Research article

Lentil protein: impact of different extraction methods on structural and functional properties

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HIGHLIGHTS

- Proteins from lentil were efficiently recovered by alkaline extraction.
- Lentil protein solubility was improved by ultrasound and enzyme extraction.
- Lentil protein surface activity was dependent on the extraction process.
- Foaming properties were improved by ultrasound extraction.
- Emulsifying properties were not influenced by the extraction process.

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ABSTRACT

Plant proteins with improved solubility, foaming, and emulsifying properties are required to meet the demand for plant-based foods. This study evaluated the influence of alkaline extraction combined with enzyme- and ultrasound-assisted extraction on lentil protein structure and functionality. Enzyme- and ultrasound-assisted extractions were not capable of increasing the protein yield compared to alkaline extraction alone. However, the purity of isolated protein was dependent on the extraction process, ranging from 82.7% to 90%. Although the molecular mass, zeta potential profiles, and denaturation temperature were not dependent on the extraction method, the enthalpy of denaturation for protein obtained solely by alkaline extraction was significantly lower than that for assisted processes, indicating that protein denaturation is caused by an alkaline process. Changes in protein structure were also suggested by solubility analyses that showed lentil proteins obtained by enzyme-assisted and ultrasound-assisted extraction have better solubility at pH 7 when compared to alkaline extraction alone. The surface activity of lentil protein was evidenced by interfacial and surface analysis, and it was influenced by the extraction process applied. We demonstrated that combining alkaline extraction with assisted processes, especially ultrasound technology, results in concentrates/isolates with higher solubility as compared to ones obtained solely by the traditional alkaline method, even though the choice of extraction method should depend on the desired functionality.

1. Introduction

Nowadays, there is a rising interest in studies on plant proteins for their subsequent use as food ingredients, because of the shift of consumer behavior towards more plant-based foods due to health concerns and ethical and/or religious preferences, besides the environmentally sustainable aspects such as producing lower greenhouse gas emissions and being less resource-intensive and less environmentally destructive as compared to animal husbandry (Fasolin et al., 2019; Lam et al., 2018). Pulse proteins are plant proteins derived from grains of the family Leguminosae that include lentils, peas, chickpeas, and beans (Shevkani et al., 2019). Lentils (Lens culinaris) have a protein content varying from 22% to 31%, of which 80% are characterized as storage proteins (Khazaei et al., 2019). The majority of the storage protein fraction in lentils is composed of globulins which are soluble in saline solution, followed by albumins, soluble in water. It may also contain glutelin and prolamins in lower proportions (Boye et al., 2010a,b).

In recent years, new protein sources have been required to replace animal protein in food formulations, aiming to meet the rising demand for plant-based products (Lam et al., 2018). The main features required in

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protein for food applications are good emulsifying and foaming properties, and solubility. Proteins can act as a surfactant in foams and emulsions due to their surface activity that allows them to be quickly adsorbed on the liquid surface, thereby reducing the surface and interfacial tension of the solution (Xiao et al., 2020). However, proteins’ surface activity depends on some intrinsic properties such as flexibility, molecular size, and charge that can be changed by the extraction process (Mitropoulos et al., 2014). Solvent exposure, heating, dehydration, homogenization, drying, and other treatments can change the protein structure and, consequently, modify the protein functionality (Zayas, 1997).

Alkaline extraction followed by isoelectric precipitation is the most common method used to obtain plant proteins. The traditional alkaline extraction of proteins is associated with simplicity, rapidity, and low cost. However, to improve yield, harsh conditions applied in this process can cause undesirable changes in the structure of the recovered protein. Denaturation, racemization, and the formation of dehydro and cross-linked amino acids are related to alkaline extraction at high pH, temperature, and exposure time, resulting in poor solubility, lower nutritional quality, and changes in functional properties such as solubility, foaming, and emulsifying properties (Sari et al., 2015; Perović et al., 2020; Wang et al., 2020a,b). Thus, combining new technologies with alkaline extraction can modulate changes in the structure of proteins that could reduce the disadvantages associated with traditional extraction and improve the functional properties of the protein obtained.

New strategies for the extraction of plant protein have been used in recent years to improve both yield and functional properties. Among the emerging techniques, the use of ultrasound- and enzyme-assisted extraction stands out. Ultrasound is a mechanical sound wave above 20 kHz able to generate bubble cavitation. This effect is based on the quick formation and collapse of vapor bubbles that create water jets, favoring the rupture of the plant cell wall and releasing the compounds embedded within the matrix (Picó, 2013; Kadam et al., 2015). Another strategy is the use of enzymes such as carbohydrases that can disrupt the cell wall by hydrolyzing the carbohydrates (cellulose, hemicellulose, and pectin) present in the plant matrix, facilitating the release of compounds bound to these macronutrients (Hnadadjev et al., 2017).

Studies with protein from beans have indicated changes in the secondary structure for protein isolates obtained by ultrasound-assisted alkaline extraction compared to those obtained by traditional alkaline extraction (Byanju et al., 2020). These results are supported by the theory that sonication can change the molecular configuration of the protein by breaking hydrogen bonds, as well as hydrophobic interactions present in the protein secondary structure (Byanju et al., 2020). In addition, studies with soy protein obtained by enzyme-assisted processes have indicated an increase in solubility compared to that of isolate obtained by alkaline extraction, which may be related to greater flexibility of the protein structure in the combined process (Perović et al., 2020).

