Effect of high-pressure treatment prior to enzymatic hydrolysis on rheological, thermal, and antioxidant properties of lentil protein isolate

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Abstract
The objective of the present work was to assess the effectiveness of high-pressure (HP) treatment on the enzymatic hydrolysis of lentil protein (LP) in order to improve functionality and antioxidant activity of hydrolysates. LP dispersions were subjected to HP treatment in the selected pressure levels (300, 450, and 600 MPa for 15 min) prior to hydrolysis with alcalase (0.5% and 1%, w/w). The postprocess samples were analyzed to assess the impact of pressure treatment on denaturation, the degree of hydrolysis (DH), and antioxidant activity of hydrolysate. HP treatment improved the %DH of the LPI ($p \leq 0.05$). The HP-assisted alcalase hydrolysis imparted significant changes on the secondary structure of protein with a shift of amide I and amide II bands. The hydrolysis of LP was further confirmed by a significant drop on the enthalpy ($\Delta H$) values in the DSC endothermic peaks and decreasing the steady shear viscosity. The pressurized protein hydrolysates attained an improvement in the foaming properties ($\approx 1.5$ times) and antioxidant activities ($\approx 2$ times) than the control hydrolysates. However, a detrimental effect was pronounced on the emulsifying activity and stability index, foam stability, and water holding capacity. These results could be useful for food industries to develop products using hydrolysates as protein supplements.

KEYWORDS
antioxidant activity, flow behavior, high-pressure treatment, lentil protein hydrolysate, SDS-PAGE

1 | INTRODUCTION

Plant-derived proteins have received extensive attention by the food industry in recent years because of their health-promoting accomplishments and regulatory functions in the human system (Carbonaro, Maselli, & Nucara, 2015). The legumes sector is undergoing a dynamic change at global, regional, and country levels, to meet the growing demand for protein. Similar to animal peptides (milk, egg, fish, and meat), the legume peptides contribute significantly to human health, in particular in bone and cardiovascular health, cancer prevention, cholesterol- and lipid-lowering effects, type 2 diabetes, and weight management (Bazzano et al., 2001;
Bean et al., 2008; Rebello, Greenway, & Finley, 2014). The legume-sourced proteins can be incorporated into innovative food product development due to their extensive range of functionalities, such as substitutes for dairy or meat products, baked goods, and extruded products (Boye, Zare, & Pletch, 2010).

Among legumes, lentil (Lens culinaris) is one of the most consumed protein source because of its high nutritional quality. The major storage proteins in lentil are oligomeric globulins (49–72%) and albumins (11–16.8%) that belong to the two groups of 7S (vicilin, ~175–180 kDa) and 11S (legumin, ~340–360 kDa; Scippa et al., 2010; Swanson, 1990). Protein isolated from lentil flour is termed as the lentil protein isolate (LPI), which can be blended with other ingredients to produce protein-enriched extruded or bakery products. The lentil processors are looking for innovative ways to increase valorization of lentils while offering healthy food products options for the consumers.

The conversion of protein isolate into hydrolysates or bioactive peptides could be the novel idea with some specific bioactive activities and, therefore, epitomize a value-added product for the food and nutraceuticals (Perreault, Héniaux, Bazinet, & Doyen, 2017). Enzyme-induced hydrolysis is used to modify and improve the physicochemical, functional, and sensory attributes of native proteins with retention of the nutritional quality (Kuiper’s et al., 2005). The functionalities of protein hydrolysates depend on multiple factors including the source of proteins, type of proteolytic enzyme, temperature, pH, reaction time, and other factors. Additionally, the hydrolysis reactions could be improved by availability of the peptide bonds accessible to the enzymatic attack (Ambrosi et al. 2016). Evangelho et al. (2017) studied the enzymatic hydrolysis of black bean protein employing alcalase and pepsin at selected hydrolysis time, and they found that the pepsin treatment yielded hydrolysates with a higher degree of hydrolysis than the alcalase treatment.

In recent years, high-pressure (HP) processing has been used for the inactivation of pathogens and enzymes to prolong the shelf life of the food product. In addition to food preservation, the HP in combination with protease enzymes has the potential for modifying allergy-relevant epitopes and protein structures by unfolding and denaturation that facilitates the action of enzymes to the cleavage susceptible peptide bonds sites (Bonomi et al., 2003; Zhao, Huo, Qian, Ren, & Lu, 2017). The HP-induced enzymatic hydrolysis increases the protein susceptibility to digestion, and thus, it is worthy of exploring the technology to improve hydrolytic products yields and make the process economically feasible. The HP-assisted enzyme hydrolysis was capable of producing peptides that exhibit in vitro antioxidant activity (Zhang, Jiang, Miao, Mu, & Li, 2012). Garcia-Mora, Peñas, Frias, Gomez, and Martinez-Villaluenga, (2015) employed a combination of HP and Savinase for the proteolysis of lentil protein, however, the focus of the study was allergens only, therefore, there is ample scope to study the functional properties of lentil proteins.

There is very little information available in the literature on the effects of HP treatment prior to enzymatically hydrolyzed lentil proteins and its influence on functional, thermal, rheological, and in vitro antioxidant properties. Therefore, the aim of this study was to determine the effectiveness of HP treatment to accelerate the enzymatic hydrolysis reaction of lentil protein, in order to improve functionality and antioxidant activity of hydrolysates.

