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Effect of Enzymatic Hydrolysis on Solubility and Emulsifying Properties of Lupin Proteins (*Lupinus luteus*)

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Abstract: Solubility and emulsifying properties are important functional properties associated with proteins. However, many plant proteins have lower techno-functional properties, which limit their functional performance in many formulations. Therefore, the objective of this study was to investigate the effect of protein hydrolysis by commercial enzymes to improve their solubility and emulsifying properties. Lupin protein isolate (LPI) was hydrolyzed by 7 commercial proteases using different E/S ratios and hydrolysis times while the solubility and emulsifying properties were evaluated. The results showed that neutral and alkaline proteases are most efficient in hydrolyzing lupin proteins than acidic proteases. Among the proteases, Protamex® (alkaline protease) showed the highest DH values after 5 h of protein hydrolysis. Meanwhile, protein solubility of LPI hydrolysates was significantly higher ($p < 0.05$) than untreated LPI at all pH analyzed values. Moreover, the emulsifying capacity (EC) of undigested LPI was lower than most of the hydrolysates, except for acidic proteases, while emulsifying stability (ES) was significantly higher ($p < 0.05$) than most LPI hydrolysates by acidic proteases, except for LPI hydrolyzed with Acid Stable Protease with an E/S ratio of 0.04. In conclusion, the solubility, and emulsifying properties of lupin (*Lupinus luteus*) proteins can be improved by enzymatic hydrolysis using commercial enzymes.

Keywords: emulsifying properties; solubility; protein hydrolysis; lupin proteins

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1. Introduction

In recent years, the food and pharmaceutical industries have turned their attention to plant-derived materials due to their considerable advantages over animal-derived ingredients, such as a low prevalence of infection and contamination, dietary beliefs and practices, vegetarian habits, versatility, and lower cost [1]. In addition, an important food trend is the formulation of foods directed to minimize the effects of animal-derived ingredients on the environment, human health, and animal welfare [2]. Thus, the consumption of plant protein ingredients, chiefly from legumes, has increased sharply in the last decade as an alternative to animal proteins [3]. Since most plant proteins are composed of a mixture of different protein fractions, which have different isoelectric points (pI) [4]. Therefore, the modification of their characteristics to enhance their functional properties is necessary [1].

Among functional properties, solubility and emulsifying properties are important protein-associated properties [5]. Protein solubility mainly determines the potential use of a protein-based ingredient in food formulations [6]. In addition, plant proteins are widely used as natural emulsifiers to stabilize oil-in-water (O/W) emulsions due to their amphiphilic properties, nutritional benefits, increased consumer acceptability, and low cost [7]. Thus, proteins are relevant due to the fact that they are the most important surface-active compounds in plant materials [8]. However, several plant proteins present low water-solubility and emulsifying properties in comparison to synthetic surfactants, which restrain their performance in many aqueous-based food formulations [9]. Therefore, some vegetable proteins must be modified to improve their functional properties and performance. In

general, protein modifications can be reached by physical, chemical, and biological methods. To date, some studies have addressed extensive or mild protein hydrolysis as a way to tailor the functional properties of plant proteins [10–19]. Liu et al. [11] found that mild hydrolysis of rice protein isolates resulted in the enhancement of emulsifying properties. In addition, Eckert et al. [14] showed that the extensive hydrolysis of fava bean proteins enhanced their solubility, foaming, and emulsifying properties. However, the plant protein modifications depend on the processing type, pH, concentration, and original structure [20].

The enzymatic hydrolysis by proteases stands out to be the most auspicious method for protein modification to formulate tailor-made foods, particularly due to reactants and by-products being innocuous [1,21]. Also, this type of modification presents some advantages over other modification methods, such as it can be reached by moderate conditions with only a few by-products, the chemical composition of the protein being preserved, and the fast enzyme reaction time and specificity [1]. Proteases based on characteristic mechanistic features are classified into six groups: cysteine, aspartate, glutamate, serine, metallo, and threonine [22]. There is a wide variety of proteases in commercial use, which range from detergent additives to therapeutics [23]. However, proteases today are becoming increasingly used to improve the functional properties of proteins. Concerning improvements in the emulsifying capacity of enzymatic hydrolysis derivatives of proteins, it has been found to depend on the protein source and hydrolysis conditions, such as the degree of hydrolysis, the type of enzyme, and the pH of the medium [24]. Currently, some research has analyzed the emulsifying properties of different proteins hydrolyzed with enzymes. For instance, Xu et al. [25] showed that partially hydrolyzed rice protein enhanced its emulsion stability. Also, Padial-Domínguez et al. [24] found that partially hydrolyzed soy protein presented improved emulsifying properties than its counterpart. Otherwise, Avramenko et al. [7] found that partially hydrolyzed lentil protein reduced their solubility and emulsifying properties as a function of the degree of hydrolysis (%).

However, the effect of enzymatic hydrolysis on the functional properties of the legume family *Lupinus* (family of *Fabaceae*) has been scarcely investigated up to now. *Lupinus* is a promising raw material due to its high amount of proteins (33 to 40% of dry weight) and excellent amino acid profile [26–28]. Amongst the sweet lupin species (*Lupinus luteus*, *Lupinus angustifolius*, and *Lupinus albus*), yellow lupin (*Lupinus luteus*) possesses very high protein content and high fibre content [29]. Thus, the lupin sweet variety Alu*Prot*-CGNA® contains around 60% (dry weight) of proteins in dehulled seeds [30]. The main seed storage lupin proteins are globular proteins (globulins), which are divided into four protein families: α-conglutin (11S globulins), β-conglutin (7S globulins), γ-conglutin (7S basic globulins), and δ-conglutin (2S sulphur-rich albumins), of which only α-conglutin, β-conglutin, and δ-conglutin are found in the lupin protein isolate (LPI) [3].

Consequently, this study aims to research the effect of enzymatic modifications of lupin proteins on the solubility and emulsifying properties produced by some commercial enzymes. Thus, enzymatic hydrolysis of lupin proteins could enhance their emulsifying properties to be used as a natural emulsifier of O/W emulsions for food formulations. It is important to mention that the effect of protein modifications of the lupin protein-rich variety Alu*Prot*-CGNA® has not been previously investigated. Therefore, the knowledge acquired from this research could be utilized to select the appropriate enzyme and hydrolysis conditions for a specific food application.

