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Increasing the Solubility and Recovery of Ara h3 Allergen from Raw and Roasted Peanut

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Abstract

Ara h3 belongs to the glycinin family of seed storage proteins and is one of the major peanut allergens. It comprises over 20% of the total peanut protein mass, making it a logical target for the detection of trace quantities of undeclared peanut contamination in foods. Both Ara h1 and Ara h3 are detected in lower quantities in cooked foods, either because of the failure to completely resolubilize the denatured proteins or because of the disruption of conformational epitopes required for monoclonal antibody recognition. A new reagent containing a proprietary non-detergent sulfobetaine (NDSB) is described which solubilizes more total protein and yields more Ara h3 protein from both raw and roasted peanut than other commonly used ELISA-compatible reagents.

Keywords: Ara h1, Ara h3, ELISA, non-detergent sulfobetaine, peanut allergy, protein solubilization

1. Introduction

In the United States, an acute food allergy reaction sends someone to the emergency room every 3 minutes [1]. While over 170 foods are known to cause allergic reaction, only eight foods are responsible for over 90% of food allergies [2]. Between 1997 and 2008, the prevalence of peanut and tree nut allergies has more than tripled in the U.S. [3, 4]. Peanut allergies are a leading cause of fatal anaphylaxis [5, 6].

The U.S. Food and Drug Administration (FDA) and the European Union have imposed strict requirements for the labeling of food ingredients. However, warnings such as “May contain peanut” or “Manufactured in a facility that processes peanuts” are voluntary and considers the possibility of contamination with peanut residues [7]. Since traces of peanut may contaminate foods supposedly free of peanuts, methods capable of reliably detecting undeclared allergens are necessary to ensure food safety [8]. Highly sensitive enzyme-linked immunosorbent assays (ELISAs) can detect low nanogram quantities of contaminating allergens in foods and biologics, whereas the rapid lateral flow assays available for consumers to test their foods are less sensitive. For example, the recently described Ara h3 ELISA [9] is about 2000 times more sensitive than the NIMA Peanut Sensor, which also targets Ara h3 [10]. To put into perspective, the ELISA could detect roughly 14 peanuts dissolved in an Olympic size swimming pool, having a volume of 2,499,330 L of water.

Proteins constitute 24–27% of the total peanut mass [11]. At least 13 different protein allergens have been identified in *Arachis hypogaea*. Of these, Ara h2 and Ara h6 are reportedly the most potent allergens [12, 13], whereas Ara h1 and Ara h3 are the two most abundant allergens, together comprising at least a third of the total peanut protein mass [14]. Ara h4 has 91.3% sequence homology with Ara h3 [8] and are considered to be the same allergen [15, 16].

Ara h3 exists as a trimer or hexamer consisting of identical 58.3 kDa subunits and having molecular masses of 180 and 360 kDa, respectively. Each subunit is derived from a single precursor which is posttranslationally cleaved to produce an acidic and a basic chain that are held together by a disulfide bond [17, 18]. Multiple subunits in the mature Ara h3 are associated via hydrophobic bonding.

The roasting of peanuts decreases the extractability of soluble protein by as much as 50% [19]. Upon heating, Ara h1 and Ara h3 may form aggregates, further decreasing their solubility [20]. Further, the denaturation of proteins drives conformational changes that can result in the loss of epitopes. Decreased antibody recognition in ELISA has been reported for both monoclonal and polyclonal antibodies [21, 22].

Lipid constitutes approximately 50% of the total peanut mass. There are conflicting reports on whether delipidation of peanuts increase protein recovery [22, 23]. Likewise, detergents such as Sarkosyl have been shown to increase protein solubility [24].

Sample preparation will be critical to the reliable quantitation of undeclared peanut allergens in foods by immunoassay, particularly in cooked foods where the denaturation of proteins renders them insoluble (e.g., imagine trying to resolubilize cooked egg whites). The failure to completely resolubilize target proteins results in their underestimation, a critical shortcoming where undeclared allergens may be present in only trace amounts.

