

## RESEARCH ARTICLE

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# Effects of enzymatic hydrolysis and ultrafiltration on physicochemical and functional properties of faba bean protein

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## Abstract

**Background and objectives:** Faba bean proteins isolates did not show sufficient functional properties for food applications due to poor solubility. Enzymatic treatments and ultrafiltration may improve solubility and functional properties of faba bean protein. The aim of the present work was to investigate the effect of different proteases (pepsin, trypsin, flavourzyme<sup>®</sup> 500 L, neutrase<sup>®</sup> 0.8 L), hydrolysis time and ultrafiltration technique on the physicochemical and functional characteristics of faba bean protein.

**Findings:** The protein solubility increased from 24.4% to 88.8% at pH 7 and 81.0% at pH 5 by pepsin hydrolysis (15 min). Their foaming capacity (FC) increased from 31.2% to 122.2% at pH 5 and 66.7% to 131.2% at pH 7 and the oil holding capacity (OHC) increased from 6.12 to 8.21 g/g by pepsin hydrolysis. Fraction I (Mw > 10 kDa) and II (Mw: 5–10 kDa) obtained after pepsin hydrolysis and ultrafiltration demonstrated further improved foaming and oil holding capacity and much improved emulsifying capacity.

**Conclusions:** Enzymatic hydrolysis and ultrafiltration provided a strategy to significantly improve faba bean protein solubility and functional properties.

**Significance and novelty:** Faba bean protein hydrolysates and ultrafiltration fractions are good sources of protein with excellent solubility and functionality.

## KEYWORDS

enzymatic hydrolysis, faba bean protein, nutritive value, physicochemical and functional properties, ultrafiltration

## 1 | INTRODUCTION

Legume proteins are widely used in new food formulations owing to their sustainability and good functionality in addition to their high nutritive value (Barbana & Boye, 2013). Among legumes, faba bean (*Vicia faba* L.) has attracted interest recently due to the excellent nitrogen-fixing capacity and high protein content (27%–34%) (Chandra-Hioe, Wong, & Arcot, 2016; Multari, Stewart, & Russell, 2015). However, current research on faba bean protein is still very limited

(Karaca, Low, & Nickerson, 2011; Vioque, Alaiz, & Girón-Calle, 2012), and consequently, faba bean is underutilized by the food industry due to incomplete data on its protein physicochemical properties and functionalities. Preliminary isolation of faba bean proteins led to protein isolates or concentrates, but they did not show sufficient functional properties for food applications primarily due to poor solubility (Karaca et al., 2011; Vioque et al., 2012). This is attributed to the compact structure of faba bean protein (El Fiel, Tinay, & Elsheikh, 2002) and also related to the alkaline conditions

(pH 10–12) used for efficient faba bean protein extraction, which led to irreversible protein denaturation and aggregation, accompanied by the reduced functionality at neutral pH (Vioque et al., 2012). While at mild acidic pH (4–5), the functional properties of faba bean proteins are impaired as pH is near to their isoelectric point (pI) (Karaca et al., 2011; Vioque et al., 2012).

Modifications of plant proteins by chemical, physical, or enzymatic treatments have been successfully used to improve their solubility and functional properties (Achouri, Zhang, & Shiyang, 1998; Tang, Sun, Yin, & Ma, 2008). Enzymatic hydrolysis is a more preferable tool to improve protein properties because of the milder processing conditions, easier control of reaction, and minimal formation of by-products (Tsumura et al., 2005). Appropriate selection of the proteolytic enzymes and control of reaction conditions is crucial for obtaining samples of desired degree of hydrolysis (DH) with enhanced physicochemical and functional properties (Barbana & Boye, 2013; Gbogouri, Linder, Fanni, & Parmentier, 2004). As reported by other researchers, the extensive enzymatic modification may impair some functional properties of food proteins while more desirable effect could be achieved through limited hydrolysis (DH < 10%) (Ghribi et al., 2015; Lqari, Pedroche, Girón-Calle, Vioque, & Millán, 2005; Muhamyankaka, Shoemaker, Nalwoga, & Zhang, 2013). Modification by a limited enzymatic hydrolysis leads to reduce protein molecular weight, changes in the protein secondary and tertiary structure, increased number of ionisable groups, and exposure of previously concealed hydrophobic groups (Muhamyankaka et al., 2013). On the other hand, proteolytic enzymes split specific peptide bonds, resulted in peptides with unique functional properties and structural characteristics (del Mar Yust, Pedroche, Carmen Millán-Linares, Alcaide-Hidalgo, & Millán, 2010; Tsumura et al., 2005). Endopeptidases break bonds of nonterminal amino acids, thus produce larger peptides, whereas exopeptidases selectively release N-terminal or C-terminal amino acid residues from polypeptides and proteins (Barrett, Woessner, & Rawlings, 2012). Therefore, in order to develop modification strategy for improved protein solubility and functionality by enzymatic hydrolysis, a systematic investigation of the effect of various proteases over the molecular structures, functional properties, and nutritive value of faba bean protein is required. However, such information has not been reported in previous research.

According to Jeon, Byun, & Kim, 1999, functionality of protein hydrolysates can be further improved by ultrafiltration (UF) which produces highly purified food-grade proteins with desired molecular size by applying of appropriate UF membranes (Jeon et al., 1999). Nevertheless, such technique has not been applied for faba bean protein hydrolysate fraction yet.

The aim of the present work was to investigate how different proteases, hydrolysis time, and ultrafiltration technique affect the physicochemical and functional characteristics of faba bean protein. The faba bean protein concentrates were hydrolyzed by four commercially available proteases, including pepsin, trypsin, neutrase, and flavourzyme, followed by ultrafiltration with membrane of different molecular weight cutoff. The protein and hydrolysate structures were systematically characterized, including amino acid composition, molecular weight distribution, conformation, surface hydrophobicity, and surface charge. The sample solubility and functional properties (foaming properties, emulsifying properties, and oil holding capacity) were evaluated at pH 5 and/or 7, representative of different food processing pHs. The faba bean protein hydrolysate structure–function relationships are carefully discussed. Furthermore, the protein, hydrolysate, and ultrafiltration fraction nutritive value (amino acid score, protein efficiency ratios, and predicted biological value) were estimated based on the amino acid composition.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

Faba bean (*Vicia faba* L.) seeds were kindly provided by W.A. Grain and Pulse Solutions with mixed varieties (Alberta, Canada). Seeds were dehulled using a Forsberg Model 2 Huller and then grounded into fine flours using a Retsch centrifugal grinding mill with screen aperture size of 0.5 mm (ZM 200, Retsch, Inc., Newtown, PA, USA). The flours were then defatted using hexane at a flour to hexane ratio of 1:3 (w/v) for 2 hr, followed by centrifugation (7,000 g, 15 min, room temperature). The defatted flours were air-dried in a fume hood and stored in a sealed plastic bags at 4°C until use. Pepsin (peptidase from porcine gastric mucosa, solid, ≥250 U/mg), Trypsin (protease from bovine pancreas, solid, ≥10,000 U/mg), Flavourzyme® 500 L (protease from *Aspergillus oryzae*, liquid, ≥500 U/g) and Neutrase® 0.8 L (protease from *Bacillus amyloliquefaciens*, liquid, ≥0.8 U/g) were purchased from Sigma Aldrich. Canola oil used for protein functionality evaluation was purchased from a local grocery store (No Name®, Loblaw Companies Ltd.). Standard protein molecule markers for SDS-PAGE (Molecular weight of 10–250 kDa) and SE-HPLC analysis were purchased from Bio-Rad and Sigma Aldrich, respectively. All other chemicals were reagent grade.

### 2.2 | Faba bean protein extraction

Proteins were extracted from faba bean (*Vicia faba* L.) seeds by alkali solution followed by an isoelectric precipitation. Briefly, defatted flours were mixed with Milli-Q™ water at a solid to solvent ratio of 1:10 (w/v), then the pH was adjusted to 10.5 using 0.1 mol/L NaOH, and the suspension was stirred

by magnetic stirring for 1 hr at temperature 23°C. The suspension was then centrifuged at 7,500 g for 15 min at 23°C, and the supernatant was collected. Supernatant was then acidified to the isoelectric point (pH 4), and the precipitated proteins were recovered by centrifugation (7,500 g for 15 min at 23°C) and freeze-dried after washing with water. The dry protein extract was stored in plastic bag at 4°C before further analysis (Karaca et al., 2011; Yang, Liu, Zeng, & Chen, 2018). The protein content of the isolated faba bean proteins was determined using a nitrogen analyzer (FP-428, Leco Corporation) calibrated with analytical reagent grade EDTA, and obtained nitrogen values were converted to protein using a conversion factor of 6.25. The fat and ash were analyzed using AOAC Official Methods (2003) 920.85 and 923.03, respectively (AOAC, 2003). The  $\beta$ -glucan content was determined by an enzymatic kit (Megazyme, Wicklow, Ireland 2016). The effectiveness of protein extraction was evaluated by estimating the protein content, defined as the percentage of protein in the isolated dry powder, and extraction efficiency (EE), defined as the percentage of protein recovered in the isolated fraction relative to the total amount of the protein in the faba bean flour.

### 2.3 | Faba bean protein hydrolysis and fractionation

Hydrolysis was carried out with 2% (w/v) protein in Milli-Q™ water and pH was adjusted to the desired value using 0.1 mol/L NaOH or 0.1 mol/L HCl depending on the enzyme used. Hydrolysis was performed by different proteolytic enzymes (pepsin, trypsin, flavourzyme, and neutrase) under continually monitored optimum conditions for each enzyme (pH 2, 38°C; pH 8, 37°C; pH 7, 50°C; and pH 7, 50°C, respectively) (Liu, Damodaran, & Heinonen, 2019; Tsumura et al., 2005). The enzyme/substrate ratio was set at 5/100 (w/w) for all treatments. The faba bean extracted protein dispersions in water were incubated with each proteolytic enzyme for 15, 30, 45, and 60 min. The proteolysis was stopped by heating the mixture at 100°C for 15 min. Then, the hydrolysates were cooled, lyophilized, and stored at 4°C until used. The obtained hydrolysates with desirable functional properties were selected for fractionation by ultrafiltration using membrane of two different molecular weight (MW) cutoff (10 and 5 kDa). Consequently, three fractions were obtained with molecular weight range of >10 kDa, 5–10 kDa, and <5 kDa. The fractions were collected, lyophilized, and stored at 4°C before further testing.

