

Comparison of major peanut allergens Ara h 1, Ara h 2 and Ara h 3 between peanut cultivars from Côte d'Ivoire and the USA using SDS PAGE and Western Blots

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Summary: Peanut is commonly consumed in many forms. The ubiquitous presence of peanut in processed food is responsible for an increasing number of allergic reactions due to accidental ingestion. The prevalence of peanut allergy seems to be underestimated in the African population possibly because of the lack of testing and clinical documentation. In this study, a comparison was made between raw and roasted peanut seeds from cultivars of Côte d'Ivoire (ARA-CI) and raw peanut seeds from the cultivar Georgia Green, grown commercially in the USA. The main objective of this study was to identify the protein profile of peanut seeds from Côte d'Ivoire and compare it with the molecular specificities of major allergens of Georgia green seeds from the USA using a combination of two methods, SDS PAGE and Western blots. Peanut protein profiles via SDS PAGE, coupled with Western blots were carried out on two collections of peanut seeds. In the raw peanut seed extracts from Côte d'Ivoire, are visible fingerprints of the major allergenic proteins Ara h 1 (63.5 kDa), Ara h 2 (17, 20 kDa), and Ara h 3 (25,36, 40 and 44 kDa) and an allergenic bands of Ara h 3 of about 36kDa. This provides evidence of the presence of the major allergens in peanut from Côte d'Ivoire, this, a presumption of a high allergenic potency peanut despite a low prevalence of peanut allergy in the country. The presence of a strongly expressed 30 kDa protein, potentially corresponding to a component of Ara h 3 in the roasted sample means that cooking processes could increase the allergenic potency of peanut. This study makes it possible to identify molecular specificity in peanut from Côte d'Ivoire for the development of local screening test adapted to the environment.

Keywords: peanuts, allergy, allergen, Ara h 1, Ara h 2 and Ara h 3, SDS-PAGE, Western blot, Côte d'Ivoire

I. Introduction

The global prevalence of allergy is reported to be in the range of 20–30% of the world's population for different forms of allergic diseases, and this global prevalence seems to have increased in the last 3 decades [1,2]. Peanuts are known to be one of the most allergenic food, often responsible for severe clinical allergic reactions through hypersensitivity mechanism with variable clinical signs in atopic subjects [3]. In the United States, as many as 15 million people (9 million adults and nearly 6 million children) have food allergies with an estimated prevalence of 0.6-1.3% of peanut allergy among the U.S. population [4,5,6,7,8]. Self-reported prevalence of allergy to common foods in Europe ranged from 0.1 to 6.0%. Prevalence of peanut allergy with 0.2% in the general European population was found to be more common among older children [9]. In a 2010, Swedish study of peanut allergy in children, the authors estimated the prevalence of clinical peanut allergy among a sensitized population to be 22.4% [10]. In France, the prevalence of peanut allergy is estimated at 1% among children aged six to ten years, and 0.2% after the age of 11 years. The prevalence of peanut allergy in the general French population was estimated between 1 and 2.5%.

In Africa, despite the limited information on allergy and allergens, most of the allergy cases in rural areas are contact allergies that may lead to mild dermatitis and pruritus. Further, people living in grasslands, in a traditional setting, with traditional dietary practices rarely suffer from allergic diseases [11,12]. Food allergy seems to be uncommon in the general population, but more frequently occurring in children (up to 8%) compared with adults (2%). About 35% of children with severe eczema experience food allergy involving IgE antibodies, and 6% of children with asthma experience food induced wheezing [13,14]. In Togo, food sensitization to trophallergens interested 36.70% of children with 19% of peanut sensitization [15]. In Côte d'Ivoire the prevalence of peanut allergy was estimated at 9.09% [16]. In Senegal, clinical cases revealed that allergic diseases seem to be on the rise. However, in a hundreds of patients, oral food challenges indicated three patients were allergic to peanut; a number which usually would not exceed two [17]. In Ghana, a skin prick test (SPT) for food (mango, banana, orange, papaya, pineapple, apple and peanut) allergy was conducted on a cohort of 1695

schoolchildren aged 5-16 years. Pineapple (~1.7%) and peanut (~1.7%) were the most frequent SPT positives, with a slightly higher proportion in urban public schoolchildren compared to their rural counterpart [18].

