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# The effects of limited enzymatic hydrolysis on the physicochemical and emulsifying properties of a lentil protein isolate

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ABSTRACT

The physicochemical and emulsifying properties of lentil protein isolates (LPI) were investigated as a function of their degree of hydrolysis (DH of 4, 9 and 20%) following exposure to trypsin/heat. Specifically, interfacial tension, surface characteristics (charge and hydrophobicity) and intrinsic fluorescence were determined. These parameters were then related to changes in the emulsification activity (EAI) and stability indices (ESI) of unhydrolyzed (u-LPI) and hydrolyzed LPI (h-LPI) in a flaxseed oil–water emulsion. Interfacial tension was found to decrease from ~6.5 to ~6.1 mN $m^{-1}$ for u-LPI and h-LPI (DH 4–20%), respectively. A similar trend was observed for surface hydrophobicity, which declined from ~30 to ~24 for the u-LPI and h-LPI (DH 4–20%), respectively. In contrast, surface charge values were similar for all materials (~−37 mV). Intrinsic fluorescence as a function of emission wavelengths (300–400 nm) indicated a slight change in the tertiary conformation of LPI upon hydrolysis, where the magnitude of fluorescence intensity declined relative to that of u-LPI. Changes in physicochemical properties upon hydrolysis had a detrimental effect on EAI and ESI values, which declined from ~51 to ~47 $m^2$ $g^{-1}$ and ~12 to ~11 min for u-LPI and h-LPI (DH 4–20%), respectively.



## 1. Introduction

In general, food proteins are effective at stabilizing oil-in-water emulsions due to their amphiphilic nature (i.e., possessing both hydrophilic and hydrophobic reactive sites on their surface) and interfacial film-forming abilities. Emulsions are defined as dispersions of two (or more) immiscible liquids which are inherently thermodynamically unstable and tend to phase separate overtime via creaming, flocculation and/or coalescence. Emulsion stability is highly dependent upon liquid droplet size and distribution, emulsion processing conditions (i.e., homogenization rates), protein characteristics (i.e., size, conformation, surface reactivity, concentration and solubility), solvent conditions (i.e., pH, salts and temperature), phase volume ratio and continuous phase viscosity (Can Karaca, Low, & Nickerson, 2011a,b; McClements, 2007). During emulsion formation, proteins diffuse from the bulk solution to the oil–water interface, where they unravel and re-orient to form a viscoelastic interfacial film around the dispersed oil droplets which acts as a physical and/or electrostatic barrier towards destabilization (Damodaran, 2005). Emulsion stability can arise from electrostatic repulsive forces between neighbouring droplets (depending on the salt and pH conditions) or steric hindrance induced by hydrophilic protein segments extending into the continuous phase that physically restricts coalescence (Can Karaca et al., 2011a).

In contrast to animal-derived proteins (e.g., whey, casein or ovalbumin), the emulsifying properties of plant proteins including legumes, have not been as extensively studied and their mechanism(s) of action are less well understood. Plant protein-based emulsifiers are attractive to the food industry for both product development and re-formulation, based on their low cost, nutritional benefits and greater consumer/market acceptability (Can Karaca et al., 2011a; Duranti, 2006). The emulsifying properties of a group of legume proteins have been studied and include those extracted from pea (Ducel, Richard, Popineau, & Boury, 2004), cowpea, (Kimura et al., 2008), faba bean (Galazka, Dickinson, & Ledward, 1999), soy (Martinez, Sanchez, Patino, & Pilosof, 2009) and lentil (Bora, 2002). Research arising from our laboratory compared the emulsifying properties of protein isolates derived from chickpea, lentil, faba bean, pea and soy produced by both isoelectric precipitation and salt extraction (Can Karaca et al., 2011a). Findings indicated that lentil protein isolates, produced by isoelectric precipitation gave the best emulsifying properties, having the highest emulsion capacity of the legumes tested, and comparable emulsifying activity/stability indices and creaming behavior to soy. Emulsion capacity refers to the maximum amount of oil that can be dispersed within a solution of emulsifiers (e.g., proteins) without the emulsion phase separating or undergoing an inversion from an oil-in-water emulsion to a water-in oil emulsion (McClements, 2007). The emulsifying activity index relates to the emulsion forming properties of the protein (Hill, 1996), and provides an estimate of the interfacial area stabilized by a given weight of protein based on turbidimetric analysis of a diluted emulsion (Pearce & Kinsella, 1978). The emulsifying stability index provides a measure of stability over a defined period of time for the same diluted emulsion (Pearce & Kinsella, 1978).

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In order to further enhance the emulsifying properties of proteins, some researchers have explored limited enzymatic hydrolysis by proteases (e.g., trypsin) as a means to enhance functionality. Limited hydrolysis can lead to a partial unravelling of the protein structure (altering its conformational stability) to expose more ionizable and hydrophobic groups, decrease protein mass, and release polypeptides/peptides into solution (Panyam & Kilara, 1996). A limited degree of protein hydrolysis (DH) (<10%) has been found by some to enhance protein functionality, however above a critical point a detrimental effect can be seen (Govindaraju & Srinivas, 2006; Guan, Yao, Chen, Shan, & Zhang, 2007; Karayannidou et al., 2007; Panyam & Kilara, 1996). Karayannidou et al. (2007) reported that trypsin-treated sunflower protein isolate (DH~10%) showed improved emulsifying and foaming properties relative to the non-hydrolyzed material. Guan et al. (2007) reported enhanced solubility, water holding, emulsifying and foaming properties for trypsin-treated oat bran protein (DH~4–8%) relative to the native protein. Also, Ventureira, Martinez, and Anon (2010) reported that trypsin-treated amaranth protein (DH of 2.2%) showed enhanced oil–water emulsion stability relative to the native form. In contrast, Govindaraju and Srinivas (2006) reported that the hydrolysis of arachin protein (DH~19%) using papain, alcalase and fungal protease resulted in a significant decrease in emulsification and, foaming capacity and stability in an oil–water emulsion. Chabanon, Chevalot, Framboisier, Chenu, and Marc (2007) reported that a 15% hydrolysis of canola protein with alcalase resulted in lower foaming capacity and stability and emulsion activity and stability in an oil–water emulsion in comparison to that of the unhydrolyzed protein.

