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Article type : Original Manuscript

Root starches enriched with proteins and phenolics from *Pachyrhizus ahipa* roots as gluten-free ingredients for baked goods

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Running title: Gluten-free protein-enriched root starches

The peer review history for this article is available at https://publons.com/publon/10.1111/ijfs.14457

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/IJFS.14457

Summary

Ahipa is a gluten-free starchy root, bearing phenolics and a protein content of ~9% db. Ahipa proteins are hydrosoluble, thus they are lost during starch extraction. The aim of this work was to recover ahipa proteins by isoelectric point (pI) precipitation to enrich ahipa and cassava starches. Both enriched starches had protein contents of ~2%, and their ATR-FTIR spectra revealed bands characteristic of ahipa proteins. Enriched starches also contained phenolics in concentrations of 18-20 μg GAE/g. Enrichment lowered the whiteness index, but it remained higher than 90. Protein-enrichment did not modify gelatinization onset and peak temperatures but reduced the gelatinization enthalpy of ahipa starch. Enriched starches produced less-rigid buns than the native ones, with smaller and more homogeneously distributed alveoli in the crumb which resulted darker than the crust. Starch slurries acidified to the pI of ahipa proteins led to novel gluten-free ingredients by an easy, low cost process.

Keywords Protein-enriched starches; phenolics; gluten-free baked goods; ahipa starch; cassava starch.

Introduction

Innovations in the field of gluten-free (GF) products tend to overcome limitations such as poor flavour, crumb and crust development, low nutritional quality, and fast staling tendency (Moroni *et al.*, 2009). Different proteins, hydrocolloids, emulsifiers, enzymes and/or fermented substrates have been used to simulate the binding between starch granules within the gluten network (Onyango *et al.*, 2009). Besides their contribution to texture, protein-rich ingredients also improve the nutritional quality of GF foods.

Roots and tubers (R&T) starches can purvey adequate techno-functional properties to GF products exhibiting diverse characteristics, which make them preferable to physically or chemically modified ones (Moorthy, 2002). Cassava (*Manihot esculenta* Crantz) is an excellent source of GF starch but has the drawback of bearing a low amount of proteins. A less explored source of GF starch is *Pachyrhizus ahipa* (ahipa) root. This species is one of the native Andean R&T crops with nutritional importance for local family farmers, which has also attracted the interest of the scientific community from different countries. Ahipa plants produce tuberous roots weighing 0.5-0.8 kg, which are their only edible organs. These roots have a fibrous skin while their flesh accumulates starch and has a relatively high protein content (7.9-11.5 %) due to their efficient nitrogen metabolism. Total phenolic

content of ahipa roots is 0.14 mg gallic acid equivalents/g (Dini *et al.*, 2013). Since ahipa proteins are highly hydrosoluble, most of them are released in the aqueous supernatant during ahipa starch extraction. However, their amount and nutritional quality may not be high enough to justify the implementation of expensive methods for their recovery as protein concentrates (Dini *et al.*, 2015).

The objectives of this work were to propose a procedure for the recovery of the proteins released during the extraction of *P. ahipa* starch and to develop ahipa and cassava protein-enriched starches, feasible to be used as novel ingredients in the formulation of GF baked goods. These composite ingredients, as well as the GF buns obtained, were characterized from a technological point of view.

Materials and Methods

Chemicals and commercial starch sample

Commercial native cassava starch (CS) Flor de Jardín® was from Jardín América Ltda (Misiones, Argentina). Gallic acid standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent, NaCl and methanol were from Anedra (San Fernando, Argentina). Citric acid was purchased from Biopack (Zárate, Argentina); Na_2HPO_4 and KH_2PO_4 from JT Baker (León, Mexico); KCl from Cicarelli (San Lorenzo, Argentina) and HCl was from Riedel-de Haën (Seelze, Germany).

Plant material

Pachyrhizus ahipa (Wedd.) Parodi plants were cropped at Paraje Esperanza Centro, Iguazú, Misiones, Argentina (25°59′51.29 LS 54°29′52.10 LW). Ahipa roots were washed and sanitized by immersion in NaClO solution (250 ppm, 10 min), then air-dried at room temperature (20°C), peeled, sliced and stored at -20 °C until further processing.

Protein extraction yield

Protein extraction from ahipa roots was assayed in a two-step procedure. In the first step, thawed ahipa root slices were diced and added with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), at 1 L/kg. The mixture was ground with a domestic grinder, kept in agitation for 1 h at room temperature, and filtered through a muslin cloth. The slurry was separated and kept at 4°C. The bagasse was subjected to the second extraction step with PBS (2 L/kg). The mixture was newly processed with a domestic grinder and then subjected to ultrasound (five pulses of 1 min at

600 W) using a Vibra-Cell™ Ultrasonic Liquid Processor (Sonics & Materials, Inc., Newtown, USA) with a CV33 probe. The suspension was agitated for 1 h and filtered. The filtrate (slurry) was added to that of the first extraction step and left 24 h at 4°C for starch sedimentation. The supernatant was separated. The starch cake was dried at 40°C and ground. The remaining bagasse, as well as peeled ahipa root slices, were dried at 105°C and milled. Nitrogen contents of the dried roots, bagasse and starch powders were determined by the Kjeldahl method (AOAC, 1990), which was applied for all nitrogen quantifications in this work. Extraction yield was calculated as follows:

$$Extraction\ yield\ (\%) = \frac{(N_{AR} - N_S - N_B)}{N_{AR}} \times 100$$

Where N_{AR}, N_S and N_B correspond to the nitrogen content (g) of the initial mass of peeled ahipa roots, the starch and the bagasse, respectively.