The physical symptoms coupled with the clinical data make it possible to confirm a diagnosis for peanut allergy. The low amount of information on the prevalence of peanut allergy and food allergy in general in Africa necessitates a much more dedicated involvement of the scientific community. However, food allergy screening tests manufactured and sold by European or American companies in Africa have a high cost. Thus, it is critical for African countries to develop local low cost screening tests and kits, for food and peanut allergies. Among the large array of panels available, serological tests to identify specific IgE antibodies to peanut may be an appropriate screening strategy to be developed large scale as suggested by some authors [19,20]. Enzyme immunoassays are used to determine not only peanut proteins but traces of allergens in food and feeds [21, 19, 22]. The main objective of this study was to identify the protein profile of peanut seeds from Côte d'Ivoire and compared the molecular specificities of the major allergens to that of Georgia green peanut seeds from the USA using a combination of SDS PAGE and western blots.

II. Materials

Three peanut cultivars were used:

- Two (2) non processed protein samples from a collection of peanut seeds from Côte d'Ivoire (ARA- CI) [16], one (1) extracted from roasted peanuts (170°C for 20 minutes^[35]) labeled sample N°2, and a second protein sample extracted from raw peanut and labeled sample N°3,
- The third protein sample was extracted from raw peanut seed of the Georgia Green variety, and provided to us by Dr. H. Dodo^[29] and labeled N°.1(Fig 1).

III. Methods

1-Extraction of Peanut Proteins

Extraction of peanut proteins from the raw and roasted peanut was performed using a modification of the protocol by Koppelman et al., 2001 [23]. 200µg of peanut seed was crushed in 2mL of 50mM Tris-HCl, pH 8.0. The mixture was stirred for 2 hrs at room temperature, and the aqueous fraction was collected by centrifugation (3,000g) for 5 min at room temperature. The aqueous phase was subsequently centrifuged (10,000g) for 15 min at room temperature to remove residual traces of oil and insoluble particles. Each extract was stored at -20°C until used to perform SDS-PAGE and Western Blots. The protein concentration of soluble peanut extracts was determined by the Bradford method [24]. All remaining extracted soluble peanut seed proteins were stored overnight at -20°C.

2 - SDS-PAGE Analysis

The SDS-PAGE was performed essentially according to Laemmli^[25] using a Mini Protein II system (Bio-Rad, Hercules, CA, USA) with 17% acrylamide gels (15x10 cm). Pre-stained molecular weight markers with standard molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, and 220 kDa were used as reference. Soluble peanut protein extracts were mixed in a 1 to 3 ratio with 4x XT buffer (BioRad) and were subsequently boiled for 5 min.

Approximately 10µg/ml of samples were loaded on the gel and let to run for 1hr at 200V. Gels were stained with Coomassie brilliant blue R-250 (Biorad) for 30 min and de-stained twice with Coomassie brilliant R-250 for 1hr. After de-staining, gel pictures were taken using Kodak EDAS-290 (BioRad).

3-Western blot analysis

Peanut proteins separated by SDS-PAGE were electrophoretically transferred to polyvinylidene-difluoride (PVDF) membranes (Bio-Rad). Blotting was performed in transfer buffer (10 mM Tris-HCl, 100 mM Gly, 10% methanol) using a Mini-Trans Blot system (Bio-Rad). A modified protocol was used for immunoblotting [23]. Membranes were blocked overnight with Tris-buffered saline plus Tween 20 (TBST; 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing nonfat 2% dry milk and subsequently incubated overnight at room temperature with the specific monoclonal antibodies for *Ara h 1*, *Ara h 2*, and *Ara h 3* diluted 1:10000, 1:20000, and 1:10000 respectively in Tris-Buffered Saline and Tween (TBST). After three washes of 10 min each with TBST, membranes were incubated with the secondary antibody peroxidase labeled Goat anti-Human IgE(ε) (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD, Cat number 074-1004 Lot QH67-1) diluted 1:10,000 in TBST. Membranes were washed as above with TBST, and antigen-antibody complexes were detected by chemiluminescence by incubating with SuperSignal^R West Pico Chemiluminescent Substrate (Pierce, Cat number 34080 kit containing SuperSignal^R West Pico Luminol/Enhancer solution and SuperSignal^R West Pico Stable Peroxidase solution) for 30 sec to 1 min. The blot was placed against the film in a cassette and exposed.