Thus, this study evaluated enzyme- and ultrasound-assisted processes combined with mild alkaline extraction of protein from lentils, aiming to overcome the drawbacks associated with extreme pH values and high temperatures. Isolated proteins were characterized in terms of chemical composition, surface charge, molecular weight, denaturation temperature, solubility, interfacial and surface tension, and emulsifying and foaming properties.

2. Material and methods

2.1. Material

Green lentils were purchased in the local market of the city of Campinas, São Paulo, Brazil. The grains were ground using a blender, with the lentil mass (50 g) and milling time (2 min) standardized for all processes to avoid the influence of particle size on the extraction procedure. The size distribution of lentil flour was bimodal between 10 and 1000 μm with a volume mean diameter (D [4,3]) of 356.85 μm and span of 1.97. The multi-enzymatic complex Viscozyme® L (100 fungal β-glucanase (FBG) units/100 g), containing arabanase, cellulase, β-glucanase, hemicellulase, and xylanase, was kindly provided by Novozymes (Denmark). Standard protein markers for SDS-PAGE (molecular weight of 6–180 kDa) were purchased from Bio-Rad (USA). Linseed oil (Vital Atman, Brazil) was used in surface tension analysis. All other materials and chemicals were of analytical grade.

2.2. Proximate composition analysis

Proximate composition analysis was performed on the lentils according to AOAC Official Methods 925.10 (moisture), 923.03 (ash), and 920.87 (crude protein by the Kjeldahl method using %N × 6.25). Lipid content was determined by the methodology of Bligh and Dyer (1959). Carbohydrate content was determined by the method of Dubois et al. (1956), and the fiber content was determined on the basis of percent differential from 100%.

2.3. Protein extraction

2.3.1. Alkaline extraction

The influence of pH on the alkaline extraction was tested at two levels (pH 8.0 and 9.0). Lentil flour was mixed with deionized water at a ratio of 1:10 (w/w). The pH value was adjusted to 8.0 or 9.0 with 1 M NaOH solution, and the mixture was stirred at 25 °C and 600 rpm for 120 min by magnetic stirring. The insoluble solids were removed by centrifugation at 10,000 × g for 20 min at 4 °C, using an Allegra 25R centrifuge (Beckman Coulter, USA). The supernatant was used for protein determination by micro-Kjeldahl methodology.

2.3.2. Ultrasound-assisted alkaline extraction

The effect of sonication power (150, 300, and 450 W) on the extraction of protein from lentils was evaluated. Lentil flour (1: 10 w/w) was dispersed in an alkaline solution (pH 9.0, conditions defined in the results obtained in Section 2.3.1) for a final volume of 200 mL. The dispersion was treated using an ultrasonic cell crusher (750 W Eco-Sonics, Ultronique, Brazil) with a 13 mm flat-tip probe at 20 kHz at different levels of power output for 10 min (determined by preliminary tests), prior to the alkaline extraction (Section 2.3.1) (Figure 1). The increase of temperature was controlled by an ice bath.

2.3.3. Enzyme-assisted alkaline extraction

The influence of enzyme concentration on the extraction of protein from lentils was tested using the multi-enzyme complex Viscozyme® L. Lentil flour was dispersed in deionized water at a ratio of 1:10 (w/w). Then, the pH value was adjusted to 6.0 with 1 M NaOH solution and the temperature was adjusted to 50 °C, the optimal enzyme condition as indicated by the supplier. Enzyme concentrations of 1%, 2%, and 4% (g enzyme/100 g lentil flour, wet basis) were tested. The enzymatic reaction occurred for 120 min in a jacketed beaker with constant agitation. Since the enzyme complex was used as an assisted process, the following steps were the same for alkaline extractions (Section 2.3.1) (Figure 1).

2.3.4. Protein precipitation

The supernatants obtained by alkaline, ultrasound-, and enzyme-assisted extraction were acidified to a pH value of 4.2 by adding 1 M HCl to precipitate the solubilized protein. Precipitates were separated by centrifugation at 10,000 × g for 20 min. The recovered protein was resuspended in ultra-pure water and neutralized to pH 7 by adding 1 M NaOH. The protein suspension was freeze-dried (LS3000, Terroni, Brazil) and stored at −18 °C until further analysis.

