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Peanut protein extraction conditions strongly influence yield of allergens Ara h 1 and 2 and sensitivity of immunoassays

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ABSTRACT

The clinical importance of peanut (*Arachis hypogaea*) allergies demands standardized allergen extraction protocols. We determined the effectiveness of common extraction conditions (20 buffers, defatting reagents, extraction time/temperatures, processing, extraction repeats) on crude protein and Ara h 1 and 2 yields. Despite similar 1D-gel profiles, defatting with *n*-hexane resulted in significantly higher yields of crude protein, Ara h 1, and Ara h 2 than with diethyl ether. The yields were affected by the composition and pH of the extraction buffers and other conditions, but crude protein yield did not always correlate with Ara h 1 and 2 yields. Denaturants, reducing agents, acidic buffers, and thermal processing of peanuts perturbed allergen quantification in ELISAs, probably via exposure of additional epitopes. Allergen detection in 2D-Western blots with PBS resulted in greater sensitivity than with TBS or Tris. We recommend that allergen extraction conditions be selected based on the research question being investigated.

1. Introduction

Among the allergenic foods, peanut (*Arachis hypogaea*) is the leading cause of anaphylactic fatalities worldwide (Bock, Muñoz-Furlong, & Sampson, 2007; Burks, 2008). There is particular concern because of the extreme hypersensitivity of some individuals for whom threshold doses are as low as 30–100 μg of peanut protein. In a study by Wensing, fifty percent of peanut-allergic individuals had an allergic reaction to only 3 mg of peanut meal (Wensing et al., 2002), a fraction of an average peanut kernel weighing 0.5–0.7 g.

A large range of peanut allergens and soluble proteins are incorporated within the structure of the kernel. When selecting an extraction protocol, possible differences in solubility, conformation and extraction yield among these proteins should be taken into account. Extraction is the basis for all subsequent measurements of the allergen content of peanuts, yet extraction conditions differ vastly in the literature. Some studies have demonstrated that the yield of crude peanut protein is highly dependent on extraction procedure and choice of buffer (Kain, Chen, Sonda, & Abu-Kpawoh, 2009; Kim et al., 2011; Poms, Capelletti, & Anklam, 2004; Sathe et al., 2009; Zeleny & Schimmel, 2010) and, recently, effects of extraction conditions on allergen detection via immunoblotting have been determined (L'Hocine & Pitre, 2016a, 2016b). However, to date there has been no empirical study to quantify specific allergen extraction yields under commonly used extraction conditions. To optimise peanut allergen extraction, there is a need to test a wide range of extraction conditions and their effects on subsequent detection methods.

Here we determined the influence of an extended range of extraction variables on the extraction yield of crude protein and Ara h 1 and 2 from peanut kernels. We quantified Ara h 1 and 2 using ELISA and performed 2D-Western blots to assess possible effects on allergen detection. This knowledge will be valuable for the optimisation of peanut protein extraction for medical researchers and peanut breeders.

Abbreviations: Ara h 1, 2, 3, etc., *Arachis hypogaea* allergen 1, 2, 3, etc.; ELISA, enzyme-linked immunosorbent assay; IEF, isoelectric focusing.

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2. Materials and methods

2.1. Peanut samples

Spanish runner peanuts (*Arachis hypogaea*) of the cultivar Walter were kindly provided by the Peanut Company of Australia and stored at 4 °C until use.

2.2. Lipid removal

Peanuts were homogenized with a mortar and pestle or an electric coffee grinder and defatted as described previously (Walczyk et al., 2013). To compare defatting reagents, either *n*-hexane or diethyl ether was used before protein extraction. In all other experiments *n*-hexane was used.