The overall goal of this research was to investigate structure-function relationships associated with the limited enzymatic hydrolysis of a lentil protein isolate (LPI), as it related to its surface characteristics, protein conformation and emulsifying properties. Lentil proteins are primarily comprised of globulins (~49%) and albumins (~16.8%) (Boye, Fatemeh, & Pletch, 2010). The salt-soluble globulin fraction consists mainly of legumin (11S, S — Svedberg Unit; ~340–360 kDa) and vicilin (7S; ~175–180 kDa) (Swanson, 1990). Legumin is a hexameric protein comprised of ~60 kDa subunits of α (~40 kDa) and β (~20 kDa) chains, whereas vicilin is a trimeric protein comprised of ~5060 kDa subunits (Swanson, 1990). In contrast, the water-soluble albumins range in size from 5 to 80 kDa, and include protease and amylase inhibitors, and lectins (Boye et al., 2010).

## 2. Materials and methods

### 2.1. Materials

Whole green lentil seeds (CDC Grandora) and flaxseed oil were provided by the Crop Development Centre (Saskatoon, SK, Canada) and Bioriginal Food and Science Corp. (Saskatoon, SK, Canada), respectively. The following materials were purchased from Bio-Rad (Mississauga, ON, Canada): Bio-Rad Broad Range Marker, Bio-Rad Tris–HCl gel (15%T), Coomassie blue R-350 and Laemmli Sample Buffer. Hexane was purchased from Fisher Scientific (Ottawa, ON, Canada). Picrylsulfonic acid (trinitrobenzenesulfonic acid (TNBS)) and trypsin (10,600 units/mg) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). The following chemicals were purchased from VWR (Edmonton, AB, Canada): hydrochloric acid, sodium dihydrogen phosphate, sodium dodecyl sulfate, sodium hydroxide, and sodium monohydrogen phosphate. All chemicals used in this study were of reagent grade except for sodium dodecyl sulfate which was ultrapure. The water used in this research was produced from a Millipore Milli-Q™ water purification system (Millipore Corp., Milford, MA, USA).

### 2.2. LPI preparation

Whole green lentil seeds were ground into a fine flour using a food processor (Cuisinart mini-prep plus grinder) (~1 min), followed by an IKA A11 analytical mill treatment (IKA Works Inc., Wilmington, NC, USA) (~1 min). The flour was then defatted by stirring in hexane (1:3 [w/v], flour: hexane) for 40 min; this procedure was repeated two additional times. Protein isolates were prepared from the defatted flour based on methods of Papalamprou, Doxastakis, and Kiosseoglou (2010) and Can Karaca et al. (2011a). In brief, 100 g of defatted flour was mixed with water at a 1:10 (w/v) ratio. The pH of the resulting suspension was adjusted to 9.00 using 1.0 N NaOH followed by mechanical stirring at 500 rpm for 1 h at room temperature (21–23 °C). The mixture was then centrifuged at 5000 ×*g* at 4 °C for 20 min using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC, USA) to collect the supernatant. The resulting pellet was re-suspended in water at a ratio of 1:5 (w/v), adjusted to pH 9.00 using 1.0 N NaOH, stirred for 1 h at room temperature, followed by centrifugation (5000 ×*g*, 20 min, 4 °C). Supernatants were pooled and adjusted to pH 4.50 with 0.1 M HCl to precipitate the protein (Bora, 2002). The LPI was washed with water, frozen at −30 °C and then freeze dried using a Labconco FreeZone 6 freeze drier (Labconco Corp., Kansas City, MO, USA). Proximate analysis of the resulting LPI was conducted according to AOAC Official Methods 925.10 (moisture), 923.03 (ash), 920.85 (lipid), and 920.87 (crude protein by using %N×6.25) (AOAC, 2003). Carbohydrate content was determined on the basis of percent differential from 100%. Proximate analyses were performed on three separate protein isolate preparations with each preparation analyzed in triplicate ($n=3$).

### 2.3. LPI hydrolysis as a function of enzyme/substrate ratio

This study included the preparation of a LPI control, hydrolyzed LPI products, and determination of a total LPI hydrolysis value.

#### 2.3.1. Preparation of the control sample (unhydrolyzed and heat treated)

One hundred milliliters of a 1.0% (w/v) lentil protein isolate in 35 mM sodium phosphate (pH 7.80) was stirred overnight at 4 °C. The resulting solution was transferred to a shaking (90 rpm) water bath (PolyScience, Niles, IL, USA) at 37 °C for 1 h. A 250 μL aliquot of the mixture was removed and added to 2.00 mL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and heated at 85 °C in a water bath for 10 min and then a 250 μL aliquot was taken and added to 2.00 mL of 35 mM sodium phosphate buffer (pH 7.80). This reaction was repeated in triplicate and analyzed employing the TNBS reaction. The LPI control sample is identified as $h_c$ in Eq. (1).

#### 2.3.2. Preparation of trypsin catalyzed protein hydrolysates

One hundred milliliters of a 1.0% (w/v) LPI in 35 mM sodium phosphate (pH 7.80) was stirred overnight at 4 °C. The resulting solution was transferred to a shaking (90 rpm) water bath at 37 °C for 1 h. Trypsin was then added to lentil protein solutions at the following enzyme/substrate (E/S [w/w]) ratios: 1/100, 1/250, 1/500 and 1/1000. A 250 μL aliquot of each E/S ratio experiment was removed at time intervals of 5, 10, 20, 30, 40, 60, 80, 100, 120 min, and were individually added to 2.00 mL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and heated at 85 °C in a water bath for 10 min to quench the hydrolysis reaction and then a 250 μL aliquot was taken and added to 2.00 mL of 35 mM sodium phosphate buffer (pH 7.80). A sample blank consisting of 250 μL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and 2.00 mL of 35 mM sodium phosphate buffer (pH 7.80) was run with each batch of hydrolysis experiments. All partial hydrolysis reactions were performed in triplicate and all samples and blanks were analyzed employing the TNBS reaction. Partially hydrolyzed LPI samples are referred to as $h_t$ in Eq. (1).