2.4. Extraction yield

The protein extraction yield for alkaline and ultrasound-assisted extraction was determined by Eq. (1), while for the enzymatic
extraction, the amount of enzyme added was deducted from the quantified amount determined by Kjeldahl (Eq. (2)).

$$\% \text{ protein yield} = \frac{M_{\text{supernatant protein}}}{M_{\text{lentil flour protein}}} \times 100 \quad (1)$$

$$\% \text{ protein yield} = \frac{M_{\text{supernatant protein}} - M_{\text{enzyme}}}{M_{\text{lentil flour protein}}} \times 100 \quad (2)$$

2.5. Analysis of protein isolate composition

The lentil protein isolate and lentil flour were analyzed to determine their proximate composition using official methods as described in Section 2.2.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic profile of the lentil proteins was determined according to the methodology of Laemmli (1970), using 12% (m/v) acrylamide separating gel and 5% (m/v) acrylamide stacking gel, both containing 0.1% (m/v) SDS. Samples (2 mg protein/mL) were diluted in a 50 mM Tris-HCl buffer at pH 6.8 (0.1% (m/v) bromophenol blue, 2% (m/v) SDS, 10% (v/v) glycerol). A reducing buffer containing 5% (v/v) β-mercaptopethanol (β-ME) was also used. Analysis was carried out at a constant voltage (110 V) using a Mini Cell Protean unit (Bio-Rad Laboratories, Hercules, CA, USA). Gels were stained using Coomassie Brilliant Blue and destained with a mixture of acetic acid, ethanol (99.5% purity), and deionized water in the proportion of 5/10/85 (v/v/v), respectively.

2.7. Zeta potential measurements

Samples extracted under different conditions were diluted in Milli-Q water (Direct-Q3, Millipore, USA) to a concentration of 0.01% (w/w) and placed in the measuring chamber of a microelectrophoresis system (Zetasizer Nano-ZS, Malvern Instruments Ltd, UK) equipped with an MPT-2 Autotitrator (Malvern Instruments, Herrenberg, Germany). Zeta potential was determined as a function of pH, between 3 and 9. The Smoluchowski model was used to convert the electrophoretic mobility measurement into zeta potential values. The samples were measured in triplicate at 25 °C.

2.8. Differential scanning calorimetry (DSC)

The thermal transition of protein isolates from lentils was examined by DSC (2920 Modulated, TA Instruments). For each protein, approximately 6 mg of the sample was weighed into an aluminum pan. The pans were hermetically sealed and heated from 20 to 130 °C at a rate of 10 °C/min. A sealed empty pan was used as reference.

2.9. Protein solubility

Protein solutions were prepared by dispersing 0.25 g of the sample in 25 mL of 0.1 M NaCl and had their pH adjusted to 10.0, 7.0, 5.0, or 3.0 with either 0.1 M NaOH or 0.1 M HCl, followed by stirring at 250 rpm for 1 h at 25 °C. Solutions were centrifuged at 10,000 x g for 20 min at 4 °C. The protein content of the supernatant was determined using the method of Bradford (1976). The protein solubility (%) was calculated by dividing the masses of protein in the supernatant and in the sample. Measurements were performed in duplicate, and average values are reported (Morr et al., 1985).

2.10. Interfacial tension

Evaluation of the interfacial properties between the protein solution (0.1% w/w) and sunflower oil was carried out using the pendant drop method using a Tracker-S tensiometer (Teclis, France), in which the oil was injected with a syringe in the aqueous phase. The results were compared to the interfacial tension between Milli-Q™ water and linseed oil (without protein). Measurements were carried out at 25 °C.
2.11. Surface tension

The adsorption behavior of lentil protein solution (1% w/w) was evaluated by measurements of surface tension at the air/water interface with time, using the pendant drop method with a Tracker-S tensiometer (Teclis, France). Measurements were carried out at 25 °C for 1 h.

2.12. Emulsifying properties

The emulsifying activity index (EAI) and emulsion stability index (ESI) were evaluated according to Pearce and Kinsella (1978) with modifications in total emulsion volume in order to reduce the required amount of lentil protein isolate. Briefly, 5 g of protein solution (0.25% w/w) and 5 g of linseed oil were homogenized at 8000 rpm for 5 min. Then, 50 μL of the emulsion was immediately taken from the bottom of the mixture and transferred to a tube with 7.5 mL of SDS solution (0.1% w/v). The absorbance at 500 nm for the diluted emulsion was recorded to estimate EAI and the absorbance after 10 min was recorded to estimate the ESI. The EAI and ESI were calculated according to Eqs. (3) and (4).

\[
EAI = \frac{2 \cdot 2.303 \cdot A_{10} \cdot N}{c \cdot \phi \cdot 10000}
\]  
\[
ESI = \frac{A_{10 \min}}{A_0} \times 100
\]

2.13. Foaming properties

The foaming properties were estimated using the method described by Miller and Groninger (1976) with modifications. Protein solutions (15 mL; 1% w/w, pH 7.0) were homogenized at 8000 rpm for 5 min. Then, the contents were immediately poured into a 50 mL measuring cylinder. The foaming capability (FC) and foaming stability (FS) were calculated using Eqs. (5) and (6).

\[
FC (\%) = \frac{\text{Foam volume}}{15} \times 100
\]

\[
FS (\%) = \frac{\text{Foam volume after 30 or 60 minutes}}{\text{Foam initial volume}} \times 100
\]

2.14. Statistical analysis

All the extraction procedures were performed in triplicate, and the analyses of functional properties were performed in duplicate. SISVAR software, a computer statistical analysis system (Ferreira, 2011), was used for analysis of variance (ANOVA). The significant differences (p ≤ 0.05) between different samples were determined by the Tukey and t-test procedures.

