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EXTRACTION AND IDENTIFICATION OF THE MAIN ALLERGENS FROM FRIED PEANUTS

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Abstract

Food allergy (FA) is a relatively new food safety issue with an increasing prevalence worldwide. Although FA-related diseases are restricted to a small category of individuals, the number of patients concerned is continuously growing in the last years. A total of eight big ingredients have been identified for most of the allergic reactions: peanuts, milk, egg, wheat, soy, tree nuts, fish, and shellfish. The peanuts allergenicity is affected by the whole ingredient composition rather than by a single allergenic protein. In general, Ara h3 accounts for approximately 28% of the total protein fraction, followed by Ara h1 (11%), Ara h2 (10%) and Ara h6 (8%). The aim of our study was to extract the main allergens from peanuts using different methods of extraction. First the fried peanuts samples were defatted by stirring with pre-cooled acetone and 0.07% β-mercaptoethanol and then extracted with different buffers. The identification of proteins was made by Biuret and BCA assay and also by electrophoretic examination.

Key words*: food allergy, peanuts, peanuts extraction, allergens*

INTRODUCTION

Food allergy (FA) is a public health problem that has consequences on a personal, social, economic and nutritional level, being a growing global problem (Loh and Tang, 2018). A recent study conducted in the United States indicates that up to 8% of children and 10.8% of adults have FA (Gupta et al., 2018). Unlike milk and eggs allergies, which are usually overcome by the age of 5-10 years, the allergy to peanuts and nuts is often lifelong, persisting into adulthood in 80% of cases (Ballmer-Weber, 2011). In the United States, peanut allergy affects 2.2% of children and 1.8% of adults. Among children with FA, those with peanut allergy report the highest rate of anaphylaxis and side effects. Moreover, compared to other allergies, the one for peanuts results in more visits to the emergency department for anaphylaxis and 1 in 4 children requires at least one visit each year to the hospital to manage allergic reactions (Parlaman et al., 2016; Motosue et al., 2018).

Avoidance of peanuts is inherently difficult because of their widespread consumption as roasted peanuts and snack products, use in food industry for oil and peanut butter production, cake decoration, extenders in meat products, soups and desserts formulations (Zhao et al., 2012). Studies designed to determine the minimum dose of peanut proteins that may cause an allergic reaction have reported subjective symptoms at a dose up to 100 µg and objective symptoms at 2 mg of ingested proteins (Al-Muhsen et al., 2003). Nowadays, some therapies have been introduced to reduce the prevalence of peanut allergy, including strict avoidance, oral immunotherapy, rescue medication upon accidental exposure to peanuts and modifying or removing allergens from foods (Wood, 2016; Togias et al., 2017).

Peanuts contain major proteins, such as albumin, globulin (arachin, conarachin), but also several minor functional proteins with a metabolic role. To date, 17 types of peanut allergens, referred to as Ara h1 - Ara h17, have been published and named by the World Health Organization and International Union of Immunological Societies (WHO/IUIS). Among these allergens, Ara h2, Ara h6 and Ara h7 are 2S albumins, while Ara h1 and Ara h3 are 7S/11S globulins (Pi et al., 2019).

Proteins have to be efficiently extracted to ensure an accurate representation of allergens from the source material. Therefore, the selection of an adequate method of extraction represents a major step for further investigations. In order to obtain an optimized peanut extraction, several parameters and buffers were tested and their effect on protein characteristics was investigated (Ma et al., 2010; Mihai (Draghici) et al., 2020).

The aim of our study was to extract the main allergens from fried peanuts using several protocols of extraction at different pH and temperature values. The quantification of proteins was performed by Biuret and BCA assays and their identification by electrophoretic examination. The data regarding the optimization of peanut protein extraction will be valuable for medical researchers and peanut breeders.

MATERIALS AND METHODS

Materials

Acetone, disodium phosphate, monosodium phosphate, boric acid, sodium hydroxide, sodium bicarbonate, sodium carbonate (anhydrous), bicinchoninic acid (BCA), sodium hydroxide, copper(II) sulfate and potassium sodium tartrate were purchased from Sigma-Aldrich (Germany). Bovine serum albumin (BSA) (synthesis grade, ≥95%) was purchased from Merck (Germany).

Peanut sample preparation

The sample (fried peanuts) was purchased from a local supermarket, washed in distilled water and dried at 40°C, in an oven.