This chapter reviews the solubility and the recovery of the Ara h3 allergen from raw and roasted peanuts extracted in several different buffers with consideration of ELISA compatibility. Since Ara h3 is one of the most abundant proteins, it is a logical target for the detection of trace quantities of peanut contamination in foods.

2. Methods

2.1 Sample preparation of raw and roasted peanuts

Raw Virginia peanuts (Hampton Farms, Severn, NC, USA) were dry roasted at 175°C for 20 minutes. Raw and roasted peanuts were shelled, and then course ground in a stainless steel coffee grinder. The resulting grounds were sifted through a 0.5 mm stainless steel mesh to provide uniform tritulates.

The peanut tritulates (53.9 ± 4.1 mg, $n = 24$) were weighed into the insert of a BioMasher centrifugal homogenizer (Omni International, Kennesaw, GA, USA). Biological replicates were extracted in 0.4 mL of each sample buffer. Sample buffers were (i) PBS pH 7.4, (ii) 0.05% Tween-20 in PBS pH 7.4, (iii), 8 M urea, 16 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in 40 mM Tris-HCl pH 8.8, and (iv) a proprietary non-detergent sulfobetaine (NDSB) from ProdigY Biosciences (Louisville, KY, USA).

Each sample was homogenized in the BioMasher for 20 seconds and the extract was collected by centrifugation at 15,000 RCF for 1 minute. The BioMasher insert was removed and centrifugation was continued at 15,000 RCF for an additional 5 minutes. Both an insoluble pellet and a floating lipid layer were observed in all samples. The supernatant was carefully aspirated from below the lipid layer and

further clarified in an UltraFree MC centrifugal microfilter with 0.22 micron pore size (Millipore-Sigma, Burlington, MA, USA) at 12,000 RCF for 5 minutes.

The homogenizer bar was withdrawn from the BioMasher insert and an additional 0.4 mL of each reagent was added and the process was repeated a second time. This enabled estimates of extraction efficiency in first and second extractions of raw and roasted peanuts, and in 0.4 and 0.8 mL extraction volumes.

2.2 Analysis

Total protein concentration was estimated using the Bradford protein assay (BioRad, Hercules, CA, USA) calibrated with a qualified BSA standard (Pierce Biotechnology, Rockland, IL, USA).

Ara h3 was quantified using the Ara h3 ELISA 2.0 Kit (Indoor Biotechnologies, Charlottesville, VA, USA) using the 1E8 and 4G9 monoclonal antibody pair and streptavidin-HRP reporter. Calibration was linear over the 2–125 ng/mL range ($r^2 = 0.9998$).

2.3 Calculation of grand average of hydropathicity (GRAVY) values

FASTA sequences of representative peanut proteins were procured from the UniProtKB Protein Knowledgebase [25, 26]. GRAVY values were based on amino acid hydropathy index [27] and calculated using the ProtParam software tools available from the ExPASy Bioinformatics Resource Portal [28].

3. Results and discussion

3.1 Total recoverable protein from raw peanut

In every case, more total protein was recovered in raw than in roasted peanut. The NDSB buffer recovered more total protein in a single 0.4 mL extraction than all of the other buffers tested. NDSB replicates averaged 14.8% total protein recovery from raw peanut triticates, nearly twice as much protein than what was extracted with urea-CHAPS. On average, NDSB extracted seven times more total protein than PBS-Tween (**Figure 1**).

3.2 Total recoverable protein from roasted peanut

Lower total protein recoveries were observed from roasted peanut. NDSB yielded 4.0% total protein from roasted peanut, compared to 4.4% from urea-CHAPS. NDSB and urea-CHAPS yielded twice as much soluble protein from roasted peanut than PBS-Tween (**Figure 2**).

3.3 Ara h3 yields from raw and roasted peanut

NDSB yielded the highest recoveries of specific Ara h3 protein in both raw and roasted peanut as measured by ELISA. On average, NDSB yielded more than twice the measurable Ara h3 from raw peanut than PBS-Tween (**Figure 1**). From roasted peanut, NDSB yielded nearly seven times more Ara h3 than PBS-Tween (**Figure 2**).

