

Proteomic Screening Points to the Potential Importance of Ara h 3 Basic Subunit in Allergenicity of Peanut

Baozhu Guo^{*,1}, Xianqiang Liang², Si-Yin Chung³ and Soheila J. Maleki³

¹*Crop Protection and Management Research Unit, Agricultural Research Service, US Department of Agriculture, Tifton, Georgia 31793, USA*

²*Institute of Crop Sciences, Guangdong Academy of Agricultural Sciences, Guangzhou, China*

³*Food Processing and Sensory Quality Research Unit, Southern Regional Research Center, Agricultural Research Service, US Department of Agriculture, New Orleans, Louisiana 70124, USA*

Abstract: Peanuts are complex storage proteins with high contents and have been identified as one of the most allergenic foods. In this review, we summarize some of the latest findings and the potential importance of the Ara h 3 basic subunit, which has been overlooked as an allergen in early literature. Some recent studies indicate that Ara h 3 basic subunit may be as significant as or even a more important allergen than the acidic subunit. For example, one clinical study found a group of children with peanut allergy who were specifically sensitized to the basic subunit of Ara h 3. Although, proteomic analysis of total peanut storage proteins has revealed limited polymorphic profiles of major proteins in diverse peanut germplasm accessions, a study reported a peanut breeding line 'GT-C9' lacking several seed protein peptides, in which the missed major proteins were basic subunits of Ara h 3. This breeding line was shown to exhibit significantly lower levels of advanced glycation end (AGE) products and IgE binding by the sera of peanut allergic patients, which implies a role for the basic subunit of Ara h 3 in the allergenicity of peanuts. Further studies are needed to investigate the contribution of Ara h 3 basic subunits to peanut allergenicity.

Keywords: *Arachis hypogaea* L., groundnut, Allergen, Ara h 3, basic subunit.

INTRODUCTION

Peanuts have been identified as one of the most allergenic foods with hypersensitivity reactions occurring in about 1% or more of children in the U.K. and the U.S. [1]. Allergic reactions to peanut are immunoglobulin E (IgE) mediated, often severe and life threatening, and resolve in only ~20% of school aged children [2]. Avoiding peanut in food products is difficult because it is used in a wide range of processed foods since peanuts have many health benefits. However, complete avoidance of peanut and peanut-containing products by allergic individuals is the most effective management strategy. Because of the health benefits of peanuts, it is important for the peanut industry and consumers that new approaches to reduce the food allergy risks associated with peanut be identified, such as breeding a peanut with reduced allergenic potential.

The majority of peanut allergens are seed storage proteins which play an important role as sources of nitrogen and amino acids during germination [3]. The International Union of Immunological Societies Allergen Nomenclature Subcommittee published 'official list of allergens' as January of 2008 and all 9 peanut allergenic proteins, Ara h 1-9, are recognized in peanuts (<http://www.allergen.org>). Peanut Ara h 1 was the first allergen described [4] and identified as a major peanut allergen [5] of 63 kDa and has gained a considerable amount of attention in recent years [6-9]. In addition to Ara h

1, 8 other peanut allergens identified are Ara h 2 [10-12] of 17 to 20 kDa that is the most potent peanut allergen [13], Ara h 3 [14,15] consisting of a series of polypeptides ranging from 14 to 45 kDa, Ara h 4 to Ara h 7 [16] of 14 to 36 kDa, Ara h 8 and Ara h 9 [17,18] that are between 7 to 18 kDa. In this review, we summarize recent developments in proteomic screening for major differences in peanut storage proteins and aim to raise the awareness of the potential importance of Ara h 3 basic subunit in peanut allergenicity.

