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Effect of pH on the inter-relationships between the physicochemical, interfacial and emulsifying properties for pea, soy, lentil and canola protein isolates

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ABSTRACT

The inter-relationships between the physicochemical, interfacial and emulsifying properties for pea, soy, lentil and canola protein isolates as a function of pHs (3.0, 5.0, and 7.0) were investigated. Surface charge, hydrophobicity, solubility, interfacial tension, rheology, droplet size and emulsion stability were all studied. Conditions that favored the protein to have a high charge, low hydrophobicity and high solubility (pH 7.0) were better able to associate with the oil–water interface to lower interfacial tension. However, conditions that fostered the protein to have a high charge, high hydrophobicity and high solubility (pH 3.0) led to stronger interfacial viscoelastic films. Findings suggest that a balance of the surface active properties is most ideal for using plant protein emulsifiers in a food application. Overall, findings from this study indicated that all proteins could form stable emulsions away from its isoelectric point (pH 3.0 or pH 7.0), although the ones formed at pH 3.0 displayed much better interfacial rheology. Of the protein-types studied, the most promising alternative to soy protein isolate as an emulsifier was lentil protein isolate because it had high charge, solubility and hydrophobicity at pH 3.0. The low solubility of pea protein at acidic pH could cause sedimentation issues in products, whereas allergen concerns are still associated with the napin protein from canola.

1. Introduction

Food emulsions are mixtures of two (or more) immiscible liquids (e.g., oil and water), where one liquid is dispersed as droplets within a continuous phase of the other, formed in the presence of emulsifiers (e.g., proteins) under mechanical shear (McClements, 2005). Although the emulsifying properties of plant proteins have been previously studied (Adebiyi & Aluko, 2011; Avramenko, Low, & Nickerson, 2013; Barac et al., 2010; Can Karaca, Low, & Nickerson, 2011a; Cheung, Wanasundara, & Nickerson, 2014; Liang & Tang, 2013), little information is available relating to how the surface properties of a protein, their interfacial characteristics (e.g., interfacial tension and interfacial rheology), and their ability to stabilize an emulsion interrelate. Knowledge of these inter-relationships involving plant proteins may be useful in emulsion preparation, prediction of long-term stability, and quality control in food products.

Plant protein-based emulsifiers typically involve the use of soy protein products (e.g., concentrates or isolates), however because of allergen concerns industry is searching for other alternatives. Some emerging protein ingredients include those derived from pea, lentil and canola. Soy, pea and lentil proteins are all dominated by 11S (S denotes a Svedberg unit; molecular mass ~350 kDa) and 7S (molecular mass ~150–180 kDa) globulin-type storage proteins (Boye et al., 2010). In soy, these are known as glycinin and β-conglycinin, respectively, whereas in pea and lentil these are referred to as legumin and vicilin, respectively. In contrast, canola proteins are dominated by a 12S globulin protein (known as cruciferin, molecular mass ~300 kDa) and a 2S albumin protein (known as napin, molecular mass ~14–16 kDa) (Wanasundara, 2011). Similar to other protein based emulsifiers, all of these proteins act by diffusing to the interface, re-orienting to become better integrated with the interface and then form a viscoelastic film to stabilize the oil droplets by either charge repulsion at pHs away from the protein's isoelectric point (pI) or through steric stabilization (Tcholakova, Denkov, Ivanov, & Campbell, 2006; Morris & Gunning, 2008).

The overall goal of this study was to investigate the effect of pH on the physicochemical, interfacial, and emulsifying properties of pea, soy, lentil and canola protein isolates separately, in order to elucidate potential inter-relationships that exist to better tailor their use in the future.

2. Materials and methods

2.1. Materials

Pea (Propulse™) (PPI), lentil (LPI) and soy (PRO-FAM 974) (SPI) protein isolates were kindly donated by Nutri-Pea Limited (Portage la Prairie, MB, Canada), POS Bio-Sciences (Saskatoon, SK, Canada) and Archer Daniels Midland Company (Decatur, IL, USA), respectively.

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Canola seeds (*Brassica napus*/variety VI-500) were kindly donated by Viterra (Saskatoon, SK, Canada) for use in preparation of the canola protein isolate (CPI). According to the Association of Official Analytical Chemists Method 920.87 (AOAC, 2003), the crude protein contents of PPI, SPI and LPI were determined to be 78.30% (wet basis, w.b.), 94.87% w.b., and 79.36% w.b. (%N × 6.25), respectively. Concentrations used in this study reflect the protein contents rather than powder weight. Canola oil used in this study was purchased from a local supermarket. Milli-Q water was obtained from a Millipore Milli-Q™ water purification system (Millipore Corporation, Milford, MA, USA).

2.2. Preparation of a canola protein isolate

Prior to use, canola seeds (stored at 4 °C in a sealed container) were initially screened using a #8 (2.63 mm) followed by a #12 (1.70 mm) Tyler mesh filters (Tyler, Mentor, OH, USA) to remove smaller seeds. The screened seed was then frozen at −40 °C for overnight before the cracking by using a stone mill (Morehouse-Cowles stone mill, Chino, CA, USA). The cotyledons were separated from the seed coat using an air classifier (Agriculex Inc., Guelph, ON, Canada). The cotyledons oil (~13%) was extracted using a continuous screw expeller (Komet, Type CA59 C; IBG Monforts Oekotec GmbH & Co., Mönchengladbach, Germany) at 59 rpm with a 3.50 mm choke. The hexane extraction (×3) at 1:3 meal to hexane ratio for 8 h was used to remove the residual oil from canola meal, and the meal was then air-dried for another 8 h to evaporate the residual hexane to prepare defatted canola meal.