Protein extraction and analysis

Protein extraction

The peanut sample was milled to a fine flour using a laboratory grinder. The peanut flour was defatted by stirring in pre-cooled acetone (1:5 ratio, w/v) at 4°C, for 3 h and then filtered using a vacuum filter. The defatting process was repeated three times. The defatted flour was dried in an oven at 40°C and stored at –20°C until further use. Peanut protein was extracted from the defatted flour using six different protocols (Protocols 1-6) by varying two parameters: the extraction buffer type and the temperature of extraction.

Protocol 1: Peanut protein extract was obtained by mixing the defatted peanut flour with 0.03M phosphate buffer, pH 7.2, in a ratio of 1:10 (w/v), at 25 °C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 2: Peanut protein extract was obtained by mixing the defatted peanut flour with 0.03 M phosphate buffer, pH 7.2, in a ratio of 1:10 (w/v), at 4°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 3: Peanut protein extract was obtained by mixing the defatted peanut flour with 1 M borate buffer, pH 8.5, in a ratio of 1:10 (w/v), at 25°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 4: Peanut protein extract was obtained by mixing the defatted peanut flour with 1 M borate buffer, pH 8.5, in a ratio of 1:10 (w/v), at 4°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 5: Peanut protein extract was obtained by mixing the defatted peanut flour with 0.05 M carbonate buffer, pH 9.6, in a ratio of 1:10 (w/v), at 25°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 6: Peanut protein extract was obtained by mixing the defatted peanut flour with 0.05 M carbonate buffer, pH 9.6, in a ratio of 1:10 (w/v), at 4°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protein quantification

Peanut protein content was investigated by Biuret and BCA assays. A standard curve was built using BSA in the range of concentrations 0.1-10 mg/ml.

Biuret assay: The principle of the method is based on the formation of a purple complex between the peptide bonds from a polypeptide chain and Cu^{2+} ion, in alkaline conditions. The intensity of the developed color is proportional to the sample protein content (Zhenga et al., 2017). Biuret reagent was prepared from an aqueous solution of potassium sodium tartrate treated with cupric sulfate and sodium hydroxide. In Biuret assay, 2 ml of sample

extract were incubated with 3 ml Biuret reagent, at 37°C, for 10 min. The absorbance was measured at 540 nm against blank (water instead of sample) using an UV-Vis spectrophotometer (Jasco V650, Japan).

BCA assay: The BCA assay determines the total concentration of protein using two step procedure by reducing Cu^{2+} to Cu^{+} ions, under alkaline conditions. Then, Cu^{+} ions react with BCA to form a colored complex (Walker, 1994). BCA reagent was prepared by mixing BCA with 4% cupric sulfate solution in a ratio of 50:1 (v/v). In BCA assay, 20 µl of sample extract were incubated with 160 µl BCA reagent, at 37°C, for 30 min. The absorbance was measured at 562 nm using the microplate reader SPECTROstar Nano (BMG Labtech, Germany).

Electrophoresis in tricine-SDS polyacrylamide gradient gel

Peanut proteins were analyzed by tricine-SDS-polyacrylamide electrophoresis, according to Schagger method (1987). The sample was denatured at 90°C, for 3 min and loaded on 10-20% tricine gels. Migration was carried out at 100 V, for 3 h using a BIO-RAD source. Wide range molecular weight marker (6.5-200 kDa) was used as standard. Gels were stained with Coomassie Brilliant Blue solution.

Statistical analysis

Experiments of extraction and protein content determination were carried out in duplicate and triplicate, respectively. All data are expressed as the mean ± standard deviation (SD). Statistically significant differences were considered at $p<0.05$.

RESULTS AND DISCUSSIONS

Extraction of the main allergens

A great challenge when studying peanut allergens is to find the most appropriate method of extraction with the highest yield and reproducibility. Several factors affect the extractability of these proteins, including the type of buffer and the extraction temperature. In our study, the allergens were extracted from fried peanuts varying the working parameters, like buffer type and temperature, in order to select the optimal ones. The extraction conditions are presented in Table 1.

Table 1. Peanut allergens extraction conditions

Sample	Extraction conditions
A I.1	0.03 M phosphate buffer; pH 7.2; 1:10 (w/v); 25°C; 4 h
A I.2	0.03 M phosphate buffer; pH 7.2; 1:10 (w/v); 4°C; 4 h
A II.1	1 M borate buffer; pH 8.5; 1:10 (w/v); 25°C; 4 h
A II.2	1 M borate buffer; pH 8.5; 1:10 (w/v); 4°C; 4 h
A III.1	0.05 M carbonate buffer; pH 9.6; 1:10 (w/v); 25°C; 4 h
A III.2	0.05 M carbonate buffer; pH 9.6; 1:10 (w/v); 4°C; 4 h

Protein quantification by BCA assay

One of the most used methods for quantifying proteins is based on their reaction with BCA, as proposed by Smith et al. (1985).