PEANUT GENETIC DIVERSITY IN STORAGE PROTEINS

One possible effort to alleviate the peanut allergy problem is to identify peanut germplasm accessions with lower levels of allergens which could be used in conventional breeding to produce a less allergenic peanut cultivar. We have examined some peanut genotypes by separation of the total storage proteins on SDS-PAGE (Fig. 1). The protein profiles revealed few major differences among the tested genotypes on SDS-PAGE. The major peanut allergens (Ara h 1, Ara h 2, and Ara h 3) are resolved as indicated in Fig. (1). All genotypes had a strong band of 63 kDa Ara h 1 and two bands of Ara h 2 in the range of 17 to 20 kDa. Majority tested peanut genotypes had three strong bands in the range of 36 to 45 kDa, which correspond to acidic subunit of Ara h 3. Several tested genotypes did not have a 36 kDa polypeptide, one of the acidic subunits of Ara h 3.

Guo *et al.* [19] reported more detailed 2-dimensional (2-D) PAGE protein profiles of total peanut storage proteins and identified one peanut breeding line 'GT-C9' lacking several protein peptides, which were identified as Ara h 3 acidic (minor spots 1,2,5,6) and basic subunits (major spots

*Address correspondence to this author at the Crop Protection and Management Research Unit, Agricultural Research Service, US Department of Agriculture, Tifton, Georgia 31793, USA;
E-mail: Baozhu.Guo@ars.usda.gov

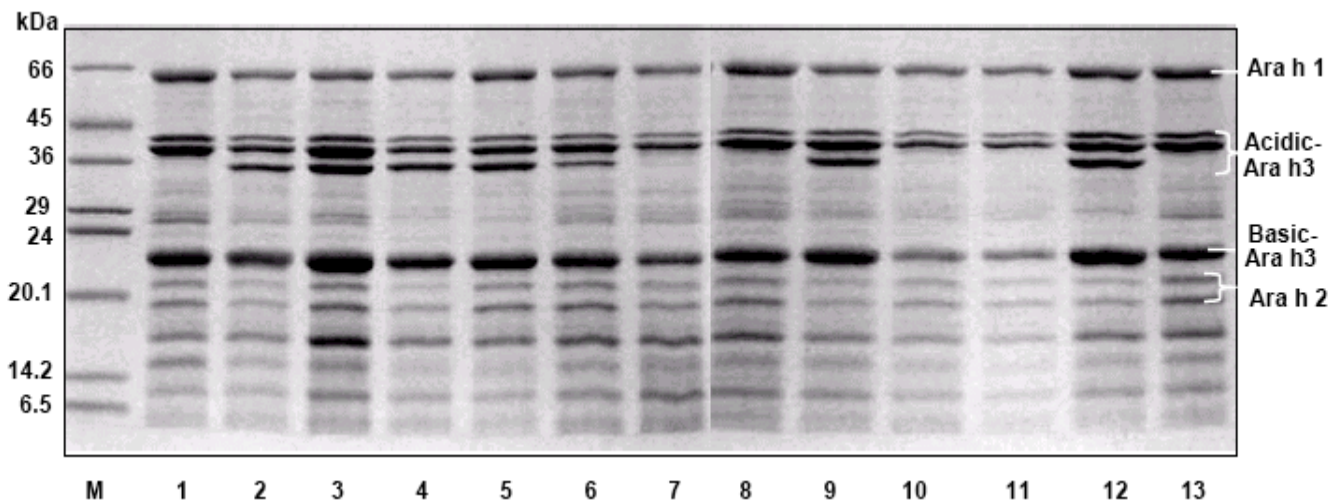


Fig. (1). One-dimensional SDS-PAGE total peanut storage protein profiles. M = molecular weight markers, 1 = YY5, 2 = C99R, 3 = IMP-3, 4 = Pinto-4, 5 = PI3374-09-05, 6 = PI337F-6, 7 = GFA-1, 8 = AR4, 9 = AR3, 10 = AR2, 11 = AR1, 12 = UF71513-9, 13 = GFA-2. Three major peanut allergens are listed on the left.