#### 2.3.3. Preparation of total protein hydrolysates

Total LPI hydrolysis was performed based the methods of Adler-Nissen (1979), Jung, Murphy, and Johnson (2005) and Barbana and Boye (2011). In brief, ~24 mg of LPI was weighed into a

20×150 mm screw cap Pyrex tube, followed by the addition of 15 mL of 6.0 N HCl. The tube was then flushed with nitrogen gas and sealed. Acid digestion was afforded by heating the sample in a forced-air oven (Yamoto Mechanical Convection DKN600, Yamoto Scientific America, Inc., CA, USA) at 110 °C for 20 h. The pH of the sample solution was adjusted to pH 7.00±0.20 with 2 M NaOH, and was filtered through Whatman Grade 3 filter paper (Whatman International Ltd., Maidstone, UK). Sample aliquots of 250 μL were added to 2.00 mL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and then a 250 μL aliquot was taken and added to 2.00 mL of 35 mM sodium phosphate buffer (pH 7.80). A sample blank was prepared by mixing volumes of 6.0 N NaOH and 6.0 N HCl to obtain a solution with a final pH of 7.00±0.20. All total LPI hydrolysis reactions were performed in triplicate and all samples and blanks were analyzed employing the TNBS reaction. Total LPI hydrolyzed samples are referred to as $h_{tot}$ in Eq. (1).

Trinitrobenzenesulfonic acid (TNBS) reaction: The TNBS reaction was conducted in accordance with the combined methods of Adler-Nissen (1979) and del Toro and Garcia-Carreno (2005) in order to determine the degree of hydrolysis. Sample/blank solutions were transferred to a covered (i.e. protected from light) shaking (90 rpm) water bath maintained at 50 °C for 1 h. To each sample/blank 4.00 mL of 0.1 N HCl was added so as to quench the derivatization reaction. Samples were then cooled to room temperature (~5 min), and their absorbance read at 340 nm (Genesys 10UV Scanning Thermo Scientific, USA). A standard curve was prepared based on the methods of Adler-Nissen (1979) and del Toro and Garcia-Carreno (2005). Briefly, a 35 mM sodium phosphate solution at pH 7.80 containing 1.5 mM glycine was prepared. A range of glycine standards (0.03, 0.04, 0.06, 0.08, 0.10, and 0.30 mM) were prepared from the stock solution by dilution to a final volume of 2.25 mL with 35 mM sodium phosphate solution (pH 7.80). As an example, the 0.03 mM glycine standard consisted of 45 μL of the 1.5 mM glycine stock solution and 2.205 mL of 35 mM sodium phosphate buffer. The sample blank consisted of 0.25 mL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and 2.00 mL of 35 mM sodium phosphate (pH 7.80). All standards and blanks were analyzed in triplicate employing the TNBS reaction.

#### 2.3.4. Degree of hydrolysis calculation

The degree of LPI hydrolysis (%DH) was calculated based on the protocols of Adler-Nissen (1979) and del Toro and Garcia-Carreno (2005) employing the following equations:

$$h = (h_t - h_c) \times DF \quad (1)$$

$$\%DH = \frac{h}{h_{tot}} \times 100 \quad (2)$$

where, $h$ is the yield of hydrolysis equivalents (of α-amino groups formed during the hydrolysis reaction; or α-$NH_2$-Gly equivalent), $h_t$ is the mM concentration of α-$NH_2$-Gly equivalent (obtained using the glycine standard curve) obtained from the trypsin catalyzed protein hydrolysis reactions, $h_c$ is the mM concentration of α-$NH_2$-Gly equivalent from the non-trypsin treated LPI (control), $h_{tot}$ is the mM concentration of α-$NH_2$-Gly equivalent from the total LPI hydrolysis, and *DF* is a dilution factor. The value used for total hydrolysis of the LPI had a mean value of 43.98±3.65 mM ($n=3$).

### 2.4. Preparation of lentil protein hydrolysates for physicochemical and emulsion stability testing

Partial protein hydrolysis was carried out using an E/S ratio of 1:250 (w/w) at times 0 (control), 5, 15 and 30 min, which corresponded to %DH of 0, 4.38, 8.78 and 19.56 (%DH 0, 4, 9 and 20), respectively, as determined by the TNBS method. Un-hydrolyzed (u-LPI) and hydrolyzed LPI (h-LPI) materials were prepared as previously described (Sec 2.3), except with a total volume of 150 mL, and an increased enzyme inactivation time that was based on sample volume. Aliquots of 30 mL were removed from the hydrolysis reaction at the aforementioned designated times, and placed directly in a hot water bath (85 °C) for 20 min to inactivate the enzyme (based on preliminary studies, not shown). Samples were then cooled to room temperature, separated into multiple sample tubes (~3–4 mL), and frozen for later use. Prior to conducting each experiment, sample tubes were removed and left to thaw overnight at 4 °C, and then allowed to warm to room temperature (21–23 °C) prior to analysis. Sample tubes were vortexed for 10 s at speed 4 prior to use. The hydrolysis reaction was conducted three separate times, to enable triplicate measurements to be performed for all treatments and analyses.

### 2.5. Sodium dodecyl sulfide polyacrylamide gel electrophoresis (SDS-PAGE)

The polypeptide profiles of u-LPI and h-LPI samples were determined by sodium dodecyl sulfide polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli (1970) method. Sample concentrations of 0.5% (v/v) (e. g. 75 μL of 1.0% sample in 75 μL water) for both the u-LPI and h-LPI were employed for SDS-PAGE analysis. Briefly, 150 μL of each sample was pipetted into separate Eppendorf tubes followed by the addition of 150 μL of Laemmli sample buffer. Samples were heated to ~95 °C for 10 min and were centrifuged (Eppendorf Centrifuge 5424, Hamburg, Germany) for 5 min at 12,000 ×*g*. Molecular weight standards (Bio-Rad Broad Range Marker) were run in conjunction with samples on a Bio-Rad Tris–HCl gel (15%) at 100–110 V for ~1.5 h. Protein bands were stained using Coomassie blue R-350.