2.3. Crude protein extraction

Unless stated otherwise, following defatting with *n*-hexane, 1.350 ml of extraction buffer was added to exactly 45 mg raw peanut flour and vortexed for 30 s. Proteins were extracted under constant agitation on a shaker. The extraction conditions were varied to determine the optimum protein yield and better conditions adopted in the experimental process (Table 1). Samples used to test different centrifugation parameters and extraction buffers (Figs. 3–5) were extracted at 40 °C for 1 h. Samples used to test different extraction temperatures were extracted for 1 h at 4, 21, 40 and 60 °C (Fig. 1b). Since an extraction at 40 °C was less effective, the effect of extraction times (30 min, 1 and 2 h) was tested at 21 °C (Fig. 4c). Furthermore, one set of samples was vortexed for 1 min (instead of 30 s) without further extraction on a shaker. Because the extraction at 30 min was most effective, all subsequent experiments (exhaustive extraction, thermally processed peanuts and defatting reagents) were performed at 21 °C, for 30 min (Figs. 1a, d and 2). After protein extraction, all extracts were centrifuged three times at 12,600*g* for 5 min. The supernatant containing the proteins was aliquoted and stored at −80 °C. Three biological replicates were used for each extraction.

2.4. Exhaustive extraction

To determine the effect of multiple extractions on yield, proteins were extracted six times consecutively with either 20 mM Tris (pH 8.5) or TBS (pH 8.5). Urea was added to the extraction buffer in the last step.

2.5. Thermal processing

Peanut kernels were roasted for 20 min at 170 °C in a conventional oven or boiled for 20 min in water before protein extraction.

2.6. Centrifugation parameters

Two centrifugation accelerations, 3000 and 12,500*g*; three centrifugation times, 5, 15 and 30 min; and 2–3 consecutive centrifugations were tested.

2.7. Protein extraction from seed coats

Seed coats (45 mg) were homogenized using a mortar and pestle for either 2 min or 15 min, and defatted before extracting with citrate (pH 4.5), 6 M urea (pH 6.7), 50 mM Tris (pH 8.5) or sodium borate (pH 9.2) using the same procedure as for the kernels.

2.8. Extraction buffers

Extractions were performed in triplicate as described above with three identical but independently prepared buffers. Buffer compositions are given in Suppl. Table 1.

2.9. Crude protein quantification

Total concentration of crude protein was determined in duplicate for each biological replicate, using the standard procedure of the 2D Quant kit (Amersham Biosciences-GE Healthcare) with a minimum pipetting volume of 10 µl to ensure reproducibility (details can be found in the manufacturer's instructions). All yields are given as mg per g defatted peanut flour.

Table 1
Extraction conditions for all experiments. The experiments are listed in chronological order. Conditions were optimised during the experimental process and bold letters indicate the chosen conditions for the consecutive steps.

Experiment	Figure	Peanut treatment	Defatting	Buffer	Centrifugation	Temperature (°C)	Time (min)	Number extractions
Centrifugations	n/a	Raw	*n*-hexane	TBS (pH 8.5)	various, incl. **12,600*g*, 5 min**	40	60	1
Buffers: general	1	Raw	*n*-hexane	Various, incl. **Tris (pH 8.5)**	12,600*g*, 5 min	40	60	1
Buffers: 2D-gels	2	Raw	*n*-hexane	Tris (pH 8.5) PBS (pH 8.0) TBS (pH 7.2)	12,600*g*, 5 min	40	60	1
Buffers: 2D-Western blots	3	Raw	*n*-hexane	Tris (pH 8.5) PBS (pH 8.0) TBS (pH 7.2)	12,600*g*, 5 min	40	60	1
Temperatures	4a	Raw	*n*-hexane	Tris (pH 8.5)	12,600*g*, 5 min	4 **21** 40 60	60	1
Times	4b	Raw	*n*-hexane	Tris (pH 8.5)	12,600*g*, 5 min	21	1 **30** 60 120	1
Defatting	4c	Raw	***n*-hexane** Diethyl-ether	Tris (pH 8.5)	12,600*g*, 5 min	21	30	1
Treatment	4d	**Raw** roasted boiled	*n*-hexane	Tris (pH 8.5)	12,600*g*, 5 min	21	30	1
Repeated extractions	5	raw	*n*-hexane	Tris (pH 8.5) TBS (pH 8.5)	12,600*g*, 5 min	21	30	5 (+1 with urea)