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ORIGINAL PAPER

Extraction and Characterization of Proteins from *Pachyrhizus ahipa* Roots: an Unexploited Protein-Rich Crop

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Abstract

Pachyrhizus ahipa is an unexploited crop known to be rich in proteins compared to other edible roots and tubers. These proteins are not prolamins, thus ahipa represents an interesting new source of ingredients for gluten-free foods. In this work, ahipa proteins (AP) were extracted and partially characterized in pursuit of their use as food ingredients. The effect of ultrasound treatment on protein extraction efficiency was evaluated. AP were characterized by their size, amino acid composition, surface hydrophobicity, intrinsic fluorescence, FTIR spectra, solubility, and thermal and emulsifying properties. AP were efficiently removed from the vegetal tissue using PBS or water, regardless of the use of ultrasound, but not easily recovered by precipitation. This protein fraction was composed of small proteins, with sizes ranging from 9 to 30 kDa, and highly polar. AP resulted particularly rich in aspartic acid (59% of the total amino acid content), for which they can be classified as Asp-rich proteins. Their elevated content of acidic groups was evidenced in the ATR-FTIR spectrum. The amide I band deconvolution as well as the low surface hydrophobicity and denaturation enthalpy indicated that these proteins are mainly unordered structures. The emulsifying properties of AP were enhanced when the concentration was increased from 0.1 to 1% (*w*/*v*) but resulted lower than those of soy protein. The high polarity, small size, and low isoelectric point make AP particularly suitable for acidic food matrices.

Keywords Acidic proteins · Aspartic acid · Gluten-free ingredients · Ahipa roots · Tuberous roots · Food proteins

Introduction

The food industry is constantly seeking new sources of proteins that could improve the technological and nutritional properties of food products. Protein-rich crops are particularly valuable due to the increasing consumer demand for plant-based protein products [1].

The Andean region is the source of countless food crops that were domesticated by indigenous communities over centuries. Some of these crops have reached universal distribution, such as potato, but many others are scarcely known, such as *Pachyrhizus ahipa*, a leguminous plant that produces starchy tuberous roots with purple dots in its flesh, due to the presence of anthocyanins [2]. *P. ahipa* shows a significant prospective, respect to its utilization as a high yielding and resilient root crop, source of starch, sugars and protein for subtropical areas. Likewise, this species can be used in interspecific crossings with the other two cultivated *Pachyrhizus* species (*P. erosus* and *P. tuberosus*) in the development of cultivars with valuable ecophysiological behavior. The crispness of ahipa succulent roots resembles that of an apple and their flavor are light, sweet, and pleasant as described for the roots of its relative *P. erosus* [3].

From a nutritional point of view, besides its starch content, one of the most notorious features of ahipa root is its protein level, relatively high compared to other edible roots and tubers (R&T) [4]. The reported values for different accessions of ahipa roots are in the range ~8–11% db [5]. Little is known about these proteins, being only recognized as non-storage [6], and non-prolamin proteins [7], thus they could be of interest for the

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production of gluten-free foods [8]. The characterization of the technological and nutritional potential of ahipa proteins could lead to a revalorization of this ancestral crop.

This work aimed to extract and partially characterize the proteins present in ahipa roots, and evaluate their emulsifying properties to assess their potential as food ingredients.

Materials and Methods

Plant Material

Locally cultivated ahipa (*Pachyrhizus ahipa* (Wedd.) Parodi) roots were obtained from a regional market in San Salvador de Jujuy (Jujuy, Argentina) (geographical coordinates: 24°11′ 40″S 65°17′49″W; average altitude: 1260 m a. s. l). Healthy roots were selected, washed with tap water, and disinfected by immersion in NaClO solution (250 ppm, 10 min). Roots were then air-dried, hand-peeled, and diced for protein extraction.

Effect of Ultrasonic Treatment in the Protein Extraction Yield

In a previous work [8] efficient protein extraction was achieved indistinctively with PBS or water in two extraction steps applying ultrasound treatment in the second step. To assess the effectiveness of the ultrasonication, two batches of 200 g of peeled and diced ahipa roots were accurately weighed and added with 200 mL of distilled water. The mixtures were homogenized with a domestic mixer, left 1 h in agitation at room temperature (RT), and then filtered through a muslin cloth. The retained bagasse was extracted again with 400 mL of distilled water and mixed. Ultrasound was applied to one of the batches (five pulses of 1 min at 600 W) according to Malgor et al. [8] using a Vibra-Cell™ Ultrasonic Liquid Processor (Sonics & Materials, Inc., Newtown, CT, USA) with a CV33 probe. Both batches were then left in agitation for 1 h at RT and filtered. The filtrates from both extraction steps were combined and left for 24 h at 4 °C to allow starch sedimentation. The starch cakes and root bagasse were dried at 50 °C and milled to fine powders. The protein extraction yield was calculated from the difference between the nitrogen content of the initial weight of peeled roots and that of the bagasse and the starch obtained after the protein extraction. Nitrogen content was determined by the Kjeldahl method according to Malgor et al. [8], and the extraction yield was expressed as percentage (%). Extractions were performed in duplicate.