2. Materials and Methods

2.1. Materials

Dehulled yellow lupin seeds (*Lupinus luteus*) variety Alu*Prot*-CGNA® were provided by CGNA (Agriaquaculture Nutritional Genomic Center, Temuco, Chile). Analytical grade chemicals such as hydrochloric acid (HCl), sodium hydroxide (NaOH), Tris, glycerol, glycine, sodium dodecyl sulphate (SDS), bromophenol blue (BPB), and 2-mercaptoethanol were acquired from Sigma (St. Louis, MO, USA). The information about commercial enzymes is listed in Table 1.

Table 1. Sources and properties of the commercial enzymes used.

Enzyme	Type	Biological Source	Supplier	Activity (Under Optimal Conditions)
Acid Stable Protease	Aspartic endopeptidase	*Aspergillus niger*	Bio-Cat (Troy, VA, USA)	4000 SAP/g
Fungal Protease A	Aspartic exo- and endopeptidase	*Aspergillus oryzae*	Bio-Cat (Troy, VA, USA)	1,000,000 HUT/g
Opti-Ziome™ P^3 Hydrolyzer™	Aspartic exo- and endopeptidase	*Aspergillus oryzae*, *Aspergillus melleus*	Bio-Cat (Troy, VA, USA)	130,000 HUT/g
Neutral Protease	Metallo endopeptidase	*Bacillus subtilis*	Bio-Cat (Troy, VA, USA)	2,000,000 PC/g
Protamex®	Serine endopeptidase	*Bacillus licheniformis*, *Bacillus amyloliquefacies*	Novozymes A/S (Bagsværd, Denmark)	1.5 AU-N/g
Alcaline Protease L	Serine endopeptidase	*Bacillus licheniformis*	Bio-Cat (Troy, VA, USA)	625,000 DU/g
Alcalase® 2.4 L FG	Serine endopeptidase	*Bacillus licheniformis*	Novozymes A/S (Bagsværd, Denmark)	2.4 AU-A/g

2.2. Preparation of Lupin Protein Isolate (LPI)

Lupin protein isolate (LPI) from lupin seeds was obtained according to the procedure described by Burgos-Díaz et al. [31] with minor modifications. The lupin seeds were milled using a rotor mill (Fritsch Mill Pulverisette 14, Indar-Oberstein, Germany) at 10,000 rpm and sieved to 200 µm size to turn into flour. Subsequently, the flour was suspended in a purified water ratio of 1:10 (w/v), and the pH was adjusted to 9.0 ± 0.1 by the addition of 1 M NaOH and stirred for 1 h at room temperature. Later, the suspension was centrifuged using a laboratory-scale centrifuge (GYROZEN 1580R, Daejeon, Korea) at 3500 rpm for 15 min at 20 °C. The supernatant containing the extracted proteins was shifted to the isoelectric point (pI) at pH 4.6 ± 0.1 using 1 M HCl to precipitate proteins and stirred for 1 h at room temperature. Afterwards, the suspension was subjected to centrifugation (GYROZEN 1580R, Daejeon, Korea) to separate the proteins under the same conditions mentioned above. The precipitated protein was neutralized by re-suspending it in purified water (1:5, w/v), bringing the pH to a value of 7.0 ± 0.1 using 1 M NaOH and frozen to subsequently freeze-dried using a lyophilizer (Liobras, Liotop LP1280, São Carlos, Brazil). Finally, the LPI powder was kept in a plastic bag and stored at room temperature until later use.

2.3. Chemical Analysis of LPI

The chemical analysis of LPI samples was carried out through the use of a Dumas Nitrogen Analyser (Dumatherm® N Pro, Königswinter, Germany) to determine the nitrogen content of LPI and then multiplied by a conversion factor of 6.25. The moisture was measured using the gravimetric method according to NCh 841 of 78.

2.4. Enzymatic Hydrolysis of LPI

LPI samples were enzymatically hydrolyzed in a 0.5 L bioreactor with pH- and temperature-adjusted to the optimal conditions of each commercial protease (Table 2). LPI suspensions were prepared by diluting LPI in purified water (1.5:10, w/v) and stirring until complete hydration. Three enzyme-to-substrate ratios (E/S) were chosen (0.01, 0.02, and 0.04% mg enzyme/g protein). Later, the enzyme was added to protein suspension and stirred while temperature and pH were kept constant. The proteins were hydrolyzed from 1 to 5 h and, after hydrolysis, heated at 90 °C for 5 min for enzyme inactivation. Subsequently, the protein hydrolysates were cooled and brought to neutral pH (7.0 ± 0.1) for neutralization. Finally, the hydrolysates samples were lyophilizer (Liobras, Liotop LP1280, São Carlos, Brazil) and milled using a rotor mill (Fritsch Mill Pulverisette 14, Indar-Oberstein, Germany) at 10,000 rpm and sieved to 200 µm size to turn into flour. The control samples used in this study were treated under the same processing conditions without the addition of the enzymes. The hydrolysis and control samples were performed in triplicate.

Table 2. Characteristics of the protease preparations during the LPI hydrolysis.

Enzyme	E/S (%)	Temperature (°C)	pH Value	Time (h)
Acid Stable Protease	0.01/0.02/0.04	55	2.5	1/2/5
Fungal Protease A	0.01/0.02/0.04	60	3.0	1/2/5
Opti-Ziome™ P³ Hydrolyzer™	0.01/0.02/0.04	60	6.0	1/2/5
Neutral Protease	0.01/0.02/0.04	55	7.0	1/2/5
Protamex®	0.01/0.02/0.04	55	8.0	1/2/5
Alcaline Protease L	0.01/0.02/0.04	55	8.5	1/2/5
Alcalase® 2.4 L FG	0.01/0.02/0.04	70	9.0	1/2/5

2.5. SDS-PAGE Analysis

The molecular weight (MW) distribution of protein dispersions was done by reducing SDS-PAGE electrophoresis in a mini-vertical gel electrophoresis unit (Mini-PROTEAN® Tetra Cell, Bio-Rad Laboratories, Inc., Richmond, CA, USA) as described by Cepero-Betancourt et al. [32]. For analyses, the sample was diluted in sample buffer (1:1), which consisted of 0.5 M Tris–HCl; pH 6.8; 2% *v*/*v* SDS; 2.5% *v*/*v* glycerol; 0.2% *v*/*v* bromophenol blue; and 0.5% *v*/*v* 2-mercaptoethanol and incubated at 90 °C for 4 min using a digital dry bath (Accu Block™, Labnet International Inc., Edison, NJ, USA). The gel bands were visualized by Coomassie staining G-250, while gel images were visualized using a digital imaging system (NUGenius, Syngene, Cambridge, UK). An amount of 15 µL of each sample and 12 µL of pre-stained standard molecular weight marker (Precision Plus Protein Kaleidoscope, Bio-Rad Laboratories, Hercules, CA, USA) were loaded on a 12% Tris–HCl Mini-PROTEAN TGX Precast Gel (Bio-Rad Laboratories Inc., Hercules, CA, USA) and SDS-PAGE was carried out at a constant voltage of 200 V.

