Studii şi Cercetări Ştiinţifice 2017, **18** (2), pp. 135 – 143
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry *ISSN 1582-540X*

ORIGINAL RESEARCH PAPER

PURIFICATION OF WATER SOLUBLE PROTEINS (2S ALBUMINS) EXTRACTED FROM PEANUT DEFATTED FLOUR AND ISOLATION OF THEIR ISOFORMS BY GEL FILTRATION AND ANION EXCHANGE CHROMATOGRAPHY

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Received: February, 02, 2017
Accepted: May, 19, 2017

Abstract: 2S albumins are water-soluble seed storage proteins present in dicotyledonous plants, including legumes. In peanuts, 2S albumins have been identified as major allergens. In this work, we aimed to study these water soluble allergenic proteins. They were extracted in water from peanut defatted flour (oilcake). It was quantified by Bradford method. The total and insoluble proteins content was determined by Kjeldahl method (% P = N x 6.25). The crude 2S albumins were purified using gel-filtration chromatography. Anion exchange chromatography analysis was applied to isolate their isoforms. The recorded values for total and insoluble proteins are 45.49 % and 36.65 % consecutively. A value of 9.99 % was determined for water soluble proteins content which correspond to 20 % compared to the total proteins. Analysis by Sephadex G-75 chromatography of soluble extract gave two majors peaks in which, the Mr ~ 25 kDa peak was predicted to be pure 2S albumin fraction. Using DAEA-cellulose chromatography, two peaks were appeared from pure 2S albumins, it were predicted that 2S albumin isoforms theoretically represent the peanut major allergens Ara h2 and Ara h6. These approaches are the basis for further studies may involve immunological analysis to understand the impact of these biomolecules on peanut allergenicity.

Keywords: *chromatography, extraction, isolation, oilcake, purification, 2S albumins, water-soluble proteins*

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INTRODUCTION

Legumes, tree nuts, and seeds such as peanut, hazelnut, walnut, sesame and mustard seeds are known to potentially induce severe food allergy [1]. Peanut (*Arachis hypogaea* L.) is highly nutritious, rich in proteins, monounsaturated and polyunsaturated fatty acids, carbohydrates, and fibers. It is also a good source of vitamins and minerals [2]. There are three kinds of storage proteins in peanut seeds: arachin, coarachin I, and coarachin II (2S albumins) [3]. Because the peanut seeds are rich of these macromolecules, it is commonly used in human food in the world under different forms: frit, boiled, raw or grilled. Peanut is a healthy food source due to its abundant proteins and essential amino acids, and is favored by many people [4]. Total protein content of peanut is represented up to 32 different proteins of which about 18 have been identified as capable of binding specific IgE (Immunoglobuline E), so to be allergenic [5]. 2S albumins are water-soluble seed storage proteins that are widely distributed in dicotyledonous plants [6]. In peanuts, 2S albumins have been identified as major allergens [1]. They have more impact on allergenicity than the globulins [7]. The Ara h2 and Ara h6 are the two allergens belonging to this family [8, 9]. 2S albumins are proteins with a molecular mass of 12 kDa to 15 kDa generally containing eight or more disulfide-bridged cysteine residues. The physiological function of these proteins is still unclear, but a role as nitrogen and sulfur donor was suggested on the basis of their amino acid composition, their high abundance in seeds, and their mobilization during germination [10, 11]. They are composed of a large and small subunits joined by one or two disulfide bridges. These two subunits result from post-translational processing of the precursor protein at several sites, close to the N-terminus, internally, and at the C-terminus [12, 13]. Due to their important nutritional role in human food and animal feeds, 2S albumins and the associated genes have been isolated from a number of plant species and characterized in great detail to facilitate protein manipulation and improvement [14, 15]. The 2S albumins are becoming of increasing interest in clinical research as they have been described as the major allergen in a number of plant foods [16]. The allergenicity of plant food proteins may be due to the amount ingested, stability to processing and gastro-intestinal tract environment, as well as their intrinsic allergenic properties. Therefore, any investigation of the structure–function relationships of allergens requires the purification, characterization and elucidation of the structure and conformation of allergens [6]. Peanut has been extensively studied [17] and is one of the most common allergenic sources of food allergies in children, but little work has been done on the water-soluble proteins of this food. It is for this reason that we searched to purify 2S albumins from water soluble proteins extract and isolate their isoforms using the chromatographic approaches.