3. Results and discussion

3.1. Proximate composition

The dry matter proximate composition of lentil flour indicates that protein and fiber were the two most important fractions at 28.1% and 48.3%, respectively. Protein content was similar to that reported by Aryee and Boye (2017) and for other pulses, such as peas, beans, and chickpeas (Boye et al., 2010a,b). The content of lipids (2.9%), carbohydrates (18.1%), and ash (1.75%) of lentils was also determined, and the values found are within the range found in the literature for different lentil varieties (Wang and Daun, 2006).

3.2. Alkaline extraction

Alkaline extraction has been used as an effective feasible procedure for solubilizing protein from vegetable sources (Shen et al., 2008). The increase in protein solubility may be related to changes in the surface charge of the protein induced by alkaline conditions. These changes can be due to dissociation of hydrogen from carboxyl and sulfate groups (Shen et al., 2008) or neutralization of side amine groups of basic amino acids such as lysine and arginine (Gao et al., 2020). In this way, the total negative charge of the peptide chain is increased, and consequently the solubility is improved. Besides that, a high hydroxyl ion content can cause swelling of the cell wall and disruption of the intermolecular bonds of the network composed of protein and polysaccharides, resulting in more protein being dissolved in the solution (Bai et al., 2020). On the other hand, extremely alkaline conditions can lead to undesirable effects such as racemization of amino acids, reduced protein digestibility, damage to some amino acids, and high salt formation (Sari et al., 2013). Hence, in order to avoid such disadvantages, moderate pH conditions (8 and 9) were selected to study the influence of pH on protein yield. These pH values have been used to obtain proteins from other plant sources, such as soybean (Sari et al., 2013) and pea (Lam et al., 2016).

The influence of pH 8.0 and 9.0 on protein solubilization (Table 1) was not significantly different (p < 0.05). Other studies indicated that alkaline conditions of pH 9.0 are enough to reach effective extraction of protein from lentils (Joshi et al., 2011; Jarpa-Parra et al., 2014) and other pulses (Boye et al., 2010a,b; Feyzi et al., 2018; Gao et al., 2020). Therefore, the results presented in our work show that lentil proteins, as well as those of other pulses, are highly extractable at moderate alkaline conditions by an increase in protein solubility or by effective loosening of the cell wall. Considering that pH 9 is the most used in the solubilization of pulse proteins (Boye et al., 2010a,b; Joshi et al., 2011; Jarpa-Parra et al., 2016), and it has resulted in high extractability, this pH value was selected for the assisted extraction processes (assisted by ultrasound and assisted by enzyme).

3.3. Ultrasound-assisted alkaline extraction

Ultrasound has been applied as an auxiliary process to alkaline extraction, aiming to improve the removal of protein from vegetable cells through its disruption by the cavitation effect (Görgüç et al., 2019). The cell wall structure is a complex matrix composed of several polysaccharides, proteins, and lipids (Chateigner-Boutin et al., 2018). Thus, the higher mass transfer and improvement of cell wall disruption promoted by the cavitation effect can help to extract proteins that are not solubilized solely by chemical processes (Tiwari, 2015). Three sonication powers were evaluated to study the influence of ultrasound on the yield of protein from lentil flour, and the results are shown in Table 2. No significant differences (p > 0.05) were observed among ultrasound-assisted processes nor between sonicated samples and the control (alkaline extraction, pH 9), even though previous studies using ultrasound-assisted extraction to obtain protein from other pulses showed an increase in protein yield (Lafarga et al., 2018). Despite several benefits associated with the cavitation effect such as higher mass transfer and cell wall degradation, disadvantages for proteins have been reported due to this effect. After the collapse of vapor bubbles, the extreme conditions (local temperature and pressure) associated with water jets give rise to homolytic water molecule cleavage, generating high-energy intermediates such as hydroxyl and hydrogen free radicals (Barteri et al., 2004). These high-energy intermediates are oxidative species that can extensively degrade protein, nucleic acids, and polysaccharides (Barteri et al., 2010).

| pH Value | Protein Yield (%) |
|----------|-------------------|
| 8.0      | 57.7 ± 6.7*       |
| 9.0      | 61.7 ± 3.6*       |

* All the data are expressed as means ± SD of three replicates. Means with the different superscript letters within the same column for each parameter are significantly different (p < 0.05) by test-t.
et al., 2004), decreasing the amount of solubilized protein in the extraction process assisted by ultrasound, as observed in this present study and in other studies with vegetable sources (Tu et al., 2015; Zhang et al., 2019). Indeed, ultrasound power may lead to protein degradation due to a strong shock wave promoting protein denaturation by shear stress (Zhang et al., 2019). Another possibility for the reduction of protein solubilization after sample sonication may be related to the lipid and carbohydrate content of the cell wall, which may have resulted in an increase in the viscosity of the extraction solution, reducing the amount of protein recovered (Byanju et al., 2020). Finally, we must also consider that, even using an ice bath during the process, ultrasound promotes a temperature increase which can denature proteins, contributing to a reduction in the amount of protein extracted (Santos et al., 2009; Edlin et al., 2018).