The BCA assay results for protein quantification in six extracts obtained from fried peanuts are showed in Table 2.

Table 2. Protein concentration in fried peanut extracts determined using BCA assay. The results are expressed as mean ± SD.

Sample	Protein concentration (mg/ml)
A I.1	2.66 ± 0.01
A I.2	1.50 ± 0.02
A II.1	14.61 ± 0.04
A II.2	11.77 ± 0.01
A III.1	26.49 ± 0.02
A III.2	21.74 ± 0.04

The results obtained by BCA assay showed that the amount of protein in fried peanut extracts varied between 1.50 and 26.49 mg/ml. The highest protein content was found in the case of A III.1 (26.49 mg/ml) and A III.2 (21.74 mg/ml) samples, while the lowest values were registered in the case of A I.1 (2.66 mg/ml) and A I.2 (1.50 mg/ml) samples. The samples A II.1 and A II.2 presented average values of protein content of 14.61 mg/ml and 11.77 mg/ml, respectively.

Protein quantification by Biuret assay

Compared with other methods for protein quantification, the Biuret assay has the advantages of easy operation, excellent precision.

The Biuret assay results for protein quantification in six extracts obtained from fried peanuts are showed in Table 3.

Table 3. Protein concentration in fried peanut extracts determined using Biuret assay. The results are expressed as mean ± SD

Sample	Protein concentration (mg/ml)
A I.1	7.04 ± 0.01
A I.2	4.66 ± 0.03
A II.1	21.14 ± 0.04
A II.2	14.00 ± 0.01
A III.1	36.69 ± 0.02
A III.2	29.88 ± 0.01

The results obtained by Biuret assay showed that the amount of protein in fried peanut extracts varied between 4.66 and 36.69 mg/ml. The highest protein concentration values were found in A III.1 (36.69 mg/ml) and A III.2 (29.88 mg/ml) samples, the lowest protein content values were registered in A I.1 (7.04 mg/ml) and A I.2 (4.66 mg/ml) samples. An average protein content was found in A II.1 (21.14 mg/ml) and A II.2 (14.00 mg/ml) samples. The results obtained in the case of Biuret assay indicated slightly higher values of protein content in fried peanut extracts, as compared to those obtained for the same extracts analyzed by BCA assay. Due to similar variation observed in both methods, the effect of extraction parameters was further discussed in relation to BCA assay. In addition values of protein content were expressed as arbitrary units (A.U) for a better comparison of the results.

Effect of the extraction buffer composition and pH on total protein content

Our study was conducted in three directions: influence of buffer pH (there is a difference of 2.4 pH units), the influence of ionic strength (the variation is between 0.03 and 1.0M) and the influence of buffer type.

The effect of buffer composition on the protein content of fried peanut extracts is presented in Figure 1.

The results show that a 10 to 14.5-fold increase of protein content was obtained in the case of fried peanut extraction using carbonate buffer, pH 9.6, compared to the case of extraction in phosphate buffer, pH 7.2. In the case of fried peanut extraction using borate buffer, pH 8.5, a 5.5- to 7.8-fold growth of protein content was observed, compared to the case of extraction in phosphate buffer, pH 7.2.

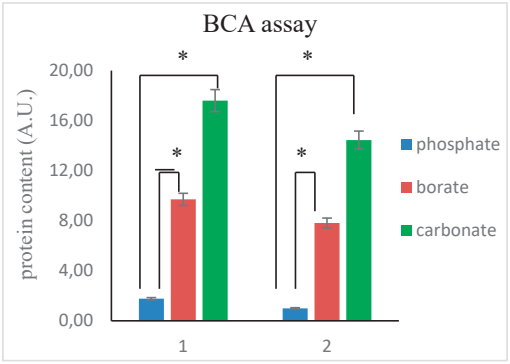

Figure 1. Effect of the extraction buffer composition on total protein content in fried peanut extracts determined using BCA. The results are expressed in arbitrary units (A.U.), as mean ± SD, *p<0.05

Statistical analysis showed significantly ($p<0.05$) higher values of protein content in extracts obtained in carbonate buffer, pH 9.6, compared to those in extracts obtained in phosphate buffer, pH 7.2. Similar, significantly ($p<0.05$) higher values of protein content were observed in borate buffer, pH 8.5, compared to those in phosphate buffer, pH 7.2.