2.6. Degree of Hydrolysis (DH)

The degree of hydrolysis (DH) attained was determined by the OPA method, according to Opazo-Navarrete et al. [33]. The OPA reagent (100 mL) was prepared the same day and stored in a bottle protected from light. L-serine was used to prepare the standard curve (50–200 mg/L). For determination, 200 µL of the sample (or standard) was mixed with 1.5 mL of OPA reagent. Finally, the samples were measured after 3 min of reaction with the OPA reagent at 340 nm using an HT Multi-Detection Microplate reader (Biotek Instruments Inc., Winooski, VT, USA). To determine the DH values of hydrolyzed and unhydrolyzed samples, absorbance values were converted to free amino groups (mmol/L) utilizing the standard curve and subtracting the free amino groups that were already present in the protein samples. Finally, serine amino equivalents (N-Terminal Serine) were utilized to express the free amino groups. Thus, the DH values were estimated using the following equations:

$$\text{DH } (\%) = \frac{\text{h}}{\text{h}_{\text{tot}}} \times 100 \quad (1)$$

$$\text{h} = \frac{\text{Serine NH}_2 - \beta}{\alpha} \quad (2)$$

where the constant values α and β used were equal to 1 and 0.4, respectively [34]. The h_{tot} value was calculated based on the concentration of each amino acid present in the lupin proteins, which was 7.73 meq/g. All the measurements were done in triplicate.

2.7. Protein Solubility

The protein solubility (%) of the hydrolyzed and unhydrolyzed LPI samples was determined in triplicate according to the method described by Morr et al. [35] with some modifications. For each measurement, was prepared a 3% (*w*/*v*) protein suspension in purified water. The pH value of suspensions was adjusted to pH 5.0, 7.0, and 9.0 by adding 0.1 M HCl or 0.1 M NaOH as appropriate. Subsequently, the protein suspensions were stirred for 1 h at room temperature, centrifuged at 15,520× *g* for 15 min at 20 °C (GYROZEN

1580R, Daejeon, Korea), and the supernatant was transferred to another tube to separate from the non-dissolved fraction. The supernatant was frozen at −20 °C and freeze-dried using a lyophilizer (Liobras, Liotop LP1280, São Carlos, Brazil). Finally, the freeze-dried sample was weight, and the protein content was determined by Dumas according to the methodology described above (Section 2.3), while protein solubility was estimated as follows:

$$\text{Protein solubility } (\%) = \frac{\text{sample mass supernatant (mg)} \times \text{protein content supernantant (\%)}}{\text{sample mass (mg)} \times \text{protein content (\%)}} \times 100 \quad (3)$$

2.8. Emulsifying Capacity (EC) and Emulsion Stability (ES)

The emulsifying capacity (EC) and emulsion stability (ES) were determined according to Burgos-Díaz et al. [36] with minor modifications. Thus, 0.2 g of LPI powder was added to 20 mL of distilled water (1%, w/v) in a 50-mL Falcon tube and shaken for 1 h at room temperature through constant stirring using an orbital shaker (Multi Reax, Heidolph Instruments, Schwabach, Germany). Subsequently, the sample was kept at 4 °C overnight. Further, the sample was adjusted to pH 7, 20 mL of sunflower oil was added (1:1), and the sample was homogenized for 2.5 min at 10,000 rpm utilizing an Ultra-Turrax (IKA-Werke GmbH & Co. KG, Staufen, Germany). After, the sample was allowed to stand at room temperature for 1 h. The emulsions were transferred to the test tubes, while total and emulsion layer height was measured at 0 and 24 h. The EC_{24} was estimated as the relation between the height of the emulsion layer at 24 h (H_{EL}) and the total height of the sample (H_T). For its part, the ES was calculated by dividing the EC_{24} by the EC at the start time (EC_0).

$$\text{EC } (\%) = \frac{H_{EL}}{H_T} \times 100, \quad (4)$$

$$\text{ES}(\%) = \frac{EC_{24}}{EC_0}. \quad (5)$$

2.9. Statistical Analysis

Statistical analysis was performed using Statgraphics Centurion XVI version 16.1.11 software (Statistical Graphics Corp., Herndon, VA, USA). The data were subjected to a one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) to determine significance differences ($p < 0.05$). All results in this research were expressed as mean values ± standard deviations.

3. Results and Discussions

3.1. Enzymatic Hydrolysis of Lupin Proteins

The Dumas analysis of LPI and their hydrolysates presented a protein content of 94.3% ± 0.2, and the dry matter was 98.3%. The degree of hydrolysis (DH) analyzed by the OPA method showed that LPI samples without adding enzymes presented a DH value of 0.82% ± 0.01. DH values differed by every enzyme treatment according to their protease specificity. The DH values of all enzymes progressed with the advancement of the hydrolysis time (Figure 1). Meanwhile, the Neutral Protease showed a fast hydrolysis rate during the first 3 h, and the rate subsequently decreased until reaching a stationary. This is typical behaviour of enzymatic reactions where the rate of hydrolysis decreases, which is due to a decrease of peptide bonds, causing the proteases and their substrates to reach a saturation state [37]. However, acidic proteases and Alcaline Protease L showed a low hydrolysis rate with a linear behaviour. Therefore, different results within the same protease family might be due to substrate specificity.