Ara h3 was not detected by ELISA in raw or roasted peanut samples extracted in urea-CHAPS. This is apparently due to the disruption of tertiary and secondary structure of the protein and the obliteration of conformational epitopes required for binding by the monoclonal antibodies used in the ELISA. While the urea-CHAPS was substantially diluted for ELISA, no restoration of antibody recognition was observed.

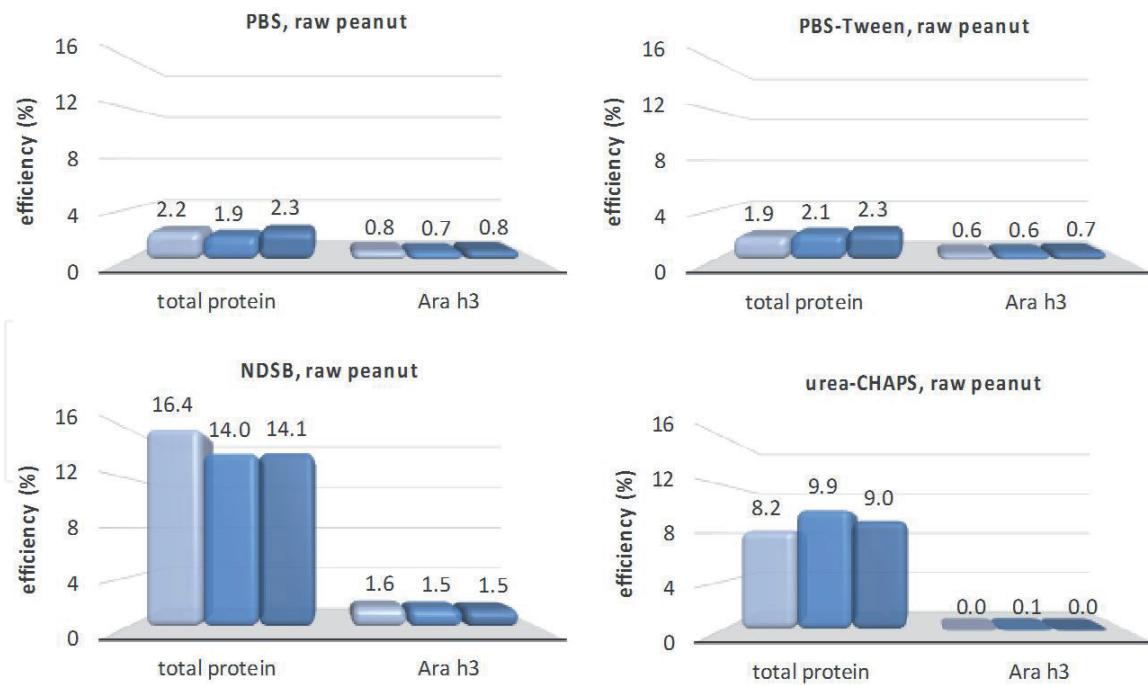


Figure 1. Normalized recovery of total protein and Ara h3 from biological triplicates of raw peanut (53.6 ± 4.9 mg, $n = 12$) extracted in 0.4 mL of PBS, PBS-Tween, NDSB, or urea-CHAPS. For extraction efficiency, total protein and Ara h3 mass are expressed in terms of their percentage of the total peanut biomass.