### 2.6. Physicochemical properties of protein hydrolysates

#### 2.6.1. Surface charge (zeta potential or ZP)

Surface charge for u-LPI and h-LPI (%DH 4, 9 and 20) materials were measured at pH 7.80, at a concentration of 0.0625% (w/w) and at room temperature (21–23 °C) using a Zetasizer Nano-ZS90 Instrument (Malvern Instruments, Westborough, MA). The electrophoretic mobility ($U_E$) of the protein solutions was used to calculate the zeta potential ($\zeta$; units: mV) by applying the Henry equation:

$$U_E = \frac{2\varepsilon \cdot \zeta \cdot f(\kappa\alpha)}{3\eta} \quad (3)$$

where $\varepsilon$ is the permittivity (units: F (Farad)/m), $f(\kappa\alpha)$ is a function related to the ratio of particle radius ($\alpha$; units: nm) and the Debye length ($\kappa$; units: $nm^{-1}$), and $\eta$ is the dispersion viscosity (units: mPa s). The Smoluchowski approximation $f(\kappa\alpha)$ was set as 1.5, as is accustom for folded capillary cells and, with particles larger than 0.2 μm dispersed in moderate electrolyte concentration (>1 mM). The Smoluchowski approximation assumes that a) concentration of particles (proteins) is sufficiently high such that such thickness of the electric double layer (Debye length) is small relative to the particle size ($\kappa\alpha \gg 1$); and b) $\zeta$ is linearly related to $U_E$. All measurements are reported as the mean±one standard deviation ($n=3$).

#### 2.6.2. Surface hydrophobicity

Surface hydrophobicity ($S_o$-ANS) for u-LPI and h-LPI (%DH 4, 9 and 20) samples was measured at pH 7.80 using a modified method of Kato and Nakai (1980). In brief, u-LPI and h-LPI (1.0% w/w) solutions were diluted with 35 mM sodium phosphate buffer to give a concentration range of 0.005% to 0.025% (w/w). For instance, 50 μL of the 1% (w/w) h-LPI stock underwent a 40-fold dilution by adding 1.95 mL of buffer to give a final concentration of 0.025% (w/w) (total volume of 2 mL). To 800 μL of these solutions, 8 μL of the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS; 8 mM ANS solution in 35 mM sodium phosphate buffer (pH 7.80)) was added, followed by vortexing for 10 s. A second set of protein solutions was also prepared without the ANS probe.

An ANS blank was also prepared which consisted of 800 μL of buffer and 8 μL of the ANS probe. Samples were kept in the dark for 5 min. Sample net fluorescent intensity (FI) was determined by subtracting the intensity measured for the protein sample without ANS and an ANS blank from that of the sample containing both the protein and ANS. All fluorescence measurements were made using a FluoroMax-4 spectrophotometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with excitation and emission wavelengths at 390 and 470 nm, respectively. Both emission and excitation slit widths were set to 1 nm. The initial slope of the plot of net FI versus % protein concentration was calculated by linear regression analysis and is used as an index of protein surface hydrophobicity. All measurements are reported as the mean ± one standard deviation ($n = 3$).

#### 2.6.3. Intrinsic fluorescence

Changes to the protein's tertiary conformation as a function of %DH was determined for u-LPI and h-LPI samples as a function of emission wavelength (300–400 nm; at 0.5 nm increments) at a constant protein concentration (0.05%, w/w) and excitation wavelength (280 nm) using a FluoroMax 4 spectrofluorometer (Aluko & Yada, 1995). Both excitation and emission slit widths were set at 5 nm. Fluorescent intensity (FI) was reported as a function of emission wavelength and maximum FI value (Klassen & Nickerson, 2012). All measurements are reported as the mean ± one standard deviation ($n = 3$).

#### 2.6.4. Interfacial tension

Interfacial tension between u-LPI and h-LPI solutions (0.25%; w/w, in the 35 mM sodium phosphate buffer (pH 7.80)) and flaxseed oil was measured using a semi-automatic tensiometer (Lauda TD2, Lauda Dr. R. Wobser GmbH & Co., Lauda-Königshofen, Germany) and a Du Noüy ring (Can Karaca et al., 2011a). Interfacial tension for a sample consisting of 35 mM sodium phosphate buffer-flaxseed oil (without protein) served as a control. Interfacial tension was calculated from the maximum force ($F_{max}$; units: milli-Newtons; instrument measures mg × gravity) using the following equation:

$$\gamma = \frac{F_{max}}{4\pi R\beta} \tag{4}$$

where, $\gamma$ is the interfacial tension (mN/m), $R$ is the radius of the ring (20 mm), $\beta$ is a correction factor that depends on the dimensions of the ring and the density of the liquid involved (McClements, 2005). All measurements are reported as the mean ± one standard deviation ($n = 3$).

### 2.7. Emulsifying properties

#### 2.7.1. Emulsion activity (EAI) and stability (ESI) indices

The emulsifying properties (*EAI*, *ESI*) for u-LPI and h-LPI were determined using the modified methods of Pearce and Kinsella (1978). In brief, 5.0 g of 0.25% (w/w) protein solution and 4.0 g of flaxseed oil were homogenized using an Omni Macro Homogenizer (Omni International, Marietta, GA, USA) with a 20 mm saw tooth generating probe at speed 4 (~7,200 rpm) for 5 min. A 50 μL emulsion sample was immediately removed from the bottom of the tube (0 min) and added to 7.5 mL of 35 mM sodium phosphate buffer (pH 7.80) containing 0.1% sodium dodecyl sulphate (SDS). A second 50 μL aliquot of the emulsion (from the bottom of the tube) was also taken at 10 min of static storage at room temperature. Sample absorbance was measured at 500 nm using a Genesys 10 UV-visible spectrophotometer (Thermo Scientific, Madison, WI, USA) using plastic cuvettes (1 cm path length). *EAI* and *ESI* were calculated using the following equations:

$$\mathrm{EAI}\left(\mathrm{m}^2/\mathrm{g}\right) = \frac{2 \cdot 2.303 \cdot A_0 \cdot N}{c \cdot \varphi \cdot 10000} \tag{5}$$

$$\mathrm{ESI}(\mathrm{min}) = \frac{A_0}{\Delta A} \cdot t \tag{6}$$

where, $A_0$ is the absorbance of the diluted emulsion immediately after homogenization, $N$ is the dilution factor, $c$ is the weight of protein per volume (g/mL), $\varphi$ is the oil volume fraction of the emulsion, $\Delta A$ is the change in absorbance between 0 and 10 min ($A_0 - A_{10}$) and $t$ is the time interval, 10 min (Gu, Campbell, & Euston, 2009; Gu, Decker, & McClements, 2005; Guzey & McClements, 2007). All measurements are reported as the mean ± one standard deviation ($n = 3$).

