

## Article

# Unveiling the Effects of Roasting Pre-Treatment on the Structural and Functional Properties of *Lupinus angustifolius* Protein Isolates and Their Impact on In Vitro Digestibility

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## Abstract

This study investigates the effects of roasting pre-treatment on *Lupinus angustifolius* protein isolate (LPI) and the resulting structure–function relationships relevant to food applications. Lupin seeds were roasted for 0, 10, 20, and 30 min prior to protein extraction, and the resulting LPI was characterized using circular dichroism (CD), Fourier-transform infrared (FT-IR) spectroscopy, intrinsic fluorescence spectroscopy, and SDS-PAGE. Unroasted LPI exhibited compact native conglutin structures with low solubility (58.64%), surface hydrophobicity (43.34  $\mu\text{g}$  BPB), emulsifying activity (30.71  $\text{m}^2/\text{g}$ ), and in vitro protein digestibility (IVPD, 82.84%). Roasting pre-treatment induced a biphasic structural response. Partial conformational changes increased solubility (up to 97.84%), exposed hydrophobic sites (peak 55.79  $\mu\text{g}$  BPB), enhanced emulsifying activity (45.37  $\text{m}^2/\text{g}$ ), doubled foaming capacity (210%), and improved IVPD (90.85%), likely due to structural changes that facilitated digestion. CD analysis showed a modest increase in  $\alpha$ -helical content (3.43  $\rightarrow$  6.74%) with minor fluctuations in  $\beta$ -sheet content, while fluorescence quenching indicated conformational loosening and partial reorganization. SDS-PAGE revealed the formation of soluble oligomers and high-molecular-weight aggregates, consistent with heat-induced association. Prolonged roasting reduced emulsion and foam stability because of aggregation, but maximized antioxidant capacity, likely associated with Maillard reaction products despite the observed depletion of amino acids. Overall, controlled roasting pre-treatment systematically modulates lupin protein structure and functionality, highlighting LPI as a competitive high-performance plant protein ingredient for food applications.

**Keywords:** lupin protein isolate; roasting; protein structural properties; functional properties



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## 1. Introduction

The global population is projected to reach 10 billion by 2050, placing unprecedented pressure on food systems and natural resources, with significant implications for food security [1]. Animal protein production contributes substantially to greenhouse gas emissions, soil degradation, and deforestation, highlighting the urgent need for sustainable protein alternatives [2]. Plant-based proteins, derived from legumes, cereals, and nuts, are increasingly recognized for their lower environmental footprint, economic value, and health benefits, as well as their compatibility with diverse dietary, ethical, and religious

preferences [3,4]. This growing interest underscores the need for innovative, sustainable, healthy, and nutritious alternatives to traditional protein sources, making plant-based proteins a promising option.

To capitalize on the current trends of increasing market demand for plant-based foods, lupin protein is well-positioned in terms of versatility and sustainability for plant-based functional foods. *Lupinus angustifolius* (narrow-leaved blue lupine) has gained commercial interest as a novel plant protein source because of its high protein content (40–45%), low fat content, fewer antinutrient factors, greater sustainability, and lower carbohydrate content than other legumes, such as peas and chickpeas [5–7]. Nutritionally, lupin consumption has been linked to various health benefits, including improved bowel function and reductions in cholesterol, blood glucose, and glycemic index [8]. Despite these advantages, including a substantially lower production cost for soy, lupin remains significantly less industrialized than soy or pea protein, representing a new frontier for the development of plant protein ingredients [6].

However, plant protein isolates encounter substantial challenges related to solubility, digestibility, and sensory properties. These challenges largely stem from the presence of anti-nutritional compounds such as phytic acid and saponins, which contribute to bitterness, as well as from their inherently compact and rigid molecular structures [9,10]. To address these limitations, various techniques have been applied to produce protein concentrates and isolates, including a combination of extraction (isoelectric precipitation (IEP), salt extraction/micellization) and pre-post-processing spanning physical, chemical, and biological methods, which have been used to improve the functional properties of proteins [7,9,11].

Roasting has emerged as a particularly promising physical modification strategy, capable of altering the functional properties of plant proteins, as demonstrated by successful studies on hemp seed, cashew nut, and sesame proteins [12–14]. Pre-roasting at 150 °C for 10 to 20 min on pea protein concentrates was reported to improve protein solubility by approximately 12–24% and enhance the emulsion ability index (EAI), mainly through alterations in tertiary protein structures [15]. Additionally, roasting enhances sensory attributes and consumer acceptance of food products through physicochemical changes in the food, including alterations in texture and color, reduction in water content, modifications in lipids, and the formation of Maillard reactions [16]. A recent study found that roasting treatments enhanced in vitro protein digestibility and reduced anti-nutritional factors in soybeans, including a 13.38% reduction in phytic acid and a 61.16% reduction in trypsin inhibitor content [17]. Despite these promising effects, the mechanistic understanding of how roasting-induced molecular changes in lupin proteins translate into functional and nutritional improvements remains limited. Specifically, the relationships among protein conformational alterations, amino acid composition, antioxidant activity, and in vitro digestibility have not been fully elucidated.

Addressing this gap, the present study investigates the effects of controlled roasting pre-treatment on *Lupinus angustifolius* seeds at varying durations prior to alkaline protein extraction, focusing on the mechanistic links between protein structure and functionality. The resulting LPI was analyzed to assess how roasting-induced conformational changes, such as partial unfolding, exposure of hydrophobic and reactive sites, and protein aggregation, affect physicochemical, techno-functional, and nutritional properties. Functional properties were evaluated, including solubility, surface hydrophobicity, emulsifying and foaming properties, in vitro protein digestibility, and antioxidant activity. By connecting molecular-level structural modifications to functional outcomes, this work provides novel mechanistic insights into LPI behavior and highlights its potential as a high-performance, sustainable plant protein for diverse food applications.

## 2. Materials and Methods

### 2.1. Materials

Split lupin (*Lupinus angustifolius*) was purchased from Benefarm Co., Ltd. (Seoul, Republic of Korea). Reagents for SDS-PAGE analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA), including a 30% Acrylamide-Bis solution (Bio-RAD, Seoul, Republic of Korea), 10% sodium dodecyl sulfate (SDS) (BIONEER, Daejeon, Republic of Korea), 10% ammonium persulfate, 1 M Tris-HCl buffers (pH 6.8 and pH 8.8), and N, N, N', N'-Tetramethylethylenediamine (TEMED). Pepsin from porcine gastric mucosa (CAS No. 9001-75-6) and pancreatin from porcine pancreas (CAS No. 8049-47-6) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemical solvents were of analytical grade and obtained from Duksan Chemicals (Ansan, Republic of Korea).

### 2.2. Lupin Protein Extraction

Split lupin seeds were divided into unroasted (UR) and roasted groups. Roasting was performed at 140 °C for 10 min (R10), 20 min (R20), and 30 min (R30), with the protocol adapted from Lao, Ye, Wang, Vongsivut and Selomulya [15] by applying a lower roasting temperature, to reduce thermal damage while still achieving sufficient roasting. Roasted and unroasted samples were milled using a laboratory grinder and defatted with hexane (1:5, *w/v*; 1 g sample per mL water) for 2 h at room temperature. Hexane was removed under vacuum, and the defatting procedure was repeated twice. The wet samples were dried overnight at room temperature. Protein extraction was conducted using a modified alkaline–isoelectric precipitation method [9]. The samples were dissolved in deionized water (1:10 *w/v*; 1 g sample per mL water) and stirred for 1 h. The mixture was alkalinized to pH 9 with 0.5N NaOH and agitated on a shaker for 60 min at room temperature to solubilize proteins. The slurry was centrifuged (Supra-22K, Hanil Science Industrial, Incheon, Republic of Korea) at 4000 rpm for 15 min at 4 °C. The protein-rich supernatant was adjusted to pH 4.5 with 1N HCl and chilled at 4 °C for 2 h to precipitate proteins. The solution was centrifuged again under the same conditions to collect the precipitated lupin protein, which was neutralized to pH 7. The neutralized isolate was dialyzed against phosphate buffer (PBS, pH 7.4) using a 13 kDa cut-off membrane. Dialysis was performed at room temperature with gentle stirring using a sample-to-buffer ratio of 1:20 (*v/v*). The buffer was replaced twice at 11 h intervals (total dialysis time 22 h). The dialyzed globulin fraction was freeze-dried and stored at 4 °C. Protein content was measured by the Lowry method.