Obtention of Ahipa Protein (AP) Concentrate

Phosphate buffered saline (PBS, pH 7.4) was added at a ratio of 1 L *per* kg of peeled roots. The mixture was homogenized with a domestic mixer, left in agitation for 2 h at 4 °C, and filtered through a muslin cloth. The filtrate was stored at 4 °C, and the bagasse was added with 1 L of PBS for a second extraction performed as described above. Filtrates from both extraction steps were combined and left for 24 h at 4 °C to allow starch sedimentation. The supernatant was separated from the starch cake, centrifuged, and filtered to remove the remaining insoluble starch. The filtrate was incubated with pancreatic alpha-amylase (K-TSTA, Megazyme, USA) at 40 °C with agitation in a temperature-controlled orbital shaker. The mixture was brought to pH 4 with 0.35 N HCl and ammonium sulfate was added at 90% of saturation. The mixture was centrifuged (10,000×*g*, 20 min, 4 °C) in a Beckman Coulter Avanti J-25 centrifuge (California, USA). The pellet was resuspended in DW, dialyzed against distilled water in a 12.4 kDa cutoff dialysis bag, and freeze-dried in a Heto FD4 (LabEquipment, Denmark) freeze-dryer. The total nitrogen content of the freeze-dried protein extract (AP) and the starch fractions (sedimented and centrifuged) obtained after the protein extraction was determined by the Kjeldahl method. The protein recovery % was calculated as the percentage of nitrogen in the AP compared to the initial amount of nitrogen in the roots.

Amino Acid Profile

Ahipa flour was obtained by peeling, slicing, drying at 60 °C, and grinding ahipa roots. The flour was defatted with hexane using the Soxhlet method. The analysis of the amino acid profile was carried out by Covance Laboratories (Madison, WI, USA). Samples were hydrolyzed in 6 N HCl for 24 h at ~110 °C, chemically stabilized, and then analyzed by HPLC after pre-injection derivatization.

Calculation of the Nitrogen-to-Protein Conversion Factor

The total nitrogen content of the defatted ahipa flour was determined by the Kjeldahl method. Ahipa flour was also analyzed for its amide nitrogen content by a 2 h digestion with 3 N HCl at 105 °C followed by alkalinization and ammonia distillation as stated in the Kjeldahl method.

The weight of total anhydrous amino acid residues (AAAw) was calculated from the amino acid profile (section 2.4). Two factors were calculated according to Mariotti et al. [9]: kp (the ratio of the AAAw to the total nitrogen content of the flour) and ka (the ratio of AAAw to the nitrogen coming from the amino acids + amide nitrogen). The conversion factor (k) was calculated as the average of ka and kp [9].

Electrophoretic Profile

AP concentrate (17 mg) was dissolved in 500 μL of sample buffer: Tris-HCl 0.185 M (pH 8.8), glycerol 12.5% (*v*/v), SDS 2% (*w*/*v*), and bromophenol blue 0.05% (w/v). The mixture was homogenized and centrifuged at 13,000×*g* for 10 min at 25 °C. The supernatant volume was divided into two equal fractions, one of which was treated with 12.5 μL of β-mercaptoethanol 5% (v/v). Polyacrylamide gel (12%, w/v) was loaded with 15–20 μg of each preparation and with a MW marker of 20.1, 30, 43, 67, and 94 kDa. Bands were revealed using Coomassie Brilliant Blue R-250 as staining agent.

Gel Filtration Liquid Chromatography

AP were analyzed by gel filtration chromatography at RT in a Superose 6B HR 10/30 column using an ÄKTA Purifier FPLC (GE-Healthcare, Uppsala, Sweden). A 20 mg sample of AP was dissolved in 1 mL of 50 mM Na_3PO_4 + 0.15 M NaCl buffer (pH = 7.4) and centrifuged at 13,000×*g* for 15 min at RT. The supernatants were separated, filtered, loaded onto the column, and eluted with the same buffer at a flow rate of 0.2 mL/min. The elution profile was obtained at 214 nm. Column calibration was performed with: blue dextran (V_0 void volume), thyroglobulin (669 kDa), alcohol dehydrogenase (150 kDa), albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa) (GE-Healthcare). Curves were processed, and data were evaluated using the Unicorn Software (GE-Healthcare).

Infrared Spectra and Data Analysis

The FTIR spectra of AP and a soy protein isolate (SP) used for comparison were determined in the range of 400–4000 cm^{-1} using an ATR diamond accessory (Thermo Scientific, MA, USA). For SP obtention, a dispersion of defatted soybean flour in water (1:10, *w*/*v*) was adjusted to pH 8, stirred for 1 h, and centrifuged at 10,000×*g* for 30 min at 4 °C. Proteins were precipitated at pH 4.5, recovered by centrifugation at 10,000×*g* for 20 min at 4 °C, resuspended in DW, neutralized, and freeze-dried.