CPI was extracted from the defatted canola meal according to Klassen, Elmer, and Nickerson (2011). In brief, defatted canola meal and 0.05 M Tris–HCl buffer (pH = 7.0) containing 0.1 M NaCl were mixed at 1:10 ratio and mechanically stirred at 500 rpm (IKAMAG RET-G, Janke & Kunkel GmbH & Co. KG, IKA-Labortechnik, Germany) for 2 h under room temperature (21–23 °C). The supernatant was then separated from the mixture using a centrifuge (Sorvall RC Plus Superspeed Centrifuge, Thermo Fisher Scientific, Asheville NC, USA) at 3000 × *g* for 1 h, followed by the filtration using #1 Whatman filter paper (Whatman International Ltd., Maidstone, England), and dialysis with Spectra/Por molecular porous membrane tubing (6–8 kDa cut off, Spectrum Medical Industries, Inc., USA) at 4 °C for 72 h in a fresh Milli-Q water (Millipore Corporation, MA, USA) environment to remove the salt. The dialyzed solution was then centrifuged (×2) at 3000 ×*g* for 1 h to collect the sediments. Finally, the sediments were freeze-dried (Labconco Corporation, Kansas City, Missouri 64132) for 24 h under a temperature difference of 35 °C to produce the CPI for the later experiments. The crude protein content of CPI was measured to be 99.11% w.b. (%N × 6.25).

2.3. Sample preparation

Protein solutions (0.05%, 0.1%, and 2.0%, w/w) were prepared by dissolving protein powders (PPI, SPI, LPI, and CPI) into Milli-Q water. The solution pH was adjusted to pH 3.0, pH 5.0, and pH 7.0 using either 1.0 M HCl or 1.0 M NaOH. The protein solutions were then mechanically stirred at 500 rpm for 2 h at room temperature (21–23 °C).

2.4. Zeta potential

A Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA) was used to measure the electrophoretic mobility (U_E) of the protein isolate solutions (0.05%, w/w), and the zeta potential (ζ, mV) was determined as a function of pH and protein type through Henry's equation (Eq. (1)):

$$U_E = \frac{2\varepsilon \cdot \zeta \cdot f(\kappa\alpha)}{3\eta} \quad (1)$$

where, ε (Farad/m) is the permittivity, $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α, nm) and the Debye length (κ, nm^{-1}), and η (mPa·s) is the dispersion viscosity (constant at 1.002 mPa·s). For this study, the Smoluchowski approximation $f(\kappa\alpha)$ equaled to 1.5 as is typically done when using folded capillary cells with point scatters larger than 200 nm in a dispersant with electrolyte concentrations of >1 mmol/L. This approximation assumes that the point scatters (i.e., the protein) is at high enough levels so that the Debye length (or thickness of the electric double layer) is small relative to the particle size ($\kappa\alpha \gg 1$). The approximation also assumes that the zeta potential is linearly related to the electrophoretic mobility. Measurements were made in triplicate, and reported as the mean ± one standard deviation (n = 3).

2.5. Solubility

Solubility was investigated as a function of pH for all isolates using the modified technique of Morr et al. (1985). In brief, a 2.0% (w/w) protein solution was transferred to a 15 mL centrifuge tube and centrifuged (Clinical 200, VWR International, Germany) at 9100 ×*g* for 10 min at room temperature (21–23 °C) to remove insoluble residues. Protein solubility was calculated based on the protein content in the supernatant divided by the protein content in the original protein sample. All measurements are reported as the mean ± one standard deviation ($n = 3$).

2.6. Surface hydrophobicity

Surface hydrophobicity for all protein isolates was measured as a function of pH using the modified method described by Kato and Nakai (1980), based on the interaction between 8-anilino-1-naphthalenesulfonate (ANS) probe and hydrophobic moieties on the protein's surface to give a fluorescent signal. In brief, the stock protein solution (0.01%, w/w) was diluted to 0.002% (w/w), 0.004% (w/w), 0.006% (w/w), and 0.008% (w/w) with Milli-Q water. 20 μL of 8 mM ANS solution was mixed with 4 mL of protein solutions by vortexing (S/P Vortex Mixer, Baxter Diagnostics Inc., USA) for 10 s, and kept in the dark for 15 min. 4 mL of protein solutions with 20 μL of Milli-Q water mixture were used as controls, and 4 mL of Milli-Q water with 20 μL of 8 mM ANS mixture was used as a blank and kept in the dark for 15 min before the test. Fluorescence intensity (FI) of samples was measured using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with the excitation wavelength at 390 nm and the emission wavelength at 470 nm at a slit width of 1 nm. Net FI was calculated by subtracting FI values for the control and blank samples from the FI value for the mixture of protein solutions with ANS. An index of relative surface hydrophobicity for the protein isolate was presented as the initial slope of the net FI versus protein concentration of the protein solutions. All measurements were reported as the mean ± one standard deviation ($n = 3$).

2.7. Interfacial tension

Interfacial tension between protein solutions (2.0%, w/w) and canola oil was determined as a function of pH using a semi-automatic tensiometer (Lauda TD2, GmbH & Co., Lauda-Königshofen, Germany) with a Du Noüy ring (20 mm diameter). In brief, a 20 mL protein solution was added into the glass sample cup (57 mm diameter), and then the Du Noüy ring was lowered into the protein solution, followed by the addition of canola oil (20 mL). The maximum force measured while the ring was pulling upwards to stretch the oil–protein interface without breaking the interface was recorded. Three consecutive maximum force readings were made on each time of interface stretching at 3 min intervals, and the measurement was stopped until the standard deviation lower than 0.10 mN/m. The interfacial tension was then calculated from the maximum force (F_{max}) using the following formula:

$$\gamma = \frac{F_{max}}{4\pi R\beta} \quad (2)$$

where γ is the interfacial tension (mN/m); R is the radius of the ring (10 mm); β is a correction factor that depends on the dimensions of the ring and the density of the liquid involved. All measurements were reported as the mean ± one standard deviation ($n = 3$).