3-4 in Fig. (2)). The levels of protein-bound end products such as advanced glycation end products (AGE) and IgE binding were tested and 'GT-C9' exhibited significantly lower levels of AGE adducts and of IgE binding [19]. The missing of the basic subunits of Ara h 3 in the peanut breeding line 'GT-C9' may contribute to this observation [19]. This finding is in agreement with the study by Restani *et al.* [20] that suggested Ara h 3 basic subunits, which have been historically neglected, may be significant, in allergy to peanut.

On the 2-D PAGE in Fig. (2), several bands that were recorded on SDS-PAGE gel as a single band (Fig. 1) were resolved into several distinct spots with different *pI* values and slightly different molecular weights (Fig. 2). The Ara h 1 with 63 kDa molecular weight by SDS-PAGE was separated into several spots with different *pI*s. The acidic isoforms of Ara h 3 were resolved with three clear bands as on SDS-PAGE ranging from 36 to 45 kDa with slightly different *pI*s on 2-D PAGE. The basic Ara h 3 subunit group with one heavy band on SDS-PAGE at about 22 kDa was separated into many spots or subunits on the 2-D PAGE with distinct *pI*s and slightly different molecular weights of 22-28 kDa (Fig. 2). Each band of Ara h 2 was separated into two spots with different *pI*s.

Therefore, because 'GT-C9' lacks several peptides on 2-D profile such as spots 3 and 4 (Fig. 2), which are the basic subunits, and has lower levels of IgE binding, this genotype could be useful towards breeding a less allergenic peanut.

PROTEIN-BOUND ADDUCTS AND ALLERGENICITY

Previous studies [8,21] reported that peanut roasting affects the allergenic properties of peanuts possibly due to the reactions of proteins with sugars to form Maillard reaction products [22]. Further investigations [23-25] established a correlation between level of IgE binding and the level of advanced glycation end products (AGE). Guo *et al.* [19] tested the levels of AGE and carboxymethyllysine (CML), a major AGE byproduct, and IgE binding in different peanut genotypes and found there were no differences in CML

among the tested genotypes. However, 'GT-C9' had the lowest levels of AGE and IgE binding, which might indicate that this genotype has reduced allergenic properties and CML, in this case, is not a major contributor to the reduction seen in IgE binding.

AGE adducts are protein-sugar or so-called Maillard reaction products that are capable of promoting an immunoglobulin G (IgG) response and cytokine production, which in turn can lead to IgE production [26-28]. The association of AGE adducts with IgE binding to peanut proteins was first demonstrated in a study in which a non-allergenic peanut protein showed IgE-binding activities after heat treatment with glucose [21]. The AGE adducts thus formed (which were attached to the protein) tended to bind or inhibit IgE in a Western blot competition assay, suggesting that IgE had an affinity for AGE adducts. This is further supported by another study [24] where polyclonal antibodies produced against AGE adducts were shown to have an inhibitory effect on IgE in ELISA. A correlation between the levels of IgE binding and AGE also existed. This data implies that AGE adducts may alter the peanut allergens in a way that influences the IgE recognition of the allergens. Maleki *et al.* [8,11,29] has demonstrated the occurrence of structural changes in peanut allergens which contain AGE adducts after roasting or reactions with various sugars. Such allergens have a higher IgE binding than those without AGEs. This is also supported by Gruber *et al.* [30] who demonstrated that thermal treatment of rAra h 2 (a major peanut allergen) in the presence of reactive carbohydrates induced a strong increase of the IgE-binding activity. The author attributed the increased IgE binding to the major epitopes which showed a distinct higher level of IgE binding when subjected to Maillard reaction, thus giving the evidence that processing induced changes might increased IgE binding of the glycosylated Ara h 2.