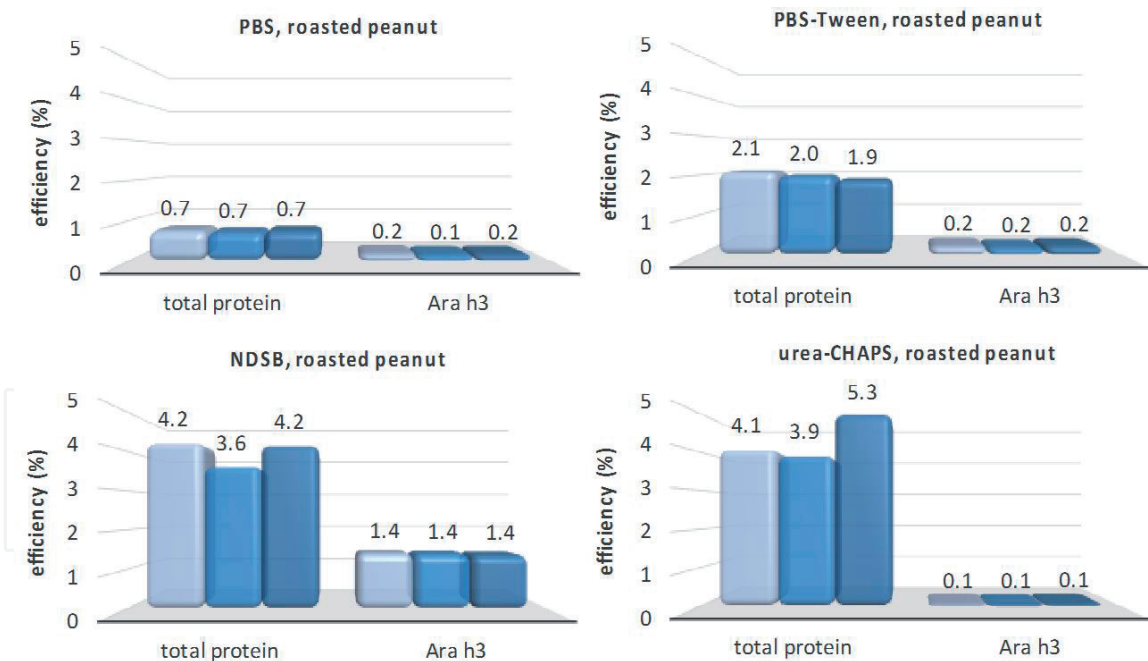


Figure 2. Normalized recovery of total protein and Ara h3 from biological triplicates of roasted peanut (53.6 ± 4.9 mg, $n = 12$) extracted in 0.4 mL of PBS, PBS-Tween, NDSB, or urea-CHAPS. Total protein and Ara h3 mass are expressed in terms of their percentage of the total peanut biomass.

3.4 Increasing total protein yields by sequential extraction

To investigate whether a second extraction would significantly improve total protein yields, an additional 0.4 mL of each buffer was added to the insoluble peanut triturate remaining in each homogenizer insert following the first extraction.

PBS extracted 50.4 and 45.3% of the total PBS soluble protein in the first extraction for raw and roasted peanut, respectively. PBS-Tween extracted 75.6 and 78.2% of the total PBS-Tween soluble protein in the first extraction for raw and roasted peanut, respectively.

NDSB extracted 83.7 and 92.0% of the total NDSB soluble protein in the first extraction for raw and roasted peanut, respectively. For raw peanut, mean efficiency of NDSB was 14.8% for the first extraction, which was increased to 17.7% when a second extraction was performed. For roasted peanut, mean efficiency of NDSB was $4.0 \pm 0.3\%$ for the first extraction, which was increased to $4.4 \pm 0.3\%$ when a second extraction was performed (**Figure 3**). In practical terms, only about 10% more protein is recovered when a second extraction is performed, but at the cost of doubling the sample volume of the isolate.

Urea-CHAPS extracted 94.5 and 89.9% of the soluble protein in the first extraction for raw and roasted peanut, respectively. For raw peanut, mean efficiency