2.8. Interfacial rheology

Oscillatory interfacial dilatational rheological properties of all isolates as a function of pH were evaluated using a rheometer (AR-G2 Rheometer, TA Instruments Ltd., New Castle, DE, USA). The interface between a protein solution (2.0%, w/w) and canola oil was subjected to be an infinitesimal sinusoidal compression and expansion surface area, and the rheological behavior of the interface was characterized by measuring the interfacial storage modulus [G_i' (Pa)] and the loss modulus [G_i'' (Pa)] through time and frequency sweep tests. The overall response of the sample against the interfacial deformation was expressed as complex modulus (G_i^*) that was calculated by the following formula:

$$G_i^* = \sqrt{(G_i')^2 + (G_i'')^2}. \quad (3)$$

A bicone geometry (diameter = 68 mm, angle = 10°) and a polytetrafluoroethylene (PTFE) cup (inner diameter = 80 mm, depth = 45 mm) were used for the experiment. The protein solution was poured into the PTFE cup at the height of 19,500 μm, followed by immersing the bicone geometry into the solution without touching the bottom. Then, the similar amount of canola oil was added to cover the exposed protein solution surface. The interface location was determined by the dramatic normal force drop while the geometry moving upwards to 15,000 μm relative to the original position. Time sweep test was carried out with the controlled strain (0.1%) and frequency (0.1 rad/s) at room temperature (21–23 °C) for 30 min to determine the formation of viscoelastic film at interface in 30 min. This was then followed immediately by a frequency sweep test over a frequency range (0.1–10 rad/s) with controlled strain (0.1%) at room temperature (21–23 °C) on the same sample to measure the strength of the viscoelastic film at interface.

2.9. Emulsion preparation

Oil-in-water emulsions were prepared by homogenizing 2.0% (w/w) protein solutions with canola oil. In brief, 9 g of 2.0% (w/w) protein solutions and 1 g of canola oil were homogenized using an Omni Macro Homogenizer (Omni International Inc., Marietta, GA, USA.) equipped with a 20 mm saw tooth at speed 4 (~7200 rpm) for 5 min to prepare 10.0% (w/w) oil-in-water emulsions in a 50 mL plastic centrifuge tube.

2.10. Droplet size distribution

Droplet size distribution of freshly prepared emulsions was determined using a Mastersizer 2000 laser light scattering instrument (Malvern Instruments Ltd., Worcestershire, UK) with a Hydro 2000S sample handling unit as described by Can Karaca et al. (2011a,b). The droplet size distribution was measured immediately after the emulsion samples were prepared. Distilled water was used as the dispersant in the sample handling unit, and the obscuration was brought up to ~14% by sample addition. The relative refractive index of emulsion, which is the ratio of the refractive index of canola oil (1.470) to the refractive index of the dispersant (1.330) was 1.105. The droplet size was reported as surface-average diameter ($d_{3,2}$) that is expressed as:

$$d_{3,2} = \frac{\sum_{i=1} n_i \cdot d_i^3}{\sum_{i=1} n_i \cdot d_i^2} \quad (4)$$

where n_i is the number of droplets of diameter (d_i) (McClements, 2005).

2.11. Emulsion stability

Emulsion stability (ES) was determined according to Liu, Elmer, Low, and Nickerson (2010) with minor modifications. In brief, homogenized samples (10 mL) were immediately filled into a 10 mL sealed graduated glass cylinders (inner diameter = 10.5 mm, height = 160 mm), and then stored for 24 h at room temperature. During storage, the emulsions separated into a cream upper layer and a serum bottom layer which included protein sediments. Emulsion stability was measured as ES (%) and expressed as:

$$ES(\%) = H_S/H_E \times 100 \quad (5)$$

where H_S is the height of the serum layer, and H_E is the height of the emulsion, as measured using a digital micrometer (Model 62379-531, Control Company, USA) having a precision of 0.01 mm. Fig. 1 gives an image of a CPI stabilized emulsion at pH 3.0 immediately after homogenization (A) and after 24 h storage (B). Fig. 1C shows an image of a destabilized CPI emulsion found to occur at pH 7.0 (see Results and discussion section).

2.12. Statistics

All experiments were performed in triplicate and reported as the mean ± one standard deviation. A two-way analysis of variance (ANOVA) was used to measure statistical differences in physicochemical properties, interfacial properties, droplet size distribution, and emulsion stability as a function of pHs (3.0, 5.0, and 7.0) and protein types (PPI, SPI, LPI, and CPI). A simple Pearson correlation was calculated to describe the relationship between different properties [i.e., solubility, charge (absolute value), hydrophobicity, interfacial tension, interfacial complex modulus, droplet size and emulsion stability] of all protein isolates as a function of pH. All statistics were analyzed using Systat 10.0 software (Systat Software, Inc., Chicago, IL).

3. Results and discussion

3.1. Physicochemical properties

Physicochemical properties, such as charge, hydrophobicity and solubility are important factors contributing to the diffusion and association of the plant proteins to an oil–water interface. Protein charge, hydrophobicity and solubility for all protein isolates (e.g., PPI, SPI, LPI, and CPI) were measured as a function of pH (Fig. 2A–C). An analysis of variance indicated that all physicochemical properties were affected by pH and protein-type, along with their interaction ($p < 0.001$). A Pearson correlation found that solubility was strongly associated with charge ($r = 0.711$; $p < 0.001$) indicating that more highly charged proteins were more soluble.

At pH 3.0, all isolates had similar net positive charges (~+32.5 mV) (Fig. 2A). Hydrophobicity at this pH was the highest relative to other pHs for all isolates, with LPI showing the greatest hydrophobicity followed by SPI, CPI and then PPI (Fig. 2B). It is presumed that the dissociation of protein subunits at pH 3.0 might contribute to the higher hydrophobicity relative to the other pHs. Differences in hydrophobicity among the proteins studied are hypothesized to reflect inherent differences in protein composition (e.g., percentage of 11S vs 7S proteins, or ratio of globulin and albumin proteins). For instance, globulin proteins tend to be more hydrophobic than albumins (Papalamprou, Doxastakis, Biliaderis, & Kiosseoglou, 2009), and 11S proteins are more hydrophobic than 7S proteins (Liang & Tang, 2013). In the case of solubility, all isolates were found to be the highest and similar in magnitude at pH 3.0 and pH 7.0, with the exception of CPI at the latter pH (Fig. 2C). At pH 3.0, CPI showed the highest protein solubility, followed by SPI, LPI, and PPI (Fig. 2C).