Determination of protein isoelectric point (pI) and recovery percentage

Peeled ahipa roots (1 kg) were subjected to protein extraction as described above. After starch sedimentation, the supernatant was separated, centrifuged at 3500×g for 5 min (Beckman Coulter Inc. centrifuge, Indianapolis, USA) to remove the remaining starch, and adjusted to pH 3.5 with 0.5M HCl. The acidified mixture was centrifuged at 9000×g for 20 min. A flocculent precipitate was observed. The supernatant was removed by aspiration and protein concentrates from each tube were pooled together and mixed. 100 µL aliquots of this suspension were adjusted to pH values from 3 to 4 (ΔpH=0.1) with 0.5M HCl, brought to 2 mL with deionized water, allowed to rest for 30 min at room temperature and analysed for their Z potential, using a SZ-100 nano particle analyser (Horiba Scientific , Kyoto, Japan). The pI was estimated from the interpolated pH value for Z potential=0.

A second protein extraction was assayed as described above, but the supernatant was acidified to the estimated pI (3.64) with 0.5M HCl, centrifuged and resuspended in distilled water, then lyophilized and analysed for its nitrogen content. Recovery percentage was calculated as:

$$Recovery\ (\%) = \frac{Np}{N_{AR}} \times 100$$

Where N_P and N_{AR} are the nitrogen contents (g) of the freeze-dried powder and the initial mass of peeled ahipa roots, respectively.

To corroborate the efficiency of the isoelectric precipitation, a new protein extraction was performed as described in Section *Protein extraction yield*. Aliquots of the supernatant

before and after pH adjustment to 3.64 and centrifugation were dried and analysed for their nitrogen content.

Effect of pH on protein-starch interaction

Interaction between ahipa starch and proteins at pH values equal to, below, and above the pI of ahipa proteins was evaluated. Starch was extracted using PBS and the starch slurry was divided into four equal parts. Three of them were adjusted to pH 3.06, 3.64 (pI) and 7.00, respectively. For pH adjustment, 1M citric acid was added under agitation. The pH of the fourth part of the slurry (pH=7.4), was not modified, and it was used for obtaining the deproteinized (washed) starch, as control. The respective starch slurries were allowed to sediment (24 h at 4 °C). The control starch cake was rinsed twice with PBS, and twice with distilled water, each washing step followed by centrifugation at 3500×*g* for 5 min, to obtain the washed starch. Supernatants were discarded and the four starch cakes were dried (40°C for 48 h). Samples were ground and analysed for their protein content.

Comparison of water and PBS as protein extraction media

Protein extraction was performed as described in Section *Protein extraction yield* using PBS or distilled water as solvents. Protein extraction yields were calculated as stated in the same section.

Protein enrichment of ahipa and cassava starches

Ahipa starch extraction

The summarized scheme is shown in Figure 1. Peeled and diced ahipa roots were added with water, grinded with a domestic mixer and kept at 4°C for 12 h (Figure 1a, contact step), filtered through a muslin cloth, and the filtrate (starch slurry) was kept for 12 h at 4°C for starch sedimentation. The bagasse retained in the muslin cloth was recovered and added with water (2 L/kg roots). The mixture was grinded and sonicated in 5 pulses of 1 min each, at 600 W (Figure 1a, second step of starch extraction). The mixture was kept at 4°C for 12 h and then filtered. The starch slurry was left to sediment (12 h at 4ºC). Sedimentation supernatants from both steps were combined and added to the bagasse. Another four extractions were performed in the same conditions described for the first step (Figure 1a) but using the combined supernatants instead of water. The final starch slurry was left to sediment to obtain the ahipa starch cake.

Protein enrichment of the starches

The ahipa starch cake was divided into three equal fractions and the supernatant from ahipa starch sedimentation (2 L) was separated into two portions. One portion of the supernatant was added to a fraction of the ahipa starch cake in a ratio of 35 g/L, and the other was added to the same amount of hydrated native cassava starch (CS). Starches were mixed with the supernatant, brought to pH 3.64 with 1M citric acid, agitated for 2 h and sedimented for 72 h at 4°C. The starch cakes were removed and dried (48 h, 40°C) and corresponded to the protein-enriched ahipa and cassava starches (AS+Cit+P and CS+Cit+P, respectively), (Figure 1b).