### 2.4 | Faba bean hydrolysate characterizations

#### 2.4.1 | Degree of hydrolysis

Degree of hydrolysis (DH%) was determined as the percentage ratio of protein soluble in 20% trichloroacetic acid (TCA)

to total protein (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003). TCA was added to hydrolysates (1:1, v/v), and after one hour of incubation at 4°C, the samples were centrifuged (5,000 g, 15 min, 4°C). The concentration of the trichloroacetic acid-soluble product in the supernatant was measured spectrophotometrically ( $\lambda = 280$  nm) (Jasco V-530 spectrophotometer, Japan), and the DH% values were calculated from the following equation:  $DH\% = [(P_1 - P_0)/S] \times 100\%$ , where  $P_1$  represents concentration of soluble proteins after hydrolysis (mg/mL),  $P_0$  represents concentration of soluble proteins before hydrolysis (mg/mL), and  $S$  represents the concentration of protein (mg/mL) in starting solution. The content of free amino acid groups (FAG) ( $\mu\text{mol/g}$ ) in hydrolysates was determined using trinitrobenzene sulfonic acid (TNBS, Sigma) according to the method by Kuchroo, Rahilly, and Fox (1983).

#### 2.4.2 | Electrophoresis

SDS gel electrophoresis was performed to determine molecular weight of faba bean protein subunits using an Amersham ECL Gel Box (GE Healthcare Bio-Sciences Corp.). Protein samples (1.5 mg/ml) were mixed with loading buffer (0.125 mol Tris-HCl, pH 6.8, 4 g/100 ml SDS, 20 ml/100 ml glycerol, 0.5 ml/100 ml 2-mercaptoethanol, and 1 g/100 ml bromophenol blue) and heated at 95°C for 5 min. After cooling, samples were loaded on a pre-cast gel (4%–20% gradient) and subjected to electrophoresis at a constant voltage of 140 V. After electrophoresis, the gels were stained with 0.1 g/100 ml Coomassie Brilliant Blue-R-250 in water, methanol, and acetic acid (4:5:1, v/v/v) for 30 min and destained with water, methanol, and acetic acid (4:5:1, v/v/v).

#### 2.4.3 | Molecular weight

Molecular weight distribution of the fractions obtained by ultrafiltration was determined by size-exclusion high-performance liquid chromatography (SE-HPLC) (Agilent series 1,100) equipped with a Biosuite™ 125/5 mm HR-SEC column (7.8 × 300 mm, Waters Corp.). Phosphate buffer (100 mM) containing 300 mM NaCl was used as mobile phase at a flow rate of 0.5 ml/min. Sample solution (50  $\mu\text{l}$ ) was injected into the HPLC system, and the protein elution was monitored at the UV wavelength of 280 nm. Standard molecular markers were used to calculate Mw of the samples. A calibration curve was obtained from the log Mw of the markers and their respective elution times ( $R^2 = 0.95$ ).

#### 2.4.4 | Peptide profiles

Peptide profiles of the hydrolysates were also studied by reversed-phase high-performance liquid chromatography (RP-HPLC). Separation was performed using the Agilent

1,100 series HPLC system on an analytical scale reversed-phase column (Eclipse XBD-C18 column,  $4.6 \times 150$  mm,  $5 \mu\text{m}$ ). A linear gradient mixture composed of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in ACN) were used at a flow rate of 1 ml/min. The gradient elution conditions were as follows: 5% solvent B for 5 min, 5%–40% B over 30 min, 40%–90% B over 10 min and then 5 min at 90% B. The absorbance of eluent was monitored at a wavelength of 230 nm, and the column temperature was  $30^\circ\text{C}$ .

## 2.4.5 | Amino acid analysis

For amino acid analysis, the extracted faba bean protein and the selected hydrolysate samples were treated under vacuum in 4 mol/L methanesulfonic acid with 0.2% (w/v) tryptamine according to the method of (Simpson, Neuberger, & Liu, 1976) with slight modifications. Glass sample tubes ( $6 \times 50$  mm) were used in the reaction vial assembly, which were then placed in the Work Station (Waters). After treating as suggested in the Work Station manual, where the contents were hydrolyzed at  $115^\circ\text{C}$  for 24 hr, the pH was adjusted to neutral with 3.5 mol/L NaOH. Amino acid analysis was performed using the Waters ACCQ-Tag method. The high-performance liquid chromatography (HPLC) system (Agilent series 1,100) consisted of an autosampler and a binary pump, a control system with a column heater maintained at  $37^\circ\text{C}$ , and a UV detector set at a wavelength of 254 nm. A reversed-phase AccQ-Tag  $150 \times 3.9$  mm C18 column with a three-eluent gradient solvent system (AccQ-Tag eluent, acetonitrile, and water) at a flow rate of 1.5 ml/min. Data acquisition was controlled by ChemStation software.

## 2.4.6 | FI-IR

The infrared spectra were recorded at temperature  $23^\circ\text{C}$  using a Nicolet 6,700 spectrometer (Thermo Scientific) at a resolution of  $4 \text{ cm}^{-1}$  and a total of 128 scans for each sample. The spectrometer was continuously supplied with nitrogen. Faba bean protein extract, hydrolysates, or ultrafiltration fractions were dissolved in  $\text{D}_2\text{O}$  solution at 5% (w/v). To ensure complete hydrogen/deuterium exchange, samples were prepared 2 days before and kept at  $4^\circ\text{C}$  prior to infrared measurements. Samples were placed between two  $\text{CaF}_2$  windows separated by 25 mm polyethylene terephthalate film spacer for FT-IR measurement. To study the amide I region of the protein ( $1600\text{--}1700 \text{ cm}^{-1}$ ), Fourier self-deconvolutions were performed using the software provided with the spectrometer (Omic 8.1.210). Band narrowing was achieved with a full width at half maximum of  $20\text{--}25 \text{ cm}^{-1}$  and with a resolution enhancement factor of  $2.0\text{--}2.5 \text{ cm}^{-1}$  (Yang et al., 2018).

## 2.4.7 | Surface charge

The surface charge of the extracted faba bean protein was determined by measuring the electrophoretic mobility (UE) of protein solutions (1% w/w) at pH range from 2 to 11. While for selected faba bean protein hydrolysates and ultrafiltration fractions, the surface charge was measured at pH 7 and 5. The study was carried on using a Zetasizer NanoS (model ZEN 1600, Malvern Instruments Ltd). The protein refractive index (RI) was set at 1.45, and dispersion medium RI was 1.33.

## 2.4.8 | Surface hydrophobicity

Surface hydrophobicity ( $H_o$ ) of extracted faba bean protein and the selected hydrolysate samples was determined using the apolar fluorescent dye, 8-anilino-1-naphthalenesulfonic acid, according to the method of (Tang et al., 2008). Briefly, the 8-anilino-1-naphthalenesulfonic acid solution ( $20 \mu\text{L}$ , 8.0 mM in phosphate buffer, pH 7) was added to serial dilutions of samples containing 0.0025%–0.0375% w/v protein. Fluorescence intensity was determined using a fluorescence spectrophotometer (Shimadzu RF-5301 spectrofluorometer) set at 390 and 470 nm as excitation and emission wavelengths, respectively, with a constant excitation and emission slit of 5 nm. The initial slope of the fluorescence intensity versus protein concentration plot was calculated by linear regression analysis and used as an index of  $H_o$ .

## 2.5 | Evaluation of protein solubility and functional properties

### 2.5.1 | Protein solubility

Solubility of faba bean protein and the hydrolysates was determined using weighting method according to (Wang et al., 2010) with modifications. Protein dispersions (2%, w/v) were prepared at pH 2 to 10 adjusted by using 1 mol/L HCl or 1 mol/L NaOH. The hydrolysate dispersions (2%, w/v) were prepared at pH 5 and 7 adjusted using 1 mol/L HCl or 1 mol/L NaOH. The solutions were mixed vigorously for 1 hr at  $23^\circ\text{C}$  and then centrifuged at  $1,500 g$  for 10 min at  $23^\circ\text{C}$ . The supernatant was removed, and the precipitate was freeze-dried and weighted. The solubility (%) was calculated from the following equation:

$$\text{Solubility (\%)} = \left( 1 - \frac{\text{mass of insoluble protein}}{\text{mass of protein in starting solution}} \right) \times 100\%$$

### 2.5.2 | Oil holding capacity

Oil holding capacity (OHC) of the faba bean protein and the selected hydrolysates was determined according to a modified version of the method described by Guan, Yao, Chen,

Shan, and Zhang (2007). The samples (1 g) were dispersed in 40 ml canola oil with a vortex mix for 30 s. After standing for 6 hr at 23°C, the dispersions were centrifuged at 2,000 *g* for 30 min at 23°C. The supernatants were discarded and the pellets weighed. OHC was expressed as g of water or oil retained per g of protein.

### 2.5.3 | Foaming properties

Foaming properties of the faba bean protein and the hydrolysates were determined according to the method of Ahmedna, Prinyawiwatkul, and Rao (1999) with a slight modification. Protein samples (0.5%, w/v) were dispersed in 50 ml of deionized water adjusted to different pHs using either 0.5 mol/L NaOH or 0.5 mol/L HCl. The solution was mixed for 2 min with a homogenizer (PowerGen 1,000, Fisher Scientific) at speed three. Volumes were recorded before and after homogenization using a graduated cylinder. The percentage volume increase was calculated as foaming capacity (FC):

$$FC = \frac{Vf_1 - Vf_0}{Vf_0} \times 100\%$$

where  $Vf_0$  and  $Vf_1$  represent the volume of the protein solution and the formed foams after homogenization, respectively.

Foam stability (FS) was determined as the volume of foams ( $Vf_2$ ) that remained after standing for 0.5 hr at 23°C expressed as a percentage of the initial foam volume:

$$FS = \frac{Vf_2}{Vf_1} \times 100\%$$

where  $Vf_2$  and  $Vf_1$  represent the volume of foam after storage for 30 min and the initial volume of foam after mixing, respectively.