ATR-FTIR spectra of AP and SP were obtained by co-adding 64 scans with 4 cm^{-1} spectral resolution. Five repetitions were performed for each sample and the recorded spectra were analyzed using the OMNIC software (version 8.3, Thermo Scientific, MA, USA). Spectra were baseline corrected and Fourier self-deconvolved in the range 1000 to 1900 cm^{-1} using a FWHH of 13 cm^{-1} and an enhancement resolution factor of 2. Peaks positions in the amide I band region (1600 to 1700 cm^{-1}) were determined from each deconvolved spectrum, and peaks were fitted to the original spectrum band using Gaussian line shape with a bandwidth of 13 cm^{-1}. For the estimation of protein secondary structure, peaks in the ranges of 1620–1638 cm^{-1} and 1680–1695 cm^{-1} were assigned to β-sheet, 1640–1650 cm^{-1} to random coil, 1650–1658 cm^{-1} to α-helix, and 1660–1680 cm^{-1} to turns and loops [10]. The percentage of each structure was calculated from the contribution of the area of peaks within each range to the total area [10].

Fluorescence Spectroscopy

AP and SP were dissolved in buffer (50 mM Na_3PO_4 + 0.15 M NaCl, pH = 7.4) to reach concentrations between 0.01 and 0.03 g L^{-1}. The mixtures were centrifuged (13,000×*g*, 15 min, 25 °C) and the intrinsic fluorescence was determined at 25 °C with a Perkin–Elmer LS 50B fluorescence spectrophotometer (Waltham-Massachusetts, USA) at λ_{ex} = 290 nm (slit width, 3 nm), an emission range of 310–550 nm (slit width, 3 nm), and a scanning speed of 300 nm min^{-1}.

Surface Hydrophobicity

Surface hydrophobicity (H_0) of AP and SP was determined as described by Kato and Nakai [11] using the 8-anilino-1-naphthalenesulfonate ammonium (ANS) fluorescent probe (Aldrich Chemical Co., Milwaukee-Wisconsin, USA). AP and SP were dissolved in phosphate buffer (35 mM KH_2PO_4, pH 8) to 16 and 14 mg mL^{-1}, respectively; stirred for 1 h at RT, and centrifuged at 9000×*g* (15 min, 20 °C). Aliquots (3 mL) of 1:50, 1:100, 1:250, and 1:400 dilutions of the AP and SP solutions were mixed with 50 μL of freshly prepared ANS solution (8 mM in phosphate buffer), and the fluorescence emission spectrum was recorded between 430 and 540 nm (λ_{ex} = 350 nm). The surface hydrophobicity was calculated as the slope of the curve of fluorescence intensity (FI) *vs* protein concentration (mg mL^{-1}). The FI was calculated by subtracting the emission spectrum (430–540 nm) of the sample from that of the ANS solution alone following the protocol of Ventureira et al. [13].

Thermal Properties

AP thermal properties were analyzed by differential scanning calorimetry (DSC) in a Q100 equipment (TA Instruments, New Castle, DE, USA). Protein aqueous suspensions (20%, *w*/w) were left for 12 h at RT, then weighed in aluminum pans and heated from 25 to 120 °C at 10 °C/min using an empty pan as reference. The denaturation peak temperature (Tp; °C) and enthalpy (ΔH; J/g) were obtained using TA Instruments Universal Analysis 2000 Software.

Effect of pH on the Solubility and Zeta Potential

AP samples (2 mg) were accurately weighed in 5 mL Falcon tubes, added with 100 μL of PBS, vortexed, and left for 2 h at RT with occasional mixing. DW (1.5 mL) was then added and the pH was adjusted to values from 1 to 8 using 1 M HCl, 0.1 M HCl, or 0.1 M NaOH. Volume was brought to 2 mL with DW and left for 30 min, vortexed every 5 min. The zeta potential of each sample was measured in an SZ-100 nanoparticle analyzer (Horiba, Kyoto, Japan).

For solubility analysis, samples were centrifuged for 10 min at 13,200×*g,* and protein content in the supernatant was determined by the Bradford method. Solubility was calculated as % of the absorbance measured at pH 12 considered as the 100% solubility.

Emulsifying Properties

AP and SP were assayed at concentrations of 0.1, 0.5 and 1%. Proteins were solubilized in alkaline medium (pH ~ 9), centrifuged at 3000×*g* for 10 min, and the supernatant was separated and brought to pH 6.2 with 0.1 M HCl. The volume was adjusted with distilled water to reach the desired protein concentration in the continuous phase.