A second fraction of the ahipa starch, and an equal amount of CS, were each added with 1 L of distilled water, brought to pH 3.64 with citric acid, agitated, sedimented and dried as described above. These samples corresponded to the controls with citric acid (AS+Cit and CS+Cit), (Figure 1b).

The third fraction of ahipa starch was directly dried at 40°C and corresponded to the native ahipa starch (AS).

All the starch samples were ground, sieved through a 35-mesh sieve and stored in sealed containers.

Characterization of protein-enriched starches

Colour measurement

Colour of the starch powders was measured using a CR-400 Konica Minolta colorimeter (Osaka, Japan). CIELAB L*, a* and b* coordinates were registered, and the whiteness index (WI) was calculated as described in Díaz *et al.* (2019):

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

Quantification of proteins and phenolics

Samples protein content was estimated from their total nitrogen using 5.1 as nitrogen-to-protein conversion factor, according to Malgor *et al.* (2019). Results were expressed as percentage (%) on a dry basis.

Since ahipa roots are a source of phenolics including anthocyanins (Díaz *et al.*, 2016), protein enrichment also led to the incorporation of phenolic compounds from the supernatant of ahipa roots starch extraction to the enriched starches. Phenolic compounds

were extracted from the starch samples (2.5 g) by adding 5 mL of methanol:water:HCl solution (80:19:1), then mixed in a vortex for 2 min, and centrifuged at 5580×*g* (10 min at 4°C). Total phenolic content (TPC) was determined by the Folin-Ciocalteu method (Kim *et al.*, 2003) using gallic acid (GA) as standard in the range 5-30 µg. Aliquots of the GA standard and the sample were brought to 2.5 mL with deionized water, added with the Folin-Ciocalteau reagent (50 µL, diluted 1:1 in deionized water) and sodium carbonate (100 µL, 20% w/v in 0.1 M NaOH), and left in the darkness at 20ºC for 90 min. The absorbance was measured at 760 nm and results were expressed as gallic acid equivalents (GAE) per g.

Thermal properties

Thermal properties of starches aqueous suspensions (20% w/w) were determined by differential scanning calorimetry (DSC) according to Dini *et al.* (2013), using a Q100 differential scanning calorimeter (TA Instruments, New Castle, DE, USA). Heating range was 10-120°C at a rate of 10 °C/min. Onset (To, °C) and peak (Tp, °C) temperatures were measured and the enthalpy of the process (ΔH, J/g) was determined.

ATR-FTIR spectroscopy

FTIR spectra of the starch powders were obtained using an ATR diamond accessory. Three scans with 64 sub-scans were performed. Each spectrum was baseline-corrected and scale normalized using the Omnic software (v.9.2). The area of the peak centred at 1633 cm^{-1} was determined in the range 1550-1720 cm^{-1}. Fourier self-deconvolution was performed in the range 2500-3500 cm^{-1} using a bandwidth of 80 and a resolution enhancement factor of 3, with a NB strong apodization function. Peak positions were determined within the deconvolved spectrum and adjusted to the original spectrum using Gaussian line shape with a spectral resolution of 0.964 cm^{-1}. The % area of the band at 2851 cm^{-1} from the deconvolved spectrum was calculated as: $\frac{\text{Peak area}}{\text{Total peaks areas in the range}} \times 100$

Preparation and characterization of gluten-free buns

Buns were formulated using 100 g of the corresponding starch sample, salt (2.5 g), xanthan gum (0.25 g), sunflower oil (15 g) and distilled water (73 mL). Dry components were mixed. The oil was then incorporated, followed by the water. The mixture was hand-kneaded until a homogeneous dough was formed, which was rolled out to 1.5 cm thick sheets and cut into 3 cm diameter discs. Dough discs were placed on a silicone baking mat

and baked (190 °C, 15 min) in a convection electric oven (MC 530, Santini, Rosario, Argentina).

Volume increase

Buns volume (mL) was determined by rapeseed displacement using a graduated cylinder. The volume increase (%) was calculated from the bun volume (V_b) and the initial volume of the raw dough disc (V_0), as: $\frac{V_b - V_0}{V_0} \times 100$

Texture analyses

A TAXT2i texturometer (Stable Micro Systems Ltd., Surrey, UK) with a cell load of 25 kg was used. Baked product texture was studied by a compression assay using the Volodkevich Bite Jaws probe penetrating 8 mm from the samples surface at a speed of 0.5 mm/s, in order to simulate the bite action of the incisive teeth on the samples, (Doporto *et al.*, 2017). Acquired data was analysed with the Texture Expert® software (v.1.2). Maximum force (N) and the area under the curves (N×s) were recorded, which are related to product firmness and the energy required in the process, respectively.