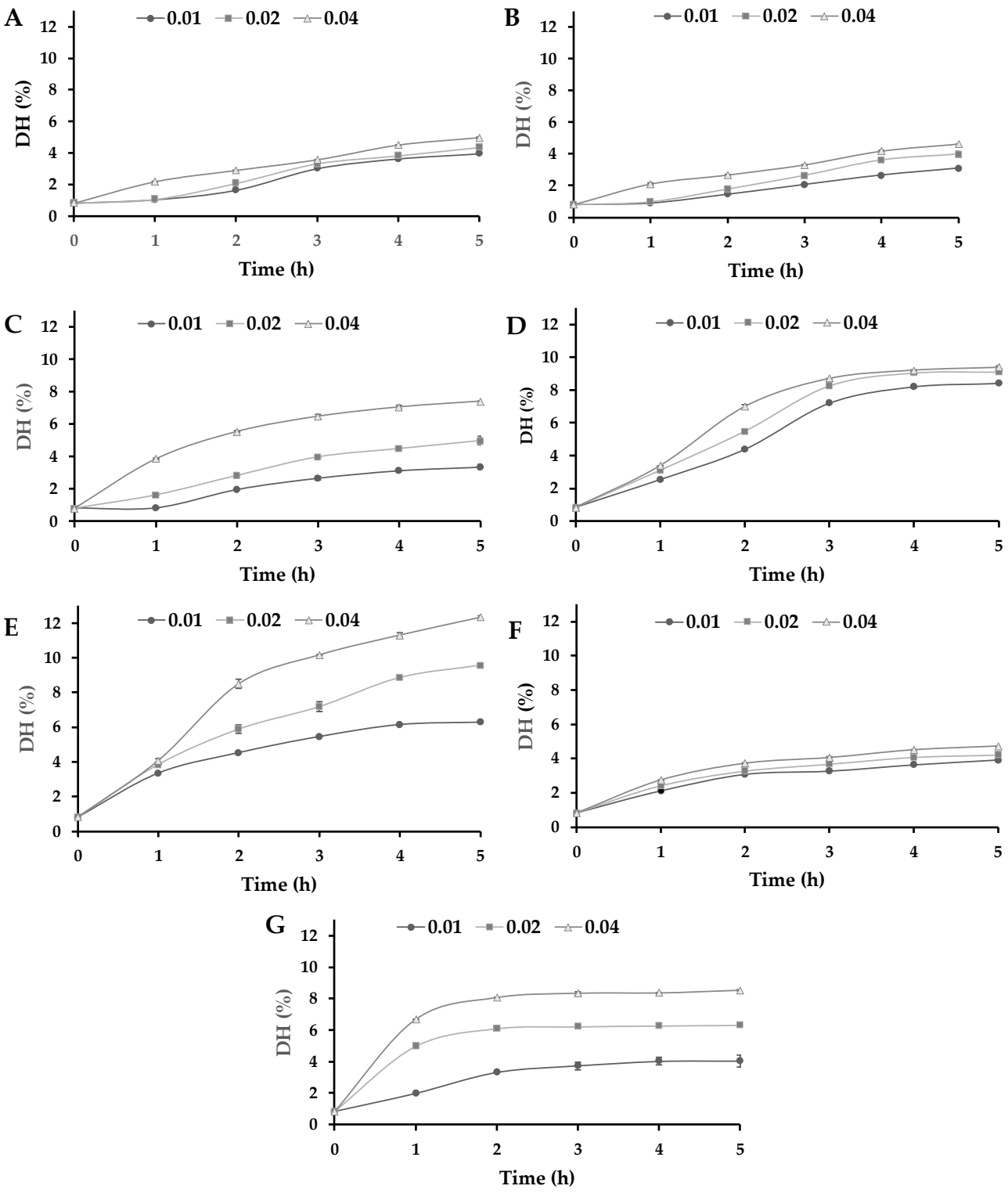

Figure 1. Degree of hydrolysis (DH) of LPI hydrolysates by different commercial enzymes. (**A**) Acid Stable Protease, (**B**) Fungal Protease A, (**C**) Opti-Ziome^{TM} P^{3} Hydrolyzer^{TM}, (**D**) Neutral Protease, (**E**) Protamex®, (**F**) Alcaline Protease L, and (**G**) Alcalase® 2.4 L FG.

The DH was strongly dependent on the E/S ratio in most of the enzymes except for aspartic proteases (Acid Stable Protease and Fungal Protease A) and a serine protease (Alcaline Protease L), where the increase in the E/S ratio does not have a great influence in the DH values. Among the proteases, Protamex®, Neutral Protease, and Alcalase® 2.4 L

FG showed higher DH values after 5 h of protein hydrolysis. This result is different from those previously found in hydrolyzed pea protein isolate (PPI) using Protamex® at an E/S ratio of 0.5% [21]. In the mentioned study, after 2 h of hydrolysis reached a DH value of 4.15%, which is lower than those found in our study (8.51%) using an E/S ratio of 0.04%. In addition, García Arteaga et al. [21] showed a higher DH value for PPI hydrolyzed by Alcalase® 2.4 L FG (9.24%) for 2 h at an E/S ratio of 0.5%, which is a 12.5-times higher E/S ratio that used in this study. However, the protein hydrolysis of LPI by Alcalase® 2.4 L FG showed to be strongly dependent on the E/S ratio. Thus, higher DH values could be expected using the same E/S ratio.

On the other hand, acidic proteases showed lower DH values in comparison with alkaline and neutral proteases. Shu et al. [38] indicated that alkaline protease-assisted reactions exhibited higher DH values compared to neutral or acid enzymes from microbial, animal, or plant origin. However, the Alcaline Protease L exhibited lower DH values than the other alkaline enzymes. This could indicate a lower activity of Alcaline Protease L to hydrolyze lupin proteins. Meanwhile, the lower DH values of the acid proteases could be a consequence of the pHs utilized to hydrolyze lupin proteins with these enzymes (2.5 and 3.0) near the pI of the lupin proteins (4.5), which could result in a certain degree of aggregation and masking of cleavage sites. This phenomenon was previously described by Abdel-Hamid et al. [39] in a study on buffalo milk proteins. Nevertheless, Alcaline Protease L seems to be an exception due to the LPI hydrolyzed by this enzyme exhibiting lower DH values in comparison with other alkaline proteases, which could be a consequence of a lower activity at these E/S relations in comparison with the other alkaline proteases used in this study.