IV. Results

SDS PAGE and Western Blot displays the profile of the proteins extracted from peanut seeds from Cote d'Ivoire (ARA-CI) and from Georgia green peanut from the USA

(B)(A)

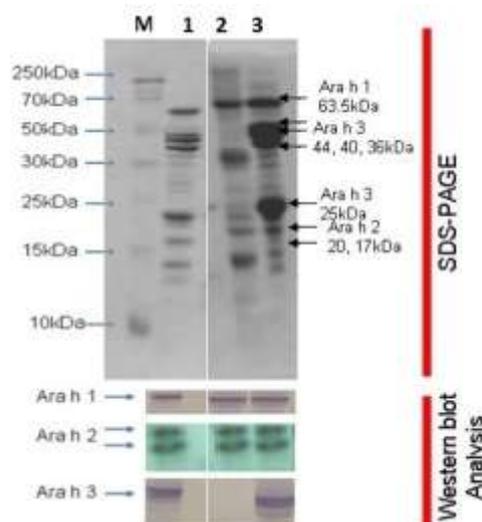


Fig.1: (A) –SDS-PAGE: Protein profile of raw (Lane 3) and roasted (Lane 2) peanut seeds from Côte d'Ivoire (ARA-CI) compared to raw **Georgia green seeds** (Lane 1) from the USA. (B) Western Blot : To detect the presence of the three major allergens (*Ara h 1*, *Ara h 2* and *Ara h 3*) in peanut seeds from Côte d'Ivoire and from the USA.

M= Molecular weight marker.

#1=control (raw peanut seed of Georgia Green market type from USA)

#2=Roasted peanut seeds from Côte d'Ivoire (ARA-CI)

3= Raw peanut seeds from Côte d'Ivoire (ARA-CI).

SDS PAGE data revealed the raw peanut seed extract of ARA-CI (Lane 3) and the raw peanut seeds extract from the USA (Lane 1) were similar in protein profile, with fingerprint bands corresponding to the molecular weights, for *Ara h 1* (63.5 kDa), *Ara h 2* (17, 20 kDa), and *Ara h 3* (25, 36, 40, 44 and kDa). The fingerprint of these three major allergenic proteins (*Ara h 1*, *Ara h 2*, *Ara h 3*) was also confirmed in Western blots. However, for the roasted peanut from Côte d'Ivoire, (ARA-CI) (Lane 2), the fingerprint bands corresponding to *Ara h 3*, with molecular weights of 25, 36, 40, and 44 kDa were present with lower intensity in both the SDS PAGE and in the Western Blot.

These isoforms of *Ara h 3* were completely destroyed by the roasting process, while *Ara h 1* and *Ara h 2* appeared to be thermostable, and were not affected by the roasting process.

On the SDS PAGE of the two ARA-CI (Lane 2 and Lane 3) the *Ara h 3* isoform of 30 kDa was strongly evident, and is highly expressed in the roasted form where it seems more thermostable than in the raw form.

The 30 kDa protein is not distinctly visible in lane 3, of the raw ARA-CI. Maybe another SDS PAGE can be run with a higher / better resolution to distinguish between the 44, 40, 36 and 30 kDa protein bands.

V. Discussion

Peanut is a legume and its seeds are a powerhouse of nutritious proteins and good quality fats. Peanut allergens are seed storage proteins and constitute about 5% of the total proteins of a cell.

Peanut allergy is caused by ingestion of crude peanut proteins and food products derived from peanuts. Three peanut proteins *Ara h 1*, *Ara h 2* and *Ara h 3* are considered major allergens because they are recognized by more than 50% of peanut allergic patients [26,27]. The three major allergens were present in the US commercial cultivar Georgia green and in the peanut cultivar collected in Côte d'Ivoire (ARA-CI) [28]. Scientific protocols such as protein profiling, SDS PAGE and ELISA reveal that allergens are not usually present as a single compound but sometime as a group of isoforms of a same protein whose structure varies slightly due to certain post-translational modifications such as glycosylation and phosphorylation [29].

Studies on protein isoforms of the major peanut allergens *Ara h 1*, *Ara h 2* and *Ara h 3* have revealed many specific information ; *Ara h 1* migrates as a 63-kDa band [30,38], *Ara h 2* migrates as a doublet at 20 and 17kDa [27,31,38] and *Ara h 3* consists of a series of polypeptides from 45 to 14 kDa [32].

Recently, an isoform of *Ara h 3*, identified as *Ara h 3-im* appears to be less allergenic than the other isoforms of *Ara h 3*, and migrates as the 36-kDa band. It was partially cloned from cDNA^[33]. This newly discovered *Ara h 3* isoform (*Ara h 3-im*) was present in the raw peanut seed from Cote d'Ivoire (ARA-CI, Lane 3) but was absent or less intense in the roasted peanut from Côte d'Ivoire, (ARA-CI, lane 2).