### 2.3. Amino Acid Profile

Amino acid composition was analyzed using an amino acid analyzer (LA-8080, Hitachi, Tokyo, Japan) equipped with an ion-exchange column (No. 2622 SCPE, 4.6 mm × 60 mm). Protein samples (0.1 g) were hydrolyzed with 6 N HCl (1 mL) under a nitrogen atmosphere at 110 °C for 24 h. Hydrolysates were vacuum-dried at 40 °C for 24 h, reconstituted in 2 mL of 0.02 N HCl, sonicated for 1 h, filtered (0.45 µm), and injected (20 µL). Amino acid content was expressed as mg/g protein [4,18]. Amino acid score (AAS), essential amino acid index (EAAI), biological value (BV), and predicted protein efficiency ratios (PER<sub>1</sub>–PER<sub>3</sub>) were calculated using Equations (1)–(6), where *a* represents amino acid content in the sample, *b* corresponds to whole egg protein standards, and *n* denotes the number of essential amino acids included.

$$\text{AAS (\%)} = \frac{\text{Essential amino acid in sample protein} \left( \frac{\text{mg}}{\text{g}} \right)}{\text{Essential amino acid recommended by FAO} \left( \frac{\text{mg}}{\text{g}} \right)} \times 100\% \quad (1)$$

$$EAAI = \sqrt[n]{\frac{Lys_a}{Lys_b} \times \frac{Tyr_a}{Tyr_b} \times \dots \times \frac{His_a}{His_b}} \quad (2)$$

$$BV = 1.09 \times EAAI - 11.7 \quad (3)$$

$$PER_1 = -0.684 + 0.456 \times Leu - 0.047 \times Pro \quad (4)$$

$$PER_2 = -0.468 + 0.454 \times Leu - 0.105 \times Tyr \quad (5)$$

$$PER_3 = -1.816 + 0.435 \times Met + 0.78 \times Leu + 0.211 \times His - 0.944 \times Tyr \quad (6)$$

## 2.4. Structural Properties

### 2.4.1. Circular Dichroism (CD)

CD spectra were recorded at 25 °C using a J-1500 spectropolarimeter (JASCO Inc., Tokyo, Japan) in the range of 190–250 nm [19]. Protein solutions (0.2 mg/mL) were prepared in PBS (pH 7.4). Measurements were conducted at a scanning speed of 100 nm/min using a 1 mm quartz cuvette. Secondary structure composition was estimated using the BestSel server <http://bestsel.elte.hu/> (accessed on 15 May 2025).

### 2.4.2. Fourier-Transform Infrared Spectroscopy (FT-IR)

The FTIR spectra of LPI were measured using a Spectrum Infrared Spectrometer (PerkinElmer, Shelton, CT, USA). Spectra were recorded from 4000 to 400 cm<sup>−1</sup> with a 4 cm<sup>−1</sup> resolution and 32 scans per sample and processed using Origin 2021 software.

### 2.4.3. Intrinsic Fluorescence Spectroscopy

Intrinsic fluorescence spectra were recorded following Wu et al. [20] with slight modifications. Protein solutions (0.1 mg/mL) were prepared in deionized water and analyzed using a SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA, USA). Samples were excited at 280 nm, and emission spectra were collected between 315 and 500 nm with excitation and emission slit widths of 5 nm.

### 2.4.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analyses were performed as described by Said and Lee [21] with slight modifications. Protein samples were prepared at a concentration of 5 mg/mL in deionized water and mixed with loading buffer at a 4:1 (v/v) ratio. The mixtures were heated at 100 °C for 10 min to ensure protein denaturation prior to electrophoresis. Aliquots (15 µL) of each sample were loaded onto a discontinuous polyacrylamide gel system consisting of a 5% stacking gel and a 12% resolving gel. Electrophoresis was carried out using a Tris-glycine buffer system containing SDS, operated at 120 V for 10 min during the stacking phase, followed by 150 V for 40 min during the resolving phase. Following electrophoresis, the gels were stained overnight with Coomassie Brilliant Blue R-250 solution (45% methanol, 10% acetic acid, 0.25% R-250) and subsequently destained with deionized water until clear protein bands were obtained. Protein molecular weights were estimated by comparison with a pre-stained protein marker (PAGE-Mark™ protein markers, 10–250 kDa, BIOMAX, Pocheon-si, Gyeonggi-do, Republic of Korea).

## 2.5. Surface Hydrophobicity

The surface hydrophobicity of LPI proteins was determined using the bromophenol blue (BPB) binding method [21]. Briefly, 200 µL of 1 mg/mL BPB solution was added to 1 mL of 1.5 mg/mL protein solution, and the mixture was vortexed. The samples were incubated in the dark for 15 min and centrifuged at 2000× g for 15 min. The supernatant was diluted 10-fold with PBS (pH 7.4), and absorbance was measured at 595 nm using a

UV/Vis spectrophotometer (UV-2075 Plus, JASCO, Tokyo, Japan). Phosphate buffer was used as the blank. BPB protein ( $\mu\text{g}$ ) was calculated using the following expression:

$$\text{Bound BPB } (\mu\text{g}) = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 200\mu\text{g} \quad (7)$$

## 2.6. In Vitro Protein Digestibility (IVPD)

LPI digestion was performed according to in vitro simulated gastrointestinal digestion (SGID) following the standardized INFOGEST method with minor modifications [22,23]. The protocol mimicked physiological conditions in the gastric and small intestinal phases. However, the oral phase was omitted because the sample consisted of a reconstituted protein isolate rather than a complex solid food matrix, and the standardized INFOGEST oral phase does not contain proteolytic enzymes but mainly provides dilution and salivary amylase activity. Therefore, its exclusion was not expected to affect protein hydrolysis markedly. Similar modified INFOGEST-based protocols omitting the oral phase have been reported by Ménard et al. [24].

Briefly, 0.4 g of protein sample was dissolved in 4 mL phosphate buffer (pH 7.4) to achieve complete solubilization and homogeneity before being subjected to the gastric phase. The LPI solutions were mixed with simulated gastric fluid (SGF) in a 1:1 volume ratio (5 mL:5 mL). The SGF electrolyte solution was prepared by combining 6.9 mL of KCl (0.5 mol/L), 0.9 mL of  $\text{KH}_2\text{PO}_4$  (0.5 mol/L), 12.5 mL of  $\text{NaHCO}_3$  (1 mol/L), 11.8 mL of NaCl (2 mol/L), 0.4 mL of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (0.15 mol/L), and 0.5 mL of  $(\text{NH}_4)_2\text{CO}_3$  (0.5 mol/L). For each digestion tube, 3.8 mL of the SGF electrolyte solution was supplemented with 0.2 mL of porcine pepsin solution (5 mg/mL prepared in 10 mM HCl; Sigma-Aldrich, Cat. No. P6887, activity 3030 U/mg), 25  $\mu\text{L}$  of 0.3 M  $\text{CaCl}_2$ , and 975  $\mu\text{L}$  of distilled water to obtain a final SGF volume of 5 mL. The pH was then adjusted to pH 3.0. The samples were incubated at 37 °C with constant shaking at 200 rpm for 2 h to ensure adequate mixing. After gastric digestion, the solution was recovered and the pH was adjusted to 7.0 using 6 M NaOH to inactivate pepsin activity. The intestinal phase was subsequently performed by transferring 10 mL of the gastric digest into a new tube and mixing it with 10 mL of simulated intestinal fluid (SIF). The SIF electrolyte solution was prepared using 6.8 mL KCl (0.5 mol/L), 0.8 mL  $\text{KH}_2\text{PO}_4$  (0.5 mol/L), 42.5 mL  $\text{NaHCO}_3$  (1 mol/L), 9.6 mL NaCl (2 mol/L), and 1.1 mL  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (0.15 mol/L), and adjusted to pH 7.0. Each post-gastric digestion tube contained 11.968 mL of the prepared SIF electrolyte solution supplemented with 5 mL of pancreatin solution (adjusted to 100 U/mL based on trypsin activity; Sigma-Aldrich, Cat. No. P7545, activity 45 U/mg), 40  $\mu\text{L}$  of 0.3 M  $\text{CaCl}_2$ , and 3.96 mL of distilled water, yielding a final intestinal phase volume of 20 mL. The mixtures were incubated at 37 °C with shaking at 200 rpm for 2 h. Enzymatic reactions were terminated by heating the samples at 95 °C for 5 min. The digested samples were freeze-dried, and the remaining protein content was determined using the Lowry method. Digestion was performed in triplicate, and protein digestibility was calculated as follows:

$$\text{Protein digestibility } (\%) = \frac{W_0 - W_1}{W_0} \times 100 \quad (8)$$

where  $W_0$  is the initial protein content (mg/g) of LPI before digestion;  $W_1$  represents the residual protein content (mg/g) after digestion.

## 2.7. ABTS<sup>+</sup>-Radical Scavenging Assay

The antioxidant capacity of the samples was determined using the ABTS radical scavenging assay as described by [21]. Briefly, a 7 mM aqueous ABTS<sup>+</sup> solution was mixed with 2.6 mM potassium persulfate and incubated in the dark at room temperature for 12 h



to generate the ABTS reagent. The ABTS<sup>+</sup> solution was diluted with distilled water to serve as a blank solution. Protein sample solutions were prepared in distilled water at concentrations ranging from 5 to 20 mg/mL. Each sample solution (50 µL) was mixed with 950 µL ABTS reagent and incubated in the dark for 30 min. The absorbance was measured at 734 nm using a UV/Vis spectrophotometer (UV-2075 Plus, JASCO, Tokyo, Japan). The scavenging activity was calculated using the following equation:

$$\text{ABTS}^+\text{scavenging activity (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100 \quad (9)$$

where  $A_0$  and  $A_1$  are the absorbance values of the blank and sample, respectively.