3.2. Molecular Weight Distributions (SDS-PAGE)

Along with the DH analysis, the molecular weight distribution of LPI hydrolysates under reducing conditions was utilized for the analysis of protein hydrolysis (Figure 2). The obtained SDS–PAGE analysis showed that enzymatic hydrolysis has an influence on molecular weight distribution, especially regarding high molecular weight fractions. Thus, all treatments hydrolyze the lupin proteins into smaller fragments with molecular sizes below 50 kD. However, acidic proteases (Acid Stable Protease and Fungal Protease A) hydrolyze the lupin proteins into polypeptides with molecular sizes below 20 kD (Figure 2A,B). Burgos-Díaz et al. [30] found that the major bands in the LPI variety Alu*Prot*-CGNA® are observed between 69 and 39 kD, while some low molecular weight bands are observed below 20 kD. In addition, they found that albumins have a high molecular weight of around 90 kD, α-conglutin has a molecular weight of around 50 kD, and β-conglutin shows a molecular weight of around 69 kD. Therefore, the results suggested that acid proteases can hydrolyze all fractions of LPI higher than 20 kD, while albumins and β-conglutin fractions are mainly hydrolyzed by neutral and alkaline proteases. This is an important issue due to polypeptides of β-conglutin with molecular weights >40 kD have been identified as the major allergens in lupin [19,40]. Thus, acid proteases showed to be the most effective enzymes for breakdown proteins into polypeptides. However, neutral proteases are most effective to hydrolyzed lupin proteins into amino acids (Figure 1).

According to the SDS-PAGE results, the E/S ratios seem to be an effect mainly in α- and β-conglutin fractions, while the time had an effect in the increase of the hydrolysis of the same fractions, which is evidenced by the faded of these bands. However, in this study, clear differences were observed among the different E/S ratios and hydrolysis times, which cannot be correlated with the DH results. These results are important because they could potentially be used to predict some functional properties of proteins.

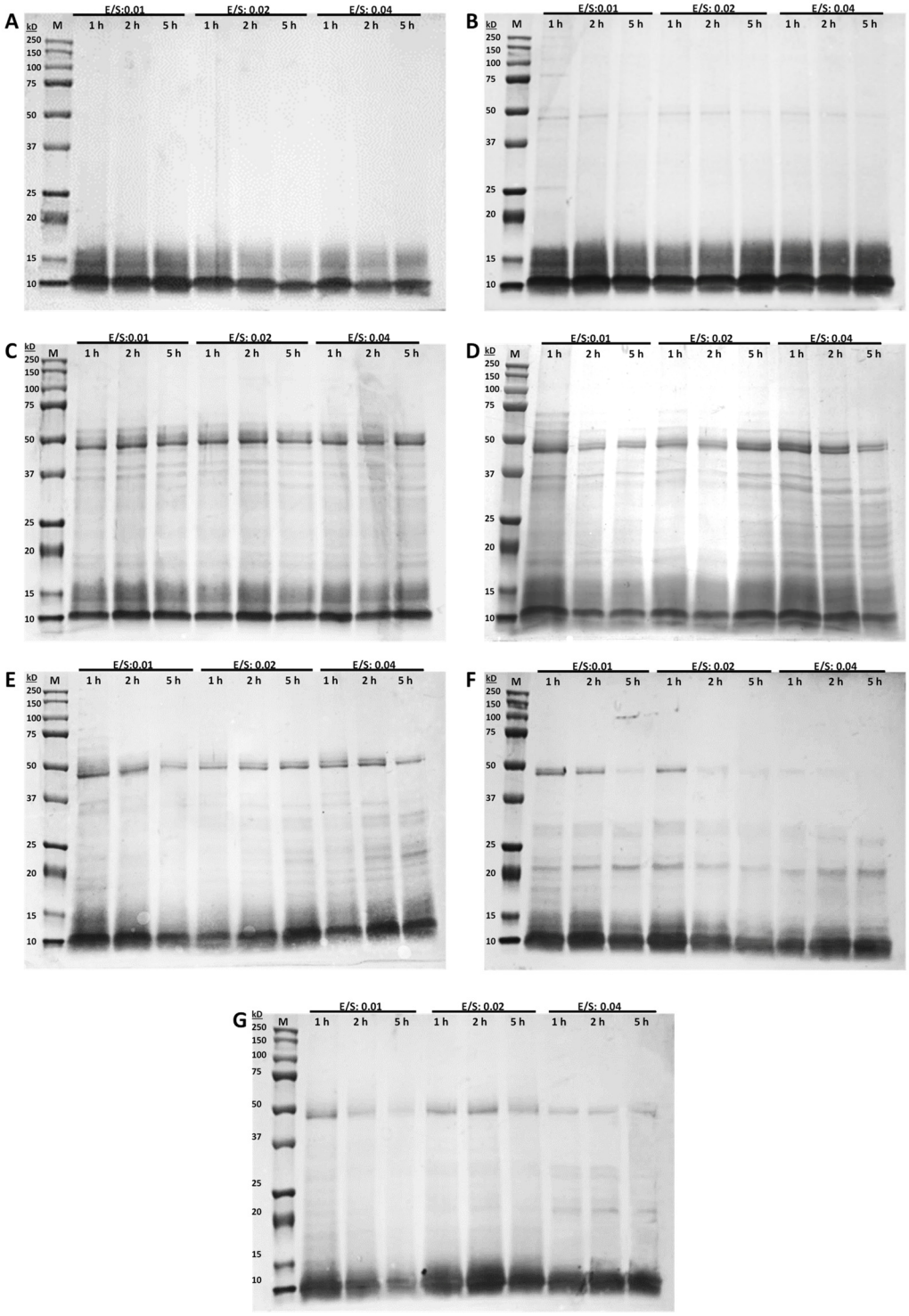

Figure 2. Molecular weight (kD) profiles of LPI hydrolysates by different commercial proteases at different E/S ratios and hydrolysis times as determined by SDS-PAGE under reducing conditions. (**A**) Acid Stable Protease, (**B**) Fungal Protease A, (**C**) Opti-Ziome^{TM} P^{3} Hydrolyzer^{TM}, (**D**) Neutral Protease, (**E**) Protamex®, (**F**) Alcaline Protease L, and (**G**) Alcalase® 2.4 L FG.

3.3. Functional Properties

3.3.1. Protein Solubility

The solubility of each LPI hydrolyzed at different E/S ratios was estimated as a function of pH at values of 5.0, 7.0, and 9.0 (Figure 3). Untreated LPI samples showed solubility of 24.4% ± 0.5 at pH 5.0, 50.1% ± 0.8 at pH 7.0, and 78.0% ± 1.5 at pH 9.0.