Crust and crumb colour

Colour parameters were determined in the buns inner and outer surface as described in the *Characterization of protein-enriched starches* section. At least three pieces of each treatment were randomly selected and ten measurements were carried out for each piece. Browning index (BI) of the crust was calculated according to Buera *et al.* (1985):

$$x = \frac{a^* + 1.75L^*}{5.645L^* + a^* - 3.012b^*}$$

$$BI = \frac{100(X - 0.31)}{0.172}$$

For the crumb, the parameters hue (H, basic tint) and Chroma (C, saturation) were calculated as:

$$H = arctan\left(\frac{b^*}{a^*}\right)$$

$$C = \sqrt{a^{*2} + b^{*2}}$$

Statistical analysis

Results were analysed 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

Obtaining of protein-enriched starches

In a previous work, PBS proved to be better than water for the extraction of ahipa proteins in a single step, achieving an extraction yield of 69.6% compared to a 62.8% reached with water (Díaz *et al.*, 2016). In the present work, the extraction of proteins using PBS assisted with sonication rendered a considerably higher extraction yield: 91.3±0.5%. Ahipa proteins are particularly rich in aspartic acid (59.1%), which leads to a remarkable low pI for these proteins (between pH 3 and 4) (Malgor *et al.*, 2019). As observed in Figure 2, the proximate pI of ahipa proteins was 3.64, while for most food proteins is in the range 4.5-5.5 (Appelqvist *et al.*, 2007).

Although protein extraction was highly efficient, the recovery by precipitation at the pI was very low (2.3±0.1%). This was corroborated by the similar (P>0.05) protein content of the supernatant before and after pH adjustment and centrifugation: 65±7 and 60±5 mg/mL, respectively. The small size and high polarity of these proteins (Dini *et al.*, 2015) make them unsuitable for precipitation methods usually used for typical storage proteins, and their concentration in the supernatant might not be high enough to justify more expensive recovery processes (i.e. spray drying). Thus, an alternative procedure for the retrieval and revalorization of these proteins was proposed.

In a previous work (Díaz *et al.*, 2016), ahipa starch extracted with water led to starch powders with protein contents of ~0.5%. To analyse the nature of starch-proteins interaction, the protein content of ahipa starch samples obtained at pHs 3.06, 3.64 (pI) or 7, and that of the washed starch was determined (Table 1). The maximum amount of proteins was registered for starches at the pI, suggesting that the interaction might be physical rather than electrostatic. At pH 7, the amount of proteins was about 25% of that obtained at the pI and washing led to a further loss of around 80% of the bound proteins, suggesting a weak interaction between ahipa proteins and starch.

The variations in the protein content of the starches at different pHs was also evidenced in their ATR-FTIR spectra (Figure 3). In a previous work, ahipa proteins showed intense peaks in the zones of 2920 and 2851 cm^{-1} due to the symmetric and asymmetric -CH_2

stretching vibrations from the abundant aspartic acid residues in ahipa proteins (Malgor *et al.*, 2019). Figure 3 shows that the protein content of the starch is reflected in the appearance of a small peak in the zone of 2851 cm^{-1}.

Starch acidification at pH=3.64 allowed to increase its protein content, but the extraction with PBS carries the problem of removing salts from the sedimented starches without losing the weakly bound proteins. The ultrasound assisted extraction was assayed in a new batch of roots using PBS or water, resulting in similar (P>0.05) extraction yields: 88.43±0.24 and 87.46±0.46%, respectively. Therefore, ahipa proteins extracted with water were used to enrich ahipa and cassava starches.

Characterization of the protein-enriched starches

Ahipa and cassava enriched starches exhibited similar protein content (P>0.05) indicating that the protein-starch interaction was independent of the botanical source, and were ~3 and 17 times higher than the native starches, respectively (Table 2). The added proteins significantly reduced (P<0.01) the WI of the starches but all samples remained in values >90, which are considered acceptable.

Total phenolic content (TPC) was similar (P>0.05) for the enriched and native ahipa starches (AS+Cit+P and AS) (Table 2), indicating that the interaction was not influenced by pH or protein content, and that TPC was not directly related to the WI. In the case of CS, since cassava roots are not a source of phenolic compounds, CS has almost no phenolics. When this starch powder is added with the extraction supernatant from ahipa roots, part of the phenolics from this supernatant are incorporated into the cassava starch cake, thus CS+Cit+P has higher phenolic content than CS. The contact of the native cassava starch (CS) with the ahipa supernatant led to a TPC for CS+Cit+P slightly lower than that of AS.

The ATR-FTIR spectra of CS and AS showed a peak that overlaps that of the amide I band of ahipa proteins (1633 cm^{-1}) (Malgor *et al.*, 2019). However, for both AS+Cit+P and CS+Cit+P the increased protein content derived in a raise of this band (Table 3). This increment resulted more pronounced for cassava than for ahipa enriched starches, in agreement with its higher difference in protein content compared to the respective native starch (Table 2). Furthermore, as previously observed for the ahipa starches at different pH values (region zoomed in Figure 3), a peak characteristic of ahipa proteins located at 2851 cm^{-1} was revealed after deconvolution of the bands of AS, AS+Cit+P and CS+Cit+P in the range 2500-3500 cm^{-1}, reflecting their protein content. Accordingly, this peak was absent for CS, CS+Cit and AS+Cit. Starch band located at 2930 cm^{-1} was also shifted towards

lower wavenumbers with increasing protein contents, indicating a contribution of the intense band of ahipa proteins at 2920 cm^{-1} (Tables 2 and 3).