## 2.8. Functional Properties

### 2.8.1. Protein Solubility

Protein solubility of LPI was determined using Lowry's method [25]. Protein samples were prepared at a concentration of 5 mg/mL in distilled water and centrifuged at 4000 rpm for 15 min to remove insoluble fractions. The supernatant was carefully collected and filtered prior to analysis. Protein content in the supernatant was measured using bovine serum albumin (BSA) as the standard, and absorbance was recorded at 750 nm using a UV/Vis spectrophotometer (UV-2075 Plus, JASCO, Tokyo, Japan). Protein solubility was calculated using the following equation:

$$\text{Protein solubility (\%)} = \frac{\text{Protein concentration in supernatant}}{\text{Protein concentration in solution}} \times 100\% \quad (10)$$

### 2.8.2. Emulsifying Properties

The emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined based on the method described by Said and Lee [21]. Protein suspensions (0.1% *w/v*) were prepared in distilled water and mixed with sunflower oil at a ratio of 4:1 (protein solution: oil, *v/v*). The mixture was homogenized at 5000 rpm for 3 min using a homogenizer (Daihan Scientific, Wonju-si, Gangwon-do, Republic of Korea). Aliquots (50 µL) of the freshly prepared emulsion were collected immediately (0 min) and after 15 min, diluted in 10 mL of 0.1% (*w/v*) SDS solution, and the absorbance was measured at 500 nm using a UV/Vis spectrophotometer (UV-2075 Plus, JASCO, Tokyo, Japan). The EAI and ESI values were calculated using the following equations:

$$\text{EAI (m}^2\text{/g)} = \frac{2 \times 2.303 \times A_0 \times D}{(1 - \varphi) \times C \times 10^4} \quad (11)$$

$$\text{ESI(min)} = \frac{A_0 \times 15}{A_0 \times A_{15}} \quad (12)$$

where  $A_0$  and  $A_{15}$  are the absorbance (500 nm) of the diluted emulsion at 0 and 15 min, respectively;  $D$  is the dilution factor;  $\varphi$  is the volume fraction of sunflower oil in the emulsion (0.25); and  $C$  is the protein concentration in the aqueous phase (g/mL).

### 2.8.3. Foaming Properties

Foaming activity (FC) and foaming stability (FS) were determined according to Zhao et al. [26]. Briefly, a 1% (*w/v*) protein solution (20 mL) was diluted with phosphate buffer (pH 7.0) and foamed at 5000 rpm for 2 min using a homogenizer (Daihan Scientific,

Gangwon-do, Republic of Korea). The foam volume was measured immediately after foaming ( $V_1$ ) and after 30 min ( $V_{30}$ ). FC and FS were calculated using the following equations:

$$FC (\%) = \frac{(V_1 - V_0)}{V_0} \times 100 \quad (13)$$

$$FS (\%) = \frac{V_{30}}{V_1} \times 100 \quad (14)$$

where  $V_0$  is the initial volume before foaming (mL),  $V_1$  is the foam volume immediately after homogenization (mL), and  $V_{30}$  is the foam volume after 30 min (mL).

### 2.9. Statistical Analysis

Statistical analysis was performed using SPSS version 26.0 (Chicago, IL, USA). The significance of differences between samples was determined by one-way analysis of variance (ANOVA), followed by Duncan's post hoc test with significance set at  $p < 0.05$ .

## 3. Results and Discussion

### 3.1. Amino Acid Profile of LPI

Roasting resulted in a clear, stepwise modification of the amino acid profile of lupin protein isolate (LPI) as shown in Table 1. Unroasted LPI (UR) showed the highest levels of all quantified amino acids, with particularly high glutamic acid (273.35 mg/g), arginine (167.27 mg/g), aspartic acid (152.68 mg/g), and leucine (124.69 mg/g), providing a nutritionally favorable baseline representative of minimally processed protein. Relative to FAO [27] patterns, UR met most EAA requirements for children and adults, while lysine remained limiting for infants, showing a specific nutritional shortfall even before roasting. With increasing roasting severity (R10 to R30), total EAA content and amino acid score (AAS) declined progressively, reflected in a drop in the EAA/TAA ratio from 30.45% (UR) to 27.60% (R30). Mild roasting (R10) already caused measurable losses in thermolabile residues, while extended roasting (R20–R30) yielded sharper reductions, especially for lysine (70.62 → 45.63 mg/g, ≈35% loss), methionine (10.36 → 4.01 mg/g, ≈61% loss), and threonine (49.53 → 34.73 mg/g, ≈30% loss). Lysine, with its reactive  $\epsilon$ -amino group, is particularly prone to Maillard condensation (formation of Amadori products and advanced glycation end products) [28,29]. At the same time, methionine showed the greatest apparent reduction across treatments, likely due to oxidative modification of its thioether group that reduces analytical recovery during acid hydrolysis [10,30]. In contrast, glutamic acid decreased only slightly (273.35 → 256.83 mg/g), and arginine remained nearly unchanged, so Glu, Asp, Arg, and Leu together still accounted for more than 50% of the total amino acid in all treatments. Leucine and isoleucine consistently exceeded FAO requirements across age groups, maintaining LPI's BCAA-rich profile. However, phenylalanine alone remained below infant reference values when considered independently (68.40 vs. 94 mg/g required) even in UR. These trends reflect the differential thermal reactivity of amino acid side chains and are consistent with observations in sesame [31], lupin [32], and soy [33,34], where Maillard reactions, oxidation, and protein unfolding reduce the availability of lysine, sulfur amino acids, and threonine.

The amino acid score (AAS) for each essential amino acid is presented in Figure 1a–c, indicating prolonged roasting decreased AAS values, with the most pronounced reduction observed in the R30. The amino acid score (AAS) was calculated based on the amino acid composition, and any amino acid with a score below 100 was considered limiting [4,27]. In infants (Figure 1a), the AAS of threonine decreased from 112.5% (UR) to 78.9% (R30). Similarly, for children (Figure 1b), lysine AAS declined from 102.3% (UR) to 66.1% (R30), falling well below the reference amino acid requirement. These reductions are likely

associated with heat-induced Maillard reactions and dehydration or oxidation of reactive side chains, particularly affecting lysine and hydroxyl-containing amino acids such as threonine [35,36]. Consistent with previous reports, protein isolates obtained from *Lupinus luteus* have been reported to be deficient in lysine, methionine, and threonine [7,37]. Overall, roasting enhances the vulnerability of already heat-sensitive amino acids; however, in this study, combined sulfur amino acids approached or met FAO/WHO/UNU reference levels for children and adults (Figure 1b,c), though they may remain limiting for infants under extended roasting conditions.

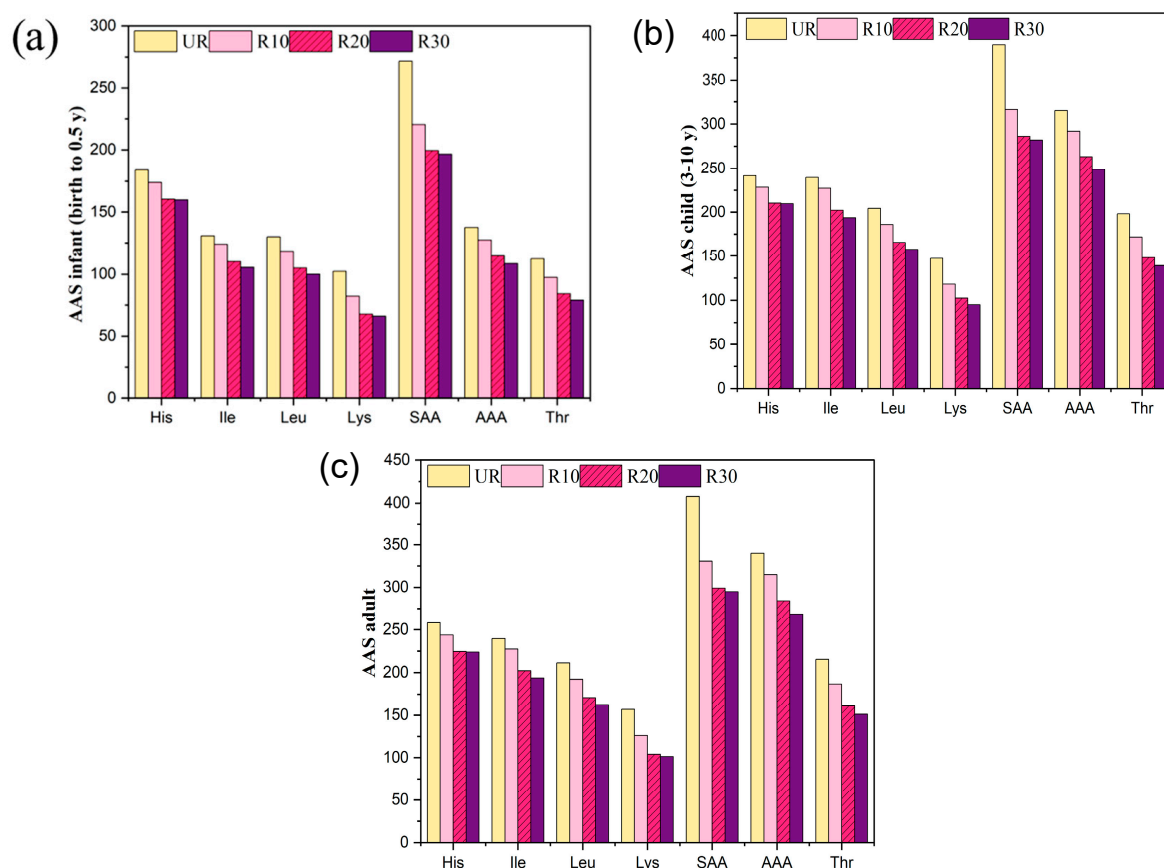
**Table 1.** Amino acid composition (mg/g protein) of LPI extracted by different roasting times.