2 | MATERIALS AND METHODS

2.1 | Raw materials

Commercially available dehulled red lentil seeds (Cv. L-4076) were procured from a local market in the State of Kuwait. Alcalase® 2.4L FG (2.4 AU/g) was kindly provided by Novozymes (Bagsvaerd, Denmark). The broad range of markers and Coomassie blue R-350 were procured from Bio-Rad, Canada. The reagents and chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

2.2 | Lentil protein isolate

Split lentil seeds were ground in a roller mill (Quadrumat®Junior, Brabender, Germany), using a 70-mesh (210-μm) sieve. Lentil protein was isolated from ground lentil flour following the methods described by Avramenko, Low, and Nickerson (2013) and Ahmed, Al-Ruwaih, Mulla, and Rahman (2018) with a slight modification. Briefly, the lentil flour was blended in the beaker containing 10× distilled water, mix well, and the pH of the dispersion was set to 9.0 with the addition of sodium hydroxide solution (1 N). The dispersion was stirred well for 30 min at 25 °C, centrifuged (4,000 × g), and the supernatant was separated. The supernatant pH was then lowered to pH 4.5 by adding hydrochloric acid (1 N), centrifuged (4,000 × g) to precipitate out the protein. The precipitated protein was freeze-dried (Virtis Genesis, NY, USA) to yield the LPI. The protein content was found to be 87% in the isolate.
2.3 | HP pretreatment

HP treatments were performed in a laboratory-scale 2L HP equipment (Avure Technologies, OH, USA) with a pressure vessel of 100 mm diameter × 254 mm height. The 5% (w/v) LPI dispersions were prepared in 0.05-M phosphate buffer (pH 7.6), stirred for an hour at 4°C, dispensed into LDPE bags (Whirl-Pak®, USA), and vacuum sealed prior to the HP treatment. Details of the HP process are reported elsewhere (Ahmed, Mulla, & Arfat, 2017). The pressure levels employed for the treatment were 300, 450, and 600 MPa for a constant holding time of 15 min. The medium water temperature increased from 20°C to 26°C with increasing the pressure from 0.101 to 600 MPa because of the adiabatic heating. The untreated nonpressurized LPI dispersion was retained as a control (0.101 MPa). After the pressurization, the dispersions were freeze-dried and pulverized into powder for subsequent experiments. All HP runs were carried out in duplicate.

2.4 | Enzymatic hydrolysis of HP-treated LPI

The alcalase hydrolysis was conducted with the addition of 1% and 5% alcalase (based on LPI protein content) on both the post-HP and untreated LPI samples with 5% (w/v) based on lentil proteins at 50°C. Some preliminary studies indicated that the enzymatic reaction time of 4 hr was sufficient for the hydrolysis. The enzymatic reaction was ceased by heating the sample at 90°C for 15 min followed by immediate cooling. The samples, thereafter, centrifuged (10,000 × g at 20°C for 20 min), freeze-dried, and placed at −20°C before further analysis or use. Alcalase was not added in one set of HP-treated LPI dispersion to understand the individual pressure effect.

2.5 | Electrophoresis

The SDS-PAGE of the LPI and hydrolysates was performed according to the method of Laemmli (1970), with some minor changes, using 12% and 4% resolving gel and stacking gel, respectively. Sample loading was done in a mini-protein gel apparatus (Bio-Rad, Richmond, CA, USA) with a 1.5-mm-thick gel and conducted at a constant voltage of 100 mV. A standard protein mixture (6.5–66 kDa, Sigma-Aldrich, MO, USA) was used as an MW marker. The gel was stained for 12 hr with 0.25% Coomassie Brilliant Blue R 250. The de-staining of the gel was done using 20% methanol and 10% acetic acid solution. To obtain the protein profiles, electrophoretic patterns were analyzed using Red Imaging system equipped with Alpha-View SA Software (Cell Biosciences Inc., Santa Clara, CA, USA).

2.6 | Degree of hydrolysis (DH)

The DH was assessed by modifying the method of Sonawane and Arya (2017). The hydrolyzed lentil protein (500 µl) was blended with 20% TCA solution (1:1) followed by incubation (30 min) and centrifugation (×10,000 rpm). The protein content in the supernatant was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The %DH was calculated from the equation given below:

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\%DH = \left(\frac{\text{Soluble protein content in 10\%TCA (mg)}}{\text{Total protein content (mg)}}\right) \times 100.
\]

2.7 | Functional properties

2.7.1 | Water holding capacity (WHC)

The WHC of LPI and LPH samples was assessed at 25°C and 70°C (Ahmed, Thomas, Taher, & Joseph, 2016), and the value was presented as the volume of water retained (g) per g dry matter of the test sample.

2.7.2 | Foaming capacity (FC) and foam stability (FS)

Both FC and FS of LPI and LPH samples were measured by following our earlier method (Al-Ruwaih, Ahmed, Mulla, & Arfat, 2019).

2.7.3 | Emulsifying activity index (EAI) and emulsion stability index (ESI)

The EAI and ESI were measured by the procedure described by Pearce and Kinsella (1978), with a slight modification. The methodology followed our earlier work on kidney bean protein hydrolysates (Al-Ruwaih et al., 2019).