MATERIALS AND METHODS

Chemicals needed for the realization of this study including hexane were from Biochem Chemopharma Company (Montreal, Quebec, Canada). Bovine serum albumin, size markers, Sephadex-G75, DEAE-cellulose, Tris and NaCl were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Biological material

Raw peanut seeds of local variety were used in this study. Peanut (*Arachis hypogaea*) cultivation is carried out in organic farming in the region of El-Kala (Algeria). Sowing takes place in April-May and harvesting is done manually in July-August. The raw peanut seeds were stored at room temperature until use.

Sample preparation

Through the use of a coffee grinder, the raw peanut seeds were ground to pass through a sieve of 1 mm until a fine paste was obtained. 50 g of this product was treated with 500 mL of hexane in a Soxhlet extractor (reflux system) for 8 hours. The defatted resulting paste was dried in open air for 48 hours and then converted into flour before the proteins extraction [18].

Peanut water-soluble proteins extraction

Two grams of defatted flour were taken up in 15 mL of distilled water. The mixture was stirred at room temperature for 20 hours. The soluble fraction containing most proteins was isolated from the insoluble fraction by centrifugation using a Sigma™ Compact Centrifuge, model 2-16 (Fisher Bioblock Scientific, Germany) at 18000 g and 4 °C for 20 minutes. The pellet was subjected to 4 successive washings in the same conditions in order to observe the extraction of almost all the proteins [19]. The protein concentrations of the supernatants are followed by reading of absorbance at 280 nm using a UV/Visible spectrophotometer UviLine 9400 (Secomam, France). Supernatants containing the majority of soluble proteins having greater than zero optical density were collected and pooled and stored at -20 °C until use whereas the final pellet obtained was dried in oven (forced convection incubator, Bio Concept BC60, Froilabo – Firlabo, France) at 60 °C for 24 hours for proteins assay.

Determination of peanut protein content

The content of total proteins of the defatted peanut flour and the water-insoluble proteins resulting from the extraction was determined by multiplying the nitrogen content by the coefficient 6.25 (the nitrogen content in proteins was estimated to average 16 %, which corresponds to a conversion coefficient of 6.25) according to the Kjeldahl method [20].
Water-soluble proteins were determined by Bradford method [21]. A calibration curve was carried through solutions of increasing concentrations vary from 0 to 1 $mg \cdot mL^{-1}$ of bovine serum albumin by measuring optical density at 595 nm using a spectrophotometer (model UviLine 9400, Secomam, France). Protein concentration in peanut extract was expressed as mg protein / mL extract.

Purification of crude 2S albumins from peanut extract

Two milliliters of filtered extract were loaded on a Sephadex G-75 size exclusion glass preparative column (1.5 cm x 120 cm) equilibrated by water and attached to a peristaltic pump (model IPC-N 12, ISMATEC, France). In this normal pressure chromatography,

the elution was performed at a 30 mL·h^{-1} flow rate at room temperature and fractions of 2 mL / tube were collected. Each fraction was assayed at 280 nm. Peaks fractions were pooled and lyophilized. The column was calibrated using a set of molecular weight markers ranging from 13.7 to 84 kDa (obtained from Sigma- Aldrich Chemical, USA).

Purification of 2S albumin isoforms

2S albumin isoforms were isolated using an anion-exchange plastic preparative column (2.5 cm x 50 cm) attached to peristaltic pump (model IPC-N 12, ISMATEC, France) and equilibrated with a binding buffer (Tris 0.02 M) to remove salts. 4 mL volume of purified 2S albumins were loaded on a DAEA-cellulose anion exchange column. In this normal pressure chromatography, the elution was performed with a NaCl gradient (0 – 0.4 M) in Tris buffer (0.02 M, pH = 8) (V/V) according to Duan *et al.* [22]. Elution is carried at a room temperature with a 2 mL / min / tube flow rate. It is followed by a measurement of the absorbance of the various fractions eluted at 280 nm. Peaks fractions were collected and lyophilized prior to further analysis.

RESULTS AND DISCUSSION

Defatted peanut flour description

In order to increase the extraction yield, it was preceded to the elimination of lipid residues by Soxhlet extraction. This process yielded a product rich in proteins named peanut oilcake whose average weight is 23.73 g for a starting weight of 50 g. It was suggested that peanut seeds are composed of about 50 % lipids and the oilcake represents the remaining 50 %.
In our study, treatment with hexane of peanut seeds brings up oilcake. It is a product of peanut seeds lipids elimination. It contains low fat level and high amounts of protein residues.