Meanwhile, protein solubility of all hydrolyzed LPI samples was significantly higher ($p < 0.05$) than untreated LPI at all pH analyzed values. Enzymatic hydrolysis provides better interaction of hydrophilic groups with the water molecules due to the decreased size of peptides, which increases protein solubility [18]. Thus, an increase in protein solubility could be a consequence of several facts, such as protein structural changes, the formation of small peptides and hydrophilic amino acids, and changes in the electrostatic forces [41].

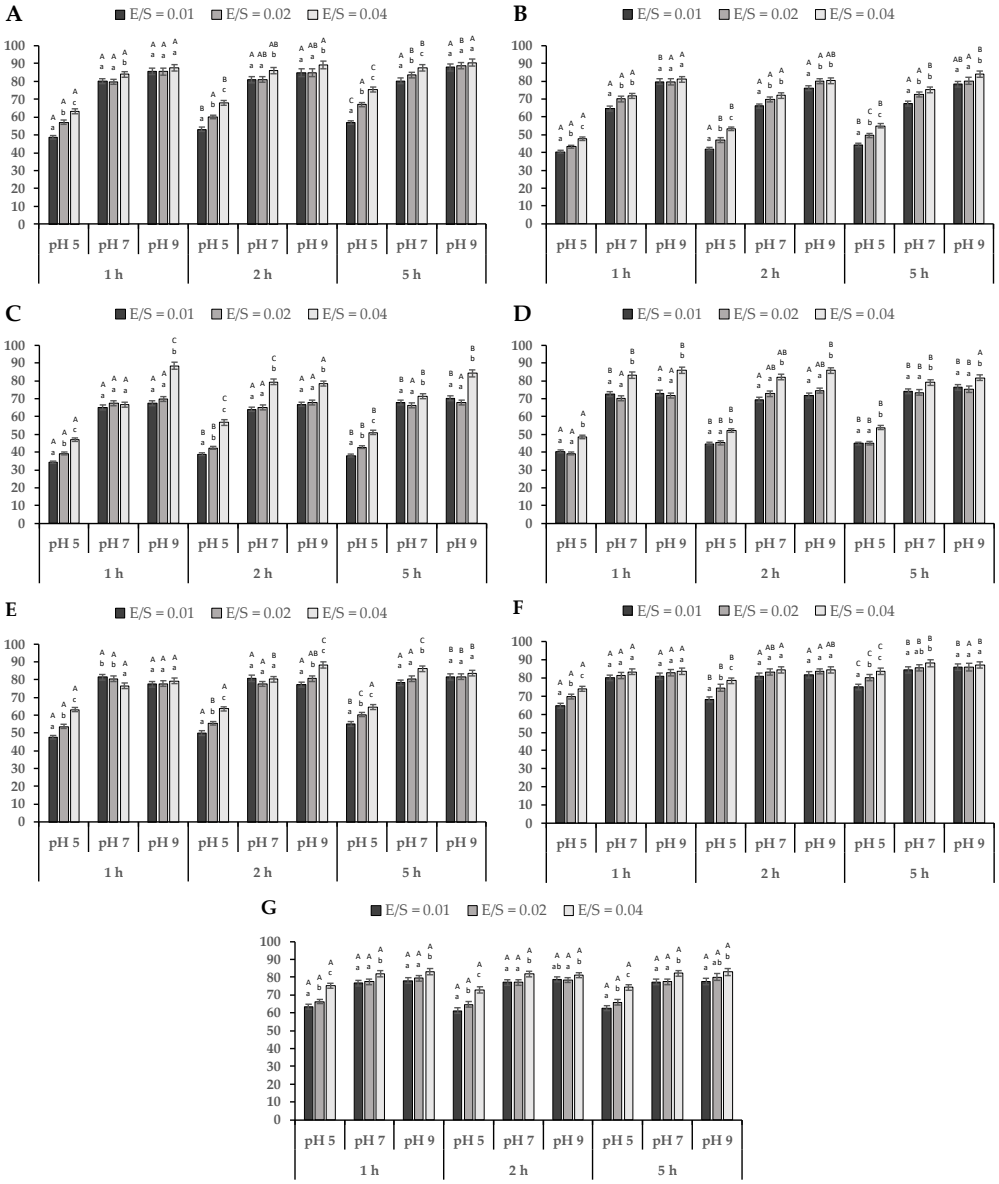

Figure 3. Protein solubility of the hydrolyzed lupin protein isolate (LPI) samples at different E/S (enzyme/substrate) ratios and hydrolysis times by different commercial proteases measured at different pHs. (**A**) Acid Stable Protease, (**B**) Fungal Protease A, (**C**) Opti-Ziome™ P^3 Hydrolyzer™, (**D**) Neutral Protease, (**E**) Protamex®, (**F**) Alcaline Protease L, and (**G**) Alcalase® 2.4 L FG. Means with different capital letters in the same row indicate significant differences in each hydrolysis time ($p < 0.05$). Means with different lowercase letters in the same column indicate significant differences in each E/S ratio ($p < 0.05$).

The protein solubility of hydrolyzed LPI samples was strongly dependent on pH obtaining higher values at pH 9.0. Conversely, the lower values were obtained at pH 5.0, near the pI of lupin proteins. The decrease in solubility at low pH is a consequence of the lack of electric charge, which increases hydrophobic aggregation and precipitation [42].

The same was found by Schlegel et al. [19], where the LPI hydrolyzed by various proteases (Neutrase 0.8 L, Corolase 7089, Papain, and Alcalase® 2.4 L FG) exhibited higher protein solubility at pH 9.0 and lower at pH 5.0. The increase in protein solubility of hydrolyzed LPI in acidic conditions compared to unhydrolyzed LPI can be attributed to the protein hydrolysis generating low molecular weight soluble peptides [43]. Meanwhile, Schlegel et al. [19] indicated that the main influence that affects the protein solubility characteristics corresponds to hydrophobic interactions, which increase protein-protein interactions and decrease solubility. Protein solubility is an important property because an increase in the water-solubility of plant proteins is commonly correlated with some functional properties such as emulsifying, gelling, and foaming [11].