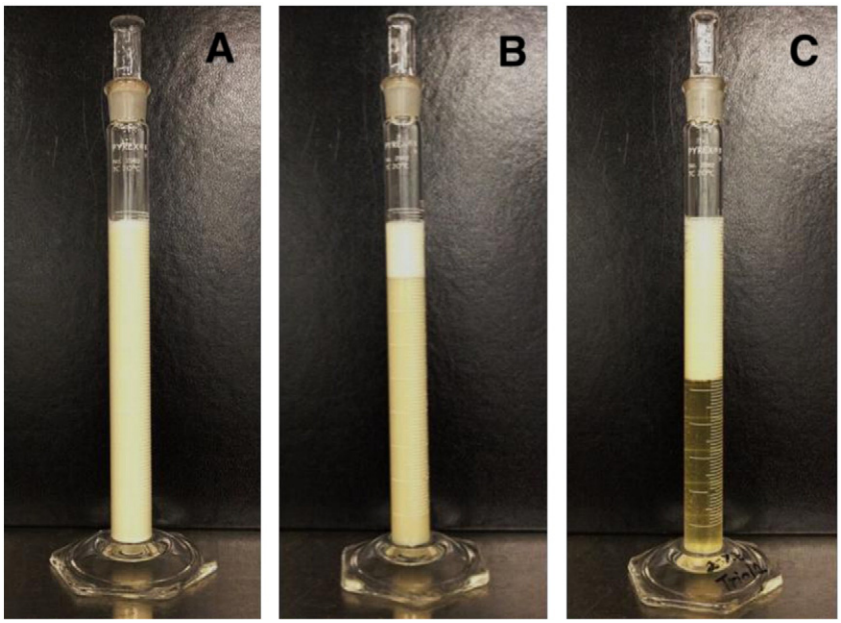

Fig. 1. An image of a freshly prepared oil-in-water emulsion stabilized with a canola protein isolate (A — regardless of the pH), and that after a 24 h storage period for an emulsion prepared at pH 3.0 (B) and pH 7.0 (C).

At pH 5.0, there were almost no net charges (~±5 mV) for PPI, SPI, and LPI indicating that proteins were near the pI values. In contrast, at pH 5.0 CPI carried a net charge of ~+18.0 mV (Fig. 2A). Net neutrality for CPI occurred near pH 6.2 (Fig. 2A). Hydrophobicity was also found to be reduced at pH 5.0 relative to pH 3.0, and followed a similar trend in terms of protein-type at pH 3.0 (Fig. 2B). Due to the reduced surface charge, protein–protein interactions dominated leading a reduction in surface hydrophobicity (i.e., hydrophobic moieties on the surface of smaller individual proteins become buried again as larger aggregates) and a minimal solubility of ~4% for SPI, LPI and PPI at pH 5.0. In the case of CPI, solubility remained near ~37% since it was still away from its pI value of 6.2.

At pH 7.0, all isolates carried a net negative charge which differed depending on the protein-type. SPI was found to display the greatest charge, followed by LPI, PPI and CPI (Fig. 2A). With the exception of CPI, all other proteins were away from their pI value. Overall hydrophobicity was found to be the lowest at pH 7.0 for all isolates relative to the other pHs, with the exception of CPI which was slightly higher than at pH 5.0. At pH 7.0, all isolates were of similar magnitude (±2 arbitrary units) (Fig. 2B). Hydrophobicity values may have been lowered at pH 7.0, because both the isolates and ANS probes carried a net negative charge. The net electrostatic repulsive forces in the solution may disrupt the interaction between aromatic moieties on protein isolates with the ANS probe to give poorer estimates of the true value (Alizadeh-Pasdar & Li-Chen, 2000). Solubility at pH 7.0 was similar to that of pH 3.0 for all isolates with the exception of CPI which was at its lowest (~23%) (Fig. 2C). Although not measured, it is presumed that solubility would be minimal for CPI at pH 6.2, which corresponds to where its surface charge was neutral.

Overall, solubility is dependent upon the balance between protein–protein and protein–solvent interactions. A Pearson correlation found that solubility was strongly associated with charge ($r = 0.711$; $p < 0.001$) indicating that more highly charged proteins (whether negative or positive) were more soluble, and that hydrophobicity was not strongly linked to solubility ($r = 0.320$; $p > 0.05$), although hydrophobic interactions are expected to play an important role in stabilizing protein–protein aggregates as they form under more neutral conditions.

3.2. Interfacial properties

During emulsion formation, proteins migrate and accumulate at the oil–water interface to lower the interfacial tension (Damodaran, 1996). Changes to the interfacial tension as a function of pH for all isolates is given in Fig. 3. An analysis of variance indicated that both pH ($p < 0.001$) and protein-type ($p < 0.001$), along with their interaction ($p < 0.05$) were significant. Overall, the addition of isolates into the aqueous phase at all pHs was found to lower the interfacial tension from ~22.5 mN/m (control, no proteins) to 8–16 mN/m. The ability for all proteins to lower the interfacial tension was similar at pH 3.0 and pH 5.0 regardless of their differences in physicochemical properties, however they significantly improved at pH 7.0 (Fig. 3). Furthermore at each pH, PPI was the most effective at reducing interfacial tension, followed by LPI and SPI which were similar, and then by CPI which was the least effective (Fig. 3). In the present study, interfacial tension was negatively correlated with surface charge ($r = -0.372$; $p < 0.05$) and positively correlated with surface hydrophobicity ($r = 0.494$; $p < 0.01$). This suggests that the effectiveness of the protein to reduce the interfacial tension (i.e., lower values) is better when the protein carries a higher charge and reduced hydrophobicity. However, it's important to note that reduced hydrophobicity does not mean any surface activity. No correlation was seen between solubility and interfacial tension ($r = 0.260$; $p > 0.05$) was found. It is hypothesized that interfacial tension is more related to an optimum balance between charge and hydrophobicity on the protein.