| Amino Acids               | UR     | R10    | R20    | R30    | FAO Reference Value<br>(mg/g Protein) |                      |                   |
|---------------------------|--------|--------|--------|--------|---------------------------------------|----------------------|-------------------|
|                           |        |        |        |        | Infant<br>(0–6 mo)                    | Children<br>(3–10 y) | Adults<br>(>18 y) |
| Essential amino acids     |        |        |        |        |                                       |                      |                   |
| Histidine                 | 38.67  | 36.55  | 33.67  | 33.56  | 21                                    | 16                   | 15                |
| Threonine                 | 49.53  | 42.91  | 37.04  | 34.73  | 44                                    | 66                   | 23                |
| Methionine                | 10.36  | 6.09   | 5.16   | 4.01   | 33                                    | 23                   | 22                |
| Lysine                    | 70.62  | 56.68  | 46.73  | 45.63  | 69                                    | 48                   | 45                |
| Isoleucine                | 71.87  | 68.17  | 60.67  | 58.10  | 55                                    | 30                   | 30                |
| Leucine                   | 124.69 | 113.35 | 100.85 | 96.02  | 96                                    | 61                   | 59                |
| Phenylalanine             | 68.40  | 63.02  | 57.33  | 54.49  | 94                                    | 41                   | 38                |
| Non-essential amino acids |        |        |        |        |                                       |                      |                   |
| Aspartic acid             | 152.68 | 147.51 | 136.95 | 129.53 |                                       |                      |                   |
| Serine                    | 57.45  | 58.17  | 54.29  | 51.58  |                                       |                      |                   |
| Glutamic acid             | 273.35 | 276.82 | 262.87 | 256.83 |                                       |                      |                   |
| Glycine                   | 62.62  | 58.50  | 51.98  | 49.14  |                                       |                      |                   |
| Alanine                   | 52.08  | 44.14  | 37.32  | 35.21  |                                       |                      |                   |
| Arginine                  | 167.27 | 176.46 | 160.64 | 160.42 |                                       |                      |                   |
| Proline                   | 86.15  | 80.68  | 70.08  | 65.73  |                                       |                      |                   |
| Cysteine                  | 79.26  | 66.69  | 60.84  | 60.66  |                                       |                      |                   |
| Tyrosine                  | 60.79  | 56.64  | 50.65  | 47.57  |                                       |                      |                   |
| Σ EAAs                    | 434.14 | 386.77 | 341.45 | 326.54 |                                       |                      |                   |
| Σ NEAAs                   | 991.65 | 965.61 | 885.62 | 856.67 |                                       |                      |                   |
| EAA/TAA (%)               | 30.45  | 28.60  | 27.83  | 27.60  |                                       |                      |                   |
| EAAI (%)                  | 134.29 | 118.88 | 105.20 | 101.35 |                                       |                      |                   |
| BV (%)                    | 134.68 | 117.89 | 102.97 | 98.78  |                                       |                      |                   |
| PER <sub>1</sub>          | 4.60   | 4.11   | 3.59   | 3.39   |                                       |                      |                   |
| PER <sub>2</sub>          | 4.55   | 4.08   | 3.58   | 3.39   |                                       |                      |                   |
| PER <sub>3</sub>          | 3.44   | 2.71   | 2.20   | 2.07   |                                       |                      |                   |

### 3.2. Nutritional Quality of LPI

The nutritional quality of LPI was evaluated based on amino acid composition (Table 1) and several protein quality indices, including the protein efficiency ratio (PER), essential amino acid index (EAAI), biological value (BV), and the ratio of essential amino acids to total amino acids (EAA/TAA). The protein efficiency ratio (PER) is a theoretical measure of a protein's ability to support growth, calculated from specific amino acid concentrations [36]. PER values below 1.5 indicate low-quality protein, 1.5–2.0 reflect medium-quality, and values above 2.0 denote high-quality protein [4]. In this study, the theoretical PER values of LPI were higher than 2.0 across all treatments, indicating high predicted nutritional quality and growth-supporting potential [38]. The unroasted sample (UR) had the highest PER values (PER<sub>1</sub> 4.60, PER<sub>2</sub> 4.55, PER<sub>3</sub> 3.44), which decreased progressively with roasting to R30 (PER<sub>1</sub> 3.39, PER<sub>2</sub> 3.39, PER<sub>3</sub> 2.07). Notably, these PER values exceeded those reported for soy protein isolates (PER<sub>1</sub> 2.63, PER<sub>2</sub> 2.91, PER<sub>3</sub> 2.72) [39], suggesting that lupin protein, even after roasting, retains high biological utilization.





**Figure 1.** Amino acid score (AAS): (a) Infant; (b) Child; (c) Adult. SAA: sulfur amino acids (cysteine + methionine); AAA: aromatic amino acids (phenylalanine + tyrosine).

The essential amino acid index (EAAI) represents the balance of essential amino acids (EAAs) in a protein relative to a reference protein and is a key indicator of protein quality. EAAI declined with increasing roasting time, with UR showing the highest value (134.29%) and R30 the lowest (101.35%). Values exceeding 100 reflect a favorable essential amino acid balance relative to the reference protein rather than absolute biological efficiency [40]. This decline reflects the loss of heat-sensitive amino acids such as lysine and methionine during roasting [10]. The biological value (BV) estimates the proportion of absorbed protein that is retained for body protein synthesis and is positively correlated with EAA content [4]. The BV of LPI decreased from 134.68% (UR) to 98.78% (R30), remaining higher than reported values for cottonseed meal isolates (70.33%) and soy protein isolates (74%) [4,41], further supporting the high nutritional quality of lupin proteins. Finally, the ratio of essential to total amino acids (EAA/TAA) provides an overall estimate of protein nutritional balance. In LPI, EAA/TAA decreased slightly from 30.45% (UR) to 27.60% (R30), but remained comparable to reported values for several plant protein sources, indicating that even after roasting, LPI can serve as a valuable source of essential amino acids.

### 3.3. Structural Properties

#### 3.3.1. Circular Dichroism (CD) Spectroscopy

CD spectroscopy was employed to investigate the effect of thermal processing on the secondary structure of the LPI. This technique measures the differential absorption of left- and right-circularly polarized light by chiral peptide bonds, allowing quantification of  $\alpha$ -helices,  $\beta$ -sheets,  $\beta$ -turns, and random coil regions, which are closely associated with protein functionality [42,43]. As indicated in Table 2, roasting induced significant alterations in the secondary structure of LPI. Compared to the unroasted sample (UR:  $\alpha$ -helix 3.43%,

$\beta$ -sheet 31.53%,  $\beta$ -turn 14.19%, random coil 50.85%), the heat-treated samples showed an increase in  $\alpha$ -helix (5.03–6.74%) and  $\beta$ -turn (11.69–14.66%) content, while the  $\beta$ -sheet (33.97–31.98%) and random coil (49.30–46.62%) fractions decreased ( $p < 0.05$ ).

**Table 2.** Secondary structure compositions of LPI extracted by different roasting times.

| Samples | $\alpha$ -Helix (%) | $\beta$ -Sheet (%)    | $\beta$ -Turn (%)  | Random Coil (%)    |
|---------|---------------------|-----------------------|--------------------|--------------------|
| UR      | $3.43 \pm 0.11^c$   | $31.53 \pm 0.31^{ab}$ | $14.19 \pm 0.15^b$ | $50.85 \pm 0.27^a$ |
| R10     | $5.03 \pm 0.61^b$   | $33.97 \pm 1.44^a$    | $11.69 \pm 0.56^d$ | $49.30 \pm 0.31^b$ |
| R20     | $5.73 \pm 0.19^b$   | $33.68 \pm 0.57^a$    | $13.05 \pm 0.01^c$ | $47.54 \pm 0.36^c$ |
| R30     | $6.74 \pm 0.24^a$   | $31.98 \pm 0.22^b$    | $14.66 \pm 0.35^a$ | $46.62 \pm 0.34^d$ |

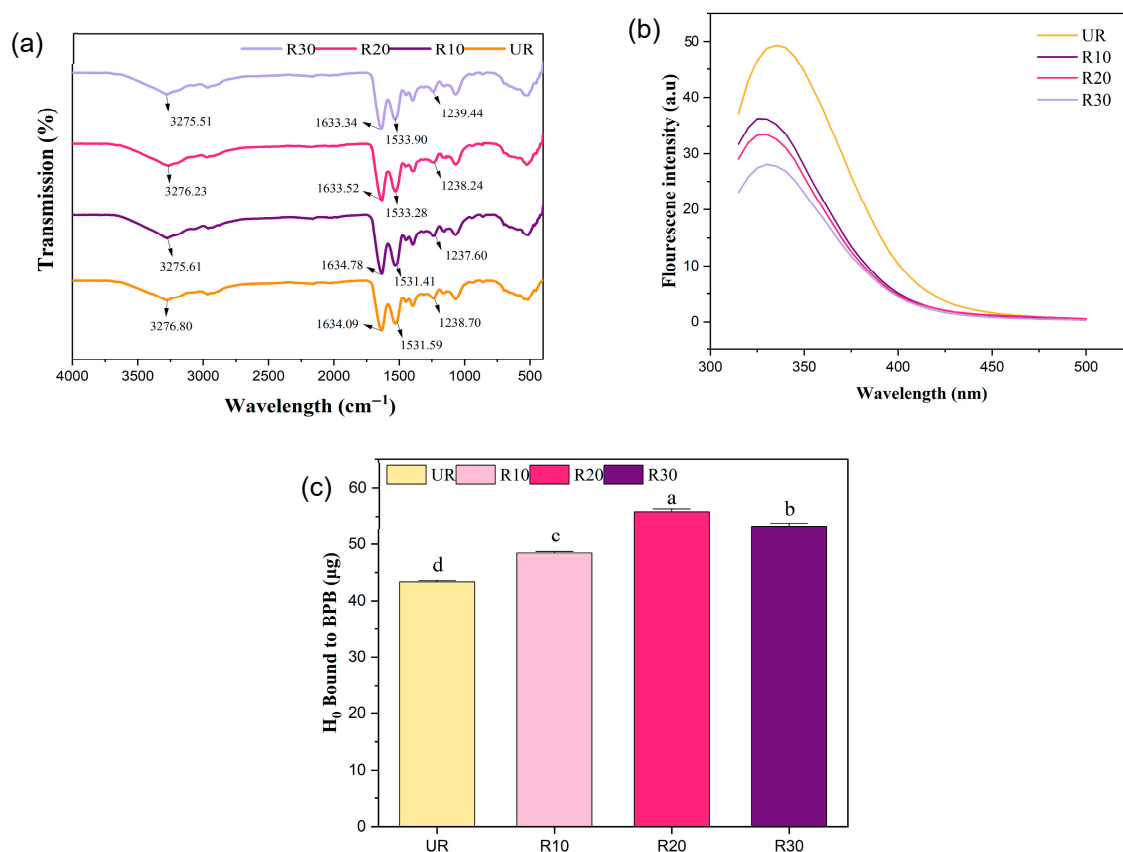
Values represent mean  $\pm$  SD ( $n = 3$ ). Different letters (a, b, c, d) indicate significant differences between samples ( $p < 0.05$ ), while the same letters indicate no significant difference.