2.7.4 | Color measurement

Color measurement of the lentil proteins was conducted on a LabScan XE colorimeter (Hunter Associates Laboratory, Reston, VA) based on three color parameters, namely, L* (0, black; 100, white), a* (−a, greenness; +a, redness), and b* (−b, blueness; +b, yellowness). The
instrument was calibrated with standard tiles (black and white) before the measurement of the lentil sample. The measurements were performed five times, and the data reported as mean values.

2.7.5 | Differential scanning calorimetric (DSC) measurement

The measurements were conducted on a TA Q 2000 calorimeter (TA Instruments, DE) to assess the thermal data for the LPI and LPH samples. The instrument was calibrated for temperature and heat capacity using indium and sapphire, respectively. The samples (8–10 mg) were heated from 0°C to 150°C (at a rate of 10°C/min) under nitrogen (flow rate, 50 mL/min) to identify the denaturation of the protein. An empty pan was taken as a reference. The protein denaturation was computed from the instrumental software.

2.7.6 | Rheological measurement

The steady flow rheological measurements of the LPI and LPH dispersions were conducted in a rheometer (Discovery Hybrid HR-3, TA Instruments, New Castle, DE, USA) fitted with a conical end (DIN) at 25 °C (±0.1°C) following our earlier methods (Ahmed et al., 2003; Al-Ruwaih et al., 2019). For two-cycle shear measurements (0 to 100 s⁻¹ and back to 0), the hydrolyzed samples (19.6 ml) were placed in the rheometer after the alcalase inactivation. The rheological parameters (shear stress, shear rate, and the apparent viscosity) were taken directly from the instrumental software. Various flow models (e.g., Newtonian, Bingham, Casson, Power, and Herschel-Bulkley) were tested for fitting the shear stress–shear rate data, and the best-fit model was chosen based on the coefficient of determination ($R^2$) and standard error (SE). Rheological measurements were conducted in duplicate.

2.7.7 | Fourier transfer infrared spectroscopy

Nicolet iS5 FTIR spectrometer (Thermo Scientific, WI) was employed to collect the FT-IR spectra (4,000 to 550 cm⁻¹). Thirty-two scans were made to accumulate the absorption with a resolution of 4 cm⁻¹.

2.7.8 | Scanning electron microscopy

JEOL scanning electron microscopy (SEM; JCM-6000 Plus, Tokyo, Japan) was used to assess the changes in the microstructure and particle dimensions of hydrolysates.

Each sample was viewed at selected magnifications (100× and 500×), whereas the software attached to the instrument estimated the dimension of the particle (length and breadth). About 15 particles were chosen to measure the dimension.

2.7.9 | 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity (DRSA)

The scavenging activities of lentil proteins against the DPPH radical were estimated by the method described by Aluko and Monu (2003) with a slight change. Detailed methodologies of DRSA are described in our earlier work (Al-Ruwaih et al., 2019).

2.8 | Statistical analysis

An analysis of variance (ANOVA) of the experimental data was conducted using Minitab software (version 17; Minitab Corp., USA), and differences were considered to be significant at $p < 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | Electrophoretic patterns of lentil proteins

The results of the SDS-PAGE of LPI, alcalase-treated (0.5 and 1%) and HP-treated hydrolysates are shown in Figure 1. For the control LPI sample, a series of visible bands with the MW between 10 and 250 kDa were observed including the subunits of 7S globulins (e.g., vicilin with 48 kDa) and 11S globulin (71 and 63 kDa; http://www.uniprot.org; Cai, McCurdy, & Baik, 2002). The sole HP treatment did not alter the electrophoretic pattern of LP significantly, even when exposed at the highest level (600 MPa for 15 min; Figure 1a,b). Generally, the covalent bonds remained unaffected by the HP, and thus, the primary protein structure did not alter after the pressurization. A similar observation was reported earlier for the HP-treated LP (Garcia-Mora et al., 2015), and those authors reported that the HP-treatment did not promote detachment of globulin subunits or association of disulfide-linked oligomers. After alcalase hydrolysis assisted by HP, the LP protein bands related to 7S and 11S globulins were completely disappeared or reduced significantly (Figure 1a,b). This finding matches with the DH values as shown in Figure 2. Furthermore, the low molecular weight bands below 10 kDa exhibited a higher intensity, which indicates break down of the peptides into much lower fragments. These observations are consistent
with the results reported by Garcia-Mora, Peñas, Frias, and Martínez-Villaluenga (2014) on alcalase-assisted hydrolysis of LP. Recently, Franck et al. (2019) reported the presence of over 75% of lower MW (below 1,500 Da) peptides and >50% between 750 and 1,500 Da for enzymatic hydrolysis assisted by HP (100 and 300 MPa for 5 and 10 min) of flaxseed proteins using trypsin.