Regarding thermal parameters of the starches (Table 3), acidification reduced the onset temperature (To) for CS. Reduction in To by acid-treatment has been previously reported for sweet potato and corn starches (Wang *et al.*, 2003; Surendra Babu *et al.*, 2016). However, the addition of proteins reversed this effect (Table 3). Similar results were observed by Yang *et al.* (2019) who reported an increase of To in corn starch with the addition of whey protein isolate. Ahipa starches exhibited the same tendency with the addition of citric acid and citric acid+proteins, but the differences resulted not significant ($P>0.05$). Gelatinization peak and onset temperatures were not affected by acid treatment or protein content, but protein enrichment reduced ($P<0.05$) the enthalpy of the process for ahipa starch. This lowering effect was also observed for corn starch added with whey proteins (Yang *et al.*, 2019) and wheat starch with increasing amounts of gluten proteins (Mohamed and Rayas-Duarte, 2003). Despite there are reports about the modification of the thermal properties of different starches by the addition of phenolic compounds (Zhu, 2015), in this case phenolics were not responsible for this change since TPC was similar for AS and AS+Cit+P.

Buns characterization

Acidification of cassava starch (CS+Cit) significantly increased ($P<0.01$) the buns volume (Table 4 and Figure 4). This is in agreement with Blanco *et al.*, (2011), who reported augmented volume of GF breads with the addition of citric acid (0.4-1.2 % on flour basis). However, citric acid added to the ahipa supernatant did not produce this effect in CS+Cit+P, probably related to the reaction of the acid with soluble compounds in the supernatant reducing its effect on the starch granules. Unlike that observed for cassava, acid treatment of AS did not modify the buns volume (Table 4 and Figure 4), as it was previously observed for spontaneously fermented ahipa and cassava starches, where the naturally released lactic acid resulted in volume-increased baked products for cassava, but not for ahipa (Díaz *et al.*, 2019).

Proteins and phenolics did not affect the baking expansion properties of the starch buns (Table 5) but rendered smaller and more homogeneously distributed alveoli in the crumb (Figure 4), evidencing a potential cross-linking effect. This was also reflected in the texture of the buns. Acidification of AS (AS+Cit) produced curves with higher slope and height than AS (Supplementary Figure 2). Likewise, maximum force and the area under the curve

increased significantly (Table 4), indicating that acidification led to a firmer structure. In the case of cassava, the control with citric acid (CS+Cit) resulted slightly higher but not significantly different in firmness and area compared to the native starch. This is probably related to a counterpoised effect between the increased rigidity of the structure and the reduced crumb density due to volume increase.

Buns from AS+Cit+P and CS+Cit+P starches exhibited the lowest maximum force and area, related to less rigid structures compared to the native and citric acid-treated starch buns. Enriched starches led to products with texture closer to that of wheat bread, with a soft crumb, while native and citric acid-treated starch buns, especially those from cassava, resulted in textures more related to extruded products such as cheese puffs. A similar effect was observed by Laswai *et al.* (2017) for pancakes made of blends of cassava starch with soy flour, where increased protein contents rendered softer sensory-evaluated textures.

Regarding buns crust colour, the addition of citric acid (AS+Cit and CS+Cit) lowered the browning index (BI) and increased the lightness (L*), while starches enrichment (AS+Cit+P and CS+Cit+P) reversed this effect for both parameters (Table 5). The BI of the AS buns was not significantly different (P>0.05) from that of the AS+Cit+P buns, despite the latter doubles the protein content of the former, indicating that phenolics and citric acid treatment are also implicated in crust colour development. According to Ou *et al.* (2019), the conversion of anthocyanins into quinones can lead to the formation of condensation products with proteins and other polyphenols that can contribute to food browning.

Furthermore, the colour of the crumb was considerably modified in the samples bearing ahipa phenolics and proteins and treated with citric acid (AS+Cit, AS+Cit+P and CS+Cit+P), resulting in lower Hue values (Table 5). This indicates a higher red component, which might be related to the colour input of ahipa anthocyanins in acidic medium (Sui *et al.*, 2015). AS, CS and CS+Cit exhibited higher Hue values associated to more yellowish tones (Table 5). The colour saturation (Chroma) of the crumb was significantly increased for the buns from the enriched starches compared to the native starch buns, which exhibited duller colours (Table 5 and Figure 4). This combined with L* values lower than the crust, provided the unusual feature of bakery products with clearer crusts than crumbs (Supplementary Figure 1), indicating that colour is not only due to Maillard reactions.