Our results align with those of Wang, Dong, Zhu, Shen, Wu and Zhang [42], who found that heating globulins increases  $\alpha$ -helix and  $\beta$ -turn content while reducing  $\beta$ -sheets, indicating that proteins undergo secondary-structure reorganization into more ordered motifs. This is because heat treatment disrupts hydrogen bonds, hydrophobic interactions, and electrostatic forces in the globulin tertiary structure, particularly in the 11S and 7S subunits. As a result, buried hydrophobic and reactive residues ( $-\text{SH}$ ,  $-\text{NH}_2$ ) become exposed, allowing the protein to interact more effectively with both water and oil [44]. The increase in  $\alpha$ -helix reflects strengthened intramolecular hydrogen bonding and formation of more stable regions, while the rise in  $\beta$ -turn indicates flexibility in loop regions connecting  $\beta$ -sheets [45]. Reductions in  $\beta$ -sheet and random coil structures suggest partial unfolding and refolding of previously disordered areas, particularly in the more heat-sensitive 7S subunits [45,46]. Tang et al. [47] observed similar changes in globulins, where heating increased  $\alpha$ -helix and  $\beta$ -turn but decreased  $\beta$ -sheet and random coil, exposing hydrophobic residues and improving solubility and emulsifying ability [19]. Longer roasting disrupts intermolecular  $\beta$ -sheet hydrogen bonds (particularly in heat-sensitive 7S subunits), allowing some intramolecular  $\alpha$ -helix/ $\beta$ -turn refolding and reducing highly disordered regions, and promoting structured intramolecular organization [15,48]. Zhao et al. [49] reported comparable results for soy protein, where heating increased the  $\alpha$ -helix content and lowered  $\beta$ -sheet and random coil fractions. Roasting induces controlled conformational rearrangements in lupin globulins, as evidenced by partial unfolding, refolding into  $\alpha$ -helices and  $\beta$ -turns, and exposure of hydrophobic residues, highlighting the functional advantages of heat pre-treatment in food applications.

### 3.3.2. Fourier-Transform Infrared Spectroscopy (FT-IR)

FT-IR spectra of LPI revealed characteristic protein absorption bands between 400 and  $4000\text{ cm}^{-1}$ , with no appearance of new peaks after roasting (Figure 2a), indicating that thermal treatment altered the existing bonds rather than forming entirely new chemical groups. The broad amide A band ( $3000\text{--}3500\text{ cm}^{-1}$ ), primarily attributed to O–H and N–H stretching vibrations, reflects hydroxyl and amino functionalities and the extent of hydrogen bonding [15]. A gradual reduction in the intensity of amide A was observed with increasing roasting duration ( $\text{UR} > \text{R20} > \text{R10} > \text{R30}$ ), whereas the peak position remained nearly constant ( $3276.80\text{--}3275.51\text{ cm}^{-1}$ ). This trend suggests changes in hydrogen bonding involving O–H and N–H groups, potentially associated with thermal reactions such as Maillard-type interactions, consistent with previous findings that thermal processing promotes dehydroxylation and alters the physicochemical properties of legumes by diminishing hydroxyl availability [50,51]. The amide I region ( $1700\text{--}1600\text{ cm}^{-1}$ ), dominated by C = O stretching ( $\sim 80\%$ ) with minor C–N contributions, is highly sensitive to protein secondary structure [15,19,52]. A red shift from  $1634.78\text{ cm}^{-1}$  (R10) to  $1633.34\text{ cm}^{-1}$  (R30) indicates altered hydrogen bonding and conformational rearrangements. These spectral

changes are consistent with CD spectroscopy results showing increased  $\alpha$ -helix and  $\beta$ -turn fractions in roasted samples, supporting the trends observed in solubility and surface hydrophobicity. The amide II band ( $1550$ – $1500$   $\text{cm}^{-1}$ ), arising from N–H bending and C–N stretching vibrations, displayed a blue shift from  $1531.41$   $\text{cm}^{-1}$  (R10) to  $1533.90$   $\text{cm}^{-1}$  (R30), reflecting altered intra- and intermolecular interactions and stabilization of protein structures [13]. Similarly, the amide III region ( $1300$ – $1200$   $\text{cm}^{-1}$ ), corresponding to C–N stretching and N–H bending, exhibited a blue shift from  $1237.60$   $\text{cm}^{-1}$  (R10) to  $1239.44$   $\text{cm}^{-1}$  (R30), indicative of backbone conformational rearrangements associated with extended thermal treatment [53]. These findings demonstrate that FTIR spectroscopy reliably detects thermal processing-induced changes in protein structure and interactions.



**Figure 2.** (a) Fourier-transform infrared spectroscopy (FT-IR); (b) Intrinsic fluorescence spectroscopy; (c) Surface hydrophobicity. Different letters indicate significant differences ( $p < 0.05$ ).

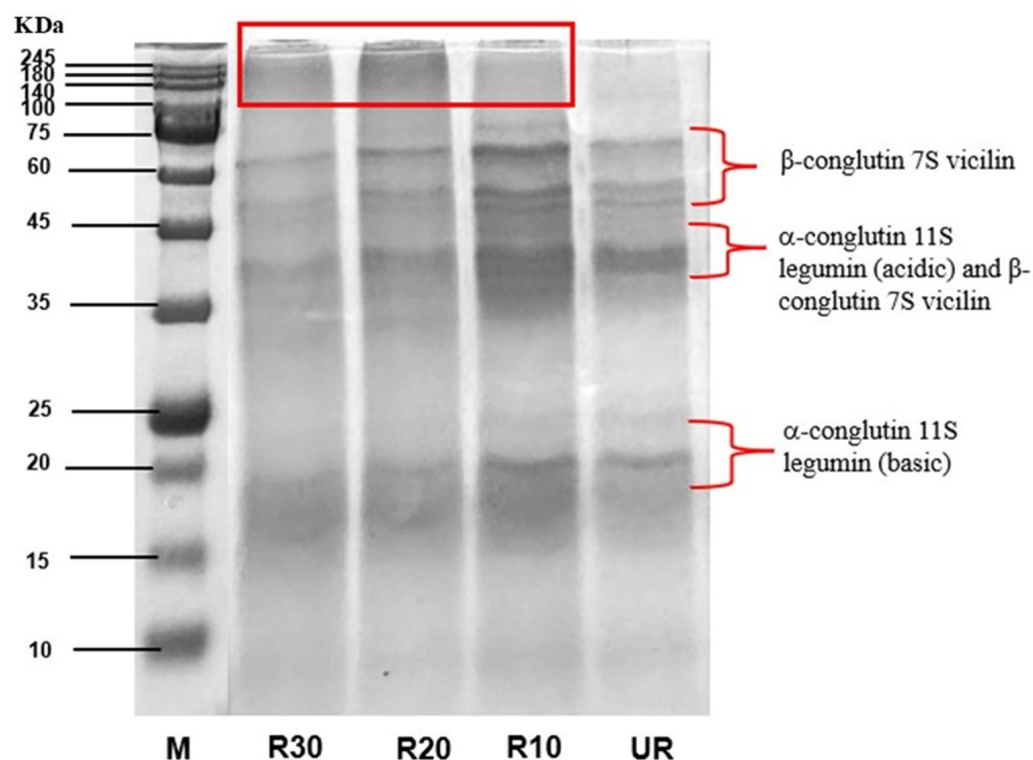
### 3.3.3. Intrinsic Fluorescence Spectroscopy

Intrinsic fluorescence spectroscopy was used to investigate tertiary structural changes in LPI induced by roasting (Figure 2b). Protein fluorescence predominantly stems from tryptophan (Trp) residues, with minor contributions from tyrosine (Tyr) and phenylalanine (Phe), functioning as intrinsic fluorophores [54]. The emission maximum ( $\lambda_{\text{max}}$ ) of Trp is highly sensitive to its local microenvironment, typically occurring at  $\sim 320$ – $330$  nm when buried within hydrophobic protein cores and shifting toward  $\sim 340$ – $350$  nm upon exposure to polar aqueous surroundings [55]. Upon increasing roasting duration, the fluorescence emission peak of LPI exhibited a subtle red shift from 330 nm (at 10 min roasting) to 335 nm (at 30 min roasting). This red shift indicates conformational loosening and partial unfolding of the protein tertiary structure, resulting in increased exposure of Trp residues to a more polar environment [13]. Such exposure stabilizes the excited state of these fluorophores, thereby lowering the emission energy and shifting the fluorescence

to longer wavelengths, which aligns with known effects of thermal processing on protein conformation [56]. Concurrent with the red shift, there was a progressive decrease in fluorescence intensity across roasted samples compared to the UR. This decline in intensity can be explained by multiple overlapping effects, such as quenching of the exposed aromatic residues through interaction with polar solvent molecules, partial aggregation of unfolded proteins leading to energy transfer and fluorescence quenching, and alterations in the local hydrophobic environment surrounding the fluorophores [56,57]. Overall, intrinsic fluorescence analysis demonstrates that roasting significantly alters the tertiary structure of lupin proteins. The observed spectral changes reveal that Trp residues, previously buried within the hydrophobic core, become increasingly exposed, highlighting unfolding events, while partial aggregation or domain rearrangements may reduce fluorescence intensity.