### 2.5.4 | Emulsifying properties

Emulsifying properties of faba bean protein and the hydrolysates were determined by the method of (Pearce & Kinsella, 1978) with minor modifications. Protein solution (0.1% w/v) was prepared using 0.01 mol/L phosphate buffer (pH 7.0). Then, 6 ml of protein solution and 2 ml canola oil were mixed and homogenized for 1 min using a homogenizer, at a setting speed 6, to form a homogenous emulsion. The emulsion (50  $\mu$ l) was added into a test tube containing 5 ml of 0.1% SDS (w/v) at 0 and 10 min after homogenizing. The absorbance of the above solutions was recorded using a spectrophotometer at a wavelength of 500 nm. Emulsifying activity index (EAI) was calculated using the following formula:

$$EAI = (2 \times 2.303 \times A_0 \times DF) / (C \times \phi \times \theta \times 10000),$$

where DF is the dilution factor (100),  $C$  is the initial concentration of protein (g/mL),  $\phi$  is the optical path (1 cm),  $\theta$  is the fraction of oil used to form the emulsion (0.25), and  $A_0$  is the absorbance of the diluted emulsions at 0 min.

Emulsion stability index (ESI) was calculated using the following equation:

$$ESI = T_0 \times \Delta t / \Delta T,$$

where  $\Delta T$  is a decrease in turbidity (absorbance) of the initial absorbance ( $T_0$ ) which occurred after 10 min ( $\Delta t$ ).

### 2.6 | Estimation of the nutritive value

The amino acid composition of the faba bean proteins, hydrolysates, and ultrafiltration fractions was applied for determination of several nutritional parameters as follows:

1. Amino acid score (chemical score): % essential amino acids in sample/% essential amino acid recommended by FAO/WHO/UNU (1985).
2. Protein efficiency ratios (PER) were calculated according to the following three equations (Alsmeyer, Cunningham, & Happich, 1974):  
 $PER_1 = -0.684 + 0.456 \times \text{Leu} - 0.047 \times \text{Pro};$   
 $PER_2 = -0.468 + 0.454 \times \text{Leu} - 0.105 \times \text{Tyr};$   
 $PER_3 = -1.816 + 0.435 \times \text{Met} + 0.78 \times \text{Leu} + 0.211 \times \text{Hys} - 0.944 \times \text{Tyr}.$
3. Predicted biological value (BV) was calculated according to (Morup & Olesen, 1976), using the following equation:

$$BV = 10^{2.15 \times \text{Lys}^{0.41} \times (\text{Phe} + \text{Tyr})^{0.60} \times (\text{Met} + \text{Cys})^{0.77} \times \text{Thr}^{0.24} \times \text{Trp}^{0.21}},$$

where each amino acid symbol represents:

% amino acid in sample/% amino acid according to FAO pattern (1985) (FAO, WHO, & UNU, 1985), if % amino acid in sample  $\leq$  % amino acid according to FAO pattern, or:

% amino acid according to FAO pattern (1985)/% amino acid in sample, if % amino acid in sample  $\geq$  % amino acid according to FAO pattern.

### 2.7 | Statistical analysis

All experiments were performed at least in three independent trials, and each sample was analyzed by at least three replicates. The results were reported as mean  $\pm$  standard deviation. One-way analysis of variance (ANOVA) was carried out using Origin 9.1 software (Origin Lab Corporation), and statistical differences among sample means were determined using Tukey's test at 95% confidence level.

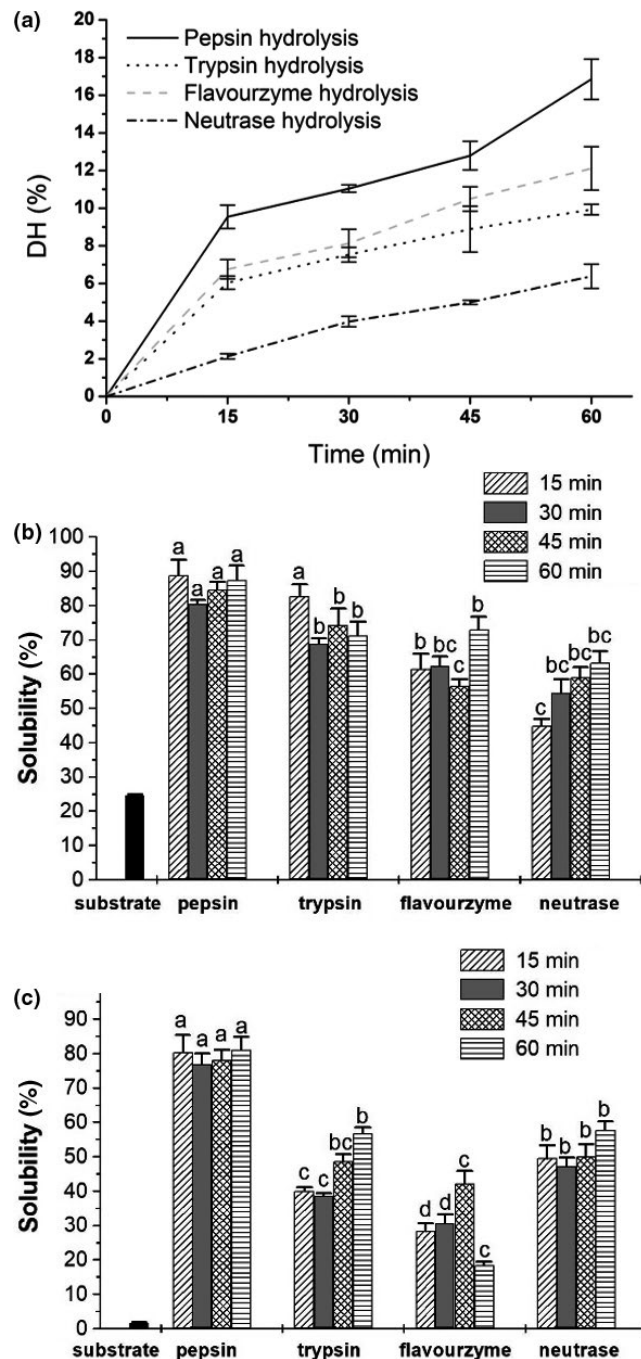


**FIGURE 1** (a) Degree of hydrolysis (DH) (%) obtained after faba bean protein treatment by pepsin, trypsin, flavourzyme, and neutrase for 15, 30, 45, and 60 min. Solubility of faba bean protein extract and the hydrolysates at (b) pH 7 and (c) pH 5. Values with different lowercase letters in the hydrolysates prepared at the same time are significantly ( $p < 0.05$ ) different

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Faba bean protein extraction

Protein alkaline extraction followed by isoelectric precipitation has been widely used for plant protein extraction and was applied in this study for faba bean protein extraction after

seed dehulling and milling (Jarpa-Parra et al., 2014; Karaca et al., 2011). The extract had a protein content of  $88.5\% \pm 0.2$  and yield of  $18.3\% \pm 0.8$  g of protein extract/100 g of faba bean flour with a protein extraction efficiency of  $55.1\% \pm 2.3$ . The remaining components include  $1.1\% \pm 0.1$   $\beta$ -glucan,  $2.9\% \pm 0.1$  ash, and less than 0.1% lipids based on established methods (AOAC, 2003). The faba bean protein extraction efficiency and the protein content in the extract are comparable to those reported for other pulses such as lentil (Jarpa-Parra et al., 2014) and two Mediterranean legumes including *Lathyrus clymenum* and *L. Annuus* (Pastor-Cavada, Juan, Pastor, Alaiz, & Vioque, 2010). Using a similar extraction method, (Karaca et al., 2011) reported a protein content of 81.9%–87.6% for chickpea, faba bean, lentil, and pea protein extracts.

SDS-PAGE electrophoresis was used to identify protein composition and subunits of the faba bean protein extract (Figure S1). The bands in the range of 34–37 kDa and 18–25 kDa correspond to the acidic and basic subunits of the 11S fraction (legumin-like), respectively (Barbana & Boye, 2013). The intense band with a molecular mass of 47 kDa correspond to subunits of vicilin-like (7S) fraction. The results of electrophoresis suggest that the extract is mainly composed of globulin proteins 11S and 7S, probably due to limited solubility of prolamins at the conditions applied for alkaline solubilization (Aydemir & Yemenicioğlu, 2013). The precipitation at pH 4 would lead to a loss of the water-soluble albumin proteins (El Fiel et al., 2002). Other bands with lower molecular mass ( $M_w < 15$  kDa) observed in the sample are likely a mixture of albumin polypeptides and/or gamma-vicilin (Barbana & Boye, 2013).

Surface charge values measured for faba bean proteins at different pH's were comparable to those reported in literature for soy (Lam, Paulsen, & Corredig, 2008) and pea protein isolates (Lam, Warkentin, Tyler, & Nickerson, 2017). pH is a major factor that affects the peptide charge, at high pH the amines are deprotonated, leaving a net negative charge while the reverse is the case at low pH, as can be seen in Figure S2A (Yuan et al., 2002). These results also agree with (Tang & Sun, 2011), who reported the isoelectric point of legume globulin proteins to be approximately pH 4.5.

Extracted proteins were characterized in terms of their solubility, as this is an essential feature in food formulations. Figure S2B shows the solubility of faba bean protein at various pH values. Solubility was the minimum at pH 4–5 as the lack of electric charge promotes hydrophobic aggregation and precipitation (Kramer, Shende, Motl, Pace, & Scholtz, 2012). The highest (nearly 50%) solubility was observed at strong alkaline conditions (pH 10–11), due to the increased protein surface charge (Figure S2A,B). Faba bean protein extract showed relatively poor solubility (24.7%) at neutral pH, much lower than proteins extracted from soy (85%), chickpea (47%), or pea (63%–75%) (Achouri et al., 1998; Ghribi et al.,