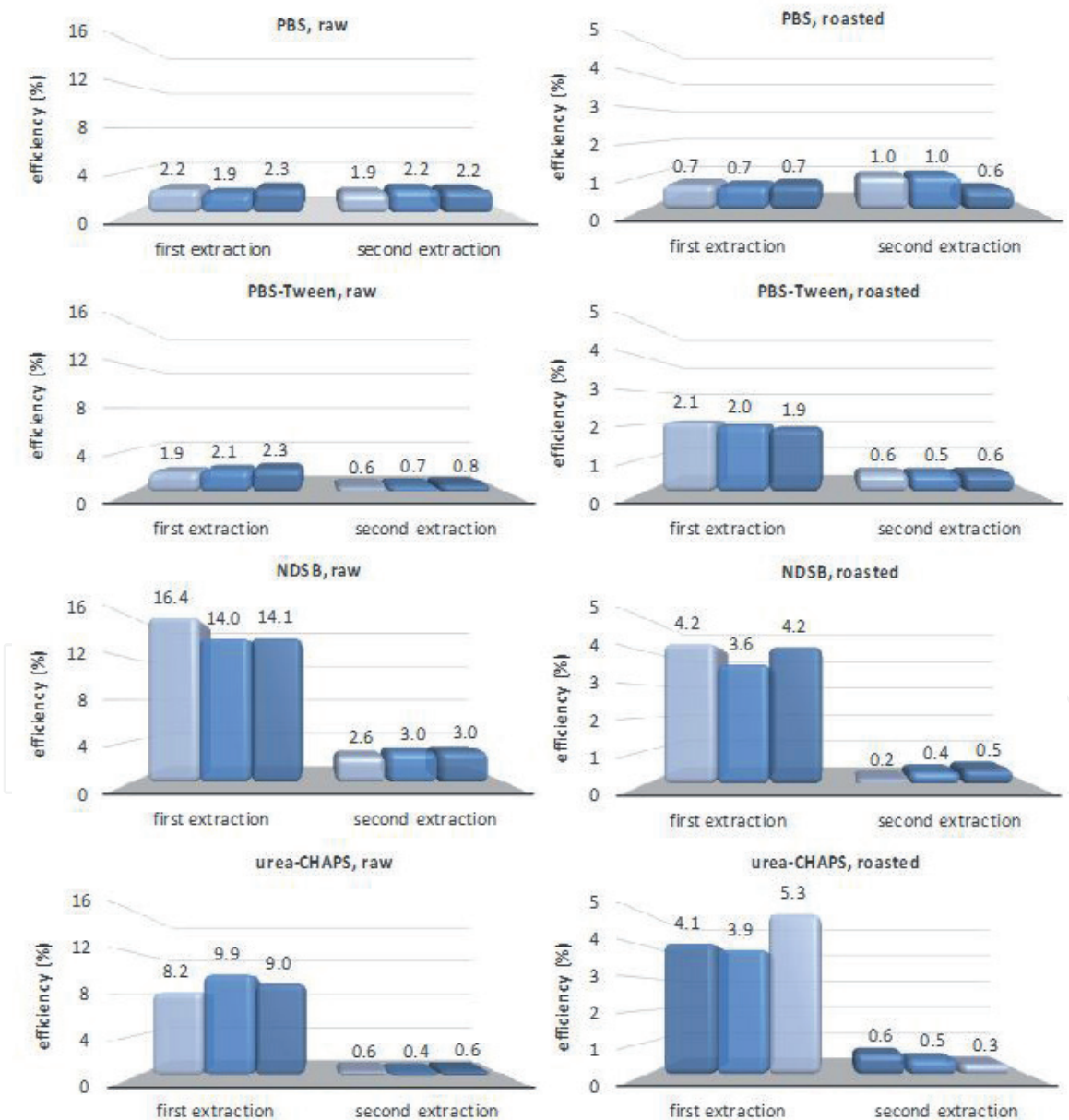


Figure 3. Normalized recovery of total protein following sequential extractions in 0.4 mL of PBS, PBS-Tween, NDSB, or urea-CHAPS. Following an initial extraction in 0.4 mL, an additional 0.4 mL of buffer was added to the insoluble peanut triturate remaining in the BioMasher insert and the extraction process was repeated. Total protein is expressed in terms of its percentage of the total peanut biomass.