Oil in water emulsions (O/W) were prepared using commercial sunflower oil (Natura®, AGD, Argentina) as dispersed phase at a ratio of 4 g oil/10 g protein solution. Mixtures were emulsified with an Ultra Turrax T25 (10,000 rpm, 2 min) followed by ultrasound treatment in a Vibra-Cell™ equipment with a CV33 probe (375 W, 3 s). Emulsion destabilization was monitored in a QuickscanCoulter® (Fullerton, USA) equipment by measuring the % backscattering (%BS) along the tube every 1 min for 60 min. The creaming destabilization zone (bottom, 7–20 mm) and phase separation zone (top, 50–65 mm) of the tube were analyzed.

Statistical Analysis

Unless specified, determinations were carried out at least in triplicate. Results were analyzed by a one-way analysis of variance (ANOVA) followed by a Fisher's least significant difference (LSD) test, at the specified significance level (p = 0.05 or p = 0.01).

Results and Discussion

In previous work, two extractions using PBS or water (both solvents giving the same extraction %) and applying ultrasound treatment in the second extraction step efficiently removed AP from the root fibrous tissue [8]. In the present work, the effect of the ultrasound treatment in the protein extraction yield was evaluated. No significant difference ($p > 0.05$) was observed in the extraction yield achieved with and without the application of ultrasound (83.89 ± 1.55 and 83.91 ± 0.44%, respectively), which is advantageous for a simpler coupling between the traditional starch extraction and the protein recovery [12].

The content and distribution of amino acids in AP are shown in Table 1. According to the amino acid profile, the total nitrogen content obtained by the Kjeldahl method (0.94 ± 0.03%) and the amount of nitrogen from amide groups (0.20 ± 0.00%), the calculated nitrogen-to-protein conversion factor for AP is 5.1. Considering this conversion factor, ahipa root protein content results slightly lower than that previously reported (where factor 6.25 was used) [5], and the corrected range would be 6.5–9.4% db or 1.0–1.6% wb. The purity of the obtained AP concentrate using 5.1 as conversion factor was 77.6 ± 1.2%. Despite root proteins were efficiently extracted with PBS, the recovery %, calculated as the amount of protein in the freeze-dried extract compared to the initial amount in the roots, only yielded 22.6%, evidencing limited effectiveness in separating the precipitate from the supernatant. However, salting out added to acidification considerably improved the separation of the precipitate compared to the acidification to the isoelectric point without the addition of ammonium sulfate, which provided a protein recovery percentage of only 2.3% [8]. The extremely low recovery % obtained using isoelectric precipitation is due to the cloudy very light precipitate formed by ahipa proteins, which after centrifugation does not provide a compact pellet which could

Table 1 Amino acid profile of ahipa protein

Amino acid	Content (mg/g)	%
Aspartic acid	31.6	59.08
Threonine	1.14	2.13
Serine	1.59	1.75
Glutamic acid	2.60	2.97
Proline	1.06	1.98
Glycine	0.936	4.86
Alanine	1.60	2.99
Valine	1.87	3.50
Isoleucine	1.13	2.11
Leucine	1.30	2.43
Tyrosine	1.28	2.39
Phenylalanine	0.980	1.83
Lysine	1.65	3.08
Histidine	1.25	2.34
Arginine	2.55	4.77
Cystine	<0.100	<0.01
Methionine	0.459	0.86
Tryptophan	0.491	0.92
Total	53.486	100.00

be easily separated from the solution. Ammonium sulphate addition improved the compactness of the precipitate, allowing a much higher amount of protein to be removed from the supernatant. *A priori* more sophisticated separation methods would not be worth since ahipa proteins have valuable but not exceptional nutritional properties, and bioactive properties have not been evaluated yet. An alternative approach to avail these proteins was to precipitate them along with the ahipa starch sedimentation to provide a value-added gluten-free ingredient, as proposed in a previous work [8].

Table 1 shows that aspartic acid (one of the amino acids with negatively charged side chains) is largely the most abundant amino acid found in the sample, accounting for 59.1% of the total amount, while each of the other amino acids represents less than 5% of the total content. Plant food protein sources recognized as rich in aspartic acid such as asparagus or avocado only reach Asp values of 17.9 and 13.1% of their total amino acid contents, respectively [14, 15]. Referring to other edible starchy R&T, proteins from a high-protein variety of sweet potato (variety 55–2) presented aspartic and glutamic acid contents of 18.5 and 9.3%, respectively [16], while potato cultivars exhibited up to 7.5 and 13.9% of Asp and Glu, respectively [17]. Yam proteins, which have Asp and Glu as their most abundant amino acids, account for 11.9 and 18.5% of the total amino acids, respectively [18]. Thus, ahipa proteins, with 59% of Asp along with 3% of Glu, become markedly acidic and different from most plant-based food proteins.