### 3.3.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of the LPI revealed a clear roasting-time-dependent progression from well-defined native congrutin bands in the unroasted sample (UR) to extensive high molecular weight (HMW) smearing in the roasted samples, indicating the formation of protein aggregates during heating, as shown in Figure 3. In UR, distinct bands corresponding to  $\alpha$ -conglutin (~20–62 kDa),  $\beta$ -conglutin (~68–75 kDa), and the  $\gamma$ -conglutin region (~47–48 kDa) were evident, confirming intact and soluble globulin subunits [58]. Following 10 min of roasting (R10), these bands became slightly broader and more diffuse, but their overall intensity remained relatively high. At 20 min (R20), intensities of all conglutin bands decreased further, and light smearing appeared above ~150–180 kDa, indicating the formation of initial HMW aggregates. After 30 min (R30), discrete  $\alpha$ -,  $\beta$ -, and  $\gamma$ -conglutin bands were almost entirely lost and replaced by dense smearing above ~180 kDa, characteristic of aggregation and reduced electrophoretic resolution.



**Figure 3.** Effect of different roasting times on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). (M) marker; (R30) Roasting 140 °C for 30 min; (R20) Roasting 140 °C for 20 min; (R10) Roasting 140 °C for 10 min; (UR) Unroasting. The red frame indicates broad stained bands in the high molecular weight (HMW) region observed in the roasted samples.

This roasting-induced evolution aligns well with the structural organization and thermal behaviour of lupin globulins.  $\alpha$ -conglutin is an 11S legumin composed of acidic (~42–62 kDa) and basic (~20–24 kDa) chains linked by disulfide bonds;  $\beta$ -conglutin is a 7S vicilin-type trimer lacking disulfide linkages; and  $\gamma$ -conglutin 7S vicilin contains intramolecular disulfide-stabilized subunits around ~47–48 kDa [9,58,59]. The sharp, intense bands in UR therefore reflect intact quaternary and tertiary structures and the presence of accessible basic residues (particularly arginine, lysine, and histidine), which strongly bind Coomassie Brilliant Blue [60]. The modest broadening observed at R10 is most consistent with early unfolding, partial dissociation, and formation of small SDS-soluble oligomers, a behaviour widely reported in globulin-rich legume and pseudocereal proteins subjected to mild heating [32,61]. By R20, the progressive attenuation of  $\alpha$ -,  $\beta$ -, and developing  $\gamma$ -conglutin region bands, together with the appearance of faint HMW smearing, suggests a shift from small oligomers to larger aggregates [32,42]. Thermal unfolding at this stage exposes hydrophobic patches and reactive side chains (particularly sulfhydryls and  $\epsilon$ -amino groups), promoting hydrophobic interactions, sulfhydryl oxidation, initial disulfide interchange reactions, along with other covalent interactions that are not fully dissociated under SDS-PAGE conditions [49,62]. Similar mid-stage transitions, band broadening followed by fading, have been documented for pea proteins as aggregates begin migrating into the HMW region [15]. The amino acid profile at R20, showing early reductions in Lys, Arg, and Cys, is consistent with the interpretation that some reactive residues are already being consumed in oxidative modification and early Maillard-type chemistry, although the most pronounced losses occur at longer roasting times.

At R30, the dominance of dense HMW smearing and near-complete disappearance of native conglutin bands indicate extensive aggregation driven by multiple covalent pathways. Beyond widespread disulfide bond formation through Cys oxidation, non-disulfide covalent cross-links such as carbonyl–amine (Maillard-derived) adducts and tyr–tyr (di-tyrosine) linkages likely contribute to network formation, consistent with mechanisms reported for roasted, glycosylated, and oxidatively modified seed proteins [62,63]. The substantial depletion of Lys, Arg, His, Tyr, Cys, Trp, and Met in the amino acid profile suggests their possible involvement in Maillard and oxidative reactions during roasting [28,29]. Similar roasting-induced transitions from sharp subunit bands to dominant HMW smears have been documented in roasted pea, hemp, and lupin proteins [13,15,32], confirming that the aggregation behaviour observed here is characteristic of thermally processed legume globulins.

### 3.4. Surface Hydrophobicity

Surface hydrophobicity serves as a key indicator of protein conformational changes and unfolding, reflecting the exposure of previously buried hydrophobic amino acid residues to the aqueous environment [56]. This parameter influences functional properties such as emulsification, foaming, and gelation by modulating protein interactions with nonpolar phases, including lipids and air interfaces [64]. The effect of roasting time on the surface hydrophobicity of LPI, expressed as bromophenol blue (BPB) binding capacity, is presented in Figure 2c.

Compared with the unroasted sample (UR; 43.34  $\mu$ g BPB bound), surface hydrophobicity increased significantly after 10 min (R10; 48.41  $\mu$ g) and 20 min (R20; 55.79  $\mu$ g) of roasting ( $p < 0.05$ ). This increase indicates partial unfolding of native conglutin structures, which disrupts stabilizing intramolecular hydrogen bonds and van der Waals interactions and exposes hydrophobic regions that were previously sequestered within the protein core. As a result, hydrophobic patches become more accessible to the BPB probe, consistent with thermally induced unfolding observed in other legume proteins [13,65].

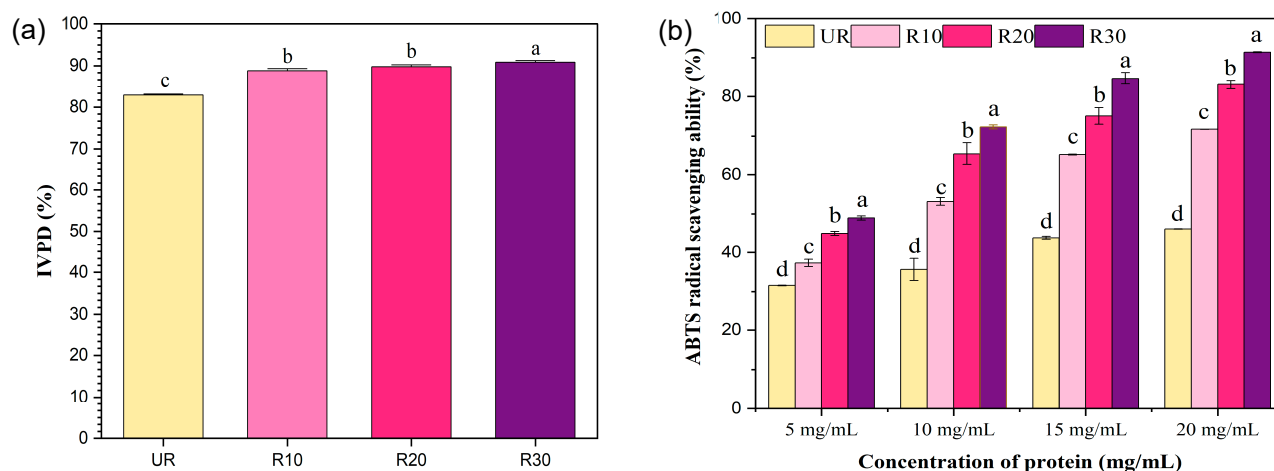


When roasting was extended to 30 min (R30; 53.20  $\mu\text{g}$ ), surface hydrophobicity decreased compared with R20 ( $p < 0.05$ ), although it remained higher than in the unroasted sample. This decrease does not mean that the proteins returned to their original structure. Instead, early roasting opens up the protein structure and exposes its water-repelling (hydrophobic) parts. With longer roasting time, these unfolded proteins tend to aggregate via hydrophobic interactions [56]. As aggregation progresses, hydrophobic residues become trapped inside the protein clumps and are no longer exposed on the surface. Because the BPB method detects only surface-accessible hydrophobic sites, fewer exposed sites are detected even though the proteins remain thermally unfolded and structurally modified, resulting in a lower hydrophobicity value at R30. This explanation is supported by SDS-PAGE results, which showed band smearing and the appearance of high-molecular-weight aggregates at longer roasting times, indicating protein clustering rather than protein breakdown [45]. Similar behavior has been reported for other heat-treated plant proteins, where prolonged heating causes proteins to stick together and hide hydrophobic regions, leading to lower measured surface hydrophobicity despite continued denaturation Chen, Zhao, Yu, Zhang, Zhu, Tong and Hao [62] and Zhao, Yuan, Chen, Fang, Li, Yao and Li [49]. Taken together, FTIR, intrinsic fluorescence, SDS-PAGE, amino acid composition, and surface hydrophobicity analyses demonstrate that roasting disrupts native conglutin structures through unfolding, aggregation, and reorganization or loss of reactive residues (particularly Lys, Met, and Thr). These coordinated structural changes are expected to modify enzyme accessibility and, consequently, influence the *in vitro* protein digestibility (IVPD) of LPI.