**TABLE 1** Amino acid composition (%) of faba bean protein extract, selected hydrolysates and their ultrafiltration fractions

| Amino acids                   | Pepsin                          |             |  | Neutrase        |            |             |             |            |                          |
|-------------------------------|---------------------------------|-------------|--|-----------------|------------|-------------|-------------|------------|--------------------------|
|                               | Faba bean<br>Protein<br>extract | Hydrolysate |  | FR II           |            | FR I        |             | FR III     |                          |
|                               |                                 | 15 min      |  | Mw:<br>5–10 kDa | Mw < 5 kDa | Mw > 10 kDa | Mw 5–10 kDa | Mw < 5 kDa | FAO/WHO/UNU <sup>b</sup> |
| Asx <sup>a</sup>              | 11.51                           | 11.68       |  | 11.84           | 11.67      | 11.72       | 11.67       | 11.44      |                          |
| Ser                           | 6.54                            | 6.54        |  | 7.26            | 7.51       | 6.64        | 6.38        | 7.82       |                          |
| Glx <sup>a</sup>              | 15.30                           | 16.24       |  | 12.26           | 10.34      | 17.58       | 16.40       | 11.62      |                          |
| Gly                           | 7.16                            | 7.38        |  | 6.94            | 6.79       | 7.19        | 7.27        | 6.81       |                          |
| His                           | 2.48                            | 2.44        |  | 1.93            | 1.56       | 2.52        | 2.53        | 1.81       | 1.9                      |
| Arg                           | 7.81                            | 7.60        |  | 7.64            | 7.60       | 8.10        | 7.27        | 8.16       |                          |
| Thr                           | 4.11                            | 3.99        |  | 3.91            | 4.41       | 3.53        | 3.90        | 3.75       | 1.4                      |
| Ala                           | 5.58                            | 5.51        |  | 6.72            | 7.11       | 5.14        | 5.62        | 7.67       |                          |
| Pro                           | 5.52                            | 5.52        |  | 5.19            | 5.21       | 5.27        | 5.46        | 5.48       |                          |
| Cys                           | 1.21                            | 1.03        |  | 0.00            | 0.00       | 0.94        | 0.87        | 0.00       |                          |
| Tyr                           | 2.96                            | 2.75        |  | 2.85            | 2.33       | 2.66        | 2.99        | 1.78       | 6.3 <sup>g</sup>         |
| Val                           | 5.93                            | 5.71        |  | 6.97            | 7.45       | 5.48        | 6.14        | 6.47       | 3.5                      |
| Met                           | 0.67                            | 0.59        |  | 0.78            | 0.81       | 0.51        | 0.78        | 0.75       | 2.5 <sup>h</sup>         |
| Lys                           | 5.83                            | 5.61        |  | 5.78            | 4.76       | 5.97        | 5.82        | 6.36       | 5.8                      |
| Ile                           | 4.81                            | 4.74        |  | 4.90            | 5.18       | 4.36        | 4.70        | 4.86       | 2.8                      |
| Leu                           | 8.34                            | 8.53        |  | 10.05           | 11.74      | 8.09        | 8.48        | 10.25      | 6.6                      |
| Phe                           | 3.95                            | 3.83        |  | 4.88            | 5.45       | 4.09        | 3.63        | 4.88       |                          |
| Trp                           | 0.3                             | 0.3         |  | 0.1             | 0.1        | 0.2         | 0.1         | 0.1        | 1.1                      |
| %EAA/TAA <sup>c</sup>         | 40.59                           | 39.52       |  | 42.15           | 43.79      | 38.35       | 39.94       | 41.01      |                          |
| AAS <sup>d</sup>              | 127                             | 124         |  | 132             | 137        | 120         | 125         | 129        |                          |
| BV <sup>e</sup>               | 62.95                           | 57.53       |  | 24.04           | 22.12      | 49.30       | 46.99       | 24.84      |                          |
| PER <sub>1</sub> <sup>f</sup> | 2.86                            | 2.95        |  | 3.65            | 4.42       | 2.76        | 2.93        | 3.73       |                          |
| PER <sub>2</sub> <sup>f</sup> | 3.01                            | 3.12        |  | 3.79            | 4.62       | 2.93        | 3.07        | 4.00       |                          |
| PER <sub>3</sub> <sup>f</sup> | 2.71                            | 3.02        |  | 4.08            | 5.82       | 2.74        | 2.86        | 5.20       |                          |

<sup>a</sup>Asx represents Asn and Asp; Glx represents Gln and Glu.

<sup>b</sup>FAO/WHO/UNU (1985).

<sup>c</sup>% Essential amino acids/total amino acids.

<sup>d</sup>Amino acids score.

<sup>e</sup>Biological value.

<sup>f</sup>Protein efficiency ratio.

<sup>g</sup>Tyr + Phe.

<sup>h</sup>Met + Cys.

2015; Lam et al., 2017). Variations in solubility have been reported for different legume isolates produced by different extraction methods (Karaca et al., 2011). Protein solubility is dependent on many extrinsic (solution pH, temperature, ionic strength) and intrinsic factors (amino acid composition and distribution, molecular flexibility, charge aggregation) (Lam et al., 2017; del Mar Yust et al., 2010). Relatively low solubility of faba bean protein in neutral pH can be attributed to their compact structure (Vioque et al., 2012). This may also be associated with the alkaline conditions (pH 10–11) used for efficient faba bean protein extraction, which lead to irreversible protein denaturation and aggregation (Vioque et al., 2012).

The limited solubility at neutral pH can significantly reduce faba bean protein functional properties and their utilization. For this reason, enzymatic hydrolysis was used in this study to address these issues. Four commercial proteases were used, including pepsin, trypsin, flavourzyme, and neutrase. The progress of the hydrolysis was analyzed by monitoring the degree of hydrolysis (DH) (%) (Figure 1) and protein–peptide profiles obtained by RP-HPLC (Figure S3).

### 3.2 | Faba bean protein enzymatic hydrolysis

Degree of hydrolysis observed in this study was affected not only by the hydrolysis time but also by specificity, selectivity, and general mode of action of the enzymes used (Barrett et al., 2012). As shown in Figure 1, the DH values vary from 2% to 16%. Pepsin preferentially cleaves at the C-terminus of phenylalanine, leucine, tyrosine, and tryptophan (Dunn, 2001). Trypsin specifically cleaves the carboxyl side of the peptide bonds like lysine or arginine but has no effect when either side of the bond is attached to proline (Barrett et al., 2012). Neutrase is a bacterial protease produced by a selected strain of *Bacillus amyloliquefaciens* that randomly hydrolyses internal peptide bonds (Tsou, Lin, Lu, Tsui, & Chiang, 2010). Flavourzyme is a protease produced by submerged fermentation of a selected strain of *Aspergillus oryzae* and may be used to prepare short chain peptides and free amino acids with no bitter taste at neutral or slightly acidic conditions (Merz et al., 2015). The highest DH was obtained after pepsin treatment (9.5%–16.9%), which was related to protein modifications at low pH, leading to partial protein unfolding and exposure of hidden peptide bonds to promote enzyme accessibility (Barrett et al., 2004). Extensive hydrolysis was also observed by flavourzyme treatment (6.8%–12.2%), which is associated with the presence of both endopeptidases and exopeptidase in the enzyme mixture (Merz et al., 2015). Hydrolysates with DH range of 6.4%–9.9% were obtained by trypsin treatment, whereas hydrolysates treated by neutrase showed the lowest DH values (2.1%–6.4%). This might be explained by different hydrolysis conditions. The alkaline

pH (8) used for trypsin hydrolysis increases the solubility of proteins, that are not soluble as well at neutral pH used for neutrase treatment (Barrett et al., 2012; Tsou et al., 2010).

Compared to the results of other pulse proteins treated by the same proteases, faba bean proteins are more resistant to enzymatic hydrolysis than lentil or pea proteins. For example, (Avramenko, Low, & Nickerson, 2013) showed that hydrolysis of lentil protein using 0.4% of trypsin (10,600 U/mg solid) resulted in DH (%) values at  $4.4 \pm 1.2$ ,  $8.8 \pm 1.7$ , and  $19.6 \pm 0.7$  after hydrolysis times of 5, 15, and 30 min, respectively. (Bajaj et al., 2017) obtained DH of 11% and 9% after 60 min hydrolysis of pea protein isolate by using 0.4% flavourzyme ( $\geq 500$  U/g solid) and neutrase, ( $\geq 0.8$  U/g solid), respectively. The low DH (%) for faba bean protein compared to that of pea or lentil might be attributed to the greater tendency of faba bean proteins to form less soluble aggregates (Avramenko et al., 2013; Lam et al., 2017).

Solubility is an essential feature for a protein to be used as a functional ingredient in food formulations and significantly affects other protein functional properties such as foaming and emulsifying properties (Moure, Sineiro, Domínguez, & Parajó, 2006). As shown in Figure 1b, after hydrolysis, the solubility at pH 7.0 improved from 24.4% to 88.8, 82.7, 72.9, and 63.1%, respectively, for pepsin (15 min), trypsin (15 min), flavourzyme (60 min), and neutrase (60 min) hydrolysates. The significantly reduced ( $p < 0.05$ ) molecular weight and surface hydrophobicity are responsible for increased solubility of hydrolysates compared to the intact protein. Considering the amino acid composition of the obtained hydrolysates (Table 1), there was an increase of negatively charged glutamic acid, as compared to faba bean protein, which have the ability to efficiently bind water molecules and thus increase the solubility (Kramer et al., 2012). The solubility was dramatically improved ( $p < 0.05$ ), especially at pH 5 and reached 81.0% after 15 min of pepsin treatment (Figure 1c). This will allow faba bean hydrolysates to be more widely used in mild acidic formulations such as juices, which is hard for pulse proteins as solubility is low near the isoelectric point. Relatively high solubility of pepsin hydrolysates compared to other samples observed at pH 5 could be attributed to the shorter peptide chain length due to higher degree of hydrolysis (del Mar Yust et al., 2010).

### 3.3 | Structure characterizations of faba bean proteins and the hydrolysates

The amino acid compositions of the faba bean protein and the hydrolysates are shown in Table 1. The most abundant amino acids in all samples are glutamine/glutamic acid (10.34/17.58%), asparagine/aspartic acid (11.44/11.84%), and leucine (8.09%–11.74%). Similar to most legume proteins, faba bean protein is poor in sulfur containing amino acids (Jarpa-Parra et al., 2014), which was confirmed by

low levels of methionine (0.51%–0.81%) and cysteine (0%–1.21%). Faba bean proteins contain higher amounts (40.45%) of hydrophilic amino acids, including glutamine/glutamic acid, asparagine/aspartic acid, lysine, and arginine, compared to 34.13% of hydrophobic residues including alanine, valine, isoleucine, leucine, phenylalanine, and proline (Alsmeyer et al., 1974). In both hydrolysates, the % of Glx increased compared to protein extract and the total hydrophilic amino acid residues increased slightly to 41.13% and 43.37% in pepsin and neutrase hydrolysate, respectively. On the other hand, the hydrophobic amino acid residues decreased from 34.84% in faba bean extract to 33.84% and 32.43% for pepsin and neutrase hydrolysate, respectively. A similar trend of changing the amino acid pattern in hydrolysates as compared to the intact protein was observed in many other studies and depend mostly on the enzyme used, the degree of hydrolysis and on protein solubility at different hydrolysis conditions (Gbogouri et al., 2004; Muhamyankaka et al., 2013; Taheri, Anvar, Ahari, & Fogliano, 2013; Wasswa, Tang, Gu, & Yuan, 2007).