Plant-based proteins rich in aspartic acid, also known as Asp-rich proteins, have also been found in *Citrus clementina* and are supposed to be related to Ca^{+2} regulation paths [19]. Asp-rich proteins are commonly found in mollusks and vertebrates and are mainly associated with biomineralization processes, such as shells assembling and dentine production [20]. Carboxyl groups in the aspartate side chains interact with calcium ions regulating the crystallization process of carbonates and oxalates providing the specific morphology for each solid mineral structure [20]. It has been previously reported that ahipa roots have relatively high amounts of oxalates [5], therefore it would be expected that ahipa proteins are involved in the formation of oxalate crystals.

Although it is not an essential amino acid, health implications related to aspartic acid consumption have been reported. Mammals have a biosynthetic pathway to produce D-Aspartate (D-Asp) from the L-enantiomer [21]. D-Asp is found in the mammalian brain as a free D-amino acid and has been related to neurological and psychiatric processes, such as cognition and affective disorders [22]. Supplementation with D-Asp orally to mice proved to enhance the regulatory processes involved in the relief of chronic pain and related psychiatric consequences [22]. D-Asp supplementation also proved to enhance testosterone levels in male animals [23]. Despite results in human trials have been inconsistent so far [23], there is an extensive market of D-asp supplements that are advertised as testosterone boosters that can help increasing muscle mass, thus new natural sources of aspartic acid such as ahipa roots would be of interest for this commercial purpose. It is worth to mention that proteins and reducing sugars in ahipa flour provide desirable characteristics in baked products due to Maillard reactions [12], but the significant amounts of aspartic acid may derive in the formation of acrylamide during cooking [24], thus this should be taken into account when using ahipa roots and its derivatives as food ingredients.

Regarding the essential acid amino acids, all of them (*i.e.*, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, and His) are present in ahipa roots, which account for 19.2% of the amino acid content.

AP were characterized by gel filtration chromatography and SDS-PAGE. Figure 1a shows that the main components of AP (Fig. 1a, I, and II) correspond to molecules of ~30 kDa and 9 kDa, respectively. These fractions are lower than those reported for potato (*Solanum tuberosum*) tuber (4.1–20.6 and 39–43 kDa) [25], also recognized among the protein-rich R&T. The low MW of AP supports the fact that the proteins might not be easily removed by precipitation.

Low-size proteins are often preferred for specific food uses therefore partial hydrolysis has been proposed to modify their functional properties within the food matrix. Extensive hydrolysis, which provides peptides in the range of 0.5 to 5 kDa, is used to increase the digestibility as well as to produce hypoallergenic hydrolysates, while less extensive hydrolysis is used for the formulation of protein supplements [26]. Partial hydrolysis leads to the improvement of functional properties such as foaming capacity and emulsion stabilization in particular proteins, but the main use of this process is to enhance protein solubility [26]. A drawback of this approach is the increase in production costs, particularly in the separation of the low MW resultant fractions from the remaining high MW ones [26]. In this sense, ahipa represents a good source of small, highly hydrosoluble proteins, without any modification needed.

The electrophoretic profile of AP showed that proteins MW are below 35 kDa (Fig. 1b), in agreement with the results reported by Forsyth and Shewry [6]. The similarity in the results obtained by gel filtration chromatography and SDS-PAGE (Fig. a, b) indicates that no significant non-covalent interactions are established between the proteins that may stabilize tertiary structures. This is to be expected in small, highly polar, and strongly negative charged proteins such as AP.

Treatment with β-mercaptoethanol showed a reduction of peptides size indicating the presence of disulfide bonds (Fig. 1b), thus cysteine residues are present, although in amounts below the detection limit of the chromatographic method (Table 1).

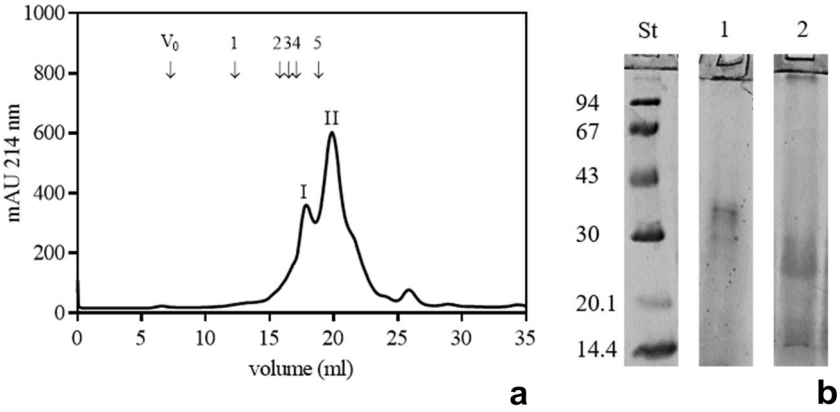

Fig. 1 **a** Gel filtration chromatography. Arrows indicate the elution volumes of standard proteins. V0: Void volume; 1: thyroglobulin (669 kDa), 2: alcohol dehydrogenase (150 kDa), 3: albumin (67 kDa), 4: ovalbumin (43 kDa), 5: ribonuclease A (13.7 kDa); **b** SDS-Page. St: standard proteins, 1: ahipa proteins, 2: ahipa proteins + β-mercaptoethanol (reducing conditions)

