Saunders et al., 2002).

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