### 2.8. Statistical analyses

A one-way analysis of variance (ANOVA) with a Scheffe post-hoc test was used to determine statistical differences among the physicochemical and emulsifying tests for the u-LPI and h-LPI materials. All statistical analyses were performed with SPSS version 17.0 software (SPSS Inc., 2008, Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Proximate composition of LPI

The mean and standard deviation values for the proximate composition of the LPI were, 87.27 ± 1.66% protein, 2.20 ± 0.20% moisture, 3.25 ± 0.03% ash, 1.12 ± 0.23% lipid and 6.16% carbohydrate; with the three separate preparations yielding a similar composition. Levels were also comparable to what others have reported for LPI produced by isoelectric precipitation (Alsohaimy, Sitohy, & El-Masry, 2007; Can Karaca et al., 2011a; Joshi, Adhikari, Aldred, Panozzo, & Kasapis, 2011). Based on literature reports, the protein concentration of the prepared LPI of 87.27 or 89.23% on a wet or dry weight basis, respectively, supports its identification as an isolate. Currently, there is no universal classification scheme to separate protein concentrates from isolates for all legume proteins (Can Karaca et al., 2011a). However, Pearson (1983) reported that a minimum protein content of 85% on a dry weight basis (6.25 nitrogen conversion factor) was required for a soy isolate designation.

### 3.2. Effect of enzyme/substrate ratio on protein hydrolysis

The degree of lentil protein hydrolysis over a 2 h time period was investigated for different E/S ratios using trypsin. The E/S ratios tested ranged from 1/100 to 1/1000 and was based on those reported in literature for other plant protein-protease reactions (Henning, Mothes, Dudek, Krause, & Schwenke, 1997; Ventureira et al., 2010; Yust, Pedroche, Millan-Linares, & Alcaide-Hidalgo, 2010). Enzyme catalyzed hydrolysis of LPI results showing the concentration of free amino groups ($h$) and corresponding degree of hydrolysis (%DH) as a function of E/S ratio and reaction time are shown in Fig. 1A and B, respectively. At the highest E/S ratio (1/100) studied, LPI hydrolysis occurred rapidly reaching a DH of ~42% within the first 40 min, which increased at a much slower rate to ~57% over the 120 min reaction period (Fig. 1B). At an E/S ratio of 1/250, the initial increase in % DH was slower reaching ~15% DH after 40 min and plateaued at ~31 DH after 60 min (i.e., 60–120 min) (Fig. 1B). At E/S ratios of 1/500 and 1/1000, protein hydrolysis proceeded very slowly over the 120 min reaction time period and reached maximums of ~17 and ~4%, respectively (Fig. 1B). Based on these results, an E/S ratio of 1/250 was selected for LPI hydrolysis as the reaction conditions could be readily controlled to achieve the desired 4–20% DH products. The %DH range selected was hypothesized to produce products that would impact functionality, specifically emulsion formation and stability, in both a positive (% DH < 10) and negative (% DH > 10) manner.

### 3.3. Characterization of LPI hydrolysates

The application of the selected E/S (1/250) ratio on a higher LPI volume (150 mL) gave % DH values for hydrolysis times of 5, 15 and 30 min of, 4.39 ± 1.21, 8.78 ± 1.66, and 19.56 ± 0.71, respectively.

These % DH values were higher than those shown in Fig. 1B and may be explained by the longer time (10 vs. 20 min) associated with trypsin inactivation due to the extra volume of the scaled up reaction.

SDS-PAGE analysis of the u-LPI and h-LPI samples is shown in Fig. 2. Un-hydrolyzed LPI (lane 2) showed multiple major bands between ~80–100 kDa, ~50 kDa, ~31 kDa and ~28 kDa. As the % DH of the h-LPI samples increased from ~4 to ~20% (lanes 3–5) a significant reduction in the ~80–100 kDa molecular weight bands with a concomitant increase in the intensity and/or appearance of protein bands in the ~30–50 kDa and <30 kDa range. Identification of major bands to known molecular weight protein sub-units (e.g., 11S or 7S) could not reliably be matched, since protein structure was already presumed altered during the deactivation of trypsin (i.e., heating to 85 °C for 20 min). These results would support the conclusion that significant changes to the protein structure of LPI occurred upon treatment with trypsin to produce h-LPI. Tryspin catalyzed reactions could influence the quaternary and tertiary conformations of proteins by cleaving peptide bonds within individual or aggregated proteins to give smaller protein sub-units and/or smaller peptides. In addition, only minor differences were evident by SDS-PAGE amongst the three hydrolyzed products (% DH 4–20).