### 3.2 Role of enzyme concentration and pressure-intensity on LPH

The efficacy of enzyme concentration (0.5% and 1%) on hydrolysis assisted by HP treatment of proteins was measured by the percent degree of hydrolysis. Figure 2 shows that the HP-treated protein did not improve the %DH much ($p > 0.05$) than the untreated hydrolysates (0.101 MPa, %DH = 4.09). The results of hydrolysis experiments conducted at ambient pressure (0.101 MPa) for 4 hr confirmed that the maximum degree hydrolysis achieved at the alcalase concentration of 1%. However, the enzymatic hydrolysis assisted by HP required only 0.5% alcalase to obtain the same degree of hydrolysis (with a minor exception at 600 MPa), indicating HP treatment facilitated the conformational changes of proteins that might increase the effectiveness of the enzymatic digestion, by providing the enzymes' access to buried sites (Chicón, López-Fandiño, Quirós, & Belloque, 2006). The enzymatic proteolysis (alcalase 0.5%) assisted by HP (300 MPa/15 min) resulted in the maximum DH (57.66%), and no substantial changes was observed in the %DH between 300 and 600 MPa ($p > .05$). Various researchers (Franck et al., 2019; Zhang et al., 2012) found a pressure of 300 MPa was optimum for the enzymatic hydrolysis of chickpea and flaxseed proteins. It has been reported that alcalase activity
improved at the vicinity of 300 MPa because of the unfolding of the protein structure, so more cleavage sites are exposed, and enhance the hydrolysis (Garcia-Mora et al., 2014; Zhang et al., 2012). A minor decrease in the DH at the pressure higher than 300 MPa could likely be associated with the non-reversible aggregation of protein produced by the pressure. Based on these observations, all LP hydrolysis works were done at 300 MPa for 15 min at the alcalase concentration of 0.5%.

3.3 | Characterization of LPH

3.3.1 | Water holding capacity (WHC)

The WHC of LPI, HP-treated and LPH samples measured at 25°C and 70°C are reported in Table 1. At both temperatures, the WHC of LPI increased with the pressurization. The increase in WHC of LPI could be associated with the increase in hydrophilic groups after pressurization. On the contrary, the WHC of the hydrolysates dropped with enzymatic digestion, and the least value was recorded for the sample treated with HP and alcalase in series. Additionally, the WHC of LPH decreased further when the temperature was increased from 25°C to 70°C. A similar drop in the WHC has been reported for the hydrolyzed soybean protein with increasing DH (Jung & Mahfuz, 2009; Mietzsch, Fehér, & Halasz, 1989). Addition of enzyme after the pressurization has been reported to reduce the ability of the proteins significantly to bind water because of the loss of soluble proteins (Jung & Mahfuz, 2009). The lowering of WHC of hydrolyzed hemp protein has been associated with the decrease of hydrophilic groups, in particular, insoluble aggregates that are present on the exterior of hemp protein components (Yin et al., 2008).

3.3.2 | Foaming properties

HP treatment did not influence the FC or the FS of the LPI. However, the enzymatic hydrolysis increased the FC considerably and dropped the FS (Table 1). Similar observations were reported for soy protein and rapeseed proteins (Chabanon, Chevalot, Framboisier, Chenu, & Marc, 2007; Puski, 1975), and those authors opined that the improved FC and decreased FS occurred because of the formation of smaller hydrophilic and/or charged peptides that facilitate their dispersion and adsorption at the interface.

3.3.3 | Emulsifying properties

The emulsifying activity index (EAI) and emulsifying stability index (ESI) of lentil proteins and hydrolysates are reported in Table 1. The EAI values were above 74%, and the maximum value was observed for the LPI
sample treated at 300 MPa for 15 min. Pressurization increased the EAI significantly, whereas the enzymatic hydrolysis dropped the value significantly. A similar increase in EAI and decrease in ESI values was observed for soy protein isolate treated at 200 MPa (Wang et al., 2008). The drop in both EAI and ESA values of hydrolysates could be associated with the creation of more hydrophilic peptides, which are poorly related with the oil–water interface or due to weak viscoelastic film formation at the interface by small peptides, which was inadequate to resist merger of adjacent droplets (Avramenko et al., 2013).

### 3.3.4 Color

The increase in lightness, $L^*$, value of the LPI with both HP treatment and enzymatic hydrolysis indicates an increase in whiteness ($p < 0.05$). Both the redness (i.e., increasing $a^*$ value) and yellowness (increasing $b^*$ value) of the lentil protein dropped significantly after the enzyme hydrolysis (Table 1). Alcalase treatment is associated with the instability of the pigments in the harsh environment where a significant change of pH occurred, and therefore, the lentil pigments affected abruptly. The observed color values were similar to those reported values for the lentil protein concentrate (Toews & Wang, 2013).

### 3.3.5 Thermal properties

During thermal scanning, two distinct irreversible endothermic peaks were detected for the lentil proteins (Table 1). The peaks I and II were ranged from 94.82 to 98.46 and 108.97 to 111.49, respectively. The observed peaks represent the peak thermal denaturation temperatures ($T_d$) of 7S and 11S of lentil proteins, namely, lectin and globulin (Cai et al., 2002; Lee, Htoon, Uthayakumaran, & Paterson, 2007). A similar range of $T_d$ has been reported previously for the lentil dispersions (Ahmed, Varshney, & Ramaswamy, 2009). The alcalase digestion influenced both the $T_d$ values with a significant drop in the $\Delta H$ values. It indicates the susceptibility of the enzyme for the protein hydrolysis. Because the drop in $\Delta H$ for $T_{d1}$ was much higher than the $T_{d2}$ suggests that the protein structure related to $T_{d2}$ was more integrated and less affected by the alcalase. On the contrary, the pressure treatment has the least effect on the $T_d$ and the corresponding $\Delta H$ values. It directs that the lentil protein’s native structure remained compact, and the globulin fraction of the protein was not completely denatured with HP.
treatment (Ahmed et al., 2009). Similar results have been reported by various researchers for hydrolyzed soy protein and black gram proteins (Ortiz & Wagner, 2002; Wani, Sogi, & Gill, 2015).