At pH 3.0, proteins have both high charge and hydrophobicity, and therefore were only moderately able to reduce interfacial tension driven most likely by the charge on the proteins. In contrast, at pH 5.0 charges were reduced to neutrality for the legume proteins (SPI, PPI and LPI) and to low levels for CPI, whereas hydrophobicity was also reduced to more moderate levels. In this case, interfacial tension was able to be reduced moderately driven most likely by the lower hydrophobicity on the proteins. At pH 7.0, proteins were more effective at reducing interfacial tension, since hydrophobicity was generally lower and charge was again high. In all cases, interfacial tension was lowered by both soluble and insoluble protein dispersed in the aqueous medium, in which the ratio of soluble-to-insoluble proteins would be pH dependent. For

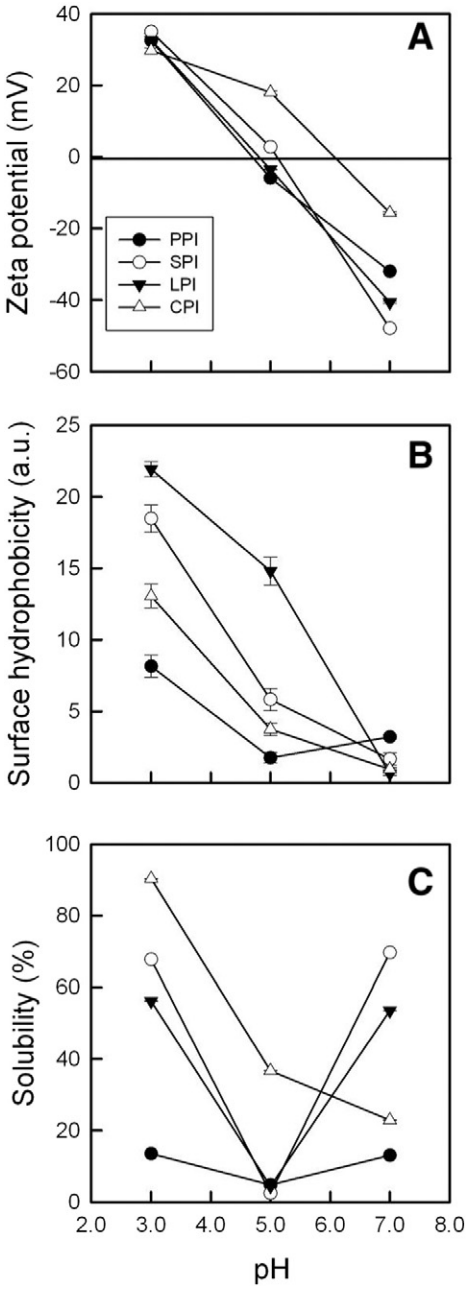

Fig. 2. Zeta potential (mV) (A), surface hydrophobicity (arbitrary units, a.u.) (B), and solubility (%) (C) for protein solutions as a function of pH and protein-type. Data represent the mean ± one standard deviation ($n = 3$). Abbreviations include: pea protein isolate (PPI), soy protein isolate (SPI), lentil protein isolate (LPI) and canola protein isolate (CPI).

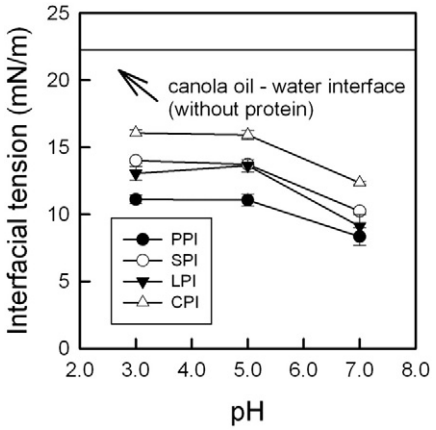

Fig. 3. Interfacial tension (mN/m) for protein solutions as a function of pH and protein-type at a canola oil–water interface. Data represent the mean ± one standard deviation ($n = 3$). Abbreviations include: pea protein isolate (PPI), soy protein isolate (SPI), lentil protein isolate (LPI) and canola protein isolate (CPI).

instance, at pH 5.0 the legume proteins were not very soluble (Fig. 2C) however they were still effective at reducing interfacial tension by the small amount of soluble protein and the insoluble protein that did not sediment yet in the time frame of the experiment. Lam and Nickerson (2014) also reported the interfacial tension of β-lactoglobulin at pH 3.0 (~17.8 mN/m) to be similar at pH 5.0 (~18.6 mN/m), even though they had large differences on the physicochemical properties.

Interfacial rheology is an important physical parameter related with the long-term stability of emulsions stabilized by proteins (Bos & Van Vliet, 2001). The dynamic interfacial storage modulus (G_i') and loss modulus (G_i'') of interfacial layers of SPI with time and frequency sweep as a function of pH are presented in Fig. 4. All other proteins (PPI, LPI, and CPI) followed a similar trend except for differences in magnitude (not shown). For time sweep data, G_i' and G_i'' showed a slight upward and downward trend, respectively over time, suggesting that SPI at pH 3.0 and pH 5.0 (Fig. 4A, C) reached the interface relatively quickly, and formed a viscoelastic film as evidenced by $G_i' > G_i''$. At the interface, it is presumed that protein–protein interactions and the rearrangement of the protein's tertiary structure lead to the formation of an intermolecular network to keep G_i' and G_i'' at the equilibrium state (Ruiz-Henestrosa, Carrera Sanchez, & Rodriguez Patino, 2008). However, for SPI at pH 7.0, $G_i' < G_i''$ indicated that no protein network was formed at the interface (Fig. 4E).