Ara h 3 EPITOPES AND BASIC SUBUNITS

The 3 major allergens, Ara h 1-3, are comprised of vicilin, conglutin, and glycinin seed storage proteins, respectively (Fig. 1). Glycinins (11S storage proteins) are initially

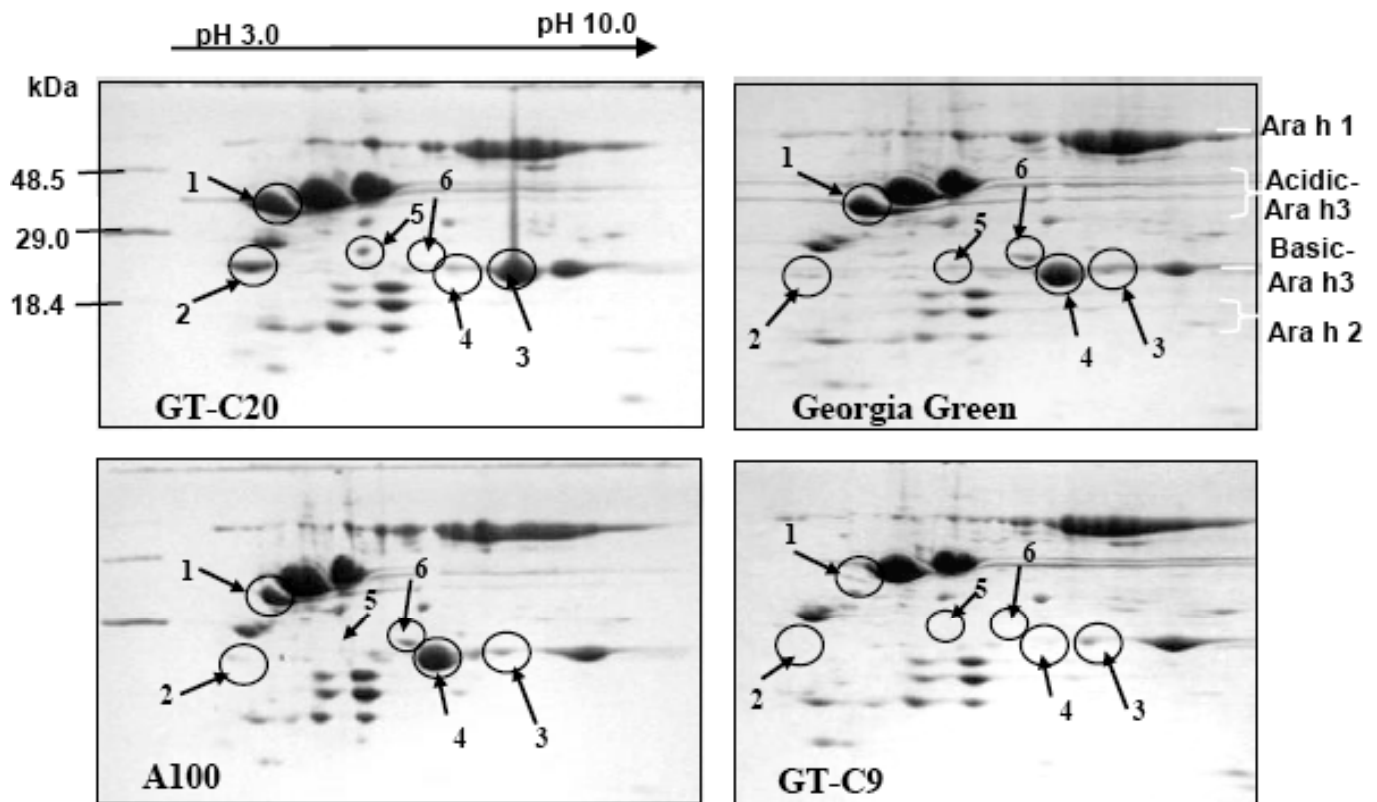


Fig. (2). Two-dimensional SDS-PAGE protein profiles [19]. For easy comparison, the spots of interest were encircled and numbered. These encircled spots were missing in peanut breeding line 'GT-C9', in which protein peptide spots 3 and 4 are Ara h3 basic subunits.