The FT-IR spectrum in the range of 1600–1700  $\text{cm}^{-1}$  is an indicator of secondary structure of proteins and peptides (Surewicz, Mantsch, & Chapman, 1993). As shown in Figure 2a, faba bean protein possessed 9 bands in amide I region, including 1695  $\text{cm}^{-1}$  ( $\beta$ -sheets/turns), 1681  $\text{cm}^{-1}$  ( $\beta$ -sheets), 1670  $\text{cm}^{-1}$  ( $\beta$ -turns), 1659  $\text{cm}^{-1}$  ( $\alpha$ -helix), 1649  $\text{cm}^{-1}$  (random coils), 1638  $\text{cm}^{-1}$  ( $\beta$ -sheets), 1628  $\text{cm}^{-1}$  ( $\beta$ -sheets), 1618  $\text{cm}^{-1}$  ( $\beta$ -sheets), and 1607  $\text{cm}^{-1}$  (vibration of amino acid residues) (Kong & Yu, 2007). The dominant secondary structure of faba bean protein in aqueous solution is  $\beta$ -sheet. After hydrolysis, the dominant  $\beta$ -sheet peak at 1638  $\text{cm}^{-1}$  disappeared, while the random coil structure increased as reflected by the increased peak at 1648  $\text{cm}^{-1}$ . In addition, a large increase in peak was observed at 1607  $\text{cm}^{-1}$  which corresponds to absorption of some amino acid backbone and side-chain

groups (especially tyrosine or glutamine), indicating they became exposed after hydrolysis. Meanwhile,  $\alpha$ -helix increased as reflected by the increased peak at 1659  $\text{cm}^{-1}$ . These results suggest that the protein was unfolded after pepsin and neutrase hydrolysis. The peak at 1618  $\text{cm}^{-1}$  is believed to be associated with the aggregation process (Surewicz et al., 1993); thus, its increase in the spectra of the hydrolysates suggests the formation of some aggregates. After protein unfolding and hydrolysis, the protein may interact with each other via the exposed hydrophobic side chains, led to some aggregation.

Surface charge values measured for faba bean protein were  $-24.6 \text{ mV} \pm 1.7$  at pH 7 and  $-14.7 \text{ mV} \pm 0.7$  at pH 5, respectively (Figure 3a). The value changed for faba bean pepsin hydrolysate to  $-27.5 \text{ mV} \pm 3.9$  at pH 7 and  $-11.8 \text{ mV} \pm 1.6$  at pH 5. The neutrase hydrolysate showed significantly ( $p < 0.05$ ) higher absolute charge values of  $-32.7 \text{ mV} \pm 3.1$  at pH 7 and  $-25.5 \text{ mV} \pm 0.7$  at pH 5. The higher negative net charge of the hydrolysates at neutral pH is caused by an increase in the number of exposed ionisable amino and carboxyl groups after protein unfolding and hydrolysis (Achouri et al., 1998). This is particularly evident in the neutrase hydrolysate (Table 1), where the highest values of absolute charge were observed at both pHs (Figure 3a).

The surface hydrophobicity values ( $H_o$ ) represent the number of hydrophobic groups on the surface of protein (Tang et al., 2008). As shown in Figure 3b, the faba bean protein showed the  $H_o$  value of  $1,387.1 \pm 33.9$ . Enzymatic treatments led to a reduction in hydrophobicity with the  $H_o$  value decreasing to  $80.6 \pm 12.4$  and  $78.8 \pm 11.2$ , for pepsin and neutrase hydrolysate, respectively. Increased number of C- and N- terminals with  $\text{COOH}^-$  and  $\text{NH}_3^+$  and charged amino acids (glutamic acid) in all the hydrolysates explained the significantly decreased surface hydrophobicity (Taheri et al., 2013).

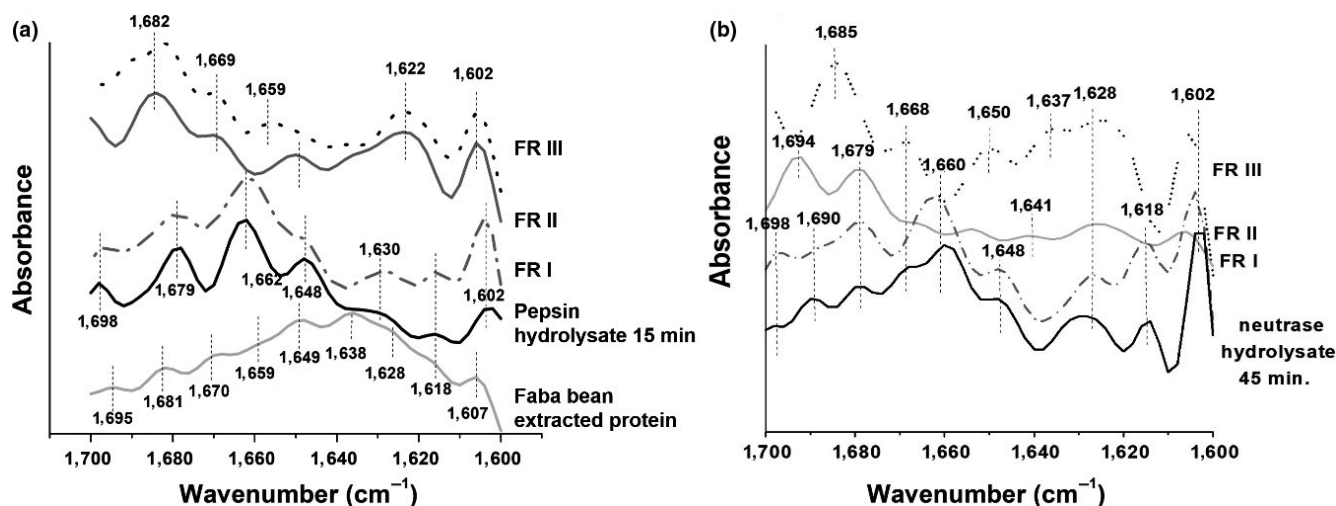


**FIGURE 2** FT-IR spectra of (a) faba bean protein, pepsin (15 min) hydrolysates and ultrafiltration fractions, and (b) neutrase (45 min) hydrolysates and ultrafiltration fractions

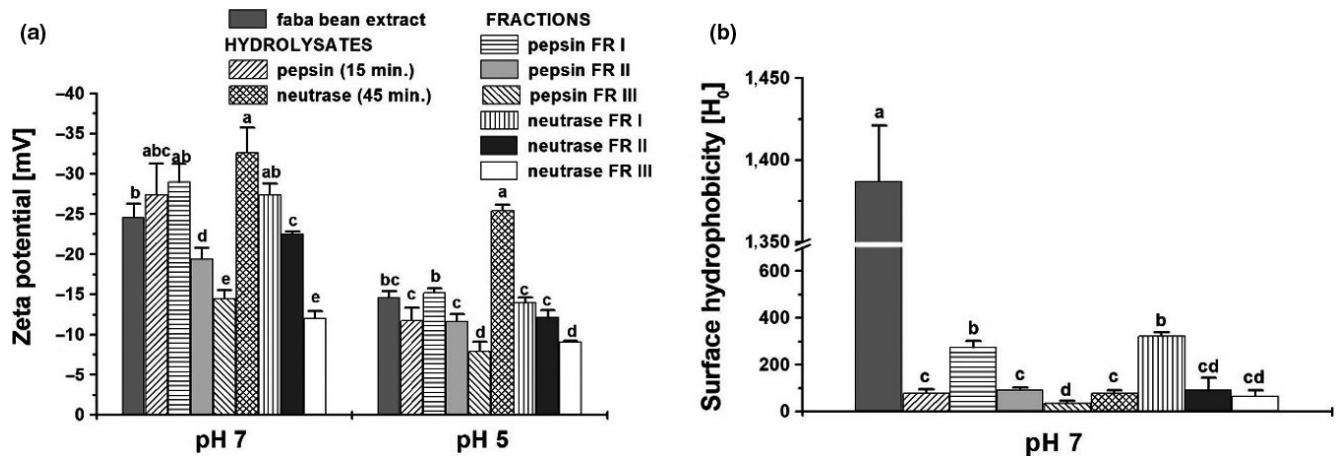


**FIGURE 3** Zeta potential (a) and surface hydrophobicity (b) of faba bean protein extract, selected hydrolysates and fractions obtained by ultrafiltration. Values with different lowercase letters for samples in the same pH are significantly ( $p < 0.05$ ) different

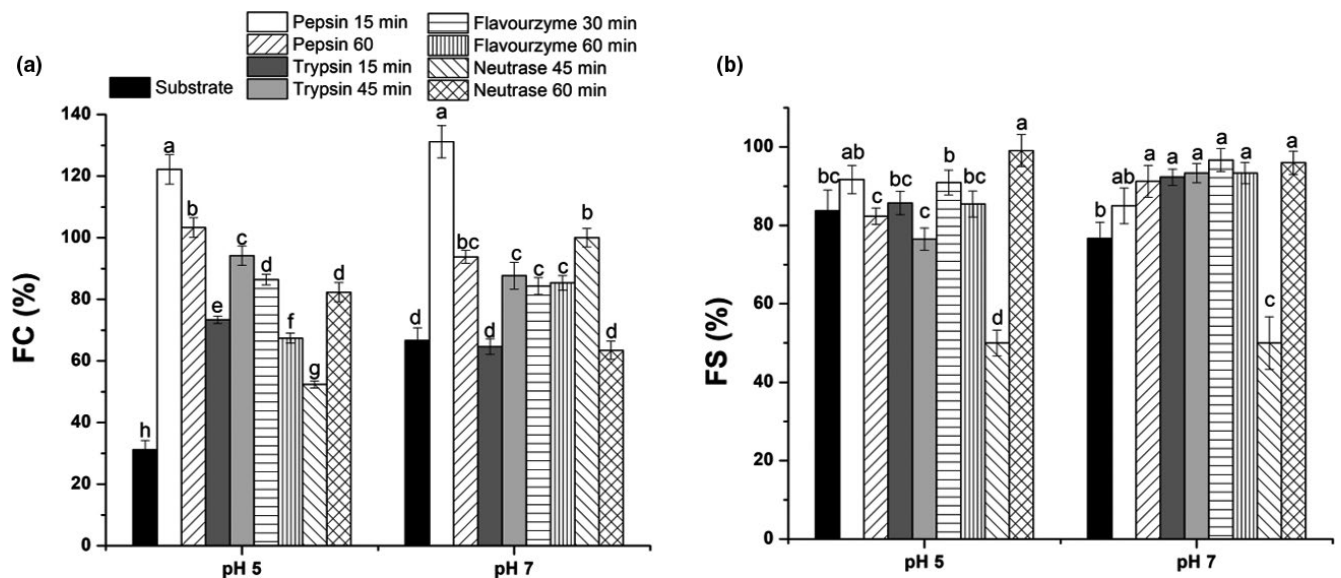


**FIGURE 4** Foaming capacity (FC) (a) and stability (FS) (b) of faba bean protein extract and the hydrolysates at pH 7 and pH 5. Values with different lowercase letters for hydrolysates at the same pH are significantly ( $p < 0.05$ ) different

### 3.4 | Evaluation of functional properties

There has been a growing interest in the food industry toward utilizing plant proteins as substitutes for animal-based proteins in new product formulations (Chandra-Hioe et al., 2016; Multari et al., 2015). Besides nutritional value, functional properties are very important characteristics of food proteins (Aydemir & Yemenicioğlu, 2013).