### 3.3.6 Steady flow measurement

The steady flow rheology of the LPI dispersions showed that the shear stress ($\sigma$) increased with increasing the shear rate ($\dot{\gamma}$) from 0.1 to 100 s$^{-1}$ (Figure 3a). After pressurization and alcalase digestion, the $\sigma$–$\dot{\gamma}$ rheograms dropped in the order of LPI + 0.5% alcalase < LPI at 300 MPa + 0.5% alcalase < LPI at 300 MPa. A close look on the rheograms exhibited the structural breakdown of the sample during forward (0 to 100 s$^{-1}$) and backward (100 to 0 s$^{-1}$) runs and generated an area loop, which mostly a measure of thixotropy. The structural breakdown continued for the HP-treated sample at 300 MPa; however, it was pronounced for both enzyme and combined HP/enzyme hydrolysis. It is postulated that the alcalase digestion of lentil proteins at 0.101 and 300 MPa produced the highest number of low MW peptides (<3 kDa) that resulted in the lowering of the viscosity at the lowest level (Figure 3b). These results are consistent with the observation of Garcia-Mora et al. (2015). The increase in the shear stress of the pressurized enzymatic hydrolysates could be related with the agglomeration of the smaller sized hydrolyzed proteins. Additionally, the particle size increased because of disruption and disorganization of weak protein bonds during proteins denaturation and aggregation (Dumay, Kalichevsky, & Cheftel, 1994; Quirós, Chichón, Recio, & López-Fandiño, 2007).

**FIGURE 3** Rheograms of lentil proteins as influenced by high-pressure and enzymatic treatment (a). Shear stress–shear rate data with thixotropic loop and (b) the apparent viscosity at a constant shear rate of 10$^{-1}$ s (LPI, lentil protein isolate; E, alcalase enzyme). Different letters indicate significant differences between means ($p < .05$) with treatment.
Various flow models were tested to describe the rheological behavior (shear stress–shear rate data) of lentil protein dispersions and hydrolysate, and it was observed that the Herschel–Bulkley model (Equation 1) fitted well ($R^2 = 0.99$). All samples exhibited a marginal yield stress ($\sigma_o$) and behaved like a Newtonian fluid with a flow behavior index ($n$) approaching unity (0.95–1.06). The $\sigma_o$ of LPI dispersion decreased with the digestion of alcalase from $9.55 \times 10^{-3}$ to $2.54 \times 10^{-4}$ Pa ($p < 0.05$), and the corresponding consistency coefficient, $K$, value dropped similarly from $2.60 \times 10^{-3}$ to $9.87 \times 10^{-4}$ Pa·s$^n$. Upon pressurization at 300 MPa, a little drop in the HB model parameters was observed ($\sigma_o$: $4.05 \times 10^{-3}$ Pa and $K$: $2.10 \times 10^{-3}$ Pa·s$^n$), whereas the addition of alcalase to pressurized samples dropped those values further ($\sigma_o$: $1.04 \times 10^{-3}$ Pa and $K$: $1.07 \times 10^{-3}$ Pa·s$^n$) confirming enzymatic hydrolysis of LPI. The $n$ value of the control LPI sample increased gradually from 0.95 to 0.99, 1.08, and 1.02 after pressurization, alcalase hydrolysis, and pressure-assisted alcalase treatment, respectively. Lamsal, Jung, and Johnson (2007) reported similar range of $n$ and $K$ values for soybean protein hydrolysates. Additionally, Tsumura et al. (2005) found that the apparent viscosity ($\eta^*$) of papain-induced hydrolyzed β-conglycinin and glycinin was less than that of the non-hydrolyzed SPI. All these data demonstrated that alcalase had a significant impact on the steady flow behavior of lentil proteins and hydrolysates.

### 3.3.7 Fourier transform infrared

The conformational changes in lentil protein as influenced by the pressure and enzymatic hydrolysis were measured by FTIR spectra (Figure 4). The lentil protein shows some intense bands in the range from 1,235 to 1,632 cm$^{-1}$ attributed to the β-sheet and β-turn structures. The bands at 1,632 cm$^{-1}$ assigned for the amide-I region representing C=O stretching (Carbonaro, Maselli, & Nucara, 2012), 1,526 cm$^{-1}$ for the amide-II region due to CN stretching and NH bending, and the band at 1,235 cm$^{-1}$ relates the amide-III region, associated with CN stretching and NH bending. Pressure treatment at 300 MPa for 15 min did not affect protein's secondary structure, and therefore, no shift was observed for the bands. Upon alcalase digestion (with/without pressure), the amide I, II, and III bands shifted to 1,634, 1,532, and 1,238 cm$^{-1}$, respectively, showing a change in the structure. A similar shift in the wavelength of amide I band was reported for the soybean protein hydrolysate because of the loss of ordered structures (Zhao, Xiong, & McNear, 2013) and formation of intermolecular β-sheet aggregates (Carbonaro et al., 2012). A shift in the absorption band at 1,517 cm$^{-1}$ for protein hydrolysate occurred due to hydrolysis of the amide band (Böcker, Wubshet, Lindberg, & Afseth, 2017). Some other broad bands that had peak absorbance at ~3,272 (amide-A region, attributable to the −NH stretching) and 2,960 to 2,874 cm$^{-1}$ [amide-B region, representing the CH antisymmetric and symmetric stretching modes of methyl (CH$_3$) and methylene (CH$_2$)] were observed. However, no major changes observed for these bands.