The curve obtained for zeta potential as a function of pH (Fig. 2a), showed that the proximate isoelectric point (pI) of the protein mixture was 3.64, lower than most of the proteins from food origin, which are normally in the range 4–7 [27]. Ahipa protein solubility at different pH values showed a typical U-shaped curve commonly found in food proteins [28], with minimum solubility around pH 3.5 (Fig. 2b). As observed, solubility is dramatically increased when pH changes from 4 to 5, which is an advantage when food uses are proposed, particularly over soy protein which has a pI rounding 4.5, which limits its application in many acidic food matrices.

The ATR-FTIR spectra of AP and SP are shown in Fig. 3. As observed, bands in the range of 2880–3000 cm^{-1} are markedly intense in the AP compared to the SP spectrum. Bands at 2851 and 2920 cm^{-1} are due to symmetric and asymmetric -CH_2 stretching vibrations, respectively, thus intense bands are indicative of the prevalence of amino acids with -CH_2 groups in their side chains, such as aspartic acid. Intense bands in this zone have been previously observed for Asp-rich proteins [29]. Bands at 1055 and 1455 cm^{-1} (Fig. 2) are associated with in-plane bending vibration of the (COH) of hydrogen-bonded Asp and Glu in the protein side chains [30]. The band at 1055 cm^{-1} could also be indicative of protein glycosylation or the presence of glycosidic groups. The shoulder at ~1735 cm^{-1}, which was also previously observed for Asp-rich proteins [29] and is absent in the SP spectrum (Fig. 3), is associated with the (COOH) vibration of Asp and Glu side chains [30], indicating the prevalence of this group within the protein structure. As observed in Table 2, the deconvolution of the SP Amide I band showed percentages of α-helix, β-sheet, turns and unordered structures within the range of values reported by other authors [31]. Despite the differences in measurement conditions leading to a high dispersion in the results informed, authors agreed in the higher proportion of β-sheet over α-helix in SP.

AP exhibited similar proportions of α-helix and turns and loops to those of SP but resulted higher in the percentage of unordered structures and lower in the amount of β-sheet. Carbonaro et al. [32] found a strong negative correlation between the amount of β-sheet and protein digestibility. Authors attributed a higher proportion of β-sheet to a better folding capability, making them less accessible to proteases. Therefore, the lower amount of β-sheet and a higher proportion of unordered structures of AP compared to SP could be associated with a better digestibility of ahipa proteins.

Thermal analysis of AP showed a broad denaturation peak centered at 81.1 ± 0.9 °C indicating that these are thermolabile structures with a ΔH of 3.5 ± 1.0 J/g, indicative of low

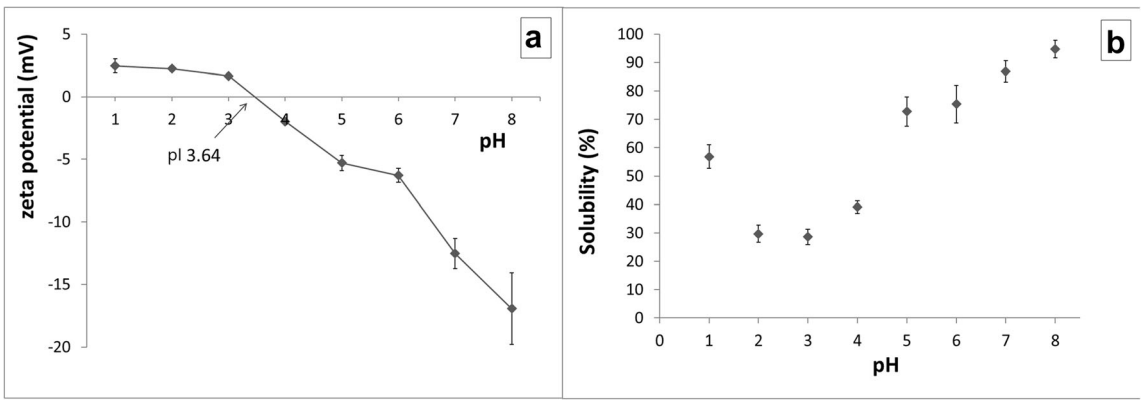

Fig. 2 Ahipa proteins **a** zeta potential and **b** solubility percentage, as a function of pH. pI: isoelectric point. From statistical analysis, LSD for zeta potential data is 2.17 and for solubility percentage is 7.05 ($p < 0.05$)