Comparing with the Georgia Green peanut cultivar (Lane 1), the apparent molecular fingerprints bands migrating approximately at 63.5 kDa for *Ara h 1*, 20 and 17 kDa for *Ara h 2*, 22, 36, 40, 44, kDa for *Ara h 3* are more clearly distinguishable than in the raw extract of peanut from Côte d'Ivoire (Lane 3). Therefore, it is very important to characterize allergens from peanut cultivars of different geographic locations to identify various isoforms of allergens. It is also critical to detect variations in the level of sensitization of various populations while using specific commercially available biological tests and provide guidance for more effective prevention against possible allergy to peanut in each region.

Quality data on the burden of peanut allergy is lacking in most of the African countries. A study of food allergy in Côte d'Ivoire^[16] showed a relatively low prevalence of 9,09% of sensitization despite a high consumption of peanuts in all forms including raw, roasted, or boiled. Explanation for the low prevalence of peanut allergy within the population of Côte d'Ivoire is not known. Koppelman proposed that it could be due to the action of gastric juices on the allergens. In fact, a literature search reveals that *Ara h 1* and *Ara h 3* were rapidly hydrolyzed by pepsin, while *Ara h 2* and *Ara h 6* were resistant to pepsin digestion, even at very high concentrations of pepsin. *Ara h 2* and *Ara h 6* are considerably more stable to digestion than *Ara h 1* and *Ara h 3*.^[44] The presence of a strong 30 kDa component of *Ara h 3* was observed in the roasted peanut of Côte d'Ivoire. This 30kDa *Ara h 3* component is usually not present. For example, in a study,^[45] the protein profile of fresh peanuts crude extracts prepared in the presence of Sodium Dodecyl Sulphate (SDS), a denaturing agent which inhibits proteases, revealed some bands of *Ara h 3* (14, 25, 42 and 45 kDa), and among others, a major band of *Ara h 1* (63 kDa), and a doublet for *Ara h 2* (17–21 kDa). The relative intensities of the *Ara h 3* bands did not change as a function of the purification procedure. Thus, it appears that the protein extraction process in this study did not alter the relative intensities of the bands corresponding to *Ara h 3* components. These data are in accordance with an earlier report on SDS-PAGE analysis of different peanut cultivars, and indicate that the molecular organization of *Ara h 3* is similar in different peanut cultivars.

The raw sample of peanut from Côte d'Ivoire revealed the presence of various isoforms of *Ara h 3* i.e. 22, 36, 40, and 44 kDa as seen in Georgia green peanut, the US cultivar.

In China, peanut allergen *Ara h 1* is of the most concern, although its level is reduced in the fried and boiled preparations^[35]. In fact, the prevalence of peanut allergy is lower in China (3,4 to 5 %) than in the USA and even lower in Africa. The consumption of peanut in China is high and is typically in fried or boiled forms^[43], which is different from the way peanut is consumed in the USA typically in dried and roasted forms. Thus, one can confirm that the type of cooking methods and type of heat treatment of peanut, does have an impact on peanut allergenicity^[35, 37, 39, 40].

A Comparison of Western blots for raw and roasted peanuts showed variations in protein recognition by custom *Ara h 1*, *Ara h 2*, and *Ara h 3* antibodies^[41]. Both the laboratory prepared and the commercial peanut flour preparations were used for the evaluation. The two ELISA kits tended to underestimate the levels of protein in samples that were subjected to elevated heat, respectively, by more than 60- to 400-fold lower for the autoclaved samples and by as much as 70- to 2000-fold lower for the dark-roast commercial flour samples^[42].

Future studies will address the proportion of allergenic proteins which may also be determined and expressed with each isoform of peanut^[28]. The difference of rate by each allergen in peanut extract from many countries may be interesting to produce local tests^[34]. But 3 findings could be applied:

- (1) Perform another SDS PAGE with protein sample from roasted peanut at a higher gel concentration to clearly separate the *Ara h* isoforms of 30, 36, 40, kDa and 44 kDa. (2) Perform a SDS PAGE with the same concentration of protein samples for roasted and non-roasted peanut; and (3) An easy way to this end is to freeze-dry the protein samples after extraction, and measure out the same quantity from each sample and dissolve it for loading.

VI. Conclusion

The comparative protein profiling between peanuts of two different origins (Côte d'Ivoire and the USA) is important to identify variations and individual specificity from one country to the next. It is therefore very interesting to know that our peanut samples from Cote d'Ivoire contains three major proteins that and may be quantified. This should therefore be taken into account in the development of quantitative bioassay screening for peanut allergy. It is very important for prevention purposes to recommend appropriate dietary measures for potentially at risk people, pregnant women, children, diabetic's type 1, and allergic patient^[36].

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