In addition, protein solubility at pH 5.0 was significantly dependent ($p < 0.05$) on the E/S ratio, proteolysis time, and commercial enzyme used to hydrolyze the LPI. Meanwhile, protein solubility at pH 7.0 for all hydrolyzed LPI samples varied between 64–67% by Fungal Protease A (E/S = 0.01) to 83–88% by Alcaline Protease L (E/S = 0.04). These results are higher than those previously found at neutral pH on faba bean protein (24.7%), chickpea (47%), and pea (63–75%) [14].

Finally, the protein solubility at pH 9.0 exhibited higher values reaching values near 90% for LPI hydrolyzed by Acid Stable Protease (E/S = 0.04). Hydrolysis by Acid Stable Protease produced polypeptides with molecular weight >20 kD (Figure 2). These results are higher than those found on rice proteins hydrolyzed by Alcalase and Papain, where values close to 50% were reached [11].

3.3.2. Emulsion Capacity (EC) and Emulsion Stability (ES) of Hydrolyzed LPI

Emulsification is one of the most important processes in the manufacturing of formulated foods. The oil-in-water (O/W) emulsions combining proteins and lipids with aqueous solutions are the most commonly used in the food industry [19]. The effect of protein hydrolysis on EC and ES of LPI is shown in Table 3. The EC of undigested LPI was 81.0% ± 1.7, which was lower than most of the hydrolysates, except for acidic proteases Acid Stable Protease and Fungal Protease A. Both acidic proteases showed lower DH values in comparison with the other proteases (Figure 1), which could be indicative that these enzymes could release more hydrophobic groups. Liu et al. [44] indicated that greater exposure by the hydrophobic groups of proteins could improve their emulsifying properties, mainly due to the enhancement of the electrostatic repulsive force between emulsion droplets. Shen et al. [45] reported that hydrophobic interactions enhanced interactions between proteins and then produced large aggregates, which increased in particle size. Furthermore, Zhang et al. [46] demonstrated that the formation of aggregates might decrease the flexibility of proteins to adsorb at the surface of lipid droplets, thus decreasing emulsifying properties. Pan et al. [47] linked the decrease in emulsifying properties to the particle size, where a larger particle size had a negative influence on EC and ES. In this study, there is an inverse correlation between the EC of proteins and protein solubility in hydrolyzed LPI samples, where less soluble samples showed a higher EC. These results are opposed to those found by Schlegel et al. [19], where a direct correlation was found between EC and protein solubility. They indicated that this behaviour was a consequence of a greater dissolution of proteins in the emulsion, which means that a higher amount of proteins is found in the oil-in-water interface during emulsification. The effect of the E/S ratio on EC did not show great changes after 2 h of hydrolysis. However, after 5 h of protein hydrolysis, an increase in E/S ratio presented a significant decrease ($p < 0.05$) in EC values, except for the hydrolyzed LPI samples with Opti-Ziome™ P^3 Hydrolyzer™ and Neutral Protease.

Table 3. Emulsifying properties of LPI hydrolyzed by different commercial enzymes.

Functional Property	Hydrolysis Time (h)	E/S (%)	Acid Stable Protease	Fungal Protease A	Opti-Ziome™ P³ Hydrolyzer™	Neutral Protease	Protamex®	Alcaline Protease L	Alcalase® 2.4 L FG
EC (%)	1	0.01	$85.7 \pm 0.6^{b,F}$	$83.3 \pm 1.5^{a,E}$	$73.3 \pm 1.2^{a,B}$	$80.0 \pm 0.0^{a,D}$	$78.0 \pm 1.0^{b,C}$	$70.3 \pm 0.6^{a,A}$	$78.0 \pm 1.0^{b,C}$
		0.02	$82.0 \pm 2.0^{a,C}$	$86.7 \pm 0.6^{a,D}$	$72.3 \pm 0.6^{a,A}$	$77.7 \pm 2.5^{a,B}$	$77.7 \pm 1.5^{b,B}$	$78.3 \pm 0.6^{b,B}$	$74.7 \pm 0.6^{a,A}$
		0.04	$83.3 \pm 1.5^{a,b,C}$	$82.7 \pm 3.2^{a,C}$	$71.7 \pm 1.5^{a,A}$	$82.7 \pm 4.0^{a,C}$	$75.0 \pm 0.1^{a,A,B}$	$77.7 \pm 2.1^{b,B}$	$75.3 \pm 0.6^{a,A,B}$
	2	0.01	$86.0 \pm 1.7^{b,E}$	$84.3 \pm 1.2^{a,D}$	$73.3 \pm 0.6^{a,A}$	$77.7 \pm 0.6^{a,B}$	$79.7 \pm 0.6^{b,C}$	$78.3 \pm 0.6^{a,B,C}$	$79.7 \pm 0.6^{b,C}$
		0.02	$86.3 \pm 1.5^{b,D}$	$82.0 \pm 1.0^{a,C}$	$74.3 \pm 0.6^{b,A}$	$79.0 \pm 1.0^{a,B}$	$79.0 \pm 1.0^{b,B}$	$77.0 \pm 2.6^{a,B}$	$79.0 \pm 1.0^{a,b,B}$
		0.04	$75.7 \pm 0.6^{a,A,B}$	$83.0 \pm 2.0^{a,C}$	$75.0 \pm 0.1^{b,A}$	$78.3 \pm 2.9^{a,B}$	$75.0 \pm 1.0^{a,A}$	$77.0 \pm 2.0^{a,A,B}$	$76.3 \pm 2.3^{a,A,B}$
	5	0.01	$86.0 \pm 2.0^{b,D}$	$84.7 \pm 1.5^{b,D}$	$71.7 \pm 1.2^{a,b,A}$	$75.3 \pm 1.2^{a,B}$	$84.0 \pm 1.7^{b,D}$	$80.3 \pm 0.6^{c,C}$	$84.0 \pm 1.7^{b,D}$
		0.02	$83.3 \pm 1.2^{b,D}$	$81.7 \pm 2.3^{a,b,D}$	$71.3 \pm 0.6^{a,B}$	$82.0 \pm 2.6^{b,B}$	$81.7 \pm 1.5^{b,D}$	$66.3 \pm 1.5^{b,A}$	$77.7 \pm 1.5^{a,C}$
		0.04	$80.3 \pm 0.6^{a,D}$	$80.3 \pm 1.2^{a,D}$	$73.0 \pm 0.0^{b,B}$	$85.3 \pm 1.2^{b,E}$	$77.7 \pm 1.5^{a,C}$	$61.3 \pm 2.3^{a,A}$	$79.3 \pm 1.2^{a,C,D}$
ES (%)	1	0.01	$81.3 \pm 1.1^{a,A}$	$88.0 \pm 2.2^{b,B}$	$95.9 \pm 1.4^{a,C}$	$97.1 \pm 4.0^{b,C,D}$	$98.7 \pm 1.3^{b,C,D}$	$90.6 \pm 1.9^{a,B}$	$99.5 \pm 0.8^{c,D}$
		0.02	$86.6 \pm 1.5^{b,B}$	$81.2 \pm 0.6^{a,A}$	$96.8 \pm 0.8^{a,E}$	$90.4 \pm 0.7^{a,b,C}$	$94.0 \pm 0.8^{a,D}$	$93.4 \pm 1.6^{a,D}$	$93.6 \pm 1.3^{b,D}$
		0.04	$84.8 \pm 2.2^{b,A}$	$84.7 \pm 2.3^{a,b,A}$	$97.7 \pm 1.6^{a,C}$	$84.8 \pm 4.4^{a,A}$	$91.0 \pm 3.3^{a,B}$	$96.6 \pm 0.8^{b,C}$	$88.9 \pm 0.8^{a,A,B}$
	2	0.01	$79.9 \pm 1.4^{a,A}$	$87.0 \pm 1.0^{a,B}$	$95.9 \pm 0.0^{a,E}$	$94.4 \pm 2.0^{b,D,E}$	$93.7 \pm 1.2^{a,D,E}$	$89.8 \pm 1.2^{a,C}$	$93.3 \pm 1.4^{a,D}$
		0.02	$82.3 \pm 2.4^{a,A}$	$88.2 \pm 1.5^{a,B}$	$95.5 \pm 3.3^{a,D}$	$94.9 \pm 0.1^{b,C,D}$	$92.4 \pm 2.1^{a,C,D}$	$91.8 \pm 1.7^{a,B,C}$	$92.4 \pm 2.1^{a,C,D}$
		0.04	$94.3 \pm 1.5^{b,B,C}$	$88.4 \pm 3.4^{a,A}$	$93.8 \pm 0.8^{a,B,C}$	$89.0 \pm 2.6^{a,A}$	$96.9 \pm 0.7^{b,C}$	$96.1 \pm 1.2^{b,C}$	$90.9 \pm 2.4^{a,A,B}$
	5	0.01	$81.4 \pm 1.0^{a,A}$	$85.5 \pm 2.5^{a,B}$	$97.7 \pm 0.8^{b,D}$	$93.1 \pm 0.9^{b,C}$	$97.9 \pm 1.9^{b,D}$	$91.3 \pm 1.3^{a,C}$	$92.1 \pm 1.7^{a,C}$
		0.02	$85.2 \pm 0.6^{b,A}$	$87.4 \pm 2.6^{a,A,B}$	$98.1 \pm 0.8^{b,E}$	$94.7 \pm 1.4^{b,D}$	$92.1 \pm 1.7^{a,C,D}$	$90.0 \pm 1.5^{a,B,C}$	$97.9 \pm 1.9^{b,E}$
		0.04	$87.6 \pm 0.1^{c,A}$	$88.0 \pm 1.6^{a,A,B}$	$93.2 \pm 2.4^{a,C}$	$87.5 \pm 1.3^{a,A}$	$89.8 \pm 1.7^{a,A,B,C}$	$91.3 \pm 4.0^{a,B,C}$	$93.3 \pm 0.7^{a,C}$