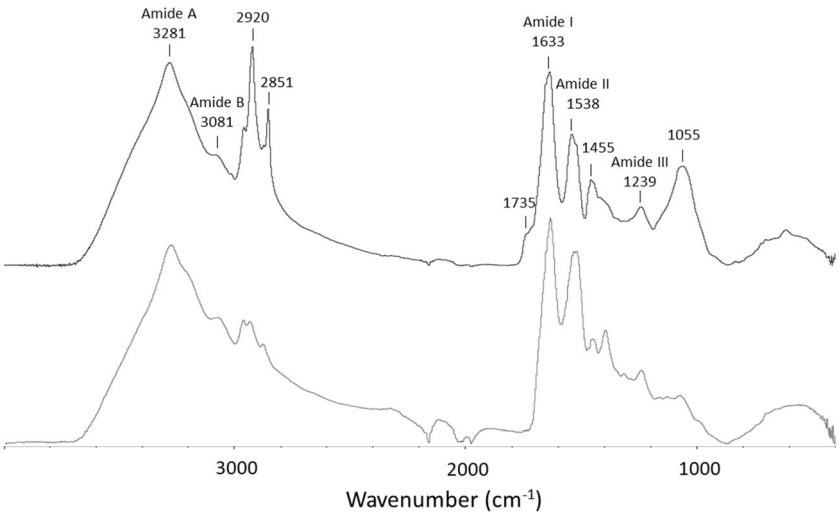

Fig. 3 ATR-FTIR spectra of ahipa (upper) and soy (lower) proteins. Bands of amide A, B, I, II and III, and specific bands discussed within the text are labeled in the ahipa protein spectrum

structured polypeptides. ΔH of AP resulted lower than that reported in the literature for SP (15.4 J/g) [33], in agreement with the higher proportion of unordered structures observed by FTIR and the lack of tertiary structures observed by size exclusion chromatography.

Fluorescence spectra showed that the spatial distribution of the tryptophan residues was different for both proteins (Table 3). In the case of AP, the tryptophan residues are more exposed to the solvent than in the SP, evidenced by a higher value of λ (Table 3), which is related to a more open and disordered structure, in agreement with the results obtained by FTIR. Nevertheless, the greater exposure of the aromatic residues of ahipa compared to those of soy proteins is not reflected in a higher surface hydrophobicity (H_0), (Table 3). A low H_0 is expected considering the Trp residues represent less than 1% of the total amino acid content of ahipa proteins (Table 1), being charged amino acids preponderant in the primary structure.

AP emulsifying properties were evaluated at different concentrations and compared to SP. As observed in Fig. 4, low concentration (0.1%) of AP and SP exhibited a similar value of initial %BS (BS_0) ($p > 0.05$), both low, indicating similar emulsion-forming properties. This value was increased when protein concentration was raised to 0.5% for both AP and SP, indicating the formation of a denser cream phase. Protein concentration of 0.5% produced a significantly higher ($p < 0.05$) BS_0 for SP than for AP but at 1% they were equated again. The good emulsification performance of AP compared to a well-known emulsifying agent such as SP might be related to its low size and high polarity, which allows AP to easily diffuse to the oil droplets surface [34]. Emulsion disruption was evidenced in the lower part of the tube (zone I) for AP, indicating that the emulsion creaming destabilization was much more pronounced than for SP, while no significant change in the %BS was observed in the upper side of the tube (zone II), indicating no change in droplets size or phase separation in that zone for either of the samples (Fig. 4). Increasing the AP concentration from 0.5 to 1% significantly improved emulsion stability (Fig. 4a), although a significant destabilization was still observed compared to SP. The low surface hydrophobicity, which may hinder the interaction with the oil phase, as well as the high number of ionizable groups, which could lead to hydrogen-bonding between adjacent proteins causing droplets aggregation, could be related to the lower stabilization properties of AP compared to SP in the assayed conditions [34]. Mu et al. [16] reported that sweet potato proteins, for which aspartic acid is the main amino acid, increased their emulsifying properties when solubility was reduced by the

Table 2 Protein secondary structures distribution from amide I band deconvolution

	α-helix	β-sheet	turns and loops	unordered
AP	14.5 ± 0.04[a]	32.8 ± 0.6[a]	19.9 ± 0.2[a]	20.9 ± 0.5[b]
SP	14.5 ± 0.2[a]	35.6 ± 0.8[b]	20.2 ± 0.7[a]	18.2 ± 0.5[a]

AP ahipa protein, *SP* soy protein

Results are expressed as average percentage ± standard deviation. Different letters within the same column indicate significant differences ($p < 0.01$)

Table 3 Protein fluorescence intensity (FI) and surface hydrophobicity (H_0)

	λ (nm)	FI per mg mL^{-1} protein	H_0
AP	338.5 ± 0.7[b]	1304 ± 16[b]	17 ± 1[a]
SP	333.5 ± 0.7[a]	788 ± 40[a]	52 ± 6[b]