Once the protein isolates were absorbed and attained the equilibrium states at the interface, the strength of the viscoelastic protein film at the interface was investigated as a function of frequency (Fig. 4B, D and F at pH 3.0, pH 5.0, and pH 7.0, respectively). G_i' and G_i'' for SPI at both pH 3.0 and pH 5.0 were relatively constant as a function of frequency, until a cross-over point was reached at ~2.24 rad/s, after which $G_i' < G_i''$ and the viscoelastic film began showing rubbery-like behavior where G_i'' increases and G_i' starts to decrease suddenly (Fig. 4B, D). At pH 7.0, no film network was formed at the interface leading to fluid-like rheological behavior of the protein network at the interface ($G_i' < G_i''$) (Fig. 4F). It is surmised that SPI at pH 3.0 and pH 5.0, PPI at pH 3.0, LPI at pH 3.0 and pH 5.0, and CPI at pH 3.0 and pH 5.0 (data were not shown) could form the viscoelastic films at the interface which is significant for the long-term stability of the emulsions, and the strength of the viscoelastic films as a function of pH and protein-type at 1 rad/s was evaluated in Table 1. An analysis of variance of complex modulus (G_i^*) data at a frequency of 1 rad/s indicated that pH ($p < 0.001$) and protein-type ($p < 0.001$), along with their interaction ($p < 0.05$) were all significant (Table 1). G_i^* expresses the energy involved at the interface through relaxation processes (Lucassen & van den Tempel, 1972; Seta, Baldino, Gabriele, Lupi, & de Cindio, 2012). G_i^* of CPI at pH 3.0 and pH 5.0 was much larger than LPI, followed by PPI and SPI at both pHs, suggesting that CPI at pH 3.0 and pH 5.0 formed stronger viscoelastic films that may result in an emulsion with better long-term stability than the others (Table 1).

A simple Pearson correlation indicated that the interfacial tension was positively correlated with G_i^* ($r = 0.705$; $p < 0.001$) suggesting that the higher the interfacial tension value (or the least effective at reducing interfacial tension the protein was) the stronger and thicker the viscoelastic film will be. Findings suggest that despite the protein's ability to lower interfacial tension further at pH 7.0, an interconnected network was unable to form possibly due to the lower surface hydrophobicity (Fig. 2B) which would stabilize protein–protein aggregation at the interface. In contrast, it is hypothesized that stronger interfacial films form at pH 3.0, since proteins experience a greater amount of protein–protein interactions as the hydrophobic forces are more abundant.

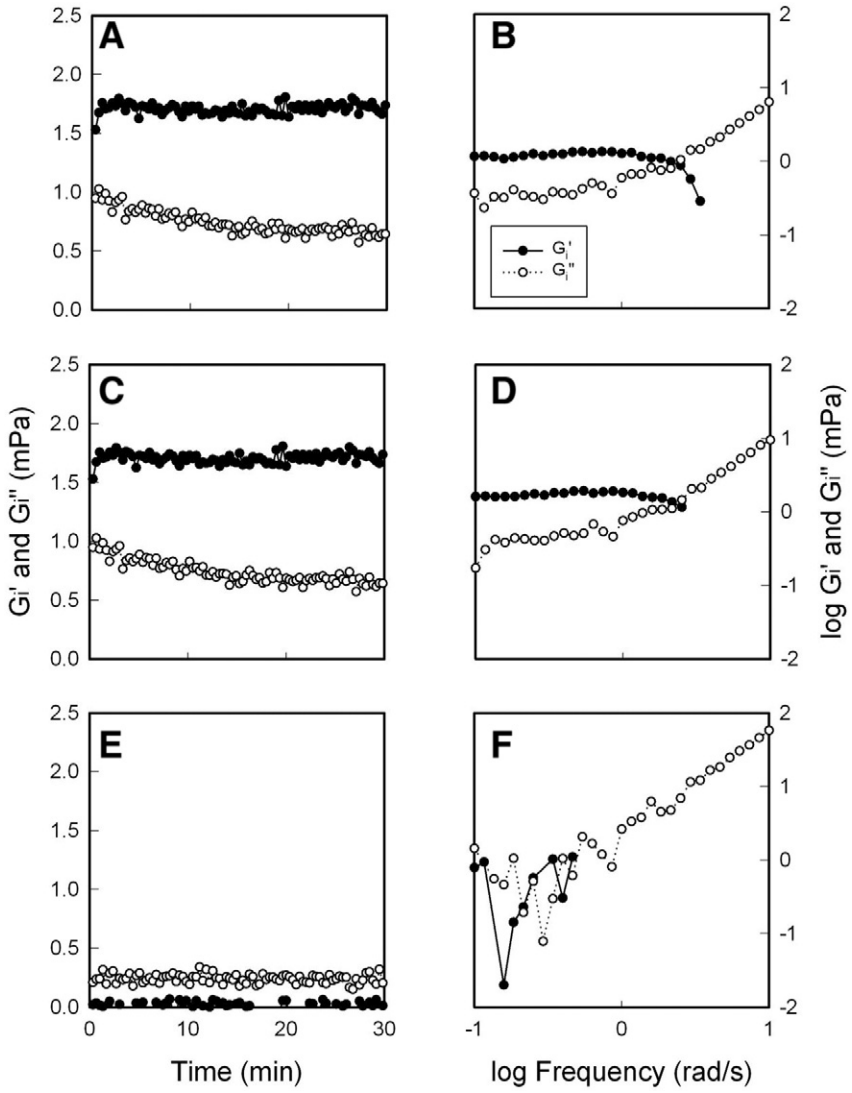

Fig. 4. Dilatational storage modulus (G_i') and loss modulus (G_i'') for SPI solutions at pH 3.0 (A and B), ph 5.0 (C and D), and ph 7.0 (E and F) at the oil–water interface as a function of time (left) and frequency (right).