### 3.5. *In Vitro* Protein Digestibility (IVPD)

The digestibility of protein isolates is a crucial factor for food applications, as it indicates the fraction of protein that can be broken down and potentially absorbed under simulated gastrointestinal conditions [66]. As shown in Figure 4a, the unroasted sample (UR) exhibited the lowest IVPD value (82.84%), whereas roasting pretreatment for 10–30 min significantly increased IVPD to 88.82–90.85% ( $p < 0.05$ ), demonstrating that thermal processing markedly enhances lupin protein digestibility. The lower digestibility of UR can be attributed to its limited exposure of reactive and cleavage-susceptible regions, as supported by its low surface hydrophobicity and intact globulin structures. These structural features restrict molecular mobility and reduce enzyme accessibility to peptide bonds, thereby limiting proteolytic efficiency [66,67]. Similar roasting-induced improvements in IVPD have been reported for silkworm pupae, soybean, and albumin proteins, where controlled heat treatment promoted protein unfolding and enhanced enzymatic hydrolysis compared with native counterparts [66,68,69].

Roasting induced progressive structural rearrangements that favor digestion. Heat-triggered disruption of stabilizing non-covalent interactions and partial unfolding of 11S conglutin proteins resulted in the formation of SDS-soluble aggregates, as evidenced in SDS-PAGE profiles [70]. Importantly, these aggregates are indicative of loosely associated protein assemblies rather than rigid, insoluble networks. Such structural reorganization increases chain mobility and reorganizes secondary motifs within aggregates, improving exposure of protease-accessible cleavage sites during gastrointestinal digestion [69,71,72]. Although SDS-PAGE did not reveal the formation of low-molecular-weight peptides prior to digestion, this is expected because proteolysis occurs during the IVPD assay rather than before electrophoretic analysis.



**Figure 4.** (a) In vitro protein digestibility (IVPD); (b) ABTS-radical scavenging assay. Different lowercase letters indicate significant differences ( $p < 0.05$ ).

In addition, legume proteins contain antinutritional factors such as phytic acid, saponins, trypsin inhibitors, and polyphenols, which can limit digestibility by binding proteins or digestive enzymes [73]. Processing methods including roasting, soaking, cooking, and ultrasound are known to reduce these inhibitors and thereby improve digestibility and nutritional quality [33,73,74]. Proteins with IVPD values around or above 80% are generally regarded as highly digestible in in vitro models [33,66]. Consistent with this, Sreechithra and Sakhare [17] reported that roasting and hydrothermal treatments increased soybean IVPD from approximately 60% to 80%, placing roasted lupin conglutins within the range of highly digestible commercial plant protein isolates. Overall, the observed increase in IVPD following roasting suggests that pretreatment alters LPI structure, which may facilitate digestion and thereby support improved nutritional utilization.

### 3.6. ABTS-Radical Scavenging Assay

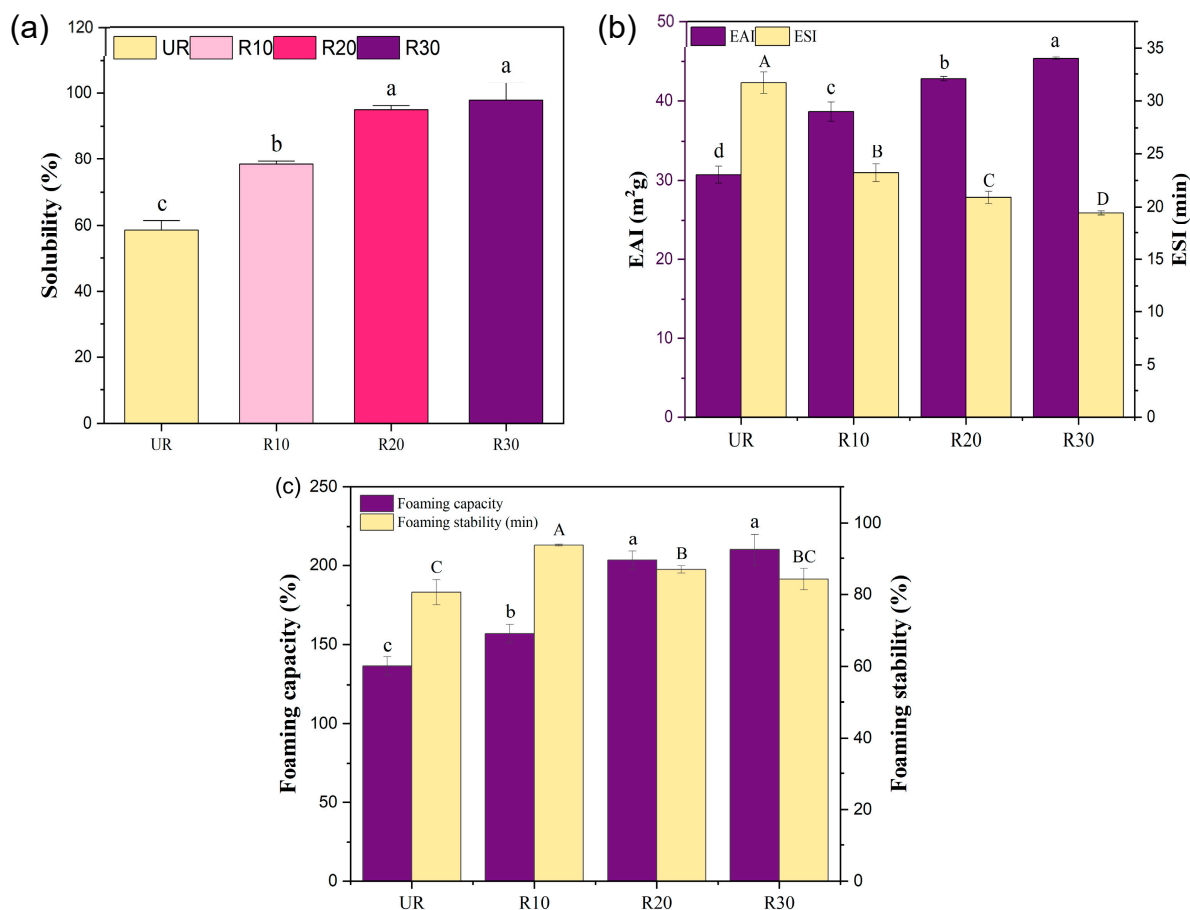
Antioxidant analysis evaluates the capacity to neutralize free radicals, providing a key indicator of the potential to prevent cellular damage and chronic diseases. The antioxidant activity of LPI obtained at three different roasting times was evaluated using the ABTS<sup>+</sup> radical scavenging assay at concentrations ranging from 5 to 20 mg/mL, as presented in Figure 4b. A significant increase ( $p < 0.05$ ) in the ABTS<sup>+</sup> radical scavenging activity of LPI was observed with increasing roasting time compared with that of native LPI. The highest antioxidant activity was recorded at 20 mg/mL for R30 (91.43%), whereas the lowest was observed at 5 mg/mL in UR (31.55%). A similar trends were reported by Wu, Lin and Chen [20] and Niu et al. [75], who found that roasting enhanced antioxidant activity compared to its native counterpart, resulting in increased radical scavenging activity. The differences in radical scavenging activity between native and roasted LPI can be primarily attributed to heat-induced structural modifications of the proteins, rather than to phenolic enrichment, as thermal processing alters protein conformation and molecular interactions [76,77]. Heat-induced unfolding and partial fragmentation increase molecular flexibility and expose redox-active amino acid residues, particularly aromatic and heterocyclic residues such as Tyr, Trp, and His, which can donate electrons or hydrogen atoms to stabilize free radicals [78]. In addition, roasting facilitates Maillard reactions between amino groups and reducing sugars, leading to the formation of melanoidins (nitrogenous brown polymers produced via non-enzymatic browning) that exhibit strong radical-scavenging and hydrogen-donating properties [79,80]. The accumulation of these melanoidins is closely associated with increased ABTS radical scavenging activity, reflecting a strong correlation

between antioxidant capacity and the formation of late-stage Maillard reaction products, particularly melanoidins.