Foaming capacity provides a desirable and unique texture to a range of aerated foods including ice-cream, bread, and cakes. In this study, faba bean protein showed poor foaming properties with the FC value of 31.2% at pH 5 and 66.7% at pH 7 (Figure 4a), which are lower than those reported for pea (167.4%–243.7%) and lentil proteins (403%–425%) (Jarpa-Parra et al., 2015; Lam et al., 2017). This is directly related to

the low solubility of faba bean protein (Vioque et al., 2012). A significant increase ( $p < 0.05$ ) in the foaming capacity was achieved for most of the hydrolysates with the highest effect observed for pepsin treatment (122.2% at pH 5 and 131.2% at pH 7). The improved FC value is directly attributed to the improved protein solubility. The highest FC value observed for pepsin hydrolysates might be closely related to their higher degree of hydrolysis. Peptides with good solubility and smaller size are able to migrate quickly to the air-water interface and form an extensive intermolecular network (peptide–peptide interactions) (Taheri et al., 2013).

Foam stability refers to the ability of a protein to stabilize a foam against stresses and maintain its volume (Phillips, Whitehead, & Kinsella, 1994). As shown in Figure 4b, the stability of the foams containing faba bean protein

extract was good, in spite of the poor foaming capacity with  $83.8\% \pm 5.3$  and  $76.7\% \pm 4.1$  of the foam remaining at pH 5 and pH 7, respectively, after 30 min of storage at room temperature. Hydrolysis further improved foam stability as some samples showed FS value of almost 100% such as neutrase hydrolysate (60 min) at pH 5 and 7, pepsin (60 min), trypsin (15 and 45 min), and flavourzyme (30 and 60 min) hydrolysates at pH 7. The exception was the neutrase hydrolysate after 45 min treatment, which showed decreased FS values (50% at both pH 5 and 7). This might be related to its lower degree of hydrolysis. However, a longer period of neutrase treatment (60 min) resulted in higher foaming stability;  $99.1\% \pm 4.1$  at pH 5 and  $96.0\% \pm 3.3$  at pH 7, respectively.

The ability of a protein to form an emulsion under a specific set of conditions is called emulsifying activity index (EAI) and emulsion stability index (ESI) (Pearce & Kinsella, 1978). EAI provides an estimation of the interfacial area stabilized per unit weight of protein based on the turbidity of a diluted emulsion, while ESI provides a measure of the stability of the same diluted emulsion over a defined time period (Pearce & Kinsella, 1978). In the present study, emulsifying properties were measured only at pH 7.0, since a number of studies have shown that legume proteins have optimal emulsifying

properties at pH values away from their isoelectric points (Ghribi et al., 2015; Karaca et al., 2011; Lam et al., 2017). As shown in Figure 5a,b, EAI and ESI values of faba bean protein were  $36.4 \text{ m}^2/\text{g} \pm 1.4$  and  $48.1 \text{ min} \pm 2.2$ , respectively. As reported by (Karaca et al., 2011), EAI values for proteins from pea, lentil, and chickpea by similar extraction method ranged between 42.9 and  $47.9 \text{ m}^2/\text{g}$ . The low solubility of faba bean protein may also explain the relatively lower EAI, together with its compact structure. According to (Pearce & Kinsella, 1978), proteins with high molecular flexibility and mobility tend to show the greatest surface activity. As shown in Figure 5, hydrolysis did not lead to marked changes ( $p < 0.05$ ) in emulsifying properties ( $41.45\text{--}29.85 \text{ m}^2/\text{g}$ ) compared to the protein extract. Flavourzyme hydrolysates showed low EAI values ( $5.90\text{--}6.96 \text{ m}^2/\text{g}$ ), while ESI values were high ( $91.6 \text{ min} \pm 2.4$  for flavourzyme (30 min) hydrolysates). Solubility increased with reduced molecular weight due to protein migration to the oil–water interface, but peptides with molecular weight that was too low could not form a strong and viscoelastic interfacial network to stabilize emulsions (Ghribi et al., 2015). The hydrolysate samples with optimal molecular size and good solubility may have increased capacity to form stronger viscoelastic films at the oil droplets surface to stabilize emulsions, although they were not able

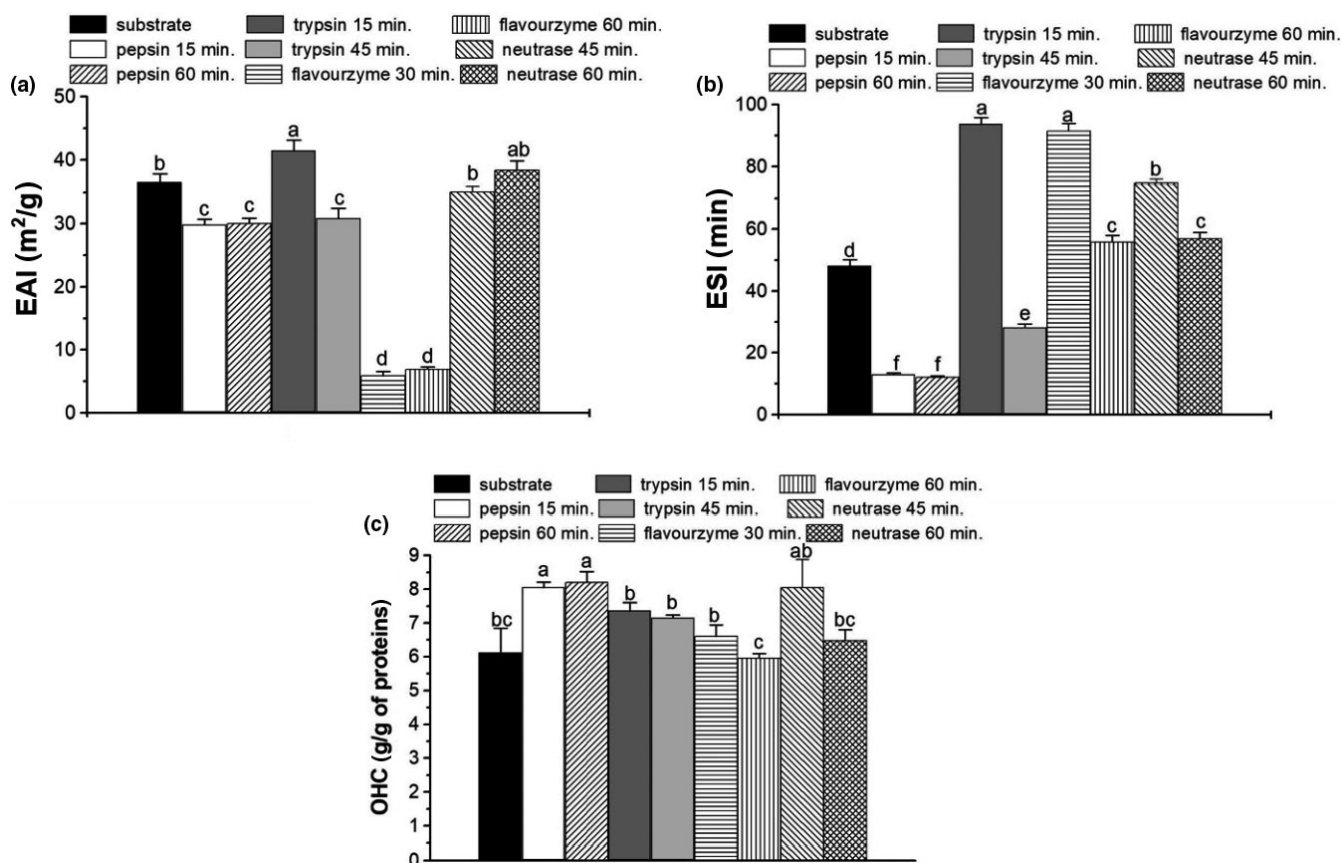


**FIGURE 5** Emulsifying activity index (EAI) (a), emulsion stability index (ESI) (b), and oil holding capacity (OHC) (c) of faba bean protein extract and the hydrolysates at pH 7. Values with different lowercase letters for different properties are significantly ( $p < 0.05$ ) different