### 3.3.8 Particle size and SEM

The particle sizes of the untreated lentil protein were irregular in shape with multiple pores on the surfaces.

*FIGURE 4* FTIR spectra of lentil proteins as influenced by high-pressure and enzymatic treatment; — LPI control (0.101 MPa); — 300 MPa treated LPI; — LPH (0.101 MPa); and — 300 MPa treated LPH (LPI, lentil protein isolate; LPH, lentil protein hydrolysate)
The particle sizes were in the range of 81.2 to 259 μm and changed marginally to the range of 75.5 to 293 μm after the HP treatment (Figure 5). Upon alcalase digestion, the particle size of lentil proteins reduced \((p < 0.05)\) with a particle size ranged between 63 and 206 μm. However, the combined treatment (HP + enzyme) produced the

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**FIGURE 5**  SEM Micrographs of a) LPI at 0.101 MPa; b) LPH at 0.101 MPa; c) LPI treated at 300 MPa and d) LPH treated at 300 MPa

**FIGURE 6**  DPPH radical scavenging activities of high-pressure and alcalase-treated lentil proteins (LPI, lentil protein isolate; E, alcalase enzyme). Different letters indicate significant differences between means \((p < 0.05)\) with treatment.
minimum particle sizes (40.2 to 168 μm). A similar reduction in particle size was reported for the fish proteins due to enzymatic hydrolysis (Foh, Kamara, Amadou, Foh, & Wenshui, 2011). The reduction of particle size could happen because of the severity of the enzyme that breaks down the larger protein molecules into smaller fragments of peptides (Lara, Adamcik, Jordens, & Mezzenga, 2011).

3.3.9 DPPH radical scavenging activity (DRSA)

DRSA assays were carried out on LPI samples after different process conditions. The hydrolysates obtained after enzymatic digestion of LPI with and without pressurization to confirm that HP treatment had an effect on the release of the bioactive compounds (Figure 6). The DPPH radical scavenging activity of LPI was lower (15.6%) than all other samples, and the value improved to 19.4% with the pressurization of LPI at 300 MPa. Upon 0.5% alcalase digestion, the DRSA value of the hydrolysate increased to 25.3% (p < 0.05), and the highest value of DRSA (about 28% higher than the LPI) was recorded for the hydrolysate produced by application of pressure (300 MPa/15 min) and alcalase (0.5%) treatment (p < 0.05). Such improvement could be attributed to the pressure-induced openness of enzyme-susceptible peptide bonds in LPI that directed to the generation of lower MW peptides with advanced DRSA (Korhonen & Pihlanto, 2003). Although HP was employed as a pretreatment step before enzymatic hydrolysis of pea protein isolate, it was observed the process was more active for the enzymatic release of DRSA peptides in comparison with the untreated sample (Girgih et al., 2015). Franck et al. (2019) found an increase of 20% antioxidant activity, whereas the pressure level was increased from 100 to 300 MPa for 10 min.

4 CONCLUSIONS

The alcalase digestion of the lentil protein to produce hydrolysates was substantially improved by employing the HP (300 MPa for 15 min) as a pretreatment step. The HP treatment prior to enzymolysis increase the openness of cleavage sites to the enzyme and improve the DH and antioxidant activity of the lentil protein. Lentil hydrolysates were characterized by SDS-PAGE, %DH, and surface properties. Flow measurement of the hydrolysates indicated the non-Newtonian behavior of the fluid and the formation of stable soluble aggregate. The change in the β-sheet secondary structure of the hydrolyzed proteins was elucidated by studying Fourier transform infrared spectra. Because of their antioxidant activity, hydrolysates could be used as dietary protein supplements or functional food ingredients. Furthermore, a good understanding between legume proteins and functional compounds could guide to develop novel strategies for increasing bioavailability, quality, and the safety of legume-based foods.

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CONFLICT OF INTEREST

Authors confirm that there is no conflict of interest in the work.

DATA AVAILABILITY

The raw data cannot be shared at this time due to technical limitations, however, available on request.

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REFERENCES

Ahmed, J., Ramaswamy, H. S., Alli, I., & Ngadi, M. (2003). Effect of high-pressure on rheological characteristics of liquid egg. IWT-Food Science & Technology, 36, 517–524.

Ahmed, J., Al-Ruwaih, N., Mulla, M., & Rahman, M. H. (2018). Effect of high pressure treatment on functional, rheological and structural properties of kidney bean protein isolate. LWT-Food Science and Technology, 91, 191–197.

Ahmed, J., Mulla, M., & Arfat, Y. A. (2017). Application of high-pressure processing and polylactide/cinnamon oil packaging on chicken sample for inactivation and inhibition of Listeria monocytogenes and Salmonella Typhimurium, and post-processing film properties. Food Control, 78, 160–168.

Ahmed, J., Thomas, L., Taher, A., & Joseph, A. (2016). Impact of high pressure treatment on functional, rheological, pasting, and structural properties of lentil starch dispersions. Carbohydrate Polymers, 152, 639–647.

Ahmed, J., Varshney, S. K., & Ramaswamy, H. S. (2009). Effect of high pressure treatment on thermal and rheological properties of lentil flour slurry. LWT-Food Science and Technology, 42, 1538–1544.