3.4. Enzyme-assisted alkaline extraction

Enzymes have been used for the extraction of protein from vegetable sources due to the increase in protein yield associated with hydrolysis of the cellular wall (Rosenthal et al., 2001). Several enzymes (carbohydrases and proteases) and complexes, such as Viscozyme L, Cellulast, hemicellulase, Alcalase, Papain, α-amylase, amyloglucosidase, Flavourzyme, and Energex have been applied to extract protein from vegetable sources (Nadar et al., 2018) and they have shown promising results (Jung et al., 2006; Puri et al., 2012; Liu et al., 2016). In the present study, Viscozyme L was selected because it is a multi-enzyme complex containing arabinase, which is responsible for degrading arabinose, the main polysaccharide present in the lentil cell wall (Brummer et al., 2015). The influence of enzyme concentration on the yield of protein from lentils is presented in Table 3. Three enzyme concentrations above 1% were evaluated, based on studies with other vegetable sources such as rapeseed cake (Niu et al., 2012) and rice bran (Hammoungiai et al., 2002), as there is still limited information about the enzymatic activity on the cell wall from legumes.

The results presented in Table 3 show that an enzyme concentration above 1% (w/w) was able to reach a similar yield of protein from alkaline extraction, but it was not able to extract more proteins as compared to the control process. The enzymatic hydrolysis was monitored by the content of soluble solids (‘Brix) (data not shown), and the results showed that even for an enzyme concentration of 4%, no increase in the content of soluble solids was observed during 120 min of hydrolysis. Even though the enzyme is highly specific to the lentil cell wall, the results show that cell wall degradation was limited and did not alter the dissolution of the compounds present in the cell matrix. Indeed, these results indicate that most of the proteins present in the lentil matrix were efficiently solubilized in alkaline conditions. Thus, the use of a multi-enzyme complex of carbohydrases, regardless of concentration, was not able to solubilize more proteins present in the plant matrix.

3.5. Proximate composition of lentil flour and protein isolates

The proximate composition of lentil protein isolate (LPI) is shown in Table 4. Evaluating the amount of solubilized protein (Sections 3.2 to 3.4), no significant differences (p > 0.05) were observed among the evaluated techniques. Although no differences were observed in the extraction yield of the proteins from lentil, the overall composition of the isolates varied (Table 4). After the protein precipitation step, LPI-AE (protein isolate obtained by alkaline extraction) and LPI-EAAE (protein isolate obtained by enzyme-assisted alkaline extraction) presented a higher protein content than LPI-UAS (protein isolate obtained by ultrasound-assisted alkaline extraction). Considering that LPI-EAAE and LPI-AE presented a similar protein content, it is possible to infer that the Viscozyme L was not efficient in favoring protein release. It has been previously demonstrated that the primary cell wall is hydrolyzed by cellulases while the secondary cell wall is digested by pectinase (Kasai et al., 2003, 2004). The results from the present study indicate that neither arabinase, cellulose, nor the other enzymes present in the enzymatic complex Viscozyme are capable of disintegrating the lentil cell wall in an efficient manner to facilitate protein release.

The LPI-EAAE sample had the lowest carbohydrate and higher ash values. Even though not influencing the protein yield, the lowest carbohydrate content may indicate that the enzymes may have released the carbohydrate bound to protein. It must be considered that, for the enzymatic process, the solution had its pH adjusted with HCl before addition of the enzymes. For the alkaline solubilization step, the solution pH was later adjusted to 9. Such acid and alkali addition resulted in the production of salts, that may have influenced the higher ash content of this sample. Thus, the data present in Table 4 show the composition on an ash-free basis.

It is possible to infer that the ultrasound-assisted process led to solubilization of other compounds, such as carbohydrates and lipids, when compared to the other extraction processes, resulting in decreased purity of the protein isolate LPI-UAS. These results could be attributed to the following hypotheses: 1) homolysis of lignin–carbohydrate bonds releasing lignin (Subhedar and Gogate, 2014); 2) an increase of the net hydrophobic character of the extraction medium, because of the cavitation effect, enabling the extraction of polar compounds (Vilkhu et al., 2008); and 3) enhancement of mass transfer due to particle size.
reduction, increasing the contact surface area between the liquid and solid phases (Subhedar and Gogate, 2014). This effect was also observed by Byanju et al. (2020), evaluating the extraction of protein from kidney beans and chickpeas. The authors reported that sonication led to the extraction of other compounds, such as oils and sugars, lowering the protein content of the isolate when compared with alkaline extraction.