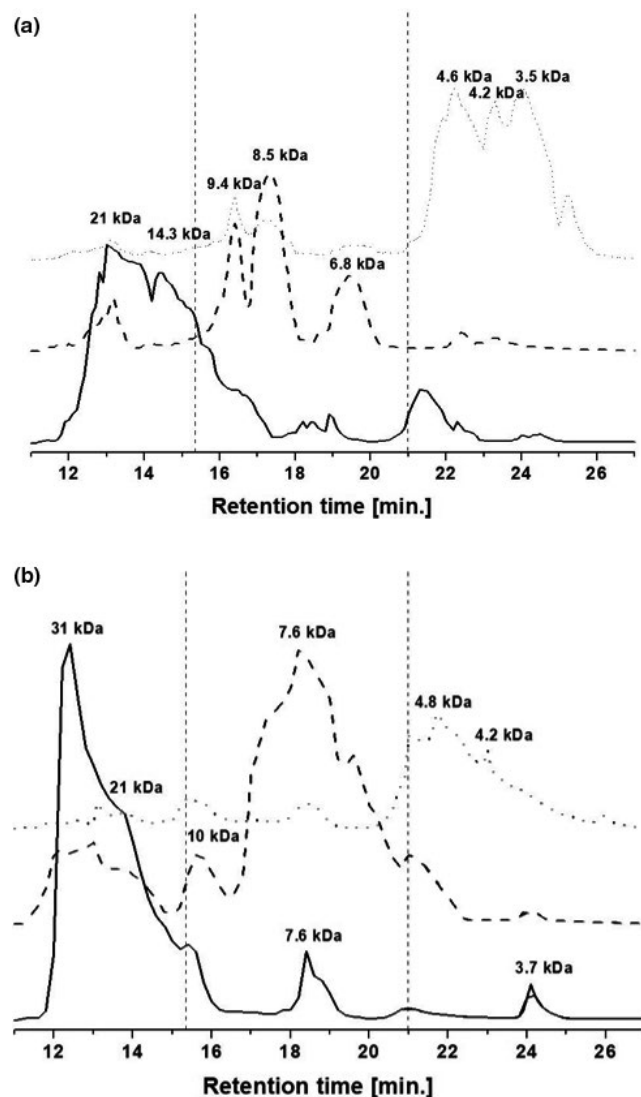


**FIGURE 6** SEC chromatogram of faba bean fractions of pepsin (15 min) (a) and neutrase (45 min) (b) hydrolysates obtained after ultrafiltration. FR I (>10 kDa)—solid line, FR II (5–10 kDa)—dash line, FR III (<5 kDa)—dot line

to increase the emulsifying capacity. This was demonstrated with the pepsin hydrolysates with larger DH between 9.5% and 16.9% that showed the lowest ESI values (Figure 5a).

Oil holding capacity (OHC) refers to the ability to imbibe and retain oil within a protein matrix and is regularly used as synonymous with oil binding capacity (Lam et al., 2017). The OHC value of faba bean protein was high (6.12 g/g of protein) (Figure 5c). Lupin protein hydrolysates, soy, and maize concentrates and hydrolysates showed OHC range of 2.59–4.72 g/g of protein (Lqari et al., 2005; Soria-Hernández, Serna-Saldívar, & Chuck-Hernández, 2015; Wasswa et al., 2007). According to (Lam et al., 2017), OHC ranged from 3.1 to 3.3 g/g in different pea cultivars. The enzyme hydrolysis further increased the fat absorption capacity, especially for the pepsin hydrolysates with an OHC value of 8.21 g/g of protein. This is likely due to the unfolding of protein

structure, that allowed more functional groups to bind hydrocarbon moieties of oil through hydrophobic, electrostatic, and hydrogen bonding (Lqari et al., 2005; Soria-Hernández et al., 2015).

### 3.5 | Fractionation of protein hydrolysates

The initial protein hydrolysates produced by enzymatic treatment contains a wide range of peptides that differ in chain length, hydrophobicity, net charge, and activity (Ghribi et al., 2015; Muhamyankaka et al., 2013). It has been proven, that the molecular weight of peptides in hydrolysates has a great impact on their functionality, hence pressure-driven membrane separations can be used as a second step to increase their specific functional properties (Berot, Popineau, Compoin, Blassel, & Chaufer, 2001; Jeon et al., 1999). Membrane separations are widely used in industry to concentrate and fractionate dairy proteins (Berot et al., 2001; D'Alvise, Lesueur-Lambert, Fertin, Dhulster, & Guillochon, 2000). Membrane fractionation of faba bean protein has never been reported. In this study, ultrafiltration was used to fractionate two selected faba bean hydrolysates with important functional properties, namely pepsin hydrolysate (15 min) with the highest solubility and foaming capacity and neutrase hydrolysate (45 min) with good emulsifying properties and oil holding capacity.

Three fractions (FR) containing peptides of different molecular weights were prepared using membranes with molecular weight cutoff (MWCO) of 10 and 5 kDa. These include FR I (Mw > 10 kDa), FR II (Mw 5–10 kDa), and FR III (Mw < 5 kDa). However, a small percentage of peptides, above or below the MWCO of each membrane were found in the analyzed fractions (Figure 6). The membranes do not perform separations with total efficiency and higher and lower molecular weight peptides can be found in several fractions (Berot et al., 2001; D'Alvise et al., 2000). Figure 6a shows the molecular weight distribution of peptides after pepsin digestion obtained after ultrafiltration. Chromatogram of FR I (Mw > 10 kDa) from pepsin hydrolysate (15 min) was characterized by major peaks at Mw of 21 kDa and 14.3 kDa, respectively. In the FR II (Mw 5–10 kDa) sample, there were three major peaks at 9.4 kDa, 8.5 kDa, and 6.8 kDa, respectively. Chromatogram of FR III (Mw < 5 kDa) was characterized by several peaks at 3.5–4.6 kDa (Figure 6a). Compared to pepsin hydrolysate fractions, the chromatogram of neutrase hydrolysate (Figure 6b) showed more large- and medium-sized peptides in FR I (peaks at 31 and 21 kDa) and FR II (peaks at 10 and 7.6 kDa), while less small sized peptides in FR III (peaks at 4.8 and 4.2 kDa). The SE-HPLC data agree with DH data that faba bean proteins were more extensively hydrolyzed by pepsin treatment (Figure 1a) to generate medium and small sized peptides.

Whereas, neutrase gradually hydrolyzed large peptides into medium-sized ones.

### 3.5.1 | Characterizations of the fraction structures

Changes in amino acid profile were also observed after ultrafiltration fractionation. For both pepsin and neutrase hydrolysate fractions, the % of hydrophilic amino acids decreased with lower peptide molecular weight. It was particularly visible for Glx residues, where the amount decreased from 15.3% in faba bean protein to 10.3% or 11.6% in FR III from pepsin or neutrase hydrolysate, respectively. In contrast, the amount of hydrophobic residues, especially Val, Leu, and Phe, increased from 5.93%, 8.34%, and 3.95% to 7.45%, 11.74%, and 5.45%, respectively, for pepsin FR III and to the level of 6.47, 10.25, and 4.88%, respectively, for the neutrase FR III (Table 1). Some hydrophobic domains might be more resistant to hydrolysis remaining in FR II and III, while the hydrophilic domains were cut into small peptides and not recovered after membrane filtration. FR II from pepsin and neutrase hydrolysates exhibited similar amounts of hydrophilic and hydrophobic segments (37.52% and 38.71% for pepsin FR II; and 39.16% and 37.75% for neutrase FR II).

Figure 2 shows the FT-IR spectra in the amide I region of the selected pepsin hydrolysate (15 min) and its fractions. The pepsin hydrolysate and FR I spectra (>10 kDa) have similar profiles and are characterized by the presence of antiparallel  $\beta$ -sheets ( $1630\text{ cm}^{-1}$ ), unordered coils ( $1648\text{ cm}^{-1}$ ), and  $\beta$ -turns ( $1679\text{ cm}^{-1}$ ) (Surewicz et al., 1993). Also, the aggregate structure remained, as shown by the peak at  $1618\text{ cm}^{-1}$ . The FR II and FR III containing the lowest molecular weight peptides showed more unfolded structures. The fractions of neutrase hydrolysates with the hydrolysate sample and FR I showed a similar profile, while FR II and FR III were more unfolded.

No significant difference ( $p < 0.05$ ) in surface charge was observed between hydrolysates and the FR I ( $M_w > 10\text{ kDa}$ ) at pH 7. Surface charge decreased with lower molecular weight fractions (FR II and III) at pH 7 and reached the values of  $-14.5\text{ mV} \pm 1.0$  and  $-12.1\text{ mV} \pm 0.9$  for FR III from pepsin and neutrase hydrolysate, respectively. This is probably caused by the decreasing number of carboxyl groups in smaller protein chains (Table 1) produced by enzymatic hydrolysis and fractionation. The variation of surface properties in the fractions was also related to their structural change and different solubility (Achouri et al., 1998). A significant decrease ( $p < 0.05$ ) was also observed at pH 5 in FR III from pepsin ( $-7.9\text{ mV} \pm 1.2$ ) and neutrase ( $-9.2\text{ mV} \pm 0.2$ ) hydrolysates, as compared to other fractions and hydrolysates (Figure 3a). Among the fractions, the highest hydrophobicity was observed for FR I of pepsin and neutrase hydrolysate at  $275.3 \pm 25.0$  and  $323.0 \pm 16.6$ , respectively. With decreasing

$M_w$ , peptide surface hydrophobicity decreased in FR II and III from pepsin and neutrase hydrolysate, and the value was between 37.9 and 67.2. Similar results were reported for protein hydrolysates from pumpkin (*Cucurbita moschata*) protein meal (Muhamyankaka et al., 2013). The peptides from FR II and III may adopt a conformation with hydrophilic groups more exposed outwards (Muhamyankaka et al., 2013); thus, lower surface hydrophobicity was observed.

### 3.5.2 | Evaluation of the fraction functional properties

The improvement of protein solubility is the most notable effect after enzymatic hydrolysis and fractionation in this study. All peptide fractions obtained by ultrafiltration exhibited higher solubility than any hydrolysates at pH 7 (Figure 7a). The highest improvement was observed in FR II and III obtained from neutrase hydrolysate (45 min), where solubility reached 100%. Generally, fractions with lower molecular weight ( $M_w$ : 5–10 kDa and  $M_w < 5\text{ kDa}$ ) were highly soluble (85%–100%) at both pH 5 and 7. However, decrease of solubility was observed for fractions containing peptides with  $M_w > 10\text{ kDa}$  ( $68.4\% \pm 2.3$  and  $40\% \pm 0.9$  for pepsin and neutrase FR I, respectively) at pH 5 ( $p < 0.05$ ) (Figure 7a). This is attributed to the presence of larger peptides with less opened structure and higher hydrophobicity, as compared to hydrolysates and other fractions (Ghribi et al., 2015; Jain, Prakash, & Radha, 2015).