Al-Ruwaih, N., Ahmed, J., Mulla, M., & Arfat, Y. A. (2019). High-pressure assisted enzymatic proteolysis of kidney beans protein isolates and characterization of hydrolysates by functional, structural, rheological and antioxidant properties. LWT-Food Science and Technology, 100, 231–236.
Aluko, R. E., & Monu, E. (2003). Functional and bioactive properties of quinoa seed protein hydrolysates. *Journal of Food Science, 68*, 1254–1258.

Ambrosi, V., Polenta, G., Gonzalez, C., Ferrari, G., & Maresca, E. (2016). High hydrostatic pressure assisted enzymatic hydrolysis of whey proteins. *Innovative Food Science & Emerging Technologies, 38*, Part B, 294–301.

Avramenko, N. A., Low, N. H., & Nickerson, M. T. (2013). The effects of limited enzymatic hydrolysis on the physicochemical and emulsifying properties of a lentil protein isolate. *Food Research International, 51*, 162–169.

Bazzano, L., He, J., Ogden, L., Loria, C., Vupputuri, S., Myers, L., & Whelton, P. (2001). Legume consumption and risk of coronary heart disease in US men and women. *Archives of Internal Medicine, 161*(21), 2573–2578.

Bean, D., Thompson, M. D., Thompson, H. J., Brick, M. A., Mcginley, J. N., Jiang, W., & Wolfe, P. (2008). Mechanisms associated with dose-dependent inhibition of rat mammary carcinogenesis by dry bean (*Phaseolus vulgaris, L.*). *Journal of Nutrition, 138*(11), 2091–2097.

Böcker, U., Wubshet, S. G., Lindberg, D., & Afseth, N. K. (2017). Fourier-transform infrared spectroscopy for characterization of protein chain reductions in enzymatic reactions. *Analyt, 142*, 2812–2818.

Bonomi, F., Fiochti, A., Frokier, H., Gaiaschi, A., Iametti, S., Poiesi, C., ... Rovere, P. (2003). Reduction of immunoreactivity of bovine β-lactoglobulin upon combined physical and proteolytic treatment. *Journal of Dairy Research, 70*, 51–59.

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International, 43*, 414–431.

Cai, R., McCurdy, A., & Bailey, B. K. (2002). Textural property of 6 legume curds in relation to their protein constituents. *Journal of Food Science, 67*, 1725–1730.

Carbonaro, M., Maselli, P., & Nucara, A. (2012). Relationship between digestibility and secondary structure of raw and thermally treated legume proteins: A Fourier transform infrared (FT-IR) spectroscopic study. *Amino Acids, 43*, 911–921.

Carbonaro, M., Maselli, P., & Nucara, A. (2015). Structural aspects of legume proteins and nutraceutical properties. *Food Research International, 76*, 19–30.

Chabanon, G., Chevalot, I., Framboisier, X., Chenu, S., & Marc, I. (2007). Hydrolysis of rapeseed protein isolates: Kinetics, characterization and functional properties of hydrolysates. *Process Biochemistry, 42*, 1419–1428.

Chicón, R., López-Fandiño, R., Quirós, A., & Belloque, J. (2006). Changes of chymotrypsin hydrolysis of beta-lactoglobulin A induced by high hydrostatic pressure. *Journal of Agricultural and Food Chemistry, 54*, 2333–2341.

Dumay, E. M., Kalichevsky, M. T., & Cheftel, J. C. (1994). High-pressure unfolding and aggregation of beta-lactoglobulin and the baroprotective effects of sucrose. *Journal of Agricultural and Food Chemistry, 42*, 1861–1868.

Evangelho, J. A., Vanier, N. L., Pinto, V. Z., De Berrios, J. J., Dias, A. R. G., & Zavareze, E. R. (2017). Black bean (*Phaseolus vulgaris L.*) protein hydrolysates: Physicochemical and functional properties. *Food Chemistry, 214*, 460–467.

Foh, M. B. K., Kamara, M. T., Amadou, I., Foh, B. M., & Wenshui, X. (2011). Chemical and physicochemical properties of tilapia (*Oreochromis niloticus*) fish protein hydrolysate and concentrate. *International Journal of Biological Chemistry, 5*, 21–36.

Franck, M., Perreault, V., Suwal, S., Marciniak, A., Bazinet, L., & Doyen, A. (2019). High hydrostatic pressure-assisted enzymatic hydrolysis improved protein digestion of flaxseed protein isolate and generation of peptides with antioxidant activity. *Food Research International, 115*, 467–473.

Garcia-Mora, P., Peñas, E., Frias, J., Gomez, R., & Martinez-Villaluenga, C. (2015). High-pressure improves enzymatic proteolysis and the release of peptides with angiotensin I converting enzyme inhibitory and antioxidant activities from lentil proteins. *Food Chemistry, 171*, 224–232.

Garcia-Mora, P., Peñas, E., Frias, J., & Martinez-Villaluenga, C. (2014). Savinase, the most suitable enzyme for releasing peptides from lentil (*Lens culinaris* var. Castellana) protein concentrates with multifunctional properties. *Journal of Agricultural and Food Chemistry, 62*, 4166–4174.