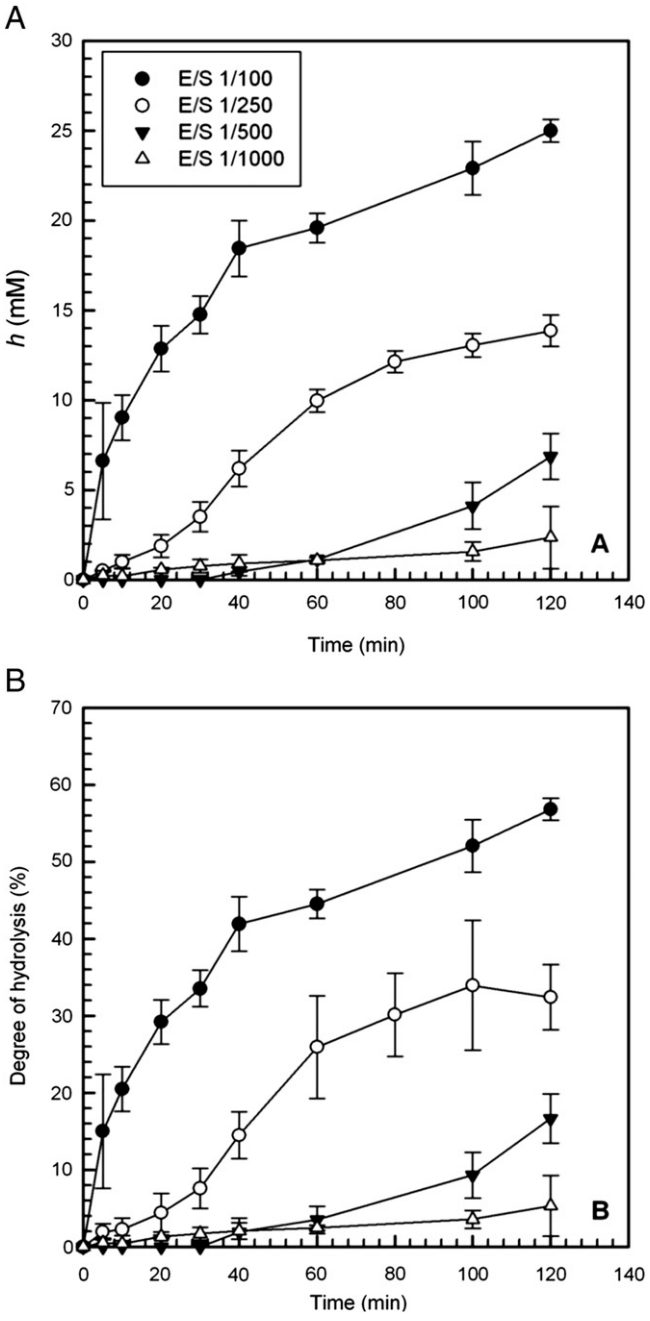


**Fig. 1.** TNBS reaction value associated with the number of free amino groups (*h*; glycine mmoles of free amino acids) (A) and degree of hydrolysis (%) (B) as a function of time (min) and E/S ratio (1:100, 1:250, 1:500 and 1:1000). Data represents the mean ± one standard deviation ($n = 3$).

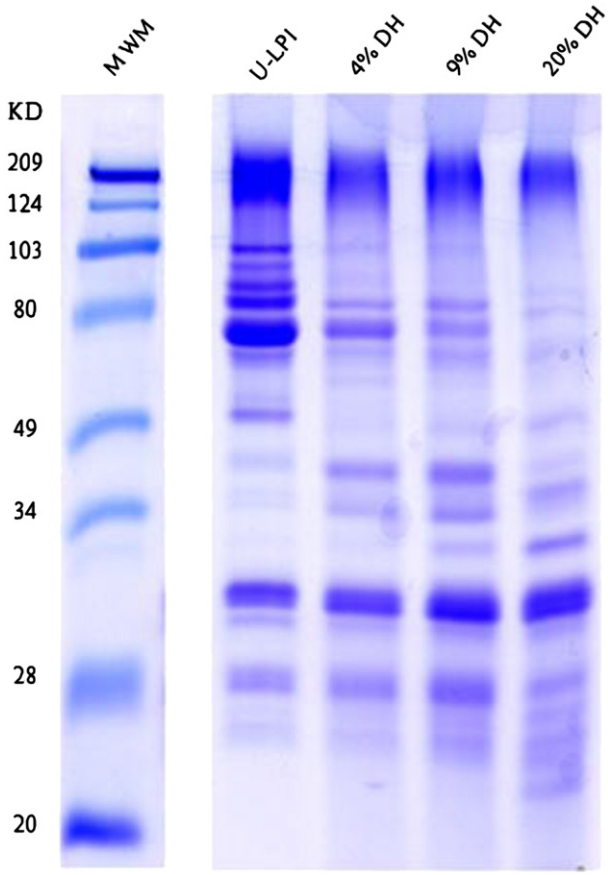


**Fig. 2.** SDS PAGE (non-reducing) of LPI as a function of degree of hydrolysis (%) (Lanes: (1) molecular weight marker (MWM); (2) u-LPI, (3) 4% DH, (4) 9% DH and (5) 20% DH).

### 3.4. Physicochemical properties of u-LPI and h-LPI

#### 3.4.1. Surface characteristics

As a prerequisite for appreciable surface activity, a protein should possess both a charge and patches of hydrophobicity on its surface. A plot of both surface hydrophobicity and charge (zeta potential) of u-LPI and the h-LPI samples as a function of % DH is shown in Fig. 3. The surface charge of all samples, as indicated by their zeta potentials, showed a net negative charge at pH 7.80, which is readily explained by the fact that this pH value was well above the isoelectric point of LPI (pI = 4.5; Bora, 2002). Sample surface charge was found to change only slightly from ~ −35 mV for the u-LPI to ~ −37 mV for h-LPI, but was found to be insignificant ($p > 0.05$). Can Karaca et al. (2011a) reported a zeta potential of −22.6 mV for an unheated and unhydrolyzed LPI at pH 7.00. In this study, the LPI was heated to 37 °C for 1–1.5 h during the hydrolysis process, followed by an additional heating at 85 °C for 20 min during the trypsin inactivation process. It is postulated that these heating steps resulted in protein unravelling which could account for the increased surface charge values obtained in this study when compared to those of Can Karaca et al. (2011a). It has been reported that it is difficult to distinguish the effects of enzymatic hydrolysis, heating and the combination of these processes on the surface properties of proteins (Panyam & Kilara, 1996).