Conclusions

Ultrasound-assisted aqueous extraction of *P. ahipa* proteins led to a good extraction yield (88.43%), and the acidification of ahipa and cassava starches at the pI of these proteins

(pH=3.64) allowed to obtain protein-enriched starches at relatively low cost. Enriched starches achieved protein contents rounding 2% and TPC in the range 18-20 μg GAE/g, with acceptable WI (>90). Thermal analyses were not significantly affected by the enrichment process. During baking, these novel ingredients resulted in less rigid structures than the native ones. Enriched-starch buns crumbs showed smaller, more regular alveoli and exhibited more reddish and vivid tones. Crumb resulted darker than the crust, providing a colour distribution contrary to that of most bakery products. Results demonstrate that these protein enriched starches are interesting novel ingredients for the formulation of premixes intended for gluten-free baked products.

Acknowledgements

This work was supported by the projects PICT 2015-0921, PICT 2015-3249 (ANPCyT), and PIP 0555 (2013-2015, prorogued). Conflict of interest: none.

Data Availability Statement

No data is available

Ethical Guidelines

Ethics approval was not required for this research.

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Legends to Figures

Figure 1. a) Flow-chart for the extraction of *P. ahipa* starch; b) Obtaining of citric acid-treated and protein-enriched ahipa and cassava starches.

Figure 2. Z potential of ahipa proteins in the pH range 3-4.

Figure 3. ATR-FTIR spectra of *P. ahipa* protein-enriched starches at different pH values.

Figure 4. Cross section of buns formulated with: a) native ahipa starch (AS); b) ahipa starch treated with citric acid (AS+Cit); c) protein-enriched ahipa starch (AS+Cit+P); d) native cassava starch (CS); e) cassava starch treated with citric acid (CS+Cit); f) protein-enriched cassava starch (CS+Cit+P).

Supplementary Figure 1. Crust and crumb colour of buns formulated with: a) native ahipa starch (AS); b) ahipa starch treated with citric acid (AS+Cit); c) protein-enriched ahipa starch (AS+Cit+P); d) native cassava starch (CS); e) cassava starch treated with citric acid (CS+Cit); f) protein-enriched cassava starch (CS+Cit+P).

Supplementary Figure 2. Maximum Force (N) versus time (s) of buns formulated with native, citric acid-treated or protein-enriched ahipa and cassava starches.

Table 1. Protein content of ahipa starches obtained at different pH values

Sample	Protein (% w/w)
Washed starch	0.12±0.04[a]
Starch pH 3.06	1.96±0.04[c]
Starch pH 3.64	2.37±0.08[d]
Starch pH 7	0.65±0.01[b]

Results are expressed as the mean±SD, n=2. Different letters indicate significant differences (P<0.01)

Table 2. Protein and total phenolics contents, and whiteness index of starch samples

Sample	Protein (% w/w)	TPC (μg GAE/g)	WI
AS	0.63±0.00[b]	19.2±0.6[b]	95.7±0.2[b]
AS+Cit	0.24±0.04[a]	11.4±1.1[a]	96.0±0.1[b]
AS+Cit+P	1.91±0.06[c]	20.3±0.9[b]	91.2±0.3[a]
CS	0.07±0.05[a]	3.2±0.3[a]	95.5±0.1[b]
CS+Cit	0.06±0.03[a]	2.2±0.7[a]	97.02±0.0[c]
CS+Cit+P	1.92±0.01[b]	17.9±1.0[b]	90.9±0.2[a]

Results are expressed as the mean±SD; protein (n=2), TPC: Total phenolics content (n=3). GAE: Gallic acid equivalents. WI: whiteness index (n=5). Different letters indicate significant differences (P<0.01) for starches from the same botanical source. AS and CS: ahipa and cassava native starches. AS+Cit and CS+Cit: citric acid treated ahipa and cassava starches. AS+Cit+P and CS+Cit+P: ahipa and cassava starches enriched with ahipa proteins.

Table 3. ATR-FTIR spectral and thermal properties of starch samples.

Sample	ATR-FTIR		DSC		
	Peak area 1633 cm^{-1}	Peak position (2920-2930 cm^{-1})	T_{onset} (°C)	T_{peak} (°C)	Enthalpy (J/g)
AS	4.3±0.3^{a,b}	2927.3±0.8^{b}	63.3±0.6^{a}	67.8±0.3^{a}	14.1±0.6^{b}
AS+Cit	4.0±0.1^{a}	2929.7±0.2^{c}	62.0±0.5^{a}	66.8±0.7^{a}	13.5±1.2^{b}
AS+Cit+P	4.7±0.3^{b}	2926.6±0.8^{a}	63.4±0.4^{a}	68.2±0.3^{a}	10.7±0.5^{a}
CS	4.3±0.2^{b}	2930.1±0.7^{b}	61.9±0.4^{b}	67.0±0.5^{a}	12.9±0.9^{a}
CS+Cit	3.7±0.3^{a}	2929.0±1.1^{b}	57.2±0.9^{a}	67.0±0.3^{a}	13.2±1.0^{a}
CS+Cit+P	6.1±0.4^{c}	2926.1±1.0^{a}	60.2±0.8^{b}	68.7±0.9^{a}	10.8±0.8^{a}

Results are expressed as the mean±SD; ATR-FTIR n=3, DSC n=2. Different letters indicate significant differences among samples from the same plant species (P<0.05). AS and CS: ahipa and cassava native starches; AS+Cit and CS+Cit: citric acid treated ahipa and cassava starches. AS+Cit+P and CS+Cit+P: ahipa and cassava starches enriched with ahipa proteins.