As shown in Figure 7b, at pH 7, FR I and II of both hydrolysates showed comparable foaming capacity to those without fractionation. Whereas at pH 5, FR I and II showed significantly ( $p < 0.05$ ) improved foaming capacity after fractionation with the FC value increasing from  $122.2\% \pm 4.8$  to  $147.1\%$ – $152.6\%$  for pepsin hydrolysates and from  $52.4\% \pm 1.1$  to  $100.0\%$ – $120.0\%$  for neutrase hydrolysates. Reduced FC values were observed for most of the FR III samples due to their lower molecular weight. FR I and II possessed better foaming capacity probably due to larger peptides (Figure 6) which could form flexible films around the air bubbles (Muhamyankaka et al., 2013). Excellent foaming stability remained for all pepsin fractions at pH 7 and for FR I at pH 5. Whereas decreased foaming stability was observed for FR II and III from pepsin hydrolysis at pH 5, and all fractions from neutrase hydrolysis at both pH (Figure 7c). According to Lqari et al. (2005), peptides with relatively small molecular weights are capable of forming films with the air interface, but those films are not strong enough to maintain their integrity.

The emulsion capacity was improved after peptide fractionation at both pH 5 and 7 (Figure 7d). FR I and II from pepsin hydrolysate increased by 115% and 104%, respectively, reaching the EAI value of  $151.2 \pm 3.1$  for FR I and  $140.7 \pm 2.5$  for FR II. Also, FR I from neutrase hydrolysis showed

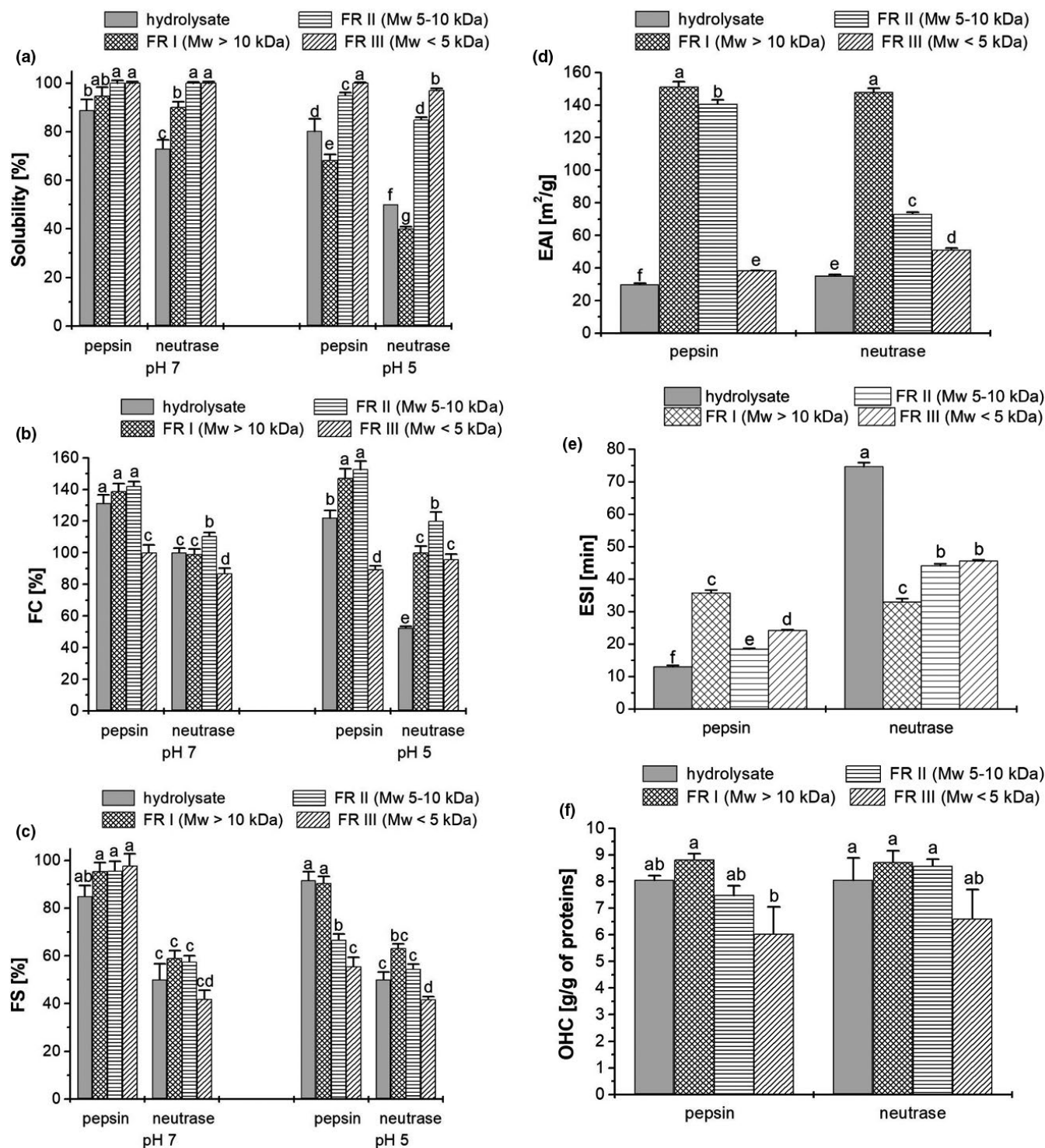


**FIGURE 7** Solubility (a), foaming capacity (FC) (b) and stability (FS) (c), emulsifying activity index (EAI) (d) and emulsion stability index (ESI) (e), and oil holding capacity (OHC) (f) of fractions obtained after ultrafiltration from pepsin (15 min) and neutrase (45 min) hydrolysates. Values with different lowercase letters for different properties at different pH are significantly ( $p < 0.05$ ) different

significant improvement of EAI (from  $35.1 \text{ m}^2/\text{g} \pm 0.8$  to  $147.9 \text{ m}^2/\text{g} \pm 2.4$ ). The lowest increase was observed for FR III from pepsin and neutrase hydrolysates, where the EAI value was  $38.2 \text{ m}^2/\text{g} \pm 0.2$  and  $51.2 \text{ m}^2/\text{g} \pm 1.0$ , respectively. It was expected that FR III would possess lower emulsifying capacity

due to its lower molecular weight. All fractions from pepsin hydrolysis showed significant ( $p < 0.05$ ) increase in ESI (from  $13.0 \text{ min} \pm 0.4$  to  $18.5\text{--}35.7 \text{ min}$ ), while the emulsifying stability was reduced for all neutrase fractions from  $74.7 \pm 1.2$  to  $33.0 \pm 1.1$ ,  $44.1 \pm 0.6$ , and  $45.6 \pm 0.4$  for FR I, II, and III,

respectively (Figure 7e). The FR I and II of pepsin hydrolysis may possess appropriate molecular weight, making them flexible and capable to form a viscoelastic membrane at the oil droplet surface, which in turn improved the emulsifying capacity (Taheri et al., 2013). FR I of pepsin and neutrase hydrolysis showed improved EAI value, but reduced ESI value, probably due to the lowest solubility among fractions (Figure 7a).

Ultrafiltration fractionation led to improvement in oil holding capacity for FR I of pepsin hydrolysate as the value increased from  $8.1 \pm 0.2$  to  $8.8 \pm 0.2$  (Figure 7f). FR I of pepsin hydrolysates and FR I and II of neutrase hydrolysates showed similar OHC values, which are also comparable to the hydrolysates without fractionation. Reduced OHC values were observed for FR III for both hydrolysates due to their low molecular weight, because smaller peptides were less capable of physically entrapping oil (Achouri et al., 1998; Taheri et al., 2013).

### 3.6 | Estimation of nutritive value based on amino acid composition

Requirements of essential amino acids were established by a report of FAO/WHO/UNU in 1985 and presented in the last column of Table 1 (FAO/WHO/UNU Energy and protein requirements, 1985). Regarding essential amino acids, it was found that all samples analyzed contained high levels of His, Thr, Val, Leu, Ile, and Phe. The nutritional qualities of the samples were also quantitatively evaluated (Table 1, lowest panel), and the results indicated that EAA/TAA values were higher than the ideal protein pattern criterion as recommended by FAO/WHO/UNU (1985), since they were all above 32%, and reached the highest value of 43.8% and 41.8% in case of FR III from pepsin hydrolysis and FR II from neutrase hydrolysis, respectively. Amino acid composition data were also used to predict several other nutritional parameters (Table 1, lowest panel) by calculating amino acid score (AAS) as well as to estimate protein efficiency ratio (PER) or theoretical biological value (BV). Interestingly, predicted theoretical biological value (BV) was the highest in faba bean protein extract, pepsin treated hydrolysates, and pepsin Fraction I. The predicted PER, which has been recognized as a good estimate of protein quality, ranged between 2.71 and 5.82 for all the investigated samples, with the highest values for FR III from pepsin and neutrase hydrolysates ranging between 4.42–5.82 and 3.73–5.20, respectively (Table 1). Based on the FAO/WHO/UNU (1985) – suggested pattern for adults (Table 1), it is possible to consider faba bean hydrolysates and obtained fractions as good sources of essential amino acids, especially His, Thr, Val, Lys, Leu, and Ile. In addition, fractionation further increased nutritive value in terms of essential amino acids like Val, Lys, Ile, and Leu. Therefore, faba bean hydrolysates and fractions obtained after ultrafiltration meet the nutritional requirements for

body performance and may be used as protein supplements to provide certain essential amino acids.

## 4 | CONCLUSIONS

This research demonstrated that enzymatic hydrolysis in combination with ultrafiltration can provide a strategy to significantly improve faba bean protein solubility and functional properties. The faba bean pepsin hydrolysate obtained after 15 min of enzymatic treatment showed better functional properties compared to the untreated sample, especially in terms of foaming and oil holding capacity. Fraction I (Mw > 10 kDa) and II (Mw: 5–10 kDa) obtained after pepsin hydrolysis and ultrafiltration demonstrated further improved foaming and oil holding capacity and much improved emulsifying capacity. It was also observed that the molecular weight has significant impact on faba bean functional properties, wherein some properties increased, some decreased with the same Mw range. In addition to Mw, other physiochemical properties impact functional properties, such as amino acid composition, hydrophobicity, and surface charge. In general, FR II and III showed high solubility due to less compact structure of the peptides, whereas FR I and II have high functional properties in general due to a better balance of the hydrophobic/hydrophilic residues and presence of higher molecular weight peptides. The FR II (5–10 kDa) from faba bean with both high solubility and good functional properties can be potentially used as functional ingredients in food formulations, such as baked goods, salad dressings, or meat products. In addition, faba bean hydrolysates and fractions have good nutritive value.

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