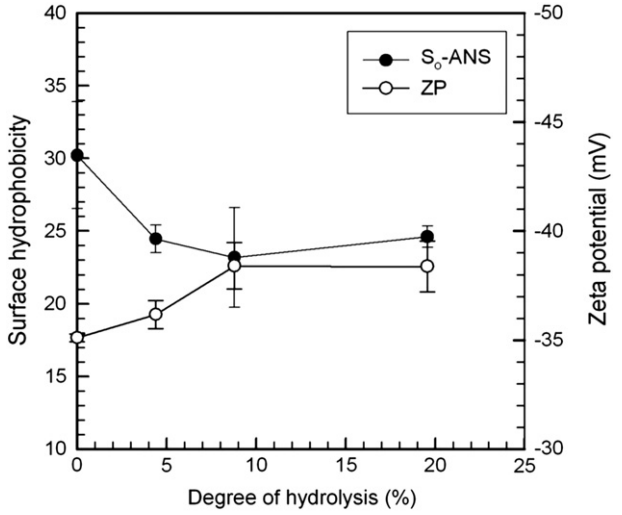


**Fig. 3.** Surface hydrophobicity ($S_o$-ANS) and zeta potential (ZP) for trypsin-treated LPI as a function of degree of hydrolysis (%) at pH 7.80. Data represent the mean ± one standard deviation ($n = 3$).

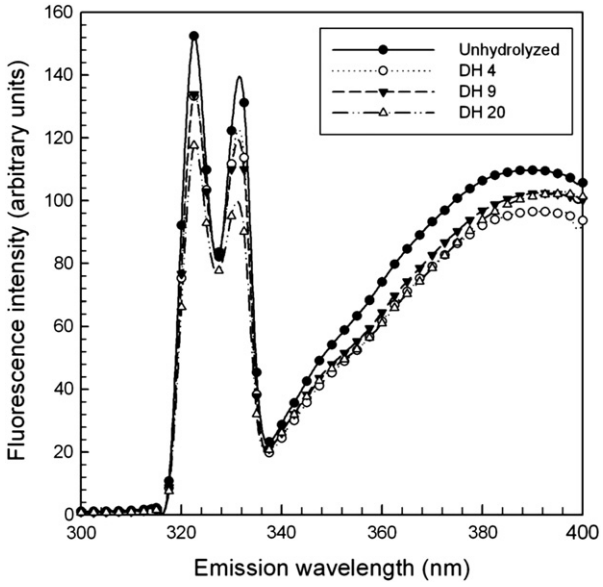


**Fig. 4.** Mean intrinsic fluorescence intensity (arbitrary units) emission scans for trypsin-treated LPI as a function of degree of hydrolysis (%) (at an excitation wavelength of 20 nm) ($n = 3$).

An analysis of variance revealed that the surface hydrophobicity of u-LPI (~30 $S_o$-ANS) was significantly higher than that of h-LPI (~24 $S_o$-ANS) ($p < 0.05$), however no differences were observed as a function of % DH (4–20%) ($p > 0.05$) (Fig. 3). It has been reported that protein surface hydrophobicity is dependent on experimental (i.e. heat/hydrolysis) conditions, protein characteristics (source, amino acid profile) and enzyme specificity; and can either increase or decrease with hydrolysis (Calderon de la Barca, Ruiz-Salazar, & Jara-Marini, 2000). For example, the limited hydrolysis of faba bean legumin with trypsin (Henning et al., 1997) and soy protein isolate with pancreatin (Hettiarachchy & Kalapathy, 1997) lead to a rise in $S_o$-ANS, which was associated with the partial unravelling of the protein's conformation and the cleaving off/or release of small polypeptides/peptides into solution; both of which exposes a greater number of hydrophobic amino groups previously buried within the interior of the protein. In contrast, others have reported a decline in $S_o$-ANS with limited hydrolysis, as was the case in the present study. As examples, a decline in hydrophobicity has been reported for trypsin-treated soy flour (Jung et al., 2005), bromelain-treated soy protein isolate (Ortiz & Wagner, 2002), extruded/un-extruded soy protein concentrate with alcalase and esperase (Surowka, Zmudzinski, & Surowka, 2004) and, an alcalase-treated brewers' spent grain protein concentrate (Celus, Brijs, & Delcour, 2007). Jung et al. (2005) proposed this decline in $S_o$-ANS has been associated with the exposure of buried hydrophobic groups (through partial unravelling or release of polypeptides/peptides), followed by their aggregation via hydrophobic interactions; effectively re-burying them within the interior of a larger aggregated structure. Conversely, Surowka et al. (2004) suggested that the partial unravelling and/or cleaving of polypeptides/peptides upon limited protein hydrolysis could lead to modifications with higher conformational entropy (or flexibility), enabling it to re-fold so that hydrophobic groups are re-buried within the interior of the new structure.

### 3.4.2. Intrinsic fluorescence

Changes in intrinsic fluorescence for u-LPI and h-LPI samples as a function of their degree of hydrolysis were studied over an emission wavelength range of 300–400 nm (Fig. 4). The fluorescent intensities for h-LPI samples (DH 4–20%) were found to be slightly lower than that observed for u-LPI at peak maximums of 322 and 332 nm (Fig. 4). Although this decrease was not significant ($p > 0.05$), the trend supported the $S_o$-ANS data for these samples. A similar trend was found between 340 nm and 400 nm, where the u-LPI material was higher than the h-LPI samples (which were similar). Fluorescence intensity is indicative of the level of exposure of aromatic amino acids (i.e. tryptophan, tyrosine and phenylalanine) to the reaction solvent, a phenomenon which is highly sensitive to a protein's tertiary conformation in solution and the neighbouring amino acids to the aromatic groups (Kronman & Holmes, 1971; Pain, 2005). The findings in the present study differ from what would typically be expected, where enzymatic hydrolysis would lead to increased exposure of buried aromatic groups to the solvent and a higher fluorescent intensity (Pain, 2005). However, a decrease in intensity would be expected if hydrolysis lead to first the exposure of aromatic groups, followed by protein aggregation (via hydrophobic interactions) which then re-buried the groups within the interior of the protein cluster; or if hydrolysis led to increased protein flexibility, which upon re-folding influenced aromatic site exposure. A similar decline in fluorescence intensity with limited enzymatic hydrolysis has been previously reported for broad bean legumin-trypsin (Braudo et al., 2006) and peanut protein-alcalase-trypsin (Zhao, Liu, Zhao, Ren, & Yang, 2011).