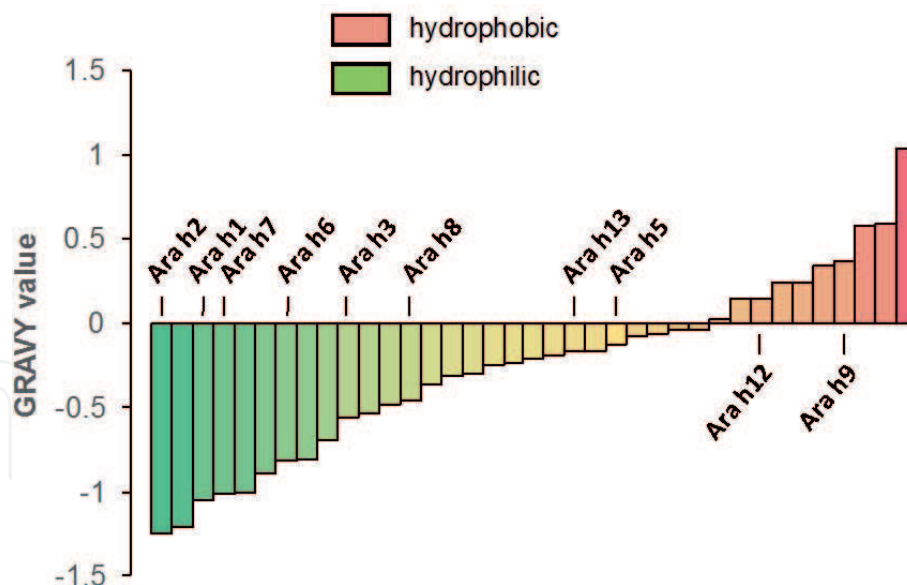


Figure 4. Relative hydrophobicity of 37 published protein sequences including 10 known allergens from *Arachis hypogaea*. Grand average of hydrophobicity (GRAVY) values were calculated from FASTA sequences where the sum of the hydrophobicity index values for each amino acid is divided by the total number of residues.

of urea-CHAPS was $9.0 \pm 0.9\%$ for the first extraction which was increased to $9.6 \pm 0.8\%$ when a second extraction was performed. For roasted peanut, mean efficiency of urea-CHAPS was $4.4 \pm 0.8\%$ for the first extraction which was increased to $4.9 \pm 0.6\%$ when a second extraction was performed. While the total protein recovered from roasted peanut extracted in urea-CHAPS or NDSB were similar, the chaotrope rendered the samples incompatible with the capture ELISA used in these studies (**Figures 3 and 4**).

4. Concluding remarks

Of the reagents tested, the NDSB reagent yielded the highest recovery of Ara h3, on average yielding seven times more allergen from roasted peanut than PBS-Tween. While the overall solubility of proteins is significantly diminished in roasted peanut, NDSB recovered nearly identical amounts of Ara h3 from both raw and roasted peanut.

ELISA and total protein values obtained from this cultivar indicated that Ara h3 comprised $34.9 \pm 1.4\%$ (CV = 0.039, n = 3), of the soluble protein derived from raw peanut and $34.6 \pm 3.0\%$ (CV = 0.085, n = 3) of the soluble protein from roasted peanut. Seed storage proteins may be overexpressed in response to growing conditions and can vary considerably between cultivars. This suggests that some cultivars may be more hyperallergenic than others. Moreover, the recovery of total soluble protein varies between cultivars, and extraction efficiencies as low as 9% based on the initial peanut mass have been reported for boiled runner peanuts [29]. The calculation of extraction efficiency, however, is influenced by variable water content. In these experiments, the NDSB reagent yielded 17.7 and 4.4% of the initial peanut mass as soluble protein from raw and dry roasted peanuts, respectively.

The described sample preparation method may be positively biased toward the solubility of this particular allergen. Further work is needed to investigate the solubility of other peanut proteins, particularly other clinically important peanut allergens.

Abbreviations

CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
ELISA	enzyme-linked immunosorbent assay
NDSB	non-detergent sulfobetaine
PBS	phosphate buffered saline.

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