synthesized as 60-kDa preproglobulins consisting of covalently linked acidic and basic polypeptides. The precursors are deposited in storage bodies where they aggregate into trimers, before being cleaved by an asparagine-dependent endopeptidase [31,32] and result in an NH₂-terminal acidic chain of ~35 kDa and a COOH-terminal basic chain of ~22-28 kDa, which later become linked by a disulfide bridge [33]. Cleavage of a conserved protease digestion site always results in an acidic (N-terminal) fragment ending with an asparagine (N) and a basic (C-terminal) fragment starting with GIEETIC which is found in all identified isoforms. The 11S storage proteins are then assembled into their mature form as hexameric oligomers consisting of six similar subunits [34].

Ara h 3 was first identified by Eigenmann *et al.* [35] as a 14 kDa peanut polypeptide and later its gene was cloned by Rabjohn *et al.* [15] encoding a protein with a molecular weight of approximately 60 kDa containing IgE binding sites. Rabjohn *et al.* [15] reported that there are four distinct IgE-recognition sites distributed throughout the primary sequence of Ara h3 and all four Ara h 3 epitopes reside within the acidic subunit (Fig. 1). There were no epitopes detected in the basic subunit by this group. These epitope motifs are designated as epitope 1 (IETWNPNNQEFECAG), epitope 2 (GNIFSGFTPEFLAQA), epitope 3 (VTVRGGLRILSPDR), and epitope 4 (DEDEYDYDEEDRG). In peanut patients that recognized Ara h 3, epitope 3 was an IgE binding site, whereas the other epitopes were active-sites in 25 to 38% of Ara h 3-allergic patients. Recently, a study [20] suggested that the basic subunit of Ara h 3 may be a major allergen in a group of children allergic to peanuts.

Restani *et al.* [20] identified a dominant allergen by using amino acid sequencing of the bands that show the strongest IgE immunoreactivity in SDS-PAGE gels and immunoblotting. Specific animal IgGs were raised against the dominant immunoreactive band in order to pinpoint the allergen(s) in peanut proteins separated by 2-D electrophoresis and immunoblotting. Further, Restani *et al.* [20] examined the peanut proteome using serum samples from the children with the unusual immunoreactivity and found a group of children with marked peanut allergy who are specifically sensitized to the basic subunit of Ara h 3. This finding of the dominant immunoreactivity of basic subunit of Ara h 3 in these patients was unexpected and not in agreement with earlier studies [15] indicating that Ara h 3 was only a minor peanut allergen and that the identified allergenic epitopes occurred only in the acidic Ara h 3 subunit. However, other studies [13,36] reconsidering the relative importance of individual peanut allergens has also shown that Ara h 3 basic subunits may be important allergenic peptides. Koppleman *et al.* [36] showed that the 25 and 28 kDa, basic subunits, of Ara h 3 were also recognized by allergic patients and therefore contain undefined IgE epitopes, which is in agreement with reports on soy glycinin.

The finding by Guo *et al.* [19] was in agreement with Liang *et al.* [3] who first reported the presence of polymorphic isoforms of Ara h 3 acidic and basic subunits, one of which was referred to as iso-Ara h 3. The characterization of deduced amino acid sequence of iso-Ara h 3 (ABI17154) demonstrated that there are some insertions and deletions between iso-Ara h 3 and Ara h 3. Because, the iso-Ara h 3 does not have the fourth identified IgE epitope it is likely to

exhibit lower IgE binding. Also, since 'GT-C9' lacks several peptides on 2-D profile such as spots 3 and 4, which are the basic subunits, and spot 1, which is an acidic subunit and has lower levels of AGE and IgE binding, this genotype might have reduced allergenicity and could be useful towards breeding a less allergenic peanut. Future studies will be designed to test the allergenicity of the peanut breeding line 'GT-C9'. Similar screening of isoforms of Ara h 1 and Ara h 2, the other major allergens, in different peanut lines would have to be performed towards breeding peanut cultivars with overall reduced allergen levels.

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