### 3.4.3. Interfacial properties

Changes to the interfacial tension of LPI as a function of % DH in a flaxseed oil–water mixture is shown in Fig. 5. An analysis of variance revealed that the interfacial tension of u-LPI (~6.5 mN $m^{-1}$) was significantly greater than that of h-LPI (~6.1 mN $m^{-1}$) ($p < 0.05$), however no differences were observed in samples as a function of % DH ($p > 0.05$) (Fig. 5). In the absence of LPI, the measured interfacial tension between the oil and water phases was 7.65 mN $m^{-1}$. The reduced interfacial tension in the presence of h-LPI relative to u-LPI may be due to the ability of the modified proteins to align with, and integrate into, the oil–water interface. Based on hydrophobicity data, it was proposed that hydrolysis of LPI lead to an initial exposure of hydrophobic groups, followed by their aggregation (via hydrophobic interactions) to form larger aggregates. Consequently, surface hydrophobicity was less for h-LPI than u-LPI materials. Interfacial tension was lower for h-LPI material possibly because of greater solubility (less hydrophobic) in the aqueous phase allowing for greater rates of diffusion to the interface. Surface charge was similar between u-LPI and h-LPI materials, therefore was not considered to be a major determinant in interfacial tension.

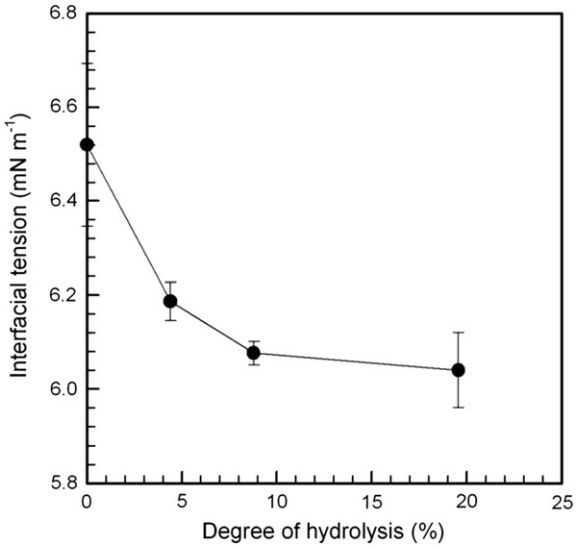


**Fig. 5.** Interfacial tension (mN m$^{-1}$) for trypsin-treated LPI as a function of degree of hydrolysis (%) at pH 7.80. Data represent the mean ± one standard deviation ($n = 3$).

### 3.5. Emulsifying properties

The emulsification activity and stability indices for LPI as a function of the % DH are shown in Fig. 6. Protein hydrolysis was found to have a negative effect on its emulsifying properties in a flaxseed oil–water system, where the EAI and ESI values declined from ~51 to ~47 m$^2$ g$^{-1}$ ($p<0.05$) and ~12 to ~11 min ($p<0.001$), respectively, for u-LPI relative to h-LPI. No significant differences were observed for either EAI or ESI as a function of % DH over the range studied ($p>0.05$) (Fig. 6). Similar results were reported by Zhao et al. (2011), where EAI values for native peanut proteins were greater than those that had undergone limited hydrolysis by alcalase. In literature, the effect of limited hydrolysis on the emulsifying properties of a protein seems to be related to both the system and extent of hydrolysis. Govindaraju and Srinivas (2006) and, Severin and Xia (2006) both reported improved emulsifying properties (i.e., capacity) for enzymatically hydrolyzed arachin protein (major oilseed protein of groundnuts) and whey proteins, respectively at low % DH, after which continued hydrolysis had a negative effect on emulsification. Severin and Xia (2006) proposed this phenomenon was related to the molecular weight distribution of peptides produced during the hydrolysis process. The initially improved emulsifying properties with increased levels of DH was presumed related to a steady decrease in molecular weight of the peptides generated, enabling greater alignment at the oil–water interface. This continued until an optimal molecular weight distribution was reached, afterwards continued hydrolysis led to reduced emulsifying properties. The authors proposed the decline could be attributed to the formation of more hydrophilic peptides that only weakly associated with the oil–water interface, or the viscoelastic film formed at the interface with the smaller peptides was insufficient to resist coalescence of neighboring droplets. In the present study, h-LPI showed reduced hydrophobicity and increased surface charge relative to the u-LPI material (Fig. 1); therefore may have greater difficulty aligning at the oil–water interface.

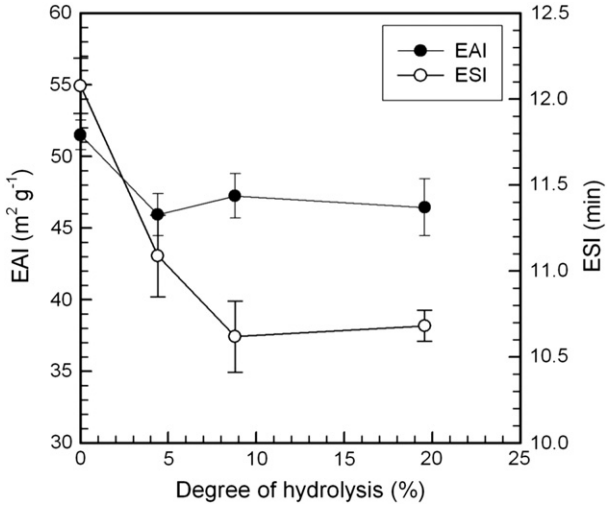


**Fig. 6.** Emulsion activity (EAI, m$^2$ g$^{-1}$) and stability (ESI, min) indices for trypsin-treated LPI as a function of degree of hydrolysis (%) at pH 7.80. Data represent the mean ± one standard deviation ($n = 3$).

## 4. Conclusions

Overall, trypsin catalyzed hydrolysis of LPI yielding % DH of 4–20 produced materials with reduced surface hydrophobicity ($S_o$-ANS) and intrinsic fluorescence relative to u-LPI. These surface characteristic changes are postulated to be due to the initial exposure of reactive aromatic amino acids, which is followed by protein aggregation to re-bury these hydrophobic sites. The observed reduction in surface hydrophobicity for h-LPI materials was thought to play a key role in reducing their emulsifying properties relative to u-LPI. No differences in physicochemical and emulsifying properties were observed among the three h-LPI (4%, 9% and 20%) materials studied. Findings from this study suggest that an unhydrolyzed LPI could be effectively used as plant-based food emulsifiers.

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