### 3.6. SDS-PAGE

Figure 2 shows the reducing and non-reducing SDS-PAGE for the lentil protein obtained by the three extraction processes evaluated in the present study (original images of SDS-page gel is presented in the supplementary material, Figure S1). No differences in SDS-PAGE profiles were observed for the different extraction processes, indicating that neither the ultrasound nor the enzymes used in the assisted processes led to changes in the protein profile found in the LPIs. Under non-reducing conditions (Columns A, B, and C), it is possible to observe bands at 64 kDa and within 15–19 kDa. The bands around 15 kDa can be related to gamma-vicilin or polypeptides resulting from the cleavage of storage proteins (Ghumman et al., 2016). The legumin protein (11S) is a hexamer with a high molecular mass of about 380 kDa. The six pairs of hexamer peptides are connected by non-covalent interactions, each of these of approximately 60 kDa (Jarpa-Parra et al., 2015). These pairs are probably responsible for the bands around 64 kDa under non-reducing conditions (Columns A, B, and C). Under reducing conditions (Columns AR, BR, and CR), a partial disappearance of the band corresponding to 64 kDa and the appearance of bands close to 49 kDa can be seen. Similar results were reported by Jarpa-Parra et al. (2014). According to the authors, the disruption of the disulfide bond, which is responsible for linking the acid α-chain (40 kDa) and basic β-chain (20 kDa) that form each part of the hexamer (Jarpa-Parra et al., 2015), could be responsible for the disappearance of the 64 kDa band and appearance of the 49 kDa band.

### 3.7. Zeta potential

The zeta potential analysis was carried out aiming to evaluate changes in protein surface charge, since it influences protein functionality, especially emulsifying properties. Early literature is inconclusive regarding how the surface charge of protein could be affected by extraction processes. Some previous studies showed that ultrasound and salt extraction influence the surface charge of plant sources due to changes in the protein secondary structure or the amino acid composition (Karaca et al., 2011a; Stone et al., 2015; Eze et al., 2022). These modifications in protein structure could affect protein protonation of carboxyl groups and de-protonation of amino groups by pH variation (Tang and Sun, 2011). Here, independent of the extraction method, the zeta potential of LPI ranged from +31.8 to −27.6 mV when the pH increased from 2 to 8. These results indicate that neither the ultrasound nor the enzyme-assisted process associated with alkaline extraction changed the surface charge of the peptide chain. The neutral charge condition of the proteins from lentils is presented in Figure 3, and no difference was observed between the three extraction processes evaluated. Thus, we conclude that alkaline extraction of lentil protein associated with enzyme and ultrasound methodologies was not able to change the surface charge of extracted protein.

### 3.8. DSC

DSC analysis provides information about the denaturation temperature (Td) and denaturation enthalpy that are related to thermal stability and the hydrophobic/hydrophilic interactions of protein molecules (Feyzi et al., 2018). Thermal properties have an important influence on functional properties. For example, Kaur and Singh (2007) studied different chickpea cultivars and observed that the samples with a higher water holding capacity were those with a higher enthalpy energy. The thermal properties of protein isolated from lentils are shown in Figure 4. All the samples presented endothermic peaks ranging between 114 and 122 °C, indicating the contribution of hydrogen bonds and van der Waals interactions in the extracted proteins (Feyzi et al., 2018). This peak is generally attributed to the thermal denaturation of globulins, which is the main protein class in lentils and has higher thermal stability (Ahmed et al., 2009).

Although the denaturation temperature was not highly affected by the applied processes, the value and width of the enthalpy peak were influenced by the association of enzymes and ultrasound with alkaline extraction. LPI-AE showed the lowest enthalpy of denaturation, indicating

![Figure 2. SDS - PAGE (reducing and non-reducing) profile of the lentil protein obtained by alkaline, enzymatic and ultrasound-assisted extraction.](image)
Figure 3. Zeta-potential of proteins isolates from lentil as a function of pH. LPI – AE: Protein isolated obtained by alkaline extraction; LPI – EAAE: Protein isolated obtained by enzyme-assisted alkaline extraction; LPI – UAS: Protein isolated obtained by ultrasound-assisted alkaline extraction.

Figure 4. DSC thermograms of protein isolates. A: LPI -AE: protein isolated obtained by alkaline extraction; B: LPI – EAAE: Protein isolated obtained by enzyme-assisted alkaline extraction; C: LPI – UAS: Protein isolated obtained by ultrasound-assisted alkaline extraction.
that the protein from the alkaline process was partially denatured due to exposure to high pH, requiring less energy to denature (Lee et al., 2007). LPI-EAAE showed an intermediate ΔH value among the evaluated isolates. However, the peak associated with this isolate is broader than that of the others (LPI-AE and LPI-UAS), which can be related to repulsion between the positive charges of the protein (Martínez and Añón, 1996). Finally, LPI-UAS was the protein isolate with the widest peak and the highest associated energy. The greater amplitude of the peak in comparison to the other isolates (LPI-AE and LPI-EAAE) may indicate cooperativity in the process of thermal denaturation due to the presence of oligomers (Martínez and Añón, 1996). These results indicate that even though the solubilization step at pH 9.0 was carried out for all extraction processes, the ultrasound and enzymatic hydrolysis may have promoted some changes in the structure of proteins, making them more resistant to denaturation under alkaline conditions.

3.9. Solubility

The protein solubility profile is important because several protein functionalities, such as emulsifying, foaming, or gelling capacity are closely associated with solubility (Kiosseoglou and Paraskevopoulou, 2011). The knowledge of protein solubility in different conditions is important to determine the best application for different protein isolates by the food industry. Figure 5 shows that the solubility of protein from lentils was pH-dependent, independent of the extraction process. This U-shaped solubility profile is characteristic of lentil protein and other proteins such as those from peas, chickpeas, and soybeans (Ladjal-Ettoumi et al., 2016). The lowest solubility at pH 4 corresponds to the neutral charge condition (pH 4–5) (Figure 3), favoring protein–protein electrostatic interactions. The high solubility observed at pH 2 (highly acid) and 9 (highly alkaline) may be due to higher net positive and negative charges favoring protein–water interactions (Ladjal-Ettoumi et al., 2016).