Table 4. Volume increase and textural parameters of gluten-free buns

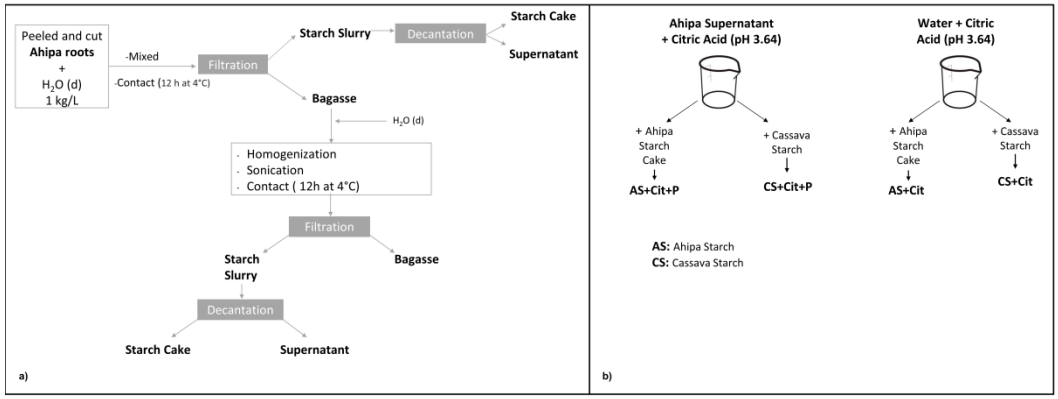
Sample	Volume increase (%)	Area under the curve (N×s)	Maximum Force (N)
AS	29 ± 10 [a]	103±29[a]	9±4[a]
AS+Cit	31 ± 13 [a]	201±72[b]	17±7[b]
AS+Cit+P	43 ± 9 [a]	80±16[a]	8±2[a]
CS	33 ± 13 [a]	244±51[b]	22±5[b]
CS+Cit	74 ± 15 [b]	287±87[b]	26±8[b]
CS+Cit+P	42 ± 10 [a]	122±67[a]	10±6[a]

Results are expressed as the mean±SD, n=3. Different letters indicate significant differences (P<0.05) for starches from the same botanical source. AS and CS: ahipa and cassava native starches; AS+Cit and CS+Cit: citric acid treated ahipa and cassava starches. AS+Cit+P and CS+Cit+P: ahipa and cassava starches enriched with ahipa proteins.

Table 5. Colour parameters of buns crust: Browning Index (BI) and lightness (L*) and crumb: lightness (L*), Hue and Chroma.

Sample	Crust		Crumb		
	BI	L*	L*	Hue (º)	Chroma
AS	31.14±1.74^{b}	71.94±0.88^{a,b}	45.58±2.31^{a}	71.22±1.28^{c}	6.81±0.79^{a}
AS+Cit	11.42±0.34^{a}	75.65±0.41^{c}	47.44±2.50^{a}	68.38±0.81^{a}	6.89±0.81^{a}
AS+Cit+P	34.55±3.27^{b}	70.94±1.03^{a}	49.53±1.76^{b}	69.72±1.68^{b}	12.57±0.44^{b}
CS	17.38±0.17^{b}	72.83±0.65^{a}	57.71±1.55^{b}	77.46±0.93^{b}	9.42±0.55^{b}
CS+Cit	8.08±0.58^{a}	79.95±2.17^{b}	55.37±3.11^{a,b}	83.73±1.04^{c}	5.83±1.21^{a}
CS+Cit+P	34.41±8.30^{c}	75.04±1.55^{a}	52.62±3.49 ^{a}	69.32±1.40^{a}	10.98±1.14^{c}

Results are expressed as the mean±SD, n=3. Different letters indicate significant differences (P<0.05) from the same botanical source. AS and CS: ahipa and cassava native starches; AS+Cit and CS+Cit: citric acid treated ahipa and cassava starches. AS+Cit+P and CS+Cit+P: ahipa and cassava starches enriched with ahipa proteins.