Peanut protein content

The Figure 1 shows the total, insoluble and water-soluble proteins content of the studied legume.
The Kjeldahl method performed to determine the total proteins content in peanut oilcake and water-insoluble proteins yielded from the protein extraction recorded values of 45.49 %, 36.65 % sequentially. This determination was expressed as percent (%) compared to total dry mass of the sample. Regarding the water-soluble proteins, a value of 9.99 % was determined by the Bradford method.
Theoretically, the peanut oilcake is rich in proteins (47 – 55 % proteins in the dry matter of oilcake) [23 – 25]. In our analysis, the total proteins content recorded for this oilcake is close to the content shown in the literature, the water-insoluble proteins representing approximately 80.56 % compared to total proteins. This value is comparable with the literature [26].

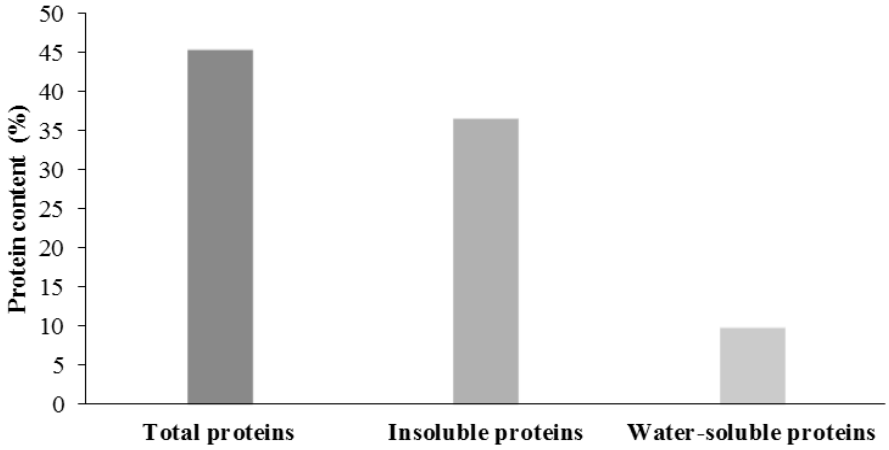

Figure 1. *Total, insoluble and water-soluble proteins content of peanut seeds defatted flour*

Relating to water-soluble proteins, the recorded value represent of 20 % compared to the total proteins. It is comparable to those reported in literature which indicates a rate between 10 and 20 % [26, 27].

Purification of crude 2S albumins and isolation of their isoforms

The analysis by gel-filtration chromatography of the water-soluble extract gave the chromatographic profile shown in Figure 2.

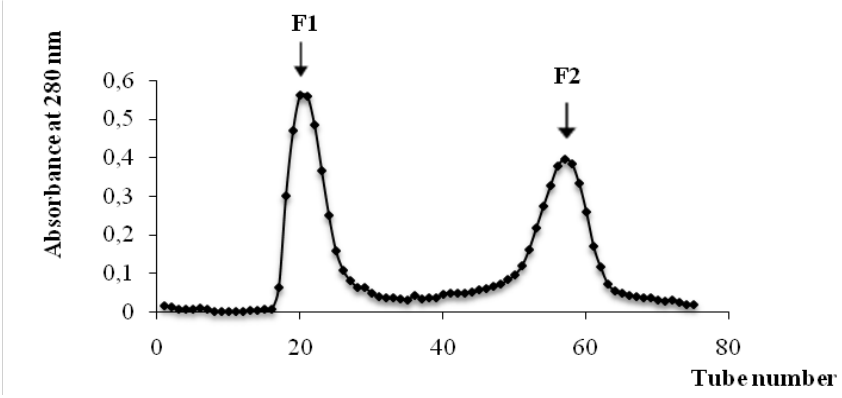

Figure 2. *Elution profile of water soluble peanut protein extract by Sephadex G-75 chromatography*

Two major peaks were obtained corresponding to two fractions designated F1 and F2 eluted in 32 – 58 mL and 92 – 132 mL volumes respectively. The molecular weight (Mr) of these peaks was calculated using standard proteins of known Mr ranging from 13.7 to 84 kDa. A distinct peak eluted at 92 – 132 mL was estimated 25 kDa, this known Mr was predicted to be peanut 2S albumins. The results of anion exchange chromatography analysis on a DEAE-cellulose column with 2 mL / min flow rate and a NaCl gradient (0 – 0.4 M) of this peak of Mr ~ 25 kDa (crude 2S fraction) are illustrated in Figure 3. The chromatographic profile obtained by the absorbance reading of elutes at

280 nm (Figure 3) gave two fractions eluted in 34 – 46 mL and 54 – 72 mL volumes respectively, it was predicted to be the 2S albumin isoforms. These entities eluted in specific volumes covering the main peaks of chromatogram were collected and stored at -20 °C for probable analysis later.