### 3.7. Functional Properties

#### 3.7.1. Protein Solubility

Solubility is an important functional attribute of proteins that strongly influences other techno-functional properties, including emulsification and foaming, through interactions between water molecules and peptide backbones, hydrogen bonds, and amino acid side chains [12,81]. As shown in Figure 5a, the protein solubility of the roasted protein sample was significantly different ( $p < 0.05$ ) from that of the unroasted sample. The solubility of roasted LPI increased markedly from 78.39% to 97.84%, whereas the unroasted sample (UR) exhibited substantially lower solubility (58.64%). Lupin proteins are predominantly globulins ( $\alpha$ - and  $\beta$ -conglutins), which typically exhibit limited solubility near their isoelectric points ( $pI \approx 4.3$ – $6.2$ ) and moderate solubility at neutral pH due to weak electrostatic repulsion and compact native packing [65]. In the unroasted (UR) sample, globulin proteins remain tightly folded, with few surface groups available to interact with water. This compact structure favors protein–protein interactions over protein–water interactions, resulting in low solubility [82]. In contrast, the soluble protein content of lupin increased progressively with increasing roasting time. Controlled thermal treatment induces partial unfolding of globular lupin proteins that become water-dispersible under alkaline extraction and neutral pH conditions, thereby disrupting non-covalent interactions such as hydrogen bonds and van der Waals forces and exposing reactive functional groups, thereby improving protein–solvent interactions, hydration, and dispersibility [26,45]. Upon unfolding, a new balance between protein–protein and protein–solvent interactions is established, which may favor the formation of soluble oligomers rather than insoluble aggregates. These soluble aggregates are stabilized by a combination of electrostatic repulsion and hydration forces, allowing them to remain dispersed in the aqueous phase [83–85]. CD spectroscopy analysis further supports this interpretation, as random coil conformations remained dominant ( $\sim 46$ – $49\%$ ) after roasting. Random coil conformations are generally more flexible and accessible to water molecules, which enhances hydration and contributes positively to solubility. The enhanced solubility of roasted LPI aligns with previous studies by Wang, Yang, Wang, Zhang and Zhao [45], which showed that roasting leads to reduced particle size, enhanced particle–water interactions, and improved protein solubility of pine kernels. Notably, roasted LPI exhibited more intense HMW bands ( $>150$  kDa) confirmed on SDS-PAGE compared with UR, indicating the formation of larger protein aggregates upon roasting [32,42]. Importantly, molecular weight alone does not imply insolubility. Heat treatment followed by alkaline extraction at neutral pH is known to generate soluble HMW oligomers, as evidenced by the retention of conglutin subunit bands alongside HMW smearing, suggesting the formation of soluble oligomeric aggregates rather than irreversible, insoluble aggregates [15,86]. A similar trend has been reported for roasted pea protein isolates, where increased solubility was attributed to the formation of charged, hydrated HMW oligomers that enhanced protein dispersibility and functionality at neutral pH [15]. Therefore, the enhanced solubility of LPI can be attributed to the predominance of soluble, hydrated oligomers and flexible secondary structures that prevent excessive insoluble aggregation. Overall, these results suggest that controlled roasting emerges as a promising and strategic approach for significantly enhancing the functional attributes of LPI.



**Figure 5.** (a) Solubility; (b) Emulsifying activity index (EAI) and emulsifying stability index (ESI); (c) Foaming capacity (FC) and foaming stability (FS). Different lowercase letters indicate significant differences for Solubility, EAI, and FC, while different uppercase letters indicate significant differences ( $p < 0.05$ ).

### 3.7.2. Emulsifying Properties

Emulsifying activity index (EAI) and emulsifying stability index (ESI) are key parameters used to evaluate the ability of proteins to form and stabilize oil-water emulsions. EAI reflects the capacity of proteins to rapidly adsorb at newly created interfaces and reduce interfacial tension, whereas ESI represents the ability of the adsorbed proteins to form a cohesive, elastic interfacial film that resists droplet flocculation and coalescence over time [49]. As shown in Figure 5b, the UR exhibited the lowest EAI ( $30.71 \text{ m}^2/\text{g}$ ), while roasting pre-treatment from 10 to 30 min progressively enhanced the EAI (from  $38.67$  to  $45.37 \text{ m}^2/\text{g}$ ) due to heat treatment ( $p < 0.05$ ). The structural rigidity of unroasted LPI reduces diffusion kinetics and delays adsorption, thereby impairing interfacial film formation [64,87]. In contrast, roasting induces partial unfolding and conformational expansion of LPI, exposing amphiphilic domains that enhance interfacial activity. The increased exposure of hydrophobic patches improves anchoring at the oil phase, while polar and charged residues orient toward the aqueous phase, collectively facilitating faster adsorption and more effective interfacial tension reduction [6,64,88]. Additionally, increased surface hydrophobicity due to prolonged roasting also contributes to better emulsification by exposing charged groups that form double-layer films around oil droplets [89]. Similar trends were observed in pea and soy protein isolates, where heat treatment facilitated structural expansion and increased emulsifying activity [15,49].

Despite the improvement in EAI, ESI (Figure 5b) decreased significantly with increasing roasting time, declining from 31.67 min in UR to 19.43 min in R30 ( $p < 0.05$ ), indicating reduced long-term emulsion stability. Roasting pre-treatment induced structural changes leading to excessive unfolding and hydrophobic aggregation, reducing their ability to form cohesive, viscoelastic interfacial films during emulsification. The interaction protein-protein limited interfacial rearrangement, thereby promoting faster droplet coalescence and accelerated phase separation [90]. SDS-PAGE results support this interpretation, showing the formation of high-molecular-weight protein aggregates at extended roasting times. Because these large protein clusters cannot spread evenly, they attach to oil droplets in patches rather than forming a continuous coating. This uneven coverage weakens droplet protection, promotes droplet clumping and coalescence, and ultimately reduces emulsion stability (ESI) [91]. Zhu et al. [92] similarly reported that large protein particles tend to aggregate randomly on droplet surfaces rather than forming an ordered structure, thereby increasing the droplet size and leading to a larger, less stable emulsion. Therefore, while heat-induced unfolding enhances the initial emulsification, excessive aggregation may hinder long-term stabilization by promoting flocculation and destabilizing the emulsion.

### 3.7.3. Foaming Properties

The effects of roasting at different times on the foaming capacity (FC) and foam stability (FS) of LPI are shown in Figure 5c. FC increased significantly from 136.66% in UR to 210% in R30 ( $p < 0.05$ ), indicating a marked improvement in foaming ability after heat treatment. In contrast, FS decreased progressively with longer roasting time, declining from 93.61% at R10 to 84.21% at R30, while UR exhibited the lowest stability (80.58%) ( $p < 0.05$ ). Studies by Zhao, Xiong, Chen, Zhu and Wang [26] similarly reported that heating disrupts disulfide bonds and partially unfolds proteins, which enhances FC but potentially reduces FS when treatment is prolonged. Native proteins typically exhibit limited foaming performance because their compact structures restrict molecular flexibility and slow diffusion, hindering efficient adsorption and rearrangement at the air-water interface around air bubbles [82]. As a result, they are less effective at reducing interfacial tension and forming stable interfacial films. Roasting induces conformational changes and promotes molten-globule-like conformations with balanced amphiphilicity, improving protein flexibility and surface activity. These changes allow proteins to migrate faster, adsorb more effectively, and align better at the air-water interface [45,93]. Consequently, this enhanced interfacial behavior contributes to the increased FC observed in roasted LPI. Moreover, higher protein solubility, surface hydrophobicity, and the flexibility of polypeptides associated with changes in net charge, facilitate diffusion and rearrangement of proteins at the interface, which further supports foam formation and increases FC [94,95].

Regarding foam stability (Figure 5c), R10 exhibited higher FS than UR because moderate roasting improves protein unfolding and interfacial adsorption, allowing proteins to form a more cohesive and elastic film around air bubbles. In contrast, prolonged roasting induces excessive aggregation and the formation of larger protein complexes, which adsorb less uniformly at the air-water interface. Instead of forming a continuous protective film, aggregated proteins attach in patches, creating weak interfacial regions that accelerate bubble coalescence and liquid drainage [90]. As a result, foam volume decreases more rapidly over time. In addition, aggregation reduces molecular flexibility and limits the ability of proteins to rearrange and recover after deformation, further lowering the resistance of the interfacial film to rupture [45,95]. Consequently, although roasting enhances foaming capacity, excessive roasting reduces the ability of proteins to stabilize the foam structure, leading to lower FS at longer roasting times. Overall, roasting modifies protein structure in a time-dependent manner, where moderate unfolding improves foam formation, whereas



extensive aggregation compromises long-term foam stability, making temperature and time critical factors governing both FC and FS.

#### 4. Conclusions

*Lupinus angustifolius* protein isolate (LPI) in its native form exhibits low solubility and limited surface activity, which restricts its application in food systems. This study demonstrates that roasting pre-treatment effectively modulates the structure–function relationship of LPI in a time-dependent manner. Although prolonged roasting caused partial losses of essential amino acids, particularly Lys and Met, it simultaneously induced beneficial molecular rearrangements that enhanced techno-functional and nutritional performance. CD and FTIR analyses revealed that roasting promoted secondary-structure reorganization rather than simple denaturation, characterized by a gradual decrease in random coil content and a concomitant increase in  $\alpha$ -helix and minor fluctuations in  $\beta$ -sheet content. These changes indicate heat-driven molecular rearrangement and intermolecular association following partial unfolding. Intrinsic fluorescence showed alterations in the microenvironment of tryptophan residues, reflecting initial exposure and subsequent re-burial during aggregation. SDS-PAGE further confirmed progressive formation of high-molecular-weight aggregates with extended roasting, consistent with heat-induced cross-linking and Maillard-type reactions. These structural modifications led to corresponding changes in physicochemical and functional behavior. Moderate unfolding increased molecular mobility, amphiphilicity, and surface hydrophobicity, thereby improving solubility, emulsifying activity, and foaming capacity through faster adsorption and more effective interfacial tension reduction. However, excessive roasting promoted aggregation, reducing interfacial packing efficiency and film elasticity, causing slight declines in emulsion and foam stability. Roasting also enhanced antioxidant capacity, as indicated by increased ABTS radical-scavenging activity, attributed to structural loosening, exposing radical-quenching residues (Tyr, Trp, His), and formation of antioxidant Maillard-derived melanoidins. In addition, roasting improved in vitro protein digestibility by altering protein conformation and reducing the impact of anti-nutritional factors. Overall, controlled roasting provides an effective strategy to tailor LPI structure for improved solubility, interfacial performance, antioxidant activity, and digestibility, supporting the development of lupin proteins as functional and sustainable food ingredients. Beyond functionality, future work on consumer acceptance, allergenicity, and anti-nutritional evaluation will further support the transformation of lupin proteins from underutilized resources into key components of innovative and sustainable food products.

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