EC: emulsifying capacity; ES: emulsifying stability. Means with different capital letters in the same row indicate significant differences in each enzyme ($p < 0.05$). Means with different lowercase letters in the same column indicate significant differences in each hydrolysis time ($p < 0.05$).

The ES can be defined as the resistance of the system to changes in its physicochemical properties with time. The ES of LPI presented a value of 94.2% ± 0.8, which is significantly higher ($p < 0.05$) than most hydrolysate LPI samples by acidic proteases, except for LPI hydrolyzed with Acid Stable Protease with an E/S ratio of 0.04. The hydrolyzed samples of LPI with alkaline proteases showed higher ES values than acidic proteases in most of the studied samples, mainly in the samples hydrolyzed for 1 or 2 h at low E/S ratios. In general, hydrolysates with low DH showed lower ES values. This is contrary to what has been mentioned by other authors, who have found a negative correlation between DH and ES when hydrolyzed whey protein using Alcalase [48]. In this study, the high emulsion instability found could be a consequence of a larger number of low-molecular-weight peptides. However, SDS-PAGE analysis showed that acidic proteases form polypeptides of smaller molecular weight than alkaline proteases (Figure 2). Therefore, the current study is in agreement with the previous findings. Regarding the E/S ratio, there is no correlation with the ES values. This could be due to the fact that during protein hydrolysis of LPI, some peptides exhibited more hydrophobic than hydrophilic areas, while as the hydrolysis time progressed, this relation changed. In this regard, several reports have suggested that there is an optimum molecular size or chain length for peptides to improve functional properties and limited hydrolysis generally leads to improved functional properties [49]. Therefore, the degree of hydrolysis should be carefully studied to achieve an improvement in functional properties by enzymatic hydrolysis of LPI.

4. Conclusions

This study demonstrated that the solubility and emulsifying properties of lupin (*Lupinus luteus*) proteins could be enhanced by controlled enzymatic hydrolysis using some specific commercial enzymes. The results show that acidic proteases used in this study are the most efficient for hydrolyzing lupin proteins to obtain polypeptides of low molecular weight. However, the alkaline proteases studied showed to be more effective in achieving free amino groups from lupin proteins, except for Alcaline Protease L. The DH (%) values

increased with the hydrolysis time, and the highest DH values were logged with Neutral Protease for the E/S ratio of 0.01 and Protamex® for E/S ratios of 0.02 and 0.04, which indicates that Protamex® has a greater ability to hydrolyze lupin proteins. The obtained results indicated that hydrolysis of LPI using the commercial acidic proteases studied can be used to improve the emulsifying capacity (EC) of lupin proteins. However, the protein hydrolysis by acidic proteases decreases the emulsifying stability (ES).

These findings demonstrate that enzymatic hydrolysis by commercial enzymes under controlled conditions is an effective way to improve the functional properties of lupin proteins and thus increase the potential use of lupin protein ingredients in food formulations.

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