The lentil protein solubility under neutral and alkaline conditions was different depending on the process employed. The proteins obtained by ultrasound- and enzyme-assisted processes showed higher solubility under neutral (pH 7) and alkaline conditions than that obtained by the alkaline process. At pH 7, ultrasound- and enzyme-assisted alkaline extraction increased protein solubility by 63.4% and 44.4%, respectively, when compared with alkaline extraction alone. For alkaline conditions, a 47.1% improvement in protein solubility was observed for both enzyme- and ultrasound-assisted processes. The lower solubility of LPI-AE under neutral and alkaline conditions can be caused by protein aggregation as indicated by Gao et al. (2020). They studied the influence of pH in the extraction of protein from yellow pea and observed that extraction carried out at pH 9.5 decreases the solubility of the isolated protein due to the formation of poorly soluble protein aggregates which was evidenced by size-exclusion chromatography analysis. On the other hand, several studies have shown that protein extraction processes using ultrasound decrease the particle size and increase dispersion, probably by the cavitation effects which increase the contact area and, consequently, increase the interactions between protein and water. These features may be related to the increase in the solubility of proteins from other vegetable sources obtained using ultrasound (Jiang et al., 2017; Bai et al., 2020). LPI-EAAE had higher solubility at pH 7 and 9 when compared with LPI-AE; however, under alkaline conditions, LPI-EAAE and LPI-UAS had similar results. These results corroborate the DSC analysis that indicated that the association of enzymes and ultrasound with alkaline extraction changed the structure of proteins and consequently exposed more hydrophilic groups, increasing their solubility. Similar results were observed by Perović et al. (2020). Using enzyme-assisted extraction of protein from soybean, the authors hypothesized that higher solubility of isolated protein obtained by carbohydrate-assisted extraction could be a result of less damage of protein structure.

3.10. Interfacial tension and emulsifying properties

Proteins can be used as emulsifiers in several food products such as creams, beverages, soups, cake batters, sauces, and ice cream (McClemens, 2010). Knowledge of the interfacial behavior of proteins at the oil–water interface is important to determine their best application. The dynamic interfacial tension between linseed oil and water, containing or not the protein isolates, is shown in Figure 6. All samples exhibited two distinct regimes during the interfacial tension analysis: a sharp decrease in the beginning, followed by slower decrease (after 500 s) before reaching the equilibrium value. Differences among the protein isolates are verified in the first phase which is related to the diffusion and adsorption of protein into the interface (Joshi et al., 2012). The LPI-AE solution showed the smallest value of interfacial tension before 500 s, followed by solutions with LPI-EAAE and LPI-UAS. The protein obtained solely by alkaline extraction was able to stabilize the oil–water interface faster than proteins obtained by enzyme- and ultrasound-assisted alkaline extraction. This fast decline in the interfacial tension of LPI-AE can be attributed to the increased hydrophobicity of the protein surface by the exposure of hydrophobic groups after alkaline processes (Primozic et al., 2017) which corroborates the solubility results (Figure 5), where

![Figure 5. Solubility of protein isolates as a function of pH. LPI–AE: Protein isolated obtained by alkaline extraction; LPI–EAAE: Protein isolated obtained by enzyme-assisted alkaline extraction; LPI–UAS: Protein isolated obtained by ultrasound-assisted alkaline extraction.](image-url)
LPI-AE had the lowest solubility at pH 7. Furthermore, the highest interfacial tension attributed to LPI-EAAE before 500 s indicates that the association of enzyme treatment prior to alkaline extraction may have contributed to preserve the hydrophobic groups inside the protein chain (as can be related to the solubility analysis; Section 3.9). This fact could favor protein–solvent interaction, delaying protein adsorption onto the oil interface. The second regime observed in the interfacial tension analysis is related to the conformational arrangement of adsorbed protein on the interface (Joshi et al., 2012). After 3000 s, interfacial tension reduced from 7.14 to 3.20 mN/m for pure water, while for lentil protein aqueous solutions, its reduction depended on the extraction process. The protein isolates obtained by alkaline-only, ultrasound-, and enzyme-assisted alkaline extraction, decreased the interfacial tension compared to the control from 6.23 to 1.23 mN/m, from 7.15 to 1.35 mN/m, and from 6.62 to 1.29 mN/m, respectively, with no significant difference (p > 0.05) in the equilibrium tension after 3000 s among the evaluated isolates. These results suggest that all systems were surface-active, since the aqueous systems containing protein isolates showed lower interfacial tension after 3000 s compared to pure water.

Similar results were found when evaluating EAI and ESI (Table 5). The EAI for lentil protein, independent of the extraction process, was around 24 m²/g, similar to that of proteins obtained from other plant sources (Karaca et al., 2011; Wang et al., 2020a,b). Indeed, high stability (above 90%) was observed in all samples after 10 min, indicating that lentil protein is surface-active, as demonstrated by interfacial tension analysis.