The higher molecular interaction between absorbed proteins at the interface could contribute to this result (Lucassen-Reynders, Lucassen, Garrett, Giles, & Hollway, 1975), which was also demonstrated by the study of interfacial properties of β-casein and β-lactoglobulin (Seta, Baldino, Gabriele, Lupi, & de Cindio, 2014).

Table 1
Effects of pH and protein-type on storage modulus (G_i'), loss modulus (G_i''), and complex modulus (G_i^*) (units: milliPascal) of the O/W model system emulsion at 1 rad/s. Data represent the mean values of triplicate samples. Standard deviations were not reported since no differences were observed until after the second decimal position. Different letters (a–d) in the column indicate significant ($p < 0.05$) differences among protein solutions. Abbreviations include: pea protein isolate (PPI), soy protein isolate (SPI), lentil protein isolate (LPI), canola protein isolate (CPI), storage modulus (G_i'), loss modulus (G_i'') and complex modulus (G_i^*).

	pH 3.0			pH 5.0			pH 7.0		
	Gi′ (mPa)	Gi″ (mPa)	Gi* (mPa)	Gi′ (mPa)	Gi″ (mPa)	Gi* (mPa)	Gi′ (mPa)	Gi″ (mPa)	Gi* (mPa)
PPI	2.3	0.7	2.4ab	–	0.3	0.4a	–	0.3	0.2a
SPI	1.3	0.6	1.4a	1.8	0.8	2.0b	–	0.3	0.3b
LPI	2.5	0.9	2.7ab	0.8	0.6	1.0c	0.3	0.4	0.8c
CPI	4.5	1.6	4.8b	4.9	2.2	5.4d	0	0.4	0.4d

3.3. Emulsifying properties

Because of droplet flocculation and excess protein aggregation in the aqueous phase, the droplet size distributions for all emulsions showed multimodal size distributions, with each of them having one prominent peak (Fig. 5). However, the magnitude and location of the peak varied based on the pH and protein-type. It is hypothesized that for the legume proteins (SPI, PPI and LPI) a shift in the distribution towards larger particles at pH 5.0 from pH 3.0 and pH 7.0 reflects protein–protein aggregation occurring due to reduced solubility. In the case of CPI, solubility continually declines as pH is raised from 3.0 to 7.0 resulting in a continuous pH-dependent shift within the size distribution. A similar multimodal size distribution was also previously reported in PPI stabilized emulsions at pH 3.0–pH 9.0 (Liang & Tang, 2013), SPI and LPI stabilized emulsions at pH 7.0 (Can Karaca et al., 2011a), and CPI stabilized emulsions at pH 7.0 (Can Karaca et al., 2011b).

The average droplet diameter ($d_{3,2}$) for all protein-stabilized emulsions as a function of pH is shown in Fig. 6. An analysis of variance indicated that pH and protein-type, along with their interaction ($p < 0.001$) were significant. Overall, oil droplets stabilized by PPI and LPI at pH 5.0 were significantly larger than those at pH 3.0 and pH 7.0 which were similar in magnitude. SPI behaved similarly, except that droplets were

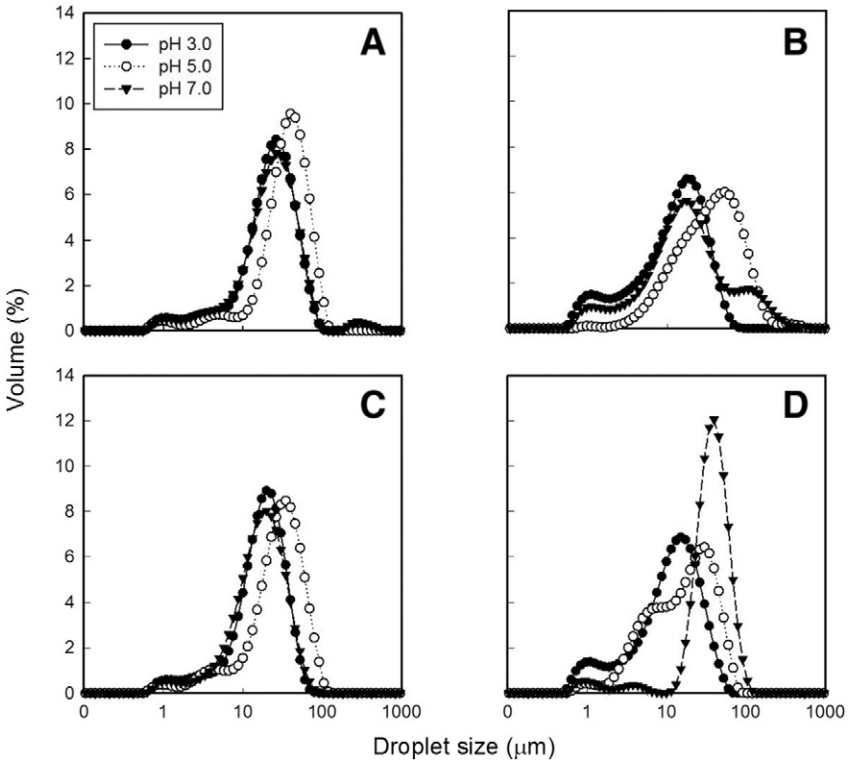

Fig. 5. Droplet size distribution of PPI (A), SPI (B), LPI (C), and CPI (D) (2.0%, w/w) stabilized emulsions prepared at a 1:9 (w/w) oil-to-water ratio with canola oil.

slightly larger at pH 7.0 than at pH 3.0 possibly. At pH 3.0, CPI and SPI produced similar size droplets (~5 μm) which were smaller than PPI and LPI stabilized oil droplets which were also similar in magnitude (~9 μm) (Fig. 6). A Pearson correlation revealed the droplet size to be negatively correlated with surface charge ($r = -0.740$; $p < 0.001$), the strength of the interfacial film (G_i^*) ($r = -0.323$; $p < 0.05$), solubility ($r = -0.817$; $p < 0.001$) and hydrophobicity ($r = -0.372$; $p < 0.05$). Findings suggest that smaller sized droplets can be obtained using proteins that: a) are highly charged to facilitate movement to the oil–water interface to lower interfacial tension and to increase charge repulsion between droplets once integrated to the interface; b) are highly soluble to allow quicker diffusion to the interface and to afford greater conformational flexibility needed to rearrange at the interface; and c) have high hydrophobicity to produce stronger interfacial films.