AP ahipa protein, *SP* soy protein

Results are expressed as mean values ±standard deviation. Different letters within the same column indicate significant differences ($p < 0.05$)

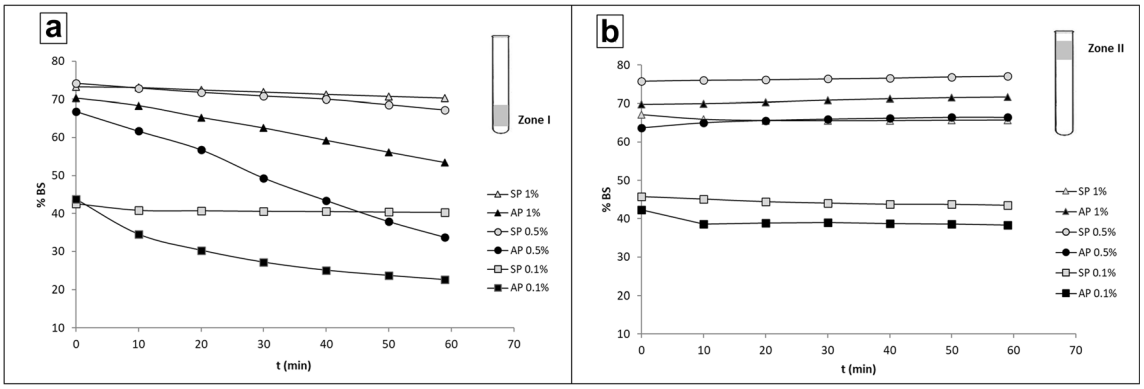

Fig. 4 Backscattering (%BS) of emulsions with 1, 0.5, and 0.1% of ahipa (AP) and soy protein (SP) as a function of time. %BS was analyzed in **a** the creaming destabilization zone (zone I); **b** the phase separation zone (zone II). From statistical analysis, LSD for % BS is 2.04 ($p < 0.05$)

addition of $CaCl_2$ or at pH close to the pI, contrary to that reported for SP [28], thus lower pH values could reduce the emulsifying performance gap between AP and SP.

Proteins can interact with anthocyanins by covalent and non-covalent bonds, and this was reported to enhance the emulsifying properties of SP [35]. As previously mentioned, ahipa root is a source of phenolics, including purple-colored anthocyanins. During the aqueous ahipa starch extraction, proteins, and anthocyanins are released and both interact with the starch granules, evidenced by a brownish dark layer over the obtained starch cake [8, 12]. In the present work, the starch cake obtained by sedimentation retained 2.7% of the initial nitrogen content of the roots, while the insoluble starch remaining in the aqueous medium, that was separated by centrifugation, bore 5.5% of the initial nitrogen content and showed a notorious surface coloration compared to the sedimented cake. This indicates that both, proteins and anthocyanins, were in higher amounts in the centrifuged sample, and suggests that they are also interacting with each other. When the supernatant is acidified to the pI the cloudy protein precipitate is white, but since ahipa anthocyanins are colorless around pH 4 [2], anthocyanins could be part of the impurities of AP. The high fluorescence intensity and the glycosidic band in the FTIR spectrum could also be suggesting the presence of small amounts of anthocyanins in AP. This interaction should be considered as a factor affecting the yield of protein recovery but could also be exploited to modify the emulsifying properties of AP or provide some antioxidant activity.

Conclusions

P. ahipa represents a new source of non-gliadin proteins, and a particularly interesting ingredient for the manufacture of gluten-free foods. Its protein content is relatively high compared to other edible roots and tubers (~6.5 to 9.5% db), and resulted outstanding in terms of its high content of aspartic acid: 59%, reflected in a particularly low pI (3.64) compared with most proteins from food origin. The protein recovery percentage obtained by isoelectric precipitation was significantly improved by combination with salting out (ammonium sulphate 90% of saturation) but further improvement needs to be done to make protein recovery scalable and economically viable.

AP is mainly composed of unordered structures, which is usually related to good digestibility. Their small size (up to 30 kDa), and high polarity led AP to be highly hydrosoluble, which is a sought feature for food proteins, particularly those intended as emulsifiers. Despite providing lower emulsion stability than soy protein (which has well-known emulsifying properties) at neutral pH, AP showed similar emulsion forming capacity than SP in the assayed conditions. Unlike SP, the good solubility of AP at pHs between 4 and 5 make these proteins interesting for being used in acidic emulsions such as dressings. The emulsifying properties of AP at different pHs and their interaction with the anthocyanins present in ahipa roots that could improve their emulsification performance are yet to be studied.

Abbreviations AP, Ahipa proteins; db, Dry basis; DW, Deionized water; FWHH, Full width at half height; PBS, Phosphate buffered saline; pI, Isoelectric point; RT, Room temperature; R&T, Roots and tubers; SP, Soy protein; wb, Wet basis

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Data Availability Not applicable.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no conflict of interest.

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