Girigh, A. T., Chao, D., Lin, L., He, R., Jung, S., & Aluko, R. E. (2015). Enzymatic protein hydrolysates from high pressure-pretreated isolated pea proteins have better antioxidant properties than similar hydrolysates produced from heat pretreatment. *Food Chemistry, 188*, 510–516.

Jung, S., & Mahfuz, A. A. (2009). Low temperature dry extrusion and high-pressure processing prior to enzyme-assisted aqueous extraction of full fat soybean flakes. *Food Chemistry, 114*, 947–954.

Korhonen, H., & Pihlanto, A. (2003). Food-derived Bioactive Peptides - Opportunities for Designing Future, Foods. *Current Pharmaceutical Design, 9*, 1297–1038.

Kuipers, B. J. H., Koningsveld, G. A., Alting, A. C., Driehuis, F., Gruppen, H., & Voragen, A. G. J. (2005). Enzymatic hydrolysis as a means of expanding the cold gelation conditions of soy proteins. *Journal of Agricultural and Food Chemistry, 53*, 1031–1038.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Lamsal, B. P., Jung, S., & Johnson, L. A. (2007). Rheological properties of soy protein hydrolysates obtained from limited enzymatic hydrolysis. *LWT- Food Science and Technology, 40*, 1215–1223.

Lara, C., Adamcik, J., Jordens, S., & Mezzenga, R. (2011). General self-assembly mechanism converting hydrolyzed globular proteins into giant multistranded amyloid ribbons. *Biomacromolecules, 12*, 1868–1875.

Lee, H. C., Hoon, A. K., Uthayakumaran, S., & Paterson, J. L. (2007). Chemical and functional quality of protein isolated from alkaline extraction of Australian lentil cultivars: Matilda and Digger. *Food Chemistry, 102*, 1199–1207.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Mietusch, F., Fehér, J., & Halasz, A. (1989). Investigation of functional properties of partially hydrolyzed proteins. *Molecular Nutrition & Food Research, 33*, 9–15.

Ortiz, S. E. M., & Wagner, J. R. (2002). Hydrolysates of native and modified soy protein isolates: Structural characteristics,
solubility and foaming properties. *Food Research International*, 35, 511–518.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry*, 26, 716–723.

Perreault, V., Hénaux, L., Bazinet, L., & Doyen, A. (2017). Pretreatment of flaxseed protein isolate by high hydrostatic pressure: Impacts on protein structure, enzymatic hydrolysis and final hydrolysate antioxidant capacities. *Food Chemistry*, 221, 1805–1812.

Puski, G. (1975). Modification of functional properties of soy proteins by proteolytic enzyme treatment. *Cereal Chemistry*, 52, 655–664.

Quirós, A., Chichón, R., Recio, I., & López-Fandiño, R. (2007). The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chemistry*, 104, 1734–1739.

Rebello, C. J., Greenway, F. L., & Finley, J. W. (2014). A review of the nutritional value of legumes and their effects on obesity and its related co-morbidities. *Obesity Reviews*, 15(S), 392–407.

Scippa, G. S., Rocco, M., Ialicicco, M., Trupiano, D., Viscosi, V., DiMichele, M., ... Scaloni, A. (2010). The proteome of lentil (*Lens culinaris* Medik.) seeds: Discriminating between landraces. *Electrophoresis*, 31(3), 497–506.

Sonawane, S. K., & Arya, S. S. (2017). Bioactive *L. acidissima* protein hydrolysates using Box–Behnken design. *3 Biotech*, 7, 218.

Swanson, B. G. (1990). Pea and lentil protein extraction and functionality. *Journal of the American Oil Chemists' Society*, 67, 276–280.

Toews, R., & Wang, N. (2013). Physicochemical and functional properties of protein concentrates from pulses. *Food Research International*, 52, 445–451.

Tsumura, K., Saito, T., Tsuge, K., Ashida, H., Kugimiya, W., & Inouye, K. (2005). Functional properties of soy protein hydrolysates obtained by selective proteolysis. *LWT- Food Science and Technology*, 38, 255–261.

Wang, X. S., Tang, C. H., Li, B. S., Yang, X. Q., Li, L., & Ma, C. Y. (2008). Effects of high-pressure treatment on some physicochemical and functional properties of soy protein isolates. *Food Hydrocolloids*, 22, 560–567.

Wani, I. A., Sogi, D. S., & Gill, B. S. (2015). Physico-chemical and functional properties of native and hydrolysed protein isolates from Indian black gram (*Phaseolus mungo* L.) cultivars. *LWT-Food Science and Technology*, 60, 848–854.

Yin, S. W., Tang, C. H., Cao, J. S., Hu, E. K., Wen, Q. B., & Yang, X. Q. (2008). Effects of limited enzymatic hydrolysis with trypsin on the functional properties of hemp (*Cannabis sativa* L.) protein isolate. *Food Chemistry*, 106, 1004–1013.

Zhao, J., Xiong, Y. L., & McNear, D. H. (2013). Changes in structural characteristics of antioxidative soy protein hydrolysates resulting from scavenging of hydroxyl radicals. *Journal of Food Science*, 78, C152–C159.

Zhao, R. J., Huo, C. Y., Qian, Y., Ren, D. F., & Lu, J. (2017). Ultra-high-pressure processing improves proteolysis and release of bioactive peptides with activation activities on alcohol metabolic enzymes in vitro from mushroom foot protein. *Food Chemistry*, 231, 25–32.

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