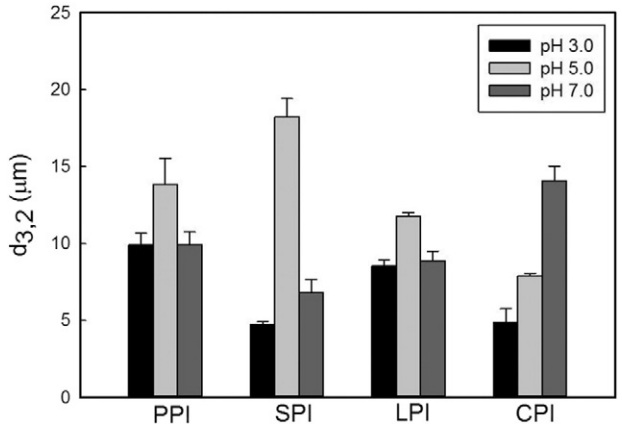

Fig. 6. Mean droplet diameter ($d_{3,2}$, μm) of different proteins (2.0%, w/w) stabilized emulsion prepared at a 1:9 (w/w) canola oil-to-water ratio. Data represent the mean ± one standard deviation ($n = 3$). Abbreviations include: pea protein isolate (PPI), soy protein isolate (SPI), lentil protein isolate (LPI) and canola protein isolate (CPI).

Gravitational separation driven by density differences between oil and aqueous phases is one of the most common mechanisms for instability (McClements, 2007). Because of the significantly ($p < 0.001$) different protein solubility, the emulsions either separated into a 2-phase emulsion or destabilized. Fig. 1 shows an example involving CPI only. It was observed that the emulsion with CPI at pH 3.0 separated into a cream layer (at the top) and a turbid serum layer (at the bottom) (Fig. 1B) after 24 h from time zero (Fig. 1A). The emulsions with PPI, SPI, and LPI at pH 3.0 and pH 7.0 separated in a similar manner. However, the emulsion prepared with CPI at pH 5.0 (not shown) and pH 7.0 (Fig. 1C) destabilized into a cloudy emulsion layer with oil–protein flocculates (at the top) and a clear serum layer (at the bottom). This destabilization occurred with emulsions prepared with PPI, SPI, and LPI at pH 5.0, which is close to the protein's isoelectric point.

Overall, ES was found to be similar in magnitude at pH 3.0 and pH 7.0 for PPI (~85% and ~87%, respectively), SPI (~90% and ~86%, respectively), and LPI (~87% and ~83%, respectively). However, all of them were found to be unstable at pH 5.0 which was close to the pI for the legume proteins. In contrast, ES for CPI at pH 3.0 was found to be ~85%, whereas the emulsions were unstable at both pH 5.0 and pH 7.0 which were closer to the isoelectric point (~pH 6.2) (Fig. 2A). Despite differences seen in the physicochemical and interfacial properties at pH 3.0 and pH 7.0 for the legume proteins, emulsion stability over the 24 h time frame remained similar. It was surmised that this may be due to the high charge (negative or positive) on the protein's surface that coated the oil droplets. Most likely the neutral charge on the protein's surface at pH 5.0 resulted in flocculation of the oil droplets during the gravitational creaming experiment. Droplet size within range of ~5 to 9 μm did not seem to play a key role in altering stability, however the small reduction in stability for SPI from 90% to 86% may be the result of a slightly smaller droplet size at pH 3.0. In the case of CPI, the charge on the protein declined as pH was raised from pH 3.0 to pH 7.0, leading to droplet flocculation and instability at pH 5.0 and pH 7.0.

4. Conclusions

The generic consensus of how a protein stabilizes an emulsion involves its migration to the interface, where it then unravels and rearranges to position its hydrophobic moieties towards the apolar phase and its hydrophilic moieties towards the polar phase. An interfacial viscoelastic film then forms by protein–protein aggregation to coat the oil droplet and stabilize the emulsion via charge repulsion (at pHs away from the protein's pI) and/or steric forces. Findings from this study indicate that the ability for a protein (specifically isolates from pea, soy, lentil and canola) to initially associate with the oil–water interface during the initial stage of emulsion formation to lower the interfacial tension requires them to have a high surface charge and low hydrophobicity (i.e., pH 7.0 in the present study). It's important to note that low hydrophobicity does not imply that the protein is not surface active. However the properties of a protein to form a strong viscoelastic interfacial film are different, where proteins require a high surface charge and high hydrophobicity (i.e., pH 3.0 in the present study). Therefore selection of an effective plant protein emulsifier really entails finding a balance between properties needed to associate at the oil–water interface with those needed to develop a strong interfacial film.

The most prudent way to find this balance is to consider the oil droplet size, where smaller sized droplets typically lead to improved stability. Findings from this study suggest that proteins should be: (i) highly charged so they can associate with the interface and to provide charge repulsion once the oil droplet is coated; (ii) have good solubility to allow for easier diffusion to the interface and conformational flexibility during the rearrangement step; and (iii) have moderately high hydrophobicity to produce stronger interfacial films. Although a long-term stability trial was not performed as part of this study, it is presumed that the stronger interfacial films formed at pH 3.0 than pH 7.0 would lead to more stable emulsion, which may find applications in protein-rich acidic beverages or similar type products. Of the protein-types studied, the most promising alternative to SPI as an emulsifier is LPI because it showed high charge, solubility and hydrophobicity at pH 3.0. The low solubility of pea protein at this pH could result in precipitation issues of the emulsifier for product developers within the continuous phase (although not impacting emulsion stability), whereas CPI would have issues lowering interfacial tension during emulsion formation and allergen concerns associated with its 2S protein.

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