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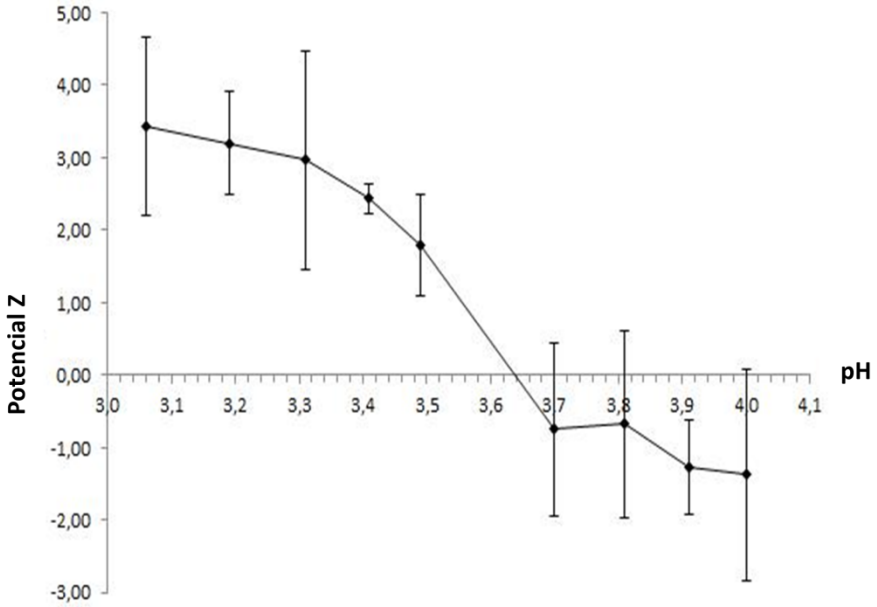

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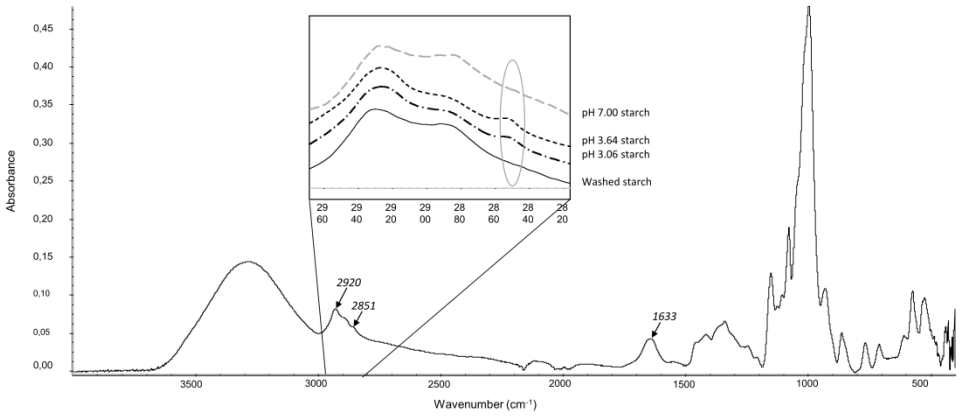

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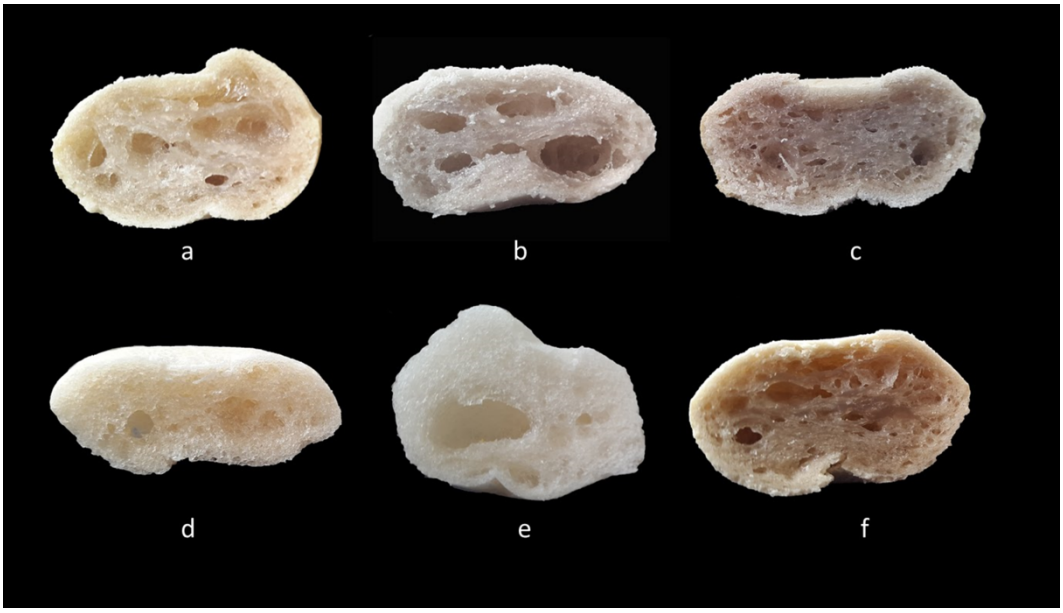

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