### 3.11. Surface tension and foaming properties

The surface properties of proteins are important since foam-based products such as ice creams, soft dough, and confectionery are very common in the food industry (Eugénie et al., 2014). Figure 7A shows the dynamic surface tension between lentil protein solution and air. LPI-AE and LPI-EAAE had similar behavior to each other, with higher surface tension reduction after 3000 s, in comparison to LPI-UAS. On the other hand, LPI-UAS showed an increase in surface tension after 1000 s. In general, the reduction of surface tension by a surfactant can be divided into the following steps: (i) proteins diffuse from the bulk solution towards the interface, (ii) adsorb to the interface, (iii) change their molecular structure, and (iv) spread at the interface (Mitropoulos et al., 2014). The LPI-EAAE surface behavior can be explained by its high solubility at pH 7, which can facilitate diffusion to the interface. However, LPI-AE had the lowest solubility at pH 7 but a similar surface behavior to LPI-EAAE. Although protein solubility is important for diffuse processes, adsorption to the interface is a process governed by hydrophobic interactions (Xiao et al., 2020). The solubility and DSC analysis indicated that LPI-AE was more denatured after extraction processes than the other isolates, exposing more hydrophobic groups that can explain the fast adsorption and large reduction of surface tension. The LPI-UAS surface behavior is probably a result of low conformational flexibility. After occupying the interface, flexible molecules tend to unfold and undergo more structural changes during adsorption (Makri and Doxastakis, 2007; Mitropoulos et al., 2014). As it is possible to observe from the DSC analysis, LPI-UAS has a more compact structure than the other isolates, indicating that lentil protein obtained by the ultrasound-assisted process was less flexible to unfold to adsorb at the liquid–air interface.

As for the surface tension analysis, the extraction processes influenced the FC and FS (Table 5). The FC of LPI-UAS was significantly (p < 0.05) higher than that of the other samples, indicating better ability to form bubbles. Eugénie et al. (2014) demonstrated that surfactants that present slow adsorption kinetics form big bubbles with a large size distribution. On the other hand, surfactants with fast adsorption kinetics produce a foam with smaller and more homogeneous bubbles (Eugénie et al., 2014). Analyzing Figure 7B (original images of foams are presented in the supplementary material, Figure S2), the foam produced with LPI-UAS seems to present big bubbles that increase the final foam volume and, consequently, the FC. Foam produced with LPI-AE and LPI-EAAE had more homogeneous bubbles that agreed with the fast absorption

![Dynamic interfacial tension of the systems composed by water - pure linseed oil and different protein isolates. LPI – AE (Protein isolated obtained by alkaline extraction); LPI – EAAE (Protein isolated obtained by enzyme-assisted alkaline extraction); LPI – UAS (Protein isolated obtained by ultrasound-assisted alkaline extraction).](image-url)
indicated by the surface tension analysis. Indeed, all the samples had an FS significantly (p < 0.05) different from that of the others. The highest stability was observed in foam produced with LPI-UAS and the lowest stability was observed in LPI-EAAE after 30 min and 1 h. The main features for a good foaming agent are its solubility and structural flexibility (Agboola et al., 2005). The results from the present study demonstrated that LPI-UAS has the highest solubility at pH 7 and its structure was modified and evidenced by DSC analysis. Thus, it is possible to infer that the highest FS is a result of better protein solubility and structural flexibility of LPI-UAS when compared with the other protein isolates, as has been shown by other studies (Ochoa-Rivas et al., 2017; Wang et al., 2020a,b). On the other hand, the low FS of LPI-EAAS can be associated with its poor flexibility due to the high content of disulfide bonds, as demonstrated by other studies with plant sources (Agboola et al., 2005; Jung et al., 2006).

4. Conclusions

In our study, the extraction method affected the physical-chemical and functional properties of lentil protein. Proteins were successfully extracted from lentils by all methods tested (alkaline, enzyme-, and ultrasound-assisted alkaline extraction). The enzyme-assisted alkaline extraction showed the highest purity as compared to the other processes. Even though the lentil protein obtained using the ultrasound-assisted alkaline process showed the lowest purity, it had better functionality compared to the other extraction processes, with the highest solubility at pH 7, foaming capacity, and stability, besides showing less damage to protein structure by the extraction process. Our results show that the best protein extraction process for future applications as food ingredients cannot be based only on protein extraction yield; it is also important to understand how the extraction process affects the functional properties of the protein isolates.

Declarations

Author contribution statement

Cristiane Grella Miranda: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Paula Speranza: Analyzed and interpreted the data; Wrote the paper.
Louise Emy Kurozawa: Conceived and designed the experiments; Analyzed and interpreted the data.
Ana Carla Kawazoe Sato: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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**Data availability statement**
Data associated with this study has been deposited at Repositório da Produção Científica e Intelectual da UNICAMP at the URL: https://doi.org/10.47749/T/UNICAMP.2020.1141248.

**Declaration of interest’s statement**
The authors declare no conflict of interest.

**Additional information**
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