Effect of the extraction temperature on total protein content

The effect of the temperature on the protein content of fried peanut extracts is presented in Figure 2.

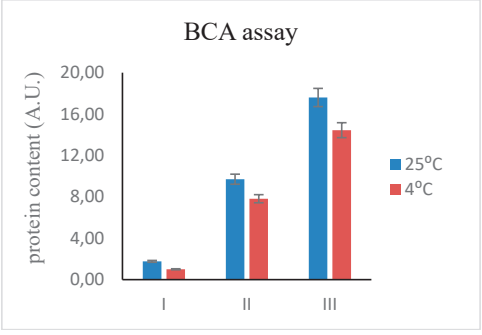

Figure 2. Effect of the extraction temperature on total protein content in fried peanut extracts determined using BCA. The results are expressed in arbitrary units (A.U.), as mean ± SD, *p<0.05

It was observed that the temperature of extraction has induced great variation of the protein content in the six fried peanut extracts. Thus the extract obtained in phosphate buffer, pH 7.2 (I), at 25°C had an 1.77-fold higher protein content than that observed in the case of fried peanut extract at 4°C. A slightly lower increase of 1.24-fold was recorded for the peanut extract in borate buffer, pH 8.5 (II), at 25°C, compared to 4°C. Similar increase of 1.22-fold was calculated for the protein content in the peanut extract obtained in M carbonate buffer, pH 9.6 (III), at 25°C, compared to 4°C.

Statistical analysis showed significantly ($p<0.05$) higher amount of protein obtained at a temperature of 25°C than that at 4°C, regardless of the used buffer type for peanut extraction.

In other studies Tris-HCl was frequently used for peanut protein extraction at different pH and molarities. It was observed that pH values higher than 8 increased the extraction of peanut allergens similar to results our present study (Mihai (Draghici) et al., 2020; Masuyama et al., 2018).

Identification of allergens from fried peanuts by electrophoresis

The main allergens from fried peanuts were identified by electrophoresis in tricine-SDS-polyacrylamide gradient gel. The migration patterns of six protein extracts from fried peanuts are presented in Figure 3. Based on the molecular weight, the main bands were assigned to Ara h6 at 15 kDa, Ara h2 at 17-19 kDa, Ara h3 at 28 kDa and 42 kDa and Ara h1 at 64 kDa. The protein extracts in borate (A II.1, A II.2) and carbonate (A III.1, A III.2) buffers presented all types of allergens, although the peanuts were subjected to high temperature processing. In turn, faint bands corresponding to identified allergens were extracted in phosphate buffer, in particular at 4°C (A I.2).

Previous studies identified and quantified the main allergens in peanuts as Ara h1 in a percentage of 12-16%, Ara h2 10%, Ara h3 3.7-4.3% and Ara h6 in a proportion of 6-9% of the total peanut proteins content (Ma et al., 2010). Due to the presence of multiple disulfide bonds, Ara h2 and Ara h6 are more resistant to high temperatures and proteases than Ara h1 and Ara h3 (Pi et al., 2019).

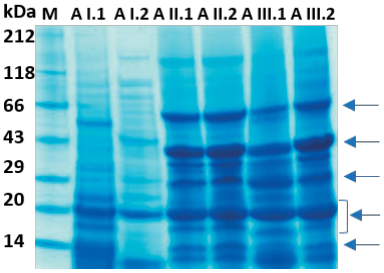

Figure 3. Tricine-SDS-polyacrylamide electrophoresis of fried peanut extracts A I.1-A III.2. The molecular weight marker (M) was migrated in the same gel

CONCLUSIONS

In this study, the influence of two extraction parameters, the buffer type and temperature was assessed, in order to identify the conditions that improved peanut allergens extraction. The protein content significantly increased in the case of using carbonate buffer at pH 9.6 as extraction solvent, compared to that obtained in phosphate buffer. The temperature also played an important role, the best results being obtained in the case of extraction at room temperature. The main allergens from fried peanuts were identified using tricine-SDS-polyacrylamide electrophoresis. Ara h1 (64 kDa), Ara h2 (17-19), Ara h3 (28 kDa, 42 kDa) and Ara h6 (15 kDa) appeared as main bands in all analyzed samples, excepting for phosphate buffer extracts. All these results presented valuable information for the optimization of allergens extraction from fried peanuts and their identification and could be valorized within medical and food industry studies.

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