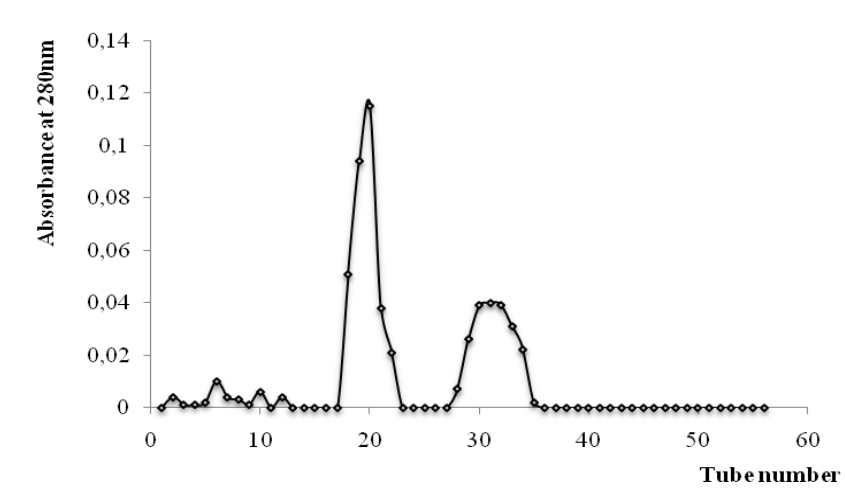

Figure 3. *Chromatogram of 2S albumin isoforms isolation on exchange anion DEAE-cellulose column*

Peanut seeds are known to potentially induce severe food allergy [1]. Peanut seeds contain abundant 2S albumin proteins which have an impact on allergenicity [7, 28]. 2S albumins have been identified as major allergens in many allergenic nuts and seeds [29]. 2S albumins are a very important family of peanut allergens which are structurally related to other plant proteins [30]. They are water-soluble proteins [22, 31]. Analysis by gel-filtration chromatography of our water-soluble extract gave two peaks of higher and lower molecular weight (Figure 2). The peak one that was eluted in the void volume (Vo) can represents the impurities or protein molecules soluble in the same conditions. It has a high molecular weight which, it was predicted to be globulins fragments. The second one peak was estimated of 25 kDa. Porterfield *et al.* [32] and Kulis *et al.* [33] showed that the fraction containing the Ara h2 and Ara h6 which was purified by gel chromatography (Sephadex G-75) has a theoretical mass molecular of 20 kDa and an interval from 13 to 25 kDa. So, our estimated value corresponds to pure 2S albumins. Generally, purification of these macromolecules (2S albumins) was the subject of numerous studies not only peanuts but also many others legumes such as Brazil nuts and sesame [34, 35], therefore, these proteins have an important place in the area research. The Ara h2 and Ara h6 are two allergens belonging to the 2S albumin family [36, 37]. Purification of these isoforms has actually begun by Burks *et al.* [38] where they isolated Ara h2 by anion exchange chromatography on a PL-SAX (BioRad) solid phase and the elution is performed with a NaCl gradient from 0 to 1.5 M. In 2002, Sen *et al.* [39] purified Ara h2 from a protein extract precipitated with 40 % to 70 % ammonium sulfate, and then fractionated by anion exchange chromatography on HighQ phase (BioRad) whose elution is performed with a linear gradient of 40 to 140 mM NaCl, Ara h6 was purified by Suhr *et al.* in 2004 [40] followed by Koppelman *et al.* in 2005 [41]. In our study, the chromatographic profile obtained by elution of pure 2S albumins fraction on anion exchange chromatography

DEAE-cellulose is resolved because it shows two distinct entities which mostly represent both 2S albumin isoforms Ara h2 and Ara h6.

CONCLUSIONS

Most of the plant seeds are rich in nutrients and energy sources, such as proteins, starches, and oils (storage matter). These reserves are mobilized to supply the seedling with energy during growth and germination. Peanut is a legume which known to potentially induce severe food allergy. In peanut, 2S albumins were identified as major allergens. In this study, the extraction, quantification, purification of water-soluble fraction (2S albumins) and isolation of their isoforms are reported. The results presented in this work show the usefulness of chromatography types to purify and isolate 2S albumins and their isoforms. Such information is crucial for future works including the structural and immunological studies to understand how these biomolecules induce the allergy appearance.

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